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REVIEW

BIOSPECIFIC INTERACTIONS THEIR QUANTITATIVE CHARACTERIZATION AND USE FOR SOLUTE PURIFICATION

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LIST OF ABBREVIATIONS

A	f-Valent solute (acceptor of ligand)
K	Equilibrium constant for solute self-association (molar scale)
K'	Equilibrium constant for solute self-association (weight scale)
L	Equilibrium constant for the association of monomer with immobilized monomer
M	Monomer undergoing self-association to polymer P
M_ι	Molecular weight of species ι
P	Polymeric form of self-associating monomer
R_f	Electrophoretic mobility of a species relative to that of bromophenol blue
$\bar{R}_f$	Relative constituent mobility of a species
S	Univalent ligand
V_ι	Elution volume of species ι
V_ι^*	Elution volume of ι in the absence of interaction with matrix
$\bar{V}_\iota$	Constituent elution volume of species ι
X	q-Valent acceptor (receptor)
c_ι	Weight concentration of uncomplexed species ι
$\bar{c}_\iota$	Total weight concentration of species ι
f	Valence of acceptor, or of partitioning solute (affinity chromatography)
$[\iota]$	Molar concentration of uncomplexed species ι
$[\bar{\iota}]$	Total molar concentration of species ι
$[\bar{\bar{\iota}}]$	Total molar concentration of species ι in partition studies
k_{ij}	Intrinsic association constant for the interaction between species ι and j

k_T	Intrinsic association constant for ternary complex formation
n	Stoichiometry of solute self-association
q	Valence of matrix sites
r	Klotz (Scatchard) binding function
r_f	Counterpart of r for an f-valent ligand
v_i	Electrophoretic mobility of species i
$\bar{v}_i$	Constituent electrophoretic mobility of species i
con A	Concanavalin A
ELISA	Enzyme-linked immunosorbent assay
EnOH	Serine-dependent enzyme
FPLC	Fast protein liquid chromatography
HPLAC	High-performance liquid affinity chromatography
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IgG	Immunoglobulin G
RIA	Radioimmunoassay

1 INTRODUCTION

Specificity is a fundamentally important characteristic that is common to vast numbers of biological interactions Indeed, it is a feature upon which reliance is placed not only for the maintenance of cells in their normal physiological state but also (in favourable circumstances) for the selective control of pathological deviations therefrom In general, biospecificity is achieved at the molecular level by the formation of non-covalent complexes between reacting species, at least one of which is macromolecular The individual hydrogen bonds, electrostatic linkages and hydrophobic interactions contributing to such complex formation in an aqueous environment are inherently weak, but the synergistic effect of many such interactions can still give rise to association constants in excess of $10^9\ M^{-1}$ (i e , dissociation constants lower than nM). As examples of biospecific phenomena we may cite the interactions of enzyme with substrate, of hormone with receptor, of antigen with antibody, of protein with lipid, drug or metabolite, and of glycoprotein with lectin, plus the multitude of nucleic acid and protein interactions responsible for gene expression and for the transfer of genetic information during cell division

Despite their collective grouping as biospecific phenomena, these interactions exhibit considerable variation in the degree of specificity. For example, albumin acts as a transport protein for a whole array of amphiphilic molecules such as fatty acids and drugs; and the site responsible for haemoglobin's functional role as a transporter of oxygen also interacts with carbon monoxide and cyanide Similarly, most enzymes are moderately specific in the sense that although catalytic action is usually restricted to one biologically significant reaction (or class of reactions), other compounds can also be substrates or

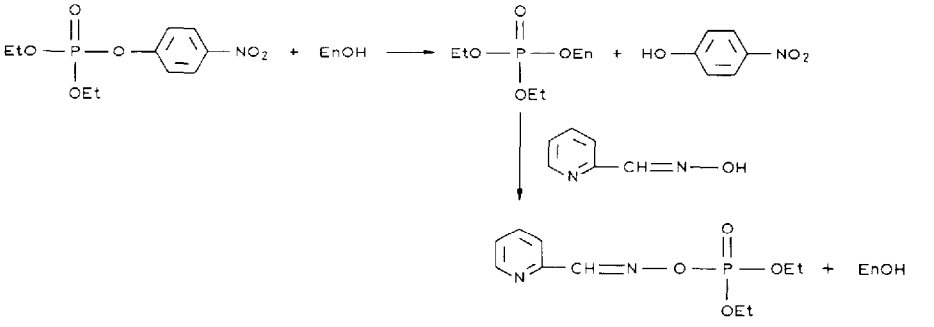
competitive inhibitors – a point illustrated by the amidase activity of plasma cholinesterase [1] Finally, there are highly specific interactions such as those between antigens and the antibodies that are the elicited response to provide biological defence mechanisms These must exhibit the species specificity characteristic of the immune response – a necessary state of affairs in the sense that antibodies elicited in response to (say) an administered protein antigen from another species must clearly have no affinity for the homologous host protein, even though the antigen and host protein are very similar in structure.

The range of equilibrium constants governing these interactions is correspondingly broad For example, the binding of long-chain fatty acids to albumin is governed by association constants in the range 10^3–10^8 M^{-1} [2], and association constants for enzyme–substrate complex formation are typically in the range 10^3–10^6 M^{-1} On the other hand, the magnitude of the equilibrium constant for the inhibition of dihydrofolate reductase by the cancer chemotherapeutic agent methotrexate is in the vicinity of 10^{11} M^{-1} [3], and the association constant for the avidin–biotin interaction is believed to be still larger by some four orders of magnitude [4] On probability grounds it seems logical to presume that the requirements for formation of sufficient non-covalent interactions to account for an association constant of 10^3 M^{-1} should place less stringent demands on the nature and topography of the reacting sites than those associated with the generation of an interaction some 10^6-fold stronger, and that it is a reasonable rule-of-thumb, therefore, to consider that the degree of specificity increases with increasing strength of the interaction There are, however, marked exceptions to this generalization For example, despite the fact that the interaction unique to catalysis by lactate dehydrogenase is that between pyruvate and the enzyme–NADH complex, its association constant (approximately 10^4 M^{-1}) is at least 10-fold smaller than that for NADH binding by enzyme [5], an interaction that is common to many dehydrogenases In similar vein, the sites on serum albumin to which warfarin [6] and dicoumarol [7] bind with relatively high affinities (10^5–10^6 M^{-1}) also interact with fatty acids and a number of other amphiphilic drugs [8,9]

There are also systems for which the biospecific interaction between species entails covalent bond formation, a phenomenon typified by the covalent binding of substrate analogues to the active site of serine-dependent esterases, proteinases and acyltransferases in a reaction that mimics the first step of the catalytic mechanism, such covalent reaction is, however, usually preceded by the formation of a non-covalent complex between the enzyme and the substrate analogue Two examples are provided

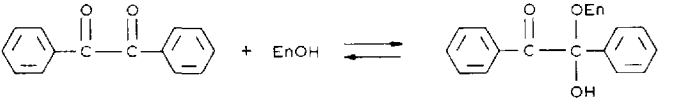
(i) The covalent reaction of the active-site serine hydroxyl group in acetylcholinesterase (EnOH) with organophosphorus compounds such as paraoxon (diethyl *p*-nitrophenyl phosphate) [10] yields an adduct which is sufficiently stable that the release of free enzyme at a significant rate can only be effected

by the addition of a good nucleophile such as N-methylpyridinium-2-aldoxime (eqn 1).



(1)

(ii) In the interaction of benzil with liver carboxylesterase to give a hemiketal adduct at the active site (eqn. 2), the formation of the



(2)

hemiketal and its decomposition both occur rapidly, the reaction being characterized by an association constant of $10^8\ M^{-1}$ [11] Substrate analogues which react specifically and covalently with groups in the active sites of enzymes have been termed suicide substrates or mechanism-based inhibitors [12]

Having indicated that the association constants for specific interactions between ligands and macromolecules can range from (say) 10^3 to $10^{15}\ M^{-1}$, some comment is necessary on the rates of association and dissociation for such complexes On the time scale of most chromatographic and electrophoretic techniques it can be assumed that attainment of equilibrium from either direction is likely to be effectively instantaneous for interactions at the low end of the energy spectrum, but that dissociation of complexes governed by large association constants is likely to be slow Affinity chromatography exemplifies an application of the biospecific approach where sufficient time must be allowed for the system to attain chemical equilibrium On the other hand, the validity of many binding assays [radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), nitrocellulose filter assays of protein–nucleic acid interactions] relies upon dissociation of the complex being sufficiently slow for effectively no interconversion to occur during the removal of uncomplexed reactants, a situation more likely to prevail when the association constant is large For either type of application it is clearly important to establish that the assumed situation represents a reasonable approximation for the particular system under study

Traditional approaches to the problem of solute purification have relied upon the existence of differences in general properties such as size and charge of

macromolecules, and on the probability that solutes with similar characteristics in one such respect may well differ in the other This rationale, which stemmed from the pioneering ultracentrifugal and electrophoretic studies of Svedberg and Rinde [13] and Tiselius [14], respectively, formed the basis of the amazing progress in protein purification that followed the development of the corresponding chromatographic procedures based on dextran gels [15] and cellulose-based ion-exchangers [16,17]. The biospecific approach to the problem of solute purification [18,19] is a logical sequel to ion-exchange chromatography in that the general electrostatic interaction between solute and ion-exchanger is replaced by an interaction related to the solute's biological function. Thus, in relation to an example already cited, dihydrofolate reductase may be selectively adsorbed from a tissue extract by chromatography on a matrix (Sepharose) to which methotrexate has been covalently attached [20] All other solutes may therefore be removed by copious elution of the column with buffer, at which stage it becomes evident that successful deployment of the biospecific approach to purification of the enzyme also depends upon the availability of conditions that diminish the strength of the interaction sufficiently for the enzyme to be eluted from the column in an undenatured and active (or activatable) state

The advantages of the biospecific approach as a preparative chromatographic procedure also extend to its use for characterizing the interactions used to effect solute purification [21–23] For example, the specificity of the interaction responsible for the one-step isolation of lactate dehydrogenase by affinity chromatography on a matrix with oxamate (a pyruvate analogue) attached [24] has also made possible the determination of binding constants for the interactions of NADH with the five lactate dehydrogenase isoenzymes present in a crude tissue extract [25]. Furthermore, for the determination of equilibrium constants by this technique, termed quantitative affinity chromatography [26] or analytical affinity chromatography [27], there seems to be no restriction to the range of magnitudes of association constants that may be measured [28,29]

Because explicit accounts have already been presented on the practical details of preparative affinity techniques [30–34], this aspect of the biospecific approach is presented in the form of an overview, the emphasis being placed on the general manner in which the problem of devising a new separation procedure is attacked. Coverage of this topic is thus illustrative rather than comprehensive, and is designed as a challenge to readers to contemplate the possibility of employing the biospecific approach for additional biomedical systems: there is ample cross-referencing to the technical details should the review be successful in that endeavour The theme of the second part of this review is the quantitative characterization of biospecific interactions by a range of chromatographic techniques, a topic with much broader scope than that of recent reviews on quantitative affinity chromatography [26,27,35]. In addition to

considering the characterization of interactions by a range of chromatographic and electrophoretic techniques, attention is given to the determination of association constants by competitive binding assay and solid-phase immunoassay (RIA or ELISA) – techniques that are used routinely in the biomedical environment but not as sources of quantitative information on (say) drug–receptor interactions or the immune response

2 BIOSPECIFIC SEPARATION AND ESTIMATION OF SOLUTES

2 1 Selection and preparation of affinity columns

An affinity resin is composed of an insoluble matrix and a ligand, the ligand being attached to the matrix by stable covalent bonds The function of an affinity resin is to present the immobilized ligand in such a way as to provide optimal access for solutes in the mobile phase Major factors in achieving this optimal access are the properties of the matrix and the method of attachment of the ligand Given adequate accessibility of the immobilized ligand to the solutes, the central factor in any affinity chromatography experiment is the interaction between the ligand and one or more of the solutes.

Before considering the various types of ligands, matrices and coupling methods, some comments on the availability of affinity resins and columns are appropriate.

(i) Many complete affinity resins are available commercially, some already packed into columns, and suitable for either conventional liquid chromatography or high-performance liquid affinity chromatography (HPLAC)

(ii) A variety of matrices is available commercially (some already prepacked in columns), and simple methods have been developed for coupling of ligands to them [31], such methods are sufficiently simple for novices in organic chemistry to complete them satisfactorily and safely

(iii) Many ligands can be obtained commercially and others can be synthesized chemically or purified from biological sources

In summary, nobody should be deterred from attempting an affinity chromatography experiment by lack of prior experience or the absence of a strong biochemical background. The reward of an affinity chromatography experiment may occasionally be the rapid, single-step purification of a desired biological molecule to homogeneity However, affinity chromatography is not the complete answer to all problems requiring separation and/or estimation of biological materials It should be considered as one of a range of powerful chromatographic and electrophoretic techniques, biospecific and otherwise, available to the research worker In many purification procedures an affinity chromatography step is one of several purification steps required to achieve homogeneity of the desired material Frequently, initial extracts (large volumes of possibly turbid solutions) are subjected to salt fractionation and/or

large-scale ion-exchange chromatography before being in a form suitable for purification by affinity chromatography On the other hand, Mattiasson and Ling [36] have adopted a different approach to allow biospecific separation to be achieved early in large-scale separation processes In this approach the ligand is covalently attached to a soluble compound chosen because its properties (e g , size, charge, partition into the organic phase of a two-phase system) allow ready separation of the solute–ligand–compound ternary complex from the rest of the components present in crude extracts techniques such as membrane filtration, ion-exchange chromatography and liquid–liquid partition have been used for this latter purpose.

2 1 1 The ligand

As indicated in the Introduction, there exist many different types of biospecific interaction, and hence we have a wide choice of ligands for use in affinity chromatography. The principal criteria to be considered in selecting a ligand for any particular separation are

(i) The specificity of the interaction: how many molecules present in the mixture being fractionated are likely to bind to the ligand?

(ii) The strength of binding is binding sufficiently strong to allow retention of the desired solute during stringent washing of the column to remove unbound or weakly ('non-specifically') bound solutes? Is binding sufficiently weak to allow elution of the solute from the column under non-denaturing conditions, or at least under conditions where the solute is only reversibly denatured?

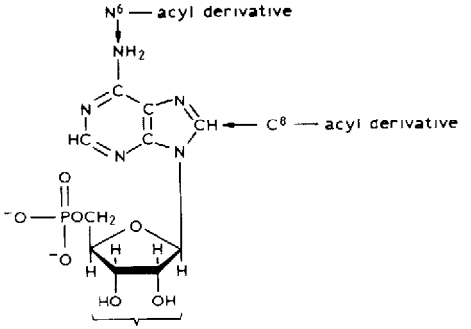
(iii) The stability of the ligand and of its attachment to the matrix under the chromatographic conditions, these should be sufficiently stable to allow multiple use of the column without deterioration in performance – a factor of particular relevance when the ligand is a macromolecule

The probability of success of an affinity chromatography experiment in separating one solute from a mixture of solutes is largely determined by criteria (i) and (ii) It may also be relevant to categorize ligands as group-specific if they interact with a group of molecules or highly specific if they are designed to interact with one or a small number of molecules Quantitatively, the value of an affinity chromatography step in the purification of a protein is assessed by the purification factor (the ratio of the specific activity after the step to that before) and the recovery or yield of activity (the percentage of the initial activity in the sample which is recovered) The more specific the ligand, the greater is the purification factor able to be achieved.

Particular examples will now be used to illustrate the different categories of ligand

2 1 1 1 AMP and related compounds AMP-containing affinity matrices are likely to bind to any enzyme which uses AMP, ADP, ATP, NAD^+, $NADP^+$ or coenzyme A as a substrate, i e , approximately 30% of all enzymes listed in the I U B. classification of enzymes [37] Despite this low degree of specificity,

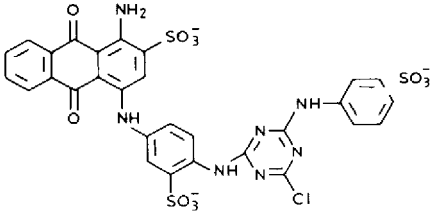
widespread use has been made of AMP resins in which the ligand has been attached to the matrix via at least three different sites (indicated by arrows) on the AMP molecule [38]. Their value in the purification of any given protein



Periodate oxidation and borohydride reduction

may be enhanced by biospecific elution (see below) or by varying the site of attachment of the AMP to the matrix to determine which gives the best result

Affinity matrices which in principle show even less specificity are those which use polycyclic dye molecules such as Cibacron Blue F3GA as ligand Such dyes



Cibacron Blue F3GA

have been shown to bind to the nucleotide-binding sites of many enzymes and have proved very useful in (i) protein purification [38] and (ii) quantitative studies of protein–ligand interactions [39] These dye columns would appear to be at the limit of what we legitimately term affinity chromatography since (i) the ligand structures are only moderately similar to the structures of the nucleotide substrates of the enzymes which bind to them and (ii) many proteins such as serum albumin which lack a specific nucleotide binding site can be purified efficiently on such columns

Some nucleotide-containing affinity resins may be more specific than described above For example, GMP-Sepharose has proved very effective in the isolation of hypoxanthine guanine phosphoribosyltransferase from human erythrocytes [40]. After preliminary ammonium sulphate fractionation and DEAE-cellulose chromatography, chromatography on GMP-Sepharose results in a 200-fold purification of the enzyme

2 1 1 2 Lectins and related proteins Among the most widely used group-

specific ligands are the lectins, proteins which bind particular carbohydrate molecules [41]. Thus, immobilized concanavalin A (con A-Sepharose) has been widely used in the purification of glycoproteins, especially those containing terminal mannose residues. The success of lectin affinity chromatography depends on the source of the glycoprotein and the pretreatment of the sample. For example, con A-Sepharose is very effective in purification of the purple phosphatase of red kidney beans [42,43]. However, if the crude salt extract of the beans is chromatographed, two problems are discerned. the extract contains large amounts of glycoproteins which rapidly overload the column and the extract contains compounds, probably lectins, which complex the purple phosphatase and prevent its interaction with the lectin column (C Clark and J de Jersey, unpublished results) Average purification factors of 5-fold to 10-fold are achieved by lectin affinity chromatography.

Lectin affinity chromatography has also been used to detect changes to the N-linked oligosaccharide moieties of glycoproteins in neoplastic tissues. For example, hepatoma γ-glutamyl transpeptidase can be distinguished from the normal liver enzyme by differential binding to erythrophytohemagglutinin-Sepharose [44]. The specificity of binding of complex-type oligosaccharides to a column of *Datura stramonium* agglutinin-Sepharose has recently been reported [45]

There are also a few examples of the fractionation of polysaccharides (glycosaminoglycans) on affinity columns with proteins such as lipoprotein lipase and laminin as ligands [46]

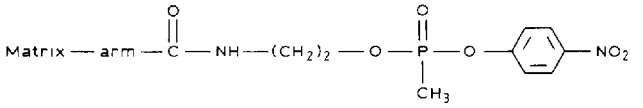
2 1 1 3 Proteinases and their protein inhibitors Many animal and plant tissues contain proteins which form tight inhibitory complexes with trypsin and other proteinases. Such proteinase inhibitors have been purified using matrices containing the relevant proteinase as ligand For example, Kortt [47,48] used trypsin-Sepharose and chymotrypsin-Sepharose to purify specific trypsin and chymotrypsin inhibitors from extracts of winged bean seed Conversely, matrices with proteinase inhibitors as ligands may be used to purify proteinases [49]. As well as being useful in protein purification, such affinity matrices have obvious applications in removing potentially harmful proteinases and their inhibitors from tissue extracts. In fact, the tight binding which ensures quantitative removal of proteinases or their inhibitors from tissue extracts may be a disadvantage when affinity chromatography is used in protein purification, since it makes elution difficult (see below).

2 1 1 4 Hormones and related compounds There are many examples of the highly successful use of hormones and hormone-related drugs as immobilized ligands in the purification of membrane receptors Here, the tight binding results in very large purification factors For example, a 5500-fold purification of the α_2-adrenergic receptor from porcine brain was obtained by a single passage of a digitonin extract of a brain membrane fraction through a yohimbine-agarose column [50], after which rechromatography on the same column gave a

further 12-fold purification to near homogeneity Another recent example of this approach was the successful use of sequential affinity chromatography on insulin-Affi-Gel 10 and insulin-like growth factor I (IGF I)-Affi-Gel 10 to separate the high-affinity IGF I receptor from lower-affinity receptors in Triton X-100 extracts of human placental membranes [51].

2 1 1 5 Antibodies and antigens In principle, any protein can be separated from all other proteins in a single step by immunoaffinity chromatography – affinity chromatography in which the ligand is an antibody of the protein being purified. Development of an immunoaffinity procedure does, of course, rely upon prior purification of the protein by a different procedure to give sufficient highly purified protein to serve as the immunogen. A further possible source of difficulty is the tight and specific binding between antigen and antibody, which can cause problems with elution of the antigen from the column (see below) The power of immunoaffinity chromatography as a purification procedure has been boosted by the development of monoclonal antibody technology, which not only allows large amounts of a particular antibody to be prepared but also permits the selection of antibodies with appropriate affinity (weaker or stronger) for the antigen, depending on the application The advantages of monoclonal over polyclonal antibodies as ligands have been discussed in detail by Calton [52]. Furthermore, ever increasing numbers of both monoclonal and polyclonal antibodies are becoming available commercially [53].

2 1 1 6 Ligands which react covalently with solutes Such ligands have been exploited in affinity separations far less frequently than ligands which bind non-covalently to macromolecules This may be due to the greater difficulties involved in preparing the derivatized ligand and coupling it to the affinity matrix, given that these ligands contain chemically reactive groups It also reflects the relative rarity of covalent interactions that give rise to stable but readily dissociable adducts The following immobilized organophosphorus compound



has been used as an affinity matrix for acetylcholinesterase [10], which can be eluted subsequently by the inclusion of N-methylpyridinium-2-aldoxime in the column eluate (eqn 1) A second example of enzyme purification by covalent affinity chromatography involves the use of dipeptidyl argininal ligands to isolate trypsin-like enzymes [54] These aldehyde-containing ligands form hemiacetal adducts with the active-site serine hydroxyl groups of the enzymes in a manner analogous to the reaction of benzil with carboxylesterase (eqn 2) Other examples of enzyme purification by covalent affinity chromatography entail the use of organomercurial-agarose [55] and thiol–disulphide interchange [56] to isolate papain, which contains an active-site thiol group

Although covalent affinity chromatography has not found widespread use in enzyme purification, it does have certain advantages over traditional affinity chromatographic methods based on non-covalent interactions between solute and immobilized ligand Because the enzyme is effectively irreversibly bound to the matrix (in the absence of a good nucleophile, reducing agent, etc), its continued attachment does not depend on maintenance of the three-dimensional structure of the macromolecule Furthermore, the fact that the covalent reaction duplicates part of the catalytic mechanism of the enzyme means that only catalytically functional enzyme molecules react In conventional affinity chromatography there is always the possibility that ligands which bind non-covalently may still interact (albeit more weakly) with a catalytically impaired enzyme active site

2 1 2 Choice of matrix and coupling method

The main criteria which govern the suitability of a matrix for affinity chromatography are (i) its mechanical and flow properties, (ii) the ease of covalent coupling of ligand to matrix and (iii) the stability of the bonds linking ligand to matrix.

As in any form of column chromatography, the best resolution is obtained if the matrix particles are uniform in size and shape and as small as possible The rigid silica or polymeric beads of diameter 5 or 10 μm used in normal-phase high-performance liquid chromatography (HPLC) are also suitable for derivatization with ligand and use in HPLAC The use of rigid beads to allow operation of columns at the high pressures needed to give high flow-rates does not give the same advantages in the chromatography of macromolecules as it does when small molecules are being separated With small molecules, diffusion is rapid, leading both to rapid equilibration of the solute molecules between stationary and mobile phases and to rapid broadening of elution zones Macromolecules equilibrate and diffuse much less rapidly, demanding lower flow-rates for optimal resolution Therefore, many affinity separations still make use of traditional gel matrices, such as cross-linked agarose and polyacrylamide On many occasions, however, this choice merely reflects greater familiarity with the methods used for the attachment of the ligand to them The better mechanical properties and durability of rigid matrices, the more uniform packing obtained in factory-packed columns and the overall ease and speed of use of HPLC equipment should ensure a trend towards the greater use of rigid matrices Another important factor in the choice of the matrix is the pore size Large pore sizes are necessary in affinity chromatography since either the ligand or the species binding to it is a macromolecule – often both are macromolecules Pore sizes of 100–400 nm are necessary to provide unhindered access of macromolecular solutes to macromolecular ligands Finally, it is preferable for the matrix beads to be stable in organic solvents as well as in the

aqueous phase so that greater flexibility is available in the selection of conditions for coupling.

Underivatized matrix beads can be obtained commercially, activated in the laboratory using one of a number of well documented procedures developed for each type of matrix, and immediately reacted with the ligand. Alternatively, matrix beads which have been activated in one of a number of ways are available in a stable form (e.g., lyophilized or as a slurry in an organic solvent) These activated matrices will react under mild conditions with chemical groups in the ligand Examples of reactive groups in activated supports include (i) imidocarbonate and cyanate groups formed by reaction of matrix hydroxyl groups with cyanogen bromide, (ii) epoxy groups formed by reaction of a bisoxirane with matrix hydroxyl or amino groups and (iii) imidazolylcarbamate groups formed by reaction of carbonyldiimidazole with matrix hydroxyl groups These reactive groups will all react with nucleophilic groups, especially amino and thiol groups, in the ligand under mild conditions.

A third alternative is to obtain a matrix into which amino or carboxyl groups have been incorporated. These functional groups may then be reacted with carboxyl and amino groups, respectively, in the ligand, coupling being achieved by the presence of a carbodiimide This type of derivatized matrix is especially useful in instances where the incorporation of a spacer arm between the backbone of the matrix and the ligand is necessary to decrease steric hindrance in the binding of ligand to solute. Spacer arms are often used to improve the accessibility of a small immobilized ligand to a macromolecular solute with which it interacts biospecifically.

In selecting a coupling method for the preparation of an affinity matrix, aim for (i) high stability of the linkage between ligand and matrix in storage and use and (ii) electrical neutrality and hydrophilicity of the linkage and the spacer arm to limit secondary interactions.

2 2 Experimental facets of preparative affinity chromatography

2 2 1 Selection of equipment

It is one of the major advantages of affinity chromatography that excellent results can be obtained with minimal equipment. Because the interaction between the immobilized ligand and the solute(s) is more specific than the interaction between solutes and the stationary phase in other forms of chromatography, it is mostly possible to obtain the desired separations using relatively small columns (bed volumes of 1–20 ml) The size of the column is determined by three factors: the amount of sample loaded, the fraction of the solute molecules likely to bind and the capacity of the affinity matrix. Commonly, a peristaltic pump is used to load the sample, wash the column and elute bound solutes at a constant flow-rate In most cases, elution is achieved by step-wise changes in the eluting solvent rather than by application of a gradient. The

eluent would usually be monitored by measuring its UV absorbance at 280 nm (for proteins), 260 nm (for nucleic acids) or some other wavelength, and by measuring the biological activity of collected fractions From these data, the specific activity of the eluate (U/mg) and hence the purification achieved by the chromatography step can be calculated, together with the percentage recovery of activity

Because of the small column size, sample loading, washing, elution and re-equilibration can usually be achieved in a period of one to a few hours This is probably satisfactory when affinity chromatography is carried out irregularly as part of a purification procedure However, considerable savings in time as well as improvements in reproducibility and resolution could be expected by the use of an integrated HPLC or fast protein liquid chromatography (FPLC) system especially in analytical work Many affinity chromatography columns suitable for HPLC are available commercially, as are prepacked columns of activated matrix (e g , activated tresyl-silica) ready for derivatization in situ with the particular ligand required

In most instances, application of the sample at a relatively low flow-rate allows sufficient time for equilibration between bound and free solute to be achieved There are systems, however, for which equilibrium is attained very slowly, especially when the initial concentration of the solute to be bound is very low To ensure that a high percentage of the solute is bound, the affinity matrix and the solute sample may be mixed, with gentle agitation, for a period of hours (batch adsorption) prior to pouring a column, washing and eluting in the normal manner [51,57]

2 2 2 Depletion of secondary interactions

The essence of affinity chromatography is the specific interaction between the derivatized matrix and the solute In principle, other non-covalent interactions (hydrogen bonding, electrostatic bonding and hydrophobic interactions) between any solute molecule and any part of the derivatized matrix should be minimized Such interactions may be with (i) the matrix itself, (ii) the linkages of matrix to spacer arm and spacer arm to ligand, (iii) the spacer arm, if present, and (iv) the ligand. Non-specific adsorption of solutes to the matrix can generally be overcome by capping of reactive matrix groups, such as hydroxyl groups in silica beads and controlled pore glass Adsorption to agarose, dextran and polyacrylamide gels is not usually a significant problem

The most likely secondary interaction is ion-exchange For example, when cyanogen bromide is used to activate the matrix, the ligand or spacer arm ($R\text{-}NH_2$) is attached via a protonated isourea linkage which is positively charged at physiological pH The affinity matrix thus becomes an anion-exchanger, i e ,

$$\text{matrix–O–C(=}{}^{+}\text{NH}_2\text{)–NH–R,}$$

whose capacity may equal or exceed that of commonly used anion-exchangers such as matrices derivatized with diethylaminoethyl groups. This is a significant disadvantage of the cyanogen bromide method of matrix activation Similarly, the presence of charged groups in the spacer arm should be avoided In many cases, the ligand itself is charged, positively or negatively, at physiological pH, and the electrostatic interaction between ligand and solute is at least partially responsible for the specific binding This situation arises, for example, with affinity matrices involving AMP (see above) or benzamidine, a competitive inhibitor of trypsin that has frequently been used as immobilized ligand in the purification of trypsin and related enzymes [58]. The obvious method to limit non-specific ion-exchange interactions between an affinity matrix and solutes is to equilibrate the column and the sample to be loaded with buffer containing a high concentration of electrolyte (say 1 *M* sodium chloride) or to wash the column with high-ionic-strength buffer after loading the sample but before specific elution of solute is attempted. Alternatively, the problem may sometimes be averted by manipulating the pH at which the experiment is conducted or by preceding the affinity chromatography experiment by an ion-exchange step designed to remove solutes for which the interaction with affinity matrix is non-specific ion-exchange. It should be noted that this phenomenon is likely to be significant in experiments with a protein as the immobilized ligand.

Significant hydrophobic interactions between spacer arm or ligand and solutes may also occur These can be minimized by using a hydrophilic (but uncharged) spacer arm and by employing a buffer of low ionic strength to diminish hydrophobic interactions In that regard it should be noted that the conditions required for overcoming non-specific ion-exchange and hydrophobic effects are mutually exclusive Resort to biospecific elution procedures (see below) also helps to obviate problems caused by secondary interactions

Although Scopes [38] has calculated that an association constant of at least $10^6\ M^{-1}$ is required to ensure effective adsorption of a solute to an immobilized ligand, a high degree of binding to an affinity column is frequently encountered with systems for which the association constants describing complex formation between solute and soluble ligand are 10^3–$10^5\ M^{-1}$, the range that applies to many complexes between enzymes and substrate analogues Part of the answer to this apparent anomaly appears to be that non-specific binding between solute and affinity matrix (e g , with the spacer arm) provides an additional contribution to the energetics of the interaction between solute and immobilized ligand For affinity systems involving weak interactions, non-specific binding may therefore be advantageous

2 2 3 Biospecific and non-specific elution

Once the sample has been loaded and the affinity column washed to remove unbound or non-specifically bound solutes, specific elution may be achieved by displacement of the equilibrium position for the binding of ligand to solute. This is usually done by elution with a soluble compound which competes with the immobilized ligand for binding sites on the solute or with the solute for binding sites on the ligand In regard to two examples of affinity matrices already mentioned, trypsin-like enzymes may be eluted from a benzamidine affinity matrix with an eluting buffer containing benzamidine [58] and red kidney bean purple phosphatase is eluted from con A-Sepharose by a buffer containing α-methyl mannoside [43]. In the latter case, the α-methyl mannoside competes with N-glycans on the phosphatase for binding sites on the ligand, con A

A further dimension in biospecific elution is demonstrated by the separation of three dehydrogenases by affinity chromatography on AMP-Sepharose [59] A mixture of malate, alcohol and lactate dehydrogenases was applied to the column and, as expected, all bound Malate dehydrogenase was specifically eluted with NADH–oxaloacetate adduct; then alcohol dehydrogenase was eluted with a mixture of NAD^+ and hydroxylamine, and finally, lactate dehydrogenase was eluted with NADH–pyruvate adduct In each case, the specific substrate or substrate analogue in combination with the common substrate (NAD^+ or NADH) effected elution of the one enzyme capable of ternary complex formation with the particular substrate (analogue)–coenzyme pair

The purification of calmodulin by chromatography on Phenyl-Sepharose provides another interesting example [60] This separation is based on the hydrophobic interaction between immobilized phenyl groups and a binding site on calmodulin, an interaction of low specificity that barely merits designation as affinity chromatography However, the procedure uses the specific effect of Ca^{2+} on the binding properties of calmodulin, the Ca^{2+} complex of which binds to the matrix After loading of the sample in the presence of Ca^{2+}, specific elution of the calmodulin is achieved by including ethylene glycol bis (β-aminoethyl ether) N,N,N′,N′-tetraacetate in the buffer to chelate the Ca^{2+} and hence abolish the binding of calmodulin to Phenyl-Sepharose This affinity procedure has been extended to determine the effects of chemical modification of calmodulin on the ability of Ca^{2+} to expose the hydrophobic regions responsible for the interaction with Phenyl-Sepharose [61]

Affinity elution of proteins from ion-exchange columns is a closely related phenomenon The principle is best illustrated by an example – the specific elution of aldolase from a carboxymethyl-cellulose column at pH 7 1 by a low concentration (0 1 mM) of fructose 1,6-bisphosphate [62,63] Each molecule of substrate bound to the enzyme adds between three and four negative charges at pH 7 1, and the effect is multiplied 4-fold since aldolase is a tetramer The aldolase–substrate complex has a net negative charge under these conditions

and is eluted, whereas other bound proteins would largely be unaffected by the presence of this low concentration of fructose bisphosphate Scopes [37,63] has outlined the factors involved in achieving successful biospecific elution from ion-exchange columns

Elution becomes a problem when there is very tight binding between immobilized ligand and solute As mentioned in the Introduction, the rate of complex dissociation may be very slow, so that there is no possibility of displacement of solute from immobilized ligand (e g , by competition between immobilized and soluble ligand) on a viable time scale To illustrate this point, consider the simple equilibrium

$$A + B \underset{k_2}{\overset{k_1}{\rightleftarrows}} AB \qquad (3)$$

where $K_{AB} = k_1/k_2 = 10^{10}\ M^{-1}$ Measured values of k_1 for the formation of enzyme–substrate and enzyme–inhibitor complexes are in the range 10^6–$10^8\ M^{-1}\ s^{-1}$ [64] The corresponding rate constant for the reaction of a macromolecule with an immobilized ligand may be 100-fold smaller, given the difference between diffusion coefficients of small and large molecules If we take $10^5\ M^{-1}\ s^{-1}$ as a reasonable estimate of k_1, k_2 becomes $10^{-5}\ s^{-1}$, corresponding to a half-life of about 20 h To quote an extreme example, the interaction of trypsin with basic pancreatic trypsin inhibitor is characterized by a k_1 of $1\ 1 \cdot 10^6\ M^{-1}\ s^{-1}$ and a k_2 of $6\ 6 \cdot 10^{-8}\ s^{-1}$ [65], corresponding to a half-life of about 120 days for dissociation of the complex

When elution using a soluble ligand is not possible, the only method available is to decrease the association constant by changing the conditions This may sometimes be achieved by a relatively small change in pH [51,57] The most common examples of very tight binding and slow dissociation are in immunoaffinity chromatography, where elution is commonly achieved by decreasing the pH to 2–3, or by incorporating a denaturing agent such as urea or guanidinium chloride in the eluting buffer [52] Such elution conditions could well lead to irreversible denaturation of the solute, which may or may not be a problem depending on the purpose of the experiment As mentioned above, the use of monoclonal antibodies, selected to bind with an intermediate association constant, can overcome these difficulties

2 3 Biomedical applications of affinity chromatography

2 3 1 Purification of proteins and nucleic acids

The purification to homogeneity of one protein species from a mixture which may contain thousands of different proteins remains one of the more formidable challenges of biomedical science Since its inception in the work of Lerman [66] in the 1950s and the major improvements made by Cuatrecasas,

Wilchek, Anfinsen and others in the late 1960s [18,19], affinity chromatography has become an important step in the purification of a large number of proteins Extensive compilations of specific affinity procedures are contained in earlier reviews, especially ref 30, and further examples appear continually in biochemical journals. Protein purification and partial sequencing in many cases precede gene cloning, DNA sequencing, mutagenesis and expression, with major developments in these areas resulting in expanding interest in protein purification and hence in affinity chromatography The need for stringent purification of proteins produced by recombinant DNA technology has also increased the importance of affinity chromatography as a preparative isolation procedure In one recent example of an expression system [67], the cDNA of the protein being studied was inserted into a plasmid vector adjacent to cDNA for glutathione S-transferase. Expression in *Escherichia coli* resulted in the formation of a fusion protein which was purified by affinity chromatography on a glutathione-containing matrix The purified fusion protein was then cleaved from the desired protein by thrombin at a cleavage site incorporated into the region linking the glutathione S-transferase cDNA and the cDNA of the protein being expressed

In the affinity chromatography of nucleic acids, two types of ligand could be used a second nucleic acid or a protein. The classical example of an immobilized nucleic acid ligand is oligo-(dT)-cellulose for isolating mRNA by virtue of base pairing between poly A tails on the mRNA and the ligand [68]. Apart from this example, little use has yet been made of ligands which contain a specific oligonucleotide sequence expected to hybridize with only one type of mRNA or single-stranded DNA in a complex mixture Similarly, little, if any, use has been made of DNA- or RNA-binding proteins as ligands to purify nucleic acids containing sequences which bind to the proteins There is clearly considerable scope for further developments in affinity chromatography of nucleic acids. The reverse procedure, where an immobilized nucleic acid ligand is used as an affinity matrix to separate nucleic acid binding proteins, has been applied frequently [30,69]. For example, purified transcription factor Sp1, a protein which binds to a particular promoter sequence in DNA (the 'GC box'), has been obtained by affinity chromatography on a Sepharose matrix containing synthetic nucleotides with the sequence 5′-GGGGCGGGGC-3′ [70].

Potentially, affinity chromatography is a powerful method for separation of different types of cells, based on the interaction between an immobilized ligand and a molecule which is present on the surface of only one or a few cell types It is a complementary procedure to cell separation using a fluorescence-activated cell sorter, which is another biospecific method. However, practical problems remain, mostly associated with the difficulty of eluting bound cells from the matrix in such a way that they remain viable [71] One promising approach has been to link the ligand to the matrix by means of a spacer arm which can be cleaved readily under mild conditions, an example of such an affinity system

being matrix–arm–Hg–S–ligand After cells have been bound to the ligand, the cell–ligand complex may be released by incorporating into the eluting medium an excess of a thiol such as dithiothreitol [71]

2 3 2 Biospecific uses of immobilized proteins

Immobilized proteins are frequently used in affinity chromatography for the purification and analysis of other proteins, and several examples have been described above Immobilized proteins have other biospecific uses, including·

(i) Catalysis of specific chemical reactions; examples include the use of glucose isomerase in the industrial-scale preparation of high-fructose corn syrup and the use of a variety of enzymes in enzyme electrodes and thermistors to allow the estimation of clinically significant metabolites [34,72].

(ii) Resolution of enantiomeric mixtures by differential binding of enantiomers, e g , resolution of N-benzoyl-DL-serine on silica-immobilized bovine serum albumin [73].

(iii) Various ELISA and RIA techniques, as well as allowing the estimation of macromolecules, progress is being made in the use of immobilized antibodies for the estimation of low-molecular-weight metabolites, such as specific steroids.

2 4 Biospecific procedures in electrophoresis

2 4 1 Biospecific identification of species

The development of blotting procedures (electro and capillary) to transfer proteins and nucleic acids from electrophoretic supports such as agarose and polyacrylamide to nitrocellulose or other types of film has stimulated major recent advances in biospecific identification of species (see ref 74 for a recent review) Blotting onto polymeric film leads to a major improvement in accessibility of the transferred macromolecules, especially to other macromolecules used in identification Identification of specific proteins after electrophoresis or isoelectric focussing (IEF) and blotting can be achieved by either (i) location of the biological (e.g., enzymatic) activity of the native protein (provided the protein has not been irreversibly denatured prior to or during the separation and blotting process) or (ii) use of an antibody able to bind specifically to the protein in either its native or denatured form, or both (viz , Western blotting).

For nucleic acids, a labelled probe with a base sequence complementary to portion of the nucleic acid molecule being located is allowed to hybridize with the nitrocellulose film onto which the nucleic acid molecules have been blotted, after first converting the bound nucleic acids from double stranded to single stranded if necessary Excess probe is washed away and the bound label is detected, usually by autoradiography The result of the overall procedure (electrophoresis, blotting and visualization) is termed a Northern blot (when RNA is identified) or a Southern blot (for DNA).

Enzymatic activity is still frequently visualized in electrophoretic and IEF gels without blotting. Specific staining procedures have been developed for a large number of enzymes and other proteins [75,76], and it can be safely assumed that direct staining of gels will continue to be widely used in addition to staining of blots. Immunoelectrophoresis is the corresponding method which has been used to locate antigens directly in gels after electrophoresis or IEF [77] In this method, antigens are first separated by electrophoresis or IEF, usually in an agarose gel, and the specific antibody solution is placed in a trough parallel to the lane in which the sample was run. Antigen and antibody then diffuse through the gel and form a precipitin line. This technique has severe limitations, especially in sensitivity, and has largely been superseded by blotting and visualizing the antigen in question by means of a radioactive or enzyme-linked antibody, or by a sandwich technique

With the explosion in the amount of DNA sequence data available, many open reading frames have been found for which the gene product is unknown. Biospecific procedures may be used to identify the gene product, as follows: gene sequence→protein sequence→synthetic peptides→antibodies against peptides→Western blotting of proteins from the appropriate cell→elution of proteins which bind to the anti-peptide antibody and confirmatory sequencing

2 4 2 Affinity electrophoresis

Affinity electrophoresis is a form of gel electrophoresis in which the gel contains a ligand for one or more of the solutes (usually proteins) [78] Mobility depends on the charge and size of the solute molecules, as in normal gel electrophoresis, but in addition, molecules which bind to the ligand are retarded. The separation pattern achieved in an affinity electrophoresis experiment is compared with that obtained in a control experiment (with no ligand) to locate bands corresponding to solutes which bind to the ligand One recent example which shows the value of the method is the separation of individual immunoglobulin G (IgG) species present in rabbit polyclonal anti-dinitrophenyl antibody [79]. A two-dimensional system was used, with IEF in the first dimension and electrophoresis in a polyacrylamide gel containing dinitrophenyl or trinitrophenyl groups covalently attached to the polyacrylamide in the second dimension Use of either affinity resin gave resolution of approximately 100 spots due to individual IgG species, whereas the analogous system without ligand failed to produce any discreet spots This separation was made feasible by the relatively weak binding between the antibodies and the haptens (association constants of 10^4–10^5 M)

In instances where the ligand is too large to migrate through the gel, affinity electrophoresis may be performed without covalent attachment of the ligand to the matrix – a situation demonstrated in a gel electrophoretic study of muscle phosphorylase on polyacrylamide impregnated with glycogen [80] Affinophoresis [81] differs from both techniques so far described in that the bio-

specific ligand is not immobilized Provided that the ligand is relatively small and bears net charge, its interaction with a particular solute in a tissue extract allows identification of that solute by virtue of its changed electrophoretic behaviour Rocket electrophoresis [77], where the gel contains an antibody to one of the components of the sample, is a variant of affinophoresis This component migrates until its concentration is the same as that of the antibody, at which point precipitation occurs The pattern of precipitation resembles a rocket, and the length of the rocket gives an indication of the concentration of the antigen in the sample Like immunoelectrophoresis, rocket electrophoresis is largely superseded by blotting, ELISA and RIA techniques

By combining the resolving powers of gel electrophoresis and affinity chromatography, affinity electrophoresis must be regarded as one of the most powerful separation techniques available to an experimenter – a point illustrated by the above-mentioned separation of individual IgG species [79] Despite this potential, affinity electrophoresis has not been used extensively, possibly because gel electrophoresis and affinity chromatography are both high-resolution techniques in their own right In that regard the fact that so much attention has been focussed on affinity chromatography undoubtedly reflects the ease with which a biospecific procedure developed for analytical estimation of a particular solute may be upgraded to a preparative procedure for isolation of that solute or, indeed, adapted to yield quantitative information on the stoichiometry and strength of the biospecific phenomenon

3 QUANTITATIVE CHARACTERIZATION OF BIOSPECIFIC INTERACTIONS

In the preceding section emphasis was placed on the advantageous use of biospecificity to effect solute fractionation and estimation by chromatographic and electrophoretic techniques We now wish to consider the adaptation of those same procedures to characterize a biospecific interaction in terms of stoichiometry and equilibrium constant – on the grounds that important features of these interactions are their non-covalent nature and the consequent dependence of their equilibrium positions upon prevailing reactant concentrations Such quantitative characterization of the biospecific phenomenon must therefore precede meaningful discussion of, for example, changes in the degree of receptor-site occupancy with variation in metabolic hormone concentration or of the likely systemic drug concentration required for effective cancer chemotherapy The possibility that the technique used to isolate (say) a protein or enzyme on the basis of biospecificity may also yield quantitative information on the biospecific interaction is clearly an attractive prospect worthy of pursuit

This section begins with a discussion of general aspects related to the determination of equilibrium constants by chromatographic and electrophoretic techniques (i) the type of experimental design that is most rewarding from the quantitative viewpoint, (ii) interpretation of the resulting experimental

measurements in terms of the energetics of the interaction being studied, and (iii) allowance for the effects of ligand multivalency – a hitherto largely ignored problem despite its relevance to the binding of oligomeric proteins to larger macromolecules. The first method considered is gel chromatography – an extremely versatile technique for the characterization of interactions, specific or non-specific, that sets the quantitative standards by which the biospecific electrophoretic and chromatographic procedures must be assessed Then follows a discussion of current gel and electrophoretic techniques, which clearly do not measure up very well against those standards Finally, consideration is given to quantitative affinity chromatography, which, as in Section 2, is accorded most extensive treatment on the grounds that it provides the best characterization of interactions that the biospecific approach has to offer.

3 1 General experimental aspects

Physiological systems abound with examples of specific macromolecular interactions in which chemical equilibrium between the participating species is rapidly established, the rapidity of equilibrium attainment being essential to current concepts of metabolic regulation in response to an ever-changing cellular environment For such systems the chromatographic or electrophoretic behaviour clearly cannot reflect directly the composition of the initial equilibrium mixture because of the continual reequilibration that must necessarily accompany the attempted separation of complex(es) from reactants by differential migration. Traditional interpretations of electrophoretic and chromatographic patterns therefore need to be supplanted by analyses which take into account this complication and which thereby render possible the quantitative characterization of rapid chemical equilibria by these techniques [82–84] The following considerations serve to emphasize (i) the nature of the revised analyses, (ii) the simplicity of the resulting chromatographic and electrophoretic methods for quantifying biospecific interactions and (iii) their ability to provide such characterization for a whole spectrum of macromolecular interactions.

3 1 1 Zonal and frontal techniques

Most isocratic chromatographic studies employ zonal analysis, which entails the application of a small zone of solute to a column, and subsequent elution with buffer As the zone migrates through the chromatographic bed it undergoes continual dilution because of axial dispersion, with the result that the concentration of the eluted zone is considerably smaller than that of the applied solution (Fig 1a). For any system in which chromatographic migration exhibits dependence upon solute concentration such dilution is clearly a complicating factor from the viewpoint of interpreting the resultant elution profile This difficulty is readily obviated by resort to frontal chromatography (Fig 1b), in which a sufficient volume of solution is applied to the column to ensure

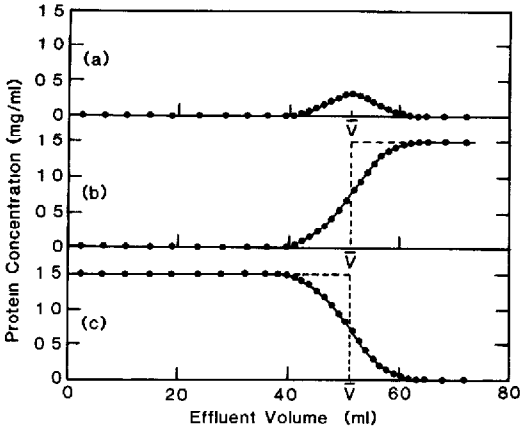


Fig 1 Gel chromatographic elution profiles for ovalbumin (1 5 mg/ml) on a Sephadex G-100 column (19 5 cm×2 4 cm) equilibrated with 0 1 *I* phosphate, pH 6 8 (a) Zonal elution profile obtained by applying 3 ml of solution (b) Advancing elution profile obtained in frontal gel chromatography of the same solution (c) Trailing elution profile generated in the same frontal experiment

the existence in the elution profile of a region where the solute composition equals that applied Thereafter, the column may be eluted with buffer to generate a second elution profile (Fig 1c). The particular advantage of the frontal technique is its provision of two independent elution profiles (advancing and trailing), both of which are related unequivocally to the chromatographic characteristics of the equilibrium mixture applied to the column

The emphasis in electrophoresis on increased versatility as a preparative procedure has led to the virtual demise of moving boundary electrophoresis [85], the frontal technique that preceded the current spate of zonal electrophoretic procedures. Consequently, the disadvantages discussed in relation to chromatography of interacting systems also apply to current electrophoretic techniques, the use of which for characterizing interactions is restricted to a particular experimental design that minimizes the complications due to continual reestablishment of chemical equilibrium within the migrating reactant zone (see later)

3 1 2 Partition equilibrium experiments

For rapidly established equilibria the elution volume derived from a column chromatographic experiment is a steady-state (time-independent) parameter, even though it is derived from a mass migration experiment [86,87] Under those circumstances, an alternative to column chromatography is therefore to conduct a series of partition equilibrium experiments in which the concentrations of solute in the liquid phase are determined for mixtures with known total concentration of solute Mixtures containing known amounts of chromato-

graphic matrix and partitioning solute are allowed to equilibrate at the temperature of interest until partition equilibrium has been established, at which stage a sample of the supernatant is obtained by filtration [23] or centrifugation [88] of each mixture at the same temperature. The weight concentration of all forms of the partitioning solute in the liquid phase, $\bar{c}_A$, is then determined by any appropriate spectrophotometric, enzymic or radiochemical means. This quantity is unequivocally the liquid phase concentration for a system with a total solute concentration, $\bar{\bar{c}}_A$, that is obtained by dividing the weight of solute added by V_A^*, the volume accessible to A in the absence of any interaction with matrix sites [23,89]. Any differences between the volumes of stationary (V_s) and liquid (V_o) phases in the various mixtures are taken into account by employing the corresponding partition coefficient K_{av}^* [90] and the relationship $V_A^* = V_o + K_{av}^* V_s$, where the volume of liquid phase may be deduced from an experiment with a solute that neither interacts with, nor penetrates, the stationary phase.

A disadvantage of such partition experiments is the need for precise determination of the weight and hence volume, V_s, of affinity matrix present in each reaction mixture [23] As noted previously [26], this requirement is a potential source of uncertainty in instances where reliance must be placed on the reproducibility with which aliquots may be taken from a concentrated slurry of chromatographic or cellular matrix A possible means of obviating this difficulty is resort to a recycling partition technique [91,92] in which the liquid phase of a stirred slurry of chromatographic matrix and partitioning solute is monitored spectrophotometrically by means of a flow-cell placed in the line returning the liquid phase to the slurry Even in the event that aliquots of liquid phase must be removed for assessment of $\bar{c}_A$ [93], this procedure still has the advantage that a number of partition experiments may be performed with the same sample of matrix material by making several additions of concentrated solute to the slurry and determining the corresponding value of $\bar{c}_A$ after each addition

3 1 3 Intrinsic binding constants

In studies of the interaction between two solutes in solution the smaller solute has been designated as the ligand, S, and the larger reactant as acceptor, A [94] Whereas the ligand is considered to be univalent in its interaction with A, the acceptor may possess several sites for interaction with S and is therefore said to be multivalent In studies of such mixtures of acceptor and ligand it is possible to define a binding function, r [94] or ν [95], as the molar amount of ligand bound to the total amount of acceptor Since both of these amounts are contained within the same volume, it follows that

$$r = ([\bar{S}] - [S])/[\bar{A}] \qquad (4)$$

where [S] denotes the equilibrium concentration of free ligand in a mixture

with total (constituent) molar concentrations $[\bar{S}]$ and $[\bar{A}]$ of ligand and acceptor, respectively For an acceptor with f sites for interaction with ligand the dependence of the binding function requires description in terms of f binding constants to account for the interaction at each site However, for many systems the acceptor sites are equivalent and independent, whereupon binding becomes described by the relationship

$$r = fk_{AS}[S]/(1+k_{AS}[S]) \tag{5}$$

in which k_{AS} is the intrinsic association constant [94] or site-binding constant [96] Experimentally, the existence of such a class of homogeneous sites is recognized by plotting results in terms of the Scatchard [95] linear transform of eqn. 5, namely,

$$r/[S] = fk_{AS} - rk_{AS} \tag{6}$$

Non-linearity of the suggested plot of $r/[S]$ versus r signifies the inadequacy of a single intrinsic binding constant to describe the system, which thus requires additional association constants to encompass either the binding of ligand to different classes of sites or the cooperativity of ligand binding to equivalent but dependent sites.

In affinity chromatography and, indeed, many biological systems the binding phenomenon of interest is the interaction of a macromolecular solute (e g., hormone, enzyme or antibody) with an immobilized (or particulate) receptor Since the interaction may also be influenced by the presence of a small metabolite, we retain the designation of A and S for the f-valent macromolecule and univalent ligand, respectively, and denote matrix sites (receptor sites) by X Such action obviates the need for a change in terminology on extension of a study of (say) the interaction between lysozyme and a cell wall preparation (in which the lysozyme could be regarded as a ligand) to include examination of the effect of N-acetylglucosamine on that interaction. It also allows account to be taken of the fact that a macromolecular solute may not be univalent in its interaction with matrix (receptor) sites. For example, it is reasonable to consider the interactions of the four coenzyme-binding sites on lactate dehydrogenase (A) with NADH (S) in terms of eqns 4 and 6 with $f=4$ [5], but these expressions do not describe the binding of the same sites to Blue Sepharose [97–99] because of their failure to make allowance for the interaction of one enzyme molecule with more than one matrix site (X). By designating the f-valent macromolecular solute as A we are not merely devising terminology to cope with the additional reactant encountered in affinity chromatography, we are also drawing attention to the necessity of modifying the basic binding expressions (eqns 4 and 6) in instances where the ligand (small or large) is multivalent in its interactions with acceptor (receptor) sites

3 1 4 Ligand multivalency

Despite the inevitable multivalency of antibodies in their interactions with specific surface antigens, results are often presented in Scatchard [95] format, i e , in accordance with eqn. 6 A disadvantage of this course of action is that ligand multivalency introduces curvilinearity into the Scatchard plot that is of the same form as that for the binding of a univalent ligand to non-equivalent or negatively cooperative receptor sites [96,98,99] A general counterpart of the Scatchard analysis that can take into account the effect of ligand multivalency has evolved from theoretical expressions developed in the context of quantitative affinity chromatography [92,98].

For the interactions of an f-valent solute (ligand), A, with q-valent receptor (acceptor), X, the binding function should be defined [99] as

$$r_f = ([\bar{A}]^{1/f} - [A]^{1/f})/[\bar{X}] \quad (7)$$

from which it is evident that the Klotz [94] binding function (eqn 4) is, indeed, that for a univalent ligand Provided that a single intrinsic association constant, k_{AX}, governs all solute–acceptor interactions, the general counterpart of the Scatchard analysis becomes [99]

$$r_f/[A]^{1/f} = qk_{AX} - fk_{AX}r_f[\bar{A}]^{(f-1)/f} \quad (8)$$

A linear plot of $r_f/[A]^{1/f}$ versus $r_f/[\bar{A}]^{(f-1)/f}$ is thus the requirement for equivalence and independence of receptor sites for a ligand that is multivalent Those familiar with the traditional Scatchard analysis of binding data may at first query the presence of a term in total ligand concentration within the abscissa parameter However, on setting $f=1$ in eqn 8 the term in question becomes unity by virtue of the power (zero) to which $[\bar{A}]$ is raised

An obvious prerequisite for application of eqns 7 and 8 to binding data is the assignment of a magnitude to the ligand valence, f Although a degree of reticence in regard to this value is certainly understandable, it must be clearly understood that any attempt to avoid the issue by resort to a conventional Scatchard analysis merely means that the researcher has selected unity as the most appropriate valence To emphasize this point we consider results obtained in a partition equilibrium study of the binding of aldolase to rabbit muscle myofibrils [88], a system for which the interactions of enzyme with myofibrillar matrix and substrate are mutually exclusive (competitive) [100] Fig 2a presents a plot of those results (Table 1 of ref 88) that is obtained by traditional Scatchard analysis, a course of action that has, by default, assigned a value of unity to f Any attempted quantitative interpretation of the curvilinear plot would presumably be in terms of myofibrillar sites that are different and/or negatively cooperative in their interactions with enzyme On the other hand, Fig 2b presents the corresponding analysis of the results in accordance with the general counterpart of the Scatchard expression (eqn 8) and a value of 4 for f, on the grounds that aldolase is a tetrameric enzyme with four equiv-

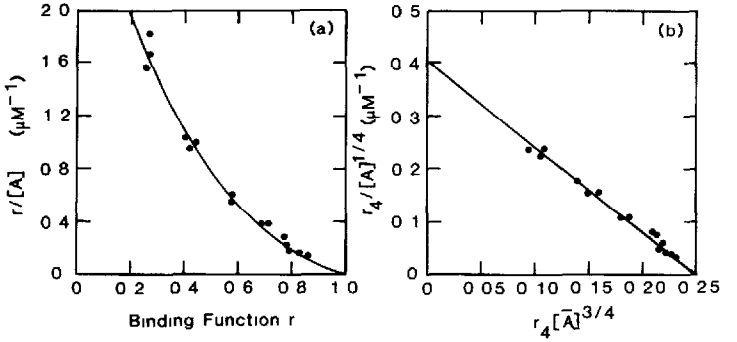


Fig 2 Effect of ligand multivalency on the analysis of binding data (a) Conventional Scatchard plot of partition equilibrium results (Table 1 of ref 88) for the interaction of aldolase with rabbit muscle myofibrils (b) Replot of the same results in accordance with eqn 8 and a value of 4 for f, the enzyme valence

alent and independent active sites [101]. The linearity of this plot and its conformity with the mandatory abscissa intercept ($1/f$) of 0.25 lead to the conclusion that an intrinsic association constant (k_{AX}) of 410 000 M^{-1} describes the binding of aldolase to a single class of matrix sites, a finding consistent with the highly ordered and regular nature of the myofibrillar matrix [102]

3 2 Gel chromatographic techniques

A major breakthrough in the development of chromatography as a means of studying biological interactions was the introduction of cross-linked dextran gels as chromatographic media [15]. For these gels and their subsequent molecular sieve counterparts chromatographic migration reflects a partition equilibrium that is (i) very rapidly established, (ii) dependent on molecular size and (iii) fairly insensitive to solute concentration These three characteristics have rendered gel chromatography an extremely versatile technique for studying a whole range of macromolecular interactions.

3 2 1 Solute self-association

Because of the relative independence of elution volume upon concentration for a non-interacting solute [103], concentration dependence of elution volume such as that observed in frontal gel chromatography of α-chymotrypsin [104] on a column of Sephadex G-100 (Fig 3a) must reflect variations in the proportions of monomeric and polymeric species with enzyme concentration as the result of reversible self-association Specifically, in frontal chromatography of an equilibrium mixture comprising monomer and single higher polymer ($n\mathrm{M} \rightleftarrows \mathrm{P}$) with total concentration $\bar{c}$, the measured elution volume, $\bar{V}$, is a weight-average quantity given by

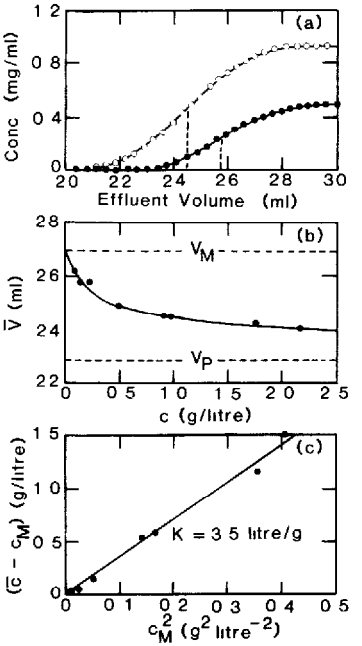


Fig 3 Characterization of α-chymotrypsin dimerization (pH 3 9, I 0 2) by frontal gel chromatography on Sephadex G-100 (a) Advancing elution profiles obtained with 0 50 mg/ml (●) and 0 93 mg/ml (○) enzyme solutions on a 32 cm×1 25 cm Sephadex column (b) Concentration dependence of the weight-average elution volume ($\bar{V}$) from a series of such experiments (data taken from Fig 5 of ref 104) (c) Analysis of the results from (b) by means of eqn 9 and the law of mass action for a monomer-dimer equilibrium

$$\bar{V} = [c_M V_M + (\bar{c} - c_M) V_P]/\bar{c} \tag{9a}$$

This expression enables the monomer concentration, c_M, to be evaluated, provided that estimates of the elution volumes of monomer, V_M, and polymer, V_P, are also available [105–107] For this purpose eqn 9a is rearranged to the form

$$c_M = \bar{c}(\bar{V} - V_P)/(V_M - V_P) \tag{9b}$$

Fig 3b presents further information on the concentration dependence of $\bar{V}$ for α-chymotrypsin [104], and Fig 3c the direct analysis of the results in terms of the law of mass action for a monomer–dimer equilibrium, namely, $c_P = (\bar{c} - c_M) = K' \ (c_M)^n$, where K' is the association constant expressed on a weight–concentration scale (l^{n-1} g^{1-n}) and $n = 2$; an alternative approach is to employ the same expression in logarithmic format [107] If required, the resulting equilibrium constant of 2 l/g that is obtained from the slope may be converted to the corresponding molar association constant, K, by means of the expression $K = [K' \ (M_M)^{n-1}]/n$, where M_M is the molecular weight of monomer

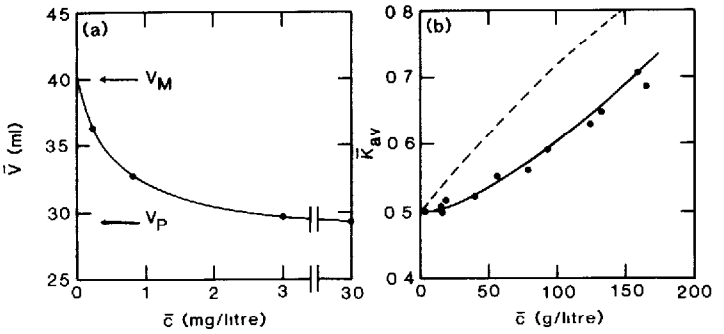


Fig 4 Versatility of frontal exclusion chromatography for the characterization of protein self-association (a) Demonstration of the dissociation of bovine aryl sulphatase at concentrations appropriate to enzymic assay, adapted with permission from ref 108 (b) Self-association of concentrated haemoglobin detected by exclusion chromatography on CPG-10-120A, the broken line is the predicted concentration dependence of the weight-average partition coefficient ($\bar{K}_{av}$) for a non-interacting $\alpha_2\beta_2$ entity, whereas the solid line has been calculated on the basis that the $\alpha_2\beta_2$ species undergoes dimerization governed by an association constant of 157 M^{-1} Adapted with permission from ref 112

Examples of self-associating systems that have been investigated by frontal gel chromatography include the enzymes α-chymotrypsin [104,106,107], thrombin [104] and arylsulphatase [108], the proteins haemoglobin [105] and β-lactoglobulin [109] and the drug chlorpromazine [110,111] Further information on the study of self-associating systems by gel chromatography is contained in an earlier review [84], which also considers the use of exclusion chromatography for studies of concentrated protein solutions [112–114] In such studies a changeover to porous glass beads is recommended to avoid the complications arising from osmotic shrinkage of a gel chromatographic medium [115–117]

Because of the availability of molecular sieving media with widely different porosities, frontal exclusion chromatography affords an extremely versatile means of studying solute self-association Furthermore, the only requirement for its application is a means of assaying the column effluent in an appropriate concentration range for detection of the equilibrium It may therefore be used to quantify self-association that is very strong, such as the monomer–tetramer equilibrium for arylsulphatase A (Fig 4a) – a system requiring solute detection by enzymic assay [108] – or self-association that is very weak, such as that of haemoglobin (Fig. 4b) – a system for which concentration dependence due to thermodynamic non-ideality outweighs that due to solute self-association [112,114]

3 2 2 Interactions between dissimilar macromolecules

In frontal gel chromatographic studies of rapidly reversible complex formation between dissimilar solutes ($A+B \rightleftarrows C$) the advancing and trailing elu-

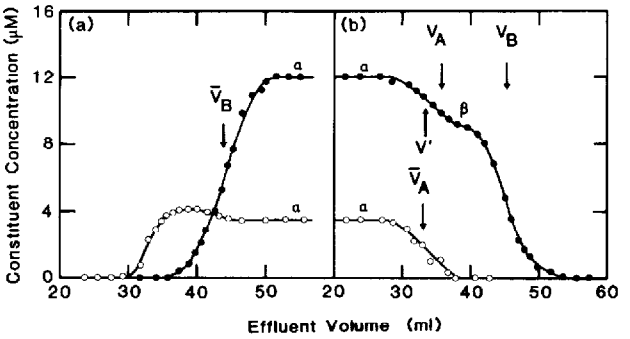


Fig 5 (a) Advancing and (b) trailing elution profiles obtained in frontal gel chromatography of a mixture of soybean trypsin inhibitor (3 5 μM) and cytochrome c (12 2 μM) on a column (32 cm × 1 5 cm) of Sephadex G-75 equilibrated with 0 01 I phosphate, pH 6 8 Open and solid symbols denote constituent concentrations of soybean trypsin inhibitor (A) and cytochrome c (B), respectively, and the arrows indicate elution volumes appropriate to the application of eqns 10 and 13 Adapted with permission from ref 121

tion profiles usually contain a reaction boundary that separates the plateau of original composition ($[\bar{A}]^{\alpha}$, $[\bar{B}]^{\alpha}$) from a second plateau corresponding to one or other of the individual reactants [118–121] For example, Fig. 5 presents such elution profiles for the two constituents in a study on Sephadex G-75 of the electrostatic interaction between soybean trypsin inhibitor (A) and cytochrome c (B), pH 6.8, I 0 01 [121] Neither pure solute boundary corresponds to its equilibrium concentration in the applied mixture; but the concentration of cytochrome c in the pure solute phase (β-phase) of the trailing elution profile ($[B]^{\beta}$) may be used to determine $[A]^{\alpha}$, the equilibrium concentration of soybean trypsin inhibitor in the applied mixture, via the expression [82,118,119,122,123]

$$[A]^{\alpha} = (V_B - V')([\bar{A}]^{\alpha} - [\bar{B}]^{\alpha} + [B]^{\beta})/(V_B - V_A) \tag{10}$$

where V' denotes the median bisector of the gradient in constituent concentration of B across the $\alpha\beta$ boundary. The magnitude of the association equilibrium constant may then be calculated as

$$K_{AB} = ([\bar{A}]^{\alpha} - [A]^{\alpha})/([\bar{B}]^{\alpha} - [\bar{A}]^{\alpha} + [A]^{\alpha}) \tag{11}$$

Alternatively, provided that complex formation is restricted to 1 1 stoichiometry, the two constituent elution volumes, viz ,

$$\bar{V}_A = (V_A[A]^{\alpha} + V_C[C]^{\alpha})/[\bar{A}]^{\alpha} \tag{12a}$$

$$\bar{V}_B = (V_B[B]^{\alpha} + V_C[C]^{\alpha})/[\bar{B}]^{\alpha} \tag{12b}$$

may be deduced from the median bisectors of the appropriate reaction boundaries in Fig 5 and be combined with the constituent composition of the applied mixture to yield K via the relationship [120,121]

$$[B]^{\alpha}=\{[\bar{B}]^{\alpha}(\bar{V}_B-V_A)-[\bar{A}]^{\alpha}(\bar{V}_A-V_A)\}/(V_B-V_A) \quad (13)$$

Eqn. 13 should provide the more accurate estimate of K inasmuch as its application requires the estimation of only two parameters ($\bar{V}_A$, $\bar{V}_B$) in addition to the elution volumes of individual reactants (V_A, V_B), whereas the use of eqn 10 requires a value of $[B]^{\beta}$, the concentration of the pure solute phase (cytochrome *c*) in the trailing elution profile Nevertheless, it is eqn. 10 that has given the biggest boost to the determination of equilibrium constants by gel chromatography In the particular circumstance that complex and faster reactant comigrate ($V_C=V_A$) the constituent and species elution volumes of A become identical ($\bar{V}_A=V_A$), a situation which ensures the identity of V_A and V' Eqn 10 then simplifies to

$$[A]^{\alpha}=[\bar{A}]^{\alpha}-[\bar{B}]^{\alpha}+[B]^{\beta} \quad (14)$$

a condition which signifies the identity of $[B]^{\alpha}$ and $[B]^{\beta}$ Thus, by employing a gel medium that excludes the larger reactant (A) and hence C, the equilibrium concentration of the smaller reactant is given directly by the concentration of B that separates as the pure solute phase in the trailing elution profile – irrespective of the valences of either reactant.

An advantage of molecular-sieve chromatography for studying macromolecular interactions is the relative ease with which this combination of elution volumes ($V_C=V_A<V_B$) may be achieved by appropriate selection of matrix. This simplified approach has been used, for example, to quantify the interaction of a lectin, con A, with Dextran T2000 by frontal exclusion chromatography on Glyceryl-CPG-170 porous glass beads [99]. Although gel chromatography thus has considerable potential for the characterization of interactions between macromolecules, its major application to date has been as a rapid alternative to equilibrium dialysis for studies of the binding of small ligands to macromolecules [5,124–126]

3 2 3 Studies of ligand binding

In biological systems the binding of a ligand to a macromolecular acceptor is an extremely common event that can have pronounced effects upon the functional state of the macromolecules and also upon the entire physiological system Equilibrium dialysis is the classical method for investigating such interactions, but, as noted above, gel chromatography also has the potential for direct measurement of the equilibrium concentration of ligand, $[S]^{\alpha}$, provided that acceptor (A) and all acceptor–ligand complexes $[AS_i]$ co-migrate [5,124–126] To create the plateau of original composition, a mixture of acceptor and ligand is added to the column until the emerging effluent has the composition of the solution being applied Elution of the column with buffer then generates a trailing elution profile such as that shown in Fig 6a for a mixture of sulphamethoxypyridazine and albumin [124] that had been predialyzed to establish

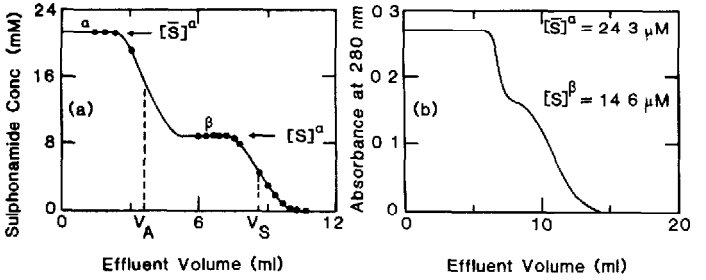


Fig 6 Quantitative studies of ligand binding by frontal exclusion chromatography (a) Trailing ligand elution profile deduced from Fig 2 and Table 1 of ref 124 for a mixture of serum albumin (14 m*M*) and sulphamethoxypyridazine (20 m*M*) on a column (30 cm×0 5 cm) of Sephadex G-25, $[S]^{\alpha}$ denotes the estimate of the free sulphonamide concentration obtained by equilibrium dialysis (b) Trailing ligand elution profile obtained in frontal chromatography of a mixture of Dextran T2000 (1 mg/ml) and concanavalin A (1 24 mg/ml) on Glyceryl-CPG 170 [99]

the equilibrium concentration of the drug. Clearly, $[S]^{\beta}$ provides a direct estimate of $[S]^{\alpha}$. Moreover, the rapidity with which equilibrium binding results may be acquired is a decided asset of the gel chromatographic technique. For example, a period of 2 h elapsed between the commencement of sample application and complete elution of the sulphonamide drug in Fig 6a; and an even shorter time period (40 min) was required for the experiment reported in Fig 6b on the interaction of con A with Dextran T2000 [99]. Further curtailment of the time factor, and also of the amount of equilibrium mixture required, can readily be achieved by resort to smaller columns and the use of more sophisticated means of effluent scanning

3 2 4 The Hummel and Dreyer technique

A disadvantage of the frontal gel chromatographic technique for studying ligand binding is the relatively large amount of acceptor–ligand mixture (at least one column volume) required to create the plateau of original composition Consequently, greater popularity has been accorded the Hummel and Dreyer procedure [127], which involves application of a small zone of acceptor to a column pre-equilibrated with a known concentration of ligand, $[S]_p$. In the resultant elution profile, shown schematically in Fig 7, the increase in constituent concentration of ligand ($[\bar{S}]$) coincident with elution of acceptor at $\bar{V}_A$ reflects the binding of ligand, whereupon it follows that the amount of ligand bound may be calculated by trapezoidal integration to find the area of this peak Since complex formation has been achieved at the expense of the pre-equilibrating ligand concentration, the elution profile necessarily exhibits a negative peak at V_S, the elution volume of ligand Furthermore, considerations of mass conservation show that the amount of S defined by the area of this valley must also correspond to the amount of complexed ligand The Klotz binding function, r, may therefore be determined as the amount of ligand bound

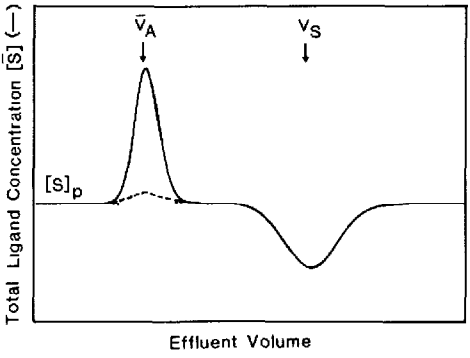


Fig 7 Schematic representation of an elution profile obtained in ligand-binding studies by the Hummel-Dryer procedure [127] The solid line denotes the constituent concentration of ligand in zonal chromatography of acceptor (A) on a column pre-equilibrated with a concentration $[S]_p$ of ligand The dotted line indicates the variation of free ligand concentration within the acceptor zone in situations where $V_A < V_{AS} < V_S$, whereas the broken line refers to systems with $V_{AS} < V_A < V_S$ [129]

divided by the total amount of acceptor applied to the column Greater economy in regard to the amount of acceptor (though not of ligand [124]) may well be an advantage of this technique, but the required trapezoidal integration places stringent demands on the accuracy with which ligand concentrations are measured and also on the accuracy with which the volume scale of the elution profile is defined As noted by Colman [128], the latter disadvantage is obviated in instances where the availability of separate assay procedures for acceptor and ligand allows the elution profile to be defined in terms of both constituent concentrations ($[\bar{A}]_V$, $[\bar{S}]_V$) as a function of effluent volume V A value of the binding function, r, is then obtained by substituting this pair of concentrations for any given volume V within the acceptor zone in the expression

$$r = ([\bar{S}]_V - [S]_p)/[\bar{A}]_V \qquad (15)$$

Since frontal gel chromatography of an acceptor–ligand mixture only provides a direct measure of the free (and hence bound) ligand concentration in instances where acceptor and complexes AS_i co-migrate, it has been argued [96] that the identity of V_A and V_{AS} must also be an inherent assumption in the evaluation of binding data by the Hummel–Dreyer procedure [127] In that regard most applications of the technique have entailed studies of protein–ligand interactions on gels such as Sephadex G-25 (or G-50) and Bio-Gel P-2, the exclusion of protein and all complexes from which ensures the validity of this implicit assumption However, its validity does not extend to the interpretation of Hummel–Dreyer elution profiles obtained in gel chromatographic studies of nucleotide–metal ion interactions on Sephadex G-10 [128], a me-

dium for which $V_A < V_{AS} < V_S$ [129] This difference between the elution volumes of nucleotide (V_A) and complex renders invalid the substitution of $[S]_p$, the pre-equilibrating ligand concentration for [S] in eqn. 15. By numerical simulation of the Hummel–Dreyer elution profiles for the ATP–Mg^{2+} system on Sephadex G-10 it has been shown [129,130] that the free ligand concentration within the acceptor zone is less than $[S]_p$ for this combination of elution volumes (dotted curve in Fig. 7), whereupon values of the binding function based on eqn 15 (or on the area of the ligand peak or valley) underestimate the true value. In similar vein, faster migration of the complex ($V_{AS} < V_A < V_S$) gives rise to a local peak in ligand concentration within the acceptor zone (dashed curve in Fig. 7) and hence overestimation of r by eqn 15 [129,130]

3 3 Electrophoretic methods

As a mass migration procedure, electrophoresis shares with sedimentation velocity and gel chromatography the ability, in principle, to yield quantitative information on interactions between dissimilar molecules [82–84] and is complementary in the sense that migration is a function of charge density (charge/size ratio) rather than of size and shape Unfortunately, the virtual disappearance of the U-tube assembly [85] has deprived electrophoresis of its best mode of attack on the problem of characterizing interactions – moving boundary electrophoresis. Consideration is therefore restricted to the use of current zonal electrophoresis techniques, some of which can be adapted for characterization of a limited range of interactions However, it transpires that equilibrium constants are usually obtained more readily and/or with greater accuracy by the gel chromatographic procedures already described or by the affinity chromatographic techniques that conclude this section on the quantification of biospecific phenomena.

3 3 1 Gel electrophoretic studies of ligand binding

The fact that gel electrophoresis is a zonal technique severely limits its adoption as a method for studying rapid, reversible interactions because of the restraints imposed by the need to minimize chemical re-equilibration as the result of the differential migration of complex(es) and reactants. In that regard we note that the standard gel electrophoretic method for the quantitative analysis of specific protein–DNA interactions [131] neglects any such re-equilibration and that the reliability of results so obtained is conditional upon the validity of the assumption that no dissociation of protein–DNA complex occurs within the time frame of the gel electrophoretic experiment In studies of ligand binding the need for making this assumption can be obviated by resort to methodology akin to the Hummel–Dreyer gel chromatographic procedure [127], wherein the net result of electrophoresis is a zone of acceptor migrating in a region of gel with a fixed concentration of ligand, $[S]_p$. In the method of

counter-ion electrophoresis [132], developed to quantify the binding of Ca^{2+} to negatively charged calcium-binding proteins, this situation was effected by adding calcium chloride to the anodic chamber reservoir and applying the protein sample to the cathodic end of the gel Since electrophoresis of the system eventually led to a steady state in which the Ca^{2+} was uniformly distributed along the gel in front of and behind the migrating zone, the same situation could have been achieved by pre-equilibration of the gel with a concentration $[S]_p$ of ligand prior to application of the protein zone The latter technique has been used in a gel electrophoretic study of the interaction between phosphate and ovalbumin [133]

As well as differing in the means used to effect gel electrophoresis of acceptor in a fixed ligand environment, the two investigations also employed different approaches to the problem of quantifying ligand binding By including radiolabelled ligand ($[^{45}Ca]$) in the calcium chloride, Ueng and Bronner [132] generated a gel electropherogram that was the exact counterpart of a Hummel–Dreyer [127] gel chromatographic elution profile (Fig 7) Trapezoidal integration was then used to obtain the amount of ligand in the peak of radioactivity co-migrating with the protein zone, and hence to obtain an apparent binding function – in complete conformity with the gel chromatographic procedure already described On the other hand, the binding of phosphate to ovalbumin was quantified by the change in mobility resulting from complex formation [133] On the basis of the Smith and Briggs [134] approximation that each successive addition of a charged ligand to the protein should give rise to a constant incremental change in acceptor mobility, the constituent mobility, $\bar{v}_A$, is related to its mobility in the absence of ligand, v_A, by the expression [133,135]

$$(\bar{v}_A - v_A)/v_A = f\delta k_{AS}[S]/(1 + k_{AS}[S]) \qquad (16)$$

in which δ is the incremental change in mobility expressed as a function of the mobility of free A in a gel chromatographic context $\bar{v}_A$ and v_A may be replaced by $\bar{R}_f$ and R_f, the corresponding mobilities expressed relative to that of bromophenol blue [133] Although such consideration of the dependence of $\bar{R}_f$ upon $[S]_p$ in terms of a rectangular hyperbola (eqn 16) provides a value of the intrinsic binding constant, k_{AS}, the second parameter evaluated by this means ($f\delta$) does not yield the number of binding sites on acceptor, except by recourse to rationalization of the maximal mobility change ($v_A f\delta$) in terms of a model of electrophoretic migration [136] to assess the charge difference between A and AS_f, and hence f on the basis of the charge borne by S

The major objection to both of these procedures is their reliance upon identification of the ligand concentration in the acceptor-free region ($[S]_p$) as the equilibrium concentration within the acceptor zone [130] The invalidity of this assumption is evident from Fig 7, which presents the analogous zonal gel chromatographic profile in situations where $V_{AS} \neq V_A$ In electrophoresis the

mobility of the complex is likely to be intermediate between those of acceptor and ligand, and the dotted line in Fig. 7 is therefore the appropriate representation of free ligand concentration within the migrating acceptor zone Failure to take into account the diminished concentration of free S within the acceptor zone clearly leads to underestimation of the binding function by trapezoidal integration of the associated peak in total ligand concentration In similar vein, the measured constituent mobility of the acceptor zone ($\bar{v}_A$ or $\bar{R}_f$) is governed by a lower concentration of ligand than $[S]_p$ Cann and Fink [130] have noted that a steady-state binding function (r_{ss}) pertaining unequivocally to $[S]_p$ may be obtained by extrapolating the measured values to zero acceptor concentration and in that regard a similar extrapolation of $\bar{R}_f$ would be required to obtain the appropriate value for substitution in eqn. 16. In counter-ion electrophoresis, however, the suggested extrapolation of the measured binding constant to zero acceptor concentration does not conclude the difficulties confronting the experimenter, who is still faced with the problem of converting the consequent steady-state binding constant to an equilibrium constant [130]

The logical conclusion to be drawn from this discussion is that gel electrophoresis is not the method of choice for studies of ligand binding because of the difficulties created by non-identity of the mobilities of acceptor and acceptor–ligand complexes Although counter-ion electrophoresis has afforded a convenient means of identifying two calcium-binding proteins in rat mucosal scrapings [132], it is inferior to gel chromatography for the quantitative characterization of their interactions with Ca^{2+} Gel chromatography would not, however, have provided any information on the ovalbumin–phosphate system because of the extremely high phosphate concentrations (1–7 mM) required to effect this interaction [133,135] For characterization of such weak interactions it is imperative that attention be switched from determinations of bound ligand based on differences between total and free ligand concentrations to determinations based on the consequent changes in an acceptor parameter In that regard electrophoretic mobility certainly fulfils that role if the ligand is charged, but it transpires that for these systems electrophoresis is again supplanted by another chromatographic technique (affinity chromatography) as the method of choice

3 3 2 Affinity electrophoresis

The technique of affinity electrophoresis in a polyacrylamide gel was introduced by Takeo and Nakamura [80] in a quantitative study of the interaction between phosphorylase and glycogen, a reactant (ligand) sufficiently large to justify the approximation being made that the uniform concentration of glycogen established within the gel during its preparation prevailed throughout electrophoresis of the phosphorylase because of inability of the polysaccharide to migrate through the gel pores Since interaction of the enzyme with glycogen thus resulted in the formation of complexes with zero velocity, the constituent

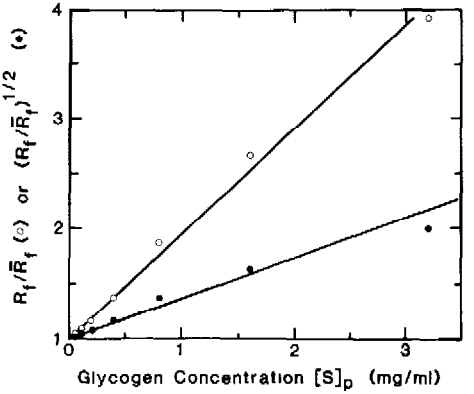


Fig 8 Quantitative characterization of the interaction between muscle phosphorylase and glycogen by affinity electrophoresis Closed and open symbols refer to plots of the experimental data (Fig 1 of ref 80) in accordance with eqns 17a and 17b, respectively

mobility of the enzyme, $\bar{v}_A$, or the corresponding relative mobility parameter, $\bar{R}_f$, reflected the proportion of uncomplexed enzyme and thus decreased with increasing glycogen concentration On the basis that the tissue extracts were from resting muscle, the enzyme would have been predominantly phosphorylase *b* and hence dimeric in its interaction with glycogen, S From the definition of the constituent mobility of enzyme for such a system, viz, $\bar{v}_A = \Sigma(v_{AS_i}[AS_i])/\Sigma[AS_i]$, $0 \leq i \leq 2$, it follows that the intrinsic binding constant, k_{AS}, for the interaction of glycogen with two equivalent and independent binding sites on phosphorylase *b* may be evaluated via the expression

$$(v_A/\bar{v}_A)^{1/2} = (R_f/\bar{R}_f)^{1/2} = 1 + k_{AS}[S]_p \qquad (17a)$$

where $[S]_p$ denotes the pre-equilibrating concentration of glycogen Alternatively, if the viewpoint is adopted that formation of the complex AS_2 is precluded on steric grounds, the corresponding expression is

$$v_A/\bar{v}_A = R_f/\bar{R}_f = 1 + 2k_{AS}[S]_p \qquad (17b)$$

Results reported in the caption to Fig. 4 of ref 80 for the muscle phosphorylase–glycogen system are presented in Fig. 8, where closed and open symbols denote the plots in accordance with eqns 17a and 17b, respectively On the basis of the former an intrinsic association constant of 0 36 ($\pm$0.14) l/g is obtained from the slope of the line joining the mandatory ordinate intercept of unity to the mean of the experimental points. Uncertainty about the feasibility of forming the AS_2 complex has no significant effect on the magnitude of the intrinsic constant evaluated, since similar treatment of the results plotted in accordance with eqn 17b yields a k_{AS} of 0.45 ($\pm$0 18) l/g In that regard the association constant of 0 9 l/g obtained by Takeo and Nakamura [80] from a comparable analysis is to be recognized as the stoichiometric constant

($K_{AS}=2k_{AS}$), since no allowance was made for the fact that there were two ways of forming a 1 1 complex between glycogen and phosphorylase *b*. The relatively large uncertainty in the k_{AS} value deduced from either plot reflects its curvilinearity, which is almost certainly due to the failure of eqn 17 to make allowance for the tetrameric nature of the small amount (10–15%) of phosphorylase *a* that is present in resting muscle.

A more conventional form of quantitative affinity electrophoresis is to immobilize a ligand, X, that interacts with electrophoretically migrating solute, A, so that competition between X and a small, uncharged ligand, S, may be examined by electrophoresis on gels pre-equilibrated with S [137] Specifically, the interactions between lectins and various sugars were studied by affinity electrophoresis on polyacrylamide gels containing immobilized sugar residues, a technique which again provided an experimental measurement of the constituent mobility of A as a function of pre-equilibrating ligand concentration, $[S]_p$. For these systems, which also involved the electrophoretic migration of a dimeric (divalent) solute, A, the expressions analogous to eqns. 17a and 17b are

$$(v_A\bar{v}_A)^{1/2}=(R_f/\bar{R}_f)^{1/2}=1+k_{AX}[X]_p+k_{AS}[S]_p \tag{18a}$$

$$v_A/\bar{v}_A=R_f/\bar{R}_f=(1+k_{AS}[S]_p)^2+2k_{AX}[X]_p \tag{18b}$$

where k_{AX} denotes the intrinsic association constant for the interaction of acceptor with immobilized ligand, present at concentration $[X]_p$. Eqn. 18b is based on the premise that the formation of AX_2 but not AS_2 is sterically precluded it is noted that the predicted non-linear dependence of $R_f/\bar{R}_f$ upon $[S]_p$ is at variance with the original analysis [137], which was based on univalence of the lectin in its interactions with both S and X

Subsequent considerations of the theoretical aspects of affinity electrophoresis [138,139] drew attention to the large number of assumptions inherent in the original quantitative analysis. Assumptions of particular concern included:

(i) The need to consider that complex formation with soluble ligand has no effect on the mobility of acceptor – a requirement that immediately restricts the application of affinity electrophoresis to studies with uncharged ligands

(ii) The requirement that the solute (A) be univalent – an assumption rectified by eqns 17 and 18

(iii) The presumption that the chemical interactions of A with S and X occur at a sufficiently rapid rate for equilibrium to prevail throughout electrophoretic migration – an entirely reasonable proposition for many reactions on the time scale of electrophoresis

(iv) The approximation that the free concentrations of immobilized ligand, [X], and soluble ligand, [S], are given with sufficient accuracy by the predetermined constituent concentrations $[X]_p$ and $[S]_p$ Theory developed to take into account some of these problems [138,139] is, in fact, covered in the

treatment of quantitative affinity chromatography, an analogous technique with far greater versatility than its electrophoretic counterpart from the viewpoint of potential applications to biospecific phenomena

3 4 Quantitative affinity chromatography

Introduced [140] at a stage when the power of affinity chromatography as a method of solute purification was well established, the quantitative adaptation of the technique was originally envisaged as a means of taking additional advantage of a chromatographic matrix developed for isolation of a solute on the basis of its biospecificity. Whereas the function of the immobilized ligand in preparative affinity chromatography is its selective interaction with a particular solute, its role in quantitative affinity chromatography is to provide competition for the ligand whose biospecificity it is mimicing, and thereby a means of quantitatively characterizing the biospecific interaction In this context affinity chromatography has been used to evaluate many equilibria involving enzyme interactions with modifiers, inhibitors or substrates [23,25,97,98,140–147], and also to study protein–drug interactions [29,148], protein–protein interactions [149–151], hormonal interactions [152–154] and antibody–antigen systems [155–158]. A shift in methodology from column chromatography to partition equilibrium studies [23,88] has led to a vast increase in the scope of the technique, the theory of which is also central to investigations of metabolite-dependent changes in the subcellular distribution of enzymes [88,159–164] and also to the use of standard RIA and ELISA procedures for quantitative characterization of immunochemical interactions [156–158]

3 4 1 Studies of ligand binding

In its chromatographic context quantitative affinity chromatography entails immobilization of a biospecific reactant group, X, on a matrix (often Sepharose) and measurement of the weight-average elution volume [26], $\bar{V}_A$, of the partitioning solute (A) in a series of experiments in which solute migrates in the presence of different concentrations [S] of ligand S that interacts specifically with A or X In most studies so far reported the dependence of $\bar{V}_A$ upon [S] has reflected either the interaction of immobilized reactant X with binary AS complex [25,140] or competition between ligand and immobilized reactant for the same A sites (e.g refs. 23, 89 and 141–148). An example of the latter type of interaction is illustrated in Fig 9a, which shows the effect of methyl-α-D-glucoside on the elution volume of con A in frontal chromatographic experiments on Sephadex G-50, the biospecific elution in this system reflects competition between the glycoside (S) and the anhydroglucose polymer chain of the Sephadex (X) for the two carbohydrate-binding sites on con A The aim of quantitative affinity chromatography is to interpret these variations in $\bar{V}_A$

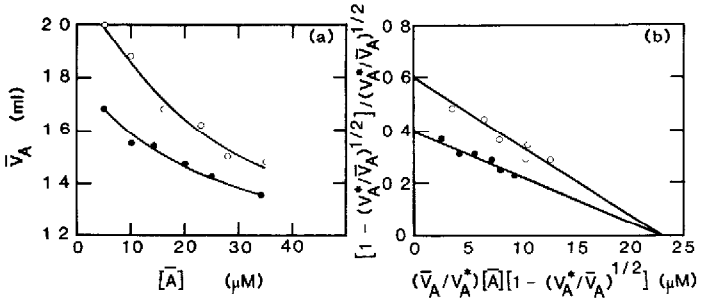


Fig 9 Characterization of the interaction between methyl-α-D-glucoside and concanavalin A by affinity chromatography on Sephadex G-50 [165] (a) Concentration dependence of the weight-average elution volume of concanavalin A on a column of Sephadex G-50 in the absence (○) and presence (●) of ligand (0 1 m*M*) (b) Replot of those results in accordance with eqn 19

in terms of the operative equilibria, frontal chromatographic data being preferred for precise delineation of the reaction mixture to which $\bar{V}_A$ refers

For a system involving competition between ligand and matrix groups for A, the affinity chromatographic behaviour of an *f*-valent solute is most conveniently expressed in the form [92,98]

$$\frac{[1-(V_A^*/\bar{V}_A)^{1/f}]}{(V_A^*/\bar{V}_A)^{1/f}}=\frac{k_{AX}[\bar{X}]}{1+k_{AS}[S]}-\frac{fk_{AX}(\bar{V}_A/V_A^*)[\bar{A}]\{1-(V_A^*/\bar{V}_A)^{1/f}\}}{1+k_{AS}[S]} \qquad (19)$$

where $\bar{V}_A$ denotes the elution volume of solute in a frontal experiment with total concentration [Ā] of solute and free (equilibrium) concentration [S] of ligand, V_A^*, the elution volume of A in the absence of any solute–matrix interaction, is clearly the void volume for con A on Sephadex G-50. Although $[\bar{X}]$ refers to the effective total concentration of matrix sites, the validity of eqn. 19 is not reliant upon the assumption, inherent in the earlier electrophoretic analyses, that this concentration approximates the free concentration of matrix sites. At a fixed value of free ligand concentration [S], realizable by prior dialysis of solute against ligand [26,28], a plot of $[1-(V_A^*/\bar{V}_A)^{1/f}]/(V_A^*/\bar{V}_A)^{1/f}$ versus $(\bar{V}_A/V_A^*)[\bar{A}][1-(V_A^*/\bar{V}_A)^{1/f}]$ from experiments with different total solute concentration should thus be linear, with a slope of $fk_{AX}/(1+k_{AS}[S])$ and an ordinate intercept of $k_{AX}[\bar{X}]/(1+k_{AS}[S])$ Fig 9b employs this format to analyze the results (Fig 9a) for the Sephadex-concanavalin system in the absence (open circles) and presence (closed circles) of methylglucoside (0 1 m*M*) The first point to note is the essential linearity of both plots, which suggests the adequacy of a single intrinsic association constant to describe the formation of AX_2 as well as of AX Secondly, comparison of either the slopes or the ordinate intercepts of these two plots leads to a value of 5000 M^{-1} for k_{AS}, which is essentially the value obtained previously by this method [165] and by equilibrium dialysis [166] The consequent values of 13 000 M^{-1} for k_{AX} and 46 μM for [X] have no absolute

significance because they are effective magnitudes of parameters defined, for convenience, in terms of a system in which matrix sites are distributed uniformly throughout the volume accessible to solute [165]

The partition equilibrium technique [26,88] provides a second situation devoid of ambiguity about the reaction mixture to which an experimental measurement refers In this case the expression analogous to eqn. 19 is [92,98]

$$\frac{1-([\bar{A}]/[\bar{\bar{A}}])^{1/f}}{([\bar{A}]/[\bar{\bar{A}}])^{1/f}}=\frac{k_{AX}[\bar{\bar{X}}]}{1+k_{AS}[S]}-\frac{fk_{AX}[\bar{A}]\{1-([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}}{1+k_{AS}[S]} \qquad (20)$$

where $[\bar{A}]$ continues to define the constituent concentration of partitioning solute in the liquid phase and $[\bar{\bar{A}}]$, the corresponding total solute concentration, is inferred from the total amount of solute added and the volume ($V_{\bar{A}}^{*}$) to which it has access Fig 10 summarizes, in appropriate format, the results of a partition equilibrium study of the effect of phosphate on the interaction of aldolase with myofibrils [88] Comparison of this plot for the phosphate-free system (open circles) with Fig 2b reveals identity of the two, and hence the fact that Figs 9b and 10 are plots of binding data in the generalized Scatchard format [99]. From the effect of phosphate concentration on either the slopes or the ordinate intercepts of Fig. 10, a value of 400 M^{-1} is obtained for k_{AS} Whereas these results emphasize the merits of quantitative affinity chromatography for characterizing interactions that are too weak for study by methods such as equilibrium dialysis or frontal gel chromatography (Section 3 2 3.), attention has also been drawn to the possible use of affinity chromatography for characterizing interactions at the other end of the energy spectrum, viz., those that are too strong for study by conventional means [26,28,29]

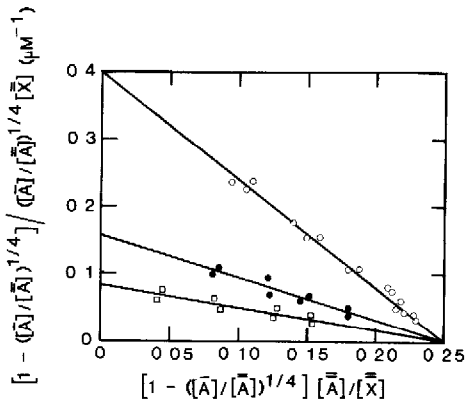


Fig 10 Characterization of the interaction between phosphate and aldolase by means of the effect of the ligand on the binding of enzyme to rabbit muscle myofibrils [88] Experimental points refer to partition equilibrium studies conducted in a phosphate-free environment (○) and in the presence of 4 mM (●) and 10 mM (□) phosphate

Ligand-facilitated elution of the partitioning solute could, of course, reflect competition between A and S for the same matrix sites, a situation for which the expression analogous to eqn 20 is [167]

$$\frac{1-([\bar{A}]/[\bar{\bar{A}}])^{1/f}}{([\bar{A}]/[\bar{\bar{A}}])^{1/f}}=\frac{k_{AX}[\bar{\bar{X}}]}{1+k_{XS}[S]}-\frac{fk_{AX}[\bar{A}]\{1-([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}}{1+k_{XS}[S]} \qquad (21)$$

where k_{XS} is the intrinsic association constant for the ligand–matrix interaction and $[\bar{A}]$ (the total solute concentration in the liquid phase) is also its free concentration due to lack of interaction with S. From the formal identity of eqns. 20 and 21 it follows that procedural aspects of data collection and analysis for the two competitive systems are identical and that the investigator must decide whether the second binding constant determined is k_{AS} or k_{XS}

Ligand-retarded elution of the partitioning solute, on the other hand, signifies ternary complex formation, either through the interaction of AS with X or of XS with A. For the former situation, as exemplified in affinity chromatography of lactate dehydrogenase on oxamate-Sepharose [25,140], the expression analogous to eqns. 20 and 21 is [167]

$$\frac{1-([\bar{A}]/[\bar{\bar{A}}])^{1/f}}{([\bar{A}]/[\bar{\bar{A}}])^{1/f}}=\frac{k_{AS}k_T[S][\bar{\bar{X}}]}{1+k_{AS}[S]}-\frac{fk_{AS}k_T[S][\bar{A}]\{1-([\bar{A}]/[\bar{\bar{A}}])^{1/f}\}}{1+k_{AS}[S]} \qquad (22)$$

where k_T is the intrinsic association constant for ternary complex formation between AS and matrix sites, X. For these systems too, the same generalized Scatchard analysis is thus appropriate, and the magnitude of k_{AS} is again determinable via the ratio of either the slopes or the ordinate intercepts of plots obtained with two different fixed concentrations of free ligand On this occasion, however, zero is not an acceptable value of [S] for one of those plots.

In conventional methods of studying ligand binding the evaluation of k_{AS} is achieved by examining acceptor–ligand reaction mixtures in which the ligand concentration is varied, a situation which contrasts markedly with the above recommendation that in quantitative affinity chromatography it is better to fix the ligand concentration and vary that of partitioning solute However, it should also be pointed out that Kasai and Ishii [142] have obtained ligand-binding constants from a series of frontal affinity chromatographic experiments with a fixed concentration of partitioning solute and varying ligand concentration. It is therefore important to enquire whether experiments of that design might be more rewarding by virtue of the simpler protocol To pursue that objective we select ligand-facilitated elution due to competition between S and X for sites on A, the system for which eqn 19 is the quantitative expression

There is no rearrangement of eqn. 19 that allows unequivocal evaluation of k_{AS} from the variation in $\bar{V}_A$ as a function of [S] However, eqn 19 may be rearranged to the form

$$\frac{(V_A^*)^{1/f}k_{AX}[\bar{\bar{X}}]}{(\bar{V}_A)^{1/f}-(V_A^*)^{1/f}}-fk_{AX}[\bar{A}](\bar{V}_A/V_A^*)^{(f-1)/f}=1+k_{AS}[S] \tag{23}$$

which, for a univalent solute, simplifies [23] to

$$\frac{1}{\bar{V}_A-V_A^*}=\frac{k_{AS}[S]}{V_A^*k_{AX}[X]}+\frac{1+k_{AX}[\bar{A}]}{V_A^*k_{AX}[X]} \tag{24}$$

From the plot of $1/(\bar{V}_A-V_A^*)$ versus [S] that is suggested by eqn. 24, a value may be obtained for $k_{AS}/(1+k_{AX}[\bar{A}])$ as the ratio of the ordinate intercept to the slope. Only in the event that $k_{AX}[\bar{A}] \ll 1$ does this procedure provide a direct measure of the ligand-binding constant [23] As noted previously [26], the problem with this approach is uncertainty about the magnitude of k_{AX}, which may differ substantially from that of k_{AS} even in instances where the immobilized reactant and ligand are essentially identical entities For example, in the phosphate-facilitated elution of aldolase from cellulose phosphate $k_{AS}=350\ M^{-1}$ whereas $k_{AX}=50\ 000\ M^{-1}$ [92].

Another way of looking at this approximation inherent in the Kasai and Ishii approach [142] is to examine the source of the $k_{AX}[\bar{A}]$ term in eqn 24 It stems from the second term on the left-hand side of eqn. 23, which originates from the amendment of $[\bar{\bar{X}}]$ to obtain the free concentration, [X], for evaluating the equilibrium constant, k_{AX} [92] Neglect of this term, which thus amounts to adoption of the approximation $[X] \cong [\bar{\bar{X}}]$, leads to disappearance of the $k_{AX}[\bar{A}]$ term in eqn. 24. For a multivalent solute the corresponding expression is

$$\frac{1}{(\bar{V}_A)^{1/f}-(V_A^*)^{1/f}}=\frac{k_{AS}[S]}{(V_A^*)^{1/f}k_{AX}[\bar{\bar{X}}]}+\frac{1}{(V_A^*)^{1/f}k_{AX}[\bar{\bar{X}}]} \tag{25}$$

This approach is thus certainly justified in situations where $[\bar{A}] \ll [\bar{\bar{X}}]$, since the assumption $[X] \cong [\bar{\bar{X}}]$ that is inherent in eqns 24 and 25 then becomes an acceptable approximation, irrespective of the magnitude of k_{AX}. This method is thus on par with the affinity electrophoretic methods [80,137], both of which employed the same approximation.

3 4 2 Zonal affinity chromatography

If eqn 24 or eqn. 25 is to be used for the analysis of data reflecting ligand-facilitated elution of solute from an affinity matrix, very little is gained by resorting to frontal chromatography, the only possible advantage of which would be the better delineation of very large elution volumes [26] These two quantitative expressions may also be used to analyze the dependence of $\bar{V}_A$ upon [S] determined by zonal affinity chromatography of solute on a column pre-equilibrated with ligand [141,143,145] In experiments of this design the absence of a clearly defined value for $[\bar{A}]$ obviously leaves the investigator with

no option but to assume that $[X] \cong [\bar{\bar{X}}]$. Although use of the zonal technique is strongly recommended [27,168,169], any result so obtained is conditional upon that assumption, and accordingly confirmation of the magnitude of k_{AS} has usually been sought by its comparison with values obtained by other methods. It is clearly preferable to perform the analysis under conditions such that the validity of this assumption is not open to question, whereupon the validity of the result is not conditional upon its verification by other means.

An obvious way to guarantee validity of the approximation that $[X] \cong [\bar{\bar{X}}]$ is to employ an affinity matrix with a very large concentration of immobilized reactant residues. Such affinity matrices are commonly used for solute purification, and the only factor mitigating against their use for characterizing solute–ligand interactions seems to be the assertion (e g , ref. 170) that preparative affinity columns are unsuitable for quantitative assessment of solute–ligand binding constants. This assertion, which clearly contradicts the present inference that a preparative affinity column should be the matrix of choice for zonal affinity chromatographic studies, can be traced to the manner in which the dependence of $\bar{V}_A$ upon ligand concentration has been analyzed.

The difficulty resided in the fact that eqn 25, with $f=1$, was the expression being used for evaluation of k_{AS} as the ratio of the slope to the ordinate intercept obtained in a plot of $1/(\bar{V}_A - V_A^*)$ versus $[S]$ Since a large value of $[\bar{\bar{X}}]$ resulted in the ordinate intercept being indistinguishable from zero, it was recommended that a lower concentration of immobilized reactant be employed to allow better delineation of the ordinate intercept. Such action certainly increases the precision with which the magnitude of k_{AS} may be determined by the application of eqn 25 to experimental data, but it also increases the chances that eqn. 25 is not a valid approximation of the complete expression (eqn. 23) The solution to this dilemma is not to repeat the experiment with a lower value of $[\bar{\bar{X}}]$, but rather to rearrange eqn 25 as [28]

$$(\bar{V}_A)^{1/f} - (V_A^*)^{1/f} = (V_A^*)^{1/f} k_{AX} [\bar{\bar{X}}] - k_{AS}\{(\bar{V}_A)^{1/f} - (V_A^*)^{1/f}\}[S] \qquad (26)$$

which indicates that k_{AS} may be evaluated as the slope of a linear plot of $(\bar{V}_A)^{1/f} - (V_A^*)^{1/f}$ *versus* $\{(\bar{V}_A)^{1/f} - (V_A^*)^{1/f}\}[S]$. This prediction is verified in Fig. 11b, which presents the suggested replot of the 'uninterpretable' results [170] shown in Fig 11a for the *p*-aminobenzamidine-facilitated elution of trypsin (for which $f=1$) from *p*-aminobenzamidine-Sepharose.

By rendering possible the quantitative analysis of results obtained under conditions where the enforced assumption that $[X] \cong [\bar{\bar{X}}]$ is most likely to be a valid approximation, eqn 26 clearly achieves the breakthrough required for unequivocal characterization of ligand binding by zonal affinity chromatography From the experimental viewpoint the zonal technique has the undeniable attraction of being more economical in terms of solute requirements. In addition, the fact that this affinity chromatographic counterpart of the Hummel–Dreyer gel chromatographic procedure [127] generates a solute zone mi-

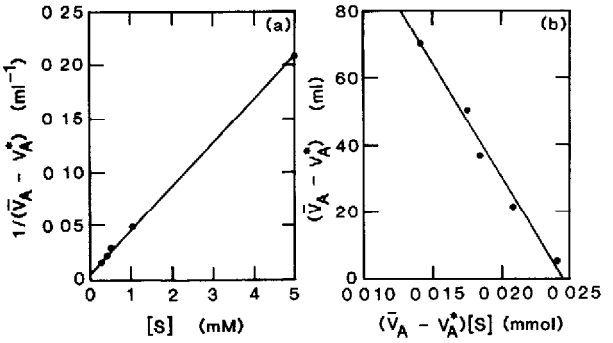


Fig 11 Quantitative evaluation of the interaction between *p*-aminobenzamidine and trypsin by zonal chromatography on *p*-aminobenzamidine-Sepharose (a) Results in the form originally presented [170], (b) their reassessment [28] in terms of eqn 26 Adapted with permission from refs 170 and 28, respectively

grating in the presence of a defined concentration of free ligand may be used to advantage in studies of interactions where solute and ligand are both macromolecular, a situation which precludes pre-establishment of [S] by dialysis. Selection of a matrix that neither solute nor ligand can penetrate ensures satisfaction of the requirement, inherent throughout quantitative affinity chromatography theory, that a common accessible volume (V_A^*) applies to A and all AS_i complexes, whereupon the only concern to be addressed in the application of eqn 25 is validity of the assumption that $[X] \cong [\bar{X}]$. The ovalbumin-facilitated elution of con A from Sephadex G-50 has been used to illustrate such use of zonal affinity chromatography for characterizing an interaction between macromolecules [151]

Finally, the ability of zonal affinity chromatography to provide information on the migration of solute in an environment with known concentration of free ligand may also be used to advantage in studies of biospecific interactions with markedly impure solute preparations, a feature demonstrated by an investigation involving the use of oxamate-Sepharose to characterize the separate interactions between NADH and all five lactate dehydrogenase isoenzymes present in a crude tissue extract [25]

3 4 3 Subunit-exchange chromatography

The specificity of monomer–monomer interactions in self-associating proteins has led to the development of subunit-exchange chromatography as a means of isolating and characterizing such proteins [171–173]. Briefly, the technique entails covalent linkage of protein monomer to a solid matrix to effect retardation of the protein during chromatography under conditions where monomeric and polymeric states coexist in self-association equilibrium From the quantitative viewpoint this is an unusual example of affinity chromato-

graphy in that the partitioning solute (protein) is also the competing ligand; but it is a competing ligand whose concentration cannot be varied independently of that of the partitioning solute. Theoretical expressions such as eqns. 19 and 20 do not therefore apply.

In subunit-exchange chromatography the two competing equilibria for a solute undergoing self-association to a single polymeric state are

$$n\mathrm{M} \rightleftarrows \mathrm{P}, \quad K = [\mathrm{P}]/[\mathrm{M}]^n \tag{27a}$$

$$(n-1)\mathrm{M} + \mathrm{X} \rightleftarrows \mathrm{XM}_{n-1}; \quad L = [\mathrm{XM}_{n-1}]/[\mathrm{X}][\mathrm{M}]^{n-1} \tag{27b}$$

where X continues to denote the immobilized reactant (monomer) and K and L refer to association constants for formation of soluble and immobilized n-mers, respectively

Subject to the proviso that the same accessible volume, $V_{\bar{A}}^*$, applies to monomeric and polymeric states of the solute, the quantitative expression describing solute retardation may be written [174]

$$[\bar{\bar{A}}]\left[\frac{(n-1)[\bar{\bar{X}}] - ([\bar{\bar{A}}] - [\bar{A}])}{[\bar{\bar{A}}] - [\bar{A}]}\right]^{1/(n-1)} = L^{-1/(n-1)} \tag{28}$$

$$+\{nK L^{-n/(n-1)}\}\left[\frac{[\bar{\bar{A}}] - [\bar{A}]}{(n-1)[\bar{\bar{X}}] - ([\bar{\bar{A}}] - [\bar{A}])}\right]$$

$[\bar{A}] = [\mathrm{M}] + n[\mathrm{P}]$ refers to the base-molar concentration of partitioning solute (weight concentration divided by monomeric molecular weight) in the liquid phase, whereas $[\bar{\bar{A}}]$ includes solute that has interacted with X This expression is in a form ready for direct application to results of partition equilibrium studies such as those reported for the light-harvesting a/b protein [175] It is also readily adapted to the frontal chromatographic situation by incorporating the mass conservation requirement that $[\bar{\bar{A}}] - [\bar{A}] = (\bar{V}_A - V_{\bar{A}}^*)[\bar{A}]/V_{\bar{A}}^*$, where $\bar{V}_A$ is the weight-average elution volume for solute present at a concentration $[\bar{A}]$ in the mobile phase; zonal data such as that reported by Swaisgood and Chaiken [154] for neurophysin self-association therefore remain quantitatively uninterpretable For a predetermined value of $[\bar{\bar{X}}]$, the total concentration of monomer covalently attached to the matrix, eqn. 28 signifies that the magnitudes of both association constants are obtainable by plotting $[\bar{A}][\{V_{\bar{A}}^*(n-1)[\bar{\bar{X}}] - (\bar{V}_A - V_{\bar{A}}^*)[\bar{A}]\}/(\bar{V}_A - V_{\bar{A}}^*)[\bar{A}]]^{1/(n-1)}$ versus $(\bar{V}_A - V_{\bar{A}}^*)[\bar{A}]/\{V_{\bar{A}}^*(n-1)[\bar{\bar{X}}] - (\bar{V}_A - V_{\bar{A}}^*)[\bar{A}]\}$, which has a slope of $nK\{L^{-n/(n-1)}\}$ and an ordinate intercept of $L^{-1/(n-1)}$.

In most quantitative applications of subunit-exchange chromatography $[\bar{\bar{X}}]$ has been deduced from the analytical composition of the matrix [171–173,175], but we prefer to retain this parameter as an operationally defined quantity. Fig. 12a presents, as classical binding data in double-reciprocal format [94], a replot [174] of results from an investigation of α-chymotrypsin dimerization

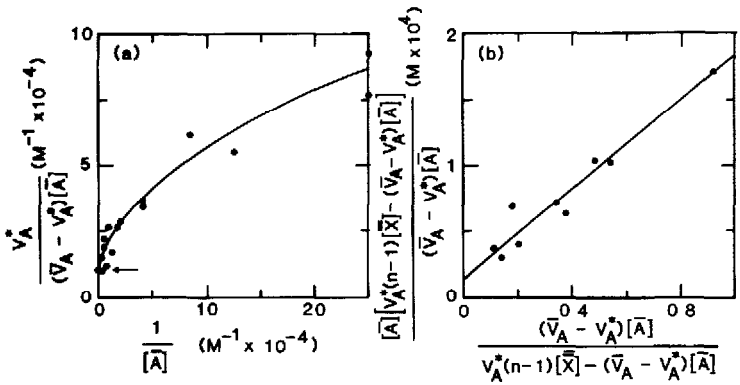


Fig 12 Characterization of α-chymotrypsin dimerization (pH 3 9, I 0 2) by subunit-exchange chromatography on Sepharose-linked α-chymotrypsin (a) Double-reciprocal plot of the concentration of enzyme bound as a function of its applied concentration [173] (b) Plot of those results in accordance with eqn 28 and the identity $([\bar{\bar{A}}]-[\bar{A}]) \equiv (\bar{V}_A - V_A^*)[\bar{A}]/V_A^*$ Adapted with permission from ref 174

by subunit-exchange chromatography [173] For this system there is clearly agreement between the effective concentration of covalently bound monomer, $[\bar{\bar{X}}]$, deducible from the reciprocal of the ordinate intercept, and the value of 108 mM based on the analytical composition (horizontal arrow in Fig. 12a). As predicted, the consequent plot of the same results in the form suggested by eqn 28 does yield a linear relationship (Fig. 12b), but the ordinate intercept $(1/L)$ is not defined with sufficient accuracy for unequivocal determination of the association constant for dimer formation with immobilized monomer However, combination of the slope, $2K/L^2$, with the value of 15 000 M^{-1} deduced for K from sedimentation equilibrium studies under comparable conditions [176,177] yields a value of 13 000 ($\pm$2000) M^{-1} for L [174]. Since immobilization of α-chymotrypsin has led to no significant effect on ability to form dimers, the original analysis of the results on the basis of such a premise [173] did yield a valid dimerization constant for the enzyme in solution

The analyses presented in Fig 12 have certainly pointed to the feasibility of using subunit-exchange chromatography for the characterization of protein self-association. However, that goal is only attained by considering the self-association characteristics of monomer to be unaffected by its covalent attachment to matrix, an assumption that may or may not be justified Clearly, subunit-exchange chromatography is not the procedure of choice for characterizing solute self-association, a phenomenon that is much better studied by frontal gel chromatography (Section 3.2 1)

3 4 4 Solid-phase immunoassays

Although solid-phase immunoassay procedures are routinely used for determining the concentrations of antigens in sera, very little attention has been

given to the possibility that essentially the same technique may also be used to characterize the biospecific antibody–antigen interaction on which the immunoassay is based [156–158,178] In principle expressions such as eqn. 20 may be used for analysis of solid-phase immunoassays (RIA or ELISA) in situations where the effect of soluble antigen (S) on the extent of interaction between partitioning antibody (A) and immobilized antigen (X) allows accurate assessment of the difference between total antibody concentration ($[\bar{\bar{A}}]$) and $[\bar{A}]$, its constituent concentration in the liquid phase [156] A problem with that approach in many immunoassays is that $[\bar{\bar{X}}]$ is so small in comparison with $[\bar{\bar{A}}]$ that there is no discernible difference between $[\bar{\bar{A}}]$ and $[\bar{A}]$. Consequently it is necessary to remove the liquid phase and then determine the concentration of adsorbed antibody, either by radioactivity measurements on the solid phase (RIA) or by means of an enzyme conjugated to an anti-idiotypic antibody (ELISA). In resorting to this procedure it is imperative that the washing regimen be closely examined to establish (i) that it is adequate for the removal of all soluble antibody (A and AS_i complexes) and (ii) that no discernible dissociation of matrix-bound antibody occurs during this procedure [156,157].

The fact that $f[\bar{\bar{A}}]$ far exceeds $[\bar{\bar{X}}]$ certainly validates the substitution of $[\bar{\bar{A}}]$ for $[\bar{A}]$, and furthermore, the extremely low concentration of matrix sites ($[\bar{\bar{X}}]$) allows the introduction of an additional approximation that formation of multiply linked antibody–matrix complexes is effectively excluded on steric grounds [23,157] With this proviso, the experimentally determined ratio of the concentrations of antibody bound to a fixed concentration $[\bar{\bar{X}}]$ of matrix sites in the absence and presence of a concentration [S] of antigen is given by the expression [157]

$$\frac{([\bar{\bar{A}}]-[\bar{A}])_o}{([\bar{\bar{A}}]-[\bar{A}])_s}=\frac{r_o[\bar{\bar{X}}]}{r_s[\bar{\bar{X}}]}=1+\frac{k_{AS}[S]}{1+fk_{AX}[\bar{\bar{A}}]} \tag{29}$$

where r_o and r_s denote the Klotz [94] binding function in the absence and presence, respectively, of antigen. Unequivocal evaluation of the intrinsic association constant for the antibody–antigen interaction (k_{AS}) from the linear dependence of r_o/r_s upon the concentration of competing antigen is clearly conditional upon prior determination of k_{AX}, a parameter obtainable from measurements of antibody partitioning in the absence of antigen via the expression [157]

$$r_o[\bar{\bar{X}}]/[\bar{\bar{A}}]=fk_{AX}[\bar{\bar{X}}]-fk_{AX}r_o[\bar{\bar{X}}] \tag{30}$$

Application of this procedure to ELISA data on the interaction between paraquat and a mouse monoclonal antibody (IgG) elicited in response to this univalent antigen is summarized in Fig 13, where concentrations of bound antibody ($r[\bar{\bar{X}}]$) are recorded in terms of absorbances reflecting catalysis by the horseradish peroxidase conjugated to the anti-IgG antibody Combination

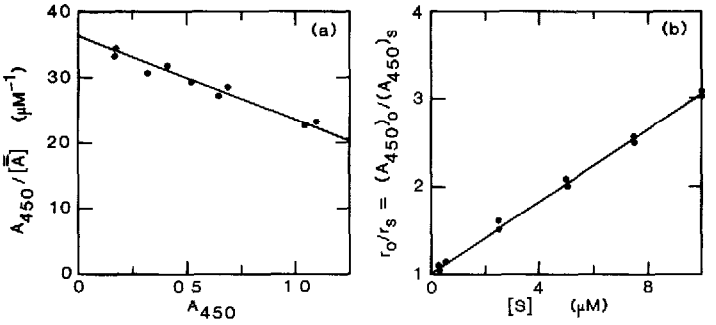


Fig 13 Determination of the intrinsic association constant for an antibody-antigen interaction by quantitative analysis of an ELISA for paraquat (a) Evaluation of k_{AX} from results for the monoclonal IgG (A) obtained in the absence of paraquat, the results being plotted in accordance with eqn 30 (b) Plot of results obtained with 24 nM IgG and a range of paraquat concentrations, [S], in accordance with eqn 29 Adapted with permission from ref 157

of the antibody–matrix interaction constant derived from the slope ($-2k_{AX}$) of the Scatchard plot (Fig. 13a) for the binding of bivalent monoclonal IgG to immobilized paraquat in the ELISA wells with the slope ($k_{AS}/(1+k_{AX}[\bar{A}])$ derived from the dependence of r_o/r_s upon [S] in experiments with 24 nM antibody (Fig 13b) yields an intrinsic association constant of 2.7 (± 0.3)·10^5 M^{-1} for k_{AS}

A requirement of this affinity chromatography approach to the characterization of an antibody–antigen interaction by solid-phase immunoassay is that the antigen (S) be univalent However, essentially the same procedure also applies to studies involving a multivalent antigen provided that the univalent Fab fragment is substituted for the monoclonal antibody (IgG or IgM) [157]. At present the concept of using solid-phase immunoassays to characterize antibody–antigen interactions is in its infancy, but clearly, such applications have the potential to add an extra dimension to the utility and scope of this widely used technique in immunochemical studies

4 SUMMARY

Biospecificity is due largely to the formation and dissociation of non-covalent complexes between small molecules and macromolecules, or between two macromolecules The first part of this review is concerned with the use of such biospecificity in the fractionation and identification of solutes Major emphasis is given to affinity chromatography, especially in regard to the practical considerations inherent in an experimental situation and to the wide range of specific interactions that can be utilized. The second part of the review considers the quantitative characterization of biospecific complex formation. The merits of frontal gel chromatography, electrophoretic methods and affinity

chromatography are discussed, and special consideration is given to the effects of ligand and/or acceptor multivalency because of its relevance to many biospecific interactions. Finally attention is drawn to the feasibility of employing quantitative affinity chromatographic theory for the determination of association constants for antigen–antibody systems by radioimmunoassay and enzyme-linked immunosorbent assay techniques.

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