AN INVESTIGATION OF THE SYNTHESES OF CELLULOSE AND AGAROSE DERIVATIVES CONTAINING SULPHATE, N-(6-AMINOHEXYL)-2-NAPHTHALENESULPHONAMIDE AND CARBOXYL GROUPS FOR THE PURIFICATION OF PROTEINS

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ABSTRACT

The syntheses of three different cellulose and agarose derivatives were investigated, namely, cellulose sulphate, N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) cellulose and cellulose and agarose with multiple carboxyl groups.

In the case of cellulose sulphate, an attempt was made to find a sulphating reagent and conditions for a commercially convenient method of preparing a cellulose derivative with a sulphate substitution level of 3.5 meq/g. This synthesis was found to require the control of at least one of the following factors: (a) water present in the system, (b) the quantity of sulphating reagent and (c) temperature. The stability of the sulphated cellulose in 0.08M sodium hydroxide at 83°C over 28 days was also evaluated. It was found that the sulphate substitution level decreased linearly over 4 weeks at the rate of 1% per day.

Two routes of preparing 2-ANS-cellulose derivative were studied, namely, (1) the coupling of 2-ANS to epoxide activated cellulose and (2) the coupling of 2-naphthalene sulphonyl chloride (2-NSCl) to diaminohexyl (DAH)-cellulose. Both methods of synthesis were found to be equally feasible. However, the former method required the prior multi-step preparation of 2-ANS, while the latter method was carried out stepwise on the cellulose matrix. The excess reagents were readily washed away before the next step was undertaken. Also, the preparation of 2-NSCl from sodium 2-naphthalene sulphonate was quantitative. The capacity of these 2-ANS-cellulose derivatives for bovine serum albumin (BSA) was also investigated. The products prepared by method 1 showed a much lower capacity (0.05 - 0.38 gBSA/g) for BSA than those prepared by method 2 (0.49 - 0.78 gBSA/g).

The syntheses of cellulose and agarose derivatives containing alpha (A)- and beta (B)-citrylhexamethylenediamine (CM_6D), aspartic acid (Asp) and 6-aminohexylaspartate (Asp-AH) groups were investigated using both epoxide and 1,1'-carbonyldiimidazole (CDI) activation procedures. The use of these products for the purification of bovine lactoferrin (Lf) was assessed. The nature of the binding action of Lf to the CM_6D-matrices was also studied. It was found that (a) high CM_6D substitution level on the matrix, (b) high porosity of the matrix and (c) the removal of additional cationic properties from the matrix by replacing the basic nitrogen linkage resulting from the epoxide activation by a non-basic urethane linkage resulting from CDI activation, led to an increase in the strength of Lf binding to the derivative. The results also suggested that the Lf binding was predominantly ionic in nature. Finally, it was found that Lf purification on A-CM_6D-agarose gave a product of higher purity than that on Asp-agarose and Asp-AH-agarose.

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A-CM$_4$D	alpha-citrylhexamethylenediamine
AH	6-aminohexanoic acid
2-ANS	N-(6-aminohexyl)-2-naphthalenesulphonamide
AR	analytical reagent grade
B-CM$_4$D	beta-citrylhexamethylenediamine
BNS	bis-N,N'-(2-naphthalene sulphonyl)-hexamethylene-diamine
BSA	bovine serum albumin
CDI	1,1'-carbonyldiimidazole
CM$_4$D	citrylhexamethylenediamine
CM-Sephadex	Carboxymethyl-Sephadex
DAH	1,6-diaminohexane
DAH-cellulose	diaminohexyl-cellulose
DEAE-cellulose	Diethylaminoethyl-cellulose
DMF	dimethylformamide
EA-aga	epoxide activated agarose
Fe-NTA	iron (III) nitrilotriacetate
HP-cellulose	hydroxypropylated cellulose
Lf	lactoferrin
Lp	lactoperoxidase
LR	laboratory reagent grade
2-NS	2-naphthalene sulphonyl group
2-NSCl	2-naphthalene sulphonyl chloride
py-SO_3	pyridine sulphur trioxide complex

SECTION 1

GENERAL INTRODUCTION

1.1 Fractionation and purification of proteins and enzymes

The fractionation and purification of proteins and enzymes are necessary in biochemical research. They require the application of high resolution and mild separative techniques. High resolution techniques are required as these molecules are usually found in highly complex mixtures. Proteins and enzymes are sensitive to changes in their environment and the techniques used, therefore, need to be mild so as not to disturb the functional properties of these molecules [1].

The separation of these biomacromolecules is most widely achieved by chromatography [2]. Chromatographic separation is based on the different rate of movement of molecules in two different phases, namely, stationary and mobile phases. The commonly used techniques include ion-exchange, gel filtration, affinity and hydrophobic interaction chromatography.

1.2 Basic principles of ion-exchange, gel filtration, affinity and hydrophobic interaction chromatography

In ion-exchange chromatography [1,3,4], the separation is achieved on the basis of the differing polarity of molecules. The ion exchanger is an insoluble matrix containing covalently bound charged groups. The mobile counter ions associated with the matrix can be reversibly exchanged with other ions of the same charge. Basically, there are two classes of ion exchangers, namely, cationic and anionic ion exchangers. Some of the common functional groups used in ion exchangers for the separation of biomacromolecules are given in Table

1.1. Strong ion exchangers are those where the functional group is completely ionized over a wide pH range while the degree of ionization of the weak ones is more dependent on pH.

Table 1.1 : Some functional groups used in ion exchangers

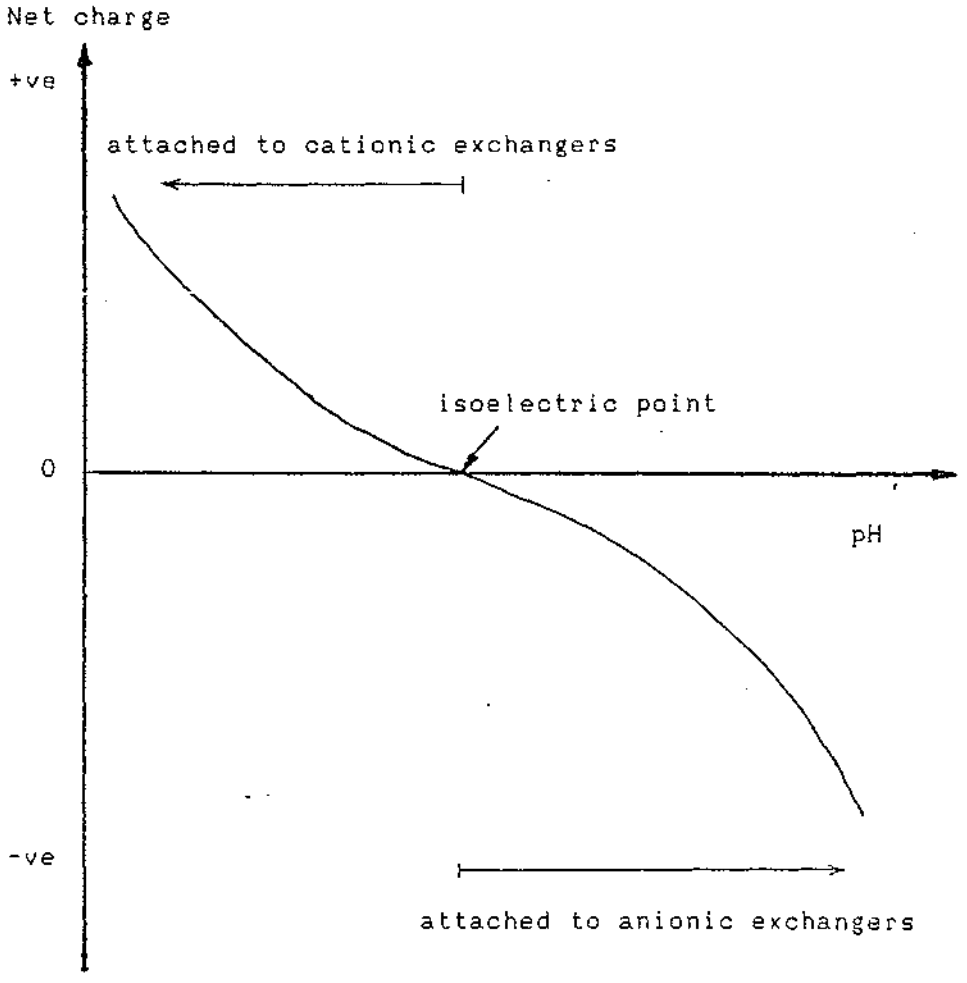
Class	Functional group	Type	Structure
cationic	sulphopropyl (SP)	strong	$-(CH_2)_3SO_3^-$
	phospho (P)	intermediate	$-OPO_3H^-$
	carboxymethyl (CM)	weak	$-CH_2COO^-$
anionic	diethylaminoethyl (DEAE)	weak	$-(CH_2)_2N^+HEt_2$
	quaternary aminoethyl (QAE)	strong	$-(CH_2)_2N^+Et_2C_3H_6OH$

Proteins and enzymes are complex molecules with differing degrees of charge depending on the number of ionic groups present in the polypeptide chain. They are amphoteric in character, that is, they can be either negatively or positively charged. The actual effective charge on them is dependent on the pH and at a particular pH, known as the isoelectric point, the net charge is zero. The class of ion-exchangers selected for the adsorption of these biomacromolecules is dependent on the pH (see Figure 1.1). The adsorption and desorption is then brought about by altering conditions such as ionic strength and pH.

In gel filtration (or size exclusion) chromatography [1,5,6], the separation of the biomacromolecules is brought about by their differing ability to distribute between the mobile phase and the liquid situated in the pores of the matrix. The volume between the

matrix particles is available to all molecules but access to the pore volume of the selected matrix is limited to molecules of smaller sizes. The very large molecules are, therefore, eluted most rapidly while the small molecules can easily enter unhindered into the available pores. They are, thus, subjected to the greatest delay. Hence, molecules are eluted in order of decreasing molecular size.

Figure 1.1 : The net charge of a protein as a function of pH [3]



The term affinity chromatography [7-9] is used to describe separations that are based on biospecific interaction between the biomacromolecules and an immobilized ligand. This ligand may be an

enzyme substrate, enzyme inhibitor, antigen or lectin. Ideally, it is covalently attached to an insoluble inert matrix via a spacer arm of usually six carbon chain length. It will then only interact with the desired biomacromolecule for which it has an affinity. When the adsorption is complete, the impurities are washed off the matrix with the starting buffer. The affinity bound material is recovered by elution with a new buffer of different pH or ionic strength. Alternatively, the elution may be carried out with a buffer containing the soluble ligand or another ligand for which the biomacromolecule has a higher affinity. The latter mode of specific elution is preferred but it is not always possible to apply in practice.

The separation of biomacromolecules by hydrophobic interaction chromatography [9-11] relies on their tendency to associate in aqueous solution. Generally, proteins and enzymes are folded in such a manner as to bury most of the hydrophobic sidechains in the interior of the molecule while most of the polar sidechains are exposed on the surface. Some hydrophobic groups remain exposed at the surface forming hydrophobic patches or pockets. These patches can interact with hydrophobic ligands immobilized onto a suitable matrix to form "hydrophobic bonds". Adsorption of the biomacromolecules by hydrophobic interaction is enhanced with increasing ionic strength and a pH which is close to the isoelectric point. Desorption of the desired protein or enzyme can be achieved by varying ionic strength, pH and the polarity of the eluent. A decrease in the polarity of the eluent, for example, by the addition of ethylene glycol weakens the hydrophobic interaction. Hofstee et al. [12] have suggested the use of an increasing hydrophobicity gradient for binding proteins so that the subsequent elution would be carried out under mild conditions without the danger of denaturation of the bound proteins.

1.3 Characteristics of matrix

The characteristics desirable in a good matrix or support for the chromatographic methods are described below [3,6-8].

1. The matrix should be insoluble under operating conditions. Generally, the isolation and purification of proteins and enzymes is carried out in an aqueous medium. Hence, the matrix should be insoluble in aqueous solution of varying ionic strength and pH.

2. The matrix should be hydrophilic. Hydrophobic interactions should be minimized as they tend to complicate the adsorption of the biomacromolecules and to cause their denaturation. It is best that the matrix is swellable or, at least, highly wettable with water.

3. The matrix should be stable to mechanical, physical, chemical and microbial degradation. This stability should extend over long periods of time and under a range of pH, temperature and ionic strength so as to ensure it can be used repeatedly. Mechanical stability ensures that it is not deformed by the forces exerted by the flow of eluent.

4. The matrix should be inert and neutral. These characteristics are desirable for they eliminate non-specific ionic interaction.

5. The matrix should consist of particles which are rigid, uniform and spherical with good flow properties. The use of irregularly shaped particles results in band broadening because of the unequal paths taken by the biomacromolecules to be separated.

6. The matrix should be porous. This permits unimpaired diffusion of the biomacromolecules and consequently, maximum interaction with the matrix surfaces. In gel filtration chromatography, the pore size of the matrix determines the fractionation range. In affinity chromatography, large pores matrices are particularly important for maximum ligand-biomacromolecule interaction.

7. The matrix should have an ample supply of chemical groups available for covalent coupling with a variety of ligands where a modified matrix is required.

8. The matrix should contain a low density of modifier groups so as to allow the elution of the biomacromolecules under relatively mild conditions and thereby, prevents their denaturation.

It is obvious that no matrix will have all these desired characteristics. There are various types of matrices in use (see Table 1.2) and a discussion on them is found elsewhere [4,6,9]. The matrices used in this project are cellulose and agarose. Their advantages and limitations are briefly discussed below.

Table 1.2 : Some chromatographic matrices in use

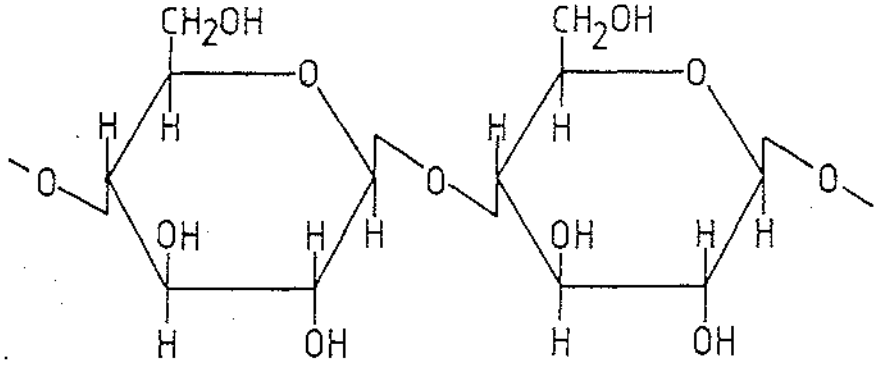
Type	Example
Biopolymers (polysaccharides)	agarose, cellulose, dextran
Synthetic polymers	polyacrylamide, polystyrene
Inorganic materials	silica
Biopolymers/synthetic copolymers	agarose polyacrylamide
Inorganic materials/organic polymers	silica/hydrophilic copolymers

1.4 Cellulose

Cellulose is a polysaccharide containing beta-1,4-linked D-glucose units with occasional 1,6-bonds [13] (Figure 1.2). Native cellulose contains microcrystalline regions interspersed with amorphous regions. The former regions result from extensive hydrogen bonding between the adjacent linear polysaccharide chains. While, in the latter regions, there is less hydrogen bonding. The early commercial native cellulose used was a dense fibrous powder of

irregular shape [3,14,15].

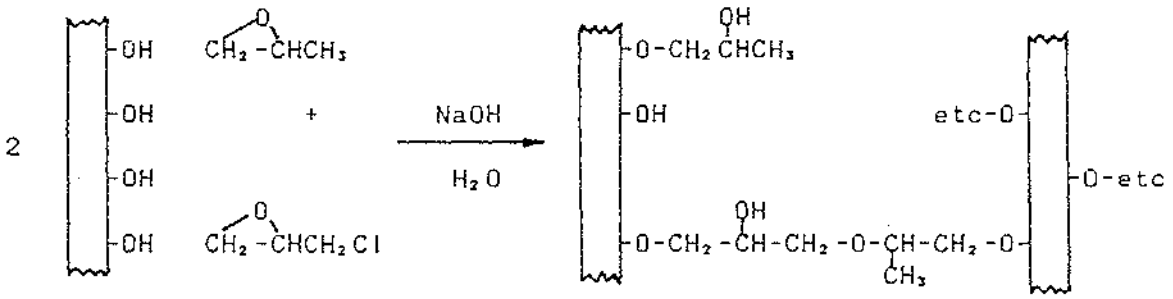
Figure 1.2 : Partial chemical structure of cellulose



Sober and Peterson [16] were the first to use cellulose for the ion-exchange chromatographic separation of proteins. Since then, other forms viz native fibrous [17], microgranular [17], microcrystalline [3] or regenerated [18,19] have become available for various chromatographic applications.

The cellulose matrix used in this project was a cross-linked hydroxypropylated cellulose (HP-cellulose) prepared from regenerated cellulose by reaction with propylene oxide and epichlorohydrin in the presence of sodium hydroxide [20,21] (Figure 1.3). Regenerated cellulose is a particularly robust, resilient and long-lasting form of cellulose capable of withstanding high operating flow rates [19]. The one which was used in this project was made via the xanthate derivative [15,21].

Figure 1.3 : Typical reactions for preparation of cross-linked-HP-cellulose [21]



Cellulose

CL-HP-Cellulose
insoluble in water

The HP-cellulose has previously been used to prepare several derivatives including DEAE-cellulose and CM-cellulose [19,22]. The advantages of using HP-cellulose are [21]:

1. It is hydrophilic and can be easily modified chemically via the OH groups.

2. It is mechanically, chemically and physically stable. It is free of noticeable compression in column applications. It is resistant to dilute aqueous sodium hydroxide and is stable to repeated handling and mechanical stirring.

3. The hydroxypropyl cross-linking of the cellulose gives it gel-like properties, enhanced porosity and stability to microbial degradation compared to the starting regenerated cellulose.

4. It is relatively cheap because cellulose is such an abundant natural material. It has good flow properties.

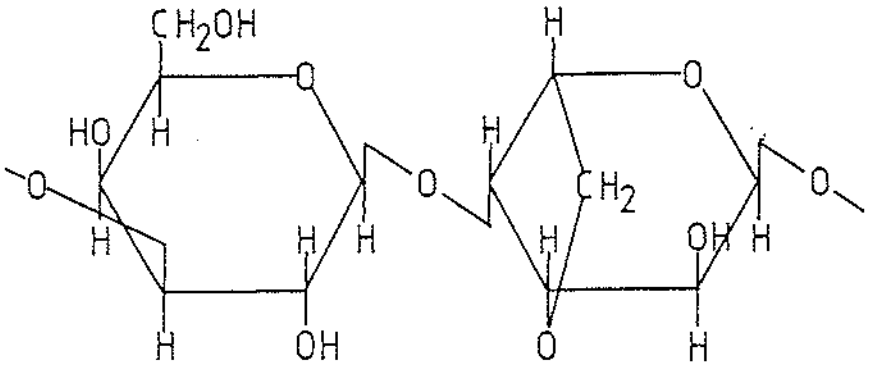
Hence, it is a good choice for industrial scale application.

However, the porosity of the HP-cellulose is much lower than that of agarose [21] which has limited its applications mainly to ion-exchange chromatography.

1.5 Agarose

Native agarose is isolated from a complex mixture of charged and neutral polysaccharides referred to as agar. It is a linear water-soluble polysaccharide consisting of alternating residues of 1,3-linked beta-D-galactose and 1,4-linked-3,6-anhydro-L-galactose [9,13] (Figure 1.4).

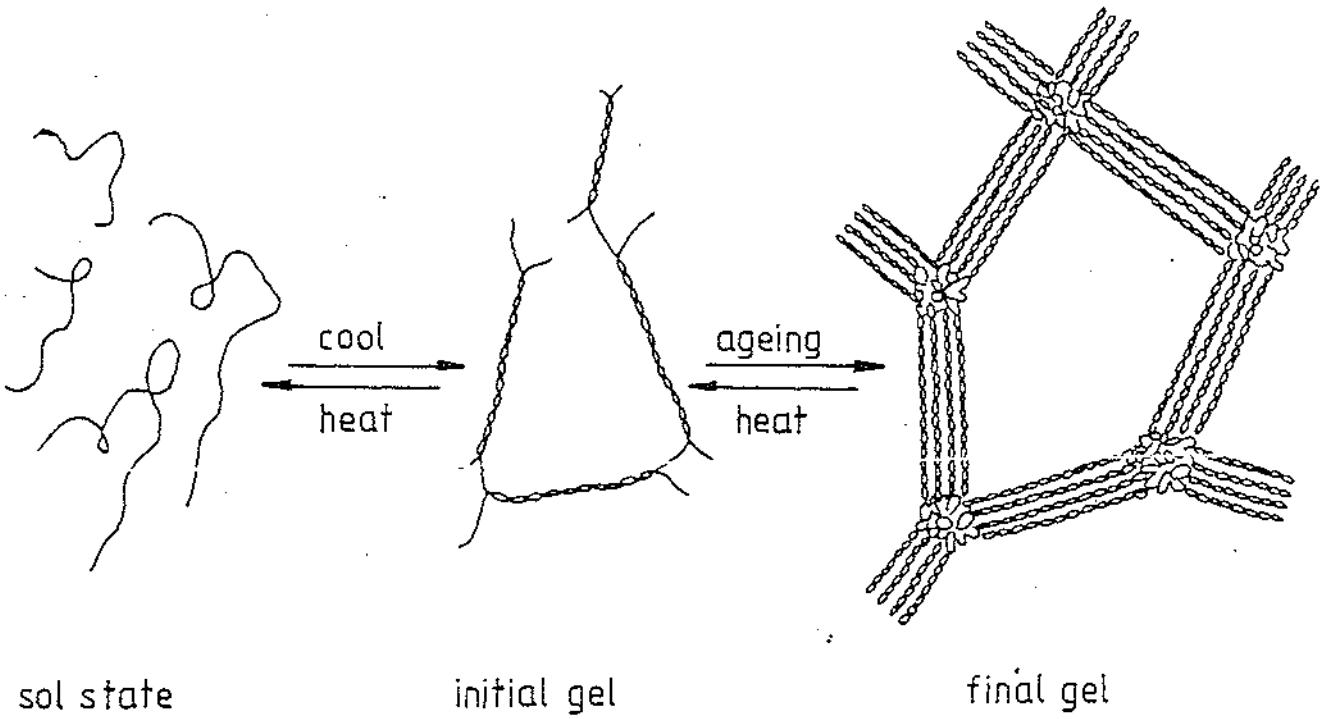
Figure 1.4 : Partial chemical structure of agarose



The agarose gels are available in bead, pellet or spherical forms which are made from the spontaneous gelation of aqueous agarose solutions cooled to below 50°C [9]. The gel structure is thought to be composed of highly ordered fibres or bundles resulting from the aggregation, through hydrogen bonding and hydrophobic interaction, of

the double helix formed by the polysaccharide chains (Figure 1.5). The resulting structure is macroporous and hence, allows the relative free diffusion of biomacromolecules [15,23].

Figure 1.5 : Schematic representation of gelation of agarose



The agarose matrices used in this project were Sepharose 6B and Sepharose 6B-CL. Sepharose 6B contains about 6% agarose [5]. Sepharose 6B-CL is the cross-linked product of Sepharose 6B. It is made from the latter by reaction with 2,3-dibromopropanol in the presence of alkali to produce cross-linking. It is then subjected to alkaline hydrolysis under reducing conditions to remove sulphate groups and give a product with an extremely low content of ionizable groups [3,5].

Like the celluloses, these matrices are hydrophilic and contain readily modifiable OH groups. They are mechanically, chemically and

physically stable (Table 1.3). Because of their macroporous structure, their porosity is much higher than that of cellulose [9]. Their molecular weight exclusion limit is of the order of 4 x 10^6 for proteins [5]. Generally, they do not exhibit non-specific binding of proteins. However, any weak non-specific interactions noted could be due either to the residual sulphate and carboxyl groups via the ester in position 6 or to hydrophobic interactions, probably resulting from the ether bridge of the anhydro-galactose unit [9]. They exhibit good flow properties. Generally, they are resistant to microbial attack due to the unusual sugar 3,6-anhydro-L-galactose. However, an antimicrobial agent is usually added in prolonged experiments and during storage in the swollen state [5].

Table 1.3 : Stabilities of Sepharose 6B and 6B-CL [5,9]

	Sepharose 6B	Sepharose 6B-CL
pH	4 - 9	3 - 14
Temperature range (°C)	below 40	below 70
Solvents	stable in aqueous solutions with high ionic strength, urea, guanidine.HCl, detergents; DMF-H_2O (1:1), Ethylene glycol-H_2O (1:1)	stable in aqueous solutions with high ionic strength, urea, guanidine.HCl, detergents over pH 3-11; organic solvents.
Sterilization	not autoclavable	autoclavable at pH 7, 120°C
Chaotropic ions (eg. KSCN)	low stability	high stability

Sepharose 6B is sensitive to extremes of pH, temperature and solvents and unstable to continuous mechanical disruption. A leakage of the covalently attached ligand may also result on prolonged washing [24]. The cross-linked product is more stable towards extremes of pH, temperature, solvents and mechanical disruption [5]. Generally, they may be hydrolyzed under oxidizing conditions [3,5]. Compared to the celluloses, they are very much more expensive and less robust to mechanical disruption. Hence, they are less suitable for industrial usage.

1.6 Nature of project

This project was an investigation of the synthesis of three different types of matrix derivatives, namely,

1. cellulose derivative containing sulphate groups (section 2),
2. cellulose derivative containing N-(6-aminohexyl)-2-naphthalene-sulphonamide groups (section 3) and
3. cellulose and agarose derivatives containing carboxyl groups (section 4).

It was the intention of the work in sections 2 and 3 to establish the optimal conditions for preparing the derivatives stated. This work was carried out with the ultimate aim of the products being useful for industrial applications. This orientation influenced the direction of the project. Whenever possible, the attempt at synthesis and the choice of starting materials were directed to devising methods of synthesis that were simple and cost effective.

The work reported in Section 4 involved not only the preparation of special carboxylic acid derivatives of various matrices but also an investigation of their use for purifying bovine lactoferrin.

SECTION 2

AN EMPIRICAL STUDY OF THE SYNTHESIS OF SULPHATED CELLULOSE

INTRODUCTION

2.1 Sulphated cellulose and its applications

The usual derivatives of cellulose used as cation exchangers for protein chromatography are phosphate, carboxylate and sulphonate. The sulphated cellulose is a less commonly used cation exchanger. But, it has been used in this laboratory for the selective removal of lipoproteins from blood plasma or serum [25-27].

Blood lipoproteins [28] play an important role in several biological activities such as lipid metabolism and the inhibition of procoagulant activity of tissue factor. Also, plasma lipoproteins concentration profile is known to alter significantly in diabetes mellitus, hypothyroidism, glycogen storage defect, obstructive jaundice and coronary artery disease. They are biochemically and medically important. The sulphated cellulose readily isolates these blood lipoproteins as well as provides a simple method for their quantification.

In addition, the fractionation and recovery of the various other types of proteins from blood is commercially important. However, the selective recovery of these other proteins is often complicated by the presence of lipoproteins. The sulphated cellulose developed is a high capacity ion exchanger capable of selective removal of lipoproteins from serum.

Recently, a cross-linked, sulphated poly (vinyl alcohol) has been reported as being used for the same purpose [29]. Also, the

sulphated cellulose has been used in the purification of viruses [30-32].

2.2 Sulphation of HP-cellulose

Sulphated polysaccharides are most often prepared by reacting the polysaccharides with a Lewis base complex of sulphur trioxide under anhydrous conditions [33]. Sulphur trioxide is a strong Lewis acid and combines readily with a Lewis base to form an "adduct" or "complex" [34]. The Lewis bases used range from very weak ones such as dioxane, DMF or DMSO to stronger ones like the tertiary amines.

$$R_3N + SO_3 \longrightarrow R_3N^+\text{-}SO_3^- \qquad (2\text{-}1)$$

The reactivity of the sulphating reagent decreased with increasing strength of the Lewis base with which the sulphur trioxide is combined. In the sulphation of polysaccharides, the sulphur trioxide is "released" and the base forms the salt of the new monoester of sulphuric acid, thereby avoiding acidic conditions which may otherwise hydrolyze the polysaccharides. For example,

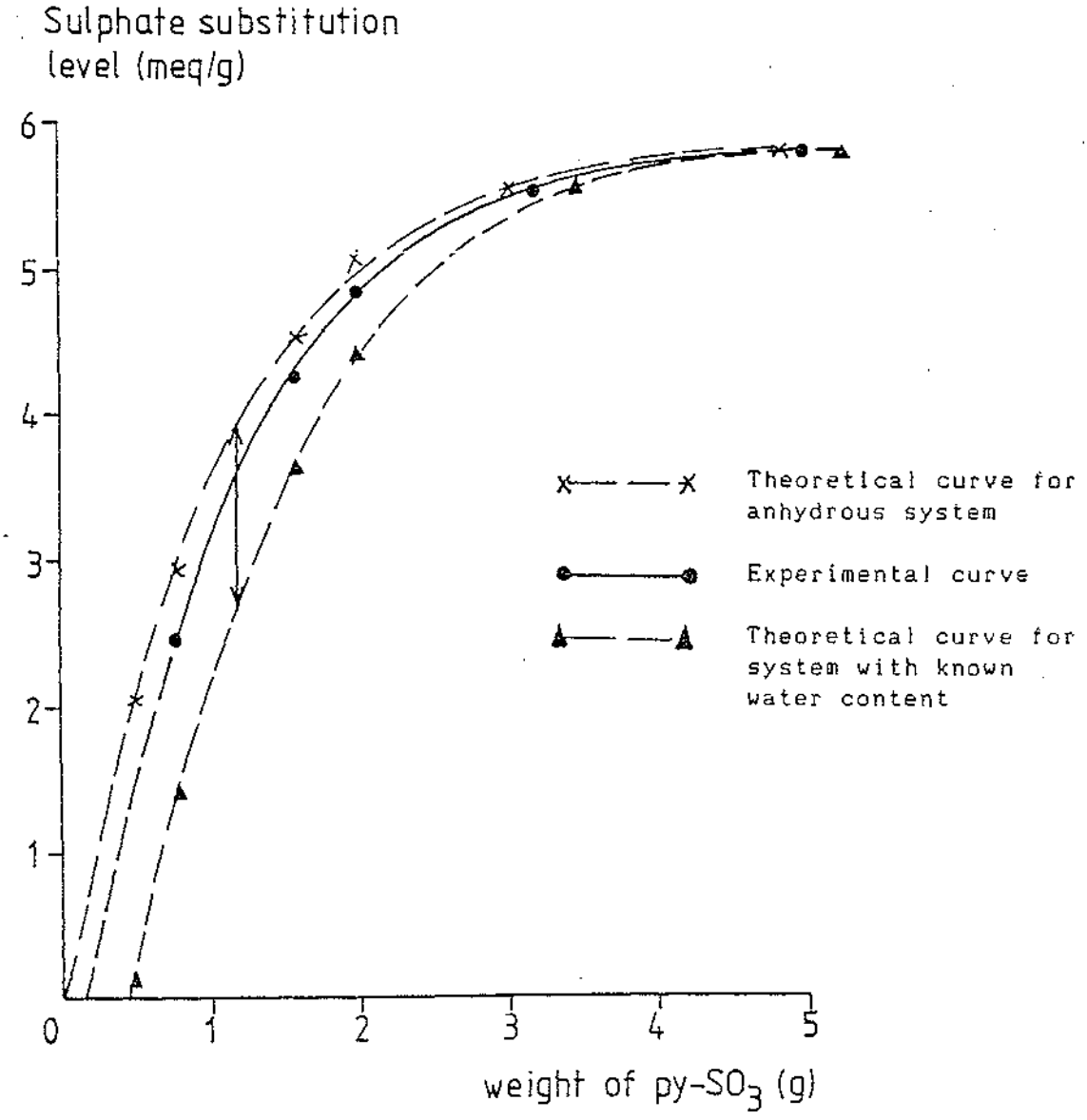
$$\text{Cell-OH} + R_3N^+\text{-}SO_3^- \longrightarrow \text{Cell-}OSO_3^-\text{-}R_3NH^+ \qquad (2\text{-}2)$$

2.3 Commercial preparation of sulphated HP-cellulose

The only insoluble sulphated celluloses currently available commercially are INDION™ sulphated celluloses (SA 5.2 and SA 3.5) manufactured by Waitaki International Biosciences Limited [35]. They are made from HP-cellulose (see Section 1.4) by reaction with pyridine sulphur trioxide complex (py-SO_3) in DMF. The extent of the sulphation of HP-cellulose is determined by the quantity of sulphating reagent

used as shown in Figure 2.1 (see also Appendix 1). The solid line shown in Figure 2.1 comes from actual experimental results based on a set of reactions of HP-cellulose (1g, batch 1248) with py-SO_3 at 20°C for 23 hours [36]. The dotted line to the left of it is a theoretical one based on a completely anhydrous system. While, the one to its right is calculated for a system where the HP-cellulose and DMF used contain 2% and 0.1% moisture respectively.

Figure 2.1 : Effect of py-SO_3 on sulphate substitution



The intercept on the horizontal axis and the position of the solid line is thus dependent on the amount of moisture in the system, for water consumes py-SO_3 [37].

$$py^+\text{-}SO_3^- + H_2O \longrightarrow pyH^+HSO_4^- \qquad (2\text{-}3)$$

The freeze-dried HP-cellulose typically contains 0.5% moisture but, if left exposed to the atmosphere for any length of time, it absorbs another 5 to 6% moisture [25]. The DMF used has a water content of less than 0.1% but it could contribute significantly to the actual position of the solid line shown in Figure 2.1.

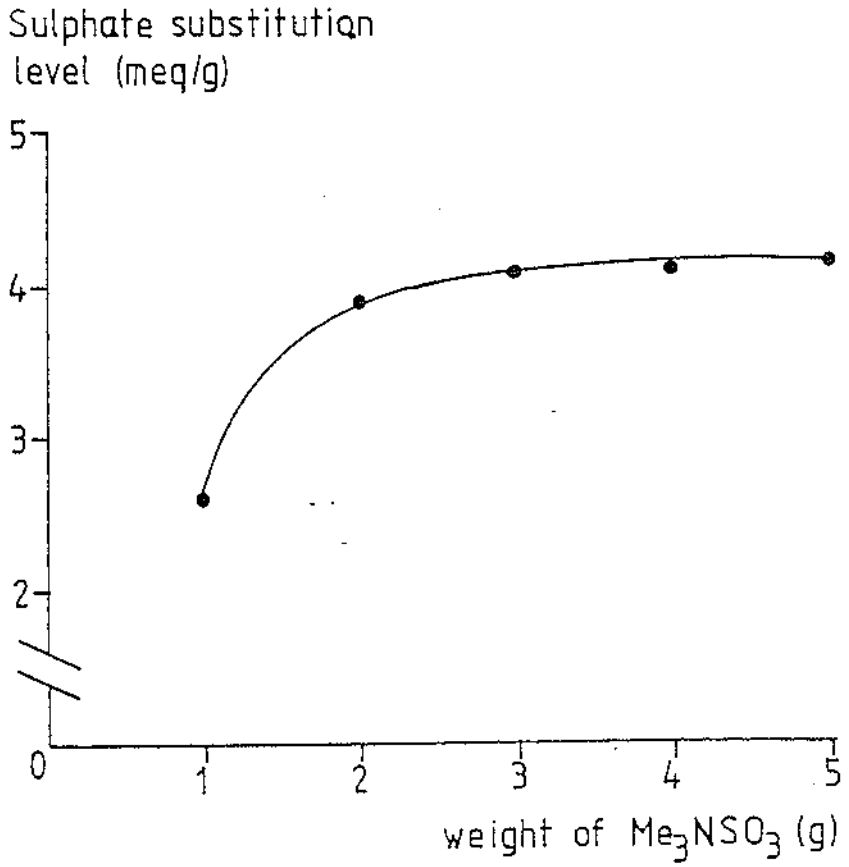
SA 5.2 made by Waitaki has a sulphate substitution of 5 to 5.5 meq/g. This level of substitution is easily prepared reproducibly by using an excess of py-SO_3 complex, that is, greater than 4g/g HP-cellulose (see Figure 2.1). The levelling off in the substitution obtainable presumably results after all the readily available hydroxyl groups have been esterified.

On the other hand, SA 3.5 has a sulphate substitution of 3 to 4 meq/g. It is much more difficult to prepare reproducibly because of the effect of small amounts of water if present. The solid line in Figure 2.1 is very steep in the 3 to 4 meq/g region. The dotted lines show the variation that could occur for the HP-cellulose with 0 to 2% moisture present and the DMF from 0.0 to 0.1% water content. A fixed amount of py-SO_3 added to the reaction, for example, 1.2g complex/g HP-cellulose could give a product with 2.8 to 3.9 meq/g depending only on the moisture content in the system.

An obvious solution to this difficulty lies in the quality control of raw materials going into the reaction. This has been achieved by Waitaki. However, previous work has indicated an

alternative possibility might exist. It has been found that the reaction of HP-cellulose with an excess of trimethylamine sulphur trioxide complex (Me_3NSO_3) in DMF gave a product with a sulphate substitution of about 4 meq/g at room temperature as shown in Figure 2.2 [36]. It was found that even when the sulphating reagent, Me_3NSO_3 was used in excess, the substitution level did not rise beyond 4 meq/g. Since Me_3N is a stronger base than pyridine, its SO_3 complex is less reactive than that of pyridine. It was hoped that by varying the base used in the reaction, it would be possible to further restrict the degree of sulphation to about 3.5 meq/g.

Figure 2.2 : Effect of Me_3NSO_3 on sulphate substitution



2.4 Aims of this study

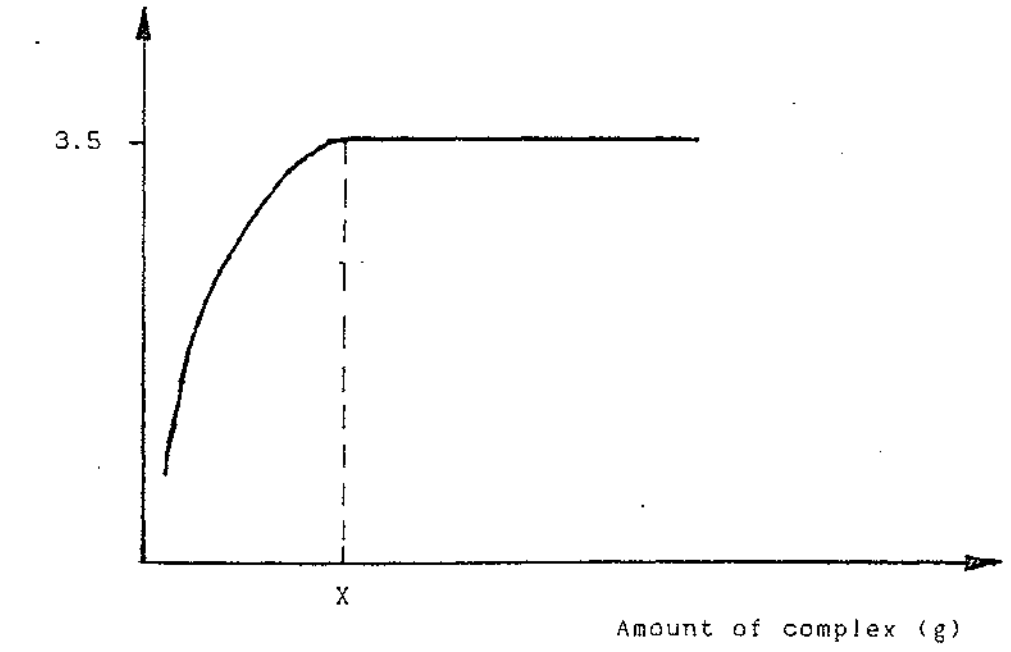
2.4.1 Preparation of 3.5 meq/g sulphated cellulose

Since the present method for preparing INDION™ SA 3.5 is critically dependent on the quantity, the purity and perhaps, the age of the sulphating reagent and the solvent (see Section 2.3), an attempt was made to find a sulphating reagent and conditions for a commercially convenient method of synthesis where:

1. an excess of the sulphating reagent could be used so as to eliminate the effect of small amounts of water, and
2. only readily available or relatively cheap reagents were used.

It was hoped that a set of reagents might be found where under certain conditions, the sulphate substitution level might level off to give a plateau region at about 3.5 meq/g as shown in Figure 2.3.

Figure 2.3 : Ideal effect of sulphur trioxide complex on sulphate substitution



The two commercially available sulphation complexes are py-SO_3 and Me_3NSO_3. They are obtained as stable but moisture sensitive powders. Since, from previous work (see Section 2.3), Me_3NSO_3 was found to be still quite reactive, the effect of the presence of other slightly stronger bases on the sulphation reaction was investigated. These bases and their pKa's are listed in Table 2.1.

Table 2.1 : Basicity constants of various amines [38]

amine	pKa (in H_2O, 20°C)
Pyridine	5.19
Trimethylamine	9.91
Dimethylethylamine	10.16
Triethylamine	10.78

2.4.2 Stability of sulphated celluloses

An additional aim of the work reported in this section was to determine the stability of the sulphated HP-cellulose as alkyl sulphates can be hydrolyzed, for example,

$$Cell\text{-}OSO_3^-Na^+ + NaOH \longrightarrow Cell\text{-}OH + Na_2SO_4 \qquad (2\text{-}4)$$

Since a potential commercial user was interested in the life of INDION™ SA products, the conditions under which they might be hydrolyzed were investigated.

EXPERIMENTAL

2.5 Materials and equipment

The HP-cellulose 8-50 which is now marketed as "INDION™ HP-10 cellulose" was obtained from Waitaki International Biosciences Ltd., Christchurch, New Zealand. Trimethylamine-sulphur trioxide complex, triethylamine and dimethylethylamine were obtained from Aldrich-Chemie, Steinheim, West Germany. Pyridine-sulphur trioxide complex was obtained from Aldrich Chemical Co., Inc., Wisconsin, USA. Sodium chloride (AR) was obtained from May and Baker New Zealand Ltd., Mount Maunganui, New Zealand. Sodium hydroxide concentrate (AR) for making 1.00M standard solution was obtained from May and Baker Ltd., Dagenham, England. Sodium borohydride (AR) was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Dimethylformamide (DMF) (AR) was obtained from Ajax Chemicals, Sydney, Australia. It was dried, degassed and redistilled over calcium hydride before use.

All titrations were carried out using an Autoburette ABU 11 from Radiometer, Copenhagen, Denmark.

2.6 Preparation of sulphated HP-cellulose

2.6.1 Method of preparation

The freeze-dried cellulose (1g, batch 1177) was soaked in DMF (25ml) for 1 to 2 hours at 60°C or overnight (16 to 20 hours) at room temperature. The sulphur trioxide complex with trimethylamine (5g) or with pyridine (5.72g) was added to the pre-swollen cellulose in DMF. A mole equivalent of Et_3N(5ml) or Me_2NEt(1.9ml) was also added when required. The reactants were allowed to mix for 24 to 48 hours at a constant temperature (4 to 60°C).

Then the cellulose derivative obtained was solvent exchanged

from DMF to water as given in Appendix 2. The derivative was converted into the sodium form for storage by washing it with 0.5M sodium chloride solution followed by deionized water to remove excess sodium chloride, for example,

$$Cell\text{-}OSO_3^-NH^+Me_3 \; + \; NaCl \longrightarrow Cell\text{-}OSO_3Na^+ \; + \; Me_3N^+HCl^- \qquad (2\text{-}5)$$

2.6.2 Determination of sulphate substitution level

The substitution level of the derivative was determined by the following method. A moist sample (5 to 10g) was washed with 1M hydrochloric acid (ca. 100ml) and then with deionized water (until neutral). It was dispersed in 0.5M sodium chloride solution (ca. 10ml) and titrated with 1.00M sodium hydroxide solution to pH 8. The sample was transferred quantitatively onto a weighed sintered glass funnel, washed with deionized water and freeze-dried. The sulphate substitution level (in meq/g) was determined (see Appendices 3 and 4).

2.7 Stability of HP-cellulose sulphate

The cellulose sulphate, (60g moist sample, batch 1191) was washed with 0.5M sodium chloride solution to ensure that it was in the sodium form followed by deionized water and drained on a filter. A portion (5g) of the sample was weighed into a vial (25ml capacity). Eight such vials were prepared. A solution (20ml) of 0.1M sodium hydroxide containing 0.1% sodium borohydride (w/v) was added to each of the eight vials. The resulting mixtures were placed in an oven maintained at 83°C. They were mixed occasionally.

At the commencement of the stability test, the substitution levels of two samples were also determined. At weekly intervals, two vials were removed from the oven and the cellulose sulphates therein

were transferred to sintered glass filters where they were washed with deionized water. The substitution level for each sample was then determined according to the method described in section 2.2.3. The moist HP-cellulose sulphate(5g) contained about 4.5g water. When this amount of water was taken into consideration, the effective concentration of sodium hydroxide in the stability test worked out to be 0.08M.

RESULTS AND DISCUSSION

2.8 Effect of variation of temperature and sulphating reagent on degree of sulphation

2.8.1 Introduction

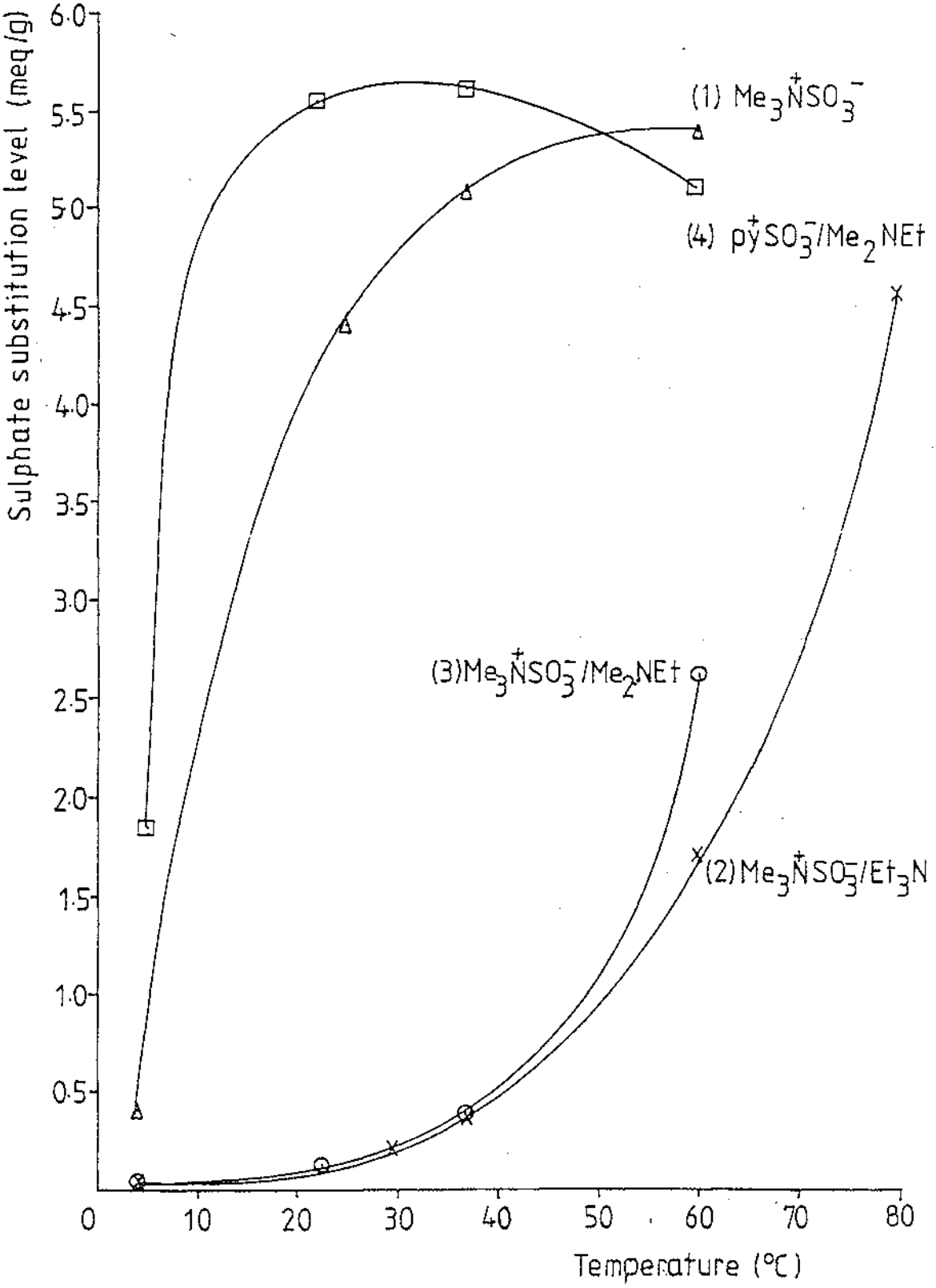
For previous work (see, for example, Figure 2.2), the sulphation reactions were studied under constant temperature. The effect of temperature on the reaction was not known. This was investigated. Using excess sulphating reagent, reactions were undertaken at temperatures from 4 to 60°C for 27 to 48 hours. The reaction at 4°C was allowed to react for 48 hours because it was expected that the reaction would be slower. Reaction at 20°C with py-SO_3 was known to be complete in 24 hours [36]. The results are shown in Table 2.2 and Figure 2.4.

Table 2.2 : Sulphation of HP-cellulose at various temperatures with different sulphating reagents

Sulphating reagent	Time (hr.)	Temperature (°C)	Substitution level (meq/g)
1. Me_3NSO_3 (5g)	48	4	0.41
	27	25	4.41
	27	37	5.08
	27	60	5.39
2. Me_3NSO_3 (5g)/ Et_3N (5ml)	48	4	0.05
	28	29.5	0.22
	28	37	0.37
	21	60	1.71
	26	80	4.59
3. Me_3NSO_3 (5g)/ Me_2NEt (1.9ml)	48	4	0.05
	27	22.5	0.14
	27	37	0.39
	27	60	2.61
4. py-SO_3 (5.72g)/ Me_2NEt (1.9ml)	48	4	1.85
	28	22	5.55
	28	37	5.61
	28	60	5.10

Notes :
1. Reaction conditions : 1g of HP-cellulose in 25ml of DMF.
2. The amines and the base.SO_3 complexes were in mole equivalence.

Figure 2.4 : Effect of temperature on sulphate substitution level using different sulphating reagents



2.8.2 Effect of temperature on esterification reaction

By using excess sulphating reagent, the reaction was no longer moisture sensitive. However, it was found that it was temperature sensitive (see graph 1, Figure 2.4). When excess Me_3NSO_3 was used, the sulphate substitution level increased rapidly with temperature initially from 0.41 to 4.41 meq/g over the range of 4 to 25°C. It continued to rise further with increasing temperature and finally levelled off at about 5.4 meq/g at 60°C. The plateau region occurred above the desired 3.5 meq/g region (cf. Figure 2.3). But, the results showed that Me_3NSO_3 could still be used in excess to achieve a substitution of 3.5 meq/g so long as the temperature for the sulphation reaction was accurately controlled at 20 ± 1°C for 27 hours.

It was assumed that the reaction was complete after 27 hours on the basis of the previous results shown in Figure 2.2 where the final concentration changes of Me_3NSO_3 beyond 2g did not affect the substitution obtained. This would indicate that the less reactive hydroxyl groups did not react further at room temperature. However, this was not confirmed. This effect of temperature had not previously been observed with py-SO_3 complex since it is more reactive and achieved the 5.4 meq/g substitution levels readily at room temperature.

2.8.3 Effect of adding other bases to the esterification reaction

It was thought that it might be possible to restrict the degree of sulphation and cause it to level off at the desired 3.5 meq/g region if a stronger base such as Et_3N were used. However, Et_3NSO_3 cannot be bought. It can be made with SO_3 (stabilized) and Et_3N but the SO_3 (stabilized) is difficult to ship and handle. Furthermore, there was the problem of air pollution associated with the preparation

of the complex. Waitaki preferred to buy the complex.

Consequently, it was decided to repeat the reaction with Me_3NSO_3 in the presence of a mole equivalent of Et_3N. Triethylamine, being a stronger base (see Table 2.1), would compete for the sulphur trioxide released and hopefully, decreased the reactivity of Me_3NSO_3. This worked but too well. It resulted in little or no reaction below 37°C (see graph 2, Figure 2.4). At higher temperatures, the sulphate substitution rose steeply through the desired 3.5 meq/g region.

The addition of a mole equivalent of Me_2NEt, which was a slightly weaker base than Et_3N, to the reaction produced essentially the same result except that the increase in sulphate substitution was even steeper beyond 37°C (see graph 3, Figure 2.4).

It would appear from these results that there was little hope of achieving 3.5 meq/g easily with any of these reagents in excess (or combinations of reagents) without strict temperature and possibly reaction time control. This being the case, the use of excess Me_3NSO_3 for the sulphation reaction would be the most suitable. It would be relatively simple to maintain a constant temperature of 20°C for up to 27 hours to produce the desired product.

One final attempt was made using a combination of excess py-SO_3 with a mole equivalent of Me_2NEt for the sulphation. But, these conditions were found to be too reactive (see graph 4, Figure 2.4). The use of excess py-SO_3/Me_2NEt in the reaction to produce the desired 3.5 meq/g product would require extensive cooling to below 10°C and again, the steepness of the slope in the 3 to 4 meq/g region indicated the need for very strict temperature control. Hence, this was abandoned.

2.8.4 Conclusion

It was not possible to find a method of preparing INDION™ SA 3.5 that could be used without controlling at least one of the following factors, (i) water present in the system, (ii) the amount of sulphating reagent in the reaction and (iii) temperature of the reaction. Further investigation would require the use of less common reagents for the reaction. This was thought not to be worthwhile.

The best alternative to Waitaki's current method of preparing INDION™ SA 3.5 with limited amount of py-SO_3 under controlled anhydrous conditions would be to use excess Me_3NSO_3 with strict temperature control around 20°C for 27 hours. Further studies would be needed to see if the reaction time was important. This alternative to Waitaki's existing method would seem to have just as many disadvantages ie. excess sulphating reagent is needed and temperature control. Both can be avoided with py-SO_3 so long as moisture content of the starting HP-cellulose (HP-INDION™) and the solvent is monitored.

2.9 Stability of sulphated HP-cellulose

2.9.1 Introduction

The stability of the ester bond in the sulphated HP-cellulose was investigated to determine how labile the sulphate groups were to hydrolysis (see reaction 2-4). Preliminary tests with HP-cellulose sulphate (batch 1191) in 0.08M sodium hydroxide at 72°C did not show any consistent trend in the loss of sulphate groups over 28 days nor in 0.8M sodium hydroxide at 72°C over 4 days [39]. This HP-cellulose sulphate had a substitution level of 4.33 meq/g and it was decided to repeat the tests at a higher temperature of 83°C. The addition of 0.1M sodium hydroxide to the moist sample of the HP-cellulose sulphate gave

a final concentration of 0.08M sodium hydroxide (see Section 2.7). The addition of 0.1% sodium borohydride (w/v) was used to limit degradation of the cellulose by reducing any aldehyde end groups present. The results of these tests are summarized in Table 2.3 and Figure 2.5.

2.9.2 Results of stability test on HP-cellulose sulphate

As shown in Figure 2.5, the decline in the substitution level over 4 weeks showed an approximately linear relationship. The overall loss of sulphate groups after 4 weeks was about 26%. It is unlikely that any practical application would require such extreme conditions for this length of time. But for cleaning purposes, it should be possible to use 0.1M sodium hydroxide safely at 60 to 65°C for short periods of time (hours) on a regular basis without significant damage to the INDION™ SA products.

2.9.3 Conclusion

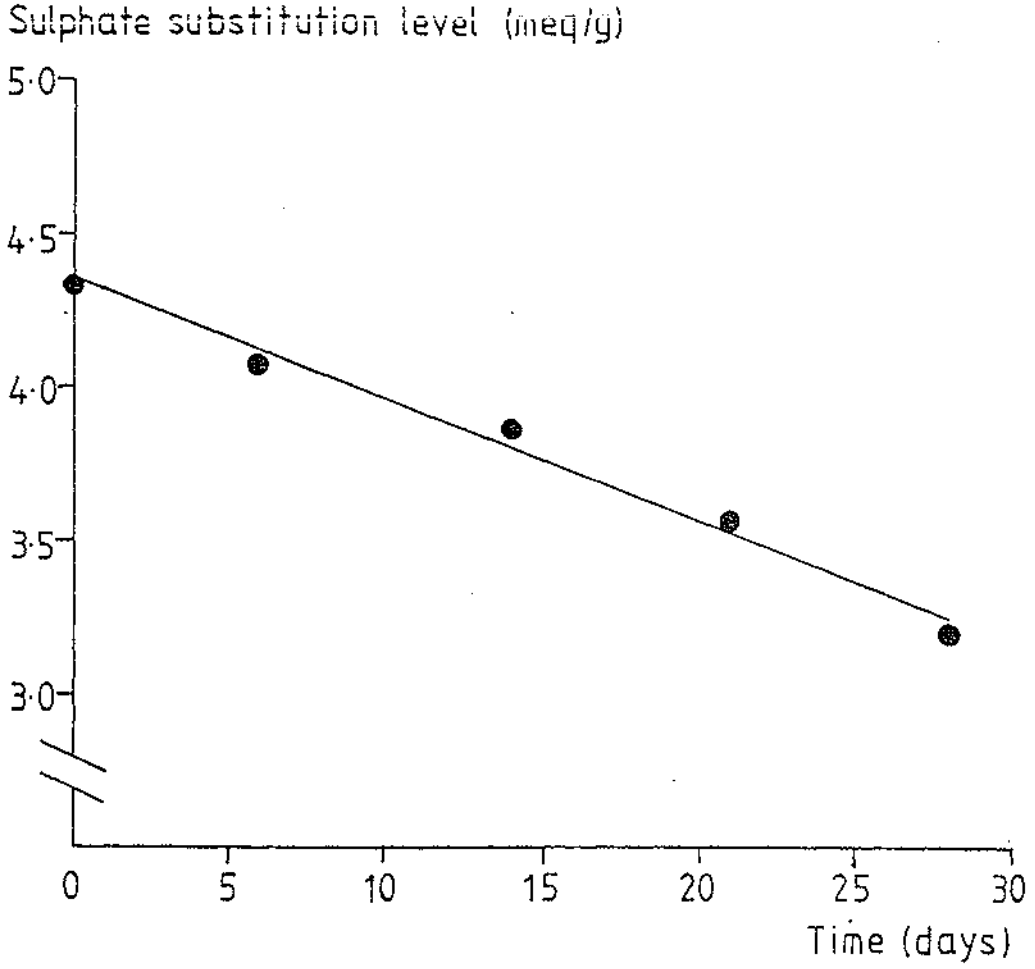
It was found that temperatures above 60°C were necessary to hydrolyze the sulphate ester groups when 0.1M sodium hydroxide was added to sulphated cellulose. Even then, days rather than hours were required to cleave a significant number of groups ie. 1% per day. This finding should allow any commercial user to clean the products at 60-65°C for short durations (hours) to remove irreversibly adsorbed proteins.

Table 2.3 : Stability of sulphated cellulose in 0.08M NaOH at 83° C

Time (days)	Sulphate substitution level (meq/g) 1	2	Mean
0	4.33	-[1]	4.33
6	4.09	4.05	4.07
14	3.76	3.98	3.87
21	3.53	3.60	3.57
28	3.12	3.28	3.20

Note :
1. A low value of 4.09 meq/g was obtained. This was discarded on the basis of previous studies (39).

Figure 2.5 : Stability of sulphated cellulose in 0.08M NaOH at 83° C



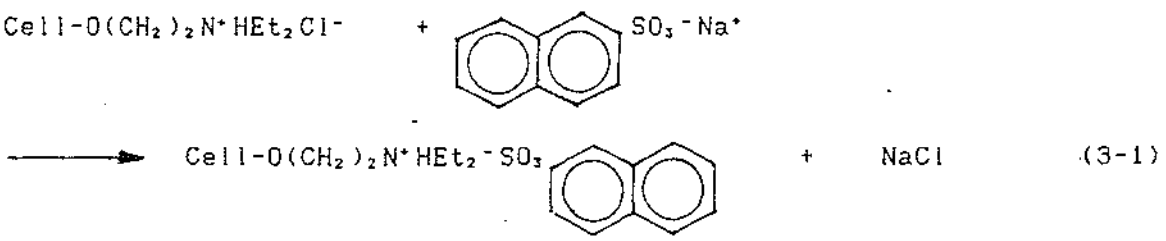
SECTION 3

AN INVESTIGATION OF THE SYNTHESIS OF N-(6-AMINOHEXYL)-2-NAPHTHALENE SULPHONAMIDE CELLULOSE

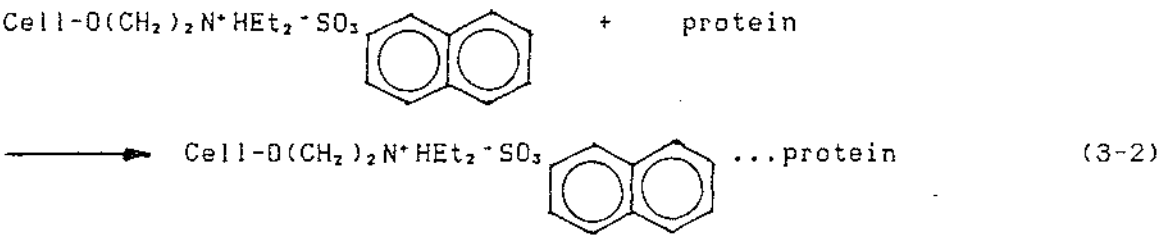
INTRODUCTION

3.1 Nature of the problem

In a potential commercial application [40], DEAE-cellulose in its 2-naphthalene sulphonate form is used to purify a protein as shown by the following scheme.



(3-1)



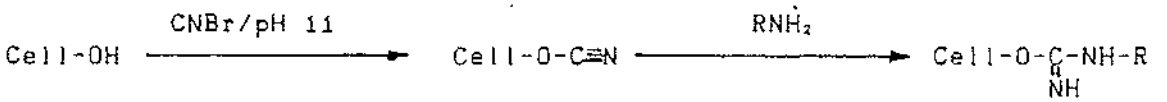
(3-2)

The identity of the protein has not been disclosed. It is believed to bind to the naphthalene ring via an affinity type interaction. When the protein is eluted off the DEAE-cellulose with a sodium chloride gradient, it is contaminated with 2-naphthalene sulphonate which is not surprising since the reverse of reaction 3-1 above takes place.

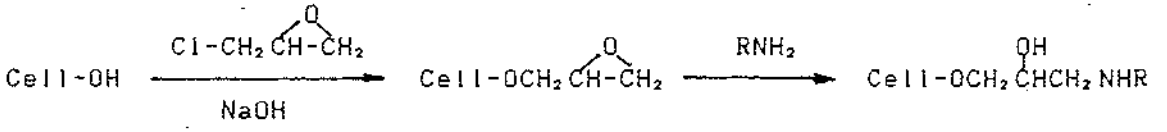
This problem of contamination of the eluted protein would be avoided by covalent immobilization of 2-naphthalene sulphonyl group onto cellulose. It was the aim of this work to achieve such a covalent link by a simple procedure which could be used on a commercial scale.

3.2 Covalent attachment of the 2-naphthalene sulphonyl group (2-NS) onto cellulose

There are many methods used for immobilizing ligands onto insoluble polysaccharides for affinity chromatography [13]. Most of them involve the conversion of some of the hydroxyl groups present to groups which are reactive towards nucleophilic amines, for example,



(3-3)



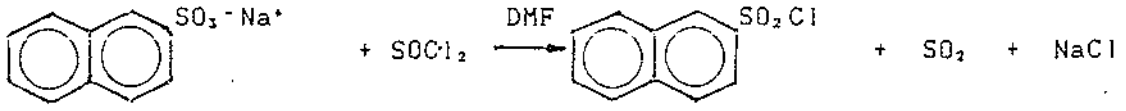
(3-4)

The latter method using epichlorohydrin was chosen for this work because it used cheap chemicals to give a relatively stable epoxide derivative (I). Furthermore, the production of the epoxide derivative of cellulose (epoxide activated cellulose) is well established by Waitaki International Biosciences Ltd. [41] and there is a considerable amount of background information available on this method of activation. By varying the ratios of cellulose, sodium hydroxide and epichlorohydrin, substitution levels up to 1.5 meq/g can be achieved [36]. For this work, the epoxide activation needed to be kept low (0.5 meq/g to 1.0 meq/g) to ensure a more porous matrix and free

interaction of the protein with the covalently bound ligand.

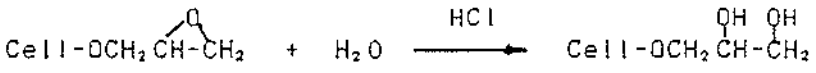
Two different ways of attaching the 2-NS groups onto HP-cellulose via the epoxide derivative were investigated. These are outlined in Figure 3.1. Both schemes give the same final product in which the 2-NS group is coupled to the cellulose via a 1,6-diaminohexane (DAH) spacer arm. This is a standard technique where affinity type interactions are involved so that the protein can gain better access and correct alignment with the ligand [9].

The synthesis of 2-naphthalene sulphonyl chloride (2-NSCl) used in both Scheme 1 and Scheme 2 (Figure 3.1) is straightforward. It is prepared by reaction between sodium 2-naphthalene sulphonate and thionyl chloride in DMF at 10 to 12°C [42].



(3-5)

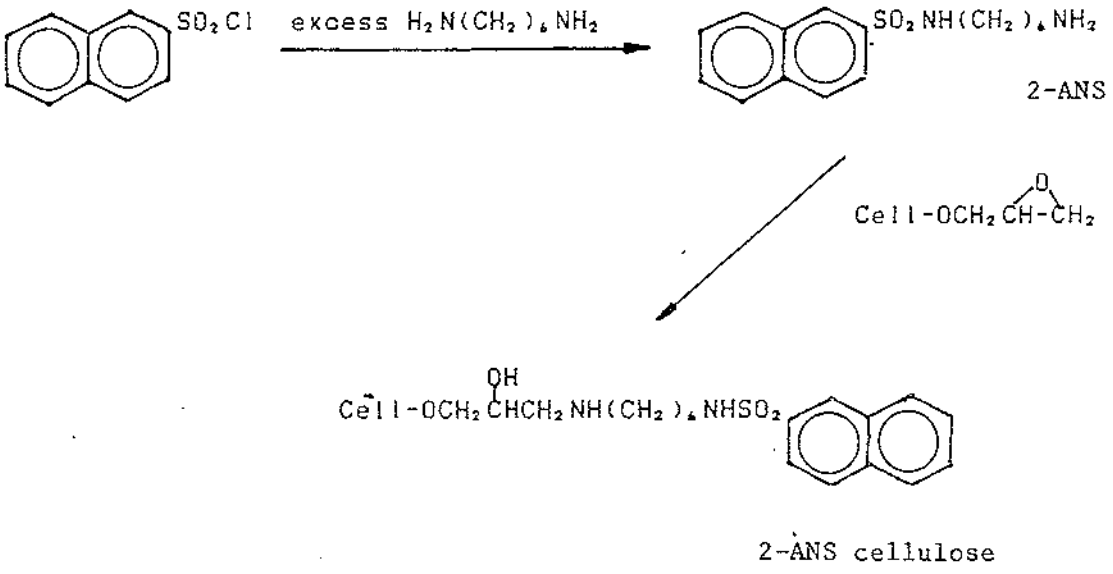
In Scheme 1 (Figure 3.1), only the desired N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) groups are coupled to the epoxide activated cellulose while any remaining unreacted epoxide groups are readily cleaved at the finish by acid hydrolysis.



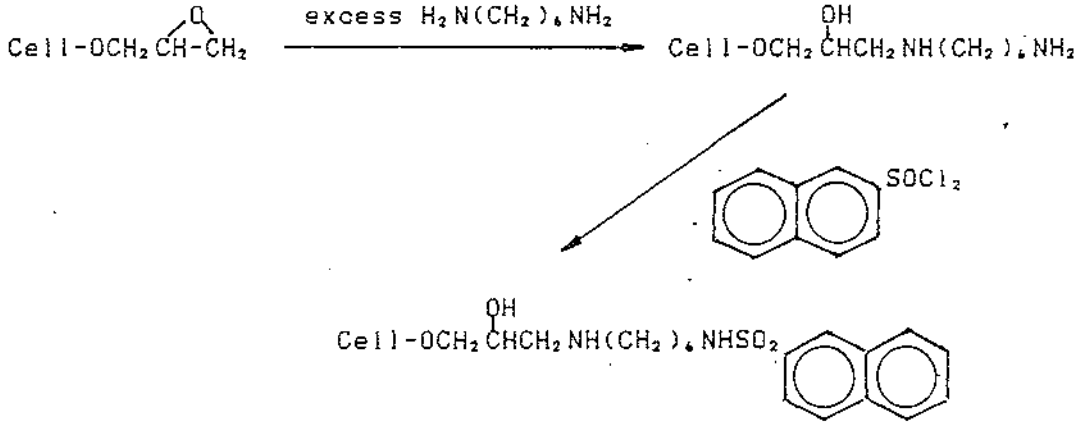
(3-6)

Figure 3.1 : Covalent attachment of 2-NS group onto cellulose

Scheme 1

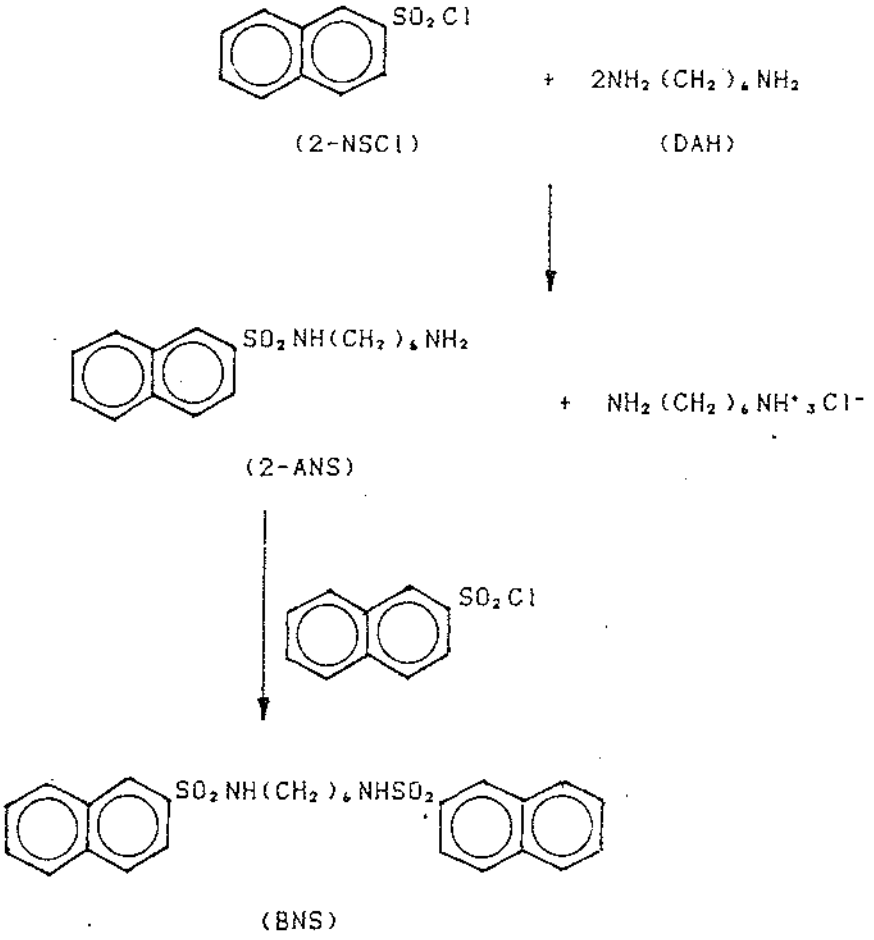


Scheme 2



The synthesis of 2-ANS is complicated. Reports in the literature show two methods of preparing 2-ANS. It has been made by reaction of 2-NSCl (1 mole) with DAH (3 moles) in dioxane [43]. The excess diamine present serves two functions. It statistically favours the formation of 2-ANS over the bis-naphthalene sulphonamide (BNS) [bis-N,N'-(2-

naphthalene sulphonyl) hexamethylenediamine]. It is also used to neutralize the hydrogen chloride liberated. Although this procedure looks straightforward, the yield of the sulphonamide, 2-ANS was low (12 to 34%) and its isolation rather laborious.



(3-7)

Similar sulphonamides have been synthesized in high yields (80%) for pharmaceutical studies [44] . To achieve this, they were prepared by reaction of the sulphonyl chloride, RSO_2Cl in chloroform at room temperature with the diamine protected at one end, ie. $R(CH_2)_6NH_2$ (where R is the phthalimido group). Although the yield was high, the method is tedious, laborious and expensive. Thus, there was no

satisfactory method for preparing 2-ANS which is simple and economical. Our aim was to find such a method so as to allow the investigation of the coupling of the product with the epoxide activated cellulose as shown in scheme 1 (Figure 3.1).

On the other hand, Scheme 2 (Figure 3.1) has the advantage of having one end of the 1,6-diaminohexane (DAH) blocked by coupling to the epoxide before reaction with 2-naphthalene sulphonyl chloride (2-NSCl) and all reagents can be washed away from the insoluble cellulose after each step in the synthesis. The coupling of DAH to the activated cellulose has been extensively studied in this laboratory. However, it was not known whether the coupling of 2-NSCl to DAH-cellulose could be accomplished efficiently.

3.3 Summary of objectives

The objectives of the work reported in this section include:

1. to find a simple method of preparing N-(6-aminohexyl)-2-naphthalenesulphonamide which could be used to couple to the epoxide activated cellulose (Scheme 1, Figure 3.1);
2. to find the conditions necessary to couple 2-naphthalene sulphonyl chloride to the diaminohexyl-cellulose (Scheme 2, Figure 3.1) and
3. to measure the capacity of these N-(6-aminohexyl)-2-naphthalenesulphonamide celluloses for bovine serum albumin (BSA), a readily available protein known to bind to hydrophobic groups; BSA was used in the absence of any knowledge regarding the particular protein or enzyme that the potential user was interested in purifying commercially.

3.4 Materials and equipment

The HP-cellulose 8-50 (batch 1217) which is now marketed as "INDION™ HP-10 cellulose" was obtained from Waitaki International Biosciences Ltd., Christchurch, New Zealand. Sodium 2-naphthalene sulphonate monohydrate (LR), thionyl chloride (AR), sodium sulphite (LR) and silica plates were obtained from Merck, Darmstadt, West Germany. Dimethylformamide (DMF) (AR), sodium hydroxide pellets (AR) and sodium metabisulphite (LR) were obtained from Ajax Chemicals, Sydney, Australia. DMF was dried, degassed and redistilled over calcium hydride before use. Sodium hydrogen phosphate (AR) and 1,6-diaminohexane were obtained from BDH, Seelze-Hannover, West Germany. Epichlorohydrin (LR), concentrated hydrochloric acid (AR), 1,2-dichoroethane (AR) and hydrochloric acid concentrate (AR) for making 1.00M standard solution were obtained from BDH, Poole, U.K. and sodium chloride from May and Baker New Zealand Ltd., Mount Maunganui, New Zealand. Anhydrous magnesium sulphate (LR), formic acid (AR), iodine and sodium hydroxide concentrate (AR) for making 1.00M standard solution were obtained from May and Baker Ltd., Dagenham, England. Toluene (LR) and hexane (LR) were obtained from Shell Oil New Zealand Ltd., New Zealand and methanol (LR) from Mobil Oil New Zealand, New Zealand. The solvents were redistilled before use. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

All titrations were carried out using an Autoburette ABU 11 from Radiometer, Copenhagen, Denmark.

3.5 Preparation of 2-naphthalene sulphonyl chloride

3.5.1 Purification of sodium 2-naphthalene sulphonate

The crude sodium 2-naphthalene sulphonate obtained commercially was recrystallized several times from 10% sodium chloride solution [45]. The anhydrous sodium salt was obtained by drying at 105°C.

3.5.2 Method for preparation of 2-naphthalene sulphonyl chloride

Thionyl chloride (25ml, 0.34 mol) was added dropwise to anhydrous sodium 2-naphthalene sulphonate (55.0g, 0.24 mol) in DMF (100ml) at 10-12°C with continuous stirring [5]. After complete addition, the mixture was stirred for another 15 minutes. Then, it was poured into ice water (ca. 600ml) with continuous stirring. The stirring of the ice water was important during addition of the reaction mixture to encourage clean crystallization rather than the formation of tacky material. The product obtained was collected on a filter and washed thoroughly with cold water. It was dried under vacuum at room temperature. The yield of colourless crystalline solid, 2-naphthalene sulphonyl chloride obtained was 53.4g (98.5%); m.p. 78-79°C (literature m.p. 79°C).

3.6 Preparation of N-(6-aminohexyl)-2-naphthalenesulphonamide

Three methods of synthesis were investigated.

Method A

2-Naphthalene sulphonyl chloride (2.0g, 8.8 mmol) in toluene (20ml) was added to 1,6-diaminohexane (6.0g, 50.4 mmol) in toluene (90ml) and the mixture was refluxed for about 2 hours. The solid residue obtained on cooling was collected on a filter and washed with toluene (100ml). The resulting toluene filtrate was set aside. The solid residue was then washed with 0.2M sodium hydroxide solution (ca. 100ml) followed by a thorough wash with water. The crude colourless crystalline solid

(0.705g) obtained was identified as bis-N,N'-(2-naphthalene sulphonyl)-1,6-hexa-methylenediamine (BNS). Found:

m.p. 161-165°C;

nmr (CD_3SOCD_3) : 7.6 (6.8H, multiplet, aromatic H), 5.2 (1.5H, singlet, NH), 2.6 (2H, singlet, CH_2), 1.1 (4.1H, singlet, CH_2). (The proton integrated values were in reasonable agreement with those expected for BNS except for the NH value.)

ir (Nujol) : 3260 (strong, NH), 3060 (weak, NH), 1585 (weak, NH), 1325 (strong, sym. SO_2), 1150 (strong, asym. SO_2) cm^{-1}.

Elemental analysis : 62.27% C, 5.42% H, 5.54% N, 12.62% S (calculated : 62.90% C, 5.65% H, 5.65% N, 12.90% S).

The toluene filtrate set aside was washed with 0.2M sodium hydroxide solution (100ml) and with water until neutral. It was dried over magnesium sulphate and evaporated to dryness under reduced pressure. The residue was recrystallized from hexane-toluene mixture. The colourless crystalline solid (0.653g, 24%) obtained was identified as N-(6-aminohexyl)-2-naphthalene- sulphonamide (2-ANS). Found:

m.p. 90-94°C;

nmr ($CDCl_3$) : 7.8 (2.3H, multiplet, aromatic H), 2.9 (1H, multiplet, NH), 2.5 (1.7H, multiplet, CH_2), 1.2 (2.8H, singlet, CH_2). (The proton integrated values were in reasonable agreement with those expected for 2-ANS except for the methylene proton next to the sulphonamide nitrogen.)

ir (Nujol) : 1585 (weak, NH), 1320 (strong, sym. SO_2), 1145 (strong, asym. SO_2) cm^{-1}.

Elemental analysis : 62.55% C, 7.00% H, 8.61% N, 10.78% S (Calculated : 62.75% C, 7.19% H, 9.15% N, 10.46% S).

Method B

2-Naphthalene sulphonyl chloride (10.0g, 44 mmol) in DMF (30ml) was added dropwise to 1,6-diaminohexane (30.0g, 259 mmol) in DMF (50ml) at room temperature. After complete addition, the mixture was stirred for another 0.5 hour. The residue obtained was filtered off. The DMF filtrate was concentrated to 1/3 of its volume by distillation under reduced pressure. Then, it was poured into cold water (ca. 100ml). The water was stirred during addition to prevent the formation of tacky material. The product was recrystallized from toluene to give a colourless crystalline solid (4.1g). It had a m.p. of 171-173°C. It was identified as bis-N,N'-(2-naphthalene sulphonyl)-1,6-hexamethylenediamine (BNS) on the basis of its improved m.p. and similar ir and nmr spectra (see Method A). Thin layer chromatography of BNS on silica in 1,2-dichloroethane-methanol-formic acid (7:3:0.5, v/v/v) showed one spot with a Rf value of 0.82. The chromatogram was visualized with iodine vapour.

Method C

2-Naphthalene sulphonyl chloride (8.0g, 35 mmol) in toluene (80ml) was added dropwise (vertical drop) to neat 1,6-diaminohexane (37.7g, 0.325 mol) maintained at 50 to 60°C. After complete addition, the mixture was stirred for another 0.5 hour. The hot reaction mixture was poured into ice water (ca. 500ml) which was stirred continuously during addition. The precipitate formed was filtered and washed with water. It was dried under vacuum at room temperature. A colourless crystalline solid was obtained in the yield of 9.1g (85%). It had a m.p. 90-94°C. It was identified as N-(6-aminohexyl)-2-naphthalene-sulphonamide (2-ANS) on the basis of its m.p. and similar ir and nmr

spectra (see Method A). Attempts to purify it by recrystallization from toluene, toluene-hexane, water and chloroform did not meet with much success. Thin layer chromatography of the crude product on silica in 1,2-dichloroethane-methanol-formic acid (7:3:0.5, v/v/v) showed two spots with Rf values of 0.61 and 0.82. The faster moving spot corresponded to bis-N,N'-(2-naphthalene sulphonyl)-1,6-hexamethylenediamine while the slower one corresponded to 2-ANS.

3.7 Epoxide activation

3.7.1 Procedure for activation

The extent of activation of the HP-cellulose is dependent on the strength of the sodium hydroxide solution used so long as the epichlorohydrin is kept in excess at all times. The volume of sodium hydroxide solution used needs to be sufficient to make a slurry which will mix well. A 1% sodium hydroxide (w/v) activation of HP-cellulose is described as an illustration of this activation procedure. A solution of 10M sodium hydroxide (6.25ml) and deionized water (149ml) were added to the moist* HP-cellulose (105g, 10g dry weight content, batch 1217) and mixed into a slurry. The mixture was cooled in ice for about 1 hour before adding the epichlorohydrin (6.2ml). It was then mixed for 24 hours at 4°C. The epoxide activated cellulose was washed with deionized water until neutral. The conditions for other activation levels are found in Appendix 5.

(* The moist cellulose was obtained by draining dry the wet cellulose on a filter.)

3.7.2 Epoxide activation level analysis

Sodium sulphite (0.25g) and sodium metabisulphite (0.25g) in deionized water (10ml) were added to a sample of the moist epoxide activated cellulose (5g). This was mixed overnight at room temperature. The

sample was washed with water, 1M hydrochloric acid (ca. 50ml) and water until neutral. It was dispersed in 0.5M sodium chloride solution (ca. 10ml) and titrated with 1.00M sodium hydroxide solution to pH 8. The sample was transferred quantitatively onto a weighed sintered glass filter and washed thoroughly with water before drying it at 60°C overnight followed by drying it at 105°C for about 2 hours. The sulphonic acid content of the product was then calculated in terms of milliequivalents per dry gram (meq/g). From this, the epoxide content of the original activated cellulose could be determined assuming a 100% conversion of epoxide to sulphonate (see Appendix 6 for more calculation details).

3.8 Coupling of the ligand to the cellulose derived matrix

3.8.1 Coupling of 1,6-diaminohexane to epoxide activated cellulose

1,6-Diaminohexane (9 to 15g) dissolved in water (150 to 170ml) was added to moist epoxide activated cellulose (158g). This was mixed for about 3 days at room temperature. The product was washed on a filter with deionized water until neutral to remove the excess diamine.

3.8.2 Coupling of N-(6-aminohexyl)-2-naphthalenesulphonamide to epoxide activated cellulose

The moist epoxide activated cellulose (5g) was solvent exchanged from water to DMF as described in Appendix 2. N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) (0.6 to 1.2g) in DMF (5-10ml) was added to the epoxide activated cellulose and was allowed to mix. The reaction was repeated at various temperatures for different duration of time. The sample was washed with DMF to remove excess 2-ANS and was then solvent exchanged from DMF back to water as described in Appendix 2.

3.8.3 Coupling of 2-naphthalene sulphonyl chloride to diaminohexyl (DAH) cellulose

The moist DAH cellulose (5g) was solvent exchanged from water to DMF as described in Appendix 2. 2-Naphthalene sulphonyl chloride (2-NSCl) (0.17 to 0.61g) in DMF (5-10ml) was added to the DAH cellulose and was allowed to mix. The reaction was repeated at various temperatures for different duration of time. The sample was washed with DMF to remove excess 2-NSCl and was then solvent exchanged from DMF back to water as described in Appendix 2.

3.8.4 Nitrogen analysis by titration

A sample of the moist DAH-cellulose [or 2-ANS-cellulose] (5g) was washed with 1M sodium hydroxide solution (ca. 50ml) [or 0.5M sodium hydroxide solution (ca. 75ml)] followed by deionized water until neutral. It was dispersed in 0.5M sodium chloride solution (ca. 10ml) and titrated with 1.00M hydrochloric acid to pH 4. It was transferred quantitatively onto a weighed sintered glass filter, washed thoroughly with deionized water and transferred to a measuring cylinder to settle overnight. Once its settled volume was recorded, it was transferred back to the same glass filter and dried at 60°C overnight followed by drying at 105°C for about 2 hours. The number of titratable nitrogen (meq/g) and the swollen volume (ml/g) of the product were then calculated (see Appendices 3, 4 and 6 for more details).

3.9 Capacity of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) celluloses for bovine serum albumin (BSA)

2-ANS-cellulose was pre-equilibrated with 0.01M sodium hydrogen phosphate (pH 7.0) buffer solution and drained on a filter. A solution (20ml) of 0.5% BSA in buffer was added to an accurately known weight of the pre-equilibrated cellulose (300 to 400mg). This was mixed for 2

hours at room temperature. The absorbance of the filtrate was measured at 280nm. By comparing the measured absorbance of the BSA stock solution at 280nm, the amount of BSA adsorbed by the cellulose was determined. The test was carried out in duplicate. A dry matter analysis of 2-ANS-cellulose was carried out separately in triplicate. Hence, the capacity in gram BSA per gram dry weight of 2-ANS-cellulose was calculated.

RESULTS AND DISCUSSION

3.10 Preparation of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS)

3.10.1 Comparison of methods of preparing 2-ANS

The preparation of 2-ANS was attempted by three methods, A, B and C (see Section 3.6). Method A involved adding 2-naphthalene sulphonyl chloride in toluene to six-fold excess of 1,6-diaminohexane in toluene and refluxing for 2 hours. The solid which crystallized out on cooling was shown to be bis-N,N'-(2-naphthalene sulphonyl)-1,6-hexamethylenediamine (BNS) by spectroscopic and elemental analysis. It had a melting point of 161-165°C. The residue recovered from the toluene layer was the desired 2-ANS which was also identified by spectroscopic and elemental analysis. It had a melting point of 90-94°C. The yield of 2-ANS prepared by this method was low (24%). The significant difference in the ir spectrum of BNS from that of 2-ANS was the absence of a strong NH absorption band at 3260 cm^{-1} in the latter. The proton nmr spectra of both compounds showed similar chemical shifts but differed in the ratios of the number of protons and the chemical shift position of the NH group.

In method B, DMF was used as the solvent for the reaction. The reactants had a much higher solubility in DMF than in toluene. Unlike method A, the preparation was carried out at room temperature. However, it gave mainly BNS. The yield for the recrystallized product was 38%. It had a melting point of 171-173°C which was an indication of higher purity. Apparently, the use of DMF, a polar solvent, favoured the formation of BNS.

The preparation by method C gave the desired product, 2-ANS in excellent yield. The crude product had a melting point of 90-94°C. In this method, the 2-naphthalene sulphonyl chloride (2-NSCl) was dissolved in minimum volume of toluene. It was added to nine-fold excess of neat 1,6-diaminohexane (m.p. 41-43°C) maintained at 50-60°C to keep it liquid. The addition of 2-NSCl in toluene to the amine was made dropwise from directly above the latter. The dropwise addition from directly above and the use of liquid DAH were thought to be crucial to the formation of 2-ANS in high yield. These factors set this method apart from the earlier two methods in that the 2-NSCl was constantly in the vicinity of a local excess of diamine. It was found that when the dropwise addition of 2-NSCl was made from the side, the product obtained was the unwanted BNS.

3.10.2 Purification of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) and bis-N,N'-(2-naphthalene sulphonyl) hexamethylenediamine (BNS)

BNS was easier to purify than 2-ANS. The former could be purified by repeated recrystallization from toluene. The attempts made at purifying 2-ANS by recrystallization from toluene, toluene-hexane, water and chloroform did not meet with much success. Thin layer chromatography showed that the impurity which persisted was BNS which was less soluble than 2-ANS in all those solvents making it difficult to remove.

Since, this impurity was not expected to interfere with the subsequent coupling reaction between 2-ANS and the epoxide activated cellulose, there was no advantage in purifying the crude product obtained. The contaminant, BNS would not react with the epoxide activated cellulose as it has no nucleophilic amino group present. Furthermore, in the coupling reaction, 2-ANS can be used in excess and so, the presence of the impurity does not matter.

3.10.3 Conclusion

Method C was the best method for preparing N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS). It was a simple, quick and relatively economical method of synthesis. The procedure was straightforward without the need for complicated and long isolation process. The starting material, sodium 2-naphthalene sulphonate was readily available and cheap. The conversion of sodium 2-naphthalene sulphonate to 2-naphthalene sulphonyl chloride was quantitative. The crude 2-ANS could be made from 2-naphthalene sulphonyl chloride in high yield and no further purification was necessary as the main contaminant, bis-N,N'-(2-naphthlene sulphonyl) hexamethylenediamine did not interfere with the subsequent coupling reaction mentioned. Therefore, method C provided a useful method of making 2-ANS as a ligand for coupling with the epoxide activated cellulose.

3.11 Preparation of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) celluloses by Scheme 1 (Figure 3.1)

3.11.1 Introduction

HP-cellulose (INDION™ HP-10) was reacted with epichlorohydrin using a slurry in either 1% or 3% aqueous sodium hydroxide. This gave two different epoxide activated celluloses with epoxide substitution levels of 0.47 and 1.24 meq/g respectively.

Before the epoxide activated celluloses could be used, they had to be solvent exchanged into an organic solvent which would both swell the cellulose matrix and dissolve the 2-ANS. DMF was found to be suitable. Preliminary coupling experiments showed that it was necessary to heat the reaction to 60-70°C in order to couple the 2-ANS whereas simple aliphatic amines normally couple at room temperatures in aqueous solution.

3.11.2 Coupling of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) to epoxide activated cellulose

A series of reactions at 64°C using a seven fold excess of 2-ANS over epoxide groups was undertaken to determine the time required for complete reaction. The results are shown in Table 3.1.

For both cellulose epoxide derivatives, the 2-ANS substitution levels reached their maximum after 24 hours and approximately 90% of the epoxide groups were converted. Because of the weight increase on the cellulose when the 2-ANS was coupled, there is a drop in the substitution levels when expressed as meq/g. But, the meq/g of epoxide converted can be calculated and expressed as coupling efficiency of 2-ANS which are shown in Table 3.1 (for calculation details, see Appendix 6)

The swollen volumes (bed volumes) of the products shown in Table 3.1 were measured with the 2-naphthyl-celluloses (BN celluloses) in

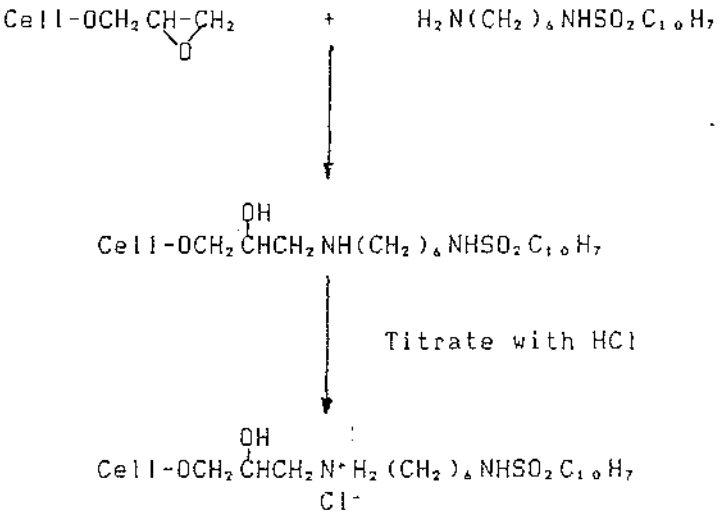
the hydrochloride form (see Figure 3.2). These volumes were observed to decrease markedly if the charge on the nitrogen was removed by washing with dilute alkali. Without the repulsion between adjacent positive charges, the hydrophobic interactions between the naphthyl groups contracted the cellulose matrix.

Table 3.1 : Effect of time on 2-ANS substitution level

Epoxide activated cellulose used[1,2]	Product number[3]	Reaction time (hr.)	Swollen volume (ml/g)	2-ANS substitution[4] Titratable nitrogen, N' (meq/g)	Coupling efficiency, E_A (%)
0.47 meq/g	BN 43	6	17.7	0.28	66
	BN 44	24	22.8	0.38	94
	BN 45	48	23.4	0.39	96
1.24 meq/g	BN 46	6	-	0.69	73
	BN 47	24	20.9	0.80	89
	BN 48	48	23.6	0.79	87

Notes :
1. Reaction conditions : 5g moist epoxide activated cellulose was solvent exchanged and used in DMF at 64°C
2. 0.6g and 1.2g of 2-ANS were used for the epoxide activated celluloses with degrees of activation(s) of 0.47 and 1.24 meq/g respectively.
3. BN was the code given to 2-ANS-celluloses obtained.
4. For calculation details, see Appendix 6.

Figure 3.2 : Coupling of 2-ANS to epoxide activated cellulose



3.11.3 Summary of findings

The results showed that the direct coupling of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) to epoxide activated cellulose was straightforward. The reaction was complete after 24 hours at 64°C with 90% of the epoxide groups converted. The large excess of coupling reagent, 2-ANS used in these reactions (see Table 3.1) could probably be reduced but this aspect was not investigated. The observed contraction in the bed volume of the BN cellulose when the charge on the nitrogen was removed would probably limit the useful pH range to pH less than 9 for protein chromatography work.

The products, BN 45 and BN 48 shown in Table 3.1 were selected for further investigation of protein capacities discussed later (see Section 3.13).

3.12 Preparation of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) celluloses by Scheme 2 (Figure 3.1)

3.12.1 Diaminohexyl (DAH) celluloses

Several DAH-celluloses were prepared from commercially available HP-cellulose (batch 1208 and 1217). The conditions used in the preparation of these derivatives and their properties are summarized in Table 3.2.

Table 3.2 : Conditions for preparation of DAH-celluloses and their properties

DAH-cellulose	20	27	28	29
(a) Conditions for preparation[1]				
moist epoxide activated cellulose (158g) made from NaOH activation (%)[2]	6.0	1.0	1.5	2.0
Weight of DAH added (g)	15.0	9.0	12.0	15.0
(b) Properties of DAH-celluloses				
Swollen volume (ml/g)	14.9	31.1	33.1	37.6
DAH substitution Titratable nitrogen, N" (meq/g)	2.04	0.81	1.10	1.44

Notes :
1. See Section 3.8.1 for details on preparation of DAH 27 to 29.
2. See Appendix 5 for conditions for NaOH/epichlorohydrin activation of HP-cellulose.

3.12.2 Preliminary determination of coupling conditions of 2-naphthalene sulphonyl chloride (2-NSCl) to diaminohexyl (DAH) cellulose

(a) Introduction

The extent of coupling of 2-NSCl to DAH cellulose was expressed in terms of coupling efficiency (%). There are two titratable nitrogens on DAH cellulose (ie. a primary and a secondary amine). On coupling of 2-naphthalene sulphonyl group to DAH-cellulose, the primary amine is converted to a sulphonamide linkage. This leaves only one titratable nitrogen left. By appropriate calculations based on the number of titratable nitrogen left (expressed in meq/g) after coupling and the consequent weight increase, the coupling efficiency of 2-NSCl may be worked out. The detailed calculations are shown in Appendix 6.

The conditions for coupling 2-NSCl to DAH 20 were investigated. In particular, the effects of the quantity of 2-NSCl added and the reaction time were studied.

(b) Effect of 2-naphthalene sulphonyl chloride (2-NSCl)

As shown in Table 3.3, it was found that the N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) substitution increased as the quantity of 2-NSCl added was increased. When a 1:1 molar ratio of 2-NSCl to diaminohexyl (DAH) groups was used, 72% of the DAH reacted. While, when a four fold excess of 2-NSCl over the primary amine group was added, about 90% of the DAH reacted. It would appear from these results that only the primary amine in DAH-cellulose reacted and not the less basic and more sterically hindered secondary amine.

Table 3.3 : Effect of 2-NSCl on 2-ANS substitution for DAH-cellulose 20

weight of 2-NSCl (g)	Product Number[2]	Swollen volume[3] (ml/g)	2-ANS substitution[4] Titratable nitrogen, N_A . (meq/g)	Coupling efficiency, E_A' (%)
0.17	BN 15	10.6	1.18	72
0.30	BN 16	9.7	1.09	79
0.61	BN 17	-	1.00	88

Notes :
1. Reaction conditions : 5g moist DAH-20; 8ml DMF; 17 hr.; 22°C.
2. BN was the code given to 2-ANS-celluloses obtained.
3. The swollen volume of 2-ANS-cellulose was measured in its hydrochloride form (see Figure 3.2).
4. For calculation details, see Appendix 6.

(c) Effect of reaction time

The results obtained in Table 3.4 showed that the coupling reaction occurred rapidly and the N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) substitution level reached the maximum value in less than one hour. The lower coupling efficiency obtained (cf. 72% in Table 3.3) was probably due to water present in the system as a result of incomplete solvent exchange. This would consume some of the sulphonyl choride and only a 1:1 ratio of 2-naphthalene sulphonyl chloride (2-NSCl) to diaminohexyl (DAH) groups was used in this case. Nevertheless, the constant coupling efficiency showed that the reaction time was obviously very fast.

Table 3.4 : Effect of reaction time on 2-ANS substitution for DAH-cellulose 20

Reaction time (hr.)	Product Number[2]	Swollen volume[3] (ml/g)	2-ANS substitution[4] Titratable nitrogen, N_R (meq/g)	Coupling efficiency, E_A' (%)
1	BN 18	13.1	1.54	39
2	BN 19	12.9	1.55	39
5	BN 20	12.7	1.57	37
24	BN 21	12.8	1.57	37

Notes :
1. Reaction conditions : 5g moist DAH 20, 0.17g 2-NSCl used, 6ml DMF, room temperature.
2. BN was the code given to 2-ANS-celluloses obtained.
3. The swollen volume of 2-ANS-cellulose was measured in its hydrochloride form (see Figure 3.2).
4. For calculation details, see Appendix 6.

The reaction was repeated for two and twenty four hours but with the following changes: (i) a two-fold excess of 2-NSCl over DAH was used to prevent the possibility of small amounts of water present from affecting the final substitution level obtained, and (ii) DAH-cellulose 29 (see Table 3.2) was used in place of DAH-cellulose 20 as it was considered not necessary or desirable to have such a high substitution level in the final product as was possible with DAH 20. The results are presented in Table 3.5 and show that about 85% of the DAH groups on cellulose are readily converted to naphthalene sulphonamide in less than two hours with a two-fold excess of 2-NSCl at 22°C in DMF.

Table 3.5 : Effect of reaction time on 2-ANS substitution for DAH-cellulose 29

Reaction time (hr.)	Product Number[2]	Swollen volume[3] (ml/g)	2-ANS substitution[4] Titratable nitrogen, N_R (meq/g)	Coupling efficiency, E_4' (%)
2	BN 35	10.3	0.76	86
24	BN 39	10.2	0.77	85

Notes :
1. Reaction conditions : 5g moist DAH 29, 0.23g 2-ANS used, 8ml DMF; 22°C.
2. BN was the code given to 2-ANS-celluloses obtained.
3. The swollen volume of 2-ANS-cellulose was measured in its free amine form.
4. For calculation details, see Appendix 6.

3.12.3 Preparation of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS)-cellulose for protein capacity tests

For large bulky groups like naphthalene sulphonyl group, there is usually an optimum substitution level for maximum protein capacity. Consequently, a range of products for testing was prepared by Scheme 2 (Figure 3.1) based on the results shown in Tables 3.2 to 3.5. The reaction was carried out at room temperature overnight using about two-fold excess of 2-naphthalene sulphonyl chloride (2-NSCl). An excess of 2-NSCl was used to ensure that the reaction would not be affected by traces of water in the system. The conditions for the preparation of the final products and their properties are summarized in Table 3.6.

Table 3.6 : Properties of 2-ANS-celluloses prepared

BN cellulose[1]	40	41	42
(a) Conditions for preparation[2]			
moist DAH-cellulose (40g) used	DAH 27	DAH 28	DAH 29
weight of 2-NSCl (g) added	0.8	1.1	1.4
(b) Properties of 2-ANS-celluloses			
Swollen volume[3] (ml/g)	16.5	12.6	8.8
2-ANS substitution[4]			
Titratable nitrogen, N_R (meq/g)	0.47	0.65	0.79
Estimated 2-ANS content, S_A' (meq/g)	0.32	0.42	0.60
Coupling efficiency (%)	78	76	83

Notes :
1. BN was the code given to 2-ANS-cellulose prepared.
2. The reaction was carried out in 50ml DMF at room temperature for 18 hr.
3. The swollen volume of 2-ANS-cellulose was measured in its free amine form.
4. For calculation details, see Appendix 6.

As shown in Table 3.6, the products obtained had 76-83% of the DAH sulphonated. The swollen volumes decreased as the 2-ANS substitution increased. The contraction in the bed volumes was caused by the increasing hydrophobic interactions due to increasing number of naphthyl groups attached.

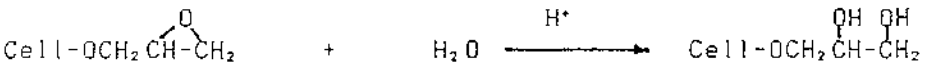
3.12.4 Summary of findings

The investigation showed that the coupling of 2-naphthalene sulphonyl chloride (2-NSCl) to diaminohexyl (DAH)-cellulose was rapid. A 90% coupling efficiency could be achieved by using at least a four fold excess of 2-NSCl. However, a two-fold excess was sufficient to sulphonate about 80% of the DAH groups. BN 40, BN 41 and BN 42 shown in Table 3.6 were selected for further investigation of protein

capacities along with BN 45 and BN 48 prepared by the other route (Scheme 1, Figure 3.1) (see Section 3.14).

3.13 Conclusion

The results showed that it was equally feasible to prepare N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS)-cellulose by either direct coupling of 2-ANS to epoxide activated cellulose (Scheme 1, Figure 3.1) or coupling of 2-naphthalene sulphonyl chloride to diaminohexyl (DAH)-cellulose (Scheme 2, Figure 3.1). The coupling conditions required for the former were more harsh than that required by the latter. The reaction was slower and required heating at 64°C for coupling to take place. Presumably, the steric hindrance resulting from the bulkiness of 2-ANS caused it to be less reactive. The present method of coupling 2-ANS to epoxide activated cellulose used a seven-fold excess of the ligand over DAH. This large excess may not be essential. It would be more economical to use a limited amount of 2-ANS to couple with a highly epoxide activated cellulose. Any unreacted epoxide could then be hydrolyzed by dilute acid. But, this was not investigated.



The advantage of preparing 2-ANS-cellulose by Scheme 1 was that the final product was expected to exhibit less non-specific ionic properties. This resulted from the absence of residual primary amine groups. However, non-specific ionic properties of the cellulose cannot be totally eliminated because of the basic secondary amine linkage found in epoxide activated cellulose. In general, 2-ANS-cellulose prepared by Scheme 1 would be expected to exhibit enhanced hydrophobic

or affinity type properties.

The investigation into the synthesis of the 2-ANS-cellulose was terminated at this point. Some of the products prepared were sent for testing by the potential customer.

3.14 Capacity of N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS)-cellulose for bovine serum albumin (BSA)

The capacity test was carried out for the following 2-ANS-celluloses :

1. BN 45 and BN 48 which were prepared by Scheme 1,
2. BN 40, BN 41 and BN 42 which were prepared by Scheme 2 and
3. DAH 27, DAH 28 and DAH 29 for comparison (see Table 3.2).

The results are summarized in Table 3.7.

The BSA capacities of 2-ANS-celluloses which were prepared by Scheme 2 (Figure 3.1) were very much higher than that which were prepared by Scheme 1 (Figure 3.1). This was surprising but might be explained by the presence of approximately 20% of the original DAH groups which remained unsulphonated and could contribute to the capacity.

For BN 40 to BN 42 shown, the capacity for BSA decreased as the 2-ANS substitution level increased. This was probably due to the contraction in swollen volume as the 2-ANS substitution was increased (see Section 3.13.3) and thereby, reducing the extent of interaction between the ligand and BSA. This shows the effect of overloading the cellulose matrix with hydrophobic groups. The more usual effect in ion exchange binding was shown by DAH 27 to DAH 29. As the DAH substitution increased, the capacity of the DAH-celluloses for BSA also increased.

Table 3.7 : Results of BSA capacity test

Scheme	Matrix	Estimated 2-ANS content (meq/g)[1]	Protein capacity (g/g)
1	BN 45	0.39	0.38
	BN 48	0.79	0.05
2	BN 40	0.31	0.78
	BN 41	0.40	0.60
	BN 42	0.56	0.49
-	DAH 27	0.81[2]	1.22
	DAH 28	1.10[2]	1.77
	DAH 29	1.44[2]	2.12

Notes :
1. See Appendix 6 for calculation details.
2. Refer to the number of titratable nitrogen in meq/g.

3.15 Conclusion

Scheme 2 (Figure 3.1) would appear to be the better route to preparing a N-(6-aminohexyl)-2-naphthalenesulphonamide derivative of cellulose. The complete synthesis was carried out stepwise on the cellulose matrix where the excess reagents could be washed away and the next step undertaken. Thus, it did not involve the prior preparation of N-(6-aminohexyl)-2-naphthalenesulphonamide. All steps were carried out at ambient temperatures.

In addition to these synthetic advantages, the bovine serum albumin capacities were higher than the corresponding products prepared by Scheme 1 (Figure 3.1).

SECTION 4

AN INVESTIGATION OF THE SYNTHESES OF CELLULOSE AND AGAROSE DERIVATIVES CONTAINING CITRATE AND ASPARTATE GROUPS FOR THE PURIFICATION OF BOVINE LACTOFERRIN

INTRODUCTION

4.1 Lactoferrin

Lactoferrin (Lf) is an iron binding protein found in mammalian milk and in a variety of mammalian exosecretions [46]. It consists of a single polypeptide chain [47] which is divided into two domains [46]. Each domain contains an iron-binding site which is similar but not identical [48]. It is capable of tight but reversible binding of two ferric ions per protein molecule with the synergistic binding of two bicarbonate or carbonate anions [49]. It exhibits bacteriocidal and bacteriostatic properties [50-52].

Bovine Lf has a molecular weight of 80000 [46]. Early studies [53,54] showed that its isoelectric point is about pH 8.0 but it could be much higher than this. The iron saturated protein gives a salmon pink colour in aqueous solution and shows an absorption maximum at 465nm [55]. The concentrations of bovine Lf in normal milk, colostrum and secretion of involuted mammary gland are found in the ranges of 0.1 to 0.3, 2 to 5 and 20 to 30 mg/ml respectively [56].

4.2 Isolation and purification of bovine lactoferrin (Lf)

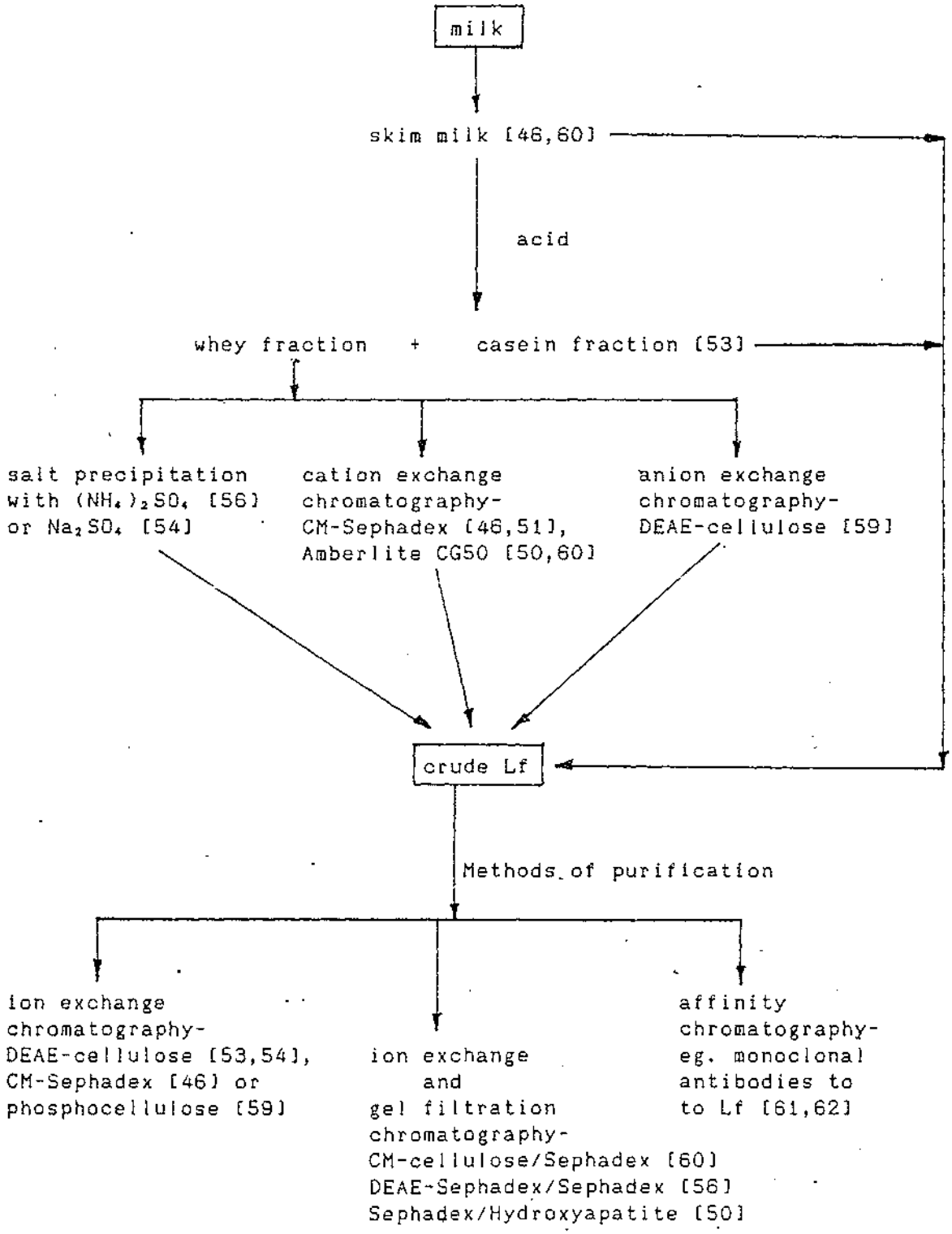
There is considerable interest in purified bovine Lf. Crystals are required for crystallographic studies as an aid to further understanding of its biochemical role. It is also a potentially

important commercial protein because of its bacteriostatic and bacteriocidal properties.

The isolation and purification of Bovine Lf is often complicated by the difficulty in removing the main contaminant, lactoperoxidase (Lp) [57]. This enzyme has a similar molecular weight (Mr = 76000 to 92000) and isoelectric point (pI = 9.6) to Lf [58].

There are several methods available for the isolation and purification of Lf from bovine milk. When casein is precipitated from milk by a shift of pH to 4.6, most of the Lf usually remains in the whey fraction. Although an early preparation of Lf used the acid precipitated casein as a source [53], it is usual nowadays to start with the whey fraction [50,51,54,56,59] or even isolate it directly from skim milk [46,60]. Lf can be obtained from whey by either salting it out with the addition of ammonium sulphate [56] or sodium sulphate [54], or separating it using ion exchange chromatography [46,51,50,60]. In the latter method, cation exchangers have been used to bind Lf at a pH below its pI ie. pH 6-8 where most other whey proteins are anionic and not bound. Such cation exchangers include CM-Sephadex [46,51] and Amberlite CG50 [50,60]. Alternatively, an anion exchanger, DEAE-cellulose [59] has been used to retain most of the whey proteins while the basic ones such as Lf and Lp are eluted off. Figure 4.1 summarizes these methods.

Figure 4.1 : General scheme for the isolation and purification of bovine Lf



Further purification of the isolated Lf is generally achieved by one of the following methods : (i) ion exchange chromatography, (ii) a combination of ion exchange and gel filtration chromatography and (iii) affinity chromatography. DEAE-Cellulose [53,54], CM-Sephadex [46] and phosphocellulose [59] columns have been used to purify Lf by the first method. In the second method, crude Lf is either passed through an ion-exchanger followed by a gel filtration column or vice versa. A combination of columns have been used. They include DEAE-Sephadex A-50/Sephadex G-200 columns [56], CM-cellulose/Sephadex G-100 columns [60] and Sephadex G-100/ Hydroxyapatite columns [50]. Finally, Lf has also been purified by affinity chromatography using, for example, monoclonal antibodies to Lf [61,62].

4.3 Criteria for purity of lactoferrin (Lf)

A criteria for determining the purity of Lf that has been frequently used is a spectroscopic method [46,55,57]. The Lf is iron saturated by adding two to three fold excess of 0.01M iron(III)nitrilotriacetate (Fe-NTA) containing 0.1M bicarbonate. The absorbance of the iron-saturated Lf was measured at 280, 410 and 465 nm. Generally, it is considered to be of acceptable purity when the absorbance ratios of:

(i) A_{280}/A_{465} is between 27 and 28 and

(ii) A_{410}/A_{465} is between 0.8 and 0.85.

4.4 Interaction between citrate and lactoferrin (Lf)

Citrate in milk has been found to inhibit the bacteriostatic activity of Lf [56,63]. This suggests that there may be some biospecific interaction between citrate and Lf; in which case, citrate could be used as an affinity ligand for the purification of Lf.

A preliminary study [64] carried out in this laboratory on the purification of Lf by a citrate-agarose derivative showed promise. It was decided that the use of the citrate ligand for the purification of bovine Lf should be further investigated.

4.5 Covalent immobilization of citrate to cellulose and agarose

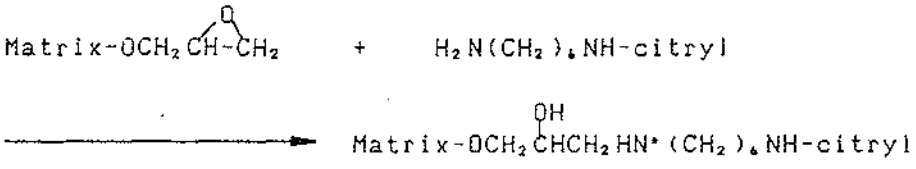
Citric acid can be covalently linked to a polysaccharide matrix via either an ester linkage or an amide linkage. Ester linkages hydrolyze fairly easily so attachment via an amide bond is the preferred method. This requires the use of an amino derivative of either the polysaccharide or the citric acid. An additional advantage of the amide linkage is that it allows the use of a spacer arm such as 1,6-diaminohexane (DAH). This is a standard procedure in affinity chromatography where the spacer arm allows the protein to gain access and correct alignment with the ligand for maximum interaction [9]. The amino derivative of citric acid, which is of interest, is citrylhexamethylenediamine (CM_6D). It can be attached to the polysaccharides via either the epoxide activated matrix (see Section 3.2) or 1,1' carbonyldiimidazole (CDI) activated matrix [65,66]. These methods of covalent attachment are shown in Figure 4.2.

The coupling of CM_6D to the epoxide activated matrix produces a basic secondary amine linkage indicated with an asterisk in reaction 4-1 (see Figure 4.2). This nitrogen is charged below pH 11 and would give the matrix some additional ionic properties, whereas, the coupling of the ligand to the CDI activated matrix leads to a nonbasic urethane derivative [65]. This nonbasic urethane linkage would not be charged and hence, would not interfere with the anionic character of the citrate ligand covalently attached in this way. Consequently, the citrate matrices prepared by these two methods could have quite

different properties.

Figure 4.2 : Covalent attachment of citrylhexamethylenediamine to polysaccharide matrix

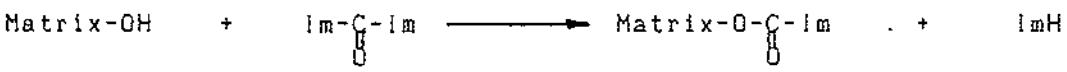
(a) via epoxide activated matrix



(4-1)

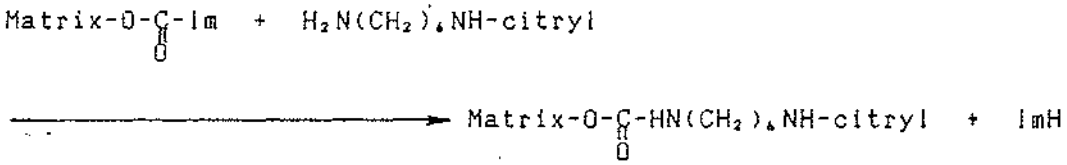
(b) via 1,1' carbonyldiimidazole (CDI) activated matrix

(i) CDI activation of matrix.

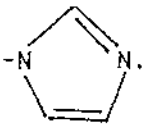


(4-2)

(ii) Covalent attachment of ligand to activated matrix.



(4-3)

where Im represents the imidazoyl group  .

The two methods of preparing CM_4D-matrices which were of interest included:

1. coupling of citric acid to diaminohexyl-matrix via a water soluble carbodiimide as shown in equation 4-4.

$$\text{Matrix-NH(CH}_2\text{)}_4\text{NH}_2 \quad + \quad \text{citric acid}$$

$$\xrightarrow{\text{carbodiimide}} \quad \text{Matrix-NH(CH}_2\text{)}_4\text{NH-citryl} \quad + \quad \text{urea} \qquad (4\text{-}4)$$

2. the preparation of the CM$_4$D ligand and coupling it to the activated matrix as shown in Figure 4.3.

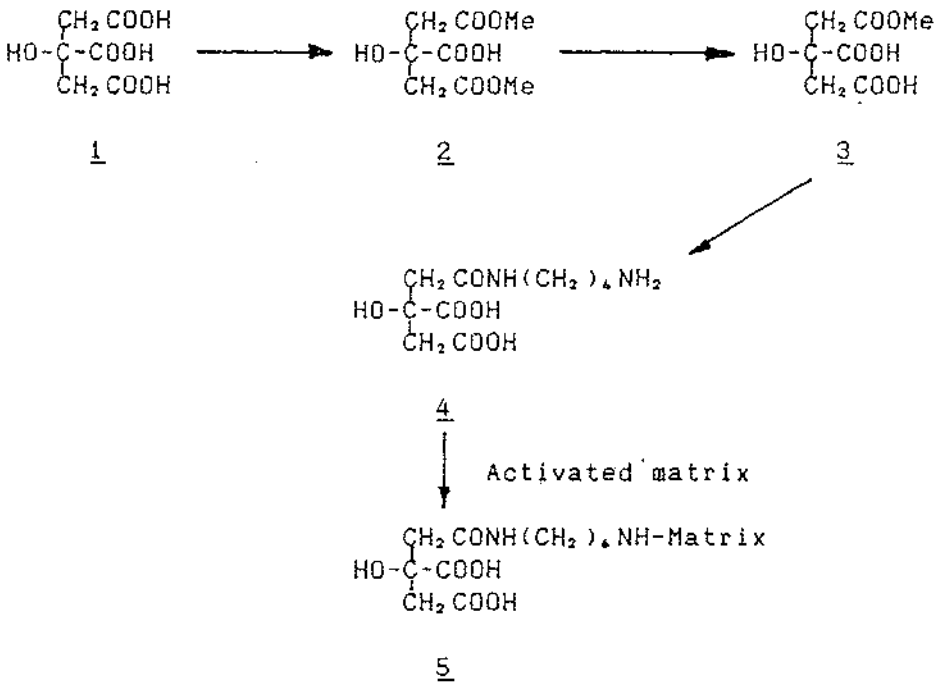
In the first method, the disadvantage is that it also forms ester bonds with hydroxyl groups on the polysaccharide matrix and there is no control over which of the two different carboxylic acid groups in citric acid is used to form the amide bond. In the latter method, it is possible to control which of the two different structural isomers of citrylhexamethylenediamine, alpha (A)-CM$_4$D or beta (B)-CM$_4$D (Structures 4 and 8 in Figure 4.3) is coupled with the activated matrix. These two compounds have been previously reported [67,68]. The method of preparing A-CM$_4$D, 4 and B-CM$_4$D, 8 are shown in Figure 4.3.

The starting material used was anhydrous citric acid, 1. Controlled esterification of citric acid gave rise to sym-dimethyl citrate, 2. Partial hydrolysis of 2 gave asym-monomethyl citrate, 3 which was then used to prepare A-CM$_4$D, 4 by reaction with an excess of 1,6-diaminohexane. Hirota et al. [68] coupled 4 with cyanogen bromide activated agarose to give A-CM$_4$D-agarose and used it for the purification of fumarase by affinity chromatography.

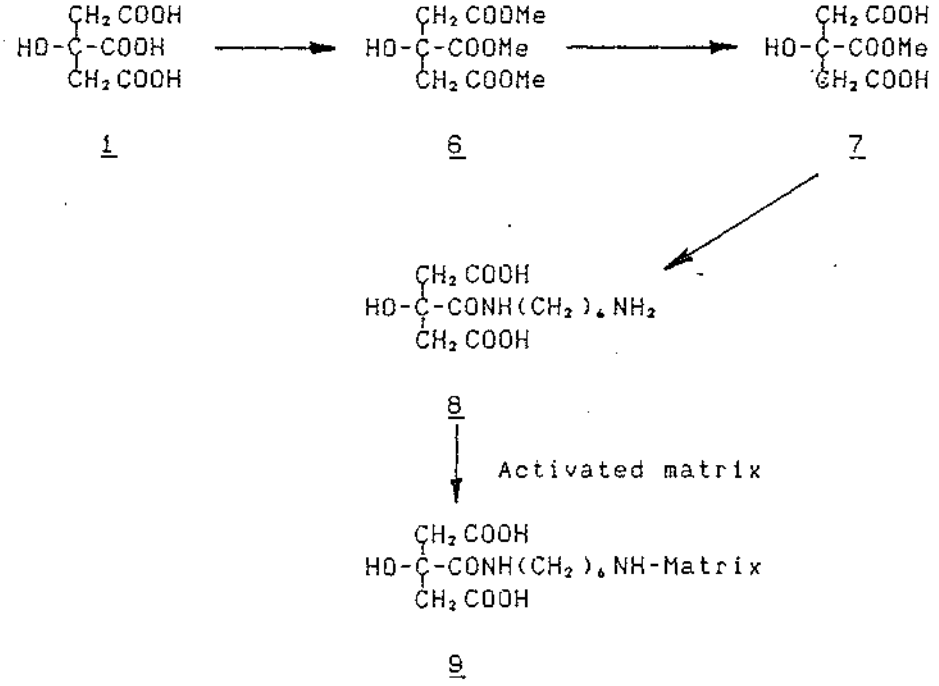
The trimethyl citrate, 6 was made by the complete esterification of 1. The partial hydrolysis of 6 gave sym-monomethyl citrate, 7. The B-CM$_4$D, 8 was prepared by reacting 7 with an excess of DAH which was then coupled to an activated matrix and the resulting B-CM$_4$D-matrix was used for affinity chromatography [68].

Figure 4.3 : Preparation of citrate matrices

Scheme 1



Scheme 2



4.6 Aims of this study

The aims of the work reported in this section were initially two fold.

1. To prepare the two citrylhexamethylenediamine compounds by the simplest route possible with good yields. The previous work reported was aimed at obtaining absolute purity of products [67,68].
2. To couple the two citrylhexamethylenediamine to activated cellulose and agarose matrices and assess the performance of these products for purifying bovine Lf.

It was also hoped that the binding action of Lf to the citrate matrices might be identified as either simply ionic attraction between the negatively charged carboxylate groups of the ligand and general cationic sites in Lf or, whether there was some specific affinity type interaction between the citrate and the bicarbonate binding sites in Lf.

EXPERIMENTAL

4.7 Materials and equipment

The HP-cellulose 8-50 which is now marketed as "INDION™ HP-10 cellulose" was obtained from Waitaki International Biosciences Ltd., Christchurch, New Zealand. Sepharose 6B-100 was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Sepharose CL-6B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex 50Wx12-200 (H^+ form), Dowex 50Wx8-40 (H^+ form), citric acid (LR), concentrated sulphuric acid (AR), concentrated hydrochloric acid (AR), 1,2-dichloroethane (AR), bromocresol green and epichlorohydrin were obtained from BDH, Poole, UK. Dowex 50Wx4-400 (H^+ form) and sodium

borohydride (AR) were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Sodium hydroxide pellets (AR), concentrated ammonia (AR), glacial acetic acid (AR) and dimethylformamide (DMF) (AR) were obtained from Ajax Chemicals, Sydney, Australia. DMF was dried, degassed and redistilled over calcium hydride before use. Formic acid (AR), anhydrous diethyl ether (AR), calcium carbonate (LR), iodine (LR) and sodium hydroxide concentrate (AR) for making 1.00M standard solution were obtained from May and Baker Ltd., Dagenham, England. Sodium chloride (AR) was obtained from May and Baker New Zealand Ltd., Mount Maunganui, New Zealand. Silica plates and 1,1' carbonyldi-imidazole were obtained from Merck, Darmstadt, West Germany. Ninhydrin (AR) was obtained from Koch-light Laboratories Ltd., Bucks, England and 1,6-diaminohexane from RDH, Seelze-Hannover, West Germany. Amberlite IRA-900 (OH^- form) was obtained from Rohm & Haas New Zealand Ltd., Auckland, New Zealand. Methanol (LR) was obtained from Mobil Oil New Zealand, New Zealand and acetone (LR) from Shell Oil New Zealand Ltd., New Zealand. Absolute ethanol (LR) was obtained from Chemby Marketing New Zealand, Wellington, New Zealand. The solvents were redistilled before use.

Tosyl salt of aspartic acid dibenzyl ester and freshly isolated bovine lactoferrin were gifts from Dr. D.H.K. Harding and Mrs. H. Baker of Massey University respectively. Purified bovine lactoferrin and bovine lactoperoxidase were gifts from Dairy Research Institute, Palmerston North, New Zealand.

All titrations were carried out using an Autoburette ABU 11 from Radiometer, Copenhagen, Denmark.

4.8 Preparation of sym-dimethyl, asym-monomethyl, trimethyl and sym-monomethyl citrate

4.8.1 Preparation of sym-dimethyl citrate (2, Figure 4.3)

The method for preparation of sym-dimethyl citrate was adapted from methods described by Hirota et al. [67], Fischer et al. [69] and Pearce [70].

Dowex 50Wx4-400 resin (120ml) was solvent exchanged to methanol and was added to anhydrous citric acid (192g, 1 mol) in methanol (500ml, 12.3 mol). The mixture was refluxed for about 3 hours (or until about 2/3 of the carboxyl groups were methylated determined by periodic titration of 0.25ml aliquots of the reaction mixture). The resin was removed by filtration and washed with hot methanol (ca. 100ml). The filtrate was concentrated to about half its volume. The product which crystallized out was collected and recrystallized from methanol to remove the trimethylcitrate. The product obtained was further recrystallized from water to remove the remaining contaminants.

The final yield of the pure sym-dimethyl citrate obtained was 152.3g (64%), m.p. 121-123°C (literature m.p. 122-124°C [67]). Thin layer chromatography (TLC) showed one spot with a Rf value of 0.68 in CH_2ClCH_2Cl:MeOH:HCOOH (7:3:0.5, v/v/v) on silica plate (cf. literature Rf: 0.69 [67]). The chromatogram was visualized with iodine vapour followed, after a time of exposure to the atmosphere to remove the iodine, by 0.1% ethanolic solution of bromocresol green.

4.8.2 Preparation of asym-monomethyl citrate (3, Figure 4.3)

The method for preparation of asym-monomethyl citrate was adapted from that described by Hirota et al. [67].

The sym-dimethyl citrate (23.8g, 0.1 mol) was suspended in water (120ml). An aqueous solution (150ml) containing sodium hydroxide (0.2

mol) was added dropwise to the suspension with vigorous stirring at room temperature over 3 hours. After the addition, the stirring was continued until all the caustic had been consumed and the mixture became neutral. It was stirred for 0.5 hour with Dowex 50Wx12-200 resin (H-type, 200ml). The resin was filtered and washed with hot water several times. Calcium carbonate (10g, 0.1 mol) was added in small portions to the filtrate with constant stirring at 80-90°C to precipitate out the calcium salt of asym-monomethyl citrate. The precipitate obtained was filtered washed and resuspended in water (ca. 100ml). It was desalted with DOWEX 50Wx12-200 resin (H-type, 200ml). After filtering off the resin, the resulting solution obtained was concentrated to dryness to give a colourless syrup. On drying under vacuum, the crude crystalline product was obtained in the yield of 11.2g (54.3%), m.p. 116-119°C (literature m.p. 116.5-117.5°C [67]). TLC showed two spots with Rf values of 0.40 and 0.11. The chromatogram was developed under the same conditions as described in Section 4.8.1.

The Rf values of 0.40 and 0.11 corresponded to asym-monomethyl citrate [67] and citric acid respectively. Assuming that citric acid was the only contaminant, the purity of the monomethyl citrate was estimated by titrating a known weight of it against standard sodium hydroxide (see Appendix 7). In the above preparation, the product was estimated to be 90% pure. Typically, the citric acid contamination was found to be in the range of 10-15%. Since citric acid did not interfere with the subsequent coupling reaction (see Section 4.9), the asym-monomethyl citrate obtained by this method was used without further purification.

4.8.3 Preparation of trimethyl citrate (6, Figure 4.3)

The trimethyl citrate was prepared by D.F. Elgar [71]. Concentrated sulphuric acid (5ml) was added to anhydrous citric acid (19.2g, 0.1 mol) in methanol (30ml, 0.74 mol). The mixture was refluxed for 7 hours. On cooling overnight in a refrigator, the crystalline solid obtained was filtered and washed with ice-cold methanol. It was suspended in water (30ml) and neutralized with sodium hydroxide to pH 7. It was then recrystallized from water to give trimethyl citrate (13.6g) in a yield of 58%, m.p. 78-80°C (literature m.p. 78-79°C [72]). TLC showed one spot with a Rf value of 0.83 (literature Rf: 0.83 [67]). The chromatogram was developed under the same conditions as given in Section 4.8.1.

4.8.4 Preparation of sym-monomethyl citrate (7, Figure 4.3) [67]

A solution (200ml) containing sodium hydroxide (0.2 mol) was added dropwise to a 70% methanol solution (150ml) of trimethyl citrate (23.4g, 0.1 mol) over 2 hours at room temperature with vigorous stirring. The solution was concentrated to about 70 ml and was then passed through a column of DOWEX 50Wx8-40 (H-type, 250ml). The column was washed with deionized water. The acidic eluate (ca. 1 l) was concentrated to dryness under reduced pressure. The residue was recrystallized from acetone to give sym-monomethyl citrate (10.9g). The concentration of the mother liquor gave a second crop (2.4g). The overall yield of sym-monomethyl citrate was 65%, m.p. 173-175°C (literature m.p. 174-175°C [67]). TLC showed one spot with a Rf value of 0.54 (literature Rf: 0.55 [67]). The chromatogram was developed under the same conditions as given in Section 4.8.1.

4.9 Preparation of alpha-citrylhexamethylenediamine (A-CM_6D) and beta-citrylhexamethylenediamine (B-CM_6D) (4 and 8, Figure 4.3) [68]

Asym- or sym-monomethyl citrate (20.6g, 0.1 mol) was added to 1,6-diaminohexane (DAH) (58.0g, 0.5 mol) in water (40ml). [For asym-monomethyl citrate, correction in weight was made according to % purity.] The resulting solution was allowed to mix overnight (at room temperature for asym-monomethyl citrate-DAH solution and at 37°C for sym-monomethyl citrate-DAH solution). The mixture was passed through a column of Amberlite IRA-900 (OH-type, 450ml) which binds CM_6D while allowing excess DAH to be washed away with deionized water (ca. 2.4l). It was then eluted with 1M hydrochloric acid (ca. 2l). The eluate was concentrated to dryness under reduced pressure at 40°C. The residue obtained was dissolved in minimum volume of deionized water. The solution was passed through a column of DOWEX 50Wx8-40 (H-type, 350ml) which binds CM_6D again while allowing citrate anions to be washed away with deionized water (ca. 1.5l). It was then eluted with 1M ammonia. The initial neutral eluate was discarded. The basic eluate (ca. 2.5l) was concentrated to dryness under reduced pressure at 50°C. The residue obtained was dissolved in minimum volume of warm acetic acid. The product was recovered by precipitation with the addition of an equal volume of diethyl ether. The resulting precipitate was recrystallized from 70% ethanol in water.

The analytical data and the yields obtained were as follows:

A-CM_6D : The yield was 8.0g (28%), m.p. 162-164°C (literature m.p. 146-148°C [68]); TLC showed one spot with a Rf value of 0.35 in EtOH:HCOOH:water (18:1:1, v/v/v) on silica plate (literature Rf: 0.35 [68]).

B-CM_6D : The yield was 2.4g (8%), m.p. 194-197°C (literature m.p. 179-181°C [68]); TLC showed one spot with a Rf value of 0.54 in

EtOH:HCOOH:water (18:1:1, v/v/v) on silica plate (literature Rf: 0.55 [68]).

The chromatograms were visualized with a solution of 0.2% ninhydrin in acetone.

4.10 Matrix activation

4.10.1 Epoxide activation

The procedure for epoxide activation of cellulose and agarose and their subsequent analysis were identical to that found in Section 3.7. In this study, 1% and 3% sodium hydroxide-epoxide activated cellulose were prepared.

Comparable epoxide activation of moist Sepharose 6B (20g) was achieved by using the following conditions:

(I) 0.25M sodium hydroxide solution (16ml) containing sodium borohydride (32mg), epichlorohydrin (1ml), 24 hr., 4°C and

(II) 1.5M sodium hydroxide solution (16ml) containing sodium borohydride (32mg), epichlorohydrin (3ml), 22 hr., room temperature.

4.10.2 1,1'-Carbonyldiimidazole (CDI) activation

(a) Procedure for CDI activation of agarose

The moist Sepharose CL-6B (10g) was solvent-exchanged from water to DMF as given in Appendix 2. CDI (0.6 to 3.0g) was added to moist Sepharose CL-6B and was mixed at room temperature for about 1 hour. It was washed with DMF and used immediately.

(b) CDI-activation analysis

The CDI-activated agarose (2g) in DMF was solvent-exchanged from DMF to water as given in Appendix 2. A solution of 1M sodium hydroxide (1 to 2ml) was added to moist sample and was left to stand overnight at room temperature. After adjusting the pH to pH 3, nitrogen was bubbled into the mixture for about 15 minutes to remove carbonic acid

as carbon dioxide. It was titrated with 1.00M sodium hydroxide to pH 5. After zeroing the autoburette, the sample was titrated from pH 5 to 8.5 to determine the amount of imidazoyl groups released. The sample was transferred quantitatively onto a weighed sintered glass funnel, washed with deionized water and dried at 60°C overnight followed by drying at 105°C for about 2 hours.. The CDI-activation level (in meq/g) was determined (see Appendix 8)

4.11 Coupling of alpha-citrylhexamethylenediamine (A-CM_6D), beta-citrylhexamethylenediamine (B-CM_6D) and 6-aminohexanoic acid (AH) to activated matrices

4.11.1 Preparation of A-CM_6D- or B-CM_6D- and AH-matrices

A-CM_6D or B-CM_6D (0.8 to 1.5g) or AH (1.9g) was suspended in some deionized water and was dissolved by titration with sodium hydroxide to pH 11. The resulting solution was made up to 10 ml and 1M sodium bicarbonate (pH 11) buffer (10ml) was added to it. The final ligand solution was added to the moist activated matrix (7.5 to 10.0g) and was mixed at room temperature for several days (see Tables 4.1 and 4.2). The product obtained was collected on a filter and washed thoroughly with deionized water.

4.11.2 Ligand substitution level analysis

A moist sample (5g) was washed with 0.1M hydrochloric acid (ca. 100ml) followed by deionized water until neutral. It was dispersed in 0.5M sodium chloride solution (ca. 10 ml). A-CM_4D- or B-CM_6D- matrix made from epoxide activated matrices were titrated with 1.00M sodium hydroxide to pH 7.5. After zeroing the autoburette, it was titrated from pH 7.5 to pH 11 to determine the nitrogen content. While the matrices activated with 1,1'-carbonyldiimidazole were simply titrated with 1.00M sodium hydroxide to pH 8 to determine the carboxyl content.

The sample was transferred quantitatively onto a sintered glass filter, washed with deionized water and transferred to a measuring cylinder to settle overnight. Once its settled volume was recorded, it was transferred back to the same glass filter and dried at 60°C overnight followed by drying at 105°C for about 2 hours. The number of titratable nitrogen or carboxyl groups (meq/g) and the swollen volume (ml/g) of the product were then determined (see Appendices 3 and 4 for more details).

4.12 Coupling of protected aspartic acid to 1,1'-carbonyldiimidazole (CDI)-activated agarose and 6-aminohexanoic acid-agarose derivative

4.12.1 Recovery of aspartic acid dibenzyl ester (Asp-OBz_2) from its tosyl salt

The tosyl salt of aspartic acid dibenzyl ester (Asp-OBz_2) (20g) was dissolved in diethyl ether (150ml). The p-toluene sulphonic acid was extracted from the ether layer with 1M sodium hydroxide (3x100ml). The ether layer was washed with water until neutral and was evaporated to dryness under reduced pressure to give an oil (11.5g). Asp-OBz_2 was used as an oil.

4.12.2 Preparation of aspartic acid-agarose derivative (Asp-agarose)

Asp-OBz_2 (6.0g) was added to CDI-activated agarose (10.0g) in DMF (10ml) and was mixed at room temperature for 24 hours. After washing the sample with DMF, it was solvent exchanged from DMF to water as given in Appendix 2. A small portion of the sample (1g) was set aside for benzyl group analysis (see Section 4.12.4).

The product was hydrolyzed by holding it at pH 12 with sodium hydroxide for 2 hours at 30 to 40°C. After the appropriate washings, the product was titrated to pH 8 with 1.00M sodium hydroxide (see Section 4.11.2). This process of hydrolysis and titration was repeated

until a relatively constant titration volume was obtained. Then, a portion of the sample was analyzed for its carboxyl content (meq/g) and swollen volume (in ml/g) as given in Section 4.11.2.

4.12.3 Preparation of agarose 6-aminohexyl aspartate derivative (Asp-AH-agarose)

Asp-OBz_2 (5.4g) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) (0.32g) were added to agarose-6-aminohexanoic acid derivative (16.0g) in DMF (10ml). This was mixed at room temperature for 2 hours. After the sample was solvent exchanged from DMF to water as given in Appendix 2, it was base hydrolyzed as described in the preceding section (Section 4.12.2). The number of carboxyl groups present (in meq/g) and the swollen volume(in ml/g) of the sample were determined as given in Section 4.11.2.

4.12.4 Benzyl group analysis [73]

Asp-OBz_2-agarose and the hydrolyzed product, Asp-agarose and Asp-AH-agarose were analyzed for the benzyl group content. The dried agarose derivative (20mg) was hydrolyzed with concentrated hydrochloric acid (1ml) at room temperature for 10 to 15 minutes. The resulting clear solution was diluted to 10 ml with methanol. A 1 ml aliquot was further diluted to 10 ml. The absorbance of this solution was read at 258 nm (λ_{max} of benzyl alcohol) against a blank of methanol acidified with 0.12M hydrochloric acid. The calibration curve for benzyl alcohol was prepared using three standard solutions of the alcohol with concentrations of 0.242, 0.483 and 0.723 mmol/l. The benzyl alcohol was dissolved in methanol acidified with 0.12M hydrochloric acid. From this standard curve obtained, the benzyl group content was estimated (in meq/g).

4.13 Stepwise elution of bovine lactoferrin and bovine lactoperoxidase

The matrix was pre-equilibrated with the buffer solution and was packed into a column (2ml). A 1% lactoferrin (Lf) or lactoperoxidase (Lp) solution (4ml) in buffer was loaded onto the column and was left to equilibrate for 0.5 hour before elution. The column was eluted with the starting buffer solution (6ml) and the eluate (10ml) was collected. Then, the column was equilibrated with 0.1M sodium chloride solution (4ml) in buffer for about 0.5 hour as before. It was eluted with another 6ml of 0.1M sodium chloride solution and the eluate (10ml) was collected. This stepwise elution was repeated with 0.2, 0.3, 0.4 and 0.5M sodium chloride. The absorbance of the Lf (or Lp) eluate at various salt strength was read at 280 (or 412) nm.

4.14 Continuous gradient elution for purification of isolated bovine lactoferrin

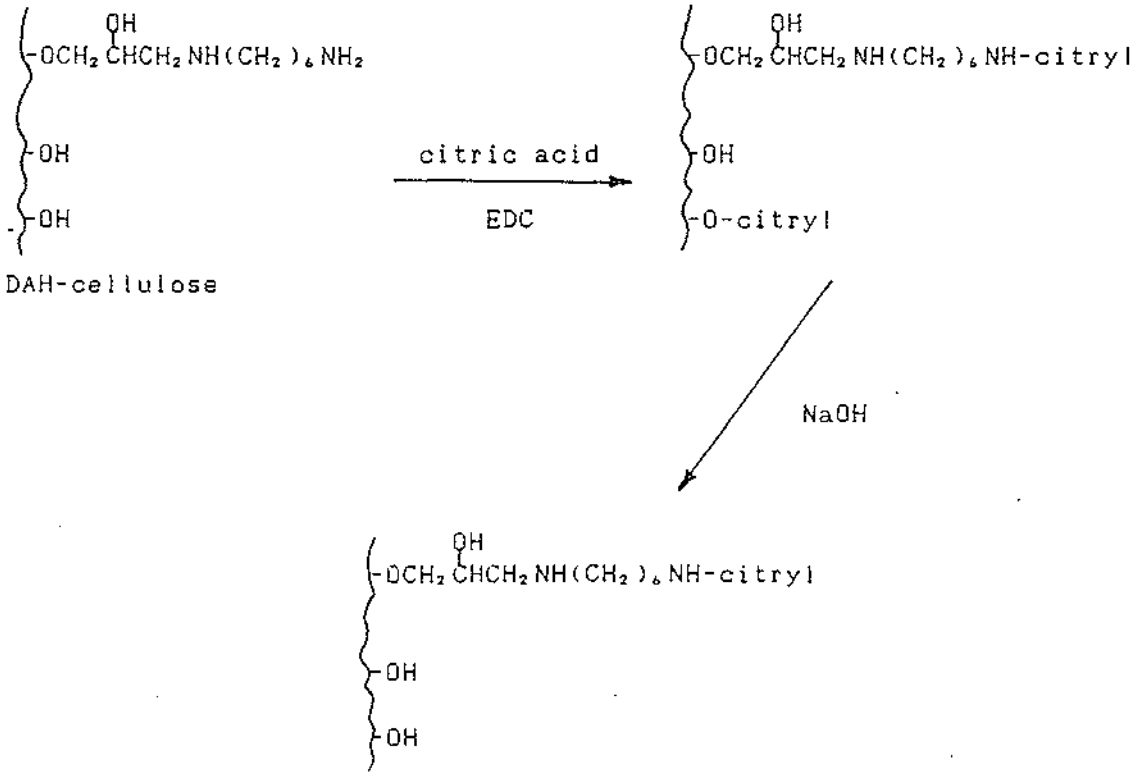
The crude lactoferrin (Lf) solution (10 mg/ml) was dialyzed against 0.025M Tris/HCl buffer (pH 7.8) solution containing 0.2M sodium chloride. The matrix was pre-equilibrated with buffer and was packed into a column (100x7 mm diameter). The column was loaded with the Lf solution (1 ml) and was eluted under a continuous salt gradient from 0.2 to 0.7M sodium chloride over 75 minutes at 1.0 ml/min. The Lf fraction collected was concentrated to about 2.5ml by ultra-filtration. It was iron saturated by adding an excess of 0.01M Fe-NTA and its absorbance at 465, 410 and 280 nm were measured. The concentration of the Lf fraction was necessary to give an absorbance reading of at least 0.1 at 465 nm. The A_{280}/A_{465} and A_{410}/A_{465} ratios were calculated.

4.15 Preliminary investigation of binding of lactoferrin (Lf) and lactoperoxidase (Lp) on citrate-cellulose derivative (CT 4)

4.15.1 Introduction

The first citrate cellulose investigated was prepared from the reaction of citric acid and diaminohexyl (DAH)-cellulose using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) as a coupling reagent [36]. The product obtained, labelled CT 4 after base hydrolysis to remove any ester bonded citryl groups, had a citrate substitution level of 1.0 meq/g (see Figure 4.4).

Figure 4.4 : Typical reactions in the preparation of CT 4



4.15.2 Elution profiles of Lf and Lp

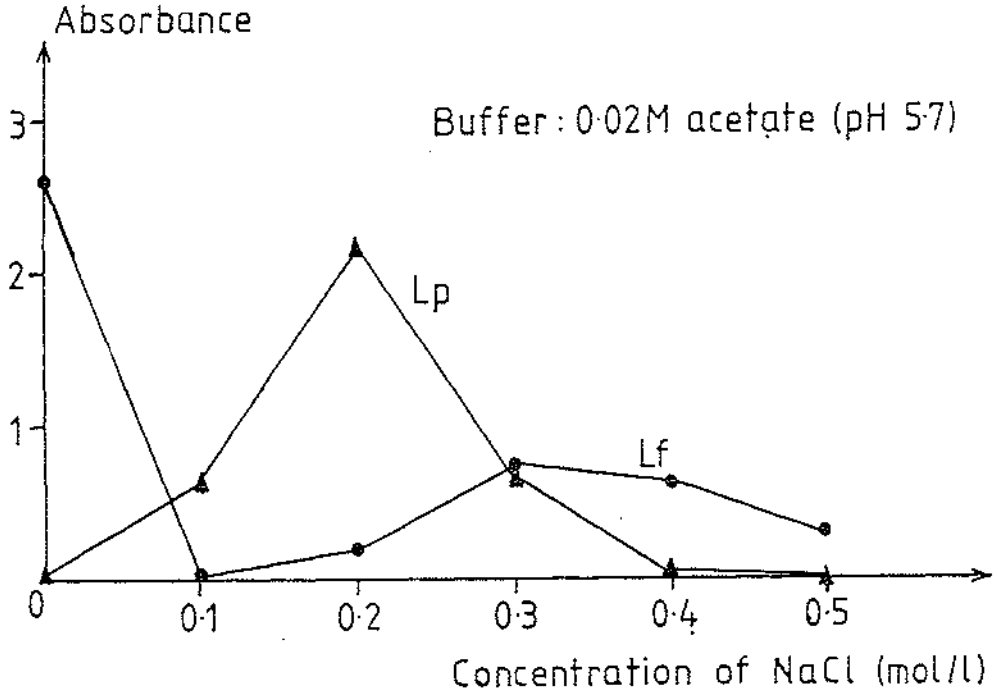
Lf (40mg) and Lp (40mg) were loaded separately onto 2ml columns of CT 4 in 0.02M acetate buffer (pH 5.7). A stepwise elution of the retained protein was carried out using 10ml of 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5M sodium chloride in the same buffer (see section 4.13). The elution of Lf and Lp from CT 4 at each of these salt strengths is shown in Figure 4.5.

The capacity of CT 4 for Lf under the loading conditions was very low as much of it came straight through. The rest of the Lf desorbed at 0.3M-0.4M sodium chloride. All the Lp was bound on the column but was eluted off at a lower ionic strength of 0.2M sodium chloride. Thus, there was some resolution between Lf and Lp on CT 4.

4.15.3 Conclusion

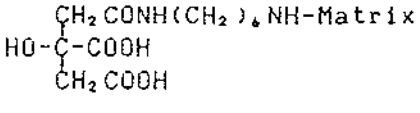
CT 4 showed that it might have some ability to resolve Lf and Lp but CT 4 was a matrix which contained a random mixture of the alpha and beta configurational isomers of citrate. The contribution made by each of these isomers of citrate to the binding of Lf was not clear. This preliminary finding prompted the preparation of the alpha-citrylhexamethylenediamine and beta-citrylhexamethylenediamine matrices (see Figure 4.3) so that the effect of each of these ligands on Lf binding could be investigated separately.

Figure 4.5 : Stepwise elution of Lf and Lp from CT 4

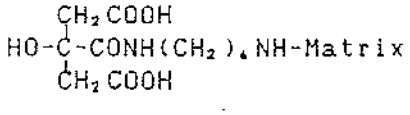


4.16 Investigation of binding of Lactoferrin (Lf) and Lactoperoxidase (Lp) on alpha-citrylhexamethylenediamine (A-CM_6D) and beta-citryl-hexamethylenediamine (B-CM_6D) matrices

4.16.1 Introduction



A-CM_6D-Matrix



B-CM_6D-Matrix

The A-CM_6D and B-CM_6D-matrices (5 and 9, Figure 4.3) were prepared as described in experimental sections 4.10 and 4.11. The binding of Lf and Lp on these matrices was then investigated. Lf (40mg) and Lp (40mg) were chromatographed separately on these matrices (2ml column) in 0.02M acetate buffer (pH 5.7) by stepwise elution of the retained protein with increasing sodium chloride concentration as

described above for CT 4. The elution profiles of Lf and Lp on these columns were studied. The effect of (i) citrate substitution level, (ii) porosity of the matrix and (iii) additional ionic properties of the matrix on the strength of Lf binding and resolution between Lf and Lp were examined.

4.16.2 Preparation of A-CM_6D- and B-CM_6D-celluloses via epoxide activation

(a) Properties of A-CM_6D- and B-CM_6D-celluloses

The A-CM_6D- and B-CM_6D-celluloses were made at two citrate substitution levels. The substitution level was determined by titrating the amino function of the CM_6D between pH 7.5 and 11 and this was used as the basis for the citrate content. The properties of these matrices prepared from their respective starting epoxide activated celluloses (EA-cell) are summarized in Table 4.1.

Table 4.1 : Properties of A-CM_6D and B-CM_6D-celluloses via epoxide activation

Code	Matrix	Swollen volume (ml/g)	Titratable nitrogen[2] (meq/g)
CT 8	A-CM_6D-cellulose[1a]	25.0	0.31
CT 9	B-CM_6D-cellulose[1a]	24.4	0.30
CT 10	A-CM_6D-cellulose[1b]	22.4	0.73
CT 11	B-CM_6D-cellulose[1b]	21.4	0.72

Notes :
1. Reaction conditions :-
(a) 7.5g moist EA-cell (0.42 meq/g); 1g A-CM_6D (0.85g B-CM_6D); 6 days; room temperature.
(b) 7.5g moist EA-cell (1.15 meq/g); 1.5g A-CM_6D (1g B-CM_6D); 4 days; 22°C.
2. Equivalent to the citrate substitution level (see Figure 4.2).

(b) Elution profiles of Lf and Lp

The elution profiles of Lf and Lp on the CM$_6$D-celluloses are shown in Figures 4.6 and 4.7. As shown in Figure 4.6, both A-CM$_6$D- and B-CM$_6$D-celluloses with low citrate substitution showed very little capacity for Lf. Also, the protein was only weakly bound to both celluloses. At 0.1M sodium chloride, most of the bound Lf was eluted off.

Lf was found to bind more strongly on both A-CM$_6$D- and B-CM$_6$D-celluloses with high citrate substitution (see Figure 4.7); in particular, on A-CM$_6$D-cellulose. However, their Lf capacities were still low (only 20mg Lf was loaded). Most of the Lp were eluted off at 0.1M sodium chloride in both celluloses. The resolution between Lf and Lp on both celluloses would be expected to be poor on these matrices.

From these results, both A-CM$_6$D- and B-CM$_6$D-celluloses were found to be of limited use in terms of their capacity for Lf and their resolution of Lf from Lp. But they showed that the strength of Lf binding could be increased by using a high substitution of citrate ligand. The poor capacity for Lf and their weak binding may be attributed to the lack of porosity of the celluloses studied.

4.16.3 Preparation of A-CM$_6$D- and B-CM$_6$D-agaroses via epoxide activation

(a) Properties of A-CM$_6$D- and B-CM$_6$D-agaroses

The A-CM$_6$D- and B-CM$_6$D-agaroses were made at two citrate substitution levels. The properties of these matrices prepared from their respective starting epoxide activated agaroses (EA-aga) are summarized in Table 4.2.

Figure 4.6 : Stepwise elution of Lf from A-CM, D-cellulose (CT 8) and B-CM, D-cellulose (CT 9)

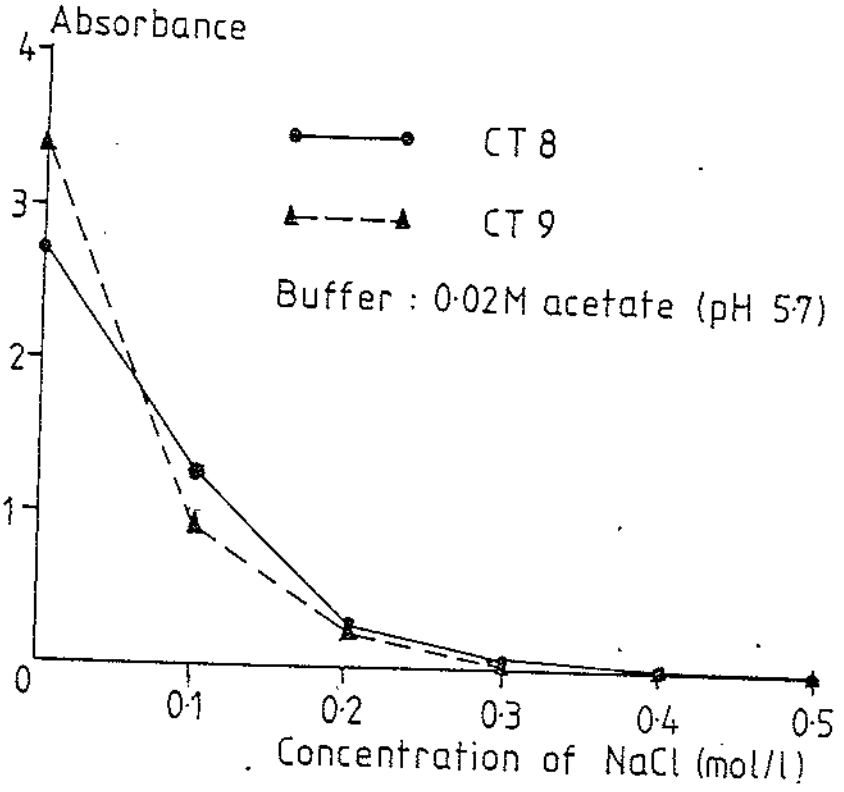


Figure 4.7 : Stepwise elution of Lf and Lp from A-CM, D-cellulose (CT 10) and B-CM, D-cellulose (CT 11)

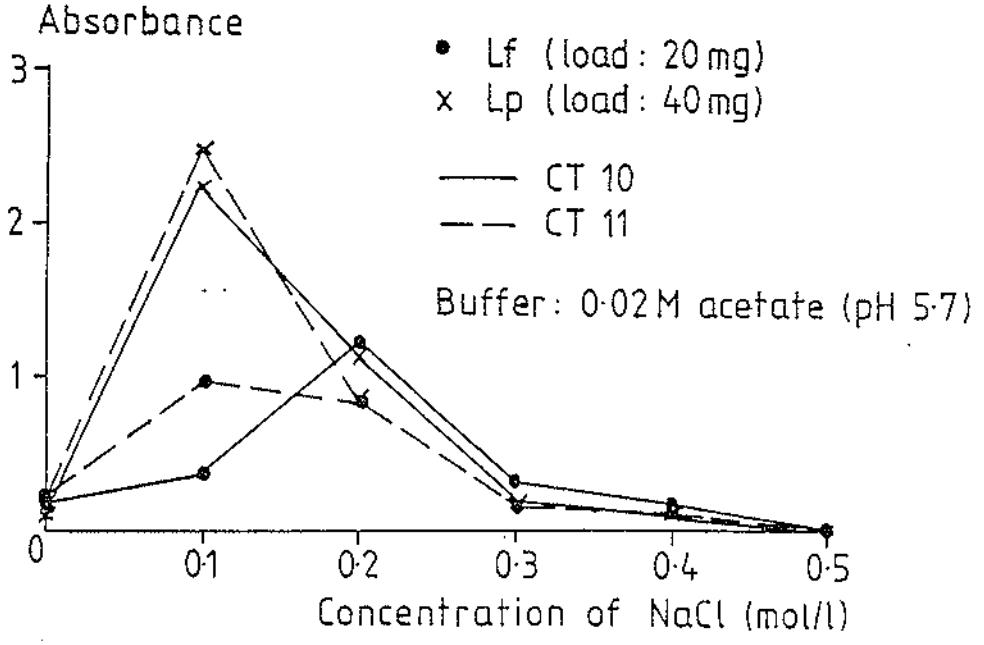


Table 4.2 : Properties of A-CM$_6$D and B-CM$_6$D-agaroses via epoxide activation

Code	Matrix	Swollen volume (ml/g)	Titratable nitrogen[2] (meq/g)
AG133-CT2	A-CM$_6$D-agarose[1a]	20.1	0.34
AG133-CT1	B-CM$_6$D-agarose[1a]	22.0	0.33
AG134-CT1	A-CM$_6$D-agarose[1b]	16.4	0.63
AG134-CT2	B-CM$_6$D-agarose[1b]	16.6	0.58

Notes :
1. Reaction conditions :-
(a) 7.5g moist EA-aga (0.48 meq/g); 1g A-CM$_6$D (0.85g B-CM$_6$D); 6 days; room temperature.
(b) 10.0g moist EA-aga (1.14 meq/g); 1.5g A-CM$_6$D (1g B-CM$_6$D); 4 days; 22°C.
2. Equivalent to the citrate substitution level (see Figure 4.2).

(b) Elution profiles of Lf and Lp

The elution profiles of Lf and Lp on CM$_6$D-agaroses are shown in Figures 4.8 and 4.9. As shown in Figure 4.8, Lf was more tightly bound on both A-CM$_6$D- and B-CM$_6$D-agaroses with low citrate substitution compared to the cellulose equivalents (cf. Figure 4.6). The capacity for Lf had increased significantly. The total Lf loaded (40mg) onto the 2ml column was adsorbed. Generally, Lf was eluted off at 0.2M sodium chloride while Lp eluted off at 0.1M sodium chloride. There was significant resolution between Lf and Lp. Much the same observations were noted for A-CM$_6$D- and B-CM$_6$D-agaroses with high citrate substitution except that both Lf and Lp eluted off at higher salt strength, 0.3M and 0.2M sodium chloride respectively (see Figure 4.9). In both cases (see Figures 4.8 and 4.9), the alpha linked citrate derivatives bound the Lf slightly more tightly than did the beta linked derivatives.

Figure 4.8 : Stepwise elution of Lf and Lp from A-CM_6 D-agarose (AG133-CT 2) and B-CM_6 D-agarose (AG133-CT 1)

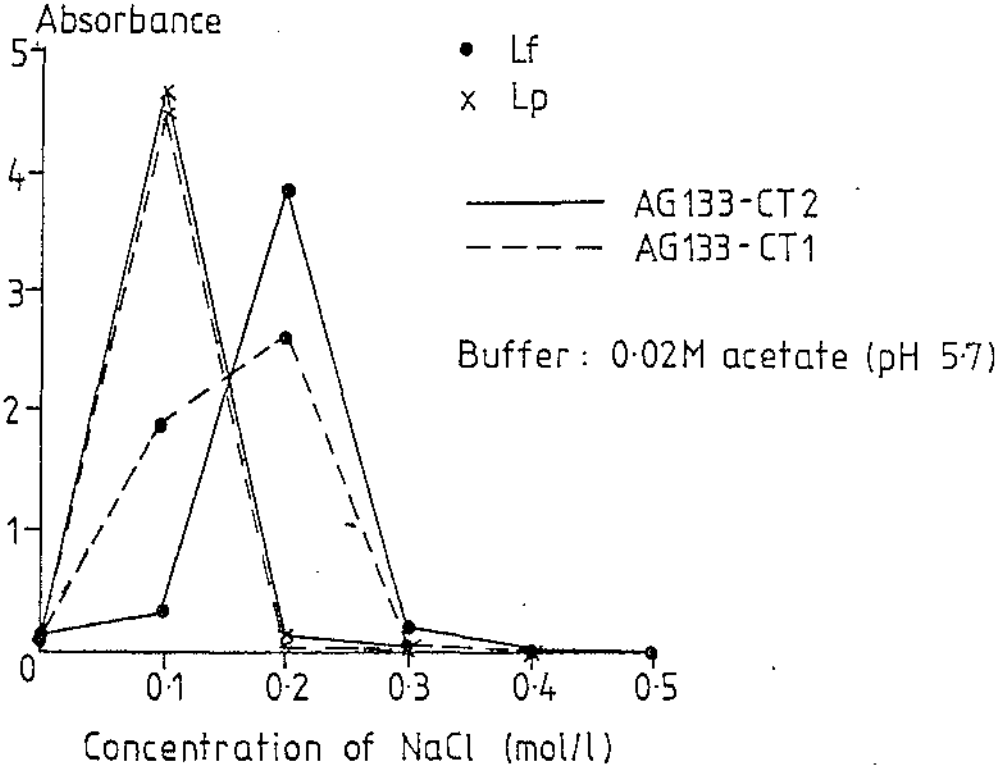
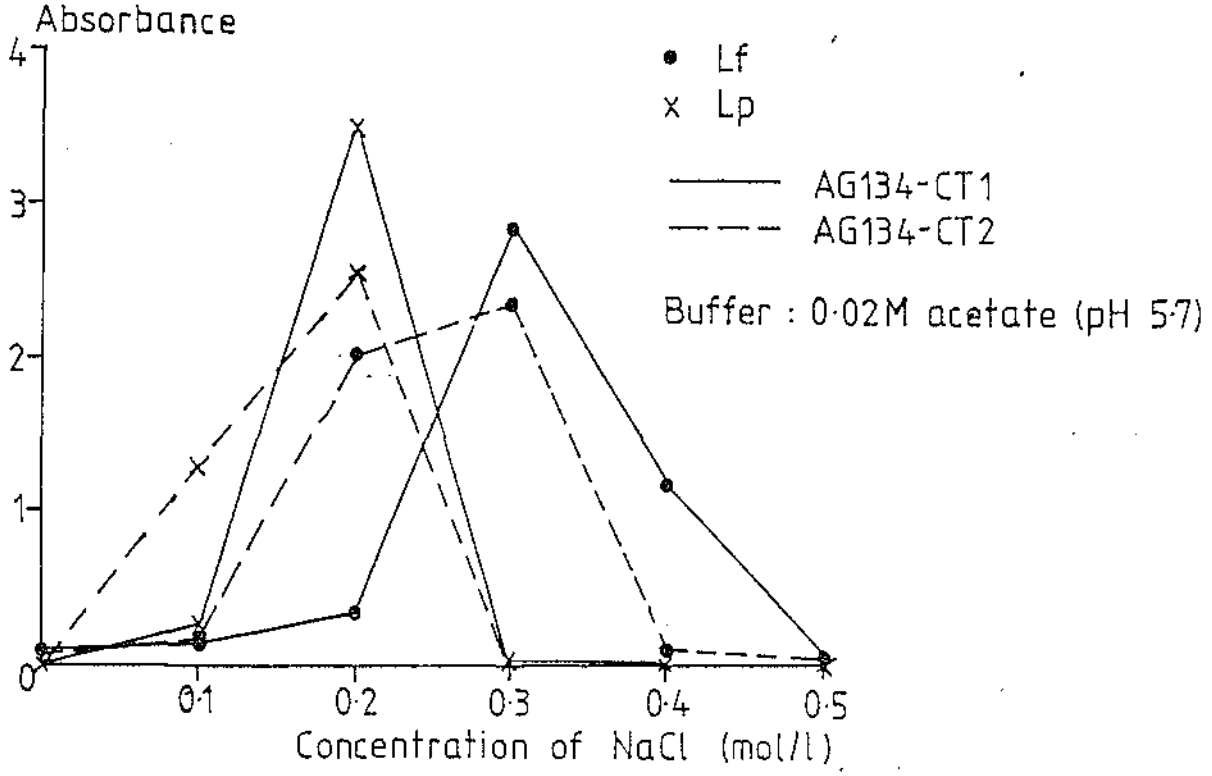


Figure 4.9 : Stepwise elution of Lf and Lp from A-CM_6 D-agarose (AG134-CT 1) and B-CM_6 D-agarose (AG134-CT 2)



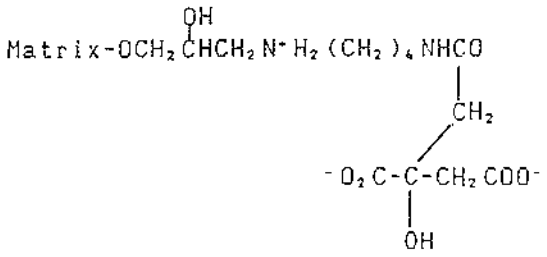
The results showed that porosity of the matrix was an important factor. The use of agarose, which has a much higher porosity than cellulose, had led to an increase in the capacity and the strength of binding of Lf. The resolution between Lf and Lp was also improved. The effect of citrate substitution level on the Lf binding was clearly illustrated. The change of the CM_6D-agaroses with low citrate substitution to high citrate substitution caused the Lf to elute off at a distinctly higher ionic strength.

4.16.4 Preparation of A-CM_6D- and B-CM_6D-agaroses via 1,1'-carbonyldiimidazole (CDI) activation

(a) Rationale

Since A-CM_6D-matrices were marginally binding Lf more tightly than B-CM_6D-matrices (see preceding two sections), it was decided that only A-CM_6D-matrices would be further studied.

The previous A-CM_6D-matrices were prepared via the epoxide activation. This mode of activation resulted in a basic secondary amine linkage which was positively charged under the conditions investigated (pH < 11). It was likely that this positive charge was tying up one of the negatively charged carboxyl groups on the citrate ligand, for example,



It was hoped that the removal of this positive charge might possibly result in a stronger binding of Lf and a better resolution of Lf and

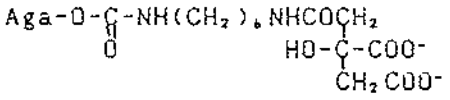
Lp.

An attempt was made to remove this positive charge by:

1. reaction of A-CM$_4$D-agarose (AG134-CT1) with acetic acid using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide as a coupling reagent and;

2. reaction of AG134-CT1 with succinic anhydride.

The attempt to remove this positive charge by introducing an amide linkage was not completely successful. Presumably, the secondary amine was in a sterically hindered position. Consequently, it was decided that A-CM$_4$D would be best coupled with CDI-activated agarose to give the nonbasic urethane derivative [65], ie.



(b) Properties of A-CM$_4$D-agarose via CDI activation

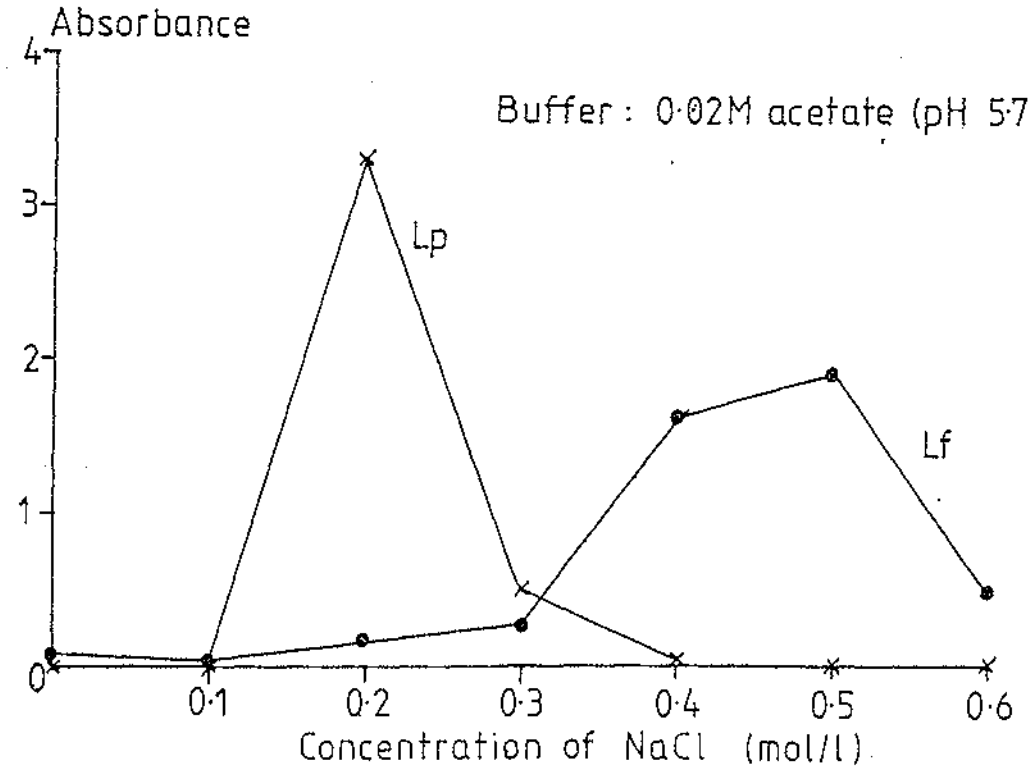
A-CM$_4$D was coupled with a moderately activated agarose (0.77 meq/g) by the CDI method (see Figure 4.2). This failed to produce a A-CM$_4$D-agarose of comparable citrate substitution level (ca. 0.6 meq/g). The coupling of A-CM$_4$D with a highly activated agarose (6.53 meq/g) gave the desired A-CM$_4$D -agarose (AG135-CT). It had a carboxyl content of 1.07 meq/g. This was approximately 0.54 meq/g of citrate groups. Because of the absence of titratable nitrogen, the carboxyl content of the matrix was determined directly. This determination was carried out by first washing the matrix with acid (0.1M hydrochloric acid) and water and then, titrating it to pH 8 to estimate the carboxyl content. Thus, about 0.54 meq/g (or 8%) of the activated groups had coupled and the rest hydrolyzed.

(c) Elution profiles of Lf and Lp

The elution profiles of Lf and Lp on A-CM-D-agarose are shown in Figure 4.10. There was a significant increase in the ionic strength (0.5M sodium chloride) required for the elution of Lf while Lp was eluted off at the same ionic strength (0.2M sodium chloride) as before (cf. Figure 4.9).

The expected increase in the strength of Lf binding and the significant improvement in the resolution of Lf and Lp were observed. Hence, it was necessary to remove the positively charged amine linkage resulting from the epoxide activation. It exerted a very significant influence on the strength of Lf binding.

Figure 4.10 : Stepwise elution of Lf and Lp from A-CM-D-agarose (AG135-CT)



4.16.5 Summary of findings

It was found that the citrate substitution level, the porosity and the additional ionic properties of the matrix were important factors in the binding of Lf and the resolution of Lf and Lp on the matrix. The strength of Lf binding was strongly dependent on the degree of citrate substitution. As the citrate substitution was increased, Lf was eluted off at higher ionic strength. The switch from cellulose to agarose matrix caused an increase in the capacity for Lf as well as the strength of Lf binding. Apparently, the higher porosity in the agarose matrix permited greater interaction between the citrate and Lf. Finally, the removal of the additional cationic properties from the matrix led to a very significant increase in the strength of Lf binding. The positive charge on the secondary amine in the epoxide linkage obviously interfered with the interaction between citrate and Lf.

4.17 Investigation of the nature of interaction between citrate and Lf

4.17.1 Introduction

An attempt was made to determine whether the interaction between the citrate ligand and Lf was ionic in nature or whether there was some special affinity type interaction involved. Previous work had suggested the latter as the esr signal for the iron atom had changed when a citrate cellulose was mixed with Lf [64]. Since the extent of charge on the protein is dependent on pH, some understanding of the interaction between the citrate ligand and Lf can be gained by studying the effect of pH on the strength of Lf binding to A-CM-D-agarose and comparing it with the corresponding Lf binding to a simple carboxymethyl agarose such as CM-Sepharose-fast flow (CM-Sep-ff). The latter is a cation exchanger similar to CM-sephadex which is used

extensively for Lf purification. CM-Sep-ff has an agarose matrix in place of the cross-linked dextran in CM-Sephadex.

The effect of pH on Lf binding to CM-Sep-ff provides an illustration of an interaction which is ionic in nature. This then served as a basis for comparing the effects of pH on Lf binding to A-CM$_4$D-agarose.

4.17.2 Effect of pH on Lf binding to A-CM$_4$D-agarose (AG135-CT)

Lf was loaded onto 2ml columns of A-CM$_4$D-agarose (AG135-CT) and CM-Sep-ff and was eluted stepwise with an increasing sodium chloride concentration as described before (see Section 4.13). Lf binding to these columns was studied at three different pH levels using the following buffers: 0.02M acetate (pH 5.7), and 0.025 Tris/HCl (pH 7.8 and 9). Lf binding to CM-Sep-ff was also studied at pH 9.5 using 0.02M borate buffer.

The effects of pH on Lf binding to A-CM$_4$D-agarose and CM-Sep-ff are shown in Figures 4.11 and 4.12 respectively. The strength of Lf binding to CM-Sep-ff decreased as the pH was increased from pH 5.7 to 9.5. This shift towards lower ionic strength for the elution of Lf as the pH was increased was expected when the binding of the protein was based on ionic interaction. The was because the extent of positive charge on Lf decreased as the pH was increased but the change was expected to be much greater especially at pH 9.5 if its pI was around pH 8.0 as reported [53,54].

The same trend in the change of the strength of Lf binding to A-CM$_4$D-agarose was observed (see Figure 4.11). In the case of A-CM$_4$D-agarose, the shift towards lower ionic strength was more distinct. Presumably, the lower carboxyl density on A-CM$_4$D-agarose (1.07 meq/g) compared to CM-Sep-ff (ca. 1.41 meq/g) was responsible for the more significant shift.

Figure 4.11 : Stepwise elution of Lf from A-CM$_6$D-agarose (AG135-CT) at different pH levels

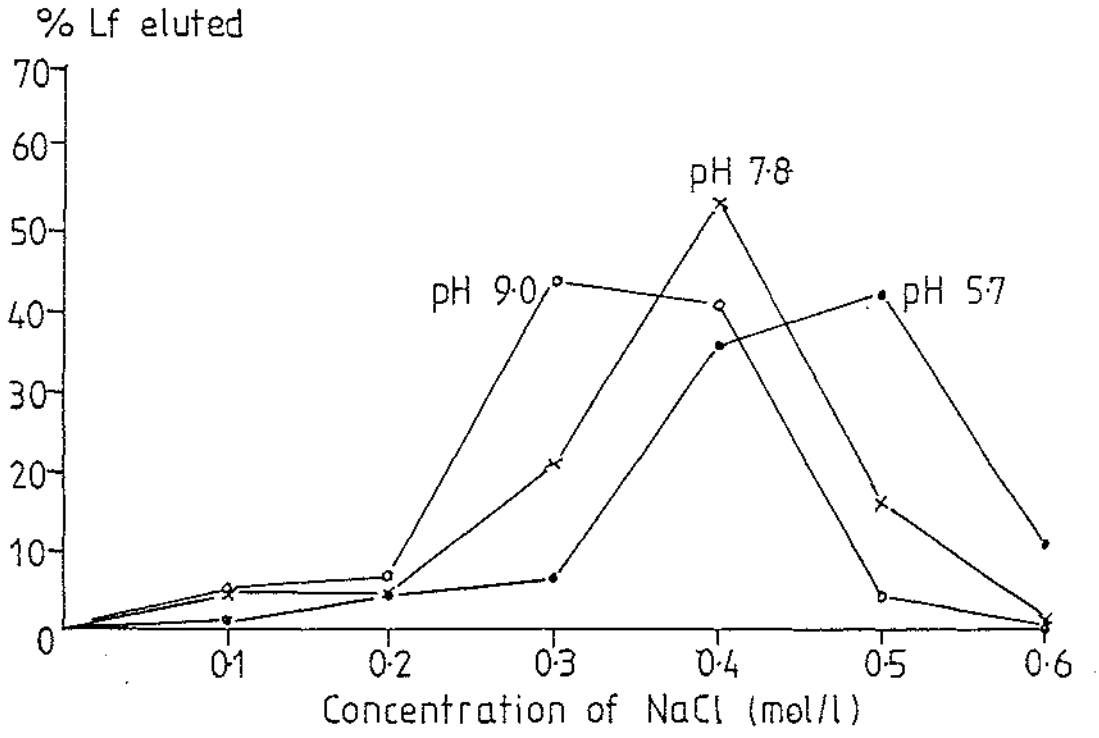
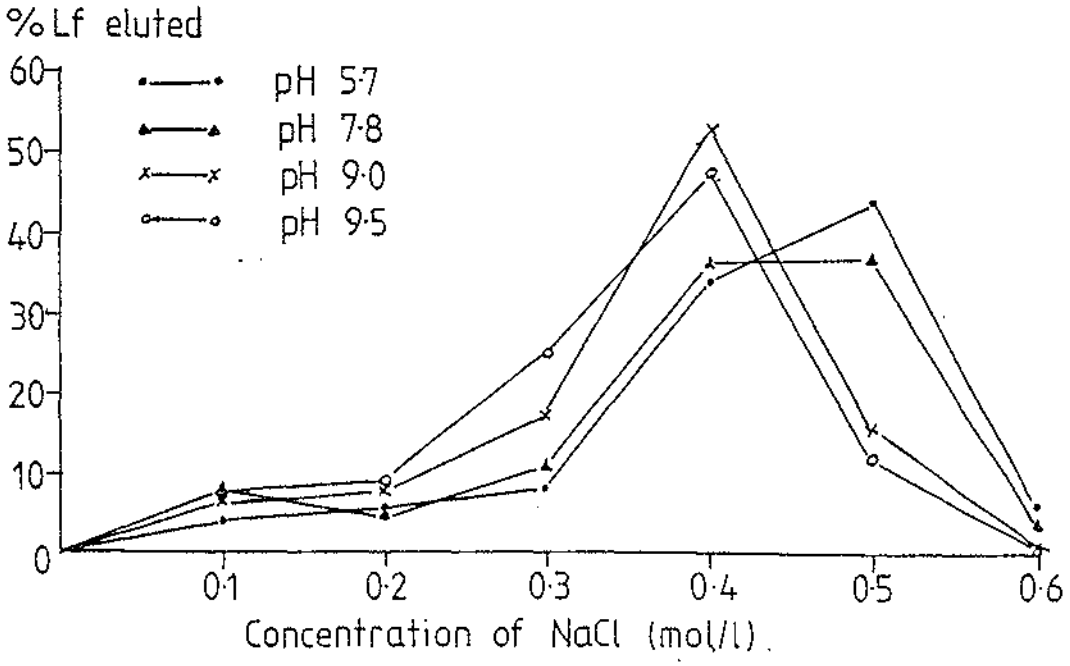


Figure 4.12 : Stepwise elution of Lf from CM-Sepharose (fast flow) at different pH levels



4.17.3 Conclusion

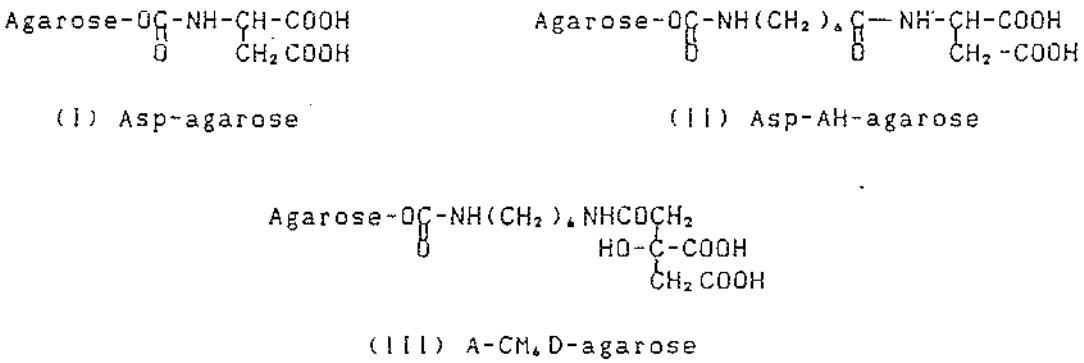
The results suggested that the Lf binding was predominantly ionic in nature. Also, the relatively strong Lf binding at pH 9 and 9.5 would indicate that the pI of the protein is much higher than that cited (cf. pH 8.0 [53,54]). However, it was recognized that there was incomplete information to fully understand the interaction between citrate and Lf. Information from other physical methods such as crystallographic studies, C-13 nmr and esr studies should be sought.

4.18 Preparation of agarose aspartate derivative (Asp-agarose) and agarose 6-aminohexyl aspartate derivative (Asp-AH-agarose)

4.18.1 Introduction

In order to further investigate the nature of the binding action of Lf and its purification on A-CM-D-agarose, compounds with similar structures to citrate in terms of the presence of two carboxyl groups were sought for comparison. One such compound is aspartic acid (Asp). When Asp is coupled to CDI activated agarose and to agarose derivative containing 6-aminohexanoic acid (AH), the products obtained are aspartate agarose (Asp-agarose) and 6-aminohexyl aspartate-agarose (Asp-AH-agarose) respectively. The structures of Asp-agarose (I) and Asp-AH-agarose (II) are shown in Figure 4.13. Asp-agarose (I) and Asp-AH-agarose (III) are similar to A-CM-D-agarose (III) in that they all have two unbound carboxyl groups. The preparation of Asp-agarose allowed the effect of the spacer arm on the purification of Lf to be studied as the coupling of citrate directly to the CDI activated agarose was not possible. The preparation of Asp-AH-agarose permited the contribution of the hydroxyl group in the citrate to Lf purification to be investigated.

Figure 4.13 : Agarose derivatives containing carboxyl groups



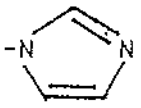
(I) Asp-agarose

(II) Asp-AH-agarose

(III) A-CM$_4$D-agarose

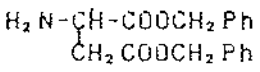
It should have been possible to prepare Asp-agarose from CDI activated agarose by the method used for CM$_4$D-agarose (see Section 4.11) as follows:

(4-5)

where Im represents the imidazolyl group, 

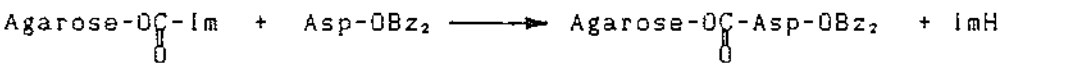
However, when Asp was coupled to CDI activated agarose (2.10 to 6.75 meq/g), the products obtained had very low carboxyl contents (0.11 to 0.31 meq/g). The properties of the Asp-agaroses prepared are summarized in Table 4.3 under Method A.

In an attempt to prepare a product of comparable carboxyl content to A-CM$_4$D-agarose (1.07 meq/g), the coupling of the dibenzyl ester or aspartic acid (Asp-OBz$_2$) to the CDI activated agarose was investigated in DMF. The structure of Asp-OBz$_2$ (IV) is shown below.

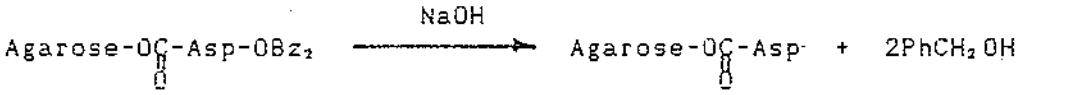


(IV) Asp-OBz_2

It was known that the benzyl group could be easily hydrolyzed by base under mild conditions [74], while the urethane linkage has been shown to be stable to 1M ammonium hydroxide at 37°C for at least 20 hours [75]. Hence, this method of preparing Asp-Aga was investigated as outlined below.



(4-6)



(4-7)

As a check on the success of the method, Asp-OBz_2 was analyzed for the benzyl group [73] by spectroscopy before and after hydrolysis.

4.18.2 Properties of Asp-agarose

Asp-OBz_2-agarose obtained from the reaction of Asp-OBz_2 with the CDI activated agarose gave, on hydrolysis, Asp-agarose with a much higher carboxyl content (0.66 meq/g). The properties of the Asp-agarose prepared are summarized in Table 4.3 under Method B.

The benzyl group analysis showed that the Asp-OBz_2-agarose prior to hydrolysis had a benzyl content of 0.64 meq/g. This was in good agreement with the carboxyl content determined by titration for Asp-agarose (see Table 4.3). It showed that the urethane linkages were stable to hydrolysis conditions. The benzyl group analysis for Asp-

agarose showed no detectable benzyl content indicating that the hydrolysis of the benzyl ester was indeed complete.

Table 4.3 : Properties of Asp-agaroses prepared

Method	Product code	Starting CDI-agarose used (meq/g)	Swollen volume (ml/g)	Carboxyl content (meq/g)
A[1]	AG-ASP1	2.10	20.7	0.11
	AG-ASP2	3.65	19.1	0.21
	AG-ASP3	3.75	18.1	0.31
B[2]	AG-ASP4	3.49	19.3	0.66

Reaction conditions :
1. 7.5g moist CDI-agarose; 4g Asp added (large excess); 20ml 0.5M bicarbonate buffer (pH 11); room temperature; 18 hours (see Section 4.11)
2. As given in Section 4.12.2.

4.18.3 Properties of Asp-AH-agarose

The AH-agarose was prepared from moist CDI activated agarose (2.10 meq/g) at room temperature over the weekend as described in Section 4.11. It had a carboxyl content of 1.12 meq/g.

Asp-OBz_2 was coupled to AH-agarose using a carbodiimide as described in Section 4.12.3. On hydrolysis, the desired Asp-AH-agarose was obtained. It had a carboxyl content of 1.72 meq/g. On the basis of the starting AH-agarose, the coupling was not 100% efficient. The derivative contained a residual amount of carboxyl groups from the unreacted aminohexanoate groups. The benzyl group analysis showed no detectable benzyl content.

4.18.4 Elution of Lf and Lp from Asp-agarose (AG-ASP4) and Asp-AH-agarose

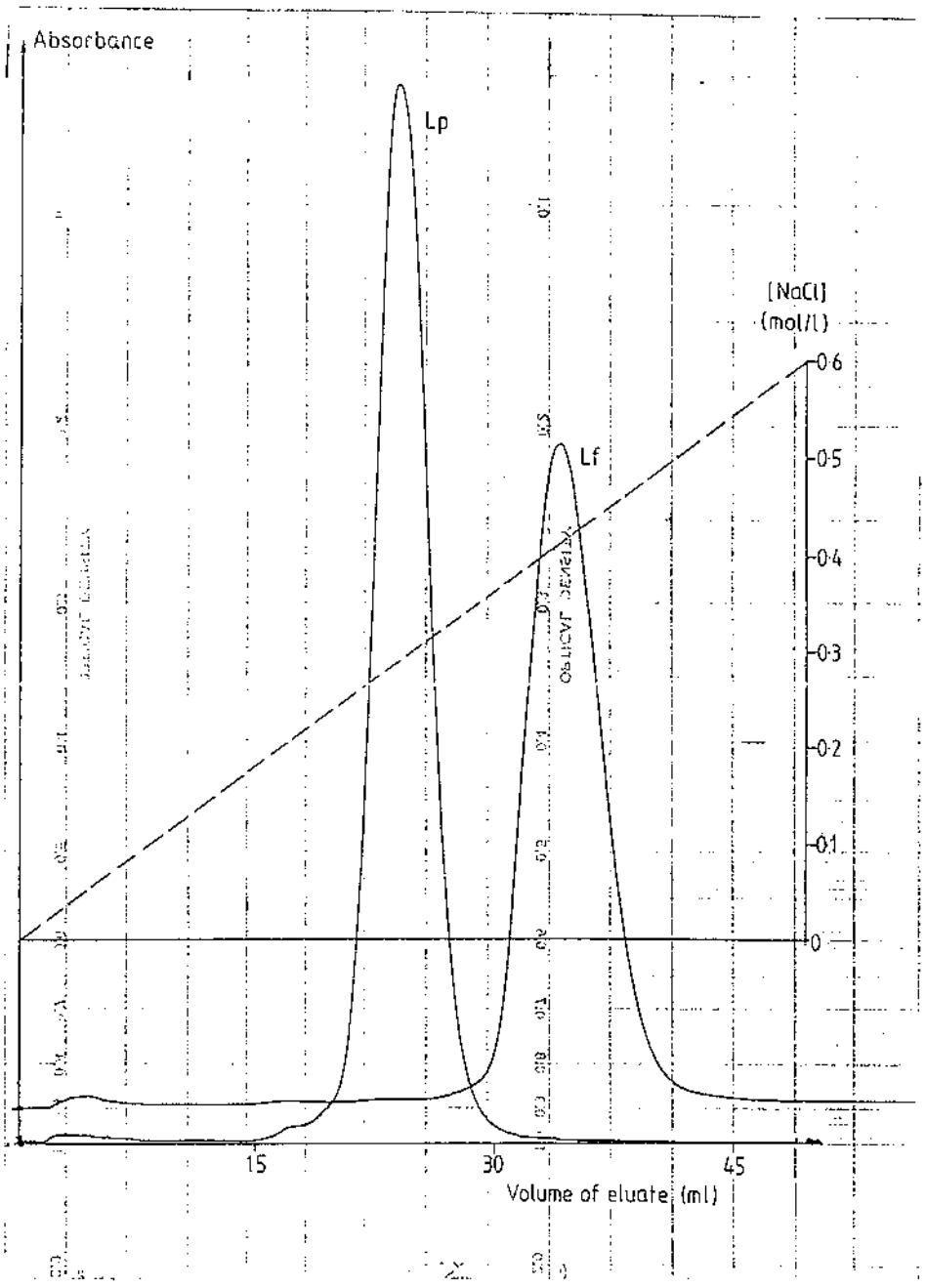
Lf (10mg) and Lp (10mg) were loaded separately onto columns (100x7 mm diameter) of Asp-agarose (AG-ASP4) and Asp-AH-agarose in 0.025M Tris/HCl buffer (pH 7.8). The retained protein was eluted under a continuous salt gradient from 0.0 to 0.7M sodium chloride at 1.0 ml/min (see Section 4.14). The elution profiles of Lf and Lp on these columns are shown in Figures 4.14 and 4.15.

Lf eluted from the Asp-agarose column at about 0.4M sodium chloride while Lp was desorbed at about 0.3M sodium chloride. With the Asp-AH-agarose column, Lf was not eluted until about 0.6M sodium chloride while Lp was desorbed at much the same position ie. 0.3M sodium chloride. The tighter binding of Lf to Asp-AH-agarose can be attributed to its higher carboxyl content of 1.72 meq/g compared with only 0.66 meq/g on Asp-agarose. The 6-aminohexanoic acid spacer arm on Asp-AH-agarose might also have allowed a stronger interaction with Lf.

4.18.5 Conclusion

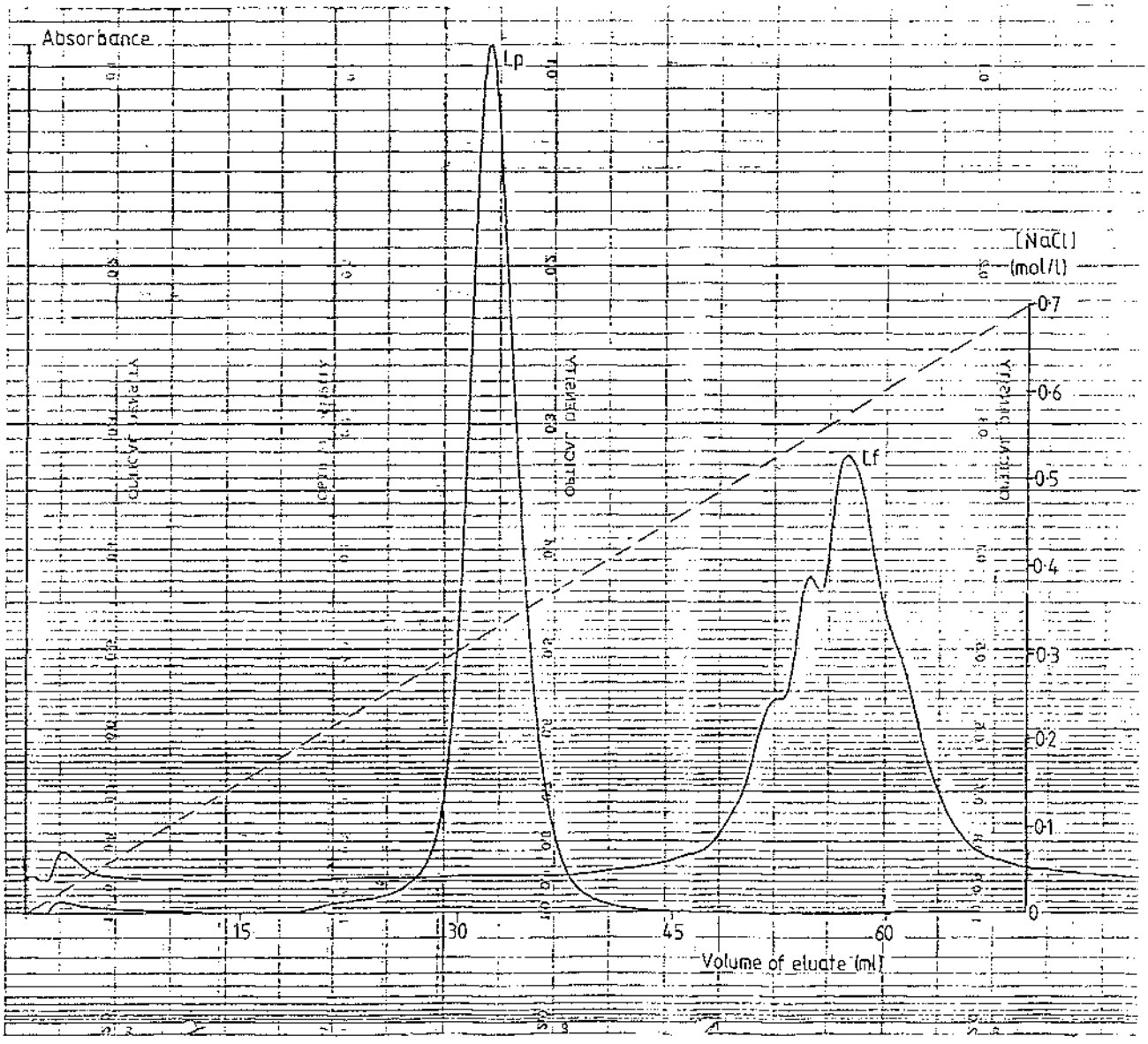
This method of preparing Asp-agarose and Asp-AH-agarose was not straightforward or simple. It was a two step process and required repeated hydrolysis. However, no attempt was made to optimize the reaction conditions. The objective was not the investigation of the method of synthesis but to produce the required derivatives for comparison to A-CM$_4$D-agarose in the purification of Lf (see next section). The results from the preceeding section showed that the elution of Lf on the two columns could be achieved under similar conditions to that of A-CM$_4$D-agarose (cf. Figures 4.10).

Figure 4.14 : Continuous gradient elution of Lf and Lp from Asp-agarose (AG-ASP4)



Buffer : 0.02SM Tris/HCl (pH 7.8)
Flow rate : 1.0 ml/min
Column dimensions : 100x7 mm diameter

Figure 4.15 : Continuous gradient elution of Lf and Lp from Asp-AH-agarose



Buffer : 0.025M Tris/HCl (pH 7.8)
Flow rate : 1.0 ml/min
Column dimensions : 100x7 mm diameter

4.19 Purification of bovine Lactoferrin (Lf)

4.19.1 Introduction

The freshly isolated bovine Lf used in this study was obtained from bovine colostrum using a CM-Sephadex column [76]. Lp and other whey proteins were removed from the column first by elution with 0.3M sodium chloride in 0.025M Tris/HCl buffer (pH 7.8) and then all the Lf was eluted with 0.6M sodium chloride. The Lf fraction used had a concentration of about 10 mg/ml. This crude Lf had ultra-violet to visible light absorption ratios of A_{280}/A_{465} and A_{410}/A_{465} of 30.9 and 0.70 respectively when iron saturated. It was purified on columns of A-CM$_6$D-agarose, Asp-agarose, Asp-AH-agarose and CM-Sep-ff as described in Section 4.14 to compare their effectiveness and investigate the aspects of the citrate ligands which contributed most to the purification of the protein. At least two runs on each column were carried out. The properties of the various columns used are summarized in Table 4.4.

Table 4.4 : Properties of matrices used in Lf purification

Matrix	Swollen volume (ml/g)	Carboxyl content (meq/g)
A-CM$_6$D-agarose (AG135-CT)	16.1	1.07
Asp-agarose (AG-ASP4)	19.3	0.66
Asp-AH-agarose	16.0	1.72
CM-Sep-ff	10.8	1.41

4.19.2 Purification of crude Lf

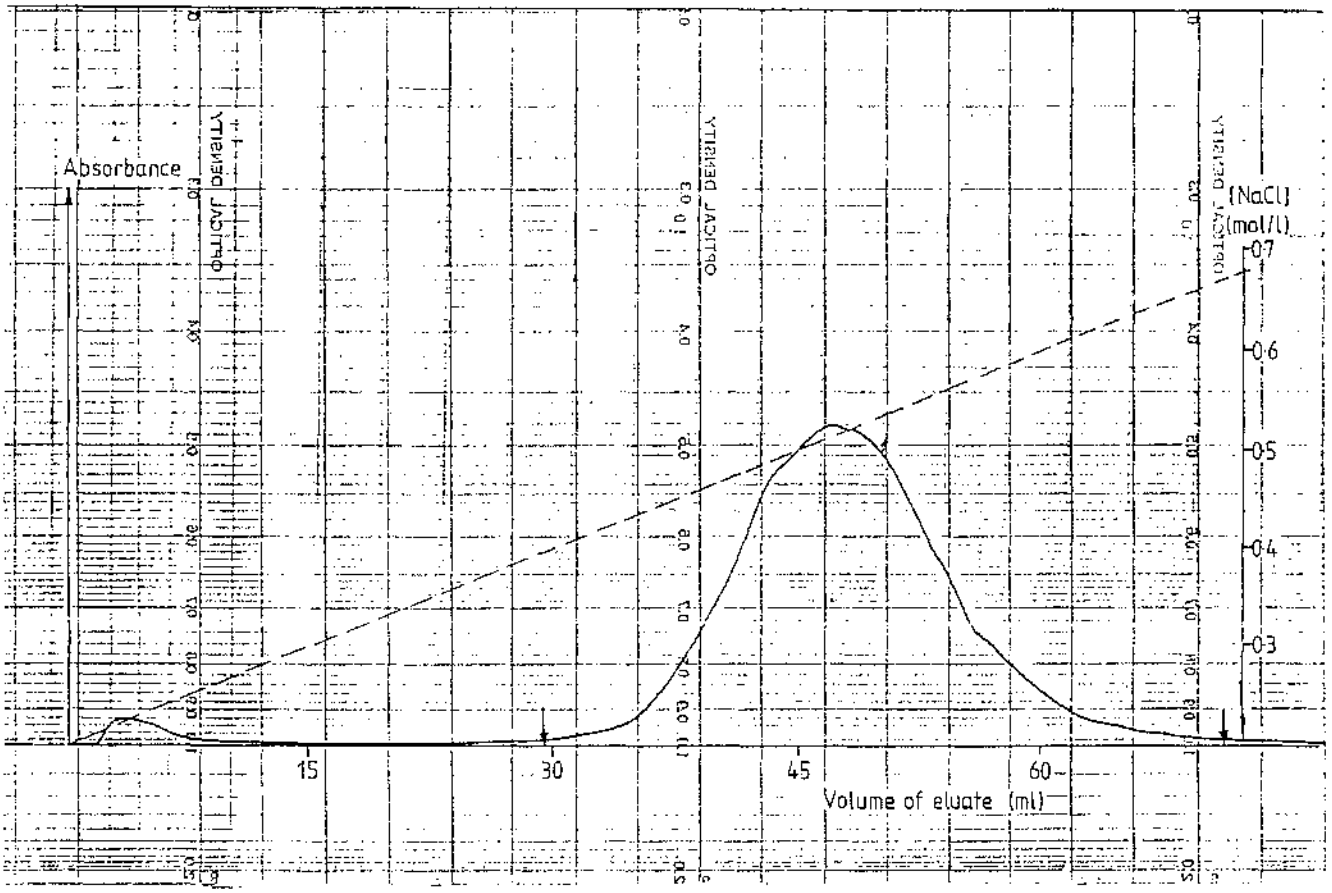
The crude Lf (10mg) was loaded onto each of the columns in 0.025M Tris/HCl buffer (pH 7.8) containing 0.2M sodium chloride and was then eluted with a sodium chloride gradient. Typical elution profiles of Lf from the columns are shown in Figures 4.16 to 4.19. A small impurity peak was eluted with 0.2M sodium chloride while Lf was eluted as a broad dilute band at higher salt concentration (0.35M to 0.6M sodium chloride). After concentrating the Lf fraction, the A_{280}/A_{465} and A_{410}/A_{465} ratios were determined. The results are presented in Table 4.5.

Table 4.5 : A_{280}/A_{465} and A_{410}/A_{465} ratios of purified Lf

	Purified Lf			
	Run #1		Run #2	
Matrix	A_{280}/A_{465}	A_{410}/A_{465}	A_{280}/A_{465}	A_{410}/A_{465}
A-CM$_6$D-agarose	26.1	0.75	25.3	0.75
Asp-AH-agarose	26.8	0.79	27.9	0.75
Asp-agarose	27.5	0.75	27.5	0.73
CM-Sep-ff	29.9	0.75	29.0	0.74

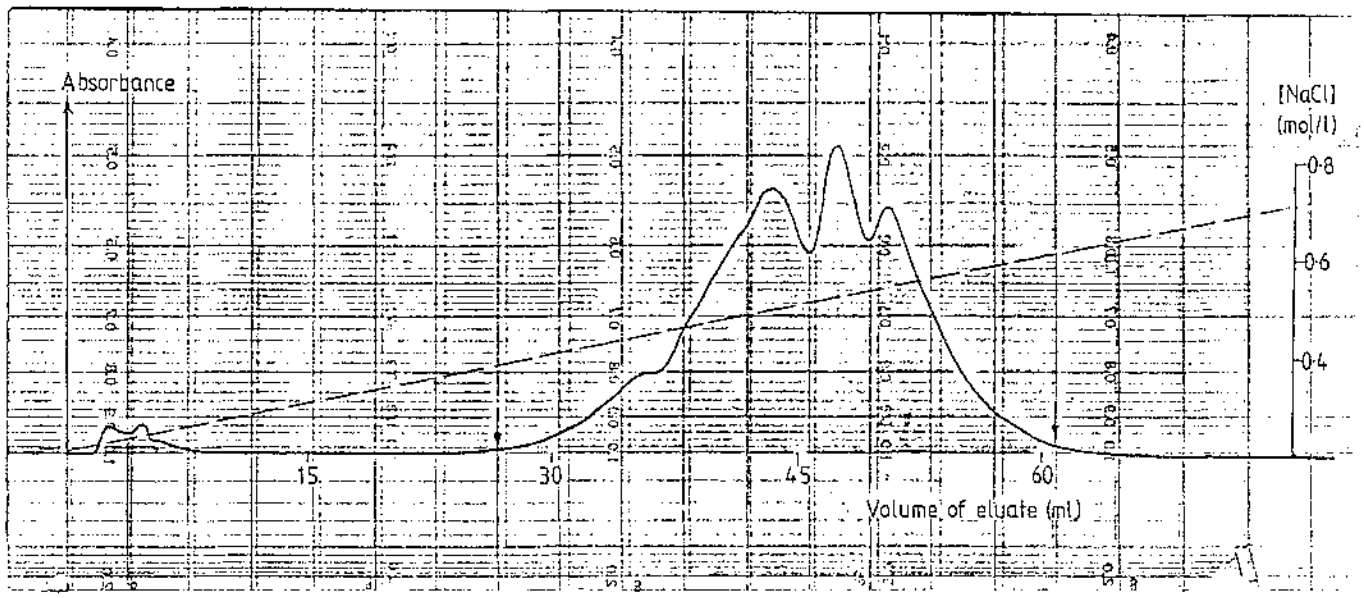
The purified Lf on all columns had A_{410}/A_{465} ratios between 0.75 and 0.77. This showed that the contamination by Lp was minimal (see Section 4.3). The Lf purified on A-CM$_6$D-agarose showed the best A_{280}/A_{465} ratio. The ratio of 25 to 26 indicates a purity better than previously accepted as the minimum. The Lf purification on Asp-AH-agarose and Asp-agarose gave a product with improved purity over the starting material while CM-Sep-ff achieved very little purification.

Figure 4.16 : Continuous gradient elution of crude Lf from A-CM_6 D-agarose



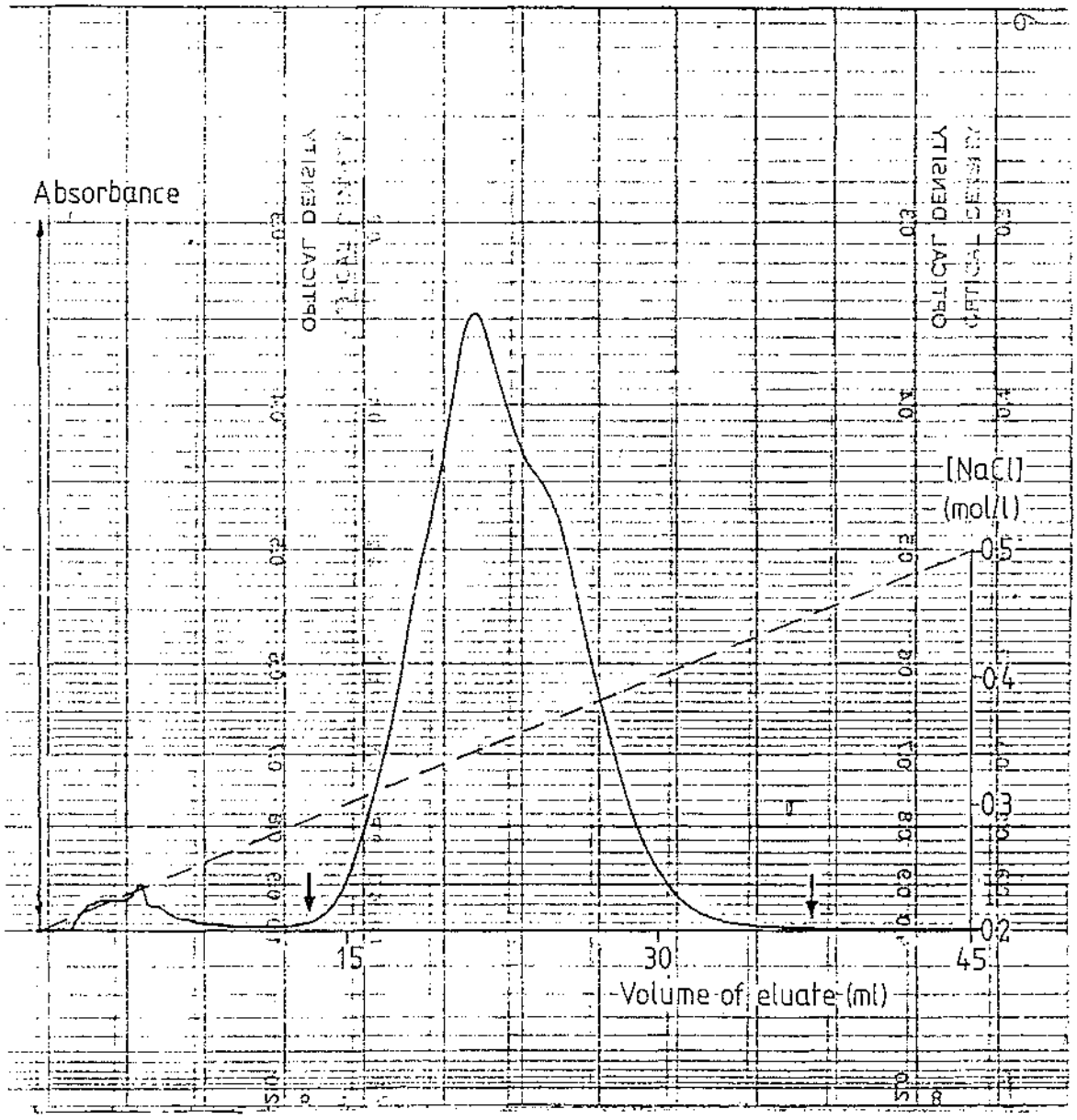
Buffer : 0.025M Tris/HCl (pH 7.8)
Flow rate : 1.0 ml/min
Column dimensions : 100x7 mm diameter

Figure 4.17 : Continuous gradient elution of crude Lf from Asp-AH-agarose



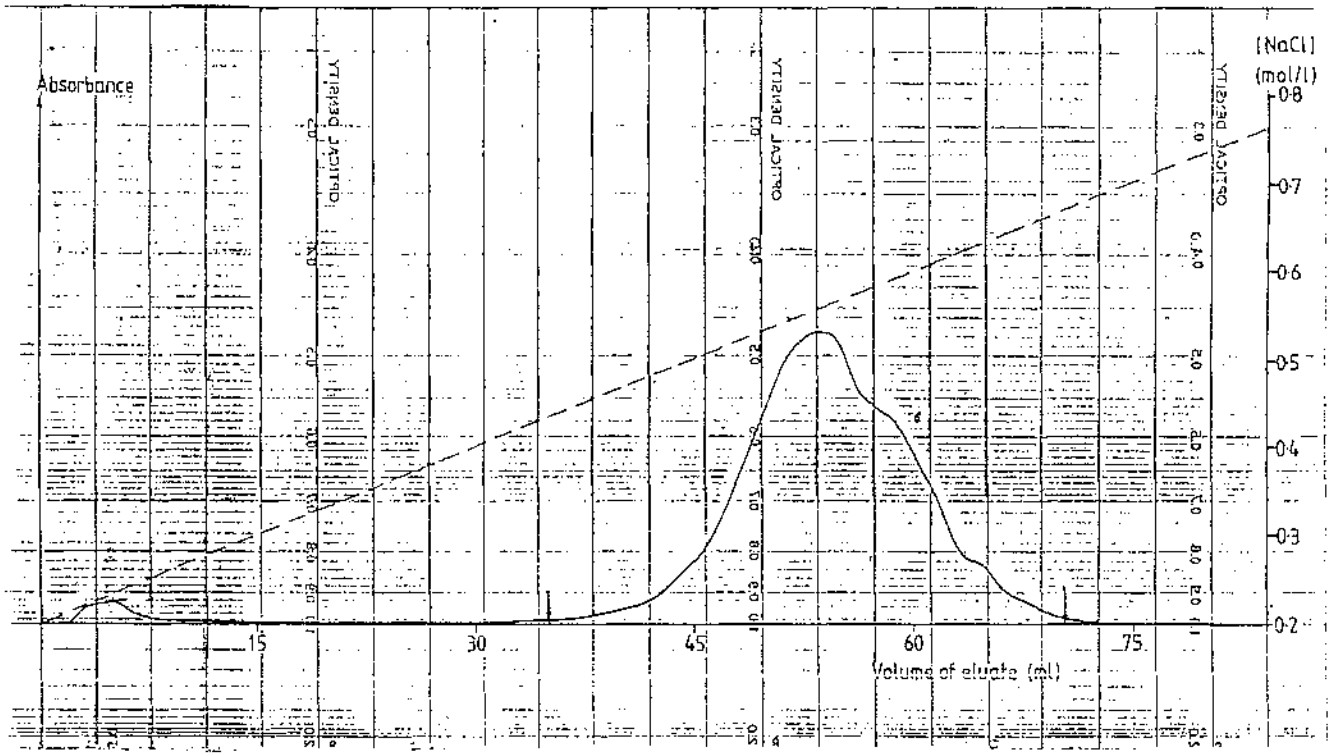
Buffer : 0.025M Tris/HCl (pH 7.8)
Flow rate : 1.0 ml/min
Column dimensions : 100x7 mm diameter

Figure 4.18 : Continuous gradient elution of crude Lf from Asp-agarose (AG-ASP4)



Buffer : 0.025M Tris/HCl (pH 7.8)
Flow rate : 1.0 ml/min
Column dimensions : 100x7 mm diameter

Figure 4.19 : Continuous gradient elution of crude Lf from CM-Sepharose-fast flow



Buffer : 0.025M Tris/HCl (pH 7.8)
Flow rate : 1.0 ml/min
Column dimensions : 100x7 mm diameter

4.20 Conclusion

The variation in the strength of Lf binding to the A-CM, D-agarose with pH suggested a predominantly ionic interaction between citrate and the protein (see Section 4.17). However, the above results showed that A-CM, D-agarose had a higher specificity for Lf than the other matrices studied. This indicated that the Lf binding to citrate cannot be purely by non-specific ion exchange mechanism. Though the Asp ligands had two carboxyl groups like the citrate, they did not show the same specificity for Lf. This provided evidence that some biospecific interaction may be involved. The binding of Lf to immobilized citrate may occur via the bicarbonate binding site of the protein or some other citrate binding site not associated with the iron atom. Further examination of the binding of Lf to the matrices using esr techniques might provide better understanding of the nature of the interaction.

Also, the results showed that Asp-agarose and Asp-AH-agarose were more efficient in purifying Lf than CM-Sep-ff (see Table 4.5). This suggested that there was definite advantage in using an immobilized ligand containing two carboxyl groups in close proximity. Apparently, a localized concentration of carboxyl groups on the ligand was a more important factor for purifying Lf than a high density of carboxyl groups distributed over the matrix as in the case of CM-Sep-ff.

From section 4.16.4, it was found that the matrix containing the alpha configurational isomer of the citrate did not differ very significantly from the beta isomer in the binding of Lf. If it could be established that the matrix containing the beta isomer would purify Lf with the same efficiency as the alpha equivalent, there would be no advantage in preparing the matrices containing all alpha or beta

isomers. It would be more simple and economical to prepare a matrix containing a random mixture of both isomers. The preparation of A-CM$_6$D or B-CM$_6$D ligand was difficult involving multiple step synthesis starting from citric acid with none of the steps being straightforward (see Section 4.5). Hence, it would be advantageous to be able to prepare the matrix containing a random mixture of both isomers. The synthesis of such a matrix merely involves the reaction between citric acid and DAH-matrix using a carbodiimide coupling reagent.

In Sections 4.16.2 and 4.16.3, it was shown that porosity of the matrix was an important factor in Lf binding. The cellulose citrate derivatives were not very useful for Lf purification and will need to be made more porous before it could be used on a large scale for commercial production of Lr with high purity.

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APPENDICES

APPENDIX 1 : Background to Figures 2.1 and 2.2

(a) Effect of py-SO_3 on degree of sulphation (Figure 2.1)

1. The sulphation of HP-cellulose with py-SO_3 was known to be quantitative ie. there was no loss of HP-cellulose or SA cellulose by dissolution during reaction or during the wash up procedure [34].

2. When pure py-SO_3 is used in a completely anhydrous system, the sulphation reaction proceeds in such a way that the total sulphate substitution on HP-cellulose (1g) varies linearly with the quantity of complex used as shown in Figure A1.1 (line 1). The slope of this line is calculated to be 6.29 (on the basis of the molar mass of py-SO_3 which is 0.159 g/mmol).

3. The experimental results of the reactions of HP-cellulose (1g, batch 1248) with py-SO_3 at 20°C for 23 hours are shown in Table A1.1 [33].

4. The total sulphate substitution (n in Table A1.1) on HP-cellulose was found to vary linearly with the quantity of py-SO_3 complex up to about 2g of complex as shown in Figure A1.1 (line 3). The slope of this line is calculated by linear regression to be 5.15. The difference in the slopes between lines 1 and 3 is an indication of the purity of the complex used. From the ratio of the slopes, the complex was found to be 82% pure. Since there are finite numbers of -OH groups present for sulphation, the line deviates from ideal beyond 2g of complex as most of these -OH groups are sulphated. The magnitude of the intercept on the x-axis showed the amount of complex consumed by water present initially in the system.

5. Based on 82% pure complex, a new theoretical line is plotted as shown in Figure A1.1 (line 2) using the data worked out in Table A1.2.

6. If the HP-cellulose (1g) contains 2% moisture and the DMF (25ml) used has 0.1% water content, then the total number of millimoles of water in the system worked out to 2.5 mmol.

$$py^{+}\text{-}SO_3^{-} \quad + \quad H_2O \longrightarrow pyH^{+}HSO_4^{-}$$

Hence, 2.5 mmol of complex would be consumed by this water. The effect of this amount of water in the system on the degree of sulphation is shown in Table A1.3.

Table A1.1 : Effect of py-SO_3 on degree of sulphation

Reaction number	py-SO_3 (g)	D.S.[1] (meq/g)	Yield of reaction[2]	n[3] (mmol)
CS 69	0.8	2.47	1.34	3.31
CS 70	1.6	4.23	1.76	7.44
CS 71	2.0	4.83	1.97	9.52
CS 72	3.2	5.50	2.28	12.54
CS 73	5.0	5.75	2.42	13.92

Notes :

1. D.S. is the degree of sulphation for the reaction.
2. The yield of reaction is given by:

$$Y = \frac{1}{1 - 0.102 D.S.}$$

   The factor 0.102 is g/mmol of weight increase resulting from the addition of $SO_3^{-}Na^{+}$ to HP-cellulose.
3. n is the number of millimoles of $SO_3^{-}Na^{+}$ added to HP-cellulose.

Table A1.2 : Effect of 82% pure py-SO_3 on degree of sulphation under completely anhydrous conditions

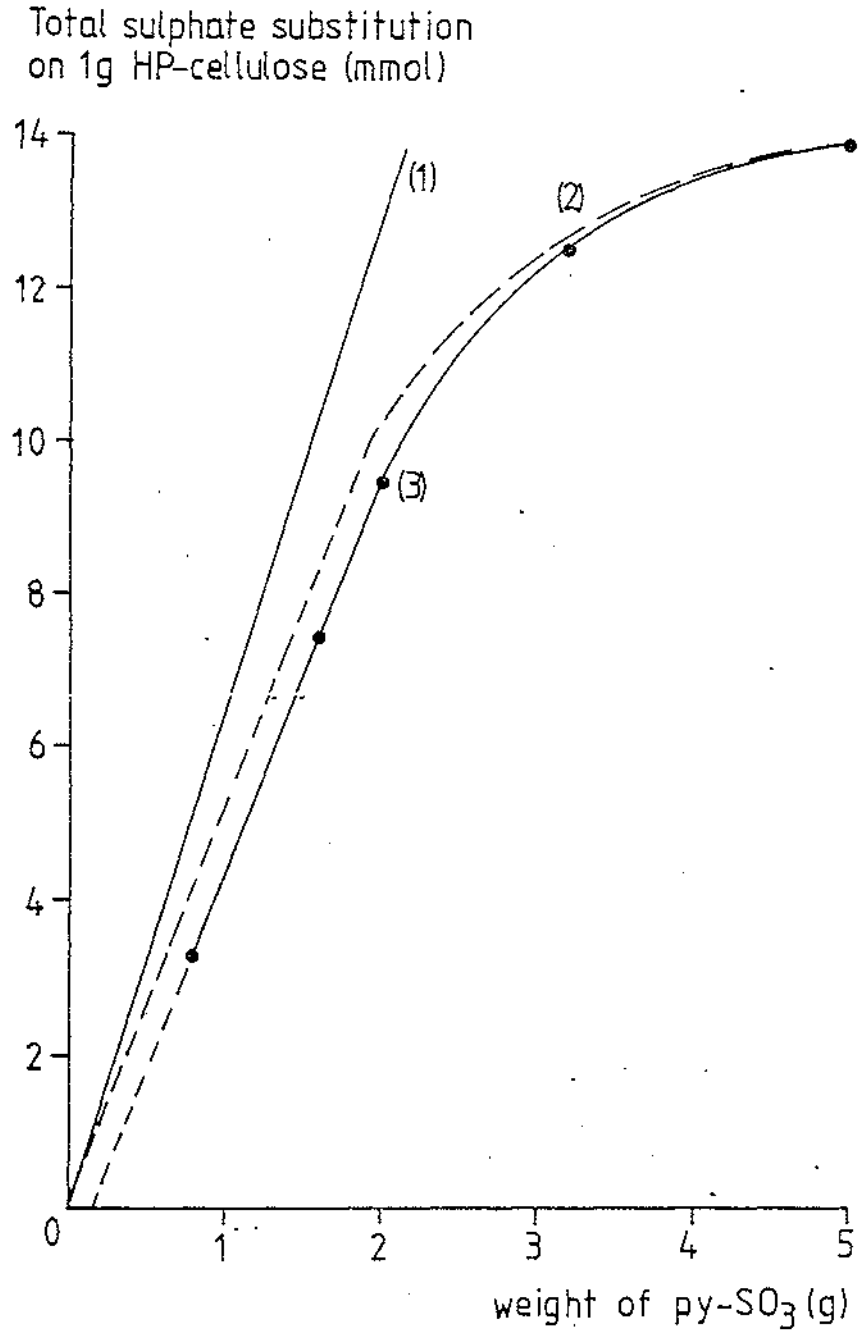
py-SO_3 (g)	Effective [py-SO_3] (mmol)	Total weight of SA (g)	Calculated D.S. (meq/g)
0.5	2.58	1.26	2.05
0.8	4.13	1.42	2.91
1.6	8.25	1.84	4.48
2.0	10.31	2.05	5.03

Note :
The points beyond 2g of complex are extraploted on the basis of the intercept.

Table A1.3 : Effect of 82% pure py-SO_3 on degree of sulphation of HP-cellulose (1g) with 2% moisture using DMF with 0.1% water content

py-SO_3 (g)	Effective [py-SO_3] (mmol)	Total weight of SA (g)	Calculated D.S. (meq/g)
0.5	0.08	1.01	0.08
0.8	1.63	1.17	1.39
1.6	5.75	1.59	3.62
2.0	7.81	1.80	4.34

Figure A1.1 : Effect of py-SO_3 on total sulphate substitution on HP-cellulose (1g)



(b) Effect of Me_3NSO_3 on degree of sulphation (Figure 2.2)

The experimental results for the reactions of HP-cellulose (1g, batch 1177) with Me_3NSO_3 at room temperature for 26 hours are shown in Table A1.4.

Table A1.4 : Effect of Me_3NSO_3 on degree of sulphation

Reaction Number	Me_3NSO_3 (g)	D.S. (meq/g)
CS 10	1.0	2.60
CS 11	2.0	3.89
CS 12	3.0	4.07
CS 13	4.0	4.08
CS 14	5.0	4.15

APPENDIX 2 : Solvent exchange of matrix

1. The matrix is generally solvent exchanged from DMF to water or vice versa in stages to prevent damage to its structure.

2. It is solvent exchanged from DMF to water (or from water to DMF) by washing sequentially with DMF, DMF-water (7:3, v/v), DMF-water (3:7, v/v) and water (or the reverse order).

APPENDIX 3 : Preparation of sample for titration

1. Before titration, the sample is generally dispersed in 0.5M NaCl (10ml). The presence of salt aids equilibration during titration.

2. The following equilibria are encountered in this project.

a. $Matrix\text{-}OSO_3^-H^+ + NaCl \longrightarrow Matrix\text{-}OSO_3^-Na^+ + HCl$

b. $Matrix\text{-}COOH + NaCl \longrightarrow Matrix\text{-}COO^-Na^+ + HCl$

c. $Matrix\text{-}NHR \text{ (or } Matrix\text{-}NH_2) + H_2O \longrightarrow Matrix\text{-}NH^+_2ROH^- \text{ (or } Matrix\text{-}NH_3OH^-)$

$Matrix\text{-}N^+H_2ROH^- \text{ (or } Matrix\text{-}N^+H_3OH^-) + NaCl \longrightarrow Matrix\text{-}N^+H_2RCl^- \text{ (or } Matrix\text{-}N^+H_3Cl^-) + NaOH$

It is HCl or NaOH "released" as a result of the equilibrium which is titrated.

3. In practice, it is simpler and equally effective to add a "pinch" of NaCl to the sample suspended in deionized water just before titration instead of dispersing it in 0.5M NaCl.

APPENDIX 4 : Calculation of titratable groups in meq/g and swollen volume in ml/g

Let the volume of the standard solution required for titration = V ml

the molarity of the standard solution = M mol/l

the settled volume of the matrix = Vs ml

the dry weight of the matrix = W g

Then,

$$\text{swollen volume} = \frac{Vs}{W} \text{ ml/g}$$

$$\text{Number of titratable groups} = \frac{MV}{W} \text{ meq/g}$$

APPENDIX 5 : Epoxide activation of HP-cellulose

1. The dry weight of 105g of moist HP-cellulose 8-50 is approximately 10g ie. it contains approximately 95g of water.

2. If the total volume of solution for the activation (including the 95ml of water in the moist cellulose) is Vml, then X% NaOH (w/v) activation requires:

$$\text{volume of 10M NaOH solution} = \frac{XV}{40} \text{ ml}$$

$$\text{volume of water} = V - \frac{XV}{40} - 95 \text{ ml}$$

3. If excess epichlorohydrin (ECH) is added (1.25 moles per mole NaOH), then

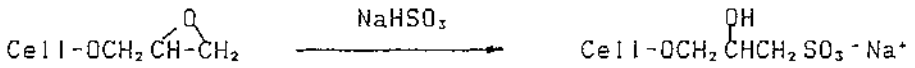
$$= \frac{\text{Volume of ECH}}{\text{density of ECH}} = \frac{1.25 \times \text{no. of moles of NaOH} \times \text{molar mass of ECH}}{\text{density of ECH}}$$

$$= 0.0245XV.$$

APPENDIX 6 : Calculation of 2-ANS substitution level and coupling efficiency

1. Degree of epoxide activation

The degree of epoxide activation is determined by converting the epoxide groups to titratable sulphonate groups



I II

The weight increase from I to II is 0.104g/meq. If w g dry weight of the starting matrix (I) gives 1g dry weight of the sulphonated matrix (II) with a degree of sulphonation of N meq/g, then,

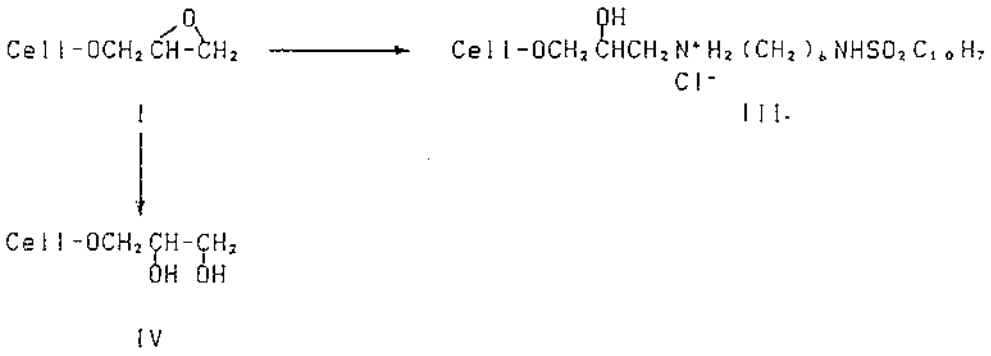
$$w = 1 - 0.104N \qquad (1)$$

Assuming a 100% conversion of epoxide to sulphonate, then the epoxide content, S of the activated matrix (I) is given by:

$$S = \frac{N}{w}$$

$$= \frac{N}{1 - 0.104N} \text{ meq/g} \qquad (2)$$

2. Degree of 2-ANS substitution for matrix prepared by direct coupling of 2-ANS to epoxide activated matrix (Scheme i, Figure 3.1)



The degree of substitution obtained in 2-ANS cellulose (III) was measured by direct titration (see Appendix 4). However, to determine the efficiency of epoxide conversion, it was necessary to calculate the number of epoxide groups present in the activated matrix which had been converted. The method of calculation is the same as that for the sulphonate conversion.

The weight increase from I to III is 0.343 g/meq. If w' g dry weight of starting epoxide activated matrix (I) gives 1g dry weight of 2-ANS-derivative (III) with a titratable nitrogen content of N' meq/g, then,

$$w' = 1 - 0.343N' \qquad (3)$$

Then this weight, w' had N' meq of epoxide groups on I which were converted to III. Therefore, the epoxide content, S_A, of the activated matrix (I) converted to 2-ANS groups is given by:

$$S_A = N'/w' \text{ meq/g} \qquad (4)$$

Then, coupling efficiency of 2-ANS, E_A is given by:

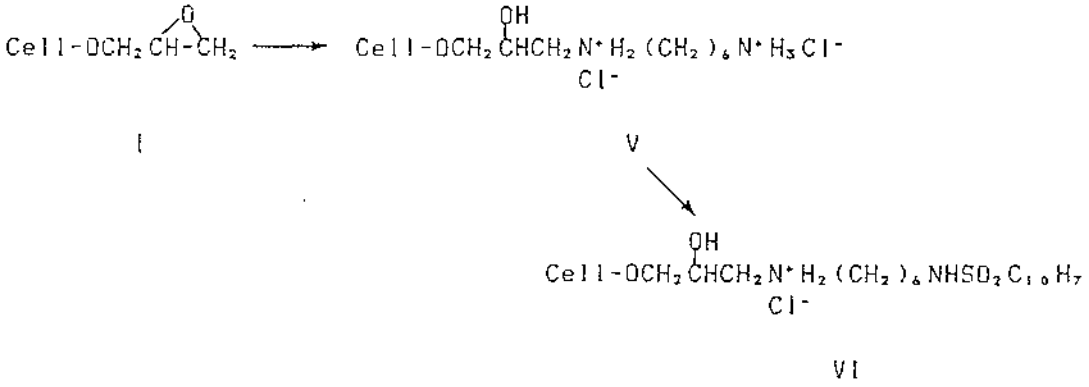
$$E_A = (S_A/S) \times 100\% \qquad (5)$$

where S is the actual epoxide content (meq/g) determined by conversion to sulphonate groups.

This calculation ignores the very small increase in weight, 0.018 g/meq, due to some epoxides being hydrolyzed to the diol (IV). This assumption is particularly valid when the coupling efficiency of 2-ANS groups is high ie. greater than 90%.

3. Degree of 2-ANS substitution for matrix prepared by coupling 2-NSCl to DAH-cellulose (Scheme 2, Figure 3.1)

For 2-ANS-cellulose in the hydrochloride form,



The weight increase from V to VI is 0.154 g/meq. If x mmol of 2-naphthalene sulphonyl groups are added to w" g dry weight of starting DAH-cellulose (V) to give 1g dry weight of the derivative (VI), then,

$$w'' = 1 - 0.154x \qquad (6)$$

If the titratable nitrogen contents in V and VI are N" and N_R meq/g respectively, then,

$$x = N"w" - N_R \qquad (7)$$

Solving equations (6) and (7), we have,

$$x = (N" - N_R)/(1 + 0.154N") \qquad (8)$$

Then, the 2-ANS content, S_A' with respect to 1g dry weight of starting DAH-cellulose (V) is given by:

$$S_A' = x/w" \quad meq/g. \qquad (9)$$

Hence, the coupling efficiency of 2-ANS, E_A' is given by:

$$E_A' = \frac{S_A'}{0.5N"} \times 100\% \qquad (10)$$

where E_A' is calculated in terms of the percentage of sulphonation of the DAH groups on V.

For 2-ANS-cellulose in the free amine form (ie. after the removal of HCl by washing with dilute alkali), it can be shown by a similar calculation as above that,

$$x = (N" - N_R)/(1 + 0.118N") \qquad (11)$$

and $$w" = 1 - 0.118x \qquad (12)$$

APPENDIX 7 : Estimation of the purity of asym-monomethyl citrate

Let the weight of the crude product sampled = w g

the weight of the asym-monomethyl citrate = w_1 g

the weight of the citric acid = w_2 g

The molar masses of asym-monomethyl citrate and citric acid monohydrate are 206 and 210 g/mol respectively.

If the number of moles of NaOH required to neutralize the sample is x, then, assuming that citric acid is the only contaminant, we have,

$$w = w_1 + w_2 \qquad (1)$$

$$2(w_1/206) + 3(w_2/210) = x \qquad (2)$$

Solving equations (1) and (2), we have,

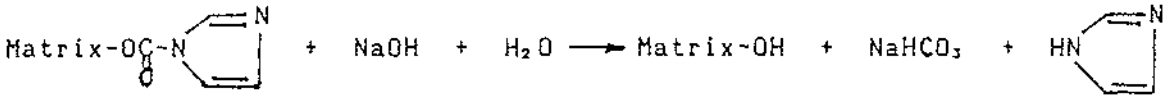
$$w_1 = \frac{103w - 7210x}{33}$$

Hence, percentage purity of asym-monomethyl citrate is:

$$\frac{w_1}{w} \times 100\%$$

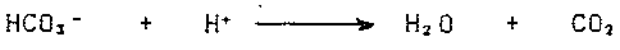
APPENDIX 8 : Preparation of CDI-activated agarose for titration

1. The CDI linkage in the sample is hydrolyzed with base.



2. The pH of the sample is adjusted to pH 3 with hydrochloric acid to neutralize the excess base.

3. The nitrogen is bubbled into it to expel the carbon dioxide formed.



4. The pH is adjusted to pH 5 with 1.00M NaOH to neutralize the excess acid.

5. The imidazole hydrochloride is titrated with 1.00M NaOH from pH 5 to pH 8.5.