Cross-Partition of Proteins. Effect of Ionic Composition and Concentration†

Harry Walter,* Shigeru Sasakawa,‡ and Per-Åke Albertsson

ABSTRACT: Aqueous–aqueous two-phase systems are obtained when solutions of dextran and of poly(ethylene glycol) are mixed above certain concentrations. Some inorganic salts partition unequally between such phases giving rise to an electrical distribution potential. Further, a ζ potential exists between the phases, the sign, and magnitude of the charge depending markedly on the salt composition of the system. It has previously been reported that a protein's isoelectric point can be determined by cross-partition, *i.e.*, by a number of partitions in phase systems containing one of two different salts and varying the pH (Albertsson, P. Å., Sasakawa, S., and Walter, H. (1970), *Nature (London) 228*, 1329). The curves obtained when plotting the partition coefficients of proteins in phases containing one of two different salts *vs.* pH cross close to the protein's isoelectric point (cross point). We have now examined the effects of ionic character and concentration on the cross-partition of several proteins. It was found that while different ions have a marked influence on the partition of proteins, the partition coefficients of proteins are virtually independent of salt composition at the cross point. Small differences in cross-point pH do exist but these appear to be of the same order as the effect of salt composition on a protein's isoelectric point as determined by electrophoresis. Similarly salt concentration (in the range of 0.1–0.6 M NaCl) has hardly any effect on either the partition coefficients of proteins or on the pH at their cross points. An exception to these findings is ribonuclease which appears to have a crossline, covering the pH range from 7.2 to 9.3, rather than a cross point. The results show that cross-partition is a reliable and simple method for the determination of isoelectric points of proteins even at high ionic strength.

When aqueous solutions of dextran and of poly(ethylene glycol) are mixed above certain concentrations liquid two-phase systems are obtained (Albertsson, 1971). Such systems can be buffered and made isotonic and have proved useful not only in the separation by partition of cells, particles, membranes, and macromolecules (Albertsson, 1970, 1971; Walter, 1969; Brunette and Till, 1971; Walter and Sasakawa, 1971) but also in providing information on some of the surface properties of the materials being partitioned (Walter *et al.*, 1968, 1972; Walter and Sasakawa, 1971; Walter and Selby, 1966).

Despite the fact that dextran and poly(ethylene glycol) are themselves nonionic, different inorganic salts partition differently between the phases (Johansson, 1970). This gives rise to an electrical potential between them (Albertsson, 1971). A ζ potential has been demonstrated between the two phases by electrophoresis of droplets of one phase in the other. The magnitude and sign of the potential was found to depend markedly on the salt composition of the phase system examined (Seaman and Walter, 1971). That the partition coefficient (K) of materials in two-polymer aqueous phases is greatly dependent on the ionic character of such systems (Albertsson, 1971) is therefore not surprising.

In the case of proteins sign of charge is involved in determining the partition coefficient in a phase system of given salt composition (Albertsson *et al.*, 1970). Also there is a direct relation between the change in net charge of a protein and the change in its partition (Johansson, 1971). The absolute K value depends on additional parameters including molecular weight (Sasakawa and Walter, 1972). Species-specific differences in the partition coefficients at the cross point have been found in the cases of hemoglobins (Walter and Sasakawa, 1971), hen and turkey egg-white lysozymes, and β-galactosidases from two different *Escherichia coli* strains (Sasakawa and Walter, 1972). Possibly the surface hydrophobic–hydrophilic character of the proteins also contributes to their partition.

When proteins are partitioned at different pH's in phases containing one of two different salts, and lines are drawn through the points obtained with each salt, the two curves cross close to the isoelectric point of the protein (Albertsson *et al.*, 1970). Since it is known that the determination of isoelectric points of proteins is often subject to the ionic composition and concentration under which the measurement is made (Alberty, 1953), we have studied the effects of these parameters on the cross-partition points obtained. It was found that the partition coefficients of proteins at their cross points are hardly affected by the ionic character and concentration.

Methods

Proteins. Horse heart cytochrome *c* and bovine pancreatic ribonuclease A were obtained from Sigma Chemical Co., St. Louis, Mo. Trypsin was purchased from Worthington Biochemical Corp., Freehold, N. J. Hen ovalbumin was either from Sigma Chemical Co. (experiments depicted in Figure 1) or from Schuchart, Munich, Germany (all other experiments). Human adult hemolysate was prepared as previously described (Walter and Sasakawa, 1971). All proteins were dialyzed against distilled water prior to use.

† From the Laboratory of Chemical Biology, Veterans Administration Hospital, Long Beach, California 90801, from the Department of Biological Chemistry, UCLA Medical School, Los Angeles, California 90024, and from the Department of Biochemistry, University of Umea, Umeå, Sweden. *Received June 10, 1972.* Supported in part by a grant from the National Institutes of Health (HE 08304) and by Malmfonden Swedish Board for Technical Developments.

‡ Present address: Laboratory of Biochemistry, Research Department, Central Blood Center, Japanese Red Cross, Tokyo, Japan.

Salts. All salts were of analytical grade.

Preparation of Phase Systems. Aqueous dextran–poly(ethylene glycol) phase systems containing salts and buffers (see below) were used. Dextran T500 (Pharmacia Fine Chemicals, N. J., or Uppsala, Sweden), lots 3202 and 5996, were used interchangeably. Poly(ethylene glycol) was purchased under the trade name Carbowax 6000 from Union Carbide, N. Y.

Phase systems were prepared in a manner analogous to that previously reported (Albertsson *et al.*, 1970; Walter and Sasakawa, 1971; Sasakawa and Walter, 1971, 1972). To summarize, stock solutions were prepared of dextran (20%, w/w), poly(ethylene glycol) (40%, w/w), a series of 0.04 M buffers (glycine or sodium phosphate) encompassing the pH range from 3.5 to 11.5, and of alkali (*i.e.*, lithium, sodium, potassium) chlorides and sulfates at a concentration four times that needed in the final phase systems (see salt concentrations in captions to figures).

A mixture containing 14% (w/w) dextran and 8.8% (w/w) poly(ethylene glycol) was prepared by weighing out appropriate quantities of the stock polymer solutions. Partition of proteins was carried out as follows: 2 g of the mixture containing 14% (w/w) dextran and 8.8% (w/w) poly(ethylene glycol), 1 g of the sodium (*or* potassium *or* lithium) chloride stock solution *or* sodium (*or* potassium *or* lithium) sulfate stock solution containing between 0.5 and 1% of a given protein, and 1 g of 0.04 M buffer were weighed into small centrifuge tubes. The mixture was well agitated. The final phase systems had, in addition to protein, the following compositions: 7% (w/w) dextran, 4.4% (w/w) poly(ethylene glycol), 0.1–0.6 M alkali *chloride* and 0.01 M glycine or sodium phosphate buffer (system I); 7% (w/w) dextran, 4.4% (w/w) poly(ethylene glycol), 0.05–0.3 M alkali *sulfate* and 0.01 M glycine or sodium phosphate buffer (system II). The phase systems were centrifuged at room temperature for 10 min at 1200*g* to hasten phase settling.

Determination of Protein Partition Coefficients. The partition coefficient, *K*, is defined as the ratio of protein concentration (or absorbance) in top phase to protein concentration in bottom phase. As described earlier (Sasakawa and Walter, 1972) 0.5 ml of top phase and 0.5 ml of bottom phase were carefully pipetted from the phase system in each tube, and each diluted by addition of 2.0 ml of water. The solution was mixed and the absorbance was measured at 280 nm (nonhemoproteins) or 540 nm (hemoproteins) against a top or bottom phase blank on a Gilford spectrophotometer (Model 240) or on a Zeiss PMQ II spectrophotometer. pH was measured directly on the remaining phases.

Results and Discussion

Effect of Ionic Composition of the Phase System on the Partition of Positively and Negatively Charged Proteins. While many parameters are involved in determining the partition of charged materials in two-polymer aqueous phases (Albertsson, 1971; Walter and Sasakawa, 1971; Sasakawa and Walter, 1972; Walter *et al.*, 1967), the most dramatic influence on their partition is the ionic composition of the phases. Thus Albertsson (1971) has been able to arrange salts in a series, analogous to the Hofmeister series, which increase (or decrease) the partition coefficients of *all* negatively charged materials in the same sequence. Walter *et al.* (1968) subsequently demonstrated that the effect of the Albertsson salt series was reversed when positively charged materials were partitioned instead of those negatively charged. As one example, negatively charged materials have higher partition coefficients in phases containing sodium sulfate rather than sodium chloride while the reverse holds for positively charged materials (Albertsson *et al.*, 1970). Furthermore, negatively charged materials have decreasing partition coefficients when the cationic series is changed from (for example) lithium to sodium to potassium (Albertsson, 1971); similarly the reverse holds for materials positively charged (Walter *et al.*, 1968).

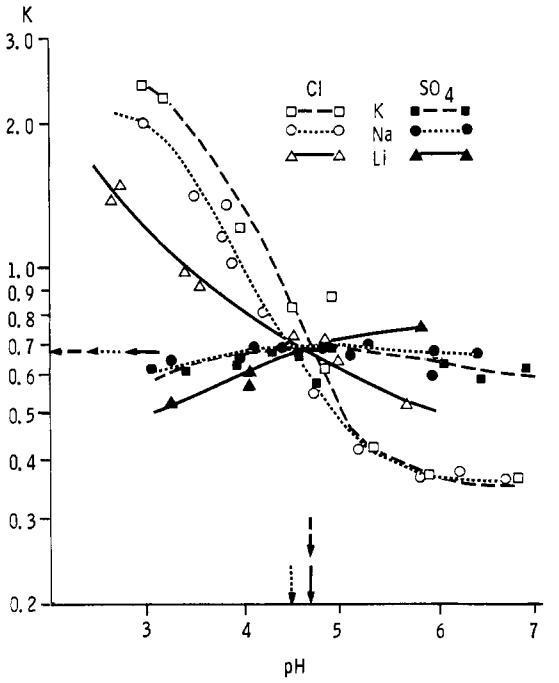

FIGURE 1: Cross-partition curves of ovalbumin (Sigma). Partition coefficients, *K*'s, in phase system I (□, containing KCl; ○, containing NaCl; and △, containing LiCl) and in phase system II (■, containing K_2SO_4; ●, containing Na_2SO_4; and ▲, containing Li_2SO_4) are plotted as a function of pH. The arrows indicate the *K* and pH of ovalbumin at the cross point in each pair of corresponding alkali chloride–alkali sulfate phase systems. For details, see text.

It follows from the above that when proteins are partitioned in phases containing sodium chloride or sodium sulfate at different pH's (*i.e.*, at points at which the protein's charge will differ) and the partition coefficients obtained are plotted *vs.* pH, one obtains two curves (one for each salt) which cross close to the isoelectric point (cross point) of the protein examined (Albertsson *et al.*, 1970). The effect of ionic composition on the pH and *K* of a protein's cross point was determined. A series of curves obtained with ovalbumin using potassium, sodium, and lithium sulfates and chlorides is depicted in Figure 1. It is evident that the marked effect and differences in partition coefficients as a function of salt composition is at a minimum at the cross point; and that the cross point is virtually independent of salt composition.

Below the cross-point pH ovalbumin has the highest *K* value in the phase system containing potassium chloride followed by sodium and lithium chlorides. Above the cross-point pH the reverse salt sequence holds. In the cases of the alkali sulfates, the potassium and sodium sulfates appear to have virtually identical effects on the partition coefficients of ovalbumin.

While all of the partition curves cross very close to one another and close to the isoelectric point of ovalbumin (Figure 1), slight differences in the cross point as a function of ionic composition of the phases are in evidence. These differences

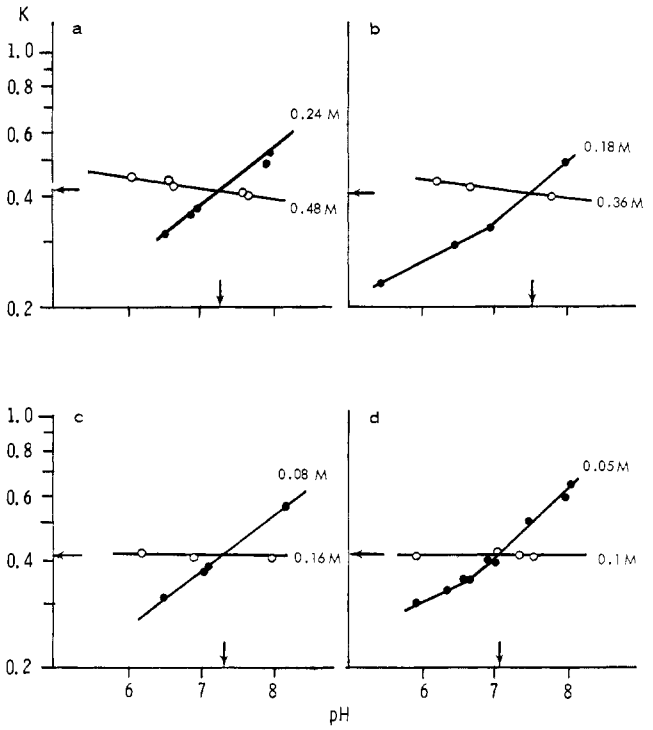

FIGURE 2: Cross-partition curves of human adult hemoglobin in phase systems having different salt concentrations. Partition coefficients, K's, in phase system I [○, containing NaCl: 0.48 M (a), 0.36 M (b), 0.16 M (c), and 0.1 M (d)] and in phase system II [●, containing Na_2SO_4: 0.24 M (a), 0.18 M (b), 0.08 M (c), and 0.05 M (d)] are plotted as a function of pH. The arrows indicate the K and pH obtained at the respective cross points.

are similar to those observed when the isoelectric point is determined by other means in different salt media.

Effect of Ionic Concentration of the Phase System on the Partition of Some Selected Proteins. Cross-partition curves,

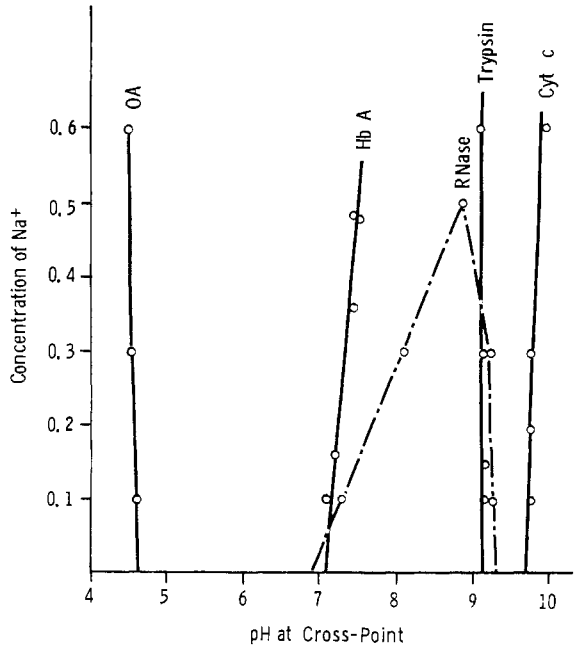

FIGURE 3: Relationship between the cross-point pH of several proteins and the Na^+ concentration of the phase system. For details, see text.

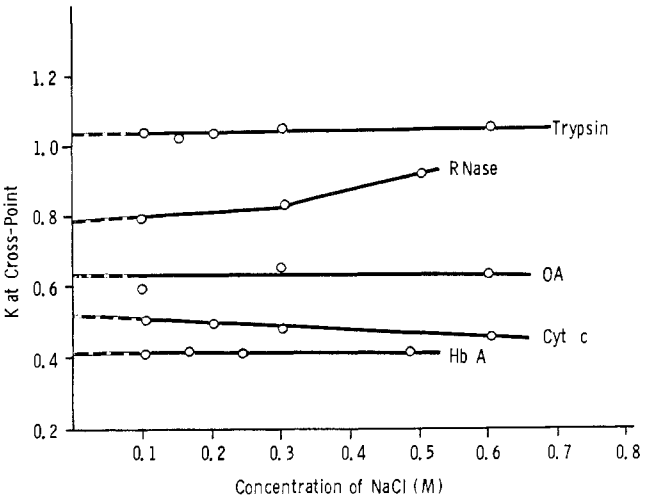

FIGURE 4: Relationship between protein partition coefficient, K, at the cross point and the sodium chloride concentration in phase system I.

obtained with human adult hemoglobin (Hb A) using different concentrations of sodium chloride and sodium sulfate in the phases, are depicted in Figure 2a–d. Salt concentrations used are indicated in Figure 2. Analogous experiments were carried out with ovalbumin, bovine pancreatic ribonuclease A, trypsin, and horse heart cytochrome *c* (cyt *c*). These results are summarized in Figures 3 and 4.

On the whole, changes in cross-point pH of proteins as a function of salt concentration were slight (Figure 3). An exception in ribonuclease (see discussion below). Variation in the determined isoelectric point pH as a function of salt concentration is of the same order (Alberty, 1953). K's at the

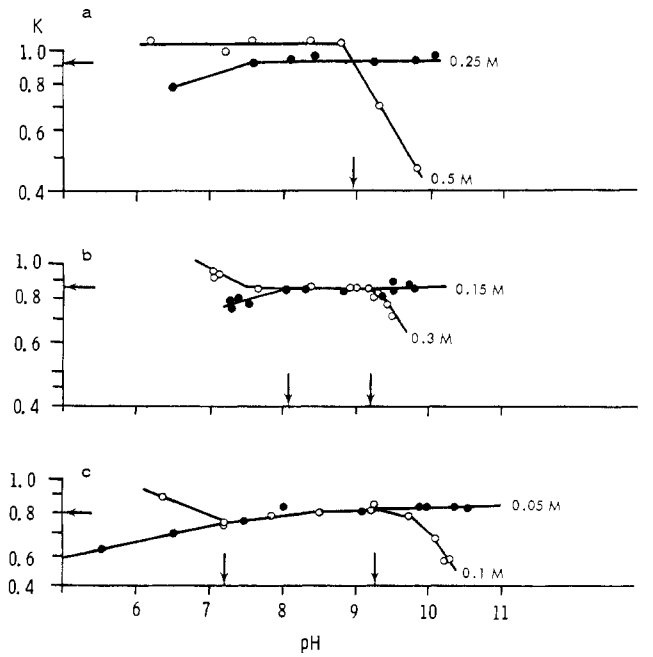

FIGURE 5: Cross-partition curves of ribonuclease A in phase systems having different salt concentrations. Partition coefficients, K's, in phase system I [○, containing NaCl: 0.5 M (a), 0.3 M (b), and 0.1 M (c)] and in phase system II [●, containing Na_2SO_4: 0.25 M (a), 0.15 M (b), and 0.05 M (c)] are plotted as a function of pH. For details, see text.

cross point appear also reasonably independent of salt concentration (except, again, for ribonuclease) as shown in Figure 4. A slight increase in K at the cross point of cyt c accompanies a decrease in salt concentration.

The cross-partition curves of ribonuclease obtained under our usual conditions (*i.e.*, with 0.1 M sodium chloride–0.05 M sodium sulfate) have been described and discussed (Sasakawa and Walter, 1972), and are presented for further consideration in Figure 5c. The cross-partition curves of ribonuclease are quite different from any obtained with other proteins. The curves rather than crossing actually join over the pH range from 7.2 to 9.3. It is of interest to point out that the isoelectric points reported for ribonuclease are from 7.8 (Rothen, 1940) to a range extending from pH 8.2 to 9.6 (Barnett and Bull, 1960). Hence the cross-partition line obtained with ribonuclease (Figure 5c) covers the range of isoelectric points reported. Johansson (1971) has shown that the change in K value with pH is directly related to the change in net charge of the protein (Johansson, 1971). We conclude therefore that there is no net change in charge of ribonuclease over the pH range in which the two partition curves join.

The effect of some other sodium chloride and sodium sulfate concentrations on ribonuclease are shown in Figure 5a,b. It is clear that salt concentration has a marked effect on the ribonuclease cross-partition curves obtained. With some salt concentrations (Figure 5a) one can get a cross point; with other salt concentrations (Figure 5b) the length of the cross-line is altered. We believe that these results indicate salt concentration-dependent conformational changes in the ribonuclease molecule that affect the molecule's surface charge and/or hydrophobic–hydrophilic surface properties. Investigations on the partition behavior of S-protein and S-peptide from ribonuclease are under way.

Conclusion

The partition coefficients of 5 proteins in two-polymer aqueous phase systems were determined as a function of pH, salt composition, and salt concentration. When the partition coefficients are plotted against pH the curves obtained in alkali chloride and those obtained in alkali sulfate cross close to the isoelectric point of the respective proteins (Albertsson *et al.*, 1970; Walter and Sasakawa, 1971). Below the cross-point pH of proteins (*i.e.*, when they are positively charged) the partition coefficients are greater in potassium than in sodium than in lithium chloride. Above the cross-point pH (*i.e.*, when the proteins are negatively charged) this sequence of ion effects is reversed. The partition coefficients and the pH's of proteins at their cross points are hardly affected by either the salt composition or concentration except for ribonuclease.

Since the cross points are fairly independent of ionic character and ionic concentration, cross-partition can be used for the determination of isoelectric points of proteins. Further, the partition coefficient of a protein at the isoelectric point can be regarded as a characteristic constant.

Acknowledgment

We thank Rita Tung for technical assistance in one of the experiments.

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