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DISTINCTION BETWEEN URINARY ACIDS ORIGINATING FROM NUTRITION AND THOSE PRODUCED IN THE HUMAN BODY

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SUMMARY

A simple procedure to distinguish between urinary acids originating from nutrition and beverages and those produced from compounds stored in the body is described. Acids originating from nutrition disappear in urine samples after a few days of a zero diet, whereas those produced from body compounds do not. The zero diet can be substituted by a diet of peeled rice and water.

INTRODUCTION

Urine contains a large number of acidic compounds. Some of these are excreted in highly increased amounts in the case of inborn errors of metabolism [1, 2]. Profiling of the acid fraction of urine has therefore become a powerful method if such an inborn error of metabolism is suspected [3]. Most of the excreted acids originate from nutrition; variations in nutrition may alter the profiles. A method is required to distinguish between compounds occurring in urine that originate from nutrition and those that are produced from compounds stored in the body, which are usually those acids that reflect an error in metabolism.

Some time ago, we tried to make such a distinction by comparing acids obtained from urine samples of a baby fed only with mother's milk and acids present in the mother's milk. Compounds found in the urine but not in mother's milk were regarded to be produced in the baby's body [4].

This is a rather complicated procedure. A much easier method was recently applied in our laboratory in order to determine whether a previously unknown compound, detected in the neutral urine fraction, was a product of metabolism of a foodstuff or of a body compound: it was found out that the compound dis-

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appeared during a zero diet [5] and reappeared if food was consumed, thus indicating its origin from nutrition. Based on this finding, we report here on the investigation into the behaviour of urinary acids in the course of a zero diet, which proved to be a simple method to determine the origin of acids.

Some of the acids taken into the body via nutrition or beverages run through the body without change, some are metabolized during the passage and some may be metabolites of compounds stored in the body. Only the latter are of interest for the detection of metabolic disorders.

EXPERIMENTAL

Reagents and solvents

Sulphuric acid, ethyl acetate, cyclohexane, diethyl ether and methanol were purchased from Merck (Darmstadt, F.R.G.). The internal standard 2-(3-carboxy-4-methyl-5-pentylfuranyl)acetic acid (II) was prepared in our laboratory [6]; 13-oxotetradecanoic acid (III) and 13-hydroxytetradecanoic acid (IV) were prepared in the laboratory of Professor H. Gerlach, University of Bayreuth.

Instruments

Gas chromatograph: HRGC Carlo Erba 4160, WCOT glass capillary columns, OV-101; temperature-programmed from 100 to 192°C at 2°C/min, from 192 to 280°C at 5°C/min, with the detector at 270°C.

Gas chromatograph—mass spectrometer: a Varian MAT 312 mass spectrometer combined with a Varian 3700 gas chromatograph.

Procedure

Urine was sampled in several portions per day in clean polyethylene bottles and kept frozen at −20°C until used.

To determine the average excretion of acids during 1 h (ml/h), the amount of sample was measured during the excretion period. The determined amount of urine collected in 1 h was transferred to a 100-ml bottle. This was filled up with distilled water to 100 ml. A 10-μl aliquot (corresponding to 30 μg) of standard II, 30 μl (corresponding to 60 μg) of standard III and 30 μl (corresponding to 90 μg) of standard IV were added (we did not determine the concentrations in fraction A; so we did not need a standard) and the solution was brought to pH 1 by addition of concentrated sulphuric acid. The acidified urine samples were extracted three times with 50 ml of ethyl acetate for 1.5 min using a shaking machine. Several millilitres of acetone were added to destroy emulsions. The combined organic layers were dried with 5 g of sodium sulphate, the sodium sulphate was filtered off and the ethyl acetate was removed by a rotary evaporator. The residue was dissolved in 10 ml of methanol and treated with 10 ml of a 5% solution of diazomethane in diethyl ether at room temperature. The excess diazomethane was removed by a stream of nitrogen.

A 100-mg quantity of silica gel 60 (Fluka, 70–230 mesh) was added to the methanolic solution. The suspension was evaporated to dryness. The sample adsorbed on the silica gel was added to the head of a silica gel column (5 g of silica gel were soaked in cyclohexane and filled into a 22 × 1 cm I.D. column).

Fraction A was eluted with 25 ml of cyclohexane—diethyl ether (95:5), fraction B with 25 ml of cyclohexane—diethyl ether (80:20), fraction C with 25 ml of cyclohexane—diethyl ether (70:30) and fraction D with 25 ml of cyclohexane—diethyl ether (50:50) [7]. Fraction A (containing fatty acids) was discarded and fractions B, C and D were brought to dryness. Each residue was

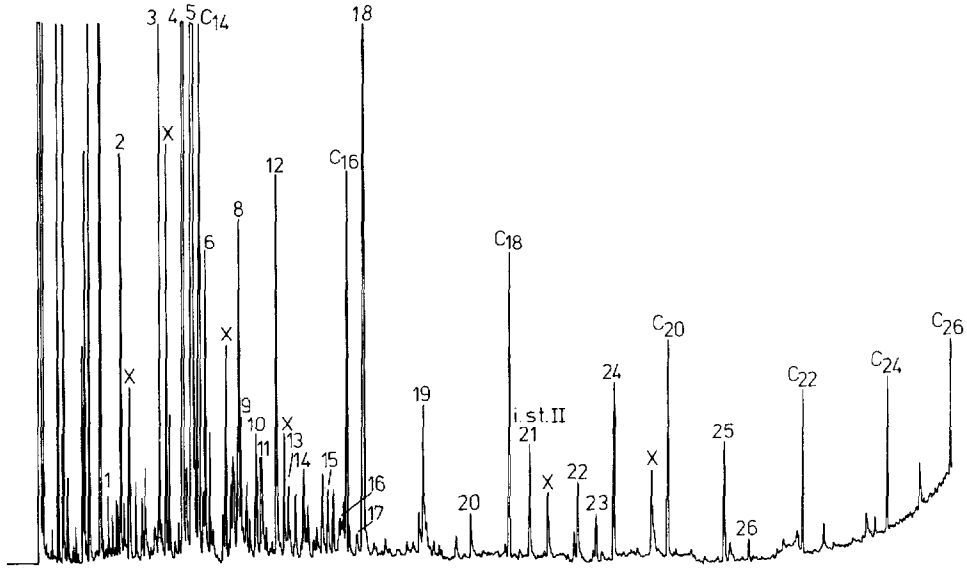


Fig. 1. Glass-capillary gas chromatogram of urinary organic acids separated as their methyl esters; fraction B; X = unknown compounds; i.st. = internal standard.

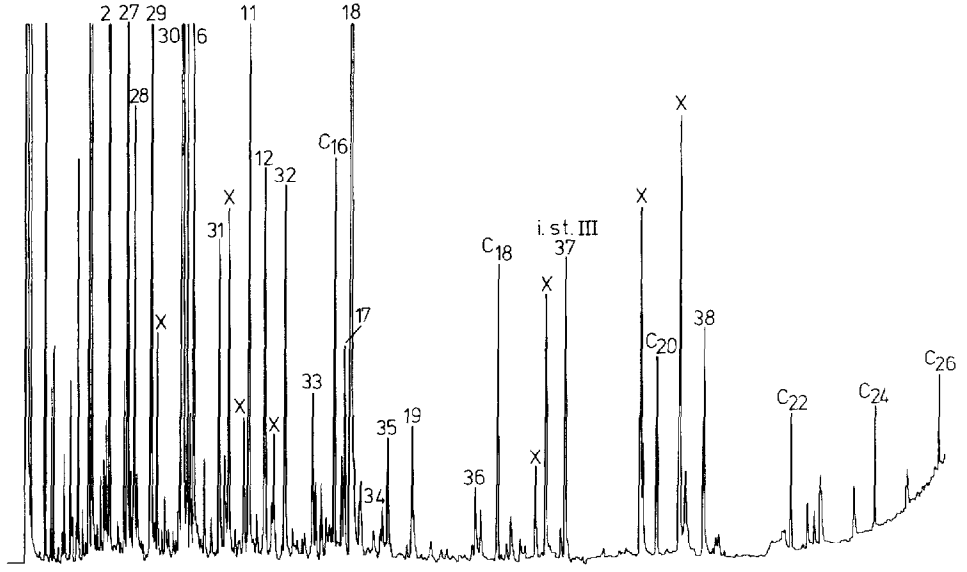


Fig. 2. Glass capillary gas chromatogram of urinary organic acids separated as their methyl esters; fraction C; X = unknown compounds; i.st. = internal standard.

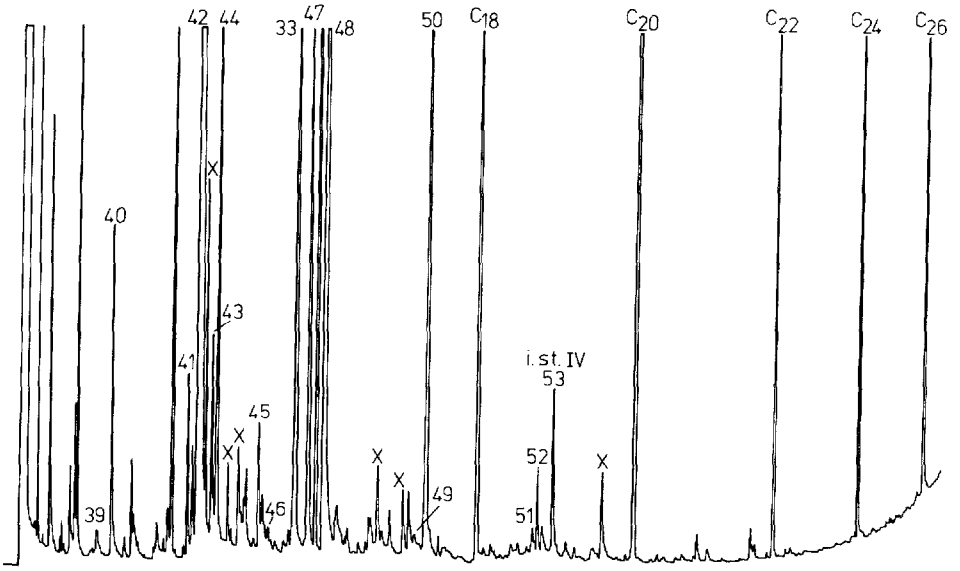


Fig. 3. Glass capillary gas chromatogram of urinary organic acids separated as their methyl esters; fraction D; X = unknown compounds; i.st. = internal standard.

dissolved in 3 ml of diethyl ether and transferred into a small glass container. The diethyl ether was removed by a stream of nitrogen.

Fraction B was then dissolved in 50 μl, fraction C in 70 μl and fraction D in 100 μl of ethyl acetate. Aliquots of these solutions (0.2 μl) were injected into the gas chromatograph. Figs. 1, 2 and 3 show the gas chromatograms of the methylated acids.

RESULTS AND DISCUSSION

Reproducibility

Ten samples of the same urine collection were analysed and the standard deviation was determined. The standard deviations (ΔX) are given in Table I as $\Delta X/X$. Quantification of fatty acids was impossible, since they were eluted in fraction A and fraction B and we did not use a standard for fraction A. Short-chain carboxylic acids showed high standard deviations due to heavy losses during evaporation. The reproducibility of the more interesting compounds, e.g. long-chain dicarboxylic acids, aromatic hydroxy acids and urofuran acids, seemed acceptable.

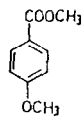
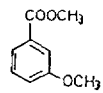
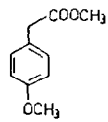
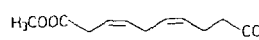
Quantification

The method used was a half-quantitative one, because we did not know all the response factors of the compounds under gas chromatography. The relation of the peak area or peak height of the compounds to the peak area or peak height of the internal standard was determined. This value is called unit/standard. This method made it possible to determine the relative alterations in excretion, but not the absolute values.

TABLE I

LIST OF URINARY ORGANIC ACIDS SEPARATED AS THEIR METHYL ESTERS

In column 5, standard deviations are given in per cent; I.S. = internal standard.

No.	Retention index	Structure	Fraction	$\Delta X/X$ (%)
1	1216	$H_3COOC-(CH_2)_4-COOCH_3$	B	108
2	1242	$H_3COOC-\underset{\displaystyle CH_3}{CH}-(CH_2)_3-COOCH_3$	B + C	86
3	1325		B	50
4	1368	$H_3COOC-\underset{\displaystyle CH_3}{CH}-CH_2-\underset{\displaystyle CH_3}{CH}-(CH_2)_2-COOCH_3$	B	24
5	1386		B	26
6	1410		B	25
7	1415	$H_3COOC-(CH_2)_6-COOCH_3$	B + C	34
8	1455	$H_3COOC-(C_7H_{12})-COOCH_3$	B	29
9	1459	$H_3COOC-CH_2-\underset{\displaystyle CH_3}{CH}-(CH_2)_4-COOCH_3$	B	18
10	1479	$H_3COOC-\underset{\displaystyle CH_3}{CH}-(CH_2)_6-COOCH_3$	B	22
11	1487	$H_3COOC-CH=CH-(CH_2)_5-COOCH_3$	B + C	19
12	1508	$H_3COOC-(CH_2)_7-COOCH_3$	B + C	12
13	1519	$H_3COOC-(C_8H_{14})-COOCH_3$	B	
14	1544	$H_3COOC-CH_2-\underset{\displaystyle CH_3}{CH}-CH_2-\underset{\displaystyle CH_3}{CH}-(CH_2)_2-COOCH_3$	B	59
15	1576	$H_3COOC-(CH_2)_3-CH=CH-(CH_2)_3-COOCH_3$	B	43
16	1592	$H_3COOC-(CH_2)_3-CH=CH-(CH_2)_3-COOCH_3$	B	43
17	1612	$H_3COOC-(CH_2)_8-COOCH_3$	B + C	12
18	1624		B + C	10

(Continued on p. 8)

TABLE I (continued)

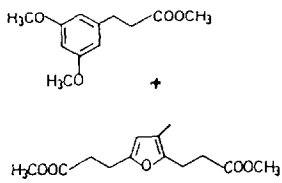
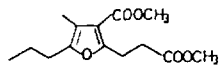
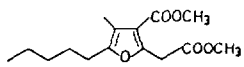
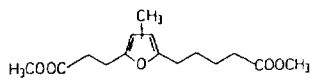
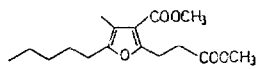
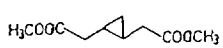
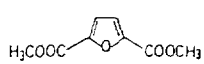
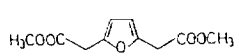
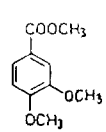
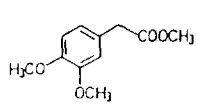
No.	Retention index	Structure		Fraction	$\Delta X/X$ (%)
19	1703			B + C	
20	1753			B	11
21	1825		I.S. II	B	
22	1887			B	20
23	1910	$H_3COOC-(CH_2)_{14}-CH_3$		I+II A + B	73
24	1934			B	9
25	2091	$H_3COOC-(CH_2)_7-CH=CH-(CH_2)_7-CH_3$		A + B	
26	2115	$H_3COOC-(CH_2)_{16}-CH_3$		A + B	83
27	1276			C	
28	1294	$H_3COOC-(CH_2)_5-COOCH_3$		C	72
29	1328			C	17
30	1391	$H_3COOC-(CH_2)_2-CH=CH-(CH_2)_2-COOCH_3$		C	101
31	1444			C	22
32	1535			C	9
33	1572			C + D	18

TABLE I (continued)

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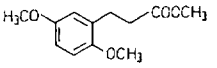
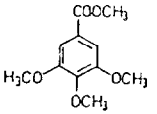
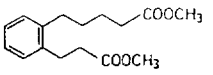
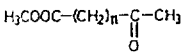
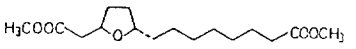
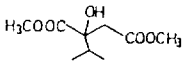
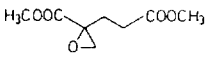
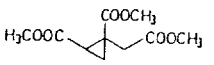
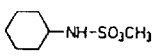
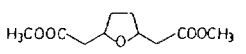
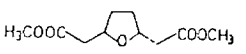
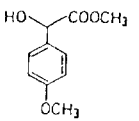
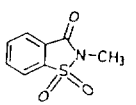
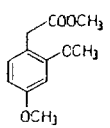
34	1660			C	8
35	1668			C	15
36	1772			C	
37	1886		I.S. III	C	
38	2074			C	22
39	1230			D	163
40	1272			D	29
41	1420			D	
42	1440			D	19
43	1461			D	13
44	1465				
45	1521			D	25
46	1534			D	52
47	1588			D	28

TABLE I (continued)

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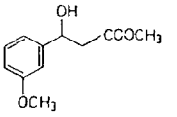
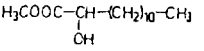
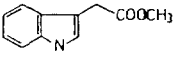
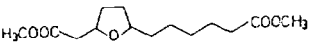
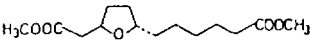
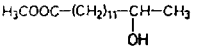
48	1611			D	17
49	1718			D	13
50	1740			D	11
51	1871			D	15
52	1878			D	17
53	1895		I.S. IV	D	

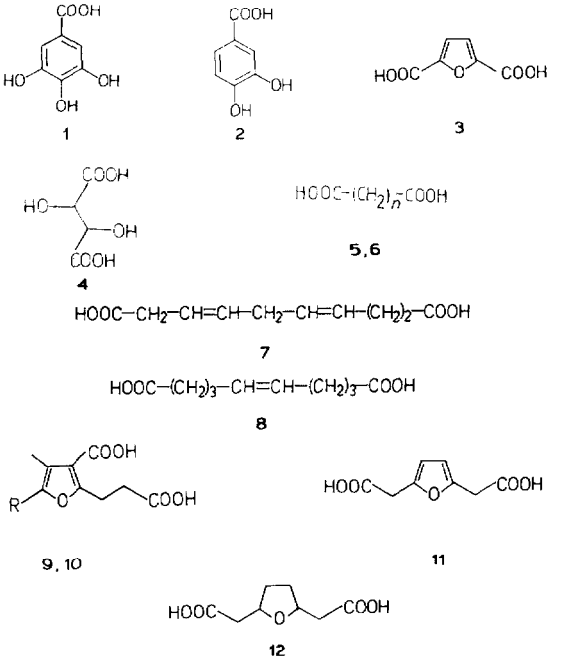
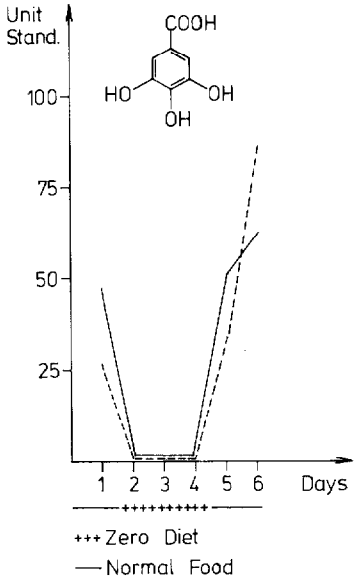


Fig. 4. Chemical structures of 3,4,5-trihydroxybenzoic acid (1), 3,4-dihydroxybenzoic acid (2), 2,5-furandicarboxylic acid (3), tartaric acid (4), nonanedioic acid (5, n = 7), heptanedioic acid (6, n = 5), deca-3,6-dienedioic acid (7), deca-5-enedioic acid (8), propylurofuran acid (9, R = C_3H_7), pentylurofuran acid (10, R = C_5H_{11}), 2,5-furandiacetic acid (11) and tetrahydrofurandiacetic acid (12).

Acids running through the body without change. Four acids ran through the body unchanged within a few hours: 3,4,5-trihydroxybenzoic acid (**1**), 3,4-dihydroxybenzoic acid (**2**), 2,5-furandicarboxylic acid (**3**) and tartaric acid (**4**) (Fig. 4). They disappear completely from the urine samples after one day of zero diet and reappear immediately after the resumption of food (see Fig. 5).

Acids that suffer metabolism. The greatest part of excreted acids belong to compounds that are degraded by the well known fatty acid metabolism. Since during a zero diet the body tries to use the available stocks of energy optimally, long-chain fatty acids are often metabolized by ω-oxidation combined with β-oxidation. Thus, dicarboxylic acids are produced. During a zero diet, we observe a reduction in excretion of longer dicarboxylic acids compared to excretion during consumption of normal food; for example, the excretion of nonanedioic acid (**5**, Fig. 4) is reduced very much (by a factor of 3 to 4). In contrast, the excretion of heptanedioic acid (**6**, Fig. 4) is increased by a similar factor, reflecting the better use of the fatty acid stock by better use of the larger fatty acids in further oxidation reactions. This finding is in agreement with starvation experiments carried out with rats (see Figs. 6 and 7) [8]. Also, two unsaturated dicarboxylic acids found in very limited amounts during normal eating are excreted in high amounts during the zero diet: deca-3,6-dienedioic acid (**7**) and deca-5-enedioic acid (**8**) (Fig. 4). The strong increase of compound **8** during the zero diet is especially remarkable.

Deca-3,6-dienedioic acid (**7**, Fig. 4) was found in increased amounts in the urine of rats treated with hypoglycin A [9]. Hypoglycin A is an unusual amino acid. The metabolites thereof [10] were found to cause Jamaica vomiting sickness [11, 12], a metabolic disease in which the blood sugar content was



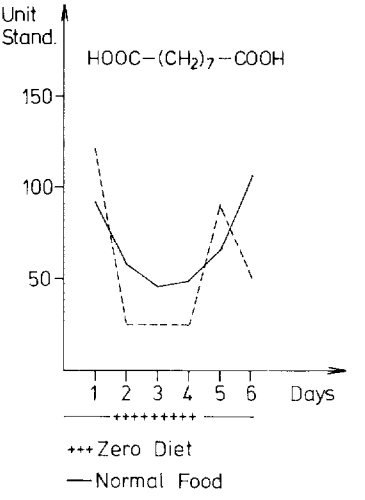
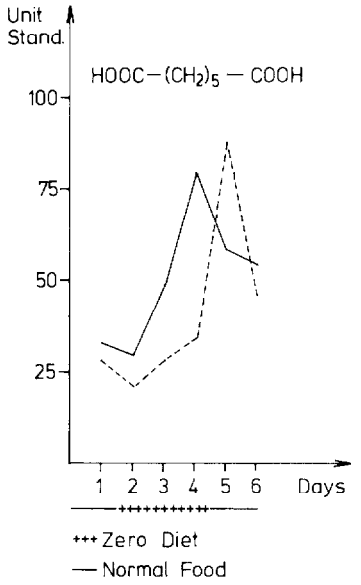


Fig. 5. Excretion of 3,4,5-trihydroxybenzoic acid before, during and after a zero diet; values from two persons.

Fig. 6. Excretion of nonanedioic acid (**5**) before, during and after a zero diet; values from two persons.



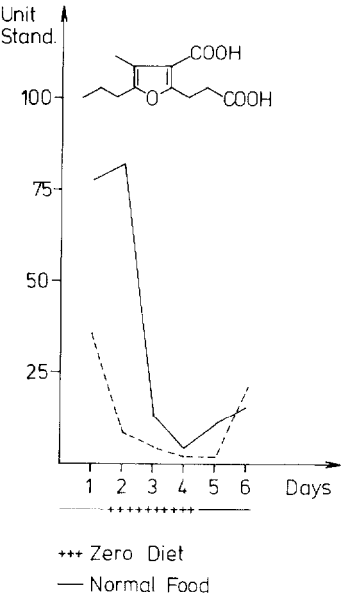


Fig. 7. Excretion of heptanedioic acid (**6**) before, during and after a zero diet; values from two persons.

Fig. 8. Excretion of propylurofuran acid (**9**) before, during and after a zero diet; values from two persons.

found to be extremely low [13] and the liver glycogen exhausted. The increase in excretion [14] of deca-3,6-dienedioic acid (**7**) in rats fed with hypoglycin A was assumed to be connected with an unknown influence of hypoglycin A. Our experiments now present a simple explanation for this finding.

Hypoglycin A is known to increase the activity of fatty acid metabolism [9]. If no liver glycogen is available to provide the energy for this increased metabolism, the stored fatty acids must provide this energy, and this would cause the increase in excretion of compound **7** in analogy to our zerodiet experiments, where an increase of compound **7** was observed too.

The precursors of compounds **7** and **8** are obviously oleic acid and linolenic acid, which seem to be degraded in the case of energy need in the body in larger amounts by ω- and β-oxidation.

Recently, we detected a previously unknown class of compounds in the body, the urofuran acids [15]. The main representatives of this group of compounds are propylurofuran acid (**9**, Fig. 4) and pentylurofuran acid (**10**, Fig. 4). Persons subjected to a zero diet excrete far less of these compounds (see Fig. 8). This would either mean that compound **9** is further degraded in higher amounts than usual during a zero diet to still unknown acids (e.g. tri- or tetracarboxylic acids by oxidative attack of the alkyl side-chain), which may escape detection due to their high water solubility, or that their precursor molecules must be provided from the limited body stock. Thus, the behaviour of these acids under zero diet is comparable with that of long-chain dicarboxylic acids (cf. Fig. 6). Interestingly, the pentylurofuran acid (**10**) behaves differently; its decrease in a zero diet is smaller compared to that of propylurofuran acid.

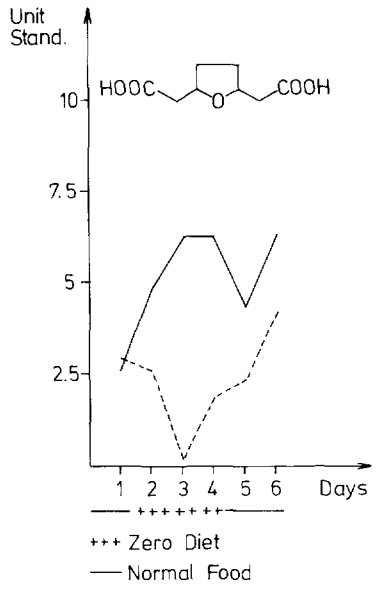


Fig. 9. Excretion of tetrahydrofurandiacetic acid (**12**) before, during and after a zero diet; values from two persons.

In contrast 2,5-furandiacetic acid (**11**, Fig. 4) is excreted in the case of a zero diet in much larger amounts than during normal uptake of food. Therefore, we can conclude that compound **11** is a final product in a chain of metabolic processes. Compound **11** must be produced from compounds stored or synthesized in the body.

Another group of excretion products are obviously not influenced during a zero diet (Fig. 9). These compounds are the *cis* and *trans* forms of tetrahydrofurandiacetic acid (**12**, Fig. 4).

CONCLUSION

The reported results allow us to classify the urinary acids into different groups: (a) Compounds introduced by nutrition or beverages, which run through the body unchanged, disappear very rapidly in the urine profiles during a zero diet. (b) Compounds taken in by nutrition or beverages, which are metabolized within the body, usually disappear in urine profiles during a zero diet more slowly than the compounds mentioned above; sometimes they may still be detected three to four days after starting the diet [5]. (c) The excretion of final products of metabolic processes derived from compounds stored in the body increases at least in the first few days of a zero diet. (d) Intermediates of metabolic processes derived from compounds stored in the body show a decreased excretion in urine during a zero diet. (e) No effect of a zero diet is typical for endogenous compounds that are not involved in the metabolism.

A long-lasting zero diet is not very comfortable, therefore, we have tried to substitute it. This can be done if a diet of peeled rice and water is consumed. Rice contains virtually no fatty acids or proteins. In addition, rice is adsorbed to a very high extent by the intestine, resulting virtually in a halt in

excretion of faeces. Thus, urine collection after a one-week rice diet is sufficient to find all the typical metabolites of body compounds, and to eliminate those taken into the body by nutrition. The rice-diet experiments confirmed the results obtained by the zero diet.

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