An Assessment of the Fractionation of Carbohydrates on Concanavalin A–Sepharose 4B by Affinity Chromatography

By **John F. Kennedy** * and **Alan Rosevear**, Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT

Assessment of a water-insoluble but active form of the lectin Concanavalin A has established that a number of monosaccharides may be separated by elution from a column of Concanavalin A immobilised on Sepharose 4B with aqueous phosphate buffer at near neutral pH values as eluant. Branched-chain polysaccharides may also be fractionated on the column. Weakly interacting polysaccharide fractions are eluted in the phosphate buffer; more tightly bound fractions may be eluted with borate buffer. The use of borate buffer for this purpose overcomes the problems arising in the use of carbohydrates for the elution of bound polysaccharide. The work demonstrates that mixtures of carbohydrates which are not separable by complex formation with Concanavalin A in solution may be separated by use of an immobilised form of the lectin.

AFFINITY chromatography has been used extensively for the purification of components which are adsorbed strongly on an immobilised adsorbent in an interaction which involves a high degree of biological specificity. Non-interacting impurity can then easily be eluted, and the purified component can subsequently be desorbed by disrupting the interaction in a way which does not cause damage. Desorption has been achieved by changes in pH [1] or ionic strength,[2] the use of an inhibitor of the interaction,[3] or a combination of these methods.[4]

Affinity chromatography has been applied to the purification of components of enzyme–inhibitor and antibody–antigen complexes,[5-8] but the technique has only been used to a limited extent for carbohydrates.

Concanavalin A (Con A), a lectin (phytohaemagglutinin) obtained from jack beans, is known to interact specifically with branched chain polysaccharides to form an insoluble complex [9] in a manner reminiscent of the antibody–antigen reaction. At pH 7 the lectin consists of two dimeric protein units with a combined molecular weight of 110,000 and having four saccharide binding units.[10] Below pH 7 the single dimer units begin to

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[6] P. Cuatrecasas and C. B. Anfinsen, *Methods Enzymol.*, 1971, **22**, 345.
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predominate.[11] Each sub-unit contains one calcium and one manganese atom [11] which are essential for the interaction of the lectin with the carbohydrate.[12] The precipitin reaction between the lectin and branched chain polysaccharides is inhibited by low molecular weight carbohydrates which do not themselves cause precipitation of the lectin.[13] This hapten-like inhibition is greatest for compounds which contain structures similar to that of 1,4-anhydro-D-arabinitol.[14]

The use of modified polysaccharides as matrices in the field of insolubilisation has been reviewed.[15] Con A has been immobilised by cross-linking the protein with glutaraldehyde [16,17] and by covalent coupling to Sepharose 4B (a commercially available macroporous agarose) activated with cyanogen bromide,[18,19] and has been used to separate strongly interacting macromolecular carbohydrates from complex mixtures. The strongly adsorbed material has been desorbed by use of a low molecular weight carbohydrate hapten inhibitor which was then removed by another purification technique. An assessment of the utility of insolubilised Con A for the separation of monosaccharides and of polysaccharides is now presented.

EXPERIMENTAL

Concanavalin A covalently bound to Sepharose 4B by the cyanogen bromide method [18] was supplied by Pharmacia Fine Chemicals AB as a suspension in 0·1M-sodium acetate buffer (pH 6·0) containing M-sodium chloride, mM-calcium chloride, mM-magnesium chloride, mM-manganese chloride, and 0·02% merthiolate. Shell fish glycogen and rat liver glycogen were obtained from Sigma Chemical Company (Types II and III, respectively). Linear dextrans (molecular weights 7×10^4 and 4×10^4) were obtained from Pharmacia Fine Chemicals AB and amylopectin was isolated from potato starch after precipitation of the amylose with butanol. A branded dextran fraction, containing (1 ⟶ 6)-linkages and (1 ⟶ 3)-branch points,[20] from *Betacoccus arabinosaceous* was provided by Professor S. A. Barker. Other carbohydrates were obtained from Koch–Light Laboratories Limited.

Elution of Carbohydrates from Con A–Sepharose 4B by Phosphate Buffer.—An aqueous slurry of the adsorbent (1·05 g dry weight) was packed into a column (116 × 0·6 cm diam.) and was washed with 0·1M-sodium phosphate buffer–M-sodium chloride (pH 7·2) at 20° at 0·175 ml min^{-1} until a steady state was reached. Solutions of carbohydrates in the same buffer (0·5 mg ml^{-1}; 0·1 ml) were applied individually to the top of the column and eluted with the same buffer. The eluate was continuously monitored for neutral carbohydrate by an automated cysteine–sulphuric acid assay.[21] The elution volume of each sample was calculated (Table 1).

Samples of maltose and of methyl α-D-mannopyranoside in the foregoing buffer (0·25—5 mg ml^{-1}; 0·1 ml) were similarly eluted and the elution volume corresponding to the peak in the elution profile was plotted against the size of the sample (Figure 3). Solutions containing two carbohydrates (0·25 mg ml^{-1} of each; 0·1 ml) were similarly eluted (Figure 1) and other samples containing two sugars were eluted at 0·350 ml min^{-1} (Figure 2).

TABLE 1

Elution of individual carbohydrates (50 μg) from Con A–Sepharose with 0·1M-sodium phosphate–M-sodium chloride (pH 7·2) at 0·175 ml min^{-1}

Compound	Elution volume (ml)
Methyl α-D-mannopyranoside	40·9
Methyl α-D-glucopyranoside	33·0
Maltose	31·8
D-Glucose	29·9
D-Galactose	29·4
Linear dextran (M 7 × 10^4)	29·9 [a]
Linear dextran (M 4 × 10^4)	28·6 [a]
Cellobiose	28·9 [b]

[a] Wide peak. [b] Eluted at 0·350 ml min^{-1}.

Solutions containing two sugars were similarly eluted at 0·175 ml min using 0·1M-sodium phosphate buffer–M-sodium chloride (pH 6·0) and also using this buffer which had been made mM with respect to calcium, magnesium, and manganese ions (Figure 1). Mixtures of two sugars were also eluted in 0·01M-sodium phosphate buffer (pH 6·0) (Figure 1).

A fresh column of Con A–Sepharose of the same dimensions was eluted with 0·01M-sodium phosphate buffer (pH 6·0) at 0·380 ml min^{-1} and solutions containing carbohydrates in the same buffer (0·50 mg ml^{-1}; 0·1 ml) were eluted indi-

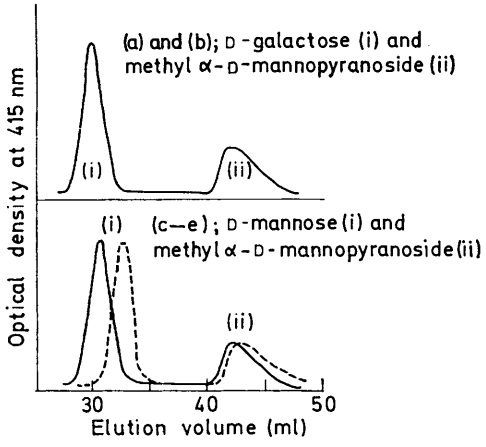

FIGURE 1 Elution of pairs of carbohydrates (25 μg each) from Con A–Sepharose at 0·175 ml min^{-1} with (a) 0·1M-sodium phosphate buffer–M-sodium chloride (pH 7·2), (b and c) 0·1M-sodium phosphate buffer–M-sodium chloride (pH 6·0), (d) 0·1M-sodium phosphate buffer–M-sodium chloride–mM-calcium chloride–mM magnesium chloride–mM manganese chloride (pH 6·0), and (e) 0·01M-sodium phosphate buffer pH (6·0); (a—d) (——), (e) (– – –)

[11] J. L. Wang, B. A. Cunningham, and G. M. Edelman, *Proc. Nat. Acad. Sci. U.S.A.*, 1971, **68**, 1130.
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[14] L. L. So and I. J. Goldstein, *Carbohyd. Res.*, 1969, **10**, 231.
[15] J. F. Kennedy, *Adv. Carbohyd. Chem. Biochem.*, 1973, **29**, in the press.
[16] E. H. Donnelly and I. J. Goldstein, *Biochem. J.*, 1970, **118**, 679.
[17] S. Avrarameas, *Compt. rend., Ser. D.*, 1970, **270**, 2205.
[18] K. Aspberg and J. Porath, *Acta Chem. Scand.*, 1970, **24**, 1839.
[19] K. O. Lloyd, *Arch. Biochem. Biophys.*, 1970, **127**, 460.
[20] S. A. Barker, E. J. Bourne, G. T. Bruce, W. B. Neely, and M. Stacey, *J. Chem. Soc.*, 1954, 2395.
[21] A. R. Law, Ph.D. Thesis, University of Birmingham, 1968.

vidually. The eluate was continuously monitored at 0·175 ml min^{-1} by the automated cysteine–sulphuric acid assay or an automated periodate–formaldehyde assay [22] (Table 2).

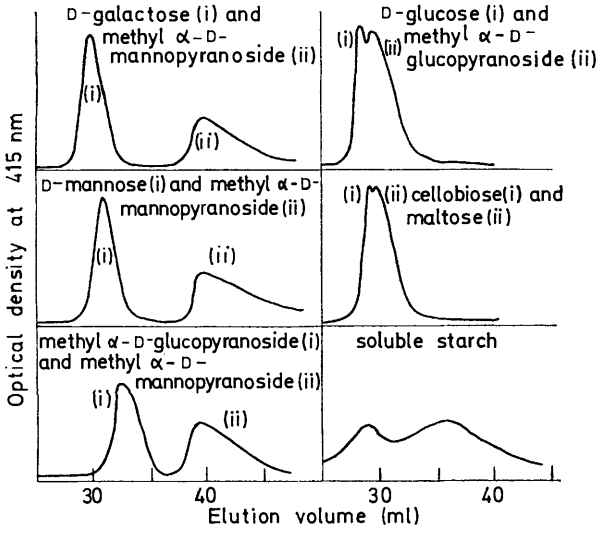

FIGURE 2 Elution of pairs of carbohydrates (25 μg each) from Con A–Sepharose at 0·350 ml min^{-1} with 0·1M-sodium phosphate buffer–M-sodium chloride (pH 7·2)

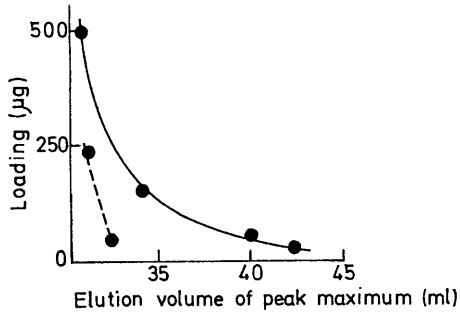

FIGURE 3 Dependence of elution volumes of methyl α-D-mannopyranoside (——) and maltose (– – –) from Con A–Sepharose upon column loading; elution at 0·175 ml min^{-1} with 0·1M-sodium phosphate buffer–M-sodium chloride (pH 7·2)

TABLE 2

Elution of individual carbohydrates (50 μg) from Con A–Sepharose with 0·01M-phosphate buffer (pH 6·0) at 380 ml min^{-1}

Compound	Elution volume (ml)
Periodate–Formaldehyde assay	
D-Mannose	33·4
D-Fructose	31·9
D-Glucose	30·4
L-Sorbose	30·4
2-Acetamido-2-deoxy-D-mannose	29·6
2-Acetamido-2-deoxy-D-glucose	29·6
2-Acetamido-2-deoxy-D-galactose	29·2
D-Mannitol	29·3
D-Glucitol	29·3
Galactitol	29·3
Erythritol	29·3
Cysteine–Sulphuric acid assay	
αα-Trehalose	34·2
Cyclohexa-amylose (α-Schardinger dextrin)	30·4
Cyclohepta-amylose (β-Schardinger dextrin)	30·4

Elution of Carbohydrates from Con A–Sepharose 4B by Borate Buffer.—A column of the type used in the foregoing experiments was eluted for several hours with 0·1M-sodium borate buffer (pH 6·0) at 0·353 ml min^{-1}. Solutions containing carbohydrates in this buffer (0·5 mg ml^{-1}, 0·1 ml) were eluted individually from the column and the eluate was monitored by the automated cysteine–sulphuric acid

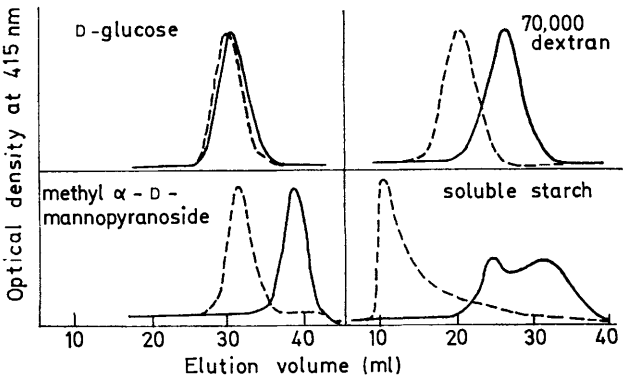

FIGURE 4 Elution of carbohydrates (50 μg each) from Con A–Sepharose at 0·350 ml min^{-1} with 0·1M-sodium phosphate buffer–M-sodium chloride (pH 7·2) (——), or 0·1M-sodium borate (pH 6·0) (– – –)

assay. (Figures 4 and 5). The recovery of each carbohydrate from the column was estimated by comparison of the assay response of the carbohydrate with the assay response of D-glucose eluted under similar conditions (Table 3).

Elution of Carbohydrates from Con A–Sepharose 4B by Phosphate Buffer followed by Borate Buffer.—The column used in the foregoing experiments was eluted for several hours with 0·1M-sodium phosphate buffer (pH 6·0) at 0·353 ml min^{-1}. A solution of shell fish glycogen in this buffer (0·5 mg ml^{-1}, 0·1 ml) was eluted from the column and a part of the eluate was monitored at 0·175 ml min^{-1} by the automated cysteine-sulphuric acid assay. When all the weakly bound carbohydrate had been eluted, the eluant was changed to 0·1M- or 0·01M-sodium borate buffer (pH 6·0),

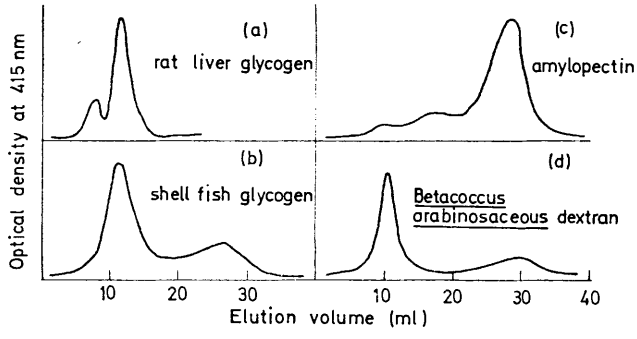

FIGURE 5 Elution of branched-chain polysaccharides (5—50 μg each) from Con A–Sepharose at 0·353 ml min^{-1} with sodium borate buffer (pH 6·0) [(a—c) 0·1M; (d) 0·01M]

and the remaining carbohydrate material was eluted at 0·175 ml min^{-1} (Figure 6).

Individual samples of D-glucose, rat liver glycogen, amylopectin and *Betacoccus arabinosaceous* dextran in

[22] O. Samuelson and H. Stromberg, *Carbohyd. Res.*, 1966, **3**, 89.

phosphate buffer (0·05—0·50 mg ml^{-1}; 0·1 ml) were similarly eluted from the column in the two buffers (Figure 6). In some cases the elution of the second major fraction was accomplished in 0·01M-sodium borate buffer (pH 6·0). The

TABLE 3

Elution of carbohydrates from Con A–Sepharose with 0·1M-sodium phosphate buffer (pH 6·0) and 0·1M- or 0·01M-sodium borate buffer (pH 6·0) at 0·353 ml min^{-1}

Compound	Wt. loaded (μg)	Recovery (%) from column [a]	Ratio of amounts of carbohydrate eluted in phosphate and borate
D-Glucose	50	100	23·9
Amylopectin	50	93	0·5
Betacoccus arabinosaceous Dextran	25	N.d.	1·0
	50	116	1·2
Linear dextran (M 7×10^4)	50	104	∞
Rat liver glycogen	5	N.d.	1·1
	25	N.d.	1·5
	50	N.d.	1·2
Shell fish glycogen	50	93	2·9

[a] Column eluted with borate only; N.d. = not determined.

ratio of the amounts of carbohydrate eluted in the two buffers was calculated by comparing the areas under the eluted peaks on the assay recorder trace (Table 3).

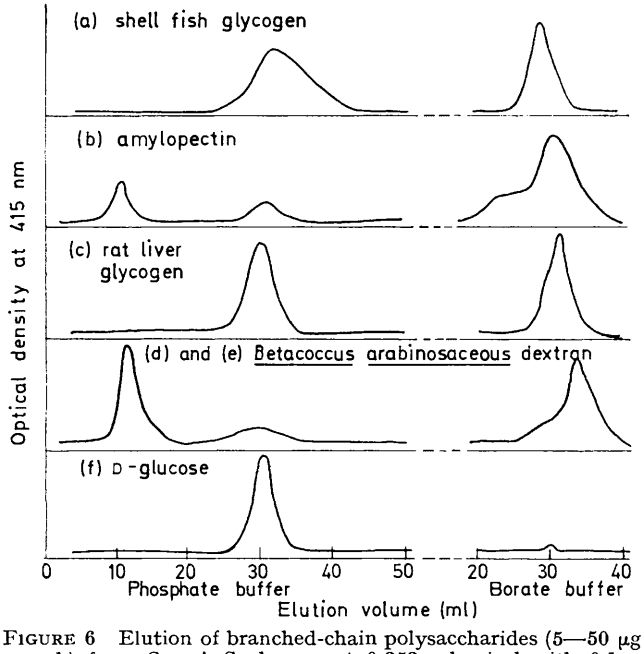

FIGURE 6 Elution of branched-chain polysaccharides (5—50 μg each) from Con A–Sepharose at 0·353 ml min^{-1} with 0·1M-sodium phosphate buffer (pH 6·0) followed by sodium borate buffer (pH 6·0) [(a—d) and (f) 0·1M; (e) 0·01M]

DISCUSSION

It was considered that the differences in the hapten inhibitory power of monosaccharides on the precipitin reaction of soluble Con A reflected differences in the strength of the interaction between the inhibitor and the active site of the lectin. This suggested that low molecular weight carbohydrates could be fractionated *on an* immobilised Con A. Carbohydrates which do not cause precipitation of the soluble lectin were eluted from the Con A–Sepharose column by 0·1M-sodium phosphate buffer (pH 7·2) M in sodium chloride, conditions which were known to lead to adsorption of branched-chain polysaccharides.[17] Differences in the elution volumes of the single compounds were apparent (Table 1) and permitted complete separations of mixtures of carbohydrates, for example a mixture of D-galactose and methyl α-D-mannopyranoside (Figure 1).

Despite the changes in the quaternary structure of Con A which are known to accompany a change in pH of the environment,[10,11] no differences in either the elution volume of methyl α-D-mannopyranoside alone, or in the separation of a mixture of the glycoside with D-mannose, was observed on changing the pH of the eluant from 7·2 to 6·0 (Figure 1). Furthermore, the presence of mM amounts of calcium, magnesium, and manganese in the eluant had no detectable effect on the separation of these two compounds despite the fact that the soluble Con A precipitin reaction is inhibited by the presence of manganese ions.[12] When the rate of elution was doubled, methyl α-D-mannopyranoside could still be separated from less strongly interacting sugars (Figure 2) but separation of other mixtures was not satisfactory with the particular size of column employed.

The retention of both D-mannose and methyl α-D-mannopyranoside was increased by elution in low ionic strength buffer (Figure 1e), although the actual separation of the two components was slightly less. It is known that M-sodium chloride inhibits the precipitin reaction of soluble Con A with some less strongly interacting carbohydrates [23] and so it was omitted from all subsequent eluants. Since the precipitin reaction of methyl α-D-mannopyranoside was greatest at pH 6·2, all subsequent elutions were performed with pH 6 buffer.

The elution volumes of a range of carbohydrates were found to parallel closely their hapten inhibitory powers [13,14] (Table 2), suggesting that these were indeed competitive inhibitors of the precipitin reaction. The ease with which methyl α-D-mannopyranoside, the strongest hapten inhibitor, and linear dextran were eluted from the column suggested that the precipitin reaction with branched-chain polysaccharide depends on many simultaneous weak interactions between the lectin and the carbohydrate rather than a few very strong interactions. It has already been suggested that the lectin interacts principally with the non-reducing ends of the carbohydrate and this has been substantiated by the observation that cyclohexa-amylose, maltose, and αα-trehalose, which have 0, 1, and 2 non-reducing ends per molecule, respectively, are retarded to increasing extents. However it appears that some weak interaction occurs between Con A and non-terminal saccharide units since, in spite of the gel permeation properties of

[23] R. J. Doyle, E. E. Woodside, and C. W. Fishel, *Biochem. J.*, 1968, **106**, 35.

Sepharose which would be expected to lead to the elution of dextrans well before D-glucose,[24] both dextrans had an elution volume similar to that of D-glucose. The inhibitory power of some simple polyols has been found to increase with the number of free hydroxy-groups.[25]

The elution of a particular carbohydrate from insoluble Con A was found to depend on the size of the sample loading, the peak in the elution profile being displaced to lower elution volume with increased loading (Figure 3). This dependence of retardation on the loading of the column above a certain value indicates that such a column can only be used for the identification of particular sugars by their elution position provided that the amounts employed are small. This is not a serious disadvantage since many component identifications have to be carried out on small scales, owing to shortage of material.

Attention was also given to assessment of insolubilised Con A for the fractionation of materials which are strongly bound to the insoluble Con A. Low molecular weight hapten inhibitors (usually methyl α-D-mannopyranoside) have generally been used to disrupt the interaction between the lectin and the carbohydrate,[18,19] but this has required a further separation to obtain the pure fractionated carbohydrates. Since the interaction between Con A and a carbohydrate is heavily dependent on the tertiary structure of the latter, a suitable means of reversibly altering the apparent shape of the carbohydrate without damaging its structure was sought. Borate ions are known to form reversible complexes with carbohydrates [26] and it is known that the precipitation of Con A by carbohydrates is inhibited slightly in borate buffer.[27] In view of the ease with which this inorganic ion can be removed from solution (*e.g.* conversion into methyl borate, which is volatile), its use for the disruption of lectin–carbohydrate interactions was investigated.

The elution of carbohydrates which do not cause precipitation of the soluble lectin was compared using phosphate and borate buffers as eluants (Figure 4). The weakly interacting D-glucose showed little change in its elution position for the two buffers, but compounds which from studies of soluble Con A were thought to interact more strongly with the lectin had much lower elution volumes in the borate buffer than in the phosphate buffer. The elution volumes of carbohydrates in borate buffer were generally similar to those expected on the basis of the gel permeation properties of agarose, although the difference in the elution volumes of D-glucose and methyl α-D-mannopyranoside (Figure 4) suggests that some interaction with the lectin still occurs.

Branched-chain polysaccharides which were known to cause precipitation of soluble Con A were eluted from the insoluble Con A in borate buffer (Figure 5). Elution of the polysaccharides was quantitative within experimental limits (Table 3), and indicated that more than one fraction of carbohydrate was present in what were considered to be chemically homogeneous samples. The borate buffer therefore appeared to be suitable for eluting polysaccharides which would be expected to bind very strongly to the lectin, and thus was suitable for fractionation studies.

When shell fish glycogen was eluted from the insoluble lectin in phosphate buffer, a weakly interacting fraction with an elution volume similar to that of dextran was detected. When the eluant was changed to borate buffer a second more strongly bound fraction was eluted (Figure 6). Rat liver glycogen was similarly separated into two fractions. Amylopectin and *Betacoccus arabinosaceous* dextran gave two weakly interacting carbohydrate fractions in the phosphate buffer. Both compounds gave fractions of more strongly bound carbohydrate when eluted with borate buffer (Figure 6).

The elution of smaller amounts of polysaccharide from the column did not change the relative distribution of polysaccharide in the fractions (Table 3), indicating that the first peak was not a result of overloading. It was found that 0·01M-borate buffer was sufficiently strong to elute the more strongly bound polysaccharide and no further fractions were eluted, even when the column was washed for 10 h with the 0·01M-borate buffer. D-Glucose was almost totally eluted from the column in phosphate buffer, only an insignificant amount of material appearing in the borate, thus confirming that the borate peaks were not artefacts of the column.

The elution of branched-chain polysaccharides from insoluble Con A by the two buffers has led to the identification of at least two fractions in what appeared, from studies of soluble Con A, to be homogeneous samples.[28] This fractionation is thought to depend on the different physical characteristics of the molecules in the sample. Polysaccharides with the same number of chain units per branch point but different molecular weights can be considered to be fractionated on the basis of molecular weight, whilst polysaccharides of the same molecular weight may be fractionated on the basis of differences in the degree of branching.

These factors could also operate in the case of the soluble lectin–carbohydrate interaction, but restrictions imposed by the immobilisation of the lectin on the matrix may modify this operation. Molecules of soluble Con A are free to move in relation to each other and can thus concentrate at points where the degree of chain branching is greatest and differences between polysaccharides may not be observed. Immobilisation of the protein would prevent this movement and consequently only carbohydrates with a particular distance between non-reducing ends would interact with the maximum possible strength. Thus, in the immobilised case, molecules may be fractionated according to differences

[24] Pharmacia, ' Gel permeation of Agarose,' Uppsala, Sweden, 1969.
[25] E. F. Plow and H. Resnick, *Biochem. Biophys. Acta*, 1970, **221**, 657.
[26] H. Weigel, *Adv. Carbohyd. Chem.*, 1963, **18**, 61.
[27] S. Svensson, S. G. Hammarström, and E. A. Kabat, *Immunochem.*, 1970, **7**, 413.
[28] A. Giblin, personal communication, 1972.

in the length of branches or the distribution of branch points which are not apparent when the complexation is carried out in solution.

It is not clear whether the borate complexes involve the carbohydrate, the lectin, or the agarose support or a combination of these three. However, the use of phosphate and borate buffers in conjunction with each other has enabled branched-chain polysaccharides to be rapidly fractionated. Thus use of insolubilised Con A provides a new criterion of purity for macromolecular carbohydrates.

We thank Professor S. A. Barker, D.Sc. for advice, Professor M. Stacey, C.B.E., F.R.S., for his interest, and the S.R.C. for a research studentship (to A. R.).

[3/476 *Received*, 2*nd March*, 1973]