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CROSS-LINKED HYDROXYPROPYLATED CELLULOSE GEL FOR CHROMATOGRAPHY

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SUMMARY

A series of insoluble cross-linked hydroxypropylated celluloses (HP-Regcell) were prepared by reacting ground regenerated cellulose with epichlorohydrin and propylene oxide in the presence of strong aqueous sodium hydroxide at a temperature of 40°C or higher. The products had gel-like properties with pore sizes up to about 110 Å and were useful as column packings for gel chromatography. They were also used to prepare ion-exchange derivatives with excellent capacities for protein adsorption.

INTRODUCTION

Cellulose has been widely used as a chromatographic material for the ion-exchange separation of proteins since Sober and Peterson introduced it in 1954[1]. Various forms of cellulose have since become available, including spherical beads, but cellulose ion exchangers have not been widely used in large industrial applications for protein purification for reasons of limited life, poor flow-rates attainable when packed in large columns or high cost.

Several attempts have been made to use modified cellulose packings. Regenerated cellulose was reported as early as 1962[2]. The tough, rigid nature of cellulose when regenerated in an insoluble form from solution should have lent it to industrial use, yet it has met with only limited success[3,4]. The main problem has been the relatively high density of the regenerated cellulose powders produced by the standard viscose-rayon process and the consequent low porosity. This has precluded their use as a separating medium for gel chromatography and limited their utility for the synthesis of ion-exchange derivatives[5,6].

The low porosity of the "viscose"-regenerated cellulose results from shrinkage of the cellulose gel during the regeneration stage of the process. New methods have been developed to prevent this shrinkage and to obtain at the same time, regeneration

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of the cellulose in spherical bead form of low density and thus suitable as a chromatographic column packing material. These methods involve forming an emulsion of the aqueous cellulose xanthate solution with a water-immiscible organic solvent and then regeneration of the insoluble cellulose by addition of an organic acid[7,8] or by heat treatment[9]. An aqueous solution of calcium thiocyanate has also been used as the solvent for cellulose prior to dispersion in an organic solvent and finally regeneration[10].

Alternative approaches have started with cellulose esters and used either emulsion techniques[11–13] or heat treatment of ester chips in silicon oil[14,15] to form beads prior to regeneration by ester hydrolysis.

Since cellulose is such an abundant natural material, it is an obvious choice for preparing an inexpensive gel matrix for industrial chromatography and many of the methods cited above have been aimed at developing this potential. However they suffer from the disadvantage that they require organic solvents or carefully controlled processes for preparation.

We wish to report on the gel chromatography performance of cellulose-derived gels resulting from a very different approach to utilising cellulose. Since large-scale protein purification does not always require sophisticated spherical beads, but only porous particles, we have taken conventionally produced regenerated cellulose (viscose process) in ground irregular form and reacted it with propylene oxide, epichlorohydrin and concentrated aqueous sodium hydroxide. This simple, one-step chemical treatment has resulted in a cross-linked hydroxypropyl cellulose with gel-like properties and enhanced porosity compared with the initial dense regenerated cellulose.

Although hydroxyalkyl cellulose gels have been made before, the processes started with an aqueous solution of the already-substituted cellulose and emulsifying it with an organic solvent before desolubilising it, either by cross-linking with epichlorohydrin[16] or by acidification[17]. These processes still suffer from the disadvantage of having to use organic solvents.

The preparation of the new hydroxypropyl cellulose gels reported here has not required the solubilisation of the hydroxypropyl cellulose at any stage. Consequently the gels, even though swollen, have retained the tough, resilient and non-fibrous nature of the initial regenerated cellulose which allows high flow-rates and long life.

EXPERIMENTAL

Materials

The regenerated cellulose used was a ground powder with 50–75-μm particles obtained from Enka (Arnhem, The Netherlands). Epichlorohydrin was obtained from BDH (Poole, U.K.) and propylene oxide from Koch-Light (Colnbrook, U.K.). The soluble dextran T fractions were purchased from Pharmacia (Uppsala, Sweden), while the two low-molecular-weight fractions 2.6 and 6.6 were gifts from Dr. K. Granath of Pharmacia. DEAE and CM Indion were obtained from Phoenix Chemicals (Nelson, New Zealand). Anthrone was AR grade while all other chemicals were reagent grade.

Reation of epichlorohydrin and propylene oxide with regenerated cellulose powder

The epichlorohydrin was dissolved in the propylene oxide and then added to 20 g of cellulose powder simultaneously with 30 ml of 30% (w/v) aqueous sodium hydroxide at 15°C. The reagents were stirred thoroughly in a stainless-steel reaction vessel (250 ml) for 2 min after which time the cellulose had swelled and absorbed all the liquid phase. The vessel was sealed, fitted with a pressure gauge when necessary, and then heated for 1.5 h in a water bath at 60°C without any additional mixing. After cooling, the reaction mixture was finely divided and sprinkled onto 1 l of water, stirred rapidly and allowed to stand for 1 h. It was then diluted with water to 4 l before collecting the derivatized cellulose and washing it thoroughly on a Büchner funnel. It was soaked overnight in very dilute hydrochloric acid pH 2–3 and de-fined by decantation. The product was finally collected on a Büchner funnel and washed thoroughly. The yield was determined by carrying out a dry matter analysis on the damp product. Samples were also solvent exchanged into methanol, dried under vacuum and then allowed to re-swell in various organic solvents for 118 h (Table IV).

The packed bed volume (ml/g) for each gel in water was determined at the conclusion of the gel chromatography experiments by drying the total gel from the column at 105°C (Table II).

Gel chromatography

The cross-linked hydroxypropylated cellulose particles were slurried in 0.3% sodium chloride and packed in a glass column fitted with flow adaptor (Pharmacia K16/70). Bed volumes were all in the range 120–128 ml and a flow-rate of 20 ml/h was used. The probe solutes (4 mg in 2 ml of 0.3% sodium chloride) were applied singly to the column and 1.7–2.2-ml fractions were collected. The diameters of the solute molecules were found by interpolation of data previously reported[18] and are listed in Table I.

Analysis of eluate

Carbohydrate and dextran fractions were analysed by the anthrone method[19].

TABLE I

PROPERTIES OF THE PROBE SOLUTE MOLECULES

Solute	*Molecular weight, MW*	*Molecular diameter in solution, Å*
Methyl β-D-glucoside	194	8
Raffinose	504	12
Dextran 2.6	$2.6 \cdot 10^3$	26
Dextran 6.6	$6.6 \cdot 10^3$	41
Dextran T10	$9.3 \cdot 10^3$	47
Dextran T20	$22.3 \cdot 10^3$	69
Dextran T40	$42.4 \cdot 10^3$	92
Dextran T70	$63 \cdot 10^3$	110
Dextran T150	$167 \cdot 10^3$	173
Dextran T500	$495 \cdot 10^3$	295
Dextran T2000	$2000 \cdot 10^3$	560

The elution volume (V_e) of each solute chromatographed was determined at their point of maximum concentration in the eluate.

Preparation of ion-exchange derivatives

Cation- and anion-exchange derivatives of HP-Regcell were prepared using standard procedures and chemicals[20].

Protein capacities of ion exchangers

Bovine serum albumin (0.5%) in 0.01 *M* Tris, pH 7.5, was used to determine the protein-binding capacity of the DEAE derivative in a 2-h batch test. Ovine haemoglobin (0.5%) in 0.01 *M* acetate buffer, pH 5.0, was used for the CM and SP derivatives.

RESULTS AND DISCUSSION

The reaction of alkali cellulose with propylene oxide or ethylene oxide forming water-soluble hydroxyalkyl cellulose is well known[21]. The intention here was to cross-link the cellulose simultaneously with hydroxyalkylation so that the bulk of the cellulose remained insoluble at all times but swelled usefully in water (Fig. 1).

Hydroxypropylation and cross-linking

The reaction of alkali cellulose with propylene oxide was easily followed by means of a pressure gauge on a sealed vessel. Fig. 2 shows the time course for this reaction at different temperatures. Calculations showed that greater than 95% of the propylene oxide had been consumed by the time the pressure dropped below atmospheric pressure (0 p.s.i.). Very little difference was seen between the products obtained from reactions at 40, 50 and 60°C for 4, 2 and 1½ h, respectively. But the product obtained by reaction at 25°C for 24 h was very different and resembled the unsubstituted starting regenerated cellulose powder. A swollen product could not be obtained at 25°C no matter how long the reaction was left. To obtain a usefully swollen cellulose derivative, reaction had to be carried out in the vapour phase above the boiling point of propylene oxide (34°C) in a pressure vessel.

A previously reported procedure[22] for preparing hydroxypropylated and cross-linked fibrous cellulose powder used reflux conditions for the reaction of alkali cellulose in liquid propylene oxide (PO) and epichlorohydrin (ECH) with an extremely high ratio of organic reagents to cellulose, *viz.* PO:ECH:cellulose = 20:4:1. Chro-

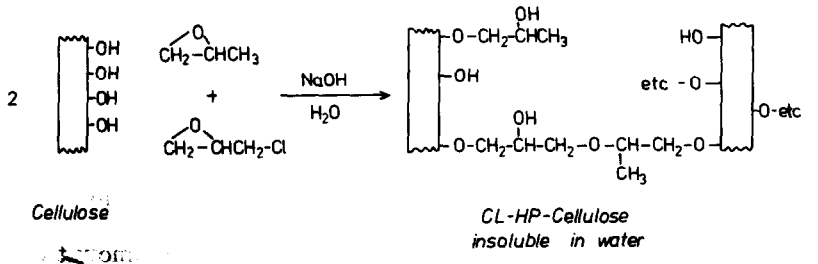

Fig. 1. Typical reactions illustrating the preparation of cross-linked hydroxypropylated cellulose.

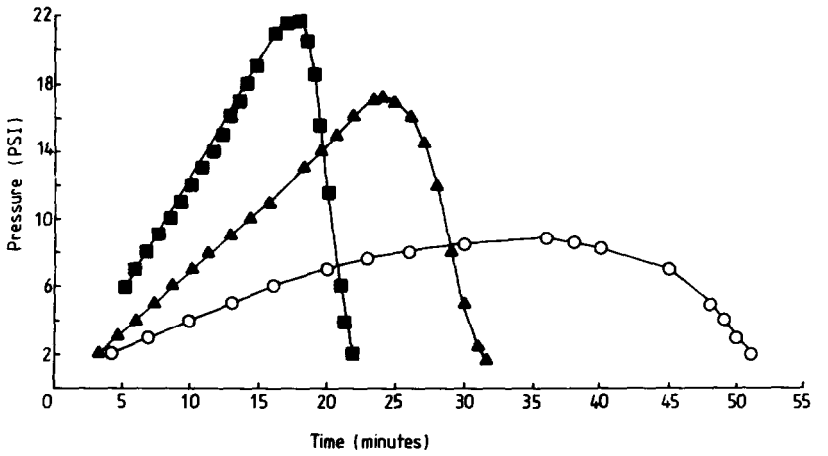

Fig. 2. Effect of water-bath temperature on pressure and time of reaction. ■, 60°C; ▲, 50°C; ○, 40°C.

matographically useful products were obtained in the present work with PO:cellulose less than 1:1 and ECH:cellulose less than 0.16:1 so long as reaction was carried out at 40°C or higher in a pressure vessel. This remarkable efficiency of the reaction in the vapour phase has been reported before for epichlorohydrin[23], and is consistent with the fact that water-soluble hydroxyethyl and hydroxypropyl cellulose are manufactured by reacting alkali cellulose with alkylene oxides at elevated temperatures and pressures[21]. All results discussed below relate to reaction products prepared at 60°C for 1.5 h. It was assumed that the cross-linking by epichlorohydrin was also complete after this time[23].

From preliminary trials it was found that the strength of the aqueous sodium hydroxide could be varied from 20 to 40% (w/v) without significant changes in the products. Likewise the water to cellulose ratio was equally effective at 1:1 and 1.5:1. Consequently 30% aqueous sodium hydroxide at a ratio of 1.5 ml per g of cellulose was used for all results reported here.

Effect of hydroxypropylation and cross-linking

A series of cross-linked hydroxypropylated regenerated cellulose powders was prepared by varying independently the amount of epichlorohydrin (ECH) and propylene oxide (PO) used in the reaction. The yields of water-insoluble products and their water swelling characteristics are listed in Table II. The products are referred to as "HP-Regcell" and coded according to the volumes (ml) of ECH and PO used per 100 g of cellulose powder.

For a given amount of propylene oxide used, the yields decreased and the bed volumes increased as the cross-linking was reduced. With 50% propylene oxide the bed volume reached a maximum of 25.5 ml/g at 2% cross-linking. A further reduction in cross-linking to 1% only resulted in more hydroxypropylated cellulose dissolving causing a lower yield of useful gel. Thus no attempt was made to prepare a product with less than 1% epichlorohydrin.

When the cross-linking was held constant, *e.g.* at 4%, and the amount of propylene oxide increased from 30 to 100% a large increase in the swelling charac-

TABLE II

PREPARATION AND PROPERTIES OF CROSS-LINKED HYDROXYPROPYL REGENERATED CELLULOSES

Regenerated cellulose (10 g) was reacted with 15 ml of 30% NaOH, epichlorohydrin (ECH) and propylene oxide (PO) at 60°C for 1.5 h.

*HP-Regcell**	*ECH (ml)*	*PO (ml)*	*Yield (g)*	*Bed volume (ml/g)*	*Internal porosity*** *(ml/g)*
16-50	1.6	5	9.8	8.2	2.4
8-50	0.8	5	9.0	13.6	6.0
4-50	0.4	5	8.2	20.0	10.0
2-50	0.2	5	5.4	25.5	15.0
1-50	0.1	5	3.8	24.0	14.5
4-30	0.4	3	9.1	14.8	5.6
4-50	0.4	5	8.2	20.0	10.0
4-100	0.4	10	6.8	35.0	21.0
0-00	0	0	8.9	6.7	–

* The water insoluble products are coded according to the volume (ml) of ECH and PO used per 100 g of cellulose.

** Calculated from gel chromatography data = $(V_e - V_0)$/dry weight of gel, where V_e = elution volume for methyl β-D-glucoside.

teristics of the products was observed as shown in Table II. Clearly, the propylene oxide increases the swollen gel-like nature of the product in water while the epichlorohydrin retains the insolubility of the product. Propylene oxide is well-known for its ability to increase the swelling of cross-linked polysaccharide matrices in non-polar organic solvents[22] because of the hydrophobic effect of the methyl group but here it has been used to enhance the aqueous swelling of cellulose.

Porosity of "HP-Regcell"

The porosities of the gels listed in Table II were determined by measuring their gel chromatography performances with a series of dextran fractions of known molecular weight plus two low-molecular-weight carbohydrates. Table III lists the partition coefficients, K_{av}*, obtained for each gel. These results reflect the pore size distributions of the gels and give rise to the calibration curves shown in Fig. 3.

Also listed in Table II are the capacity ratios V_i/V_0** for each gel. Increasing the propylene oxide from 30 to 100% lifted the capacity ratio from 0.70 to 1.70. The latter figure is typical of materials regularly used for gel chromatography. A value of 1.78 has been reported[10] for Sepharose CL-2B. Surprisingly though, propylene oxide over this same range of 30–100% had little effect on the pore size distribution

* $K_{av} = \dfrac{V_e - V_0}{V_t - V_0}$, where V_e = elution volume of solute, V_0 = void volume, V_t = total volume of column.

** V_i = internal volume of gel (calculated from the elution volume for methyl glucoside, $V_e - V_0 = V_i$).

TABLE III

PARTITION COEFFICIENTS, K_{av} FROM GEL CHROMATOGRAPHY

Standard solute	*K_{av} for HP-Regcell matrices*						
	16-50	*8-50*	*4-50*	*2-50*	*1-50*	*4-30*	*4-100*
Dextran T-2000	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T-500					0.03		
T-150				0.01	0.08		
T-70			0.02	0.09	0.18		
T-40		0.03	0.04	0.16	0.26	0.04	0.04
T-20		0.04	0.13	0.28	0.36	0.12	0.11
T-10	0.02	0.09	0.30	0.48	0.56	0.29	0.33
6.6	0.06	0.14	0.35	0.58	0.62	0.35	0.43
2.6	0.17	0.31	0.56	0.73	0.75	0.55	0.61
Raffinose	0.61	0.71	0.86	0.90	0.90	0.79	0.89
Me β-D-Glu	0.69	0.76	0.89	0.94	0.93	0.84	0.93
V_i/V_0*	0.51	1.02	1.08	1.40	1.26	0.70	1.70

* V_i = internal volume of pores and was calculated from the elution volume V_e for methyl β-D-glucose; $V_i = V_e - V_0$.

even though there is an increase of nearly 400% in the internal porosity of the gel. The vertical bars on the curve for the 4% cross-linked products in Fig. 3 cover the K_{av} values obtained for all three products (4-30, 4-50, and 4-100) and show this similarity in pore structure. Reducing the cross-linking, on the other hand, does open

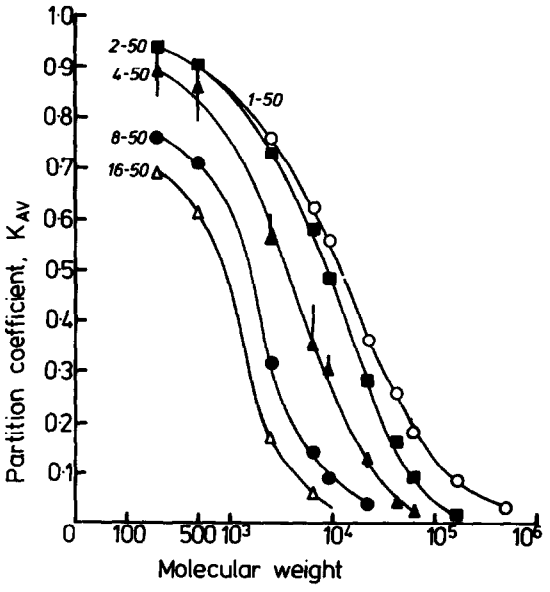

Fig. 3. Partition coefficient curves for HP-Regcells from gel chromatography data. ▲, Range of values for 4-30, 4-50 (▲) and 4-100 products.

up the pores, at least as far as allowing a dextran molecule of 70,000 molecular weight (mol. diameter = 110 Å) to start entering them.

Several attempts were made to obtain the corresponding K_{av} values for the unsubstituted regenerated cellulose that had only been treated with 30% sodium hydroxide, *i.e.* Regcell 0-00. These were unsuccessful as the dextran peaks were not clearly defined. This demonstrates further the advantages of using propylene oxide to form gel-like derivatives from regenerated cellulose.

Other properties of "HP-Regcells"

The mechanical stability of the more highly cross-linked gels such as HP-Regcell 8-50 was good. They did not compress noticeably when packed in a column and used over ten runs. The more highly swollen gels, especially HP-Regcell 1-50 did suffer from physical instability with changing pressure and flow-rates. This same gel also showed some evidence of slow dissolution in water and hence traces of column bleed. Eluate from the column gave a positive anthrone test for carbohydrate after standing overnight in 0.3% sodium chloride.

The ease and method of drying the HP-Regcells without affecting the ability to re-swell in water was dependent on the amount of cross-linking present. HP-Regcell 4-50 for example regained more than 97% of its former swollen volume even after air drying the aqueous moist gel at 65°C. HP-Regcell 2-50 on the other hand regained only 70% after freeze-drying or after methanol exchange followed by vacuum drying. Swelling in organic solvents was more rapid for the freeze-dried material but the final settled volumes were the same for both drying methods as shown in Table IV.

The residual carboxyl groups, usually present in cellulose, were determined by pH titration and found to be 20 μmoles per gram of dry HP-Regcell 2-50. This low level of charged groups is typical of many polysaccharide gels. Even so it was sufficient to give HP-Regcell 2-50 gel an ion-exchange capacity of 19 mg/g for cytochrome *c* in 2 m*M* ammonium acetate at pH 4.1[16]. However, all results here were obtained

TABLE IV

SETTLED VOLUMES OF HP-REGCELL 2-50 IN ORGANIC SOLVENTS

Solvent	*Settled volume (ml/g)*						
	Methanol dried; swelling time (h)				*Freeze-dried; swelling time (h)*		
	0.25	*3*	*70*	*118*	*0.25*	*3*	*16*
DMF	2.2	2.4	7.2	9.2	7.2	8.8	9.4
DMSO				39			
Water				18			
DMSO–dioxane (1:1)				12.8			
DMF				9.2			
Pyridine				7.2			
Methanol				4.8			
Chloroform				2.8			
Dil[illegible]				2.8			

TABLE V

DEXTRAN MOLECULAR WEIGHT EXCLUSION LIMITS FOR HP-REGCELL GELS

HP-Regcell	*Exclusion limit dextran (MW)*	*Sephadex equivalent*
16-50	5000	G-25
8-50	10,000	G-50
4-50	30,000	G-50 to G-75
2-50	70,000	G-75 to G-100
1-50	150,000	G-150

from gel columns run in 0.3% sodium chloride to prevent any adsorption of the solutes used.

Table V summarises the molecular weight exclusion limit for dextran for each of the HP-Regcell gels and lists for comparison the equivalent Sephadex products widely used for protein separations. Fig. 4 shows the complete separation of bacitracin (MW 1400), myoglobin (MW 17,000) and catalase (MW 225,000) obtained on HP-Regcell 4-50. Although these new cellulose gels are not likely to be used for high-resolution gel chromatography because of their irregularly shaped particles they would be an excellent choice of gel for crude industrial separations such as desalting. Their main advantage is their resistance to damage by microbial contamination and the high flow-rates obtainable with them.

Ion-exchange derivatives of "HP-Regcell"

To date, the main use of the hydroxypropylated cellulose gels has been for the synthesis of high protein capacity ion-exchangers[24]. Table VI shows the properties of the diethylaminoethyl (DEAE), carboxymethyl (CM) and sulphopropyl (SP) derivatives. Included in the table are the protein capacities for DEAE and CM Indion

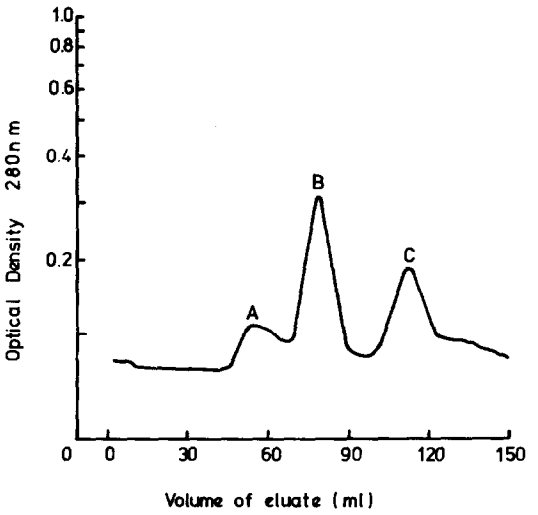

Fig. 4. Gel chromatography of catalase (A) 6 mg, myoglobin (B) 4 mg and bacitracin (C) 5 mg in 0.3% NaCl on a 128-ml column of HP-Regcell 4-50.

TABLE VI

PROPERTIES OF ION EXCHANGERS FROM REGENERATED CELLULOSE AND HP-REGCELL

Ion-exchange derivative	*Ion-exchange capacity (mequiv./g)*	*Protein capacity*	
		(g/g)	*(mg/ml)*
DEAE HP-Regcell 8-50	1.2	1.28	134
DEAE HP-Regcell 7-50	1.2	1.55	155
CM HP-Regcell 8-50	1.3	1.39	156
CM HP-Regcell 6-50	1.2	1.61	120
SP HP-Regcell 4-50	1.1	1.58	160
DEAE Indion (Regcell 1-00)	1.1	0.37	60
CM Indion (Regcell 1-00)	1.0	0.40	66

which are the ion-exchange derivatives prepared from regenerated cellulose powders without the use of propylene oxides[3]. These latter values are typical of those obtained for most cellulosic ion exchangers and highlight the 4–5-fold increase in capacity obtained with the aid of propylene oxide.

The values listed in Table VI for the hydroxypropylated cellulosic ion exchangers are as high as the 160 mg/ml reported for DEAE-Sephacel[25]. The latter is an ion exchanger prepared from high-porosity cellulose beads by a sophisticated regeneration process involving emulsion techniques using organic solvents as mentioned in the Introduction.

Another advantage observed for the new HP-Regcell ion exchangers was their excellent chemical resistance to aqueous sodium hydroxide solutions. This is shown in Table VII. Although improved protein capacities were obtainable with only 30% propylene oxide, a minimum of 50–70% was necessary to obtain excellent stability in alkali. Some products have not shown any detectable weight loss on soaking in 1% sodium hydroxide at 65°C for one week. The ion exchangers have also shown a high resistance to physical attrition which means that the production of fines does not occur on repeated handling or mechanical stirring. Consequently the ion exchangers can be re-used many hundreds of times by periodically cleaning them with hot 1% alkali to remove irreversibly adsorbed protein.

TABLE VII

CHEMICAL STABILITY OF DEAE HP-REGCELL

Weight loss (%) after soaking in 10% NaOH for 2 h at 25°C.

Propylene oxide (%)	*Epichlorohydrin (%)*				
	5	*6*	*7*	*8*	*9*
30	16	13	9	6	
50		8	6	3	2
70		4	1	0	1

CONCLUSION

The new cellulosic chromatographic materials described here have been prepared from conventionally regenerated cellulose, in powder form, by cross-linking and hydroxypropylation. By so doing, the disadvantages caused by the high density and low porosity of the starting regenerated cellulose have been overcome without resorting to special regeneration techniques. The products have shown utility in both gel chromatography and as a starting matrix for the synthesis of high protein capacity ion exchangers*. Other applications have also been investigated and will be reported separately.

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* These ion exchangers are now manufactured and marketed by Phoenix Chemicals, C/- Waitaki NZ Refrigerating, Nelson, New Zealand as part of their range of *ind*ustrial *ion* exchangers (Indion Products).