Esterification, Identification, and Gas Chromatographic Analysis of Krebs Cycle Keto Acids

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Esterification and gas chromatographic analysis of the keto acids of the Krebs cycle has demonstrated that the problem is more complex than originally believed. Examination of the ester products with both hydrogen flame and electron absorption detectors provides sufficient information to facilitate their identification. The identification of these esters may provide assistance with future determinations of these acids; furthermore, this information suggests suitable derivatives which might possibly avoid the complication of multiple ester products.

THE BIOLOGICAL IMPORTANCE of the Krebs cycle has encouraged the use of gas liquid chromatography (GLC) as a potentially practical method for the rapid analysis of its various members (*1–11*). It is customary to convert the free acids of the cycle to volatile methyl esters prior to chromatographic analysis, a practice which is satisfactory for most of the non-keto acids. The keto acids of the cycle are, however, unusually labile and may yield unexpected products in addition to the anticipated simple esters. Various authors (*4*, *8–11*) have recognized the difficulties which are inherent in the analysis of such acids as oxaloacetic, α-ketoglutaric, and pyruvic; however there appears to be little or no agreement as to the number and nature of the esters produced. In a recent paper, Estes and Bachmann (*11*) have reexamined the problem, but unfortunately achieved only limited success in the analysis of these ester products, which still appear to have eluded precise characterization.

Our interest in the Krebs cycle originated from the observation by Lovelock (*12*) that the alternate intermediates of the cycle possess unusually large cross sections for the absorption of thermal electrons. It was therefore considered that some preliminary diagnostic information might be gained by examining esters of the keto acids with both hydrogen flame and electron capture ionization detectors. We have esterified pyruvic, oxaloacetic, and α-ketoglutaric acids with both methanol–HCl and diazomethane. The use of diazomethane has been criticized where the methylation of polyfunctional compounds is anticipated (*13*). However, such distinct advantages as a gaseous by-product, excellent yields, and the elimination of any elaborate procedure for the recovery of the esters appear to be indispensable to several workers (*4*, *7*, *10*, *11*). Preliminary information obtained by an examination of the ester mixtures with the dual detection system, encouraged the isolation of the individual esters by preparative scale gas chromatography. The isolated esters have been subjected to infrared, mass spectral, elemental, and nuclear magnetic resonance (NMR) analysis in order to elucidate their precise structure. The complication of several ester products arising from individual keto acids may be elegantly avoided by derivative formation. Horii *et al.* (*14*) have recently described the formation of the appropriate oxime followed by conversion of the acids to trimethyl silyl ethers. Derivatives which may be promising have also been investigated during the course of this study.

EXPERIMENTAL

Apparatus. The Barber-Colman Model 5320 gas chromatograph equipped with a flame ionization detector was used as the basic instrument. The electron capture ionization detector was a design previously reported by Lovelock (*15*); the detector was adapted to fit into the oven of the Barber-Colman instrument. The polarizing potential for the electron capture detector was supplied by a Datapulse Model 102 pulse generator. Ionization currents were measured by a Cary Model 31 vibrating reed electrometer, in terms of the potential developed across a known high resistance. Chromatographic separations were achieved on two 200-foot 0.03-inch i.d. stainless steel capillary columns (Handy and Harmon Tube Co., Norristown, Pa.). One capillary column was coated with a 10% solution of Apiezon L, the second column was coated with a 10% solution of Ucon 50 HB 2000. Both columns were conditioned for 24 hours in an atmosphere of nitrogen prior to chromatography.

Preparative scale separations of the reaction mixtures were performed with a Varian Aerograph Model 712 chromatograph, using a 50-foot × 1/4-inch diameter column packed with 40–60 mesh Gas Chrom P coated with SE 30 silicone phase.

Mass spectra were taken with the LKB 9000 mass spectrometer-gas chromatograph (LKB Produkter, AB., Stockholm, Bromma 1, Sweden).

NMR spectra were recorded on a Varian Model HA-100 nuclear magnetic resonance spectrometer. Infrared data were collected with a Beckman Model IR 10 spectrophotometer. Elemental analyses were performed by Huffman Laboratories Inc. (Wheatridge, Colo.).

Reagents. Reagents used in the esterification of the keto acids were prepared as follows: Diazomethane freshly pre-

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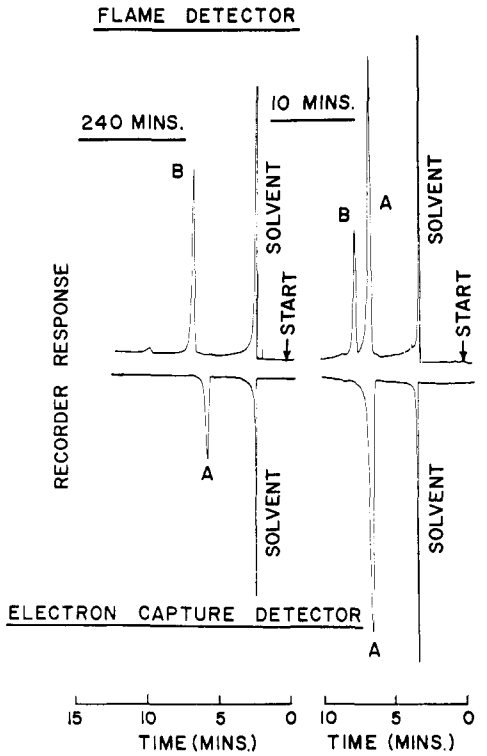

Figure 1. Methyl esters of α-ketoglutaric acid formed by reaction with diazomethane

Column 200 feet × 0.03-inch i.d. capillary coated with 10% Ucon 50 HB 2000. Isothermal 135°C. Nitrogen flow rate 30 ml/min

pared in ethereal solution from "Diazald" (*N*-methyl *N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co.) by the method of DeBoer (*16*).

Methanol–HCl was prepared fresh every two days by bubbling dry HCl gas into anhydrous Spectrograde methanol until the concentration was 10% by weight.

The keto acids used in this study were purchased from two sources, K & K Chemical, N. Y. and Calbiochem Corp., California.

Procedure. Diazomethane was used in excess, and the progress of the reaction was monitored by injection of 1-μl aliquots of the reaction mixture into the gas chromatograph at 5-minute intervals; the reaction was observed for a total of 4 hours. After reaction with diazomethane, the excess reagent and ether solvent were removed in a stream of dry nitrogen.

In the preparation of esters from methanol–HCl, 1 gram of the free acid was weighted into a 50-ml flask, and 25 ml of the 10% methanol–HCl was added and the mixture refluxed for 6 hours on an oil bath maintained at 70° C. After reaction the volume of the mixture was concentrated to approximately 2 ml using a rotary vacuum pump. Esters were recovered by the dilution of the reaction mixture with 15 ml of distilled water followed by extraction with five separate 5-ml volumes of diethyl ether. The combined ethereal fraction was subsequently dried over anhydrous magnesium sulfate. Promotion of the methanol–HCl esterification with 2,2-dimethoxypropane (*17*) was also studied using a modification previously reported by these authors (*18*). Ester mixtures from all esterification procedures were separately analyzed with both hydrogen flame and electron absorption detector systems.

Samples fractionated by preparative scale GLC were rechromatographed on the two analytical columns in order to reference the isolated esters to a specific peak in the reaction mixture.

Preparation of Derivatives. DIMETHYL KETALS. Two-hundred milligrams of the keto acid was dissolved in 20 ml of 10% methanol–HCl. Then 2 ml of redistilled trimethyl orthoformate was added and the mixture was refluxed for 6 hours on an oil bath maintained at 70° C. After reaction the mixture was neutralized by addition of saturated sodium bicarbonate solution, and the volume was concentrated to approximately 10 ml by evaporation on a rotary vacuum pump. The residue was extracted with four separate 5-ml volumes of ether, and the combined ether fractions were dried over anhydrous magnesium sulfate.

RESULTS

The analysis of α-ketoglutaric esters produced by reaction with diazomethane is illustrated in Figure 1. The upper chromatogram is the response from the flame detector, while the lower chromatogram, which has been inverted, represents the response of the electron absorption detector. It was soon observed that the ratio of the two products (i.e., peaks *A* and *B*, 10-minute reaction) varied with the time allowed for reaction with diazomethane. Two peaks are observed during the first hour but the proportion of the second peak *B* increased steadily as the reaction progressed until the first peak *A* was almost entirely converted into *B* (240-minute reaction). It can also be seen that peak *A* absorbs electrons intensely while peak *B* has no affinity for thermal electrons. The intensity of electron absorption by ester *A* was particularly noticeable in the four-hour reaction where no trace of *A* appears on the hydrogen flame channel while a significant signal still appears on the electron capture chromatograph, even though the latter sample was diluted 1000-fold. This information is significant since compound *A* must contain one or more of the "electrophore" groups discussed by Lovelock (*12*); conversely peak *B* should contain no electrophore. The electrophore present in dimethyl α-ketoglutarate (i.e., esterified only at the two terminal carboxylic acid groups) would be the —CO·CO— structure, an intense electron absorber. Simply from a negative standpoint it is unlikely that peak *B* is dimethyl α-ketoglutarate.

Esters *A* and *B* have been separated by preparative scale GLC and subjected to NMR, infrared, and elemental analysis. Table I lists the NMR and elemental analysis data for the various ester products.

The H′ NMR spectrum of compound *A* indicated the structure was dimethyl α-ketoglutarate. The ester mixture has been analyzed by the combination LKB mass spectrometer–gas chromatograph, which permitted a mass spectrum to be taken for individual esters. The mass spectrum of compound *A* showed a parent ion peak at *m/e* 174, and a base peak at *m/e* 115 which suggests the loss of either terminal carbomethoxy group (i.e., M-59).

The mass spectrum of compound *B* showed a parent ion peak at *m/e* 188 which suggests that this structure differs from *A* by the presence of an additional methylene group.

The H′ NMR spectrum showed a pair of doublets at δ 2.78 ppm and 3.02 ppm ($|J_{AB}| = 3$ cps) which may be assigned to an AB proton case. A complex pattern centered at δ 2.25 ppm may be assigned to an AA′BB′ system. This compound is therefore believed to be methyl 2-(carbomethoxyethyl) glycidate. Elemental analysis supports this view. Further-

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Table I. NMR and Elemental Analysis Data for α-Keto Acids

Structure	NMR Data (H^1 NMR Spectra), Varian HA 100	Elemental analysis
A and *C*. $CH_3OOCCOCH_2CH_2COOCH_3$ (a, b, c)	(a or b) Pair triplets δ2.6 ppm and 3.08 ppm [Area = 2] (c) Two single resonance peaks at δ3.65 3.85 ppm [Each peak area = 3]	Calcd for $C_7H_{10}O_5$: C 48.27, H 5.79, O 45.94 Found: C 48.1, H 5.93, O 45.97
B. $CH_3OOC-C(H(a))(H(b))-CH_2CH_2COOCH_3$ (e, c, d, e)	H (a); H (b) Pair of doublets at δ2.78 and 3.02 ppm (c and d) Complex pattern centered at δ2.25 ppm which may be assigned to an AA'BB' system (e) Two single absorptions at δ3.6 and 3.7 ppm [Each peak area = 3]	Calcd for $C_8H_{12}O_5$: C 51.06, H 6.42, O 42.52 Found: C 51.38, H 6.52, O 42.10
D. $CH_3OOCC(OCH_3)_2CH_2CH_2-COOCH_3$ (d, c, a, b, d)	(a and b) Multiplet symmetrical centered at δ2.04 ppm (c) Single resonance at δ3.12 ppm [Area = 6] (d) Two single absorptions at δ3.51 and 3.61 ppm [Each peak area = 3]	Calcd for $C_9H_{16}O_6$: C 49.08, H 7.33, O 43.59 Found: C 49.14, H 7.22, O 43.64
E and *G*. $CH_3COCOOCH_3$ (a, b) *F*. $CH_3-C(H(a))(H(b))-COOCH_3$ (c, d)	H (a); H (b) Pair of doublets at δ2.56 2.94 ppm [Area = 2] $\lvert J_{AB}\rvert$ = 3 cps (c) Singlet at δ1.49 ppm [area = 3] (d) Single resonance at δ3.65 ppm [Area = 3]	Known compound
H. $CH_3C(OCH_3)_2COOCH_3$ (a, b, c)	(a) Singlet at δ1.4 ppm [Area = 3] (b) Single resonance peak at δ3.18 ppm [Area = 6] (c) Single resonance peak at δ3.64 ppm [Area = 3]	Calcd for $C_6H_{12}O_4$: C 48.64, H 8.17, O 43.19 Found: C 48.74, H 8.11, O 43.15
I (trans) $CH_3OOCC(OCH_3)=CHCOOCH_3$ (a, a, a, b)	(a) Three single resonance peaks at δ3.66, 3.76, and 3.84 ppm [Each peak area = 3] (b) Single sharp resonance peak at δ5.99 ppm [Area = 1] vinylic proton Note: NMR taken of mixture	Mixture, *I*, *J*, and *K* unfractionated No elemental analysis reported
J. $CH_3OOCC(H(a))(H(b))-CH_2COOCH_3$	H (a); H (b) Pair of doublets at δ2.78 & 3.03 ppm $\lvert J_{AB}\rvert$ = 3 cps Mixture of *I*, *J*, and *K* did not permit more precise analysis Note: NMR taken of mixture	
K. (cis) $CH_3OOCC(OCH_3)=CHCOOCH_3$		
L. (trans) $CH_3OOCC(OH)=CHCOOCH_3$ (a, b, c, a)	(a) Two single resonance peaks at δ3.73 and 3.77 ppm (Each peak area = 3) (b) Singlet at δ5.91 ppm (Area = 1) Vinylic proton (c) Broad absorption at δ11.38 ppm (Area = 1) Enolic OH and intramolecularly hydrogen bonded	Calcd for $C_6H_8O_5$: C 45.00, H 5.04, O 49.96 Found: C 45.09, H 5.26, O 49.65
M. $CH_3OOCC(OCH_3)_2CH_2COOCH_3$ (c, b, a, c)	(a) Singlet at δ2.78 ppm (b) Single resonance peak at δ3.17 ppm [Area = 6] (c) Two single resonance peaks at δ3.54 and 3.66 ppm [Each peak area = 3]	Calcd for $C_8H_{14}O_6$: C 46.60, H 6.84, O 46.56 Found: C 46.43, H 6.96, O 46.61
N. (cis) $CH_3OOCC(OH)=CHCOOCH_3$		

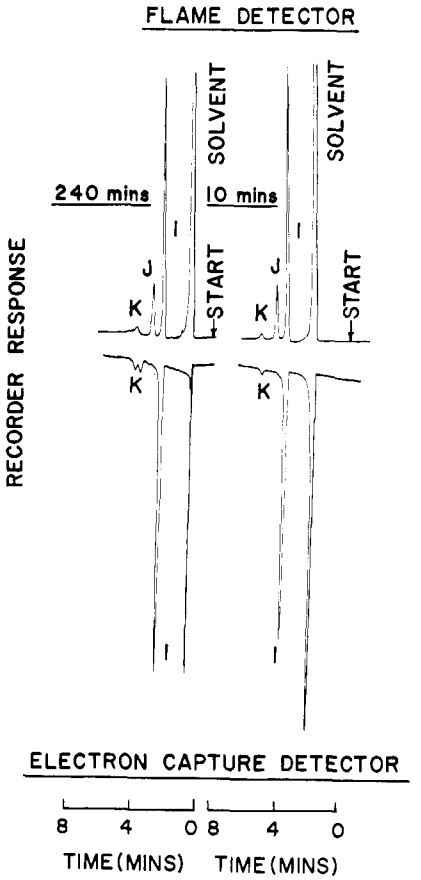

Figure 2. Methyl esters of oxaloacetic acid formed by reaction with diazomethane

Conditions as in Figure 1

more, it is interesting that no electron absorption is predicted for cyclic ethers (*19*).

Reaction of α-ketoglutaric acid with methanol–HCl yielded two major products in approximately equal amounts. The first peak eluted *C* gave a strong electron capture response, and was found to have the same NMR and mass spectrum as well as the same retention time as dimethyl α-ketoglutarate (Compound *A*). The second peak eluted *D* gave no electron capture signal, and preparative scale GLC fractionation followed by NMR analysis indicated the structure was a dimethyl ketal. The elemental analysis confirmed that compound *D* was dimethyl 2,2-dimethoxyglutarate.

Although pyruvic acid is not strictly a part of the Krebs cycle, it is often analyzed with other Krebs cycle components because of its central role in intermediary metabolism. Reaction of pyruvic acid with diazomethane was strikingly similar to that of α-ketoglutaric acid. Two principal products were observed during the first 2 hours and again the first compound eluted from the chromatograph *E* was rapidly converted into the second product eluted *F*.

The first component *E* was therefore predicted to be methyl pyruvate. Its infrared spectrum was superimposable with the infrared spectrum of an authentic sample of methyl pyruvate. The second compound *F* was purified as was *E* by preparative scale GLC. A lack of electron capture response and the analytical data classified compound *F* as methyl 2-methylglycidate. This ester was expected to be a major product from the data of Arndt *et al.* (*20*).

Pyruvic acid was also similar to α-ketoglutaric acid in its reaction with methanol–HCl. Two major products were formed in approximately a 50:50 ratio. From the analytical data the first component *G* eluted from the GLC was found to be methyl pyruvate. The second component *H* was predicted to be a ketal, as may be deduced from a lack of electron capture response and the similarity of the reaction to α-ketoglutaric acid. This prediction was again substantiated by the analytical data. Compound *H* is most likely to be methyl 2,2-dimethoxypyruvate.

Oxaloacetic acid has previously proved to be the most difficult of the keto acids to analyze. The products of the reaction with diazomethane are shown in Figure 2; the proportion of the two major products do not change significantly with exposure to excess diazomethane over a 4-hour period. Peak *I* absorbed electrons intensely and constituted approximately 85% of the total yield. Preparative scale GLC was unfortunately unable to resolve this mixture under the conditions used. The H′ NMR spectrum of the mixture at low dilution showed three separate resonance peaks at δ 3.66, 3.76, and 3.84 ppm. The only other resonance was at δ 5.99 ppm which may be attributed to a vinylic proton.

Infrared analysis gave a distinct absorption band at 1645 cm^{-1} which is characteristic for the $-\overset{OCH_3}{\overset{|}{C}}=CH-$ structure. The mass spectrum of peak *I* showed a parent ion at *m/e* 174; it was also found that peak *K* gave a very similar mass spectrum which suggested that peaks *I* and *K* were probably cis-trans isomers. Unfortunately the trace amount of peak *K* precluded any precise determination.

They are believed to be dimethyl 2-methoxymaleate and dimethyl 2-methoxyfumarate. Peak (*I*) is preferred as the trans isomer for two reasons: it is thermodynamically the more favored isomer, and on both analytical columns dimethyl fumarate has a shorter retention time than the corresponding cis isomer.

The mass spectrum of peak (*J*) also showed a parent ion at *m/e* 174; but the rest of the spectrum did not agree with the spectra of peaks *I* and *K*. However, careful examination of the NMR spectrum of the mixture at greater sensitivity showed a pair of doublets at δ 2.78 and 3.02 ppm $|J_{AB}| = 3$ cps which is reminiscent of the AB proton pair present in the structure of the epoxide ring. It seems reasonable that some methyl 2-(carbomethoxymethyl)glycidate might be formed from attack of the keto form of oxaloacetic acid by diazomethane. The fact that compound (*J*) was transparent to thermal electrons supports the view that the structure probably contains an epoxide ring.

The reaction of oxaloacetic acid with methanol–HCl is illustrated in Figure 3. Peaks (*L*) and (*N*), which were found to have similar mass spectra, were formed in significant amounts when the esterification was performed at ambient temperature and the methylation was promoted by 2,2-dimethoxypropane. When the free acid was refluxed only with methanol–HCl on an oil bath at 70° C the major product was compound (*M*) and only traces of (*L*) and (*N*) were detected. Fractionation by preparative scale GLC and subsequent NMR analysis showed that peak (*L*) is dimethyl oxaloacetate, or

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perhaps more correctly dimethyl 2-hydroxyfumarate. Crystals of this compound melted at 75°–77° C which is in good agreement with the melting point reported for dimethyl oxaloacetate by Fenton and Jones (*21*). Peak *N* is predicted to be the corresponding cis isomer although it is present only in trace amount and no definite identification has been achieved. Peak *L* is preferred as the trans isomer for the reasons which applied in the case of the cis and trans methoxy diesters. The major product *M* showed two separate resonance peaks at δ 3.54 and 3.66 ppm, in the H′ NMR spectra, which can be attributed to two methoxy groups. Two single absorptions at δ 2.78, 3.17 ppm are attributed to a single methylene and a ketal group, respectively. The infrared analysis was consistent with the structure proposed, dimethyl 2,2-dimethoxysuccinate. A lack of electron capture response for this compound as would be predicted was also observed.

In the preparation of derivatives, both oxaloacetic and α-ketoglutaric acids formed ketals smoothly, when prepared by the method described in the experimental section. Extraction into ether solution gave yields of 80 and 68% for ketals of α-ketoglutaric acid and oxaloacetic acid, respectively. No attempt has been made in this investigation to optimize the synthesis and extraction of ketal derivatives, although some loss of product is believed to occur during the extraction procedure.

DISCUSSION

It is apparent that simple methylation of biologically active intermediates can produce unexpected products. Furthermore the electron absorption detector can be quite successfully employed as an additional monitor of biologically interesting compounds. The success which can be achieved is clearly demonstrated in the case of the oxaloacetic esters. Previous data (*12*) indicated that the —CO—CH=CH—CO— structure which is present in dimethyl fumarate absorbs thermal electrons intensely. This conjugated electrophore will also be present in dimethyl oxaloacetate (i.e., dimethyl 2-hydroxy fumarate) and dimethyl 2-methoxy fumarate, as well as their cis isomers; and these compounds should elicit a large signal from the electron absorption detector. Conversely the absence of such conjugated electrophores as in dimethyl 2,2-dimethoxysuccinate and methyl 2-(carbomethoxymethyl)glycidate should make these compounds transparent to the passage of thermal electrons. Although highly sophisticated analytical techniques may be required later to completely establish the structure of an unknown compound, an electron absorption detector may provide substantial clues as to the presence or absence of certain structures within the molecular framework. It can be of considerable value in those cases where a biological extract is believed to contain a certain active intermediate, since if the structure contains one or more of the electrophore groups discussed by Lovelock (*12*) a large electron capture response should be observed. The use of electron absorption detectors in this context has been seldom exploited but the technique would appear to have considerable potential

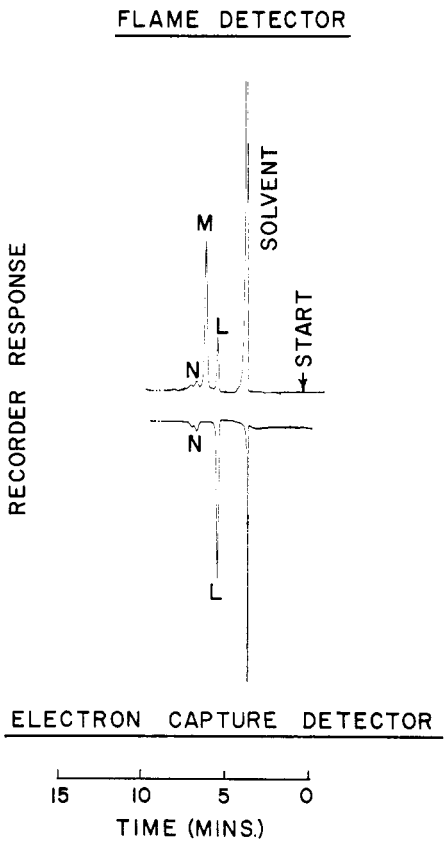

Figure 3. Methyl esters of oxaloacetic acid formed by reaction with methanol–HCl

Conditions as in Figure 1

The similarity in the reactions of pyruvic and α-ketoglutaric acids with the methylating agents is best explained if both acids are considered as true keto acids. The lack of any appreciable enolization would thereby account for the easy formation of the epoxide rings, upon reaction with diazomethane (*22*).

In contrast oxaloacetic acid is believed to be considerably enolized and consequently yields predominately the enol ester after reaction with diazomethane.

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