Isolation and characterization of 3-[(carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid from human urine and preparation of its proposed precursor, *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteine

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3-[(Carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid (I) was isolated from healthy human urine by using ion-exchange column chromatography, and characterized by physicochemical analyses involving i.r., m.s. and n.m.r. spectrometries as well as chemical synthesis. The urinary content was 0.04–0.07 $\mu\text{mol/l}$. Compound (I) was synthesized by the addition of mercaptoacetic acid to urocanic acid. In order to establish the origin of the compound, *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteine (II) and *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione (III) were produced by similar reactions of urocanic acid with cysteine and GSH respectively. The yield of compound (II) was markedly increased by sunlight irradiation of the reaction mixture or by the use of *cis*-urocanic acid rather than the *trans* isomer. Incubation of compound (II) with rat liver homogenate in a phosphate buffer, pH 7.40, formed a major and some minor products of enzymic degradation, one of which was identified with compound (I). Exposure of rats to the sunlight for 2 days resulted in increase of the epidermal content of *trans*-urocanic acid from the normal value of 0.38 to 1.70 $\mu\text{g/mg}$ wet wt. of skin, accompanied by formation *de novo* of the epidermal *cis* isomer. After sunlight irradiation, the content of the *trans* isomer decreased at a constant rate of 0.03 $\mu\text{g/mg}$ wet wt. of skin per day, whereas the *cis* isomer was eliminated more quickly, having a phase of rapid decrease in the early period. From these results we suggest that compound (I) may participate in the metabolism of urocanic acid and natural thiol compounds such as cysteine and GSH.

INTRODUCTION

In the course of studies on the metabolism of histidine and sulphur-containing amino acids, an unidentified urinary compound was found on a high-voltage paper electrophoretogram sprayed with Pauly's reagent. This compound has now been determined to be 3-[(carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid (I). The present paper describes the isolation, determination of the structure and the proposed precursor of the urinary compound.

MATERIALS AND METHODS

Chemicals

Ion-exchanger Diaion SK no. 1 (H^+ form, a sulphonated cation-exchanger; 100–200 mesh) and Diaion SA no. 100 (acetate form, a strong anion-exchanger; 100–200 mesh) were purchased from Mitsubishi Kasei Co. (Tokyo, Japan), Dowex 50W X8 (H^+ form; 200–400 mesh) and Dowex 1 X8 (acetate form; 200–400 mesh) were from Dow Chemical Co. (Midland, MI, U.S.A.) and Amberlite CG-50 type 1 (H^+ form) was from Organo Co. (Tokyo, Japan). Imidazol-4-yl-lactic acid [2-hydroxy-3-(1*H*-imidazol-4-yl)propanoic acid], *trans*-urocanic acid [*trans*-3-(1*H*-imidazol-4-yl)acrylic acid] and mercaptoacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *cis*-Urocanic acid [*cis*-3-(1*H*-imidazol-4-yl)acrylic acid] was made by illuminating the *trans* compound, which gave partial isomerization (Masuda, 1956), and purified by ion-exchange column chromatography on Dowex 50 and Dowex 1: ^1H n.m.r. δ (p.p.m.) ($^2\text{H}_2\text{O}$) 6.49 (1H, d, J 16 Hz, CHCHCO), 7.31 (1H, d, J 16 Hz, CHCHCO), 7.46 (1H, s, N^2HCHC) and 8.55 (1H, s, N^2HCHN);

m.s. m/z 138 [M^+ (the molecular ion)]. Pauly's reagent (Pauly, 1904; Macpherson, 1946), a diazotized sulphanilic acid reagent for the detection of imidazole compounds, was made before use by mixing 3 ml of 0.2% (w/v) sulphanilic acid in 0.48 M-HCl, 3 ml of 0.2% NaNO_2 and 6 ml of 10% (w/v) Na_2CO_3 . Chloroplatinate reagent for the detection of sulphur-containing compounds was prepared by the standard method (Toennies & Kolb, 1951) as follows: 4 ml of 2 mM- Na_2PtO_6 , 0.25 ml of 1 M-KI, 0.4 ml of 2 M-HCl and 76 ml of acetone were mixed in that order. The ninhydrin reagent used was 2% (v/v) pyridine/1% (w/v) ninhydrin/acetone solution.

Spectroscopy

^1H -n.m.r. (100 MHz) spectra were recorded with a JEOL JNM-FX 100 Fourier-transform n.m.r. spectrometer in $^2\text{H}_2\text{O}$ with sodium 3-trimethylsilyl[2,2,3,3- $^2\text{H}_4$]propionate as an internal standard. M.s. was carried out on a Shimadzu LKB-9000 gas chromatograph-mass spectrometer at 70 eV with a direct-inlet system. I.r. spectra (KBr pellet) were recorded with a Japan Spectroscopic A-102 i.r. spectrophotometer.

Amino acid analysis

Automatic amino acid analyses were carried out on a Hitachi KLA-5 amino acid analyser fitted with a 0.9 cm $\times$ 55 cm column packed with Hitachi Custom ion-exchange resin 2613 with 0.2 M-sodium citrate buffer, pH 3.25, at 55 $^\circ\text{C}$ and a flow rate of 60 ml/h.

Paper electrophoresis

High-voltage paper electrophoreses were performed on Toyo-Roshi no. 51 paper in pyridine/acetic acid/water buffer,

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(1:20:179, by vol.), pH 3.1, at a potential gradient of 100 V/cm for 45 min (Ubuka, 1962). The paper was cooled by immersion in *n*-hexane at 0–5 °C. After electrophoresis, the paper was dried with an air-flow drier at 100 °C for 30 min. The dried paper was sprayed with Pauly's reagent, chloroplatinate reagent or ninhydrin reagent. Relative mobilities of compounds on the paper electrophoretogram, termed the mR_{ILA} value, were determined by comparison of the mobility for imidazol-4-yl-lactic acid as 1.00. In these electrophoretic conditions mR_{ILA} values of *cis*-urocanic acid, *trans*-urocanic acid and histidine were 0.90, 1.52 and 2.02 respectively.

T.l.c.

T.l.c. was carried out on silica gel 60 t.l.c. plates (Wako Chemicals Co., Osaka, Japan), developed in the solvent system indicated for each experiment: solvent I, butan-1-ol/acetic acid/water (12:3:5, by vol.); solvent II, butan-1-ol/pyridine/water (40:25:12, by vol.); solvent III, propan-2-ol/85% (v/v) formic acid/water (16:1:4, by vol.); solvent IV, pyridine/acetone/25% (v/v) conc. NH_3 /water (9:6:2:1, by vol.). The t.l.c. plate was dried with the drier at 55 °C for 30 min, and sprayed with Pauly's reagent, chloroplatinate reagent or ninhydrin reagent.

Enzymic degradation

Male Wistar rats weighing 250–300 g were used. Rat liver was homogenized with 3 vol. of 0.1 M-potassium phosphate buffer, pH 7.40, containing 5 mM-EDTA in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was used as the enzyme source without further purification. The enzymic degradation of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-cysteine {3-[(2-amino-2-carboxyethyl)thio]-3-(1*H*-imidazol-4-yl)-propanoic acid (II)} as a substrate was carried out by incubating 6 ml of a reaction mixture containing 5.0 ml of the homogenate, 0.5 ml of the substrate (25 μmol) and 0.5 ml of 0.1 M-potassium phosphate buffer, pH 7.40, containing 5 mM-EDTA at 37 °C for 2 h. The reaction was terminated by the addition of trichloroacetic acid at a final concentration of 10% (w/v). After termination, the reaction mixture was centrifuged at 1800 *g* for 30 min to obtain the supernatant as a sample for determination of the products. Reaction mixtures without the substrate or with 0.5 ml of trichloroacetic acid (a final concentration of 10%, w/v) instead of the phosphate buffer were used for comparative experiments of this enzymic degradation.

Determination of the content of *trans*-urocanic acid and *cis*-urocanic acid in rat skin exposed to sunlight

Experiments were carried out on male Wistar rats weighing 250–300 g in summer. Four rats were used for each experiment. After the fur on the dorsal skin of the rats had been clipped with a Thrive model 900 electric clipper fitted with a blade no. 40 (Daito Electric Mfg. Co., Osaka, Japan), they were exposed to sunlight for 2 days at an irradiation time of 10 h/day. On the day indicated, rats were killed by decapitation and then 500 mg each of the dorsal skin and of the abdominal skin was obtained from each rat with a fine scissors. Then the skin was cut into small pieces. This skin was always handled with forceps to avoid contamination from human sweat and skin. The skin samples were homogenized with 4 ml of conc. NH_3 (Levy *et al.*, 1969) in a Potter–Elvehjem homogenizer with a glass pestle. The entire homogenate was centrifuged at 1800 *g* for 20 min. The supernatant was desalted on a Dowex 50 column (1 cm $\times$ 15 cm) with 50 ml of 2 M- NH_3 as eluent. The aq. NH_3 eluate was evaporated to dryness under reduced pressure, and the residue was dissolved in 1.0 ml of water. The solution was used as a sample to evaluate the epidermal content of *trans*-urocanic acid and *cis*-urocanic

acid. The recovery of these isomers in the above procedure was $90 \pm 5\%$.

For evaluation, 10 μl of the sample was applied on to a t.l.c. plate together with 8.96 μg and 4.48 μg of the authentic *trans* and *cis* isomer respectively. The t.l.c. plate was chromatographed with solvent I, dried and sprayed with Pauly's reagent. Yellow–reddish spots of the *trans* and the *cis* isomers were measured with a Shimadzu CS-900 dual-wavelength t.l.c. scanner at 410 nm. A calibration curve between 0.30 and 13.0 μg was obtained by standard solutions of both isomers, and the minimum value for content of urocanic acid that could be determined was assessed as being 0.06 $\mu\text{g}/\text{mg}$ wet wt. of skin.

RESULTS

Isolation of compound (I) from human urine

Isolation of the urinary compound was carried out from a total of 600 litres of healthy human urine. Each 10 litres of urine was collected from several adults in bottles containing 10 ml of 6 M-HCl and 5 ml of toluene per litre of urine. The collected urine (20 litres) was filtered, and the filtrate was transferred to a Diaion SK no. 1 column (9 cm $\times$ 50 cm). The column was washed with 10 litres of water and then eluted with 15 litres of 2 M- NH_3 . The aq. NH_3 eluate was evaporated to dryness under reduced pressure. The dried residues obtained from 40 litres of the collected urine were combined in 1 litre of water and applied to a Diaion SA no. 100 column (9 cm $\times$ 50 cm). The column was treated with 10 litres each of water and of 0.02 M-acetic acid and then with 20 litres of 0.2 M-acetic acid. The 0.2 M-acetic acid eluate was dried, and the residue was dissolved in 200 ml of water. The solution was transferred to a Dowex 50 column (3 cm $\times$ 17 cm) followed by chromatography with 500 ml each of water, 0.2 M-acetic acid, water and 2 M- NH_3 , in that order. The aq. NH_3 eluate was dried to obtain the desired compound desalted.

After repetition of the above procedures five times, the residues were combined in 100 ml of water and applied to a Dowex 1 column (1.8 cm $\times$ 53 cm). The column was washed with 500 ml of 0.02 M-acetic acid, and this was followed by fractionation with 0.2 M-acetic acid with 25 ml portions being collected. After being concentrated to an appropriate volume, each fraction was checked by paper electrophoresis. The desired compound was found between 150 and 450 ml of the eluate. The eluate was desalted on a Dowex 50 column (1.5 cm $\times$ 30 cm). The desalted residue was dissolved in 3 ml of water and applied to a Dowex 1 column (1.5 cm $\times$ 36 cm). After the column had been washed with 250 ml of 0.02 M-acetic acid, fractionation with 0.2 M-acetic acid was performed with 10 ml portions being collected. As checked by paper electrophoresis, the desired compound was obtained with little contamination between 120 and 170 ml of the eluate.

Further purification of the compound was carried out by high-voltage paper electrophoresis. After electrophoresis, a part of the paper containing the desired compound, the mR_{ILA} value of which was 0.54, was cut out with scissors and cut into small pieces. After repetition of the paper electrophoresis about 30 times, the compound was extracted from the pieces with 2 M-acetic acid. The extracts were combined, concentrated to an appropriate volume and applied to a Dowex 50 column (1.0 cm $\times$ 13 cm). The column was washed with 2 M-acetic acid, and then the compound was eluted with 100 ml of 2 M- NH_3 . The aq. NH_3 eluate was evaporated to dryness and the residue was dissolved in 1 ml of water. The solution was finally fractionated on an Amberlite CG-50 column (0.5 cm $\times$ 15 cm) with water. Eluates between 3 and 6 ml were combined and desalted on a Dowex 50 column (0.5 cm $\times$ 6 cm).

Compound (I): R = -S-CH₂-CO₂H

Compound (II): R = -S-CH₂-CH(NH₂)-CO₂H

Compound (III): R = HO₂C-CH(NH₂)-[CH₂]₂-CO-NH-S-CH₂-CO-NH-CH₂-CO₂H

Fig. 1. Structural formulae of the synthesized imidazole compounds 3-[(carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid (I), *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteine (II) and *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione (III)

By repeating the above procedures, approx. 6 mg of crystals of the compound was obtained from 600 litres of human urine, and this was followed by recrystallization from ethanol/water. The purity of crystals was determined by high-voltage paper electrophoresis and t.l.c.

Characterization of the urinary compound

The isolated compound gave positive reactions with Pauly's reagent and the chloroplatinate reagent but the ninhydrin test was negative, suggesting that the compound is an imidazole derivative having a sulphur-containing side chain. On t.l.c. the compound had R_f 0.03 in solvent I, R_f 0.34 in solvent II and R_f 0.05 in solvent IV. The crystals melted at 201–203 °C. Analytical data found: C, 41.8; H, 4.35; N, 12.08 %. These data indicated a molecular formula of C₈H₁₀N₂O₄S (M_r 230). The mass spectrum of the isolated compound showed a peak at m/z 212 at a relative intensity of 32 %, corresponding $M^+ - H_2O$ of the above formula. The i.r. spectrum of the isolated compound was identical with that of compound (I) synthesized as described below.

Urinary content of compound (I)

The concentration of compound (I) in human urine was too low for determination of the urinary content without a preceding concentration step. Therefore the collected urinary samples from four adults were treated on the successive ion-exchange columns SK no. 1, SA no. 100, Dowex 1, Dowex 50 and Amberlite CG-50 as described above in the isolation procedure. The recovery of this compound in the concentration step was nearly 80 %. The concentrated samples were subjected to t.l.c. with the solvent system II. After development, the t.l.c. plate was sprayed with Pauly's reagent. By scanning the reddish spot (R_f 0.34) on the t.l.c. plate, the urinary content was determined to be 0.04–0.07 $\mu\text{mol/l}$ (average 0.052 $\mu\text{mol/l}$).

Chemical synthesis of compound (I)

Compound (I) was made by the addition of mercaptoacetic acid (92 g, 1 mmol) to *trans*-urocanic acid (166 mg, 1.2 mmol); these reactants, in a glass tube, were dissolved in 20 ml of deoxygenated water, and 320 mg (3 mmol) of Na₂CO₃ was added in small portions under N₂. After the glass tube had been sealed, the reaction mixture was stirred for 1 week in a dark room at room temperature. After reaction, the reaction mixture was adjusted to pH 4.0, and filtered. The filtrate was applied to the successive ion-exchange columns Dowex 50 and Dowex 1. The product was purified finally on a Dowex 1 column (1.5 cm $\times$ 40 cm) by fractionation with 0.2 M-acetic acid with 10 ml portions being collected. Eluates between 130 ml and 190 ml were combined and evaporated to obtain 181 mg (yield

Fig. 2. Mass spectrum of compound (I)

79 %) of compound (I) followed by recrystallization twice from ethanol/water. The chemical structure is shown in Fig. 1. This compound gave positive reactions with both Pauly's reagent and the chloroplatinate reagent, but the ninhydrin test was negative.

The compound had m.p. 201–203 °C, unchanged by admixture with the isolated urinary compound. Analytical data calc. for C₈H₁₀N₂O₄S: C, 41.73; H, 4.38; N, 12.17 %. Found: C, 41.82; H, 4.26; N, 12.02 %. Signals on the n.m.r. spectrum were assigned as follows: δ (p.p.m.) (²H₂O) 2.95 (2H, d, J 8 Hz, CHCH₂CO), 3.30 (2H, s, SCH₂CO), 4.51 (1H, t, J 8 Hz, CHCH₂CO), 7.43 (1H, s, N²HCHC) and 8.67 (1H, s, N¹HCHN). In the mass spectrum (Fig. 2) peaks were assigned as follows: m/z 212 ($M^+ - H_2O$), 138 (C₃H₃N₂-CH-CH₂-CO₂⁺), 94 (base, C₃H₃N₂-CH=CH₂⁺) and 74 (S-CH₂-C=O⁺). This compound behaved identically with the isolated urinary compound on the high-voltage paper electrophoresis and t.l.c., and had an identical i.r. spectrum. From these data the urinary compound was determined to be 3-[(carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid (I).

In order to make clear the origin of the compound, *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteine (II) and *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione (*N*-{*S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-*N*-L- γ -glutamyl-L-cysteinyl}glycine (III)) were synthesized, and the enzymic degradation of the former compound was carried out as described below.

Chemical synthesis of compound (II)

By modifying the synthetic method for *S*-(2-carboxyisopropyl)cysteine (Kodama, 1968), compound (II) was made by the addition of L-cysteine (363 mg, 3 mmol) to *trans*-urocanic acid (496 mg, 3.6 mmol) in similar reaction conditions to those

used for compound (I). After reaction, the reaction mixture was adjusted to pH 4.0 with acetic acid, and filtered to remove unchanged *trans*-urocanic acid. The filtrate was desalted on a Dowex 50 column (2.6 cm $\times$ 18 cm), and chromatographed on a Dowex 1 column (2.6 cm $\times$ 18 cm) with 25 ml portions of the 0.02 M-acetic acid eluate being collected. As checked by paper electrophoresis, the product was found between 150 and 350 ml of the eluate. Further purification of the crude product was carried out on an Amberlite CG-50 column (1.3 cm $\times$ 15 cm) with elution with water. Crystals were obtained from the 185–380 ml fraction of the eluate. The crystals were recrystallized twice from ethanol/water to give 152 mg (yield 19.2%) of compound (II). It decomposed at 155 °C. The compound gave positive reactions with Pauly's reagent, the chloroplatinate reaction and the ninhydrin reagent.

All the experimental procedures described above were carried out in the dark-room, since *trans*-urocanic acid was isomerized to the *cis* form by u.v. irradiation. The presence of the *cis* isomer in the reaction mixture complicated the above isolation step employed for compound (II) because of the similar behaviour the two compounds on ion-exchange chromatography. Moreover, various minor products were formed in the reaction mixture it was exposed to u.v. irradiation.

However, the yield of compound (II) was increased by exposing the reaction mixture to sunlight. When a reaction mixture containing *trans*-urocanic acid (0.12 mmol), L-cysteine (0.1 mmol), Na₂CO₃ (0.3 mmol) and deoxygenized water (2 ml) in a sealed vessel was stirred while exposed to sunlight irradiation for 5 days (40 h of the effective irradiation time), 0.089 mmol of compound (II) (yield 89%) was produced, compared with the lower yield of 21% for the reference experiment that was carried out at the same time in a sealed vessel covered with aluminium foil. *cis*-Urocanic acid was detected in the u.v.-irradiated reaction mixture, but was not found in the reference reaction mixture.

When *cis*-urocanic acid was used instead of the *trans* isomer in the dark-room in a similar reaction to that described above, compound (II) was generated in 2.5 times the yield obtained for the reaction with *trans*-urocanic acid, suggesting a higher reactivity of the *cis* isomer for cysteine.

Analytical data calc. for C₈H₁₃N₃O₅S: C, 41.69; H, 5.05; N, 16.21%. Found: C, 41.93; H, 5.07; N, 16.32%. Signals on the n.m.r. spectrum were assigned as follows; δ (p.p.m.) (²H₂O) 2.84 (2H, d, *J* 9 Hz, CHCH₂CO), 2.98 (2H, d, *J* 8 Hz, SCH₂CH), 3.89 (1H, t, *J* 8 Hz, SCH₂CH), 4.48 (1H, t, *J* 9 Hz, CHCH₂CO), 7.46 (1H, s, N²HCHC) and 8.63 (1H, s, N²HCHN). The compound had *mR*_{ILA} 1.15 and *R*_F values of 0.20, 0.12 and 0.02 in solvents I, III and IV respectively. In the automatic amino acid analysis, two peaks at 195 and 203 min with similar shapes were recorded. It has not been established why two peaks were observed for this compound. Similar peaks at 195 and 203 min were observed for the product obtained by hydrolysis of compound (III) by 6 M-HCl at 110 °C for 12 h as described below.

Chemical synthesis of compound (III)

Compound (III) was synthesized by the addition of GSH (308 mg, 1 mmol) to *trans*-urocanic acid (165.2 mg, 1.2 mmol) in reaction conditions similar to those used for compound (I). The reaction mixture was chromatographed on the successive ion-exchanger columns Dowex 50, Dowex 1 and Amberlite CG-50 and finally crystals of compound (III) were obtained (78 mg, yield 17.5%). Its *mR*_{ILA} value was 0.66; the retention time on the amino acid analyser was 141 min. Compound (III) gave positive reactions with Pauly's reagent and the ninhydrin reagent.

Acid hydrolysis of the crystals (1.12 μ mol) by 6 M-HCl supported the chemical structure of compound (III): glutamic acid (0.97 μ mol) at a retention time of 79 min, glycine (1.06 μ mol)

at 108 min and compound (II) (0.98 μ mol) at 195 and 203 min were determined with the amino acid analyser.

Enzymic degradation of compound (II)

Enzymic degradation of compound (II) was performed by incubating it with rat liver homogenate in 0.1 M-potassium phosphate buffer, pH 7.4, containing 5 mM-EDTA as described in the Materials and methods section. After reaction, the reaction mixture was centrifuged at 1800 *g* for 30 min to remove denatured proteins. The supernatant was desalted on a Dowex 50 column (1 cm $\times$ 13 cm) followed by chromatography on a Dowex 1 column (1 cm $\times$ 12 cm). The column was treated with 50 ml each of water, 0.02 M-acetic acid and 2 M-acetic acid successively. The 2 M-acetic acid eluate was concentrated to a small volume and subjected to high-voltage paper electrophoresis. On the paper electrophoretogram sprayed with Pauly's reagent, a major product at *mR*_{ILA} 0.38 and a few minor products at *mR*_{ILA} 0.54, 1.60 and 2.10 were observed. These compounds gave a positive reaction with the chloroplatinate reagent but the ninhydrin test was negative.

The major product at *mR*_{ILA} 0.38 was unstable in acid. When a solution of the product in 6 M-HCl was heated at 80 °C for 30 min, it was changed in part into the compound having *mR*_{ILA} 0.54. This compound was identical with compound (I) on paper electrophoresis and t.l.c.

From these results, we assumed that the isolated urinary compound may participate in metabolism of urocanic acid. Therefore we investigated the change in the content of epidermal urocanic acid after sunlight irradiation of rats as described below.

Rise and fall of *trans*-urocanic acid and *cis*-urocanic acid contents in rat skin exposed to sunlight

Contents of *trans*-urocanic acid and *cis*-urocanic acid in rat epidermis were measured with the t.l.c. scanner as described in the Materials and methods section. On the t.l.c. chromatogram of the aq. NH₃ extract of skin samples, spots of *trans*-urocanic acid (*R*_F 0.54) and *cis*-urocanic acid (*R*_F 0.45) separated clearly from some other spots with *R*_F values of 0.31, 0.17 and < 0.05. The skin of untreated normal rats had only the *trans* isomer at an average concentration of 0.38 μ g/mg wet wt. of skin. After exposure of rat to sunlight for 2 days, the content of the *trans* isomer in the dorsal skin was increased to 1.70 μ g/mg wet wt. of skin. At that time the *cis* isomer was newly formed at a concentration of 0.87 μ g/mg wet wt. of skin (34% in total of urocanic acid). In the abdominal skin the *trans* isomer was little increased (average 0.42 μ g/mg wet wt. of skin) and the *cis* isomer was scarcely formed at all, suggesting that the increasing formation of urocanic acid and the *trans*-*cis* isomerization occurred in the part affected by u.v. light. Fig. 3 shows the curves for the change in the contents of both the *trans* isomer and the *cis* isomer in the dorsal skin after sunlight irradiation. The content of the epidermal *trans* isomer decreased gradually from 1.70 to 1.29 μ g/mg wet wt. of skin at a rate of approx. 0.03 μ g/mg wet wt. of skin per day. On the other hand, two different phases were observed in the curve for the decrease in the epidermal *cis* isomer: a remarkable phase of rapid decrease in the early segment and a following slow phase having a similar rate as that for the *trans* isomer. On 12th day after sunlight irradiation the *cis* isomer showed only a trace on the t.l.c. plate, hardly measurable with the t.l.c. scanner (below 0.06 μ g/mg wet wt. of skin).

These results indicate a difference between epidermal *trans*-urocanic acid and *cis*-urocanic acid in their behaviour with respect to accumulation and removal under conditions of sunlight irradiation.

Fig. 3. Curves of the change in the epidermal content of *trans*-urocanic acid and *cis*-urocanic acid after exposure of rats to sunlight

After exposure of rats to sunlight for 2 days, the dorsal skin was extracted with conc. NH_3 and then applied to t.l.c. on a silica-gel plate with solvent I followed by spraying with Pauly's reagent. The contents of *trans*-urocanic acid (●) and *cis*-urocanic acid (○) were determined by use of the t.l.c. scanner at 410 nm. N indicates the contents for untreated rats. Data were not corrected for the recovery of $90 \pm 5\%$.

DISCUSSION

Judging from the chemical structure of compound (I), we postulate that cysteine and urocanic acid are its precursors.

In the transaminative pathway of L-cysteine metabolism, it is well known that mercaptopyruvate is formed from cysteine by the action of cysteine aminotransferase, which was purified from rat liver and identified with aspartate aminotransferase (EC 2.6.1.3) (Ubuka *et al.*, 1978). Mercaptopyruvate is also generated by oxidative deamination of cysteine. This compound is unstable in acid, decomposing in part to mercaptoacetic acid. If the reaction of urocanic acid with natural thiol compounds such as cysteine or GSH occurs *in vivo*, and if the products are metabolized like cysteine, compound (I) may be formed.

In the present work it has been shown that urocanic acid reacts with cysteine and GSH *in vitro* to produce compounds (II) and (III) respectively. In addition, one of the products formed by enzymic degradation of compound (II) by rat liver homogenate has been shown to be identical with compound (I).

In the first step of the catabolism of L-histidine, only *trans*-urocanic acid is formed by histidase (histidine ammonia-lyase, EC 4.3.1.3), which is found mostly in only liver and epidermis (Zannoni & La Du, 1963). In liver, urocanic acid is further metabolized to culminate in complete degradation to CO_2 and water. In epidermis, however, the metabolism of histidine terminates with the formation of urocanic acid, since urocanase (urocanate hydratase, EC 4.2.1.49), the enzyme degrading urocanic acid, is absent from the skin (Baden & Pathak, 1967). Thus urocanic acid accumulates in epidermis, although its content

changes in response to various factors, e.g. the ethnic origin of the subject (Kral *et al.*, 1968) and the extent of exposure to u.v. light (Hais & Štrych, 1968; Wadia *et al.*, 1975). Exposure of skin to u.v. light results in a formation of *cis*-urocanic acid from the *trans* isomer (Anglin *et al.*, 1961). The functions of the accumulation of urocanic acid and of the *trans*-*cis* isomerization in epidermis have not been established, although the widespread belief is that urocanic acid absorbs u.v. to protect skin from various kinds of damage by sunlight.

Elimination of urocanic acid from the skin under conditions of u.v. irradiation has not been clear. In the present experiments differences in the accumulation and in the removal of the epidermal *trans*-urocanic acid and *cis*-urocanic acid were demonstrated by exposure of rats to sunlight. The rapidly decreasing phase observed in the curve of the change in the epidermal content of the *cis* isomer may be caused by the good solubility and/or by the higher reactivity than the *trans* isomer. In preliminary experiments, the solubility of the *cis* isomer in water at 37°C was 487 mg/100 ml of water, compared with the low solubility of the *trans* isomer (14.3 mg/100 ml of water). Moreover, it has been shown that, in the chemical synthesis of compound (II), the yield was markedly increased by exposure of the reaction mixture to sunlight, or by the use of the *cis* isomer rather than the *trans* one. From these results, it is possible that some portions of epidermal *cis*- and/or *trans*-urocanic acid penetrate into the body fluid, and conjugate with thiol compounds such as cysteine and GSH.

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