*Journal of Solid-Phase Biochemistry, Vol. 4, No. 3, 1979*

# AFFINITY CHROMATOGRAPHY, CARRIERS, AND LIGAND BINDING[1]

**W. BRÜMMER**

*E. Merck, Darmstadt*
*Department of Biochemical Research*
*W. Germany*

Accepted May 22, 1979

Affinity chromatography is a certain type of adsorption chromatography. Figure 1 shows how affinity chromatography works. It is based in most cases on a biospecific interaction between a ligand, which is bound by a spacer to an insoluble matrix, and a macromolecule in solution. The spacer is necessary to prevent a steric hindrance of the complex formation. When a solution that contains a macromolecular substance is poured over a column that contains a corresponding affinity resin, the macromolecules are retained when the dissociation constant of the complex is $10^{-4}$ M or less. Contaminations are washed from the column by the starting buffer. In order to elute the macromolecules, the complex must be decomposed. Therefore, the pH value of the elution buffer is altered or its ionic strength or its temperature is raised. Competitive elution is possible too, e.g., the elution of an enzyme by its coenzyme or its substrate. If the complex is very tight, denaturing substances such as urea or guanidinium chloride will be useful under the condition that they do not destroy irreversibly the macromolecules to be isolated.

The most important prerequisite for the preparation of a good affinity resin is an appropriate carrier, which must have the following properties. The carrier must be insoluble in water but hydrophilic, that is, it is easily wetted. The carrier must have good mechanical properties, that is, particle size and compressibility must allow good flow properties of the packed column; the matrix must not be too fine and too soft. The carrier must be macroporous, so that the ligands which are bound inside the pores are also accessible to the macromolecules. The carrier must be inert, and especially it should not be hydrophobic and not an ionic exchanger. This has nothing to do with the spacer, as discussed below.

[1]This paper was presented at the symposium on "Hydrophobic and Biospecific Affinity Chromatography" held at Ruhr-Universität Bochum, West Germany, October 4, 1978.



0146-0641/79/0900-0171$03.00/0 

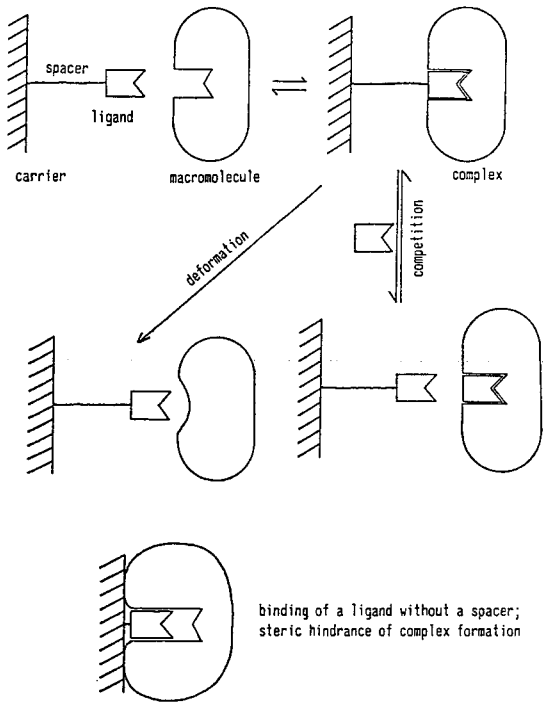


FIG. 1. Scheme of affinity chromatography.

The carrier must be stable chemically and biochemically, and especially it must not be hydrolyzed at pH values between 2 and 12 and must be inert against microbial digestion. For binding ligands, the carrier must have enough functional groups. These groups must be located at the ends of spacers in order to prevent as much as possible steric hindrance between the carrier bound ligand and the macromolecule in solution. Of course, this part of the ligand, which is important for the interaction, must be freely available after the ligand is bound to the matrix. Thus, the point of attachment of the ligand to a carrier can influence the complex formation. Table 1 shows the interaction of some dehydrogenases with the general ligand AMP, the "half-molecule" of NAD, which is bound in different ways to agarose: in one case through the amino group in position 6, and in another case by the carbon atom in position 8 ($C_8$). The table shows that alanine-DH and MDH from *B. subtilis* form stronger complexes with AMP, which is bound by the $C_8$ atom, than with AMP, which is bound through the amino group. On the other hand, ADH, LDH, and MDH from animal tissues form stronger

TABLE 1. **Complex Formation of Some Dehydrogenases with Two Different AMP-Agaroses**

| Enzyme | Binding point of AMP | |
|---|---|---|
| | C(8) | N(6) |
| Alanine-DH | + + + | + |
| ADH | + | + + |
| LDH ($M_4$) | + | + + + |
| LDH ($H_4$) | + | + + |
| MDH | − | + + |
| MDH (B. subt.) | + | − |
| D-Gal-DH | − | − |

complexes with AMP, which is bound through the amino group in position 6.

Adenosine deaminase, which catalyzes the change of the amino group at the purine ring (adenosine) to a hydroxyl group (inosine), forms a stable complex with carrier bound adenosine only in the case that the purine ring is freely available. That means adenosine must be bound through the ribose rest. Also, the spacer, which was initially introduced to prevent steric hindrance of the complex formation, can influence the course of affinity chromatography by its hydrophilic or hydrophobic character. Table 2 shows the influence of different spacers on the complex formation of LDH with AMP-agarose. The spacers are hexamethylene diamine, adipic acid dihydrazide, and 2,3,4,5-tetrahydroxy-1,6-diaminohexane. Though the four

TABLE 2. **Influence of Spacer and Charge on Complex Formation**

| Affinity resin | Conditions of elution |
|---|---|
| (1) A———O—C(=$^{\oplus}$NH$_2$)—NH—$(CH_2)_6$—NH~AMP($N_6$) (pK = 9.4) | KCl + NADH |
| (2) A———O—C(=NH)—NH—NH—C(=O)—$(CH_2)_4$—C(=O)—NH—NH~AMP($N_6$) (pK = 4.2) | KCl |
| (3) A———O—C(=$^{\oplus}$NH$_2$)—NH—$CH_2$—(CH(OH))$_4$—$CH_2$—NH~AMP($N_6$) (pK = 9.4) | No binding |

spacers are all equal in length, the complexes that the resins form with LDH have different strengths. The reason is that the spacers have different hydrophilicity or hydrophobicity and either have or lack ionic groups.

The resin that contains AMP bound by hexamethylene diamine forms the strongest complex, as the conditions of elution show. Raising the salt concentration of the elution buffer is not enough; NADH must also be present in order to remove the enzyme by competition. The strength of this complex is explained by an additional hydrophobic interaction between the hydrophobic spacer and a hydrophobic part of the enzyme molecule as well as by an additional ionic interaction between the positive charge of the isourea group (pK 9.4) and a negative charge of the enzyme. These interactions are present at pH 7, the pH value of the working buffer. In addition, AMP, which is bound by hydrophobic hexamethylene diamine, stands away from the particles of the resin and is easily accessible.

If adipic acid dihydrazide is used as a spacer, there is no positive charge at pH 7, because of the low pK value of 4.2. Thus, the complex gets weaker. It is enough to raise the salt concentration for eluting the enzyme. When the hydrophilic tetrahydroxyaminohexane is used as a spacer, the affinity resin no longer binds LDH. Indeed, the positively charged isourea group (pK 9.4) appears again, but the hydrophilic side chain is closely adsorbed to the surface of the gel particles, so that the ligand is not accessible to the macromolecule.

Thus, the main biospecific interaction may be strengthened by ionic and/or hydrophobic interactions in some cases in a desired manner, a phenomenon called *compound affinity*. Of course, the biospecific component must be dominant in affinity chromatography. This can be demonstrated by control experiments (see below). A separation that is realized mainly by hydrophobic interaction is called hydrophobic chromatography (see the paper of H. P. Jennissen in this issue). From these considerations about the spacer and the binding manner of the ligand, it follows that every affinity resin can be optimized by varying the chemical nature of the spacer arm and the binding manner of the ligand.

Agarose is the carrier that is mostly used in affinity chromatography. Dextran, cellulose, polyacrylamide, porous glass, nylon, and vinyl polymers are also employed for this purpose. None of these carriers shows all properties an ideal carrier should have (see above).

Functional groups of the carriers that are convenient for binding ligands are as follows. First, there are the amino, the hydroxyl, and the carboxyl groups, which can form carbonic acid amide and carbonic acid ester bonds. Next, there is the sulfhydryl (—SH) group, which can form thioester bonds and disulfide (—S—S—) bridges. Third, there is the vinyl group, to which, e.g., amines can be added. Fourth, there is the aldehyde group, which can,

for instance, be formed by perjodate oxidation of carbohydrate residues on polysaccharides (e.g., starch) and which can form azo methines (Schiff's bases) with amines. Finally, there is the oxirane ring, which is introduced in hydroxyl groups containing carriers by epichlorhydrine. The three-membered ring can be opened by nucleophilic reagents; for instance, by amines, and by aromatic amino and aromatic hydroxyl groups, which can be diazotized and used for diazo couplings. In the following chapter, some reactions are discussed that are used mainly for activating carriers and for covalently binding amino groups containing spacers and macromolecules.

The activation of agarose and dextran or cellulose by cyanogen bromide is shown in Fig. 2. Recent investigations (J. Kohn and M. Wilchek, personal communication), show that the hydroxyl groups of the carriers are converted to cyanate ester groups, which react directly, or in the case of dextran and cellulose via imidocarbonate, with amino groups containing substances to form isourea derivatives. High molecular weight amino groups containing ligands, which must not be attached to a spacer, can be bound directly to cyanogen bromide activated carriers. The reaction with $\alpha,\omega$-diamino alkane or with $\varepsilon$-aminocaproic acid introduces spacers with amino or carboxyl groups at their free ends. $\alpha,\omega$-Diaminoalkane must be used abundantly in this case, so that only one of the two amino groups may react with the activated carrier, while the second one remains free for coupling a ligand. Thus, crosslinking of the carrier is avoided, which would diminish its porosity. Binding ligands by canogen bromide results mainly in the formation of isourea derivatives. This may be proved by aminolytic cleavage of carrier-bound ornithine, which is fixed by its $\delta$-amino group and mainly forms free arginine. Besides derivatives of isourea, derivatives of carbamate and imidocarbamate are formed too.

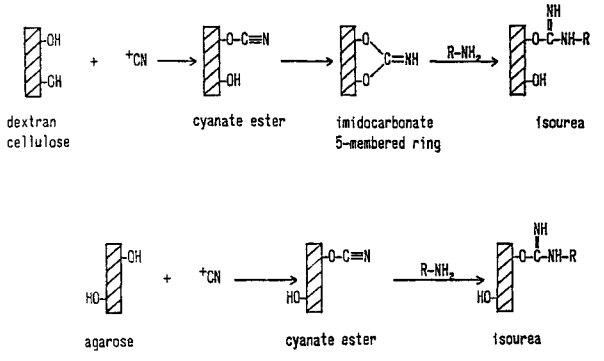


FIG. 2. Activation of agarose, dextran, and cellulose by cyanogen bromide (CNBr) [after KOHN, J., and WILCHEK, M. (1978) Biochem. Biophys. Res. Commun. 84 : 7–14].

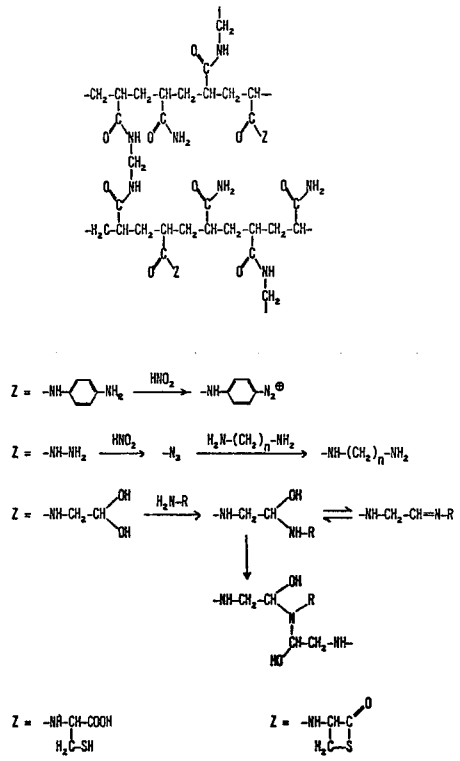

FIG. 3. Crosslinked polyacrylamide.

A disadvantage of agarose resins is their increasing hydrolysis at pH values above 7, mainly in the presence of nucleophilic buffer substances such as triethanolamine. The application of multivalent spacers that form several bonds with the carrier avoids bleeding of ligands. It is also useful to avoid nucleophilic buffer substances.

Figure 3 shows crosslinked polyacrylamide. "Z" means functional groups that are suitable for covalently binding spacers and macromolecular ligands. Some functional groups are described as follows. First, there is the aromatic residue, which may be diazotized and/or coupled to other aromatic groups. Second, there is the carbonic acid hydrazide rest, which can be converted to a reactive acid azide group by nitrous acid. The reaction with $\alpha,\omega$-diaminoalkane forms spacers with amino groups at their free ends in this case too. The acetic aldehyde group in the hydrated form reacts with amino groups containing ligands to form azo methines (Schiff's bases), which may be reduced to stable amines by sodium borohydride. Two aldehyde

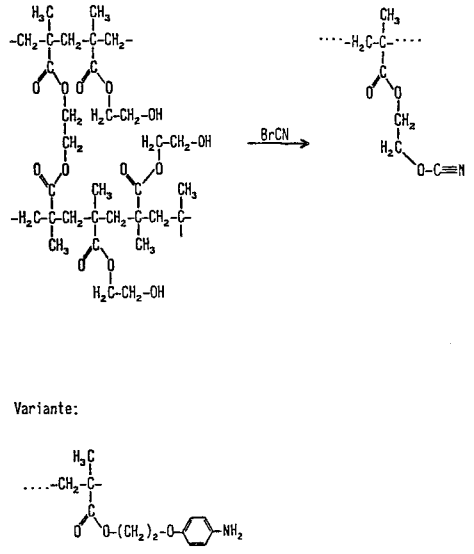


FIG. 4. Crosslinked polyhydroxyethylmethacrylate.

groups may react with one amino group to form a tertiary amine; the cysteine residue may form disulfide (—S—S—) bridges with sulfhydryl (—SH) groups containing substances; and the reactive thiolactone may be opened by nucleophilic reagents such as amines to form carbonic acid amides.

Figure 4 shows polymethacrylates, which are rich in hydroxyethyl groups and may be activated by cyanogen bromide, corresponding to agarose. In this case, functional groups may be introduced by copolymerizing with appropriate acrylic acid derivatives, as is shown here for the aromatic amino group. This method has the advantage of avoiding the hydrolyzable isourea derivatives that are formed by the cyanogen bromide activation.

Figure 5 shows the derivation of cellulose carriers. Cellulose is changed to carboxymethyl cellulose by chloroacetic acid. Then carboxymethyl cellulose is esterified by hydrochloric acid in methanole, and the ester is transformed to the carbonic acid hydrazide by hydrazine, which is converted to the carbonic acid azide by nitrous acid. Reaction with amines forms carbonic acid amides. The reaction with abundant $\alpha,\omega$-diaminoalkane again introduces spacers with amino groups at their free ends.

Cellulose can also be activated with triazines such as cyanuric chloride or dichlorotriazines, which contain solubilizing groups such as carboxymethoxy or carboxy-methylamino groups. The reaction proceeds in two

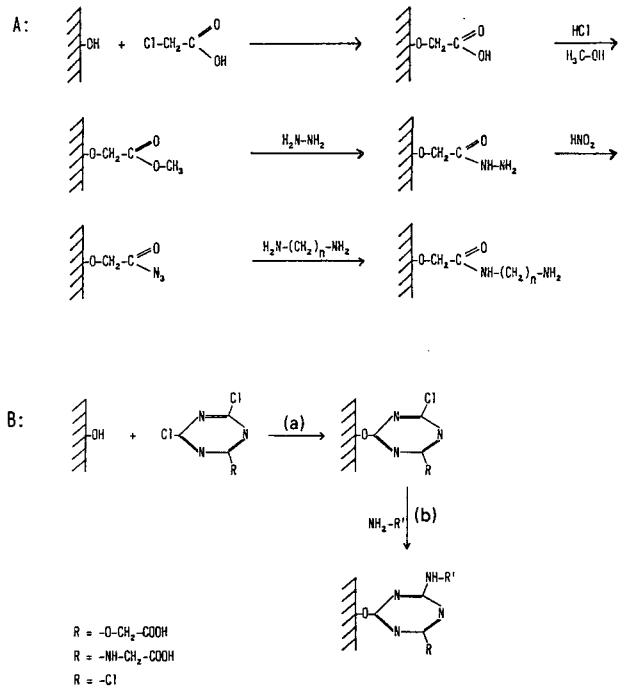


FIG. 5. Derivation of cellulose: (A) via carboxymethyl cellulose; (B) via triazine.

steps: (a) rapid reaction of one of the chlorines in a few minutes with the cellulose matrix to give monochloro-s-triazinyl-cellulose, and (b) a slower reaction with strong nucleophiles such as the primary amino groups of spacers or proteins.

Figure 6 shows the reaction by which an amino group containing spacer is bound to porous glass. Hydroxyl groups on the surface of the glass particles react with $\gamma$-aminopropyltriethoxy-silane to form the so-called "alkylamino-glass."

If nylon (as a net or a tube) is used as a carrier, the surface of the nylon is first edged on by a hot methanolic solution of calcium chloride. Then the

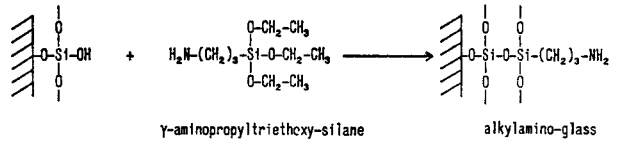


FIG. 6. Conversion of glass to alkylamino-glass.

nylon is partially hydrolyzed by hydrochloric acid to get functional carboxyl and amino groups.

From the group of vinylpolymeric carriers, polystyrene and polyvinyl alcohol may be mentioned. Functional groups at the benzene ring of polystyrene are, for instance, amino and isothiocyanato groups. Polyvinyl alcohols, which are crosslinked by terephthalic aldehyde, may be activated by triazines, as already described for cellulose. However, polystyrene and polyvinyl alcohol are less qualified for affinity matrices because of their relatively strong hydrophobic character.

A presentation of reactions that are important for binding ligands to amino groups containing carriers is shown in Fig. 7. Carboxyl groups containing ligands can be bound directly by water soluble carbodiimides. For binding amino groups containing ligands, first the amino groups of the carrier must be derived. There are different methods for doing so. Carboxyl groups may be introduced by succinylation. Then, the amino groups containing ligands can also be bound as carbonic acid amides by water soluble carbodiimide. Another method is the preparation of the bromo-acetamidoalkyl carrier with O-bromo-acetyl-N-hydroxysuccinimide. The amino group of the ligand reacts with the bromoacetyl rest while hydrobromic acid is split off.

Bifunctional reagents are also used for binding amino groups containing ligands. When the amino groups of the carrier are reacted with an abundant amount of divinylsulfone or glutaraldehyde, one of the two functional groups reacts with the amino group of the carrier. The second functional group remains available for binding the ligand. If the activation is done by glutaraldehyde, azo methines (Schiff's bases) are formed, which may be reduced to stabile alkylamines by sodium borohydride. A thiol (—SH) group may be introduced by reacting the amino group of the carrier with N-acetylhomocysteine thiolactone. Thereby the thiolactone ring is opened. Thiol groups containing affinity resins retain thiol groups containing substances by forming disulfide (—S—S—) bridges. Thiol (—SH) groups containing macromolecules that are isolated in this manner are bound covalently to the resin. Thus, this sort of affinity chromatography is also called "covalent chromatography."

The introduction of an aromatic amino group is done by p-nitrobenzoic acid azide. The nitro group is reduced to the amino group by sodium dithionite ($Na_2S_2O_4$). The aromatic amino group can be diazotized and coupled to aromatic rests of ligands. It may also be converted to isothiocyanate by thiophosgene. Then the amino group of a ligand may be added to the isothiocyanate. This is only a partial presentation of methods for synthesizing affinity resins (Fig. 7). In principle, any chemical reaction may be used that does not alter the carrier in an unsuited manner.

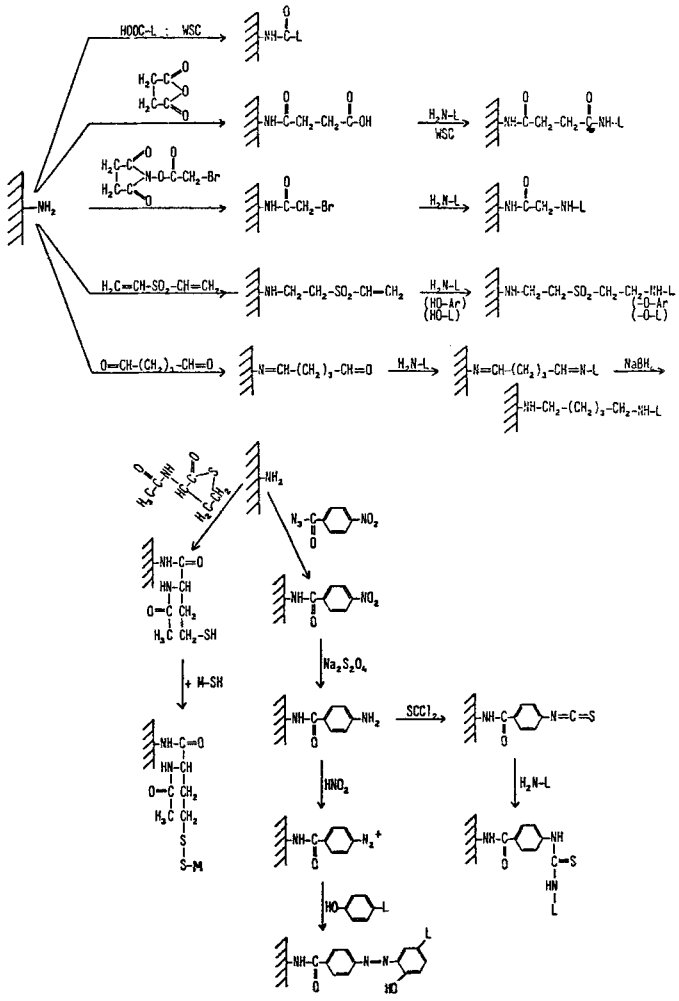

FIG. 7. Binding ligands to amino groups containing carriers.

In our laboratories at E. Merck, Darmstadt, we use a special cellulose carrier that is made from a nonfibrous, highly substituted, water soluble CM-cellulose. The degree of substitution is 0.85 carboxyl groups per glucose unit, which means 3.2 meq per g of substance. The CM-cellulose has a degree of polymerization that is 1,500 units of glucose per cellulose molecule. The CM-cellulose is converted to aminododecyl-CM-cellulose ($n$ = 12) by the reactions shown in Fig. 5A. Carboxyl groups as functional groups may be introduced by succinylation (see also, Fig. 7). The carrier con-

veniently satisfies the conditions listed earlier in this paper. It is mechanically stable, hydrophilic, macroporous, and insoluble in water. The spacer and the ligand are bound to the cellulose by stable ether and carbonic acid amide bonds, and therefore resist hydrolysis at pH values from 2 to 12. The spacer, which has an amino or a carboxyl group at its free end, is a hydrophobic hydrocarbon arm, standing away from the cellulose particles. Ligands that are bound to its ends are very accessible.

The macroporosity of the carrier was determined by comparison with an equal agarose resin. Figure 8 shows the striking experiments. L-arginine-4-nitroanilide (Argpa), a low molecular weight substrate of trypsin, was covalently bound to succinylated aminohexyl agarose (SAHA) and to succinylated aminohexyl CM-cellulose (SAHC). The carrier-bound L-arginine-4-nitroanilide was hydrolyzed chemically by sodium hydroxide (NaOH) and enzymatically by trypsin. The amounts of 4-nitroaniline, which were set free by sodium hydroxide, were taken as 100% in both cases. Then the kinetics of the enzymatic hydrolysis were measured. In both cases the concentrations of substrate and enzyme were the same. Again, in both cases, 65% of the carrier bound substrate was split off after several hours, which means that 65% of the carrier bound 4-nitroanilide is accessible for trypsin. (The endpoint for the tryptic hydrolysis of SAHA-bound Argpa is not shown in Fig. 8.) The tryptic hydrolysis in the case of the SAHC-bound substrate is evidently faster than in the case of the SAHA-bound one, which means that the substrate molecules bound inside the pores of the cellulose

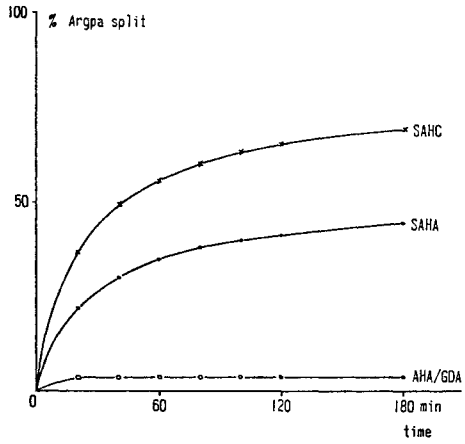


FIG. 8. Demonstration of the macroporosity of the cellulose carrier by enzymatic hydrolysis of three samples of carrier bound L-arginine-4-nitroanilide by trypsin.

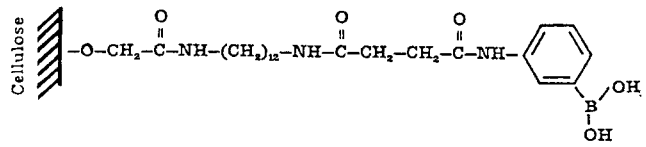


FIG. 9. SADC-bound 3-aminophenyl boronic acid.

carrier are more accessible for typsin that those bound inside the pores of agarose. Thus, the pores of the cellulose carrier are larger than those of the agarose.

The third curve of this figure shows the hydrolysis of Argpa, which was bound to aminohexyl agarose by glutaraldehyde activation. In this case, only 3% of the insoluble substrate could be hydrolyzed enzymatically, although the time of incubation was several hours. This means that the carrier was crosslinked in spite of the abundance of glutaraldehyde, and its pores became inaccessible for the trypsin molecules. The same is the case when divinylsulfone is used as activator.

Finally, I describe an affinity resin that works in two different ways: 3-aminophenyl boronic acid covalently bound to succinylated aminododecyl-CM-cellulose (SADC). On the one hand, the resin works by forming complexes with hydroxyl groups containing substances such as ribose and its derivatives; on the other hand, it is an inhibitor of serine proteases such as chymotrypsin, subtilisin, etc., and retains these proteases.

The ligand 3-aminophenyl boronic acid is bound to the carrier (SADC) by the carbodiimide reaction as carbonic acid amide (Fig. 9). The degree of substitution, which is determined by boron analysis, is 780 $\mu$mol of 3-aminophenyl boronic acid per g of dry substance or 80 $\mu$mol/ml bed volume, as 1 g of dry resin corresponds to 10 ml bed volume. Figure 10 shows how the SADC-bound 3-aminophenyl boronic acid forms complexes

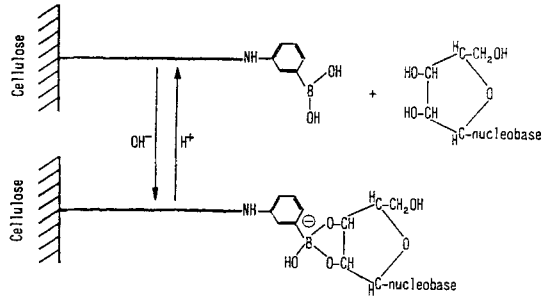


FIG. 10. Complex formation of SADC-bound 3-aminophenyl boronic acid with derivatives of ribose that contain two free hydroxyl groups in the 2'- and the 3'- positions.

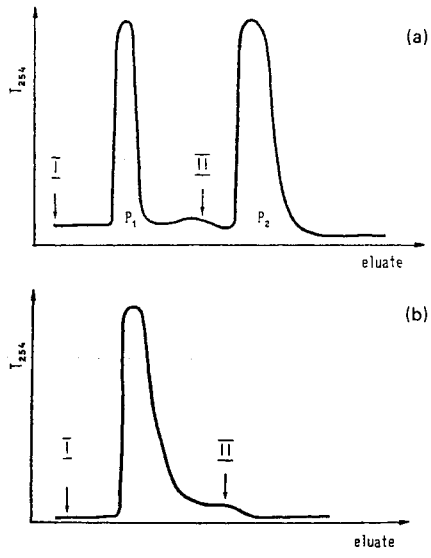


FIG. 11. (a) Separation of two nucleosides, adenosine and 2′-deoxythimidine, by affinity chromatography on SADC-bound 3-aminophenyl boronic acid. (b) Control experiment with a "mock resin"; for details, see text. I: solution of adenosine and 2′-deoxythymidine in 0.05 M morpholine buffer, pH 8.5. II: Elution of adenosine with 0.05 M MES buffer, pH 5.0.

with substances that contain 2 vicinal, cis-standing hydroxyl (—OH) groups. The complexes are formed in weak alkaline solution, for instance, at pH 8.5, and are split again by weak acid solution, for instance, at pH 5. Strong complexes are formed with carbohydrates in the furanose form, which is also the cyclic conformation of ribose. Thus, components of nucleic acids may be separated by this resin: ribonucleosides with free hydroxyl groups in the 2′- and 3′- position from those which are substituted in these positions [2′-deoxyribonucleosides; 2′, (3′)-ribonucleotides]. Also, oligo- and polynucleotides up to the molecular size of t-RNA may be separated by this boronic acid resin.

Figure 11 shows in its upper part the separation of two nucleosides, namely, 2′-deoxythymidine and adenosine. 2′-deoxythymidine passes through the column at pH 8.5 without any retardation ($P_1$), while adenosine is retained. For its elution, an acid buffer (pH 5.0) is necessary ($P_2$). The lower part of the figure shows the chromatography of the same nucleosides at a so-called "mock resin," which contains ethanolamine instead of 3-

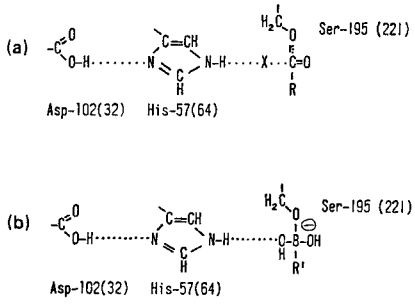


FIG. 12. (a) Substrate complex. (b) Inhibitor complex of boronic acid with chymotrypsin and subtilisin (numbers in brackets).

aminophenyl boronic acid as a ligand. The unspecific interactions (hydrophobic and ionic interactions) between the "mock resin" and the substances to be separated are the same as in the case of the affinity resin. Of course, the specific interaction is missing. The nucleosides are not separated by the "mock resin," which means that the separation by the affinity resin is based on the specific interaction between the boronic acid and the hydroxyl groups of the ribose moiety and not on unspecific interactions. The performance of an analogous control experiment is necessary for each affinity chromatographic separation, in order to exclude the possibility that a separation is based on unspecific interactions, and not on the desired biospecific ones.

The described interaction between the boronic acid resin and the ribose derivatives is applied in the analysis of rare nucleobases in the urine of cancer patients, for the isolation of ADP-ribosylated nucleoproteins, for the determination of the activity of ribonucleoside-disphosphate reductase, and for the isolation of epinephrine and norepinephrine from urine.

Figures 12 and 13 show the affinity chromatographic purification of serine proteases with the insoluble 3-aminophenyl boronic acid resin, which depends on the fact that the ligand is an inhibitor of serine proteases. Figure 12 shows the substrate and the inhibitor complex with boronic acid as inhibitor. Both complexes occur in the case of chymotrypsin and subtilisin, and are formed by the same amino acid residues, namely, serine, histidine, and asparaginic acid. Besides chymotrypsin and subtilisin, the boronic acid resin also retains proteinase K. This is an extracellular serine protease produced by *Tritirachium album*, which is isolated from the culture broth of this organism. The enzyme is useful for the isolation of macromolecular nucleic acids. Its purification by affinity chromatography is shown in Fig. 13. Proteinase K is retained by the SADC-bound 3-aminophenyl boronic acid at pH 6.5 and eluted at pH 11.

As already mentioned, the affinity resins based on CM-cellulose are very stable. Thus, one and the same contents of a chromatography column

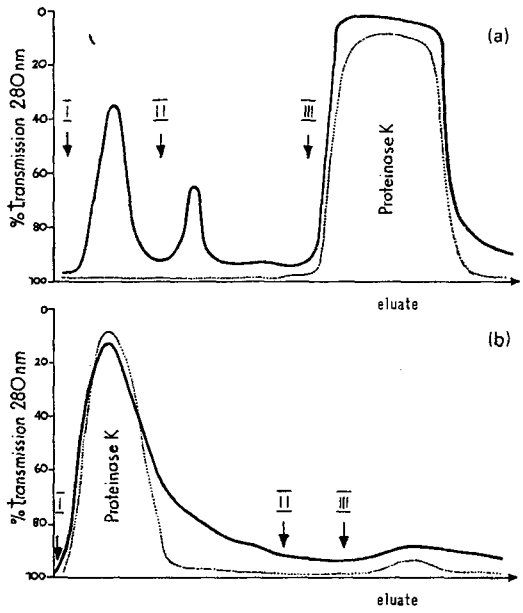


FIG. 13. (A) Affinity chromatography purification of proteinase K on SADC-bound 3-aminophenyl boronic acid. (B) Control experiment with a "mock resin" (see also, Fig. 11). I: solution of proteinase K in 0.1 M tris/HCl buffer, pH 6.5, containing 1 mM $CaCl_2$. II: washing with 0.1 M morpholine/HCl buffer, pH 9. III: elution with 0.1 M triethylamine/HCl buffer, pH 11.0.

was used more than 50 times within 2.5 years without any reduction of capacity or in achievement of separation.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADH | alcohol dehydrogenase |
| AHA | aminohexyl agarose |
| AMP | adenosine 5'-monophosphate |
| Argpa | arginine 4-nitroanilide |
| CM-cellulose | carboxymethyl cellulose |
| DH | dehydrogenase |
| GDA | glutaraldehyde |
| LDH | lactate dehydrogenase |
| MDH | malate dehydrogenase |
| NADH | dihydronicotinamide adenine dinucleotide |
| NAD | nicotinamide adenine dinucleotide |
| SAHA | succinylated aminohexyl agarose |
| SAHC | succinylated aminohexyl CM-cellulose |

## BIBLIOGRAPHY


1. AXEN, R., PORATH, J., and ERNBACK, S. (1967) Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature 214 : 1302–1304.
2. BRÜMMER, W. (1974) Affinitätschromatographie. Kontakte (Merck) 1/74, 23–28; 2/74, 3–13.
3. CUATRECASAS, P. (1972) Affinity chromatography of macromolecules. *In* Advances in Enzymology, Vol. 36, MEISTER, A. (ed.), Wiley, New York, pp. 29–89.
4. CUATRECASAS, P. (1970) Protein purification by affinity chromatography. Derivations of agarose and polyacrylamide beads. J. Biol. Chem. 245 : 3059–3065.
5. CUATRECASAS, P., and ANFINSEN, C. B. (1971) Affinity chromatography. Ann. Rev. Biochem. 40 : 259–278.
6. CUATRECASAS, P., and ANFINSEN, C. B. (1971) Affinity chromatography. *In* Methods in Enzymology, Vol. 22, JAKOBY, W. B. (ed.), Academic, New York, pp. 345–378.
7. CUATRECASAS, P., and PARIKH, J. (1972) Adsorbents for affinity chromatography. Use of N-hydroxysuccinimide esters of agarose. Biochemistry 11 : 2291–2299.
8. DUNLAP, R. B. (ed.) (1974) Advances in Experimental Medicine and Biology, Vol. 42, Immobilized Biochemicals and Affinity Chromatography, Plenum, New York.
9. ER-EL, Z., ZAIDENZAIG, Y., and SHALTIEL, S. (1972) Hydrocarbon coated Sepharose; use in the purification of glycogen phosphorylase. Biochem. Biophys. Res. Commun. 49 : 383–390.
10. FEINSTEIN, G. (1971) Affinity chromatography of biological macromolecules. Naturwissenschaften 58 : 389–396.
11. FUCHS, S., and SELA, M. (1973) Immunoadsorbents. *In* Handbook of Experimental Immunology, 2nd ed., WEIR, D. M. (ed.), Blackwell Scientific Publications, Oxford, U.K., pp. 11.1–11.5.
12. HOFSTEE, B. H. J. (1973) Hydrophobic affinity chromatography of proteins. Anal. Biochem. 52 : 430–448.
13. INMAN, J. K., and DINTZIS, H. M. (1969) The derivation of cross-linked polyacrylamide beads. Controlled introduction of functional groups for the preparation of special-purpose biochemical adsorbents. Biochemistry 8 : 4074–4082.
14. JAKOBY, B. W., and WILCHEK, M. (eds.) (1974) Methods in Enzymology, Vol. 34, Affinity Techniques, Enzyme Purification, Part B, Academic, New York.
15. JENNISSEN, H. P., and HEILMEYER, L. M. G. (1975) General aspects of hydrophobic chromatography. Adsorption and elution characteristics of some skeletal muscle enzymes. Biochemistry 14 : 754–759.
16. LOWE, C. R. (1977) Affinity chromatography: the current status. Int. J. Biochem. 8 : 177–181.
17. LOWE, C. R., and DEAN, P. D. G. (1974) Affinity chromatography, Wiley, London.
18. MORGAN, M. R. A., BROWN, P. J., LEYLAND, M. J., and DEAN, P. D. G. (1978) Electrophoresis: a new preparative desorption technique in affinity chromatography. FEBS Lett. 87 : 239–243.
19. MOSBACH, K. (ed.) (1976) Methods in Enzymology, Vol. 44, Immobilized Enzymes, Academic, New York.
20. MOSBACH, K. (1978) Immobilized coenzymes in general ligand affinity chromatography and their use as active coenzymes. *In* Advances in Enzymology, MEISTER, A. (ed.), Vol. 46, Wiley, New York, pp. 205–278.
21. O'CARRA, P., BARRY, S., and GRIFFIN, T. (1974) Spacer arms in affinity chromatography. Use of hydrophilic arms to control or eliminate non-biospecific adsorption effects. FEBS Lett. 43 : 169–175.

22. OCHOA, J. L. (1978) Hydrophobic (interaction) chromatography. Biochimie 60 : 1–15.
23. ORTH, H. D., and BRÜMMER, W. (1972) Carrier-bound biologically active substances and their application. Angew. Chemie, Int. Ed. 11 : 249–260.
24. PORATH, J. (1973) Conditions for biospecific adsorption. Biochimie 55 : 943–951.
25. PORATH, J., and KRISTIANSEN, T. (1975) Biospecific affinity chromatography and related methods. *In* The Proteins, Vol. I, NEURATH, H. (ed.), Academic, New York, pp. 95–178.
26. POTUZAK, H., and DEAN, P. D. G. (1978) Affinity chromatography on columns containing nucleic acids. FEBS Lett. 88 : 161–166.
27. REINER, R. H., and WALCH, A. (1971) Affinity chromatography—specific separation of proteins. Chromatographia 4 : 578–587.
28. ROSENGREN, J., PAHLMAN, S., GLAD, M., and HJERTEN, S. (1975) Hydrophobic interaction chromatography on noncharged Sepharose derivatives. Biochim. Biophys. Acta 412 : 51–61.
29. SCOUTEN, W. H. (1974) Affinity chromatography. Bioselective adsorption on inert matrices. Int. Lab. 23 : 13–29.
30. TURKOVA, J. (1974) Affinity chromatography. J. Chromatogr. 91 : 267–291.
31. WEETALL, H. H. (1972) Insolubilized antigens and antibodies. *In* Chemistry of Biosurfaces 2 : Marcel Dekker, New York, pp. 597–631.
32. WILCHEK, M., and HEXTER, C. S. (1976) The purification of biologically active compounds by affinity chromatography. *In* Methods of Biochemical Analysis, Vol. 23, GLICK, D. (ed.), Wiley, New York, pp. 347–385.
33. WILCHEK, M., and MIRON, T. (1976) On the mode of adsorption of proteins to "hydrophobic columns." Biochem. Biophys. Res. Commun. 72 : 108–113.
34. YON, R. J. (1974) Enzyme purification by hydrophobic chromatography: an alternative approach illustrated in the purification of aspartate transcarbamoylase from wheat germ. Biochem. J. 137 : 127–130.