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HYDROXYCARBOXYLIC AND OXOCARBOXYLIC ACIDS IN URINE: PRODUCTS FROM BRANCHED-CHAIN AMINO ACID DEGRADATION AND FROM KETOGENESIS

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SUMMARY

Hydroxy- and oxomonocarboxylic acids in urine of healthy individuals and of patients with diabetic ketoacidosis are analysed as methyl esters and methyl esters/O-methyloximes, respectively, by gas chromatography and gas chromatography—mass spectrometry. The derivatives are pre-fractionated by thin-layer chromatography.

The acids originate mainly from ketogenesis and from the metabolism of valine, leucine and isoleucine. The amino acid metabolites fall into three groups: the 2-oxocarboxylic acids (2-oxoisovaleric acid, 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid); the 2-hydroxycarboxylic acids (2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid); and the 3-hydroxycarboxylic acids (3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, 3-hydroxy-2-ethylpropionic acid, *threo*-3-hydroxy-2-methylbutyric acid and *erythro*-3-hydroxy-2-methylbutyric acid). The *threo* form of 3-hydroxy-2-methylbutyric acid is the major constituent within the diastereomeric pair. Of the three groups of amino acid metabolites, the 3-hydroxycarboxylic acids in particular are elevated during ketoacidosis.

The characteristic general features of the mass spectrometric fragmentation of the derivatives of the identified components are systematically described. The discussion of the fragmentation includes constituents of low concentrations, such as 3-oxocaproic acid, 4-oxobutyric acid and 5-oxocaproic acid, which can be detected only when the pre-fractionation technique is applied.

INTRODUCTION

Hydroxy- and oxomonocarboxylic acids have been described as urinary excretion products in a number of congenital and acquired metabolic disorders; some have been found in normal urines too. 2-Oxocarboxylic acids and 2-hydroxycarboxylic acids were reported to be characteristic metabolites in maple syrup urine disease [1—3]; 3-oxocarboxylic acids and 3-hydroxycarboxylic acids have been described in propionic acidaemia [4, 5]; and several 3-

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hydroxycarboxylic acids in cases of ketoacidosis [6, 7]. Even though some of the acids have been reported as normal urinary constituents [8—10], they have been described only in small groups and under specific aspects. This paper deals with a systematic investigation of hydroxy- and oxomonocarboxylic acids in urine of healthy individuals and of patients with diabetic ketoacidosis, with the emphasis on those acids that originate from ketogenesis and from the metabolism of the branched-chain amino acids.

EXPERIMENTAL

Sample preparation

The sample preparation procedure included the reaction of the urine samples with O-methylhydroxylamine hydrochloride to form the O-methyloximes from the oxocarboxylic acids, the isolation of the organic acids by anion-exchange using Amberlyst A-26, the formation of the methyl esters with diazomethane, and the pre-fractionation of the derivatives by preparative thin-layer chromatography (TLC). The details of the method have been described [11]. Only the following modifications were employed. The urine sample (66 ml) was added slowly and under shaking to 134 ml of isopropanol, and any precipitated proteins were removed by centrifugation. The supernatant solution was added to 330 mg of O-methylhydroxylamine hydrochloride, kept at 65°C for 1 h and then applied in equal portions to two 25-cm long anion-exchange columns. For small samples (10 ml of urine), TLC plates with a 0.25-mm thick layer of silica gel were sufficient; the larger samples used in this study required a gel thickness of 2 mm. Instead of pre-fractionating the acid derivatives into the previously described four fractions, a total of eight fractions was obtained by dividing fraction 2 into two equally broad subfractions 2a and 2b, and fraction 3 into four equally broad subfractions 3a—d.

Gas chromatographic and mass spectrometric analysis

Fractions 2a, 2b and 3a—d were analysed by gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS). The GC separations were performed on a Model 3700 gas chromatograph with a flame-ionization detector (Varian, Darmstadt, F.R.G.) under the following conditions: 25 m × 0.2 mm I.D. fused-silica column, coated with OV-1701 (Scientific Glass Engineering, Weiterstadt, F.R.G.); carrier gas, nitrogen at 4 ml/min; column temperature, 40°C for 10 min, then programmed at 2°C/min; injector block temperature, 250°C; sample size, 1 μl at a splitting ratio of 1:20. Under the chosen conditions the derivatives of the hydroxycarboxylic and oxocarboxylic acids appeared in the early portions of the chromatograms (within 40 min).

For the GC—MS analyses a combination of a Model 2700 gas chromatograph, CH 5 mass spectrometer and Spectrosystem 100 MS computer (Varian-MAT, Bremen, F.R.G.) was used. The gas chromatograph and the mass spectrometer were interfaced by an open coupling system. By automatic repetitive scanning, the mass spectra were recorded over the mass range *m/e* 15—300 and stored on magnetic tape. Helium was used as the carrier gas. Otherwise, the same GC conditions were used as described for the GC separations. The MS conditions were as follows: ionization by electron impact; ionization energy,

70 eV; accelerating voltage, 3 kV; multiplier voltage, 2.25 kV; emission current, 300 μA; ion source temperature, 220°C; interface temperature, 220°C; resolution, 600.

Reference substances

The described hydroxycarboxylic and oxocarboxylic acids were identified on the basis of the mass spectra and the GC retention indices of the derivatives of the urinary components and of reference substances. The reference compounds were either purchased or synthesized. Glycolic acid, methyl lactate, sodium salt of 2-hydroxybutyric acid, 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid, 3-hydroxypropionic acid, pyruvic acid, 2-oxobutyric acid, 2-oxovaleric acid, 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid were purchased from Fluka (Neu-Ulm, F.R.G.), methyl 2-hydroxyisobutyrate and 5-oxocaproic acid from EGA-Chemie (Steinheim, F.R.G.), sodium salt of 2-hydroxy-3-methylvaleric acid, 2-oxoisovaleric acid and 4-oxobutyric acid from Sigma Chemie (Munich, F.R.G.), methyl 3-hydroxybutyrate and methyl 3-oxobutyrate from E. Merck (Darmstadt, F.R.G.). The identification of 3-hydroxyisobutyric acid and 3-hydroxyisovaleric acid was based on reference spectra of the methyl esters from the literature only [7, 12]. Methyl 3-hydroxy-2-ethylpropionate, methyl 3-hydroxy-2-methylbutyrate and methyl 3-oxocaproate were synthesized.

Methyl 3-hydroxy-2-ethylpropionate was synthesized by reduction of ethylmalonic acid with $LiAlH_4$, methylation of the resulting 3-hydroxy-2-ethylpropionic acid with diazomethane and purification of the methyl ester by preparative TLC.

Methyl 3-hydroxy-2-methylbutyrate (mixture of the two diastereomers) was prepared from methyl 3-oxo-2-methylbutyrate by reduction with $NaBH_4$ and purification on an Extrelut® column (Merck) using ethyl acetate as the eluent. By this method about equal amounts of the *threo* and *erythro* forms were obtained.

Methyl *erythro*-3-hydroxy-2-methylbutyrate was synthesized by reaction of the acetoxymercury adduct from tiglic acid with hydrogen sulphide [13].

Methyl 3-oxocaproate was prepared by ester condensation between methyl butyrate and methyl acetate, resulting in a mixture of methyl 3-oxobutanoate, methyl 3-oxocaproate, methyl 2-ethyl-3-oxobutanoate and methyl 2-ethyl-3-oxocaproate. This mixture was suitable to determine the mass spectrum and the retention behaviour of the 3-oxocaproic acid derivative.

All hydroxycarboxylic compounds were used in the form of their methyl esters, all oxocarboxylic compounds as methyl ester/O-methyloxime derivatives. The carbonyl functions were transformed into the O-methyloximes; free carboxyl groups were methylated according to the procedure described for the urinary constituents. Sodium salts were passed through a cation-exchange column filled with Dowex 50W-X8 (Serva) to form the free acids, which were then derivatized.

RESULTS AND DISCUSSION

The analysis of the hydroxy- and oxomonocarboxylic acids within the com-

plex mixture of organic acids in urine involves several considerable problems. Apart from 3-hydroxybutyric acid and 3-oxobutyric acid which are very major components in cases of increased ketogenesis, most of the other acids are (except in cases of some inborn errors of the metabolism of amino acids) excreted into the urine in low amounts. Their separation from the major components and from other organic acids is for some acids difficult to achieve by GC, unless

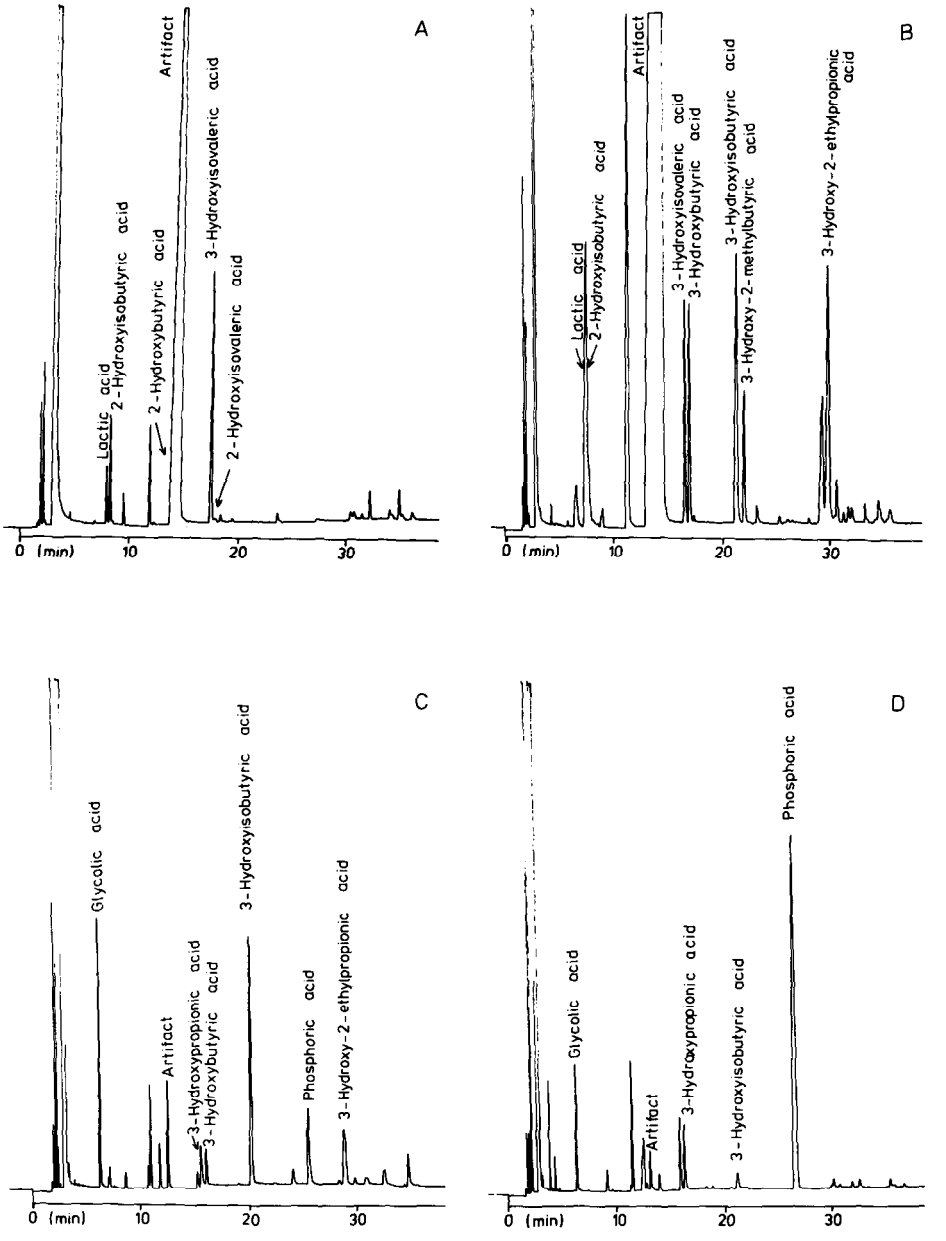


Fig. 1. Early portions of the gas chromatograms of fraction 3a (A), fraction 3b (B), fraction 3c (C) and fraction 3d (D) of the derivatives of the organic acids in urine of a normal individual.

a pre-fractionation procedure is applied to partially separate otherwise unresolved constituents. A second problem is encountered with respect to the oxocarboxylic acids. They require derivatization of the carbonyl group to prevent decomposition during the analytical procedure. The O-methyloxime derivatives have proved to be suitable, even though some of them give *syn—anti* isomeric peaks.

Pre-fractionations

By modifying the previously described TLC pre-fractionation method [11], fraction 2 containing the oxocarboxylic acids was further fractionated into 2a and 2b, and fraction 3 containing the hydroxycarboxylic acids, was divided into 3a—d. Proceeding from 2a to 3d, the substances show increasing polarity.

Fig. 1 exemplifies the early portions of the chromatograms of fractions 3a—d of the organic acids in a normal urine. These early portions contain the hydroxycarboxylic acids. Because of the limited separating efficiency of the TLC plate, most hydroxycarboxylic acids appear in more than one subfraction. Because this complicates quantitative determinations, the refined subfractionation may be omitted for the quantification of those constituents which are sufficiently separated. The least polar acids, such as lactic acid, 2-hydroxyisobutyric acid, 2-hydroxybutyric acid, 2-hydroxyisovaleric acid and 3-hydroxyisovaleric acid are part of fractions 3a and 3b, the more polar acids, such as glycolic acid

TABLE I

DISTRIBUTION OF THE OXOCARBOXYLIC AND HYDROXYCARBOXYLIC ACID DERIVATIVES IN THE TLC FRACTIONS

Substance	Fraction
Pyruvic acid	2a
2-Oxobutyric acid	2a
2-Oxoisovaleric acid	2a
2-Oxovaleric acid	2a
2-Oxo-3-methylvaleric acid	2a
2-Oxoisocaproic acid	2a
3-Oxobutyric acid	2a, 2b
3-Oxocaproic acid	2a
4-Oxobutyric acid	2b
5-Oxocaproic acid	2b
2-Hydroxyisobutyric acid	2b, 3a, 3b, (3c)
2-Hydroxyisovaleric acid	2b, 3a
2-Hydroxyisocaproic acid	2b
3-Hydroxyisovaleric acid	2b, 3a, 3b
Lactic acid	3a, 3b
2-Hydroxybutyric acid	3a
2-Hydroxy-3-methylvaleric acid	3a
3-Hydroxybutyric acid	3a, 3b, 3c, (3d)
3-Hydroxyisobutyric acid	3a, 3b, 3c, 3d
3-Hydroxy-2-methylbutyric acid	3a, 3b
3-Hydroxy-2-ethylpropionic acid	3a, 3b, 3c
Glycolic acid	3c, 3d
3-Hydroxypropionic acid	3d

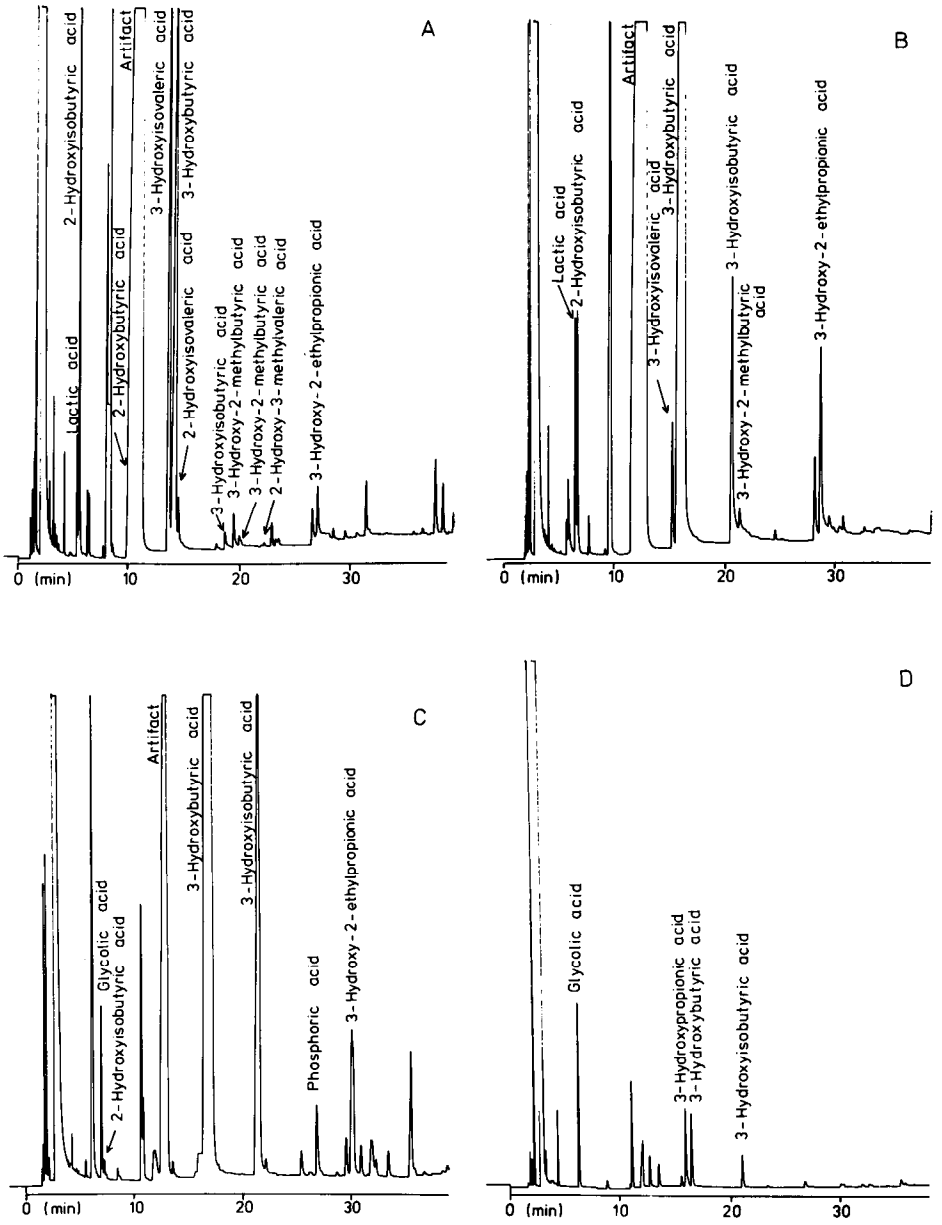


Fig. 2. Early portions of the gas chromatograms of fraction 3a (A), fraction 3b (B), fraction 3c (C) and fraction 3 d (D) of the derivatives of the organic acids in urine of a diabetic patient with ketoacidosis. The first peak of 3-hydroxy-2-methylbutyric acid in fraction 3a is the *threo* form, the second peak the *erythro* form.

and 3-hydroxypropionic acid, part of fractions 3c and 3d. 3-Hydroxybutyric acid has its maximum in 3b. The distribution of all of the hydroxycarboxylic acids in the different fractions is indicated in Table I. Fractions 3a and 3b, in some cases also 3c, contain an artifact which is introduced during the sample preparation, and which so far can not be eliminated. In the chromatograms it partially covers the peak of 2-hydroxybutyric acid.

The advantage of the subfractionation of fraction 3 becomes clear when the hydroxycarboxylic acids are investigated in urine of patients with ketoacidosis. Under this condition, 3-hydroxybutyric acid is by far the main hydroxy acid. Without subfractionation, it would cover 3-hydroxyisovaleric acid, 3-hydroxypropionic acid and 2-hydroxyisovaleric acid. Fig. 2 is an example of the hydroxycarboxylic acids in urine of a patient with ketoacidosis. When the ketoacidosis is very pronounced, the width of the 3-hydroxybutyric acid peak represents several minutes, even when the separating efficiency of the GC column is high and no peak tailing occurs (Fig. 3). Another advantage of the subfractionations is an enrichment effect for acids occurring in very low amounts, e.g. 2-hydroxy-3-methylvaleric acid in fraction 3a (Fig. 2) or *erythro*-3-hydroxy-2-methylbutyric acid which is the diastereomer of the diastereomeric pair of 3-hydroxy-2-methylbutyric acid that occurs in a lower concentration and which appears in some urines as a small peak after the major diastereomer, i.e. *threo*-3-hydroxy-2-methylbutyric acid (Fig. 2A).

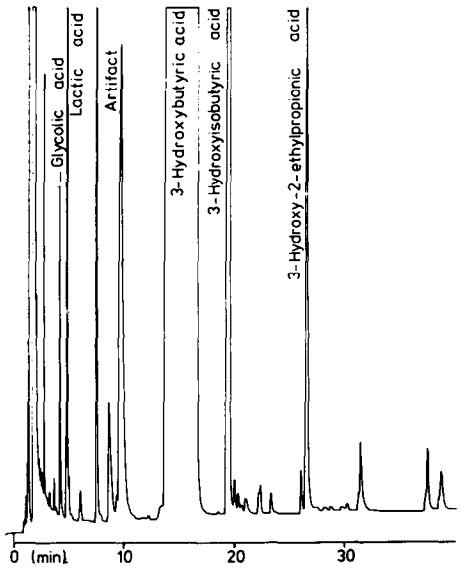


Fig. 3. Early portion of the gas chromatogram of fraction 3c of the derivatives of the organic acids in urine of a diabetic patient with ketoacidosis.

By subfractionating fraction 2 into 2a and 2b, most of the oxocarboxylic acids, except 3-oxobutyric acid, are enriched in 2a. By this procedure it is possible to detect 3-oxocarboxylic acids occurring in low concentrations, such as 3-oxocaproic acid in fraction 2a (Fig. 4) in cases of ketoacidosis. As a fraction slightly less polar than 3a, fraction 2b contains also portions of the least polar hydroxycarboxylic acids, i.e. 2-hydroxyisobutyric acid, 2-hydroxyisovaleric acid, 3-hydroxyisovaleric acid and 2-hydroxyisocaproic acid (Fig. 4).

Classification of the hydroxycarboxylic acids

Except for glycolic acid, 2-hydroxyisobutyric acid, 2-hydroxybutyric acid and 3-hydroxypropionic acid, whose formation is not completely clear, the bio-

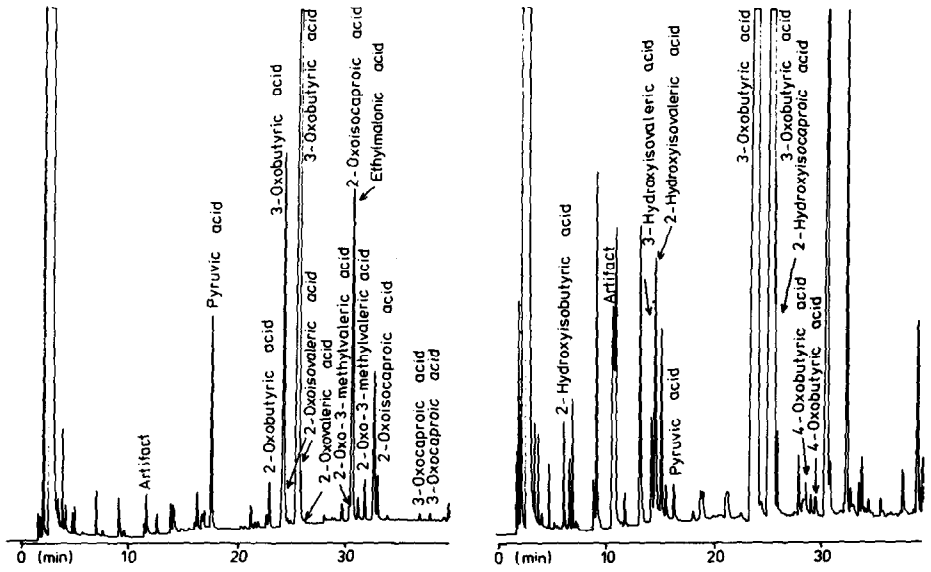
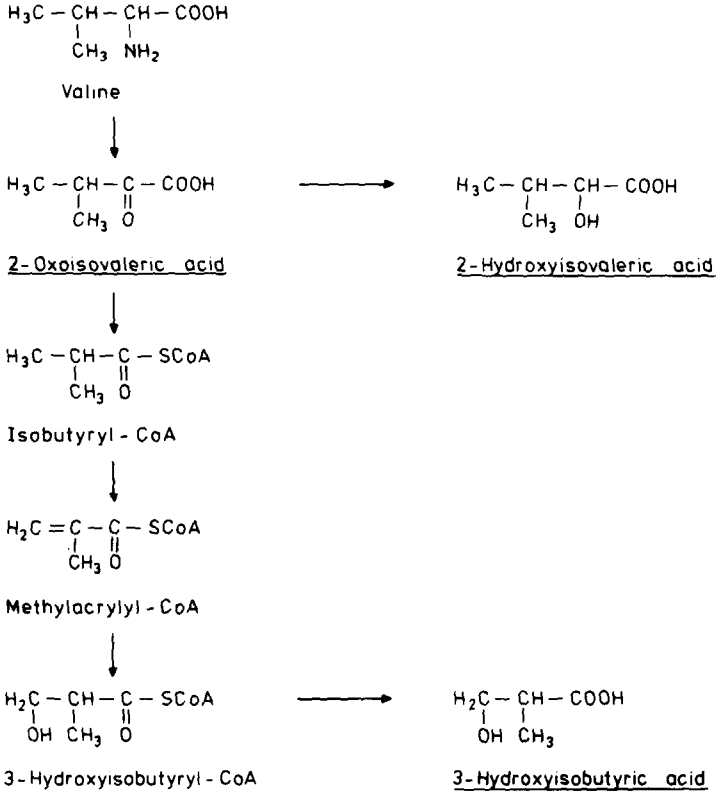
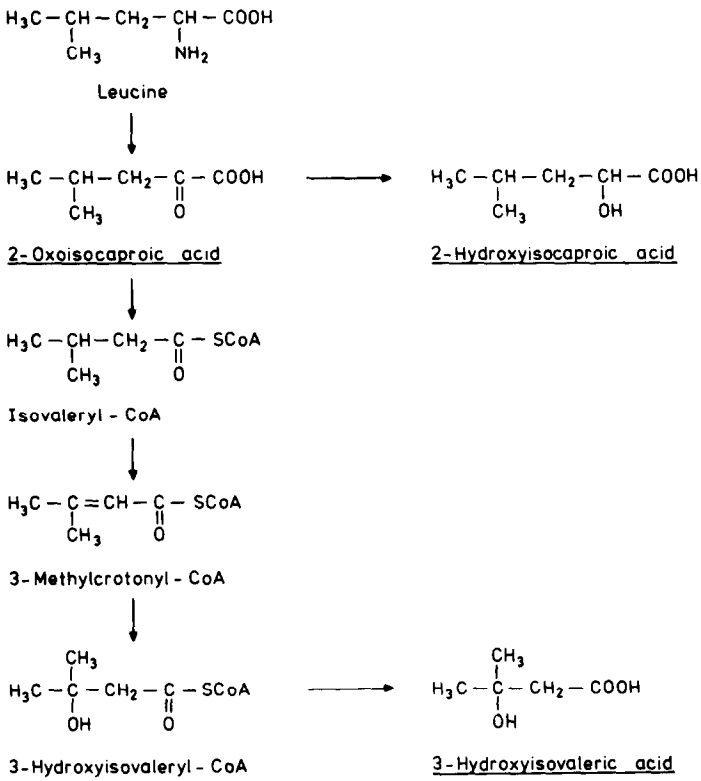


Fig. 4. Early portions of the gas chromatograms of fraction 2a (left) and 2b (right) of the derivatives of the organic acids in urine of a diabetic patient with ketoacidosis. The O-methyloxime derivatives of 2-oxoisovaleric acid, 2-oxoisocaproic acid, 2-oxo-3-methylvaleric acid, 3-oxobutyric acid, 3-oxocaproic acid and 4-oxobutyric acid occur as *syn—anti* isomers.

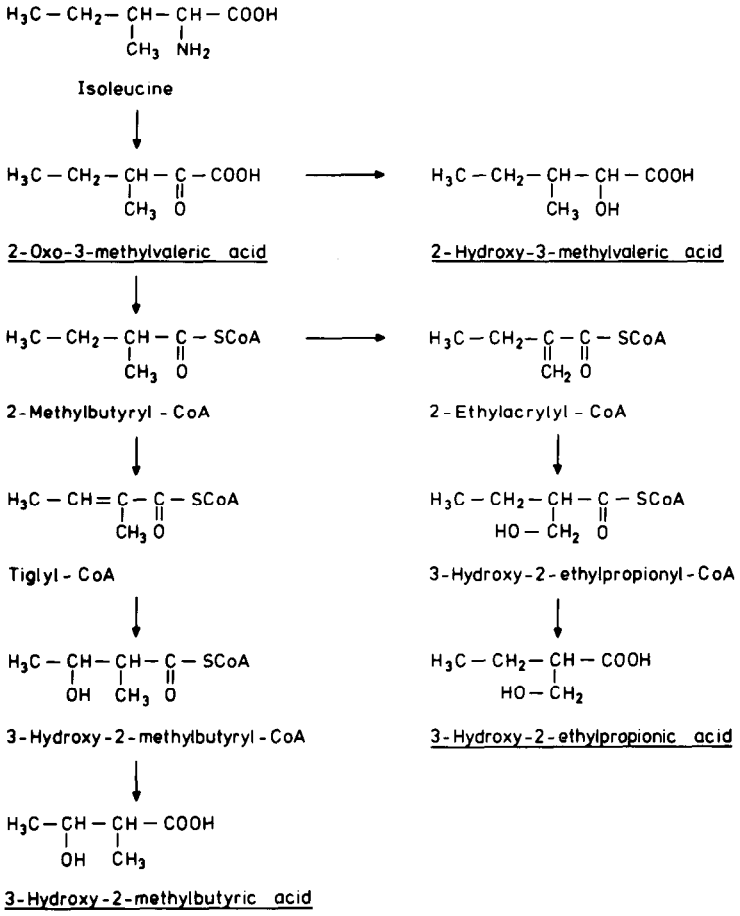


Scheme 1. Metabolism of valine. The underlined substances are found in urine.



Scheme 2. Metabolism of leucine. The underlined substances are found in urine.

chemical origin of the other hydroxycarboxylic acids is easily classified. Lactic acid is the reduction product of pyruvic acid and is formed under the conditions of anaerobic glycolysis. 3-Hydroxybutyric acid is generated by ketogenesis. All the other hydroxycarboxylic acids are metabolites of the branched-chain amino acids valine, leucine and isoleucine. 2-Hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid are formed by reduction of 2-oxocarboxylic acids, which themselves result from the oxidative desamination of the amino acids valine, leucine and isoleucine, respectively (Schemes 1—3). 3-Hydroxycarboxylic acids are products from the amino acids as well, and are formed in a reaction including several steps. 3-Hydroxyisobutyric acid stems from valine (Scheme 1), 3-hydroxyisovaleric acid from leucine (Scheme 2) and 3-hydroxy-2-methylbutyric acid and 3-hydroxy-2-ethylpropionic acid from isoleucine (Scheme 3). The formation of two different 3-hydroxycarboxylic acids from isoleucine is possible because the elimination of hydrogen in 2-methylbutyryl-CoA (coenzyme A) and the subsequent addition of water can be orientated towards two sides (Scheme 3). In the metabolization of isoleucine both the *threo* and the *erythro* forms of 3-hydroxy-2-methylbutyric acid are produced. However, the major diastereomer is the *threo* form. This has been proved by preparing the reference compound *erythro*-3-hydroxy-2-methylbutyric acid by stereospecific synthesis.



Scheme 3. Metabolism of isoleucine. The underlined substances are found in urine.

Classification of the oxocarboxylic acids

Pyruvic acid originates from glucose and is produced in the glycolytic process. 2-Oxoisovaleric acid, 2-oxoisocaproic acid and 2-oxo-3-methylvaleric acid stem, as mentioned above, from the oxidative transamination of valine, leucine and isoleucine (Schemes 1—3). 3-Oxobutyric acid is, besides 3-hydroxybutyric acid, the main product of ketogenesis, which means that it is formed from acetyl-CoA according to the 3-hydroxy-3-methylglutaryl-CoA cycle. For the homologous 3-oxocarboxylic acid, i.e. 3-oxocaproic acid, the biochemical pathway is not clear. However, since 3-oxocaproic acid occurs in a detectable amount only in cases of ketoacidosis, a condensation mechanism analogous to the ketogenesis pathway can be assumed, with butyryl-CoA and acetyl-CoA as starters. Acetyl-CoA would act as the acidic compound to react with the thioester group of butyryl-CoA. The formation of 2-oxobutyric acid and 2-oxovaleric acid cannot be easily explained. The same holds true for 4-oxobutyric acid and 5-oxocaproic acid which are often found in urine of ketoacidotic patients. 4-Oxobutyric acid has been described as a metabolite of γ-aminobutyric acid in a patient with neurological abnormalities [14].

Mass spectrometric fragmentation and identification of the hydroxycarboxylic acid methyl esters

The mass spectrometric fragmentation of the methyl esters of the hydroxycarboxylic acids is characterized by a number of general features. Even though the spectra of several of these esters have been published, it appears useful to point out these features systematically. Table II shows the intensities of the fragments.

The molecular ion can be distinguished as a small peak only in the methyl esters of glycolic acid, lactic acid and 3-hydroxypropionic acid. In the substances with higher molecular weight it is completely missing. $(M-1)^+$ occurs with low intensity in the esters of 3-hydroxypropionic acid and 3-hydroxybutyric acid. It is absent in all the other compounds.

Several major fragment peaks originate from α-cleavage processes with respect to the C=O group of the carbomethoxy unit. In the spectra of the methyl esters of the 2-hydroxycarboxylic acids, the ion $(M-COOCH_3)^+$ reaches a high intensity; in some cases it is the base peak. In the 3-hydroxy compounds this fragment peak is very small. Instead, the formation of the ions $(M-OCH_3)^+$ and $(COOCH_3)^+$ is favoured in the fragmentation of the 3-hydroxycarboxylic acid methyl esters. These processes are, with the exception of lactic acid methyl ester, of little importance in the 2-hydroxy compounds. In the same context the ion $(M-H_3COH, -CH_3)^+$ can be seen. It is favoured in the 3-hydroxycarboxylic acid methyl esters, except in 3-hydroxypropionic acid, where no methyl group is available.

The loss of water, resulting in the ions $(M-H_2O)^+$ and $(M-CH_3, -H_2O)^+$ with relative intensities up to 18%, is observed only for the 3-hydroxycarboxylic acid methyl esters, apparently because of the easy formation of a C=C double bond conjugated with the C=O group. The olefinic ion $(M-COOCH_3, -H_2O)^+$ is more generally formed and occurs in a broad range of relative intensities in the 2-hydroxy and 3-hydroxy compounds.

The fragment ion *m/e* 43 is observed in all of the hydroxycarboxylic acid methyl esters and is in several cases the base peak. In the methyl esters of 2-hydroxyisovaleric acid and 2-hydroxyisocaproic acid, *m/e* 43 probably corresponds to the isopropyl ion; in lactic acid, 2-hydroxyisobutyric acid, 3-hydroxybutyric acid, 3-hydroxy-2-methylbutyric acid and 3-hydroxyisovaleric acid, *m/e* 43 is assumed to correspond to the acetyl ion.

Very indicative for the interpretation of the spectra of the hydroxycarboxylic acid methyl esters are ions from McLafferty rearrangement processes. The ion *m/e* 90 (fragment a in Scheme 4) is formed from the 2-hydroxycarboxylic acid methyl esters with a carbon chain of four or more C atoms. In the shorter-chain esters of glycolic acid, lactic acid and 2-hydroxyisobutyric acid, this process is not possible. For the 3-hydroxy compounds, two different McLafferty rearrangements are possible, one involving the hydrogen from an alkyl group (Scheme 5), the other the hydrogen from the OH group (Scheme 6). Both processes lead to the fragment b in Schemes 5 and 6, with the masses *m/e* 74, *m/e* 88 or *m/e* 102 depending on the structure. In the 3-hydroxycarboxylic acid methyl esters with carbon chains of four or more C atoms, both rearrangements are possible and cannot be differentiated from the mass spectra. In the esters with a straight chain of three carbon atoms, i.e. 3-hy-

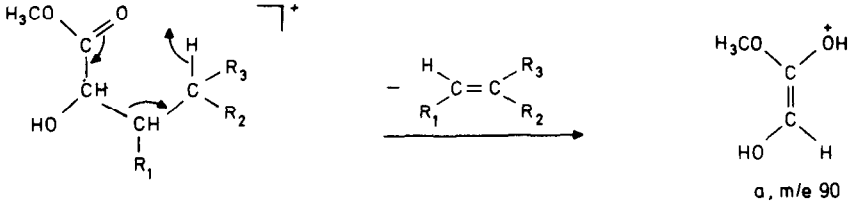
TABLE II

MASS SPECTROMETRIC FRAGMENTATION OF THE METHYL ESTERS OF HYDROXYCARBOXYLIC ACIDS

The values represent the relative intensities (%) of the fragments listed.

Substance	MW	M^+	$(M-1)^+$	$(M-COOCH_3)^+$	$(M-OCH_3)^+$	$(COOCH_3)^+$
Glycolic acid	90	8	–	100		20***
Lactic acid	104	2	–	100	1	53
2-Hydroxybutyric acid	118	–	–	100*	–	
2-Hydroxyisobutyric acid	118	–	–	100*	1	
2-Hydroxyisovaleric acid	132	–	–	100	–	7
2-Hydroxyisocaproic acid	146	–	–	43	–	10
2-Hydroxy-3-methylvaleric acid	146	–	–	65	–	7
3-Hydroxypropionic acid	104	2	6	14	81**	18
3-Hydroxybutyric acid	118	–	2		23	25*
3-Hydroxyisobutyric acid	118	–	–		40**	31*
3-Hydroxyisovaleric acid	132	–	–	12	8	80
3-Hydroxy-2-methylbutyric acid: *threo*	132	–	–	9	19	37
erythro		–	–	3	17	15
3-Hydroxy-2-ethylpropionic acid	132	–	–	8	19**	14

*Superimposition of $(M-COOCH_3)^+$ and $(COOCH_3)^+$.
**Superimposition of $(M-OCH_3)^+$ and $(M-HOCH_2)^+$.
***Superimposition of $(M-OCH_3)^+$ and $(COOCH_3)^+$.



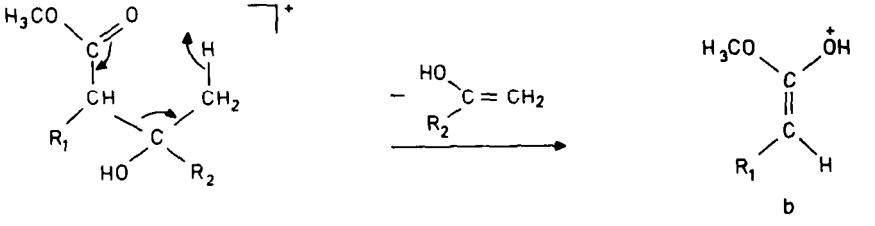
R_1	R_2	R_3	
H	H	H	2-Hydroxybutyric acid
CH_3	H	H	2-Hydroxyisovaleric acid
H	CH_3	CH_3	2-Hydroxyisocaproic acid
CH_3	H	CH_3	2-Hydroxy-3-methylvaleric acid

Scheme 4. McLafferty rearrangement of 2-hydroxycarboxylic acid methyl esters.

droxypropionic acid, 3-hydroxyisobutyric acid and 3-hydroxy-2-ethylpropionic acid, only the rearrangement involving the OH group is feasible (Scheme 6).

Because of its ethyl substituent, the spectrum of the methyl ester of 3-hydroxy-2-ethylpropionic acid shows some features that are not observed in the other substances. Most characteristic in the spectrum is the fragment

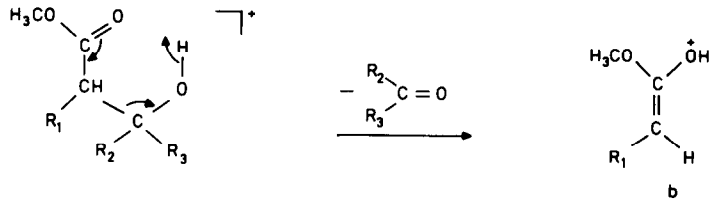
m/e 61	$(M-CH_3, -H_3COH)^+$	$(M-H_2O)^+$	$(M-CH_3, -H_2O)^+$	$(M-COOCH_3, -H_2O)^+$	*m/e* 43	$(M-CH_3)^+$	a	b
18	–	–	–	–	4	–	–	–
54	–	–	–	–	92	25	–	–
13	–	–	–	18	32	–	4	–
5	–	–	–	14	77	6	–	–
7	2	–	–	32	20	–	60	–
7	–	–	–	84	100	–	17	–
5	–	–	–	27	19	–	100	–
8	–	18	15	–	58	–	–	100
35	46	9	17	24	100	32	–	86
5	9	12	11	10	15	4		100
1	73	–	–	4	100	52	–	37
5	24	3	4	26	56	7	–	100
5	19	1	4	16	32	4	–	100
3	–	3	3	85	30	–	–	47



R_1	R_2	
H	H	3-Hydroxybutyric acid
H	CH_3	3-Hydroxyisovaleric acid
CH_3	H	3-Hydroxy-2-methylbutyric acid

Scheme 5. McLafferty rearrangement of 3-hydroxycarboxylic acid methyl esters involving an alkyl group.

group *m/e* 101, 102, 103, 104 and the ion *m/e* 87 (Fig. 5). The fragment *m/e* 101 can be explained by $(M-OCH_3)^+$ and $(M-HOCH_2)^+$, *m/e* 102 and *m/e* 104 by two McLafferty rearrangement processes, and *m/e* 103 by the loss of the ethyl group. The base peak *m/e* 87 results from the McLafferty product, *m/e* 102 by γ-cleavage (Scheme 7).



b

R_1	R_2	R_3	
H	H	H	3-Hydroxypropionic acid
C_2H_5	H	H	3-Hydroxy-2-ethylpropionic acid
CH_3	H	H	3-Hydroxyisobutyric acid
H	H	CH_3	3-Hydroxybutyric acid
H	CH_3	CH_3	3-Hydroxyisovaleric acid
CH_3	H	CH_3	3-Hydroxy-2-methylbutyric acid

Scheme 6. McLafferty rearrangement of 3-hydroxycarboxylic acid methyl esters involving the OH group.

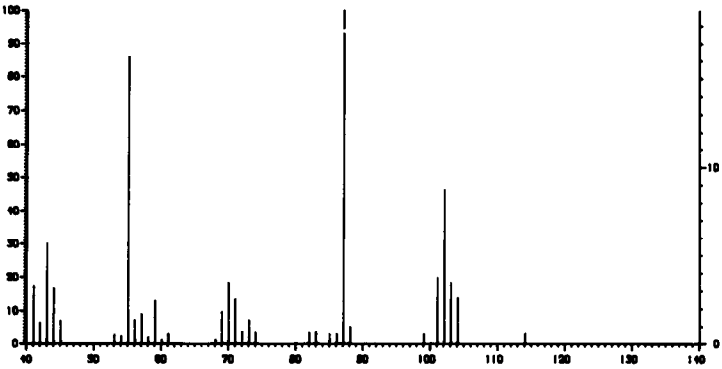
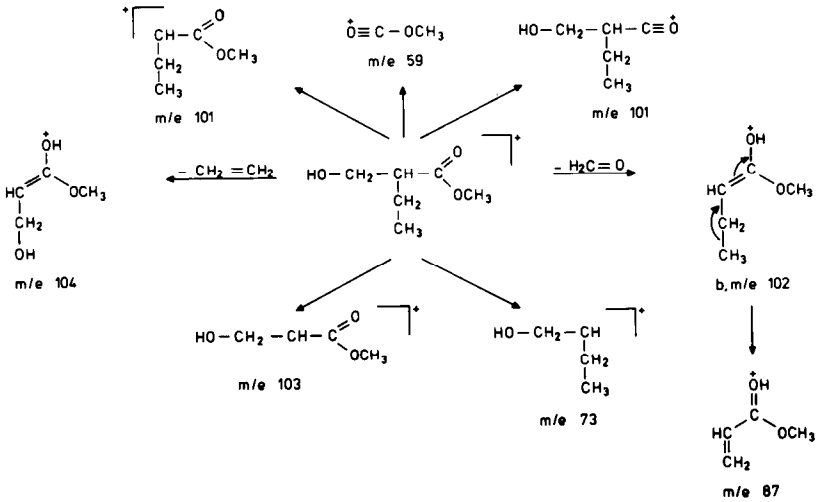


Fig. 5. Mass spectrum of the methyl ester of 3-hydroxy-2-ethylpropionic acid.



Scheme 7. Mass spectrometric fragmentation of the methyl ester of 3-hydroxy-2-ethylpropionic acid.

Mass spectrometric fragmentation and identification of the O-methyloxime derivatives of the oxocarboxylic acid methyl esters

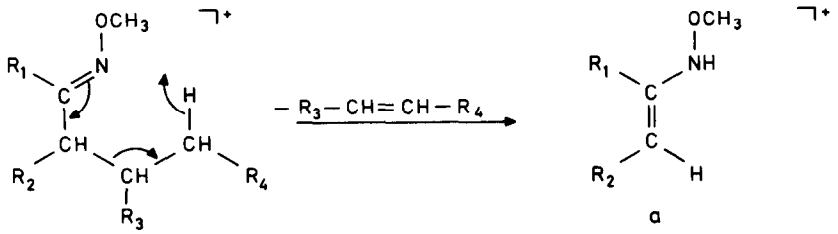
The mass spectrometric fragmentation of this group of substances has also some characteristic general features. As in the case of the hydroxy compounds, these characteristics are systematically described, even though some spectra are published. The relative intensities of the ions are listed in Table III. The *syn—anti* isomers of the O-methyloxime derivatives give very similar mass spectra. The intensities given in Table III refer to the first GC peak of the isomeric pair.

The intensity of the molecular ion decreases with increasing molecular weight. It is lower in the 2-oxo than in other oxocarboxylic acid derivatives.

Among the α-cleavage processes with respect to the C=O function of the carbomethoxy unit, the formation of $(M-COOCH_3)^+$ is strongly favoured in the 2-oxocarboxylic acid derivatives, and in some cases it leads to the base peak. It is also the main fragment in the spectrum of the derivative of 4-oxobutyric acid. The ions $(M-OCH_3)^+$ and $(COOCH_3)^+$ are generally produced from the oxocarboxylic acid derivatives. The formation of the ion $(M-OCH_3)^+$ may involve the carbomethoxy unit and the O-methyloxime group. Besides the methoxy group, in some instances the molecular ions lose methanol.

The ion $(M-CH_2COOCH_3)^+$ generated by a β-cleavage process, occurs with high abundance or even as the base peak in the mass spectra of the 3-oxocarboxylic acid derivatives. It is also formed when the O-methyloxime group is more distant from the carbomethoxy group, i.e. in the spectra of the 4-oxobutyric acid and 5-oxocaproic acid derivatives.

In the spectra of most of the oxocarboxylic acid derivatives, four ions are observed with the compositions $(M-H_3COH, -CH_3)^+$, $(M-H_3COH, -OCH_3)^+$, $(M-COOCH_3, -CH_3)^+$ and $(M-COOCH_3, -H_3COH)^+$. The relative intensities of these fragments vary strongly, the abundance of $(M-COOCH_3, -H_3COH)^+$ normally being pronounced.



R_1	R_2	R_3	R_4	
$COOCH_3$	H	H	H	2-Oxovaleric acid
$COOCH_3$	CH_3	H	H	2-Oxo-3-methylvaleric acid
$COOCH_3$	H	CH_3	H	2-Oxoisocaproic acid
$CH_2-COOCH_3$	H	H	H	3-Oxocaproic acid
CH_3	H	H	$COOCH_3$	5-Oxocaproic acid

Scheme 8. McLafferty rearrangement of oxocarboxylic acid derivatives involving the O-methyloxime group.

TABLE III

MASS SPECTROMETRIC FRAGMENTATION OF THE METHYL ESTERS OF OXOCARBOXYLIC ACIDS

The values represent the relative intensities (%) of the fragments listed.

Substance	MW	M^+	$(M-COOCH_3)^+$	$(M-OCH_3)^+$	$(M-CH_2COOCH_3)^+$	$(COOCH_3)^+$
Pyruvic acid	131	35	100	16	–	44
2-Oxobutyric acid	145	16	100	60	–	33
2-Oxovaleric acid	159	1	47	48	–	52
2-Oxoisovaleric acid	159	4	100	23	–	28
2-Oxo-3-methylvaleric acid	173	–	100	34	–	85
2-Oxoisocaproic acid	173	–	24	18	–	62
3-Oxobutyric acid	145	90	6	38	100	56
3-Oxocaproic acid	173	27	10	17	80	46
4-Oxobutyric acid	145	18	100	74	12	48
5-Oxocaproic acid	173	14	27	48	100	93

As in the mass spectra of the hydroxycarboxylic acid methyl esters, McLafferty ions are indicative for the fragmentation pattern of the oxocarboxylic acid derivatives. Two rearrangement processes are possible, one involving the O-methyloxime group, the other the carbomethoxy group. The first process leads to fragment a (Scheme 8). In the 2-oxocarboxylic acid derivatives, it requires a straight carbon chain of at least five atoms, and in 3-oxocarboxylic acid derivatives a chain of at least six carbon atoms. The second process results in fragment b, *m/e* 74, which corresponds to the regular McLafferty ion from methyl esters. In the 3-oxocarboxylic acid derivatives, a chain length of four or more carbon atoms is necessary for this fragmentation. The process is not possible in the 2-oxo compounds and the 4-oxobutyric acid derivative.

Comparison of the excretion of hydroxycarboxylic and oxocarboxylic acids in urine of normal individuals and diabetic patients with ketoacidosis

Ten of the thirteen hydroxymonocarboxylic acids described in Table I are regularly found in normal urine and can be recognized in Fig. 1. The other three substances, i.e. 2-hydroxyisovaleric acid, 2-hydroxyisocaproic acid and 2-hydroxy-3-methylvaleric acid, occur in very low amounts and are often below the detection limit. In the normal urine depicted in Fig. 1, of these three acids only 2-hydroxyisovaleric acid was identified. In healthy individuals, ketogenesis is reflected in the urinary excretion of 3-hydroxybutyric acid; the degradation of the branched-chain amino acids is shown in the excretion of a series of 3-hydroxycarboxylic acids, i.e. 3-hydroxyisobutyric acid, 3-hydroxyisovaleric acid, 3-hydroxy-2-methylbutyric acid and 3-hydroxy-2-ethylpropionic acid. The series of the 2-hydroxycarboxylic acids, which are the direct reduction products from the 2-oxocarboxylic acids, are of little importance in normals.

In diabetic patients with ketoacidosis, the urinary excretion of the ketone

$(M-H_3COH, -CH_3)^+$	$(M-H_3COH)^+$	$(M-H_3COH, -OCH_3)^+$	$(M-COOCH_3, -CH_3)^+$	$(M-COOCH_3, -H_3COH)^+$	a	b
–	3	–	1	–	–	–
–	20	2	10	18	–	–
3	1	11	–	45	100	–
2	3	6	–	33	–	–
8	–	20	20	72	34	–
2	–	12	73	46	46	–
3	78	25	3	71	–	58
100	18	10	7	27	20	35
–	70	80	2	91	–	–
17	–	8	22	22	36	24

body 3-hydroxybutyric acid is naturally increased as compared to normals. In accordance with elevated levels of the branched-chain amino acids, i.e. valine, leucine and isoleucine, in the case of diabetic ketoacidosis [15, 16], their metabolites are also increased. It is observed that the 3-hydroxy series in particular is affected. Considering the two possible metabolites of the 3-hydroxy series resulting from isoleucine, 3-hydroxy-2-ethylpropionic acid is the major product (Figs. 2 and 3). For 3-hydroxy-2-methylbutyric acid, which is the other metabolite from isoleucine, an elevation is not always found. The three components of the 2-hydroxy series of the amino acid metabolites are increased (Figs. 2 and 4). However, their absolute amounts remain low.

The excretion of oxocarboxylic acids in normal urine is very low. Only pyruvic acid is found in considerable but varying amounts [11]. In patients with ketoacidosis, a large quantity of the ketone body 3-oxobutyric acid is found in urine as a result of strong ketogenesis. All three 2-oxocarboxylic acids as metabolites of valine, leucine and isoleucine, are raised, too. However, as in the case of the 2-hydroxycarboxylic acids, their absolute quantities in urine are low as compared to serum [11]. Apparently the 2-oxocarboxylic acids are reutilized and, in contrast to the ketone bodies, the organism loses only small amounts of these constituents.

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