Effects of Salts on the Partition of Proteins in Aqueous Polymeric Biphasic Systems

GÖTE JOHANSSON

Department of Biochemistry, Umeå University, S-901 87 Umeå, Sweden

1. The influence of salts on the partition of ovalbumin and lysozyme in an aqueous biphasic system containing dextran and poly(ethylene glycol) has been studied. The logarithm of the partition coefficient of protein is almost linearly related to the net charge of the protein. The slope of the line varies with the salt included in the system.

2. The separatory capacity of this biphasic system has been characterized by comparing the partition of the two proteins. The separatory capacity varies markedly with the salt used and can be divided into two independent parts. One part depends on the difference in the net charge of the two proteins and is sensitive to cosolute electrolytes. The other part is determined by the solvation properties of the phases for proteins and is independent of the electrolytes in the system. The ions of the salt included in the system function independently in determining the charge-sensitive part of the separatory capacity.

3. The data on partition of a protein and of the salt that steers the protein partition conform to an earlier proposed model. According to this, the unequal affinity of the ions of the salt causes an interfacial potential which in turn affects the partition of charged macromolecules.

4. The interfacial potential has been experimentally determined by independent methods. Its value is in the range of 0–7 mV.

Biological materials such as proteins, nucleic acids, cell organelles, and even cells can be included, without changes in their biological activity, in the aqueous polymeric biphasic systems introduced by Albertsson.¹ Substances, depending on their molecular structure or surface characteristics, usually partition unequally between the two liquid phases of the system. The latter can therefore be used for separation of biomaterials. The partition behav-

iour of both biopolymers and particles is sensitive to the presence of salts in the system. It is further affected in different ways by salts which are chemically very similar, *e.g.* LiCl and NaCl.¹ Various alkali salts have been used to steer the partition of biomaterials in the biphasic system. It has been observed that salts also affect the resolving power of the systems.² In a previous communication³ it was shown that alkali salts partition unequally between the two phases.

A theoretical model to describe the influence of salt on the partition of proteins has been formulated by Albertsson.⁴ According to this model, the presence of several kinds of ions which differ in their relative affinity for the two liquid phases gives rise to an electrical potential across the interface between the phases. This proposed interfacial potential, ψ , that arises when the salt $A_{\nu_+}B_{\nu_-}$ is included in the system is given by

$$\psi = \frac{RT}{(\nu_+ + \nu_-)F} \ln \frac{K_-}{K_+} \quad (1)$$

where R is the gas constant, T the absolute temperature and F the Faraday constant. K_+ and K_- represent hypothetical partition coefficients which the positive and the negative ion, respectively, should have if every single ion could partition independently of other electrically charged species in the system. K_+ and K_- are related to the resultant partition coefficient of the salt, K_s , by

$$\ln K_s = \frac{1}{\nu_+ + \nu_-} \ln (K_+^{\nu_+} K_-^{\nu_-}) \quad (2)$$

If a protein with the net charge Z is partitioned together with an excess of salt, the partition coefficient of the former, K_p , is given by

$$\ln K_p = \ln K_p^0 + \frac{Z}{\nu_+ + \nu_-} \ln \frac{K_-}{K_+} \quad (3)$$

where K_p^0 is the partition coefficient of the protein, when partitioned with an excess of a salt for which $K_- = K_+$, which means that $\psi = 0$.

The aim of the present work was to study the mechanism of the salt effect and to test the theoretical model. For this purpose two well known proteins, ovalbumin and lysozyme, for which the charge can be predicted from pH, have been partitioned in the biphasic systems.

The separatory effect of different salts included in the biphasic system has also been determined by comparing the partition of the two proteins under identical conditions, and the interfacial potential has been measured.

The results are in agreement with the model and the partition of proteins as regards the salt effect can therefore be explained and predicted in a rational way.

MATERIAL AND METHODS

Chemicals. Dextran T 500, mol. wt. 500 000, was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Poly(ethylene glycol), mol. wt. 4000, was obtained from Union Carbide Chemicals Co., New York, as Carbowax 4000. Salts, acids, and bases were of analytical grade. Water was double distilled in quartz. Proteins were obtained from Sigma Chemical Co., St. Louis, Missouri: hen ovalbumin, grade V, and lysozyme from egg white, grade I.

Biphasic systems were obtained by mixing aqueous solutions of dextran, 20 % (w/w) and poly(ethylene glycol), 40 % (w/w) with protein and/or salt solutions in test tubes. The protein solutions, 5 g/l lysozyme or 10 g/l ovalbumin, were predialyzed against 5 mM potassium phosphate buffer, pH 6.8, at 3 °C overnight. The pH of the solutions of neutral salts was adjusted to 6.8 by adding minute amounts of HCl or NaOH solutions. Ammonium salts were adjusted to the same pH by adding dilute ammonia.

The biphasic systems were mixed and placed in a thermostat bath, 25 ± 0.2 °C. After attaining the bath temperature, the system was mixed by inverting 40 times and then left for one hour in the thermostat bath to separate. The pH measured directly in the upper phase by means of a glass electrode was close to 6.70.

Partition coefficient of a protein. The protein

concentration in each phase was measured in terms of absorbance. From each phase an aliquot was withdrawn with a 500 μ l constriction pipette. The phase was transferred to 2.000 ml water and the inside of the pipette was washed ten times with the diluted phase to remove all of the viscous solution. The same pipette was used for both phases to ensure equal volumes. The absorbance of the diluted phases was measured at 280 nm with a Zeiss spectrophotometer, PMQ II, using a blank prepared from a system containing no protein, but the same concentration of salt. The partition coefficient, K_p , was calculated as the ratio between the absorbance determined for the upper and lower phase, respectively.

The partition coefficient as function of the net charge of the protein was determined by preparing 20 g phase system containing protein and neutral salt but no buffer. The system was titrated with either NaOH, Tris, HCl or H_3PO_4 solution, 0.1–1.0 M, at constant temperature, 25 ± 0.2 °C. After each addition the system was shaken and two aliquots, 2 and 1 ml were withdrawn. The 2 ml portion was allowed to settle and the partition coefficient, K_p , was determined as above. The 1 ml portion was diluted with 1.5 ml water and pH of the diluted solution was measured using a pH-meter (Radiometer, pHM-26) equipped with a glass electrode (Radiometer GK 2026C).

The partition coefficients of salt, K_s , was determined for chlorides, bromides, iodides, and thiocyanates by potentiometric titration using a Radiometer titrigrath fitted with one silver and one $Hg-Hg_2SO_4$ -electrode (Radiometer, type P4011 and K 601). 1.000 ml upper or lower phase was mixed with 5 ml water and titrated with 15 mM $AgNO_3$. Partition of fluoride, sulfate, nitrate, and perchlorate was determined by direct conductometric measurements. 2.000 ml of one phase was mixed with 2.000 ml of the opposite phase from a phase system containing no salt and with 10.00 ml water. The conductivity of the solution was measured at 25 °C with a Metrohm conductometer E 382. Corrections were made by subtracting the conductivity of a similarly diluted mixture of phases from a system without salt. The conductivity is assumed to be proportional to the salt concentration in these diluted solutions. To obtain equal volumes of the two phases the same method as described earlier for dilution of the phases was used.

Measurements of interfacial potential. The arrangement for measuring the difference in interfacial potential is shown in Fig. 1. The potential was measured with a Radiometer TTT2 titrator used as mV-meter and connected with a Servogor potentiometric recorder, type RE 520. The whole apparatus was kept in a thermostated room at 25 °C. The measured potential was corrected for the potential that appeared between two identical systems, both containing potassium chloride.

Fig. 1. Arrangement for measuring the interfacial potential difference between two biphasic systems containing different salts. Each U-tube-shaped vessel contains a single biphasic system where (1) is the upper phase and (2) the lower phase. The two lower phases are brought into electrical contact *via* a salt bridge consisting of 0.5 M NH_4NO_3 in 20 % (w/w) dextran solution (3). Calomel electrodes, Radiometer type K 100, (4), with open liquid junction and containing 12 % (w/w) poly(ethylene glycol) saturated with KCl are inserted in the upper phases. The potential difference between the two calomel electrodes is measured with the aid of a millivoltmeter (5).

RESULTS AND DISCUSSION

Dependence of protein partition on the electrical charge of the protein. Ovalbumin or lysozyme were partitioned in a biphasic system containing either K_2SO_4 , KF, or KBr. The variation of the protein partition coefficient with the protein net charge is shown in Fig. 2. The net charges were estimated from the measured pH values by using the titration curves of the proteins given by Tanford and Wagner⁵ and Kenchington.⁶ The logarithm of the partition coefficient of the protein is found to be linearly related to its net charge, Z . The slopes of the lines for the two proteins are almost identical when partition was carried out in the presence of a given salt. Some deviations from linearity are observed for ovalbumin when it is negatively charged. On the other hand, the slope of the line varies with the salt. This is in agreement with eqn. (3). For a single protein, the lines

Fig. 2. Relationship between protein partition coefficient, K_p , and the net charge of the proteins, Z . The latter was obtained from titration curves and isoelectric points for the proteins. 1, lysozyme with 0.05 M K_2SO_4 ; 2, lysozyme with 0.1 M KF; 3, lysozyme with 0.1 M KBr; 4, ovalbumin with 0.05 M K_2SO_4 ; 5, ovalbumin with 0.1 M KF; and 6, ovalbumin with 0.1 M KBr, partitioned in systems containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C. The concentrations of protein were 1 g/l for lysozyme and 2 g/l for ovalbumin. Broken lines indicate linear extrapolations.

obtained when it is partitioned with different salts intersect where the net charge $Z=0$. Albertsson *et al.*⁷ have taken advantage of this fact and from the plot of $\log K_p$ versus pH estimated the isoelectrical points for a series of proteins.

The deviation from a straight line may indicate either a change in molecular weight of the protein due to association or dissociation, or changes in its effective charge due to interaction with ions other than H^+ and OH^- . The deviation could also be due to a difference in the extent of ionization of the protein in water as compared with that in polymer solutions. The effective Z may consequently differ from that calculated from titration curves in aqueous solutions. Deviations may also result from conformational changes which affect the K_p^0 in eqn. (3). Sasakawa and Walter⁸ have shown that K_p at the isoelectric point, *i.e.* K_p^0 , varies

Fig. 3. Variation of protein partition coefficient, K_p , with salt concentration.

a. Lysozyme, 1 g/l (black spots) and ovalbumin, 2 g/l (white spots) were partitioned at 25 °C in a system containing 8 % (w/w) dextran, 8 % (w/w) poly(ethylene glycol), 1 mM sodium phosphate buffer, pH 6.8, and various concentrations of KF, O; potassium acetate, □; and KBr, △.

b. Theoretical curves for lysozyme, black spots ($Z=7.3$, $K_p^0=0.89$ and $C_p=0.0705$ mM) and

strongly with the protein and is also related to the molecular weight of the protein.

When K_2SO_4 is included in the system used here, the partition of a given protein is almost independent of Z . This salt therefore gives a system in which the interfacial potential is close to zero.

Dependence of protein partition on the concentration of salt. When ovalbumin and lysozyme are partitioned at very low salt concentrations their partition coefficients are close to one, but with increasing salt concentration K_p asymptotically approaches limiting values, Fig. 3a. At pH=6.7 and at high salt concentration the positively charged lysozyme and the negatively charged ovalbumin have affinity for opposite phases.

The dependence of K_p on the salt concentration, according to the theoretical model, can be derived from eqn. (1), Fig. 3b, by combination with expressions for electroneutrality and mass balance which yields eqn. (4).

$$\frac{1 - 1/(1 + K_-y) + Z(2C_p - x)/2C_s}{Zx/2C_s + 1/(1 + K_-y)} = \frac{K_+}{y} \quad (4)$$

where

$$y = [(2C_p - x)/xK_p^0]^{1/2}$$

C_p , and C_s are the respective molar concentrations of protein and salt in the system, and x is the molar concentration of protein in the lower phase. K_p^0 , K_+ and K_- have been defined in connection with eqn. (1)–(3).

The validity of (4) is restricted to a system where the two phases have equal volume and the salt is a 1–1-electrolyte. Furthermore the protein must be negatively charged. Z is the numerical value of the protein net charge. Eqn. (4) was numerically scanned over a range of x values $0 < x < 2C_p$, with an increment of 0.0001 mM to obtain the value of x that best fitted the equation. This was done with aid of a Hewlett Packard electronic calculator 9100. K_p is related to x via eqn. (5).

$$K_p = (2C_p - x)/x \quad (5)$$

ovalbumin, white spots ($Z = -7.3$, $K_p^0=0.71$ and $C_p=0.0445$ mM) when partitioned with KBr, △, or KF, O. $K_+=0.828$ (K^+), $K_-=1.202$ (Br^-) or 0.904 (F^-). The broken lines indicate the limit partition of the proteins at infinite salt concentration.

The theoretical and experimental curves, Fig. 3, have the same shape. At very low salt concentrations the presence of 1 mM phosphate buffer will increasingly affect the partition of the protein as salt concentration decreases. This has not been corrected for in the calculations. The contribution of the protein to the interfacial potential will grow smaller and smaller as the concentration ratio between salt and protein increases. Therefore the partition of the protein will be increasingly determined by the partition of the salt ions when the concentration of the latter increases. The limiting partition coefficient for a protein in the presence of excess salt is given by eqn. (3). It is evident that to obtain the largest possible difference in the partition of the two proteins the salt concentration must be sufficiently high. In the present experiments the protein concentration in the systems was 2 g/l or less. When 25 mM 1-1-electrolyte is used, the difference in $\log K_p$ for the two proteins was about 90 % of the maximal one.

Above a minimum salt concentration the separation effect is almost independent of the salt concentration. This is in contrast to other separation methods which utilize charge differences between proteins, *e.g.* ion exchange chromatography or electrophoresis. In these separation procedures the ionic strength plays an important role in determining the separation.

In partition, however, ionic strength can be chosen within a very wide range of values without affecting the degree of separation. Partition at high ionic strength may therefore be used for resolving proteins or subunits which otherwise interact strongly.

The separatory effect of various salts. The degree of separation of two substances by partition is measured by a separation factor, β . This is defined as the ratio between the partition coefficients of the two substances⁹ and the order of the coefficients is chosen so that $\beta \geq 1$. It has here been found more suitable to use a logarithmic expression.

According to eqn. (3) the partition coefficient of a protein can be split into two parts; one, K_p^0 , depending on the relative solvation of the protein in the two phases, the other part, $K_p^{el} = (K_-/K_+)^{z/(v_+ + v_-)}$, depending on the net charge of the protein. The difference in $\log K_p$, $\Delta \log K_p$, of two proteins partitioned under equal conditions can be split up in the same way:

$$\Delta \log K_p = \Delta \log K_p^0 + \Delta \log K_p^{el} \quad (6)$$

where $\Delta \log K_p^{el}$ depends on the difference in net charge between the two proteins. The value $\Delta \log K_p$ is a measure of the separatory capacity of the biphasic systems and is related to the separation factor β , *via* $\log \beta = |\Delta \log K_p|$. The part of the separatory capacity that depends

Table 1. The charge-dependent part of the separatory capacity, $\Delta \log K_p^{el}$, obtained when ovalbumin or lysozyme, respectively, was partitioned with different salts in a biphasic system containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C.

Salt	Salt concentration (mM)	$\Delta \log K_p^{el}$	Salt	Salt concentration (mM)	$\Delta \log K_p^{el}$
KF	25	-0.25	NaF	25	-0.23
KCl	25	-0.87	NaCl	25	-0.83
KBr	25	-1.06	NaBr	25	-1.04
KI	25	-1.36	NaI	25	-1.26
KSCN	25	-1.49	NaSCN	25	-1.36
K ₂ SO ₄	20	0.04	Na ₂ SO ₄	20	0.05
KClO ₄	25	-1.73	NaClO ₄	25	-1.58
NH ₄ F	25	0.04	LiF	20	0.29
NH ₄ Cl	25	-0.60	LiCl	25	-0.35
NH ₄ Br	25	-0.81	LiBr	25	-0.54
NH ₄ I	25	-1.14	LiI	25	-0.86
NH ₄ SCN	25	-1.21	LiSCN	25	-0.92
(NH ₄) ₂ SO ₄	20	0.22	Li ₂ SO ₄	20	0.31
KNO ₃	100	-0.81	LiClO ₄	25	-1.15

on the net charges of the proteins should, according to the theory, be the difference between $\Delta \log K_p$ and $\Delta \log K_p^0$. $\Delta \log K_p [= \log K (\text{ovalbumin}) - \log K (\text{lysozyme})]$ for the protein pair ovalbumin and lysozyme (partitioned separately) at pH 6.7 has been determined using biphasic systems which contained different salts. $\Delta \log K_p^0 = 0.10$ for this pair of protein has been determined from Fig. 2, as the difference in $\log K_p$ at $Z=0$ when the proteins are partitioned in the presence of K_2SO_4 . The resulting $\Delta \log K_p^{cl}$ values are given in Table 1.

When the salt used is a 1-1-electrolyte, the $\Delta \log K_p^{cl}$ values obtained, can be expressed as the sum of two constant terms, $\Delta \log K_C$ and $\Delta \log K_A$, which are characteristic for the cation and anion respectively,

$$\Delta \log K_p^{cl} = \Delta \log K_C + \Delta \log K_A \quad (7)$$

Setting $\Delta \log K_C$ for the potassium ion arbitrarily equal to zero, $\Delta \log K_C$ will be 0.03 for Na^+ ; 0.28 for NH_4^+ ; and 0.53 for Li^+ and $\Delta \log K_A$ will be -0.25 for F^- , -0.87 for Cl^- , and -1.07 for Br^- . The maximum deviation for $\Delta \log K_A$ or $\Delta \log K_C$ calculated for an ion from data concerning different salts containing that ion is less than ± 0.02 . The additivity holds less well for I^- , SCN^- , and ClO_4^- , for which $\Delta \log K_A$ is found to be -1.36 ± 0.07 , -1.45 ± 0.06 , and -1.67 ± 0.06 , respectively.

If the negative ions are arranged in order of their $\Delta \log K_A$ values, the series obtained corresponds to the well-known Hofmeister's series. The most extreme charge-depending separatory

effect is shown by salts containing chaotropic ions, SCN^- or ClO_4^- , which makes these salts attractive for enhancing the separatory capacity of the biphasic systems. However, such salts should be used with care, since high concentrations of the chaotropic ions may denature proteins.

By using salts for which $\Delta \log K_p^{cl} = 0$ the pH-dependent separation effect can be nullified. In the system studied here and at the operating temperature NH_4F as well as K_2SO_4 have this property. Therefore in a system containing one of these salts the partition coefficient of a protein should be independent of the protein net charge and equal to K_p^0 . Variations in K_p^0 with pH would indicate structural changes in the protein molecule.

Depending on the salt used, a small yet significant variation in K_p^0 has been noted. It is here assumed that the shift in $\log K_p^0$ is the same for the two proteins studied so that the difference $\Delta \log K_p^0$ is independent of salt. The good agreement between experiment and theory indicates that this is a valid approximation. The relative variation of K_p^0 with salt has been approximated by the change in the means of $\log K_p$ of ovalbumin and lysozyme. The increase in $\log K_p^0$ goes hand in hand with increasing partition coefficient of the salt, given in Table 2. The order of increasing K_p^0 is $F^- < Cl^- < Br^- < I^- < SCN^- < ClO_4^-$ and $K^+ < Na^+ < NH_4^+ < Li^+$. The shifts in $\log K_p^0$ seem to be unique for each ion and additive just as in the case of $\Delta \log K_p^{cl}$.

Table 2. Partition coefficients, K_s , of salts in a biphasic system containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C.

Salt	Conc. (mM)	$\log K_s$	Salt	Conc. (mM)	$\log K_s$
LiI	20	0.0622	NH_4F	20	-0.0386
NH_4I	20	0.0484	NaF	20	-0.0575
NaI	20	0.0310	KF	20	-0.0640
KI	20	0.0257	LiF	20	-0.0255
$LiBr$	20	0.0314	KSCN	20	0.0342
NH_4Br	20	0.0224	$KClO_4$	20	0.0362
$NaBr$	20	0.0043	KNO_3	20	0.0000
KBr	20	0.0009	K_2SO_4	10	-0.0964
$LiCl$	20	0.0212			
NH_4Cl	20	0.0095			
$NaCl$	20	-0.0123			
KCl	20	-0.0173			

The zero-potential partition of ions. By combining the partition coefficients for a salt and those for proteins partitioned with excess of this salt, the K_+ and K_- values for the ions of the salt can be calculated. Eqn. (3) yields

$$\log \frac{K_-}{K_+} = \frac{v_+ + v_-}{\Delta Z} \Delta \log K_p^{\text{el}} \times 1.11 \quad (8)$$

where ΔZ is the difference in net charge of the two proteins. The correction factor 1.11 estimated from Fig. 3 is introduced since $\Delta \log K_p$ at the salt concentration used is close to, but not identical with, the limiting value obtained with excess of salt. For 1-1-electrolytes eqn. (2) can be written as

$$\log (K_+ K_-) = 2 \log K_s \quad (9)$$

K_+ and K_- , Table 3, column b, have been calculated for alkali, ammonium, and halide ions from (8) and (9) and using $\Delta \log K_p^{\text{el}}$ and K_s from Tables 1 and 2. The charge difference, ΔZ , used in the calculations has been obtained from the titration curves and the isoelectric points. The latter were taken to be 4.75 for ovalbumin and 11.0 for lysozyme.⁸

On this basis, at pH 6.7, Z is -12.0 and 7.5 for ovalbumin and lysozyme, respectively, and therefore $\Delta Z = -19.5$.

A considerable variation is observed in the values of K_+ or K_- for a given ion. In addition,

there is also a systematic variation in the values for the various counter ions. The K_+ and K_- have also been calculated *via* eqn. (9) solely from the partition coefficients of salts given in Table 2. This is possible because one of the salts, NH_4F , only gives rise to very small interfacial potential, as can be seen from the data given in Table 1. K_+ and K_- for the ions of this salt must therefore be almost equal to each other and also equal to the partition coefficient of the salt. From these two values, K_+ and K_- for the other ions have been calculated *via* (9) and are recorded in Table 3, column d. These K_+ and K_- differ considerably from the values obtained from $\Delta \log K_p^{\text{el}}$.

The K_- has also been directly estimated for chloride, bromide, and iodide by partition of salts containing these ions in a system containing K_2SO_4 in large excess. This salt gives only rise to a very low interfacial potential since $\Delta \log K_p^{\text{el}}$ is close to zero, Table 1. These experimental K_- values, Table 3, column a, agree well with the values calculated on the basis of $K_+ = K_-$ for NH_4^+ and F^- , Table 3, column d. K_+ for the cations, Table 3, column e, has been calculated from the experimental values concerning Br^- and Cl^- .

The observed deviation of K_+ and K_- calculated from the $\Delta \log K_p^{\text{el}}$ data when compared with the values obtained experimentally or

Table 3. Logarithms of partition coefficients (K_+ and K_-) of ions when interfacial potential is zero, in a biphasic system containing 8 % (w/w) dextran and 8 % (w/w) poly(ethylene glycol) at 25 °C. Salt concentration, 10–25 mM.

a, 10 mM halide in a system containing excess of K_2SO_4 , 50 mM.

b, calculated from $\Delta \log K_p^{\text{el}}$ and K_s using eqns. (8) and (9) with $\Delta Z = -19.5$.

c, calculated as in b but using $\Delta Z = -14.6$.

d, calculated from K_s *via* eqn. (9), assuming K_+ for ammonium to be equal to K_- for fluoride.

e, K_+ calculated from K_s *via* (9) using K_- for Cl^- and Br^- given under a. K_- for F^- is likewise calculated from K_+ so obtained.

Ion	Experi- mental $\log K_-$	Calculated $\log K_+$ or $\log K_-$			
	a	b	c	d	e
K^+		-0.062 ± 0.016	-0.082 ± 0.004	-0.089	-0.084 ± 0.002
Na^+		-0.056 ± 0.014	-0.073 ± 0.007	-0.076	-0.076 ± 0.002
NH_4^+		-0.025 ± 0.012	-0.037 ± 0.002	-0.039	-0.036 ± 0.003
Li^+		$+0.002 \pm 0.011$	-0.005 ± 0.003	-0.012	-0.015 ± 0.005
I^-	$+0.151$	$+0.107 \pm 0.006$	$+0.130 \pm 0.005$	$+0.136$	
Br^-	$+0.083$	$+0.063 \pm 0.005$	$+0.080 \pm 0.007$	$+0.084$	
Cl^-	$+0.051$	$+0.038 \pm 0.006$	$+0.051 \pm 0.004$	$+0.058$	
F^-		-0.044 ± 0.006	-0.044 ± 0.004	-0.039	-0.040 ± 0.004

via the partition coefficients of the salts indicates that the estimated net charges for ovalbumin and lysozyme are not correct. By using eqn. (3) the net charges have been calculated from the partition data of the two proteins in a system containing KBr and assuming $\log K_p^0$ to be -0.05 and -0.15 for lysozyme and ovalbumin, respectively. The net charge is found to be 7.3 for lysozyme and -7.3 for ovalbumin. The former is in good agreement with the value, 7.4 , obtained from the titration curve, while the value for ovalbumin is considerably less than the titrimetrical one, -12.0 . If the net charges, calculated via (3), are used for calculating K_+ and K_- from $\Delta \log K_p^{el}$ the result, Table 3, column c, is in excellent agreement with the values obtained from the partition coefficients of the salts, Table 3, columns a, d, and e. The assumption of a smaller Z value for ovalbumin at $\text{pH}=6.7$ is consistent with the data for this protein in Fig. 2. Ovalbumin shows a deviation from the linear dependence of Z when negatively charged. If, however, the net charge at $\text{pH}=6.7$ is estimated by extrapolation to the assumed linear relation, one obtains $Z = -7.5$ in the case of KF and $Z = -8.0$ in the case of KBr.

In a biphasic system the ions of a salt would attain the partition coefficients K_+ and K_- if the demand of electroneutrality did not require

that their actual partition coefficients are the same. Only in a thin layer around the interface may an unequal distribution of cation and anion exist, a so called Stern layer.¹⁰ K_+ and K_- express the relative affinities of the ions for the two phases. The affinity of ammonium and alkali ions for the dextran-rich lower phase is greater than that for the PEG-rich upper phase; $K_+ < 1$. The affinity for the lower phase increases in the order $\text{Li}^+ < \text{NH}_4^+ < \text{Na}^+ < \text{K}^+$. The above finding indicates that these ions, especially K^+ and Na^+ , form weak complexes with dextran in water solution. It has further been shown that the IR-spectra of dextran¹¹ in aqueous solution are influenced by potassium and sodium salts.

The halide ions except F^- have a greater affinity for the upper phase and this affinity increases in the order $\text{Cl}^- < \text{Br}^- < \text{I}^-$. This is in agreement with the electrophoretic mobility of PEG in salt solutions containing these ions.²

Effect of salt concentration on K_s . The partition coefficient of the studied salts are found to be independent of the salt concentration in the interval $2-100$ mM.

Interfacial potential, ψ , was calculated using the following expression (10), obtained by combining (1), (3), and (6).

Table 4. Values of interfacial potential, ψ , in a biphasic system containing 8 % (w/w) dextran, 8 % (w/w) poly(ethylene glycol) and 20 mM alkali or ammonium halide at 25°C .

Salt	$\psi(\text{mV})$ meas. ^a	$\psi(\text{mV})$ calc. ^b	$\psi(\text{mV})$ calc. ^c
KF	-2.6	-1.1	-1.3
KCl	-4.0	-3.9	-4.0
KBr	-4.5	-4.8	-4.9
KI	-5.6	-6.1	-6.5
NaF	-1.1	-1.0	-1.1
NaCl	-2.9	-3.7	-3.8
NaBr	-3.6	-4.7	-4.7
NaI	-4.2	-5.7	-6.3
NH_4F	-1.4	+0.2	+0.1
NH_4Cl	-3.3	-2.7	-2.6
NH_4Br	-3.8	-3.7	-3.5
NH_4I	-4.6	-5.1	-5.1
LiF	-0.2	+1.3	+0.7
LiCl	-1.5	-1.6	-2.0
LiBr	-2.5	-2.4	-2.9
LiI	-3.0	-3.9	-4.5

^a Measured relative to KCl and recalculated to absolute values by assuming the potential to be -4.0 mV in the case of KCl. ^b Calculated from $\Delta \log K_p^{el}$. ^c Calculated from K_+ and K_- .

$$\psi = \frac{RT}{F} \frac{1}{\Delta Z} \Delta \ln K_p^{\text{el}} (\text{excess of salt}) \quad (10)$$

with $\Delta Z = -14.6$ and introducing the correction factor 1.11 mentioned earlier, in connection with (8), yields

$$\psi = - \frac{59.2}{14.6} \Delta \log K_p^{\text{el}} \times 1.11 \quad (11)$$

The interfacial potentials calculated for alkali and ammonium halides are given in Table 4.

The interfacial potential has also been measured relative to a system containing KCl. The results are presented in Table 4. For anions the potential varies in the expected sequence I^- , Br^- , Cl^- , F^- . For cations the sequence expected is K^+ , Na^+ , NH_4^+ , Li^+ , but that observed experimentally is K^+ , NH_4^+ , Na^+ , Li^+ . This deviation may be due to polarization phenomena at the contact surfaces in the electrical element, the so called junction potentials. These could perhaps be decreased by using another salt in the salt bridge between the two biphasic systems. NH_4NO_3 , which has been used here, is ideal in water solution because of the equal transport number of the two ions. The same is not necessarily true in 20 % (w/w) dextran solution, used in these experiments. The experiments show, however, that the interfacial potential is of the predicted order.

Measurements of the interfacial potential in an aqueous biphasic system containing phosphate and chloride has recently been published by Reitherman *et al.*¹² The values of the potentials were of the same order as those here described.

CONCLUSIONS

The effect of electrolytes on the separatory capacity of the aqueous biphasic systems is due to a difference in the affinity of the constituent ions for the two phases.

This leads to the formation of an electrical double-layer and consequently an interfacial potential. When a biphasic system contains an excess of salts the induced interfacial potential depends only on the salt and this will affect the partition of proteins according to their charges.

The separatory capacity of a biphasic system for a given pair of proteins can be split into

two parts. One part reflects the solvation of the proteins in the two phases and is almost independent of the presence of salts in the system. The other part is related to the difference in net charges of the two proteins. This charge-depending part of the separatory capacity is characteristically affected by the ions of the salt in the system. The effects of the ions are algebraically additive. By choosing a salt with ions having large effects on the separatory capacity it may be possible to resolve (even complex) protein mixtures by liquid-liquid extraction. Since the separatory capacity induced by a salt is constant throughout a considerably wide range of salt concentrations, separations can be carried out at a suitable ionic strength without any loss in the efficiency of separation.

The experimental data are in excellent agreement with the equations for interfacial potential put forward by Albertsson. They can therefore be used with confidence for quantitative predictions. Thus, partition studies can be used for determination of physico-chemical properties of proteins, *e.g.* net charge. It should also be possible to detect conformational changes, protein-protein associations and binding of low-molecular weight substances to proteins, from partition studies.

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