THE SPECIFIC PURIFICATION OF EQUINE DIPHTHERIA AND TETANUS ANTIBODIES FROM HYPERIMMUNE SERUM

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ABSTRACT

Antisera from horses hyperimmunized against Clostridium tetani and Corynebacterium diphtheriae toxins are purified to reduce the amount of antigenic horse specific proteins. The antisera can then be administered to humans as an immunoglobulin preparation for therapeutic use against tetanus or diphtheria infections. The antisera for human use must comply with the W.H.O. requirements pertaining to units per mg total protein in the final product. This study compared two methods of isolating tetanus and diphtheria antibodies to optimize the degree of purification so the final product had a maximum antibody potency with a minimum total protein content. For the first method, an anion exchanger, DEAE-Sephadex, was used, which separated the serum protein fractions on the basis of their charge. The immunoglobulin fraction with the smallest overall negative charge at a pH of 6,5 passed through the gel while the other serum protein fractions were bound to the anion exchanger. This resulted in a heterogenous mixture of tetanus or diphtheria antibodies with the naturally occurring equine antibodies. The second method used two different immunoadsorbent gels, CNBr-Sepharose 4B and a derivative of Spherosil, prepared with the relevant toxoids which were used to isolate those antibodies that would bind specifically to the toxoids.

The degree of purification obtained for each method was compared. The results showed that the immunoaffinity methods used to purify diphtheria antisera gave a range of mean purification factors of 2,06 to 2,35 compared with an average purification factor of 1,33 for the ion exchange method. Subsequent electrophoresis showed the immunopurified diphtheria antisera produced a more defined band of immunoglobulins when compared with diphtheria anti isolated by ion exchange.

The Spherosil immunoadsorbent purified tetanus antisera by a factor of 2,75 compared to factors of 1,01 to 1,55 for CNBr-Sepharose 4B immunoadsorbents and 1,31 for ion exchange purified tetanus antisera. Double diffusion assays confirmed that the purity of both the diphtheria and tetanus antisera was improved by the chromatography processes and also that the purified antisera contained tetanus and diphtheria antibodies identical to those present in the raw plasma.

It was concluded that affinity chromatography is a more selective *purification system suitable* for improving the purity, with respect to units/mg protein, of both diphtheria and tetanus equine antisera. However, a cost analysis and comparison of the affinity chromatography methods with the ion exchange *method does not* recommend affinity chromatography for diphtheria antisera purification on an industrial scale. Based on a comparison of the chromatographic results, there is a potential for large scale purification of tetanus antisera with the Spherosil derived immunoabsorbant.

DECLARATION

[illegible] is my own work. It is being [illegible] University of [illegible] submitted for any [illegible]

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I wish to dedicate this dissertation to my parents, Brian and Phyllis Burt, for their support, encouragement and love.

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LIST OF ABBREVIATIONS

A.N.O.V.A.	analysis of variance
F.T.	flocculation toxin
I.A.U. or units or u	International Antibody Units
IgG	immunoglobulin G
Lf	Limes flocculationus (measure of titre of toxoids)
P.F.	purification factor
P.N.	protein nitrogen
S.A.I.M.R.	South African Institute for Medical Research
S.D.	standard deviation
W.H.O.	World Health Organisation

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1. INTRODUCTION

1.1 THERAPEUTIC EQUINE ANTISERA

Purified antisera from hyperimmunised horses is commonly used for therapeutic purposes against Clostridium tetani and *Corynebacterium diphtheriae infections* in humans. The exotoxins produced by these bacterial organisms are neutralised by passive immunisation with horse tetanus or diphtheria antibodies.

Infections of Cl.tetani and C.diphtheriae can be treated with antibiotics as the bacteriocidal agent and the relevant isolated immunoglobulin preparation to neutralise the exotoxins. Human antitetanus immunoglobulins purified using cold ethanol fractionation are available and are used in preference to equine antitetanus. These preparations contain only human proteins and are safer to use than equine immunoglobulins. There is minimal risk of anaphylaxis, serum sickness or sensitising the recipient which could cause hypersensitive reactions if a further injection is required. Diphtheria or tetanus antitoxins are usually administered therapeutically to patients who have developed diphtheria or tetanus. They may sometimes be used prophylactically if the patient has been exposed to possible infection and has no previous history of active immunisation with the relevant vaccine. Passive immunisation with the immunoglobulin preparation is important for therapeutic purposes as it avoids the latent period required for an active immune response to vaccination.

The advantage of using the horse as a source of antibodies is that large quantities of antisera can be obtained after immunisation of the animal. However there are disadvantages in using equine antisera, as the serum proteins are antigenic when administered to humans. The risk of serum reactions are reduced when purified antitoxins are used. Serum sickness may occur 7 to 14 days after the injection. It is characterised by itching rashes, a rise in temperature and joint pains. It can be treated with antihistamines or corticosteriods. Acute serum reaction, or anaphylaxis, is rare but may occur and should be treated with adrenalin. The horse serum must be purified to reduce the amount of foreign protein being administerred to the patient which will reduce the risk of adverse side effects. The purified antisera also permits the administration of a large amount of antibodies in a small volume and is more stable than whole serum during long-term storage.

1.2 PURIFICATION OF EQUINE IMMUNOGLOBULINS

The horses are immunised against diphtheria or tetanus toxins by administering increasing dosages of diphtheria or tetanus toxoid. The subsequent production of tetanus and diphtheria antibodies is monitored by testing blood samples for antibody titre. When the horses have been immunised, they are bled and the plasma obtained by plasmaphoresis is purified in the Serum and Vaccine Department of the S.A.I.M.R.

The first step in the purification of antibodies from hyperimmune horse serum is the enzymatic digestion of the immunoglobuline followed by salting out of those immunoglobulins with ammonium sulphate and dialysis of the precipitate.

The method used is a modified method of that described by Pope (1939a), Pope (1939b) and Pope and Stevens (1951). The immunoglobulins are digested by pepsin. The pepsin acts on the IgG molecule by degrading the heavy chains. Degradation begins at the carboxyl terminal end and proceeds to the region of the interchain disulphide bridge. The Fc portion is destroyed by splitting it into small peptides. Pepsin digestion leaves a *fragment that possesses* two antigen binding sites and therefore can still precipitate antigen. This fragment is the portion of the antibody molecule that is required for toxin neutralisation and is called the $F(ab')_2$ *fragment. The* $F(ab')_2$ *fragment has* lost most of the specific antigenic determinants of IgG since most of these are located in the carboxyl-terminal half of the heavy chain, or the Fc fragment (Heimer et al. 1967; Bernier 1978; Nisonoff 1982). The non-antitoxic portion of the IgG is easily denatured in an acid solution whereas the antitoxic portion is far less readily denatured. After pepsin digestion, any further enzyme action is stopped by increasing the pH of the plasma above the optimal pH required for enzyme activity. The plasma is then heated to a temperature at which only the non-antitoxic portion is denatured and precipitated. Lipids are *removed by adding toluene to the plasma* before heating. The lipids are precipitated out with the coagulated protein fraction when the plasma is heated (Pope 1939b). The antitoxin remaining in solution is then salted out with additional ammonium sulphate. The salting out fractionation will separate a heterogenous mixture of gammaglobulins. Albumin is discarded in the final filtrate in which it is soluble. and fibrinogen is discarded in the first precipitate. The final fraction obtained will contain trace amounts of all the serum proteins (Haurowitz 1977; Scopes 1982).

The diphtheria and tetanus antibody titres are measured in International Antibody Units per ml (I.A.U./ml or units/ml).

The forementioned purification step gives a recovery yield of approximately 90 % with respect to diphtheria or tetanus antibody units and a purification factor of approximately 2 with respect to units/mg protein.

The risk of adverse serum reactions in humans from equine antisera is reduced by using purified antisera. The enzymatic digestion of the immunoglobulins followed by heat denaturation and subsequent salt fractionation of the serum proteins reduces the amount of species specific protein in the final product.

1.3 OBJECTIVES

The objective of the present study is to isolate the immunoglobulins that will bind specifically to diphtheria or tetanus exotoxins, from the heterogeneous mixture of immunoglobulins present in the equine plasma. The result of this will be to produce a safer product by eliminatating the naturally occurring antibodies in the antisera. This in turn will reduce the amount of species specific protein present and will improve the purification with respect to units/mg protein present in the final product. The final product has to comply with the requirements specified by the World Health Organisation pertaining to units/ml protein present in an immunoglobulin preparation for human use. By improving the purification factor, horse plasma that has an antibody level of less than 450 units/ml (diphtheria or tetanus) after immunination may be purified to the required potency. With the present method of ion exchange purification this antisera will not reach W.H.O. requirements and therefore cannot be used.

It is hoped that a selective purification system can be developed that does not affect the biological activity of the final product and ensures a high yield. The laboratory scale processes will be theoretically scaled up to industrial scale processing, taking into account the raw material to be processed, the amount and frequency of the product required and the regeneration of the adsorbant for repeated usage. The feasibility of using affinity chromatography on the commercial scale for the production of antisera will depend largely on the cost of the process in the long term compared with the present method of ion exchange chromatography.

Purification of the antitoxin was investigated in the present study using chromatographic techniques. Methods of ion exchange chromatography and affinity chromatography were used to purify the pepsin treated equine antisera. The four chromatographic matrices that were employed were diethyl amino ethyl Sephadex (DEAE-Sephadex), cyanogen bromide activated Sepharose 4B (CNBr-Sepharose 4B), 6-aminohexyl Sepharose 4B (AH-Sepharose 4B) and a derivative of porous silica beads. The methods of immunoaffinity chromatography that were used have all been described by previous workers. Immunoadsorbents have not been previously used in our laboratories to purify equine antisera as ion exchange chromatography purifies high titred plasma to the required specifications. With the accumulation of low titred plasma it would be advantageous to have a method that will increase the purification factor. The selective purification should also produce a product with a lower risk of serum sickness and anaphylaxis when administered.

After preparing suitable affinity gels, the optimal coupling and elution conditions were determined. The antibody concentration

was measured using flocculation tests (Hamon 1922) to find the potency of each antisera and protein determinations (Peters et al., 1976) to find the degree of purification. The diphtheria antisera samples were also analysed using skin tests to determine the potency and neutralising capacity of the antisera (British Pharmacopeia 1980). The isolated immunoglobulins were compared qualitatively using electrophoretic techniques.

1.4 CHROMATOGRAPHIC PURIFICATION

1.4.1 ION EXCHANGE CHROMATOGRAPHY

Proteins can be separated by ion exchange chromatography on the basis of the charge. The chromatographic resin is a charged insoluble matrix that will reversibly bind proteins by electrostatic bonds if the proteins have a charge opposite to that of the ion exchanger groups. These proteins can be displaced by increasing the ionic strength of the eluant. The displacement is dependent on the charge of the proteins which is a function of the pH. This charge varies for different protein molecules depending on their isoelectric point so that the separation of proteins can be achieved. At a pH of 6,5, the serum proteins are negatively charged. The immunoglobulin fraction has a higher isoelectric point than the other serum protein fractions and will have the smallest overall negative charge at a pH of 6,5. Therefore the immunoglobulins have the lowest affinity of the serum proteins for the anoin exchanger . The anion exchanger will bind the serum proteins other than the immunoglobulins which will pass through the matrix. DEAE-Sephadex A-50 will separate a heterogeneous mixture of immunoglobulins from other serum proteins.

In this study a batch method as described by Christensen P.A. and Andersen C.G. (unpublished) was used to isolate immunoglobulins from antisera obtained from horses hyperimmunised against diphtheria and tetanus. The antisera were initially digested with pepsin and precipitated with ammonium sulphate as described in the previous paragraph. The ion-exchange purification of horse immunoglobulins was compared qualitatively and quantitatively with the specific purification of diphtheria and tetanus immunoglobulins using affinity chromatography.

Previous workers have used DEAE-Sephadex to fractionate immunoglobulins from sera. A batch method for the preparation of immunoglobulins from undialysed normal human serum using DEAE-Sephadex A-50 has been described by Baumstark et al., (1964). They mixed serum with DEAE-Sephadex and washed the serum-gel with phosphate buffer pH 6,5. The immunoglobulins were recovered in the filtrate and other serum protein components were bound to the gel and only eluted with an increase in the ionic strength of the influent buffer. They reported that optimal separation of immunoglobulins occurred at a pH of approximately 6,5.

Porper et al., (1967) modified the method, described above, for the purification of gammaglobulins from horse anti-lymphocytic serum. Undialysed horse serum was used and DEAE-Sephadex A-50 was employed as the anionic exchanger using the batch method. It was found that if the pH or the osmolarity of the buffer was varied, other serum proteins were washed through the gel and appeared with the IgG fraction. The sephadex was equilibrated with 0,01 M phosphate buffer pH 6,5. The final preparation was electrophoretically pure and structurally unaltered as shown by in vivo studies, and immunoelectrophoretic analysis.

Einstein et al. (1968) adapted the method of Baumstark et al.,(1964) to prepare immunoglobulins from human sera. The sera were pipetted onto DEAE-Sephadex A-50 packed in columns and eluted with phosphate buffer pH 7.0. After chromatographic treatment, the eluates were assayed by electrophoresis, double diffusion and immunoelectrophoresis. Only immunoglobulins were found to be present.

1.4.2 AFFINITY CHROMATOGRAPHY

The separation of certain proteins by affinity chromatography is dependent on the biological specificity of a protein-ligand interaction. The ligand is irreversibly bound to a supporting matrix and will, theoretically, retain specific proteins from a heterogeneous mixture. These proteins must then be eluted by dissociation of the protein-ligand interaction. The principle of affinity chromatography can be applied to the separation of specific antibodies from other serum proteins because of the specificity of antigen-antibody interactions (Cuatrecasas and Anfinsen, 1971; Wilchek et al., 1984).

Immunoaffinity matrices were used in this study to isolate equine antibodies that interact with diphtheria or tetanus toxoids bound to the affinity gels. Three methods of preparing immunoadsorbents for the specific purification of antibodies were used. Purified diphtheria and tetanus toxoids were used as protein ligands in the preparation of each immunoadsorbent. These toxoids were prepared from the bacterial exotoxins produced by C.diphtheriae and Cl.tetani organisms. The toxin was treated with formaldehyde which converted the toxin to a non toxic form without the loss of immunogenicity. The toxoids were subsequently purified by ammonium sulphate precipitation and ultrafiltration.

The first method that was investigated involved coupling the purified toxoids to a matrix called AH-Sepharose 4B. AH-Sepharose 4B contains 6-carbon spacer arms and free amino groups. AH-Sepharose 4B can be activated with glutaraldehyde to give a stable intermediate derivative. After the removal of excess glutaraldehyde this derivative covalently binds proteins to give immunoadsorbents with a 13-atom long spacing arm between the Sepharose and the protein (Cambiaso and Masihi et al.,1975). Cambiaso et al.,(1975) bound human IgG, I_1 and serum albumin to AH-Sepharose 4B glutaraldehyde derivatives and isolated specific antibodies against each from rabbit antisera. Van Wezel et al.,(1982) purified polio virus with immobilised antibodies coupled to the glutaraldehyde activated derivative of AH-Sepharose 4B using the method described by Cambiaso. This immunisation was applied to the preparation of immunoadsorbents using diphtheria toxoid and tetanus toxoid.

CNBr-Sepharose 4B was the second matrix that was used to prepare suitable affinity gels for purifying specific horse antibodies. The method reported by Pharmacia, Sweden for the coupling of protein ligands to CNBr-Sepharose 4B was used to couple diphtheria and tetanus toxoids to the gel. These immunoadsorbents were used for the specific isolation of equine diphtheria and tetanus antibodies from pepsin treated and ammonium sulphate precipitated antisera.

Proteins are spontaneously coupled to CNBr-Sepharose 4B via primary amino groups to form a chemically stable matrix (Axen et al.,(1967). The activation of agarose by cyanogen bromide has been described by March et al.,(1974). Agarose is a commonly used matrix for affinity chromatography because of its relative stability, biological inertness and reproducibility of the coupling capacity of activated gels.

The matrix is commercially available in an activated form. The coupling of protein ligands to CNBr-Sepharose 4B has been used by several authors for the immunopurification of specific equine antibodies (Sidorova et al.,1975; Gonyea 1977; Buchowicz et al.,1986). Sheppard et al.,(1987) purified tetanus toxin using an affinity gel derived from polyclonal equine antitoxin coupled to CNBr-Sepharose 4B. Hughes et al.,(1974) isolated tetanus toxoid from polymerised horse antitoxin immunoadsorbant columns. All authors reported that their final product was purer with respect to contaminating proteins and was biologically unaltered.

The third chromatographic gel that was used in this investigation was a derivative of porous silica beads. Spherosil silica beads have negative charges in their surface due to the acidic character of the silanol function present. DEAE dextran will bind to these charges to give a positive charge to the porous silica beads. These cationic silica beads bind proteins at a pH where the proteins are negatively charged. This layer of protein is fixed by ionic interaction with the Spherosil cationic internal surface and is then cross-linked with glutaraldehyde. This provides a stable protein layer without affecting the immunological properties of the molecule (Tayot et al.,1978). The different types of Spherosil available have varying physical properties. The ideal Spherosil with the largest pore size is the XOC005 type with a pore size of 300 nm. The pore diameter allows free access to protein layers. This type of Spherosil was used to prepare immunoadsorbents using the method described by Tardy et al.,(1978) for the preparation of tetanus toxoid immunoadsorbents and the method was adapted to prepare a diphtheria toxoid immunoadsorbent.

Tardy et al.,(1978) reported that they purified pepsin digested immunoglobulins raised in horses immunised with tetanus toxoid using the abovementioned affinity matrix.

They purified both equine tetanus antisera by a factor of 1,5 with 14% activity yield and pepsin digested equine tetanus antisera by a factor of 2,9 with 43% activity yield. The gel had a tetanus toxoid capacity of 4 200 Lf/g gel. They reported that *the Sepharose must have an average pore* size of at least 125 nm to fix tetanus toxoids in large amounts. The molecular weight of tetanus toxoid is 150 000 (Bizzini 1984) and that of diphtheria toxoid is 62 000 (Pappenheimer 1977).

Tayot et al.,(1980a, 1981) documented the same techniques for the attachment of the ganglioside G_{M1} to this Sepharose derivative and its application to cholera toxin purification. Nelyveld (1981) reported the isolation of pure antibodies by immunoaffinity chromatography on Sepharose derivatives.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

The following is a general list of chemicals used in the experiments.

Diethyl amino ethyl Sephadex A-50, (DEAE Sephadex);
Cyanogen bromide activated Sepharose 4B, (CNBr-Sepharose 4B);
Diethyl amino ethyl dextran, (DEAE-dextran);
Amino-hexyl Sepharose 4B, (AH-Sepharose 4B).

The above were obtained from Pharmacia Fine Chemicals, Uppsala, Swedan.

Spherosil XOC005 obtained from Rhone Poulenc Chimie Fine, Paris, France.
Paragon Electrophoresis Kit from Beckman Instruments.
Biuret Reagent from Laboratory Reagents and Diagnostics, S.A.I.M.R.
Protein Standards from Boehringer Mannheim.
Ponceau S from Merck.
Mini-trol ES Level 1 Chemistry Control from Aserical Dade.
Glutaraldehyde from Merck.
Agarose from B.D.H.
Glycine from B.D.H.

All other chemicals mentioned in the text were obtained from Merck.

2.1.2 BIOLOGICALS

Purified diphtheria toxoid (20 800 Lf/ml: protein content 64 mg/ml) and purified tetanus toxoid (14 400 Lf/ml: protein content 46 mg/ml) were supplied by the Serum and Vaccine Department, South African Institute for Medical Research.

Equine antidiphtheria and antitetanus plasma were supplied by the Serum and Vaccine Department, S.A.I.M.R.

Standard diphtheria flocculating antitoxin, a freeze dried powder reconstituted in 50 ml sterile saline (36 u/ml) was obtained from W.H.O. Copenhagen. It was used to standardise the flocculating diphtheria toxin, F.T.17.

Flocculating diphtheria toxin, F.T.17 (2) Lf/ml) was supplied by the Serum and Vaccine Department, S.A.I.M.R. It was used to standardise the flocculating diphtheria antitoxin, D.10, and to flocculate antitoxin samples.

Standard equine diphtheria flocculating antitoxin, D.10, (4 000 units/ml) was supplied by the Serum and Vaccine Department, S.A.I.M.R. It was used to flocculate toxoid samples.

Standard diphtheria antitoxin (10 units/ml) which was diluted 1 in 50 with Schick buffer for Lf/100 dose was supplied by W.H.O Copenhagen. It was used to standardise diphtheria toxin for use as a standard toxin in guinea pig skin tests.

Standard diphtheria toxin was supplied by the Serum and Vaccine Department, S.A.I.M.R. It was tested against W.H.O. standard antitoxin Lf/100 dilutions to determine the antitoxin equivalent dose of the toxin.

Standard tetanus flocculating antitoxin was supplied by W.H.O. Copenhagen. It was used to standardise the flocculating tetanus toxin, A.1.

Flocculating tetanus toxin A.1, 42 Lf/ml, was supplied by the Serum and Vaccine Department, S.A.I.M.R. It was used to standardise the flocculating tetanus antitoxin, T.34, and to flocculate the antitoxin samples.

Standard equine tetanus flocculating antitoxin, T34, (1 500 units/ml) was supplied by the Serum and Vaccine Department, S.A.I.M.R. It was used to flocculate tetanus toxoid samples.

2.1.3 ANIMALS

Female Duncan Hartley guinea pigs, approximately 1,0 kg in weight. The experiment were cleared on the 24.11.1987 by the Animal Ethics Control Committee of the University of the Witwatersrand, clearance no. 87/180.

2.2 METHODS

2.2.1 ION EXCHANGE CHROMATOGRAPHY

DEAE-Sephadex A-50 was preswollen in distilled water and equilibrated with 0,01 M phosphate buffer pH 6,8 containing 0,035 M sodium chloride. Pepsin digested and ammonium sulphate precipitated equine antisera from horses immunised with tetanus toxoid or diphtheria toxoid was dialysed against distilled water for three days. These antisera were mixed with 5% w/v of 0,01 M phosphate buffer pH 6,8. The Sephadex was added to the buffered antisera and stirred mechanically for two hours. The entire procedure was conducted at room temperature. The supernatant was filtered under suction through Whatmans Grade 113 filter paper on a Buchner funnel. The filtrate was retained and the Sephadex was stirred with a further 1 litre of 0,01 M phosphate buffer/kg Sephadex for 90 minutes, Christiansen P.A and Andersen C.G. (unpublished). The filtration was repeated and the combined filtrates were concentrated by evaporation. The filtrate was dialysed against distilled water for three days and 0,3 % cresol was added to the antisera as a preservative. The Sephadex was regenerated for further use by washing the gel with 1 litre of 2M sodium chloride/kg Sephadex for 90 minutes and filtering the supernatant. This procedure was repeated. The Sephadex was then washed four times with 1 litre of distilled water/kg Sephadex. The Sephadex was re-equilibrated with 0,01 M phosphate buffer pH 6,8 containing 0,035 M sodium chloride and 0,1 % sodium azide. (Pharmacia Handbook 1970). The Sephadex was stored at $4^{o}C$.

2.2.2 AFFINITY CHROMATOGRAPHY

2.2.2.1 CYANOGEN BROMIDE ACTIVATED SEPHAROSE 4B

A known amount (5-10 g) of CNBr-Sepharose 4B was preswollen in 1 M hydrochloric acid, and the gel equilibrated with the coupling buffer which was a 0,1 M borate buffer pH 8,3 containing 0,5 M sodium chloride. A solution of the protein ligand, diphtheria toxoid or tetanus toxoid, was diluted with the coupling buffer so as to contain 4,3-10 mg protein/ml. The gel was added to the protein ligand solution and left for 2 hours in a shaking water bath at 20°C. To optimise the method to be used, some samples were left at 4°C for 16 hours and the coupling results were compared. The gel was then transferred to 0,2 M glycine solution pH 8 and left in the shaking water bath for 2 hours at 20°C. The gel was repeatedly washed with the coupling buffer followed by 0,1 M acetate buffer pH 5,5 containing 0,5 M sodium chloride until protein was absent from the washings, (Pharmacia Handbook, 1979). All washings were retained. The gel was either packed into a column, 15 mm in diameter x 250 mm in length, or left on a sintered glass filter for batch processing.

Pepsin treated and ammonium sulphate precipitated equine antisera, (Pope 1959a; Pope 1959b; Pope and Stevens 1951), were diluted 1 in 5 with coupling buffer and added to the gel in a flask for batch processing or applied undiluted to the column and washed through the column with coupling buffer. The gel was left overnight at room temperature. Unadsorbed antisera was washed out of the column with coupling buffer and the protein content of the eluent was monitored with a Gilson U.V. detector. When all the unadsorbed protein was removed, 2 ml 0,2 M glycine buffer (adjusted to pH 2,8 with hydrochloric acid) per gram gel was washed through the column for 15 minutes to elute diphtheria antibodies.

For tetanus immunopurification the elution step was carried out using 0,1 N hydrochloric acid (Tayot et al., 1980b). The eluted protein was again monitored with the U.V. detector and immediately neutralised with 0,1 M borate buffer pH 8,3. For the batch method, unadsorbed protein was washed out of the gel by washing the gel on a sintered glass filter funnel under vacuum with a total of 10 ml coupling buffer per gram of gel. The protein content of successive washes was determined by the Biuret method to find the volume of buffer required to remove all unadsorbed protein. The gel was then washed for ten minutes with 2 ml 0,2 M glycine buffer, pH 2,8, per gram of gel to elute diphtheria antitoxin or 2ml 0,1 M hydrochloric acid per gram of gel to elute tetanus antitoxin. The supernatant was removed under suction through the sintered glass filter. The eluted protein was neutralised with 0,1 M borate buffer pH 8,3.

The gels in the column and in the batches were re-equilibrated with the coupling buffer before repeating the purification of more antisera. The eluted protein solutions were evaporated down approximately 2s % of their original volume and dialysed against distilled water for 24 hours at 4^{0}C. A comparison was made between column chromatography and batch chromatography for the purification of diphtheria and tetanus antitoxins on diphtheria toxoid immunoadsorbents and tetanus toxoid immunoadsorbents respectively, using CNBr-Sepharose 4B as the adsorbent.

2.2.2.2 AH-SEPHAROSE 4B

A known amount (5-10 g) of AH-Sepharose 4B was swollen and washed on a sintered glass filter with 0,5 M sodium chloride solution. The gel was then washed with 0,1 M carbonate buffer pH 8,5 and packed into a column, 15 mm in diameter x 250 mm in length. A 0,5 % solution of glutaraldehyde in 0,1 M carbonate buffer was applied to the column and the mixture left at room temperature for 90 minutes.

The glutaraldehyde solution was washed out with buffer (Cambiaso et al.,1975). A protein ligand solution of diphtheria or tetanus toxoid was prepared which contained 10 mg protein/ml gel. The protein ligand was added to the activated gel and left at room temperature for 15 minutes. After the incubation period, the gel was washed with buffer to remove unwanted protein. The coupling procedure was repeated. The protein coupling period was increased and the protein concentration varied to determine the optimal amount of protein coupled per ml of gel. The protein content of all washings was determined by the Biuret method and subtracted from the amount of protein applied in order to obtain the amount of protein coupled to the gel. The amount of diphtheria and tetanus toxoid coupled to the gel was compared with that coupled to CNBr-Sepharose 4B and the Spherosil derivative.

The coupling capacity of the gel for diphtheria toxoid was lower than that of the other two gels. The amount of tetanus toxoid coupled to the gel was negligable. For this reason, AH-Sepharose 4B was not used to purify equine antibodies.

2.2.2.3 DEAE DEXTRAN DERIVATIVE OF SEPHEROBIL SILICA BEADS

Spherosil XOC005 silica beads were impregnated with DEAE-Dextran by soaking 8,0 g of Spherosil XOC005 in 50 ml of 7,5 % DEAE-Dextran in 0,01 M phosphate buffer pH 6,8 for at least 12 hours. The gel was packed into a glass column 15 mm in diameter x 250 mm in length, or left on a sintered glass funnel for batch processing. Excess DEAE-Dextran was washed off with 200 ml of 0,01 M phosphate buffer pH 6,8. The protein ligand, diphtheria or tetanus toxoid, was diluted with 0,01 M carbonate buffer pH 8,3 to contain 5-10 mg protein/ml, applied to the gel and left overnight at room temperature.

Unadsorbed toxoid was washed out with 0,01 M carbonate buffer pH 8,3. Adsorbed protein was crosslinked with 0,5 % glutaraldehyde in carbonate buffer pH 8,3 which was circulated for 15 minutes. The glutaraldehyde solution was washed out and the gel rinsed with 0,2 M glycine buffer pH 8,2 (adjusted with 1M sodium hydroxide), followed by 0,05 M sodium citrate buffer pH 3,5 and re-equilibrated with 0,2 M glycine pH 8,2 (Tardy et al.,1978; Tajrot et al.,1980).

Pepsin treated and ammonium sulphate precipitated equine antisera were applied to the gel in 0,01 M carbonate buffer pH 8,3 and left overnight. Unadsorbed protein was washed out using 0,05 M sodium citrate and a decreasing pH gradient of 5,5 to 3,5. Adsorbed diphtheria antibodies were eluted with 0,2 M glycine-HCl buffer pH 2,6. Adsorbed tetanus antibodies were eluted with 0,1 M hydrochloric acid. The eluents were neutralised with 0,1 M carbonate buffer pH 8,3.

The gel was re-equilibrated with 0,01 M carbonate buffer pH 8,3 before the process of antisera purification was repeated. Eluted samples were evaporated down approximately 80 % of their original volume and dialysed overnight in distilled water at 4^{0}C. A comparison was made between column and batch chromatography for the purification of diphtheria and tetanus antitoxins.

2.2.3 FLOCCULATION TESTS

The potency of diphtheria toxoids and tetanus toxoids is expressed as the amount of international flocculation units per ml, abbreviated as Lf/ml.

The diphtheria toxoid samples were tested against an equine antiserum, batch number D.10, which had a potency of 4 000 units/ml. A constant volume of the toxoid was added to 1,0 ml saline to which was added increasing volumes of the standard antiserum, D.10. The tetanus toxoid samples were tested as described above against an equine antiserum, batch number T.34, which had a potency of 1 500 units/ml. The tubes were incubated in a shaking water bath at 37°C. At intervals the tubes were illuminated by a direct light source and observed through a 10x magnification lens. The order in which the tubes flocculate was recorded. The mixture that flocculated first was that which contained the most nearly equivalent quantities of toxoid and antitoxin (British Pharmacopeia, 1980).

The potency of diphtheria and tetanus antitoxins expressed as International Antitoxin Units/ml (IAU/ml or units/ml). The diphtheria antitoxin samples were tested against the diphtheria flocculating toxin, batch number F.T.17, which had a potency of 21 Lf/ml. Increasing volumes of the antitoxin were added to 1,0 ml of F.T.17 and the tubes were incubated in a shaking water bath at 37°C. The first tubes to flocculate were recorded as described in the previous paragraph. The tetanus antitoxin samples were tested, as described for diphtheria antitoxin, but using the tetanus flocculating toxin, batch number A.1, which has a potency of 42 Lf/ml. The mixture that flocculated first was that which contained the most nearly equivalent quantities of toxin and antitoxin (British Pharmacopeia, 1980). The results from the flocculation tests were expressed as in vitro titre, in units/ml.

2.2.4 BIOLOGICAL ASSAY OF DIPHTHERIA ANTITOXIN

The neutralising capacity of diphtheria antitoxin was determined *in vivo* by comparing the dose necessary to protect guinea pigs against the erythrogenic effects of a fixed dose of diphtheria toxin with the quantity of the standard preparation of antitoxin necessary to give the same protection (British Pharmacopeia, 1963).

The titre of antitoxin samples was determined by flocculation tests. The samples were then diluted with sterile saline to contain 2 units/ml. A series of dilutions were made using 0,1 ml of the antitoxin at 2 units/ml and increasing volumes of saline ranging from 1/6 to 1/14. To each antitoxin dilution was added an equal volume of the Lf/100 standard toxin dose. A control was set up using equal volumes of Lf/100 standard toxin dose and the W.H.O. standard antitoxin (0,2 units/ml). Female Duncan Hartley guinea pigs were shaved on their flanks. One guinea pig was used for two samples of antisera. Each sample required five injection sites on one flank of the guinea pig. One control injection site was run for each guinea pig. For each dilution, 0,1 ml was injected intradermally. The injected dose of the control antitoxin contained 0,01 units. The guinea pigs were housed in cages with a water bottle and commercial rabbit pellets. Thirteen guinea pigs were used altogether. The guinea pigs were checked every 24 hours after they had been injected. After 48 hours the guinea pigs were examined and the injection sites compared with those of the control. The potency of the antitoxin was calculated from the smallest antitoxin dilution that gave a positive erythematous reaction, a small, characteristic skin reaction. At this site, the injected dose was taken to contain 0,01 units of antitoxin, and after accounting for the dilution, the results were expressed as the *in vivo* titre in units/ml.

The Lr/100 dose was the smallest quantity of the toxin which when mixed with 0.01 units of antitoxin and injected intradermally into guinea pigs caused a small reaction at the site of injection (British Pharmacopeia, 1980).

2.2.5 PROTEIN DETERMINATIONS

The total protein concentration of samples was determined by the Biuret method (Peters et al., 1978). The optical densities were read on a *Pye Unicam Spectrophotometer* at 540 nm against a reagent blank solution. The total protein concentration was found by multiplying by the conversion factor from a standard protein sample read under the same conditions. *The protein* standards obtained from Boehringer Mannheim were diluted before use to contain 4, 6 and 8 mg protein/ml.

The protein nitrogen content of the samples was estimated from the total protein content using the assumption that the average nitrogen content of protein is 16 % (Scientific Tables 1962).

The protein content in the column washes and eluents was monitored using a *Gilson U.V. detector*.

2.2.6 ELECTROPHORESIS

All electrophoresis experiments were performed using a Gelman microelectrophoresis chamber with a Vokam stabilised DC power supply and using Beckman's Paragon Protein Electrophoresis Kit.

The samples were applied to the agarose gel, supplied in the kit, through template slots. All samples were initially diluted with saline to contain 0.8 mg protein nitrogen/ml. The samples were allowed to diffuse into the gel and then electrophoresed for 2½ minutes at 100 volts with 0,075 M barbital buffer pH 8,6 in the electrophoresis chambers. Upon completion of electrophoresis the gel was removed from the electrophoresis chamber and placed in a solution of 3 volumes methanol : 2 volumes glacial acetic acid for 5 minutes. The gel was dried at 90°C in an oven for approximately 20 minutes or until the gel was dry. The protein was stained with Paragon Blue Protein Stain. The gel was then washed in 5 % glacial acetic acid until the background was cleared.

2.2.7 IMMUNODIFFUSION

A 1 % agarose solution in saline with 0,1 % sodium azide added as a bacteriostat, was prepared. The hot agarose solution was poured onto glass slides to give a layer of agarose 2-3 mm in depth. When set, perspex templates were placed on the agarose and were used to punch out holes in the agarose gel approximately 6-7 mm apart. Samples were placed in the holes through the template and air bubbles were removed with a needle. The gels were left for 48 hours in a humid developing chamber at room temperature. The templates were removed and the gels were washed with saline. The gel was placed in the oven at 90°C for approximately 30 minutes or until the gel had dried. The immunodiffusion lines were stained by placing the gels in 0,2 % Ponceau R, 3,5 % trichloroacetic acid for 10 minutes. Background staining was removed by placing the glass slides in 5 % acetic acid until the background was clear.

3. RESULTS

3.1 ION-EXCHANGE CHROMATOGRAPHY

The ion-exchange purification was studied uping the batch method. Table 1 shows the results obtained from purifying six batches of diphtheria antisera. This method gave a mean recovery of 94,1 % ± 9,56 with respect to the quantity of diphtheria antibodies measured by in vivo analysis. The mean purification factor was 1,33 ± 0,06 calculated from the specific activity of the antitoxin. The specific activity is expressed as diphtheria antibody units/mg protein nitrogen.

Table 2 shows the results from purifying six batches of tetanus antisera. This method gave a mean recovery of tetanus antibodies of 82,0 % ± 6,92 as measured by in vitro flocculation tests. The mean purification factor was 1,31 ± 0,.2. between each purification the Sephadex ion exchanger was regenerated. In this study the efficiency of the Sephadex for purifying the antisera did not drop with repeated use. Twelve batches of antisera were purified with the same batch of ion exchanger.

Where the antibody titre results in all the tables are expressed as in vivo (i.e. seroneutralisation tests), the in vitro results gave the correspnding titres. Where the results are expressed as in vitro only flocculation tests were performed on these samples.

TABLE 1 The purification of pepsin digested e, ine diphtheria antisera using DEAE-Sephadex A-50

	Before Purification			After Purification			
	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	5 100,0	27,3	187,0	6 630,0	26,3	252,0	1,35
	2 700,0	25,1	117,0	4 400,0	28,0	157,0	1,34
	4 465,0	22,0	203,0	6 780,0	26,5	258,0	1,27
	1 965,0	16,0	104,0	3 430,0	24,7	139,0	1,34
	2 492,0	19,5	124,0	3 560,0	25,6	139,0	1,12
	2 170,0	17,2	126,0	4 360,0	24,7	176,0	1,40
Mean	3 120,0	21,2	143,5	4 926,7	25,9	189,5	1,33
±S.D	1 332,2	5,0	40,9	1 421,9	1,2	52,1	0,06

TABLE 2 The purification of pepsin digested equine tetanus antiserin using DEAE-Sephadex A-50

	Before Purification			After Purification			
	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	1 800,0	19,4	93,0	4 128,0	33,6	123,0	1,32
	1 900,0	14,6	103,0	3 666,0	31,6	116,0	1,13
	1 950,0	13,2	148,0	3 900,0	21,3	183,0	1,24
	1 700,0	19,2	89,0	3 300,0	27,7	119,0	1,35
	1 720,0	16,6	104,0	3 700,0	23,7	156,0	1,50
	2 357,0	19,5	121,0	4 500,0	28,4	158,0	1,31
Mean	1 842,8	17,1	109,0	3 865,3	27,7	142,5	1,31
±S.D	291,3	2,7	21,8	414,3	4,6	27,2	0,12

3.2 OPTIMAL CONDITIONS FOR THE PREPARATION AND USE OF IMMUNOADSORBANTS

3.2.1 STABILITY OF PURIFIED TOXOIDS AND EQUINE ANTISERA

The stability of diphtheria and tetanus toxoids and antisera at various pH levels was tested. Their stability was measured in the buffers and solutions that were used in the immunoadsorbent columns. Pepsin digested equine antisera and purified toxoids were tested by flocculation assays after 1 and 24 hours at room temperature in each buffer. The antisera and toxoids were found to be stable in saline, 0,1 M borate buffer pH 8,3, 0,1 M carbonate buffer pH 8 and 0,5 % glutaraldehyde solution in carbonate buffer after 24 hours. Tetanus antisera and tetanus and diphtheria toxoids were stable in 0,2 M glycine/HCl pH 2,8 for 24 hours. Diphtheria antisera were stable in 0,2 M glycine/HCl pH 2,8 for 1 hour but denatured after 24 hours. One hour exceeds the maximum elution time during which the antisera are exposed to 0,1 M HCl. Tetanus antisera were stable in 0,1 M HCl after 1 hour. After 24 hours the tetanus antisera were partially denatured. One hour exceeds the time required to elute the tetanus antibodies.

3.2.2 THE COUPLING OF PROTEIN LIGANDS TO THE CHROMATOGRAPHY MATRICES

The coupling of protein ligands to CNBr-Sepharose 4B is shown in Table 3. The diphtheria toxoid was diluted with borate buffer pH 8,3 to contain 4,3, 5,0 and 10 ,0 mg protein/ml gel. It was assumed that 1 g of dry gel swells to 3,5 ml (Pharmacia, 1979). From the results shown in Table 3 the immunoadsorbent columns were prepared using a protein ligand concentration of approximately 5 mg protein/ml gel.

All subsequent CNBr-Sepharose 4B immunoadsorbents were prepared at room temperature using a coupling time of 2 hours for the protein ligand and a blocking time of 2 hours with 0,2 M glycine buffer pH 8.

TABLE 5 Optimisation of protein ligand coupling to CNBr-Sepharose 4B. Diphtheria toxoid was used as the protein ligand and diluted with 0,1 M borate buffer pH 8,5.

Diphtheria Toxoid Added Lf (total)	Protein mg/ml	Coupling Time hrs	Coupling Temp C	Blocking Time hrs	Diphtheria Toxoid Adsorbed Lf	% Toxoid Adsorbed
14 560	4,0	2	20	2	12 250	84
14 560	4,0	2	20	2	12 250	84
18 200	5,0	2	20	2	13 992	77
18 200	5,0	2	20	2	14 538	79
18 200	5,0	16	4	2	12 961	71
18 200	5,0	16	4	2	12 456	68
34 400	10,0	2	20	2	19 330	56
35 400	10,0	2	20	2	14 330	39

The Spherosil immunoadsorbents were prepared as described by Tardy et al.,(1976).

3.2.3 DESORPTION FROM IMMUNOADSORBENTS

A suitable dissociating agent was found by monitoring the eluent from each column with a U.V. monitor. The fractions collected from each peak were analysed by flocculation tests and protein determinations. For diphtheria antisera, 0,2 M glycine buffer adjusted to pH 2,8 with HCl was found to be a suitable dissociating agent. For tetanus antitoxin 0,1 M HCl was required to dissociate the antitoxin from the toxoid. For all Spherosil immunoadsorbents a decreasing pH gradient from pH 5,5-3,5 was passed through the gel before the eluting buffer. All eluents were neutralised by collecting them in borate buffer pH 8,3.

3.3 IMMUNOPURIFICATION OF EQUINE ANTISERA

3.3.1 THE COUPLING CAPACITY OF IMMUNOADSORBENTS

The coupling capacity of each gel for diphtheria toxoid is shown in Table 4. The coupling capacity of AH-Sepharose 4B for diphtheria toxoid was approximately 35 % that of CNBr-Sepharose 4B and approximately 42 % that of Spherosil XOC005 derivative. Table 4 also shows the coupling capacity of each gel for purified tetanus toxoid. The AH-Sepharose immunoadsorbent gels were not used for the purification of antitoxins because of their low coupling capacity for the toxoids.

TABLE 4 Immunoadsorbtion properties of affinity matrices. The coupling capacity of each matrix as determined from Lf of toxoid/g of gel ± S.D.

Matrix	Diphtheria Toxoid Lf/g	n	Tetanus Toxoid Lf/g	n
CNBr-Sepharose 4B	12 979 ± 893	6	10 671	1
Spherosil EC00005	10 203 ± 1851	4	10 549 ± 1060	2
AH-Sepharose 4B	4 300 ± 345	3	0	3

n = Sample size

7.3.2 PURIFICATION OF ANTISERA BY AFFINITY CHROMATOGRAPHY

Immunopurification was studied using small scale column and batch methods for each gel. In Table 5 the results are shown for the purification of diphtheria antisera on CNBR-Sepharose 4B immunoadsorbent using a batch method. This method gave a mean purification factor of 2,33 ± 0,21. Table 6 shows the results of using a column method for purifying diphtheria antisera on CNBr-Sepharose 4B. This method gave an average purification factor of 2,06 ± 0,22. The results of purifying diphtheria antisera on the Spherosil derivative using a batch and a column methods are shown in Table 7 and 8 respectively. The batch method gave a mean purification factor of 2,17 ± 0,31. The column method gave a mean purification factor of 2,07 ± 0,32.

TABLE 5 The purification of pepsin digested equine diphtheria antisera using CNBr-Sepharose 4B, a batch method

	Before Purification			After Purification			
	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	2 275,0	18,4	123,6	156,0	0,62	251,6	2,04
	2 275,0	18,4	123,6	224,0	0,81	276,5	2,24
	2 275,0	18,4	123,6	195,0	0,61	319,7	2,59
	2 275,0	18,4	123,6	144,0	0,47	306,4	2,48
	2 275,0	18,4	123,6	208,0	0,73	284,7	2,31
Mean	2 275,0	18,4	123,6	185,4	0,65	287,7	2,33
±S.D	0	0	0	34,2	0,13	26,7	0,21

TABLE 6 The purification of pepsin digested equine diphtheria antisera using CNBr-Sepharose 4B, a column method

	Before Purification			After Purification			
	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	1 775,0	17,6	100,8	190,0	0,8	237,5	2,36
	1 775,0	17,6	100,8	170,0	0,8	212,5	2,11
	1 775,0	17,6	100,8	150,0	0,7	214,3	2,11
	1 775,0	17,6	100,8	830,0	4,3	193,0	1,91
	1 775,0	17,6	100,8	400,0	2,2	181,8	1,80
Mean	1 775,0	17,6	100,8	348,0	1,8	207,8	2,06
±S.D	0	0	0	287,6	1,6	21,5	0,22

TABLE 7 The purification of pepsin digested equine diphtheria antisera using DEAE-dextran derivative of Spherosil silica beads, a batch method.

	Before Purification			After Purification			
	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	2 672,0	25,7	103,9	540,0	2,69	208,0	2,00
	2 672,0	25,7	103,9	467,0	2,40	194,6	1,87
	2 672,0	25,7	103,9	520,0	2,36	219,0	2,04
	2 672,0	25,7	103,9	480,0	1,89	254,0	2,54
	2 672,0	25,7	103,9	260,0	0,77	260,0	2,50
Mean	2 672,0	25,7	103,9	437,4	1,94	227,7	2,19
±S.D	0	0	0	108,5	0,76	32,0	0,31

TABLE 8 The purification of pepsin digested equine diphtheria antisera using DEAE-dextran derivative of Spherosil silica beads. A column method.

	Before Purification			After Purification			
	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vivo titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	2 275,0	18,4	123,6	165,0	0,56	294,6	2,38
	2 275,0	18,4	123,6	302,0	1,15	262,6	2,12
	2 275,0	18,4	123,6	210,0	0,78	269,2	2,18
	2 275,0	18,4	123,6	189,0	0,71	266,2	2,15
	2 275,0	18,4	123,6	150,0	0,80	187,5	1,52
Mean	2 275,0	18,4	123,6	203,2	0,80	256,0	2,07
±S.D	0	0	0	59,8	0,22	40,3	0,32

The degrees of purification obtained from each method employed were compared statistically using A.N.O.V.A. Table 9 shows the mean purification factors ± S.D. for each method. The degree of purification obtained by affinity chromatography was found to be significantly different from that obtained by ion exchange chromatography. There was no significant difference between the purification obtained for each method of affinity chromatography. These results are illustrated graphically in Fig. 1.

TABLE 9 The purification factors obtained for diphtheria antisera were analysed using an A.N.O.V.A. test. The results shown are the mean purification factors ± S.D. obtained for each method used to purify diphtheria antisera.

Matrix	n	Mean ± S.D.	P<0.05
CNBr-Sepharose 4B (batch)	5	2,33±0.21	NS
Spherosil derivative (batch)	5	2,19±0.31	NS
Spherosil derivative (column)	5	2,07±0.32	NS
CNBr-Sepharose 4B (column)	5	2,06±0.23	NS
DEAE-Sephadex	6	1,32±0.05	S

NS : not significant at the 5 per cent level
S : significantly different at the 5 per cent level

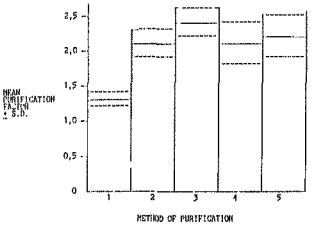

FIG.1 A COMPARISON OF THE MEAN PURIFICATION FACTORS ±S.D. OBTAINED FROM EACH METHOD USED TO PURIFY DIPHTHERIA ANTIBODIES

1. ION EXCHANGE CHROMATOGRAPHY
2. CNBr-SEPHAROSE 4B - COLUMN CHROMATOGRAPHY
3. CNBr-SEPHAROSE 4B - BATCH CHROMATOGRAPHY
4. SPHEROSIL DERIVATIVE - COLUMN CHROMATOGRAPHY
5. SPHEROSIL DERIVATIVE - BATCH CHROMATOGRAPHY

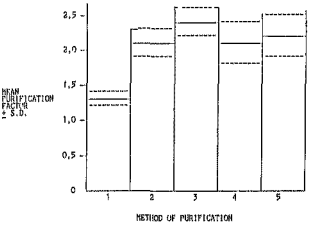

FIG.1 A COMPARISON OF THE MEAN PURIFICATION FACTORS ±S.D. OBTAINED FROM EACH METHOD USED TO PURIFY DIPHTHERIA ANTIBODIES

1. ION EXCHANGE CHROMATOGRAPHY
2. CNBr-SEPHAROSE 4B - COLUMN CHROMATOGRAPHY
3. CNBr-SEPHAROSE 4B - BATCH CHROMATOGRAPHY
4. SPHEROSIL DERIVATIVE - COLUMN CHROMATOGRAPHY
5. SPHEROSIL DERIVATIVE - BATCH CHROMATOGRAPHY

When considering the difference between the specific activity of the antisera before purification and after purification, the ion exchange method was found to give a significantly lower difference than the affinity chromatography methods. These differences in specific activity were analysed by A.N.O.V.A. and the results are shown Table 10 .

s in the specific activity of
ers for each method of purification
were co ...tistically using an A.N.O.V.A. test.
The table ... the mean values ± S.D. for the
specific activity of diphtheria antisera before and
after purification, and the mean increases in specific
activity.

Matrix	Initial S.A. (Mean±S.D) I.U/mg P.N	Final S.A. (Mean±S.D) I.U/mg P.N	Diff. S.A. (Mean±S.D) I.U/mg P.N	P<0.05
INFA Septacorn 48 (batch)	123,4±0	287,7±26,7	164,2±26,9	S
Spherosil derivative (column)	123,6±0	256,0±40,3	132,4±36,1	NS
Spherosil derivative (batch)	103,9±0	227,7±32,0	124,6±32,0	NS
INFA Septacorn 48 (column)	190,9±0	317,4±21,5	107,0±21,5	NS
IFEAE Septacel	143,5±40,7	189,5±52,1	46,0±12,9	S

S.A. : specific activity
S : significantly different at the 5 per cent level
NS : not significantly different at the 5 per cent level

The W.H.O. stipulates that the antisera for human therapeutic use must have a potency of 4 000 u/ml and a maximum total protein content of 16,8 %. This means that the final product must have a minimum specific activity of 148,8 units/mg protein nitrogen.

The results of purifying tetanus antisera using CNBr-Sepharose 4B and tetanus toxoid as the immunoadsorbent are shown in Tables 11 and 12. The batch method, Table 11, gave a purification factor of 1,01 ± 0,03. The column method, Table 12, gave a purification factor of 1,55 ± 0,14. The Spherosil material immunoadsorbent was more successful for purifying tetanus antisera. The batch method, Table 13, gave a mean purification factor of 1,84 ± 0,11 whereas the column method, shown in Table 14, gave a mean purification factor of 2,75 ± 0,13.

TABLE 11 The purification of pepsin digested equine tetanus antisera using CNBr-Sepharose 4B. A batch method.

	Before Purification			After Purification			
	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	1 000,0	16,6	90,3	105,0	1,1	95,5	1,06
	1 000,0	16,6	90,3	190,0	1,7	88,2	0,98
	1 000,0	16,6	90,3	175,0	1,9	92,1	1,02
	1 000,0	16,6	90,3	81,0	0,9	89,0	0,99
	1 000,0	16,6	90,3	62,0	0,7	88,6	0,98
Mean	1 000,0	16,6	90,3	114,6	1,3	90,7	1,01
±S.D	0	0	0	47,1	0,5	3,1	0,03

TABLE 12 The purification of pepsin digested equine Lelanus antisera using CNBr-Sepharose 4B. A column method.

	Before Purification			After Purification			
	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	1 500,0	16,6	90,3	131,0	0 92	142,4	1,58
	1 500,0	16,6	90,3	88,0	0,56	157,1	1,74
	1 500,0	16,6	90,3	98,0	0,70	140,0	1,61
	1 500,0	16,6	90,3	95,0	0,68	139,7	1,55
	1 500	16,6	90,3	70,0	0,55	127,3	1,41
	1 500,0	16,6	90,3	60,0	0,48	125,0	1,38
Mean	1 500,0	16,6	90,3	85,7	0,60	139,4	1,55
S.D	0	0	0	27,5	0,18	11,9	0,14

TABLE 13 The purification of pepsin digested equine Lelanus antisera using a DEAE-Dextran derivative of Spherosil silica beads. A batch method.

	Before Purification			After Purification			
	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	1 500,0	16,6	90,3	1 ,0,0	0,86	162,8	1,80
	1 500,0	16,6	90,3	296,0	1,48	175,0	1,93
	1 500,0	16,6	90,3	460,0	2,57	179,0	1,98
	1 500,0	16,6	90,3	300,0	1,82	164,0	1,82
	1 500,0	16,6	90,3	300,0	1,80	153,8	1,70
Mean	1 500,0	16,6	90,3	251,2	1,49	166,5	1,84
S.D	0	0	0	1,1,7	0,65	9,7	0,11

TABLE 14 The purification of pepsin digested equine tetanus antisera using a DEAE-Dextran derivative of Spherosil silica beads. A column method.

	Before Purification			After Purification			
	In vitro Protein titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	In vitro titre I.U/ml	Protein Nitrogen mg/ml	Specific Activity I.U/mg P.N	Purification Factor
	1 500,0	14,4	104,2	175,0	0,64	273,4	2,62
	1 500,0	14,4	104,2	168,0	0,55	305,7	2,93
	1 500,0	14,4	104,2	191,0	0,67	285,1	2,74
	1 500,0	14,4	104,2	194,0	0,45	275,6	2,64
	1 500,0	14,4	104,2	191,0	0,45	291,1	2,80
Mean	1 500,0	14,4	104,2	156,6	0,35	286,2	2,75
±S.D	0	0	0	28,6	0,10	13,1	0,13

The degree of purification of tetanus antisera was analysed statistically using A.N.O.V.A. Each method of purification employed gave significantly different purification factors from other methods. The results are shown in Table 15.

TABLE 15 A statistical analysis of the purification factors obtained for tetanus antisera. The results were analysed using analysis of variance.

Matrix	n	Mean±S.D.	PCO,05
Spherosil derivative (column)	5	2,75 ± 0,13	S
Spherosil derivative (batch)	5	1,84 ± 0,11	S
CNBr-Sepharose 4B (column)	6	1,55 ± 0,14	S
DEAE-Sephadex	6	1,37 ± 0,12	S
CNBr-Sepharose 4B (batch)	5	1,01 ± 0,0	S

S : significantly different at the 5 % level
n : sample size

The mean purification factors are illustrated in a bar graph in Fig.2. Table 15 shows the mean differences between the initial specific activity of tetanus antisera and the purified specific activity of the antisera. The Spherosil derivative gave a significantly higher increase in purification when compared with the other methods by A.N.O.V.A. However each method gave, statistically, significantly different increases in the mean specific activity of the antisera. The average specific activities of the antisera before and after each method of protein purification are shown in Table 16.

TABLE 16 The mean increases in the specific activity of tetanus antisera for each method of purification were compared statistically using A.N.O.V.A. test. The table shows the mean values ± S.D. for the specific activity of tetanus antisera before and after purification, and the mean increases in specific activity.

Matrix	Initial S.A. (Mean±S.D) I.U/mg P.N	Final S.A. (Mean±S.D) I.U/mg P.N	S.A. (Mean±S.D)	P<0,05
Spherosil derivative (column)	104,2± 0	286,2±13,1	182,0±13,1	S
Spherosil derivative (batch)	90,3± 0	166,5± 9,7	76,2± 9,7	S
CNBr-Sepharose 4B (column)	90,3± 0	139,4±11,9	49,1±11,9	S
HEAC-Sepharose	109,0±31,8	142,5±27,2	32,8±12,6	S
CNBR-Sepharose 4B (batch)	90,3± 0	90,3± 3,1	0,1± 3,1	S

S.A : specific activity
S : significantly different at the 5 % level

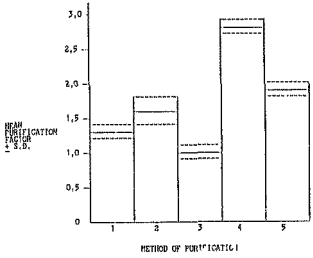

FIG.2 A COMPARISON OF THE MEAN PURIFICATION FACTORS ± S.D. OBTAINED FROM EACH METHOD USED TO PURIFY TETANUS ANTIBODIES

1. ION EXCHANGE CHROMATOGRAPHY
2. CNBr-SEPHAROSE 4B - COLUMN CHROMATOGRAPHY
3. CNBr-SEPHAROSE 4B - BATCH CHROMATOGRAPHY
4. SPHEROSIL DERIVATIVE - COLUMN CHROMATOGRAPHY
5. SPHEROSIL DERIVATIVE - BATCH CHROMATOGRAPHY

3.4 ELECTROPHORETIC ANALYSIS

The chromatographically purified antisera samples were compared with a standard serum control, hyperimmune horse plasma and pepsin digested equine antisera. All the samples were diluted with saline to contain approximately 0,8 mg P.N/ml. Fig.3 shows the standard serum control, Min.-Trol, compared with plasma from a hyperimmunised horse and plasma that has been digested enzymatically and fractionated with ammonium sulphate. Fig.4 shows the electrophoretic pattern obtained from various diphtheria antisera samples. Figs. 5 and 6 show the electrophoretic patterns obtained from tetanus antisera samples and the standard serum control.

After pepsin digestion of the horse plasma, the band of immunoglobulins is more concentrated with trace amounts of the other serum protein bands present. After ion exchange purification, a diffuse band of immunoglobulins is present. The traces of albumin and alpha-globulins have disappeared. After affinity chromatography of diphtheria antisera the band of immunoglobulins is more defined. The electrophoretic patterns were interpreted by a visual comparison. According to Figs. 4, 5 and 6, the patterns indicate that only immunoglobulins were eluted from the chromatographic gels. A comparison of the immunoglobulin bands in Fig. 4 shows that the antisera eluted from the affinity gels produced a more defined and narrower band of immunoglobulins than that eluted from the ion exchange gel.

The samples applied to the agarose gels had the same protein content suggesting that the ion exchange purified antisera contained a more heterogenous mixture of immunoglublins than the affinity purified antisera. Figs. 5 and 6 indicate that the antisera eluted from the Sephadex and CNBr-Sepharose 4B gels contained a more heterogenous mixture of immunoglobuline compared with the antisera eluted from the spherosil derived gel.

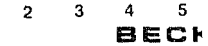

FIG.5 Serum samples electrophoresed on agarose gel

2. Standard serum control
3. Hyperimmune horse plasma
4. Pepsin digested horse antisera

FIG.4 Diphtheria antisera samples electrophoresed in agarose gel

1. Hyperimmune horse plasma
2. Pepsin digested antisera
3. Diphtheria antisera purified by ion exchange chromatography
7. Diphtheria antisera purified on CNBr-Sepharose 4B column
8. Diphtheria antisera purified batchwise on CNBr-Sepharose 4B
9. Diphtheria antisera purified on Spherosil derivative column
10. Diphtheria antisera purified batchwise on Spherosil derivative

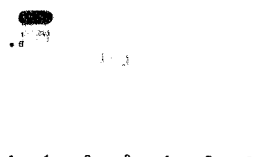

Fig.5 Tetanus antisera samples electrophoresed on agarose gel

1. Standard serum control
2. Hyperimmune horse plasma
3. Pepsin digested antisera
4. Tetanus antisera purified on CNBr Sepharose 4B column
5. Tetanus antisera purified on CNBr Sepharose 4B (batch)
6. Tetanus antisera purified on Spherosil derivative column

6 7 8 9 10

FIG.6 Tetanus antisera samples electrophoresed on agarose gel

6. Hyperimmune horse plasma
7. Papain digested horse plasma
8. Tetanus antisera purified by ion exchange chromatography
9 & 10. Tetanus antisera purified on Sepharose derivative immunoadsorbents

3.5 IMMUNODIFFUSION

The purified antisera were investigated by immundiffusion against their corresponding toxoids. Figs.7 and 8 both had reactions of identity between the antisera samples which showed the presence of identical antibodies in the raw plasma and the purified antitoxins. A comparison of the precipitation lines produced shows that the chromatographically purified antisera samples gave fewer lines of precipitation when compared with the raw plasma and the pepsin digested plasma. This may be an indication that the purity of the samples was improved by the ion exchange and affinity gels. It cannot be considered as conclusive proof as the raw plasma may have produced multiple precipitation lines and false spur phenomena which were absent from the purified samples.

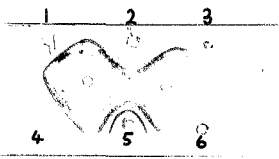

FIG.7 Double diffusion of diphtheria antisera and toxoid

Central wells contain diphtheria toxoid.

1 & 3. Hyperimmune horse plasma
2. Pepsin digested antisera
3. Ion exchange purified antisera
4. CNBr-Sepharose 4B immunosorbent
6. Sphérosil immunosorbent

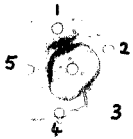

FIG.8 Double diffusion of tetanus antisera and toxoid

Central wells contain tetanus toxoid.
1. Hyperimmune horse plasma
2. Pepsin digested antisera
3. Ion exchange purified antisera
4. CNBr-Sepharose 4B immunosorbent
5. Spherosil immunosorbent

3.6 COMPARISON OF RECOVERY OF ANTISERA FROM CHROMATOGRAPHIC MATRICES

The recovery of diphtheria and tetanus antibodies from chromatography matrices is shown in Tables 17 and 18 respectively. The purification factors for each method has been included in the tables to compare the yield and the purity obtained.

TABLE 17 Recovery and degree of purification of diphtheria antibodies from chromatography matrices. The recovery was determined from the number of units of antibody applied to the gels and the number eluted. The recoveries and purification factors are shown as the mean ± S.D.

Matrix	n	Recovery Percentage	Purification Factor
DEAE-Sephadex A-50 (batch)	6	94,10± 9,96	1,33±0.06
CNBr-Sepharose 4B (column)	5	45,96± 9,65	2,06±0.22
CNBr-Sepharose 4B (batch)	5	49,76±17,88	2,33±0.21
Spherosil derivative (column)	5	31,02± 5,07	2,07±0.32
Spherosil derivative (batch)	5	53,02± 3,03	2,19±0.31

n : sample size

TABLE 18 Recovery and degree of purification of tetanus antibodies from chromatography matrices. The recovery was determined from the number of units of antibody applied to the gels and the number eluted. The recoveries and purification factors are shown as the mean ± S.D.

Matrix	n	Recovery Percentage	Purification Factor
DEAE-Sephadex A-50 (batch)	6	83,00±6,52	1,5±0.12
CNBr-Sepharose 4B (column)	6	31,70±8,49	1,95±0.14
CNBr-Sepharose 4B (batch)	5	28,86±6,87	1,01±0.03
Spherosil derivative (column)	5	45,52±6,69	2,75±0.13
Spherosil derivative (batch)	5	39,90±3,76	1,94±0.11

n : sample size

After purifying diphtheria and tetanus antibodies by ion exchange chromatography, there was a minimal loss of antibodies when compared with the loss of antibodies when the antisera was purified by affinity chromatography methods. Excluding DEAE-Sephadex, the highest yield of active diphtheria or tetanus antibodies was obtained after purifying the antisera with the Spherosil derivative immunoadsorbent.

4. DISCUSSION

The objective of the work described was to assess immunopurification as a technique which could be used on a production scale for the purification of equine antisera for therapeutic use. The methods of affinity chromatography developed were compared with the present method of antisera purification by ion exchange chromatography.

The degree of purification was compared using the purification factor (P.F.) obtained for each method calculated from the difference in specific activity (units/mg P.N.) of antisera before and after each method of purification. Where the immunoaffinity chromatography resulted in higher P.F. than the presently used method of ion exchange chromatography, the P.F. was considered to be good. A P.F. equal to or less than that from ion exchange purification of the antisera was considered to be a poor P.F.

The CNBr-Fepharose 4B immunoadsorbent prepared with diphtheria toxoid gave a significant increase in the final purification factor of the diphtheria antisera using both column and batch methods.

The difference between the mean purification factors obtained from the column and from the batch was insignificant. The CNBr-Sepharose 4B and the tetanus toxoid adsorbent resulted in little or no purification of tetanus antisera compared to the other methods. Tayot et al.,(1980) report that when CNBr activated agarose is used there is a risk of some hydrolysis, under pH 3, of the bonds between the proteins and the matrix.

In this study, 0,1 M hydrochloric acid was required to dissociate tetanus antibodies from the tetanus toxoid. The poor purification may be the result of hydrolysis of the tetanus toxoid molecules bound to the gel. Tetanus toxoid was not detected in eluate using flocculation assays and double diffusion tests. This indicates that the protein ligand may have been partially denatured. The batch method gave a particularly low purification factor and low yield. The electrophoretic pattern showed a narrower band of immunoglobuline when compared with ion exchange chromatography of the tetanus antisera although the samples contained the same protein content. From the electrophoresis results the antisera was purified, however the specific activity of these samples showed little purification, and in some samples, no purification. The protein content was determined using Biuret Reagent. The higher protein content in these apparently "purified" samples may be due to tetanus toxoid that has leaked off the gel and has been biologically altered by the acid so it cannot be detected by reactions that involve precipitation with the antitoxin. It will however increase the total protein content in the eluate. The column method did show improved specific activity when compared with the batch method. One explanation could be the technique involved. The columns were not run dry before the elution step.

In the batch method the buffer was sucked off the gel, under a vacuum, removing any buffering solution before the dissociating agent was added. The gel in the batch method was more exposed to the dissociating agent than the gel in the column. This may have caused greater leakage of the tetanus toxoid from the adsorbent gel which lowered the specific activity of the eluate. The coupling capacity of the gel for antibodies did not drop. The percentage recovery of antibody units was lower than that for ther methods of affinity chromatography. The gel did not appear to be saturated by the amount of antisera applied.

The immunoadsorbents prepared with CNBr-Sepharose 4B and tetanus toxoid do not appear to be suitable for efficient purification of tetanus antisera. For the diphtheria antisera affinity purification a less acid solution was used as the eluting buffer. This method improved the purification factor and the specific activity of diphtheria antisera when compared with ion exchange chromatography.

Other dissociating agents (e.g. urea, guanidine hydrochloride, chaotropic ions or polarity reducing agents such as dioxane), may have been suitable for the desorption of tetanus antibodies. These were not employed as the final product is intended for human therapeutic use. This eliminated the need to test the final product for the presence of residual toxic substances introduced in the eluting buffer.

In this study it was found that using 0.1 M hydrochloric acid and 0,2 M glycine/HCl buffer pH 2,8 as dissociating agents gave a good purification factor and a high specific activity of the final antitoxin. Double diffusion experiments using the eluate against a range of antitoxin concentrations to test for the presence of toxoid in the eluate did not produce any lines of precipitation. Flocculation tests on the eluate for the presence of toxoid were also negative. These three points support the work reported by Tardy et al.,(1980) which stated that glutaraldehyde stabilises the binding of the toxoid to the Sepharose and prevents protein ligand leakage from the gel. The electrophoretic patterns of the tetanus antitoxins supports the indication that Sepharose derivative immunoadsorbents prepared with tetanus toxoid give a purer final product compared with those from ion exchange chromatography and CNBr-Sepharose 4B immunoadsorbents. A purer product will contain less protein per unit of antibody.

DEAE-dextran has anion exchange properties. The DEAE-dextran Sepharose immunoadsorbents were washed with a decreasing pH gradient, before the eluting buffer, to dissociate proteins that were bound to the gel according to their charge. A purer product with respect to Units/mg P.N. was then eluted.

The results of purifying diphtheria antisera on Sepharose derivatives are comparable with those from CNBr-Sepharose 4B. The statistical analysis showed no significant differences between the mean purification factors obtained from each method. The sample sizes are however small and greater samples sizes may point to one method giving a significantly better purification factor.

The immunopurified diphtheria antisera all showed significantly greater purification factors than ion exchange chromatography. The improved purification was also supported by the visual comparison of the electrophoretic patterns obtained from the affinity purified antitoxins. For the purpose of the theoretical scaling up to industrial scale production, discussed in the next chapter, the *CNBr-Sepharose* 4B batch method will be used as the example to compare with ion exchange chromatography.

When coupling the toxoid to the gel the protein ligand:gel ratio was not found to influence the amount of toxoid coupled. A protein ligand concentration of 4,0-10,0 mg/ml was used to prepare the immunoadsorbents. By increasing this concentration there was a decrease in the amount of the toxoid coupled as a percentage of toxoid applied. A *protein concentration* of 4, -5,0 mg/ml gave an optimal coupling capacity and an average adsorption of 76,0% of the toxoid applied. The coupling was done according to the method of Tardy et al.,(1978). They reported that the binding of the toxoid also depended on the average pore diameter of the Spherosil particles and the smaller particles will exclude the toxoid. Diphtheria toxoid has a molecular weight of 62 000 *and albumin has a* molecular weight of 69 000. Tetanus toxoid has a molecular weight of 150 000. Spherosil type X00005 was reported to be suitable for binding *tetanus toxoid* and albumin. It was assumed in this study that it would be suitable for diphtheria toxoid which is comparable in size to *albumin*.

AH-Sepharose 4B had a low coupling capacity for diphtheria toxoid and an undetectable coupling capacity for tetanus toxoid. AH-Sepharose 4B may be suitable for purifying small quantities of diphtheria antibody in the laboratory. However it was impractical for large scale use because of the quantity of the gel that would be required to bind sufficient toxoid for purifying large volumes of antisera.

To convert diphtheria and tetanus toxins to their toxoid form they were treated with formaldehyde. This reaction results in intermolecular bridges within the toxin. Amino acids, peptides and proteins that are present in the media are irreversibly linked to the toxin during the toxoiding process (Pappenheimer 1963). This lowers the specific diphtheria or tetanus antigen content. This same toxoid was used to prepare the columns and to immunise the horses. Hence the equine serum obtained from the horses, hyperimmunised with this toxoid, contains antibodies to non-specific antigens other than the toxin. The antisera immunopurified will also contain some non-diphtheria or non-tetanus toxin antibodies that will bind to the non-specific antigens on the column.

The neutralising capacity of antisera cannot be determined from the flocculation tests. In vivo as well as in vitro tests were done on the purified diphtheria antisera. The results corresponded which showed that the neutralising capacity of the diphtheria antisera had not been destroyed by the immunoaffinity purification. Before the immunopurified tetanus antigen can be used therapeutically, the neutralising capacity for tetanus toxin must be tested in vivo by serumneutralisation tests in mice. Serumneutralisation tests measure the true capacity of a given preparation to neutralise the toxin in vivo.

The low yield of antibodies from affinity gels could be due to a combination of two factors. Pepsin digested equine antisera was stable in the dissociating agents for the period of time required for elution. However the more concentrated products may be susceptible to acid denaturation. There could also be inefficient elution of the antibody. The coupling capacity of the gels for the antibodies did not drop between successive runs. The maximum capacities of these gels does not appear to have been attained by the amount of antisera initially applied. This is important for scaling up these columns for industrial processes.

5. SUMMARY AND CONCLUSION

5.1 COLUMNS VERSUS BATCHES FOR LARGE SCALE METHODS

Small scale laboratory procedures are simpler to conduct in columns than batchwise. However with the larger volumes required for production, batch processes can often be simpler to manipulate. DEAE-Sephadex A-50-anion exchanger changes volume during the regeneration steps and therefore is used batchwise. The CNBr-Sepharose 4B and Spherosil derivative adsorbents do not change volume with changes in ionic strength or pH so can readily be used in columns. A change in volume would affect the packing of the gel and subsequently the flow rate. Column methods have certain advantages. The closed system makes sterility easier to maintain which is essential for a therapeutic product. With the use of a pump, the antisera can be recirculated to optimise antigen-antibody binding. A pH gradient for elution from the Spherosil adsorbents is simpler to apply to columns whereas a drop in pH stepwise is necessary for batch methods. In this study both methods of decreasing the pH worked adequately. A pH gradient was first optimised using the column and then applied as a stepwise drop [illegible] to the batches.

From the results obtained in this study, the batch methods of purifying tetanus antisera by affinity chromatography gave lower purification factors when compared with the column methods. Column chromatography requires a short and wide column to reduce the time required for the elution step, because the eluting buffer may denature the column components if left in the column for too long. A shorter and wider column makes the elution time and flow rate easier to control. When purifying the equine diphtheria antisera, there was no significant difference between the purification factors obtained from column methods or batch methods. Both column and batch methods are suitable when considering the degree of purification obtained. This may be because the buffers used in diphtheria purification are less acid than those used in tetanus purification and therefore there is less probability of the proteins being denatured during the process.

5.2 LARGE SCALE PURIFICATION BY CHROMATOGRAPHY METHODS

From the above discussion it can be seen that some of the methods studied for purifying antisera by affinity chromatography resulted in a better purification compared with the method based on ion exchange chromatography. However for affinity chromatography to be applied to large scale purification of antisera, other factors must be considered. Each step that has been optimized in the above study must be scaled up. The cost of the material must be considered. For the purpose of this discussion the research studies will be theoretically scaled up to meet the present production requirements in our laboratories.

The cost of purifying diphtheria antitoxin with CNBr-Sepharose 4B adsorbent and the cost of purifying tetanus antitoxin with DEAE dextran derivative of Spherosil adsorbent is compared with ion exchange chromatography of the antisera. Tables 19 and 20 show the size of each column required for diphtheria and tetanus purification respectively. The column capacity required is determined from the ratio of antitoxin (units) bound to antigen (Lf) in the column and the number of antitoxin units to be purified. After pepsin digestion and ammonium sulphate purification of the horse plasma, the average titre of the concentrated plasma was taken to be 1 900 u/ml for both diphtheria and tetanus antisera.

TABLE 19 Mass of gel required to purify batches of diphtheria antisera, using CNBr-Sepharose 4B immunoadsorbent.

Diphtheria toxoid adsorbed	12 975 Lf/g gel
Diphtheria antitoxin adsorbed	3 244 u/g gel
Ratio toxoid : antitoxin	4 : 1
No. of horses immunized	2
Duration of immunization course	3 months
No. of bleeding per year/horse	12
Total volume plasma	132 litres
Total volume concentrated antisera	18 litres
Batch sizes/year	2,6 litres x 4
2,6 litres diphtheria antisera ! 900 u/ml	3 960 000 units
Column capacity	15 600 000 Lf
Column mass	! 202 g gel

TABLE 20 *Mass of gel required to purify batches of tetanus antisera, using Spherosil derivative immunosorbent.*

Tetanus toxoid adsorbed	10 347 Lf/g gel
Tetanus antitoxin adsorbed	2 607 u/g gel
Ratio toxoid : antitoxin	4 : 1
No. of horses immunised	2
Duration of immunisation course	7 months
No. of bleeding per year/horse	12
Total volume plasma	152 litres
Total volume concentrated antisera	10 litres
Batch sizes/year	2,5 litres x 4
2,5 litres diphtheria antisera 1 500 u/ml	3 900 000 units
Column capacity	15 600 000 Lf
Column mass	1 479 g gel

The immunisation *course referred* to in Tables 19 and 20 includes the immunisation of the horse and the subsequent bleeding and recovery time. Each horse is bled three times after each immunisation. It is assumed that an average of 5,5 litres of plasma is obtained from each horse per bleed. Table 21 shows the initial cost of the gels.

TABLE 21 *The cost of each gel is compared using the amount of CNBr-Sepharose 4B required to purify diphtheria antitoxin and the amount of Spherosil required to purify tetanus antitoxin*

	Sephadex	CNBr-Sepharose 4B	Spherosil TOYOSO	DEAE-Sephrose
Mass of matrix (g)	1 560	1 202	1 479	180
Cost of matrix (R)	5 101	32 214	N.A	560
Total toxoid (Lf)	-	15,6 x 10	15,6 x 10	

N.A : cost not available
R : S.A. Rands

The prices quoted correct at time of printing.

The initial cost of preparing CNBr-Sepharose 4B is far greater than that of Sephadex. Sephadex also has the advantage that it can be used for purifying both diphtheria and tetanus antitoxins whereas immunoaffinity columns are specific for each antitoxin. Diphtheria antisera can be efficiently purified using DEAE Sephadex and a batch method. This method gives a high recovery of above 90% of diphtheria antibodies. The purification factor obtained with this method is sufficient to purify the diphtheria antisera so that it meets W.H.O. requirements concerning potency and maximum protein content in the final product. The cost of the process is minimal when compared with that of affinity chromatography methods. The disadvantage of this method is that the final product has a heterogenous mixture of the diphtheria antibodies and the naturally occurring antibodies in equine plasma. The cost of immunoaffinity methods make them impractical to use for the large scale production of equine diphtheria antibodies.

The horses immunised against diphtheria toxin develop a higher antibody against the immunising toxin than the horses immunised against tetanus toxin. It would be advantageous to use immunoaffinity columns for purifying tetanus antisera. The short term cost of preparing an immunoaffinity column for purifying tetanus antisera would be balanced by the long term cost of keeping the horses, immunising and bleeding the horses at regular intervals and then not being able to utilise the antisera if it does not reach minimum requirements. Hence the Spherosil immunoadsorbent prepared with tetanus toxoid has a potential use. The disadvantages of affinity chromatography are the cost and the approximately 50% loss of the tetanus antibodies. The advantages are the higher purification factor and therefore the higher probability that the antisera will pass the W.H.O. requirements, and also the elimination of naturally occurring antibodies from the antisera leaving a heterogenous mixture of immunoglobulins that react specifically with tetanus toxoid.

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