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PHYSICO-CHEMICAL FACTORS GOVERNING PARTITION BEHAVIOUR OF SOLUTES AND PARTICLES IN AQUEOUS POLYMERIC BIPHASIC SYSTEMS

III. FEATURES OF SOLUTES AND BIOLOGICAL PARTICLES DETECTED BY THE PARTITION TECHNIQUE

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SUMMARY

The partition behaviour of human serum albumin and oxyhaemoglobin and several amino acids and small peptides was studied in the aqueous Ficoll–dextran biphasic system as a function of the ionic composition and pH. The partition coefficients of the solutes were expressed in terms of the equivalent number of CH_2 groups, n^{CH_2}, and the equivalent number of carboxyl groups, m. The physical meaning of these two parameters and of the relationships found between them and pH for the proteins examined are discussed.

A correlation was established between the difference in the relative hydrophobicities of the individual phases of various water–organic solvent systems and the interfacial tension, γ_{12}, of the systems. It is argued that a relation of a similar type exists for the aqueous polymeric biphasic systems.

The possibility of estimating the relative intensity of Van der Waals and hydration interactions of a solute and particle surface by examination of their partitioning in a biphasic system calibrated for the hydrophobic and hydration properties of the phases is discussed.

INTRODUCTION

It has been shown in Parts I[1] and II[2] that at least two main features of aqueous polymeric biphasic systems should be taken into consideration when examining the partition behaviour of solutes or particles. These features are the hydrophobic and hydration properties of the individual phases of the biphasic system having a given polymeric and ionic composition.

Different polymeric compositions of the two phases and an unequal distribution of the ions present seem to result in different structuring of water in these phases. Moreover, the difference in the relative hydrophobicities of the phases can be attributed to the different relative strengths of the water–water interactions of the

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phases. Now, the affinity of a solute or particle surface for an aqueous environment is known to depend upon two types of interactions: those between the water molecules and those between water and a solute molecule (or particle surface). For a non-polar solute the affinity depends upon the water–water interactions and the surface area of the molecule[3]; for a polar solute the affinity depends upon both water–water and water–polar group interactions. The hydration property of a given phase of a biphasic system was defined[2] as the ability of water to participate in interactions with polar groups as mediated by the presence of neighbouring polymers and ions. An approach to estimate this property was also proposed in Part II[2].

In the present work this approach was employed to account for the partition behaviour of two proteins, human serum albumin and oxyhaemoglobin, and of several amino acids and small peptides distributed in the aqueous Ficoll–dextran biphasic system under various ionic compositions. The results obtained are discussed with regard to the nature of the features of solutes and particles detected by the partition technique.

MATERIALS AND METHODS

Materials

Ficoll-400 (Lot 11069) was obtained from Pharmacia (Sweden); Dextran 70 (Lot 580870), trade-name Polyglucinum, was obtained from Minmedprom (U.S.S.R.).

Human serum albumin, Cohn fraction V, was purchased from ICN Pharmaceuticals (U.S.A.), human oxyhaemoglobin from Serva (G.F.R.).

Amino acids were obtained from Sigma (U.S.A.). All the peptides used in this work were kindly provided by Dr. M. I. Titov (All-Union Research Center for Cardiology). Their purity was verified by thin-layer chromatography in four different solvent systems and by amino acid analysis of acid hydrolysates.

Partition experiments

The aqueous Ficoll–dextran biphasic system containing 12.5% (w/w) Ficoll 400, 10.8% (w/w) Dextran 70 and various amounts of NaCl and sodium phosphate buffer as indicated below was prepared as described earlier[1,2]. The amounts of NaCl and sodium phosphate buffer used are related by

$$C_{\text{buffer}} = 0.11 - 0.67\, C_{\text{NaCl}}$$

where C_{buffer} and C_{NaCl} are concentrations in mol/kg. The concentration of NaCl was varied from zero up to 0.15 *M*.

The partition experiments were carried out as described elsewhere[1,4–6]. The phases were allowed to settle at 25°C for 24 h, after which the solute concentration of aliquots of both phases was determined. The oxyhaemoglobin concentration was determined by absorbance measurements at 410 nm. The concentrations of amino acids, peptides and serum albumin were determined by the fluorescamine technique[7].

The partition coefficient, *K*, in the biphasic system is defined as the ratio of the solute concentration in the Ficoll-rich phase to that in the dextran-rich phase. Partition coefficients were measured for each solute over approximately ten-fold concen-

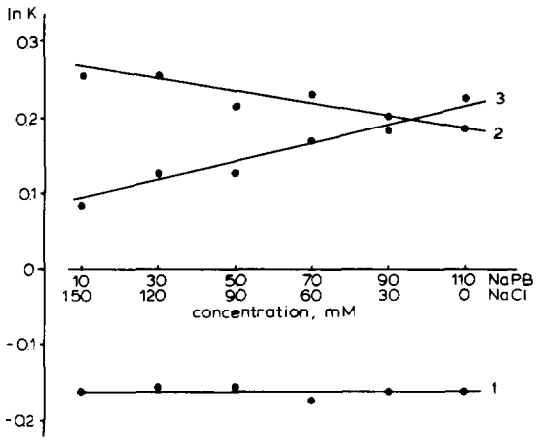

Fig. 1. Logarithm of the partition coefficient, K, as a function of ionic composition in the Ficoll–dextran phase system containing NaCl and sodium phosphate buffer, pH 7.4. Peptides: 1 = Tyr-Gly-Leu-Arg-OH; 2 = Tyr-D-Ala-Gly-Phe(NO_2)-OH; 3 = Tyr-D-Ala-Gly-Phe-N_2H_2-Leu.

tration ranges and were found to be independent of the solute concentration. The values were the means of two measurements on three dilutions from each partition experiment carried out three or four times in a given biphasic system. The deviation from the mean did not exceed 3% for all the solutes examined.

RESULTS

Fig. 1 shows some typical relationships between the logarithm of the partition

TABLE I

CHARACTERISTICS OF THE PARTITION BEHAVIOUR OF AMINO ACIDS AND PEPTIDES IN FICOLL–DEXTRAN BIPHASIC SYSTEMS, A AND B, AND CHARACTERISTICS OF THE SOLVENT INTERACTIONS OF THE COMPOUNDS, n^{CH_2} and m

The relationship between the logarithm of the partition coefficient and the ionic composition of the system under the conditions employed is given in the text. Parameter n^{CH_2} characterizes the relative intensity of the Van der Waals interactions of a solute with an aqueous environment, and m specifies the relative intensity of the hydration interactions of the solute (see text).

Compound	A	B	n^{CH_2}*	m
Gly	−0.205 ± 0.05	0	−7.6	0
Trp	0.014 ± 0.018	0	0.52	0
Tyr-Arg-OH	−0.118 ± 0.028	0	−4.4	0
Leu-Gly-OH	−0.106 ± 0.016	0	−3.93	0
Phe-Leu-Gly-OH	0.040 ± 0.020	0	1.5	0
Gly-Gly-Gly-OH	−0.150 ± 0.027	0	−5.6	0
Ile-His-Pro-Phe-OH	0.001 ± 0.014	0	0.04	0
Tyr-D-Ala-Gly-Phe(NO_2)-OH	−0.077 ± 0.015	1.01 ± 0.06	1.51	0.62 ± 0.04
Tyr-Pro-Phe-Pro-Gly-OH	0.081 ± 0.018	0	3.01	0
Tyr-D-Ala-Gly-Phe-N_2H_2-Leu	0.392 ± 0.009	−0.711 ± 0.034	11.4	−0.44 ± 0.02
Tyr-D-Ala-Gly-Phe(NO_2)-N_2H_2-Gly	0.139 ± 0.029	0	5.2	0

* To calculate the error in n^{CH_2} the corresponding error in A should be divided by 0.027.

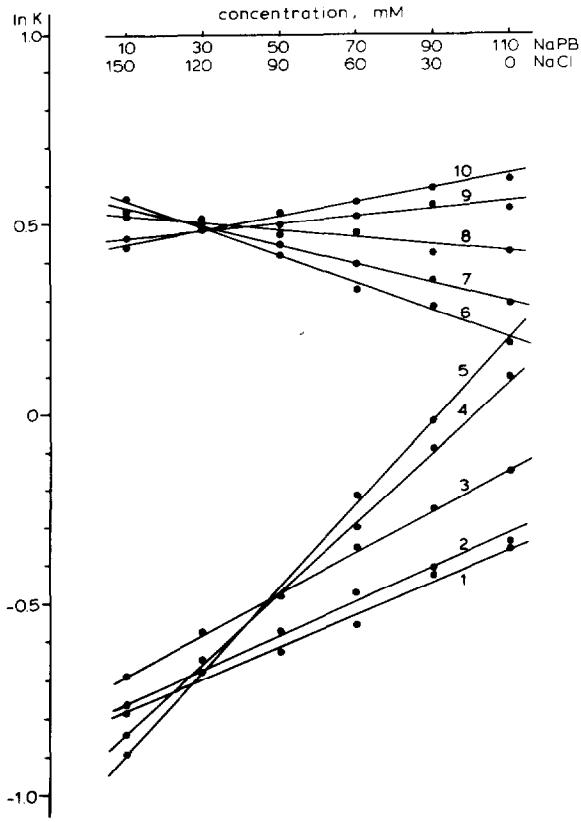

Fig. 2. Logarithm of the partition coefficient of human serum albumin (curves 1–5) and human oxyhaemoglobin (curves 6–10) as a function of the ionic composition in the Ficoll–dextran phase system containing NaCl and sodium phosphate buffer at different pH: 6.15 (1 and 6); 6.4 (2 and 7); 6.8 (3 and 8); 7.4 (4 and 9); 7.8 (5 and 10).

coefficient of a peptide in the Ficoll–dextran biphasic system and the concentration ratio NaCl/sodium phosphate buffer (NaPB), pH 7.4. If the above ratio is determined by the corresponding ionic strength value, (varied from 0.176 to 0.288 *M*), the relationships can be described by

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

where A and B are constants. The A and B values calculated from the experimental relationships obtained for the amino acids and peptides examined are presented in Table I.

The partition behaviour of human serum albumin as a function of the NaCl/NaPB concentration ratio in the Ficoll–dextran biphasic system was studied at different pH values. The results obtained are given in Fig. 2 (curves 1–5). The data obtained for human oxyhaemoglobin are also shown (curves 6–10).

It was shown earlier[2] that the partitioning of solutes in this biphasic system depends on many factors, in particular the ionic strength of the sodium phosphate buffer and the $HPO_4^{2-}/H_2PO_4^-$ concentration ratio. Therefore the partition be-

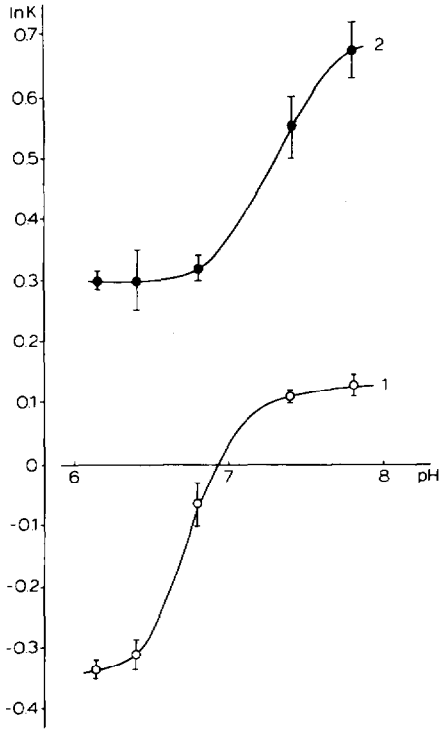

Fig. 3. pH-dependence of the logarithm of the partition coefficient of human serum albumin (1) and human oxyhaemoglobin (2) in the Ficoll–dextran phase system containing sodium phosphate buffer of constant ionic strength, 0.165 *M*.

haviour of the proteins was examined at constant ionic strength (0.165 *M*) at various pH values (and thus buffer concentrations) in the absence of NaCl. The data obtained are presented in Fig. 3.

DISCUSSION

It has been shown previously[1,4] that the partitioning of a non-polar solute does not depend on the hydration properties of the phases and can be described by

$$\ln K = n^{CH_2}E \tag{2}$$

where n^{CH_2} is the equivalent number of CH_2 groups[4] and E represents the difference in the relative hydrophobicities of the two phases of a given biphasic system[1] which is related to the free energy of transfer of a CH_2 group from one phase to the other, $\Delta G_{tr}^{CH_2}$, according to

$$\Delta G_{tr}^{CH_2} = -RTE$$

where R is the gas constant and T the absolute temperature.

The partitioning of a polar molecule participating in both polar and non-polar

Van der Waals interactions with an aqueous environment in the phases, as proposed earlier[2], can be described by

$$\ln K = n^{CH_2}E + mC \tag{3}$$

where C represents the logarithm of the partition coefficient of 2,4-dinitrophenyl (DNP)-glycine chosen as a semiquantitative measure of the hydration property of the phases[2] and m is the equivalent number of carboxyl groups —this parameter indicates the strength of the total hydration interactions characteristic of the solute relative to that of DNP-glycine.

It has been shown earlier[1] that C changes with ionic composition over the salt concentration range used according to

$$C = A_c + B_c I \tag{4}$$

where A_c and B_c are constants. It follows from eqns. 1 and 4 that

$$\ln K = A - \frac{B}{B_c} \cdot A_c + \frac{B}{B_c} \cdot C \tag{5}$$

or

$$\ln K = \alpha + mC \tag{6}$$

where $\alpha = A - (B/B_c)A_c$ and $m = B/B_c$. Eqn. 6 takes the form of eqn. 3 provided $n^{CH_2} = \alpha/E$, and it is evident that the partition coefficient of a solute can be described by:

$$(\ln K)/E = n^{CH_2} + m(C/E) \tag{7}$$

The difference in the relative hydrophobicities between the two phases seems to be essential for the formation of a biphasic system (*i.e.*, $E \neq 0$). It is possible, however, that the hydration properties of both phases in relation to a given polar group are similar; if the carboxyl group is the one under consideration this means that $C = 0$. In this case the partition coefficient of a solute would characterize only the non-polar Van der Waals interactions of the solute with the phases used.

The fact that the parameter $n^{CH_2} = \alpha/E = (A - mA_c)/E$ includes m seems to indicate that the presence of polar groups in a solute molecule affects its ability to participate in non-polar interactions. This is in accord with current concepts of the rôle of molecular structure in hydrophobic properties[8–10].

If the hydration properties of the phases are dissimilar, *i.e.*, $C \neq 0$ but parameter $m = 0$, eqn. 3 takes the form of eqn. 2 which means that the solute being partitioned is non-polar or that the hydration energies of the solute's polar groups are counterbalanced. The latter case seems to be typical of the amino acids with non-polar side chains and di- and tripeptides examined in this work.

The data given in Table I indicate that the partitioning of amino acids and di- and tripeptides is independent of the hydration properties of the phases over the

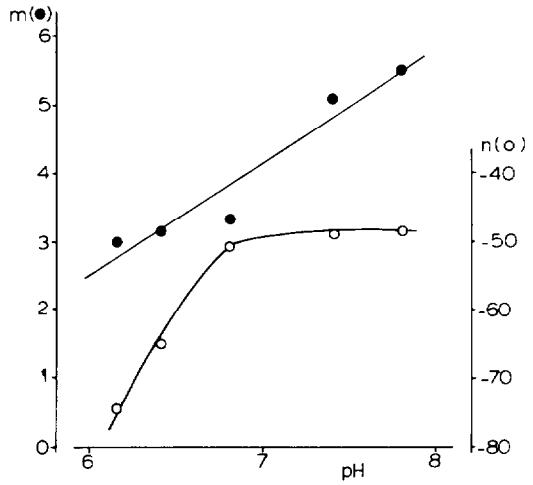

Fig. 4. pH-dependence of the characteristics n^{CH_2} and m for human serum albumin. For explanation see text.

range of concentrations of NaCl and sodium phosphate buffer, pH 7.4 ($B = 0$), *i.e.*, the hydration energies of carboxyl and amino groups are counterbalanced. In contrast the partitioning of some tetra- and pentapeptides is affected by alteration in the hydration properties of the phases under the conditions employed. This effect is probably related to the conformations of the peptides. The results obtained appear to

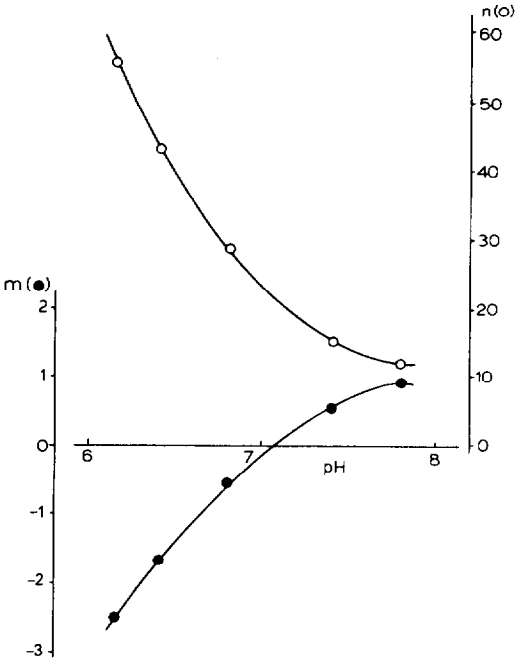

Fig. 5. pH-dependence of the characteristics n^{CH_2} and m for human oxyhaemoglobin. For explanation see text.

agree with the known chain-length dependence of the exact folding of a peptide[11] and can be taken as an indication of the possibility of using the partition technique in conformational studies.

The data shown in Fig. 2 (curves 1–5) for human serum albumin were treated according to eqn. 7 using the E and C values determined earlier[1,2]. The parameters n^{CH_2} and m were calculated and are plotted against pH in Fig. 4. The data indicate that the albumin surface is of hydrophilic character over the whole pH range (6.15–7.8) examined. The relative strength of the Van der Waals interactions of the protein surface appears to change from -76.8 to -49.5 equivalent CH_2 groups as the pH changes from 6.15 to 6.8, but further increase of pH is not followed by an alteration of the above feature of the macromolecular surface.

The observed increase in the equivalent number of carboxyl groups (parameter m) when going from pH 6.15 to 7.8 seems to be in line with an increase of the net negative charge of the protein.

It seems possible to assume that the observed changes in the n^{CH_2} value in the pH range noted indicate some conformational changes of the protein affecting the macromolecular surface features. This assumption, however, has no experimental justification since the effect of solute conformational changes on n^{CH_2} and m remains to be elucidated.

The data given in Fig. 2 (curves 6–10) for human oxyhaemoglobin were also treated according to eqn. 7. The relationships between parameters n^{CH_2} and m and the pH of the medium are shown in Fig. 5. The relative strength of the Van der Waals interactions of the oxyhaemoglobin surface appears to decrease from *ca.* 55 to *ca.* 12

TABLE II

FREE ENERGIES OF TRANSFER OF A CH_2 GROUP FROM THE AQUEOUS TO THE ORGANIC PHASE AND THE INTERFACIAL TENSION OF THE WATER–ORGANIC SOLVENT BIPHASIC SYSTEMS

The $\Delta G_{tr}^{CH_2}$ values are taken from ref. 19 and the interfacial tension, γ_{12} from ref. 18.

Organic solvent	$-\Delta G^{CH_2}$ *(cal/mole CH_2)*	γ_{12} *(dyn/cm)*
n-Butanol	542 ± 58	1.80
Isoamyl alcohol	604 ± 12	4.91
n-Amyl alcohol	700 ± 11	4.11
n-Octanol	727 ± 17	8.84
Diethyl ether	732 ± 101	10.48
Octane*	768 ± 53	51.01
Oils**	757 ± 16	18.37
Benzene	842 ± 66	33.60
Heptane*	827 ± 12	50.85
Chloroform	846 ± 24	31.39
Carbon tetrachloride	922 ± 41	43.40
Toluene	959 ± 126	35.70
Hexane	1010 ± 31	50.89
Cyclohexane*	1127 ± 43	51.01

* The values were omitted in deriving eqn. 10 for the reason discussed in the text.

** The $\Delta G_{tr}^{CH_2}$ value was taken from ref. 21.

equivalent CH_2 groups when going from pH 6.15 to 7.8. The equivalent number of carboxyl groups, m, increases in the same pH range. It should be particularly noted that the m value is zero at a pH close to the isoelectric point of the protein, pH = pI = 7.1 (ref. 12).

As the data given in Fig. 3 indicate the pH-dependence of the total hydrophobic character of serum albumin and oxyhaemoglobin, it is impossible to estimate the relative contributions of different solvent interactions of a macromolecule from these data. Hence, it seems more promising to study the partitioning of a solute at various hydration properties of the phases as shown in Fig. 2.

Eqn. 3 is similar in appearance to the equation suggested by Albertsson[13] to describe the partitioning of a charged protein in the presence of an interfacial potential difference between the phases of a given biphasic system

$$\ln K = \ln K^0 + (FZ/RT) \cdot \Delta\psi \tag{8}$$

where K and K^0 are the partition coefficients of the protein when partitioned in the presence and in the absence of the potential difference, $\Delta\psi$, between the phases, respectively, Z is the net charge of the protein macromolecule and F is the Faraday constant. This is reasonable as K^0 in eqn. 8 is supposed to represent the relative hydrophobic character of the macromolecular surface[13–15]. We have shown[1] that the corresponding term, $n^{CH_2}E$, in eqn. 3 represents the relative strengths of the Van der Waals interactions of a solute with the two phases. It has also been shown[2] that the interfacial potential difference, $\Delta\psi$, measurable in the biphasic system is solely a partial characteristic of the difference in the hydration properties of the phases, which should be estimated by parameter C instead of $\Delta\psi$ as considered earlier[2].

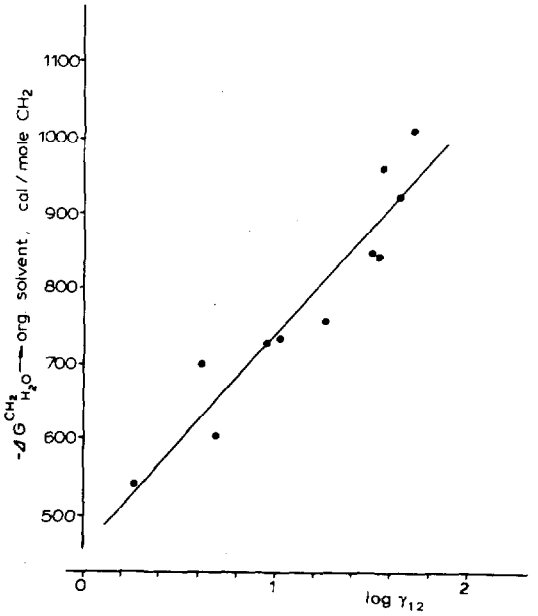

Fig. 6. Relationship between the interfacial tension, γ_{12}, and the free energy of interfacial transfer of a CH_2 group, $\Delta G_{tr}^{CH_2}$, for water–organic solvent biphasic systems.

The suggested approach also seems to be suitable for examination of the partitioning of cell particles. Gerson[16,17] expressed the partitioning of cells in the presence of the interfacial potential difference between the phases as

$$\ln K_{cell} = \alpha' \cdot \gamma_{12} \cdot \cos\theta + \delta \cdot \Delta\psi + \beta \tag{9}$$

where θ is the contact angle of a drop of the dense phase resting on a cell layer immersed in the light phase of the biphasic system, γ_{12} is the interfacial tension between the phases ($\gamma_{12} \cdot \cos\theta = -\Delta\gamma$, which is the difference between the surface energies of the cells in each phase), K_{cell} = the partition coefficient of the cell particles, α', δ and β are empirical constants which include all non-idealities in the parameter with which they are associated.

It can be seen that if the interfacial tension between the two phases, γ_{12}, is related to the difference in the relative hydrophobicities of the phases (expressed as $\Delta G_{tr}^{CH_2}$ or E in eqn. 3) Gerson's eqn. 9 should take a form similar to that of eqn. 3. In order to verify this the interfacial tension values of different water–organic solvent biphasic systems[18] were examined together with the free energies of transfer of a CH_2 group between the phases of these systems, $\Delta G_{tr}^{CH_2}$, reported previously[19]. The γ_{12} and $\Delta G_{tr}^{CH_2}$ values are listed in Table II, and the correlation established between them is presented in Fig. 6. This correlation is described by:

$$-\Delta G_{tr}^{CH_2} = (457 \pm 11) + (284 \pm 33) \cdot \log \gamma_{12} \tag{10}$$

$$n = 11,\ r = 0.944,\ s = 51$$

Most of the biphasic systems formed by water and hydrocarbons do not fit relation 10 probably due to the apparent insensitivity of the interfacial tension value to slight differences in the solvent features, in contrast to that of the free energy of transfer of a CH_2 group.

It should be emphasized that there is no direct evidence that relation 10 is valid for the aqueous polymeric biphasic systems under consideration. Data reported earlier[20,21], however, indicate that there seems to be no fundamental difference between the features of the common water–organic solvent partitioning systems and those of the aqueous polymeric biphasic systems. It is known also that a shift in the critical point of a given polymeric biphasic system induced by the presence of a salt is accompanied by an alteration of the interfacial tension value[22] as well as by a change in the $\Delta G_{tr}^{CH_2}$ value[20]. Hence, it seems possible to assume that there is a correlation between the $\Delta G_{tr}^{CH_2}$ (or E) and γ_{12} values for aqueous polymeric biphasic systems which is likely to be similar in form to that of eqn. 10. If that is the case Gerson's eqn. 9 becomes

$$\ln K_{cell} = \alpha^* \cdot (n^{CH_2})^* \cdot E + \delta^* \cdot m^* \cdot C + \beta^* \tag{11}$$

where $(n^{CH_2})^*$ is the equivalent number of CH_2 groups per particle surface, m^* is the equivalent number of carboxyl groups for the particle's surface, α^*, δ^* and β^* are empirical constants which include all non-idealities in the parameter with which they are associated as well as corrections for using soluble substances for rating the properties of the phases employed in the partition of cell particles.

It is clear that there are many difficulties in the use of eqn. 11 for estimation of the non-polar and hydration features of the cell surface. These difficulties remain to

be solved, but the above considerations seem to indicate that the physico-chemical factors governing the partition behaviour of both solutes and cell particles in aqueous polymeric biphasic systems are similar. The suggested approach allows one to examine the partition behaviour of cell particles and solutes from the same point of view, *i.e.*, the features detected by the partition technique are the relative strength of Van der Waals interactions and of polar hydration interactions with the aqueous environment in the two phases of the system used.

At present, the most difficult problem in the use of this approach for the study of cell particles seems to be an inadequacy of the rating with the soluble markers of the properties of the phases used for partitioning. Our choice of markers was made arbitrarily and is probably far from optimal. It seems likely that some better ways for rating the properties of the phases will be found in the near future. Nevertheless, understanding of the principles governing the partition behavior of cell particles and biological macromolecules can be achieved by application of simple physico-chemical approaches, providing certain restrictions are borne in mind.

REFERENCES

1 B. Yu. Zaslavsky, L. M. Miheeva, N. M. Mestechkina and S. V. Rogozhin, *J. Chromatogr.*, 253 (1982) 139–148.
2 B. Yu. Zaslavsky, L. M. Miheeva, N. M. Mestechkina and S. V. Rogozhin, *J. Chromatogr.*, 253 (1982) 149–158.
3 A. Ben-Naim, *Hydrophobic Interactions*, Plenum, New York, 1980.
4 B. Yu. Zaslavsky, N. M. Mestechkina, L. M. Miheeva and S. V. Rogozhin, *J. Chromatogr.*, 240 (1982) 21–28.
5 B. Yu. Zaslavsky, N. M. Mestechkina and S. V. Rogozhin, *Biochim. Biophys. Acta*, 579 (1979) 463–465.
6 B. Yu. Zaslavsky, L. G. Shchyukina and S. V. Rogozhin, *Mol. Biol. (U.S.S.R.)*, 15 (1981) 1315–1320.
7 P. Böhlen, S. Stein, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys.*, 155 (1973) 213–219.
8 R. F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1978.
9 J. F. M. Kinkel, E. Tomlinson and P. Smit, *Int. J. Pharm.*, 9 (1981) 121–136.
10 E. Tomlinson, H. Poppe and J. C. Kraak, *Int. J. Pharm.*, 7 (1981) 225–243.
11 B. A. Levine and R. J. P. Williams, *Proc. R. Soc. London, Ser. A*, 345 (1975) 5–22.
12 D. Malamud and J. W. Drysdale, *Anal. Biochem.*, 86 (1978) 620–647.
13 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist & Wiksell, Stockholm, 2nd ed., 1971.
14 G. Johansson, *Mol. Cell. Biochem.*, 4 (1974) 169–180.
15 P.-Å. Albertsson, *J. Chromatogr.*, 159 (1978) 111–122.
16 D. F. Gerson, *Biochim. Biophys. Acta*, 602 (1980) 269–280.
17 D. F. Gerson, *Immunol. Methods*, 2 (1981) 105–138.
18 *Handbook of Chemistry (in Russian)*, Vol. 1, GCI, Moscow, 1963, pp. 1026–1028.
19 B. Yu. Zaslavsky, L. M. Miheeva and S. V. Rogozhin, *J. Chromatogr.*, 216 (1981) 103–113.
20 B. Yu. Zaslavsky, L. M. Miheeva, N. M. Mestechkina, L. G. Shchyukina, M. A. Chlenov, L. I. Kudrjashov and S. V. Rogozhin, *J. Chromatogr.*, 202 (1980) 63–73.
21 B. Yu. Zaslavsky, L. M. Miheeva and S. V. Rogozhin, *J. Chromatogr.*, 212 (1981) 13–22.
22 S. Bamberger, G. V. F. Seaman and D. E. Brooks, *Proc. 2nd Int. Conf. on Partitioning, Sheffield, April 5–8, 1981*, Biological Sciences Duplicating Centre, University of Sheffield, 1981, p. 3.