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AFFINITY CHROMATOGRAPHY

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SUMMARY

This review describes, in the individual chapters, the principles of affinity chromatography, the choice of attached affinants and the required characteristics of adsorbents for affinity chromatography. It also gives a brief survey of the most common solid supports. The conditions of the adsorption and desorption of the products isolated on insoluble affinants are discussed. The last chapters deal with commercially available insoluble affinants and briefly discuss the merits of the method. A table is included summarizing the use of affinity chromatography for the isolation of various biologically active products. The table lists more than 140 references which are mainly from the years 1972 and 1973.

PRINCIPLE OF AFFINITY CHROMATOGRAPHY

Affinity chromatography is based on the exceptional ability of biologically active substances to bind specifically and reversibly other substances, generally called ligands or especially for the purpose of affinity chromatography affinants, according to Reiner and Walch[1]. If an affinant is attached by covalent bonding to a solid support and a solution containing the biologically active products to be isolated is passed through a column of this affinant, then all compounds which under the given experimental conditions have no affinity for the affinant will pass through unretarded; in contrast, products showing affinity for the insoluble affinant are adsorbed on the column. They can be released later from the specific complex with the attached affinant, *e.g.* by a solution of a soluble affinant or by a change of solvent composition. The dissociation of the complex can often be achieved by a change in pH, ionic strength or temperature, or alternatively by dissociating agents, as will be shown later.

The principle of affinity chromatography has been known for more than twenty years. Campbell *et al.*[2] were the first to use this principle in 1951, for the isolation of antibodies on a column of cellulose with covalently attached antigen. Affinity chromatography was first used in the isolation of enzymes by Lerman[3] in 1953, in isolating tyrosinase on a column of cellulose with etherically bound resorcinol residues. In subsequent years the method of affinity chromatography was employed only rarely, the reason obviously being the character of insoluble supports which did not offer enough possibilities of complex formation between the product to be isolated and the attached

affinant. Non-specific adsorption was often observed when supports with hydrophobic or ionogenic groups were used. The past few years, however, have witnessed an extensive development of this method. A milestone in this development was the method of affinant attachment to agarose activated with cyanogen bromide, developed by Porath and co-workers[4–6]. Cuatrecasas and Anfinsen[7] have shown that agarose (most often the commercial product Sepharose) possesses almost all of the characteristics of an ideal support. In 1968, Cuatrecasas *et al.*[8] successfully employed affinity chromatography for the isolation of nuclease, chymotrypsin and carboxypeptidase A. This study, in which the term affinity chromatography was used for the first time, stimulated an extensive use of this method in the isolation of enzymes, their inhibitors, antibodies and antigens, nucleic acids, transport and repressor proteins, hormones and their receptors, and of a great number of other products, as evidenced by many literature references. Studies in the field of affinity chromatography have been reviewed by Cuatrecasas and Anfinsen[7] in *Methods in Enzymology* and by Friedberg[9] in *Chromatographic Reviews* already in 1971.

CHOICE OF ATTACHED AFFINANT

Any compound is a suitable affinant for the isolation of biologically active products if it will bind these products specifically and reversibly. Hence, depending on the different nature of biologically active products, affinants represent very different types of chemical compounds. Their classification can therefore be based on biochemical function rather than chemical structure.

Competitive inhibitors can be used as affinants for the isolation of enzymes. The affinity chromatography of chymotrypsin on Spheron 300 with an attached trypsin inhibitor[10], shown in Fig. 1, can be given as an example (Spheron is a trade mark

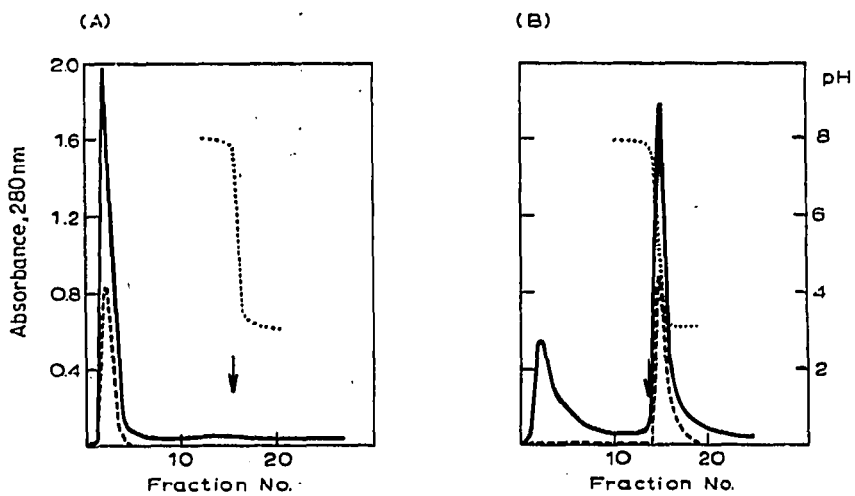


Fig. 1. Chromatography of chymotrypsin[10] on Spheron 300 (A) and Spheron 300–trypsin inhibitor (B) 10 × 1 cm I.D. columns. 300 mg chymotrypsin were applied to the columns and 6-ml fractions were collected at 10-min intervals. ———, absorbance at 280 nm; — — —, proteolytic activity; ....., pH value. Vertical arrow, elution buffer changes from pH 8.0 (0.05 *M* Tris–HCl buffer) to pH 3.1 (approximately 0.1 *M* acetic acid).

of a commercial Czechoslovak hydroxyalkyl methacrylate gel). The dissociation of the enzyme–inhibitor complex was achieved by a change in pH. The same chymotrypsin preparation was chromatographed on a column of unmodified Spheron to check the non-specific adsorption. As can be seen in Fig. 1, active chymotrypsin was separated from inactive contaminants by affinity chromatography. Attached substrates, effectors or coenzymes can also be used as affinants in the isolation of enzymes. As an example, the affinity chromatography of a mixture of two dehydrogenases and serum albumin on Sepharose with attached nicotinamide-adenine dinucleotide (NAD^+)[11] is shown in Fig. 2. NAD^+ forms binary complexes with various NAD^+-dependent dehydrogenases. When the column was eluted by solutions of cofactors, glyceraldehyde 3-phosphate dehydrogenase emerged as the second peak, lactate dehydrogenase as the third peak and both were separated from the inactive serum albumin.

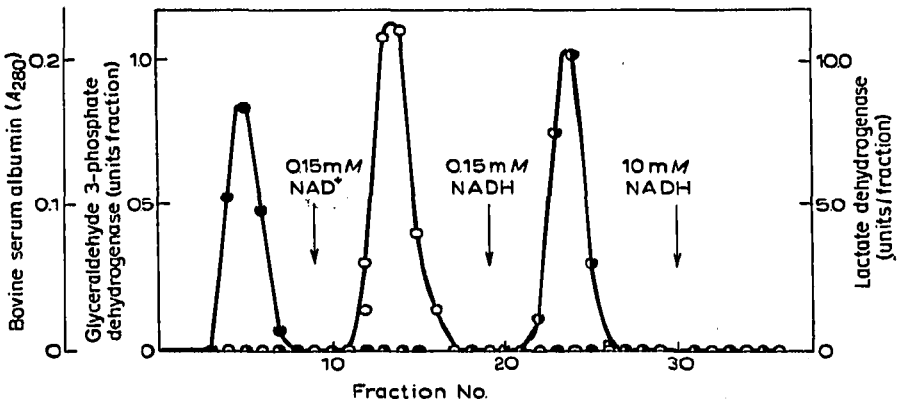


Fig. 2. Chromatography of a mixture of two dehydrogenases and bovine serum albumin[11] on an NAD^+–Sepharose 10.3 × 1 cm I.D. column equilibrated with 0.1 *M* sodium phosphate buffer at pH 7.0. The flow-rate was 1 ml/8 min and 2-ml fractions were collected. —●—, bovine serum albumin; —○—, glyceraldehyde 3-phosphate dehydrogenase activity; —◑—, lactate dehydrogenase activity.

The corresponding enzymes are used as affinants for the isolation of inhibitors. The isolation of potato chymotrypsin inhibitor on a column of hydroxyalkyl methacrylate gel with attached chymotrypsin[10] is shown in Fig. 3. The dissociation of the complex between the inhibitor to be isolated and the attached enzyme was again achieved by a change in pH; the possibility of non-specific adsorption to the gel was again checked with the unmodified gel.

Polynucleotides attached to cellulose serve as insoluble affinants in the separation of, *e.g.*, octanucleotides. An example taken from the sequential studies on nucleic acids by Gilham and Robinson[12] is shown in Fig. 4. DNAs are also suitable affinants for the isolation of specific gene mRNAs, DNA polymerases, etc.

Fig. 5 shows the isolation of nitrotyrosyl peptides from a tryptic digest of nitrotyrosyl lysozyme by Helman and Givol[13]; antityrosyl antibodies attached to Sepharose are used here as the affinant. If an enzymatic digest of the nitrated protein is passed through a column containing the attached nitrotyrosine antibody, all peptides (except those containing nitrotyrosine) emerge as the first peak. The nitrotyrosine peptides are then eluted by 1 *M* ammonia. The procedure described can be used in

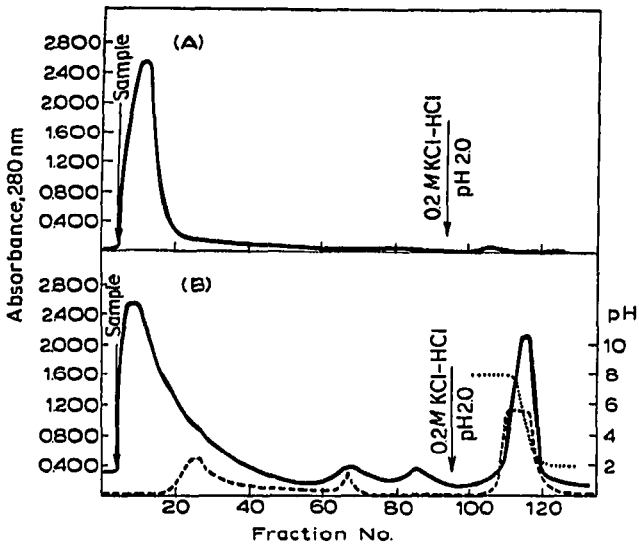


Fig. 3. Chromatography of a crude extract of potatoes[10] on Spheron 300 (A) and Spheron 300–chymotrypsin (B) 20 × 1.8 cm I.D. columns. 3 g of crude extract of potatoes were applied to the column and 10-ml fractions were collected at 1-h intervals. ———, absorbance at 280 nm; — — —, inhibitor activity; ....., pH value. Vertical arrow, elution buffer changes from pH 8.0 (0.2 *M* Tris–HCl buffer) to pH 2.0 (0.2 *M* KCl/HCl).

topographical studies aimed at the determination of tyrosine residues located on the surface of the molecule. Peptides containing 2,4-dinitrophenylsulphenyl (DNPS)-tryptophan can be isolated from the digest of DNPS-protein using the antibody to DNPS-tryptophan as affinant by a similar procedure[14]. The antialbumin antibodies attached to Sepharose may serve as an affinant for the isolation of albumin[15], as shown in Fig. 6. *Vice versa*, a sorbent with covalently attached insulin was used by Cuatrecasas[16] for the isolation of antibodies to insulin. This author[17] attached these antibodies again to Sepharose at pH 6.5 and pH 9.5. The proteins bind to Sepharose

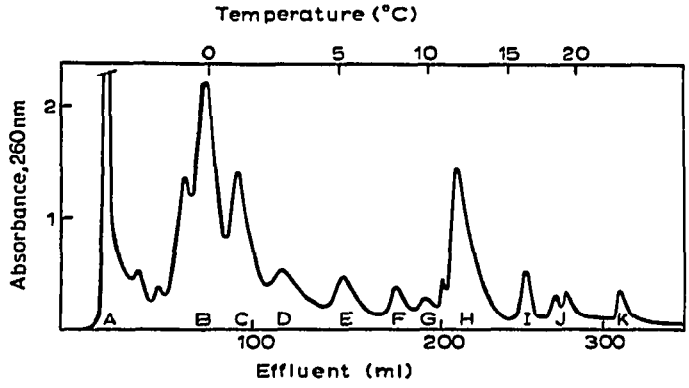


Fig. 4. Separation of heptanucleotides[12] on a temperature-controlled 40 × 1 cm I.D. column of thymidine polynucleotide–cellulose. Elution was carried out with a 1 *M* sodium chloride–0.01 *M* sodium phosphate solution, pH 7.0, at a flow-rate of 1–2 ml/h.

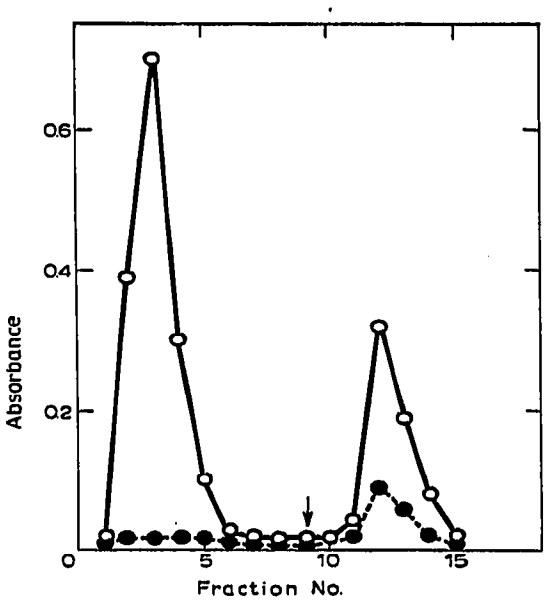


Fig. 5. Isolation of nitrotyrosyl peptides from the tryptic digest of nitrotyrosyl lysozyme[13] on a 6 × 1 cm I.D. column of antinitrosyl antibody–Sepharose. The column was washed with 0.1 *M* NH_4HCO_3 and the yellow nitrotyrosyl peptides were eluted with 1 *M* NH_3 (arrow). —○—, absorbance at 280 nm; —●—, absorbance at 381 nm.

through unprotonated forms of amino groups. A decrease in pH also causes a decrease in the number of binding groups. Hence, as a result of the difference in pH, the first derivative was able to bind almost 80% of the theoretical capacity for insulin, whereas only 7% of the theoretical capacity for insulin was bound to the derivative prepared by the attachment of insulin at pH 9.5. As the total content of the attached affinant was the same in both derivatives, the second derivative necessarily contained immunoglobulin which could not bind effectively to the antigen. Because of the great number of amino groups bound, the natural three-dimensional structure was obviously impaired.

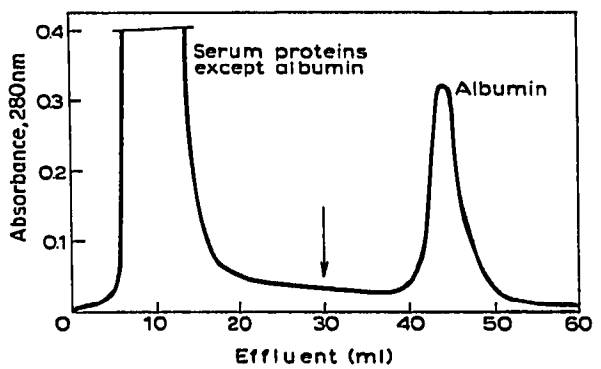


Fig. 6. Isolation of albumin from human serum[15] using antialbumin antibodies coupled to Sepharose. Sample, 3 ml human serum; bed volume, 10 ml; vertical arrow, elution buffer changes from pH 8 (0.2 *M* Tris–HCl containing 0.5 *M* NaCl) to pH 2.8 (0.2 *M* glycine–HCl containing 0.5 *M* NaCl).

Concanavalin A is a suitable affinant for the isolation of polysaccharides or glycoproteins with the glycosyl terminal group.

An example of the isolation of a protein with a free SH-group, taken from the study by Sluyterman and Wijdenes[18], is shown in Fig. 7. The proteins are bound by

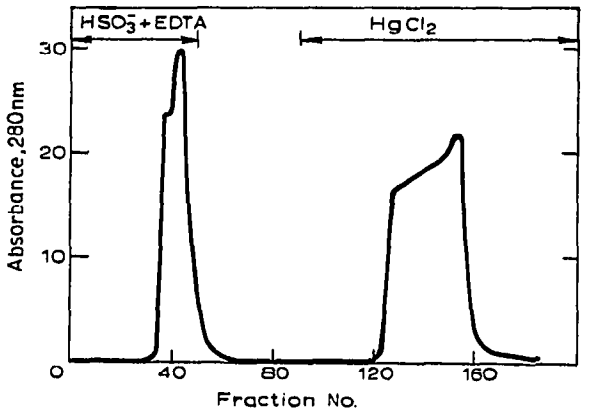


Fig. 7. Chromatography of papain[18] on a 28 × 2.5 cm I.D. agarose mercurial column. 2.6 g papain were applied to the column and 7-ml fractions were collected at a flow-rate of 150 ml/h. The chromatography was carried out in the standard buffer (*i.e.* 10% dimethyl sulphoxide, 0.5% butanol, 0.1 *M* KCl and 0.05 *M* acetate, pH 5.0). Arrows indicate the additions to the standard buffer.

their SH-groups to Sepharose with attached *p*-aminophenylmercuric acetate. The full capacity of the column was used by passing the papain solution through the column until absorbance of the effluent was the same as the absorbance of the sample applied. After all the products adsorbed non-specifically had been washed out, active papain was displaced by a solution of mercuric chloride.

REQUIRED CHARACTERISTICS OF ADSORBENTS FOR AFFINITY CHROMATOGRAPHY

The required properties of adsorbents for affinity chromatography can be summarized briefly as follows: (a) minimum non-specific adsorption, (b) good flow characteristics, (c) a sufficient number of active groups suitable for the attachment of the affinant, (d) chemical and mechanical stability, (e) high porosity, and (f) high specific gel surface.

Some of these requirements will be discussed in more detail. To minimize non-specific adsorption, the insoluble affinant must be prepared by a procedure which will yield a support with covalently attached affinant molecules only. Molecules of the affinant which are not attached through a covalent bond must be washed off. This is difficult with supports which strongly adsorb the affinant molecules. Similarly, if products are to be isolated which form specific and reversible complexes with the attached affinant, it is important that these products only be retained by the column of the insoluble affinant and only in a specific complex with the attached affinant. This is one of the main reasons why supports containing ionogenic groups, *e.g.* a copolymer of ethylene with maleic anhydride having free carboxyl groups after the affinant has been attached, did not find such an application in affinity chromatography as

neutral agarose. The chemical and mechanical stabilities determine the possibility of repeated use of the specific adsorbent. For the adsorption on the attached affinant of the product to be isolated it is necessary that the support be porous enough, thus providing for the formation of the specific complex. The adsorption of β-galactosidase to adsorbents prepared by the attachment of an inhibitor, *p*-aminophenyl-β-D-thiogalactopyranoside, through a hydrocarbon link both to a polydextran gel (Sepharose) and to a polyacrylamide gel (Bio-Gel P-300), carried out by Steers *et al.*[19], is an example. The content of the inhibitor attached was practically the same in both cases, yet in spite of that the isolation of β-galactosidase was successful only on Sepharose. The enzyme was not retained at all in the Bio-Gel P-300 column, even though its inhibitor content was high (50 μmoles/ml). This can be explained by the very high volume of the β-galactosidase tetramer (mol.wt. 540,000) which could not enter the pores of Bio-Gel. By contrast, Bio-Gel P-300 was found to be a suitable carrier for the isolation of a staphylococcal nuclease of molecular weight 17,000. High porosity of the support is also necessary for the isolation of products of relatively weak affinity for the affinant attached (dissociation constant $\geq 10^{-5}$). The concentration of the affinant attached and its free accessibility to the product to be isolated must be very high in this case, in order that a relatively strong interaction be achieved which would physically retain the flow of the products isolated through the column.

Another factor involved in the choice of gel in addition to its pore size is its specific surface. Table I shows the quantity of chymotrypsin and glycine bound to 1 ml of hydroxyalkyl methacrylate gels of different pore sizes, depending on the value of exclusion molecular weights and of the different specific surfaces[10]. It is obvious from the data shown in Table I that the quantity of bound chymotrypsin directly depends on the size of the specific surface, which is largest with Spheron 300 and 500. The quantity of bound glycine indicates that there are relatively small differences in the number of reactive groups.

However, the important factors are not only the character of the matrix and the modification of the affinant but also changes in its steric accessibility. In view of the different structures of the products to be isolated, there does not exist a general rule governing the minimum distance between the affinant and the surface of the solid

TABLE I

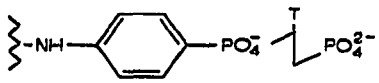
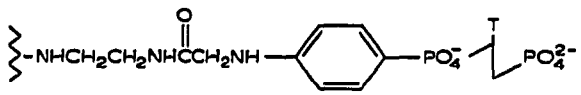
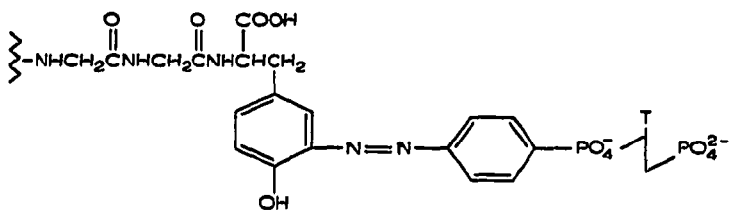
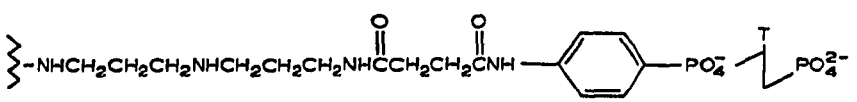
THE AMOUNT OF CHYMOTRYPSIN AND GLYCIN BOUND TO HYDROXYALKYL METHACRYLATE GELS (COMMERCIAL NAME SPHERON) IN DEPENDENCE ON THE MAGNITUDE OF THEIR SURFACE

Gel	*Exclusion mol.wt.*	*Specific surface area (m^2/ml)*	*Amount of bound glycine (mg/ml)*	*Amount of bound chymotrypsin (mg/ml)*	*Relative proteolytic activity (%)*
Spheron 10^5	10^8	0.96	0.5	0.73	—
Spheron 10^3	10^6	5.9	3.1	7.8	44
Spheron 700	700 000	3.6	2.8	6.7	49
Spheron 500	500 000	23	2.6	17.1	37
Spheron 300	300 000	19.5	3.15	17.7	44
Spheron 200	200 000	0.6	2.3	6.9	53
Spheron 100	100 000	0.2	2.6	2.6	38

support. The affinant, however, must be located at a distance from the support surface which will guarantee that formation of the complex will be possible without a deformation of the product to be isolated. The effect of the distance between the affinant 3'-(4-aminophenylphosphoryl)-deoxythymidine 5'-phosphate and the surface of the solid support, either Sepharose 4B or Bio-Gel P-300, on the capacity of the gel for affinity chromatography of staphylococcal nuclease[17] is shown in Table II. In case A the inhibitor has been attached directly to the matrix, in the other cases a link of varying length has been placed between the inhibitor and the gel surface.

TABLE II

CAPACITY OF COLUMNS CONTAINING VARIOUS AGAROSE AND POLYACRYLAMIDE ADSORBENTS FOR STAPHYLOCOCCAL NUCLEASE

Type of bond between inhibitor and matrix	*Capacity on the derivative (mg of nuclease/ml of gel)*	
	Sepharose-4B	*Bio-Gel P-300*
A 	2	0.6
B 	8	2
C 	8	3
D 	10	—

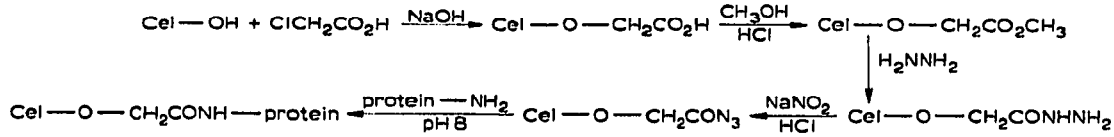
The course of affinity chromatography is affected also by the concentration of the affinant on the matrix. When Steers *et al.*[19] isolated β-galactosidase by affinity chromatography on agarose with a low content of *p*-aminophenyl-β-D-thiogalactopyranoside attached, the adsorbed enzyme was eluted by buffers containing the substrate. When the concentration of the attached affinant was high, the β-galactosidase adsorbed was not eluted by substrate-containing buffers; on the contrary, the enzyme remained active even after attachment and cleaved the substrate contained in the buffer on its passage through the column. Kalderon *et al.*[20] have found that if the concentration of the attached affinant [N-(ε-aminocaproyl)-*p*-aminophenyl]trimethylammonium bromide is raised over 0.16 μmole/ml of support, the specificity of the adsorbent for the binding of acetylcholinesterase decreases. This phenomenon can be accounted for by non-specific adsorption to the adsorbent, which acquires the properties of an ion-exchange resin after the number of ammonium groups has been increased. The au-

thors originally assumed that the enzyme could be adsorbed selectively on the support at a high ionic strength when non-specific electrostatic interactions do not play a role. However, it was shown later that this approach is impossible because of the decreased affinity of acetylcholinesterase inhibitors for the enzyme at high ionic strength values.

The capacity of the attached affinant also depends on its conformation, which should be changed as little as possible. This has already been demonstrated by Cuatrecasas[17] in his experiments with the isolation of insulin by affinity chromatography on a Sepharose column with antibodies to pig insulin attached at both pH 6.5 and 9.5.

BRIEF SURVEY OF COMMON SOLID SUPPORTS AND A PROCEDURE FOR AFFINANT ATTACHMENT

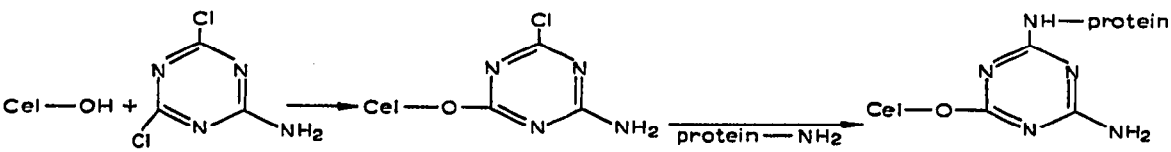
Some of the first supports to be used for covalent attachment of proteins and supports still in use are cellulose and its derivatives. The method of attaching substances containing a free amino group to cellulose was developed by Micheel and Ewers[21] in 1949.



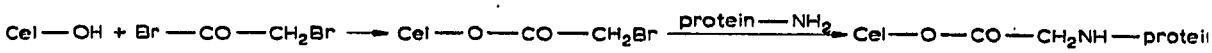
When carboxymethylcellulose azides are prepared by the Curtius rearrangement, isocyanate is formed to which, *e.g.*, a protein can bind through its amino groups. At present this method is mostly used in a modified form (Hornby *et al.*[22]).

Affinants with a free amino group can also be coupled to the carboxyl groups of cellulose with carbodiimides, as described by Weliky *et al.*[23].

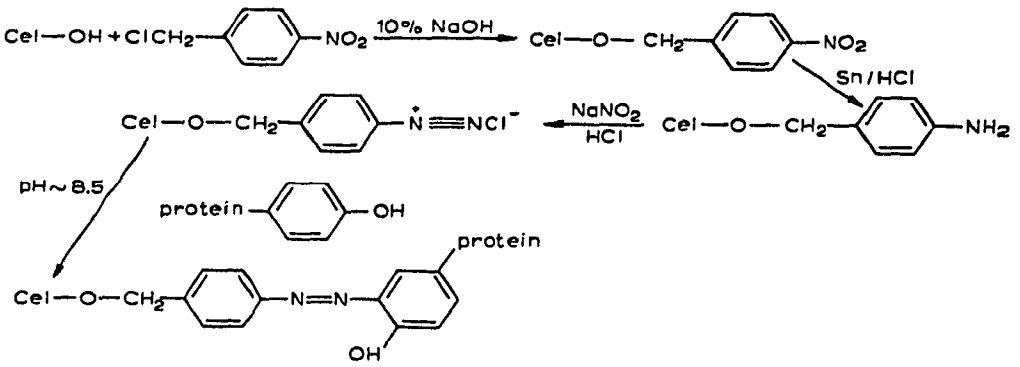
Water-soluble carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate, are preferred at present. A triazine method of protein attachment has been developed by Kay and Lilly[24]; 2-amino-4,6-dichloro-*s*-triazine is coupled to the hydroxyl groups of cellulose and then reacts with the amino groups of proteins.



A method of protein attachment by acylation of the hydroxyl groups of cellulose with bromoacetyl bromide followed by the alkylation of the amino groups of proteins has been developed by Jagendorf *et al.*[25].

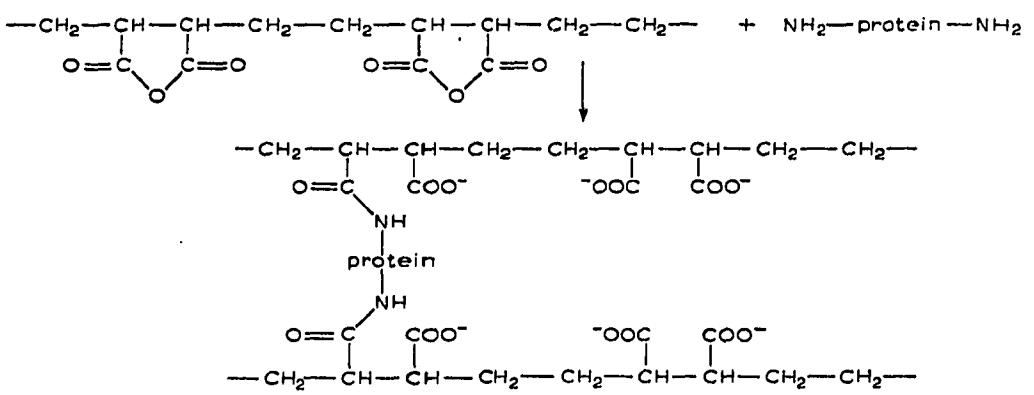


One of the first methods of attachment of proteins to cellulose was the method developed by Campbell *et al.*[2] using diazonium groups.



The proteins are coupled through their aromatic residues, predominantly those of tyrosine and histidine, but also non-specifically through their amino groups[26,27].

The attachment of proteins to a copolymer of ethylene with maleic anhydride was worked out by Levin *et al.*[28] in 1964. The proteins bind to the anhydride groups of the polymer by their amino groups.



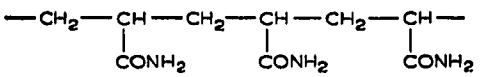
During the attachment of protein, carboxyl groups are released and the carrier acquires a polyanionic character.

Agarose, a polydextran carrier, is the most common support in affinity chromatography to date. The method of attachment of proteins to agarose activated by cyanogen bromide was developed by Porath and co-workers[4–6]. The proteins are attached through primary aliphatic or aromatic amino groups. Axén and Ernback[6] proposed a two-stage activation scheme. In the first stage, a labile intermediary cyanate is formed by the action of cyanogen halides and in the second stage it is converted into an inert carbamate and a reactive imidocarbonate. The amino group of the protein is bound to the latter in a slightly alkaline medium and a stable covalent bond is

formed. In view of the finding that agarose does not contain vicinal hydroxyl groups, and from studies on model reactions of methyl-4,6-O-benzylidene-α-D-glucopyranoside[29], this proposed scheme appears improbable. The prevailing view at present is that attachment of the proteins to the carrier proceeds mainly via derivatives of isourea. In addition to the methods described above, the triazine method developed by Kay and Lilly[24] is also used for the attachment of proteins to agarose.

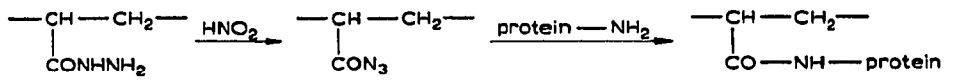
Cuatrecasas[17,30] has reported the preparation of a great number of agarose derivatives and various groups can therefore be employed for the binding. Those derivatives are now available as commercial products. They include AH-Sepharose 4B (agarose with covalently bound 1,6-diaminohexane) and CH-Sepharose 4B (agarose with covalently attached 6-aminohexanoic acid) which bind compounds either through their carboxyl or free amino groups via soluble carbodiimides. Because the affinants are attached at the end of a long hydrocarbon chain at a sufficient distance from the surface of the solid carrier, they become sterically more accessible.

Polyacrylamide gels consist of a hydrocarbon backbone to which carboxyamide groups are attached.

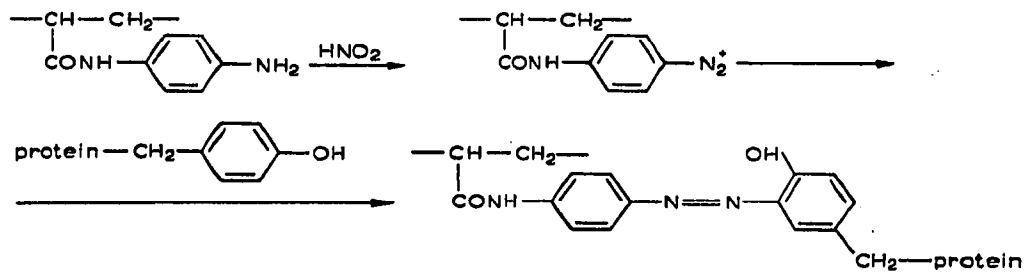


Reaction with appropriate compounds[31] gives aminoethyl derivatives and hydrazide derivatives; *p*-aminobenzamidoethyl derivatives can be prepared from their aminoethyl derivatives.

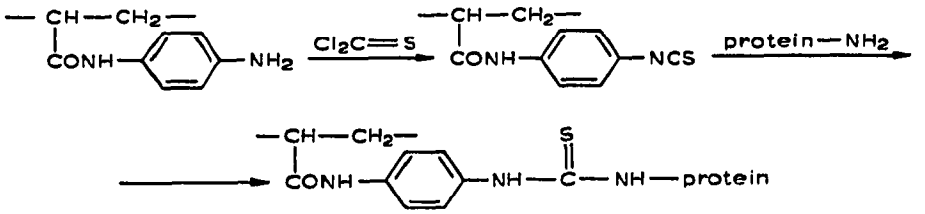
The hydrazide derivatives when activated by nitrous acid bind proteins through their amino groups



After diazotation polyacrylamide gels containing aromatic acid residues bind proteins mostly through their aromatic residues (predominantly via tyrosine)

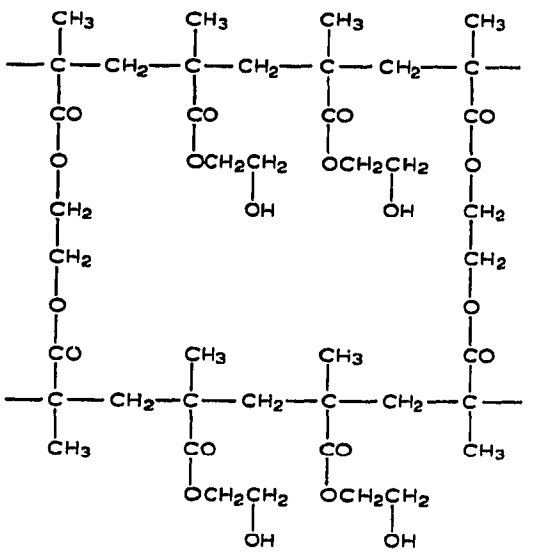


After activation by thiophosgene the same gels bind proteins through their free amino groups.



Polyacetal derivatives[32] of polyacrylamide gels bind proteins through their amino groups, polythiol derivatives[32] through their sulphydryl groups and polythiolactone derivatives[32] through their hydroxyl groups. These derivatives, however, have been used very rarely in affinity chromatography.

The chemical structure of hydrophilic hydroxyalkyl methacrylate gels is as follows:



The hydroxyl groups of the gel show properties analogous to those of the hydroxyl groups of agarose. After activation with cyanogen bromide, they bind affinants through their amino groups in the same manner as agarose[10]. The number of reactive groups, porosity and size of the specific surface of these gels[33] can be varied over a wide range during their production. The dependence of the quantity of attached high-molecular-weight substance (chymotrypsin) and of low-molecular-weight compound (glycine) on the size of the specific surface is shown for seven commercial products (Spheron) in Table I. NH_2-Spheron, prepared by the attachment of 1,6-hexamethylenediamine, and COOH-Spheron, prepared by the attachment of ε-aminocaproic acid, can be employed to advantage for the preparation of insoluble peptidic affinants by methods of peptide synthesis in organic solvents[34]. The basic series of

Spheron gels can be modified by copolymerization of monomers containing reactive functional groups or groups which can be activated for the binding of an affinant by a conversion analogous to polymerization[35,36].

Glass which has been allowed to react with γ-aminopropyltriethoxysilane becomes a support suitable for the binding of proteins[37–40]. Proteins can be attached to the amino groups of the modified glass through their carboxyl groups via soluble carbodiimides, through their amino groups after activation with thiophosgene and through their aromatic residues via an azo bond to the arylamide derivative of the glass support.

ADSORPTION AND DESORPTION OF ISOLATED PRODUCTS ON INSOLUBLE AFFINANTS

The choice of adsorption conditions for the products to be isolated on an insoluble affinant entirely depends on the optimum conditions of complex formation between the affinant and the product; in addition to pH and ionic strength it depends also on the content of metal ions or of other specific factors. A high ionic strength of the starting buffer decreases non-specific adsorption of polyelectrolytes to the charged groups which may be present in the attached affinant or solid support. Therefore an approximately 0.5 *M* concentration of NaCl is recommended, although only in cases where an increase of ionic strength does not decrease the affinity of the product to be isolated for the attached affinant, as shown by Kalderon *et al.*[20] in their experiments with the isolation of acetylcholinesterase. It is quite common in affinity chromatography to combine the batchwise with the column arrangement. This combination prolongs the period necessary not only for the contact of the product to be isolated with the attached affinant but also for the proper orientation of the binding sites.

The isolated product can be released from the complex with the attached affinant by a number of procedures. Porath and Sundberg[41] isolated chymotrypsin and trypsin from a column of agarose with attached soya bean trypsin inhibitor (Fig. 8). The adsorbed chymotrypsin was released by a solution of tryptamine which specifically released only chymotrypsin, whereas trypsin was specifically released by a solution of benzamidine. The dissociation of the complex can also be brought about by

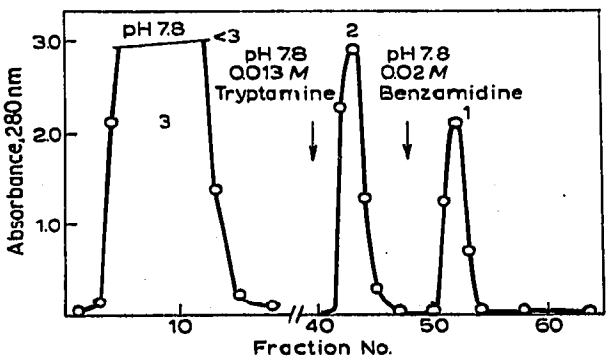


Fig. 8. Affinity chromatography of pancreas extract[41] on an agarose–soya bean trypsin inhibitor column (volume: 30 ml). Stepwise elution was accomplished with specific inhibitor. 1 = Trypsin; 2 = chymotrypsin; 3 = pancreas extract.

a change in the composition of the solvent. Fig. 9 shows the isolation of chymotrypsin and trypsin by Porath and Sundberg[41]; in this case the authors used a change of pH of the buffer for the dissociation of the complex. By using a pH gradient they were able to first release (at pH 4.5) chymotrypsin from the complex of enzymes with the insoluble inhibitor; the dissociation of the complex of trypsin with the inhibitor was achieved at a lower pH. If affinity chromatography on a column with attached trypsin inhibitor is used only for the separation of contaminating products from chymotrypsin, the course of affinity chromatography is simpler, as shown in Fig. 1. A batch processing arrangement can be used to advantage in cases where the affinity of the product to be isolated for the affinant is high. The isolation process can then be regarded as precipitation rather than chromatography.

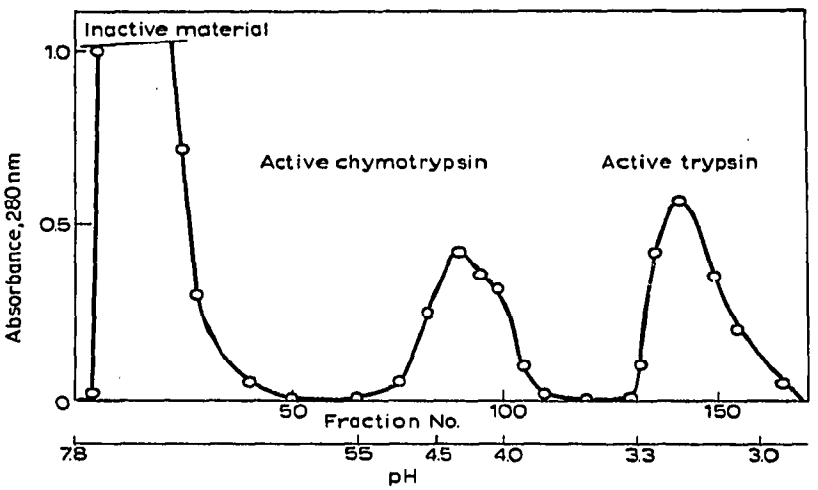


Fig. 9. Affinity chromatography of pancreas extract[41] on an agarose–soya bean trypsin inhibitor column (volume: 30 ml). The chromatogram was developed with a pH gradient.

The adsorbed material can also be released from the insoluble affinant by increasing the ionic strength. The affinity chromatography of *Escherichia coli* RNA polymerase on DNA–agarose carried out by Nüsslein and Heyden[42] is shown in Fig. 10. From 95 ml of a fraction of RNA polymerase, which had been obtained by chromatography on Bio-Gel A, inactive material was separated by affinity chromatography and emerged in the first peak; after subsequent application of a linear gradient of 0.25–1.25 *M* KCl two different RNA polymerases were obtained. Cuatrecasas and Wilchek[43], who isolated egg-white avidin by affinity chromatography on a column of Sepharose with covalently attached biocytine, were able to dissociate the complex only after the application of a drastic dissociating agent, 6 *M* guanidine hydrochloride. This procedure can be used only if the affinant and the product to be isolated do not loose their activities in guanidine hydrochloride. The product to be isolated can also be released from the complex by increasing the temperature; an example is given in Fig. 4, which shows the affinity chromatography of nucleic acid fragments on DNA–cellulose carried out by Gilham and Robinson[12].

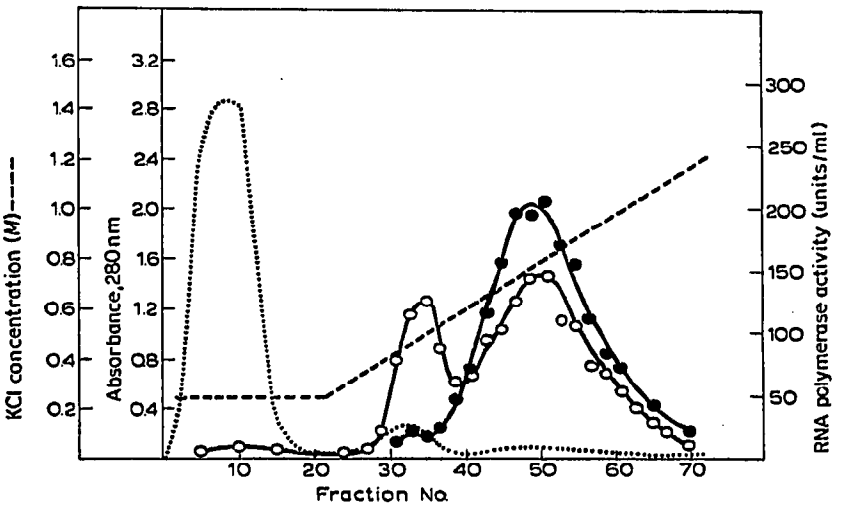


Fig. 10. Affinity chromatography of RNA polymerase[42] on DNA–agarose. The column (15 × 2 cm I.D.) was equilibrated and chromatographed with standard buffer (0.01 *M* Tris, pH 8.0; 10^{-3} *M* EDTA, 10^{-4} M dithioerythritol, 5% glycerol) and 0.25 *M* KCl. Elution of adsorbed protein was carried out with a 600-ml linear gradient of 0.25–1.25 *M* KCl in standard buffer. The fraction of RNA polymerase (95 ml) was applied to the column. 10-ml fractions were collected and assayed for RNA polymerase activity, using calf thymus DNA (○—○) and T4 DNA (●—●) as template. The total recovery of the RNA polymerase activity from the column was 80%. ....., absorbance at 280 nm.

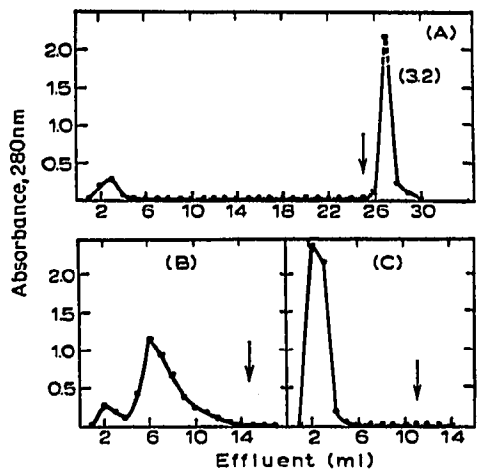


Fig. 11. Affinity chromatography of α-chymotrypsin[8] on inhibitor–Sepharose columns. (A) Sepharose coupled with ε-aminocaproyl-D-tryptophan methyl ester; (B) Sepharose coupled with D-tryptophan methyl ester; (C) unsubstituted Sepharose. The columns (5 × 0.5 cm I.D.) were equilibrated and run with 0.05 *M* Tris–HCl buffer, pH 8.0. Each sample (2.5 mg) was applied in 0.5 ml of the same buffer. The columns were run at room temperature with a flow-rate of about 40 ml/h. Fractions containing 1 ml were collected. Vertical arrows, elution buffer change (0.1 *M* acetic acid, pH 3.0). First peaks in A and B were devoid of enzyme activity. ——, absorbance at 280 nm.

A few practical suggestions follow from the work of Cuatrecasas *et al.*[8]. If a product having a high affinity for the attached affinant is to be isolated, the volume of the sample applied has no effect. In contrast, affinity chromatography can be used for the concentration of extremely dilute solutions. If the product to be isolated shows a low affinity, the volume of the sample applied should not exceed 5% of the hold-up volume in order that simultaneous elution of the product and the unadsorbed material be prevented. As an example, Fig. 11 shows the affinity chromatography of α-chymotrypsin on Sepharose with attached ε-aminocaproyl-D-tryptophan methyl ester (A), on Sepharose with attached D-tryptophan methyl ester (B) and on unsubstituted Sepharose (C) (ref. 8). In the first case (A) the attached inhibitor shows a high affinity for α-chymotrypsin. The enzyme can be released from the complex only by decreasing the pH of the eluting buffer. The chymotrypsin fraction can be eluted by 0.1 *M* acetic acid and emerges as a sharp peak; the volume in which chymotrypsin emerges does not depend in this case on the volume of the sample applied to the column. In the second case (B) the inhibitor bound directly to the Sepharose has a far lower affinity for the α-chymotrypsin to be isolated because of steric hindrances. The elution of the enzyme can be achieved in this case without a buffer change and, as can be seen in

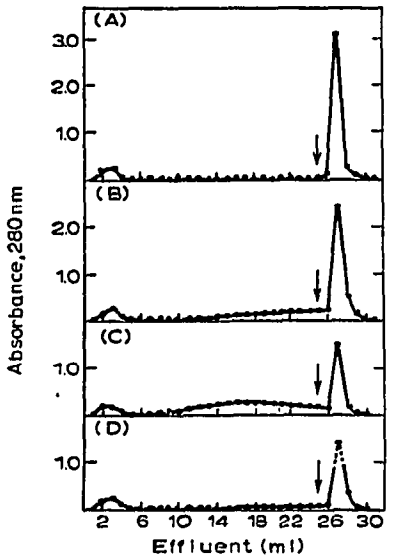


Fig. 12. Effects of pH and ionic strength on affinity adsorption of α-chymotrypsin[8] on a column of Sepharose derivative substituted with ε-aminocaproyl-D-tryptophan methyl ester. The columns (5 × 0.5 cm I.D.) were equilibrated and run with (A) 0.05 *M* Tris, pH 8.0; (B) 0.05 *M* Tris, pH 7.4; (C) 0.05 *M* Tris, pH 6.8; (D) 0.01 *M* Tris, pH 6.8. The other conditions are identical to those shown in the legend to Fig. 11.

the graph, the enzyme emerges in a far larger volume immediately after the inactive material. Chromatography of α-chymotrypsin on the unsubstituted support (C) was carried out to determine whether there is non-specific adsorption under the given experimental conditions.

The effect of pH and ionic strength on the affinity adsorption of α-chymotrypsin to Sepharose columns with attached ε-aminocaproyl-D-tryptophan methyl ester is

shown in Fig. 12. The first peak (fractions 2 – 4) contained in all cases material free of chymotryptic activity. The specific activity of the chymotrypsin emerging in the subsequent fractions was constant. It is therefore obvious from Fig. 12 that a decrease in the pH of the starting buffer results in a decrease in the stability of the complex of α-chymotrypsin with the attached inhibitor. As a result, α-chymotrypsin is slowly eluted by the first buffer. The elution of α-chymotrypsin from the insoluble affinant proceeds faster at a higher ionic strength.

TABLE III

REVIEW OF INSOLUBILIZED ENZYMES PRODUCED BY THE FIRM MILES-SERAVAC AND MILES-YEDA

Enzyme	*Enzymes bound to (I) CM-cellulose or (II) DEAE-cellulose*	*Percentage of protein*	*Enzymes bound to copolymer of ethylene with maleic anhydride (EMA)*	*Percentage of protein*	*Enzymes bound to agarose*
Trypsin	Enzite–trypsin (I)	5–10	Enzite–EMA–trypsin	65–70	Enzite–agarose–trypsin
Chymotrypsin	Enzite–chymotrypsin (I)	5–10	Enzite–EMA–chymotrypsin	65–70	Enzite–agarose–chymotrypsin
Papain	Enzite–papain (I)	5–10	Enzite–EMA–papain	60–65	Enzite–agarose–papain
Protease (*Streptomyces griseus*)	Enzite–protease (I)	1–10			Enzite–agarose–protease
Subtilopeptidase			Enzite–EMA–subtilopeptidase A	50–55	
			Enzite–EMA–subtilopeptidase B	50–55	
Leucine aminopeptidase	Enzite–leucine aminopeptidase (II)	5–10			
Bromelain	Enzite–bromelain (I)	5–10			
Ficin	Enzite–ficin (I)	5–10			
Alcohol dehydrogenase	Enzite–YADH* (II)	1–5			
Glucose oxidase	Enzite–glucose oxidase (II)	5–10			
Peroxidase	Enzite–peroxidase (I)	1–10			
Ribonuclease	Enzite–RNAse (I)	5–10			
Urease	Enzite–urease (II)	5–10			
Amylase	Enzite–amylase (I)	1–5			
Cytochrome C	Enzite–cytochrome C (I)	5–10			

* YADH = yeast alcohol dehydrogenase.

COMMERCIALLY AVAILABLE INSOLUBLE AFFINANTS

To illustrate the rapid development of affinity chromatography, a survey of commercially available specific adsorbents, in this case of insoluble enzymes produced exclusively by Miles-Seravac and Miles-Yeda, (Maidenhead, Great Britain), is presented in Table III. This company also produces ε-aminocaproyl-D-tryptophan methyl ester coupled to agarose for the isolation of chymotrypsin, agarose with the attached tetrapeptide Gly–Gly–Tyr–Arg(OBZ = O-benzyl) for the isolation of papain, agarose–

OBZ

5'-(4-aminophenyl)-uridine-2'-(3')-phosphate for the isolation of ribonuclease, agarose–L-tryptophan and agarose–D tryptophan for the isolation of tryptophan-binding proteins, and agarose–L-phenyl-alanine, agarose–L-tyrosine and agarose–thyroxine for the isolation of proteins binding phenylalanine, tyrosine and thyroxine, respectively. Concanavalin A attached to agarose is supplied under the name Glycosylex A. The company also produces immuno-sorbents for the isolation of antibodies, *i.e.* agarose with attached antigens for the isolation of antibodies and agarose with attached antibodies for the isolation of antigens.

This survey is given only as an example, a number of insoluble affinants also being supplied by Pharmacia (Uppsala, Sweden), Merck (Darmstadt, G.F.R.), etc. The ever increasing number of commercially available insoluble affinants is the best evidence of the rapid development and important role of this method.

TABLE IV

USE OF AFFINITY CHROMATOGRAPHY FOR THE ISOLATION OF BIOLOGICALLY ACTIVE PRODUCTS — SUMMARY OF PAPERS PUBLISHED IN 1972 AND 1973

Substances isolated	*References*
Enzymes	
Acetylcholinesterase	46, 49–53
β-N-Acetylhexosaminidase	54
Alginase	55
Arylsulphatase	56
ATPase	57
Carbonic anhydrase	58, 59
Deoxyribonuclease	60, 61
Dehydrogenases	
Alcohol dehydrogenase	62–64
Glucose 6-phosphate dehydrogenase	63
Glyceraldehyde 3-phosphate dehydrogenase	11, 63–66
D-3-Hydroxybutyrate dehydrogenase	63
Lactate dehydrogenase	63, 64, 67, 68
Lipoamide dehydrogenase	69
Malate dehydrogenase	63
Oestradiol-17β dehydrogenase	70
Tetrahydrofolate dehydrogenase	71–75
L-Threonine dehydrogenase	63
Endopolygalacturonase	76
α-Galactosidase	77
β-Galactosidase	77–79
β-Glucuronidase	80
Glycogen phosphorylase	81

TABLE IV *(continued)*

Substances isolated	*References*
Glycosidase	82
β-Lactamase	83
Lipoxygenase	84
Penicillinase	85
Phenylalanine:tRNA ligase	86
Phosphofructokinase	87
Polymerases	
DNA polymerase	88, 89
RNA polymerase	42, 90
Proteinases	
Acrosomal proteinase	91
Alkaline proteinase from *Aspergillus oryzae*	92
Bromelain	93
Carboxypeptidase	44
Chymotrypsin	10
Insulin-specific protease	94
Kallikrein	95, 96
Neutral protease from *Bacillus subtilis*	97
Neutral protease from *Clostridium histolyticum*	98
Plasminogen	99–101
Renin	102, 103
Thermolysin	97
Thrombin	104, 105
Trypsin	105–107
Protocollagen proline hydroxylase	108
Pyruvate kinase	109
Reductases	
Glutathione reductase	63, 110
Phage T4-induced ribonucleotide reductase	111
Ribonuclease	112, 113
Synthetases	
Aminoacyl-tRNA synthetase	114–119
Dihydroneopterin triphosphate synthetase	120
Glycogen synthetase	121
Thymidylate synthetase	122
Threonine deaminase	123
Transferases	
Aspartate aminotransferase	124
Choline acetyl transferase	125
Galactosyl transferase	126
Nucleoside deoxyribosyl transferase	127
Tyrosine aminotransferase	128
Xanthine oxidase	129
Inhibitors	
α_1-Antitrypsin	130, 131
Chymotrypsin inhibitor from potatoes	10
Ovomucoid	132
Polyvalent inhibitors of trypsin, chymotrypsin, plasmin and kallikrein	133
Proteinase inhibitors	134
Soya bean trypsin and chymotrypsin inhibitors	135
Trypsin inhibitor (Kunitz)	136

(Continued on p. 286)

TABLE IV *(continued)*

Substances isolated	*References*
Specific peptides	
Haeme peptide	137
Nitrotyrosine-containing peptides	47
Tryptophan-containing peptides	14
Nucleic acids and nucleotides	
Adenosine 3':5'-monophosphate	138
Globin messenger RNA	139
Messenger RNA from KB-cells	140
Mononucleotides	141
Oligonucleotides	141
Ovalbumin messenger RNA	142
Transfer RNA	141, 143, 144
Antibodies and antigens	
Antiserum to α-foetoprotein	145
α-Foetoprotein	146, 147
Glucagon-like immunoreactivity	148
Interferon	149
Protein A from *Staphylococcus aureus*	150
Thioredoxin	151
Receptors and transport proteins	
Acetylcholine receptors	152–154
Aldosterone-binding macromolecules	155
Cholinergic receptor	156
Folate-binding protein	157
L-Fucose-binding proteins	158
Glycoprotein receptors	159
Penicillin-binding components	160
Thiamine-binding protein	161
Steroid receptor	162, 163
Vitamin B_{12}-binding proteins	164, 165
Agglutinins and haemagglutinins	
Galactose-binding haemagglutinins	166
Galactose-binding phytoagglutinins	167
Haemagglutinins	168
Phytohemagglutinins	169–171
Soya bean agglutinin	172
Wheat germ agglutinin	173,174
Lipids	
Lipids from plasma	175
Others	
Concanavalin A-binding proteins	176, 177
Erythrocyte membrane protein	178
Histone fraction	179
Human intrinsic factor	180
Ovine luteinizing hormone	48
Polysomes	181
Serum albumin	182
Teichoic acid	183
Tubulin	184
Vasopressin	185
Virus of Aleutian mink disease	186
Virus glycoproteins	187

CONCLUSIONS

Purification of biologically active products by classical methods based on differences in the number of polar and non-polar groups, in the size and shape of their molecules, etc., is non-specific, very often elaborate, and incomplete; the yields obtained are very low. In contrast, the isolation of biologically active products by affinity chromatography makes use of the extraordinary ability of these products to form specific, stable and reversible complexes. Such complexes are, for example, enzymes with their inhibitors, substrates, cofactors or effectors, antibodies with antigens, hormones with receptors or their transport proteins, nucleic acids with nucleic acid polymerases or specific gene messenger nucleic acids, etc., concanavalin with polysaccharides and glycoproteins containing glycosyl terminal groups, and products with free SH-groups with mercury compounds. If one of the components forming the complex is covalently attached to a solid support, a specific adsorbent (the so-called insoluble affinant) is thus obtained. To this affinant the second component of the complex will be adsorbed from the medium. After products bound non-specifically have been washed out, this second component can be released from the complex by a suitable procedure. The isolation of the product thus becomes simpler and faster, and the yields increase considerably. Often one chromatographic step will provide the pure, or at least considerably concentrated, biologically active product, as demonstrated in Figs. 8 and 9, which illustrate the experiments of Porath and Sundberg[41], in which highly active chymotrypsin and trypsin were obtained from a pancreatic extract by one chromatographic run. Similarly, the isolation of carboxypeptidase from activated pancreatic juice[44] or the isolation of acetylcholinesterase from a tissue extract[45,46] may be mentioned. Peptides containing modified tyrosine[13,47] or modified tryptophan[14] can be isolated in one step from an enzymatic digest; luteinizing hormone[48] can also be obtained in one chromatographic experiment. This easier isolation of biologically active products in high yield and at considerably lower cost offers additional possibilities for use of these products in medicine, the analytical field or industry. To illustrate the rapid development of this method, a summary of studies on the use of affinity chromatography which have been published during the last two years is presented in Table IV.

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