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Bead cellulose derivatives as supports for immobilization and chromatographic purification of proteins

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ABSTRACT

Characteristic data are presented for Divicell, a macroporous bead cellulose with excellent flow parameters. The preparation of Divicell derivatives and their properties are described with respect to their application as chromatographic supports. The ion exchangers Divicell DEAE and Divicell CM were manufactured in two types with different exclusion limits and an available capacity for proteins of up to 100 mg/ml gel. Divicell Blue is a bead cellulose with covalently bound Cibacron Blue F3G-A and was found to be a very suitable adsorbent for the selective separation and purification of human serum albumin. Activation of Divicell with sodium periodate, epichlorohydrin and 5-norbornene-2,3-dicarboximido carbonochloridate provided activated supports used for immobilization of ligands in organic solvents and in aqueous solutions. Coupling of amines, diamines, amino acids, carbohydrates and proteins is described. The immobilized ligands retained their biological activity as determined by their specific adsorption of proteins. Divicell alkyl derivatives were tested in hydrophobic interaction chromatography with bovine serum albumin as a model. Examples are presented of the application of Divicell derivatives to the purification of biomacromolecules such as immunoglobulins and lectins by affinity chromatography. The results were comparable to those obtained using the corresponding Sepharose-derived adsorbents.

INTRODUCTION

Hydrophilic polymers, especially cross-linked agarose and dextran, play a dominant role as supports for chromatographic procedures in biosciences and biotechnology. During the last 10 years, cellulose has achieved increasing importance in the form of highly porous beads owing to its mechanical and chemical stability. Various techniques have been described for the preparation of macroporous cellulose beads [1–4]. Bead cellulose has been tested as a packing material in gel chromatography [3,5]. The hydroxyl groups of bead cellulose can be converted by the usual chemical reactions applied to other hydrophilic matrices, *e.g.*, fibrous cellulose and agarose. The preparation of ion-exchange derivatives of bead cellulose partly cross-linked and their use in chromatographic separation and purification processes was described by

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Štamberg *et al.* [1,2], Motozato and Hirayama [6] and Gemeiner *et al.* [7]. A further application of bead cellulose was investigated in dye–ligand chromatography for the purification of proteins [7–9].

Different methods have been used for the introduction of active groups and ligands into bead cellulose. First, Turková *et al.* [10] described the immobilization of trypsin to dialdehyde bead cellulose. The activation of cellulose beads with chloroformates was reported by Drobník *et al.* [11]. Several other activation methods with cross-linked bead cellulose for the purification of immunoglobulins were evaluated by Peng *et al.* [12]. Recently, a novel carbonochloridate (chloroformate) for the activation of hydrophilic supports, especially of bead cellulose was described by Büttner *et al.* [13]. Various methods have been employed for immobilization of biospecific ligands to bead cellulose used in the chromatographic purification of proteins and in biotransformation processes [14–19].

In this work, Divicell, a macroporous bead cellulose produced by Leipziger Arzneimittelwerk (Leipzig, Germany), was investigated with respect to its characterization and use as a chromatographic support, especially for immobilization of ligands and purification of biopolymers. The aim of this work was the preparation of various Divicell derivatives with regard to different activation methods and the development of biospecific sorbents possessing high efficiencies for adsorption and desorption of proteins. The suitability of Divicell sorbents as chromatographic supports was tested using several model systems of protein chromatography based on hydrophobic interaction and affinity of proteins to coupled biospecific ligands.

EXPERIMENTAL

Materials

Bead cellulose Divicell (particle size 80–200 μm) was obtained from Leipziger Arzneimittelwerk, Sepharose 2B, 4B, 6B, and CL-6B, CNBr–Sepharose 4B, Con A–Sepharose and Blue Dextran 2000 from Pharmacia (Uppsala, Sweden), 5-norbornene-2,3-dicarboximido carbonochloridate (Cl-CO-ONB) from the Institute for Drug Research (Berlin, Germany), immunoglobulins and antisera from the State Institute for Immunopreparations and Nutrient Media (Berlin, Germany), Cibracron Blue F3G-A, lysozyme and human γ-globulin from Serva (Heidelberg, Germany), trypsin from Boehringer (Mannheim, Germany), human and bovine serum albumin (HSA and BSA, respectively) from the Institute of Vaccines (Dessau, Germany) and porcine insulin from Berlin-Chemie (Berlin, Germany). Penicillin acylase was a gift from Dr. B. Rockstroh (Research Centre of Biotechnology, Berlin, Germany). The following proteins were prepared by standard methods: human immunoglobulin G (human IgG) [20], crude hen egg ovalbumin [21], human methaemoglobin (MetHb) [22], concanavalin A (Con A) [23], crude wheat germ (WGA) [18], soybean (SBA) [24] and peanut (PNA) [25] agglutinins. All other reagents were of analytical-reagent grade.

Determination of exclusion limit

The exclusion limit of Divicell was determined with Blue Dextran 2000. A 50-ml volume of Divicell was equilibrated in a column (25 × 1.6 cm I.D.) with 0.1 *M* phosphate buffer (pH 7). Blue Dextran (5 mg in 0.5 ml of phosphate buffer) was

loaded on the column and chromatographed at a flow-rate of 6–8 ml/h. Blue Dextran splits off into two fractions, one excluded and the other non-excluded. The exclusion limit was determined by means of a calibration graph (ratio of the excluded to non-excluded amounts of Blue Dextran from Sepharose 2B, 4B and 6B *versus* their exclusion limits of $4 \cdot 10^6$, $20 \cdot 10^6$ and $40 \cdot 10^6$ dalton, respectively).

Determination of porosity

Pore-size distribution was measured with a Type 2000 mercury porosimeter (Carlo Erba, Milan, Italy). Samples of Divicell were dehydrated by stepwise transfer into water–ethanol mixtures, followed by ethanol and finally diethyl ether. The diethyl ether was removed at room temperature.

Electron microscopy

Transmission electron micrographs were obtained with a BS 500 electron microscope (Tesla, Prague, Czechoslovakia). Dried samples of Divicell were embedded in methyl polymethacrylate. After dissolving the polyester, ultra-thin sections were contrasted with Pt/Ir–C at an angle of 45°C.

Preparation of Divicell DEAE and Divicell CM ion exchangers

The ion exchangers were prepared by conversion of Divicell with 2-chloroethyldiethylamine and chloroacetic acid [26], respectively and were characterized as described previously [27].

Divicell DEAE-H. Wet Divicell (205 g, cellulose content 40.5 g) was mixed with 20 g of 2-chloroethyldiethylamine hydrochloride dissolved in 100 ml of water for 30 min at room temperature. The mixture was dried under vacuum (water-jet pump) at 60°C until the weight was constant. After cooling to room temperature, 12 g of sodium hydroxide in 100 ml of water were added dropwise with stirring. The mixture was then heated at 90°C for 30 min followed by acidification with 0.5 *M* hydrochloric acid at room temperature and washing with water.

Divicell DEAE-L. The same procedure was applied with 40 g of 2-chloroethyldiethylamine hydrochloride, 20 g of sodium hydroxide and 2.3 g of epichlorohydrin in 100 ml of water.

Divicell CM-H. Divicell (205 g wet weight) was converted into the CM derivative with 20.2 g of sodium monochloroacetate in 100 ml of water at room temperature for 30 min. After drying, 8 g of sodium hydroxide in 73 ml of water were added as described above. The mixture was then heated at 70°C for 2 h. After cooling to room temperature, the support was washed with water.

Divicell CM-L. The procedure was carried out with 30.3 g of sodium chloroacetate, 12 g of sodium hydroxide and 2.3 g of epichlorohydrin in 100 ml of water as described for the H-derivative.

Preparation of Divicell Blue

A 20-g amount of wet Divicell was suspended in 36 ml of water and the mixture was heated in a 500-ml flask at 60°C with stirring. A solution of 0.32 g of Cibacron Blue F3G-A (C.I. 61211) in 12.8 ml of water was added dropwise. Stirring was continued at 60°C for 1 h, then 16 g of sodium chloride were added. After a further 1 h the temperature was increased to 90°C and 1.28 g of sodium carbonate were added to the

stirred suspension. Two hours later the mixture was allowed to cool to room temperature, then the unbound dye was removed by washing with water and determined spectrophotometrically ($\varepsilon_{615} = 1.3 \cdot 10^5$ l mol^{-1} cm^{-1}). The content of covalently fixed dye was 6–8 mg/ml gel.

Conversion of Divicell with sodium periodate into Divicell Dialdehyde

A 100-ml volume of settled Divicell was transferred into a 500-ml vessel and 120 ml of 0.05 or 0.1 *M* sodium periodate were added. The suspension was stirred at room temperature for 2, 4 or 6 h. After addition of 30 ml of ethylene glycol the mixture was stirred for 1 h and then filtered. The support was washed with 2 l of distilled water and stored in a refrigerator in the presence of 0.02–0.04% sodium azide. The dialdehyde content (see Table III) was determined via stoichimetric consumption of hydroxyl ions under definite conditions as a consequence of the Cannizzaro reaction of dialdehyde groups [28,29].

Activation of Divicell with epichlorohydrin to Divicell Epoxy

Divicell Epoxy was prepared by a modified procedure according to Matsumoto *et al.* [30]. Briefly, a mixture of 40% (v/v) wet Divicell and 12% (v/v) epichlorohydrin in 1 *M* sodium hydroxide solution (final concentration) was agitated at 50°C for 30 min. After cooling to room temperature, the support was washed exhaustively with water on a sintered-glass funnel. The support was transferred into acetone or isopropanol and stored at 4–8°C.

The content of epoxy groups was determined as described by Sundberg and Porath [31]. Divicell Epoxy contained 20–25 μmol of active groups per millilitre sedimented gel. An additional run through the above activation procedure increased the content of epoxy groups of this gel to 40–50 μmol/ml.

Activation of Divicell with Cl-CO-ONB to Divicell ONB-Carbonate

This Divicell derivative was prepared as described recently [13]. A 100-ml volume of Divicell sedimented in water was transferred into acetone and washed thoroughly with acetone (water content *ca.* 0.3%). Approximately 2 g of Cl-CO-ONB dissolved in 100 ml of acetone were added to the slurry. The suspension was gently agitated at room temperature for 16–20 h. The solvent was sucked off and the support was washed with a tenfold volume of acetone.

The content of active ONB-carbonate groups determined spectrophotometrically at 270 nm [13] was 25 μmol/ml gel. For the preparation of low- and high-activated supports the amount of Cl-CO-ONB was reduced and increased, respectively. Divicell ONB-Carbonate was stored at room temperature in acetone in the absence of humidity.

Coupling of proteins to Divicell Dialdehyde

A 1-ml volume of activated gel sedimented in water was equilibrated with 0.1 *M* phosphate buffer (pH 7). After removing the supernatant, 1 ml of protein solution (8–10 mg/ml in the above buffer) was added and the suspension was stirred at room temperature for 6 h. The unbound protein was washed out using the coupling buffer.

For inactivation of remaining aldehyde groups, the Divicell derivative was equilibrated with 0.1 *M* phosphate buffer (pH 8) and 1 ml of ethanolamine adjusted

to pH 8 was added. After stirring for 24 h at 2°C or room temperature, the support was thoroughly washed with buffer and finally with water.

To convert the initially formed carbinolamine or the corresponding Schiff's base into the more stable amine, the gel was equilibrated with 0.1 *M* borate buffer (pH 9). Then 5 mg of sodium borohydride in 1 ml of buffer were added and the suspension was stirred for 30 min at room temperature. An additional 5 mg of sodium borohydride were added and stirring was continued for 30 min.

Finally, the support was washed exhaustively with 0.1 *M* phosphate buffer (pH 7) or suitable application buffer.

Immobilization of ligands to Divicell Epoxy

Divicell Epoxy was transferred from acetone or isopropanol to water. The suction-dried gel was added to the coupling solution in a 1:1 to 1:2 (v/v) ratio. This suspension was gently agitated at an appropriate temperature for 1–3 days. After washing out the unbound material with coupling buffer or water (at least ten bed volumes), unreacted epoxy groups were deactivated by incubation with 1 *M* ethanolamine (pH 8–11) for 24 h at room temperature. Finally, the gel was thoroughly washed with water, 0.1 *M* borate buffer (pH 8.0) and again with water. The ligand-containing gel should be stored at 4–8°C in the presence of 0.02–0.04% sodium azide.

The immobilization of N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-galactosamine (GalNAc) was carried out according to Uy and Wold [32].

For coupling of heparin, the polysaccharide was precipitated with isopropanol from a commercial solution. The precipitate was dissolved in water (80 mg/ml) and adjusted for coupling to pH 11 with sodium hydroxide.

Coupling of iminodiacetic acid (IDA) was performed as described by Porath and Olin [33].

Divicell IDA–Cu^{2+} and IDA–Zn^{2+} were prepared by loading of solutions of copper (II) chloride and zinc acetate (5 mg/ml) on Divicell IDA as described previously [34]. The contents of Cu^{2+} and Zn^{2+} bound to the support were calculated from differences between the amounts of ions added and unbound. The determination of Cu^{2+} was carried out after treatment of the Cu^{2+} solutions with concentrated ammonia by measurement of the absorbance at 620 nm. Zn^{2+} was determined by titration with 0.01 *M* EDTA in the presence of Eriochrome Black T.

Immobilization of ligands to Divicell ONB-Carbonate

Coupling of ligands was carried out by the same procedure as described for the immobilization to Divicell Epoxy; however, the immobilization to Divicell ONB-Carbonate was complete after 1–20 h at 4°C or room temperature.

Residual ONB-carbonate was deactivated by hydrolysis in 0.1 *M* borate buffer (pH 8) (room temperature, 16 h) or by blocking with 0.5—1 *M* Tris, glycine and ethanolamine, respectively, at pH 7–9 (4°C or room temperature, 16 or 2 h.

For immobilization of low-molecular-mass ligands, the molar ratio of offered ligand to active groups was 1.1:1 to 20:1 (see Table V). The amounts of proteins applied for immobilization varied from 1 to 25 mg per millilitre of coupling solution and millilitre of activated gel (see Table VI).

Determination of immobilized ligands

The amount of immobilized protein was most often calculated from the spectrophotometrically determined unbound protein prior to and after immobilization. To suppress the absorption at 270 nm of released HONB, the protein solutions were acidified to pH 2–3. Fixed protein was also determined after treatment of the support with 1 *M* sodium hydroxide solution at room temperature for 16 h in the alkaline supernatant according to Lowry *et al.* [35].

The activity of immobilized trypsin and penicillin acylase was determined according to Hummel [36] with tosyl-L-arginine methyl ester (TAME) and according to Balasingham *et al.* [37] with benzylpenicillin as substrates, respectively.

The determination of coupled heparin was performed by the method of Smith *et al.* [38].

Immobilized GlcNAc, GalNAc or GalN were determined after hydrolysis of the support with 6 *M* hydrochloric acid at 100°C for 6–8 h by the modified Morgan–Elson method [39].

The amount of aminohexyl residues was calculated from the determination of NH_2 groups according to Schmitt and Walker [40] or by acidimetric titration using an AT 3 autotitrator (MLW, Dresden, Germany).

Coupled amino acids or alkyl- and arylamines were determined using the Kjeldahl method [41].

Chromatographic purification of proteins

All chromatographic separations were performed with glass columns at low pressure and at 4°C or room temperature using a Microperpex peristaltic pump, a Uvicord S UV monitor and a Redirac fraction collector from LKB (Bromma, Sweden). Proteins were loaded on the respective support in the appropriate equilibration/binding buffer if not stated otherwise. Unbound protein was washed off with the same buffer (at least a fivefold column volume).

Generally, flow-rates were *ca.* 1–2 column volumes per hour. The UV profiles were recorded using the absorption at 280 nm. The purification was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or in an SDS-free system (PAGE) and by specific analysis of the isolated proteins.

Determination of binding capacity for proteins on Divicell sorbents

A 0.5–10-ml volume of the Divicell sorbent was equilibrated in a column with an appropriate buffer. A defined protein solution was applied until the absorbance at 280 nm of the effluent was the same as that of the sample. Then the column was washed with the equilibration buffer until the absorption of the eluate was <0.01. All protein-containing fractions were pooled. The capacity was calculated from the difference between applied and unboud protein amounts. This value was compared with the amount of protein that was eluted specifically (see Table VII).

Hydrophobic interaction chromatography (HIC) of BSA

For example, Divicell Pentyl was prepared by coupling of *n*-pentylamine to Divicell ONB-Carbonate (34 μmol active groups/ml gel) with a molar ratio of 1.1 mol of amine per mole of ONB-carbonate in acetone for 1 h at room temperature. The coupling was quantitative with respect to the active groups.

Columns (25 × 1 cm I.D.) with the respective HIC support were equilibrated with 0.05 *M* phosphate buffer (pH 7.4) containing 2 *M* ammonium sulphate. A 2-ml volume of BSA in equilibration buffer (50 mg/ml) was loaded and subsequently eluted with 5 ml of equilibration buffer, 10 ml of 0.05 *M* phosphate buffer (pH 7.4), 10 ml of 6 *M* urea in water and 10 ml of 0.05 *M* phosphate buffer (pH 7.4) containing 2 *M* potassium thiocyanate (flow-rate 15.3 cm/h). Protein elution was monitored by UV absorption 280 nm. BSA was determined in the urea-containing fractions. The capacity of support was 23.0 mg protein/ml gel and the recovery was $\geqslant 95\%$.

Examples of other HIC supports derived from Divicell ONB-Carbonate are given in Table VIII. The binding capacities for lysozyme were comparable to those for BSA, but elution was effected with less chaotropic eluents as for BSA (data not shown).

The process operating with Divicell Pentyl was simulated by two series of ten adsorption–elution cycles of BSA (elution with 6 *M* urea). Each series was followed by a cycle with ten gel volumes of 1 *M* sodium hydroxide solution at room temperature ("cleaning-in-place").

Isolation of HSA from human serum using Divicell Blue

A 10-ml volume of Divicell Blue was equilibrated in a column (15 × 1.1 cm I.D.) with 0.05 *M* Tris–HCl buffer (pH 8) containing 0.5 *M* sodium chloride. A 2-ml volume of human serum was added and the non-adsorbed serum proteins were removed with equilibration buffer. Then the adsorbed HSA was eluted with 0.05 *M* Tris–HCl buffer (pH 8) containing 1 *M* potassium thiocyanate. The amount of HSA was calculated from its absorbance at 279 nm ($A_1^{1\%}{}_{cm} = 0.531$).

Fractions of washed-out proteins and eluted HSA were pooled, dialysed against 0.01 *M* phosphate buffer (pH 7) and concentrated by ultrafiltration (UM 10 membrane; Amicon, The Netherlands).

Removal of HSA was monitored by cellulose acetate foil (CAF) electrophoresis of the starting material and the pooled and concentrated washings (see Fig. 6).

Purification of anti-human IgG from rabbit antiserum

The affinity support was prepared by coupling of 10 mg of human IgG to 2 ml of Divicell ONB-Carbonate (55 μmol active groups/ml gel); 4.8 mg of human IgG were immobilized per millilitre of gel. A 1.5-ml volume of the support was packed into a column (7 × 0.6 cm I.D.) and washed with twenty column volumes each of 0.1 *M* glycine–HCl (elution buffer), water and phosphate-buffered saline (PBS, binding buffer).

After equilibration with PBS, 1 or 2 ml of rabbit anti-human IgG serum was diluted with an equal volume of PBS containing 0.05% Tween 20 and loaded onto the column at 4°C. The flow-rate was 2 and 4 ml/h, respectively. The rabbit anti-human IgG was eluted first with 0.1 *M* glycine–HCl buffer (pH 2.8), followed by the same buffer (pH 2.2). The flow-rate in all steps was about 2–3 ml/h. Fractions of 1 ml were collected and adjusted to pH 7 with 0.5 *M* K_2HPO_4. Antibody-containing fractions were pooled and used for analysis.

Purification of human IgG from human serum

The immobilization of goat anti-human IgG to Divicell using Divicell ONB-

Carbonate (4.3 and 42.3 μmol active groups/ml gel, respectively) resulted in supports with 2.7 mg goat IgG/ml gel in each instance. Affinity chromatography was performed as described for purification of rabbit anti-human IgG. A 1-ml volume of human serum was loaded onto 2 ml of gel. Human IgG was eluted with glycine-HCl buffer (pH 2.5).

Isolation of wheat germ agglutinin (WGA) on Divicell GlcNAc

For preparation of WGA, Divicell GlcNAc (7 μmol GlcNAc/ml gel) was synthesized starting from Divicell Epoxy. A 1000-ml volume of a wheat germ extract dialysed against PBS containing 5 g of total protein were loaded onto a Divicell GlcNAc column (20 × 1.5 cm I.D.) at room temperature. WGA was eluted with 0.5 *M* formic acid at a flow-rate of 30 ml/h. Breakthrough fractions and washings were rechromatographed. Pooled fractions containing WGA were dialysed against distilled water and lyophilized. The purity of WGA was tested by SDS-PAGE and its biological acitivity by haemagglutination.

Purification of hen egg ovalbumin on Divicell Con A

Divicell Con A (8.8 mg concanavalin A/ml gel) was packed into a column (9 × 1.5 cm I.D.) and equilibrated with 0.1 *M* sodium acetate (pH 6.5) containing 0.2 *M* sodium chloride, 1 m*M* calcium chloride and 1 m*M* manganese(II) chloride. A 10-ml portion of crude ovalbumin (10 mg/ml equilibration buffer) was loaded on the column at room temperature. The specific elution was performed with equilibration buffer complemented with 0.2 *M* D-glucose (flow-rate 12 cm/h). Fractions containing ovalbumin were combined, dialysed against distilled water and lyophilized. The purity of ovalbumin was checked by SDS-PAGE. It was found that 66% of the applied protein could be recovered as electrophoretically pure ovalbumin.

Testing of purified proteins

All purified proteins were subjected to PAGE (non-denaturating system No. 1 according to Maurer [42], T = 7.5%) and 7.5–15% T gradient SDS-PAGE according to Laemmli [43], staining with Coomassie Brilliant Blue G-250.

Isolated antibodies were tested by single (SRID) [44] and double radial immunodiffusion (DRID) [45]. Titres of purified IgGs were determined by an enzyme-linked immunosorbent assay (ELISA) using 1:10 to $1:10^6$ serial dilutions of the starting and purified material. The second antibody was conjugated with alkaline phosphatase. The substrate for the indicator reaction was *p*-nitrophenyl phosphate. The conditions used for ELISA were as described by Peng *et al.* [12].

A haemagglutination assay for WGA was performed in two-fold serial dilutions with trypsinized human type A erythrocytes according to Osawa and Matsumoto [46].

RESULTS AND DISCUSSION

Characteristic properties of Divicell

Divicell swollen in water or organic solvents represents a support of regular formed beads with a cellulose content of 70–90 mg/ml. After removal of solvents and careful drying at room temperature, the beads showed distinct shrinkage. However,

TABLE I

CHARACTERISTIC PARAMETERS OF DIVICELL

Parameter	Value
Particle diameter	80–200 μm
Swelling volume	10–15 ml/g cellulose
Porosity	70–90%
Exclusion limit	$5 \cdot 10^6$–$20 \cdot 10^6$ dalton
Flow-rate at 0.2 MPa	⩾ 1000 cm/h

reswelling in solvents yielded beads with about 80% of the orginal swelling volume. Divicell accomplishes the essential parameters of a matrix for chromatographic purposes (see Table I). Depending on the preparation conditions for Divicell, the swelling volume, porosity and exclusion limit can be varied in the limits given in Table I.

The pore-size distribution was determined by mercury porosimetry and is demonstrated in Fig. 1. Although the samples of bead cellulose were dried, the results indicate the macroporosity of the matrix. From the differential curves (not shown), the mean pore diameter was calculated and was *ca.* 30 and 100 nm for the samples with exclusion limits of $5 \cdot 10^6$ and $20 \cdot 10^6$ dalton, respectively. The highly porous structure of Divicell is also evident from the electron micrograph (Fig. 2) of a sample with an exclusion limit of $5 \cdot 10^6$ dalton for the water-swollen beads. Pores with diameters up to 1 μm can be seen.

The mechanical stability and rigidity of Divicell are remarkable for a bead-formed biopolymer without cross-linking, as is demonstrated by the dependence of flow-rate on the operating pressure (Fig. 3). The flow-rate of Divicell (curve A) considerably exceeds that of cross-linked agarose (Sepharose CL-6B, curve D). A higher flow-rate, suggesting increased stability, was observed after activation and coupling of ligands (curves B and C). A reason could be a cross-linking of the matrix after activation with epichlorohydrin or by multi-point attachment of ligands. Generally, a

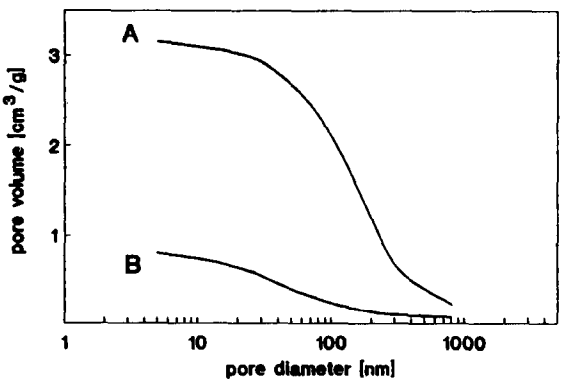

Fig. 1. Pore-size distribution for Divicell (integral curves) determined by mercury porosimetry of dried samples. Exclusion limit of Divicell swollen in water was (A) $20 \cdot 10^6$ dalton and (B) $5 \cdot 10^6$ dalton.

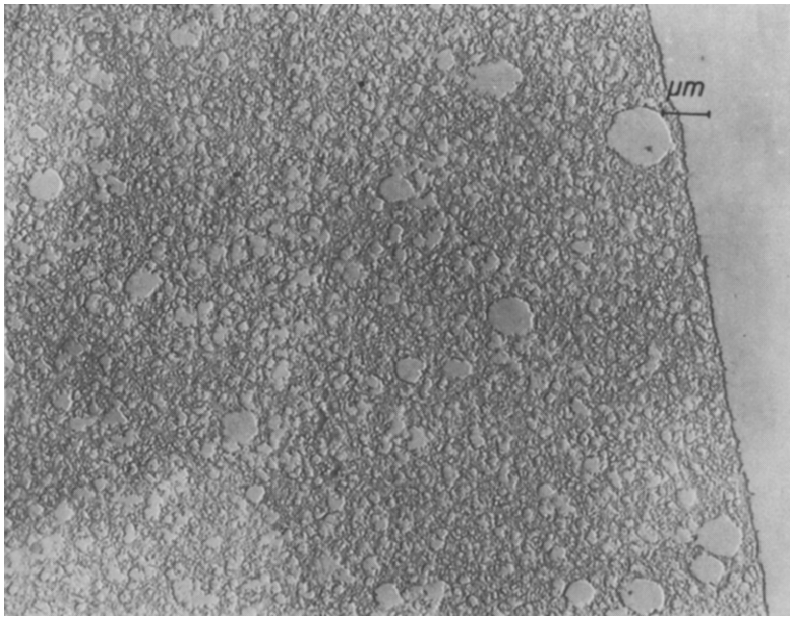

Fig. 2. Transmission electron micrograph of Divicell (ultra-thin section) showing the pore structure of the cellulose matrix.

linear increase in flow-rate was observed up to 0.2 MPa. The curves have smaller slopes for flow-rates at pressures >0.2 MPa, but, the gel bed did not collapse even at 2.0 MPa. With smaller Divicell beads (50–80 µm diameter), the linear range extended to 0.8 MPa. In addition, Divicell beads remain stable during stirred batch processes for at least 1000 h if sodium azide is added as a preservative. This high mechanical

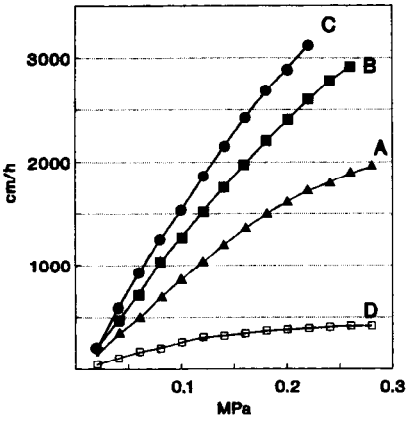

Fig. 3. Flow characteristics of Divicell supports in comparison with Sepharose CL-6B. The flow-rates were determined on a column (32 × 0.8 cm I.D.) with distilled water as mobile phase. Supports: (A) Divicell; (B) Divicell GlcNAc; (C) Divicell Con A; (D) Sepharose CL-6B.

stability recommends the application of bead cellulose supports in large-scale processes, *e.g.*, in biotechnology.

Like fibrous cellulose, Divicell shows a high resistance to many chemicals (data not shown). For instance, it is stable in 0.1 *M* hydrochloric acid or 0.1 *M* sodium hydroxide solution for 1 week at room temperature. No changes in bead size, bead shape or flow-rate could be observed after treatment of unmodified Divicell with 1 *M* sodium hydroxide solution at least 6 h at room temperature. Also, the use of concentrated salt solutions or chaotropic substances, *e.g.*, 3 *M* sodium chloride, 3 *M* potassium thiocyanate, 8 *M* urea or 6 *M* guanidinium hydrochloride, or detergents such as sodium dodecyl sulphate or Triton X-100 did not alter these parameters. The thermal stability allows autoclaving at 120°C.

Irreversible interactions could not be detected between unmodified cellulose beads and proteins. When 2 ml of human serum per millilitre of gel were loaded in buffers (ionic strength >0.1, pH >4), all proteins could be recovered in the flow-through within the detection limit of UV monitoring or the Lowry protein assay.

All these advantageous properties suggest that Divicell should be suitable for the preparation of derivatives for chromatography, *e.g.*, ion-exchange, dye–ligand or activated supports for immobilization of ligands.

Divicell DEAE, CM and Blue

Partial etherifications of hydroxyl groups of bead cellulose were performed with reactive chlorocompounds, *e.g.*, chloroethyldiethylamine, chloroacetic acid or the triazine dye Cibacron Blue F3G-A, in the presence of bases. The resulting supports were the anion exchanger Divicell diethylaminoethyl (Divicell DEAE), the cation exchanger Divicell carboxymethyl (Divicell CM) and the dye matrix Divicell Blue. They were characterized by their swelling volumes, contents of charged or dye groups and binding capacities for selected proteins such as HSA, insulin, methaemoglobin (MetHb) and lysozyme (Table II). For ion-exchange chromatography (IEC), in each instance two types (H and L) were prepared by cross-linking of the matrix with epichlorohydrin. As a consequence of the smaller exclusion limit of L-types, these supports should be used preferably for purification of peptides and proteins with molecular masses up to 30 000. H-types can be applied for purification of large proteins (MW $>$ 30 000). The ion-exchange capacity was 1–2 mequiv./g dry cellulose (0.07–0.3 mequiv./ml gel). The binding capacities for proteins were 75–200 mg/ml sedimented gel, depending on the applied support or the protein. In chromatographic columns the operating pressure can be raised to 0.1 and 0.3 MPa for the H- and L-derivatives, respectively. In stirred reactors with suspended paddles, Divicell DEAE and CM were stable over a period of 500 h at 300 r.p.m.

Divicell Blue was prepared by modifying the usual procedure [47] (see Experimental). By varying the experimental conditions the amount of coupled dye could be stretched over a wide range (2–15 mg dye/ml gel). The covalent linkage between dye and cellulose is more stable than that in other polysaccharides, *e.g.*, dextran or agarose. Acid hydrolysis of Divicell Blue produces no quantitative cleavage of the dye–cellulose bond under conditions described for other polysaccharides [9,48].

Immobilized Cibacron Blue F3G-A possesses an affinity to nucleotide cofactor-dependent enzymes and blood proteins [8]. For instance, Travis and co-workers [49,50] purified albumin both on Blue dextran-Sepharose and on a material where the

TABLE II

SELECTED DATA FOR DIVICELL DEAE, CM AND BLUE

The swelling volume was calculated from the content of cellulose per millilitre of sedimented gel (drying at 105–110°C for 24 h). The capacity of ion exchangers was determined by titration. For determination of the binding capacity for proteins the following buffers were used for binding/elution: (1) 0.05 *M* Tris–HCl (pH 8)/the same buffer containing 1 *M* NaCl; (2) 0.05 *M* Tris–HCl with 50% (v/v) isopropanol (pH 8)/the same buffer containing 1 *M* NaCl; (3) and (4) 0.05 *M* acetate (pH 6)/the same buffer containing 2 *M* NaCl; (5) 0.01 *M* phosphate (pH 5)/the same buffer containing 1 *M* KSCN.

No.	Divicell derivative	Swelling volume (ml/g cellulose)	Ion-exchange capacity (mequiv./g cellulose)	Binding capacity (mg protein/ml gel)	
1	DEAE-H[a]	12–14	1.2	HSA	100
2	DEAE-L[b]	6– 8	2.0	Insulin	75
3	CM-H[a]	12–14	1.0	MetHb	100
4	CM-L[b]	7– 9	1.4	Lysozyme	200
5	Blue	10–15	5-10[c]	HSA	50

[a] Exclusion limit $1 \cdot 10^6$ dalton.
[b] Exclusion limit $3 \cdot 10^4$ dalton.
[c] mg dye/ml gel.

dye was bound directly to agarose (Blue Sepharose). When the dye was coupled to fibrous cellulose, albumin was not separated from other proteins [51,52]. With Divicell Blue, however, it was possible to adsorb HSA selectively [8]. The binding capacity for a support with 3.6 mg of dye was *ca.* 45 mg/ml gel if HSA was diluted in 0.01 *M* phosphate buffer (pH 5). The amount of adsorbed HSA was lower using 0.05 *M* Tris–HCl (pH 8). An example of the application of Divicell Blue for the isolation of HSA from human serum is shown later in Fig. 6.

Activation of Divicell with sodium periodate and immobilization of proteins to Divicell Dialdehyde

By treatment of Divicell with sodium periodate, the vicinal hydroxyl groups in positions 2 and 3 of the glucose unit are oxidized to aldehyde groups with splitting of the C–C bond (Fig. 4). The activation procedure is very simple and can be stopped by addition of ethylene glycol. The degree of oxidation can be adjusted over a broad range by variation of the reaction time or temperature and the concentration of sodium periodate. Divicell Dialdehyde could be stored over a period of 2 years as an aqueous suspension in the presence of 0.02–0.04% sodium azide at 4°C. During that time no decrease in the degree of activation or coupling capacity for proteins was observed [28]. The mechanical stability of the activated gel expressed as pressure–flow behaviour was not diminished in comparison with the unmodified material (Fig. 3, curve A). This finding was surprising, as it had been expected that the support would lose its excellent flow properties because of cleavage of glucose rings [10,53].

The immobilization of proteins to Divicell Dialdehyde was carried out under mild conditions (6 h at room temperature). Using constant protein concentrations for coupling, not only the amount of immobilized protein but also the coupling yield increased proportionally to the degree of activation (Table III). Of course, the cou-

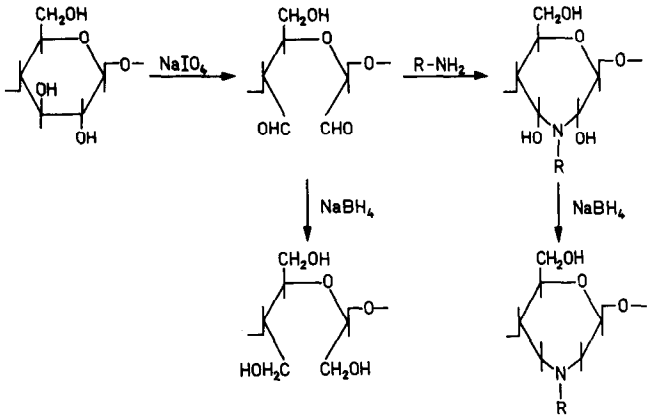

Fig. 4. Reaction scheme for periodate oxidation of glucose units of cellulose and subsequent covalent binding of ligand (RNH_2), completed by reduction with $NaBH_4$.

pling yield also depends on the kind of protein. Applying the same conditions, the yield for immobilization of MetHb was double that for human γ-globulin. The maximum yield was up to 82% of the offered MetHb. After coupling of proteins, the unreacted aldehyde groups can be blocked with ethanolamine. A reduction with sodium borohydride (see Fig. 4) is recommended to stabilize the covalent bond between ligand and matrix by conversion of the carbinolamines or Schiff's bases formed into stable C–N bonds. Immobilized MetHb was able to bind oxygen reversibly and showed a pseudo-peroxidatic activity as measured by oxidation of aniline to 4-aminophenol.

Activiation of Divicell with epichlorohydrin and immobilization of ligands to Divicell Epoxy

The activation of supports with epoxy compounds, *e.g.*, epichlorohydrin or 1,4-butanediol diglycidyl ether, has been described previously [30–33, 54]. The reac-

TABLE III

IMMOBILIZATION OF PROTEINS TO DIVICELL DIALDEHYDE AS A FUNCTION OF CONCENTRATION OF ACTIVE GROUPS

Standard conditions were used as described under Experimental.

Preparation of Divicell Dialdehyde			Protein	Immobilized amount (mg/ml gel)	Coupling yield (%)
Amount of $NaIO_4$ (mg/ml gel)	Reaction time (h)	Amount of active groups (μmol/ml gel)			
13	2	21	MetHb	3.9	48
13	6	40	MetHb	4.9	60
26	4	71	MetHb	6.7	82
13	6	48	Human γ-globulin	2.8	28

tivity of epoxy groups is relatively low. Therefore, for coupling of ligands strong alkaline conditions, elevated temperatures, high concentrations of ligands and long reaction times were used. Advantages of epoxy-activated gels are the increased stability by cross-linking of the matrix during activation, the ability to couple compounds containing hydroxyl groups and the high stability of the ether or C–N bond between the support and attached ligands (minimized leakage). The degree of activation of Divicell Epoxy could be varied from 20 to about 50 μmol/ml gel (Table IV). The epoxy support could be stored in isopropanol or acetone at 4°C for 1 and 2 years, respectively, without a substantial decrease in activity.

The hydrolysis of active groups was investigated at pH 9.5 and 11.0 at 40°C and compared with that of epoxy-activated Sepharose 6B [55]. It was found that the rate of hydrolysis of epoxy groups for the bead cellulose derivative was significantly lower. After 24 h the percentage of epoxy groups remaining on the Divicell support was 80% (pH 9.5) and 40% (pH 11) and on the corresponding Sepharose support 50% and 14%, respectively. Complete deactivation of Divicell Epoxy was achieved in 0.1 *M* sodium hydroxide solution at room temperature after 7 days or by reaction with 1 *M* ethanolamine (pH 9) for 24 h at room temperature.

Similar flow properties were obtained for Divicell Epoxy and Divicell GlcNAc prepared by coupling of N-acetyl-D-glucosamine (GlcNAc) to the epoxy support (Fig. 3, curve B).

A large excess of ligands was necessary for immobilization to Divicell Epoxy (Table IV). Using low-molecular-mass ligands, such as 4-nitroaniline, 1,6-diaminohexane (1,6-DAH), IDA, GalNAc and GlcNAc, up to a fifteenfold molar excess with respect to the active groups was added. High immobilization rates of coupled ligands

TABLE IV

IMMOBILIZATION OF LIGANDS TO DIVICELL EPOXY

Coupling medium: (1) ethanol; (2) 0.1 *M* borate (pH 8.3); (3) 2 *M* Na_2CO_3; (4) and (5) 0.1 *M* NaOH; (6) 1 *M* potassium phosphate (pH 8); (7) water adjusted to pH 11 with 0.1 *M* NaOH; (8) 1 *M* potassium phosphate (pH 7.5). For abbreviations, see text.

No.	Amount of epoxy groups (μmol/ml gel)	Ligand	Added amount of ligand (μmol/ml gel)	Temperature (°C)[a]	Time (h)	Immobilized ligand (μmol/ml gel)	Coupling yield relative to to active groups (%)
1	20	4-Nitroalinine	72	r.t.	48	20	100
2	20	1,6-DAH	200	r.t.	72	7.6	38
3	46	IDA	680	65	24	35	76
4	40	GlcNAc	600	37	24	7	18
5	46	GalNAc	300	37	24	9	20
6	21	Human γ-globulin	50[b]	r.t.	65	6.9[b]	14[c]
7	40	Heparin	80[b]	r.t.	72	0.8[b]	1[c]
8	35	Penicillin acylase	115[d]	r.t.	48	46[d]	40[c]

[a] r.t. = Room temperature.
[b] mg/ml gel.
[c] With respect to amount of ligand added.
[d] Units/ml gel.

depended on the degree of activation. With GlcNAc, 2, 4 and 6 μmol were bound on supports with 10, 20 and 30 μmol epoxy groups/ml gel, respectively. The fixation of GlcNAc or GalNAc to highly activated gels yielded supports that were comparable to the corresponding Sepharose-derived supports [32,56]. The Divicell derivatives obtained were used successfully for the purification of lectins (see Table IX).

The maximum yield of coupled proteins was 40%. The enzyme activity of immobilized penicillin acylase was 530 units/g cellulose, which is about half of that found by Burg *et al.* [57] after coupling to an epichlorohydrin-activated synthetic polymer.

Activation of Divicell with Cl-CO-ONB and immobilization of ligands to Divicell ONB-Carbonate

The activation of supports with Cl-CO-ONB was described recently by Büttner *et al.* [13]. The activation was performed in anhydrous organic solvents and, in general, highly activated gels were used for coupling of ligands. However, in our investigations the activated supports were always prepared by conversion of Divicell with Cl-CO-ONB in non-dried solvents, especially acetone. The resulting Divicell ONB-Carbonate (Fig. 5) was stored in acetone containing *ca.* 0.3% of water. Only a small decrease (maximum 5–10%) in active groups was observed over a period of 2 years. The amount of ONB-carbonate groups could be reproducibly adjusted in the range of 4–80 μmol/ml gel.

Covalent binding of ligands was carried out both in organic solvents and in aqueous solutions (Tables V and VI). Owing to the higher reactivity of ONB-carbonate groups compared with epoxy groups, the immobilization of ligands takes place under milder conditions (lower temperature and pH, shorter coupling times). The attachment of compounds with only hydroxyl groups failed. Immobilization of ligands bearing amino groups resulted in high coupling yields. In most instances, for immobilization of small molecules an excess of ligands was applied in up to twenty times the amount of active groups (Table V). Coupling of 1,6-DAH gave higher yields in isopropanol than in an aqueous medium.

Data obtained by testing the flow characteristics led to the assumption that the Divicell matrix was cross-linked by 1,6-DAH. The flow-rate was similar to that found for Divicell Con A (Fig. 3, curve C), whereas when ethanolamine was coupled the flow-rates were identical with those for the unmodified Divicell (Fig. 3, curve A).

Results for immobilization of proteins are given in Table VI. The coupling conditions were tested over the pH range 5.0–8.5 in different buffers for between 1 and 16 h. The coupling yield was 70–90% in most instances. Only for coupling of human γ-globulin at pH 5.0 and of BSA and protein A using low-activated supports

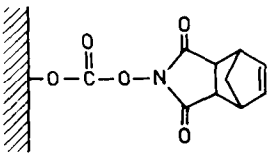

Fig. 5. Structure of active groups on Divicell ONB-Carbonate (NB = 5-norbornene-2,3-dicarboximidyl residue).

TABLE V

IMMOBILIZATION OF LIGANDS TO DIVICELL ONB-CARBONATE

Coupling medium: (1) acetone; (2) dioxane; (3) 0.1 *M* $NaHCO_3$ (pH 9); ; (4) isopropanol; (5) 0.1 *M* borate (pH 8.5); (6) 0.1 *M* borate (pH 8.3); (7) 0.1 *M* $NaHCO_3$ (pH 9); (8) 0.1 *M* borate (pH 8.9).

No.	Amount of active groups (μmol/ml gel)	Ligand	Added amount of ligand (μmol/ml gel)	Temperature (°C)[a]	Time (h)	Immobilized ligand (μmol/ml gel)	Coupling yield relative to active groups (%)
1	34	*n*-Pentylamine	38	r.t.	1	34	100
2	34	Benzylamine	38	r.t.	1	27	78
3	80	1,6-DAH	800	4	20	16	20
4	26	1,6-DAH	520	r.t.	2	17	65
5	44	GalN[b]	150	4	18	27	61
6	60	Glycine	180	r.t.	16	60	100
7	30	ε-ACS[c]	600	r.t.	18	30	100
8	39	L-Lysine	390	r.t.	2	23	60

[a] r.t. = Room temperature.
[b] D-Galactosamine.
[c] ε-Aminocaproic acid.

TABLE VI

IMMOBILIZATION OF PROTEINS TO DIVICELL ONB-CARBONATE

Coupling buffer: (1) PBS; (2) and (3) 0.1 *M* borate (pH 8.3); (4) 3 m*M* phosphate (pH 7.0); (5) 0.1 *M* acetate (pH 5.0); (6) and (7) 0.07 *M* phosphate (pH 7.4) containing 0.15 *M* NaCl; (8) and (9) 0.1 *M* borate (pH 8.3); (10) 0.1 *M* acetate (pH 6) containing 0.2 *M* NaCl; (11) 0.1 *M* phosphate (pH 7.5); (12) 0.1 *M* phosphate (pH 8.0); (13) 0.1 *M* borate (pH 8.5) containing 10 m*M* $CaCl_2$

No.	Amount of active groups (μmol/ml gel)	Protein	Added amount of protein (mg/ml gel)	Temperature (°C)[a]	pH	Time (h)	Immobilized protein (mg/ml gel)	Coupling yield (%)
1	4.3	Goat anti-human IgG	3.6	4	7.3	16	2.7	75
2	8.8	Human IgG	5.0	4	8.3	16	4.6	92
3	51	Human IgG	5.0	4	8.3	16	4.8	96
4	6.7	Human IgG	1.1	r.t.	7.0	2	1.0	91
5	30	Human γ-globulin	12.0	r.t.	5.0	2	7.2	60
6	4.4	BSA	1.0	4	7.4	2	0.4	40
7	30	BSA	10.0	4	7.4	2	8.7	87
8	26	Con A	10.0	r.t.	8.3	1	7.1	71
9	65	Con A	25.0	r.t.	8.3	16	17.5	70
10	26	Con A	10.7	4	6.0	2	8.8	88
11	7.5	Protein A	3.0	r.t.	7.5	3	1.5	50
12	26	Protein A	2.0	r.t.	8.0	16	1.8	90
13	80	Trypsin	350[b]	r.t.	8.5	6	300[b]	86

[a] r.t. = Room temperature.
[b] Units/ml gel.

(4.4 and 7.5 μmol of active groups/ml gel, respectively) was the yield diminished to 40–60%. The amounts of covalently bound proteins ranged from 0.4 up to 17.5 mg/ml gel.

After coupling of proteins, the supports also possess improved flow properties, as shown for immobilized Con A in Fig. 3 (curve C). The biological activities of immobilized proteins were retained at all times (see Tables VII and IX). An application of sorbents in affinity chromatography is demonstrated for coupled IgG, Con A and protein A. Immobilized enzymes proved to be highly effective biocatalysts. The maximum activities of bound trypsin and penicillin acylase were 3300 and 1900 units/g cellulose, respectively. The specific activities are significantly higher than those reported for trypsin–agarose from Sigma, trypsin coupled to sodium periodate-activated bead cellulose [10] and for penicillin acylase immobilized to a synthetic polymer [57], and corroborate the successful application of Divicell supports for biotransformation processes. Trypsin–bead cellulose was used for the catalytic conversion of porcine insulin into human insulin ethyl ester [15,16] and coupled penicillin acylase for the manufacture of 6-aminopenicillanic acid from culture broths [19]. No loss of activity was found for either support after multiple reuse over a period of 500 h. These results correspond to the finding that the stability of immobilized trypsin was much higher than that of the soluble enzyme, as described by Guisan and Blanco [58].

Sorbents containing ligands coupled via ONB-carbonate groups can also be used with advantage because of the minimum leakage due to the stable urethane linkage formed between ligand and matrix [13]. Splitting off of [^{14}C]glycine immobilized to Divicell ONB-Carbonate was similar as for Divicell Epoxy under acidic (pH 4) or alkaline (pH 8.3) conditions (< 1% after 1200 h at room temperature). In comparison, a leakage of 1% of [^{14}C]glycine was observed after 40 h (pH 8.3) and 220 h (pH 4), respectively, with a support prepared by coupling to cyanogen bromide-activated Sepharose [59].

Binding capacity for proteins on Divicell sorbents

The binding capacity of Divicell derivatives was tested by both static and dynamic methods. The amounts of bound proteins were determined by application either of a single protein (Table VII) or by isolation of proteins from crude products, such as seed protein extracts or sera (see Table IX). The binding capacities ranged from 1 to 30 mg/ml gel, depending on the sorbent and the applied protein.

The capacities of Divicell sorbents were compared with those for commercially available Sepharose supports. For instance, Lysine–Sepharose 4B (4–5 μmol lysine/ml gel) binds about 0.6 mg plasminogen/ml gel (Pharmacia data sheet). The higher capacity for Divicell Lysine as indicated in Table VII can be attributed to the greater amount of immobilized lysine.

Protein A–Sepharose CL-4B (2 mg protein A/ml gel) adsorbs about 20 mg human IgG/ml gel (Pharmacia data sheet). Probably the lower binding of human IgG to Divicell Protein A (example 7, Table VII) is due to possible steric hindrance during the formation of the protein A–human IgG complex. By decreasing the immobilized amount of protein A to bead cellulose to 0.5 mg/ml gel, the reversible adsorption of human IgG is also diminished (example 8, Table VII), but calculated with respect to 1 mg of attached protein A this is of the same order of magnitude as for Sepharose supports.

TABLE VII

BINDING CAPACITY OF DIVICELL SORBENTS FOR PROTEINS

The following buffers were used for binding/elution of proteins: (1) 0.05 *M* phosphate (pH 7.4) containing 2 *M* $(NH_4)_2SO_4$/6 *M* urea; (2) and (3) PBS/0.5 *M* formic acid; (4) 0.1 *M* phosphate (pH 7.5)/the same buffer containing 0.015 *M* ε-ACS; (5) 0.05 *M* phosphate (pH 7.0)/ 50 m*M* glycine; (6) 0.1 *M* acetate (pH 6.0)/50 m*M* EDTA; (7) and (8) 0.1 *M* phosphate (pH 7.2) containing 0.15 *M* NaCl/0.1 *M* glycine–HCl (pH 2.2); (9) 0.1 *M* acetate (pH 6.0) containing 0.15 *M* NaCl, 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$/the same buffer containing 0.2 *M* methyl-α-D-glucoside.

No.	Divicell derivative	Immobilized ligand per ml gel ml gel	Bound protein	Binding capacity (mg/ml gel)
1	Pentyl	34[a]	BSA	23.0
2	GalN	37[a]	SBA	2.4
3	Ovalbumin	6.0[b]	WGA	2.0
4	Lysine	23[a]	Plasminogen	1.5
5	IDA–Cu^{2+}	36[c]	Con A	24
6	IDA–Zn^{2+}	35[c]	Plasminogen	14
7	Protein A	1.5[b]	Human IgG	9.4
8	Protein A	0.5[b]	Human IgG	4.7
9	Con A	6.6[b]	Ovalbumin	3.1

[a] μmol.
[b] mg.
[c] μg-atoms.

The following results were obtained by comparison of the respective concanavalin A derivatives: Divicell Con A adsorbs 3.1 mg ovalbumin/ml gel (see Table VII) and Con A–Sepharose (13 mg Con A/ml gel) has a capacity of 2.8 mg, although its content of immobilized Con A is about twice as high as for the corresponding Divicell derivative.

Cu^{2+} and Zn^{2+} chelates have frequently been used in immobilized metal ion chromatography for the purification and separation of human serum proteins, enzymes and lectins [33,34,60–63]. Therefore, Divicell IDA–Cu^{2+} and –Zn^{2+} were prepared by loading of IDA-bead cellulose with Cu^{2+} and Zn^{2+} salts. By testing their reversible adsorption of proteins, Con A and plasminogen were selected for the Cu^{2+} and Zn^{2+} chelates, respectively. In either instance the binding capacity was found to be the highest compared with the other Divicell affinity supports.

Hydrophobic interaction chromatography (HIC) on Divicell alkyl supports

HIC is a useful tool in the separation of proteins [64]. We tested the suitability of hydrophobic Divicell supports prepared from Divicell ONB-Carbonate for adsorption and desorption in a model system using BSA (Table VIII). The density of ligands was 27–34 μmol/ml gel. The binding capacity of BSA to sorbents containing short-chain alkyl residues was 21–23 mg/ml gel. It increased to 29 (octyl derivative) and 47 mg/ml gel (decyl or dodecyl support).

In accordance with other workers [65] who described an increase in interaction forces between an HIC support and proteins with increasing alkyl chain length, in our system the elution conditions for supports with longer alkyl residues had to be much

TABLE VIII

BINDING CAPACITIES AND RECOVERY FOR BOVINE SERUM ALBUMIN

Preparation of supports was carried out according to the pentyl derivative (see Table V). The coupling yield was 90–100% except for Divicell Benzyl. In each instance 100 mg of BSA were loaded on a column (2.5 × 1 cm I.D.) at room temperature. The binding buffer was 0.05 *M* phosphate (pH 7.4) containing 2 *M* $(NH_4)_2SO_4$.

Divicell derivative	Immobilized ligand (μmol/ml gel)	Capacity for BSA (mg bound/ml gel)	Recovery (%)	Elution medium
Propyl	32.3	23.5	99	0.05 *M* phosphate (pH 7.4)
Butyl	30.6	22.4	99	0.05 *M* phosphate (pH 7.4)
Pentyl	34.0	23.0	95	6 *M* urea
Hexyl	34.0	21.3	95	50% acetonitrile[a]
Octyl	34.0	28.7	85	50% acetonitrile[a]
Decyl	27.1	47.0	90	80% methanol[a]
Dodecyl	27.1	47.2	90	80% methanol[a]
Benzyl	26.7	29.7	90	6 *M* urea

[a] Mixture with water (v/v).

more drastic, whereas variation of the ligand density over a certain range (10–55 μmol/ml gel) had no substantial effect.

Reuse of Divicell Pentyl was tested with a series of 22 loading–elution cycles. The support was regenerated with 1 *M* sodium hydroxide solution after ten cycles each time. For each loading–elution cycle the recovery of BSA was 93.0 ± 1.5% (n = 20, determined by UV absorbance). Only traces of protein were eluted during regeneration (simulation of cleaning-in-place). No significant changes in binding capacity, recovery or flow-rate could be observed during these experiments.

Application of Divicell sorbents in affinity chromatography

Purification of proteins. Selected examples of the application of Divicell sorbents are presented in Table IX. Sorbents derived from activated supports were prepared by coupling of ligands to Divicell ONB-Carbonate (Nos. 2–6) and to Divicell Epoxy (Nos. 7–9). Each purified protein was checked by electrophoresis (CAF, PAGE and SDS-PAGE) and fulfilled the purification criteria.

Starting from biological material, the amount of bound protein per millilitre of sorbent was lower than expected from the binding capacity determined using a defined protein solution (Table VII). For instance, to the lysine support 1.5 mg (Table VII) and 0.8 mg (Table IX) of plasminogen were bound by application of a plasminogen solution and human serum, respectively, and to the protein A support 4.7 mg (Table VII) or 4.2 mg (Table IX) of human IgG were adsorbed when a human IgG solution or human serum was used.

Generally, the binding capacities were sufficient for the isolation of 10–500-mg amounts of the proteins listed in Table IX using small columns.

Dye–ligand affinity chromatography on Divicell Blue. The application of Divicell Blue for purification of HSA from human serum is demonstrated in Fig. 6. Under the conditions used only HSA was specifically adsorbed on the support. The other serum proteins could be washed out with equilibration buffer (first peak). This was proved

TABLE IX

DIVICELL SORBENTS FOR CHROMATOGRAPHIC PURIFICATION OF PROTEINS

The following buffers were used for binding/elution of proteins: (1) 0.05 *M* Tris–HCl (pH 8.0) containing 0.5 *M* NaCl/the same buffer containing 1 *M* KSCN; (2) PBS containing 0.05% Tween 20/0.1 *M* glycine–HCl (pH 2.8 and 2.2); (3) PBS containing 0.05% Tween 20/0.1 *M* glycine–HCl (pH 2.5); (4) 0.1 *M* phosphate (pH 7.2) containing 0.15 *M* NaCl/0.1 *M* glycine–HCl (pH 2.3); (5) 0.1 *M* acetate (pH 6.5) containing 0.2 *M* NaCl, 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$/the same buffer containing 0.2 *M* D-glucose; (6) 0.1 *M* phosphate (pH 7.5)/the same buffer containing 0.015 *M* ε-ACS; (7)–(9) PBS/0.5 *M* formic acid.

No.	Divicell sorbent	Immobilized ligand (mg/ml gel)	Applied material[a] per ml gel	Purified protein	Bound protein (mg/ml gel)
1	Blue	6–8	0.2 ml human serum	HSA	10–15
2	Human IgG	4.8	2 ml rabbit antiserum	Rabbit anti-human IgG	2.5
3	Goat anti-human IgG	2.7	1 ml human serum	Human IgG	1.5
4	Protein A	0.5	0.5 ml human serum	Human IgG	4.2
5	Con A	8.8	6.7 mg crude ovalbumin	Ovalbumin	4.4
6	Lysine	23[b]	15 ml human serum	Plasminogen	0.8
7	GlcNAc	7[b]	125 mg total protein[c]	WGA	7.2
8	GalNAc	9[b]	75 mg total protein[c]	SBA	2.6
9	GalNAc	9[b]	150 mg total protein[c]	PNA	10.0

[a] Dissolved in binding buffer.
[b] μmol /ml gel.
[c] From plant seed extract.

by CAF electrophoresis (see insets in Fig. 6). More than 95% of the HSA was removed from human serum with only low non-specific adsorption of other proteins. The recovery for HSA was about 80%.

Immunoaffinity chromatography. The model system human IgG–anti-human IgG was chosen. First, human IgG was immobilized (4.8 mg/ml gel) to purify the corresponding antibodies from antiserum. The chromatographic profile is shown in Fig. 7. The total amount of purified rabbit anti-human IgG was 2.5 mg/ml gel.

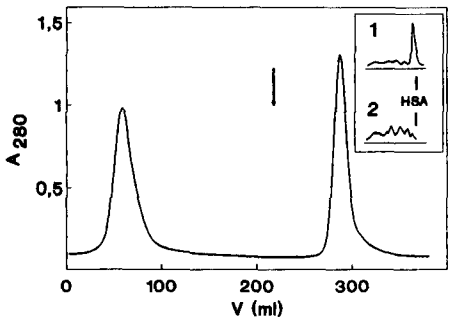

Fig. 6. Isolation of HSA from human serum using Divicell Blue (see Experimental). The arrow indicates the elution of HSA with Tris–HCl buffer containing 1 *M* KSCN. In the insets the densitogram for cellulose acetate foil (CAF) electrophoresis of human serum is shown (1) before and (2) after affinity chromatography.

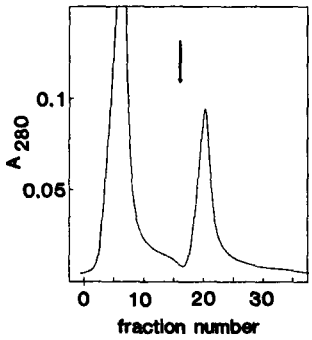

Fig. 7. Purification of rabbit anti-human IgG by affinity chromatography on a column of human IgG immobilized to Divicell (see Experimental). A 2-ml volume of rabbit antiserum were applied. After washing the antibodies were eluted with 0.1 *M* glycine–HCl buffer (pH 2.8) (arrow).

Approximately 90% of the antibodies were eluted at pH 2.8 and 10% by subsequent elution at pH 2.2. The purity of the antibodies was tested by PAGE (Fig. 8).

Electrophoresis suggests that pure IgG was eluted. For comparison with an agarose support, human IgG–Sepharose was prepared from CNBr–Sepharose 4B. The immobilized amount was 5 mg human IgG/ml gel and the yield of antibodies was 2.8 mg. Also, no contamination was detectable by PAGE (Fig. 8, gel No. 5).

The amounts of IgG eluted were also determined by SRID with sheep anti-rabbit IgG and rabbit IgG as standard. The activity of antibodies was checked by

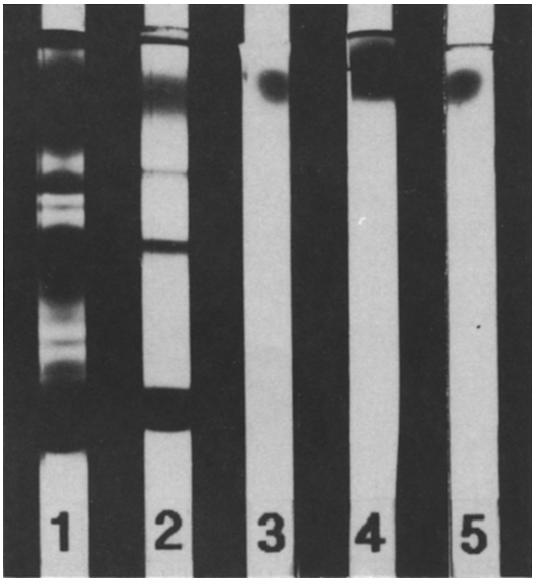

Fig. 8. PAGE of (1) rabbit anti-human IgG serum, (2) PBS-washing fraction, (3) 15 μg and (4) 100 μg of rabbit anti-human IgG eluted from a Divicell column at pH 2.8 and (5) 15 μg of rabbit anti-human IgG eluted from a human IgG–Sepharose column at pH 2.8.

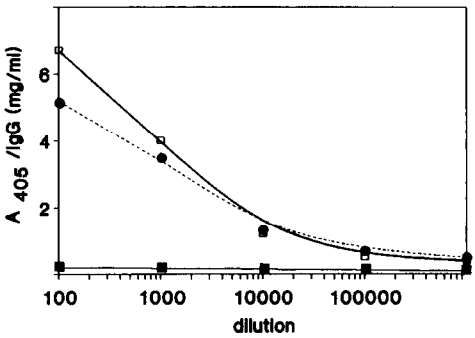

Fig. 9. Determination of the activity of rabbit anti-human IgG eluted at pH 2.8 by an ELISA. Serial dilutions were made from (□) antibodies (360 μg/ml) eluted from a Divicell column, and (●) antibodies (400 μg/ml) eluted from a Sepharose column and (■) from the starting rabbit anti-human IgG serum. First human IgG was bound on microtitre plates, then diluted samples were added to the wells. After binding of antibodies, goat anti-rabbit IgG conjugated with alkaline phosphatase was used as the second antibody. The substrate was 4-nitrophenylphosphate. Amounts of 4-nitrophenolate formed were measured spectrophotometrically at 405 nm.

DRID and ELISA. The results of ELISA are demonstrated in Fig. 9. The graphical representation shows that the antibodies eluted from the Divicell column gave higher titres per milligram of IgG than those eluted from the Sepharose sorbent. A similar relationship was obtained by comparison of the eluates at pH 2.2. However, the specific affinity of antibodies against human IgG was about 50% higher in the pH 2.2 eluates for both Divicell and Sepharose adsorbents, showing that the antibodies with the highest affinity were desorbed under stronger elution conditions.

For the opposite example, goat anti-human IgG was coupled to Divicell to isolate human IgG from human serum. The amount of IgG eluted was 1.5 mg/ml gel when the activation level of Divicell ONB-Carbonate was low (4.3 μmol active groups/ml gel) and decreased to 0.35 mg/ml when a high-activated support (42.3 μmol/ml gel) was used for immobilization of antibodies.

Our results conflict with those of Peng *et al.* [12], who found that high binding efficiencies were only achieved when the concentration of goat anti-human IgG immobilized to a cross-linked bead cellulose was less than 0.2 mg/ml gel. A reduction in the efficiency from 55 to 5% was reported when the concentration of the antibody was increased to more than 2.0 mg/ml gel. In this attempt the efficiency of 100% was fixed at a 1:1 ratio of immobilized antibody to adsorbed human IgG. In our experiments we determined an efficiency of 55% for 2.7 mg of immobilized goat anti-human IgG. We assume that this apparent contradiction is due to the higher macroporosity of Divicell, which diminished steric effects caused by the matrix, or to the lower activation level of the support, preventing multi-point attachment of immobilized antibody, which may decrease its efficiency.

Purification of lectins. The affinity of many proteins to carbohydrates can be used for the chromatographic separation and purification of lectins [66]. For this purpose GlcNAc and GalNAc immobilized to Divicell Epoxy were utilized for purification of WGA, SBA and PNA from the corresponding plant seed extracts (see Table IX). For instance, the elution profile of WGA is shown in Fig. 10. The isolated

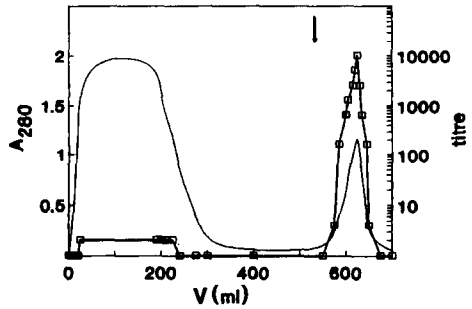

Fig. 10. Purification of WGA from a crude wheat germ extract on Divicell GlcNAc (see Experimental). Chromatography was monitored by measuring absorbance at 280 nm for determination of protein and by a haemagglutination assay presented as a titre. The arrow indicates elution with 0.5 *M* formic acid.

amount of WGA was 420 mg from 5 g of total protein in the crude extract obtained from 1 kg of wheat germs. The binding capacity of WGA is comparable to that described for Sepharose affinity sorbents [32,56]. The eluted WGA was electrophoretically pure (SDS-PAGE) and showed a high biological activity. The titre in the haemagglutination assay was about 2000 in dilution series starting with 10 mg WGA/ml.

With reuse of Divicell GlcNAc, the amount of WGA eluted decreased after 8 cycles to 0.2 mg/ml gel. Probably deacetylation of bound GlcNAc by formic acid was partly responsible for the diminished efficiency, because after reacetylation and further use of the sorbent the WGA eluted increased to 2.4 mg/ml gel.

The purification of SBA and PNA on Divicell GalNAc was carried out under similar conditions to those described for WGA (see Table IX). When Divicell GalN prepared from Divicell ONB-Carbonate was applied, a binding capacity for SBA of 2.4 mg/ml gel was obtained.

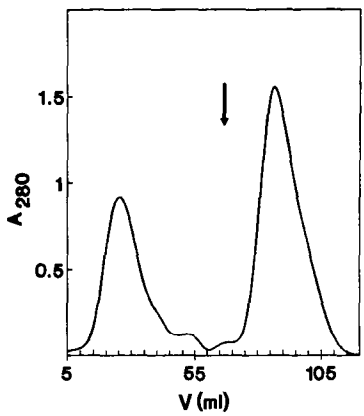

Fig. 11. Affinity chromatographic purification of ovalbumin on Divicell Con A (see Experimental). Ovalbumin was eluted with equilibration buffer complemented with 0.2 *M* D-glucose (arrow).

Purification of ovalbumin. The high biological activity of Con A attached to Divicell ONB-Carbonate could be demonstrated (see above). Therefore, this sorbent is suitable for the purification of glycopeptides or glycoproteins containing oligosaccharides with glucose and mannose residues [67]. We used Divicell Con A in the chromatography of ovalbumin from hen eggs as a model (see Fig. 11). Elution of adsorbed protein with buffer containing D-glucose led to electrophoretically pure ovalbumin (recovery 66%) and was similar to results that were obtained using Con A–Sepharose.

CONCLUSIONS

The bead cellulose Divicell has been demonstrated to be a support with advantageous properties. Further modifications of the matrix were possible with respect to the preparation of many different supports and for use of these Divicell derivatives in ion-exchange, hydrophobic interaction and affinity chromatography or related techniques. Chemical modifications by activation and coupling of ligands did not change the macroporosity and the advantageous chromatographic properties. Because of the very good mechanical and chemical stability, the high biological activity of immobilized ligands and the high yields of purified proteins, Divicell supports are especially suitable for the use in large-scale protein separations.

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