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平松, 直子

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神戸大学博士論文

NUTRITIONAL STUDIES ON DRUG-METABOLIZING SYSTEM IN THE LIVER OF RAT ORALLY ADMINISTERED AUTOXIDIZED LINOLEATE

（自動酸化リノール酸投与ラット肝の薬物代謝系に関する栄養学的研究）

平成３年１月

平　松　直　子

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I GENERAL INTRODUCTION

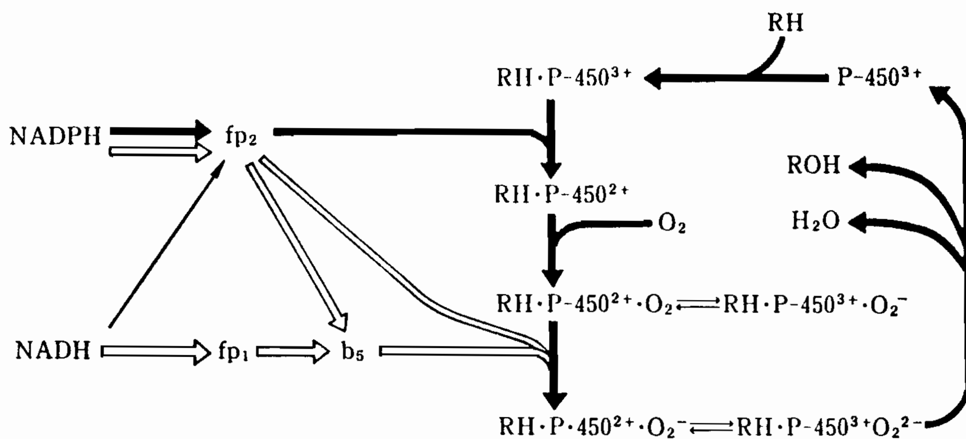
Oxygen, for the living body, has two-facedactions of both an efficient energy source and a toxic substance, *i. e.*, active oxygens such as $O_2{}^{\cdot -}$, H_2O_2 and $^{\cdot}OH$. These active oxygens are intermediates in the reduction of molecular oxygen *in vivo* (*1-15*). In other words, organisms produce active oxygens with toxicity as an inevitable consequence, resulting in the damages of lipid-constituted biomembranes. However, the organisms have several systems for detoxication or protection against active oxygens, for example, the systems of catalase (*7, 19-21*), superoxide dismutase (*7, 16-19*), and glutathione peroxidase (*7, 20, 21*), or reductants such as vitamins E and C (*22-26*). They have been acquired through the long process of evolution. An imbalance between active oxygens and the protective systems may bring on human beings a variety of serious deseases such as hepatitis, myocardial ischemia, atherosclerosis, cancer (*27-31*).

The deterioration of lipids by oxygen occurs not only in the living body but also in the foods including polyunsaturated lipids. Polyunsaturated fatty acids constituting lipids readily react with atmospheric

oxygen to form first their hydroperoxide *(27, 32)* and subsequently a mixture of completely oxidized products *(27, 33)*, termed secondary oxidation products (SP), containing polymers, acids, and aldehydes. These substances occur in foods, although in small amounts. If these are given to rats, they accumulate transiently in internal organs, especially in the liver *(34, 35)*. It is, therefore, very important to clarify the effects of autoxidized fatty acids on various metabolic system in the liver. These substances may be also recognized as some kinds of xenobiotics for the body.

It had been formerly recognized that the most important xenobiotics were drugs. The metabolizing

Scheme I-1



mechanism of lipophilic drugs has been studied by many workers *(36-41)*. It is generally well known that lipophilic drugs are oxidized in the first step by the enzymes of hepatic microsomal drug-metabolizing system (Scheme I-1). The oxydized drugs are, in the second step, altered to the more water-soluble substances by conjugation with glucronic acid, sulfate, glycine and so on (Scheme I-2). Subsequently, they can be excreted in the bile or urine. The drug-metabolizing system is composed of cytochrome P-450 which plays a central role, cytochrome b_5, NADPH-cytochrome *c* reductase, and NADH-cytochrome *c* reductase, and require molecular oxygen and NADPH or NADH as an electron donor, as shown

Scheme I-2

ROH **RO-X**

X = -glycine

$-SO_3OH$

-glucuronic acid

etc.

Abbreviation:

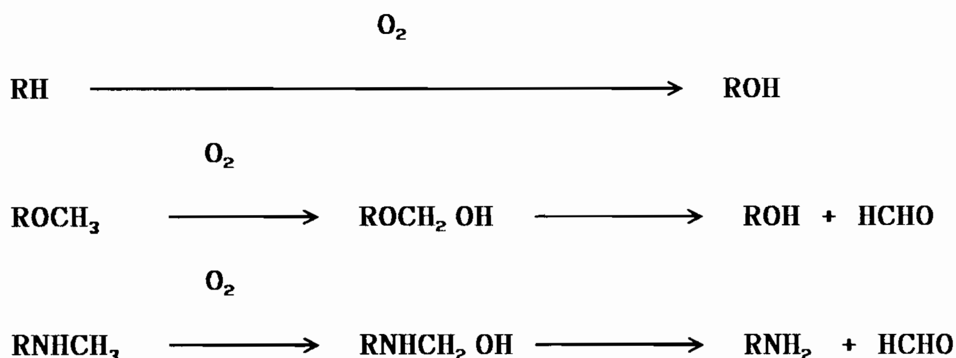
fp_1 = NADH-cytochrome *c* reductase
fp_2 = NADPH-cytochrome *c* reductase
b_5 = Cytochrome b_5
RH = Substrate
ROH = Product

$P\text{-}450^{3+}$ = Cytochrome P-450 (Fe^{3+})

$P\text{-}450^{2+}$ = Cytochrome P-450 (Fe^{2+})

in Scheme I-1. The enzyme system can react with various lipophilic xenobiotics, and can be also induced by them. The enzyme system not only reacts with foreign compounds to the body but also participates in a steroids metabolism *(42-51)*, ω-oxidation of fatty acids *(52-57)* *in vivo*, and so on.

The system is involved not only in oxidation of drugs but also in hydroxylation, *O*- or *N*-dealkylation of drugs. Though these reactions have been generally



recognized as a major detoxicating mechanism, these reactions do not necessarily reduce toxicities of drugs, and indeed there are many drugs which represent carcinogenesis or mutagenesis through the system like those of benz(α)pyrene, afratoxin B_1 and 2-acetylamino-fluorene (2-AAF) *(58-60)*.

It is, therefore, very interesting to know if the

autoxidized fatty acids ingested by animals influence drug-metabolizing system as xenobiotics. However, there have been only a few studies investigating the effect of autoxidized fatty acids on drug-metabolizing system in rat liver microsomes *(61-63)*. Brown *et al.* reported that dietary fatty acids peroxides, unlike peroxidized sterols, did not stimulate the demethylation of 3-methyl-4-monomethylaminoazobenzene in a rat liver homogenate system *(61)*. On the other hand, Arakaki and Ariyoshi *(62)* reported that the aniline hydroxylase activity decreased significantly when a single dose (0.4 ml/100 g body weight) of autoxidized corn oils (PV=400 meq/kg) was given, but the activity increased with a single dose (0.4 ml/100 g body weight) of autoxidized corn oils (PV=1,100 meq/kg). They also reported that after repeated oral doses (0.4 ml/100 g body weight/day) of autoxidized corn oil with 800 meq/kg and 1,100 meq/kg of peroxide values for 10 days and 7 days, respectively, the aniline hydroxylase activity and the cytochrome P-450 content increased at PV=1,100 meq/kg administration, while the activity of drug-metabolizing enzyme and the contents of cytochrome P-450 and b_5 decreased slightly at PV=800 meq/kg administration, in male Wistar rats. I found,

previously, that the enzyme activity of the drug-metabolizing system (S-9 activity in Ames' test) in the liver of male Wistar rats decreased when the autoxidized linoleate was orally administered at a daily dose of 0.25 ml/100 g body weight for 5 successive days, though the cytochrome P-450 content did not decrease *(63)*.

These incompatible findings suggest that the effect of autoxidized oils on drug-metabolizing activity in rat liver may change in accordance with differences in the autoxidized levels of oils, administration periods, dose levels and so on.

In addition, the drug-metabolizing system is variously affected by nutritional status *(64-70)* such as quantity and quality of dietary proteins, carbohydrates, lipids, vitamins, and minerals. The effect of nutrient deficiency on the drug-metabolizing system had been mainly investigated in the past. It has been known in recent years that the induction of drug-metabolizing system by xenobiotics changes a requirement for nutrients *in vivo*. From the standpoint of nutritional science, there are several reports *(71-83)* concerning the effects of xenobiotics, mainly polychlorinated biphenyls (PCB), and vitamins with

antioxidative property on the drug-metabolizing system and lipid peroxide formation *in vivo*. It has been reported that ingestion of xenobiotics increases a requirement of vitamins A (71-73) or C (74-80) accompanied by the increase of drug-metabolizing activity. It has been also reported that, in the liver of PCB-dosed rats, lipid peroxides increased (79-81) and was effectively depressed by the increasing dietary vitamin E (73, 82), and the interaction between ascorbic acid and vitamin E in PCB-exposed animals was an antioxidative synergy against lipid peroxidation (82, 83). Thus, it has been gradually realized that the changes in drug-metabolizing system and endogenous lipid peroxidation was closely associated with the *in vivo* contents of vitamins with antioxidative property. However, there are few studies about the effect of dietary pantethine with antioxidative property *in vivo* 84-87) on the enzyme activity of drug-metabolizing system.

In this study, first of all, I investigated the effects of dose levels and dose periods of autoxidized linoleate (AL) on drug-metabolizing system in the liver of male Wistar rat. In the next step, I investigated the effect of dietary pantethine levels on the

drug-metabolizing system and the extent of the *in vivo* lipid peroxidation in the liver of orally AL-administered rats.

In Chapter II, AL with 800 meq/kg of peroxide value (PV) and 1,700 meq/kg of carbonyl value (CV) was given in repeated oral doses at a daily dose level of 0 - 0.75 ml/100 g to the rats for 5 successive days, and the effect of increasing AL dose on the drug-metabolizing system was investigated in rat liver microsomes and S-9 fractions. Next, AL with the same character as used in Chapter II was given in repeated oral doses for 1-15 days at a daily dose level of 0.25 ml/100 g to the rats and the effect of AL on drug-metabolizing system was investigated periodically in liver microsomes. It was described in Chapters II and III that both the contents and activities of the constituents in drug-metabolizing system were increased by a low level of AL dose (0 - 0.25 ml/100 g) and decreased by a high level of AL dose (0.35 - 0.75 ml/100 g), and that the content and the activity were decreased by the elongation of the dose period even in the case of a low level of AL dose. It was presumed that a low level of AL dose caused *in vivo* lipid peroxidation and the drug-metabolizing system was induced in order to

dispose them, and that a high level of AL dose occasioned drastically *in vivo* lipid peroxidation and thereby the contents and activities of the constituents in drug-metabolizing system were lowered markedly.

Next, I studied on the effect of the levels of dietary pantethine and oral dose of AL on the growth and on the other symptoms in the rats in Chapter IV. The rats in pantethine-deficient (pantothenic acid deficient) group showed typical pantothenic acid deficient symptoms. The rats in both pantethine -adequate and -excess groups grew up normally, and no difference among the two groups was observed externally. In Chapter V the effect of dietary pantethine levels on drug-metabolizing system in the liver of rats orally administered varying amounts of AL was described, and I presumed that pantethine relieved the effects of both a low and a high level of AL doses on the drug-metabolizing system in rat liver. In Chapter VI, under the same conditions, the effect of dietary pantethine levels on the contents of fatty acids and thiobarbituric acid (TBA) reactive substances in the liver of rats orally administered varying amounts of AL was investigated. It was suggested that dietary pantethine depressed *in vivo* lipid peroxidation

caused by AL administration as an antioxidant, pantetheine derived from pantethine. It was also suggested that pantethine was closely related to lipid metabolism and made it smoothly through the biochemical functions of coenzyme A and acyl carrier protein because pantetheine, the reduced type of pantethine, is a constituent of them.

II EFFECTS OF DOSE LEVELS OF AUTOXIDIZED LINOLEATE ON DRUG-METABOLIZING SYSTEM IN RAT LIVER

II-1 INTRODUCTION

There have been few studies on the effect of autoxidized oil on drug-metabolizing activity in the rat liver microsomes, except some papers reported by Brown *et al.* (*61*), Arakaki *et al.* (*62*) or Hiramatsu *et al.* (*63*). These reports have included some incompatible findings, as described in General Introduction. These incompatible findings suggest that the effect of autoxidized oil on drug-metabolizing activity in the rat liver may change in accordance with differences in the autoxidized levels of oil used, dose levels, the administration periods, *etc.*

In order to better understand the effect of autoxidized fatty acid on the drug-metabolizing system, in this chapter, changes in cytochrome P-450 and b_5 contents, the enzyme activities of electron transfer system and drug-metabolizing activities were investigated in liver microsomes or S-9 fractions of male Wistar rats administered increasing levels of AL for 5 successive days.

II-2 MATERIALS AND METHODS

Chemicals. Linoleic acid was of extra-pure reagent grade from Nakarai Chemical Co. (Kyoto), and its purity as determined by gas chromatography was about 95 %. NADPH and NADH were purchased from Oriental Yeast Co. (Tokyo). Bovine serum albumin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and cytochrome *c* from horse heart were obtained from Sigma Chemical Co. (USA). Nutrient broth and agar powder were purchased from Difco (USA). The other chemicals were of guaranteed reagent grade from Nakarai Chemical Co. (Kyoto). Pantethine (60 % solution) was kindly supplied by Daiichi Seiyaku Co. (Tokyo).

Animals and diet. Male Wistar rats (JCL, Tokyo, Japan) aged 3 weeks were divided into 6 groups (5 rats/group), and were fed for one month on the diet (Clea Japan Inc., Tokyo) shown in Table II-1. The drinking water contained 6.25 mg pantethine/100 ml, because the diet contained no pantothenic acid and its related compounds. The diet and drinking water were provided *ad libitum*. No difference in diet and water

Table II-1. Composition of diet.

Dietary component	% by weight
Vitamin-free casein	24.5
Corn starch	45.5
Granulated sugar	10.0
Corn oil	6.0
Avicel (powdery cellulose)	3.0
KC flock (flocky cellulose)	2.0
α-Starch	1.0
Vitamin mixture*	1.0
Mineral mixture**	7.0

*Vitamin			**Mineral		
Vitamin A	1,200	I.U.	$CaCO_3$	1,355.4	mg
D_3	240	I.U.	KH_2PO_4	1,730	mg
E	20	mg	$CaHPO_4 \cdot 2H_2O$	1,500	mg
K_3	0.3	mg	$MgSO_4 \cdot 2H_2O$	800	mg
B_1	1.5	mg	NaCl	600	mg
B_2	1.56	mg	$FeC_6H_5O_7 \cdot 5H_2O$	190	mg
B_6	1.02	mg	$5ZnO \cdot 2CO_2 \cdot 4H_2O$	6	mg
B_{12}	5	mg	$CuSO_4 \cdot 5H_2O$	1.26	mg
Biotin	0.5	mg	$CoCl_2 \cdot 6H_2O$	0.4	mg
p-Aminobenzoic acid	10.15	mg	$Ca(IO_3)_2$	1.54	mg
Niacin	10.15	mg	$MnSO_4 \cdot 4H_2O$	15.4	mg
Inositol	15	mg	Corn starch	800	mg
Folic acid	0.2	mg			
Choline-Cl	300	mg			
Corn starch	632.22	mg			

* and ** are amounts of vitamins and minerals, respectively, in 100 g of diet.

intakes and growth were observed between the groups before the administration of AL.

Preparation of AL. Linoleic acid (12.5 ml) was poured into a 12.5-cm diameter petri dish and autoxidized at 37 ^{o}C for 30 days in an incubator. During the progress of autoxidation, the peroxide value (PV) (*88*) and carbonyl value (CV) (*89, 90*) were determined periodically. The AL that showed 800 meq/kg of PV and 1,700 meq/kg of CV at autoxidation for 20 days was used for the experiments.

Administration of AL. After the initial feeding for one month, AL was orally administered to the rats in each group at a daily dose of 0 (control), 0.15, 0.25, 0.35, 0.5 and 0.75 ml/100 g body weight. The AL administered to the rats during the administration period was prepared by shifting the first day of autoxidation so that linoleic acid was autxidized for 20 days at 37 ^{o}C.

Preparation of S-9 and microsomes. The S-9 and microsomes were prepared from rat liver by a modification of the method of Yahagi (*91*) and Omura and

Sato (*38*). After decapitation, the rat liver was excised and thoroughly perfused with autoclaved 1.15 % KCl solution. The liver was finely chopped with a razer, and homogenized with 3 volumes of 1.15 % KCl solution. The homogenate was centrifuged at 9,000×*g* for 15 min and the supernatant was used as S-9. One part of the supernatant was centrifuged at 105,000×*g* for 70 min with Hitachi 55P-72 automatic preparative ultra-centrifuge. The firmly packed microsome pellet was resuspended in distilled water with a Potter homogenizer. The resultant microsomal suspensions were stored at 4 °C and used within 2 to 3 days. All the treatments were carried out at 0-4 °C and S-9 was prepared under sterile conditions and stored at -80 °C.

Measurements of cytochrome b_5 and cytochrome P-450 contents. The contents of cytochrome b_5 and cytochrome P-450 in rat liver microsomes were determined by the method of Omura and Sato (*38*) The difference spectrum between NADH-reduced and air-saturated microsomes was scanned from 450 nm to 400 nm in a Shimadzu UV-200 spectrophotometer. The cytochrome b_5 content was estimated using the following equation: $\Delta E_{424-409} \times 1{,}000/185 \times$ protein (mg/ml) =

cytochrome b_5 (nmol/mg protein), where $\Delta E_{424-409}$ is the absorbance difference between 424 nm and 409 nm of the difference spectrum. The same microsomal sample was then used for measurement of cytochrome P-450. Carbon monoxide gas was admitted into the sample cell, followed by the addition of a few mg of hydrosulfide into both the reference and sample cells. Thus, the difference spectrum between reduced cytochrome P-450 and its CO complex was scanned from 500 nm to 400 nm. The cytochrome P-450 content was estimated using the following equation: $\Delta E_{450-490} \times 1{,}000/91 \times$ protein (mg/ml) = cytochrome P-450 (nmol/mg protein), where $\Delta E_{450-490}$ is the absorbance difference between 450 nm and 490 nm of this difference spectrum.

Measurements of the enzyme activities in electron transfer system. The NADPH-cytochrome *c* reductase activity was determined by the method of Omura and Takesue *(92)*. NADPH and cytochrome *c* from horse heart were added to the liver microsomal suspension, and an increase in absorbance at 550 nm (ΔE_{550}) was determined. The specific activity was calculated according to the following equation : specific activity (μmol/min/mg protein)=ΔE_{550}/min $\times$ volume (ml) of

reaction mixture / 21.1 × mg protein in reaction mixture.

The NADH-ferricyanide reductase activity was determined by the method of Takesue and Omura (*93*). NADH, potassium ferricyanide and liver microsomal suspension were used as electron donor, electron acceptor and enzyme respectively. The specific activity was calculated according to the following equation : specific activity (μmol/min/mg protein) $=\Delta E_{420}$/min × volume(ml) of reaction mixture / 1.02 × mg protein in the mixture, where ΔE_{420} is a decrease in absorbance at 420 nm in the reaction system.

The NADH-cytochrome *c* reductase activity was determined by the method of Takesue and Omura (*94*). NADH and cytochrome *c* from horse heart were added to the liver microsomal suspension, and an increase in absorbance at 550 nm (ΔE_{550}) was determined. The specific activity was calculated according to the following equation : specific activity (μmol/min/mg protein)$=\Delta E_{550}$/min × volume(ml) of reaction mixture /21.1 × mg protein in the mixture.

Measurement of the S-9 activity. Ames' test was carried out according to the method of Yahagi (*91*) with

a slight modification in the order of 2-AAF addition. *Salmonella typhimurium* TA 1538 strain was cultured overnight at 37 ^{o}C in Difco nutrient broth medium. The mixture containing 0.7 % agar and 0.6 % sodium chloride was prepared as top agar and autoclaved. A sterile solution (10 ml) of 0.5 mM L-histidine and 0.5 mM biotin was added to 100 ml of the melted top agar. The culture strain (0.1 ml) was mixed thoroughly with 0.01 % 2-AAF-dimethylsulfoxide solution (0.1 ml), NADPH generating system (0.35 ml) containing 8 μmole of magnesium chloride, 33 μmole of potassium chloride, 5 μmole of glucose-6-phosphate, 0.25 unit of glucose-6-phosphate dehydrogenase, 4 μmole of NADPH and 100 μmole of sodium phosphate buffer (pH 7.4) and S-9 fraction (0.15 ml), and incubated for 20 min at 37 ^{o}C, and then, 2 ml of the melted top agar were added to the mixture. The mixture is poured onto minimal-glucose agar-medium, which contained 1.5 % agar and 2 % glucose in Vogel-Bonner medium E containing magnesium sulfate (2 g), citric acid monohydrate (20 g), potassium phosphate dibasic (anhydrous) (100 g) and sodium ammonium phosphate (35 g) per 1000 ml medium. After incubation at 37 ^{o}C for 2 days, revertant colonies were counted. The number of revertants of *Salmonella typhimurium* TA

1538 caused by mutagenic metabolites of 2-AAF metabolized by S-9 fraction was used as a measure of the drug-metabolizing activity.

Measurement of aminopyrin-N-demethylase activity. As an indication of the drug-metabolizing activity of cytochrome P-450, aminopyrin-N-demethylase activity in the liver S-9 fraction was determined according to the method of Kato *et al.* (*74*). The reaction mixture contained 1.2 ml of 100 mM Tris-HCl buffer (pH 7.4), 0.3 ml of 50 mM semicarbazide hydrochloride-100 mM Tris-HCl buffer (pH 7.4), 0.7 ml of H_2O, 0.3 ml of NADPH generating system, 0.3 ml of S-9 fraction and 0.5 ml of 150 mM KCl-25 mM Tris-HCl buffer (pH 7.4). After the mixture was incubated at 37 ^{o}C for 5 min, the reaction was allowed to proceed for 15 min at 37 ^{o}C by the addition of 0.2 ml of 30 mM aminopyrin. Formaldehyde produced was measured according to the method of Nash (*95*).

Other measurement methods. In this study, protein was determined by the procedure of Lowry *et al.*(*96*) with bovine serum albumin as the standard.

Statistical analysis. The statistical

significance of differences among values was analyzed by one-way analysis of variance (ANOVA). When main effect was significant, least significant difference (LSD) test was performed. A probability of <0.05 was taken as the level of significance.

II-3 RESULTS

Changes in PV and CV of the autoxidized linoleic acid.

Linoleic acid was autoxidized at 37 °C, as described above. The changes in PV or CV during the autoxidation period are shown in Fig. II-1. PV

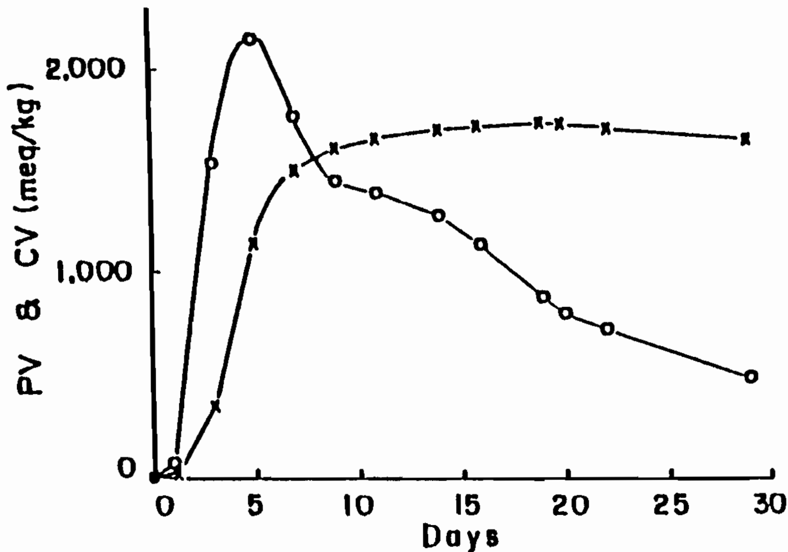


Fig. II-1. Changes in peroxide value and carbonyl value during autoxidation of linoleic acid. o, PV; ×, CV.

gradually decreased after it reached a peak 4 to 5 days after the initiation of autoxidation. In contrast, CV increased even after PV reached a peak and remained constant at about 1,700 meq/kg from 10 days after the beginning of autoxidation. Studies on the toxicity of autoxidized fatty acids have shown that SP is more toxic than the fatty acid hydroperoxides (*97-101*). Thus, autoxidized fatty acids with high CV, although with low PV, may be generally more toxic than those with low CV and high PV. In consideration of these facts, I chose AL which showed about 800 meq/kg of PV and about 1,700 meq/kg of CV at autoxidation for 20 days at 37 ^{o}C.

Lethal dose level.

All the rats of 0.75 ml AL/100 g body weight group had died by the fourth day of consecutive oral doses. Two rats of the 0.5 ml AL/100 g body weight group died on the fourth day of consecutive oral doses, and the other rats died on the fifth day. Two rats of the 0.35 ml AL/100 g body weight group died on the fifth day, and the other rats lived until the morning after the fifth day of consecutive oral doses. On the fourth day of the doses, the groups of a daily dose of 0.5 ml

AL/100 g body weight and 0.75 ml AL/100 g body weight were, respectively, supplemented with 5 rats. Thus, the effects of AL dose on drug-metabolizing system were investigated for the groups of a daily dose of 0 - 0.35 ml AL/100 g body weight for 5 successive days and for the groups of a daily dose of 0.5 - 0.75 ml AL/100 g

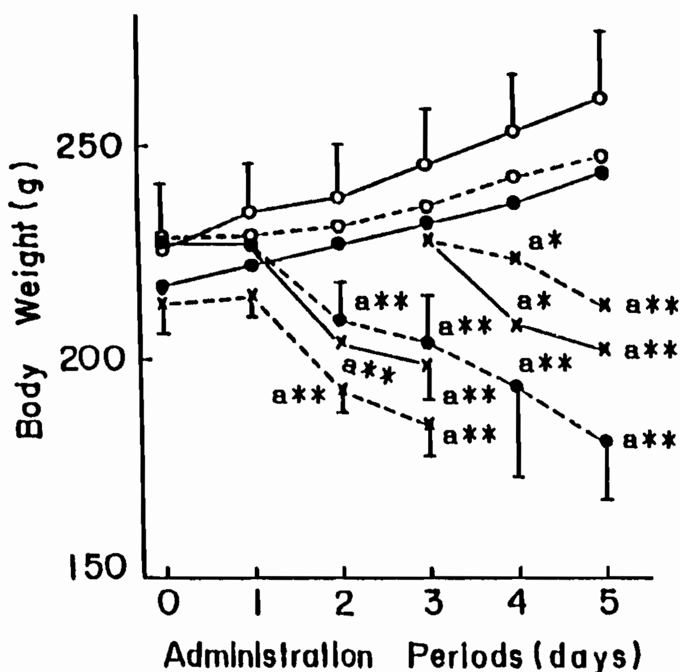


Fig. II-2. Changes in body weight by increasing AL dose. —o—, control (without AL dose); ---o---, 0.15 ml AL/100 g body weight; —●—, 0.25 ml AL/100 g body weight; ---●---, 0.35 ml AL/100 g body weight; —x—, 0.50 ml AL/100 g body weight; ---x---, 0.75 ml AL/100 g body weight. [a]significantly different from control group. *, $p<0.05$; **, $p<0.01$.

body weight for 2 successive days.

Changes in body weight.

No difference in growth among the six groups of rats was observed during the initial feeding period. Even after AL administration, body weights in the 0.15 ml AL/100 g body weight and 0.25 ml AL/100 g body weight groups were not significantly different from that of the control group, though a retardation of body weight gain occurred on the first day at a daily dose of 0.25 ml AL/100 g body weight (Fig. II-2). The body weights in the 0.35 - 0.75 ml AL/100 g body weight groups kept on decreasing after the second day of consecutive oral doses.

Changes in cytochrome P-450 and cytochrome b_5 contents.

The cytochrome P-450 and b_5 contents were measured after AL administration. The change in cytochrome P-450 content is shown in Fig. II-3. The maximal content was obtained by the dose of 0.25 ml AL/100 g body weight, but the content decreased sharply and significantly in the 0.35 ml AL/100 g body weight group in comparison with control, and the greater decrease was observed in the dose of 0.5 and 0.75 ml AL/100 g body weight. The

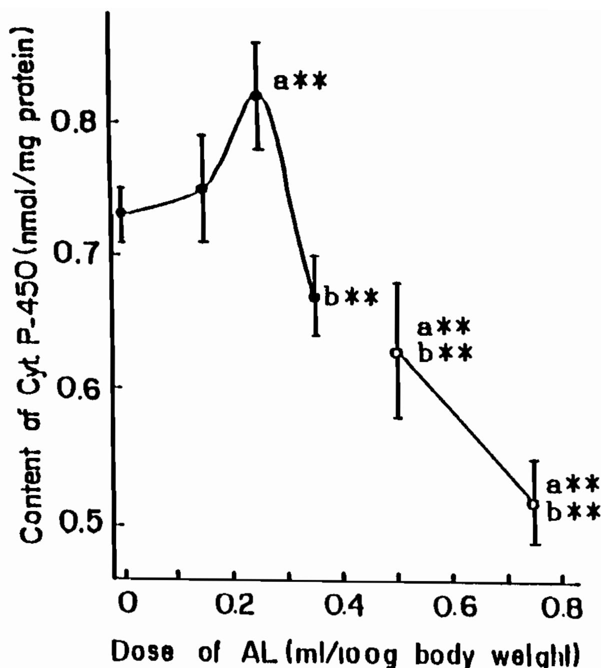


Fig. II-3. Changes in cytochrome P-450 content in rat liver microsomes by increasing AL dose. ●, AL doses for 5 successive days; o, AL doses for 2 successive days. [a]significantly different from control group. [b]significantly different from 0.35 ml AL/100 g body weight group. **, *p*<0.01.

changes in cytochrome b_5 content are shown in Fig. II-4.

The cytochrome b_5 content increased significantly in the 0.15 - 0.35 ml AL/100 g body weight groups. The maximal content was observed in the 0.25 ml AL/100 g body weight group. Unlike cytochrome P-450, the cytochrome b_5 content did not significantly decrease in

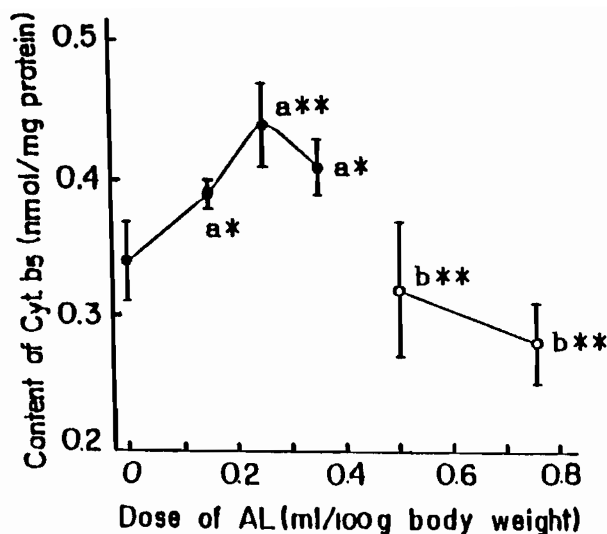


Fig. II-4. Changes in cytochrome b_5 content in rat liver microsomes by increasing AL dose. o, AL doses for 5 successive days; o, AL doses for 2 successive days. [a]significantly different from control group. [b]significantly different from 0.25 ml AL/ 100 g body weight group. *, *p*<0.05; **, *p*<0.01.

the 0.5 and 0.75 ml AL/100 g body weight groups in comparison with the control, although it decreased significantly in comparison with the 0.25 ml AL/100 g body weight group.

Changes in enzyme activities of electron transfer system.

As shown in Fig. II-5, the NADPH-cyt.*c* reductase activity increased significantly at a daily dose of

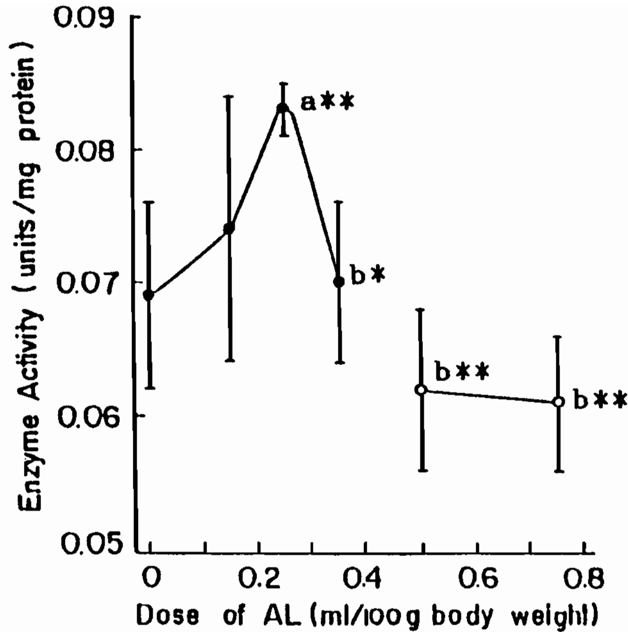


Fig. II-5. Changes in NADPH-cytochrome *c* reductase of electron transfer system by increasing AL dose. ●, AL doses for 5 successive days; o, AL doses for 2 successive days. [a]significantly different from control group. [b]significantly different from 0.25 ml AL/100 g body weight group. *, $p<0.05$; **, $p<0.01$.

0.25 ml AL/100 g body weight in comparison with the control, and the maximal activity was obtained at this dose level. However, the enzyme activity decreased significantly in the 0.35 ml AL/100 g body weight group and 0.5 and 0.75 ml AL/100 g body weight group in comparison with the group of the maximal activity, although no difference was observed compared with the

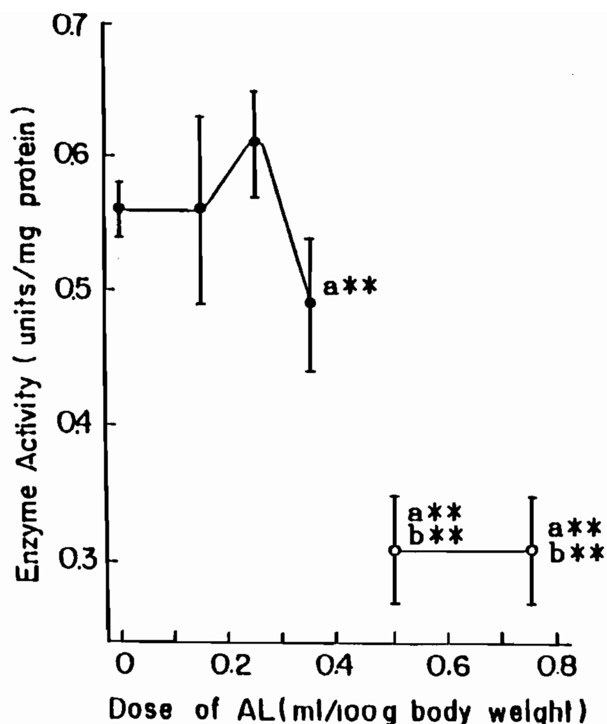


Fig. II-6. Changes in NADH-cytochrome *c* reductase of electron transfer system by increasing AL dose. ●, AL doses for 5 successive days; o, AL doses for 2 successive days. [a]significantly different from 0.25 ml AL/100 g body weight group. [b]significantly different from 0 - 0.35 ml AL/100 g body weight groups. **, $p<0.01$.

control.

The mean of NADH-cyt.*c* reductase activity showed the maximal activity in the 0.25 ml AL/100 g body weight group, but the value was not significantly different from both the control and 0.15 ml AL/100 g

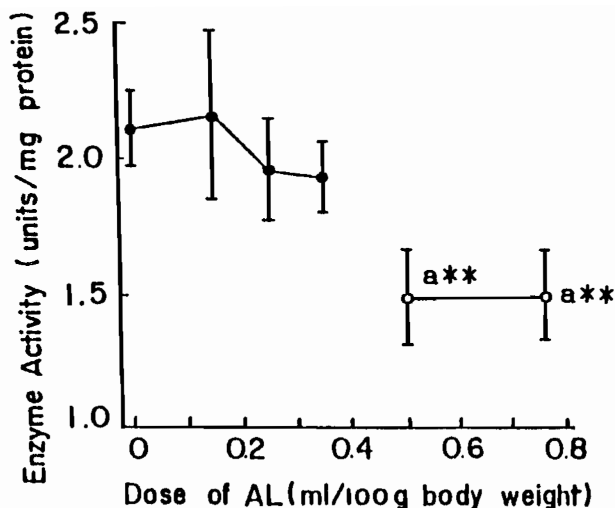


Fig. II-7. Changes in NADH-ferri (CN)reductase of electron transfer system by increasing AL dose. o, AL doses for 5 successive days; o, AL doses for 2 successive days. [a]significantly different from control group. **, $p<0.01$.

body weight groups (Fig. II-6). However, the enzyme activity decreased significantly in the 0.35 ml AL/100 g body weight group in comparison with the 0.25 ml AL/100 g body weight group, and also decreased significantly in the 0.5 and 0.75 ml AL/100 g body weight group in comparison with the groups of a daily dose of 0 - 0.35 ml AL/100 g body weight.

The NADH-ferri(CN) reductase activity decreased gradually by a daily dose of 0 - 0.35 ml AL/100 g body

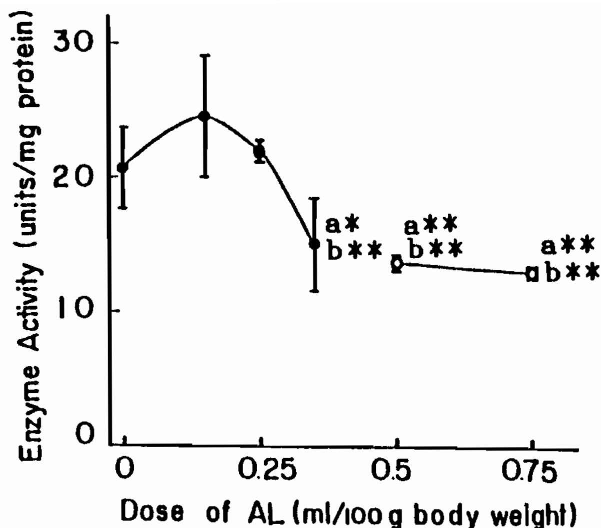


Fig. II-8. Changes in aminopyrin-N-demethylase activity in rat liver S-9 fraction by increasing AL dose. ●, AL doses for 5 successive days; o, AL doses for 2 successive days. [a]significantly different from control group. [b]significantly different from 0.15 and 0.25 ml AL/100 g body weight groups. *, $p<0.05$; **, $p<0.01$.

weight for 5 days, but the enzyme activity decreased suddenly and significantly by a daily dose of 0.5 ml AL/100 g body weight for 2 days, compared with those of the groups of 0 - 0.35 ml AL/100 g body weight (Fig. II-7).

Changes in aminopyrin-N-demethylase activity.

Aminopyrin-N-demethylase responded to AL orally

dosed, as shown in Fig. II-8. The maximal mean value of aminopyrin-N-demethylase activity was obtained by the dose of the 0.15 ml AL/100 g body weight, but it was not significantly different from the values of the 0 and 0.25 ml AL/100 g body weight groups. The enzyme activity of the 0.35 ml AL/100 g body weight group was significantly lower than those of the 0.15 and 0.25 ml AL/100 g body weight groups. At a daily dose of 0.5 and 0.75 ml AL/100 g body weight for 2 days, the activities decreased significantly in comparison with those of the 0 - 0.25 ml AL/100 g body weight groups, though no difference was observed between the above groups and the dose group of 0.35 ml AL/100 g body weight for 5 days.

Changes in S-9 activity.

As shown in Fig. II-9, the S-9 activity rose significantly at a daily dose of 0.15 and 0.25 ml AL/100 g body weight for 5 successive days, compared with the control group, but decreased with greater dose levels of AL. Thus, the activity in the 0.35 ml AL/100 g body weight group was almost the same as that of the control group, and was significantly lower than that of the 0.15 and 0.25 ml AL/100 g body weight groups.

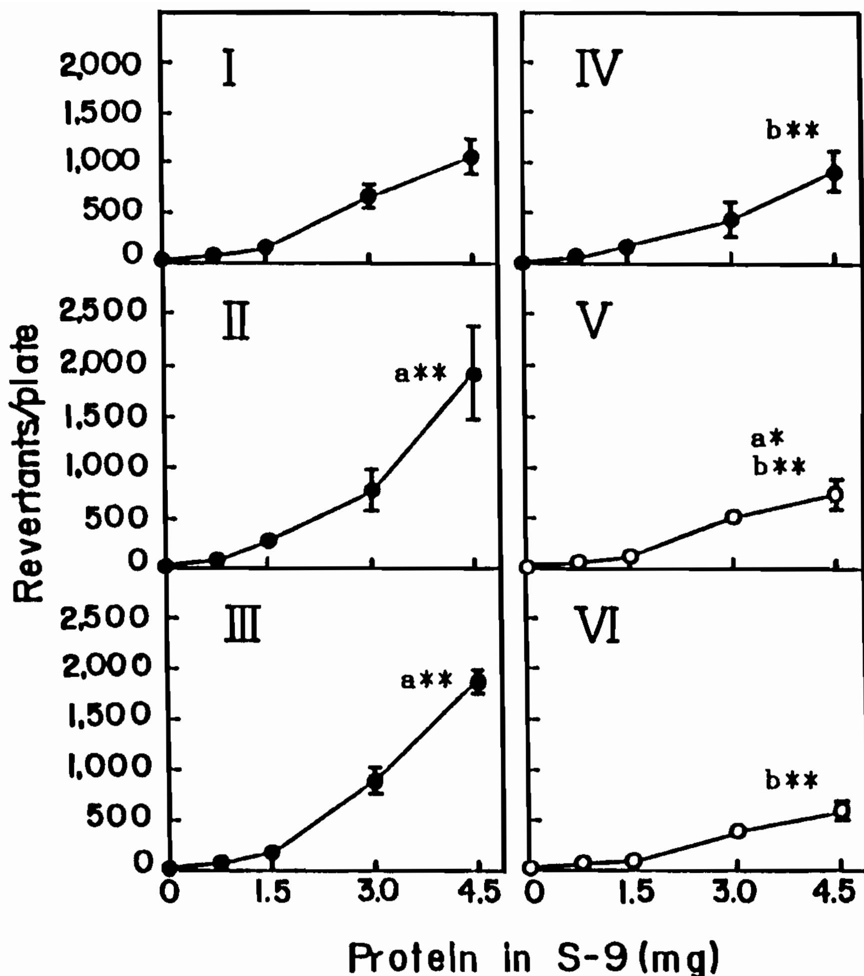


Fig. II-9. Changes in S-9 activity in rat liver S-9 fraction by increasing AL dose. I, control (without AL dose); II, 0.15 ml AL/100 g body weight for 5 days; III, 0.25 ml AL/100 g body weight for 5 days; IV, 0.35 ml AL/100 g body weight for 5 days; V, 0.5 ml AL/100 g body weight for 2 days; VI, 0.75 ml AL/100 g body weight for 2 days. [a]significantly different from control group. [b]significantly different from 0.15 and 0.25 ml AL/100 g body weight groups. *, $p<0.05$; **, $p<0.01$.

Furthermore, at the higher dose level of 0.5 and 0.75 ml AL/100 g body weight for 2 days, the additional decreases of S-9 activities were observed.

II-4 DISCUSSION

In preliminary experiments, it was recognized that the effect of SP (0.2 ml/100 g body weight/day ; prepared by column chromatography from AL with about 75 % yield) on the drug-metabolizing system was essentially similar to that of AL (0.25 ml/100 g body weight/day) administered for 5 days. Therefore, the AL was used as an autoxidized oil administered orally in the present experiment.

In the present chapter, the responses of drug-metabolizing system to dosed AL were investigated in liver microsomes or S-9 fractions from rats administered increasing levels of AL. The maximal contents of cytochrome P-450 and b_5 were observed at a daily dose of 0.25 ml AL/100 g body weight for 5 days, but the cytochrome P-450 content decreased significantly at a daily dose of 0.35 ml AL/100 g body weight for 5 days and at a daily dose of 0.5 and 0.75 ml AL/100 g body weight for 2 days (Fig. II-3). On the other hand,

the content of cytochrome b_5 was scarcely decreased by the dose of 0.35 ml AL/100 g body weight for 5 days and 0.5 ml AL/100 g body weight for 2 days (Fig. II-4). These results suggest that cytochrome P-450 is easier to break down than cytochrome b_5 by a relatively high dose of AL. Jeffry *et al.* (*102*) have also reported that linoleic acid hydroperoxide destroys cytochrome P-450 in microsomes without destroying cytochrome b_5 *in vitro*. Thus, the decrease of cytochrome P-450 content might be caused by the endogenous lipid hydroperoxide from the *in vivo* lipid peroxidation accelerated by a high dose of exogenous AL. In addition, Patzelt-Wenczler (*103*) has reported that the cytochrome b_5 content increased in liver microsomes of vitamin E-deficient rats, and that it was lowered by applications of vitamin E to the control level. These phenomena were very similar to the results of the present experiment. It can be concluded from these facts that a rise of *in vivo* lipid peroxidation induces cytochrome b_5. It is likely that this increase of cytochrome b_5 is probably due to the necessity of accelerating the desaturation of fatty acids and of protecting the microsomal membrane.

The results shown in Figs. II-5, II-6 and II-7

suggest that each of the enzymes in electron transfer system is also impaired by a high level of AL dose. However, NADPH-cytochrome *c* reductase is more difficult to break down as compared with the other enzymes in electron transfer system, because no difference was observed between 0.5 and 0.75 ml AL/100 g body weight groups and the control group, though the reason was not clear.

The changes in aminopyrin-N-demethylase and S-9 activities by increasing dose levels of AL were essentially the same as those observed for the cytochrome P-450 content, *i.e.*, the drug-metabolizing activities increased in a low level of AL dose, and decreased in a high level of AL dose (Figs. II-8 and II-9). We inferred from these facts that the decrease of drug-metabolizing enzyme activities after a high level of AL dose resulted either from a disorder of the microsomal membrane and the lowering in the content of cytochrome P-450 which plays a central role in the hydroxylation of 2-AAF at the membrane, or from the inactivation of acetyltransferase which forms the final mutagen, though the possibility exists that the lowering in the cytochrome P-450 content and in drug-metabolizing activities by a high level of AL dose

might arise indirectly from the reduction in digestion and absorption abilities owing to the injury of digestive organs.

In the administration period of 5 consecutive days, the cytochrome P-450 content and drug-metabolizing enzyme activity were significantly increased by a daily dose of 0.25 ml AL/100 g body weight, compared with control. A small part of the ingested AL is accumulated in the rat liver *(34, 35)*, and the AL contains some kinds of xenobiotics such as aldehydes including malonaldehydes, hydroperoxyalkenals, hydroperoxyepoxides, and hydroperoxide polymers. It is well known that the cytochrome P-450 content and drug-metabolizing activity are increased by ingesting lipophilic xenobiotics, and also there have been some reports *(104-108)* that both cytochrome P-450 and b_5 act as peroxidase on lipid hydroperoxide. Therefore, it seems that these phenomena are reasonable responses against xenobiotics which are metabolized by the drug-metabolizig system in rat liver. Within this dosage (0.25 ml AL/100 g body weight), the ingested AL and *in vivo* lipid peroxidation caused by AL may be able to be disposed by *in vivo* antioxidation mechanisms including catalase (*19*), glutathione peroxidase (*21*),

superoxide dismutase (*19*), cytochrome P-450 (*104-106*) and b_5 (*107, 108*), or by antioxidants such as vitamin E and C (*26*). However, the cytochrome P-450 contents and drug-metabolizing functions were lowered even with this dose level by the elongation of dose period, as will be mentioned in the next chapter. In the present experiments, the cytochrome P-450 content and drug-metabolizing activities were also decreased by the high level of AL administration. These findings show that the damages by both the given AL and *in vivo* lipid peroxide caused by AL may exceed the peroxide disposition resulting from the *in vivo* antioxidants or antioxidation mechanisms, when dose period was elongated, or a high level of AL dose was given. In the next chapter, I will describe the effect of dose period of AL on drug-metabolizing system.

III EFFECTS OF DOSE PERIODS OF AUTOXIDIZED LINOLEATE ON DRUG-METABOLIZING SYSTEM IN RAT LIVER.

III-1 INTRODUCTION

As described in Chapter II, the cytochrome P-450 and b_5 contents, enzyme activities in electron transfer system, aminopyrin-N-demethylase activity and S-9 activity in the drug-metabolizing system changed essentially in a similar manner. Namely, both the contents and activities of enzymes in the system were increased by a low level of AL dose and decreased by a high level of AL dose, though the cytochrome b_5 was more stable than cytochrome P-450. In consequence, it is clear that the effect of autoxidized oil on drug-metabolizing system in rat liver changes in accordance with the difference in the dose levels of AL.

In this chapter, in order to understand more closely the effect of AL on drug-metabolizing system, the effect of dose periods of AL on the system was investigated in the liver microsomes and S-9 fraction of male Wistar rats.

III-2 MATERIALS AND METHODS

Chemicals. All the chemicals used in this chapter were purchased from the same pharmaceutical companies as described in Chapter II.

Animals and diet. Male Wistar rats (JcL, Tokyo, Japan) aged 3 weeks were divided into 8 groups (6 rats/group), and were fed for one month on the diet (Clea Japan Inc., Tokyo), as described in Chapter II. The drinking water contained 6.25 mg pantethine/100 ml. The diet and drinking water were provided *ad libitum*. No difference in diet and water intake was observed among the groups.

Preparation of AL. The AL having 800 meq/kg of PV and 1,700 meq/kg of CV was prepared from linoleate according to the way described in Chapter II.

Administration of AL. After the initial feeding for one month, AL was orally administered to the rats in one group for 15 days at a daily dose of 0.25 ml/100 g body weight. Four days after the initial dose, to another group of rats, AL was administered in the same

manner for 11 days. Thereafter, the administration was given every other day for the remaining 5 groups. Thus, the rats in these 7 groups orally received AL at a daily dose of 0.25 ml/100 g body weight for 15, 11, 9, 7, 5, 3, and 1 successive days, respectively. The administered AL to the rats in each group were prepared by shifting the start of autoxidation so that linoleic acid was autoxidized for 20 days at 37 ^{o}C. Rats in the control group were fed with the diet shown in Table II-1 without AL administration.

Preparation of S-9 and microsomes. After the AL administration, all rats in the 8 groups were treated on the same day. The S-9 and microsomes were prepared from rat liver by a modification of the method of Yahagi (*91*) and Omura and Sato (*38*), as described in Chapter II.

Measurements of cytochrome b_5 and cytochrome P-450 contents. The contents of cytochrome b_5 and cytochrome P-450 in rat liver microsomes were determined by the method of Omura and Sato (*38*), as described in Chapter II.

Measurement of the S-9 activity. As mentioned in Chapter II, Ames' test was carried out according to the method of Yahagi (*91*) with a slight modification in the order of 2-AAF addition. The number of revertants of *Salmonella typhimurium* TA 1538 caused by addition of 2-AAF to S-9 fractions was calculated and used as a measure of the drug-metabolizing activity.

Measurement of aminopyrin-N-demethylase activity. As an indication of the drug-metabolizing activity of cytochrome P-450, aminopyrin-N-demethylase activity in the liver S-9 fraction was determined according to the method of Kato *et al.* (*74*), as described in Chapter II.

Statistical analysis. The statistical significance of differences among values was analyzed by the same way as described in Chapter II.

III-3 RESULTS

Changes in body and liver weights during repeated AL doses.

No difference in growth between the 8 groups of rats was observed during the initial pretreatment

month. After AL administration, body weights in all experimental groups were lower than those of the control groups (Fig. III-1). A gradual increase in body weight was observed in the rats in two groups (AL-15 and AL 11 days administration), although a retardation of body weight gain occurred during the first one or two days at a daily dose of 0.25 ml AL/100 g body

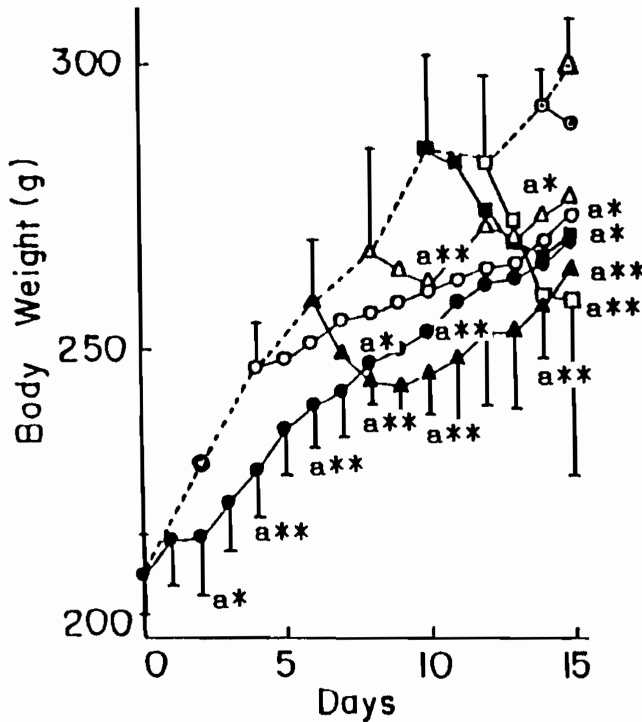


Fig. III-1 Effect of repeated oral doses of AL on the body weight of rats. ●, AL-15 days; o, AL-11 days; ▲, AL-9 days; Δ, AL-7 days; ■, AL-5 days; □, AL-3 days; ⊙, AL-1 days; A, control. [a]significantly different from control group. *, p<0.05; **, p<0.01.

weight. In the rats of the other groups, although there was a considerable decrease in body weight until 3 to 4 days after the beginning of the consecutive AL doses, afterward body weights began to increase gradually. As shown in Fig. III-2, the ratio of liver weight to body weight showed a tendency to increase each day during the administration periods. The increase of the ratio was statistically significant by the consecutive AL

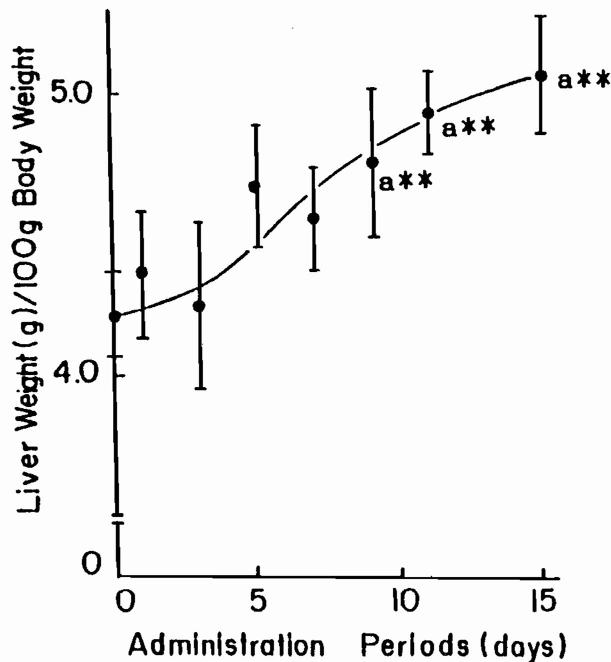


Fig. III-2 Changes in liver weight (g) to 100 g body weight during repeated oral doses of AL. The value at 0 days of administration is that from rats of the control group without AL administration. [a]significantly different from control group. **, $p<0.01$.

administration for more than 9 days, compared with that of the control group.

Changes in cytochrome P-450 and cytochrome b_5 contents by repeated AL doses.

The time course of cytochrome P-450 content was

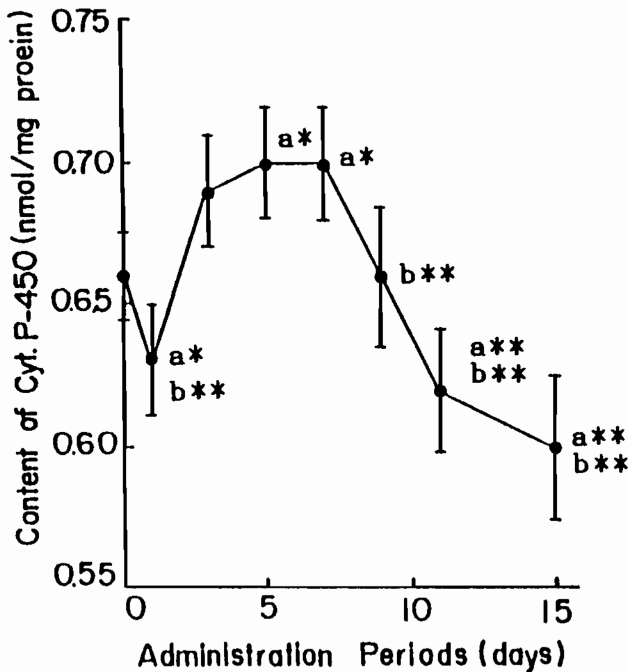


Fig. III-3 Changes in cytochrome P-450 content in rat liver microsomes during repeated oral doses of AL. The value at 0 days of administration is that from rats of the control group without AL administration. [a]significantly different from control group. [b]significantly different from AL-7 days (maximal content) group. *, *p*<0.05; **, *p*<0.01.

followed during repeated AL administration and the results obtained are shown in Fig. III-3. The maximal contents were observed 3 to 7 days after AL administration, and thereafter the content decreased gradually. Thus, the cytochrome P-450 content was significantly decreased by repeated AL administration for 11 to 15 days, compared with that of the control group. Changes in cytochrome b_5 content are shown in

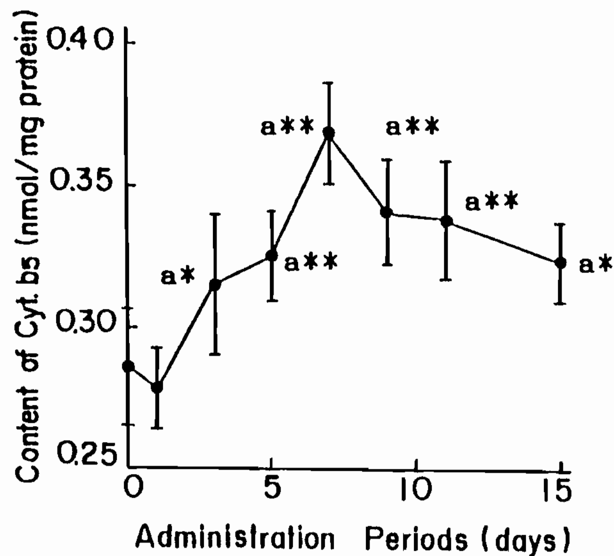


Fig. III-4 Changes in cytochrome b_5 content in rat liver microsomes during repeated oral doses of AL. The value at 0 days of administration is that from rats of the control group without AL administration. [a]significantly different from control group. *, $p<0.05$; **, $p<0.01$.

Fig. III-4. The maximal content was observed on 7 day of AL administration, while the content of cytochrome b_5 was slightly decreased by repeated AL administration for 9 to 15 days. Unlike cytochrome P-450, the cytochrome b_5 content, even in rat liver microsomes of repeated AL administration for 15 days, was appreciably higher than that of the control group.

Changes in drug-metabolizing activities on rat liver S-9 fraction.

The aminopyrin-N-demethylase activity in the rat liver S-9 fraction was also determined as a measure of drug-metabolizing activity. As shown in Fig. III-5, there was no significant difference in the activities between the control and the groups receiving repeated oral doses for less than 3 days. On the other hand, the activities were significantly increased by repeated oral doses of AL for more than 5 days during the experiment, compared with that of the control group.

The metabolic activation of 2-AAF with the S-9 fraction was measured by Ames' test. The number of revertants of *Salmonella typhimurium* TA 1538 was used as a measure of the total drug-metabolizing activities. The activities increased with the increasing volume of

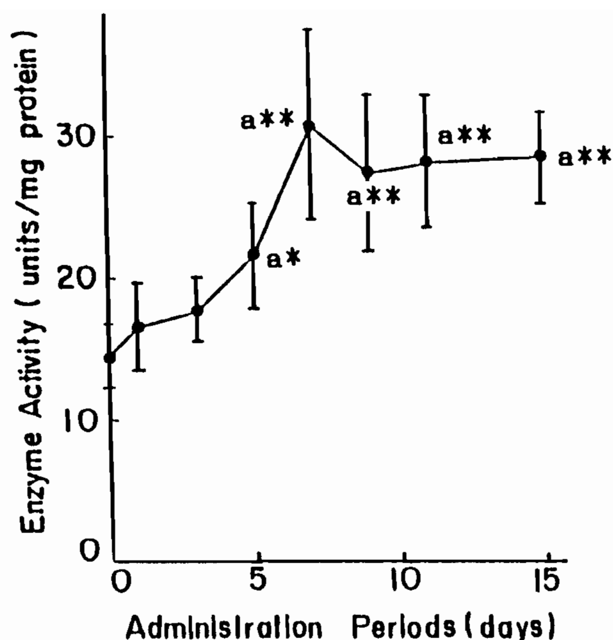


Fig. III-5 Changes in aminopyrin-N-demethylase activity in rat liver S-9 fraction during repeated oral doses of AL. The value at 0 days of administration is that from rats of the control group without AL administration. [a]significantly different from control group. *, $p<0.05$; **, $p<0.01$.

the S-9 fraction in the control and single oral dose groups, as shown in Fig. III-6. The S-9 activity decreased a little by repeated oral doses of AL for 3 days, and gradually decreased with dosages for more than 5 days. The activities were lost almost completely by consecutive doses of AL for more than 9 days.

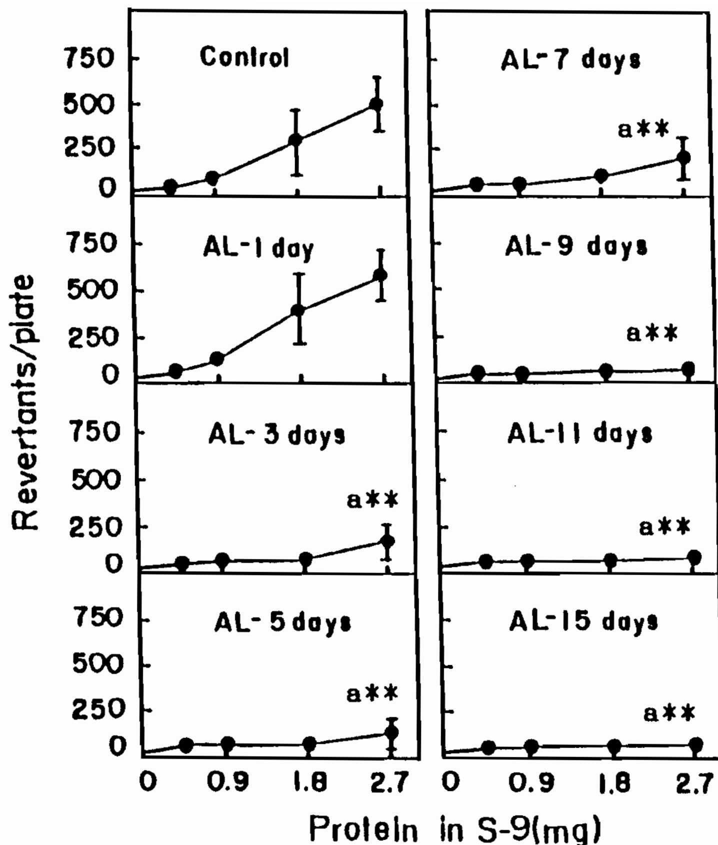


Fig. III-6 Changes in S-9 activity in rat liver S-9 fraction during repeated oral doses of AL.
[a]significantly different from control group. **, $p<0.01$.

III-4 DISCUSSION

In Chapter II, it was recognized that a daily dose of 0.25 ml/100 g body weight of AL (PV=800 meq/kg and CV=1,700 meq/kg) was suitable as the maximum dose

level, since this dose did not decrease body weight gain due to diarrhea or anorexia in AL-administered rats for 5 successive days.

In the present study, the periodic changes in the cytochrome P-450 and b_5 contents and in the drug-metabolizing activities were investigated for the liver microsomes from rats receiving AL for various periods. The administration period for each group was altered by changing progressively the start of AL administration with the progress of growth, and finally by decapitating all rats at the same time.

As shown in Fig. III-1, the decrease in body weight, which occurred immediately after the beginning of AL administration for 9, 7, 5, 3, and 1 days, was not observed in the two groups of rats administered AL for 11 and 15 days. These facts show that more severe effects of AL might appear immediately when a relatively large amount of AL is suddenly given to rats with larger body weight in later growth stages. However, because the mean ratio of liver weight to body weight increased from day to day during the administration periods (Fig. III-2), it was judged that the method of the present experiment was generally well suited for studing the effects of consecutive AL doses

on the drug-metabolizing system in rat liver.

Figure III-3 shows that the cytochrome p-450 content in rat liver microsomes increased at first (induction period) and then decreased (reduction period) by the consecutive oral dose of AL for 15 days. Now we can better explain the incompatible phenomena reported previously (*61-63*) for the changes in the cytochrome P-450 content by repeated AL administration. The increase and decrease of the cytochrome P-450 content by repeated AL administration previously reported probably correspond to the observations in the induction and reduction periods, respectively.

The cytochrome b_5 content increased by repeated AL administration for 3 to 7 days; afterward it scarcely decreased, unlike the cytochrome P-450 content (Fig. III-3). Patzelt-Wenczler (*103*) has reported that the cytochrome b_5 content increased in liver microsomes of vitamin E-deficient rats, and that it was lowered by applications of vitamin E to the control level. It can be concluded from these facts that a rise of *in vivo* lipid peroxidation induces cytochrome b_5. It seems likely that this increase of cytochrome b_5 is probably due to the necessity of accelerating the desaturation of fatty acids and of protecting the microsomal

membrane.

The results shown in Figs. III-3 and III-4 suggest that cytochrome P-450 is easier to break down than cytochrome b_5 during successive oral doses of AL for relatively long periods. Jeffry *et al.* (*102*) have reported that linoleic acid hydroperoxide destroys cytochrome P-450 in microsomes without destroying cytochrome b_5 *in vitro*. Thus, the decrease of cytochrome P-450 content might be caused by the endogenous lipid hydroperoxide from the *in vivo* lipid peroxidation accelerated by a large amount of exogenous AL that arose from the elongation of the dose period.

The changes in aminopyrin-N-demethylase activity do not necessarily coincide with that of the cytochrome P-450 content, as shown in Figs. III-3 and III-5. Because there are multiple forms of cytochrome p-450 (*109*) and one of them, which plays an essential role in N-demethylation of aminopyrin, might be scarcely damaged by consecutive AL doses for 9-15 days, although the total content of cytochrome P-450 decreases.

In the Ames' test, 2-AAF is converted into N-hydroxide by cytochrome P-450 in liver microsomes. The final mutagen is formed through deacetylation of the N-hydroxide by acetyltransferase in the liver

cytosol (*110, 111*). To estimate the total drug-metabolizing activity, the authors calculated the number of revertants of *Salmonella typhimurium* TA 1538 caused by the final mutagen for the S-9 fraction prepared from each rat. Unlike the contents of cytochrome P-450 and b_5, and aminopyrin-N-demethylase activity, the S-9 activity decreased gradually during consecutive oral doses of AL for 3-7 days, and was completely lost by doses for more than 9 days, although the activity did not change by the administration for one day (Fig. III-6). However, the S-9 activities in Chapters II and V did not decrease at a daily dose of both 0.25 ml and 0.2 ml AL/100 g body weight for 5 successive days, respectively (Figs. II-9 and V-7). The early decrease of S-9 activity in this chapter might be caused by partial inactivation of S-9 fraction occurred during either refrigeration, storage or dissolution by using in Ames' test after the S-9 preparation. But, the S-9 activity changed essentially in a similar manner. Namely, the activity was decreased by increases of AL load in any experiment. I also inferred that the disappearance of S-9 activity after repeated long-term AL administration was resulted either from a disorder of the microsomal membrane, where cytochrome p-450

plays a central role in the hydroxylation of 2-AAF, or from the inactivation of acetyltransferase which forms the final mutagen.

Now we can better understand the effect of AL on the drug-metabolizing system. Namely, it can be concluded from the facts obtained in Chapters II and III that a low level of AL dose induces the drug-metabolizing system in rat liver, and that the system is damaged and lowered by the elongation of AL dose period or by the increase of AL dose level. It is known that nutritional conditions affect the drug-metabolizing system. From the standpoint of nutritional science, it is interesting to know how dietary pantethine with antioxidative property *in vivo*, affects the drug-metabolizing system in the liver of rat orally administered varying amounts of AL. Before answering this question, I will describe in detail in the next chapter the effect of dietary pantethine levels on the growth and appearance of rats orally administered varying amounts of AL.

IV EFFECTS OF DIETARY PANTETHINE LEVELS ON THE GROWTH PHASE OF RATS ORALLY ADMINISTERED VARYING AMOUNTS OF AUTOXIDIZED LINOLEATE

IV-1 INTRODUCTION

Pantethine, D-bis-(pantothenyl-β-aminoethyl) disulfide, is a dimer of pantetheine which is a derivative of pantothenic acid and cysteamine (*112-114*). The pantothenic acid-related compounds participate in the various metabolisms *in vivo* (*115-118*) as a portion of each structure of coenzyme A or acyl carrier protein. Though physiological function of pantothenic acid compounds has never yet been found out except coenzyme A and acyl carrier protein, there have been some reports that pantethine showed the repairing effect for pathological changes by lipid peroxides (*84-87*). It should be recognized from these findings that pantothenic acid compounds like pantethine, converting to pantetheine containing -SH group by redox system *in vivo*, play a role as an antioxidant against *in vivo* lipid peroxidation.

In this chapter, some experiments were carried out in order to observe whether the rats fed on the

pantothenic acid-deficient diet showed the so-called pantothenic acid-deficient symptoms such as reduced growth rate, roughening of the coat, loss of hair (*119-123*), and also in order to investigate the effect of the levels of dietary pantethine and oral dose of AL on the growth and the other symptoms in the rats.

IV-2 MATERIALS AND METHODS

Chemicals. AL was prepared by autoxidizing linoleic acid at 37 ^{o}C for 20 days as described in Chapter II. S.TA-Test Wako kit was purchased from Wako Pure Chemical Industries Ltd., (Osaka). All the other chemicals were purchased from the same pharmaceutical companies as described in the preceeding chapters.

Animals. Male Wistar rats, 3 weeks old and weighing about 40 g (JCL, Tokyo), were initially divided into 3 groups (15 rats/group), and each of the rats was housed alone to avoid cannibalism at approximately 23 ^{o}C with a light and dark cycle of 12 h each. They were fed for one month on the same diet (Clea Japan Inc., Tokyo) as well as in Chapter II. The drinking water containing 0, 6.25, or 125 mg

pantethine/100 ml was given to the rats which were accordingly designated the pantethine-deficient, -adequate, and -excess groups, respectively. The diet and drinking water were provided *ad libitum*.

Administration of AL. After the initial feeding for one month, AL prepared by the same way as

Table IV-1. Experimental conditions.

Group Number	Pantethine mg /100ml drinking water	Pantethine status	AL ml /100 g B.W.
1	0		0
2	0	Deficient	0.2
3	0		0.35
4	6.25		0
5	6.25	Adequate	0.2
6	6.25		0.35
7	125		0
8	125	Excess	0.2
9	125		0.35

The AL which showed 800 meq/kg of PV and 1,700 meq/kg of CV was prepared by autoxidizing 12.5 ml of linoleic acid at 37 ^{o}C for 20 days in a 12.5-cm diameter petri dish.

described in Chapter II was orally administered to the rats of each group of the pantethine levels at a daily dose of 0, 0.2 (low dose), and 0.35 (high dose) ml/100 g body weight for 5 successive days. The experimental dose condition of pantethine and AL are shown in Table IV-1.

Observations of rats. During the whole feeding period before and after AL administration, the changes in the intakes of water and diets, body weight gains and the other appearances or symptoms were observed in each rat of pantethine-deficient, -adequate, and -excess groups.

Other measurement method. The activities of glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in serum were measured according to the method of Reitman and Frankel (*124*).

Statistical analysis. The statistical significance of differences among values was analyzed by two-way analysis of variance (ANOVA), or one-way ANOVA in the case of the constant level of one factor. When main effect and/or interaction were significant,

least significant difference (LSD) test was performed. A probability of <0.05 was taken as the level of significance.

IV-3 RESULTS

Ingestion of diet and drinking water.

Before AL administration, no difference among pantethine-excess and -adequate groups was observed in the ingestion of both diet and drinking water. The ingestion of diet in pantethine-deficient group was significantly less than those of the pantethine -adequate and -excess groups from about 1 week after the start of feeding, as shown in Fig. IV-1. The intakes of drinking water in pantethine-deficient group was also significantly less than those of the other groups from about 2 weeks after the start of feeding, as shown in Fig. IV-2.

After the low level of AL dose for 5 successive days, the amount of diet ingestion kept about 80 % for those of respective non-AL group in any pantethine groups, but the amount of respective diet ingestion in the three groups were remarkbly lowered by the high level of AL dose, as shown in Figs. IV-3, -4 and -5.

The lowering extent of diet ingestion in the pantethine-deficient group was the most remarkable in comparison with those of the other pantethine level groups.

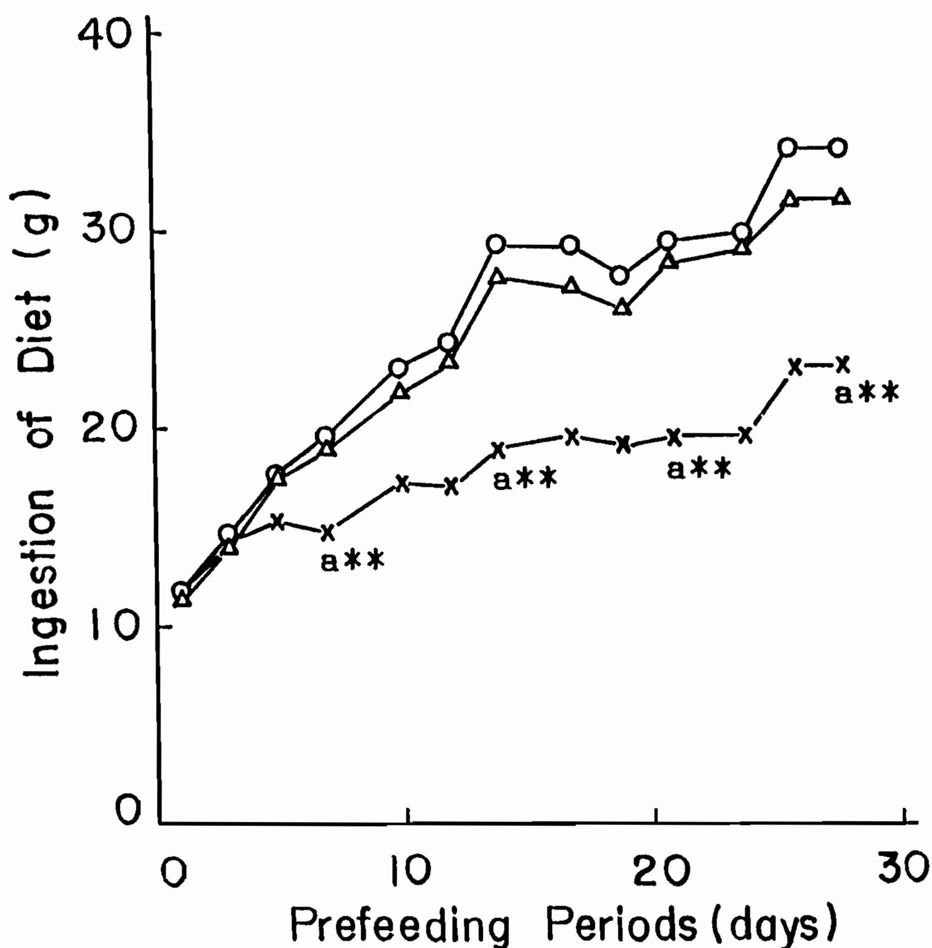


Fig. IV-1 Changes in diet ingestion during prefeeding periods. ×, Pantethine-deficient group; Δ, Pantethine-adequate group; o, Pantethine-excess group. Each value is the mean of 15 rats. [a]Significantly different from the pantethine-adequate and -excess groups. **, $p<0.01$.

Unlike diet ingestion, the ingestion of drinking water increased in the early period of AL administra-

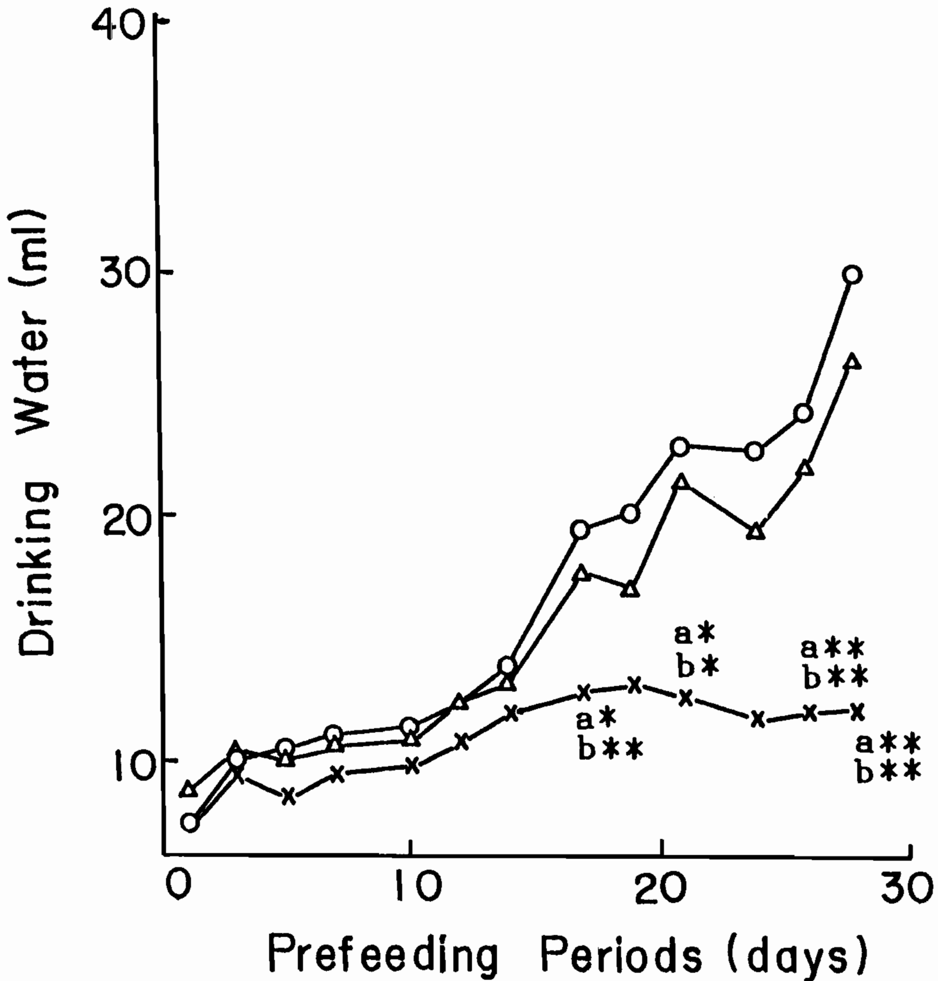


Fig. IV-2 Changes in drinking water ingestion during prefeeding periods. ×, Pantethine-deficient group; Δ, Pantethine-adequate group; o, Pantethine-excess group. Each value is the mean of 15 rats. [a]Significantly different from the pantethine-adequate group. [b]Significantly different from the pantethine-excess group. *, p<0.05; **, p<0.01.

tion in any pantethine level. After for 4 or 5 days of the high level of AL administration, the rats in the pantethine-deficient and -adequate groups had not been

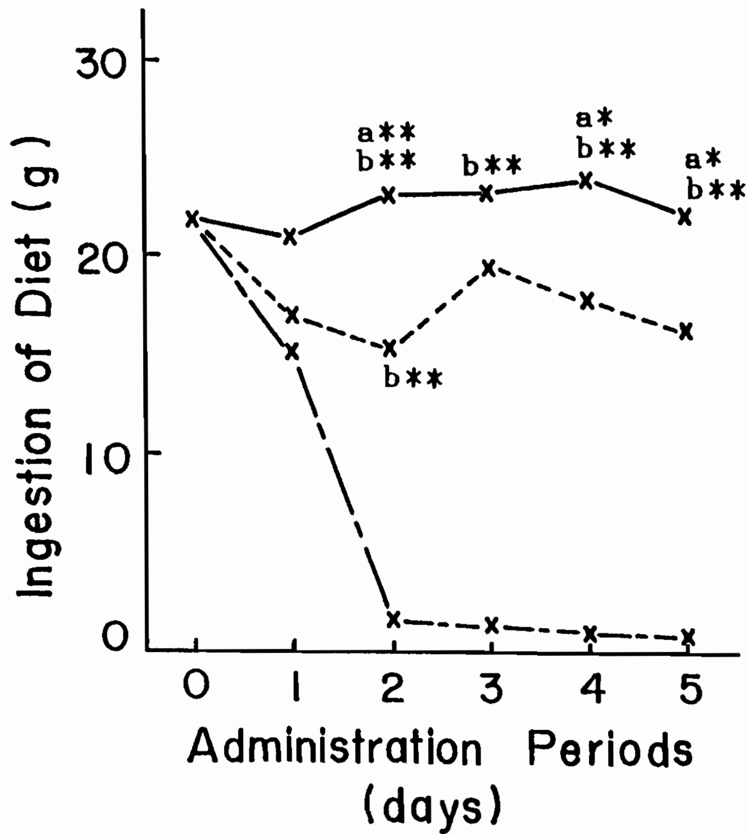


Fig. IV-3 Changes in diet ingestion in the pantethine-deficient group during the AL administration periods. ———, Non AL; -----, Low AL; —-—, High AL. Each value is the mean of 5 rats. [a]Significantly different from the low dose of AL group. [b]Significantly different from the high dose of AL group. *, p<0.05; **, p<0.01.

able to both eat (Figs. IV-3 and IV-4) and drink (Figs. IV-6 and IV-7), whereas the rats of the pantethine

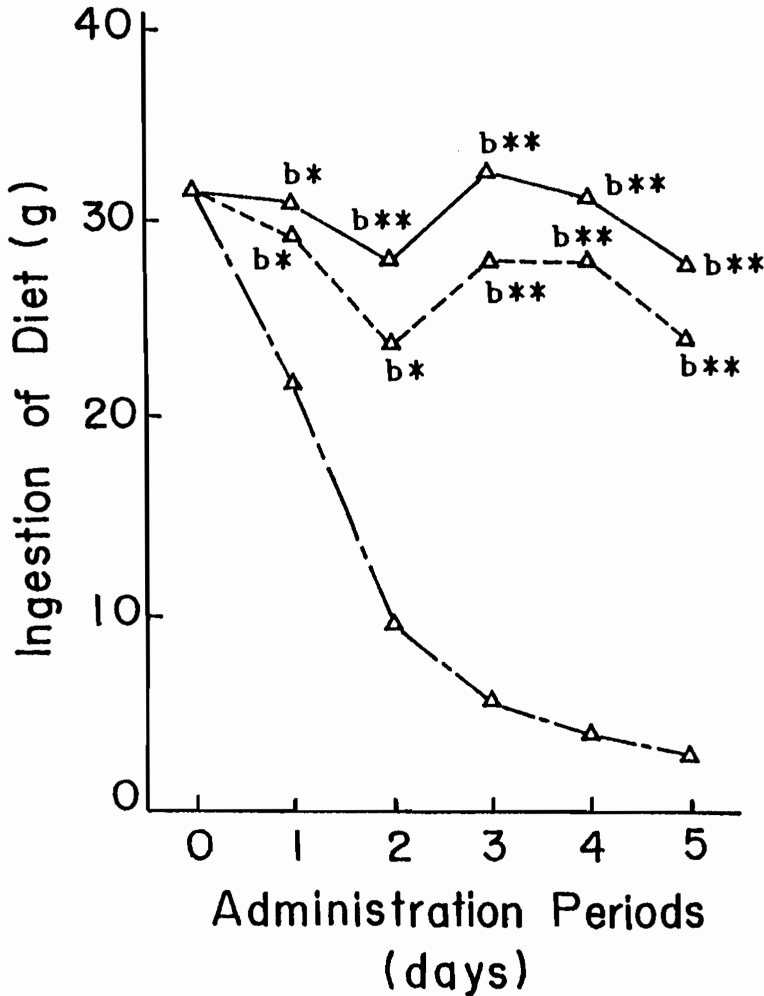


Fig. IV-4 Changes in diet ingestion in the pantethine-adequate group during the AL administration periods. ———, Non AL; -----, Low AL; —-—-—, High AL. Each value is the mean of 5 rats. [a]Significantly different from the low dose of AL group. [b]Significantly different from the high dose of AL group. *, $p<0.05$; **, $p<0.01$.

-excess group could drink water sufficiently (Fig. IV-8).

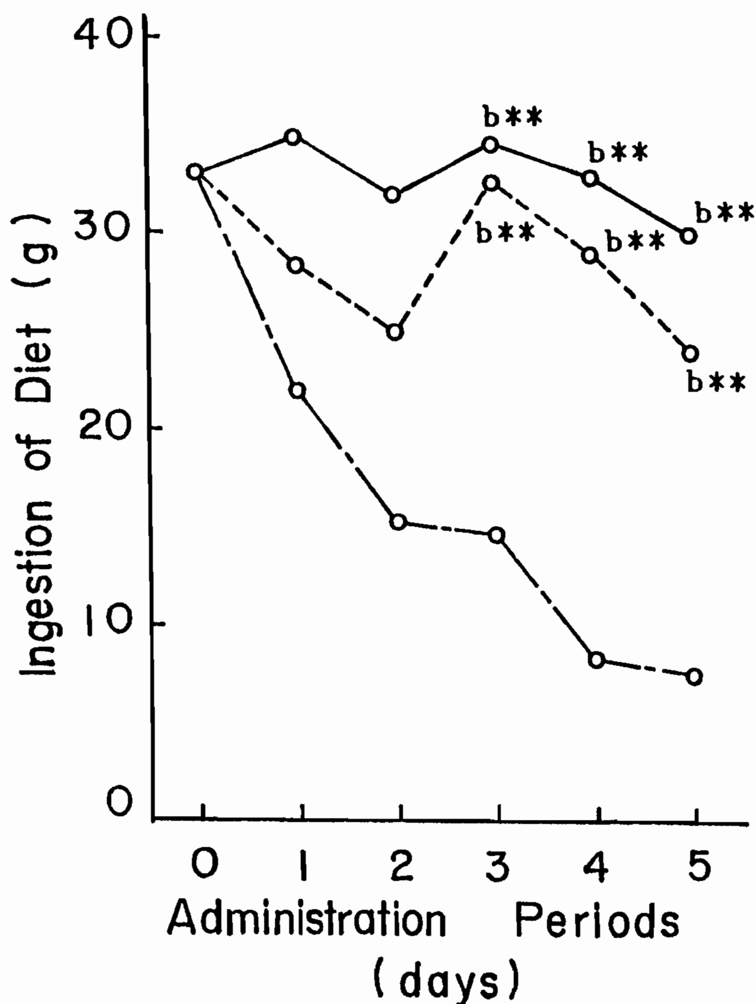


Fig. IV-5 Changes in diet ingestion in the pantethine-excess group during the AL administration periods. ———, Non AL; -----, Low AL; —-—, High AL. Each value is the mean of 5 rats. [a]Significantly different from the low dose of AL group. [b]Significantly different from the high dose of AL group. **, $p<0.01$.

Changes in body weight.

No difference between the pantethine-adequate and

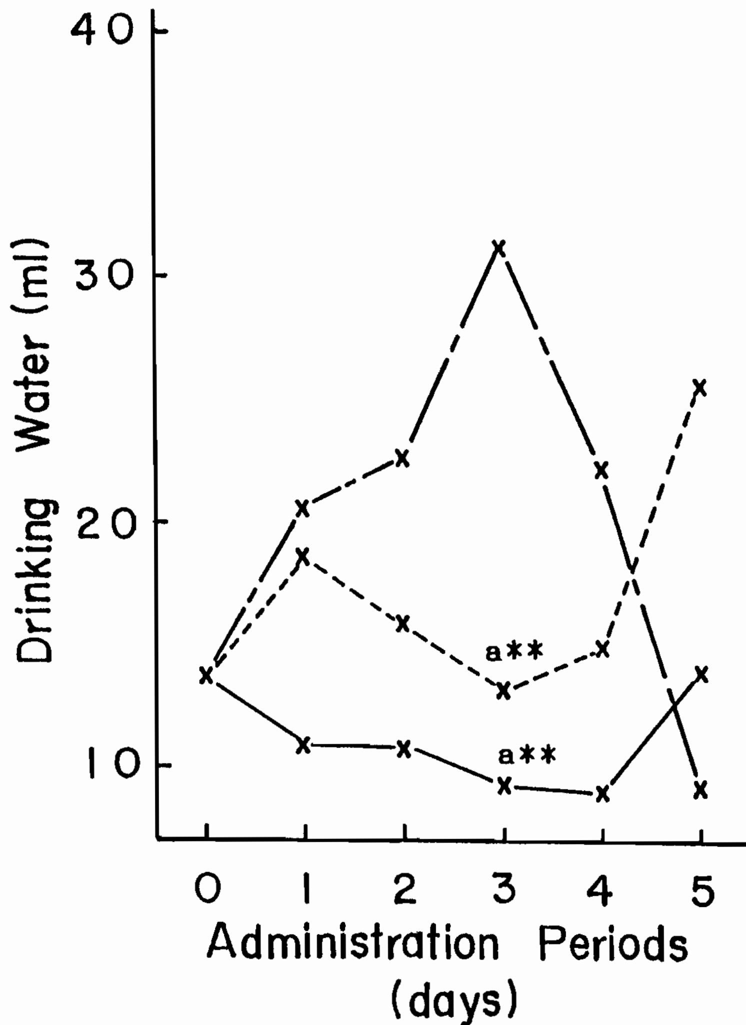


Fig. IV-6 Changes in drinking water ingestion in the pantethine-deficient group during the AL administration periods. ———, Non AL; -----, Low AL; — — —, High AL. Each value is the mean of 5 rats. [a]Significantly different from the high dose of AL group. **, $p<0.01$.

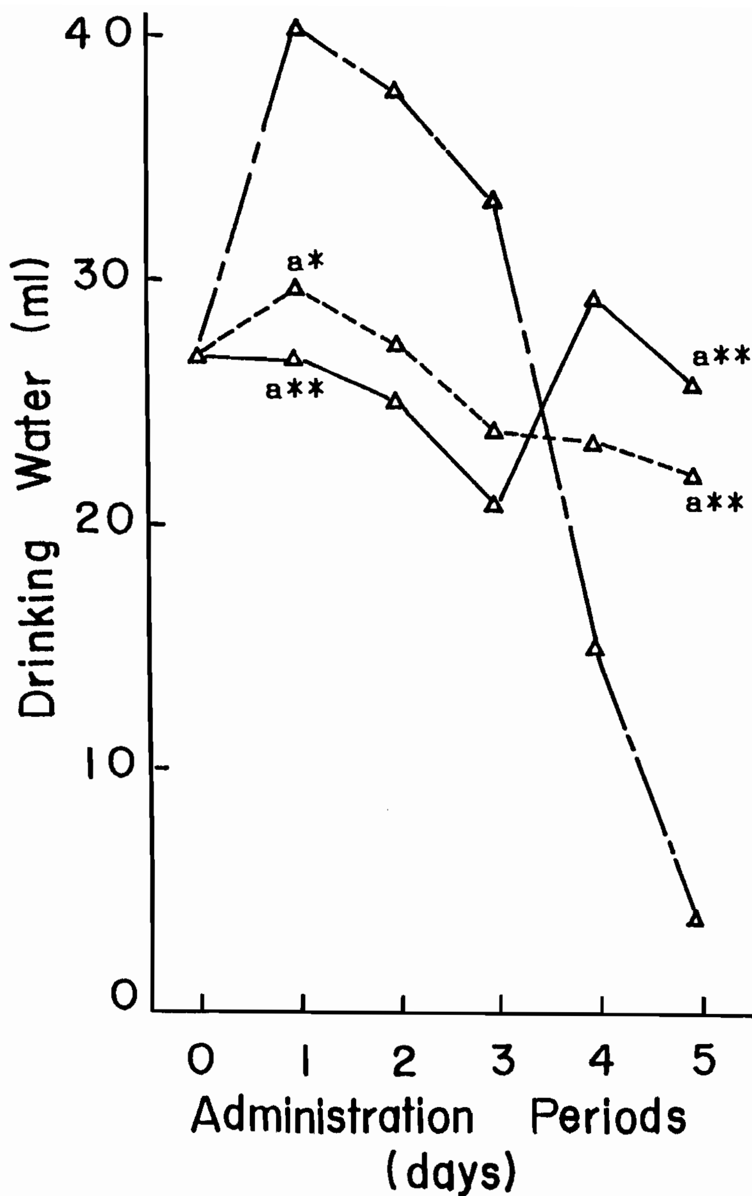


Fig. IV-7 Changes in drinking water ingestion in the pantethine-adequate group during the AL administration periods. ———, Non AL; -----, Low AL; —-—, High AL. Each value is the mean of 5 rats. [a]Significantly different from the high dose of AL group. *, $p<0.05$; **, $p<0.01$.

-excess groups was observed in the growth of rats during the prefeeding for one month, but the growth of rats in the pantethine-deficient group was significantly different from the pantethine-adequate and -excess

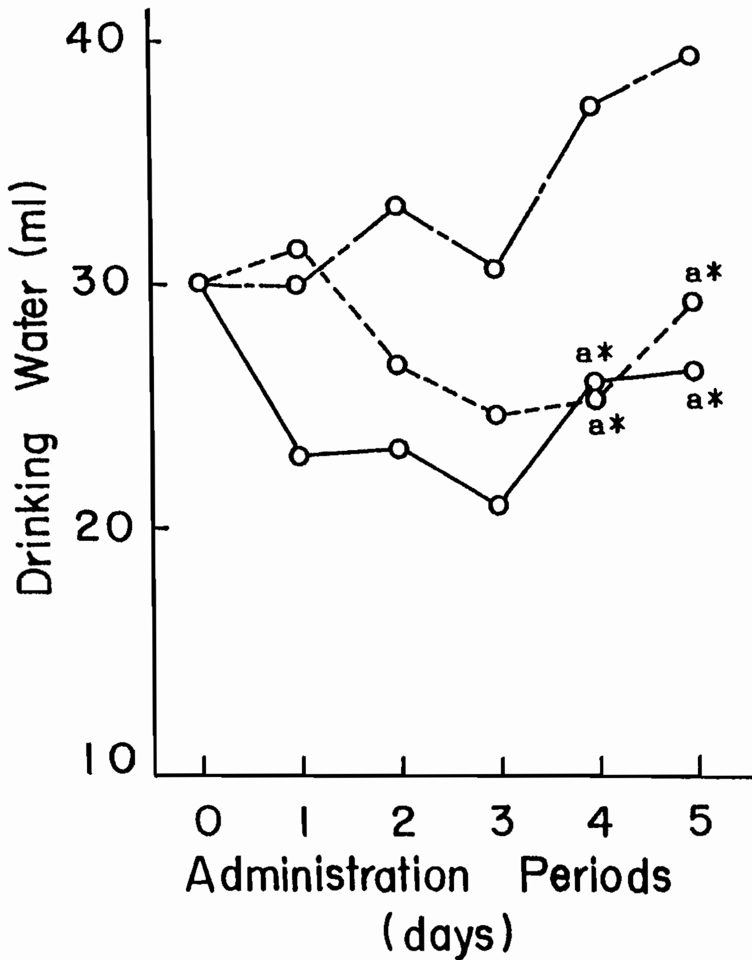


Fig. IV-8 Changes in drinking water ingestion in the pantethine-excess group during the AL administration periods. ———, Non AL; -----, Low AL; —·—, High AL. Each value is the mean of 5 rats. [a]Significantly different from the high dose of AL group. *, *p*<0.05;

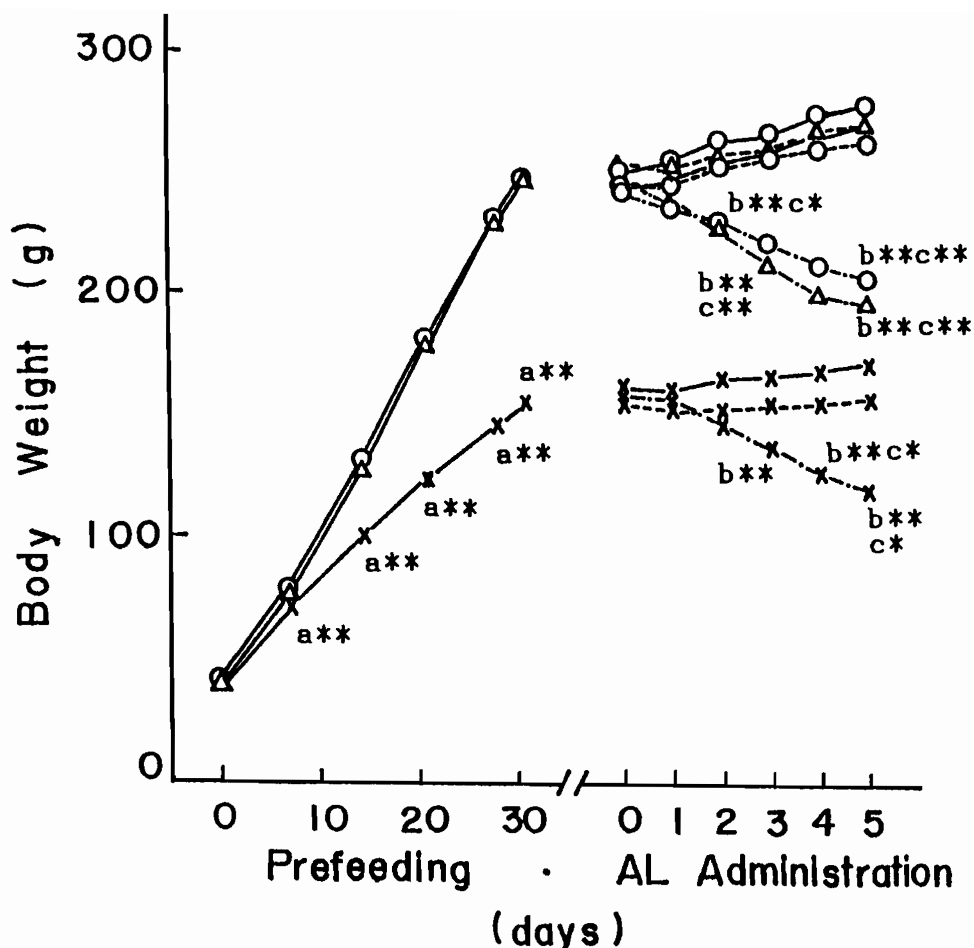


Fig. IV-9 Changes in body weights of rats during the whole experimental periods. ×, Pantethine-deficient group; Δ, Pantethine-adequate group; o, Pantethine-excess group. ———, Non AL; -----, Low AL; —-—, High AL. Each value is the mean of 15 rats during prefeeding periods. Each value is the mean of 5 rats during AL administration periods. [a]Significantly different from the pantethine-adequate and -excess groups. [b]Significantly different from non AL group in the same diet groups. [c]Significantly different from the low dose of AL group in the same diet groups. *, $p<0.05$; **, $p<0.01$.

groups, as shown in Fig. IV-9.

With the low level of AL dose for 5 successive days, the body weight of rats kept increasing similarly to the respective non-AL groups in any pantethine level group. With the high level of AL dose for 5 successive days, the body weight of rats kept decreasing in contrast with the respective non-AL and the low level of AL dose groups in any pantethine level groups (Fig. IV-9).

(1) (2) (3)

Fig. IV-10 Diet scattered by rats.
(1) Pantethine-deficient group. (2) Pantethine-adequate group. (3) Pantethine-excess group.

Fig. IV-11 Rat fed on pantethine-deficient diet for one month.

Behavior of rats.

Rats in the pantethine-adequate and -excess groups grew normally without abnormal actions and symptoms. On the other hand, rats in the pantethine-deficient group showed abnormal action and various symptoms. That is, the pantethine-deficient rats scattered vigorously the diet after 20 days of the pre-feeding (Fig. IV-10). After about 24 days of the pre-feeding, the rats

deficient in pantethine (*i.e.* pantothenic acid) began to exhibit loss of hair, which became remarkably within three or four days after that (Fig. IV-11). The hardening of the rat skin in the pantethine-deficient group was observed at the blood drawing from the heart, for a needle of injector was hard to stick in the skin, compared with those of the pantethine-adequate and -excess groups. After the AL administration, the survival period of the rats was longer in the pantethine-excess group rather than in the pantethine-adequuate group. That is, three rats died by the high AL dose (0.4 ml/100 g body weight) in the pantethine-adequate group, but all the rats in the pantethine-excess group were vigorous even under the same condition of the AL administration.

Changes in liver weight.

As shown in Table IV-2, the effect of AL dose level on liver weight is statistically significant, and the effect of dietary pantethine level and interaction were not significant. Namely, the liver weights increased by the low level of AL dose in comparison with non-AL, and lowered by the high level of AL dose in comparison with the low level of AL dose. The

Table IV-2. Changes in liver weight.

Group No.	(g/100 g body weight)	ANOVA	
1	4.51 ± 0.12	AL	**
2	4.98 ± 0.34	PaSS	NS
3	4.78 ± 0.33	AL×PaSS	NS
4	4.61 ± 0.14		
5	4.91 ± 0.14		
6	4.63 ± 0.23		
7	4.56 ± 0.20		
8	4.96 ± 0.18		
9	4.69 ± 0.39		

Groups 1-9, see Table IV-1. AL, autoxidized linoleate. PaSS, pantethine. Values are mean ± SD for 7 rats in each group. NS, not significant. **, $p<0.01$.

Table IV-3. The activities of GOT and GPT in serum after the prefeeding for one month.

Pantethine status	GOT		GPT	
Deficient	148.5 ± 19.0[a,b]	(n=7)	29.4 ± 5.6[a,b]	(n=7)
Adequate	108.0 ± 7.2	(n=4)	20.1 ± 2.2	(n=7)
Excess	102.6 ± 5.6	(n=6)	17.1 ± 2.6	(n=7)

Values are mean ± SD. [a]significant difference from the pantethine-adequate group at $p<0.01$. [b]significant different from the pantethine-excess group at $p<0.01$.

increase of liver weight of rat by the low level of AL dose indicate hypertrophy of the liver. The slight decrease of liver weight of rat by the high level of AL dose may be recognized as an indication of hepat-atrophy.

Measurement of GOT and GPT.

As shown in Table IV-3, significant differences were observed in both serum GOT and GPT of rats between the pantethine-deficient group and the other groups.

III-4 DISCUSSION

Studies on pantothenic acid was originated with the study of Bios by Wildier in 1901, and had been carried on by many workers from multi-sided fields. In each step, pantothenic acid was given various names, such as Liver filtrate factor, Yeast filtrate factor, Chick antipellagra factor, Antidermatitis factor, or Chick filtrate factor. Because of its wide distribution in tissues of diverse species, in 1933 R. J. Williams (*125*) named it 'Pantothenic acid', which is from the Greek, meaning 'from everywhere'.

Workers in the early days had been studying on the

deficiency of pantothenic acid (*119-123*). However, since Lipman found out in 1947 that pantothenic acid was included in coenzyme A for acetylation of amine (*126*) and determined chemical structure of coenzyme A (*127, 128*), the biochemical studies on pantothenic acid had been rapidly developed. It seemed as if all the role of pantothenic acid compounds in the living body had been explained through coenzyme A. Coenzyme A, which consists of pantothenic acid compounds, is necessary for many kind of biochemical reactions such as TCA cycle, metabolism of fatty acids or amino acids, and biosyntheses of choresterol (*115, 116*), porphyrin (*117*), and acetylcholine (*118*). Another biochemical role of pantothenic acid compounds was also evidenced in 1965 in addition to coenzyme A. Majerus *et al.* (*129*) found out that phosphopantetheine was contained in the active site of acyl carrier protein essential for fatty acid synthase system. Thus, since pantothenic acid compounds participate in comprehensive metabolisms *in vivo*, pantothenic acid deficiency in animals causes not only dermatitis but also complicated morbid states, such as gastrointestinal disturbances, reduced antibody production, and impaired adrenal function, which may be resulted from assemblage of various metabolic

disturbances.

The animals deficient in pantothenic acid exhibit some characteristic appearances, such as pollution around eyes termed 'spectacle eye' by porphyrins pigment, dermatitis, alopecia, besides reduced growth rate or loss of body weight observed also in the case of the other vitamins-deficiency.

In the present chapter, some typical symptoms from pantothenic acid-deficiency was also observed; namely, the rats of pantethine-deficient (*i.e.* pantothenic acid deficient) group showed not only reduced growth rate, roughness of hair, alopecia but also hardening of skin and hepatopathy. It has been reported by Kanke *et al* (*130*) that pantothenic acid increased the hyaluronic acid content in male mouse skin. It is well known that hyaluronic acid has the property of moisture keeping, and thereby participates in a lubricating function in joint or a softness of skin. In this experiment, the hardening of rat skin in the pantethine-deficiet group might be due to the decrease of hyaluronic acid content in rat skin. The rats of both pantethine-adequate and -excess groups grew up normally during the prefeeding. No difference between the two groups was also observed in the appearance of rats.

Then to the rats of each group fed on the diets having three levels of pantethine were administered 0, 0.2, or 0.35 - 0.4 ml AL/100 g body weight/ day for 5 successive days, respectively. With the low level of AL dose for 5 successive days, the body weight of rats in any pantethine levels kept increasing almost similarly to the respective non-AL groups. With the high level of AL dose for 5 successive days, the body weight in any pantethine level kept decreasing in contrast with the respective non-AL and the low level of AL dose groups. In the pantethine-adequate, three rats died by the high level of AL administration for 4 days, but all the rats in the pantethine-excess group were vigorous even under the same condition. Thus, pantethine-excess level in diet seems to relieve the toxicity caused by the high level of AL dose. In the next chapter, I will discuss the changes in the drug-metabolizing system under the same conditions as described in this chapter.

V EFFECTS OF DIETARY PANTETHINE LEVELS ON DRUG-METABOLIZING SYSTEM IN THE LIVER OF RATS ORALLY ADMINISTERED VARYING AMOUNTS OF AUTOXIDIZED LINOLEATE

V-1 INTRODUCTION

Pantethine decreased the injury in the rat liver caused by carbon tetrachloride (*84*), and improved a dermatopathy developed by an *in vivo* lipid peroxide (*85, 86*), and also decreased a cardiotoxicity produced by an administration of adriamycin (*87*). Although the pantethine itself does not have a reducing property, the metabolite of pantethine may function as one of the antioxidants against *in vivo* lipid peroxidation which occurs mainly at the biomembrane.

The enzymes of drug-metabolizing system are also present in biomembrane, especially in the electron transfer system of liver microsomes. It seems likely that the system is affected by both lipid peroxides yielded from *in vivo* metabolism of ingested xenobiotics and the endogenous scavengers against the lipid peroxides. There are several reports (*74, 75, 80, 83, 131, and 132*) on the effects of xenobiotics (mainly polychlorinated biphenyl) and scavengers (mainly

vitamin E, ascorbic acid or vitamin A) on the changes in drug-metabolizing system and lipid peroxide formation *in vivo*. However, there have never been any studies on the effect of the ingestion of pantethine on drug-metabolizing activity in the liver microsomes of orally AL-administered rats.

From the standpoint of nutritional science, I planned to investigate the effects of dietary pantethine levels on the drug-metabolizing system in rat livers with both a low and a high level of AL doses. Thus, in the present study, the changes in cytochrome P-450 and b_5 contents, the enzyme activities of electron transfer system, and drug-metabolizing activities were determined with microsomes or the postmitochondrial 9,000 x *g* supernatant (S-9 fraction) prepared from the liver of male Wistar rats administered a low or a high level of AL dose for 5 successive days.

V-2 MATERIALS AND METHODS

Chemicals. All the chemicals were purchased from the same pharmaceutical companies as described in the preceding chapters.

Animals and diet. Male Wistar rats, 3 weeks old and each weighing about 40 g (JCL, Tokyo, Japan), were divided into 9 groups (5 rats/group), and were housed at approximately 23 ^{o}C with a light and dark cycle of 12 h each. They were fed for one month on the same diet (Clea Japan Inc., Tokyo) as described in Chapter II. As shown in Table IV-1 in Chapter IV, the drinking water containing 0, 6.25, or 125 mg pantethine/100 ml was given to the rats, which were accordingly designated the pantethine-deficient, -adequate, and -excess groups, respectively. The diet and drinking water were provided *ad libitum*.

Administration of AL. After the initial feeding for one month, AL prepared by the same way as described in Chapter II was orally administered to the rats of each group of the three pantethine levels at a daily dose of 0, 0.2, and 0.4 ml/100 g body weight for 5 successive days, as shown in Table IV-1 in Chapter IV.

Preparation of S-9 and microsomes. The S-9 and microsomes were prepared from rat liver as noted

in Chapter II.

Measurements of the enzyme activities in electron transfer system. NADPH-cytochrome *c* reductase, NADH-ferricyanide reductase, and NADH-cytochrome *c* reductase activities were determined by the methods of Omura and Takesue (*92-94*) as described in Chapter II.

Other measurement methods. Cytochrome P-450 and cytochrome b_5 contents were determined by the method of Omura and Sato (*38*) as described in Chapter II. The S-9 activity and the aminopyrin-N-demethylase activity were determined according to the methods of Yahagi (*91*) and Kato *et al.* (*75*), respectively.

Statistical analysis. Statistical significance of differences among values was analyzed by the same way as described in Chapter IV.

V-3 RESULTS

Changes in cytochrome P-450 and cytochrome b_5 contents.

The changes in the cytochrome P-450 content are shown in Fig. V-1. The effects of AL dose level on the

cytochrome P-450 content were statistically significant, but both the effect of dietary pantethine

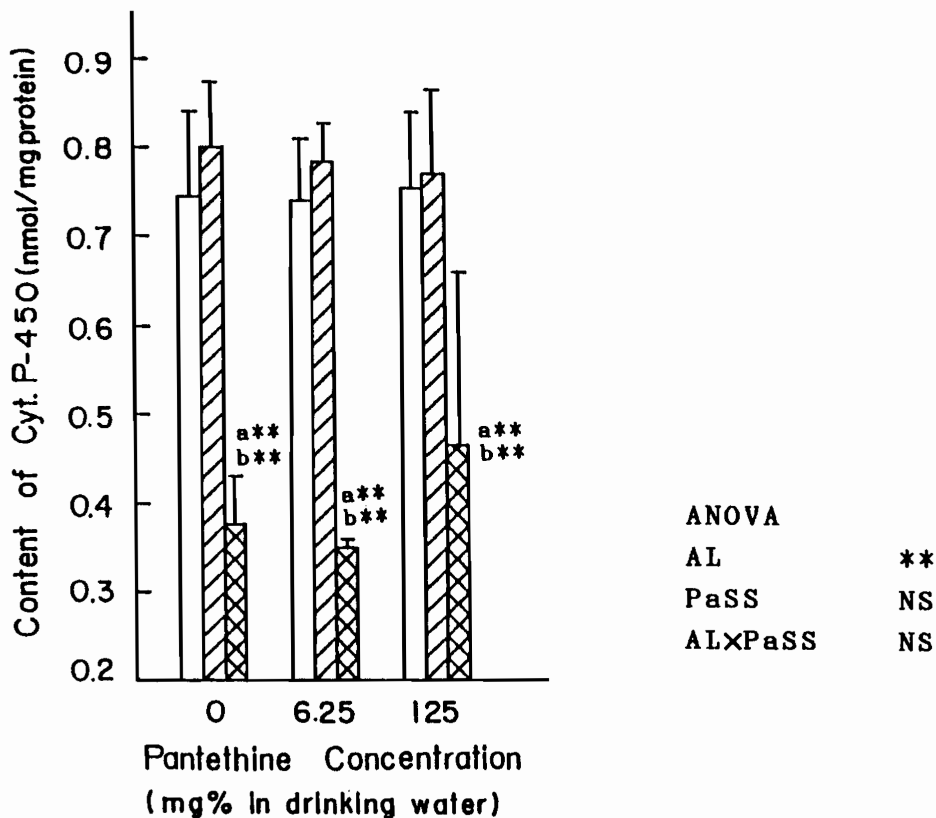


Fig. V-1. Changes in cytochrome P-450 content after AL administration. □, Non-AL; ▨, 0.2 ml AL/100 g body weight for 5 successive days; ⊠, 0.4 ml AL/ 100 g body weight for 5 successive days. [a]Significantly different from the non AL group in the same level of dietary pantethine. [b]Significantly different from the low dose of AL group in the same level of dietary pantethine. *, $p<0.05$; **, $p<0.01$.

level and the interaction between AL and pantethine on the content was not significant. The content showed a tendency to increase by the low level of AL dose in any

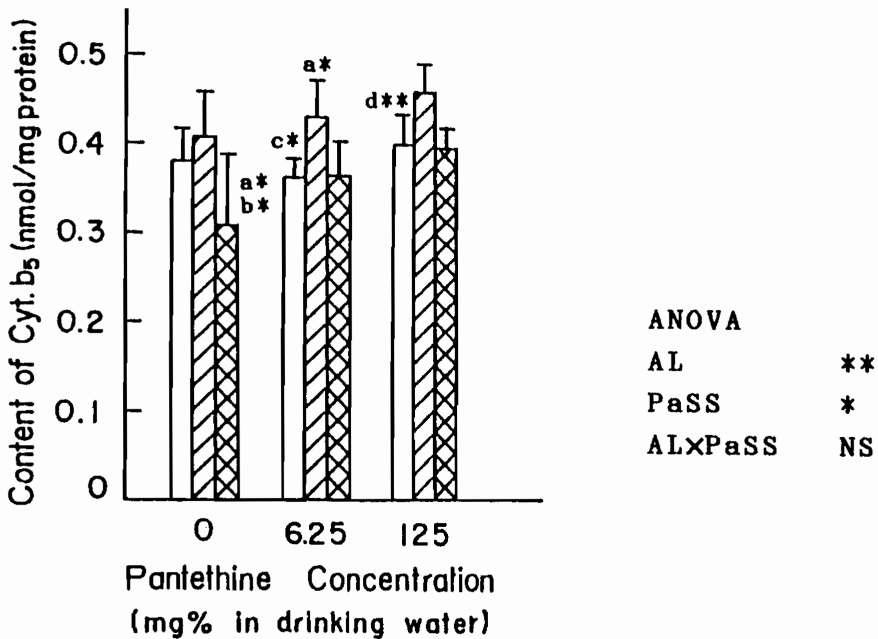


Fig. V-2. Changes in cytochrome b_5 content after AL administration. □, Non-AL; ▨, 0.2 ml AL/100 g body weight for 5 successive days; ⊠, 0.4 ml AL/ 100 g body weight for 5 successive days. [a]Significantly different from the non AL group in the same level of dietary pantethine. [b]Significantly different from the low dose of AL group in the same level of dietary pantethine. [c]Significantly different from the pantethine-deficient group in the same level of AL dose. [d]Significantly different from the pantethine-adequate group in the same level of AL dose. *, $p<0.05$; **, $p<0.01$.

level of dietary pantethine, but it was not significant. The content was markedly decreased by the high level of AL dose in any dietary pantethine level. The content in the pantethine-excess group showed a higher level than that of the pantethine-adequate group, though it was not significant.

The changes in the cytochrome b_5 content are shown in Fig. V-2. The effects of AL dose level and dietary pantethine level on the cytochrome b_5 content was statistically significant, but the interaction of them was not significant. The content was significantly increased by the low level of AL dose in the pantethine adequate group, and was decreased by the high level of AL dose only in the pantethine-deficient group. Unlike cytochrome P-450, significant decreases of cytochrome b_5 contents in the pantethine-adequate and -excess were not observed even by the high level of AL dose in comparison with non-AL and the low level of AL dose. This stability in the change of cytochrome b_5 content was also recognized in Chapters II and III.

Changes in enzyme activities of electron transfer system.

As shown in Fig. V-3, the effects of both AL dose

level and dietary pantethine level on the NADPH-cyt.*c* reductase activity were statistically significant, but

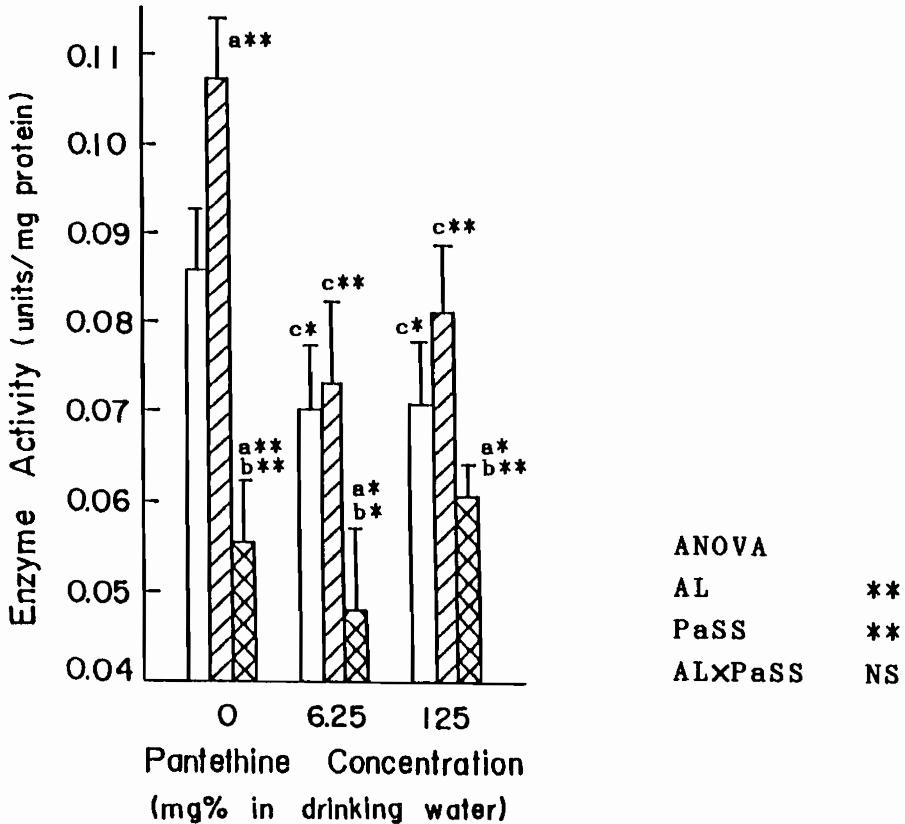


Fig. V-3. Changes in NADPH-cytochrome *c* reductase in electron transfer system after AL administration.
□, Non-AL; ▨, 0.2 ml AL/100 g body weight for 5 successive days; ⊠, 0.4 ml AL/100 g body weight for 5 successive days. [a]Significantly different from the non AL group in the same level of dietary pantethine. [b]Significantly different from the low dose of AL group in the same level of dietary pantethine. [c]Significantly different from the pantethine-deficient group in the same level of AL dose. *, $p<0.05$; **, $p<0.01$.

the interaction between them was not significant. The enzyme activity was significantly increased by the low

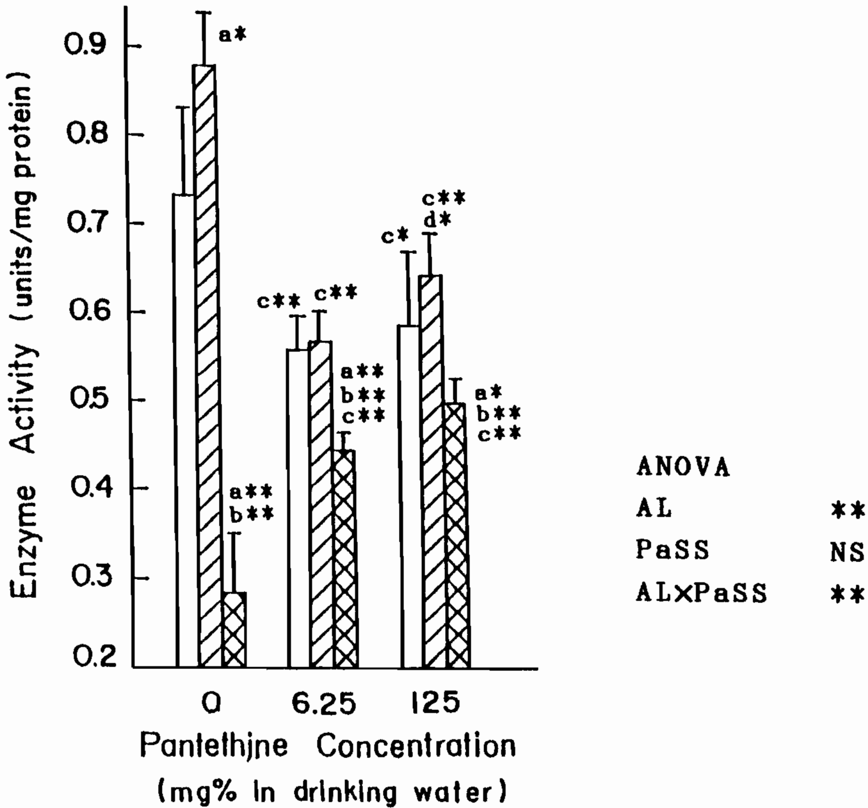


Fig. V-4. Changes in NADH-cytochrome *c* reductase in electron transfer system after AL administration.
□, Non-AL; ▨, 0.2 ml AL/100 g body weight for 5 successive days; ⊠, 0.4 ml AL/100 g body weight for 5 successive days. [a]Significantly different from the non AL dose group in the same level of dietary pantethine. [b]Significantly different from the low dose of AL group in the same level of dietary pantethine. [c]Significantly different from the pantethine-deficient group in the same level of AL dose. [d]Significantly different from the pantethine-adequate group in the same level of AL dose. *, $p<0.05$; **, $p<0.01$.

level of AL dose in the pantethine-deficient group, and decreased remarkably by the high level of AL dose in any level of dietary pantethine. In both the non-AL and the low level of AL dose, the enzyme activity was significantly higher in the pantethine-deficient group than in the pantethine-adequate and -excess groups.

As shown in Fig. V-4, the effect of AL dose level and the interaction with dietary pantethine on the NADH-cyt. *c* reductase activity were statistically significant, but the effect of dietary pantethine level on the enzyme activity was not significant. The enzyme activity was significantly increased by the low level of AL in the pantethine-deficient group, and decreased remakably by the high level of AL dose in any level of dietary pantethine. In both the non-AL and the low level of AL dose, the enzyme activity was significantly higher in the pantethine-deficient group than in the pantethine-adequate and -excess groups.

The effect of AL dose level on the NADH-ferricianide reductase was statistically significant, but both the effect of dietary pantethine level and the interaction with AL was not significant, as shown in Fig. V-5. The NADH-ferricianide reductase activity was significantly lowered by the high level of AL dose in

the pantethine-deficiet group.

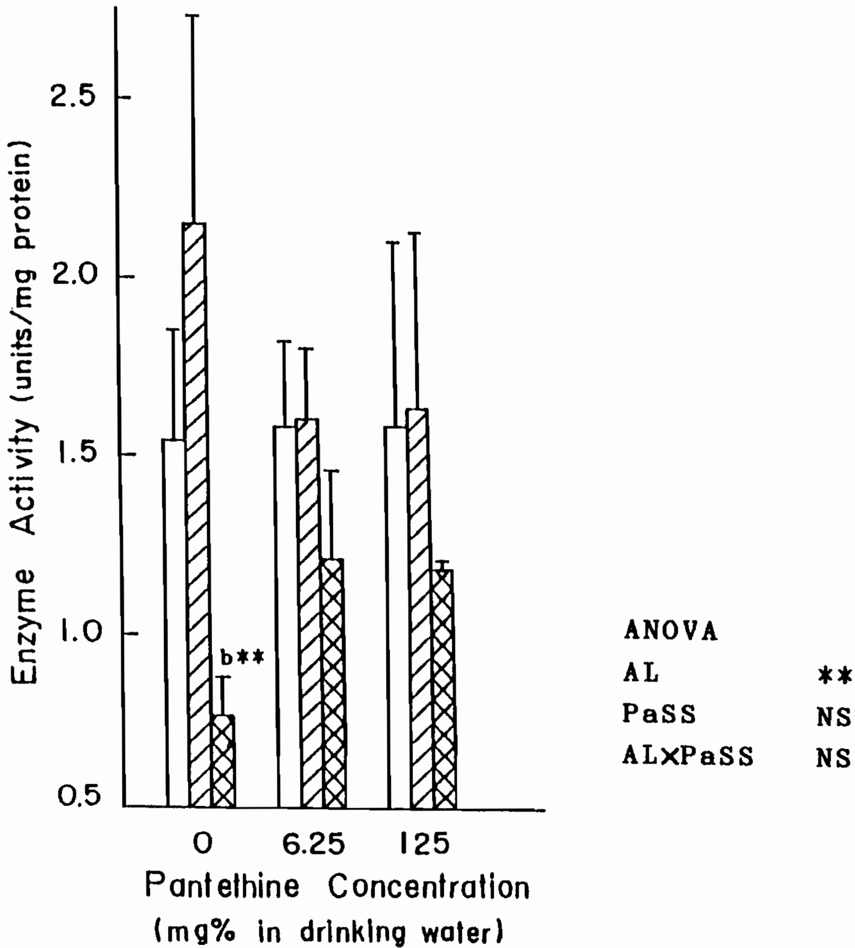


Fig. V-5. Changes in NADH-ferricyanide reductase in electron transfer system after AL administration.
□, Non-AL; ▨, 0.2 ml AL/100 g body weight for 5 successive days; ⊠, 0.4 ml/100 g body weight for 5 successive days. [b]Significantly different from the low dose of AL group in the same level of dietary pantethine. **, $p<0.01$.

Changes in drug-metabolizing enzyme activity.

Both the effect of AL dose level and the interaction between AL and pantethine were statistically significant, but the effect of dietary pantethine

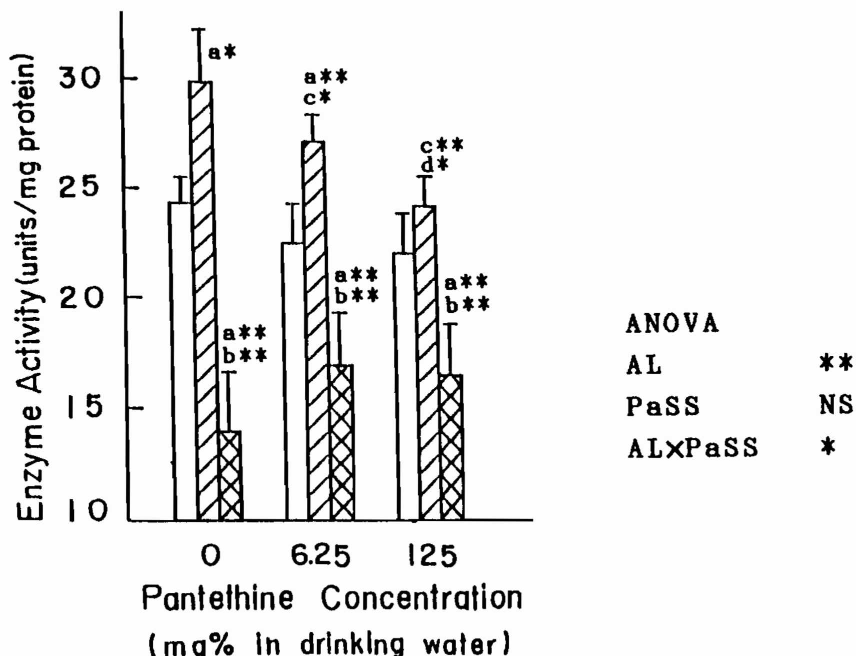
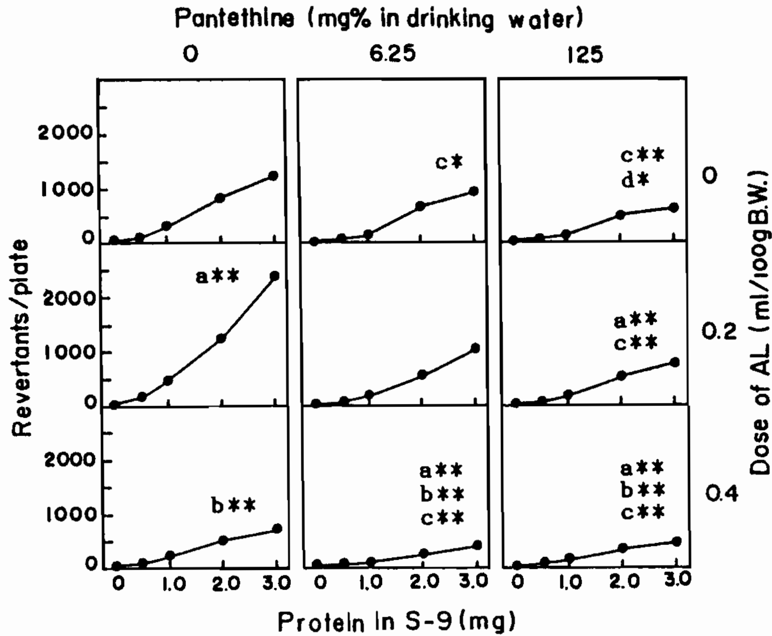


Fig. V-6. Changes in aminopyrin-N-demethylase activity in liver S-9 fraction after Al administration.
□, Non-AL; ▨, 0.2 ml AL/100 g body weight for 5 successive days; ⊠, 0.4 ml AL/100 g body weight for 5 successive days. [a]Significantly different from the non AL group in the same level of dietary pantethine. [b]Significantly different from the low dose of AL group in the same level of dietary pantethine. [c]Significantly different from the pantethine-deficient group in the same level of AL dose. [d]Significantly different from the pantethine-adequate group in the same level of AL dose. *, $p<0.05$; **, $p<0.01$.

level was not significant, as shown in Fig. V-6. The enzyme activity was significantly increased by the low



ANOVA	
AL	**
PaSS	**
AL×PaSS	**

Fig. V-7. Changes of S-9 activity in liver S-9 fraction after AL administration. [a]Significantly different from the non AL group in the same level of dietary pantethine. [b]Significantly different from the low dose of AL group in the same level of dietary pantethine. [c]Significantly different from the pantethine-deficient group in the same level of AL dose. [d]Significantly different from the pantethine-adequate group in the same level of AL dose. *, $p<0.05$; **, $p<0.01$.

level of AL dose in both the pantethine-deficient and -adequate groups, and remakably decreased by the high level of AL dose in any dietary pantethine level. In both the non-AL and the high level of AL dose, the effect of dietary pantethine level was not recognized on the enzyme activity. In the low level of AL dose, the enzyme activity showed significantly lower value corresponding to the increase of dietary pantethine level.

The effects of both AL dose level and dietary pantethine level, and the interaction between them were statistically significant on the S-9 activity (Fig. V-7). The activity was increased by the low level of AL dose in both the pantethine-deficient and -excess groups, and decreased by the high level of AL dose in any dietary pantethine level. In any AL dose level, the activity was higher in the pantethine-deficient group than the groups of the other dietary pantethine level.

V-4 DISCUSSION

The characteristic changes in the cytochrome P-450 and b_5 contents, the enzyme activities of electron transfer system, and the activities of drug-metabo-

lizing enzymes were discussed in detail in Chapters II and III.

In the present chapter, I also recognized at any pantethine level that the low level of AL dose increased the contents and the activities of drug-metabolizing enzymes and that the high level of AL dose decreased them. Therefore, it has become clear that the effects of AL dose levels on drug-metabolizing system change essentially in the same way, in any pantethine levels.

In this chapter, I focus on and discuss the difference in induction levels of drug-metabolizing system among pantethine-deficient, -adequate, and -excess groups. No difference was observed in the contents of cytochrome P-450 and b_5 both by the non-AL and by the low level of AL dose in any pantethine level (Figs. V-1, V-2). However, even under the condition of non-AL, the activities of NADPH-cyt. *c* reductase and NADH-cyt. *c* reductase rose in the pantethine-deficient group rather than in the pantethine-adequate and -excess groups (Figs. V-3, V-4). Their induction levels in the pantethine-deficient group, moreover, rose by the low level of AL dose in comparison with the other groups. The reason why, in spite of no difference in

the cytochrome P-450 content, the drug-metabolizing activities rose in the pantethine-deficient group rather than in the other groups might have been attributable to the rise of these enzyme activities in the electron transfer system.

As mentioned in the introduction section, it has been reported *(84-87)* that pantethine has an antioxidative effect against lipid peroxide *in vivo*. The absorbed pantethine, not having a reducing property by itself, might be reduced to pantetheine *via* the redox system *in vivo*, and the resultant pantetheine might have depressed lipid peroxidation *in vivo* by the reducing property originating from its sulfhydryl group. In the rats fed on a diet deficient in both pantothenic acid and its related compounds, the drug-metabolizing system was induced in order to decompose the lipid peroxides resulting from the *in vivo* redox system because radical scavengers decreased, and thereby *in vivo* lipid peroxidation would be more accelerated. The rise of drug-metabolizing activity in the pantethine-deficient non-AL group may be a reasonable response of the system against *in vivo* elevated accumulation of lipid peroxide. Presumably, symptoms such as alopecia or scleroderma observed in

the pantethine-deficient non-AL group would appear as results of *in vivo* lipid peroxidation. The drug-metabolizing activities in the pantethine-deficient group was increased more by the low level of AL dose in comparison with those of the other groups. These experimental facts suggest that, in such an extent of AL dose level as the body weight did not decrease, the less the scavenger such as pantetheine exist, the more the drug-metabolizing activities rise.

The high level of AL dose decreased the drug-metabolizing activity in any pantethine level. In this experiment, the rats in all the pantethine levels were severely damaged by the high level of AL dose. Under such a drastic condition, it was difficult to observe the difference of reduction in drug-metabolizing system because the system was injured markedly in any pantethine level. However, the survival period of the rats in the pantethine-excess group was longer than that of the pantethine-adequate group, and the cytochrome P-450 content of the pantethine-excess group showed a higher level than that of the pantethine-adequate group. In addition, I also found in preliminary experiments that the contents and activities of enzymes of drug-metabolizing system in

the liver of AL-administered rats were well maintained in the pantethine-excess level diet group rather than in the adequate level diet group, when male Wistar rats were fed on commercial feeds (Clea Japan Inc., Tokyo, type CE-2 which contains 3.0 mg pantothenic acid per 100 g diet) and drinking water containing 0 mg (adequate level diet) or 125 mg pantethine (excess level diet)/100 ml. These observations suggest that the pantethine-excess diet may effectively protect drug-metabolizing system from damage by AL.

In conclusion, it seems likely that pantethine relieves the effect of both a low and a high level of AL doses on drug-metabolizing system in the rat liver. In the next chapter, I will describe the effect of dietary pantethine levels on *in vivo* lipid peroxidation in the liver of AL-administered rats.

VI EFFECTS OF DIETARY PANTETHINE LEVELS ON CONTENTS OF FATTY ACIDS AND THIOBARBITURIC ACID REACTIVE SUBSTANCES IN THE LIVER OF RATS ORALLY ADMINISTERED VARYING AMOUNTS OF AUTOXIDIZED LINOLEATE

VI-1 INTRODUCTION

The contents and activities of the enzymes in the drug-metabolizing system were induced by the low level of AL dose for 5 successive days and lowered by the high level of AL dose for the same period, compared with respective non-AL groups in each of the three pantethine levels. In groups of either non-AL or the low level of AL dose, the enzyme activities of electron transfer system in the rat liver microsomes, the aminopyrin-N-demethylase activity, and the metabolic activation of 2-AAF in the S-9 fraction were significantly higher in the pantethine-deficient group than in the pantethine-adequate and -excess groups. These results suggested that the pantethine-deficiency and the low level of AL dose accelerated lipid peroxidation *in vivo*, followed by the induction of drug-metabolizing system in order to depress the enhanced *in vivo* lipid peroxidation. In the high level

of AL dose, the pantethine-excess diet seemed to effectively protect drug-metabolizing system in comparison with the pantethine-adequate diet, though the enzyme activities in the system decreased significantly in each pantethine level group. It was concluded from these results that pantethine might have relieved the effect of either a low or a high level of AL dose on the drug-metabolizing system in the rat liver.

In the present chapter, I planned to investigate the effect of dietary pantethine level on the contents and compositions of fatty acids and the extent of lipid peroxidation in the liver and the S-9 fraction of rats orally administered varying amounts of AL. Thus, fatty acid contents and TBA values are determined with liver homogenates and the S-9 fraction including drug-metabolizing system on the same experimental conditions as described in Chapter IV.

VI-2 MATERIALS AND METHODS

Chemicals. Linoleic acid was of extra-pure reagent grade from Nakarai Chemicals Ltd, Kyoto, and its purity as determined by gas chromatography was about 95 %.

Fatty acid standards (palmitic, stearic, oleic, linoleic, linolenic, arachidonic, and docosahexanoic acids) were purchased from Gasukuro Kogyo, Tokyo. Five % trifluoro-methylphenyltrimethyl-ammonium hydroxide in methanol (TFM-PTAH) was from Nishio Kogyo, Tokyo. The column packing was 15 % diethyleneglycol succinate (DEGS) on acid washed and dimethylchlorosilane (DMCS) treated Chromosorb W (60-80 mesh) (Gasukuro Kogyo). All other reagents used for gas chromatography were of analytical reagent grade. Bovine serum albumin was obtained from Sigma Chemical Co. St. Louis, MO. Pantethine (60 % solution), D-bis-(pantothenyl-β-aminoethyl) disulfide, was kindly supplied from Daiichi Seiyaku Co., Tokyo. The other chemicals were of guaranteed reagent grade from Nakarai Chemicals Ltd.

Animals and diet. Male Wistar rats, 3 weeks old and each weighing about 40 g (JCL, Tokyo), were divided into 9 groups (5 rats/group), and were housed at approximately 23 ^{o}C with a light and dark cycle of 12 h each. They were fed for one month on the same diet (Clea Japan Inc., Tokyo) as described in Chapter II. The drinking water containing 0, 6.25, or 125 mg pantethine/100 ml was given to the rats, which were

accordingly designated the pantethine-deficient, -adequate, and -excess groups, respectively, as described in Chapter IV (Table IV-1). The diet and drinking water were provided *ad libitum*.

Administration of AL. After the initial feeding for one month, AL prepared by the same way as described in Chapters II was orally administered to the rats of each group of the pantethine levels at a daily dose of 0, 0.2, and 0.35 ml/100 g body weight for 5 successive days, under the same conditions as shown in Table IV-1 in Chapter IV.

Preparation of liver homogenate and its S-9 fraction. The liver homogenate and its S-9 fraction were prepared by a modification of the method of Yahagi (*91*), as described in Chapters II, III and V.

Extraction of lipid and methylation. Lipids were extracted from 1 ml of the rat liver homogenate or S-9 fraction with 3 ml of a mixture of chloroform : methanol (1:2, v/v) containing 50 ppm of butylated hydroxytoluene (BHT), and were centrifuged at 3,000 rpm for 5 min. The precipitate was extracted again with 3.8

ml of a mixture of chloroform : methanol : water (1:2:0.8, v/v/v) containing 50 ppm of BHT, and were centrifuged again at 3,000 rpm for 5 min. The first and second supernatants were combined, and 2 ml of chloroform and 2 ml of water were added to the combined extract. Then, the extract was allowed to stand overnight. After removal of water soluble substances from the extracts, lipid samples were obtained after drying in a stream of nitrogen.

A 0.5 ml of standard solution and/or sample in benzene was pipetted into a 2-ml screw-capped test tube. To this was added 200 μl of TFM-PTAH. The tube was capped and left at room temperature for 15 min. In this way, the esterification of fatty acids was performed completely. An aliquot (4 μl) of this solution was analyzed by gas chromatography.

Gas chromatography. The chromatographic system consisted of a Hitachi Model 163 gas chromatograph with a flame ionization detector (FID) and a data processor (Shimadzu Model C-R3A). A glass column (200 x 0.3 cm I.D.) was packed with 15 % DEGS. The column temperature was set at 180 ^{o}C. Other details: the temperatures of injector block and detector were 210 ^{o}C; The flow rate

of carrier gas (nitrogen) was 70 ml/min. Peaks were identified by comparison with those of standards. The content of each fatty acid in the sample was calculated by comparison with peak area of the corresponding standard fatty acids.

Measurement of TBA value. The TBA values in liver homogenate and the S-9 fraction were measured by the method of Masugi and Nakamura (*133*). To liver homogenate or S-9 fraction (0.25 ml) in a tube was added 0.25 ml of 0.05 M phosphate buffer (pH 7.0), 0.2 ml of 7 % sodium dodecylsulfate, 2.0 ml of 0.1 N HCl, 0.3 ml of 10 % phosphotungstic acid, and 1.0 ml of 0.5 % TBA. The air in the tube was replaced by a stream of nitrogen gas, and the tube was capped. After boiling for 45 min, the reaction mixture was cooled in ice bath and 5.0 ml of 1-butanol was added to the mixture with vigorous shaking. Then the mixture was centrifuged at 3,000 rpm for 5 min. The absorption at 532 nm was measured for the butanol extract. Using *tetra*-ethoxypropane as the standard, TBA value was expressed as malondialdehyde content in liver homogenate or S-9 fraction. In the liver homogenate, the TBA value was calculated per g liver weight or mg fatty acids. In the

S-9 fraction, the TBA value was calculated per mg protein or mg fatty acid.

Statistical analysis. Statistical significance of differences among values was analyzed by the same way as described in Chapter IV.

VI-3 RESULTS

Changes in fatty acids compositions and contents.

The changes in fatty acid compositions and contents in the liver homogenate and the S-9 fraction are shown in Table VI-1 and VI-2, respectively. The effects of the levels of AL dose ($p<0.01$) and dietary pantethine ($p<0.01$), and the interaction ($p<0.01$) between them were statistically significant on the total fatty acid contents both in the liver homogenate and in the S-9 fraction.

In the case of non-AL, no difference in the total fatty acid contents of liver homogenates was observed between the pantethine-deficient (Group 1) and -adequate (Group 4) groups, whereas the contents of palmitic acid ($p<0.01$) and total fatty acids ($p<0.01$) in liver homogenates of the pantethine-excess group

(Group 7) were significantly higher than those of the pantethine-deficient and -adequate groups (Table VI-1).

Table VI-1. Composition of fatty acids in liver homogenates. **(Continued on the next page.)**

Group No.	C16:0	C18:0 (%)	C18:1
1	9.2±0.5	18.1±1.2	13.3±0.6
2	9.2±0.5	17.3±0.9	12.5±0.9
3	11.5±1.5	19.5±2.4	8.0±1.0
4	9.6±0.6	17.2±2.0	14.9±0.6
5	10.0±0.3	14.4±1.4	12.9±1.6
6	10.0±0.5	16.6±1.9	8.8±0.4
7	11.6±0.5	19.3±0.7	14.3±0.7
8	11.3±0.2	19.0±0.5	12.6±0.5
9	10.7±0.7	20.8±0.5	12.1±0.8
ANOVA for content			
AL	**	**	**
PaSS	**	**	**
AL×PaSS	NS	*	**
ANOVA for composition			
AL	*	*	**
PaSS	**	**	**
AL×PaSS	**	NS	*

Groups 1-9, see Table IV-1. AL, autoxidized linoleate. PaSS, pantethine. Values are mean ± SD for 5 rats in each group. NS, not significant. *, p<0.05; **, p<0.01.

In the S-9 fraction, the contents of total fatty acids of the pantethine-deficient group (Groups 1-3) were

Table VI-1. Composition of fatty acids in liver homogenates. (Continued from the previous page.)

Group No.	C18:2	C20:4 (%)	C22:6	Total (mg/g liver)
1	14.7±0.7	33.2±0.9	4.5±0.6	16.8±0.3
2	15.0±1.0	34.8±0.9	4.2±0.4	15.1±0.4
3	17.7±2.2	32.1±5.5	4.2±0.5	10.3±1.3
4	12.7±1.2	34.0±0.6	4.6±0.4	16.6±0.8
5	15.2±0.9	35.9±0.7	4.5±0.3	15.7±0.3
6	17.8±1.3	35.5±1.7	4.2±1.1	13.6±0.3
7	13.0±0.6	31.1±1.1	3.8±0.4	18.0±0.4
8	13.6±0.3	32.6±0.7	4.1±0.2	17.4±0.4
9	13.4±0.5	31.8±0.7	4.1±0.3	16.7±0.9
ANOVA for content				
AL	*	**	**	**
PaSS	NS	**	NS	**
AL×PaSS	**	**	*	**
ANOVA for composition				
AL	**	**	NS	
PaSS	**	**	NS	
AL×PaSS	**	**	NS	

Group 1-9, see Table IV-1. AL, autoxidized linoleate. PaSS, pantethine. Values are mean ± SD for 5 rats in each group. NS, not significant. *, $p<0.05$; **, $p<0.01$.

significantly lower ($p<0.01$) than those of the pantethine-adequate (Groups 4-6) and -excess (Groups

Table VI-2. Composition of fatty acids in S-9 fraction. (Continued on the next page.)

Group No.	C16:0	C18:0	C18:1
1	18.9±1.4	17.3±1.4	18.1±1.0
2	22.3±0.3	19.0±0	12.6±0.3
3	17.6±0.1	19.8±0.2	8.8±0.2
4	17.8±0.1	17.1±0.1	19.9±0
5	18.7±0.2	16.6±0.1	13.4±0.2
6#	20.5	20.1	8.0
7	20.9±0.1	14.5±0	25.0±0.2
8	20.8±0.5	17.9±0.4	21.8±0.6
9#	20.6	18.0	21.7
ANOVA for content			
AL	*	NS	**
PaSS	**	**	**
AL×PaSS	**	**	*
ANOVA for composition			
AL	*	**	**
PaSS	**	*	**
AL×PaSS	**	NS	*

Groups 1-9, see Table IV-1. AL, autoxidized linoleate. PaSS, pantethine. Values are mean ± SD for 5 rats in each group. #Values are mean of two samples. NS, not significant. *, $p<0.05$; **, $p<0.01$.

7-9) groups in any AL dose level (Table VI-2).

Corresponding to the dose level of AL, the total

Table VI-2. Composition of fatty acids in S-9 fraction. (Continued from the previous page.)

Group No.	C18:2	C20:4	C22:6	Total (mg/g protein)
1	15.5±1.3	21.4±2.0	2.0±0.4	31.9 ± 1.0
2	15.4±0.6	21.7±0.6	2.1±0.1	23.7 ± 0.4
3	14.6±0.2	29.8±0.4	2.5±0.3	15.1 ± 0.3
4	15.6±0.3	20.7±0.3	2.0±0	40.9 ± 0.3
5	18.0±0.3	24.5±0.2	1.9±0	39.5 ± 0.9
6#	17.6	24.1	2.7	37.6
7	14.1±0	15.9±0.1	2.2±0.1	46.2 ± 1.8
8	13.8±0.1	16.6±0.5	2.3±0.1	45.6 ± 2.5
9#	12.0	18.2	2.5	48.9
ANOVA for content				
AL	**	NS	NS	**
PaSS	**	**	**	**
AL×PaSS	**	**	*	**
ANOVA for composition				
AL	NS	**	*	
PaSS	**	**	NS	
AL×PaSS	NS	**	NS	

Groups 1-9, see Table IV-1. AL, autoxidized linoleate. PaSS, pantethine. Values are mean ± SD for 5 rats in each group. #Values are mean of two samples. NS, not significant. *, $p<0.05$; **, $p<0.01$.

fatty acid contents decreased in the rat liver homogenate of pantethine-deficient and -adequate groups and in the S-9 fraction of pantethine-deficient group. On the contrary, the decrease of fatty acids except palmitic acid and oleic acid in the liver homogenate of pantethine-excess group was not significant even by the large dose of AL. Therefore, the total contents of fatty acids in the liver homogenate of the group (Group 9) maintained the same level as those in the case of non-AL in pantethine-deficient (Group 1) and -adequate (Group 4) groups. The decreases of oleic acid contents by the high level of AL dose were significantly ($p<0.01$) more remarkable in the both pantethine -deficient (Group 3) and -adequate (Group 6) groups than in the pantethine-excess group (Group 9) in both the liver homogenate and the S-9 fraction (Tables VI-1 and VI-2).

Evidently, the fatty acid composition was affected by both AL dose level and dietary pantethine level, except docosahexanoic acid in liver homogenate and linoleic acid and docosahexanoic acid in S-9 fraction. Especially, oleic acid content and composition lowered correspondingly to the increasing level of AL dose in the pantethine-deficient (Groups 2 and 3) and -adequate

(Groups 5 and 6) groups. On the contrary, the decrease rates of the other fatty acid contents were less than that of oleic acid, and their compositions showed a tendency to increase corresponding to the increasing level of AL dose. Unlike the pantethine-deficient and

Table VI-3. Thiobarbituric acid value of liver homogenate.

Group No.	MDA nmol /g wet liver	MDA nmol /mg fatty acid
1	144 ± 29	8.5 ± 1.6
2	178 ± 16	11.0 ± 0.9
3	201 ± 43	20.0 ± 6.0
4	124 ± 30	7.5 ± 2.1
5	189 ± 36	12.0 ± 2.5
6	178 ± 27	13.1 ± 2.1
7	155 ± 19	8.6 ± 1.2
8	178 ± 27	9.9 ± 2.9
9	191 ± 48	11.5 ± 3.3
ANOVA		
AL	*	**
PaSS	NS	NS
AL×PaSS	NS	NS

Groups 1-9, see Table IV-1. AL, autoxidized linoleate. PaSS, pantethine. Values are mean ± SD for 5 rats in each group. NS, not significant. *, $p<0.05$; **, $p<0.01$.

-adequate groups, the fatty acid contents in both liver homogenate and S-9 fraction of the pantethine-excess group showed only a little decrease, and the fatty acid compositions little changed (Table VI-2).

Changes in thiobarbituric acid value.

Table VI-4. Thiobarbituric acid value of S-9 fraction.

Group No.	MDA nmol /mg protein	MDA nmol /mg fatty acid
1	0.77 ± 0.08	24.6 ± 2.5
2	1.27 ± 0.17	53.2 ± 5.4
3	1.15 ± 0.14	72.3 ± 10.4
4	0.65 ± 0.04	15.5 ± 1.3
5	1.76 ± 0.62	41.1 ± 10.3
6	1.18 ± 0.22	35.0 ± 4.2
7	0.97 ± 0.21	20.8 ± 4.3
8	1.10 ± 0.18	24.1 ± 5.8
9	1.31 ± 0.48	27.0 ± 10.5
ANOVA		
AL	*	**
PaSS	NS	**
AL×PaSS	NS	**

Groups 1-9, aee Table IV-1. AL, autoxidized linoleate. PaSS, pantethine. Values are mean ± SD for 5 rats in each group. NS, not significant. *, $p<0.05$; **, $p<0.01$.

The TBA values are expressed as malondialdehyde (MDA) nmol per wet liver weight (g) or per liver fatty acid (mg) in liver homogenate and as malondialdehyde (MDA) nmol per protein (mg) or per fatty acid (mg) in the S-9 fraction, respectively, in Tables VI-3 and VI-4. The TBA values expressed as amounts of MDA either per wet liver weight in the liver homogenate or per mg protein in the S-9 fraction showed the significant difference only on AL dose level ($p<0.05$). On the other hand, the TBA values expressed as MDA nmol/mg fatty acid in liver homogenates showed more clearly the significant difference on AL dose level ($p<0.01$). Among the values of MDA nmol/mg fatty acid, the effects of AL dose level ($p<0.01$) and dietary pantethine level ($p<0.01$), and the interaction between them ($p<0.01$) were statistically significant in the S-9 fractions. Namely, the TBA value in S-9 fraction increased correspondingly with increasing dose level of AL, and the increase was repressed markedly with the increase of dietary pantethine level.

VI-4 Discussion

It has been reported that pantethine has a

relieving effect against various injuries caused by lipid peroxidation *in vivo* (*84-87*). The absorbed pantethine, not having a reducing property by itself, might be reduced to pantetheine *via* the redox system *in vivo*, and the resultant pantetheine might have depressed lipid peroxidation *in vivo* by the reducing property originating from its sulfhydryl group.

As described in Chapter V, the drug-metabolizing activities in the cases of either non-AL or a low level of AL dose were significantly higher in the pantethine-deficient group than in the pantethine-adequate and -excess groups. It was inferred from this result that, in the pantethine-deficient non-AL group, drug-metabolizing system was induced (*104-106*) in order to decompose the lipid peroxides resulted from *in vivo* redox system because of the decrease in the content of radical scavengers, and thereby *in vivo* lipid peroxidation would be more accelerated. It was also presumed that the induction of drug-metabolizing system by the low level of AL dose might be a reasonable response of the system against the elevated accumulation of lipid peroxides *in vivo*. Thus it seems likely that the ingestion of pantethine relieves the induction of drug-metabolizing system because the

pantethine derivative having antioxidative property depressed the lipid peroxidation *in vivo*.

In order to better understand the state of lipid peroxidation in biological systems, it is important to measure the loss of fatty acids by a direct analysis of the tissue lipids themselves and to evaluate the products resulted from peroxidation. In the present chapter, I determined the changes in the contents and compositions of fatty acids and TBA values in the liver and the S-9 fraction obtained from rats grown under the same condition as described in Chapter IV.

The total fatty acids contents in the liver and the S-9 fraction were decreased correspondingly with the increase of AL dose in pantethine-deficient and -adequate groups, and it was especially remarkable in the pantethine-deficient group. In the liver homogenate of pantethine-excess group, the decrease of fatty acids contents except oleic acid was not significant in spite of the high level of AL dose (Table VI-1). The TBA values in liver homogenate and the S-9 fraction raised with the increase of AL dose level, especially in the pantethine-deficiet group, and lowered with the increase of the dietary pantethine level even in the high level of AL dose (Table VI-3 and VI-4). These

changes in the fatty acid contents and TBA values showed that fatty acids in tissue and subcellular fraction were damaged by the dosed AL and that pantethine relieved the damage. I have also found in this connection that it is suitable to express TBA value as MDA nmol per mg fatty acid constituting lipids rather than per g liver weight or mg protein in the S-9 fraction because TBA value expresses the level of lipid peroxidation. These results supported our presumption mentioned in Chapter V on the changes of drug-metabolizig system produced by AL dose and dietary pantethine.

It is well known *in vitro* that the increase in the number of double bond of fatty acid is accompanied by the increase of reactivity in lipid peroxidation (*134, 135*). On the other hand, much of the work on biological effects of lipid peroxidation is based on inferential evidences (*27*) and has not been clarified enough for mechanism of *in vivo* lipid peroxidation (*136*). Thus, the reason why oleic acid content decreased remarkably by increasing AL dose to the rats of pantethine -deficient and -adequate groups could not be explained from the hitherto obtained knowledges of lipid peroxidation. From the view points of fatty acid

metabolism, I would like to speculate the reason for the remarkable decreasing of oleic acid as follows.

In the present experiments, the rats lost their appetite by increasing AL dose, and starved. Fatty acids taken up from blood stream to the liver cells may be mainly either oxidized to provide energy or resynthesized into phospholipids to repair the damaged biomembrane. In this connection, oleic acid is a major component in hepatic neutral lipid, and on the contrary, is a minor component in hepatic phospholipid in comparison with the other polyunsaturated fatty acids such as linoleic acid and arachidonic acid (*137*). Consequently, the rat liver in state of starvation may consume more oleic acid than the other fatty acids. Furthermore, endogenous oleic acid in the rat liver is synthesized through Δ9-desaturation of stearic acid by cyanide sensitive factor which is the terminal enzyme in microsomal electron transfer system (*138*). It was reported by Imai *et al.* (*139-141*) that the biosynthesis of oleic acid by the Δ9-desaturation of stearic acid decreased markedly in the liver of starved or diabetic rats. Thus, the production of oleic acid remarkably decreases, but the consumption of oleic acid increases in the liver of starved rat. The Δ9-desaturation of

stearic acid in the rat liver requires thioesterification of the fatty acid with CoA, and the yielded oleyl CoA is incorporated to phospholipid or triglyceride (*140-141*). It has been reported that the level of Co A in the rat liver was depressed by administration of secondary autoxidation products of linoleic acid (*142*). Probably, the decrease of oleic acid content caused by the depression of Δ9-desaturation may be more remarkable than those caused by the *in vivo* lipid peroxidation. Thus, the Δ9-desaturation reaction in the liver of AL-administered rat which was deficient in pantothenic acid, a constituent of Co A, might be furthermore depressed because of the severe decrease of Co A. However, because the rats of pantethine-excess group were sufficiently supplied with CoA from pantethine, the biosynthesis of oleic acid might be maintained in the rat liver. Moreover, the supplement of fatty acids from the adipose tissue to the liver *via* bloodstream might be prevented by increasing AL dose in the rats of pantethine-deficient and -adequate groups. These seem to be the reasons why the oleic acid content decreased remarkably in the rat liver homogenate and S-9 fraction of the pantethine -deficient and -adequate groups.

In conclusion, it appears that sufficient dietary pantethine depresses *in vivo* lipid peroxidation and maintains normally the lipid metabolism, even under the condition of high level of AL administration.

VII SUMMARY

There have been only a few papers which reported the effect of autoxidized oil on drug-metabolizing system in the rat liver. These included a few incompatible findings. In one case the dosed autoxidized oil induced drug-metabolizing system, and in the other case reduced the system. I assumed that the effect of autoxidized oil on drug-metabolizing system in the rat liver changes in accordance with differences in the autoxidized levels of oil used, the administration periods, dose levels, *etc*. In order to better understand the effect of autoxidized fatty acid on drug-metabolizing system, I investigated in detail the effects of dose levels and dose periods of AL on drug-metabolizing system in the rat liver.

AL having 800 meq/kg of peroxide value and 1,700 meq/kg of carbonyl value was given in repeated oral doses at a daily dose of 0 (control) - 0.35 ml/100 g body weight to male Wistar rats for 5 successive days. All the rats died at a daily dose of 0.5 - 0.75 ml/100 g body weight after the third day of consecutive oral doses. The cytochrome P-450 and b_5 contents, enzyme activities in electron transfer system, aminopyrin

-N-demethylase activity and S-9 activity in drug-metabolizing system changed essentially in a similar manner, namely, both the contents and the activities were increased by a low level of AL dose, and were decreased by a high level of AL dose. The cytochrome P-450 was more unstable than cytochrome b_5 against AL dose.

In addition, AL (PV=800 meq/kg and CV=1,700 meq/kg) was given in repeated oral doses for 1 - 15 days at a daily dose of 0.25 ml/100 g body weight to male Wistar rats. The contents of cytochrome P-450 and b_5 were increased by consecutive oral doses for 3 - 7 days. Thereafter, the content of cytochrome P-450 decreased gradually, while the b_5 decreased slightly. Thus, after administration for 11 - 15 days, the cytochrome P-450 content lowered significantly, but the cytochrome b_5 content was rather high in comparison with the control group. The aminopyrin-*N*-demethylase activity was not reduced even after repeated oral doses of AL for 15 days. The S-9 activity decreased gradually by the administration for more than 7 days.

In the first conclusion, both the contents and the activities of enzymes in the drug-metabolizing system in the rat liver were increased by a low level of AL

dose, and were decreased by a high level of AL dose, although they were decreased by the elongation of the dose period even in the case of a low level of AL dose. The contradiction of the findings reported was resolved in this study.

Next, the effect of dietary pantethine levels on the growth phase of rat, drug-metabolizing system and the fatty acid content in the liver were investigated under administration of 0 (non), 0.2 (low dose) and 0.35 - 0.4 (high dose) ml of AL/100 g body weight /day for successive 5 days. AL was dosed to the rats of each group given drinking water containing 0 (deficient), 6.25 (adequate) and 125 (excess) mg pantethine/100 ml.

The pantethine-deficient group was different from the pantethine-adequate and -excess groups not only in the growth of rat but also in the diet and water intakes, behaviors, and some symptoms of rat.

The effect of AL dose level was statistically significant on all the components of drug-metabolizing enzyme system, but the effect of dietary pantethine level was statistically significant only on the cytochrome b_5 content, the NADPH-cytochrome *c* reductase activity, and the S-9 activity in the enzyme system. The results suggest that the effect of dosed AL on

drug-metabolizing system in the rat liver was relieved corresponding to the increase of dietary pantethine. I assumed that the absorbed pantethine, not having a reducing property by itself, is reduced to pantetheine *via* the redox system *in vivo*, and the resultant pantetheine depresses lipid peroxidation. This is the reason why both the induction by the lowl level of AL dose and the reduction by the high level of AL dose in drug-metabolizing enzyme system are more depressed in the pantethine-excess group in comparison with the other groups.

The effects of AL dose level and dietary pantethine level, and the interaction of them were statistically significant on the total content of fatty acids in both the liver homogenate and the S-9 fraction, and TBA values. These results support the assumption described above on the changes in drug-metabolizing system.

In addition, the lowering of oleic acid content by the high level of AL dose was the most remarkable in measured fatty acids contents, especially in the pantethine-deficient group, which was markedly relieved in the pantethine-excess group. I speculate a few reasons as follows: (1) The starvation owing to the

loss of appetite by the high level of AL dose accelerates the utilization of oleic acid as an energy source because of a major component in triglyceride. (2) The desaturation from stearyl CoA to oleyl CoA is damaged on account of the decrease of CoA. (3) The fatty acid supplement from the adipose tissue to the liver *via* bloodstream is prevented by the high level of AL dose.

In conclusion, the sufficient dietary pantethine depresses apparently *in vivo* lipid peroxidation and maintains normally the lipid metabolism, even under the condition of high level of AL administration.

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