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Selective adsorption of immunoglobulins and glucosylated proteins on phenylboronate–agarose

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ABSTRACT

Aminophenylboronate-substituted agarose in 20 m*M* N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, pH 8.5, selectively adsorbs immunoglobulins and complement factors C3 and C4 from human serum. The selectivity of binding is strongly influenced by the presence of magnesium chloride in the sample buffer. Adsorbed immunoglobulins are quantitatively eluted by sorbitol, but only partially by ethylene glycol or methylcellosolve. Aniline–agarose of a similar degree of substitution shows only weak adsorption of serum proteins under similar experimental conditions, thus indicating the important contribution of the boronate moiety to this interaction. Immunoglobulin adsorption seems not to be due to the *cis*-diol complexation used extensively for the chromatographic determination of non-enzymatically glucosylated proteins. Hydrophobic and π–π interactions with the aromatic structure of the ligand seem also to contribute to protein binding. The behaviour of aminophenylboronate-liganded agarose is, in some respects, rather similar to that of the so-called "thiophilic adsorbents".

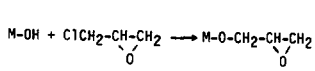
INTRODUCTION

Boronate is known to reversibly esterify certain carbohydrates containing 1,2-*cis*-diol structures, and this is also the case with organic boronic acids [1,2]. Aminophenylboronic acid coupled to agarose (PBA) is therefore a potential adsorbent for carbohydrate-containing proteins. It has been used successfully for monitoring diabetes [3,4] since glucosylated haemoglobin and glycosylated albumin and other serum proteins are markers of blood glucose control [5]. The non-enzymatically glucosylated proteins interact via the exposed glucose groups.

However, non-glucose-containing glycoproteins and other proteins may also interact with the aromatic boronate ligand. The present investigation was undertaken to find out whether other serum proteins might be adsorbed and, if so, to reveal the nature of the interaction.

The following reaction schemes summarize the steps employed for the synthesis of aminophenylboronate–agarose:

(1) Activation of the agarose matrix.



(1)

(2) Coupling of *m*-aminophenylboronic acid.

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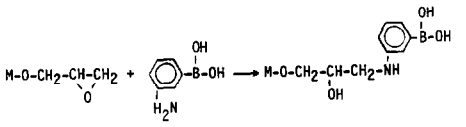
(2)

MATERIALS AND METHODS

Materials

Agarose (Ultrogel A-6) was purchased from LKB (Bromma, Sweden); *m*-aminophenylboronic acid hemisulphate, aniline, sodium borohydride and epichlorohydrin were obtained from Fluka (Buchs, Switzerland) and Glico Gel B was from Pierce (Rockford, IL, USA). N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) and nicotinamide adenine dinucleotide (NAD^+) were products from Sigma (St. Louis, MO, USA). Human serum albumin (HSA) was kindly supplied by Kabi Vitrum (Stockholm, Sweden). Human total serum was obtained from the University Hospital in Uppsala (Sweden). Antisera against human serum proteins were kindly supplied by Professor C.-B. Laurell (Malmö Hospital, Malmö, Sweden). All other chemicals used were of reagent or analytical grade.

Analytical procedures

The distribution of proteins in column effluents was determined spectrophotometrically at 280 nm. Electrophoretic analysis was done on 1% agarose gels at pH 8.6. Serum proteins were immunologically identified by the conventional Ouchterlony immunodiffusion techniques using monospecific antibodies [6].

Glucosylation of human serum albumin

The glucosylation procedure was carried out essentially as described by Mereish *et al.* [7] by incubating for 15 days at 37°C solutions of albumin (47.5 mg/ml) in 0.01 *M* sodium phosphate, pH 7.4, containing 0.1 *M* D-glucose. Glucosylated albumin was monitored at 279 nm assuming an absorbance of 0.531 for a 1 mg/ml solution.

Synthesis of phenylboronate agarose

Activation. Agarose was activated essentially as described by Porath and Fornstedt [8]. A 100-g aliquot of Ultrogel A-6, filter dried and washed with water, was suspended in 64 ml of 2.5 *M* sodium hydroxide containing 100 mg of sodium borohydride. Epichlorohydrin (8 ml) was added and the mixture was shaken at room temperature for a total time of 18 h. The gel was then washed with water until neutral and immediately used for coupling.

Coupling. The activated gel was incubated for 48 h at room temperature with 6 g of *m*-aminophenylboronic acid hemisulphate dissolved in 200 ml of 1 *M* sodium carbonate, pH 10. The gel was then washed consecutively with 0.1 *M* sodium hydrogencarbonate, pH 8.5, water, 95% ethanol, water, 10% (v/v) acetic acid and finally water.

Deactivation. To hydrolyse residual epichlorohydrin-activated groups the gel suspension was incubated in a boiling water bath for 30 min. The excess liquid was filtered off and the moist gel was stored in 0.05 *M* sodium acetate, pH 5.0, at 4°C until use.

Synthesis of aniline–agarose

The activation of agarose was carried out as described above. A 60-g aliquot of filter-dried epoxi-activated agarose was suspended in 120 ml of 0.2 *M* sodium carbonate buffer, pH 9.5, and 10 ml of freshly distilled aniline were added dropwise with gentle shaking. Incubation was performed for 16 h at room temperatrue. The gel was then thoroughly washed as indicated above.

Ligand content

The total ligand contents of the phenylboronate and aniline gels were determined by micro-Kjeldahl nitrogen analysis.

Determination of binding capacities towards carbohydrates

Low-molecular-weight cis-diols: NAD. The capacity was determined essentially as reported by Maestas *et al.* [9]. Packed gel beds were fed with 0.1 m*M* NAD^+ in 0.05 *M* HEPES, pH 8.5–0.1 *M* magnesium chloride until saturation (a Uvicord unit equipped with a 254-nm UV detector and a strip chart recorder were used). After exhaustive washing with HEPES buffer, bound NAD^+ was eluted with 0.2 *M* borate buffer, pH 10.2, and the absorbance at 260 nm was measured. A molar extinction coefficient of $1.78 \cdot 10^4$ was used to calculate the amount of bound NAD^+ per ml of packed gel.

High-molecular-weight cis-diols: glucoproteins. The capacities were determined by saturating the

gel beds with glucosylated human serum albumin in 0.25 *M* ammonium acetate, pH 8.5. After washing with several column volumes of acetate buffer, the albumin was eluted with 0.2 *M* sorbitol in the same buffer and quantified spectrophotometrically.

Chromatography of serum proteins

Phenylboronate–agarose. Human serum was diluted ten-fold with the following application buffers: (a) 0.02 *M* HEPES, pH 8.5 (standard conditions); (b) 0.02 *M* HEPES, pH 8.5, containing 0.01–0.16 *M* magnesium chloride; (c) 0.02 *M* HEPES, pH 7.2; and (d) 0.25 *M* ammonium acetate, pH 8.5. The serum samples were applied at a flow-rate of 16 ml/h into 3 ml gel beds (1.0 × 4.0 cm) at room temperature (22°C). After sample application the column was washed with several volumes of application buffer. Elution was effected with 0.2 *M* sorbitol in 0.25 *M* ammonium acetate buffer, pH 8.5, unless otherwise specified. Before re-use the columns were washed with 6 *M* urea.

Aniline–agarose. Serum samples diluted in 0.02 *M* HEPES, pH 8.5, with or without 0.04 *M* magnesium chloride, were applied under the above-mentioned conditions. After washing the gel beds with several volumes of application buffer, elution was effected with 30% ethyleneglycol and finally with 6 *M* urea.

RESULTS

Characterization of synthesized phenylboronate–agarose

The adsorbent was obtained as a brownish gel that was stable for at least several months. The average ligand densities for several preparations of phenylboronate– and aniline–agarose gels, as estimated by nitrogen analysis, were 43 μmol and 37 μmol per ml of packed gel bed, respectively (Table I).

The binding capacity of phenylboronate–agarose for a low-molecular-weight *cis*-diol as determined by saturating the adsorbent with NAD^+ was 5.0 ± 0.2 μmol per ml of packed gel.

For comparison, a commercially available phenylboronate–agarose (Glico Gel B, containing 17 μmol of ligand per ml of packed gel, according to the manufacturer) was analysed (Table I). As expected from the lower degree of substitution, the adsorption capactity was only 40% of ours, *i.e.* 2.2 μmol/ml.

Less than 4% of human serum albumin was adsorbed in 0.25 *M* ammonium acetate, pH 8.5. However, after extensive *in vitro* non-enzymatic glucosylation, the albumin became quantitatively adsorbed under the same conditions. It was eluted with near 100% recovery by 0.2 *M* sorbitol. The adsorption capacity for this glucosylated protein was 46 mg (0.7 μmol) per ml of packed gel, which on a molar basis correponds to 1–2% utilization of the ligands (Table I) assuming single-point adsorption.

The adsorbent can be stored for at least 3 months at 4°C without significant decrease in adsorption capacity. Gel beds can be used repeatedly after washing with 6 *M* urea.

Aniline–agarose does not bind NAD^+ or glucosylated HSA under the above conditions, proving that the boronate moiety is essential for these interactions.

TABLE I

GEL-BINDING CAPACITIES FOR LOW- AND HIGH-MOLECULAR-WEIGHT *cis*-DIOLS

See Materials and methods section for details. Values reported (±S.D.) are the average of five determinations.

Gel derivative	Ligand content (μmol/ml)	Binding capacity for	
		NAD (μmol/ml)	Glycosylated HSA (mg/ml)
Phenylboronate agarose (this study)	43	5.01 ± 0.2	46.0 ± 2.3
Glico Gel B	17	2.2 ± 0.1	35.5 ± 1.8
Aniline–agarose	37	Negligible	Negligible

TABLE II

CHROMATOGRAPHY OF SERUM PROTEINS ON PHENYLBORONATE–AGAROSE: INFLUENCE OF BUFFERS

A 30-ml aliquot of diluted serum sample (corresponding to 3 ml of serum or approximately 200 mg of protein) was chromatographed on 1 × 4 cm gel beds in the indicated buffers. In the case of 0.02 *M* HEPES, pH 7.2, 150 ml of diluted serum sample were applied (overloading conditions). Desorption was achieved by including 0.2 *M* sorbitol in 0.25 *M* ammonium acetate buffer, pH 8.5. Protein was estimated by 280 nm absorption and in some runs identified by electrophoresis and immunodiffusion (Figs. 1 and 2). About 19% of the applied protein was adsorbed

Adsorption conditions		Desorbed by sorbitol elution	
Buffer	Magnesium chloride (*M*)	Percentage of total protein	Proteins identified
0.05 *M* HEPES, pH 8.5	0.00	14.5	IgG, IgA, C3, C4
0.02 *M* HEPES, pH 8.5	0.01	16.5	IgG, IgA, C3, C4, small amounts α_2-macroglobulin, HSA, transferrin, haptoglobin
0.02 *M* HEPES, pH 8.5	0.04	14.7	
0.02 *M* HEPES, pH 8.5	0.16	9.6	
0.02 *M* HEPES, pH 7.2	0.00	Overloading conditions	IgG, IgA, C3, C4, small amounts α_2-macroglobulin, transferrin, haptoglobin
0.25 *M* ammonium acetate pH 8.5	0.00	10.4	

Chromatography of human serum proteins

In 0.02 *M* HEPES buffer, pH 8.5 (standard conditions), essentially only immunoglobulins and the complement factors C3 and C4 were adsorbed (Table II). HEPES has been reported to enhance *cis*-diol interactions and was therefore chosen as the adsorption buffer [4,10]. Sorbitol elution in this buffer did not give sharp peaks. Excellent elution profiles were obtained with sorbitol included in 0.25 *M* ammonium acetate, pH 8.5. Sorbitol was necessary for the desorption. An additional 1–2% of the protein was recovered upon a final washing of the column with 6 *M* urea. About 95% of serum proteins were accounted for.

Influence of pH, magnesium chloride and decreased polarity on protein adsorption

The use of a lower pH, pH 7.2, only marginally affected the chromatographic performance (Table

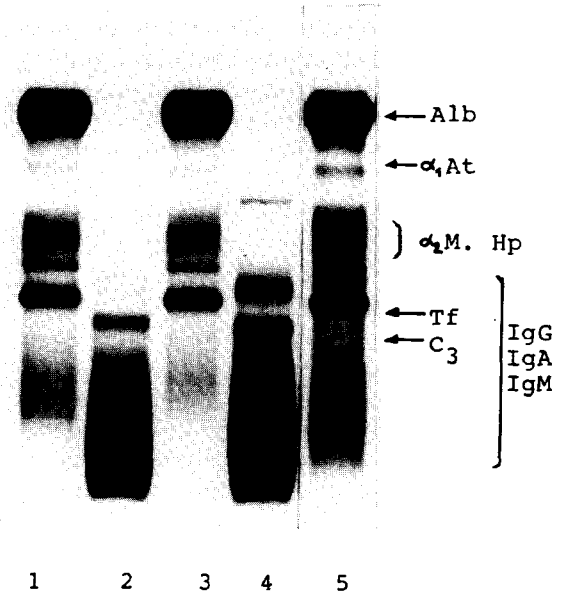

Fig. 1. Electrophoretic analysis of human serum proteins chromatographed on phenylboronate–agarose. Effect of pH of the application buffer. In each run the proteins that passed unretarded through the column are shown on the left and those eluted by 0.2 *M* sorbitol in 0.25 *M* ammonium acetate, pH 8.5, are shown on the right. Lanes: 1 and 2 = 0.02 *M* HEPES, pH 8.5 (overloading conditions); 3 and 4 = 0.02 *M* HEPES, pH 7.2 (overloading conditions); 5 = serum reference. Alb = Albumin; α_1At = α_1-antitrypsin; α_2M = α_2-macroglobulin; Hp = haptoglobulin; Tf = transferrin; C_3 = complement factor 3; Ig = immunoglobulin.

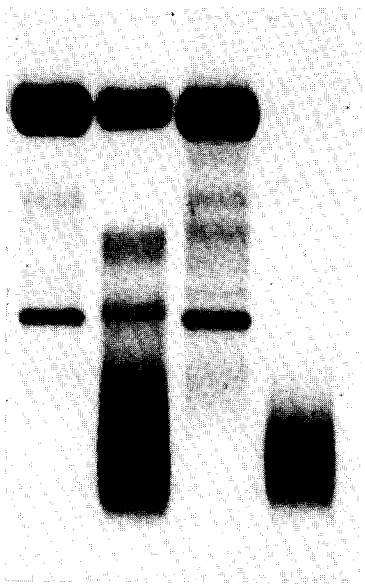

Fig. 2. Electrophoretic analysis of human serum proteins chromatographed on phenylboronate–agarose. Effect of including magnesium chloride in the application buffer. In each run, the proteins that passed unretarded through the column are shown on the left and those eluted by 0.2 *M* sorbitol in 0.25 *M* ammonium acetate, pH 8.5, are shown on the right. Lanes: 1 and 2 = 0.02 *M* HEPES, pH 8.5, containing 0.02 *M* magnesium chloride; 3 and 4 = 0.02 *M* HEPES, pH 8.5.

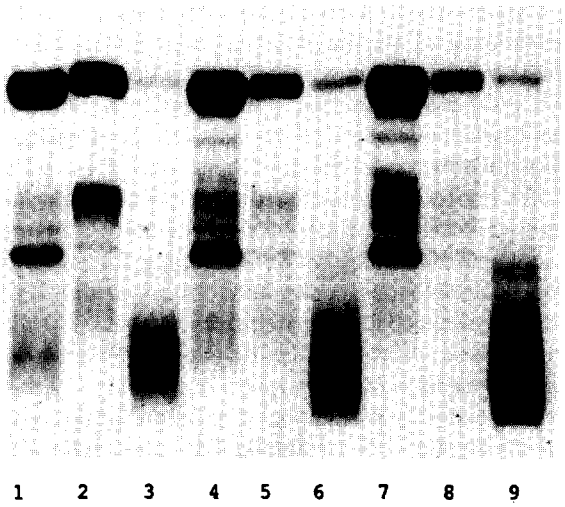

Fig. 3. Electrophoretic analysis of human serum proteins chromatographed on phenylboronate–agarose. Effect of deleting magnesium chloride on the desorption of adsorbed proteins. The proteins were applied in 0.02 *M* HEPES, pH 8.5, containing varying concentrations of magnesium chloride and eluted first by passing HEPES buffer without magnesium chloride and finally by 0.2 *M* sorbitol in 0.25 *M* ammonium acetate, pH 8.5. In each run the proteins that passed unretarded are shown on the left, those eluted by buffer lacking magnesium chloride in the middle and those eluted by sorbitol on the right. In each of the three experiments, the initial magnesium chloride concentrations were as follows: lanes: 1–3 = 0.16 *M*; 4–6 = 0.04 *M*; 7–9 = 0.01 *M*.

II and Fig. 1), but a few more proteins were adsorbed at the lower pH.

The presence of magnesium chloride in the application buffer increased the adsorption capacity and decreased the selectivity. Additional proteins became adsorbed, namely serum albumin, α_2-macroglobulin, transferrin and haptoglobin (Fig. 2). Most of these proteins were eluted simply by deleting magnesium chloride (Fig. 3).

TABLE III

EFFECTS OF LOW-POLARITY AGENTS ON ELUTION OF ADSORBED SERUM PROTEINS (IMMUNOGLOBULINS) FROM PHENYLBORONATE–AGAROSE

A 10-ml aliquot of 10-fold diluted human serum, corresponding to a total of 70 mg of protein and about 10–15 mg of immunoglobulin, was applied on the column (1 × 4 cm) in 0.02 *M* HEPES buffer, pH 8.5. The column was eluted as indicated in the two experiments. Protein was estimated by 280 nm absorption. About 19% of applied protein was adsorbed

Experiment	Elution conditions	Applied protein eluted (%)
(1) First eluent	40% Ethyleneglycol	8.4 (broad peak)
Second eluent	0.2 *M* Sorbitol in 0.25 *M* ammonium acetate, pH 8.5	8.6 (total 17.0)
(2) First eluent	30% Methylcellosolve	7.3 (broad peak)
Second eluent	40% Ethyleneglycol	1.7 (broad peak)
Third eluent	0.2 *M* Sorbitol in 0.25 *M* ammonium acetate, pH 8.5	7.9 (total 16.9)

The possible influence of hydrophobic effects on the binding of serum proteins to phenylboronate ligand was investigated under conditions that favour *cis*-diol interactions (0.02 *M* HEPES, pH 8.5) and ethyleneglycol and 2-methoxyethanol (methylcellosolve) were tested for their ability to elute hydrophobically bound proteins (immunoglobulins) (Table III). Relatively high concentrations of these agents (30–40, v/v) were required for elution; broad and extended peaks were obtained, and only 50% of the adsorbed protein was recovered. Aniline–agarose with a similar degree of substitution only adsorbed 4–6% of applied serum proteins under the same experimental conditions. This compares with the approximately 19% of protein adsorbed in standard conditions on the PBA. The adsorbed proteins were eluted by 30% ethyleneglycol and 6 *M* urea.

DISCUSSION

Immobilized aminophenylboronate ligand readily forms complexes under mild conditions with molecules containing two vicinal hydroxyl groups in the *cis* configuration. 1,2-*cis*-Diol compounds, such as sorbitol, act as displacers [11]. The adsorbent described herein behaves accordingly, as demonstrated by its ability to bind NAD^+ and *in vitro* glucosylated HSA.

Immunoglobulins and complement factors C3 and C4 are adsorbed selectively from human serum upon chromatography under conditions that favor *cis*-diol interactions (20 m*M* HEPES buffer, pH 8.5). Unexpectedly, however, protein binding also occurs at pH 7.2, at which pH the boronate–carbohydrate interaction is supposed to be negligible [9,12].

This observation and the fact that many serum glycoproteins, *e.g.* ceruloplasmin and transferrin, were not adsorbed while immunoglobulins in which the carbohydrate moieties is not readily available stayed on the column indicate that *cis*-diol binding to carbohydrate is of little importance here. Since glucose-containing albumin binds, we conclude that the carbohydrates in the oligosaccharide side chains in the glycoproteins which lack glucose do not interact significantly with the boronate moiety.

It is clear (Table II) that under standard conditions the proteins which are absorbed by the boronate gel generally are the same as those which are absorbed by thiophilic adsorbents with the uncharged ligands 2-hydroxy-ethylsulphonyl [13] and 2-pyridine-sulphido groups [14], but there are differences. α_2-Macroglobulin has a lower affinity for the boronate gel than for the thiophilics. In any case, ionic interactions may not necessarily be involved. This assumption is supported by the fact that only a smaller part of the protein adsorption at pH 8.5 is lost by adding 0.25 *M* ammonium acetate. This concentration is high enough to efficiently suppress ionic interactions.

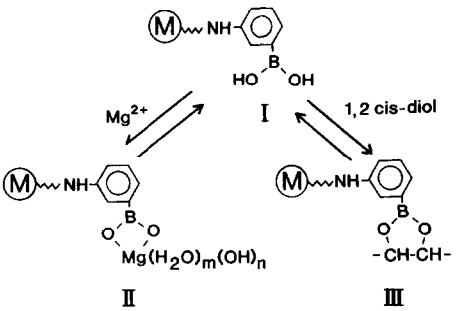

Fig. 4. Postulated interconvertible structures of ligand involved in various adsorption phenomena observed on aminophenylboronate–agarose gel.

We observed that the adsorption capacity of aniline–agarose was very low. The boronate substitution makes the ligand more hydrophilic but, paradoxically, the adsorption, which appears to be related to hydrophobicity, increases.

Let us discuss the protein–aminophenylboronate adsorption in terms of equilibrium conditions between the three forms, I, II and III, in Fig. 4. Adsorption may be essentially due to form I, and the contribution of form II may be negligible. This could explain why sorbitol is also a more efficient elution agent with respect to the hydrophobic/thiophilic part of the adsorption. Sorbitol reacts with the ligand to give III.

Upon addition of magnesium chloride to 0.02 *M* HEPES buffer the adsorption increases to a maximum (Table II). Mg^{2+} at high concentrations lowers the adsorption capacity and selectivity at the same time, as evidenced by the increasing amount of serum albumin in the desorbed fraction (Fig. 3). Magnesium chloride is a chaotropic salt and the effect at high concentration is therefore expected.

However, there must be another explanation for the behaviour of the magnesium-loaded adsorbent (form II, Fig. 4). In the range 0.01–0.16 *M* the effects of chaotropic magnesium chloride and the antichaotropic magnesium sulphate were similar (results not shown), suggesting that it is not the anion but the metal ion that is important for adsorption. Mg^{2+} and the boronate group may form an ion pair. Magnesium chloride has been reported to increase the stability of the phenyl–boronate–carbohydrate (nucleotide) interaction [15] and is currently used in standard buffers for carbohydrate–boronate chromatography. Proteins show some kind of rather non-specific affinity for magnesium. Carboxylic oxygen may enter into the coordination sphere of the immobilized magnesium. Magnesium is also likely to cause a redistribution of the π electron cloud over the ligand, thus possibly affecting π–π complexation. An alternative, perhaps more likely, explanation is that Mg^{2+} interacts with the proteins, diminishing ionic repulsion between protein carboxylates and the boronate ligand.

The complexity of the overall adsorption is further revealed by the experiments referred to in Table III. Hydrophobic and hydrophilic interaction may explain the effect of ethylene glycol and methylcellosolve on the desorption. The incomplete and slow desorption by these solvents points to additional operational factor(s). The efficient elution of the proteins by sorbitol clearly shows that boronate–protein interaction is involved. In conclusion, we suggest that the behaviour observed is the combined effect of several kinds of interactions: boronate interactions, hydrophobic and π–π interactions and possibly hydrogen bonding. All of these interactions are abolished when sorbitol is included in the medium. A weak ionic interaction may play a role at low ionic strength.

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REFERENCES

1 Ch. A. Zittle, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 12 (1951) 495.
2 J. P. Lorand and J. O. Edwards, *J. Org. Chem.*, 24 (1959) 769.
3 B. J. Gould and P. M. Hall, *Clin. Chim. Acta*, 163 (1987) 225.
4 F. A. Middle, A. Bannister, A. J. Bellingham and P. D. G. Dean, *Biochem. J.*, 209 (1983) 771.
5 S. F. Kemp, R. H. Creech, and T. R. Horn, *J. Pediatr.*, 105 (1984) 394.
6 A. Milford-Ward, in R. A. Thompson (Editor), *Techniques in Clinical Immunology*, Blackwell, Oxford, 1981, p. 1.
7 K. A. Mereish, H. Rosemberg and J. Cobby, *J. Pharm. Sci.*, 71 (1982) 235.
8 J. Porath and N. Fornstedt, *J. Chromatogr.*, 51 (1970) 479.
9 R. Maestas, J. Prieto, G. Kuehn and J. Hageman, *J. Chromatogr.*, 189 (1980) 225.
10 P. D. G. Dean, F. A. Middle, C. Lonstaff, A. Bannister, J. J. Dembinski, in I. M. Chaiken, M. Wilcheck and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1983, p. 433.
11 S. Hjertén and J. P. Li, *J. Chromatogr.*, 500 (1990) 543.
12 A. M. Yurkevich, I. I. Kolodkina, E. A. Ivanova and E. I. Pichuzkina, *Carbohydr. Res.*, 43 (1975) 215.
13 J. Porath, F. Maisano and M. Belew, *FEBS Lett.*, 185 (1985) 306.
14 J. Porath and S. Oscarsson, *Macromol. Chem., Macromol. Symp.*, 17 (1988) 359.
15 V. Boutoris, I. J. Galpin and P. D. G. Dean, *J. Chromatogr.*, 210 (1981) 267.