Communications to the Editor

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Enzymatic Oxidation of Methylhexabital

In the enzymatic study of methylhexabital (5-cyclohexenyl-3,5-dimethylbarbituric acid) (MHB), Cooper and Brodie[1] repotred that the enzyme system in the rabbit liver which oxidized cyclohexenyl in MHB to yield keto-MHB-I was located in the microsomes of liver cells and required both oxygen and reduced triphosphopyridine nucleotide. However, further details of oxidation mechanism have not been elucidated as yet. Under a similar experimental condition, Tochino[2] observed that hydroxylation of cyclohexenyl ring occurred and hydroxy-MHB was produced. In both cases, unchanged MHB was extracted and assayed by the method of Brodie, *et al.*[3] and enzyme activity was determined by the value of disappeared MHB.

Previous work from this laboratory[4,5] showed that two diastereoisomeric α- and β-3-OH-MHB (α- and β-5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid), 3-keto-MHB (5-(3-oxo-1-cyclohexenyl)-3,5-dimethylbarbituric acid), and three additional products were excreted in the urine of rabbit administered MHB, and that 3-OH-MHB and 3-keto-MHB were interconvertible *in vivo*.[6]

Table I. Cellular Localization of Enzyme Activity metabolizing MHB, α- and β-3-OH-MHB, and 3-keto-MHB

Substrate	MHB			α-3-OH-MHB		β-3-OH-MHB		3-keto-MHB	
Metabolite	MHB	3-OH-MHB*	3-keto-MHB	3-OH-MHB*	3-keto-MHB	3-OH-MHB*	3-keto-MHB	3-keto-MHB	3-OH-MHB*
Cell fractions	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Whole homogenate	20.4	16.3	36.2	31.6	44.4	34.2	46.9	54.5	16.5
Nuclei	59.2	12.6	19.5	39.3	50.5	26.6	55.5	75.1	10.3
Supernatant fraction (600*g*, 10 min.)	12.9	22.9	47.4	54.5	28.5	56.1	30.6	64.2	4.0
Mitochondria	95.2	1.8	0.8	80.5	4.9	84.7	5.8	104.0	3.4
Supernatant fraction (9,000*g*, 20 min.)	11.4	25.6	39.8	55.1	21.6	59.2	26.1	72.0	6.9
Microsomes	101.2	1.8	0.0	84.0	4.4	89.0	4.9	86.0	2.6
Soluble fraction (80,730*g*, 60 min.)	89.0	3.3	3.3	48.5	35.6	59.5	37.0	39.5	30.0
Microsomes + soluble fraction	14.5	26.1	44.7	69.5	22.5	65.5	26.9	74.0	7.9

* The two diastereoisomeric α- and β-3-OH-MHB have the same Rf values and cannot be distinguished by the chromatographic procedure used.

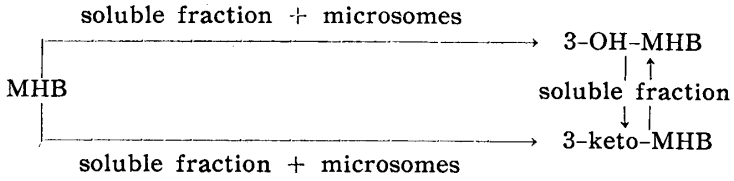
Each fraction, equivalent to 2.66 g. of the liver, prepared in 8 cc. of 0.1*M* phosphate buffer (pH 7.4). Mitochondria and microsome fraction were washed with homogenization medium and recentrifuged.

Condition : One incubation flask contained 4 cc. of cell fraction, 100 μmoles of nicotinamide, 50 μmoles of $MgCl_2$, 3 μmoles of substrate, and 0.1*M* phosphate buffer (pH 7.4) to a final volume of 8 cc. Incubation was carried out for 2 hr. at 37°. The data shown above were obtained from a combination of two incubation mixtures which were incubated under the same condition.

1) J. R. Cooper, B. B. Brodie : J. Pharmacol. Exptl. Therap., **114**, 409(1955).
2) Y. Tochino : Wakayama Med. Repts., **7**, 150(1956).
3) B. B. Brodie, J. J. Burns, L. C. Mark, P. A. Lief, E. Bernstein, E. M. Papper : J. Pharmacol. Exptl. Therap., **109**, 26(1953).
4) H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, **4**, 368(1956).
5) H. Yoshimura : *Ibid.*, **5**, 561(1957).
6) H. Tsukamoto, S. Toki, K. Kaneda : *Ibid.*, **7**, 651(1959).

Present study was undertaken in order to clarify the MHB oxidation mechanism and metabolic pathway *in vitro*. MHB and synthetic α- and β-3-OH-MHB and 3-keto-MHB were incubated individually with rabbit liver homogenate and its cell fractions prepared by differential centrifugation. The method of identification and determination of each product was similar to that reported previously.[6~8] After extraction of the incubation mixture with ethyl acetate under acid condition (pH 6), each metabolite was separated by buffered paper chromatography and estimated spectrophotometrically.

Cellular localization of enzyme activity is shown in Table I. The activity of metabolizing MHB to 3-keto-MHB and 3-OH-MHB was found to remain in the supernatant fraction (9,000*g*) which contains microsomes and soluble fraction of the cells. Neither microsomal nor soluble fraction alone showed considerable activity but it was restored when recombined together. Both α- and β-3-OH-MHB were oxidized to 3-keto-MHB in every fraction. These fractions showed that the α-isomer was metabolized to about the same extent as the β-isomer. When 3-keto-MHB was incubated with the soluble fraction alone, the activity of reduction to 3-OH-MHB was markedly increased and it was diminished by the addition of microsomes to this fraction.



Accordingly, it will be considered that 3-OH-MHB and 3-keto-MHB are interconvertible in the soluble fraction but microsomes interfere to some extent in the reduction of a ketone to hydroxyl group by soluble fraction. In connection with these results, 3-OH-MHB from MHB in the supernatant fraction (600*g* and 9,000*g*) might have formed directly from MHB and not via 3-keto-MHB. It is still not clear whether another MHB metabolite, 3-keto-MHB, is derived via 3-OH-MHB or from MHB directly, or from either.

Further details of these experiments will be reported in the near future.

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7) H. Tsukamoto, H. Yoshimura, S. Toki : *Ibid.*, **6**, 15(1958).
8) *Idem* : *Ibid.*, **6**, 88(1958).