Esterification and Gas Chromatography of Some Acids of the Tricarboxylic Acid Cycle

SIR: A number of workers (*1, 2, 6–9, 11, 14*) have studied the gas chromatographic separation of esters of various acids of the tricarboxylic acid (TCA) cycle. In most cases diethylene glycol succinate, or a similar polyester, was used as the stationary phase. The six nonketo TCA cycle acids were studied by Luke *et al.* (*10*) but these workers were unable to separate all of the diazomethane esterification products on diethylene glycol succinate. They reported isomerization of fumaric acid and *cis*-aconitic acid and anomalous retention times for the esters of these acids.

This paper describes the gas chromatographic separation of the methyl esters of the nonketo TCA cycle acids on diethylene glycol succinate and the results of esterification of these acids by three different procedures.

EXPERIMENTAL

Separations were performed on a Research Specialties Co. Series 600 gas chromatograph equipped with a hydrogen flame detector. To avoid decomposition of the methyl esters during chromatography, column and vaporization block temperatures were kept as low as possible. A short column with a packing material containing the minimum amount of liquid phase was used to give rapid separations.

Column Preparation. A 2-foot glass column of $\frac{1}{4}$-inch o.d. was packed with 5% diethylene glycol succinate (LAC-728) on Chromosorb W. The packing material was prepared by adding 0.25 gram of the succinate polyester in 25 ml. of chloroform to 5 grams of Chromosorb W and evaporating the mixture to dryness in a rotary evaporator. The column was equilibrated for 48 hours at 180° C. before use. When used at temperatures up to 180° C., the column was usable for about 4 weeks. During this time, retention values decreased by about 25% as a result of bleeding of the polyester liquid phase.

Preparation of Methyl Esters. The methyl esters of fumaric acid, succinic acid, L-malic acid, *cis*-aconitic acid, citric acid, and DL-isocitric acid lactone were prepared singly and in combination by three methods. The esters of maleic acid and *trans*-aconitic acid were also prepared.

DIAZOMETHANE at 25° C.: Ten-milligram quantities of the acids were dissolved in 1 ml. of methanol and a slight excess of diazomethane in ether was added. The diazomethane was prepared from nitrosomethylurea by the method of Arndt (*3*). After standing for about 30 seconds, the reaction mixture was evaporated on a rotary evaporator to a small volume (about 0.5 ml.), transferred to a 10-ml. volumetric flask, and made to volume with ether.

DIAZOMETHANE at −70° C.: Ten-milligram quantities of the acids were dissolved in 1 ml. of methanol and the solution was cooled to −70° C. in a dry ice–acetone bath. Diazomethane in ether, also at −70° C., was added slowly until a slight excess was present as indicated by the yellow color of the diazomethane. The excess was then immediately destroyed by adding dropwise 10% acetic acid in methanol. The reaction mixture was then evaporated to a small volume, transferred to a 10-ml. volumetric flask, and made to volume with ether.

METHANOL–SULFURIC ACID: Ten-milligram quantities of the acids were dissolved in 10 ml. of 5% sulfuric acid in methanol and let stand at 25° C. for 16 hours. The solution was neutralized with anhydrous sodium carbonate, diluted with 20 ml. of ether, and filtered. The filtrate was evaporated to a small volume, transferred to a 10-ml. volumetric flask and made to volume with ether.

Gas Chromatography. CONDITIONS: helium carrier gas at 1.5 p.s.i.g.; air and hydrogen at 18 and 8 p.s.i.g., respectively; vaporization block 160° C.; column temperatures, 100° C., 180° C., and 100°–180° C. using linear temperature programming (80°/24 minutes); attenuation, see Figure 1; sample volume, 5 μl. Prior to the programmed runs, the column oven was heated to 180° C., then let cool slowly to 100° C. (required about 40 minutes). An extraneous peak was obtained if the column was cooled rapidly before programming.

All esters were chromatographed singly and in combination. Pure dimethyl fumarate (m.p. 102° C.), dimethyl succinate (m.p. 19° C.), *trans* trimethyl aconitate (syrup), dimethyl L-malate (syrup), trimethyl citrate (m.p. 79° C.), dimethyl isocitrate lactone (m.p. 106°–107° C.) were prepared and used as standards to determine yields obtained by the esterification procedures.

RESULTS AND DISCUSSION

Succinic acid, malic acid, and citric acid all gave the corresponding methyl esters and these gave single chromatographic peaks with retention times in the expected order (Table I). Yields were quantitative with diazomethane at 25° C. and at −70° C. Low yields were obtained if the esterification was carried out in an ether solution. The addition of methanol greatly enhanced the yields, as was previously noted by Schlenk and Gellerman (*15*). The yields obtained using methanol–sulfuric acid were 90%, 84%, and 87%, respectively.

Fumaric acid gave the dimethyl ester in good yield (86%) with methanol–sulfuric acid. Upon esterification of fumaric acid using diazomethane at 25° C., no isomerization to dimethyl maleate, which shows a higher retention time (8.3 minutes at 100° C.) than both the fumaric and succinic esters, was observed, in contradiction to Luke *et al.* (*10*). A white crystalline product (m.p. 95°–96° C.) was obtained which showed a retention time of 52.0 minutes (100°–180° C. temperature programmed). Maleic acid gave the same product. It is probable that addition across the double bond occurred with formation of 4,5-dicarbomethoxy-pyrazoline [m.p. 97°–98° C. (*5*)]. The preparation of pyrazolines by the addition of diazomethane to double bonds adjacent to carbonyl or carboxyl groups has been reported (*4, 12, 13*). It seems likely that the unexpectedly high retention times for fumarate and maleate reported by Luke *et al.* were for the pyrazoline. At −70° C., with a limited exposure to excess diazomethane, the addition re-

Table I. Retention Times for the Methyl Esters of TCA Cycle Acids

Ester	Retention times (min.) 100° C. (isothermal)	180° C. (isothermal)	100–180° C. (programmed, 80°/24 min.)
Dimethyl fumarate	3.9		2.8
Dimethyl succinate	5.5		3.5
Dimethyl malate	47.7	1.8	10.8
cis and *trans*-Trimethyl aconitate	...	3.3	16.2
Trimethyl citrate	...	7.8	24.5
Trimethyl isocitrate	...	11.6	28.5
Dimethyl isocitrate lactone	...	21.1	38.5
4,5-Dicarbomethoxy pyrazoline (fumaric acid)	...	34.0	52.0

action was effectively halted and fumaric acid was quantitatively converted to the dimethyl ester.

cis-Aconitic acid (and its *trans*-isomer) appeared also to form a pyrazoline on prolonged exposure to excess diazomethane at 25° C. A syrupy product was obtained which gave no well-defined chromatographic peak. However, with a brief exposure to a slight excess of diazomethane, or by conducting the esterification at −70° C., the trimethyl ester was quantitatively obtained. Methanol–sulfuric acid also gave the trimethyl ester but in poor yield (45%).

The trimethyl esters prepared from *cis*- and *trans*-aconitic acid both gave a single chromatographic peak with the same retention time. No separation could be obtained even at low column temperatures (ca. 130° C). It appeared that *cis*→*trans* isomerization was taking place either during chromatography or spontaneously at room temperature. The esters were prepared by the three procedures and isolated as syrups. These were hydrolyzed with 1*N* NaOH at room temperature, then neutralized with 1*N* H_2SO_4. The hydrolysates were evaporated to dryness and the liberated acids taken up in methanol and chromatographed on Whatman 3MM paper using amyl alcohol–25% aqueous formic acid (1:1) (*4*). Both the *cis* and *trans* hydrolysates showed only the presence of *trans*-aconitic acid. Authentic *cis*-aconitic acid was run through the hydrolysis procedure and showed no conversion to the *trans* form. It is concluded that the trimethyl ester of *cis*-aconitic acid is highly unstable and undergoes spontaneous isomerization to the *trans* form.

DL-Isocitric acid lactone on treatment with diazomethane gave dimethyl isocitrate lactone (m.p. 85°–7° C.) which gave a single chromatographic peak. Esterification of the lactone with methanol–sulfuric acid also gave the dimethyl ester but the chromatograms of the esterification mixture also showed a trace second peak presumably for trimethyl isocitrate.

Gas chromatograms of the methyl esters as prepared by esterification with diazomethane at 25° C. and −70° C. are shown in Figure 1.

It is concluded that diazomethane is a suitable reagent for the quantitative esterification of the five nonketo TCA cycle acids and isocitric acid lactone but precautions must be observed to avoid the formation of pyrazolines from fumaric and aconitic acid. All esters can be separated by gas chromatography. Fumaric acid can be determined as a pyrazoline but aconitic acid cannot. Using the methods described in this paper, *cis*-aconitic acid can only be determined as the *trans* ester.

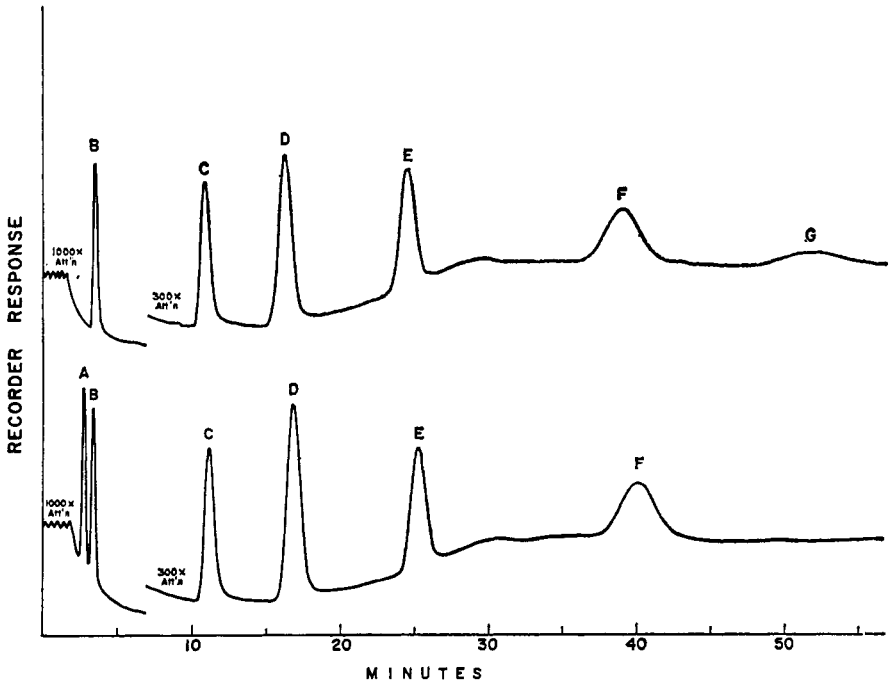

Figure 1. Gas chromatograms of equal amounts of TCA cycle acids and isocitric acid lactone esterified with diazomethane at 25° C. (upper chart) and −70° C. (lower chart)

Sepd. on 2-foot col. of 5% diethylene glycol succinate on Chromosorb W; temp. programmed from 100°–180° C. (180° C. reached after 24 minutes)
A, dimethyl fumarate; *B*, dimethyl succinate; *C*, dimethyl malate; *D*, *trans*-trimethyl aconitate; *E*, trimethyl citrate; *F*, dimethyl isocitrate lactone; *G*, 4,5-dicarbomethoxypyrazoline

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