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NON-IONIC ADSORPTION CHROMATOGRAPHY OF PROTEINS

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CONTENTS

1. INTRODUCTION

Until relatively recently it was assumed that nearly all hydrophobic amino acid side-chains of a protein are located in the interior of the molecule. The occurrence of accessible hydrophobic groups was looked upon as rather unusual. However, several years ago, Klotz concluded from X-ray data[1] that the exterior of a number of proteins is much more hydrophobic than had been assumed. This is in agreement with subsequent findings that at an NaCl concentration of 1 *M* or higher, *i.e.*, under conditions which tend to quench charge effects[2], many proteins are bound by certain agarose-bound amines and that the extent of binding increases with increasing hydrophobicity of the ligand[3–5]. Furthermore, several proteins are bound by hydrophobic adsorbents that, in contrast to the positively charged amino-agaroses[6], carry no charge[7–10] or are neutralized by the presence of a negative charge, *e.g.*, as in the case of agarose substituted with an amino acid[11,12]. Hydrophobic binding actually was found to be stabilized by certain salts, *e.g.*, NaCl[7,13]. These observations are also in accord with the more indirect but overwhelming earlier evidence obtained by Hansch and co-workers on the frequent occurrence of hydrophobic phenomena in biochemical interactions (*e.g.*, see ref. 14). Early observations in this laboratory, discussed in ref. 15, showed that chemically so-called "inert" hydrophobic groups in the substrate often play an important role in enzyme reactions and affect not only the binding process, as reflected in the Michaelis constant (K_m), but also the rate of subsequent breakdown of the enzyme–substrate complex as expressed by the maximal rate (V_m).

All of these findings suggest that hydrophobicity probably plays an important

role in the biological function of proteins and in the binding and transport of metabolites (and drugs). Hydrophobic effects in adsorptive binding involving nucleic acids are also indicated (see ref. 2).

Studies of the interactions of proteins and of other biochemically important structures with adsorbents carrying covalently bound hydrophobic groups are of great importance as "models" for "solid-state" chemical processes occurring *in vivo*[16]. However, under favorable conditions, *e.g.*, in the presence of relatively high concentrations of certain salts, such adsorbents can also be used for the chromatographic separation of proteins based on differences in their hydrophobic properties. The present communication is concerned with the separation of proteins by this means (also see refs. 3, 12, 17). Some preliminary data on another non-ionic but apparently non-hydrophobic parameter (presumably hydrogen bonding) involved in the adsorption of certain proteins, are also presented.

2. EXPERIMENTAL

The *n*-alkylamino-agaroses were prepared via CNBr activation[18,*] from Sepharose 4B or CL-Sepharose 4B (Pharmacia) and either aged for several weeks or heated for 1–2 h at 100° at pH 4–5 before use[19]. The alkylamines were crystallized as the hydrochlorides from ethanol through the addition of ethylether at −15°. DEAE-agarose was obtained by courtesy of BioRad Labs. Before equilibration with the experimental medium, the adsorbents were exhaustively washed with 50% ethylene glycol (EG) or dimethylformamide (DMF) in 0.3–1.0 *M* NaCl. Their relative degrees of substitution were estimated from the extent of irreversible binding of Ponceau S in the absence of salt[17].

Blood serum was dialyzed against and individual proteins were dissolved in the applied medium. In order to prevent excessive packing, the columns were supported, if necessary, with siliconized glass beads of appropriate size[20]. The loaded columns, which were kept at *ca.* 5°, were washed with the ambient medium until, as indicated by the absorbance or fluorescence of the effluent, little or no further material was released, and the bound protein was subsequently eluted by means of 50% EG or 50% DMF in 0.3–1.0 *M* NaCl.

SDS electrophoresis was carried out as described by Weber and Osborn[21] in 10% polyacrylamide gels at *ca.* 3 mA per gel for about 19 h. Before electrophoresis, the salt and the EG or DMF of the eluates were removed by drying the mixture at *ca.* 5° in a dialysis bag placed in a strong solution of polyethylene glycol (Carbowax 4000, Union Carbide), followed by reconstitution with an amount of water sufficient to produce a suitable protein concentration. Experimental details are described in the legends to the Figures.

3. EFFECT OF LIGAND HYDROPHOBICITY AND OF SALT (NaCl) CONCENTRATION ON BINDING OF SERUM ALBUMIN

Fig. 1, reproduced from an earlier publication[16], shows the effect of the hydrophobicity of the ligand and of the concentration of NaCl in the medium on the

* CNBr control: unsubstituted, inactivated, CNBr-treated agarose. C_0: CNBr-activated agarose treated with ammonia. C_{1-8}: agarose substituted via CNBr activation with *n*-alkylamines of varying (1–8) hydrocarbon chain lengths.

extent of binding of serum albumin by agarose carrying a diethylaminoethyl-, *n*-hexylamino-, *n*-heptylamino-, 4-phenylbutylamino(PBA)-, or *n*-octylamino group. Since the alkylamines keep their basic properties in the bound state[6] and, since their p*K* is *ca.* 10 (ref. 9), they are positively charged at the pH values usually employed. Therefore, at low ionic strength the adsorbents behave as ion exchangers and they all strongly bind the negatively charged serum albumin at the applied pH of 8.

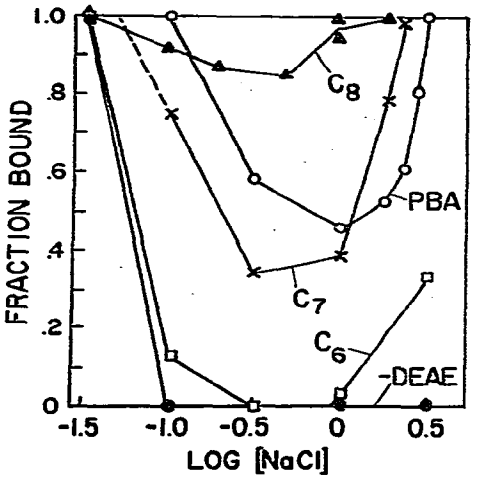

Fig. 1. Effect of salt (NaCl) concentration on the fractional binding of serum albumin by *n*-caprylamino(*n*-C_8)-, 4-phenyl-*n*-butylamino(PBA)-, *n*-heptylamino(*n*-C_7)-, *n*-hexylamino(*n*-C_6)-, or diethylamino-ethyl(DEAE)-agaroses. A few milligrams of the protein were applied to a 1 ml column of an adsorbent equilibrated with 0.01 *M* Tris–HCl (pH 8). The loaded column was washed exhaustively with the buffer alone and subsequently with buffer containing NaCl. Reproduced from previously published results[16] by permission of Marcel Dekker, Inc.

As can be seen (Fig. 1), if the salt concentration is increased, ionic binding begins to be reversed but upon further increase of the salt concentration binding increases again, presumably due to hydrophobic interaction. It should be noted that the hydrophobicity of the phenyl group of PBA is equivalent to that of only 3–4 straight-chain hydrocarbons[22–24].

It is clear that at intermediate, including physiological, NaCl concentrations binding is through a combination of ionic and hydrophobic forces (see also refs. 4, 25, 26). In this region of the salt concentration partial elution will be obtained by raising of the ionic strength *per se*. However, a salt more "chaotropic" than NaCl would be more effective since such a salt may reverse hydrophobic as well as ionic binding. Together with the fact that in this region the binding increases with increasing hydrophobicity of the ligand (Fig. 1), this could erroneously be interpreted as hydrophobic binding that is reversed by the addition of NaCl, whereas in fact the extent of hydrophobic binding increases with increasing NaCl concentration[7,13] and, presumably, only electrostatically bound protein is released (see also ref. 27).

4. CHARGED *VERSUS* UNCHARGED ADSORBENTS

From a physiological point of view, the region of the salt concentration where

hydrophobic as well as ionic binding can occur is the most interesting, but for studies of hydrophobic phenomena *per se*, it is advantageous to separate them from charge effects. For this reason, neutral adsorbents have been prepared (see Introduction). However, for the case of the positively charged amine-substituted agaroses[6], one can carry out the experiments in the presence of high concentrations of a salt such as NaCl, which quenches the charge effect and at the same time enhances hydrophobic bonding. Thus, with the same adsorbent one can study hydrophobic binding at high, ionic binding at low, and combined hydrophobic and charge effects at intermediate salt concentrations.

Another advantage of the charge on the adsorbent-bound amine is that it presents a convenient means for determining the binding capacity of the adsorbent from the amount of a negatively charged dye, such as Ponceau S, that is "irreversibly" bound in the absence of salt[17]. For this purpose, the column is saturated with a salt-free aqueous solution of the dye, followed by washing with water until no further color is released, elution by means of a suitable agent (*e.g.*, 50% EG or DMF in 0.3 *M* NaCl), and spectrophotometric determination of the amount of dye at 525 nm. The evidence[17] indicates that this procedure essentially measures the density of the charged amino group that is introduced together with the hydrophobic group*. An example of the usefulness of the Ponceau procedure is shown in Fig. 2.

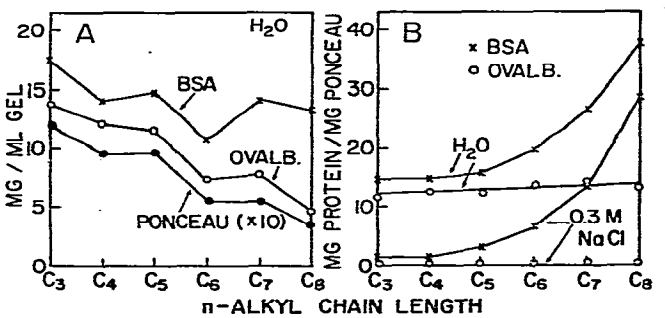

Fig. 2. Effect of C-chain length (C_3–C_8) of *n*-alkylamine-substituted agaroses on their binding capacities for bovine serum albumin (BSA) and ovalbumin (OV) as related to the capacities for binding of Ponceau S in the absence of salt. After saturation of the adsorbents (*ca.* 1 ml) with protein in the presence of 0.01 *M* Tris–HCl (pH 8) and exhaustive washing with water, the columns were first eluted with 10 ml of 0.3 *M* NaCl in 0.01 *M* Tris–HCl, followed by elution with 10 ml of the same solution containing 50% DMF. A, binding capacities of the adsorbents for BSA, OV and Ponceau S in the presence of water without additions. B, binding capacities in water and those in 0.3 *M* NaCl expressed as the amount of protein bound relative to the amount of Ponceau S bound in the absence of salt.

* As was previously noted[4], unsubstituted aged CNBr-treated agarose preparations often are positively charged to some extent. Consequently, they also bind Ponceau S. However, dye-binding of CNBr-treated CL-Sepharose is abolished by preheating the gel for 1 h at 100°. Preliminary results also indicated that heating of the substituted cross-linked agaroses (C_6–C_4) for either one or two hours at 100°, decreased their Ponceau binding capacities by *ca.* 60%, regardless of the degree of substitution (unpublished observations).

5. NORMALIZATION OF PROTEIN BINDING CAPACITIES OF ADSORBENTS

Fig. 2A shows the amounts of bovine serum albumin (BSA), ovalbumin (OV) and Ponceau S bound by a homologous series of *n*-alkylamine-substituted adsorbents under saturating conditions. These data seem rather meaningless until they are plotted as mg protein bound per mg Ponceau *versus* the C-chain length of the adsorbent (Fig. 2B). Plotted in this manner they show that the hydrophobicity of the adsorbent has little or no effect on the binding capacity for OV but that above C_5 the binding of serum albumin is greatly affected. This normalization procedure not only circumvents the effect of differences in the degree of substitution of the adsorbents but also that of slight differences in column size.

The data of Fig. 2B also indicate that electrostatic binding involving the positive charge on the ligand is completely or nearly completely quenched in 0.3 *M* NaCl and that any interaction above this level of the salt concentration is non-ionic. Such binding occurs with BSA but not in the case of OV. Above 0.3 *M* the effect of NaCl can still be studied over a wide range of concentrations without interference of charge effects. Elution can be achieved with the aid of an agent such as EG[3], if necessary, in combination with a limited decrease in ionic strength.

TABLE 1

PROTEIN BINDING BY *n*-HEXYLAMINO-AGAROSE IN THE PRESENCE OF 3.3 *M* NaCl

Percent of $\leq$ 5 mg protein held by 25 ml of the adsorbent at *ca.* 5° after washing with 2–3 bed volumes of the medium (3.3 *M* NaCl in 0.01 *M* Tris–HCl, pH 8) and subsequently eluted by 50% EG in buffer containing 1 *M* NaCl.

Protein	*Protein binding (%)*
7S γ-Globulin	100
Serum albumin	100
β-Lactoglobulin	100
Chymotrypsinogen	100
α-Chymotrypsin	$>$ 90
DNase I	$>$ 90
RNase	$\approx$ 75
Cytochrome *c*	$\approx$ 25
α-Lactalbumin	$\approx$ 15
Myoglobin	$<$ 10
Ovalbumin	$<$ 5

6. GENERALITY OF NON-IONIC PROTEIN BINDING

Table 1 shows the relative extent of binding of a number of arbitrarily chosen proteins by *n*-hexylamine-substituted agarose in the presence of 3.3 *M* NaCl. In contrast to Fig. 2, where small (*ca.* 1 ml) columns were saturated with protein, only a few milligrams of a protein were applied to a 25-ml column.

It can be seen that under the experimental conditions employed, *i.e.*, with relatively small amounts of protein on a rather large column and in the presence of NaCl in high concentration, all or nearly all of the proteins are bound, at least to some extent. The fact that positively charged protein species (*e.g.*, chymotrypsinogen), as

well as negatively charged species (*e.g.*, serum albumin), may be strongly bound indicates the absence of charge effects. This is also indicated by the finding that OV, which like serum albumin is negatively charged, shows little or no binding.

It should be noted that with columns more highly substituted and/or ligands more hydrophobic than *n*-hexylamine, some or all of the proteins that show little binding under the conditions of Table 1, may also be extensively bound. For instance, it has been shown that with adsorbents of higher hydrophobicity, even OV which displays little hydrophobicity under the conditions of Table 1, is also bound to a considerable extent[28].

In any event, these and other results[17] suggest that hydrophobicity is a general property of proteins that varies from one protein to the next and can thus be applied as an independent parameter for protein separation in addition to other parameters such as electrical charge or molecular size. The advantage of hydrophobic over ionic adsorption chromatography is that the hydrophobic factor is much more selective because widely varying degrees of hydrophobicity can be employed. Qualitatively, for ion exchange the choice is limited to either positive or negative.

7. DETERMINATION OF RELATIVE HYDROPHOBICITIES OF PROTEINS (HYDROPHOBICITY "SCALE")

The data of Table 1 are sufficient to distinguish the more hydrophobic proteins from the less hydrophobic ones. However, they do not allow for assignment of a relative hydrophobicity value to each individual protein. For this purpose, it is necessary for the more hydrophobic proteins at the top of Table 1 to determine the relative extent of binding on adsorbents less hydrophobic than hexylamino-agarose. Conversely, for more accurate determination of the hydrophobicities of the proteins at the bottom of Table 1 more hydrophobic adsorbents are needed.

It should also be ascertained that the observed binding in each case is determined solely by hydrophobic effects and that other non-ionic parameters (*e.g.*, hydrogen bonding, see below) are not involved.

Since the binding of proteins by the adsorbents would be favored by multiple-point attachment (see refs. 16 and 17) the molecular size of the protein would be a factor. It may be noted (Table 1) that cytochrome *c*, α-lactalbumin and myglobin, which have relatively low molecular weights (M.W.), also show relatively little binding. Furthermore, the protein with the highest M.W. (7S γ-globulin) shows extremely strong binding. On the other hand, this does not hold true for OV, which shows little or no binding but has an M.W. which is higher than that of the other proteins of Table 1, with the exception of 7S γ-globulin and serum albumin. Such a positive relationship between M.W. and extent of binding also seems to be absent for most of the other proteins.

Results from P.-Å. Albertsson's laboratory on the relative strength of binding of several proteins by the monopalmitoyl ester of polyethyleneglycol[29,30] indicated the following relationship for the binding constants of different proteins: BSA $>$ β-LG $>$ myoglobin $>$ ovalbumin $\approx$ chymotrypsinogen A. With the exception of chymotrypsinogen, these results are not unlike our own. Since multiple point attachment to the palmitoylester would be unlikely, this does not appear to be a factor in this case.

In any event, eventually it may be possible to determine exact hydropho-

bicity indices for proteins in general. Such values could be of equal importance as those of other properties such as isoelectric point and molecular weight.

8. PROTEIN SEPARATION ON HYDROPHOBICITY GRADIENTS

It would seem, that with the aid of a column such as the *n*-hexylamino-agarose column of Table 1, separation would be possible by loading it with a protein mixture in the presence of high concentrations of a salt such as NaCl, followed by differential elution, *e.g.*, with a gradient of increasing EG concentrations in combination with a limited decrease in ionic strength (see above). However, in view of possible extremely strong binding, ascribed to multiple-point interaction of the protein with several adsorbent binding sites ("chelation"), application of a mixture to a column of an adsorbent of arbitrarily chosen hydrophobicity may result in binding of the more hydrophobic proteins that is too strong for subsequent elution by a mild eluent, *i.e.*, recovery may not be possible without denaturation (see also refs. 31 and 32). On the other hand, the less hydrophobic proteins may not be held by the column. For this reason, the use of hydrophobicity gradients has been proposed[12]. Such a gradient consists of a series of interconnected columns of increasing hydrophobicity through which the protein mixture is pumped starting with the least hydrophobic one. In this manner, each protein tends to be held by the column that provides the minimum degree of hydrophobicity required for binding. Thus, after extensive washing with the ambient medium, each of the subsequently disconnected columns may be separately eluted with a relatively mild eluant such as EG[3] which even in 50% concentration appears to have little or no irreversible effect on most proteins[33]. A similar procedure for charcoal chromatography has been proposed by Porath[34].

9. SEPARATION OF BLOOD SERUM PROTEINS ON HYDROPHOBICITY GRADIENTS

The data of Fig. 3 show the extent of binding of serum proteins by a hydrophobicity gradient of the C_4, C_5, C_6, C_7 and C_8 adsorbents. It can be seen that relatively little protein is bound by the C_4 column. A peak appears at C_5, followed again by an increase in binding with increasing chain length from C_6 to C_8.

The SDS electrophoresis patterns of the eluates as well as the patterns of the protein solution after (rec) and before (ser) recycling through the gradient, are shown in the insert. Before electrophoresis all of these solutions were dialyzed and dried against polyethylene glycol and reconstituted with water (see Experimental). A control of an untreated solution of human γ-globulins (Fr.II) is also included.

It should be emphasized that the intensity of the electrophoretic bands cannot be compared quantitatively from one gel to another because the eluates were concentrated to different extents based on their A_{280} values, as shown in the curve. For instance, little γ-globulin is seen in the pattern of the C_8 eluate despite the fact that the two corresponding bands of the heavy and light chains are still present in the pattern of the recycled solution and therefore all of the columns must have been in contact with this protein. The reason could be that this solution, because of its high absorbance, was concentrated the least (see legend to Fig. 3). Another possibility is that competition with the albumin is involved.

Although the mobilities of the bands do not correspond exactly to those of the

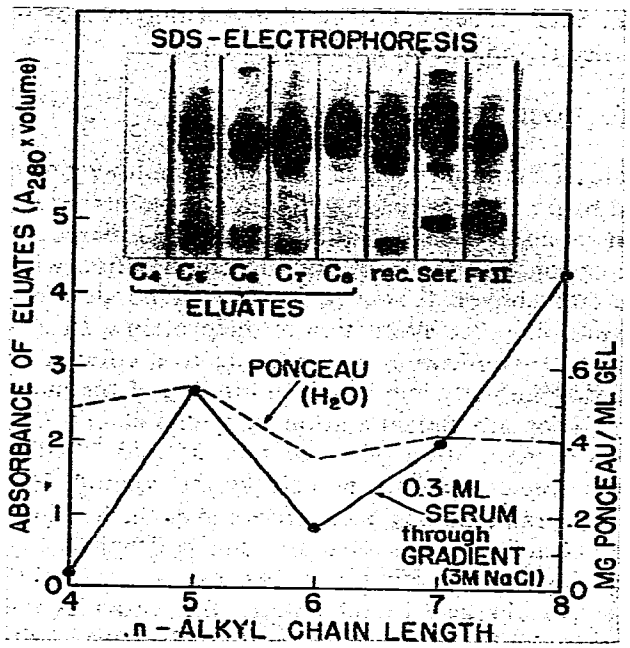

Fig. 3. Adsorption chromatography of 0.3 ml of human blood serum on a hydrophobicity gradient consisting of interconnected 1 ml columns of *n*-butyl(C_4)-, *n*-pentyl(C_5)-, *n*-hexyl(C_6)-, *n*-heptyl(C_7)- and *n*-octyl(C_8)amino-agaroses prepared via CNBr activation from CL-Sepharose 4B. The adsorbents, preheated for 1 h at 100° and pH 4–5, were equilibrated at *ca.* 5° with 3 *M* NaCl in 0.01 *M* Tris–HCl (pH 8). The serum, diluted 1:50 with and dialyzed against the buffer–salt medium, was recycled about ten times through the gradient in the direction of increasing hydrophobicity. This was followed by exhaustive washing with the ambient medium and subsequent elution of the disconnected columns by means of 50% ethylene glycol in buffer containing 0.3 *M* NaCl (see Experimental). Before electrophoresis the eluates of the C_4, C_5, C_6, C_7 and C_8 adsorbents were desalted and concentrated approximately 10-, 4-, 10-, 7-, and 2.5-fold, respectively (see Experimental).

controls (ser, Fr.II), the data indicate that the major component of the protein fraction bound by and eluted from the C_5 adsorbent, as opposed to the C_6, C_7, and C_8 adsorbents, consists of γ-globulin, and that this adsorbent, in contrast to the more hydrophobic ones, binds little or no albumin. Other bands, relatively stronger than those on the Fr.II control, can also be noted.

In any event, separation of the two major serum protein fractions *i.e.*, albumin and γ-globulin, seems to be possible by means of the pentylamine-substituted agarose (see also ref. 8). Similar results have been previously obtained by means of phenylalanine-substituted agarose[11,12]. It is of interest that the hydrophobicity of the phenylalanine side-chain corresponds to that of 4–5 straight-chain hydrocarbons[22,24].

The spilling over of an abundant protein, such as albumin in the case of serum, onto columns more hydrophobic than required for binding of that protein, may defeat the purpose of the hydrophobicity gradient and, in fact, could render these columns useless. The results of Fig. 3 indicate that this is likely to occur in attempts to isolate minor components from whole serum by hydrophobic gradient chromatography on columns more hydrophobic than butylamino-agarose. Therefore, the run shown in Fig. 4 was carried out with less hydrophobic adsorbents. In order to allow minor

components to accumulate, columns four times larger were used and 5 ml instead of 0.3 ml serum was applied. Also, the degree of substitution of the C_4 adsorbent, as indicated by the Ponceau values, was approximately twice as high as that of the C_4 column of Fig. 3. Thus potentially much more protein could accumulate on this column. As indicated by A_{280}, it can indeed be seen that more than 100 times as much was bound. However, the amount rapidly decreases with decreasing C-chain length of the adsorbent (Fig. 4).

The surprising finding is that the CNBr-control binds more than the C_0, C_1 and C_2 and about the same as the C_3 column. A striking feature of the electrophoretic patterns of Fig. 4 is that those of the eluates of the C_0, C_1 and C_2 columns are about the same and that corresponding bands also occur in the eluates of the C_3 and C_4 columns. This indicates that the binding of some of the material in serum is not dependent upon the hydrophobicity of the adsorbent. On the other hand, the CNBr-control as well as the C_3 and C_4 gels seem to contain components that do not occur on the other adsorbents. For C_3 and C_4 this can be ascribed to increased hydro-

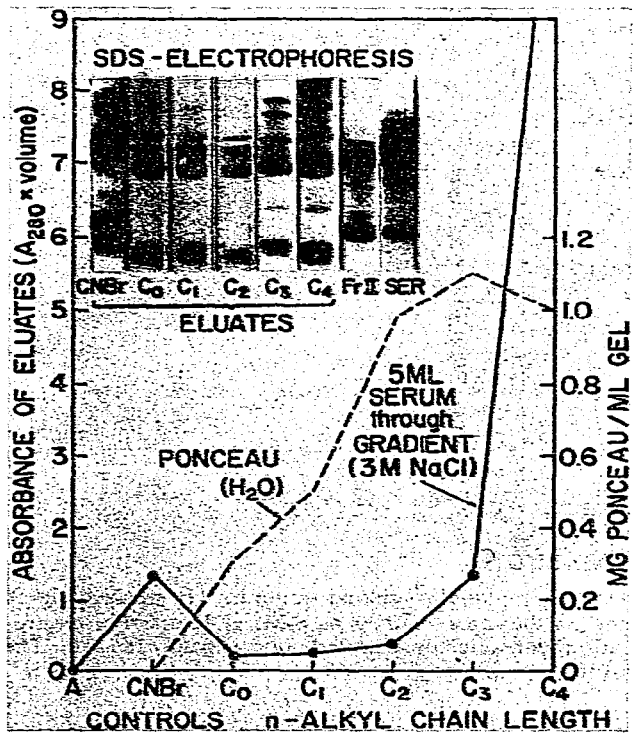

Fig. 4. Adsorption chromatography of 5 ml of human blood serum on a hydrophobicity gradient consisting of *ca.* 4-ml columns of preheated CL-agarose (A), CNBr-activated CL-agarose (CNBr) and CNBr-activated CL-agarose treated with ammonia (C_0) or with methyl (C_1)-, ethyl(C_2)-, *n*-propyl (C_3)- or *n*-butylamine(C_4). The experimental conditions were generally the same as those for Fig. 3, except that the serum was diluted only ten-fold. Before electrophoresis (insert) the eluates of the CNBr, C_0, C_1, C_2, and C_3 adsorbents were desalted and concentrated approximately 8-, 30-, 30-, 20-, and 8-fold respectively. The C_4 eluate was similarly treated but reconstituted to its original volume (see Experimental).

phobicity, but the protein bound by the CNBr-control, as well as the bands common to all these gels, may be due to another type of binding, most likely involving hydrogen bonds (see below).

In any event, if a component appears on a particular column and not on subsequent columns of the series, this indicates that such a component, occurring in trace amounts in the serum, has accumulated on that particular column which apparently provides the minimum degree of affinity required for binding. This is the case, for instance, for some components on the CNBr-control, on the C_3 and possibly on the C_4 column.

Another striking feature of these patterns is that there are two bands, similar to those of the light and the heavy chain of γ-globulin, that occur in all of the electrophoretic patterns, suggesting that all of the corresponding columns bind γ-globulin. This is confirmed by the results of Fig. 5.

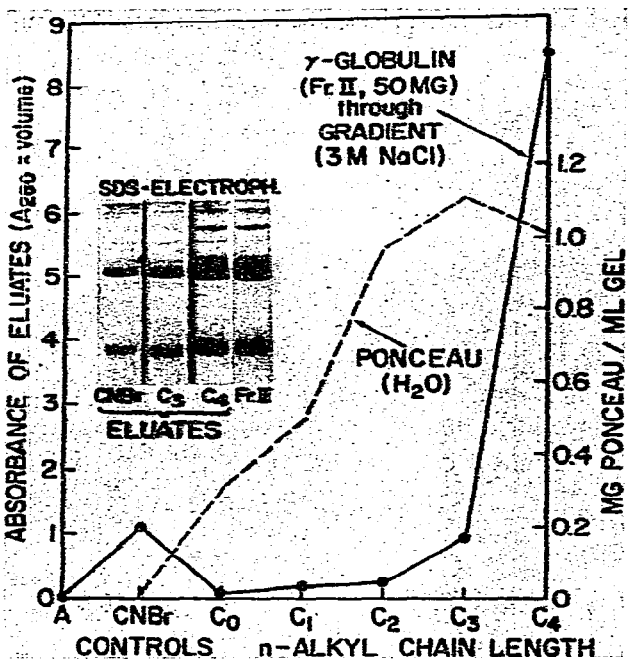

Fig. 5. Adsorption chromatography of 50 mg of "γ-globulin" (blood serum Fr. II), dissolved in 50 ml medium, under the same conditions and on the same columns as those of Fig. 4. For electrophoresis the eluates of the CNBr and C_3 adsorbent were concentrated approximately ten-fold. The C_4 eluate was not concentrated (see legend to Fig. 4).

10. NON-IONIC NON-HYDROPHOBIC BINDING OF γ-GLOBULIN COMPONENT(S)

The shape of the absorbance curve of Fr.II (Fig. 5) strongly resembles that of the whole serum of Fig. 4, suggesting that the curve for whole serum is largely determined by the γ-globulin. The electrophoretic patterns of the proteins bound by CNBr control and by the C_3 and C_4 adsorbents (Fig. 5) seem to be qualitatively identical to the Fr.II control in every detail. This indicates that the same protein unit or units are

bound by all of these gels. As one could expect, in view of the numerous antigenic compounds that can be bound by γ-globulin, this protein in particular should be capable of binding in various ways, *e.g.*, hydrophobically by *n*-alkyl groups larger than C_3 and perhaps by hydrogen-bonding in the case of the CNBr control column and to a lesser extent by the substituted columns as well.

That this binding of γ-globulin by the CNBr control is predominantly non-hydrophobic is also indicated by the data of Fig. 6 which show that very little reversal is obtained by lowering the salt concentration from 3 to 0.3 *M*. On the other hand, binding by the C_4 column is to a large extent reversed by lowering the salt concentration indicating a much larger hydrophobic effect. This holds true for Fr.II (which is mostly γ-globulin) as well as for a preparation of 7S γ-globulin.

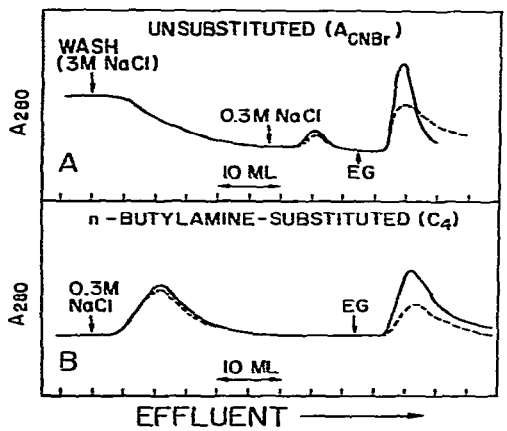

Fig. 6. Elution profiles of 7S γ-globulin (—) and of "Fraction II" (— — —) peaks only, after adsorption on 3–4 ml columns of the CNBr and the C_4 adsorbents (see Fig. 5). Protein solution (10 ml) with an A_{280} value of 0.3–0.4 were recycled several times through a column until the absorbance of the effluent was constant. The column was then washed with 3 *M* NaCl (shown only in the upper part), subsequently with 0.3 *M* NaCl and finally with 50% ethylene glycol (EG) in 0.3 *M* NaCl. Arrows indicate the points of change of medium.

Fig. 6 also indicates that the non-hydrophobic type of binding, like the hydrophobic type, is readily reversed by 50% EG. If the binding by A_{CNBr} is indeed mostly through hydrogen bonding, this could have a bearing on the theory[35] that hydrophobic bonding ultimately depends on the intermolecular structure of water which, in turn, also depends on hydrogen bonding.

It should be noted that the binding capacity of the CNBr column of Fig. 5 for γ-globulin is not more than *ca.* 0.2 mg protein per ml of the settled gel. It would be of interest, therefore, to test other adsorbents with potentially stronger hydrogen bonding capabilities.

11. ACKNOWLEDGEMENTS

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12. SUMMARY

Evidence is presented indicating that protein separation by adsorption chromatography, based on differential non-ionic interaction with immobilized hydrophobic ligands, potentially is as generally applicable as ion-exchange chromatography. A procedure for the normalization of binding capacities of amine-substituted agaroses has been presented. Attempts have been made at the separation of proteins in normal human blood serum by means of hydrophobicity gradients consisting of series of interconnected columns of *n*-alkylamino-agaroses of increasing hydrophobicities and equilibrated with 3 *M* NaCl. The γ-globulin fraction, or components thereof, can be bound hydrophobically as well as through another type of salt(NaCl)-stable but non-hydrophobic binding.

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