THE CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF ORGANIC ACIDS AND THEIR APPLICATION TO YEAST

by J. N. LADD AND P. M. NOSSAL

(From the Department of Biochemistry, University of Adelaide).

(Accepted for publication 15th December, 1953.)

The necessity for separating organic acids of biological interest, particularly for studies involving radioactive isotopes, has been met in recent years by the development of quantitative silica gel or Celite column chromatography. It was soon realised (Isherwood, 1946) that separation of complex acid mixtures could not be obtained on one column with one solvent system of fixed composition.

Bulen, Varner and Burrell (1952) used more than one column, developed with solvents differing in composition and in the proportion of their constituents, to separate the organic acids of plant tissues. Phares *et al.* (1952) isolated many biosynthetic organic acids and several inorganic acids by similar methods. Several systems have been proposed in which only the one column is employed, but the solvent system is increased in polarity in a number of discrete steps throughout the run. Marvel and Rands (1950) separated many organic acids in this way, but did not include some of the more commonly occurring acids of biological interest. Frohman, Orten and Smith (1951) developed a system whereby all the acids of the Krebs cycle could be separated and estimated quantitatively in animal tissues.

The idea was further developed by Donaldson, Tulane and Marshall (1952), who designed an apparatus for gradually and automatically increasing the polarity of the solvent throughout the run. In the present work, the latter technique was extended to the quantitative separation of several additional acids and applied to an investigation of the organic acids in yeast. Two-dimensional paper chromatography was used to identify the individual acid fractions obtained.

EXPERIMENTAL.

Column chromatography.

Apparatus and reagents. Silica gel was prepared according to Isherwood (1946). The apparatus used was identical with that of Donaldson *et al.* (1952), the solvent reservoir being subjected to pressure from a nitrogen cylinder to maintain a constant rate of flow of solvent throughout the experiment. The solvent system, size of column, and amounts of silica gel and aqueous phase used were the same as those described by Marshall, Donaldson and Friedberg (1952).

The acids investigated were acetic, succinic (B.D.H., Analar), fumaric, glycollic, maleic, malonic, tartaric (B.D.H., Laboratory Reagent), α-ketoglutaric, L-malic, tricarballylic (Light's), citric (Baker's A.R.), glutaric (Hopkins and Williams), lactic (Judex, A.R.), formic (F. H. Faulding), oxaloacetic, pyruvic and *cis*-aconitic. The oxaloacetic acid was prepared from sodium diethyloxaloacetate (Krampitz and Werkman, 1941) and was shown to be pure by the aniline citrate method (Edson, 1935). Pyruvic was freshly distilled *in vacuo* and kept as a molar, unneutralized solution. *Cis*-aconitic acid was prepared from *trans*-aconitic acid (Malachowski and Maslowski, 1928).

The silica gel column was supported by a chromatographic tube, 35 cm.×6 mm., having one end bevelled and constricted to a diameter of 2 mm. The effluent was cut into fractions by an automatic fraction collector operating on a time basis.

Preparation of acids for the column. All acids applied to the column, whether as standards to determine their order of elution or in yeast extracts, were dissolved in a 40 p.c. *n*-butanol-60 p.c. chloroform mixture (Marvel and Rands, 1950). The filtrate of a boiled 1:1 aqueous yeast extract (Effront Yeast Co., Victoria) was acidified to pH 1 with 5 N sulphuric acid and 3 ml. pipetted on to 25 gm. anhydrous sodium sulphate in a mortar, mixed thoroughly, transferred quantitatively to a Soxhlet thimble and extracted with freshly distilled anaesthetic ether for six hours. The ethereal extract was evaporated to dryness at room temperature by a current of air, the residue dissolved in the chloroform-butanol mixture and the appropriate amount (usually equivalent to 0·5 gm. wet weight of yeast) applied to the column.

Procedure. 1·1 gm. silica gel was added directly to 0·8 ml. 0·05 N sulphuric acid, mixed thoroughly and sieved (85 mesh). Chloroform was added until a lump-free slurry was obtained and this was poured into the chromatographic tube, which had at the constricted end a filter paper disc to support the column. 10 ml. chloroform were passed through the column under a pressure of 3 lb./sq. inch, taking care not to allow the chloroform level to fall below the level of the gel. The column was gently tamped down with a glass rod, excess chloroform removed and the appropriate amount of sample was added. This was allowed to pass into the column under slight pressure, again avoiding drying out the top of the gel. The column was then connected to the apparatus containing the developing solvent. The solvent reservoir was charged with a 1:1 mixture of 30 p.c. *n*-amyl alcohol and 30 p.c. *tert*-amyl alcohol in chloroform, which was delivered into the mixing vessel originally containing 50 ml. chloroform. 220 one-minute, followed by two-minute fractions, were collected, the pressure being adjusted to give a rate of flow of 22 drops/minute (≐ 0·3 ml.). One drop 0·02 p.c. phenol red was added and each fraction was titrated with caustic soda, the tube contents being vigorously mixed by a stream of CO_2-free air. The fractions containing the individual neutralized acids were pooled.

Paper chromatography.

Both ascending and descending chromatograms were run.

Apparatus. Whatman No. 1 filter paper was used throughout. For descending chromatograms, drain pipes similar to those of Consden, Gordon and Martin (1944) were used as chromatographic chambers. The pipes stood in close-fitting trays to which they were sealed by pitch. Weighted glass plates rested firmly on the top of the pipes, making the chambers air-tight. Papers 6 in. × 24 in. were suspended from stainless steel troughs containing the developing solvent, and run in the conventional manner. Ascending chromatograms were run in battery jars, the tops of which were also sealed with weighted glass plates. Papers 12 in. × 12 in. were stapled to form cylinders (Stark, Goodban and Owens, 1951) and stood in Petri dishes. The developing solvents were poured into the troughs or dishes through a small hole drilled in the centre of the glass plate.

Solvents. (1) Alkaline solvent: 8 vol. 95 p.c. ethyl alcohol, 1 vol. water, 1 vol. 7·5 N ammonium hydroxide. (2) Acidic solvent: water-poor phase from 3 vol. *n*-butyl alcohol, 6 vol. *iso*-butyl alcohol, 9 vol. water, 3 vol. formic acid (90 p.c.). Both phases of the acidic solvent remained in mutual contact with frequent shaking for eight hours before use. The choice of solvents was made after a wide range of described and new solvent mixtures had been investigated by the capillary ascent test-tube method of Rockland and Dunn (1949).

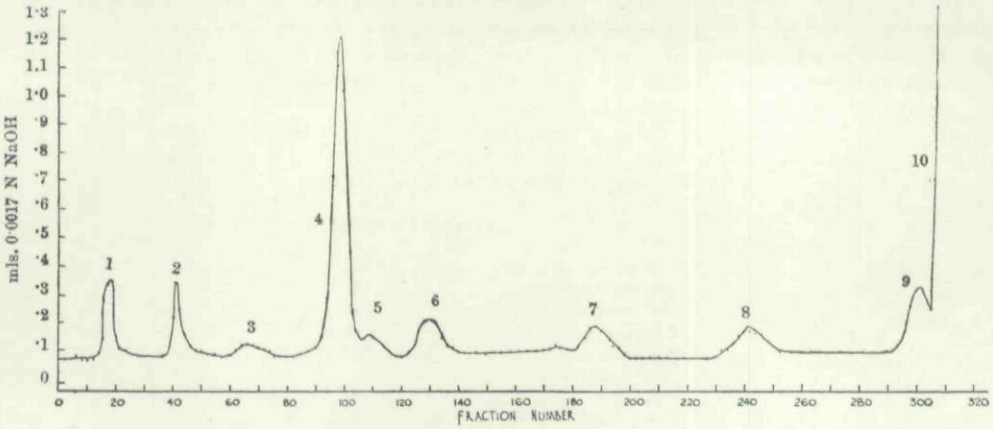

Fig. 1. Column chromatogram of yeast extract. 1 and 2, unknown acid giving a barely visible spot on a paper chromatogram (Fig. 4A, No. 7); 3, fumaric acid; 4, succinic acid; 5, α-ketoglutaric acid; 6, unknown acid running as a monocarboxylic acid in the alkaline solvent; 7, unknown acid running as a dicarboxylic acid in the alkaline solvent; 8, malic acid; 9, citric acid; 10, sulphuric acid eluted off column.

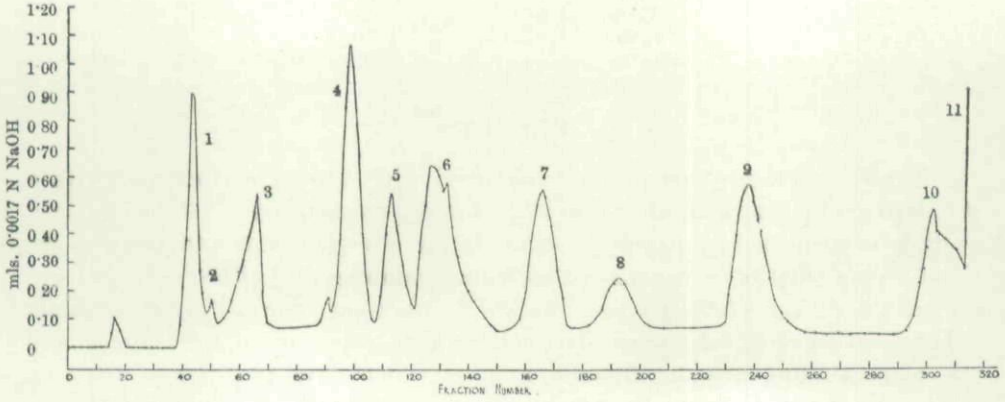

Fig. 2. Column chromatogram of a standard acid mixture. 1, acetic acid; 2, lactyl lactic acid; 3, fumaric acid; 4, succinic and lactic acids; 5, α-ketoglutaric acid; 6, oxaloacetic and *cis*-aconitic acids; 7, glycollic acid; 8, tricarballylic acid; 9, L-malic acid; 10, citric acid; 11, sulphuric acid eluted off column.

Procedure. A total volume of 6 μl. of 0·1 N aqueous solutions of standard acids was applied to the paper in a succession of spots, 4 mm. in diameter, each spot being dried with a hot air blower before the next was applied. To identify the acids from the column, the pooled fractions of each were evaporated to dryness at 70° C., air being drawn continuously over their surfaces. The sodium salts of the acids were then applied to the paper. Irrespective

of whether the chromatogram was 1- or 2-dimensional, the papers were equilibrated with the solvent system (with both phases of the acidic solvent) for at least 8 hours and usually overnight before the developing phase was added. Careful equilibration was essential for good chromatograms. To remove the acidic solvent after development, the papers were suspended in an oven, the bottom of which was covered with a tray containing water. A fan and an electric heater created a very moist atmosphere in the oven, and the temperature was kept below 50° C. After 20 minutes, fan and heater were turned off and the papers allowed to remain in the oven for a further 30 minutes. Only the fan was necessary (30 minutes) to remove the alkaline solvent completely from the papers. The spray used was 0·04 p.c. bromo-cresol green in 95 p.c. alcohol, brought to pH 6 with NaOH.

TABLE 1.

Position of acids eluted from column.

Acid	Mean fraction number
Acetic	43
Lactyl-lactic	50
Glutaric	54
Pyruvic	54
Formic	59
Fumaric	65
Lactic	100
Succinic	100
Maleic	110
α-Ketoglutaric	115
Malonic	124
cis-Aconitic	130
Oxaloacetic	133
Glycollic	167
Tricarballylic	190
L-Malic	242
Citric	303

RESULTS.

Typical column chromatograms obtained with a yeast extract and with a standard acid mixture are shown in Figs. 1 and 2 respectively. All the standard acids were identified by paper chromatography, except formic and acetic acids. These, being volatile, were removed with the swamping acid of the acidic solvent during the drying of the papers. Volatile acids were identified by their established location on the column. The acids which were run on the column, and the corresponding average fraction number which gave the highest titration figure, are listed in Table 1. The fraction at which an acid is eluted from a column is only a pointer to its identity when unknown extracts are investigated. This is demonstrated in Figs. 1 and 2. Two acids in the yeast extract (Fig. 1, Nos. 6 and 7) ran identically with oxaloacetic acid and tricarballylic acid respectively on columns prepared and run under the same conditions. In fact, pooled fractions from the columns of these yeast acids, after development on paper chromatograms run in the acidic solvent, gave spots again located in the same positions as oxaloacetic and tricarballylic acids (Fig. 4A, Nos. 3 and 5). How-

ever, in the alkaline solvent (where the R_F values of the various acids are markedly different from those in the acidic solvent) the acid in yeast which appeared to be identical with oxaloacetic ran as a monocarboxylic acid, and that apparently identical with tricarballylic acid as a fast moving diacarboxylic acid. These two acids in the yeast are as yet unidentified.

The percentage recovery of some of the acids run on the column is shown in Table 2.

TABLE 2.

Recovery of acids run on the column.

Acid	Added (μg.)	Recovered (μg.) (calculated from titration figures)	Percentage recovery
Acetic	620	625	101
cis-Aconitic	244	245	100
Citric	260	253	97
Formic	831	793	95
Fumaric	241	244	101
Glycollic	320	326	102
α-Ketoglutaric	292	257	88
L-Malic	282	286	101
Oxaloacetic	264	216	82
Pyruvic	748	688	92
Succinic	248	250	101

The lower recovery of pyruvic, oxaloacetic and α-ketoglutaric acids is probably due to their instability. In the case of α-ketoglutaric acid, the error was accentuated by a 5 p.c. impurity of succinic acid, as measured manometrically with succinoxidase (Krebs, 1937).

Different yeast samples yielded varying amounts of acids per unit weight of yeast. This variation was particularly marked with succinic acid (Table 3).

TABLE 3.

Variation of acid content in different yeast samples.

Acid	μ equiv. acid per gm. wet weight yeast			
	Sample 1	Sample 2	Sample 3	Sample 4
Fumaric	0·82	0·91	2·27	1·18
Succinic	10·9	34·4	1·38	17·9
α-Ketoglutaric	0·40	0·91	3·40	1·64
Unknown (Fig. 1, No. 6)	10·8	2·97	7·55	3·08
Unknown (Fig. 1, No. 7)	3·6	2·74	3·08	2·79
Malic	2·26	3·78	10·4	3·38
Citric	—	—	—	6.50

Although citric acid was present in all four yeast samples, it is only occasionally well separated from sulphuric acid, which is also eluted from the column at the end of the run.

A two-dimensional ascending chromatogram of a standard acid mixture is shown in Fig. 3, the alkaline solvent first separating the various acids into their classes according to the number of carboxyl groups in the molecule, and the acidic solvent permitting differentiation of the acids within their classes. The R_F values of the acids run in these two solvents are shown in Table 4.

TABLE 4.

R_F values of standard acids.

Acid	R_F values × 100	
	Alkaline solvent	Acidic solvent
cis-Aconitic	4	90
Citric	2	41
Fumaric	21	96
Glutaric	22	92
Glycollic	34	53
α-Ketoglutaric	15	65
Isocitric	2	39
Lactic	46	77
Maleic	11	59
L-Malic	12	49
Malonic	9	71
Oxaloacetic	12	56
Succinic	17	82
Tartaric	9	24
Tricarballylic	3	72

Although a two-dimensional chromatogram is necessary for more positive identification of the acids in the yeast, a one-dimensional chromatogram, developed in the acidic solvent, was sufficient to separate all the detectable acids present. Use of the two solvents gave no increase in the number of spots on the paper. The one-dimensional technique has the added advantage of being less time consuming and giving spots which are more compact and sharply defined.

One-dimensional descending chromatograms of an appropriate amount of the same yeast extract used for Fig. 1 and of a standard acid mixture are shown in Fig. 4.

DISCUSSION.

The results of the column chromatograms are essentially the same as those obtained by Marshall *et al.* (1952), except that lactic and succinic acids could not be separated under any circumstances. These two acids ran together, both as individual acids on separate columns and on paper chromatograms. By slowing the rate of increase of polarity of the solvent, the two acids were partially separated, but only very slightly. The small rise in titre occurring between acetic and fumaric acids shown in Fig. 2 may be due to lactyl-lactic acid, which

always accompanies lactic acid. When lactic acid was omitted from the acid mixture, no such rise in titre occurred, and, conversely, if lactic acid alone was run, two peaks were obtained. The larger peak was identified as lactic acid; the smaller corresponds to the slight peak found between acetic and fumaric acids.

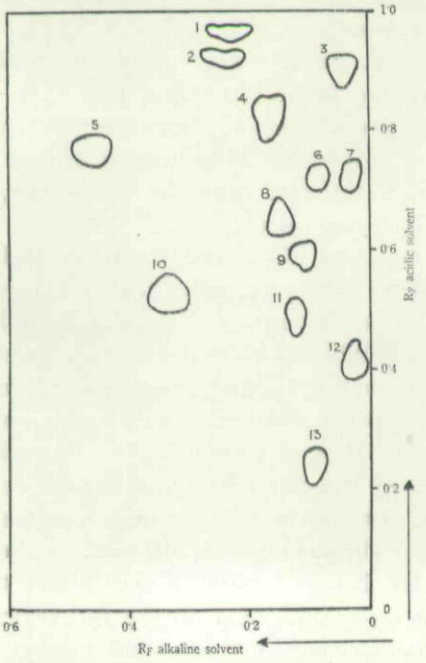

Fig. 3.

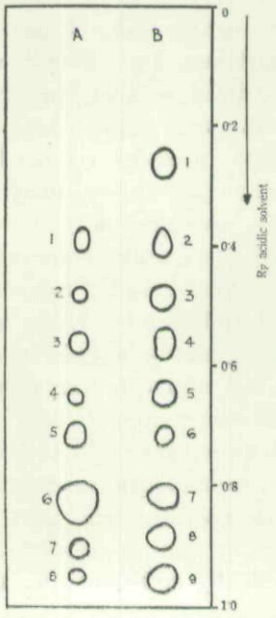

Fig. 4.

Fig. 3. Two-dimensional chromatogram of a standard acid mixture. 1, fumaric acid; 2, glutaric acid; 3, *cis*-aconitic acid; 4, succinic acid; 5, lactic acid; 6, malonic acid; 7, tricarballylic acid; 8, α-ketoglutaric acid; 9, maleic acid; 10, glycollic acid; 11, L-malic acid; 12, citric acid; 13, tartaric acid.

Fig. 4. One-dimensional paper chromatogram of A, yeast extract (≡100 mg. wet weight yeast); B, standard acid mixture.

Acids in A: 1, citric acid; 2, malic acid; 3, unknown acid running as a monocarboxylic acid in the alkaline solvent; 4, α-ketoglutaric acid; 5, unknown acid running as a dicarboxylic acid in the alkaline solvent; 6, succinic acid; 7, very faint spot given by peaks 1 and 2 in Fig. 2; 8, fumaric acid.

Acids in B: 1, tartaric acid; 2, citric acid; 3, L-malic acid; 4, oxaloacetic acid; 5, α-ketoglutaric acid; 6, tricarballylic acid; 7, succinic acid; 8, *cis*-aconitic acid; 9, fumaric acid.

Oxaloacetic acid was found to run together with *cis*-aconitic acid on the column, whereas Marshall *et al.* (1952) obtained good separation of oxaloacetic from the other acids run. Both these differences can probably be attributed to the different properties of individual batches of silica gel.

The extraction of organic acids from yeast extract in Soxhlets is far less time consuming than in liquid-liquid extractors (Umbreit, Burris and Stauffer,

1951). The method was proved quantitative in two ways: (a) known amounts of standard acids were extracted from aqueous solution and run on a silica gel column, the recoveries being estimated by titration of the individual acids; (b) known amounts of succinic acid and L-malic acid were estimated manometrically after extraction, using succinoxidase (Krebs, 1937) and a suspension of *Lactobacillus arabinosus* (Nossal, 1951) respectively.

Although the method of preparing and running the column is carefully standardized so that the fraction giving the highest titration figure for any particular acid peak rarely deviates from the average by more than five tubes, the initial pressure required to achieve a flow of 22 drops/minute varied considerably (4-7 lb./sq. inch). To maintain the rate over the whole run, the pressure was gradually increased to 9-13 lb./sq. inch.

The two peaks occurring between fractions 15-20 and fractions 35-45 in Fig. 1 (Nos. 1 and 2 respectively) could be due to the same acid in the yeast. Marvel and Rands (1950) showed that an acid, which is only slightly soluble in water, highly soluble in alcohol and insoluble in chloroform, may give two peaks. We have found with fumaric acid that when the amount placed on the column was increased, the normal peak between fractions 55-65 and a second peak between fractions 15-20 were obtained. Both of these gave two-dimensional paper chromatograms identical with that of fumaric acid. But neither of the first two peaks in yeast gave an acid spot, or at the best a very faint spot moving just faster than *cis*-aconitic acid, on paper chromatograms, although applied in theoretically sufficient quantities (Fig. 4A, No. 7). Neither peak seems to be a volatile acid which is removed with the swamping acid during the drying of the papers, since the peaks themselves and a whole aqueous yeast extract contained no volatile acid (Bartley, 1953). When the neutralized column fractions of these two yeast peaks were pooled, evaporated to dryness and re-applied to a new column instead of a paper chromatogram, only a 20 p.c. recovery was obtained. The sodium salt of the yeast acid is therefore unstable to mild heating in aqueous solution, and the failure to identify it by paper chromatography is due to its decomposition during the evaporation of the pooled column fractions.

Different batches of yeast vary markedly in the absolute amounts of the various acids present, particularly succinic acid. It has become obvious that identification by comparing the number of fractions required to elute these acids and suspected known acids from the column, or even co-chromatography with known acids, is by no means infallible. The unknown acid and the suspected acid should give identical spots on a two-dimensional paper chromatogram, using solvents giving widely different R_F values for the particular acids, and, if possible, this evidence should be supplemented with chemical and physical data.

SUMMARY.

Nine organic acids of biological interest have been completely separated on a silica gel column, making use of a solvent which automatically and gradually increases in polarity. The positions of eight other acids have been established.

Except for the keto acids, the acids are recovered to an extent of 95-102 p.c.

The maximal amount of each acid which can be applied to the column and yet completely separated varies with the position at which the acid is eluted. For those acids eluted well apart from any other, up to 30 μ equiv. have been added and quantitatively recovered. For those acids which are eluted close together, no more than 5 μ equiv. of each could be added for complete separation.

The acids are identified by two-dimensional paper chromatography. These techniques have been applied to a study of the non-volatile organic acids of yeast. Eight acids occur, of which five are fumaric, succinic, α-ketoglutaric, malic and citric acids. The other three acids remain unknown.

Acknowledgments. The authors wish to thank Dr. H. J. Rodda, of the Chemistry Department, for his assistance in the preparation of oxaloacetic acid, and the Effront Yeast Co., South Yarra, Victoria, for a regular supply of yeast.

REFERENCES.

Bartley, W. (1953): Biochem. J., 53, p. 305.
Bulen, W. A., Varner, J. E. and Burrell, R. C. (1952): Anal. Chem., 24, p. 187.
Consden, R., Gordon, A. H. and Martin, A. J. P. (1944): Biochem. J., 38, p. 224.
Donaldson, K. O., Tulane, V. J. and Marshall, L. M. (1952): Anal. Chem., 24, p. 185.
Edson, N. L. (1935): Biochem. J., 29, p. 2082.
Frohman, C. E., Orten, J. M. and Smith, A. H. (1951): J. biol. Chem., 193, p. 277.
Isherwood, F. A. (1946): Biochem. J., 40, p. 688.
Krampitz, L. O. and Werkman, C. H. (1941): *Ibid.*, 35, p. 595.
Krebs, H. A. (1937): *Ibid.*, 31, p. 2095.
Malachowski, R. and Maslowski, M. (1928): Ber. dtsch. chem. Ges., 61B, p. 2521.
Marshall, L. M., Donaldson, K. O. and Friedberg, F. (1952): Anal. Chem., 24, p. 773.
Marvel, C. S. and Rands, R. D., Jr. (1950): J. Amer. chem. Soc., 72, p. 2642.
Nossal, P. M. (1951): Biochem. J., 50, p. 349.
Phares, E. F., Mosbach, E. H., Denison, F. W., Jr. and Carson, S. F. (1952): Anal. Chem., 24, p. 660.
Rockland, L. B. and Dunn, M. S. (1949): Science, 109, p. 539.
Stark, J. B., Goodban, A. E. and Owens, H. S. (1951): Anal. Chem., 23, p. 413.
Umbreit, W. W., Burris, R. H. and Stauffer, J. F. (1951): Manometric Techniques and Tissue Metabolism, 2nd Ed., p. 158. Burgess Publishing Co.