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Satoshi Toki and Tamiko Takenouchi : Hexobarbital Metabolism. Formation of α-1,5-Dimethyl-5-(3-hydroxy-1-cyclohexen-1-yl)-barbituric Acid Glucuronide.

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It has been previously reported[1,2] that diasteroisomeric α- and β-1,5-dimethyl-5-(3-hydroxy-1-cyclohexen-1-yl)barbituric acids (α- and β-3-OH-HB), 1,5-dimethyl-5-(3-oxo-1-cyclohexen-1-yl)barbituric acid (3-keto-HB) and three additional metabolites were excreted in the urine of rabbits administered hexobarbital (1,5-dimethyl-5-(1-cyclohexen-1-yl)barbituric acid). The main metabolic pathway of hexobarbital was further elucidated by *in vivo* and *in vitro* experiments.[3,4] During the course of above investigation, it was postulated that hexobarbital is also metabolized by glucuronide formation, and that, in this case, glucuronic acid will be conjugated on N in barbituric acid ring and/or on O of hydroxyl group in 3-OH-HB.

In earlier papers concerning the metabolism of barbiturates glucuronide formation, some workers found that the amount of hydroxyl derivative of phenobarbital[5,6] or pentobarbital[7] was increased after hydrolysis of urine from animals administered each of the parent compound. In the metabolism of barbital, glucuronide was detected only by a paper chromatography method.[8] However, none of these glucuronides has yet been isolated.

In the present investigation, one glucuronide was isolated and purified from the urine of rabbit administered hexobarbital. And it was demonstrated to be O-glucuronide of α-3-OH-HB (5-(α-3-O-β-D-glucociduronicacid-1-cyclohexen-1-yl)-1,5-dimethyl-barbituric acid) (α-3-OH-HB-GA).

Methods and Results

Paper Chromatography——For the chromatography of 3-OH-HB-GA, BuOH-AcOH-H_2O (4:1:5) was used as a solvent mixture in an ascending system, using Toyo Roshi No. 51. For 3-OH-HB, the paper which was treated with borate buffer (pH 10) was used and chromatographed in BuOH-borate buffer (pH 10) (1:1). The spot of metabolites and related compounds were detected on the paper chromatogram by examining it under an ultraviolet lamp having a maximum intensity of 2536 Å or by spraying it with 1% cobalt nitrate in EtOH, then drying and exposing it to ammonia vapour. The metabolites may also be detected by spraying the chromatogram with an aqueous solution containing 1% $NaIO_4$ and 1% $KMnO_4$ solution. The results are shown in Table I.

Isolation of 3-OH-HB-GA——The procedure was partly modified from the method of Kamil, Smith and Williams.[9] A 24 hr. urine specimen was collected from rabbits which were administered a total of 15.7 g. of hexobarbital[*2] (400 mg./kg. body wt.). It was filtered through cotton wool and adjusted to pH

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*2 A freshly prepared aq. solution containing 1.1 equiv. NaOH.

1) H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, **4**, 368 (1956).
2) H. Yoshimura : *Ibid.*, **5**, 561 (1957).
3) H. Tsukamoto, S. Toki, K. Kaneda : *Ibid.*, **7**, 651 (1959).
4) S. Toki, K. Toki, H. Tsukamoto : *Ibid.*, **10**, 709 (1962).
5) E. J. Algeri, A. J. McBay : Science, **123**, 183 (1956).
6) T. C. Butler : J. Pharmacol. Exptl. Therap., **116**, 326 (1956).
7) E. Titus, H. Weiss : J. Biol. Chem., **214**, 807 (1955).
8) S. Golgschmidt, R. Wehr : Z. Physiol. Chem., Hoppe-Seyler's, **308**, 9 (1957).
9) I. A. Kamil, J. N. Smith, R. T. Williams : Biochem. J., **50**, 235 (1951).

Table I. Paper Chromatography of 3-OH-HB-GA and Related Compounds

Compounds	Rf values A	Rf values B	Location reagent
3-OH-HB-GA	0.44	—	a, b, c
Glucuronic acid	0.07	—	b
Glucuronolactone	0.25	—	b
3-OH-HB[a)]	—	0.5	a, b, c

Solvent: A. BuOH-AcOH-H_2O, 4:1:5
B. BuOH-Borate buffer (pH 10), 1:1 (Buffered paper)
Reagent: a. Cobalt nitrate-Ammonia (+: Purple)
b. $NaIO_4$-$KMnO_4$ (+: Yellow)
c. UV lapm (+ Dark spot)
a) The diastereoisomeric α- and β-3-OH-HB have identical Rf values and cannot be distiguished by the chromatograpic procedure used.

4.0 with glacial AcOH. Saturated aqueous lead acetate was added to the filtrate. The mixture was centrifuged and the precipitate was discarded. The supernatant solution was collected and adjusted to pH 8.0 with NH_4OH and was then treated again with saturated aqueous basic lead acetate in excess. The precipitate was collected by centrifugation and was suspended in 600 ml. of MeOH. The suspension was saturated with H_2S. After removal of PbS by filtration, the solution was evaporated to dryness at 35° under reduced pressure. The residue was dissolved in 40 ml. of water and washed three times with 40 ml. of AcOEt. The aqueous solution was evaporated to dryness at 35° under reduced pressure and residue was extracted with 240 ml. of EtOH-ether (1:3, v/v). The insoluble substance was removed by filtration. The filtrate was evaporated to dryness under reduced pressure at 35°. The residue was dissolved in 30 ml. of EtOH, and then, 50% aqueous $Ba(AcO)_2$ was added to the solution until the precipitation was complete. The precipitate was collected by centrifugation, washed repeatedly with a 20 ml. portion of EtOH and dissolved in 30 ml. of water. Then the solution was filtered and adjusted to pH 7.0 with NH_4OH. A lead salt, which was obtained by the addition of saturated aqueous basic lead acetate, was treated with H_2S in the same way as described above, *i.e.*, H_2S was bubbled into a suspension of lead salt in MeOH. The precipitated PbS was collected by filtration and MeOH was removed by evaporation. The free glucuronide residue (2.2 g.) was dissolved in 30 ml. of EtOH. The Na salt of this glucuronide was precipitated by the addition of ethanolic NaOH to the solution and it was separated by centrifugation. The precipitate salt was, then, washed thoroughly with EtOH and dissolved in 30 ml. of MeOH. The resulting solution was, then, filtered. The filtrate was evaporated to dryness at 35° and the residue was dissolved in 25 ml. of water. The solution was adjusted to pH 7.0 and saturated aqueous basic lead acetate was added. Lead was removed from the lead salt of glucuronide by H_2S as shown above and, in this manner, 1.017 g. of free glucuronide was isolated as a white or pale yellow amorphous solid.

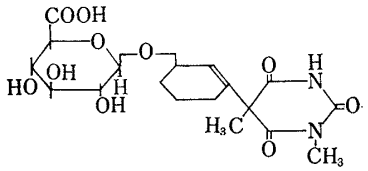
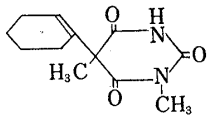
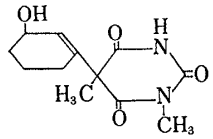
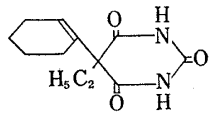
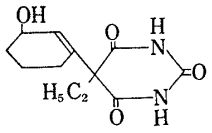
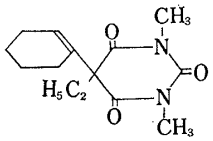
Purification and Characterization of 3-OH-HB-GA by Silica Gel Chromatography——In order to remove an impurity which occurred persistently in the final preparation, the isolated glucuronide was treated as following: 1.017 g. of the glucuronide was dissolved in AcOEt saturated with water. After the solution was concentrated to about half of the volume, it was chromatographed on silica gel (20 g.) and eluted stepwise with 1) 90 ml. of AcOEt, 2) 60 ml. of MeOH-AcOEt (1:20, v/v), 3) 60 ml. of MeOH-AcOEt (1:10), 4) 60 ml. of MeOH-AcOEt (1:10), and 5) 60 ml. of MeOH-AcOEt (1:5). Most of the glucuronide was contained in the eluate in solution 3; the first part of MeOH-AcOEt (1:10), this was confirmed by paper chromatography. This eluate was evaporated to dryness. 832 mg. of residue was collected, taken up in EtOH-AcOEt (1:20), and the resuting solution was concentrated. The pure 3-OH-HB-GA was isolated as white amorphous powder, the yield being about 800 mg. The attempt to crystalize the compound was unsuccesful. $[\alpha]_D^{20}$ −61°(c=1, H_2O). *Anal.* Calcd. for $C_{18}H_{24}O_{10}N_2 \cdot \frac{1}{2}H_2O$: C, 49.65; H, 5.76; N, 6.40. Found: C, 49.48; H, 5.78; N, 6.24.

This compound showed a single spot on paper chromatograms and the Rf value was 0.44 (Table I). The area containing this spot was cut out and extracted with water. The naphthoresorcinol test on this extract was positive.

Ultraviolet Absorption Spectra——Ultraviolet absorption spectra were measured by the Shimazu recording spectrophotometer SV 50 with standard 10 mm. square quartz absorption cells. Borate buffer (pH 10) was used as the solvent and concentration of the solution was 10 γ/ml. in all cases.

The absorption peak of 5,5-disubstituted barbituric acid and its 1-alkyl derivative appeared approximately at 240 mμ and 245 mμ respectively and its 1,3-dialkyl derivative afforded no absorption peak. These spectra are shown in Table II. 3-OH-HB-GA exhibited virtually identical characteristic absorption peak of 1-alkyl-5,5-disubstituted barbituric acid, strongly suggesting that the site of glucuronic acid conjugation is not on the ring amide of barbituric acid, but on the hydroxyl group in 3-OH-HB.

Table II. Ultraviolet Absorption Spectra of 3-OH-HB-GA and Related Compounds

Compounds	Structure	$\lambda_{min}^{pH\ 10\ Borate\ buffer}$ (mμ)	$\lambda_{max}^{pH\ 10\ Borate\ buffer}$ (mμ)
3-OH-HB-GA		225.5	245 (ε : 9625)
Hexobarbital		225	244.5
3-OH-HB		225	245
Cyclobarbital		220	239.5
5-Ethyl-5-(3-hydroxy-1-cyclohexen-1-yl)-barbituric acid		220.5	240.5
1,3-Dimethyl-5-ethyl-5-(1-cyclohexen-1-yl)-barbituric acid[a]		none	none

a) 1,3-Dimethyl-5-ethyl-5-(1-cyclohexen-1-yl)barbituric acid was kindly supplied from Dr. Y. Kuroiwa.

Acid Hydrolysis of 3-OH-HB-GA——The purified sample of 3-OH-HB-GA (0.52 mg.) was hydrolyzed by 1 ml. of 5% HCl for 4 hr. at 37°,*3 and the reaction mixture was shaken with 2×10 ml. of AcOEt to extract 3-OH-HB. Both the aqueous layer and AcOEt extract were concentrated and applied onto a paper chromatogram. 3-OH-HB, glucuronolactone and glucuronic acid were identified on the chromatogram as shown in Table I. Under the same conditions, 340 mg. of glucuronide was hydrolyzed and the crystal was obtained which were identified as α-3-OH-HB (m.p. 213~215°), the melting point remained underpressed on admixture with an authentic sample prepared according to the method of Tsukamoto, *et al.*[10,11]

Enzymatic Hydrolysis——In a 50 ml. Erlenmeyer flask, 4.27 mg. of 3-OH-HB-GA and 3×10^3 units of β-glucuronidase*4 were introduced. The final volume of the reaction mixture was made to 10 ml. with 0.1*M* acetate buffer. Incubation was conducted at 37° for 48 hr. The reaction mixture was, then, extracted with AcOEt. The aglycone, 3-OH-HB, was identified by paper chromatography method using buffered paper in conjunction with ultraviolet spectrophotometry.[4,12] The yield of 3-OH-HB was 20.3%.

Discussion

The important role of glucuronide conjugation in the biotransformation of foreign

*3 It was confirmed by preliminaly experiments that most of the aglycone, 3-OH-HB, was decomposed to unidentified compounds when the reaction was prolonged at 37° or the reaction mixture was heated at 85° for 10 min.

*4 β-Glucuronidase was purchased from the Sigma Chem. Co.

10) H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, **4**, 364 (1956).
11) H. Yoshimura : *Ibid.*, **6**, 13 (1958).
12) H. Tsukamoto, H. Yoshimura, S. Toki : *Ibid.*, **6**, 88 (1958).

compounds, especially in connection with detoxication mechanism, is well known. Five main types of glucuronide conjugation were elaborated by various investigators[13] : Glucuronic acid was linked : (1) on the O of hydroxyl groups in phenols and in primary, secondary or tertiary alcohols; (2) on the O of carboxyl groups in aromatic or certain aliphatic compounds; (3) on N in some aliphatic or aromatic amino compounds; (4) on S in certain sulfur compounds such as thiophenol and mercaptobenzothiazole; or (5) on O of hydroxyl groups of carbohydrates.

Accordingly, it was postulated that hexobarbital would be metabolized directly by the formation of N-glucuronide and/or metabolized by the hydroxylation of hexobarbital, affording the formation of O-(or N-) glucuronide of 3-OH-HB. On the metabolism of barbiturates by glucuronide conjugation, Algeri and McBay,[5] and Butler[6] observed that phenobarbital was converted to *p*-hydroxyl derivative prior to conjugation with glucuronic acid, since the acid hydrolysis or β-glucuronidase treatment of urine afforded an increased yield of a hydroxylated compound. Titus and Weiss[7] studied the metabolism of pentobarbital-^{14}C, and suggested that between the two diasteroisomers of 5-ethyl-5-(1-methyl-3-hydroxy-buthan-1-yl)barbituric acid, the dextrorotatory form appears to be preferentially conjugated with glucuronic acid.

In our experiments, the glucuronide of 3-OH-HB was isolated from the urine of rabbits administered hexobarbital and was purified by silica gel chromatography. The product exhibited a characteristic ultraviolet absorption spectrum of 1-alkyl-5,5-disubstituted barbituric acid. This fact shows that one proton in barbituric acid ring of the glucuronide is free as in hexobarbital or 3-OH-HB and that position of glucuronic acid conjugation in 3-OH-HB is on the hydroxyl group.

Moreover, this glucuronide was split by β-glucuronidase or hydrochloric acid to 3-OH-HB and glucuronic acid and crystal of α-3-OH-HB was obtained through acid hydrolysis. It was well known, through the work of Tsukamoto, *et al.*, that hexobarbital was metabolized to the two diasteroisomers, α- and β-3-OH-HB, and that both of the hydroxylated compounds were oxidized further to 3-keto-HB. However, the present work demonstrated that another pathway of hexobarbital metabolism was in the selective conjugation of α-3-OH-HB by glucuronic acid.

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Summary

α-3-OH-HB-GA was isolated from the urine of rabbit administered hexobarbital.

The position of the conjugated glucuronic acid was suggested by ultraviolet absorption spectrum, and the structure of aglycone was confirmed by enzymatic and acid hydrolysis.

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13) G. J. Dutton : "Proceedings of the First International Pharmacological Meeting" Vol. **6**, p. 39 (1962), Pergamon Press, Oxford.