AN INVESTIGATION OF CELLULOSE ION EXCHANGERS

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Melvin John PETERSEN

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ABSTRACT

The aim of this thesis was to prepare a range of polysaccharide
ion exchangers and to explore their potential for use in chromatographic
methods and their ability to remove protein from solution.

The ion exchangers were prepared from regenerated cellulose cross
linked with epichlorohydrin and hydroxyalkylated with propylene
oxide. The preparations of the DEAE -, CM- and SP- derivatives were
investigated and the products shown to be chemically stable, to allow
high flow rates and to have excellent capacities for adsorbing protein.

Practical applications of these ion exchangers were demonstrated.
The DEAE-derivative was used for the chromatographic fractionation
of serum proteins and an enzyme purification. The CM- and SP- deriv-
atives were found to be useful for removing protein from whey. The
conditions, such as pretreatment of whey and pH, were investigated
to find the conditions necessary for good protein uptake and from
the results a new process was developed for efficient recovery of
protein from whey by ion exchange.

ACKNOWLEDGEMENTS

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PART A

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INTRODUCTION

1.1   Background

An ion exchanger is an insoluble material containing chemically
bound charged groups and mobile counter ions. If the matrix carries
positive groups the counter ions will be negative. Such an ion
exchanger will exchange negative ions and is therefore termed an
anion exchanger. In the same way, if the matrix carries negative
groups the counter ions will be positive. Since the positive ions
are exchangeable the term cation exchanger is used.

Ion exchangers have been around for a long time. As early as
1850, Thompson (1) and Way (2) reported the ability of soils to
exchange ions, such as ammonium for calcium. Other early developments
involved the demonstration that a number of natural minerals, part-
icularly the zeolites are capable of ion exchange (3-4). While research
continued on these points it was not until 1905 that Gans (5-6),
synthesized examples of inorganic ion exchangers. In 1935, one of the
most important events in the history of ion exchangers was the recognition
by Adams and Homes (7-8) that synthetic resins have ion exchange properties.
Since 1936 patents in this field have proliferated.

The ion exchangers which were first obtained by polycondensation
came to be replaced increasingly by polymerization products after 1945,
when d'Alelio (9) succeeded in incorporating sulfonic acid groups into
a cross-linked polystyrene resin. Further developments dealt with
improvements and the production of special resins with specific ion
exchange properties.

The phenomenon of separating specific compounds from a mixture
with the aid of ion exchangers was first called base exchange and was
interpreted as a chemical process in 1856 (10). The mechanism by which
separation is obtained on an ion exchanger is one of reversible adsorption.
In two stages there is the binding of substances to the ion exchanger
followed by the removal of these one at a time, separated from eachother.
Separation is possible since substances normally have different electrical

properties and are released from the ion exchangers by change in ionic strength or a shift in pH. Such a separation is today referred to as ion exchange chromatography.

The problem with synthetic ion exchangers with a few exceptions was their failure to be established as useful media for polyelectrolyte fractionation. Being specifically designed for application to problems involving inorganic ions, their molecular structure is inaccessible to polyelectrolytes of higher molecular weight. To have sufficient ion exchange groups accessible to polyelectrolytes on a macrosurface, would necessitate reducing the material to an impracticably small size. Also, synthetic resins show irreversible adsorption to proteins by forming too many electrostatic bonds with the protein, preventing the disruption of these bonds under elution conditions consistent with maintaining the configuration of the protein.

The use of macroreticular ion exchange resins in adsorbing biological substances has been subjected to a number of restrictions. Using the macroreticular ion exchange resin, Amberlite IRC-50, Pollio and Kunin (11) found the exchanger to be limited to substances of low molecular weight (m.w. 10,000 - 70,000). In comparing both Cytochrome C, (m.w. 11,000 - 13,000) and Lysozyme (m.w. 14,000 - 19,000) it was found that such factors as the size of the organic molecule and resin particle size had a great effect on the rate and capacity of these types of ion exchangers. For larger molecules, for example Haemoglobin (m.w. 68,000) adsorption was considerably more difficult (11, 12). Overall macroreticular synthetic ion exchangers have a limited capacity for proteins.

1.2   Celluloses with Ion Exchange Properties.

Early cellulose based ion exchangers included, Oxycellulose (a weakly acidic carboxyl type ion exchanger) (13, 14), cellulose succinic half esters (15) and a variety of treated cottons (16, 17). The diethyl-aminoethyl ether of cellulose was prepared in 1930 (18) and subsequently by Hoffpauir and Guthrie (16) by heating a mixture of β-chloroethyldiethyl-amine with alkali cellulose.

Scheme 1

CH_2OH

etc

OH

CH_2OH

OH

CH_2OH

OH

etc

$\xrightarrow{\text{NaOH}}$ Cellulose $-O^-$ Na^+

Cellulose

Alkali Cellulose

Cellulose $-O-CH_2-CH_2-N\begin{cases} C_2H_5 \\ C_2H_5 \end{cases}$

$Cl-CH_2CH_2N(C_2H_5)_2$

DEAE-Cellulose

Cellulose-O^-Na^+

$ClCH_2CO_2H$ $\longrightarrow$ Cellulose-$O-CH_2CO_2H$
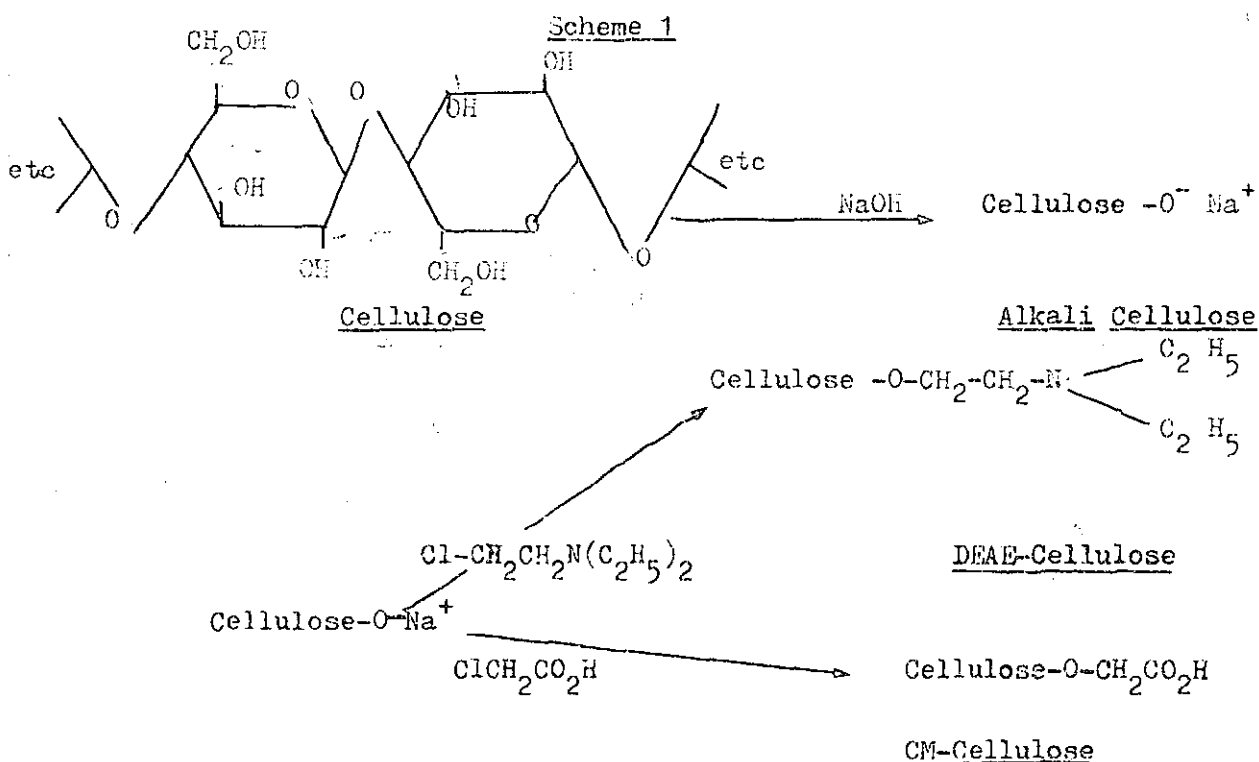
CM-Cellulose

However, the chromatographic separation of polyelectrolytes such as proteins did not really become possible until Peterson and Sober (19-21) prepared the cation exchanger, carboxymethyl-cellulose (CM) by treating strongly alkali cellulose with chloroacetic acid and the anion exchanger, diethylaminoethyl-cellulose (DEAE) by treating strongly alkali cellulose with 2-chlorotriethylamine hydrochloride. Both the phospho-cellulose (P) and epichlorotriethanolamine-cellulose (ECTEOLA) were prepared as well. Using their preparations. made with cellulose powder, they used the column situation in the separation and concentration of proteins.

Since this initial work others have studied cellulose based ion exchangers. Guthrie and Black (22), found ion exchange celluloses able to combine a relatively low total binding strength with an adequate capacity for polyelectrolytes. The products being finer, presented a larger surface area than ordinary resins and also their open and porous structure allowing larger molecules to enter. Fractionation of serum proteins using DEAE-Cellulose as investigated by James and Stanworth (23), who observed an increase in specific adsorption capacities with degree of substitution. A relatively high capacity was found. They did not elaborate whether increased capacity was due to an increased number of adsorption sites on the ion exchanger or to an increased selectivity. Peterson and Sober performed similar experiments but with a lower capacity being obtained.

TABLE 1

Sephadex derivatives available

TYPE	USEFUL pH RANGE	FUNCTIONAL GROUPS	IONIC FORM	DESCRIPTION
DEAE	2-9	Diethylaminoethyl	$-C_2H_4N^+(C_2H_5)_2H$	Weak base:anion exchanger
QAE	2-10	Diethyl-(2-hydroxy-propyl)amino-ethyl.	$-C_2H_4N^+(C_2H_5)_2$ $CH_2CH(OH)CH_3$	Strong base:anion exchanger
CM	4-10	Carboxymethyl	$-CH_2COO-$	weak acid:cation exchanger
SP	2-10	Sulphopropyl	$-C_3H_6SO_3$	strong acid:cation exchanger

Staelhelim et al (24) and Tenser et al (25) studied the strength of interaction between ion exchanger molecules and the adsorbed polyelectrolytes and found this to be primarily dependent on the cumulative electrostatic binding between oppositely charged sites on the two.

There are a number of cellulose derivatives available today as ion exchangers. The most important of these are the DEAE and CM Celluloses. The diethylaminoethyl cellulose is prepared by heating alkali cellulose with B-chloroethyldiethylamine hydrochloride (CED). The carboxyl cellulose is prepared by reacting alkali cellulose with chloroacetic acid as shown in scheme 1.

There are a number of DEAE celluloses commercially available, with small ion capacities in the range of 0.1 - 1.1 meq/g. Other available celluloses include the triethylaminoethyl (TEAE), $-O-CH_2-CH_2-N^+(C_2H_5)_3$; quaternary aminoethyl (QAE), $-C_2H_4N^+ (C_2H_5)_2CH_2CH(OH)CH_3$; epichlorotriethanolamine (ECTEOLA); and phospho (P), $-O - \overset{O}{\underset{OH}{\overset{|}{P}}} - OH,$

with various small ion capacities.

The other main ion exchangers used for protein fractionation are those based on spherical beads of cross-linked dextran and marketed under the name of Sephadex. The derivatives available are shown in Table 1.

Other polysaccharides such as Starch and Agarose have also been used as the matrix for ion exchangers after being stabilized by crosslinking.

1.3 Choice of Ion Exchanger and Conditions of Use

The choice of ion exchanger depends on the net charge of the substance to be chromatographed. Substances which carry both negatively and positively charged groups are amphoteric and their net charge is thus dependent on pH. At low pH the net charge is positive, at high pH it is negative. At the point of zero net charge, the isoelectric point (IEP), the substances are not bound to any type of ion exchanger. Proteins are amphoteric polyelectrolytes and can normally be bound to both anion and cation exchangers by a suitable choice of conditions. The net charge on a typical protein as a function of pH is shown in Figure 1.

Figure 1

The net charge of a protein as a function of pH
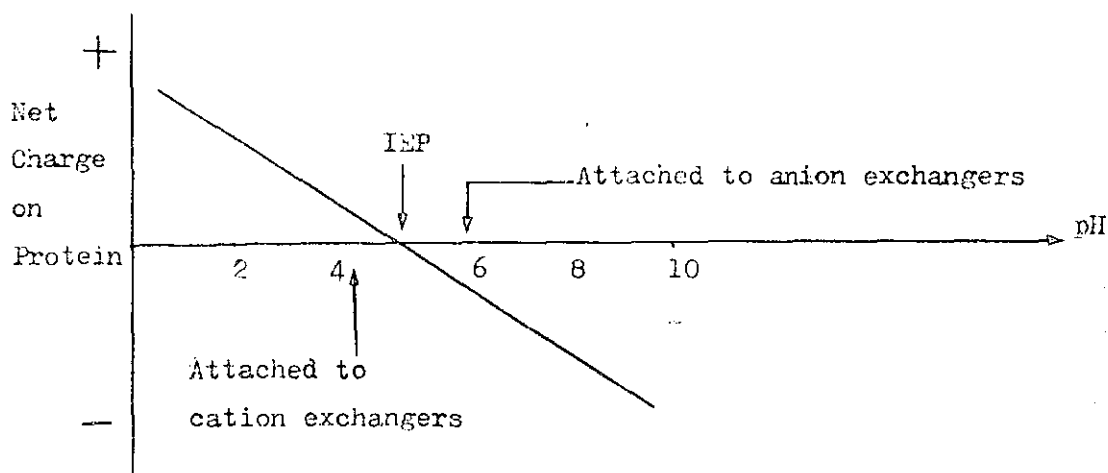
It can be seen that below the IEP the protein has a net positive charge and is therefore adsorbed by cation exchangers. Above the IEP the protein can be adsorbed by anion exchangers since it carries a net negative charge. The choice of ion exchanger maybe determined by the range of stability of the protein. The ion exchange derivative used will be one that has the correct charge to bind the protein within the pH range of stability of the protein used.

The protein adsorbed on the ion exchanger can be eluted from the ion exchanger by shifting the pH so that the charge on the protein is changed or by raising the ionic strength which increases the competition for charged groups on the ion exchanger and thus reduces the interaction between the ion exchanger and the protein thereby causing their elution.

1.4 Regenerated Cellulose Ion Exchangers

The use of cellulose based ion exchangers is restricted due to their fibrous structure. These ion exchangers usually suffer from the disadvantage of poor hydraulic properties. They generally have low flow rates and tend to become easily clogged by particles of suspended matter. These difficulties were overcome by Grant (26) who used regenerated cellulose containing chemical cross-links as a matrix to which ionizable groups were attached.

Regenerated cellulose is prepared from natural cellulose by anyone of several processes. The Xanthate process, discovered in 1893 (27), involves rendering cellulose soluble by reaction with NaOH and carbon disulphide to form sodium xanthate. The sodium cellulose xanthate is soluble in caustic soda solution and gives a solution known as "viscose".

<u>Xanthation</u>   R Cell ONa + CS_2 ---------------> R Cell OCSSNa

<u>Regeneration</u>   2R Cell OCSSNa + H_2SO_4 ----------->2R CellOH +Na_2SO_4+$2CS_2$
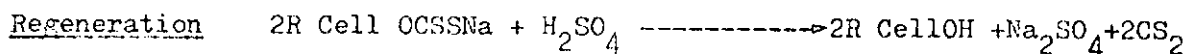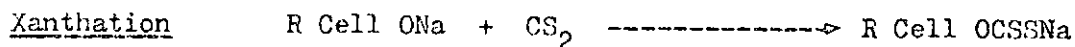
Regeneration may be affected by heat or acid and it is possible to considerably modify the microstructure and physical properties of the regenerated cellulose by varying the "viscose" composition and regeneration conditions.

Regenerated cellulose was proposed as an ion exchange material in 1959 (28). It was of no practical value since no cross-linking was proposed. The use of cross-linking agents to inhibit solubilization of regenerated cellulose ion exchangers was investigated by Selegny <u>et al</u> in 1966 (29). Murphy (30) investigated the reaction with isocyanate and amines to produce cross-linked ion exchange material from natural cellulose and regenerated cellulose.

The major work on use of regenerated cellulose for ion exchange preparation was that introduced in 1968 by Grant (26). Grant proposed the making of an ion exchanger comprising the introduction of cross-linking residues into regenerated cellulose together with or followed by the introduction of groups capable of anion or cation exchange. Groups capable of anion exchange suggested by Grant were: amino, alkylamino and quaternary ammonium groups. Groups capable of cation exchange were sulphonic acids, phosphate and carboxyl groups.

According to Grant, for both the cross-linking reaction and the introduction of exchange groups, the water content of the reaction

mixture should be carefully controlled to give optimum results. In
general the water content preferably should be in the range, 50 to 100
percent of the weight of regenerated cellulose. It should be introduced
with the exchange groups and depends somewhat on the grain size of the
cellulose used.

Since Grant's patent other patents have appeared for ion exchangers
based on cross-linked regenerated cellulose. These involve a modif-
ication of the Xanthate process. One of these (31), involved the
introduction of ionizable groups at the soluble Xanthate stage before
regeneration of the cellulose. This allows the regenerated cellulose
exchangers to be produced in a variety of forms such as sponges, fibres,
rods, filaments and yarn as well as particles as used by Grant. Other
patents (32, 33) describe the production of "cellulose pearls" by re-
generating the cellulose after an emulsion of the xanthate solution
had been formed in an organic solvent. This gave highly swollen beads
with excellent protein capacities (1000 - 2000 mg/g) but the flow
rate through the ion exchangers when packed in a column are not as
high as those obtainable with Grant's product.

Since 1969 the commercial development of the Grant ion exchangers
has been hampered by their low protein capacity, i.e. 200 - 500 mg/g.,
but the potential is there for large scale application because of their
robustnature, low attrition properties and high flow rates of ground
regenerated cellulose particles.

Sheerin (34) first noted the ability of propylene oxide to swell
regenerated cellulose. Instead of using the difficult regeneration
procedure of Pharmacia (32, 33) with organic solvents to produce highly
swollen regenerated cellulose the effect of propylene oxide on particles
of ground regenerated cellulose has been investigated further in this
thesis to see if improved protein capacities could be obtained for
the ion exchangers. Although many commercial ion exchangers are available
for work in extracting protein from solution, ones with hydroxypropyl
groups attached to the cellulose back bone have not been reported before.
The anion exchangers, DEAE-hydroxypropylated regenerated cellulose, and
cation exchangers, CM- and Sulphopropyl hydroxypropylated regenerated
cellulose have been investigated to find preparations with good protein
capacity, reasonable water volume and stability to repeated use. The
preparation of the cation exchangers were investigated for use in
extracting protein from whey by use of a large column of ion exchanger.

SECTION 2

EXPERIMENTAL

2.1 Materials

Regenerated cellulose (ENKA, 75 - 150 u) and DEAE-Protion were obtained from Tasman Vaccine Laboratories Ltd; epichlorohydrin from BDH Chemicals Ltd; Propylene oxide from Koch Light Laboratories Ltd; B-chloroethyldiethylamine hydrochloride (CED) from Sigma Chemical Company; Monochloroacetic acid from May and Baker Ltd; 1,3-propane Sultone from Aldrich Chemicals Ltd and SP-Sephadex-G25 from Pharmacia, Sweden. Vistec C_1 was obtained from Koch Light Laboratories Ltd.

2.2 Methods of Ion-Exchanger Preparation

2.2.1 DEAE-Hydroxypropylated Regenerated Cellulose, (DEAE-HP-Regcel)

Regenerated cellulose (10 g) was mixed with a reagent containing propylene oxide (5 ml), epichlorohydrin (0.4 - 0.8 ml) and aqueous sodium hydroxide (10 - 15 ml, 20 - 30% w/v). Mixing was thorough and was continued until all the moisture had been absorbed. The mixture was then transferred to a screw capped steel bomb (4 x 8 cm), and was placed in a water bath at 60° C for 1 hr and then cooled. A solution of CED (4 - 7 g) in water (10 ml) was thoroughly mixed with the contents of the bomb, and then the mixture was returned to the bomb and allowed to stand in a water bath at 100° C for 1 hr.

The product was then slowly added to a large excess of water (1 l.), filtered and washed with 0.1 M HCl (200 ml). It was soaked with 0.1 M HCl (200 ml) for 30 minutes and then washed with deionized water until the pH of the washings was 4.5. Final treatment was a wash with 0.1 M NaOH (400 ml), followed by deionized water until the washings were at pH 9.8.

The products were stored wet at 4°C after being sucked dry on a sintered glass filter funnel.

2.2.2 DEAE-Hydroxyethylated Regenerated Cellulose, (DEAE-HE-Regcel)

Modifications were made to the DEAE-HP-Regcel method because of the use of the volatile ethylene oxide.

The regenerated cellulose (10 g) was mixed with aqueous sodium hydroxide (15 ml, 30% w/v) and allowed to cool in the freezer for 1 hr. The ethylene oxide (5 ml) was cooled and mixed with the epichlorohydrin (0.8 ml) and then carefully mixed into the swollen cellulose. The mixture

was then transferred to a screw capped steel bomb and placed in a water
bath at 65°C for 1 hr. A solution of CED (6 g.) in water (10 ml.) was
mixed with the contents of the bomb, and the mixture returned to the
bomb and allowed to stand in a water bath at 100°C for 1 hr.

The washing and regeneration procedure was that followed for the
DEAE-HP-Regcel preparation.

2.2.3   CM-Hydroxypropylated Regenerated Cellulose (CM-HP-Regcel)

In a one step reaction, regenerated cellulose (10 g.) was mixed with
a reagent containing propylene oxide (5 ml.), epichlorohydrin (0.25 –
0.8 ml.), aqueous sodium hydroxide (10 – 15 ml, 20 – 30% w/v) and
monochloroacetic acid (1.5 – 4.5g). Mixing was done thoroughly until
the cellulose had absorbed all the moisture. The mixture was then
transferred to a screw capped steel bomb and placed in an oven at
60° C for 1 hr, followed by placing in a water bath at 100° C for 1 hr.

The mixture was then slowly added to an excess of water (1 l.),
filtered and washed with 0.1 M HCl (1 l.). Final treatment was by
washing with deionized water until the pH of the washings was 3.6.

The products were stored wet at 4°C, after excess water had been
removed on a sintered glass funnel.

2.2.4   SP-Hydroxypropylated Regenerated Cellulose, (SP-HP-Regcel)

The method used was similar to that used for the CM-HP-Regcel
preparations above. Special care was taken in handling the carcinogenic
1,3-propane sultone.

A mixture containing propylene oxide (5 ml), epichlorohydrin
(0.4 – 0.8 ml) and 1,3-Propane sultone (3 g) was added to an aqueous
sodium hydroxide solution (8 – 15 ml, 20 – 40% w/v), and then thoroughly
mixed into regenerated cellulose (10 g). When all the moisture had been
absorbed, the mixture was transferred to a steel capped steel bomb
and placed in an oven at 60° C for 1 hr, then into a water bath at
100° C for 1 hr.

After cooling, the mixture was slowly added to an excess of water
(1 l.), filtered and then treated with 0.5M HCl (500 ml) and washed
with deionized water until the pH of the washings was 3.6. Final
treatment was by washing with 0.5 M NaOH (500 ml), followed by deionized
water until the pH of the washings was 9.8.

The products were stored wet at 4°C after being sucked dry on a
sintered glass filter funnel.

2.3　　　　　Determination of Ion Exchange Capacity (meq/g)

In these determinations analysis by potentiometric titration was chosen. In order to determine the moisture content of the ion exchangers, duplicate wet samples (1 g) were accurately weighted into small beakers and allowed to air dry overnight at $65^{o}C$.

2.3.1　　　　　Anion Exchangers: DEAE-HP-Regcel

A sample of the ion exchanger in the wet state (1 g) in the free base form, was mixed with 0.25M NaCl (20 ml) and titrated to pH 4 with 0.1 M HCl. The ion exchange capacity (meq/g) was calculated on the basis of the dry weight content of the sample used.

2.3.2.　　　　　Cation Exchangers: CM- and SP- HP- Regcel

A sample of the ion exchanger in the wet state (1 g), in the hydrogen form, was mixed with 0.25M NaCl (20 ml) and titrated to pH 9 with 0.1M NaOH. The ion exchange capacity was calculated as meq/dry g.

2.4　　　　　Determination of Chemical Stability of Ion Exchangers.

Initially different conditions of increasing severity were used to try to dissolve the ion exchanger. These were:

(a) 2.5% NaOH, at room temperature for 1 hr.

(b) 2.5% NaOH. at 60° C for 1 hr.

(c) 10% NaOH, at room temperature for 2 hrs.

The following procedure was used.

Duplicate samples (approximately 1g) of wet ion exchanger were accurately weighed out into small screw capped vials and the sodium hydroxide solution (15 ml) added. The sample was degassed for 30 minutes and then turned end over end for 2 hrs at room temperature (or left standing at $60^{o}C$). The samples were then each washed on sintered glass filter funnels and air dried at $65^{o}C$ overnight. The dry weight of the original samples was determined by taking another pair of samples and drying them under the same conditions.

2.5　　　　　Preparation of Haemoglobin Solution.

The red cells were collected from fresh citrated ovine blood by centrifugation and washed twice with 0.9% saline solution. They were then haemolysed by dialysis against distilled water. The haemoglobin solution was removed from the cell debris by centrifugation and stored frozen until required. It was diluted down to approximately 0.5% with 0.1 M Acetate buffer; pH 5, before use.

2.6   Determination of Protein Capacity of Ion Exchangers

The CM-HP-Regcel was pre-equilibrated with 0.01 M sodium acetate buffer, pH 5, but the others were used unequilibrated as it was found not to be necessary. Only 10 mg/10 ml of protein solution was used and the pH never shifted by more than 0.1 pH units on addition of the unequilibrated ion exchanger to the protein solution.

2.6.1.   Cation Exchanges: Batch Method using Haemoglobin

A haemoglobin solution was used to determine the capacity of both CM- and SP-HP-Regcel. A sample of the wet ion exchanger equivalent to 10 mg. dry weight was shaken end over end with the haemoglobin solution for 2 hrs. The suspension was allowed to settle and a 250 μl sample was drawn off and diluted to 5 ml with deionized water for an optical density reading at 280 nm. The protein concentration remaining was determined from a standard curve prepared by dilution of the original haemoglobin solution.

In the case of CM-HP-Regcel, samples were also taken at other times up to 4 hrs to determine the rate of uptake of protein.

2.6.2.   Anion Exchangers: Batch Method using Bovine Serum Albumin

A sample of the wet ion exchanger equivalent to 40 mg dry weight was mixed end over end with 20 ml of 0.5% BSA in 0.01 M TRIS buffer; (pH 7.5) for 2 hrs. The suspension was allowed to settle and 250 μl was withdrawn and made up to 5 ml in a volumetric flask with deionized water for an optical density reading at 280 nm. The protein concentration was determined from a standard curve.

2.7   Methods Used for Loading Columns with Protein: Column Capacity

2.7.1   DEAE-HP-Regcel

Into disposable Pasteur pipettes (2 ml) the ion exchanger was loaded so as to occupy approximately three quarters of the volume of the pipette. Equilibration of the column was carried out with 0.2 M Phosphate buffer (pH 7), followed by 0.005M phosphate buffer (pH 7). A protein solution, 1% BSA in 0.005M phosphate was then passed through the column until near saturation of the column was indicated by the optical density reading at 280 nm of the eluate being the same as that of the solution loaded. Unbound protein was washed from the column by loading buffer. The protein which passed through the column was collected in a volumetric flask (25 ml). The protein bound to the column was stripped from the column using 1MNaCl. Analysis of the protein stripped from the column, to determine capacity, was done by diluting the protein solution (1 ml) to 10 mls with deionized water and measuring the O.D at 280 nm and comparing the result with a standard curve.   The weight of ion exchanger used in each determination

was determined by washing the ion exchanger from the glass pipette into weighed sintered glass funnels and drying overnight in an oven at $65^{\circ}C$.

2.7.2.   CM-HP-Regcel. Vistec C_1 and CM-Protion.

Into disposable Pasteur pipettes (2 ml), samples of the ion exchanger preparations (1 ml in volume) were packed and then equilibrated with 0.01M sodium acetate buffer at pH 5. Using a peristaltic pump to control flow rate, a haemoglobin solution of 3.3 mg/ml in 0.01M acetate buffer pH 5 was loaded onto the column to a stage where near saturation was obtained (determined by visual observation). The bound protein was then stripped using the stripping agent, (0.5 M $NaHCO_3$ plus 0.5M NaCl ; pH 9.0), at a flow rate rate (0.2 - 0.3 ml/min) and made up to 5 ml in a small volumetric flask. Analyses of protein was done by diluting 1 ml of protein solution to 10 ml with deionized water and taking an optical density reading at 280 nm.

2.8   Fractionation of Serum using DEAE-HP-Regcel

The method followed was that introduced in 1966 by Himmelhoch and Peterson (35) with a few modifications.

2.8.1   Serum Preparation

Blood (450 ml) was obtained in a transfusion pack from Palmerston North Hospital. This pack also contained glucose and disodium citrate (2 g) in 70 ml of solution to prevent clotting. Plasma (70 ml) was removed from the pack and calcium nitrate (0.22 g) was dissolved in it. A dialysis sack, prepared according to Peterson and Chiazze (36) was used to dialyse the plasma against 0.1% NaCl for 3 hrs at $4^{\circ}C$, followed by dialysis overnight at $4^{\circ}C$ against starting buffer, 0.04M Tris plus 0.005 M Succinic acid, pH 8.3. The precipitated fibrinogen was centrifuged out at 3,000 r.p.m. for twenty minutes and the pale pink serum collected.

2.8.2   Equilibration of the Column

The DEAE-HP-Regcel (4.5 g dry weight) was packed in a column (1.6 x 40 cm) in 0.5M NaCl and washed with 100 ml of final buffer (0.3 M Tris and 0.3 M Succinic acid; pH 4.3). It was then equilibrated with starting buffer (0.04 M Tris and 0.005 M Succinic acid; pH 8.3).

2.8.3   Serum Loading and Elution

The serum (4 ml) was applied through the flow adaptor to the top of the column at a flow rate of 1 ml/min. Elution with starting buffer was continued until peak 1 (Himmelhoch and Peterson, Fig. 1A, p. 387)

appeared. Normally about 60 - 80 ml were required. A gradient system was then used, comprising a mixing chamber of starting buffer (400 ml) and a side chamber of final buffer (80 ml) at a flow rate of 80 ml/hr. At the end of the gradient, final buffer (150 - 170 ml) was passed through the column. The adsorbance of the column eluate at 280 nm was recorded.

2.9   Purification of Aldehyde Dehydrogenase on DEAE-HP-Regcel.
2.9.1   Method of Enzyme Preparation according to Crow et al (37)
2.9.2   Equilibration of DEAE-HP-Regcel

The buffers used were prepared according to Dawson et al (38) with a few modifications.

The ion exchanger (27.8 gm wet) was added to a solution of 0.1 M KH_2PO_4 containing 0.1% Mercapto ethanol and then adjusted to pH 4.5 with concentrated HCl. The suspension was then placed in a Buchner flask and degassed. Sufficient 0.1 M NaOH was then added to the suspension to alter the pH back to 7.3. The ion exchanger was then packed into a column (1.6 x 40 cm) and equilibrated with the following solutions, all containing 0.1% mercapto ethanol and at pH 7.3.

   (a)   100 ml of 0.05M NaH_2PO_4 plus 0.05M NaOH.
   (b)   100 ml of 0.01M NaH_2PO_4 plus 0.01M NaOH.
   (c)   100 ml of 0.005M NaH_2PO_4 plus 0.005M NaOH.

2.9.3.   Purification of Aldehyde Dehydrogenase

The enzyme preparation (42 ml) was loaded via a flow adaptor to the top of the column at a flow rate of 0.3 ml/min using a peristaltic pump. A wash (60 ml) of loading buffer, (0.005M Phosphate, pH 7.3), was passed through at the same flow rate. A gradient system was then used; the mixing chamber containing loading buffer, 100 mls. of 0.005M phosphate buffer, and the side chamber, 100 mls of 0.1 M phosphate buffer. This was passed through at a flow rate of 1 ml/min. The eluate from the column was collected in 6 ml fractions. A final wash of buffer, 0.1 M phosphate, pH 7.3 (100 ml) followed by 1M NaCl (100 ml) were passed through the column. Absorbance at 400 nm, protein concentration, conductivity and enzyme activity were measured on the fractions collected.

2.9.4   Method of Protein Determination

The protein concentrations in the fractions were estimated by the method of Lowry et al (39).

2.9.5   Assay for Aldehyde Dehydrogenase Activity

The method of Crow et al (37) was used to determine the enzyme activity in the fractions.

SECTION 3

RESULTS AND DISCUSSION

3.00   Preparations and Properties of HP-Regcel Ion Exchangers

3.1   DEAE-HP-Regcel

3.1.1   Introduction

Previous investigations carried out on the production of Protion, a
commercially available DEAE-ion exchanger prepared from regenerated cellulose,
have shown that the amount of water present when the functional groups are
attached is critical. This was part of the basis of Grant's Patent (26),
but the protein capacities exhibited by these products were still low,
e.g. 300 - 500 mg./g. The capacity of protion was improved by sodium
hydroxide swelling, but this gave an unstable product to caustic solutions
(40).

Hydroxypropylation was used successfully by Sheerin to swell regen-
erated cellulose (34). Consequently, hydroxypropylation was investigated
in this thesis to see if more swollen ion exchangers could be prepared from
regenerated cellulose. The main property required of these products was
an improved protein capacity greater than $1g/1g$, and yet it had to be
achieved without loss of chemical stability or too great a loss of flow
rate through the product. The reason for maintaining chemical stability
is to enable the regeneration and revitalization of the ion exchangers to
be carried out many times without loss of product. The high flow rates
possible through columns of Protion ion exchangers were their main
advantage over other commercially available ion exchangers.

The DEAE groups cannot be introduced at the same time as hydroxy
propylation or the DEAE groups are quaternized, either before or after
attachment to the cellulose matrix (41). (See Reaction Scheme II).

SCHEME II

R-OH + Cl-CH$_2$-CH$_2$-NEt$_2$ $\xrightarrow{\text{NaOH}}$ R-O-CH$_2$-CH$_2$-NEt$_2$

(CED)

CH$_3$-CH-CH$_2$ CH$_3$-CH-CH$_2$
       \O/   \O/

                   + Et   + Et
                   | |
R-OH + Cl-CH$_2$-CH$_2$-N-CH$_2$-CH-CH$_3$ $\xrightarrow{\text{NaOH}}$ R-O-CH$_2$-CH$_2$-N-CH$_2$-CH-CH$_3$
                   | | \Et |
                   Et   OH   OH

R = Cellulose matrix
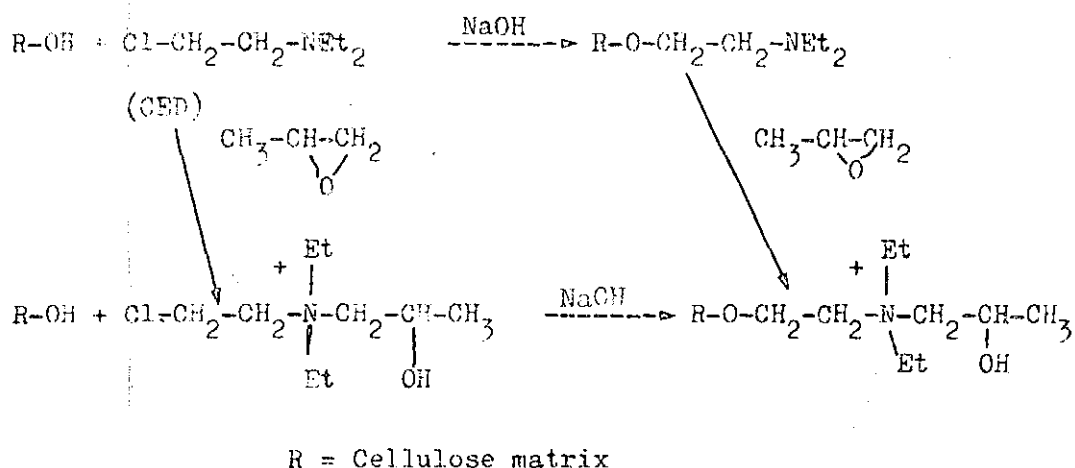
Consequently hydroxypropylation was carried out prior to DEAE group introduction. To do this the procedure of Sheerin (34) was used to cross-link and hydroxypropylate the regenerated cellulose with propylene oxide and epichlorohydrin in the presence of 30% sodium hydroxide and then the β-chloroethyldiethylamine hydrochloride (CED) was added. Very little of the sodium hydroxide was consumed in the first step and so there was sufficient present to both neutralize the hydrochloride and cause the cellulose to react with the CED in the second step as shown in Reaction Scheme III.

SCHEME III

HO-R-OH + CH$_3$-CH-CH$_2$ $\xrightarrow{\text{NaOH}}$ R-O-CH-CH-CH$_3$
                \O/   OH | OH

                                                       | Cl-CH$_2$-CH$_2$-NEt$_2$
                                                       | NaOH
                                                       ↓

    R-O-CH$_2$-CH-CH$_3$ or   R-O-CH$_2$-CH-CH$_3$
    OH | | OH
             O   O
             | |
             CH$_3$-CH-NEt$_2$ CH$_2$-CH$_2$NEt$_2$

R = cellulose matrix (crosslinks not shown)
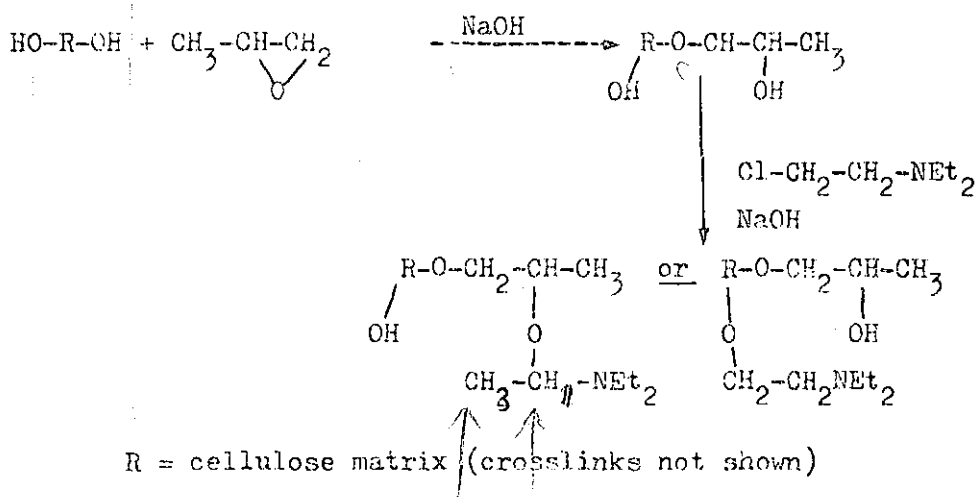
3.1.2.   Introduction of DEAE Groups.

Preliminary experiments were carried out to determine if the amount of water used in the reaction to introduce DEAE groups was critical. This was done as described in section 2.2.1 using 4 g. of CED which was,

(a)   added as a dry powder

(b)   added as a solution in 10 ml. of water

The protein capacities were similar in the two cases, (a) 0.53 g/g. and (b) 0.63 g/g. Consequently all further preparations used 10 ml of water to dissolve the CED.

3.1.3   Effect of the Amount of Reagent (CED) in the Reaction

Following the basic recipe of section 2.2.1, using 15 ml of 30% NaOH, 8% crosslinking and 50% hydroxypropylation, the amount of CED was varied in order to obtain a sufficient degree of substitution of charged groups on the matrix i.e. 1-2 meq/g. The results of this are shown in Table 2.

TABLE 2

Effect of the amount of CED used

Regenerated cellulose : 10 g
30% NaOH   : 15 ml.
Epichlorohydrin   : 0.6 ml.
Propylene Oxide   : 5 ml.

PREPARATION	CED (g)	YIELD (g)	Meq/g.	CAPACITY g/g.[1]
1	4	-	0.86	0.63
2	5	8.8	1.15	1.12
3	6	8.8	1.43	1.28
4	7	8.8	1.65	1.30

1. Protein capacity determined as in sec. 2.7.1.

It is evident that at least 5 g. of CED is required under these conditions. Larger amounts increase the ion exchange capacity towards 2 meq/g but this has little effect on the protein capacity. This levelling off in protein capacity could be a result of a decrease in the amount of excess NaOH left at the end of the reaction. There is also the fact that the introduction of further groups does not always introduce further sites for protein binding.

Another series of preparations was carried out using less caustic i.e. 10 ml of 30% NaOH, instead of 15 ml. The results are shown in Table 3.

TABLE 3

Effect of the Amount of CED used

Regenerated cellulose:	10 g.
30% NaOH :	10 ml.
Epichlorohydrin :	0.8 ml
Propylene Oxide :	5 ml.

PREPARATION	CED (g)	Meq/g.	CAPACITY g/g[1]
1	4	1.16	1.12
2	5	1.35	0.9
3	6	1.54	0.9

1. Protein capacity determined as in sec. 2.7.1.

The drop off in capacity is probably a result of the CED-hydrochloride neutralizing too much of the NaOH to swell the product. Consequently preparations using 15 ml. of 30% NaOH were used for further investigation, (sec. 3.1.6.).

3.1.4.   Comparison of Hydroxyalkylating Reagents: Propylene and Ethylene Oxide.

According to the procedure outlined in sec. 2.2.1, preparations using 15 ml. of 30% NaOH, 8% crosslinking and 50% hydroxyalkylation (either propylene oxide or ethylene oxide) were prepared. The results obtained gave yields of 8.6 g for DEAE-HP-Regcel and 12 g for DEAE-HE-Regcel. Both preparations had similar swollen volumes of 8.6 ml/g and similar ion exchange capacities of 1.2 meq/g. Either ethylene oxide or propylene oxide can be used satisfactorily. But we used the latter as it was more convenient to handle.

3.1.5   Chemical Stability Tests

Since these ion exchangers need to stand up to repeated use and regeneration which often requires strong alkali a simple test was required in order to determine the products likely stability under such conditions.

When 1% NaOH plus 3.5% NaCl was used to soak the ion exchangers the losses were so small that the test needed to be repeated 10 - 20 times to detect them. Consequently an accelerated chemical test was required and Table 4 shows the results of three different conditions tried.

TABLE 4

Effect of alkali on stability of the Ion Exchanger: DEAE-HP-Regcel 6 -50

CONDITION	LOSS (%)
1. 2.5% NaOH at R.T. for 1 hr.	-
2. 2.5% NaOH at 60°C for 1 hr.	4
3. 10% NaOH at R.T. for 2 hrs.	8

4-5% NaOH at 60° C was used by Tasman Vaccine Laboratories to revitalize the Protion after every 20 - 50 cycles of operation and was known to slowly dissolve it. 10% NaOH was more damaging than this so was used as a measure of the likely relative stabilities of the products made.

3.1.6 Variations in Cross-linking and Hydroxypropylation.

With preliminary investigations completed it was decided to run a series of preparations with different amounts of propylene oxide and epichlorohydrin to optimize the reaction conditions. The results of these preparations are shown in table 5.

TABLE 5

Effect of E.C.H. and P.O. on the Reaction Product

Regenerated cellulose : 10 g.

30% NaOH : 15 ml.

Epichlorohydrin : 0.5 - 1.0 ml.

Propylene oxide : 3 - 7 ml.

CED : 6 g.

PREPARATION[a]		E.C.H. (ml)	P.O. (ml)	YIELD (g)	SWOLLEN VOLUME (ml/g)	CAPACITY[b] g/g.	STABILITY(%)[c] loss
1.	10 - 70	1	7	10.6	9.6	1.13	1
2.	9 - 70	0.9	7	10.2	9.6	1.08	0
3.	8 - 70	0.8	7	10.1	10.2	1.38	1
4.	7 - 70	0.7	7	10.1	10.9	1.41	4
5.	9 - 50	0.9	5	10.5	7.6	0.54	2
6.	8 - 50	0.8	5	10.3	9.5	1.28	3
7.	7 - 50	0.7	5	10.9	10.0	1.57	6
8.	6 - 50	0.6	5	10.4	9.3	1.44	8
9.	8 - 30	0.8	3	10.4	7.5	0.3	7
10.	7 - 30	0.7	3	10.0	8.0	0.58	9
11.	6 - 30	0.6	3	10.0	7.8	0.63	13
12.	5 - 30	0.5	3	10.5	8.2	1.03	16
13	DEAE-PROTION	0.105	-	9.9	5.5	0.37	12

a.   Code: % crosslinking: % hydroxypropylation

b.   Capacity determined as in sec: 2.6.2.

c.   Stability   was determined as in sec. 2.4

There is no significant variation in the yields obtained. The necessity of using propylene oxide for both capacity and stability is evident. Although the swollen volume per gram increases from 5.5 to 8 with 30% propylene oxide the stability and capacity are only slightly improved over Protion (preparations 10 and 13). If preparations 3 and 6 are compared it can be seen that there is a four fold increase in protein capacity over Protion, and an improved chemical stability.

It is clearly evident that by attaching hydroxypropyl groups to regenerated cellulose both the protein capacity and the stability of ion exchangers from regenerated cellulose can be improved. This is a result of the greater swollen volume of the products. It would appear from the results in Table 5 that a swollen volume greater than 8 ml/g is required.

3.1.7   Comparison of Flow Rates for DEAE-Protion and DEAE-HP-Regcel 7-50.

Another property which needed to be considered was the hydraulic properties of the new products. The flow rate was measured for DEAE-HP-7-50 (Preparation 7, Table 5) and compared to DEAE-Protion using a range of hydrostatic pressures across the column. The results are shown in Figure 2 and it can be seen that the flow rate has decreased by half for the new product. The slower flow rate shown by DEAE-HP-7-50 is offset by the much higher capacity since a column of only half the height would be required to do the same job. In both cases there was noticed a 2% shrinkage at maximum pressure used.

3.1.8   Conclusion

An improved product can be obtained using 15 ml. of 30% NaOH and 6 gm of CED per 10 g of regenerated cellulose and using between 50 and 70% propylene oxide and 7 and 8% epichlorohydrin.

This work was carried out using Enka viscose of 100 - 200 mesh particle size and the optimum may not be the same for other particle sizes or for regenerated cellulose from other sources. If the preparation is scaled up it will also have to be reinvestigated to find the optimum conditions as the losses of propylene oxide from these small scale reactions by evaporation while mixing were probably quite high.

The results for both serum fractionation and an enzyme purification using preparation 2 (Table 2) are presented later.

3.2   CM-HP-Regcel

3.2.1   Introduction

DEAE ion exchangers is the derivative of major use for protein separations. Approximately 70% of all uses for cellulose ion exchangers have been with the DEAE derivative. The CM ion exchangers are the next most useful so it was important that the derivative of hydroxypropylated regenerated cellulose was also investigated.

Figure 2 Flow rate for DEAE-HP-Regcel-7-50,
DEAE-protion and SP-HP-Regcel

Pressure (cm H₂O)
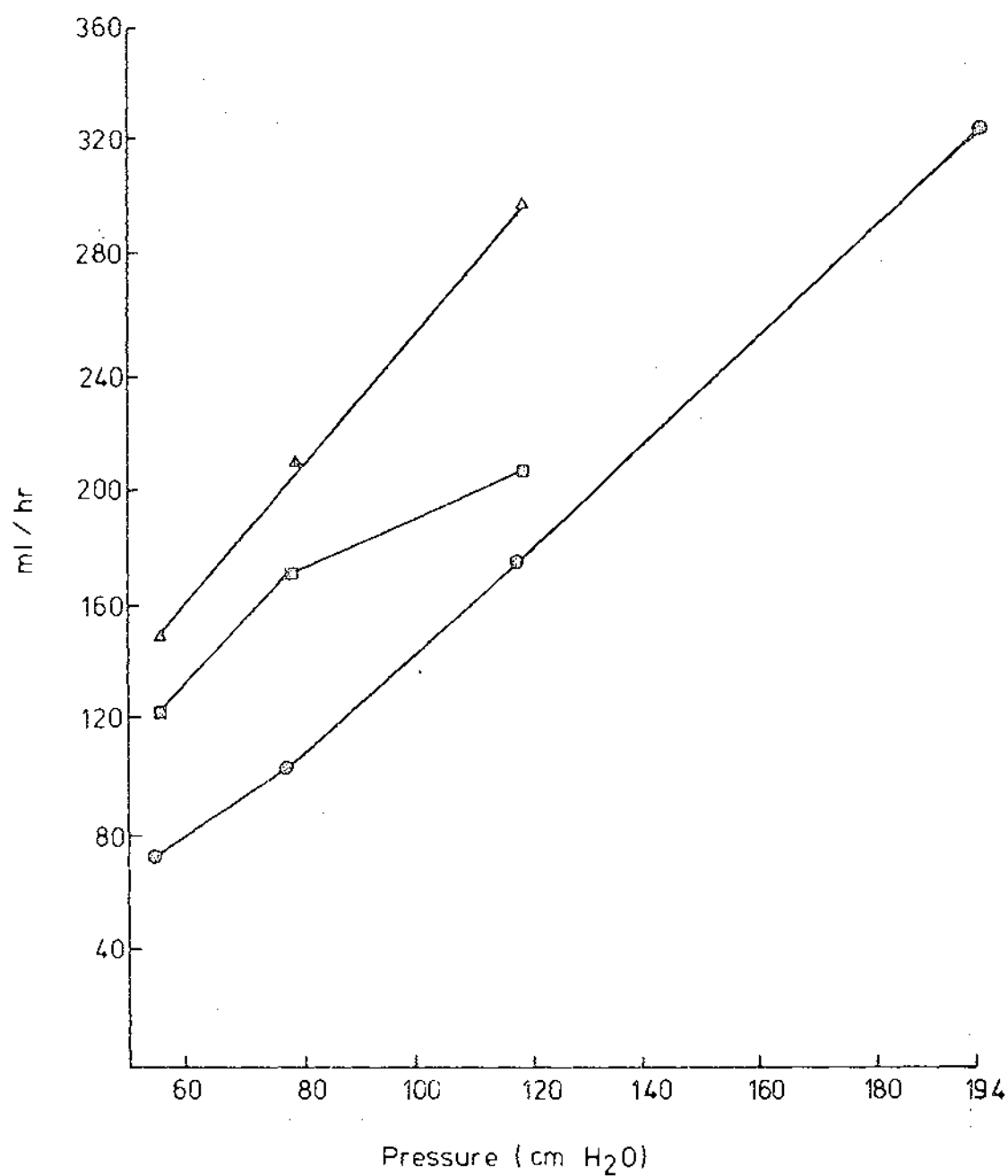
Column Dimensions: 15 cm × 1·6 cm
DEAE-protion : △—△
DEAE-HP-Regcel -7-50 : ○—○
SP-HP-Regcel : ▱—▱

3.2.2   Preparation of CM-HP-Regcel

Several preparations were tried in an attempt to achieve a range of ion exchange capacities as well as protein capacity greater than that possible with the Grant patented product (CM-Protion), usually around 0.5 g/g for haemoglobin. The carboxymethyl groups were introduced into regenerated cellulose at the same time as crosslinking and hydroxypropylation as one was not expected to interfere with the other as was the case for the DEAE derivative.

The reactions involved in the preparations are those shown in Scheme IV.

Scheme IV

$$HO-R-OH + CH_3-CH-CH_2 + ClCH_2CO_2H \quad \xrightarrow{\quad NaOH \quad}$$
$$\underset{O}{\diagdown\diagup}$$

$$NaO_2C-CH_2-O-R-O-CH_2-CH-CH_3$$
$$\overset{|}{O}H$$

$$\underline{OR} \quad HO-R-O-CH_2-CH-CH_3$$
$$\overset{|}{O}-CH_2-CO_2^- Na^+$$
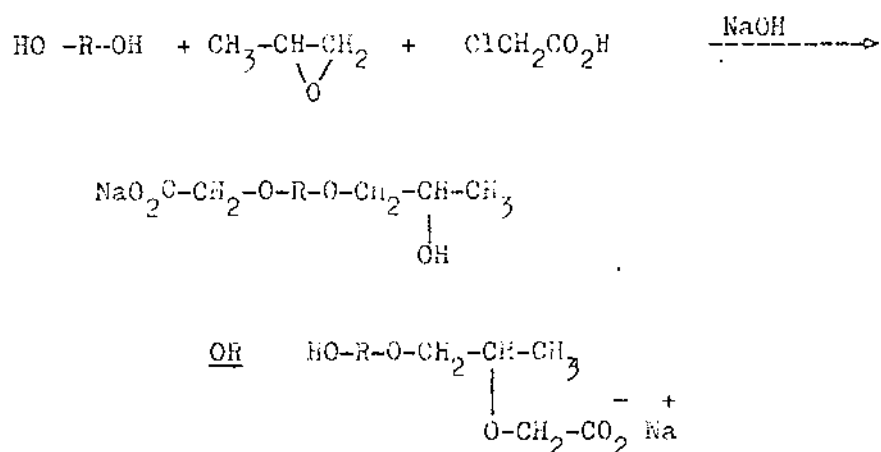
R = Regenerated Cellulose matrix (crosslinks not shown)

The results of several reactions are shown in Table 6.

TABLE 6

Preparation of CM-HP-Regcel

Regenerated cellulose: 10 g.

PREPARATION	ECH (ml)	P.O. (ml)	30% NaOH (ml)	ClCH$_2$CO$_2$H (g)	YIELD (g)	Meq/g	SETTLED VOLUME ml/g	CAPACITY g/g$_c$
1 [a.]	0.25	–	7.2	1.5	11.2	1.4	11.7	0.45
2	0.8	5	15	3.0	12.4	1.21	8.9	1.39
3	0.8	5	15	4.0	11.0	1.7	7.3	–
4	0.8	5	15	4.5	11.3	1.9	8.8	–
5	0.6	5	15	3.0	11.6	1.1	13.4	1.61
6 [b.]	–	–	8	2.0	11.4	1.1	17.2	1.68

a. Grant patent product (CM-Protion)

b. Used 10 g. HP-Regcel-8-50 prepared separately and dried.

c. Protein capacity determined as in sec. 2.6.1

As with DEAE-HP-Regcel preparations. 15 ml of 30% NaOH per 10 g of regenerated cellulose gave good results. The use of 3 g. chloroacetic acid was satisfactory for the 1 meq/g incorporation of ion exchange groups usually required for good protein adsorption. The ion exchange capacity was raised to 1.9 meq/g by simply increasing the ClCH$_2$CO$_2$H to 4.5 g. Decreasing the crosslinking from 8% to 6% helped the capacity.

Preparation 1 (Table 6) was a preparation according to the Grant Patent (26) and although it had a high swollen volume its capacity was low compared with the swollen volume and capacity of preparation 2. This suggests that it is not so much the swollen volume which is important for protein capacity but also the interior volume (pores) of the particles which need to be accessible to the protein. Obviously the hydroxyalkylation reaction helps improve this.

Although not necessarily the optimum preparations, preparations 2 and 4 were chosen to work with.
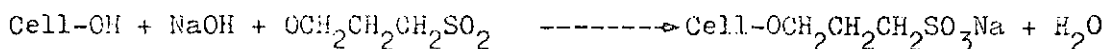
3.3   SP-HP-Regcel

3.3.1   Introduction

Although not used very widely it was anticipated that this could be a
useful derivative for the isolation of whey proteins so its preparation
was investigated.

Various reagents have been used to prepare sulphonic acid
derivatives of cellulose e.g.: Haloalkyl-sulphanates, sodium chloro-
ethyl-sulphonate, sodium chloropropyl sulphonate and 1, 3-propane sultone.
The early sulfoethyl celluloses prepared by Karner et al (42) and Timell
(43) based on fibrous starting materials were not useful owing to the
high consumption of basic reagents and the low effectiveness of the
main etherification reaction. These were improved using epichlorohydrin
crosslinked microcrystalline cellulose to give good ion exchange capacity
and flow rate, but if in preparation,temperatures above 65° C were
used, the cellulose was known to decompose (44). Using cellulose beads,
Detterman et al (45) had prepared SP-Celluloses for use in chromatographic
separations. The Sulfomethyl cellulose derivatives had come under invest-
igation (46), but no sulphonic acid derivative of cellulose or regenerated
cellulose is available commercially.·

The strong acid derivative of crosslinked dextran originally sold
commercially was an SE-Sephadex ion exchanger product marketed by
Pharmacia Fine Chemicals Ltd but it had bad flow properties. This has
been replaced since 1970 by Sulpho propyl (SP) Sephadex prepared using
1,3-propane sultone.

Consequently to prepare a sulphonic acid derivative of HP-Regcel,
1,3-propane sultone was chosen as the reagent.

Cell-OH + NaOH + OCH$_2$CH$_2$CH$_2$SO$_2$ --------→Cell-OCH$_2$CH$_2$CH$_2$SO$_3$Na + H$_2$O

3.3.2   Preparation of SP-HP-Regcel

From experience with the preparation of CM-HP-Regcel it was
decided to start off using 10 ml of 30% NaOH per 10 g of regenerated
cellulose rather than 15 ml, as the reaction consumes 1 mole of NaOH
per mole of 1,3-Propane sultone whereas CED and ClCH$_2$CO$_2$H both consumed
2 moles.

A series of preparations were tried to achieve a range of products,
keeping the regenerated cellulose, propane sultone and hydroxypropylation
constant and varying both crosslinking and NaOH.

The results are shown in Table 7.

TABLE 7

Preparations of SP-HP-Regcels

Regenerated cellulose: 10 g

1,3 Propane sultone : 3 g

Propylene oxide : 5 ml.

PREPARATION	ECH (ml)	P.O. (ml)	NaOH	YIELD (g)	Meq/g	SWOLLEN VOLUME (ml/g)	CAPACITY g/g[b].	STABILITY %Loss [c].
1	0.4	5	10 ml 30%	11.4	1.1	10	1.58	1
2	0.6	5	10 ml 30%	11.2	1.0	7.8	0.78	0
3	0.8	5	10 ml 30%	11.9	1.0	5.9	0.39	4
4	0.8	5	8 ml 30%	12.5	1.0	5.0	0.19	0
5	0.8	5	10 ml 20%	12.5	1.2	5.1	0.25	0
6	0.8	5	15 ml 20%	11.8	0.8	8.1	1.18	5
7	0.8	5	10 ml 40%	10.8	0.7	8.4	1.16	4
8[a].	0.6	8	10 ml 30%	11.0	1.1	9.9	1.30	3
SP-Sephadex	–	–	–	–	2.3	9.1	0.95	5

a. Two step process, where crosslinking and hydroxypropylation carried out in the first step at 60° C for 1 hr in an oven. Followed by addition of propane sultone in 3 mls of P.O. in a second step for reaction at 100° C for 1 hr in a water bath.

b. Protein capacity determined as outlined in sec. 2.6.1.

c. Stability determined as outlined in sec. 2.4.

It is evident that (Preparation 1), using 10 ml, of 30% NaOH and only 4% crosslinking gives a product with excellent protein capacity and chemical stability. At 8% crosslinking a reduction in NaOH (preparations 3, 4 and 5) did not improve the protein capacity. Equal or greater amounts of NaOH as in preparations 6 and 7 give reasonable protein capacities but the ion exchange capacity has been reduced.

The SP-Sephadex[1] was considerably less stable than the products described here.

Footnote: 1. This is the only sulphonic acid derivative available commercially for use with proteins.

Increased amounts of propane sultone (4 and 5 g.) did not increase the ion exchange capacity as has been possible with DEAE and CM derivatives. Further work is required to obtain higher substitution. Although only a small number of formulations were tried here, preparation 1 (Table 7) had excellent properties, i.e. ion exchange capacity, protein capacity and chemical stability, and this preparation was used further to assess the usefulness of the SP-derivatives.

3.3.3   Effect of Pressure on flow rate of SP-HP-Regcel

The flow rate was measured using a range of hydrostatic pressures across the column. The results are shown in Figure 2 and it is evident that the SP-derivative does have good flow properties.

3.4   Protein Capacity of CM-HP-Regcel
3.4.1   Protein Capacity by the Batch Method

Some of the preparations listed in Table 6 were tested for protein capacity. The results given in Table 6 were for a 2 hr batch test but small samples were also withdrawn at other time intervals up to 4 hours to see if the protein uptake was slow or rapid in comparison with Protion and other ion exchangers (47). The results for preparations 1,2,5 and 6 of Table 6 are shown in Table 8.

TABLE 8

Capacity of CM-HP-Regcels to Haemoglobin (g/g)

PREPARATION	TIME IN HRS WITH CAPACITY (g/g).			
	½ hr	1 hr	2 hrs	4 hrs
1	0.24	0.40	0.45	0.49
2	0.71	0.93	1.39	1.60
5	0.71	1.07	1.61	1.87
6	0.93	1.29	1.68	2.30

Uptake is slow and protein continues to be adsorbed for at least 4 hours. The rate is similar in all cases with roughly 50% being adsorbed in the first half hour. This is similar to that reported for ion exchangers based on cross linked dextran, but is much slower than fibrous and microgranular cellulose ion exchangers.(47).

The most important point is that the amount of protein adsorbed is far greater for the HP-Regcel derivative than CM-Protion whether the time period is 0.5 or 4 hours.

3.4.2. Column Capacity

With the slow rate of uptake of protein shown for these derivatives it might have been thought that these ion exchangers of improved capacity might not be very useful in a column situation unless very slow flow rates were used. This was shown not to be the case by passing a solution of haemoglobin through a 1 ml column of CM-HP-Regcel (preparation 2, table 6) at two different flow rates, both of them very high, 1 ml/min and 0.2 ml/min. The results can be seen in column 3 of plates 1 and 2. The higher flow rate did have the effect of reducing the capacity, but at 0.2 ml/min the haemoglobin was bound in a well defined tight band at the top of the column and the breakthrough contained no trace of colour. Even this flow rate, 0.2 ml/min, (1 bed Volume in 5 minutes), is faster than is normally useful in ion exchange chromatography.

The other two columns in plates 1 and 2 contained the only 2 ion exchangers available capable of high flow rates; CM-Protion and Vistec C_1. Both of these were inferior to CM-HP-Regcel particularly at the higher flow rate especially the CM-Protion where only 2.5 ml of haemoglobin was passed through the column before colour was noticed in the breakthrough. Clearly columns of CM-HP-Regcel can be used effectively to remove protein from solution in spite of the slow uptake observed in the batch tests.

The desorption of protein from CM-HP-Regcel and Vistec Cl achieved in 30 minutes was 82% and 77% respectively. Complete desorption could not be completed quickly and it was found that 1-2 hrs were required for efficient recovery.

3.5 Ion Exchange Chromatography using DEAE-HP-Regcel

3.5.1 Introduction

Although these new DEAE-HP-Regcel ion exchangers had excellent capacities for protein, it was also desirable to show that they were useful for column chromatographic separations of proteins and enzymes. This was especially so since DEAE-Protion had been deficient in this respect (48, 49).

Two systems were chosen to test the DEAE-HP-Regcel; (1) An enzyme separation which had been well characterized by other workers within the department and for which DEAE-Protion was useless; (2) the fraction-ation of serum. This latter fractionation has been used in the past (35) to characterize DEAE celluloses and is often published for new ion

Plate 1. <u>Column Capacity of CM-Ion Exchangers for Haemoglobin:</u>
<u>flow rate 1 ml/min.</u>
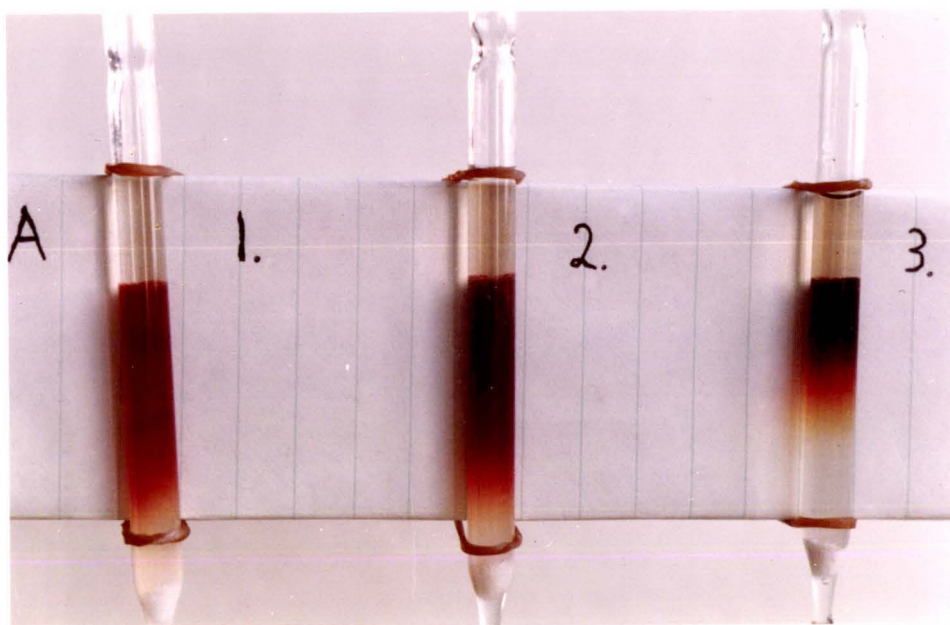
1. CM-Protion (2.5 ml)
2. Vistec C₁ (10 ml)
3. CM-HP-Regcel (10 ml)

<u>Column</u>
Plate 2. <u>Capacity of CM-Ion Exchangers for Haemoglobin:</u>
<u>flow rate: 0.2 ml/min.</u>
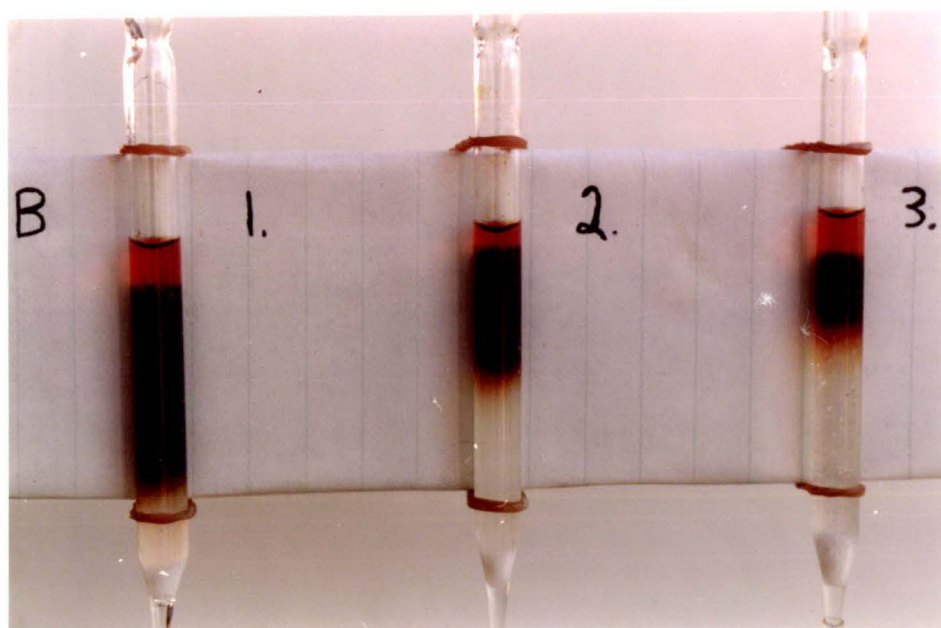
1. CM-Protion (10 ml)
2. Vistec C₁ (10 ml)
3. CM-HP-Regcel (10 ml)

exchangers when they are made available commercially.

3.5.2. Serum Fractionation

This was carried out on 4 ml of serum under conditions as close as possible to those of Himmelhoch and Peterson (35) except that a less complex gradient was used. The result is shown in Figure 3 along with the best resolution obtained by Himmelhoch and Peterson on DEAE-Cellulose. All the peaks observed on DEAE-Cellulose are present in the fractionation on DEAE-HP-Regcel. With DEAE-Protion only peaks 1, 4, 6, 9 and 10 were observed under the same conditions.

The performance of DEAE-HP-Regcel has been improved with respect to DEAE-Protion.

3.5.3 Purification of Aldehyde Dehydrogenase

(a) Introduction

Even though DEAE-Protion had the same capacity for bovine serum albumin as DEAE-Cellulose, the aldehyde dehydrogenase enzyme breaks through Protion columns even when the column is 10 times larger than DEAE-Cellulose (48). This is a more severe test for Protion as the molecular weight for aldehyde dehydrogenase is 212,000 and Protion is known to have a cut off in porosity below this value. Sharman (40) was able to overcome this deficiency of Protion by giving it a soak in 8 - 10% NaOH to swell it and increase its porosity. However this led to a chemically unstable product.

(b) Enzyme Purification using DEAE-HP-Regcel

Using the same enzyme preparation method and column operating conditions as outlined by Crow et al (37), DEAE-HP-Regcel was used to purify aldehyde dehydrogenase. The results of this purification are shown in Figure 4.

Table 9 lists the results in comparison with those obtained by K. Crow for DEAE-Protion, DEAE-Cellulose and Protion treated with 8% NaOH for 20 minutes and 1 hr.

Figure 3a : Serum fractionation on DEAE-Cellulose

Figure 3b: Serum fractionation using DEAE – HP – Regcel.
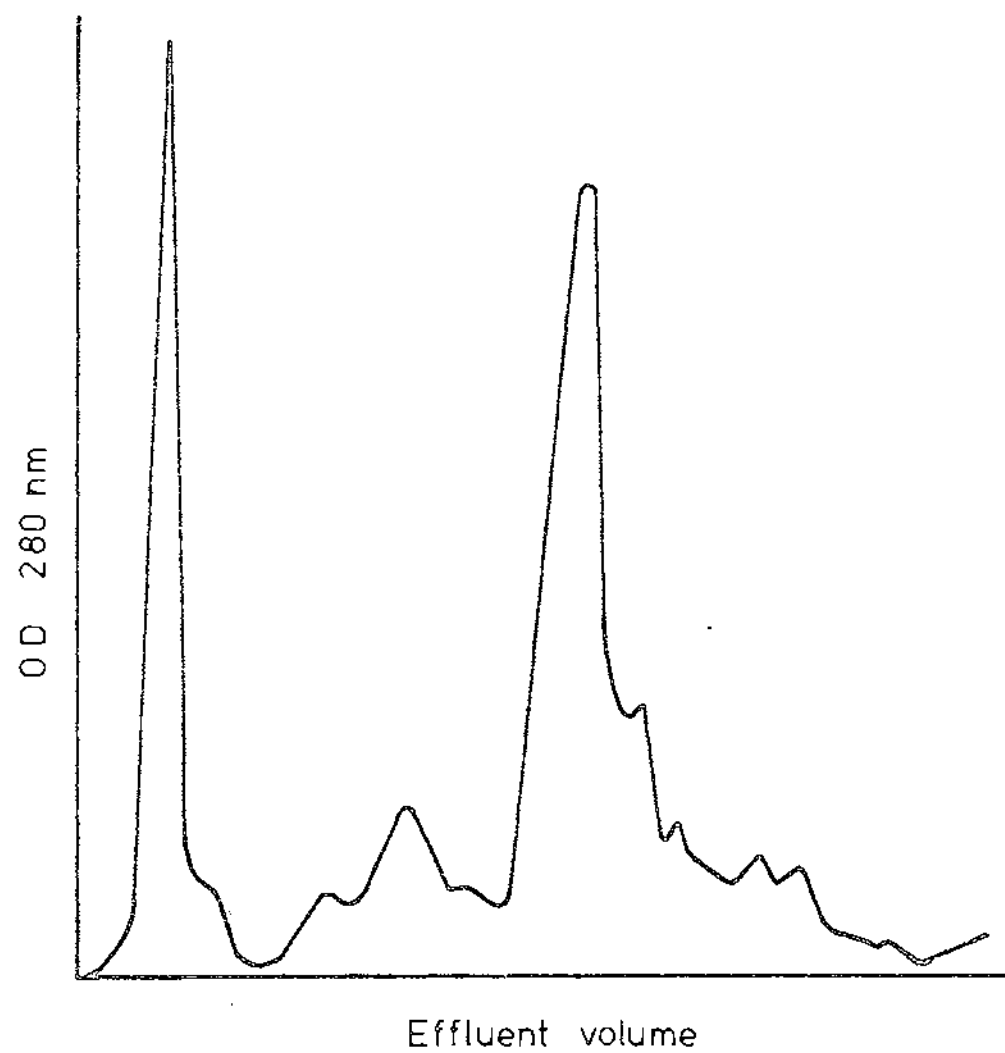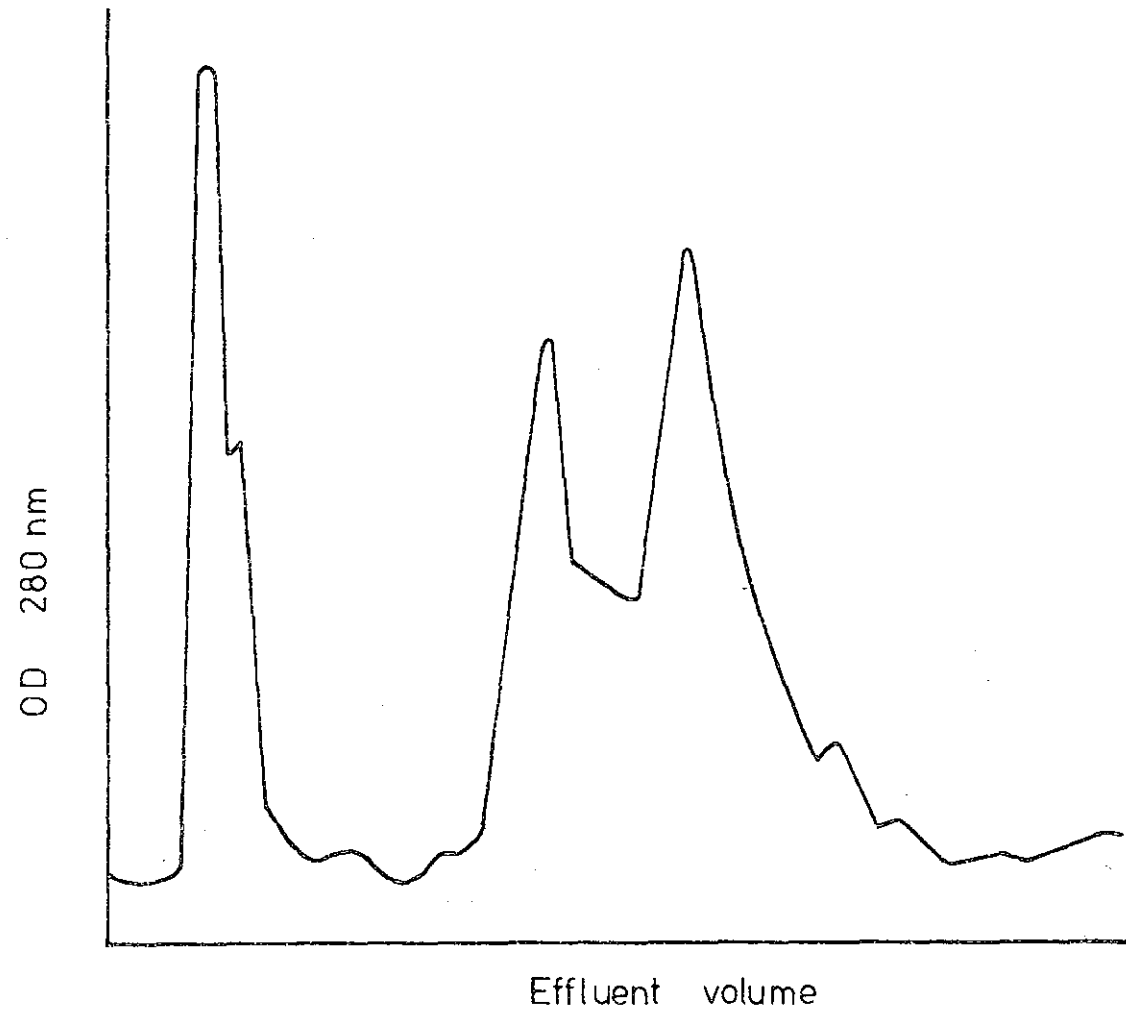
OD 280 nm

Effluent volume

Figure 4 : Purification of Acetaldehyde Dehydrogenase using DEAE-HP-Regcel

Column Dimensions : 32 cm × 1·6 cm
UV λ = 280 nm : ○—●
units enzyme activity : ⊘—⊘
UV λ = 400 nm : ▲—▲
conductivity : ⊟—⊟
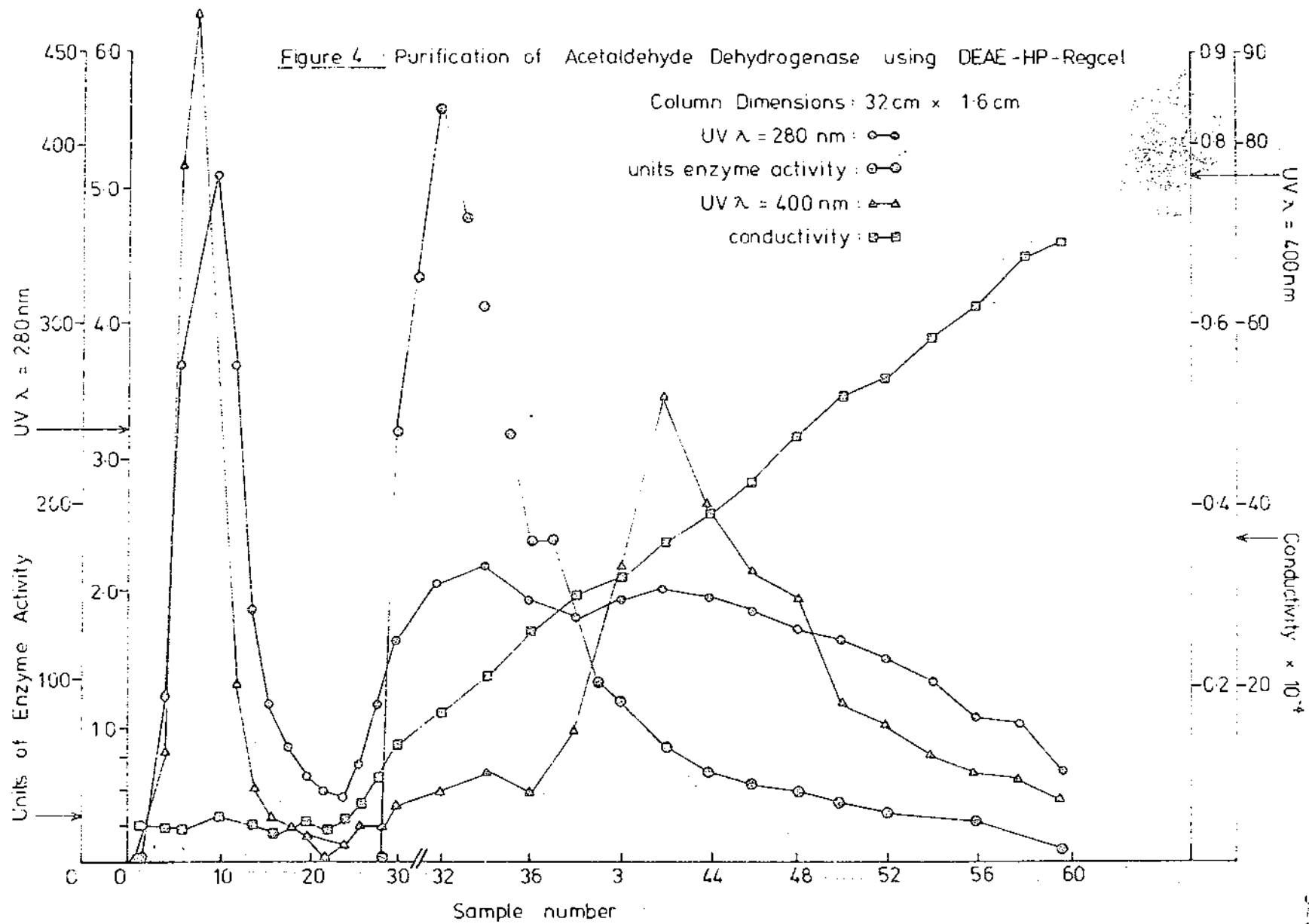
TABLE 9

Comparison of results using DEAE-ion exchangers

COLUMN FEATURES	DEAE-PROTION	DEAE-CELLULOSE	8% NAOH TREATED PROTION		DEAE-HP-REGCEL
			20 minutes	1 hr	
Column Volume (ml)	480	37	38	40	51
Protein Loaded (g)	0.90	0.37	0.37	0.37	0.92
Column Loading (g/ml)	0.0019	0.01	0.01	0.009	0.018
Activity Overload	10%	None	12%	None	None
Length of green band (cm)	--	6.7	16.5	12	12
Volume column occupied by green band (ml)	--	18.2	38	29	19.6

(c) Discussion

There is no enzyme breakthrough even when the resin loading was 10 times that used for Protion. Boehringer also reported enzyme overload at very low loadings on DEAE-Protion.

The Volume of the column occupied by the green protein band was smaller even than that of the NaOH treated Protion and was similar to that observed for DEAE-Cellulose.

The resolution was far superior to DEAE-Protion and similar to the DEAE-Cellulose apart from the longer tailing.

The total activity of the enzyme preparation loaded onto the column was 16,800 units with a specific activity of 18.4 units/mg. At the end of the purification a 84% recovery of enzyme was obtained with a specific activity of 117 units/mg. This amounted to a 6.4 fold purification and was similar to that achieved by Crow on DEAE-Cellulose columns

Clearly these DEAE-HP-Regcel ion exchangers can be used in ion exchange chromatography and will be particularly useful where rapid separations are desired. The separation above was carried out at twice the flow rate used for DEAE-Cellulose.

PART B

WHEY   PROTEIN   ISOLATION

SECTION 1

INTRODUCTION

According to McKenzie (50) the term whey protein used loosely
signifies the noncasein protein occuring in milk in an appreciable amount.
In skim milk approximately 80% of the nitrogen is in the form of casein
and can be recovered as such during manufacture of this protein. The
remaining 20% is lost in the whey in the form of whey protein, about
15%, and non-protein nitrogen, about 5%.

For many years it was generally believed that B-lactoglobulin was
the predominant "whey" protein in the milk of mammals. It is not surp-
rising that B-lactoglobulin has been the most intensively studied of the
whey proteins. ∝-lactalbumin is the next most prolific of the ruminant
"whey" proteins and it is the dominant one in human milk and occurs in
milk of other mammals. The so called "minor" proteins of milk are
transferrin. immunoglobulins, serum albumin and lactoferrin.   Along
with a number of enzymes e.g.: nucleases. lactoperoxidase, lipases and
phosphatases. they are a rapidly expanding area of research.

Table 10 shows a typical distribution and concentration of the
major proteins found in bovine whey.

TABLE 10

Typical protein distribution and concentration of Bovine

SPECIES	CONCENTRATION (g/1)	% of WHEY PROTEINS	M.W.
B-lactoglobulin	3	60	35,500
∝-lactalbumin	1	20	16,000
Serum Albumin	0.3	6	65,000
Immunoglobulins	0.7	14	180,000

This is only approximate because whey is produced by a variety of
processes resulting in a variety of wheys, e.g.; cheddar cheese whey,
lactic casein whey, rennet casein whey, co-precipitate whey, acid (HCl or
H_2SO_4) whey and lactalbumin whey.

The whey composition varies with each process as can be seen for

representative examples shown in Table 11.

TABLE 11

Typical Whey Composition (%), (51)

	RENNET WHEY	HCl WHEY	LACTIC CASEIN WHEY
Total Protein	1.01	0.78	0.95
Non Protein Nitrogen	0.042	0.031	0.048
True Protein (TPN-NPN)x6.38	0.74	0.58	0.64
Ash	0.46	0.68	0.72
Lactose	5.08	4.65	4.47
Acidity	0.10	0.37	0.64
Total solids	6.55	6.11	6.79
pH	6.5	4.5	4.5
Calcium (ppm)	565	1500	1655

Because of their amino acid profile whey proteins are superior nutritionally to casein, being equal to or better than whole egg protein (52). However, until recently whey proteins have been available only as about 10% of the solids in liquid or dried whey, or as a heat denatured insoluble powder with the trade description, lactalbumin. The use of dried whey in foods and animal feeds has been developed over recent years but still represents only a relatively small proportion of the world total for whey solids. With increasing emphasis on environmental pollution much more attention is being given to whey utilization with particular emphasis on the proteins. Research workers and dairy products manufacturers are collaborating in these efforts (53 - 56).

The various processes which have been developed to utilize the whey protein have been reviewed by Muller (57). The most important of these is ultrafiltration. In this process an "open" membrane is used so that

with low applied pressure a separation of whey into a protein "free"
permeate of water, minerals, lactose and the non-protein nitrogen
components and a retentate containing the proteins dispersed in a solution
of similar composition to the permeate. As the percentage of the feed
removed as permeate increases so does the concentration of protein in
the retentate and the ratios of protein to lactose to ash change (58).
As a result, a wide range of products of varying composition can be
manufactured.

A more recent development has been the use of an ion exchange
process. Jones (31) and Palmer (59), used "Vistec ion exchange cellulose
media" to extract protein from solution in a stirred tank reactor (STR).
The whey is pumped into the STR containing the cellulose ion exchange
media. It is mixed and the pH adjusted to 3.2 with HCl. The deprotein-
ized liquor is then drained from the STR system using a filter screen.
The ion exchange media is washed with water and then the protein desorbed
by adjusting the pH to 9 with NaOH. The concentrated protein solution
obtained is drained and then concentrated and de-ashed by ultrafiltration
ready for spray drying.

A major disadvantage of this process has been the large amount of
ion exchange media required for processing the whey and the specific
pH of 3.2 required for optimum performance.

As a result of the improved ion exchangers based on regenerated
cellulose (described in Part A), it was decided to investigate their
potential for isolating whey protein.

PART B

SECTION 2

EXPERIMENTAL

2.1   Materials

Spray dried low heat skim milk powder was obtained from the
N.Z.D.R.I. Amberlite MB-3 monobed ion exchange resin, (mesh 14-52)
was obtained from Ajax; Amberlite IR-120 (H^+), ('R') cation exchange
resin from BDH Chemicals and SP-Sephadex-C-25 from Pharmacia, Sweden.

2.2   Preparation of Whey

Skim milk was reconstituted from spray dried low heat powder,
200 g, by dissolving it in 2 l. of deionized water with slow stirring.
The pH was then adjusted to 4.6 with 2M H_2SO_4. The acidified mixture
was placed in a water bath at 50° C, until the temperature of the
mixture reached 50° C. The curds were then separated from the whey
by vacuum filtration using either Whatman no. 40 or 41 filter paper.
The whey was stored in the refrigerator and used within three days.

2.3   Whey Demineralization

The whey was demineralized using a 500 ml column of Amberlite MB-3
self indicating monobed ion exchange resin as described by Webb (60).
The resin was regenerated for reuse as described in the BDH manual (61).

2.4   Cation Exchange procedure for Whey

The Amberlite IR-120(H^+) cation exchange resin was washed thoroughly
with water and then excess water removed by draining it on a filter
with suction. Sufficient resin was added to the whey to reach the
required pH.

For the large column run on SP-HP-Regcel the whey was passed
through a column of Amberlite IR-120(H^+).

2.5   Protein Determination

The amount of protein in whey was determined by nitrogen analysis using
a micro-Kjeldahl method (62). The percentage protein equals the percentage
of nitrogen multiplied by 6.38. Whey (1 - 5 mls) was mixed with 0.8 g
of catalyst (K_2SO_4/$CuSO_4$/Na_2SeO_4; 10: 1 : 0.4) and 1 ml. of concentrated
H_2SO_4 and then digested for 1-4 hrs. The nitrogen was distilled off as
ammonia in a Markham apparatus and collected in 5 ml of 3% boric acid.

This was then titrated back to pH 5 on a Radiometer TT2 automatic titrator with 0.01M HCl.

The non-protein nitrogen was determined by precipitating the protein from a 5 ml sample with 20 ml of 15% Trichloroacetic acid. The precipitate was filtered off (using Whatman No. 42) after 30 minutes and a 5 ml sample of the filtrate analysed for nitrogen as described above.

All determinations of protein and non-protein nitrogen were carried out in duplicate.

2.6   Capacity of Ion Exchangers for Whey: Batch Tests

The capacities of CM-HP-Regcel and SP-HP-Regcel for whey were determined as follows. A sample of the wet ion exchanger equivalent to 80 mg dry weight was mixed end over end with whey solution (20 ml) for 2 hrs. The suspension was allowed to settle briefly and was then filtered through cotton wool plugs to make sure no ion exchanger was present in the samples which were then withdrawn for nitrogen analysis.

It was not necessary to pre-equilibrate the ion exchanger owing to the small amount used, but the pH was measured at the end of the 2 hr test as in some cases there was a shift of up to 0.1 pH unit.

2.7   Deproteination of Demineralized Whey with CM-HP-Regcel (1.2 meq/g): Column Tests

CM-HP-Regcel (2 ml) was loaded into small glass columns. A demineralized whey sample was adjusted to pH 3.5 with $2M$ H_2SO_4 and loaded onto the column at 1 ml/min. As the whey passed through the column, fractions (10 ml) were collected and their optical density at 280 nm and pH recorded.

2.8   Deproteination of Whey with SP-HP-Regcel

2.8.1   Small Column tests

Equal volumes (80 ml) of whey were taken and adjusted to pH's 2.5, 2.0 and 1.5 by the addition of Amberlite IR-120(H^+) prepared as described for the batch tests. Another 80 ml whey sample was passed through a small column of Amberlite MB-3 to demineralize it and all the whey collected by rinsing the column with deionized water. The pH of the demineralized whey was adjusted to 2.5 with $2M$ H_2SO_4.

Each of these whey solutions was passed through a 5 ml column (0.6 x 7.5 cm) of SP-HP-Regcel at a flow rate of 1 ml/min using a

peristaltic pump and the unbound protein displaced from the column
with 0.01M NaCl. The total whey eluate was collected in 100 ml.
volumetric flasks and analysed for nitrogen and non-protein nitrogen.
The original whey from which all samples were taken was similarly
analysed and the protein which passed through the column determined
as a % of that loaded.

2.8.2   Deproteination of Lactic Acid Whey

Lactic acid whey (180 mls) was adjusted to pH 2.0 by the addition
of Amberlite IR-120(H^+) cation exchange resin. This whey solution was
passed through a 5 ml. column (0.6 x 7.5 cm) of SP-HP-Regcel at a flow
rate of 1 ml/min using a peristatic pump. As the whey passed through
the column, fractions (20 mls) were collected and the O.D at 280 nm
and pH recorded.

2.8.3   Complete analysis of performance of a 50 ml SP-HP-Regcel
           Column

SP-HP-Regcel (Na^+ form) was packed in a 2.5 diameter column to a
depth of 10 cm (50 ml volume) in distilled water. Cation exchanged whey
(1550 ml) at pH 1.3 was loaded onto the column at a flow rate of 10 ml/min
using a hydrostatic pressure across the column of 1 metre. The pH and O.D
at 280 nm of the eluate were monitored throughout. Deionized water (100 ml)
was used at the finnish to displace the remaining whey from the column.
The total volume of deproteinated whey (1680 ml) was mixed thoroughly
and samples taken for protein and non-protein analyses as well as electro-
phoresis. Another sample (50 ml) was taken and concentrated to 5 ml by
vacuum dialysis for further electrophoretic study. The protein was removed
from the column using 0.02M Na_2HPO_4. (40 ml) and circulating this contin-
uously through the column. Before returning the eluate to the top of the
column it was adjusted to pH 8 continuously with 10% NaOH using a Radiometer
TT 2 automatic titrator set to pH stat the circulating solution. When constant
pH was obtained after 45 minutes the column was left to stand for 1 hr
to complete desorption of protein at pH 8. The protein was finally
collected in 100 ml by displacing the concentrated solution from the column
with water and then freeze dried. The column was washed with a further 100 ml
of water which was also analysed for protein content along with the ion
exchanger itself.

2.9   Polyacrylamide Gel Electrophoresis (P.A.G.E.)

The procedure followed was that of Davis (63) and Groves (64).

2.9.1   Stock Solutions and Gel Composition

                 Abreviations: TRIS - Tris(hydroxymethyl)aminomethane.

                                TEMED - N,N,N',N'-tetramethylethylene-diamine.

                                MBA - N,N' - methylenebisacrylamide.

Stock Separating Gel Composition

(a)   16.1 ml of 1M HCl)) made up to 50 ml with deionized water
       12.3 g of TRIS)
       0.057 ml of TEMED)

(b)   15 g of Acrylamide) made up to 50 ml with deionized water
       0.4 g of MBA)

(c)   0.07 g of ammonium persulfate made to 50 ml with deionized water, and made fresh daily.

Stock Stacking Gel Composition

(d)   25.6 ml of 1MHCl)) made up to 50 ml with deionized water
       2.99 g of TRIS)
       0.23 ml of TEMED)

(e)   5 g of Acrylamide) made up to 50 ml with deionized water
       1.25 g of MBA)

(f)   2 mg of Riboflavin made up to 50 ml with deionized water

Separating Gel Composition (10% Acrylamide)

   2.5 ml (a)
   2.0 ml (b)
   3.0 ml (c)

Stacking Gel Composition (2.5% Acrylamide)

   1 ml (d)
   2 ml (e)
   1 ml (f)
   4 ml distilled water

2.9.2   Electrode buffer composition

For Gel electrophoresis at pH 9.5; TRIS (3g) and Glycine (14.4 g) was made up to 1 l with deionized water and adjusted to pH 9.5 with NaOH pellets.

2.9.3   Gel casting and running procedure

Into a syringe (10 ml) was drawn the separating gel, aerated for
1 minute and then carefully transferred to the glass electrophoresis
tubes which were stoppered with rubber bungs at one end. A small layer
of water was overlayed using a 250 ul pipette to prevent a miniscus
forming and polymerization occured on standing for forty minutes.

After polymerization the small water layer was removed using
filter paper. The stacking gel was then mixed in a syringe (10 ml),
aerated for two minutes and then overlayed on top of the separating
gel to a height of 1 cm. A small water layer was again applied to prevent
miniscus formation. Photopolymerization occured after about twenty
minutes and was indicated by a cloudy colour developing in the stacking
gel.

The protein sample (0.1 - 0.4 mg) was mixed up in a small tube
with 0.1% Bromophenol blue stain (10 ul) and 1 drop of 10% sucrose
solution. From these mixtures, the protein solution (100 ul) was
carefully overlayed on top of each gel which were fitted in a Pleuger
"Acrylophor" disc electrophoresis vessel. Each of the two compartments
in the vessel were filled to the levels indicated with the buffer.

The initial power setting of 10 m.a. per tube was continued for
ten minutes, then reduced to 5 m.a. for the duration of the migration of
the staining front, (0.5 cm from the bottom of the gel).

The gels were removed from the tubes by the use of a long syringe
needle, circulating a stream of water between the gel and the glass tube.

2.9.4   Protein fixing procedure.

Each gel when removed from their tubes were allowed to stand in a
solution of 20% Sulphosalycylic acid for 2 hr.

2.9.5   Protein stain composition and procedure

Originally, both Davis (63) and Gr°ves (64) suggested the use of either
Amline blue or Amido Black 10B. But various papers by Fish et al (65)
in 1969 and Darling et al (66) in 1976 compared these two stains with
Comassie Brilliant Blue R250 for whey protein staining and found
Comassie Blue comparable if not better.

A solution comprising 0.125% Comassie blue made up in deionized water
was used to stain the gels for 2 hr.

2.9.6.   Method of destaining gels

The background stain was allowed to diffuse out in a 7.5% acetic
acid solution.

PART B

SECTION 3

RESULTS AND DISCUSSION

3.1   Protein Removal from Whey using CM-HP-Regcel

3.1.1   Introduction

The main disadvantage with the Vistec process (31) is that it is a batch process necessitated by the precise pH control which is required, otherwise a rapid fall off in the capacity of the Vistec cellulose media for protein occurs. Consequently the recovery of protein from whey is only 60 - 75% (67). Palmer (59) suggested that the protein adsorption in the STR should be repeated to ensure removal of the bulk of the protein.

To achieve greater efficiencies a column process for stripping protein from whey would be more desirable. The use of a column is not possible using the conditions developed by Viscose Group Ltd., because the column would have to be pre-equilibrated with buffers so that pH would not drift. This would not be acceptable in a commercial process.

Jones (31) showed that for the Vistec cellulose media, background salt concentration affected capacity. Whey is high in salts so three possibilities were investigated to see if capacity could be improved and the pH optimum broadened sufficiently to allow a column process using CM-ion exchangers.

The possibilities investigated were:-

(a)   Salt removed by demineralization

(b)   pH shifted to the acid region by removing cations only

(c)   charge density on the ion exchanger increased to reduce the effect of high salt concentrations in whey.

3.1.2   Determination of Optimum pH for Removal of Protein from Whey

To investigate the effect of these changes on the ion exchangers protein capacities, batch tests were employed over a range of pH. The CM ion exchangers were used with different small ion exchange capacities, 1.2 and 1.9 meq/g (Preparations 2 and 4 of Table 6), under three different conditions.

(a)   The pH of the whey was adjusted to the required value with 2M H_2SO_4 (or 2 MNaOH for pH values greater than 4.5)

(b)   The pH was lowered by the addition of sufficient cation exchange resin in the hydrogen form to reach the required pH value.

(c)   The whey was demineralized and then the pH adjusted to

Figure 5 : Protein adsorbed from whey as a function of pH for CM-HP-Regcel (1·2 meq/g)
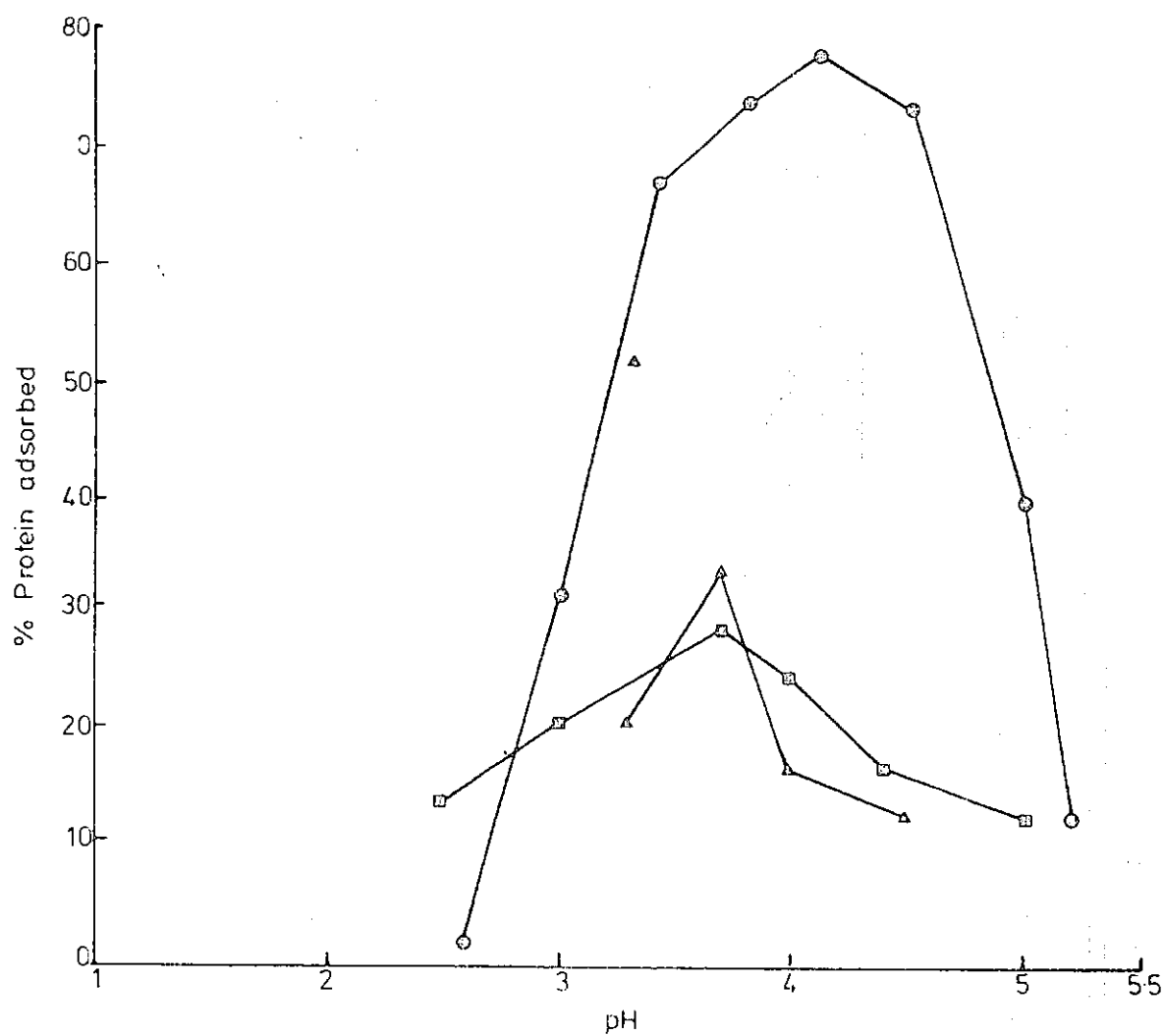
H₂SO₄ adjusted △—△ , Cation exchanged ▣—▣ , Demineralised ⊙—⊙

Figure 6: Protein adsorbed from whey as a function of pH for CM-HP-Regcel (1.9 meq/g)
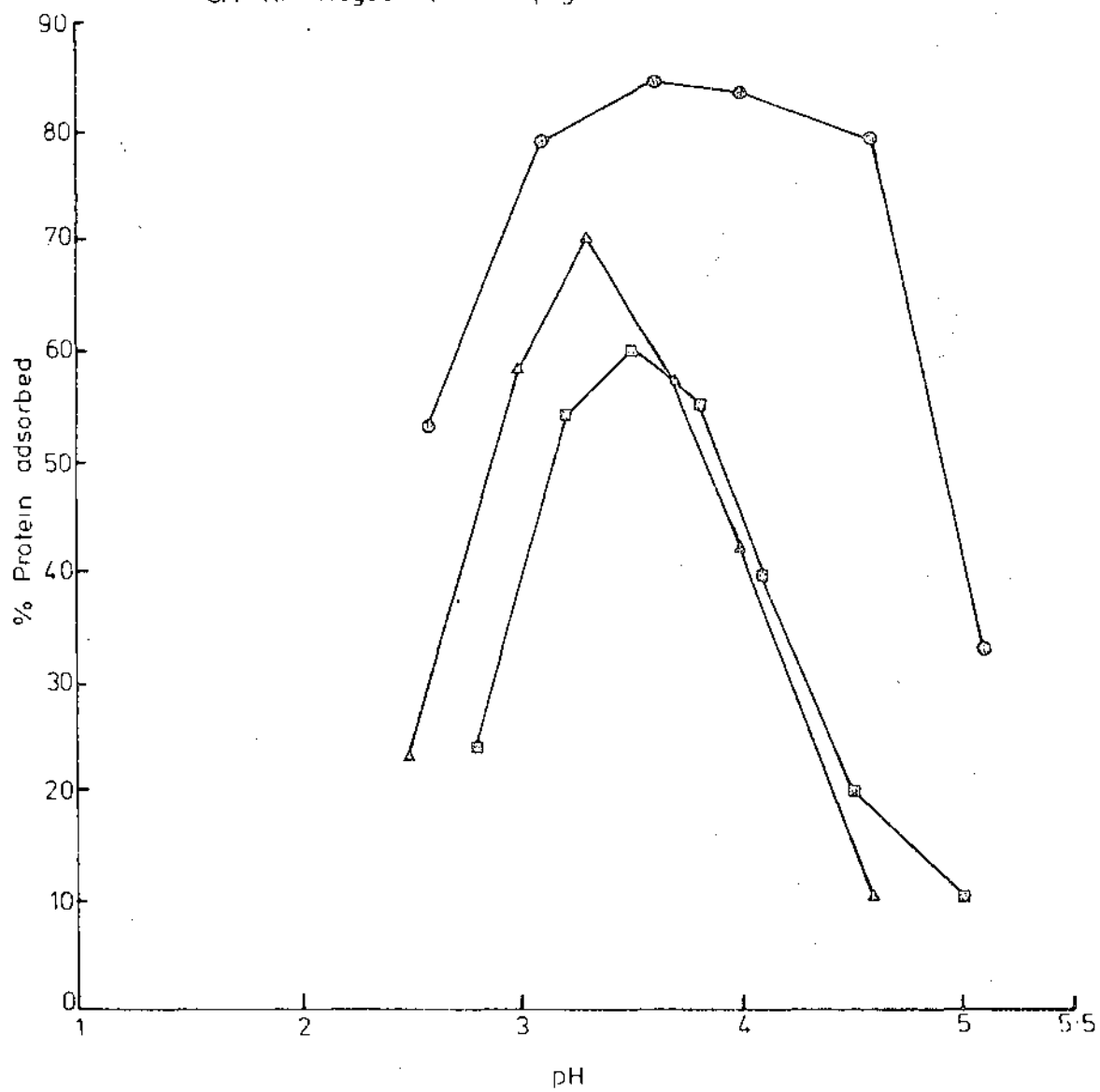
H₂SO₄ adjusted △—△, Cation exchanged □—□, Demineralised ○—○

the required value by addition of $2M H_2SO_4$.

The results are shown in Figures 5 and 6, and Table 12.

TABLE 12

Optimum pH for removal of protein from whey using CM-HP-Regcel

FORM OF WHEY	pH FOR OPTIMUM CAPACITY	
	1.2 meq/g	1.9 meq/g
a. H_2SO_4 adjusted	3.3	3.3
b. Cations removed	3.7	3.5
c. Demineralized	3.4 - 4.4	3.0 - 4.5

From figures 5 and 6 and Table 12 it can be seen that for carboxymethyl (CM) ion exchangers there is a very narrow pH range for optimum capacity. This is in agreement with the findings of Jones (31), who reported an optimum pH of 3.2 for their CM Vistec Cellulose media on hydrochloric acid adjusted whey.

Adjusting the pH by cation replacement with hydrogen ions did not improve the capacity profile either.

There was a marked improvement in capacity for both the acid adjusted and cation exchanged whey when a CM ion exchanger of higher carboxymethyl content was used ie 1.9 meq/g in place of 1.2 meq/g. However the pH range for optimum capacity remained very narrow and it is obvious a column of CM ion exchanger could not be used under these conditions to remove protein from either acid adjusted or cation exchanged whey without first equilibrating the column at the optimum pH.

With demineralized whey there was a marked improvement in both protein uptake capacity and the range in pH at which optimum capacity was observed. Only in this case was it considered that it might be possible to use a column of CM ion exchanger to remove protein from whey. Consequently this possibility was investigated using whey which had been demineralized and adjusted to pH 3.5 with acid.

3.1.3   Column Deproteination of Demineralized Whey with CM-HP-
                          Reagel (1.2 meq/g).

The column situation was investigated with the ion exchanger in both
the hydrogen ion and sodium ion form and with or without some buffer in
the whey. The columns were not equilibrated with buffer at the optimum
pH before use.

The results of these tests are shown in Figures 7 a - d for the
following four columns investigated:

      a.   Column in Na^+ form, unbuffered whey at pH 3.5.

      b.   Column in Na^+ form, buffered whey at pH 3.5.

      c.   Column in H^+ form, unbuffered whey at pH 3.5.

      d.   Column in H^+ form, buffered whey at pH 3.5.

For Column (a) the removal of protein from whey is not very
efficient. At the start a lot of protein was still present in the break-
through which is probably due to the high pH, which resulted from the
column being initially in the Na^+ form. The conditions for Column (b)
were similar to those of Column (a) except that the whey was buffered with
trisodium citrate buffer. A more efficient protein removal and pH
adjustment was observed along with a more efficient pH at the start. For
Column (c), which is in the hydrogen ion form to start with, a marked
improvement in both protein removal and maintenance of required pH is
shown. The conditions for Column (d) were similar to those of Column (c)
except that the whey was buffered. There is no improvement shown by
Column (d) over Column (c) by having citrate buffer present.

The best conditions for removing protein from demineralized whey would
appear to be having the ion exchanger in the Hydrogen form at the start.
Unfortunately this means giving the column an acid wash each time before
reuse, which could prove costly.

3.1.4   Tandem Columns

It was decided to try a tandem situation where the eluate from the
first column would be passed directly onto a secondcolumn as a method
of equilibrating the second column so that all columns after the first
could start off in the sodium ion form. Whey could also be loaded through
the first column after protein breakthrough (point x, Figure 7c) as the
remaining protein would be retained by the second column. Fresh whey
would then be introduced to the second column. The result of this

Figure 7 Protein Adsorption from Whey with Columns of CM- HP- Regcel.

Figure 7a Optical Density and pH of eluate from colum in Na⁺ form.
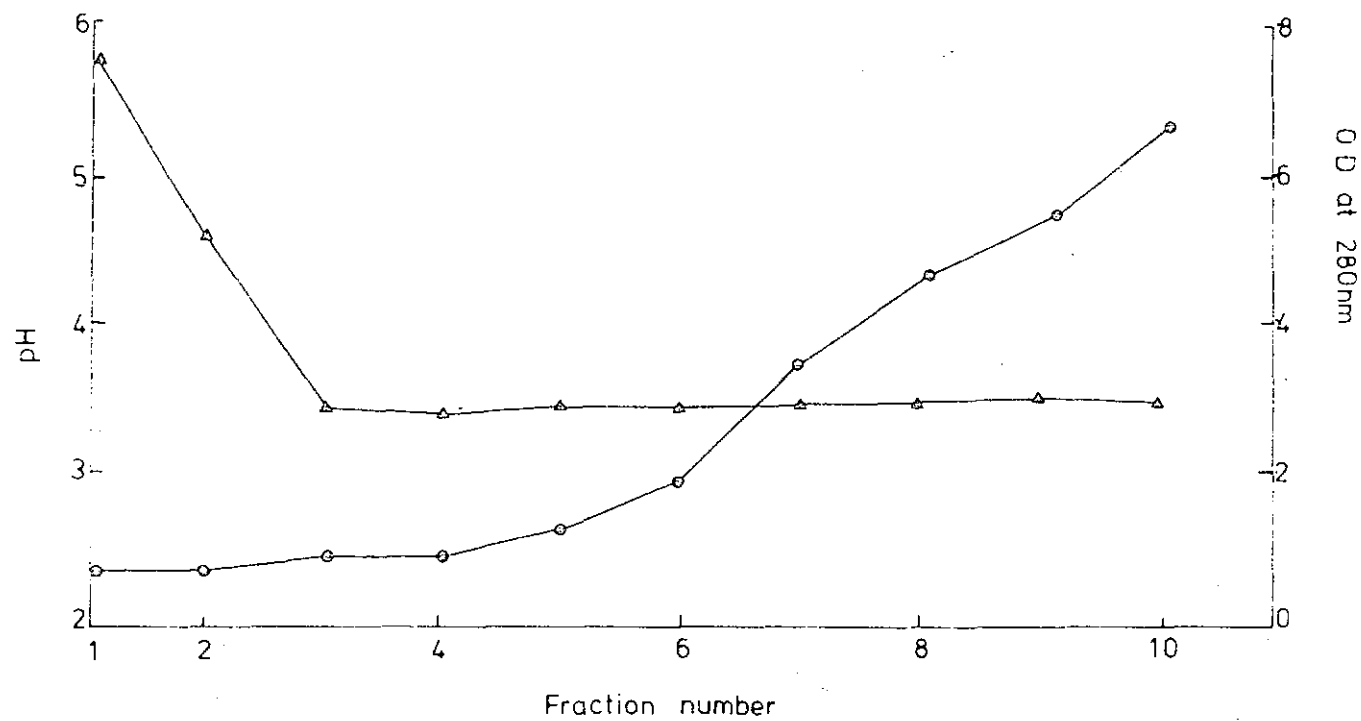

OD at 280 nm : o—o

pH : △—△
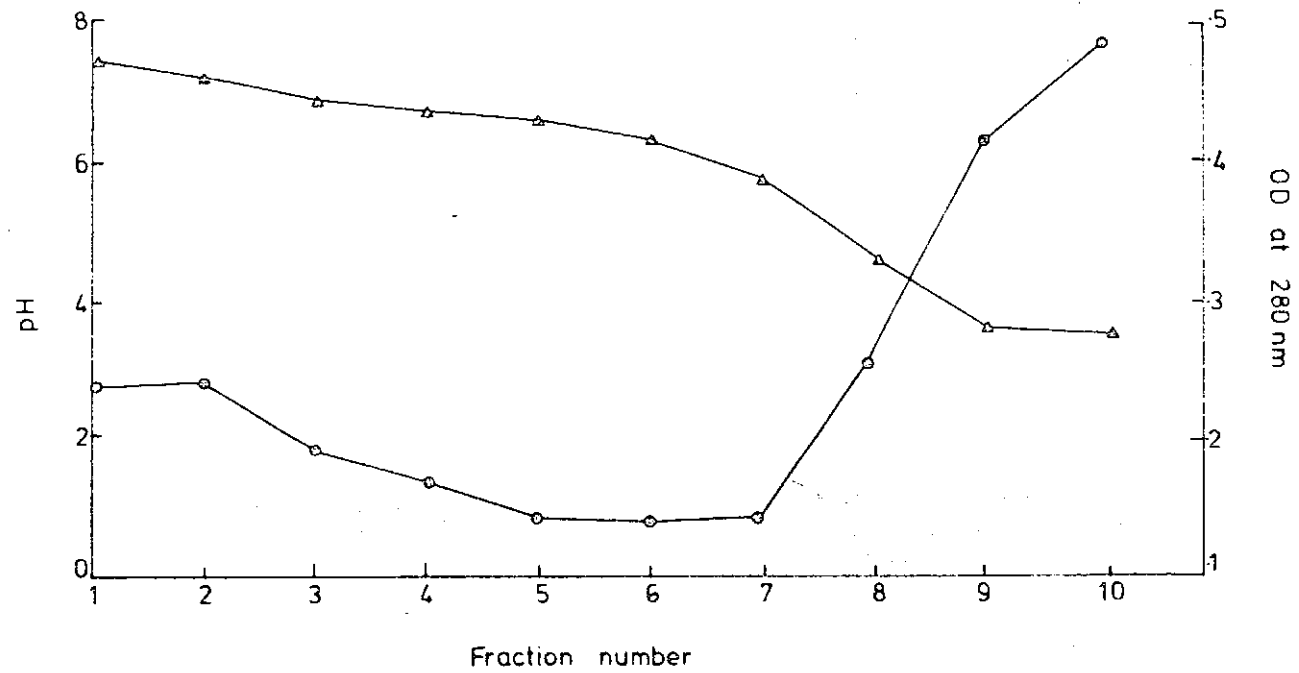
Ion exchanger in Na⁺ form.
Whey pH = 3·5
O D load = 0·675

Figure 7b : Optical Density and pH of eluant from column in Na$^+$ form : Whey buffered

OD at 280 nm : ⊶⊶

pH : ⊶⊶

Ion exchanger in Na$^+$ form.

Whey 0·01M in trisodium citrate, pH 3·5

O D load = 0·675

Figure 7c: Optical Density and pH of eluant from column in H⁺ form.

Fraction number
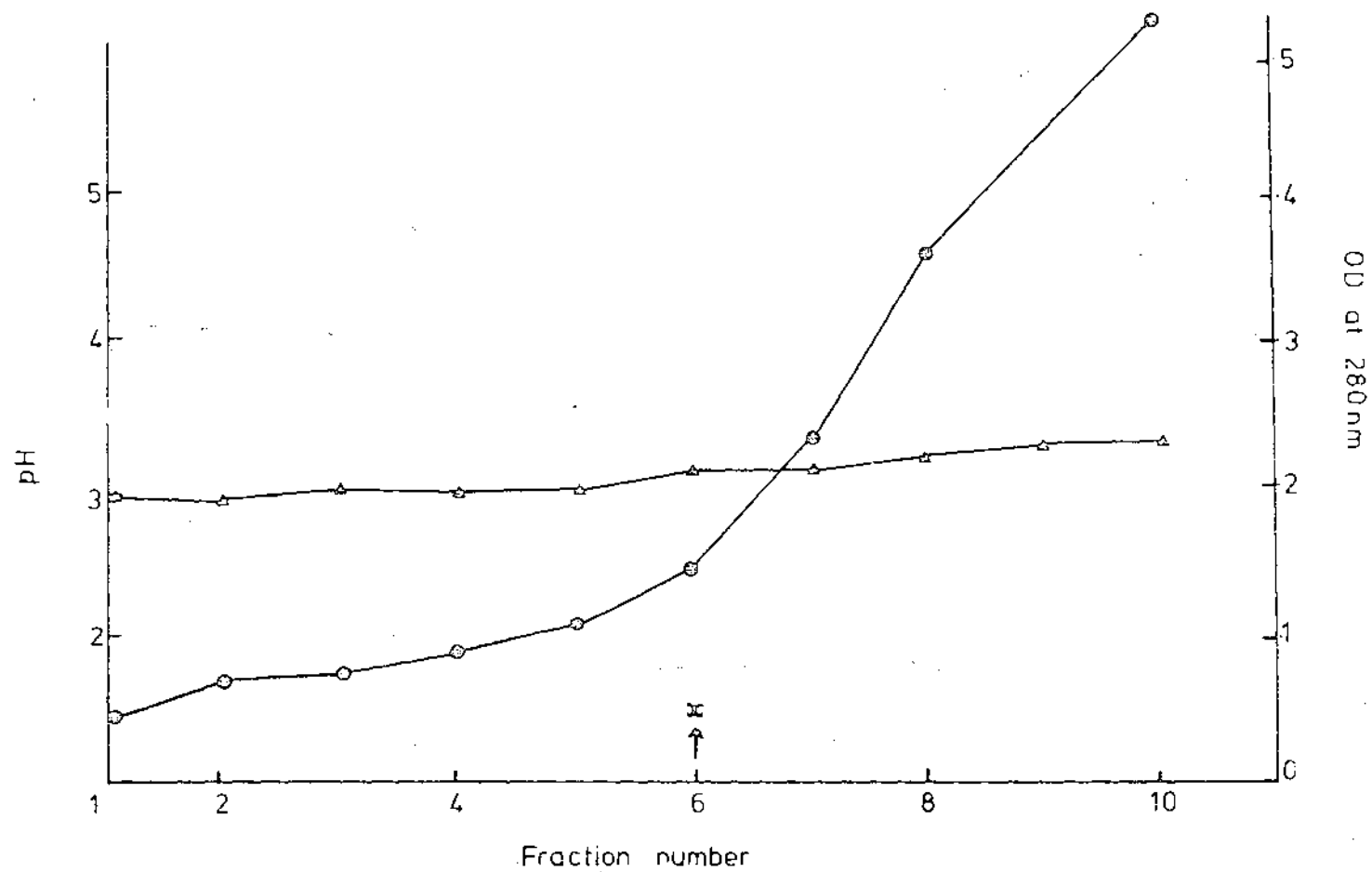
OD at 280nm: ⊘—⊘
pH: △—△   Ion exchanger in H⁺ form
OD load: 0·675   Whey pH: 3·5

Figure 7d Optical Density and pH of eluant from column in H⁺ form :
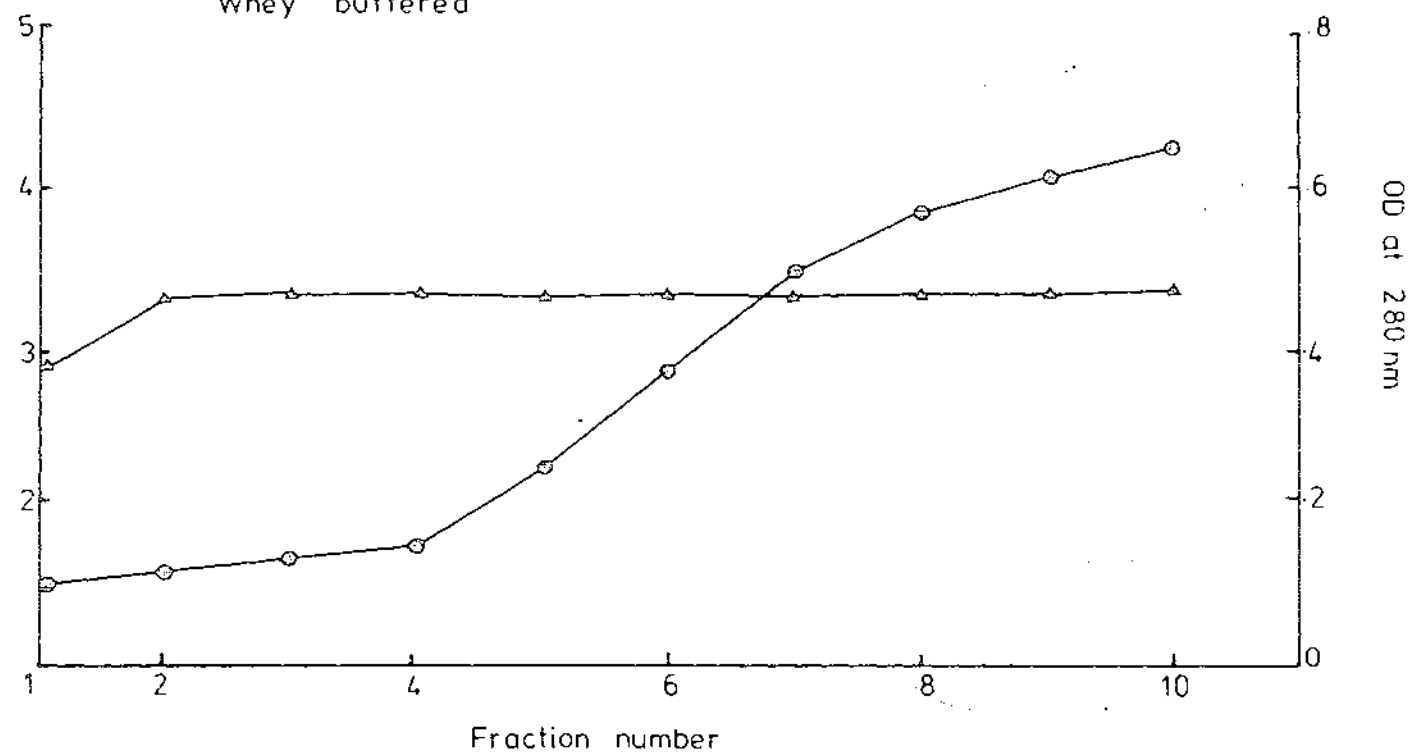Whey buffered

OD at 280nm: o—o

pH : △—△

OD load : 0·675

Ion exchanger in H⁺ farm

Whey 0·01 M in Trisodium citrate, pH 3·5

procedure is shown in Figure 8a.

It can be seen that protein breaking through the first column does take time to equilibrate the second column as far as obtaining the optimum pH, but the removal of protein is still efficient.

Fractions 8 - 16 collected from the second column were passed through a third column (again in the Na^+ form). Figure 8b shows the volume required to reach optimum pH, but the protein removal is again efficient.

It would appear then that the deproteinated whey can be used to equilibrate the next column without having to give it a separate acid wash.

3.1.5   Summary of the use of CM-Ion Exchangers

Although the CM-ion exchangers could with care be used to remove protein from demineralized whey, the column process has two main disadvantages.

(a)   It is not easy to control the pH at which the column is operating.

(b)   The necessary demineralization of the whey is an expensive process.

The difficulty in maintaining a constant pH would appear to be the result of the weak acid nature of the CM-ion exchangers. The carboxyl groups change form between pH 5 and 3 by removing hydrogen ions from the whey and upsetting the pH.

$$-O-CH_2-CO_2^- + H^+ \rightleftharpoons - O-CH_2-CO_2H \qquad pKa \quad 4\text{---}4.5$$
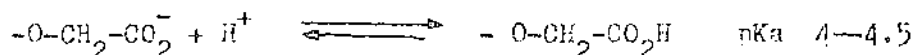
This change also causes the fall off in protein capacity below pH 3.3 causing the narrow pH optimum observed for acid adjusted and cation exchanged whey. It was hoped that by using a strong acid ion exchanger such as phosphoric acid or a sulphonic acid derivative, these difficulties would be overcome.

3.2   Protein Removal from Whey using SP-HP-Regcel

3.2.1   Determination of Optimum Adsorption pH by batch tests

In order to determine the protein binding properties of the SP ion exchangers, batch tests were carried out as for the CM ion exchangers. In the preparation of the SP-HP-Regcel it was not possible to obtain

Figure 8a: Two columns of CM-HP-Regcel in Tandern.
column 1 : H⁺ form
column 2 : Na⁺ form

OD at 280nm : O—O

pH : △—△
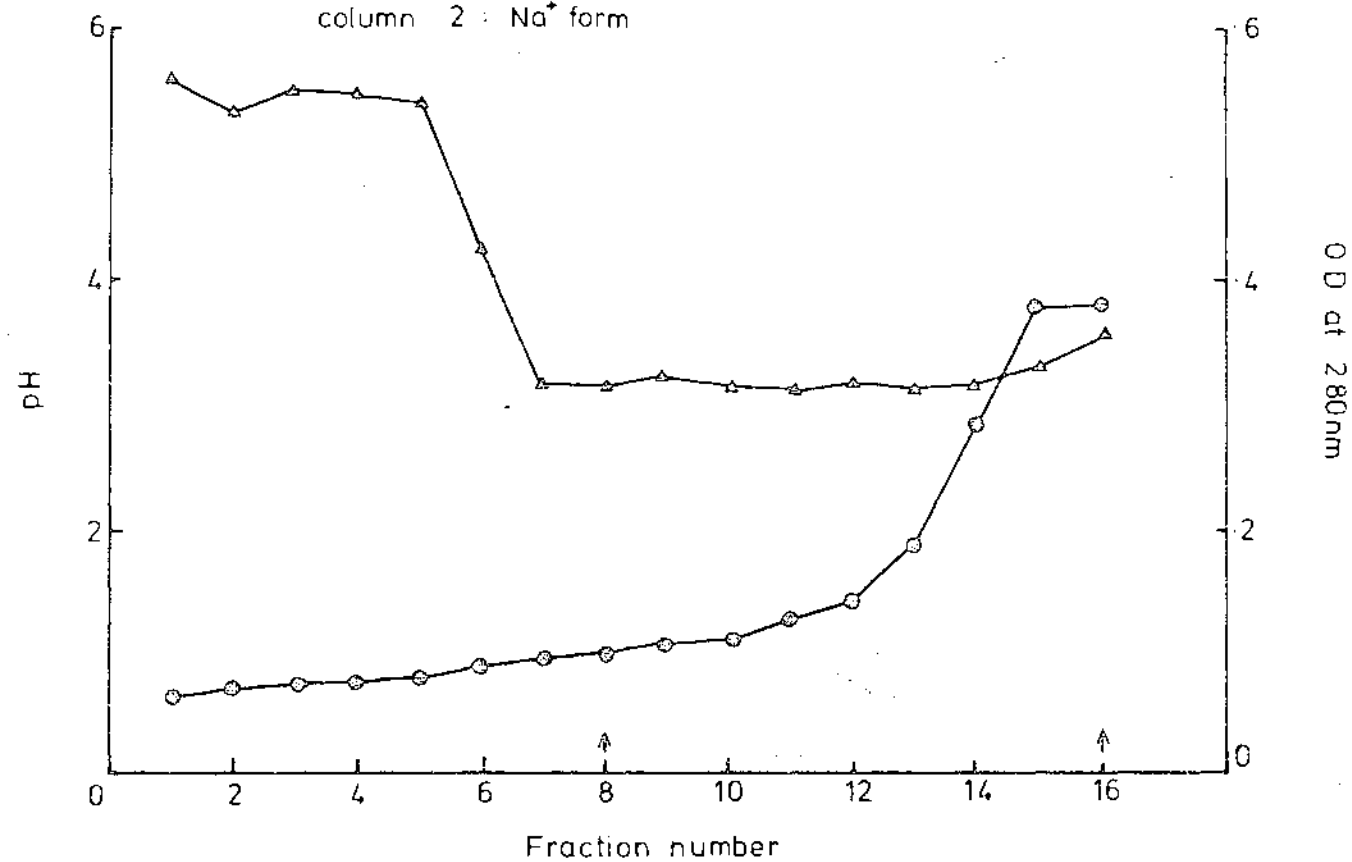
OD load : 0.375

OD at 280nm : ⊶⊶

pH : ⊶⊶

OD load : 0·375

Figure 9 : Protein adsorbed from whey as a function of pH for SP-HP-Regcel (1·1 meq /g)
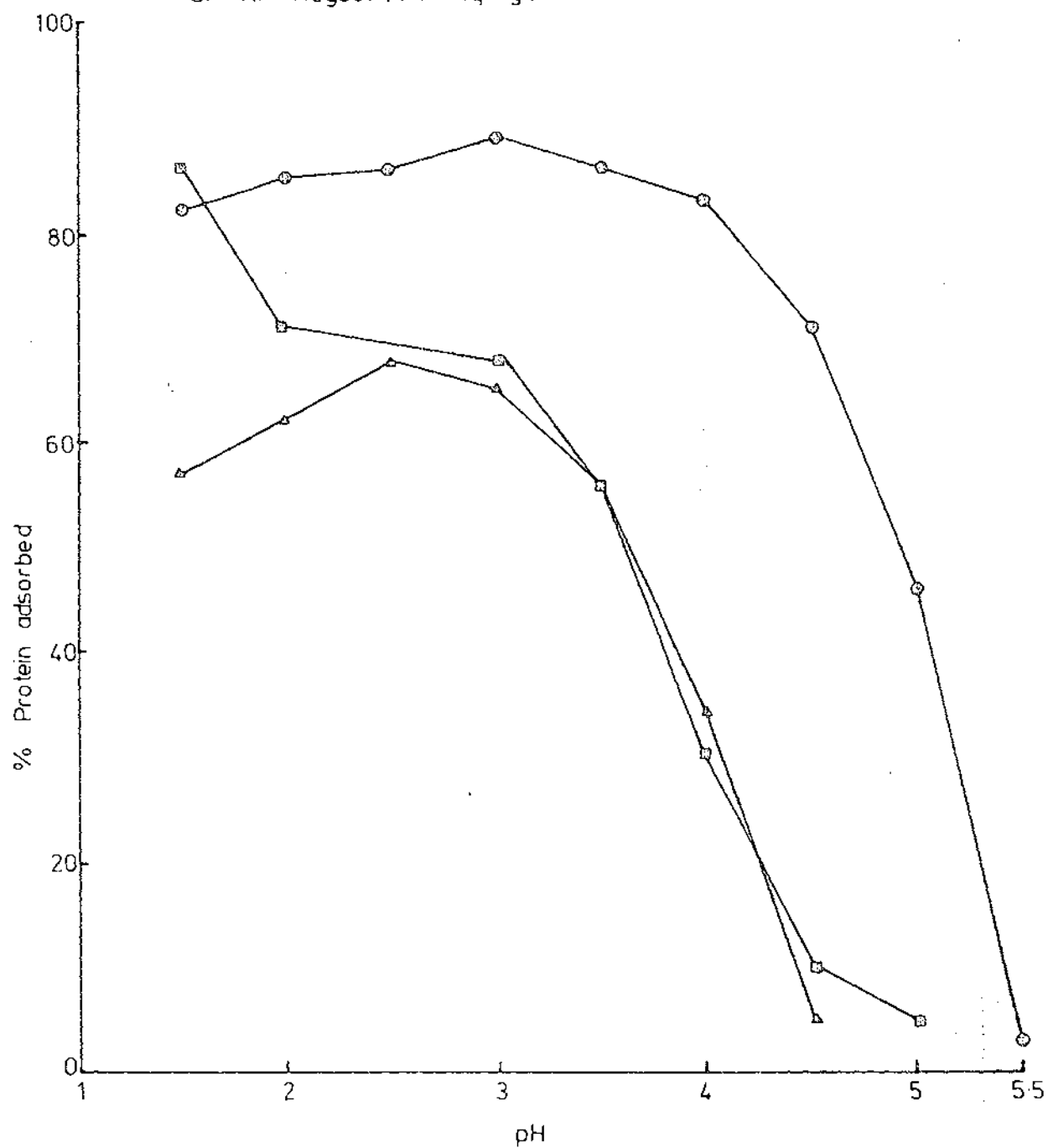
H₂SO₄ adjusted ▵—▵ . Cation exchanged ▯—▯ . Demineralised ⊙—⊙

Figure 10 : Protein adsorbed from whey as a function of pH for SP - Sephadex (2·3 meq/g)

H₂SO₄ adjusted ⌂—⌂ ., Cation exchanged ▫—▫ ., Demineralized ⊙—⊙

substitution of sulphopropyl groups much higher than 1 meq/1g.
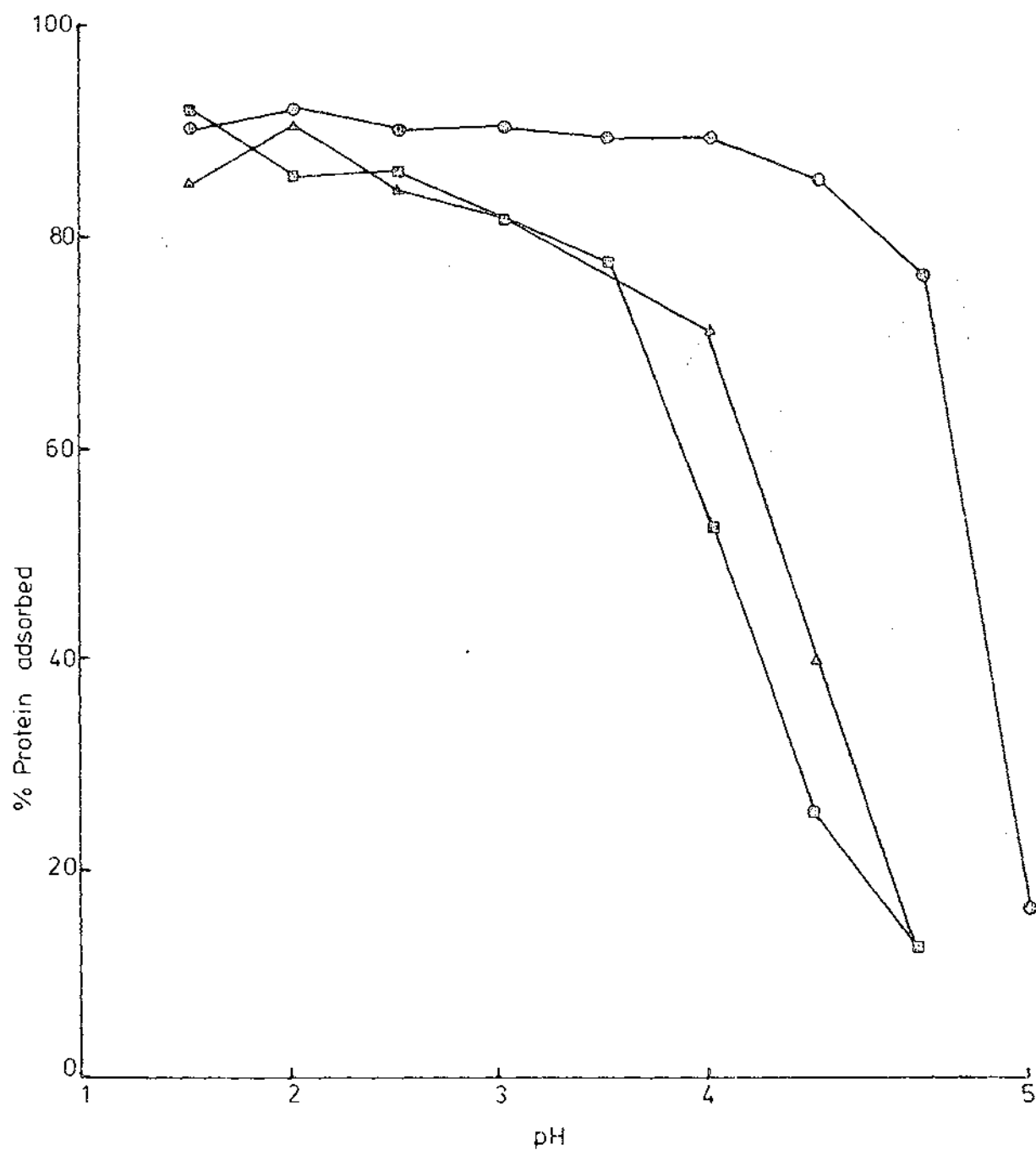Consequently SP-Sephadex-C-25 at 2.3 meq/g was also used for comparison
with a SP-HP-Regcel of 1.1 meq/g. The results of these batch tests are
shown in Figures 9 and 10 and Table 13.

TABLE 13

Optimum pH for removal of protein from whey using SP-derivatives

FORM OF WHEY	pH for Optimum Capacity	
	1.1 meq/g	2.3 meq/g
a. H_2SO_4 adjusted	1.5 - 3.5	1.5 - 4.0
b. Cations removed	1.5 - 3.5	1.5 - 4.0
c. Demineralized	1.5 - 4.5	1.5 - 5.0

The results show that good protein capacity below pH 3 occurs in
all cases and a pH range of at least 2 units with useful protein
capacity is shown. For the CM ion exchangers the fall off in protein
capacity at low pH is indeed a result of the carboxyl group losing its
charge.

There is very little difference between the acid adjusted and
cation exchanged whey except at the very low pH of 1.5 where the latter
was more efficient. In particular it was as good as the demineralised whey
at its optimum pH.

The SP-Sephadex with its higher substitution of sulphopropyl groups
reduced the difference between the three whey solutions used and in each
case extended the pH range for optimum capacity. This was similar to the
effect observed with the CM ion exchangers when a higher degree of
substitution of carboxyl groups was used.

With the large pH range available for efficient protein uptake it
was considered highly likely that a column could be used effectively
in the pH range of 2-3 without the sulphopropyl groups interfering with
the pH of the whey. This possibility was investigated.

3.2.2.   Column Deproteination of Whey with SP-HP-Regcel (1.1 meq/g)

Whey (80 ml) was passed through a small column of SP-HP-Regcel (4 ml) in the sodium ion form, at a constant flow rate of 1 ml/min, under a variety of conditions to compare the capacity and efficiency of the columns. The conditions used and results obtained are shown in Table 14. In the cases of cation exchanged whey the wet weight of Amberlite IR-120(H^+) used is also given in the table. The protein breakthrough % was determined by collecting the total eluate from the columns and analysing for true protein nitrogen.

TABLE 14

Deproteination of whey on SP-HP-Regcel

Column: 0.6 x 7.5 cm

Whey :   80 ml, 0.3% True Protein

Load :   317 mg protein

WHEY pH	IR-120(H^+) (g)	CAPACITY[a] (cm)	PROTEIN BREAKTHROUGH (%)	COLUMN SHRINKAGE (%)
2.5[b]	–	5.0	8	13
2.5	1.7	5.3	13	13
2.0	2.1	5.5	13	13
1.5	5.8	4.0	10	20

a.   Column length of bound protein visible on the column.

b.   Demineralized whey adjusted to pH 2.5 with 0.16 ml of $2MH_2SO_4$

Demineralized whey is only marginally more efficient than cation exchanged whey when using SP-HP-Regcel to remove protein. The column capacity is similar for the two wheys. There does not appear to be any advantage in using complete demineralization of whey now.

With cation exchanged whey there was a noticeable improvement in capacity of the column in going from pH 2.0 to 1.5. This was indicated in the results of the batch tests shown in Figure 9. Although 2 to 3 times as much IR-120(H^+) cation exchange resin is required to reach pH 1.5 as pH 2 it could be advantageous on a large scale because of

the improved efficiency of the SP-column for removing protein.

3.2.3 Deproteination of Lactic Acid Whey at pH 2

A trial column run was also carried out using lactic acid whey (supplied by the Dairy Research Institute), in place of the sulphuric acid whey used for all other tests. 12.6 gs of Amberlite IR-120(H^+) were required to adjust 210 mls of lactic acid whey to pH 2.0. 180 mls of this pH adjusted lactic acid whey were passed through a SP-HP-Regcel column (5 ml), at a flow rate of 1 ml/min. The pH and O.D at 280 nm were recorded and are shown on Figure 11.

3.2.4 Performance of a 50 ml Column of SP-HP-Regcel.

Because of the difficulty experienced in obtaining a protein mass balance from the small test columns, a final column was run on ten times the scale. The whey was cation exchanged first by passage through an Amberlite IR-120 (H^+) column. The pH of the whey after complete removal of the cations was 1.3. The solution was then loaded onto the SP-HP-Regcel in the Na^+ form. Samples of the eluate were taken for pH measurement and O.D readings at 280 nm and the results are shown on Figure 12.

With the column in the Na^+ form, pH control on the column is not a problem as shown by the pH of the eluate dropping rapidly to pH 1.2. A similar result was obtained with lactic acid whey (Figure 11).

This shows the main advantage of a strong acid ion exchanger such as the sulphopropyl derivative. When the protein is removed from the column at the finish with NaOH, the ion exchanger is left in the Na^+ form and is immediately ready for reuse.

The O.D. readings at 280 nm (mainly a result of non-protein compounds) remained fairly constant until 1400 ml when there was a rapid upswing as a result of greater amounts of protein breakthrough.

After 1550 ml had been loaded onto the column it was washed with water before removing the protein from the column. Normally this would be done by using either a solution of high ionic strength or high pH or a combination of both. In order to recover the protein from the ion exchanger in a highly concentrated solution with a low ash content it was decided to remove the protein from the column by using the minimum volume of dilute buffer but circulating it continuously. The circulating solution also had to be adjusted continually back up to pH 8. In this way

Figure 11: Optical Density and pH of eluant from column of SP-HP-Regcel (1.1 meg/g) for Lactic Acid Whey.
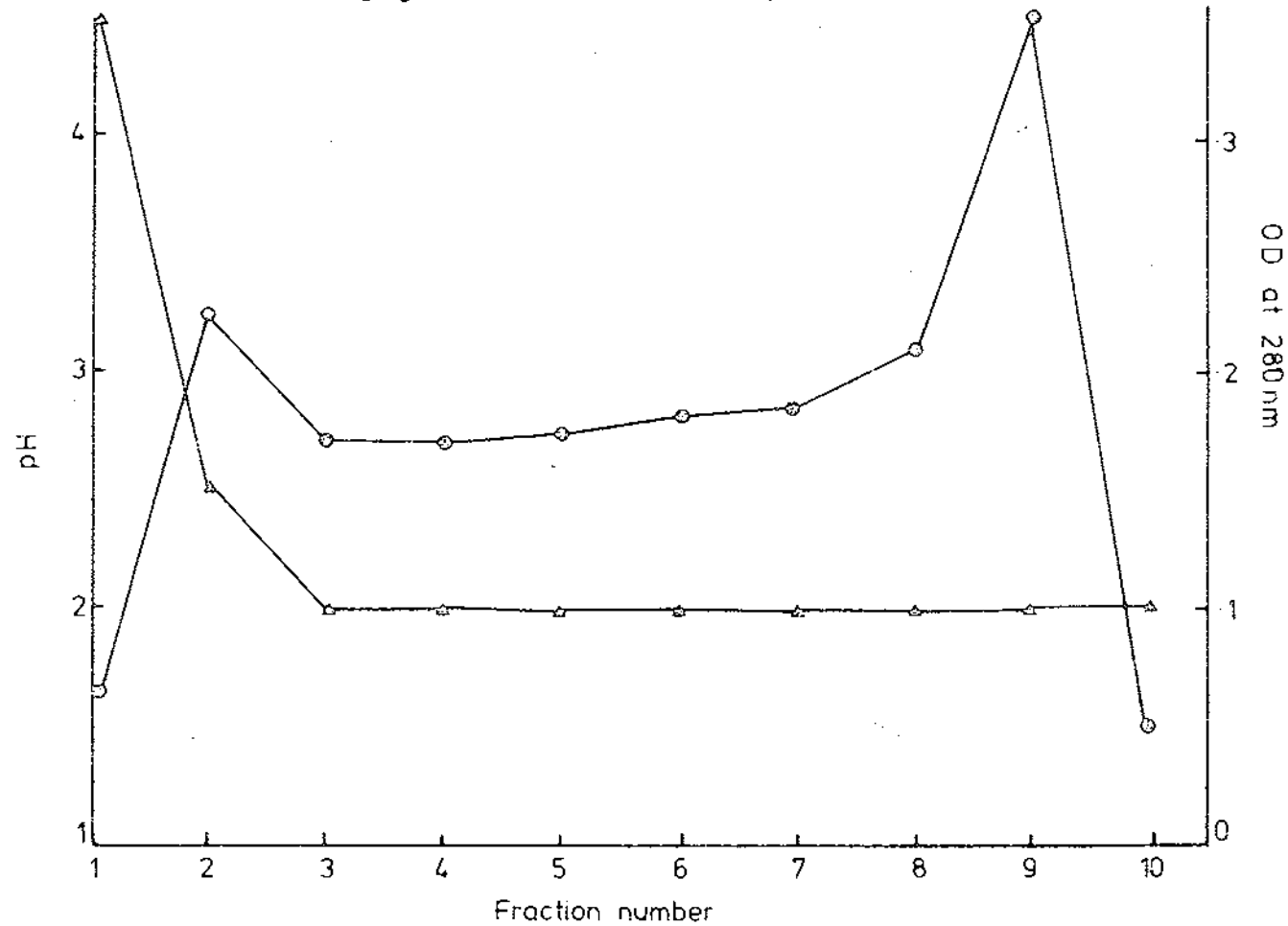
OD at 280 nm: ⊖—⊖   Protein loaded   610 mg   Column Dimensions : 0.6 × 9 cm

pH: △—△   Protein breakthrough : 92 mg, 15%   Flow rate : 1 ml/min

OD load 0.445   Protein stripped   : 458 mg, 75%   Whey :180 ml, 0.34% protein at pH 2.0

Figure 12 : Optical Density and pH of eluate from 50 ml column of SP - HP - Regcel (1·1 meg / g)
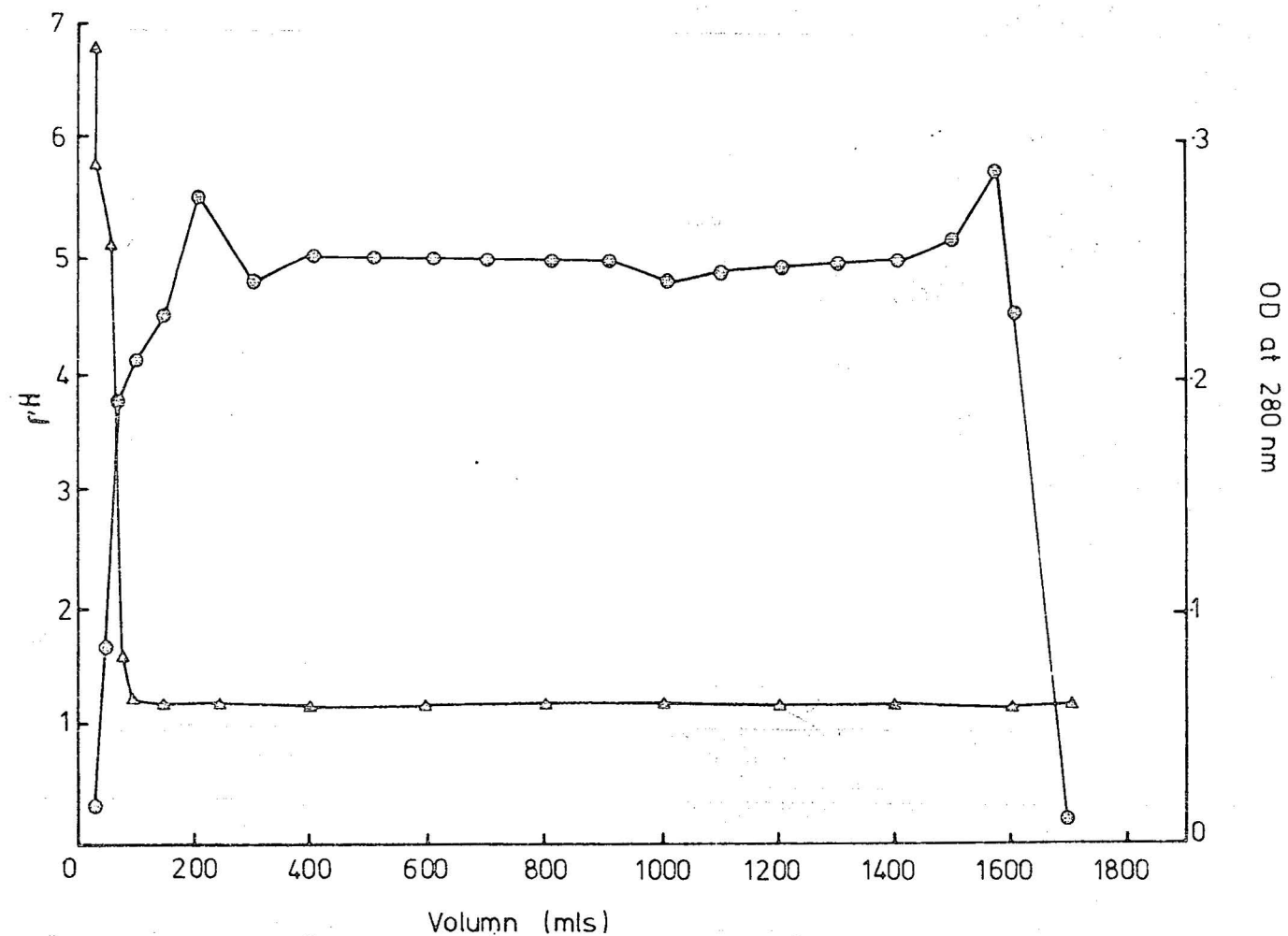
OD at 280 nm: ⊙—⊙   Flow rate   : 10 ml / min
                pH : △—△   Whey   : 1550 ml of 0·35% protein at pH 1·3
Column Dimensions : 2·5 × 10 cm (50 ml)   , OD load   : 0·415

the pH of the whole column was raised from pH 1.3 to 8 and the protein removed.

This required 3.35 mls of 10% NaOH over forty five minutes before the pH remained constant at 8. Displacement of the protein solution from the column with water after standing 1 hr. gave a 100 ml solution of 4.67% protein, a concentration of 13 fold over the whey loaded. Once the protein solution had been displaced from the column with water the column was ready again for reuse. In this case though the ion exchanger was removed from the column, mixed and analysed for protein still bound. The results of the protein analyses are shown below in table 15 and 98% of the protein is accounted for.

TABLE 15

Protein Analyses

PROTEIN SOLUTIONS	WEIGHT PROTEIN (g)	PERCENTAGE
Protein loaded: 1550 ml at 0.354%	5.49	100
Protein in breakthrough: 1680 ml at .0023%	0.48	9
Protein stripped: 100 ml at 4.67%	4.67	85
Protein on ion exchanger	0.22	4
	TOTAL :	98

Not all the protein remaining on the ion exchanger was irreversibly bound as half of it was later removed on standing in a sodium chloride solution. The protein solution removed from the column was freeze dried. This freeze dried powder (5.3 g) gave the following results on analysis.

Analysis of freeze-dried Protein Powder

Protein	89.3%
Ash	4.9%
Lactose	1.3%
Water	2.8%
TOTAL:	98.3%

This powder has a 92% protein content (calculated on dry basis) compared with 96% obtained by the Viscose Group Ltd (31, 59, 67). However they required ultrafiltration of the protein solution after removal from their ion exchange media in order to remove the high salt content and obtain the purity of 96%.

3.3   Polyacrylamide Gel Electrophoresis

In all columns run a small amount of protein came through the column. This was investigated by polyacrylamide gel electrophoresis (PAGE) to see if one particular protein or a small amount of all the whey proteins were breaking through the column. In addition the protein recovered from the column was also compared with the original starting whey on electrophoresis. The results are shown on Plates 3 and 4.

Gel (a) of Plate 3, shows the typical pattern for whey proteins. B-lactoglobulin polymorphs A and B bands are visible as are ∝-lactalbumin and serum albumin. Other minor whey protein bands are faintly visible. Gels (b) and (c) of Plate 3 show the proteins present in the whey eluate from the column. The same amount of whey was used for Gel (b) as the amount used for the original starting whey in Gel (a) and clearly shows that the bulk of the protein has been removed. The whey eluate was concentrated ten times and rerun to show the proteins still present in the whey eluate, Gel (c) of Plate 3. Most of the bands present in the original starting whey can still be seen. The most significant difference is that one of the minor whey proteins, possibly Transferrin, is present in higher concentrations and appears not to have bound to the SP-HP-Recel.

Plate 4, shows the proteins present in the original starting whey, Gel (a), and the proteins removed from the column, Gels (b) and (c). It can be seen that B-lactoglobulin, ∝- lactalbumin and serum albumin are all present. Palmer (59), reported that the Vistec cellulose media, in a batch process gave pure ∝-lactalbumin and B-lactoglobulin. With the columns process using SP-HP-Recel we have clearly isolated the serum albumin in addition to B-lactoglobulin and∝-lact albumin, and all three proteins appear to be undenatured, even though the column was run at the very low pH of 1.3

Plate 3.   Polyacrylamide gels of original starting whey and breakthrough

a.   b.   c.
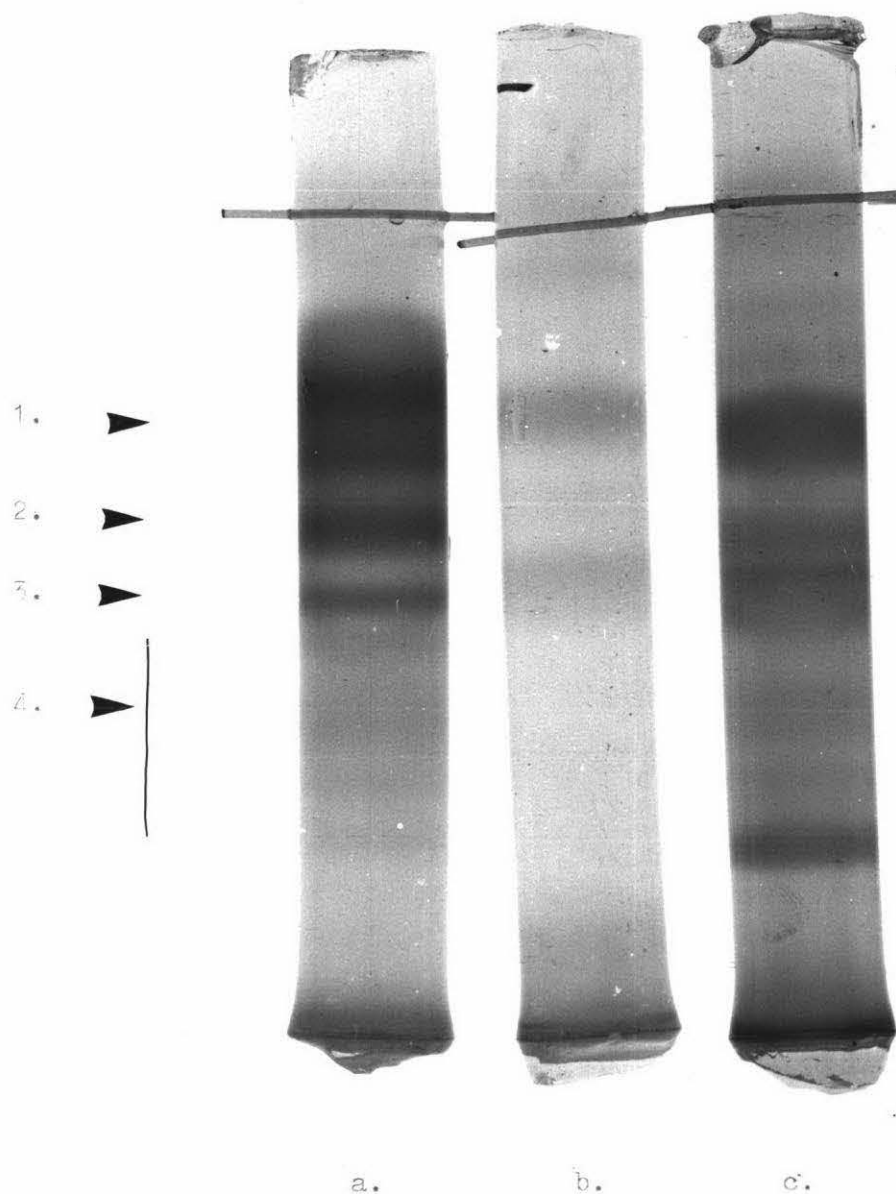
a.   original starting whey: 100 μl
b.   unconcentrated breakthrough   100 μl
c.   concentrated breakthrough   100 μl

1. B-lactoglobulin polymorphs A and B
2. α -lactalbumin
3. Serum albumin
4. Minor whey proteins

Plate 4. Polyacrylamide gels of original starting whey and
recovered proteins
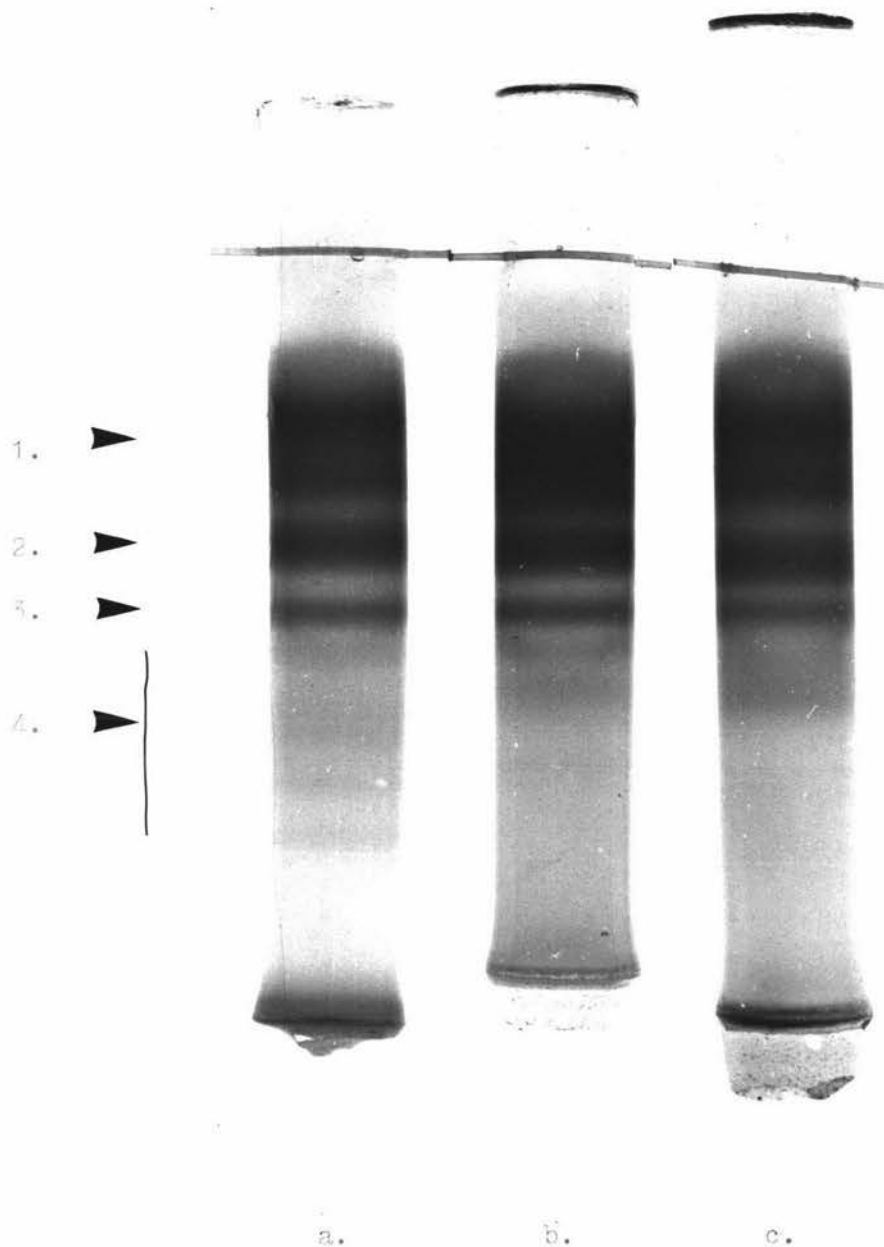
a.　　　　　　　b.　　　　　　　c.

a. original starting whey　　100 µl
b. stripped　　　　　　　　100 µl
c. stripped　　　　　　　　100 µl

1. β-lactoglobulin polymorphs A and B
2. α-lactalbumin
3. Serum Albumin
4. Minor whey proteins

CONCLUSION

Ion exchangers, both anionic and cationic have been prepared
from regenerated cellulose with the aid of hydroxyalkylating agents.
These new ion exchangers have higher protein capacities and greater
stability to caustic solutions than those already available from
regenerated cellulose eg.: Protion. They are also superior for
enzyme and protein purifications.

Although the DEAE-, CM- and SP- derivatives of hydroxypropylated
regenerated cellulose were prepared, only in the case of DEAE was any
attempt made to find the optimum formulation. This still needs to
be determined for the CM- and SP-derivatives. The optimum formulation
will depend on the particular regenerated cellulose used and
the scale of production. The loss of propylene oxide when making
the ion exchangers on a small scale was probably quite significant,
so on a large scale less propylene oxide could be required.

Because of this dependence on the source of regenerated
cellulose and the scale of operation only a limited effort was
made to find the optimum formulations. The aim of this thesis was
to show that improved ion exchanges could be obtained using hydroxy-
alkylating agents and this was achieved.

The SP-derivative has been shown to be a particularly useful
ion exchanger for whey protein isolation and a new process developed
on a laboratory scale to do this. The potential of this process
now needs to be explored on a larger scale.

APPENDIX

Commercially available Xerogel ion exchangers·

TABLE 1

Xerogel based Cation Exchangers

SUBSTITUENT	PRODUCT	MATRIX	TOTAL CAPACITY (meq/g)	SUPPLIER [a]
Carboxymethyl	Bio-Gel CM-2	Bio-Gel P-2+	-	1
Carboxymethyl	Cellex CM	Cellulose	0.7	1
Carboxymethyl	CM 22 (Std)	Cellulose	0.6	7
Carboxymethyl	CM 23 (fines)	Cellulose	0.6	7
Carboxymethyl	CM 32	Cellulose	1.0	7
Carboxymethyl	CM 52	Cellulose	1.0	7
Carboxymethyl	CM-Bio Gel A	Bio Gel A +	1.5	1
Carboxymethyl	CM-Cellulose	Cellulose	1.0	8
Carboxymethyl	CM-Cellulose	Cellulose	1.0	6
Carboxymethyl	CM-Sephadex C-25	Sephadex C-25+	4.5	4
Carboxymethyl	CM-Sephadex C-50	Sephadex C50+	4.5	4
Carboxymethyl	CM-Sepharose CL6B	Sepharose CL6B+	12 meq/100 ml	4
Carboxymethyl	CM-Glycophase	Glycophase	0.1	6
Carboxymethyl	CM-Cellulose 2100	Cellulose	0.7	9
Carboxymethyl	Vistec Cl	Cellulose	0.9	2
Carboxymethyl	Vistec Cl (5)	Cellulose	0.9	2
Sulphonic	Spheron S1000	Sepharon 1000+	1.5	2
Sulphonic	SP-Glycophase	Glycophase +	0.05	6
Sulphopropyl	Sp-Sephadex C-25	Sephadex C-25+	2.3	4
Sulphopropyl	Sp-Sephadex C-25·	Sephadex C-50+	2.3	4

a. Suppliers are listed at the end of Table 2.

b. Particle size dry (u)

TABLE 2

Xerogel based Anion Exchangers

SUBSTITUENT	PRODUCT	MATRIX	TOTAL CAPACITY (meq/g)	SUPPLIER
DEAE	DE-22	Cellulose	1.0	7
DEAE	DE-23	Cellulose	1.0	7
DEAE	DE-32	Cellulose	1.0	7
DEAE	DE-52	Cellulose	1.0	7
DEAE	DEA-Bio-Gel	Bio-Gel A+	1.0	1
DEAE	DEAE-Cellulose 1300	Cellulose	0.7	8
DEAE	DEAE-Cellulose 1300	Cellulose	0.7	8
DEAE	DEAE-Cellulose	Cellulose	–	11
DEAE	DEAE-Sephadex A-25	Sephadex G-25+	3.5	4
DEAE	DEAE-Sephadex A-50	Sephadex G-50+	3.5	4
DEAE	DEAE-CL6B	Sepharose CL6B+	13 meq/100 ml	4
DEAE	FN 2100 DEAE Cellulose	Cellulose	0.7	9
DEAE	DEAE Glycophase	Glycophase+	0.1	6
DEAE	Vistec D1	Regcel	1.0	2
DEAE	Vistec D.I 5 Grade	Regcel	1.0	2

DEAE – Diethylaminoethyl

+ – particle size dry (u)

SUPPLIERS:
1. Bio-rad Laboratories Ltd
2. Koch-Light Laboratories Ltd
4. Pharmacia (G.B.) Ltd
6. Pierce and Warriner (U.K.) Ltd
7. Whatman Ltd.
8. Anderman and Co. Ltd.
9. Camlab Ltd.
11. Chromatographic Services Ltd.

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