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# Studies on Ergothioneine. XI.[1] Inhibitory Effect on Lipid Peroxide Formation in Mouse Liver

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Ergothioneine was examined *in vitro* for ability to inhibit lipid peroxide formation. Lipid peroxide formation in mouse liver homogenate was inhibited in a dose-related manner by 5—250 mM ergothioneine. Carnitine and histidine had no such effect. Even when lipid peroxide formation was enhanced by reduced nicotinamide adenine dinucleotide phosphate (NADPH) or ascorbic acid treatment, addition of 50 mM ergothioneine inhibited that formation by 50%. It was found that ergothioneine (1) remarkably enhanced glutathione peroxidase activity from liver cytosol, (2) increased the activity of mitochondrial Mn-superoxide dismutase, and (3) inhibited NADPH-cytochrome c reductase from rat liver microsomes. Thus, ergothioneine might be one of the heat-stable factors inhibiting lipid peroxide formation in the liver cytosol.

**Keywords**——ergothioneine; inhibition of lipid peroxide formation; glutathione peroxidase activity; glutathione reductase activity; Mn-superoxide dismutase activity; NADPH-cytochrome c reductase activity

The present series of studies has been conducted to clarify the physiological significance of ergothioneine(2-mercaptohistidine trimethylbetaine; Erg). Erg is taken up from the diet and contained in large amounts in the liver, being mostly distributed in the cytosol fraction.[2] The previous paper made clear that Erg plays a role in the recovery from ethionine-induced injury of the liver,[1] in which Erg is more abundant than in any other organ.[3] It was also found that liver injury induced by a single injection of ethionine was inhibited by preadministration of Erg for 7 d, that Erg reduced the lipid peroxide (LPO) formation in the liver induced by ethionine, and that the LPO level was significantly increased in the Erg-deficient rats, while it was low in Erg-excessive rats. It has been proposed that various chemicals, phenobarbital,[4] paraquat,[5] vinylidene chloride,[6] diethylmaleate,[6] ethionine[6] and phorone,[7] commonly cause cellular injury by inducing lipid peroxidation.[8] It is thus interesting that Erg inhibited LPO formation, and the present investigation was carried out to determine the mechanism by which Erg decreased LPO *in vitro*.

## Materials and Methods

**Chemicals**——L-Erg($2H_2O$), cytochrome c (beef heart, Type IV), glutathione (GSH), xanthine, GSH reductase (yeast, Type III) and xanthine oxidase (grade I, from buttermilk) were purchased from Sigma Chemicals Co. GSH peroxidase (bovine erythrocyte) was a gift of Toyobo Co., Ltd. and superoxide dismutase (SOD) was a gift of Toyo Jozo Co., Ltd. All other chemicals were of extra-pure analytical grade.

**Animals**——Male ICR mice (20—30 g) were used in most of the experiments; they were maintained at 21 ± 1°C, humidity 50—60%, light period 12 h/d. They were fed standard laboratory chow (CE-2; manufactured by Japan Clea Co., Ltd.), and water was given *ad libitum*. For the preparation of NADPH cytochrome c reductase, male Wistar rats (220—240 g) were maintained under the same conditions as the mice.

**Preparation of Hepatic Microsomes**——Mice were sacrificed under ether anesthesia. The livers were perfused with saline and homogenized with 0.1 M phosphate buffer (pH 7.4) to prepare 10 or 20% liver homogenates. Microsomes were obtained from 9000 *g* supernatant by centrifugation at 105000 *g* for 60 min. The microsome suspension was prepared so as to contain 170 mg equivalent of liver tissue/ml of reaction mixture.

**Determination of Lipid Peroxidation**——Lipid peroxidation was estimated by means of the thiobarbituirc acid (TBA) reaction as described by Tappel *et al.*[9] The reaction mixture (2 ml), containing 1 ml of 10% liver homogenate or 170 mg liver equivalent of microsomes in 0.1 M phosphate buffer (pH 7.4), was incubated at 37°C. After incubation, 3 ml of 10% trichloroacetic acid (TCA) solution was added. Two ml of the supernatant was heated with 3 ml of 0.75% TBA solution for 10 min in a boiling water bath. Lipid peroxidation was expressed in terms of the optical density at 532 nm.

**GSH Peroxidase and GSH Reductase Activities**——A cytosol fraction was prepared from 20% liver homogenate by centrifugation at 105000 *g* for 60 min. The resulting supernatant was used as the enzyme solution. It was diluted appropriately for determination of enzyme activities. The GSH peroxidase activity was estimated by the method of Lawrence *et al.*[10] The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $NaN_3$, 0.15 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 unit/ml GSH reductase, 1 mM GSH and 0.25 mM $H_2O_2$ in a total volume of 1 ml, including the enzyme source. The GSH reductase activity was estimated with oxidized GSH (GSSG) by measuring the oxidation of NADPH.[11]

**SOD Activity**——The mitochondria (9000 *g*, 30 min) and the cytosol (105000 *g*, 60 min) fractions were obtained after centrifugation of the 20% liver homogenate in 0.1 M phosphate buffer (pH 7.4) at 900 *g* × 10 min. The washed mitochondria was subjected to sonication, and to centrifugation. The resulting supernatant was used as mitochondrial enzyme solution. The enzyme activity was estimated by the method of McCord *et al.* using xanthine–xanthine oxidase, and expressed as percentage inhibition of the reduction of cytochrome c estimated at 550 nm.[12]

**NADPH-cytochrome c Reductase Activity**——The enzyme was prepared from rat liver microsomes by the method of Omura *et al.* with Sephadex G-10 and diethylaminoethyl (DEAE)-cellulose columns.[13] The enzyme activity was estimated in the presence of NADPH by measuring the reduction of cytochrome c at 550 nm, and expressed as the quantity per μmol of reduced cytochrome c per min per mg of protein.

## Results

### Inhibition of LPO Formation in Liver Homogenate

Fig. 1 shows the relationship between the concentration of Erg added to the reaction mixture and the amount of LPO formed. An inhibitory effect was exerted by Erg at concentrations above 5 mM. There was a dose-response relationship up to an Erg concentration 250 mM.

Cysteine and GSH, which are thiol compounds, exhibited a stronger inhibitory effect than Erg on LPO formation (Fig. 2), though histidine and carnitine produced some increase. The inhibitory effects of cysteine and GSH have already been demonstrated by Christophersen in

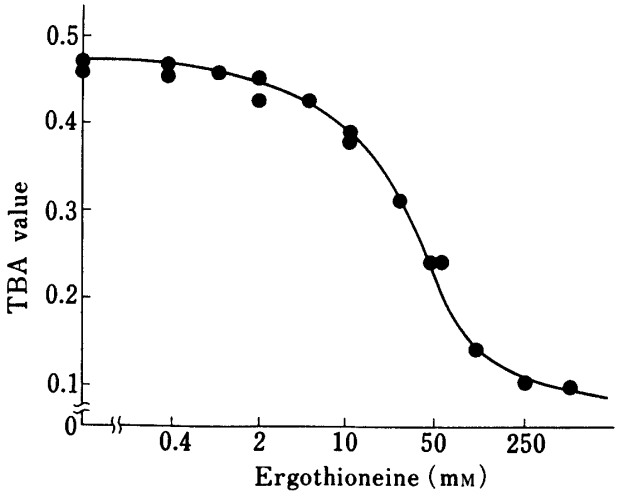



Fig. 1. Inhibitory Effect of Ergothioneine on Lipid Peroxide Formation in Mouse Liver Homogenate

The reaction mixture, containing 1 ml of 10% liver homogenate in 0.1 M phosphate buffer (pH 7.4) in a total volume of 2 ml, was incubated at 37°C for 60 min.

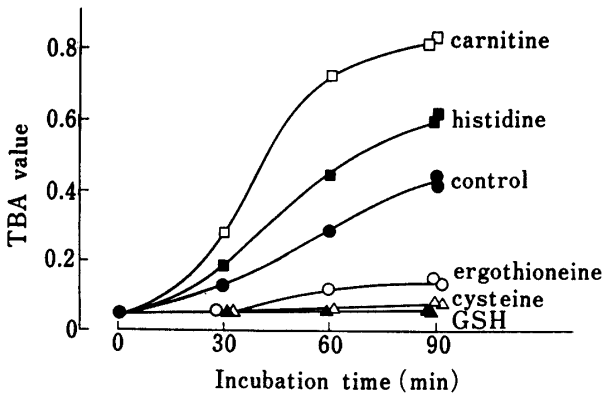



Fig. 2. Effect of Ergothioneine-related Compounds on Lipid Peroxide Formation in Mouse Liver Homogenate

The reaction mixture, containing 1 ml of 10% liver homogenate in 0.1 M phosphate buffer (pH 7.4) in a total volume of 2 ml, was incubated at 37°C with 50 mM of each compound.

rat microsomes and mitochondria.[14] In order to confirm that Erg has no effect on the colorimetric procedure in the TBA reaction, various concentraions of Erg were added to 4 or 8 nmol of malonedialdehyde. Erg exerted no influence at all on the color development at 532 mn.

**Inhibitory Effect of Erg on LPO Formation by NADPH and Ascorbic Acid**

Figures 3 and 4 show the inhibitory effect of addition of 50 mM Erg on LPO formation in the presence of NADPH or ascorbic acid. The lipid peroxidation of microsomes by NADPH proceeded linearly, but was clearly inhibited by Erg. Ascorbic acid stimulated LPO formation both with fresh microsomes and with heated microsomes. According to Wills, non-enzymatic lipid peroxidation can occur after the heat treatment of microsomes.[15] The inhibitory effect of Erg was seen only with fresh microsomes (more than 50% inhibition), and therefore Erg presumably acts on enzymatic systems.

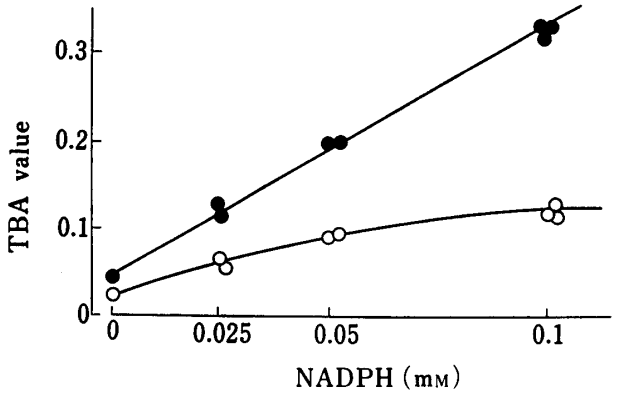



Fig. 3. Effect of Ergothioneine on Lipid Peroxide Formation in Mouse Microsomes in the Presence of NADPH

The reaction mixture, containing 1 ml of liver microsome suspension (equivalent to 170 mg of liver wet weight) in 0.15 M KCl, various concentrations of NADPH, and 25 mM phosphate buffer (pH 7.4) in a total volume of 2 ml, was incubated at 37°C for 30 min. ●——●, control; ○——○, addition of 50 mM ergothioneine.

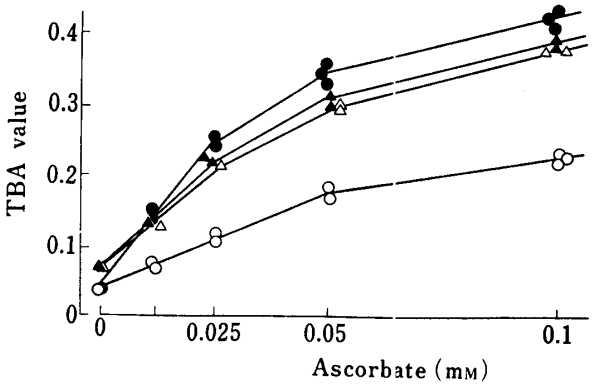



Fig. 4. Effect of Ergothioneine on Lipid Peroxide Formation in Mouse Microsomes in the Presence of Ascorbic Acid

The conditions of the standard reaction were the same as in Fig. 3 except for NADPH. ●——●, microsomes were fresh; ○——○, microsomes were fresh and 50 mM ergothioneine was added; ▲——▲, microsomes were heated in boiling water for 5 min; △——△, microsomes were heated and 50 mM ergothioneine was added.

**Effect of Erg on Enzymes related to LPO Formation in Liver**

We next investigated the effect of Erg on the enzymes related to LPO formation in liver, *i.e.*, SOD, GSH peroxidase and NADPH-cytochrome c reductase. As shown in Fig. 5, Erg markedly increased the GSH peroxidase activity in mouse liver. The maximum effect was seen at 12.5 mM Erg in the case of GSH reductase activity, which is linked to GSH peroxidase. Mitochondrial SOD activity was almost doubled by addition of 12.5 mM Erg (Fig. 6), but Erg had no influence on the activity of cytosolic Zn–Cu SOD. Erg appears to stimulate specifically the Mn SOD activity of mitochondria.

On the other hand, an increase in LPO formation after phenobarbital administration may be induced by an increase in NADPH-cytochrome c reductase activity, and the formation of active oxygen from molecular oxygen by this enzyme system is dependent on NADPH-cytochrome c reductase activity.[16] Thus, an experiment was conducted to clarify the effect of Erg upon this enzyme activity *in vitro*. Since the enzyme has been prepared mostly from rat, rabbit, pig or beef microsomes but not from mouse tissues, we extracted and partially purified it from rat liver according to the method of Omura *et al.*[13] Fig. 7 shows the inhibition of the enzyme by Erg. The data are not shown, but 10 mM GSH was needed to inhibit the activity by 50%, and when 10 mM cysteine was added, the inhibition failed to reach 50%. In contrast, 5 mM Erg inhibited the activity by 50%.

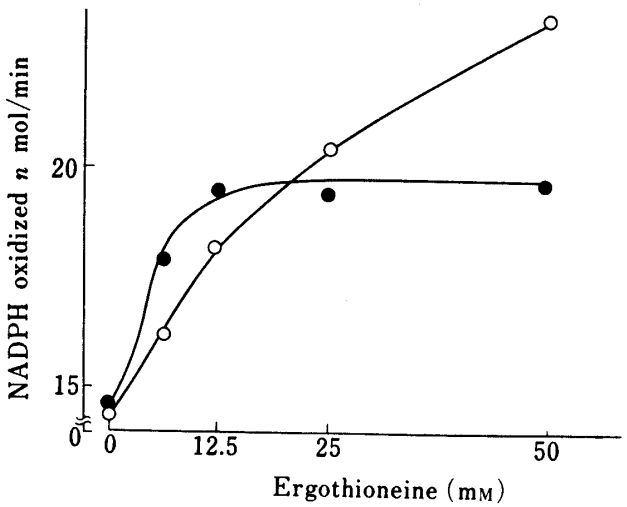



Fig. 5. Effect of Ergothioneine on GSH Peroxidase and GSH Reductase Activities in Mouse Liver Cytosol

The cytosol was prepared by centrifugation of 20% liver homogenate at 105000 *g* for 1 h. Various concentrations of ergothioneine were added to the enzyme assay mixture. GSH peroxidase and GSH reductase activities were determined with 100-fold-diluted cytosol. ○——○, GSH peroxidase; ●——●, GSH reductase.

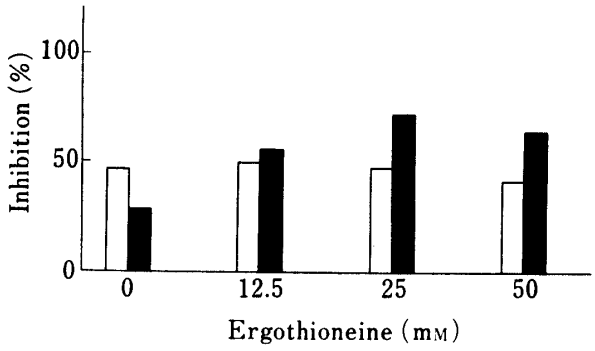



Fig. 6. Effect of Ergothioneine on Superoxide Dismutase Activity in Mouse Liver

Twenty % liver homogenate was incubated with various concentrations of ergothioneine at 37°C for 2h. The precipitate resulting from centrifugation at 9000 *g* for 30 min was suspended in 0.1 M phosphate buffer (pH 7.4). The cytosol was prepared by centrifugation at 105000 *g* for 60 min. Mitochondrial fraction equivalent to 200 μg of protein and cytosol equivalent to 48 μg of protein were assayed for superoxide dismutase activity by the xanthine-xanthine oxidase method. □, cytosol SOD; ■, mitochondrial SOD.

## Inhibitory Effect of Erg as a Cytosol Factor

Rat liver homogenate was used in this experiment so that the results would be comparable with other data. Sato *et al.* reported that a substance, obtained by heat treatment of a soluble fraction of rat liver, inhibited the lipid peroxidation of microsomes.[17] To see whether this substance might be Erg, various concentrations of Erg were heated in boiling water for 5 min before addition to the reaction mixture of lipid peroxidation. Heated Erg had the same inhibitory action as unheated Erg.

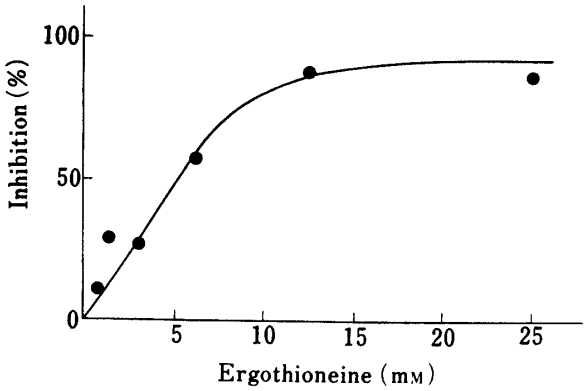



Fig. 7. Effect of Ergothioneine on NADPH-Cytochrome c Reductase Activity in Rat Liver

Various concentrations of ergothioneine were added to the enzyme assay reaction mixture. The data are given as inhibition (percent) by ergothioneine with respect to the control activity.

## Discussion

In the preceding part of the present series of studies, we found that one of the actions of Erg *in vivo* was to inhibit LPO formation in liver. Therefore, *in vitro* studies were done to clarify the mechanism of inhibition of LPO by Erg in the present work, .

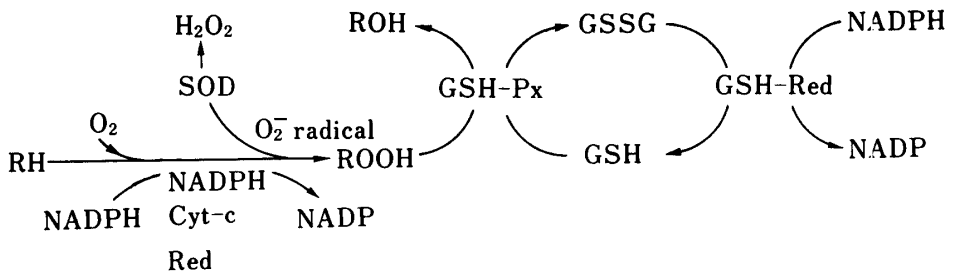
To inhibit lipid peroxidation by 50% in liver homogenate, it is necessary to add 50 mM Erg, which is much higher than the physiological concentration of this substance.[3] However, the ability of Erg to inhibit LPO formation was noticed even when LPO formation was stimulated by phenobarbital administration *in vivo* or by NADPH or ascorbic acid addition *in vitro*. There are two types of mechanism of LPO formation. One is a non-enzymatic formation

which is stimulated by ascorbic acid, $Fe^{2+}$ or adenosine diphosphate (ADP).[14,18,19] The other is an NADPH-dependent enzymatic reaction in microsomes.[20] Molecular oxygen is required for both types. In the living body, such enzymatic systems as SOD, GSH peroxidase and catalase are known to be involved in inhibiting the deposition of LPO. Christophersen[14] and Wills[15,21] suggested that LPO formation in microsomes by NADPH and ascorbic acid was inhibited by GSH through an enzymatic mechanism. Our results indicate that Erg inhibits an enzymatic process.

Recently attempts have been made to find inhibitory factors of LPO formation in the soluble fraction of the liver.[22] The thermostable inhibitor may be GSH exerting its effect *via* GSH reductase or GSH peroxidase.[17] As the liver contains a large amount of endogenous Erg (generally more than 1/10 of GSH in mol ratio), it is presumed that Erg may be involved in the effect of GSH. The results in Fig. 5 confirm that Erg is one of the factors contained in the soluble fraction possessing an inhibitory effect on LPO formation.

Since Erg is supposed to act on an enzymatic system, an experiment was designed to study the possible participation of NADPH-cytochrome c reductase as an enzyme of LPO formation, of GSH peroxidase as an enzyme degrading LPO formed, and of SOD actvity as an enzyme scavenging active oxygen. Erg inhibited NADPH-cytochrome c reductase activity remarkably, and stimulated GSH peroxidase activity and GSH reductase activity in the cytosol. Erg had no effect on Cu–Zn SOD, but did stimulate Mn SOD activity of mitochondria. It is known that Erg can chelate with metalloenzymes[23] and has affinity for metals in the following order: Cu>Hg>Zn>Cd>Co>Ni.[24] As Erg has a strong affinity for Cu–Zn, it may deactivate SOD. In general, LPO formation is inhibited by EDTA and chelating reagents.[19]

Based on the above data, we propose a working hypothesis for the mechanism by which Erg inhibits LPO formation (Chart 1). Erg both inhibits LPO formation and stimulates the decomposition and disappearance of LPO formed.




Erg. { increasing effect → { degradation system ·····GSH–Px; inhibition system ·····SOD } ; inhibitory effect → formation system ·····NADPH–Cyt-c-Red }

Chart 1. Enzymatic Depression Effect of Ergothioneine on Lipid Peroxide Formation

It is natural for GSH to exert an inhibitory effect on LPO formation, since GSH is a substrate for GSH peroxidase. Erg increases this enzyme activity. As mentioned above, it is a problem that rather high concentrations of Erg were required to exert the inhibitory effect. One of the reasons may be that we used the homogenate or cytosol, which includes stimulatory factors for LPO formation. It remains necessary to examine whether the inhibitory effect of Erg on LPO formation is truly enzymatic in a pure enzyme system, and to determined whether Erg is a direct enzyme stimulator, or whether the enzymatic activity is enhanced by the co-factor function of Erg, as well as GSH. Further work is also necessary to elucidate the scavenger mechanism of superoxide by Erg itself; this may well be a non-enzymatic process.

## References and Notes


1) Part X: H. Kawano, K. Cho, Y. Haruna, Y. Kawai, T. Mayumi and T. Hama, *Chem. Pharm. Bull.*, **31**, 1676 (1983).
2) H. Kawano, M. Otani, K. Takeyama, Y. Kawai, T. Mayumi and T. Hama, *Chem. Pharm. Bull.*, **30**, 1760 (1982).
3) T. Mayumi, H. Kawano, Y. Sakamoto, E. Suehisa, Y. Kawai and T. Hama, *Chem. Pharm. Bull.*, **26**, 3772 (1978).
4) K. Fukuzawa and M. Uchiyama, *J. Nutr. Sci. Vitaminol.*, **19**, 433 (1973); R. Nilsson, S. Orrenius and L. Ernster, *Biochem. Biophys. Res. Commun.*, **17**, 303 (1964).
5) H. Shu, R.E. Talcott, S.A. Rice and E.T. Weiz, *Biochem. Pharmacol.*, **28**, 327 (1979).
6) M. Younes and C.P. Siegers, *Res. Commun. Chem. Path. Pharmacol.*, **27**, 119 (1980).
7) M. Younes and C.P. Siegers, *Chem. Biol. Interaction*, **34**, 257 (1981).
8) A.L. Tappel, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **32**, 1870 (1973).
9) A.L. Tappel and H. Zalkin, *Arch. Biophys. Biochem.*, **80**, 326 (1959).
10) R.A. Lawrence and R.F. Burk, *Biochem. Biophys. Res. Commun.*, **71**, 952 (1976).
11) R.D. Mavis and E. Stellwagen, *J. Biol. Chem.*, **243**, 809 (1968).
12) J.M. McCord and I. Fridvich, *J. Biol. Chem.*, **244**, 6049 (1969).
13) T. Omura and S. Takesue, *J. Biochem.* (Tokyo), **67**, 249 (1970).
14) B.O. Christophersen, *Biochem. J.*, **106**, 515 (1968).
15) E.D. Wills, *Biochem. J.*, **113**, 315 (1969).
16) S.D. Aust, D.L. Roerig and T.C. Pederson, *Biochem. Biophys. Res. Commun.*, **47**, 1133 (1972).
17) M. Sato, Y. Amikura, S. Suzuki and T. Murata, *Yakugakuzasshi*, **98**, 1156 (1978).
18) R.C. McKnight, F.E. Hunter and W.H. Oshlert, *J. Biol. Chem.*, **240**, 3439 (1965).
19) E.D. Wills, *Biochem. J.*, **113**, 325 (1969).
20) P. Hochstein and L. Ernster, *Biochem. Biophys. Res. Commun.*, **12**, 388 (1963).
21) E.D. Wills, *Biochem. J.*, **113**, 333 (1969).
22) T. Kamataki, O. Sugita, M. Kitada, S. Naminohira and H. Kitagawa, *Res. Commun. Chem. Pathol. Pharmacol.*, **17**, 265 (1977); P.B. McCay, O.D. Gibson and K.R. Hornbrook, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **40**, 199 (1981).
23) D.P. Hanlon, *J. Med. Chem.*, **14**, 1084 (1971).
24) N. Motohashi, I. Mori, Y. Sugiura and H. Tanaka, *Chem. Pharm. Bull.*, **22**, 654 (1974).