USE OF THE SURFACTANT 3-(3-CHOLAMIDOPROPYL)-DIMETHYL-AMMONIOPROPANE SULFONATE IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS

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SUMMARY

Isocratic hydrophobic interaction chromatography of five proteins has been carried out using mobile phases containing the surfactant 3-(3-cholamidopropyl)-dimethylammoniopropane sulfonate (CHAPS). Linear relationships were found between $\log k'$ and ammonium sulfate concentrations for all the proteins with CHAPS in the submicellar concentration range. The slope of such a plot decreases mono-tonically as CHAPS concentration is increased. To a first approximation, the effect of CHAPS on protein retention can be explained in terms of a competitive binding model. However, CHAPS does show differential effects on the elution of proteins, sub-stantially altering selectivity. The use of a normalized capacity factor, k'/k'_0, proves useful for comparing retention times of different proteins as a function of CHAPS concentration. The magnitudes of k'/k'_0 were found to be inversely correlated with the slopes of plots of $\log k'$ vs. ammonium sulfate concentration in the absence of CHAPS. Adsorption isotherms for CHAPS were determined over the working range of ammonium sulfate. The binding of CHAPS to the SynChropak Propyl stationary phase and its effects on retention were found to be readily reversible. For each protein, plots of k'/k'_0 vs. surface concentration of CHAPS were superposable for data obtained at different salt concentrations. These findings support a competitive binding model. A simple geometric argument for stationary phase occupancy provides a qualitative explanation for the observed surfactant selectivity.

INTRODUCTION

High-performance liquid chromatography (HPLC) in recent years has become an important technique for the separation of proteins. Most commonly, reversed-phase packings such as alkyl C_{18}, C_8, C_4, and phenyl-type functionalities have been used[1-5]. However, proteins, and particularly membrane proteins, tend to bind very strongly to such packings. Consequently, in the application of HPLC to proteins, the additives commonly used to elute proteins from such columns (*e.g.*, acetonitrile and propanol, often with acids such as trifluoracetic acid or phosphoric acid) are also denaturing toward many proteins. Thus there is a need for combinations of mobile and stationary phases capable of eluting proteins under non-denaturing conditions.

Recently, increasing emphasis has been placed on hydrophobic interaction chromatography (HIC) as a number of different, commercial high-performance packings have become available for this mode of chromatography[6–10]. Elution in this mode of chromatography is achieved with decreasing salt (usually ammonium sulfate) concentration gradients under relatively mild, non-denaturing conditions.

As might be expected, surfactants were shown to be capable of interacting with HIC and reversed-phase stationary phase surfaces[11–16]. Occasional use of mild surfactants in protein chromatography has been reported[17,18], but systematic studies are lacking. Ionic surfactants are known to be capable of interacting with proteins[19], some even at concentrations below the critical micelle concentration (CMC). In contrast, non-ionic and dipolar ionic (zero net charge) surfactants interact less strongly with proteins, and with less likelihood of denaturation than ionic surfactants[20].

Therefore, zero net charge surfactants are expected to be useful in hydrophobic interaction chromatography either as eluting agents or at least to provide useful selectivity effects[21]. This study is an examination of the effects of the surfactant 3-(3-cholamidopropyl)-dimethylammoniopropane sulfonate (CHAPS) on retention of proteins in high-performance HIC.

EXPERIMENTAL

Materials

Carbonic anhydrase B (human erythrocyte, lot 10F-9320), ribonuclease A (bovine pancreatic, lot 101F-0561), bovine pancreatic trypsin inhibitor (BPTI, lot 104F-8035), and enolase (Yeast Type III, lot 101F-8125) were obtained from Sigma (St. Louis, MO, U.S.A.). Lysozyme (hen egg white, lot 7069) was obtained from Miles Labs. (Elkhart, IN, U.S.A.). The proteins were used without further purification.

Ammonium sulfate (Ultra Pure) was obtained from Schwarz Mann (Cambridge, MA, U.S.A.) and potassium phosphate (ACS reagent) was obtained from Fisher (Fair Lawn, NJ, U.S.A.). HPLC-grade water was produced with a Milli Q purification system.

CHAPS was synthesized according to the procedure of Hjelmeland *et al.*[22] and twice recrystallized from methanol.

Chromatographic procedures and equipment

The chromatograpic system consisted of a Varian Model 5000 pumping system (Palo Alto, CA, US.A.), a Valco (Houston, TX, U.S.A.) six-port injection valve with 25-μl injection loop, a Varian Varichrom variable-wavelength UV detector, and a Hewlett-Packard Model 3390a integrator. The chromatographic column was 15 cm × 4.6 mm I.D. packed with 6.5-μm SynChropak Propyl HIC packing (SynChrom, Linden, IN, U.S.A.). The column was thermostated with a circulating water bath at 30.0°C.

Ammonium sulfate solutions of varying concentrations were prepared in pairs, one member of the pair containing CHAPS at 0.0015 *M* and the other containing no CHAPS. All mobile phases contained 0.02 *M* potassium phosphate buffer at pH 6.1. CHAPS concentration in the chromatographic mobile phases was controlled by varying the proportion of the CHAPS-containing mobile phase with the HPLC pumping system. Isocratic elution of proteins was accomplished at a flow-rate of

1 ml/min, and monitored at a detector wavelength of 215 nm. Protein solutions were prepared at a concentration of *ca.* 1 mg/ml in mobile phase without CHAPS and filtered through a 0.5-μm filter prior to injection. Equilibration of the column in the presence of surfactant was verified by repeated injections of ribonuclease A until constant retention times were observed. After each series of measurements with surfactant, the column was flushed with water, methanol, and water. The column was then reequilibrated with the starting salt solution and the retention times for the five proteins were remeasured to verify reversibility of the column to surfactant exposure. Adsorption isotherms for CHAPS were measured by frontal chromatography using UV detection at 215 nm. Mobile phase concentrations of CHAPS were chosen to be below the CMC as determined fluorimetrically with a Perkin-Elmer 650-10s spectro-fluorimeter (Table I). Mobile phase containing $3 \cdot 10^{-5}$ *M* 7-diethylamino-4-methyl coumarin was titrated with successive additions of a concentrated CHAPS solution, monitoring fluorescence emission at 450 nm (excitation at 370 nm). A rapid increase in fluorescence emission begins at the CMC.

TABLE I

CRITICAL MICELLE CONCENTRATIONS FOR CHAPS AT 30.0 ± 0.5°C

The CMC values were determined fluorimetrically with a estimated uncertainty of ± 0.3 m*M*.

Ammonium sulfate (mol/l)	CMC (mmol/l)
0	8.2
0.50	5.3
0.75	3.7
1.00	2.6
1.20	2.1
1.30	1.7
1.40	1.0

RESULTS AND DISCUSSION

Data for the retention of the five proteins in this study were examined by plotting k' *vs.* CHAPS concentration at constant ammonium sulfate concentration (Fig. 1). As can easily be seen from Fig. 1, addition of CHAPS to HIC mobile phases causes definite reductions in k' for all the proteins studied. Similar results were seen for all ammonium sulfate concentrations in the range 1.1–1.4 *M*. Furthermore, it should be noted that, on this stationary phase, the effect of CHAPS on retention is selective in that some proteins are affected to a greater extent than others. For some of the proteins changes in elution order occur as CHAPS concentration is increased.

It is instructive to compare the relative effect of CHAPS at different salt concentrations. In order to do this, k' values were normalized as follows. First k' in the absence of surfactant is defined as k'_0. Then the k' values in the presence of CHAPS are divided by k'_0 to yield the normalized retention parameters, k'/k'_0, which were then plotted as a function of CHAPS concentration in the mobile phase. Typical plots are found in Fig. 2.
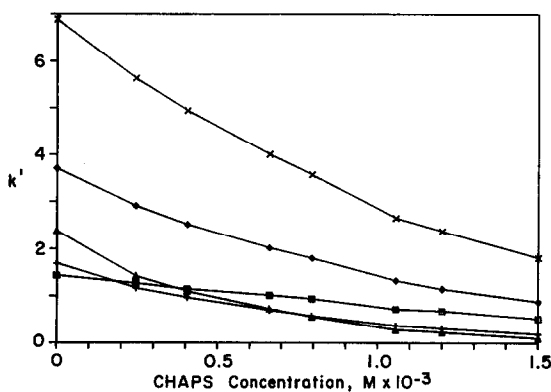
Fig. 1. Isocratic k' dependence on CHAPS concentration for five proteins. ($\times$) Lysozyme; ($\Diamond$) BPTI; ($\square$) RNase A; ($+$) carbonic anhydrase; ($\triangle$) enolase. $(NH_4)_2SO_4$ concentration: 1.30 M.

The normalized retention parameter k'/k'_0 can be useful for expressing retention in this mode of HIC, as the relative order of the plots is independent of salt concentration over the range employed in this study. That is, at all salt concentrations examined the relative magnitude of k'/k'_0 for the five proteins increases in the order of enolase < carbonic anhydrase < BPTI < lysozyme < ribonuclease A. Put another way, the effect of addition of CHAPS is always greatest on enolase and least on ribonuclease A. This effect occurs even though changes in order of retention occur as the ammonium sulfate concentration changes. The effect of CHAPS on any given protein's retention increases (*i.e.*, k'/k'_0 decreases) as the salt concentration is increased, as shown in Fig. 2 for RNase and enolase.

In discussing the meaning of these findings, a good starting place is the solvophobic model of retention of proteins. In a number of recent articles retention of proteins in the framework of this model have been discussed[23–25]. In this model, protein retention is related to the change in free energy which occurs on binding of
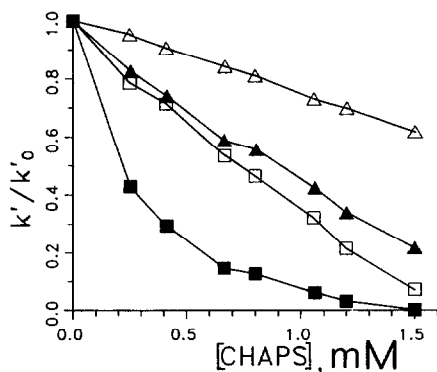

Fig. 2. Normalized retention parameter (k'/k'_0) dependence on CHAPS concentration for RNase A and enolase at two salt concentrations. ($\square$) Enolase at 1.10 M $(NH_4)_2SO_4$; ($\blacksquare$) enolase at 1.40 M $(NH_4)_2SO_4$; ($\triangle$) RNase A at 1.10 M $(NH_4)_2SO_4$; ($\blacktriangle$) RNase A at 1.40 M $(NH_4)_2SO_4$.

a protein to the stationary phase. From this viewpoint it was shown[23] that retention of proteins in hydrophobic interaction chromatography is predicted to obey an equation of the following form:

$$\ln (k'/k_0') = Bm^{\frac{1}{2}}/(1+Cm^{\frac{1}{2}}) + D\mu m + \Delta A_s \sigma m + vm + \text{constant} \qquad (1)$$

where B, C and D are constants related to the protein, μ is the protein dipole moment, v is a constant related to the particular salt, protein and stationary phase, m is the salt concentration and ΔA_s is the change in surface area of the stationary phase and protein which occurs on binding of the protein to the stationary phase. Note also that σ is the partial molal surface tension increment of the salt. At sufficiently high salt concentrations, the solvophobic model simplifies to the following equation:

$$\ln k' = \lambda m + Q \qquad (2)$$

In this equation m is the salt concentration and Q is the extrapolated value of $\ln k'$ at zero salt concentration. The coefficient λ is a linear function of the hydrophobic area of the protein in contact with the stationary phase; it is also a linear function of the molal surface tension increment, which is characteristic of the particular salt employed. Thus $\ln k'$ is expected to show a linear dependence on salt concentration, with a slope proportional to the protein hydrophobic contact area. It is worth noting that eqn. 2 is essentially the same as the empirical Setschenov equation describing the salting-out of non-electrolytes[26].

To examine our experimental results in the framework of this model, we obtained values of λ and Q by linear regression analysis of plots of $\ln k'$ vs. ammonium sulfate concentration. Values of λ and Q were obtained from each of the five proteins at each of the CHAPS concentrations used. Linear plots of $\ln k'$ vs. ammonium sulfate concentration were obtained for all five proteins, both with and without CHAPS in the mobile phase, as shown in the example of RNase A, in Fig. 3. This figure shows that increasing CHAPS concentration has the effect of decreasing the slope, λ, and increasing the intercept, Q. In Fig. 4 the values of the slope, λ, are plotted as a function of CHAPS concentration for all five proteins. Here it is shown that for all the proteins
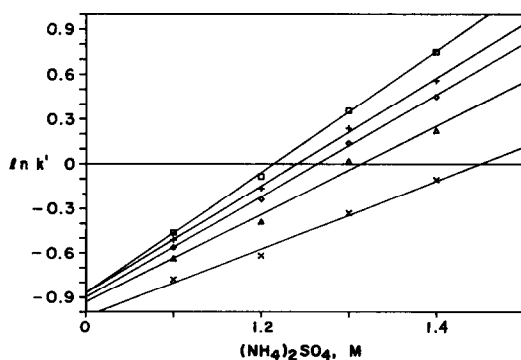

Fig. 3. Retention of RNase A as a function of $(NH_4)_2SO_4$ concentration, at several submicellar concentrations of CHAPS. ($\square$) 0 mM; ($+$) 0.20 mM; ($\diamond$) 0.60 mM; ($\times$) 1.00 mM.
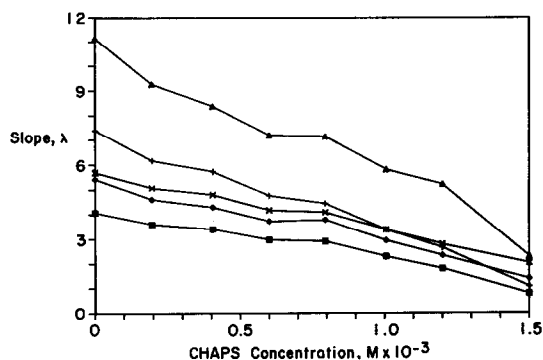
Fig. 4. Effect of CHAPS concentration on λ, the slope of a plot of ln k' vs. $(NH_4)_2SO_4$ concentration, for five globular proteins. ($\times$) Lysozyme; ($\Diamond$) BPTI; ($\square$) RNase A; ($+$) carbonic anhydrase; ($\triangle$) enolase. The range of $(NH_4)_2SO_4$ concentration for determining λ is 1.10–1.40 M.

an increase in CHAPS concentration leads to a decrease in λ, but to a different degree for each protein. Note that there is an inverse correlation between the magnitude of λ in the absence of CHAPS, and the magnitude of k'/k'_0 in the presence of CHAPS. Proteins whose retention is more strongly dependent on salt concentration are more strongly eluted by the addition of CHAPS to the mobile phase. This generalization is supported both by the data of Table II and Fig. 4. In the framework of the solvophobic model this would mean that the proteins with greater hydrophobic contact area are more strongly eluted by increasing CHAPS concentration.

Can the decrease in k' be accounted for through the surface tension of the mobile phase, as expressed in the solvophobic model? It was shown that surface tension of a surfactant solution can be related to surfactant concentration by a logarithmic relationship[27]. That is, at concentrations below the CMC, a plot of surface tension vs. log surfactant concentration will be a straight line. Other workers have shown for HIC in the absence of surfactants[23,24], and eqn. 1 predicts, that log k' is linearly related to the surface tension of the solution. Therefore we would expect to see a linear relation between log k' and log surfactant concentration if mobile phase surface tension is the dominant factor in controlling k'. However, plots of log k' vs. log CHAPS concentration are found to be definitely nonlinear. This is observed for the data

TABLE II

INVERSE RELATIONSHIP BETWEEN λ AND k'/k'_0

The slope, λ, from eqn. 2 was determined in the absence of CHAPS, while k'/k'_0 was determined for 1.30 M $(NH_4)_2SO_4$ and 0.40 mM CHAPS. A similar inverse relationship is found for other salt and CHAPS concentrations.

Protein	λ	k'/k'_0
RNase A	4.08	0.80
BPTI	5.69	0.68
Lysozyme	5.70	0.72
Carbonic anhydrase B	7.40	0.57
Enolase	11.15	0.46

obtained in this study and has also been seen in earlier results from this laboratory[21]. Therefore, with respect to surface tension, the solvophobic treatment of eqn. 1 is clearly inadequate. This is not surprising, given the highly non-ideal nature of surfactant solutions. A physically more realistic argument might be generated in terms of interfacial tensions between stationary and mobile phases. These are difficult to measure, but some encouragement is seen in recent reports[29,30]. Our continued use of eqn. 2 assumes that while the linear surface tension component of λ is here inapplicable, the linear hydrophobic contact area component of λ is valid.

Another possibility for interpreting decrease in k' with increasing CHAPS concentration is in terms of competition of CHAPS for protein-binding sites on the stationary phase, as has been done for reversed-phase chromatography[28]. If CHAPS is bound to the stationary phase and is bound with roughly the same affinity as the protein molecules, an effect similar to that shown in Fig. 5 might be observed. In this model, strength of binding of a protein to the stationary phase is related to the size of the hydrophobic contact area. In general, we expect that large proteins have larger hydrophobic surface patches than small proteins, but exceptions probably exist. The presence of CHAPS decreases the area of stationary phase available for hydrophobic contact with proteins. As the load of CHAPS on the column is increased, the number of large binding sites decreases more rapidly than the total number of binding sites. This is the natural consequence of a statistical distribution of occupancy of surfactant-binding sites. Thus this model predicts that the larger the protein hydrophobic contact area, the greater will be the effect of surfactant in reducing k'. Since the value of λ is proportional to the size of the protein hydrophobic contact area, the greater the value of λ in the absence of CHAPS, the greater will be reduction of k' in the presence of CHAPS (*i.e.* k'/k'_0 will be smaller). Table II shows that this correlation obtains for the five proteins in this study. The same trend is seen over a broader range of conditions in Fig. 4.

The above competitive binding model assumes no association between surfactant and protein in the mobile phase. This appears to be a reasonable assumption in
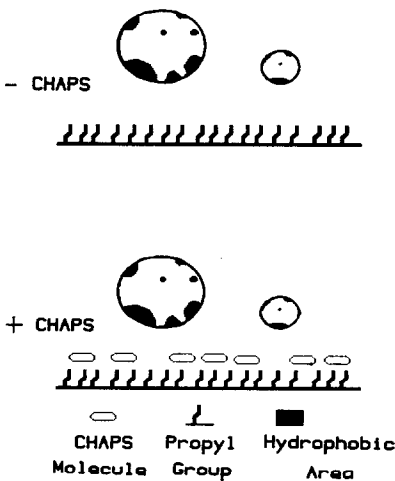
Fig. 5. Scheme to suggest how competitive binding between proteins and CHAPS could lead to selectivity.

view of the results of published binding studies[31]. Attempts to demonstrate mobile phase binding of CHAPS to this group of proteins showed no evidence of such binding[32].

A testable prediction of the above model is that the reduction in retention of a particular protein will depend only on the amount of surfactant bound to the stationary phase, irrespective of the salt concentration. Preliminary to testing this prediction we determined the binding isotherms for CHAPS at different salt concentrations by frontal chromatography (Fig. 6). It can be seen that isotherms of steeper slope are obtained at higher salt concentrations. Clearly the equilibrium between bound and mobile phase surfactant is shifted toward bound by increasing the ammonium sulfate concentration. The first premise of the competitive binding model is that, as CHAPS concentration on the stationary phase increases, decreases in k' for all the proteins will result. Fig. 7 shows this to be the case.
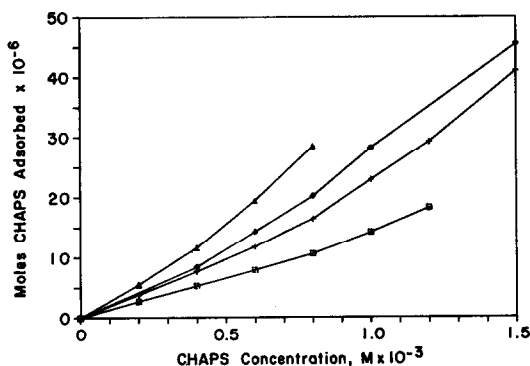

Fig. 6. CHAPS adsorption isotherms at four concentrations of $(NH_4)_2SO_4$. The isotherms were determined by frontal chromatograpy at 30.0°C on a SynChropak Propyl column, 15 × 0.46 cm I.D., pH 6.1. (□) 1.10 M; (+) 1.20 M; (◊) 1.30 M; (△) 1.40 M.

Further, if k'/k'_0 values for a protein are plotted against the surface concentration of CHAPS, the data from four different salt concentrations fall on a common curve. Fig. 7 shows that this is the case for each of the five proteins exained. This contrasts with the divergence in curve resulting from similar plots of k'/k'_0 vs. CHAPS concentration in the mobile phase, as shown by the examples in Fig. 2. Thus a testable prediction of the competitive binding model has been tested and verified. Considering that values of k' range from two-to-five fold (RNase A, smallest range), and from 14- to > 50- fold (enolase, largest range), the fit of the retention data to a single curve for each protein is evidently significant. These results are what would be predicted if a given protein competes for binding sites with bound CHAPS, and if each protein always binds on its same hydrophobic surface or distribution of surfaces. If some other mechanism for decreasing k' were playing a significant role, such as surfactant–protein association in the mobile phase, it is unlikely that the correlations observed in Fig. 7 would obtain.

It should be noted that for simplicity, the competitive binding model discussed here is based on a static picture of the chromatographic interface. A more realistic but
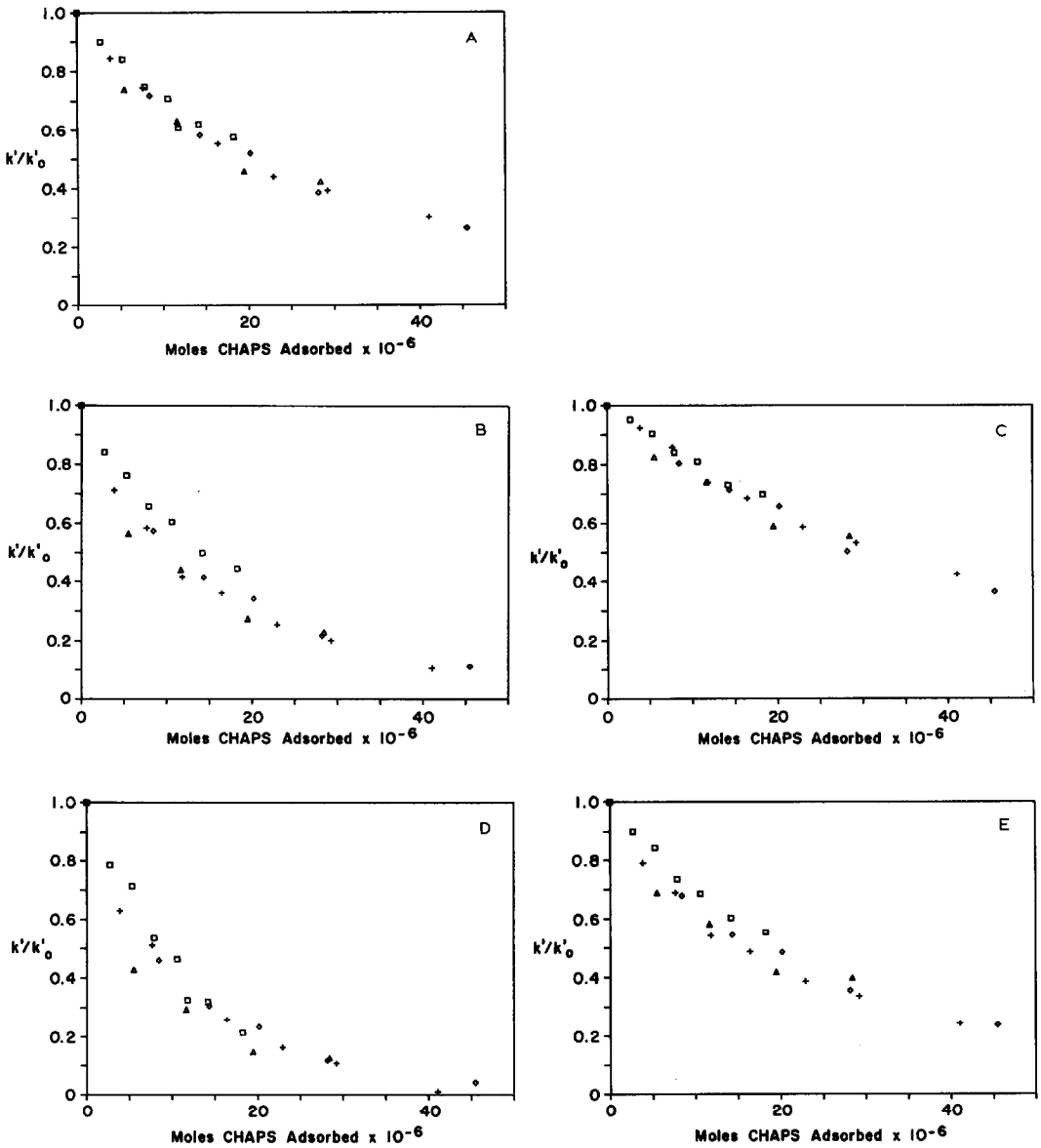
Fig. 7. Normalized retention (k'/k'_0) of five globular proteins as a function of adsorbed CHAPS from Fig. 6. Each panel displays the retention data for a particuar protein at four different $(NH_4)_2SO_4$ concentrations; (□) 1.10 M; (+) 1.20 M; (◇) 1.30 M; (△) 1.40 M. (A) Lysozyme; (B) carbonic anhydrase; (C) RNase A; (D) enolase; (E) BPTI.

more complex dynamic model involving the kinetics of association of both CHAPS and protein on and off the stationary phase might be developed; we believe that it would lead to similar conclusions. The possibility of CHAPS binding to some protein

is not excluded by the present model, but it appears unlikely to be significant in the present studies.

The effect of CHAPS on retention was found to be readily reversible, in contrast to the common experience that many surfactants bind irreversibly to reversed-phase columns[11–16,33]. After exposure of the column to CHAPS-containing mobile phases, the column could be restored to original retention characteristics by simply flushing with approximately eight column volumes of water followed by eight column volumes of methanol. Table III gives values of k' obtained on a SynChropak Propyl column before and after exposure to CHAPS-containing mobile phase. After such exposure, the retention characteristics of the column are essentially unchanged. Additional evidence in support of the conclusion that CHAPS is bound reversibly was encountered in the frontal analysis determination of binding isotherms.

TABLE III

REVERSIBILITY OF HIC COLUMNS TO CHAPS EXPOSURE

Conditions: 1.30 M $(NH_4)_2SO_4$; 0.02 M phosphate buffer pH 6.1; 1.00 ml/min; UV at 215 nm, 0.5 a.u.f.s.

Protein	k' before CHAPS exposure	k' after CHAPS exposure
RNase A	2.34	2.35
Carbonic anhydrase	2.82	2.85
BPTI	3.40	3.54
Enolase	6.19	6.10
Lysozyme	7.04	7.12

The use of CHAPS did not lead to any obvious band-broadening in these studies. Mobile phases containing ammonium sulfate and CHAPS appear to be non-denaturing for enolase, carbonic anhydrase, and RNase A[32]. The issue of chromatographic efficiency and the possibility of anomalous chromatographic behavior in the neighborhood of the CMC of CHAPS are presently under investigation. The use of CHAPS in various gradient models is also being explored as is the use of other mild surfactants. These issues will be addressed in forthcoming publications.

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