

CHROM. 15,253

GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC IDENTIFICATION OF *n*-BUTYLATION PRODUCTS OF SOME COMMON KETO CARBOXYLIC ACIDS

L. MARAI and A. KUKSIS*

Banning and Best Department of Medical Research, University of Toronto, 112 College Street, Toronto M5G 1L6 (Canada)

(Received July 29th, 1982)

SUMMARY

Mass spectra were obtained for the *n*-butylation products of 2-ketopropionic, 2-ketosuccinic and 2-ketoglutaric acids by combined gas chromatography-mass spectrometry (GC-MS). The esterification reaction was accompanied by the formation of variable amounts of *n*-butyl ketals, which were resolved by GC and identified by MS. The fragment structures were further confirmed by means of the perdeuterated *n*-butyl derivatives.

INTRODUCTION

Gas-liquid chromatographic (GLC) estimation of Krebs' cycle and related acids has remained of much interest in biomedical applications despite the need for preliminary extraction and derivatization with possible loss of volatiles and formation of artifacts¹. The popularity of GLC analysis is due to its high speed, high sensitivity of detection of the acids^{2,3} and the ease of combination with mass spectrometry (GC-MS) for exact identification of components^{4,5}.

In recent years increasing use has been made of the butyl esters as derivatives for GLC^{6,7} and for GC-MS^{8,9}, because of their lower volatility and the possibility of resolution of enantiomers as the diastereoisomers, when employing enantiomeric isobutanol in the derivatization^{10,11}. Furthermore, the butyl esters can be purified by thin-layer chromatography (TLC) prior to GC-MS, which is not possible with the commonly used methyl esters due to volatility or with the trimethylsilyl esters due to volatility and chemical instability¹². The esterification of the keto acids, however, leads to by-products, which may be easily confused with the normal esters. In the following communication we have determined the mass spectra of *n*-butylation products of the common keto acids as a means of distinguishing between normal esters and ketals generated during the esterification reaction, and have proposed the revision of some previously reported structures.

EXPERIMENTAL

α -Ketoglutaric acid was obtained from Aldrich, Milwaukee, WI, U.S.A. and oxalacetic acid from Nutritional Biochemicals, Cleveland, OH, U.S.A., while pyruvic, oxalsuccinic and adipic acids were supplied by Sigma, St. Louis, MI, U.S.A. Perdeuterated *n*-butanols ($C^2H_3C^2H_2C^2H_2C^2H_2O^2H$ and $C^2H_3C^2H_2C^2H_2C^2H_2OH$) were purchased from Merck, Sharpe and Dohme, Montreal, Canada. Other reagents and solvents were of Certified Reagent Grade supplied by Fisher Scientific, Don Mills, Canada.

Preparation of n-butyl esters

The *n*-butyl esters were prepared by treating the free acids or their sodium salts (5–15 mg) with sufficient 3–6% (v/v) sulphuric acid in *n*-butanol to cover the dry residue (0.25–0.5 ml). The esterification was completed by heating the tightly closed vials at 80°C for 15–240 min¹³. After cooling the contents of the vials were diluted with water and the butylation products extracted with chloroform. Deuterated *n*-butyl esters were prepared similarly.

Thin-layer chromatography

The butyl esters of the organic acids were purified by TLC on silica gel H (Merck, Darmstadt, G.F.R.) plates (20 × 20 cm), 250 μ m thick layer, using petroleum ether (b.p. 30–60°C)–diethyl ether–formic acid (80:30:1) as the developing solvent. The separated components were located by exposing the plates to iodine vapour. They were recovered by extracting the gel with chloroform and washing the extracts with water.

Gas chromatography–mass spectrometry

Combined GC–MS analyses of the *n*-butyl esters of the organic acids were made with Varian MAT CH-5 single focusing mass spectrometer coupled to a Varian Data 620i computer previously described for work with fatty acid methyl esters¹⁴. The GLC separations were made with a Varian Model 2700 Moduline gas chromatograph equipped with a 180 × 0.3 cm I.D. glass tube filled with 3% Silar 5CP on Gas-Chrom Q. The carrier gas was helium at 10 ml/min. The gas chromatograph which did not contain a separate detector was temperature-programmed from 100 to 275°C at 6°C/min with the injector at 250°C. The transfer line was maintained at 275°C. All spectra taken over the GLC peak were corrected for background by the computer using Varian Module SUB. Alternately, mass spectra were obtained for all compounds on a Hewlett-Packard 5985B quadrupole mass spectrometer coupled via a jet separator to a 5840A gas chromatograph, equipped with a similar Silar 5CP column or with a 25-m SP-2100 fused-silica capillary column. The mass spectrometer was operated in the electron impact (EI) mode. The mass spectrometer was coupled to an HP-1000E computer, HP 7906 disk drive, HP 2648A graphics terminal and a Tectronix 4632 video hard copy unit.

RESULTS AND DISCUSSION

Preparation and chromatographic properties

The simple mono-, di- and tricarboxylic acids are stable compounds and give quantitative yields of the *n*-butyl esters under a variety of reaction conditions, while the keto acids are known to enolize and to produce variable amounts of mono- and diketals as by-products of the esterification¹⁵. The *n*-butyl derivatives of these acids possess excellent TLC and GLC properties and are resolved into pure species on the basis of molecular weight and overall polarity (results not shown). Table I gives the R_F values on silica gel H and the relative retention times on a variety of liquid phases for the simple esters and the alkylation products of the different keto acids. In all instances the mono- and diketals are clearly resolved from each other and from the corresponding simple esters. In addition, the two geometric forms of the monoketals of oxalacetic and α -ketoglutaric acid esters are also resolved by GC. The quantitative yields of the esters and of the alkylation products for these keto acids are summarized in Table II. The yields are given relative to that of the *n*-butyl ester of adipic acid which was quantitatively derivatized at all experimental conditions tested. It is seen that pyruvic and α -ketoglutaric acids reach maximum yields of the ester within 15 min at 80°C with 3–6% H_2SO_4 . The formation of the *n*-butyl pyruvate is accompanied by a parallel dialkylation of about 40% of the ester. The proportion of the dialkyl ester of α -ketoglutarate under these conditions is about 20%. In both instances only small amounts of the monoketal were formed. There is no significant change in the proportions of these products over the next 120–240 min. The β -keto acids (oxalacetic and oxalsuccinic) undergo extensive decarboxylation to the lower homologous keto compounds, which also are subject to alkylation. Thus, the oxalacetic acid yields about 65–70% of the diketal and 2% monoketal along with 30% of the diketal and trace of monoketal of pyruvic acid, and 1–2% of the simple *n*-butyl pyruvate. None of the

TABLE I
CHROMATOGRAPHIC BEHAVIOUR* OF *n*-BUTYL DERIVATIVES OF SELECTED KETO ACIDS

Derivatives	TLC (R_F values) Silica gel H	GLC** (relative retention times)		
		3% OV-1	3% Silar SCP	SP-2100
Pyruvate	1.17	0.35	0.07	0.13
Monoketal	1.35	0.51	0.41	0.36
Diketal	1.55	0.83	0.54	0.75
Ketoglutarate	1.00	1.00	1.00	1.00
Monoketal	1.25	1.29	1.076/1.10	1.247/1.254
Diketal	1.44	1.49	1.14	1.41
Oxalacetate	ND***	0.88	0.82	0.82
Monoketal	1.15	1.23	1.11	1.10/1.125
Diketal	1.32	1.36	1.05	1.23

* Relative to *n*-butyl α -ketoglutarate.

** Other chromatographic conditions as given in text.

*** ND = not determined.

TABLE II

RELATIVE YIELD (MOLE%) OF *n*-BUTYLATION PRODUCTS OF COMMON KETO ACIDSYields relative to *n*-butyl adipate formed in 100% yield at all conditions.

Reaction time (min at 80°C)	Pyruvic*			α -Ketoglutaric**			Oxalacetic*		
	Ester	Monoketal	Diketal	Ester	Monoketal	Diketal	Ester	Monoketal	Diketal
15	56	Trace	44	95	<1.0	4	—	—	—
30	59	Trace	41	89	1.0	10	—	—	—
60	63	1.0	36	81	1.0	13	—	—	—
120	65	1.0	34	79	1.0	20	Trace	<2.0	65.70
240	63	1.0	36	79	1.0	20	—	—	—

* 6% H₂SO₄ in *n*-butanol.** 3% H₂SO₄ in *n*-butanol.

oxalsuccinic acid remained intact during the acid reaction conditions, 100% of it being converted into the corresponding simple *n*-butyl ester of α -ketoglutaric acid and its mono- and diketal derivatives in proportions previously recorded for the α -ketoglutaric acid itself. In all instances the diketal derivatives were present in much higher concentration than the corresponding monoketals. The identity of the various GLC peaks was confirmed by GC-MS of the *n*-butyl and the deuterated *n*-butyl derivatives as explained below.

Mass spectrometric properties

The overall electron impact fragmentation patterns of the keto acid esters are well established¹⁶ although those of their alkylation products have remained in doubt. Except for one attempt⁸, the mass spectra of the *n*-butyl esters of the keto carboxylic acids have not been reported. Fig. 1 gives the mass spectra of the three products recovered from the *n*-butylation of pyruvic acid. The simple *n*-butyl ester (Fig. 1A) is recognized from the parent ion at m/z 144 and characteristic fragments. The base peak at m/z 43 represents the CH₃CO portion of the original acid, as previously shown by Cronholm *et al.*¹⁷ for methyl pyruvate. The ion at m/z 85 represents the carbon from the carboxyl group along with the *n*-butyl group. It was shifted 9 mass units higher following esterification with perdeuterated *n*-butanol. The ion at m/z 57 represents the butyl group and those at m/z 41 and m/z 29 further breakdown products of it, as indicated by appropriate shifts in the masses of the corresponding ions in the spectra of the perdeuterated *n*-butyl esters. The monoketal of *n*-butyl pyruvate gave a molecular ion at m/z 200 (Fig. 1B) with the base peak at m/z 57. Other prominent ions (m/z 145, 99, 89) are generated by a loss of 55, 101 and 111 mass units due to the elimination of CH₃CH₂C=CH₂ (2-but-1-enyl), CH₃(CH₂)₃OC=O (carboxybutyl), and CH₃CH₂C=CH₂ + CH₃CH₂CH=CH₂ (1-butane) fragments, respectively, from the molecular ion as confirmed by means of the deuterated *n*-butyl derivative. The diketal of *n*-butyl pyruvate failed to give a molecular ion, but yielded a significant ion corresponding to M - 73 (m/z 211) due to a loss of a butoxy [CH₃(CH₂)₃O] group (Fig. 1C). Further cleavages produced ions at m/z 173, 145, 117, 89 and 61, which resulted from loss of masses 56, 73 and 101 in a variety

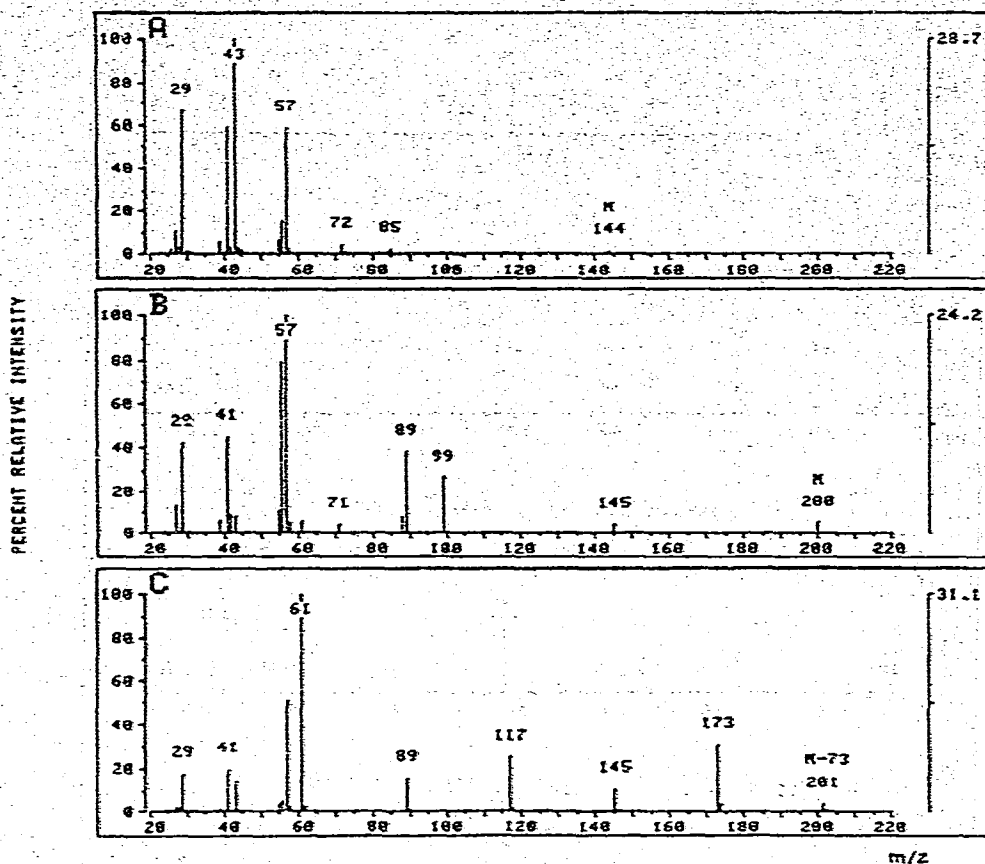


Fig. 1. GC-MS spectra of the *n*-butylation products of pyruvic acid. (A) *n*-butyl ester; (B) *n*-butyl monoketal; (C) *n*-butyl diketal.

of sequences. The base peak at m/z 61 is specific for ketals and the formation of this fragment is confirmed by the absence of a molecular ion¹⁶. The mass spectrum reported by Choudhary and Moss⁸ for authentic *n*-butyl pyruvate including a base peak at m/z 61 is therefore in error, as it must have been derived from the fragmentation of the ketal. In such a case, the fragment assigned a mass of m/z 144 by these authors should have had a mass of m/z 145, as shown here. Cronholm *et al.*¹⁷ have also suggested that the derivative formed from methyl pyruvate is probably 2,2-dimethoxypropionate. In any event the base peak at m/z 61 cannot be derived from the simple *n*-butyl ester of pyruvate.

Fig. 2 gives the mass spectra obtained for the *n*-butyl α -ketoglutarate and its mono- and diketal. The simple *n*-butyl ester (Fig. 2A) gives a small molecular ion at m/z 258. The base peak is at m/z 101 and represents multiple losses of fragments [$M - (101 + 56)$] and includes one rearranged hydrogen, as indicated by a shift of one mass unit (m/z 102) in the perdeuterated *n*-butyl ester. The ion at m/z 129 is due to a loss of a butoxy radical plus a butene group ($M - 129$) and contains one rearranged

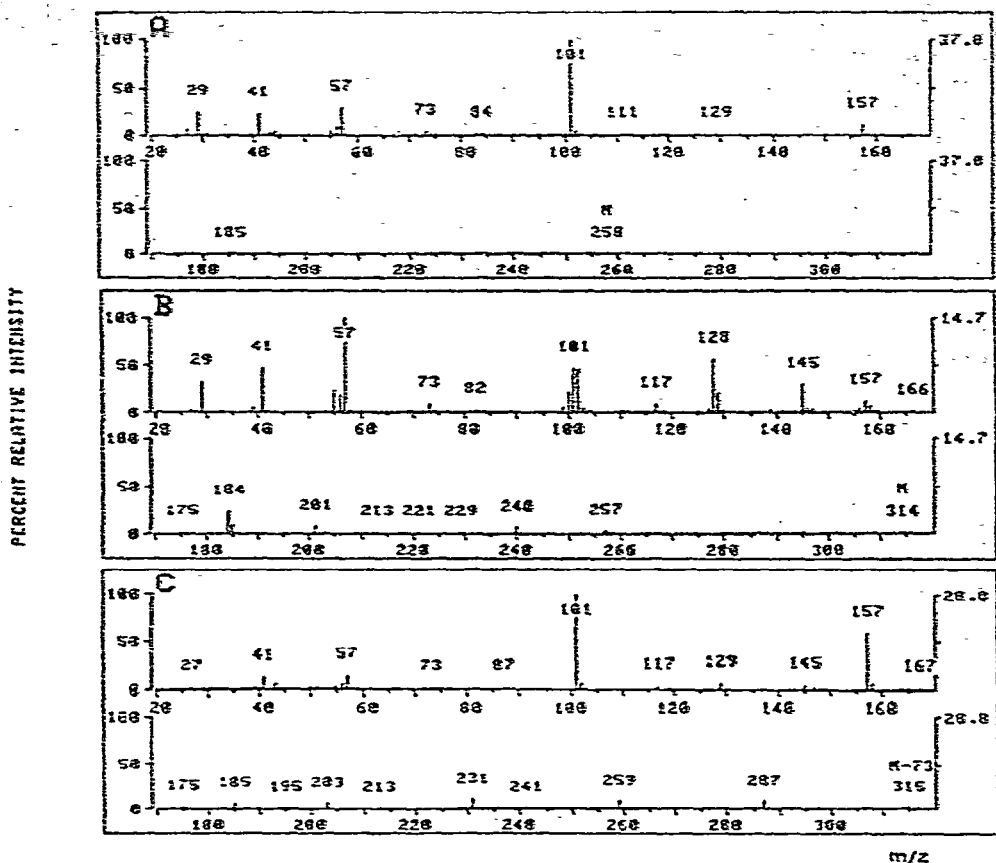


Fig. 2. GC-MS spectra of the *n*-butylation products of α -ketoglutaric acid. (A) *n*-butyl diester; (B) *n*-butyl monoketal; (C) *n*-butyl diketal.

hydrogen as indicated by a shift of one mass unit, when analyzing the perdeuterated ester. This hydrogen arises from the side chain as shown for butyl propionate¹⁸. The ions at m/z 157 and 185 represent loss of 101 and 73 mass units, due to the elimination of carboxybutyl and butoxy groups, respectively. These fragments are shifted by 9 mass units, when derived from the deuterated ester, confirming the presence of an intact butyl ester group in each of them. The monobutyl ketal of *n*-butyl α -ketoglutarate gives a molecular ion at m/z 314 (Fig. 2B). It has a base peak at m/z 57. A loss of 74 mass units provides an ion at m/z 240 representing the elimination of an intact butanol group probably from the ketal function which includes one hydrogen from the adjacent methylene carbon. Other ions are generated by the loss of mass 101 (m/z 213) and via combined losses of butyl, butoxy and carboxybutyl groups in a variety of sequences to yield fragments at m/z 201, 184, 157, 145, 128 and 101, with relative intensities ranging from 15 to 75%. The correctness of these assignments was confirmed by appropriate shifts in the masses of the ions from the corresponding derivatives prepared with deuterated *n*-butanol. The two forms of the monoketal give identical mass spectra. The dibutyl ketal of the *n*-butyl α -ketoglutarate yielded no

molecular or $M - 55$ ion (Fig. 2C). It gave a base peak at m/z 101, which has the same structure as that found in the simple ester and is formed via combined losses of groups [$M - (101 + 73 + 57 + 56)$]. The other ions can be recognized on the basis of systematic multiple losses of common fragments consistent with the fragmentation pattern proposed by Simmonds *et al.*¹⁹ for the dimethyl ketal derivative of methyl α -ketoglutarate. This identification of the various ions is again supported by the fragmentation pattern observed for the deuterated *n*-butanol reaction products.

Fig. 3 gives the mass spectra for the *n*-butyl derivatives of oxalacetic acid. The simple *n*-butyl ester of oxalacetic acid was not recovered by TLC among the reaction products. However, on GLC of the diketal, a peak was obtained, which possessed all the ions anticipated for the simple ester. This peak showed (Fig. 3A) a molecular ion at m/z 244, and other characteristic ions at m/z 189 ($M - 55$) and m/z 171 ($M - 73$) in low intensity. The ion at m/z 143 represents a fragment formed by a loss of one carboxybutyl radical ($M - 101$). Combined losses of one butoxy radical and one butene group ($M - 129$) yield an ion at m/z 115. The base peak at m/z 87 is produced via the concerted loss of one carboxybutyl and one butene group. The ion at m/z 69 is

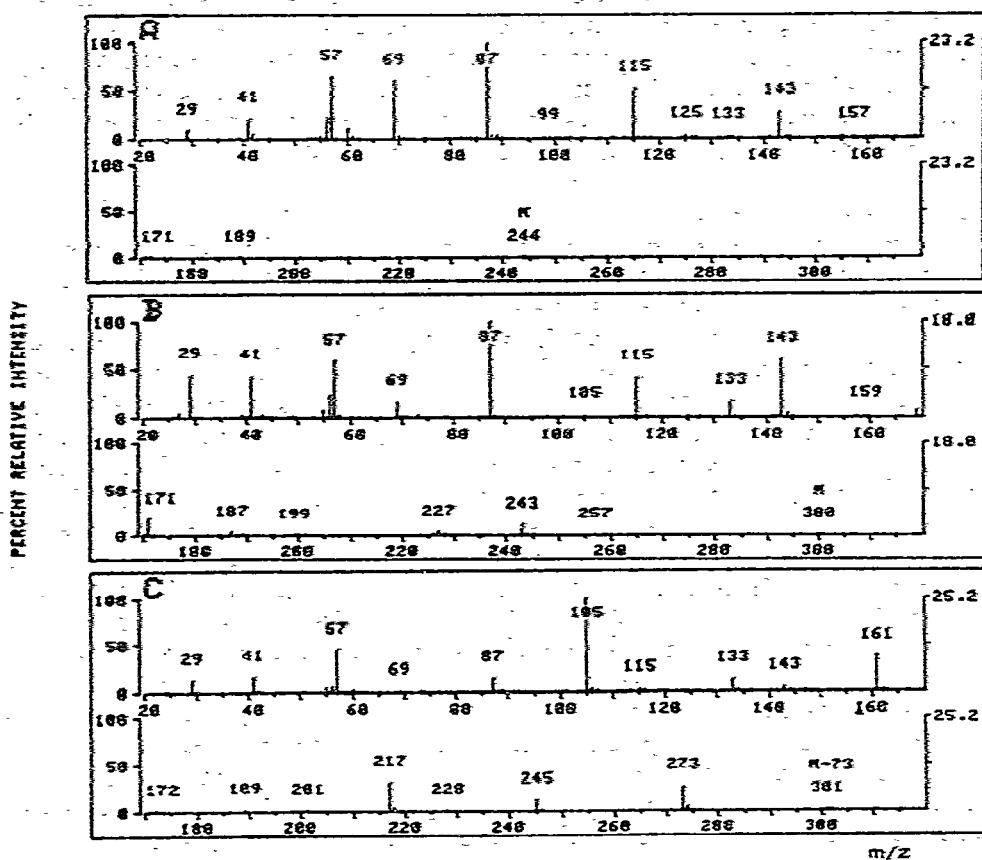


Fig. 3. GC-MS spectra of the *n*-butylation products of oxalacetic acid. (A) *n*-butyl diester; (B) *n*-butyl monoketal; (C) *n*-butyl diketal.

generated by elimination of one carboxybutyl and one butanol group. The derivatives prepared with deuterated *n*-butanol yielded the anticipated corresponding mass shifts. Furthermore, the molecular weight of the simple ester was confirmed by methane chemical ionization spectrum. The monobutyl ketal of *n*-butyl oxalacetate yielded a small molecular ion at m/z 300 (Fig. 3B). It gave a base peak at m/z 87 and characteristic major ions at m/z 115, 143 and 171, which possessed the same structures as the corresponding ions from the simple *n*-butyl ester. These ions are produced via multiple cleavages or losses of common fragments, as confirmed by the mass spectra of the corresponding deuterated *n*-butyl derivatives. The two forms of the monobutyl ketal possessed identical mass spectra. The spectrum of Fig. 3B corresponds closely to spectrum ID NO-EJ 140 (Peak 1) reported for a methyl ether of methyl oxalacetate by Markey *et al.*⁹, which may therefore be identified as that of the monomethylketal of oxalacetic acid. The dibutyl ketal of *n*-butyl oxalacetate gave no molecular ion (Fig. 3C) as expected for a diketal¹⁰. It had a base peak at m/z 105, which results from loss of a carboxybutyl and three butene groups. The other fragment ions are formed by multiple cleavages or losses of common fragments, the identity of which again is confirmed by the mass spectra of the corresponding derivatives prepared with deuterated butanol. This spectrum has similarities to the spectrum ID NO-EJ 141 (Peak 2) reported by Markey *et al.*⁹ for another methyl ether of methyl oxalacetate, which can now be identified as that of the dimethylketal of oxalacetate. However, the ion at m/z 175 must have been mistaken previously for m/z 174. As noted above for the pyruvate derivative, it is clearly a dialkyl ketal spectrum.

Deuterium-hydrogen exchange

The activated hydrogens in the methyl group of the pyruvate and in the methylene group of oxalacetate undergo deuterium-hydrogen exchange during esterification of the acids with *n*-butanol containing deuterium in the hydroxyl function. Fig. 4A shows the mass spectrum obtained for the simple *n*-butyl ester of pyruvic acid prepared with perdeuterated *n*-butanol. The ion at m/z 43 represents the $\text{CH}_3\text{C}=\text{O}$ portion of the original acid, while the ions at m/z 44–46 in the spectrum represent the deuterium-hydrogen exchange products on the methyl group. It can be calculated that the methyl group contained 25% $^2\text{H}_0$, 43% $^2\text{H}_1$, 26% $^2\text{H}_2$ and 5% $^2\text{H}_3$ species. Comparable enrichment of the deuterium content of the methyl group was found in the mono- and diketal derivatives of pyruvic acid prepared with perdeuterated *n*-butanol. The deuterium-hydrogen exchange in the methyl group of the pyruvate served to support the identification of the ion at m/z 61 by the formation of ions at m/z 64 and 65 in the perdeuterated *n*-butyl diketal derivative (Fig. 4B), with one and two deuterium atoms in the methyl group and two deuterium atoms associated with the oxygen function (see Scheme 1).

Fig. 4C shows the mass spectrum obtained for the diketal of oxalacetic acid derived from esterification with perdeuterated *n*-butanol. It can be seen that all the fragments possessing the methylene group contain 1 or 2 deuterium atoms arising from a deuterium-hydrogen exchange on the methylene group during derivatization. It was calculated from several fragments containing the methylene group (*e.g.* $M - 101$, $M - 129$) in the simple ester and in the mono- and diketal derivatives that the extra deuterium averaged 44% $^2\text{H}_1$ and 31% $^2\text{H}_2$ with 23% $^2\text{H}_0$ species. A similar deuterium-hydrogen exchange was noted when the pyruvic and oxalacetic acids were

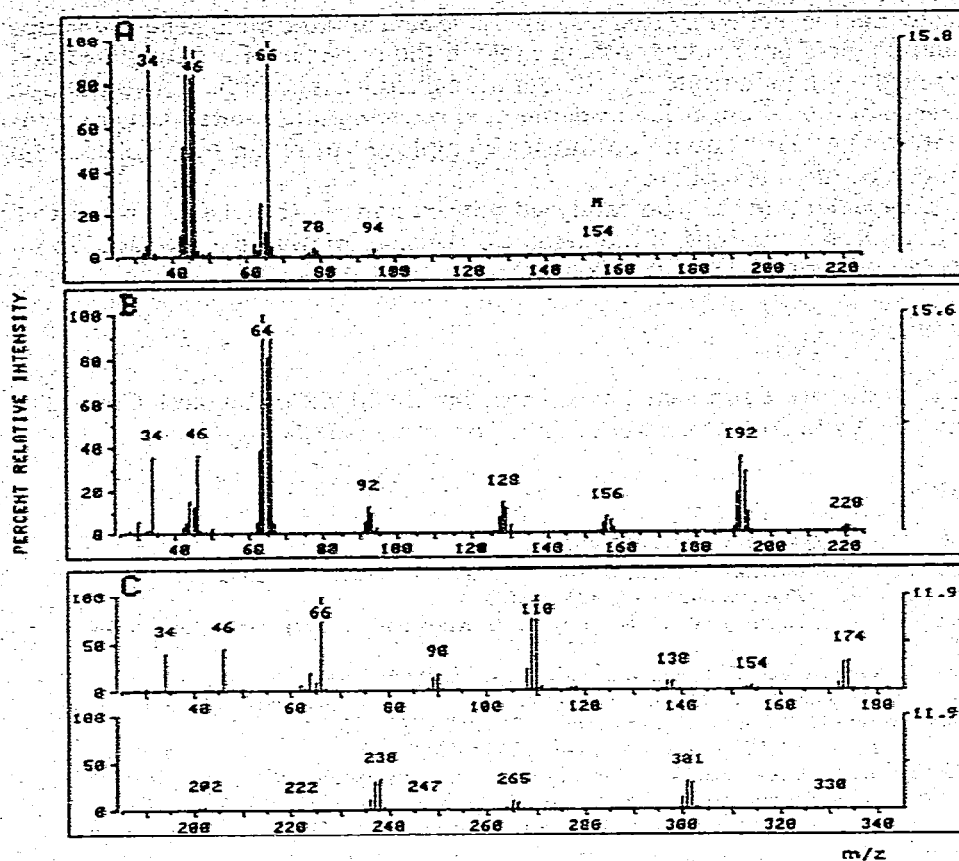
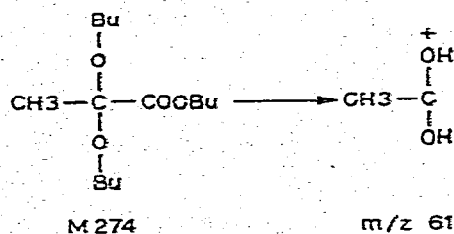


Fig. 4. GC-MS spectra of the reaction products of pyruvic and oxalacetic acids with perdeuterated *n*-butanol. (A) perdeuterated *n*-butyl pyruvate; (B) perdeuterated *n*-butyl diketal of pyruvate; (C) perdeuterated *n*-butyl diketal of oxalacetate.

butylated in the presence of $^2\text{H}_2\text{SO}_4$. No such deuterium exchange was noted with *n*-butanol containing all the deuterium bound to carbon atoms (*n*-butanol $^2\text{H}_9$, with undeuterated OH group). As noted by Djerassi and Fenselau¹⁸ the deuterium atoms from the side chain could contribute to the mass of certain ion fragments (e.g. McLafferty rearrangement ions) aside from any contribution of the deuterium in the methyl group of the pyruvate or of the methylene group of the oxalacetate. These observa-



Scheme I.

tions indicate that the methyl hydrogens in pyruvic acid and the methylene hydrogens in the oxalacetic acid are readily exchangeable in the presence of a strong acid and cannot be relied upon to indicate their deuteration during a metabolic transformation in the presence of deuterium oxide or deuterated reducing nucleotides. Other means of *n*-butylation (e.g. diazobutylation) must be employed to retain the deuterium in these positions of the metabolites.

It is concluded that the acid catalyzed preparation of *n*-butyl esters is not well suited for quantitative analysis of the keto acids because of multiple product formation and partial or complete destruction of the original acids.

ACKNOWLEDGEMENT

These studies were supported by grants from the Medical Research Council of Canada and the Ontario Heart Foundation, Toronto, Canada.

REFERENCES

- 1 E. Jellum, *J. Chromatogr.*, 143 (1977) 427-462.
- 2 A. Kuksis and P. Vishwakarma, *Can. J. Biochem. Physiol.*, 41 (1963) 2353-2362.
- 3 D. T. Canvin, *Can. J. Biochem.*, 43 (1965) 1281-1287.
- 4 C. E. Daigliesh, E. C. Horning, M. G. Horning, K. L. Knox and K. Yarger, *Biochem. J.*, 101 (1966) 792-810.
- 5 B. Eliasson, S. Linstedt and G. Steen, *J. Lipid Res.*, 17 (1976) 637-646.
- 6 J. B. Brooks, D. S. Kellogg, L. Thacker and E. M. Turner, *Can. J. Microbiol.*, 17 (1971) 531-543.
- 7 M. A. Lambert and C. W. Moss, *J. Chromatogr.*, 74 (1972) 335-338.
- 8 G. Choudhary and C. W. Moss, *J. Chromatogr.*, 128 (1976) 261-270.
- 9 S. P. Markey, W. G. Urban and S. P. Levine (Editors), *Mass Spectra of Compounds of Biological Interest*, Vol. 2, U.S. Atomic Energy Commission, Oak Ridge, TN, 1975, p. 130.
- 10 J. G. Lawless, B. Zeitman, W. E. Pereira, R. E. Summons and A. M. Duffield, *Nature (London)*, 251 (1974) 40-42.
- 11 B. Zeitman, S. Chang and J. G. Lawless, *Nature (London)*, 251 (1974) 42-43.
- 12 Z. Hosil, M. Makita and Y. Tamura, *Chem. Ind. (London)*, (1965) 1494.
- 13 A. Kuksis and W. C. Breckenridge, *J. Amer. Oil Chem. Soc.*, 42 (1965) 978-982.
- 14 J. J. Myher, L. Marai and A. Kuksis, *Anal. Biochem.*, 62 (1974) 188-203.
- 15 J. B. Hendrickson, D. J. Cram and G. S. Hammond, *Organic Chemistry*, McGraw-Hill, New York, 4th ed., 1970, pp. 328-329.
- 16 F. H. Beynon, R. A. Saunders and A. E. Williams, *The Mass Spectra of Organic Molecules*, Elsevier, New York, 1968, p. 176.
- 17 T. Cronholm, H. Matern, S. Matern and J. Sjovall, *Eur. J. Biochem.*, 48 (1974) 71-80.
- 18 C. Djerassi and C. Fenselau, *J. Amer. Chem. Soc.*, 87 (1965) 5756-5762.
- 19 P. G. Simmonds, B. C. Petit and A. Zlatkis, *Anal. Chem.*, 39 (1967) 163-167.