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DEVELOPMENT OF MODERN BIOAFFINITY CHROMATOGRAPHY (A REVIEW)

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SUMMARY

The principles of bioaffinity chromatography are presented and illustrated by some applications. The practicability of bioaffinity chromatography depends very much on the solid support (matrix) and the methods chosen for covalent attachment of the ligands that serve as adsorption centres. Some of the significant developments in this area that led to a breakthrough in modern bioaffinity chromatography and the technical developments that ensued are discussed. Future developments are considered.

INTRODUCTION

Several centuries ago a skilful university professor was reputed to be omniscient regarding the accumulated scientific facts and truths of his time. From a Chair in Natural Science he could advance through law and medicine to a position as professor in theology or even to become a bishop. How times have changed! Now we are hardly able to master completely a narrow sector of a specified branch of science.

Consider chromatography, for example, a limited area of separation science which in itself is only a minor division of chemistry. Nobody can keep pace with the output of papers in chromatography, and if one is rash enough to try, there will be no spare time left for one's own research. High-performance liquid chromatography (HPLC) and affinity chromatography are two rapidly developing branches and most participants in this Symposium are experts in HPLC. This is the background, I suppose, for the invitation to me to present a survey of affinity chromatography at this session. I shall outline the main concepts and the scope of bioaffinity chromatography, and shall take the liberty of exemplifying applications by frequent reference to contributions made by my co-workers and myself. This will undoubtedly make my presentation personally biased but perhaps also more authoritative. Those who wish to delve deeper into the subject may consult excellent books[1–3] or collections of published lectures in symposia books and review articles[4–7].

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GENERAL PRINCIPLES AND POTENTIAL APPLICATION AREAS

Objections have been raised to the term "affinity chromatography" as encompassing a broader area than was originally intended. In this review I shall cover *bio*affinity chromatography in the restricted sense that the adsorbents used consist of inert solid supports to which naturally occurring substances or closely related compounds of high selectivity have been introduced by covalent coupling to act as adsorption centres. The interactions involved in the adsorption process are usually similar or identical to interactions that occur in nature.

It is indeed true that bioaffinity chromatography in its various forms can be extremely effective and extraordinarily selective. What makes the "new" technique so fascinating is the fact that it takes advantage of nature's own ways of selectively joining, separating and regulating its elements of matter: to form molecular complexes and aggregates of lower or higher order, be it a simple enzyme–inhibitor complex, complicated multienzyme units, antigen–antibody complexes, hormone–receptor complexes or other membrane components or viruses. Indeed, by its nature, biospecific affinity chromatography offers not only means for the rapid and effective isolation of desired biomolecules but also for the study of molecular interactions.

Biologically functioning complexes are formed under the influence of familiar molecular forces and interactions that are systematized under the terms ionic bonds, hydrophobic interactions, hydrogen bonding, Van der Waal's forces, London dispersion forces, dipole–dipole interactions, charge-transfer interactions, etc. Some of these interactions, which take place in aqueous media, often in or at the phase boundaries, are not so well understood. Although there is nothing mysterious about biospecificity it is a concept with blurred or ill-defined boundaries.

Affinity chromatography implies the use of affinity adsorbents, although sometimes the term is used also for the affinity elution of non-specifically adsorbed substances. A chosen matrix substance (matrix substance forms the solid macroreticular network) of natural origin may itself specifically adsorb substances from biological extracts or exudates. For example, polysaccharide-based gels may in rare instances adsorb lectins with affinity for monosaccharide units of the matrix. Usually the affinity adsorbent is prepared by covalently coupling one of the complex-forming partners to the matrix. The covalently immobilized substance is called a "ligand" or adsorption centre and its soluble complement a "ligate". The ligand–ligate complex is an "adsorption complex" (Figs. 1, 4 and 8).

The anchoring of the ligand molecule to the matrix reduces its freedom to interact with the ligate. To counteract this effect, a spacer arm may be introduced between the matrix and the ligand. An important fact that was not recognized in the early days is that the spacer arm should not impart any adverse properties to the adsorbent. For example, it should not act as an adsorption centre itself. In addition, the coupling reaction may result in an immobilized derivative of lower or even negligible biospecificity (meaning the ability to form a specific complex as in free solution).

In its most schematic form, the adsorption–desorption of the ligate may be described by the law of mass action applied to the reaction

$$L + l \rightleftarrows Ll \qquad (1)$$

where L is the ligand and l the ligate. The upper limit (presumably) of the distribution

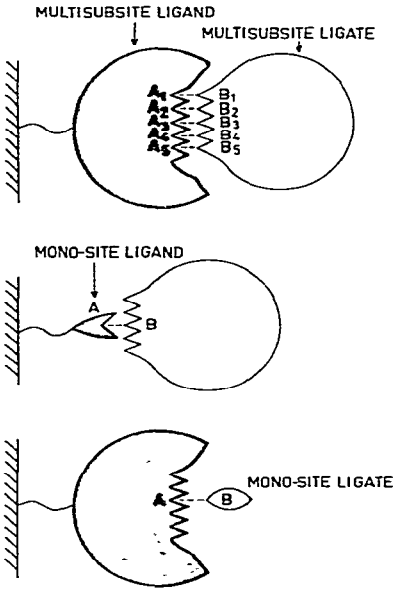

Fig. 1. Schematic representation of the principle of bioaffinity chromatography. An adsorption complex is formed between an insoluble, matrix-bound ligand and a solute (the ligate). Biospecific adsorption occurs as the sum total of many cooperating (unit) interactions within a region of high complementarity (indicated by five interaction sites in the upper figure). Monosite ligands and ligates (if they indeed exist in a strict sense) are likely to form weak, less specific complexes.

constant is the affinity or association constant, K, for L and I in free solution. When $K > 10^5\ M^{-1}$ the ligand concentration in the solid phase may be kept low and still permit good chromatographic performance. K for an enzyme–inhibitor or –substrate complex is usually in the range 10^4–$10^8\ M^{-1}$ and the same is true for many antigen–antibody systems.

Usually the adsorption involves multipoint interactions, and the law of mass action in its simple form gives only a very crude account of the thermodynamics of the complex formation and dissociation. Theories on affinity chromatography based on mass action-governed equilibria do not deviate from those of ordinary chromatography. In fact, no theory has been advanced that is uniquely adapted for bioaffinity chromatography. Such a theory should describe chromatographic behaviour in terms of molecular interaction parameters, which means that in addition to all of the complexities encountered in describing molecular interactions of polymers in solution the restrictions caused by the matrix should also be included. It is especially important to clarify, at least in a semiquantitative way, the importance of water. Water plays a fundamental role as a mediator in the ligand–ligate and ligate–matrix interaction.

Yon[8] introduced the concept "unit interactions" to describe individually weak non-covalent bonds acting to form the complex and to adsorb the ligate to non-specific sites in the adsorbent. Unit interaction, although not possible to define strictly, is a useful descriptive term to account for multipoint adsorption. A protein is adsorbed to a cellulosic-type ion exchanger primarily (in certain pH ranges, perhaps exclusively) by one type of unit interaction, namely, by formation of ionic bonds (perhaps ion exclusion caused by electrostatic repulsion should also be considered as

a kind of unit interaction?). In "pure" amphiphilic agarose adsorbents protein adsorption occurs almost exclusively by unit interactions that are hydrophobic in character.

The same kinds of unit interactions operate in biospecific and non-specific adsorption. However, biospecific adsorption occurs as a result of numerous cooperative unit interactions with localized, close-fitting surface regions on complex-forming biomolecules. In other words, biospecificity depends on the complementarity between the ligand and the ligate molecules with respect to surface geometry and spatial distribution of binding sites within the contact areas. Small deviations from the natural structures of the binding sites occasionally strengthen but usually weaken the adsorption. Attenuation of the interaction by appropriate derivatization may sometimes be recommended to facilitate mild and complete desorption.

In bioaffinity chromatography we exploit some of nature's own information channels. Proteins provide an almost unlimited array of molecular structures. Complementary adaptation to almost any conceivable substance, be it natural or artificial, has therefore been possible. Nature makes use of such adaptative interactions between proteins, enzyme cofactors, nucleic acids, carbohydrates, etc., in catalytically governed reactions and in its molecular signal systems and regulatory devices.

Bioaffinity chromatography may be used as an *in vitro* method to discover unknown biological interactions. For example, a suspected cancerogen may be coupled to agarose and allowed to make contact with liver extract to discover possible interference with liver proteins. Bioaffinity chromatography can also serve to screen biological extracts for the presence of promoters or inhibitors of vital functions, *e.g.*, hormones, nerve impulse transmitters, antihumoral factors and antibiotics.

Adsorbents may be designed that are capable of sensing individual species of very closely related substances. An antibody directed toward a particular unique antigen may thus be singled out from a mixture containing thousands of "sister molecules", all so closely similar in their molecular characteristics that only a biological recognition system can distinguish the substance of interest from the "impurities".

Biospecific hybridization, used in phylogenetic studies and "genetic engineering", depends on base pairing between complementary nucleotide sequences. In fact, such interactions are the background for the functioning of nucleic acids as biological information molecules. Hybridization may be used for affinity chromatographic fractionation of nucleic acids and their degradation products. Complications may be encountered due to complex formation among the nucleotides present together in the liquid phase.

To give some further insight into the potential of bioaffinity chromatography I shall borrow an example from the work of D. E. Koshland and co-workers in which the "induced fit hypothesis" is used to enlighten the complicated key interactions in chemotaxis[9,10]. Fig. 2 is a schematic representation of how interactions are thought to occur. The small-sized substances, in the concentration gradient in which the bacteria move, interact directly with a membrane protein (as with aspartic acid) or indirectly (as with carbohydrates) by formation of ternary adsorption complexes in which a protein serves to link a substance of small molecular size to another protein. The figure also indicates induced long-distance conformational changes that may be transmitted through the membrane, 30–40 Å or more away from the surface to the

interior of the cell, with the result that carboxylic groups become internally exposed and can react with cytoplasmic proteins. Such changes are suggested to occur in "domino" fashion where amino acid residues slide from one side to the other. This mechanism illustrates how nature makes use of affinity adsorption. Koshland and co-workers used immunoprecipitation to elucidate some of the interactions, but it is easy to visualize how adsorbents carrying the appropriate ligands may be used to isolate free or membrane fragment-bound proteins. In terms of our nomenclature the interlinking protein may be considered either as a secondary ligand or a primary ligate and we have a "sandwich-type" adsorbate of another kind than that to be discussed later.

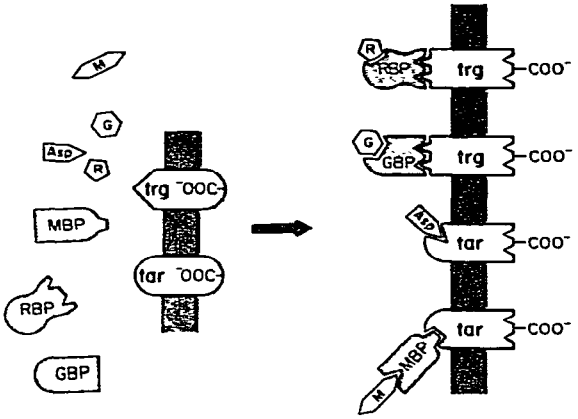

Fig. 2. Hypothetical scheme for induced conformational changes that are involved in the mechanism of bacterial chemotaxis according to Koshland *et al.* It illustrates a system of naturally occurring very complicated ligand–ligate interactions. Methyl-accepting chemotaxis proteins (MCP), coded by the genes tar and trg, are located in the bacterial membrane. Upon ligand binding the proteins change conformation. As a result, carboxylic groups are exposed on the inner side of the membrane and can interact with cytoplasmic methyltransferase. Aspartic acid binds directly to the primary receptor protein in the membrane while the low-molecular-size solutes galactose, ribose and maltose bind indirectly to the transmembrane proteins via periplastic solute-binding proteins denoted by GBP, RBP and MBP, respectively. Reproduced by permission of the authors [E. A. Wang and D. E. Koshland, Jr., *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 7157].

DEVELOPMENT OF MODERN BIOAFFINITY CHROMATOGRAPHIC METHODS

Sometimes it is said that chromatography was never discovered, as chromatographic processes occur in the earth's crust when minerals are formed and deposited. If we take this attitude towards the concept of chromatography, we may also maintain that bioaffinity chromatography indeed occurs on a macroscale in nature, *e.g.*, when humus and other debris from rotting plants, microbes or animals filter down through the soil. In the laboratory, occasional attempts have been made to use bioaffinity chromatography during more than half a century before the breakthrough at the end of the 1960s. Those interested in the early contributions in the field should consult our review[11] and other books[1–3].

The introduction of Sephadex and agarose as supporting media and cyanogen bromide as a coupling agent were contributions that paved the way for the advent of modern bioaffinity chromatography. The methods involved were first briefly de-

scribed in 1967[12] and 1968[13] and later, together with my co-workers, in more detail[14,15].

The term "affinity chromatography" was coined in 1968[16] with the intention of covering a "new" form of chromatography based on biological recognition. This paper by Cuatrecasas *et al.* signalled an immediate breakthrough for affinity separation techniques. Suddenly, all kinds of short cuts to the isolation of biomaterials seemed to be opened. I should point out, however, that our research in Uppsala, which was aimed at the conversion of the molecular sieves Sephadex and Sepharose to a variety of derivatives that could be useful as biospecific adsorbents and for the immobilization of proteins, was a prerequisite for the classical paper of Cuatrecasas *et al.*[16].

The biotin–avidin complex was chosen early as a suitable model for developmental work[12]. It also stimulated me to develop charge-transfer chromatography in aqueous solvent systems, a topic that will not be discussed here. The association constant is very large, which makes adsorption extremely efficient but renders elution difficult. The adsorption and desorption of [^{14}C]biotin went smoothly with quantitative recovery. The reverse procedure, *i.e.*, with biotin as ligand, was a disappointment. We could concentrate avidin directly from diluted hen's eggwhite and obtained in a single-step operation more than a 10,000-fold purification. However, the yield was low and, worse, the adsorption capacity decreased rapidly upon each exposure. The gel thus became useless after two or three adsorption–desorption cycles. The low yield was due to unintended ionic adsorption. The adsorbent had been prepared by coupling avidin to isothiocyanato-Sephadex, which in turn originated from amino-Sephadex. There was thus an excess of free amino groups. This example convinced me that removal of non-specific adsorption centres was an obligatory condition for the preparation of adsorbents of the extreme specificity desired. The fact that cross-linked dextran with a low matrix content is too soft forced me to abandon Sephadex in favour of agarose as a supporting matrix[15]. Even with the technical improvements made shortly thereafter, the biotin–avidin model was not easily managed; Cuatrecasas *et al.*[16] used 6 *M* guanidinium chloride to accomplish desorption.

The state of the art at the end of 1967 was described in a paper in *Nature*[13], from which I cite, "Particularly interesting, however, are the specific adsorbents that can be synthesized by binding adsorption active substances to "Sephadex" or agarose. Substances present in trace amounts in tissue extracts or biological fluids might be purified ten thousand times or more in a single step by specific adsorption. For this reason specific adsorbents, especially immunosorbents, based on cellulose, polystyrene, polyamino acids and other polymers have been prepared in many laboratories. Desorption, however, is a bottle-neck problem. Unsatisfactory desorption may depend on several factors, one of the most important being the presence of fixed charged groups in the polymer used for the preparation of the adsorbents. In my opinion, therefore, "Sephadex" and agarose, particularly the latter, are superior base materials for specific adsorbents... Activation of the carbohydrate with cyanogen bromide in alkali followed by treatment with proteins (amino acids, peptides and so on) in alkaline, neutral or slightly acidic solution will result in coupling of the solute, via its amino group(s), to "Sephadex", agarose or any other carbohydrate. The yields are high under optimum conditions." I have also taken the next example of early applications from the same paper.

Partially deacetylated blood group substance A was covalently coupled to a gel of 2% swollen agarose. A 30-ml volume of human plasma was introduced into the bed of the bioadsorbent which in volume (1 × 10 cm) was smaller than the sample. The anti-A-isoagglutinin [immunoglobulin(s)], together with some other proteins, were adsorbed on to the column. Most of the non-specifically adsorbed proteins were removed by decreasing the pH from 7.4 to 5.0. Further lowering of the pH to 3.5 caused the desorption of isoagglutinin. The protein concentration was too low to determine by spectrophotometric measurements, but calculation indicated a purification factor in excess of 25,000. The chromatogram is shown in Fig. 3. A more detailed account of our early work may be found in refs. 17 and 18.

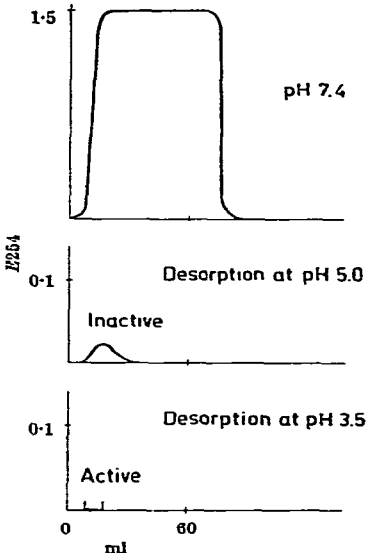

Fig. 3. Isoagglutinin A purification. Human plasma (30 ml) was subjected to bioaffinity chromatography on a column (1 × 10 cm) of agarose with blood group substance A as ligand. The purification was estimated to be well over 25,000-fold. This experiment was carried out together with T. Kristiansen and L. Sundberg. J. Porath, *Nature (London)*, 218 (1968) 837.

Immunoaffinity chromatography, as just exemplified by the purification of haemagglutinin, deserves some discussion to indicate its potentialities. I shall confine myself to simple complex formation between an antigen (Ag) and immunoglobulin (IgG). The IgG molecule consists of a pair of identical short peptide chains and a pair of identical long chains. The four chains are interconnected so as to give two identical combining sites for the antigen. In other words, the antibody is divalent with respect to the antigen. It is possible to evoke antibodies, in humans or any other mammal, against almost any foreign biopolymer (in the natural state or a derivative, such as a protein to which a low-molecular-weight substance has been coupled as a "hapten").

If an antibody, IgG (anti Ag), *i.e.*, directed toward the antigen, Ag, is coupled to an insoluble carrier, an adsorbent is obtained that can specifically adsorb Ag:

$$Ⓜ\text{–IgG (anti Ag)} + \text{Ag} \rightleftarrows Ⓜ\text{–IgG (anti Ab)}\cdots\text{Ag} \qquad (2)$$

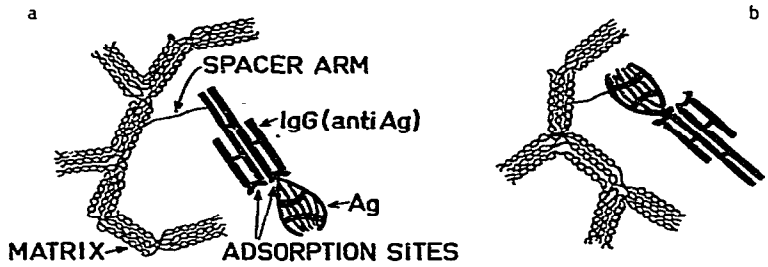

Fig. 4. Schematic representation of the adsorption of (a) antigen, Ag, to its immobilized antibody, Ig (anti Ag) and (b) antibody to immobilized antigen.

The adsorption is shown schematically in Fig. 4a.

It is possible, of course, to attach the antigen (or part of an antigen) to the matrix and thus to obtain an antibody-selective adsorbent, as in the experiment referred to in Fig. 3 and represented schematically in Fig. 4b, according to the reaction:

$$Ⓜ\text{–Ag} + \text{IgG (anti Ag)} \rightleftarrows Ⓜ\text{–Ag}\cdots\text{IgG (anti Ag)} \qquad (3)$$

It is also possible to make antibodies directed toward the whole IgG class of antibodies, for example, anti-human IgG. Such an antibody can thus in turn be coupled to the antigen–antibody complex to form a ternary adsorption complex —a "sandwich complex" (Fig. 5a). The formation of such complexes is utilized in certain types of immunoassays.

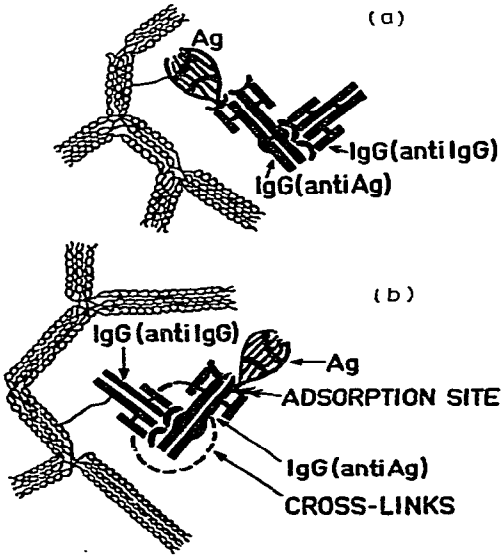

Fig. 5. Schematic representation of the sandwich-type adsorption of (a) immunoglobulin, IgG (anti Ag) against the antigen (Ag) followed by adsorption of antibody to the whole class of immunoglobulin IgG and (b) specific adsorption of antigen (Ag) to a complex ligand formed by covalent fixation of IgG (anti IgG) and subsequent binding of an antibody against antigen. The composite ligand may be cross-linked to prevent elution of IgG (anti Ag).

The flexibility in the use of immunoadsorption methods is perhaps even more apparent if we reverse the order of coupling and anchor IgG (anti IgG) to the matrix (Fig. 5b). We have now prepared an adsorbent directed against Ag but different in nature from that indicated in Fig. 4a.

IgG (anti IgG) may be replaced by Protein A from *Staphylococcus aureus*. Protein A is thus a specific ligand for the class of IgG immunoglobulins[19]. It is commercially available under the trade name Protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) as a ready-to-use adsorbent.

Sandwich systems of various kinds are useful for more than the fractionation and isolation of desired substances. For example, multienzyme systems and proteins composed of many subunits may be coupled to the matrix with attachment points in only one or a limited number of the polypeptide chains. The multicomponent system. thus immobilized, is packed to form a bed. Buffers and solvents are passed through the column. Dissociation may be followed by identifying the eluted components. The residual ligands may then be used to study reconstitution of the original complex. Such operations may be regarded as a particular form of bioaffinity chromatography. A useful survey of immunoaffinity methods was given by Ruoslahti[20].

We realize now that bioaffinity chromatography is closely related to enzyme immobilization, to hybridization methods with one of the nucleic acid partners adsorbed on to paper or other solid supports and to immunoassay techniques with granular gels. The last technique was first introduced by us prior to the cyanogen bromide method[21].

Anti IgG-agarose is a group-specific adsorbent binding all kinds of antibodies belonging to the class IgG. Group-specific adsorbents are very useful. The affinity of lectins for carbohydrates has recently been extensively exploited by means of affinity chromatography. Lectins are proteins, often found in plants, which are characterized by their abilities to bind specified hexoses and hexosamines. They also form complexes with many polymers and other derivatives containing hexose or hexosamine residues (glucosides, oligo- and polysaccharides, glycopeptides, glycoproteins and glycolipids). Lectins are therefore effective tools for the isolation of biologically important polymolecular complexes and particles such as viruses, bacteria, whole plant and animal cells and cell fragments.

Agrawal and Goldstein[22] in 1967 were the first to isolate a lectin by affinity chromatography, followed not much later by another lectin purification in our laboratory[23]. In both cases, the plant extract was passed through a bed of Sephadex and the adsorbed lectin was eluted by including glucose in the buffer. Concanavalin A and a lectin from *Vicia cracca*, respectively, were thus isolated in single-step procedures. It is worth pointing out that the matrix itself exhibits the affinity for the lectin ligate. In the same manner, agarose may be used to "fish out" certain galactose-specific lectins. However, when the affinity requires terminal galactose units it may be advisable to hydrolyse the gel partially or preferably to couple galactose or lactose to the gel. In this way, Ersson *et al.*[24] increased the adsorption capacity of agarose gel for *Crotalaria juncea* lectin (Fig. 6).

The converse procedures with coupled lectin as a specific adsorbent for glycoproteins were first published in 1970 by Lloyd[25] and independently by Aspberg and myself[26].

Many viruses carry binding sites for lectins. In our laboratory Kristiansen has

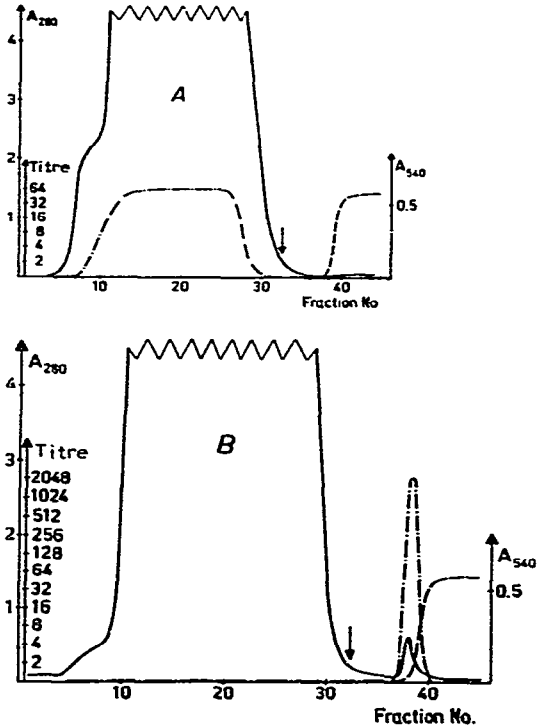

Fig. 6. Bioaffinity chromatography of sunn hemp seed extract. The lectin forms a complex with terminal galactose units. Partially hydrolysed and cross-linked Sepharose 6B is therefore an excellent adsorbent for the lectin (B), whereas untreated gel is not (A). Samples of 40 ml of extract were applied to each column (1.9 × 10 cm) in 0.05 *M* sodium phosphate buffer (pH 7.5) containing 1 *M* sodium chloride (to suppress protein–protein interactions in the solution). After washing, the lectin was eluted biospecifically with 0.1 *M* lactose included in the buffer. Lactose at that high concentration competes effectively with the galactose residues in the gel. Duration of the experiment: 8 h (at 4°C). ———, $A_{280\,nm}$ ("protein"); –·–·–, haemagglutinin titre; - - - - -, $A_{540\,nm}$ (sugar). B. Ersson, K. Aspberg and J. Porath, *Biochim. Biophys. Acta*, 310 (1972) 446.

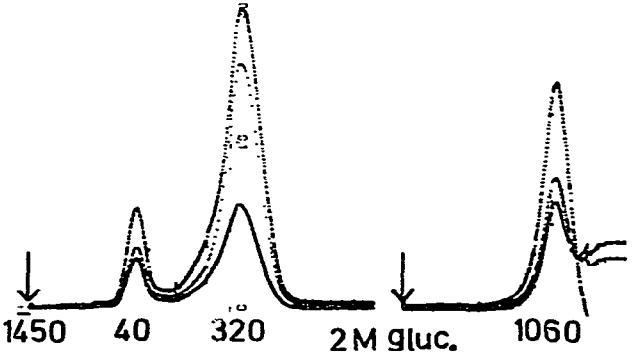

Fig. 7. Bioaffinity chromatography of influenza virus on *Vicia ervilia* lectin-Sepharose 2B. Column volume: 50 ml. Amount of immobilized lectin: 50 mg. The sample (5 ml) was applied at the point indicated by the left-hand arrow and 2 *M* glucose was then included in the buffer for biospecific affinity elution (right-hand arrow). The numbers refer to haemagglutination titres. The solid lines indicate concentration of solute. With author's permission. T. Kristiansen, *Protides Biol. Fluids*, 23 (1976) 663.

purified whole influenza virus on *Vicia ervilia* lectin Sepharose (Fig. 7). The same column was used more than 20 times with recoveries up to 100%[27]. Kristiansen and co-workers have also prepared vaccines by lectin affinity chromatography. The virus and its glycoprotein split products adsorb more strongly to concanavalin A-Sepharose than to the *Vicia ervilia* lectin adsorbent, but the yield is much lower. Too strong an interaction is thus a disadvantage, a statement which is true generally in bioaffinity chromatography.

To illustrate the principle as applied to the formation of an enzyme–inhibitor complex, I have selected our isolation of human carbonic anhydrase (Fig. 8). Carbonic anhydrase is the enzyme that catalyses the hydration of carbon dioxide. Simple as this reaction is, it plays a key role in cellular respiration. The enzyme structure has been elucidated by Nyman and collaborators in Gothenburg (primary structure) and by Strandberg, Kannan and their associates in Uppsala (secondary and tertiary structure).

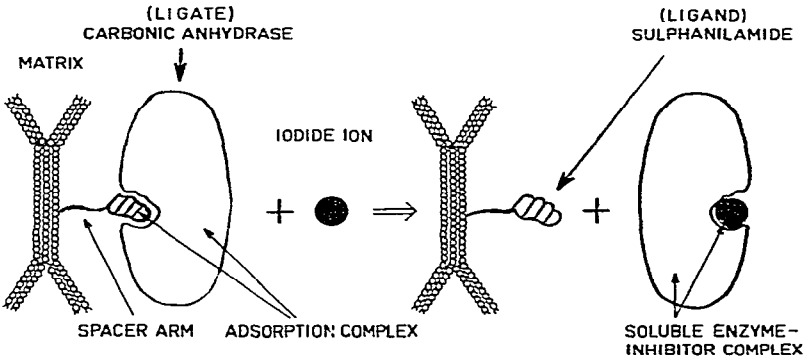

Fig. 8. The principle of using an inhibitor ligand for specific adsorption of an enzyme (carbonic anhydrase) and another inhibitor (iodide ion) for specific desorption. The binding site is often located at an indented region ("cleft" or "cavity") at the protein surface.

The molecule consists of a single peptide chain of 259 amino acid residues folded so as to create a catalytically active site in a 15 Å deep cavity. The cavity has the form of a cone, half the surface of which is hydrophobic and the other half hydrophilic. A zinc atom is located at the bottom of the groove. Inhibitors penetrate into the groove and block the site by replacing water liganded to the zinc. The active site has been mapped in detail. Fig. 9 shows a spacefilling model with the active site in a deep groove[28]. Halide ions are stronger inhibitors the larger are the ions[29]. Certain sulphonamides are also strong inhibitors. The binding is reversible. This extensive knowledge of the structure and properties of the enzyme was used by us to design procedures for its isolation[30].

Carbonic anhydrase exists in two forms (isoenzymes) in human erythrocytes. The problem is to eliminate all other proteins, including haemoglobin, except the desired enzyme. To this end the cells are first burst by an osmotic shock and the lysed cells are centrifuged to remove the membranes. The clear, strongly red-coloured solution is drained into a bed of Sephadex or Sepharose to which sulphanilamide has been coupled. A chromatogram from such an experiment is shown in Fig. 10. The chromatogram was developed by passage of buffer through the bed followed by the

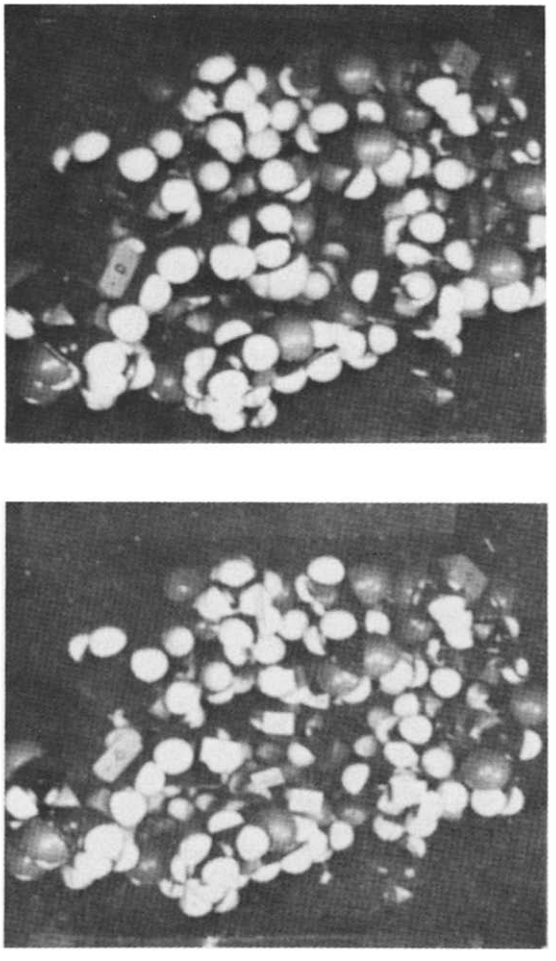

Fig. 9. Packing model of the active site of human carbonic anhydrase C and the same site (cavity) partially occupied by a sulphonamide inhibitor (indicated by the arrows). I. Vaara (1974), *Thesis*, Uppsala University, Uppsala, Sweden. With the author's permission.

same buffer to which sodium iodide had been added. The iodide displaced the sulphonamide from the active site and mobilized the carbonic anhydrase B isoenzyme, leaving the type C enzyme still adsorbed. By exchanging iodide for cyanate the C isoenzyme was easily eluted. Both the B and C isozymes were recovered in pure form and in quantitative yield. The purification factors were of the order of 200 and 4000, respectively.

A very slight modification of the active site may change the adsorption behaviour profoundly. Fig. 11 shows the separation of human carbonic anhydrase B from its monocarboxymethylated derivative.

Sulphanilamide is a "general ligand" for carbonic anhydrase and inhibits the enzyme from any organism. For example, the carbonic anhydrase from the bacterium *Neisseria sicca* can also be isolated from a crude extract by chromatography on

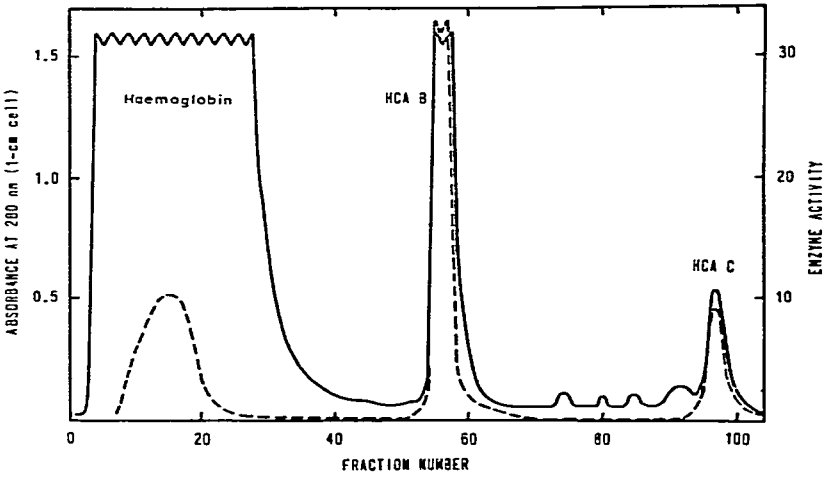

Fig. 10. Chromatogram showing isolation of two human carbonic anhydrase isoenzymes in a haemolysate of erythrocytes (red blood corpuscles). A 25-ml volume of haemolysate was applied to a column (total volume 47 ml) of sulphanilamide-Sephadex G-150 with 0.1 *M* Tris–sulphate as equilibrating buffer. The isoenzymes were separated by including first 0.1 *M* sodium iodide and then 0.01 *M* KOCN in the buffer. Protein concentration, ———; enzyme activity, – – – –. S. O. Falkbring, P. O. Göthe, P. O. Nyman, L. Sundberg and J. Porath, *FEBS Lett.*, 24 (1972) 229.

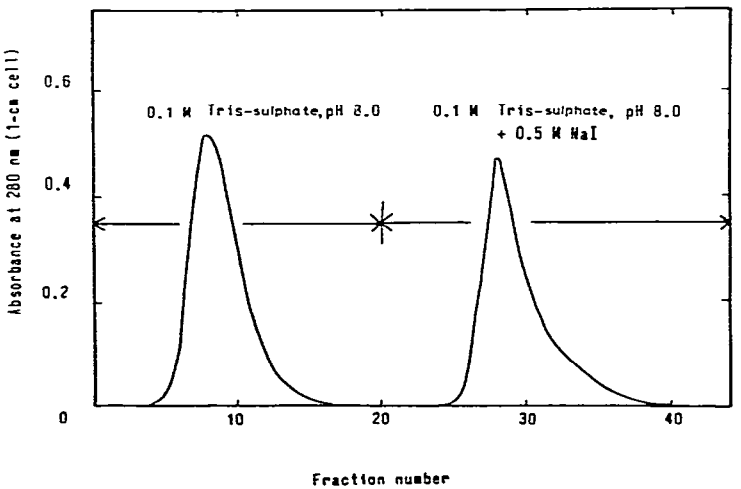

Fig. 11. Chromatogram showing the separation of human carbonic anhydrase B from its derivative in which a histidine side-chain in the active site region has been carboxymethylated. The derivatization reaction was interrupted halfway before completion. The modified protein is not retarded and the native protein requires a competing inhibitor for desorption. In molecular weight the protein and its derivative differ by only about 0.2%. The separation is even more remarkable, however, considering that the outer parts of the surface area are identical for the two molecular species. The separation is almost complete. Adsorbent and reference as in Fig. 10.

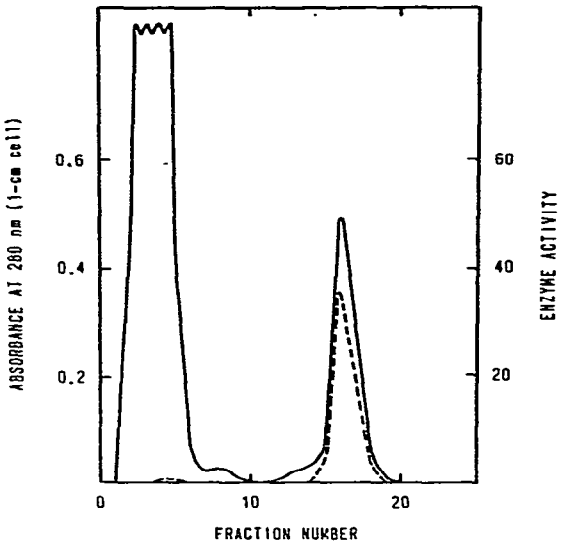

Fig. 12. Chromatogram showing isolation of bacterial carbonic anhydrase on the same adsorbent as in Figs. 10 and 11. The crude extract of *Neisseria sicca* was applied to a 10 × 1.2 cm I.D. column. Flow-rate, 18 ml/h; fraction volume, 3 ml. Equilibrating buffer, 0.1 *M* Tris–sulphate, pH 7.5; displacer, 0.01 *M* KOCN in the equilibrating buffer. Enzyme activity, – – – –; protein concentration, ———. Reference as in Fig. 10.

sulphanilamide-Sephadex (Fig. 12). The bacterial and human enzymes resemble each other in their inhibition properties and therefore presumably also in their active site.

TECHNICAL ASPECTS

The bioaffinity principle is attractive, simple and almost self-evident. Why, then, did it take so long before affinity chromatography became a routine method in biochemists' laboratories? The reason is trivial: no suitable matrix substance had been found. The introduction of cyanogen bromide as a coupling agent was also of significant importance. Agarose is superior to Sephadex and, according to my experience, also to other matrix media, including polyacrylamide. It is not ideal but has been gradually improved. Many of its assumed weak points seem to have been deliberately exaggerated to favour other products. For example, it has been claimed or insinuated that agarose, being of natural origin, will be microbially degraded. In fact, agarose is attacked by very few organisms[31] and when cross-linked the gel appears to be resistant even to isolated agarases. Another weakness often pointed out is mechanical instability. Certainly granules of agarose are elastic and will be damaged by harsh treatment. However, the gels are considerably strengthened by cross-linking with agents of suitable length[32], but admittedly, even such gels cannot resist the pressures used for HPLC. Are such high pressures necessary or desirable?; possibly for very rapid analysis, but in many instances batch procedures may be preferable and less expensive, as for example for serial immunoassays as practiced in hospitals.

A new type of agar/agarose gel has recently been prepared in my laboratory,

on the basis of our old ideas (Fig. 13). Particles or macromolecules that can be dissolved under conditions that leave agarose gel untouched are included in agarose beads of low matrix concentration. The water is displaced by washing the gel with a suitable organic solvent, *e.g.*, methanol. Upon evaporation of the solvent the gel beads shrink but retain their shape. The gel is then cross-linked in a medium in which the shrunken gel does not swell. The included particles, *e.g.*, silica or protein molecules, are then dissolved out to leave a spongy gel. Such a gel combines the advantages of mechanical strength with high permeability and efficient ligate–ligand interaction.

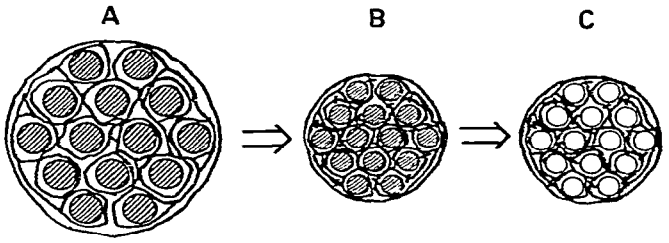

Fig. 13. Schematic representation of how a strong, highly permeable agarose gel may be produced. Small particles or large molecules are included in a gel matrix. The gel is contracted (see text) and cross-linked. The included material is dissolved, leaving "imprints" in the gel (unpublished work).

Porous ceramics and silica gels seem to be the matrix material of choice when extreme mechanical stability is needed. However, non-specific adsorption must be minimized, or preferably eliminated completely, so that protein yields exceeding 99% can be attained. Glyceropropylsilylation, as suggested by Regnier and Noel[33], seems to be promising.

The gels formed by agar and agarose are, owing to their open structure and inner stabilization due to arrangement of the polysaccharide chains into dense bundles of helices, very suitable as starting materials for biopolymer adsorbents. Interestingly, agarose adsorbs proteins and nucleic acids at high salt concentrations. The weak hydrophobicity is presumably due to the ether structure of the anhydrogalactose residues. In my opinion, the adsorption caused by bound water acts as a screen which keeps the solutes away from the matrix and thus provides the biopolymer with a mild environment.

If affinity binding is very weak, the cooperative effect of non-specific ligands may perhaps be an advantage. However, when devising coupling methods we have tried to avoid the introduction of extra adsorption centres that may lower the specificity and complicate desorption. Selection of simple methods has been another guiding principle. Additional demands are that the covalent ligand–matrix linkage should be stable, the coupling yield should be high and the ligand not unduly damaged.

The cyanogen bromide method, which has been criticized as not fulfilling the demand of giving completely stable coupling, is still the most popular method for preparing affinity adsorbents. I prefer oxirane coupling with halohydrins, bis- or trisoxiranes[34] and divinylsulphone[35]. Oxiranes give extremely stable ether, thioether or amine linkages, the drawback being the slow and/or incomplete reactions at low alkalinity. Divinylsulphone and quinone coupling[36] have not yet been optimized. As

we would stray too far from the main theme if we go deeper into this subject, I shall instead refer to reviews[1–7,34].

Detailed studies aimed at revealing the role of the spacer arm have been carried out by Cuatrecasas *et al.*[16], Lowe[37] and O'Carra *et al.*[38]. The early belief that the spacer arm serves only to reduce steric hindrance received a hard blow when β-galactosidase was observed to adsorb to a "mock affinity" gel with non-ionic spacer arms but without affinity ligands[38]. The desirability of using hydrophilic non-ionic spacer arms is now generally agreed upon. Such spacer arms of extreme length can be obtained by coupling butanediol bisglycidyl ether to the gel and then converting the terminal oxirane into a sulphydryl group followed by repeated coupling with the same bisglycidyl ether under more mildly alkaline conditions. The hydrophilic spacer arm will then contain 25 atoms with a terminal oxirane group (unpublished work).

If the affinity binding is very strong, desorption may become a serious problem, as was discussed for the biotin–avidin case. Drastic elution procedures may be necessary. For example, in order to dissociate antigen–antibody adsorption complexes extreme pH or chaotropic ions of high concentrations may be tried. There is a considerable risk of damage to the ligate, the ligand or both. Morgan *et al.*[39] devised a very mild procedure in which the ligate is eluted electrophoretically by application of an electric field across the gel. Excellent yields were claimed.

Few large-scale applications have been published so far. At the Biochemical Separation Centre in Uppsala Ersson and Brink are routinely processing hundreds of litres of plant extracts for the isolation of lectins on affinity columns with a volume of *ca.* 1 l.

To improve the separation power of bioaffinity methods further we should strive for higher chemical selectivity rather than for increased plate numbers. The distribution is almost always one-sided, *i.e.*, the ligate is found quantitatively in one

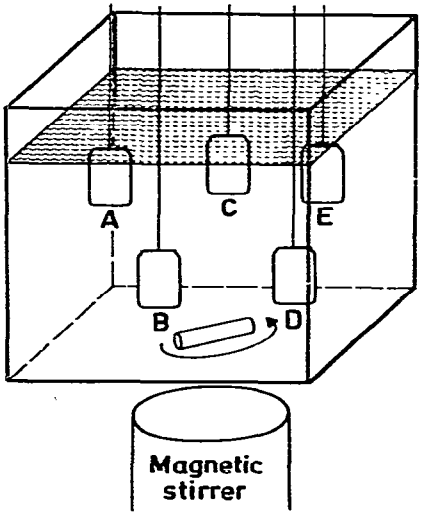

Fig. 14. The "tea bag" procedure for simultaneous adsorption of many solutes. Each nylon net bag is filled with different granular adsorbents and immersed in the solution or suspension containing the desired substances. The bags are removed after equilibrium has been attained and placed in funnels that fit the bags, and elution is executed with suitable displacers.

of the phases: in solution or adsorbed. Isocratic elution is seldom practicable in bioaffinity chromatography, which means that bed procedures are used more for convenience than of necessity. At the extremes, where very small or large volumes are to be handled, batch procedures are more appropriate. Extraction from a solution only 1 μl or less in volume may be easier by means of a single particle or a slip of paper with covalently bound ligand followed by washing. For large amounts, when many substances are to be isolated, the "tea bag" principle[11] can be used (Fig. 14). Industrial application may require fluidized bed techniques.

"Magnetogels" with permanent magnets, usually iron oxide particles, in the gel may be included to facilitate collection of the gel from the suspension[40], but the risk of extra adsorption and other adverse effects should not be neglected.

RECENT DEVELOPMENTS AND FUTURE PROSPECTS

Hydrophobic interaction, charge-transfer and metal-chelate adsorption chromatography as applied to biological materials are all closely related to the topic of this review, but I shall not discuss them here. Covalent chromatography may also be included among the affinity methods that are in a phase of rapid development. Chromatography is thus joined to solid-phase synthesis, carrier-bound drugs and the use of immobilized enzymes as tools in biotechnology. The synthesis of ligands of high stereoselectivity is in an initial stage of development. Polymer-attached crown ethers and other ion-selective substances should be mentioned, as well as adsorbents for resolving racemic mixtures. In the end we may perhaps synthesize extremely selective adsorbents suitable for fractionation work in organic solvents.

Chryobiology seems to have enormous potentialities and will no doubt be even more exploited in the future. Relevant to my subject is the pioneering work carried out by Douzou and Balny[41,42], who have begun to perform subzero bioaffinity chromatography. Denaturation is retarded upon lowering the temperature and ceases almost completely well below zero. Isolation may then take place in hydroorganic solvents. A new field of application opens up, wherein it might be possible, for example, to isolate enzyme–substrate complexes which at ordinary temperatures exist with half-lives of the order of milli- or microseconds.

An increasing demand for extremely selective methods for the isolation and analysis of highly complex natural, reconstituted or semi-synthetic biologically functioning entities including cells and modified cells can be anticipated. For such purposes chromatography is likely to be replaced by other methods. Batch procedures based on biospecific adsorption have often been used in the past. The elegant nylon fibre method of Edelman *et al.*[43] should be mentioned. Liquid–liquid extraction with polymer phase systems as introduced by Albertsson[44] is another attractive technique of great potential. High selectivity in the extraction can be obtained by including polymeric bioaffinity carriers in the system ("bioaffinity partition"). The conversion of such methods into bioaffinity partition chromatography is a challenging task, perhaps not entirely hopeless if only a suitable support can be found. In any case, accumulated knowledge from the developmental work aimed at the refinement of bioaffinity chromatography will also serve to improve the related methods mentioned.

CONCLUDING REFLECTIONS ON THE ART OF SEPARATION

When enjoying a masterpiece of painting we usually think of the painter but never give a thought to the inventor of the paint; when we listen to a symphony we never give a thought to those who made music possible, the instrument makers. Artists are more likely to be recognized than the artisans, who used to be anonymous. Yet art could never have come into existence without invented tools. We may expand the analogy to other fields, to science and technology. The increase in our knowledge of the structure and function of living matter on the molecular level that we have witnessed during the last 25 years would hardly have been possible without the introduction and refinement of physical and chemical methods for the fractionation of biological material down to its molecular entities. This simple fact is seldom appreciated by those who receive the credit for progress in molecular biology.

The points I have just made will perhaps be interpreted in terms like the cook's appraisal of his own food. This is not my intention. In a world of diminishing understanding and support of research, especially in natural sciences, and, as a consequence of that attitude, the harder struggle for financial backing and personal recognition, it becomes necessary that we who devote our time and labour to the art of fractionation, advertise our achievements and struggle for a better general understanding of the key role of our specialities, and by doing so, contribute to the further advance of other more illustrious branches of natural science.

Separation science, especially as applied to biology in the form of affinity methods, offers thrilling adventures and challenging strategic problems often closely associated with biologically highly significant and important phenomena. In addition, separation science is justified in itself, being a branch of "scheikunde" (the Dutch name for chemistry).

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