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Note

Thin-layer separation of citric acid cycle intermediates, lactic acid, and the amino acid taurine

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The *in vivo* redistribution of the ^{14}C-label from D-[U-^{14}C]glucose has been studied in the gill tissue of marine molluscs in this laboratory. One phase of the study required the separation of organic acids isolated by an ion-exchange technique. The method of separation needed to separate cleanly the citric acid cycle intermediates, lactic acid and the amino acid taurine. The separation technique needed to be inexpensive, capable of handling a fairly large sample volume (10–20 μl), relatively rapid, and needed to allow for the easy and quantitative recovery of separated ^{14}C-labeled compounds.

Several thin-layer methods for separating organic acids have been described previously[1–5]. One-dimensional methods suffer from lack of resolution[1–3]. Two-dimensional methods which utilized crystalline cellulose layers[4] were difficult to handle and exhibited considerable tailing. Separation of underivatized acids on silica gel layers resulted in crowding of acids near the origin[5]. Also, sample removal from silica gel layers can be exceedingly tedious.

Thin-layer separation methods have also been described which made use of silica gel–cellulose mixed layers[2,3,6]. This paper describes a two-dimensional mixed-layer method for separating citric acid cycle intermediates, lactic acid and the amino acid taurine. The method cleanly separates all citric acid cycle intermediates tested, excepting citric acid and isocitric acid. The solvents are inexpensive and the method allows for the rapid and quantitative removal of isolated ^{14}C-labeled acids by utilizing a simple cellulose acetate stripping mixture[7].

EXPERIMENTAL

Thin-layer plates (250 μm) were prepared according to the technique described by Turner and Redgwell[6]. Approximately 1 cm was scraped from the two edges parallel to the direction the plates were poured, to remove the excess layer from the edges; otherwise solvent flow distortion resulted. Three solvent systems were used: solvent I was 85% ethanol–1 *N* NH_4OH (4:1); solvent II was chloroform–*tert.*-amyl alcohol–90% formic acid–water (136:24:30:80), using only the lower phase; solvent

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III was anhydrous diethyl ether–90% formic acid–water (7:2:1). Ethanol, chloroform and *tert.*-amyl alcohol were glass distilled. Diethyl ether, NH_4OH and formic acid were reagent grade.

A 10–20 μl sample (50% acetone in water) was spiked with 2 μl of a 0.2 *N* standard mixture (0.4 μequiv. of each acid in 50% acetone) containing *cis*-oxalacetic acid, citric acid, isocitric acid (sodium salt), *cis*-aconitic acid, malic acid, succinic acid, α-ketoglutaric acid, fumaric acid, and lactic acid. The entire sample (12–22 μl) was loaded on the plate under a stream of warm air.

The layer was developed in the first dimension with solvent I to within 0.5 cm of the top of the layer (4–6 h). After air-drying for *ca.* 30 min the layer was developed in the second dimension with solvent II to the top of the layer (*ca.* 1 h), air dried for *ca.* 30 min and then rerun in the same dimension with solvent III to within 1 cm of the top (*ca.* 1 h). The plate was air-dried overnight in a hood and then sprayed with 0.04% bromcresol green in 95% ethanol. The pH of the indicator solution was adjusted with 0.1 *N* NaOH until a blue coloration just appeared. Amino acids may be visualized with 0.5% ninhydrin in 95% ethanol after other acids have been removed. The organic acids appeared as yellow spots against a blue background. The plate was then coated with a cellulose acetate stripping mixture and the spots removed and counted as described by Redgwell *et al.*[7].

RESULTS AND DISCUSSION

Citric acid and isocitric acid were the only citric acid cycle intermediates which were not resolved (Fig. 1). The method yielded consistent R_{malic} values (Table I) and distinct separations under all conditions despite large sample size and significant contamination with other organic acids and acidic amino acids. Contaminants were usually concentrated near the origin and the high R_F values prevented interference.

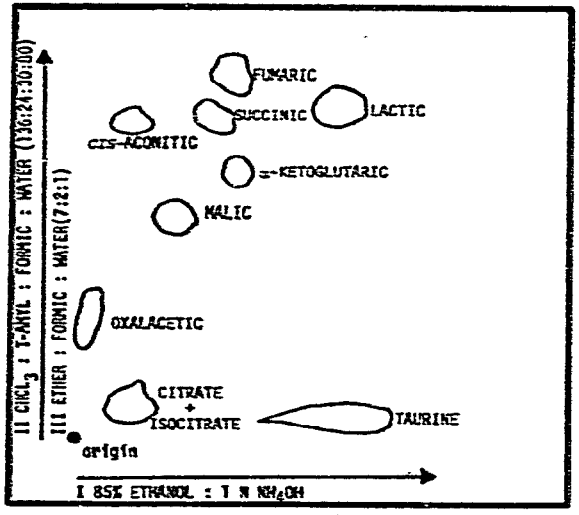

Fig. 1. Two-dimensional thin-layer chromatogram of citric acid cycle intermediates, lactic acid, and the amino acid taurine.

TABLE I

R_{malic} VALUES FOR CITRIC ACID CYCLE INTERMEDIATES, LACTIC ACID AND THE AMINO ACID TAURINE

Compound	$R_{malic} \pm 2$ S.E.M.*	
	Dimension I (Solvent I)	*Dimension II (solvents II and III)*
cis-Oxalacetic	0.44 ± 0.03	0.64 ± 0.01
Citric/Isocitric	0.60 ± 0.04	0.21 ± 0.03
cis-Aconitic	0.64 ± 0.04	1.43 ± 0.04
Malic	1.00	1.00
Succinic	1.45 ± 0.06	1.46 ± 0.03
α-Ketoglutaric	1.61 ± 0.07	1.20 ± 0.02
Fumaric	1.71 ± 0.10	1.58 ± 0.04
Taurine	2.18 ± 0.19	0.14 ± 0.02
Lactic	2.87 ± 0.24	1.47 ± 0.03

* S.E.M. = standard error of mean ($n = 8$).

Recovery of [2,3-^{14}C]succinic acid (Amersham) from two-dimensional chromatograms, using a cellulose acetate stripping mixture in the present study[7], was 96.3%. The recovery of [3-^{14}C]pyruvic acid (Amersham) was found to be very low. The recovery of oxalacetic acid is also highly questionable. The recovery of α-ketoglutaric seemed to remain consistent based on the spot size and intensity of the same standard used routinely over a 6-month period. If the recovery of keto-acids is crucial to a particular study, then the keto-acids would be best chromatogramed utilizing the derivitization procedure of Whereat *et al.*[5].

The advantages of the present method over other methods are that it does not require expensive solvents, separation is better than any other reported method, even when the sample size is large and contamination considerable. Also, the use of a mixed (silica–cellulose) layer permits the use of a simple, inexpensive stripping formulation for liquid scintillation counting of labeled compounds.

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