APPLICATIONS OF CELLULOSIC ION EXCHANGERS

A thesis presented in partial

fulfilment of the requirements

for the degree of

Master of Science

in Chemistry

at

Massey University

New Zealand

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1983

ABSTRACT

Two possible commercial applications for a new range of ion exchanger, based on regenerated cellulose were investigated.

Equilibration studies showed that strongly basic (QAE) and strongly acidic (SP) derivatives can be equilibrated quicker and more easily than weakly basic (DEAE) and weakly acidic (CM) derivatives. This makes QAE and SP derivatives those of first choice, for use in commercial ion exchange processes.

The new QA and DE Cellulose derivatives were investigated for their possible use in the commerical purification of rennet. It was found that they were unable to bind rennet with sufficient capacity, within it's pH stability limits, to be of any use in this process. This finding was surprising at first since these new ion exchangers showed improved BSA adsorption capacities over those currently employed for rennet purification. An explanation for this low capacity was proposed and verified with model studies on BSA adsorption. From these model studies it was also found that the new DE Cellulose has a more even distribution of charged groups resulting in sharper and more symmetrical peaks in the elution profiles of BSA, than those obtained from some DEAE celluloses commercially available.

The new QA Cellulose was investigated for it s possible use in the commercial extraction and purification of heparin, but was found to have insufficient density of charged groups to bind heparin at the high ionic strength used in some extraction processes. Several reaction schemes were devised and used to produce quaternary ammonium cellulose derivatives containing groups with two or three positively charged nitrogens as a means of increasing the charged density on the cellulose to match repeating negatively charged sulphate groups in heparin. The products obtained showed a dramatic increase in their binding strength for heparin but unfortunately there was a decrease in their capacity for heparin. None the less several potentially useful new cellulose derivatives for ion exchange chromatography can now be made.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. J.S. Ayers for the help he has given me throughout the course of this study.

I would also like to thank all members of the Chemistry-Biochemistry Department for their help.

Thanks also is extended to Mrs Stewart, Susan Hewitt and Janine Atkins for their assistance in preparing this text and to Jenny Trow for her assistance in preparing the diagrams.

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INTRODUCTION

1.1 Background

With the increased interest in separating proteins and other macromolecular polyelectrolytes during the 1950's, it was found that the existing polystyrene resins were unsuitable for ion exchange chromatography of such materials. The synthetic ion exchange resins had small pore sizes, which prevented large polyelectrolytes diffusing into them. This made only the external surface of the resins available for ion exchange and resulted in low protein binding capacity. The high substitution (meq/g) of charged groups on these ion exchange resins resulted in very strong interactions developing with the polyelectrolytes. Some proteins were found to be denatured by the strong interactions or the high salt strengths required for elution.

In 1956 Peterson and Sobers(1) produced the first of the modern cellulosic ion exchangers and used them to fractionate plasma proteins(2). They made several derivatives of cellulose powder (See Table 1.1) which had improved protein binding ability over synthetic resins and this made possible for the first time the chromographic resolution of a protein by ion exchange.

Table 1.1

Cellulose Derivatives and Reagents used in their Preparation

Derivative	Group	Conventional Reagent
DEAE	$-CH_2CH_2NEt_2$ Diethylaminoethyl	$ClCH_2CH_2\overset{+}{N}HEt_2\ Cl^-$
ECTEOLA	Reportedly unknown	$ClCH_2\underset{\diagdown\diagup}{O}CHCH_2$ and $N(CH_2CH_2OH)_3$
CM	$-CH_2COOH$ Carboxymethyl	$ClCH_2COOH$
Phospho	$-OPO_3H_2$	$POCl_3$ or H_3PO_4

These cellulose materials, when packed in a column allowed only poor flow rates to be used, compared with the conventional polystyrene resins but this was not a significant disadvantage in view of the new ability to resolve protein mixtures resulting from the cellulose ion exchanger's high protein capacities.

Today a wide range of ion exchange derivatives are available as fibrous and microgranular cellulose powders(3) and some typical examples are summarised in Table 1.2. Fibrous cellulose ion exchangers are those whose physical structure is not significantly different to that of native cellulose. Such ion exchangers tend to have good flow rates, but have low resolving ability. Microgranular cellulose ion exchangers are those which are prepared from cellulose, from which much of the amorphous regions has been removed and the remaining structure chemically crosslinked to prevent excessive swelling on the addition of the charged groups. The product obtained packs more tightly in columns than the fibrous forms, resulting in it having higher resol-ving ability but also higher resistance to flow. Microgranular cellulose ion exchangers are more prone to mechanical degradation than standard fibrous derivatives. The weakly basic anion exchangers (DEAE) and the weakly acidic cation exchangers (CM) are by far the most commonly used derivatives. This may be due to historical reasons, since they were the first derivatives made and used. They are also easier to make and tend to have slightly higher substitutions than the other derivatives. In addition, even now there are few manufacturers of strongly acid (SE or SP) and strongly basic (QAE) derivatives to rival the CM and DEAE derivatives.

Over the last 2 decades several methods have been developed for preparing bead cellulose, and a summary of this technology was published in 1981(4). Most of these methods involve solubilizing cellulose, then regenerating it by various means into bead form. In 1969 Determann et al(5) prepared the first cellulose ion exchangers in bead form, and since then two companies have placed bead cellulose ion exchangers on the market. Pharmacia market a DEAE derivative under the name DEAE - Sephacel, and Serva market both DEAE and CM derivatives under the names Servacel DEAE 80 and Servacel CM 80 respectively.

Table 1.2

Cellulose Ion Exchangers Available

Derivative	Chemical Structure	Cellulose Type	Suppliers*
CM	$-CH_2COO^-Na^+$	Fibrous	2,3,4,5,10
		Microgranular	3,10
		Rods	1
		Beads	20
Phospo	$-OPO_3H_2$	Fibrous	1,3,4,5,10
SE	$-CH_2CH_2SO_3^-Na^+$	Fibrous	10
		Microgranular	3
		Beads	10
DEAE	$-CH_2CH_2NEt_2$	Fibrous	1,2,3,4,5,10,11,12
		Microgranular	3,10,11
		Beads	7,10
ECTEOLA	Unknown	Fibrous	1,4,5,10
TEAE	$-CH_2CH_2N^+Et_3\ OH^-$	Fibrous	1,4,8,10
QAE	$-CH_2CH_2N^+Et_2CH_2CHOHCH_3\ Br^-$	Fibrous	1,11
AE	$-CH_2CH_2NH_2$	Fibrous	10
PEI	$-(NHCH_2CH_2)_n\ NH_2$	Fibrous	10
BD	Benzylated DEAE	Fibrous	1,6,10
BND	Benzylated, Naphthalated DEAE	Fibrous	10
CHELATE	$-N(CH_2CO_2^-Na^+)_2$	Fibrous	1,10
DHB	Boronate	Fibrous	6,9,10
PAB	$-CH_2C_6H_4NH_2$	Fibrous	10

* Suppliers
1. Bio-Rad Lab. Ltd; 2. Koch-Light Lab. Ltd;
3. Whatman Ltd; 4. Anderman & Co Ltd; 5. Camlab Ltd;
6. BDH Chemical Ltd; 7. Pharmacia Ltd
8. Chromatography Services Ltd;
9. Aldrich Chemical Co. Ltd; 10.Serva; 11.Brown Company;
12. Eastman, Organic Chemicals Dept.

These ion exchangers have several advantages over the conventional cellulose ion exchangers. The rigid bead structure allows the use of high flow rates, and little variation in the degree of swelling occurs with changes in pH and ionic strength. The spherical shape also allows the beads to pack closer together and more evenly than the conventional cellulose particles, giving columns of bead cellulose ion exchangers high resolving capacities. The bead cellulose ion exchangers are among the best cellulose ion exchangers currently available.

Besides cellulose, other naturally occuring polysaccharide materials have been used to manufacture ion exchangers for similar applications. A summary of these was compiled and published by Williams in 1977(6). The main ones are summarised in Table 1.3.

Table 1.3

Other Polysaccharides Derivates for Ion Exchangers

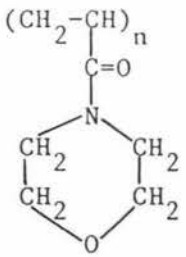
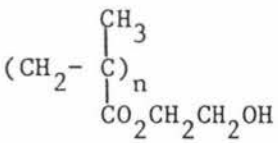
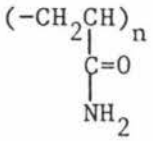
Material	Example	Derivatives Available
Crosslinked dextran	Sephadex	DEAE, CM, SP, QAE
Crosslinked agarose	Sepharose-CL	DEAE, CM
	Bio-Gel A	DEAE, CM

As far as we know these derivatives have been prepared using the conventional reagents (see Table 1.1). In addition, Williams also listed several totally synthetic gels designed for ion exchange chromatography of macromolecular polyelectrolytes. These gels were all weakly acidic cation exchangers and are summarised in Table 1.4.

All these products (both polysaccharide and synthetic gels) have found widespread use in the purification of proteins, nucleic acids and other macromolecular polyelectrolytes.

Table 1.4

Synthetic Gels for Ion Exchangers

Product	Parent Matrix	Partial Structure* of Parent Gel	Method of Group Manufacture
Enzacryl Gel CO_2H	Enzacryl Gel K2		Partial hydrolysis of amide
Spheron C1000	Spheron 1000		Partial oxidation of $-CH_2OH$ to COOH
Bio-Gel CM-2	Bio-Gel P-2		Partial hydrolysis or by derivatization of amide

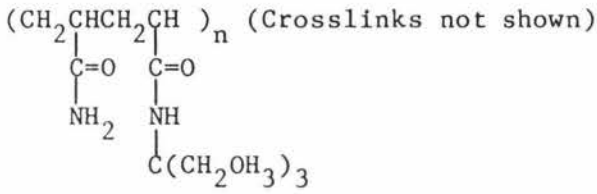
* crosslinks not shown

Since 1977 new products have continued to come on the market, for use in ion exchange chromatography. Most of these have been made by chemical means rather than using natural products as the starting gel or matrix. Some examples of these are as follows:-

Trisacryl

DEAE and CM Trisacryl are hydrophilic ion exchange gels made by the copolymerisation of N[tris(hydroxymethyl)methyl]acrylamide (CH_2=CHCONHC(CH_2OH)$_3$), acrylamide (CH_2=$CHCONH_2$) and bisacrylamide (CH_2=CHCONHCOCH=CH_2). The DEAE and CM groups are then attached via the alcoholic hydroxyl groups using conventional reagents. These gels have good porosity, high protein capacity and have the advantage that they can be sterilised in an autoclave. They have found application in the commercial fractionation of plasma proteins.

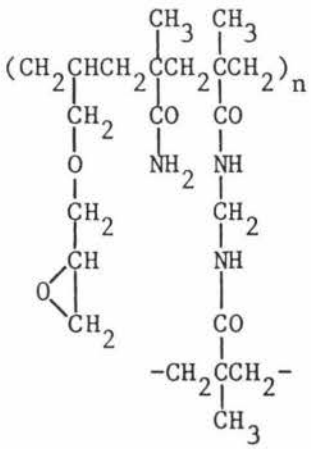
Partial Structure of Trisacryl



Eupergit

Eupergit C, a copolymer of glycidyl methacrylate (CH_2=$CMeCOOCH_2$  $CHCH_2$), allylglycidylether (CH_2=$CHCH_2OCH_2$  $CHCH_2$), methacrylamide (CH_2=$CMeCONH_2$) and N,N methylene-bismethacrylamide (CH_2=$C(Me)CONHCH_2NHCOC(Me)$=CH_2), is a hydrophilic matrix containing reactive epoxide groups. Though primarily designed for enzyme immobilisation and affinity chromatography, it could be reacted with amines and inorganic salts to produce ion exchange derivatives.

Partial Structure of Eupergit C



Styrene and Acrylic Polymers

Two ion exchangers based on highly crosslinked porous polystyrene are now available from Bio-Rad. These are called macroporous, anion (AG-MP1) and cation (AG-MP 50) exchangers, and are reported to be useful for separating, isoenzymes, and antibiotics. Another matrix which is reported to be useful for factionation of peptides, proteins and enzymes is Bio-Rex 70. This is described as a weakly acid cation exchanger, with carboxylic acid groups on a macroreticular acrylic polymer lattice.

Spherosil

Spherosil is a range of products based on porous silica beads that have been impregnated with polymer coatings with ion exchange properties. These coatings include DEAE dextran, substituted polystyrenes and substituted polyacrylamides. These ion exchangers have good flow properties, high surface area and due to their inert rigid core, do not change volume with changes in salt strength.

Some of these newer ion exchangers are now being used in large scale applications, eg Spherosil for the recovery of protein from whey(7). This is a result of their improved physical properties. Spherosil, CL-Sepharose, Sephacel and Servacel all resist dimensional changes with changing ionic strength and pH and their bead form allows high resolution of protein mixtures and reasonable flow rates when they are packed in a column. However they tend to be fairly expensive and it has been estimated(8) that it would cost $200,000 to charge a reactor with 600 kg of QMA-Spherosil to process 10,000 litres of whey per cycle (10 - 12 cycles/day). This initial capital outlay would be prohibitive to many potential users.

In contrast with these recent developments, the cellulose ion exchangers available since 1956 allow only poor flow rates in columns, although they give good resolution. On a large scale they have to be used in a batch process using a centrifuge to drain and wash the cellulose particles. They break down rapidly producing fines and can usually only be used a few times before being discarded(9).

In 1967 Grant(10) developed a range of ion exchangers on a particularly tough and resilient form of cellulose know as regenerated cellulose. This regenerated cellulose was ground into particles and then chemically crosslinked, before being treated with the conventional reagents to form cationic (CM) and anionic (DEAE) exchangers. These ion exchangers, known as the Grant resins, had the unique property of both binding protein and allowing high flow rates when packed in a column. It was hoped that these ion exchangers would make it possible to treat the total effluent

from a meat works by an ion exchange process, recovering potentially valuable protein and at the same time reducing pollution. In 1977 McNaughton (11) summarised most of the early history of the Grant resins.

The considerable promise which the Grant resins showed in the early 1970's has not been realized. The only significant application for them was at the New Zealand Co-op Rennet Company Ltd at Eltham, where they are still being used to separate mucoproteins from the rennet extract(20). Their use in processing proteinaceous effluents was not economically feasible and their application in the purification of the other proteins such as enzymes has been very limited. A major deficiency of the Grant resins is their low capacity for absorbed proteins. Enzyme producing companies like Boehringer Manheim in West Germany tested the Grant resins in 1972 and showed them to have only 1 - 10% of the necessary capacity for their enzymes. This limitation was noticed many times at Massey University but their high flow characteristics were an incentive to find a way of improving their protein capacities.

Listed in Table 1.5 are the protein capacities for the Grant DEAE resin along with some other widely used ion exchangers. The protein capacities can be expressed two different ways. One is in terms of weight of protein bound per weight of ion exchanger which is important from a manufacturing point of view. The other is the weight of protein that can be bound on a given volume of ion exchanger which is important when using it in a process.

Table 1.5
Protein Capacities of DEAE Ion Exchangers

Ion Exchanger		Protein Capacity (mg/g)	Protein Capacity (mg/ml)
DEAE-Protion	(Grant Resin)	300	50
DE-52 Cellulose	(Whatman)	600	100
DE-Sephadex-A25	(Pharmacia)	500	80
DE-Sephadex-A50*	(Pharmacia)	5,000	160
DEAE-Sepharose	(Pharmacia)	-	100
DEAE-Sephacel	(Pharmacia)	-	150
DEAE-HP-Cellulose	(Phoenix Chem.)	1,800	150

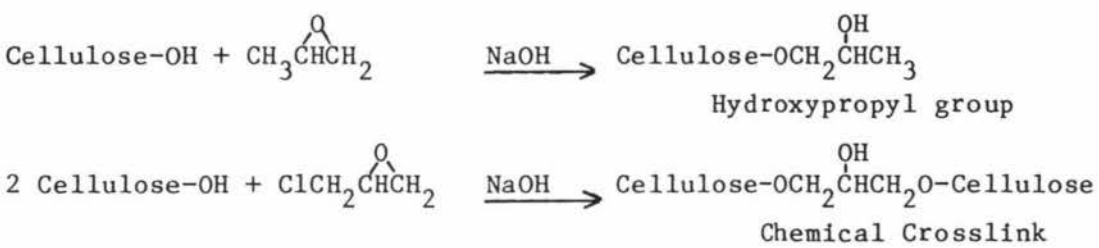
*This product changes its volume up to 100% with changing ionic strength and pH.

Since the original Grant developments, Massey University with the support of the Development Finance Corporation (DFC) has become involved in the field of making ion exchangers from regenerated cellulose. The DFC now holds the patent on the Grant resins and have licenced Phoenix Chemicals Ltd to manufacture DEAE and CM derivatives.

The last ion exchanger listed in Table 1.5 is one of a new range of modified cellulose ion exchangers that Phoenix Chemicals Ltd are licenced to manufacture as a result of the research activities at Massey University over the last 10 years. It s protein capacity is as good as any and it still has flow rates superior to most other cellulose ion exchangers, except the Grant resins.

These new, modified cellulose ion exchangers have been developed by starting with the same regenerated cellulose particles that Grant used, but reacting them first with propylene oxide and epichlorohydrin to produce a crosslinked hydroxypropylated cellulose onto which are then attached the ion exchange groups(12). The effect of the hydroxypropyl groups is to swell the cellulose and increase it's porosity and internal accessibility to protein molecules. Sheerin(13) first used propylene oxide to swell regenerated cellulose and produce a crosslinked hydroxypropylated cellulose matrix, referred to as the "modified cellulose matrix." He sulphated this matrix to produce a novel derivative for separation of lipoproteins.

Typical groups introduced into the "modified cellulose matrix" during manufacture are shown below.



Peterson(14) used this modified cellulose matrix as the basic matrix for the preparation of the three most important standard ion exchange derivatives using conventional chemical as shown below.

Modified Cellulose Matrix = M-Cellulose-OH

$$\text{M-Cellulose-OH} + \underset{\text{Chloroethyldiethylamine}}{\text{ClCH}_2\text{CH}_2\text{NEt}_2} \xrightarrow{\text{NaOH}} \text{M-Cellulose-OCH}_2\text{CH}_2\text{NEt}_2 \ \underline{\text{DEAE}}$$

$$\text{M-Cellulose-OH} + \underset{\text{Chloroacetic acid}}{\text{ClCH}_2\text{COOH}} \xrightarrow{\text{NaOH}} \text{M-Cellulose-OCH}_2\text{COO}^-\text{Na}^+ \ \underline{\text{CM}}$$

$$\text{M-Cellulose-OH} + \underset{\text{Propane Sultone}}{\text{CH}_2\text{CH}_2\text{CH}_2\text{-SO}_2\text{-O}} \xrightarrow{\text{NaOH}} \text{M-Cellulose-OCH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-\text{Na}^+ \ \underline{\text{SP}}$$

Peterson was able to produce ion exchange derivatives with useful substitution levels in the range 1-2 meq/g and with much superior protein absorption capacities(12,14) compared with the original Grant resins and without losing the high flow characteristics of the original regenerated cellulose particles. Unfortunately the reagent used in the preparation of the SP derivative, ie propane sultone, is a low melting point solid, difficult to handle and very highly suspect as a carcinogenic chemical. For these reasons its use was discontinued. (Pharmacia also changed their method of synthesis for SP derivatives during the late 1970's).

Bethell(15) tried several other known reagents for producing sulphonate derivatives of cellulose, such as $\text{BrCH}_2\text{CH}_2\text{SO}_3^-\ \text{Na}^+$ and $\text{ClCH}_2\text{CHOHCH}_2\text{SO}_3^-\text{Na}^+$ but was unable to obtain substitution levels high enough to be useful for many applications involving ion exchange chromatography of proteins. This meant that only the CM and DEAE derivatives were directly obtainable from the modified cellulose matrix in the same way that the CM and DEAE Grant resins were produced from the original regenerated cellulose.

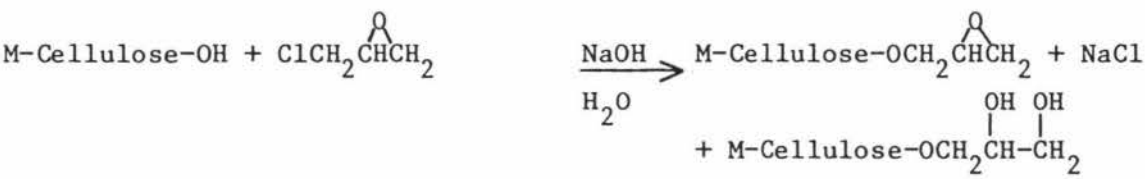
Quaternary amino derivatives have never been reported in the literature as being made successfully from reagents of the type $\text{X-(CH}_2)_n\text{-}\overset{+}{\text{N}}\text{R}_3$ or $\text{CH}_2\overset{\text{O}}{\diagup\diagdown}\text{CHCH}_2\overset{+}{\text{N}}\text{R}_3$ except with very low degrees of substitution.

It was in search of an alternative route to the SP derivative that the epoxide derivative was developed(16) with substitution levels of up to 1.5 mmol/g. It was found that these epoxide groups could be converted to sulphonic acid groups (and others) so efficiently that the epoxide derivative (activated matrix) has proved to be a very versatile intermediate in the production of a whole range of useful derivatives, including the SP and QAE derivatives(16,17). These developments are summarised in Figure 1.1

In summary the production of these ion exchangers from regenerated cellulose (via the activated matrix) is a three step process. Firstly the regenerated cellulose is swollen with propylene oxide and crosslinked with epichlorohydrin in the presence of sodium hydroxide. The ratio of epichlorohydrin to propylene oxide determines the properties of the modified cellulose matrix. Secondly the modified cellulose matrix is further reacted in the presence of sodium hydroxide with an excess of epichlorohydrin to give rise to the epoxypropyl cellulose (the activated matrix). Thirdly the activated matrix is reacted with an excess of amines, inorganic salts or other nucleophilic compounds to give a variety of derivatives for use in ion exchange, affinity and hydrophobic chromatography.

Preparation of the Activated Matrix

(M-Cellulose-OH = modified matrix)



Conversion of the Activated Matrix to Ion Exchange of Derivatives

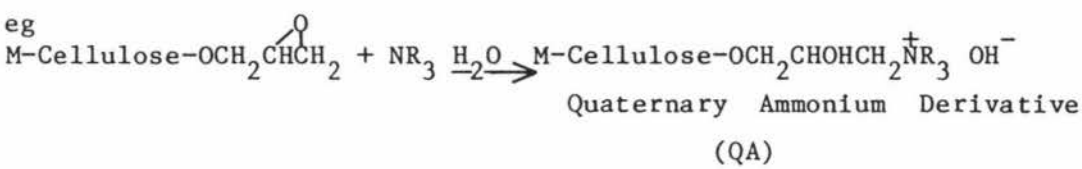
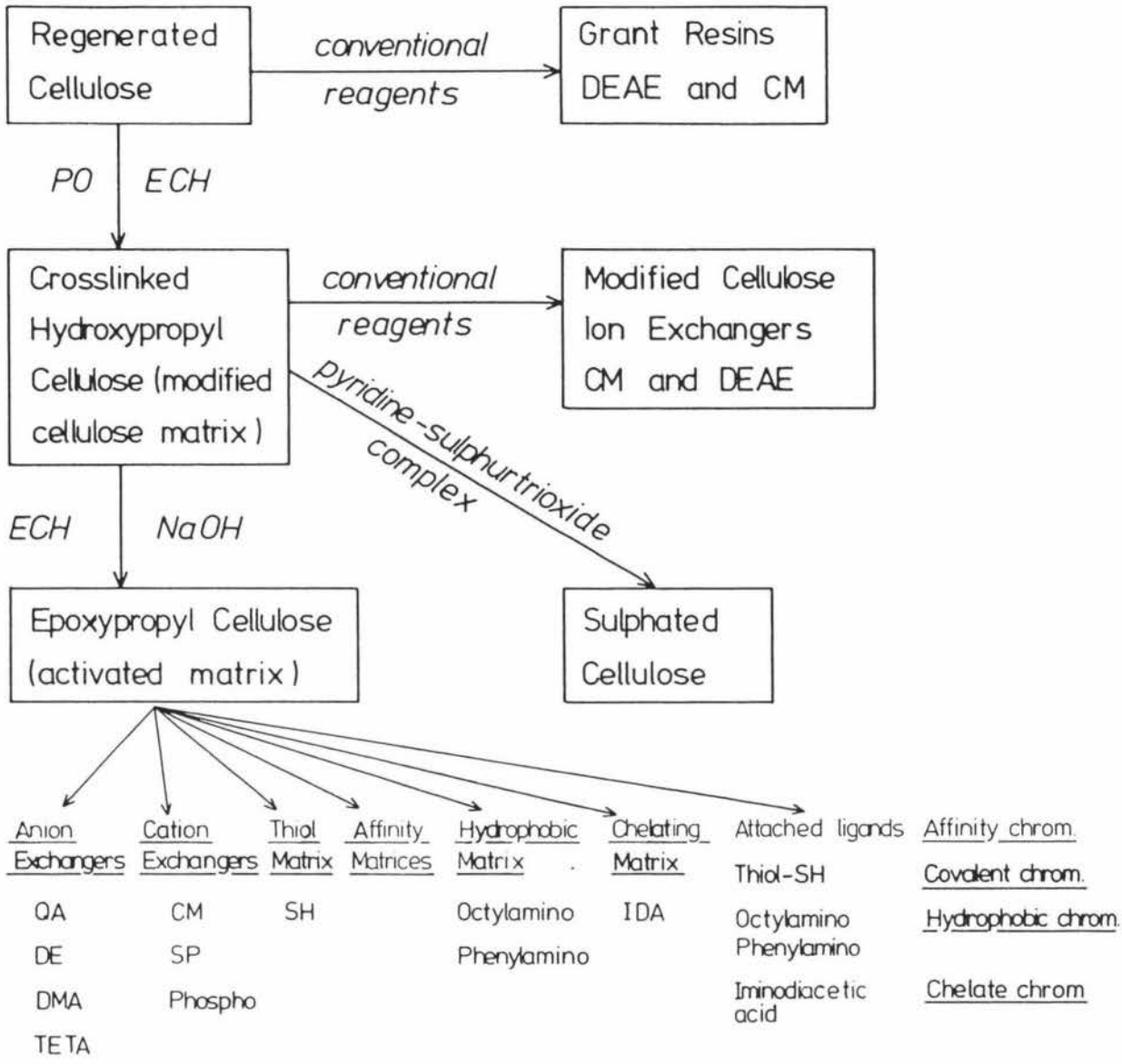


Figure 1.1
Summary of Products Obtained from Regenerated Cellulose



DMA = dimethylamino

TETA = triethylenetetramine

1.2 Aim of Thesis

As a result of these developments at Massey over the last 10 years it is now possible to produce a whole range of cellulosic deravitives for ion exchange, affinity, hydrophobic, covalent and chelate chromatography. In particular the four "classic" derivatives for ion exchange can be readily prepared. These four are the weakly basic, DEAE; strongly basic, QA; weakly acidic, CM and strongly acidic, SP derivatives, prepared either directly from the "modified matrix", ie CM and DEAE or via the activated matrix, ie QA and SP. Titration curves for these four derivatives are shown in Figure 2.1, 2.4 and 2.9. The weakly basic diethylamino derivative(DE) can also be prepared via the activated matrix. This gives a product with slightly different properties from the DEAE as can be seen from a comparison of the two titration curves in Figure 2.1

Structures of Diethylamino Groups

DEAE	DE
$-OCH_2CH_2NEt_2$ and	$-OCH_2CHOHCH_2NEt_2$
$-OCH_2CH_2\overset{+}{N}Et_2CH_2CH_2NEt_2$	
Via Conventional Route	Via Epoxypropyl Cellulose

All these ion exchangers have been shown to have the following properties(14,17)

(a) high protein capacities (greater than 1g/1g)
(b) high flow rates
(c) long life even under violent stirring (1200 rpm) continuously for more than 1 month (18)
(d) cheap enough for large scale commercial use.

It was the aim of the work reported in this thesis to look at potential applications for them, starting with a brief study of their ease of equilibration ready for protein absorption.

SECTION 2

EQUILIBRATION OF CELLULOSIC ION EXCHANGERS

2.1 INTRODUCTION

The operating conditions for ion exchange chromatography are usually characterised by pH and ionic strength. The pH determines the charge on both the ion exchanger and the polyelectrolyte, while the ionic strength determines the amount of competition, between small ionic molecules and the polyelectrolytes for the binding sites. Both these parameters are therefore critical to the affinity an ion exchanger has for a particular polyelectrolyte. They are controlled by equilibrating an ion exchanger with a buffer solution at the desired pH and concentration.

Most books on ion exchange chromatography of proteins and manufacturer's handbooks give a good description of how to find the necessary starting conditions for pH and ionic strength. Pharmacia(19) go so far as to consider the choice of the buffer substance itself, stressing that it is preferable to "use (a) cationic buffers with anion exchangers and (b) anionic buffers with cation exchangers: For if the buffering ions carry a charge opposite to that of the functional groups of the ion exchanger, they will take part in the ion exchange process and cause local disturbances in pH".

In spite of such descriptions and comments little consideration is given to the length of time required to effect equilibration of an ion exchange column. Although this may not be so important in a laboratory where resolution is often the most important property, in a large scale commercial process the cost of the chemicals and time required for equilibration can be of the utmost importance.

The aim of this section was to demonstrate the differences in the ease of equilibration of different ion exchangers with various buffer systems. We used cationic and anionic buffer systems for both anion and cation exchangers. The buffers were made up at their pKa's so that they would all have the same buffering capacities.

2.2 RESULTS AND DISCUSSION

2.2.1 Equilibration of DEAE Cellulose

Owing to the fact that DEAE ion exchangers are weak bases and to the method by which they are made, they have some buffering capacity throughout most of their working pH range. This can be seen in the titration curve for DEAE Cellulose shown in Figure 2.1. This means that in order to equilibrate a DEAE ion exchanger some of it's groups must be neutralised.

Figure 2.2 shows the effect of equilibrating the DEAE Cellulose from its free base form, which is uncharged, ie - NEt_2, with cationic buffers (curves 1 and 3), and with anionic buffers (curves 2 and 4). The results are also summarised in Table 2.1 below.

Table 2.1

Equilibration of the Anion Exchanger, DEAE Cellulose, from the Free Base Form

Buffer Type	Buffer and pH	Volume of Buffer Required (ml)
Cationic	0.05M Tris, pH 7.92	80
Cationic	0.05M Imidazole, pH 7.0	90
Anionic	0.05M Barbitone, pH 8.0	100
Anionic	0.05M Maleic acid pH 6.15	140

It can be seen that the column equilibrates more readily with the cationic buffers although there is not a significant difference. This suggests that the neutralisation of the groups is the major factor in determining the amount of buffer required.

Figure 2·1

Titration Curves of DEAE Cellulose (A_2 Protion) and DE Cellulose.

(curves run under dry N_2)

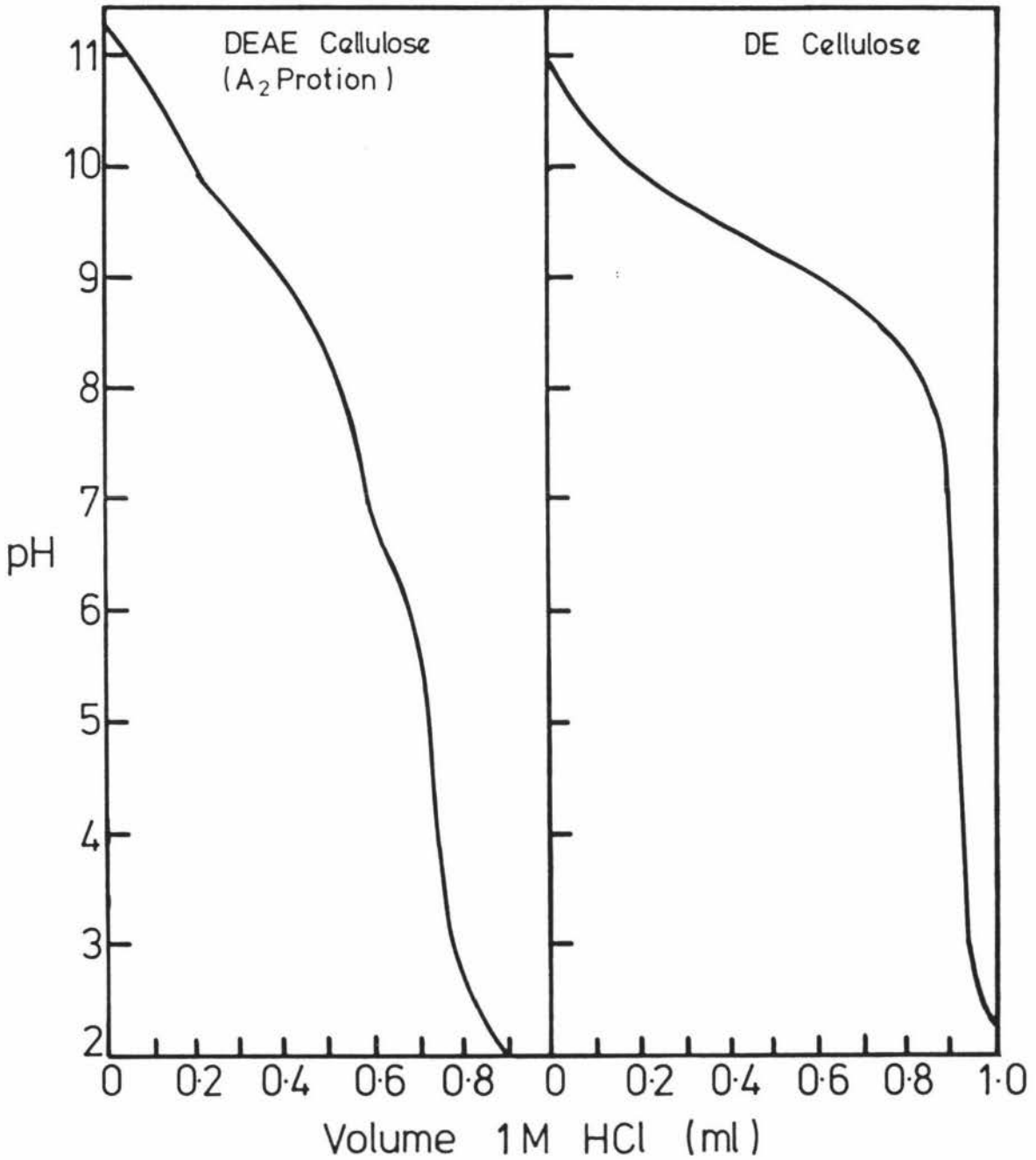
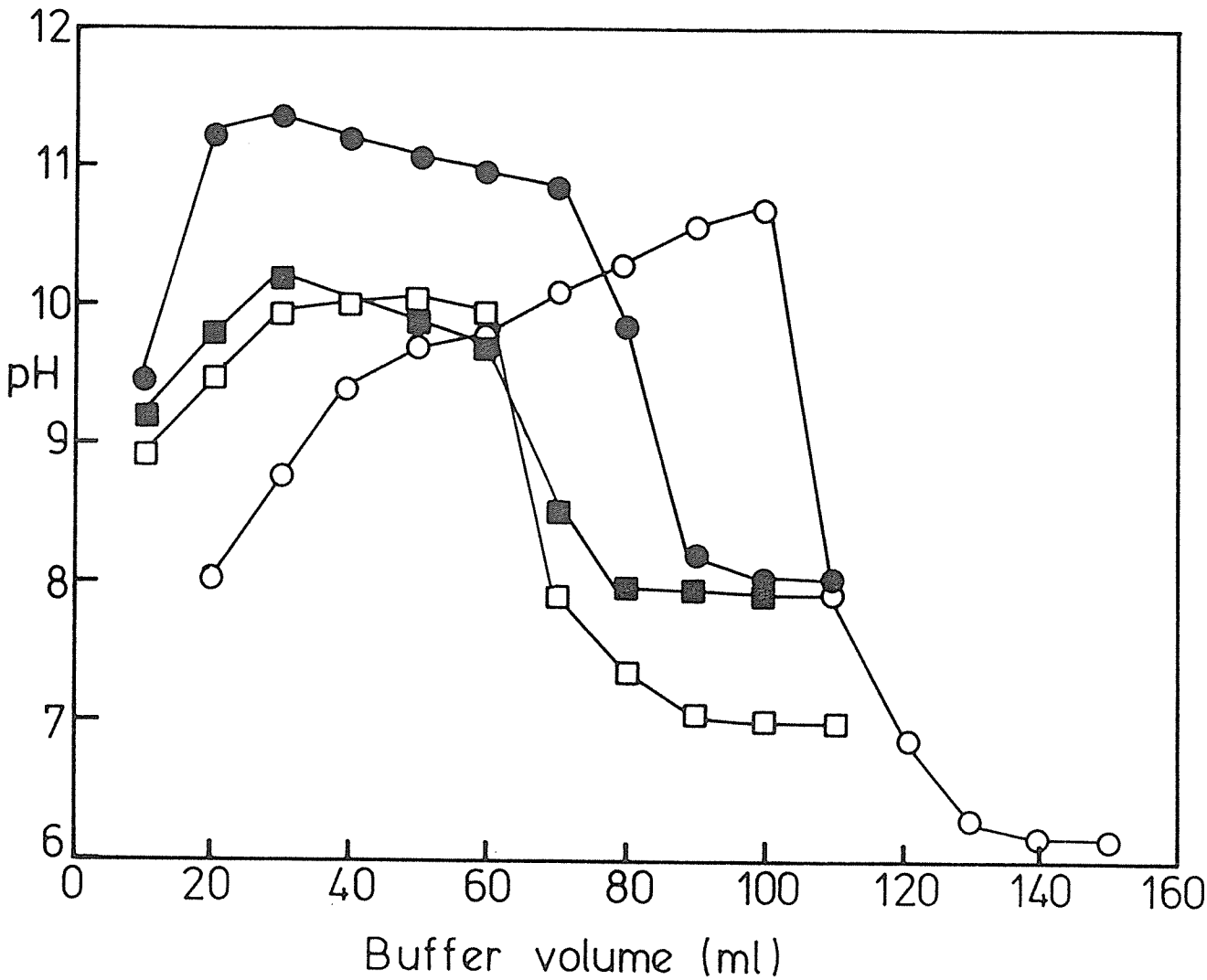


Figure 2·2

Equilibration of DEAE Cellulose from Free Base Form.



1. ■—■ 0·05M Tris, pH 7·92
2. ○—○ 0·05M Maleic acid, pH 6·15
3. □—□ 0·05M Imidazole, pH 7·0
4. ●—● 0·05M Barbitone, pH 8·0

EQUILIBRATION EQUATIONS

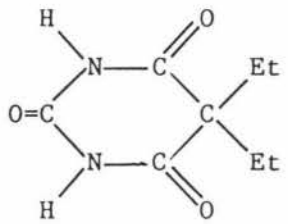
Cationic Buffer

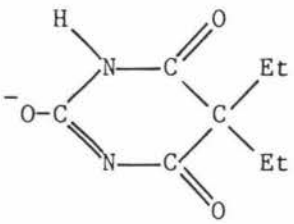
$$\text{Cellulose-OCH}_2\text{CH}_2\text{NEt}_2 \xrightleftharpoons{\text{pH 8}} \text{Cellulose-OCH}_2\text{CH}_2\overset{+}{\text{N}}\text{HEt}_2\ \ \text{Cl}^-$$

$$+\ (\text{CH}_2\text{OH})_3\text{C}\overset{+}{\text{N}}\text{H}_3\ \text{Cl}^- \qquad\qquad +\ (\text{CH}_2\text{OH})_3\text{CNH}_2$$

Anionic Buffer

$$\text{Cellulose-OCH}_2\text{CH}_2\text{NEt}_2 \rightleftharpoons \text{Cellulose-OCH}_2\text{CH}_2\overset{+}{\text{N}}\text{HEt}_2$$

+ 



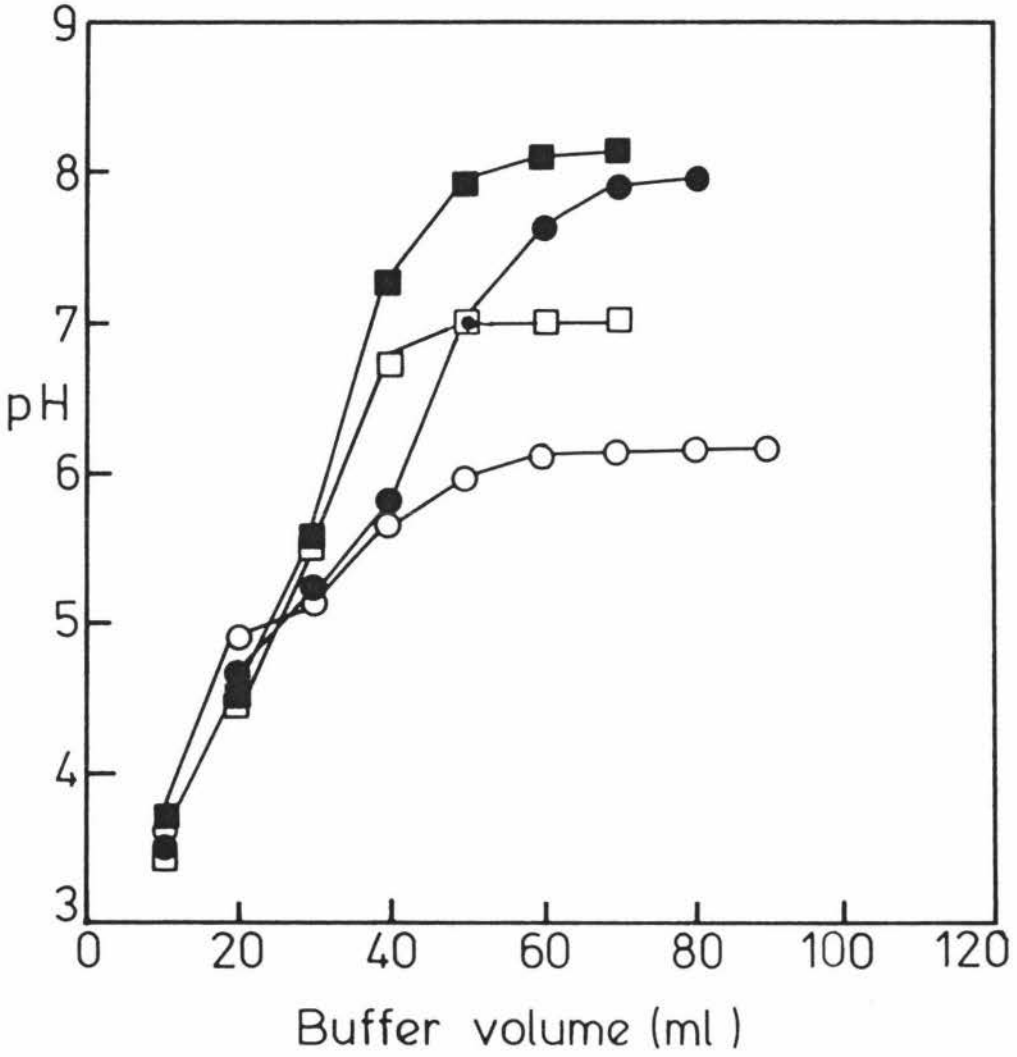
$(C_8H_{12}N_2O_3)$ $\qquad\qquad$ $(C_8H_{11}N_2O_3^-)$

The two anionic buffers probably require greater volumes because the buffering anion also acts as the counter ion to the charged groups generated on the ion exchanger during equilibration, i.e. takes part in the ion exchange process (see Equilibration of QA Cellulose later). The lower the pH of the equilibrating buffer the more buffer will be required, since more groups on the ion exchanger will require conversion from the basic to acidic form.

When neutralising from the hydrochloride form, i.e. $-\overset{+}{\text{N}}\text{HEt}_2\ \text{Cl}^-$ results similar to that above were obtained as shown in Figure 2.3 and they are summarised in Table 2.2.

Figure 2·3

Equilibration of DEAE Cellulose from the Hydrochloride form.



1. ■—■ 0·05 M Tris, pH 8·1
2. ○—○ 0·05 M Maleic acid, pH 6·15
3. □—□ 0·05 M Imidazole, pH 7·0
4. ●—● 0·05 M Barbitone, pH 7·95

Table 2.2

Equilibration of Anionic Exchanger, DEAE Cellulose from the Hydrochloride Form

Buffer Type	Buffer and pH	Volume of Buffer Required (ml)
Cationic	0.05M Tris, pH 8.1	60
Cationic	0.05M Imidazole, pH 7.0	50
Anionic	0.05M Barbitone, pH 7.95	80
Anionic	0.05M Maleic acid pH 6.15	60

Again it can be seen that the anionic buffers require slightly greater volumes to bring about complete equilibration as they still participate in the ion exchange process (as well as buffering the column) as shown in the equilibration above. This time the lower pH buffers, within the same buffer type, require the smaller volume because there is a smaller percentage of the groups on the cellulose to be neutralised at lower pH. This factor also explains the smaller volumes recorded in Table 2.2 than Table 2.1 since the pH 6-8 is closer to the protonated form of the DEAE groups than their free base form (see Figure 2.1). From these equilibration studies we can conclude that:

(a) when equilibrating a DEAE Cellulose in the top half of it's buffering pH range, less buffer will be required if equilibration proceeds from the free base form.

(b) when equilibrating a DEAE Cellulose in the lower half of it's buffering pH range less buffer will be required if equilibration proceeds from the hydrochloride form.

(c) since neutralisation of the groups is the major contributor to the amount of buffer required, there is only a slight advantage in using cationic in preference to anionic buffers.

It should be noted that the Diethylamino Cellulose (DE) made via the Epoxypropyl Cellulose has very little buffering capacity in the pH range 4-7 (compare titration curves in Figure 2.1). Thus this new derivative should equilibrate very rapidly from the hydrochloride form with cationic buffers, since there is no neutralisation of groups required in the pH range 3-7 (see similar result later for QA in the chloride form).

The effect of the presence of a few quaternary nitrogen groups in DEAE Cellulose,

i.e. Cellulose-$OCH_2CH_2\overset{+}{N}Et_2CH_2CH_2NEt_2$ Cl^-

has not been taken into consideration in the above discussion but these could also make a minor contribution to the observed differences in Table 2.2 between cationic and anionic buffers as will be shown in the next section on Quaternary Ammonium Celluloses.

2.2.2 Equilibration of QA Cellulose

The Quaternary Ammonium ion exchanger, (QA), carries a positively charged nitrogen at all pH's and has no acid or base properties itself. It is only the counter ion, A^-, associated with the QA group which may have buffering action, if it is a weak or strong base.

i.e. Cellulose-$\overset{+}{N}R_3$ A^-

where $A^- = Cl^-$ or SO_4^{2-} there is no buffering action to resist equilibration of the ion exchanger but if $A^- = CH_3CO_2^-$ or OH^- then these groups will require neutralisation during equilibration (see Figure 2.4).

Figure 2.5 shows the effect of equilibrating a QA Cellulose column in the hydroxide form with the four buffers used previously. In each case the hydroxide ion is completely neutralised by the buffers and in this case, anionic and cationic buffers require very similar volumes to achieve the equilibration.

Figure 2·4

Titration Curve of QA Cellulose in OH^- form.

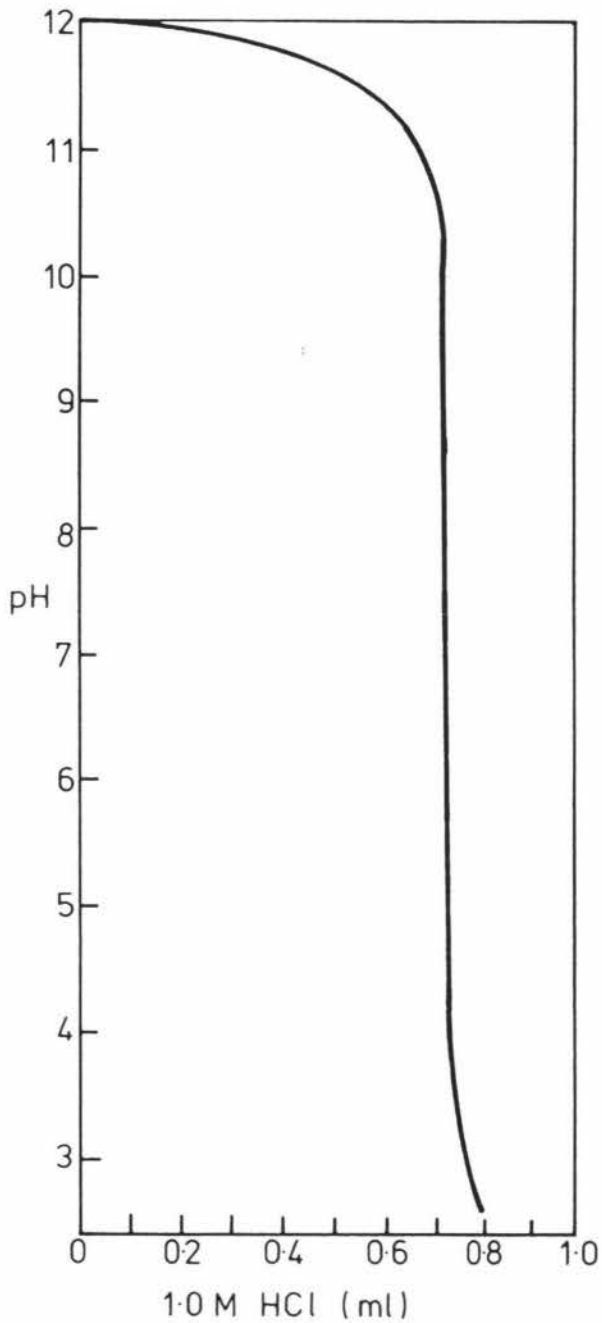
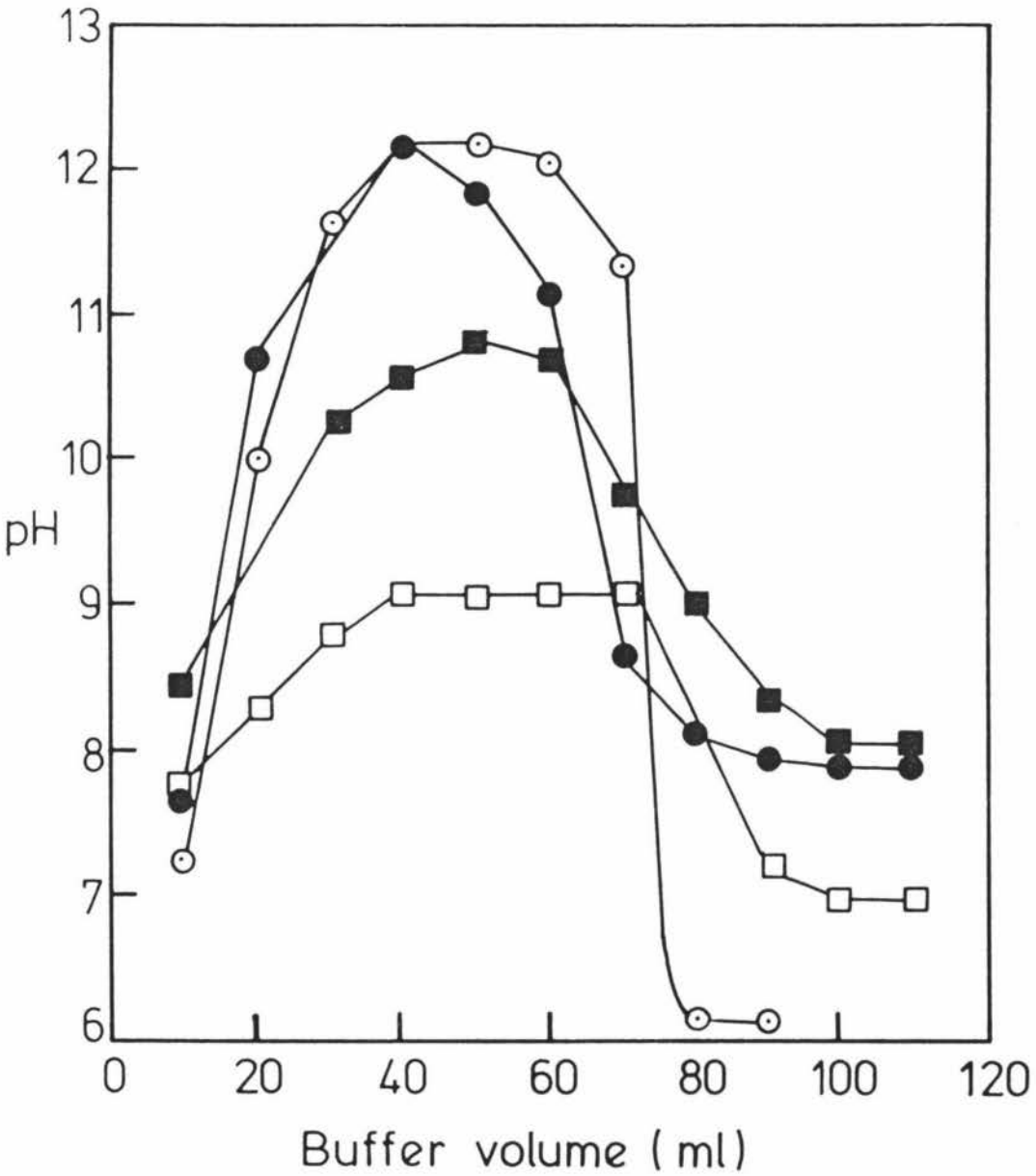


Figure 2·5

Equilibration of QA Cellulose from the Hydroxide form.



i.e. Cationic buffer

R = Cellulose-

$$R\text{-}OCH_2CHOHCH_2\overset{+}{N}(CH_3)_2CH_2CH_2OH\ OH^- \rightleftharpoons R\text{-}OCH_2CHOHCH_2\overset{+}{N}(CH_3)_2CH_2CH_2OH\ Cl^-$$
$$+ (CH_2OH)_3C\overset{+}{N}H_3\ Cl^- \qquad + H_2O + (CH_2OH)_3CNH_2$$

Anionic buffer

$$R\text{-}OCH_2CHOHCH_2\overset{+}{N}(CH_3)_2CH_2CH_2OH\ OH^- \rightleftharpoons R\text{-}OCH_2CHOHCH_2\overset{+}{N}(CH_3)_2CH_2CH_2OH$$
$$+ C_8H_{12}N_2O_3 \qquad C_8H_{11}N_2O_3^- + H_2O$$

This result is similar to that when equilibrating a DEAE ion exchanger. The neutralisation of, in this case of the counter ion, determines the amount of buffer required, while for the DEAE, it was the neutralisation of the weak amino groups.

When equilibrating QA Cellulose from the chloride form, a very different picture is observed (see Figure 2.6). There is now no neutralisation of the counter ion groups (i.e. Cl^-) and if the right buffer is used equilibration takes place very quickly and easily, taking less than two column volumes of buffer. This is only possible when the chloride salt of a cationic buffer is used .

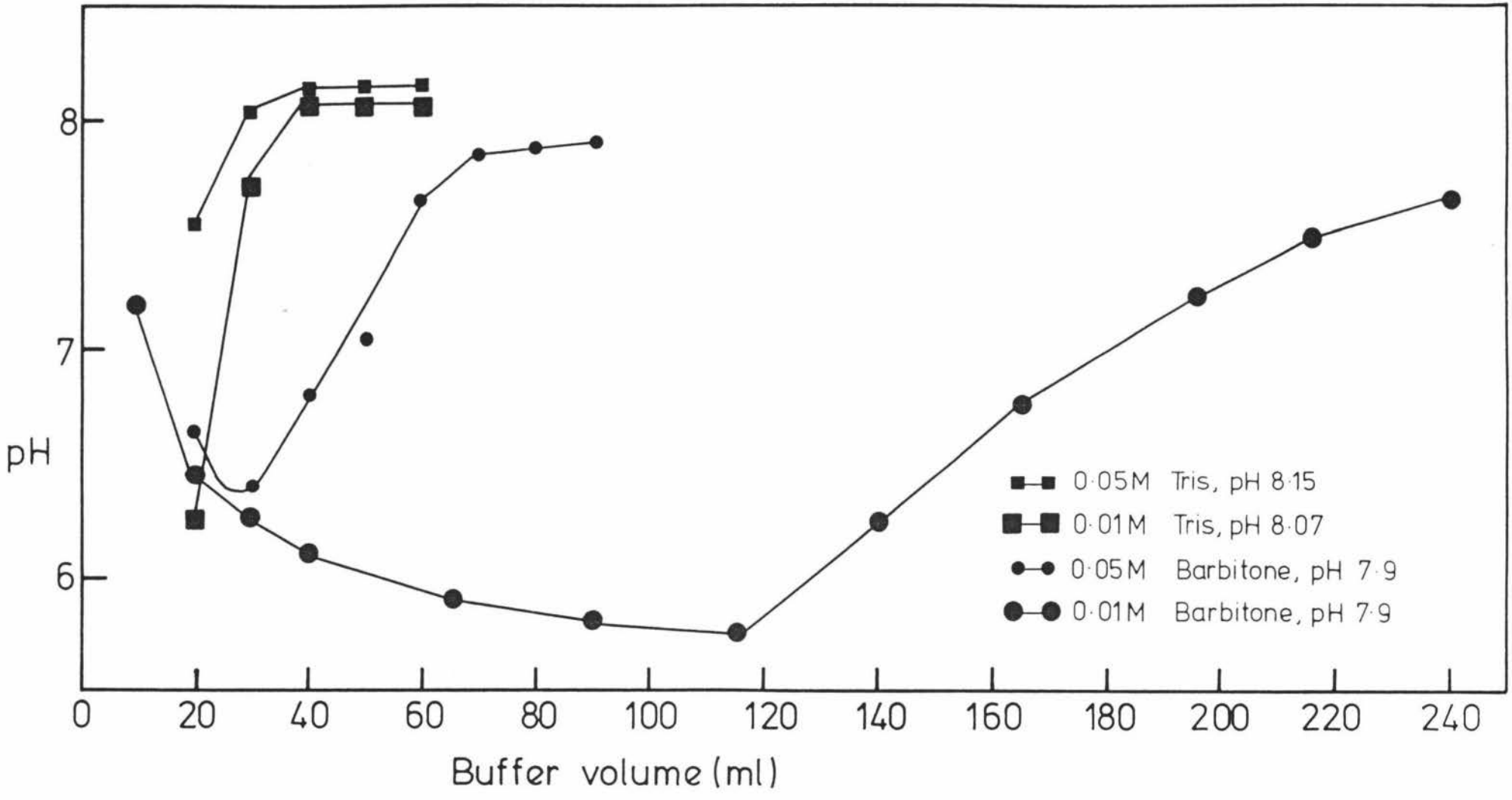
i.e. Cationic Buffer

$$Cellulose\text{-}OCH_2CHOHCH_2\overset{+}{N}(CH_3)_2CH_2CH_2OH\ Cl^- \rightleftharpoons \text{No change}$$
$$+ (CH_2OH)_3C\overset{+}{N}H_3Cl^-$$

In this case there is no change in the nature of the groups on the cellulose nor their counter ions, so it is only the aqueous environment surrounding the cellulose particles and within their pores that needs to be displaced before the column is equilibrated. This is further confirmed by reducing the concentration of the tris buffer from 0.05 mol/l to 0.01 mol/l. There is no increase in the volume of buffer required to achieve the equilibration (see Figure 2.6).

Figure 2·6

Equilibration of QA Cellulose from the Chloride form.



When equilibrating the QA derivative from the chloride form with an anionic buffer, e.g. barbitone, it is a very different picture. There is a change in the state of the matrix from chloride to the anion component of the equilibrating buffer, and the volume of buffer, needed is similar to that required when equilibrating from the hydroxide form. If the concentration of the barbitone buffer is reduced 5 fold to 0.01 mol/l there is almost a proportional increase in the volume of buffer required. These findings are summarised in Table 2.3.

TABLE 2.3

Equilibration of Anion Exchanger, QA Cellulose

Buffer Type	Buffer	Concentration (mol/l)	Volume of Buffer Required (ml)	
			From OH^- Form	From Cl^- Form
Cationic	Tris,	0.05	100	40
		0.01	-	40
Anionic	Barbitone,	0.05	100	70
		0.01	-	>260

It can therefore be concluded that equilibrating a QA ion exchanger from the chloride form using a cationic buffer is far easier than equilibrating either, a QA ion exchanger by any other method or a DEAE ion exchanger by any method.

There seem to be several advantages of using a QA in preference to a DEAE ion exchanger of similar swollen volume and substitution. These include:

(i) wider working pH range, especially in the higher pH region

(ii) more even charge density over the entire working pH range

(iii) rapid equilibration with cationic buffers

(iv) shifting the pH for elution of proteins would also occur readily with cationic buffers.

The disadvantages in using a QA in preference to a DEAE are:

(i) DEAE is more chemically stable, especially at high pH

(ii) The weak base groups in the DEAE, may be necessary to achieve the desired resolution of proteins in certain situations.

In situations where cation buffers are either not available or are unsuitable it is possible to equilibrate the anion exchangers (QA and DEAE) by having sodium chloride present along with a very dilute anionic buffer. Such is the situation in the Rennet process(20) where a DEAE Cellulose is used and equilibrated with 0.1 mol/l NaCl; 0.005 mol/l citrate buffer, pH 5.5.

In this process the anion exchanger needs to be equilibrated from the free base form. Figure 2.7 shows the greater ease with which the QA derivative is equilibrated compared to the DEAE. This is because chloride displaces hydroxide from the QA groups and the anionic citrate buffers the column quickly even though very dilute.

$$\text{i.e. Cellulose}\overset{+}{\text{-}}\text{NR}_3\ \overset{-}{\text{OH}} \xrightarrow[0.005\ \text{mol/l citrate}]{0.1\ \text{mol/l NaCl}} \text{Cellulose-}\overset{+}{\text{N}}\text{R}_3\ \text{Cl}^-$$

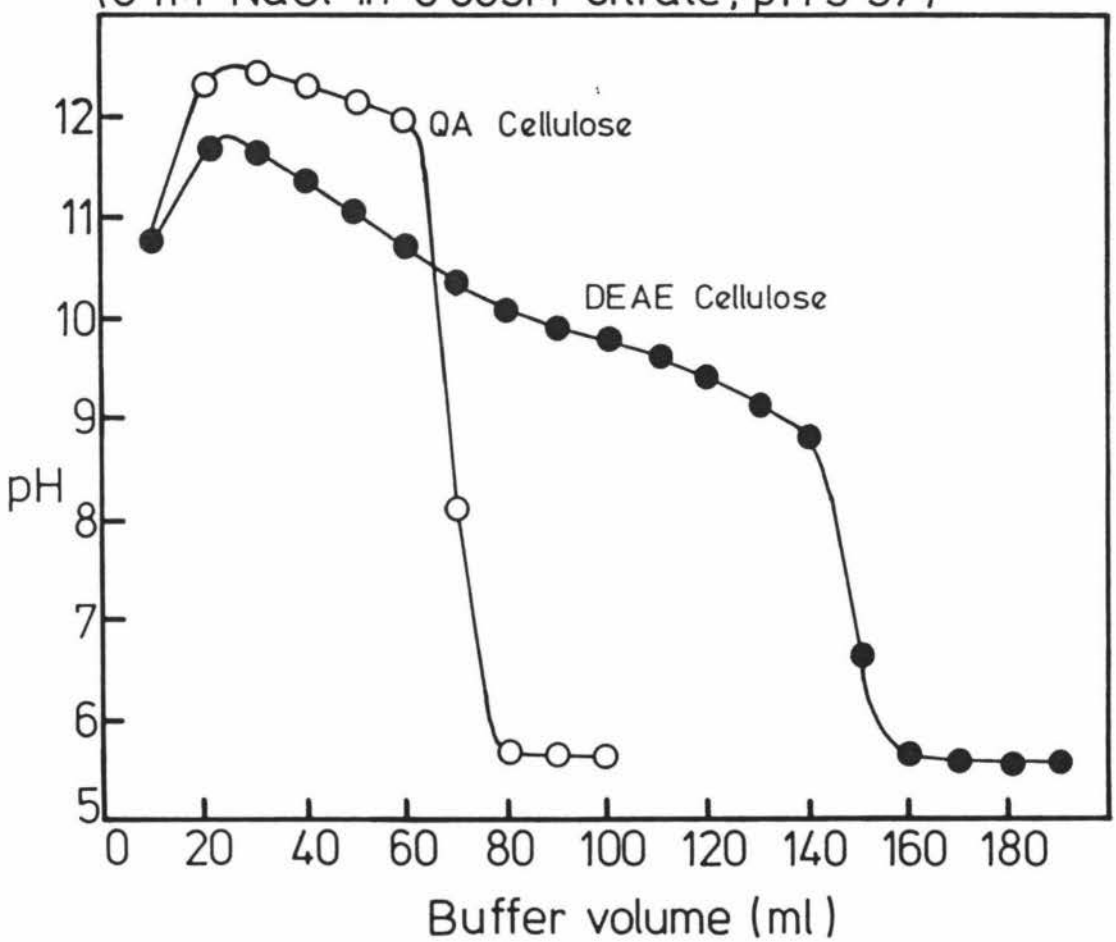
For the DEAE derivative from the free base form, the tertiary amino groups need to be protonated and the 0.1 mol/l sodium chloride does not assist this process. The only proton source is the ammonium citrate buffer which is dilute and hence equilibration takes much longer.

$$\text{i.e. Cellulose-NEt}_2 \xrightarrow[\substack{0.005\ \text{mol/l citrate} \\ \text{pH 5.5}}]{0.1\ \text{mol/l NaCl}} \text{Cellulose-}\overset{+}{\text{N}}\text{HEt}_2\ \text{Cl}^-$$

Clearly a QA Cellulose would be an easier ion exchanger to use in the rennet process if all other aspects are equal.

Figure 2·7

Equilibration of DEAE Cellulose from the free base form and QA Cellulose from the hydroxide form.
(0·1M NaCl in 0·005M citrate, pH 5·57)



2.2.3 Cation Exchangers: CM and SP Celluloses

The situation with these two ion exchange derivatives should be analogous to the DEAE and QA derivatives. The best buffers for these will be anionic so that they do not enter into the ion exchange process. For example, the cationic buffer tris/HCl would be involved in the exchange process shown below for the sulphonate derivative in the sodium form.

i.e. $\text{Cellulose-SO}_3^- \text{ Na}^+ + (\text{CH}_2\text{OH})_3\overset{+}{\text{C}}\text{NH}_3 \text{ Cl}^- \rightleftharpoons \text{Cellulose-SO}_3^- \overset{+}{\text{N}}\text{H}_3\text{C}(\text{CH}_2\text{OH})_3 + \text{NaCl}$

For this reason the equilibration tests investigated here with cationic exchangers were restricted to the use of anionic buffers only.

Both Carboxy Methyl (CM) and Sulphopropyl (SP) Celluloses in their Na^+ forms were equilibrated with two separate buffers, barbitone at pH 8 and acetate at pH 4.9. The results are shown in Figure 2.8.

(a) CM Cellulose

CM Cellulose has buffering capacity below pH 7 (See titration curve in Figure 2.9).

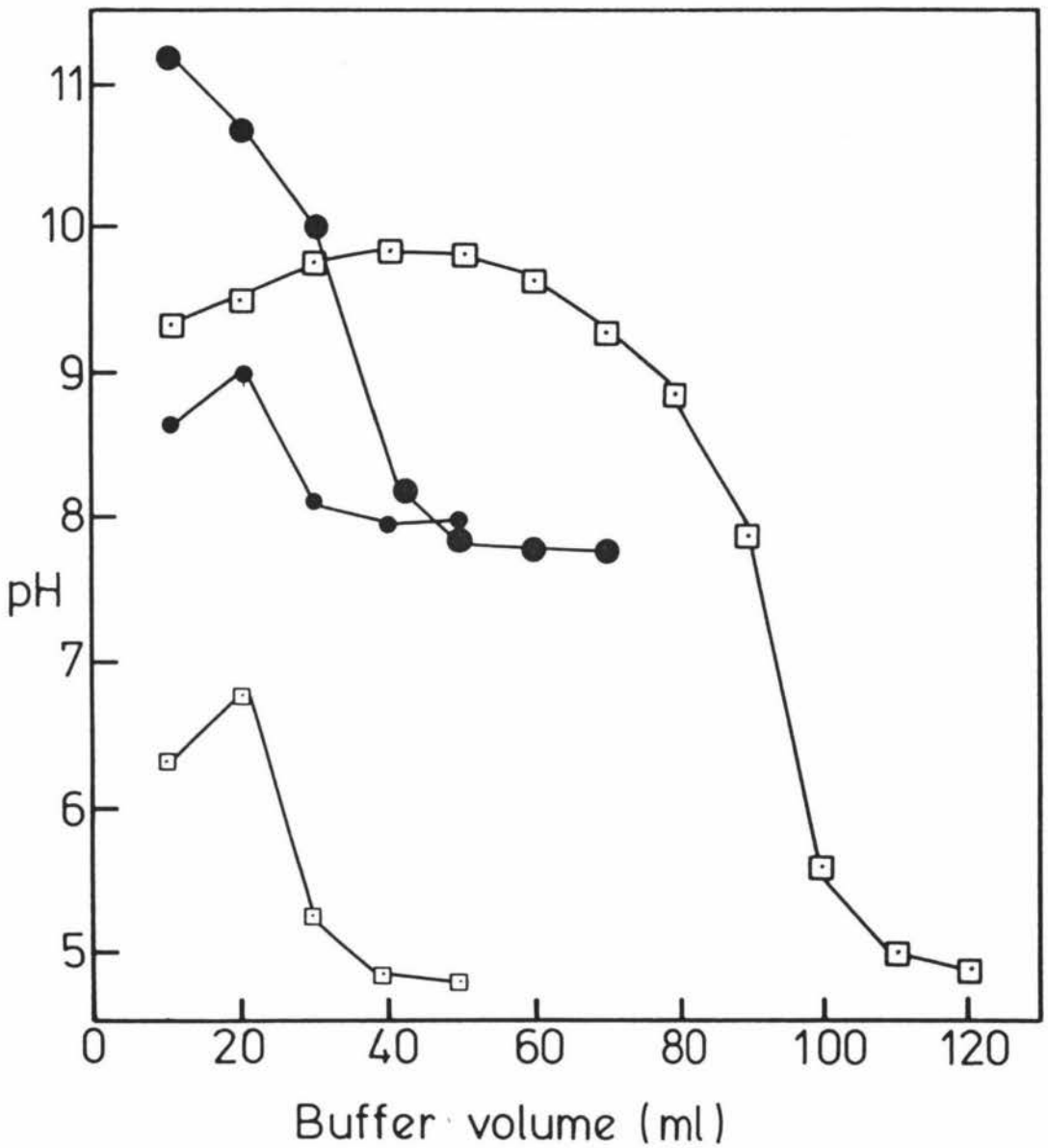
If equilibrating from the sodium form with anionic buffer above pH 7, equilibration will be rapid. This is because there is no change in the state of the matrix and only the external solution must be equilibrated (See curve 1, Figure 2.8).

i.e. Anionic Buffer pH 7 eg barbitone pH 8.0 ($B^- = C_8H_{11}N_2O_3^-$)

$\text{Cellulose-OCH}_2\text{CO}_2^- \text{ Na}^+ + \text{HB/Na}^+\text{B}^- \rightleftharpoons \text{Cellulose-OCH}_2\text{CO}_2^- \text{ Na}^+ + \text{HB/Na}^+\text{B}^-$

Figure 2·8

Equilibration of CM and SP Celluloses.



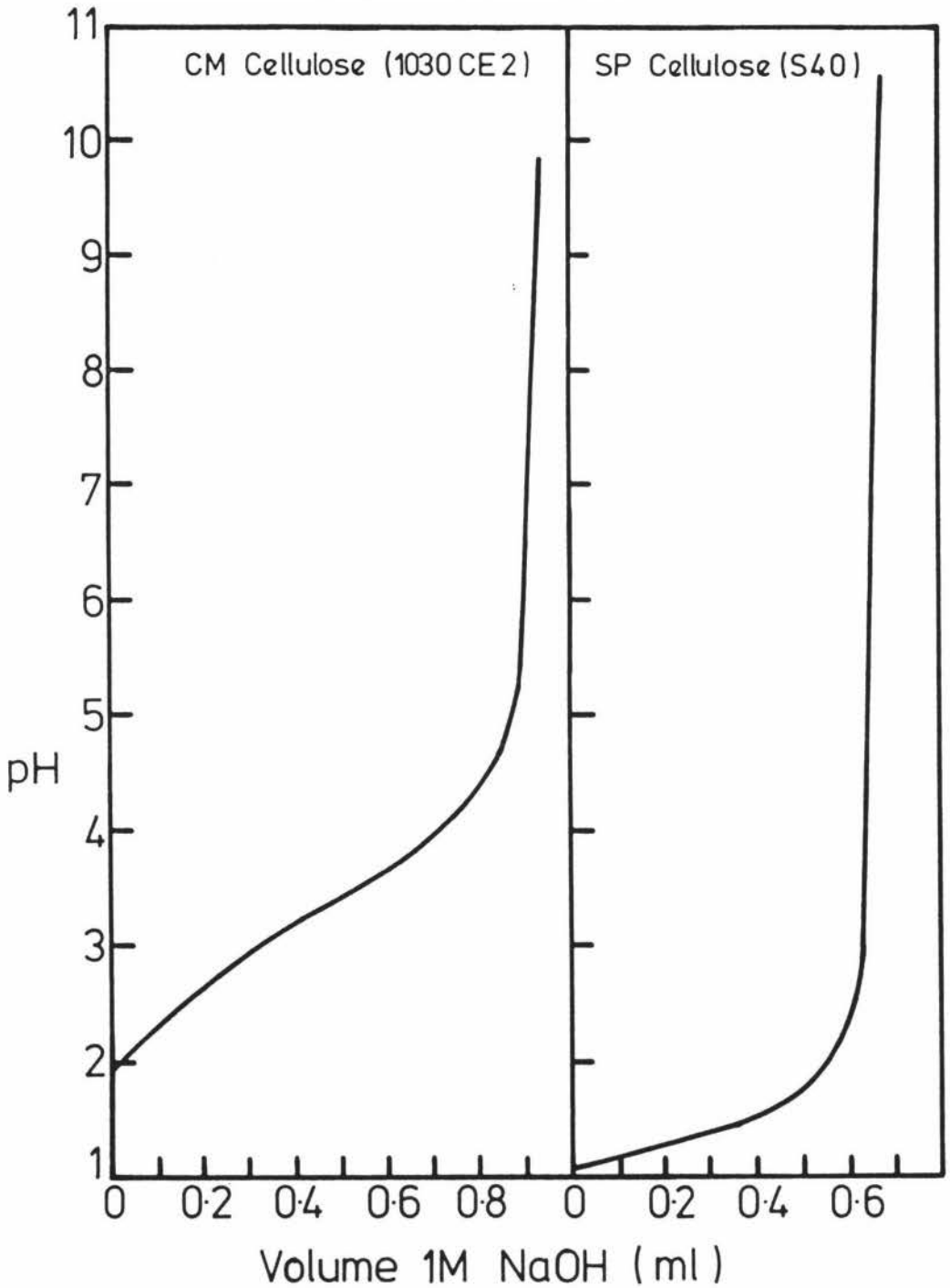
1 ●—● CM cellulose + 0·01M Barbitone, pH 7·75

2 ⊡—⊡ CM cellulose + 0·05M Acetic acid, pH 4·9

3 ●—● SP cellulose + 0·01M Barbitone, pH 7·95

4 ⊡—⊡ SP cellulose + 0·01M Acetic acid, pH 4·8

Figure 2·9
Titration Curves of CM Cellulose (1030CE2) and SP Cellulose (S40).



If the operating conditions are in the pH 3-7 range some of the groups have to be neutralised before equilibration can occur and much more buffer will be required.

i.e. Anionic Buffer pH 7 eg acetic acid pH 4.9

$$\text{Cellulose-OCH}_2\text{CO}_2^- \text{ Na}^+ \rightleftharpoons \text{Cellulose-OCH}_2\text{CO}_2\text{H}$$

$$+\text{CH}_3\text{CO}_2^- \text{ Na}^+/\text{CH}_3\text{CO}_2\text{H} \qquad + 2\ \text{CH}_3\text{CO}_2^- \text{ Na}^+$$

This is certainly observed with acetate buffer (curve 2) where not only has the buffer strength been increased 5 fold (to 0.05 mol/l) but the volume of buffer required increases to 120 ml for complete equilibration. This being approximately a 10 fold increase over that required for the equilibration above pH 7 (i.e. barbitone).

(b) SP Cellulose

Since SP Cellulose (Na^+ form) has no buffering capacity above pH 1 equilibration down to pH 1 with anionic buffer will be rapid. Operating conditions below pH 1 are very unlikely, so SP Cellulose would be easy to equilibrate, over it's entire working range, using anionic buffers and this is demonstrated for both pH 8 and pH 4.9 in Figure 2.8.

i.e. Anionic Buffer

$$\text{Cellulose-SO}_3^- \text{ Na}^+ \rightleftharpoons \text{Cellulose-SO}_3^- \text{ Na}^+$$

No change

$$+\ \text{HA/Na}^+\text{A}^- \qquad + \text{HA/Na}^+\text{A}^-$$

The advantages of a SP over a CM derivative for ion exchange chromatography are the same as listed previously for a QA over a DEAE derivative.

It is obvious from the above findings that the QA and SP ion exchangers should be the ones of first choice for trying in any protein purification of commercial importance. The QA can be kept in the chloride form at any pH with cationic buffers. While the SP in the sodium form can be used at any pH with anionic buffers. The only reason for shifting to the DEAE and CM derivatives is in cases where a better resolution is obtained and is necessary.

For historical reasons, DEAE and CM have been the major ones used in the past(21) since they have been easier to make and even today are still more widely available (See Table 1.2). Even the Grant resins are only available as DEAE and CM derivatives. However with the recent developments set out in the introduction to this thesis, new SP and QA derivatives can now be made as well as the DEAE and CM and should find many applications.

SECTION 3

APPLICATION OF THE NEW MODIFIED ION EXCHANGERS TO THE PURIFICATION OF RENNET

3.1 INTRODUCTION

Rennet, also known as chymosin is a proteolytic enzyme found in the gut of calves. It s primary function is to cleave soluble casein, to form insoluble products (curd), that can then be attacked by other proteolytic enzymes in the gut. It has a molecular weight of 35,600 and an isoelectric point of 4.6(22).

The New Zealand Co-op Rennet Co Ltd at Eltham operates a plant which extracts and purifies rennet from the abomasums of calves. Involved in this process(20) is an ion exchange step which uses a DEAE Cellulose, A_2 Protion, manufactured according to the Grant patent from regenerated cellulose(10) by Tasman Vaccine Laboratories Ltd. (The same DEAE Cellulose is now being made by Phoenix Chemicals Ltd and sold under the Trade name of Indion). In the Rennet Co process the DEAE Cellulose is used in a column to absorb the rennet enzyme from the process stream, while allowing all the mucoproteins which normally give the product a cloudy appearance, to pass straight through the column. The rennet is then recovered from the column in high concentration as a crystal clear solution by eluting it with a sodium chloride solution of high concentration. This is the only commercial use of DEAE Protion, within New Zealand, that is known to us.

The new modified ion exchangers discussed in the introduction have superior protein capacities (g/g), to that of A_2 Protion (see Table 1.5). It was expected that these new exchangers would perform better than A_2 Protion in the rennet purification, since a much smaller amount should be able to absorb the rennet.

Having already concluded from our equilibration work that a QA ion exchanger should be used in preference to a DEAE ion exchanger where possible, we decided to test the ability of the new QA Cellulose to bind rennet under the conditions used commercially, i.e. 0.005 mol/l ammonium citrate;0.1 mol/l NaCl at pH 5.5.

3.2 RESULTS AND DISCUSSION

In Table 3.1 are listed some of the properties of three ion exchangers studied in this section.

Table 3.1

Propeties of Ion Exchangers, A_2 Protion QA Cellulose and DE Cellulose

Ion Exchanger	Small Ion Capacity (meq/g)	Swollen Volume (ml/g)	Protein Capacity (BSA) (g/g)
A_2 Protion	1.1	5.5	0.5
DE Cellulose	1.17	11.3	1.2 - 1.4
QA Cellulose	1.14	12.7	1.46

A small column (2 ml) of the new modified QA Cellulose (Q4) was packed and equilibrated with 0.1 mol/l NaCl;0.005 mol/l citric acid adjusted to pH 5.5 with concentrated ammonia. This was loaded with 10 ml of a rennet solution which had been dialysed against more of the same solution used to equilibrate the column. The amount of rennet loaded in 10 ml was expected to require only a fraction of the column's capacity. However the results in Table 3.2 show that the column was considerably overloaded under these conditions.

Table 3.2

Column Chromatography of Rennet on QA Cellulose (2ml)
(0.005 mol/l citrate; 0.1 mol.1 NaCl at pH 5.5

Fraction*	Salt Strength* (mol/l)	Volume of Fraction (ml)	% of Loaded Activity+
Breakthrough	0.1	10	25
Wash	0.1	10	34
Elution 1	0.2	5	16
Elution 2	0.3	5	6
Elution 3	0.4	5	0.5

* All fractions contained 0.005 mol/l ammonium citrate at pH 5.5.
+ 19% of the loaded activity was unaccounted for, see experimental section.

These results were very puzzling, since QA Cellulose has a superior protein capacity to A_2 Protion, under conditions of low ionic strength.

The QA Cellulose column was re-equilibrated with 0.005 mol/l ammonium citrate; 0.05 mol/l NaCl. It was then loaded with a rennet solution comprised of 5 ml of dialysed rennet as used for the first column and 5 ml of 0.005 mol/l ammonium citrate pH 5.5. The results in Table 3.3 show that while all the rennet did bind to the column under these conditions, 0.1 mol/l NaCl was of sufficient ionic strength to elute off some of the bound rennet.

Table 3.3

Column Chromatography of Rennet on QA Cellulose (2ml)
(0.005 mol/l citrate; 0.1 mol.l NaCl at pH 5.5

Fraction*	Salt Strength* (mol/l)	Volume of Fraction (ml)	% of Loaded Activity+
Breakthrough	0.05	10	-
Wash	0.05	10	1
Elution 1	0.075	5	1.6
Elution 2	0.1	5	11
Elution 3	1.0	5	59

* All fractions contained 0.005 mol/l ammonium citrate at pH 5.5.
\+ 27% of the loaded activity was unaccounted for.
N.B. In this case only half as much rennet was loaded as for Table 3.2.

From the results outlined in Tables 3.2 and 3.3 it was obvious that the new modified cellulose ion exchanger, QA Cellulose, was behaving very differently to that of A_2 Protion used in the commercial rennet purification process. For this reason batch tests were carried out to compare the capacities of A_2 Protion, QA Cellulose (Q4) and DE Cellulose (D11) for rennet. The conditions chosen were the same as those in Table 3.3 i.e. 0.005 mol/l ammonium citrate; 0.05 mol/l NaCl at pH 5.5. The results in Table 3.4 show the amount of rennet activity not absorbed by the ion exchanger after mixing for two hours. Clearly netiher D11 nor Q4 works as well as A_2 Protion inspite of their superior BSA capacities shown in Table 3.1.

Table 3.4

Rennet Capacity Tests - Batch Method pH 5.5

(Ion Exchanger mixed with 10 ml of rennet in 0.005 mol/l ammonium citrate; 0.05 mol/l NaCl)

Wt of Ion Exchanger (mg dry weight)	% Unbound rennet activity in supernatant		
	A_2 Protein	Q4	D11
100	<1	-	2
50	<1	10	4
20	1	50	-
10	10	>70	>30

These results tend to suggest that the density of charged groups on the new modified ion exchangers is not sufficient to bind rennet in the presence of high concentrations of salt, i.e. 0.05 mol/l NaCl. The major difference between A_2 Protion and the new modified ion exchangers is their swollen volumes. This means that the density of charged groups (meq/ml) on the new modified ion exchangers is only approximately half that of A_2 Protion (see Table 3.1). To confirm this effect, of low charge density resulting in poor protein binding ability in the presence of salt, a series of protein capacity tests were carried out, over a range of pH's, with various salt concentrations. These tests are described below under the heading "pH profile studies". DE Cellulose was used instead of QA Cellulose so that a more direct comparison could be made with A_2 Protion, i.e. similar ion exchange groups.

3.3 pH PROFILE STUDIES

3.3.1 Basis of Method

The basis of the method used in this study involved contacting a known weight of equilibrated ion exchanger, with a known amount of protein in solution, under various conditions. After a standard two hour mixing period the protein which remained in solution, i.e. not bound to the ion exchanger, was measured and the amount bound to the ion exchanger calculated. The test conditions studied ranged from pH 5 to 8 and salt concentration of 0.0 to 0.1 mol/l NaCl.

3.3.2 Choice of Protein

The protein we chose to use for the pH profile studies was bovine serum albumin (BSA). There were several reasons for this choice, the main one being that BSA has an isoelectric point of 4.7(23) which is very similar to that of rennet. BSA is readily available from commercial sources and is cheap and easy to work with. It's absorbance at 280 nm follows a Beer's Law Plot up to at least an absorbance of 2 (see Figure 3.1) and this makes it easy to assay.

3.3.3 Choice of Buffer

There were several reasons why 0.005 mol/l imidazole;0.005 mol/l pyridine was selected as the buffering system for the pH profiles studies. Firstly, since anion exchangers were being investigated, cationic buffers were selected for the reasons outlined in Section 2. Secondly, buffering over a three pH unit range from pH 5 to pH 8 was required and a one buffer system would not be effective over this range. Initial runs used the buffers 0.01 mol/l pyridine for the lower pH's, i.e. pH 5-6.2, and 0.01 mol/l imidazole for the higher pH's, i.e. pH 6.0-7.0. This resulted in a break in profile where the two systems overlapped (see Figure 3.2), especially at low ionic strength. This we attributed to the fact that at pH 6.1, the bulk of the pyridine is uncharged while the bulk of the imidazole is charged. This means that if a profile is done at 0.025 mol/l NaCl, the effective salt strength in 0.01 mol/l pyridine at pH 6.1 is 0.025 mol/l, while in 0.01 mol/l imidazole at the same pH and sodium chloride concentration, the effective salt strength will be approaching 0.035 mol/l. This results in quite a difference in protein capacity especially for the new modified ion exchangers which have a low density of charged groups. To overcome the break in the pH profiles, we made a combined buffer system consisting of 0.005 mol/l pyridine and 0.005 mol/l imidazole. To overcome the change in ionic strength with pH, the dilute buffer system used was prepared from a concentrated stock solution of 0.5 mol/l pyridine; 0.5 mol/l imidazole at pH 4.0, using sodium hydroxide to shift them to the required pH. This enabled a constant ionic strength to be achieved over the whole pH range of the profile study, since neutralisation of the buffering ions with NaOH results in the production of NaCl so that there is no change in the effective ionic strength.

Figure 3·1

Beer's Law Plot
(BSA in 0·01M Tris, pH 8·0)

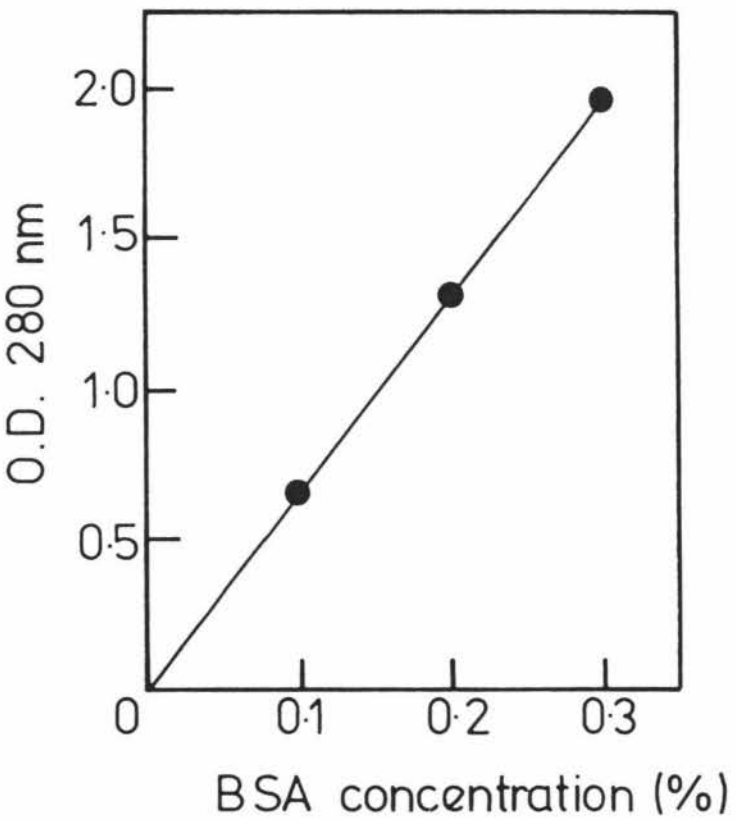
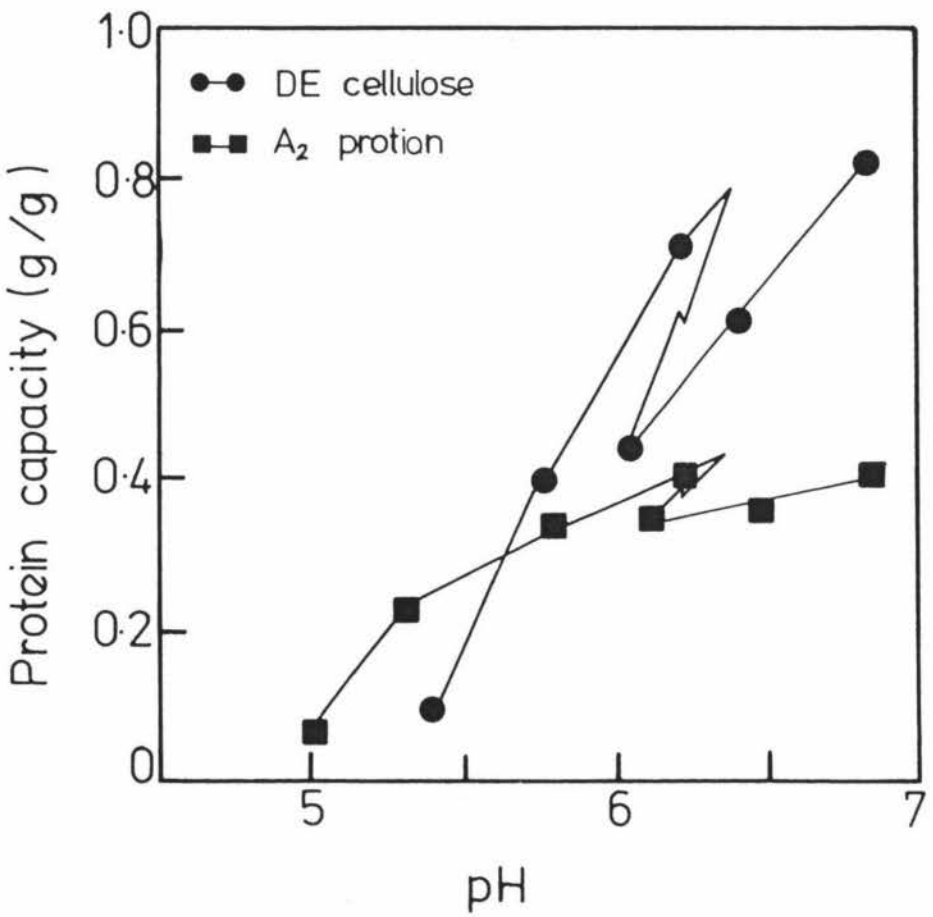
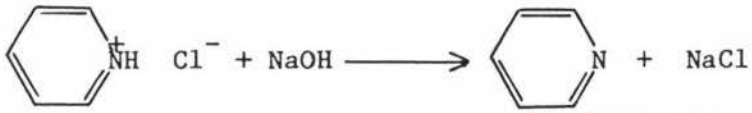


Figure 3·2

pH Profile of DEAE Celluloses in 0·025M NaCl.
(0·01M pyridine, pH 5-6·2
0·01M Imidazole, pH 6·0-7·0)



i.e. from hydrochloride form



No change in concentration of ionic species with increasing pH

3.3.4 Scale of Operation

Several factors were taken into account when choosing the scale on which the pH profiles were to be carried out. Sufficient protein must be used, so that some remains in the supernatant at the end of the test. This is to ensure that the maximum capacity of the ion exchanger, under the test conditions, is realised. It was endeavoured to use an amount of protein, such that a maximum of 50-65% of that available was bound.

If 100 ml of a 0.1% BSA solution was used it would not require dilution prior to measuring the absorbance at 280 nm. The solution would contain 100 mg of protein, of which 65 mg maximum, by the above guideline, could be bound to the ion exchanger. The maximum amount of ion exchanger, that can therefore to be used is approximately, 50 mg dry weight for the DE Cellulose and 100 mg dry weight for A_2 Protion, since DE Cellulose has a BSA capacity of 1.2 - 1.4 g/g and A_2 Protion has a BSA capacity of 0.5 g/g. With their difference in dry matter composition, approximately 350 - 400 mg of equilibrated ion exchanger, for both A_2 Protion and DE Cellulose would be required. This amount of material is easy to weigh accurately.

3.3.5 Results and Discussion

The results of pH profile studies, with DE Cellulose are shown in Figure 3.3, while those for A_2 Protion are shown in Figure 3.4. From these results, it can be seen that as the salt concentration increases the protein capacity of both ion exchangers decreases. This effect is most noticeable at lower pH's. The increase in salt

Figure 3·3

pH Profiles of DE Cellulose. (0·005M pyridine / 0·005M imidazole)

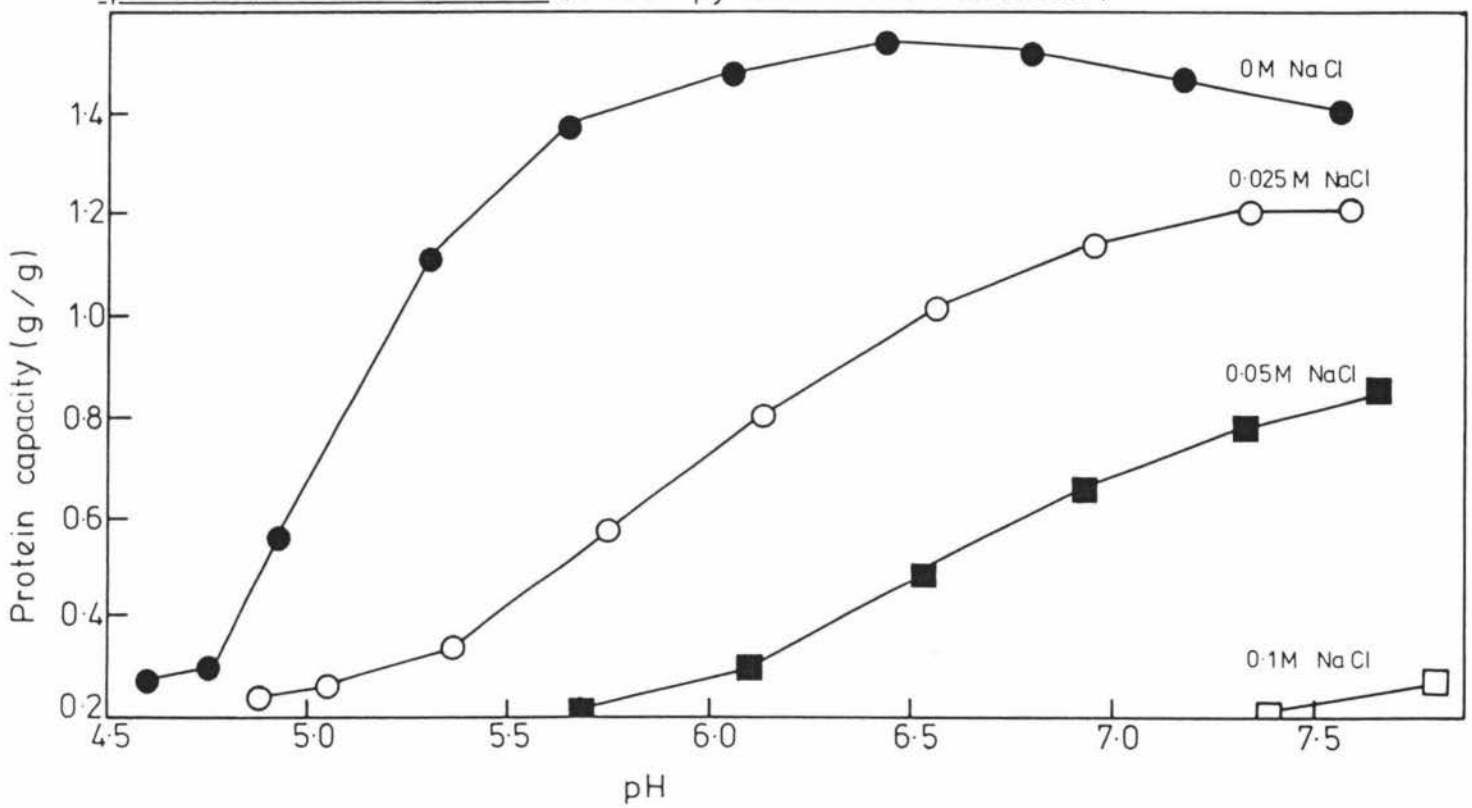
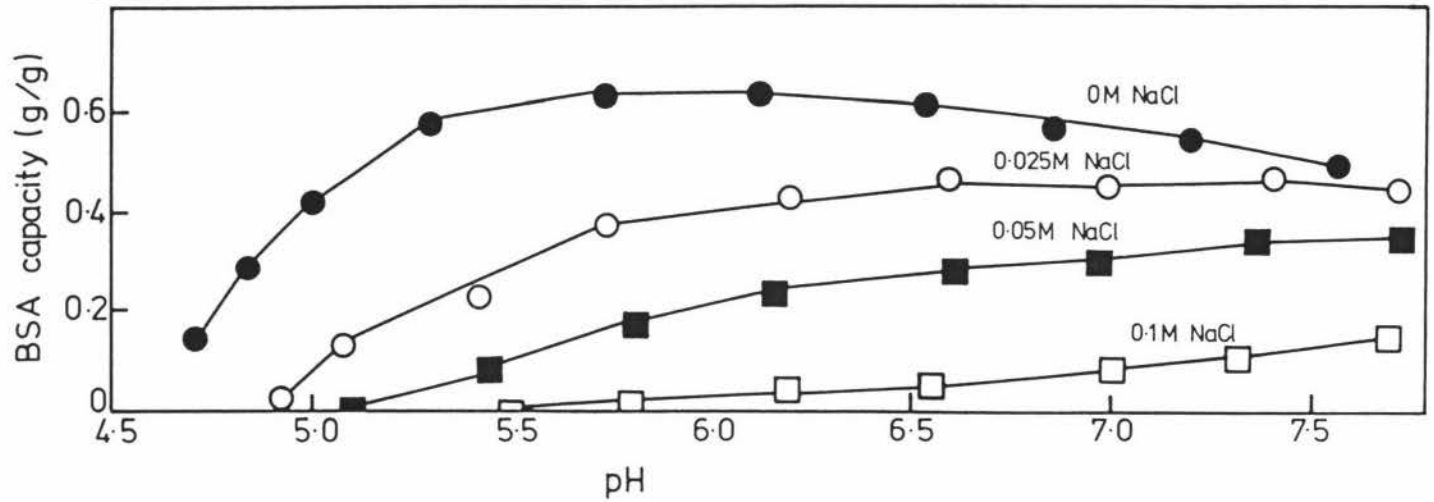


Figure 3·4

pH Profiles of A_2 Protion. (0·005M pyridine / 0·005M imidazole)



concentration increases the competition between protein molecules and chloride ions, for the binding sites, i.e. charged groups on the ion exchangers. The protein molecules are more competitive at higher pH's, since they are further away from their isoelectric point and have a higher density of charged groups which can interact with the groups on the ion exchangers. This means that the higher the ionic strength, the further we need to get away from the IEP of the protein to get good protein capacities. This is particularly true for the new modified ion exchangers.

The pH profiles at 0.05 mol/l NaCl for both A_2 Protion and DE Cellulose are shown in Figure 3.5. It can be seen that as the pH increases there is a cross over where the protein capacity of the DE Cellulose becomes better than that of A_2 Protion. This occurs at approximately pH 6.5. This cross over in capacity is not observed at low ionic strength. With 0.05 mol/l NaCl present, the new modified cellulose DE ion exchanger needs to be used at a pH further away from the protein's isoelectric point than is necessary for A_2 Protion. This was the situation we observed when we tried to bind rennet to the DE Cellulose (and QA Cellulose) at pH 5.5 in 0.1 mol/l NaCl. It was too close to the IEP of 4.6 for rennet to observe the optimum capacity for the new ion exchangers.

In 0.05 mol/l NaCl at pH 6.0, A_2 Protion has about 50% of it's maximum capacity for BSA and about three times the capacity shown by DE Cellulose which only has about 10% of it's maximum at this pH (see Figure 3.5). This is similar to the situation observed for rennet at pH 5.5 (Table 3.4). Rennet being a smaller protein and having a slightly different IEP must bind tighter than BSA, to the ion exchanger and this would shift pH profiles with rennet slightly to the left compared with those with BSA. To confirm these findings rennet capacity tests were carried out at pH 6.6 in 0.05 mol/l NaCl. In this case DE Cellulose now had a higher capacity for rennet that did A_2 Protion as expected (see Table 3.5).

Figure 3·5

pH Profile of A_2 Protion and DE Cellulose in 0·05M NaCl.
(0·005M pyridine / 0·005M imidazole)

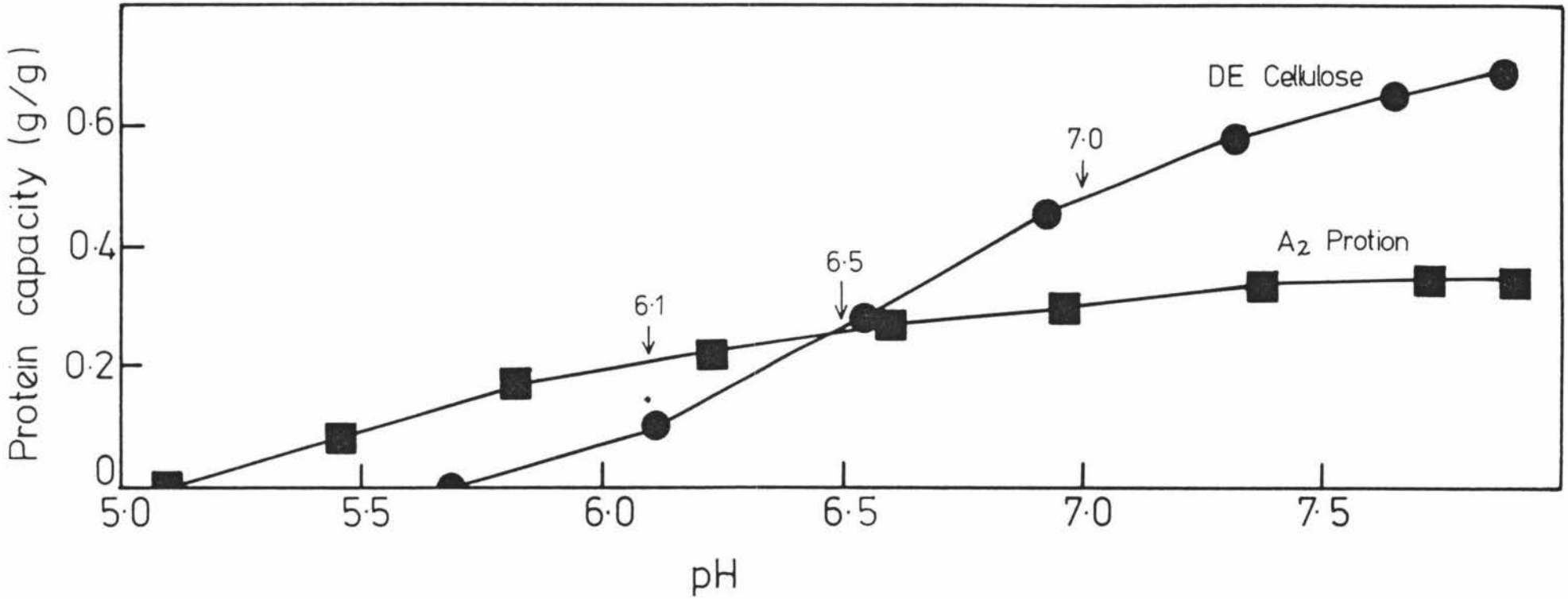


Table 3.5

Rennet Capacity Tests - Batch Method, pH 6.6

(Ion Exchanger mixed with 10 ml of Rennet in 0.005 mol/l Imidazole; 0.05 mol/l NaCl)

Weight of Ion Exchanger (mg dry wt)	% Unbound Rennet Activity in Supernatant	
	A_2 Protion	D11
100	<1	<1
50	<1	<1
20	<1	<1
10	3	<1

Rennet is a pH sensitive enzyme, which is stable in acidic conditions but unstable above pH 7. While rennet is relatively stable at pH 5.5 once the pH is shifted above pH 7 activity drops off markedly. This activity can not be regained by shifting back to more acidic conditions. This means that the conditions where rennet can be bound to the new modified ion exchangers, i.e. pH 6.6 or above, are not feasible for use in the purification of rennet since it is too close to the point of irreversible denaturation. We therefore have to conclude that for this particular purification, A_2 Protion is a better suited ion exchanger than the new modified ion exchanger. The main reason for this being the inability of these new ion exchangers to bind rennet within it's pH stability limits.

This example shows the danger of replacing one ion exchanger with another, under conditions which have been optimised for the first. If the replacement ion exchanger does not work under these conditions it is all to easy to write if off as being no good. To give a new ion exchanger a fair trial, the conditions where it performs best in that process, must be determined and then it's performance at these conditions, compared with that of the original ion exchanger under it's optimised conditions. Only then should a new ion exchanger be judged on whether or not it is suitable for that particular process. While the QA and DE Cellulose have been

found to be unsuitable for the rennet process, they are still potentially very useful, with their good flow rates and high protein capacities.

3.4 DISTRIBUTION OF CHARGED GROUPS

Another notable result from the pH profile study was the observation that A_2 Protion exhibited a low binding capcity over a wide pH range in the presence of 0.1 mol/l NaCl. Even at pH 6.0 there was a small amount of BSA absorbed whereas the DE Cellulose (D11) did not bind BSA until pH 7.5. This tends to suggest that there are a few areas of the cellulose matrix in A_2 Protion which have a higher affinity for BSA than others. This would occur if there was an uneven distribution of charged groups on the matrix. Such, an uneven distribution could arise from the heterogeneous nature of the reaction mixture during the manufacturing process of the A_2 Protion with the CED reagent. It is a semi-dry sticky mixture during the cellulose derivatization stage. D11 on the other hand was prepared in the laboratory on a small scale where better mixing is possible and also via the new modified process which is a uniform slurry when the diethylamino (DE) groups are attached.

If A_2 Protion had a few regions on the particles where there was a high concentration of DEAE groups we would expect to find a very limited capacity for BSA in 0.1 mol/l NaCl closer to the isoelectric point as observed in Figure 3.4.

Two other commercial products were tested, namely Whatman DE 52 and A_2 Indion* (Batch 1020AE2) and these gave similar results as shown in Figure 3.6. Whatman DE-52 Cellulose has a swollen volume

* This is the new trade name for the Grant resins now being manufactured by Phoenix Chemicals Ltd., so it should be similar to A_2 Protion.

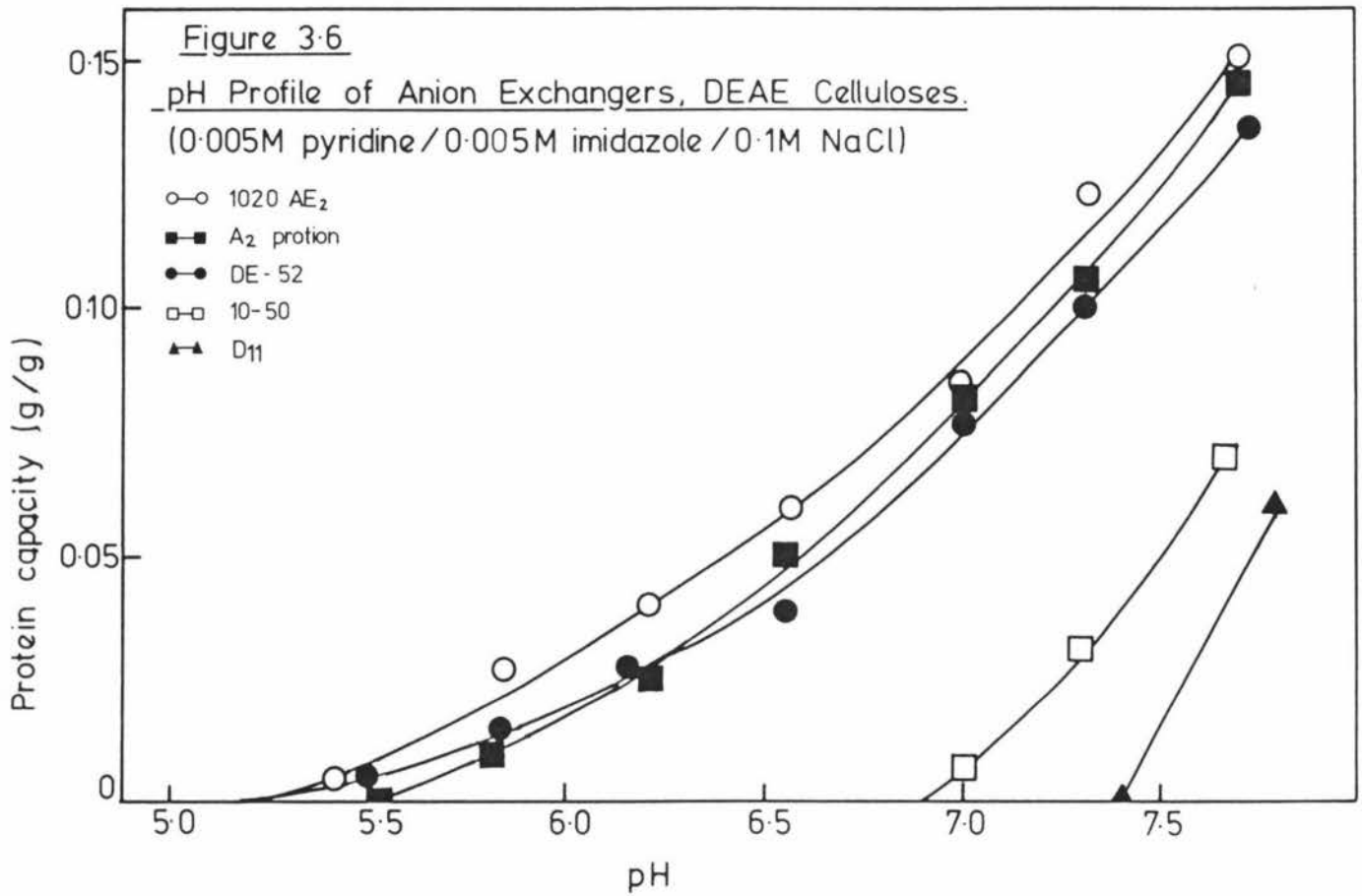


Figure 3·6
pH Profile of Anion Exchangers, DEAE Celluloses.
(0·005M pyridine / 0·005M imidazole / 0·1M NaCl)
1020 AE2
A2 protion
DE-52
10-50
D11
Protein capacity (g/g)
0·15
0·10
0·05
0
5·0
5·5
6·0
6·5
7·0
7·5
pH

and substitution level of DEAE groups very similar to those of A_2 Indion and A_2 Protion, ie 5-6 ml/g and 1.1-1.2 meq/g, so the charge densiry (average) would be much the same. DE-52 is also manufactured using CED and in the early days manufacturers had a lot of difficulty with uniformity and reproducibility of substitution(24).

The other two curves in Figure 3.6 are for the new cellulosic ion exchangers prepared from the modified matrix, Dll via the activated matrix and slurry process and 10-50 DEAE via the use of the conventional CED reagent and the more difficult mixing operation. Both these products had similar swollen volumes, so it would appear that the slurry process, produces a better product as far as evenness of substitution goes. However those latter two products were both laboratory products and for this reason make for unfair comparison with the commercial products.

It would appear that this pH profile study in 0.1 mol/l NaCl could be used as a test for the evenness of substitution as the new products are put into full scale production. As soon as the first commercial batch is made it would be of value to compare it with Dll and 10-50 DEAE.

3.5 COLUMN CHROMATOGRAPHY OF BSA

If the residual BSA capacity of the commercial products at pH 6-7 shown in Figure 3.6 is indeed the result of the uneven distribution of groups in the cellulose, this should cause some tailing of protein peaks when eluted from a column either with a salt gradient or pH gradient, and consequently cause some loss of resolution in a protein mixture. The new modified DE Cellulose (Dll) should then give sharper and more symmetrical peaks in comparison with A_2 Protion and the other commercial products.

3.5.1 Choice of Column Conditions

In order to compare D11 and A_2 Protion we wanted conditions where they had similar protein capacities and from which the protein would be eluted at a similar salt concentration. In 0.05 mol/l NaCl, A_2 Protion has a similar BSA capacity (mg/ml) at at pH 6.1 to that of D11 at pH 7. For this reason A_2 Protion and Whatman DE-52 Cellulose were loaded and eluted at pH 6.1 while pH 7.0 was used with D11 and 10-50 DEAE Celluloses. The same salt gradient of 0.0-0.3 mol/l NaCl was the used in all cases to elute the BSA peak from the columns. The buffer used was 0.01 mol/l imidazole since both pH 6.1 and pH 7.0 fall within it's buffering range.

Figure 3.7 shows the protein peak profiles resulting from these tests and it can be seen that D11 gave the most symmetrical peak with the least tailing of BSA from the solumn. 10-50 DEAE also gave a reasonably sharp peak but with more tailing. However DE-52 and A_2 commercial products both gave broad peaks with considerable tailing. This is in keeping with the findings of the pH profile studies in 0.1 mol/l NaCl and predictions made earlier.

To show that the broad peak from A_2 Protion and DE-52 were not caused by the lower pH of 6.1, DE-52 was rerun at pH 7 as for D11 and still found to give rise to a broad unsymmetrical peak (see Figure 3.8)

It would appear that these new DEAE modified cellulosic ion exchangers have the potential for superior resolution of protein mixtures, but this aspect will require further verification with real protein mixtures once the modified ion exchangers go into commercial production.

Figure 3·7

Column Chromatography of BSA on DEAE Celluloses.
(100mg BSA loaded in 0·01M imidazole, eluted by 0–0·3M NaCl gradient)

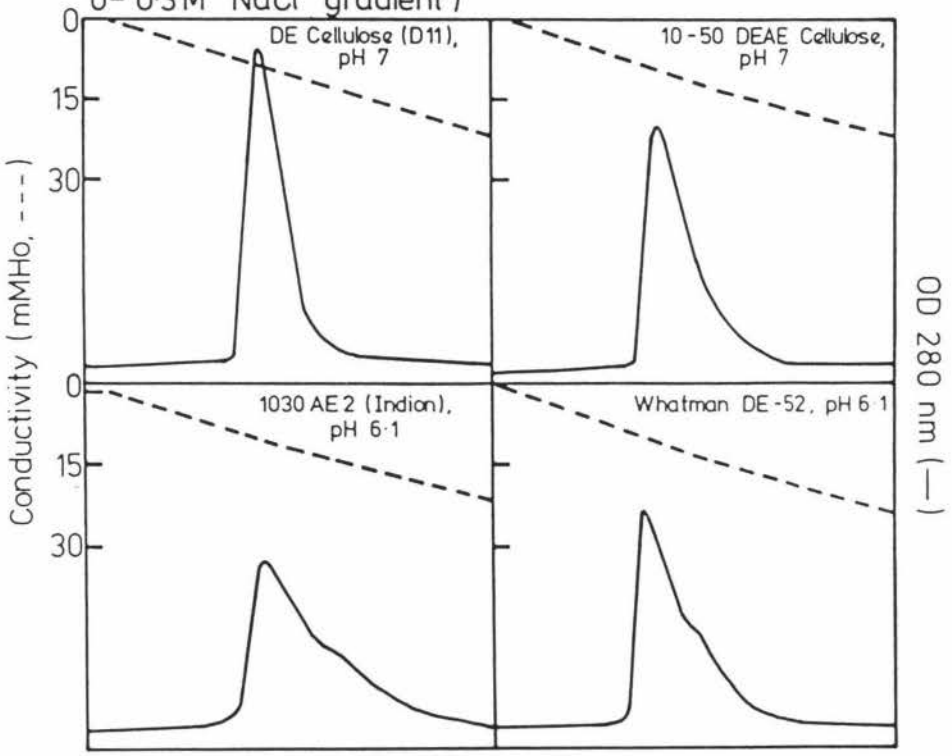
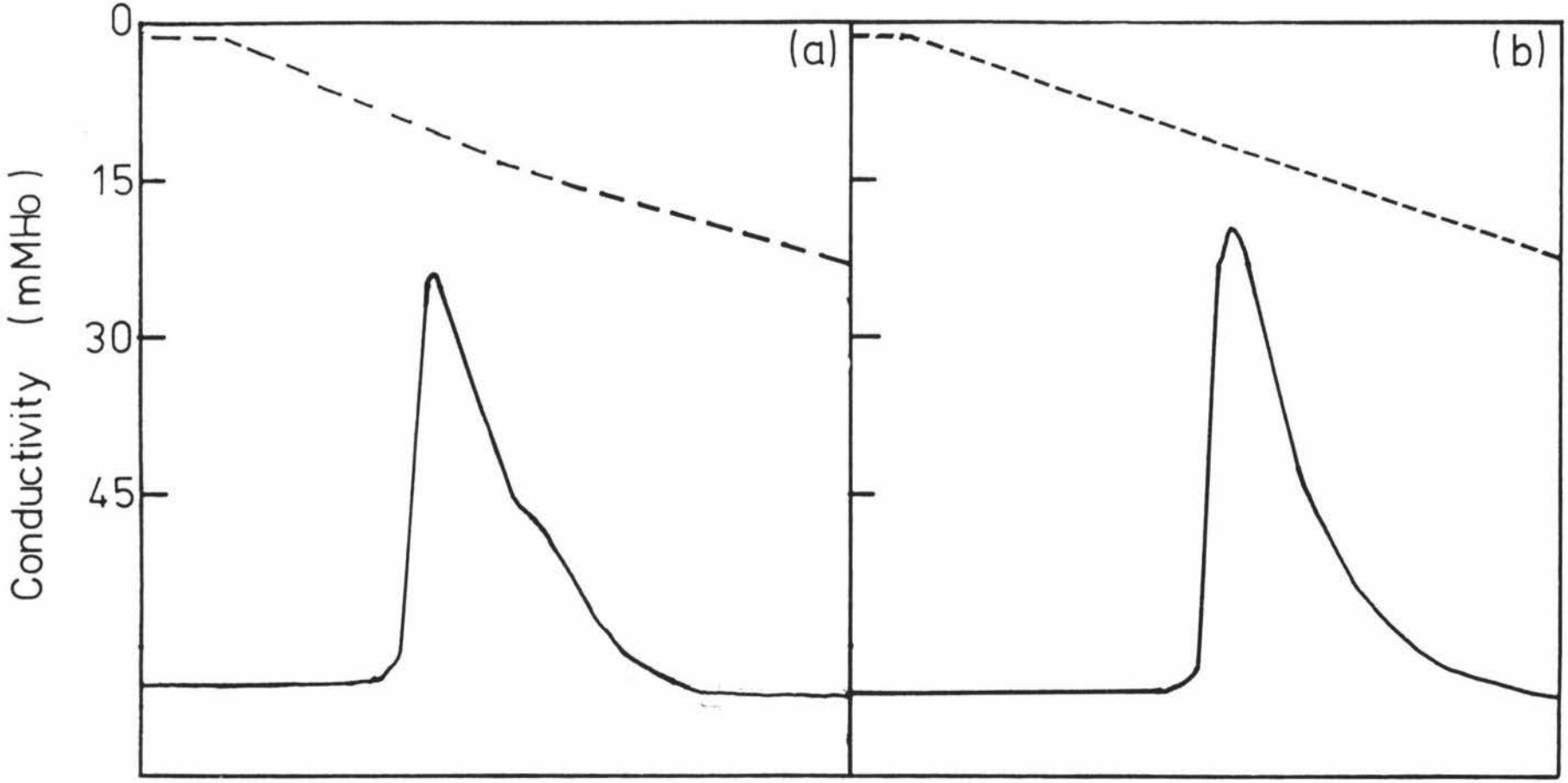


Figure 3·8

Column Chromatography of BSA on Whatman DE-52. (100mg BSA loaded in 0·01 M imidazole (a) pH 6·1, (b) pH 7·0. Elution conditions 0 - 0·3M NaCl in 0·01M imidazole (a) pH 6·1, (b) pH 7·0)

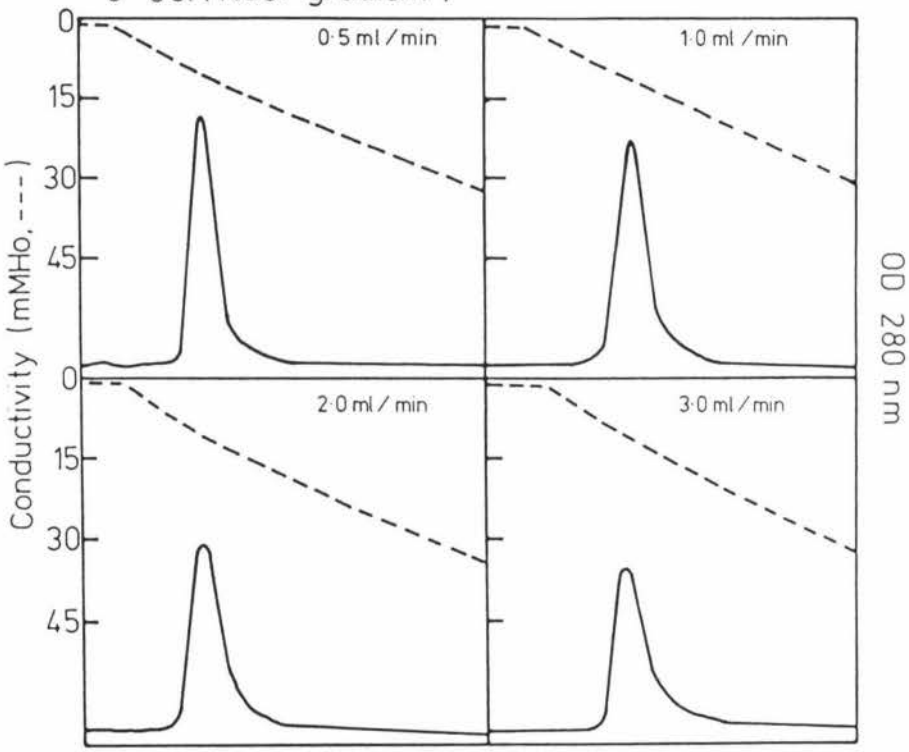


Besides this improved resolution, the other advantage of the new modified ion exchangers is the higher flow rates which are possible than for most other ion exchangers. However it is not always advisable to use the maximum flow rates, since absorption and desorption rates are finite and not instananeous. Dll was eluted with several different flow rates and Figure 3.9 shows a change in peak shape with increasing flow, especially above 1 ml/minute. However even at 3 ml/minute the BSA peak is still sharper than that obtained on DE-52 and A_2 Protion at 0.5 ml/minute.

The full advantage of the higher flow rates of Dll can not be used if maximum resolution is required but at least the preparation time for equilibration of the column can be speeded up and in some situations adequate resolution obtained with very high flow rates.

Figure 3·9

Column Chromatography of BSA on DE Cellulose (D11).
(50 mg BSA in 5ml 0·01M imidazole pH 7·0,
0–0·5M NaCl gradient)



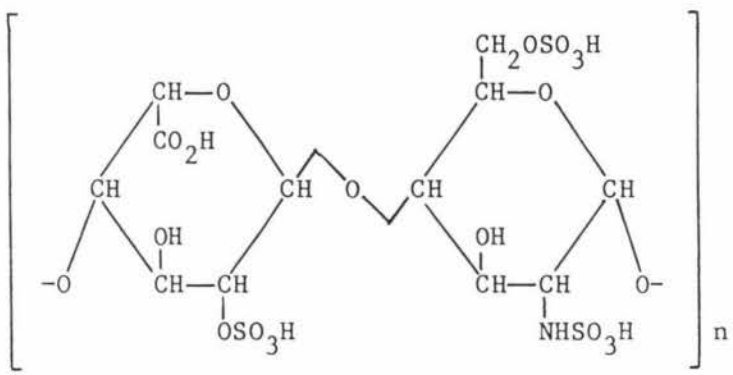
SECTION 4

ION EXCHANGE OF HEPARIN

4.1 INTRODUCTION

Heparin belongs to a class of compounds known as acid mucopolysaccharides. It is a polydisperse group of closely related molecules with a molecular weight range of 6,000-20,000(25). It is composed mainly of partially sulphated disaccharide units of iduronic acid or glucuronic acid and glucosamine. Each disaccharide unit has 1 to 3 sulphate groups and this degree of sulphation, along with chain length, determine most of the heparin's properties.

i.e. Partial structure of Heparin - showing major disaccharide unit.



Heparin is polyanionic in nature and can therefore be bound to anion exchangers. This has been the basis of several patented methods(26-29) for the extraction and purification of heparin. Strongly basic anion exchangers have tended be used, since autolysis in alkaline conditions is a common method for releasing heparin from animal tissues and weakly basic anion exchangers are uncharged in such strongly alkaline conditions.

The quaternary ammonium ion exchanger used must be able to bind heparin with good capacity under conditions of high ionic strength and pH, typically 0.5 mol/l NaCl or 2% $Na_2S_2O_5$ at pH 12. This is frequently possible because of the strong binding of heparin resulting from its polyanionic nature. The quaternary exchanger also needs to be stable at temperatures of 50-60^{o}C in the above conditions.

It was the aim in this section to develop an ion exchanger which would not only satisfy, the above conditions but also have a high capacity for heparin. If this was successfully achieved it could then be made on a large scale for use by New Zealand Pharmacuticals Limited at Linton in their heparin extraction process. We already had the ability to make a quaternary ammonium (QA) anion exchanger from regenerated cellulose as outlined in Section 1 so initially we tested it s ability to bind heparin in the conditions outlined above.

4.2 RESULTS AND DISCUSSION

4.2.1 QA Cellulose: Its Usefulness in the Heparin Process

A column of QA Cellulose (5 ml) was equilibrated with 0.01 mol/l tris pH 8.0 and loaded with 10 mg of heparin dissolved in 10 ml of this buffer. The column was then eluted with a salt gradient from 0-2.0 mol/l NaCl in 0.01 mol/l tris pH 8.0. In Figure 4.1 it can be seen that the heparin begins to elute from the column at a conductivity of 25 mMHO, which corresponds to a salt concentration of approximately 0.4 mol/l NaCl. The relationship between conductivity and salt concentration is shown in Figure 4.3. The column of QA Cellulose was then equilibrated with a solution of 2% $Na_2S_2O_5$ adjusted to pH 12. Heparin (10 mg) dissolved in 10 ml of this same solution was loaded onto the column. It was washed with several column volumes of the starting solution, before being eluted by a salt gradient of 0-1.5 mol/l NaCl in 2% $Na_2S_2O_5$, pH 12. In Figure 4.2 it can be seen that no heparin bound to the QA Cellulose under these conditions.

From these results it is clear that this QA Cellulose is unsuitable for the heparin process outlined in the introduction.

To bind heparin under conditions of high ionic strength, a matrix with a higher density of charged groups, i.e. meq N/ml, is required. The evidence for this being:

(a) the synthetic polystyrene resins commonly used to bind heparin at high salt strength have substitution levels of 3-4 meq/g (i.e. 1 meq/ml) whereas the QA Cellulose used had only 1.14 meq/g (i.e. 0.1 meq/ml)

(b) proteins bind more tightly to ion exchangers as the substitution level of charged groups is increased.

Figure 4·1

Elution Profile of Heparin (10mg) from QA Cellulose (5ml).

(load conditions - 0·01M Tris, pH 8·0

elution conditions - 0–2M NaCl gradient in 0·01M Tris, pH 8·0)

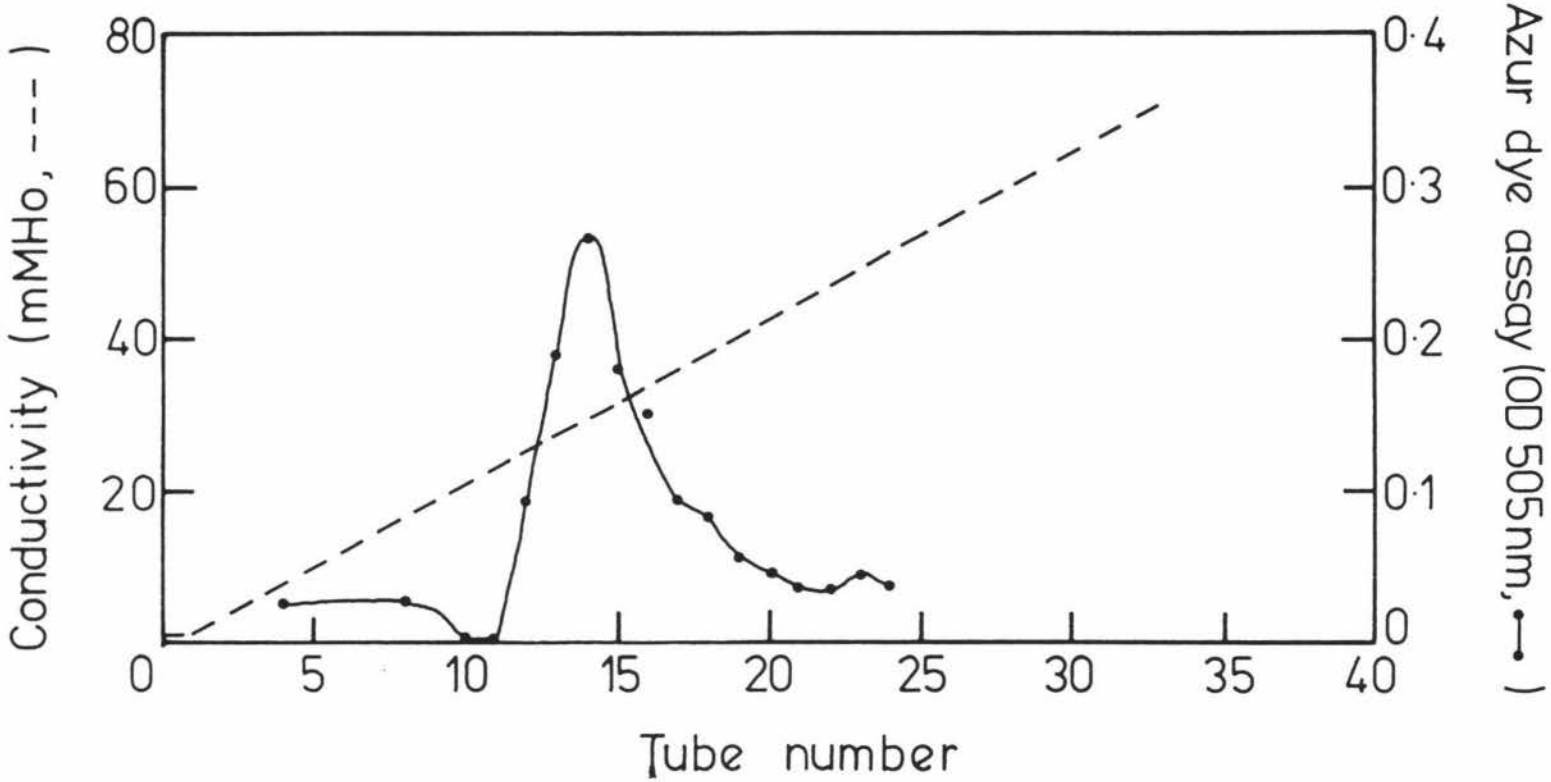


Figure 4·2

Elution Profile of Heparin (10mg) from QA Cellulose (5ml)

(load conditions- 2% $Na_2S_2O_5$, pH 12
elution conditions - 0–1·5M NaCl gradient in 2% $Na_2S_2O_5$, pH 12)

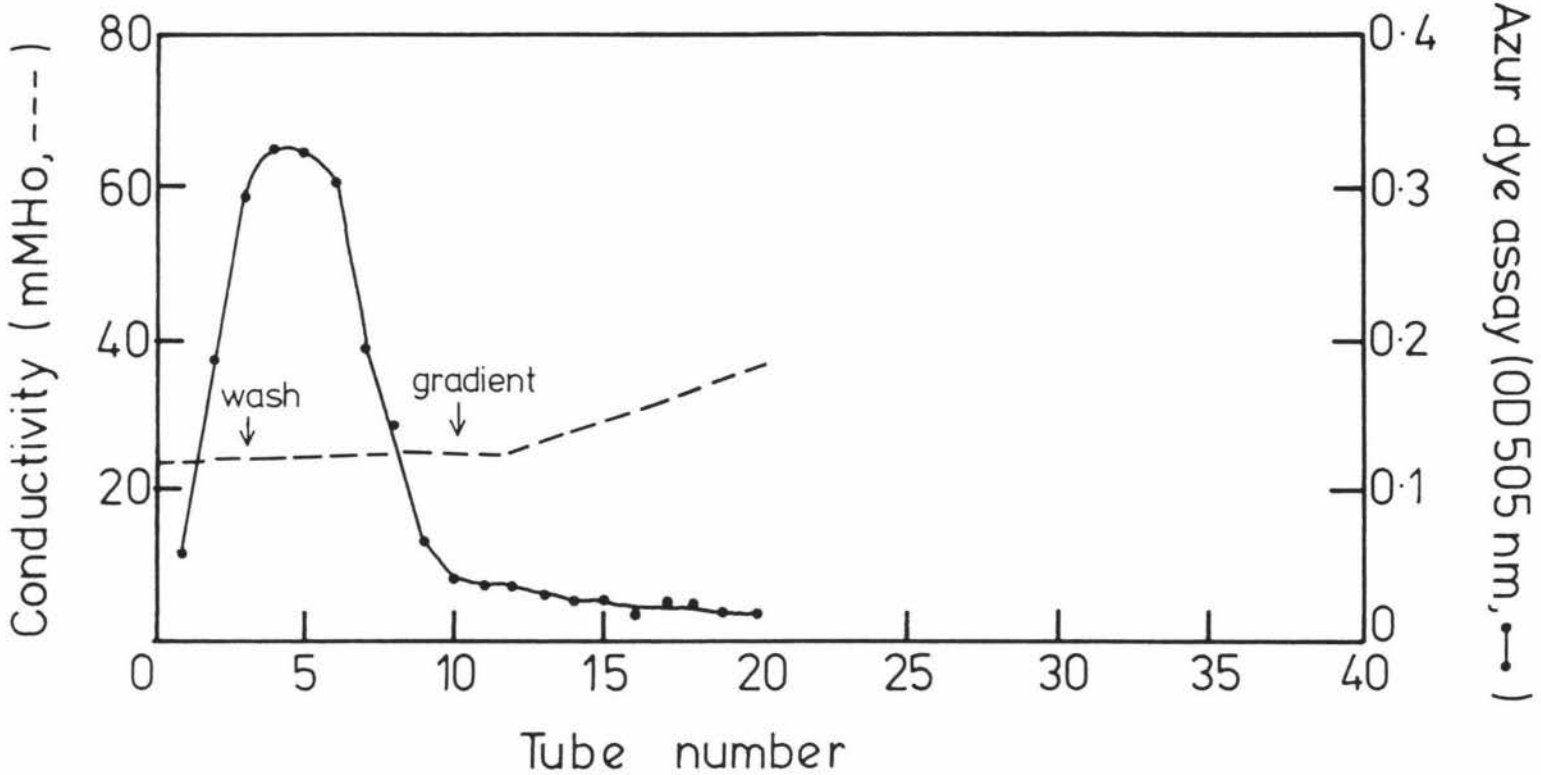
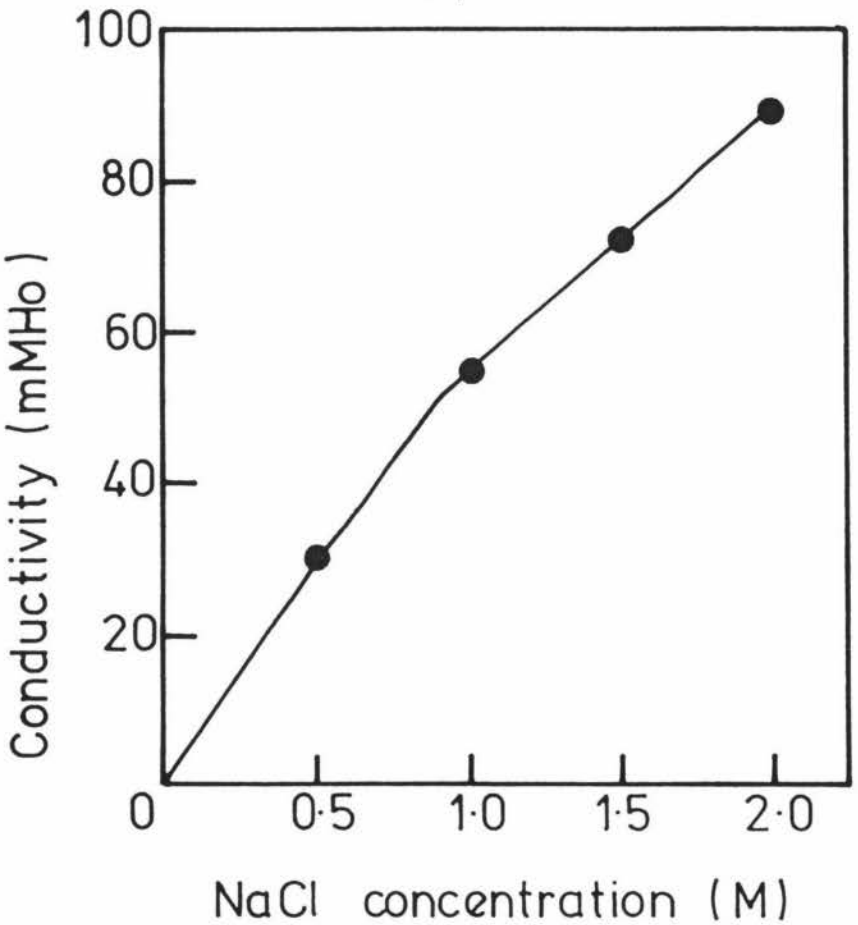


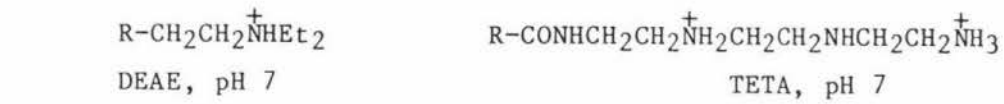
Figure 4·3

Conductivity v Concentration of NaCl.

(0·01M Tris, pH 8·0)



(c) When the DEAE group on cellulose was replaced with a TETA group at the same substitution level, heparin was found to bind effectively in salt concentrations greater than 1 mol/l NaCl(30) which was not possible with the DEAE Cellulose.



DEAE, pH 7 TETA, pH 7

The multiple charges on the TETA group gave stronger electrostatic interactions with the closely spaced sulphate groups present in heparin.

4.2.2 Possible Methods of Increasing the Density of Charged Groups

There were three methods by which the density of charged groups could be increased. These were;

(a) reduction of the swollen volume (ml/g) of the ion exchanger,
(b) a reprocessing method to increase the substitution level,
(c) the incorporation of closely spaced dimeric or trimeric quaternary ammonium groups onto the cellulose matrix, rather than a monomeric quaternary group.

(a) Reduction of the Swollen Volume

The cellulosic ion exchangers discussed in this thesis have a major proportion of their properties determined by the ratio of reagents used in the first stage of production, i.e. the preparation of the modified matrix (see Figure 1.1). This first stage involves the similtaneous hydroxypropylation and crosslinking of the regenerated cellulose powder. An increase in the amount of epichlorohydrin used as crosslinking agent results in a reduction in the swollen volume of the final product, e.g. the QA Cellulose. It has been found that reducing the swollen volume of these cellulosic ion exchangers below approximately 11 ml/g for the SP derivative and 13-14 ml/g for the QA derivate results in a marked drop in their protein binding capacities. Table 4.1 shows this rapid drop in protein capacity

when the swollen volume of the QA Cellulose is reduced from 14 ml/g to 8.2 ml/g.

To make a significant increase in the density of charged groups on the cellulose (meq N/ml) to a level where heparin could bind under the conditions outlined earlier, then the swollen volume would have to be reduced significantly below 10 ml/g and the protein capacity would be very low as shown in the Table 4.1. The same could be expected for their heparin capacities.

Table 4.1

QA Cellulose: Protein Capacity as a Function of Swollen Volume

Swollen Volume (ml/g)		Substitution Level		Protein Capacity	
Modified Matrix*	QA Cellulose	meq N/g	meq N/ml	g/g	mg/ml
12.1	20.8	1.0	48	2.45	118
11.7	18.8	1.11	59	2.29	122
10.5	16.4	1.12	68	2.15	131
8.4	13.9	1.03	74	1.60	115
7.3	12.7	1.03	81	1.29	102
6.7	11.4	1.0	88	0.99	87
5.7	8.2	0.92	112	0.48	60

* This modified matrix was manufactured on the pilot plant and separated into fractions with different swollen volumes by elutriation.

(b) Reprocessing

Reprocessing the QA Cellulose ion exchanger to incorporate further groups involves repeating Stages II and III (See Figure 1.1) of the manufacturing process.

Reprocessing has been shown to increase the density of charged groups, in the case of the SP ion exchanger, from 1.0 to 1.6 meq/g but this decreased the capacity for β-lactoglobulin from 1.5 to 0.7 g/g. This drop in capacity appeared to be far greater than could be accounted for by the additional crosslinking observed as a side reaction since the swollen volume only dropped from 12.5 to 10.8 ml/g(16). Secondly the reprocessed ion exchangers appeared to be less resilient to mechanical agitation than the ion exchanger from which they were made(31). For these reasons reprocessing of QA Cellulose was not attempted.

(c) Incorporation of Dimeric and Trimeric Quaternary Ammonium Groups

Due to the versitility of the epoxide matrix (activated matrix), it is very easy to incorporate into the cellulose matrix organic ligands with two or more closely spaced quaternary ammonium functional groups. The simplest of these is a diquaternary ammonium group with a two carbon spacer between the nitrogens. Such a derivative was prepared according to the following scheme.

Scheme 1

$$Me_2NCH_2CH_2NMe_2 \xrightarrow[\text{benzene}\ 24/hr]{MeBr} Me_2NCH_2CH_2\overset{+}{N}Me_3\ Br^-$$

A

$$\text{Cellulose-OCH}_2\overset{O}{\hat{C}}\text{HCH}_2 + A \xrightarrow{1\text{-}2\ \text{weeks}} \text{Cellulose-OCH}_2\text{CHOHCH}_2\overset{+}{N}Me_2CH_2CH_2\overset{+}{N}Me_3$$

Epoxide Matrix — DQAE Cellulose

2-Dimethylaminoethyl trimethyl ammonium bromide (A) was readily prepared from N,N,N',N'-tetramethylethylenediamine and methyl bromide in high yield(32). Coupling of this compound (A) via its tertiary amino group was rather slow because the base strength and hence nucleophilicity of the tertiary amine has been dramatically reduced by having the quaternary ammonium group close by (see Table 4.2). Consequently the coupling reaction was left for two weeks at room temperature. Inspite of the long time taken for this reaction, the coupling was still reasonably efficient (approximately 70%, see Table 4.3). In comparison dimethylethanolamine (B) required only a

few hours to give the Quaternary Ammonium Cellulose, QA-Cellulose with 100% efficiency.

Table 4.2

Base Strengths of Organic Ligands

Compound	pKa Literature(33)	pKa Experimental
$Me_2NCH_2CH_2OH$ (B)	9.3	9.4
$Me_2NCH_2CH_2NMe_2$	9.1 & 5.7	-
$Me_2NCH_2CH_2\overset{+}{N}Me_3$ (A)	-	6.1
$Me_2NCH_2CHOHCH_2\overset{+}{N}Me_3$ (C)	-	8.0
$Me_2NCH_2CHOHCH_2NMe_2$ (D)	-	9.2* & 8.0*

* Estimated from values for B and C

The epoxide groups on the matrix have been found to hydrolyse slowly in water in the pH 4-10 range with approximately 30% loss of reactive groups in two weeks at 25°C(17). (Outside this pH range the hydrolysis is more rapid because of acid and base catalysis).

i.e. Cellulose-$OCH_2\underset{\diagdown\,O\,\diagup}{CH}CH_2$ + H_2O $\xrightarrow{H^+ \text{ or } OH^-}$ Cellulose-$OCH_2CHOHCH_2OH$

With a slow coupling reaction a significant number of epoxide groups were hydrolysed resulting in less than 100% efficiency of conversion and a product which has a slightly lower substitution level. It was found that the derivative made via Scheme 1 had a substitution of 1.42 meqN/g (ie 0.7mmol of dimer/g). The epoxide content of the activated matrix was normally 1.35 mmol/g which should give 1.16 mmol/g for QA Cellulose and 1.06 mmol/g for DQAE Cellulose in

Table 4.3

Summary of Properties of Cellulose Derivatives Prepared via Schemes 1 - 8

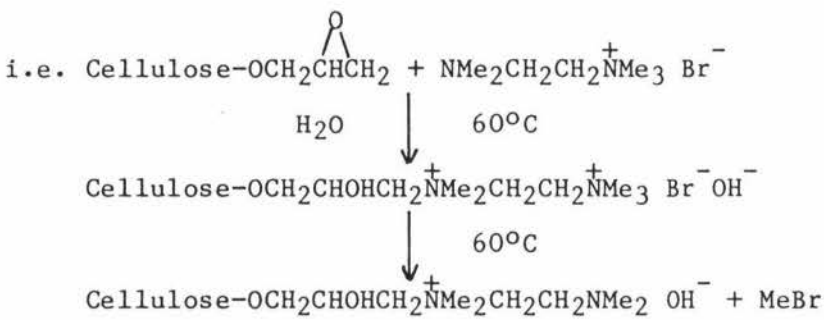
Cellulose Derivative	Scheme	Batch No	Substitution Level meq N/g	Substitution Level mmol/g Actual	Substitution Level mmol/g Calc+	Swollen Volume ml/g
QA	-	Q4	1.14	1.14	1.16	12.7
DQAE	1	$DQAE_1$/E11	1.42	0.71	1.06	-
	1	$DQAE_2$/E11	1.52	0.76		-
DQAP	2	$DQAP_1$/E11	1.69*	0.85		
		$DQAP_3$/E24	1.80	0.90	1.03	24.6
TMDAP	3	TMDAP/E25	2.11	1.05		-
	3	TMDAP/102C	1.82			15.8
DQAP	3	DQAP/E25	1.98	0.99	1.03	
DMA	4	DMA_1/E25	1.20	1.20	1.22	
		DMA/102B	1.19	1.19	1.22	18.3
DQAP'	4	DQAP/E25	1.88	0.94	1.00	-
DE	5	DE/E25	1.07	1.07	1.17	
DQ/DE	5	DQ/E25	1.39	0.70	0.97	
PA	6	PA/E23	1.05	1.05	1.26	12.6
BDQ	6	BDQ/E23	1.4	0.70	0.77**	
DAP	7	DAP/E25	2.18	1.09	1.13	
DQ/DAP	7	DQ4/E25	1.92	0.96	1.04	
TA	8	TA/E19	-	-		
TQA	8	TQA2/E19	2.25	0.75	0.92	9.18

* The epoxide matrix E11 was already one month old when DQAP, was made and had probably lost 10% of its active groups.

** Based on PA of 1.05 meq/g.

\+ All calculated values assume an epoxide matrix with 1.35 mmol/g.

the chloride forms in which they were dried*. Consequently the conversion to the DQAE derivative was less than 70% efficient. Attempts to increase the rate of coupling by raising the temperature so that a higher substitution might be obtained resulted in a product which was not 100% quaternary added in nature (see $DQAE_2$ in Table 4.4). This suggested that the dimeric derivative was notstable at elevated temperatures, especially in the presence of bromide ions which are good nucleophiles.



The presence of the hydroxide counter ion may also have produced tertiary amines by either substitution or elimination reactions.

The slow coupling reaction with only 70% efficiency substitution and the added extra weight of the dimeric ligand compared with the monomer meant that this diquaternary ammonium ion exchanger had a charge group density which was not much higher than that of the QA Cellulose i.e. only 1.52 instead of 1.14 meq/g (see Table 4.3).

4.2.3 Stability of QA and DQAE Celluloses

Since DQAE Cellulose appeared to be unstable at elevated temperatures, stability tests were carry out on this derivative and the QA derivative. Conditions harsher than those encountered in the heparin extraction process were chosen to accelerate any degradation that may occur. Samples of the ion exchangers were treated with;

* Substitution level for the cellulose derivative.

$$= \frac{1.35}{1 + 1.35\ y} \quad \text{mmol/g}$$

where y is the molar mass in the chloride form of the substituent

(a) 0.5 mol/l NaOH

(b) 2% $Na_2S_2O_5$, pH 12.

In both cases they were heated at 80^{o} for one week. The analysis of the ion exchangers was undertaken by running titration curves under carbonate free conditions, before and after treatment. The results of these tests are given in Table 4.4.

Table 4.4

Stability Tests - QA, DQAE and DQAP Celluloses
(treated for 1 week at 80^{o})

Matrix	Treatment	Total meq/g	Quaternary N(%)	Tertiary N(%)
QA	UNTREATED	1.2	100	-
QA	2% $Na_2S_2O_5$, pH 12	1.14	100	-
QA	0.5 mol/l NaOH	1.6	83	17
DQAE	UNTREATED	1.42	100	-
DQAE	2%$Na_2S_2O_5$, pH 12	0.56	43	57
DQAE	0.5 mol/l NaOH	1.54	45	55
$DQAE_2$*	UNTREATED	1.52	91	9
DQAP	UNTREATED	1.69	100	-
DQAP	2%NaS_2O_5, pH 12	1.33	71	29
DQAP	0.5 mol/l NaOH	1.74	77	23

* $DQAE_2$ was heated during the coupling reaction.

The QA Cellulose was found to be exceptionally stable, with no loss of groups detected as a result of the sulphite treatment. With the caustic treatment the formation of some weakly basic groups (17%) was detected but due to hydrolysis of the cellulose backbone changing the dry matter composition, we could not ascertain whether there was any loss of nitrogen from the matrix.

The DQAE Cellulose on the other hand was found to have undergone major changes with both the sulphite and hydroxide tests. With both hydroxide and sulphite a similar distribution of strongly basic to weakly basic groups was observed, i.e. 45% strongly; 55% weakly basic. In addition a massive loss (approximately 60%) of groups from the matrix was detected after the sulphite treatment.

With the hydroxide test, hydrolysis of the cellulose backbone prevented us from determining the group loss by this treatment. These results for the DQAE Cellulose, indicate that the sulphite treatment has caused more damage than the hydroxide treatment. Sulphite is a good nucleophile but is only weakly basic, while hydroxide is a poorer nucleophile but is strongly basic. This suggests that the predominant degradation reaction is most likely a nucleophilic substitution.

i.e. $R_3\overset{+}{N}R'\ OH^- + SO_3^{2-} \longrightarrow R_3N + R'SO_3^-$

Quaternary (strongly basic) Tertiary (weakly basic)

The major difference between the groups in QA and DQAE Cellulose would appear to be in the nature of the tertiary amine leaving group if either a substitution or elimination reaction takes place.

e.g. from QA the leaving group would be of the type.

$Cellulose\text{-}OCH_2CHOHCH_2NMe_2$ pKa = 9.3

whereas from DQAE it could be

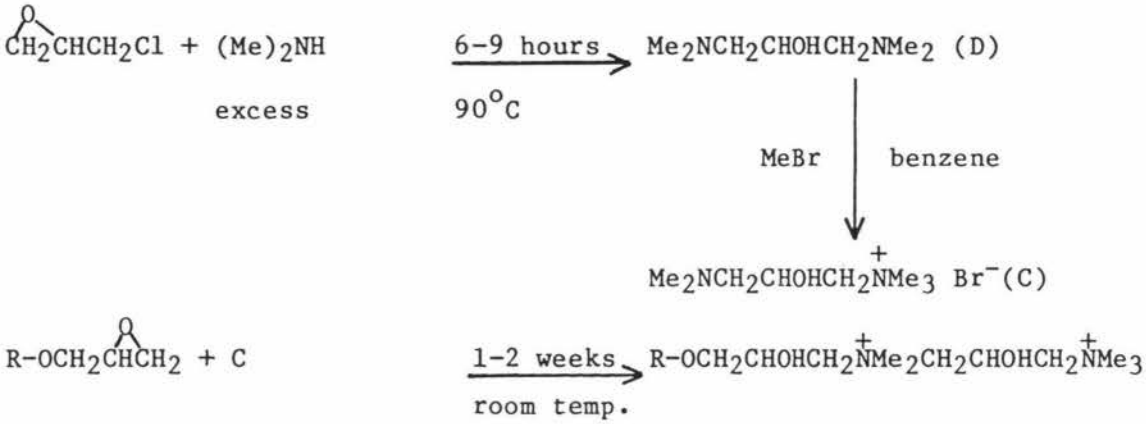
$Cellulose\text{-}\overset{+}{N}Me_2CH_2CH_2NMe_2$ pKa = 6.1

The lower pKa of the latter group (weaker base) would make it a better leaving group. If this is true, to make a dimeric derivative which is more stable, the base strength of the tertiary amine of the ligand must be increased.

This was done by increasing the distance between the tertiary amine and the quaternary ammonium group in the ligand. The ligand had a three carbon spacer between the tertiary and quaternary nitrogens and was prepared as outlined in Scheme 2.

Scheme 2

R = Cellulose-



The ligand C is more basic than A because of the extra carbon between the nitrogens. It's pKa was measured and found to be 8.0 whereas for A it was 6.1 (see Table 4.2). Consequently it would be expected to be more nucleophilic and couple faster with the epoxide matrix resulting in higher substitution than in the case of A. In addition to this the tertiary amine left on dealkylation or from elimination would be more basic and a poorer leaving group so the DQAP Cellulose should be more stable. These predictions were borne out by the preparation of DQAP, (Table 4.3). The results of stability tests are reported in Table 4.4. The DQAP derivative has both a higher substitution of quaternary nitrogens and is considerably more stable to both sulphite and hydroxide. Although this matrix is not as stable as QA Cellulose it is probably stable enough for use in the heparin process, considering the harshness of the test conditions compared to those that would be encountered in the heparin process. The higher substitution levels should also allow the heparin to bind in 0.5 mol/l NaCl.

4.2.4 DQAP Cellulose - Heparin Binding Ability

Columns similar to those run with QA cellulose were run with DQAP Cellulose. The results of these columns are shown in Figures 4.4 and 4.5, and it can be seen that heparin can be bound in the conditions outlined earlier. The heparin did not begin to elute with a NaCl gradient until the conductivity had reached 40 mMho corresponding to 0.7 mol/l NaCl. Likewise it bound when loaded in 2% $Na_2S_2O_5$, pH 12.

A sample of this ion exchanger was supplied to New Zealand Pharmaceticals Ltd for trial, and they reported that it had a poor heparin capacity. We found that this product had a low swollen volume and a poor protein capacity. An increase in swollen volune was expected to increase the protein capacity (and hopefully the heparin capacity) without decreasing the charged group density significantly.

A method was therefore sought by which we could make dimeric quaternary celluloses with substitution levels of about 1.8 meqN/g or higher from chemicals that could be obtained cheaply and quickly either from within New Zealand or via air freight from the USA.

Figure 4.6 outlines the various synthetic routes devised and tested in the laboratory. These include trimeric nitrogen derivatives in an attempt to increase the substitution levels even further.

Figure 4·4

Elution Profile of Heparin (10mg) from DQAP Cellulose (5ml)

(load conditions - 0·01M Tris, pH 8·0
elution conditions - 0-2M NaCl gradient in 0·01M Tris, pH 8·0)

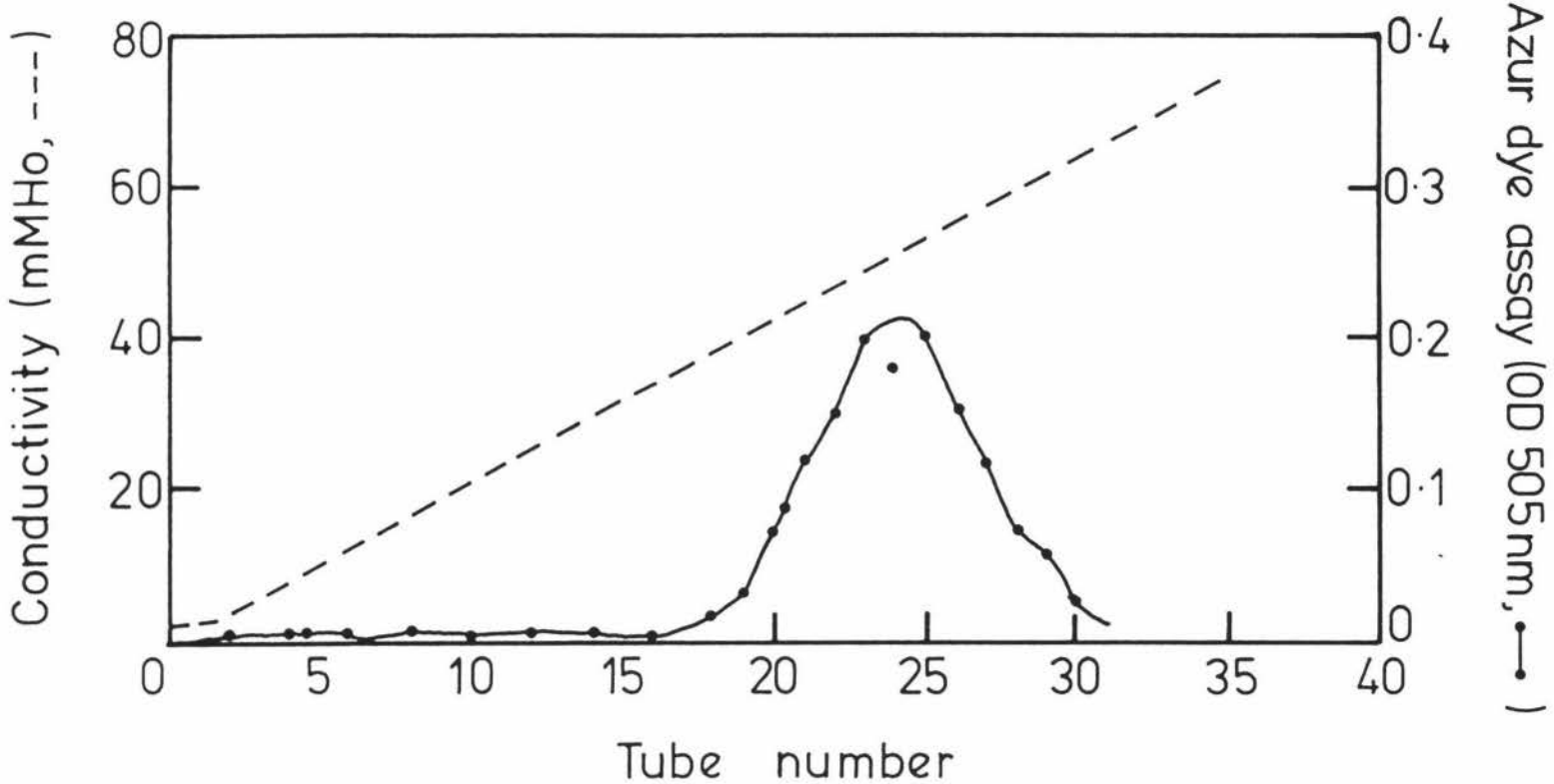


Figure 4·5

Elution Profile of Heparin (10mg) from DQAP Cellulose (5ml).

(load conditions - 2% $Na_2S_2O_5$, pH 12

elution conditions - 0 - 1·5M NaCl gradient in 2% $Na_2S_2O_5$, pH 12)

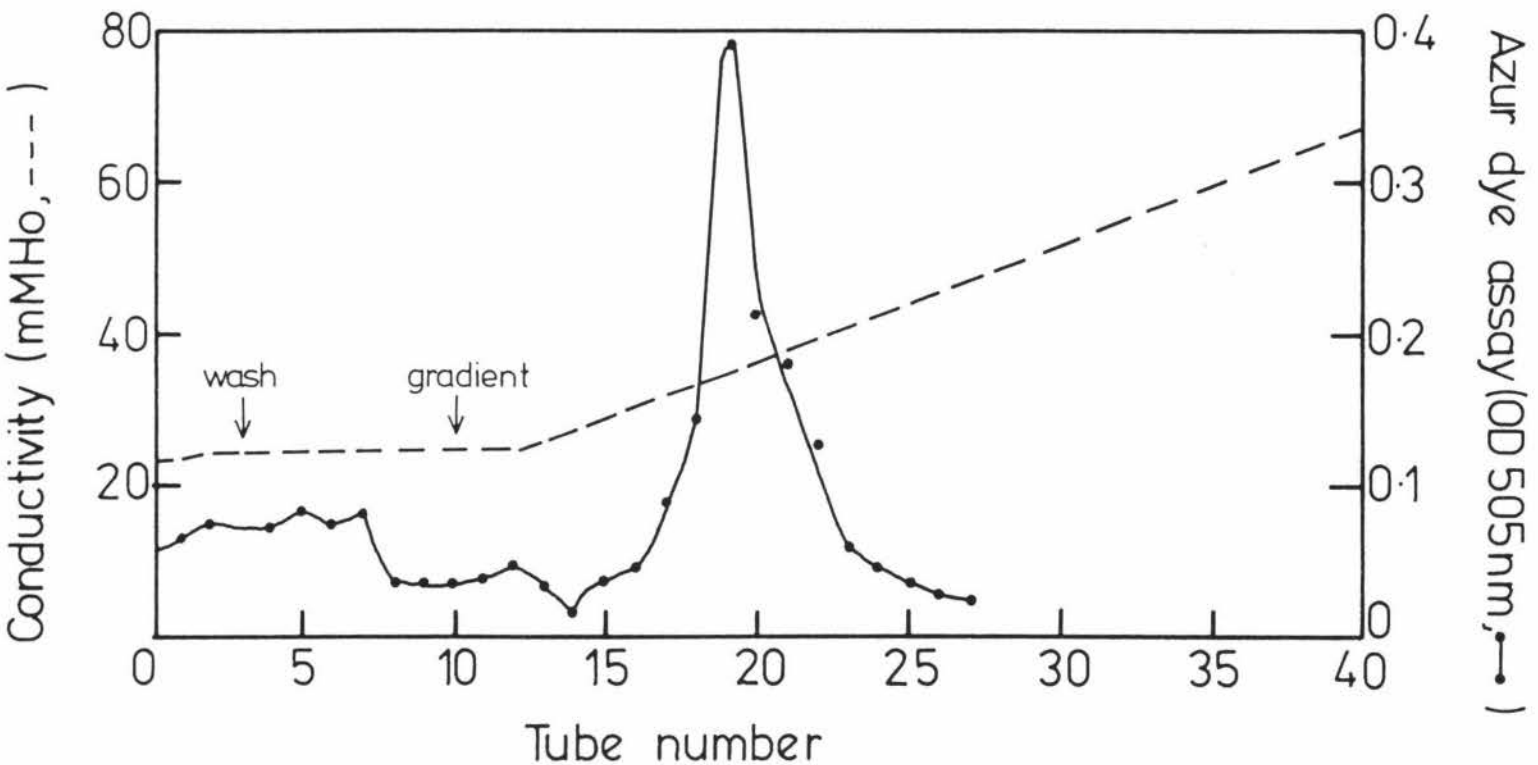
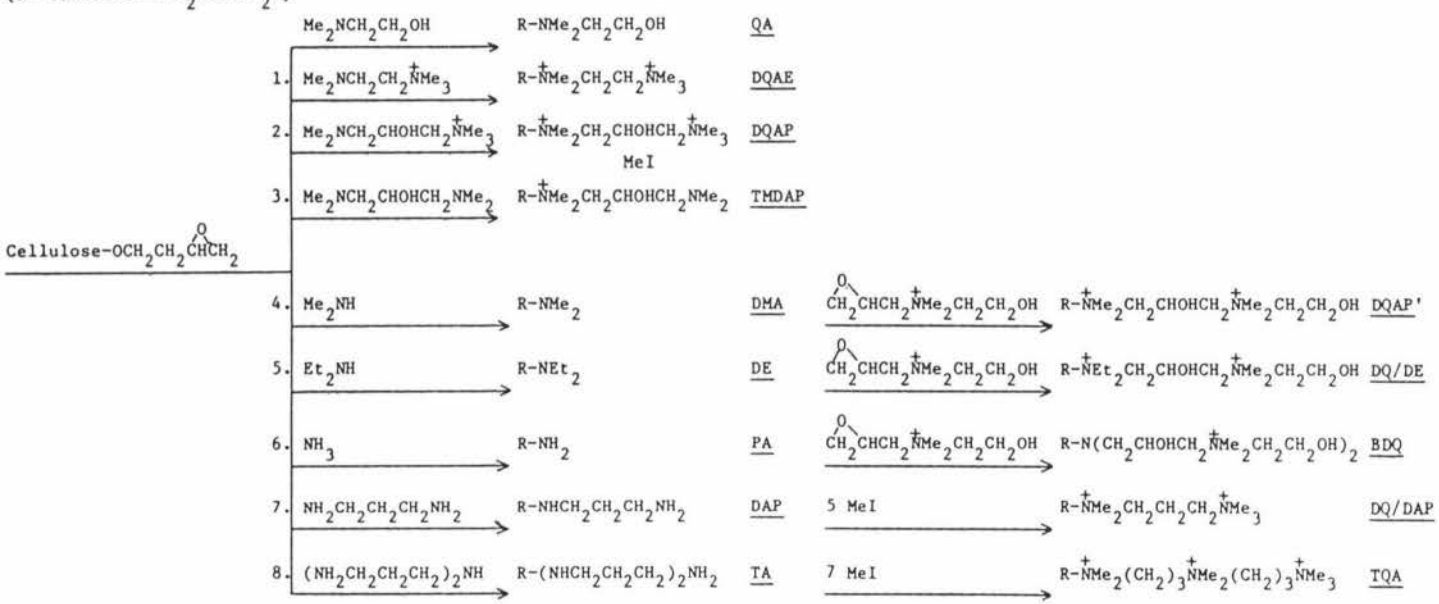


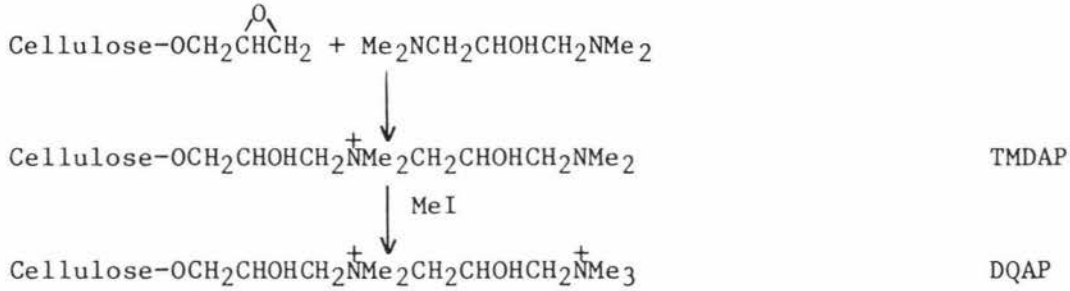
Figure 4.6

Summary of Schemes 1 to 8

(R= Cellulose-$OCH_2CHOHCH_2$-)



Scheme 3



This scheme is a variation on Scheme 2, in that the methylation step used in this scheme comes after the coupling of the ligand on to the matrix rather than prior to coupling as in Scheme 2. By coupling the ligand, i.e. 1,3-bisdimethylamino-2-propanol, it was hoped that a higher substitution would be achieved. Having a more basic tertiary amine (see Table 4.2) than that on the ligand (C) used in Scheme 2, it was anticipated that it would couple much faster and as a result there would be less hydrolysis of epoxide groups giving rise to a higher substituted product. The coupling reaction was carried out overnight instead of one week and typical products were found to have substitutions of 1.9-2.1 meqN/g.

Crosslinking is unlikely to occur when coupling this ligand, since when the first tertiary amine reacts, it forms a quaternary ammonium group, which dramatically reduces the base strength of the second tertiary amine. This drop in base strength reduces it's nucleophilicity and therefore it's reactivity. In addition to this a five fold excess of ligand was usually employed. Evidence for the lack of crosslinking can be seen from the titration curve of TMDAP (see Figure 4.7) which has 1:1 distribution of quaternary to tertiary groups.

The methylation step was carried out using an excess of methyl iodide(34) and the reaction went to completion. No tertiary amine groups were detected in the titration curve (see Figure 4.8).

The DQAP Cellulose prepared by this route had a slightly improved substitution but had the disadvantage of involving a costly solvent exchange procedure prior to methylation with methyl iodide or

Figure 4·7
Titration Curve of TMDAP Cellulose. (3g wet matrix in hydroxide form titrated in 10cm^3 1M NaCl under dry N_2 with 1M HCl)

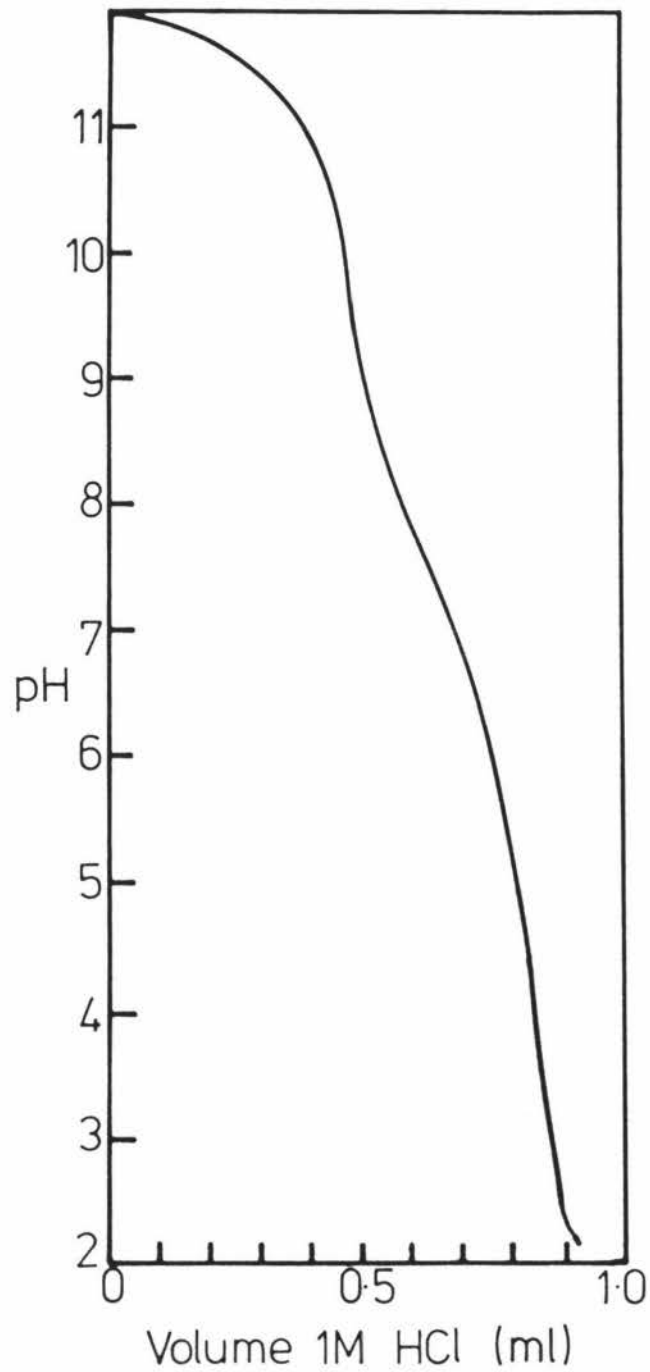
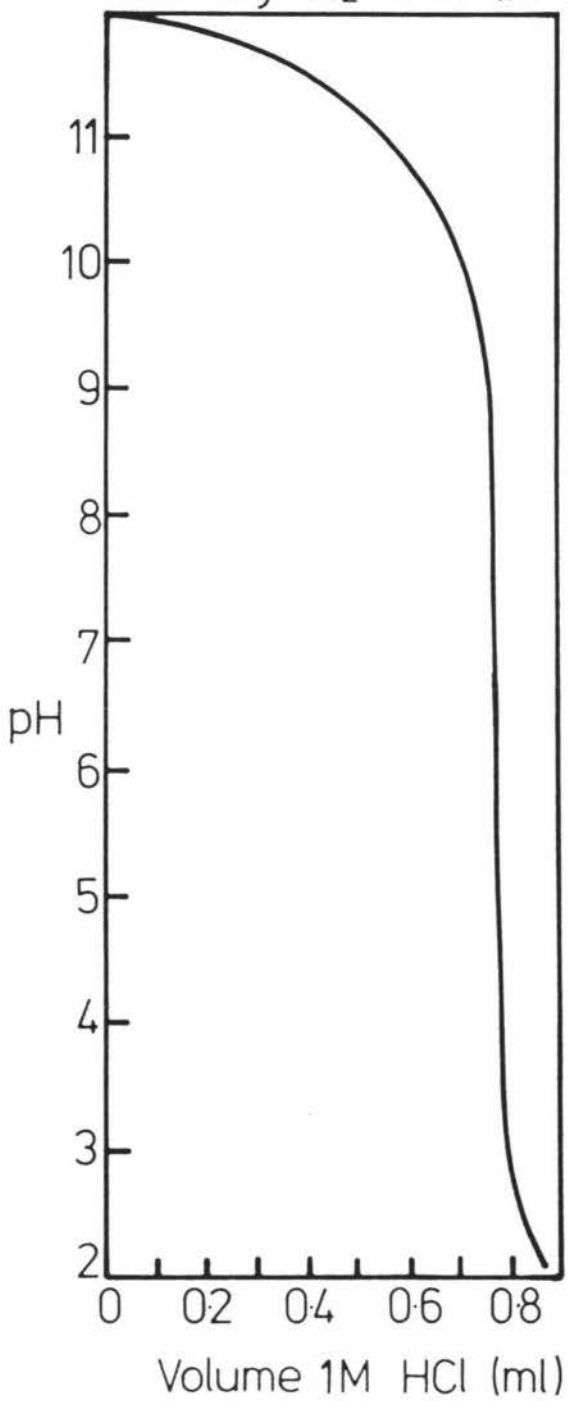


Figure 4·8

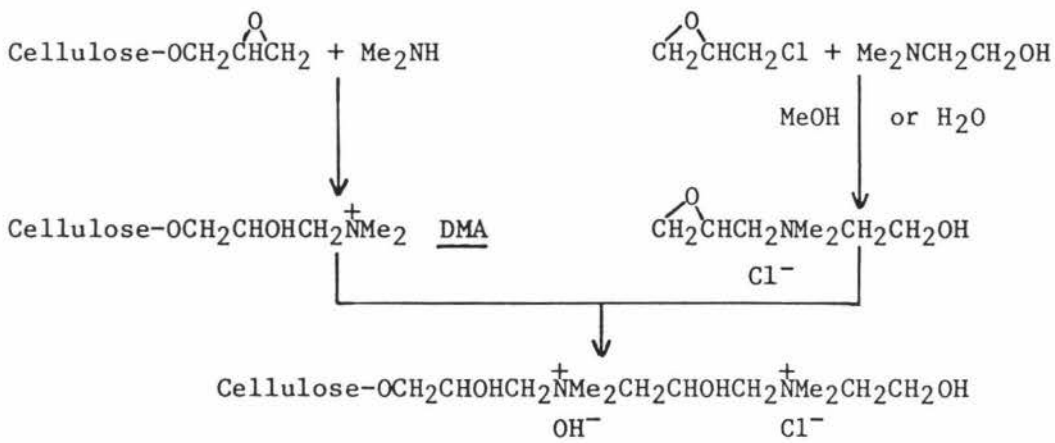
Titration Curve of DQAP Cellulose made via Scheme 3.

(3g wet matrix in the hydroxide form titrated with 10cm^3 1M NaCl under dry N_2 with 1M HCl)



bromide. It's main advantage was that the coupling step was complete in 24 hours whereas the direct coupling of the quaternary salt (Scheme 2) took one week.

Scheme 4



The product of this scheme while not being the same as that from Schemes 2 and 3 is very similar. (It has a hydroxyethyl group in place of a methyl on the end nitrogen).

In this scheme the quaternising agent, not only quaternises the dimethylamino group on the cellulose (DMA Cellulose) but also carries with it a quaternary ammonium group thereby building up the dimeric quaternary ammonium cellulose in situ.

The preparation of the 1,2-epoxypropyl trialkyl quaternary ammonium salts was reported in the literature albert with very little detail and only as solutions of the reagent in unspecified yields.

For the present purposes we used N,N-dimethylethanolamine instead of trimethylamine because it was readily available in New Zealand in large quantities and is in fact the same amine used to make the standard QA Cellulose. The dimethylamino derivative of cellulose (DMA) was easily prepared. Dimethylamine is a very good nucleophile and couples rapidly to the activated matrix. Typical products had substitutions of 1.2 meq N/g which is virtually 100% conversion of the epoxide groups.

4.2.5 Preparation and Use of the 1,2-epoxypropyl trialkyl ammonium Reagent

According to Doughty's(35) patent the reaction of a equal molar mixture of trialkylamine and epichlorohydrin in methanol at room temperature to form a glycidyl quaternising reagent is greater than 95% completed in two weeks. This was determined by ionized chloride present. We prepared quaternising reagents using both methanol and water as the solvent and followed the reaction by determining the free amine content and the epoxide content.

The free amine content was determined by periodically withdrawing a 1 ml sample from the reation mixture, diluting it with deionised water (10ml) and titrating it to an endpoint of pH 7 with 0.1 mol/l HCl

$$\text{i.e. } R_3N + HCl \longrightarrow R_3\overset{+}{N}H \ Cl^-$$

(The amine content was found to drop rapidly in the early stage of the reaction then levelled off at a value which was less than 1% of the starting concentration. This result is in agreement with Doughty's findings). The epoxide content was then determined by adding a solution of 2 mol/l sodium thiosulphate at pH 7 (10ml) to the neutralised sample (pH 7) mentioned above and titrating the liberated hydroxide as outlined by Axen et al(36).

$$R\text{-}\underset{\diagdown O \diagup}{CH}CH_2 + Na_2S_2O_3 + H_2O \xrightarrow{\text{pH 7}} R\text{-}CHOHCH_2S_2O_3Na + NaOH$$

$$0.01 \text{ mol/l } HCl \text{ to maintain pH 7} \downarrow$$

$$NaCl + H_2O$$

The epoxide content should not change much, since during the reaction no epoxides are consumed.

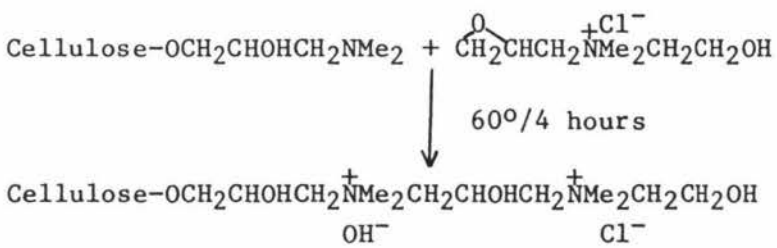
$$\text{i.e. } \underset{\diagdown O \diagup}{CH_2CHCH_2Cl} + R_3N \longrightarrow \underset{\diagdown O \diagup}{CH_2CHCH_2}\overset{+}{N}R_3 \ Cl^-$$

The only drop in epoxide content should arise from the slow solvolysis of the epoxide groups. This however is not what was observed as can be seen in Figure 4.9. In less than 2 days the epoxide content dropped rapidly to 20% of the theoretical amount. After that there was a slow decrease in the amount of epoxide present over the following two weeks which would appear to be the result of slow solvolysis.

No attempt was made to unravel the chemistry of these reactions because of the compexity of them reported by Doughty. (Reactions carried out in aprotic organic solvents gave adducts which either precipitated or oiled out and had no quaternising ability at all).

Doughty claimed that the adduct he obtained in methanol was stable and useful for introducing quaternary ammonium groups onto polysaccharides. It was found that the adduct prepared in water was similar but slightly less efficient as a quaternising agent. This is in keeping with it's lower epoxide content shown in Figure 4.9. In neither case was the product stable as prepared and should be used between 1 and 2 weeks after preparation.

The quaternising ability, of the adducts prepared in water and methanol were determined by reaching various volumes of the solutions (1-4 ml) with Dimethylamino Cellulose (DMA, 5g wet weight) in water at 60°C for 4 hours,



It was found that for complete quaternisation of the Dimethylamino Cellulose (5g moist cake) usually 3 ml was sufficient for the adduct made in methanol, while 4 ml was required with the adduct prepared in water (see Table 4.5).

Figure 4·9

Epoxide Content of the 1,2-epoxypropyltrialkylammonium chloride.

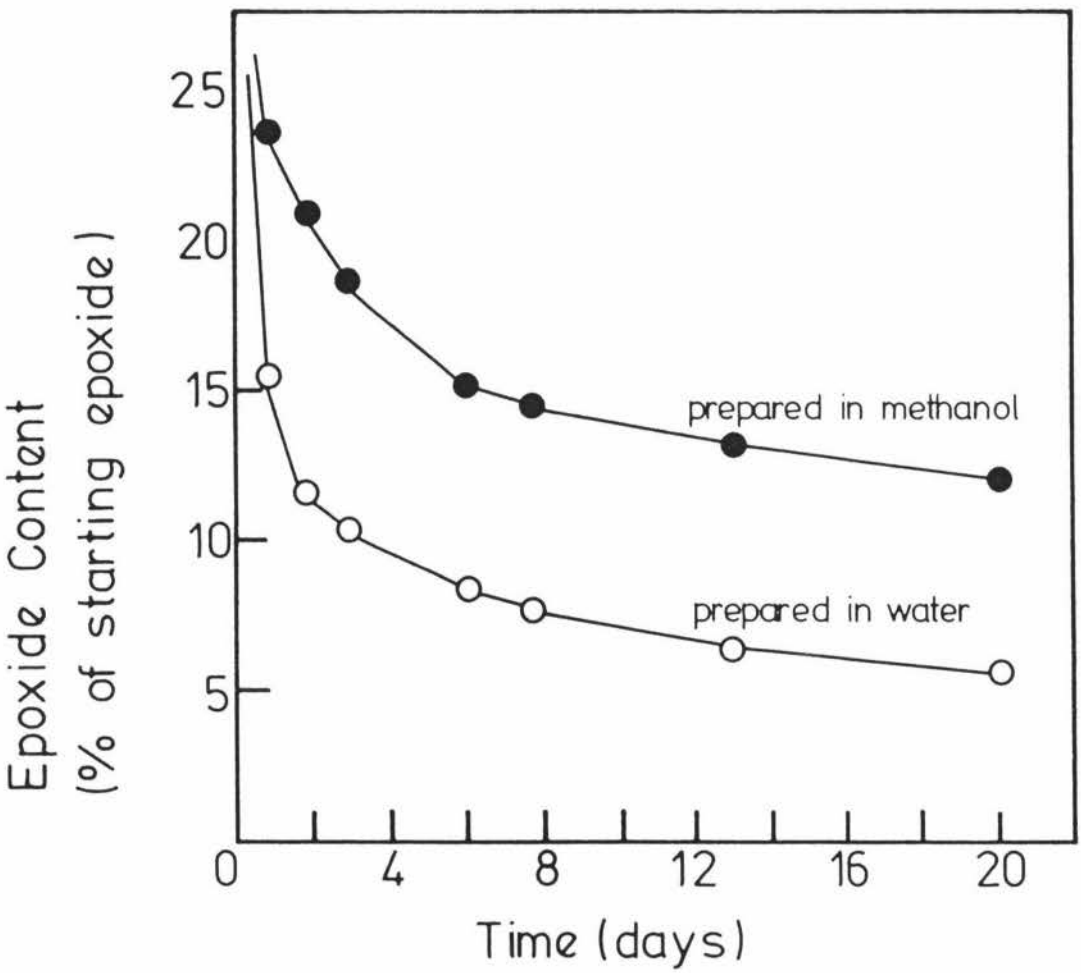


Table 4.5

Quaternisation of DMA Cellulose (5g wet weight)

(Conditions: Reagent two weeks old

Reaction 4 hours at 60^{o})

Volume of Reagent	Substitution (meq N/g)			
	Methanol Reagent		Water Reagent	
(ml)	Quat.N.	Total	Quat.N.	Total
1	-	-	0.87	1.46
2	1.64	1.78	1.50	1.78
3	1.72	1.85	1.65	1.81
4	-	-	1.77	1.89
5	-	-	1.75	1.84

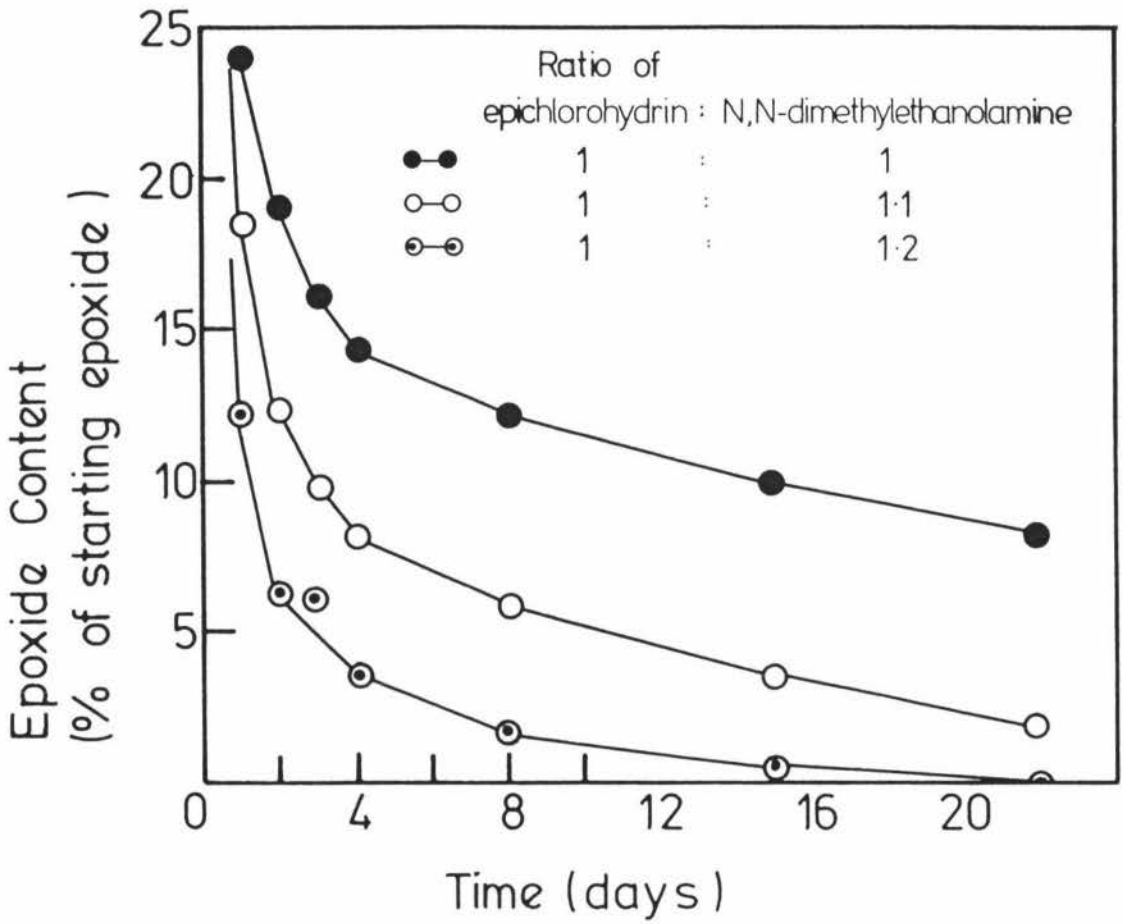
It appears obvious that this reaction is poorly understood. We experienced difficulty in reproducibility, from one preparation of the reagent to the next. This may have been a result of slight variations in the ratio of amine to epichlorohydrin from an exact 1:1 ratio. Figure 4.10 shows the dramatic effect of a 10% excess of amine over epichlorohydrin.

In spite of the lack of understanding of all the reactions which take place in the formulation of the quaternising reagent from epichlorohydrin and tertiary amines, it proved to be an effective route to the preparation of a diquaternary derivative of cellulose. The reactions on the cellulose are both complete in a very short time and so long as an excess of the glycidyl ammonium reagent is used, products with substitutions of 1.8 meq/g can be prepared.

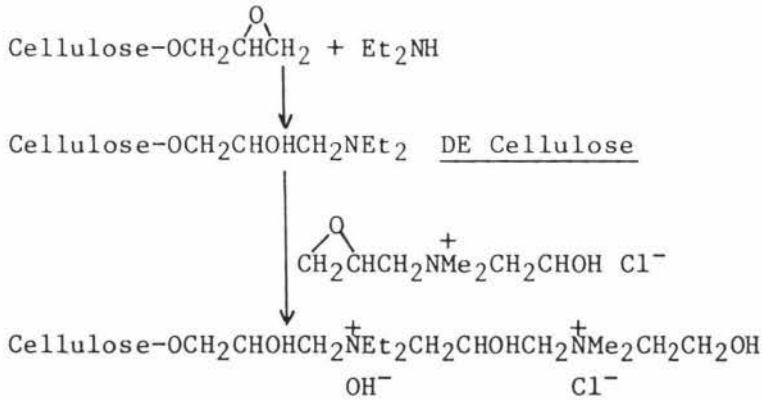
This reaction scheme was not attempted on a large scale because of a six month delay in obtaining bulk quantities of dimethylamine. Since sufficient quantities of diethylamine were available though, it's use was investigated as outlined in Scheme 5.

Figure 4·10

Change in Epoxide Content in Methanolic Solution of 1,2-epoxypropyltrialkylammonium chloride with Time.



Scheme 5



The Diethylamino Cellulose was made with a substitution of 1.07 meq N/g. When quaternisation of the DE Cellulose was attempted by the above scheme it was found that little quaternisation was achieved (see Table 4.6).

Table 4.6

Quaternisation of DE and DMA Celluloses with Glycidyl Reagent

(Equivalent of 10g dry Matrix Reacted with 45ml of 5 Days old Aqueous Reagent for 4 hours at 60^{o})

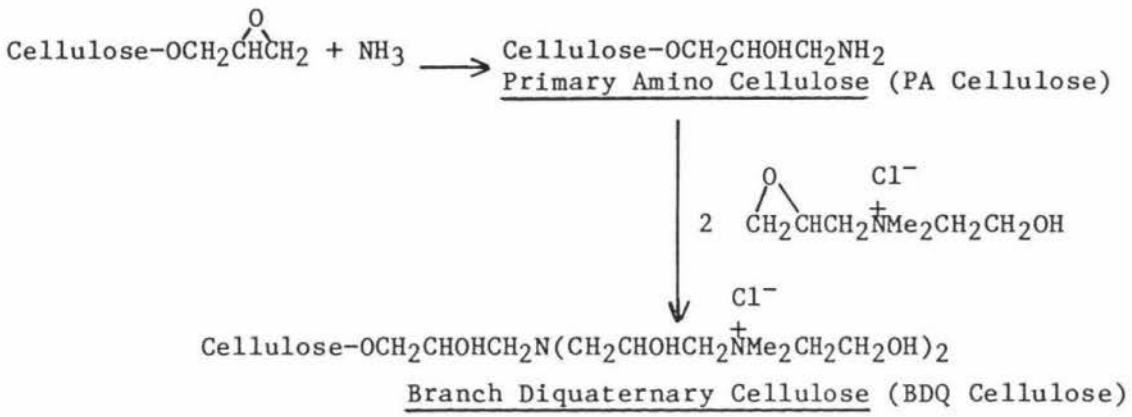
Diquaternary derivative of	meq N/g		Percent quaternisation (%)
	Quat.N.	Total	
DMA	1.65	1.88	88
DE	0.62	1.39	45

The difference in reactivity between the DMA and DE Cellulose towards the quaternising reagent must be due to the difference in the size of the groups bonded to the amino group. The more bulky ethyl groups make the nitrogen of the diethylamino group more sterically hindered than the nitrogen of the dimethylamino group. This means it is less accessible and reacts more slowly, giving the 1,2-epoxypropyl trialkyl ammonium salt a chance to hydrolyse. Large quantities of it would have to be used to achieve complete quaternisation of the DE groups. This reflects the difficulty

generally observed in trying to quaterise DEAE groups. Propylene oxide is the only reagent reported in the literature to achieve this and even then vast excesses were used(37).

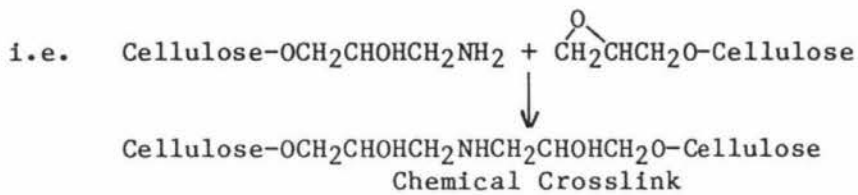
An alternative way of using the 1,2-epoxypropyl trialkyl ammonium salt to prepare a diquaternary derivative of cellulose is outlined in Scheme 6.

Scheme 6



This scheme is comprised of three separate reactions. The preparation of the quaternising reagent as already discussed. The other two reactions are, the reaction of Epoxypropyl Cellulose with ammonia, and the reaction of the PA-Cellulose with the 1,2-epoxypropyl trialkyl ammonium salt reagent.

The reaction of the ammonia with the Epoxypropyl Cellulose is rapid and results in a Primary Amine Cellulose product, with a substitution of 1.05 meq N/g. The substitution is not as high as expected (1.26 meq N/g if 100% efficient) because some crosslinking occurs.



The swollen volume of the PA Cellulose was found to be considerably less than other products made from the same epoxypropyl matrix as can be seen in Table 4.7.

Table 4.7

Comparison of Swollen Volumes and Small Ion Capacities of PA and SP Celluloses made from the same Epoxide Matrix (E23)

Matrix	Swollen Volume (ml/g)	Small Ion Capacity (meq/g)
E23-SP	18	1.17
E23-PA	12.6	1.05

The other reaction involved in this scheme, the reaction of the PA Cellulose with the quaternising reagent, was found to proceed readily to give the derivative outlined in Scheme 6. The ratio of the quaternary (strongly basic) groups to weakly basic groups was found to be approximately 2:1 as can be seen in Figure 4.11. The primary amine derivative being reasonably basic and sterically unhindered reacts readily with the 1,2-epoxypropyl ammonium reagent to give a tertiary amino group to which are bonded two alkyl chains which have quaternary ammonium groups or their end carbon. Exhaustive alkylation of the primary amino group through to a quaternary ammonium group does not occur, as the addition of each alkyl group with it's quaternary ammonium group, increases the steric hinderance about the amino group and also decreases its basicity. The result of this is a dramatic drop in it nucleophilicity (and hence reactivity). From the titration curve it can be seen that its pKa is approximately 5.5 and there would appear to be no further reaction with the 1,2-epoxypropyl trialkyl ammonium reagent.

4.2.6 Stability of BDQ Cellulose

This branched diquaternary derivative was subjected to the same stability tests as used for the DQAE and DQAP derivatives (see Table 4.4). The results of these tests are shown in Table 4.8.

Figure 4·11

Titration Curve for BDQ Cellulose.

(5g wet matrix in 10 cm^3 1M NaCl titrated under dry N_2 with 1M HCl)

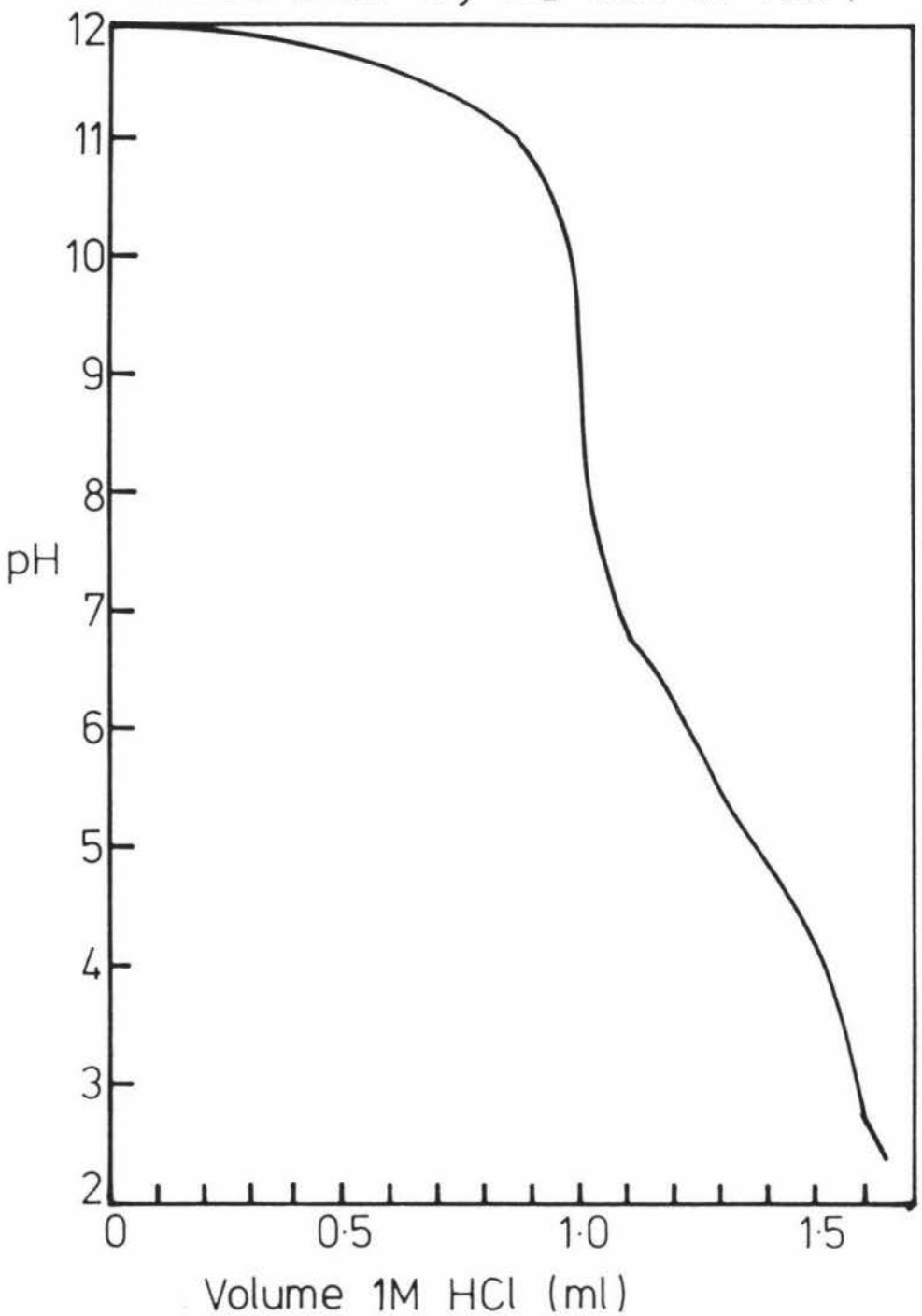


Table 4.8

Stability of BDQ Cellulose

(treated at 80°C for one week)

Conditions	Substitution			
	meq N/g (% Total)			
	Quaternary N (pH 10.3)	Tertiary N (pH 10.3 - 7)	Weak tertiary (pH 7)	Total
UNTREATED	1.10 (60)	0.08 (4)	0.66 (36)	1.84
2% $Na_2S_2O_5$, pH 12	1.04 (49)	0.18 (8)	0.92 (43)	2.15
0.5 mol/l NaOH	1.03 (45)	0.37 (16)	0.88 (39)	2.28

Though some degradation of groups is apparent, this matrix stood up to the stability tests as well as the DQAP Cellulose. However, the main disadvantage with this product was the low 1.1 meq/g substitution of quaternary groups.

An alternative reagent to the 1,2-epoxypropyl trialkyl ammonium reagent was prepared from 1,2-dibromoethane and N,N-dimethylethanolamine as described by Barnhurst(38).

$$BrCH_2CH_2Br + Me_2NCH_2CH_2OH \xrightarrow[4\ \text{days}]{37^\circ C,\ \text{Toluene}} BrCH_2CH_2\overset{+}{N}Me_2CH_2CH_2OH\ Br^-$$

2-bromoethyl dimethyl-
2'-hydroxyethylammonium bromide)

This was then reacted with the Primary Amino Cellulose.

$$\text{Cellulose-}OCH_2CHOHCH_2NH_2 + 2\ BrCH_2CH_2\overset{+}{N}Me_2CH_2CH_2OH\ Br^-$$

$$\downarrow$$

$$\text{Cellulose-}OCH_2CHOHCH_2N(CH_2CH_2\overset{+}{N}Me_2CH_2CH_2OH)_2\ Br^- + 2\ HBr$$

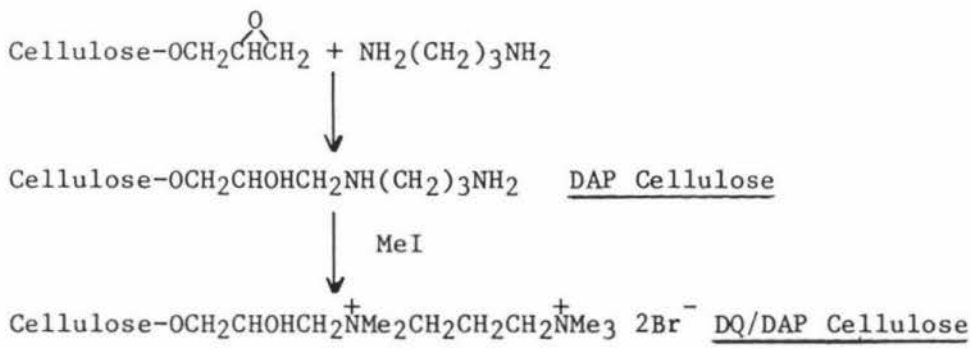
The reaction was only attempted once and little quaterisation of the DMA Cellulose was achieved. The supernatant of the reaction mixture became very acid due to the formation of HBr thus preventing the primary amine from reacting completely,

i.e. $RNH_2 + HBr \longrightarrow R\overset{+}{N}H_3\ Br^-$

The addition of Na_2CO_3 would probably help this reaction but this was not pursued.

Two further routes to quaternary products were tested using readily available primary amines, followed by quaternisation with methyl iodide. These are outlined in Schemes 7 and 8 and both gave rise to high substitution levels around 2 meq N/g.

Scheme 7



This scheme is again, a two step sythesis, with the coupling of a ligand, i.e. 1,3-diaminipropane, being the first step. This ligand couples rapidly due to the fact that it is a good nucleophile, being basic [pKa 10.4 and 8.6(33)] and a primary amine. The coupled ligand remains just as reactive as the uncoupled ligand and some crosslinking occurs. Crosslinking can be minimised by using a large excess of ligand.

The second step in this synthesis involves the methylation reaction as in Scheme 3, but in this case 5 times the amount of methyl iodide was required.

The intermediate DAP Cellulose had a substitution of 2.18 meq N/g (i.e. 1.09 mmol/g) and after exhaustive methylation resulted in a product with 1.92 meq N/g (i.e. 0.96 mmol/g). The observed decrease can be explained largely by the increase in weight after introducing 5 methyl groups in place of hydrogens. The weight increase is 70 mg/mmole of diaminopropane introduced.

i.e.

$$\underset{\text{DAP Cellulose}}{R\overset{+}{N}H_2CH_2CH_2CH_2\overset{+}{N}H_3 \quad 2Cl^-} \longrightarrow \underset{\text{DQ/DAP Cellulose}}{R\overset{+}{N}Me_2CH_2CH_2CH_2\overset{+}{N}Me_3 \quad 2Cl^-}$$

Scheme 8

$$\text{Cellulose-OCH}_2\overset{\text{O}}{\overset{\wedge}{\text{CH}}}\text{CH}_2 + NH_2(CH_2)_3NH(CH_2)_3NH_2$$

$$\downarrow \quad 3,3'\text{-Iminobispropylamine}$$

$$\text{Cellulose-OCH}_2\text{CHOHCH}_2NH(CH_2)_3NH(CH_2)_3NH_2 \qquad \underline{\text{TA Cellulose}}$$

$$\downarrow \quad \text{MeI}$$

$$\text{Cellulose-OCH}_2\text{CHOHCH}_2\overset{+}{N}Me_2(CH_2)_3\overset{+}{N}Me_2(CH_2)_3\overset{-}{N}Me_3 \quad 3I^- \qquad \underline{\text{TQA Cellulose}}$$

This scheme is similar to Scheme 7 except that a ligand with 3 amino groups was employed. Again some crosslinking would occur on coupling this ligand, reducing the swollen volume of the product. The ligand is expensive and therefore a large excess of it can only be used, (to help minimise crosslinking), if a method of ligand recovery, for use in later batches, is undertaken.

We found that this product (TA Cellulose) could not be completely methylated in one step and that to achieve exhaustive methylation the partially methylated product needed to be reacted a second time with methyl iodide. The final product had a substitution level of only 2.25 meq N/g which was not a significant increase on previous methods. Larger excesses of 3,3'-iminobispropylamine would have to be used to achieve substitutions of 2.5 - 3.0 meq N/g and less crosslinking. Overall Scheme 8 was not a satisfactory method of producing a highly substituted quaternary ammonium cellulose. In spite of that the product had some interesting properties.

4.2.7 TQA Cellulose - Heparin Binding

Heparin was bound and eluted from a column of TQA Cellulose under identical conditions to those used for QA and DQAP Cellulose. The result of these columns are shown in Figures 4.12 and 4.13. It can be seen from these results that heparin is bound tighter to TQA Cellulose than either QA or DQAP Cellulose (see Figure 4.14). The same dramatic increase in binding strength was not observed for proteins, such as BSA (see Figure 4.15 and Table 4.9).

Table 4.9

Properties of QA, DQAP, TQA Celluloses

Matrix	Substitution (meqN/g)	Conc. NaCl (mol/l) to Start Elution*	
		Heparin	BSA
QA Cellulose	1.2	0.4	0.1
DQAP Cellulose	1.69	0.7	0.1
TQA Cellulose	2.25	1.0	-

* 0-2.0 mole/l gradient for heparin
0-0.5 mole/l gradient for BSA

It would seem that the closely spaced positively charged nitrogens present in the DQAP and TQA Celluloses match repeating negatively charged sulphate groups present in the heparin structure. No such uniform distribution of charged groups are present on the surface of globular proteins like BSA and consequently the DQAP products have a lesser effect on the binding strength of proteins.

Figure 4·12

Elution Profile of Heparin (10mg) from TQA Cellulose (5ml).

(load conditions - 0·01M Tris , pH 8·0
elution conditions - 0-2M NaCl gradient in 0·01M Tris)

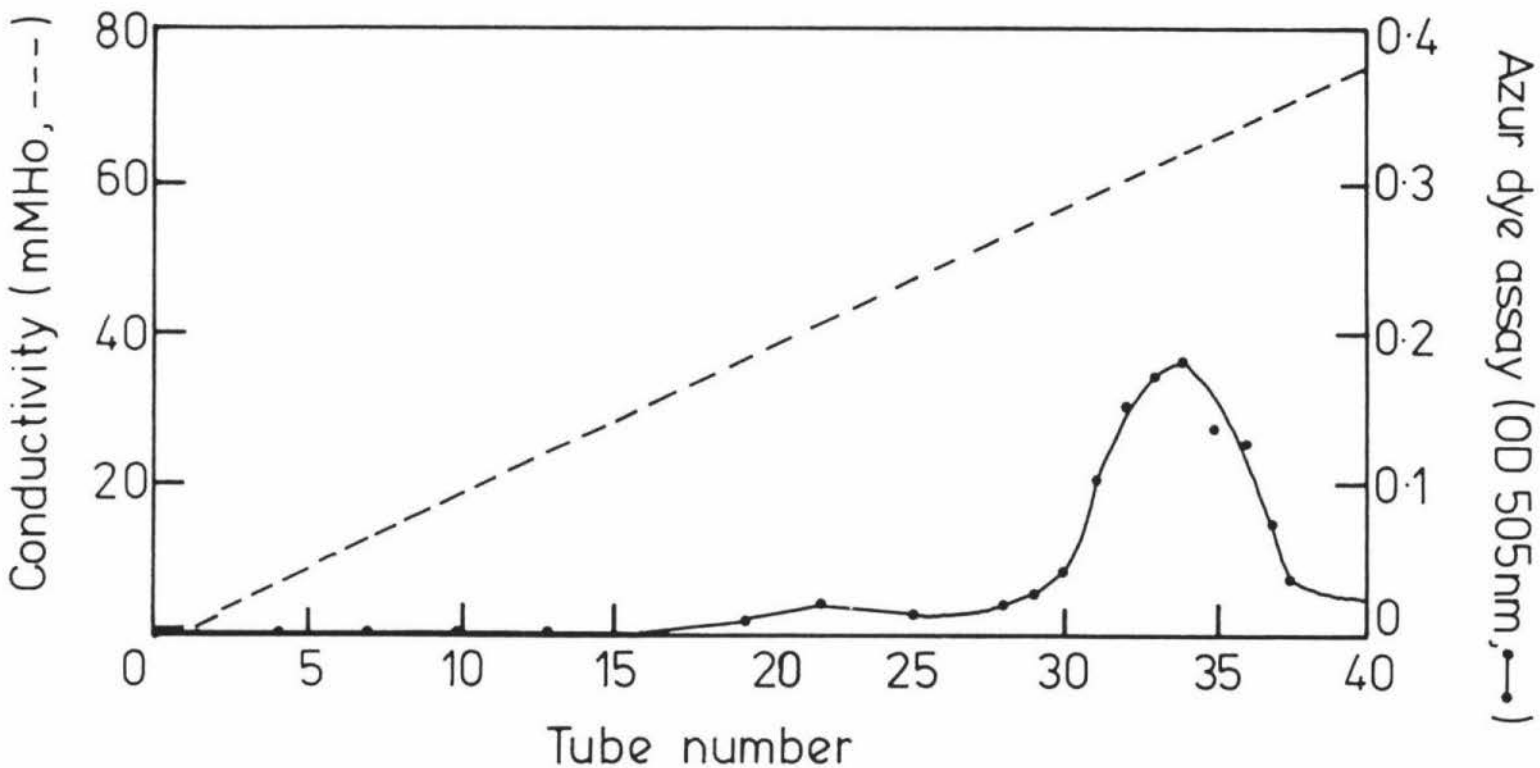


Figure 4·13

Elution Profile of Heparin (10mg) from TQA Cellulose (5ml).

(load conditions - 2% $Na_2S_2O_5$, pH 12
elution conditions - 2M NaCl in 2% $Na_2S_2O_5$, pH 12)

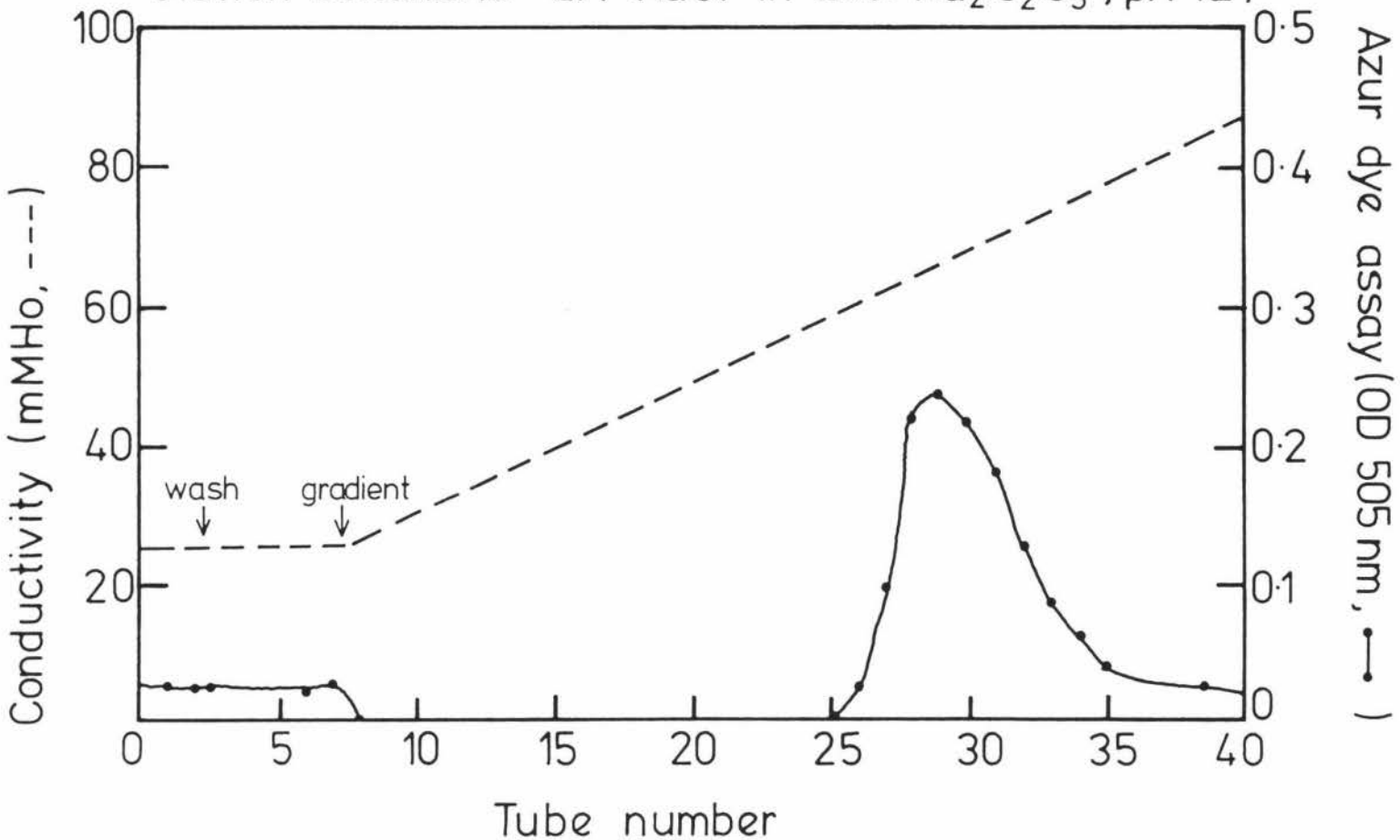


Figure 4·14

Elution Profile of Heparin (10mg) from QA, DQAP and TQA Cellulose (5ml).

(load conditions - 0·01M Tris, pH 8·0
elution conditions - 0-2·0M NaCl gradient in 0·01M Tris, pH 8·0)

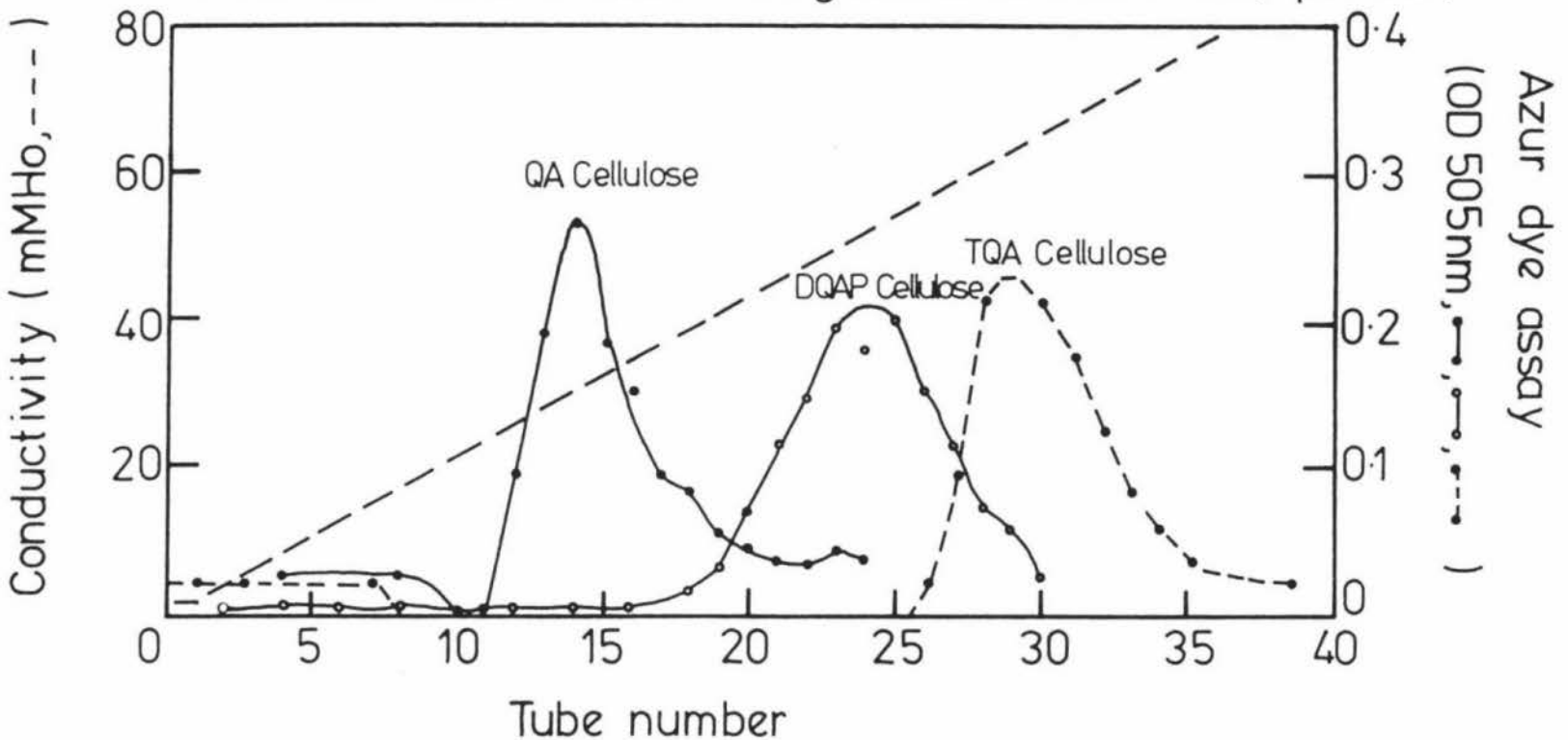
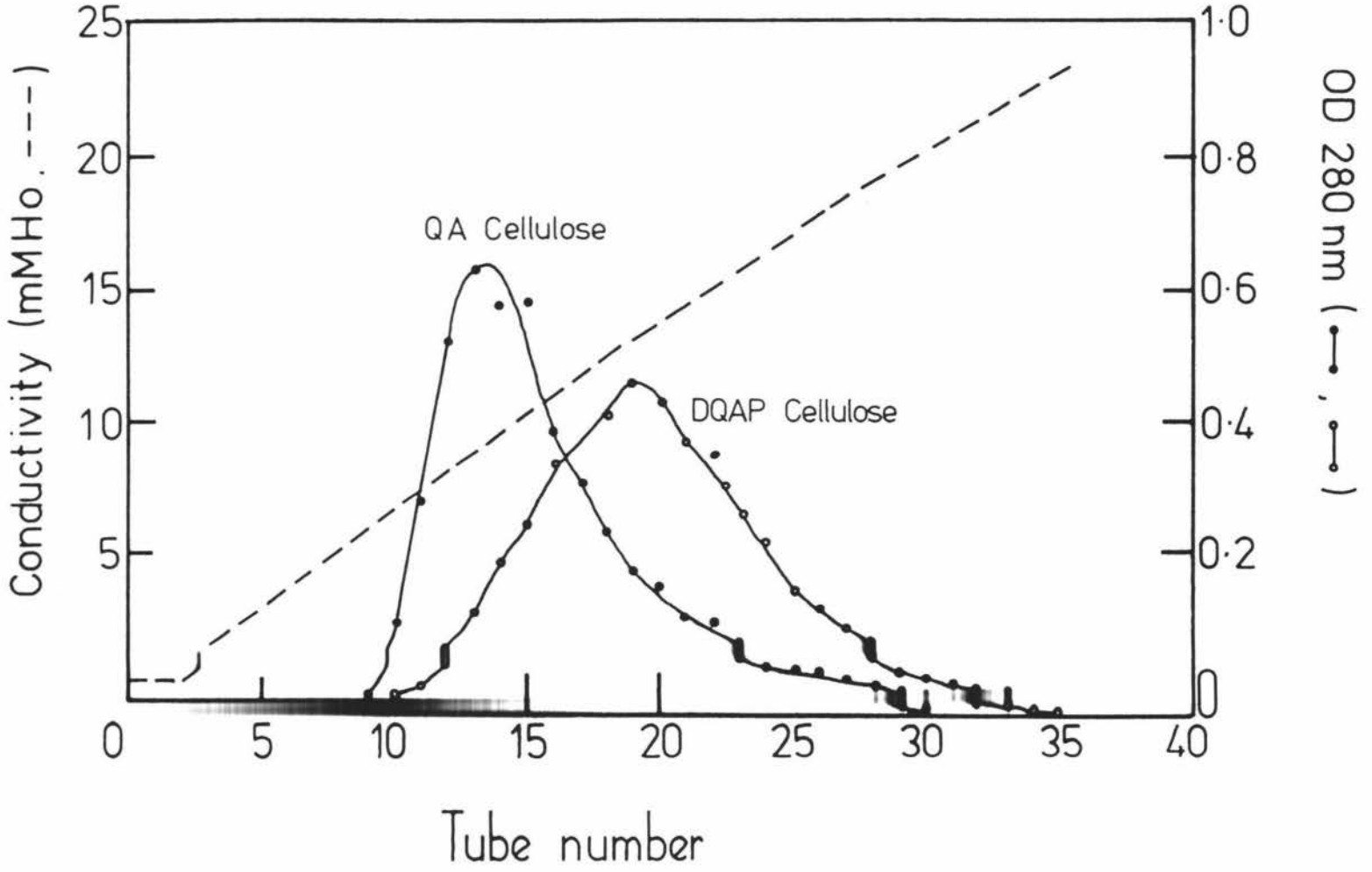


Figure 4·15

Elution Profile of BSA (50mg) from QA Cellulose and DQAP Cellulose.

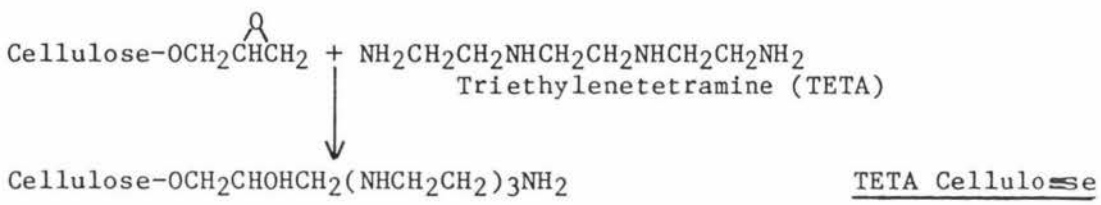
(load conditions - 0·01M Tris, pH 8·0
elution conditions - 0-0·5M NaCl gradient in 0·01M Tris, pH 8·0)



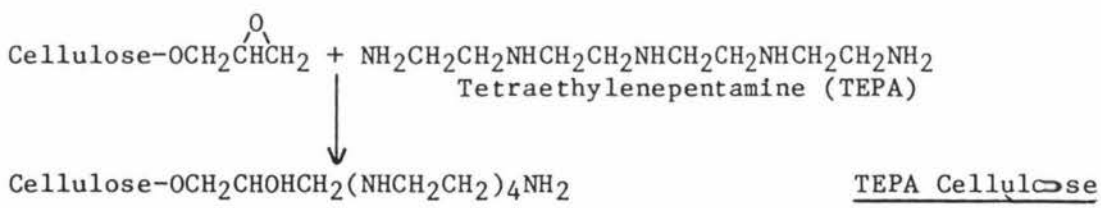
4.2.8 Multiple Nitrogen Ligands

All the schemes outlined above have been aimed at producing stable quaternary ammonium derivatives of cellulose for the adsorption of heparin at high pH, e.g. pH 12. At much lower pH's, i.e. 4 - 7 it would be possible to use primary, secondary and tertiary aliphatic amines coupled to cellulose since at these pH values their nitrogens would also be positively charged. For example DEAE Cellulose has been used for the purification of heparin(39,40). Three readily available chemicals with multiple amino groups were coupled to the activated cellulose matrix to give products with high nitorgen content. These routes are outlined in Schemes 9-11 below.

Scheme 9



Scheme 10



Scheme 11

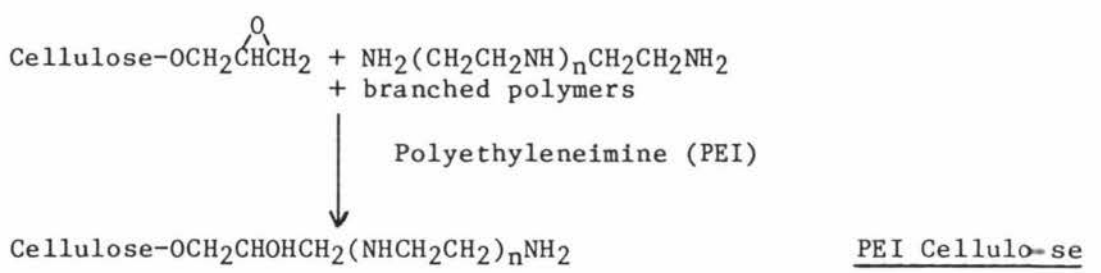


Figure 4.16 shows a typical titration curve obtained for TETA Celluloses and Table 4.10 the properties of the products obtained in comparison with those for the dimethylamino derivative prepared from the same activated matrix.

Table 4.10

Comparison of Properties of Various Nitrogen Containing Cellulose Derivatives Prepared on Batch 100

Cellulose Derivative	S.I.C.	Swollen Volume	Protein Capacity
	meqN/g	ml/g	g/g
DMA	1.35	13.6	1.06
TETA	2.88	9.2	0.5
TEPA	3.4	10.7	0.4
PEI*	3.59	8.7	0.0

*This matrix was made from a modified matrix (M1) which had a swollen volume of 18.4 ml/g.

The high substitution levels of nitrogen obtained would be expected to cause binding of heparin in salt concentrations greater than 1.0 mol/l. However, the difficulty we experienced with these derivatives, i.e. TETA, TEPA and PEI, is that because of the number of reactive amino groups in the ligands used to make them a great deal of crosslinking occurs. This crosslinking seriously reduces the swollen volumes of the resulting ion exchange derivatives, reducing their porosity and ability to bind large polyelectrolytes, like proteins and heparin. The larger the ligand the worse the effect became. PEI Cellulose was found to bind no BSA under the normal test conditions and very little heparin. Similar observations were made for many of the other derivatives. Table 4.11 shows the results of BSA and heparin capacities determined for several products prepared via schemes 1-11.

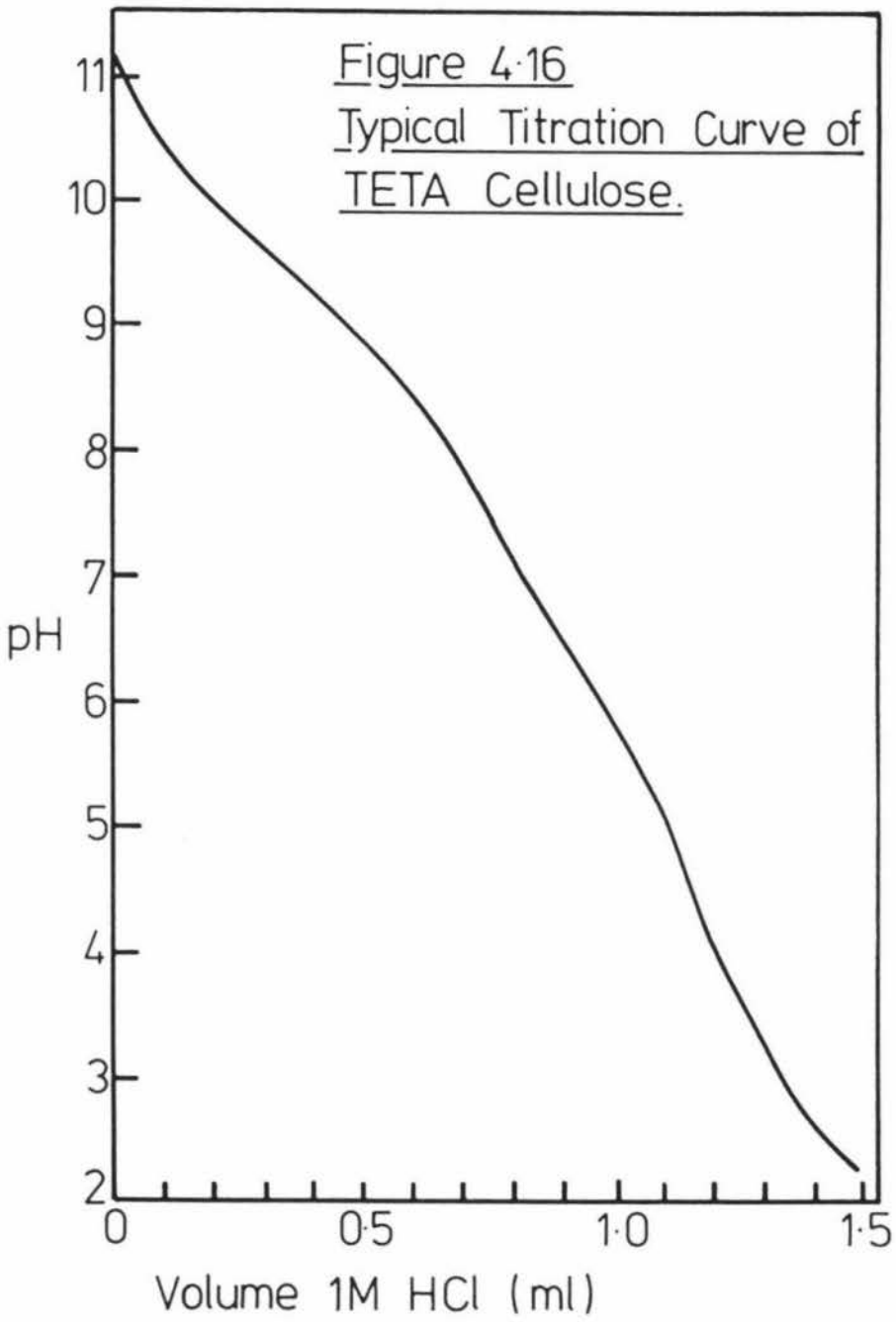


Figure 4·16
Typical Titration Curve of
TETA Cellulose.
pH
11
10
9
8
7
6
5
4
3
2
0
0·5
1·0
1·5
Volume 1M HCl (ml)

Table 4.11

Properties of Cellulose Derivatives Prepared Via Schemes 1 - 11

Cellulose Derivative	Scheme	Batch No	SIC meqN/g	Swollen Volume ml/g	BSA Capacity g/g	Heparin Capacity g/g
QA	-	Q4	1.14	12.7	1.46	-
DQAP	2	DQAP/E22	1.49	-	0.24	0.13
DQAP	2	DQAP/E24	1.80	24.6	1.33	0.22
DMA	4	DMA/102B	1.19	18.3	1.47	0.26
DMA	4	DMA/100A	1.35	13.6	1.06	-
DQAP'	4	DQAP'/102B	1.55	14.1	0.90	0.18
TQA	8	TQA/E19	2.25	-	-	0.12
TETA	9	TETA/100D	2.88	9.2	0.50	0.16
TETA	9	TETA/102D	2.10	12.4	-	0.19
PEI	11	PEI/M1	3.59	8.7	0.02	0.0

Heparin Capacity Tests

Most of the products in the above table showed reasonable capacities for BSA although none were as high as for Q4 unless its swollen volume was considerably greater than that of Q4, i.e. 12.7 ml/g. On the other hand their capacities for heparin measured under identical conditions, that is very dilute buffer (0.01 mol/l phosphate) at pH 7.0, were all less than 0.3 g/g, which is low. Part of the reason why such a variety of routes were investigated was because of the low heparin capacities observed for each of them.

One of the derivatives, DQAP/E24, was retested for heparin capacity under the same buffer conditions, but in the presence of increasing concentrations of sodium chloride, i.e. 0-0.5 mol/l. It was found (see Table 4.12) that the heparin capacity markedly improved with the addition of even 0.1 mol/l NaCl and appeared to peak at approximately 0.4 mol/l NaCl.

This improvement of capacity with increasing salt concentration, which is contrary to what we usually might expect (see pH profiles, Section 3) can be rationalised by considering the nature of the heparin molecules. The heparin molecule is an extended linear structure with a large number of negatively charged groups spaced out along its length. In the absence of salt, the heparin molecule becomes elongated as all the negatively charged groups repel each other, making it more difficult for it to diffuse into the pores in the particles of ion exchanger. The presence of even a low concentration, i.e. 0.1 mol/l, of an electrolyte species masks this repulsion between the charged groups of the heparin and it condenses into a much smaller, denser packed structure which is then able to penetrate into the particles of ion exchanger.

Table 4.12

Heparin Capacities in the presence of NaCl

(0.01 mol/l phosphate pH 7.0)

Cellulose Derivative	Concentration of NaCl (mol/l)	Heparin Capacity (g/g)
DQAP/E24	0.0	0.18
DQAP/E24	0.1	0.43
DQAP/E24	0.2	0.56
DQAP/E24	0.4	0.67
DQAP/E24	0.5	0.53
DMA/102B	0.0	0.26
DMA/102B	0.1	0.45
PEI/M1	0.0	0.0
PEI/M1	0.4	0.25

The heparin capacity begins to drop-off at 0.5 mol/l NaCl because it is approaching the salt concentration at which the heparin would begin to elute off a column (see Figure 4.4) and the number of binding sites on the cellulose which can hold the heparin in 0.5 mol/l NaCl is beginning to decrease. [It is well known that the capacity of an ion exchanger for proteins decreases markedly once conditions are reached where the protein would elute slowly from the ion exchanger(41)].

4.3 CONCLUSION

For amine derivatives of cellulose to exhibit good capacities for heparin the salt concentration needs to be of the order of 0.4 mol/l. In order to retain heparin at this concentration the cellulose should have a nitrogen substitution level of 1.8 - 2.0 meq/g. The swollen volume of the cellulose needs to be somewhat higher than that used for the SP derivative (10 - 14 ml/g) and probably in the range of 15 - 20 ml/g.

The best route for obtaining such a product would appear to be Scheme 4 so long as the 1, 2-epoxypropyl trialkylammonium chloride can be made reproducibly on a large scale. Alternatively Scheme 3 could be used but the reagent, 1,3-bisdimethylamino-2-propanol would have to be prepared from epichlorohydrin and excess dimethylamine on site because of the cost of purchasing it (US $1350/10Kg).

If the heparin purification process is conducted at pH 4 - 7 then Scheme 9 would be the simplest and cheapest route since triethylenetetramine is available at US $107/12 kg plus air freight.

SECTION 5

EXPERIMENTAL

5.1 MATERIALS

Deionised water was used throughout.
A_2 Protion was obtained from Tasman Vaccine Ltd.; A_2 Indion (Batch 1030AE2) from Phoenix Chemicals Ltd., DE 52 Cellulose Anion Exchanger from Whatman Ltd and Viscose (75-150μ) from Viscose Group Ltd.

Bovine Serum Albumin (Fraction V) was obtained from Sigma; Low heat treated skim milk powder from the Dairy Research Institute; Renco from New Zealand Rennet Co-op Ltd; and Azur 1 and Heparin (acetone precipitated from New Zealand Pharmacuticals Ltd.

The following chemicals were obtained from the companies indicated: 1,3-Diaminopropane and tetraethylenepentamine from Aldrich; Diethylamine (lab. grade) and dimethylamine (25% aqueous) from Ajax Chemicals Ltd.; Propylene Oxide from J. T. Baker; Barbitone sodium (lab. grade), citric acid (lab, grade), imidazole (lab. grade), methyl bromide (lab. grade), and sodium hydrogen carbonate ('AR' grade) from the British Drug Houses Ltd.; Epichlorohydrin from DOW; N,N,N',N'-tetramethylethylenediamine from Fluka; Ammonia (35% aqueous) from Koch-Light Laboratories Ltd; 1,2-Dibromoethane (lab. grade), maleic acid (lab. grade), pyridine ('AR' grade), sodium hydroxide ('AR' grade) and sodium sulphite ('AR' grade) from May and Baker; Calcium chloride-6-hydrate, triethylenetetramine and methyl iodide from Riedel-De-Haen, and 3,3'-Iminobispropylamine, N,N - dimethyl ethanolamine, polythyleneimine (50% aqueous), sodium chloride (Sigma grade) and tris(hydroxymethyl)aminomethane (Sigma grade) from Sigma.

1,3-Bisdimethylamino-2-propanol was prepared by the method of Campbell *et al*(42).

5.2 PREPARATION OF CELLULOSE DERIVATIVES

5.2.1 Crosslinked Hydroxypropylated Regenerated Cellulose - The Modified Matrix

Regenerated cellulose (10g) was mixed with propylene oxide (4-5 ml), epichlorohydrin (0.8 - 1.2 ml) and aqueous sodium hydroxide (15 ml,30% w/v) at 15°C. After thorough mixing and all the moisture had been absorbed (2 minutes), the mixture was transferred to a screw capped, mild steel bomb with glass liner, and placed in a water bath at 65°C for 90 minutes. After cooling under tap water for 45 minutes the contents of the bomb were either added to water and washed on a scintered glass funnel or used immediately for the preparation of the modified DEAE Cellulose or the activated matrix.

5.2.2 Modified DEAE Cellulose

A solution of diethylaminoethylchloride hydrochloride (15g) in water (10ml) was thoroughly mixed with the contents of the bomb, i.e. the modified matrix, and the mixture transferred back into the bomb. It was placed in a water both at 100°C, for 60 minutes and after cooling the product was transferred into water (1 litre) and then collected on a scintered glass funnel, and washed thoroughly with water.

5.2.3 Epoxypropylated Cellulose - The Activated Matrix

The contents of the bomb, i.e. the modified matrix, was added to a aqueous sodium hydroxide solution (3.25g NaOH in 110-120 ml water) at 4°C, in a screw top jar. To this mixture was added epichlorohydrin (18-20ml). The jar was then rolled on a ball mill for 16-20 hours in a cold room (4-5°C) and then at room temperature for a further 4 hours. The product was collected on a scintered glass funnel and washed free of excess reagents with water.

5.2.4 Derivatives from the Activated Matrix

(a) Most of the derivatives made from the activated matrix were prepared by the following procedure. To an aqueous slurry of the activated matrix was added the ligand to be coupled. This mixture was rolled overnight at room temperature. The resulting derivative was collected and washed with water. In Table 5.1 is summarised the amount of ligand used in each preparation per 10g regenerated cellulose used. In some cases $NaHCO_3$ was added as a buffer and this is also recorded.

Table 5.1

Preparation of Derivatives from the Activated Matrix (10g)
(Reaction Conditions:- Mixed overnight at room temperature)

Cellulose Derivative	Ligand	Amount of Ligand	Amount of $NaHCO_3$
SP	Na_2SO_3	15g	-
DE	Et_2NH	10ml	1.2
QA	$Me_2NCH_2CH_2OH$	10ml	1.2
DMA	Me_2NH	20-25ml(25% aqueous)	1.2
DAP	$NH_2(CH_2)_3NH_2$	10ml	1.2
TMDAP	$Me_2NCH_2CHOHCH_2NMe_2$	10ml	1.2
TA	$NH(CH_2CH_2CH_2NH_2)_2$	8ml	-
TETA	$NH_2(CH_2CH_2NH)_2CH_2CH_2NH_2$	15ml	-
TEPA	$NH_2(CH_2CH_2NH)_3CH_2CH_2NH_2$	20ml	-
PEI	$NH_2(CH_2CH_2NH)_nCH_2CH_2NH_2$	20g (50% aqueous)	-
PA	NH_3	15ml (35% aqueous)	2.5

(b) Carboxy Cellulose - CM Cellulose

To an aqueous slurry of the activated matrix was added an aqueous solution of sodium cyanide (10g). This was mixed overnight at room temperature. The cyano cellulose product was collected, washed with water and returned to the screw top jar. Aqueous sodium hydroxide (1 mol/l, 200ml) was added and the mixture heated at 80°C for 6 - 8 hours. The resulting carboxy cellulose derivative was collected and washed free of sodium hydroxide.

(c) DQAE Cellulose (Scheme 1)

To the activated matrix (15g moist cake) was added a solution of 2-dimethylaminoethyl trimethylammonium bromide (3g) dissolved in water (50ml). It was mixed for 3 days at room temperature and the product was collected on a filter and washed with water.

(d) DQAP Celluose (Scheme 2)

To a slurry of activated matrix (approximately 5g dry weight) was added 3-dimethylamino-2-hydroxypropyl trimethylammonium bromide (10g). This was mixed at room temperature for 1-2 weeks and the product collected on a scintered glass funnel and washed with water.

5.2.5 Methylation of TMDAP, DAP and TA Cellulose (Schemes 3,7,8)

The method used to methylate these cellulose derivatives was described in a confidential private communication from New Zealand Pharmacuticals Ltd to Dr J. S. Ayers. It used methyl iodide, with increasing amounts being required for the DAP and TA derivatives. The TA derivative in fact required a two step methylation to give a product, ie TQA Cellulose, whose groups were 100% quaternary in nature.

5.2.6 Preparation of Diquaternary Ammonium Celluloses (Schemes 4 and 5)

(a) From DMA Cellulose

To DMA Cellulose (90g moist cake) was added water (50ml) and 1,2-epoxypropyl dimethyl-2'-hydroxyethylammonium chloride solution (aqueous or methanolic, 45ml) which had been prepared 1-2 weeks earlier as described later. This was heated for 4 hours at 60°C in a water bath fitted with a flask shaker. The product was collected on a scintered glass funnel and washed with water.

(b) From DE Cellulose

To DE Cellulose (80g moist cake) was added water (50ml) and aqueous 1,2-epoxypropyl dimethyl-2'-hydroxyethylammonium chloride solution (44ml, 1-2 weeks old). Further processing was as described above for DMA Cellulose.

5.2.7 Preparation of Branched Diquaternary Ammonium Cellulose

(a) BDQ Cellulose via Scheme 6

To PA Cellulose (25g moist cake) was added aqueous 1,2-epoxypropyl dimethyl-2'-hydroxyethylammonium chloride solution (50ml, 9 days old). This was then mixed for 20 hours at 37°C and the product collected on a funnel and washed with water.

(b) From PA Cellulose and 2-bromoethyl dimethyl-2'-hydroxyethylammonium bromide

To PA Cellulose (5g moist cake) was added water (10ml) and 2-bromoethyl dimethyl-2'-hydroxyethylammonium bromide (2.2 g). The reaction was mixed at 37°C for 6-48 hours. (The product was 23% quaternary at 6 hours and 0% quaternary at 24 hours)

5.2.8 Preparation of Ligands and Quaternising Reagents

(a) 2-Dimethylaminoethyl trimethylammonium bromide

This ligand was prepared by a patented method (32) as follows. Methyl bromide (11ml) was slowly added with stirring to a solution of N,N,N',N'-tetramethylethylenediamine (30ml) in benzene (300ml). Stirring was continued for 24 hours at room temperature. The white precipitate was collected, washed with benzene and transferred to a desiccator. The remaining benzene was removed by vacuum pump. The yield was approximately 90%. The product was hygroscopic so it was stored in a desiccator.

(b) 3-Dimethylamino-2-hydroxypropyl trimethylammonium bromide

This was prepared from methyl bromide and bis-1,3-dimethylamino-2-propanol, by the same method as outlined for the preparation of 2-dimethylaminoethyl trimethylammonium bromide, using a molar ratio for diamine to methyl bromide of 1:1. A similar yield, ie approximately 90%, was obtained.

(c) 2-Bromoethyl dimethyl-2'-hydroxyethylammonium bromide

This was prepared by the method described by Barnhurst(38). To 1,2-dibromoethane (17.3 ml) in dry toluene (20ml) was added slowly N,N-dimethylethanolamine (20ml). This was stirred at 37^{o}C for 4 days, The product was collected on a buchner funnel, washed with ether and ethanol and then recrystallised from methanol;ethyl acetate.

(d) 1,2-Epoxypropyl dimethyl-2'-hydroxyethylammonium chloride

Preparation

Aqueous and methanolic solutions of this compound were prepared by the method described by Doughty et al(35). To a solution of

epichlorohydrin (20ml) in methanol or water (25ml), in a 100ml round bottom flask (cooled in a ice bath), was added, over 1 hour, with stirring a solution of N,N-dimethylethanolamine (25ml) in methanol or water (25ml). On completion of the addition, the mixture was stirred at room temperature for 24 hours and then allowed to stand for 1-2 weeks until it was ready to use.

Analysis

(i) Amine content

The amine content was determined by withdrawing a sample (1ml) of the reaction mixture, diluting it with water (10ml) and titrating to an endpoint of pH7 with 0.1 mol/l HCl.

(ii) Epoxide content

This was determined by the method outlined by Axen et al(36). To the above neutralised solution at pH 7 was added sodium thiosulphate solution (2 mol/l, pH7, 10ml) and the liberated hydroxide was titrated with 0.1 mol/l HCl by maintaining the solution at pH7. Approximately 2 hours were required to reach the endpoint of this titration.

(iii) Quaternising ability

To DMA Cellulose (5g moist cake) was added the aqueous or methanolic solution of 1,2-epoxypropyl dimethyl-2'-hydroxyethylammonium chloride (1-5ml) and water (9-5ml). It was shaken in a water bath at 60°C for 4 hours. The products were analyse by titration to endpoint pH 10.3 then to an endpoint of pH 3 (see Determination of Ion Exchange Capacity).

5.2.9 Stability Tests - Quaternary Ammonium Cellulose

Quaternary ammonium cellulose, ie QA, DQAE, DQAP and BDQ, (2 x 10g moist cake) was weighed into 100ml screw top jars. To these jars was added either (a) 50 ml 0.5 mol/l NaOH

or (b) 50 ml 2% $Na_2S_2O_5$, pH 12

The jars were loosely covered and heated at 80°C for 1 week. The celluloses were then washed and assayed by titration.

5.2.10 Determination of Ion Exchange Capacity (meq/g)

In these determinations, analysis was by potentiometric titration. The dry matter of the titrated ion exchanger was determined by collecting it on a preweighed dry scintered glass funnel, washing it with water and air drying overnight at 80-90°C. The ion exchange capacity was calculated on the basis of mmoles of groups per dry gram of ion exchanger.

(a) Cation Exchangers - CM and SP Celluloses

The ion exchanger was washed with 0.5 mol/l HCl and then water to convert it to the acid form. A sample (3-5g moist cake) was mixed with 0.5 mol/l NaCl (5ml) and titrated to pH 8 with 1N NaOH.

(b) Anion Exchangers (1) weakly basic:- DEAE, DE, DMA, TA, DAP, TETA, PA and PEI Celluloses.

The ion exchanger was washed with 0.5 mol/l NaOH and then water to convert it to the free base form. A sample (3-5g moist cake) was mixed with 0.5 mol/l NaCl (5ml) and titrated to pH 3 with 1 mol/l HCl.

(2) strongly basic:- QA, DQAP, DQAE, TMDAP, BDQ and TQA Celluloses.

A sample of ion exchanger (3-5g moist cake) in the hydroxide form was mixed with 0.5 mol/l NaCl (5ml) and 1.00 mol/l HCl (2.0 ml). Dry N_2 was bubbled through the solution for 10 minutes to remove any carbon dioxide. Sodium hydroxide (2.0ml, 1.00 mol/l) was added and mixed for 5 minutes, and then the solution was titrated to pH 10.3, then onto pH 3 with 1 mol/l HCl under a dry N_2 atmosphere.

(c) Titration Curves

Titration curves were run under the above conditions, but with the following modifications. The titration of the ion exchangers was carried out by the incremental addition (0.01 ml) of acid or base controlled by a programmable timer. The time between additions was such that equilibration of the ion exchanger with the solution occurred before the next increment was added. The titration curves were recorded on a Radiometer Servograph (REC 51) chart recorder. Titration curves for weakly acidic and weakly basic ion exchangers usually took 2-3 hours to run. Titration curves of strongly acidic and stongly basic ion exchangers tended to take 30-45 minutes.

5.3 EQUILIBRATION OF CELLULOSIC ION EXCHANGERS (SECTION 2)

5.3.1 Column Procedure

A Pharmacia K16/20 column was packed with cellulose ion exchanger to a bed height of 10 cm (20ml volume). A flow adaptor was fitted and the ion exchanger was pretreated prior to equilibration with each of the buffer systems. A flow rate of 1ml/minute was maintained throughout by the use of a peristaltic pump. Fractions (10ml) were collected and the pH of each was measured.

5.3.2 Pretreatment

Prior to each equilibration the ion exchanger was washed with either 0.1 mol/l NaOH or 0.1 mol/l HCl (50 ml), then with water (50 ml), to ensure all the groups were in the required form (see Table 5.2)

Table 5.2

Pretreatment of Ion Exchangers

Derivative	Required Form	Pretreatment (0.1 mol/l)
SP and CM	Sodium	NaOH
DEAE	Free Base	NaOH
QAE	Hydroxide	NaOH
DEAE	Hydrochloride	HCl
QAE	Chloride	HCl

5.3.3 Preparation of Buffer Solutions

(a) 0.05 mol/l buffer solutions

To make 1 litre of 0.05 mol/l buffer solution, the required weight of buffer was dissolved in water (900ml) and the pH of the solution adjusted to it's pKa with either (a) HCl for cationic buffers or (b) NaOH for anionic buffers. The solution was then made up to 1 litre with water in a volumetric flask.

(b) 0.01 mol/l buffer solutions

The above buffer solution (200 ml) was added to water (750 ml) and the pH adjusted, if necessary, to the pKa of the buffering species. The volume was then made upto 1 litre in a volumetric flask.

(c) 0.005 mol/l ammonium citrate;0.1 mol/l NaCl, pH 5.5

Citric acid (1.05g) and sodium chloride (5.84g) were dissolved in water (900ml) and the pH of the solution was adjusted to pH 5.5 with aqueous ammonia before making the volume up to 1 litre in a volumetric flask.

All buffer solutions were degassed prior to use.

5.4 ION EXCHANGE OF RENNET (SECTION 3)

5.4.1 Column Chromatography

(a) QA Cellulose (2ml) was packed into a disposable pasteur pipette and equilibrated with 0.1 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5. Dialysed rennet solution (10ml, dialysed against column buffer) was loaded on to the column at a flow rate of 8ml/hour and the breakthrough collected in a volumetric flask (10ml). The column was washed with equilibration buffer (10ml) and this also was collected. Elution was achieved by stepwise washing the column with the following solutions (5ml)

(i) 0.2 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5
(ii) 0.3 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5
(iii) 0.4 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5

Each fraction was assayed for rennet activity.

(b) The above column was equilibrated with 0.05 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5. A rennet solution, consisting of dialysed rennet as used above (5 ml) and 0.005 mol/l ammonium citrate, pH 5.5, was loaded and the breakthrough collected as above. The column was washed with column buffer (10 ml). Elution was achieved by stepwise washing the column with the following solutions (5 ml)

(1) 0.075 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5
(ii) 0.1 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5
(iii) 1.0 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5

Fractions were assayed for rennet activity.

5.4.2 Assay of Rennet Activity

The method of assay used was that described by Berridge(43) and modified by Clarke(20).

To a sample of milk substrate (10 ml) in a boiling tube incubated at $30^{o}C$ for 20 minutes was added the rennet solution (1 ml) to be assayed. The boiling tube was slowly rotated about it's vertical axis on a 30^{o} angle. The time taken from the addition of the enzyme to the first sign of gel formation was recorded.

5.4.3 Preparation of Milk Substrate

The milk substrate was made fresh each day by dissolving low heat treated skim milk powder (12g) in standard 0.01 mol/l $CaCl_2$ (100ml).

5.4.4 Preparation of 0.01 mol/l $CaCl_2$

This was prepared from a stock solution of approximately 0.1 mol/l $CaCl_2$ which had been standardised by the Mohr titration(44).

5.4.5 Rennet Capacity Tests

(a) Equilibraton of Ion Exchangers

Ion exchanger (QA, DE and A_2 Protion, 10-20 ml) in the hydrochloride (or chloride) form was washed 0.05 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5, on a scintered glass funnel, until equilibration was achieved. This being determined by comparing the pH and conductivity of the washings with that of the buffer solution. The matrix was then sucked dry on the funnel and transferred to a air tight storage container.

(b) Determination of Dry Matter

Triplicate samples (1g moist cake) of the equilibrated ion exchanger was accurately weighed into preweighed beakers (10 ml) and air dried in an oven at 80-90°C overnight. After cooling, the beakers were reweighed and the dry matter calculated on the basis of mg of dry ion exchanger per gram of moist cake.

(c) Capacity Test Procedure

Samples of equilibrated ion exchanger (equilivalent to 10,20,50 and 100mg dry weight) were weighed into screw capped vials (25 ml). Rennet (10ml), dialysed against more of the same buffer as the ion exchanger was equilibrated with, was added to each vial. After mixing for 2 hours at room temperature the vials were allowed to stand so that the ion exchangers settled to the bottom. The solution was assayed for rennet activity.

(d) Rennet Capcity Tests at pH 6.6

This was carried out as described above, except that 0.05 mol/l NaCl;0.005 mol/l imidazole, pH 6.6 was used instead of 0.05 mol/l NaCl;0.005 mol/l ammonium citrate, pH 5.5.

5.5 pH PROFILE STUDIES-BSA CAPACITY TESTS ON DEAE CELLULOSES (SECTION 3)

5.5.1 Equilibration of DEAE Celluloses

DEAE Celluloses (40ml) in the hydrochloride form were equilibrated on scintered glass funnels with 0.05 mol/l imidazole;0.05 mol/l pyridine, pH 7.0, then with 0.005 mol/l imidazole;0.005 mol/l pyridine, pH 7.0. The equilibrated DEAE Celluloses were sucked dry and stored in air tight containers. Their moisture content was determined by air drying triplicate samples (approximately 1g moist cake) in an oven at 80-90°C overnight.

5.5.2 Preparation of Buffer Solutions

(a) 0.5 mol/l imidazole;0.5 mol/l pyridine, pH 4.0

Imidazole (8.51g) and pyridine (9.6 ml) were dissolved in water (200 ml). Hydrochloric acid (2 mol/l) was used to shift the pH to 4.0 and finally water was added to make up the volume to 250 ml.

(b) 0.05 mol/l imidazole;0.05 mol/l pyridine, pH's 4.6 - 7.8

The above buffer (10ml) was added to water (80ml) and the pH shifted to pH 4.6 with NaOH (2 mol/l) before making up the volume to 100ml in a volumetric flask with water. This was repeated for pH's 5.0, 5.4, 5.8, 6.2, 6.6, 7.0, 7.4 and 7.8 to give a series of buffer solutions.

5.5.3 Prepartion of 0.5% BSA Solution

Bovine serum albumin (0.5g) was dissolved in water and made up to 100 ml in a volumetric flask.

5.5.4 pH Profile Test Procedure

Equilibrated DEAE Cellulose (approximately 400 mg moist cake) was accurately weighed into a screw top bottle (105ml). Water (60-70ml), buffer solution (10ml, 0.05 mol/l imidizole/0.05 mol/l pyridine, pH's 4.6-7.8), NaCl (0-10 ml, 1 mol/l) and BSA solution (20 ml, 0.5%) were added to give a total volume of 100ml of 0.1% BSA at the required pH and salt strength. The bottles were packed into a tin and mixed for 2 hours by rolling on a ball mill. The solutions were filtered through dry scintered glass funnels and the optical density 280 nm of the filtrate was measured against 0.005 mol/l imidazole;0.005 mol/l pyridine, pH 7 as the blank. Also measured was the pH of the filtrate. The protein capacity was calculated on the basis of grams of BSA removed/gram of dry ion exchanger.

5.6 COLUMN CHROMATOGRAPHY OF BSA ON DEAE CELLULOSES (SECTION 3)

5.6.1 Column Preparation

DEAE Celluloses were equilibrated with 0.01 mol/l imidazole, (for A_2 Protion and Whatman DE 52 at pH 6.1 and for DE Cellulose, the modified DEAE Cellulose and Whatman DE 52 at pH 7.0) on scintered glass funnels. The equlibrated DEAE celluloses were packed into a Pharmacia K16/20 column to a bed height of 15 cm (30ml bed volume). The columes were washed with 0.5 mol/l NaCl;0.01 mol/l imidazole, pH 6.1 (or pH 7.0) then re-equlibrated with 0.01 mol/l imidazole, pH 6.1 (or pH 7.0).

5.6.2 Preparation of 1% BSA Solutions

BSA (1g) was dissolved in 80ml of 0.01 mol/l imidazole, pH 6.1 (or pH 7.0), the pH was shifted back to the starting pH of the buffer and the solution was then made up to 100ml with 0.01 mol/l imidazole, pH 6.1 (or pH 7.0).

5.6.3 Column Procedure

BSA solution (10ml) was loaded onto the equlibrated DEAE Cellulose column and washed in with 30 ml of 0.01 mol/l imidazole, pH 6.1 (or pH 7.0). The column was then connected to a Pharmacia GM-1 gradient mixer and the bound protein eluted by a linear 0 - 0.3 mol/l NaCl gradient in 0.01 mol/l imidazole, pH 6.1 (or pH 7.0). In the mixing chamber was 200 ml of 0.01 mol/l imidazole, pH 6.1 (or pH 7.0) and in the limit buffer chamber was 200 ml of 0.3 mol/l NaCl;0.01 mol/l imidazole, pH 6.1 (or pH 7.0). A flow rate of 0.5 ml/minute was maintained by use of a peristalic pump. The conductivity and optical density 280nm were monitored continuously and recorded on a LKB 6 channel chopper bar recorder (chart speed 10 mm/hour).

5.6.4 Effect of Flow Rate on Resolution

1% BSA solution (5ml, in 0.01 mol/l imidazole, pH 7.0) was loaded onto the equlibrated DE Cellulose column. After washing with starting buffer (30 ml) the bound protein was eluted by the same gradient as described above, at a flow rate of 0.5 ml/minute. Further runs were carried out at flow rates of 1,2 and 3 ml/minute. The chart speed was also altered by the same factors so that identical lengths of chart paper were used in each run.

5.7 ION EXCHANGE ON QUATERNARY AMMONIUM CELLULOSES (SECTION 4)

5.7.1 Column Chromotography

The ion exchangers (QA, DQAP and TQA Celluloses, 5 ml) were packed into small glass columns (ID 0.9 cm). A constant flow rate of 0.85 ml/minute was maintained by a peristalic pump. Fractions were collected on a LKB fraction collector, set on 5 minute tube change. The conductivity of each fraction was recorded and the heparin or BSA content was determined. Elutions were NaCl gradients (200 ml)

(a) Heparin

(1) The above columns were equilibrated with 0.01 mol/l tris, pH 8.0. Heparin (10mg), dissolved in this buffer (10ml), was loaded and eluted with a linear NaCl gradient (0-2 mol/l in 0.01 mol/l tris, pH 8.0) generated by a Pharmacia GM-1 gradient mixer with 100ml in each chamber.

(2) The columns were equilibrated with a solution of 2% $Na_2S_2O_5$, pH 12. Heparin (10 mg), dissolved in this solution (10ml), was loaded. The columns were washed with 2% $Na_2S_2O_5$, pH 12 (25ml). Elution was by a linear NaCl gradient (0-1.5 mol/l for QA and DQAP and 0-2 mol/l for TQA, all in 2% $Na_2S_2O_5$, pH 12) generated by a GM-1 gradient mixer.

(b) BSA

The QA and DQAP columns were equilibrated with 0.01 mol/l tris, pH 8.0. BSA solution (10ml, 0.5% in 0.01 mol/l tris, pH 8.0) was loaded and eluted by a linear NaCl gradient (0-0.5 mol/l in 0.01 mol/l tris, pH 8.0). The BSA elution was followed by measuring the optical density at 280 nm of each fraction against 0.01 mol/l tris, pH 8.0 as blank.

5.7.2 Assay of Heparin

The method used was that described by Jaques and Wollin(45), with the following modifications. Instead of scanning the visible spectrum from 400-700 nm, the optical density at 505 nm was measured and, since the azur dye was only approximately 40% pure (approximately 60% methylene blue) the stock solution (100mg dye/100 ml) was diluted 25 ml to 100 ml instead of 9 ml to 100 ml.

5.7.3 Heparin Capacity Tests

(a) In the absence of salt

The ion exchangers were equilibrated with 0.005 mol/l Na_2HPO_4, pH 7 sucked dry and approximately 300 mg of the damp ion exchanger (moist cake) was accurately weighed into screw top vials (25 ml). Heparin (20 ml, 0.5% in 0.005 mol/l Na_2HPO_4, pH 7.0) was added to the vials and mixed on a end over end mixer for 2 hours. The ion exchangers were washed out of the vials, with phosphate buffer, into dry tared scintered glass funnels. The ion exchangers were washed with more buffer, sucked dry then air dried in a oven at 85°C overnight. Duplicate analysis was carried with each ion exchanger. The dry weight of the equilibrated ion exchanger used was determined by oven drying triplicate, 0.5g moist cake, samples overnight at 85°C. Heparin capacity (g/g) was calculated by weight difference.

(b) In the presence of salt

The effect of salt concentration, on heparin capacity, was investigated using a similar procedure as outlined above, with the following modifications. A series of sodium chloride solutions (0-0.5 mol/l in Na_2HPO_4, pH 7.0) were prepared and used to equilibrate samples of DQAP Cellulose. To equlibrated ion exchanger (300 mg moist cake) was added heparin solution (20ml, 0.5% in 0-0.5 mol/l NaCl in 0.005 mol/l Na_2HPO_4,pH 7.0) After mixing for 2 hours the ion exchanger was collected as above, washed with the appropriate solution, then dried as described above.

5.7.4 BSA Capacity Tests

The quaternary ammonium celluloses were equlibrated with 0.05 mol/l Na_2HPO_4, pH 7.0 then with 0.01 mol/l Na_2HPO_4, pH 7 on a scintered glass funnel. They were sucked dry and stored in air tight containers. Approximately 400 mg of equilibrated ion exchanger was accurately weighed into screw top vials (25 ml). Buffer (0.01 mol/l Na_2HPO_4, pH 7.0) was added (10ml), followed by BSA solution (10ml, 1% in 0.01 mol/l $NaHPO_4$, pH 7.0). This was mixed for 2 hours and then filtered through dry scintered glass funnels. The fitrate (2ml) was diluted to 10ml with buffer and the optical density at 280 mm measured against a buffer blank. The dry matter of ion exchanger used was determined by drying triplicate (0.5 - 1g moist cake) samples. Protein capacities calculated as grams of protein bound per gram of dry ion exchanger.

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