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CHROMATOGRAPHIC BEHAVIOUR AND CHEMICAL STRUCTURE

I. THIN-LAYER CHROMATOGRAPHY OF ALIPHATIC ACIDS

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SUMMARY

About sixty aliphatic acids, predominantly fatty acids, hydroxy acids, amino acids and halogen acids, were separated on cellulose layers with the solvent mixture *n*-butanol–diethylamine–water (85:1:14). The R_M values so obtained illustrate in most cases the validity of MARTIN's theoretical postulates for the relationship between chemical structure and R_F value in partition chromatography. Thus when R_M is plotted against the number of substituent groups of any one kind a close approximation to linearity results, except in the case of acids with vicinal hydrophilic groups. The calculation of group constants and binding increments was done by direct comparison of the R_M values of substances differing by only one group or increment. These data facilitate the chromatographic elucidation of structural problems; only very small amounts of the substances which do not even need to be isolated in pure form are required.

INTRODUCTION

In the course of our investigations of radiation induced carboxylation of organic acids with ^{14}C labelled CO_2 an analytical procedure was required to separate the organic acids formed by this process. The method was also required to indicate how many COOH-groups were introduced into the model substrate and in what position relative to other functional groups, they were located. A chromatographic procedure appeared to be especially suited, since there were already a number of papers on the relationship between molecular structure and chromatographic behaviour[1–18].

Based on predictions of CONSDEN *et al.*[19] and MARTIN[20], a linear relationship between the R_M value and the number of identical groups has been proved experimentally by many authors[1–18,21–29]:

$$R_M = G_0 + nG_X + mG_Y + \cdots\cdots\cdots \quad (1)$$

$G_X, G_Y, \cdots\cdots$ = group constant of X, Y, $\cdots\cdots$

G_0 = basic constant.

Some of these group constants are known in the case of paper chromatography (PC)[1,4–6,23], but for TLC which is better suited for the separation of organic acids[28,30,31].

only a few constants have been published[10,13]. Furthermore, in TLC the contribution of one group to the R_M value of the molecule is dependent on the other groups in the molecule, as was shown by PATAKI[13] who compared the R_M values of amino acids differing by specific groups.

It is the aim of this and a following paper[32] to establish the group constants from the R_F values of more than a hundred organic acids. In addition, the effect of criteria so far hardly considered, for instance binding increments, such as double bonds, chain branching, ring closure and the relative positions of functional groups are taken into account. These data can facilitate the elucidation of structural problems.

EXPERIMENTAL

The separation of the acids was carried out on cellulose layers for two reasons, First, the best results for the chromatography of acids were obtained on cellulose layers[33-35]. Secondly, cellulose layers correspond well with PC in their chromatographic behaviour, thus facilitating the transferability of PC experience to TLC[36]. Cellulose layers, however, have the advantages of giving better separations, and having shorter development times and greater sensitivity than PC.

Preparation of the plates

Fifteen grams cellulose powder HR 300 (Macherey, Nagel) were stirred vigorously with 75 ml water. Adsorbent layers of 0.25 mm thickness were prepared on 20 × 20 cm glass plates with Desaga equipment, (Heidelberg). The plates were air-dried horizontally, at room temperature overnight. Almost all the acids were spotted as their diethylamine salts, dissolved in methanol, along a line 2.5 cm from the lower end of the plate. Approximately 10 μg of test substance (1 μl of 1% solution) were used for each spot. The plates were then equilibrated for 4 h at constant temperature (23° ± 1°) and developed by the ascending technique in a tank lined with solvent-soaked filter paper.

Solvent system

System: *n*-butanol–diethylamine–water (85:1:14). The duration of development is 85 min. After that time the front is at a distance of 10 cm from the starting line. After development the solvent is allowed to evaporate at 60°, which is below the decomposition temperature of the diethylamine salts.

The use of diethylamine as the volatile alkali instead of the otherwise more common ammonia was first proposed by JONES *et al.*[37] for the PC of some volatile organic acids. It prevents the partial separation of the eluting system possible under certain conditions, since diethylamine dissolves well in both phases. Furthermore it facilitates the identification of the acids as diethylamine salts after spraying with ninhydrin reagent.

Detection reagent

Various reagents were tried, *e.g.*, pH-indicators such as bromocresol purple or bromocresol green, sodium nitroprusside and ninhydrin reagent. The best results, however, for minute amounts of even very weak acids were obtained with ninhydrin. After spraying with ninhydrin the plates were warmed for 30 min at 60°. Ninhydrin

reveals the acids as blue violet spots on a white or pale rose background. The diethylamine salts appear as more durable and more clearly defined spots than the ammonium salts. The detection limit is about 0.5 μg acid.

To correlate the chromatographic behaviour of substances with their chemical structure an accurate knowledge of the R_F values is necessary. Recently, new techniques for the exact determination of R_F values have therefore been proposed, such as tankless or flatbed chromatography in the case of PC[11]. We worked with conventional equipment, but strictly observed the following points to achieve reproducible and accurate results: constant temperature, time of saturation and size of spots. A control substance, in most cases glycolic acid, was always run alongside and if its R_F value differed by more than 0.02 from the standard value, the run was discarded.

RESULTS AND DISCUSSION

Monocarboxylic acids

The separation of organic acids by chromatography has been reported in a number of publications. Some of them make use of PC, *e.g.*, the work by LONG *et al.*[22], HOWE[27] and HARTLEY[38], other authors however prefer TLC. The separation of straight chain carboxylic acids by TLC was carried out by HROMATKA AND AUE[28] as well as by LYNES[39] and BRAUN AND VORENDOHRE[40], who obtained good results. Branched carboxylic acids however have been investigated less.

TABLE I

UNSUBSTITUTED MONOCARBOXYLIC ACIDS

No.	Acid	R_F exp.	R_M measured	R_M calc.	R_F calc.	ΔR_M	ΔR_F
1	Formic	0.41	0.16	0.30	0.33	0.14	0.08
2	Acetic	0.45	0.09	0.08	0.45	0.01	0.00
3	Propionic	0.58	−0.14	−0.14	0.58	0.00	0.00
4	Butyric	0.69	−0.35	−0.36	0.70	0.01	0.01
5	Valeric	0.79	−0.58	−0.58	0.79	0.00	0.00
6	Caproic	0.86	−0.79	−0.80	0.86	0.01	0.00
7	Enanthic	0.92	−1.06	−1.02	0.91	0.04	0.01
8	Caprylic	0.95	−1.28	−1.24	0.95	0.04	0.00
9	2-Methylpropionic	0.71	−0.39	−0.39	0.71	0.00	0.00
10	2-Methylbutyric	0.80	−0.60	−0.61	0.80	0.01	0.00
11	2,2-Dimethylpropionic	0.81	−0.63	−0.64	0.81	0.01	0.00
12	2,2-Dimethylbutyric	0.88	−0.87	−0.86	0.88	0.01	0.00
13	2-Ethylbutyric	0.87	−0.83	−0.83	0.87	0.00	0.00
14	2-Methyl-2-ethylbutyric	0.92	−1.06	−1.08	0.92	0.02	0.00
15	2,2-Dimethylvaleric	0.92	−1.06	−1.08	0.92	0.02	0.00
16	2-Ethylpropionic	0.95	−1.28	−1.27	0.95	0.01	0.00
17	3,3-Dimethylbutyric	0.78	−0.55	−0.52	0.77	0.03	0.01
18	4-Methylvaleric	0.82	−0.66	−0.66	0.82	0.00	0.00
19	2,2,4,4-Tetramethylcaproic	0.97	−1.51	−1.46	0.96	0.05	0.01
20	Acrylic	0.53	−0.05	−0.05	0.53	0.00	0.00
21	Crotonic	0.64	−0.25	−0.27	0.65	0.02	0.01

The experimental R_F and R_M values of straight chain and branched monocarboxylic acids, as well as the R_M and R_F values calculated by means of the established group constants, are given in Table I (Nos. 1–19). The differences, *i.e.* ΔR_M and ΔR_F, are presented in the last two columns. It can be seen that only formic acid shows a discrepancy between the experimental and calculated R_M value. This can be explained by the fact, that the calculated R_M value of formic acid is obtained by the sum of the basic constant and the R_M constant of the carboxylic group. Thus the hydrogen atom of formic acid is not accounted for.

The method described does not give a satisfactory separation of formic and acetic acid. This is in agreement with BAYZER[41] who found that for the complete separation of the alkali derivatives of C_1–C_5 carboxylic acids a combination of TLC and electrophoresis is necessary. The separation of the hydroxamates however can be achieved by TLC alone.

When the R_M values of the fatty acids are plotted against their carbon number a close approximation to linearity is obtained. The difference of the R_M values between these homologues gives the constant for the CH_2-group, $G_{(CH_2)} = -0.23$. Based on the data of other homologous compounds, especially hydroxy acids and dicarboxylic acids[32], the R_M constant of the CH_2-group as well as of any other aliphatic C-atom was established as -0.22 (Table VI). For an example might be mentioned:

$$G_{(CH_2)} = R_{M\ \text{leucine}} - R_{M\ \text{valine}} = -0.22 \qquad (2)$$

The calculation of other group constants was carried out in the same manner, by using the difference between the R_M values of two compounds, differing only by the group in question.

The R_M constants for chain branching were determined by way of the R_F values of branched fatty acids (Nos. 9–19) and branched amino acids. For a branching in the α-position the value is -0.03, for any other branching it is $+0.12$ (Table VI). The data available were not sufficient to establish these values very accurately or to distinguish between chain branching in the β- and γ-position. One can assume that these positions would also show rather different values for chain branching, an assumption which is supported by the special value of the α-position. A comparison

TABLE II

HYDROXY ACIDS

No.	Acid	R_F exp.	R_M measured	R_M calc.	R_F calc.	ΔR_M	ΔR_F
22	Glycolic	0.25	0.48	0.57	0.21	0.09	0.04
23	Lactic	0.31	0.35	0.35	0.31	0.00	0.00
24	3-Hydroxypropionic	0.27	0.43	0.42	0.27	0.01	0.00
25	2-Hydroxybutyric	0.42	0.14	0.14	0.42	0.00	0.00
26	3-Hydroxybutyric	0.38	0.21	0.20	0.39	0.01	0.01
27	4-Hydroxybutyric	0.28	0.41	0.40	0.28	0.01	0.00
28	2-Hydroxyvaleric	0.55	−0.09	−0.09	0.55	0.00	0.00
29	2-Hydroxycaproic	0.66	−0.29	−0.31	0.67	0.02	0.01
30	2-Hydroxyhexanoic	0.77	−0.52	−0.53	0.77	0.01	0.00
31	Glyceric	0.12	0.87	0.91	0.11	0.04	0.01

between the R_M values of acrylic and crotonic acid and the corresponding saturated compounds leads to a mean value of +0.09 for the R_M constant of the double bond.

Hydroxy acids

The R_F and R_M values of 10 hydroxy acids (Nos. 22–31), predominantly α-hydroxy acids are reported in Table II. With the exception of the first member of the series, glycolic acid, and glyceric acid, there is a good agreement between the experimental and calculated R_F values. Exceptions from the rule of additivity have already been noted before in the case of vicinal groups, as in glycolic and glyceric acid.

Although REICHL[4], from the very limited data available to him, differentiated between primary and secondary hydroxy groups, it appears to be much more important to take into account the position of the hydroxy group, if one regards the data given in this paper.

In calculating the R_M value of the α-hydroxy group, glycolic acid was not accounted for due to its vicinal groups. The differences between R_M values of the other α-hydroxy acids and the corresponding unsubstituted carboxylic acids lead to a $G_{(\alpha\text{-OH})}$ of +0.49. 3-Hydroxy propionic acid and 3-hydroxy butyric acid were used to calculate the constant for the β-OH group:

$$G_{(\beta\text{-OH})} = R_{M\ \text{3-hydroxy propionic}} - R_{M\ \text{propionic}} = 0.57 \quad (3)$$

$$G_{(\beta\text{-OH})} = R_{M\ \text{3-hydroxy butyric}} - R_{M\ \text{butyric}} = 0.56 \quad (4)$$

According to the above equations it does not appear to matter whether the β-OH groups are primary or secondary. Owing to the lower β-OH value of the amino acid pair threonine/2-aminobutyric acid, the $G_{(\beta\text{-OH})}$ was defined as 0.56. Using 4-hydroxy butyric acid according to the method described above, $G_{(\gamma\text{-OH})}$ was found to be +0.76.

Amino acids

A thorough study of the relationship between molecular structure and the R_M values of the amino acids has been carried out by SCHAUER AND BULIRSCH[5], PATAKI[13] and TRZASKA AND KOWKABANY[20]. The latter authors also used butanol in

TABLE III

AMINO ACIDS

No.	Acid	R_F exp.	R_M measured	R_M calc.	R_F calc.	ΔR_M	ΔR_F
32	Glycine	0.07	1.12	1.06	0.08	0.06	0.01
33	Alanine	0.13	0.83	0.84	0.13	0.01	0.00
34	2-Aminobutyric	0.19	0.62	0.62	0.19	0.00	0.00
35	3-Aminobutyric	0.17	0.69	0.68	0.17	0.01	0.00
36	4-Aminobutyric	0.14	0.79	0.78	0.14	0.01	0.00
37	2-Aminoisobutyric	0.21	0.58	0.59	0.20	0.01	0.01
38	Norvaline	0.27	0.43	0.40	0.28	0.03	0.01
39	Valine	0.22	0.55	0.54	0.22	0.01	0.00
40	Leucine	0.32	0.33	0.32	0.32	0.01	0.00
41	Isoleucine	0.31	0.35	0.32	0.32	0.03	0.01
42	6-Aminocaproic	0.14	0.79	0.78	0.14	0.01	0.00

their solvent system but in an acid solution, *n*-butanol–acetic acid–water (4:1:5), and obtained a similar mean value for $G_{(CH_2)}$ of −0.20. Due to the small number of compounds investigated, they did not calculate any other group constants.

Table III shows the R_F and R_M values of 11 amino acids (Nos. 32–42). The values of phenylalanine and tyrosine are listed in the second part of this work[32] under aromatic acids in order to make it possible to calculate the R_M constant of the phenyl group. In this other paper the data of other amino acids run in methanol solutions can also be found.

Comparison of experimental and calculated R_M values shows that a high discrepancy is only observed with glycine due to its vicinal groups. The calculation of the NH_2-group constants was done in the same way as for hydroxy compounds. Here too, emphasis was put on the relative positions of the amino and carboxyl groups (Table VI). The high positive value of $G_{(\varepsilon\text{-}NH_2)}$ which was obtained according to the following equation is notable:

$$G_{(\varepsilon\text{-}NH_2)} = R_{M\ \varepsilon\text{-amino caproic}} - R_{M\ \text{caproic}} = +1.58 \qquad (5)$$

A similar relation between the group constants for the NH_2-group was observed by SCHAUER AND BULIRSCH[5]. They too, found $G_{(\alpha\text{-}NH_2)}$ and $G_{(\beta\text{-}NH_2)}$ to be close together, whereas the group constants of the remaining positions showed distinct differences.

Halogen acids

The halogen acids (Nos. 43–53) display a rather complex behaviour. Chloro-, bromo- and iodoacetic acid for instance have almost identical R_F values. The R_F

TABLE IV

HALOGEN ACIDS

No.	Acid	R_F exp.	R_M measured	R_M calc.	R_F calc.	ΔR_M	ΔR_F
43	Difluoroacetic	0.67	−0.31	−0.28	0.66	0.03	0.01
44	Trifluoroacetic	0.78	−0.55	−0.50	0.76	0.05	0.02
45	Chloroacetic	0.52	−0.04	−0.06	0.53	0.02	0.01
46	2-Chloropropionic	0.64	−0.25	−0.28	0.65	0.03	0.01
47	2-Chlorobutyric	0.75	−0.48	−0.50	0.76	0.02	0.01
48	Dichloroacetic	0.64	−0.25	−0.28	0.66	0.03	0.02
49	Trichloroacetic	0.74	−0.45	−0.50	0.76	0.05	0.02
50	Trichlorolactic	0.69	−0.35	−0.31	0.67	0.04	0.02
51	Bromoacetic	0.54	−0.07	−0.06	0.56	0.01	0.01
52	Tribromoacetic	0.75	−0.48	−0.50	0.73	0.02	0.01
53	Iodoacetic	0.54	−0.07	−0.06	0.53	0.01	0.01

values obtained experimentally lead to the conclusion, that the number of halogen atoms rather than kind and position of the halogens are of more importance for the R_F value. Therefore an attempt was made to obtain good agreement between measured and calculated R_F values with a small number of group constants and to keep the calculation of the theoretical R_F values of halogen acids as simple as possible. As a first approximation it appears to be sufficient to introduce, apart from the group

constant of the first halogen atom in α-position ($G = -0.14$), only one more constant for any further halogen atom independent of its position with respect to any other functional group in the compound ($G = -0.22$). This simplified approach only causes greater deviations in the case of the trisubstituted acids, whereas good agreement is obtained with the other compounds.

Other acids

R_F and R_M values of six organic acids (Nos. 54–59) with different functional groups were also investigated (Table V). The first three contained a carbonyl group. The successful TLC separation of ketocarboxylic acids on cellulose layers has already been described: RINK AND HERRMANN[42] separated the acids as rhodamine derivates, and CHIARI AND RÖHR[43] separated the 2,4-DPHs of α-ketocarboxylic acids.

TABLE V

OTHER ACIDS

No.	Acid	R_F exp.	R_M measured	R_M calc.	R_F calc.	ΔR_M	ΔR_F
54	Glyoxylic	0.30	0.37	0.37	0.30	0.00	0.00
55	Pyruvic	0.49	0.02	0.01	0.49	0.01	0.00
56	Levulinic	0.57	−0.12	−0.13	0.57	0.01	0.00
57	Ethoxyacetic	0.55	−0.08	−0.09	0.55	0.01	0.00
58	Thioglycolic	0.52	−0.04	−0.05	0.53	0.01	0.01
59	Butane-1-sulfonic	0.64	−0.25	−0.25	0.64	0.00	0.00

The calculation of the group constant for α-CHO could not be done by direct comparison of the R_M values of formic acid and glyoxylic acid, since, as already mentioned, the R_M value measured for formic acid differs greatly from the theoretical. Therefore $G_{(\alpha\text{-CHO})}$ was directly determined from glyoxylic acid:

$$G_{(\alpha\text{-CHO})} = R_{M\ \text{glyoxylic}} - G_{(\text{COOH})} - G_o = +0.07 \qquad (6)$$

The difference between the R_F values of pyruvic and acetic acid leads to a $G_{(\alpha\text{-CO})}$ of −0.07, that between levulinic and butyric acid to a $G_{(\gamma\text{-CO})}$ of +0.23. Here too, one can observe, that the hydrophilic properties of a group are greatly weakened, when in the α-position.

The group constant for the ether group was obtained by subtracting the R_M value of butyric acid from the R_M of ethoxyacetic acid ($G_{\text{ether}} = +0.27$).

Thioglycolic acid, when compared with acetic acid, yields a $G_{(\alpha\text{-SH})}$ of −0.13; and butane-1-sulfonic acid a value for $G_{(SO_3H)} = +1.59$.

Calculation of the basic constant

For the calculation of group constants and binding increments by direct comparison of the R_M values of substances differing by only one group or binding increment, the knowledge of the value for the basic constant is not necessary. For its determination, straight chain fatty acids, hydroxy acids and other simple acids were

TABLE VI

BASIC CONSTANT, GROUP CONSTANTS AND BINDING INCREMENTS

Adsorbent: Cellulose HR 300 (Macherey, Nagel). Solvent: *n*-Butanol–diethylamine–water (84:1:14). Temperature: 23° ± 1°.

Basic constant	−0.96
Aliphatic C-atom	−0.22
Chain branching, in α-position	−0.03
in other positions	+0.14
C = C double bond	+0.09
OH in α-position	+0.49
in β-position	+0.56
in γ-position	+0.76
NH_2 in α-position	+0.98
in β-position	+1.04
in γ-position	+1.14
in ε-position	+1.58
CHO in α-position	+0.07
CO in α-position	−0.07
in γ-position	+0.23
COOH	+1.26
Halogen atom, the first if in α-position	−0.14
any other one	−0.22
—O—, ether groups	+0.27
SH in α-position	−0.13
$-SO_3H$	+1.59

E.g. the R_M value for valine is calculated thus: $R_M = G_o + G_{(COOH)} + 4G_{(C)} + G_{(\alpha\text{-}NH_2)} + G_{(branching)}$. $R_M = -0.96 + 1.26 - 0.88 + 0.98 + 0.14 = +0.54$ (see No. 39).

used. The calculation was done by inserting the experimental R_M value and the group constants determined (Table VI) in equation (1). For instance one can determine the basic constant G_o from butyric acid and alanine in the following way:

$$R_{M\ butyric} = G_o + G_{(COOH)} + 3G_{(C)} \qquad G_o = -0.96 \qquad (7)$$

$$R_{M\ alanine} = G_o + G_{(COOH)} + G_{(\alpha\text{-}NH_2)} + 2G_{(C)} \qquad G_o = -0.96 \qquad (8)$$

The mean value obtained from 14 different acids by this method was found to be $G_o = -0.96$.

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