

THE CHROMATOGRAPHIC BEHAVIOUR OF SOME STRUCTURAL ANALOGUES OF DICARBOXYLIC ACIDS OF THE KREBS CYCLE

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Much of what is known about the relationships between the biological activity of a compound and its chemical structure has been derived from investigations on structural analogues of metabolic substances. This is the case with studies on enzymic specificity, accumulation of intermediates, inhibitory actions and adaptation processes. When studying the possible biological activity of epoxysuccinic, mercaptosuccinic and mercaptomalic acids, which are structural analogues of oxaloacetic, succinic and malic acids, it became apparent that the chromatographic behaviour of these compounds, in standard conditions, provides a useful means of detecting and separating them.

MATERIALS AND METHODS

Epoxysuccinic, mercaptosuccinic and mercaptomalic* acids were chromatographically pure. Oxaloacetic, succinic, malic, fumaric and tartaric acids, used as reference substances, were all of the best grade available. Mixed solutions of the acids and supernatants of reaction mixtures from biochemical experiments were investigated, and various substances were identified by comparing the R_F values of the unknown spots with those of 1% standard solutions, using 20 μ l per spot.

Ascending chromatography on Macherey-Nagel No. 210 filter paper was employed. Neutral, alkaline and acid solvents were tested; the composition of the solvents, together with the references and development conditions are given in the tables of R_F values. In the experiments with neutral and alkaline solvents, the sodium salts of the acids were also used for comparative tests.

For the detection of the spots, a bromocresol green solution according to PANEK¹, was used as spraying reagent, after the chromatograms had been dried at 50° until constant weight. Higher temperatures were avoided because of the instability of the mercaptoacids. As specific reagents, a 2% FeCl_3 solution was used for the epoxy-, mercapto- and keto-acids, and alkaline silver nitrate for the hydroxyacids.

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RESULTS AND DISCUSSION

The R_F values of the free acids in neutral and alkaline solvents were low, although higher than the R_F values of the respective sodium salts, as can be seen in Table I.

TABLE I
 R_F VALUES OF DICARBOXYLIC ACIDS IN NEUTRAL AND ALKALINE SOLVENTS

Acids	$R_F \times 100$ in neutral solvents						$R_F \times 100$ in alkaline solvents				
	A		B		C		D		E		F
	acid	salt	acid	salt	acid	salt	acid	salt	acid	salt	acid
Epoxy succinic	77;19	10	48;13	08	21;42	06	36	24	59	10	00
Succinic	78	08	15;10	12	06	—	35	28	57	11	09
Mercaptosuccinic	24	09	08	04	14	05	14	11	41	06	00
Mercaptomalic	45;20	21	68;08	04	21	03	23	16	46	06	00
Malic	54	29	68	21	42	—	29	22	53	10	00
Fumaric	49	25	06	04	—	—	42	24	66	13	00

Solvents: A = 95% ethanol²; B = *n*-butanol-ethanol-water (4:1:1 v/v)²; C = phenol-water²; D = ethanol-conc. NH₄OH-water (80:5:15 v/v)³; E = *n*-propanol-conc. NH₄OH (70:30 v/v)¹; F = *n*-butanol-conc. NH₄OH (90:40 v/v)⁴.
Conditions: A, D, E: 7 h, 27°; B, C, F: 18 h, 27°.

“Comets” and double spots were frequent; this had already been observed for citric acid⁵. These inconveniences disappeared when acid solvents were used, as shown in Table II.

TABLE II
 R_F VALUES OF DICARBOXYLIC ACIDS IN ACID SOLVENTS

Acids	$R_F \times 100$					
	G	H	I	J	K	L
Epoxy succinic	61	72	77	78	84	61
Succinic	77	79	68	88	88	72
Mercaptosuccinic	85	83	87	88	83	84
Mercaptomalic	83	83	87	90	79	78
Fumaric	91	90	92	92	96	92
Oxaloacetic	29	—	17	—	—	16
Tartaric	30	32	54	63	48	13

Solvents: G = *n*-butanol-formic acid-water (10:2:5 v/v)⁴; H = butan-2-ol-formic acid-water (85:5:10 v/v)⁵; I = ethyl ether-acetic acid-water (13:3:5 v/v)⁴; J = ethyl acetate-acetic acid-water (2:1:1 v/v)⁶; K = methyl ethyl ketone-acetone-formic acid-water (80:4:2:12 v/v)²; L = methyl isobutyl ketone-formic acid-water (40:20:20 v/v).
Conditions: G, H, L: 20 h, 27°; I, J, K: 7 h, 27°.

The best results were obtained with solvents containing formic acid, BRAY and coworkers⁹ referred to similar experiences in the resolution of hydroxyacids. Fumaric acid exhibited the highest R_F value in the solvents tested, as is usual for unsaturated acids while oxaloacetic and tartaric acids had the lowest values. The R_F values of

epoxysuccinic acid were in general lower than those of succinic acid, but the R_F values of the mercaptoacids were always higher than those of the respective analogue. These differences are shown in Fig. 1.

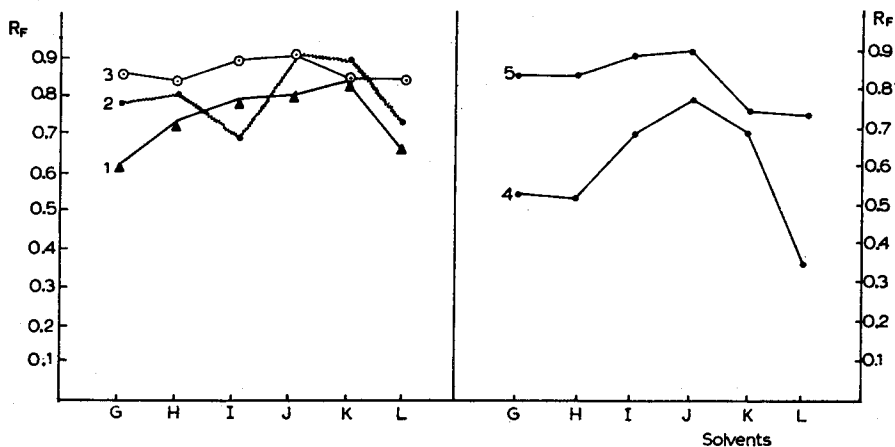
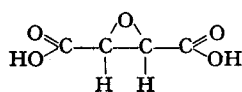
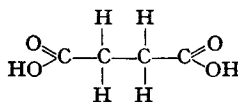


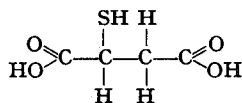
Fig. 1. R_F values of dicarboxylic acids in various solvents.



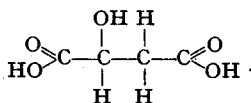
(1) Epoxysuccinic acid



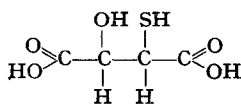
(2) Succinic acid



(3) Mercaptosuccinic acid



(4) Malic acid



(5) Mercaptomalic acid

Solvent L gave the best resolution in all the experiments; a typical chromatogram is reproduced in Fig. 2.

For organic acids REIO⁸ used a solvent containing methyl isobutyl ketone and formic acid, but in different proportions and prepared by a more complicated procedure; the R_F values obtained for the same acids are lower in REIO's solvent.

The R_F values of the mercaptoacids were very close together, the acids showing a tendency to ascend together, and good separations could only be achieved by means of two-dimensional chromatography. A pair of solvents was investigated, solvent A (95% ethanol) in the first direction and solvent L (methyl isobutyl ketone-formic acid-water, 40:20:20 v/v) in the second. A typical two-dimensional chromatogram is represented in Fig. 3.

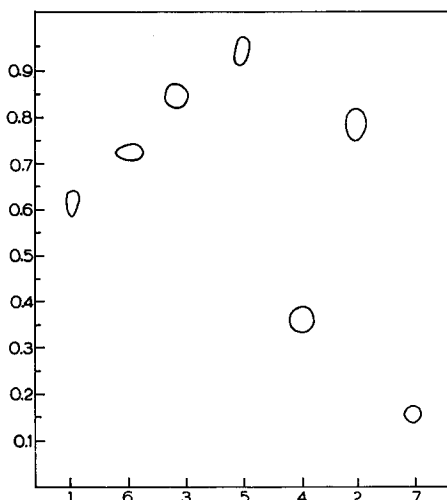


Fig. 2. Chromatogram of various dicarboxylic acids, with methyl isobutyl ketone-formic acid - water (40:20:20 v/v) as solvent. Conditions: 20 h at 27°. (1) Epoxysuccinic acid; (2) mercaptomalic acid; (3) mercaptosuccinic acid; (4) malic acid; (5) fumaric acid; (6) succinic acid; (7) oxaloacetic acid.

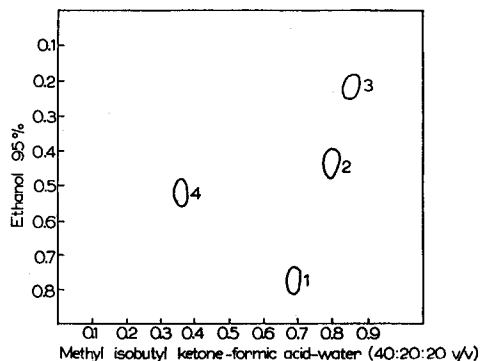


Fig. 3. Two-dimensional chromatogram of: (1) succinic acid; (2) mercaptomalic acid; (3) mercaptosuccinic acid; (4) malic acid.

Recently HOWE¹⁰ found that there is a definite relationship between the R_M value, calculated according to BATE-SMITH AND WESTALL'S formula, $R_M = \log (1/R_F - 1)$, and the molecular structure of an acid. By calculating the R_M value, it is possible to obtain some indication of the substituent groups present in a molecule. In an attempt to get some information about the influence of epoxy and sulphhydryl groups in the molecule of the various analogues, the R_M values of the acids in the acid solvents used were calculated and these are given in Table III.

The introduction of the SH group in the molecule of the respective analogues lowered the R_M values. On the other hand, the epoxy group raised the R_M values in comparison with the analogous succinic acid.

TABLE III
 R_M VALUES OF DICARBOXYLIC ACIDS IN ACID SOLVENTS

Acids	R_M					
	G	H	I	J	K	L
Epoxysuccinic	-0.19	-0.41	-0.52	-0.55	-0.72	-0.19
Succinic	-0.52	-0.57	-0.33	-0.86	-0.86	-0.41
Mercaptosuccinic	-0.75	-0.79	-0.83	-0.86	-0.79	-0.72
Mercaptomalic	-0.79	-0.79	-0.83	-0.95	-0.57	-0.55
Malic	-0.05	-0.14	-0.35	-0.55	-0.35	+0.27
Fumaric	-1.00	-0.95	-1.06	-1.06	-0.93	-1.06
Oxaloacetic	+0.39	—	+0.69	—	—	+0.72
Tartaric	+0.37	+0.33	-0.07	-0.23	+0.68	+0.83

Oxygenated groups behaved similarly also in the keto-acid and in tartaric acid. This agrees with the observations of HOWE¹⁰ on the OH group, using other solvents.

CONCLUSIONS

1. Acid solvents are more suitable for the separation of the dicarboxylic acids investigated.
2. Solvents containing formic acid gave the best resolutions.
3. Epoxysuccinic acid is easily separated in one-dimensional chromatograms, the most suitable solvent being methyl isobutyl ketone-formic acid-water (40:20:20 v/v).
4. Mercaptosuccinic and mercaptomalic acids can only be separated by two-dimensional chromatography; a procedure was developed using 95 % ethanol in one direction and methyl isobutyl ketone-formic acid-water (40:20:20) in the other.
5. In the solvents examined, the SH group raises the R_M value of the respective analogue and the epoxy group lowers it.

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SUMMARY

A paper chromatographic procedure is described for the separation of epoxysuccinic, mercaptosuccinic and mercaptomalic acids from their structural analogues involved in the Krebs cycle, succinic, fumaric, oxaloacetic and malic acids. Several solvents were assayed and the R_F values recorded. The best critical conditions are discussed and also the influence of the substituent groups on the R_M values of the metabolic compounds.

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