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EXCRETION OF URINARY ACIDS DURING INVERTED SLEEP–WAKING RHYTHM

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SUMMARY

Some organic acids that occur in human urine are excreted in very different amounts during the day and the night. The day–night rhythm in the excretion rate of citric acid and propylurofuran acid changes only gradually if the normal rhythm of life is inverted, e.g., if a person sleeps during the day and works at night (shift of 12 h), paralleling the gradual adjustment of steroid metabolism under the same conditions. In contrast, the typical rhythm in the excretion rate of tetrahydrofuran acids and tartaric acid inverts immediately if the sleep–waking rhythm of a person is inverted. In contrast to propylurofuran acid, pentylurofuran acid is excreted unrhythmically. No change in the excretion rate of amino acids was observed if the daily rhythm was inverted. Quantifications were achieved by liquid–liquid extraction, derivatization and gas chromatography computerized peak area integration.

INTRODUCTION

Adaptation of the human organism to changes in the usual sleep–waking rhythm by west- or east-bound long-distance flights [1] or shift work [2] is a well known phenomenon. Elucidation of individual physiological ailments was tried by studying variations in the typical circadian rhythm of body temperature [3], plasma cortisol [3] or excretion of 17-hydroxycorticosteroids in the urine [3]. These factors are known to be different in activity and sleep. For instance, the cortisol level in blood is highest in the early morning [3] and the excretion rates of 17-hydroxysteroids reach the highest value at noon [3].

Recently, we detected that some acids are excreted in urine in a circadian rhythm [4]. The determination of acid levels by gas chromatography (GC) is easy if the acids are converted into their methyl derivatives.

We have studied the relative amounts of citric acid, urofuran acids and tetrahydrofuran acids excreted if the sleep–waking rhythm was inverted (shift of 12

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h) and compared the rhythm of the excretion of these acids with that of steroids and amino acids which were quantified simultaneously.

EXPERIMENTAL

Diet

For the rhythm-inversion experiment, two healthy male subjects followed the diet listed below for fourteen days.

8.00 a.m.: two cups of milk with cocao powder, one piece of rye-bread, margarine, fruit jam (blackcurrant).
11.30 a.m.: one steak (pork, 150 g), roasted with palm oil, flavoured with salt, pepper, red pepper and curry; two baked potatoes, salad, 0.5 l of beer.
2.00 p.m.: 0.7 l of mineral water.
3.00 p.m.: one chocolate bar.
4.00 p.m.: one apple.
5.00 p.m.: two pieces of rye-bread, margarine, ham (125 g), 0.5 l of beer.
9.00 p.m.: 0.5 l of beer.

Sampling

Urine was collected at 8.00 a.m., 1.00 p.m., 5.30 p.m. and 10.30 p.m. in clean polyethylene bottles and stored at −20 °C until work-up.

Quantitative analysis of organic acids

Reagents and solvents. DEAE-Sephadex A-25 was purchased from Sigma (Munich, F.R.G.), pyridine, acetic acid, sulphuric acid, ethyl acetate, cyclohexane, diethyl ether and methanol from Merck (Darmstadt, F.R.G.) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Macherey and Nagel (Düren, F.R.G.). The internal standard 3,3-dimethylglutaric acid was purchased from Merck. The internal standard 2-(3-carboxy-4-methyl-5-pentylfuranyl)acetic acid (II) was prepared in our laboratory; 13-oxotetradecanoic acid (III) and 13-hydroxytetradecanoic acid (IV) were prepared in the laboratory of Professor H. Gerlach (University of Bayreuth). Pyridinium acetate solutions were prepared freshly each week as pyridine–acetic acid (1:1) in water.

Instruments. Gas chromatograph 1 (used in the course of the anion-exchange method) utilized HRGC 4160 (Carlo Erba, Hofheim/Ts., F.R.G.) with glass WCOT capillary columns (25 m × 0.3 mm I.D.) coated with OV-101. The following conditions were used: carrier gas, hydrogen; flow-rate, 2 ml/min; detector, flame ionization; injector temperature, 260 °C; detector temperature, 280 °C; temperature programme, 2 °C/min starting at 100 °C.

Gas chromatograph 2 (used in the course of the ethyl acetate extraction method) utilized L 402 (Siemens, Nürnberg, F.R.G.) with glass WCOT capillary columns (25 m × 0.3 mm I.D.) coated with OV-101. The following conditions were used: carrier gas, hydrogen (0.8 bar = $8 \cdot 10^4$ Pa); splitting ratio, 1:10; injector temperature, 270 °C; detector, flame ionization; detector temperature, 280 °C.

Gas chromatography–mass spectrometry was carried out with a Model 3700

gas chromatograph (Varian, Darmstadt, F.R.G.) on a glass WCOT capillary column (25 m × 0.3 mm I.D.) coated with OV-101 with helium as carrier gas at 2 ml/min, combined with a Varian-MAT 312 double-focusing mass spectrometer with reverse Nier–Johnson geometry; the ionization energy was 70 eV and the scan interval was 2.3 s; a Varian SS 200 data system was used (PDP 11/30 computer).

Quantitative analysis of urinary tartaric acid and citric acid. Tartaric acid and citric acid were determined according the procedure of Thomson and Markey [5] with some modifications. DEAE-Sephadex A-25 anion-exchange resin was prepared by soaking 20 g in distilled water for one day. The adsorbent was then washed with 0.5 *M* hydrochloric acid (500 ml), water (until neutral), 0.5 *M* sodium hydroxide (500 ml) and water (until neutral). The resin was stored in distilled water until used.

After this preparation, the resin was packed into a 22 cm × 1 cm I.D. column to a height of 8 cm and washed with 40 ml of 0.5 *M* pyridinium acetate solution. Of the amount of urine sampled in 1 h, 1% was added to the head of the anion-exchange column. Then 100 μg of the internal standard (in 10 μl of water) were allowed to drain into the resin, 30 ml of water were passed through and the acids were eluted with 18 ml of 1.5 *M* pyridinium acetate. The eluate was collected, frozen and lyophylized (0.5 Torr, −10°C). The residue was dissolved in 3 ml of methanol, transferred into a Reactivial (Pierce, Rockford, IL, U.S.A.) and the solvent removed with a stream of nitrogen.

The samples were trimethylsilylated for GC analysis by adding 50 μl of pyridine–tetrahydrofuran (50:50) and 250 μl of MSTFA. The solution was kept at 60°C for 15 h. A 0.6-μl aliquot of the solution was injected into the gas chromatograph.

Quantitative analysis of 3,6-epoxydodecanedioic acid, propylurofuran acid and pentylurofuran acid. To determine the average excretion (ml/h) of these acids, the amount of sample was measured during the excretion period. The determined amount of urine collected in 1 h was transferred into a 100-ml bottle, which was filled up to 100 ml with distilled water. 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 adjusted 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 over 5 g of sodium sulphate, the sodium sulphate was filtered off and the ethyl acetate was removed with 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 of diazomethane was removed with a stream of nitrogen.

A 100-mg amount of silica gel 60 (Fluka, Neu-Ulm, F.R.G.) (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

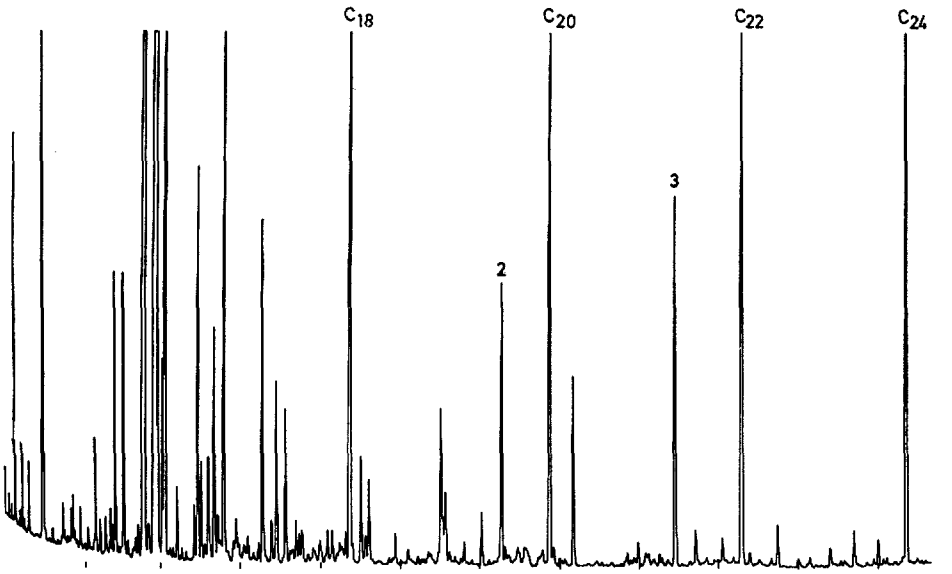


Fig. 1. Glass capillary gas chromatogram of urinary organic acids separated as their methyl esters. Fraction B: 2 = propylurofuran acid; 3 = pentylurofuran acid.

(5 g of silica gel were soaked in cyclohexane and packed into a 22 cm × 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). Fraction A (containing fatty acids) was discarded and fractions B, C and D were evaporated to dryness. Each residue was dissolved in 3 ml of diethyl ether and transferred into a small glass container. The diethyl ether was removed with 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. Fig. 1 shows the gas chromatogram of the methylated acids of fraction B, where pentyl- and propylurofuran acid were eluted.

Quantification. The method used was semi-quantitative, because we did not know all the response factors of the compounds involved. The relationship between the peak area or peak height of the compounds and the peak area or peak height of the internal standard was determined. This value is called the unit/standard. This method made it possible to determine the relative alterations in excretion, but not the absolute values.

The urinary organic acids separated as their trimethylsilyl derivatives and as their methyl esters were listed earlier [4,6].

Quantitative analysis of steroids

Enzymatic hydrolysis. A 10-ml volume of urine, 15 ml of 0.5 mol/l sodium acetate buffer (pH 4.76), 0.2 ml of β-glucuronidase–arylsulphatase (Boehringer, Mannheim, F.R.G.) and 10 μl of standard solution (20 mg of epi-etiocholanolone in 10 ml of ethanol) were stirred for 40 h at 37°C.

Extraction. The mixture was extracted twice for 3 min with 35 ml of ethyl acetate. The combined organic phases were extracted twice for 45 s with 15 ml of a solution of sodium hydrogencarbonate and sodium chloride (5% and 10%, 1:1, v/v) and once for 15 s with 15 ml of saturated sodium chloride solution. The organic phase was dried with anhydrous sodium sulphate and the solvent was removed with a rotary evaporator.

Derivatization. The residue was dissolved in 1.5 ml of methanol, 0.6 ml of the solution was transferred into a test-tube and the solvent was removed with a rotary evaporator. After addition of 2.5 μl of absolute pyridine, 2.5 μl of absolute tetrahydrofuran and 25 μl of MSTFA, the mixture was kept for 30 min in an ultrasonic bath. Then 10 μl of this solution were heated to 60°C for 15 h over 1 mg of anhydrous sodium acetate in a fused melting-point tube.

GC analysis. About 0.5–1 μl of the resulting samples were subjected to GC analysis using chromatograph 2 as described above and computerized peak area integration with a Kontron Anacomp 220 computer system and Kontron INTEGR system software (Kontron, Eching, F.R.G.). The individual relative steroid amounts were calculated from the ratio (20 × peak area)/standard area = amount of steroid (in μg).

GC–MS analysis. In order to confirm the correctness of the assignment of the individual steroids to the respective peaks in the steroid profile, every twentieth sample was routinely measured by GC–MS using the conditions described above.

Quantitative analysis of amino acids

The twenty proteinogenic amino acids, 1-methylhistidine, 3-methylhistidine, 3-hydroxyproline and N-α-methyllysine were purchased from Sigma (Munich, F.R.G.). Amberlite IR-120 ion-exchange resin (20–80 mesh) was obtained from Serva (Heidelberg, F.R.G.). Cycloleucine, norleucine and *trans*-4-(aminomethylcyclohexane)carboxylic acid (Aca) were purchased from EGA (Steinheim, F.R.G.). Standard solutions of amino acids (10 mmol/l) were prepared by dissolving the amino acids (Sigma) in 0.1 *M* hydrochloric acid containing several drops of ethanethiol.

Reagents and solvents. Methanol, *n*-butanol, 25% ammonia solution, ethanethiol, heptafluorobutyric anhydride (HFBA), chloroethyl formate, acetyl chloride and hydrochloric acid (32%) were of analytical-reagent grade from Merck. Dichloromethane was distilled and chromatographed on Alumina Woelm B, activity I (25 g per 100 ml). Trifluoroacetic anhydride (TFAA) and trifluoroacetic acid (TFA) were purchased from EGA.

GC–MS analysis. For GC a Carlo Erba 4160 analytical gas chromatograph was equipped with a flame ionization detector, either a home-made WCOT capillary column (I), 30 m × 0.33 mm I.D., coated with OV-101 according to the static method, or a fused-silica WCOT capillary column (II), 50 m × 0.22 mm I.D.

with chemically bonded phase OV-1701 (permanent deactivation, coating efficiency 80%) purchased from Chrompack (Middelburg, The Netherlands). The carrier gas was hydrogen at a flow-rate of 2 ml/min. Peak areas were determined with the Kontron Anakomp 220 computer system and the Kontron INTEGR system software.

Chromatographic parameters. The injector temperature was 260°C, the detector temperature 280°C and the column temperature was programmed at 3°C/min from 80 to 280°C (column I) or from 130 to 260°C (column II). Mass spectral data were calculated with a Varian-MAT 312 mass spectrometer equipped with the MAT SS 200 data system (PDP 11/34 computer). The ionization energy was 70 eV and the total ion current was registered at 20 eV. The mass spectrometer was connected to a Varian 3700 gas chromatograph equipped with the capillary columns mentioned above.

Clean-up of urine for amino acid analysis. A 5-ml volume of urine was adjusted to pH 1.8–2.0 by addition of 50% hydrochloric acid. The internal standard, 10 μmol of Aca (norleucine or cycloleucine), dissolved in 100 μl of water–methanol (1:1), was added. The acidified urine was poured on to an ion-exchange column (5 cm × 1 cm I.D.) of Amberlite IR-120 (20–80 mesh) in the hydrogen form and the column was rinsed with 200 ml of water. Then the amino acids and peptides were eluted with 100 ml of 1 *M* aqueous ammonia. The solution was concentrated under reduced pressure in a round-bottomed flask to a volume of ca. 2 ml on the rotary evaporator. The concentrate was transferred to a Reactivial with a Pasteur pipette. The rotary evaporator flask was rinsed with 1 ml of water and washings were added to the concentrate in the Reactivial. Finally, the water was removed by freeze-drying.

Conversion of amino acids into the heptafluorobutyric-n-butyl esters (HBB) and trifluoroacetyl-n-butyl esters (TAB). The freeze-dried sample was dissolved in 1 ml of 2 *M* methanolic hydrochloric acid (prepared by mixing equal amounts of methanol and acetyl chloride). The tightly closed Reactivial was heated for 20 min at 120°C in a Reactitherm module (Pierce). After cooling to room temperature, the excess of methanolic hydrochloric acid was evaporated off under a stream of nitrogen at 60°C. The residue was transesterified with 3 *M* butanolic hydrochloric acid prepared in the same way as methanolic hydrochloric acid, and heated at 120°C for 40 min. The excess of reagents was evaporated under a stream of nitrogen, a few drops of methylene chloride were added and the solvent was removed under nitrogen. A 50-μl volume of dichloromethane and 150 μl of HFBA (or TFAA) were added. After ultrasonication for a few minutes, the closed vial was placed in the Reactitherm module at 150°C for 12 min (if acylated with HFBA) or for 5 min (if acylated with TFA). After cooling to room temperature, the mixture was evaporated under a stream of nitrogen, 150 μl of dichloromethane were added and the solvent was removed under nitrogen to remove traces of water. Finally, the residue was dissolved in 20 μl of dichloromethane and 0.5 μl of this solution was analysed by GC.

Quantitation. For quantitation, an equimolar amount of the internal standard mixture was used to determine the relative molar response (RMR) of each amino acid with respect to the internal standard. The RMR was used to determine the

amino acid concentration in urine samples:

$$\text{RMR} = \frac{\text{peak area for AA}}{\text{peak area for I.S.}}$$

where AA denotes amino acid and I.S. denotes internal standard.

The amount of each amino acid was determined from

$$\text{concentration} = \frac{\text{peak area for AA}}{\text{RMR} \times \text{peak area for I.S.}} \times \text{concentration of I.S.}$$

The recovery of amino acids after ion exchange was determined as follows. An aliquot of an amino acid standard solution (10 μmol/ml) was placed on the ion-exchange column and eluted with 1 *M* ammonia solution (100 ml). After addition of internal standard (10 μmol/ml) to the eluate, the sample was lyophilized and derivatized as described above. The sample was dissolved in dichloromethane and 0.5 μl of this solution was chromatographed. The peak areas were determined and correlated with peak areas obtained after derivatization without the ion-exchange step. The recovery of protein amino acids was 97–99%.

RESULTS

The excretion of tartaric acid measured by GC after converting the acid into its tetra(trimethylsilyl) derivative during regular food intake is shown in Fig. 2; excreted amounts taken immediately after the sleeping period are indicated by black bars. Maximum excretion under normal conditions (work during the day, sleep at night) is observed between noon and the start of the sleeping period (10.00 p.m.)

If the sleeping period is inverted (work at night, sleep during the day) the excretion changes immediately; maximum excretion of tartaric acid now occurs between 1.30 a.m. and 10.30 a.m. (Fig. 3). Obviously this change in excretion is caused only by the shift in nutrition intake for 12 h. During a rice diet, the excretion of tartaric acid drops to zero, as rice does not contain even traces of tartaric acid (Fig. 4).

Similar but less pronounced excretion is observed for both isomers of 3,6-epoxydodecanedioic acid (1) measured in the form of its dimethylates. If the sleeping period is inverted (first Friday to Saturday, Fig. 5) the main amount was excreted in the period between midnight and the morning. The excretion of 1 was markedly reduced during a rice diet.

A very different excretion behaviour compared with tartaric acid and the tetrahydrofuran acid 1 is typical of citric acid. As reported earlier [4], citric acid is excreted in a circadian rhythm. If the usual sleep–waking rhythm is inverted, the average maximum amount is not decreased and occurs during the sleeping period during the day, but becomes more and more arrhythmic during the period of inverted sleep–waking rhythm. If the usual rhythm is reinstalled, the rhythmic excretion is recognized again (Fig. 6). During a rice diet excretion of the citric acid continues but becomes arrhythmic. [4].

The excretion of propylurofuran acid (2) is very similar to that of citric acid.

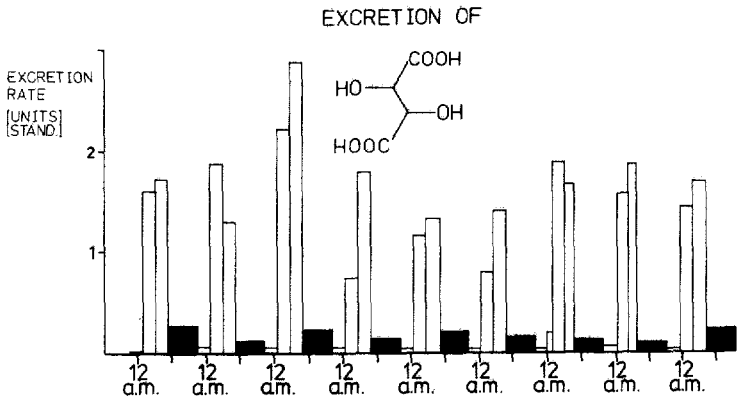


Fig. 2. Excretion of tartaric acid during constant food intake (same food at the same time of day). ■, Sleep; □, waking.

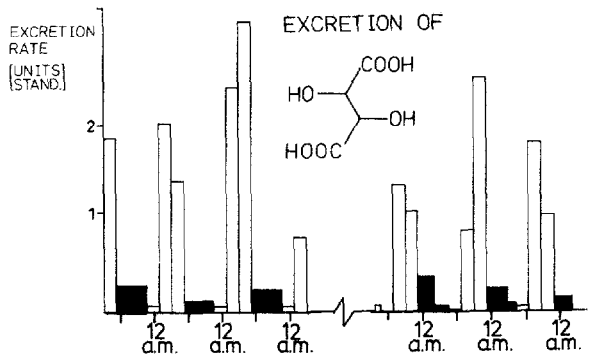


Fig. 3. Excretion of tartaric acid before and after inversion of sleep-waking rhythm. ■, Sleep; □, waking.

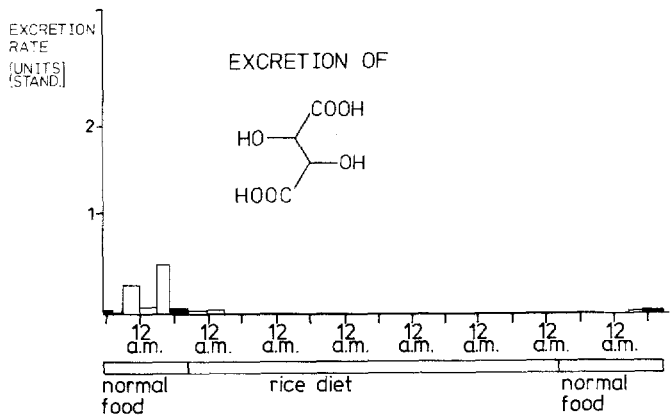


Fig. 4. Excretion of tartaric acid before, during and after rice diet. ■, Sleep; □, waking.

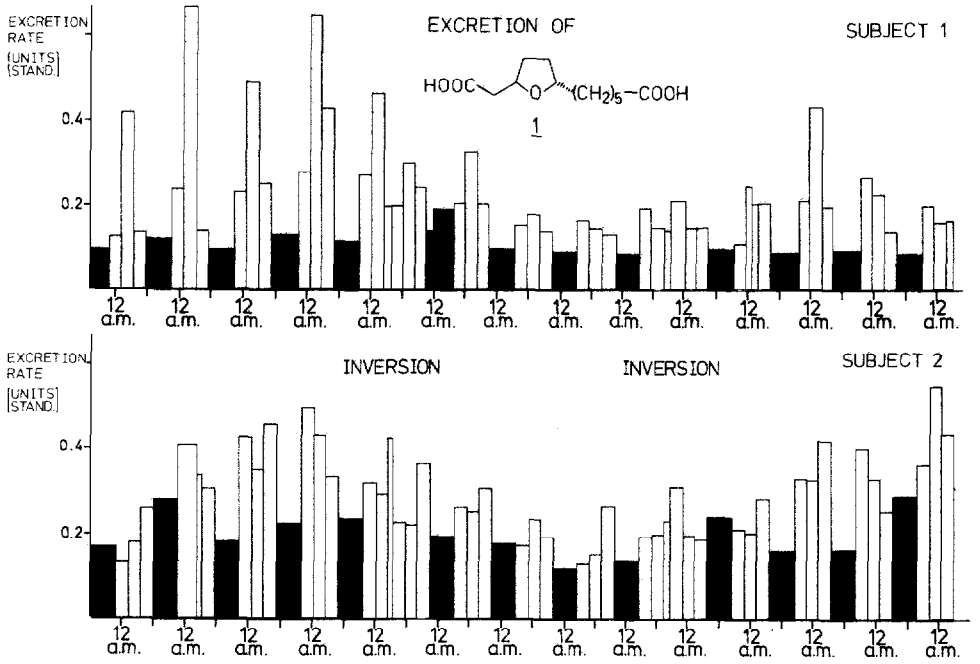


Fig. 5. Excretion of 3,6-epoxydodecanedioic acid (1) by two healthy male subjects before, during and after inversion of sleep-waking rhythm. ■, Sleep; □, waking.

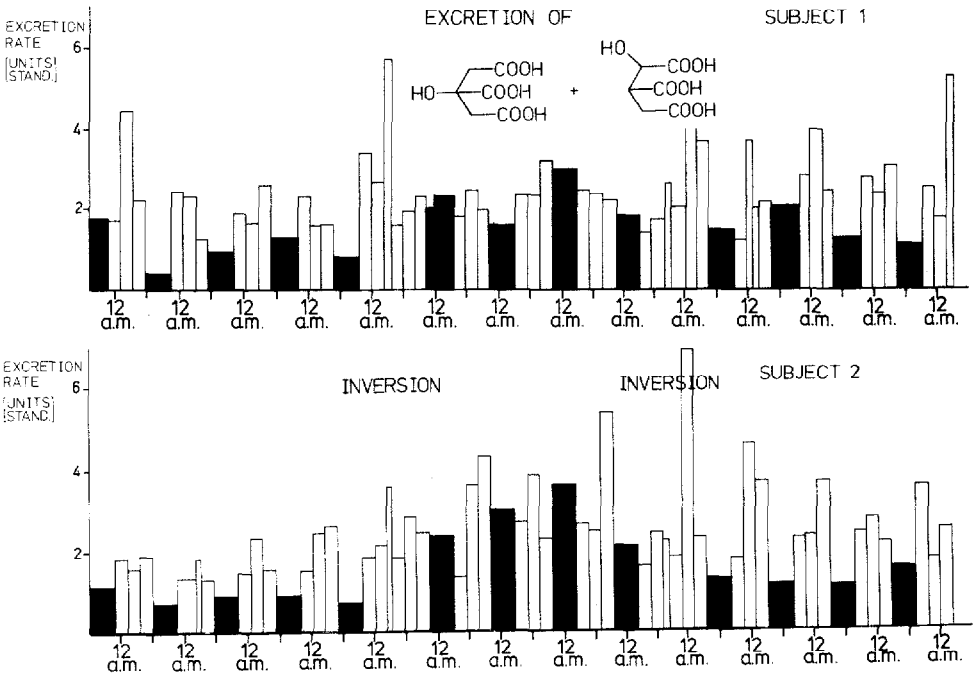


Fig. 6. Excretion of citric acid by two healthy male subjects before, during and after inversion of sleep-waking rhythm. ■, Sleep, □, waking.

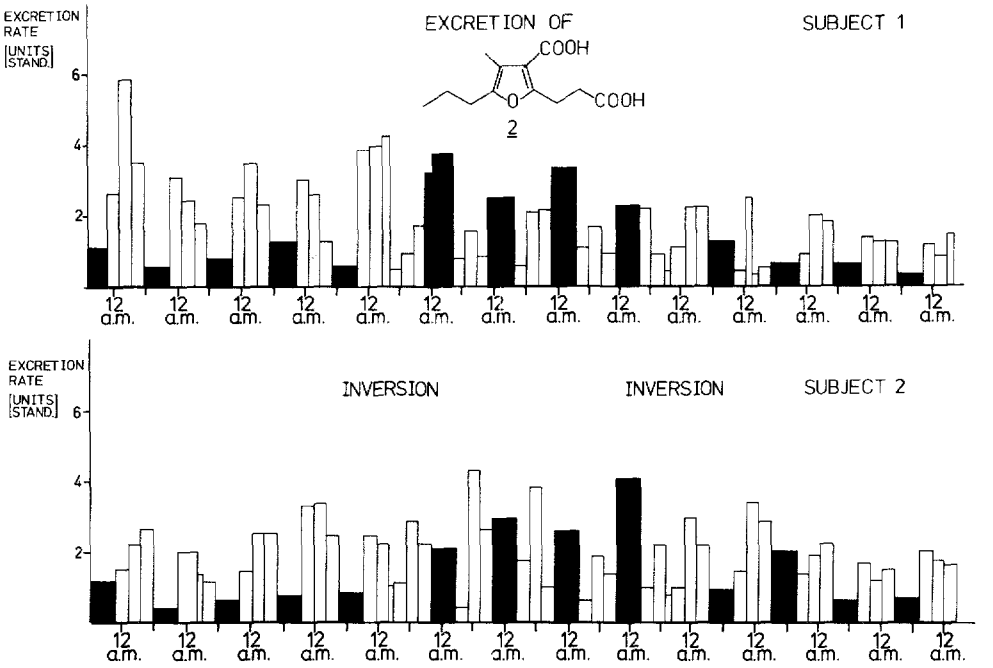


Fig. 7. Excretion of propylurofuran acid (2) by two healthy male subjects before, during and after inversion of sleep-waking rhythm. ■, Sleep; □, waking.

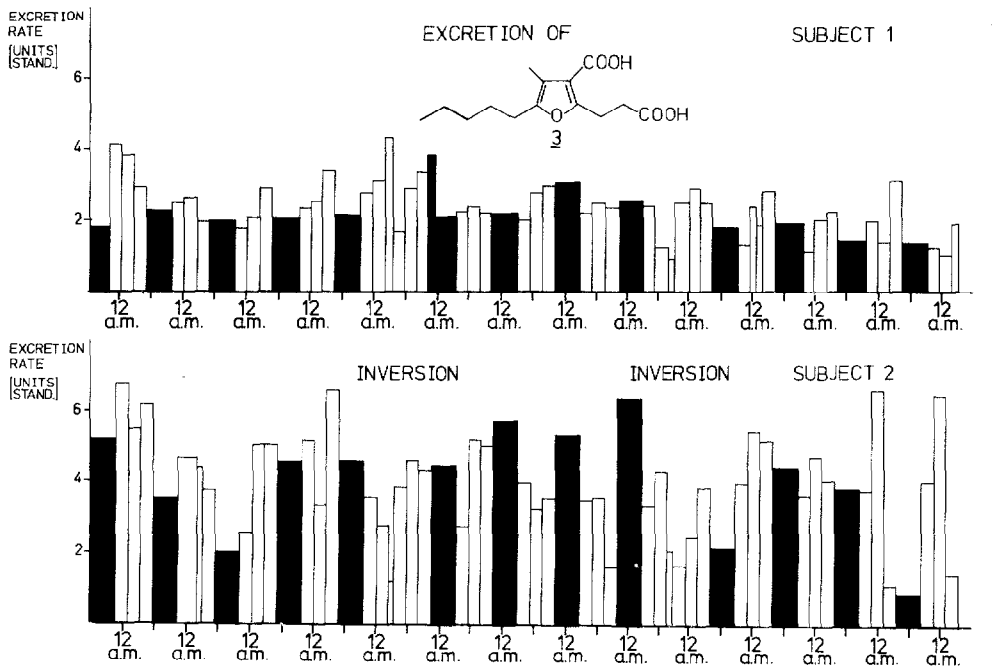


Fig. 8. Excretion of pentylurofuran acid (3) by two healthy male subjects before, during and after inversion of sleep-waking rhythm. ■, Sleep; □, waking.

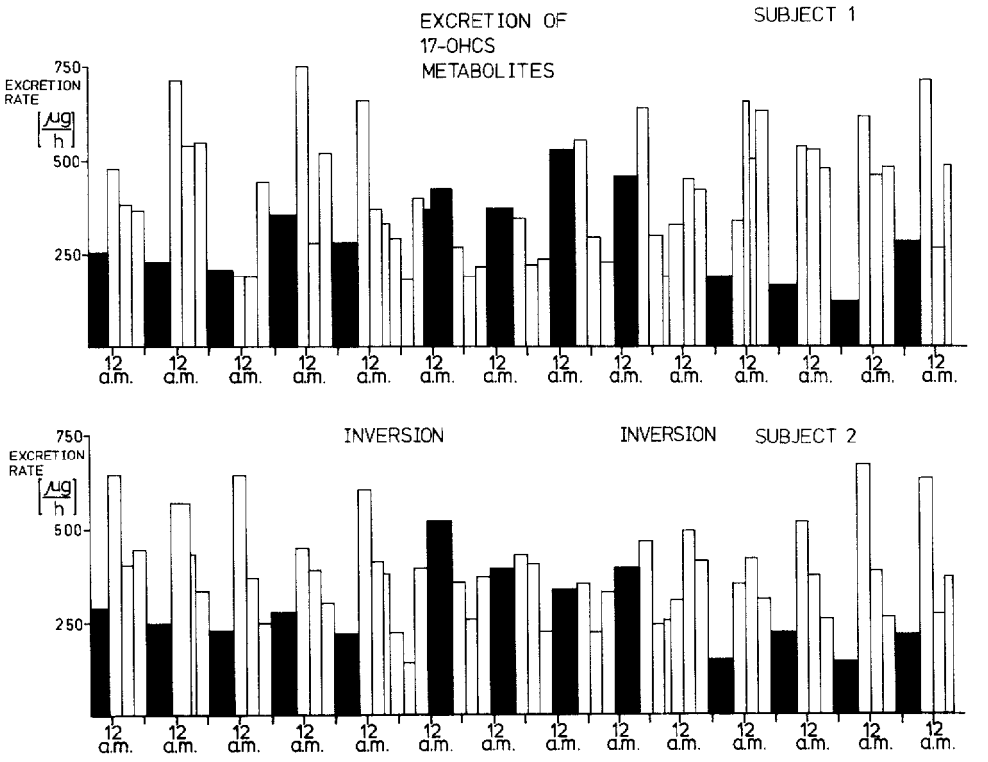
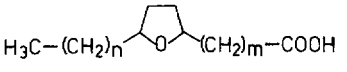


Fig. 9. Excretion of 17-hydroxycorticosteroid (17-OHCS) metabolites by two healthy male subjects before, during and after inversion of sleep-waking rhythm. ■, Sleep; □, waking.



	m	n
4	5	7
5	4	8

4,5

Fig. 10. Structures of 9, 12- and 10,13-epoxystearic acid (4 and 5).

If the sleep –waking rhythm is inverted the maximum excretion remains constant and always occurs in the afternoon (Fig. 7).

In spite of the structural similarity and obviously identical biogenetic origin of propylurofuran acid (2) and pentylurofuran acid (3), the latter unexpectedly is excreted in a much less pronounced rhythmic style (Fig. 8).

For comparison, the excretion rhythms of steroid metabolites and amino acids were determined simultaneously. The adjustment of the excretion of 17-hydroxy-corticosteroid (17-OHCS) metabolites was reported to require two to three days if the sleep–waking rhythm is inverted [7]. Obviously the excretion of 17-OHCS metabolites (Fig. 9) parallels the behaviour of citric acid and urofuran acid: when the sleep–waking rhythm was inverted the main excretion occurred during the sleeping period or was at least increased at night in comparison with the normal rhythm (work during the day, sleep at night). The excretion behaviour of the androgen metabolites is less pronounced.

No changes in the excretion of amino acids and even no rhythm were observed during the experiment.

DISCUSSION

It is assumed that there exists a master "Zeitgeber" [8] which should synchronize the individual biological clock. Therefore, we should expect that biological functions controlled by this biological clock should increase or decrease in the same way at a given time.

The finding that the excretion of urofuran acids is subject to the same "Zeitgeber" as that directing the excretion of steroids seems remarkable. It ranks this group of compounds into the class of substances which are important for the functioning of the body. Propylurofuran acid but not pentylurofuran acid was recently shown to be accumulated in the plasma of uraemic patients [9]. It was found to be a compound with the highest known tendency for albuming binding [10]. Precursors of urofuran acids are the F-acids, first detected by Glass et al. in fish [11] but now also found to occur in mammals [12], including man [13]. Their biological function is still under investigation.

The excretion of tetrahydrofuran acid obviously follows another probably exogenic "Zeitgeber", as it accommodates easily to an inverted sleep–waking rhythm.

Abbott et al. [14] reported in 1970 that linolic acid is converted by *p*-toluenesulphonic acid in methanol mainly into 4 and 5. It must be assumed that a similar reaction could occur enzymatically and therefore compound 4 may be the precursor molecule for 1 (Fig. 10). This assumption is further corroborated by the detection of 4, 5 and related compounds in human blood [15] and fat [16]. At present, we have no information as to whether tetrahydrofuran acids are of biological importance or not.

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