Hydrophobic (interaction) chromatography (*)

J.-L. OCHOA **.

Instituto de Quimica.
Universidad Nacional Autonoma
de Mexico (UNAM),
Ciudad Universitaria,
Mexico, D.F.

Introduction.
The isolation and purification of macromolecules by biochemical fractionation techniques, like ion-exchange chromatography, gel filtration (molecular-sieve chromatography), affinity chromatography, electrophoresis, etc., is primarily dependent on their biological and physicochemical properties [1-5]. Based on biospecific interactions [6], affinity chromatography has been considered as one of the most effective separating methods. However, serious disadvantages are found upon its application, due mainly to undesirable non-biospecific adsorption [7] attributed to the characteristics of the matrix or support, and the nature of the ligand and spacer-arm introduced to bridge the ligand from the matrix backbone. Moreover, once a protein is attached to an immobilized ligand, for which it shows affinity, the properties of the support may change and become like those of an ion-exchanger, interfering with the chromatographic process.
The belief that such interferences could be adequately controlled by eliminating ionic groups in the matrix and/or in the spacer-arm, brought as a consequence other types of undesirable effects closely related to the extent of hydrophobicity of the spacer-arm employed [57]. Yon [9], and Er et al. [10], reported that by coupling different types of spacer-arms (varying in hydrophobicity) to an inert matrix, potent adsorbents for proteins were obtained. The adsorption mechanism seems to be based, fundamentally, on hydrophobic interactions between the protein and the adsorbent, and it has been demonstrated that it can be positively exploited for separation purposes [11, 105]. In this way, a novel technique which takes advantage of the hydrophobicity of biomolecules, has been introduced as a complement to the other, routinely employed, separating methods mentioned above.
Since the separation is based on hydrophobic interactions, this technique receives a number of names which intend to describe the principle and the parameters involved in the separation process, though all of them are entirely or partly dealing with the concept of hydrophobicity:
Hydrophobic chromatography [11]; Hydrophobic (interaction) chromatography [51]; Hydrophobic salting-out chromatography [93]; Phosphate-induced protein chromatography [54]; Repulsion controlled chromatography [94]; Detergent protein-interaction [95, 96]; Hydrophobic affinity chromatography [128]; etc.
It has been shown [12] that a large part of the non-polar residues of the amino acids in proteins are exposed to water interface, as opposed to the expected preferential location of the hydrophobic amino acids in the interior of the biomolecule. These non-polar amino acids are found in the protein surface forming «patches» of distinct hydrophobic character which can account for the biospecific conformation of the protein [13] as well as for its ability to complex or aggregate to other types of molecules for instance, lipids. Presumably, these hydrophobic «patches» are randomly distributed on the surface of the biomolecule. Their number (possible number of interacting sites) and the extent of their hydrophobicity (type and distribution of the non-polar amino acids) should be a characteristic of each macromolecule. Therefore, their specific separation should be possible with an adequate hydrophobically coated support or matrix.

I. THE BIOLOGICAL ROLE OF THE HYDROPHOBICITY IN BIOMOLECULES.

There is no doubt that the hydrophobic interactions play an important role in biological systems. The membranes in the living cell are made...
up of mainly hydrophobically interacting lipid-lipids and lipid-proteins. The sub-units of large proteins are often held together by hydrophobic bonds, and it is well accepted that they are important in supporting the tertiary protein structure [13].

Functionally, hydrophobic interactions seem to be involved in recognition processes, as for example in the case of some enzyme-substrate complexes and antigen-antibodies associations, etc. [14-15, 27-30]. Except for the case of membranes, in which an exclusive structural function has been attributed [31], there is not much work in correlating the hydrophobic properties of the biomolecules with their functions [32, 99-100]. Therefore, it could be interesting to find out whether these hydrophobic regions in the biomolecules are related to their intrinsic biological properties, and/or to their location in the cell.

Perhaps one of the reasons why few systematic studies of hydrophobic effects have been made, is that the hydrophobic compounds possess low solubility in water. If they are made soluble by the introduction of polar groups, they tend to form micelles as exemplified by the case of soaps. Although molecular dispersion may be obtained through the addition of polarity reducing agents to the medium, such agents would also reduce the interaction of the hydrophobic compounds with proteins and even alter the protein structure, which generally depends on the integrity of the hydrophobic core [13].

A means to obtain molecular dispersion of a hydrophobic ligand in aqueous milieu, without the addition of polarity reducing agents, is to attach the ligand to a hydrophilic but insoluble polymer such as agarose. Consequently, hydrophobic supports can be used not only for separation purposes, but also to study the hydrophobicity of the biomolecules and their modes of interaction. In turn, this may lead to a clearer understanding of their functions.

II. THE PHYSICOCHEMICAL CONCEPT OF HYDROPHOBICITY.

Perrin [16] was the first to utilize the terms «hydrophilic» and «hydrophobic» when studying colloids. He considered them hydrophilic if their stability was relatively insensitive to the addition of electrolytes, or hydrophobic if they exhibit extreme sensitivity to added electrolytes. Later, Langmuir [17], in a classical experiment, discussed polar molecules (fatty acids) in terms of hydrophilic and hydrophobic groups. Since then, these terms have been in every day use.

In general, when an apolar group (or hydrophobic) is inserted into water, several effects can be observed (for review see ref. 18):

— A negative unitary entropy change (—ΔS), which implies an overall increase in the degree of order. The entropy distribution becomes relatively important as the molecular weight of the apolar group increases.

— A small enthalpy change, usually negative (—ΔH), which reflects an energetic component in the interaction between water and an apolar group and opposes the negative unitary effect favouring mixing of the hydrocarbon in water.

— An increase in heat capacity (+ΔCp), which implies either an increased degree of freedom of the vibrational and rotational motion within the existing structure, or a progressive alteration of an existing structure as the temperature is raised.

— A decrease in volume (—ΔV), which indicates that the apolar groups and the water molecules have packed together with a contraction of some structure, or that the apolar groups are accommodated into open spaces or voids preexisting within the water structure itself. The former is unlikely in view of the small enthalpy change involved.

— Finally, the presence of apolar groups in water results in an increase in the number or strength of hydrogen bonds within the water molecules, as has been demonstrated by spin lattice relaxation studies and further supported by Raman measurements [60]. Its probable reason is that the hydrocarbon chains restrict the mobility of water molecules in such a way that the covalent character of the hydrogen bond is increased [92].

On the other hand, the molecular picture of the hydrophobic interaction is the reverse of that obtained when introducing apolar groups into water (see mechanism below). The withdrawal of the apolar groups from the aqueous phase removes restrictions on hydrogen-bond bending and thus, achieves a positive unitary entropy. This supports the observation that the hydrophobic interaction is a spontaneous process. This assumption was substantially demonstrated by Frank and Evans in 1945 [20]. Some years later, it has been shown [19] that the free energy values of the transfer of aliphatic hydrocarbons from an apolar medium to a polar one, like water, increases linearly with increasing numbers of (CH2) methylene groups, and thus the hydrophobicity of the molecule.
III. Determination of the Hydrophobicity of Biomolecules.

It is well known that most proteins contain a relatively high proportion of amino acids with non-polar chains (table I). Tanford [21], and Nozaki and Tanford [22], formulated an experimental procedure based more or less on the Kauzmann [13] conception which permits the estimation of the amino acid hydrophobicity. The method consisted, essentially, in determining the solubilities of the amino acids in water as well as in progressively increasing concentration of some organic solvents, such as ethanol in water. The solubilities of the amino acids were extrapolated to pure organic solvents and then the free energy value of the transfer for the amino acid from pure organic solvent to water was calculated. Using as a reference glycine, and subtracting its free energy transfer value from that of all the other amino acids, it was possible to formulate a hydrophobicity scale for amino acid residues where their free energy transfer values become more and more positive as the hydrophobic character of the compounds increases [18].

Other attempts in scaling the amino acid hydrophobicity can be illustrated by the works of Bull and Breese [23] and Bigelow [24]. The former studied the effect of the amino acid on the surface tension of water and the latter calculated the average hydrophobicity of several proteins by their amino acid composition according to data of Tanford and of Bull and Breese.

A different approach was done in terms of the frequency of non-polar side chains in proteins [25], in which was found a variation from 0.21 to 0.47. Separately, Fisher [26] employed the ratio of the volume of the polar groups to those of the non-polar as a hydrophobicity degree, but this idea has the inconvenience, like the one mentioned above [25], to consider that a group is either polar or non-polar, without any gradation between these two extremes.

Recently, a method for studying the magnitude of the interaction between protein and aliphatic hydrocarbon chains, and thus indirectly the hydrophobicity, was reported [50]. It is based on the partition of proteins in an aqueous two phase system containing dextran and polyethylene glycol and different fatty esters of polyethylene glycol. However, the measurements depend largely on a critical chain length which, by this technique, should be greater than 8 carbons.

Finally, an approach to localize the hydrophobic sites on the surface of the proteins by means of interacting with small molecules, has been attempted using fluorescent probes [27]. It is not difficult to speculate that with this set of information, a better comprehension of many biological phenomena is near. We do not know yet how the complex enzymatic systems are organized, nor how the recognition between proteins occurs to constitute such enzymatic complexes after protein synthesis. Neither do we have a good explanation for the transport of many substances, including proteins, through the hydrophobic core of the membrane. And furthermore, it is possible to believe that hydrophobic «patches» in the membrane (either out or inside) are needed to make possible many of the most common biolo-
IV. THE MECHANISM OF THE HYDROPHOBIC INTERACTION.

The hydrophobic interaction is the result of the adherence of two non-polar groups. The case of detergents can be considered as an example where negative enthalpy changes are observed during the micelle formation in aqueous solvents. If the adsorption of proteins to hydrophobic matrices is considered to be a process of limited micelle formation, a negative change in the enthalpy value would not preclude the hydrophobic nature of the binding. In addition, this change must be negligible as compared to the value of increasing entropy of the system. Furthermore, before the interaction the water molecules are forced to keep in order around the hydrophobic entities (fig. 1) as compared to the order in the bulk. When the hydrophobic sticks come in contact with each other, the ordered water molecules will be excluded and will adopt the less ordered bulk water state which is equivalent to an increase in entropy. This hypothesis is supported experimentally by the study of antigen-antibody complex formation where a relative insensitivity to dissolution of a preformed antigen-antibody precipitate is observed, suggesting that once the complex is formed, the solvent is largely excluded in the regions of contact.

Thermodynamically, the free energy value $\Delta G$ of a hydrophobic interaction is a function of $\Delta H$ and $\Delta S$, according to equation:

$$\Delta G = \Delta H - T \Delta S \quad \quad \quad \quad \quad (N^\ast 1)$$

Since $\Delta H$ is small as compared to $T \Delta S$ value, the process is fundamentally determined by the change in entropy. In these conditions the reaction proceeds spontaneously. In other words, the input of energy or chemical work is not necessary to make possible the interaction between two hydrophobic molecules in aqueous solutions.

The contact between two different molecules, like the sub-units in the case of some proteins, is largely dependent on the surface areas occupied by the residues which participate in the interaction. The concept of accessible surface area describes the extent to which protein atoms can form contacts with water, and is related to hydrophobic free energies. In any case, the association of protein sub-units, whether by van der Waals contacts, electrostatic forces and/or hydrophobic interactions, leads to a reduction of the surface area accessible to the solvent when the two molecules associate. Evidence against the hydrogen bond as a major contribution to the free energy of the protein-protein interaction has been obtained thermodynamically. On the other hand, van der Waals interactions, though they are more numerous as they involve all the pair of neighbouring atoms, are much less energetic and their overall contribution is small. The hydrophobic contribution is largely dependent upon the entropy gained by water due to the smaller accessible protein surface area when the protein molecules form a complex. The hydrophobic interaction seems to be entirely unspecific as compared to the complementarity of the surfaces involving hydrogen bonds and van der Waals contacts; however, they decide which proteins can recognize each other.

V. THE SPECIFICITY OF THE ADSORPTION OF BIOMOLECULES ON HYDROPHOBIC SUPPORTS.

Biospecific affinity, whether involving an « active site » or not, presumably depends to a large extent on the complementarity of the contours of the interacting molecules. In the absence of any specific effect, the binding of proteins to substituted agaroses is greatly affected by the overall charge of the biomolecule.

The lack of activity in the adsorbed state of some proteolytic enzymes, indicates that the binding site is occupied by the hydrophobic group of the substituted agarose. Whether the hydrophobic group interacts with the hydrophobic pockets of the active sites of the enzymes or acts by a less specific way can be questioned. In any event, both cases may contribute to the adsorption phenomena.
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In other examples, the addition of the specific substrate accompanying the enzyme during the chromatography (to mask the specific binding sites) did not alter the binding of the enzyme to the hydrophobic matrix, indicating that the adsorption takes place through sites other than the specific substrate binding site [97].

It is interesting to note that glutamine synthetase and other three proteins involved in the regulation of glutamine metabolism are all retained by the same amino-alkyl agarose derivatives [99] (see table II). Although, as Shaltiel and coworkers have signaled, this could be fortuitous, it might reflect a mutual biospecific affinity among these proteins since they must interact with each other in order to effect their regulatory functions in the highly integrated glutamine synthetase cascade system.

The models proposed by Shaltiel [11] and Jennissen [78] explain in a different manner the mechanism by which the interaction between the protein and the adsorbent occurs. For neutral supports, Jennissen [86] has presented evidence in favor of the idea that the adsorption of proteins to alkyl-agarose derivatives takes place at a critical alkyl group density and is a function of its hydrophobicity. In other words, this hypothesis considers that the protein needs to present multiple attachment points in order to be adsorbed by a determinate member of the series of alkyl-agarose derivatives. This is in agreement with an earlier observation made by Hjertén et al. [52]. Shaltiel [1] on the other hand, suggests that the adsorption of the protein is due to the interaction of an alkyl residue of specific length (« yard-stick ») with a hydrophobic pocket of the protein. Evidently, and as compared to Jennissen's model, the latter implies a more specific mechanism. However, it has been demonstrated [58] that at high chain length, when the interactions are stronger, the binding is also less specific.

The « positive cooperative interaction » [84] through the multivalent binding, may explain the increased free energy value of adsorption of a determinate member of the alkyl-agarose series. That is, a given protein may be adsorbed by a certain member of agarose-substituted series only if the number of « contacts » is big enough to effect its retention, and this can be done by variation of the degree of substitution. Comparatively, amino-alkyl agaroses present a « negative cooperative » effect towards the adsorption of phosphorylase b, possibly due to a different mechanism of interaction which does not necessarily exclude the multivalent attachment [84].

It has been observed, in the case of some hydrophobic alkyl- and alkylamino supports, that certain « strong » binding sites are occupied first, and that others of decreasing affinity become occupied when more protein is fed to the column. This is shown by the fact that only part of the protein can be eluted by increasing the salt concentration, whereas the remained is dislodged by the addition to the eluant of a polarity reducing agent such as ethylene-glycol. It should be emphasized that this problem is mostly related to those hydrophobic supports which possess both hydrophobic and ionic groups, resulting from the coupling [70, 47] procedure or the nature of the ligand [96], like in the case of amino-alkyl derivatives. This apparent inhomogeneity of both matrix and protein can lead to possible wrong interpretations about the purity and characteristics of many proteins [98, 105]. For instance, the recovery of rechromatographed materials is improved up to 95 per cent, as compared to that of 70 per cent obtained when the crude extract is applied into the column [98]. In other cases, desorption of purified material requires the variation of the eluant, as pointed above. It seems

BIOCHIMIE, 1978, 60, n° 1.
### Table II. Hydrophobic matrices.

<table>
<thead>
<tr>
<th>Type</th>
<th>Coupling procedure</th>
<th>Representation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Agarose derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Un-substituted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Amino-alkyl</td>
<td>BrCN</td>
<td>(A)-CH-NH-(CH₂)n-NH₂ + NH₂</td>
<td>[9-11, 94, 113, 114, 58]</td>
</tr>
<tr>
<td>3. Alkyl</td>
<td>BrCN</td>
<td>(A)-CH-NH-(CH₂)-(CH₃) + NH₂</td>
<td>[11, 94, 113, 114]</td>
</tr>
<tr>
<td>4. Amino acid</td>
<td>BrCN</td>
<td>(A)-glycine</td>
<td>[97, 115]</td>
</tr>
<tr>
<td>5. Other derivatives</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acids</td>
<td>BrCN (azide)</td>
<td>(A)-glycine</td>
<td>[97, 115]</td>
</tr>
<tr>
<td>Acetyl-N-amino-alkyl</td>
<td>BrCN (carbodiimide)</td>
<td>(acetylation of amino-alkyl-(A))</td>
<td>[57, 94, 117]</td>
</tr>
<tr>
<td>Aniline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzyl-ether</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl-N-butyl-amine-NAD⁺⁻⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxy-alkyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diverse aromatic-alkyl</td>
<td>BrCN and glycidyl ether</td>
<td>(A)-(CH₂)n-OH</td>
<td>[58, 112]</td>
</tr>
<tr>
<td>derivatives</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>II. Dextran derivatives</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Sephadex)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Acetyl-Sephadex</td>
<td>acetylation</td>
<td>CH₃-CO-(S)</td>
<td>[130]</td>
</tr>
<tr>
<td>2. Methyl ether-(S)</td>
<td>acetylation</td>
<td>CH₃-O-(S)</td>
<td>[91]</td>
</tr>
<tr>
<td>3. Hydroxy-propyl-ether-(S)</td>
<td></td>
<td>HO-CH₂-CH₂-CH₃-O-(S)</td>
<td>[91]</td>
</tr>
<tr>
<td>4. Hydroxy-alkyl-(S)</td>
<td></td>
<td>HO-(CH₂)n-O-(S)</td>
<td>[91]</td>
</tr>
<tr>
<td>5. LH-20-(S)</td>
<td></td>
<td>from commercial sources</td>
<td>[91]</td>
</tr>
<tr>
<td><strong>III. Cellulose derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Diethyl amino ethyl</td>
<td>from commercial sources</td>
<td>DEAE-(C)</td>
<td>[47, 71]</td>
</tr>
<tr>
<td>cellulose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Carboxy methyl-(C)</td>
<td>from commercial sources</td>
<td>CM-(C)</td>
<td>[47, 71]</td>
</tr>
<tr>
<td>3. Benzoylated-DEAE-(C)</td>
<td>benzoyl chloride</td>
<td>BD-(C)</td>
<td>[103]</td>
</tr>
<tr>
<td>4. Esters of alkyl and aryl-</td>
<td>phenoxy acetylation</td>
<td></td>
<td>[101]</td>
</tr>
<tr>
<td>(C) (paper, cotton)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IV. Glass derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Alkyl-silated-(G)</td>
<td>amino-silanization and amide bond formation with the corresponding alkyl chloride</td>
<td>[118]</td>
<td></td>
</tr>
<tr>
<td>2. Propyl-lipoamide-(G)</td>
<td></td>
<td></td>
<td>[107]</td>
</tr>
</tbody>
</table>

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Table II.

<table>
<thead>
<tr>
<th>Type</th>
<th>Coupling procedure</th>
<th>Representation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. Others supports</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Polyamino methyl-styrene (polystyrene, polyamide, Dowex I-X8)</td>
<td>from commercial sources</td>
<td></td>
<td>[71]</td>
</tr>
<tr>
<td>2. Alkyl-amino of polyacrylic resins (butyl, capryl, lauryl, palmityl, stearyl, oleyl, linoleyl)</td>
<td>chlorination for amide bond formation</td>
<td></td>
<td>[76]</td>
</tr>
</tbody>
</table>

that this discrepancy depends on the hydrophobicity of the protein and the adsorbent, and moreover, that the binding sites are not identical in a given carrier (which is true for most of the adsorbents employed in chromatography in general).

The multiple point attachment binding is most likely to occur in a cavity of the adsorbent than on its surface, particularly when the protein molecule fits into the cavity (fig. 2). Conversely, at points on the matrix where the bound ligands are distributed over a protruding area, binding would be less strong. Since the surface of the adsorbent may be assumed to be irregular, many different situations in addition to these two hypothetical cases would be obtained. Additionally, small amounts of protein bind more homogeneously on a particular column than a large amount. This could mean that by reducing the amount of applied protein, the stronger binding sites might predominate in the binding.

If multiple point attachment were one of the reasons for strong non-specific adsorption, one way to reduce it would be to lower the degree of substitution to the point where the distance between the substitution groups is larger than the diameter of the protein molecule. This would not affect the specific «one-to-one» interaction as that between an enzyme active site and an immobilized substrate analogue.

VI. The factors involved in hydrophobic (interaction) chromatography.

A. The matrix hydrophobicity.

1. Matrices and supports.

Proteins differ in their hydrophobicity as a function of their primary structure, that is, in the sequence and amino acid composition. Hence, it is not surprising that the first type of hydrophobic supports ever employed were derivated from coupling various non-polar amino acids to an inert support or matrix like agarose [51]. Other supports have also been employed: cellulose, glass, dextran, etc. (see table II), but the ideal matrix without secondary non-biospecific adsorption effects has not been found.

All the different types of supports and matrices utilized at the present possess undesirable interferences attributed to their chemical composition. Additional effects are obtained in such a way that is difficult to obtain a pure hydrophobic interaction chromatography after the introduction of the spacer-arm or hydrophobic ligand.

In table II, the various types of matrices employed in HIC, as well as the different types of ligands coupled, have been summarized. References are given to illustrate specific applications.

As has been demonstrated, un-substituted agarose is sufficiently non-polar presumably due to the 3-6 methylene diether bridges present in every second galactose residue of the polysaccharide chain [67] with respect to the retention of halophilic proteins [47] and nucleic acids [41, 119], at high salt concentrations (2.5 M ammonium sulfate). Though the halophilic proteins are highly negatively charged [48], as a result of an excess of acidic groups [49], it is believed that the high salt concentration eliminates the possible ionic interactions.

When DEAE- and CM-derivatives, either of cellulose or agarose, have been used, the elution pattern shifts to higher or lower salt concentrations than those required when the neutral derivative is used as a support. This is explained by...
electrostatic attraction-repulsion forces between the negatively charged haloproteins and the nature of the charge on the matrices.

With few exceptions, agarose is the type of matrix most commonly used (table II), and the kind of ligands or spacer-arm introduced are in a wide range of hydrophobicities and structures. Consequently, the hydrophobicity of the matrix depends on the type of the ligand [78], and on the degree of substitution [56, 86]. In this respect, the properties of the ligand should be discussed first.

2. The role of the ligand in the hydrophobicity of the matrix.

As pointed out above, HIC was born (in many cases) as a consequence of non-biospecific adsorption found in affinity chromatography systems and attributed to the type of spacer-arm used to bridge the ligand to the matrix. Nevertheless, evidences exist that prove that HIC was conceived by Hjerlén and his group in a different way, while studying the solubilization of membrane proteins. They attempted their separation using a hydrophobic support to which a detergent (SDS) was coupled. In this case, however, the adsorption was so strong that the proteins could not be desorbed by any non-denaturing system (unpublished results, personal communication). When Cuatrecasas [87] showed that β-galactosidase could only be fractionated if the ligand was sufficiently separated from the matrix, and thus avoiding sterical hindrance, the idea of separating ligands from the matrix backbone by use of spacer-arms rapidly spread. Very soon, it became clear, that many proteins were adsorbed nonspecifically because the presence of the arm introduced non-biospecific adsorption centers. For instance, a spacer-arm carrying charged groups gives origin to electrostatic interactions [106]. The substitution of the charged arms by other neutral chemical analogs was thought to be an excellent alternative but then, other type of non-specific interactions, hydrophobic in nature, turned out to be important [7].

Yon [9], Er-el et al. [10] found that protein could bind substituted agarose matrices without any specific ligand. The adsorption was attributed to the presence of the diamino-alkyl group employed as a spacer. This observation were further supported by O’Carra and coworkers [57, 88], who found that the presence of the ligand did not always account for the retardation effect and again the spacer-arm was considered as responsible. Yon [9] suggested that it was possible to take advantage of such non-biospecific adsorption and showed that certain proteins could be selectively adsorbed on decyl-agarose derivatives differing in the ionic character of the spacer-arm. Later, Shaltiel and his group [10, 11] reported purification of several enzymes on a series of alkyl and amino-alkyl agarose derivatives. Almost simultaneously, Hjerlén and his group [52] reported, for the first time, the preparation of different substituted agaroses with uncharged groups that could illustrate an exclusive hydrophobic mechanism of adsorption.

The effect of the hydrophobicity of the spacer-arm in the purification of various proteins [8-11] has been possible thanks to the development of the «mock affinity systems». Steers [8] demonstrated that the adsorption of β-galactosidase was strongly related to the length of the spacer-arm. The series of alkyl and amino-alkyl derivatives of agarose prepared by Shaltiel have also been successfully applied in many cases [11].

The difference of a single C-atom in agarose bound N-alkyl groups may have a large effect in the hydrophobic binding of a particular protein [75]. Therefore intermediary hydrophobic compounds between two consecutive members of a homologous series of ligands are needed. Such intermediate hydrophobicities can be obtained, for instance, through the introduction of a charged or other hydrophilic group, e.g., hydroxyl group. The introduction of double bonds or of branching of the chain, reduces also the hydrophobicity as compared to the corresponding saturated straight chains [13]. Weiss and Bucher [76] have prepared some alkyl agarose derivatives of increasing insaturation for this purpose. In addition aromatic derivatives may possess intermediate hydrophobicities between two consecutive members of the alkyl-agarose series. Benzene, for example, is equivalent to that of 3-4 straight chain hydrocarbon [19].

Although Tanford [19] has showed that the hydrophobicity of a linear aliphatic carbon increases linearly with increasing number of CH₂ groups, Shanbhag [50] considers that the effective hydrophobicity depends also on the flexibility of the hydrocarbon chain and consequently on the degree of interaction within such chains, specially for long chains. Hofstee [75], on the other hand, assures that neither the difference in molecular shape of the N-alkyl ligands and the side chain of aromatic compounds like phenylalanine or tryptophan, nor the difference in net charge of the adsorbent is a determining factor for protein binding. It seems then, that the me-
The mechanism of the adsorption of proteins to hydrophobic matrices follows some very complex rules. This problem can be exemplified by the case of the adsorption of erythrocytes on a series of alkyl-agarose derivatives [110] in which case a decrease in adsorption between C6-C8 is noticed to occur without a reasonable explanation.

The combination of the principles of ionic-exchange chromatography and HIC has been successfully applied in some cases [9, 41]. The use of ligands containing both ionic and hydrophobic characters have been recommended in order to avoid the denaturing effect detected in the use of alkyl-agaroses [108]. It was also argued that the elution procedure might be milder than in the case of pure hydrophobic spacer-arms or ligands [9] and furthermore, that the use of charged arms was capable of finer discrimination between lipophilic proteins. The protein, in this case, interacts hydrophobically, involving the alkyl chains, and electrostatically, involving the terminal ionic groups. With this type of adsorbents, if the chromatography is carried out at a pH equivalent to the isoelectric point (IP) of the protein, the adsorption will be due to hydrophobic binding alone. By changing the pH to introduce in the protein a net charge of the same sign as the charge of the adsorbent, the repulsion effect will decrease the adsorptive force due to hydrophobic bonding, making possible the desorption. Obviously, a drawback in using this type of adsorbent arises from the problem that frequently the IP of the biomolecule of interest is unknown, and that many proteins precipitate at their IP. Moreover, the simultaneous presence of extraneous ionic and hydrophobic groups in affinity adsorbents, has been found to cause a substantial non-specific protein binding thus resulting in a reduced bi-specificity of these materials [106].

3. The influence of the degree of substitution on the hydrophobicity of the matrix.

The degree of substitution on Sepharose 4B with alkyl-amines of different hydrophobicities is a critical parameter in the adsorption of proteins. About 10^{12} alkyl residues per Sepharose sphere appears to be a critical degree of substitution for the adsorption of enzymes like phosphorylase kinase, phosphorylase phosphatase, 3'5'-cAMP dependent protein kinase, glycogen synthetase, and phosphorylase b which are successively adsorbed when the hydrophobicity of the Sepharose is increased. In addition, the degree of substitution determines the capacity of the gel [78].

The critical hydrophobicity needed to adsorb proteins can be obtained by either increasing the degree of substitution or by elongating the employed alkyl-amine chain at a constant degree of substitution. Consequently, as the hydrophobicity of the gel is increased, higher binding affinities result and the desorption requires more and more severe conditions. In the case of neutral alkyl-agaroses [34], elution of proteins from the hydrophobic matrix can be described in terms of salting-in phenomena, since desorption is dependent on the type of salt employed and not on the ionic strength alone.

In all cases, a minimal chain length seems to be required in order to obtain the adsorption of a given protein. This fact has been interpreted as fitting a hydrophobic group into a hydrophobic pocket of the protein. By variation of the concentration of cyanogen bromide in the activation mixture, the amount of hydrophobic residues may be varied. Thus, the corresponding hydrophobicity may be increased such that the amount of adsorbed material increases exponentially when the degree of substitution of the gel is enhanced. Control experiments with methyl-agarose show that similarly increased degrees of substitution, and thus similar numbers of positive charges introduced by the coupling procedure [117], do not affect the adsorption of the assayed protein. Therefore, the adsorption of larger amounts of material, determined by increasing the degree of substitution, is not a function of the additional number of charges [78]. One may conclude that, if in a series of gels of different hydrophobicities a crude extract containing hydrophobically differing proteins is applied, the one with the highest hydrophobicity will be adsorbed by the gel of the lowest degree of substitution. Then as the number of alkyl residues increases on the matrix, proteins of lower hydrophobicities are adsorbed.

A direct method of determining the degree of substitution for charged hydrophobic groups has been proposed by Hofstee [89]. The method describes the use of Ponceau S, a dye which carries hydrophobic groups in conjunction with an overall negative charge bound in an irreversible fashion to the ligand. Under a given set of conditions, and after application of a saturating amount of dye, a certain amount will remain bound even after extensive washing. Eighty-five percent of the Ponceau S binding capacity is lost after a period of almost 5 months indicating that the degree of substitution decreases gradually upon storage. In some cases, 40 per cent was lost in only 40 days.
The data also suggest that the adsorbents with the highest degree of substitution are the least stable [89].

An estimation of the relative degree of substitution can be obtained from the acid-base treatment of the adsorbent [89], by a nucleophilic attack of N-substituted isourea (derivated from the coupling procedure with BrCN) with an active chromogenic substance [70] ; or more indirectly, by measuring the capacity of the adsorbent with a coloured protein like cytochrome C [90] or phycocerythrin [56]. A much more sophisticated but accurate method utilizes NMR spectra [58].

Finally, it is important to mention that a consequence of the degree of substitution is the shrinkage of the gel owing to a decrease in hydrophilicity due to the hydrophobic character of the ligand. Depending on the special structure of the agarose gel, the shrinkage seems to be much less important than with other gels like Sephadex or polyacrylamide [90].

B. The influence of salt.

The influence of salt on the adsorption of proteins to hydrophobic supports is probably due to a number of factors acting on the protein as well as on the matrix. It has been demonstrated that neutral salts induce conformational and structural changes in biomolecules [34]. These studies have been carried out by circular dichroism spectra of the protein at constant ionic strength. It has been concluded that salt-out ions cause conformational but not structural changes whereas salt-in ions cause sometimes severe structural changes which may be one reason of their denaturing effect [34].

The «structure forming» properties of salt-out ions enhance intramolecular, as well as intermolecular, hydrophobic bonding as reflected by a stabilization of the hydrophobic core of the biomolecule [35, 43]. The effect of specific ions on macromolecules was first noticed by Hofmeister [36]. He found that salts differ greatly in their ability to salt-out proteins at a given salt concentration. At high concentrations of salt-out ions, the solubility of the protein is adversely affected by decreasing the availability of water molecules in the bulk and increasing the surface tension of water, resulting in an enhancement of the hydrophobic interactions [31, 37]. Accordingly, the influence of neutral salts on the adsorption phenomena determines to a large extent the degree of adsorption and correlates closely with the Hofmeister series [63, 58]. Salting-in ions, on the other hand, are «structure-breaking» ions, and thus do not favour hydrophobic interactions. This effect can be regarded as an inevitable consequence of the new order imposed by the ion orienting water molecules in such a way that water cannot undergo further positive entropy change, as it is required for the formation of hydrophobic bonds [18]. These ions have also been termed «chaotropic» [44] because they provoke unfolding, extension and dissociation of the macromolecules. In this respect, they might be used in the elution of strongly adsorbed materials on hydrophobic supports.

Already in 1948, Tiselius [38] noticed that proteins and other substances (e.g., dyes) that could be precipitated by high concentrations of neutral salts could be adsorbed (at much lower salt concentration) to common adsorbents, whereas in the absence of salts those adsorbents showed no affinity for the substance. In the years afterwards, some attempts have been made to purify proteins on solid supports using gradients of ammonium sulfate [39, 40, 54]. In general, proteins which precipitate at low ammonium sulfate concentrations should likewise be retained on hydrophobic supports at low salt concentrations ; and similarly, proteins which precipitate at high concentrations of ammonium sulfate would require relatively higher concentrations of salt to obtain their retention [40].

The purification of nucleic acids [41] has also been reported using unsubstituted agarose with high concentrations of ammonium sulfate. It should be emphasized that the adsorption occurs at concentrations below which the macromolecules precipitate out of solution. Since the adsorption is controlled by salt concentration, rather than by the hydrophobicity of the column, the names of «salt-mediated hydrophobic chromatography» and hydrophobic (salt-out) chromatography have been employed elsewhere [42, 54, 67]. However, the hydrophobicity of the gel and the effect of the salt concentration may be combined efficiently to improve the adsorption phenomena and/or the purification [64], especially of proteins which may be affected by the high salt concentration required for its adsorption on the given gel [46]. If the use of high salt concentrations is limited by the enzyme activity or stability for instance, a comparatively longer alkyl-chain must be employed in order to obtain sufficient capacity. Because a similar salt-out/salting-in effect has been noticed in the case of high concentrations of sugars, namely sugaring-out and sugaring-in effect [45], the possibility in using...
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sugars instead of salts should be considered. However, this effect is probably not general.

C. Effect of temperature and pH.

From the equation N° 1, it is clear that the temperature may influence, in a positive way, the adsorption of proteins on hydrophobic matrices [52, 55]. However, temperature may also provoke other effects, such as increased solvation and decreased surface tension, which may withstand the ability of molecules like proteins to interact with hydrophobic supports [53].

The protein molecules retain their biological activity or capacity to function only within a limited range of temperature and pH. Their exposure to extremes of pH and temperature causes them to undergo denaturation; the most visible effect is a decreased solubility of globular proteins. Since the covalent chemical bonds in the peptide backbone of the protein are not broken during denaturation, it has been concluded that it is due to the unfolding of the characteristic conformation of the native form of the protein molecule. The refolding of a denatured protein does not require the input of chemical work from outside. It proceeds spontaneously, provided that the conditions of temperature and pH are adjusted to be compatible with the stability of the native conformation of the protein. In this respect, the process is similar to the one in hydrophobic bond formation.

The strength of hydrophobic bonds should increase with rising temperature up to above 60°C, at which point the additional stability arising from hydrogen bonding, electrostatic forces between charges or dipoles, van der Waals interactions and disulfide bridges disappear, and turn against the favoured hydrophobic interaction [18, 30, 59] of the protein.

A pH effect on hydrophobic bonding is observed in the case, for example, of bovine serum albumin binding alkanes at pH 4. It was observed that at low pH the site of hydrophobic bonding is disrupted [61], and that the degree of aggregation of the globulin does not affect the degree of binding. The conclusion was that the sites associated with aggregation are relatively non-hydrophobic and that any conformational changes, resulting from the polymerization, do not exert any effect at the hydrophobic binding site of alkanes [62].

Decreasing pH below pK value of the amino group of adenylic and cytidylic acid residues of tRNA changes the negative charge of the molecule, altering its tertiary structure [65] and provoking its desorption from the charged alkyl amine agarose columns. On the other hand, binding at pH 7.5 does not occur probably due to other conformational changes in the molecule, which could mask key sites involved in the binding [41].

Lysozyme, a basic protein, becomes more and more retarded on C10 (alkyl Sepharose) as the pH of the eluant is lowered, and binds to this column at pH 1.5 [66]. Cytochrome C is also highly pH-dependent in its adsorption to neutral ethers of agarose derivatives [67].

No influence of pH has been observed on the adsorption of polysaccharides to substituted agaroses [68]. In this case, the retention is mostly determined by the hydrophobicity of the column and by the size of the polymer.

Naturally, pH plays an important role in the adsorption of biomolecules on hydrophobic matrices carrying charged groups. With this type of adsorbents, pH may modify the charge either of the biomolecule or of the adsorbent. By changing the pH, a net charge may be introduced in protein, which can be of the same sign, or opposite of the charge on the adsorbent. In the first case, the repulsion effect will affect negatively the adsorption phenomena. As was pointed out previously, a maximal hydrophobic adsorption will thus occur at the isoelectric point of the protein [9].

VII. METHODS OF DESORPTION OR ELUTION OF ADSORBED MATERIALS ON HYDROPHOBICS MATRICES.

The elution of adsorbed materials from hydrophobic gels can be obtained in a number of ways depending on the type of adsorbent employed, the conditions in which the adsorption occurred, and the properties of the biomolecule.

When proteins have been adsorbed at high salt concentrations, a decreasing ionic strength of the eluant will usually result in the removal of the material from neutral adsorbents [71, 79]. If the support carries charges introduced either by the coupling procedure [70], or by the nature of the ligand, electrostatic interactions will become important at low ionic strength, and the elution will not be possible [47, 105]. In such cases, changes in the pH [9, 65], buffer composition [47], temperature, or the addition of non-polar organic substances may be quite useful procedures.
The fact that in many cases elution is possible by raising salt concentration, demonstrates the presence of cooperative electrostatic interactions in the overall adsorption phenomena in the case of charged-hydrophobic supports. Nevertheless, it has been show that in those situations, the mechanism of hydrophobic adsorption at high salt concentration prevents the electrostatic interactions of being the main driving force involved in the process.

The use of denaturing agents, such as urea, guanidine-HCl etc, has been successfully applied in desorption procedures. Their mechanism of action appears to be involved in both direct interaction with the side-chains and peptide groups and disruption of hydrophobic interactions between side chains [23, 69, 77, 109].

Chaotropie ions, on the other hand, are known for their ability to impart lipophilic properties to water [72] and to alter the structure of biomolecules [44]. Their application for the elution of proteins from high members of alkyl-agarose derivatives has been reported to be successful [73]; however, it is important to bear in mind their property of dissolving agarose gels and denaturing proteins. In this case, cross-linked agaroses, which are known to support more severe conditions (pH, temperature, ionic strength etc) than the normal agarose gel, are to be employed.

As has been mentioned before, by increasing the hydrophobicity of the gel tighter binding results, and desorption of proteins requires ever more drastic conditions [78]. The combination of salt gradients with « polarity-lowering » agents (e.g., ethylene glycol), variation in pH or temperature, etc., may give an increased selectivity of the elution. As an illustration, it can be mentioned the case of serum albumin, where elution occurs at pH 3 with 50 per cent ethanol in the buffer [79]. Similarly, the use of mixed gradients of buffer solution with high salt concentration, and buffer without salt but containing 50 per cent of ethylene glycol, have been successfully applied to desorb enzymes such as chymotrypsin and trypsin from hydrophobic matrices [90].

A simple pure salt gradient may result in a strongly diluted peak in the elution because the conditions in which the equilibrium for the hydrophobic interaction is attained are slow. Only lower flow rates could provide more favourable results. In addition, it should be emphasized that in some cases the elution with ethylene glycol, in the absence of salts, may be inefficient due to the presence of electrostatic interactions [98] when adsorbents involving mixed effects are employed.

The use of detergents [127] is usually the last resource, when the elution by other milder conditions has not been possible. The problem in using detergents is their intrinsic denaturing effect and the difficulty of their removal from the columns during the regeneration step [127]. A wide range of different detergents has been used varying in their efficiency to eliminate strongly adsorbed materials [127]. In this respect, the more ionic ones are to be preferred for the reasons discussed above.

Finally, flat curves are obtained by gradient elution [75], presumably due to a postulated irregularity of the matrix (see fig. 2) and the occurrence of a wide range of binding sites of varied strengths [74]. For this reason, attempts should be made to fractionate through « differential-adsorption » [123] (as opposed to differential-elution) on a series of adsorbents of increasing hydrophobicities. In this way, each protein tends to be adsorbed or bound to the column that provides the minimum degree of hydrophobicity required for binding, and complete elution of the materials can be accomplished with mild eluants. Another alternative consists of the use of a high member of the alkyl agarose series, which may retain a high percentage of the protein content of a particular mixture, and allows the elution of the molecule of interest under mild conditions while most of the other protein remain adsorbed on the column [123]. Obviously, this possibility can be utilized only when the biomolecule in question has a low hydrophobicity. One should note that in principle it should be possible to achieve purification by HIC, not only by a selective retention of a given protein, but also by exclusion of a desired protein when most of the other proteins in the mixture are adsorbed [114].

VIII. APPLICATIONS, FUTURE AND IMPLICATIONS OF HIC.

The relevance of HIC lies in the fact that it is perhaps the first technique which takes advantage of the hydrophobic properties of the biomolecules.

The hydrophobic character of biomolecules should be a specific property, similar to the ionic character, since this is a function of the primary structure in the case of proteins and nucleic acids. So, it is not unrealistic to consider that

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Hydrophobic (interaction) chromatography implies the consideration of three main factors:
- the nature and hydrophobicity of the adsorbent and of the protein,
- the conditions of the adsorption,
- the conditions of the elution of the adsorbed material.

In conclusion, the correct application of hydrophobic chromatography implies the consideration of three main factors:

The application of HIC to the purification of nucleic acids using matrices of varied hydrophobicities according to the type of ligand attached or to its degree of substitution awaits for further studies which may greatly improve the application of this technique. This aspect is especially interesting when the molecules are not stable or soluble at either too high or too low salt concentrations.

The separation of particles, like sub-cellular fractions or entire cells [110, 121] has been reported, and it seems to be a very promising application of HIC, since membranes can be considered as aggregates of molecules of varied hydrophobicities. A similar application in a quite different field of biochemistry is preparation of enzymatic reactors through the immobilization of enzymes on hydrophobic supports [32, 101, 122, 126]. The basic idea is the possibility to have a reactor which can be periodically recycled, when the adsorbed material loses its activity by elution and adsorption of freshly active substances (which may be of the same or of another biological activity, but similarly adsorbed on the hydrophobic support). Thus, the hydrophobic matrix may function as an universal support of easier handling than those systems in which the immobilization necessitates a covalent linkage between the biomolecule and the adsorbent, with their corresponding limitations in function and utility.

It should be reminded that some hydrophobic ligands denature proteins through a « detergent-like » action [96]. This effect can be reduced by employing hydrophobic ligands of milder influence, or by introducing additional polar or ionic groups in the hydrocarbon chain [9]. Finally, the problem of « inhomogeneity », which constitutes another important drawback in protein separation by chromatography on hydrophobic columns, must be considered. If inhomogeneity occurs in the adsorbent, that is if the interacting sites on a given member of the substituted-agarose series are different in their strength of interaction with a particular protein (fig. 2), elution by decreasing salt concentration or increasing concentration of « polarity-lowering » agents, will never result in a narrow peak but rather in flat curves, which increase contamination of the sample by other proteins. Some suggestions to avoid this phenomenon have already been mentioned taking advantage of the hydrophobicity of the matrix.

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