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PREPARATION OF CHELATING EXCHANGERS WITH A POLYSACCHARIDE NETWORK AND LOW CROSS-LINKAGE

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SUMMARY

Iminodiacetate is readily carboxymethylated by bromoacetate (a second-order reaction with a rate constant of about 0.7×10^{-2} litre $mole^{-1}$ min^{-1}), and this reaction was used for the synthesis of low-cross-linked chelating resins by treatment of bromoacetamido-butyryl-Sepharose or bromoacetamido-butyryl-CM-Sephadex with an excess of iminodiacetate. Resins so synthesised and equilibrated with copper(II) ions retained amino acids similarly to Chelex A-100X10, but did not differentiate transfer ribonucleic acid (tRNA) from aminoacylated tRNA. The resins can be useful in the ligand-exchange fractionation of proteins.

INTRODUCTION

For several years, ligand-exchange chromatography[1–6] has found a variety of applications, mainly for isolation or fractionation of low-molecular-weight compounds; in particular, amines[1,2], amino acids[3,4], alkaloids[5] and nucleosides[6] have been successfully fractionated by this method. The high degree of cross-linking of commercially available exchangers has hitherto prevented their use for the fractionation of large molecules; however, ligand-exchange chromatography can be a valuable complementary technique to the methods most commonly used for separating proteins.

In this paper, we describe the conversion of polysaccharides into a ligand-exchanger material containing iminodiacetate chelating groups. As it is known that amines and amino acids are strongly bound to Chelex A-100X10 (Cu^{2+}), it was originally thought that the modified (and Cu^{2+} equilibrated) polysaccharides would bind aminoacylated transfer ribonucleic acids (aminoacyl-tRNA) strongly enough to permit their separation from non-aminoacylated tRNA. It appears that the aminoacyl moiety of aminoacyl-tRNA does not form complexes with exchanger-bound cupric ions, but, as shown by Porath *et al.*[7] and by our preliminary results, low-cross-linked chelating resins can be useful in the fractionation of proteins.

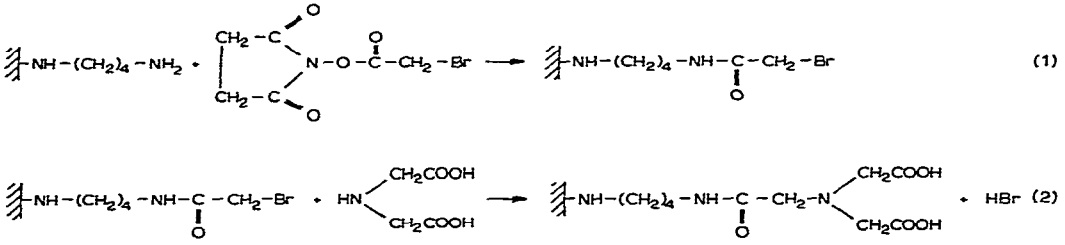
EXPERIMENTAL

Materials

Sepharose and CM-Sephadex C-50 were obtained from Pharmacia (Uppsala, Sweden), N,N'-dicyclohexylcarbodiimide (DCC) from Merck (Darmstadt, G.F.R.), N-hydroxysuccinimide from Sigma (St. Louis, Mo., U.S.A.), [^{14}C]-L-valine from The Radiochemical Centre (Amersham, Great Britain), and iminodiacetic acid from BDH (Poole, Great Britain). The tRNA was isolated from barley embryos[8] and other chemicals were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Scheme of synthesis

The general approach to the synthesis of the desired chelating resin is as follows:



Conditions for the reaction of bromoacetate with iminodiacetate have not been described in the literature, and it was therefore necessary to determine the reaction kinetics.

Reaction kinetics of bromoacetate with iminodiacetate

Aqueous solutions of iminodiacetic acid were adjusted to pH 7.5, 9.0, 10.0 or 11.0, then mixed with bromoacetic acid solutions adjusted to the same pH. Each mixture was placed in a thermostatically controlled cell connected to a pH stat (Radiometer, Copenhagen, Denmark), and the time-course of each reaction was measured by titration of the hydrobromic acid liberated. For calculations of the reaction-rate constant and order of reaction, the following combinations of reactant concentrations (bromoacetate and iminodiacetate, respectively) were used in a final volume of 4 ml: 0.1 *M* and 1.0 *M*; 1.0 *M* and 0.1 *M*; 0.5 *M* and 1.0 *M*; 1.0 *M* and 0.5 *M*; 1.0 *M* and 1.0 *M*. The rate constants calculated for 20-min intervals during a reaction time of 180 min were extrapolated to zero time to eliminate error due to changes in the volume of the reaction mixture (uptake of sodium hydroxide solution). A typical curve of the consumption of sodium hydroxide by the reaction mixture is shown in Fig. 1; there was no measurable uptake of alkali when the mixture, under otherwise identical conditions, contained only bromoacetate.

The carboxymethylation of iminodiacetate with bromoacetate fulfils the equation of a second-order reaction; the calculated rate constants are shown in Table I.

Derivatisation of Sepharose

A 30-ml portion of bromoacetamido-butyryl-Sepharose, prepared as described by Cuatrecasas[9], was suspended in 100 ml of water, the pH of the suspension was

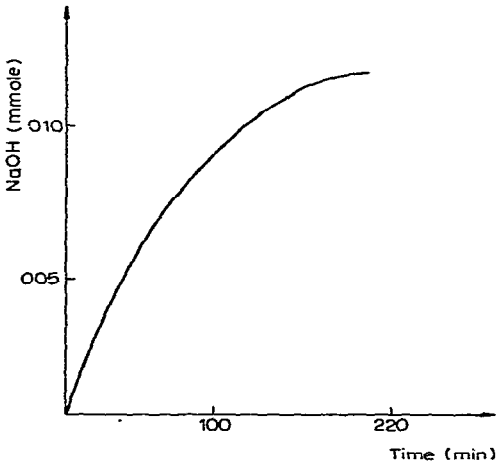


Fig. 1. Time-course of reaction of bromoacetate (2 mmoles) with iminodiacetate (4 mmoles) at pH 11.0 as evidenced by uptake of sodium hydroxide.

TABLE I

RATE CONSTANTS OF CARBOXYMETHYLATION OF IMINODIACETATE WITH BROMOACETATE

pH	*Rate constant, litre/mole/min*
7.5	$(0.35 \pm 0.04) \cdot 10^{-2}$
9.0	$(0.72 \pm 0.06) \cdot 10^{-2}$
10.0	$(0.78 \pm 0.07) \cdot 10^{-2}$
11.0	$(0.76 \pm 0.06) \cdot 10^{-2}$

adjusted to 1 l with sodium hydroxide solution, and the suspension was mixed with 100 ml of 1 *M* sodium iminodiacetate adjusted to the same pH. The mixture was connected to a pH-stat, and the pH was kept constant by automatic additions of 0.1 *M* sodium hydroxide. The reaction was judged to be complete when consumption of alkali ceased. Excess of iminodiacetate was then removed by washing the modified Sepharose with a large volume of 0.5 *M* sodium hydroxide and then with water. The product was equilibrated with cupric chloride as described by Goldstein[6] for Chelex A-100X10 and used for packing the chromatography columns.

Derivatisation of CM-Sephadex C-50

For the binding of diamine to CM-Sephadex, we activated the carboxyl groups by using DCC. Well-swollen CM-Sephadex C-50 (50 ml; H^+) was suspended in 100 ml of 60% aqueous ethanol and the pH was adjusted to 4.7 with sodium hydroxide, then a tenfold excess of butane-1,4-diamine in 60% ethanol adjusted to pH 4.7 was added, followed by an equimolar amount of DCC. The amounts of the amine and DCC were calculated according to the carboxyl-group content of the CM-Sephadex. The mixture was set aside overnight at room temperature, with continuous slow stirring,

and the product was then washed successively with 60% ethanol, 0.05 *M* sodium hydroxide, 0.05 *M* hydrochloric acid and water to remove the excess of reagents.

Iminodiacetate was covalently bound to the modified Sephadex in the way described for Sepharose.

CHELATING PROPERTIES OF THE EXCHANGERS

The following tests were used to ascertain the chelating capacities of the synthesised resins:

(a) The resin was equilibrated with cupric chloride in 1 *M* ammonia solution and exhaustively washed with 1 *M* ammonia solution followed by 1 *M* sodium chloride in 0.05 *M* sodium acetate buffer of pH 4.7; 1 ml of this resin was then mixed with 5 ml of 1 *M* hydrochloric acid and the suspension was set aside overnight, with continuous stirring. The suspension was then filtered, 1 ml of the filtrate was mixed with 2 ml of 1 *M* ammonia solution, and the absorbance (at 625 nm) of the resulting solution was compared with that of a standard ammoniacal cupric chloride solution. It was assumed that the number of Cu^{2+} ions washed from the resin with 1 *M* hydrochloric acid was equivalent to the number of chelating groups of the exchanger.

(b) The ability of the resin (Cu^{2+}) to bind amino acids at alkaline pH was compared with that of Chelex A-100X10. A solution (final concentration 1 mg/ml) of tyrosine and/or ε-(dinitrophenyl)-lysine in 0.05 *M* sodium chloride and 0.01 *M* magnesium chloride (adjusted to pH 8.0 with sodium hydroxide) having A_{280} (for tyrosine) or A_{365} (for the lysine derivative) values of 5 optical units was applied to a column (12 cm × 8 mm) of each resin (Cu^{2+}) that had been equilibrated with the same solvent. Each column was then washed with 15 void volumes of the alkaline solvent, and the amino acids were desorbed with 0.2 *M* sodium acetate buffer of pH 4.7. The same procedure was carried out with a Chelex A-100X10 column.

CHROMATOGRAPHY OF MACROMOLECULES ON THE POLYSACCHARIDE CHELATING RESINS

Transfer-RNA and aminoacylated transfer-RNA

The tRNA was de-aminoacylated as described by Guderian *et al.*[10], dialysed against 0.05 *M* sodium chloride–0.01 *M* magnesium chloride of pH 8.0, and approximately 20 A_{260} optical units were applied to a column (12 cm × 8 mm) of modified CM-Sephadex C-50. The column was washed with 15 void volumes of the alkaline solvent and then with 30 ml of 0.2 *M* sodium acetate buffer of pH 4.7. The same chromatographic procedure was used for tRNA aminoacylated with [^{14}C]valine.

Aminoacylation of tRNA was performed as described elsewhere[11]. For a single chromatographic run, a sample equivalent to 60,000 c.p.m. of [^{14}C]valyl-tRNA was used.

Chromatography of tRNA and aminoacyl-tRNA on the modified Sepharose was performed in the same manner, except that the concentration of sodium chloride in the solvents was increased to 1 *M* to overcome the strong ionic interaction between RNA and Sepharose modified by activation with cyanogen bromide.

Chromatography of proteins

Pepsin or bovine serum albumin was dissolved in 0.05 *M* sodium chloride of pH 8.0, and approximately 2 A_{280} optical units of protein were applied to a column (12 cm × 8 mm) of the modified CM-Sephadex; the procedure was then as described above for tRNA.

RESULTS AND DISCUSSION

As shown in Fig. 1 and by the calculated rate constants, bromoacetate at alkaline pH readily reacts with iminodiacetate. The reaction provides an easy way of covalently linking chelating iminodiacetate groups to polysaccharides to which bromoacetyl groups have already been bound. The second-order kinetics of this reaction permit rapid substitution of bromine in the modified matrix by using high concentrations of iminodiacetate.

Another approach to the synthesis of Sepharose-based chelating resins was used by Porath *et al.*[7], who used oxiran-activated Sepharose[12] for the covalent binding of iminodiacetate.

The chelating properties of exchangers obtained by the oxiran method and by the method described in this paper seem to be much the same: the former method gave a gel adsorbing 20 μmoles of cupric ions per ml[7]; the Sepharose-based exchangers described in this paper could bind about 10 μmoles, and the CM-Sephadex derivative about 70 μmoles of cupric ions per ml.

The exchangers described here could bind amino acids only when loaded with copper, which indicates that they contain N-bis(carboxymethyl) groupings. As shown in Fig. 2, tyrosine and/or ε-(dinitrophenyl)-lysine was strongly retained by our modified resins, the behaviour being similar to that of Chelex A-100X10 (Cu^{2+}). Protonation of amino acids by applying an acidic buffer solution to the column caused

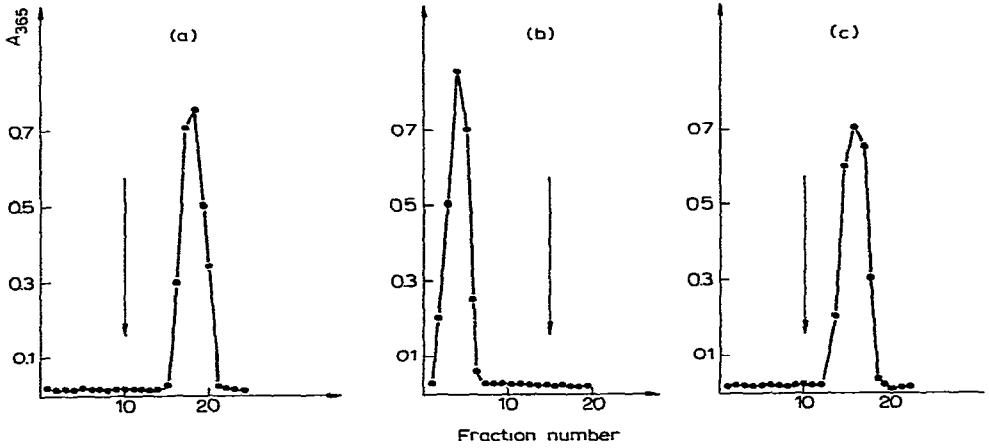


Fig. 2. Chromatography of ε-(dinitrophenyl)-lysine applied at pH 8.0 to: (a) derivatised CM-Sephadex equilibrated with Cu^{2+} and with acetate buffer of pH 4.7 as eluent; (b) derivatised CM-Sephadex not equilibrated with Cu^{2+}; (c) Chelex A-100X10 equilibrated with Cu^{2+} and with acetate buffer of pH 4.7 as eluent. The arrows indicate the point at which eluent was applied. Similar curves were obtained with tyrosine.

their elution. When the columns were not equilibrated with copper ions, no binding of amino acids was observed.

None of these modified exchangers could differentiate between the amino-acylated and non-aminoacylated-tRNA (see Fig. 3). Both tRNA samples were eluted from CM-Sephadex-based exchangers with the front of 0.2 *M* sodium chloride of pH 8.0. For elution of tRNA from the modified Sepharose, 1 *M* sodium chloride had to be used, as cyanogen bromide-treated Sepharose carries a positive charge and binds tRNA by simple ionic interactions[13]. Aminoacylation of tRNA caused no change in its eluting position.

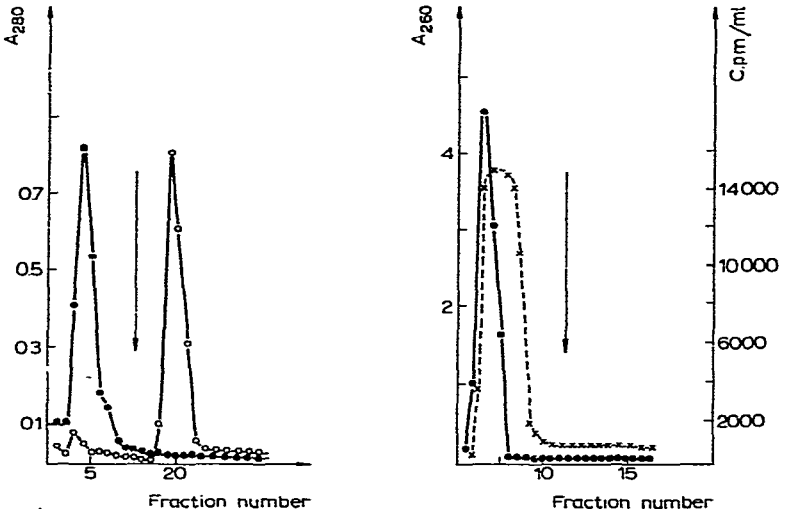


Fig. 3. Composite chromatogram of proteins on derivatised CM-Sephadex equilibrated with Cu^{2+}: ●—● pepsin, applied and eluted at pH 8.0; ○—○ bovine serum albumin applied at pH 8.0 and eluted at pH 4.7. The arrow indicates the point at which eluent was applied.

Fig. 4. Chromatography of tRNA (●—●) and aminoacyl-tRNA (×—×) on derivatised CM-Sephadex; see text for details. The arrow indicates the point at which eluent was applied.

The reasons for the inability of the amino-group of aminoacyl-tRNA to form a complex with the resin-bound copper is not clear. However, this might be due to protonation of the aminoacyl amino-group by a neighboring phosphate group in the tRNA chain. Nevertheless, the experiments with aminoacyl-tRNA showed that copper ions were sufficiently strongly chelated by the resins to prevent their catalytic effect on the hydrolysis of the ester linkage of aminoacyl-tRNA. If hydrolysis of this compound had taken place, the liberated amino acid would be strongly bound to the column at pH 8.0 and would be released only after decreasing the pH of the eluent.

These low-cross-linked chelating resins can be useful in the fractionation of proteins. As shown in Fig. 4, at pH 8.0, pepsin was not retained on the modified CM-Sephadex, whereas bovine serum albumin was retained and could be eluted only with the acidic buffer. None of the proteins was adsorbed at the ionic strength used unless the exchanger was saturated with copper ions; thus, the fractionation was again due to ligand-exchange effects.

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