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A Relationship between Charge and Distribution Constants of Compounds[1a]

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The distribution constants of a number of amino acids, peptides and proteins in various 2-butanol–0.1 *N* acid systems are reported. A relationship was noted between the charge on the compound, the value of the distribution constant of the compound and the distribution constant of the acid in each system. A theoretical basis of the relationship is proposed. The potential uses and limitations of the relationship are discussed.

In connection with a general study on the partition column chromatography of insulin,[2] it was noted that a relationship existed between the distribution constant of strong acids in 2-butanol–water systems and the distribution constant of insulin in 2-butanol–0.1 *N* acid systems as measured by the method of partition column chromatography.[3] When the distribution constant of insulin in each 2-butanol–0.1 *N* acid system was plotted against the distribution constant of the acid between 2-butanol–water, the points fell on a smooth curve. The curve was useful in predicting the distribution constant of insulin in other 2-butanol–acid systems and as such took some of the empiricism out of the selection of solvent systems for partition column chromatography and countercurrent distribution studies. In order to determine the nature of the relationship as well as the extent of its applicability, the present study on model compounds was instigated.

In essence the procedure followed was to measure the distribution constant of trace amounts of amino acids, peptides and proteins in various 2-butanol–0.1 *N* aqueous acid systems. The distribution constants of the compound so obtained were compared with the distribution constants of the acids in the systems. For theoretical reasons the acids studied were limited to strong, highly ionized acids including hydroxy-ethylsulfonic (isethionic), methylsulfonic, hydrochloric, hydrobromic, nitric and perchloric acids. The distribution constants of the acids in the 2-butanol–water system increased in the order given.

A similar but more limited study was also performed in a solvent system which was modified by the incorporation of some methanol to increase the degree of miscibility of the butanol–water mixtures. In order to correlate the experimental results with a theoretical treatment, some information about the nature of the solvent systems was needed. The amount of water in each phase of each 2-butanol–0.1 *N* acid system was measured as well as the dependence of the distribution constant of the acid on the concentration of the acid in the systems.

(1) (a) This work was supported in part by grant A-608 of the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health. Presented at the 133rd National Meeting of the American Chemical Society, San Francisco, California, April, 1958. (b) Fellow of the Belgian American Educational Foundation (1955–56) on leave from Institut pour la Recherche Scientifique en Afrique Centrale, Belgian Congo.

(2) F. H. Carpenter and G. P. Hess, THIS JOURNAL, **78**, 3351 (1956).

(3) F. H. Carpenter, *Archiv. Biochem. Biophys.*, in press.

Experimental

Materials.—With the exception of L-arginyl-L-leucine and L-arginyl-L-glutamic acid which had been prepared in this Laboratory,[4] the amino acids, di- and tripeptides were commercial samples. Crystalline zinc–insulin[5] (Lilly No. 972-519) of bovine origin and crystalline lysozyme (Armour and Company) were used. Purified samples of bacitracin-A, gramicidin S-A, polypeptin-A and tyrocidin-A were kindly furnished by Dr. Lyman C. Craig.[6] Commercial 2-butanol was refluxed with and distilled from calcium hydride.[3] Variable amounts of impurities present in different batches of 2-butanol purified in this manner had a measurable effect on the miscibility with water. Only those batches were selected for use which had the same degree of miscibility with water. The acid solutions were made by dilution of commercial preparations with the exceptions of hydrobromic acid which was made from a constant boiling fraction and hydroxy-ethylsulfonic (isethionic) acid which was made by passing a solution of the sodium salt, prepared according to Lauer and Hill,[7] through a sulfonic acid ion-exchange resin (Amberlite IR-120 in the acid form).

Equilibrations.—All equilibrations were performed in a water-bath at 25 ± 0.5°. *The systems are designated according to their composition before equilibration.* 2-Butanol or 3% (by volume) methanol in 2-butanol was added to an equal volume of 0.1, 0.05 or 0.01 *N* acid in a glass-stoppered cylinder. The cylinder was inverted 15 times, placed in the bath for 2 hr., inverted another 100 times and placed in the bath for at least another 8 hr. before samples were removed from the upper and lower layers for analyses. In the distribution studies on the amino acids and peptides the initial concentration of the compounds was 0.0005 *M* in the 0.1 *N* acids. For the insulin, lysozyme and bacitracin-A, the initial condition was a 0.01% solution of these compounds in the 0.1 *N* acids while for gramicidin S-A, polypeptin-A and tyrocidin-A, the initial condition was a 0.01% soluton in 2-butanol. At least duplicate equilibrations were performed.

Analyses. Acids.—Aliquots (5–10 ml.) were removed from each phase and titrated with standard sodium hydroxide to the phenolphthalein end-point.

Amino Acids and Small Peptides.—Appropriate size aliquots (3 from each phase of each equilibration) were placed in test-tubes (18 × 180 mm.). After the addition of 0.5 *M* potassium carbonate (0.05 to 0.1 ml.), the test-tubes were placed in a vacuum desiccator and the solvent was removed *in vacuo* at room temperature. The compounds were estimated in the residue by the quantitative ninhydrin procedure of Moore and Stein.[8] Blanks for the color determination were obtained from control equilibrations in which the compound had been omitted.

Large Peptides and Proteins.—Appropriate size aliquots were evaporated to dryness as above. Constant boiling hydrochloric acid (1 ml.) was added to each tube and the tubes were placed in a steam-bath for 18 hr. The solvent was removed *in vacuo* and the amino acids in the residue were estimated by the ninhydrin color reaction.[8] In addi-

(4) D. T. Gish and F. H. Carpenter, THIS JOURNAL, **75**, 5872 (1953).

(5) We wish to thank Dr. O. K. Behrens of Eli Lilly and Company for furnishing the crystalline insulin.

(6) The authors wish to take this opportunity to thank Dr. Craig of the Rockefeller Institute for Medical Research for these samples.

(7) W. M. Lauer and A. Hill, THIS JOURNAL, **58**, 1873 (1936).

(8) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

TABLE I

DISTRIBUTION CONSTANTS OF COMPOUNDS IN 2-BUTANOL–0.1 *N* ACID SYSTEMS

Compound \ System	Distribution constants HO-EtSO$_3$H	CH_3SO_3H	HCl	HBr	HNO_3	$HClO_4$	Const. of equation *n*	*k*
Acid	0.262	0.282	0.305	0.397	0.482	0.789		
Ammonia	.067	.079	.082	.114	.137	.231	1.09	0.303
Glycine	.081	.095	.098	.128	.152	.256	1.00	.322
Alanine	.095	.111	.115	.151	.182	.302	1.02	.386
α-Aminobutyric acid	.128	.144	.161	.207	.247	.420	1.04	.538
Norvaline	.225	.258	.289	.375	.452	.709	1.01	.925
Arginine	.023	.027	.032	.053	.069	.183	1.85	.281
Lysine	.015	.018	.022	.035	.046	.124	1.86	.190
Ornithine	.015	.019	.020	.032	.043	.118	1.81	.174
Histidine	.013	.018	.021	.028	.031	.136	1.91	.177
Arginylleucine	.118	.138	.178	.293	.397	1.04	1.94	1.675
Arginylglutamic acid	.036	.038	.049	.079	.110	0.293	1.92	0.458
Glutathione disulfide	.024	.028	.031	.051	.065	153	1.67	.226
Histidylhistidine	...	....	.009	.020	.040	.133	2.83	.277
Gramicidin S-A	9.60	12.5	16.4	14.7[a]	35.6	36.6[a]	2.03	161.8
Polypeptin-A	2.28	2.30	3.25	4.63	6.20	12.5	1.57	18.84
Bacitracin-A	0.488	0.540	0.848	1.54	2.35	5.00	2.14	9.64
Insulin	0.029	0.042	0.084	0.273	0.563	4.50	4.51	14.62
Lysozyme	...	....	....	....	....	5.7	...	....

[a] These points were omitted in calculating the slope of the line.

tion the modified Folin reaction of Lowry, *et al.*,[9] was applied in some cases (insulin and lysoyzme) on the residues before hydrolysis. In these instances the color developed did not follow Beer's law and a standard curve was run with each experiment.

Water.—Aliquots (1 ml.) were removed from each layer and added to a tared, 50-ml. volumetric flask. The flask was weighed again and then diluted to the mark with absolute methanol. Aliquots of this solution (10 ml. when dealing with the organic layer and 5 ml. when dealing with the aqueous layer) were titrated with the Karl Fischer reagent to the first appearance of a brownish-red color. A blank titration was made on a similar aliquot of absolute methanol. The water equivalence of the reagent was determined by titrating an accurately weighed quantity of water dissolved in methanol.

Calculations.—The distribution constants (K) are expressed as the ratio of *concentration* (wt./vol.) of solute in the organic (upper) layer to that in the aqueous (lower) layer. The precision with which the ratios could be determined varied with their size and with the type of analysis used. For the amino acids and small peptides, distribution constants in the range 50 to 0.02 could be determined with a fair degree of reproducibility. For the large peptides and proteins, where several additional steps were involved in the analysis, good reproducibility was generally limited to K values in the range 20 to 0.05. In all cases, the values near the extremes noted above are not as reliable as those in the center of the range.

Results

The results of the measurements of the distribution constants of a number of compounds in the various 2-butanol–0.1 *N* acid systems are shown in Table I. The distribution constants of the acids in the 2-butanol–water systems are shown in the first line of Table I. Since the distribution constant of tyrocidin-A was greater than 20 in all of these systems and could not be determined with any degree of reproducibility, it is not included in the table. When one makes a plot of the distribution constant of the compound in each one of the 2-butanol–acid systems against the distribution constant of the acid in the system, one obtains a regular curve for all of these compounds as is illustrated for a few examples in Fig. 1. In some cases the plot gives a straight line while in others a curved line is obtained. In all cases extrapolation of the line would indicate that it goes through the origin. These results indicate a regular relationship between the way in which the acids distribute in the system and the way in which the solutes distribute.

The nature of this relationship and especially the reason for the shape of the curves is revealed in a log-log plot. Figure 2 shows a plot in which the distribution constants of the compounds are plotted against the distribution constants of the acids both on a logarithm scale. In the examples shown, the points for each compound fall rather closely along straight lines. However, the slope of these lines differs. The points for alanine and ammonia fit quite well with lines of unit slope. The points for arginine follow a line with slope of 2 while the points for histidyl-histidine fall along a line with a slope of 3. The most obvious difference among these compounds which can be correlated with the slopes of the lines in the log-log plot is the number of positive charges present on the molecules in acid solution. Alanine and ammonia have one positive charge, arginine has two while histidyl-histidine has three positive charges. This correlation is verified for most of the other compounds in Table I. The next to the last column gives the slope of the best fitting line, as calculated by the method of least squares,[10] for the points in the log-log plot. It will be noted that with the exception of a few of the large molecules, the slope of the best fitting line in the log-log plot agrees quite well with the expected number of positive charges on the molecules in acid solution.

The relationship between the distribution constants of the compound and the acids in the log-log plot can be expressed by means of the equation

$$\log K_{A^{n+}} = n \log K_{H^+} + \log k \qquad (1)$$

(9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(10) D. P. Bartlett, "General Principles of the Method of Least Squares with Applications," Rumford Press, Rumford, N. H., 1915.

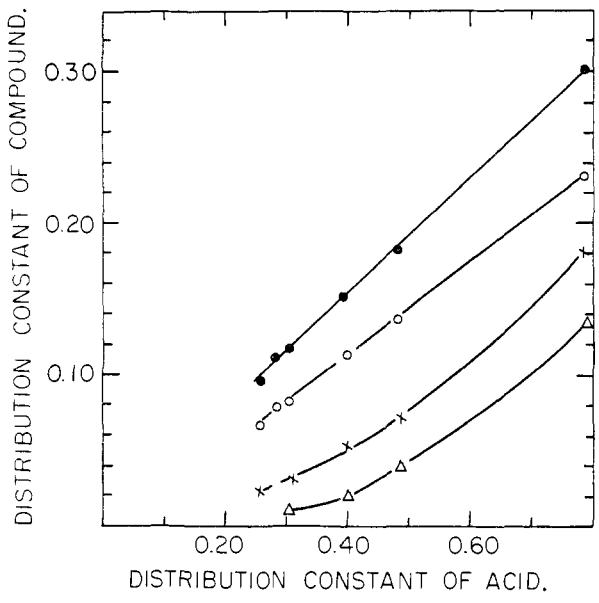

Fig. 1.—A plot of the distribution constants of several compounds as a function of the distribution constants of various strong acids in 2-butanol–water systems: ●, alanine; O, ammonia; ×, L-arginine; △, L-histidyl-L-histidine.

where $K_{A^{n+}}$ is the distribution constant of the compound (A^{n+}), K_{H^+} is the distribution constant of the acid (H^+), n is a constant which is approximately equal to the number of positive charges on the compound and k is a proportionality constant whose value depends on the nature of the compound and the solvent system and which would be equal to $K_{A^{n+}}$ in an acid system where K_{H^+} is equal to unity. The equation takes the form shown in (2) for the regular plot. The values for the

$$K_{A^{n+}} = k(K_{H^+})^n \qquad (2)$$

proportionality constant (k) as calculated from the experimental data (including the experimental slopes rather than the theoretical number of positive charges) are shown in the last column of Table I.

In order to obtain more information on this relationship, the solvent system was modified by the incorporation of some methanol. The results of a somewhat more limited study of the distribution constants in the system 3% methanol in 2-butanol–0.1 N aqueous acids are shown in Table II. It will be noted that although the values of the distribution constants of the acids and compounds are different from that in the 2-butanol–0.1 N acid systems, the relationship between charge and slope of the line in the log-log plot appears to hold quite well. The value of the proportionality constant (k) for each compound is somewhat larger in the methanol containing system than in the straight 2-butanol system.

Proteins and Large Peptides.—The results obtained with some of the proteins and large peptides in the 2-butanol–0.1 N acid systems deserve special mention. The distribution constant of lysozyme could be measured in only one system, the perchloric acid system. If it were assumed that the compound followed the relationship of equation

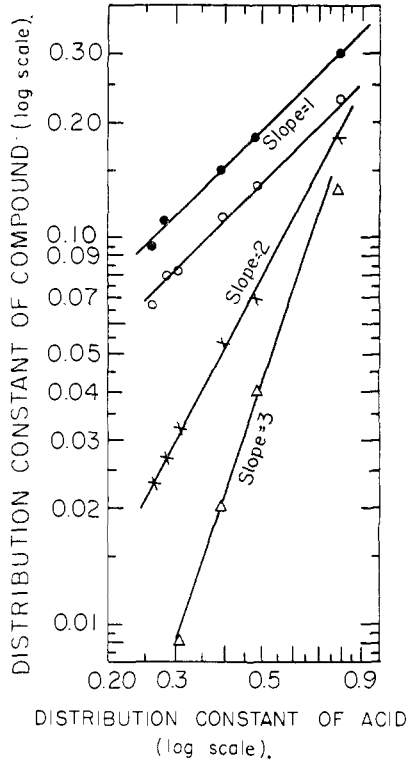

Fig. 2.—A plot of the distribution constants of several compounds as a function of the distribution constants of various strong acids (both on logarithm scales) in 2-butanol–water systems: ●, alanine; O, ammonia; ×, L-arginine; △, L-histidyl-L-histidine.

2 and that it contained 19 positive charges in acid solution,[11] one calculates that its distribution constant in all of the other systems studied would be too low to measure which was the experimental finding.

The results with the other polypeptides and small proteins, as shown in Fig. 3, are not entirely in agreement with the relationship of equation 2. Whereas most of the points in the log-log plot fall along straight lines, the results obtained in the perchloric acid system were always somewhat lower than one would predict on the basis of the results in the other systems. Most of the points for bacitracin-A with the exception of the value in the perchloric acid system fall along a curve with a slope of 3 (Fig. 3) which is in agreement with the number of positive charges expected for this compound on the basis of what is known about its structure.[12] With gramicidin S-A, in addition to the low value in perchloric acid, the value in hydrobromic acid was also lower than what would have been predicted on the basis of the other four values which fall fairly well along a line with slope of 2 in agreement with the number of positive charges predicted for this compound on the basis of its structure.[13] The low result obtained in the hydrobromic acid system may have been due to

(11) J. C. Lewis, N. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

(12) L. C. Craig, W. Hausmann and J. R. Weisiger, *ibid.*, **200**, 765 (1953); THIS JOURNAL, **76**, 2839 (1954).

(13) A. R. Battersby and L. C. Craig, *ibid.*, **73**, 1887 (1951).

TABLE II

DISTRIBUTION CONSTANT OF COMPOUNDS IN 3% METHANOL IN 2-BUTANOL–0.1 *N* ACID SYSTEMS

Compound \ System	Distribution constants						Const. of equation	
	$HO\text{-}EtSO_3H$	CH_3SO_3H	HCl	HBr	HNO_3	$HClO_4$	*n*	*k*
Acid	0.343	0.377	0.399	0.500	0.588	0.864	..	...
Ammonia[a]	...	...	.139	.168	.199	.333	1.14	0.382
Alanine[a]	...	...	.179	.212	.272	.435	1.18	.508
Cystine[a]	...	...	.062	.099	.119	.260	1.83	.333
Histidyl-histidine[a]	...	...	.029	.048	.071	.222	2.65	.313
Lysine	0.043	0.052	.063	.096	.119	.286	2.00	.373
Ornithine	.045	.052	.061	.093	.121	.283	1.98	.367
Histidine	.043	.056	.066	.101	.130	.320	2.10	.428
Arginine	.058	.067	.081	.124	.158	.357	1.96	469

[a] The values of the distribution constants in the hydroxy-ethylsulfonic and methylsulfonic acid systems were not measured.

analytical difficulties. Lack of material prevented a thorough study of this point. It should be noted that in all of these systems the distribution constant of gramicidin S-A was of such a magnitude as to preclude accurate determination of the values.

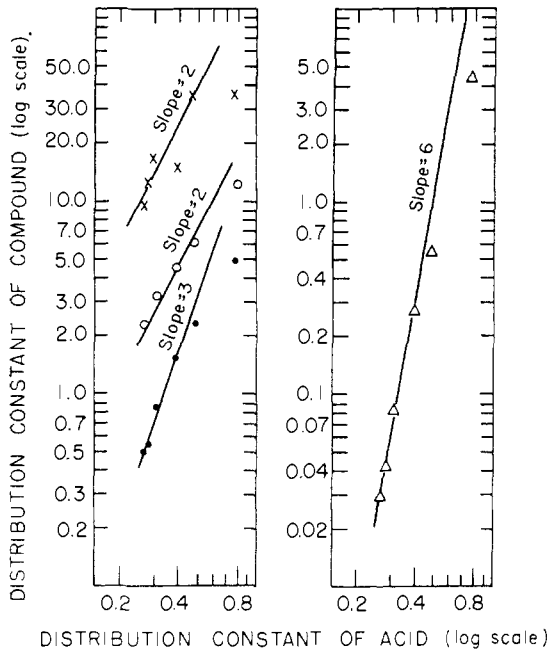

Fig. 3.—Distribution constants of several large peptides and insulin as a function of the distribution constants of acids (both on logarithm scales) in 2-butanol–water systems: ●, bacitracin-A; O, polypeptin-A; ×, gramicidin S-A; Δ, insulin.

The results obtained with polypeptin-A are definitely contradictory to what is expected for this compound. Although the complete structure of polypeptin-A is not known, what knowledge is available would indicate that it contains three positive charges in acid solution.[14] Even with the exclusion of the value in perchloric acid, the remaining five values for the distribution constants of this compound fall close to a line with slope of 2 rather than 3. Although it is known that polypeptin-A undergoes some sort of transformation in acid solution[14] which may account for these results, definite conclusions on the significance of these results will have to await further studies on the structure of polypeptin-A.

Insulin (Fig. 3) represents rather a unique case in that the values of the distribution constants increased over a hundred fold in going from the hydroxy-ethylsulfonic acid system to the perchloric acid system. Although most of the points in the log-log plot fell along a line with a slope of 6, as would be predicted on the basis of the structure,[15] again the value in the perchloric acid system was somewhat lower than one would predict on the basis of the results in the other systems. When all of the points are included, the best fitting straight line by the method of least squares has a slope of 4.5 instead of 6 in the log-log plot.

Composition of the Solvent Systems.—In order to gain further insight as to the nature of the solvent system, the two layers in the various systems were analyzed for their water content (Table III). Ignoring the contribution of the acids to the number of molecules in solution, the mole fraction per cent. of water in each of the layers was calculated. It can be seen that in terms of the number of molecules present both the organic (*ca.* 66% N_{H_2O}) and aqueous (*ca.* 94% N_{H_2O}) layers were quite rich in water. Furthermore, the water content of these layers changed very little in going from one acid system to another. This was reflected in the distribution constant for water which remains practically constant (0.36) for the systems from hydroxyethylsulfonic acid up through hydrobromic acid and then increased slightly for nitric and perchloric acid systems (next to last column of Table III). The changes in the distribution constant of the water were relatively minor as compared to that of the acids in going from one system to another (last column of Table III).

Similar results were obtained on water analysis of the various layers in the 3% methanol in 2-butanol–0.1 *N* aqueous acid systems as shown in Table IV. Because of the presence of methanol it was not possible to calculate the mole fraction of water in each layer but on a weight/volume or weight/weight basis it appears that the upper layer is enriched while the lower layer is somewhat depleted in water as compared to the comparable systems in the absence of methanol. This is in agreement with the findings on the distribution constants of the various compounds which were

(14) W. Hausmann and L. C. Craig, *J. Biol. Chem.*, **198**, 405 (1952).

(15) A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **60**, 541 (1955).

TABLE III

WATER CONTENT OF THE VARIOUS 2-BUTANOL–0.1 *N* ACID SYSTEMS

Acid	Organic layer: Density, g./ml.	Organic layer: Water content, By vol., g./ml.	Organic layer: Water content, N_{H_2O}, %	Aqueous layer: Density, g./ml.	Aqueous layer: Water content, By vol., g./ml.	Aqueous layer: Water content, N_{H_2O}, %	Distribution constants: H_2O	Distribution constants: Acid
HO–$EtSO_3H$	0.861	0.269	64.9	0.968	0.766	94.0	0.351	0.262
CH_3SO_3H	.865	.278	66.0	.966	.771	94.3	.360	.282
HCl	.860	.282	66.6	.966	.783	94.5	.361	.305
HBr	.863	.285	66.9	.967	.780	94.5	.361	.397
HNO_3	.864	.291	67.6	.962	.763	93.9	.381	.482
$HClO_4$	.869	.311	69.6	.960	.756	93.8	.411	.789
None	.864	.285	66.7	.962	.768	94.3	.367	...

TABLE IV

WATER CONTENT OF THE VARIOUS 3% METHANOL IN 2-BUTANOL–0.1 *N* ACID SYSTEMS

Acid	Organic layer: Density, g./ml.	Organic layer: Water content, By wt., g./g.	Organic layer: Water content, By vol., g./ml.	Aqueous layer: Density, g./ml.	Aqueous layer: Water content, By wt., g./g.	Aqueous layer: Water content, By vol., g./ml.	Distribution constants: H_2O	Distribution constants: Acid
HO–$EtSO_3H$	0.874	0.364	0.318	0.966	0.786	0.759	0.419	0.343
CH_3SO_3H	.866	.372	.322	.951	.796	.757	.425	.377
HCl	.880	.373	.328	.969	.796	.771	.425	.399
HBr	.884	.388	.343	.965	.779	.752	.456	.500
HNO_3	.877	.394	.345	.957	.779	.745	.463	.588
$HClO_4$	.889	.428	.381	.951	.756	.719	.530	.864
None	.868	.363	.315	.966	.796	.769	.410	...

larger in the methanol containing systems than in the straight 2-butanol systems.

Effect of Concentration on the Distribution Constants of the Acids.—In another study the variation of the distribution constant of the acids with concentration was measured over a limited range from 0.01 *N* to 0.1 *N* (original concentration in water). The results (see Fig. 4) indicated that the distribution constants changed with dilution to a variable extent in this range. The distribution constants of hydroxy-ethylsulfonic, methylsulfonic and hydrochloric acids changed very little and at about the same rate with dilution. The amount and rate of change of the distribution constants with dilution becomes increasingly greater in going from hydrobromic, to nitric, to perchloric acids. The amount and rate of change appears to be correlated with the size of the distribution constants of these acids in the 0.1 *N* systems, *i.e.*, increasing values for the distribution constant of the acids in 2-butanol–water is correlated with increasing change in these distribution constants on dilution from the 0.1 *N* systems.

Theory

Since both the upper and lower layers in the systems studied were quite rich in water and since all of the acids studied are considered to be highly dissociated, an approach from the theory of strong electrolytes appears justified. Considering for the moment the distribution of hydrochloric acid between the two layers (u for upper and l for lower), the equation for the various equilibria present in and between the two layers can be expressed as

$$u_{(H^+)}u_{(Cl^-)}/l_{(H^+)}l_{(Cl^-)} = u_{(HCl)}/l_{(HCl)} \qquad (3)$$

where parenthesis are used to indicate the activities of the various species. When the ratio, $u_{(H^+)}/l_{(H^+)}$ is replaced by the apparent distribution constant (K_{H^+}) and the ratio of activity coefficients of the hydrogen ion in the two layers ($^u\gamma_{H^+}/^l\gamma_{H^+}$) and when the other ratios are replaced by their thermodynamic distribution constants (K^0), one obtains

$$K_{H^+}K^0_{Cl^-}\,{}^u\gamma_{H^+}/{}^l\gamma_{H^+} = K^0_{HCl} \qquad (4)$$

Now consider the distribution of *trace* amounts of the chloride salt of a cation (ACl_n) between the

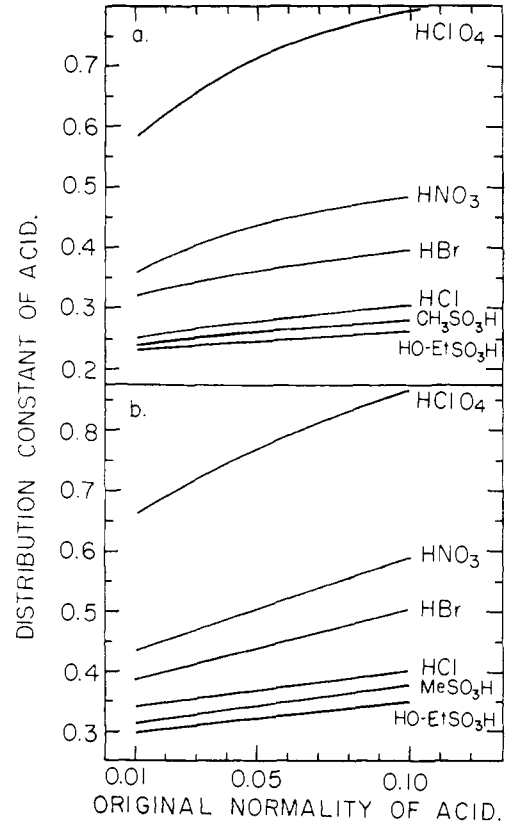

Fig. 4.—Distribution constants of some strong acids as a function of concentration: (a) 2-butanol–water; (b) 3% methanol in 2-butanol–water.

two layers of the hydrochloric acid system and again assume complete dissociation. By a procedure similar to that used above, one obtains an expression for the distribution of the salt in the system. When the terms of equation 4 are raised

$$K_{A^{n+}}(K^0_{Cl^-})^n \, {}^u\gamma_{A^{n+}}/{}^l\gamma_{A^{n+}} = K^0_{ACl_n} \qquad (5)$$

to the nth power and the result divided into equation 5, an expression is obtained for the distribution of the salt in terms of the distribution of the acid. The two terms on the left of equation 6

$$K_{A^{n+}}/(K_{H^+})^n = \frac{K^0_{ACl_n}}{(K^0_{HCl})^n}\left(\frac{{}^u\gamma_{H^+}}{{}^l\gamma_{H^+}}\right)^n \frac{{}^l\gamma_{A^{n+}}}{{}^u\gamma_{A^{n+}}} \qquad (6)$$

are the expressions which were actually measured in the distribution experiments; *i.e.*, $K_{A^{n+}}$ is the measured distribution constant of the compound and K_{H^+} is the measured distribution constant of the acid.

By a procedure similar to that used above, the expression for the distribution of the bromide salts of the compound (ABr_n) in the hydrobromic acid system is obtained. A prime

$$K'_{A^{n+}}/(K'_{H^+})^n = \frac{K^{0\prime}_{ABr_n}}{(K^{0\prime}_{HBr})^n}\left(\frac{{}^u\gamma'_{H^+}}{{}^l\gamma'_{H^+}}\right)^n \frac{{}^l\gamma'_{A^{n+}}}{{}^u\gamma'_{A^{n+}}} \qquad (7)$$

is used to differentiate the terms for the various quantities in the hydrobromic acid system from those in the hydrochloric acid system.

In order for the empirical relationship of equation 2 to be valid, the following identities must hold

$$K_{A^{n+}}/(K_{H^+})^n = K'_{A^{n+}}/(K'_{H^+})^n = k \qquad (8)$$

$$k = \frac{K^0_{ACl_n}}{(K^0_{HCl})^n}\left(\frac{{}^u\gamma_{H^+}}{{}^l\gamma_{A^+}}\right)^n \frac{{}^l\gamma_{A^{n+}}}{{}^u\gamma_{H^{n+}}} = \frac{K^{0\prime}_{ABr_n}}{(K^{0\prime}_{HBr})^n}\left(\frac{{}^u\gamma'_{H^+}}{{}^l\gamma'_{H^+}}\right)^n \frac{{}^l\gamma'_{A^{n+}}}{{}^u\gamma'_{A^{n+}}} \qquad (9)$$

where k is the proportionality constant of equation 2. Similar identities would have to hold for each one of the systems studied. Concentrating for the moment on the two systems noted, the ratios of thermodynamic constants shown in equation 9 would be equal *providing that the nature of the solvent*

$$K^0_{ACl_n}/(K^0_{HCl})^n = K^{0\prime}_{ABr_n}/(K^{0\prime}_{HBr_n})^n \qquad (10)$$

system does not change appreciably in going from one acid system to another.[16] Stated in other words, if the degree of mutual miscibility of the water and butanol is affected to a very minor extent by the presence of the acids, then the standard state condition for each acid in the upper layer (and also for each acid in the lower layer) refers to a solvent of the same composition.

Since the concentration of the distributed compound was never greater than 5×10^{-4} molar, it is safe to assume that the ratio of activity coefficients of the compound ${}^l\gamma_{A^{n+}}/{}^u\gamma_{A^{n+}}$ shown in equation 9 was constant and probably unity in all the solvent systems studied. With regard to the ratio of activity coefficients of the hydrogen ion that appears in equation 9, it should be noted that in going from the hydroxy-ethylsulfonic to the perchloric acid systems the concentration of the acid changes from 0.08 to 0.056 N in the lower layer and from 0.02 to 0.044 N in the upper layer. One would not expect the activity coefficients to change very markedly over this limited concentration range. However, it should be noted that the ratio of hydrogen ion activities appears as a power term. Small variations in this ratio may not be apparent when distributing mono- or bivalent cations but would become increasingly exaggerated as the number of charged groups on the molecule increased.

From the above considerations it is apparent that the empirical relationship of equation 2 can be derived from theoretical considerations providing the following assumptions are made: (a) The solutes and acids behave as strong electrolytes in both layers. (b) The composition of the solvent system does not change appreciably in going from one acid system to another. (c) The compound under investigation is present in trace amounts. (d) The ratio of activity coefficients of the hydrogen ion remains constant in going from one acid system to another.

Discussion

The derivation just presented was based on the theory of strong electrolytes and ignored the possibilities of incomplete dissociation or ion-pairing in either one of the phases. Inclusion of these possibilities gives rise to an equation which will not simplify to the form of equation 2 without making a number of further assumptions. On the basis of conservation of assumptions the approach from the view of strong electrolytes appears justified. The fact that most of the compounds studied followed the form of equation 2 would indicate that the assumptions made in deriving the equation from theoretical considerations must be essentially valid. However, certain of the compounds (primarily the large peptides) failed to follow the form of equation 2 in all solvent systems. This failure could be attributable to a number of things such as heterogeneity of samples, unrecognized incomplete dissociation, partial ion-pairing or molecular associations as well as failure of the assumptions made in deriving equation 2 to hold in all systems. Although none of these possibilities can be unequivocally eliminated, it is perhaps advisable to examine the results with particular reference to the assumptions made in deriving equation 2 from the theory of strong electrolytes.

Small compounds with one charge yielded results in which the found n was in close agreement with the theoretical number of charges per molecule. Divergence between theory and found values for n became apparent with compounds of two or more charges. The degree of divergence appeared to increase with charge and to a certain extent with the size of the molecules. Because of the good agreement with the uni-charged compounds, it appears that the cause of the divergence in the multi-charged compounds must occur as a power term function of the number of charges so that small discrepancies only become apparent upon being raised to some power. It should also be noted that where divergence between theory and found values for n occurred, this divergence could largely be traced to the results obtained in the perchloric acid system (Fig. 3).

(16) This is apparent when the terms of equation 10 are replaced by their identities

$$\frac{K^0_{A^{n+}}(K^0_{Cl^-})^n}{(K^0_{H^+})^n(K^0_{Cl^-})^n} = \frac{K^{0\prime}_{A^{n+}}(K^{0\prime}_{Br^-})^n}{(K^{0\prime}_{H^+})^n(K^{0\prime}_{Br^-})^n}$$

Of the assumptions involved in the derivation, the one involving constant composition of the two layers of the system and the one involving a constant ratio of hydrogen ion activities in the two layers appear most open to criticism. The results of the water analyses of the equilibrated layers, as shown in Table III, show that the composition of the upper and lower layer is practically the same for all of the acid systems except the perchloric acid system where a significant difference is observed. Thus the standard state condition of the compounds in the perchloric acid system would refer to a solvent of slightly different composition than that of the other systems and equality of the terms in equation 10 would be questionable. One part of the term of equation 10, namely (K^0_{HX}) is raised to a power which is a function of the number of charges on the molecule. Consequently small divergencies in this term would be magnified in multi-charged compounds.

The assumption of a constant ratio of activity coefficients for the hydrogen ion between the two layers cannot be expected to hold strictly under the concentration conditions studied. Although this ratio also appears as a power term in equation 9, attributing the divergence noted in the perchloric acid system to a change in this ratio necessitates the assumption of an abrupt change in the ratio in going to the perchloric acid system which is not evident in the other systems.

Regardless of the fact that the relationship of equation 2 does not hold strictly for all compounds in all of the systems studied, it should, nevertheless, prove quite useful as a guide in the selection of solvent systems for countercurrent distribution and partition column chromatographic studies. Given the number of positive charges on a molecule and its distribution constant in one of the acid systems, one can predict with fair reliability what the distribution constant of the compound will be in any one of the other acid systems. On the other hand, the relationship may also prove to be of use in determining the number of positive charges on an unknown compound. This information could be obtained by measurement of the distribution constant in each of a series of acid systems and determining the slope of the line in the log-log plot as is illustrated in Figs. 2–3. Such information combined with a measured value for a neutral equivalence could serve as the basis for a molecular weight determination on an unknown cationic acid.

It should also be noted that a relationship similar to that reported here between the distribution constants of strong acids and their positively charged salt ions should also exist between the distribution constants of strong bases and their negatively charged salt ions. Although no experimental work has been done on this problem in this Laboratory, the theoretical derivation would follow along the same lines as the one proposed here and would involve essentially the same assumptions.

In all cases the conditions which would appear to put the greatest restraints on the general applicability of the relationship are (a) complete dissociation, (b) constant solvent composition on changing the distributing acid and (c) constant ratio of hydrogen ion activities on changing the distributing acid. The number of solvent systems which form two phases both of which are rich enough in water to allow complete dissociation are probably quite limited. In addition to the one described here, one might predict that certain glycol ethers (or mixtures thereof) could possibly form the organic constituent of such systems. Changes in solvent composition with changes in the distributing acids conceivably could be eliminated by proper experimental conditions. By adjustment of the concentration of the acids in the various systems, it should be possible to obtain a series of acid systems in which all of the lower layers (and all of the upper layers) have the same relative composition of water and butanol. If this can be done while still maintaining a large excess of the distributing acid over the distributing salt, one should eliminate the possibility of change in solvent system and make the equality of equation 10 strictly valid. Maintenance of a constant ratio of hydrogen ion activities may be approximated by confining the study to a series of acids whose distribution constants vary over a limited range.

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