CHROM. 3614

Thin-layer chromatography of citric acid cycle intermediates, pyruvate and lactate

The *in vivo* steady state rates of the individual steps of the Krebs tricarboxylic acid cycle have been studied in isolated mitochondria in this laboratory. We have evaluated a number of methods for the separation and assay of each of the component acids after extraction from mitochondrial preparations.

Several paper chromatographic methods have been described (review, ref. 1) but these have been found wanting in several respects. The development of the paper chromatogram is slow; two-dimensional systems may consume several days. The sample size must be relatively large and elution of the material from the paper for enzymatic or radioassay entails large and variable losses.

A number of thin-layer chromatographic methods have been described[2–11]. These have proved much more satisfactory because of greatly enhanced sensitivity, speed of development and discreteness of separation. This report describes a system of thin-layer chromatography of Krebs cycle intermediates which has provided the best resolution of the largest number of component acids of all of the previously published methods.

Experimental

The thin-layer chromatographic plates were prepared according to STAHL[12] using Silica Gel G (Merck, Darmstadt). The gel was water-spread to 20 × 20 cm glass plates using a Desaga (Brinkmann) spreader providing a layer thickness of 0.25 mm. The plates were activated at 110° for 45 min.

Solvent system I (first dimension) was absolute ethyl alcohol–7 *N* ammonium hydroxide–water in the ratio of 200:9:40. Solvent system II was isopentyl formate–formic acid–water in the ratio of 110:20: between 5 and 7. In the latter system (II) water was added to the first two components until the resulting mixture, after shaking, remains barely turbid (*viz.* usually between 5–7 ml). The first dimension was allowed to migrate 15 cm (1 1/2 h). The plates were then dried in a 75° oven for 30 min and turned at right angles for development in the second dimension. The second dimension solvent mixture travelled 15 cm in 45 min. Better separation was achieved when filter paper was *not* placed alongside the walls of the developing tank. Final drying of the plate took about 45 min to volatilize all the formic acid. A solution of Brom Cresol Green (0.1 % in ethanol) was made very slightly alkaline by adding NaOH until the color was just barely blue-green. The plates were sprayed with this indicator after dilution to four times its volume with acetone. The acids became visible as yellow spots on a blue background. Caution must be exercised in entirely ridding the plate of formic acid when using this indicator for identification of the spots. It was found that application of 0.25 μmoles of the individual acids in pyridine provided an easily identifiable spot with little tailing. Smaller sample sizes 0.02–0.03 μmoles) could similarly be detected by using thinner (0.10 mm) layers of Silica Gel G or, alternatively, sensitivity could be enhanced by spraying with 2′,7′-dichlorofluorescein (Merck, Darmstadt) and detection of the spots in U.V. light. We preferred

not to use the fluorescein dye because of color quenching with liquid scintillation assay of radioactivity.

Methoximes were prepared by adding 10 mg of methoxylamine to individual α-keto acids or to samples of mixtures of acids in 1 ml of pyridine (dried over KOH). The reaction was carried out in a heating block for 30 min at 45°. Radioassay was

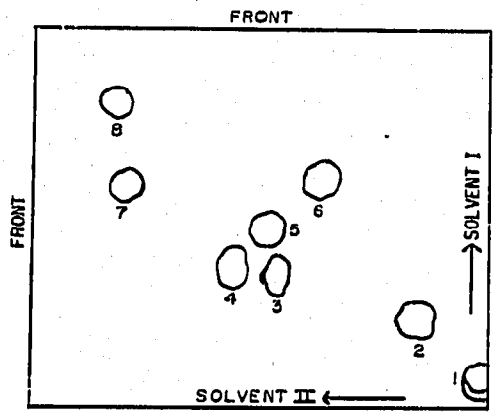

Fig. 1. Two-dimensional thin-layer chromatogram of intermediates of the citric acid cycle plus pyruvic and lactic acids. (1) = citric acid; (2) = malic acid; (3) = methoxime of oxaloacetic acid; (4) = succinic acid; (5) = methoxime of α-ketoglutaric acid; (6) = lactic acid; (7) = fumaric acid; (8) = methoxime of pyruvic acid.

performed in a Packard Tricarb liquid scintillation spectrometer, using a scintillator consisting of toluene containing a mixture of 4.0 g/l of 2,5-diphenyloxazole and 0.05 g/l of 1,4-bis[2-(5-phenyloxazolyl)]-benzene. Succinic-2,3-^{14}C acid was obtained from New England Nuclear Corporation.

Results and discussion

Fig. 1 illustrates the separation achieved by this method. The α-keto acids (α-ketoglutaric, oxaloacetic, and pyruvic) could be separated using the same solvent systems and gave discrete spots. However, the latter spots were relatively close to the origin and, as a result, the chromatogram became quite crowded in that area. Moreover, chromatography of the α-keto acids was unsatisfactory due to their instability. Oxaloacetic acid was the most elusive and often would disappear from the chromatogram and appear as pyruvic acid. Recovery of all three α-keto acids after elution from the silica gel was poor and variable. Various derivatives were prepared, including 2,4-dinitrophenylhydrazones, oximes and methoximes. Methoximes were the most satisfactory in our hands and did not interfere with liquid scintillation spectrometry as the colored nitrophenylhydrazones did. Accordingly, the illustration (Fig. 1) is a mixture of Krebs cycle acids plus pyruvate and lactate, in which the α-keto acids have been converted to their respective methoximes. Derivatization, therefore, not only stabilized these acids but also improved their chromatographic behavior by moving them further away from former close neighbors. Exposure to methoxylamine did not alter the position of the acids which did not contain carbonyl groups. Some impurity in the methoxylamine gave an acidic spot close to the solvent (I) front on the origin side and far removed from any spots of interest.

The only lack of success was the complete separation of citrate and isocitrate.

Citrate has a slightly greater R_F value than isocitrate in solvent II so that two spots could be seen, but the spots overlapped to a large extent.

Table I lists the average R_F values for each of the two dimensions for the acids studied.

It was important for us to be able to assure ourselves of consistent recoveries for radioassay. Several μC of succinate-2,3-^{14}C were chromatographed in our thin-layer system and eluted from the single succinate spot with methanol (5 ml). The latter was used as a chromatographically-pure standard. Aliquots of this standard

TABLE I

SEPARATION OF PYRUVATE, LACTATE AND INTERMEDIATES OF THE CITRIC ACID CYCLE

Acid	R_F	
	Solvent I	*Solvent II*
Citric	0.07	0.03
Malic	0.24	0.20
Oxaloacetic (MeOx)[a]	0.37	0.47
Succinic	0.39	0.56
α-Ketoglutaric (MeOx)	0.47	0.50
Lactic	0.58	0.38
Fumaric	0.60	0.72
Pyruvic (MeOx)	0.80	0.78

[a] MeOx refers to the methoxime of the respective acids.

were chromatographed both alone and in mixtures of other acids. The entire plate was assayed for radioactivity by scraping silica gel from the regions where spots were present and from the remainder of the plate. Using the chromatographically-pure succinate-2,3-^{14}C, 99.7 % of the recovered radioactivity was found in a single spot with the R_F values of succinate.

Radioassay of chromatographed isotopic (^{14}C) acids was performed by scraping silica gel from the plate directly into scintillation counting vials containing toluene and scintillator. The efficiency of counting of our samples was about 60 % due to the ^{14}C self-absorption. Elution of samples from the silica gel before counting afforded poor recovery. The method of SNYDER AND STEPHENS[13], utilizing Cab-O-Sil (Packard), a thixotropic agent, was tried but did not improve the efficiency of counting of our samples.

BARNESS and his coworkers[14] in this institution have used this solvent system for paper chromatography of Krebs cycle intermediates. The same solvent systems for thin-layer chromatography of these intermediates plus the methoximes of the α-keto acids has proved very useful and advantageous for metabolic studies. The R_F values are consistent, the separation is always distinct, the development in both dimensions is rapid and the methoximes of the α-keto acids are stable and easily separable.

Acknowledgements

This work was supported by Grant-in-aid 65 G 15 from the American Heart Association and grants HE 08805, HE 05239 and AM 2231 from the USPHS. One of

the authors (A.F.W.) is a Career Development Awardee of the USPHS (2-K3-HE 7495).

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Received May 22nd, 1968

J. Chromatog., 36 (1968) 390–393

CHROM. 3621

Chromatography of riboflavin decomposition products

Part VI. Demonstration of non-volatile contaminants of ^{14}C-acetate by thin-layer chromatography*

It is well known that commercial radiochemicals may contain considerable amounts of contaminants which are due, at least partly, to self-irradiation of solid samples.[2] This does not apply only to such reactive organic substances as methionine, but, *e.g.*, to phosphate or iodide as well. We should like to report an observation which may serve as a warning to those concerned with the conversion of acetate into other products, especially in the case of enzyme-catalyzed reactions.

We have been trying to characterize a substance which was called 69 CX in one

* For the preceding communication, see ref. 1.