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REVIEW

GAS CHROMATOGRAPHIC PROFILING OF KETONE BODIES AND ORGANIC ACIDS IN DIABETES[*]

H.M. LIEBICH

Medizinische Universitätsklinik, D-7400 Tübingen (F.R.G.)

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[*]Dedicated to the 60th birthday of Professor Dr. med. M. Eggstein.

1. INTRODUCTION

Diabetes mellitus is the most common of the metabolic diseases, its frequency in the general population being estimated to be more than 2%. It has no distinct and definable pathogenesis, etiology, invariable set of clinical findings and generally accepted laboratory tests. The disease is characterized by a number of hormone-induced metabolic abnormalities and by long-term complications.

Because abnormalities of glucose levels have been easiest to measure and were discovered first, diabetes mellitus has been primarily defined as a defect in the glucose metabolism. However, the metabolisms of lipids and amino acids are also affected. The development of the techniques for metabolite analysis, gas chromatographic—mass spectrometric (GC—MS) methods being the most flexible and probably the most powerful, has contributed to our understanding of the pathophysiological alterations in the glucose, lipid and amino acid metabolisms. Such alterations occur during situations of decompensation of the disease and diabetic ketoacidosis, but certain abnormalities are also found when diabetic patients are under good therapeutic control.

Diabetic patients are susceptible to an acute metabolic complication, viz., diabetic ketoacidosis. This profound alteration of metabolism results from a deficiency of insulin action. During diabetic ketoacidosis ketone bodies and four classes of organic acids, viz., fatty acids, dicarboxylic acids, oxocarboxylic acids and hydroxycarboxylic acids, are elevated in blood and in urine. They reflect increased lipolysis, ω-oxidation of fatty acids, ketogenesis and degradation of amino acids, which are the causes of ketoacidosis. The levels of the metabolites normalize when the ketoacidotic situation is overcome.

Another organic acid, 2-ethyl-3-oxohexanoic acid, which is also an oxocarboxylic acid but behaves differently to the ketone bodies and the other oxocarboxylic acids, is increased in therapeutically well controlled diabetic patients.

Because of the variety of metabolic changes in diabetes mellitus, profile analyses of the metabolites are particularly suited to biochemical and diagnostic studies of the disease. With profile analyses it is possible to distinguish clearly between a metabolic situation where the diabetes is sufficiently balanced and a metabolic situation where the diabetes is decompensated. Certainly such information can also be obtained by measuring blood and urinary glucose, pH and the conventional ketone bodies, and this is done routinely. Profile analyses, however, give a much more detailed picture of the metabolic pathways involved, such as glucose metabolism only, or glucose metabolism and lipolysis, or in addition ketogenesis, or in addition metabolism of amino acids, or in addition lactic acidosis. Moreover, profile analyses supply information on the extent of the pathophysiological deviation and on the success of the therapeutic approach in rebalancing the diabetic patient.

Apart from this more diagnostically oriented application, profile analyses are helpful in biochemical studies of the metabolic pathways, e.g., in investigating if ketogenesis produces only acetoacetic acid, 3-hydroxybutyric acid and acetone, or other ketones as well. Profile analyses may also help to answer the question of whether the metabolism of a diabetic patient whose blood glucose is well controlled is otherwise normal.

2. PROFILING PROCEDURES

2.1. Methods of choice

Because of the complexity of the ketone bodies and the organic acids in blood and in urine, GC is the method of choice for the separation of these compounds. High-performance liquid chromatographic (HPLC) procedures are less generally applicable because of their limitations with regard to the detectability of many of the aliphatic components. Other disadvantages of HPLC are the separation efficiency, which is usually not adequate for organic acids, and the restrictions arising from coupling with MS.

This review covers methodical aspects and alternatives, and the results obtained with GC and GC—MS procedures, emphasizing profile analyses.

2.2 Ketones

2.2.1. Isolation of ketones

Of the neutral non-acidic ketones, acetone is one of the three classical ketone bodies. It is formed by decarboxylation of acetoacetic acid, which itself arises from ketogenesis. In addition to acetone, a number of higher molecular weight ketones occur in blood and in urine. Like acetone, most of them are methyl ketones.

The isolation of these ketones can be achieved by gas-phase extraction and adsorption on a solid material. Other volatile endogenous constituents, e.g., alcohols, are also isolated and can be studied together with the ketones by profile analysis. Several years ago a number of variations of this isolation technique were suggested [1—7], most of them using Tenax GC, a porous polymer of 2,6-diphenyl-p-phenylene oxide, as the adsorbing material. The method is applicable to serum and urine. The efficiency of the gas-phase extraction is 95—100% for ketones with five or more carbon atoms. It decreases with increasing polarity of the substances and is only 50% for acetone. The extent of adsorption on the Tenax GC adsorbent is 95—100% for ketones with four or more carbon atoms. Strongly polar and hydrophilic substances such as acetone are incompletely adsorbed (50—80%).

Other isolation techniques, such as solvent extraction, have been applied in quantitative determinations of specific ketones. For profile analyses they are less suitable than the gas-phase extraction and adsorption procedure, because solvent impurities cause interferences in this low-molecular-weight range.

2.2.2. Gas chromatographic profiling of ketones

For GC analysis, the components must be desorbed from the adsorbing material. Usually thermal desorption is chosen, the adsorbent trap being inserted into the GC injector block. The components are recondensed in a cooled pre-column prior to separation on a capillary GC column [4]. The quantitative desorption of the ketones requires a temperature of approximately 300°C. Substances with higher polarity are desorbed at 220°C and below.

The GC separation can be achieved on a mildly polar column, e.g., a 100 m X 0.5 mm I.D. stainless-steel column coated with Emulphor ON-870. The

column temperature was 60°C for 16 min, then programmed to 175°C at 2°C/min. Other stationary phases such as OV-17 and SE-30 can be used, as the separation of the ketones is not critical. The identification of the substances is performed by GC—MS.

Using the described profile analysis of volatile constituents in serum and urine, the ketones have been recognized as regular metabolites. In conjunction with the observation that the levels of the methyl ketones increase in diabetic or fasting ketoacidosis, these ketones have been identified as products arising from a general ketogenesis pathway. Another achievement of profile analysis of the volatile substances is the finding that aliphatic alcohols often reach elevated levels in the serum and urine of diabetic patients [8].

More insight into the pathophysiological situation has been obtained by complementing the profile analyses through quantitative determinations of the low-molecular-weight substances. For quantifications of ketones and alcohols, mass fragmentographic techniques have been developed [9—11].

2.3. Organic acids

2.3.1. Isolation of organic acids

Two basic methods are used for the isolation of the organic acids: solvent extraction and isolation by anion-exchange chromatography. In solvent extraction, acceptable results have been obtained using diethyl ether [12, 13] or ethyl acetate [14—20], or a mixture of these solvents [21, 22]. Other solvents, such as dichloromethane [23] and hexane—diethyl ether (1:1) [24], have been less frequently used. To improve the extraction, the urine or serum sample can be saturated with salts, such as sodium chloride. The methods are not time consuming, as normally they are carried out batchwise and only in a few instances continuously. However, for many organic acids the procedure is not quantitative. This is especially true for polyhydroxycarboxylic acids and other very polar acids. Protein-containing fluids, especially serum, have to be deproteinated prior to extraction. The removal of the proteins can be achieved by dialysis or ultrafiltration [19, 25] or by precipitation [26]. As protein-bound organic acids are partially lost during dialysis or ultrafiltration, precipitation of the proteins is often preferable [27]. However, some organic acids may coprecipitate with the proteins.

Isolation of organic acids by anion-exchange chromatography is widely used for studying acid profiles. Usually DEAE-Sephadex [28—31], Amberlyst A-26 [32, 33] or Dowex [25] is employed. Isolation by anion exchange is more laborious than solvent extraction procedures. However, it is more quantitative, and it is chosen when detailed profiles of various classes of organic acids, including hydroxycarboxylic acids, are being investigated. The improvement in the isolation of polyhydroxycarboxylic acids achieved by using DEAE-Sephadex instead of diethyl ether—ethyl acetate has been described [34]. Good results for profiles have also been obtained on isolation using Extrelut columns [35].

2.3.2. Derivatization of carboxyl groups

Generally, the carboxyl groups of organic acids are converted into either the

trimethylsilyl esters or the methyl esters. Trimethylsilylation is usually achieved with bis(trimethylsilyl)trifluoroacetamide (BSTFA) [16, 18, 19, 21, 31, 36—38] or bis(trimethylsilyl)acetamide (BSA) [14, 15, 17, 20, 28, 39—41]. For the methylation of organic acids, most laboratories use the reaction with diazomethane [22, 25, 32, 33, 35, 42—47]. Both derivatives have advantages and disadvantages. When using GC—MS to identify the acids, the mass spectra of the methyl esters are easier to interpret than those of the tri-methylsilyl esters, as the fragmentation of the methyl esters is often simpler and more predictable. When the necessary working precautions are followed, methylation with diazomethane is a very simple and rapid procedure. However, it does have the disadvantage that the hydroxy groups of phenolic acids are also methylated, making it impossible to distinguish between aromatic hydroxy and methoxy groups in the original compound. Another disadvantage is the possible reaction of diazomethane with double bonds, carbonyl groups and amino groups [48] to produce artificats. To establish the original structure of the substances and to differentiate between genuine compounds and artifacts, control analyses with GC—MS have been performed using deuterated diazo-methane in the sample preparation [47, 48].

Apart from trimethylsilyl and methyl esters, in some instances other deriva-tives of the carboxyl groups, such as pentafluorobenzyl esters [23], have been suggested.

In addition to the carboxyl group, other functional groups in organic acids can be derivatized. Whereas the hydroxy groups of hydroxycarboxylic acids are converted into the trimethylsilyl ethers when the organic acids are tri-methylsilylated, they are usually left underivatized when the organic acids are methylated.

2.3.3. Derivatization of carbonyl groups

The carbonyl groups of oxocarboxylic acids are usually derivatized because many oxocarboxylic acids are not stable enough for GC analysis. Suitable derivatives are oximes [14, 17, 41 49], O-methyloximes [18, 33, 50, 51] and O-ethyloximes [28, 37]. 2-Oxocarboxylic acids have been converted into O-trimethylsilylquinoxalinols [52—54].

2.3.4. Pre-fractionation of organic acids

Because of the complexity of the mixture of acidic components in serum and in urine, frequently pre-fractionation of the total mixture of organic acids is favoured. It becomes essential whenever constituents present in low concen-trations are to be studied. Pre-fractionation is effectively achieved either by preparative thin-layer chromatography (TLC) [32, 33, 46, 47, 50, 51] or by HPLC [46].

In the investigation of organic acids in samples from patients with diabetes mellitus, pre-fractionation of the acid derivatives by TLC into four fractions using Kieselgel 60 F_{254} plates was applied [33]. Methyl stearate, methyl phenylacetate, methyl indolylbutyrate and dimethyl tartrate as reference sub-stances were applied on the right-hand side of the plate to mark the fractions. The plates were developed with two solvent mixtures in succession. The first [n-heptane—diethyl ether—chloroform (40:30:30)] was allowed to run to the

upper edge of the plate. After marking the position of methyl indolylbutyrate under a UV lamp (254 nm), the plate was developed with the second solvent mixture [cyclohexane—chloroform—methanol (40:55:5)] up to the marked position of methyl indolylbutyrate. With the left-hand side of the plate covered, the right-hand side was treated with 2,7-dichlorofluorescein in order to make the reference substances visible at 366 nm.

Fraction 1 (zone between methyl stearate and methyl phenylacetate) contained mainly fatty acids, fraction 2 (zone between methyl phenylacetate and methyl indolylbutyrate) contained dicarboxylic acids, the derivatized oxocarboxylic acids and aromatic acids, fraction 3 (zone between methyl indolylbutyrate and dimethyl tartrate) contained hydroxycarboxylic acids, aromatic acids, nitrogen-containing acids and acid conjugates and fraction 4 (zone between dimethyl tartrate and the starting line) contained additional acid conjugates. For the analysis of the oxocarboxylic acids and the hydroxy-carboxylic acids, further fractionation of fraction 2 in two subfractions (2a, 2b) and fraction 3 in four subfractions (3a—3d) gave more detailed information on these two classes of organic acids in diabetes mellitus [50].

An example of the analytical procedure for the organic acids, including pre-fractionation by preparative TLC, is shown in Fig. 1.
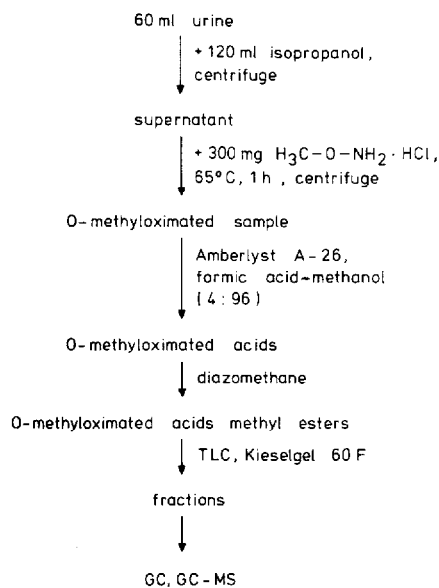
60 ml urine
| + 120 ml isopropanol,
↓ centrifuge

supernatant
| + 300 mg $H_3C-O-NH_2 \cdot HCl$,
↓ 65°C, 1 h , centrifuge

O-methyloximated sample
| Amberlyst A-26,
| formic acid—methanol
↓ (4 : 96)

O-methyloximated acids
↓ diazomethane

O-methyloximated acids methyl esters
↓ TLC, Kieselgel 60 F

fractions
↓

GC, GC-MS

Fig. 1. Analytical procedure for the organic acids.

2.3.5. Gas chromatographic profiling of organic acids

The isolated and derivatized organic acids can be analysed by GC or GC—MS, either as a total profile or as partial profiles after pre-fractionation of the sample. Packed columns that were formerly used for the GC separation were usually replaced by glass or fused-silica capillary columns. Frequently used stationary phases include OV-17, OV-101, SE-30, OV-1701, DB-1 and DB-1701. The columns are usually 25 m long.

Total profiles of organic acids have been widely studied in conjunction with

the detection and characterization of pathophysiological situations and altered metabolic pathways leading to organic acidaemias and acidurias. Many of these biochemical alterations are sufficiently pronounced to be recognizable in the overall acid profile. Typical examples are inborn errors of metabolism. The defect, caused by an insufficient enzyme activity at a certain step of the metabolism, often of the metabolism of amino acids, results in specific and clear changes in the acid profile [55]. It has been proposed that screening for such organic acidurias can be achieved with GC alone and without mass spectrometry when a dual-column GC method is employed [17, 20]. Total profiles have also been used in the investigation of normal ranges of organic acids [22, 31], of abnormal levels of organic acids in patients with tumours such as phaeochromocytoma or neuroblastoma [56] and in the study of the metabolic changes in patients with diabetes mellitus, especially during diabetic ketoacidosis [57]. In the last example, 25 m X 0.2 mm I.D. glass capillary columns coated with OV-17 and fused-silica columns coated with OV-1701 have been used. The column temperature was 40°C for 10 min, then programmed to 230°C at 2°C/min.

The acid fractions are separated on the same stationary phases as the total profiles. In addition to flame ionization detectors, specific detectors have been applied, such as the thermionic specific detector for nitrogen-containing acids and acid derivatives, and the mass spectrometer in the mass fragmentographic mode. The thermionic specific detector has proved useful in the analysis of oxocarboxylic acids when the carbonyl function is converted into the O-methyloxime [33]. The identification of the organic acids is based on their mass spectra and on their GC retention indices. The levels of the different blood and urinary acids analysed by GC and identified by GC—MS are between 10 μg/dl and 100 mg/dl in serum or urine.
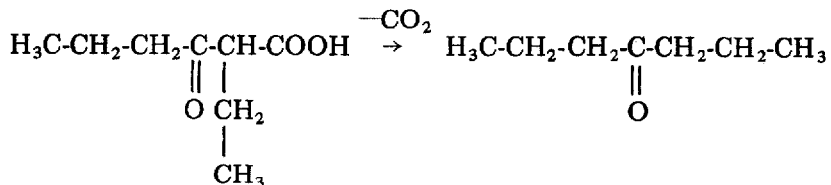
The advantages of the pre-fractionation procedure over the total profile method, such as improvement in separation and enrichment of acids present in very small amounts, have been described and exemplified [50].

It must be pointed out that in the analysis of organic acids the possibility of artifact formation as a source of error must be critically considered. Artifacts may be produced at any step of the sample preparation procedure and during the separation. Another source of error is interferences from drugs and medications. Many examples of artifacts and other interferences have been reported [40, 48, 55, 58—60].

3. APPLICATION TO DIABETES MELLITUS

3.1. Diabetic patients under good therapeutic control

Profile analyses of serum and urine samples of diabetic patients who are therapeutically well controlled have shown that the levels of the ketones and the organic acids are not different from those of normal individuals, except for the ketone 4-heptanone and the organic acid 2-ethyl-3-oxohexanoic acid. Because of the chemical and biochemical relationship between these two components, the sum of 4-heptanone and 2-ethyl-3-oxohexanoic acid is called total 4-heptanone. The ketone is formed from the labile 3-oxocarboxylic acid by decarboxylation according to the reaction

$$H_3C\text{-}CH_2\text{-}CH_2\text{-}\overset{\|}{\underset{O}{C}}\text{-}\overset{|}{\underset{\underset{CH_3}{|}}{\underset{CH_2}{C}}}H\text{-}COOH \quad \overset{-CO_2}{\rightarrow} \quad H_3C\text{-}CH_2\text{-}CH_2\text{-}\overset{\|}{\underset{O}{C}}\text{-}CH_2\text{-}CH_2\text{-}CH_3$$

The decarboxylation partly occurs in vivo, analogously to the decarboxylation of acetoacetic acid to form acetone. In vitro, heat enhances this reaction [9].

The acid is detected by GC–MS within a fraction of the organic acids either in the form of its methyl ester with the carboxyl group left underivatized, or in the form of its methyl ester with the carbonyl group derivatized to the O-methyloxime [61, 62]. The ketone is detected within the profile of low-molecular-weight components by GC [2, 4, 62]. Total 4-heptanone is determined after thermal decarboxylation of the 3-oxocarboxylic acid [62].

The biochemical pathway of the formation of 2-ethyl-3-oxohexanoic acid is not completely clear. However, the findings suggest that the biosynthesis of the acid runs parallel to the synthesis of fatty acids, with butyryl-coenzyme A as the starter. This hypothesis is supported by the observation that together with elevated 2-ethyl-3-oxohexanoic acid, ethylmalonic acid, whose coenzyme A form is an intermediate of this mechanism, is also increased [63].

A study with 28 healthy individuals, 143 patients without metabolic diseases, 109 diabetic patients of type I and 125 diabetic patients of type II revealed increased levels of total 4-heptanone in the urine of the diabetic patients. Whereas the mean values for total urinary 4-heptanone in healthy individuals and non-diabetic patients were 207 μg per 24 h and 246 μg per 24 h, respectively, the mean urinary excretion of 2-ethyl-3-oxohexanoic acid plus 4-heptanone in the 234 diabetic patients was 1073 μg per 24 h (diabetics of type I 1215 μg per 24 h and diabetics of type II 987 μg per 24 h) [63]. In a longitudinal study over 30 days, the total 4-heptanone values of two healthy controls were 80–410 μg per 24 h.

It has also been observed that the urinary total 4-heptanone decreases during diabetic ketoacidosis, which is in accordance with the postulated biochemical origin of 2-ethyl-3-oxohexanoic acid. From the findings it is concluded that the excretion of total 4-heptanone is elevated in diabetes mellitus when the test is performed outside hyperglycaemic and ketoacidotic periods. It is further concluded that the excretion of total 4-heptanone is a sensitive indicator of the interplay between fatty acid oxidation and fatty acid synthesis. A decrease is observed during fatty acid oxidation and an increase during fatty acid synthesis [64].

3.2. Diabetic patients with ketoacidosis

3.2.1. Ketones

In the profile of volatile metabolites a number of higher molecular weight methyl ketones are regularly found in addition to acetone. The main components are 2-pentanone, 3-penten-2-one and 2-heptanone (Fig. 2). During ketoacidosis their urinary excretion and their concentrations in serum are
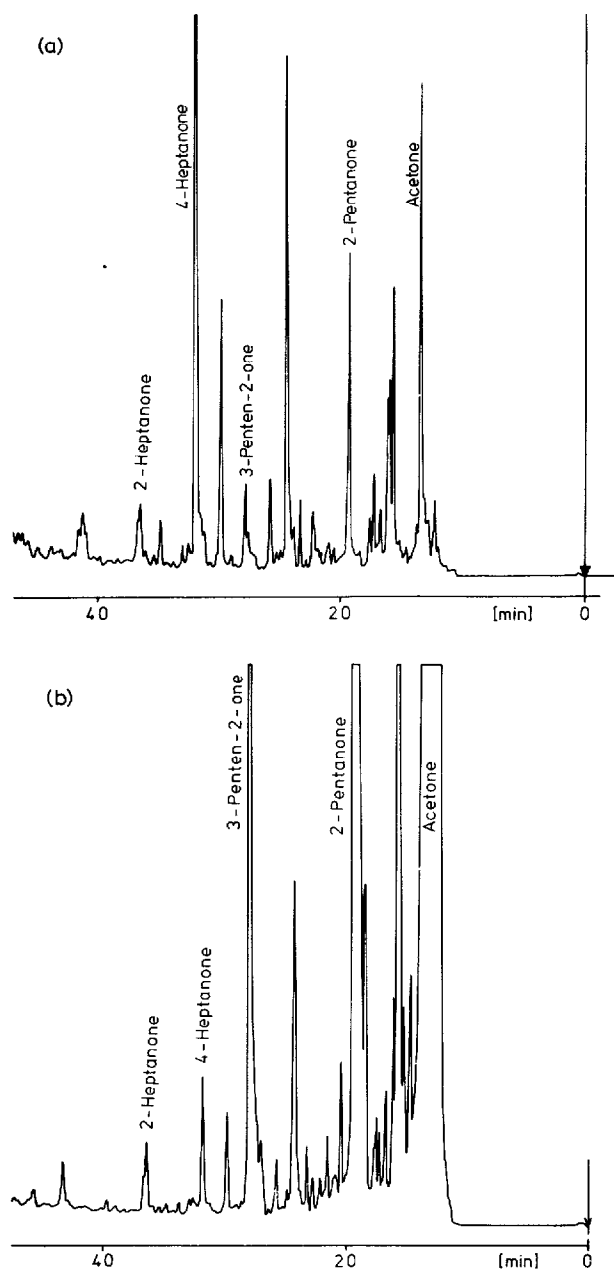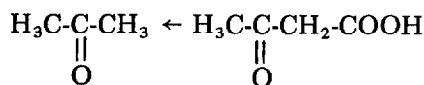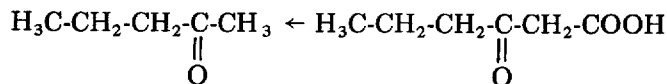
Fig. 2. (a) Gas chromatogram of the methyl ketones in the urine of a normal individual. (b) Gas chromatogram of the methyl ketones in the urine of a patient with diabetic keto-acidosis. Column, 100 m × 0.5 mm I.D., stainless steel, coated with Emulphor ON-870; column temperature, 60°C for 16 min, then programmed to 175°C at 2°C/min.
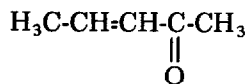
drastically increased. Acetone is formed by spontaneous decarboxylation from its precursor, acetoacetic acid. The corresponding precursor of 2-pentanone, i.e., 3-oxohexanoic acid, has been found within a fraction of the organic acids [50].
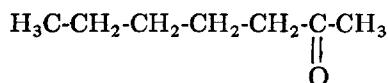
H₃C-C-CH₃ ← H₃C-C-CH₂-COOH

$$H_3C-\overset{\underset{\displaystyle O}{\|}}{C}-CH_3 \quad\leftarrow\quad H_3C-\overset{\underset{\displaystyle O}{\|}}{C}-CH_2-COOH$$

Acetone   Acetoacetic acid

$$H_3C-CH_2-CH_2-\overset{\underset{\displaystyle O}{\|}}{C}-CH_3 \quad\leftarrow\quad H_3C-CH_2-CH_2-\overset{\underset{\displaystyle O}{\|}}{C}-CH_2-COOH$$

2-Pentanone   3-Oxohexanoic acid

$$H_3C-CH{:}CH-\overset{\underset{\displaystyle O}{\|}}{C}-CH_3$$

3-Penten-2-one

$$H_3C-CH_2-CH_2-CH_2-CH_2-\overset{\underset{\displaystyle O}{\|}}{C}-CH_3$$

2-Heptanone

Even though the precursors of 3-penten-2-one and 2-heptanone have not yet been detected, probably because of insufficient concentrations, a general concept for ketogenesis is likely. By analogy with the formation of the three conventional ketone bodies acetoacetic acid, 3-hydroxybutyric acid and
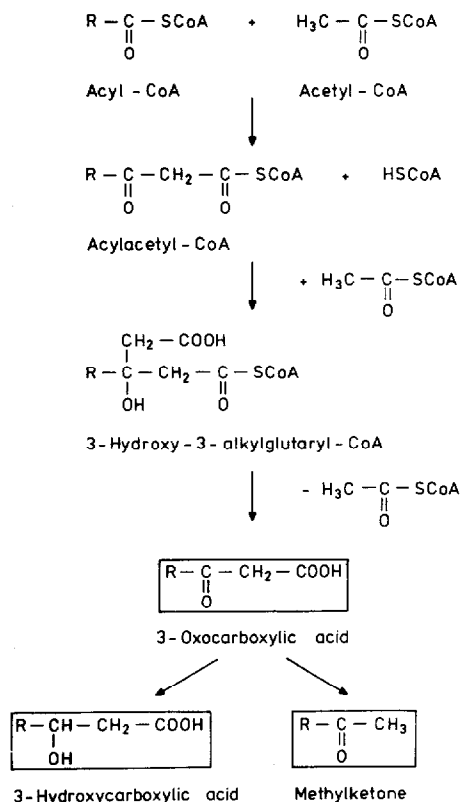

Fig. 3. Hypothetical general pathway for ketogenesis.

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Fig. 4. Fraction 2b of the methyl esters of the organic acids in the urine of a normal individual. Column, 25 m × 0.2 mm I.D., fused silica, coated with OV-1701; column temperature 40°C for 10 min, then programmed to 230°C at 2°C/min. 3-Methylglutaconic acid occurs in the form of the cis—trans isomers.

tion. Fig. 4 shows fraction 2b of the organic acids in the urine of a normal individual, with some dicarboxylic acids labelled. Most of the other substances in fraction 2b are aromatic acids. In serum dicarboxylic acids are also found, but in much smaller concentrations than in urine. Therefore, some of the acids have not been detected so far. On the other hand, the higher molecular weight saturated and unbranched dicarboxylic acids (C_{12}—C_{18}) are identified in serum only.

During diabetic ketoacidosis mainly the saturated acids with even-numbered carbon chains are elevated, especially succinic, adipic, suberic and sebacic acids.
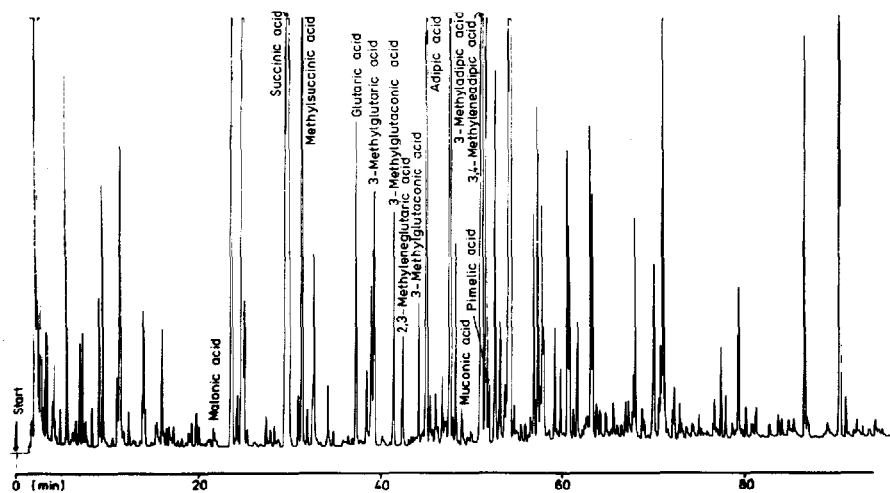

Fig. 5. Fraction 2b of the methyl esters of the organic acids in the urine of a diabetic patient with ketoacidosis. GC conditions as in Fig. 4.

The increase in succinic acid and adipic acid is exemplified in Fig. 5 (cf., Fig. 4). Suberic acid and sebacic acid are not observed as components of fraction 2a.

The methyl-branched dicarboxylic acids and the acids with a cyclopropane ring are little affected by ketoacidosis. They are formed by ω-oxidation of methyl-branched or methylene monocarboxylic acids [68, 69], which are assumed to be products of the microflora of the intestinal tract. It was shown by experiments with deuterated diazomethane that the methylene dicarboxylic acids are indeed of endogenous origin and are not artifacts produced with diazomethane.

3.2.4. Aliphatic oxocarboxylic acids

Oxomonocarboxylic acids (listed in Table 2 according to increasing methylene units) originate from different biochemical sources. Pyruvic acid is produced from glucose in the glycolytic process, 2-oxoisovaleric, 2-oxoiso-caproic and 2-oxo-3-methylvaleric acids stem from the oxidative transamination of the amino acids valine, leucine and isoleucine (Figs. 6—8). 3-Oxobutyric acid is a 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-oxohexanoic acid, which occurs in detectable amounts in urine only during ketoacidosis, a condensation mechanism analogous to the ketogenesis pathway can be assumed (Fig. 3, with butyryl-CoA and acetyl-CoA as starters). The reduction product, 3-hydroxyhexanoic acid, which was found by Niwa et al. [19] in the urine and serum of keto-acidotic patients and was explained as an intermediate of β-oxidation, could be better understood as resulting from this condensation mechanism. 3-Oxo-2-ethylhexanoic acid is assumed to be formed according to a mechanism analogous to the fatty acid biosynthesis [63]. The formation of glyoxylic, 2-oxobutyric and 4-oxobutyric acids cannot be easily explained. The same applies to 2-oxovaleric and 5-oxohexanoic acids, which are often found in the urine of ketoacidotic patients.

In the serum of normal individuals the main oxomonocarboxylic acids are pyruvic and 2-oxoisocaproic acids [33]. In urine only pyruvic acid is excreted in considerable but varying amounts, the excretion of the other oxocarboxylic acids being low (Fig. 9). The derivatives of all of the oxomonocarboxylic acids appear in the early portions of the gas chromatograms of fractions 2a and 2b.

In patients with ketoacidosis, the ketone body 3-oxobutyric acid and all

TABLE 2

OXOMONOCARBOXYLIC ACIDS IN NORMAL URINE

No.	Acid	No.	Acid
1	Glyoxylic acid	7	4-Oxobutyric acid
2	Pyruvic acid	8	2-Oxo-3-methylvaleric acid
3	2-Oxobutyric acid	9	2-Oxoisocaproic acid
4	3-Oxobutyric acid	10	3-Oxohexanoic acid
5	2-Oxoisovaleric acid	11	5-Oxohexanoic acid
6	2-Oxovaleric acid	12	3-Oxo-2-ethylhexanoic acid

H₃C — CH — CH — COOH
     | |
    CH₃ NH₂

Valine

↓

H₃C — CH — C — COOH
CH₃ O

→

H₃C — CH — CH — COOH
CH₃ OH

2 - Oxoisovaleric acid   2 - Hydroxyisovaleric acid

↓

H₃C — CH — C — SCoA
     | ||
    CH₃ O

Isobutyryl - CoA

↓

H₂C = C — C — SCoA
      | ||
     CH₃ O

Methylacrylyl - CoA

↓

H₂C — CH — C — SCoA   →
 | | ||
OH CH₃ O

H₂C — CH — COOH
OH CH₃

3 - Hydroxyisobutyryl - CoA   3 - Hydroxyisobutyric acid
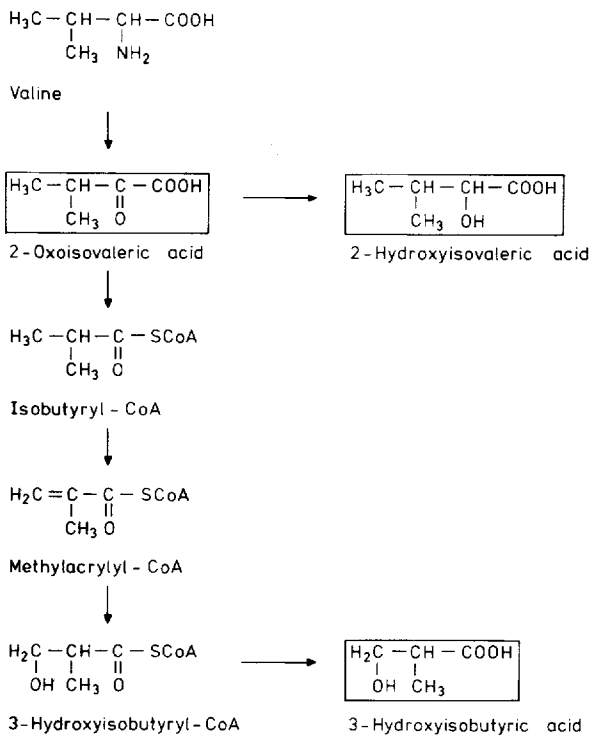
Fig. 6. Oxo- and hydroxycarboxylic acids derived from valine.

H₃C — CH — CH₂ — CH — COOH
     | |
    CH₃   NH₂

Leucine

↓

H₃C — CH — CH₂ — C — COOH
CH₃   O

→

H₃C — CH — CH₂ — CH — COOH
CH₃   OH

2 - Oxoisocaproic acid   2 - Hydroxyisocaproic acid

↓

H₃C — CH — CH₂ — C — SCoA
     | ||
    CH₃   O

Isovaleryl - CoA

↓

H₃C — C = CH — C — SCoA
     | ||
    CH₃   O

3 - Methylcrotonyl - CoA

↓

       OH
       |
H₃C — C — CH₂ — C — SCoA   →
    | ||
  CH₃   O

OH
H₃C — C — CH₂ — COOH
CH₃

3 - Hydroxyisovaleryl - CoA   3 - Hydroxyisovaleric acid
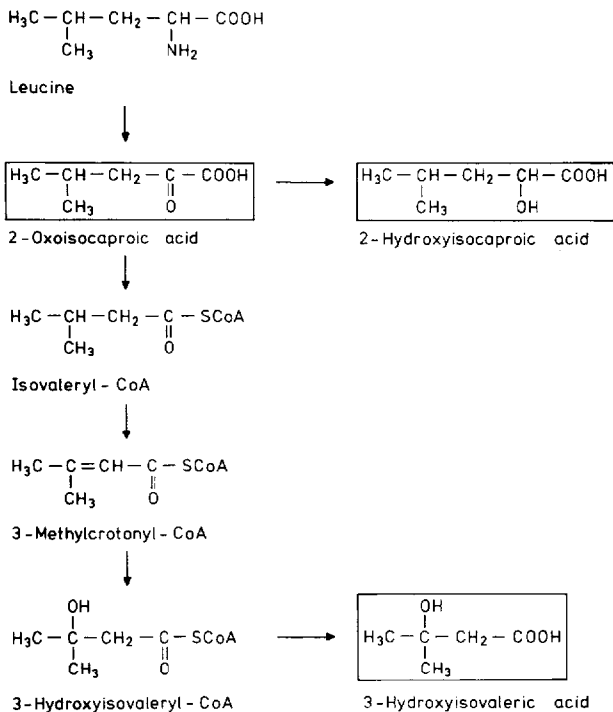
Fig. 7. Oxo- and hydroxycarboxylic acids derived from leucine.

$H_3C-CH_2-CH-CH-COOH$
$\quad\quad\quad\quad |\quad\quad |$
$\quad\quad\quad\quad CH_3\ NH_2$

Isoleucine

$H_3C-CH_2-CH-C-COOH$
$\quad\quad\quad\quad\quad |\quad\ ||$
$\quad\quad\quad\quad\quad CH_3\ O$

2 – Oxo - 3 – methylvaleric acid

$H_3C-CH_2-CH-CH-COOH$
$\quad\quad\quad\quad\quad\quad |\quad\quad |$
$\quad\quad\quad\quad\quad\quad CH_3\ OH$

2-Hydroxy-3-methylvaleric acid

$H_3C-CH_2-CH-C-SCoA$
$\quad\quad\quad\quad\ |\quad\ ||$
$\quad\quad\quad\quad\ CH_3\ O$

2- Methylbutyryl – CoA

$H_3C-CH_2-C-C-SCoA$
$\quad\quad\quad\quad\ ||\ ||$
$\quad\quad\quad\quad\ CH_2\ O$

2 -Ethylacrylyl – CoA

$H_3C-CH=C-C-SCoA$
$\quad\quad\quad\quad\ |\quad\ ||$
$\quad\quad\quad\quad\ CH_3\ O$

Tiglyl – CoA

$H_3C-CH_2-CH-C-SCoA$
$\quad\quad\quad\quad\quad |\quad\ ||$
$\quad\quad\quad\ HO-CH_2\ O$

3 - Hydroxy - 2 - ethylpropionyl - CoA

$H_3C-C-CH-C-SCoA$
$\quad\quad\ |\quad\ |\quad\ ||$
$\quad\quad\ OH\ CH_3\ O$

3-Hydroxy-2-methylbutyryl – CoA

$H_3C-CH_2-CH-COOH$
$\quad\quad\quad\quad\quad |$
$\quad\quad\quad\ HO-CH_2$

3-Hydroxy - 2 - ethylpropionic acid

$H_3C-CH-CH-COOH$
$\quad\quad\ |\quad\ |$
$\quad\quad\ OH\ CH_3$

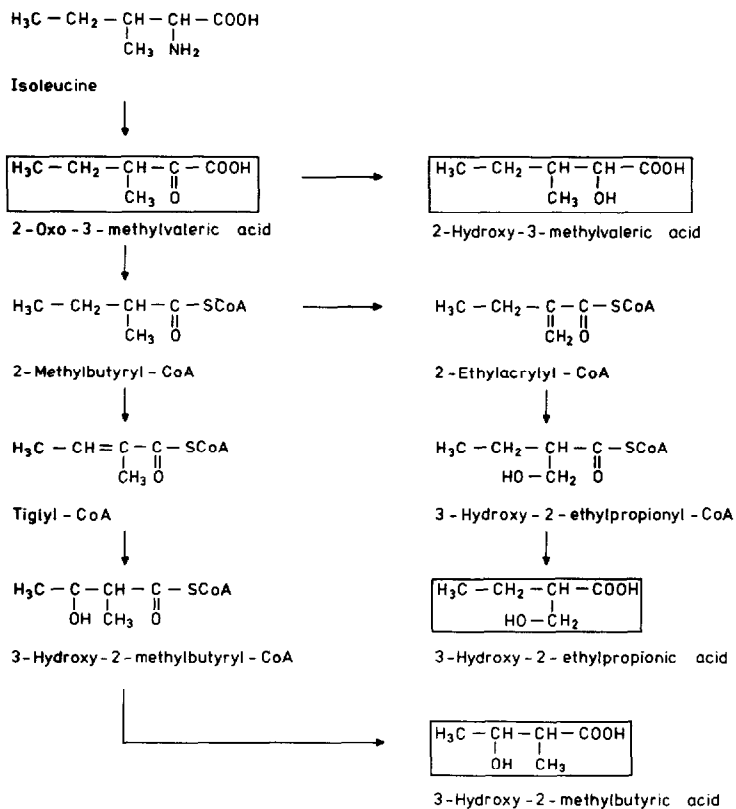3-Hydroxy - 2 - methylbutyric acid

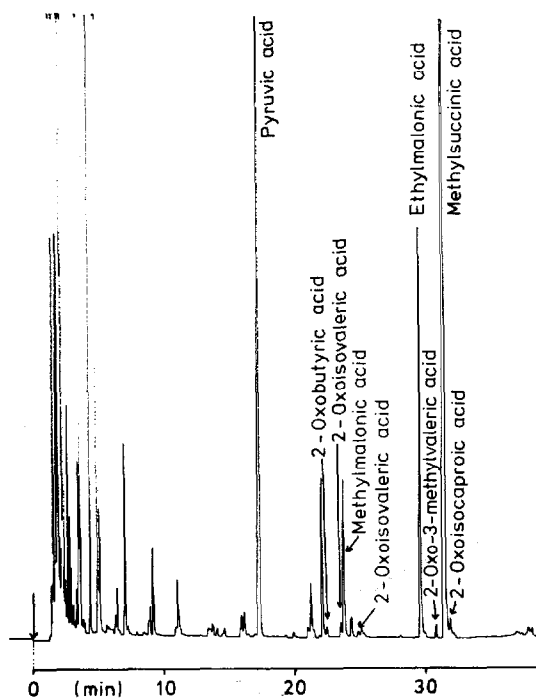Fig. 8. Oxo- and hydroxycarboxylic acids derived from isoleucine.

Fig. 9. Early portion of the gas chromatogram of fraction 2a of the derivatives of the organic acids in the urine of a normal individual. The O-methyloxime derivatives of 2-oxoisovaleric acid occur as *syn—anti* isomers. GC conditions as in Fig. 4.
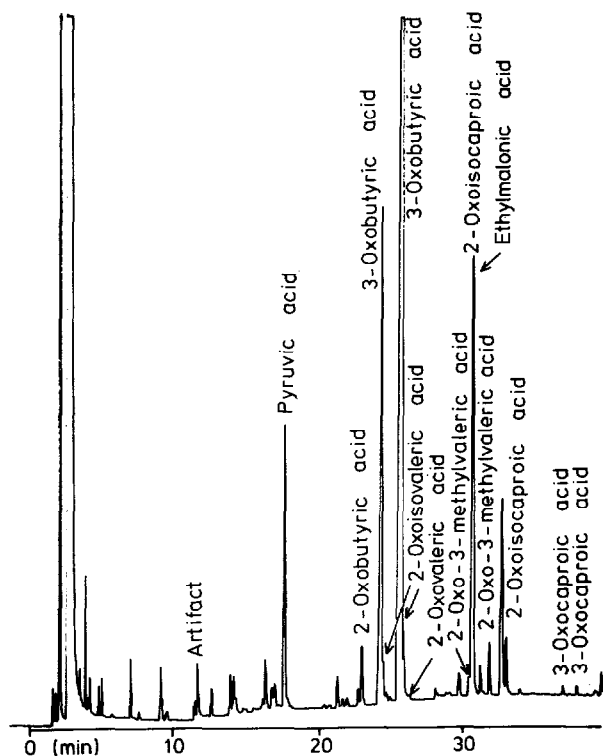
Fig. 10. Early portion of the gas chromatogram of fraction 2a of the derivatives of the organic acids in the urine of a diabetic patient with ketoacidosis. The O-methyloxime derivatives of 2-oxoisovaleric, 2-oxoisocaproic, 2-oxo-3-methylvaleric, 3-oxobutyric and 3-oxohexanoic (3-oxocaproic) acids occur as *syn—anti* isomers. GC conditions as in Fig. 4. (Reproduced from ref. 50.)

three 2-oxocarboxylic acids as metabolites of valine, leucine and isoleucine are raised [33]. The elevated level of the amino acid metabolites is not surprising as the branched-chain amino acids are also increased during ketoacidosis [70]. In urine a large amount of 3-oxobutyric acid is excreted. 2-Oxoisovaleric, 2-oxoisocaproic and 2-oxo-3-methylvaleric acids are also raised (Fig. 10). However, their absolute amounts in urine are low compared with serum. Apparently the 2-oxocarboxylic acids are reutilized and, in contrast to the ketone bodies, the organism loses only small amounts of these constituents.

3.2.5. Aliphatic hydroxycarboxylic acids

The hydroxymonocarboxylic acids detected in urine are listed in Table 3 according to increasing methylene units. With the exception of glycolic, 2-hydroxyisobutyric, 2-hydroxybutyric and 3-hydroxypropionic acids, the formation of which is not completely clarified, the biochemical origin of the other hydroxymonocarboxylic acids is easily traced. Lactic acid is produced by reduction of pyruvic acid under the conditions of anaerobic glycolysis. 3-Hydroxybutyric acid is formed by ketogenesis. The other hydroxymonocarboxylic acids are degradation products of valine, leucine and isoleucine. 2-Hydroxyisovaleric and 3-hydroxyisobutyric acids stem from valine (Fig. 6), 2-hydroxyisocaproic and 3-hydroxyisovaleric acids from leucine (Fig.

TABLE 3

HYDROXYMONOCARBOXYLIC ACIDS IN NORMAL URINE

No.	Acid	No.	Acid
1	Glycolic acid	8	2-Hydroxyisovaleric acid
2	Lactic acid	9	3-Hydroxyisobutyric acid
3	2-Hydroxyisobutyric acid	10	3-Hydroxy-2-methylbutyric acid
4	2-Hydroxybutyric acid	11	2-Hydroxy-3-methylvaleric acid
5	3-Hydroxypropionic acid	12	2-Hydroxyisocaproic acid
6	3-Hydroxyisovaleric acid	13	3-Hydroxy-2-ethylpropionic acid
7	3-Hydroxybutyric acid		

7) and 2-hydroxy-3-methylvaleric, 3-hydroxy-2-methylbutyric and 3-hydroxy-2-ethylpropionic acids from isoleucine (Fig. 8). 3-Hydroxy-2-methylbutyric acid is found as a diastereomeric pair, the *threo*-form being the main diastereomer.

The hydroxymonocarboxylic acids appear in the four subfractions 3a—3d [50]. Without the pre-fractionation their identification would be difficult, because some of them overlap in the GC separation, especially with samples from ketoacidotic patients, where 3-hydroxybutyric acid is by far the major component.

In patients with ketoacidosis, 3-hydroxybutyric acid and all the hydroxy metabolites of the branched-chain amino acids are elevated. Figs. 11 and 12 show as examples the fractions 3c of the hydroxymonocarboxylic acids in the
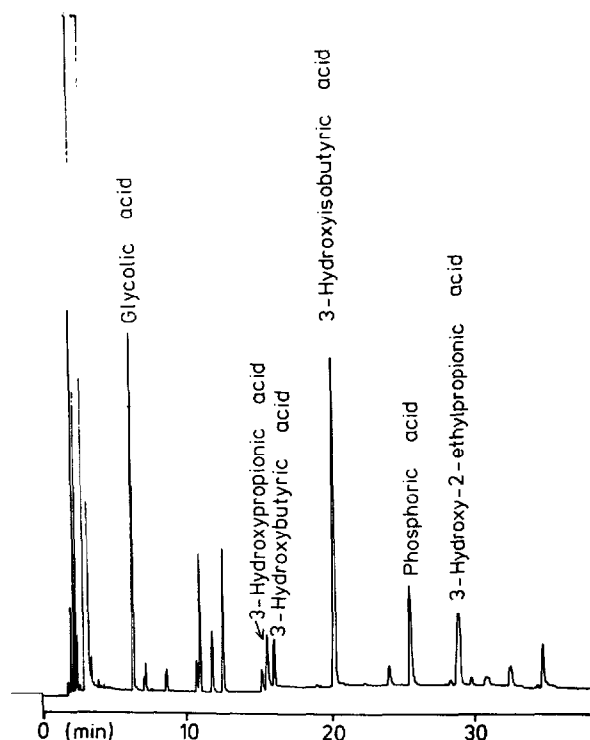

Fig. 11. Gas chromatogram of the methyl esters of the hydroxycarboxylic acids in fraction 3c from a normal individual. GC conditions as in Fig. 4. (Reproduced from ref. 50.)
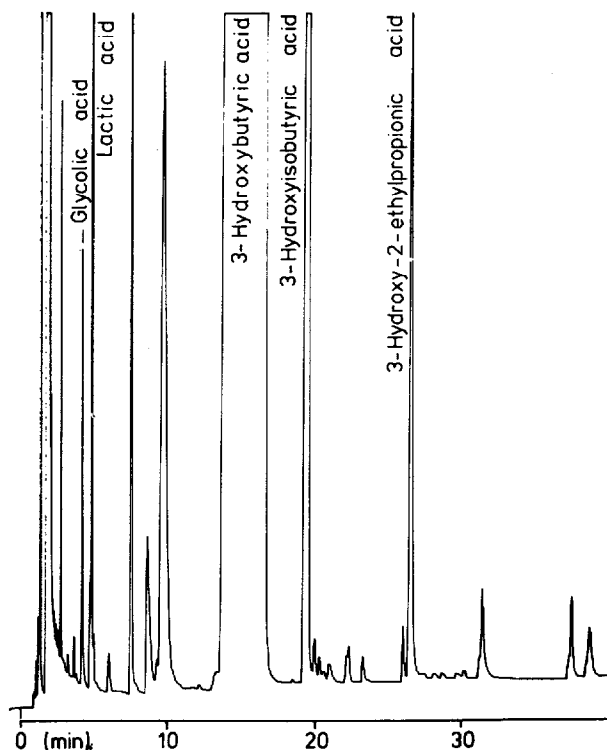
Fig. 12. Gas chromatogram of the methyl esters of the hydroxycarboxylic acids in fraction 3c from a diabetic patient with ketoacidosis. GC conditions as in Fig. 4. (Reproduced from ref. 50.)

urine of a normal person and a ketoacidotic patient, respectively. The remainder of the hydroxycarboxylic acids are found in fractions 3a, 3b and 3d. It is observed that the 3-hydroxy series is particularly affected by ketoacidosis. Of the two possible metabolites of the 3-hydroxy series resulting from isoleucine, 3-hydroxy-2-ethylpropionic acid is the major product. For 3-hydroxy-2-methylbutyric acid an elevation is not always found. The three components of the 2-hydroxy series of the amino acid metabolites are increased. However, their absolute amounts remain low. In normal urines 2-hydroxyisocaproic acid is often not even detectable. The behaviour of the hydroxymonocarboxylic acids in serum appears to be similar to that in urine.

4. SUMMARY

Diabetes mellitus is a defect not only in glucose metabolism, but also in the metabolism of lipids and amino acids. Gas chromatographic and gas chromatographic—mass spectrometric profile analyses have contributed much to the understanding of the metabolic changes connected with this defect. Ketones are isolated by a gas-phase extraction and adsorption technique and profiled after thermal desorption. Organic acids are isolated by solvent extraction or anion exchange, derivatized and separated either as total acid profiles or subprofiles after pre-fractionation of the acid derivatives.

The main results are as follows. (a) Increased total 4-heptanone is inherently connected with diabetes mellitus. Its urinary levels are elevated in therapeutically well controlled patients. (b) A general ketogenesis pathway leads to higher molecular weight ketone bodies in addition to the conventional ketone bodies. (c) During diabetic ketoacidosis, in addition to the fatty acids the following acids are elevated in serum and in urine: dicarboxylic acids resulting from ω- and β-oxidation of monocarboxylic acids; oxomonocarboxylic acids as metabolites of the amino acids valine, leucine and isoleucine and as products of ketogenesis; and hydroxymonocarboxylic acids, also originating from amino acids and from ketogenesis.

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