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PREPARATION OF AFFINITY ADSORBENTS WITH TOYOPEARL GELS

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SUMMARY

The optimal conditions for the activation of Toyopearl by epichlorohydrin and subsequent immobilization of ligands were investigated. The optimal conditions for the activation were very different from those for agarose gel. The concentration of epoxy groups introduced was as high as 330 μmole per gram of wet gel for Toyopearl HW-55 and 150 μmole per gram of wet gel for Toyopearl HW-65 (diol type). Epoxy-activated Toyopearl was converted into amino and carboxyl derivatives and was subsequently coupled with various ligands. Glycamyl Toyopearl was prepared in a much shorter time (6 h) than agarose gel (800 h), because a higher reaction temperature could be used. The adsorbents obtained were successfully used for the affinity chromatography of lectin and trypsin. However, their adsorption capacities were lower than those of the agarose adsorbents prepared by the same methods.

INTRODUCTION

The preparation of adsorbents for affinity chromatography requires suitable matrices and effective coupling methods for attachment of ligands. Beaded agarose gel has been most widely used as a carrier because of its porous, hydrophilic properties and abundance of hydroxyl groups that can be activated by the CNBr method or the epoxy method. Though the CNBr method discovered by Axén *et al.*[1] has contributed greatly to the recent development of affinity chromatography, it does not completely satisfy demands such as the formation of stable linkages between ligands and matrix[2,3], no introduction of charged groups in the linkage region[4,5] and high yield. The epoxy method, in which a ligand or a spacer having an amino group or a hydroxyl group is readily coupled to epoxy-activated agarose[6,7], gives adsorbents with better properties in these respects[7,8]. Though the epoxy method has other disadvantages, such as the requirements of high temperature[6], high pH[6,7] and a large excess of ligand during coupling step[7], these problems were circumvented by derivatizing epoxy groups of activated agarose into more reactive groups, which are able to react with ligands under mild conditions[9].

However, agarose gel is not a completely satisfactory carrier especially for an industrial use, because it has several unfavourable properties: instability to high temperature and organic solvents that are useful for derivatization reactions; poor

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mechanical strength; inability to give a high flow-rate on the column; and sensitivity to degradation by micro-organisms. On the other hand, a hydrophilic vinyl polymer Toyopearl[10], which is now available as a carrier for gel filtration and high-performance liquid chromatography, does not have these disadvantages. In this paper, we report an investigation of the optimal conditions for the activation of Toyopearl by the epoxy method, the subsequent derivatization and the immobilization of various ligands. The affinity adsorbents obtained were successfully used for the affinity chromatography of lectin and trypsin.

EXPERIMENTAL

Materials

Toyopearls HW-55, -65, -65 (diol type) and -75 were obtained from Toyo Soda. epichlorohydrin, succinic anhydride, lactose, trypsin and 2,4,6-trinitrobenzene sulfonate (TNBS) from Wako. D-galactosamine hydrochloride (GalNHCl) from Seikagaku Kogyo, and sodium cyanoborohydride ($NaCNBH_3$), 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) from Nakarai Chemicals. Galactose oxidase was purchased from Sigma, soybean trypsin inhibitor (STI) from Miles Chemicals and benzoyl L-arginine ethylester (BAEE) from Fluka. Crude soybean agglutinin (SBA) and *Ricinus communis* agglutinin (RCA) were prepared by the method of Allen and Johnson[11].

Epoxy activation of Toyopearl

The activation of Toyopearl with epichlorohydrin was performed as follows. A 1-ml volume of 15 *M* NaOH and 2.5 ml of epichlorohydrin were added to 1.3 g of Toyopearl HW-55. The final concentrations were as follows: 50% (v/v) epichlorohydrin, 3 *M* NaOH. The suspension was incubated at 50°C for 2 h with shaking. The gel was then washed extensively with water. To 1.3 g of Toyopearl HW-65 (diol type) suspended in 1 ml of water, 1 ml of 10 *M* NaOH solution and 1.5 ml of epichlorohydrin were added. The final concentrations were 30% (v/v) epichlorohydrin, 2 *M* NaOH. The suspension was incubated at 50°C for 1 h with shaking. To find the optimal conditions, a series of experiments were carried out with one of the parameters modified in each experiment. The content of epoxy groups in the gel was determined according to the method of Sundberg and Porath[6] and expressed as μmoles per gram of wet gel. The volume per gram of wet Toyopearl gels was 1.1–1.2 ml.

Amination and succinylation of epoxy-activated Toyopearl

Amino Toyopearl was prepared by the same method for the preparation of amino Sepharose described previously[9]. The epoxy-activated Toyopearl was suspended in 1.5 volumes of concentrated ammonia solution. The suspension was incubated at 40°C for 1.5 h with shaking. Determination of amino groups introduced into the gel was performed with respect to the nitrogen content by the micro Kjeldahl method[12].

Succinyl Toyopearl was prepared by the same method for preparing succinyl Sepharose described previously[9]. Amino Toyopearl was washed with 0.1 *M* NaCl and suspended in 1.5 volumes of 0.1 *M* NaCl. Small portions of powdered succinic anhydride (0.08 g per gram of wet amino Toyopearl) were added gradually. The pH

of the suspension was maintained at 6 by the addition of 20% NaOH. The suspension was allowed to stand at room temperature overnight. The substitution of free amino groups was ascertained by the negative result of the TNBS colour test. To remove labile carboxyl groups, the washed succinyl Toyopearl was incubated with 0.1 *M* NaOH for 30 min at room temperature.

Coupling of lactose by reductive amination with amino Toyopearl

First, 2 g of suction-dried amino Toyopearl HW-65 (diol type) were suspended in 1.25 ml of 0.2 *M* K_2HPO_4 containing 0.2 g of lactose and 0.2 g of $NaCNBH_3$. The suspension was refluxed for 6 h. Then the free amino groups that remained in the gel were acetylated by the method previously described[9]. The amounts of lactose immobilized on the gel were determined as galactose liberated after hydrolysis with 0.5 *M* H_2SO_4 at 100°C for 4 h by the galactose oxidase method[13].

Coupling of GalNHCl with succinyl Toyopearl

First, 3 g of suction-dried succinyl Toyopearl HW-65 (diol type) was mixed with 6 ml of 0.5 *M* NaCl containing 0.2 g of GalNHCl. Then, after the pH had been adjusted to 5, the suspension was mixed with 0.1 g of EDC and was incubated at room temperature. After 24 h, another 0.1 g of EDC was added and the incubation was continued for 3 days. To determine the amounts of GalN immobilized on the gel, a part of the gel was hydrolysed with 3 *M* HCl at 100°C for 16 h and then the concentration of GalN in the hydrolysate was determined by the Elson–Morgan method[14].

Coupling of STI with succinyl Toyopearl

First, 8 g of suction-dried succinyl Toyopearl HW-55 was mixed with 16 ml of 0.5 *M* NaCl containing 0.4 g of STI. Then, after the pH had been adjusted to 5, the suspension was mixed with 0.25 g of EDC and was incubated at room temperature for more than 2 days. The gel thus obtained was then washed with water, 0.1 *M* sodium carbonate buffer (pH 9.5), water, 0.1 *M* glycine buffer (pH 3.0), and 0.05 *M* Tris–HCl buffer (pH 7.8). The amount of immobilized STI was estimated from the absorbance of the supernatant at 280 nm, using $A_{280}^{1\%} = 20.5$.

RESULTS

Optimal conditions for the activation of Toyopearl by epichlorohydrin

Effect of the concentrations of NaOH and epichlorohydrin. Samples of suction-dried Toyopearl were mixed in a round-bottomed flask with water and NaOH solution of various molarities. The reaction was started in an incubator with shaking by the addition of various amounts of epichlorohydrin. After 2 h the gel was washed on a glass filter with 50 ml of water, the amount of epoxy groups in the gel was then determined. The maximum amount of epoxy groups introduced into Toyopearl HW-55 was found at an initial concentration of 3 *M* NaOH and that of 30% epichlorohydrin as shown in Fig. 1, and that for Toyopearl HW-65 (diol type) was found at initial concentrations of 2 *M* NaOH (Fig. 2A) and of 30% epichlorohydrin (Fig. 2B).

Effect of reaction time and temperature. The time course of activation was studied at four different temperatures: 30, 40, 50 and 60°C, as shown in Figs. 3 and 4.

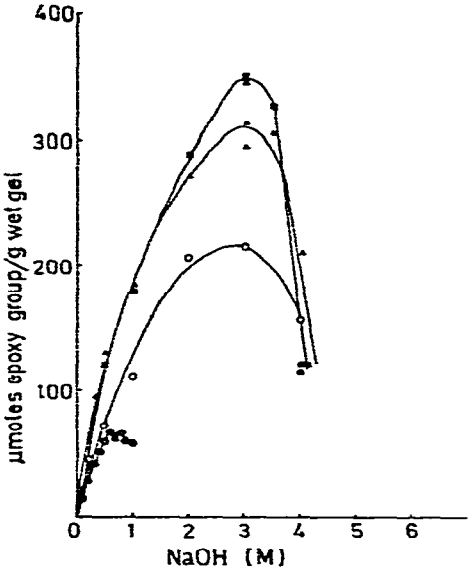

Fig. 1. Effect of the concentrations of NaOH and epichlorohydrin (ECH) on the incorporation of epoxy groups into Toyopearl HW-55. Curves: ■, 50% ECH, 50°C, 2 h; ▲, 50% ECH, 40°C, 2 h; ○, 20% ECH, 50°C, 2 h; ●, 5% ECH, 40°C, 2 h.

The higher the reaction temperature, the quicker was the time for maximum incorporation and for degradation. At 30°C the reactions were slow; the maximum was reached after 20 h for both carriers. At 60°C the maximum was reached in less than 1 h and then the epoxy groups introduced were hydrolysed quickly. At 50°C the highest amount of epoxy groups was introduced in 2 h for Toyopearl HW-55 (330 μmole per gram of wet gel) and in 1 h for Toyopearl HW-65 (diol type) (160 μmole per gram of wet gel).

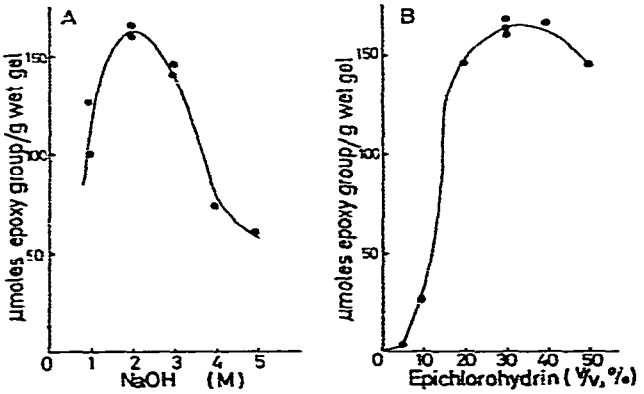

Fig. 2. Effect of the concentrations of NaOH (A) and epichlorohydrin (B) on the incorporation of epoxy groups into Toyopearl HW-65 (diol type). (A), 30% epichlorchydrin, at 50°C for 2 h; (B), 2 *M* NaOH, at 50°C for 2 h.

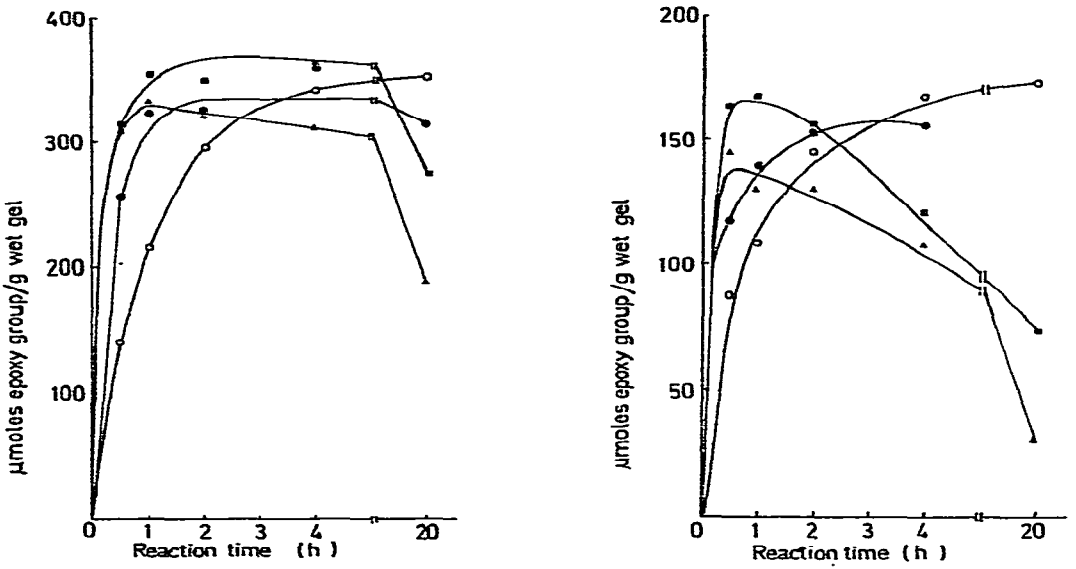

Fig. 3. Effect of reaction time and temperature on the incorporation of epoxy groups into Toyopearl HW-55. Curves: ○, at 30°C; ●, at 40°C; ■, at 50°C; ▲, at 60°C.

Fig. 4. Effect of reaction time and temperature on the incorporation of epoxy groups into Toyopearl HW-65 (diol type). Curves: ○, at 30°C; ●, at 40°C; ■, at 50°C; ▲, at 60°C.

Derivatization of epoxy-activated Toyopearl

The amination of epoxy-activated Toyopearl was performed in concentrated ammonia solution at 40°C for 1.5 h. The introduction of amino groups into the gel was detected by the TNBS colour test, and the content of amino groups was determined by the micro Kjeldahl method. The content of amino groups introduced was 200 μmole per gram of wet gel for Toyopearl HW-55 and 100 μmole per gram of wet gel for Toyopearl HW-65 (diol type). The conversion of epoxy groups into amino groups was not complete and some epoxy groups remained, as was observed in case of agarose gel. The remaining epoxy groups may be less reactive and should not covalently adsorb the solute non-specifically during the course of affinity chromatography[7].

Coupling of ligands with derivatized Toyopearl

Direct reductive amination of lactose and amino Toyopearl HW-65 (diol type) with sodium cyanoborohydride was performed in 6 h because the gel could be boiled. The content of lactose immobilized on the gel was 30 μmole per gram of wet gel, which was comparable with that in the agarose gel obtained by the same reaction at room temperature for 800 h (ref. 15). Couplings of STI and GalN with Toyopearls were performed by the aid of water-soluble carbodiimide, EDC. Some 40 mg of STI and 24 μmole of GalN were immobilized per gram of wet gel.

Affinity chromatography of lectins on Toyopearl adsorbents

Affinity chromatograms of lectins from castor bean and soybean are shown in Figs. 5 and 6. Crude lectin solution was applied to the affinity column, and effluents

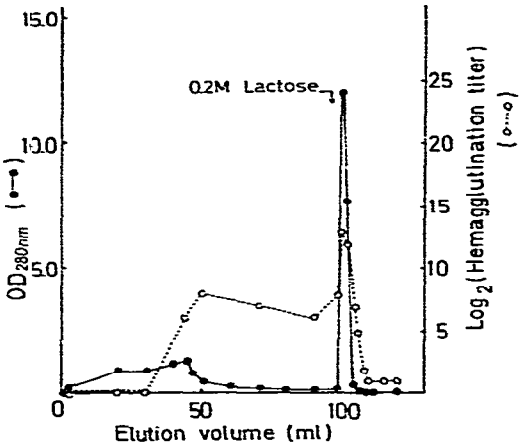

Fig. 5. Affinity chromatography of *Ricinus communis* agglutinins on a lactamyl Toyopearl HW-65 (diol type) column. Crude agglutinins (200 mg) were loaded on a column (2.4 × 1.3 cm I.D.) and washed with phosphate-buffered saline. The adsorbed agglutinins were then eluted with 0.2 *M* lactose. Fractions of 2 ml were collected at a flow-rate of 6 ml/h at 4°C. The absorption at 280 nm (●) and hemagglutinating activity (○) of eluted fractions were measured.

were assayed for absorbance at 280 nm (protein) and for hemagglutinating activity. After the hemagglutinating activity of the effluent became as high as that of the original lectin solution, each column was washed with PBS to remove unbound proteins and then elution was started with 0.2 *M* lactose. Fractions were assayed for protein, and the hemagglutinating activity of each fraction was determined after exhaustive dialysis against PBS to remove lactose. The adsorption capacities of lactamyl Toyopearl HW-65 (diol type) and GalN-Toyopearl HW-65 (diol type) were 3.7 mg RCAs and 2.4 mg SBA per ml of gel, respectively. The purity of the lectins ob-

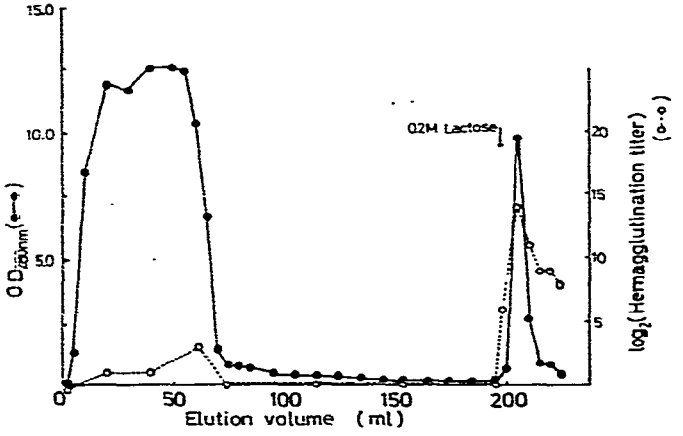

Fig. 6. Affinity chromatography of soybean agglutinin on a GalN-Toyopearl HW-65 (diol type) column (10.4 × 1.0 cm I.D.). A solution of crude soybean agglutinin (50 mg/ml) was applied to the column until the hemagglutinating activity became as high as that of the original solution. Hemagglutination titer was measured using trypsin-treated human erythrocytes.

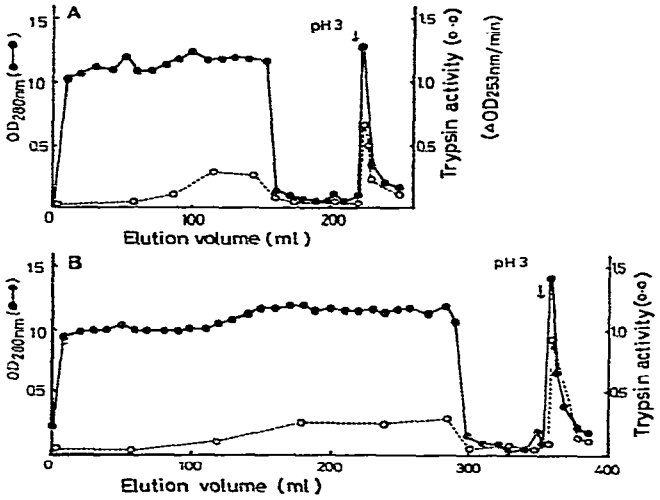

Fig. 7. Affinity chromatography of trypsin on an STI-Toyopearl HW-55 column (2.1 × 1.25 cm I.D.) (A) and an STI-Sepharose 4B column (2.8 × 1.25 cm I.D.) (B). A solution of crude trypsin (16.7 mg/ml 0.05 *M* Tris–HCl–0.5 *M* NaCl, pH 7.8) was applied to both columns until the trypsin activity of the effluent became as high as that of the original trypsin solution. The columns were then washed with 0.05 *M* Tris–HCl–0.5 *M* NaCl, pH 7.8, and citrate-phosphate buffer (pH 3) was started. Fractions of 3 ml were collected at a flow-rate of 10 ml/h at 4°C. The absorption at 280 nm (●) and the trypsin activity (○) were measured.

tained was examined by polyacrylamide gel electrophoresis[16]. Purified SBA gave a single band, whereas purified RCA gave two bands as had been expected[17].

Affinity chromatography of trypsin on STI-Toyopearl

As shown in Figs. 7A and 7B, affinity chromatography of trypsin was performed on STI-Toyopearl HW-55 (described in the section on coupling of ligands) and STI-Sepharose 4B prepared by the same procedures as STI-Toyopearl except the conditions for epoxy activation. A solution of crude trypsin was continuously applied to the affinity columns until the trypsin activity of the effluent reached that level of the original solution. Enzyme activity was determined with BAEE as substrate, the change in absorption at 253 nm being measured. After the column had been washed, the adsorbed trypsin was eluted with citrate–phosphate buffer (pH 3). STI-Toyopearl had a lower adsorption capacity for trypsin than STI-Sepharose containing almost the same amount of STI.

DISCUSSION

The optimal conditions for the epoxy activation of Toyopearls were very different from those for agarose gels. Under the optimal conditions for Sepharose 6B gel (5% epichlorohydrin, 0.4 *M* NaOH, 40°C, 2 h)[7], only a low amount of epoxy groups (67 μmole per gram of wet gel) was introduced on Toyopearl HW-55, as shown in Fig. 1. Because Toyopearl gel can withstand stronger conditions (higher temperature and higher concentrations of NaOH and epichlorohydrin) a sufficient amount of epoxy groups was introduced into the gel.

The optimal conditions for epoxy activation varied with the pore size of the Toyopearl. Toyopearls HW-65 and HW-75 were activated with epichlorohydrin under the optimal conditions for Toyopearls HW-65 (diol type), and 51 and 36 μmole per gram of wet gel of epoxy groups were introduced into Toyopearls HW-65 and HW-75, respectively. As the pore size increased, the amount of epoxy groups introduced decreased. It may be due to a lower amount of hydroxyl groups in Toyopearls with a large pore size.

Because Toyopearl gels withstand higher temperatures than agarose gel, it was also possible to perform reductive amination of lactose and amino-Toyopearl in a far shorter time. However, because Toyopearl gels were not solubilized by 70% acetic acid at 100°C or even by 6 *M* HCl at 100°C overnight, it was impossible to determine the content of amino groups introduced in the gel by the TNBS method. The micro Kjeldahl method was used, but a very long degradation time was required.

As had been expected, the affinity adsorbents obtained with Toyopearl gel were very stable, and a flow-rate of the columns was maintained under the increased pressure. The adsorption capacities of these columns, however, were lower than those of extremely high capacity agarose adsorbents[15] prepared by the same method and with the same amount of ligand. It is well known that affinity adsorbents prepared with BioGel also have lower binding capacities than those prepared with agarose gel[15,18,19]. It may be due to the less hydrophilic properties of Toyopearl and BioGel.

REFERENCES

1 R. Axén, J. Porath and S. Ernback, *Nature*, 215 (1967) 1302.
2 J. Ludens, I. R. De Vries and D. D. Fanstiel, *J. Biol. Chem.*, 247 (1972) 7533.
3 V. Sica, E. Nola, I. Parikh, G. A. Puca and P. Cuatrecasas, *J. Biol. Chem.*, 248 (1973) 6543.
4 R. Lamed, Y. Levin and A. Oplatka, *Biochim. Biophys. Acta*, 305 (1973) 163.
5 A. H. Nishikawa and P. Bailon, *Arch. Biochem. Biophys.*, 168 (1975) 576.
6 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87.
7 I. Matsumoto, Y. Mizuno and N. Seno, *J. Biochem.*, 85 (1979) 1091.
8 R. F. Murphy, J. M. Conlon, A. Imam and G. J. C. Kelly, *J. Chromatogr.*, 135 (1977) 427.
9 I. Matsumoto, N. Seno, A. M. Golovtchenko-Matsumoto and T. Osawa, *J. Biochem.*, 87 (1980) 535.
10 Y. Kato, K. Komiya, T. Iwaeda, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 211 (1981) 383.
11 H. J. Allen and E. A. Z. Johnson, *Carbohydr. Res.*, 50 (1976) 121.
12 Y. Nagai, *Seikagaku Kenkyuho* (in Japanese), Asakura Shoten, Tokyo, 1976, pp. 444–445.
13 H. Roth, S. Segal and D. Bertoli, *Anal. Biochem.*, 10 (1965) 32.
14 S. Gardell, *Acta Chem. Scand.*, 7 (1953) 207.
15 I. Matsumoto, H. Kitagaki, Y. Akai, Y. Ito and N. Seno, *Anal. Biochem.*, 116 (1981) 103.
16 R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature*, 195 (1962) 281.
17 G. L. Nicolson, J. Blaustein and M. E. Etzler, *Biochemistry*, 13 (1974) 196.
18 P. Cuatrecasas, *J. Biol. Chem.*, 245 (1970) 3059.
19 R. J. Baues and G. R. Gray, *J. Biol. Chem.*, 252 (1977) 57.