Chemical Aspects of Affinity Chromatography

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1 Introduction

The use of biospecific adsorbents in biochemical purification has rapidly become widely accepted since the term 'affinity chromatography' was first coined by Anfinsen and co-workers in 1968.[1] This technique constitutes a conceptual departure from traditional methods, which generally depend on a particular physicochemical parameter such as size or charge, in utilizing the specific interaction between the macromolecule of interest and its substrate, cofactor, allosteric effectors, or inhibitors. In principle, a ligand is attached covalently to a water-insoluble matrix to form a tailor-made chromatographic material suited to adsorb from a mixture just those components having an affinity for the ligand. All other 'non-complementary' constituents pass through the adsorbent unrestrained, leaving the adsorbed molecules to be eluted after some appropriate change in conditions. The method thus closely parallels the use of insolubilized antigens as immunosorbents for the purification of antibodies.[2]

Biospecific adsorbents have been used to purify a variety of substances, *viz.* enzymes; binding, transport, and repressor proteins; peptide substrates, antigens, and fragments from proteolytic degradation of affinity-labelled proteins; glycoproteins and carbohydrates; nucleic acids; intact cells and polysomes; and viruses. Appropriate insoluble ligands can prime the synthesis of specific nucleic acids, function as cofactors, hormones, or effectors, or be used as molecular probes for mechanistic studies. Affinity chromatography has advantages over conventional separation techniques apart from its specificity. As a consequence of the tiny proportion of total protein adsorbed from a crude mixture, a relatively small amount of adsorbent is required. The adsorbed material is rapidly separated from proteolytic enzymes and may be stabilized by ligand binding at the 'active' site. The adsorbent can usually be regenerated many times. Affinity chromatography has been reviewed by several authors,[3] with much duplication and restatement of data and opinions. All emphasize the biological aspects, chemical considerations being subsidiary and fragmented. In this review an attempt is made to highlight the chemical aspects of affinity chromatography, to note

[1] P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.A.*, 1968, **61**, 636.
[2] I. H. Silman and E. Katchalski, *Ann. Rev. Biochem.*, 1966, **35**, 873.
[3] A useful selection includes (*a*) P. Cuatrecasas in 'Biochemical Aspects of Reactions on Solid Supports', ed. G. R. Stark, Academic Press, New York and London, 1971, p. 79; (*b*) P. Cuatrecasas and C. B. Anfinsen in 'Methods in Enzymology', ed. S. P. Colowick and N. O. Kaplan, Academic Press, New York and London, 1971, vol. 22, p. 345; (*c*) R. H. Reiner and A. Walch, *Chromatographia*, 1971, **4**, 578.

recent important advances,[4] and to focus attention on a field of great biological and medical interest in which the talents of the chemist are much in demand.

2 Designing the System

A. General Considerations.—The requirements of a suitable system for affinity chromatography are dictated by the moiety to be purified, the coupling of ligand to matrix, and the working milieu. Enzyme purification imposes rigorous restraints on the choice of bioadsorbent, which must be stable both to the enzyme and to conditions under which the protein is not denatured. Limitations on extreme conditions become significant when the separated material is eluted; very strong adsorption is not always an unqualified advantage. The very 'lock-and-key' specificity that is the cornerstone of the technique requires that no great modification of the ligand occurs either during attachment to the support or under the experimental conditions. Not only must the key be correctly cut, it must be of suitable length such that the binding determinants of the ligand are accessible to the lock, *e.g.* the active site of an enzyme. For many research purposes details of molecular parameters may be vital: knowledge and reproducibility of coupling modes, matrix characteristics, and ligand–matrix–medium interactions then assume paramount importance. For large-scale production in particular, mechanical stability, rapid flow-rate, resistance to microbial attack, and re-use are important factors.

B. Choice of Matrix.—For many years immunosorbents have been prepared by coupling antigens to cellulose or polystyrene derivatives. The requirements of these adsorbents are, however, not very stringent because of the large number of antigenic determinants, often readily accessible owing to the size of many of the coupled proteins, and the high avidity of antibodies for antigens (dissociation constants are commonly lower than 10^{-7} mol l^{-1}). The greater rigour of affinity chromatography has necessitated optimization of conditions, including finding more suitable carriers. In addition to the criteria cited above, the matrix must be capable of mild chemical modification without undergoing gross structural changes (particularly shrinkage), be free of ionic residues which would cause non-specific interactions with proteins, have a loose lattice structure of sufficient porosity to allow macromolecules unimpeded access to bound ligands, and be hydrophilic enough to permit interaction between the two phases. Beaded agarose, polyacrylamide, and glass approach this ideal, can be used with certain non-aqueous solvents, and, having the additional virtue of commercial availability, are now used almost exclusively. Cellulose has been superseded but is important historically for its use in the pioneering studies of McCormick[5] and Lerman, who purified tyrosinase with the first biospecific adsorbent, diazotized

[4] For a comprehensive review of earlier studies see N. Weliky and H. H. Weetall, *Immunochemistry*, 1965, **2**, 293.

[5] (*a*) D. B. McCormick, *Analyt. Biochem.*, 1965, **13**, 194; (*b*) C. Arsenis and D. B. McCormick, *J. Biol. Chem.*, 1964, **239**, 3093.

aminophenol coupled through resorcinol to a cellulose carrier.[6] Cellulose offers little advantage over agarose, and its fibrous, heterogeneous nature results in low porosity and poor flow-rate. Cellulose also often bears a significant proportion of carboxy-residues. Polystyrene and similar polymers are unsuitable not only because they are highly lipophilic, but because they strongly and non-specifically adsorb many proteins.

Occasionally an unmodified matrix is useful as a biospecific adsorbent. Dextran cross-linked with epichlorohydrin (Sephadex®) has been used to purify several saccharide-binding proteins, *e.g.* anti-A agglutinin from *Helix pomatia*[7] and a fructosan-specific precipitating protein from nurse shark serum.[8] This polymer, however, is insufficiently porous to find much application in affinity chromatography.

C. Choice of Ligand.—The ligand must interact specifically and reversibly with the molecule to be purified. If this be an enzyme, a substrate analogue, inhibitor, or cofactor should serve. Binding proteins have been purified on immobilized substrates, antigens and other substrates on immobilized antibodies and binding proteins, and nucleic acids on immobilized complementary oligonucleotides. Interactions involving dissociation constants of greater than 10^{-3} mol l^{-1} are likely to be too weak. The ligand must be suitable for coupling to a matrix with the minimum of modification to that part of its structure essential for binding. It will often be necessary to space the ligand from its support. This may be achieved either by coupling the ligand to one end of an 'arm', the other end of which is subsequently attached to the carrier, or by coupling it to an arm already modifying the matrix. This precaution will be superfluous if the ligand is bulky enough to make its binding site(s) available, *e.g.* if it is a protein, polynucleotide, or other large molecule. Thyroxine coupled directly to agarose through its α-amino-group is effective in extracting thyroxine-binding globulin from serum[9] because binding involves the di-iodophenol ring more remote from the point of attachment. Spacing of ligand from matrix is likely to be particularly important for purification of large proteins such as β-galactosidase[10] (mol. wt. $\sim 4 \times 135000$).

D. Coupling of Ligand to Matrix.—(i) *Nature of Coupling.* Almost all affinity chromatography systems involve a covalent bond between ligand and matrix. Ionic bonds or physical adsorption are prone to leakage or displacement of the ligand from its carrier.

(ii) *Stability.* It must be ensured that the ligand–carrier complex is stable to the experimental conditions, otherwise there may be elution of ligand during

[6] L. S. Lerman, *Proc. Nat. Acad. Sci. U.S.A.*, 1953, **39**, 232.
[7] I. Ishiyama and G. Uhlenbruck, *Z. Immun.-Forsch.*, 1972, **143**, 147.
[8] V. Harisdangkul, E. A. Kabat, R. J. McDonough, and M. M. Sigel, *J. Immunol.*, 1972, **108**, 1244.
[9] J. Pensky and J. S. Marshall, *Arch. Biochem. Biophys.*, 1969, **135**, 304.
[10] M. R. Villarejo and I. Zabin, *Nature New Biol.*, 1973, **242**, 50, and refs. therein.

chromatography. If this occurs to a sufficient degree, all the material of interest could elute complexed to free ligand. This may lead to completely erroneous conclusions, especially if fractions are assayed by substrate binding capacity.[11] Proteins present in very low concentration are particularly susceptible to this hazard.

Ligand–carrier complexes which have not been adequately processed after the coupling reaction are open to similar criticisms. Before use, the bioadsorbent must be extensively washed in several cycles with buffers of as wide a range of pH and as high an ionic strength as the stability of the covalent bonds will allow. Detergents are sometimes also advisable. All matrices have some non-specific binding properties, particularly after activation procedures and introduction of 'spacers'. Adequate washing of gels is therefore essential to remove non-covalently bound ligand. There are many reports of investigations where these precautions are conspicuous by their absence and therefore conclusions based on such work must be regarded with some scepticism (*cf.* Section 3Aiv).

(iii) *Amount of Ligand Coupled to Carrier.* Some estimate of the success of a coupling reaction comes from measuring the amount of ligand not attached but, for a more precise determination of coupling yield, a direct assay is desirable. In favourable cases the ligand can be chemically removed intact from its support, *e.g.* by hydrolysis from glass or by dithionite reduction if a diazo-linkage is involved. Solubilizing a complete ligand–agarose, –dextran or –cellulose system, either by acidic or enzymic digestion, allows direct optical density measurement or amino-acid analysis (for peptide ligands). Electronic spectra of the intact complex can be measured by suspending the gel in a viscous medium such as 0.5% polyethylene oxide. Attaching an isotopically labelled ligand allows a very accurate assay of coupling yield.

The amount of ligand on the carrier represents the maximum theoretical binding capability and should not be equated with the capacity as a bioadsorbent. In practice, only a fraction of the molecules coupled may be accessible for binding, since the matrix may have non-ideal porosity. Also, once a macromolecule is adsorbed, it may mask adjacent ligands. Therefore, although raising the ligand concentration on a matrix improves most bioadsorbents up to a point, there is usually a limit above which the capacity no longer increases and may begin to fall again.[12]

3 Chemistry of the Matrix

Much of the chemistry involved in fixing ligands to matrices has not been developed specifically for the purpose but is an adaptation of conventional methods to the biphasic nature of ligand-carrier coupling. Many procedures used for preparing immunosorbents and insoluble enzymes, however, are unsuitable for affinity chromatography because matrices such as carboxymethyl-, amino-

[11] (*a*) J. H. Ludens, J. R. DeVries, and D. D. Fanestil, *J. Biol. Chem.*, 1972, **247**, 7533; (*b*) G. I. Tesser, H.-U. Fisch, and R. Schwyzer, *F.E.B.S. Letters*, 1972, **23**, 56.

[12] N. Kalderon, I. Silman, S. Blumberg, and Y. Dudai, *Biochim. Biophys. Acta*, 1970, **207**, 560.

ethyl-, phospho-, or *p*-aminobenzyl-cellulose or *p*-aminostyrene carry a residue of ionizable groups even after coupling. Agarose, polyacrylamide, and glass are carriers of choice partly because of their minimal, non-specific interaction with proteins. The chemistry of assembly of a biospecific adsorbent should preserve this character insofar as is possible.

Three variations on the theme of assembly must be considered: (*a*) direct linkage of ligand to carrier; (*b*) extension of a ligand by an 'arm' which is subsequently attached to the matrix; and (*c*) derivatization of the carrier with a spacer, the free end of which has a functionality suitable for attaching the ligand. Clearly, a system may be designed so that the functional group to be coupled to the matrix is the same for almost all cases covered in (*b*) and (*c*) and some of those in (*a*). Thus, for any given matrix, one coupling procedure could be generally applicable.

A. Polysaccharides.—A general and by far the most widely employed method for linking ligands to agarose, dextran, or cellulose involves (*a*) activation of the carrier and (*b*) coupling of primary aliphatic or aromatic amines to the activated matrix.

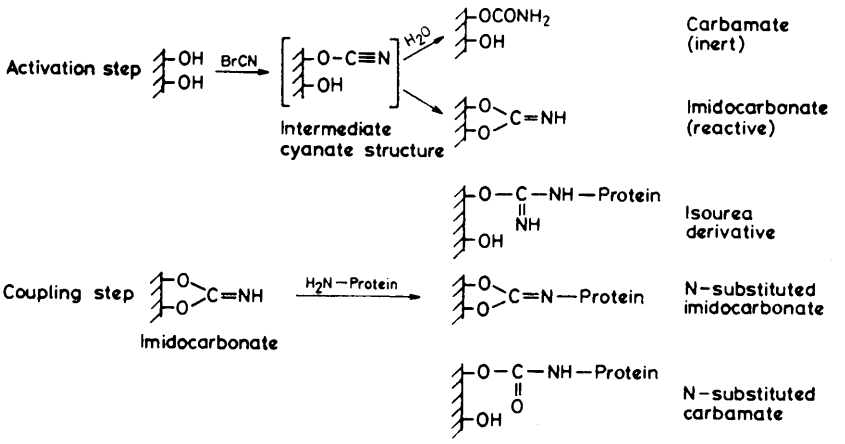
(i) *Cyanogen Halide Activation.* Activating polysaccharides with cyanogen bromide* at high pH was developed by Axén and co-workers[13] for coupling peptides and proteins specifically through their amino-groups, particularly to Sephadex, the polymer used in most definitive studies. However, comparisons with other polysaccharides have shown the method and its chemistry to be quite general. At pH 11, the optimum, activation is complete in a few minutes,† and the degree of activation increases with the amount of cyanogen bromide. Up to 6% nitrogen and a trace of bromine are incorporated into the active polymer. Scheme 1 ('Activation step') shows the probable course of events.[14] The initial (and least well-defined) step is the formation of labile cyanate which can interact with an adjacent hydroxyl to form (in the case of Sephadex) either a *trans*-fused five-membered cyclic imidocarbonate or a cross-linked imidocarbonate involving an adjacent dextran chain. Cross-linking is apparent from the decrease in swelling capacity of the gel which accompanies activation.[13] The cyanate can also be hydrolysed to an inactive carbamate; prolonged, mild alkaline hydrolysis converts activated Sephadex into the completely inactive carbamylated polymer. It is therefore important to conduct the activation for the short time prescribed. After mild acid hydrolysis, which liberates 'labile' (*i.e.* imidocarbonate) nitrogen as ammonium ion, five-membered cyclic carbonates can be detected as well as carbamate. Liberation of ammonia parallels loss of most of the coupling capacity

* Cyanogen iodide behaves very similarly except that activation takes longer.

† This is the pH of the bulk medium. The pH at the interface remains to be studied.

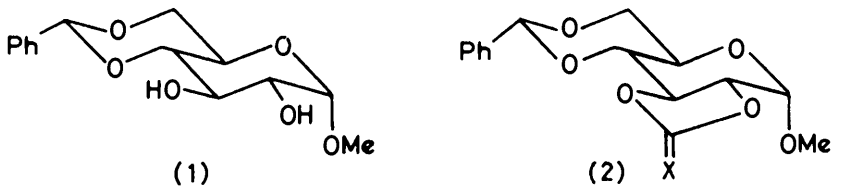
[13] R. Axén, J. Porath, and S. Ernback, *Nature*, 1967, **214**, 1302.

[14] (*a*) R. Axén and S. Ernback, *European J. Biochem.*, 1971, **18**, 351; (*b*) R. Axén and P. Vretblad in 'Protides of the Biological Fluids', Proceedings of the 18th Colloquium, Bruges, 1970, ed. H. Peeters, p. 383; (*c*) R. Axén and P. Vretblad, *Acta Chem. Scand.*, 1971, **25**, 2711.



Scheme 1 *Chemical activation of polysaccharides by means of cyanogen halides and chemical coupling of proteins to cyanogen halide-activated polysaccharides* (Reproduced by permission from *European J. Biochem.*, 1971, **18**, 352)

but there is some acid-resistant residual activity, due either to the presence of bromotriazine groups on the polymer (arising from trimerization of cyanogen bromide) or to the reactivity of the strained cyclic carbonate. Small amounts of peptides can be coupled to Sephadex-carbonate.[14]‡ A model study of the reaction between cyanogen bromide and methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (1) bears out this scheme. Good evidence is adduced for the formation of the *trans*-fused cyclic 2,3-imidocarbonate (2; X = NH) with smaller proportions of carbonate (2; X = O) and 2- and 3-carbamates.[15]

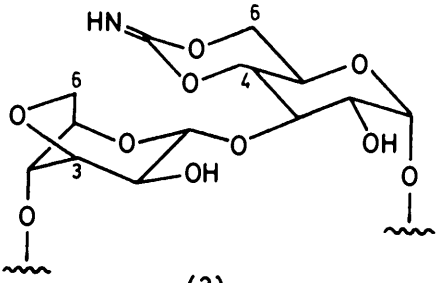


Agarose, consisting of alternating (1→3)-linked β-D-galactopyranose and (1→4)-linked 3,6-anhydro-α-L-galactopyranose residues, cannot form such a five-membered ring, so that the major active intermediate is probably the six-

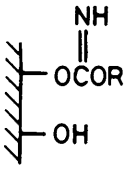
‡ Poly(allyl carbonate) has recently been described and used for insolubilizing enzymes [S. A. Barker, J. F. Kennedy, and A. Rosevear, *J. Chem. Soc.* (*C*), 1971, 2726]. This polymer might be suitable for affinity chromatography since it is highly substituted with neutral, hydrophilic groups (it contains ethoxycarbonyl residues, eight-membered cyclic carbonate rings, and, after coupling through amide bonds, hydroxyl residues).

[15] L. Ahrgren, L. Kågedal, and S. Åkerström, *Acta Chem. Scand.*, 1972, **26**, 285.

membered cyclic 4,6-imidocarbonate (3).* Pre-activated agarose has recently become available as a dry powder, stabilized with lactose and dextran, and can be used directly for coupling.†



(3)



(4)

(ii) *Organic Cyanates.* Activation of polysaccharides with organic cyanates is optimal at pH 10—11 and is even more rapid than with cyanogen bromide. The active intermediates are imidoesters (4)[16] and (again) cross-linked imidocarbonates.

(iii) *Coupling to Activated Polysaccharides.* Unprotonated primary amines couple to cyanogen bromide-activated agarose and dextran, often with high efficiency. Coupling yields can be increased by using excess ligand or cyanogen halide for activation, although the relationship is complex because of steric and interligand repulsions. The reaction medium should be chosen to give a pH above the pK_a of the ligand ($\sim$ 10 is the upper limit), *e.g.* 7—8 for aromatic amines ($pK_a \sim 5$), 9.5—10 for amino-acids ($pK_a^{\alpha\text{-}NH_2} \sim 8$) and 10 for aliphatic amines.[3b] Cyanate-activated polymers behave similarly but more rapidly.

The derivatives formed are of isourea and N-substituted imidocarbonate or carbamate (Scheme 1, 'Coupling step'). The mechanism of the coupling reaction is but poorly studied and understood. When alanine ethyl ester is attached to activated dextran, ammonia is liberated (suggesting hydrolysis of an isourea or nucleophilic displacement from imidocarbonate) but not when the ligand is glycine. Similarly, diethyl imidocarbonate forms the *N*-ethoxycarbonylmethyl derivative with ethyl glycinate but *O*-ethyl-*N*-carboxymethylisourea with the free acid.[17]

(iv) *Stability of the Complex.* The importance of a stable ligand–carrier complex

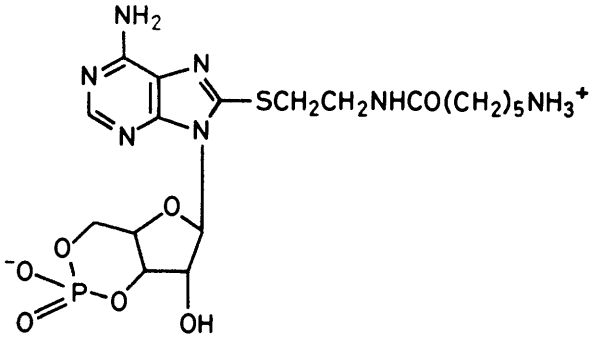
* Aminopolymers (*e.g.* aminoethyl-Sephadex, Enzacryl® AA) can similarly be activated, optimally at pH 8—9. The intermediates are cyanamides or carbodi-imides and coupling to amines probably involves guanidino bonds (which preserve overall charge).[13,14] Although this is unlikely to be useful in affinity chromatography because of the anionic nature of the polymer, it has been surprisingly little exploited for immobilizing enzymes.

† Even after prolonged coupling, residual activated groups sometimes remain (especially with bulky ligands). It is advisable to incubate the adsorbent with excess ethanolamine or 1-aminopropane-2,3-diol after coupling, since unreacted imidocarbonates and isoureas confer ionic character to the matrix.

[16] L. Kågedal and S. Åkerström, *Acta Chem. Scand.*, 1970, **24**, 1601.

[17] P. Vretblad, Thesis, Uppsala University, 1971.

has been emphasized and, for most affinity chromatography, systems prepared by the cyanogen bromide method are adequate in this respect. However, a study of stability of agarose complexes with [^{14}C]alanine and with a radioactive adenosine 3′,5′-cyclic phosphate (cAMP) analogue (5)[11b,18] demonstrated some



(5)

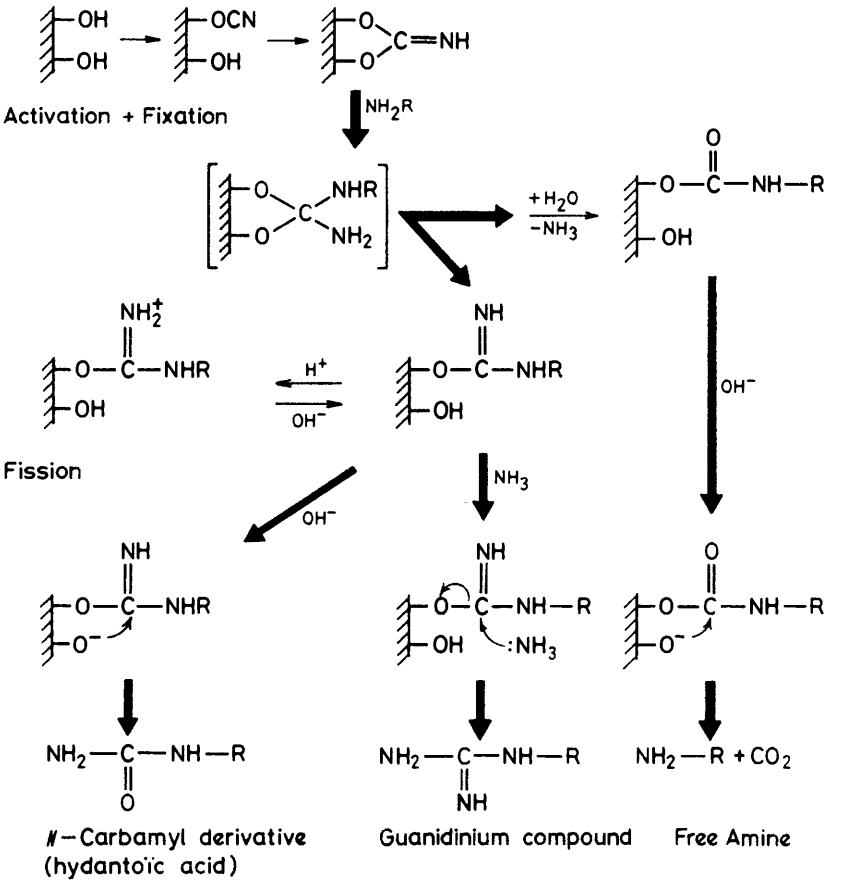
leakage at pH's greater than 5.‡ Products varied with conditions: in tris buffer, pH 8, a mixture of *N*-alanylguanidine and alanine (1:4) was liberated from [^{14}C]alanylagarose; at pH 9 (same buffer) the ratio was 2:1. At pH 11 in aqueous triethylamine (no NH_2) only *N*-carbamylalanine and a trace of 4-methylhydantoin could be detected. A summary and rationale of these observations is presented in Scheme 2. This problem becomes significant if the protein to be purified is present in such low concentration that ligand leakage is of comparable magnitude. Coupled proteins seem more stable, perhaps because several lysyl residues may be involved, possibly in more sterically hindered bonds.

B. Polyacrylamide.—This matrix is a cross-linked copolymer of acrylamide *NN′*-methylenebisacrylamide, comprising a hydrocarbon framework carrying carboxamide side-chains which are resistant to hydrolysis in the pH range 1—10. It is available in spherical beads of various sizes and porosities. Inman and Dintzis[19] have developed a wide range of mild methods for modifying this structure with chemical groups suitable for ion-exchange and covalent bonding. Some of their derivatives are particularly suitable for preparing biospecific adsorbents because overall neutrality is preserved. The amido-NH_2 is readily displaced by other amino-derivatives in what is essentially acylation of an amine by an amide. Scheme 3 illustrates the principal activation pathway. Treatment of polyacrylamide with aqueous hydrazine produces an acyl hydrazide gel (6)

‡ 0.5 nmol h^{-1} at pH 6 and 2.2 nmol h^{-1} at pH 8, per gram of gel containing 1200—5000 nmol of ligand.

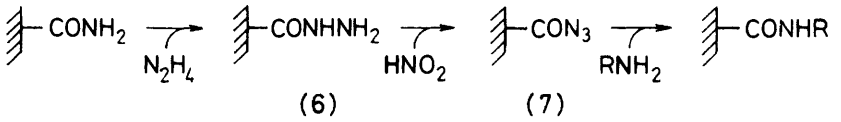
[18] G. I. Tesser, H.-U. Fisch, and R. Schwyzer, Abstracts of the 8th F.E.B.S. Conference, Amsterdam, 1972, Abstract 795.

[19] J. K. Inman and H. M. Dintzis, *Biochemistry*, 1969, **8**, 4074.



Scheme 2 *Liberation of ligands from polysaccharide gels: a tentative scheme based on the coupling mechanism proposed by Axén and Ernback (European J. Biochem.*, 1971, **18**, 351)

(Reproduced by permission from a lecture by G. I. Tesser at the 8th F.E.B.S. Conference, Amsterdam, 1972)

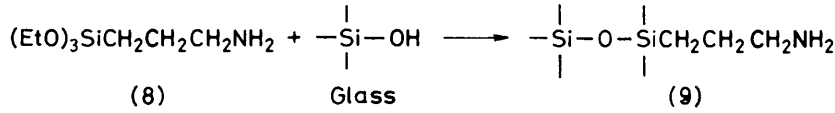


(6) (7)

Scheme 3

which is converted by nitrous acid into the key acyl azide intermediate (7). This can be coupled either to amino-ligands or to spacers which can be modified by methods analogous to those used for agarose (*vide infra*). Unreacted acyl azide is reconverted into carboxamide with ammonium ion. The only disadvantage with this procedure is that azide formation is accompanied by gel shrinkage which reduces porosity, limiting the usefulness of polyacrylamide for affinity chromatography of large proteins. Polyacrylamide can also be functionalized by treatment with glutaraldehyde, to which ligands are attached as Schiff's bases.[20] Polyacetal–Enzacryl® is also suitable for this type of coupling.

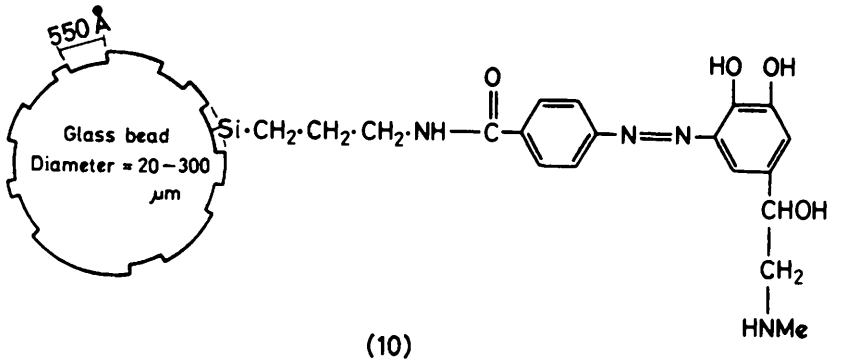
C. **Glass Beads.**—Porous glass has been introduced as a matrix for insolubilizing enzymes as a result of a search for carriers suitable for large-scale production processes. Glass is very resistant to changes in acidity and solvent, mechanical damage, and microbial attack. Functionality is introduced on to the glass surface by using silyl coupling reagents, including 3-aminopropyltriethoxysilane (8) (Scheme 4). As well as the simple covalent bond depicted, there may be a coating



Scheme 4

of aminopropylsilyloxy-groups around the bead due to polymerization of (8). There are few published data on the use of glass in affinity chromatography. Glutaraldehyde has been allowed to react with (9), leaving a free aldehyde group to be attached to glycyl-D-phenylalanine, an inhibitor of carboxypeptidase A. This enzyme was selectively adsorbed from a mixture of proteins on to the modified beads, from which it could be eluted with 10^{-2} M-acetic acid, pH 3.[21] The *N*-hydroxysuccinimide ester of succinylaminoalkyl-glass has been prepared, and behaves as the analogous agarose-active ester towards amino-ligands (*vide infra*). Other modified glass carriers, already used for insolubilizing enzymes and suitable for preparing biospecific adsorbents, include isothiocyanatoalkyl- and diazotized *p*-aminobenzamidoalkyl-glass.[22] Biologically active glass-bound catecholamines have been prepared. Immobilized L-epinephrine (10) and isoproterenol [1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol] retain their ability to accelerate and immobilized propranalol [1-isopropylamino-3-(naphth-1-yloxy) propan-2-ol] its ability to decelerate the heart-beat rate of dogs and chick embryons.[23]

[20] T. Ternynck and S. Avrameas, *F.E.B.S. Letters*, 1972, **23**, 24.
[21] P. J. Robinson, P. Dunnill, and M. D. Lilly, *Biochim. Biophys. Acta*, 1971, **242**, 659.
[22] H. H. Weetall, *Research and Development*, 1971, **22**, 18.
[23] J. C. Venter, J. E. Dixon, P. R. Maroko, and N. O. Kaplan, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 1141.

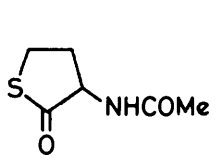


(10)

(Reproduced by permission from *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 1141)

4 Matrix Modifications and Spacers

It will usually be necessary to separate small ligands from the gel by a 'spacer'; also, some ligands will not have an NH_2 group suitable for direct coupling. Activated agarose and polyacrylamide can be modified with bifunctional reagents of the general structure NH_2—R—X, where X is a functional group and R, chemically inert, determines rigidity, hydrophilicity, and maximum length. Useful in combining the function of spacer with the introduction of a new reactivity are α,ω-diaminoalkanes, di-(3-aminopropyl)amine, ε-aminocaproic acid, benzidine, lysine, and small peptides, *e.g.* Gly-Gly-Tyr. Aminoalkylagaroses are versatile gels which can be further extended and modified. Succinic anhydride introduces a carboxy (*cf.* Scheme 5), *N*-acetylhomocysteine thiolactone (11) a thiol, *O*-bromoacetyl-*N*-hydroxysuccinimide (12) a bromoacetyl, and *p*-nitro-



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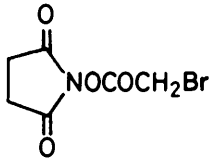


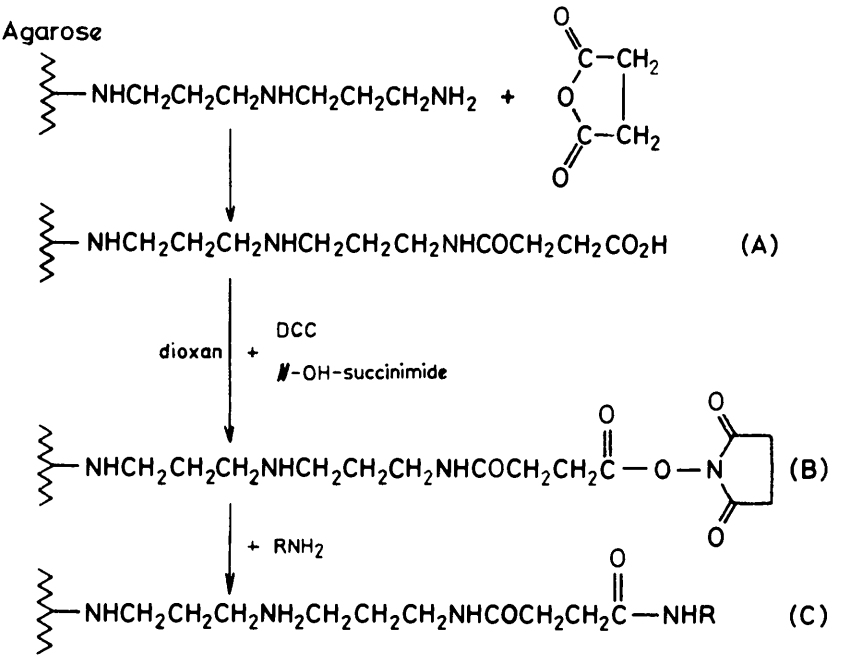
(12)

benzoyl azide a *p*-aminobenzoyl group (after dithionite reduction). Earlier reviews and papers by Cuatrecasas[3a,3b,24] include extensive surveys of these manipulations. The disadvantage of some of the derivatives is the lack of selectivity of the end product. Aminoalkyl and succinylaminoalkyl gels are usually coupled to ligands by using di-imides, so that polyfunctional amino-acids and nucleotides have to undergo elaborate blocking–deblocking procedures to ensure specific coupling modes and to prevent cross-linking between ligand molecules. Bromoacetamidoalkyl gels alkylate amino, histidyl, and phenolic compounds,

[24] P. Cuatrecasas, *J. Biol. Chem.*, 1970, **245**, 3059.

and diazotized *p*-aminobenzamidoalkyl gels fail to discriminate between histidyl and tyrosyl residues. More selective (and rapid) methods are especially desirable for coupling peptides and proteins. Some elegant solutions to this problem are discussed below.

(i) N-*Hydroxysuccinimide Esters.* *N*-Hydroxysuccinimide esters of, *e.g.*, succinylated diaminodipropylaminoagarose react rapidly and specifically in the pH range 6—9 with unprotonated amino-groups (Scheme 5). Esterification is carried out in anhydrous dioxan, which does not much affect agarose. Of a wide range of N-protected amino-acids, only *N*-acetylcysteine competed with alanine in the displacement step, so that only amino- and sulphydryl groups of unprotected peptides and proteins should participate in insolubilization. It is also possible to

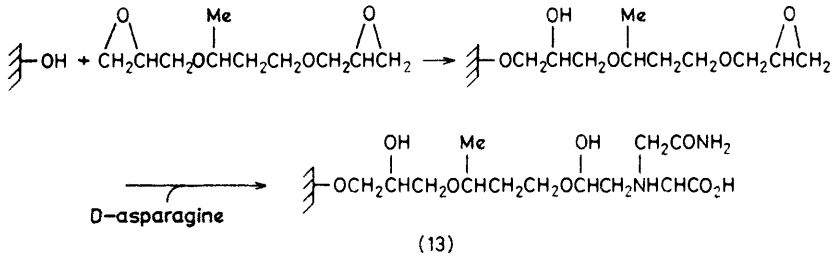


Scheme 5 *Reactions involved in the preparation and use of* N-*hydroxysuccinimide esters of agarose. Diaminodipropylaminoagarose is treated with succinic anhydride in saturated sodium borate buffer to obtain the corresponding succinylated derivative* (A). *The latter is treated with* NN'-*dicyclohexylcarbodiimide* (*DCC*) *and* N-*hydroxysuccinimide in dioxan to yield the active agarose ester* (B). *After removing dicyclohexylurea and the unreacted reagents* (*dioxan and methanol washes*) *the active ester of agarose is treated in aqueous medium with ligands or proteins to yield stable amide-linked derivatives* (C)

(Reproduced by permission from *Biochem.*, 1972, **11**, 2293)

discriminate in favour of α- over ε-NH_2 groups at low pH to allow coupling of lysine-containing peptides at the *N*-terminal residue only. This method seems advantageous, however, only for small ligands for, if no spacer were required, the adsorbent could be prepared by direct coupling to the activated gel. It cannot be applied to polyacrylamide, which is not resistant to dioxan, but may find wide application with glass supports.

(ii) *Epoxide Reagents.* Alkaline 2,3-epoxy-1-(*p*-nitrophenoxy)propane introduces the 2-hydroxy-3-(*p*-nitrophenoxy)propyl substituent into polysaccharides. Dithionite reduction and reaction with thiophosgene produces an isothiocyanato gel which forms thioureas with amino-ligands.[25] Gels with the oxiran functionality are formed by using either alkaline epichlorohydrin[26] or excess α,ω-diepoxides, *e.g.* butane-1,3-diol diglycidyl ether.[27] Epoxidized carriers are excellent for specific coupling of polyfunctional amino-ligands, *e.g.* D-asparagine (Scheme 6). The adsorbent (13) was successfully used in the purification of L-asparaginase.



(13)

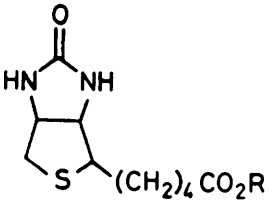
Scheme 6

(iii) *Other Methods.* Sephadex has been functionalized by periodate oxidation, which liberates aldehyde groups for coupling through azomethine bridges, or involvement with isocyanides for attaching amino- or carboxy-ligands under neutral conditions in an Ugi-type reaction.[17,28]

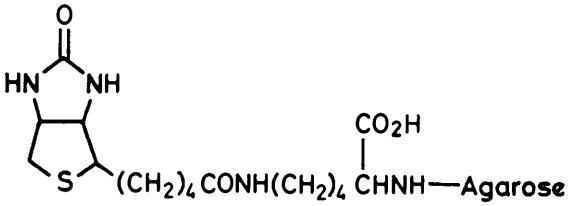
(iv) *Spacers.* That many small ligands must be spaced from their carriers to be accessible to a macromolecular substrate and provide a viable adsorbent is well established. A dramatic example is provided by a comparison of two attempts to purify avidin, the egg-white protein which binds strongly to biotin (14; R = H) with a dissociation constant of 10^{-15} mol l^{-1}. In spite of the enormous avidity of the protein for its substrate, avidin is only weakly retarded by biotinyl-cellulose (14; R = cellulose).[5a] By contrast, when the binding determinants are accessible as in ε-(*N*-biotinyl)-L-lysylagarose (15), avidin is adsorbed so strongly that 6M-

[25] Swedish P. 1192784/1970.
[26] J. Porath and N. Fornstedt, *J. Chromatog.*, 1970, **51**, 479.
[27] M. Einarsson, Thesis, Uppsala University, 1972.
[28] R. Axén, P. Vretblad, and J. Porath, *Acta Chem. Scand.*, 1971, **25**, 1129.

guanidinium chloride, pH 1.5, is needed as eluant.[29] Similarly, tyrosine aminotransferase from mouse hepatoma tissue culture cells is unretarded by pyridoxamine 5′-phosphate coupled directly to cyanogen bromide-activated agarose but is strongly and specifically adsorbed when this ligand is spaced from the matrix (Scheme 7), thus providing a rapier-like means of plucking out from a crude extract a polysome capable of synthetizing a single enzyme.



(14)



(15)

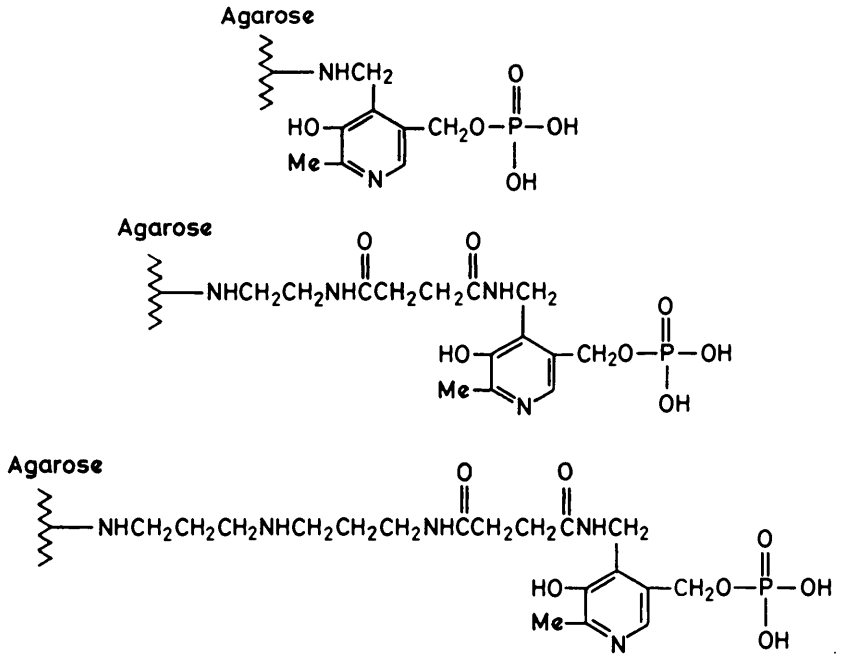
The greater effectiveness of ligands when attached to spacer-arms is generally ascribed to their increased steric availability to the protein. Although this is doubtless the major factor, there are others to consider. The ligands themselves may be more separated when on a long, mobile chain, so that possible masking of those adjacent by an adsorbed protein molecule is minimized. Also, controls have rarely been run to ascertain whether the arm alone has any affinity for the protein, although, even if there were an additional effect, it would often be desirable, provided it were specific.

In choosing a spacing system, the major consideration is often preparative simplicity but, although hydrocarbon chains are conveniently accessible, this should not be an overriding consideration. It should be recognized that a two-dimensional, linear picture of a polymethylene array is unrealistic. Such a lipophilic assembly will have a strong tendency to coil in aqueous milieu, thus reducing its effective length. The same criticism may be levelled at some peptides. Polyamino arms, though more hydrophilic, introduce ionic character on to the bioadsorbent. Something approaching the ideal may be the polyhydroxy-ethers

[29] P. Cuatrecasas and M. Wilchek, *Biochem. Biophys. Res. Comm.*, 1968, 33, 235.

arising from the bisepoxides described above (*cf.* Scheme 6). An alternative approach is to use a rigid spacer such as benzidine or a steroid.

Use of an arm of greater than a certain limiting 'length' does not seem to give improved binding capacity. For instance, immobilized *N'*-(6-aminohexyl)- and *N'*-(12-aminododecyl)-pyridoxamine 5'-phosphates exhibit equal affinity for *apo*-glutamic-oxalacetic transaminase.[30]



Scheme 7 *Biospecific adsorbents prepared by coupling pyridoxamine phosphate to agarose through spacers of varying length*

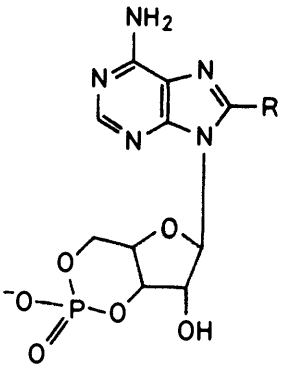
(Reproduced by permission from *Biochim. Biophys. Acta*, 1972, **276**, 408)

5 General Ligands

Much attention has been focused recently on immobilized ligands, such as nucleotides, of general application not only in affinity chromatography but as biologically active cofactors or effectors.[31] Glass-bound catecholamines were mentioned above. Adenine nucleotides are of particular interest. These molecules have a number of sites capable of chemical modification; synthesis of analogues suitable for immobilization so that the mode of attachment is specific and proven has been a considerable chemical challenge, not always adequately met.

[30] R. Collier and G. Kohlhaw, *Analyt. Biochem.*, 1971, **42**, 48.
[31] K. Mosbach, H. Guilford, R. Ohlsson, and M. Scott, *Biochem. J.*, 1972, **127**, 625.

A. Adenosine 3′,5′-Cyclic Phosphate.—This ligand (16; R = H) poses the least problems since the phosphate diester is stable, limiting the number of potential sites for either attachment of an arm or direct coupling to a matrix. Anhydride acylation of, successively, the 2′-OH and the 6-NH_2, followed by selective hydrolysis of the ester, affords N^6-acyl-cAMP's. Wilchek has used bis-(*N*-benzyloxycarbonyl)-ε-aminocaproic anhydride to make (after hydrogenolysis) N^6-(ε-aminocaproyl)-cAMP, suitable for coupling to cyanogen bromide-activated agarose.[32] An alternative approach arises from the observation that the carbon atom with the highest electron density in the adenine ring is at position 8, so that cAMP can be brominated there specifically. The halogen atom is readily displaced by nucleophiles, including cysteamine in the first step of the synthesis[11b] of (5) from 8-bromo-cAMP (16; R = Br) and 1,6-diaminohexane, which gives



(16)

8-(6-aminohexyl)amino-cAMP [16; R = $NH(CH_2)_6NH_2$].[33] The latter coupled to agarose provides a useful bioadsorbent for purifying intact cAMP-dependent kinases.[34]

B. Adenosine 5′-Monophosphate.—Adenosine 5′-monophosphate offers more scope for chemical modification and hence more problems of selectivity. The vicinal diol group of the ribose moiety allows periodate cleavage to a dialdehyde, convenient for coupling to amino supports. This method is applicable to other ribonucleotides.[35] 8-(6-Aminohexyl)amino-AMP is available from 8-bromo-AMP by a synthesis analogous to that of the similar cAMP derivative.[34]

These methods take the ribonucleotides as the starting material: a synthesis

[32] M. Wilchek, Y. Salomon, M. Lowe, and Z. Selinger, *Biochem. Biophys. Res. Comm.*, 1971, **45**, 1177.
[33] H. Guilford, P.-O. Larsson, and K. Mosbach, *Chem. Scripta*, 1972, **2**, 165.
[34] B. Jergil, H. Guilford, and K. Mosbach, in preparation.
[35] P. T. Gilham in 'Methods in Enzymology' ed. S. P. Colowick and N. O. Kaplan, Academic Press, New York and London, 1971, vol. 21, p. 191.

of a tailor-made, immobilized AMP ligand from a simple nucleoside is summarized in Scheme 8. The unambiguous character of N^6-(6-aminohexyl)-AMP (17) lends a rigour and specificity to this approach which is lacking in some others. A practical disadvantage, however, of the method as outlined is the tedium of separation of the huge excess of 1,6-diaminohexane necessary to suppress the formation of N^1N^6-bis(nucleotide)hexane. New reagents developed to overcome this drawback are 1-amino-ω-trifluoracetamidoalkanes (18),[36] in which only one NH_2 group can participate in the displacement step, the other being subsequently regenerated by mild hydrolysis of the protecting group.

$$CF_3CONH(CH_2)_nNH_2$$

(18)

The availability of the same ligand immobilized in different ways allows some insight into which functional groups are necessary for binding. For instance, immobilized N^6-(6-aminohexyl)-AMP binds strongly to many nicotinamide adenine dinucleotide (NAD^+)-dependent dehydrogenases, but weakly or not at all to those for which $NADP^+$ is cofactor.* Some NAD^+-dependent dehydrogenases do not seem to be adsorbed on Sepharose-bound 8-(6-aminohexyl)-amino-AMP. Neither adsorbent retards a wide range of kinases.

C. Nicotinamide Adenine Dinucleotide.—Bioadsorbents with nicotinamide adenine dinucleotide as ligand have been prepared by carbodi-imide coupling to agarose substituted with caproyl spacers.[37] Several ester and amide linkages seem to be involved. NAD^+-dependent dehydrogenases can be chromatographed on this preparation. Coupling, probably by electrophilic attack at C-8 of the purine ring, either to diazotized *p*-aminobenzamidoalkyl-glass or through benzidine attached to a polysaccharide *via o*-hydroxyaniline, offers alternatives.[38] Succinic anhydride reacts with NAD^+ to form a hemisuccinamide with the purinyl 6-amino-group suitable for attachment to polyethyleneimine or, for affinity chromatography, ω-aminohexylagarose.[39] All these preparations retain a measure of cofactor activity. Ethylene oxide quaternizes the N-1 position of the purine ring with a 2-hydroxyethyl substituent which can be rearranged on to the N-6 position in alkali (the cofactor must first be reduced to the base-stable NADH).[40]

* *I.e.* NAD^+ with a phosphate group on the 2′-OH of the adenylribose.

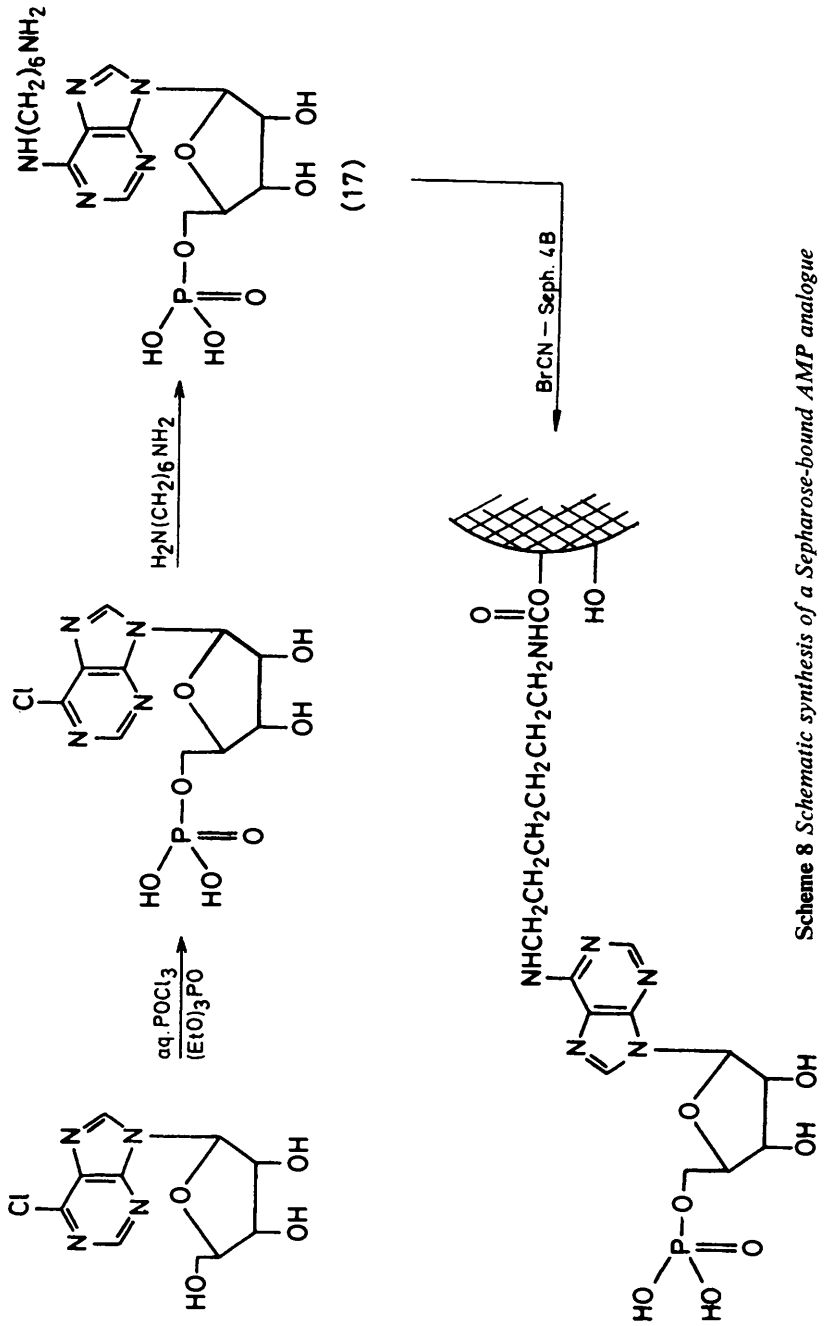
[36] H. Guilford, in preparation.

[37] P.-O. Larsson and K. Mosbach, *Biotechnol. Bioeng.*, 1971, **13**, 393.

[38] (*a*) M. K. Weibel, H. H. Weetall, and H. J. Bright, *Biochem. Biophys. Res. Comm.*, 1971, **44**, 347; (*b*) C. P. Lowe and P. D. G. Dean, *F.E.B.S. Letters*, 1971, **14**, 313.

[39] J. R. Wykes, P. Dunnill, and M. D. Lilly, *Biochim. Biophys. Acta*, 1972, **286**, 260.

[40] H. G. Windmueller and N. O. Kaplan, *J. Biol. Chem.*, 1961, **236**, 2716.



Scheme 8 *Schematic synthesis of a Sepharose-bound AMP analogue*

This N^6-(2-hydroxyethyl) substituent should be capable of selective attachment to an appropriate arm, *e.g.* isocyanatoalkyl.

D. Other General Ligands.—B_{12} coenzyme and other cobalamins have been coupled to substituted agarose either *via* the phosphate group[41] or by amide formation at one of the side-chains.[42] Intrinsic factor, transcobalamins and B_{12}-dependent enzymes bind to these adsorbents. Immobilized methotrexate[43] (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid) and folic acid[44] have been used in purification of, respectively, dihydrofolate reductase, of great interest in cancer research, and folate-binding protein from milk. Pyridoxal phosphate analogues were mentioned above. Thiamine pyrophosphate has been coupled to succinylaminoalkylagarose by using di-imide.[45] P^1-(6-Amino-1-hexl)-P^2-(5′-uridine) pyrophosphate has been described.[46]

6 Polynucleotides

Immobilized polynucleotides are used in two kinds of affinity chromatography. One depends on the ability of single-stranded nucleic acids to hydrogen-bond specifically with complementary strands. Thus, immobilized poly(U) adsorbs KB-cell polysomal messenger RNA which has a poly(A) segment that can 'base-pair' with the adsorbent. The ribosomal and transfer RNA's lack this segment and are not retained.[47] The other is the orthodox approach by which enzymes are chromatographed on nucleic acid substrates. Several aminoacyl-tRNA synthetases have been purified on columns of immobilized tRNA's.[48]

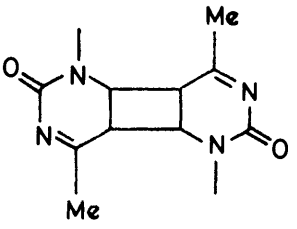
A variety of methods have been described for attaching polynucleotides to carriers.

A. DNA Embedded in Agar.—A rare example of a practical biospecific adsorbent not involving a covalent bond between ligand and carrier results from dispersing single-stranded DNA in liquid agar. When the homogenate cools, the DNA strands are firmly entrapped in the gel.[49]

B. Di-imide Coupling.—Many oligonucleotides have been linked to paper strips by di-imide-mediated ester formation at the terminal phosphate.[50]

[41] H. Olesen, E. Hippe, and E. Haber, *Biochim. Biophys. Acta*, 1971, **243**, 66.
[42] (*a*) R.-H. Yamada and H. P. C. Hogenkamp, *J. Biol. Chem.*, 1972, **247**, 6266; (*b*) R. H. Allen and P. W. Majerus, *ibid.*, p. 7695.
[43] P. C. H. Newbold and N. G. L. Harding, *Biochem. J.*, 1971, **124**, 1.
[44] D. N. Salter, J. E. Ford, K. J. Scott, and P. Andrews, *F.E.B.S. Letters*, 1972, **20**, 302.
[45] A. Matsuura, A. Iwashima, and Y. Nose, *Biochem. Biophys. Res. Comm.*, 1973, **51**, 241.
[46] R. Barker, K. W. Olsen, J. H. Shaper, and R. L. Hill, *J. Biol. Chem.*, 1972, **247**, 7135.
[47] U. Lindberg and T. Persson, *European J. Biochem.*, 1972, **31**, 246.
[48] (*a*) O. D. Nelidova and L. L. Kiselev, *Mol. Biol.*, 1968, **2**, 60; (*b*) S. Bartkowiak and J. Pawelkiewicz, *Biochim. Biophys. Acta*, 1972, **272**, 137; (*c*) P. Remy, C. Birmelé, and J. P. Ebel, *F.E.B.S. Letters*, 1972, **27**, 134.
[49] H. Schaller, C. Nüsslein, F. J. Bonhoeffer, C. Kurz, and I. Nietzschmann, *European J. Biochem.*, 1972, **26**, 474.
[50] (*a*) P. T. Gilham, *J. Amer. Chem. Soc.*, 1964, **86**, 4982; (*b*) I. E. Scheffler and C. C. Richardson, *J. Biol. Chem.*, 1972, **247**, 5736.

C. Photochemical Methods.—Gels are formed when polynucleotides alone are irradiated with u.v. light. The strands become cross-linked through pyrimidine pairs, *e.g.* the thymine dimer (19). If, however, irradiation of poly(U) or poly(C) is carried out in the presence of cellulose, nylon, or polyvinyl beads, the strands become immobilized, even at an irradiation dose level at which the polynucleotides alone are unaffected.[51] Coupling presumably proceeds through a free-radical mechanism, initiated probably by electron abstraction from the carrier.



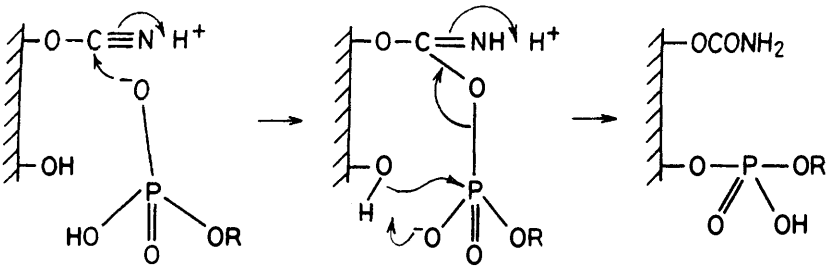
(19)

D. Periodate Oxidation.—The vicinal 2′,3′-diol group of the riboses in ribonucleic acids is readily oxidized by periodate. The modified ligand is then coupled to an amino carrier through azomethine bridges,[35] or as a hydrazone to acyl hydrazide residues on a modified agarose.[52] The latter has also been used to prepare immobilized guanosine 5′-triphosphate (GTP) for affinity chromatography of the folate-synthetizing enzyme D-erythrodihydroneopterin triphosphate synthetase from *Lactobacillus plantarum*.[53] Periodate oxidation cannot be applied to 2′-deoxyribonucleotides which lack the diol system.

E. Coupling to Cyanogen Bromide-activated Agarose.—Weissbach and co-workers studied the coupling of various DNA's and RNA's to activated agarose at pH 8.[54] Single-stranded nucleic acids, especially poly(A), were coupled well but most double-stranded DNA's were not covalently linked unless there were single-stranded extensions at each end. Native HeLa DNA with 2.2% single-strand was coupled to the extent of 4.3%, whereas 24% was coupled where there was 8.3% single-strandedness. It was assumed that the coupling involved the 6-amino-groups of the adenines, but little evidence for this was presented. The reactivity of these groups towards activated agarose varies with its environment: in adenosine and cAMP it is unreactive but NAD^+ can be coupled in this fashion. One report describes the immobilization of ribonucleotides at one clearly defined site, the terminal 5′-phosphate.[55] Cyanogen bromide-activated agarose

[51] R. J. Britten, *Science*, 1963, **142**, 963.
[52] D. L. Robberson and N. Davidson, *Biochem.*, 1972, **11**, 533.
[53] R. J. Jackson, R. M. Wolcott, and T. Shiota, *Biochem. Biophys. Res. Comm.*, 1973, **51**, 428.
[54] M. S. Poonian, A. J. Schlabach, and A. Weissbach, *Biochem.*, 1971, **10**, 424.
[55] A. F. Wagner, R. L. Buganiesi, and T. Y. Shen, *Biochem. Biophys. Res. Comm.*, 1971, **45**, 184.

and poly(I) or poly(C) were incubated at pH 6, chosen to be low enough to preclude 'amino-group participation'. It was suggested that the mechanism involves addition of the terminal group to cyanate to give an active adduct able to react with an adjacent hydroxyl on the carrier (Scheme 9). It is not clear,



Scheme 9

however, that lowering the pH would have much effect on the adenyl ring substituent since, having a pK_a of ~ 3.5—5 (depending on ionic strength), the 6-amino-group will be largely unprotonated anyway. Conversely, it is plausible that many couplings of adenine nucleotides, *e.g.* other reports of insolubilized nucleic acids and the immobilization of ADP,[56] may be *via* the terminal phosphate group even under conditions designed to facilitate amino-group participation.

7 Concluding Remarks

Affinity chromatography has become an accepted part of biochemical methodology and has facilitated the isolation of many interesting macromolecules hitherto inaccessible by less sophisticated techniques. Its success will doubtless continue. It should be realized, however, that even a tailor-made ligand–spacer–carrier system does not necessarily constitute a biospecific adsorbent. It has usually been tacitly assumed that spacers play little part in the chromatographic process and that inhibitors and substrates exhibit similar affinity characteristics in the free state and when modified to render them sterically available. Recent studies show that these assumptions are not always valid.[34,57] Kinetic parameters should be established for modified ligands, and non-specific effects must be evaluated in control experiments using matrix–spacer gels lacking a ligand.

The next logical steps in the development of affinity chromatography are a theoretical treatment on which quantitative evaluations of biospecific adsorbents can be based and extensive investigations into the problems associated with the evolvement of a research tool into a useful production method. The multidisciplinary nature of affinity chromatography will thus be extended even further, beyond the present need for collaboration between synthetic chemist and enzymologist.

[56] J. T. Neary and W. F. Diven, *J. Biol. Chem.*, 1970, **245**, 5585.
[57] P. O'Carra, Abstracts of the F.E.B.S. Conference, Dublin, 1973, Abstract 7.

Note Added in Proof. A number of investigations within the scope of this review have come to the author's notice since the preparation of the manuscript:
(i) The results of a study of the mechanism of cyanogen bromide-activation of cellulose, using *trans*-cyclohexane-1,2-diol as a model, are consistent with Scheme 1 (G. J. Bartling, H. D. Brown, L. J. Forrester, M. T. Koes, A. N. Mather, and R. O. Stasiw, *Biotechnol. Bioeng.*, 1972, **14,** 1039).
(ii) A high-capacity, ultrastable 'polyvalent handle' [11b] has been prepared by immobilizing polylysine by multipoint attachment to activated agarose (M. Wilchek, *F.E.B.S. Letters*, 1973, **33,** 70).
(iii) t-Butyloxycarbonylhydrazide derivatives of peptides have been introduced for coupling haptens to carriers (J. K. Inman, B. Merchant, and S. E. Tacey, *Immunochemistry*, 1973, **10,** 153).
(iv) Immobilized colchicine derivatives have been used for affinity chromatography of a brain microtubule, tubulin (N. D. Hinman, J. L. Morgan, N. N. Seeds, and J. R. Cann, *Biochem. Biophys. Res. Comm.*, 1973, **52,** 752).
(v) Alkylamino-glass was used as a carrier for lipoic acid in purifying lipoamide dehydrogenase (W. H. Scouten, F. Torok, and W. Gitomer, *Biochem. Biophys. Acta.* 1973, **309,** 521).
(vi) A hydroxamate assay has been developed for quantifying coupling yields (J. S. Wolpert and M. L. Ernst-Fonberg, *Analyt. Biochem.*, 1973, **52,** 111).
(vii) Rigorous, comparative studies have been made, based on a semi-quantitative treatment, of non-specific adsorption and spacers (P. O'Carra, S. Barry, and T. Griffin, *Biochem. Soc. Trans.*, 1973, **1,** 289; *cf.* S. Barry and P. O'Carra, *Biochem. J.*, 1973, in the press).

It is a pleasure to express my thanks to my colleagues at the University of Lund, Sweden, especially Dr. Klaus Mosbach who first fostered my interest in affinity chromatography.