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49. Hiroyuki Ide, Hidetoshi Yoshimura, and Hisao Tsukamoto : Metabolism of Drugs. LII.*1 Enzymatic Study on Secobarbital Metabolism.

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ω- and (ω-1)-Oxidation of secobarbital was studied with cell free systems of rabbit liver. By gas chromatography, secobarbital was found to be metabolized into (ω-1)-hydroxysecobarbital, ω-carboxysecobarbital, and (ω-1)-oxosecobarbital by incubation with 9000 *g* supernatant fraction of liver homogenate.

When secobarbital was incubated with microsomes with addition of NADPH and oxygen, formation of (ω-1)-hydroxysecobarbital was detected, but not that of ω-hydroxysecobarbital.

Oxidation of ω-hydroxysecobarbital and (ω-1)-hydroxysecobarbital with the soluble fraction was demonstrated.

Both horse liver alcohol dehydrogenase and 3-hydroxymethylhexabital dehydrogenase were not concerned with metabolism of secobarbital in rabbit liver.

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A number of work on the metabolism of barbiturate performed *in vivo* and *in vitro* have shown that alkyl chains attached to the barbiturate ring are mainly oxidized in the positions of ω- and (ω-1)-carbon atoms in a mammalian body,[1~6] but there still remains the question of whether the same enzyme system catalyzes the oxidation on both carbon atoms or not.

The present investigation is aimed at clarifying the relationship between ω- and (ω-1)-oxidation, by following the metabolic pathways of secobarbital in rabbit liver.

It has previously reported that two metabolites, ω-carboxysecobarbital (5-allyl-5-(1-methyl-3-carboxypropyl)barbituric acid) and (ω-1)-hydroxysecobarbital (5-allyl-5-(1-methyl-3-hydroxybutyl)barbituric acid) were isolated from a urine of rabbits administered secobarbital (5-allyl-5-(1-methylbutyl)barbituric acid).[5] The present work described has shown that secobarbital is oxidized mainly to ω-carboxysecobarbital and (ω-1)-hydroxysecobarbital in supernatant fraction of rabbit liver and the oxidation to (ω-1)-hydroxysecobarbital is catalyzed by NADPH-dependent enzymes in microsomes, but that ω-hydroxylation does not proceed by microsomal fraction in the presence of NADPH. On the other hand, a supposed intermediate of ω-oxidation, namely ω-hydroxysecobarbital (5-allyl-5-(1-methyl-4-hydroxybutyl)barbituric acid) is shown to be oxidized easily to ω-carboxysecobarbital in the soluble fraction.

Experimental

Materials

Secobarbital was supplied by Yoshitomi Pharmaceutical Ind., Ltd. (ω-1)-Hydroxysecobarbital (m.p. 167°, $[\alpha]_D^{15}$ +19.3°), and ω-carboxysecobarbital (m.p. 197°) were obtained from the urine of rabbits administered secobarbital.[5] α-3-Hydroxymethylhexabital, m.p. 213~215°(decomp.), was prepared by the Meerwein-

*1 Part LI. This Bulletin, **14**, 939 (1966).
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1) E. W. Maynert, J. M. Dawson : J. Biol. Chem., **195**, 389 (1952).
2) E. Titus, H. Weiss : *Ibid.*, **214**, 808 (1955).
3) J. R. Cooper, B. B. Brodie : J. Pharmacol. Exptl. Therap., **120**, 75 (1957).
4) E. W. Maynert : Federation Proc., **22**, 479 (1963).
5) H. Tsukamoto, H. Yoshimura, H. Ide : This Bulletin, **11**, 9 (1963).
6) H. Tsukamoto, *et al.* : *Ibid.*, **11**, 427 (1963).

Ponndorf reduction of 3-oxomethylhexabital.[7] NAD, NADH, NADP, NADPH, and horse liver alcohol dehydrogenase were purchased from the Sigma Chemical Company (U. S. A.).

Synthesis of ω-Hydroxysecobarbital (see Chart 1)

1) 4-Bromopentanol——An ether solution of 10 g. of 4-bromopentanoic acid (b.p_3 92°) was treated with excess of CH_2N_2. Eleven g. of colorless liquid (b.p_2 36°) obtained by removal of the solvent and CH_2N_2 was added dropwise under stirring to a suspension of 1.2 g. of $LiAlH_4$ in anhyd. ether. After refluxing the mixture for 2 hr., excess of $LiAlH_4$ was decomposed with 20 ml. of 0.1*N* HCl. Ether layer was separated and aq. acid layer was extracted repeatedly with ether. The extracts were combined with the initial ether layer, washed with water, dried, and distilled *in vacuo*. The yield of the product was 6.5 g. (b.p_2 58°). IR λ_{max}^{liquid} μ : 2.75 (ν_{OH}). *Anal.* Calcd. for $C_5H_{11}Br$: C, 35.09; H, 6.60. Found : C, 34.63; H, 6.64.

2) 1-(2-Tetrahydropyranoxy)-4-bromopentane——To a mixture of 6.4 g. of 4-bromopentanol and 3.2 g. of 2,3-dihydro-4*H*-pyran a drop of conc. HCl was added and the mixture was shaken at room temp. for 1 hr. After addition of a piece of NaOH, the reaction mixture was distilled *in vacuo* and 3.8 g. of an oily substance (b.p_2 87°) was obtained. *Anal.* Calcd. for $C_{10}H_{19}O_2Br$: C, 47.78; H, 7.50. Found : C, 47.75; H, 7.87.

3) Diethyl 2-Allyl-2-[1-methyl-4-(2-tetrahydropyranoxy)butyl]malonate——To a solution of EtONa, prepared from 0.25 g. of Na and 50 ml. of anhyd. EtOH, 50 ml. of redistilled diethyl carbonate and 3.6 g. of diethyl allylmalonate were added. The mixture was distilled under reduced pressure until EtOH was removed. 1-(2-Tetrahydropyranoxy)-4-bromopentane (2.8 g.) was added dropwise to the residual oil and the mixture was refluxed for 16 hr. The reaction mixture was poured into 10 g. of ice and diethylcarbonate was evaporated under reduced pressure. The residue was neutralized with dil. HCl and extracted with isopropyl ether exhaustively. The extracts were combined, dried and distilled *in vacuo*. Yield of the product, 1.2 g. (b.p_2 156~158°). *Anal.* Calcd. for $C_{17}H_{30}O_6$: C, 64.84; H, 9.25. Found : C, 64.34; H, 9.06.

4) 5-Allyl-5-(1-methyl-4-hydroxybutyl)barbituric Acid——To a solution of EtONa, prepared from 0.25 g. of Na and 20 ml. of anhyd. EtOH, 0.8 g. of dried finely powdered urea and 0.9 g. of diethyl 2-allyl-2-[1-methyl-4-(2-tetrahydropyranoxy)butyl]malonate were added. After the mixture was refluxed for 20 hr., it was poured into 10 g. of ice and EtOH was distilled off under reduced pressure. The residual solution was acidified with 1*N* HCl, extracted with ether, and the solvent was exporated from the extract. Resulting oily substance (0.8 g.) showed characteristic UV and IR spectra of a barbiturate. A mixture of 0.8 g. of this oily substance, 15 ml. of water, 5 ml. of EtOH, and 0.1 ml. of conc. HCl was heated on a water bath maintained at about 80° for 1 hr. The solution was extracted with AcOEt and the solid material (0.48 g.) obtained after evaporation of AcOEt was dissolved in benzene and chromatographed on alumina column (20 g. of Al_2O_3), from which fractions were separated by a stepwise elution with benzene and acetone. The fraction of benzene-acetone (1:1) was recrystallized from a mixture of benzene and AcOEt to colorless crystals, m.p. 117°(160 mg.). Paper chromatographic Rf value of this compound was 0.63~0.65 with the solvent system of BuOH-EtOH-conc. NH_4OH (4:2:1.2).[8] UV $\lambda_{max}^{borate\ buffer\ (pH\ 10)}$ mμ : 240. IR λ_{max}^{Nujol} μ : 2.75 (ν_{OH}), 3.10, 3.22 (ν_{NH}), 5.66, 5.78, 5.88 ($\nu_{C=O}$), 6.05 ($\nu_{C=C}$), 9.95, 10.86 ($\delta_{CH=CH_2}$). *Anal.* Calcd. for $C_{12}H_{18}O_4N_2$: C, 56.68; H, 7.14; N, 11.02. Found : C, 56.67; H, 6.99; N, 10.89.

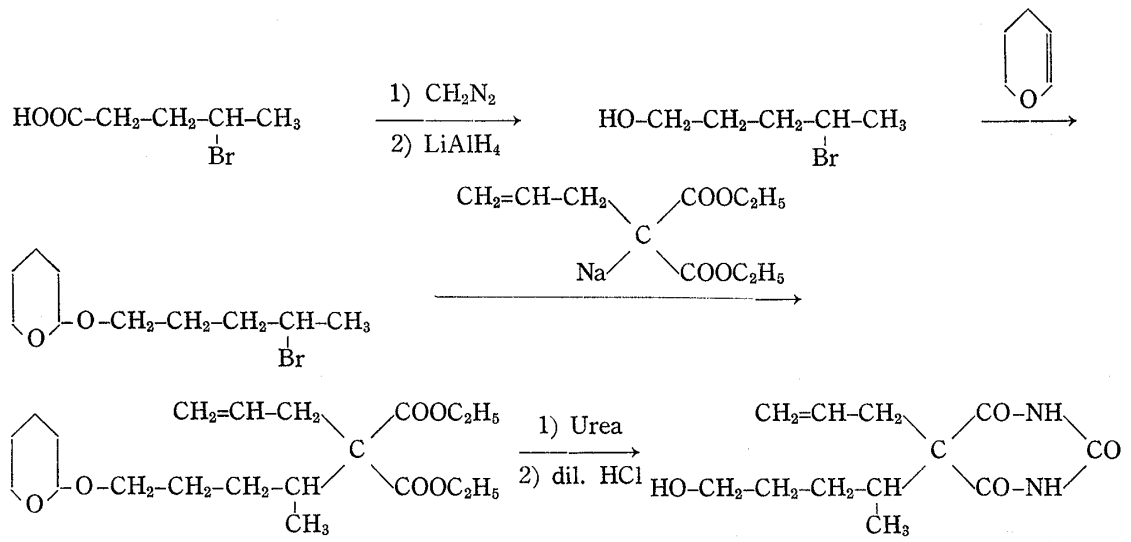

Chart 1. Synthesis of ω-Hydroxysecobarbital

7) H. Yoshimura : This Bulletin, **6**, 13 (1958).
8) H. Tsukamoto, H. Ide, E. Takabatake : This Bulletin, **8**, 236 (1960).

Synthesis of (ω-1)-Oxosecobarbital (5-Allyl-5-(1-methyl-3-oxobutyl)barbituric Acid)

A solution of (ω-1)-hydroxysecobarbital (37 mg.) and CrO_3 (22.3 mg.) in 0.4 ml. of glacial AcOH containing a drop of H_2O was warmed on a water bath maintained at about 40° for 2 hr. with occasional shaking, diluted with 20 ml. of water, and extracted with AcOEt. The extract was washed with water, dehydrated over Na_2SO_4, and the solvent was evaporated. The residue was recrystallized from $CHCl_3$ to colorless crystals of m.p. 110°(26 mg.). The Rf value (0.53~0.55) of this compound was the same as that of an unknown metabolite of rabbit administered with secobarbital.[8] UV $\lambda_{max}^{borate\ buffer\ (pH\ 10)}$ mμ : 240. IR λ_{max}^{Nujol} μ: 3.10, 3.24 (ν_{NH}), 5.70~5.80 ($\nu_{C=O}$), 9.94, 10.85 ($\delta_{CH=CH_2}$). *Anal.* Calcd. for $C_{12}H_{16}O_4N_2$: C, 57.13; H, 6.39; N, 11.11. Found : C, 57.15; H, 6.44; N, 10.98.

Gas Chromatographic Method[*3]

The column was operated at 175°. The flow rates of N_2 and H_2 were both 40 ml./min. on the pretreated SE-30 column (1.5% SE-30 on Chromosorb W; 4 mm. × 225 cm.). The pretreatment was performed by injection of about 50 ml. of hexamethyldisilazane into the column maintained at 150° with a slow N_2 flow rate (20 ml./min.) during 6 hr.[9]

To each sample of secobarbital and its derivatives excess of CH_2N_2 was added and the mixture was allowed to stand in a refrigerator for 16 hr. After removal of CH_2N_2 the residue was dried under reduced pressure and dissolved in acetone. One % solution of these methylated barbiturate in acetone (0.5~10 μl.) was introduced with a Hamilton microsyringe. The retension time is shown in Table I.

Table I. Relative Retention Time of Methylated Products of Secobarbital and Its Derivatives

Barbiturate	Relative retention time	Barbiturate	Relative retention time
Secobarbital	1.00[a)]	ω-Hydroxysecobarbital	2.81
(ω-1)-Oxosecobarbital	1.71	ω-Carboxysecobarbital	2.96
(ω-1)-Hydroxysecobarbital	1.94		

a) Retention time of methylated secobarbital is 2.8 min. The column (1.5% SE-30 on Chromosorb W) was treated with hexamethyldisilazane Carrier gas : nitrogen, 40 ml./min. Temp. of injection port, column, and detector : 220°, 175°, and 190°, respectively.

The peak areas of the gas chromatogram of the methylated barbiturates were copied on an aluminum foil, cut out, and weighed accurately. Relationship between weight of the pieces of aluminum foil and amount of barbiturates was in a linear quantitative curve.

Preparation and Fractionation of Liver Homogenate

Animals used were male albino rabbits weighing about 3 kg. and preparation of the liver fractions was carried out below 5°. The supernatant fraction was obtained by centrifugation at 9000 *g* of the liver homogenate which was prepared in 2 volumes of 0.1*M* phosphate buffer containing 37.5 m*M* of nicotinamide and 18.75 m*M* of $MgCl_2$. The 9000 *g* supernatant fraction containing microsomes and the soluble fraction was centrifuged at 105000 *g* for 1 hr. Microsomes were separated, washed with the phosphate buffer, and recentrifuged at 105000 *g* for 1 hr. The washed microsomes were resuspended in the phosphate buffer equivalent to the volume of the original homogenate.

Enzymatic Assay

Secobarbital, (ω-1)-hydroxysecobarbital, or ω-hydroxysecobarbital was incubated with various preparations of liver in 10 ml. glass centrifuge tube at 37° for 1 hr. In most studies of enzymatic oxidation of barbiturates, one tube contained 0.3 ml. of enzyme preparation, 0.1 ml. of substrate solution (0.25 μ moles), and 0.4 ml. of 0.1*M* phosphate buffer containing 37.5m*M* of nicotinamide and 18.75 m*M* of $MgCl_2$ to a final volume of 0.8 ml. After the incubation, 0.1 g. of NaCl, 0.1 ml. of 2*N* HCl, and 7 ml. of AcOEt were added immediately to the incubation mixture. Then the tube was stoppered, shaken mechanically for 30 min., and centrifuged. A 6-ml. aliquot from the AcOEt layer was transferred into a fresh tube and washed with 0.5 ml. of water by shaking for 5 min. and centrifuged. A 5-ml. portion of the AcOEt layer was transferred into another tube, to which 0.05 g. of activated charcoal and 0.5 g. of Na_2SO_4 were added. The mixture was allowed to stand for 1 hr. and 4 ml. of AcOEt layer was pipetted out after centrifugation. The solvent was evaporated from this extract. Excess of CH_2N_2 was added to this residue and the mixture was allowed to stand in a refrigerator for 16 hr. After evaporation of CH_2N_2 the residue was further dried under a reduced

*3 Shimadzu GC-1B with hydrogen flame ionization detector was used.

9) E. C. Horning, *et al.* : "Method of Biochemical Analysis," **11**, 69 (1963), Interscience Publishers, Inc., New York-London.

pressure, dissolved in 0.05 ml. of acetone, and gas chromatographed by the method mentioned above. For the requirement of the base line of gas chromatography, the blank test was carried out in parallel. The recovery by this procedure was 84.7% for secobarbital, 85.4% for (ω-1)-oxosecobarbital, 84.9% for (ω-1)-hydroxysecobarbital, 80.1% for ω-hydroxysecobarbital, and 79.5% for ω-carboxysecobarbital.

Activity of Alcohol Dehydrogenase

The activity of alcohol dehydrogenase was measured by the procedure described in "Method in Enzymology."[10] One-tenth ml. of 96% EtOH, 3 ml. of 0.1*M* glycine buffer (pH 9.6), and 1 mg. of NAD^+ were mixed in a quarz cell, about 6 μg. of the enzyme preparation was added, and absorbance of NADH was measured at 340 mμ with intervals of 3, 5, 10, and 20 min.

Activity of 3-Hydroxymethylhexabital Dehydrogenase

Purification of the enzyme and estimation of the activity were carried out according to the methods described in the previous paper.[11]

Examination of Urine of the Mice Administered ω-Hydroxysecobarbital

ω-Hydroxysecobarbital was injected intraperitoneally to mice weighing about 17 g., in a dose of 125 mg./kg., but no pharmacological effect was observed, although injection of 25 mg./kg. of secobarbital showed a marked hypnotic activity. The urine (3.2 ml.) was collected for 24 hr. after the medication into a bottle containing a few drops of dil H_2SO_4 (5.78 mg. of ω-hydroxysecobarbital was administered to mice in total.). To the combined urine was added 0.5 g. of NaCl and it was extracted with 50 ml. of AcOEt. After washing with small amounts of NaCl-saturated water and drying over Na_2SO_4, the AcOEt layer was evaporated under a reduced pressure. The urine extract was then examined by gas chromatography.

Results

Intracellular Localization of Enzyme Activity

The enzyme activity of various preparations of the liver was determined by gas chromatographic analysis and representative data are shown in Table II.

Table II. Intracellular Distribution of Enzyme Activity of Secobarbital Metabolism[a]

Metabolite / Fraction	Unchanged yield		(ω-1)-OH yield		(ω-1)-Oxo yield		ω-COOH yield		Total yield
	(γ)	(%)	(γ)	(%)	(γ)	(%)	(γ)	(%)	(%)
Homogenate	28.1	47.1	12.4	19.5	2.2	3.4	14.7	22.0	92.0
Supernatant	29.0	48.9	13.0	20.5	2.3	3.6	15.2	22.8	95.8
Soluble fraction	57.0	95.7	2.4	3.8	—	—	—	—	99.5
Microsomes	57.9	97.2	1.8	2.8	—	—	—	—	100.0
Soluble fraction + microsomes	30.9	51.8	12.9	20.3	1.9	3.0	14.5	21.7	96.8

a) Various cell fractions equivalent to 0.1 g. of rabbit liver were incubated with 0.25 μmole of secobarbital as described in the text.

Almost the same activity as that observed in the whole homogenate was found to remain in the 9000*g* supernatant fraction of the liver. The supernatant fraction was separated into microsomes and the soluble fraction of the cell by centrifugation at 105,000*g*. Neither of these fractions alone demonstrated appreciable activity but when added together, activity was markedly restored.

Unchanged secobarbital and the three metabolites, (ω-1)-oxosecobarbital, (ω-1)-hydroxysecobarbital, and ω-carboxysecobarbital were identified with the authentic samples by gas chromatography. The homogenate experiment was also checked by paper chromatography,[8] in which formation of (ω-1)-hydroxysecobarbital and ω-carboxysecobarbital was clearly indicated.

10) R. K. Bonnichsen, N. G. Brink: "Method in Enzymology," **1**, 495 (1955), Academic Press, New York.
11) K. Toki, H. Tsukamoto: J. Biochem. (Tokyo), **55**, 142 (1964).

Factors Affecting Secobarbital Metabolism in the Supernatant Fraction

By addition of NADPH to the supernatant fraction, the formation of (ω-1)-hydroxysecobarbital and ω-carboxysecobarbital considerably increased and was inhibited by SKF 525-A, a common inhibitor of drug metabolizing enzymes in liver microsomes. No activity was observed under anaerobic condition. These results are summarized in Table Ⅲ.

Table Ⅲ. Effect of Pyridine Nucleotides and Inhibitors on Secobarbital Metabolism with 9000 *g* Supernatant Fraction

Addition \ Metabolite	Unchanged yield (γ)	Unchanged yield (%)	(ω-1)-OH yield (γ)	(ω-1)-OH yield (%)	(ω-1)-Oxo yield (γ)	(ω-1)-Oxo yield (%)	ω-COOH yield (γ)	ω-COOH yield (%)	Total yield (%)
Control	29.0	48.9	13.0	20.5	2.3	3.6	15.2	22.8	95.8
in Nitrogen	55.1	92.5	2.0	3.1	—	—	2.0	3.0	98.6
+SKF-525A ($1\times10^{-3}M$)	42.5	71.4	8.2	12.9	0.8	1.2	8.3	12.4	97.9
+Cl-Promazine-HCl ($1\times10^{-3}M$)	37.0	62.2	9.2	14.5	1.8	2.8	11.5	17.2	96.7
+NADPH (1 μmole)[a]	8.9	15.0	24.0	38.0	3.6	5.6	23.5	35.2	93.8
+NADH (1 μmole)[a]	29.5	49.6	14.0	21.6	2.5	4.0	15.0	22.5	97.7
+$NADP^+$ (1 μmole)[a]	24.0	40.3	16.6	26.2	2.5	4.0	17.5	26.1	96.6
+NAD^+ (1μmole)[a]	30.0	50.4	13.2	20.8	2.4	3.8	15.0	22.5	97.5

Supernatant fraction equivalent to 0.1 g. liver was incubated with 0.25 μmole of secobarbital as described in the text.
a) Co-factors (0.25 μmole) added in five-minute intervals.

Hydroxylation of Secobarbital by Microsomal Enzyme System

As shown in Table Ⅳ, secobarbital was metabolized to (ω-1)-hydroxysecobarbital by microsomes with NADPH in aerobic condition. However, simultaneous formation of ω-hydroxysecobarbital which was considered as an intermediate in ω-oxidation of secobarbital, was not observed at the same time.

Table Ⅳ. Requirement for Co-factors in Secobarbital Metabolism by Microsomes

Addition \ Metabolite	Unchanged yield (γ)	Unchanged yield (%)	(ω-1)-OH yield (γ)	(ω-1)-OH yield (%)	ω-OH yield (γ)	ω-OH yield (%)	Total yield (%)
Control	57.9	97.2	1.8	2.8	—	—	100.0
+NADPH (1 μmole)[a]	50.5	84.4	10.2	16.1	—	—	100.9
+NADH (1 μmole)[a]	57.5	97.2	1.6	2.5	—	—	99.7
+NADH (1 μmole)[a]+$FeSO_4$ (1 μmole)	58.1	97.5	1.6	2.5	—	—	100.0
+NADPH (1 μmole)[a]+Glutathione(3 μmole)	49.0	82.3	9.4	14.9	—	—	97.2
+NADPH (1 μmole)+Cysteine (3 μmole)	49.8	83.7	10.0	15.8	—	—	99.5
+NADPH (1 μmole)[a]+Glutathione(1 μmole) +Ascorbic acid (1 μmole)	47.0	79.6	9.4	14.8	—	—	100.0

Microsomes equivalent to 0.1 g. liver were incubated with 0.25 μmole of secobarbital as described in the text.
a) Co-factors (0.2 μmole) were added in five-minute intervals.

Formation of ω-Carboxysecobarbital from ω-Hydroxysecobarbital

The urine extract of mice administered ω-hydroxysecobarbital was examined by gas chromatography and about 65% of injected ω-hydroxysecobarbital was found to have been converted into ω-carboxysecobarbital as the only metabolite.

The *in vitro* experimental results are shown in Table V.

Table V. Requirement for Co-factors[a] on Activity of ω-Hydroxysecobarbital Metabolism by Dialyzed Soluble Fraction

Fraction \ Metabolite	Unchanged yield (γ)	Unchanged yield (%)	ω-COOH yield (γ)	ω-COOH yield (%)	Total yield (%)
Soluble fraction	—	—	132.9	99.7	99.7
Dialyzed soluble fraction	122.1	96.0	3.7	2.8	98.8
Dialyzed soluble fraction + NAD^+ (4 μmole)[a]	—	—	134.7	101.0	101.0
Dialyzed soluble fraction + $NADP^+$ (4 μmole)[a]	—	—	133.1	100.8	100.8

Soluble fraction equivalent to 0.1 g. liver was incubated with 0.5 μmole of ω-hydroxysecobarbital as described in the text.
a) Co-factors (0.8 μmole) were added in five-minute intervals.

In the 105,000*g* soluble fraction, ω-hydroxysecobarbital was completely oxidized into ω-carboxysecobarbital. When the soluble fraction was dialyzed against water for 24 hr. at 0°, its activity was markedly decreased. However, the activity was restored by the addition of NAD or NADP.

Formation of (ω-1)-Oxosecobarbital from (ω-1)-Hydroxysecobarbital in the Soluble Fraction

One-half micromoles of (ω-1)-hydroxysecobarbital was incubated with 105,000*g* soluble fraction. Only a portion of (ω-1)-hydroxysecobarbital was oxidized to (ω-1)-oxosecobarbital in the soluble fraction. When (ω-1)-hydroxysecobarbital was incubated with the dialyzed soluble fraction, oxidative activity of this alcohol never decreased. Moreover, the addition of NAD or NADP to the dialyzed soluble fraction exhibited no effect for the oxidation of (ω-1)-hydroxysecobarbital.

Table VI. Requirement for Co-factors on Activity of (ω-1)-Hydroxysecobarbital Metabolism by Dialyzed Soluble Fraction

Fraction \ Metabolite	Unchanged yield (γ)	Unchanged yield (%)	(ω-1)-Oxo yield (γ)	(ω-1)-Oxo yield (%)	Total yield (%)
Soluble fraction	114.1	90.5	8.0	6.9	97.4
Dialyzed soluble fraction	119.3	93.8	6.1	4.7	98.5
Dialyzed soluble fraction + NAD^+ (4 μmole)[a]	117.4	92.3	7.1	5.5	97.8
Dialyzed soluble fraction + $NADP^+$ (4 μmole)[a]	118.2	93.0	6.8	5.2	98.2

Soluble fraction equivalent to 0.1 g. liver was incubated with 0.5 μmole of (ω-1)-hydroxysecobarbital as described in the t ext.
a) Co-factors (0.8 μmole) were added in five-minute intervals.

Dehydrogenation of ω-Hydroxysecobarbital and (ω-1)-Hydroxysecobarbital by Horse Liver Alcohol Dehydrogenase

Using the assay method described in the Experimental part, it was confirmed that ω-hydroxysecobarbital and (ω-1)-hydroxysecobarbital were not dehydrogenated by horse liver alcohol dehydrogenase.

Dehydrogenation of ω-Hydroxysecobarbital by 3-Hydroxymethylhexabital Dehydrogenase

The result of a DEAE-cellulose column chromatography showed that 3-hydroxymethylhexabital dehydrogenase could be separated from the fraction which possessed

Table VII. Substrate Specificity of Alcohol Dehydrogenase

Time (min.)	Absorbance at 340 mμ × 10^3 (25°)				
	3	5	10	15	20
Ethanol	25	64	115	156	197
ω-Hydroxysecobarbital	67	59	57	57	52
(ω-1)-Hydroxysecobarbital	79	75	72	66	61

To a mixture of 3 ml. of 0.1*M* glycine buffer (pH 9.6), 0.1 ml. of the NAD^+ solution (1 mg.) and 0.1 ml. of ethanol or 0.1 ml. of barbiturate solution (3 μmoles), 6 μg. of enzyme was added.

the activity of dehydrogenating ethanol and ω-hydroxysecobarbital. Dehydrogenation of neither (ω-1)-hydroxysecobarbital,[6] nor ω-hydroxysecobarbital was catalyzed by 3-hydroxymethylhexabital dehydrogenase.

Table VIII. Activity of Chromatographic Fraction on DEAE-cellulose Column

Specific Activity (U × 10^3)[a] / Fraction	Ethanol	ω-OH-Secobarbital	3-OH-MHB
Soluble fraction	90.0	44.9	35.2
0.005*M* Phosphate buffer fraction	236.7	204.1	5.6
0.05*M* Phosphate buffer fraction	—	—	64.4

The incubation mixture consisted of 1.5 μmoles of NAD, 0.5 μmole of barbiturates, or 0.1 ml. of 50% EtOH, 0.1*M* glycine buffer (pH 9.6), and a suitable quantity of enzyme in a final volume of 1.5 ml.
a) One unit (U) is the amount of enzyme which forms 1 μmole of NADH per minute at 25°.

Discussion

By incubation with supernatant fraction of a liver homogenate, secobarbital formed mainly two products, (ω-1)-hydroxysecobarbital and ω-carboxysecobarbital, in about equal amounts and (ω-1)-oxosecobarbital in only a small amount. Addition of NADPH to the above system increased the formation of all of three metabolites. This observation suggested that NADPH might play an important role in both ω- and (ω-1)-oxidation. In fact, the formation of (ω-1)-hydroxysecobarbital was found to be catalyzed by microsomal enzymes in the presence of NADPH and oxygen, so called drug metabolizing enzyme systems.[12,13] However, the same microsomal fraction could not catalyze the oxidation of secobarbital to either ω-hydroxy- or ω-carboxysecobarbital in the presence of NADPH and oxygen.

The presumption that the enzyme activity for ω-hydroxylation might be lost during the procedure for isolation of microsomes was ruled out, since the microsomes, without the activity of ω-oxidation under the condition described above, restored the full activity with addition of the soluble fraction. The soluble fraction alone could not catalyze ω-hydroxylation of secobarbital, although it had a strong dehydrogenase activity converting ω-hydroxysecobarbital, which was synthesized chemically, into ω-carboxysecobarbital.

These facts strongly indicated that microsomal fraction could catalyze not only (ω-1)-hydroxylation but also ω-hydroxylation, but some other factors in the soluble

12) B. B. Brodie, J. Axelrod, *et al.* : Science, **121**, 603 (1955).
13) B. B. Brodie, J. R. Gillette, B. N. LaDu : Ann. Rev. Biochem., **27**, 427 (1958).

fraction besides NADPH should be necessary for the latter reaction. Several biological reducing agents such as glutathione, cysteine, and ascorbic acid were examined to see whether one or two of them are concerned with this enzyme system but none of these agents activated ω-hydroxylation of the methylbutyl side-chain.

Cooper and Brodie[3] reported that pentobarbital, possessing an ethyl side-chain instead of the allyl group in secobarbital, was metabolized to ω-carboxylic acid and (ω-1)-alcohol in rabbit liver supernatant fraction, and assumed that the same microsomal enzyme system catalyzed both ω- and (ω-1)-oxidation. However, this assumption does not seem quite correct since in the study with microsomal enzyme systems they did not detect the formation of these metabolites, but only estimated it by the disappearance of pentobarbital from the incubation mixture.

ω-Hydroxysecobarbital was easily oxidized into ω-carboxysecobarbital in the soluble fraction of liver, and this enzyme system requires NAD or NADP as the cofactor. (ω-1)-Hydroxysecobarbital was also oxidized into (ω-1)-oxosecobarbital in the soluble fraction but enzyme activity for this dehydrogenation was very low.

It has been shown by Gillette[14] that alcohol dehydrogenase in the soluble fraction catalyzed the oxidation of *p*-nitrobenzyl alcohol. He suggested that this enzyme might be concerned with the oxidation of hydroxylated metabolites of foreign compounds, but in this experiment, neither (ω-1)-hydroxysecobarbital nor ω-hydroxysecobarbital did act as a substrate for alcohol dehydrogenase of horse liver.

Previous study in this laboratory[11] showed that 3-hydroxymethylhexabital dehydrogenase could be separated from alcohol dehydrogenase by the chromatographic fractionation of the soluble fraction of rabbit liver on DEAE-cellulose column, and this enzyme did not dehydrogenate (ω-1)-hydroxysecobarbital. ω-Hydroxysecobarbital synthesized in the present work was also found not to be metabolized by this enzyme. From the consideration of these results, it seems reasonable to conclude that there should be some type of enzymes for the dehydrogenation of hydroxylated metabolites of foreign compounds in the soluble fraction of liver cells.

On the basis of these data, the possible metabolic pathways of secobarbital in rabbit liver would be summarized as shown in Chart 2.

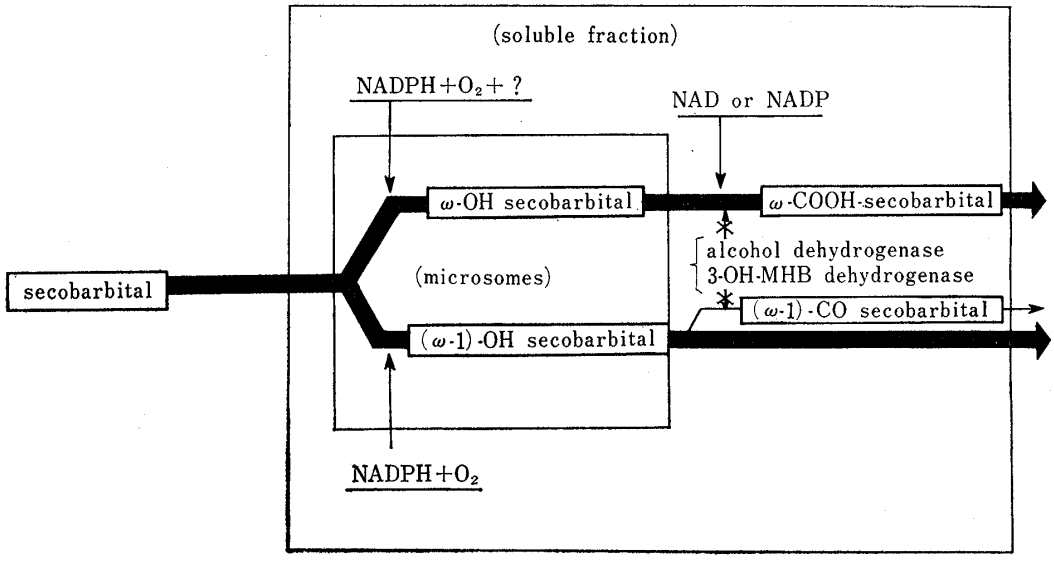

Chart 2. Possible Metabolic Pathways of Secobarbital in Rabbit Liver

14) J. R. Gillette : J. Biol. Chem., **234**, 139 (1959).

Waddell[15,16] reported recently that the allyl side chain of secobarbital was also oxidized in mammalian bodies. In our *in vivo*[5] and *in vitro* experiments it was found that the allyl group remained intact in all of the metabolites.

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15) W. J. Waddell : Federation Proc., **22**, 480 (1963).
16) *Idem* : J. Pharmacol. Exptl. Therap., **149**, 23 (1965).