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41. Hisao Tsukamoto, Hiroyuki Ide, and Eigo Takabatake: Metabolism of Drugs. XXII.*1 The Metabolic Fate of Thiamylal. (1).

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Thiamylal (5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid) is a widely used intravenous anesthetic but little is known of its metabolic fate. Spector and Shideman[1] reported the *in vitro* removal of sulfur atom from this drug by minced liver of a rat, producing secobarbital (5-allyl-5-(1-methylbutyl)barbituric acid). Cooper and Brodie[2] stated that thiobarbiturates such as thiamylal and thiopental were also metabolized by the kidney and brain. This paper presents results obtained from paper chromatography of the urine extract of rabbits receiving thiamylal or secobarbital. The isolation and characterization of a major metabolite of thiamylal are also described.

Experimentals and Results

Drug Administration and Urine Extraction—Thiamylal-Na in the doses of 200 mg./kg. body wt. was administered by a stomach tube to rabbits weighing about 2.5 kg. Nothing except water was given for 24 hr. before and after the medication. The urine was collected into bottles containing a few drops of dil. H_2SO_4 and by catheter 48 hr. after the medication. The urine was saturated with $(NH_4)_2SO_4$ at pH 5, heated at 60° for 1 hr., and filtered by the aid of Hyflo Super-Cel. The clear filtrate was adjusted to pH 2 with H_2SO_4 and continuously extracted with AcOEt for 20 hr. After washing with small amounts of acidic water, the AcOEt layer was concentrated under a reduced pressure and shaken with dil. NaOH at pH 10~11. The aqueous phase was washed with Et_2O and extracted with petr. ether containing 2.5% of iso-AmOH at pH 7 to separate more nonpolar substances such as unchanged thiamylal or secobarbital produced from other metabolites. The materials were returned to an aqueous phase by shaking with dil. NaOH and re-extracted with Et_2O at pH 2. After the Et_2O extract was dehydrated over Na_2SO_4 and evaporated, an oily brown substance was obtained as TA-fraction.

The aqueous phase from which the TA-fraction was removed by extraction with iso-AmOH-petr. ether was adjusted to pH 2 with HCl, saturated with NaCl, and extracted with Et_2O. After dehydration over Na_2SO_4 and evaporation of Et_2O, the TB-fraction was obtained as an oily brown substance.

Secobarbital was also administered to rabbits and their urine was treated similarly as in the case of thiamylal to separate SA- and SB-fractions, and compared with TA- and TB-fractions.

Paper Chromatography—Each fraction was dissolved in MeOH, refluxed for 1 hr. with activated charcoal, filtered, and chromatographed on a filter paper (Toyo Roshi No. 50), using the solvent system of BuOH:EtOH:conc. NH_4OH (4:2:1.2 v/v) by the ascending technique. The following reagents were used for the location of paper chromatogram. (1) (Mn) $NaIO_4$ and $KMnO_4$[3]; for the detection of unsaturated bond such as allyl side-chain in thiamylal. (2) (Cu) $CuSO_4$ and pyridine[2]; thiobarbiturates gave yellowish green spots, color reaction for barbiturates was violet but insensitive. (3) (Hg) $HgNO_3$[4]; both thiobarbiturates and barbiturates gave gray to black spots. (4) (Co) $Co(NO_3)_2$ and ammonia[5]; thiobarbiturates gave light green spots but barbiturates gave faint violet spots.

Paper Chromatogram of Urine Extract—The spots detected are shown in Table I. Since spot 1 is of a barbiturate (Cu, −, Hg, +) and appeared in both TB and SB fractions, it is assumed to be one of desulfurized metabolite of thiamylal. Spot 2 was established as that of thiamylalcarboxylic acid, as will be shown below. Spot 3 is positive only in (Mn) test and assumed to be a metabolite without the ring of barbituric acid. Spots 4 and 5 are of barbiturates and markedly apparent in SB but faintly in the TB-fraction. The area of Rf 0.69~0.81 was considerably difficult to detect, based

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on the differences of color located by (Cu) or (Co) reagent, but it was identified that there were 3 spots in this area; spots 6, 7, and 8 are respectively a thiobarbiturate metabolite, secobarbital, and unchanged thiamylal. Spot 9 is assumed to be a metabolite with destroyed ring similar to spot 3.

Table I. Paper Chromatogram of Urine Extract

Spot No.	Rf	Mn	Cu	Hg	Co	TA	TB	SA	SB	Identification
1	0.14~0.15	+	−	+	v	−	+	−	+	Barbiturate metabolite
2	0.22~0.23	+	+	+	g	+	++	−	−	Thiamylalcarboxylic acid
3	0.27~0.30	+	−	−	−	+	+	−	−	Ring-destroyed metabolite
4	0.53~0.55	+	−	+	v	−	+	−	+	Barbiturate metabolite
5	0.62~0.63	+	−	+	v	−	+	−	+	〃
6	0.69~0.70	+	+	+	±	−	+	−	−	Thiobarbiturate metabolite
7	0.72~0.73	+	−	+	v	+	−	+	−	Secobarbital
8	0.80~0.81	+	+	+	g	+	−	−	−	Unchanged thiamylal
9	0.83~0.85	+	−	−	−	−	+	−	+	Ring-destroyed metabolite

v : violet g : green

Isolation and Characterization of a Metabolite—The recrystallization of TA-fraction from MeOH gave white plates, m.p. 129°. This compound was established as unchanged thiamylal because the m.p. was not depressed by admixture with medicated sample and of the similarity to paper chromatography. From the urine of rabbits receiving a total dose of 13.5 g. of thiamylal, about 350 mg. of unchanged drug was recovered.

TB-Fraction was dissolved in Et_2O and extracted with 5% $NaHCO_3$. The aqueous solution was acidified, saturated with NaCl, and extracted with Et_2O. The residue after evaporation of Et_2O was recrystallized from water. After standing in a refrigerator for some days, the deposited solid substance was washed with a small amount of $CHCl_3$ and recrystallized from 30% EtOH to crystals of m.p. 158°. 140 mg. of purified metabolite was obtained from the urine of rabbits given a total dose of 3.6 g. of thiamylal. Ultraviolet absorption spectrum of this metabolite shown in Fig. 1 was

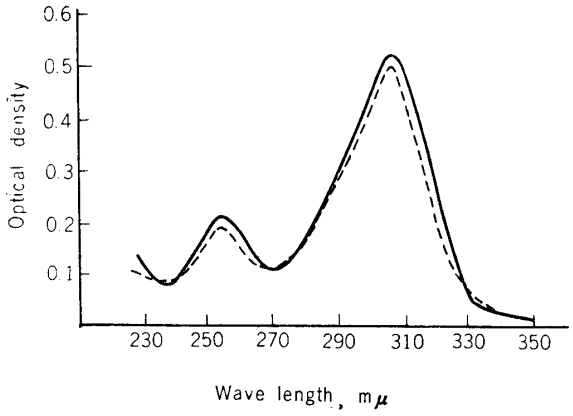

Fig. 1. The Ultraviolet Absorption Spectra of Thiamylal and its Carboxylic Acid

—— Thiamylal
------ Thiamylalcarboxylic acid
5 γ/cc. borate buffer of pH 10

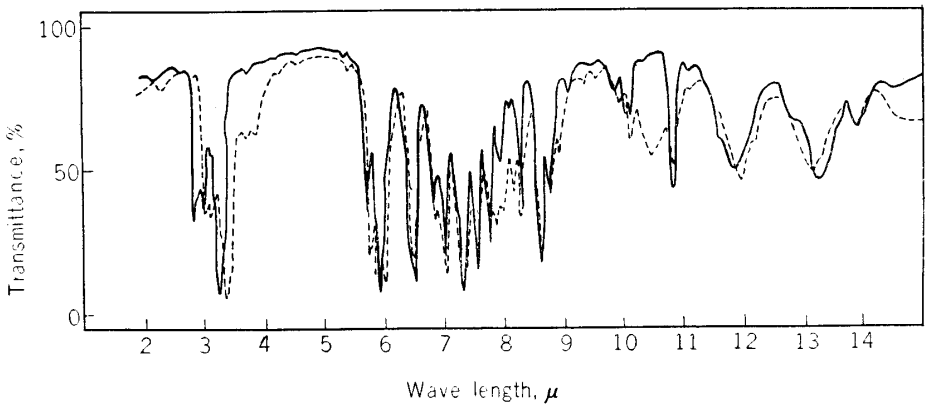

Fig. 2. Infrared Absorption Spectra of Thiamylal and its Carboxylic Acid

—— Thiamylal ------ Thiamylalcarboxylic acid

indistinguishable and indicated that it was a thiobarbiturate as it possessed 2 peaks at 256 and 305 mμ in borate buffer (pH 10). This was established by the color reaction with $CuSO_4$ and pyridine. From the infrared absorption spectrum shown in Fig. 2, this metabolite was assumed to be a carboxylic acid because of its absorptions at 3.4~3.6, 5.9, and 10.9 μ. Hydroxamic acid-iron reaction for carboxylic acid was positive and elemental analyses agreed with calculation for the structure derived from thiamylal by oxidation of one of the methyl groups in the side chain to carboxyl group. *Anal.* Calcd. for $C_{12}H_{16}O_4N_2S$: C, 50.69; H, 5.67; N, 9.85. Found : C, 50.90; H, 5.87; N, 9.58. Rf value of this metabolite on the paper chromatogram was 0.22~0.23 and agreed with spot 2 in Table I by mixed chromatography.

Thiamylalcarboxylic acid was injected intraperitoneally into mice weighing about 15 g., in the doses of 2.0~4.5 mg./10 g. but no pharmacological effect was observed, although thiamylal of 0.5 mg./10 g. was markedly hypnotic. This metabolite was also isolated with the aid of column chromatography with Dowex-1, details of which will be reported in the future.

Discussion

There are some reports on the metabolism of thiopental (5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid) which possesses an ethyl side-chain instead of an allyl group in thiamylal. A main metabolite was isolated from the urine of man receiving thiopental[6] and from the incubation mixture with rabbit liver microsome.[2] The structure of this metabolite was identified as 5-ethyl-5-(3-carboxy-1-methylpropyl)-2-thiobarbituric acid.[7] On the other hand, pentobarbital, the oxygen analog of thiopental, was said to be converted to (ω-1)-hydroxyl compounds[8] and to ω-carboxylic acid.[9] In the case of thiamylal, a carboxylic acid possessing both thiobarbituric acid ring and unsaturated bond of allyl side-chain was isolated as a main metabolite, but hydroxyl derivatives have not yet been isolated though the presence of thiobarbiturate metabolites other than carboxylic acid was detected on the paper chromatogram.

The position of carboxyl group in this metabolite has not yet been determined but it is assumed from analogy with thiopental that ω-position of methylbutyl side-chain was oxidized.

In was shown by Spector and Shideman,[1] Winter, *et al.*,[10] and by Raventos[11] that the biological removal of sulfur atom of thiobarbiturate occurred in the matabolic process and that some analogous oxygen metabolites were produced. Dietz and Soehring[3] found some desulfurized metabolites on the paper chromatogram of urine of man and dog given thiobarbiturate such as thiopental. Based on the paper chromatogram of urine extract of the rabbit receiving thiamylal, it is apparent that barbiturate metabolites were also produced *in vivo*. These metabolites agreed with secobarbital and its metabolites. Their isolation is now in progress. Furthermore, the occurrence of some metabolites with destroyed rings was estimated on the paper chromatogram.

From the results of this experiment, it is apparent that thiamylal was metabolized to compounds not only with thiobarbituric acid ring and its oxygen homolog but also without the ring structure.

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Summary

Thiamylal was orally administered to rabbits and its metabolites excreted in urine were examined, mainly by paper chromatography. One of the metabolites was isolated and characterized as a carboxylic acid with thiobarbituric acid ring and allyl side-chain. Comparing the paper chromatogram of urine of rabbits receiving thiamylal or secobarbital, it was apparent that thiamylal was metabolized to the compounds not only with thiobarbituric acid ring and its oxygen homologs but also without the ring structure.

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42. Keitaro Kato, Kazuo Yoshida, Hisao Tsukamoto*1; Masashi Nobunaga, Tomiichi Masuya, and Toichiro Sawada*2 : Synthesis of *p*-Nitrophenyl β-D-Glucopyranosiduronic Acid and Its Utilization as a Substrate for the Assay of β-Glucuronidase Activity.

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Phenolphthalein-mono-β-D-glucosiduronic acid has been utilized as a substrate for the assay of the hydrolytic activity of β-glucuronidase.[1] This substrate is generally prepared biosynthetically from the urine of rabbits to which phenolphthalein has been administered. *p*-Nitrophenyl β-D-glucosiduronic acid was synthesized in this laboratory for this purpose. The chemical synthesis provides a useful chromogenic substrate for β-glucuronidase which should be more practicable and more readily available than the tedious biosynthetic preparation of phenolphthalein mono-β-D-glucosiduronic acid currently in use. The latter compound has not been obtained in crystalline form but is only available as a crude cinchonidine salt. *p*-Nitrophenyl β-D-glucosiduronic acid is rapidly hydrolyzed by β-glucuronidase and the free *p*-nitrophenol may be readily determined photocolorimetrically in alkaline solution. The method of assay is similar in principle to that used for sodium phenolphthalein-mono-β-D-glucosiduronate.

The synthesis of *p*-nitrophenyl β-D-glucosiduronic acid was accomplished by alkaline hydrolysis of methyl (*p*-nitrophenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (I), which was prepared in a good yield by the condensation of methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate and *p*-nitrophenol using acetonitrile and freshly prepared silver oxide. By the treatment of potassium *p*-nitrophenoxide with methyl 1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranuronate in acetone-water or the fusion of *p*-nitrophenol with methyl (tetra-O-acetyl-β-D-glucopyranosid)uronate in the presence of *p*-toluenesulfonic acid, (I) was obtained only in a small amount. The method of Helferich and Berger[2] was adopted in alkaline hydrolysis of (I).

The present paper describes the use of *p*-nitrophenyl β-D-glucosiduronic acid as a chromogenic substrate for the assay of the hydrolytic activity of β-glucuronidase and preparation of the substrate.

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