Metabolism of β -Ionone

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF THE METABOLITES IN THE URINE OF RABBITS

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1. Rabbits dosed orally with β -ionone excreted in the urine unchanged β -ionone, 3-oxo- β -ionone, 3-oxo- β -ionol, dihydro-3-oxo- β -ionol and 3-hydroxy- β -ionol. 2. Excretion products were isolated as 2,4-dinitrophenylhydrazone derivatives (β -ionone, 3-oxo- β -ionone, 3-oxo- β -ionol and dihydro-3-oxo- β -ionol) and as *p*-nitrobenzoate derivatives (3-oxo- β -ionol, dihydro-3-oxo- β -ionol and 3-hydroxy- β -ionol), which were characterized and identified by comparison with the synthetic authentic compounds. 3. The glucuronides of 3-oxo- β -ionol and dihydro-3-oxo- β -ionol were also detected in the urine. The latter compound was isolated as free glucuronide, sodium salt and 2,4-dinitrophenylhydrazone.

β -Ionone is used in perfumery and as a starting material of vitamin A synthesis. In a study of the metabolic fate of β -ionone, Bielig & Hayashida (1940) administered β -ionone orally to rabbits, and isolated β -ionol and dihydro- β -ionol as the reduction products of β -ionone. Further, they described that three hydroxylated metabolites were excreted in the urine. Prelog & Meier (1950) repeated this experiment. They isolated 3-oxo- β -ionone, 3-hydroxy- β -ionol and another metabolite, which was presumed either 3-oxo- β -ionol or 3-hydroxy- β -ionone, from the urine of rabbits receiving β -ionone. However, no reduction of the double bonds of β -ionone was observed in this case.

In order to elucidate the metabolism of β -ionone further, we followed the same experiment. The present work confirmed the excretion of unchanged β -ionone, 3-oxo- β -ionone, 3-oxo- β -ionol, dihydro-3-oxo- β -ionol, 3-hydroxy- β -ionol and glucuronides of 3-oxo- β -ionol and dihydro-3-oxo- β -ionol in the urine of rabbits receiving β -ionol.

EXPERIMENTAL

All melting points are uncorrected. All boiling points are expressed by bath temperature, because the quantities of the samples were very small and micro rectifiers were used for the distillation.

Materials. β -Ionone [2,6,6-trimethyl-1-(3-oxo-1-but-1-enyl)cyclohex-1-ene] was obtained from E. Merck A.-G., Darmstadt, Germany. Its 2,4-dinitrophenylhydrazone, crystallized from 80% methanol, had m.p. 129°C (Braude & Jones, 1945). Acetyl- β -ionol [1-(3-acetyl-1-but-1-enyl)-2,6,6-trimethylcyclohex-1-ene] (b.p. 132–136°C/14 mm-Hg) was prepared by a modification of the method of Kandel (1937): β -ionone was reduced by NaBH₄ to β -ionol, then treated with acetic anhydride in pyridine.

Acetyldihydro- β -ionol [1-(3-acetyl-1-butyl)-2,6,6-trimethyl cyclohex-1-ene] (b.p. 139–141°C/12 mmHg) was prepared by the methods of Naves & Bachmann (1943) and Naves & Ardizo (1949). 3-Hydroxy- β -ionone [3-hydroxy-2,6,6-trimethyl-1-(3-oxo-1-but-1-enyl)cyclohex-1-ene] (b.p. 132°C/0.02 mmHg) was prepared by the method of Henbest (1951); its u.v. spectrum had $\lambda_{\max}$ 287 nm (ϵ 8000). Its phenylsemicarbazone, crystallized from methanol, had m.p. 155°C (Found: C, 70.5; H, 7.8; N, 12.2. Calc. for C₂₀H₂₇N₃O₂: C, 70.5; H, 8.0; N, 12.3%). Its *p*-nitrobenzoate, crystallized from 80% methanol, had m.p. 79°C; its u.v. spectrum had $\lambda_{\max}$ 265 nm (ϵ 28 800) (Found: C, 67.5; H, 6.5; N, 3.9; C₂₀H₂₃NO₅ requires C, 67.2; H, 6.5; N, 3.9%). Manganese dioxide (B type) was prepared by the method of Henbest, Jones & Owen (1957). β -Glucuronidase (*Helix pomatia*; 100 000 units/ml) was obtained from Endo Laboratories Inc., New York, N.Y., U.S.A.

Spectroscopy. The u.v. spectra of the compounds in 95% (v/v) ethanol were measured with a Beckman model DB spectrophotometer. The i.r. spectra in NaCl or KBr were recorded with a Hitachi model EPI-G spectrophotometer. The n.m.r. spectra in CDCl₃ solution, with tetramethylsilane as a reference standard, were run at 24°C on a JEOL model JNM-C-60H high-resolution spectrometer at 60 MHz.

T.l.c. For the detection of the free metabolites and their derivatives, t.l.c. was carried out on silica gel HF₂₅₄ (E. Merck A.-G.) with *n*-hexane-ethyl acetate (1:1, v/v) and (7:3, v/v). The compounds were detected on the plate as dark spots under a u.v. lamp (253.6 nm), as the brown spots after spraying them with 1% KMnO₄ solution, or as the dark-brown spots by treatment with conc. H₂SO₄-ethanol (1:1, v/v).

Chromatograms of glucuronides were developed with chloroform-methanol-water (13:7:2, by vol.) and detected as purple spots against a reddish-pink background when the plate was sprayed with a solution composed of 0.2% (w/v) naphtharesorcinol in ethanol–85% phosphoric acid (5:1, v/v) and heated at 120–130°C for

Scheme 1. Syntheses of the β -ionone metabolites.

10 min. Chromatograms of the 2,4-dinitrophenylhydrazone derivative of dihydro-3-oxo- β -ionol glucuronide were developed with *n*-hexane-ethyl acetate (1:1, v/v).

Oral administration. β -Ionone in aqueous suspension with gum arabic was administered by stomach tube to a male albino rabbit (approx. 3.0 kg body wt.) at the dose of 1 g/kg per day for 7 days (total dose 23 g). The urine was collected daily during the administration period and for 4 days after the last administration.

Extraction and fractionation of the free metabolites. The urine was adjusted to pH 6.0 with conc. HCl and extracted continuously with ether for 20 h. The ether extract was

washed with the same volume of saturated NaHCO_3 solution and saturated NaCl solution, and dried over anhydrous Na_2SO_4 . After evaporation of the ether under reduced pressure, the gummy residue (approx. 2.1 g) was suspended in *n*-hexane and chromatographed on a column containing 105 g of silicic acid (100 mesh; Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.). Elution was started with *n*-hexane, and *n*-hexane-ethyl acetate (9:1, v/v), (4:1, v/v), (7:3, v/v), (3:2, v/v) and (1:1, v/v) were used as subsequent eluents.

Syntheses of the free metabolites. The route for the syntheses of the β -ionone metabolites is shown in Scheme 1.

(a) 3-Oxo- β -ionol [1-(3-hydroxy-1-but-1-enyl)-2,6,6-trimethyl-3-oxocyclohex-1-ene]. A pentane solution (10ml) of acetyl- β -ionol (1g) was percolated through MnO_2 (20g). First about 60% of the starting material was removed by washing the mixture with an additional amount of pentane (250ml). The mixture was then extracted with ethyl acetate, the solvent was removed under reduced pressure and acetyl-3-oxo- β -ionol [1-(3-acetyl-1-but-1-enyl)-2,6,6-trimethyl-3-oxocyclohex-1-ene] (210mg) was obtained. The acetyl-3-oxo- β -ionol had b.p. 155°C/8mmHg; its i.r. spectrum had ν_{max} 1675, 1600, 1460, 1360, 1240, 1170 and 970 cm^{-1} in NaCl. Its 2,4-dinitrophenylhydrazone, crystallized from 90% ethanol, had m.p. 103–104°C (decomp.) (Found: C, 58.7; H, 6.0; N, 13.0; $\text{C}_{21}\text{H}_{26}\text{N}_4\text{O}_6$ requires C, 58.6; H, 6.1; N, 13.0%). The acetyl-3-oxo- β -ionol (150mg) was refluxed with KHCO_3 (200mg) in 50% (v/v) ethanol (50ml) for 30 min. After evaporation of the solvent under reduced pressure, the residue was extracted with ether and the ether removed; 3-oxo- β -ionol (103mg) was then obtained. The 3-oxo- β -ionol had b.p. 132–134°C/0.08mmHg; its i.r. spectrum had ν_{max} 3420, 2960–2870, 1670, 1595, 1450, 1355, 1340, 1200, 1180 and 970 cm^{-1} in NaCl; its u.v. spectrum had λ_{max} 267nm (ϵ 11400); its n.m.r. spectrum had τ (numbers of protons in parentheses) 3.71 (1) doublet, 4.29 (1) quartet, 5.35–5.75 (1) multiplet, 7.38–8.25 (5) multiplet, 8.19 (3), 8.64 (3) doublet and 8.83 (6) (Found: C, 74.8; H, 9.7; $\text{C}_{13}\text{H}_{20}\text{O}_2$ requires C, 75.0; H, 9.7%). Its 2,4-dinitrophenylhydrazone, crystallized from 80% ethanol, had m.p. 167°C (decomp.); its i.r. spectrum had ν_{max} 3420, 2970–2850, 1660, 1160 and 970 cm^{-1} in KBr; its u.v. spectrum had λ_{max} 391nm (ϵ 34200) (Found: C, 58.3; H, 6.3; N, 14.4; $\text{C}_{15}\text{H}_{24}\text{N}_4\text{O}_5$ requires C, 58.8; H, 6.2; N, 14.4%). Its *p*-nitrobenzoate, crystallized from 90% methanol, had m.p. 67°C; its i.r. spectrum had ν_{max} 2980–2860, 1670, 1120 and 980 cm^{-1} in KBr; its u.v. spectrum had λ_{max} 262nm (ϵ 17600) (Found: C, 66.9; H, 6.8; N, 3.7; $\text{C}_{20}\text{H}_{23}\text{NO}_3$ requires C, 67.2; H, 6.5; N, 3.9%).

(b) Dihydro-3-oxo- β -ionol [1-(3-hydroxy-1-butyl)-2,6,6-trimethyl-3-oxocyclohex-1-ene]. A mixture of acetyldihydro- β -ionol (600mg) and MnO_2 (20g) in pentane (100ml) was stirred for 20h. After the pentane had been filtered off, the MnO_2 was shaken with ethyl acetate (500ml). The ethyl acetate extract was concentrated and chromatographed on silicic acid (30g). Elution with *n*-hexane-ethyl acetate (9:1, v/v) gave acetyldihydro-3-oxo- β -ionol [1-(3-acetyl-1-butyl)-2,6,6-trimethyl-3-oxocyclohex-1-ene] (140mg). The acetyldihydro-3-oxo- β -ionol had b.p. 105–108°C/0.04mmHg; its i.r. spectrum had ν_{max} 1680, 1600, 1460, 1360, 1240 and 1170 cm^{-1} in NaCl. Its 2,4-dinitrophenylhydrazone, crystallized from 90% ethanol, had m.p. 116°C (decomp.) (Found: C, 58.7; H, 6.4; N, 13.0; $\text{C}_{21}\text{H}_{28}\text{N}_4\text{O}_6$ requires C, 58.3; H, 6.5; N, 13.0%). The acetyldihydro-3-oxo- β -ionol (120mg) was refluxed with KHCO_3 (200mg) in 50% (v/v) ethanol (10ml) for 2h. After evaporation of the ethanol under reduced pressure, dihydro-3-oxo- β -ionol (76mg) was obtained by ether extraction. The dihydro-3-oxo- β -ionol had b.p. 135–137°C/0.04mmHg; its i.r. spectrum had ν_{max} 3420, 2970–2870, 1660, 1605, 1450, 1355, 1340, 1205 and 1180 cm^{-1} in NaCl; its u.v. spectrum had λ_{max} 249nm (ϵ 13000); its n.m.r. spectrum had τ (numbers of protons in parentheses) 6.07–6.39 (1) multi-

plet, 7.42–8.49 (9) multiplet, 8.23 (3), 8.76 (3) doublet and 8.82 (6) (Found: C, 74.0; H, 10.8; $\text{C}_{13}\text{H}_{22}\text{O}_2$ requires C, 74.2; H, 10.5%). Its 2,4-dinitrophenylhydrazone, crystallized from 80% ethanol, had m.p. 163°C (decomp.); its i.r. spectrum had ν_{max} 3400, 2960–2860, 1650 and 1160 cm^{-1} in KBr; its u.v. spectrum had λ_{max} 387nm (ϵ 20200) (Found: C, 58.7; H, 6.8; N, 14.3; $\text{C}_{19}\text{H}_{26}\text{N}_4\text{O}_5$ requires C, 58.5; H, 6.7; N, 14.4%). Its *p*-nitrobenzoate, crystallized from 90% methanol, had m.p. 126°C; its i.r. spectrum had ν_{max} 2980–2850, 1640 and 1120 cm^{-1} in KBr; its u.v. spectrum had λ_{max} 252nm (ϵ 11600) (Found: C, 66.7; H, 6.7; N, 3.8; $\text{C}_{20}\text{H}_{25}\text{NO}_3$ requires C, 66.8; H, 7.0; N, 3.9%).

(c) 3-Oxo- β -ionone [2,6,6-trimethyl-3-oxo-1-(3-oxo-1-but-1-enyl)cyclohex-1-ene]. This compound was prepared by the method of Henbest (1951). To a solution of 3-hydroxy- β -ionone (500mg) in acetone (10ml), chromic acid (200mg) in 20% (v/v) H_2SO_4 solution (1ml) was added with stirring at 0°C. Then 5 min later water (25ml) was added and 3-oxo- β -ionone was extracted with ether (100ml). 3-Oxo- β -ionone, crystallized from 50% methanol, had m.p. 54°C (yield 370mg); its i.r. spectrum had ν_{max} 2990–2860, 1660, 1600, 1455, 1365, 1350, 1205, 1175 and 975 cm^{-1} in KBr; its u.v. spectrum had λ_{max} 277 and 222nm (ϵ 10200 and 8900 respectively) (Found: C, 75.7; H, 8.9. Calc. for $\text{C}_{13}\text{H}_{18}\text{O}_2$: C, 75.7; H, 8.8%). Its bis-2,4-dinitrophenylhydrazone, crystallized from ethanol, had m.p. 264°C (decomp.); its i.r. spectrum had ν_{max} 2970–2860, 1670, 1180 and 970 cm^{-1} in KBr; its u.v. spectrum had λ_{max} 389nm (ϵ 17000) (Found: C, 53.0; H, 4.7; N, 19.6. Calc. for $\text{C}_{25}\text{H}_{26}\text{N}_8\text{O}_8$: C, 53.0; H, 4.6; N, 19.8%).

(d) 3-Hydroxy- β -ionol [3-hydroxy-1-(3-hydroxy-1-but-1-enyl)-2,6,6-trimethylcyclohex-1-ene]. To a suspension of 3-oxo- β -ionone (250mg) in 50% (v/v) ethanol, fine powdered NaBH_4 (15mg) was added with stirring, and the stirring was continued for 1h at room temperature. A few drops of dil. acetic acid was added to the reaction mixture, which was evaporated to dryness under reduced pressure. The residue was suspended in ether (50ml), washed with saturated NaCl solution and dried over anhydrous Na_2SO_4 . After evaporation of the ether, 3-hydroxy- β -ionol (243mg) was obtained. The 3-hydroxy- β -ionol had b.p. 125–127°C/0.04mmHg; its i.r. spectrum had ν_{max} 3420, 2965–2860, 1640, 1450, 1020 and 965 cm^{-1} in NaCl; its u.v. spectrum had λ_{max} 227nm (ϵ 6200); its n.m.r. spectrum had τ (numbers of protons in parentheses) 3.69 (1) doublet, 4.27 (1) quartet, 5.33–5.73 (1) multiplet, 5.91–6.10 (1) multiplet, 8.02–8.58 (6) multiplet, 8.20 (3), 8.70 (3) doublet, 9.00 (3) and 10.25 (3) (Found: C, 74.0; H, 10.8; $\text{C}_{13}\text{H}_{22}\text{O}_2$ requires C, 74.2; H, 10.5%). Its bis-*p*-nitrobenzoate, crystallized from ethanol, had m.p. 182–183°C; its i.r. spectrum had ν_{max} 2980–2860, 1120 and 960 cm^{-1} in KBr; its u.v. spectrum had λ_{max} 260nm (ϵ 19100) (Found: C, 63.7; H, 5.6; N, 5.5; $\text{C}_{27}\text{H}_{28}\text{N}_2\text{O}_8$ requires C, 63.8; H, 5.6; N, 5.5%).

Fractionation of the glucuronides. The method of Kamil, Smith & Williams (1951) was applied to isolate the glucuronides of the β -ionone metabolites. All the processes were carried out at below 10°C to prevent decomposition of unstable glucuronides. After the free metabolites had been thoroughly extracted with ether, the urine was adjusted to pH4 with acetic acid, and

saturated lead acetate solution was added until precipitation was complete. The precipitate was removed by centrifugation and discarded. The supernatant was adjusted to pH 8 with conc. NH_3 , and saturated basic lead acetate solution was added in excess. The resulting precipitate was collected by centrifugation, washed twice with methanol and then suspended in methanol. The methanol suspension was saturated with H_2S . After removal of PbS by filtration, the filtrate was evaporated to dryness under reduced pressure. The gummy residue (approx. 5.4g) was dissolved in water and chromatographed on a column containing 200g of charcoal-Celite (1:1, w/w). The column was washed with water and methanol, and eluted with 0.28% (w/v) NH_3 in methanol followed by stepwise increases in the concentration of NH_3 .

Hydrolysis of the glucuronides by β -glucuronidase. A portion of each glucuronide gum (1mg) obtained from charcoal column chromatography was dissolved in 3ml of 0.2M-sodium acetate buffer, pH 4, and incubated with β -glucuronidase (5000 units) at 37°C for 20 h.

RESULTS

Detection of the free metabolites. T.l.c. revealed the presence of the five products in the urinary extract. These products were excreted for 96h after the administration of β -ionone. The R_F values of each spot were 0.92 (metabolite 1), 0.74 (metabolite 2), 0.45 (metabolite 3), 0.40 (metabolite 4) and 0.32 (metabolite 5) with *n*-hexane-ethyl acetate (1:1, v/v) as the solvent system. All the products were detected as the dark spots under a u.v. lamp and showed a positive reaction with potassium permanganate solution or concentrated sulphuric acid-ethanol (1:1, v/v). Although metabolites 3 and 4 were located very close to each other, multiple development of the chromatogram with the same solvent helped to separate the two spots.

A part of the urinary extracts was treated with 2,4-dinitrophenylhydrazine, or *p*-nitrobenzoyl chloride, and the derivatives of the metabolites were chromatographed on a thin-layer plate with *n*-hexane-ethyl acetate (7:3, v/v). Four spots were detected as the 2,4-dinitrophenylhydrazones (R_F 0.90, 0.57, 0.52 and 0.09) and three spots were

detected as the *p*-nitrobenzoates (R_F 0.91, 0.60 and 0.55). These results suggested that two of the five products possess both carbonyl and hydroxyl groups in their structures.

Fractionation and isolation of the free metabolites.

The urine extract (2.1g) obtained from a rabbit given 23g of β -ionone was fractionated by silicic acid column chromatography. As shown in Table 1, stepwise increases in the concentration of ethyl acetate in *n*-hexane separated the extract into six fractions. In this experiment the eluates were examined by t.l.c., and the tubes that contained the same metabolite were combined. Fraction 1, the eluate of 800ml of *n*-hexane-ethyl acetate (9:1, v/v), contained metabolite 1. This fraction was purified further by alumina column chromatography with benzene, and 18mg of metabolite 1 was obtained as a colourless oil. Fraction 2, the eluate of 300ml of *n*-hexane-ethyl acetate (4:1, v/v), contained metabolite 2. The residue of this fraction was recrystallized from 50% methanol and 12mg of metabolite 2 (m.p. 53°C) was isolated. Fraction 3, the eluate of 600ml of *n*-hexane-ethyl acetate (7:3, v/v), contained 650mg of metabolite 3 as an oil. Fraction 4, the mixture of metabolites 3 and 4, was obtained by further elution of the column with the same solvent, but most of the mixture was separated into 90mg of metabolite 3 and 250mg of metabolite 4 by rechromatography of this fraction under the same conditions. Fraction 5, the eluate of 550ml of *n*-hexane-ethyl acetate (3:2, v/v), contained 590mg of metabolite 4 as an oil. Finally, fraction 6, the eluate of 500ml of *n*-hexane-ethyl acetate (1:1, v/v), contained 110mg of metabolite 5 as a viscous oil.

Characterization and identification of the free metabolites. The structures of all the metabolites were characterized and identified by comparison with the synthetic authentic compounds. The R_F values (Table 2) and the u.v. and i.r. spectra of the following metabolites, including those of the 2,4-dinitrophenylhydrazone and *p*-nitrobenzoate derivatives, were compared with the respective authentic compounds, and identical results were obtained.

Table 1. Isolation of the β -ionone metabolites from the urinary extract by silicic acid column chromatography

Fraction no.	Solvent <i>n</i> -hexane-ethyl acetate	Volume (ml)	Nature of residue on evaporation	Yield (mg)
1	9:1 (v/v)	800	Oil (metabolite 1)	18
2	4:1 (v/v)	300	Crystals (metabolite 2)	12
3	7:3 (v/v)	600	Oil (metabolite 3)	650
4	7:3 (v/v)	300	Oil (metabolites 3 and 4)	340
5	3:2 (v/v)	550	Oil (metabolite 4)	590
6	1:1 (v/v)	500	Oil (metabolite 5)	110

Table 2. R_F values of β -ionone metabolites and their derivatives on t.l.c.

β -Ionone metabolites and their 2,4-dinitrophenylhydrazones or *p*-nitrobenzoates or both were chromatographed on thin-layer plates of silica gel HF₂₅₄ with the following systems: β -ionone metabolites, *n*-hexane-ethyl acetate (1:1, v/v); derivatives, *n*-hexane-ethyl acetate (7:3, v/v). Compounds were detected under a u.v. lamp.

	R_F		
	Original compound	2,4-Dinitrophenylhydrazone	<i>p</i> -Nitrobenzoate
β -Ionone	0.92	0.90	—
3-Oxo- β -ionone	0.74	0.09	—
3-Oxo- β -ionol	0.45	0.57	0.60
Dihydro-3-oxo- β -ionol	0.40	0.52	0.55
3-Hydroxy- β -ionol	0.32	—	0.91

(a) Metabolite 1. The 2,4-dinitrophenylhydrazone derivative of metabolite 1, crystallized from ethanol, had m.p. 129°C (decomp.), not depressed by mixture with the same derivative of β -ionone.

(b) Metabolite 2. This metabolite, crystallized from 50% methanol, had m.p. 53°C, undepressed on admixture with 3-oxo- β -ionone. Its bis-2,4-dinitrophenylhydrazone crystallized from methanol, had m.p. 264°C (decomp.), undepressed on admixture with the same derivative of 3-oxo- β -ionone (Found: C, 52.8; H, 4.8; N, 19.3. Calc. for C₂₅H₂₆N₈O₈: C, 53.0; H, 4.6; N, 19.8%).

(c) Metabolite 3. This metabolite formed both 2,4-dinitrophenylhydrazone and *p*-nitrobenzoate derivatives. The 2,4-dinitrophenylhydrazone, crystallized from 80% ethanol, had m.p. 166°C (decomp.), undepressed on admixture with the same derivative of 3-oxo- β -ionol (Found: C, 58.4; H, 6.3; N, 14.3; C₁₉H₂₄N₄O₅ requires C, 58.8; H, 6.2; N, 14.4%). The *p*-nitrobenzoate, crystallized from 90% methanol, had m.p. 67°C, undepressed on admixture with the same derivative of 3-oxo- β -ionol (Found: C, 67.3; H, 6.5; N, 3.7; C₂₀H₂₃NO₅ requires C, 67.2; H, 6.5; N, 3.9%).

(d) Metabolite 4. This metabolite also formed both 2,4-dinitrophenylhydrazone and *p*-nitrobenzoate derivatives. The i.r. spectra were very similar to those of the derivatives of metabolite 3 except that they lacked the characteristic absorption due to >C=C< (*trans* form) at about 965 cm⁻¹. The 2,4-dinitrophenylhydrazone, crystallized from 90% ethanol, had m.p. 163°C (decomp.), undepressed on admixture with the same derivatives of dihydro-3-oxo- β -ionol (Found: C, 58.5; H, 6.7; N, 14.4; C₁₉H₂₄N₄O₅ requires C, 58.5; H, 6.7; N, 14.4%). The *p*-nitrobenzoate, crystallized from 90% methanol, had m.p. 126°C, undepressed on admixture with the same derivative of dihydro-3-oxo- β -ionol (Found: C, 67.0; H, 7.0; N, 4.0; C₂₀H₂₃NO₅ requires C, 66.8; H, 7.0; N, 3.9%).

(e) Metabolite 5. The bis-*p*-nitrobenzoate of this metabolite, crystallized from ethanol, had m.p. 184°C, undepressed on admixture with the same derivative of 3-hydroxy- β -ionol (Found: C, 63.5; H, 5.7; N, 5.5; C₂₇H₂₈N₂O₈ requires C, 63.8; H, 5.6; N, 5.5%).

Metabolites 1, 2, 3, 4 and 5 are thus identified as β -ionone, 3-oxo- β -ionone, 3-oxo- β -ionol, dihydro-3-oxo- β -ionol and 3-hydroxy- β -ionol respectively.

Fractionation and detection of the glucuronides. The glucuronides of β -ionone metabolites were separated by using the method of Kamil *et al.* (1951) and fractionated by charcoal column chromatography. Two fractions obtained from the eluates of 0.28% and 0.56% (w/v) ammonia in methanol (fractions A and B respectively) contained glucuronides. Both fractions were collected separately and evaporated to dryness. From the total dose of 19.2g of β -ionone, 208mg of fraction A and 517mg of fraction B were obtained as colourless gummy residues. The residues were dissolved in methanol and subjected to t.l.c. with chloroform-methanol-water (13:7:2, by vol.) as solvent. Each fraction gave a single spot with R_F 0.70. The spot was detected under a u.v. lamp, and the colour tests with 1% potassium permanganate solution and with 0.2% naphtharesorcinol-phosphoric acid solution were positive. A portion of fraction A was treated with β -glucuronidase and the reaction products were extracted with ether. Two aglycones corresponding to 3-oxo- β -ionol and dihydro-3-oxo- β -ionol were detected by t.l.c. with *n*-hexane-ethyl acetate (1:1, v/v) as solvent. This result was further confirmed by the formation of the *p*-nitrobenzoate derivative of the each aglycone. Both derivatives had R_F values identical with those of the derivatives of authentic 3-oxo- β -ionol and dihydro-3-oxo- β -ionol with *n*-hexane-ethyl acetate (7:3, v/v) as solvent. A portion of fraction B was also treated with β -glucuronidase, and the aglycone

was detected by t.l.c. as a single spot corresponding to dihydro-3-oxo- β -ionol.

Isolation and identification of (dihydro-3-oxo- β -ionol β -D-glucosid)uronic acid. A trace amount of impurity was removed from fraction B by silicic acid column chromatography with chloroform-methanol-water (13:7:2, by vol.). The eluate containing glucuronide of dihydro-3-oxo- β -ionol was collected and evaporated to dryness under reduced pressure. This gummy compound is soluble in water, ethanol and acetone. Its i.r. spectrum had $\nu_{\max}$. 3450, 2980–2880, 1755, 1660, 1600, 1400, 1355, 1340, 1320, 1210, 1170 and 1090–1030 cm^{-1} in NaCl (Found: C, 59.2; H, 8.2; $\text{C}_{19}\text{H}_{30}\text{O}_8$ requires C, 59.1; H, 7.8%). Its 2,4-dinitrophenylhydrazone, crystallized from 50% ethanol, had m.p. 169°C (decomp.); it had R_F 0.12 in *n*-hexane-ethyl acetate (1:1, v/v); its i.r. spectrum had $\nu_{\max}$. 3420, 2970–2920, 1780, 1640, 1595 and 1140 cm^{-1} in KBr (Found: C, 53.3; H, 6.1; N, 9.6; $\text{C}_{25}\text{H}_{34}\text{N}_4\text{O}_{11}$ requires C, 53.0; H, 6.1; N, 9.9%).

The sodium salt of (dihydro-3-oxo- β -ionol β -D-glucosid)uronic acid was prepared as follows. The glucuronide gum (200mg) was dissolved in water (1ml) and the solution was adjusted to pH 6 with dilute sodium hydroxide solution at 0°C. Cold acetone was added to this solution and crystals of the sodium salt appeared. After recrystallization from acetone-water (4:1, v/v), 162mg of white shiny granules was obtained. It had m.p. 138°C (decomp.) and $[\alpha]_D^{18} - 2.6^\circ$ (c 2 in water); its u.v. spectrum had $\lambda_{\max}$. 253nm (ϵ 2100); its i.r. spectrum had $\nu_{\max}$. 3420, 2980–2880, 1660, 1610–1600, 1440, 1400, 1355, 1340, 1315, 1220, 1165 and 1090–1030 cm^{-1} in KBr (Found: C, 52.6; H, 7.1; $\text{C}_{19}\text{H}_{29}\text{O}_8\text{Na} \cdot 1.5\text{H}_2\text{O}$ requires C, 52.4; H, 7.4%).

DISCUSSION

The results showed that five free products were excreted in the urine of rabbits that had been given β -ionone. These products were isolated and identified as 3-oxo- β -ionone, 3-oxo- β -ionol, dihydro-3-oxo- β -ionol, 3-hydroxy- β -ionol and unchanged β -ionone by comparison with the synthetic authentic compounds. None of these 3-oxo and 3-hydroxy derivatives exhibit any kind of odour. In addition to the above products, two glucuronides of β -ionol metabolites were detected. Both glucuronides were hydrolysed by β -glucuronidase, and the structures of the aglycones were confirmed as 3-oxo- β -ionol and dihydro-3-oxo- β -ionol. Although (dihydro-3-oxo- β -ionol β -D-glucosid)uronic acid was isolated, the quantity of 3-oxo- β -ionol glucuronide was too small for a pure product to be obtained.

Bielig & Hayashida (1940) studied the metabolic

fate of β -ionone in rabbits and claimed the detection of unchanged β -ionone, β -ionol, dihydro- β -ionol, hydroxy- β -ionone, hydroxy- β -ionol and dihydro-hydroxy- β -ionol. When Prelog & Meier (1950) administered β -ionone orally to rabbits and examined the urine, they obtained three metabolites: 3-oxo- β -ionone as the major metabolite and 3-oxo- β -ionol and 3-hydroxy- β -ionol as minor metabolites. The structure of 3-oxo- β -ionol was confirmed by Henbest (1951). In our laboratory, however, 3-oxo- β -ionol was obtained as a major metabolite as well as dihydro-3-oxo- β -ionol, which is an entirely newly discovered metabolite, and only a trace amount of 3-oxo- β -ionone was isolated. This difference in the yield of 3-oxo- β -ionone might be due to the instability of 3-oxo- β -ionol and 3-hydroxy- β -ionol. Both metabolites would be easily converted into 3-oxo- β -ionone during the fractional distillation procedure adopted by Prelog & Meier (1950) to purify the metabolites. This conversion does not occur during separation and purification of the metabolites by silicic acid column chromatography followed by the formation of 2,4-dinitrophenylhydrazones or *p*-nitrobenzoates.

Of the five free products only dihydro-3-oxo- β -ionol, the reduction product of the side-chain double bond, did not exhibit the absorption band of >C=C< (*trans* form) at about 965 cm^{-1} in its i.r. spectrum and lacked the signals of olefinic protons in the region τ 3.7–4.2 in its n.m.r. spectrum. Dihydro-3-oxo- β -ionol and 3-oxo- β -ionol, and their 2,4-dinitrophenylhydrazone and *p*-nitrobenzoate derivatives migrated close to each other, but could be separated, on t.l.c.

To establish the structure of metabolite 3 as 3-oxo- β -ionol, and to distinguish it from its structural isomer, 3-hydroxy- β -ionone, the latter compound was synthesized by the method of Henbest (1951) and its chemical properties compared with those of the synthetic 3-oxo- β -ionol. Both compounds exhibited identical R_F values on t.l.c., but their *p*-nitrobenzoate derivatives showed different R_F values (3-oxo- β -ionol: 0.60; 3-hydroxy- β -ionone: 0.65).

It was presumed that β -ionone is metabolized by hydroxylation of the ring system at the carbon atom α to the ring double bond and then oxidation of the hydroxyl group to 3-oxo derivatives, as well as other cyclohexenyl compounds; for example, hexobarbital and cyclobarbitol. In the present work, attempts to detect the formation of 3-hydroxy- β -ionone in the urine of rabbits were unsuccessful; however, preliminary results have shown that the rabbit liver microsomal enzyme system catalyses the production of this compound from β -ionone (H. Ide & S. Toki, unpublished work).

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