# USE OF SOLUTE PARTITION FOR COMPARATIVE CHARACTERIZATION OF SEVERAL AQUEOUS BIPHASIC POLYMERIC SYSTEMS

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## SUMMARY

Eight biphasic ficoll–dextran systems differing in the molecular weight of the latter polymer and in the polymer composition were studied. The effect of the salt composition on the phase diagrams for all systems was examined and it is shown that salts present in the polymer mixture should be considered as essential components of the mixture involved in the separation of phases.

The partition behaviour of a number of proteins and dinitrophenyl-amino acids in the systems was studied. It is shown that the partition coefficients obtained in different phase systems can be correlated using the relation $\ln K_i = a \ln K_0 + b$, where $K_i$ and $K_0$ are the partition coefficients of a solute in the system in question and in the system chosen as the reference, respectively, and $a$ and $b$ are the scaling factors independent of the chemical nature of the solutes partitioned.

The difference in the relative hydrophobicities between the two phases was determined experimentally in two phase systems and calculated for six other systems. It is shown that it is possible to compare the partition results obtained in various polymer phase systems using any solutes chosen as "partition markers".

## INTRODUCTION

The partition of biological macromolecules and particles in aqueous biphasic polymeric systems is commonly used as an extremely sensitive method for the separation of cells and biopolymers[1,2]. It has been shown that the partition technique can be used as an analytical method for determining the relative hydrophobicities of the distributed macromolecule or particle surface[3–7]. We previously proposed[8] a ficoll–dextran phase system which has several advantages over the common dextran–polyethylene glycol (PEG) system, particularly for analytical studies of both macromolecule and cell surface properties.

The analytical application of the method, however, appears to be limited owing to minor variations in polymer composition and batch variations in polymer characteristics, which are known to have large effects on the partition behaviour of a

given substance in the dextran–PEG phase system[9–11]. Similar effects have been observed by us when using the ficoll–dextran phase system.

It is obvious that the analytical application of the partition technique depends greatly on the possibility of comparing different data obtained by various workers generally using polymers of different batches and manufacture. Some factors are required for scaling of the partition values obtained in phase systems with different polymer compositions and formed from various polymer samples.

It was the purpose of this work to find the factors for scaling of the partition values obtained in the phase systems formed by ficoll and various dextran batches.

## EXPERIMENTAL

### Materials

*Polymers.* Ficoll 400 (lots 3004, 6594, 7868 and 11069) and dextran T 40 (lot 2771) were purchased from Pharmacia (Uppsala, Sweden). Dextrans of molecular weight about 40,000 were obtained from Polfa (Kutno, Poland) (lot 325-71), Loba (Vienna, Austria), Ferak (Berlin, G.F.R.) and under the trade-name Reopolyglucinum (lot 190677) from Minmedprom (Moscow, U.S.S.R.). Dextrans of molecular weight about 70,000 under the trade-name Polyglucinum (lots 310670, 390476 and 580870) were obtained from Minmedprom.

*Proteins.* Equine heart cytochrome *c* and horse skeletal muscle myoglobin were purchased from Calbiochem (Los Angeles, CA, U.S.A.). Human serum albumin, Cohn fraction V, was obtained from ICN Pharmaceuticals (Cleveland, OH, U.S.A.). Human plasma was obtained as described earlier[7].

*Amino acids.* Dinitrophenylated amino acids (DNP-L-Ala, DNP-L-Phe, DNP-Gly) were obtained from Serva (Heidelberg, G.F.R.). 2,4-Dinitrofluorobenzene was purchased from Calbiochem. L-Norleucine and DL-norvaline were obtained from Reanal (Budapest, Hungary) and DL-2-amino-*n*-octanoic acid from BDH (Poole, Great Britain). The amino acids were dinitrophenylated as described in ref. 12.

*Chemicals.* Sodium alkyl sulphates, $R_nOSO_3Na$ ($n = 8, 10, 12$), were prepared by the method of Dreger *et al.*[13]. All salts and chemicals used were of analytical-reagent grade.

### Determination of molecular weight distribution and other properties of dextran samples

The molecular weight distribution of the dextran samples was measured by high-performance liquid chromatography (HPLC) in the size-exclusion mode. A DuPont Model 830 liquid chromatograph provided with a refractive index detector (LDC, Model 1107) and a Rheodyne fixed-volume loop injector (20 $\mu$l) was used. Two DuPont high-performance size-exclusion columns (SE-100, SE-500; 25 cm × 6.2 mm I.D.) loaded with small (8-$\mu$m) porous silica particles were connected in series. Ethanol (2%) filtered through MF-Millipore filters (0.45 $\mu$m) was used as the eluent in the solvent delivery system. The solvent flow-rate was 0.9 ml/min and the recorder speed was set at 1.5 cm/min. A 1% solution of the sample in the mobile phase was used for injection.

Dextran standards and glucose were used for column calibration. The dextran standards used (T 10, T 20, T 40, T 70, T 110, T 150, T 250, T 500 and T 2000) were obtained from Pharmacia with weight-average molecular weights ($\overline{M}_w$), number-

average molecular weights ($\overline{M}_n$) and molecular weight distribution (MWD) curves supplied by the manufacturer. As the dextran standards have wide MWD [dispersity (1.4–3.0)], a calibration graph was obtained from the geometric mean of the $\overline{M}_w$ and $\overline{M}_n$ values and peak elution volumes[14]. Using the calibration graph the $\overline{M}_w$ and $\overline{M}_n$ values of the dextran samples were determined as described elsewhere[14,15]. The dextran samples were analysed 2–3 times, always together with all of the standards.

The degree of branching characteristic for the dextran samples was estimated by use of $^{13}$C NMR spectroscopy as described by Seymour et al.[16]. A Brucker WP-80 spectrometer was employed in the Fourier-transform mode.

Near-infrared measurements on the dextran samples in water (10% solutions) were carried out as described by Aizawa et al.[17].

*Preparation of phase systems*

Stock solutions of ficoll (ca. 40%, w/w) and dextran (ca. 35%, w/w) in water were prepared. A mixture containing the amounts of polymers indicated below was prepared by weighing appropriate amounts of the stock polymer solutions. The appropriate amounts of 0.44 $M$ sodium phosphate buffer (pH 7.4) and 0.6 $M$ sodium chloride in 0.04 $M$ sodium phosphate buffer (pH 7.4) were added so as to achieve the required ionic composition (ionic strength) and the polymer composition of the phase system. The amounts of sodium chloride and sodium phosphate buffer (pH 7.4) in a given phase system can be calculated from the equations $C_{NaCl} = (0.288 - I)/0.75$ and $C_{buffer} = 0.11 - 0.67C_{NaCl}$, where $C_{NaCl}$ and $C_{buffer}$ are the sodium chloride and sodium phosphate buffer concentrations, respectively, and $I$ is the ionic strength in the phase system.

The phase diagrams were constructed as described by Albertsson[1].

*Phase systems.* The final phase systems used had the following polymer compositions (all in weight-percent):

System    I: 14% ficoll (lot 6594), 12% Reopolyglucinum;
System   II: 14% ficoll (lot 6594), 12% dextran 40 (Polfa);
System  III: 14% ficoll (lot 6594), 12% dextran T 40 (Pharmacia);
System  IV: 14% ficoll (lot 11069), 12% dextran 40 (Loba);
System   V: 14% ficoll (lot 6594), 12% Polyglucinum (lot 390476);
System  VI: 11.5% ficoll (lot 6594), 10% Polyglucinum (lot 390476);
System VII: 12.5% ficoll (lot 7868), 10.8% Polyglucinum (lot 310670);
System VIII: 12.5% ficoll (lot 11069), 10.8% Polyglucinum (lot 580870).

*Determination of partition coefficients of solutes*

As the top phase was ficoll-rich in systems I–V and dextran-rich in systems VI–VIII, the partition coefficient, $K$, in this work is defined as the ratio of sample concentration in the ficoll-rich phase to sample concentration in the dextran-rich phase. After settling of the phases (settling time 24 h) aliquots of both phases (0.1–0.2 ml) were carefully pipetted from the phase system, each was diluted by addition of an appropriate volume of water and the concentrations of the partitioned solute were determined.

The concentrations of sodium alkyl sulphates were measured by the method described by Hayashi[18]. The serum albumin concentrations were determined using the Coomassie G-250 technique[19]. The cytochrome $c$ and myoglobin concentrations

were determined by absorbance measurements at 410 nm, and those of the dinitrophenylated amino acids were determined at 360 nm. In all instances the correspondingly diluted pure phases were used as blank solutions.

The partition coefficient for each solute was determined at six ionic strengths as the mean of two measurements on two or more dilutions from each partition carried out 2–4 times at a given ionic strength. The deviation from the mean $K$ value did not exceed 3% for any of the solutes studied.

## RESULTS

### Polymer characteristics and phase diagrams

Fig. 1 shows the phase diagrams for the ficoll–dextran systems with different dextran samples in the presence of 0.15 $M$ sodium chloride in 0.01 $M$ sodium phosphate buffer (pH 7.4). It can be seen that the higher the weight-average molecular weight of dextran (see Table I), the lower is the total polymer concentration required for phase separation. It is evident that the previously used polymer composition of 14% (w/w) ficoll and 10% (w/w) dextran 40[4–8] does not provide phase separation in all of the polymer mixtures under study. In particular, the dextran batch from Polfa (lot 325-71) did not give phase separation at the above polymer composition, in contrast to the dextran sample from the same manufacturer used previously[7]. Hence, the polymer composition of 14% ficoll and 12% dextran was chosen for most of the
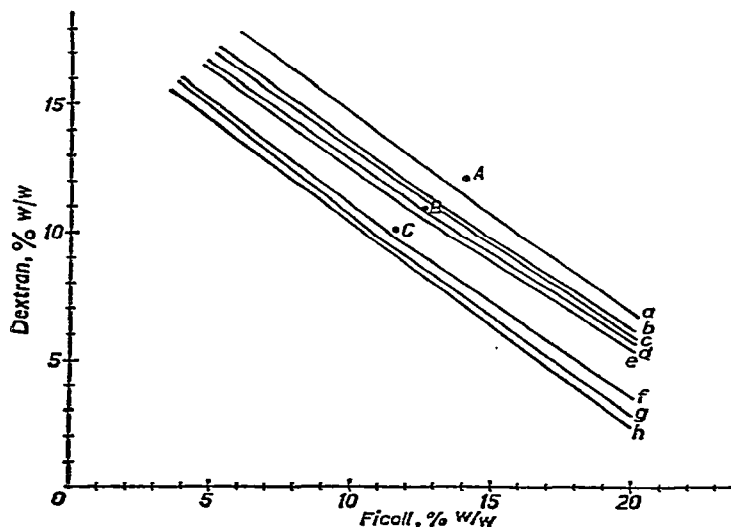


Fig. 1. Phase diagrams for biphasic ficoll–dextran systems in the presence of 0.15 $M$ NaCl in 0.01 $M$ sodium phosphate buffer (pH 7.4). (a) Ficoll (lot 3004 or 6594), Reopolyglucinum; (b) ficoll (lot 6594), dextran 40 (Polfa, lot 325-71); (c) ficoll (lot 3004 or 6594), dextran T 40 (Pharmacia, lot 2771); (d) ficoll (lot 11069), dextran 40 (Loba); (e) ficoll (lot 3004), dextran 40 (Ferak); (f) ficoll (lot 7868), Polyglucinum (lot 310670); (g) ficoll (lot 11069), Polyglucinum (lot 580870); (h) ficoll (lot 3004 or 6594), Polyglucinum (lot 390476). Point A corresponds to the polymer composition 14% (w/w) ficoll, 12% (w/w) dextran, point B to 12.5% (w/w) ficoll and 10.8% (w/w) dextran and point C to 11.5% (w/w) ficoll, 10% (w/w) dextran.

systems as this composition gives rise to phase systems with similar volumes of both phases. It should be noted, however, that the densities of the two phases in systems I–IV with the above composition are very close and one obtains the so-called isopycnic phases[1], which hinders the partition experiments.

In order to characterize the phase systems under study, the systems with the above polymer composition in the presence of 0.15 $M$ sodium chloride in 0.01 $M$ sodium phosphate buffer (pH 7.4) and in the presence of 0.11 $M$ sodium phosphate buffer (pH 7.4) were transformed into a one-phase system and the polymer compositions at the binodial (corresponding to a ficoll to dextran ratio of 1.16) were determined. The total polymer concentrations characteristic of these compositions are given in Table I together with the molecular weight characteristics of the dextran samples. The molecular weights of the ficoll samples were not determined. However, the phase diagrams and the binodial polymer compositions were compared for the systems formed by Reopolyglucinum and Polyglucinum (lot 390476) with two ficoll batches (lots 3004 and 6594), and no difference could be detected.

TABLE I

MOLECULAR WEIGHT DISTRIBUTION CHARACTERISTICS OF DEXTRAN SAMPLES AND TOTAL CONCENTRATIONS OF FICOLL 400 AND DEXTRAN REQUIRED FOR SEPARATION OF PHASES IN POLYMER MIXTURES

The total polymer concentrations required for separation of phases in the polymer mixtures were determined as characteristic for the polymer compositions at the corresponding binodial (corresponding to a ficoll/dextran ratio of 1.16). The polymer concentrations are given in weight-percent.

| Dextran | $\bar{M}_w \cdot 10^{-3}$ [*] | $\bar{M}_n \cdot 10^{-3}$ | $\bar{M}_w/\bar{M}_n$ | C | |
|---|---|---|---|---|---|
| | | | | Ionic strength 0.176 $M$ [**] | Ionic strength 0.288 $M$ [***] |
| Reopolyglucinum | 34.5 | 18.3 | 1.89 | 25.4 | 23.2 |
| Dextran 40 (Polfa, lot 325-71) | 39.4 | 28.9 | 1.37 | 24.1 | 21.7 |
| Dextran T 40 (Pharmacia, lot 2771) | 41.5 | 28.8 | 1.44 | 23.8 | 21.4 |
| Dextran 40 (Loba) | 41.4 | 25.0 | 1.66 | 23.4 | 21.4 |
| Dextran 40 (Ferak) | 44.8 | 29.6 | 1.51 | 23.1 | 20.5 |
| Polyglucinum (lot 310670) | 58.6 | 23.2 | 2.52 | 21.2 | 19.9 |
| Polyglucinum (lot 580870) | 64.5 | 22.7 | 2.84 | 20.7 | 19.4 |
| Polyglucinum (lot 390476) | 68.8 | 23.5 | 2.93 | 20.5 | 19.1 |

[*] The deviation from the mean $\bar{M}_w$ value is ca. 5%.
[**] In the presence of 0.15 $M$ NaCl in 0.01 $M$ sodium phosphate buffer (pH 7.4).
[***] In the presence of 0.11 $M$ sodium phosphate buffer (pH 7.4).

The degree of dextran branching as detected by the ^{13}C NMR technique was 3–6% for all of the dextran samples examined, and cannot be regarded as a property that affects phase separation in the polymer mixtures or governs the partition properties of the phase systems. Therefore, this characteristic will not be considered further.

An attempt to detect any differences in the dextran solutions using near-infrared measurements[17] failed completely.

*Partition of proteins*

It has been shown earlier[4,7] that the relationship between the logarithm of the

protein partition coefficient and the ionic strength of the phase system can be described by

$$\ln K = A + BI \tag{1}$$

where $I$ is the ionic strength and $A$ and $B$ are constants (the ionic strength was varied as indicated above from 0.176 to 0.288 $M$).

A least-squares treatment of the partition data led to the $A$ and $B$ values listed in Table II for the proteins examined. It can be seen that the $A$ and $B$ values are different and depend on the phase system used in the partition experiments. It should be noted that the ratio $B/A$ appears to be specific for a given protein, independent of the system used. This seems reasonable according to the physical meaning of $A$ and $B$[4–7]. The $B/A$ value is $-3.78 \pm 0.17$ for human serum albumin and $-12.88 \pm 0.90$ for cytochrome *c*. The myoglobin partition appears to be independent of the ionic strength, and hence the $B/A$ value for this protein is zero. The observed differences in the partition behaviour of the proteins require separate discussion, which is beyond the scope of this paper.

TABLE II

CHARACTERISTICS OF THE PARTITION BEHAVIOUR OF PROTEINS IN AQUEOUS FICOLL–DEXTRAN BIPHASIC SYSTEMS

The logarithm of the partition coefficient of a given protein (ln $K$) depends on the ionic strength of the phase system ($I$) as ln $K = A + BI$. Parameters $A$ and $B$ calculated from the experimental $K$ values are presented as the means $\pm$ standard deviation with the correlation coefficient values given in parentheses. The ionic strength was varied from 0.176 to 0.288 $M$ as described in the text. Polymer compositions of the phase systems are indicated in the text.

| Phase system | Human serum albumin | | Myoglobin* | Cytochrome c | |
|---|---|---|---|---|---|
| | $-A$ | $B$ | $-A$ | $A$ | $-B$ |
| I | 1.64 ± 0.04 (0.998) | 6.0 ± 0.19 | 0.32 ± 0.04 (0.998) | 0.28 ± 0.25 (0.892) | 3.44 ± 1.0 |
| II | 2.86 ± 0.04 (0.999) | 10.38 ± 0.19 | 0.33 ± 0.02 (0.989) | 0.22 ± 0.04 (0.996) | 2.83 ± 0.16 |
| III | 2.92 ± 0.10 (0.997) | 11.4 ± 0.41 | 0.34 ± 0.02 (0.997) | 0.24 ± 0.14 (0.958) | 3.39 ± 0.59 |
| IV | 2.28 ± 0.09 (0.996) | 8.2 ± 0.37 | 0.38 ± 0.01 (0.999) | 0.30 ± 0.26 (0.892) | 4.18 ± 1.06 |
| V | 1.97 ± 0.17 (0.980) | 7.3 ± 0.74 | 0.35 ± 0.04 (0.997) | 0.28 ± 0.06 (0.994) | 3.75 ± 0.25 |
| VI | 1.13 ± 0.09 (0.987) | 4.62 ± 0.38 | 0.22 ± 0.03 (0.989) | 0.20 ± 0.04 (0.990) | 2.28 ± 0.18 |
| VII | 2.01 ± 0.06 (0.998) | 7.72 ± 0.25 | 0.27 ± 0.04 (0.999) | 0.20 ± 0.08 (0.974) | 2.50 ± 0.34 |
| VIII | 2.28 ± 0.09 (0.996) | 8.81 ± 0.38 | 0.27 ± 0.03 (0.999) | 0.21 ± 0.04 (0.971) | 2.63 ± 0.16 |

* Partition of myoglobin is independent of the ionic strength in the $I$ range used, hence the $B$ value is zero in all phase systems examined.

The total plasma proteins, being a complex multi-component system, were distributed in phase systems VII and VIII. The $A$ values are $-1.61 \pm 0.11$ and $-1.70 \pm 0.12$, respectively, and the $B$ values are $6.88 \pm 0.45$ and $7.73 \pm 0.51$, respectively.

*Partition of sodium alkyl sulphates*

The partition behaviour of sodium alkyl sulphates as a function of the ionic strength of the system was studied in phase systems I–VI. It has been shown previ-

ously[4] that the effect of ionic strength and alkyl chain length on the partition of surfactants can be expressed as

$$\ln K = C + BI + Em \qquad (2)$$

where $m$ is the number of $CH_2$ groups in the alkyl chain of the solute molecule, $E$ characterizes the difference in the relative hydrophobicity between the two phases of a given system and $C$ and $B$ are constants.

The main purpose of these experiments was to establish the $E$ values needed to characterize the phase systems under study. Unfortunately, the indirect method for the measurement of surfactant concentration[18] did not allow us to obtain reliable results. We have just managed to find that the $E$ values are different and vary from 0.03 to 0.06 in phase systems I–VI.

*Partition of dinitrophenylated amino acids*

The concentrations of DNP-amino acids can be measured directly. Hence these compounds can be used for evaluating the relationship between hydrophobic character and partition coefficient in biphasic polymeric systems. Unfortunately, this method for determining the $E$ values came to our attention when the polymer batches needed for systems I–VI had been used up. Therefore, the DNP-amino acids were partitioned in phase systems VII and VIII only.

The relationship between the logarithm of the partition coefficient and the ionic strength of the phase system can be described by eqn. 1. The corresponding $A$ and $B$ values are given in Table III. The $A$ value as a function of the number of $CH_2$ groups in the amino acid aliphatic side-chain is presented in Fig. 2.

TABLE III

CHARACTERISTICS OF THE PARTITION BEHAVIOUR OF DNP-AMINO ACIDS IN TWO FICOLL–DEXTRAN PHASE SYSTEMS

Logarithm of the partition coefficient depends on the ionic strength ($I$) of the system as $\ln K = A + BI$ (for details see text). The $B$ values for the DNP-amino acids are $1.624 \pm 0.01$ and $1.500 \pm 0.050$ in phase systems VIII and VII, respectively. The polymer compositions of systems VIII and VII are given in the text.

| DNP derivative | $-A$ | |
| --- | --- | --- |
| | Phase system VIII | Phase system VII |
| Glycine | 0.190 | 0.194 |
| Alanine | 0.152 | 0.163 |
| Norvaline | 0.107 | 0.128 |
| Norleucine | 0.087 | 0.106 |
| 2-Amino-*n*-octanoic acid | 0.019 | 0.056 |
| Phenylalanine | −0.001 | 0.039 |

DISCUSSION

*Phase separation in ficoll–dextran mixtures*

The data in Fig. 1 and Table I indicate that the ionic composition and/or ionic strength of the polymer mixture and the molecular weight of dextran influence phase
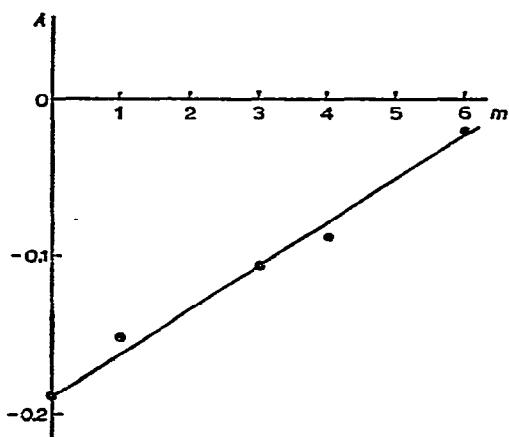
Fig. 2. Value of parameter $A$ as a function of the aliphatic side-chain length ($m$) of dinitrophenylated glycine, alanine, norvaline, norleucine and 2-amino-$n$-octanoic acid.

separation in the polymer mixtures studied. The dependence of the total polymer concentration required for phase separation on the dextran weight-average molecular weight ($\overline{M}_w$) can be described as

$$C_\Sigma = \alpha + \beta \log \overline{M}_w \tag{3}$$

where $C_\Sigma$ represents the total concentration of both polymers needed for phase separation and $\alpha$ and $\beta$ are constants depending on the ionic composition and/or ionic strength of the polymer mixture. $\alpha$ and $\beta$ are $97.8 \pm 4.1$ and $-16.0 \pm 0.9$, respectively, in the presence of $0.15$ $M$ sodium chloride in $0.01$ $M$ sodium phosphate buffer (pH 7.4) (correlation coefficient $r^2 = 0.991$). In the presence of $0.11$ $M$ sodium phosphate buffer (pH 7.4), the corresponding $\alpha$ and $\beta$ values are $76.4 \pm 6.8$ and $-11.9 \pm 1.5$, respectively ($r^2 = 0.958$). It should be noted that eqn. 3 is valid only for the dextran $\overline{M}_w$ range $30 \cdot 10^3$–$70 \cdot 10^3$ and it may be invalid for dextrans of other molecular weights.

Our data indicate that salts present in a given polymer mixture and their concentration cannot be considered as factors that affect solely the partition behaviour of solutes and particles in the phase systems formed. The salts appear to be the essential components of the system, governing its formation and its separation properties. This seems to be at variance with the accepted point of view[1] that phase separation in mixtures containing only non-ionic polymers occurs independently of the salt composition and concentration. Nevertheless, the data obtained in this study seem to justify the above conclusion.

*Partition of solutes and factors of scaling*

In order to compare the partition coefficients of solutes determined in two different solvent pairs, the following equation has to be used[20,21]:

$$\ln K_t = a \ln K_0 + b \tag{4}$$

where $K_t$ and $K_0$ denote the partition coefficient in the two phase systems and $a$ and $b$ are constants.

In order to correlate the partition coefficients obtained in various phase systems, the results should be scaled by selection of any system as a reference. System VIII was selected here as large amounts of the polymer batches needed for this system were available.

As parameter $A$ in eqn. 1 is the ln $K$ value at zero ionic strength in the medium, eqn. 4 can be rewritten as

$$A_t = aA_0 + b \qquad (5)$$

where $A_t$ is the $A$ value for a given solute in the $i$th phase system, $A_0$ is the $A$ value for the same solute in system VIII and $a$ and $b$ are constants.

It is clear that the values of $a$ and $b$ can be calculated according to eqn. 5 from the ln $K$ values for any two solutes partitioned in phase systems $i$ and VIII. The $A$ values obtained for albumin and myoglobin were treated according to eqn. 5 to determine $a$ and $b$ for the phase systems studied. The $a$ and $b$ values are listed in Table IV. The $A$ values for cytochrome $c$ ($A_i^{cyt}$) in all of the phase systems were calculated from the corresponding $a$ and $b$ values and the $A_0^{cyt}$ value for cytochrome $c$ in phase system VIII. The calculated $A_i^{cyt}$ values are listed in Table IV together with those found experimentally, and it can be seen that they agree well. The data for the total plasma proteins given above indicate that the parameters $a$ and $b$ can be used for complex protein mixtures as well as for individual proteins.

TABLE IV

FACTORS FOR SCALING THE PARTITION VALUES OBTAINED IN VARIOUS PHASE SYSTEMS ($a$ AND $b$), CHARACTERISTICS OF THE DIFFERENCE IN THE RELATIVE HYDROPHOBICITIES BETWEEN THE TWO PHASES OF THE SYSTEMS ($E$) AND THE RELATIVE HYDROPHOBICITIES OF PROTEINS EXPRESSED IN TERMS OF EQUIVALENT CH$_2$ GROUPS ($n$)

| Phase system | $a$ | $b$ | $A_{calc.}^{cyt}$[*] | $A_{exp.}^{cyt}$[**] | $E$[***] | $n = (A - b)/E$ | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | | | Albumin | Myoglobin | Cytochrome c |
| I | 0.66 | −0.143 | 0.28 | 0.28 ± 0.25 | 0.018 | −83.2 | −9.83 | 7.61 |
| II | 1.27 | 0.010 | 0.26 | 0.22 ± 0.04 | 0.034 | −84.4 | −10.00 | 6.76 |
| III | 1.28 | 0.007 | 0.26 | 0.24 ± 0.14 | 0.035 | −83.6 | −9.91 | 7.06 |
| IV | 0.94 | −0.125 | 0.32 | 0.30 ± 0.26 | 0.025 | −86.2 | −10.20 | ·7.00 |
| V | 0.81 | −0.132 | 0.30 | 0.28 ± 0.06 | 0.022 | −83.5 | −9.91 | 6.73 |
| VI | 0.45 | −0.098 | 0.19 | 0.20 ± 0.04 | 0.012 | −86.0 | −10.17 | 8.50 |
| VII | 0.81 | −0.041 | 0.22 | 0.20 ± 0.08 | 0.022 | −84.4 | −10.00 | 7.78 |
| VIII[‡] | — | — | — | 0.21 ± 0.04 | 0.027 | −85.6 | −9.96 | 6.91 |

[*] $A$ values for cytochrome $c$ calculated as described in the text from the experimental $A$ value found in phase system VIII and the corresponding $a$ and $b$ values.

[**] Experimental $A$ values found for cytochrome $c$.

[***] $E$ values were determined experimentally in phase systems VII and VIII and calculated for the other phase systems (for details see text).

[‡] Phase system VIII was chosen as the reference system.

It has been suggested previously[5] that the factor $a$ in eqn. 4 reflects the difference in the relative hydrophobicities between the two phases of a given phase system with respect to that specific for the reference phase system. In order to check this assumption, the partition behaviour of the DNP-amino acids was examined in phase systems VII and VIII.

The $A$ and $B$ values for the amino acid derivatives are given in Table III. The results show that the $a$ and $b$ values calculated from the protein partition coefficients can be used for comparison of the partition behaviour of the DNP-amino acids in systems VII and VIII. This fact indicates that $a$ and $b$ can be used for scaling the partition results obtained in various phase systems for any solutes independent of their chemical nature. The effect of an additional $CH_2$ group on the ln $K$ value, *i.e.*, parameter $E$ in eqn. 2, can be determined by comparison of the $A$ values for the DNP derivatives of glycine, alanine, norvaline, norleucine and 2-amino-*n*-octanoic acid. The $E$ values are 0.022 and 0.027 in phase systems VII and VIII, respectively.

It can be seen that parameter $a$ in eqn. 4 is related to $E_{VII}$ and $E_0$ specific for the systems VII and VIII, respectively, by

$$a_{VII} = E_{VII}/E_0 \qquad (6)$$

Hence, the $E_i$ values for phase systems I–VI can be calculated according to eqn. 6 in the following form:

$$E_i = a_i E_0 \qquad (7)$$

The calculated $E_i$ values for the phase systems studied are listed in Table IV.

It was assumed earlier[4-7] that the relative hydrophobicity of any solute or particle can be expressed in terms of the equivalent number of $CH_2$ groups (experimental proof will be published later) using the equation

$$n = A/E \qquad (8)$$

where $n$ is the equivalent number of $CH_2$ groups the total hydrophobicity of which is equal to that produced by the compound in question at zero ionic strength in the medium and $A$ and $E$ are as defined above.

Thus, the hydrophobic character of the proteins can be determined from the $A_0$ and $E_0$ values found in phase system VIII, and from the corrected $A_i$ values, *i.e.*, $A_i - b_i$, and $E_i$ values obtained in phase systems I–VII. The relative hydrophobicity of cytochrome $c$ at zero ionic strength in the medium is equivalent to that of 7.29 $\pm$ 0.6 $CH_2$ groups, human serum albumin is characterized by $-84.6 \pm 1.2$ $CH_2$ groups and myoglobin by $-10.0 \pm 0.13$ $CH_2$ groups.

The data reported seem to justify the conclusion that it is possible to compare the partition coefficients obtained in various aqueous polymer biphasic systems. For this correlation any solutes can be chosen as "partition markers" for calibration of the phase system under study relative to that selected as the reference system. This fact allows one to conclude that the partition technique can be used as an analytical tool in various fields of biochemical and biomedical research.

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