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"SALTING-OUT" CHROMATOGRAPHY: EMPIRICAL EQUATIONS RELATING R_F VALUES TO ATOMIC REFRACTION CONSTANTS*

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Investigations by TISELIUS[1] have shown that dyestuffs and some proteins show a very marked increase of adsorption onto filter paper and silica gel in the presence of salts in large concentrations. The underlying principle, called 'salting-out' adsorption[2], has since been applied in a number of instances in chromatographic procedures for proteins[3] and in a few instances in the chromatography of smaller compounds[2,4,5]. In the present study of paper chromatography based on the salting-out principle, compounds of various types were chromatographed using saturated ammonium sulfate as the developing solvent. This report lists the R_F values and presents equations relating them to atomic refraction constants.

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References p. 185.

TABLE I

COMPARISON OF EXPERIMENTAL AND CALCULATED R_F VALUES

Experimental R_F values are the average of 6 to 8 determinations. The average deviation for each compound was 0.02–0.03 R_F units. Calculated R_F values were determined from equations in the text. △ is defined as 100 (Exptl. R_F—Calc. R_F).

Compound	MR_D	Exptl. $RF \times 100$	Calc. $RF \times 100$	△	Compound	MR_D	Exptl. $RF \times 100$	Calc. $RF \times 100$	△
L-Phenylalanyl-L-phenylalanine	86.8	17	15	+2	3-Hydroxy-L-kynurenine	55.7	27	30	−3
Glycylglycine	29.1	87	90	−3	3,5-Diiodo-L-tyrosine	72.4	7	5	+2
Glycyl-DL-alanine	33.8	87	88	−1	α-Amino*iso*butyric acid	25.8	80	85	−5
Glycyl-L-leucine	47.6	69	71	−2	L-Methionine	38.4	74	72	+2
Glycylglycylglycine	41.8	90	91	−1	L-Ethionine	43.0	63	62	+1
DL-Alanylglycine	33.8	88	86	+2	L-Cysteine	28.8	86	88	−2
DL-Alanylglycylglycine	46.4	88	88	0	L-Cystine	56.3	86	85	+1
DL-Leucylglycine	47.6	70	71	−1	L-Homocystine	65.5	69	68	+1
DL-Leucylglycylglycine	60.2	71	69	+2	S-Phenyl-L-cysteine	53.2	37	36	+1
DL-Alanyl-DL-alanine	38.4	87	82	+5	*p*-Aminobenzoic acid	53.2	25	28	−3
Glycyl-L-tryptophan	69.1	25	35	−10	*o*-Aminobenzoic acid	46.1	23	28	−5
Glycyl-L-tyrosine	59.4	56	52	+4	*o*-Acetaminobenzoic acid	46.1	9	13	−4
Glycyl-DL-valine	43.0	79	77	+2	*o*-Aminoacetophenone	39.9	11	9	+2
Glycyl-DL-phenylalanine	57.9	53	54	−1	N-Naphthylethylenediamine	59.1	5	6	−1
Glycyl-DL-asparagine	42.1	91	93	−2	N-α-Acetyl-L-kynurenine	61.2	6	4	+2
DL-Alanyl-DL-asparagine	46.8	90	88	+2	Indolpropionic acid	53.0	5	7	−2
DL-Alanyl-DL-methionine	56.6	66	58	+8	Homogentisic acid	41.2	43	41	+2
DL-Alanyl-DL-norvaline	47.6	68	71	−3	*p*-Hydroxyphenylacetic acid	38.6	39	35	+4
DL-Alanyl-DL-phenylalanine	62.5	44	46	−2	Picric acid	57.7	6	4	+2
L-Leucyl-L-tryptophan	87.6	14	13	+1	Acetanilide	37.5	14	11	+3
DL-Alanyl-DL-valine	47.6	69	71	−2	Benzaldehyde	28.5	22	25	−3
L-Leucyl-L-leucine	66.1	43	40	+3	β-Naphthol	43.2	8	11	−3
L-Leucyl-L-phenylalanine	76.3	30	25	+5	Acetyl-L-tryptophan	63.5	12	11	+1
Glycyl-L-cysteine	41.5	80	79	+1	Tryptamine	48.1	17	17	0
L-Valyl-L-valine	56.9	64	56	+8	*p*-Nitrobenzoyl-DL-serine	60 8	18	21	−3
Histidylhistidine*	82.3	83	78	+5	α-Ketobutyric acid	22.2	63	66	−3
L-Aspartic acid	27.3	89	93	−4	α-Ketovaleric acid	26.8	50	55	−5
L-Glutamic acid	31.9	86	90	−4	α-Keto*iso*caproic acid	31.5	38	41	−3
L-Threonine	26.2	91	90	+1	α-Keto-β-methylvaleric acid	31.5	42	44	−2
L-Serine	21.6	95	94	+1	Pyruvic acid	17.6	72	75	−3
L-Phenylalanine	45.3	53	55	−2	α-Ketoglutaric acid	28.4	68	73	−5
L-Tyrosine	46.8	53	52	+1	Oxalacetic acid	23.8	82	81	+1
L-Lysine dihydrochloride	38.4	92	88	+4	α-Keto*iso*valeric acid	26.8	54	55	−1
L-Histidine*	43.1	87	91	−4	Guanine	48.5	9	7	+2
L-Citrulline	32.8	77	74	+3	Adenine	46.1	7	5	+2
L-Ornithine	33.9	97	92	+5	Uracil	31.1	45	41	+4
L-Arginine*	46.1	85	85	0	Pyridoxal*	44.3	48	49	−1
Glycine	16.5	93	94	−1	Pyridoxamine*	47.8	70	75	−5
L-Alanine	21.1	93	94	−1	*o*-Toluic acid	37.1	31	28	+3
L-Valine	30.4	82	78	+4	L-Asparagine	29.5	90	94	+4
L-Leucine	35.0	74	69	+5	L-Tryptophan	56.5	20	19	+1
L-Isoleucine	35.0	74	69	+5	L-Kynurenine sulfate	54 2	27	30	−3
DL-Norleucine	35.0	69	69	0					
L-Proline	28.4	84	86	−2					
L-Hydroxyproline	29.9	87	86	+1					
2-Hydroxy-DL-tryptophan	58.0	26	26	0					

* 20 was subtracted from the MR_D values for each imidazol or guanidine group in the compound; 15 was subtracted for each pyridine nucleus.

EXPERIMENTAL

26 peptides, 32 α-amino acids and 30 miscellaneous organic compounds were chromatographed at room temperature (24°C ± 2°) by the ascending method on Whatman No. 1 filter paper using as the developing solvent saturated aqueous solutions of ammonium sulfate.

Aliphatic amines were detected on the paper by spraying with 0.1% ninhydrin solution in water-saturated *n*-butanol followed by heating for 5 to 10 minutes at 100°C. Longer heating resulted in a pink coloration of the paper, due probably to reaction of ninhydrin with ammonium sulfate. To detect α-keto acids, the sheets were sprayed with 0.05 % *o*-phenylenediamine dihydrochloride in 10% trichloracetic acid solution and after heating at 100°C for 2 minutes were inspected for fluorescence under ultraviolet light[6]. All of the other compounds tested were detected either by their fluorescence or by their absorption of ultraviolet light.

RESULTS

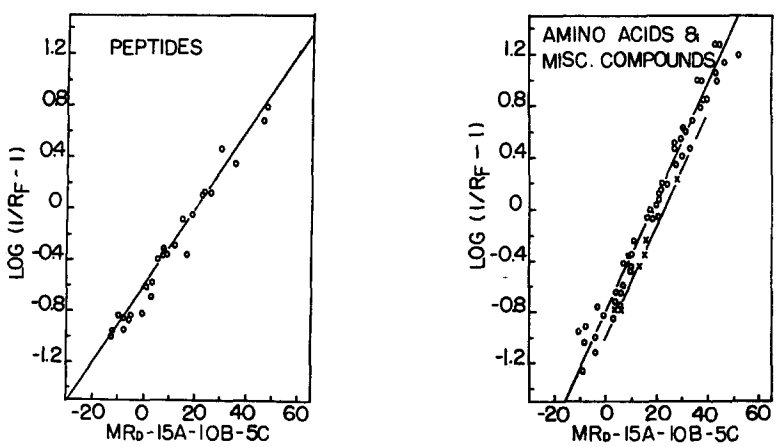
HAGDAHL AND TISELIUS[2] noted in paper chromatograms of 7 amino acids and 6 peptides developed with concentrated phosphate buffer solutions that the R_F values were *inversely* proportional to the molecular weights in contrast to the generally direct relationship obtained using organic solvents. Such an inverse relationship is apparent also in the R_F values obtained in the present study using saturated ammonium sulfate solutions (Table I). A more precise relationship was achieved in the present study, however, by relating the R_F values to molecular refraction (MR_D) values as indicated in Equations 1, 2 and 3. Equation 1 is applicable to the peptides, Equation 2, to the amino acids containing one or two sulfur atoms per molecule, and Equation 3, to the other amino acids and miscellaneous compounds.

$$\log (1/R_F - 1) = -0.61 + 0.030\,(MR_D - 15A - 10B - 5C) \quad (1)$$

$$\log (1/R_F - 1) = -1.02 + 0.045\,(MR_D - 15A - 10B - 5C) \quad (2)$$

$$\log (1/R_F - 1) = -0.80 + 0.045\,(MR_D - 15A - 10B - 5C) \quad (3)$$

The three equations were derived from Figs. 1 and 2, which, in turn, were based on the data in Table I. In each equation, A represents the number of aliphatic amino and



Figs. 1 and 2. Relationship of R_F values to structural characteristics of the solutes. A, B, C and MR_D are defined in the text. Crosses in Fig. 2 represent sulfur-containing amino acids; all other compounds are represented by circles.

References p. 185.

amide (including peptide) groups, B, the number of carboxyl groups and C, the number of hydroxyl groups. MR_D values were obtained by adding atomic refraction constants[7]. Additional constants were assumed for pyridine, guanidine and imidazol groups as indicated in Table I. Since few representatives of each of these types were available for testing, the latter constants must be regarded as tentative.

R_F values calculated by the use of Equations 1, 2 and 3 are presented in Table I for comparison with the experimental values. Good agreement (deviations of 0.05 R_F units or less) was found for 85 of the 88 compounds tested. Therefore, the equations appear to be valid for the types of compounds studied.

DISCUSSION

Use of pre-determined group-constants as illustrated above permits the prediction of R_F values of compounds of known structure. Conversely, experimental R_F values can provide information concerning the structures of unknown substances of the general types of those listed in Table I even without preliminary isolation and purification.

MARTIN *et al.*[8,9] derived a theory of partition chromatography according to which the R_F value of a substance is related to its partition coefficient, a, in the two phases of the solvent system and to the ratio of the volume of the organic phase (A_l) to the volume of the aqueous phase (A_s) as shown in Equation 4.

$$a = A_l/A_s\,(1/R_F - 1) \tag{4}$$

Difficulties in evaluating a as well as A_l/A_s have undoubtedly limited the use of this equation. Such difficulties are avoided to a large extent in Equations 1, 2 and 3, since the R_F values are related in these equations to published atomic constants[7] and readily determined group constants.

The term, log $(1/R_F - 1)$, appearing in Equations 1, 2 and 3 was suggested by consideration of MARTIN's[8,9] treatment of partition chromatography. Why this term should also appear in equations concerning salting-out chromatography or why molecular refraction is a decisive factor is not at present apparent to us.

SUMMARY

Peptides, α-amino acids and miscellaneous organic compounds were chromatographed on filter paper using saturated ammonium sulfate solution as the developing solvent. Equations relating R_F values to molecular refraction constants were derived empirically. Applications of such equations in predicting R_F values and obtaining information concerning molecular structure are discussed briefly.

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