Liquid Chromatographic Studies of the Effect of Phosphate on the Binding Properties of Silica-Immobilized Bovine Serum Albumin

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Abstract

High-performance liquid chromatography has been used to examine how phosphate ions affect the binding properties of bovine serum albumin (BSA) immobilized to porous silica. In doing this, the time dependence of the protein to reach conformational equilibrium is measured as a function of the concentration of phosphate in the eluent using the D- and L-isomers of tryptophan and kynurenine as solutes. The overall binding and chiral selectivity ($\alpha_{D,L}$) of the protein toward these solutes appear to be related to two types of effects: one being those that are site-selective and only influence the retention of the L-isomers and the other being those that are nonselective and influence the retention of both enantiomers. An interesting feature of the concentration-dependent data is a maximum in $\alpha_{D,L}$ at intermediate phosphate concentrations (i.e., 10 to 50mM phosphate) indicative of both cooperative and antagonistic binding effects. Phosphate eluents within this concentration range provide selectivity advantages, and those at higher concentrations decrease the time required for the protein or column to reach equilibrium. A final set of studies has also been carried out using four alternate buffer systems (i.e., borate, carbonate, acetate, and arsenate eluents). Although the borate eluents affect the BSA's binding properties and $\alpha_{D,L}$ similar to the phosphate eluents, the other buffers result in poor separations. Observations from this study are useful in helping to optimize separations carried out on immobilized BSA as well as addressing biological and mechanistic questions related to how anions influence the native binding properties of serum albumins.

Introduction

Stereochemical analysis has become increasingly important in pharmaceutical development because the two enantiomeric forms of many chiral compounds often exhibit different physiological properties. Typically, three chromatographic approaches are used to resolve enantiomers: (*a*) preanalysis conversion of the enantiomers to diastereomers followed by their separation using achiral stationary and mobile phases (1), (*b*) the separation of enantiomers on an achiral stationary phase in combination with a chiral mobile phase additive (2), and (*c*) direct resolution of enantiomers using chiral stationary phases (CSPs) (3). Of these three approaches, the latter has become the more popular.

CSPs can be classified into five major groups (4,5): pirkle-type (donor–acceptors), inclusion (chiral cavity), helical polymer, ligand exchange, and protein-based phases. In the latter instance, chiral recognition is a native property of many proteins, and when they are immobilized properly some, if not most, of their indigenous binding affinities are preserved. Protein-based CSPs have been produced using a number of proteins including α_1-acid glycoprotein (6), ovomucoid (7) α-chymotrypsin (8), avidin (9), cellobiohydrolase (10), ovotransferrin (11), lysozyme (12), and various types of serum albumin (13–15). It is not surprising that among these, serum albumin (i.e., bovine) was the first to be used to produce chiral phases because it is the most abundant protein in blood plasma and serves as a depot and transport agent for numerous endogenous and exogenous compounds (16–18).

Although the exact number of distinct binding locations in bovine serum albumin (BSA) is still open to debate, the general consensus is that there are two principal sites for small heterocyclic and aromatic carboxylic acids, at least two to three for long-chain fatty acids that are unique and separate from the binding sites for small anionic compounds, and two metal binding sites (one involving Cys-34 and the other N-terminus) (17). Several of these binding sites (i.e., site I and II for small heterocyclic and aromatic carboxylic acids) have been identified crystallographically and reside within subdomains IIA and IIIA (19). Under aqueous conditions, BSA is highly flexible and thermally stable up to approximately 45°C (20). It exists in several conformational states, and transitions between them (because of pH) are usually fast and reversible (21). Below a pH of 3.5 the protein's length (i.e., the expanded form) is 65% greater than it is at neutral pH, and it contains only 35% α-helix. Between pH 4 and 4.5 BSA exists in two other conformations, a "fast" form (F) and a "normal" form (N). During the transition between the F and N forms, the helical content

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increases from 45% to 55% (18). At pH values above 7, two additional isomerizations occur: a normal-base transition (N-B) between pH 7 and 9 resulting in a drop in the helical content to 48% and a base-aged transition at pH values above 9.6.

Various spectrometric methods have been used to study protein–ligand interactions (22–26) that are often accompanied by detectable conformational changes in the protein. Walji et al. (22) have studied how drugs such as aspirin, carbenicillin, and testosterone affect human serum albumin (HSA) and found that the protein's high-affinity binding sites are conformationally labile. Similarly, Manfait et al. (26) have examined the interaction of HSA with fatty acids and observed significant differences in the infrared spectra before and after binding that are consistent with an earlier model proposed by Karush (27,28), which suggests that certain ligands trigger the formation of new protein binding sites by a loss of helical structure.

Ligand-induced conformational changes can result in both a cooperative and antagonistic binding effect on other ligands. Cooperative binding can be either homotropic (in which two different ligands interact or function on the same site to enhance their individual binding) or heterotropic (in which the interaction of the secondary at an alternate site results in a structural change that enhances the binding of the primary ligand). Examples of cooperative binding effects include the enhancement of the interactions of progesterone and testosterone by the palmitate ion (29) and bilirubin by the laurate or myristate ions (30) as well as the increased binding of warfarin following the addition of a long-chain fatty acid (31,32). In contrast to cooperative binding, antagonistic binding has been proposed to explain why low concentrations of long-chain fatty acids decrease the binding of chlorophenoxyisobutyrate (33), halofenate (33), and diazepam (34).

In terms of its chromatographic applications, Stewart was the first to use agarose-immobilized BSA to resolve D,L-tryptophan via discontinuous elution (35). Subsequently, silica has been the immobilization substrate of choice, and a number of important chromatographic and mechanistic properties of this system have been examined. Wainer et al. (36–38) have determined that when HSA is attached to silica it does not significantly lose its binding properties nor its ability to alter its conformation in response to ligands. In addition, the influence of other parameters such as temperature and the addition of small amounts of organic cosolvents on the binding properties of serum albumin (20,39–41) as well as other immobilized proteins such as hen and turkey-egg-white lysozymes (12,42–44) have been examined. In some of these studies the species dependence (i.e., the influence of subtle differences in the amino-acid sequences) on binding has been evaluated (12,40,44).

The purpose of this study has been to investigate how phosphate influences the binding and chromatographic properties of silica-immobilized BSA. The potential insights to be gained from such a study are relevant not only because silica-immobilized serum albumins in combination with phosphate eluents have been used to separate a variety of chiral compounds, but phosphate systems are important in biological processes and are commonly used in biological and chemical research. In addition, studies have been carried out under similar conditions in order to evaluate how other common inorganic anions (i.e., buffers) influence the indole binding and separation properties of BSA.

Experimental

Materials

The LiChrospher Si-300 silica (10-µm particle diameter) used to prepare the albumin phase was obtained from EM Separations (Gibbstown, NJ). The γ-aminopropyl triethoxysilane was from Hüls-Petrarch (currently United Chemical Technologies, Bristol, PA), and the 25% aqueous solution of glutaric dialdehyde was from Aldrich Chemical Co. (Milwaukee, WI). Sodium cyanoborohydride; sodium phosphate; sodium carbonate; sodium borate; boric acid; arsenic acid (sodium salt and heptahydrate); and the test solutes D,L-tryptophan, L- tryptophan, D,L-kynurenine, and L-kynurenine were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. Phosphoric acid, glacial acetic acid, and toluene were from Fisher Scientific (Pittsburgh, PA). The deionized water was prepared in-house using a Labconco (Kansas City, MO) water system.

The different phosphate, borate, and acetate buffer eluents were prepared by adjusting a given amount of the appropriate salt to a desirable pH using the corresponding acid. In the case of the carbonate buffers the pH was adjusted to its value using dry ice, and the arsenate buffer was prepared from arsenic acid and sodium hydroxide. A Fisher Scientific Model 15 pH meter was used to monitor these preparations. Prior to use all buffer eluents were degassed via stirring under vacuum for 15 min.

Synthesis

The silica-immobilized BSA stationary phase was synthesized similar to a previously reported three-step procedure (20,39–44). Each step in this procedure was monitored by the microelemental analysis of carbon and nitrogen using a LECO (St. Joseph, MI) Model CHNS-932 analyzer.

The aminopropyl intermediate was prepared as follows. Five grams of silica was suspended in approximately 100 mL deionized water by swirling and sonicating for 5 min and then allowed to settle for 3 h. The water was carefully decanted off and the silica was dried at 110°C overnight. The purpose of this initial treatment was to control the amount of physisorbed water presented. Subsequently, the silica was transferred into a specially designed glass reaction flask and equilibrated with 200 mL water-saturated toluene using a stream of dry nitrogen to stir the slurry. After equilibration, the silica was allowed to settle for 1 h, and 100 mL of the solvent was removed. This was followed by the addition of 20 mL of γ-aminopropyltriethoxysilane, which was allowed to react with the silica overnight under refluxing conditions. The solvent was removed and the modified silica was washed by resuspending it in 100 mL toluene, refluxing for 1 h, and removing the toluene. This washing procedure was repeated three additional times. The resulting material was dried under vacuum for two days. A

small sample was removed for analysis.

The amino-derivatized silica was transferred to a 250-mL round-bottom flask. A 5% glutaric dialdehyde solution in a coupling buffer (0.05M phosphate buffer, pH 7.0, 100 mL buffer, and 25 mL of a 25% aldehyde solution in water) was added as well as a solution of 2.5 g sodium cyanoborohydride in 125 mL of the coupling buffer. The reaction was allowed to proceed for 3 h at room temperature during the rotation of the flask at an angle in order to stir the solution and quench foaming. A stir bar was not used in order to prevent the silica particles from being broken by its grinding action. The contents of the flask were transferred to conical centrifuge tubes (50 mL) and the coupling media removed by centrifugation at 1200 rpms for 5 min (IEC CRU-5000 centrifuge, Damon/IEC Division, Needham Heights, MA). The activated silica was washed ten times with deionized water by resuspending and then centrifuging, followed by decanting the solvent. Again, a small sample was removed for analysis.

The carbonyl activated material from step 2 was transferred into a 250-mL round-bottom flask using 88 mL of the coupling buffer. During the swirling of the solution, 0.11 g of sodium cyanoborohydride was added. This was followed by the dropwise addition of a solution of 302 mg BSA in 72 mL of the coupling buffer by employing an addition funnel. The funnel was washed with an additional 18 mL of buffer, and 0.11 g more of sodium cyanoborohydride was added. The mixture was rotated for 20 h at room temperature before transferring it to 50-mL centrifuge tubes and centrifuging for 5 min at 1200 rpms. The supernatant was decanted off and the modified silica was washed sequentially with 45 mL of a 0.2M KCl solution in coupling buffer and deionized water. The final material was lyophilized using a Labconco Model 8 freeze dryer, and a small sample was used to carry out microelemental analysis. Based on both the carbon and nitrogen analysis data, a coverage of 95 mg BSA per gram (approximately 1.4 µmol BSA per gram) of silica was obtained.

Column packing

Stainless steel columns having dimensions of 0.21- × 15-cm i.d. were made with tubing purchased from the Handy & Harman Tube Company (Norristown, PA). The column blanks were thoroughly cleaned, endfittings were attached, and the modified silica was packed into them using a previously described slurry procedure (45) as follows. Approximately 0.55 g silica was suspended in approximately 25 mL 2-propanol. This slurry was then transferred into a high-pressure dynamic reservoir assembly, which was filled to volume (i.e., 30 mL) with 2-propanol and sealed. The mixture was stirred during the entire packing process, which was carried out in an upward fashion. The column included a metal frit (2.0-µm pore diameter) at the top end to retain the silica. The packing system was pressurized to approximately 6000 psi using a Haskel (Burbank, CA) Model DSTV-122 air-driven fluid pump. Methanol was used as the carrier solvent, and the packing was continued for approximately 3 h.

Chromatographic measurements

After the packing procedure was completed and prior to carrying out any chromatographic separations, all columns were conditioned overnight at a flow rate of 0.5 mL/min with deionized water. Following this preparation and before use, each column was conditioned at the desired flow rate and temperature until the baseline was stable.

The liquid chromatographic system consisted of a Varian (Walnut Creek, CA) Model 9002 series isocratic pump, a Spectra Physics (San Jose, CA) Model Spectra 100 series variable wavelength UV detector set to 230 nm, and a Model 4400 Chromjet integrator connected to a PC for data collection. The samples were injected using a Rheodyne (Berkeley, CA) Model 7125 six-port injection valve equipped with a 20-µL sample loop. The temperature of the column and the injection valve were controlled to ± 0.1°C using a Fisher Scientific Model 9500 Isotemp refrigerated circulator bath, and the flow rate was monitored by a Phase Separation (Queensferry, Clwyd., U.K.) Model FLOSOA1 digital flowmeter.

Results and Discussion

An initial set of experiments was carried out using three different types of phosphate buffer eluents that were prepared from the ammonium, potassium, and sodium salts. Phosphate buffers were selected because they are known to be useful for resolving such enantiomers as D,L-tryptophan, D,L-kynurenine, and other related compounds on silica-immobilized BSA in which the L-isomers selectively bind at the indole site (39–41 and other cited references). Furthermore, they are important biochemically and are used often in solution protein-binding studies. Previously, it has been demonstrated that chiral resolution is obtainable with phosphate buffer eluents in the pH range of 7.2 to 8.0 in which BSA undergoes its N-B transition and the chiral selectivity ($\alpha_{D,L}$) increases with increasing pH. In contrast, the chemical stability of silica-based bonded phases is best at lower pH values, and eluents in the pH range of 7.4 to 7.8 are often used to carry out chiral separations (39).

Shown in Figure 1 are representative chromatograms illustrating the chiral resolution that is obtainable using a 50mM pH 7.8 sodium phosphate buffer eluent. In terms of the non-site-specific binding isomers, D-tryptophan was retained slightly longer (peak 1, Figure 1A) than D-kynurenine (peak 1, Figure 1B), but the site-specific binding was greater for L-kynurenine (peak 2, Figure 1B) than L-tryptophan (peak 2, Figure 1A). These results are consistent with previously published work (39–41) and establish a reference set of data for the remaining experiments that will be discussed.

Even in the relatively narrow pH range in which BSA undergoes its N-B transition and below the pH in which silica-immobilized phases are chemically unstable, there were significant differences in terms of solute retention (k') and $\alpha_{D,L}$ for enantiomers such as tryptophan, kynurenine, and related compounds. This is illustrated in Figure 2, which shows plots of k' versus pH for D- and L-tryptophan separated using 50mM buffers prepared from the corresponding potassium, ammonium, and sodium phosphate salts (for reference purposes, the single data points appearing at pH 7.8 in curves 1c and 2c

were calculated from the chromatogram in Figure 1A). In general, the particular cation had a relatively minor but similar nonselective effect on the solute retention for both the nonbinding and binding isomers (i.e., relative position of curves a, b, and c in Figure 2) that is consistent with simple ion-exchange and charge effects. Although not shown, identical trends were observed for D- and L-kynurenine. Interestingly, the $\alpha_{D,L}$ (as reflected by the data summarized in Table I) followed the reverse trend for the three buffer systems (i.e., $\alpha_{D,L}$ for the K^+ eluent > $\alpha_{D,L}$ for the NH_4^+ eluent > $\alpha_{D,L}$ for the Na^+ eluent) from the overall retention order shown in Figure 2 (i.e., k' for the Na^+ eluent > k' for the NH_4^+ eluent > k' for the K^+ eluent). Although the intent of this work was not to study in detail the influence of nonselective cation effects on the binding properties of BSA, the data demonstrated that $\alpha_{D,L}$ can be improved by reducing background solute-protein sorption.

Except for the pH studies discussed previously, the remaining phosphate buffer experiments were carried out using pH 7.4 eluents in order to maximize the phase stability. This was important because several time-dependence studies required a careful measurement of k' over extended periods. Under these eluent conditions, slightly less than baseline resolution was obtained for D- and L-tryptophan, but the peak maxima were clearly separated and peak overlap was sufficiently small enough so that retention measurements could be made on both isomers from a single injection. In the case of D,L-kynurenine (in which the L-isomer has a higher protein binding constant than L-tryptophan) baseline resolution was obtainable even at pH 7.4.

The time-dependence of the immobilized protein that was needed to reach conformational equilibrium was studied as follows. After packing, each column was conditioned overnight with deionized water at a flow rate of 0.75 mL/min. Then, the mobile phase was switched to either 6.7mM or 50mM (sodium phosphate buffer and retention measurements made as a function of time). Changes in the retention for D- and L-tryptophan are summarized in Figure 3A. For the 50mM phosphate buffer (bottom set of curves in Figure 3A), protein equilibrium was established quickly as noted by the constant values of k' as a function of time. However, for the 6.7mM buffer (top set of curves in Figure 3A) equilibrium was reached over a relatively long time, as noted by an initial slight increase in the solute retention and then the very gradual decrease in k' through the first 40 h of exposure to the eluent. The initial rise in k' most likely was caused by simple eluent changeover effects (i.e., water to buffer), and the resulting pH induced an N-B conformational change in the protein and its effect on binding because it occurred in a similar time period as the higher concentration curves. However, the gradual decrease in k' over many hours of exposure to the phosphate ion was caused by a kinetically slow process resulting from additional conformational changes in BSA. After reaching equilibrium the final k' values for the L-tryptophan were 1.65 and 3.70 for the 50mM and 6.7mM phosphate buffer eluents, respectively. Similarly, the equilibrium k' values for D-tryptophan were 0.65 and 1.50. An interesting observation is that although the overall retention was significantly higher for the 6.7mM data (curves b)

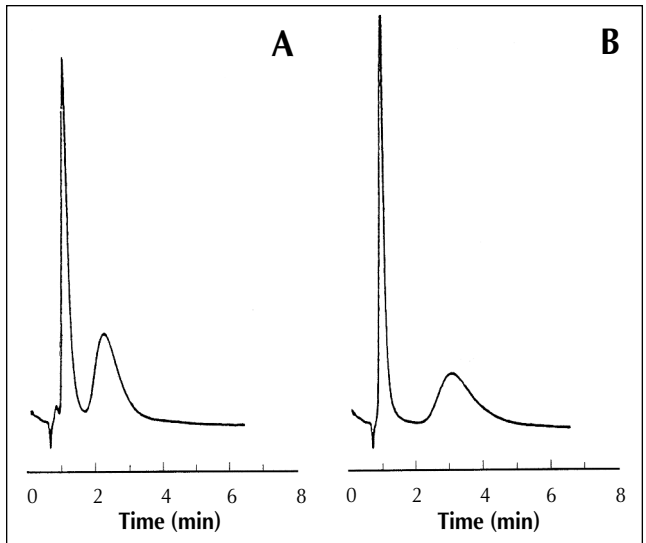

Figure 1. Representative chromatograms showing the resolution (A) D-tryptophan in peak 1 and L-tryptophan in peak 2 and (B) D-kynurenine in peak 1 and L-kynurenine in peak 2 on silica-immobilized BSA using 50mM pH 7.8 sodium phosphate as the eluent.

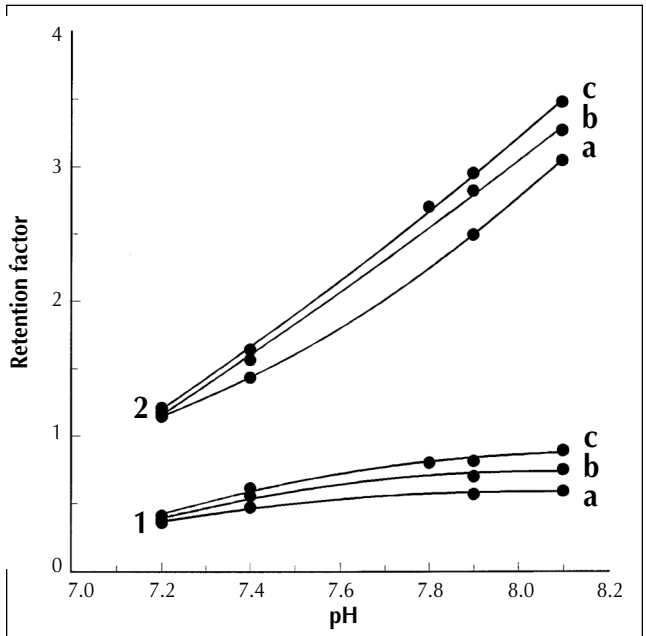

Figure 2. Influence of pH on k' for (1) D- and (2) L-tryptophan for 50mM phosphate buffer eluents prepared using (a) potassium, (b) ammonium, and (c) sodium phosphate salts.

Table I. $\alpha_{D,L}$ for D- and L-Tryptophan Separated Using 50mM pH 7.4 Phosphate Buffers

pH	$\alpha_{D,L}$ for the K^+ buffer	$\alpha_{D,L}$ for the NH_4^+ buffer	$\alpha_{D,L}$ for the Na^+ buffer
7.2	3.2	3.1	3.0
7.4	3.1	2.9	2.7
7.9	4.4	4.0	3.6
8.1	5.2	4.4	3.9

compared with the 50mM data (curves a), the calculated $\alpha_{D,L}$ was experimentally identical (2.5 + 0.04) after equilibrium was established. This can be more easily seen by the time-dependent selectivity plots appearing in Figure 3B.

Mechanistically, the data in Figure 3 are explainable by a combination of two types of effects, those that are site-selective and those that are nonselective. The latter case results in a reduction in background sorption with the presence of increasing amounts of the sodium phosphate buffer resulting from either simple charge effects or nonselective binding. In terms of site-selective effects, the presence of the phosphate ion appears to play an important role in the binding properties (i.e., chiral recognition) of the indole site by inducing additional non-pH-related conformational changes in the protein. This

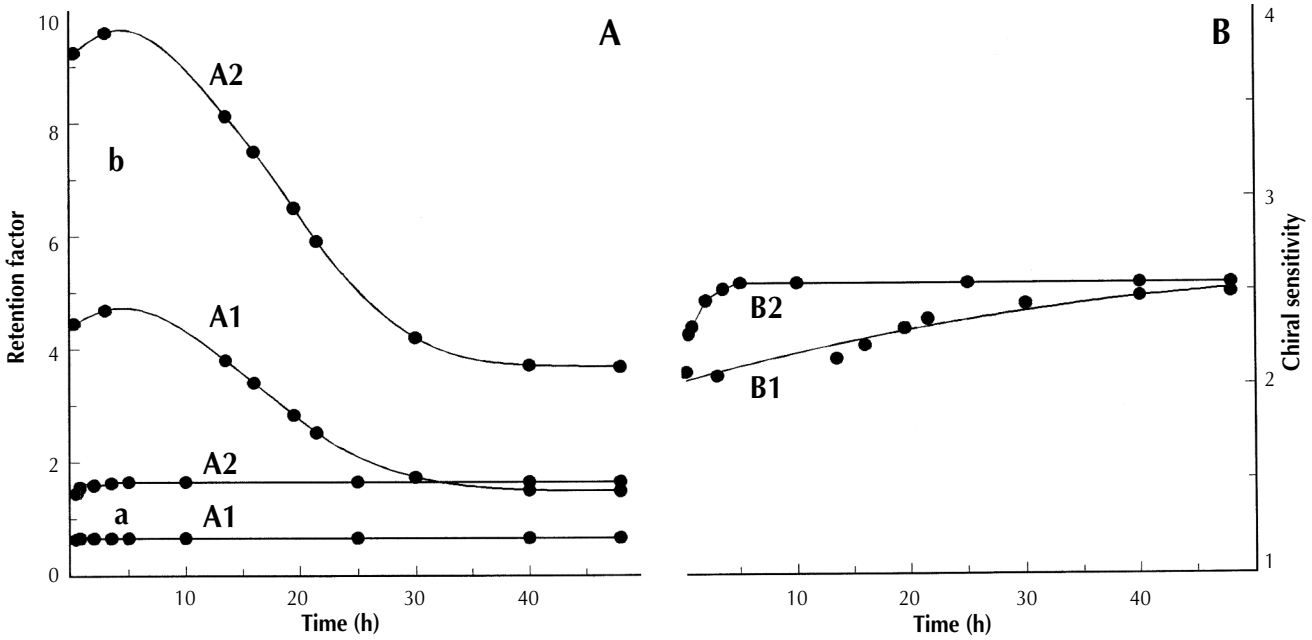

Figure 3. Influence of equilibrium time on (A) k' for (A1) D- and (A2) L-tryptophan and (B) $\alpha_{D,L}$ as a function of eluent concentration for pH 7.4 buffers: (a and B2) 50mM sodium phosphate and (b and B1) 6.7mM sodium phosphate.

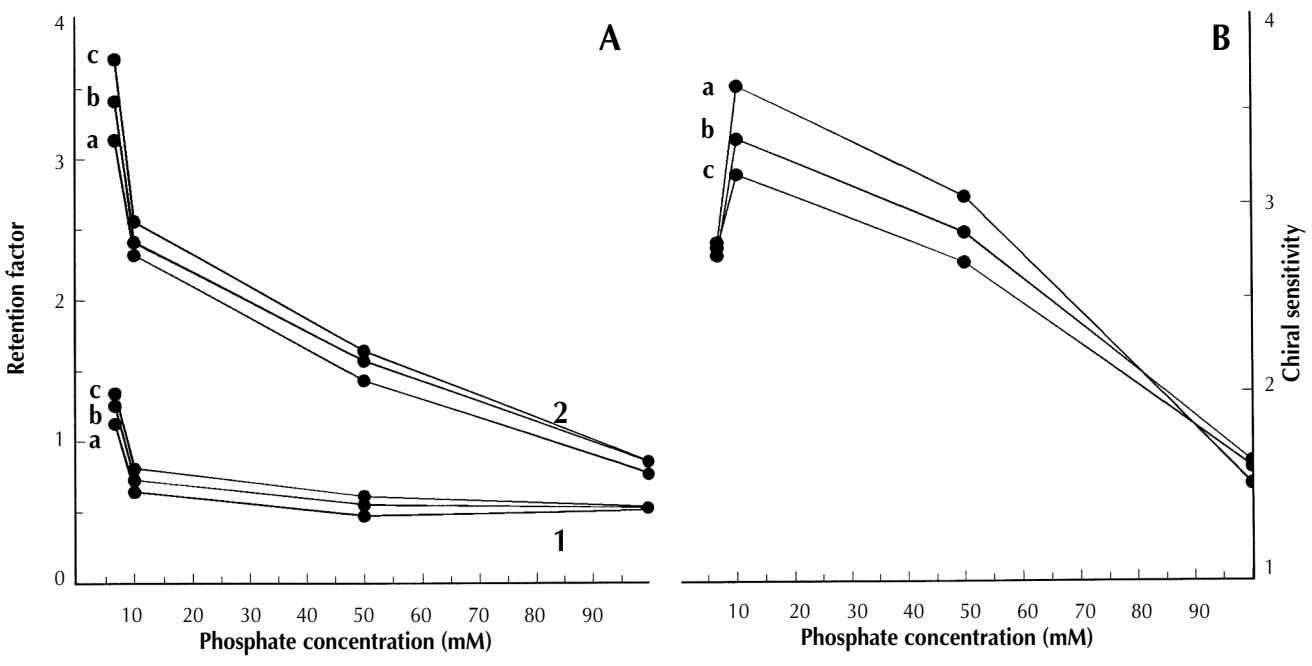

Figure 4. Influence of phosphate concentration on (A) k' and (B) the selectivity for D- and L-tryptophan for pH 7.4 phosphate buffer eluents prepared using (a) potassium, (b) ammonium, and (c) sodium phosphate salts.

model is also consistent with the observed differences in protein equilibration rates with phosphate concentration and the general anion binding properties of serum albumins in which a variety of anions are known to interact with the protein through a small number of strong binding sites and a larger number of weaker interactions (46–49).

Similar studies to those previously referenced were carried out using 100mM sodium phosphate buffer as the eluent. However, although the immobilized-protein phase was found to reach equilibrium quickly, after relatively short exposure times (i.e., within the first 8–10 h) k' was found to begin to decrease gradually. Similar changes in the retention of immobilized protein phases at a high buffer concentration have been observed by others and have been attributed to phase instability (50). As such, in collecting the 100mM data (discussed in a later section), a number of columns were used, and the reported values were within the time period before k' began to decrease.

In addition to the concentration studies noted that were made using only sodium phosphate buffers as eluents, additional concentration studies were carried out with the other buffer systems. Shown in Figure 4A are plots of k' versus the phosphate concentration for D- and L-tryptophan. The three sets of results (potassium, ammonium, and sodium phosphate buffers) that were collected using pH 7.4 eluents to maximize phase stability show again the same cation effect observed in the initial pH studies. However, the more interesting feature of the concentration-dependent data shown in Figure 4A was an apparent maximum in the $\alpha_{D,L}$ at intermediate buffer concentrations occurring between the 6.7mM and 50mM phosphate. This is illustrated by the plots of the selectivity versus concentration appearing in Figure 4B. Of the data shown, $\alpha_{D,L}$ was greatest with the 10mM phosphate eluents. However, because other data were not collected between them and the 50mM buffers, it was not possible to give a more exact value for the optimum buffer concentration.

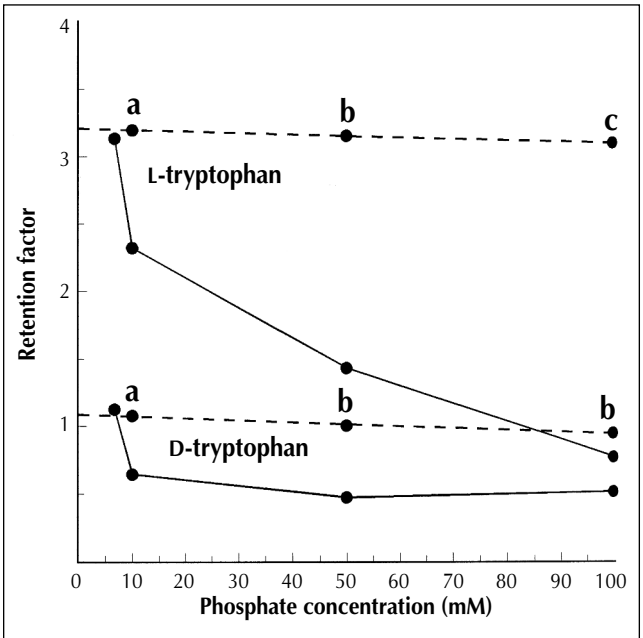

Figure 5. Influence of ionic strength on k' for the D- and L-tryptophan for pH 7.4 sodium phosphate buffer eluents. Solid lines and corresponding points represent the original data shown in Figure 4. Dashed lines and corresponding points were obtained using a 6.7mM sodium phosphate buffer adjusted to the equivalent ionic strength of the corresponding (a) 10mM, (b) 50mM, and (c) 100mM phosphate buffer with sodium sulfate.

Based on these results and previously discussed cation effects it is unclear whether the maximum in $\alpha_{D,L}$ was the result of an optimum amount of phosphate or the result of an optimum charge/ionic strength effect. In order to examine these two possibilities, another set of measurements was made in which the phosphate concentration was maintained at a constant low level and the ionic strength of the eluents was adjusted using sodium sulfate. Sodium sulfate was chosen to control ionic strength because the sulfate ion is known to bind very weakly with BSA (46). The results from this experiment are shown in Figure 5, which contains plots of k' versus the phosphate concentration for pH 7.4 buffers prepared with sodium phosphate as well as similar k' data for D- and L-tryptophan chromatographed using 6.7mM pH 7.4 sodium phosphate buffer eluents adjusted to the equivalent ionic strength of the corresponding 10mM, 50mM, and 100mM phosphate buffers with sodium sulfate. Although not shown, similar results were obtained when these same measurements were repeated using D- and L-kynurenine as test solutes. It is apparent from this study and the data in Figures 3 and 4 that the presence of phosphate is important for chiral recognition at the indole binding site and that a significant reduction in site-selective binding was observed by the addition of increasing amounts of phosphate ions beyond a given level (i.e., as noted by the decrease in selectivity shown in Figure 3B). Furthermore, this reduction does not appear to be a result of simple ionic strength effects based on the nearly constant k' values when sodium sulfate was used to make ionic strength adjustments to the phosphate buffers. Also, the data suggest that phosphate has a cooperative binding effect at lower concentrations but an antagonistic binding effect at higher concentrations that is likely because of a few strong site-selective interactions and a larger number of less specific interactions.

In an effort to more fully understand the role phosphate has on chiral recognition, a final set of experiments were car-

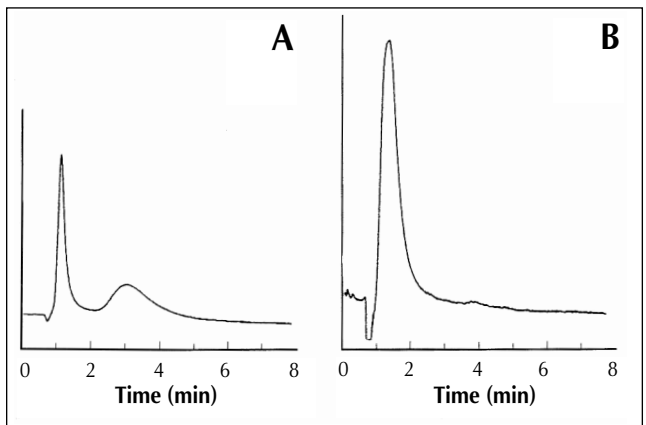

Figure 6. A representative chromatogram of D-kynurenine in peak 1 and L-kynurenine in peak 2 on silica-immobilized BSA using (A) a 50mM pH 7.8 borate buffer as the eluent and (B) a 50mM pH 7.8 carbonate buffer eluent.

ried out in order to evaluate the influence of several other common anions on the chromatographic properties of silica-immobilized BSA. In doing this, the selection of other buffers was limited to borate, carbonate, acetate, and arsenate systems because most organic anions are known to bind with BSA. Under the same buffer conditions (i.e., pH and concentration), the chiral resolution ($\alpha_{D,L}$) was dramatically different. As in the case of the phosphate eluents, borate buffers were also found to be useful for resolving the enantiomers of tryptophan and kynurenine. This is illustrated in Figure 6A, which shows the separation of D- and L-kynurenine using 50mM pH 7.8 borate eluent. However, the D- and L-isomers could not be resolved when either the carbonate (Figure 6B) or acetate buffers were used as eluents. The lack of chiral resolution in the carbonate eluent was of particular interest, because it is a commonly used and important physiological buffer (51). Although carbonate and acetate buffers with different pH values (7.2–8.0) and concentrations (6.7–100mM) was tried as eluents, no significant differences in chiral resolution were obtainable. In addition, it was found that when the immobilized protein was first exposed to the arsenate buffer (i.e., for approximately 15 min), the D,L-isomers initially eluted from the column and were resolvable, but the system was unstable and after relatively short exposure to the arsenate ion no peaks were found. Because other pHs and buffer strengths gave equivalent results, further experiments with this buffer were discontinued following the initial set of experiments.

Conclusion

In this study no attempt was made to produce columns that were optimized in terms of their chromatographic efficiency. Instead, liquid chromatography was used to study how phosphate ions influence the native binding properties of BSA at its indole site. Because BSA is a structurally dynamic protein, its binding properties are known to be highly pH dependent and they can change when it interacts with strongly binding ligands. Besides these known effects, the current data suggest that phosphate ions play an important role in the selective recognition of the L-isomers of tryptophan, kynurenine, and structurally similar compounds by way of induced changes at the indole site that are kinetically slow to develop when the protein is treated with low concentrations of phosphate. In addition, borate ions appear to have a similar effect on the indole site in BSA to phosphate, whereas the other anions studied such as carbonate do not. These latter results also demonstrate anion-related binding differences. Additional studies are in progress to further characterize these effects as well as other related conformational effects.

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Manuscript accepted December 28, 2000.