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BEHAVIOUR OF BIOMOLECULES IN WATER-ORGANIC SOLVENT-IN-ORGANIC SALT TWO-PHASE TERNARY SYSTEMS

II*. MECHANISM OF DISTRIBUTION OF SUBSTANCES IN THE SYSTEMS

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INTRODUCTION

The distribution of 3-aminophthalimide in the two-phase ternary system formed by salting out of *n*-propanol by sodium chloride from an aqueous solution was studied. High-performance liquid chromatography, spectrofluorimetry and equilibrium distribution methods showed that the distribution is determined by the ratio between the volume proportions of the alcohol in co-existing phases.

INTRODUCTION

It was previously demonstrated¹ that two-phase ternary systems formed by salting out of polar organic solvents from their aqueous solutions may be used for the fractionation of various classes of biomolecules. Taking into account the possibilities and the advantages in using such systems, an investigation of the mechanism and the factors that affect the distribution is desirable.

The distribution of a substance in two-phase systems is related, as a rule, to its solubility in co-existing phases. Solubility, in its turn, is determined by the solvating strength or polarity of the phases. Therefore, by determining the polarity it is possible to estimate the character of the distribution and to analyse the factors that affect it.

Owing to complexity of the concept of polarity, this generalized property is usually described by physico-chemical characteristics of the solvent. A connection of the solvent polarity with the electronic spectral shift of some substances has been established, allowing one to suggest the following empirical solvent polarity scales: Kosower's *Z*-scale² and Zelinskii's *S*-scale³. According to the latter scale, each solvent is characterized by an empirical value *S*, which is proportional to the difference in the frequencies of the maxima of the fluorescence bands of 4-amino-*N*-methylphthalimide observed in the gas phase and in the present solvent. The connection

* For Part I, see ref. 1.

between the polarity and the location of fluorescence maxima of phthalimide derivatives was substantiated theoretically by Bakhshiev⁴, based on an analysis of the influence of both non-specific and specific substance-solvent interactions on the electronic spectral shift.

A systematic and detailed experimental investigation and theoretical considerations therefore make it possible to estimate the polarity of co-existing phases in two-phase ternary systems according to the shift of the fluorescence spectral maxima. 3-Aminophthalimide (3-API), whose spectral characteristics are well known, may serve as a fluorescence probe.

An alternative method for the determination of the polarity or solvating strength of the solvent is based on a study of the dependence of the retention factors, k' , on the composition of the eluent (solvent) in HPLC with the use of reversed phases. As shown by Snyder and co-workers^{5,6}, the retention factor is related to the volume proportion (φ) of organic solvent in an eluent by the equation: $\log k' = \log K_w + S\varphi$, where S characterizes the solvating strength of the solvent and K_w is the extrapolated value of k' for $\varphi = 0$. Hence the determination of S values for systems with different solvents and salt contents can also be used for determining the polarity of the co-existing phases.

In this work, the effect of several factors on the solvating strength of co-existing phases and, hence, on the distribution of a substance in two-phase ternary systems, has been investigated by fluorescence and HPLC methods for 3-API. The system water-*n*-propanol-sodium chloride was selected.

EXPERIMENTAL

Materials

n-Propanol purified as described in ref. 7, deionized water with a specific resistance of ≥ 15 M Ω /cm and sodium chloride of extra-purity grade (Reakhim, U.S.S.R.) were used. 3-API was prepared according to ref. 8 from pure-grade 3-nitrophthalic acid (Reakhim). The homogeneity of 3-API was demonstrated by thin-layer chromatographic and fluorescence methods. A column (25 \times 4 mm I.D.) packed with Silasorb C-18 (Lachema, Czechoslovakia) (7.5 μ m) was used in all the experiments without preliminary treating.

Spectrofluorimeter

Fluorescence spectra were recorded on a SIM-4800S spectrofluorimeter (U.S.A.) over the wavelength range 450–550 nm, with an excitation monochromator slit width of 16 nm and an emission monochromator slit width of 1 nm; the wavelength of the exciting light was 395 nm. Spectral correction of the fluorescence spectra was not performed.

HPLC

Retention factors, calculated according to the equation $k' = (t - t_0)/t_0$, where t and t_0 are retention times of the retained and non-retained substances, respectively, were determined with the use of a Hitachi 658-50 chromatograph. t_0 was determined by retention of *n*-propanol in all the systems under study using a Hitachi 300-10 differential spectrophotometer as the detector. Retention times were determined in an automatic regime with an accuracy of ± 0.02 min.

TABLE I
COMPOSITION OF EQUILIBRIUM PHASES

System	Top phase (% w/w)				Bottom phase (% w/w)			
	NaCl	H ₂ O	C ₃ H ₇ OH	φ_1^*	NaCl	H ₂ O	C ₃ H ₇ OH	φ_2^*
1	1.00	17.00	82.00	0.86	21.10	74.00	4.90	0.08
2	1.20	21.80	77.00	0.82	17.00	75.70	7.30	0.11
3	1.60	25.80	72.60	0.78	14.40	76.90	8.70	0.12
4	2.25	32.50	65.25	0.71	11.75	77.00	11.25	0.15
5	2.50	37.50	60.00	0.67	9.75	74.00	16.25	0.21

* φ_1 and φ_2 are volume concentrations in the top and bottom phases, respectively.

Measurement of distribution coefficients

The distribution of 3-API in systems of different phase composition was performed as follows. The required amount of 3-API, dissolved in a calculated amount of *n*-propanol, was added to a definite amount of an aqueous solution of sodium chloride. The system was allowed to stand at $25 \pm 0.1^\circ\text{C}$ for 2–3 h; after separating the system into layers, aliquots of co-existing phases were taken, in which the substance concentration was determined by absolute calibration⁹ by liquid chromatography using a Hitachi 650-IOLC fluorescence spectrophotometer as a detector.

The distribution coefficient (*P*) was calculated according to the equation

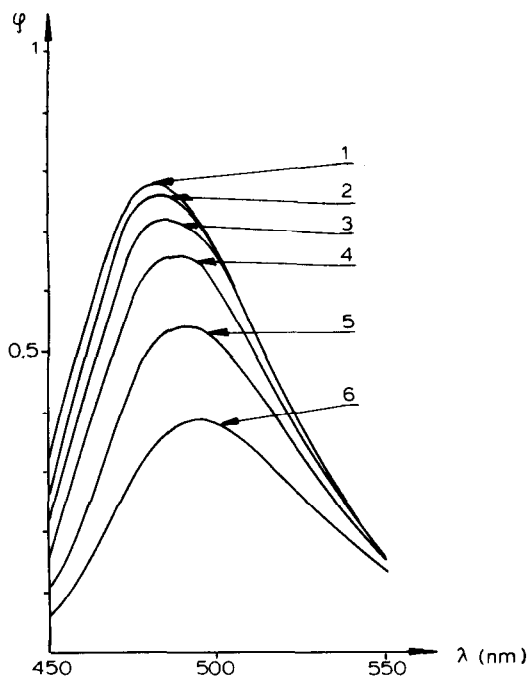


Fig. 1. Fluorescence spectra of 3-API in *n*-propanol-aqueous solutions. Volume parts of *n*-propanol (φ): 1, 1.0; 2, 0.8; 3, 0.6; 4, 0.4; 5, 0.2; 6, 0.

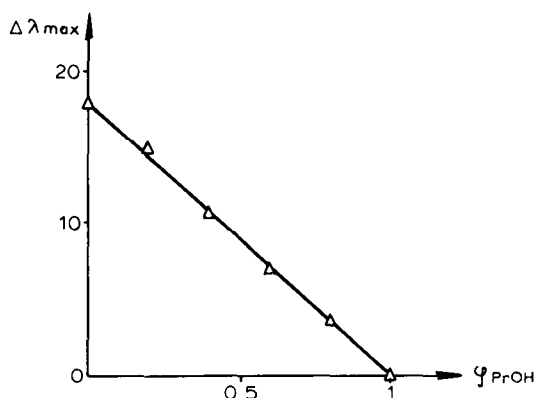


Fig. 2. Dependence of the shift of the fluorescence maximum on volume parts of *n*-propanol. Experimental error in λ_{max} determination: ± 1 nm.

$P = C_1/C_2$, where C_1 are the concentration of 3-API in the top and bottom phases, respectively. P values were calculated as means values of not less than three separate measurements. The determination error was $\leq 5\%$.

Determination of composition of co-existing phases

The phase composition, presented in Table I, was determined by the method described in ref. 1. The determination error was $\leq 2.5\%$. Mathematical treatment of the experimental results was carried out on a Hewlett-Packard 9815 S computer.

RESULTS AND DISCUSSION

Fluorescence spectra of 3-API in alcohol-aqueous solutions, containing various concentrations of alcohol (φ), are presented in Fig. 1.

With increase in alcohol concentration in the system, a regular short-wave shift and a noticeable increase in the fluorescence quantum yield are observed. The dependence of the shift of the fluorescence maximum, $\Delta\lambda_{\text{max}}$, on the concentration of alcohol in the system (φ) is shown in Fig. 2.

A monotonic variation of the fluorescence maximum shift with variation in the concentration of alcohol in solution may indicate the absence of specific 3-API-solvent interactions in the system under consideration¹⁰.

TABLE II

DEPENDENCE OF φ_{max} ON THE COMPOSITION OF THE SYSTEMS

$\varphi_{\text{alc.}}$	C_{NaCl} (%, w/w)	λ_{max} (nm)	C_{NaCl} (%, w/w)	λ_{max} (nm)	C_{NaCl} (%, w/w)	λ_{max} (nm)	C_{NaCl} (%, w/w)	λ_{max} (nm)
0.0	1	494	2	496	3	496	15	494
0.2	1	491	2	491	3	491	—	—
0.4	1	489	2	488	3	489	—	—
0.6	1	485	2	483	3	484	—	—
0.8	1	482	2	—	3	—	—	—

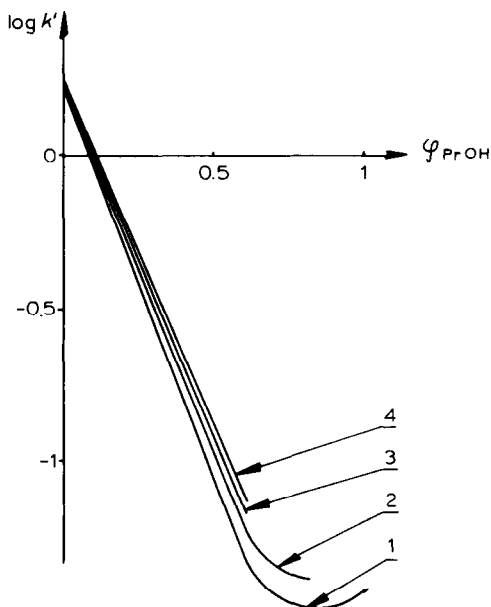


Fig. 3. Dependence of retention factors on eluent composition. Concentration of sodium chloride (% w/w): 1, 0; 2, 1; 3, 2; 4, 3.

The influence of sodium chloride in water-organic systems at concentrations that do not cause separation into layers is shown by the results in Table II.

The results indicate that, when sodium chloride is introduced, it does not have a great effect on the λ_{\max} position (in the determination of λ_{\max} , the variations do not exceed the experimental error of ± 1 nm).

The theoretical concepts established⁴ and the overall fluorimetric data allow one to suggest that in the system under study, the nearest solvating environment of 3-API molecules is mainly determined by the water to alcohol ratio in the system, with a weak dependence on the sodium chloride concentration.

HPLC data on the determination of the dependence of the retention factors of 3-API on RP-18 on the eluent composition are presented in Fig. 3. The composition of the eluents was similar to that of the systems used in fluorescence measurements.

It can be seen from Fig. 3 that up to 0.6 volume parts of alcohol in systems containing different amounts of sodium chloride, the relationship between $\log k'$ and ϕ remains linear. Treatment of the data obtained on the linear dependence section by the least-squares method leads (with a regression coefficient of 0.96) to the following Snyder equations:

$$\log k'_1 = (0.24 \pm 0.01) - 2.63\phi \quad (C_{\text{NaCl}} = 0\%, \text{ w/w}) \quad (1)$$

$$\log k'_2 = (0.24 \pm 0.01) - 2.41\phi \quad (C_{\text{NaCl}} = 1\%, \text{ w/w}) \quad (2)$$

$$\log k'_3 = (0.24 \pm 0.01) - 2.31\varphi \quad (C_{\text{NaCl}} = 2\%, \text{ w/w}) \quad (3)$$

$$\log k'_4 = (0.24 \pm 0.01) - 2.29\varphi \quad (C_{\text{NaCl}} = 3\%, \text{ w/w}) \quad (4)$$

The retention factors of 3-API are the result of approximation of the $\log k'$ values to zero alcohol concentration. Comparison of the S values in the equations obtained shows a change of 0.12 unit with increase in the sodium chloride concentration by a factor of 3. It is known that this change is 0.6 unit if methanol is replaced with ethanol, which has similar properties⁵. Consequently, similar S values may indicate that the solvating strength does not change much when a salt is introduced. It should also be taken into account that the variation in S values may be caused by an alteration of the properties of the reversed phase rather than a change in the solvating strength of the solvent^{11,12}.

As the fluorescence data indicate that sodium chloride has a weak effect on the solvation of 3-API, which in turn apparently conditions the slight dependence of S on the sodium chloride concentration, one may assume that the distribution coefficient of 3-API in the two-phase water-*n*-propanol-sodium chloride system is determined by the ratio of the volume parts of alcohol in the co-existing phases.

This suggestion was substantiated by direct determination of the distribution coefficient in systems with different compositions of the co-existing phases (see Table I). The dependence of $\log P$ on the ratio of volume parts of the alcohol is shown in Fig. 4.

It can be seen that the distribution coefficient is actually determined by the ratio between the volume parts of alcohol in the equilibrium phases, in spite of the considerable difference in the sodium chloride concentration in the co-existing phases. Treatment of the experimental data by the least-squares method results in the equation $\log P = 0.31 + 0.12 \varphi_1/\varphi_2$ (with a regression coefficient of 0.94). Hence the distribution of 3-API in the water-*n*-propanol-sodium chloride two phase system is mainly determined by the ratio between the volume parts of alcohol in the co-existing phases.

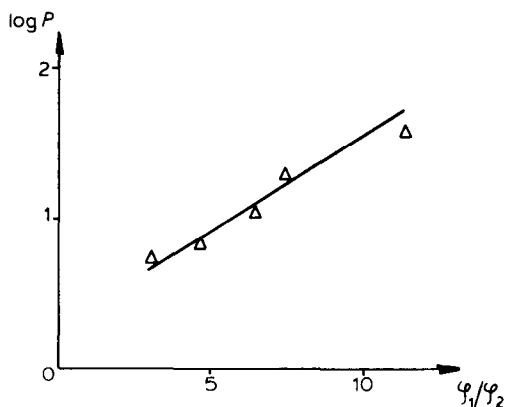


Fig. 4. Dependence of distribution coefficient on the ratio of volume parts of *n*-propanol in co-existing phases.

One may conclude that in systems formed by salting-out a polar organic solvent from their aqueous solutions by neutral (1:1) salts, similar regularities will also be observed for other substances, provided that the solvating strength of such systems with respect to the distributed component is determined primarily by the organic solvent to water ratio.

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