SYNTHESIS OF ALKYL QUATERNARY AMINO CELLULOSES AND AN INVESTIGATION OF THEIR POTENTIAL AS BILE ACID SEQUESTRANTS

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ABSTRACT

One method of treatment for patients with elevated serum cholesterol levels (hypercholesterolemia) is the oral administration of an anion exchange resin to adsorb bile acids in the gastrointestinal tract. The resins used successfully to date have been mainly synthetic ones, whereas anion exchange derivatives of cellulose have not been shown to be effective.

Alkyl quaternary amino (QA) cellulose ion exchangers were prepared from hydroxypropylated regenerated cellulose (HP Indion^{R}). Their capacities for cholate anions were measured in the presence of a background of chloride ions at physiological concentration. It was shown that butyl QA cellulose was ineffective at binding cholate anions. However, alkyl QA celluloses with alkyl groups longer than butyl (octyl and dodecyl) bound cholate anions in preference to the chloride ions and greatly improved capacities were obtained. The capacities of the octyl and dodecyl QA celluloses were dependent on the cross-linking present in the HP Indion used. If the cross-linking was greater than the 15% used in HP5 Indion, then the capacity for binding cholate dropped off. A significant increase in substitution level and decrease in swollen volume were obtained by reprocessing the alkyl QA cellulose ion exchangers and this improved their cholate binding capacities.

The alkyl QA celluloses were all prepared by first attaching chemically reactive epoxide groups to HP Indion and then reacting those epoxide groups with a tertiary amine (N,N-dimethyl-N-alkylamine) to form the quaternary amino cellulose. These two steps were repeated for the reprocessed products. The optimum conditions for epoxidation of HP5 Indion were shown to be 48 hours at 4-6°C in 6% sodium hydroxide with excess epichlorohydrin. In the reprocessing step, where this reaction was used a second time, 24 hours was sufficient to reach maximum epoxidation levels. When coupling the N,N-

dimethyl-N-alkylamine to the epoxide cellulose it was necessary to use aqueous ethanol as a solvent. The ethanol concentration was dependent on the size of the alkyl group. For N,N-dimethyl-N-dodecylamine a concentration of greater than 60% ethanol was required. For larger amines considerable hydrolysis of the epoxide groups occured during coupling. This was avoided almost completely in the first coupling step by half-neutralising the amine with hydrochloric acid, to lower the pH and buffer the reaction. The optimum coupling time and temperature were shown to be 10 hours at 70°C.

As a result of optimising these processing steps the substitution level obtained for dodecyl QA cellulose was increased from 0.83 to 1.14 meq/g at the end of the first stage and from 1.18 to 1.50 meq/g with the reprocessed product.

A range of alkyl QA celluloses were prepared using the optimum conditions found for dodecyl QA cellulose. Octyl and dodecyl QA celluloses prepared on HP7 Indion were shown to have higher capacities for cholate than cholestyramine and colestipol (Colestid^{R}) at cholate concentrations less than 6 mmol/L. The dodecyl QA cellulose had superior capacities at all cholate concentrations (0-15 mmol/L) when measured on the basis of the volume of the ion exchanger used. In contrast, cholestyramine and colestipol had higher capacities for deoxycholate anions than either of the cellulose products, except when the residual concentration of deoxycholate was very low (less than 0.5 mmol/L). Cholestyramine and colestipol bound deoxycholate efficiently (97-99%) when the residual concentration was between 5-10 mmol/L, but not cholate (29-48%). However the alkyl QA celluloses bound deoxycholate and cholate with very high efficiency (82-138%).

To compare the efficiencies of the ion exchangers at low concentrations a more sensitive test was developed utilising a mixture of conjugated bile salts, similar in composition to that found in the human duodenum. This was equilibrated with

samples of each ion exchanger and then analysed by HPLC. Methyl and butyl QA celluloses were the least effective at reducing the concentrations of the bile salts, however they still performed as well as colestipol. The capacity of octyl QA cellulose was much better than the methyl or butyl QA celluloses, indicating the importance of the hydrophobic alkyl chain. Cholestyramine had a similar capacity to the octyl QA cellulose, except that it was not as effective at binding glycocholate. The most effective product was found to be dodecyl QA cellulose, which reduced the concentration of all of the bile salts to lower levels than any other ion exchanger.

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ABBREVIATIONS

BBS	Bicarbonate Buffer System
CHD	Coronary Heart Disease
CL	Cross-Linking
Conc.	Concentration
DEAE	2-Diethylaminoethyl
DMB-Cellulose	Butyl Quaternary Amino Cellulose
DMDo-Amine	N,N-dimethyl-N-dodecylamine
DMDo-Cellulose	Dodecyl Quaternary Amino Cellulose
DMO-Cellulose	Octyl Quaternary Amino Cellulose
ECH	Epichlorohydrin
FH	Familial Hypercholesterolemia
GC	Glycocholate
GDC	Glycodeoxycholate
HDL	High Density Lipoprotein
HP Indion	Hydroxypropylated Regenerated Cellulose
HP3 Indion	25% Cross-Linked HP Indion Cellulose
HP5 Indion	15% Cross-Linked HP Indion Cellulose
HP7 Indion	10% Cross-Linked HP Indion Cellulose
HPLC	High Performance Liquid Chromatography
LDL	Low Density Lipoproteins
meq	Milliequivalent/s
ODS	Octadecyl Silica
% aq	Percentage Aqueous (v/v)
QA	Quaternary Amino
RT	Room Temperature
SD	Standard Deviation
SP-Cellulose	Sulphopropyl Cellulose
TMA-Cellulose	Methyl Quaternary Amino Cellulose
TC	Taurocholate
TDC	Taurodeoxycholate
TPC	Total Plasma Cholesterol
UV	Ultraviolet
VLDL	Very Low Density Lipoproteins

CHAPTER 1. INTRODUCTION

1.1 OUTLINE

The object of this thesis was to produce a quaternary amino (QA) cellulose ion exchanger with potential for use as a bile acid sequestrant in the treatment of hypercholesterolemia.

This introduction backgrounds the debilitating effects of hypercholesterolemic disorders and gives a brief summary of the clinical methods which are used to treat them. Treatments involving bile acid sequestrants, their mode of action and chemistry are reviewed. The procedures previously developed at Massey University for the preparation of cellulose ion exchangers are summarised. A rationale for the type of exchanger to be synthesised is given and the modifications considered necessary for promoting the selective and efficient binding of bile acids are discussed.

1.2 EFFECTS OF ELEVATED PLASMA CHOLESTEROL

It is now widely accepted that elevated plasma cholesterol is associated with coronary heart disease (CHD)[1]. In normal caucasians the total plasma cholesterol (TPC) level is approximately 200 mg/dL[2], but only a small fraction of this circulates as free cholesterol in the blood. Between 50-70% of TPC is transported between tissues as a low density lipoprotein (LDL) fraction, 20-45% as a high density lipoprotein (HDL) fraction, 5-10% as a very low density lipoprotein (VLDL) fraction and an insignificant amount in the chylomicrons[3]. Abnormally high levels of LDL (β-lipoproteins), and therefore cholesterol, can be associated with a number of diseases. This condition is classified as hyper-β-lipoproteinemia and there are two recognised subtypes: type IIa and type IIb[4]. Both of these subtypes result in premature CHD[5].

Familial hypercholesterolemia (FH) is a relatively common inherited form of hyper-β-lipoproteinemia, which can be identified at birth. FH individuals have significantly elevated TPC and LDL levels (2-3 times greater than normal in heterozygotes and 4-6 times greater than normal in homozygotes). The clinical consequences of unmanaged FH are well documented and include arcus coronae, xanthomas, marked atherosclerosis and premature CHD[3].

1.3 GENERAL APPROACH TO CLINICAL MANAGEMENT OF HYPERCHOLESTEROLEMIA

Substantial reductions, sometimes resulting in normalisation of TPC and LDL concentrations, can now be made in all forms of hyper-β-lipoproteinemia except for homozygous FH[6]. Initial treatment involves adherence to a strictly controlled diet, followed by administration of hypolipidemic drugs where necessary (Table 1.1). Altered diets (which limit cholesterol intake to less than 300 mg per day and are low in saturated fat) can be expected to reduce TPC and LDL levels by up to 10%[7], but normalisation is still not achieved in some cases. Recent studies[6,8,9] indicate that the most promising treatment of severe hyper-β-lipoproteinemia probably lies with combined treatment regimens, incorporating a bile acid sequestrant with complimentary drugs.

TABLE 1.1 Hypolipidemic Drugs Used in the Management of Plasma Cholesterol[6]

Drug	Mode of Action
Nicotinic acid	Inhibits secretion of VLDL (a precurser for LDL synthesis)
Compactin Mevinolin	Cause competitive inhibition of the major rate limiting step of cholesterol biosynthesis
Clofibrate Gemfibrozil	Cause hydrolysis of VLDL triglycerides by lipoprotein lipase
Neomycin	Inhibits reabsorption of bile acids (used where bile acid sequestrants are poorly tolerated)

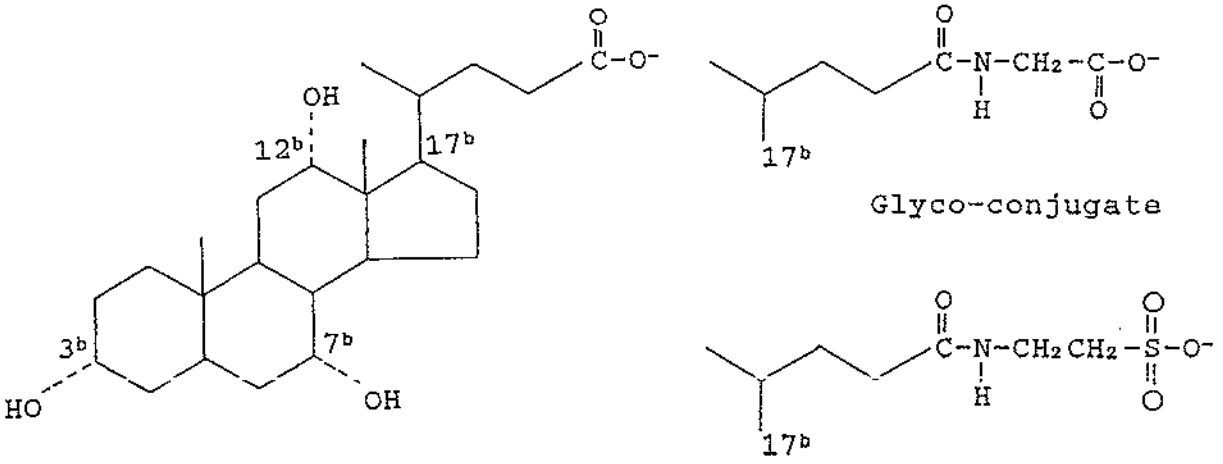
1.4 USE OF BILE ACID SEQUESTRANTS TO REDUCE PLASMA CHOLESTEROL LEVELS

One well established method of treating hypercholesterolemia involves oral administration of non-absorbable anion exchangers which function as bile acid sequestrants. These materials have basic functional groups attached to a semi-rigid polymer backbone and they operate by exchanging chloride ions for bile acid anions in the intestinal lumen[10,11].

Bile acids are secreted into the duodenum in response to food intake, where they promote the absorption of dietary fat. The structure of the major bile acids and their distribution in human bile is given in Figure 1.1[12]. Ingestion of bile acid sequestrants increases the faecal excretion of bile acids and lowers their concentration in the intestine. This results in decreased absorption of dietary cholesterol and other lipids[13]. It also causes an indirect decrease in plasma cholesterol, resulting from disruption of the enterohepatic circulation by which bile acids are reabsorbed. In healthy individuals approximately 95% of bile acids are actively reabsorbed from

the distal ileum and the remaining 5% are compensated for by new synthesis[12]. However, under the conditions of bile acid sequestrant therapy fewer bile acids are reabsorbed, so an increased rate of synthesis must occur. Plasma cholesterol (from LDL) is the precurser for all new liver synthesis of bile acids[14], therefore when bile acid sequestrants are used there is increased removal of plasma LDL and a corresponding decrease in TPC[15].

Figure 1.1 Major Bile Acids Present in Human Bile[a]



Cholate

Bile Acid	3[b]	7[b]	12[b]	Proportion (%)
Cholate	OH	OH	OH	50
Chenodeoxycholate	OH	OH	H	30
Deoxycholate	OH	H	OH	15
Lithocholate	OH	H	H	5

[a] Bile acids are usually present in human bile as sodium or potassium salts, and they are conjugated with either glycine or taurine

[b] Numbers refer to positions in the sterol ring

1.5 BILE ACID SEQUESTRANTS CURRENTLY AVAILABLE

The ion exchangers which have been used for therapy are:

A. Cholestyramine

Cholestyramine, the most commonly used bile acid sequestrant, is or has been available as Cuemid (83% cholestyramine[16]), Questran (45% cholestyramine[16]) and Quantalan[17]. It is a copolymer of styrene and 2% divinylbenzene, with quaternary amino groups attached using trimethylamine and balanced with chloride counterions (Figure 1.2A).

B. Colestipol Hydrochloride

Colestipol hydrochloride is a copolymer of diethylenetriamine and epichlorohydrin with approximately 20% of the available nitrogens titrated with hydrochloric acid (Figure 1.2B). It is sold under the proprietary name Colestid[17].

C. Diethylaminoethyl (DEAE) Sephadex

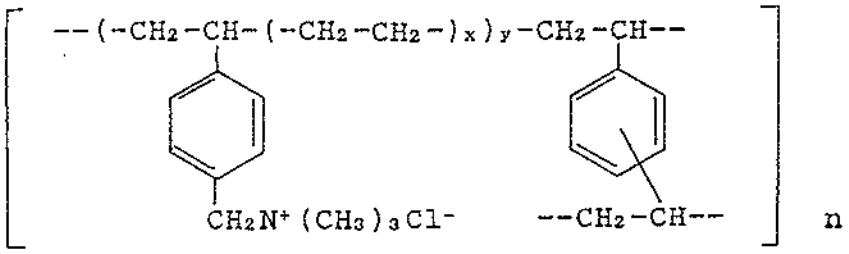
Polydexide or PDX chloride (formerly Secholex) is 79% DEAE-Sephadex[16]. It is a dextran cross-linked with epichlorohydrin and orthosubstituted with 2-diethylaminoethyl (DEAE) groups, some of which are quaternised with 2-diethylaminoethyl chloride[17] (Figure 1.2C).

Cholestyramine based bile acid sequestrants (Questran and Cuemid) are usually preferred over other products (Colestid and Secholex) because they have superior capacity and binding kinetics for bile acids in vitro[16,20]. Laboratory studies have demonstrated that Questran binds 40% more bile acids than Cuemid after mixing with bile for 2 hours at 37°C[16]. In contrast, clinical studies show that the different bile acid sequestrants produce similar hypolipidemic effects in vivo[21,-22,23]. Typically, the TPC in patients can be expected to drop by 20-25% more than that achieved with dietary control alone. This drop occurs in a very short time after administration of

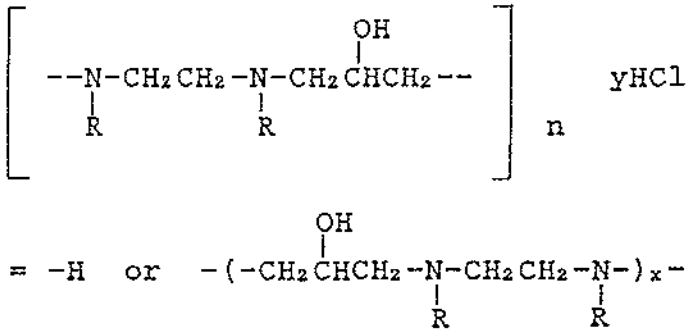
the sequestrant and can be sustained indefinitely with continued use.

Figure 1.2 Partial Structures of Bile Acid Sequestrants Used in Therapy

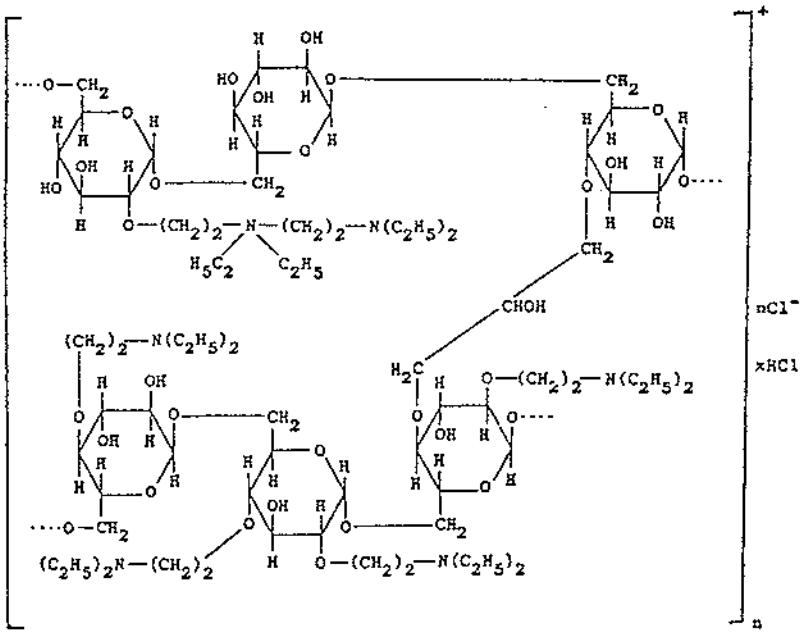
A. Cholestyramine[18]



B. Colestipol[18]



C. DEAE Sephadex[19]



1.6 SOME PROBLEMS ASSOCIATED WITH THE USE OF BILE ACID SEQUESTRANTS

Bile acid sequestrants are nonspecific and, as a result, a number of anions are able to compete with the bile acid anions for the available binding sites. The in vitro efficiency for binding bile acids in duodenal fluid is low for all of the sequestrants mentioned in Section 1.5, with only about 50% of the available ion exchange capacity being used under physiological conditions[16]. The efficiency may be even less in vivo. Also small reductions of plasma cholesterol are compensated for by an increase in cholesterol biosynthesis[15]. Thus for effective therapy prolonged and/or large dose rates are required[17].

Since the ion exchangers are not absorbed from the gastro-intestinal tract no systemic ill effects can be expected. Nevertheless, prolonged therapeutic treatment (e.g. 16-20 g cholestyramine per 24 hours) commonly results in constipation (10-50% incidence) and can sometimes lead to nausea, vomiting and other gastrointestinal discomfort. Because normal fat absorption is impaired, malabsorption of fat soluble vitamins may also occur, leading to side-effects including lypocalcemia, hypothrombonemia and/or folic acid deficiency[10,11]. Considerable work has therefore focused on making bile acid sequestrants more effective.

The bile acid binding capacity of cholestyramine is enhanced by the addition of taurine and its 'pharmaceutically accepted' salts[25], ascorbic acid[26] and some essential fatty acids[27]. Cholestyramine resins with different mesh sizes have been tested, however while finer particle sizes were less gritty and more palatable, their in vivo efficiency in binding bile acids was not improved over the standard grade[28]. More recent studies have attempted to make cholestyramine more palatable by the addition of CM-cellulose, Primogel, lactose and flavours[29]. Also, formulations incorporating dietary fiber and

citric acid have been shown to eliminate the mouthfeel and taste of ion exchange drugs[30]. These studies point to the need for the development of new sequestrants which have superior ion exchange capacity and higher selectivity for binding bile acids.

1.7 THE USE OF HP INDION CELLULOSE MATRIX FOR THE PREPARATION OF ION EXCHANGERS

Earlier work by Dr J.S. Ayers at Massey University has demonstrated that the chemical modification of cellulose can produce materials suitable for derivatisation and subsequent use as chromatography media. These studies demonstrated that hydroxypropylated regenerated cellulose (now available as HP Indion[a]) was an acceptable starting matrix for the preparation of ion exchangers[31]. Some of the HP Indion ion exchangers produced locally have proved to be ideal for large scale protein separation[32], because they are resiliant to both chemical and physical attrition, and have high capacities for binding macromolecules[31].

HP Indion is prepared from regenerated cellulose, which is first ground and then graded into different particle sizes. Next it is swollen with propylene oxide and cross-linked with epichlorohydrin in the presence of sodium hydroxide, according to the method first described by Sheerin[33]. Typical groups present in the HP Indion cellulose matrix are shown in Equation 1.1. HP Indion is available in a range of particle sizes and degrees of cross-linking[34].

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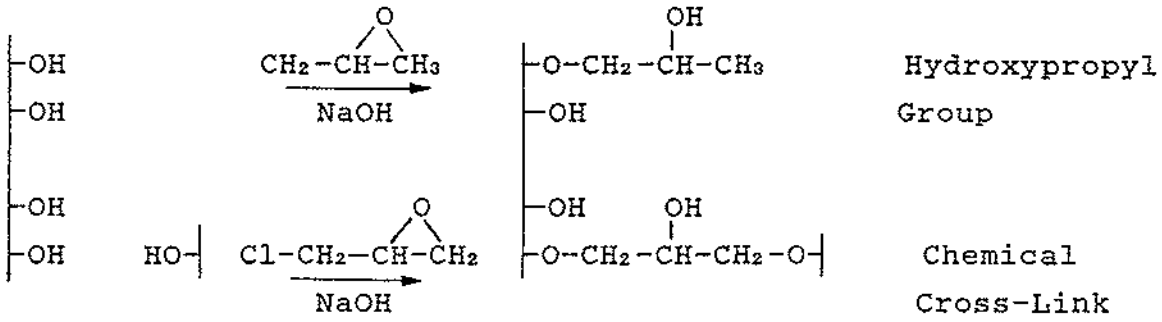
[a] Waitaki International Biosciences, Christchurch

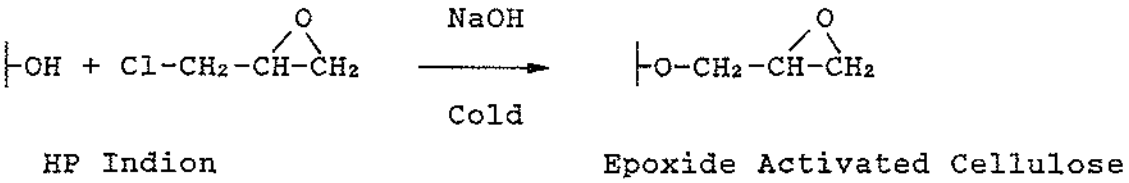
Equation 1.1 Preparation of HP Indion from Regenerated Cellulose



1.8 EPOXIDE ACTIVATION OF HP INDION CELLULOSE MATRIX

One very effective method of preparing ion exchangers from the cellulosic HP Indion starting matrix involves epoxide activation (Equation 1.2) and coupling (Equation 1.5).

Equation 1.2 Epoxide Activation

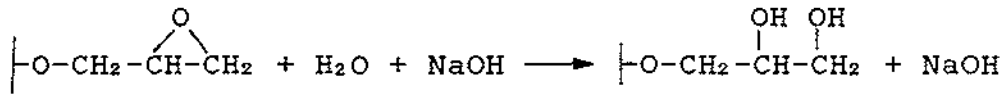


The conditions needed to substitute the maximum number of epoxide groups onto the cellulose matrix have been thoroughly investigated[35]. The critical factor affecting the level of epoxidation relates to the ratio of sodium hydroxide and epichlorohydrin used. Sodium hydroxide is consumed by epichlorohydrin in a 1:1 mole ratio in order to react with the cellulose matrix, and also through hydrolysis (Equation 1.3).

Equation 1.3 Hydrolysis of Epichlorohydrin

Once the sodium hyroxide has been consumed further activation is not possible no matter how much epichlorohydrin is used. However, if excess sodium hydroxide is used hydrolysis of the active epoxide groups occurs (Equation 1.4).

Equation 1.4 Hydrolysis of Activated Cellulose by Excess Sodium Hydroxide



Epoxide Activated Cellulose

Hydrolysis Product

In order to produce the maximum number of active epoxides a 25% molar excess of epichlorohydrin over sodium hydroxide must be used. A 6-8% w/v solution of sodium hydroxide is also essential: above this concentration extra weight is added to the cellulose without a significant increase in the number of epoxides produced (indicating that hydrolysis had occured); but when less than 6% soidum hydroxide is used limited epoxidation results. The actual volume of sodium hydroxide solution, and the number of active epoxides produced, ultimately depends on the physical properties of the HP Indion used. Other factors influencing the number of epoxides produced are the temperature and time of the reaction. Maximum epoxidation (up to 1.4 meq/g) is obtained by activating at low temperatures, however the time required for the reaction to reach completion at 4°C is in excess of 30 hours.

It is possible to couple a wide variety of functional groups to epoxide activated HP Indion (Table 1.2). Previous work demonstrated that amino cellulose ion exchangers were moderately effective at binding bile acid anions in vitro[36]. However, this work was limited and synthetic methods for optimising the ion exchange function were not investigated.

Table 1.2 Ion Exchangers Prepared from Epoxide Activated HP Indion

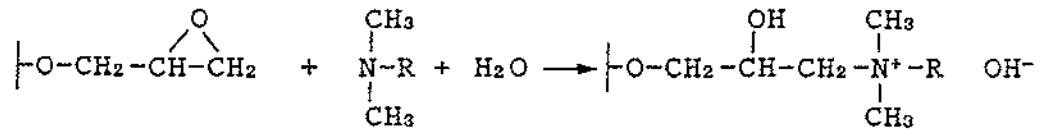
Ion Exchanger	Structure	Function
Sulphopropyl (SP) cellulose	$-SO_3^-Na^+$	Cation Exchange
Phosphate (PT) cellulose	$-PO_4^-Na^+$	
Quaternary Amino (QA) cellulose	$-NR_3^+Cl^-$	Anion Exchange
Tertiary Amino (DE) cellulose	$-NHR_2^+Cl^-$	
Primary Amino (PA) cellulose	$-NH_3^+Cl^-$	

1.9 AIM OF THESIS

Bile acid sequestrants operate by exchanging negatively charged counterions (usually chloride ions) for bile acid anions, hence it was anticipated that quaternary amino (QA) cellulose ion exchangers would be effective because they remain charged at all pH levels. Therefore it was decided to prepare a range of dimethylalkyl QA celluloses by coupling N,N-dimethyl-N-alkylamines to epoxide activated HP Indion as shown in Equation 1.5.

The aim was to identify the most effective alkyl group, and then optimise the synthesis of the cellulose ion exchanger. It was hoped that the capacity of the resultant product to bind bile acids in vitro would match or exceed that of cholestyramine. Ultimately it was hoped that a cellulose based drug would alleviate some of the side-effects which occur with the synthetic polystyrene matrix of cholestyramine.

Equation 1.5 Preparation of Quaternary Amino (QA) Cellulose (Coupling)



Epoxide Activated Cellulose — Dimethylalkyl QA Cellulose

CHAPTER 2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

HP3 Indion (Grade 3, Batch 1185, 25% cross-linking), HP5 Indion (Grade 3, Batch 1184, 15% cross-linking) and HP7 Indion (Grade 3, Batch 1183 and 1259, 10% cross-linking) were obtained from Waitaki International Bio-Sciences, Christchurch, New Zealand.

Cholestyramine resin was obtained from Sigma Chemical Company, St Louis, Missouri, USA; and colestipol hydrochloride (Colestid^{R}) from The Upjohn Company, Kalamazoo, Michigan, USA.

Acetonitrile (Far UV, HPLC), dimethylamine (26% aqueous), ethanol (AR), ethanol (AR, 95% aqueous), hydrochloric acid (Volumetric Concentrate^{R}), hydrochloric acid (AR), sodium bicarbonate (AR), sodium hydroxide (AR), sulphuric acid (AR), trimethylamine (32% aqueous) and trimethylamine (33% in ethanol) were obtained from BDH Chemicals Ltd., Poole, England; orthophosphoric acid (AR), sodium chloride (AR), sodium hydroxide (Volucon^{R}) and sodium metabisulphite (Lab. grade) from May and Baker Ltd., Dagenham, England; sodium cholate, sodium deoxycholate and sodium glycocholate from New Zealand Pharmaceuticals, Palmerston North, New Zealand; sodium glycodeoxycholate, sodium taurocholate and sodium taurodeoxycholate from Sigma Chemical Company; N,N-dimethyl-N-octylamine and n-dodecylamine from Aldrich Chemical Company, Milwaukee, Wisconsin, USA; 1-bromobutane from Riedel De Haen, Seelze-Hannover, West Germany; N,N-dimethyl-N-dodecylamine from Polysciences Inc., Warrington, England; epichlorohydrin from DOW Chemical Company, Midland, Michigan, USA; indicator paper from Whatman Ltd., Maidstone, Kent, England; nitrogen (dry industrial compressed) from New Zealand Industrial Gases,

Lower Hutt, New Zealand; and sodium sulphite (anhydrous) from Merck Ltd., Darmstadt, West Germany.

De-ionized water (Milli-Q^R) was used to wash all of the resins and for the preparation of all buffers and aqueous reagents.

2.1.2 Equipment

All titrations were carried out using an ABU II Autoburette, TT2 Titrator and REA 300 Titragraph, all from Radiometer, Copenhagen, Denmark.

Ultraviolet and visible absorption were measured on a PYE Unicam PV 8610 Kinetics Spectrophotometer from Philips, Cambridge, Great Britain.

Analysis of conjugated bile acids by HPLC was carried out using: a Zorbax^R ODS (Octadecyl Silica) column (4.6 mm x 250 mm) from Dupont, Wilmington, Detroit, USA; two Model 510 HPLC pumps, a Model 680 Automated Gradient Controller, a Model 450 Variable Wavelength Detector and a Model 730 Data Module, all from Waters Associates, Milford, Massachusetts, USA.

2.2 DRY MATTER ANALYSIS

All ion exchangers were washed with water or equilibrated with buffer, drained thoroughly on a sintered glass filter and stored at 4°C in a sealed container until required. In order to determine the dry matter of each resin, three samples were accurately weighed into small beakers of known mass and oven-dried overnight at 65°C, followed by a further 2 hours at 100°C. After cooling in a desiccator they were weighed and the dry matter was calculated as follows:

$$\text{Dry Matter (mg/g)} = \frac{\text{Dry weight of ion exchanger (mg)}}{\text{Wet weight of ion exchanger (g)}}$$

A mean of the three values was used.

2.3 GENERAL METHOD FOR PREPARING CELLULOSE ION EXCHANGERS

The novel ion exchangers were prepared exclusively from hydroxypropylated regenerated cellulose with 10%, 15% or 25% cross-linking (HP7, HP5 or HP3 Indion respectively). These were first suspended in water and the fine particles removed by decantation. Each cellulose was then drained thoroughly on a sintered glass filter and stored in a sealed container at 4-6°C. The dry matter of each was determined as described in Section 2.2.

2.3.1 Activation of HP Indion Cellulose

The moist HP Indion cellulose (10 g dry matter) was placed in a screw-top jar (250 mL). To this was added enough water and 40% w/v aqueous sodium hydroxide to make a thick slurry, with a final sodium hydroxide concentration of 5.6 to 6%. (The water content of the starting matrix was taken into consideration in calculating this final sodium hydroxide concentration.) This mixture was cooled to below 6°C before epichlorohydrin was added to it. (The molar ratio of epichlorohydrin to sodium hydroxide used was always 1.25:1.) Finally the jar was sealed and placed on a roller for 21 to 67 hours in a cold room at 4-6°C. The consistency of the matrix was such that good mixing was obtained, but not so thin that large amounts of epichlorohydrin were used unnecessarily.

Table 2.1A shows the actual amounts and the conditions used to activate the various HP Indion celluloses. Also included in the Table are the amines and coupling conditions used to prepare the alkyl QA cellulose ion exchanger from each activated cellulose (Section 2.3.2).

2.3.2 Coupling Activated Celluloses with Tertiary Amines.

At the end of the reaction of HP Indion with epichlorohydrin the activated cellulose was washed on a sintered glass filter

with water, 30% ethanol and 60% ethanol (approximately 500 mL of each in several lots). The gel was drained thoroughly and returned to the jar. To this was added the amine to be coupled (10 to 15 mL), dissolved in 30 to 95% ethanol (30 to 50 mL). (The number of moles of amine used was at least 3 times greater than the number of epoxides present on the activated matrix. The choice of solvent depended on the solubility of the particular amine used.) The jar was sealed and the amine was coupled for 120 hours at room temperature, or a combination of 6 hours at room temperature, 65 hours at 37°C and 1-3 hours at 70°C. The coupling at room temperature and 37°C was carried out on a roller. The coupling at 70°C was achieved for small batches by shaking the mixture in a water bath. Larger batches (greater than 10 g dry matter) were held in a water bath and mixed by a paddle stirrer.

After coupling, the ion exchanger was washed on a sintered glass filter with 60% ethanol containing 1 mol/L hydrochloric acid, 30% ethanol and finally water (approximately 500 mL of each in several lots). Acidified aqueous alcohol washes were used to remove the excess amine. To ensure that the ion exchanger was completely free of amine and that any unreacted epoxides were hydrolysed it was given further washes with 1 mol/L hydrochloric acid, water and 1 mol/L sodium hydroxide. Soaking times of up to 1 hour were used for each of the washes to ensure complete removal of the uncoupled amine. The wash with sodium hydroxide converted the ion exchanger into the hydroxide form ready for analysis by titration (Section 2.6.1) and reprocessing (Section 2.4) where necessary. Finally the ion exchanger was washed with water until the washings were neutral to pH 6-8 indicator paper and then drained thoroughly.

2.4 REPROCESSING

Some of the ion exchangers shown in Table 2.1A were activated for a second time with epichlorohydrin and further coupled with amine. The second processing step was carried out using similar techniques to those used in the first step (Section 2.3). The details for particular ion exchangers are given in Table 2.1B. For example, DMDo 3 (10 g dry matter) was reactivated in 4% sodium hydroxide (113 mL) with epichlorohydrin (11 mL). After washing with 60% aqueous ethanol this was coupled with N,N-dimethyl-N-dodecylamine (15 mL) dissolved in 95% ethanol (40 mL), to give DMDo 4.

2.5 OPTIMISATION OF THE SYNTHESIS OF DODECYL QA CELLULOSE

2.5.1 Coupling Temperature

Moist HP5 Indion (10 g dry matter) was placed into a screw-top jar (250 mL). To this was added water (50 mL) and 40% aqueous sodium hydroxide (18 mL) to give, in total, 120 mL of 6% aqueous sodium hydroxide. This mixture was cooled in ice to below 6°C before adding epichlorohydrin (18 mL) to it. The jar was sealed and rolled for 24 hours in a cold room at 4-6°C. The activated cellulose was collected on a sintered glass filter, washed with water, 30% ethanol and 60% ethanol (approximately 500 mL of each in several lots), and then drained of excess liquid.

The damp activated matrix was divided into 5 equal parts to investigate different coupling conditions. Each part (2/10 of total) was coupled with N,N-dimethyl-N-dodecylamine (DMDo-amine) (3.0 mL) dissolved in 95% ethanol (10 mL) for various times at temperatures from 20 to 70°C. After coupling, each of the dodecyl QA celluloses were collected on a sintered glass filter and washed with: 1 mol/L hydrochloric acid in 60% ethanol; 30% ethanol; water; 1 mol/L hydrochloric acid; water; 1 mol/L sodium hydroxide; and then rinsed with water

TABLE 2.1 Summary of the Alkyl QA Cellulose Ion Exchangers Prepared in Chapter 3

Properties			Starting Materials				Activation[a]			Coupling[a]		
Ion Exchanger (Code)	Substitution Level (meq/g)	Swollen Volume (mL/g)	Amine $N(CH_3)_2R$ (R)	Starting Matrix (Indion)	Cross-Linking (%)	Dry Matter (mg/g)	Vol./Conc. NaOH (mL/% aq)	Volume ECH[b] (mL)	Time (4-6°C) (h)	Volume Amine (mL)	Vol./Conc. Ethanol (mL/% aq)	Time/ Temp. (h/°C)
Table 2.1A Single Processed Ion Exchangers												
DMB 6	1.09	6.5	Butyl	HP3	25	183	85/5.6	12	21	[c]	50/30	120/RT
DMO 6	0.77	5.7	Octyl	HP3	25	183	85/5.6	12	21	13	37/75	120/RT
DMO 8	0.84	7.5	Octyl	HP5	15	163	113/6.0	17	41	14	30/60	[d]
DMO 10	0.95	11.6	Octyl	HP7[e]	10	131	150/6.0	23	42	15	50/60	[d]
DMDO 2	0.60	5.2	Dodecyl	HP3	25	183	85/5.6	12	21	15	37/75	120/RT
DMDo 3	0.71	6.5	Dodecyl	HP5	15	163	113/6.0	17	41	15	30/95	[d]
DMDo 7	0.83	8.3	Dodecyl	HP7[e]	10	131	150/6.0	23	67	15	50/95	[d]
Table 2.1B Double Processed Ion Exchangers												
DMO 9	1.27	5.2	Octyl	DMO 8	15	169	113/4.0	11	65	13	40/60	[d]
DMO 11	1.38	7.2	Octyl	DMO 10	10	119	150/4.0	15	65	10	50/60	[d]
DMDo 4	1.16	3.7	Dodecyl	DMDo 3	15	188	113/4.0	11	65	15	40/95	[d]
DMDo 8	1.18	3.7	Dodecyl	DMDo 7	10	182	150/4.0	15	64	15	50/95	[d]

[a] Amount used per 10 g (dry matter) of the starting matrix activated
[b] Epichlorohydrin
[c] N,N-dimethyl-N-butylamine hydrobromide (17 g) neutralised to pH 11 with 40% w/v aqueous sodium hydroxide
[d] Coupling was for 6 hours at room temperature, 65 hours at 37°C and 1-3 hours at 70°C
[e] Batch Number 1183

until the washings were neutral to pH 6-8 indicator paper. They were then sucked dry and samples were taken for titration analysis (Section 2.6.1).

2.5.2 Half-Neutralisation of DMDo-Amine

A batch of activated matrix was prepared from HP5 Indion (4 g dry matter) as described in Section 2.5.1. After washing with 60% ethanol it was drained and divided into 2 equal parts. To each part was added a solution made up of DMDo-amine (3.0 mL), concentrated hydrochloric acid (0.48 mL) and absolute ethanol (9.5 mL). (The acid was added to the ethanol first and the mixture cooled before adding the amine.) One part was coupled for 48 hours at room temperature, followed by 2 hours at 70°C. The other part was coupled for 3 hours at 70°C. Both parts were then collected and washed as described in Section 2.5.1.

2.5.3 Coupling Time at 70°C

A batch of activated matrix was prepared from HP5 Indion (20 g dry matter) as described in Section 2.5.1, and divided into parts. One part (1/20 of total) was coupled with trimethylamine (Section 2.5.3a). Five parts (each 2/20 of total) were coupled with half-neutralised DMDo-amine (Section 2.5.2) at 70°C for 1 to 5 hours. A further part (2/20 of total) was coupled with non-neutralised DMDo-amine (Section 2.5.1) for 2 hours at 70°C.

Each of the parts which were coupled with DMDo-amine were washed with 60% ethanol and divided in half. One half was washed as normal (Section 2.5.1). The other half was reacted further with trimethylamine (Section 2.5.3a).

2.5.3a Coupling with Trimethylamine in Ethanol

The activated matrix (prepared from approximately 1 g dry matter of HP Indion) was coupled with trimethylamine (1.25 mL

of a 33% solution in ethanol) in 95% ethanol (5 mL) by reaction for 24-48 hours at room temperature. The product was then collected on a sintered glass filter and washed as described in Section 2.5.1.

2.5.4 Coupling Time at 22°C

A batch of activated matrix was prepared from HP5 Indion (20 g dry matter) as described in Section 2.5.1, and divided into parts. One part (1/20 of total) was coupled with trimethylamine (Section 2.5.3a). Four parts (each 3/10 of total) were coupled with half-neutralised DMDo-amine (Section 2.5.2) at 22°C for 24 to 96 hours. A further part (3/20 of total) was coupled with non-neutralised DMDo-amine (Section 2.5.1) for 48 hours at 22°C.

Each sample that was reacted with DMDo-amine was washed with 60% ethanol and divided into 3 equal parts (each 1/20 of the total matrix activated). The first part was coupled for a further 4 hours at 70°C with DMDo-amine. The second part was further coupled with trimethylamine (Section 2.5.3a). The third part was washed as usual (Section 2.5.1).

2.5.5 Optimum Time for Activation

Moist HP5 Indion (3 g dry matter) was activated for 24 hours at 4-6°C as described in Section 2.5.1. Two further reactions were carried out for 48 and 72 hours. Each activated matrix was washed with water, drained and processed in the following way. One third was coupled with sulphite (Section 2.5.5a). The remaining two thirds were washed with 60% ethanol and divided in half. One half was coupled with trimethylamine (Section 2.5.3a). The other half was mixed with half-neutralised DMDo-amine (Section 2.5.2) for 14 hours at room temperature and 10 hours at 70°C, and then cooled overnight (17 hours). The resulting product was washed in the usual way (Section 2.5.1).

2.5.5a Coupling with Sulphite

The activated matrix (prepared from approximately 1 g dry matter of HP Indion) was reacted with a mixture of sodium sulphite (0.5 g) and sodium metabisulphite (0.5 g) in water (5 mL) for 24 hours at room temperature. The product was washed thoroughly with water and 1 mol/L hydrochloric acid, and then rinsed with water until the washings were neutral to pH 6-8 indicator paper, ready for titration analysis (Section 2.6.2).

2.5.6 Reinvestigation of Coupling Time at 70°C

Moist HP5 Indion (12 g dry matter) was activated with epichlorohydrin in 6% sodium hydroxide (Section 2.5.1) for 48 hours at 4-6°C. After washing with 60% ethanol the activated matrix was divided into 6 equal parts (each 2/12 of total). One part was coupled with trimethylamine (Section 2.5.3a). The remaining parts were all coupled with half-neutralised DMDo-amine (Section 2.5.2) for 2 to 20 hours at 70°C. Each of the samples coupled with DMDo-amine were then washed with 60% ethanol and divided in half. One half was further coupled with trimethylamine (Section 2.5.3a) and the other half was washed in the usual way (Section 2.5.1).

2.5.7 Large Scale Preparation of Dodecyl QA Cellulose (DMDo 72)

Moist HP5 Indion (100 g dry matter) was activated with epichlorohydrin (176 mL) in 6% aqueous sodium hydroxide (total volume, 1200 mL) for 48 hours at 4-6°C as described in Section 2.5.1. The product was washed with water and a sample (1/100 of total) was removed and coupled with sulphite (Section 2.5.5a). After washing with 60% ethanol a further sample (1/100 of total) was removed and coupled with trimethylamine (Section 2.5.3a). The rest was coupled with DMDo-amine (150 mL) half-neutralised with concentrated hydrochloric acid

(23.8 mL) in absolute ethanol (475 mL), for 12 hours at room temperature and 10 hours at 70°C. It was then cooled overnight (17 hours). The product was collected on a sintered glass filter and washed as described in Section 2.5.1.

2.5.8 Coupling Conditions for Trimethylamine

Moist HP5 Indion (10 g dry matter) was activated as described in Section 2.5.1. It was washed with water and divided into 10 equal parts. One part was coupled with sulphite (Section 2.5.5a). Three parts were each coupled with trimethylamine (1.65 mL of a 32% aqueous solution) in water (6.0 mL); three parts were each coupled with trimethylamine (3.30 mL of a 32% aqueous solution) in water (6.0 mL); and three parts were each coupled with a solution made up of trimethylamine (1.65 mL of a 32% aqueous solution), concentrated hydrochloric acid (0.33 mL) and water (6.0 mL). Coupling was for 12, 24 and 48 hours in each case. The products were washed thoroughly with water, 1 mol/L hydrochloric acid, water and 1 mol/L soidum hydroxide, and then rinsed with water until the washings were neutral to pH 6-8 indicator paper, ready for titration analysis (Section 2.6.1).

2.5.9 Conditions for Activating Dodecyl QA Cellulose

Samples (each 2 g dry matter) of moist dodecyl QA cellulose (DMDo 72, Section 2.5.7) were activated with epichlorohydrin in 4%, 6% and 8% sodium hydroxide (Section 2.3.1) for 24, 48 and 72 hours. The total volume of aqueous sodium hydroxide used was 24 mL in each case.

After activation each of the nine matrices was washed with water and divided into 2 equal parts. One part was coupled with trimethylamine (Section 2.5.9a). The second part, after washing with 60% ethanol, was coupled with half-neutralised DMDo-amine (Section 2.5.2) for 15 hours at room temperature

and 10 hours at 70°C, and then cooled overnight (17 hours). The products were then washed as usual (Section 2.5.1).

2.5.9a Coupling with Trimethylamine in Water

After washing with water, the activated matrix (prepared from approximately 1 g dry matter of HP Indion) was reacted with 9% aqueous trimethylamine (7.0 mL) for 24 hours at room temperature. The product was then washed with water, 1 mol/L hydrochloric acid, water and 1 mol/L sodium hydroxide, and then rinsed with water until the washings were neutral to pH 6-8 indicator paper, ready for titration analysis (Section 2.6.1).

2.5.10 Coupling Conditions for Reactivated DMDo 72

Moist DMDo 72 (30 g dry matter) was activated with epichlorohydrin (45 mL) in 6% sodium hydroxide (300 mL total volume) for 24 hours at 4-6°C (Section 2.5.1). After washing with water two samples were coupled with trimethylamine. The first sample (8/30 of total) was coupled with trimethylamine in water (Section 2.5.9a). The second sample (2/30 of total) was coupled with a solution containing trimethylamine (4.0 mL of a 32% aqueous solution), concentrated hydrochloric acid (0.19 mL) and water (12.0 mL). Both of these samples were coupled for 48 hours at room temperature and then washed as described in Section 2.5.9a.

After washing the remaining activated matrix with 60% ethanol two samples were removed (each 8/30 of the total matrix activated). One of these samples was coupled with half-neutralised DMDo-amine (Section 2.5.2) for 18 hours at room temperature and 10 hours at 70°C, and then cooled overnight (15 hours). The other sample was coupled with DMDo-amine in the same way, except that the volume of concentrated hydrochloric acid was increased from 19.2 to 28.8 mL. Both samples were washed with 60% ethanol, 30% ethanol and water, and then a small part of each (each corresponding to

approximately 1/30 of the total matrix activated) was coupled with trimethylamine (Section 2.5.9a). The rest of the product was washed as usual, ready for titration analysis (Section 2.6.1).

2.6 DETERMINATION OF SUBSTITUTION LEVEL AND SWOLLEN VOLUME

2.6.1 Anion Exchangers

A small sample (approximately 5 g wet weight) of the ion exchanger was washed with 1 mol/L sodium hydroxide to fully convert it to the hydroxide form, and then rinsed with water until the washings were neutral to pH 6-8 indicator paper. (In the case of cholestyramine and colestipol 2 mol/L sodium hydroxide was used.) It was then placed in a titration vessel and covered with 10 mL of water. To this was added about 0.5 g of sodium chloride to cause hydroxide ions to dissociate from the ion exchanger. The sample was titrated to pH 4.0 with 1.00 mol/L hydrochloric acid. It was then washed into a sintered glass filter of known mass. After further washing to remove all of the sodium chloride the sample was transferred into a 25 mL measuring cylinder, topped up with water and allowed to settle for at least 6 hours. The cylinder was tapped occasionally until a constant volume was recorded. The sample was then returned to the sintered glass filter, drained thoroughly and oven dried overnight at 65°C. It was dried for a further 2 hours at 100°C, allowed to cool in a desiccator and weighed. The substitution level and swollen volume were then calculated as follows:

$$\text{Substitution Level (meq/g)} = \frac{\text{Volume 1.00 mol/L hydrochloric acid (mL)}}{\text{Dry weight of ion exchanger (g)}}$$

$$\text{Swollen Volume (mL/g)} = \frac{\text{Settled volume of ion exchanger (mL)}}{\text{Dry weight of ion exchanger (g)}}$$

2.6.2 Sulphopropyl Celluloses (SP-Celluloses)

A small sample of the SP-cellulose (approximately 5 g wet weight) was washed with 1 mol/L hydrochloric acid and then rinsed with water until the washings were neutral to pH 6-8 indicator paper. The sample was placed in a titration vessel along with 10 mL of water and about 0.5 g of sodium chloride. It was titrated to pH 8.0 with 1.00 mol/L sodium hydroxide. The sample was then washed into a sintered glass filter of known mass. After further washing the substitution level and swollen volume of the ion exchanger were determined as described in Section 2.6.1. The number of epoxide groups (meq/g) converted to sulphopropyl was calculated when necessary from the expression:

$$\text{Number of epoxides (meq/g)} = \frac{X}{(1-0.104X)}$$

where X was the substitution level of the SP-cellulose (meq/g).

2.7 TITRATION CURVES

A small sample, with approximately 1 g dry matter of the ion exchanger was converted to the hydroxide form (Section 2.6.1) and then transferred to a titration vessel. Sodium chloride (10 mL, 1 mol/L) was then added to cause hydroxide ions to dissociate from the ion exchanger. The solution was acidified to less than pH 3.0 using a known volume of 1.00 mol/L hydrochloric acid. Nitrogen was bubbled through the solution for several minutes to remove carbon dioxide. An identical volume of 1.00 mol/L sodium hydroxide was added (to neutralise the 1.00 mol/L hydrochloric acid) and the mixture was maintained under a nitrogen atmosphere. The titration curve was then recorded using a Radiometer autotitration assembly. Small increments (0.01 mL) of 1.00 mol/L hydrochloric acid

were injected at time intervals great enough to allow complete equilibration after each increment, usually 3-5 minutes.

2.8 BILE ACID CAPACITY TESTS

2.8.1 Capacity for Cholate and Deoxycholate

The bile acid binding capacities of selected ion exchangers were determined according to the method of Krasopolous et al.[37]. The capacity tests were carried out in Bicarbonate Buffer System (BBS). BBS was prepared by mixing together an aqueous solution (50 mL) of sodium bicarbonate (4.2 g), and an aqueous solution (1000 mL) of sodium chloride (9.0 g). The resulting solution was then adjusted to pH 8.0 prior to use.

The ion exchanger was equilibrated to pH 8.0 by washing it with BBS on a sintered glass filter and then draining it thoroughly. Samples were then dried to determine the dry matter as described in Section 2.2. Five samples of the moist equilibrated ion exchanger with dry matter in the range 15 to 200 mg were then weighed into screw-top vials (25 mL). To each of these was added a 15 mmol/L stock solution of sodium cholate or sodium deoxycholate (20 mL), prepared by dissolving the bile salt in BBS and readjusting the pH to 8.0. The vials were sealed securely and mixed for 2 hours (Chapter 3) or 25 hours (Chapter 6) at room temperature. This procedure was always carried out in duplicate. After mixing, each sample was filtered and the residual concentration of cholate or deoxycholate in the filtrate determined spectroscopically[38] as follows:

An aliquot (20 µL) of the filtered solution was transferred to a test tube with a ground glass stopper, using a Pederson pipette. (Duplicate analyses were carried out on each of the filtered solutions.) Sulphuric acid (6.0 mL, 65% aqueous solution) was then added to the tube and the chromophore developed. For cholate samples, the UV absorption at 318 nm

was measured after 20 minutes at room temperature. For deoxycholate samples, the absorption at 384 nm was measured after heating them for 20 minutes at 60°C. Each time the bile acid capacity tests were done it was necessary to prepare a standard curve for absorption from 0 to 15 mmol/L, by making appropriate dilutions of the stock solution and analysing them as above. The standard curve was then used to determine the residual concentration of the bile salt remaining in each of the filtered solutions. The chromophores were stable for more than 6 hours at room temperature, and Beers Law was found to be obeyed by both bile salts up to concentrations of 15 mmol/L.

The capacity of the ion exchanger for binding cholate or deoxycholate was then calculated as follows:

$$\text{Capacity (mg/g)} = \frac{\text{Mass of bile acid anion bound (mg)}}{\text{Dry weight of ion exchanger (g)}}$$

$$\text{Capacity (mg/mL)} = \frac{\text{Capacity (mg/g)}}{\text{Swollen volume of ion exchanger (mL/g)}}$$

The capacity (mg/g or mg/mL) was plotted against the residual concentration of the bile salt (mmol/L) in the filtrate at the end of the test.

The binding efficiency of the ion exchanger at various residual concentrations was calculated as follows:

$$\text{Binding Efficiency (\%)} = \frac{\text{Capacity (mg/g)}}{M_r \text{ (g/mole)}} \times \frac{100}{\text{Substitution level (meq/g)}}$$

where M_r is the molar mass of the bile acid anion.

2.8.2 Capacity for Conjugated Bile Acid Anions

A stock solution (500 mL) containing a mixture of bile salts, each 2.0 mmol/L, was prepared by dissolving sodium

taurocholate (0.538 g) sodium taurodeoxycholate (0.522 g), sodium glycocholate (0.488 g) and sodium glycodeoxycholate (0.472 g) in BBS (Section 2.8.1), and readjusting the pH to 8.0. Aliquots (20 mL) of this solution were then mixed with samples (15 to 160 mg dry matter) of the ion exchanger, previously equilibrated with BBS (Section 2.8.1). After 25 hours mixing at room temperature the supernatant was clarified by filtration through a sintered glass filter and the residual concentration of each bile salt remaining in solution was measured by HPLC analysis (Section 2.8.2a).

2.8.2a HPLC Analysis of Bile Salts

Samples containing conjugated bile salts were diluted appropriately with water and filtered through a 2 µm filter disk before analysis. A reverse phase octadecyl silica column (Zorbax ODS) was used to separate the bile acids using the following buffers:

Buffer A - acetonitrile, 5% : water, 95% : orthophosphoric acid, 0.1%;

Buffer B - acetonitrile, 80% : water, 20% : orthophosphoric acid, 0.1%.

Separation required 30 minutes using a linear gradient from 30% B to 100% B, with a flow rate of 1.0 mL/minute. Detection of the bile acids was by UV absorption at 200 nm. The order of elution was: taurocholic acid, taurodeoxycholic acid, glycocholic acid and glycodeoxycholic acid. The concentrations were determined from a working curve, which was prepared by injecting standards containing 0.00 to 0.08 µmoles of each bile salt. The detector response was calibrated daily.

2.9 PREPARATION OF N,N-DIMETHYL-N-BUTYLAMINE

1-Bromobutane (50 mL, 0.47 moles) was dissolved in absolute ethanol (50 mL) and added dropwise to a stirred solution of dimethylamine (240 mL, 26% aqueous solution, 1.4 moles) dissolved in absolute ethanol (100 mL). After 2 days the hydrobromide salt of N,N-dimethyl-N-butylamine was crystallised by evaporating the solvent and excess dimethylamine under reduced pressure. The crude product was recrystallised from absolute ethanol and dried under vacuum. The yield was 70%.

CHAPTER 3. PRELIMINARY STUDIES

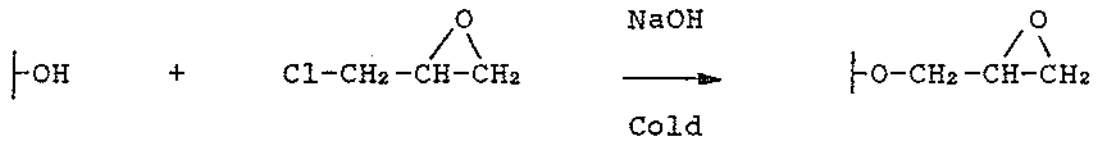
3.1 INTRODUCTION

A preliminary investigation was undertaken to determine whether or not amino cellulose ion exchangers were likely to be useful as bile acid sequestrants. Although various amino celluloses had been prepared previously, only a few of these bound bile acid anions effectively[36] and optimisation of synthetic methods was not attempted. Consequently, it was necessary to identify the ion exchanger with the greatest potential and then try to improve it by optimising the method of preparation. This chapter discusses several amino celluloses which were prepared and their ability to bind cholate anions. From this an ion exchanger worthy of further synthetic investigation was identified.

3.2 PREPARATION OF QUATERNARY AMINO CELLULOSE

The activation of hydroxypropylated regenerated cellulose (HP Indion) with epichlorohydrin in a slurry of 4-6% sodium hydroxide (Equation 3.1) has been extensively studied[35].

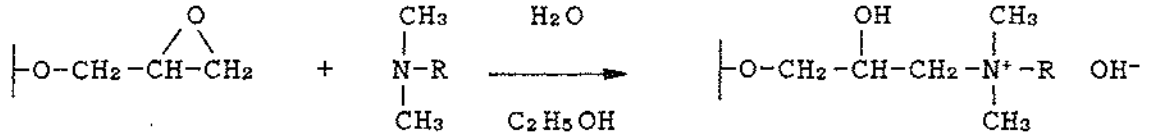
Equation 3.1 Activation of HP Indion



HP Indion Epichlorohydrin Epoxide Activated Cellulose

These epoxide activated celluloses have been used in the synthesis of a variety of quaternary amino (QA) celluloses by coupling N,N-dimethyl-N-alkylamines to the epoxide groups (Equation 3.2).

Equation 3.2 Coupling



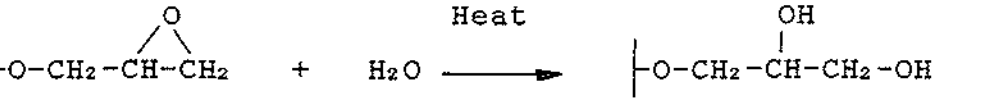
Epoxide Activated Cellulose

Quaternary Amino (QA) Cellulose

When the alkyl group R is small, for example $-CH_3$, $-CH_2CH_3$ or $-CH_2CH_2OH$, the coupling reaction occurs almost quantitatively in water after 16 hours at room temperature[39]. However it was anticipated that coupling would be more difficult for larger amines due to steric effects, and that ethanol would need to be used as a co-solvent because of the limited solubility which these compounds were found to have in water.

For these preliminary studies initial activation was carried out with 6% sodium hydroxide for 21 to 67 hours at 4-6°C to maximise the number of epoxides produced[35]. Coupling was carried out for up to 120 hours at room temperature or 37°C, followed by up to 3 hours at 70°C to make sure the reaction was complete[40]. These times were excessive and not the optimum conditions for coupling, however high temperatures were avoided early in the reaction to minimise hydrolysis of the epoxides (Equation 3.3) which is known to take place very rapidly above 60°C[39]. (A complete summary of the procedures and conditions used is given in Section 2.3 and Table 2.1A.)

Equation 3.3 Hydrolysis



Epoxide Activated Cellulose

Hydrolysis Product

Initial investigation centred on the effects of altering the size of the amine coupled, and the amount of cross-linking present in the HP Indion starting matrix. HP Indions with 10%, 15% and 25% cross-linking were used to synthesise butyl,

octyl and dodecyl QA celluloses. The physical properties of these ion exchangers are summarised in Table 3.1.

TABLE 3.1 Properties of the Alkyl QA Celluloses

Amine $N(CH_3)_2R$ (R)	Substitution Level (meq/g)			Swollen Volume (mL/g)		
	10%CL[a]	15%CL[a]	25%CL[a]	10%CL[a]	15%CL[a]	25%CL[a]
Butyl	-	-	1.09	-	-	6.5
Octyl	0.95	0.84	0.77	11.6	7.5	5.7
Dodecyl	0.83	0.71	0.60	8.3	6.5	5.3

[a] CL = Cross-Linking

The data reported in Table 3.1 show that as the length of the alkyl group was increased from butyl to dodecyl there was a corresponding decrease in the substitution level attained for the ion exchangers. This decrease occurred for all three levels of cross-linking. This was due to the lower reactivity of the larger amines and the extra weight they added to the product per milliequivalent. For example, every millequivalent of N,N-dimethyl-N-dodecylamine (M_r 214) coupled to the activated matrix produced a weight increase of 0.232 grams (Equation 3.2), compared with 0.119 grams for N,N-dimethyl-N-butylamine (M_r 101). This caused the substitution level of a butyl QA cellulose to be greater than that of a dodecyl QA cellulose when an identical number of epoxide groups were converted. The number of epoxide groups in each of the activated matrices, which were converted to QA groups, are given in Table 3.2.

TABLE 3.2 Number of Epoxide Groups Converted to QA Groups During Coupling[a]

Amine $N(CH_3)_2R$ (R)	Epoxides Converted (meq/g)		
	10%CL[b]	15%CL[b]	25%CL[b]
Butyl	-	-	1.28
Octyl	1.16	1.00	0.91
Dodecyl	1.05	0.86	0.71

[a] Epoxides converted (meq/g) = $Y/(1-XY/1000)$
where Y = the substitution level of the QA cellulose (meq/g)
X = the molar mass of $(CH_3)_2N(CH_2)_nCH_3$ plus HCl

[b] CL = Cross-Linking

The substitution levels also decreased for each of the amines as the level of cross-linking in the HP Indion was increased from 10% to 25%. Presumably this was because the matrices with less cross-linking were more open[31], allowing higher activation levels to be achieved and causing the epoxides to be more accessible to the approaching amines.

The swollen volumes of the ion exchangers (Table 3.1) displayed similar related trends. As the length of the alkyl group was increased there was a decrease in the swollen volumes of the ion exchangers. This was because the larger alkyl groups were more hydrophobic, and probably interacted with each other to exclude water and contract the cellulose matrix. The swollen volumes also decreased when a more highly cross-linked cellulose was used, because these were inherently less swollen to begin with[31].

3.3 CHOLATE CAPACITY TESTS

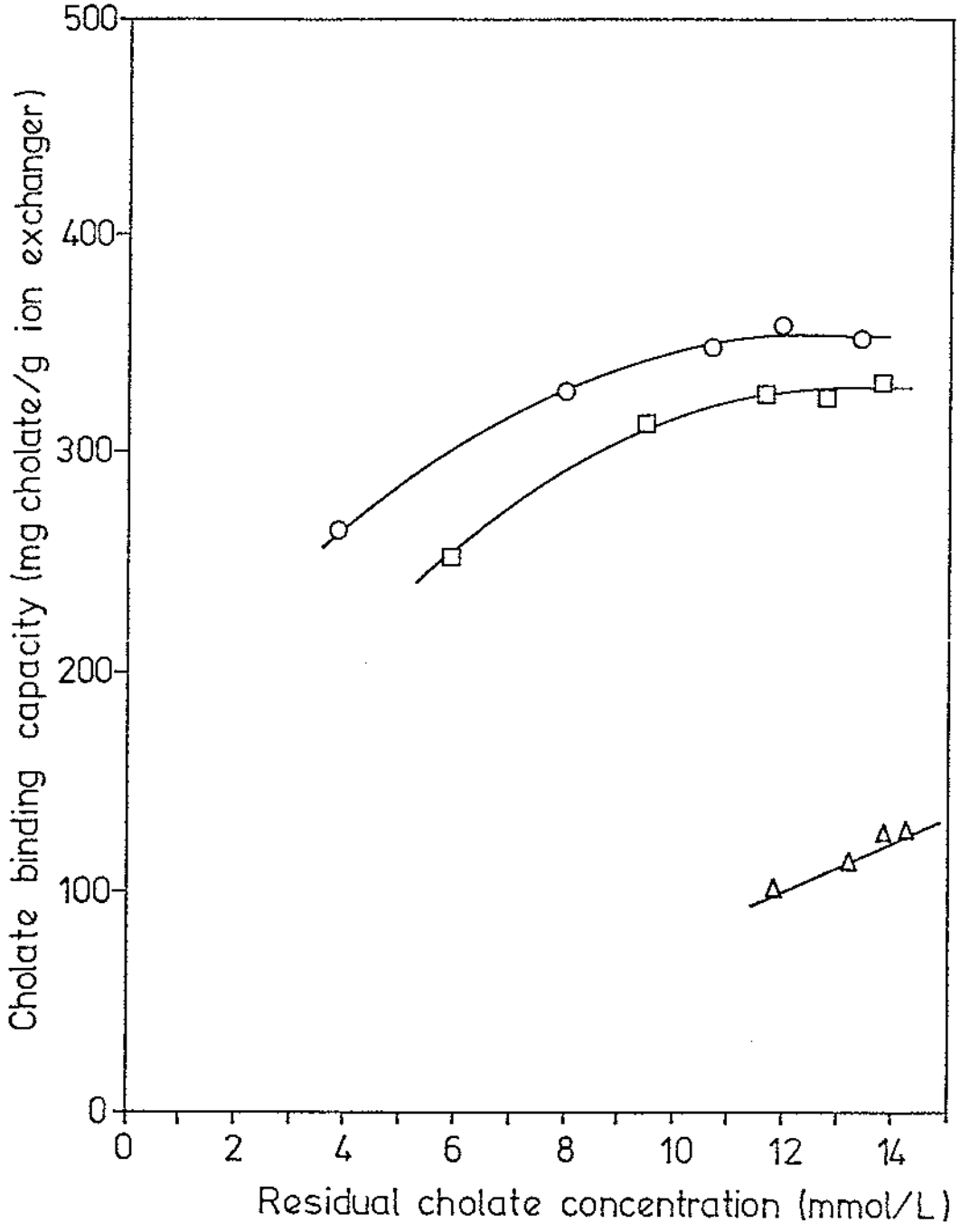
Batch capacity tests (Section 2.8.1) were carried out on some of the ion exchangers listed in Table 3.1 in order to determine which of them bound cholate anions most effectively

in vitro. Each ion exchanger was first equilibrated with Bicarbonate Buffer System (BBS), a physiological bicarbonate buffer at pH 8.0 containing chloride ions (approximately 150 mmol/L). These conditions were used to mimic those of the intestinal tract[37]. At physiological saline concentrations of 150 mmol/L there is strong competition from the chloride ions for the exchange sites on the cellulose. (The total concentration of bile anions in vivo is only 6-8 mmol/L[16].) Several different sized samples of the equilibrated ion exchanger were mixed with 15 mmol/L sodium cholate in BBS (20 mL), for 2 hours at room temperature. The capacity of the ion exchanger for cholate was calculated after measuring the residual concentration of sodium cholate at the end of the test. The results are shown in Figures 3.1 and 3.3.

3.3.1 The Effect of the Length of the Alkyl Group

A comparison of the effectiveness of the butyl, octyl and dodecyl QA celluloses (all prepared on 25% cross-linked HP Indion) for binding cholate anions is given in Figure 3.1. The capacity to bind cholate (mg) was calculated with respect to the dry weight (g) of the ion exchanger used. The figure clearly illustrates that even though the substitution levels decreased as the length of the alkyl group was increased, there was a dramatic increase in the binding capacity (mg/g) for cholate anions. At the point where there was a background concentration of 12 mmol/L sodium cholate in solution, the binding efficiencies (Section 2.8.1) for the butyl, octyl and dodecyl QA celluloses were 23%, 104% and 143% for cholate respectively. These efficiencies dropped off in favour of chloride as the residual cholate concentration in solution decreased. If every amine group on the ion exchanger bound one cholate anion the binding efficiency would be 100%, which was approximately the case for the octyl QA cellulose. This indicates that there could be some kind of favourable interaction between the cholate anions and the long hydrophobic carbon chains of the amine groups, because the

Figure 3.1 The Effect of Alkyl Chain Length on Cholate Capacity

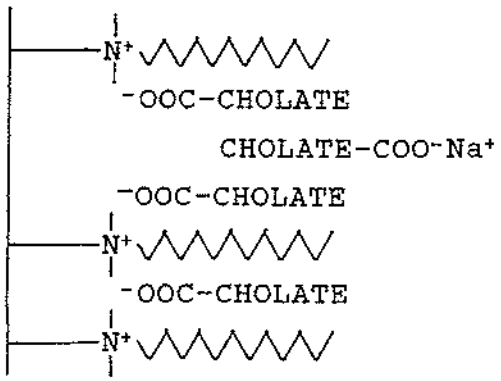


○ 25% cross-linked dodecyl QA cellulose (DMDo 2, 0.60 meq/g)
□ 25% cross-linked octyl QA cellulose (DMO 6, 0.77 meq/g)
Δ 25% cross-linked butyl QA cellulose (DMB 6, 1.09 meq/g)
Conditions: small samples of each ion exchanger were mixed with a 15 mmol/L solution of sodium cholate in BBS (20 mL) at pH 8.0 for 2 hours at room temperature

concentration of chloride (150 mmol/L) was at least ten times the concentration of cholate (15 mmol/L). If there was no preference at all for cholate anions the binding efficiency for cholate would be less than 10%.

The butyl QA cellulose bound cholate to only 23% of its available positively charged nitrogen groups, which demonstrated that it had little selectivity for cholate anions over chloride ions. For this reason further investigation of butyl QA cellulose ion exchangers for use as bile acid sequestrants was suspended. However, both the octyl and dodecyl QA cellulose derivatives showed the desired selectivity for cholate over chloride. The binding efficiency considerably greater than 100% for the dodecyl QA cellulose was surprising because it suggested that an average of 43% of the amine groups were binding more than one cholate anion. This may have been due to the formation of micelle-like structures within the cellulose matrix, with cholate anions stacking up between the dodecyl groups as indicated in Figure 3.2.

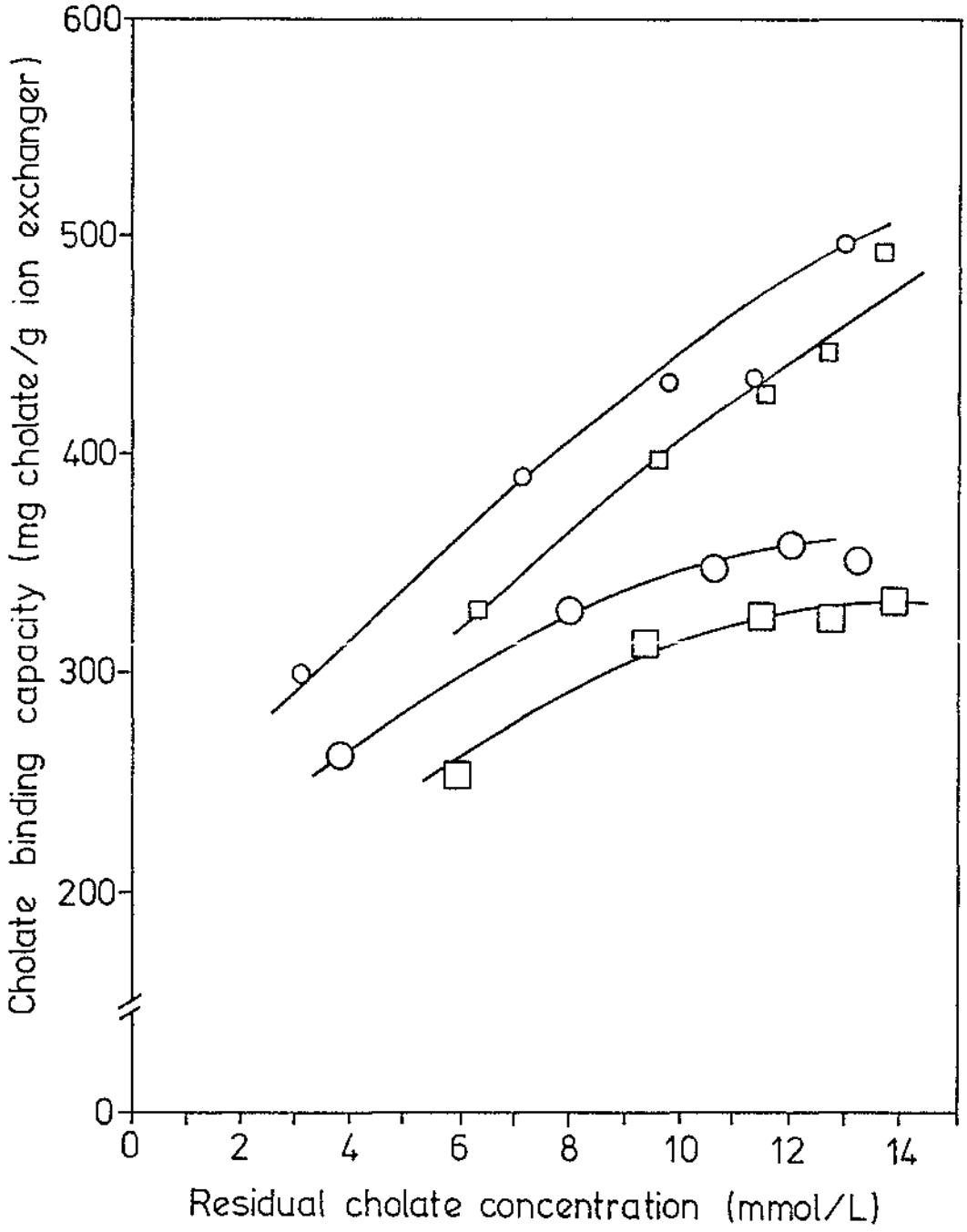
Figure 3.2 A Proposed Theory to Account for Binding Efficiencies Greater than 100%



3.3.2 The Effect of Cross-Linking

Figure 3.3 shows that when the cross-linking was reduced from 25% to 15% for the octyl and dodecyl QA celluloses there was

Figure 3.3 The Effect of Cross-Linking on Cholate Capacity



○ 15% cross-linked dodecyl QA cellulose (DMDo 3, 0.71 meq/g)
○ 25% cross-linked dodecyl QA cellulose (DMDo 2, 0.60 meq/g)
□ 15% cross-linked octyl QA cellulose (DMO 8, 0.84 meq/g)
□ 25% cross-linked octyl QA cellulose (DMO 6, 0.77 meq/g)

Conditions: as for Figure 3.1

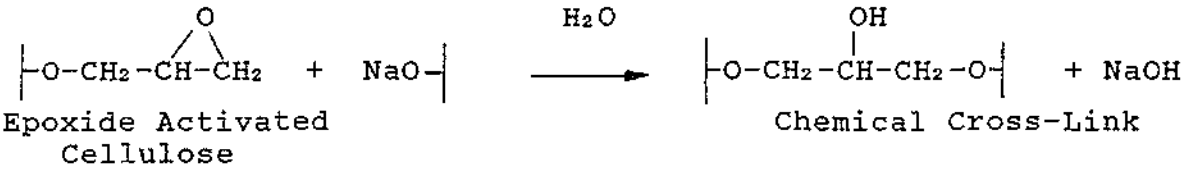
an increase in the cholate capacity for these ion exchangers, reflecting the higher substitution levels obtained for the 15% cross-linked products. The binding efficiencies for the octyl and dodecyl QA celluloses, at a residual concentration of 12 mmol/L sodium cholate, were 129% and 166% respectively for the ion exchangers prepared on the 15% matrix, compared with 104% and 143% for those prepared on the 25% matrix. This may have been because the QA celluloses prepared on the 15% matrix had higher swollen volumes, allowing more room for the formation of the micelle-like structures proposed in Figure 3.2. For both the 15% and 25% cross-linked QA celluloses the dodecyl derivatives had the highest capacities for binding cholate anions.

3.4 THE EFFECT OF REPROCESSING

The substitution levels of 0.60-0.95 meq/g for the octyl and dodecyl QA cellulose derivatives (Table 3.1) were not very satisfactory when compared with most other cellulose ion exchangers which have 1.0-1.5 meq/g[34,41,42], cholestyramine which was determined to have 3.81 meq/g and colestipol which was determined to have 7.59 meq/g (Section 4.10). For this reason the 10% and 15% cross-linked octyl and dodecyl QA celluloses were reactivated (Equation 3.1) and recoupled with the appropriate amine (Equation 3.2) to try and increase their substitution levels. Details of the procedures and conditions used are given in Section 2.4 and Table 2.1B.

All of the products in Table 3.1 were activated in a 6% sodium hydroxide slurry, however for reprocessing 4% sodium hydroxide was used to try and minimise extra cross-linking (Equation 3.4) without substantially reducing the number of active epoxides produced[18]. This was because extra cross-linking could render some of the binding sites on the cellulose matrix inaccessible to bile acid anions.

Equation 3.4 Formation of Extra Cross-Links During Activation



The physical properties of the reprocessed ion exchangers are given in Table 3.3. The values in parenthesis show the corresponding properties for the single processed ion exchangers (Table 3.1) from which each product was made.

TABLE 3.3 Properties of the Reprocessed Alkyl QA Celluloses[a]

Amine $N(CH_3)_2R$ (R)	Substitution Level (meq/g) 10%CL[b]	Substitution Level (meq/g) 15%CL[b]	Swollen Volume (mL/g) 10%CL[b]	Swollen Volume (mL/g) 15%CL[b]
Octyl	1.38	1.27	7.2	5.2
	(0.95)	(0.84)	(11.6)	(7.5)
Dodecyl	1.18	1.16	3.7	3.7
	(0.83)	(0.71)	(8.3)	(6.5)

[a] Numbers in parenthesis refer single processed ion exchangers (Table 3.1)

[b] CL = Cross-Linking

The results in Table 3.3 illustrate the effectiveness of reprocessing. The substitution levels for the four ion exchangers increased by more than 40% in each case, and as a consequence of this the swollen volumes decreased by up to 55%. Both of these factors were important since the main problems associated with bile acid sequestrants stem from the large doses required to significantly reduce the concentration of bile acids in the intestinal lumen[17]. This can lead to poor patient compliance because of their unpleasantness and gastrointestinal discomfort related to the bulk of the ion exchangers ingested[24].

The physical properties of the reprocessed ion exchangers exhibited similar trends to those observed for the parent ion exchangers. The reprocessed octyl QA celluloses prepared on the 10% cross-linked matrix gave higher substitution levels and swollen volumes than those prepared on the 15% cross-linked matrix. But in the case of the dodecyl QA cellulose derivatives the cross-linking made very little difference. Also the substitution levels of the octyl QA celluloses were significantly higher than the dodecyl QA celluloses. Although it might appear that a limit had been reached for the dodecyl QA celluloses at around 1.2 meq/g, later work (Chapter 4) showed that significantly higher substitution levels could in fact be reached with this amine.

3.5 CHOLATE CAPACITY TESTS

Cholate capacity tests (Section 2.8.1) were carried out on some of the ion exchangers reported in Table 3.3. The results are shown in Figures 3.4, 3.5 and 3.6.

3.5.1 The Effect of Reprocessing on the Cholate Capacity

The effect of reprocessing was best demonstrated by the cholate capacity curves (mg/g) for the 15% cross-linked octyl and dodecyl QA celluloses shown in Figure 3.4. These capacity curves show that reprocessing was more effective at increasing the cholate capacity of the octyl QA cellulose than its dodecyl QA cellulose counterpart. This was because there was a much larger decrease in the binding efficiency of the double processed dodecyl QA cellulose than the double processed octyl QA cellulose. For example, at a residual concentration of 12 mmol/L sodium cholate the binding efficiency of the octyl QA cellulose ion exchanger dropped from 129% to 99% after reprocessing, compared with a drop from 166% to 104% for the dodecyl QA cellulose exchanger. The drop in efficiency for the reprocessed ion exchangers may have been associated with the large decrease in their swollen volumes which occurred

after reprocessing (Table 3.3), because this could eliminate the formation of micelle-like structures within the cellulose matrix (Figure 3.2).

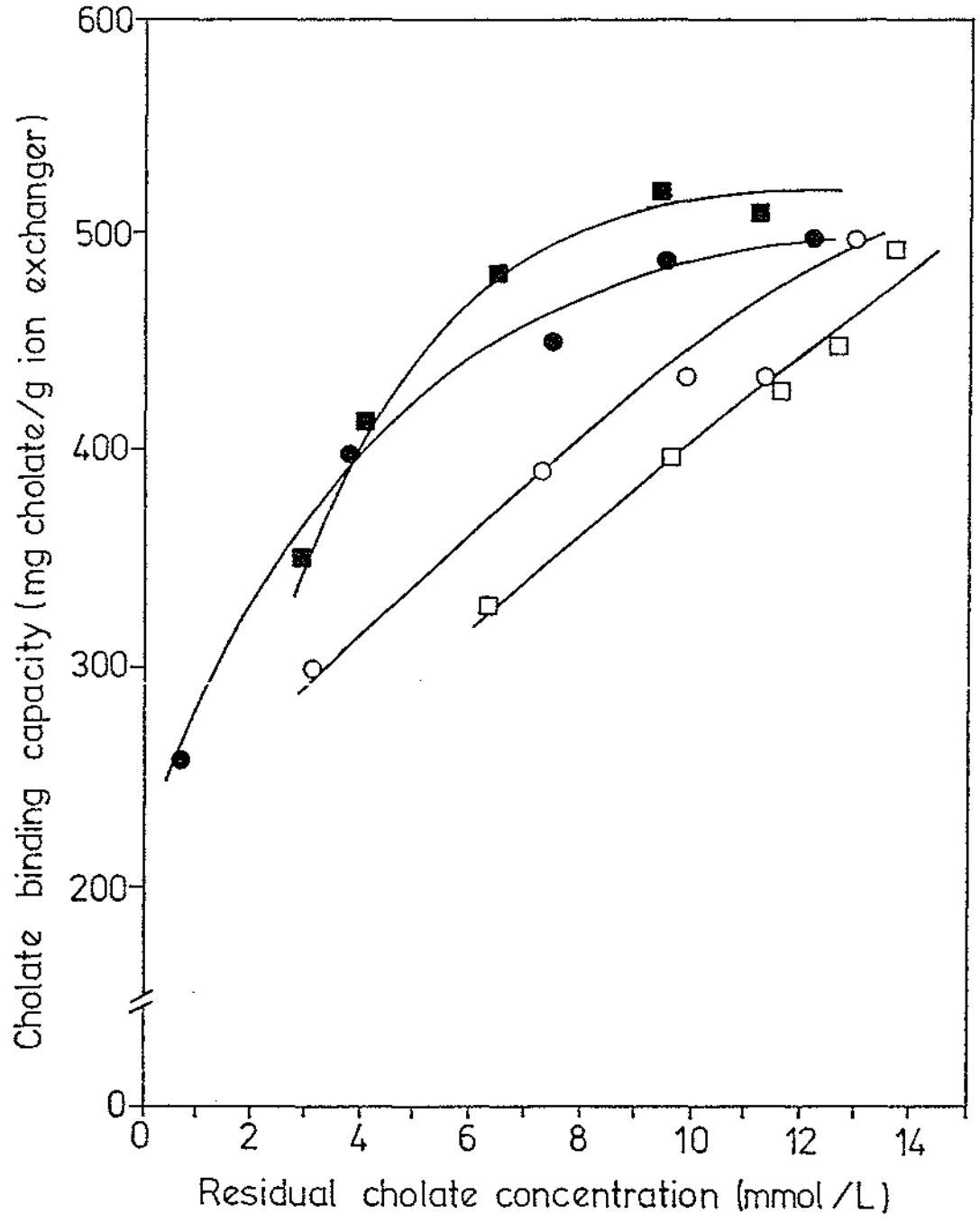
In spite of the decrease in efficiency, the decrease in the swollen volumes of the double processed ion exchangers had a favourable effect on the bulk required to bind cholate, compared with the single processed ion exchangers. This can be seen from the cholate capacity curves in Figure 3.5 where the capacity to bind cholate (mg) has been calculated with respect to the volume (mL) of ion exchanger used, instead of the dry weight as in Figure 3.4. On the basis of volume the double processed ion exchangers had much higher cholate capacities (mg/mL) than the single processed ion exchangers, because their swollen volumes were much lower. Also the double processed dodecyl QA cellulose was much more effective than the double processed octyl QA cellulose. This could be very important from a clinical point of view because any discomfort experienced by a patient taking this kind of medication would be exacerbated if the volume of ion exchanger they were required to take was increased.

(Animal testing later showed that reprocessing dodecyl QA cellulose increased the performance of the ion exchanger even on a weight basis[18]. The very high efficiency of 166% found in the laboratory test for the single processed product may not be attained in the animal model if some of this binding is reversible in the gut of the animal.)

3.5.2 The Effect of Cross-Linking on Cholate Capacity

The difference between the cholate capacity curves for the 10% and 15% cross-linked dodecyl QA celluloses shown in Figure 3.6 were negligible, which reflects the fact that their substitution levels were similar. The small improvement in substitution level achieved by using 10% cross-linked HP Indion, instead of 15%, to prepare octyl QA cellulose had

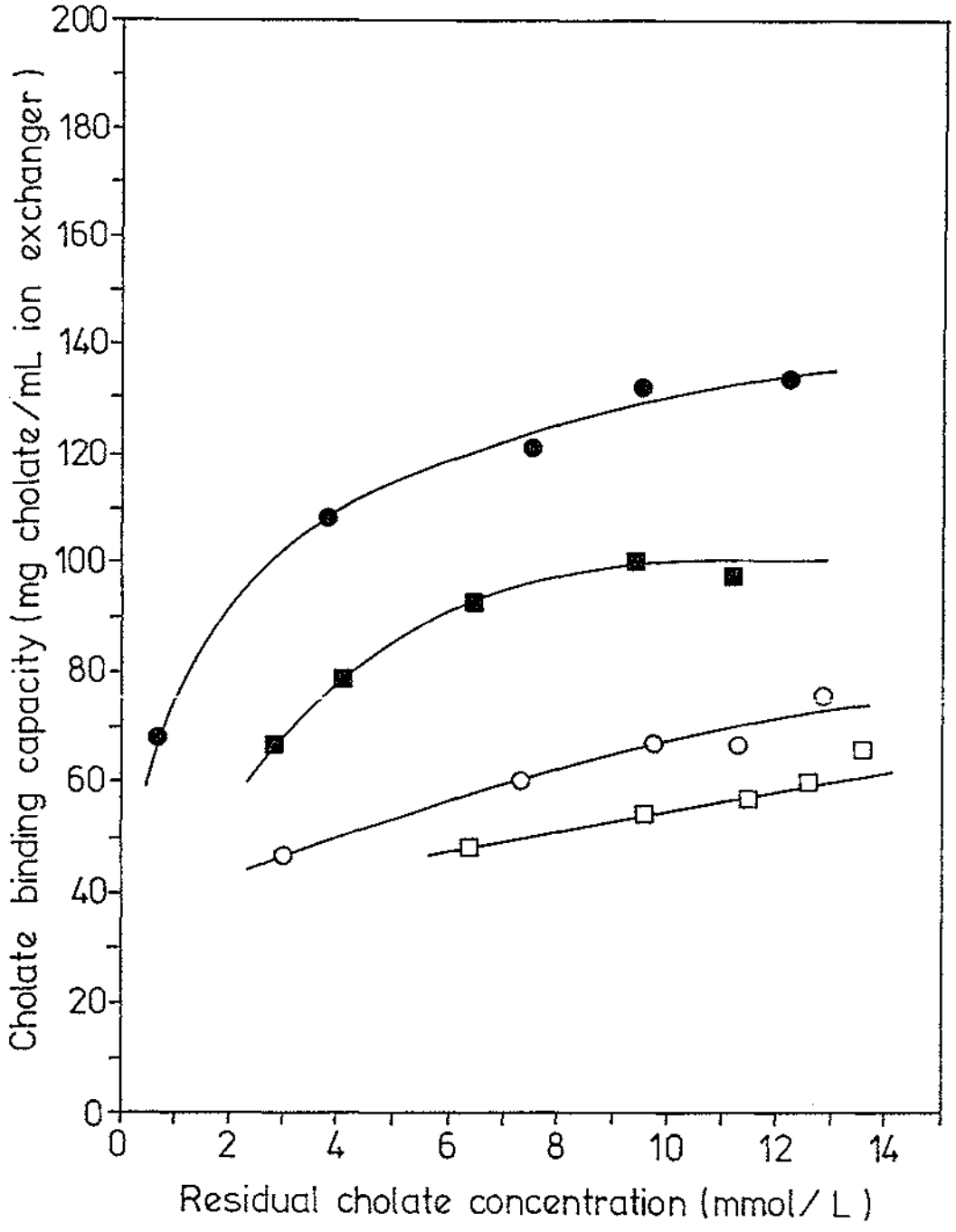
Figure 3.4 The Effect of Reprocessing on Cholate Capacity (mg/g)



● double processed, 15% cross-linked, dodecyl QA cellulose (DMDo 4, 1.16 meq/g)
○ single processed, 15% cross-linked, dodecyl QA cellulose (DMDo 3, 0.71 meq/g)
■ double processed, 15% cross-linked, octyl QA cellulose (DMO 9, 1.27 meq/g)
□ single processed, 15% cross-linked, octyl QA cellulose (DMO 8, 0.84 meq/g)

Conditions: as for Figure 3.1

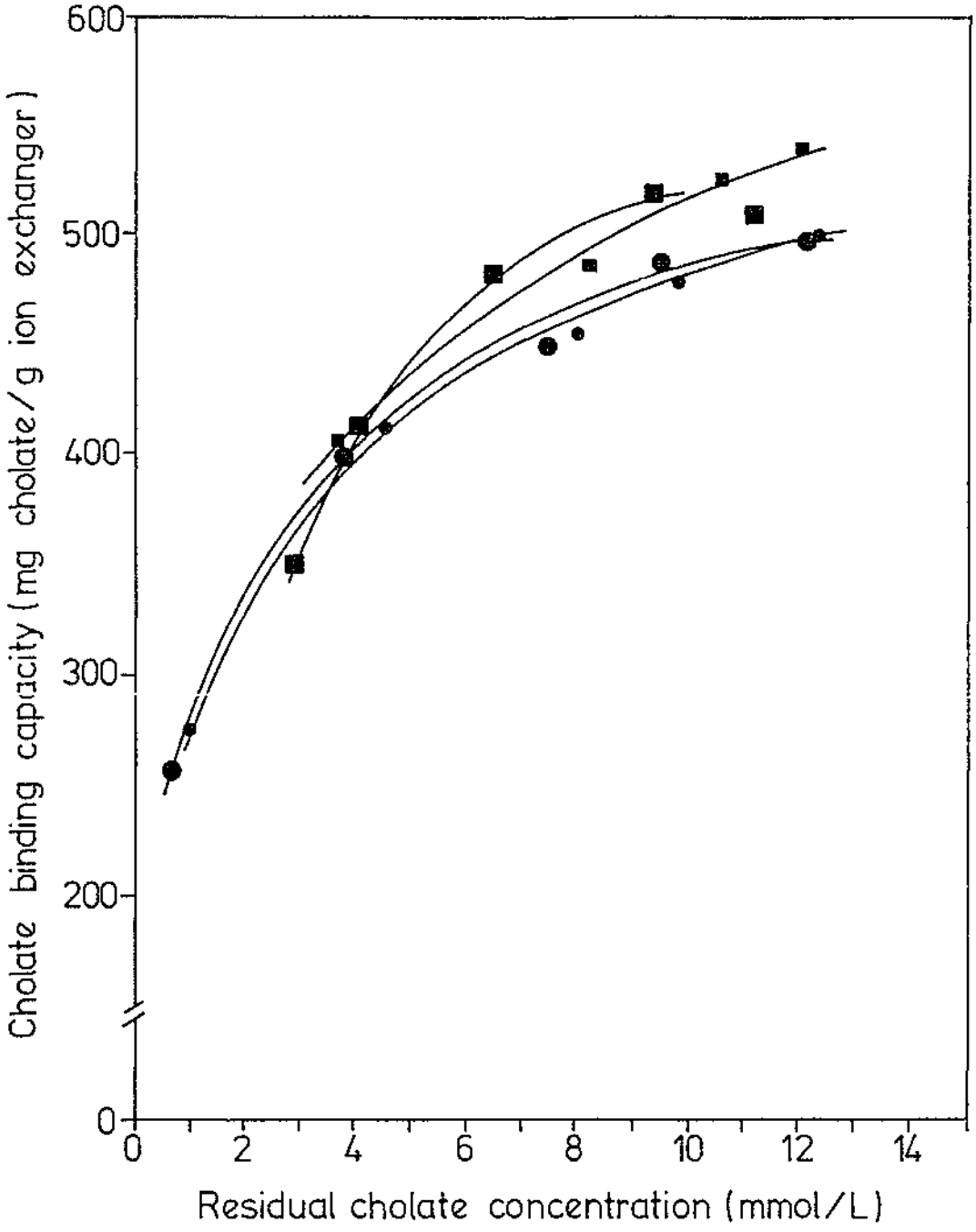
Figure 3.5 The Effect of Reprocessing on Cholate Capacity (mg/mL)



● double processed, 15% cross-linked, dodecyl QA cellulose (DMDo 4, 3.7 mL/g)
○ single processed, 15% cross-linked, dodecyl QA cellulose (DMDo 3, 6.5 mL/g)
■ double processed, 15% cross-linked, octyl QA cellulose (DMO 9, 5.2 mL/g)
□ single processed, 15% cross-linked, octyl QA cellulose (DMO 8, 7.5 mL/g)

Conditions: as for Figure 3.1

Figure 3.6 The Effect of Cross-Linking on Cholate capacity



- ● double processed, 15% cross-linked, dodecyl QA cellulose (DMDo 4, 1.16 meq/g)
- • double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 8, 1.18 meq/g)
- ■ double processed, 15% cross-linked, octyl QA cellulose (DMO 9, 1.27 meq/g)
- ▪ double processed, 10% cross-linked, octyl QA cellulose (DMO 11, 1.38 meq/g)

Conditions: as for Figure 3.1

little effect on their ability to bind cholate. The dodecyl derivatives appeared to work better at very low cholate concentrations, the octyl at high concentrations.

The 15% cross-linked matrix was selected to investigate the preparation of these alkyl QA cellulose derivatives. It was thought that the extra cross-linking present could help the product to be more resistant against degradation in the gut. The swollen volumes were also lower. However, after optimising the process, distinct advantages appeared for the 10% cross-linked matrix as discussed in Chapters 4 and 5.

3.6 CONCLUSION

From these preliminary studies various cellulose based ion exchangers appeared to have potential for use as bile acid sequestrants. The most promising ion exchanger was a reprocessed N,N-dimethyl-N-dodecylamino cellulose prepared on 15% cross-linked HP Indion cellulose matrix. Subsequent investigation, into the optimisation of the synthetic methods and in vitro testing, initially centred on this ion exchanger.

CHAPTER 4. PREPARATION OF N,N-DIMETHYL-N-DODECYLAMINO CELLULOSE

4.1 INTRODUCTION

In Chapter 3 N,N-dimethyl-N-dodecylamino cellulose (dodecyl QA cellulose) prepared from 15% cross-linked HP Indion was shown to be the most effective ion exchanger for binding cholate anions in the presence of a 10-1000 fold excess of chloride ions. This was also the most difficult of the QA cellulose ion exchangers to produce with a high substitution level and stood to benefit the most from any improvements made to the synthetic scheme. Therefore it was decided to concentrate on optimising the synthesis of this ion exchanger to achieve the maximum possible substitution level. It was hoped that by increasing the substitution level a parallel increase in bile acid anion binding would also be achieved.

4.2 THE EFFECT OF TEMPERATURE ON COUPLING

All of the dodecyl QA celluloses reported in Chapter 3 were prepared by coupling N,N-dimethyl-N-dodecylamine (DMDo-amine) with epoxide activated HP Indion (Equation 3.2). Coupling was for up to 120 hours at room temperature or 37°C, followed by up to 3 hours at 70°C. This was based on previous experience of coupling the epoxide activated cellulose with other nucleophiles[40]. To find the best temperature and reaction time for coupling a single batch of epoxide activated cellulose was prepared, washed with water and 60% ethanol, and then split into five equal portions, which were coupled with DMDo-amine under various conditions (Section 2.5.1). These conditions, and the properties of the products, are shown in Table 4.1. All reactions were given 2 hours at 70°C at the finish to make sure they were complete.

TABLE 4.1 The Effect of Temperature on Coupling

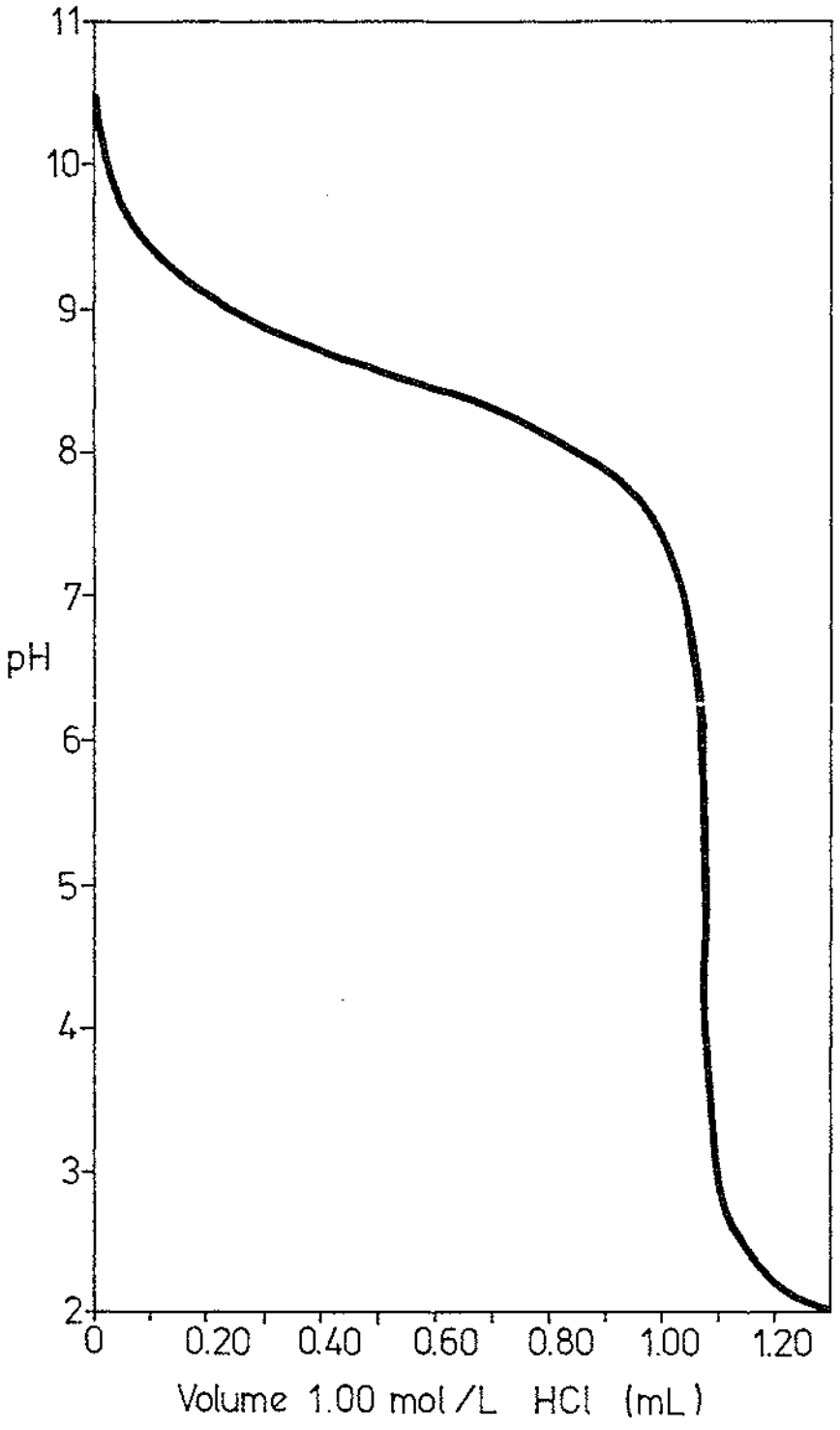
Ion Exchanger DMDo	Coupling Conditions Temp (°C)	Time (h)	Temp (°C)	Time (h)	Substitution Level (meq/g)	Swollen Volume (mL/g)
11	RT	48	70	2	0.74	6.3
12	34	24	70	2	0.71	6.2
13	50	24	70	2	0.68	6.3
14	60	6	70	2	0.66	6.1
15	70	3	-	-	0.64	5.9

It can be seen that as the temperature was increased the substitution levels decreased. The most significant evidence of this comes from a comparison of DMDo 12 and 13, because these ion exchangers were coupled for exactly the same length of time but at different temperatures. DMDo 12 had a higher substitution level than DMDo 13 which indicates that the rate of hydrolysis of the epoxides (Equation 3.3) was increased relative to the rate of substitution (Equation 3.2) at the higher temperature.

4.3 THE EFFECT OF HALF-NEUTRALISATION OF DMDo-AMINE ON COUPLING

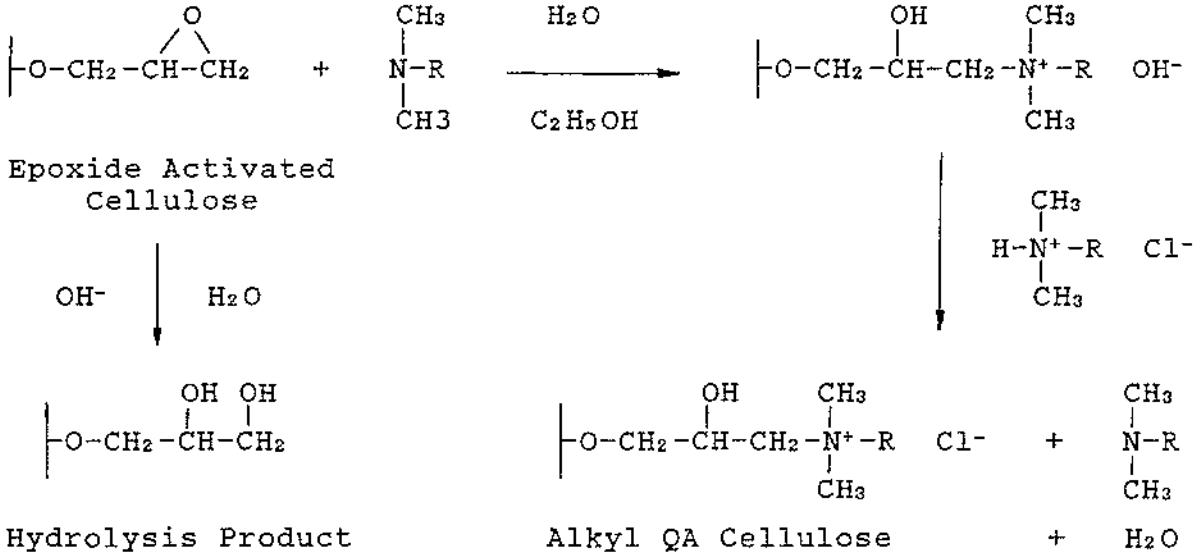
The stability of the epoxide activated cellulose in water has been shown to be independent of pH between pH 4 to 10. However above pH 10 it is hydrolysed increasingly rapidly at room temperature[39]. The titration curve of DMDo-amine in approximately 66% aqueous ethanol is given in Figure 4.1. This shows that half-neutralisation of DMDo-amine reduced the pH of the solution by about 2 pH units from pH 10.5 to 8.5. For this reason it was decided to investigate the effect of half-neutralising DMDo-amine during the coupling step. This reduced the concentration of free amine by approximately half, but it also reduced the concentration of hydroxide by about 100 fold. By doing this it was hoped to significantly reduce hydrolysis of the epoxides on the activated cellulose without impeding the coupling reaction (Equation 4.1).

Figure 4.1 Titration Curve for DMDo-Amine in 66% Aqueous Ethanol



Conditions: increments of hydrochloric acid (0.01 mL) were added at regular time intervals to DMDo-amine (0.3 mL) in 66% aqueous ethanol (60 mL)

Equation 4.1 Competition Between Hydrolysis and Coupling



The activation and coupling conditions used were exactly the same as those used in Section 4.2 except that sufficient concentrated (11.6 mol/L) hydrochloric acid was added to the coupling reagents to half-neutralise the DMDo-amine (Section 2.5.2). The coupling conditions and properties of the products are given in Table 4.2, along with the corresponding products from Table 4.1 where hydrochloric acid was not present during coupling.

TABLE 4.2 The Effect of Half-Neutralisation on Coupling

Ion Exchanger DMDo	½N HCl	Coupling Conditions Temp (°C)	Time (h)	Temp (°C)	Time (h)	Substitution Level (meq/g)	Swollen Volume (mL/g)
11	No	RT	48	70	2	0.74	6.3
21	Yes	RT	48	70	2	0.91	5.1
15	No	70	3	-	-	0.64	5.9
22	Yes	70	3	-	-	0.86	5.6

The substitution levels achieved for DMDo 21 and 22 were 24% and 34% greater than those for DMDo 11 and 15 respectively, which was a significant improvement. It is interesting to

note that the improvement was greatest for the ion exchanger coupled at 70°C, so much so that there appeared to be very little advantage in coupling for long periods of time at room temperature. On the strength of these results all further investigations with DMDo-amine were carried out with half-neutralisation of the free amine during coupling.

4.4 INVESTIGATION OF THE COUPLING TIME REQUIRED FOR HALF-NEUTRALISED DMDo-AMINE

4.4.1 Introduction

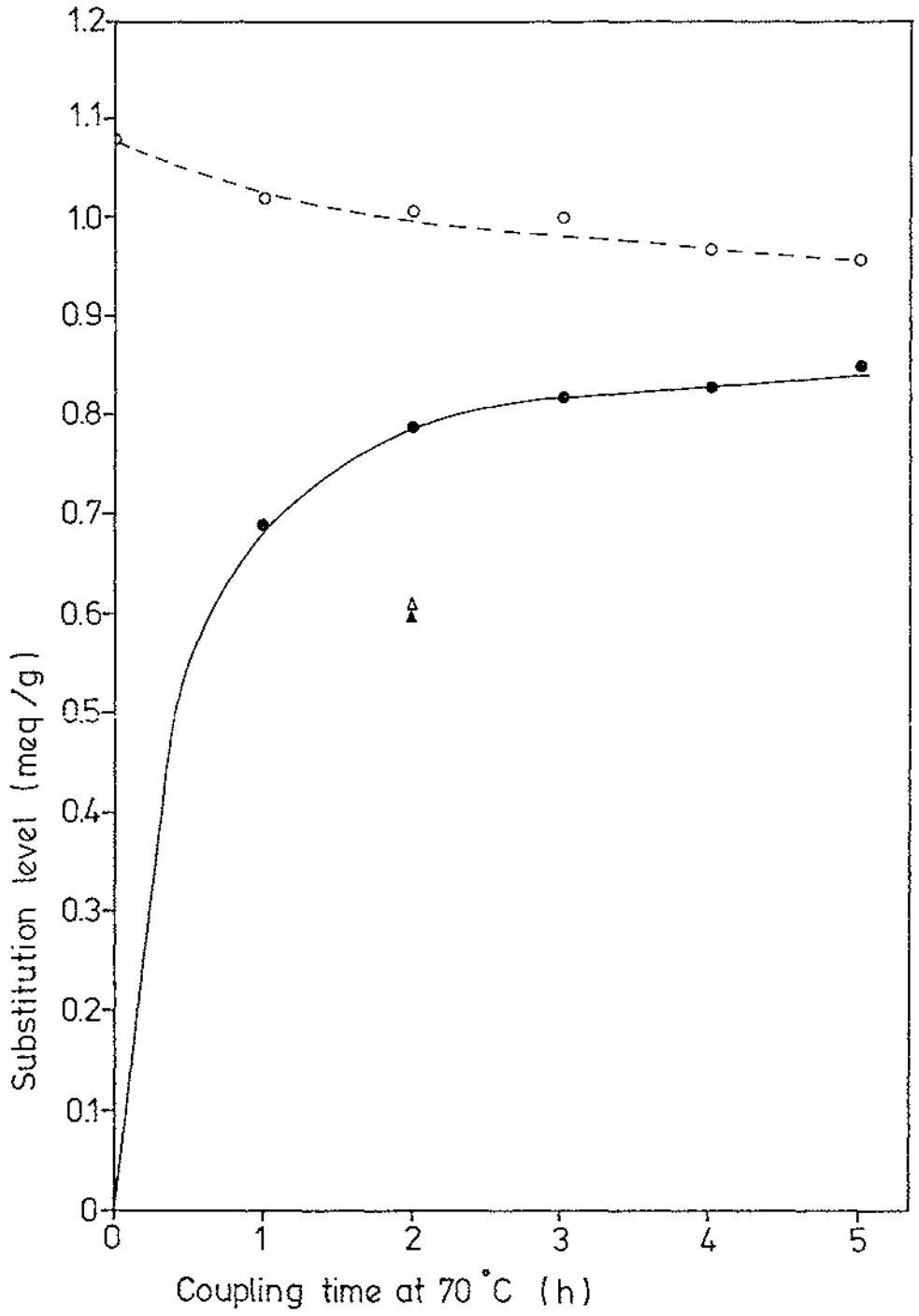
The object of these experiments was to ascertain the length of time required for coupling half-neutralised DMDo-amine at 70°C and room temperature, and to find out whether or not the temperature of coupling affected the final substitution levels obtained.

4.4.2 Coupling Time at 70°C

The solid line in Figure 4.2 shows the substitution levels obtained at 70°C for coupling times ranging from 1 to 5 hours on 15% cross-linked HP Indion (Section 2.5.3). The bulk of the reaction was over in 2-3 hours, but the substitution level steadily increased up to 5 hours. It may have continued to increase even after 5 hours because there were still a number of unreacted epoxide groups on the cellulose as indicated by the upper dashed line in Figure 4.2.

The residual epoxide groups were detected in each dodecyl QA cellulose prepared by taking half of the product, washing it, and then reacting it further with trimethylamine for 24 hours at room temperature (Section 2.5.3a). This introduced additional quaternary amino groups onto the cellulose which were then determined, along with the quaternary dodecylamino groups, by titration analysis. Trimethylamine was able to

Figure 4.2 Coupling Time for Half-Neutralised DMDo-Amine at 70°C

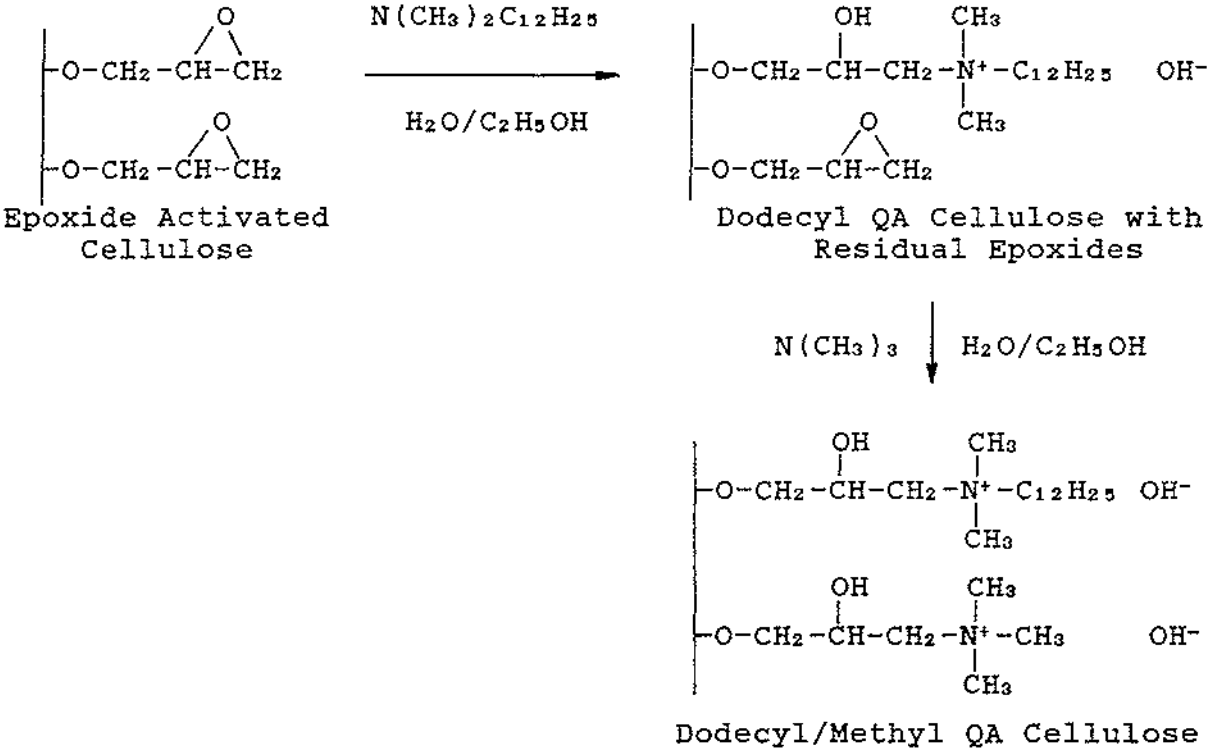


● dodecyl QA cellulose
○ dodecyl/methyl QA cellulose
▲ dodecyl QA cellulose without addition of hydrochloric acid
△ dodecyl/methyl QA cellulose without addition of hydrochloric acid

Activation: 15% cross-linked HP Indion in 6% sodium hydroxide for 24 hours at 4-6°C

gain access more easily than DMDo-amine to the residual epoxide groups (Equation 4.2). The gap between the two lines indicates that the coupling reaction for DMDo-amine takes longer than 5 hours at 70°C.

Equation 4.2 Determination of Residual Epoxides with Trimethylamine



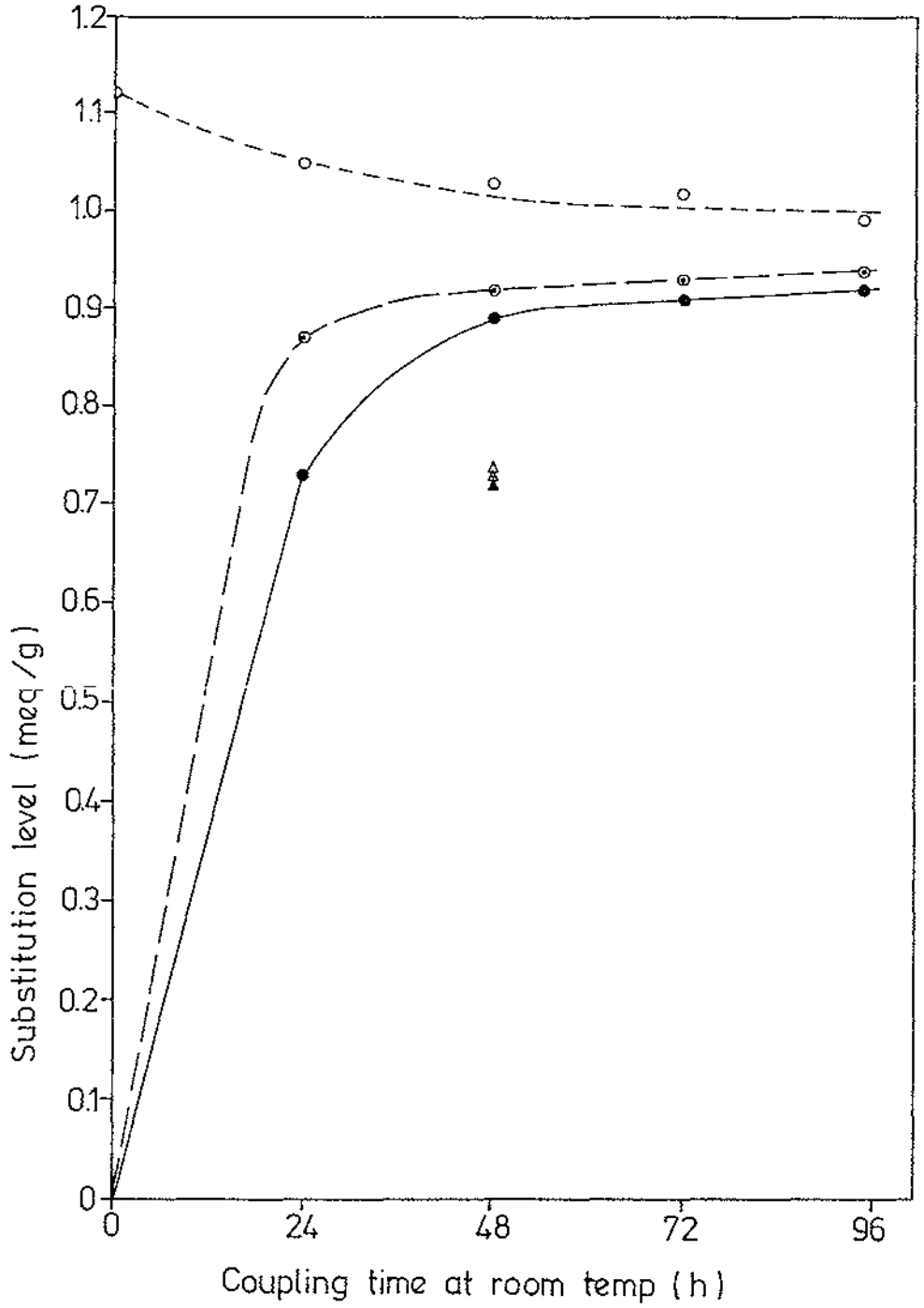
To verify the importance of buffering the reaction at the pKa of DMDo-amine by half-neutralisation the 2 hour reaction was also carried out without addition of the half mole ratio of hydrochloric acid. The two isolated points on Figure 4.2 show that the results achieved were significantly lower than the corresponding 2 hour buffered reaction. Furthermore there were no epoxide groups left after 2 hours at 70°C indicating that many of them must have been hydrolysed.

4.4.3 Coupling Time at Room Temperature

The solid line in Figure 4.3 shows the substitution levels achieved by coupling DMDo-amine to epoxide activated HP Indion (15% cross-linked) at 22°C for 24 to 96 hours (Section 2.5.4). This shows that the substitution level increased rapidly up to 48 hours and then levelled off. The upper dashed line corresponds to dodecyl QA celluloses which were washed and further coupled with trimethylamine. The gap between these two lines indicates that there were still a number of active epoxides left even after coupling DMDo-amine for 96 hours at 22°C. However the substitution level of the dodecyl QA celluloses only increased very slowly after 48 hours. The middle broadly dashed line corresponds to dodecyl QA celluloses which were heated for a further 4 hours at 70°C after coupling at 22°C. Heating did raise the substitution level further, but after 48 hours at 22°C the increase was small. It would seem that some of the epoxides present in the activated matrix are very resistant to coupling with DMDo-amine. Fortunately though, under the buffered conditions being used, they are also fairly stable to hydrolysis at both 22 and 70°C.

The ion exchangers coupled at 22°C were made on a different activated matrix than those coupled at 70°C (Section 4.4.2), but the conditions used for activation were identical. There is a slight advantage in coupling at 22°C, because after 96 hours a dodecyl QA cellulose was obtained with a substitution level of 0.94 meq/g, whereas only 0.85 meq/g was achieved after heating for 5 hours at 70°C. However, a long reaction time was required which may be unacceptable for industrial use. It would commit expensive equipment for extended periods and achieve only a 10% increase in substitution over that obtained at 70°C. Furthermore the reaction at 70°C was obviously not complete after 5 hours and might have been further improved upon.

Figure 4.3 Coupling Time for Half-Neutralised DMDo-Amine at 22°C



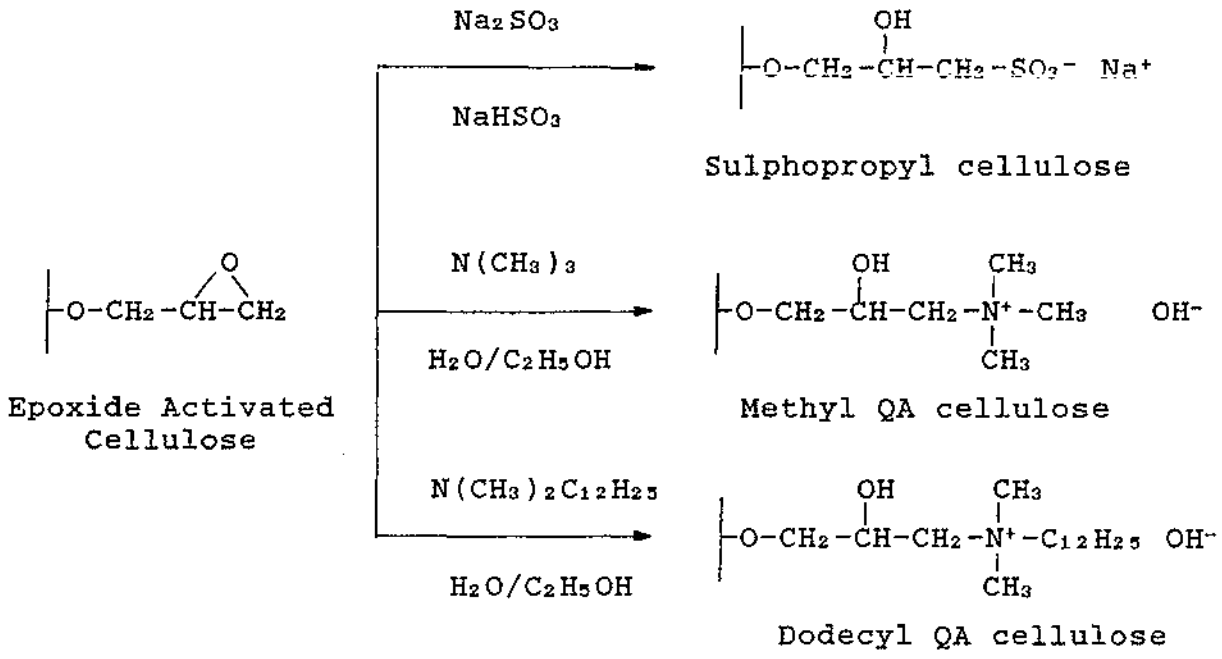
● dodecyl QA cellulose at 22°C
⊙ dodecyl QA cellulose at 22°C, followed by 4 hours at 70°C
○ dodecyl/methyl QA cellulose
▲△△ the corresponding derivatives prepared without addition of hydrochloric acid

Activation: as for Figure 4.2

4.5 OPTIMUM TIME FOR ACTIVATION

All of the products reported in Figures 4.2 and 4.3 were prepared by activating 15% cross-linked HP Indion with epichlorohydrin in a slurry of 6% sodium hydroxide for 24 hours at 4-6°C. This activation was repeated along with similar activations carried out for 48 and 72 hours to determine the length of time required to substitute the maximum number of active epoxides onto the cellulose matrix. The activated matrices were then compared by dividing each one into three fractions (Section 2.5.5) and converting them through to sulphopropyl cellulose (SP-cellulose), methyl QA cellulose (TMA-cellulose) and dodecyl QA cellulose (DMDo-cellulose) derivatives (Equation 4.3). The properties of these products are shown in Table 4.3.

Equation 4.3 Cellulose Derivatives



Sulphite couples quantitatively to the epoxide groups on the activated matrix[39]. Hence SP-celluloses can be used to determine the epoxide content of the cellulose (Section 2.6.2). The SP-celluloses in Table 4.3 indicate that maximum activation was achieved after 48 hours. This was also

TABLE 4.3 Optimum Time for Activation

Activation Time (h)	Ion Exchanger	Swollen Volume (mL/g)	Substitution Level (meq/g)	Epoxides Converted[a] (meq/g)	Epoxides Converted[b] (%)
24	SP	9.3	1.22	1.40	100
24	TMA 17	7.6	1.08	1.20	86
24	DMDo 69	5.0	0.93	1.21	86
48	SP	8.6	1.35	1.57	100
48	TMA 18	7.7	1.16	1.30	83
48	DMDo 70	4.9	1.02	1.37	87
72	SP	8.5	1.35	1.57	100
72	TMA 19	7.7	1.18	1.33	85
72	DMDo 71	4.9	1.04	1.41	90

[a] Epoxides Converted (meq/g): = x/(1-0.104x) for SP;
= y/(1-0.095y) for TMA;
= z/(1-0.250z) for DMDo;
where x, y and z are the substitution levels for the SP, TMA and DMDo derivatives respectively

[b] It is assumed that sulphite reacts quantitatively with the epoxide groups[39]

reflected in the substitution levels for the methyl and dodecyl QA celluloses. Surprisingly DMDo-amine coupled to approximately 90% of the available epoxides, matching that of trimethylamine which is a much smaller molecule (similar in size to the sulphite nucleophile). However the trimethylamine was not half-neutralised and this may have resulted in more rapid hydrolysis of the epoxides compared to the DMDo-amine (Section 4.3). Quantitative coupling conditions for trimethylamine are discussed in Section 4.7.

4.6 REINVESTIGATION OF COUPLING TIME AT 70°C

The substitution levels of the ion exchangers reported in Figure 4.2 which were coupled at 70°C indicated that the reaction required longer than 5 hours to reach completion. Therefore the coupling time was reinvestigated for up to 10

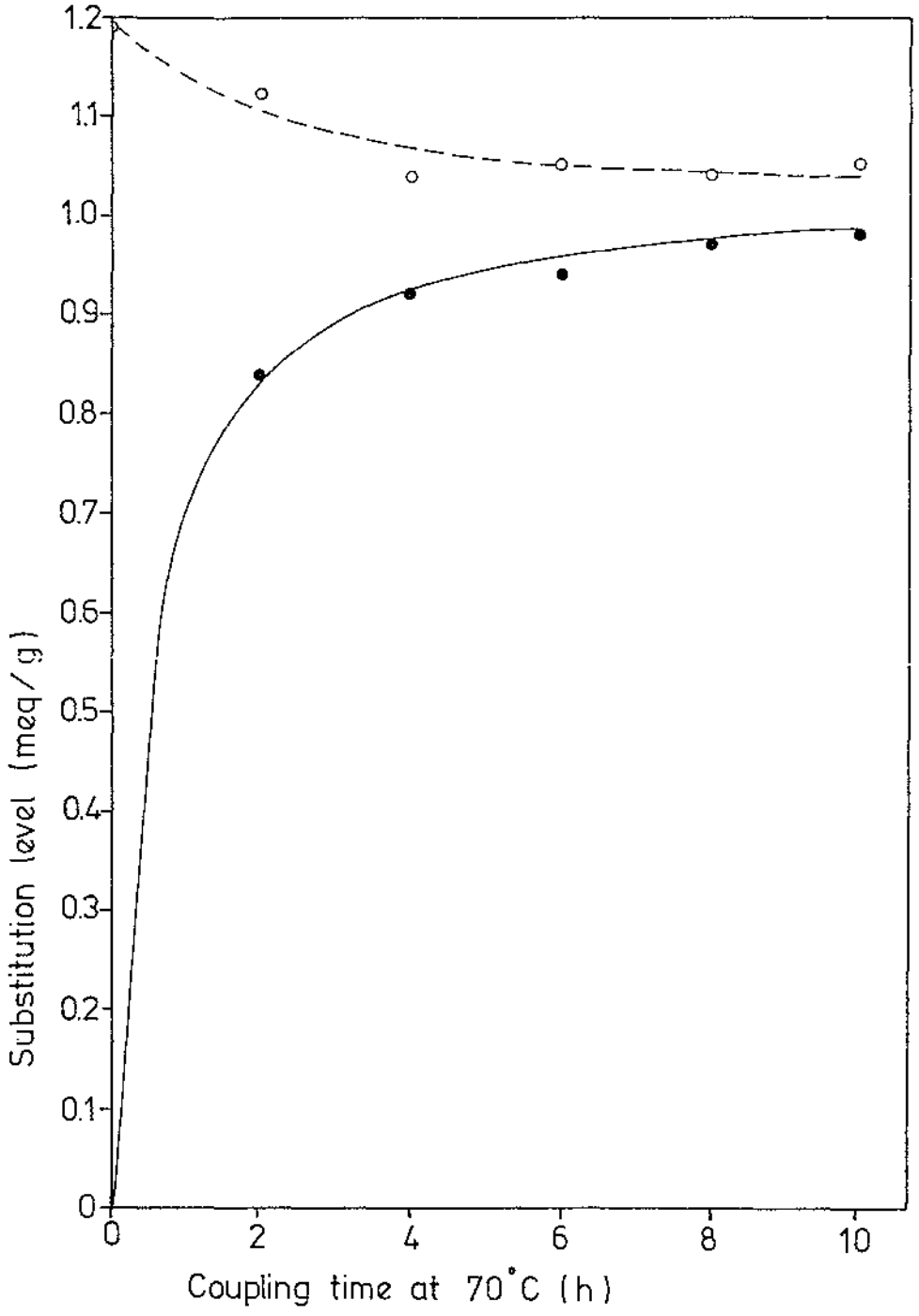
hours at 70°C (Section 2.5.6) and the results are shown in Figure 4.4. A change was also made in the preparation of the epoxide activated cellulose. To achieve the maximum number of active epoxides the reaction time for activation of the 15% cross-linked HP Indion was increased to 48 hours at 4-6°C, instead of 24 hours which was used to prepare the ion exchangers in Figures 4.2 and 4.3. This gave a slightly higher epoxide content for the activated matrix in agreement with the results reported in Section 4.5. (See the substitution levels for the methyl QA celluloses at zero time in Figures 4.2, 4.3 and 4.4).

The solid line in Figure 4.4 shows that further increases in the substitution levels for the dodecyl QA celluloses were achieved by increasing the coupling time at 70°C beyond 5 hours. The substitution level was still increasing slowly at up to 10 hours at 70°C. The broken line, representing dodecyl/methyl QA celluloses (Equation 4.2), indicated that there were still residual epoxides remaining. Hence even 10 hours at 70°C may not be long enough for maximum coupling to take place.

4.6.1 Preparation of a Large Batch of DMDo-Cellulose (DMDo 72)

The product obtained with the 48 hour activation at 4-6°C, followed by coupling for 10 hours at 70°C (0.98 meq/g, Figure 4.4), was an improvement on the 24 hour activation and 96 hour coupling at room temperature (0.94 meq/g, Figure 4.3). Consequently the standard procedure used for further work was a 2 day activation at 4-6°C, with the bulk of the coupling carried out at 70°C. For the production of large laboratory batches it was more convenient to couple them overnight at room temperature before heating them at 70°C for 10 hours on the following day, and then cool them overnight before washing up the product. This gave a total coupling time of 40 hours, of which at least 10 hours were at 70°C. This was the

Figure 4.4 Coupling Time for Half-Neutralised DMDo-Amine at 70°C



● dodecyl QA cellulose at 70°C
○ dodecyl/methyl QA cellulose
Activation: 15% cross-linked HP Indion in 6% sodium hydroxide for 48 hours at 4-6°C

procedure used to prepare the first large batch of dodecyl QA cellulose (DMDo 72) from 100g of 15% cross-linked HP Indion (Section 2.5.7). The results of this preparation are summarised in Table 4.4. (Small samples of the activated matrix were converted to sulphopropyl and methyl QA cellulose derivatives for comparison.) Of particular interest is the fact that with DMDo 72, 97% coupling efficiency of the epoxides was achieved, producing a product with 1.08 meq/g of quaternary nitrogen groups. This was nearly up to the substitution level of 1.16 meq/g obtained in the preliminary work by double processing (Table 3.3), and reflects the significant improvements made by optimising the various steps in the synthetic process. The yield for this reaction was 167 grams which indicates a weight increase to the cellulose of 67%.

TABLE 4.4 Preparation of DMDo 72 from 100 g of HP Indion

Ion Exchanger	Swollen Volume (mL/g)	Substitution Level (meq/g)	Epoxides Converted[a] (meq/g)	Epoxides Converted[b] (%)	Yield (g)
SP	8.6	1.31	1.52	100	-
TMA 20	10.6	1.18	1.33	88	-
DMDo 72	8.2	1.08	1.48	97	167

[a] and [b] See Table 4.3 Footnotes

DMDo 72 was used to optimise the conditions needed for reprocessing to achieve even higher substitution levels (Section 4.8 and 4.9). However before this was done the failure of trimethylamine to react quantitatively with the epoxide groups (Table 4.3 and 4.4) was investigated.

4.7 QUANTITATIVE COUPLING CONDITIONS FOR TRIMETHYLAMINE

A batch of HP5 Indion was activated as for TMA 17 in Table 4.3. Fractions of it were then converted to methyl QA

celluloses with trimethylamine under various conditions (Section 2.5.8), as indicated in Table 4.5. In addition a sulphopropyl cellulose derivative was prepared to determine the epoxide content.

TABLE 4.5 Quantitative Conversion of Epoxides with Trimethylamine

Ion Exchanger TMA	Trimethylamine Coupling Conditions Excess	½N HCl	Time (h)	Substitution Level (meq/g)	Epoxides Converted[a] (meq/g)	Epoxides Converted[b] (%)
(SP)	-	-	-	1.24	1.42	100
21	5	No	12	1.21	1.37	97
22	5	No	24	1.21	1.37	97
24	5	Yes	12	1.24	1.41	99
25	5	Yes	24	1.23	1.39	98
27	10	No	12	1.22	1.38	97
28	10	No	24	1.22	1.38	97

[a] and [b] See Table 4.3 Footnotes

It can be seen from Table 4.5 that it did not matter whether there was a 5 or 10 fold excess of trimethylamine over the epoxide groups, or whether or not the amine was half-neutralised, because the conversion of epoxides was always at least 97% efficient. Also the reaction was complete in less than 12 hours at room temperature.

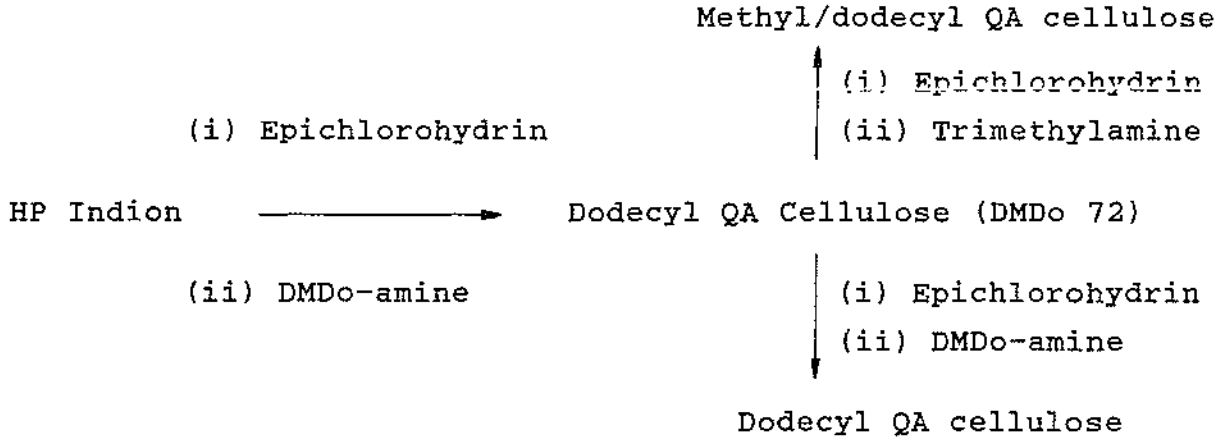
These results were in contrast to those obtained in Tables 4.3 and 4.4 where the conversion of epoxides was only 86 to 90% efficient. The only way this can be explained is by the fact that the reactions here were carried out in aqueous solution, whereas those reported earlier were in 60-95% ethanol. Half-neutralisation of trimethylamine may well have made a difference in aqueous ethanol as it did for DMDo-amine, however it was not necessary to investigate this because methyl QA celluloses were more conveniently prepared in water. Hence water was used for all further reactions with trimethyl-

amine. (Unfortunately DMDo-amine could not be coupled in water because it required at least 65% ethanol to remain soluble.)

4.8 OPTIMUM ACTIVATION CONDITIONS FOR REPROCESSING

DMDo 72 was reactivated with epichlorohydrin in 4%, 6% and 8% aqueous sodium hydroxide slurries for 24, 48 and 72 hours at 4-6°C (Section 2.5.9). Fractions of the nine activated products were then coupled with trimethylamine or DMDo-amine (Equation 4.4), and analysed to determine the optimum activation conditions for double processing. The conditions used for activation and coupling, and the properties of the products, are summarised in Table 4.6.

Equation 4.4



The substitution levels obtianed for the dodecyl and methyl/dodecyl QA celluloses were all independent of the activation time in the range 24 to 72 hours. Therefore a 1 day activation was adequate for reprocessing. The substitution levels were also almost independent of the strength (%) of the sodium hydroxide used, but when the results for the three activation times were averaged it could be seen that there was a small increase in the substitution level when this was increased from 4% to 8%. However this increase was only 5% for the dodecyl QA celluloses and 10% for the methyl/-dodecyl QA celluloses which was hardly significant in view of

TABLE 4.6 Optimum Activation Conditions for Reprocessing

Ion Exchanger DMDo	Activation NaOH(aq)/Time (%aq/h)	Amine Coupled	Substitution Level (meq/g)	Average Substitution (meq/g)	Swollen Volume (mL/g)
75	4/24	DMDo-amine	1.34		3.7
77	4/48	"	1.32	1.32	3.5
79	4/72	"	1.30		3.3
76	4/24	Trimethyl-	1.61		4.2
78	4/48	amine	1.63	1.62	3.9
80	4/72	"	1.62		3.7
81	6/24	DMDo-amine	1.35		3.3
83	6/48	"	1.38	1.35	3.2
85	6/72	"	1.33		3.2
82	6/24	Trimethyl-	1.69		3.9
84	6/48	amine	1.76	1.72	3.8
86	6/72	"	1.71		3.7
87	8/24	DMDo-amine	1.39		3.3
89	8/48	"	1.41	1.38	3.2
91	8/72	"	1.35		3.2
88	8/24	Trimethyl-	1.75		3.9
90	8/48	amine	1.81	1.78	3.6
92	8/72	"	1.78		3.5

TABLE 4.7 Preparation and Properties of Alkyl QA Celluloses Prepared from DMDo 72

	Coupling Conditions					
	DMDo-amine		Trimethylamine			
Ion Exchanger DMDo	Time/Temp (h/°C)	Neut.[a] (%)	Time/Temp (h/°C)	Neut.[a] (%)	Substitution Level (meq/g)	Swollen Volume (mL/g)
101	-	-	24/RT	0	1.70	3.8
102	-	-	24/RT	50	1.72	3.7
95	18/RT,10/70, 17/RT	50	-	-	1.37	3.3
96	As 95	50	24/RT	0	1.57	3.5
99	As 95	75	-	-	1.31	3.3
100	As 95	75	24/RT	0	1.59	3.2

[a] Neutralisation (%) of the amine during coupling

the fact that twice as much sodium hydroxide and epichlorohydrin were used.

For the 24 hour products there was a contraction in the swollen volumes from 3.7 mL/g to 3.3 mL/g when the sodium hydroxide strength was increased from 4% to 6%. For this reason 6% sodium hydroxide slurries were used to reprocess all subsequent ion exchangers, making it the same as the initial activation.

Probably the most significant differences in Table 4.6 occurred between the substitution levels obtained for the dodecyl QA celluloses and methyl/dodecyl QA celluloses. The latter had much higher substitution levels which reflected the difficulty which DMDo-amine had in gaining access to the active epoxide groups. This made it necessary to investigate the coupling conditions for DMDo-amine in the reprocessing stage, and also to consider using the ion exchanger coupled with DMDo-amine after the first activation and trimethylamine after the second activation for in vitro testing as a bile acid sequestrant.

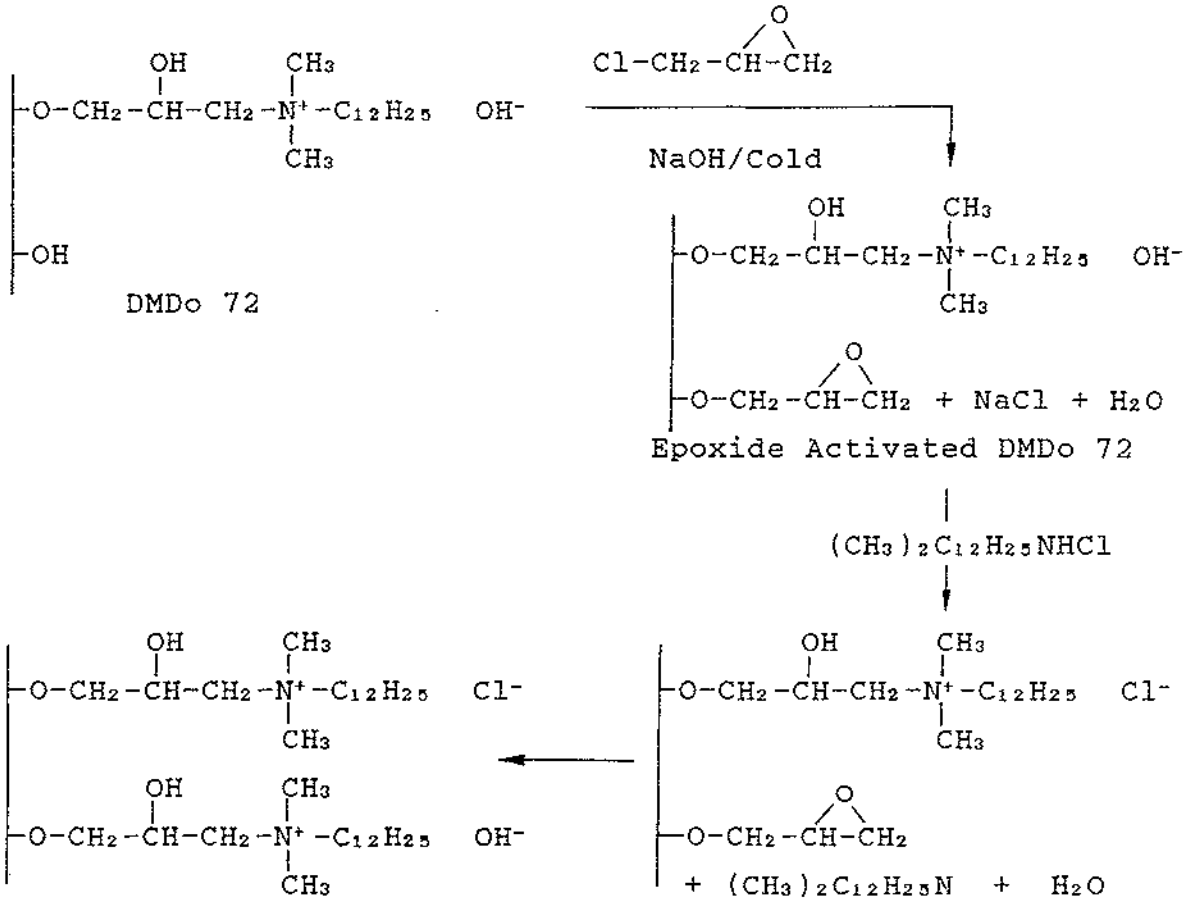
4.9 COUPLING CONDITIONS FOR REACTIVATED DMDo 72

DMDo 72 was activated in 6% sodium hydroxide for 24 hours at 4-6°C. Fractions of this were then coupled with trimethylamine, DMDo-amine, or DMDo-amine followed by trimethylamine. In some cases the amine was 50% or 75% neutralised with hydrochloric acid to buffer the coupling reaction (Section 2.5.10). The conditions and results are given in Table 4.7 (page 61).

The results shown in Table 4.7 are consistent with others obtained earlier in this Chapter. DMDo-amine coupled very slowly, because even after 10 hours at 70°C there were still some epoxide groups which could be coupled with trimethylamine (see substitution levels for DMDo 95 and 96). This indicates

that these epoxides may have been difficult to reach, which is probably due to the fact that the swollen volume of the products became extremely small. (Higher temperatures than 70°C may be necessary in order to finish coupling DMDo-amine or destroy the remaining epoxide groups.) Neutralising 75% of the DMDo-amine instead of 50% was tried because the starting matrix (DMDo 72) was in the hydroxide form after epoxide activation, and the hydroxide ions would have neutralised some of the amine hydrochloride (Equation 4.5). However 75% neutralisation did not increase the substitution level obtained over that for the 50% neutralised product (see substitution levels for DMDo 95 and 99).

Equation 4.5 Reprocessing DMDo 72



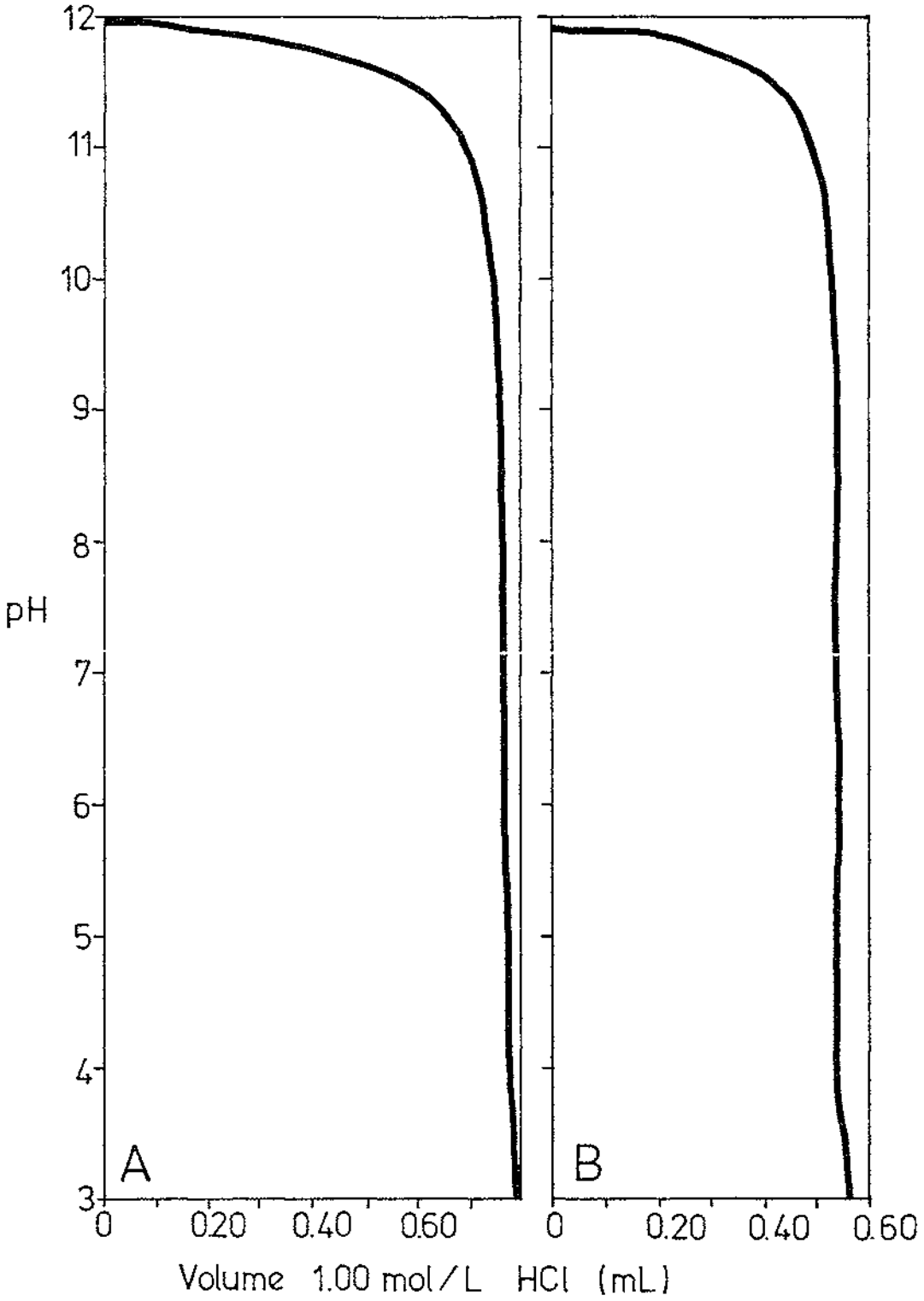
The highest substitution levels, 1.70 meq/g, were obtained when trimethylamine was coupled in water at room temperature,

and it made no difference whether it was buffered (DMDo 102) or unbuffered (DMDo 101). When coupling was carried out with just DMDo-amine the highest substitution level achieved was 1.37 meq/g (DMDo 95), which only corresponded to about 45% of the epoxides coupled with just trimethylamine.
(Epoxides coupled:
for DMDo 95 = (b-a)/(1-0.25(b-a)) = 0.313 meq/g, and
for DMDo 101 = (c-a)/(1-0.095(c-a)) = 0.659 meq/g,
where a, b and c are the substitution levels for DMDo 72, 95 and 101 respectively.) This indicated that many of the epoxides were hydrolysed during coupling. However, reprocessing was still worthwhile because the substitution level of 1.37 meq/g was 27% higher than that of the starting matrix (DMDo 72) which had only 1.08 meq/g. The decrease in the swollen volume from 6.5 mL/g (DMDo-72) to 3.3 mL/g (DMDo 95) was also very significant. Cholate binding capacity curves for DMDo 72, 95 and 101 are compared in Chapter 6.

4.10 TITRATION CURVES

The substitution levels of the various alkyl QA celluloses reported in this Chapter were all determined by converting a sample of the ion exchanger to the hydroxide form and then titrating it to a fixed end point of pH 4.0 with 1.00 mol/L hydrochloric acid (Section 2.6.1). In selected cases full titration curves were produced by adding increments of acid (0.01 mL) to the mixture and allowing it to equilibrate after each addition (Section 2.7). The result for a dodecyl QA cellulose (DMDo 95, Section 4.9) and a methyl QA cellulose (TMA 33, Appendix I) are shown in Figure 4.5. Both of these titration curves had a single buffering region followed by a very sharp end point, which indicated that only quaternary amine groups were present. The buffering region was between pH 11 to 12 for both of the titration curves, because in each case the species being titrated was the hydroxide counterion

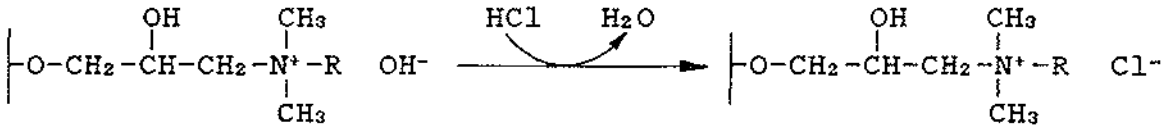
Figure 4.5 Titration Curves for Alkyl QA Cellulose Ion Exchangers



A: methyl QA cellulose (TMA 33, 1.89 meq/g)
B: dodecyl QA cellulose (DMDo 95, 1.37 meq/g)
Both ion exchangers were double processed on 15% cross-linked HP Indion

of the quaternary amine (Equation 4.6). This type of titration curve was obtained for all of the ion exchangers produced by coupling tertiary amines to cellulose, regardless of the length of the alkyl chain, the cross-linking of the starting matrix, whether the ion exchanger was single or double processed, or the temperature of coupling. This indicated that the alkyl QA celluloses were being produced without undergoing degradation under the coupling conditions used.

Equation 4.6 Titration of Alkyl QA Cellulose



Hydroxide Form of QA Cellulose

Chloride Form of QA Cellulose

The corresponding curves for cholestyramine and colestipol are shown in Figures 4.6 and 4.7. Cholestyramine is a copolymer of styrene and 2% divinylbenzene to which are attached quaternary amino functional groups (Section 1.5). For this reason the titration curve for cholestyramine is very similar to those of the QA cellulose ion exchangers shown in Figure 4.5. The substitution level determined for cholestyramine was 3.81 meq/g, and its swollen volume was 5.2 mL/g.

Colestipol is a copolymer of diethylenetriamine and epichlorohydrin, therefore it is rich with secondary and tertiary amine groups in a wide range of different environments (Section 1.5). Because of this the titration curve is almost continuous from relatively strong bases at pH 10 right through to very weak bases below pH 3. The small buffer region above pH 11 corresponds to titration of the hydroxide counterions of the very few quaternary amine groups present.

Figure 4.6 Titration Curve for Cholestryamine

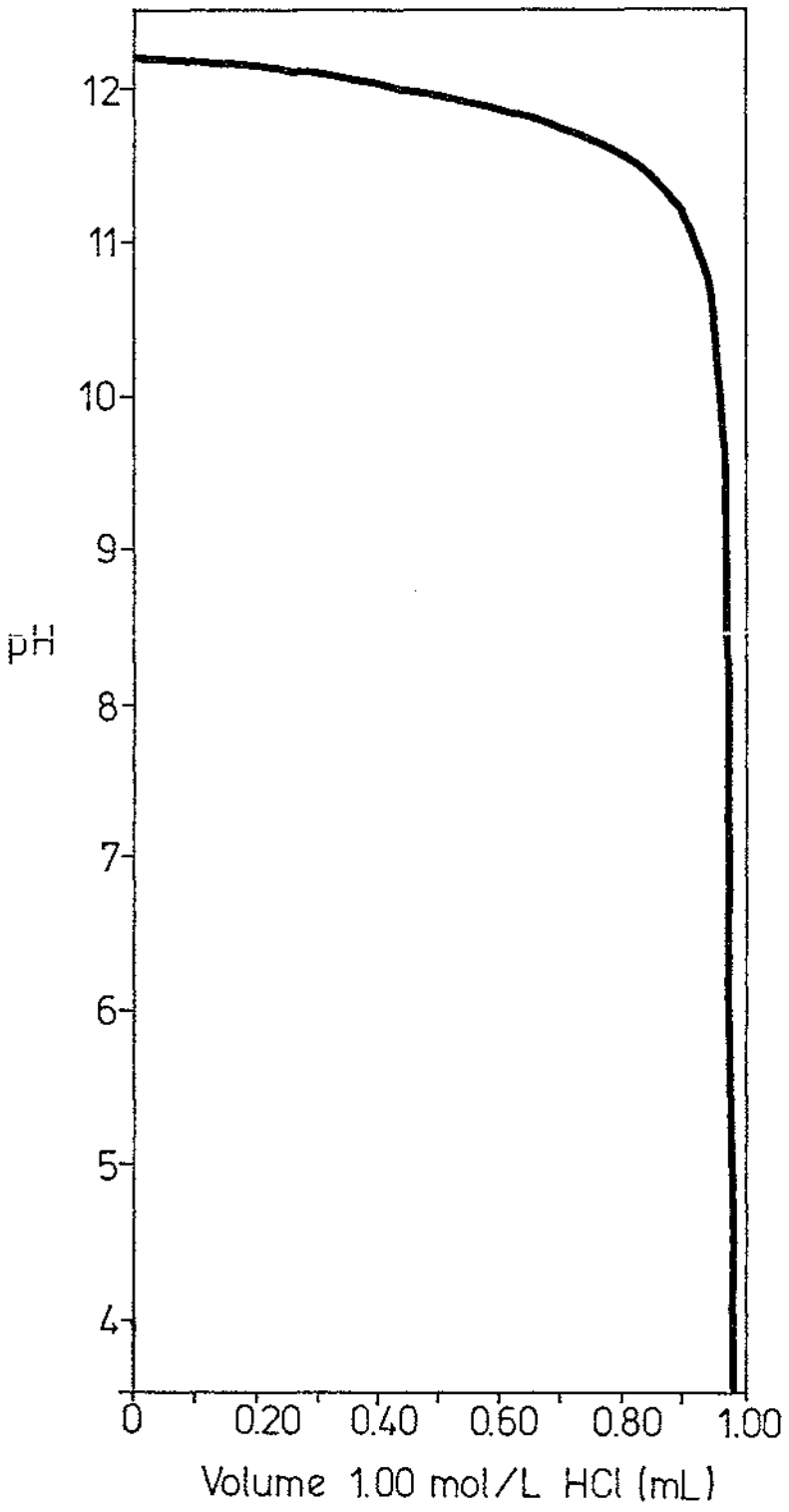
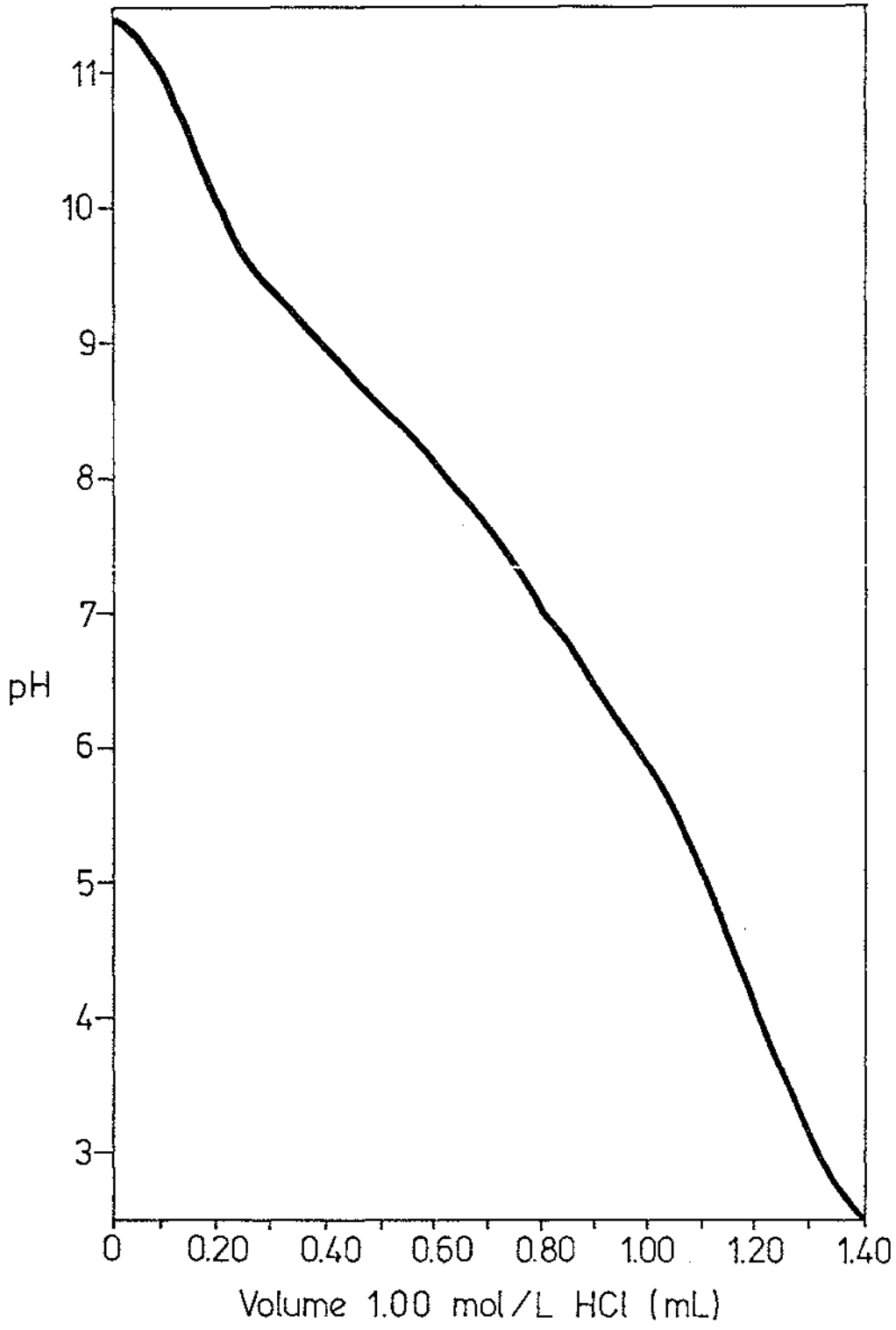
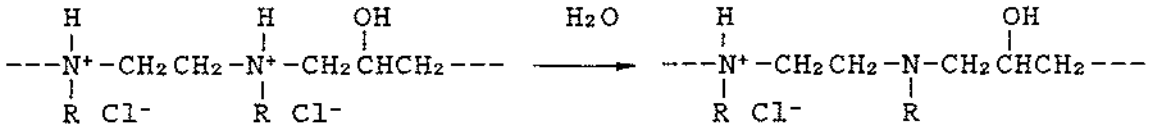


Figure 4.7 Titration Curve for Colestipol



The nitrogen content of colestipol was determined to be 7.59 meq/g at an end point of pH 2.5, but only if the sample was washed with 0.01 mol/L hydrochloric acid before determining its dry weight. If the sample titrated was washed extensively with water, hydrolysis of the amine hydrochlorides (Equation 4.7) lowered its dry weight considerably and elevated the apparent substitution level. This only happens with very weak amines (pKa 2-6 region) not normally present in ion exchangers[18].

Equation 4.7 The Effect of Washing Colestipol Hydrochloride[a]



Hydrochloride Form of Colespipol + H_3O^+ + Cl^-

[a] For a more complete structure see Figure 1.2B

What was clear from the titration curve for colestipol was that only about half of the nitrogens were charged at pH 6-8, which is the pH range in the intestine where they are required to bind bile acid anions. For this reason it would be more meaningful to quote an exchange capacity using an end point in the pH 6-8 range. This was done and a value of 4.03 meq/g was arrived at for pH 8.0. (Figure 4.5 and 4.6 show that for the alkyl QA celluloses or cholestyramine, titration to any pH less than 10.0 would give exactly the same result.)

The hydrolysis of hydrochloride from colestipol also made it difficult to determine its swollen volume in the usual way (Section 2.6.1). Hence it was decided to swell 1 g of colestipol in water as supplied. The sample swelled to 8.0 mL, and hence a swollen volume of 8.0 mL/g was subsequently used for colestipol.

4.11 SUMMARY

In this chapter it was demonstrated that by half-neutralising DMDo-amine during the coupling reaction it was possible to greatly increase the substitution level of the final product. It was also shown that long activations, combined with short coupling times at high temperatures, gave the highest substitution levels. For example, DMDo 72 was prepared by activating 15% cross-linked HP Indion with epichlorohydrin in 6% sodium hydroxide for 48 hours at 4-6°C, and then carrying out most of the coupling for 10 hours at 70°C after mixing overnight at room temperature. The efficiency of coupling by this method was 97% and the substitution level obtained was 1.08 meq/g. When this work began, substitution levels of 1.1 meq/g could only be obtained by double processing (Table 3.3).

It was shown that trimethylamine could be coupled quantitatively in water without half-neutralisation in less than 12 hours at room temperature (but not in ethanol). By using trimethylamine to indicate the number of active epoxides achieved when DMDo 72 was reprocessed, it was demonstrated that a 24 hour activation at 4-6°C using 6% sodium hychoxide was sufficient to achieve the highest substitution level. Using these conditions a methyl/dodecyl QA cellulose (DMDo 101) was produced with a substitution level of 1.7 meq/g. When DMDo-amine was coupled using the coupling conditions previously developed for DMDo 72, the product (DMDo 95) had a substitution level of 1.37 meq/g, and a desirable low swollen volume of only 3.3 mL/g. Only about 45% of the epoxides which coupled with trimethylamine coupled with DMDo-amine, however this still represented a significant increase in the substitution level. Further optimisation of the reprocessing step for dodecyl QA cellulose would be very difficult and possibly not worthwhile. Coupling at temperatures higher than 70°C might improve the efficiency but this has not been investigated. Apart from this possibility there seems little hope for further improving the substitution

level for a dodecyl QA cellulose. Even at 1.4 meq/g, 35% of the product is no longer cellulose but dimethyldodecylammonium chloride added onto the matrix.

For these reasons it was decided to synthesise a range of ion exchangers using these methods (Chapter 5), and then measure their ability to bind bile acid anions (Chapter 6), before attempting any further synthetic investigation.

CHAPTER 5. PREPARATION OF ION EXCHANGERS FOR IN VITRO BILE ACID CAPACITY TESTS

5.1 INTRODUCTION

A range of alkyl QA cellulose ion exchangers (where alkyl = C_1, C_4, C_8 and C_{12}) were prepared on HP Indion using the synthetic methods developed in Chapter 4. All of the amines were half-neutralised in the coupling step, except for trimethylamine where half-neutralisation was shown to be unnecessary. Because N,N-dimethyl-N-octylamine and N,N-dimethyl-N-butylamine were likely to be as or more reactive than DMDo-amine for steric reasons, the optimum conditions for preparing dodecyl QA cellulose were applied directly to the synthesis of octyl and butyl QA celluloses without further synthetic investigation. This yielded acceptable results. In addition, several 'special' ion exchangers were prepared using similar conditions.

5.2 PREPARATION OF SINGLE AND DOUBLE PROCESSED ION EXCHANGERS

The products reported in Chapter 4 were all prepared on 15% cross-linked HP Indion. For in vitro bile acid capacity tests it was decided to prepare products on both the 15% and the 10% cross-linked matrices (HP5 Indion and HP7 Indion respectively). The properties of the various products prepared are summarised in Tables 5.1 and 5.2. The double processed ion exchangers in Table 5.2 were prepared from the corresponding single processed ion exchangers in Table 5.1. (Complete details of the reaction conditions used are summarised in Appendix I.)

As found in earlier work, it was more difficult to couple the epoxide matrix with the amine as the size of the alkyl group increased from methyl to dodecyl, especially for the double processed products (Table 5.2). Double processing produced a

TABLE 5.1 Single Processed Ion Exchangers

Amine $N(CH_3)_2R$ (R)	Substitution Levels (meq/g) 10% CL[a]	Substitution Levels (meq/g) 15%CL[a]	Swollen Volumes (mL/g) 10%CL[a]	Swollen Volumes (mL/g) 15%CL[a]
Methyl	-	1.25	-	8.6
Butyl	-	1.10	-	8.4
Octyl	1.20	1.07	9.9	8.0
Dodecyl	1.14	1.08	8.0	8.2

[a] CL = Cross-Linking

TABLE 5.2 Double Processed Ion Exchangers[b]

Amine $N(CH_3)_2R$ (R)	Substitution Levels (meq/g) 10% CL[a]	Substitution Levels (meq/g) 15%CL[a]	Swollen Volumes (mL/g) 10%CL[a]	Swollen Volumes (mL/g) 15%CL[a]
Methyl	-	1.89	-	7.2
Butyl	-	1.68	-	6.5
Octyl	1.70	1.59	5.1	4.9
Dodecyl	1.50	1.37	3.3	3.3

[a] CL = Cross-Linking

[b] The double processed ion exchangers were prepared from the single processed ion exchangers reported in Table 5.1

significant reduction in the swollen volumes of the products. The reduction in swollen volume was greatest for the largest alkyl groups, presumably because of hydrophobic interactions which excluded water and contracted the cellulose matrix. The cross-linking present in the starting matrix (HP Indion) only had a small effect on the substitution levels obtained. Slightly higher substitution levels were obtained on the 10% cross-linked matrix compared to the 15% cross-linked matrix.

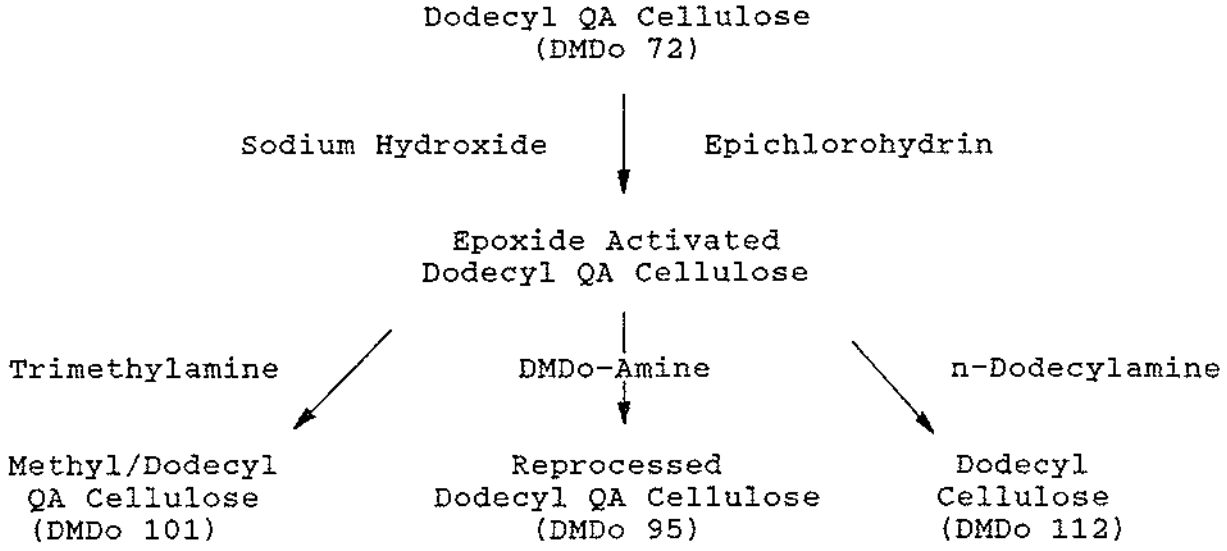
5.3 'SPECIAL' ION EXCHANGERS

Three special ion exchangers were prepared to determine whether a product could be produced with a better capacity for bile acid anions than those synthesised in Section 5.2. (Preparative details are included in Appendix I).

5.3.1 Reprocessing Dodecyl QA Cellulose with Either Trimethylamine or n-Dodecylamine

It was observed in Section 4.9 that much greater substitution levels could be obtained for alkyl QA celluloses by coupling trimethylamine, instead of DMDo-amine, to dodecyl QA cellulose in the reprocessing step (Equation 5.1). The dodecyl QA cellulose ion exchanger referred to in Table 5.1 was reactivated and coupled to trimethylamine producing the methyl/dodecyl QA cellulose referred to in Table 5.3 (DMDo 101). This had a substitution level of 1.7 meq/g which was considerably greater than the double processed dodecyl QA cellulose which only had 1.37 meq/g.

Equation 5.1



It was observed in Section 3.3.3 that QA cellulose ion exchangers with short alkyl chains (such a butyl QA cellulose) were ineffective at binding cholale anions. However it was hoped that the presence of the quaternary dodecylamino groups already on the cellulose matrix would provide a suitable hydrophobic environment to enable some of the quaternary methylamino groups to be effective. For example, two adjacent charged centres, a quaternary methylamino group and a quaternary dodecylamino group, might each bind a bile acid anion and share the long hydrophobic dodecyl chain. For this reason the methyl/dodecyl QA cellulose was included with the ion exchangers whose cholate binding capacities are reported in Chapter 6.

TABLE 5.3 'Special' Ion Exchangers Prepared by Reprocessing DMDo 72

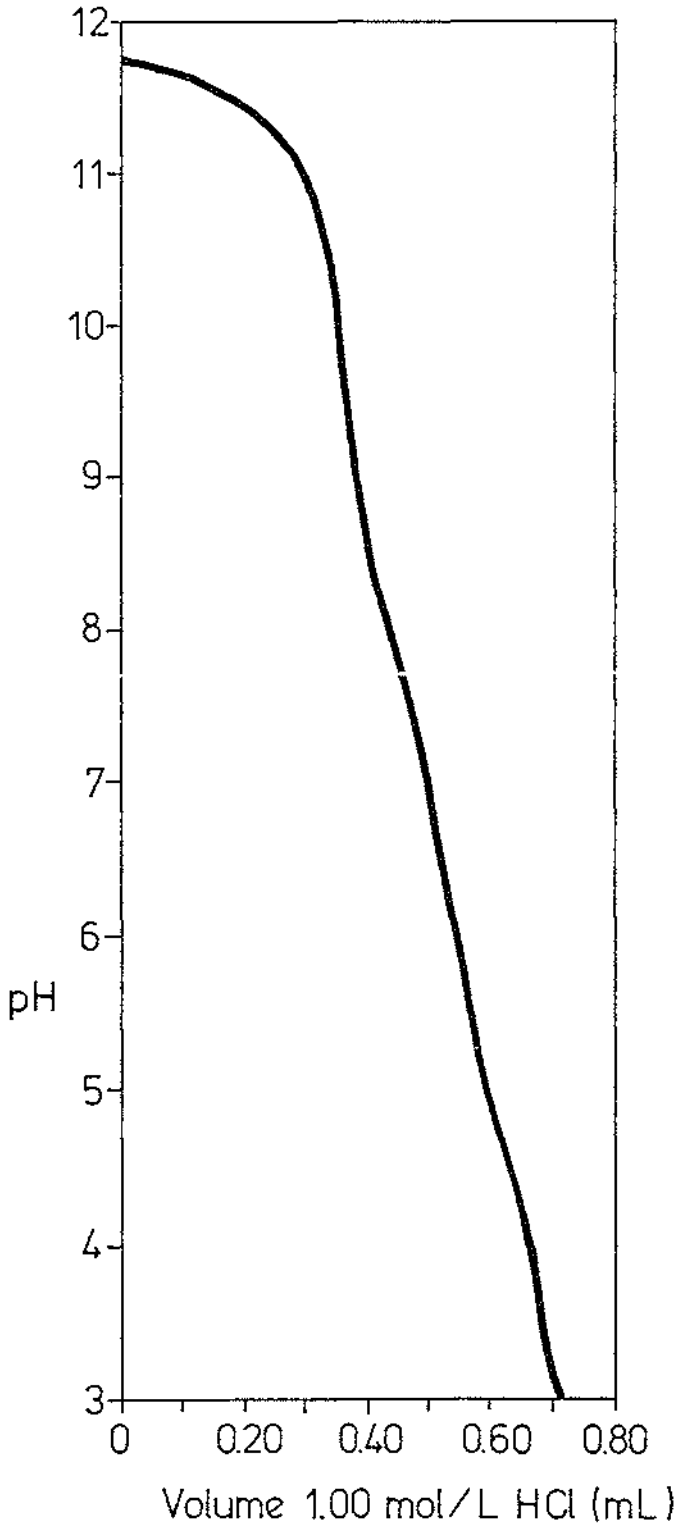
Ion Exchanger DMDo	Amine Coupled[b]	Substitution Level (meq/g)	Swollen Volume (mL/g)
95[a]	DMDo-amine	1.37	3.3
101	Trimethylamine	1.70	3.8
112	n-Dodecylamine	1.42	3.2

[a] From Table 5.2

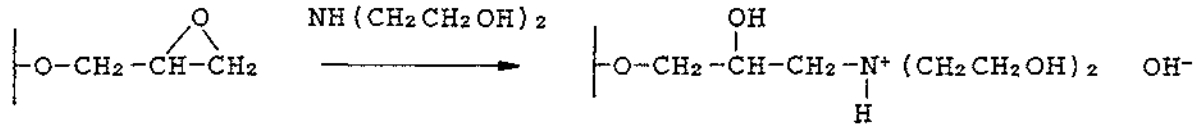
[b] Amine coupled in the reprocessing step

Coupling reactivated dodecyl QA celluloses with DMDo-amine was only about 45% efficient compared to trimethylamine (Section 4.9). Hence it was decided to try and couple n-dodecylamine to the reactivated dodecyl QA cellulose (Equation 5.1) in an attempt to elevate the substitution level without losing the long hydrophobic carbon chain. The primary amine is less sterically hindered and should be more reactive. A sample of DMDo 72 was reactivated and coupled with half-neutralised n-dodecylamine in the usual way. The properties of this product (DMDo 112) are given in Table 5.3. Unfortunately the nitrogen content, determined by titrating to an end point of pH 3.0, was only slightly higher than that achieved by coupling the corresponding tertiary amine. Furthermore the titration curve (Figure 5.1) revealed that nearly all of these nitrogens were very weakly basic and were uncharged at pH 8.0. (Titration to an end point of pH 8.0 indicated that the number of charged nitrogens at this pH was only 0.90 meq/g). This was surprising since the pKa's of primary and secondary alkyl amines coupled to epoxide activated cellulose usually lie between the pH 8-9 region. For example, even $NH(CH_2CH_2OH)_2$ coupled to epoxide activated cellulose (Equation 5.2) has a pKa of 8.2[18].

Figure 5.1 Titration Curve for Dodecyl QA Cellulose Reprocessed with n-Dodecylamine



Equation 5.2

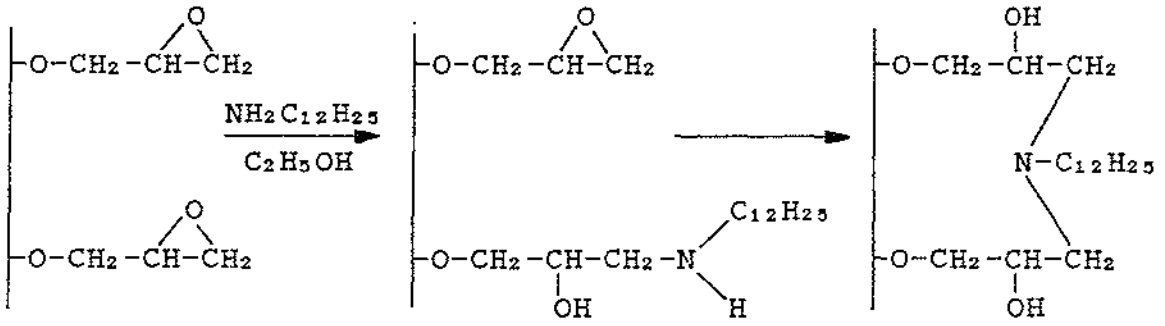


Epoxide Activated Cellulose

pKa 8.2

The most likely explanation for the low substitution level of the ion exchanger, and the low pKa of the amine groups attached, was that some of the secondary amine groups which formed when n-dodecylamine coupled to the epoxide activated matrix may have been able to react with further epoxide groups (Equation 5.3). This would introduce further cross-linking into the ion exchanger and produce weakly basic tertiary amine groups. Also the close proximity of the positively charged quaternary amine groups to the secondary and tertiary nitrogens would further lower the pKa values. Whatever the explanation, this product was not tested for cholate binding capacity.

Equation 5.3



Epoxide Activated DMDo 72

Secondary Amine

Tertiary Amine

5.3.2 Triple Processing of Octyl QA Cellulose

Table 5.2 indicates that the swollen volumes of the reprocessed octyl QA celluloses were quite large compared to the reprocessed dodecyl QA celluloses. For this reason it was

decided to carry out a further reprocessing step on the double processed octyl QA cellulose with 10% cross-linking, to determine whether the substitution level could be increased. The activation and coupling conditions used to prepare the triple processed octyl QA cellulose were similar to those used to prepare the single and double processed ion exchangers (Section 5.2). The physical properties of all three products are reported in Table 5.4. The substitution level for the triple processed ion exchanger was 1.98 meq/g compared with 1.70 meq/g for the double processed ion exchanger, which represented a small but significant increase. Also the swollen volume of the ion exchanger dropped from 5.1 mL/g to 3.8 mL/g on triple processing, to come more in line with the double processed dodecyl QA celluloses which had swollen volumes of about 3.3 mL/g (Table 5.2). Because of the favourable substitution level and swollen volume of the triple processed ion exchanger it was included in the cholate binding capacity studies reported in Chapter 6.

TABLE 5.4 Octyl QA Cellulose Ion Exchangers

Number of Processes	Starting Matrix	Ion Exchanger DMO	Substitution Level (meq/g)	Swollen Volume (mL/g)
1	HP7[a]	12	1.20	9.9
2	DMO 12[b]	14	1.70	5.1
3	DMO 14[c]	25	1.98	3.8

[a] 10% cross-linked HP Indion cellulose

[b] Table 5.1

[c] Table 5.2

CHAPTER 6 INVESTIGATION OF BILE ACID BINDING CAPACITY IN VITRO

6.1 INTRODUCTION

This section describes and compares the bile acid binding capacities of various QA cellulose ion exchangers prepared using the synthetic methods developed in Chapters 4 and 5. The corresponding capacities of cholestyramine and colestipol (two ion exchangers marketed as bile acid sequestrants in the pharmaceutical products Questran and Colestid respectively) were determined for comparison. Capacity tests were initially carried out with unconjugated bile acid anions (sodium cholate and sodium deoxycholate). Then selected ion exchangers were equilibrated with mixtures of the glyco- and tauro-conjugates of cholate and deoxycholate. Analysis of the supernatant by HPLC showed the effectiveness of the various ion exchangers for binding the bile acid anions in such a mixture, resembling the conditions found in the duodenum.

6.2 CHOLATE CAPACITY TESTS

The procedure used to determine the cholate binding capacity of the ion exchangers was basically that described by Krasopoulos et al.[37]. Small samples of the ion exchanger, previously equilibrated, were mixed with a 15 mmol/L solution of sodium cholate in a Bicarbonate Buffer System (BBS) at pH 8.0, containing approximately 150 mmol/L sodium chloride. The amount of cholate taken up by the ion exchanger was then determined from the residual cholate concentration in the mixture when equilibrium was reached, using the method of Sjovall[38].

One aspect of this test which needed to be investigated for the new cellulose ion exchangers was how long the samples needed to be mixed in order to reach equilibrium. This was determined by centrifuging one of the samples and removing

aliquots (20 µL) of the supernatant for spectroscopic analysis at regular intervals and then resealing the vials for further mixing. (A 20 µL sample represented only 0.1% of the total volume and so even when several samples were removed the volume change was inconsequential.) The same equilibrium capacities were obtained irrespective of whether the tests were carried out at room temperature or 30°C. In no case did it take longer than 12 hours to reach equilibrium at room temperature and the bulk of the ion exchange was usually over in two hours. However the capacity studies reported herein were all mixed for 25 hours at room temperature for the sake of convenience and to be confident that equilibrium capacities had been reached.

6.2.1 The Effect of Reprocessing Dodecyl QA Celluloses With and Without Half-Neutralisation of the Amine

The three cholate capacity curves (mg/g) shown in Figure 6.1 all correspond to dodecyl QA cellulose ion exchangers prepared on 15% cross-linked HP5 Indion. The middle curve corresponds to the double processed dodecyl QA cellulose which was prepared without half-neutralisation in Section 3.4 (DMDo 4) and shown to be the most promising ion exchanger on which to base further investigation. The upper and lower curves correspond to dodecyl QA celluloses coupled with half-neutralisation according to the methods developed in Chapter 4. The lower curve is for the single processed product (DMDo 72) and the upper curve is for the double processed product (DMDo 95) prepared from it.

Figure 6.1 demonstrates that all three of these ion exchangers have similar capacities on a dry weight basis for binding the cholate anion. This was in spite of the fact that the dodecyl QA cellulose double processed with half-neutralisation of the coupling reagent had a substitution level of 1.37 meq/g, compared to the 'non-neutralised' ion exchanger with only 1.16 meq/g, and the single processed ion exchanger with

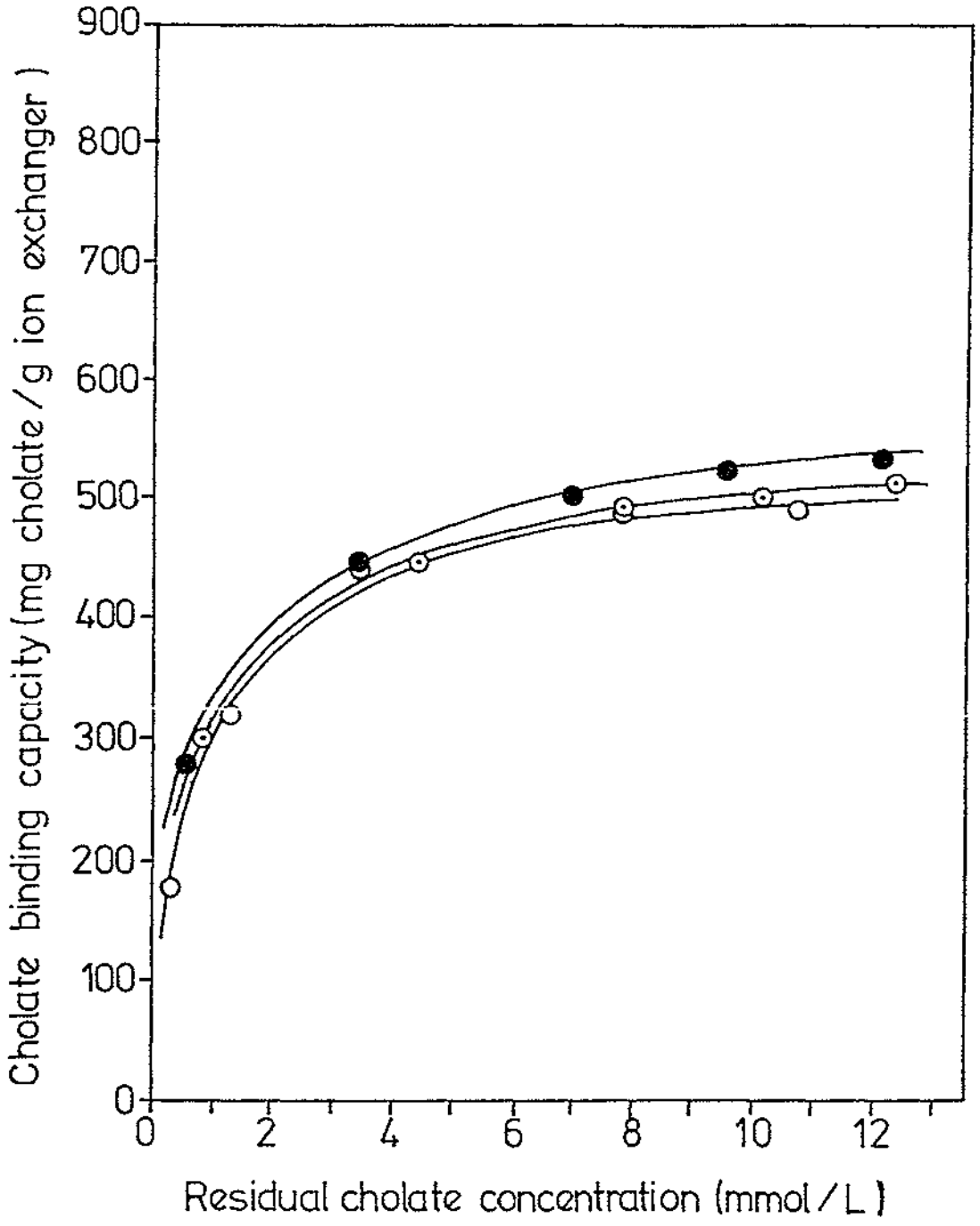
1.08 meq/g. This arose because the efficiency with which these ion exchangers bound cholate decreased as the substitution level increased. For example at equilibrium the binding efficiencies of the above ion exchangers at a residual concentration of 10 mmol/L sodium cholate were 94%, 107% and 111% respectively. This was unfortunate because it meant that any synthetic advances made for a particular ion exchanger did not proportionally enhance its cholate capacity. However tests on poultry did show superior performance of DMDo 95 over DMDo 72[43].

When the cholate capacity curves for these ion exchangers were compared on a volume basis (mg/mL) as shown in Figure 6.2 the two reprocessed ion exchangers had considerably greater capacities because of the contraction of their swollen volumes (mL/g) upon reprocessing. The superior capacity shown in Figure 6.2 for DMDo 95 (coupled with half-neutralised amine) compared with DMDo 4 (coupled without half-neutralised amine) also resulted from a swollen volume effect. DMDo 95 had a swollen volume of only 3.3 mL/g, whereas DMDo 4 had a swollen volume of 3.7 mL/g. For this reason, only reprocessed ion exchangers made using half-neutralised amine were investigated further.

6.2.2 Comparison of Octyl and Dodecyl QA Celluloses Prepared on 10% and 15% Cross-Linked HP Indion

The cholate capacity curves (mg/g and mg/mL) for the reprocessed octyl and dodecyl QA celluloses prepared on 10% and 15% cross-linked HP Indion are shown in Figures 6.3 and 6.4. Figure 6.3 gives the cholate capacities with respect to the dry weight of the ion exchangers. This shows that, in agreement with Section 3.5.1, the octyl QA celluloses had slightly greater cholate capacities at high residual concentrations of sodium cholate. However, at low residual concentrations (<4 mmol/L) the dodecyl QA celluloses performed better. The efficiency of the quaternary amine groups to bind

Figure 6.1 Cholate Capacity Curves (mg/g) for Dodecyl QA Celluloses Coupled With and Without Half-Neutralisation of DMDo-amine



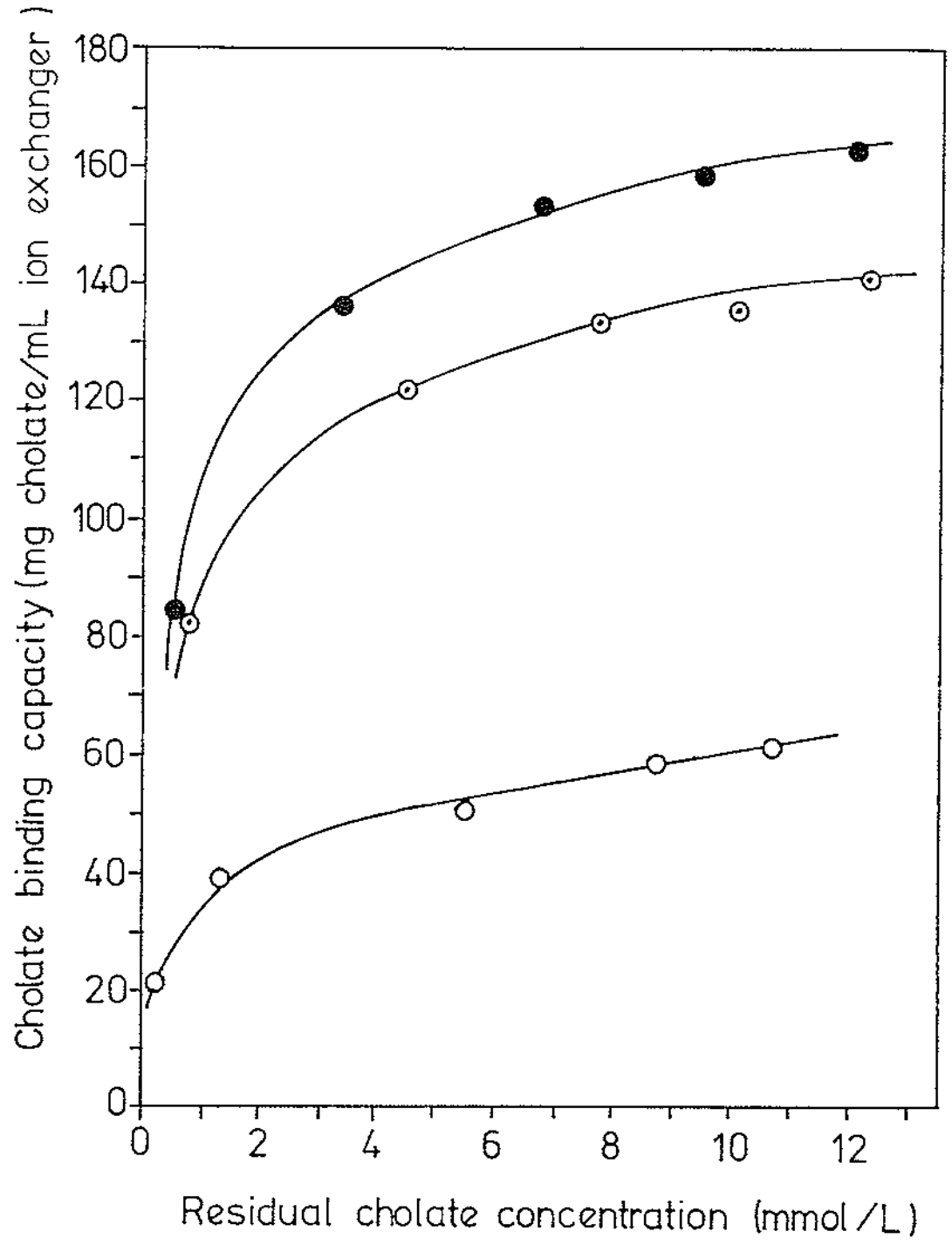
● 15% cross-linked dodecyl QA cellulose, double processed with half-neutralised DMDo-amine (DMDo 95, 1.37 meq/g)

⊙ 15% cross-linked dodecyl QA cellulose, double processed without half-neutralisation of DMDo-amine (DMDo 4, 1.16 meq/g)

○ 15% cross-linked dodecyl QA cellulose, single processed with half-neutralised DMDo-amine (DMDo 72, 1.08 meq/g)

Conditions: small samples of each ion exchanger were mixed with 15 mmol/L sodium cholate in BBS (20 mL) at pH 8.0 for 25 hours at room temperature

Figure 6.2 Cholate Capacity Curves (mg/mL) for Dodecyl QA Celluloses Coupled With and Without Half-Neutralisation of DMDo-amine



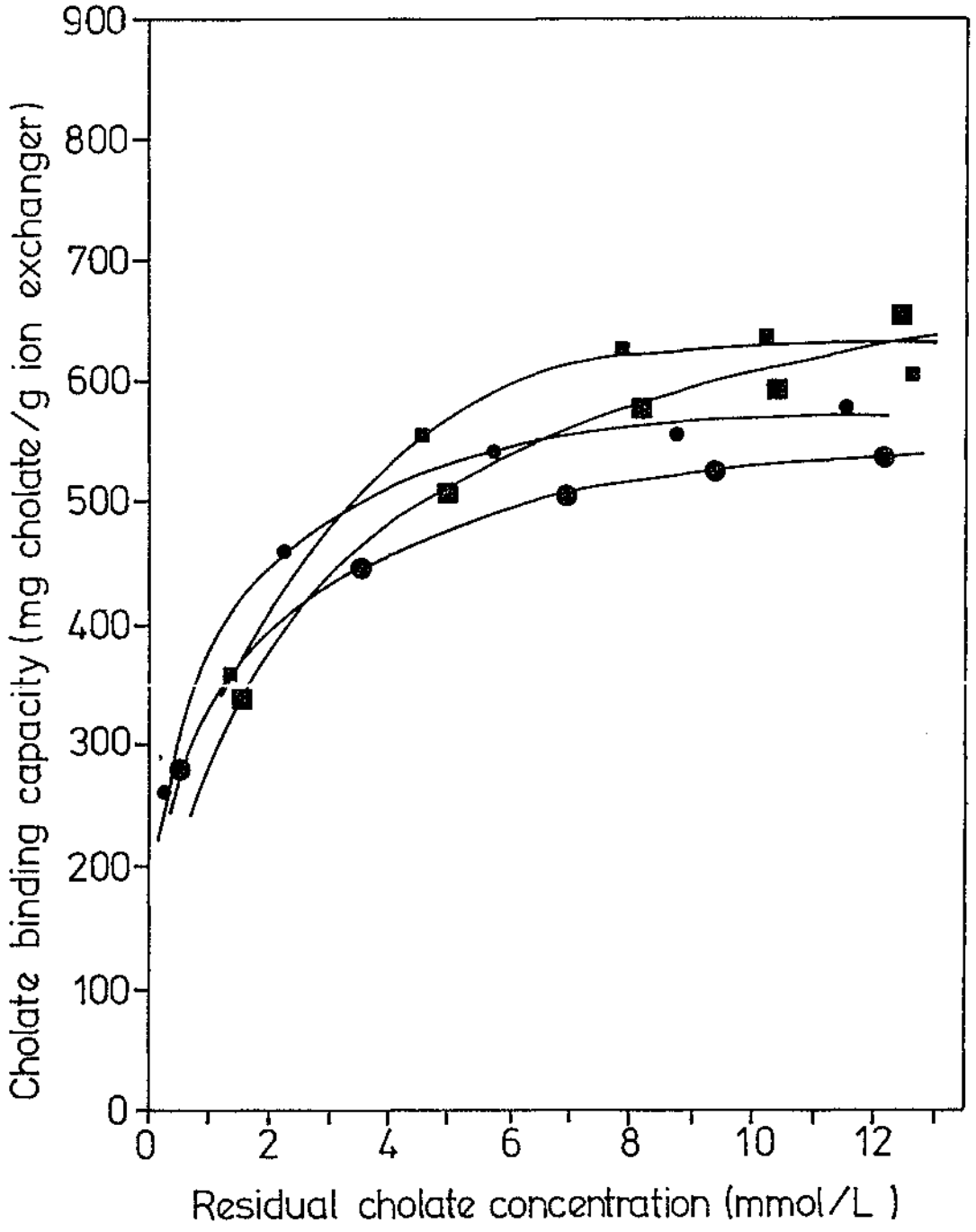
● 15% cross-linked dodecyl QA cellulose, double processed with half-neutralised DMDo-amine (DMDo 95, 3.3 mL/g)

◉ 15% cross-linked dodecyl QA cellulose, double processed without half-neutralised DMDo-amine (DMDo 4, 3.7 mL/g)

○ 15% cross-linked dodecyl QA cellulose, single processed with half-neutralised DMDo-amine (DMDo 72, 8.2 mL/g)

Conditions: as for Figure 6.1

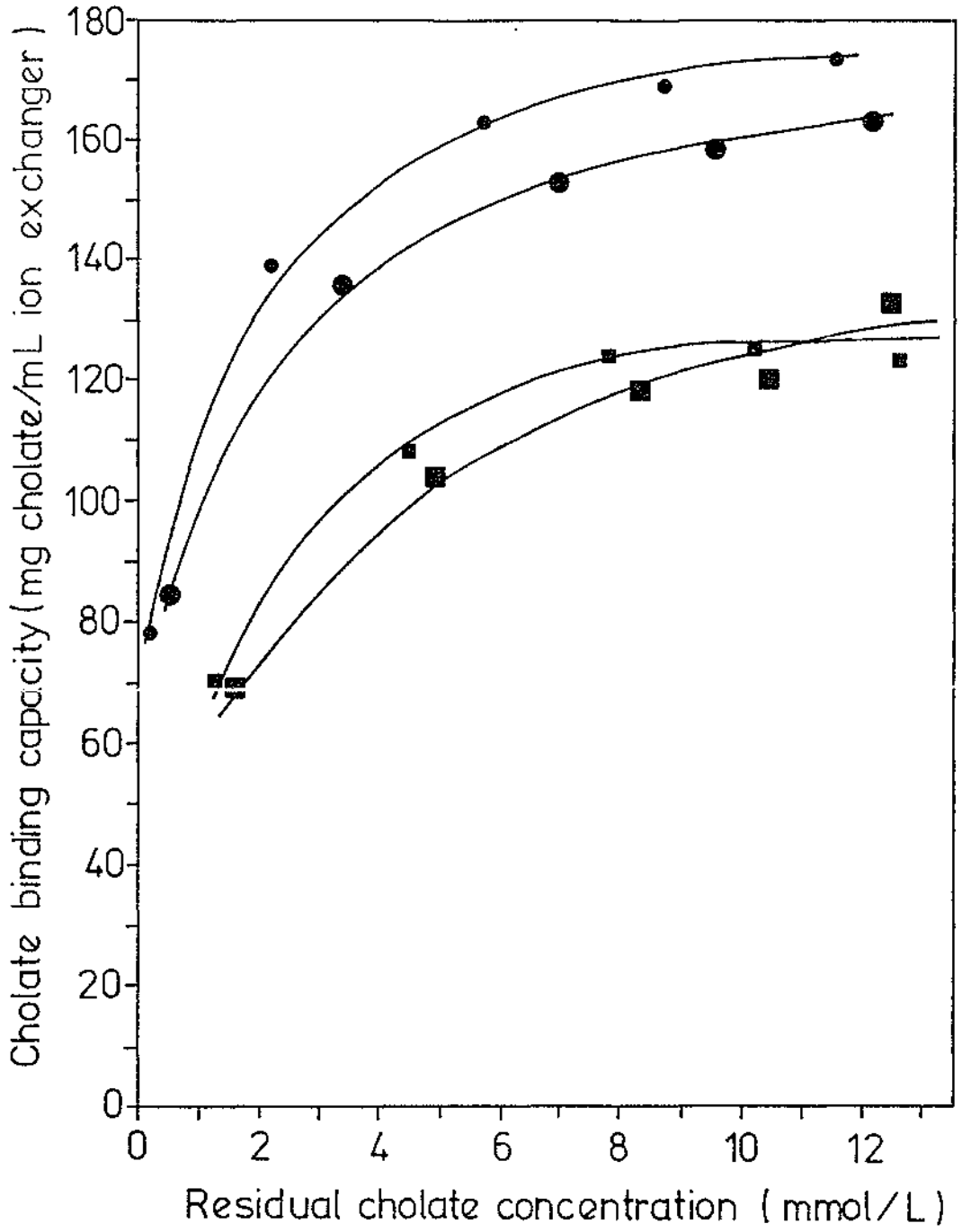
Figure 6.3 Cholate Capacity Curves (mg/g) for 10% and 15% Cross-Linked Octyl and Dodecyl QA Celluloses



- ● double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 109, 1.50 meq/g)
- ● double processed, 15% cross-linked, dodecyl QA cellulose (DMDo 95, 1.37 meq/g)
- ■ double processed, 10% cross-linked, octyl QA cellulose (DMO 14, 1.70 meq/g)
- ■ double processed, 15% cross-linked, octyl QA cellulose (DMO 17, 1.59 meq/g)

Conditions: as for Figure 6.1

Figure 6.4 Cholate Capacity Curves (mg/mL) for 10% and 15% Cross-Linked Octyl and Dodecyl QA Celluloses



- ● double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 109, 3.3 mL/g)
- ● double processed, 15% cross-linked, dodecyl QA cellulose (DMDo 95, 3.3 mL/g)
- ■ double processed, 10% cross-linked, octyl QA cellulose (DMO 14, 5.1 mL/g)
- ■ double processed, 15% cross-linked, octyl QA cellulose (DMO 17, 4.9 mL/g)

Conditions: as for Figure 6.1

cholate at a residual concentration of 10 mmol/L were all similar, being 93% and 94% for the 10% and 15% cross-linked dodecyl QA celluloses, and 93% and 90% for the octyl QA celluloses respectively. Therefore the slight advantage the octyl QA celluloses had at high concentrations of sodium cholate arose because they had slightly greater substitution levels (Table 5.2).

The curves in Figure 6.4 give the cholate capacities calculated with respect to the volume of the ion exchangers used. These curves show that the dodecyl QA celluloses have far superior cholate capacities than the octyl QA celluloses on a volume basis. This could be very important when considering these ion exchangers for use as bile acid sequestrants, because if a patient were required to take a very large volume of an ion exchanger orally, this would increase the level of gastrointestinal discomfort.

On both a volume and dry weight basis the 10% cross-linked ion exchangers had higher cholate capacities than the 15% cross-linked ion exchangers. This was due to the fact that they had slightly greater substitution levels (Table 5.2). From these observations the dodecyl QA cellulose ion exchanger prepared on 10% cross-linked HP Indion cellulose matrix appeared to have the greatest potential for use as a bile acid sequestrant. (Earlier work in Section 3.5.2 did not show any significant increase in the cholate capacity of ion exchangers when the cross-linking of the starting matrix was decreased from 15% to 10%. However the samples used in the capacity tests in Chapter 3 were only mixed for 2 hours and equilibrium may not have been reached.)

6.2.3 The Effect of Reactivating Dodecyl QA Cellulose and Coupling Trimethylamine on Cholate Binding

When single processed dodecyl QA celluloses were reprocessed it was shown (Section 5.3.1) that significantly higher

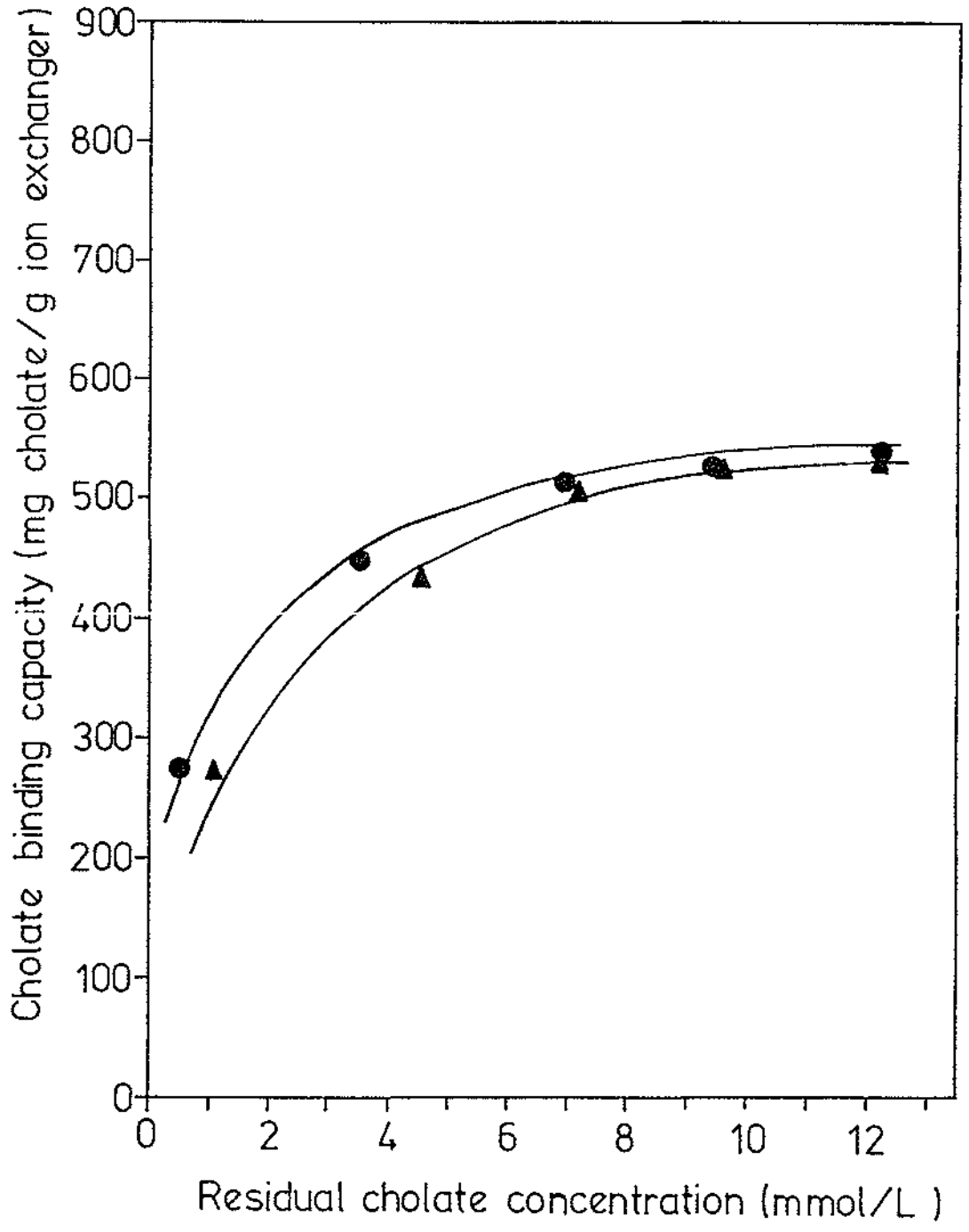
substitution levels could be obtained by coupling trimethylamine in place of DMDo-amine. For example the trimethylamino derivative (DMDo 101) prepared on 15% cross-linked dodecyl QA cellulose (DMDo 72) had a substitution level of 1.70 meq/g, whereas the double processed dodecyl QA cellulose (DMDo 95) had a substitution level of 1.37 meq/g (Table 5.3). Cholate binding capacity tests were run on both of these ion exchangers and the results are given in Figure 6.5. Clearly these two ion exchangers have very similar capacities (mg/g) to bind cholate, and the more highly substituted product appeared to have no advantage. These results were confirmed by in vivo tests on poultry[18].

The quaternary trimethylamino groups did not bind cholate efficiently even though there were plenty of hydrophobic dodecylamino groups present as well. At a residual concentration of sodium cholate of 10 mmol/L the binding efficiency for the trimethylamino derivative corresponded to only 75% of the total number of quaternary amine groups, compared to 94% for the product double processed with DMDo-amine. This was consistent with earlier experiments which indicated that QA celluloses with short alkyl chains were ineffective at binding cholate anions (Section 3.3.1). Since there was no significant increase in cholate binding capacity for the ion exchanger reprocessed with trimethylamine, further investigation on mixed alkyl QA celluloses of this type were not carried out.

6.2.4 The Effect of Triple Processing Octyl QA Celluloses on Cholate Binding

In Section 5.3.2 it was shown that the substitution level of the double processed octyl QA cellulose prepared on 10% cross-linked HP Indion could be raised from 1.70 meq/g to 1.98 meq/g by adding a third processing step. The cholate capacity curves (mg/g) for these two products are shown in Figure 6.6. It is obvious from this that there is no advantage to be

Figure 6.5 Cholate Capacity Curves for Dodecyl QA Celluloses Reprocessed with Trimethylamine and DMDo-Amine

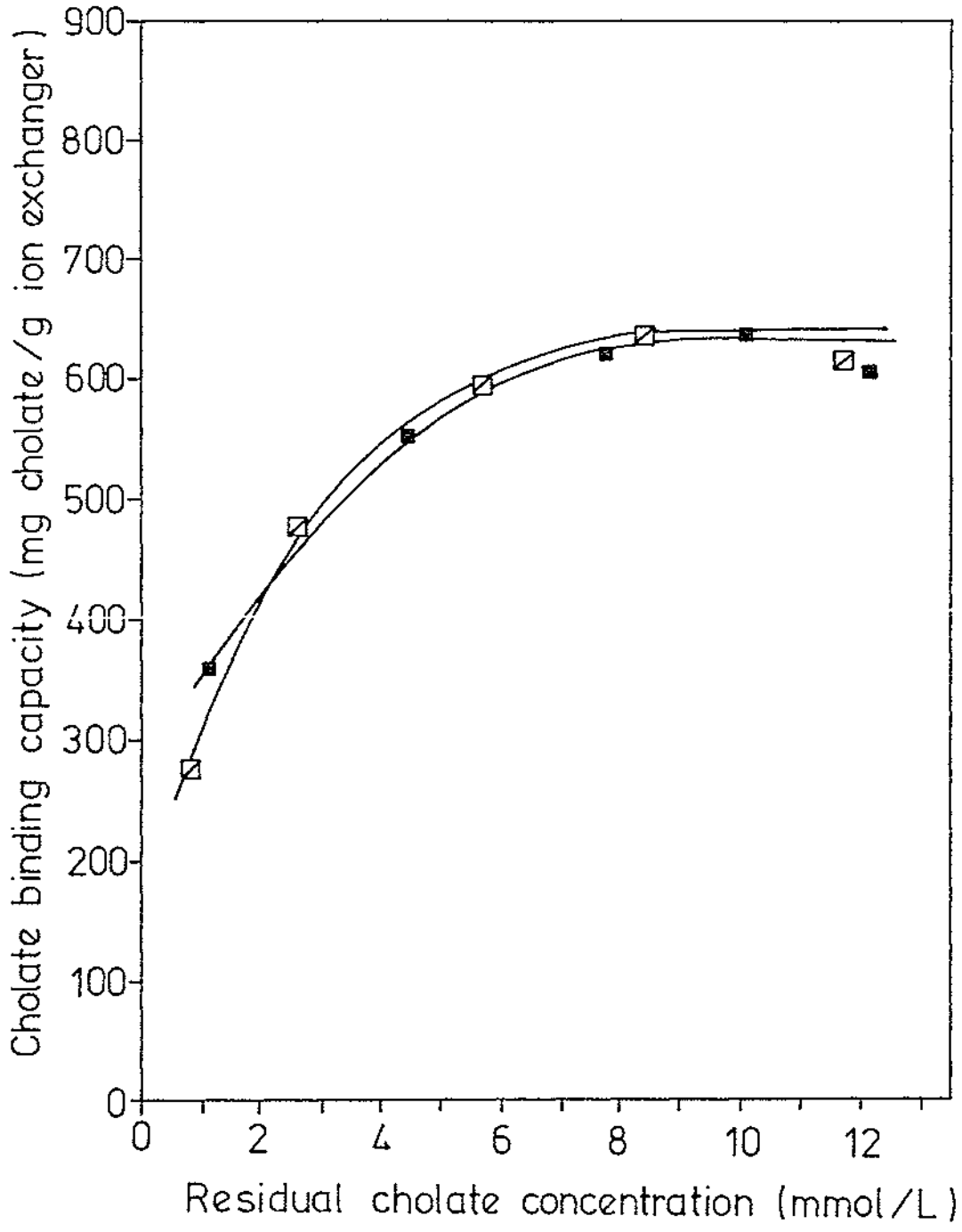


● 15% cross-linked dodecyl QA cellulose (DMDo 72) reporcessed with DMDo-amine (DMDo 95, 1.37 meq/g)

▲ 15% cross-linked dodecyl QA cellulose (DMDo 72) reprocessed with trimethylamine (DMDo 101, 1.07 meq/g)

Conditions: as for Figure 6.1

Figure 6.6 Cholate Capacity Curves for Double and Triple Processed Octyl QA Celluloses



■ double processed, 10% cross-linked, octyl QA cellulose (DMO 14, 1.70 meq/g)

☑ triple processed, 10% cross-linked, octyl QA cellulose (DMO 25, 1.98 meq/g)

Conditions: as for Figure 6.1

gained by triple processing the octyl QA cellulose. Even though the number of quaternary amine groups increased they were not all accessible to the cholate anions, since the cholate binding efficiency dropped from 93% to 80% (measured at a residual sodium cholate concentration of 10 mmol/L at equilibrium).

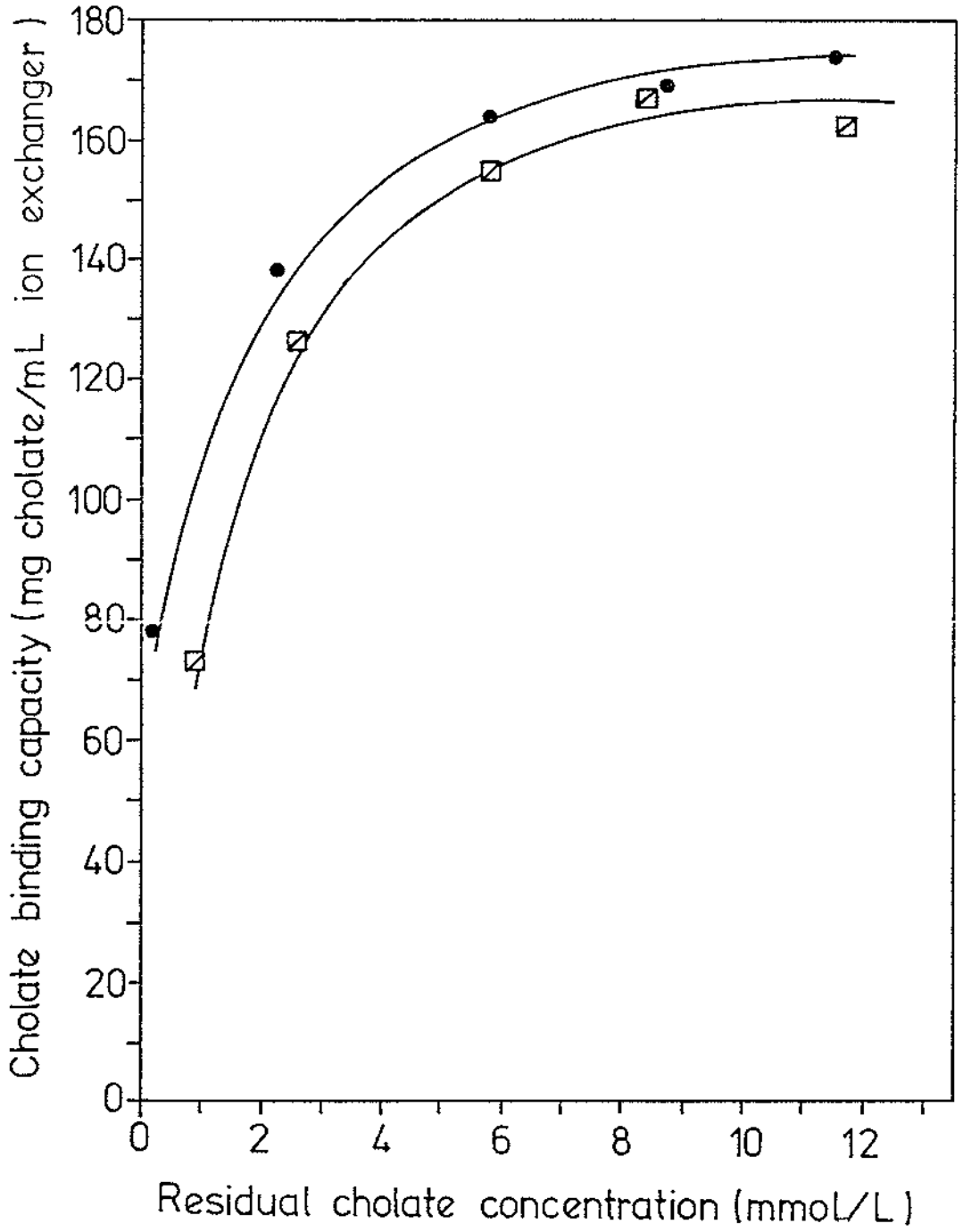
The only advantage of the triple processing was a significant reduction in the swollen volume of the product from 5.1 mL/g to 3.8 mL/g, bringing it more into line with that of the double processed dodecyl QA celluloses (Table 5.2). In fact the triple processed octyl QA cellulose had a cholate capacity on a volume basis similar to that of the dodecyl QA cellulose prepared on the 10% cross-linked matrix, which had a swollen volume of 3.3 mL/g (Figure 6.7). However its production required an additional step without gaining any benefit over the double processed dodecyl QA cellulose product. Therefore this ion exchanger was not investigated further.

6.2.5 Cholate Capacity Curves of Cholestyramine and Colestipol

The results of the cholate capacity tests in Sections 6.2.1 to 6.2.4 above show that the most promising ion exchangers for use as bile acid sequestrants are the octyl and dodecyl QA celluloses prepared on 10% cross-linked HP Indion. Therefore these ion exchangers were compared with two commercially available bile acid sequestrants, cholestyramine and colestipol. The cholate capacity curves (mg/g and mg/mL) of these four ion exchangers are given in Figures 6.8 and 6.9.

In Figure 6.8 the cholate capacities shown were calculated with respect to the dry weight of the ion exchanger concerned. Cholestyramine and colestipol were effective at binding cholate when there was a high background concentration of cholate at equilibrium (greater than 6-10 mmol/L), but much

Figure 6.7 Cholate Capacity Curves for Double Processed Dodecyl QA Cellulose and Triple Processed Octyl QA Cellulose

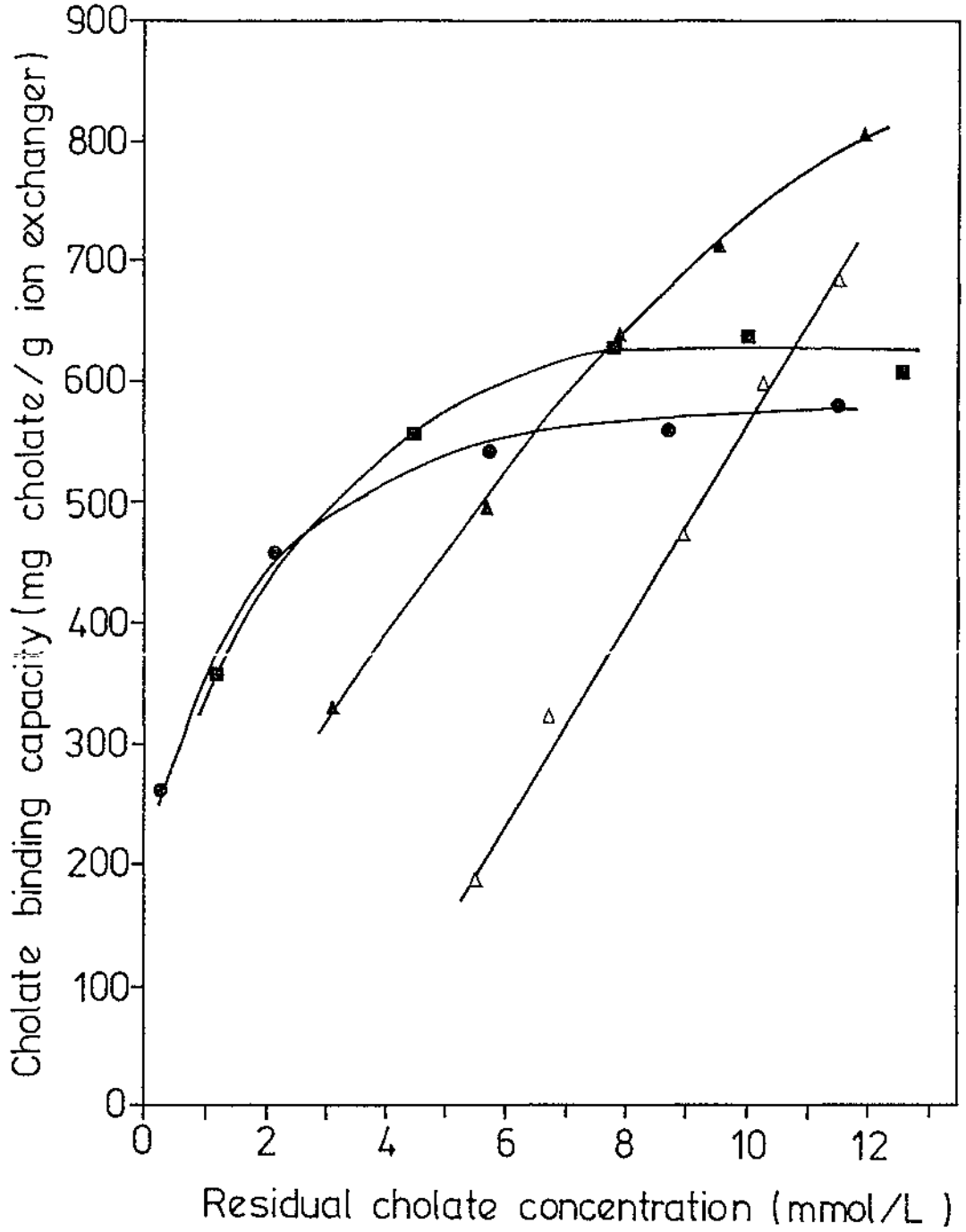


☒ triple processed, 10% cross-linked, octyl QA cellulose (DMO 25, 3.8 mL/g)

● double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 109, 3.3 mL/g)

Conditions: as for Figure 6.1

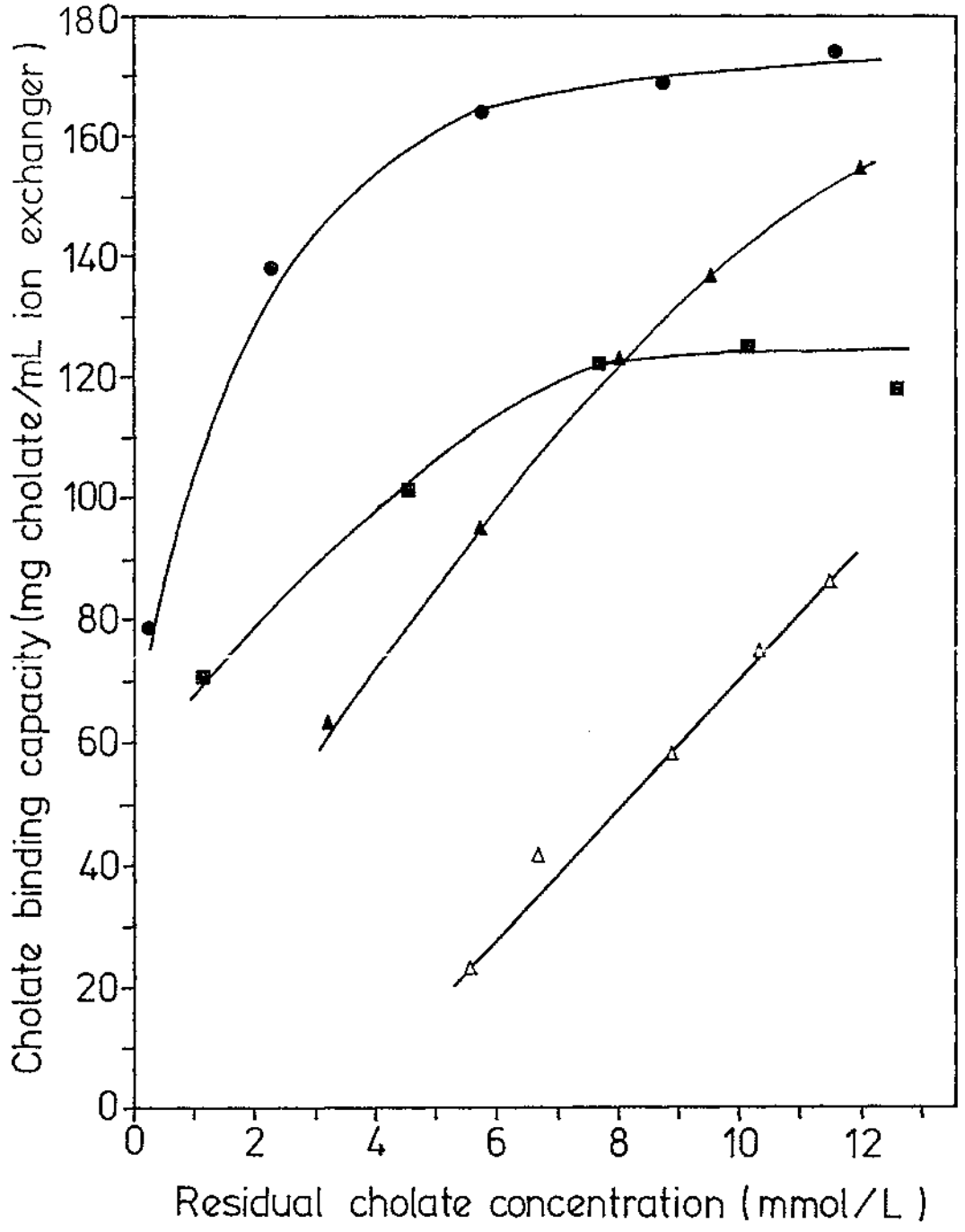
Figure 6.8 Cholate Capacity Curves (mg/g) for Octyl and Dodecyl QA Celluloses, Cholestyramine and Colestipol



▲ cholestyramine (3.81 meq/g)
△ colestipol (4.03 meq/g)
● double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 109, 1.50 meq/g)
■ double processed, 10% cross-linked, octyl QA cellulose (DMO 14, 1.70 meq/g)

Conditions: as for Figure 6.1

Figure 6.9 Cholate Capacity Curves (mg/mL) for Octyl and Dodecyl QA Celluloses, Cholestyramine and Colestipol



▲ cholestyramine (5.2 mL/g)
△ colestipol (8.0 mL/g)
● double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 109, 3.3 mL/g)
■ double processed, 10% cross-linked, octyl QA cellulose (DMO 14, 5.1 mL/g)

Conditions: as for Figure 6.1

less effective when the residual concentration of cholate was low. Also the cholate capacity of these two ion exchangers continued to increase above 10 mmol/L, whereas the octyl and dodecyl QA celluloses levelled off at this point. This was because cholestyramine and colestipol had very high substitution levels, but they were not very effective at binding cholate ions in the presence of excess chloride ions. (The considerably greater concentration of chloride (150 mmol/L) over cholate (15 mmol/L) favours the binding of chloride.)

The efficiencies of the four ion exchangers at different residual cholate concentrations are set out in Table 6.1. The hydrophobic QA celluloses bound cholate with greater than 80% efficiency even at 5 mmol/L residual cholate, when there was a greater than thirty fold excess of chloride. The dodecyl QA cellulose in particular reduced the residual concentration of sodium cholate to very low levels (well below 1 mmol/L), which were unobtainable with cholestyramine or colestipol. This was important because when bile is injected into the duodenum from the gall bladder and mixed with food, the total bile acid concentration is about 8 mmol/L[16]. Most (95%) of these bile acids are reabsorbed via the enterohepatic circulation[12], so the concentration of bile acids by the time the food reaches the distal ileum is very small indeed. Therefore these new cellulose ion exchangers appeared promising as bile acid sequestrants and were put forward for in vivo studies[18].

The capacity curves for the four ion exchangers were recalculated on a volume basis and the results are given in Figure 6.9. This demonstrates dramatically the superior performance of the dodecyl QA cellulose at all concentrations of bile acids likely to be encountered in vivo. These results were very encouraging and indicated that further work should be carried out in vitro and in vivo on alkyl QA cellulose ion exchangers.

TABLE 6.1 Efficiency of Ion Exchangers for Binding Cholate Against a Background of 150 mmol/L Sodium Chloride at pH 8.0

Ion Exchanger	Substitution Level (meq/g)	Efficiency at 5 mmol/L (%)	Efficiency at 10 mmol/L (%)
Dodecyl QA Cellulose	1.50	87	93
Octyl QA Cellulose	1.70	82	93
Cholestyramine	3.81[a]	29	48
Colestipol	4.03[b]	-	35

[a] Section 4.10

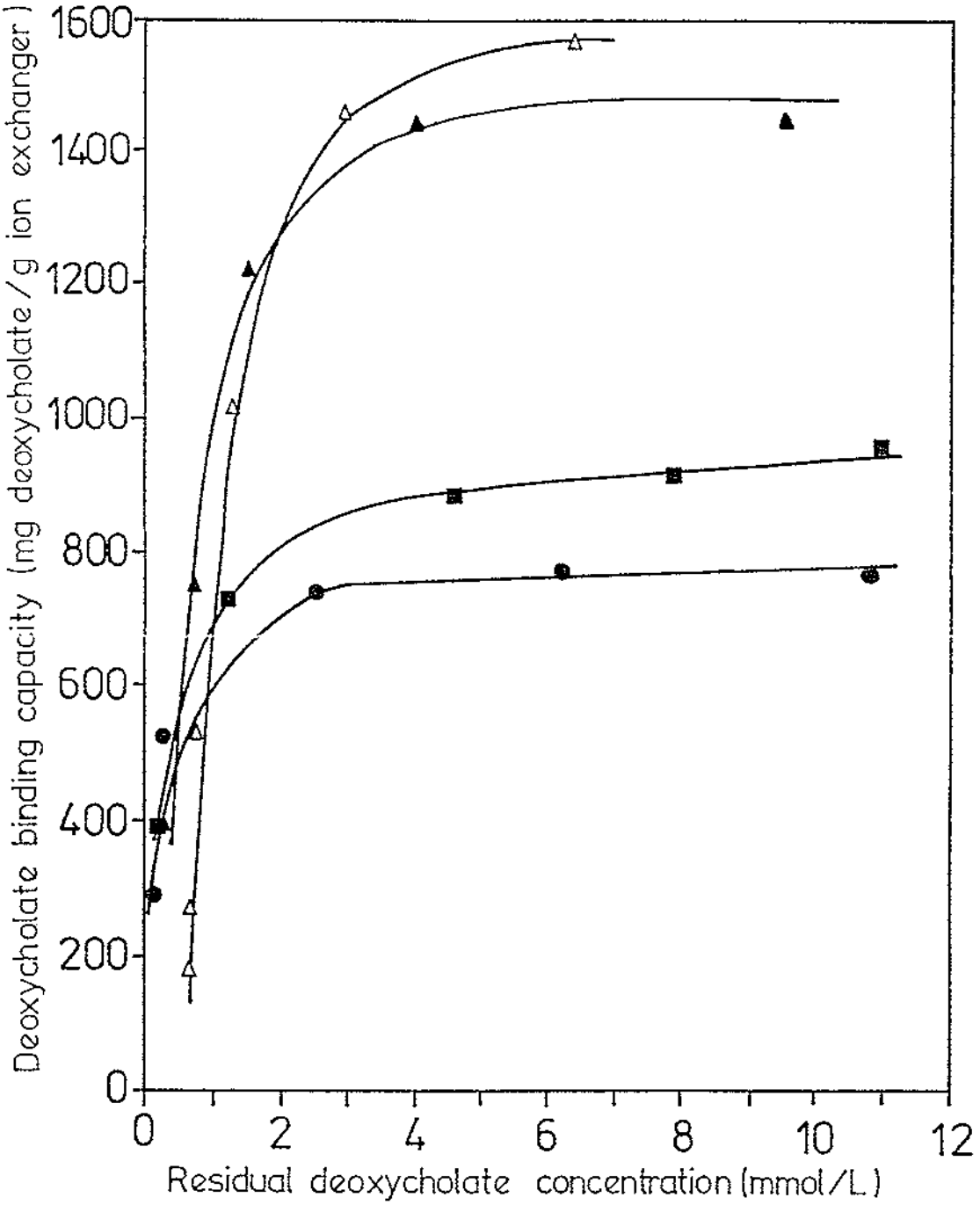
[b] Corresponds to the number of charged nitrogens at pH 8.0 per gram of colestipol (Section 4.10)

6.3 DEOXYCHOLATE CAPACITY TESTS

It has been demonstrated in vitro that ion exchangers are usually more effective at binding one bile acid anion than another[16,44,45]. For this reason it was decided to measure the deoxycholate capacity of cholestyramine, colestipol and the octyl and dodecyl QA celluloses prepared from 10% cross-linked HP Indion. The method was the same as that used for the cholate capacity tests given earlier (Section 6.2). The deoxycholate capacity curves (mg/g and mg/mL) of the four ion exchangers are given in Figures 6.10 and 6.11.

The most striking feature of these Figures is the excellent capacity of colestipol and cholestyramine for deoxycholate, compared with cholate (Figures 6.8 and 6.9). Even at low concentrations the positively charged nitrogens bound deoxycholate effectively. The deoxycholate capacity curves for the octyl and dodecyl QA celluloses were similar to those obtained for cholate (Figures 6.8 and 6.9), except that the capacities were greater for deoxycholate. The large capacities of the synthetic ion exchangers (Figure 6.10) reflect their very high nitrogen substitution levels (Table 6.2). However on a volume

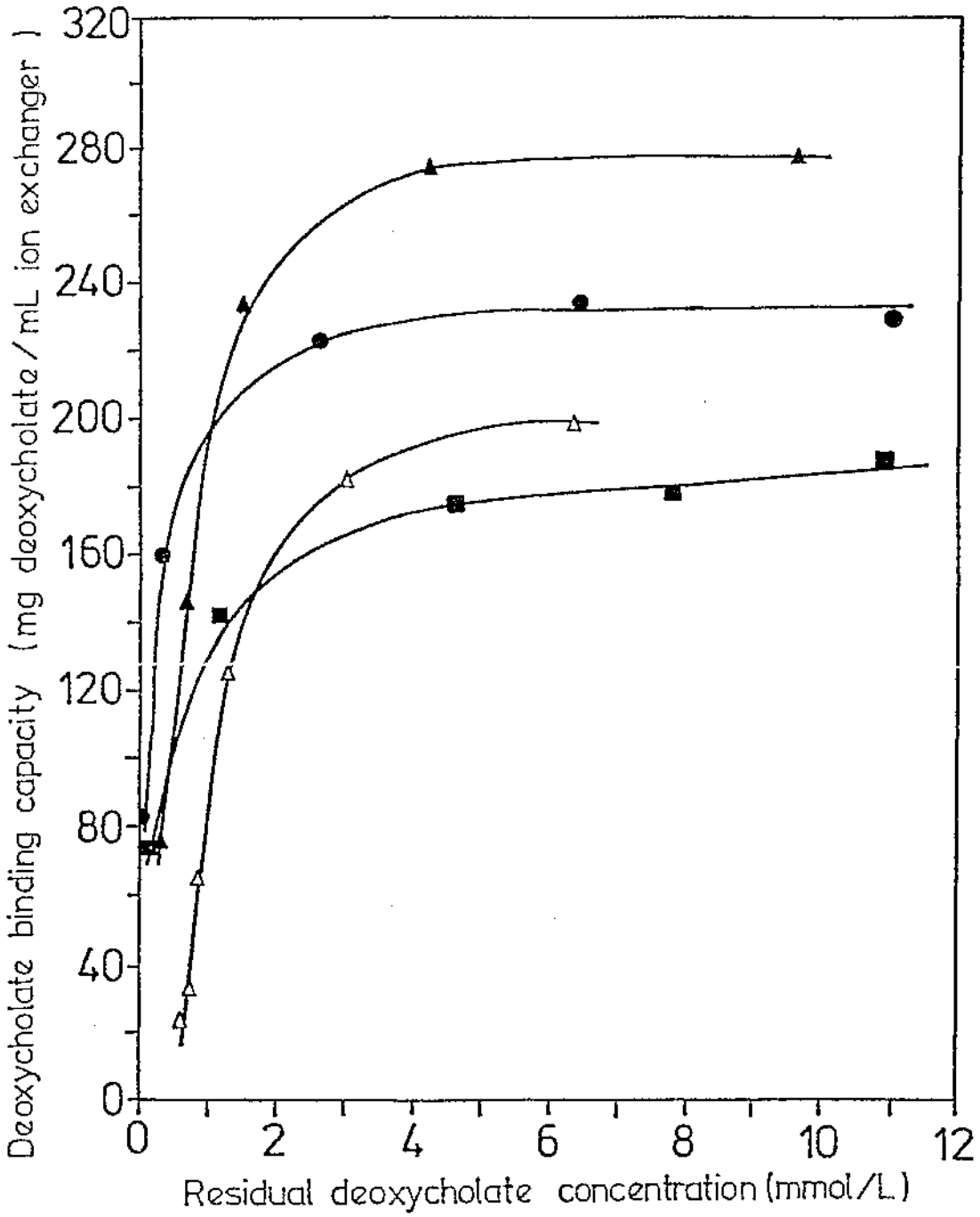
Figure 6.10 Deoxycholate Capacity Curves (mg/g) for Octyl and Dodecyl QA Celluloses, Cholestyramine and Colestipol



▲ cholestyramine (3.81 meq/g)
△ colestipol (4.03 meq/g)
● double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 109, 1.50 meq/g)
■ double processed, 10% cross-linked, octyl QA cellulose (DMO 14, 1.70 meq/g)

Conditions: small samples of each ion exchanger were mixed with 15 mmol/L sodium deoxycholate in BBS (20 mL) at pH 8.0 for 25 hours at room temperature

Figure 6.11 Deoxycholate Capacity Curves (mg/mL) for Octyl and Dodecyl QA Celluloses, Cholestyramine and Colestipol



▲ cholestyramine (5.2 mL/g)
△ colestipol (8.0 mL/g)
● double processed, 10% cross-linked, dodecyl QA cellulose (DMDo 109, 3.3 mL/g)
■ double processed, 10% cross-linked, octyl QA cellulose (DMO 14, 5.1 mL/g)

Conditions: as for Figure 6.10

basis (Figure 6.11) the capacity of the dodecyl QA cellulose to bind deoxycholate was between those of cholestyramine and colestipol, except at very low deoxycholate concentrations (less than 1 mmol/L) where it outperformed both of them.

The efficiencies of the four ion exchangers for binding deoxycholate are summarised in Table 6.2. This shows that all of the ion exchangers had a greater binding efficiency for deoxycholate than cholate (Table 6.1). The greatest improvements were for cholestyramine and colestipol which bound deoxycholate with close to 100% efficiency. Both of the cellulose ion exchangers bound deoxycholate at about 130-140% efficiency. (No attempt was made to determine the nature of this greater than one to one binding, or whether or not it was reversible.)

TABLE 6.2 Efficiency of Ion Exchangers for Binding Deoxycholate Against a Background of 150 mmol/L Sodium Chloride at pH 8.0

Ion Exchanger	Substitution Level (meq/g)	Efficiency at 5 mmol/L (%)	Efficiency at 10 mmol/L (%)
Dodecyl QA Cellulose	1.50	129	129
Octyl QA Cellulose	1.70	132	138
Cholestyramine	3.81[a]	97	99
Colestipol	4.03[b]	98	-

[a] Section 4.10

[b] Corresponds to the number of charged nitrogens at pH 8.0 per gram of colestipol (Section 4.10)

The deoxycholate curves in Figures 6.10 and 6.11 did not flatter the cellulose ion exchangers as much as the results obtained for cholate in Figures 6.8 and 6.9. However the cellulose ion exchangers still bound deoxycholate well and showed greater capacities at very low concentrations. Also, the ratio of deoxycholate to cholate is only about 30% in

human bile[12] and therefore the results obtained here for deoxycholate may be less important than those obtained previously for cholate (Section 6.2.5). The fact that the absence of one hydroxyl group in deoxycholate (a dihydroxy bile acid) compared to cholate (a trihydroxy bile acid) can make such a big difference to the relative ability of an ion exchanger to bind them efficiently had important implications. For example, it was necessary to try and duplicate the composition of human bile as closely as possible when developing a more sensitive test for ion exchangers in vitro, in order to get a more accurate assessment of whether improvements made to the ion exchangers were likely to have favourable effects in vivo. Such a test is outlined in Section 6.4.

6.4 BINDING STUDIES WITH CONJUGATE MIXTURES

About 7% of human bile is composed of a mixture of bile acids. These bile acids are present as sodium or potassium salts, and they are usually conjugated with either glycine or taurine[12]. Typical concentrations of the major bile salts found in human duodenal fluid are given in Table 6.3[16]. Studies have shown that ion exchangers have a different affinity for different bile acid anions in duodenal fluid and the overall effectiveness of an ion exchanger often depended on its ability to bind glycocholate, which was the least strongly bound[16]. These studies also indicated that a mixture of bile salts with a total concentration of about 7 mmol/L in an appropriate buffer would serve equally well as duodenal fluid to test the effectiveness of ion exchangers in vitro.

TABLE 6.3 Concentration of Bile Acids in Human Duodenal Fluid[16]

Bile Acid	Concentration (±S.D.[a])
Taurocholic acid	0.91 ± 0.02[b]
Taurodeoxycholic acid and Taurochenodeoxycholic acid	1.48 ± 0.04[c]
Glycocholic Acid	1.46 ± 0.03[b]
Glycodeoxycholic acid and Glycochenodeoxycholic acid	2.52 ± 0.22[b]

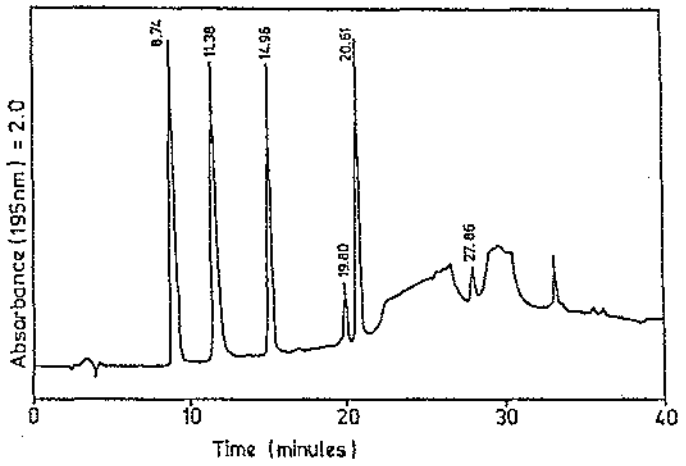
[a] Standard Deviation, [b] N = 4, [c] N = 3

6.4.1 Development of HPLC Method for Determination of Conjugated Bile Acids

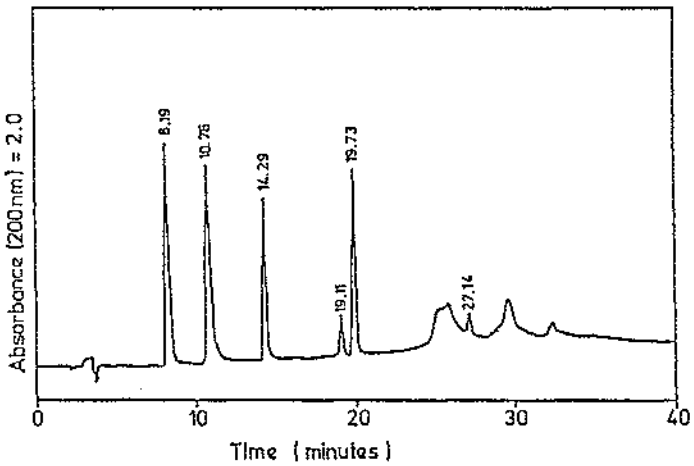
It was found that bile acid conjugates could be separated by reverse phase, linear gradient HPLC. Details of the optimum conditions for separation, and the hardware used, are given in Section 2.8.2a. Three different wavelengths for detecting the bile acids were initially investigated. These were 195, 200 and 205 nm, and the elution patterns produced are shown in Figures 6.12A, B and C respectively. In each case a solution (100 µL) containing taurocholate (TC) (50 µg), taurodeoxycholate (TDC) (50 µg), glycocholate (GC) (50 µg), and glycodeoxycholate (GDC) (50 µg) was injected. The sensitivity of detection of the bile acids increased at shorter wavelengths, but at 195 nm the interference from additional peaks, resulting from solvent impurities, became unacceptable. Therefore 200 nm was the most favourable wavelength to use. Figure 6.12D shows the elution pattern obtained at this wavelength when 100 µL of water was injected. The noise is well clear of the first 20 minutes which is needed to analyse the first four large peaks in Figure 6.12B, corresponding to the conjugated bile acids. Each of these was identified by injecting a mixture of the four conjugated bile acids (10 µg), and further samples in which the mixture was spiked with 10 µg of each bile acid in turn. The results and assignments of the peaks are shown in Table 6.4.

Figure 6.12 Detection of Bile Acid Conjugates by UV Absorption

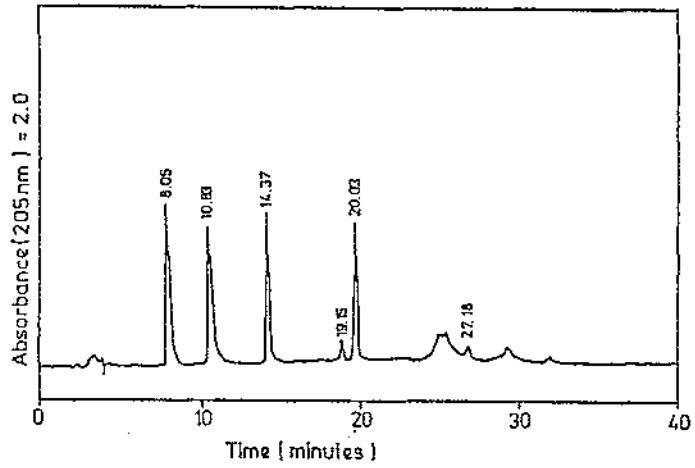
6.12A Detection of Bile Acids at 195 nm



6.12B Detection of Bile Acids at 200 nm



6.12C Detection of Bile Acids at 205 nm



6.12D Blank at 200 nm

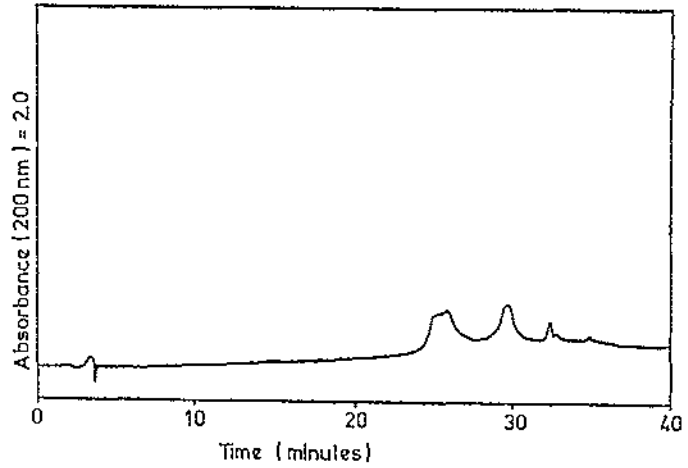


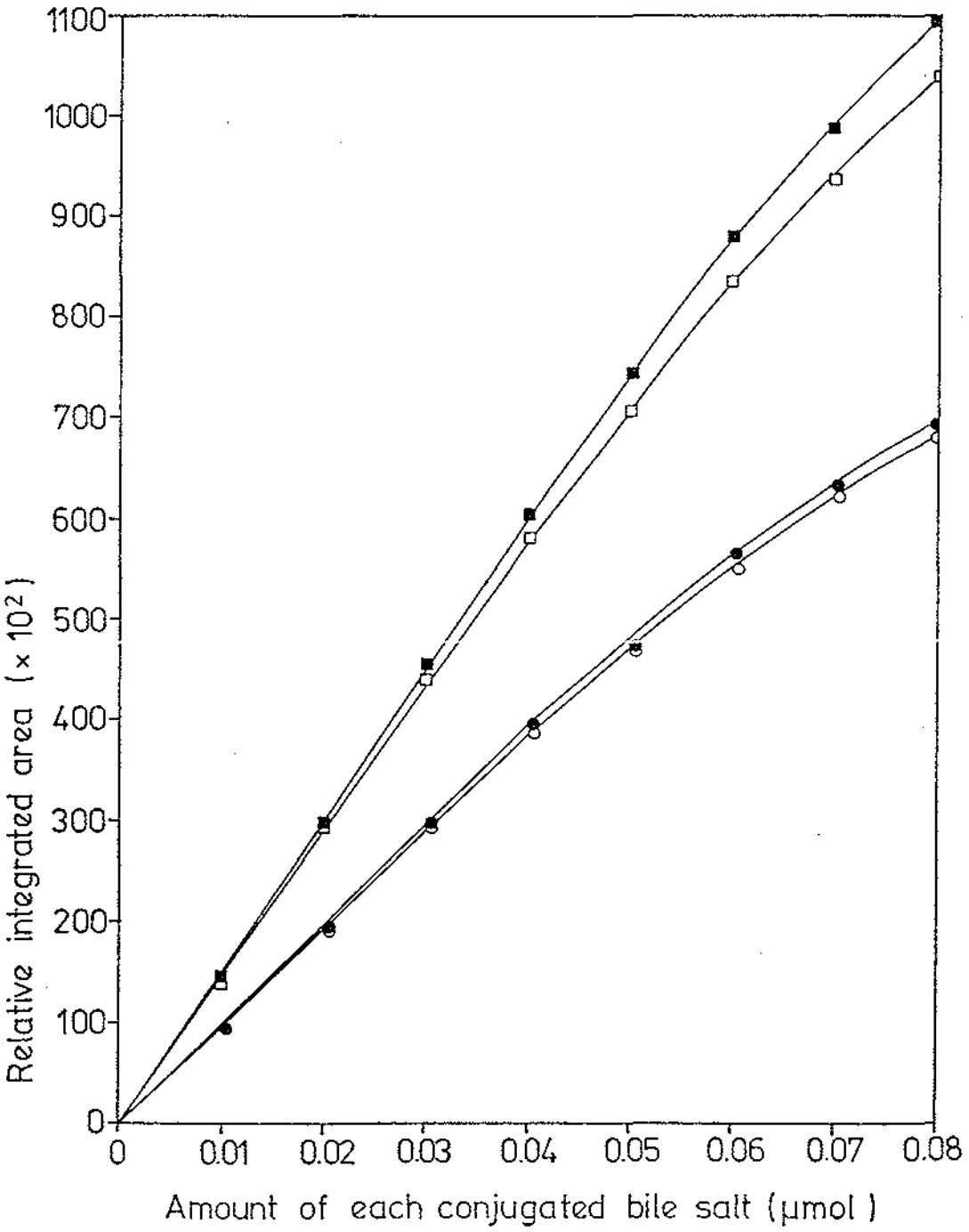
TABLE 6.4 Identification of Conjugated Bile Acids by HPLC

Run Number	Spike (+10 µg)	Relative Integrated Area of Peaks Average Retention Time of Peaks (Minutes) 9.38	11.87	15.18	20.78
1	-	268	284	194	206
2	TC	**515**	282	195	211
3	TDC	273	**559**	197	208
4	GC	269	282	**378**	205
5	GDC	265	279	195	**400**

Runs 1 to 5 in Table 6.4 were conducted without using an internal standard, but in spite of this the results were very reproducible. For example the peaks at 9.38 minutes in Run 1,3,4 and 5 had a mean area of 269 units, and the maximum deviation of any peak area from this mean was less than ±1.5%. Similar reproducibilities were obtained for the three other peaks. In light of this it was decided not to use an internal standard. A working curve was constructed from 0.00 to 0.08 µmoles of TC, TDC, GC and GDC (Figure 6.13). Beers Law was obeyed for up to 0.05 µmoles of each conjugate, but with higher amounts a negative deviation from Beers Law occurred.

Refrigerated solutions were found to be stable for up to five days. The peak areas showed little variation indicating that hydrolysis of the conjugates was negligable (Table 6.5).

Figure 6.13 Working Curve for Bile Acid Conjugates



□ taurocholate
■ taurodeoxycholate
○ glycocholate
● glycodeoxycholate

Conditions: Section 2.8.2a

TABLE 6.5 Stability of Refrigerated Standards Containing 0.05 µmoles of Each Conjugate per 100 µL

	Relative Integrated Area of Peaks Average Retention Time of Peaks (Minutes)			
Day	9.38	11.87	15.18	20.78
1	709	746	470	471
3	702	731	459	462
4	690	714	448	442
5	720	744	469	478
Error(%)[a]	±2.1	±2.7	±3.0	±4.5

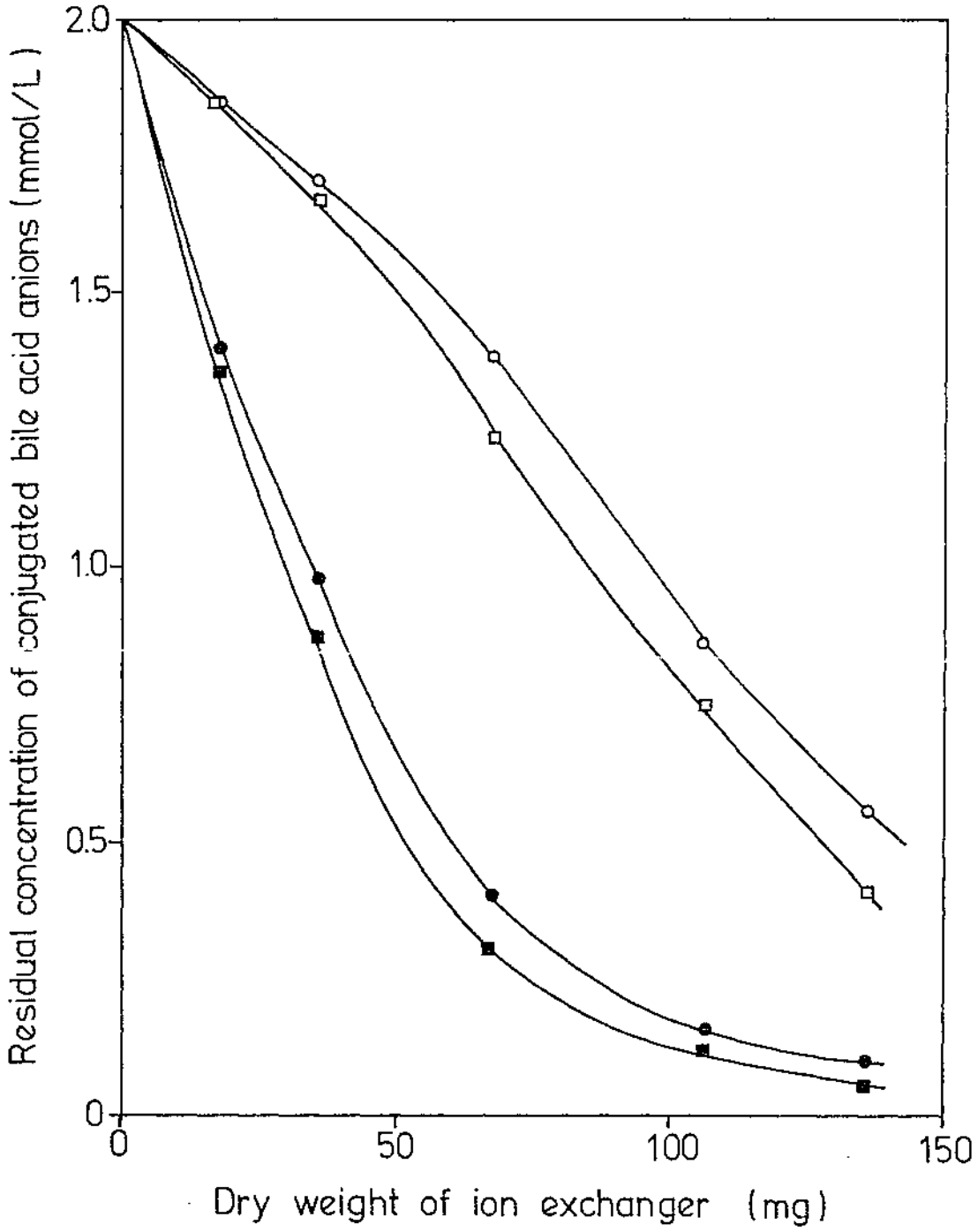
[a] Maximum deviation of any peak area from the mean of the values above

6.4.2 Measurement of Capacity for Binding Conjugated Bile Acid Anions

It was decided to measure the effectiveness of several alkyl QA cellulose ion exchangers for binding conjugated bile acid anions present in a mixture and compare them with cholestyramine and colestipol. The mixture used contained 2 mmol/L of the sodium salts of glycocholate, glycodeoxycholate, taurocholate, and taurodeoxycholate in Bicarbonate Buffer System (BBS) at pH 8.0. (BBS provided a background of chloride ions seventy five times greater than the concentration of individual bile acid anions, to compete for binding sites on the ion exchangers.) Samples of the various ion exchangers (equilibrated in BBS), with predetermined dry matters, were mixed for 25 hours with 20 mL of the conjugate mixture (Section 2.8.2). The residual concentration of each conjugate was determined by HPLC (Section 2.8.2a). These concentrations were then plotted against the dry matter of the ion exchanger used.

A typical pattern of results is shown in Figure 6.14. This shows the effectiveness of a single processed dodecyl QA cellulose, prepared on 10% cross-linked HP Indion (DMDo 72),

Figure 6.14 Conjugated Bile Acid Binding to Single Processed Dodecyl QA Cellulose (DMDo 72, 1.08 meq/g)



○ glycocholate
□ taurocholate
● glycodeoxycholate
■ taurodeoxycholate

Conditions: small amounts of ion exchanger were equilibrated in 8.0 mmol/L conjugate solution (GC:TC:GDC:TDC 1:1:1:1) in BBS (20 mL) at pH 8.0 for 25 hours at room temperature

for binding conjugated bile acid anions. Two points of interest emerge. There was a marked preference for the glyco- and tauro-conjugates of deoxycholate, compared to the conjugates of cholate. There was also a slight preference for the adsorbtion of the tauro-conjugates compared with the glyco-conjugates in the case of both deoxycholate and cholate. Thus the order of binding was TDC > GDC > TC > GC, the same as that reported for a number of ion exchangers[16]. This order was found for all of the QA cellulose products reported here, as well as cholestyramine and colestipol (Figures 6.14 to 6.18, 6.20 and 6.21).

6.4.3 The Effect on Capacity of Reprocessing Dodecyl QA Cellulose

Reprocessing dodecyl QA cellulose lifted the number of charged nitrogen groups from 1.0-1.2 meq/g to 1.3-1.5 meq/g (Tables 5.1 and 5.2), but preliminary capacity tests with 15 mmol/L sodium cholate solutions did not reflect this increase (Figure 6.1). Since the cholate concentration used in the early tests was very high, the test was insensitive to differences in capacity between the ion exchangers in the 0-2 mmol/L range. Figure 6.15 shows the effectiveness of a double processed dodecyl QA cellulose, prepared on 10% cross-linked HP Indion (DMDo 109), with a mixture of conjugated bile salts. When compared with Figure 6.14 an improvement in performance can be seen over the single processed product. In particular the curves were steeper reflecting a higher capacity (on a dry matter basis) and with larger amounts of ion exchanger much lower concentrations of bile acid conjugates were reached. These differences are summarised in Table 6.6 and show the advantages obtained by reprocessing, even though the amine groups were not used quite as efficiently.

TABLE 6.6 Binding Characteristics of Single Processed and Double Processed Dodecyl QA Celluloses[a]

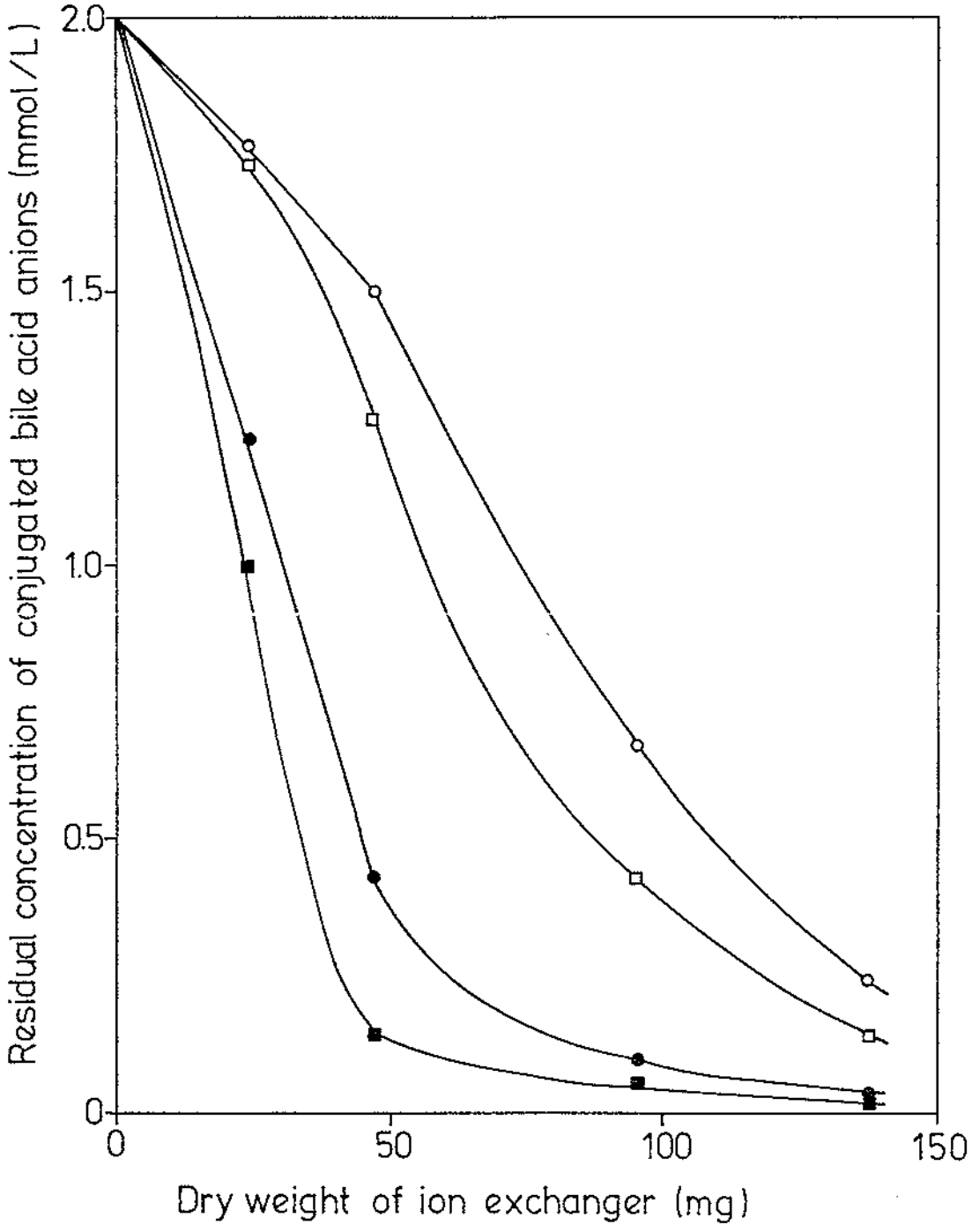
Ion Exchanger	Single Processed (DMDo 72)	Double Processed (DMDo 109)
Substitution Level (meq/g)	1.08	1.50
Dry Weight (mg)	100	100
Residual Concentration of All Bile Acids (mmol/L)	2.08	0.96
Residual Concentration of All Bile Acids (% of Loading)	26	12
Total Conjugates Bound (mmol)	0.118	0.141
Available Nitrogens (meq)	0.108	0.150
Efficiency (%)	109	94

[a] Effectivness of the ion exchangers for binding conjugated bile acid anions at the 100 mg dry weight point in Figures 6.14 and 6.15

6.4.4 Effect on Capacity of the Alkyl Group in the Alkyl QA Celluloses

The conjugate binding curves for the double processed dodecyl and octyl QA celluloses prepared on 10% cross-linked HP Indion, and butyl and methyl QA celluloses prepared on 15% cross-linked HP Indion are given in Figures 6.15 to 6.18. The 15% cross-linked HP Indion was used for the butyl and methyl QA celluloses because their swollen volumes were closer to those for the dodecyl and octyl QA celluloses than if they were all prepared on the 10% cross-linked matrix. From these curves it is obvious that the dodecyl and octyl QA celluloses worked much better than the butyl or methyl QA celluloses, demonstrating that alkyl groups larger than butyl are necessary to bind bile acid anions effectively, in agreement with previous results (Section 3.3.1).

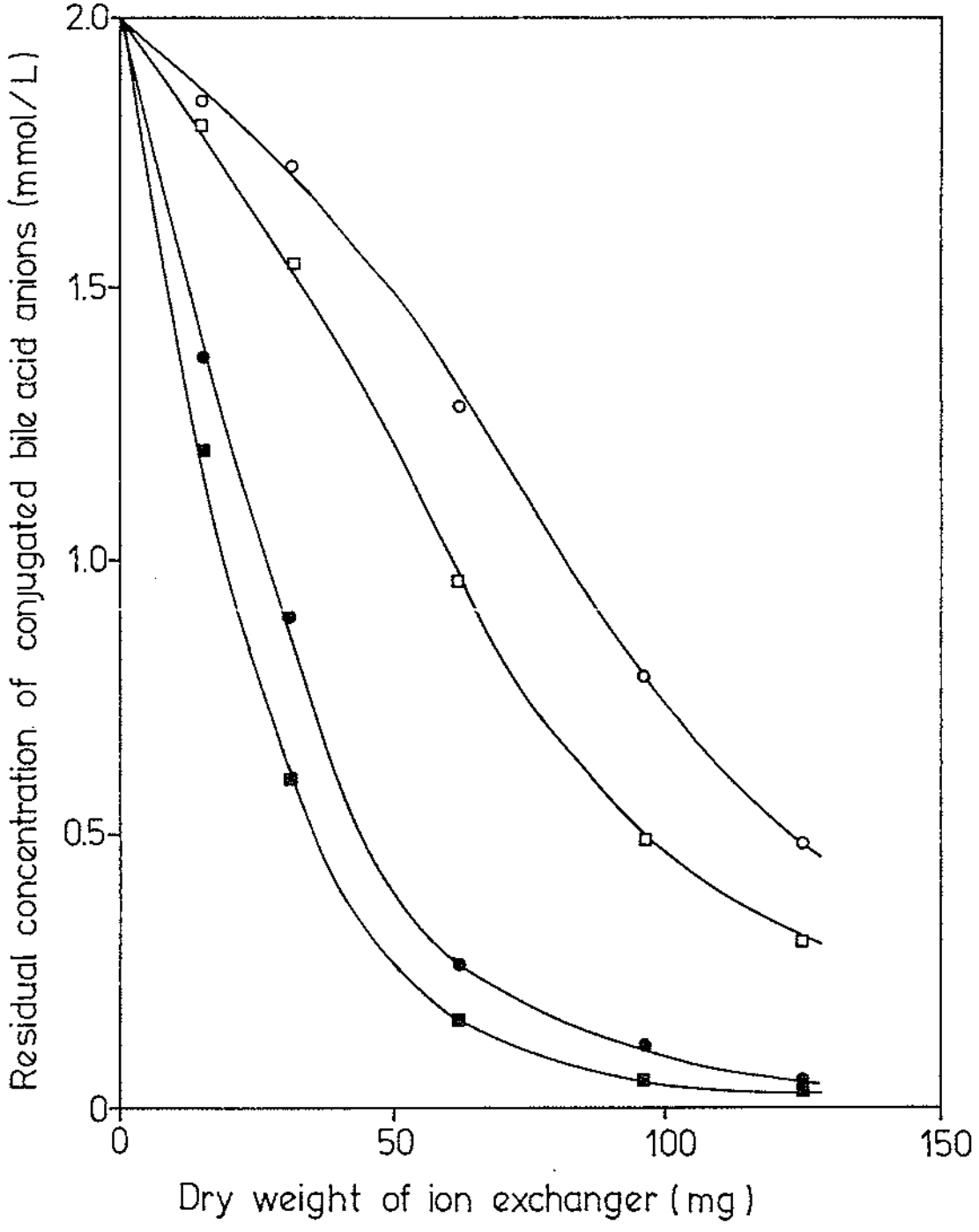
Figure 6.15 Conjugated Bile Acid Binding to Double Processed, 10% Cross-Linked, Dodecyl QA Cellulose (DMDo 109, 1.50 meq/g)



○ glycocholate
□ taurocholate
● glycodeoxycholate
■ taurodeoxycholate

Conditions: as for Figure 6.14

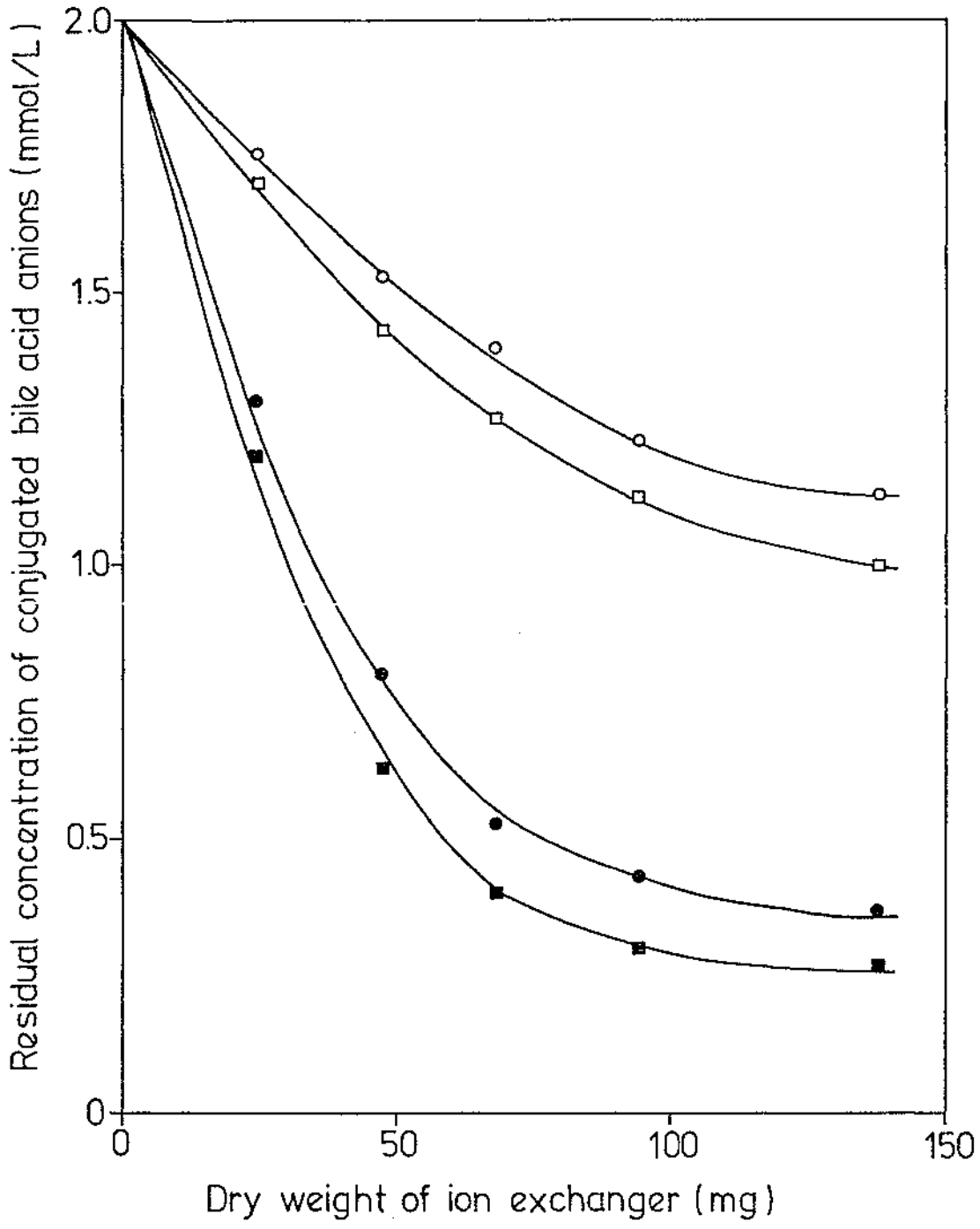
Figure 6.16 Conjugated Bile Acid Binding to Double Processed, 10% Cross-Linked, Octyl QA Cellulose (DMO 14, 1.70 meq/g)



○ glycocholate
□ taurocholate
● glycodeoxycholate
■ taurodeoxycholate

Conditions: as for Figure 6.14

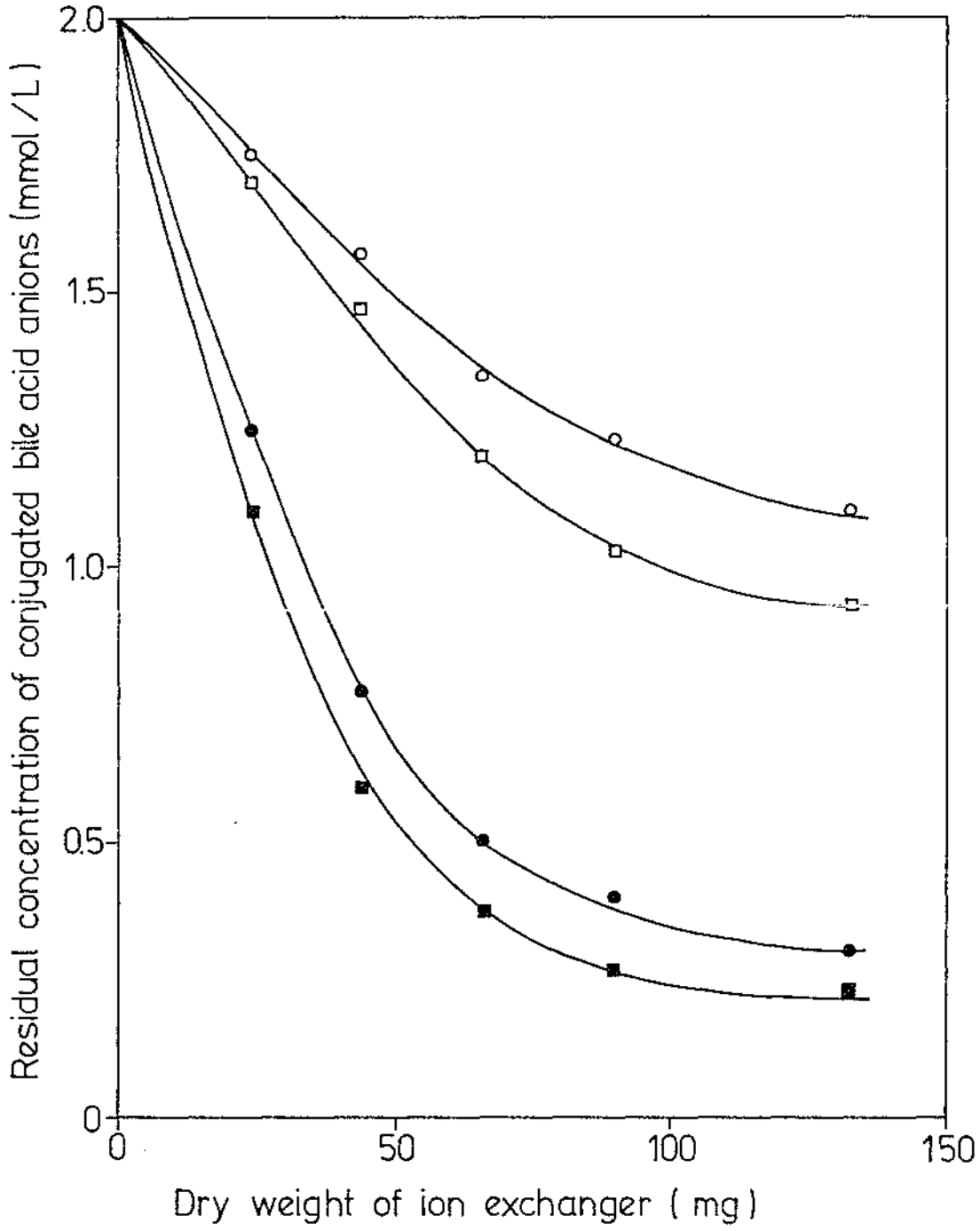
Figure 6.17 Conjugated Bile Acid Binding to Double Processed, 15% Cross-Linked, Butyl QA Cellulose (DMB 9, 1.68 meq/g)



○ glycocholate
□ taurocholate
● glycodeoxycholate
■ taurodeoxycholate

Conditions: as for Figure 6.14

Figure 6.18 Conjugated Bile Acid Binding to Double Processed, 15% Cross-Linked, Methyl QA Cellulose (TMA 33, 1.89 meq/g)



○ glycocholate
□ taurocholate
● glycodeoxycholate
■ taurodeoxycholate

Conditions: as for Figure 6.14

Table 6.7 and Figure 6.19 summarise the results for the four ion exchangers at the 100 mg dry weight point in Figures 6.15 to 6.18, and further emphasise the need for an alkyl group with greater than four carbons to be present on the amino substituted cellulose. This was in spite of the unfavourable trend of decreasing substitution levels as the alkyl groups increased in size. The slightly greater overall effectiveness for the methyl QA cellulose compared to the butyl QA cellulose, seen in Figure 6.19, was most likely to be a result of this greater nitrogen content.

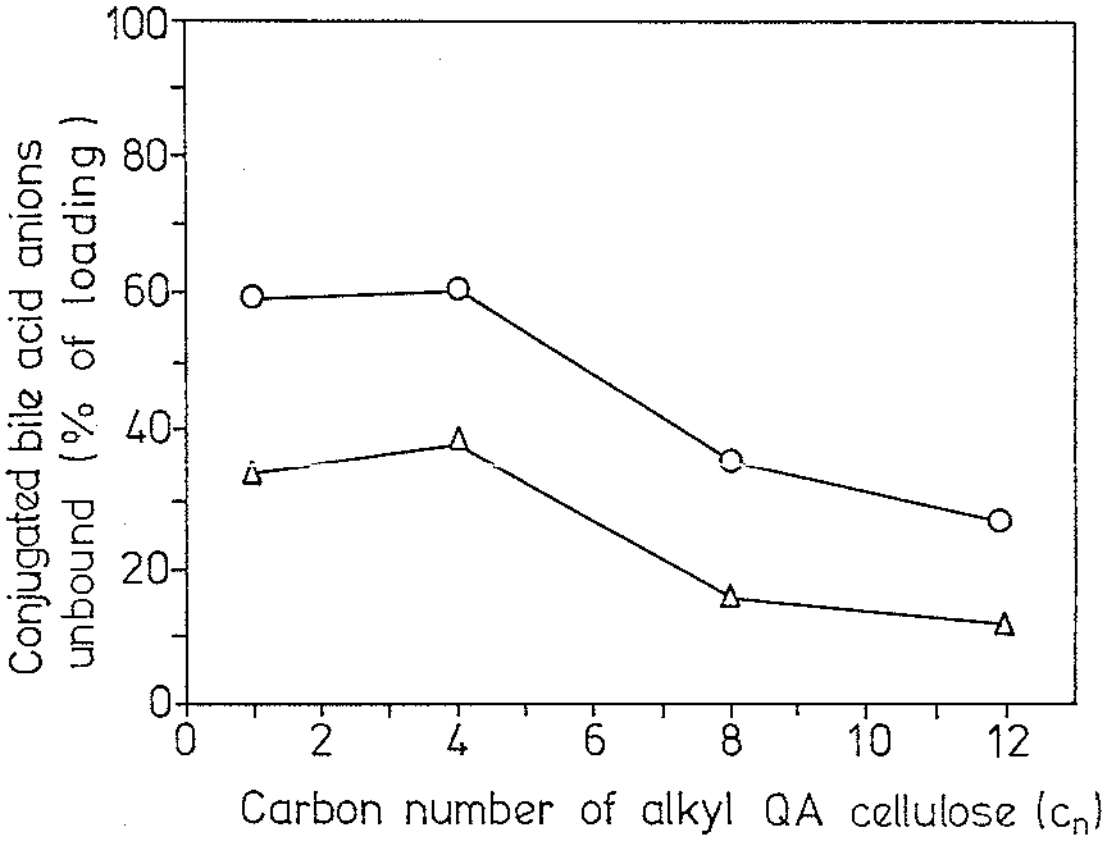
The effectiveness of the four alkyl QA celluloses for binding glycocholate is singled out in Table 6.7 and Figure 6.19 because this is reported to be the major bile acid present in humans[16]. It is also obviously the most difficult bile acid anion to bind in the face of competition from chloride ions and other bile salts. The most effective ion exchanger at binding glycocholate was dodecyl QA cellulose and if enough of it was used it bound almost all of the glycocholate present.

TABLE 6.7 Binding Characteristics of the Alkyl QA Celluloses[a]

Ion Exchanger	Dodecyl	Octyl	Butyl	Methyl
Substitution Level (meq/g)	1.50	1.70	1.68	1.89
Dry Weight (mg)	100	100	100	100
Residual Concentration of All Bile Acids (mmol/L)	0.96	1.27	3.02	2.79
Total Conjugate Concentration of All Bile Acids (% of Loading)	12	16	38	34
Residual Concentration of Glycocholate (mmol/L)	0.54	0.72	1.20	1.18
Residual Concentration of Glycocholate (% of Loading)	27	36	60	59

[a] See Footnotes for Table 6.6 and Figures 6.15 to 6.18

Figure 6.19 Binding Characteristics of Alkyl QA Celluloses



○ % of glycocholate remaining in solution at end of test
Δ % of total bile salts remaining in solution at end of test

Effectiveness of methyl QA cellulose (n=1), butyl QA cellulose (n=4), octyl QA cellulose (n=8) and dodecyl QA cellulose (n=12) at reducing the concentration of conjugated bile acid anions at the 100 mg dry weight point in Figures 6.15 to 6.18 respectively

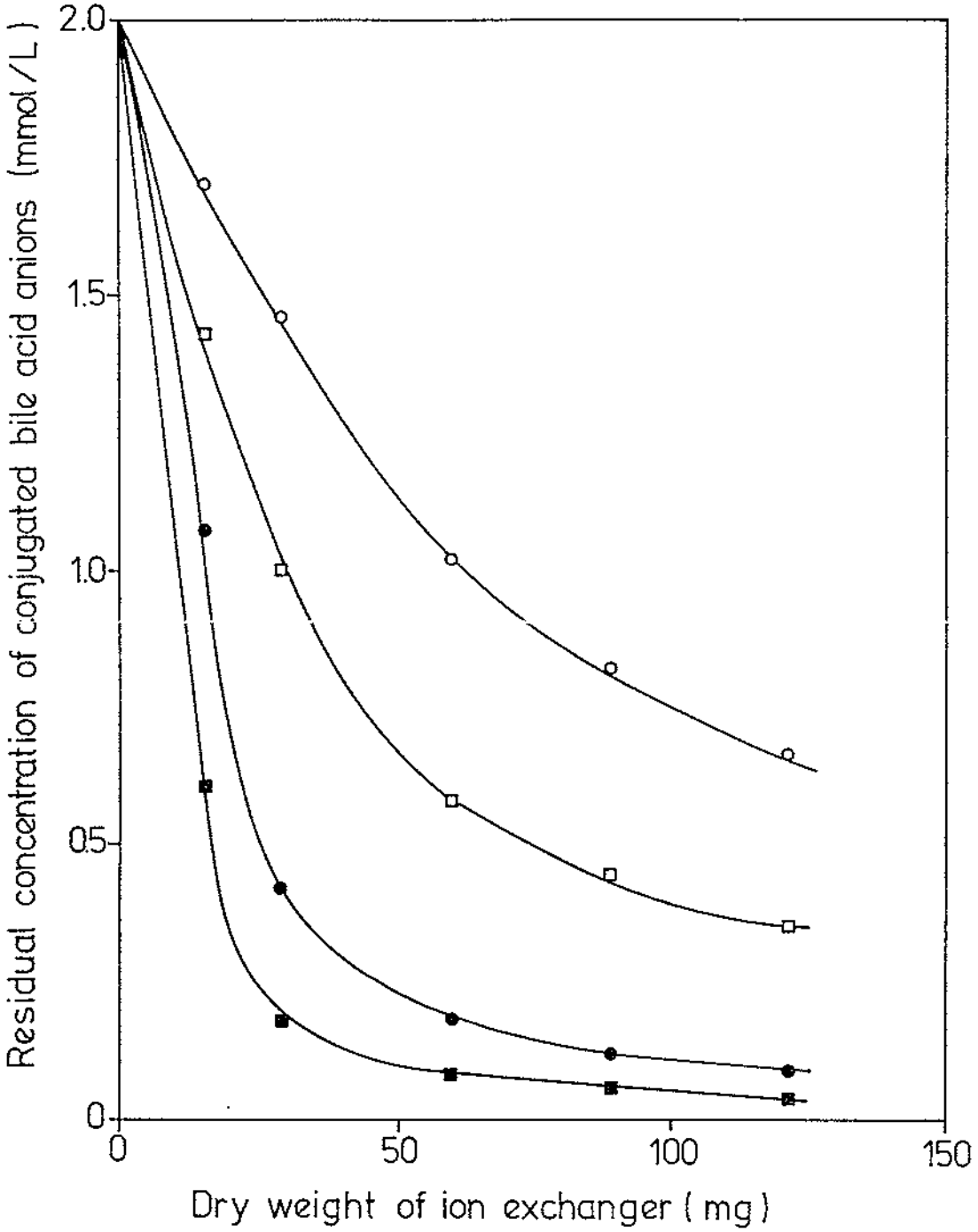
6.4.5 Cholestyramine and Colestipol

The conjugate binding curves for cholestyramine and colestipol are given in Figure 6.20 and 6.21 respectively. The order of binding of these ion exchangers was the same as for the cellulose ion exchangers - TDC > GDC > TC > GC. Hagerman et al. also found that the absolute capacity of cholestyramine for these conjugated bile salts in isotonic saline decreased in this order[45].

From a comparison of Figure 6.20 and 6.15 it would appear that cholestyramine was more effective than dodecyl QA cellulose when small amounts of ion exchanger were used, because the curves in Figure 6.20 are much steeper at loadings of 0-50 mg of ion exchanger. However, at higher loadings the dodecyl QA cellulose was the more effective. This difference resulted from the higher exchange capacity of the cholestyramine, but less specific nature of the binding compared with the dodecylated quaternary nitrogen groups on the cellulose ion exchanger.

The colestipol curves (Figure 6.21) were also very steep initially but levelled out at much higher residual bile salt concentrations than either cholestyramine or dodecyl QA cellulose. It was particularly ineffective at binding glycocholate and taurocholate. This is very similar to the methyl QA cellulose (Figure 6.18), which is not surprising because both of these ion exchangers have only hydrophilic groups in, and on, the matrix. Cholestyramine on the other hand has benzene rings in its polystyrene matrix, which gives it considerable hydrophobic character. The differences between the binding for cholestyramine, colestipol, methyl and dodecyl QA cellulose are summarised in Table 6.8.

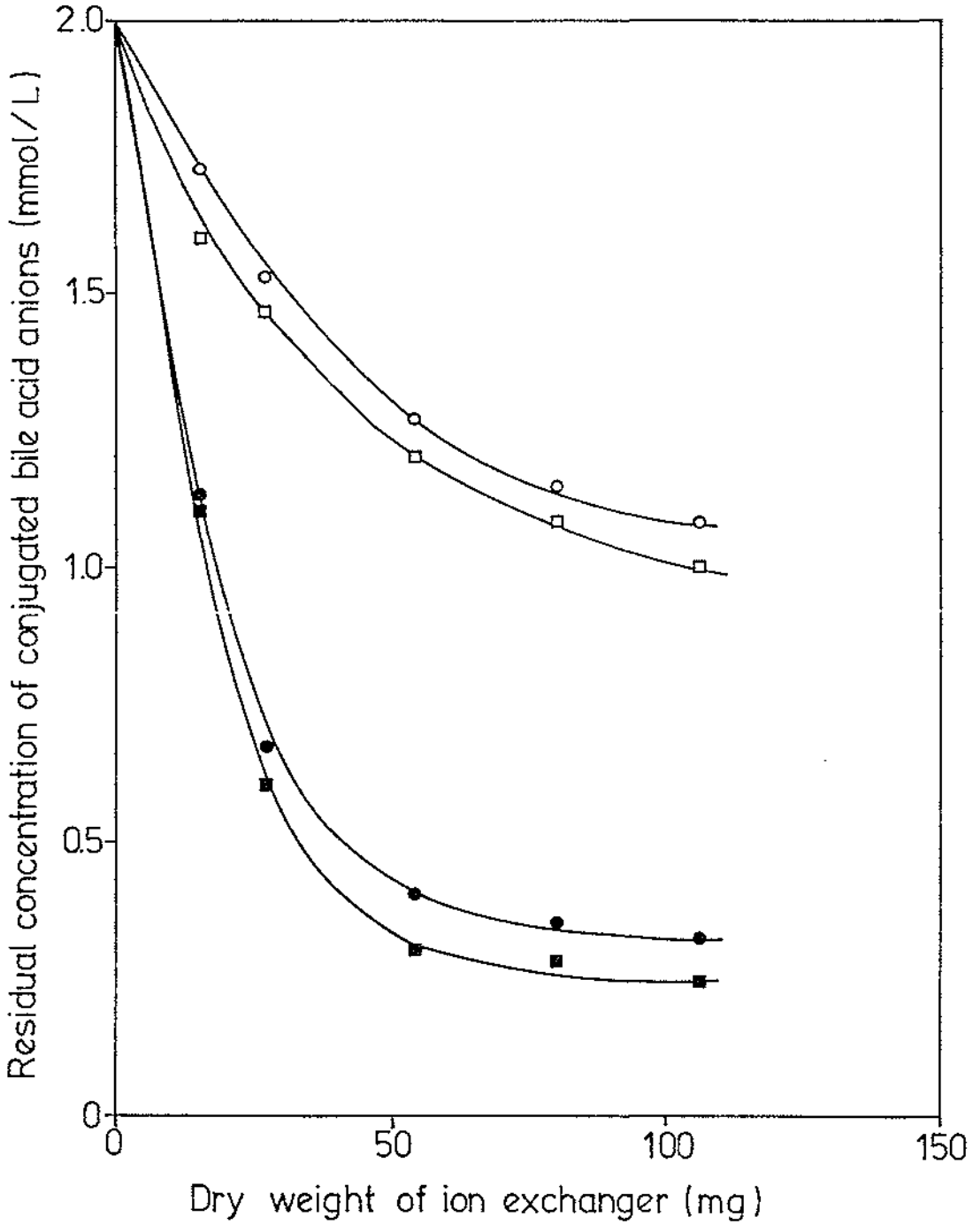
Figure 6.20 Conjugated Bile Acid Binding to Cholestyramine



○ glycocholate
□ taurocholate
● glycodeoxycholate
■ taurodeoxycholate

Conditions: as for Figure 6.14

Figure 6.21 Conjugated Bile Acid Binding to Colestipol



○ glycocholate
□ taurocholate
● glycodeoxycholate
■ taurodeoxycholate

Conditions: as for Figure 6.14

TABLE 6.8 Comparison of Methyl and Dodecyl QA Celluloses, Cholestyramine and Colestipol[a]

Ion Exchanger	Dodecyl QA Cellulose	Cholestyr-amine	Colestipol	Methyl QA Cellulose
Substitution level (meq/g)	1.50	3.81	4.03[b]	1.89
Part A: 50 mg ion exchanger				
Residual Conc.(mmol/L) of All Bile Acids	3.38	2.14	3.28	4.10
Residual Conc. of All Bile Acids (% of Loading)	42	27	41	51
Total Conjugates Bound (mmol)	0.092	0.117	0.124	0.078
Available Nitrogens (meq)	0.075	0.191	0.202	0.095
Efficiency (%)	122	61	47	82
Part B: 100 mg ion exchanger				
Residual Conc.(mmol/L) of All Bile Acids	0.96	1.31	2.68	2.79
Residual Conc. of All Bile Acids (% of Loading)	12	16	33	34
Total Conjugates Bound (mmol)	0.141	0.134	0.106	0.104
Available Nitrogens (meq)	0.150	0.381	0.403	0.189
Efficiency (%)	94	35	26	55

[a] Conditions: as in the Footnote to Table 6.6, but at 50 and 100 mg dry weight points in Figures 6.15, 6.18, 6.20 and 6.21

[b] Corresponds to the number of charged nitrogens at pH 8.0 per gram of colestipol (Section 4.10)

6.5 CONCLUSION

Quaternary amino celluloses have been prepared using improved synthetic schemes, and shown to be effective at binding conjugated bile acid anions in vitro, under conditions likely to be encountered in the human intestinal tract (i.e. at concentrations less than 2 mmol/L against a background of 150 mmol/L sodium chloride at pH 8.0). Methyl and butyl QA celluloses were unable to substantially reduce the concentration of the conjugated bile acid anions. Octyl QA cellulose was considerably better than the short chain alkyl QA celluloses, demonstrating that alkyl groups with greater than four carbon atoms are necessary to be effective. Dodecyl QA cellulose was shown to be particularly effective and this effectiveness was greatly enhanced if the ion exchanger was double porcessed. It was slightly better than cholestyramine even though it had only 40% of the exchange capacity for small ions. Colestipol did not work nearly as well as dodecyl QA cellulose or cholestyramine in the batch capacity tests involving conjugate mixtures.

These findings were confirmed by in vivo studies on 6-18 week old cockerels, when the ion exchangers were fed at a dose rate of 1% by weight of their diet[43]. The blood serum cholesterol level reductions obtained after 4 days are summarised in Table 6.9. Cholestyramine and double processed dodecyl QA cellulose both reduced the serum cholesterol by a significant amount. Although it was hoped to produce a product with superior performance than those already commercially available, the results obtained here were very encouraging and suggested that further work should be carried out to develop alkyl QA celluloses for use as bile acid sequestrants. The result for colestipol was surprising, but it was in keeping with the results obtained in vitro using bile acid conjugates. In both systems colestipol worked only as well as the single processed dodecyl QA cellulose, which indicates that the in vitro test developed gives a good indication of the likely performance of

these ion exchangers in vivo. However a true measure of the hypocholesterolemic activity of these ion exchangers must eventually be obtained by careful clinical trials.

TABLE 6.9 The Effect of Ion Exchangers on Serum Cholesterol Concentrations in Cockerels[a]

Ion Exchanger	Reduction of Serum Cholesterol
Cholestyramine (Questran^{R})	-17%
Dodecyl QA Cellulose	
Single Processed	-6%
Double Processed	-14%, -17%, -17%, -19%
Colestipol (Colestid^{R})	-4%

[a] For 6-18 week old cockerels, at a dose rate of 1% by weight of the diet after 4 days[43]

APPENDIX I Preparation and Properties of the Ion Exchangers Discussed in Chapters 5 and 6

Properties			Starting Materials				Activation[a]			Coupling[a]			
Ion Exchanger (Code)	Substitution Level (meq/g)	Swollen Volume (mL/g)	Amine $N(CH_3)_2R$ (R)	Starting Matrix (Indion)	Cross-Linking (%)	Dry Matter (mg/g)	Vol./Conc. NaOH (mL/% aq)	Volume ECH[b] (mL)	Time (4-6°C) (h)	Volume Amine (mL)	Volume HCl[c] (mL)	Vol./Conc. Ethanol (mL/% aq)	Time/ Temp. (h/°C)
IA Single Processed Ion Exchangers													
TMA 32	1.25	8.6	Methyl	HP5	15	163	120/6	18	48	16.5[d]	[d]	50/0	24/RT
DMB 7	1.10	8.4	Butyl	HP5	15	163	120/6	18	48	6 g[e]	[e]	64/50	[f]
DMO 15	1.07	8.0	Octyl	HP5	15	163	120/6	18	48	15	3.15	47.5/100	[f]
DMO 12	1.20	9.9	Octyl	HP7[g]	10	134	150/6	23	48	15	3.15	47.5/100	[f]
DMDo 72	1.08	8.2	Dodecyl	HP5	15	163	120/6	18	48	15	2.38	47.5/100	[f]
DMDo 107	1.14	8.0	Dodecyl	HP7[g]	10	134	150/6	23	48	15	2.38	47.5/100	[f]
IB Double Processed Ion Exchangers													
TMA 33	1.89	7.2	Methyl	TMA 32	15	169	100/6	15	24	16.5[d]	[d]	50/0	24/RT
DMB 9	1.68	6.5	Butyl	DMB 7	15	187	100/6	15	24	8 g[e]	[e]	84/50	[f]
DMO 17	1.59	4.9	Octyl	DMO 15	15	189	100/6	15	24	15	3.15	47.5/100	[f]
DMO 14	1.70	5.1	Octyl	DMO 12	10	141	125/6	19	24	15	3.15	47.5/100	[f]
DMDo 95	1.37	3.3	Dodecyl	DMDo 72	15	286	100/6	15	24	15	2.38	47.5/100	[f]
DMDo 109	1.50	3.3	Dodecyl	DMDo 107	10	258	125/6	19	24	15	2.38	47.5/100	[f]
IC 'Special' Ion Exchangers													
DMDo 101	1.70	3.8	Methyl	DMDo 72	15	286	100/6	15	24	16.5[d]	[d]	50/0	48/RT
DMDo 112	1.42[h]	3.2	n-Dodecyl	DMDo 72	15	286	100/6	15	24	10.2 g	2.38	47.5/100	[f]
DMO 25	1.98	3.8	Octyl	DMO 14	10	191	104/6	15	24	15	3.15	47.5/100	[f]

APPENDIX I - Footnotes

[a] Amount used per 10 g (dry matter) of the starting matrix
[b] Epichlorohydrin
[c] Concentrated (11.6 mol/L) hydrochloric acid
[d] 32% aqueous trimethylamine coupled in water without addition of hydrochloric acid
[e] Dimethylbutylamine hydrobromide titrated to pH 10.1 with 1.00 mol/L hydrochloric acid, to which was added an identical volume of absolute ethanol to give a 50% aqueous solution
[f] Coupling was for 12-18 hours at Room Temperature, 10 hours at 70°C and the ion exchanger was cooled overnight (12-15 hours) at room temperature
[g] Batch Number 1259
[h] Titrated to pH 3.0 with 1.00 mol/L hydrochloric acid

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