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Relation between Paper Chromatographic R_f-Values and Two Phase-Distribution Ratios of Phosphate Ions

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An easy and useful method reflecting the behaviors of phosphate ions in paper chromatography was proposed. Under some assumption, the correlations between R_f in the paper chromatography and the distribution ratio D_s or D_f in the solvent extraction or in the filter paper batch equilibration were investigated. When the water-miscible solvents are used, D_f-values may reflect R_f-values considerably well. If the water-immiscible solvents are used, rather D_s-values reflect R_f-values well. Although the partition only has so far been considered in these types of paper chromatography, the results showed that the adsorption phenomena cannot entirely be ignored in some solvent systems like a basic benzyl alcohol system.

In paper chromatography, parition and adsorption are considered as the two main types of retention of solutes onto paper material through developing. Since the adsorption sites of paper are usually enough covered (hydrated) with water or other hydrophilic solvent molecules supplied from a developing solvent, the direct adsorption on paper fiber have often been neglected in the case where a sample amount treated is not very large. For the partition type the following equation has been derived by Martin and Synge1) from the theory of countercurrent distribution,

$$R_f = 1/\{1 + (\alpha/K)\} \tag{1}$$

where K is a partition coefficient, and α is a filter paper constant represented by the volume ratio of the stationary phase to the mobile phase. K is kept constant in a fixed system as far as the concentration of a solute is low. First, it has been tried to ascertain this equation by using several phosphates (ortho-, di-, tri- and long-chain phosphates) and to find the relation between their paper chromatographic and solvent extraction behaviors. This seems essentially to be a fundamental problem on the paper chromatographic separation. Second, a technique, "a filter paper batch equilibration," has been introduced, which may serve to know quickly the chromatographic behaviors of phosphates. In this study R_f-values, distribution ratios D_s and D_f in the solvent extraction and in the filter paper batch equilibration have been determined and compared under various conditions. To correlate these quantities with each other the developing solvents used in paper chromatography should be those immiscible at

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1) A. J. P. Martin and R. L. M. Synge, *Biochem, J.*, **35**, 1358 (1941).

least partly with an aqueous solution. Otherwise D_s cannot be attained, while D_f can be determined in any medium. A highly immiscible solvent may have difficulties in the development of ionic species in paper chromatography. Accordingly, the selection of a solvent system appropriate to this purpose is considerably limited. After preliminary tests on this point, benzyl alcohol - water and *s*-butyl alcohol - water systems have been taken as models, though other systems such as isopropyl alcohol - water and ethyl alcohol - water systems have also been employed for comparison.

In addition to the constants in Eq. (1), other factors must be taken into account; factors due to the filter paper effect and an agitation effect have been introduced to interpret the data. The type of equation like Eq. (1) seems still convenient for the present study. Since K is the ratio of the concentration of the mobile phase to that of the stationary phase, then Eq. (1) becomes

$$R_f = 1/\{1 + (\alpha C_s/C_m)\} \tag{2}$$

where C_s and C_m denote the concentrations of the stationary and mobile phases. The ratio C_s/C_m cannot directly be determined. On the other hand the distribution ratio in the solvent extraction D_s is measurable and written as follows.

$$D_s = C_o/C_w \tag{3}$$

where C_o and C_w are the concentrations of the organic and the aqueous phases, respectively. Although C_o and C_w correspond to C_m and C_s in forms, K may deviate somewhat from D_s by the filter paper effect. If this deviation is expressed by a factor β, Eq. (2) is rewritten as follows.

$$R_f = 1/\{1 + (\alpha/\beta D_s)\} \tag{4}$$

One might easily come to an idea that the partition coefficient K is equal to D_f in "a filter paper batch equilibration." However, D_f may not actually be equal to K because of the fact that the stationary aqueous layer can partly removed from the paper surface chemically and mechanically by the bulk of an organic layer during equilibration. Therefore, γ as "the agitation effect" has been introduced by the following relation,

$$D_f = K\gamma/V_s = D_s\beta\gamma/V_s \tag{5}$$

where V_s is the volume of the stationary phase per gram of dry filter paper and D_f can be determined experimentally as follows.

$$D_f = \frac{\text{amount of P/supernatant solution (m}l\text{)}}{\text{amount of P/filter paper (g)}} \tag{6}$$

Substituting Eq. (5) into Eq. (4), one gets

$$R_f = 1/\{1 + (\gamma/V_m D_f)\} \tag{7}$$

where V_m is the volume of the mobile phase per gram of dry filter paper.

The volumes of the stationary and mobile phases per gram of dry filter paper, V_s and V_m, have the following relation with the total weight G of liquid adhered to one gram of dry filter paper,

$$G = V_m d_m + V_s d_s \tag{8}$$

where d_m and d_s express the specific gravities of the mobile and stationary phases. G can be determined experimentally.

Experimental

Phosphates. Sodium dihydrogen orthophosphate dihydrate, $NaH_2PO_4 \cdot 2H_2O$ (1P), tetrasodium diphosphate decahydrate, $Na_4P_2O_7 \cdot 10H_2O$ (2P), and pentasodium triphosphate hexahydrate, $Na_5P_3O_{10} \cdot 6H_2O$ (3P), were of special grade. For sodium triphosphate hexahydrate a further purification was done by recrystallization. Glassy sodium metaphosphate, $(NaPO_3)_n$ (*n*P), which is mainly composed of long-chain phosphates, was prepared by heating sodium dihydrogen orthophosphate. The average chain length of it was determined to be 70.[2,3]

Solvents. For this study it is desirable to use a developing solvent containing a single organic constituent. After preliminary tests, ethyl alcohol, isopropyl alcohol, *s*-butyl alcohol and benzyl alcohol were selected. Acetic acid and concentrated ammonia were used for acidic and basic media.

Solvent Extraction. For the *s*-butyl or benzyl alcohol system, distribution ratios in the solvent extraction were determined as follows. Standard sample solutions of ortho-, di-, tri- and long-chain phosphates each containing 30 μg P/m*l* were prepared. Mixing ratios of the solvent, water, the sample solution and acetic acid (or concentrated ammonia water) were 50 : 20 : 10 : 20 (A series), 50 : 15 : 30 : 5 (B series) and 50 : 19 : 30 : 1 (C series), respectively. After shaking to equilibrium, 10 m*l* of the organic phase was taken into a 50 m*l* beaker and evaporated nearly to dryness on a water bath. In the case of benzyl alcohol, back extraction stripping technique was used to determine phosphorus concentrations, since it has a relatively high boiling point of 205°C. After evaporation concentrations of phosphorus were determined by colorimetry using a Mo(V)–Mo(VI) reagent described by Lucena-Conde and Prat.[4] A photoelectric colorimeter "Hirama IIB type" with a cell of 10 mm and a filter of 800 mμ was employed.

Filter Paper Batch Equilibration. Filter paper Toyo No. 51A was cut into pieces of an about 5×5 mm square. Two grams of them was taken into an Erlenmeyer flask. For the *s*-butyl or benzyl alcohol system 30 m*l* of the equilibrated organic layer obtained in the solvent extraction described above was added to the flask. For the ethyl or isopropyl alcohol system, was added 30 m*l* of a mixture of the solvent, water, the sample solution and acetic acid or concentrated ammonia, the ratios of which were the same as those described in the last section. Only in the case of the basic isopropyl alcohol system, the volume ratio of the

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[2] J. R. Van Wazer, E. J. Griffith and F. F. McCullough, *Anal. Chem.*, **26**, 1755 (1954).

[3] E. J. Griffith, *ibid.*, **28**, 525 (1956).

[4] F. Lucena-Conde and L. Prat, *Anal. Chim. Acta.*, **16**, 473 (1957).

alcohol to water was made 60 to 40, because the solvent with the above composition gave the most satisfactory spots in the paper chromatography, while the solvent with the volume ratio of 50 to 50 gave the spots with tailing. The mixture was agitated mechanically for one hour at a room temperature of 20±2°C. Then the content was filtered using a glass filter, and 10 m*l* of the filtrate was analyzed for phosphorus by the colorimetric method described above.

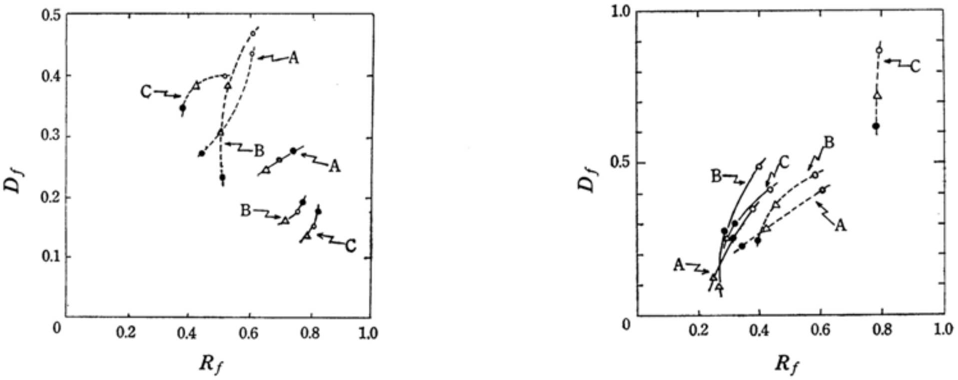
Paper Chromatography. The sample solution containing about 3 mg P/m*l* was prepared for each phosphate. Filter paper of Toyo No. 51A was cut into 5×40 cm for a cylindrical jar or 20×15 cm for a rectangular battery jar. The sample solutions were applied on the starting points and allowed to dry for a few minutes. After the filter paper was preexposed to the saturated vapor of the developing solvent in the jar for about 12 hr, the lower end of the paper was immersed in the developing solvent, which had the same composition of the organic solvent, water and the acid or the base as that described in the last section. The development was carried out at a room temperature of 20±2°C. Phosphate spots were detected by spraying a molybdate reagent described by Hanes and Isherwood.⁵⁾

Measurement of G, d_m and d_s. In order to calculate the values of V_m and V_s, the following procedure was taken for the solvent containing *s*-butyl or benyzl alcohol. In this experiment a chromatographic jar was set in a glove box saturated with the vapor of the chromatographic solvent to be used. After developing for all day, the wet part of the paper was cut at a constant length inside the glove box, put into a weighing bottle immediately, and then weighed. Next it was dried at 110°C for 5 min, put into a desiccater, and weighed again. G was calculated from the difference of the two weights. Since d_m and d_s are considered to be nearly equal to the specific gravities of organic and aqueous phases in contact, respectively, they were measured using a hydrometer.

Results and Discussion

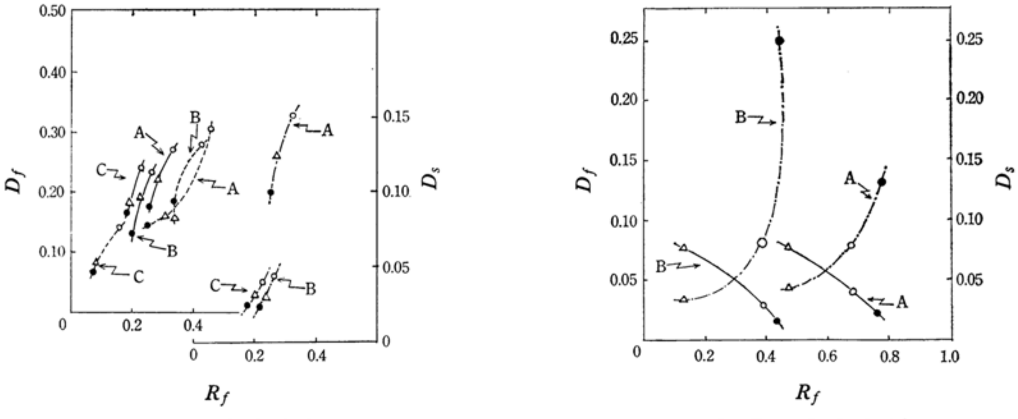
The relations between D_f or D_s and R_f are diagrammed in Fig. 1.

Water-miscible Solvent System. The results for ethyl alcohol and isopropyl alcohol are given in Tables 1 and 2. Here D_s and other



a. Ethyl alcohol

b. Isopropyl alcohol



c. *s*-Butyl alcohol

d. Benzyl alcohol

Fig. 1. Relations between D_f or D_s and R_f. ○, 1P; △, 2P; ●, 3P; ——, D_f, basic solvent; ----, D_f, acidic solvent; —·—·—, D_s, basic solvent.

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5) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

quantities derived from D_s are not written, because D_s is unmeasurable in these systems. In the basic ethyl or isopropyl alcohol system the order of R_f-value was tri->ortho->di- or ortho->tri->di-, respectively, under the conditions studied. This behavior seems unusual, because in the other systems so far studied by many workers,[6,7] the ordinary order of ortho->di->tri- has been

TABLE 1. D_f, R_f AND γ/V_m VALUES FOR THE ETHYL ALCOHOL SYSTEM

Series	Sample	Basic solvent			Acidic solvent		
		D_f	R_f	γ/V_m	D_f	R_f	γ/V_m
A	1P	0.253	0.70	0.11	0.436	0.61	0.28
	2P	0.240	0.66	0.12	0.310	0.51	0.30
	3P	0.270	0.75	0.09	0.270	0.46	0.32
	*n*P	0.213	0	—	0.025	0	—
B	1P	0.172	0.76	0.06	0.469	0.62	0.29
	2P	0.162	0.72	0.06	0.387	0.54	0.33
	3P	0.190	0.78	0.06	0.227	0.52	0.21
	*n*P	0.187	0	—	0.036	0	—
C	1P	0.149	0.81	0.03	0.391	0.54	0.33
	2P	0.141	0.79	0.04	0.388	0.43	0.52
	3P	0.173	0.83	0.03	0.344	0.41	0.50
	*n*P	0.145	0	—	0.028	0	—

TABLE 2. D_f, R_f AND γ/V_m VALUES FOR THE ISOPROPYL ALCOHOL SYSTEM

Series	Sample	Basic solvent			Acidic solvent		
		D_f	R_f	γ/V_m	D_f	R_f	γ/V_m
A	1P	0.359	0.38	0.59	0.406	0.61	0.26
	2P	0.113	0.25	0.34	0.287	0.42	0.40
	3P	0.264	0.31	0.59	0.235	0.35	0.44
	*n*P	0.284	0	—	0.029	0	—
B	1P	0.485	0.41	0.70	0.458	0.59	0.32
	2P	0.108	0.26	0.31	0.368	0.45	0.45
	3P	0.274	0.30	0.64	0.256	0.42	0.35
	*n*P	0.265	0	—	0.205	0	—
C	1P	0.414	0.44	0.53	0.868	0.80	0.22
	2P	0.272	0.30	0.63	0.721	0.79	0.19
	3P	0.307	0.32	0.62	0.620	0.79	0.17
	*n*P	0.312	0	—	0.382	0	—

TABLE 3. PHASE COMPOSITIONS AT EQUILIBRIUM

Solvent		Series	Concentration of ammonia or acetic acid, N		Volume, m*l* (total: 100 m*l*)	
			Organic phase	Aqueous phase	Organic phase	Aqueous phase
s-Butyl alcohol	Acidic	C	0.15	0.19	38.3	61.7
	Basic	A	1.94	3.14	69.5	30.5
		B	0.48	1.01	59.7	40.3
		C	0.09	0.20	58.7	41.3
Benzyl alcohol	Basic	A	2.16	4.27	55.7	44.3
		B	0.33	0.84	54.5	45.5
		C	0.05	0.15	51.8	48.2

TABLE 4. D_s, D_f, R_f, α/β, V_m, V_s, γ/V_m AND γ VALUES FOR THE ACIDIC *s*-BUTYL ALCOHOL SYSTEM

Series	Sample	D_s	D_f	R_f	α/β	V_m	V_s	γ/V_m	γ
A	1P	—	0.302	0.47	—	—	—	0.34	—
	2P	—	0.158	0.32	—	—	—	0.34	—
	3P	—	0.147	0.26	—	—	—	0.42	—
	*n*P	—	0.027	0	—	—	—	—	—
B	1P	—	0.280	0.45	—	—	—	0.34	—
	2P	—	0.158	0.33	—	—	—	0.32	—
	3P	—	0.185	0.34	—	—	—	0.36	—
	*n*P	—	0.015	0	—	—	—	—	—
C	1P	0.217	0.142	0.17	1.1	1.0	1.1	0.69	0.7
	2P	0.186	0.083	0.09	1.9	0.7	1.3	0.84	0.6
	3P	0.175	0.081	0.09	1.8	0.7	1.3	0.82	0.6
	*n*P	0.074	0.026	0	—	—	—	—	—

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6) H. Grunze and E. Thillo, *Sitz. Ber. Deut. Akad. Wiss. Berlin, Kl. Math. u. Allgem. Naturw.*, No. 5, 26 (1953).

7) H. Hettler, *J. Chromatog.*, **1**, 389 (1958).

TABLE 5. D_s, D_f, R_f, α/β, V_m, V_s, γ/V_m AND γ VALUES FOR THE BASIC *s*-BUTYL ALCOHOL SYSTEM

Series	Sample	D_s	D_f	R_f	α/β	V_m	V_s	γ/V_m	γ
A	1P	0.153	0.254	0.34	0.30	1.04	0.31	0.49	0.51
	2P	0.125	0.224	0.29	0.31	1.03	0.32	0.55	0.57
	3P	0.101	0.179	0.27	0.27	1.06	0.29	0.48	0.51
	*n*P	0.024	0.110	0	—	—	—	—	—
B	1P	0.044	0.233	0.27	0.12	1.24	0.15	0.63	0.78
	2P	0.032	0.198	0.24	0.10	1.27	0.13	0.63	0.80
	3P	0.027	0.133	0.22	0.10	1.27	0.13	0.47	0.60
	*n*P	0.016	0.067	0	—	—	—	—	—
C	1P	0.041	0.241	0.24	0.13	1.26	0.16	0.76	0.96
	2P	0.029	0.189	0.21	0.11	1.27	0.14	0.72	0.91
	3P	0.028	0.170	0.20	0.11	1.27	0.14	0.68	0.86

obtained. On the other hand, for all acid systems the common order of ortho->di->tri- was obtained. In acidic media where phosphates may present mainly as neutral acid molecules R_f-values will decrease with the increasing order of molecular size. In basic media, however, the dissociations into anions with various charges will make the chromatographic behaviors more complicated. As far as this problem is concerned, it may be said that the higher the charge of phosphate anions per phosphorus atom, the greater the amount of the anions in the stationary phase that contains more water than the mobile phase does. It may be, therefore, reasonable to consider that the coupling of these two factors, moleccular size and ionic charge, produces the unusual order of R_f-values described above. With a few exceptions the γ/V_m-value is roughly constant in each series irrespective of phosphate species. It may be concluded that D_f-values reflect R_f-values considerably well in this system.

TABLE 6. D_s, D_f, R_f, α/β AND γ/V_m VALUES FOR THE BASIC BENZYL ALCOHOL SYSTEM

Series	Sample	D_s	D_f	R_f	α/β	γ/V_m
A	1P	0.080	0.041	0.68	0.04	0.02
	2P	0.047	0.078	0.47	0.06	0.09
	3P	0.133	0.024	0.78	0.04	0.01
	*n*P	0.047	0.076	0	—	—
B	1P	0.082	0.030	0.39	0.13	0.05
	2P	0.036	0.075	0.13	0.24	0.50
	3P	0.252	0.018	0.44	0.32	0.02
	*n*P	0.012	0.037	0	—	—
C	1P	0.106	0.032	0	—	—
	2P	0.038	0.143	0	—	—
	3P	0.257	0.017	0	—	—
	*n*P	0.054	0.024	0	—	—

Water-Immiscible Solvent System. For the acidic or basic *s*-butyl and basic benzyl alcohol systems the volume and acetic acid or ammonia concentration of each phase at equilibrium after shaking are given in Table 3. A considerable amount of acetic acid, ammonia or water moves from the aqueous phase to the organic phase. These equilibrated organic layers were used as developing solvents in the paper chromatography or as organic solvents in the batch equilibration. In these systems, all the quantities described before are tabulated in Tables 4 and 5 for *s*-butyl alcohol and Table 6 for benzyl alcohol. Blanks in the columns of series A and B of Table 4 mean that the two phases could not be attained here. The acidic benzyl alcohol system was omitted, because the spots of phosphates did not move at all in this condition. In the acidic or basic *s*-butyl alcohol systems, both D_f and D_s reflect R_f-values considerably well. In the basic benzyl alcohol system, however, D_f does not follow R_f, and here rather D_s well predicts the tendency of R_f. The γ/V_m-values are nearly constant for the acidic and basic *s*-butyl alcohol system, while for the basic benzyl alcohol system these are quite different from each other, particularly in diphosphate. Since V_m must be constant within a given series, this variation should come from the change of γ. This phenomenon may be considered as follows; by agitation the stationary phase of the filter paper is torn off and the filter paper interacts directly with phosphate species. This interaction depends on the adsorption type power of each species affected by ionic charge, molecular size, and hydration power.

Interpretation of the Values of α/β, V_m, V_s and γ. The measured values of G, d_m, d_s are given in Table 7. Now we can obtain the values of α/β from Eq. (4) and γ/V_m from Eq. (7). The calculated values are given in Table 1, 2, 4, 5 and 6. In the acidic or basic *s*-butyl alcohol system the values of α/β are nearly constant in any series in spite of the difference of the species. If the values of α/β vary in the same series, the effect of the filter paper must be considered. In the acidic or basic *s*-butyl alcohol system, hence, the filter paper may be considered to be only a supporter of the stationary phase. In this case, β can be put unity,

TABLE 7. G, d_m AND d_s VALUES

Series	*s*-Butyl alcohol G (g)	d_m (g/m*l*)	d_s (g/m*l*)	Benzyl alcohol G (g)	d_m (g/m*l*)	d_s (g/m*l*)
	(acidic solvent)			(acidic solvent)		
C	1.90	0.900	0.968	—	—	—
	(basic solvent)			(basic solvent)		
A	1.21	0.889	0.928	1.29	1.030	0.974
B	1.24	0.881	0.962	1.19	1.041	0.993
C	1.26	0.878	0.972	—	—	—

and the values of V_m, V_s and γ can be calculated. On the other hand, in the basic benzyl alcohol system the values of α/β are fairly different from each other, so the values of V_m, V_s and γ can not be obtained, since β is not presumable. The uniqueness in the basic benzyl alcohol system indicates that the adsorption power cannot wholly be neglected even in the paper partition chromatography. Another notable result is seen in Table 6, *i. e.*, in the basic benzyl alcohol system the R_f-value of diphosphate is quite smaller than those of ortho- and triphosphate. This may have an important meaning to the analytical problem of phosphate mixtures, *i. e.*, small amounts of ortho- and triphosphate can be separated from a large amount of diphosphate without any serious interference using the basic benzyl alcohol system.