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An Alternative Coupling Procedure for Preparing Activated Sepharose for Affinity Chromatography of Penicillinase

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Abstract

Most applications of affinity chromatography employ the cyanogen bromide activation scheme first devised by Axèn *et al.* (1967). Porath and Sundberg (1972) reported an alternative procedure in which phloroglucinol and divinylsulphone are used in activating reactions. The advantages of this scheme and parameters relevant to the activating reactions are reported here. Conditions for the attachment of various ligand molecules to sepharose using a divinylsulphone activation method are defined, and a comparison with cyanogen bromide activating and coupling techniques is drawn.

α-Chymotrypsin is immobilized by covalent attachment to activated sepharose. The optimum coupling pH is 8·0–8·6 and the reaction is virtually complete after 20 h at room temperature. Conjugates containing as much as 2 g of enzyme per gram dry weight of polymer were obtained. The immobilized enzyme retained 41% of the free enzymic activity.

An affinity column of divinylsulphone-activated methicillin–sepharose was used to demonstrate the reversible adsorption of penicillinase.

Introduction

Affinity chromatography is essentially a new biochemical technique and has achieved widespread use only during the past several years. In principle, ligand molecules are attached to an insoluble support enabling reversible, specific adsorption of proteins or other macromolecules. An enzyme, for example, may be reversibly adsorbed onto an insoluble support to which is attached a specific substrate or inhibitor of the enzyme. The method thus exploits the unique biological specificity of macromolecules.

The methodology has been extended to include almost any specific interaction. Affinity chromatography has proved useful for the isolation and purification of many enzymes, antibodies and antigens, nucleic acids, polysaccharides, lymphocytes, viruses, and cellular particles such as ribosomes. This versatility is dependent upon the choice of insoluble polymer, the activation of the polymer matrix, the nature of the ligand or protein, and the method of attachment of the ligand or protein to the activated matrix.

Sepharose beads display virtually all the desirable characteristics of a matrix for the immobilization of biologically active molecules (Cuatrecasas *et al.* 1968). They are readily activated with cyanogen halides (Axèn *et al.* 1967; Porath *et al.* 1967) thereby enabling the coupling of ligands while retaining good flow properties. Enzymes and other biological particles can be fixed to sepharose gels with substantial retention of activity (Axèn *et al.* 1969, 1971; Kay and Lilly 1970).

Although many successful applications of affinity chromatography have been reported, these have usually employed the cyanogen bromide activation reaction first described by Axèn *et al.* (1967). Porath and Sundberg (1972) briefly reported a novel activation reaction in which phloroglucinol and divinylsulphone are coupled to the insoluble matrix. Little is known of parameters such as reaction time, pH, temperature, and concentrations of ligands and reactants controlling the activating and coupling reactions for this newer activation scheme. A further problem, and one which is common to all coupling methods, is the quantitative estimation of the polymer-bound ligand concentration.

This paper attempts to define the best conditions for the attachments of various ligand molecules to sepharose using a divinylsulphone activation procedure. A comparison with cyanogen bromide activating and coupling techniques is drawn. Conditions for the immobilization of α-chymotrypsin are evaluated and the reversible adsorption of commercial penicillinase to an affinity column of divinylsulphone-activated methicillin–sepharose is demonstrated.

Materials and Methods

Chemicals

Epichlorohydrin and divinylsulphone were from Koch–Light Laboratories; phloroglucinol, *p*-nitrophenol and *p*-nitrophenyltrimethylacetate from Aldrich; sepharose 4B and cyanogen bromide (CNBr)-activated sepharose 4B from Pharmacia; Penicillinase B grade and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride from Calbiochem (Aust.); α-chymotrypsin (bovine pancreas) type II from the Sigma Chemical Company; Celbenin (methicillin sodium for injection) from Beecham (Aust.); benzyl-penicillin injection (sodium salt) from Commonwealth Serum Laboratories (Aust.). All other chemicals were of analytical grade.

Instruments

All experiments requiring spectrophotometric readings, standard calibration curves and enzyme assays were performed with the use of a Varian Techtron 635 Double Beam UV-VIS Spectrophotometer, incorporating a Digital Multimeter (Marconi Instruments).

Phloroglucinol Assay

The method of Pen (1965) was used to estimate the amount of phloroglucinol recovered in the gel washings.

Divinylsulphone Assay

Divinylsulphone was estimated titrimetrically as the total number of 'available' vinyl groups, using the method of Davies *et al.* (1970) modified in the following way. Divinylsulphone-coupled sepharose was thoroughly washed with distilled water on a glass filter. An aliquot (1 ml) of the divinylsulphone gel was taken and stirred for 30 min with 1 M NaOH (10 ml) and 0·097 M β-mercaptoethanol (10 ml), the latter previously standardized with iodine–thiosulphate using starch as indicator. The suspension was washed and the washings sucked through a glass funnel into a large hypodermic syringe. The filtrate was then acidified with 1 M HCl and the excess β-mercaptoethanol back-titrated with 0·1 M iodine solution. A blank was prepared by using 1 ml of unactivated sepharose 4B under the same conditions.

Qualitative Test for the Presence of Free Amino Groups on the Gel

The method of Bartos (1964) was adapted and applied as a qualitative test for the presence of free primary amino groups on derivatized sepharose gels. Activated sepharose beads containing free aliphatic amino groups (yellow) develop a crimson red colour when tested.

Quantitative Estimation of Bound Ethylenediamine

Aliquots (100 mg) of washed ethylenediamine–sepharose were added to 10 ml of 0·01 M HCl (previously standardized with borax). The suspensions were left at room temperature for 15 min

and then filtered and washed with small amounts of distilled water, the filtrate being drawn into a hypodermic syringe. Unreacted HCl in the filtrate was back-titrated with 0·009 M NaOH (previously standardized with 0·01 M HCl) using methyl orange as indicator. A blank was prepared using divinylsulphone–sepharose (100 mg) under the same conditions.

Estimation of Bound Methicillin

The amount of methicillin coupled to each gel was taken as the difference between the amount recovered in the washings and that quantity originally added to the reaction mixture. For this purpose a standard methicillin curve (0–0·1 mg/ml) was calibrated at 280 nm.

Estimation of Bound Enzyme

The quantity of α-chymotrypsin immobilized was calculated as the difference between the amount recovered in the washings and the amount originally added to the coupling solution. A standard enzyme curve (0·0–0·5 mg/ml) was calibrated at 280 nm (ε was calculated as $4{\cdot}45 \times 10^4$ M^{-1} cm^{-1}).

Measurement of α-Chymotrypsin Activity

The method of Bender *et al.* (1967) was adapted for the calculation of free and immobilized enzymic activities. Velocities were estimated from a standard curve for *p*-nitrophenyltrimethylacetate ($0{-}5 \times 10^{-5}$ M) in 0·1 M NaOH, calibrated at 400 nm (ε was calculated as $1{\cdot}835 \times 10^4$ M^{-1} cm^{-1}).

Penicillinase Assay

The micro-iodometric method of Novick (1962) was used for all cuvette enzyme assays. The reagent solutions contained benzylpenicillin (200 μM), iodine (20 μM), penicillinase (0·2–0·8 units*) and methicillin (80–200 μM).

Results

Activating and Coupling Reaction Sequences

Figs 1*a* and 1*b* are flow schemes for the divinylsulphone activation of sepharose and subsequent covalent attachment of methicillin or protein. In Fig. 1*a* methicillin is coupled to ethylenediamine by an amide linkage. In Fig. 1*b* protein is coupled either directly to divinylsulphone via an amino residue or to ethylenediamine via a carboxyl residue.

Coupling of Phloroglucinol to Sepharose 4B

(i) *Amount-dependence*

Reaction mixtures contained sepharose 4B (5 ml settled gel), 1 M NaOH (20 ml), epichlorohydrin (48, 96, 147, 220 or 367 mg), and phloroglucinol (65, 130, 200, 300 or 500 mg). The contents of each reaction mixture were stirred gently for 2 h at room temperature. The suspensions were then washed with distilled water on a glass filter until the washings were neutral. The amount of covalently bound phloroglucinol was calculated as the difference between the quantity of phloroglucinol recovered in the washings and that amount originally added to the reaction mixtures. The amounts of phloroglucinol bound to sepharose 4B were 17, 50, 95, 114 and 250 mg in the five mixtures respectively.

The quantity of phloroglucinol bound increases as the amounts of epichlorohydrin and phloroglucinol added are increased. This result is consistent with the observation of Cuatrecasas *et al.* (1968) for the CNBr activation reaction.

* One enzyme unit hydrolyses one unit of benzylpenicillin (0·6 μg) per minute at 25°C and pH 7.

(ii) *Time-dependence*

Reaction mixtures contained 0·5 ml sepharose 4B, 37 mg epichlorohydrin, 50 mg phloroglucinol, and 5 ml 1 M NaOH. The contents of eight such mixtures were shaken gently at room temperature for 30, 60, 90, 120, 150, 180, 210 or 240 min. The suspensions were washed as before and the amounts of bound phloroglucinol estimated by difference.

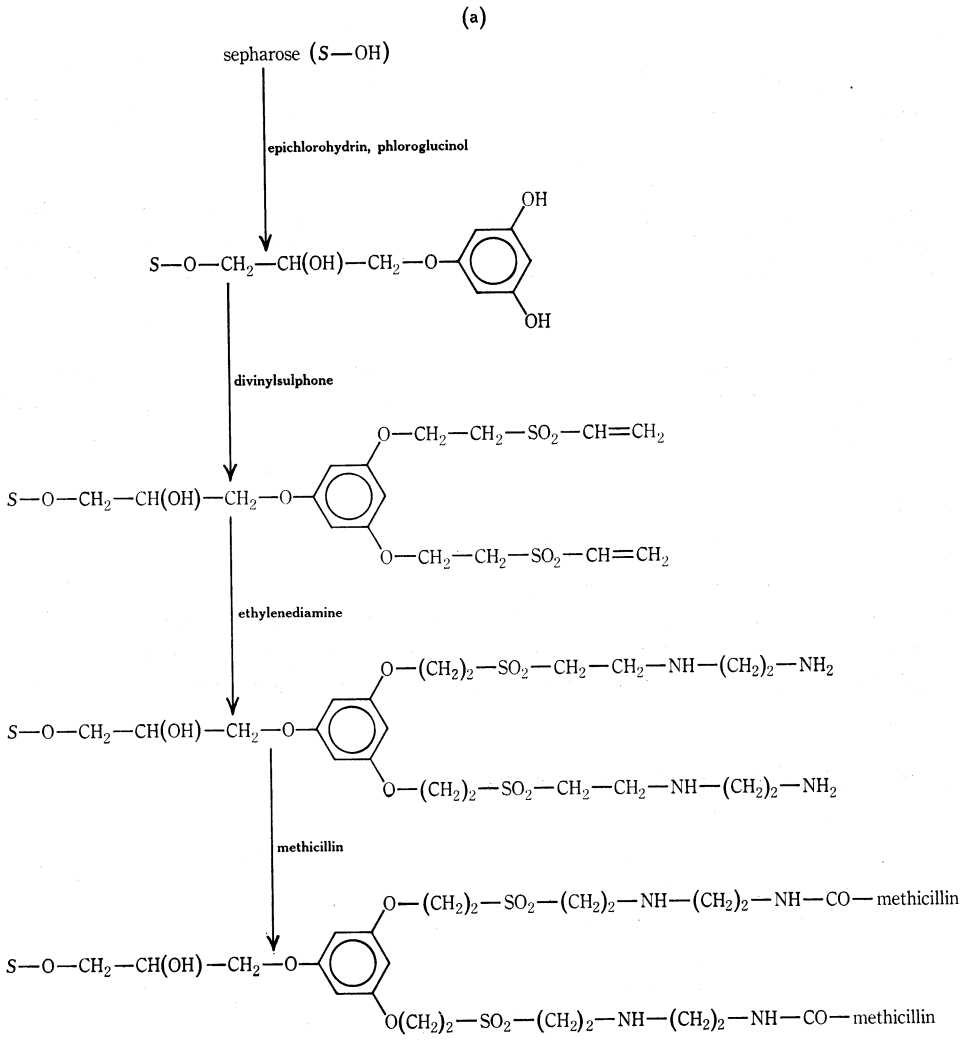

Fig. 1(*a*). Scheme for the activation of and attachment of methicillin to divinylsulphone-activated sepharose.

The amounts of phloroglucinol bound were found to be 44·4, 58·0, 60·8, 61·4, 60·4, 61·2, 60·1 and 60·8 mg in the eight mixtures respectively. It is seen from these results that a reaction time of about 2 h is necessary for phloroglucinol to couple under the conditions used.

Coupling of Divinylsulphone to Phloroglucinol–Sepharose

Phloroglucinol–sepharose (10 ml) was stirred gently at room temperature or 40°C in 5% (w/v) divinylsulphone (40 ml). The pH was adjusted to and maintained at 11 by the dropwise addition of 0·1 M NaOH. After the elapsed reaction time, the brownish orange suspension was washed on a glass filter with distilled water.

The results (Table 1) indicate that the divinylsulphone reaction requires about 30 min for maximum coupling to occur. About 30% more divinylsulphone was bound at 40°C than at room temperature. Coupling at higher temperatures was not attempted because of the risk of partially solubilizing, and therefore irreversibly damaging, the gel matrix. Divinylsulphone was estimated titrimetrically as the total number of 'available' vinyl groups (see Methods).

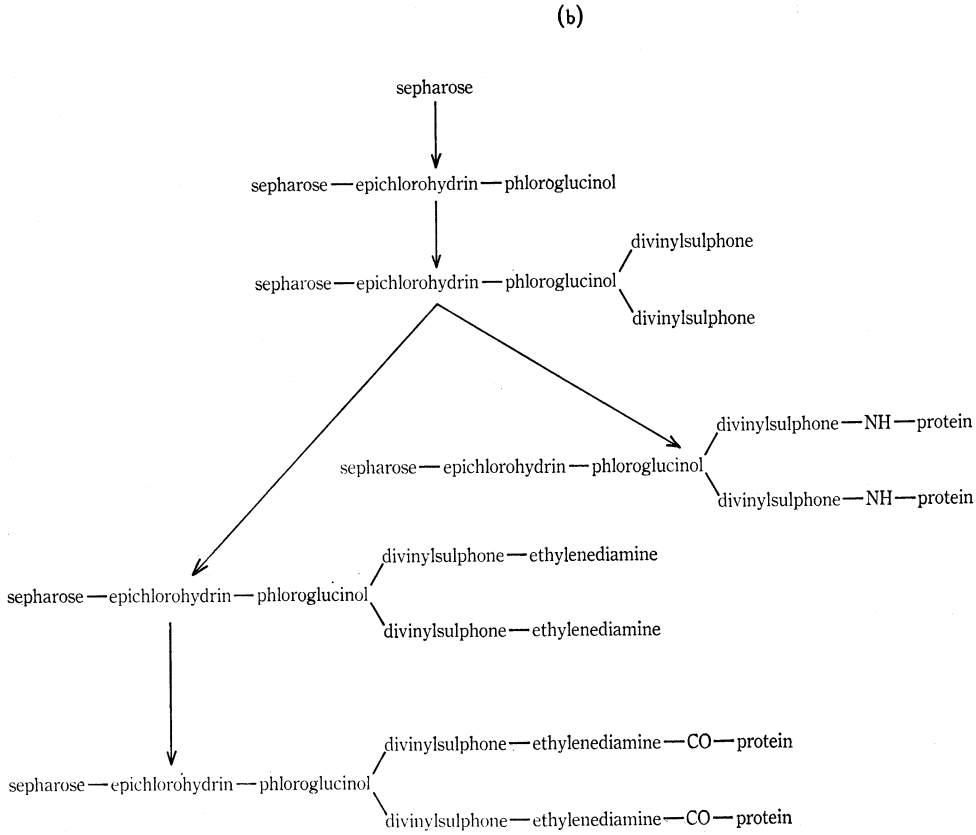

Fig. 2(*b*). Scheme for the activation of and attachment of protein to divinylsulphone-activated sepharose.

Coupling of Ethylenediamine to Divinylsulphone–Sepharose

The polymer 'arm' can be extended beyond divinylsulphone by attaching, for example, ethylenediamine. The advantages of the extension are (1) the availability of primary amino groups for the subsequent coupling of proteins and small molecules by the formation of amide bonds (Figs 1*a* and 1*b*), and (2) the positioning of ligand molecules at a sterically free distance from the polymer backbone.

(i) *Effect of varying the amount of added ethylenediamine*

In a typical experiment, solutions of ethylenediamine (0·1, 0·5, 1·0 or 2·0 g) in distilled water (20 ml) were adjusted to pH 10 with 6 M HCl and then added to washed divinylsulphone–sepharose (10 ml settled volume). The suspensions were stirred gently at room temperature or 4°C for 2 h. The now yellow suspensions were then thoroughly washed with distilled water on a glass filter. The amounts of ethylenediamine bound per millilitre of divinylsulphone–sepharose were calculated as 6·0, 8·4, 12·0 and 14·4 μmol in the four mixtures respectively. These results show that the amount of ethylenediamine bound to divinylsulphone–sepharose increases as the amount of ethylenediamine added to the reaction mixture is increased, but the amount coupled is ultimately limited by the number of available vinyl groups on the polymer.

Table 1. Effect of time and temperature on the coupling of divinylsulphone to phloroglucinol–sepharose

Time (min)	Divinylsulphone coupling (mg bound/ml polymer)		Time (min)	Divinylsulphone coupling (mg bound/ml polymer)	
	Room temp.	40°C		Room temp.	40°C
10		7·38	40		8·35
15	6·40		45	6·20	
20		8·10	50		8·58
30	6·48	8·58	60	6·45	

(ii) *Effect of reaction time*

Solutions containing ethylenediamine (2 g) in distilled water (20 ml) were adjusted to pH 10 with 6 M HCl and added to divinylsulphone–sepharose (10 ml). The suspensions were stirred gently at room temperature for 1, 2 or 3 h. The amounts of ethylenediamine bound per millilitre of divinylsulphone–sepharose were 12, 14 and 14 μmol in the three mixtures respectively, indicating an optimum coupling time of about 2 h. Cuatrecasas (1970) reported 12 μmol of ethylenediamine bound per millilitre of CNBr-activated sepharose.

Coupling of Ethylenediamine and 1,6-Diaminohexane to CNBr-activated Sepharose

Commercially available CNBr-activated sepharose was used to couple ethylenediamine and 1,6-diaminohexane. CNBr-activated sepharose (10 ml) was added to ethylenediamine (1 g) or 1,6-diaminohexane (2 g) in distilled water (10 ml). The diamine solutions were previously adjusted to pH 10 with 6 M HCl. The suspensions were stirred gently for 2 h at room temperature then thoroughly washed with distilled water on a glass filter. 11·05 μmol of ethylenediamine and 12 μmol of 1,6-diaminohexane were bound, respectively, per millilitre of CNBr–sepharose. These values are consistent with those reported by Cuatrecasas (1970) for the same derivatives. 1,6-Diaminohexane–CNBr–sepharose ('AH-sepharose') is commercially available and contains 6–10 μmol of 1,6-diaminohexane per millilitre of polymer.

Covalent Attachment of Methicillin to Sepharose Derivatives

Table 2 summarizes experiments in which methicillin was attached to various derivatives of sepharose by a carbodiimide coupling procedure: 5-ml samples of

divinylsulphone–sepharose, ethylenediamine–divinylsulphone–sepharose, ethylenediamine–CNBr–sepharose and 1,6-diaminohexane–CNBr–sepharose were added to 50-ml final volume solutions containing methicillin (50–250 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (50–250 mg). The pH of each solution was adjusted and maintained at the pH value indicated in Table 2 by the dropwise addition of 0·1 M NaOH. The temperature was 4°C or room temperature. At the completion of the allocated reaction time (3–20 h) the suspensions were thoroughly washed with distilled water on a glass filter. Divinylsulphone–sepharose was the least successful and divinylsulphone–ethylenediamine–sepharose the most successful derivative for the attachment of methicillin. The temperature of the coupling reaction appears to be unimportant, the same results being achieved at 4 and 23°C. 1,6-Diaminohexane–CNBr–sepharose coupled more methicillin than ethylenediamine–CNBr–sepharose, presumably because in the latter case the length of the spacer arm might not have been long enough to avoid steric interference by the gel matrix during the coupling reaction (Cuatrecasas *et al.* 1968).

Table 2. Coupling of methicillin to various activated sepharoses

DVS, Divinylsulphone; ED, ethylenediamine; DH, 1,6-diaminohexane; CNBr, cyanogen bromide; EDC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride

Sepharose type	Methicillin added (mg)	EDC added (mg)	pH	Time (h)	Temp. (°C)	Methicillin coupling (μmol bound/ ml sepharose)
DVS–sepharose	50	50	6·2	6	4	0
DVS–sepharose	50	50	6·2	6	23	0
DVS–sepharose	50	50	8·4	6	4	0·425
DVS–sepharose	50	50	8·4	6	23	0·425
ED–DVS–sepharose	50	50	6·5	20	4	3·5
ED–DVS–sepharose	50	50	6·5	20	23	3·5
ED–DVS–sepharose	250	250	6·5	20	23	5·0
ED–DVS–sepharose	100	200	6·5	3	23	3·125
ED–DVS–sepharose	100	200	6·5	5	23	3·5
ED–DVS–sepharose	100	200	6·5	20	23	4·4
ED–CNBr–sepharose	100	200	6·5	20	23	2·5
DH–CNBr–sepharose	100	200	6·5	20	23	3·42

Generally, the carbodiimide-mediated reaction of methicillin and derivatized sepharose follows the pattern of other ligand coupling reactions. That is, the number of ligand molecules attached to sepharose is limited by the number of available activated groups on the polymer, the concentration of reactants, the pH of the reaction mixture, and the reaction time.

The stability of the β-lactam rings of sepharose-attached methicillin molecules was tested by applying the iodometric method of Alicino (1946). In all experiments involving the coupling of methicillin to sepharose it was found that approximately 90% of the attached molecules retained intact β-lactam rings. However, this value was reduced to about 70% after storage of the methicillin gels at 4°C in pH 6·5 sodium phosphate buffer for 1 week.

The experiments summarized in Table 2 were designed for the purpose of categorizing the effectiveness of various activated sepharoses in coupling methicillin, since the preparation of methicillin conjugates has not been previously reported.

Immobilization of α-Chymotrypsin

(i) *Effect of pH on the coupling of α-chymotrypsin to divinylsulphone–activated ethylenediamine–sepharose*

Reaction mixtures contained 50 mg enzyme, 1 ml ethylenediamine–sepharose, and 10 ml buffer (pH 6·0–10·0). The reaction mixtures were shaken gently at room temperature for 4 h and then washed thoroughly on a glass filter with (1) sodium acetate buffer, pH 4, 0·5 M NaCl, and (2) sodium borate buffer, pH 8, 0·5 M NaCl.

Fig. 2*a* depicts the amount of α-chymotrypsin immobilized as a function of pH. The optimum pH for the covalent attachment of enzyme is 8·0–8·6. Axèn and Ernback (1971) coupled α-chymotrypsin to CNBr-activated sepharose at pH 8·3. Stage and Mannik (1974) coupled macromolecules to sepharose at pH 8 because this value was consistent with availability of unprotonated amino groups and also with retention of native conformation and biological activity of macromolecules.

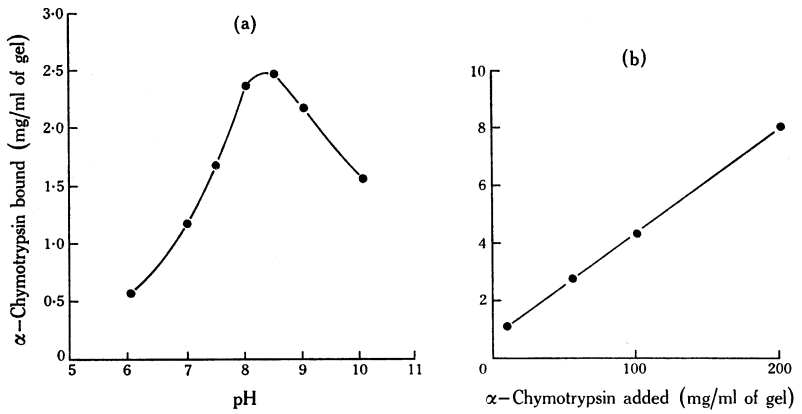

Fig. 2. Effect of (*a*) pH and (*b*) enzyme concentration on the coupling of α-chymotrypsin to ethylenediamine–sepharose.

(ii) *Effect of enzyme concentration on the coupling of α-chymotrypsin to divinylsulphone-activated ethylenediamine–sepharose*

Reaction mixtures contained 0·1 ml ethylenediamine–sepharose and 10–200 mg of enzyme. The suspensions, in 10 ml of 0·1 M $NaHCO_3$ buffer, pH 8·5, were shaken gently at room temperature for 4 h and then washed as before.

Fig. 2*b* indicates that the amount of enzyme coupled increases as the enzyme concentration in the reaction mixture is increased. This can result in conjugates containing as much as 2 g of enzyme per gram dry weight of polymer.

(iii) *Effect of time on the coupling of α-chymotrypsin to divinylsulphone–sepharose*

Three reaction mixtures containing divinylsulphone–sepharose (1 ml) and α-chymotrypsin (10 mg) were shaken gently at room temperature for 2, 5 or 20 h at pH 8·5 and in 0·1 M $NaHCO_3$ buffer containing 0·5 M NaCl (10 ml). The suspensions were washed on a glass filter as before.

From Fig. 3 it is seen that the coupling of enzyme to divinylsulphone–sepharose at room temperature proceeds almost linearly for the first 5 h and then gradually levels out. The reaction is virtually complete after 20 h have elapsed.

Calculation of Immobilized Enzyme Activity

The method of Bender *et al.* (1967) was adapted for the estimation of bound enzyme activity.

Samples of 100 mg of α-chymotrypsin–divinylsulphone–sepharose (0·7 mg enzyme/100 mg gel) were incubated at 37°C with 0·1 ml *p*-nitrophenyltrimethylacetate (final concentration 5×10^{-5} M) in 4·8 ml of 0·01 M tris buffer, pH 8·5 (final enzyme concentration $2 \cdot 18 \times 10^{-5}$ M). Readings of liberated *p*-nitrophenol were taken at 5-min intervals to 40 min and the results were expressed as velocity against time. Extrapolation of the linear portion of the curve to the ordinate axis yields the actual enzyme concentration (Kèzdy and Bender 1962).

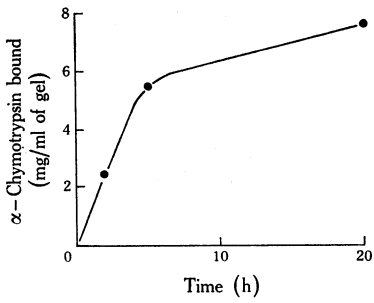

Fig. 3. Effect of time on the coupling of α-chymotrypsin to divinylsulphone–sepharose.

Pregrinding of the enzyme–gel conjugate increases the enzymic reaction velocity, presumably by facilitating the diffusion of substrates into, and products out of, the matrix (Kay and Lilly 1970). Hence the two curves in Fig. 4 represent recovered activity of the unground enzyme–sepharose conjugate and recovered activity of the preground enzyme–sepharose conjugate. The attached enzyme in the two preparations had 32 and 41%, respectively, of the free enzymic activity.

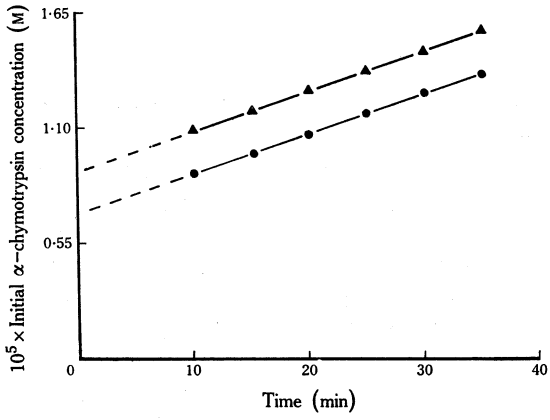

Fig. 4. Calculation of immobilized enzyme activity. ▲ Preground enzyme–sepharose conjugate. ● Unground enzyme–sepharose conjugate.

Affinity Chromatography of Penicillinase

The elution profile of penicillinase on a column of methicillin–sepharose (Fig. 5) demonstrates affinity chromatography. Sodium phosphate buffer (0·5 ml, 0·05 M, pH 6·5) containing 3500 penicillinase units was applied to a column of bed dimensions

0·9 by 25 cm; the flow rate was 30 ml/h. The first protein peak, eluted at 30 ml, has certainly been retarded because the same protein was eluted from a sepharose column at 10 ml and the total bed volume of either column is only 15 ml. This first protein band represents 23% of the total amount of enzyme added to the column. When the elution volume reached 60 ml the ionic strength of the eluant was increased by the addition of NaCl to a concentration of 1 M. A sharp protein peak was eluted at 70 ml. This second band contained 64% of the protein originally added to the column. The total recovery of protein was 87%.

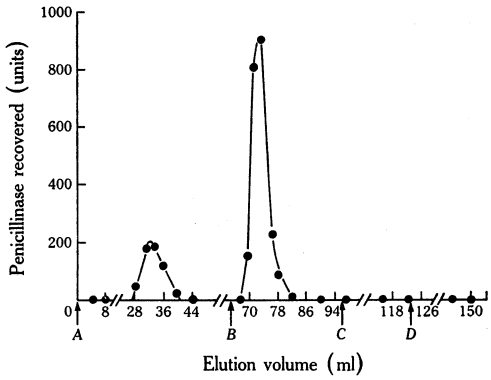

Fig. 5. Elution profile of penicillinase on methicillin–sepharose. *A*, 0·05 M sodium phosphate buffer, pH 6·5. *B*, 0·05 M sodium phosphate buffer, pH 6·5, containing 1 M NaCl. *C*, 0·05 M sodium phosphate buffer, pH 6·5, containing 2 M NaCl. *D*, Acetate buffer, pH 4·0, containing 0·5 M NaCl.

Representative proteins are not influenced to any great extent by the derivatized sepharose in their passage through the affinity column. The elution volumes and percentage recoveries were 14 ml and 96% for α-chymotrypsin, and 12 ml and 93% for bovine serum albumin on a sepharose column; and 15 ml and 95% for α-chymotrypsin, and 16 ml and 92% for bovine serum albumin on a methicillin–sepharose column.

The first protein band in Fig. 5 is probably the result of incomplete solubilization of the sample applied to the column, or overloading of the column.

Discussion

The intermediate activating and coupling reactions are necessary before the attachment of the specific ligand molecule to sepharose. The presence of a chemical extension arm between the gel matrix and the attached ligand is needed to minimize steric interference by the gel matrix on the ligand–macromolecule interaction (Cuatrecasas 1970) and to avoid the diffusional barrier of ordered water molecules surrounding the matrix backbone (Lowe *et al.* 1973). However, extension of the spacer arm beyond 10 carbon units may result in decreased binding, presumably by folding back of the spacer to bring the ligand back into the sphere of influence of the matrix backbone.

In the present report, a divinylsulphone activation scheme (Porath and Sundberg 1972) has been compared to the more conventional CNBr activation method (Axèn *et al.* 1967; Cuatrecasas *et al.* 1968). In the divinylsulphone scheme phloroglucinol is cross-linked to sepharose by epichlorohydrin (Fig. 1*a*). The advantages of this reaction are: (1) an increase in the total number of available hydroxyl groups, (2) the introduction of hydroxyl groups of higher reactivity, and (3) sterically more suitable location of the hydroxyl groups. Following activation with the bifunctional reagent

divinylsulphone, reactive vinyl groups are available for coupling to the free amino groups of small ligands or proteins.

Reaction parameters relevant to the divinylsulphone activation and coupling of sepharose have been described and some conclusions may be drawn from the results. The coupling yields of diamines, methicillin and α-chymotrypsin reported here are the same or greater than for similar molecules attached after CNBr activation. In general, higher coupling yields can be obtained if the concentrations of reactants are increased, but the initial activating reactions are important in that they place a limitation on the number of ligand molecules that may be attached in subsequent reactions.

A large number of gel activating groups can be a disadvantage, for example in the coupling of some sensitive molecules such as enzymes. A protein should be attached to a matrix by the fewest possible bonds (Cuatrecasas 1969), therefore increasing the probability that the bound macromolecule retain its tertiary structure and the properties of the same native protein in solution. Axèn and Ernback (1971) reported that the specific activity of α-chymotrypsin was inversely related to the density of the enzyme conjugated to sepharose. In the present paper a substantial percentage of the inherent activity of α-chymotrypsin is retained upon immobilization (Fig. 4). Full manifestation of fixed enzyme activity is probably prevented by steric factors (Axèn and Ernback 1971) or diffusional barriers (Kay and Lilly 1970). These observations are supported by the increase in recovered activity of preground α-chymotrypsin–sepharose (Fig. 4).

The experiment summarized in Fig. 5 demonstrated that a methicillin–sepharose conjugate can be used to effect the reversible adsorption of pencillinase by affinity chromatography. The enzyme is retained on the column by specific interaction with methicillin and is released by increasing the ionic strength of the eluant. This experiment suggests a general procedure whereby any penicillinase or cephalosporinase could be purified by reversible adsorption to an appropriate penicillin or cephalosporin. A forthcoming publication will demonstrate the isolation and purification of a bacterial penicillinase using an affinity column prepared by the procedures described above.

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