THE EFFECT OF pH ON THE R_F VALUES OF ORGANIC ACIDS IN PAPER CHROMATOGRAPHY WITH NEUTRAL SOLVENTS

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INTRODUCTION

In enzymology the substances taking part in a reaction are generally suspended in a buffer solution, and at the final stage the proteins are precipitated with trichloroacetic acid (TCA). When studying the paper chromatographic behaviour of the products resulting from such a reaction, we observed that the R_F values of some substances alter considerably when the pH is altered.

These observations led us to carry out an investigation with the object of determining which substances behave in this manner and the conditions under which these changes occur. As our attention had first been drawn to this problem when investigating an enzymic reaction involving amino- and keto-acids, our research was directed to the study of the behaviour of organic acids in general.

EXPERIMENTAL

The organic acids were used in 0.1 % or 1.0% solutions according to the pH we wished to obtain. Each sample was investigated at an acid and an almost neutral pH, the solutions being prepared in water, in 0.2 *M* phosphate buffer of pH 8.0, and in the same buffer treated with 30 % trichloroacetic acid, or *N* hydrochloric acid, or *N* sulphuric acid until the pH reverted to the acid zone.

Ascending paper chromatograms were run on Macherey-Nagel No. 261 paper at room temperature for 20 hours in several neutral solvent systems. In order to find the system that gives the best results, the following neutral mixtures were tested [1,2]:

(a) 1-Butanol–water (85:15).
(b) Acetone–water (80:20).
(c) 2-Butanol–amyl alcohol–water (4:4:1).
(d) Water-saturated amyl alcohol.
(e) 1-Propanol–water (70:30).
(f) 95 % ethanol.
(g) 77 % ethanol.

For aliphatic dicarboxylic, hydroxy- and keto-acids, the best results were obtain-

ed with 1-propanol–water (70:30), whereas 77% ethanol was most convenient for amino acids.

For locating the compounds on the paper, the following spraying reagents were used[1,3]:

(a) 0.1 % ninhydrin solution in isopropanol for amino acids.
(b) Bromocresol green solution in ethanol for acids in general.
(c) Ammoniacal silver nitrate for reducing compounds.
(d) 2 % ferric chloride for keto-acids.
(e) 1 % potassium permanganate for hydroxy-acids.

RESULTS

The effect of using trichloroacetic acid, hydrochloric acid and sulphuric acid to adjust the pH of the various compounds was investigated and the R_F values obtained are given in Table I.

TABLE I

INFLUENCE OF TCA, HCl AND H_2SO_4 ON THE R_F VALUES OF AMINO ACIDS

Amino acids adjusted to pH = 1.0	R_F		
	TCA	*HCl*	*H_2SO_4*
Alanine	0.51	0.50	0.47
Valine	0.65	0.63	0.62
Glycine	0.34	0.35	0.35
Leucine	0.60	0.60	0.58
Glutamic acid	0.54	0.54	0.56
Aspartic acid	0.40	0.39	0.40

Since in our experiments it was often necessary to dissolve some of the acids in a phosphate buffer of pH 8.0, in order to obtain a final pH between 5.0 and 6.0, the possible influence of the buffer upon the migration rate of the compounds tested had to be examined.

TABLE II

INFLUENCE OF PHOSPHATE BUFFER ON THE R_F VALUES OF AMINO ACIDS

Amino acid at pH = 5.0	R_F		*Amino acid at pH = 1.0*	R_F	
	H_2O	*Buffer*		*TCA*	*Buffer + TCA*
Aspartic acid	0.22	0.24	Aspartic acid	0.41	0.42
Glutamic acid	0.32	0.30	Glutamic acid	0.54	0.55

As it was found that under our working conditions the buffer did not interfere at all with the migration of the spots on paper, we could proceed with our experiments on the different types of organic acids.

Amino acids

The complete series of the most common mono- and dicarboxylic amino acids, as well as the amides of the latter, were investigated as regards their R_F values when the pH of the solution was altered (Table III).

TABLE III

INFLUENCE OF pH ON THE R_F VALUES OF AMINO ACIDS

Amino acids	R_F		
	pH 5.0	*pH 3.0*	*pH 1.0*
Glycine	0.37	0.37	0.37
Alanine	0.47	0.49	0.50
Valine	0.60	0.60	0.61
Leucine	0.58	0.59	0.61
Serine	0.41	0.41	0.42
Threonine	0.53	0.53	0.54
Cysteine	0.10	0.11	0.12
Lysine	0.32	0.32	0.35
Glutamic acid	0.29	0.41	0.57
Aspartic acid	0.22	0.25	0.40
Glutamine	0.27	0.27	0.33
Asparagine	0.12	0.13	0.17

Aliphatic acids

Dicarboxylic aliphatic acids, as well as some keto- and hydroxy-acids, were tested as to their behaviour on paper chromatography when the pH of the solution is altered (Table IV). The monocarboxylic aliphatic acids with a short carbon chain were excluded from our work because they could not be tested in neutral solvents owing to their volatility.

TABLE IV

INFLUENCE OF pH ON THE R_F VALUES OF ORGANIC ACIDS

Organic acid	R_F	
	pH 5.0–6.0	*pH 1.0*
Oxalic	0.21	0.31
Malonic	0.48	0.59
Succinic	0.78	0.81
Adipic	0.90	0.89
Lactic	0.34	0.68
Malic	0.16	0.64
Tartaric	0.22	0.55
Citric	0.12	0.57
Pyruvic	0.46	0.69
Oxaloacetic	0.12	0.49
α-Ketoglutaric	0.15	0.63

The R_F values at pH 3.0 were omitted from Table IV, because they are always intermediate values and do not contribute any further information.

DISCUSSION

From Tables I and II it is evident that when the pH of the sample is adjusted to 1.0 the R_F values of the samples remain constant, irrespective of whether trichloroacetic, hydrochloric or sulphuric acid is used, or whether the initial dilution is made in water or phosphate buffer.

From the series of amino acids (Table III) it can be seen that a variation of the R_F is evident only for the dicarboxylic acids, and does not occur with the monocarboxylic acids even when other groups are present in the molecule. It should be noted that for glutamine and asparagine, which are amides of dicarboxylic amino acids, the magnitude of the variation is considerably diminished.

The differences in the R_F values can be accentuated still more if an excess of the auxiliary acid is used, and this may even lead to slight alterations in the cases that were considered as negative here.

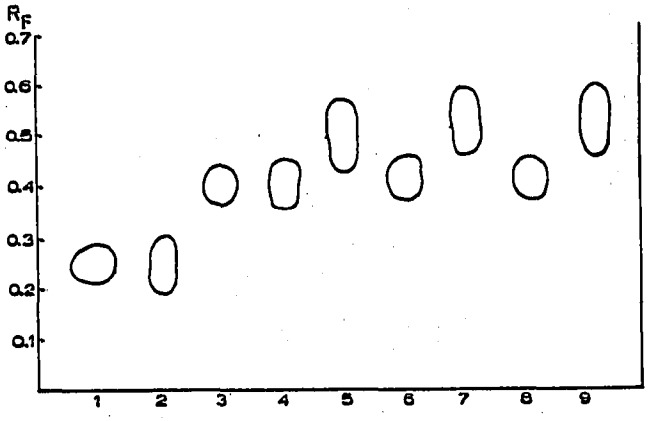

Fig. 1. Chromatogram of aspartic acid in: (1) water, pH = 4.0; (2) buffer, pH = 4.5; (3) TCA, pH = 1.0; (4) buffer and TCA, pH = 1.0; (5) buffer and an excess of TCA; (6) buffer and HCl, pH = 1.0; (7) buffer and an excess of HCl; (8) buffer and H_2SO_4, pH = 1.0; (9) buffer and an excess of H_2SO_4. Solvent: 77% ethanol.

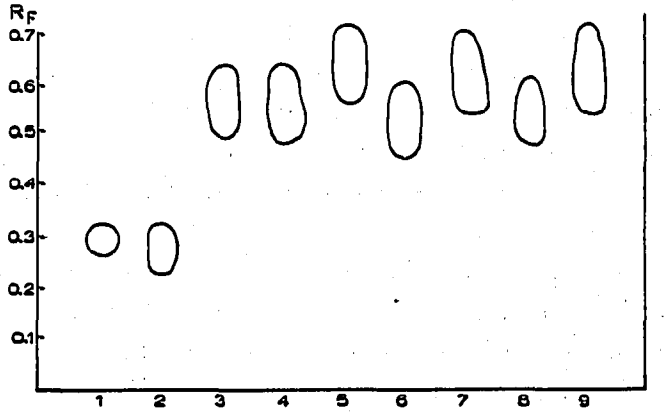

Fig. 2. Chromatogram of glutamic acid in: (1) water, pH = 4.0; (2) buffer, pH = 5.0; (3) TCA, pH = 1.0; (4) buffer and TCA, pH = 1.0; (5) buffer and an excess of TCA; (6) buffer and HCl, pH = 1.0; (7) buffer and an excess of HCl; (8) buffer and H_2SO_4, pH = 1.0; (9) buffer and an excess of H_2SO_4. Solvent: 77% ethanol.

The results obtained with the dicarboxylic aliphatic acids were unexpected because of the lack of regularity. Only the first two acids of the series, those with two and three carbon atoms, behaved in this fashion. On the other hand, there is strong evidence for believing that the presence of hydroxyl and carbonyl groups is responsible for the change in the R_F values when the pH is altered (Table IV). For instance, the influence of the hydroxyl groups of tartaric acid can be clearly seen in Fig. 3, where this acid has been chromatographed together with succinic and malonic acid.

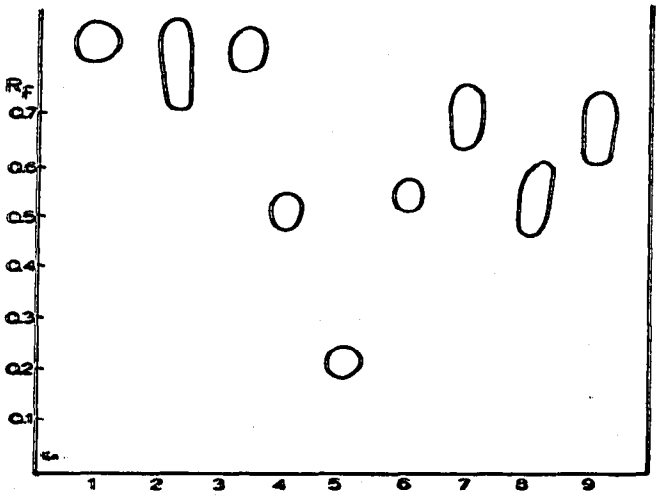

Fig. 3. Chromatogram of: (1) succinic acid in water, pH = 1.0; (2) succinic acid in buffer, pH = 6.0; (3) succinic acid in buffer and TCA, pH = 1.0; (4) tartaric acid in water, pH = 2.0; (5) tartaric acid in buffer, pH = 6.0; (6) tartaric acid in buffer and TCA, pH = 1.0; (7) malonic acid in water, pH = 1.0; (8) malonic acid in buffer, pH = 6.0; (9) malonic acid in buffer and TCA, pH = 1.0. Solvent: 1-propanol–water (70:30).

On comparing the results obtained with those for the amino acids, it can be observed that the R_F values of the hydroxy-monocarboxylic acids alter considerably when the pH is altered. Not only the hydroxyl-group but also the carbonyl-group in mono- and dicarboxylic acids seems to influence the R_F values. This can be easily seen when succinic acid, which shows no differences, is compared with oxaloacetic acid for which the R_F changes from 0.12 to 0.49 when the pH is altered from 6.0 to 1.0. Fig. 4 represents one of the chromatograms of the keto-acids studied.

To explain these facts we first thought of the possibility of cyclization products being formed, although our working conditions were not the same as those applied for the preparation of these products. Thus, in the case of glutamic acid the lactam, pyrrolidone-carboxylic acid could be formed, which is not detected by ninhydrin. The spot obtained after altering the pH was, however, located on the paper by means of the ninhydrin spray[4]. Further support for abandoning this supposition came from the observation that when our sample was dissolved in water at a concentration sufficient to obtain a pH of 1.0, its R_F was the same as that obtained when the sample was dissolved in the buffer solution and the pH then adjusted to 1.0 with one of the auxiliary acids, a treatment which could lead to the formation of a new compound (Fig. 5).

We are, therefore, inclined to believe that all the variations observed are a conse-

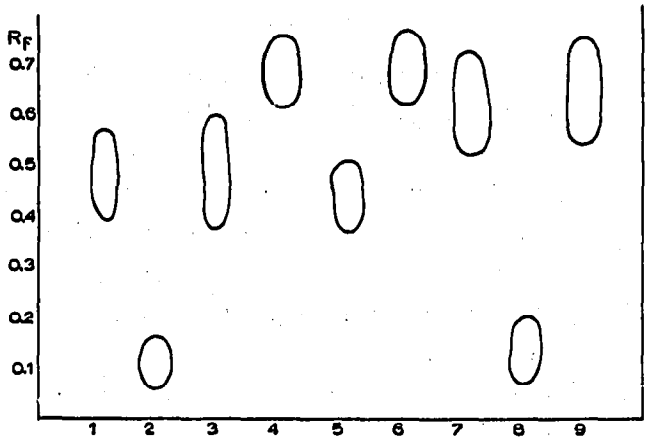

Fig. 4. Chromatogram of: (1) oxaloacetic acid in water, pH = 1.0; (2) oxaloacetic acid in buffer, pH = 6.0; (3) oxaloacetic acid in buffer and TCA, pH = 1.0; (4) pyruvic acid in water, pH = 1.0; (5) pyruvic acid in buffer, pH = 5.0; (6) pyruvic acid in buffer and TCA, pH = 1.0; (7) α-ketoglutaric acid in water, pH = 1.0; (8) α-ketoglutaric acid in buffer, pH = 6.0; (9) α-ketoglutaric acid in buffer and TCA, pH = 1.0. Solvent: 1-propanol–water (70:30).

quence of alteration of the pH. The irregularities of the phenomena must still be considered. The results obtained for the dicarboxylic aliphatic acids furnish strong evidence for the assumption that the dissociation constant of the compounds studied is responsible for the variations of the R_F values. In view of the fact that at a very acid pH almost no

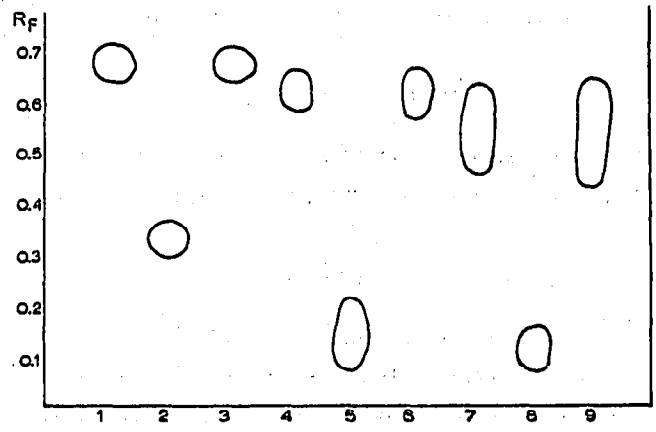

Fig. 5. Chromatogram of: (1) lactic acid in water, pH = 1.0; (2) lactic acid in buffer, pH = 6.0; (3) lactic acid in buffer and TCA, pH = 1.0; (4) malic acid in water, pH = 1.0; (5) malic acid in buffer, pH = 6.0; (6) malic acid in buffer and TCA, pH = 1.0; (7) citric acid in water, pH = 1.0; (8) citric acid in buffer, pH = 6.0; (9) citric acid in buffer and TCA, pH = 1.0. Solvent: 1-propanol–water (70:30).

dissociation occurs, oxalic and malonic acids, which have higher dissociation constants than succinic and adipic acids, must undergo a greater change in dissociation between pH 6.0 and pH 1.0, which is the cause of the R_F variations observed. The results obtained with hydroxy-acids provide further support for this hypothesis, since it is a well-established fact that a hydroxyl group at the alpha position increases the dissociation constant.

CONCLUSIONS

From what has been stated above, it may be concluded that:

(a) The acids used to adjust the pH do not influence the migration of the samples spotted on the paper.

(b) The use of a phosphate buffer does not affect the results.

(c) In the series of amino acids studied, only the dicarboxylic acids show a variation in their R_F values when the pH of the solution is altered.

(d) Blocking of one of the carboxyl groups by another amino group reduces the magnitude of the variation considerably.

(e) In the series of dicarboxylic aliphatic acids, only the first two members of the series show some variations.

(f) Hydroxy- and keto-, mono- and dicarboxylic acids exhibit considerable variations.

(g) The variations observed are the result of alterations in the pH of the solutions and not of the formation of new compounds; they are probably due to dissociation phenomena.

Finally, it should be noted that in chromatographic analysis these observations are of importance, especially when a comparison of reaction products with standard solutions might lead to erroneous interpretations if the variations due to alteration of the pH during the reaction are not taken into account. This is precisely the case in enzymic reactions in which minute concentrations of the reagents are suspended in a buffer solution and very often treated with trichloroacetic acid which is not always completely eliminated.

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SUMMARY

The variations of the R_F values of organic acids due to changes in the pH of the solution were studied, as well as the influence upon the R_F of the use of trichloroacetic acid, hydrochloric or sulphuric acids for the adjustment of the pH.

Amino acids, as well as two of their amides, aliphatic dicarboxylic acids, hydroxy- and keto-, mono- and dicarboxylic acids were examined in various neutral solvent systems and their chromatographic behaviour in relation to pH variations was registered.

Practical applications of the results obtained, as well as possible theoretical explanations of the phenomena are discussed.

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