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GAS CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF MICROQUANTITIES OF THE ESTERS OF THE TRICARBOXYLIC ACID CYCLE ACIDS AND RELATED COMPOUNDS

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SUMMARY

A column was developed which separated and determined microquantities of the methyl esters of seven tricarboxylic acid cycle acids, *viz.* fumaric, succinic, malic, α-ketoglutaric, *cis*-aconitic, citric, and isocitric acid. The 2 ft. × 1/4 in. O.D. 10% Reoplex 400 on acid-washed Chromosorb W column also separated the key intermediate of the dicarboxylic acid cycle, glyoxylate, from the tricarboxylic acid cycle acids. In addition, the column allowed determination of microquantities of the esters of pyruvic, lactic, glycollic, oxalic, malonic, maleic, itaconic, adipic, tartaric and acetoacetic acids. Of all these acid esters, only glycollic, oxalic and glyoxylic acid esters, and itaconic and maleic acid esters, could not be separated from each other. Using the dual hydrogen flame ionization detector on a Beckman GC-M, a helium flow rate of 100 ml/min and temperature programming between 50 and 200° at a rate of 5°/min, were selected as optimum conditions for the separation and determination of the acid esters. Under these conditions, all seven tricarboxylic acid cycle acid esters with the exception of α-ketoglutarate and isocitrate, could be standardized down to 0.25 μg; α-ketoglutarate and isocitrate were standardizable to 25 μg. In addition, all of the esters could be estimated in amounts as low as 0.1 μg, with *cis*-aconitate estimable to 0.05 μg.

The boron trifluoride–methanol esterification procedure was tested, and found to produce 90–100% recovery for the dimethyl esters, and 50–55% recovery for the methyl and trimethyl esters of the above-mentioned acids.

INTRODUCTION

The tricarboxylic acid cycle (TCA cycle) has been accepted for over three decades as the final common pathway for the aerobic oxidation of carbohydrates, fats and proteins in most biological systems. This cycle also plays an important role in the biosynthesis of cell material in anaerobes grown on an organic compound of low molecular weight as their sole carbon source, *e.g.* acetate. A study of the meta-

bolism of glucose requires the simultaneous determination of lactic acid, pyruvic acid, and the TCA cycle acids. Glycollate, oxalate, malonate and tartrate are important carbon sources for the growth of microorganisms. These compounds are utilized to form TCA cycle acids, so that the latter may sometimes be regarded as end products, although they are most commonly intermediates, of bacterial metabolism[1].

A lack of rapid and accurate analytical methods for these compounds, however, has remained a problem, deterring the progress of biochemical and physiological studies. The enzymatic, manometric, colorimetric and fluorimetric techniques developed for the determination of these acids are laborious and timeconsuming, and are subject to considerable error. In addition, they do not lend themselves to a simultaneous determination of all acids. Over the past few years many efforts have been made to incorporate the gas chromatograph into the analytical procedures[2–17]. However, the resolution of α-ketoglutarate[13] and oxaloacetate[6,10,12,14,16,17], the separation of succinate from fumarate[10], and the decomposition of some of the mono-, di- and tricarboxylic acids on methylation[5,10,17] has hindered the use of this technique in metabolic studies.

In this study, a comprehensive review of previous literature was made, followed by the development of a method which allows a highly sensitive and quantitative determination of eighteen mono-, di-, and tricarboxylic acids, including the seven TCA cycle acids, by temperature-programmed gas chromatography.

MATERIALS AND METHODS

Gas chromatograph

The instrument employed was a Beckman GC-M linear-programmed research gas chromatograph equipped with thermoconductivity cell and dual flame ionization detector. The recorder was a 0–100 mV Honeywell recorder.

Columns

The following columns, which were all made of stainless steel of 1/4 in. O.D., were used:

(1) 6 ft. 5% DEGS on acid-washed Chromosorb W (60–80 mesh);

(2) 6 ft. 5% DEGS on acid-washed hexamethyldisilazane (HMDS)-treated Chromosorb W;

(3) 8 ft. 15% DEGS on acid-washed HMDS-treated Chromosorb W;

(4) 6 ft. 5% DEGA on acid-washed Chromosorb W (60–80 mesh);

(5) 4 ft. 10% Carbowax 20M on acid-washed Chromosorb W (60–80 mesh);

(6) 6 ft. 4% Carbowax 20M on acid-washed HMDS-treated Chromosorb W;

(7) 4 ft. 15% Apiezon L coated with 0.1% PEG 6,000 on acid-washed HMDS-treated Chromosorb W;

(8) 6 ft. 15% SE-30 on acid-washed Chromosorb W (60–80 mesh);

(9) 4 ft. 4% SE-30 on acid-washed Chromosorb W (60–80 mesh);

(10) 6 ft. 25% E-301 (silicone elastomer) on acid-washed Celite 545 (30–80 mesh);

(11) 4 ft. 10% Reoplex 400 on acid-washed Chromosorb W (60–80 mesh);

(12) 2 ft. 10% Reoplex 400 on acid-washed Chromosorb W (60–80 mesh);

(13) 2 ft. 10% Reoplex 400 on acid-washed HMDS-treated Chromosorb W.

In order to keep the sample small, the Beckman 22 400 liquid sampler with its minimum volume size of 0.005 ml (5 μl) or a 10 μl microsyringe with 1/10 divisions from Scientific Glass Engineering Pty Ltd., Melbourne, Australia was used.

Reagents

From the British Drug Houses Ltd., London, England, were obtained: boron trifluoride (BF_3)–methanol complex (51% BF_3); chloroform, A.R.; diethyl ether, A.R.; ethyl acetate, A.R.; lactic acid, A.R.; *dl*-malic acid, L.R.; malonic acid, L.R.; methanol, A.R.; pyruvic acid, L.R.; sodium sulfate, A.R.; D(+)-tartaric acid, A.R.; adipic acid and succinic acid, A.R. Methyl citrate was purchased from City Chemical Corporation, New York, U.S.A.; the dimethyl esters of succinate, oxalate, α-ketoglutarate, maleate, itaconate and the methyl esters of acetoacetate and pyruvate in the purest grade from Fluka A.G., Buchs, Switzerland; methyl lactate, dimethyl fumarate, dimethyl malate and triethyl citrate from K & K Laboratories Inc., Plainview, N.Y., U.S.A.; *cis*-aconitic acid, *cis*-oxaloacetic acid and trisodium isocitric acid from Sigma Chemical Comp., St. Louis, Mo., U.S.A. and glyoxylic and glycollic acid from Calbiochem, U.S.A.

Column material

Chromosorb W, acid-washed (60–80 mesh), HMDS-treated Chromosorb W (60–80 mesh), diethylene glycol adipate (DEGA), Dow-Corning silicone grease 11, SE-30, and SE-52 were obtained from Analabs Inc., Hambden, Conn., U.S.A.; Carbowax 20M from Applied Science Lab. Inc., State College, Pa., U.S.A.; Apiezon L from Associated Electrical Industries Ltd., England; Celite 545 (30–80 mesh) from British Drug Houses Ltd., London, England; diethylene glycol succinate (DEGS), Reoplex 400 (polypropylene glycol adipate), and E-301 from Griffin and George Ltd., London, England; and polyethylene glycol (PEG) 6,000 from Koch-Light Laboratories, Colmbrook, Bucks., England. The 6 ft. 15% SE-30 on Chromosorb W (42–60 mesh) columns were purchased from Beckman Industries, Munich, Germany.

Column efficiency

Calculations of column efficiency in terms of the number of theoretical plates (N) in a column, and the height equivalent to a theoretical plate (HETP)[18,19] were made with respect to the fumarate ester peak on all columns, using the following formulae:

$$N = \frac{(\text{Injection point to centre of peak})^2}{\text{Width of peak at base}} \times 16 = \frac{(R_t)^2}{W} \times 16$$

$$\text{HETP} = \frac{\text{column length (ft.)}}{N} \times 305 \text{ mm} = \frac{L}{N} \times 305 \text{ mm}$$

Esterification

The acids (10–100 mg) were dissolved in 3 ml of 51% BF_3–methanol in a stoppered pyrex specimen bottle or a 150 × 20 mm capped test tube. Methylation was carried out either by shaking the tubes overnight in a 28° water bath shaker, or by heating the tubes directly in a boiling water bath for 2–$\frac{3}{4}$ min, followed by

immediate cooling in an ice-bath. The BF_3–methanol complex was then hydrolyzed by the addition of 5 ml of distilled water. The esters were extracted twice with 2 ml of chloroform by shaking vigorously for 2 min each time. The esters must be extracted no later than 5 min after the addition of the distilled water. The combined extracts were dried over anhydrous sodium sulfate, and were injected directly into the gas chromatograph.

RESULTS AND DISCUSSION

Preliminary investigations were carried out under both isothermal and temperature-programming conditions, and with varying temperatures of injection port and column oven, type of carrier gas and carrier gas flow rate, to determine which of the twelve tested columns, many of which were selected from literature, would allow optimum separation and sensitivity for the esters of the mono-, di- and tricarboxylic acids. Special emphasis was given to the resolution of the TCA cycle acids and the allied acids, lactate and pyruvate. The results of this survey are summarized in Table I.

Isothermal gas chromatography[2,3,5,9] was found to be unsatisfactory for the determination of a range of acids with such widely separated boiling points as the

TABLE I

A SURVEY OF TWELVE COLUMNS OF DIFFERENT SIZES AND WITH DIFFERENT LIQUID PHASES FOR THE SEPARATION

Substance	*5% DEGS–Chromosorb W (60–80) 6 ft. × ¼ in. O.D.*	*5% DEGS–Chromosorb W, HMDS (60–80) 6 ft. × ¼ in. O.D.*	*15% DEGS–Chromosorb W, HMDS (60–80) 8 ft. × ¼ in. O.D.*	*5% DEGA–Chromosorb W (60–80) 6 ft. × ¼ in. O.D.*	*10% Carbowax 20 M–Chromosorb W (60–80) 4 ft. × ¼ in. O.D.*	*4% Carbowax 20 M–Chromosorb W, HMDS (60–80) 6 ft. × ¼ in. O.D.*
Pyruvate	two peaks	single peak	decomposed	two peaks	single peak	
Lactate	not tested	not tested	not tested	not tested	not tested	as 4 ft. column
Succinate	good separation	good separation	good separation	good separation		
Fumarate	good separation	good separation	good separation	good separation	good separation	
Malate	one peak with citrate	one peak with citrate	close to citrate	good separation		
2-Oxoglutarate	decomposed	unstable	decomposed	decomposed	unstable	unstable
Citrate	one peak with malate	one peak with malate	close to malate	good separation	good separation	
Isocitrate	no single peak			insensitive	insensitive	
Column efficiency (H.E.T.P.)	0.34 mm	0.26 mm		0.45 mm	0.87 mm	
Theoretical plate number (*N*)	5350	7056		4096	1403	

TCA cycle acids, and lactate and pyruvate. The most widely used liquid phase, diethylene glycol succinate (DEGS), proved unsatisfactory for the analysis of the ten organic acids for several reasons. Methyl pyruvate produced two peaks on DEGS columns of all lengths and liquid loadings. At an injection port temperature of 220°, this decomposition was found to be greater than at 100°, which agreed with earlier reports[9,17]. α-Ketoglutarate also decomposed on all DEGS columns tested. This finding was also observed by McKEOWN AND READ[14], FERRAZ AND RELVAS[15] and KUKSIS AND PRIORESCHI[17], but was disputed by others[9,16], including ESTES AND BACRUANN. However, the peak illustrated by the latter workers in their chromatograph was non-symmetrical, and resembled the hump which was obtained on a 5% DEGS on Chromosorb W (60–80 mesh) column. Dimethyl fumarate produced a discrete, well-resolved peak just prior to that of succinate, which was in contrast to reports that dimethyl fumarate has a prolonged retention time on DEGS[8]. It is suspected that these workers may have been analyzing a decomposition product of dimethyl fumarate, which is formed by diazomethylation, 4,5-dicarboxymethoxypyrazoline. It was not possible to obtain a single peak for oxaloacetate on DEGS.

Diethylene glycol adipate (DEGA) also proved unsatisfactory for the analysis of the organic acids. Methyl pyruvate produced two peaks, one large and one small, and α-ketoglutarate decomposed extensively as reported by ALCOCK[12].

AND HIGHEST SENSITIVITY OF THE ESTERS OF THE TRICARBOXYLIC ACID CYCLE ACIDS

5% Apiezon + 0.1% PEG ,000 on Chromosorb W, HMDS (60–80) 1 ft. × ¼ in. O.D.	*15% SE-30–Chromosorb W (60–80) 6 ft. × ¼ in. O.D.*	*4% SE-30–Chromosorb W (60–80) 4 ft. × ¼ in. O.D.*	*25% Silicone Elastomer–Celite 545 (30–80) 6 ft. × ¼ in. O.D.*	*10% Reoplex 400–Chromosorb W (60–80) 4 ft. × ¼ in. O.D.*	*10% Reoplex 400–Chromosorb W (60–80) 2 ft. × ¼ in. O.D.*
ne peak with olvent	single peak			good separation	
ot tested	not tested				
no separation	no separation	no separation	no separation	good separation good separation	very good separation
ood separation	insensitive				
ingle symmetrical peak	good peak	as 15% SE-30		unstable	single symmetrical peak
ood separation	together with *cis*-aconitate				good separation
hree peaks	three peaks				
0.44 mm	1.5 mm			0.16 mm	0.16 mm
2750	1170			7369	3685

A Carbowax 20M column allowed excellent resolution of the methyl esters of pyruvate, succinate, fumarate, malate and citrate. However, α-ketoglutarate decomposed, and appeared as two small peaks and a larger central hump. A single peak for dimethyl α-ketoglutarate was reported on a 10% Carbowax 20M column by SPENCER[7], but unfortunately only a few operating parameters were incorporated in the report. Columns with Teflon supports[6,17] exhibited far lower efficiency in the separation of pyruvate from lactate and succinate from fumarate than those with Chromosorb W supports.

In the case of Apiezon L coated with 0.1% polyethylene glycol 6,000, methyl pyruvate emerged too closely to the solvent peak to make accurate quantitation possible, even with a low starting temperature of 50°. In addition, fumarate and succinate were not separated, and diethyl oxaloacetate decomposed extensively on the column. On the other hand, α-ketoglutarate produced a single symmetrical peak on Apiezon L.

Silicone elastomer columns have also been extensively tested for the analysis of organic acids. Methyl pyruvate was found to produce a peak incompletely resolved from that of the solvent, and malate and α-ketoglutarate were not well separated. In addition, malate produced an insensitive peak on the SE-30 columns tested, and succinate and fumarate emerged as a single peak.

The final liquid phase tested was Reoplex 400 (polypropylene glycol adipate). In the initial experiments, a 4 ft. 10% Reoplex 400 on Chromosorb W (60–80 mesh) column was used. All TCA cycle acid esters were well resolved on this column with the exception of α-ketoglutarate, which decomposed extensively, and oxaloacetate, which could not be detected. In addition, although large amounts of isocitrate produced a discrete peak, small quantities could not be detected. Methyl pyruvate was well resolved from the solvent peak, and the succinate and fumarate esters displayed very good separation. Variations in carrier gas flow rate, temperature-programming rate and injection port temperature, were unsuccessful in improving the α-ketoglutarate peak.

Column efficiency calculations on the 4 ft. 10% Reoplex 400 column revealed a very high number of theoretical plates (Table I) and the HETP value for the column was 0.16 mm. These results indicated that the Reoplex 400 stationary phase was operating more efficiently than any of the other liquid phases tested. This was evidenced in practice by the resolution of closely related esters such as succinate and fumarate, and fumarate and maleate, and by the sharp, symmetrical peaks that were produced. As Reoplex 400 allowed greater sensitivity to the TCA cycle acid esters than had any of the remaining liquid phases, a shorter column was prepared to determine whether the decomposition of α-ketoglutarate, and the apparent decomposition of isocitrate, could be lessened by a decreased retention time within the high temperatures of the column. The column prepared consisted of 2 ft. 10% Reoplex 400 on acid-washed Chromosorb W (60–80 mesh). With an injection port temperature of 150°, a helium flow rate of 100 ml/min, a temperature-programming rate of 5°/min between 50 and 200°, a hydrogen flow rate of 35.5 ml/min and an air flow rate of 40 p.s.i., all of the TCA cycle acids with the exception of oxaloacetate produced sharp, symmetrical and single peaks (Fig. 1). The chromatogram in Fig. 1 also shows the retention times and temperatures at which the individual esters appeared. The presence of oxaloacetate in the extract did not result in interference with any of the

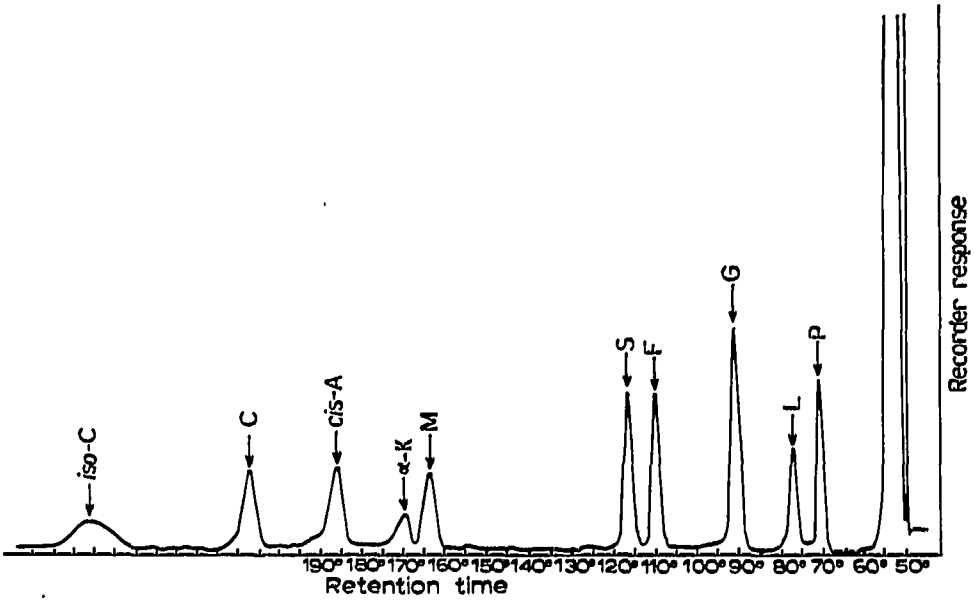


Fig. 1. Separation of the methyl esters of pyruvate (P), lactate (L), glyoxylate (G), fumarate (F), succinate (S), malate (M), α-ketoglutarate (α-K), *cis*-aconitate (*cis*-A), citrate (C) and isocitrate (*iso*-C) by temperature-programming using a Beckman GC–M gas chromatograph under the following conditions: injection port temperature, 150°; detector room temperature, 250°; carrier gas, helium; helium flow rate, 100 ml/min; dual hydrogen flame ionization detector; hydrogen flow rate, 35.5 ml/min; air flow rate, 40 p.s.i.; attenuation, 2×10^3; inoculation size, 5 μl; dual column, 2 ft. × $^1/_4$ in. O.D. Reoplex 400 on acid-washed Chromosorb W (60–80 mesh); temperature programming rate, 50–200° at 5°/min starting at the time of injection.

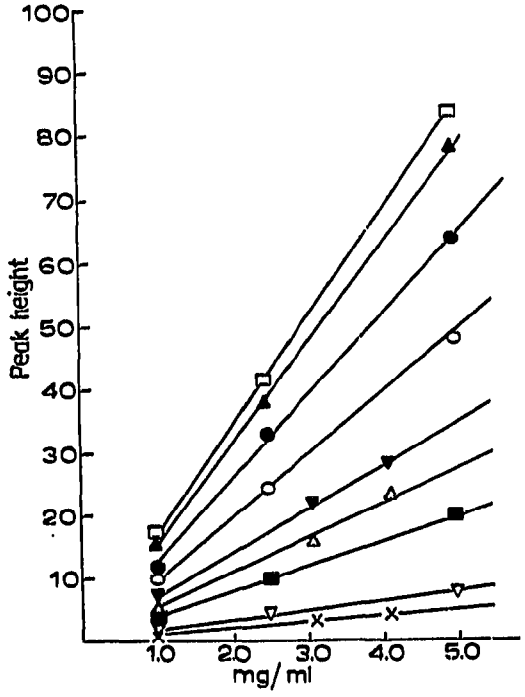


Fig. 2. Standard curves for the methyl esters of pyruvate (●—●), lactate (○—○), glyoxylate (△—△), fumarate (▲—▲), succinate (□—□), malate (■—■), α-ketoglutarate (▽—▽), *cis*-aconitate (▼—▼), citrate (■—■) and isocitrate (×—×). The values of the 5 μl injection size have been converted to mg/ml.

TABLE II

RETENTION TIMES AND PEAK CORRECTION FACTORS (DIMETHYLFUMARATE = 1.0) FOR TEMPERATURE-PROGRAMMING CONDITIONS ON GC–M AND DUAL HYDROGEN FLAME IONIZATION DETECTORS

Carrier gas flow rate, 100 ml/min; starting temperature, 50° with a rate of 5°/min up to 200°; columns, as in Fig. 1.

Methyl ester of	*Retention time (min)*	*0.25–0.5 μg* (1×10^2)	*0.5–2.5 μg* (2×10^2)	*2.5–5.0 μg* (1×10^3)	*5.0–25 μg* (2×10^3)	*25–125 μg* (1×10^4)	*125–250 μg* (2×10^4)
Pyruvate	4.2	1.5593 ± 0.3335	0.9684 ± 0.0941	0.9792 ± 0.0146	0.8886 ± 0.0553	0.9528 ± 0.0201	0.8512 ± 0.0255
Lactate	5.5	1.1411 ± 0.1089	0.6323 ± 0.0363	0.7308 ± 0.0813	0.6073 ± 0.0017	0.6540 ± 0.0215	0.6613 ± 0.0099
Oxalate	8.9	1.3629 ± 0.1371	0.6895 ± 0.1230	0.7240 ± 0.0456	0.5786 ± 0.0175	0.6227 ± 0.0056	0.6322 ± 0.0254
Succinate	14.0	1.2477 ± 0.0380	1.1207 ± 0.0542	1.0808 ± 0.0101	1.0561 ± 0.0228	1.0735 ± 0.0251	1.0898 ± 0.0241
Fumarate	12.6	1.0	1.0	1.0	1.0	1.0	1.0
Malate	23.2	0.2200 ± 0.0057	0.2320 ± 0.0054	0.2385 ± 0.0038	0.2365 ± 0.0143	0.2983 ± 0.0499	0.2531 ± 0.0065
α-Ketoglutarate	24.2	—	—	—	0.0807 ± 0.0085	0.0901 ± 0.0053	0.1343 ± 0.0110
Citrate	32.0	0.4166 ± 0.0263	0.3165 ± 0.0271	0.2801 ± 0.0229	0.2523 ± 0.0198	0.2789 ± 0.0111	0.2950 ± 0.0128

remaining TCA cycle acids, the three decomposition products of this ester appearing as small peaks between glyoxylate and fumarate, succinate and malate, and *cis*-aconitate and citrate. The appearance of these peaks in a chromatogram may be taken as an indication of the presence of oxaloacetate in a mixture; this acid ester can then be quantitated on a 6 ft. 15% SE-30 on acid-washed Chromosorb W (60–80 mesh) column.

The linearity of the peak height *vs.* concentration of the TCA cycle acid esters on attenuation 2×10^3 for the range of 5–25 μg is illustrated in Fig. 2. The peak heights were determined from the point of ascension towards the peak of the compound according to the method of CRIPPEN AND SMITH[20], which proved to be extremely satisfactory for these acid esters. Using attenuation 1×10^2, a sample of 0.25 μg was readily determinable and 0.1 μg was detectable. The linearity in the ester range of 0.25–5.0 μg was comparable with that obtained for the concentrations shown in Fig. 2. Comparisons of the detectable concentrations of the esters of pyruvate, lactate, fumarate, succinate, malate, α-ketoglutarate and citrate were made using the weights of the corresponding esters. In the case of glyoxylate, *cis*-aconitate and isocitrate, these comparisons were made on the basis of the weight of acid used for esterification. The variations in peak height which were obtained for those acids purchased as their methyl esters, are presented in Table II in the form of peak correction factors[21], calculated for each attenuation separately. The variation was very low, and was far smaller than that obtained for the C_1–C_7 branched- and straight-chain saturated fatty acids[22].

Following the quantitation of the standard methyl esters, it was of interest to learn the efficiency with which the free acids were esterified with BF_3 in methanol. During these investigations it was found that the standard methylation procedure outlined in the section MATERIALS AND METHODS does not esterify more than 100 mg of free acid; larger amounts of acid yielded inconsistent recoveries. With 100 mg of acid, however, the recoveries indicated in Table III were obtained. The dimethyl esters gave a high recovery of between 90 and 100%, with a replicate variation of only ± 2%. The methyl and trimethyl esters, however, yielded a constant 50–55%

TABLE III

PER CENT RECOVERY OF THE ACIDS OF THE TRICARBOXYLIC ACID CYCLE AFTER METHYLATION OF THE FREE ACIDS WITH THE BF_3–METHANOL COMPLEX METHOD AND GAS CHROMATOGRAPHICAL ANALYSIS

Conditions, as described in Fig. 1.

Free acid	% *recovery*
Pyruvate	52
Lactate	55
Oxalate	100
Succinate	100
Fumarate	96
Malate	90
α-Ketoglutarate	102
Citrate	52

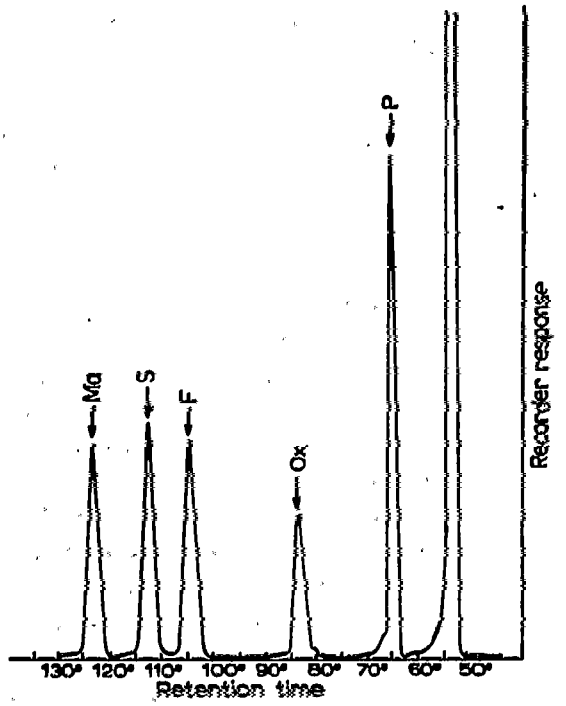


Fig. 3. Separation of the methyl esters of pyruvate (P), oxalate (Ox), fumarate (F), succinate (S) and maleate (Ma) under the conditions described in Fig. 1.

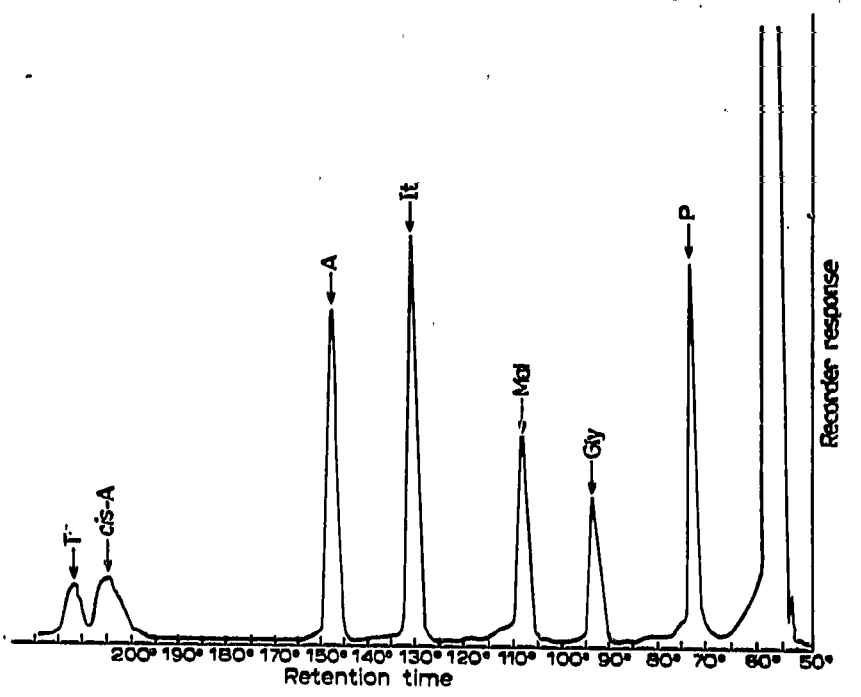
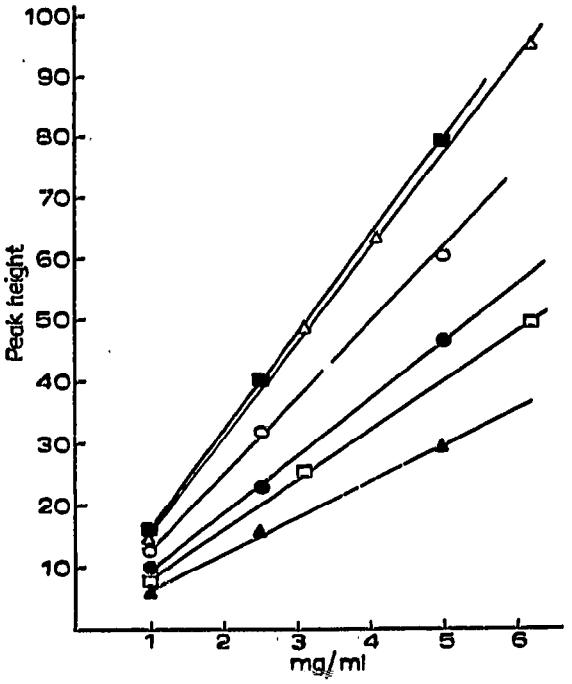


Fig. 4. Separation of the methyl esters of pyruvate (P), glycollate (Gly), malonate (Mal), itaconate (It), adipate (A), *cis*-aconitate (*cis*-A), and tartrate (T) under the conditions described in Fig. 1.

recovery, with a variation of ± 3% amongst the replicates. The method of esterification by shaking overnight, rather than heating for short periods in a boiling water bath, resulted in at least 10% higher recoveries of the esters.

Due to the high efficiency of methylation obtained for the dicarboxylic acids of the TCA cycle, the investigation was extended to include a number of other acids of importance in bacterial metabolism. Oxalate, glycollate, itaconate and tartrate are important carbon sources for many microorganisms, and adipic acid is a key intermediate in hydrocarbon metabolism[1]. The methyl esters of each of these acids produced a single, discrete peak on the 2 ft. Reoplex 400 column; in addition, all of these peaks were well resolved from those of the TCA cycle acid esters (Figs. 3 and 4). In fact, of the eighteen acids investigated, only glycollate, oxalate and glyoxylate, and itaconate and maleate eluted as combined peaks in a mixture of the eighteen esters. The inhibitor of the TCA cycle enzymes, malonate, as well as the isomer of fumarate, maleate, were well resolved from the remaining esters tested. Malonate produced a peak prior to that of fumarate; that of maleate appeared approximately 2 min after succinate.

In the preparation of methyl esters of the non-TCA cycle acids, efficient esterifications were obtained for all but glycollic and tartaric acids, which were difficult to dissolve. The linearity of the peak height *vs.* concentration plots for those acids obtained as their methyl esters is illustrated in Fig. 5 for the ester range of 5–25 μg at an attenuation of 2×10^3. Using an attenuation of 1×10^2, a sample of 0.3 μg



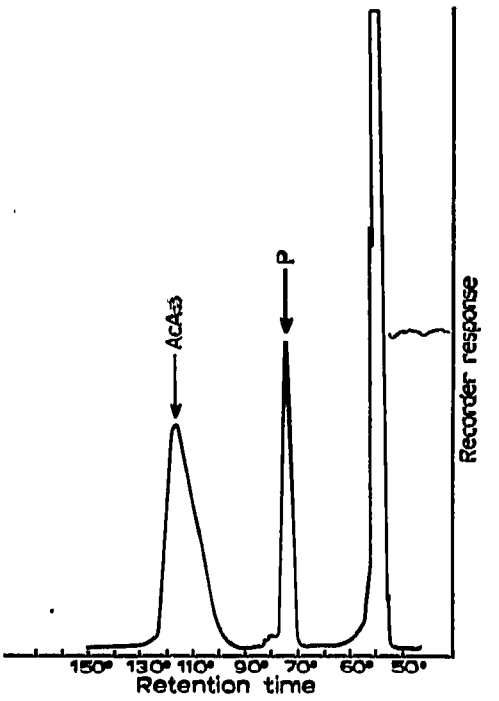


Fig. 5. Standard curves for the methyl esters of oxalate (●—●), maleate (○—○), malonate (□—□), itaconate (■—■), adipate (△—△) and acetoacetate (▲—▲). The values of the 5 μl injection size have been converted to mg/ml.

Fig. 6. Separation of the methyl esters of pyruvate (P) and aceto-acetate (AcA) under the conditions described in Fig. 1, but the rate of temperature programming was increased to 10° /min from 5 min after injection.

TABLE IV

SENSITIVITIES OF EIGHTEEN MONO-, DI- AND TRICARBOXYLIC ACIDS AS MEASURED AND ESTIMATED DURING THE STANDARDIZATION ON THE GAS CHROMATOGRAPH

Conditions, as described in Fig. 1 and for acetoacetate in Fig. 6.

Methyl esters of	*Sensitivities*	
	Measured (μg)	*Estimated (μg)*
Pyruvate	0.25	0.1
Lactate	0.25	0.1
Glyoxylate	0.30	0.2
Glycollate	—	—
Oxalate	0.25	0.1
Malonate	0.30	0.1
Fumarate	0.25	0.1
Succinate	0.25	0.1
Malcate	0.25	0.1
Itaconate	0.25	0.1
Adipate	0.30	0.1
Malate	0.25	0.2
α-Ketoglutarate	25.00	1.0
cis-Aconitate	0.30	0.05
Tartrate	—	—
Citrate	0.25	0.6
Isocitrate	25.00	0.6

or 300 ng was readily detectable. The linearity in the other concentration ranges was comparable with that shown in Fig. 5.

One additional acid is often encountered in the anaerobic carbohydrate metabolism of bacteria. Acetoacetate was the last of the acids investigated. Since this acid is formed mainly from pyruvate, the separation of these two acids was of prime interest, and was satisfactorily achieved, as shown in Fig. 6. The peak was broader and slightly less sensitive than those of the other acids investigated. An improved peak for acetoacetate was obtained by increasing the temperature programming rate to 10°/min immediately after the elution of the pyruvate peak.

The measured and estimated sensitivities of all the acids tested are summarized in Table IV. The values for tartaric and glycollic acid esters have been omitted due to the variation which occurred in esterification of these acids.

The esters were injected into the gas chromatograph both individually, and in mixtures for this standardization, no significant variation in peak heights being noted apart from tartrate and glycollate. The reproducibility was between 1 and 3%, and was comparable, therefore, to that obtained for alcohols[23] and the C_1–C_7 branched- and straight-chain fatty acids[22].

The use of HMDS-coated Chromosorb W instead of untreated, acid-washed Chromosorb W, deteriorated the resolution of the acids, and significantly reduced the sensitivity of the column.

Since the retention times of the more volatile esters were found to vary with slight changes in operating temperatures, it was necessary to ensure accurate equilibration of the starting temperature at 50° to avoid changes in retention time from run to run.

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