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EXTRACTIVE FRACTIONATION OF EQUINE HYPERIMMUNE PLASMA

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ABSTRACT

Aqueous two-phase partition and triphasic systems containing thiophilic and metal ligands were used to produce anti–snake venom serum using the fractionation of hyperimmune equine plasma with neutralizing activity against *Bothrops spp*. venom as the model.

In poly(ethyleneglycol)(PEG)/salt systems, PEG molecular weight, system pH, and the amount of polyvinylpirrolidone (PVP) added were optimized in order to promote selective immunoglobulin recovery. From these studies a PEG 1500/ammonium sulfate system, pH 8.0, a tie-line length parameter (TLL) 21%, and a 2% PVP K-12 were preliminarily selected. The immunoreactive fraction (ELISA) mainly preferred the top phase, with a theoretical yield of >97% and a purification factor of 3.5. Practical recoveries were, however, hampered by limited product solubility and an erratic global process performance.

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In order to overcome these limitations, we moved to a system formed by derivatives of cellulose and starch, which contained an affinity solid phase. This three-phase system was compatible with the use of thiophilic or immobilized metal ion affinity (IMAC) supports, the first showing the higher selectivity. The system composed of 2% Methocel, 2% Reppal, 1.0 mol/kg ammonium sulfate, and 5–10% 2S-thioether sulfone agarose allowed recovery of 52% of the immunoreactive fraction from hyperimmune equine plasma at a total protein concentration of 0.25%. This process strategy also allowed simple operation and easy of affinity ligand recycling.

INTRODUCTION

The specific treatment of snakebite poisoning consists of the administration of antivenoms, an immunotherapeutic approach that has led to reduced mortality and morbidity among subjects treated. Antivenom sera are usually prepared from horse (hyperimmune) plasma rich in antibodies directed against venom protein components. However, these preparations are also responsible for acute or delayed allergic reactions due to the injection of heterologous antibodies and their often low purity degree (1,2).

The development of new antivenoms by using different immunized animals e.g., sheep instead of horses) and/or improved immunoglobulin purification or processing strategies has been proposed to prevent immediate undesired reactions. For example, pepsin or papain treatment of immunoglobulins yields fragments with neutralizing capacity (Fab, $F(ab')_2$) but without effects mediated by complement activation by the classical pathway, characteristic of the Fc moiety (3). However, the introduction of complex process schedules (ultrafiltration, ion exchange, affinity chromatography, and pasteurization) has meant an increased cost of the final product (4). As a consequence, in many developing countries, protocols for antivenom sera production are still based on the low-resolution method of Pope, that is, selective precipitation of whole immunoglobulins (or their fragments after pepsin digestion) with ammonium sulfate (5). In the last few years, some improvement in product quality was achieved through a negative precipitation technique using caprylic acid (6). However, and despite their simplicity, both methods have the inherent limitations of coarse fractionation operations. In this frame, there is room enough to pursue the development of easy-to-operate and economical technologies for antisera preparation.

Aqueous two-phase systems (ATPS), such as those composed by two polymers or a polymer and a salt, are formed spontaneously when certain concentrations

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of both components are reached (7). Due to their high water content (over 85%), these ATPS provide a favorable environment for biological materials, such as cells, organelles, or proteins. Furthermore, the unique surface properties of these entities allow separation by differences in their partition behavior (8). This strategy was successfully employed in protein purification. High-recovery, easy scale-up, continuous processing capability and high benefit/cost ratio are among the main advantages of this method (9). For technological applications, polymer/salt ATPS e.g., poly(ethyleneglycol), PEG, and phosphate, sulfate or citrate are usually preferred (10). However, two-polymer ATPS, such as those formed by PEG and ethylhydroxyethyl cellulose, hydroxypropyl starch, crude or fractionated dextran, or polyvinylpirrolidone (PVP), can also be used (11).

Macromolecule partition in ATPS depends on their molecular weight, surface charge, and hydrophobicity. The concentration, type, and molecular weight of the phase-forming polymers, the pH and temperature of the system, and the presence of added ions were all found to influence solute partition (12). Partition of a protein can also be modified by attaching an affinity ligand to a phase-forming polymer or to a solid phase (13,14). Immunoglobulin (particularly IgG) purification usually employs protein A as the ligand, even at an industrial scale. In addition to its high cost, this ligand has several disadvantages, such as contamination of the final product and degradation under regeneration and/or cleaning-in-place procedures. Moreover, the poor binding ability of protein A toward equine immunoglobulins is well known. As an alternative, robust group-specific ligands of low molecular weight were developed to purify immunoglobulins from different classes and species (15). The original T-ligand (16) and some related structures (17), which typically comprise two neighboring sulfur atoms acting as an antagonistic electron donor–acceptor pair, are proposed as selective ligands for IgG purification.

The aim of this work is to study the partitioning behavior of the proteins from equine hyperimmune plasma, especially those having immunoreactivity against *Bothrops spp*. snake venom. We have also studied the use of group-specific ligands on solid matrices for immunoglobulin purification in ATPS. Several strategies to integrate phase partition and affinity capture on solid supports were explored. Based on the results obtained, a potentially useful plasma fractionation procedure for antivenom sera preparation is proposed.

MATERIALS AND METHODS

Materials

PEG 600, 1000, 1540, 3350, and 6000 were from Fluka Chemicals (Buchs, Switzerland). Reppe Glycos AB (Vaxjo, Sweden) donated Hydroxypropyl starch

(Reppal PES-100). Ethylhydroxyethyl cellulose (Methocel E-15) was from Biosciences Inc., USA, and polyvinylpirrolidone (PVP K-12 and K25) were from BASF, Germany. Sepharose CL 4B, Sephacryl HR-300, Dextran T-500, and PD-10 columns were from Pharmacia Biotech (Uppsala, Sweden). DEAE-Affi-Blue was from Bio-Rad (Hertfordshire, UK). Peroxidase-labeled rabbit antihorse IgG antibodies, *o*-phenylenediamine (OPD), and α-chloro naphtol were from Sigma Chemical Co. (St. Louis, MO). All other reagents were of AR grade. Laboratorio de Biológicos Alfredo Gutiérrez (Buenos Aires, Argentina) provided normal equine plasma, and Instituto Nacional de Microbiología Carlos G. Malbrán (Buenos Aires, Argentina) supplied plasma from horses immunized with *Bothrops spp*. venom.

Methods

Aqueous Two-Phase Systems Preparation

ATPS were built from concentrated polymer solutions (PEG 600, 100%; PEG 1000, 80%; PEG 1500, 50%; PEG 3350, 40%; PEG 6000, 25%; Dx T-500, 20%; Reppal PES-100, 20%; Methocel E-15, 5%; PVP K-12 and K-25, 25%) and/or salts (phosphate, 40 wt.-%; ammonium sulfate, saturated solution, potassium sulfate, 10 wt.-%; magnesium sulfate, 23 wt.-%; sodium citrate, 20 wt.-%). The appropriate stock solutions, distilled water, and protein sample (e.g., plasma) were added into graduate tubes in the amount required to form a 10-g system.The tubes were sealed and mixed in an overhead shaker (60 min, 25°C). Phase separation was speeded up by centrifugation (4000 rpm, 5 min). The final protein content was typically around 1 g protein/L of system. In all cases, the volume phase ratio was approximately 1, and the amount (in g) of water to be added to 1 g of ATPS to transform it into a monophasic one was between 0.05 and 0.15. These values correspond to tie-line lengths in the range of 12–17%, under the experimental conditions here reported. This particular method for standardization—which accounts for the degree of robustness of a certain system—helped us to prevent protein precipitation at the interphase, even at higher protein loads (up to 2.5 g protein/liter ATPS). The upper and bottom phases were transferred to clean tubes with a Pasteur pipet for subsequent analysis. When precipitated material was noticed at the interphase, it was also saved and—after dissolution with distilled water—analyzed in order to account for the protein balance in the system. The apparent pH of the upper phase was recorded using a semimicro glass combination electrode (Cole-Palmer, Vernon Hills, IL).

Triphasic systems were also obtained by adding 5–10 wt.-% of a solid affinity matrix to the ATPS in order to capture immunoglobulins. These systems were prepared and mixed as previously described, but afterwards were placed in a water

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bath at 25°C for additional 120 min order to allow phase separation and protein binding. Phases were analyzed as usual. The affinity matrix was recovered in small chromatographic columns and washed. The bound protein (if any) was eluted—under selected experimental conditions for thiophilic or IMAC adsorbents (refer to the following text)—and also subjected to analysis.

Equine Plasma Immunoglobulin Fraction Preparation

Protein from 5 mL of equine hyperimmune plasma was precipitated by the addition of 5 mL of saturated ammonium sulfate solution (pH 7.2). Precipitated proteins were collected by centrifugation (30 min 3000 g, 4°C), dissolved in 10 mL of distilled water and the solvent changed to 20 m*M* Tris–HCl, pH 8.0, 28 m*M* NaCl in PD-10 columns (2.5 mL/column). The effluent of each column was directly loaded on a DEAE-Affi-Blue column (10-mL bed volume). The immunoglobulin that passed through was recovered in the column wash (20 mL) and lyophilized. Nonimmunoglobulin contaminants retained in the column were then washed away by a regeneration procedure using a 1 *M* NaOH/2 *M* NaCl solution. Normal horse IgG for binding experiments was obtained according to Steinbuch and Audran (18).

Synthesis and Characterization of Group-Specific Adsorbents

T-gels (divinyl sulfone-activated Sepharose CL 4B modified with β-mercaptoethanol) were synthesized as described by Hermanson et al. (19) and IMAC (immobilized metal ion affinity chromatography) gels as per Sundberg and Porath (20), using Sepharose CL 4B and/or Sephacryl HR-300 as support matrices. Affinity adsorbents always partitioned to the upper phase.

The maximum capacity (q_m) and the dissociation constant (K_D) for equine IgG were calculated from the equilibrium adsorption isotherms, as described by Chase (21): In 50-mL Erlenmeyer flasks, 9.0 mL of equine IgG at various concentrations (0.01, 0.05, 0.1, 0.25, 0.5, 2.5 mg/mL in the adsorption buffer) and 1 mL of chromatographic matrix slurry (1:1 in the same buffer) were added. The flasks were gently shaken for 24 h at 20°C, and IgG concentration in the supernatants was determined spectrophotometrically at 280 nm ($\varepsilon = 12.8$). The isotherm was plotted and parameters were calculated by nonlinear regression. The adsorption buffer for thiophilic matrices was 100 m*M* Tris–HCl, 0.75 *M* ammonium sulfate, pH 8.0, whereas a 25 m*M* sodium phosphate/0.2 *M* NaCl buffer (pH 7.0) was used for IMAC adsorbents.

To assess the selectivity of the synthesized absorbents, open columns of 2-mL bed volume were utilized. After loading a 0.2-mL sample in the adsorption buffer, the column was washed with 4 vol. of the same buffer. Bound proteins were

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eluted with 2 vol. of 100 m*M* Tris–HCl buffer, pH 8.0 (thiophilic adsorbents), or a 100 m*M* sodium acetate buffer, pH 3.0, 0.2 *M* NaCl (IMAC adsorbents). Total protein and immunoreactivity were determined in the fractions collected.

The effect of the phase-forming polymers on immunoglobulin binding onto affinity supports was studied in a low-medium pressure chromatographic system (house assembled), consisting of a peristaltic pump (Pharmacia, Uppsala, Sweden), a glass column (9-mL bed volume, Sigma, St. Louis, MO), and a UV monitor (Pharmacia, Uppsala, Sweden). Fractions (1 mL) were collected for further analysis. The system was run at a constant flow rate (1 mL/min). Experiments were performed with an adsorption buffer (as above) or polymer-containing buffers at a 1% concentration.

Analytical Techniques

Total protein was determined by the BCA method (Pierce, Rockford, IL), according to the manufacturer's instructions. Sulfate determination was carried out according to the recommendations of the "Standard Methods for the examination of water and wastewater" (21).

SDS-PAGE was carried out in a Bio-Rad Mini Protean II (Bio-Rad, Hercules, CA) according to Laemmli (23). Gels were stained with Coomassie Blue by the standard method. In those gels where proteins were electroblotted onto nitrocellulose membranes, bands were revealed with antiequine IgG serum (rabbit) conjugated with peroxidase. Enzyme activity on the bands was evidenced with α-chloro naphtol as per the instructions of Sigma Immunochemicals (St Louis, MO).

Solid-phase ELISA was utilized for immunoreactivity assessment in different samples (24). Microplates (Dako, Roskilde, Denmark) were coated with whole *Bothrops spp*. venom (0.5 μg/well). The 100-μL samples (diluted 1:40 in PBS-1% BSA) were incubated with the antigen for 1 h at room temperature. After four washings with PBS-0.1% Tween, 100 μL of peroxidase-conjugated rabbit antiequine immunoglobulin fraction (1:15000) was added. Washing was repeated with PBS-Tween (4 times), and peroxidase activity was revealed by the addition of 100 μL of OPD (1 mg/mL in sodium citrate buffer, pH 5.5)—hydrogen peroxide (10 μL of a 30 vol. solution/mL). The reaction was allowed to proceed for 15 min and stopped by adding 50 μL of 2 *M* sulfuric acid (0.5% sodium sulfate added). The absorbance at 492 nm was measured with a microplate reader MR 5000 (Dynatech, Denkendorf, Germany). Antibody titres were determined as the reciprocal of the highest dilution that causes an absorbance over 0.100 under the assay conditions, to avoid the influence of nonspecificd reactions.

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RESULTS AND DISCUSSION

Selection of the Aqueous Two-Phase System

Preliminary experiments were designed to choose between the different available ATPS with regard to protein solubility and partition behavior. In doing so, the partition behavior of equine plasma components in PEG 1000/salt, PEG 6000/Reppal PES 100 and Methocel/Reppal PES 100 was studied. Equivalent systems, i.e., those having the same volume phase ratio and same tie-line length, were employed. Table 1 shows results obtained after measurement of the (apparent) partition coefficient and visual inspection for precipitate formation. Calculated (theoretical) yields (Y%) are also presented for comparison with actual (experimental) recoveries (R%). In PEG/salt systems, a strong influence of the salt on the protein partition coefficient was evident. PEG/phosphate and PEG/ammonium sulfate systems were the most effective for immunoglobulin extraction into the top phase and also the most selective. Albertsson (7) also observed increased solubility for IgG in selected PEG 1000/ammonium sulfate systems, and several authors

Table 1. Partition Behavior of Equine Plasma and Its Immunoglobulin Fraction in Different Aqueous Two-Phase Systems

		IgG			Protein			
System	pH	K_{IgG}	Y%	R%	K_{TEP}	Y%	R%	Precipitate
PEG 1000/ phosphate	7.28	6.1	85.5	84.8	2.1	67.6	66.1	–
PEG 1000/ magnesium sulfate	7.40	0.72	41.8	23.7	0.10	9.1	8.8	+
PEG 1000/ ammonium sulfate	7.35	8.7	90.1	90.0	1.3	55.6	54.9	–
PEG 1000/ sodium citrate	7.40	0.45	31.1	0.60	0.35	25.9	18.3	++
PEG 6000/ Reppal PES-100	7.38	0.52	34.2	33.9	0.59	37.2	37.1	–
Methocel E-15/ Reppal PES-100	7.40	0.47	32.1	31.8	0.57	36.4	36.2	–

K_{IgG} and K_{TEP} are the partition coefficients for IgG and total equine plasma protein respectively. In all ATPS the tie-line length parameter was 17%. K is the apparent partition coefficient. Y (as percent) is the theoretical yield calculated from the K values and assuming a top-to-bottom phase volume ratio equal to one. R is the experimental recovery (as percentage of protein or IgG load). pH was measured in the top phase.

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Table 2. Influence of the Tie-line Length on the Partition Behavior of Equine Plasma and Its Immunoglobulin Fraction in PEG 1000/Phosphate Aqueous Two-Phase Systems

	SYSTEM		IgG			Protein			
Number	TLL	pH	K_{IgG}	Y%	R%	K_{TEP}	Y%	R%	Precipitate
1	12–14	7.21	7.2	87.7	87.5	1.2	53.8	54.1	−
2	17	7.28	6.1	85.5	84.8	2.1	67.5	66.1	−
3	21	7.20	2.9	74.6	44.2	3.7	78.7	59.0	+
4	27	7.24	2.6	72.5	12.7	2.4	70.9	38.9	++

TLL is the tie-line length parameter, expressed as %. K_{IgG} and K_{TEP} are the partition coefficients for IgG and total equine plasma protein respectively. K is the apparent partition coefficient, Y% is the theoretical yield and R% is the experimental recovery. pH was measured in the top phase.

proposed the use of PEG 1500/phosphate systems for monoclonal antibody purification (25,26). On the other hand, when other phase-forming salts were used, the plasma proteins as a whole preferred the bottom phase, thus precluding an effective extraction. In addition, precipitation phenomena at the interphase were evident in PEG 1000/magnesium sulphate and PEG 1000/sodium citrate systems. This effect was also observed in PEG/phosphate systems when the tie-line length was raised over 17%. As a consequence, the modification of this operational parameter was restricted to low values; otherwise, it would have been expected to promote extensive protein precipitation (and product loss) at the interphase. Table 2 shows the influence of the tie-line-length on normal equine plasma protein partition in PEG 1000/phosphate systems. Although the shortest TLL assayed (12–17%) produced the best result as regards differential partition, this is not as good as that obtained with the PEG/ammonium sulphate system. At long tie-line lengths (21 and 27%), a gross protein precipitation at the interphase occurred, and the partition coefficient became distorted.

In polymer/polymer systems (Table 1) the increase in tie-line lengths up to 21% did not result in protein precipitation at the interphase, and the partition coefficient did not increase either. At longer TLL, precipitation was noticed, and the addition of betaine (50 mmol/kg) helped to prevent such phenomenon. This type of zwitterionic agent is known to inhibit IgG aggregation, making this protein less susceptible to precipitation by polymers like PEG. A similar result was described by Hansson et al. (27), who encouraged the use of 100 mM glycine at pH 9 in order to improve IgG, IgA, and IgM solubility during liquid-liquid partition chromatography in PEG/dextran systems. However, the use of precipitation inhibitors did not improve the separation between IgG and its contaminants in equine plasma. Results obtained, taken as a whole, allowed to select the PEG/ammonium sulphate ATPS for further experimentation.

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Partition of Equine Plasma Proteins in PEG/Ammonium Sulphate ATPS

Plasma (pool from five batches) from horses immunized with *Bothrops spp*. venom was used. The protein mixture was fractionated by DEAE Affi-Blue chromatography, the immunoglobulins were isolated, and they were subsequently tested in ATPS. For comparison, partition experiments were also performed with total (unfractionated) hyperimmune plasma.

Modifications in the molecular weight of the phase-forming polymers were early recognized as a key parameter affecting protein partition in ATPS (7). Figure 1 shows the effect of PEG molecular weight on the (apparent) partition coefficients of the equine plasma proteins at pH 7.3. A similar trend for both immunoglobulin fraction and total plasma samples was clearly observed: K_{IGs} dropped from 6.1 to 0.10 and K_{TPP} from 1.3 to <0.10 when the PEG molecular weight increased from 600 to 6000. In other words, in this case the partition coefficient depends on PEG molecular weight irrespective of the protein class considered, thus meaning lack of selectivity. However, the addition of PVP K-12 to the above systems modified protein partition behavior, opening the possibility to achieve a differential partition at PEG molecular weights around 1000–3000 (refer to following discussion).

In order to keep contaminants in the bottom phase and taking the previous results into account, PEG 1500 was used for further experimentation. The pH of PEG/salt systems has also proven to influence protein partition behavior, but can also impair protein solubility (28). Figure 2 shows the effect of the system pH on

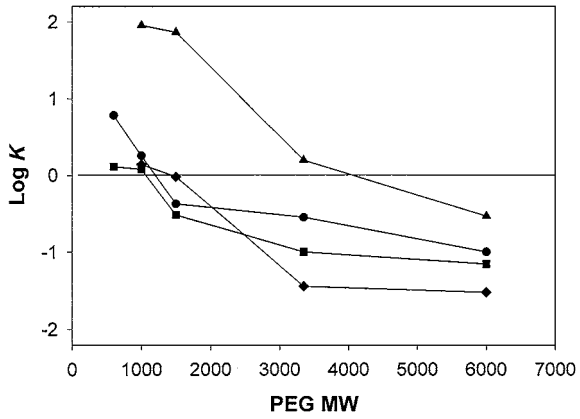

Figure 1. Effect of the PEG molecular weight on the partition of immunoglobulin and total protein from equine plasma in a PEG/ammonium sulphate ATPS at pH 7.3, in the presence and absence of 5 wt.-% PVP K-12. Immunoglobulin, ATPS with PVP (▲) and without PVP (●). Total protein, ATPS with PVP (◆) and without PVP (■).

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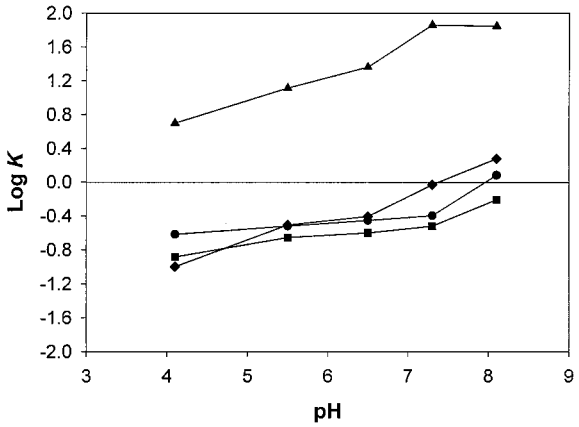

Figure 2. Effect of the system pH on the partition of immunoglobulin and total protein from equine plasma in a PEG 1500/ammonium sulfate ATPS, in the presence and absence of 5 wt.-% PVP K-12. Immunoglobulin, ATPS with PVP (▲) and without PVP (●). Total protein, ATPS with PVP (◆) and without PVP (■).

equine plasma protein partition in the PEG 1500/ammonium sulfate system. A pH shift from 4.2 to 8.1 brought about an increase in protein partition coefficients: 0.24 to 2.2 for the immunoglobulin fraction and 0.13 to 0.81 for total protein. It was not until alkaline pH values were reached that some extent of differential partition was achieved. Despite the low protein concentration (1 mg/g system) and the short tie-line length (14%) used, protein precipitation at the interphase was observed at the working pHs, except for pH 5.5 and 8.1. These results partly agree with those of Andrews et al. (26) for monoclonal antibody purification in ATPS; they found less IgG precipitation in PEG/phosphate systems at pH 5 in comparison with pH 7 or 9. The beneficial effect of working with ATPS at pH 9 on IgA and IgM solubility has also been described (29).

Additive Effect on Partition

It is now accepted that surface hydrophobicity is the main force driving macromolecules to top phase in PEG/salt aqueous two-phase systems (30). Charge effects are less relevant (31). In this way, salt addition to ATPS can provide—by salting-out like mechanisms—an important tool for obtention of protein selective partition (32). However, due to solubility limitations, in this case we preferred to study the effect of additives on immunoglobulin partition in selected systems. Following this approach, Müller (29) reported an improvement in the solubility of immunoglobulins by PVP addition to PEG/dextran systems, and Miranda and

Cascone (33) reported the PVP effect on peroxidase partitioning in PEG/salt systems.

As mentioned above, Figure 1 shows the effect of PVP K-12 addition (5 wt.-%) to systems composed of PEG (molecular weight between 600 and 6000) and ammonium sulfate, at pH 7.3. Although an important increase in K_{IGs} was observed, the polymer added only brought about a slight rise in K_{TPP}; this is probably due to the contribution of the immunoglobulin mass. However, it should be noted that only PEG 1500-containing systems showed a good resolutive capacity, where immunoglobulin mainly partitioned to the top phase ($K_{IGs} \geq 70$) whereas the whole plasma proteins preferred the bottom phase ($K_{TPP} \approx 0.9$). On the other hand, it was observed that in PEG 600 or PEG 1000-containing ATPS there was a general partition trend to the top phase, and that in PEG 3350 to 6000-containing systems all proteins preferred the bottom phase. Therefore, it was not possible to obtain an acceptable separation degree between immunoglobulins and the other proteins in ATPS containing such PEGs.

Further modification of system pH (between 4.2 and 8.0) in PEG 1500/ammonium sulphate containing PVP K-12 (5 wt.-%) caused an increase in the partition constant of both immunoglobulin and total plasma proteins (from <0.1 to 1.9 for total plasma protein and from 5.0 to 71 for immunoglobulin). No definite selectivity was achieved in these experiments, and thus pH was regarded as an irrelevant variable toward immunoglobulin purification, even in the presence of PVP (Fig. 2). Changes observed in K_{IGs} in the presence of PVP may reflect, at least, a better immunoglobulin solubilization in the upper phase. In a previous work (33) it was observed that PVP partitionates preferentially to the top phase in PEG/salt systems and, therefore, an affinitylike mechanism should also be considered to explain these results.

In order to optimize the required amount of PVP K-12, a concentration between 1 and 50 wt.-% of this polymer was added to a PEG 1500/ammonium sulfate ATPS at pH 7.3. Figure 3 shows that PVP concentrations up to 10 wt.-% improve the separation whereas higher concentrations have a deleterious effect on selectivity. This fact could be explained by taking into account that, at low concentrations, PVP behaves as an additive enhancing immunoglobulin solubility and partition constant, but at higher concentrations it becomes a phase-forming polymer thus altering the properties of the original ATPS.

Despite the improvements previously described for PEG/salt systems with regards to hyperimmune plasma fractionation, low reproducibility of the process as a whole was later observed. This was due to erratic product losses (measured as immune reactivity) during the procedure. The need to keep tie-line length within a narrow range, to avoid protein precipitation at higher system load, impaired process robustness and made it difficult to control. Consumption of polymers was also high, thus bringing about a significant increase in the process costs. Summarizing, the disadvantages associated with this one-step extraction prompted us to evaluate

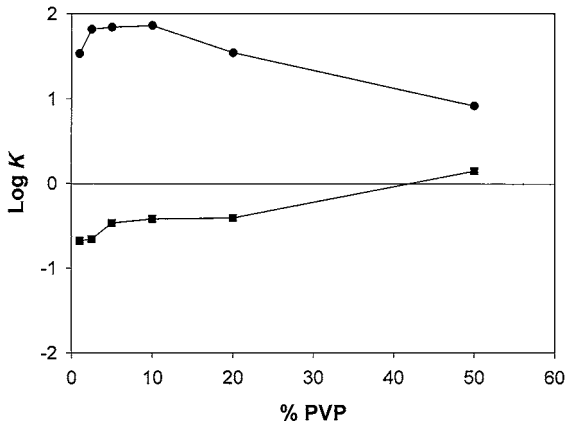

Figure 3. Effect of the PVP K-12 concentration on the partition of immunoglobulin and total protein from equine plasma in a PEG/ammonium sulfate ATPS at pH 7.3. Immunoglobulin (●); total protein (■).

alternative polymer-polymer ATPS for immunoglobulin recovery. However, the use of these less-selective systems requires the introduction of an affinity ligand (either as a modified soluble polymer or as a solid phase) in order to shift the target protein to the desired phase. In the following section this further development is presented.

Immunoglobulin Recovery by Affinity Adsorption in ATPS

Product capture with an affinity-modified solid phase in ATPS has been proposed to increase selectivity in aqueous two-phase systems. Kondo et al. (34) proposed the purification of antibodies and fusion proteins with (immuno) affinity nonporous latex microspheres. Thse authors were also able to show the successful integration between target protein adsorption and partition in PEG/phosphate systems. Furthermore, Mattiasson and Ling (14) developed a purification scheme for dehydrogenases based on a similar principle but using triazine dye-modified Sepharose beads in PEG/dextran systems. In both cases, the solid phase was selected so that it partitioned to the upper phase and the system environment was designed in order to prevent possible interference with the protein-ligand binding mechanism. Positively charged submicron-sized polymeric particles were used by Kim et al. (35) to increase purification efficiency and simplify the recovery process of β-galactosidase during liquid–liquid extraction.

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Affinity Ligands and Solid Phases

A family of structurally related thiophilic ligands (17) and IMAC ligands were immobilized onto well-known carbohydrate backbone matrices. As phase-forming polymers could interfere with IgG binding to the immobilized ligand (25), their effect on equine immunoglobulin binding to T-gel was assessed. Figure 4 shows the elution profile observed when an equine immunoglobulin sample was chromatographed in a T-Gel column. When 1% of PEG 6000, Reppal PES-100 or PVP K-12 was included in the adsorption buffer, the chromatographic behavior remained unchanged in comparison with that of a standard buffer run, thus indicating that PEG and Reppal did not interfere (at least at a 1% concentration) with the binding mechanism of the equine immunoglobulin fraction to the thiophilic ligand. PVP K-12, in contrast, was able to inhibit to some extent the binding of immunoglobulin to the T-ligand. The effects observed for PVP may be related to its influence on the relative hydrophobic character of an aqueous medium and to its well-known complex-forming properties (30).

In order to compare the performance of various thiophilic and immobilized-metal adsorbents, an open-column screening trial with T-gel, NT-gel, ST-gel, IDA-Cu^{2+} gel, and IDA-Zn^{2+} gel was performed, employing hyperimmune equine plasma as the sample. Table 3 shows the corresponding results, whereas Figure 5

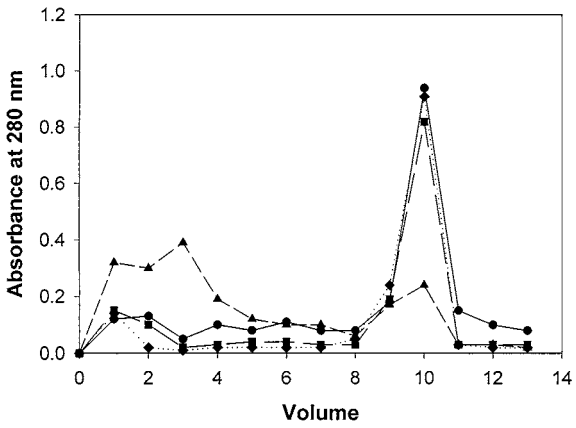

Figure 4. Effect of various phase-forming polymers on the elution pattern of an immunoglobulin sample chromatographed in a T-Gel column. 1% PEG 6000 (●) or Reppal PES 100 (■) or PVP K-12 (▲) were added to 100 m*M* Tris-HCl, 0.75 *M* ammonium sulfate, pH 8.0, (adsorption buffer), and 1 mg immunoglobulin was loaded onto a T-Gel column (9-mL bed volume). 1 mL fractions were collected at a flow rate of 1 mL/min and the absorbance of the eluent was monitored at 280 nm. (◆) Adsorption buffer without polymer addition.

Table 3. Comparative Performance of Pseudobioaffinity Matrices in the Capture of the Immunoreactive Fraction from Hyperimmune Equine Plasma

Affinity Matrix	Protein Recovery (%)	Activity Recovery (%)	Purification Factor	K_D (mol/l)	q_m (mg/ml)
T-gel	23.0	52	2.3	1.4×10^{-5}	30
NT-gel	8.0	38	4.7	5.0×10^{-5}	24
ST-gel	14.1	57	4.1	1.1×10^{-7}	38
IDA-Cu^{2+}	7.5	22	2.9	4.2×10^{-6}	45
IDA-Zn^{2+}	2.6	7	2.8	ND	ND

Ligand chemical structure is shown in Figure 5. The T-gel, the NT-gel, and the ST-gel are thiophilic adsorbents; IDA: iminodiacetic acid with immobilized metal ion; ND: not determined; K_D is the dissociation constant; and q_m is the maximum capacity calculated from batch experiments (isotherms) employing purified horse IgG.

shows the chemical structures of the ligand moieties employed. The three tiophilic adsorbents displayed a good selectivity toward horse antibodies, yielding purified preparations free from the two major contaminants albumin and transferrin, as judged by SDS-PAGE analysis (data not shown). However, some contaminants could be still observed after T-gel chromatography, a finding that agrees with the low purification factor. On the other hand, the NT-gel and the ST-gel were found to be more selective. Moreover, the ST-gel combines the two positive properties of selectivity (PF 4.1 out of a maximum of about 4.8 attainable) and good recovery (57% expressed as immunoreactivity). The two IMAC adsorbents differed in their selectivity for equine plasma proteins. IDA-Cu^{2+} showed moderate recovery of immunoreactive material, but the immunoglobulin recovered was extensively contaminated, especially with albumin. IDA-Zn^{2+} presented more selectivity, a fact that was not reflected by the calculated purification factor, probably due to interference in low-level protein quantification, but clearly seen after electroforetic analysis (data not shown). The latter, however, displayed a very low recovery. The low capacity usually observed in IMAC supports can be explained by taking into account a possibly different binding behavior among different immunoglobulin subclasses.

The adsorbents were also compared according to some of their thermodynamic properties. Maximum capacity (q_m) and dissociation constant (K_D) were calculated from equilibrium adsorption isotherms using purified normal horse IgG for each of the affinity supports under investigation (Table 3). All curves displayed the typical hyperbolic shape of equilibrium adsorption isotherms and allowed the calculation of the above parameters. Results obtained confirmed that the performance of ST-gel is the best, as it has good capacity and high affinity to IgG in

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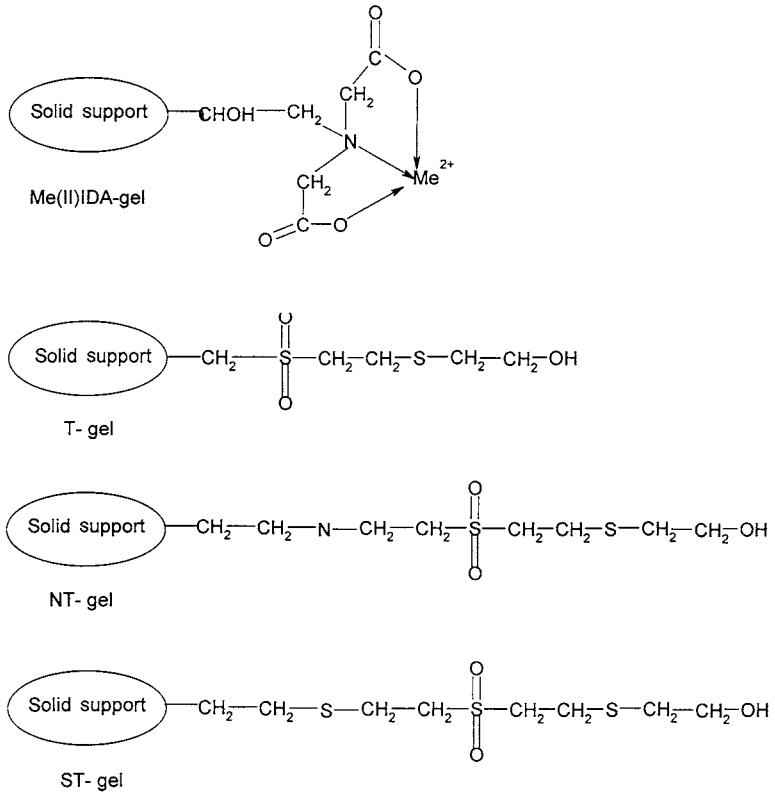

Figure 5. Chemical structures of the ligands employed in this work.

comparison to that of the other thiophilic ligands studied. This is most probably due to the presence of an additional thioether group in the ST-gel (17). On the other hand, IDA-Cu^{2+} showed good parameter values but they did not translate in adequate actual performance due to poor selectivity to IgG in the frame of the plasma protein mixture.

Affinity Triphasic Systems

To extend our observations under the proposed process scenario, typical adsorbents representing both families were assessed on the basis of their binding capacity to the equine immunoglobulin fraction in various types of aqueous two-phase systems. Table 4 shows the distribution of immunoglobulins in the top phase, bottom phase, or solid phase in PEG 600/ammonium sulfate, PEG 4000/Reppal

Table 4. Adsorption of Equine Immunoglobulin Fraction to ST-gel or IDA-Cu^{2+} in Various Aqueous Two-Phase Systems

	PEG 600/AS			PEG 4000/Reppal			Methocel/Reppal		
Matrix	Top Phase (%)	Bottom Phase (%)	Solid Phase (%)	Top Phase (%)	Bottom Phase (%)	Solid Phase (%)	Top Phase (%)	Bottom Phase (%)	Solid Phase (%)
None	$K = 27$–100			$K = 0.78$–0.84			$K = 0.42$–0.54		
ST-gel	72	0.1	0.1	21	33	25	10	34	51
IDA-Cu^{2+}	8.3	0.1	68	24	32	20	8.3	18	66

PEG: poly(ethyleneglycol); AS: ammonium sulfate; Methocel, E-15; Reppal, PES-100.

(containing ammonium sulfate) and Methocel/Reppal (containing ammonium sulfate) ATPS, where the solid phase was ST-gel or IDA-Cu^{2+} Sepharose. In the PEG/salt system only IDA-Cu^{2+} could bind immunoglobulin efficiently. As in this case, the adsorbents partitioned to the top phase; this effect could be attributed to this particular environment. It is well documented that the affinity of IgG to a thiophillic ligand is a strong function of the ammonium sulfate concentration in the liquid phase. In our particular case (upper phase from ATPS), the salt concentration may be well insufficient for protein binding. This is due to the typical phase diagrams of PEG/salt systems, where the salt is found predominantly in the bottom phase. Thus, the sulfate concentration in the PEG-enriched (top) phase was measured. Values in the range 0.44–0.51 *M* were found, which are in the lower recommended sulfate concentrations for protein adsorption to the T-Gel (i.e., 0.5–0.8 *M*). This situation may impair immunoglobulin adsorption to this particular thiophilic ligand. However, those values are well in the range required for ligands with higher affinity like the ST-Gel (i.e., 0.3–0.6 *M*). It can be concluded that, at least in the case of the ligands with a lower dissociation constant, a high PEG concentration seems to specifically inhibit immunoglobulin binding (Table 3). These results help to explain the previous observation of Sulk et al. (25) during monoclonal antibody purification, where the ATPS top phase was found to interfere with product binding to a thiophilic matrix. On the other hand, in PEG/Reppal systems, both chromatographic matrices captured a 20–25% of immunoglobulin whereas in Methocel/Reppal systems product recovery was higher (51% recovery for the thiophilic support and 66% for the IMAC support). Irrespective of the previous considerations, ATPS without PEG were found to provide an adequate environment for product capture by salt-promoted adsorption onto solid supports.

As it can be expected from the above results, when equine hyperimmune plasma was fed in a Methocel/Reppal system, the immunoglobulin fraction

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recovered from IDA-Cu^{2+} was strongly contaminated with several plasma proteins—mainly albumin and transferrin—as revealed by SDS-PAGE analysis (not shown). Under the same conditions, ST-gel yielded a highly pure (>90%) immunoglobulin preparation. Despite the known disadvantages of cellulose-derived polymers (e.g., high viscosity and low solubility at high ion strength), Methocel industrial utilization is favored by the possibility of building ATPS at a low polymer concentration and due to its safety for pharmaceutical use (11). Our preliminary results have shown the possibility to reduce Methocel viscosity by radiation under specific conditions (unpublished data), yielding a polymeric product able to form ATPS with dextran or hydroxypropylstarch.

Both adsorption onto selective affinity porous supports (e.g., ST-Gel) and extraction in Methocel/Reppal ATPS have been successfully integrated in order to obtain a immunoreactive protein fraction from horse hyperimmune plasma. The process comprises the following steps: i) Contacting (2–3 h) of the 1:9 diluted equine plasma with the thiophilic matrix (10% w/v). An adsorption buffer (100 m*M* Tris–HCl, pH 8, 0.5 *M* ammonium sulfate) was used for dilution. ii) Addition of the phase-forming polymers (20% Reppal solution and 5% Methocel dispersion to reach a 2% final concentration of each component in the system) and mixing. Phase separation was allowed to occur under gravity overnight at room temperature. iii) Adsorbent beads were collected from the interphase and transferred to a cylindrical percolation device. The beads were washed with adsorption buffer and the product eluted under appropriate conditions (PBS buffer: 20 m*M* phosphate, pH 7.4, 0.85% NaCl).

The process is innocuous due to the biodegradability of the polymers involved and economical due to the easy recycling of the solid matrix. In addition, it possesses a high capacity and can be easily adapted to the production scale (14,36,37).

It must be pointed out that we did not perform in vivo neutralization studies with the active fractions obtained by the process proposed. However, it should be noted that several authors (38,39) reported a good correlation between the in vivo potency assessment and ELISA measurements.

Results herein obtained may be in principle extended—with the appropriate modifications—to the preparation of other passive vaccination sera, e.g. for the treatment of diphteria, tetanus, herpes zoster, gas gangrene, botulism, rabies, or hepatitis B.

Finally, it should be considered that results shown in this study regarding PEG/salt systems include only one-step partitioning. However, the use of interacting polymers in combination with multistep extractions (i.e., countercurrent distribution) may be envisioned as an attractive strategy for plasma fractionation (40). Two-stage extraction processes are also worthy of further investigation (41).

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