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MODIFYING FACTORS IN HYDROPHOBIC PROTEIN BINDING BY SUBSTITUTED AGAROSES

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SUMMARY

Determinations have been made of the relative extent of non-ionic binding of a number of proteins by agaroses carrying *n*-alkyl-ligands with and without terminal phenyl-groups. Some proteins were preferentially bound by the aliphatic-, others by the aromatic adsorbents of about the same hydrophobicity and ligand content, whereas the binding of still others was determined by the ligand hydrophobicity alone. The effect of ligand structure and other modifying factors, such as ligand density and composition of the medium, that were also found to affect the relative extent of binding, provide a variety of parameters for protein separation by hydrophobic adsorption chromatography in addition to hydrophobicity *per se*, but are not conducive to the exact determination of relative hydrophobicities from the extent of binding under a fixed set of conditions.

INTRODUCTION

In previously established procedures for the separation of proteins by adsorption chromatography, *i.e.*, those based on the use of ion-exchangers[1–4] and in particular those using charcoal as the adsorbent (for quotations of the early work from Tiselius' laboratory, see ref. 5), hydrophobic effects may have played a role, but systematic studies of such effects were not made until about 1970. Furthermore, the first studies of this type most often included (salt-reversible) interaction with charges on the adsorbent, either introduced deliberately[6] or coincidentally[7–11] together with the hydrophobic group, *i.e.*, the positive charge on amine-substituted agaroses[12]. Purely hydrophobic binding was not dealt with until after the finding that the protein-complexes with such adsorbents are stable under charge-quenching conditions, *e.g.*, in $\geqslant M$ NaCl, and that the stability against the salt increases with increasing hydrophobicity of the ligand[9–11,13]. In fact, hydrophobic, in contrast to ionic interaction, is enhanced by an increase in the concentration of a "structure-forming" salt[14,15]. Further evidence for hydrophobic bonding of proteins in the absence of charge effects can be derived from the results with amino-acid substituted agaroses[16,17], where at the usually applied pH-values the positive charge on the amino-group is

neutralized by the ionized carboxyl group, and from the results with adsorbents that carry little or no charge at all[13,18–20].

In the meantime, the possibility of protein separation by non-ionic interactions other than hydrophobic, *e.g.*, through "aromatic" (π–π) interaction or hydrogen bonding, has been noted[21] and separation on the basis of metal chelate affinity has been explored[22]. An example of non-ionic, salt-stable but apparently also non-hydrophobic protein adsorption, is the more or less selective binding of γ-globulin by aged CNBr-activated agarose in the presence of 3 *M* NaCl, assumed to be caused by hydrogen bonding[23].

In the present investigations, several instances were encountered of the operation of hydrophobic in conjunction with aromatic effects. This phenomenon and the occurrence of "specific" effects of *n*-alkyl- or of phenyl-groups are the main subjects of the present report. However, other factors that may alter the relative extent of observed hydrophobic binding of different proteins, especially the composition of the medium and the ligand content of the adsorbent have also been investigated.

EXPERIMENTAL

Materials

Proteins. Chymotrypsinogen A (Worthington, Freehold, N.J., U.S.A.; CGC, 5× cryst.), cytochrome *c* (Miles-Seravac, Kankakee, Ill., U.S.A.; Grade 1, 90–100%), deoxyribonuclease I (Worthington D-6GA, 1× cryst.), γ-globulin (Miles, Fr. II, bovine, 98% γ-globulins), hemoglobin (Calbiochem., Los Angeles, Calif., U.S.A.; 3745, 2× cryst.), β-lactoglobulin A (Miles-Pentex, cryst., > 95% A, < 5% B), β-lactoglobulin B (Miles-Pentex, 98%), myoglobin (Miles-Seravac, 1× cryst., 95–100%), ovalbumin (National Biochemical, Chicago, Ill., U.S.A.; 5× cryst.), ribonuclease A (Worthington, RAF 6072, purest grade), serum albumin (Miles-Pentex, bovine, 99.6%).

Adsorbents. Phenyl-ethyl-, *n*-hexyl-, 4-phenyl-*n*-butyl- and *n*-octyl-amino-agaroses were prepared via CNBr-activation[24] from CL-Sepharose 4B (Pharmacia, Piscataway, N.J., U.S.A.). The amines were obtained from Eastman Kodak (Rochester, N.Y., U.S.A.) and were recrystallized as the hydrochlorides from ethanol with the aid of ethyl ether at −15°. The Ponceau values (mg/ml gel) of the aged preparations in the absence of salt, which are a measure of their relative degree of substitution[25], were 1.7, 1.8, 1.8 and 1.4, respectively. Based on previous determinations[26] of the increase in titer due to the positively charged agarose-bound amino group (pK_a value ≈ 10) and compared to an aged CNBr-treated but unsubstituted agarose control, this corresponds to a ligand concentration of 10–13 μmol/ml gel. The adsorbents were stored at $\approx 5°$ in 10^{-4} *N* HCl containing 0.02% sodium azide. Under these conditions little change in dye-binding capacity was observed over a period of several months. (For a comprehensive literature review on the preparation and properties of this type of adsorbent, see ref. 27.)

Phenyl- and *n*-octyl-glycidyl-agaroses[18] were obtained from Pharmacia (courtesy of Dr. T. Låås). According to the manufacturer's information, the ligand concentration of both of these gels is about 40 μmole/ml.

Methods

A series of 1-ml columns of an adsorbent were prepared in disposable pipets plugged with glass wool and equilibrated at ≈5° with 3.0 *M* NaCl in 0.01 *M* Tris–HCl, pH 8. The columns were charged with 2 mg of a protein dissolved in 2 ml of the salt-buffer solution and washed with this ambient medium. This was followed by washing with 0.3 *M* NaCl in buffer and subsequently with other media containing ethylene glycol, dimethylformamide, *n*-octylamine or urea as indicated in the legends to the figures. The whole procedure was carried out in a refrigerated box open at the top. The amount of protein in the filtrates was determined from the light absorbance at 280 nm as compared to that of the applied protein solution. For these measurements the corresponding filtrates of a blank column without protein were used as the references.

It may be assumed that at the low temperature applied and in the presence of 0.3–3.0 *M* NaCl, little or no denaturation of most of the proteins takes place. Ethylene glycol, even in 50% concentration, in general seems to have no irreversible effect[28]. Although denaturation in urea, applied in 7 *M* concentration, can be expected, no information is available on the effects of dimethylformamide or *n*-octylamine. However, it has been found that even *n*-decylsulfate has little or no irreversible effect on serum albumin[29,30] despite the fact that the internal structure of this protein is relatively weak[31].

For comparing the results obtained with the *n*-alkyl-agaroses with those carrying phenyl groups, it is of importance to point out that the hydrophobicity of a phenyl group has been found to correspond to 3–4 straight-chain methylene groups[30,32,33]. Thus, the hydrophobicity of the phenyl-ethyl ligand is close to that of the *n*-hexyl- and that of phenyl-*n*-butyl- is close to that of the *n*-octyl-residue.

RESULTS

Evidence for hydrophobic binding

For all of the proteins tested, the extent of binding by *n*-octyl-amino-agarose in 3.0 and/or 0.3 *M* NaCl is invariably greater than in the case of the corresponding less hydrophobic *n*-hexyl-adsorbent (Fig. 1). A similar effect of the ligand C-chain length is observed for the phenyl-ethyl- and phenyl-*n*-butyl-amino agaroses. In view of their Ponceau-binding capacities (see Experimental), differences in the degree of substitution cannot account for these observations. Furthermore, binding by any of the adsorbents of Figs. 1 and 2 is invariably diminished by lowering the NaCl concentration from 3.0 to 0.3 *M* and/or the addition of EG*, which also is indicative of hydrophobic bonding[9]. The absence of a significant effect of the salt concentration alone on the binding of some of the proteins by the strongly hydrophobic *n*-octyl- and phenyl-butyl-derivatives (Figs. 1 and 2) indicates that in these cases hydrophobic binding occurs even without the aid of the salt.

It is of interest that hydrophobic binding apparently occurs with all of the

* Abbreviations: EG = ethylene glycol; DMF = dimethylformamide; PBA = 4-phenyl-*n*-butylamine; BSA = bovine serum albumin; OV = ovalbumin; ChT = α-chymotrypsin; ChTng = chymotrypsinogen A; γ-G = (immuno)-γ-globulin; LG = β-lactoglobulin; Hglb = hemoglobin; RNase = ribonuclease A; DNase = deoxyribonuclease I.

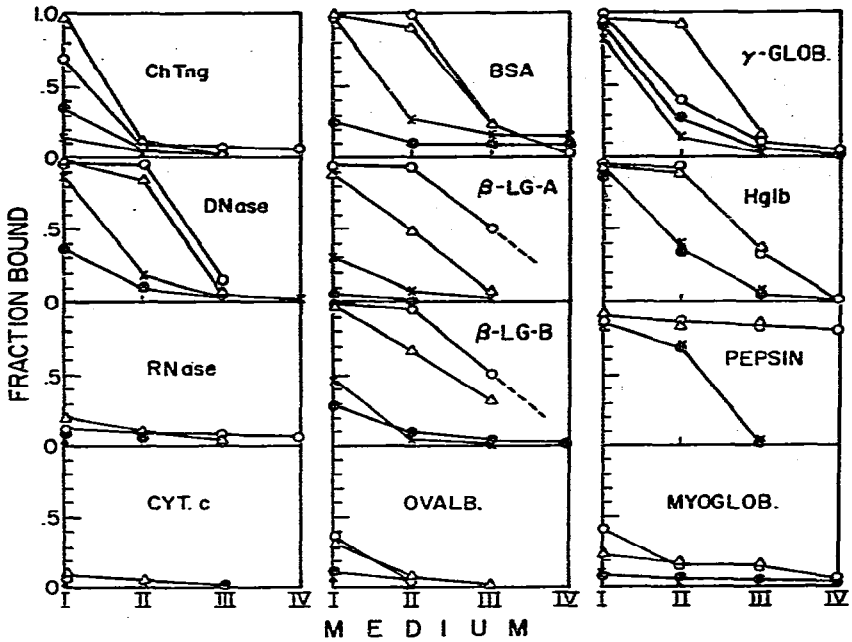

Fig. 1. Relative extents of binding of various proteins by phenyl-ethyl- (●), *n*-hexyl- (×), 4-phenyl-*n*-butyl- (△) or *n*-octyl- (○) amino agaroses. After application of a protein (see *Methods)*, the column was washed successively with 8 bed volumes of 3.0 *M* NaCl (I), 8 bedvolumes of 0.3 *M* NaCl (II), 6 bedvolumes of 50% 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). In the case of β-lactoglobulin A or B, the application of medium IV resulted in a turbid filtrate.

proteins tested, although to different extents. Less than 2% of any of the applied proteins (with the exception of γ-G, see ref. 23) was bound by columns of aged CNBr-activated but unsubstituted agarose, even in the presence of 3 *M* NaCl. Whenever tested, the same was true for untreated agarose (see ref. 34). This further confirms the generality of the accessibility of hydrophobic groups on native proteins under mild conditions[35] and the general applicability of protein separation by hydrophobic adsorption (affinity, interaction) chromatography[25].

Modifying factors in hydrophobic interactions

Aromatic (π–π) and "specific" n-alkyl-effects. Although the degree of substitution of the phenyl-ethyl-NH-agarose of Table I is slightly lower than that of the *n*-C_6-adsorbent and the degree of its hydrophobicity is about the same (see Experimental), ChTng and also γ-G to some degree, are more extensively bound by the aromatic ligand. At least for ChTng, a similar effect is evident from a comparison of the *n*-octyl- and phenyl-butyl-NH-adsorbents. Of the twelve proteins tested, ChTng, γ-G and possibly RNase are the only ones that show this effect.

With the much more highly substituted glycidyl-agaroses the phenyl-effect of ChTng seems to be greatly increased and also appears to manifest itself in the case of DNase and even for OV. In fact, binding of DNase by the phenyl-ligand is more extensive than that by the much more hydrophobic and equally highly substituted

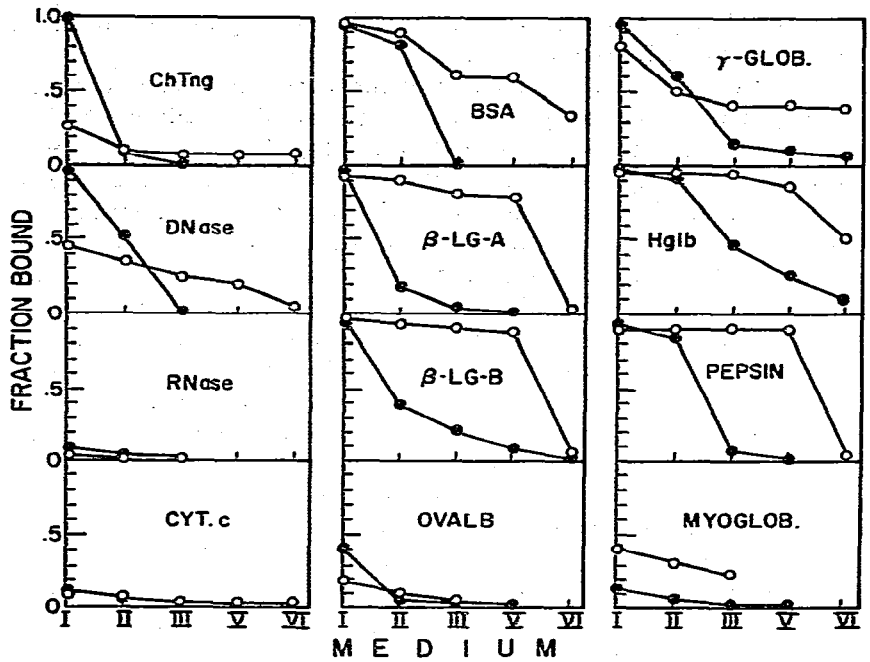

Fig. 2. Binding of the proteins of Fig. 1 by phenyl- (●) and by *n*-octyl- (○) glycidyl-agaroses. The washings with media I, II, and III (see legend to Fig. 1) were followed by 4 or 6 bedvolumes of 50% dimethylformamide in 0.3 *M* NaCl (V) and subsequently by 4 or 6 bedvolumes of 7 *M* urea in water (VI).

TABLE I

PROTEIN BINDING BY SUBSTITUTED AGAROSES

Binding (%) of 2 mg of various proteins by 1-ml columns of *n*-hexyl (C_6)-, phenyl-ethyl (C_2-phe)-, *n*-octyl (C_8)- or 4-phenyl-*n*-butyl (C_4-phe)-amino-agaroses and by 3–4 times as highly substituted *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.

Protein	*Amino-agaroses*				*Glycidyl-agaroses*	
	n-C_6	*C_2-Phe*	*n-C_8*	*C_4-Phe*	*n-C_8*	*-Phe*
BSA	100*	28	100	100***	98	95***
Hemoglobin	96	88	97	95	98	98
DNase	86*	36	97	99***	47§	97***
Pepsin	86	87	87	87.5	90	91
γ-Globulin	84	96**	97	95***	81§	98***
β-Lactoglobulin	45*	28	98	100***	97	97***
β-Lactoglobulin A	31*	6	95	91***	98	96.5***
ChTng A	18	37**	70	100**	28.5§	100**
RNase	8.5	9.5	12	18**	4	10**
Ovalbumin	8	10	33	28	23§	41
Myoglobin	6	6	39*	24.5	39*	23
Cytochrome *c*	2	3.5	8	10	10	12

* Preference for *n*-alkyl-ligand.
** Preference for aromatic ligand.
*** Preference masked by high hydrophobicity and/or high ligand density.
§ Binding by *n*-octyl-adsorbent inhibited at high ligand density.

n-C_8-derivative. It is more likely, however, that at least the binding of the enzyme is inhibited by the high ligand density (see below).

The relative extent of binding of different proteins, even when the interaction is purely hydrophobic, is not necessarily a true measure of their relative hydrophobicities. For instance, as can be seen from Table I and Fig. 1, with the *n*-C_8-NH-adsorbent the two forms of LG appear as hydrophobic as BSA, but in the case of *n*-C_6-NH-agarose, the binding of the albumin in 0.3–3.0 *M* NaCl far outstrips that of the lactoglobulins. It is also noted that the extent of binding of BSA by the *n*-C_6-derivative, as well as that of the two lactoglobulins, is several times larger than that shown by the nearly equally hydrophobic phenyl-ethyl-adsorbent. This indicates that both proteins have a preference for n–alkyl groups (for the albumin, see also ref. 36), but in the case of LG, the binding increases faster with the increase in C-chain-length than is the case for the albumin. This could mean, however, that BSA merely is more hydrophobic than LG. On the other hand, for Hglb and pepsin, the binding appears to depend on hydrophobicity of the adsorbent *per se*, regardless of whether or not the hydrophobicity is derived, in part, from a phenyl group.

The need for comparative studies under varying conditions to determine binding preference with respect to the type of ligand, *i.e.*, hydrophobic *versus* aromatic, is further demonstrated as follows. For instance, the relative extents of binding of ChTng and DNase by *n*-octyl- and phenyl-glycidyl agaroses in 3 *M* NaCl (Table I) suggest in both cases a preference for the phenyl-derivative as compared to the more hydrophobic and equally highly substituted *n*-octyl-derivative. However, with the less highly substituted *n*-hexyl- and phenyl-ethyl-amino-agaroses, the preference of DNase is for the *n*-alkyl- and that of ChTng is for the phenyl-ethyl-ligand. Taking into consideration the hydrophobicities *per se* and the relative degrees of substitution, it appears that for DNase, but not for ChTng, the preference is masked by high ligand hydrophobicity, *i.e.*, in the case of the *n*-C_8- and phenyl-butyl-NH-adsorbents.

It is emphasized that the apparent specific aromatic effect of the adsorbent phenyl-group on the binding of ChTng in 3 *M* NaCl (Figs. 1 and 2), is readily reversed by lowering of the salt concentration to 0.3 *M*. The aromatic (π–π) effect *per se* presumably is a direct electronic interaction and not a lyotropic effect as in the case of hydrophobic binding and is not necessarily affected by the salt concentration. It is possible, however, that π–π interaction and hydrophobic interaction of the phenyl-group merely reinforce each other, and that either one alone cannot produce the observed strong binding.

Effects of ligand density and of molecular size of protein. If it is assumed that the extent of binding is determined only by the ligand proper and not by its mode of attachment to the agarose matrix, the effect of the ligand concentration on the binding of the proteins can be determined from a comparison of the results with the *n*-octyl-amino- and the more highly substituted *n*-octyl-glycidyl-agaroses (Table I, Figs. 1 and 2). Such a comparison of the extents of binding in the region of 3.0–0.3 *M* NaCl might indicate that under the experimental conditions the higher ligand concentration has little effect on the binding of most proteins and sometimes even is inhibitory, *e.g.*, for ChTng, DNase and possibly for γ-G and OV. However, it may also be noted that the extent of reversal, in particular by 50% EG (medium III), most often is much greater for the less highly substituted amino-agarose, indicating weaker binding.

It is evident that the opportunity for multiple point interaction and thus the

extent and strength of binding of a protein molecule by an adsorbent, depends on the molecular size of the protein and on the density of the interacting sites of the two reactants. However, once all of the pertaining protein groups are in contact with the corresponding adsorbent sites, an increase in the density of the latter would have no further positive effect. It may be noted (Table I) that the extents of binding of RNase, cytochrome *c* and myoglobin, all of relatively low molecular weight, generally are not less for the *n*-octyl-NH- than for *n*-octyl-glycidyl-agarose. This suggests that the relatively low extent of binding of these proteins is not related to their relatively small molecular size*. Nonetheless, in order to counteract the effect of molecular size on the extent of binding of proteins of very low MW it is expedient to use relatively highly substituted adsorbents to ensure participation of all of the pertaining protein binding sites.

Efficacies of eluting solutions. Even more generally shown by the present data than the eluting effect of lowering the NaCl-concentration is the effect of EG, which most often greatly weakens the binding further, but by no means always to the same extent for all of the proteins tested. For instance, in contrast to any of the other proteins, hardly any pepsin is released from *n*-C_8- or phenyl-butyl-NH-agarose by any of the applied eluants, although complete elution by 50% EG readily occurs in the case of the *n*-C_6- and phenyl-ethyl-NH-agaroses (Fig. 1, medium III). Furthermore, although pepsin is not removed from the highly hydrophobic amino-agaroses by *n*-octylamine (Fig. 1, medium IV), elution of this protein from C_8-glycidyl-agarose readily occurs with 7 *M* urea (Fig. 2, medium VI). However, the opposite seems to be true for the case of Hglb.

It is of interest that the application of 50% DMF (medium V) after EG has little or no further effect on the binding of most of the proteins by the *n*-octyl-adsorbent of Fig. 2, even when considerable amounts of protein still are bound. Subsequent addition of 7 *M* urea is very effective as an eluant for the β-lactoglobulins and pepsin, moderately effective for BSA and hemoglobin, but in the case of γ-globulin has almost no further effect beyond that of EG or DMF. This difference could be related to the relative stability of the protein structures against urea. In any event, the elution patterns of Figs. 1 and 2 are more or less characteristic for each protein. This is emphasized by the fact that aside from the indication of a slightly higher hydrophobicity of the B-form, the patterns for β-lactoglobulins A and B are about the same and different from those of all of the other proteins. On the other hand, a similarity between the patterns of DNase and BSA would indicate a similarity in the availability

* Multiple point attachment can only occur when the distance between the adsorbent-bound ligand molecules is smaller than the diameter of the protein molecule. Calculations based on even distribution of the ligand molecules over the solid material of the gel would indicate that this condition is not fulfilled for the proteins with the smallest molecular weights applied to the adsorbents of Fig. 1. The finding that in the present case an increase in the ligand content, nonetheless, does not increase the extent of binding can be explained on the basis of heterogeneity of binding sites (*e.g.*, see refs. 25 and 37). For instance, a cavity on the absorbing surface of a size and shape similar to that of the protein molecule would offer better opportunities for attachment than a flat or a protruding area. Thus, at least with subsaturating amounts of protein, the possibilities for multiple point attachment would be better than indicated by the calculated average distance between ligand molecules.

of hydrophobic groups on the molecular surfaces of these otherwise disparate proteins.

Applications of the observed "specific" effects to protein separation by hydrophobic adsorption chromatography

Since the modifying factors discussed above often affect different proteins to different extents, they may contribute a degree of "specificity" to the binding. Although this is not conducive to the exact determination of relative protein hydrophobicities from the extents of binding, these factors may, on the other hand, provide additional parameters for protein separation by hydrophobic adsorption chromatography. In summary, such possible applications are: (1) The relatively strong aromatic effect noted in the binding of ChTng (Table I, Figs. 1 and 2) could be exploited in the isolation of this and possibly other proteins by hydrophobic adsorbents carrying aromatic groups (see also ref. 38); (2) The apparent preference of serum albumin for straight chain hydrophobic- as opposed to phenyl-groups and the opposite being true for the case of γ-G (Table I, Fig. 1), may be used for the separation of these two proteins in blood serum. In fact, this has already empirically been applied to the separation of these proteins[15,16]. (3) The observation of a certain degree of specificity of binding with respect to the C-chain length of immobilized *n*-alkyl-groups is another factor that could be of use in the separation in certain cases. For instance, although BSA and LG both are strongly bound by the *n*-octyl-NH-adsorbent (Fig. 1), the former is much more strongly bound by the corresponding *n*-hexyl derivative than the latter. This merely emphasizes previous observations[16,25] that for determination of relative hydrophobicities and for protein separation purposes differential adsorption on several adsorbents of different hydrophobicities is preferable to differential elution from one adsorbent of arbitrarily chosen hydrophobicity. (4) Comparison of the *n*-C_8-NH-adsorbent of Fig. 1 with the *n*-C_8-glycidyl derivative of Fig. 2 also shows a degree of specificity of binding with respect to the ligand concentration. For instance, by lowering of the salt concentration γ-G is more readily eluted than BSA from the *n*-octyl-NH-adsorbent, whereas the difference is much less in the case of the more highly substituted *n*-octyl-glycidyl-derivative. From a comparison of Figs. 1 and 2, and as already noted for ChTng and DNase, it can be seen that in 3 *M* NaCl some proteins, in contrast to others, are to varying degrees more extensively bound by the less highly substituted *n*-C_8-adsorbent. As suggested above, this could merely be the result of inhibition by excess ligand hydrophobicity. In any event, the findings suggest the possibility of protein separation by differential adsorption on preparations carrying the same ligand but in different concentrations, which might also be applicable to ion exchange chromatography. (5) The observed opposite effects of urea and of *n*-octylamine on the elutions of Hglb and pepsin demonstrates another type of "specificity" that could possibly be exploited in certain cases.

DISCUSSION

"Irreversible" versus reversible protein binding

With respect to protein adsorption by solid materials the term "irreversible" refers to the absence of noticeable protein release by washing with large amounts of the ambient medium over long periods of time (days, weeks). However, the distinction

between reversible and "irreversible" binding is not a sharp one. In intermediate cases, the protein may be released at a slow but noticeable rate*. Particularly in such cases the results depend greatly on the experimental conditions. For instance, the sequence of several of the proteins of Table I arranged according to their extent of binding by the column of *n*-C_6-NH-agarose is not identical with such an arrangement based on the results with the same proteins and the same adsorbent presented previously[35]. This earlier investigation (see also ref. 23), which was designed to demonstrate the generality of hydrophobic protein binding in 3.3 *M* NaCl rather than to determine relative hydrophobicities *per se*, was carried out with about the same amounts of the proteins as used in the present investigation but applied to a 25-ml column and the wash-in with the strong NaCl solution was with only 2–3 bedvolumes (as compared to 1-ml columns and washing with 8 bedvolumes as in the present case). Thus, the previous studies may have emphasized binding of some of the less hydrophobic proteins. It is imperative, therefore, that for comparative purposes the binding studies are carried out under identical conditions. This requirement is fulfilled especially for the applied procedure (see Experimental), whereby a large number of columns can be treated exactly the same.

In any event, under favorable conditions a protein may practically be immobilized by mere adsorptive (*i.e.*, non-covalent) interaction with a properly substituted matrix.

Adsorptive protein immobilization

The interactions of proteins with ligands covalently bound to an insoluble matrix are virtually of a different nature than the corresponding interactions in free solution. In the latter case, the interaction usually is a "one-to-one" process, whereas in the case of the immobilized ligands opportunities obtain for multiple point attachment of the protein to the solid matrix, which may add orders of magnitude to the stability of the complex.

In the present as well as in previous investigations[37], many instances of immobilization of proteins through non-ionic adsorption with substituted agaroses were encountered. Virtually irreversible binding because of the "absence of finite equilibrium" also has been noted in the case of protein interaction with ion exchangers[40]. With respect to the present results, the extent of this effect, aside from the presence of salt, appears to depend on (1) the degree of substitution of the adsorbent, *i.e.*, the ligand density; (2) the number (density) of accessible hydrophobic and/or aromatic sites on the proteins and (3) the degree of hydrophobicity of the interactants. However, other factors, such as the above mentioned hydrogen bonding and metal chelate affinity, may add to the stability of this type of binding *in vivo*.

Although most of the present investigations were carried out at relatively high (non-physiological) ionic strengths in order to quench ionic effects and simultaneously to enhance hydrophobic interactions, at physiological salt concentrations ionic interactions also may play a role, in particular when the charges are shielded by hydrophobic groups[14]. Furthermore, specific effects, *e.g.*, with respect to aromatic groups or ligand *n*-alkyl-C-chain length (Table I), in addition to complementarity

* Conversely, it has been observed that under conditions that favor binding the association process also may be extremely slow[39].

(molecular "fit"), also may enhance the stability of binding and further obviate the need for high salt concentrations. The occurrence of such specific interaction with groups outside the active center of an enzyme is suggested by the above results with ChTng and will be elaborated upon elsewhere.

As pointed out previously[37], the phenomenon of multiple-point "irreversible" attachment could have far reaching biological implications. This type of binding could not only have a bearing on the attachment of proteins to cell membranes and on the formation of the membranes themselves but also may be involved in the stabilization of other macromolecular structures such as viruses and other protein-nucleic acid complexes which usually depends on adsorptive rather than co-valent interaction (for artificial complexes of this type, see refs. 41–44). The view that the protein-matrix interactions of the present and previous investigations may be looked upon as "models" for the formation of such structures *in vivo* is supported by earlier observations on the "irreversible" binding (immobilization) of several intracellular enzymes by substituted agaroses and in particular by the finding that the binding often occurs with retention of activity[45]. Complexes of this type are also reminiscent of the "far from equilibrium" structures assumed to underlie the formation and evolvement of living systems[46].

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