A PAPER CHROMATOGRAPHIC INVESTIGATION OF THE HOMOLOGOUS SERIES OF AMINO ACIDS FROM GLYCINE TO α-AMINO-*n*-TETRADECANOIC ACID

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The concept of paper chromatography as a liquid–liquid distribution process is based on the theories of MARTIN AND SYNGE[1], CONSDEN and co-workers[2], MARTIN[3] and BATE-SMITH AND WESTALL[4]. Application of these theories to experimental results can give a measure of the importance of the competing processes of adsorption and ion exchange in the operation of the chromatogram.

In checking the purity of a homologous series of amino acids to be used for other investigative purposes, an opportunity was afforded to test the applicability of the theories of MARTIN and BATE-SMITH AND WESTALL to the paper chromatographic behaviour of the homologous series of aliphatic amino acids from glycine to α-amino-*n*-tetradecanoic acid.

According to CONSDEN and co-workers[2], the following relation holds for the distribution coefficient, α, and the R_F value (ratio of the distance the chromatographic spot has moved to the distance the solvent front has moved):

$$\alpha = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right)$$

where A_L/A_S = ratio of the volumes of the moving and stationary phases in the chromatogram.

Distribution coefficients calculated in this manner showed good agreement with those experimentally determined for a number of amino acids. MARTIN[3] considered the chemical potential, μ, for a solute A distributed between two immiscible phases in equilibrium with each other.

Using the relation of α to the chemical potential, μ, he concluded that addition of a group X (*e.g.*, —CH_2—) to the molecule A should change α by a given factor which depends on the nature of the group and the pair of phases employed but *not* on the rest of the molecule.

Extending these ideas, BATE-SMITH AND WESTALL[4] have shown that log $(1/R_F - 1)$, designated R_M, should change in a regular way for a series of compounds differing only in the number of substituents of a given kind.

$$RT \ln K\left(\frac{1}{R_F} - 1\right) = \Delta\mu_1 + d\Delta\mu_x + e\Delta\mu_y \ldots$$

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where $\Delta\mu$ = chemical potential for a given group or substituent in the molecule and d and e = the number of groups or substituents in the molecule. They tested this relation with a group of phenolic substances differing in the number of hydroxyl groups. The rule was obeyed, giving a straight line-plot in a large number of series. Exceptions were noted, particularly in the case of vicinal groups.

BREMNER AND KENTEN[5] undertook a similar study with aliphatic amines. FRENCH AND WILD[6], also JEANES and co-workers[7], have studied the relation between structure and chromatographic behavior of oligosaccharides. POLSON[8] studied the two-dimensional chromatographic behavior of a series of amino acids. The R_M relation was determined by SERCHI[9] for a homologous series of amino acids in *n*-butyl alcohol–acetic acid–water. PARDEE[10] related the R_F values of peptides to their constituent amino acids.

An R_M study of the homologous series of aliphatic amino acids from glycine to α-amino-*n*-tetradecanoic acid in a number of solvent developers is reported here.

PROCEDURE

General

Whatman No. 1 paper was treated with a 0.05 % solution of potassium ferrocyanide (0.05 g salt in 70 ml methanol and 40 ml water) and air dried. The salt treatment prevents bearding of spots. Aqueous methanol reduced crinkling and warping of the paper (as contrasted to a water solution) when the paper dried. The paper was cut into tapered strips, 28 cm long, 2.3 cm wide at the top, and 1.5 cm wide at the base. Amino acid solution was spotted 1.5 cm from the narrower end. Some runs were made on strips of 13 cm length[11]. Approximately 0.2 μl of solution containing

TABLE I

SOLVENT DEVELOPERS*

Solvent no.	*Solvent composition, v/v*	
1	Phenol–H_2O	(9:1, w/w)
2	2,4,6-Collidine–2,4-lutidine–H_2O	(1:1:2)
3	*n*-Butyl alcohol–acetic acid–H_2O	(4:1:5)
4	*n*-Butyl acetate–*n*-butyl alcohol–acetic acid–H_2O	(47:9:28:16)[12]
5	*n*-Butyl alcohol–dioxane–H_2O	(4:1:saturated)
6	*tert.*-Amyl alcohol–dioxane–H_2O	(4:1:saturated)
7	*tert.*-Amyl alcohol–dioxane–H_2O	(3:1:1)
8	*tert.*-Amyl alcohol–dioxane–H_2O	(5:3:2)
9	*tert.*-Amyl alcohol–dioxane–H_2O	(5:1:2)
10	*tert.*-Amyl alcohol–dioxane–H_2O	(9:3:4)
11	*tert.*-Amyl alcohol–dioxane–H_2O	(2:1:1)
12	*tert.*-Amyl alcohol–pyridine–H_2O	(2:1:1)
13–16*	*tert.*-Amyl alcohol–dioxane–H_2O	(8:7:5)
17	*tert.*-Amyl alcohol–dioxane–H_2O	(9:3:5)
18	*tert.*-Amyl alcohol–dioxane–H_2O	(5:2:3)
19	*tert.*-Amyl alcohol–dioxane–H_2O	(4:3:3)
20	*tert.*-Amyl alcohol–dioxane–H_2O	(11:3:6)
21	*tert.*-Amyl alcohol–dioxane–H_2O	(1:1:1)
22	*tert.*-Amyl alcohol–dioxane–H_2O	(8:5:7)

* Solvent developers are numbered to correspond to strip numbers in Fig. 1.

2 to 4 μg of each amino acid was spotted on the paper. The strips were not conditioned before ascending development in test tubes 30 cm long, at room temperature. When the developer reached the top of the strip, the strip was removed and air dried.

Amino acid solutions

The α-amino acid series from glycine through α-amino-*n*-nonanoic acid was dissolved in glacial acetic acid. Corresponding derivatives of *n*-decanoic through *n*-tetradecanoic acid were dissolved in formic acid (98 %).

Solvent developers

See Table I, which is keyed to Fig. 1.

Color revelation

Air dried strips were sprayed with 0.25 % ninhydrin in acetone. The color was allowed to develop at room temperature. Duplicate runs were made for all R_F values recorded in this study. R_F value deviations were within $\pm$ 0.02.

DISCUSSION

Fig. 1 shows typical chromatographic patterns, but not necessarily typical R_F values, for the solvent developers used. Table I numbers the solvent developers tried and is keyed to the corresponding strip numbers shown in Fig. 1. Developers will be referred to by the numbers shown in Table I. The following discussion will be concerned, first, with separations of the amino acids, *i.e.*, R_F values, and secondly, with the R_M study.

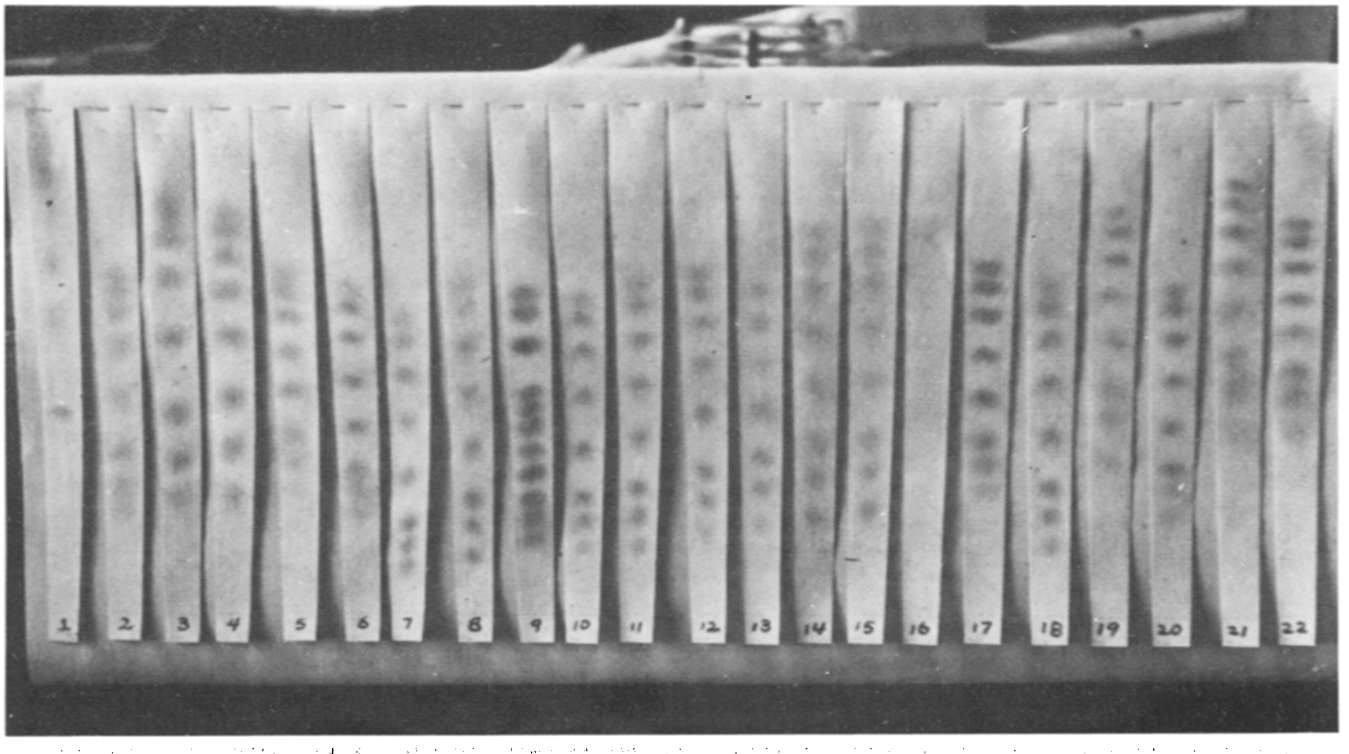

Fig. 1. Paper chromatograms of amino acids. Chromatogram numbers are keyed to solvent developers in Table I.

Attempts to separate the α-amino derivatives of the series *n*-decanoic through *n*-tetradecanoic acid were unsuccessful. The series glycine–α-amino-*n*-nonanoic acid was separated with the following solvent developers: Nos. 2, 4, 5, 6, 7, 8, 9, 10, 11, 14, 17, 18, 19, 20, 21, 22 in Table I. In solvent 3, the octanoic and nonanoic derivatives overlap. Although the series decanoic through tetradecanoic defied resolution, discrete spots could be obtained for these compounds in a number of developers when the acids were chromatographed *individually*. See Table II for individual R_F values in solvent No. 17.

TABLE II

R_F VALUES OF STRAIGHT CHAIN AMINO ACIDS RUN INDIVIDUALLY

Solvent developer: *tert.*-amyl alcohol–dioxane–water (9:3:5, v/v).

Amino acid	R_F *value*
Hexanoic	0.53
Heptanoic	0.58
Octanoic	0.64
Nonanoic	0.67
Decanoic	0.68
Hendecanoic	0.71
Dodecanoic	0.71
Tridecanoic	0.72
Tetradecanoic	0.72

The hendecanoic through tetradecanoic series gave identical R_F values within experimental error and hence were not separable when run as a mixture.

Strips 13–16 were run in the same developer. Strip 13 was not treated with ferrocyanide. Separation is poorer than on strip 14 which was treated. Strip 16 contrasts a decanoic spot run alone with the glycine–decanoic series on strip 15. The R_F value of the decanoic spot is not altered in the presence of the other amino acids.

The *tert.*-amyl alcohol–dioxane–water mixtures were studied in some detail. These mixtures, in differing proportions, were eminently successful, not only in separating the amino acids but also in giving well defined spots. In some of these mixtures, the spots having higher R_F values were elliptical or "football" shaped, the minor axis of the ellipse being parallel to the direction of solvent flow. (See strips Nos. 9, 21, and especially No. 22.) This is contrary to the usual behavior of fast running spots, which tend to streak in the direction of solvent flow, and highly desirable in separating spots having closely similar R_F values.

The 5:1:2 mixture, solvent No. 9 in Table I separated not only the straight chain series from glycine through α-amino-*n*-nonanoic but also valine, leucine and isoleucine. See strip No. 9 in Fig. 1 which shows clear separation of eleven amino acid spots. An unambiguous separation of leucine and its two isomers is normally quite difficult. In accord with the expectation that the greater the degree of branching in the chain, the greater the water solubility and therefore the lower the R_F values, the sequence observed is isoleucine, leucine, norleucine in order of increasing R_F values. This expectation is based on distribution of the amino acids, according to solubility, between a stationary water phase and a mobile organic solvent phase.

TABLE III

R_F, R_M, AND ΔR_M VALUES

Amino acid	*Collidine–lutidine–water (1:1:2)*			*n-Butanol–acetic acid–water (4:1:5)*			*n-Butyl acetate–n-butanol–acetic acid–water (47:9:28:16)*		
	R_F	R_M	ΔR_M	R_F	R_M	ΔR_M	R_F	R_M	ΔR_M
Glycine	0.20	0.60		0.16	0.72		0.20	0.60	
Alanine	0.24	0.50	0.10	0.22	0.55	0.17	0.30	0.37	0.23
n-Butyric	0.30	0.37	0.13	0.31	0.35	0.20	0.39	0.19	0.18
Norvaline	0.41	0.16	0.21	0.46	0.07	0.28	0.52	—0.04	0.23
Norleucine	0.52	—0.04	0.20	0.58	—0.14	0.21	0.61	—0.19	0.15
Heptanoic	0.60	—0.17	0.13	0.66	—0.28	0.14	0.68	—0.33	0.14
Octanoic	—	—	—	—	—	—	0.72	—0.41	0.08
Nonanoic	—	—	—	—	—	—	0.74	—0.46	0.05

TABLE IV

R_F, R_M, AND ΔR_M VALUES

Amino acid	*Solvent developer proportions v/v, tert.-amyl alcohol–dioxane–water*					
	*5:1:2**			*8:7:5*		
	R_F	R_M	ΔR_M	R_F	R_M	ΔR_M
Glycine	0.15	0.75	—	0.21	0.58	—
Alanine	0.19	0.63	0.12	0.29	0.39	0.19
n-Butyric	0.22	0.55	0.08	0.35	0.27	0.12
Norvaline	0.31	0.35	0.20	0.46	0.07	0.20
Norleucine	0.41	0.16	0.19	0.56	—0.10	0.17
Heptanoic	0.49	0.02	0.14	0.64	—0.25	0.15
Octanoic	0.55	—0.09	0.11	0.70	—0.37	0.12
Nonanoic	0.58	—0.14	0.05	0.73	—0.43	0.06

* R_F value of valine = 0.27, isoleucine = 0.35, leucine = 0.38.

TABLE V

COMPARISON OF PYRIDINE WITH DIOXANE IN SOLVENT DEVELOPER

Amino acid	*tert.-Amyl alcohol–dioxane–water (2:1:1)*			*tert.-Amyl alcohol–pyridine–water (2:1:1)*		
	R_F	R_M	ΔR_M	R_F	R_M	ΔR_M
Glycine	0.16	0.72	—	0.17	0.69	—
Alanine	0.22	0.55	0.17	0.23	0.53	0.16
n-Butyric	0.26	0.45	0.10	0.29	0.39	0.14
Norvaline	0.36	0.25	0.20	0.40	0.18	0.21
Norleucine	0.46	0.07	0.18	0.50	0.00	0.18
Heptanoic	0.54	—0.07	0.14	0.58	—0.14	0.14
Octanoic	0.59	—0.16	0.09	0.64	—0.25	0.11
Nonanoic	0.63	—0.23	0.07	0.68	—0.33	0.08

Some tentative generalizations may be made regarding solvent developer composition R_F values. As the mole fraction of the alcohol in the *tert.*-amyl alcohol mixtures decreased, R_F values generally increased. Also, the higher the mole fraction of water the higher the R_F values. Comparing developers 9, 10, 11 and 14, it appears that as the mole fraction of dioxane increases and that of the alcohol decreases, that of water being nearly constant, the R_F values rise. The differences in some instances are quite small, however. Developers 17–20 have the same percentage of water and about the same mole fraction of water. Here, too, a marked increase in dioxane mole fraction raises R_F values for developer 19 compared to 17, 18 and 20. See Fig. 1 or Fig. 5.

Strips 11 and 12 and Table V contrast dioxane with pyridine in the *tert.*-amyl alcohol–water developer. Pyridine gives slightly higher R_F values but spot appearances are comparable. Both pyridine and dioxane increase the miscibility of the alcohol and water.

The results in the R_M study are shown in Tables III–V and Figs. 2–6. A straight line relation between R_M and the number of methylene groups would be strong support for the liquid–liquid distribution theory and would bear out therefore the theories of MARTIN and BATE-SMITH AND WESTALL. As can be seen, a very nearly linear relation was observed for the series glycine–α-amino-*n*-nonanoic acid in a variety of *tert.*-amyl alcohol mixtures as well as in collidine–lutidine and the *n*-butyl alcohol containing mixtures. The first member of the series, glycine, is arbitrarily graphed as having no methylene groups. Fig. 6 shows that R_M (and therefore R_F) is independent of whether the amino acids were run together or chromatographed individually. Compared to the shorter chain compounds somewhat larger deviations from linearity are to be noted for the derivatives of *n*-octanoic and *n*-nonanoic acids.

While ΔR_M would be expected to be a constant for a given group, —CH_2— in

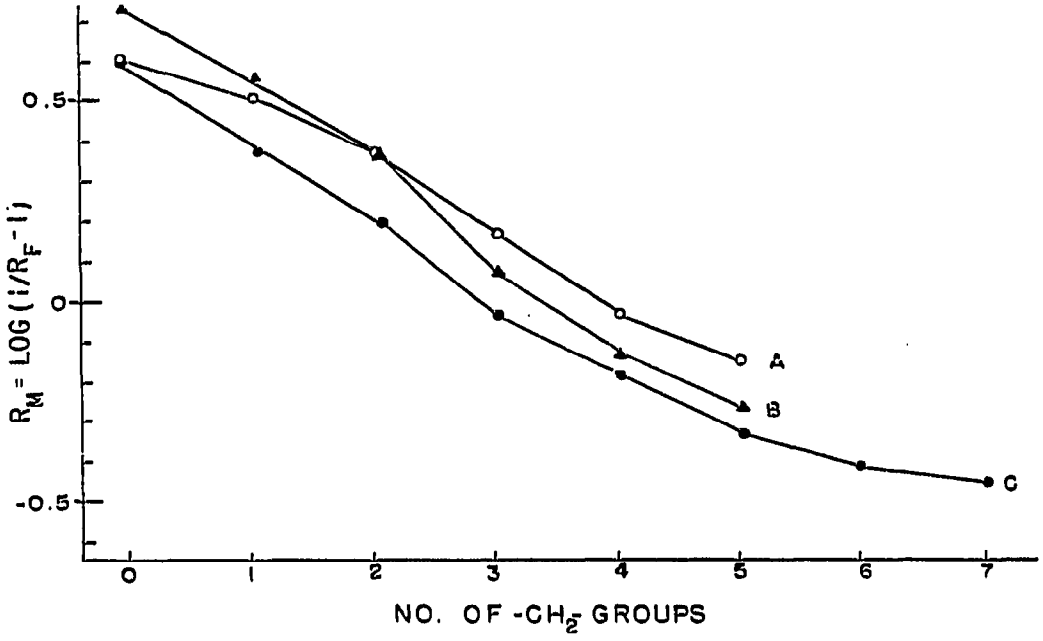

Fig. 2. R_M values for glycine–α-amino-*n*-nonanoic acid. A = Collidine–lutidine–water (1:1:2, v/v); B = *n*-butyl alcohol–acetic acid–water (4:1:5, v/v); C = *n*-butyl acetate–acetic acid–*n*-butyl alcohol–water (47:28:9:16, v/v).

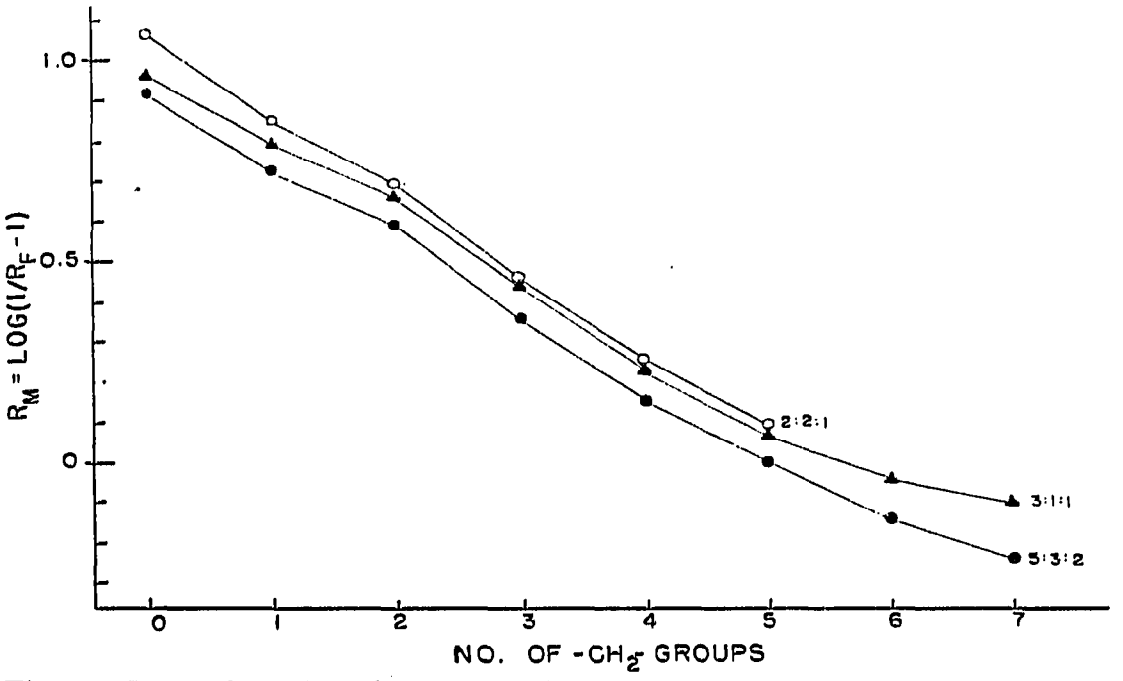

Fig. 3. R_M values for glycine–α-amino-*n*-nonanoic acid in *tert.*-amyl alcohol–dioxane–water mixtures (v/v).

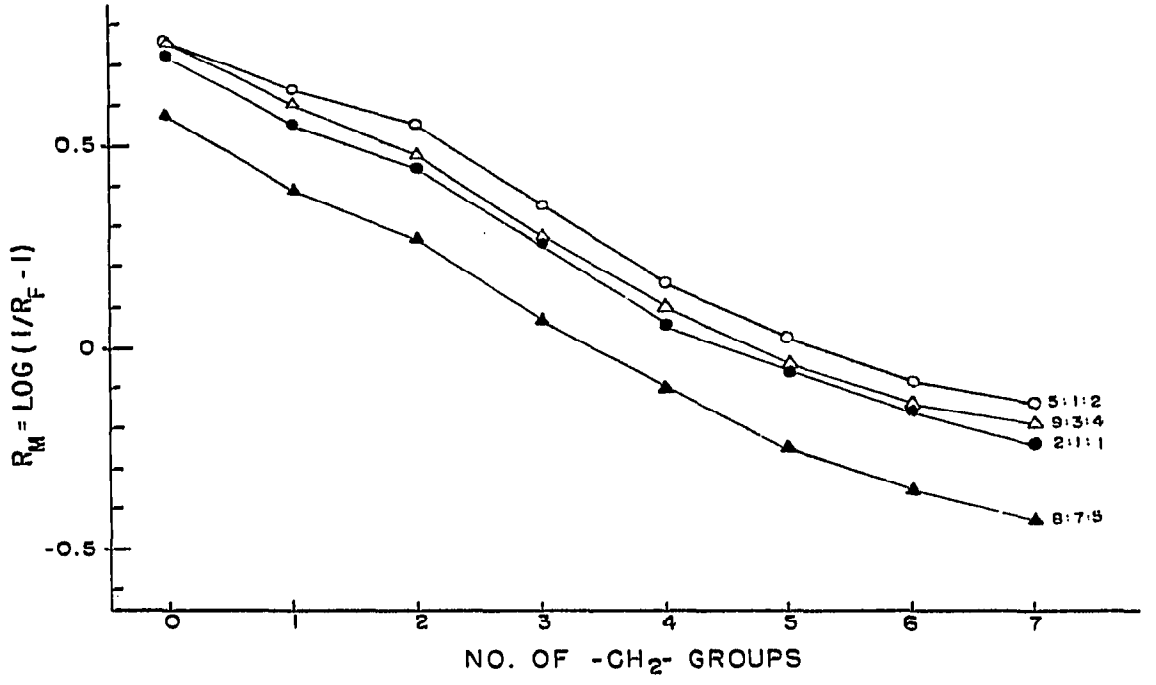

Fig. 4. R_M values for glycine–α-amino-*n*-nonanoic acid in *tert.*-amyl alcohol–dioxane–water mixtures (v/v).

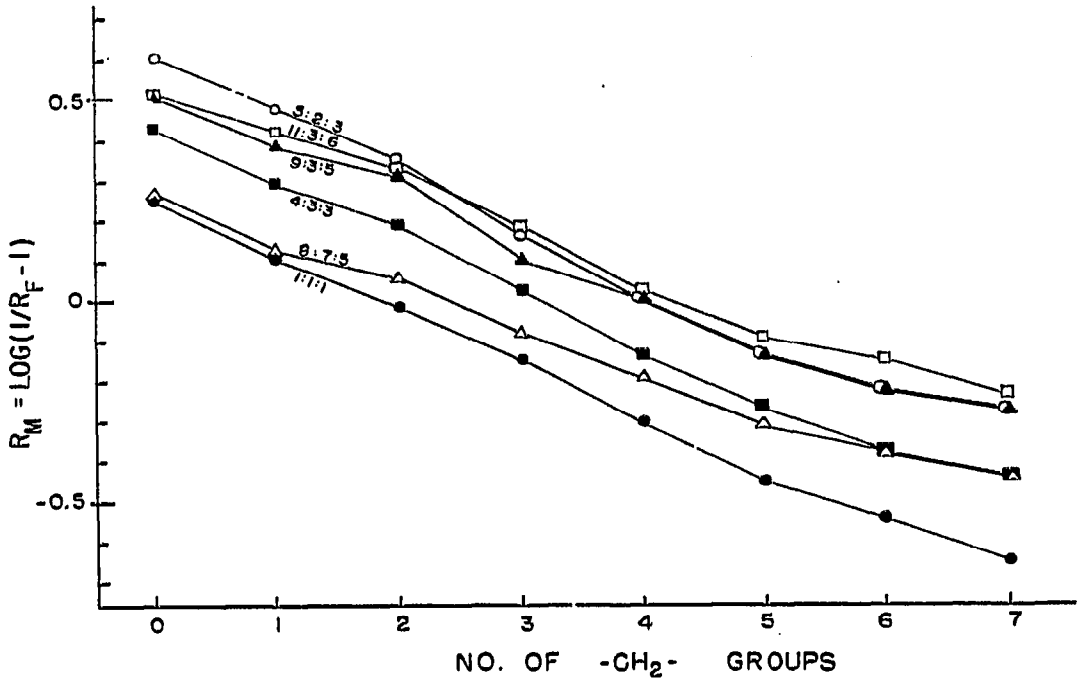

Fig. 5. R_M values for glycine–α-amino-*n*-nonanoic acid in *tert.*-amyl alcohol–dioxane–water mixtures (v/v).

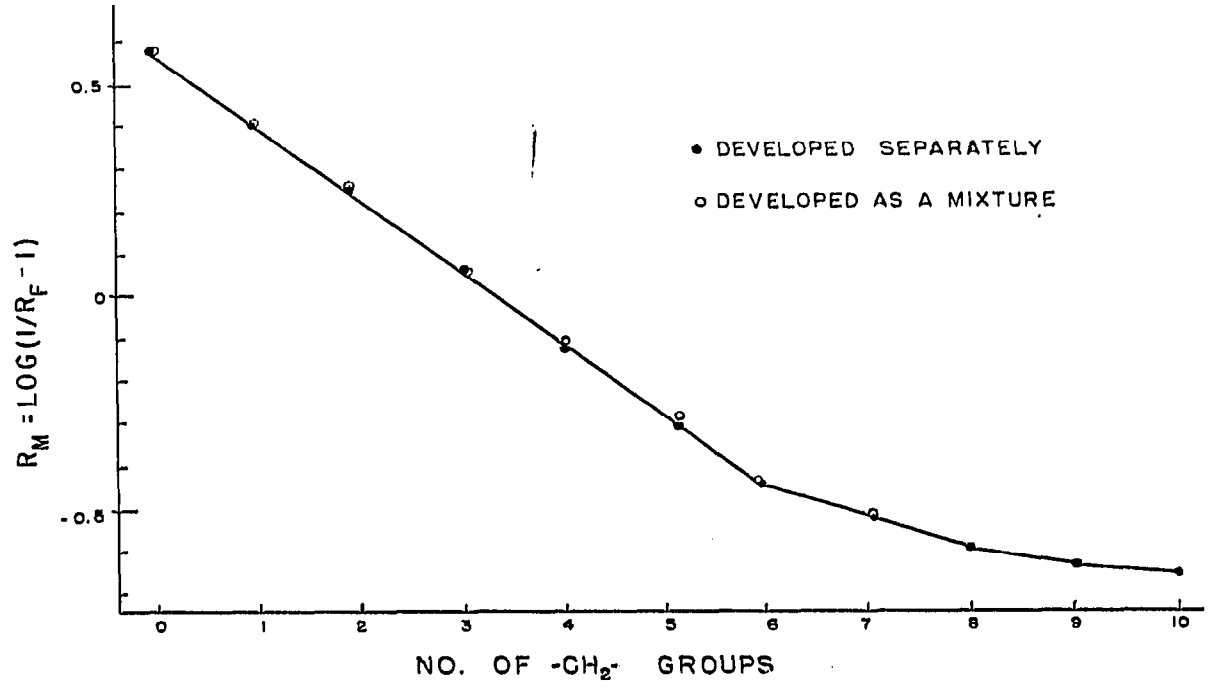

Fig. 6. R_M values for glycine–α-amino-*n*-dodecanoic acid in *tert.*-amyl alcohol–dioxane–water (8:7:5, v/v).

this case, examination of Tables III, IV and V, shows variations in ΔR_M values. However, small variations in R_F values cause large variations in ΔR_M values. Application of experimental errors of $\pm$ 0.02 to R_F values will show that the ΔR_M values are constant within experimental error in most cases. Deviations beyond experimental error do occur for the highest members of the homologous series as the graphs indicate.

The deviations from linearity of the R_M plot for the higher acids and inability to separate the amino acid series from decanoic to tetradecanoic may be due to adsorption effects competing with the liquid–liquid distribution process. TRAUBE[13] noted that with the homologous series of fatty acids there was a regular increase in adsorption for each methylene group added to the chain. LANGMUIR[14] confirmed this observation noting that adsorption increased in the order in which solubility of the acids decreased. MCKEEKIN, considering amino acid solubilities, proposed the rule that the "ratio of solubility in alcohol to that in water is increased threefold for each —CH_2— group in the hydrocarbon chain terminating in methyl groups"[15]. However, the solubilities of the acids in question are so slight in water that a threefold increase would be insignificant. The fact that the R_F values seem to converge for the longer chain compounds so that the C_{10}–C_{14} compounds all have the same R_F values suggests adsorption of the long chain compounds to the cellulose of the chromatogram. It was observed that only small amounts of the long chain amino acids could be applied to the paper without resultant streaking of the spot. Streaking suggests adsorption on the limited surface (and therefore low adsorptive capacity) of the paper.

Fig. 6 sheds light on possible adsorption phenomena. One indication of adsorption would be marked changes in R_F values depending on whether the amino acids were chromatographed individually or as a mixture. No appreciable differences were noted in this case.

ISHERWOOD AND JERMYN[16] noted a linear relation between R_M and -log mole fraction of water in the solvent developer for sugars in a variety of solvent developers. A similar relation was observed for the amino acids in this study. However, the solvent developers used were not as varied as those tested by ISHERWOOD AND JERMYN.

SUMMARY

The R_F values and R_M values for the homologous series of amino acids glycine–α-amino-*n*-tetradecanoic acid were determined in a number of solvent developers. The results are in accord with a liquid–liquid distribution process except for the longer chain amino acids having 10–14 carbon atoms. For these amino acids there is evidence of adsorption. An excellent solvent developer, *tert.*-amyl alcohol–dioxane–water (5:1:2, v/v), cleanly separates not only the homologous series glycine–α-amino-*n*-nonanoic acid but also valine, leucine and isoleucine.

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