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HYDROPHOBIC EFFECTS IN ADSORPTIVE PROTEIN IMMOBILIZATION

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INTRODUCTION

In an aqueous environment, noncovalent polar bonding (i.e., through ionic, charge transfer, or hydrogen bonds) may be assumed to be relatively weak because of the strong charge-solvating and hydrogen-bonding ability of water. It has been suggested, therefore, that apolar (hydrophobic) forces may be the most important single factor providing the driving force for noncovalent intermolecular interactions in an aqueous milieu [1, 2]. However, in aqueous solutions electrostatic interaction may become important when the pertaining charges are shielded by accompanying hydrophobic groups from the quenching effect of water [1]. In this connection it may be pointed out that, on account of the low dielectric constant of the hydrophobic interior of a protein, intramolecular interaction of oppositely charged surface groups can be strong indeed [3].

Shielding from the quenching effect of water may also be assumed to occur when the binding of a particular molecule takes place in a complementary cavity of a larger entity, e.g., in the case of the interaction of a substrate with an

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[1] With the technical assistance of N. Frank Otillio.

enzyme or of the binding of a protein in a cavity on a solid surface (see below). Furthermore, it is possible that the interaction of two oppositely charged molecules, in both of which the charge is shielded by a hydrophobic group, may be enhanced by a mutual reinforcing effect of (long-range) electrostatic and (short-range) hydrophobic forces. From these points of view the two types of interaction may be of equal importance in biochemical processes.

Enhanced binding through the postulated shielding of charges by hydrophobic groups is a phenomenon entirely different from the formation of hydrophobic "bonds." The former remains essentially electrostatic and can be reversed by salt, whereas hydrophobic bond formation may be stabilized by salt, e.g., NaCl in concentrations of 1 to 4 M [4-10]. There is no reason to assume that in the interaction of oppositely charged compounds with relatively large hydrophobic groups the two effects could not occur at the same time.

The study in aqueous solution of hydrophobic bond formation between compounds with large hydrophobic groups has been hampered by the inherently low solubility of such compounds. Chemical studies in general can be properly carried out only with the interacting compounds in a state of molecular dispersion. However, highly hydrophobic and insoluble compounds may be molecularly dispersed in an aqueous milieu by their covalent attachment to an insoluble but hydrophilic (wettable) matrix. Although adsorbents with hydrophobic groups have previously been applied to chromatographic separations (for literature quotations, see Ref. 9), systematic studies of hydrophobic phenomena by means of such adsorbents and initiated by Yon [11] have been made only relatively recently [4-13].

Because of recent advances in the preparation and chemical substitution of polymers, many compounds can be attached to a hydrophilic carrier suitable for chromatographic procedures, e.g., beaded agarose [14-17]. A major part of the work on

protein binding thus far has been carried out with agarose substituted via CNBr activation with various ligands carrying primary amino groups. In the final product the amino group retains its basic properties [18] and is positively charged at the pH values at which most of the experiments usually are carried out.

A charge on the adsorbent is necessary for study of combined hydrophobic and electrostatic effects. Furthermore, such a charge may be assumed not to interfere with studies of true hydrophobic bonding which is favored at high salt concentrations where charge effects generally would be quenched. Nevertheless, it is of interest to carry out studies with adsorbents that are uncharged [10, 19] or where the positive charge on the agarose-bound amino group is neutralized by the negative charge of an ionized carboxyl group, as in the case of a substituting amino acid [20, 21].

The current series of investigations [4-8, 20, 21] was originally started on the basis of the conclusion that hydrophobic forces are involved in the interaction of the active site of many enzymes with their substrates or other specific ligands [22]. In view of the generally nonspecific nature of hydrophobic effects per se [23], it was postulated [4] that the apparently accessible hydrophobic sites in the active center would exhibit nonspecific affinity for many other hydrophobic ligands. In the course of these investigations it became apparent, however, that depending on the hydrophobicity of the immobilized ligands most of the proteins that were tested, including BSA,[2] γ-G, and β-LG, were capable of forming

[2] The abbreviations used are: BSA, bovine serum albumin; OV, ovalbumin; γ-G, 7S γ-globulin; β-LG, β-lactoglobulin; EG, ethylene glycol; A-C_{1-8}, adsorbents prepared from CNBr-activated agarose (Sepharose 4B) through substituion with an n-alkylamine with a C-chain length varying from C_1 to C_8; A-PBA, agarose substituted via CNBr-activation with 4-phenyl-n-butylamine: XO, xanthine oxidase; LDH, lactate dehydrogenase.

salt-stable hydrophobic bonds with n-alkylamine-substituted agaroses. These findings are in accord with recent observations [21, 24] indicating frequent occurrence of hydrophobic groups on the surface of protein molecules. It appears that the occurrence of such groups is not limited to relatively small ones such as the $-CH_3$ side-chain of alanine, but includes the larger hydrophobic side-chains such as those of phenylalanine and tryptophan as well [24]. In any event, the possibilities for hydrophobic bonding of proteins by immobilized hydrophobic ligands are much more widespread than originally surmised. In fact, it appears now that hydrophobicity is a general characteristic of proteins and at least in the case of enzymes, may play an important role in their attachment to intracellular matrices as well as in their biological function per se. With respect to chromatographic protein purification, hydrophobicity may be of equal importance as properties such as isoelectric point or molecular weight which underlie ion-exchange and exclusion chromatography, respectively [4, 20].

GENERAL EXPERIMENTAL APPROACH AND OBJECTIVES

The investigations described below deal with the binding of highly purified proteins by agarose (Sepharose 4B) substituted with ligands carrying hydrophobic groups. Studies with known proteins are prerequisite to possible applications of hydrophobic phenomena, e.g., in new procedures for the separation of proteins in mixtures of unknown composition [4, 20], or in adsorptive enzyme immobilization for reactor-type experimentation [6]. For this reason, investigations have been made of factors such as the degree of hydrophobicity and the electric charge of the protein and of the adsorbent. Studies have also been made of the apparent "irreversibility" of binding (immobilization) and possible inhomogeneity of the adsorbent binding sites and of the relationship of these factors to the degree of substitution of the adsorbents. Experimental details

are described in previous publications [4-8] and in the legends to the figures presented below.

Frequent use is made of the known difference in hydrophobicity of OV and BSA [25]. OV has little or no ability to form hydrophobic bonds stable at high salt concentrations. By contrast, BSA readily forms such bonds. Other proteins that are relatively hydrophobic in this respect are γ-G and β-LG [7,25]. It is a rather simple matter to separate BSA and OV on the basis of hydrophobicity (see Ref. 4), but it is much more difficult to attain good separation of the more hydrophobic proteins from each other. In order to achieve this goal, suitable adsorbents and eluants must be found and the proper experimental conditions established.

On the basis of the finding that certain salts stabilize hydrophobic bonding [26], separation should in principle be obtained by means of a gradient of decreasing concentration of such a salt (e.g., see Ref. 10). However, in order to reverse hydrophobic bonds and also to circumvent possible electrostatic binding at the lower salt concentrations, it would seem logical to apply a gradient of increasing concentrations of a "chaotropic" agent [27]. A difficulty here is the apparent nonuniformity of the binding of a pure protein by a particular adsorbent [4, 7] which also was observed with several ion-exchangers [28-30]. Although this difficulty does not apply to reactor-type experiments in which enzymes are immobilized through adsorption by certain substituted agaroses [6], it must be resolved before application of hydrophobic phenomena can be made to efficient chromatographic protein separation.

Most often the adsorbents used were prepared by substitution of CNBr-activated agarose [15, 16, 31] with n-alkylamines of varying C-chain lengths. As was noted, the bound amino group retains its basic properties. In some experiments the adsorbent was DEAE-agarose (Bio Rad) where the ligand is bound to the agarose via an ether linkage.

A convenient means for determining the degree of substitution of the applied adsorbents has been worked out on the basis of irreversible binding of Ponceau S at low ionic strength [7]. For a homologous series of n-alkylamines (C_1 to C_8) it was found that the binding capacity with respect to saturation by the dye was proportional to the binding capacity for OV under similar conditions. The constancy of the dye:protein ratio indicates that the binding of the dye and of the protein is to the same adsorbent sites and occurs in the same or a similar manner. The great disparity in structure of the dye and of the protein emphasizes the absence of specificity of their interactions with the adsorbents.

Chromatographic analyses of protein mixtures on columns, in particular when carried out with the aid of a concentration gradient of an eluant, are best achieved by continuous application of the eluant solution and, if possible, continuous monitoring of the eluate. However, with one such set-up only one analysis can be made at the time. Chromatographic information on a single pure protein, e.g., the relative effect of a series of eluants, can be obtained much more rapidly by means of a series of identical columns eluted by manual applications of the eluant and manual assay of the eluates, e.g., measurement of protein content by spectrophotometry. A major portion of the data discussed below were obtained through the use of series of 1 ml columns in disposable pipets (Van-Lab) and provided with a small plug of glass wool. The amount of protein in the filtrates was determined from the light absorbance at 280 nm.

DUALITY OF NONSPECIFIC PROTEIN BINDING BY n-ALKYLAMINO-AGAROSES

Previous results [7], compiled in Fig. 1, indicated that the lower members (A-C_{1-6}) of a homologous series of n-alkylamine-substituted agaroses (A-C_{1-8}) strongly bind negatively charged proteins (e.g., OV, BSA, and β-LG) by electrostatic (salt

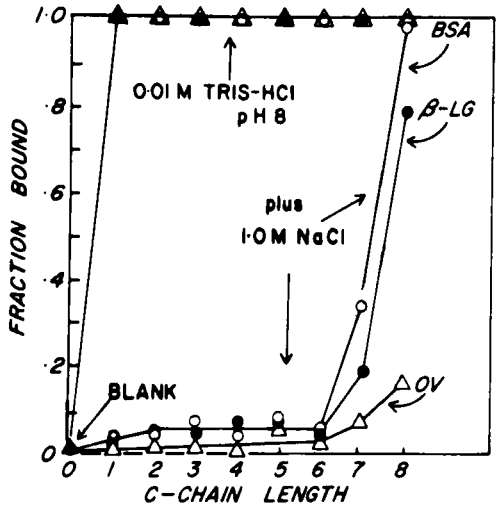

FIG. 1.
Effect of the C-chain length of n-alkylamino-agaroses on their fractional binding of bovine serum albumin (BSA), β-lactoglobulin (β-LG), or ovalbumin (OV) in 0.01 M Tris-HCl buffer, pH 8, in the absence or in the presence of 1.0 M NaCl. Aged CNBr-activated but unsubstituted agarose was used as a blank. Two milligrams of a protein were applied to a 1-ml column of an adsorbent equilibrated at ≈5° with the buffer. The fraction of the protein bound refers to "irreversible" adsorption (see text). (Data compiled from previous results [7] with permission of Publisher.)

reversible) interaction. In addition, the larger n-alkyl groups (>C_6) appear to form hydrophobic (salt stable) "bonds" with a more hydrophobic protein, such as BSA. The following presentation is prompted by the results of further studies on these two aspects of protein binding by substituted agaroses.

Electrostatic (Salt Reversible) Binding

Since the amino groups of the substituting ligands are positively charged under the experimental conditions [18], one might assume that the salt reversible binding of the applied negatively charged proteins by the short chain (<C_6) ligands (Fig. 1) could be the result of the presence of this positive

charge. Confirmation of this proposition might be derived from the fact that under the same conditions and in the absence of specific interaction, positively charged proteins are generally not bound by these adsorbents [4-7]. Also, binding occurs as readily with relatively nonhydrophobic OV as with much more hydrophobic BSA of β-LG (see Fig. 1). Furthermore, previous data [7] showed little or no difference in the salt (NaCl) concentration at which OV or BSA are eluted either from C_1 or C_4. In addition, recent observations [32] indicate that the mere treatment of CNBr-activated agarose with ammonia, that is, in the absence of any hydrophobic group, endows the material with a high capacity for electrostatic protein (OV, BSA) binding.

For the interpretation of a possible effect of hydrophobic shielding on electrostatic interactions, it should be taken into consideration that such shielding not necessarily requires the presence of hydrophobic groups on the protein. An electrostatic bond could be shielded by a hydrophobic group in conjunction with the charge on one of the reactants only. As noted above, further protection against quenching by the aqueous medium may occur when the binding is in a cavity on the adsorbent, in particular in the case of a close fit between the protein molecules and the cavity (see also below).

An entirely different interpretation of the effect of hydrophobic groups on electrostatic binding could be based on the recent suggestion by Lewin [33] that the structured water around a hydrophobic group is oriented with the hydrogen outward. This could present opportunities for hydrogen bonding and preferential binding of anions. It should be noted that also in this case binding would not necessarily depend on the hydrophobicity of the protein.

Hydrophobic (Salt Stable) Bonding

It is clear from the data of Fig. 1 that with the applied proteins and under the experimental conditions, hydrophobic

(salt-stable) "bonding" occurs primarily when the C-chain length of the adsorbent is $>C_6$. This type of binding is much stronger for BSA and β-LG than for OV. It had previously been shown by McClure and Edelman, through binding studies with naphthalene derivatives [25], that BSA and β-LG are much more hydrophobic than OV, which confirms that the binding depends on hydrophobic interaction between the protein and the ligand.

The data of Fig. 2 show that, whereas at low salt concentration (<0.1 M) BSA is bound by any of the adsorbents tested, at high salt concentration (>1 M) the extent of binding of the protein is greatest for the more hydrophobic ligands, e.g., n-C_8 > n-C_7 > n-C_6. The data suggest, furthermore, that the hydrophobicity of -DEAE is less than that of n-C_6 (see also Ref. 8) and that the hydrophobicity of the PBA-ligand is intermediate between those of the n-C_7 and n-C_8 ligands.[3] Since the extent of binding would also depend on the degree of substitution, it should be noted that the order of the latter (determined by saturation with Ponceau S, see above) was n-C_6 > n-C_7 $\gtrsim$ DEAE > PBA > n-C_8, which at least for the n-alkyl adsorbents is the opposite of the order of fractional BSA-binding (Fig. 2). Thus binding is determined by the hydrophobicity of the ligand rather than by its concentration on the matrix.

At the highest NaCl concentration (3.3 M) that was applied (Fig. 2), only the PBA, n-C_7, and n-C_8 adsorbents form the salt-stable type of hydrophobic bond with BSA. Even at this high ionic strength the immobilized n-C_6 ligand apparently forms this type of bond only with part of the protein, whereas -DEAE does not form such bonds at all under the applied conditions.

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[3] This observation is in accord with early findings [55, 56] that, with respect to micelle formation of certain fatty acid esters, the hydrophobicity of the benzene ring corresponds to 3 to 4 straight chain methylene groups [see also Ref. 57].

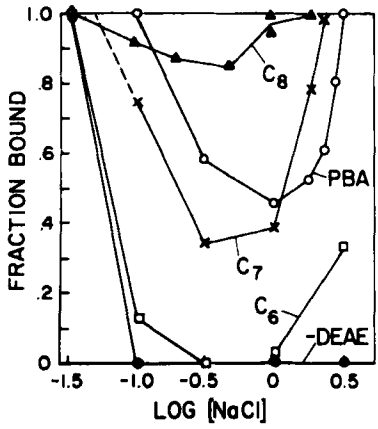

FIG. 2.
Effect of salt (NaCl) concentration on the fractional binding of bovine serum albumin by n-caprylamino(n-C_8)-, 4-phenyl-n-butylamino(PBA)-, n-heptylamino(n-C_7)-, n-hexylamino(n-C_6)-, or diethylaminoethyl(DEAE)-agaroses under the same general experimental conditions as for Fig. 1.

APPARENT "IRREVERSIBILITY" OF BINDING

More than 30 bed volumes of low ionic strength eluant can be passed through a 2-ml DEAE-agarose column loaded with 2 mg OV, without any protein appearing in the filtrate. Elution does not occur unless the ionic strength is raised to a certain level. Earlier results [7] showed that this also holds true for n-alkylamine-substituted agaroses. Furthermore, the data of Fig. 3A indicate that 1 mg OV remains bound on a 2-ml A-PBA column after washing with more than 600 ml (≃ 300 bed volumes) of 0.01 M Tris-HCl buffer, pH 8, over a period of ≃ 15 hr. Also, the elution curve is not significantly affected by the amount of low ionic strength buffer that is washed through the loaded column prior to starting the salt gradient. Furthermore, large differences in the rate at which the salt gradient is applied (from ≃ 10 to ≃ 40 ml/hr, Fig. 3B) have only relatively little effect on the results. Thus under the ambient conditions the strength of binding is tantamount to immobilization of

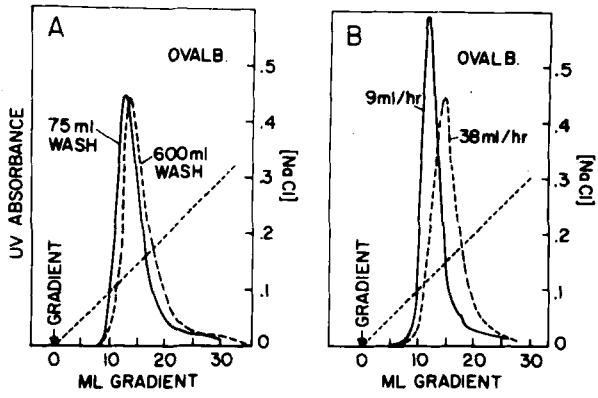

FIG. 3.

Elution pattern (A_{222}) of ovalbumin from 4-phenylbutylamino-agarose. One milligram of the protein was applied to about 2 ml of the adsorbent in a 5-mm wide column equilibrated at ≈5° with 0.01 M Tris-HCl, pH 8, and eluted by means of a linear NaCl gradient in the buffer. (A) Effect of washing of the loaded column with buffer alone prior to applying the NaCl gradient. The washing was either with 75 ml (solid curve) or with 600 ml (dashed curve) of the buffer. The flow rate in each case was ≈40 ml/hr. (B) Effect of the flow rate of the salt gradient either 9 ml/hr (solid curve) or 38 ml/hr (dashed curve).

the protein. This phenomenon has been applied to the immobilization of enzymes in reactor-type experiments (Ref. 6, see also below).

It would seem unlikely that the observed strong binding by mere adsorption could be the result of a single bond per protein molecule, even if the bond were formed through the combined effects of hydrophobic and electrostatic forces. Evidence has been presented that nonspecific binding as opposed to the "one-to-one" specific type of binding, such as that of a substrate by the active center of an enzyme, sharply decreases when the degree of substitution of the adsorbent is lowered to the point where the distance between the ligand molecules is larger than the diameter of the protein molecule [34]. Therefore, as also suggested by others [28, 29] it is

assumed that strong nonspecific binding is the result of multiple point attachment of the same protein molecule to several ligand molecules on the matrix. That the number of binding sites involved need not be large is indicated also by the fact that binding of Ponceau S by these adsorbents is "irreversible." The molecule of the dye contains only four negative charges in conjunction with hydrophobic groups. In any event, it appears that the simultaneous interaction of only a few sets of opposite charges may result in strong, often virtually irreversible, interaction.

It should be emphasized that the term "irreversible" is used here in a strictly experimental sense. The term merely indicates that under the applied conditions the protein cannot be washed off the column by the ambient medium without changing the composition of the latter. Evidently, in adsorptive binding an equilibrium must obtain between bound and unbound protein. However, the equilibrium apparently is often in favor of binding, even to the extent that no free protein can be detected by the applied procedures.

FACTORS UNDERLYING APPARENT INHOMOGENEITY OF BINDING OF PURE PROTEINS

Inhomogeneity of Adsorbent Binding Sites

Previous results [7] indicated that the homogeneity of a particular preparation of OV seemingly was not always the same with respect to column chromatography on an adsorbent such as n-butylamino-agarose. The elution pattern depended on the protein:adsorbent ratio (i.e., the protein load) and the degree of substitution. These observations are further substantiated by the results of Fig. 4 where DEAE-agarose was saturated with OV in 0.001 M Tris-HCl buffer, pH 8, and washed with the buffer alone until no protein could be detected in the filtrate. The data indicate that a small fraction of the

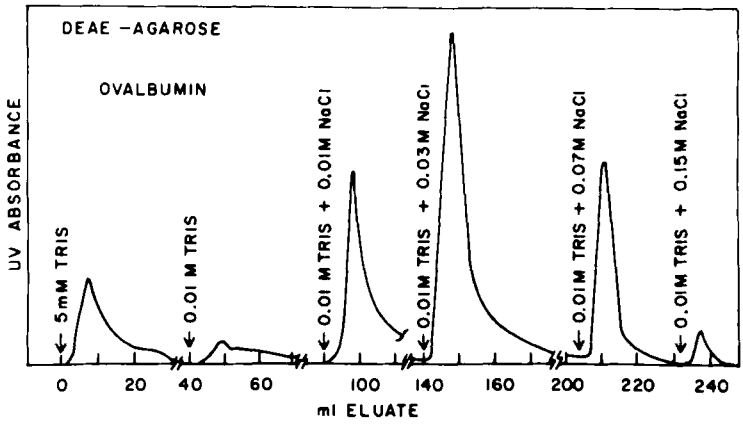

FIG. 4.

Elution patterns (A_{280}) of ovalbumin (5X crystallized and nearly homogeneous by isoelectric focusing) from DEAE-agarose by means of stepwise increase of the salt concentration. A saturating amount of the protein (≈75 mg) was applied to a 5 X 100 mm column of the adsorbent equilibrated at ≈5° with 1.0 mM Tris-HCl buffer, pH 8. About 60 mg remained bound upon subsequent exhaustive washing with the buffer alone. The stepwise applications of eluant solutions of varying composition are indicated by the arrows. (From previously published data by author [8]).

OV bound in 0.001 M Tris can be eluted with 0.005 M buffer, after which the UV absorbance of the filtrate returns to base line. Another small fraction can be eluted with 0.01 M buffer, and more is eluted by stepwise increase of the ionic strength of the eluant until no protein is left on the column. With respect to isoelectric focusing, the 5X crystallized OV used here showed only one major and one minor fraction [35].

It is important to note (Fig. 4) that after each successive step the protein content of the eluate returns to zero. Although this point often is reached very slowly, it indicates that at the pertaining level of the salt concentration the remaining protein is "irreversibly" bound and, under the ambient conditions, in fact, is immobilized on the column. Thus these results confirm previous indications that

these as well as other types of adsorbent [see Ref. 28] may be endowed with binding sites having varying degrees of affinity for a particular protein and that, at least with high protein loads, apparent inhomogeneity may indicate inhomogeneity of the adsorbent binding sites rather than of the protein. Similar conclusions have been reached by Hixson and Nishikawa [36] and by Nishikawa et al. [37].

A consequence of the inhomogeneity of binding sites would be that not only the binding capacity of an adsorbent but also the strength of binding of a fixed amount of protein would depend on the number of available binding sites, i.e., on the amount of adsorbent applied and on its degree of substitution. The data of Fig. 5 show that about 65% of the applied amount of BSA is washed off by 0.33 M NaCl from a 1-ml column of n-octylamine-substituted agarose. The degree of substitution of the adsorbent was relatively low and the applied salt concentration unfavorable for binding (see Fig. 2). It is of interest, however, that under the same conditions all of the protein remains irreversibly bound by a larger(5 ml) column. If only one type of binding site were involved, one would expect that the extent of the elution by the same number of bed volumes of the eluant (0.33 M NaCl) would be the same for the two columns. The results clearly show that this is not the case. Apparently only the larger column contains a sufficiently large number of "strong" binding sites to bind all of the protein irreversibly under the applied conditions.

It should be noted that for both columns the major portion of the protein that remains irreversibly bound in 0.33 M NaCl can subsequently be eluted by including ethylene glycol in the eluant (see below). Thus these data further confirm the proposition that nonuniformity of the adsorbent binding sites may result in apparent chromatographic inhomogeneity of a pure protein. It may be assumed that the surface of the adsorbent matrix is irregular and presents cavities of varying size and shape. An extremely tight binding would occur when such a

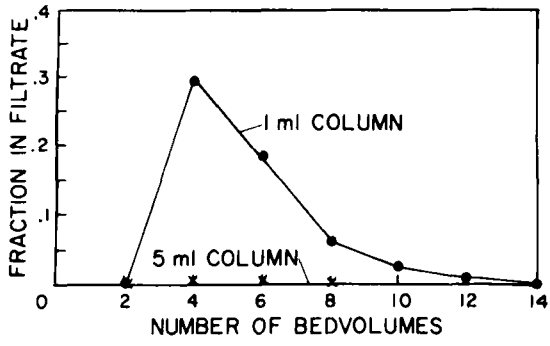

FIG. 5.

Effect of the adsorbent:protein ratio on the strength of binding of bovine serum albumin by n-octylamino-agarose. The two columns (1 and 5 ml, both about 4 cm high), were equilibrated at ≈5° with 0.33 M NaCl in 0.01 M Tris-HCl, pH 8, and each was charged with ≈1.7 mg of the protein, followed by washing with the ambient NaCl-buffer solution. The volumes of the filtrates are expressed as the number of bedvolumes for each column, i.e., as multiples of 1 or 5 ml, respectively.

cavity contains several ligand molecules properly placed and provided with the proper charges and/or hydrophobic groups, in particular when the shape and size of the cavity fits that of the protein molecule (see also Refs. 36 and 37). In this manner simultaneous binding of a protein molecule with several ligand molecules can take place in a three-dimensional fashion.

The data are consistent with the assumption of a wide range of binding sites of varying binding strengths but nevertheless all being able to bind protein "irreversibly" under the proper experimental conditions (see also below).

Simultaneous Salt Reversible (Electrostatic) and Salt Stable (Hydrophobic) Binding.

When 3 mg BSA is applied to a column of 5 ml PBA-agarose, part of the protein is removed in a 0 to 1.0 M NaCl gradient. However, a second fraction can subsequently be eluted by a solution of 50% ethylene glycol in 1 M NaCl. By contrast,

under the same conditions all or nearly all of the much less hydrophobic OV can be eluted by salt alone [4]. The fact that elution of the second fraction of BSA requires an agent such as ethylene glycol suggests that hydrophobic bonding is involved [4]. This type of apparent inhomogeneity is not necessarily the result of inhomogeneity of the adsorbent binding sites, which most often manifests itself primarily at much higher protein loads than were applied here. A possible interpretation is as follows: At low ionic strength the negatively charged BSA binds irreversibly through electrostatic interaction as discussed above. As the salt concentration is raised, this type of binding is reversed and the protein begins to be released. However, at the same time "true" hydrophobic binding, which increases with the salt concentration (see Fig. 2), becomes more and more predominant. Thus part of the protein would remain on the column by virtue of true hydrophobic bonding at the higher salt concentrations and can then be removed by an agent that weakens hydrophobic bonds (e.g., ethylene glycol).

ADSORPTIVE IMMOBILIZATION OF ENZYMES

General Observations

The electrostatic and the salt-stable hydrophobic type of protein binding are both of an unspecific nature and most probably involve charged and/or hydrophobic groups that could occur on the molecular surface of any protein [21, 24]. The nonspecificity of the binding is emphasized by the finding [7] that binding by the present adsorbents of a protein such as OV or a dye such as Ponceau S occurs in a similar fashion. From this it follows that adsorptive binding of a biologically active protein, e.g., an enzyme, may occur through a site or sites other than the active center. In fact, the binding of enzymes to intracellular matrices, which does not seem to depend on covalent bonds, must occur in a manner that leaves

the active center free to react with substrate. One could even speculate that the in vivo binding of such enzymes occurs through electrostatic and hydrophobic forces similar to the binding of BSA or OV to the present adsorbents. There is considerable evidence that both these forces indeed play a role in the intracellular binding of certain enzymes [2, 38]. Although presumably of the adsorptive type, the binding nevertheless appears to be extremely strong and virtually "irreversible" under intracellular conditions.

Strong adsorptive binding of enzymes to a variety of artificial solid matrices has been observed on many occasions (e.g., for reviews see Refs. 39 and 40). In view of possible leakage problems, the reviewers express some skepticism as to the use of this type of binding in enzyme "reactors." However, thus far attempts at adsorptive binding have been mainly empirical. The more systematic studies discussed above show virtually irreversible binding of enzymes and other proteins by agaroses substituted with primary amines carrying hydrophobic groups. A crucial factor, in addition to the presence of hydrophobic groups, appears to be a high degree of substitution, allowing multiple point attachment of the protein molecule to the supporting matrix (see also below).

Another point that should be taken into consideration here is the evidence that a particular adsorbent appears to be endowed with binding sites that may range from extremely weak to extremely strong. Although under a given set of conditions part of the protein may be reversibly bound, it is often impossible to remove all of the protein, even by exhaustive washing with the ambient medium. Apparently a residue of the protein remains bound to the stronger binding sites. For the less hydrophobic amine-substituted adsorbents this holds true only at relatively low ionic strength, but for adsorbents with the larger hydrophobic groups irreversible binding may occur at any salt concentration (see Fig. 2). In any event if

initial leakage occurs under a given set of conditions, this does not necessarily mean that under those conditions eventually all of the protein will be washed off the adsorbent.

It is possible that under certain conditions, e.g., those required for optimum activity of an enzyme, only relatively few sites on the adsorbent bind the protein in an irreversible fashion, i.e., only a small fraction of a given amount of protein may remain bound by a given amount of adsorbent. As shown by Fig. 5 more protein will be bound irreversibly simply by increasing the amount of adsorbent.

Application to Enzyme "Reactors"

On the basis of the above considerations and in view of the nonspecific but strong binding of several proteins by the present type of adsorbent, the binding of a number of enzymes by n-butyl- or n-octylamino-agarose was investigated [6]. It was noted that in 0.01 M Tris-HCl, pH 8, several enzymes with isoelectric points in the acid region (xanthine oxidase, lactate dehydrogenase, DN-ase I, alkaline phosphatase, and urease) are strongly, virtually irreversibly, bound by these two adsorbents. By contrast, RNase, the isoelectric point of which lies at pH values >9, showed little or no binding. In the presence of 1 M NaCl, the acidic enzymes, negatively charged at pH 8, were invariably more strongly bound to A-C_8 than to A-C_4. The binding to A-C_4 of all of the enzymes could be largely reversed by 1 M NaCl, indicating a predominantly electrostatic type of interaction. On the other hand, binding to A-C_8 could most often only partly be reversed by 1 M NaCl. Little or no reversal by salt occurred for the case of DNase, although most of the enzyme could be eluted from A-C_8 by a combination of 1 M NaCl and 50% ethylene glycol. This indicates the involvement of the salt-stable hydrophobic type of binding.

The major finding of these investigations [6] was that for all of these enzymes, conditions can be found under which virtually irreversible binding (immobilization) occurs by one

or the other n-alkyl amino-agarose, often even at high ionic strength. It was also found that in most, if not all, cases the adsorbent-bound enzyme retained at least part of its activity, confirming the supposition that binding does not necessarily involve the active center. In fact, the high frequency of this noninvolvement for the five arbitrarily chosen enzymes could mean that these enzymes are endowed with binding sites, "nonfunctional" with respect to their catalytic action, but necessary for their proper attachment to intracellular matrices. In any event, protein binding by adsorbents of this type may present not only models for protein binding in vivo, but also may provide opportunities for the immobilization of enzymes in "reactor"-type experiments.

Reactor-type (long-term) experiments with several enzymes were carried out with 1 to 2 mg of, most often, highly purified enzyme preparations adsorbed on 1 to 2 ml columns of either A-C_4 or A-C_8, equilibrated with 0.01 M Tris-HCl buffer of pH 8. After exhaustive washing of the column, a substrate solution was filtered through and the presence of bound active enzyme detected by the continuous appearance of product in the filtrate during extended time periods [6].

For xanthine oxidase the substrate solution was 0.1 mM xanthine in 0.01 M Tris-HCl, pH 8, which was applied at ≈ 5° to the C_4-adsorbent containing the enzyme. It was found through measurement of the increase in absorbance at 290 nm that after establishment of a steady state, at least 90% of the substrate was converted continuously into uric acid over a time period of several days and the passage of about 500 bed volumes of the xanthine solution. Similar results were obtained with LDH and with urease. It can be seen (Fig. 6) that in the presence of 0.01 M Tris-HCl, XO, LDH, and DNase are also immobilized on DEAE-agarose. As was found with the A-C_4 adsorbent, in each case most of the enzyme protein could be eluted by 1 M NaCl.

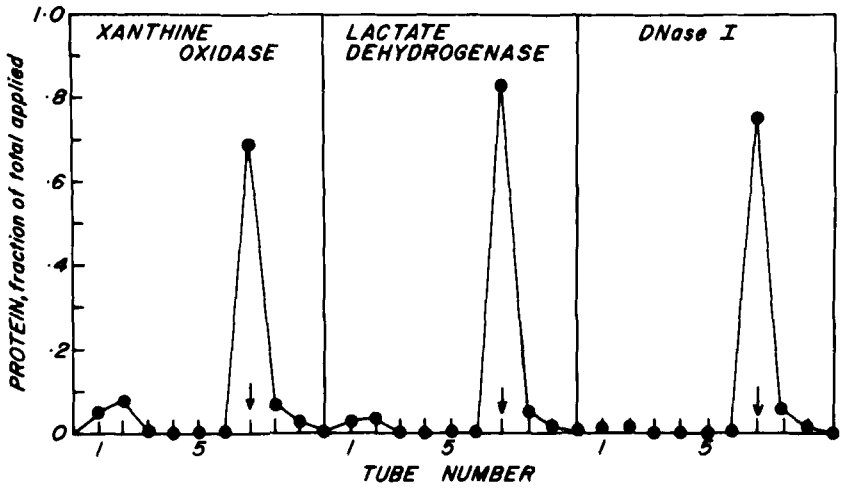

FIG. 6.
"Irreversible" adsorption (immobilization) of various enzymes by DEAE-agarose. Of the order of 1 mg of XO (Miles Seravac 36/611), LDH (Miles-Seravac 36-301), or DNase (Worthington, DSV, Standard), dissolved in 2 ml of 0.01 M Tris-HCl, pH 8, was applied at ≈5° to a 1-ml (8.7 X 16 mm) column of the adsorbent which then was washed 5 times with a 2-ml portion of the buffer and subsequently 4 times with 2 ml buffer containing 1.0 M NaCl (beginning at the arrow). The protein contents of the filtrates were determined from the light absorbance at 280 nm. (From previously published results by author [8]).

The results of a reactor-type experiment with 0.5 to 1 mg of XO on a 0.5 x 10 cm cooled (≈ 5° C) DEAE-agarose column were similar to the ones with A-C_4. With moderate flow rates (e.g., 25 ml/hr), no decrease in activity was noted after more than 50 hr of continuous operation of the column during which period more than 1500 ml (>750 bed volumes) of substrate solution (0.1 mM xanthine in 0.01 M Tris-HCl, pH 8) were passed through. At this time and as determined from the UV absorbance, more than 90% of the amount of enzyme protein originally bound could be recovered by elution with 1 M NaCl. However, after this treatment the column still was enzymatically active. The data of Fig. 6 indicate that a small

part of the enzyme remains bound in 1 M NaCl, presumably due to the above discussed inhomogeneity of the adsorbent binding sites. In any event, it would seem that under the applied conditions little or no enzyme protein was lost during more than 2 days of continuous operation of the reactor.

Under conditions favorable for activity, i.e., in the presence of 0.01 M $MgCl_2$ and at pH 5, DNase (Worthington, DSV, Standard) remains bound to A-C_8 in toto and in part also to A-C_4 [6]. However, reactor-type experiments with high molecular DNA as the substrate were hampered by binding of the negatively charged nucleic acid by the positively charged adsorbent.

In the case of alkaline phosphatase (Worthington PC), the major portion of ≈ 1 mg of the enzyme preparation was bound by 1 ml of the A-C_4 as well as by the A-C_8 adsorbent in 0.01 M Tris HCl, pH 8, in the absence of additions [6]. On the other hand, most of the enzyme protein was washed off these columns by 0.01 M glycine buffer, pH 9.5, containing 0.01 M $MgCl_2$, a medium favorable for activity of this enzyme. Nevertheless, after continued washing with the glycine-$MgCl_2$ mixture until no enzyme could be detected in the filtrate, the column remained active as was indicated by the hydrolysis of o-carboxyphenyl phosphate, measured through an increase in the absorbancy of the filtrate at 300 nm [41]. Although the efficiencies gradually decreased, activity was still observed after passage of ≈ 100 and more than 300 bed volumes of glycine-$MgCl_2$ through the A-C_4 and A-C_8 columns, respectively, during a period of 16 hr at room temperature.

The strong binding of only a relatively small amount of alkaline phosphatase under conditions favorable for activity (i.e., at pH 9.5 and in the presence of $MgCl_2$) may be ascribed to the presence of only a limited number of sufficiently strong binding sites under those conditions. Apparently other sites are present that bind the enzyme strongly at pH 8 and in the absence of $MgCl_2$. In any event, the presumed inhomogeneity

of the binding sites (see above), which may be a drawback for chromatographic protein separation [4], is not necessarily a disadvantage in the use of these adsorbents for enzyme reactors. Provided the rate of the enzyme reaction is sufficiently large, no extremely large columns would be required to provide a sufficient number of "strong" binding sites in order to immobilize the relatively small amount of enzyme that would be needed. Furthermore, in view of recent developments, in particular those on the stabilization of hydrophobic binding at high concentrations of "structure forming" salts, the capacities for irreversible protein binding of an adsorbent such as A-C_8 might be considerably increased. Under such conditions, binding is also independent of the overall charge of the protein [20, 21].

Immobilization of enzymes by adsorptive means not only is less cumbersome, but also is less likely to result in irreversible alteration of the enzyme structure than may be assumed to be the case during chemical manipulation needed for covalent binding. Also, depending on the hydrophobicity of the adsorbent, the enzyme can be recovered by changing the composition of the medium.

The above noted unexpectedly high frequency of retention of activity, shown by a series of arbitrarily chosen enzymes after binding by the adsorbent, may be explained as follows: In view of the nonspecificity of the binding of protein by the present adsorbents, it can be expected that not all of the protein molecules are bound in the same manner. Binding may be assumed to be possible through several ionic and/or hydrophobic groups located in different areas on the protein surface, including the active center of an enzyme. However, if binding would occur through the active center, the presence of substrate would tend to prevent the latter mode of binding and the enzyme would tend to become attached through a site other than its active center, i.e., in a fashion not susceptible to reversal by the substrate. Thus in the presence of substrate

the enzyme would be bound preferentially in a manner in which the active center is available for interaction with the substrate. If this supposition is correct, adsorptive binding would have an additional advantage over covalent binding, because in the latter case binding involving the active center would inactivate the enzyme irreversibly.

PROTEIN SEPARATION BY HYDROPHOBIC ADSORPTION CHROMATOGRAPHY

The principle of separation of proteins on the basis of differences in their ability to form hydrophobic bonds is demonstrated by the data of Fig. 1. It can be seen that at pH 8 and in the presence of 1 M NaCl, all of the BSA is bound by A-C_8, whereas little OV is bound under such conditions. In view of the fact that the isoelectric points of the proteins do not greatly differ, separation of BSA and OV would be difficult to achieve by electrochemical procedures alone. Thus the hydrophobic factor presents an additional parameter for separation of proteins with similar electrochemical properties. The same would apply to proteins with similar molecular weights which would make them difficult to separate by exclusion chromatography.

It has been tentatively suggested [4] that the rather strong binding, e.g., of BSA by A-C_8, might be the result of cooperation between electrostatic and hydrophobic binding. The major point of evidence for this supposition was that binding of BSA to A-C_8 in 0.01 M Tris was reversed by the combination of 1 M salt and 50% EG but not by the salt alone or by the EG alone. However, from the above observations it is now clear that in the presence of 1 M NaCl alone strong hydrophobic binding is possible, whereas in 0.01 M Tris-HCl and with EG alone strong electrostatic binding takes place.

The results of Fig. 2 show overlap of the regions of the salt concentration in which the electrostatic and the true hydrophobic type of binding predominate. As a result, all or

most of the BSA remains bound to A-C_8 at any salt concentration, although in an intermediate range of the latter the binding appears relatively weak. These findings do not preclude elution by means of a decreasing salt concentration gradient, especially when protein release is enhanced by the presence of an agent such as EG. Under such conditions all of the protein may be released before the onset of electrostatic binding, which does not begin until the salt concentration has dropped to relatively low values ($\lesssim$0.3 M).

Electrostatic effects can also be circumvented by employing neutral adsorbents. These can be obtained through the introduction of a negative charge that neutralizes the positive charge on the amino group, e.g., the ionized carboxyl group in the case of amino acids [20, 21, 42]. For the same purpose, adsorbents free of charge have also been prepared [10, 19]. However, the positively charged amine-substituted agaroses, which have been used for studying electrostatic effects at low ionic strength, can be used for "true" hydrophobic protein binding, merely by carrying out the procedures in the presence of a "structure forming" salt in a concentration that not only quenches electrostatic effects but at the same time enhances hydrophobic bonding. This has recently been applied to a procedure for protein separation by means of a hydrophobicity gradient of a series of connected columns of agaroses substituted with ligands of increasing hydrophobicities and equilibrated with $\simeq$ 3 M NaCl. The protein mixture is washed-in with the salt solution and the subsequently disconnected columns are eluted with 50% ethylene glycol in 1 M NaCl [20]. Use of a hydrophobicity gradient has the advantage that a particular protein is bound by the adsorbent that provides the minimum degree of binding strength that is required. Thus elution is likely to be achieved by means of a relatively mild eluant [see Ref. 20].

It can be seen (Fig. 7) that the fraction of a protein (BSA) that can be eluted by EG decreases with increasing

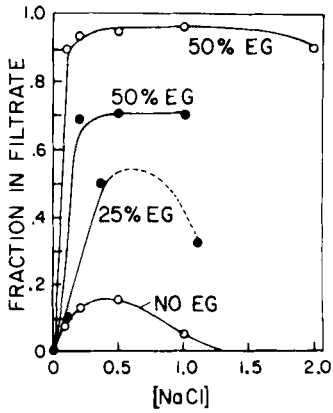

FIG. 7.
Effect of the concentrations of salt (NaCl) and of ethylene glycol (EG) on the elution of bovine serum albumin from preparations of n-octylamino-agaroses with different ligand contents. The experimental conditions were generally the same as those for Figs. 1 and 2. The open and closed circles refer to adsorbent preparations with different degrees of substitution (Ponceau values of about 0.47 and 0.72 mg/ml adsorbent respectively, see text).

degree of substitution. This could be interpreted on the basis of the assumption that with increasing degree of substitution additional opportunities for multiple point (three-dimensional) attachment become available, resulting in the formation of "stronger" binding sites (see above). Thus lowering the degree of substitution would facilitate elution, and under such conditions an eluant such as EG may completely counteract hydrophobic binding at high salt concentrations. The same result is obtained by lowering the hydrophobicity of the ligand instead of its concentration. In concentrations as high as 50%, EG seems to have no irreversible effect on most proteins [43]. Further details of systematic investigations of this type and of actual applications to protein separation will be presented elsewhere [44].

NONSPECIFIC VERSUS SPECIFIC PROTEIN BINDING

Studies of the nonspecific hydrophobic effects discussed above are useful not only with respect to their potential applications to protein immobilization or separation, but also with respect to the possible interference of these nonspecific effects with the isolation of biologically active proteins by biospecific affinity chromatography.

It was shown [4] that an adsorbent "designed" for the specific adsorption of a particular enzyme may bind a wide variety of unrelated proteins (including enzymes) in a nonspecific manner involving hydrophobic effects in conjunction with electrostatic forces. Subsequently, it was suggested [e.g., Refs. 5, 12, and 45] that such nonspecific interaction could occur through the n-alkylamine "arm" by which the ligand usually was attached to the matrix. However, nonspecific binding also could occur through hydrophobic and/or charged groups on the ligand itself, even when the latter is bound by an inert arm to an inert matrix. This type of binding is schematically depicted in the upper and lower parts of Fig. 8. Since nonspecific one-to-one binding may be assumed to be generally weaker than specific binding, the latter is favored when the distance between the matrix-bound ligand molecules is larger than the diameter of the enzyme molecule (Fig. 8, middle part). This condition prevents multiple point nonspecific attachment which, by contrast, may be assumed generally to be much stronger than the one-to-one specific type of binding. Figure 8 also attempts to show that the mode of multiple point binding tends to be more dependent on the locale of the binding site and, in contrast to the one-to-one type, is not necessarily the same for all molecules of a particular protein. This could be the basis of the above discussed false chromatographic inhomogeneity of a protein.

Specific interaction, e.g., that between an enzyme and a substrate or substrate analog, presumably involves complementarity

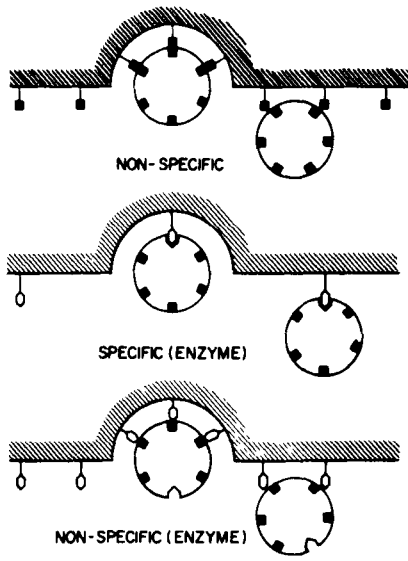

FIG. 8.
Schematic presentation of nonspecific multiple point protein binding by an immobilized ligand (upper part), as compared to the specific "one-to-one" binding of an enzyme with an immobilized substrate analog (middle part). Nonspecific multiple point binding of an enzyme with retention of activity is depicted in the lower part. An attempt is also made to show different modes of nonspecific binding of two molecules of the same protein. The nonspecific type of binding is assumed to take place through electrostatic-hydrophobic interaction of the immobilized ligand (possibly the substrate analog in the case of an enzyme) with corresponding groups of the protein which not necessarily are located in the "active center." "Specific" binding presumably depends on complementarity of the molecular contours of the ligand and the active center of the enzyme, but is assumed often to be weaker than nonspecific multiple-point binding.

(i.e., fit) of molecular contours of the ligand and the active center of the enzyme. For instance, α-chymotrypsin activity appears to depend on the interaction of the aromatic moiety of the substrate (usually a derivative of tyrosine, phenylalanine, or tryptophan) with the "tosyl hole" of the enzyme active center [46]. There may also be assumed to be complementarity with respect to bond formation, including the formation of

electrostatic and hydrophobic bonds. The same could apply to interaction of an antibody with the antigen or hapten [47]. It should be emphasized that for an enzyme at least, specificity of interaction not necessarily relates primarily to strength of binding, but rather to the imposition of a particular orientation with respect to the substrate molecule as a whole as well as with respect to its intramolecular conformation [e.g., see Ref. 22].

One may assume that the binding of chymotrypsin by PBA-agarose [4, 48] is due to the fact that the ligand is a substrate analog. In this case both the protein and the adsorbent carry a positive overall charge and binding occurs <u>despite</u> apparent electrostatic repulsion. Chymotrypsinogen, also positively charged, showed little affinity for PBA-agarose [4]. Furthermore, the enzyme and other positively charged proteins, including chymotrypsinogen, lysozyme, and RNase, were not strongly bound by n-alkylamine-substituted agaroses [5], presumably because of the absence of specificity. On the other hand, and as already noted above, at relatively low ionic strength (0.01 to 0.05) the positively charged alkylamine-substituted agaroses generally strongly bound all <u>negatively</u> charged protein species that were tested, including a number of enzymes and other unrelated proteins such as BSA, OV, β-LG, and γ-G [4-7]. The latter unspecific type of binding seems to depend on the overall charge of the protein.

As suggested previously [7], in addition to lowering the ligand concentration on the matrix (Fig. 8), the absence of a stringent dependence on overall charge in the case of specific affinity might present another opportunity to circumvent nonspecific binding during attempts to isolate proteins by means of biospecific affinity. A procedure that may have more or less general application is as follows: The protein mixture containing the enzyme is filtered over an ion exchanger that carries the same overall charge as the enzyme to be isolated and that does not have a specific affinity for the latter,

e.g., DEAE-agarose in the case of chymotrypsin. The filtrate, which in this case contains only positively charged protein species, then is applied to an adsorbent with a positively charged ligand that binds the enzyme specifically. The contaminating positively charged protein species generally will be repelled by the adsorbent but the enzyme would be specifically bound, despite its positive charge. After washing the adsorbent-bound enzyme with the buffer solution alone, elution may be achieved, e.g., by changing the ionic conditions [see Ref. 4]. For a negatively charged enzyme a negatively charged ion exchanger and a negatively charged specific adsorbent would be used.

POSSIBLE IMPLICATIONS AND FUTURE APPLICATIONS OF HYDROPHOBIC EFFECTS

Hydrophobic affinity per se or in combination with electrostatic effects is relevant not merely to the immobilization or the separation and purification of proteins as discussed above. As exemplified by the virtually irreversible adsorption of a dye such as Ponceau S by the applied adsorbents, this type of interaction may be assumed to be involved in the binding of relatively low molecular compounds as well.

The general importance of hydrophobicity in drug action is indicated by the work of Hansch and co-workers [see Ref. 49]. The implication of hydrophobic effects in narcosis, as expressed in the Meyer-Overton theory, has been known since the turn of the century. A tentative listing and discussion of other biochemical processes in which hydrophobic effects may play a role and of their possible future applications, some of which are as yet highly conjectural, is presented as follows.

The Interaction of Enzymes with Substrates or Effectors (Cofactors, Inhibitors, Drugs)

It has been shown that small changes in the hydrophobic moiety of a substrate may have a large effect on the activity

of esterolytic enzymes (for a discussion, see Ref. 22). Since a change in hydrophobicity of the substrate most often affects the V_m of the reaction, but not necessarily results in a change in K_m, it was concluded that at least in such cases the effect is on the orientation and conformation of the substrate molecule in the enzyme-substrate complex rather than on the enzyme-substrate affinity.

Hydrophobic effects are not limited to esterolytic enzymes and seem to play a role in many enzymatic reactions including certain redox systems and several other types [see Ref. 50]. Exact studies on the requirements with respect to size and shape of the hydrophobic moiety of the substrate or cofactor might aid in the "design" of inhibitors and could contribute to the elucidation of the mechanism of action of enzymes in general. Such inhibitors (e.g., substrate analogs) covalently bound to an insoluble matrix can be of use in the isolation of the enzyme from crude extracts by biospecific affinity chromatography. A typical example is the strong binding of α-chymotrypsin by 4-phenyl-butylamino-agarose [48].

Interaction of Antibodies and Antigens

Similar to the case of enzyme-substrate interactions, complex formation between antibodies and antigens (or haptens) often seems to involve hydrophobic effects [47]. It is of interest in this respect that γ-G is strongly bound by some of the hydrophobic adsorbents, e.g., PBA-agarose [4], used in the present investigations. Rabbit immunogloblin is bound by L-phenylalanine-Sepharose at high salt concentrations, which also suggests hydrophobic binding [42].

Binding of Metabolites (and Drugs) by Serum Albumin as a Transport Protein

Extensive studies have been made on the binding of various hydrophobic ligands by serum albumin [e.g., Refs. 23, 51, 52].

The binding of long-chain fatty acids to serum albumin indicates the presence of at least 8 hydrophobic binding sites per protein molecule [53]. The hydrophobicity of serum albumin is also indicated by fluorescence studies with derivatives of naphthalene sulfonate [25]. The hydrophobic character of BSA relative to certain other proteins, e.g., OV, is confirmed by the present results (see Fig. 1). Thus it may be assumed that hydrophobic forces play an important role in the binding by BSA of metabolites and drugs, which also often are endowed with hydrophobic properties.

Association of Enzyme Subunits

Although as yet conjectural, it would seem reasonable to assume that hydrophobic forces also might be involved in the association of enzyme subunits that seem to occur through noncovalent binding [e.g., see Ref. 2].

Immobilization of Enzymes in vivo

As pointed out above, there is a strong possibility that the immobilization of certain enzymes on intracellular matrices, which also seems to occur without covalent binding, is through hydrophobic interaction, presumably in cooperation with electrostatic forces. Since the enzymes are active, binding must be through sites that are not in the (substrate-binding) active center. Thus the adsorptive immobilization of various enzymes on n-alkylamino-agaroses, which often occurs without blocking the active center [6], might present a "model" for enzyme immobilization in vivo. Such studies would be relevant to the possibility that intracellular enzymes may be endowed with binding sites away from the active center and for the purpose of "specific" attachment of the enzymes to the corresponding sites on the proper membrane.

Structure of Cell Membranes

There seems to be no doubt that hydrophobic phenomena play a role in maintaining the structure of cell membranes (for a recent review of the involvement of hydrophobic forces, see Ref. 2). The great interest in this field is, in part, due to the realization that most intracellular biochemical processes appear to occur on the surface of membranes, which is entirely different from the conditions in classical "solution" chemistry.

Formation of Protein-Nucleic Acid Complexes

Previous findings [54] indicate that positively charged proteins tend to enter into complex formation with nucleic acids. For instance, at low ionic strength chymotrypsin forms a stoichiometric complex with native double-stranded calf thymus DNA. The complex, for which the protein: DNA ratio is 20:1 by weight, sediments by ultracentrifugation as a stable enzymatically active unit. Electron micrograms suggested that the DNA is coated with a monomolecular layer of protein [54]. Although the complex dissociates at relatively low salt concentrations, indicating a primarily electrostatic type of binding involving the charged phosphate groups, recent suggestions as to the possible hydrophobic properties of nucleic acids [e.g., see Ref. 1], could mean that hydrophobic forces also are involved.

The greater general stability of viruses as compared to the artificial complexes could merely be the result of more pronounced hydrophobic properties of the virus proteins and a stronger hydrophobic intermolecular interaction of the latter and/or between the protein and the nucleic acid bases.

Possible Slow Release of Topically Applied Drugs

The observation that, depending on the experimental conditions, a dye such as Ponceau S may be extremely strongly

bound by the present type of adsorbent suggests that this may also be the case for certain drugs. One could assume that the rate of release of such a drug from the complex with the adsorbent could be regulated (e.g., by changing the relative hydrophobicity of the ligand) and adapted to certain environmental conditions. Therefore, it is reasonable to assume that adsorbents could be designed that would bind the drug and when applied topically release it at a desired rate under the conditions at the site of application. The adsorbent-bound drug should be contained in a suitable permeable bag. Such a device might be inserted directly into a body cavity or be implanted subcutaneously.

SUMMARY

Evidence has been presented for the duality of nonspecific protein binding by agaroses substituted with primary amines carrying hydrophobic groups. It appears that the binding by these positively charged adsorbents may occur 1) through a more or less general salt-reversible electrostatic effect or 2) through hydrophobic salt stable "bonding," the latter occurring predominantly in the case of the more hydrophobic ligands interacting with the more hydrophobic proteins, e.g., bovine serum albumin as opposed to ovalbumin.

Further evidence is presented that in contrast to electrostatic binding "true" hydrophobic bonding is stabilized with increasing NaCl concentrations. It appears that this is one of the reasons that a highly purified protein may seem chromatographically inhomogeneous when attempts are made at elution by means of a salt gradient of increasing concentration. "Irreversible" protein binding, in conjunction with nonuniformity of adsorbent binding sites, may constitute another factor underlying the often observed and presumably false chromatographic inhomogeneity of a pure protein.

Nonspecific irreversible binding, which is ascribed to simultaneous multiple point attachment of a protein molecule to two or more adsorbent binding sites, may occur with any highly substituted adsorbent that carries charged and/or hydrophobic groups. The latter even could be provided by a ligand attached with an inert spacer to an inert matrix. Although generally a drawback for protein separation by column chromatography, nonspecific irreversible binding is the basis for adsorptive protein immobilization, e.g., for the purpose of reactor-type experiments in the case of enzymes.

Previous results with enzymes immobilized by adsorption on n-alkylamine-substituted agaroses have been augmented with DEAE-agarose as the adsorbent. A number of arbitrarily chosen enzymes with isoelectric points in the acid region were investigated and in each case at least part of the enzyme remained irreversibly bound under conditions favorable for activity.

In view of the apparent nonuniformity of binding sites, postulated to range from extremely "weak" to extremely "strong," initial "leakage" of an enzyme from a column does not necessarily mean that all of the enzyme will be released upon continued washing with the ambient medium.

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