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Enzymatic Hydrolysis of N-(α-Methylbenzyl)linoleamide[1]

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It has been reported that N-(α-methylbenzyl)linoleamide (MBLA) had a remarkable cholesterol-lowering effect on the experimental hypercholesterolemia in rabbits and rats.[3]

There are three optical isomers of MBLA: D-, L- and DL-MBLA because of an asymmetric carbon atom in the amine moiety of MBLA. And the potency of the cholesterol-lowering effect was found to be the following order: D->DL->L-MBLA.[4]

Fukushima[5] also found that the respiratory excretion of ^{14}C after oral administration of carboxy ^{14}C-labeled L-MBLA to rats was significantly greater than that of D- or DL-MBLA, whereas the ratio of urinary ^{14}C excretion of D-MBLA was higher than that of L- or DL-MBLA. These results suggest that the difference of the cholesterol-lowering potency observed among the MBLA isomers might be closely related with the difference of biological disposition of the isomers.

The present paper describes the results of *in vitro* studies on the enzymatic hydrolysis of the amide linkage of MBLA isomers.

Method and Material

Male Wistar rats weighing about 200 g were used.

Tissue homogenates were prepared with 4 volumes of 0.15 M phosphate buffer, pH 7.4, or of distilled water in a teflon-glass homogenizer. A crude enzyme solution was obtained as supernatant solution of tissue homogenates after centrifugation at $9000 \times g$ for 10 minutes at 4°. Optical isomers of ^{14}C-labeled MBLA (linoleic-1-^{14}C) with a specific radioactivity of 200 mCi/M were synthesized in this laboratory. For the *in vitro* experiment, ^{14}C-labeled MBLA was suspended in 0.15 M phosphate buffer, pH 7.4, containing 6 mM sodium taurocholate. Unless otherwise stated, the substrate concentration was 40.7 μM.

The reaction mixture was incubated at 37.5° in air phase for various time intervals. Enzyme activity was determined by measuring ^{14}C radioactivity of the linoleic acid spot after separating the chloroform–methanol (2:1, v/v) extract of the reaction mixture on Silica gel G plate (Kieselgel nach Stahl, E. Merck AG), which was prepared to 0.25 mm thickness and activated for 1 hour at 110°. The solvent system used for the thin-layer chromatographic separation of the extract was a mixture of petroleum ether–diethyl ether–acetic acid (85:15:3, by volumes).

After detection of radioactive spots with a radio thin-layer chromatogram scanner (Aloka, TRM-1), the spots were scraped into glass vials. Radioactivity was determined by a Beckman LS-133 liquid scintillation counter with counting efficiency of about 70 to 85% by the external channel ratio method.

Result and Discussion

The amide linkage of DL-MBLA was hydrolyzed by about 10% with 5% rat liver homogenates while practically no hydrolysis was observed with boiled homogenates under the conditions described in Table I.

Preliminary experiment with the simulated intestinal and stomach fluid (USP XVIII) showed no cleavage of the amide linkage, and the amide linkage was not hydrolyzed in acidic

1) This work was presented at the 90th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, July, 1970.
2) Location: *Takatsukasa 4-2-1, Takarazuka, Hyogo.*
3) H. Fukushima, K. Toki, and H. Nakatani, *J. Atheroscler. Res.*, **9**, 57 (1969); H. Fukushima, S. Aono, and H. Nakatani, *J. Nutr.*, **96**, 15 (1968).
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Table I. Enzymatic Hydrolysis of the Amide Linkage of DL-MBLA

Enzyme solution[a]	Hydrolysis (%)[b]
5% liver homogenate	10.0
Boiled liver homogenate	0.4
5% duodenum homogenate	1.6

a) Two ml of 9000×*g* supernatant fraction of rat tissue homogenate in 0.2M phosphate buffer, pH 7.4, was incubated with 2 ml of the buffer containing 0.1 μ mole of ^{14}C-labeled DL-MBLA for 5 hours at 37.5°.

b) The rate of hydrolysis was expressed as percentage ratio of radioactivity of linoleic acid spot to total radioactivity on thin-layer chromatogram.

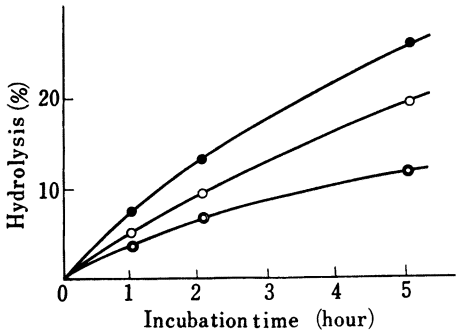

Fig. 1. Enzymatic Hydrolysis of D-, DL- and L-MBLA

The reaction mixture consisted of 0.5 ml of 9000×*g* supernatant fraction of rat liver homogenate and 0.5 ml of 0.1M phosphate buffer, pH 7.4, containing 0.15 μ mole of D-, DL- or L-MBLA and 3 μ mole of sodium taurocholate.

—●—: L-MBLA —○—: DL-MBLA
—◎—: D-MBLA

or alkaline medium, either.

Figure 1 shows that the rate of enzymatic hydrolysis of the amide linkage was markedly different in the order of L-, DL- and D-MBLA.

Since the order of biological potency was reverse to the order of hydrolysis, it is reasonable to assume that the cholesterol-lowering potency of the MBLA isomers is depending on the difference of metabolic rate and fate of the isomers.

The enzyme activity was also found in the small intestine and kidneys, but not so much greater than that in the liver (Table II). The optimal pH for the enzyme reaction was found to be 9.2 with 0.05 M borate buffer (Fig. 2).

Table II. Distribution of the Enzyme Activity

Tissue	Enzyme activity (mμ moles/ mg protein)[a]
Liver	0.307
Kidneys	0.139
Intestine	0.077
Spleen	0.004
Plasma	0.001

a) The activity is expressed as mμ moles of linoleic acid formed in 2 hours incubation with DL-MBLA as the substrate. Protein concentration was determined by the method of Lowry, *et al.*[6] For the other procedure see Methods and Materials.

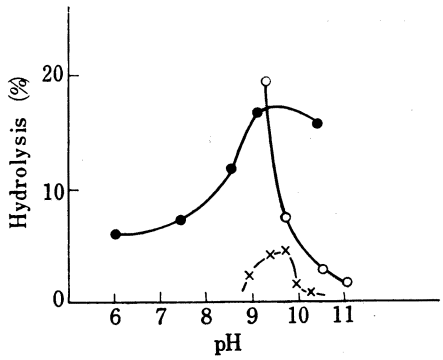

Fig. 2. pH-Activity Curves

Mixture of 0.5 ml of 9000×*g* supernatant fraction of rat liver homogenate and 0.5 ml of buffer solutions containing 40.7 mμ mole of DL-MBLA was incubated for 4 hour at 37.5°.

—●—: 0.1M phosphate buffer
—○—: 0.05M borate buffer
—×—: 0.05—0.1M bicarbonate buffer

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6) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).