

NON-IONIC ADSORPTION CHROMATOGRAPHY AND ADSORPTIVE IMMOBILIZATION OF PROTEINS

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Abstract. Hydrophobic and other non-ionic factors provide many new parameters for the separation of proteins by differential adsorption in addition to the mere difference in charge employed in ion-exchange chromatography. Their application also greatly broadens the range of conditions under which adsorptive protein immobilization, e.g., for the preparation of enzyme-reactors, is possible.

INTRODUCTION

In the present communication the emphasis is on non-ionic interactions, because recent evidence indicates the importance and generality of this type of interaction as a means for chromatographic separation and for adsorptive immobilization of proteins. The interactions to be discussed pertain especially to hydrophobic effects but also to other non-ionic types of binding, collectively referred to as "charge transfer" effects, including hydrogen-bonding (1). Such effects may have been involved to some extent in the binding of proteins by certain ion-exchangers and particularly in charcoal chromatography. However, systematic studies of these non-ionic factors in the absence of electrostatic effects have been initiated only relatively recently.

An important characteristic of hydrophobic bonding is its stabilization by "structure forming" salts (2,3). By contrast, binding of proteins by ion-exchangers usually is reversed by raising the ionic strength (unless non-electrostatic binding is also involved). This applies similarly to the immobilization of proteins by charged particles such as kaolin (4-6) and to the complexes of positively charged proteins with nucleic acids (7-9). The electrostatic nature of such interactions is confirmed by the finding that the binding of ChT (Note a) by kaolin (5) as well as in the case of the ChT-DNA-complex (9) causes the pH-activity curve to shift towards the alkaline region. These shifts in pH-activity curves are similar to those observed for enzymes covalently bound to charged polymers (10,11). It is of importance to note that, at least for the case of ChT-DNA, the maximal activity for $[H^+] \rightarrow 0$ is about the same as that of the free enzyme (9), i.e., the immobilization does not affect the potential activity, indicating that the binding does not involve the active center of the enzyme (see also below).

Little information is available on the effect of salt on charge transfer reactions. However, since these are direct interactions and unlike hydrophobic bonding are not lyotropic phenomena, they are not necessarily affected by salt.

A crucial observation in studying the effect of salt (NaCl) on the binding of certain enzymes by n-alkyl-amino-agaroses of varying hydrocarbon chain length was that generally the stability against addition of the salt increased with increasing hydrophobicity of the n-alkyl-ligand (12). It had been shown earlier that the binding of several proteins by other adsorbents of this type was unexpectedly stable in 1 M NaCl (13). At about the same time, similar observations were made by others (14,15). In any event, it is now clear that at relatively low ionic strength the amine-substituted agaroses, which carry a positive charge at pH \approx 9, due to the ionized amino-group (16), behave as anion-exchangers but that with increasing concentration of the salt and increasing hydrophobicity of the ligand hydrophobic binding increases at the cost of electrostatic interaction (see below).

Note a. Abbreviations: ChT, α -chymotrypsin; DFP-ChT, diisopropylfluorophosphate-inactivated ChT; ChTng, chymotrypsinogen A; BSA, bovine serum albumin; OV, ovalbumin; γ -G, γ -globulin; C_n, n-alkyl-chain with n C-atoms; PBA, 4-phenyl-n-butylamine; EG, ethylene glycol; DMF, dimethylformamide.

The main purpose of the present communication is to show that hydrophobic and other types of non-ionic interaction provide a variety of new parameters for the chromatographic separation and immobilization of proteins, including the adsorptive immobilization of enzymes with retention of activity.

EXPERIMENTAL

The adsorbents used were agaroses substituted with n-alkyl-amines with or without terminal phenyl groups, prepared via CNBr-activation (17) of Sepharose 4B (Pharmacia). Phenyl- and n-octyl-glycidyl-derivatives of (CL)-Sepharose 4B (18), obtained from Pharmacia, also were used. The employed proteins were the purest commercially available preparations.

The methods employed for determination of the extent of binding by various adsorbents and under varying conditions were exceedingly simple. Typical binding studies were performed with the aid of small (≈ 1 ml) columns of the adsorbents in disposable pipets plugged with glasswool, placed in a refrigerated ($\approx 5^\circ$) box open at the top and equilibrated with a particular medium. A few mg of a protein, dissolved in 2 ml of the medium, was applied and washed-in with several 2 ml samples of the cooled medium. The amount of protein in the first filtrate and in subsequent washings was determined from the absorbance at 280 nm as compared to that of the original protein solution. The spectrophotometric measurements in the filtrates were made with the corresponding filtrates of a blank column without protein as the references. The amount of protein subsequently released by changing the composition of the medium, was determined in the same manner. The advantage of this step-wise procedure with manual determination of the absorbance of the filtrates is that a large number of runs can be made at the same time under identical conditions. By contrast, automated procedures, with continuous application of the medium and automatic monitoring of the filtrate are limited to one or only a few runs at the time. However, the latter type of procedure was used for long-term experiments, such as the reactor-type runs described below. Experimental details are described in the legends to the Table and the Figures.

SALT-STABLE HYDROPHOBIC VERSUS SALT-REVERSIBLE ELECTROSTATIC EFFECTS.

Although in many *in vivo* binding processes ionic as well as non-ionic effects may be assumed to play a role, for systematic investigations it is an advantage to have them separated. For the purpose of studying hydrophobic interactions *per se*, adsorbents virtually free of charge, such as the above mentioned glycidyl-derivatives, can be used. However, the positive charge on the extensively employed agaroses substituted with hydrophobic amines can be neutralized by the introduction of a carboxyl group, as in the case of amino acids (19,20), or simply be quenched through the use of relatively high salt concentrations, e.g., 1.0 - 3.0 M NaCl (13), which at the same time also enhances hydrophobic binding.

The presence of the positive charge on the amine-substituted agaroses has the advantage that estimation of the relative degree of substitution can be made simply through the extent of irreversible binding of a negatively charged dye such as Ponceau S in the absence of salt (21,22). The usefulness of the dye-binding procedure has been shown on several occasions (21-23). It provides a convenient measure for routine estimation of the relative degree of substitution, which must be known for the correct interpretation of experimental data.

Although the charge on the adsorbent limits the range of salt concentrations where studies of hydrophobic effects can be made in the absence of electrostatic effects, previous results (23) have shown that, at least for OV and BSA, the binding by a homologous series of n-alkylamino-agaroses is purely hydrophobic above about 0.3 M NaCl. This still leaves more than a 10-fold range of NaCl concentrations at which studies of the ionic strength on hydrophobic binding can be made. Elutions of the proteins can be achieved with the aid of agents such as EG and DMF, if necessary in combination with a limited decrease in ionic strength. Nonetheless, in the work described in the present communication the commercially available uncharged n-octyl- and phenyl-glycidyl derivatives of agarose have also been employed, in particular since their degrees of substitution, determined by the manufacturer through NMR-measurement (18) - a procedure impractical for routine determinations - are considerably higher (≈ 40 $\mu\text{mol/ml}$) than those of the employed amino-agaroses (10-13 $\mu\text{mol/ml}$). This provides opportunities for studying the effect of ligand concentration on the binding (see below).

Early work on hydrophobic protein binding by n-alkylamino-agaroses suggested that this type of binding was counteracted by the addition of NaCl. Also, a "chaotropic" salt such as KCNS was more effective than NaCl (24). Since the assumption of reversal of hydrophobic binding by NaCl is in conflict with the observed stabilizing effect on such interaction, there is the possibility that shielding of the positive charge on the adsorbent from a quenching effect by the aqueous medium, which would increase with increasing hydrophobicity of the agarose-bound ligand, was involved. The possibility of enhanced electrostatic binding through polarization of water around a hydrophobic group (25), which may be assumed to

increase with the size of the latter, was also considered (see ref. 22). However, an alternative interpretation is as follows.

As shown previously (21), at pH 8 and low ionic strength negatively charged BSA, β -LG and OV are strongly bound by all of the positively charged n-alkylamino-agaroses, regardless of the ligand C-chain length. However, for the lower members of the homologous series of adsorbents binding of all three proteins is reversed by the addition of M NaCl. On the other hand, with increasing hydrophobicity of the adsorbent and depending on the hydrophobicity of the protein (BSA and β -LG are more hydrophobic than OV, see below) binding becomes stable in the presence of the salt, indicating hydrophobic interaction. It may be assumed that at intermediate NaCl concentrations binding is in part electrostatic and in part hydrophobic. Under such conditions reversal of binding by a certain increase in the NaCl concentration would become less with increasing ligand hydrophobicity because more of the protein would become hydrophobically bound at the cost of (salt reversible) electrostatic binding. Chaotropic salts would be especially effective because of their ability to counteract hydrophobic as well as electrostatic binding. A more detailed discussion of this matter has been presented in ref. 26.

Systematic studies on the inter-relationships between hydrophobic and electrostatic effects in the binding of proteins by polymers carrying charges as well as hydrophobic groups (see also ref. 27) are of importance because of the possibility of synergism between ionic and non-ionic interactions, which could play a role at intermediate salt concentrations, e.g., in in vivo processes.

NON-SPECIFIC VERSUS SPECIFIC BINDING

It may be assumed that the same forces (hydrophobic, ionic, "charge transfer") are involved in "non-specific" as in "specific" interactions, and a sharp distinction between the two types of binding cannot always be made. However, in the case of a particular molecule interacting with a much larger entity specific, in contrast to non-specific, interaction usually is assumed to involve complementarity between the interactants with respect to contour of interacting surfaces (molecular fit) and distances between the points of interaction. This would apply, for instance, to a substrate interacting with an enzyme but, in turn, possibly also to the interaction of the enzyme with a macromolecular matrix, e.g., as in the case of intracellular enzymes adsorptively immobilized with retention of activity (see below).

It can be seen from the following example that depending on the conditions, the interaction of a substrate or substrate-analogue with an enzyme may be either "specific" or non-specific and that the latter type of binding may be much stronger than the former. Figure 1 shows that in the presence of 3 M NaCl, ChT, DFP-ChT as well as ChTng are strongly, virtually irreversibly, bound by 4-phenyl-n-butylamino-agarose. However, it can be seen that upon lowering of the salt (NaCl) concentration to 0.3 M, the two inactive species (ChTng, DFP-ChT) are readily released, whereas the active enzyme is considerably more strongly held, although by no means as strongly as is the case for all three protein species at the higher salt concentration. The obvious interpretation is that in 3 M NaCl all three proteins are bound through non-ionic binding stabilized by the salt, whereas in 0.3 M NaCl this type of binding is largely reversed and the interaction is primarily through the active center of the enzyme which is blocked in DFP-ChT and not fully developed in the zymogen.

Although the non-specific binding in 3 M NaCl here is primarily through aromatic-hydrophobic rather than through "purely" hydrophobic interaction (see below), the data of Figure 1 demonstrate not only that an enzyme may bind non-specifically as well as specifically with the same ligand, but also that depending on the conditions, the former type of binding may supersede the latter. However, it should be pointed out that the non-specific type of interaction is not always as readily counteracted by mere lowering of the salt concentration as is the case for the chymotrypsins under the conditions of Figure 1. In the course of our investigations many cases of strong non-specific interaction under "normal" conditions such as required for enzyme activity have been encountered. For instance, it was found that an adsorbent carrying a ligand "designed" for the specific binding of a particular enzyme also was able to strongly bind through non-specific interaction a large number of other and unrelated proteins (13). Such non-specific interactions may constitute a serious drawback in the isolation of biologically active proteins through specific adsorption ("affinity") chromatography (see also refs. 28-30).

IRREVERSIBILITY AND INHOMOGENEITY OF BINDING

The "irreversible" type of non-specific binding (immobilization) of a protein, e.g., as demonstrated in Figure 1 by the binding of the chymotrypsins by PBA-agarose in the presence of 3 M salt, is not reversed by extensive washing with the ambient medium. Nonetheless, as also shown in Figure 1, reversal often may readily be achieved merely by changing the composition of the medium, e.g., by lowering the salt concentration as in this case. This type of virtually irreversible interaction may occur through any means of adsorption, in-

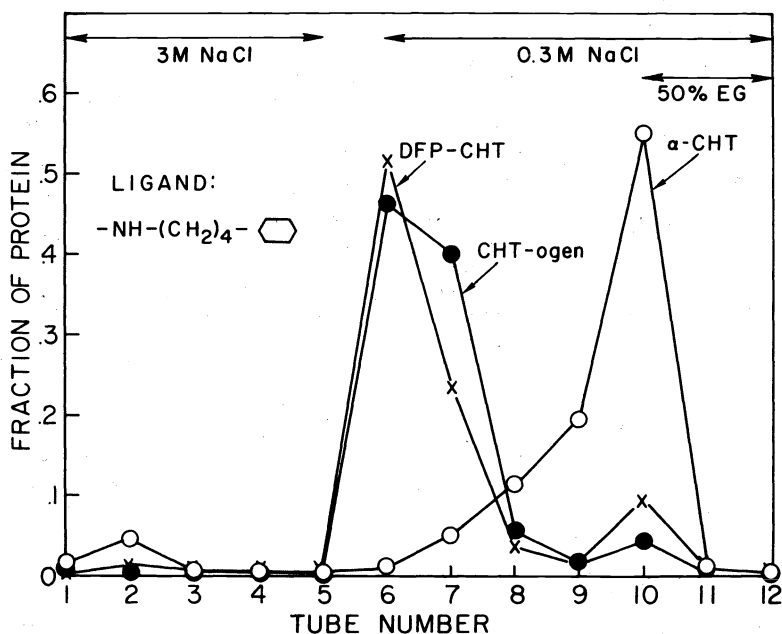


Figure 1. Binding of α -chymotrypsin (α -ChT), chymotrypsinogen A (CHT-ogen) and diisopropyl-fluorophosphate-inactivated α -chymotrypsin (DFP-ChT) by phenyl-n-butyl-amino-agarose in 0.01 M tris-HCl, pH 8, containing 3 M NaCl, 0.3 M NaCl or 0.3 M NaCl and 50 percent ethylene glycol (EG). Temp. $\approx 5^\circ$.

volving ionic, hydrophobic and/or charge transfer which, as noted above, are the same forces that are operative in the specific type of interaction. However, the difference is that specific binding usually involves only one site per protein monomer (e.g., the active center in the case of an enzyme), whereas non-specific binding may occur through the simultaneous interaction of several binding sites of the protein with several ligand-molecules on the adsorbent. Such multiple point interaction is assumed to be the cause of apparent irreversibility, because it is improbable that dissociation would occur at several sites simultaneously. Nonetheless, if the multiple point binding is through a single parameter, reversal can be expected by the addition or removal of a single component to or from the medium, e.g., the addition of salt in the case of electrostatic interaction or the removal of salt (or the addition of a "chaotropic" agent) in the case of hydrophobic interaction. For mixed type of interactions application of more than one agent may be needed.

It is obvious that the multiple point irreversible type of interaction is a consequence of the immobilization of the ligand and is inherent in "solid state" as opposed to classical solution chemistry. Another factor inherent in chemical interactions occurring on solid surfaces, at least artificial ones, is inhomogeneity of binding sites, which may result in one "irreversibly" bound molecule of a particular protein being more readily detached from the adsorbent by an eluant than another also "irreversibly" bound molecule.

These two parameters, i.e., irreversibility and inhomogeneity of binding, which for the case of proteins have been extensively treated on several previous occasions (21,22,31), are serious drawbacks for any type of adsorption chromatography of materials of relatively large molecular size unless means to counteract them can be found (see below).

GENERILITY OF HYDROPHOBIC PROTEIN BINDING

Similar to previous results (23,32), the data of Table 1 show that a large number of proteins are bound by n-hexylamino-agarose in the presence of 3 M NaCl. Under such charge-quenching conditions (2) the interaction may be assumed to be largely or purely hydrophobic, in particular since untreated agarose and inactivated CNBr-treated agarose usually show little or no binding. This is further confirmed by the fact that the extent of binding invariably is higher in the case of the more hydrophobic, n-octyl-NH-agarose of about the same ligand content. Increased binding with increase in adsorbent hydrophobicity, i.e., hydrocarbon chain length, is also seen for the phenyl-ethyl- and phenyl-butylamine-substituted agaroses. However, in these cases the binding, at least for some proteins, is not necessarily purely hydrophobic (see below). In any event, the fact that virtually every protein tested so far shows a degree of affinity for the n-alkyl-amino-agaroses indicates the generality of the role of hydrophobic effects in protein binding and confirms

TABLE 1. Protein binding by substituted agaroses

Binding (per cent) of 2 mg of various proteins by 1 ml columns of n-hexyl(C₆)-, phenyl-ethyl(phe-C₂)-, n-octyl(C₈)- or 4-phenyl-n-butyl(phe-C₄)-amino agaroses and by n-octyl- and phenyl-glycidyl-agaroses in 3 M NaCl containing 0.01 M tris-HCL, at pH 8 and 5°, after washing with 8 ml of the salt-buffer solution.

	Amino-				Glycidyl-	
	n-C ₆	phe-C ₂	n-C ₈	phe-C ₄	n-C ₈	phenyl
Bov. ser. alb.	100 ^{b)}	28	100	100 ¹⁾	98	95 ¹⁾
Hemoglob.	96	88	97	95	98	98
DN-ase	86 ^{b)}	36	97	99 ¹⁾	47 ²⁾	97 ¹⁾
Pepsin	86	87	87	87.5	90	91
γ-Glob.	84	96 ^{a)}	97	95	81 ²⁾	98
β-Lactogl. B.	45 ^{b)}	28	98	100 ¹⁾	97	97 ¹⁾
β-Lactogl. A.	31 ^{b)}	6	95	91 ¹⁾	98	96.5 ¹⁾
ChTogen A	18	37 ^{a)}	70	100 ^{a)}	28.5 ²⁾	100 ^{a)}
RN-ase	8.5	9.5	12	18 ^{a)}	4	10 ^{a)}
Ovalbumin	8	10	33	28	23 ²⁾	41
Myoglob.	6	6	39 ^{b)}	24.5	39 ^{b)}	23
Cytochr. c	2	3.5	8	10	10	12

a) Preference for phenyl-.

b) Preference for n-alkyl-.

1) Preference masked by high hydrophobicity and/or high ligand density.

2) Binding by n-octyl- inhibited at high ligand density.

the conclusion that accessible hydrophobic groups occur much more generally on the surface of proteins than had been assumed (e.g., see ref. 33). This generality and the apparently wide differences in the extent of binding of one protein to the next intimate, in turn, the possibility of wide application of protein separation by chromatography based on differential hydrophobic adsorption.

It should be pointed out that the relative extents of binding of those proteins of Table 1 that previously had been tested on an n-hexylamino-agarose column (23,32) is not the same as those obtained with the present column of the same adsorbent. It must be noted however, that for those earlier results a 25 ml n-C₆-column was used as compared to the 1 ml column of Table 1. Furthermore, the washing-in of the proteins into the larger column was with only 2-3 column bedvolumes, compared to 8 bedvolumes in the present case. Thus, the earlier experiment, which was designed to show the generality of hydrophobic binding rather than to determine relative protein hydrophobicities, tended to show even a small degree of retardation whereas the data of Table 1 often refer to virtual irreversible binding. For this reason, the relative extent of binding of some proteins was exaggerated in the earlier data.

THE AROMATIC-HYDROPHOBIC EFFECT

In the first four columns of Table 1 the extent of binding of the applied proteins by n-alkylamino-agaroses is compared with the binding by adsorbents of about the same degree of substitution, but whereby a phenyl-group is substituted for the three terminal hydrocarbons. Since the hydrophobicity of a phenyl group is equivalent to about 3-4 straight-chain hydrocarbons (34,35), such substitution does not significantly alter the hydrophobicity of the adsorbent. Nonetheless, it can be seen that, at least in the case of ChTng, the substitution greatly enhances the extent of binding. As seen from the last two columns of Table 1 this also holds true in a comparison of the binding of ChTng by the n-octyl- and phenyl-glycidyl-agaroses. Although the degrees of substitution of these two adsorbents is about the same, the hydrophobicity of the phenyl- is much less than that of n-octyl-residue. That, nonetheless, the extent of binding of ChTng by the phenyl-substituted adsorbent is

very much larger than in the case of the n-alkyl-ligand indicates a strong preference for aromatic binding in which hydrophobicity per se may play only a supporting role.

For γ -globulin a similar aromatic effect, although very much less extensive, is indicated by a comparison of the n-C₆- and phenyl-C₂- adsorbents. The apparent absence here of the aromatic effect in the case of phenyl-C₄- as compared to the n-C₈-amino-adsorbent could be the result of masking by strong hydrophobic interaction. A preference of this protein for the phenyl-group is also indicated by comparison of the C₈- and phenyl-glycidyl-adsorbent. However, it can also be seen that for several proteins (including ChTng and γ -G) binding by the C₈-glycidyl-derivative is less than by the less highly substituted C₈-amino-agarose, which may indicate inhibition by excess ligand density (see ref. 18).

A slight preference for the phenyl-group may also be operative for RN-ase. However, of all the proteins of Table 1 only ChTng shows a strong preference for aromatic interaction. This indicates a much less general occurrence of the latter type of binding than is the case for hydrophobic interaction per se. As shown in Figure 1 the aromatic type of binding, at least in the case of ChTng and ChT is, similarly to hydrophobic interaction, dependent on the presence of a non-chaotropic salt such as NaCl. It would seem, however, that aromatic (π - π) binding which, as noted above, is a direct interaction and not an indirect lyotropic effect, would not be dependent on the presence of salt. Therefore, it is possible that it depends on synergism with the hydrophobicity that is inherent in aromatic compounds.

OTHER MODIFYING FACTORS OF HYDROPHOBIC INTERACTION

The aromatic effect is not the only factor that may alter the relative extent of binding of proteins by a hydrophobic adsorbent. For instance, whereas ChTng shows a preference for the phenyl-group as compared to the n-alkyl-ligand of about the same hydrophobicity, the opposite is true for serum albumin (Table 1). Here the extent of binding by the phenyl-C₂- is very much less than in the case of n-C₆-adsorbent, indicating a strong preference for the straight hydrocarbon chain. The fact that the lesser binding by the phenyl-derivative does not similarly manifest itself in the case of phenyl-C₄-amino-agarose would indicate that here the higher hydrophobicity alone suffices to achieve complete binding. Nonetheless, as indicated by the elution data of Figure 2 reversal by certain eluants is more readily achieved here than in the case of n-C₈, indicating weaker binding. The same holds true for the two glycidyl-agaroses which under the experimental conditions bind this protein to about the same extent, but where also the combination with the phenyl-derivative is less stable than in the case of n-C₆ (36). A similar preference of serum albumin for n-alkyl-chains has been observed by others (37).

A preference for n-alkyl groups is also indicated for DN-ase. However, binding of this protein, in contrast to BSA, appears to be inhibited by high ligand density of the adsorbent as can be seen from a comparison of the results with the n-C₈-amino-agarose and the more highly substituted n-C₈-glycidyl-derivative (Table 1). The behavior of the two β -lactoglobulins is similar to that of BSA, i.e., a preference for the n-alkyl-ligand that is masked by high hydrophobicity but not inhibited by excess ligand, is in evidence. Although generally less extensively bound, myoglobin also shows a preference for the n-alkyl-adsorbents, at least in the case of n-C₆. On the other hand, hemoglobin and pepsin do not show a preference one way or the other, i.e., in these cases the binding seems to depend only on the hydrophobicity of the ligand regardless of whether this is derived from n-alkyl- or phenyl-groups (Table 1, Fig. 2).

The finding that the binding of some proteins (e.g. DN-ase) is inhibited by high ligand density, whereas this is not the case for others (e.g., BSA, β -LG) by itself is another modifying factor that may affect the relative extent of binding of different proteins under a given set of conditions. Furthermore, unless the ligand density of an adsorbent is sufficiently high to allow contact with all available binding sites on each of the proteins of which the relative extent of binding is being determined, the molecular size of the proteins also could be a factor. However, a comparison of the extent of binding of the proteins of Table 1 by the n-C₆-amino- and the more highly substituted n-C₈-glycidyl-adsorbent indicates that this factor is not of great importance in this case (Note b).

Note b: Calculations of the average distance between ligand molecules on the solid fraction of the adsorbent, assuming even distribution, indicate that for the proteins of Table 1 with the lowest molecular weights the distance between ligand molecules is larger than the molecular diameter of the protein, which would exclude multiple-point attachment. However, in view of the above discussed inhomogeneity of binding sites and with a sub-saturating amount of protein, the opportunities for such attachment would be better than indicated by the calculated distance.

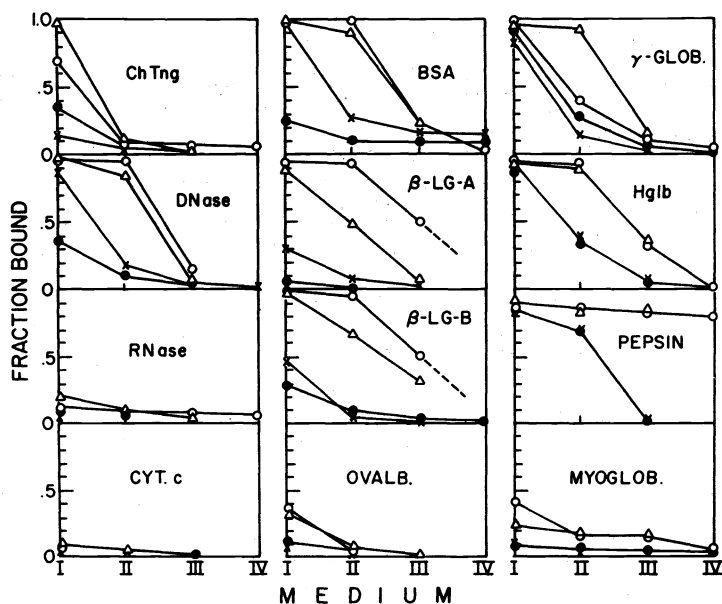


Figure 2. Relative extents of binding of various proteins by phenyl-ethyl- (●), n-hexyl- (X), 4-phenyl-n-butyl- (Δ) or n-octyl- (○) amino-agaroses. After application of a protein (see Experimental), the column was washed successively with 8 bedvolumes of 3.0 M NaCl (I), 8 bedvolumes of 0.3 M NaCl (II), 6 bedvolumes of 50 percent ethylene glycol in 0.3 M NaCl (III) and, if indicated, with one bedvolume of the latter mixture containing 0.5 M n-octylamine (IV). All solutions contained 0.01 M tris-HCl, pH 8. Temp. $\approx 5^{\circ}$. In the case of β -lactoglobulin A or B, the application of medium IV resulted in a turbid filtrate. The Ponceau-values of the adsorbents were 1.7, 1.8, 1.8 and 1.4, respectively, corresponding to ligand contents of 10-13 $\mu\text{mol/ml}$ gel (21,22).

A more extensive account of all experimental data on the various factors affecting the relative extent of hydrophobic protein binding will be presented elsewhere (36). In any event, the occurrence of all of these modifying factors of hydrophobic bonding in addition to hydrophobicity *per se*, is not conducive to the exact determination of relative hydrophobicities of proteins from the relative extent of binding by hydrophobic adsorbents under a fixed set of conditions.

SELECTIVITY OF "NON-SPECIFIC" PROTEIN BINDING

The data of Table 1, showing a clear preference for binding by n-alkyl-groups in the case of some proteins as opposed to a preference for the aromatic group for other proteins, indicate the need for further elaboration on the term "specificity".

Since the selective binding of inactive ChTng and DFP-ChT as well as of the active ChT in 3 M NaCl by a phenyl-group (Fig. 1, Table 1) apparently is not due to complementary binding involving the enzyme active center, another kind of "specificity" seems to be involved here. It is of interest that ChT is specific for the hydrolysis of peptide bonds adjacent to aromatic amino acid residues, i.e., phenylalanine, tyrosine and tryptophan. Usually this is ascribed to the specific interaction of the side chain of these amino acids with the hydrophobic "tosyl hole" of the enzyme active center (38). However, the present results indicate that at least in the presence of high NaCl concentrations the specific attachment of the enzyme to the aromatic amino acids is not necessarily through the active center *per se*. Although the strong irreversible type of binding of ChT and its derivatives as shown in Figure 1 would be unlikely to occur at physiological salt concentrations, such "specific" binding through sites outside the active center is not necessarily excluded for other enzymes under physiological conditions.

The difference in relative affinities of different proteins for n-alkyl- and phenyl-groups presents additional opportunities for protein separation. For instance, in the separation of serum albumin and γ -globulin by hydrophobic adsorption chromatography, the introduction of a phenyl-group in an alkyl-ligand slightly increases binding of γ -globulin and greatly decreases binding by the albumin (see Table 1). This parameter has already empirically

been applied to the "specific" separation of these two proteins by chromatography on phenylalanine-substituted agarose (19,20).

NON-IONIC VERSUS ION-EXCHANGE CHROMATOGRAPHY

Adsorption chromatography based on differences in protein hydrophobicity has the advantage over ion-exchange chromatography of much greater selectivity, i.e., potentially an unlimited "scale" of adsorbents is available for the differential binding of proteins. By contrast, for ionic interaction the choice is qualitatively limited to either positive or negative. This is the basis for the use of hydrophobicity gradients (see below) for which there is no counterpart in ion-exchange chromatography. The possibility for applying differences in ligand density to the separation of proteins varying in the number per molecule of available hydrophobic groups or charges respectively, are the same in the two cases.

The generality of the applicability of the hydrophobic procedure also compares favorably with that of ion-exchange chromatography. This is indicated by the general occurrence of hydrophobic protein binding as shown by Table 1 (see also ref. 23), which is in accord with the frequent occurrence of accessible hydrophobic groups on proteins of known three dimensional structure (33) and with the less direct but overwhelming evidence for the near ubiquitous role of hydrophobic phenomena in various biological processes, accumulated by Hansch and co-workers (39,40).

Although probably of less general applicability (see Table 1), the aromatic effect adds another parameter to protein separation by non-ionic adsorption chromatography. Yet another possibility is adsorption chromatography based on differences in hydrogen bonding. A possible example of this is found in the binding of γ -globulin by inactivated CNBr-treated agarose. As seen in Figure 3 this type of binding decreases by prior substitution of the activated agarose with *n*-alkylamines of low hydrophobicity (C_1 - C_2). Increased binding by the C_4 -derivative is ascribed to hydrophobic bonding. It would seem reasonable to assume that the binding in the absence of hydrophobic groups is through hydrogen bonding involving carbonyl-amide groups which represent up to 50 percent of the total N-content in CNBr-treated agarose and which play a role in the substitution process (41). It is of interest that this type of bonding, like hydrophobic interaction is counteracted by EG (23). This would be in accord with the assumption that hydrophobic bonding is reversed by agents that weaken the intermolecular water structure which also depends on hydrogen bonding. Thus, from this point of view, ethylene glycol could be looked upon as a "chaotropic" agent (see also ref. 22).

In any event, all of these non-ionic factors vastly increase the possibilities of protein separation by "non-specific" adsorption chromatography for which previously primarily only ion-exchange has been applied.

PROTEIN SEPARATION ON HYDROPHOBICITY GRADIENTS

As noted above, the phenomena of apparent irreversibility and inhomogeneity of binding are drawbacks to the efficient separation of proteins by differential adsorption, including the hydrophobic type. However, as opposed to the case of electrostatic binding (where, as already noted, qualitatively the choice is limited to either positive or negative) the hydrophobic factor is susceptible to gradation. Although it would be feasible to establish a gradient of varying degrees of aromaticity, this would also affect the degree of hydrophobicity. For hydrophobicity per se, gradation can readily be achieved merely through changing the size of the hydrophobic group, e.g., in the case of a homologous series of *n*-alkyl-groups, without simultaneously changing or introducing other variables. The advantage of such a gradient is as follows.

If a protein mixture is applied to an adsorbent of an arbitrarily chosen degree of hydrophobicity, the less hydrophobic proteins may not be held at all, whereas the most hydrophobic ones may be bound with such tenacity that they cannot be recovered without the aid of eluants that tend to denature them. In order to prevent such strong binding, the mixture may be led through a gradient consisting of interconnected columns of adsorbents of increasing hydrophobicity (20). In this procedure each protein tends to be held by the adsorbent that provides the minimum degree of hydrophobicity required for binding. After application of the mixture to the first column of the series, the ambient medium containing charge quenching salt, is pumped through until little or no further protein appears in the filtrate of the last column.

It should be noted that although excessively strong binding is avoided by the use of a hydrophobicity gradient, the binding of the proteins is nonetheless of the irreversible type, i.e., the proteins become immobilized with respect to binding in the ambient medium. However, elution is usually achieved by means of a relatively mild agent such as 50 percent ethylene glycol (13) which has no irreversible effect on many proteins tested (42). Although the use of a hydrophobicity gradient also tends to decrease the range of strengths

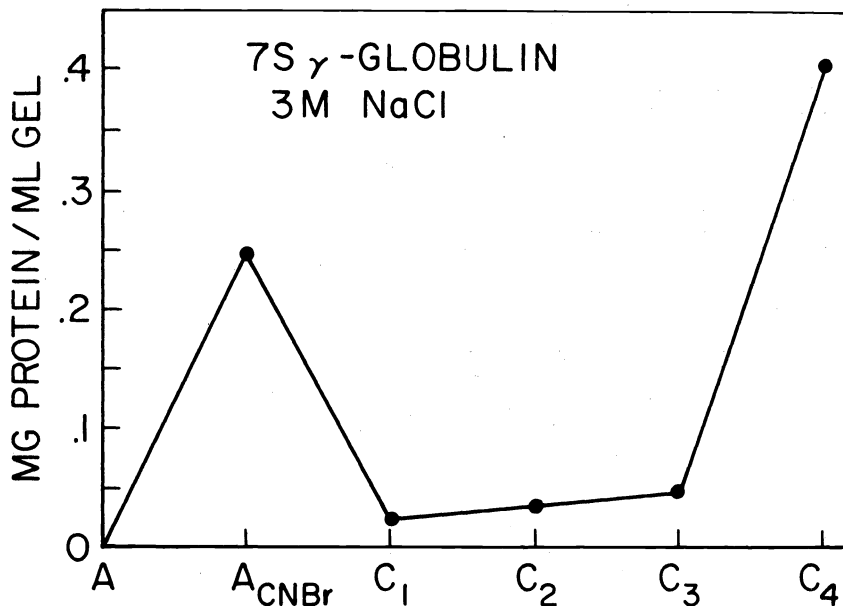


Figure 3. Salt (NaCl)-stable binding of 7S γ -globulin by 1 column of CL-agarose (A), unsubstituted CNBr-treated A, and CNBr-treated A substituted with n-alkylamines of varying C-chain lengths (C₁-C₄). The preparations were heated for 1 hour at 100° and pH 4 before use. About 4 mg of the protein in \approx 30 ml 3 M NaCl containing 0.01 M tris-HCl, pH 8, was applied to the first (A) of the inter-connected columns and recycled \approx 20 times through the series. Each of the subsequently disconnected columns was washed with 10 ml. of the salt solution and then eluted with 50 percent ethylene glycol in 0.3 M NaCl. Temp. \approx 5°.

of binding sites a particular protein is exposed to, it does not solve the problem of inhomogeneity of binding. For this reason it would not be possible, for instance, that through the use of a very shallow gradient a mixture of proteins could be sharply separated with each protein found only on a particular adsorbent. Problems also arise when some proteins are present in the original mixture in much higher concentrations than others (e.g., as in the case of blood serum, see ref 23). Nonetheless, one can expect to find generally the more hydrophobic proteins on the lower members of the gradient and the less hydrophobic ones on the more hydrophobic members, whereas those of extremely low hydrophobicity will be found in the filtrate of the last column. The procedure can also be expected to separate an already partially purified protein from contaminating protein species regardless of similarities in molecular weights and/or isoelectric points. A similar procedure based on gradient chromatography and also for the purpose of avoiding excessively strong binding of the proteins, has been used by Porath in the case of charcoal chromatography (43).

ADSORPTIVE IMMOBILIZATION OF ENZYMES

As noted in the introduction, on numerous occasions proteins have been immobilized through the irreversible, multiple point type of electrostatic interaction with oppositely charged adsorbents (e.g., see refs. 44, 45). This also applies to the immobilization of enzymes with retention of activity (for a recent review, see ref. 46). However, it was found only relatively recently that immobilization with retention of activity also is possible through hydrophobic and other types of non-ionic interaction. For instance the binding of several negatively charged enzymes by a preparation of n-butyl-amino-agarose in 0.01 M tris-HCl, pH 8, is largely reversed by the addition of 1 M NaCl, indicating predominantly electrostatic binding involving the positively charged agarose-bound amine. However, in the case of the more hydrophobic n-octyl-amino-agarose, a considerable fraction of several of these enzymes remained bound, even upon continued washing with 1 M NaCl (12). In particular, since the octyl-derivative was less highly substituted than its butyl-counterpart, this indicated that depending on the hydrophobicity of the ligand, strong hydrophobic binding may occur in the absence of electrostatic effects. This also applies to proteins carrying the same overall charge as the adsorbent, whereby the electrostatic repulsion may be canceled through the addition of a non-chaotropic salt (e.g., NaCl), which simultaneously enhances hydrophobic interaction.

A most important additional finding of these studies on adsorptive binding of enzymes is that in each case tested the enzyme retained at least part of its activity (12). It is noted, that in particular when the enzyme is added in the presence of substrate, i.e., when the active center is blocked, binding is apt to occur through sites on the enzyme protein that are not located in the active center. The obvious significance of these observations is that hydrophobic as well as electrostatic interaction may be employed in "reactor"-type experimentation whereby the substrate, pumped through such an enzyme-containing adsorbent, may be continuously transformed into its product over considerable periods of time. This was found to be the case for several of the enzymes tested (12).

The hydrophobic factor greatly increases the range of conditions under which adsorptive enzyme immobilization is possible. The results of Figure 1, which aside from a small contaminating fraction show irreversibility of the binding of ChT in 3 M NaCl by PBA-agarose, and the evidence (Table 1, Fig. 2) that the binding is largely caused by aromatic interaction, further adds to this range of possibilities. Figure 4 shows that 1 mg of ChT adsorbed on a 4 ml PBA-agarose column remains bound after pumping \approx 4 liters of 0.005 M acetyltyrosine ethylester (ATEE) through the "reactor". As judged by the decrease in absorbance at 235.5 nm, the substrate was continuously and completely hydrolyzed during the entire period (several weeks) of the run. As can be seen, similar results were obtained with a column of more highly substituted but less hydrophobic and uncharged phenyl-glycidyl-agarose.

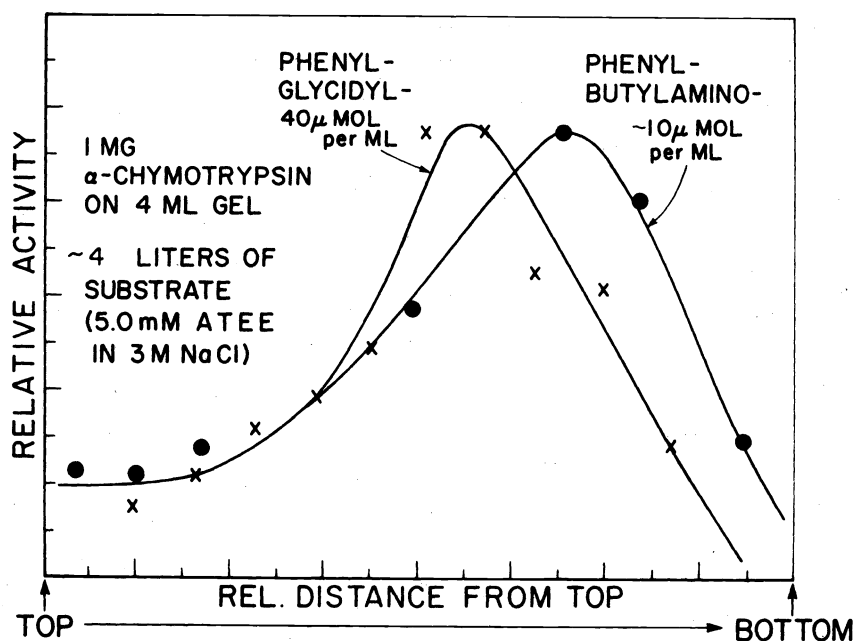


Figure 4. Relative degrees of α -chymotrypsin activity remaining at varying distances from the top of 5.8 x 150 mm columns of phenyl-glycidyl or 4-phenyl-n-butylamino-agaroses after application of 1 mg of the enzyme followed by \approx 4 liters (\approx 1000 bedvolumes) of 5.0 mM acetyltyrosine ethylester (ATEE). The entire procedure was carried out at \approx 5° in 3 M NaCl containing 0.01 M tris-HCl, pH 8. Activity determinations on sections of the columns were carried out in a pH-stat in $<$ 0.3 M NaCl with ATEE as the substrate. Gel volumes were measured in hematocrit tubes.

The above observations are of great possible practical significance. The fact that chemical manipulation for achieving co-valent binding of the enzyme is not necessary, not only is time saving but also is apt to avoid possible inactivation more likely to occur in chemical than in mild adsorptive processes. Furthermore, in contrast to the case of co-valent binding, adsorptively bound enzyme often may be recovered simply by changing the composition of the medium (see above). It should be noted that in the preparation of such a reactor, initial leakage from the column not necessarily means that eventually all of the enzyme will be washed off by the ambient medium. In such a case the capacity of the column for irreversible binding of the enzyme may not be large enough and a larger column or a lesser amount of enzyme should be employed. In this connection it should also be pointed out that the enzyme applied to the top of the column and washed-in with a particular medium will move down the column until eventually all of it is bound by sites that bind sufficiently strongly for irreversible attachment to occur under the given conditions. For the

case of Figure 4 the enzyme eventually might have been washed off the column if the run had been continued for several more weeks. However, this would have been prevented simply by using less enzyme and/or a larger column.

In any event, particularly in view of the many potential parameters suggested by the results described above and which can be employed with or without simultaneous electrostatic effects, it would seem that for almost any enzyme an adsorbent can be found that binds sufficiently strongly to prepare a reactor without the chemical manipulation needed for co-valent binding.

NON-IONIC INTERACTIONS IN IN VIVO PROCESSES

Because of the fact that electrostatic effects tend to be quenched in the aqueous, salt containing, in vivo environment, it has been suggested (47) that hydrophobic bonding for which such an environment is favorable, may be the most important factor in the formation of biological structures, at least in the case of micelles and cell membranes. In view of the above results it would appear that the aromatic-hydrophobic factor and other types of salt-stable interaction also could be involved. Furthermore, it would seem reasonable to assume that such interactions also play a role in the formation of many other in vivo complexes, not only in the case of enzyme-substrate interaction but also in interactions such as those between antigens and antibodies, protein and nucleic acids (viruses, chromosomes) and in the formation of intracellular organelles (see also ref. 31).

Reactor-type studies with enzymes adsorptively immobilized on substituted agaroses, as described above, may be looked upon as "models" for in vivo reactions catalyzed by enzymes immobilized on intracellular matrices. It is of interest that such in vivo combinations are of the "irreversible" type similar to the artificial complexes studied in the present investigations. In fact, structures of this type are strongly reminiscent of the "far from equilibrium" but stable structures that appear to underly the formation and evolution of living matter in general (48). From this point of view, it seems that the significance of systematic studies of the present type may go far beyond their possible application to chromatographic protein separations and the preparation of enzyme reactors.

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