Action of Malotilate on Reduced Serum Cholesterol Level in Rats with Carbon Tetrachloride-Induced Liver Damage

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Accepted May 1, 1985

Abstract—The mode of action of malotilate in normalizing serum cholesterol in hypocholesterolemic rats with fatty liver was examined by determination of biosynthesis, catabolism and excretion of cholesterol. Fatty liver was produced by subcutaneous injection of CCl₄ at the dose of 1 ml/kg into male rats (SLC-SD) twice a week for 3 weeks. Daily administration of malotilate (100 mg/kg) in rats with hypocholesterolemia resulted in a rapid normalization of lowered serum cholesterol. Such a recovery of cholesterol level in serum coincided in time with normalization of the decreased cholesterol level of each lipoprotein fraction, VLDL-triglycerides secretion and the decreased apolipoprotein A₁ value. Histopathological improvement in liver was also confirmed by a decrease in the size of fat droplets stored within the hepatocytes. The malotilate treatment gave a tendency to facilitate hepatic cholesterol synthesis in the rats with fatty liver. Malotilate at a concentration of 0.5–2 ½g/ml also stimulated cholesterol biosynthesis in cultured normal hepatocytes. The drug had the action to accelerate the catabolic excretion of ³H-labeled cholesterol into feces. These results suggest that the mode of action by which serum cholesterol is normalized in rats with fatty liver is probably due to a stimulative effect of malotilate on hepatic cholesterol synthesis and cholesterol secretion from the liver.

Recently, a double blind study was conducted concerning the effects of malotilate on patients with chronic hepatitis and liver cirrhosis. Results of this study revealed a marked improvement in reduced cholesterol level, albumin level and cholinesterase activity in the serum of these patients through their response to malotilate administration (1). This in turn brought attention to malotilate’s ability to facilitate protein and cholesterol synthesis. The administration of malotilate in rats facilitated the incorporation of ¹⁴C-leucine into liver microsomal protein (2) and induced proteins in the cytochrome b₅ electron transport system (3, 4). This suggests that malotilate affects the facilitation of protein synthesis in the liver. The incorporation of ¹⁴C-acetic acid into cholesterol in the liver was also demonstrated (M. Katoh et al., unpublished observation) as being facilitated by malotilate treatment. These actions probably represent fundamental effects of malotilate on the liver. Recently, the action of malotilate in normalizing serum cholesterol level shown in the clinical trial was further elucidated in experimental hypocholesterolemia of rats with liver damage (M. Katoh et al., unpublished observation). Since the improvement in serum cholesterol corresponded to the improvement in the histopathological findings of the liver, the effect on serum cholesterol may be a useful index for demonstrating improvement of liver function. However, the mode of action in normalization of serum cholesterol level is not yet clear. More studies are required for a better understanding of the mode of action of malotilate. The present paper describes the effect of malotilate on hepatic and intestinal cholesterol synthesis and catabolic excretion.
of cholesterol in hypocholesterolemic rats with fatty liver.

Materials and Methods

Preparation of rats with carbon tetrachloride-induced liver damage: Male rats of the SLC-SD strain (body weight of 150 g; supplied by the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) were maintained on a F-II diet (Funabashi Farm Co., Ltd., Chiba, Japan) for 1 week. Then an equal mixture of CCl₄ (Kishida Chemicals Co., Ltd., Osaka, Japan) and olive oil (Kishida Chemicals, Co., Ltd., Osaka, Japan) was injected subcutaneously at the dose of 2 ml/kg into the back twice a week (Monday and Thursday or Tuesday and Friday) for 3 weeks. At 24 hr after the last injection, a blood sample of approximately 0.1 ml was taken from the tail vein with a glass capillary tube (Terumo Co., Ltd., Tokyo, Japan). Rats with sufficiently low serum cholesterol values (weighing approximately 250 g) were divided into groups of 5 rats. Immediately upon completion of the CCl₄-olive oil mixture-treatment in the 4th week, malotilate (Lot No. KT1017, Nihon Nohyaku Co., Ltd., Tokyo, Japan) suspended in 0.5% CMC (50 mg/ml) was orally administered at a dose of 100 mg/kg once daily for 3 days. Rats of the group having only the administration of CCl₄ and the control group were given 0.5% CMC under the same conditions. The animals were then sacrificed on the 4th day by exsanguination from the abdominal aorta under pentobarbital anesthesia (40 mg/kg). Under pentobarbital anesthesia the animals were sacrificed by exsanguination from the abdominal aorta 1 hr later to obtain blood samples and to remove the liver and intestines. The removed intestine includes the entire portion from the duodenum to the ileum. The weight of intestines is expressed as a weight of scraped mucosa and submucosa. Lipids were extracted from the serum and each tissue with 20 volumes of a chloroform-methanol (2:1) mixture. An aliquot of the extract was applied on a silica gel 60 F₂₅₄ TLC plate (Merck & Co., Inc., NJ, U.S.A.) and developed with a solvent mixture consisting of n-hexane: diethyl ether: acetic acid (73:25:2). Spots corresponding to free cholesterol and cholesterol ester were scraped off, and a scintillator containing toluene was added to measure the incorporated radioactivity. The cholesterol content of the extract was measured at the same time. A 0.5% solution of CMC was administered to the rats treated with CCl₄ alone and the control rats.

Measurement of the cholesterol biosynthesis from ¹⁴C-acetic acid in vivo: Rats with carbon tetrachloride-induced liver damage were divided into groups of 5 animals, and each received 100 mg/kg of malotilate suspended in 0.5% CMC once daily. At 8 and 24 hr after the initial administration (malotilate at a dose of 100 mg/kg was administered once again 2 hr prior to the intravenous injection of ¹⁴C-acetic acid) and 24 hr after the final dose of malotilate given on the third day, ¹⁴C-acetic acid dissolved in physiological saline (54.9 mCi/mmol, New England Nuclear, Boston, U.S.A.) was intravenously injected into the femoral vein at a dose of 100 μCi/kg. Under pentobarbital anesthesia the animals were sacrificed by exsanguination from the abdominal aorta 1 hr later to obtain blood samples and to remove the liver and intestines. The removed intestine includes the entire portion from the duodenum to the ileum. The weight of intestines is expressed as a weight of scraped mucosa and submucosa. Lipids were extracted from the serum and each tissue with 20 volumes of a chloroform-methanol (2:1) mixture. An aliquot of the extract was applied on a silica gel 60 F₂₅₄ TLC plate (Merck & Co., Inc., NJ, U.S.A.) and developed with a solvent mixture consisting of n-hexane: diethyl ether: acetic acid (73:25:2). Spots corresponding to free cholesterol and cholesterol ester were scraped off, and a scintillator containing toluene was added to measure the incorporated radioactivity. The cholesterol content of the extract was measured at the same time. A 0.5% solution of CMC was administered to the rats treated with CCl₄ alone and the control rats.
damage. At five hours after the initial administration, ³H-labeled cholesterol (40.7 mCi/mmol, New England Nuclear, Boston, U.S.A.) in 20% ethanolic physiological saline was injected into the femoral vein at a dose of 100 µCi/ml/kg, followed by blood sampling from the tail vein at the indicated times. The same dose of malotilate was orally administered thereafter at 24 hr intervals. The animals were sacrificed 6 days after the malotilate administration by the method described already. The liver, kidneys, intestine and lungs were removed in addition to whole blood sampling. The lipids were extracted from aliquots of serum and each organ with 20 time volumes of chloroform-methanol mixture (2:1). Radioactivity and cholesterol contents in the extracts were measured. The half life of radioactivity in the blood and the Fractional Catabolic Rate (FCR) were calculated according to Notari's method (6). A 0.5% solution of CMC was administered in the animals treated with CC14 alone and control animals.

Preparation of histopathological specimens: The liver was removed from rats under pentobarbital anesthesia (40 mg/kg) at the time of sacrifice. A portion of the lateral aspect of the right lobe and part of the middle lobe were fixed in neutral formalin (pH 7.4), embedded in paraffin using a standard procedure and cut into thin sections. These sections were stained by Hematoxylin-Eosin and Azan for histopathologic examination.

Preparation of hepatocytes: Using male SLC-SD rats (body weight of 200 g) (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan), hepatocytes were prepared by the method of Nakamura et al. with a minor modification (10). The rats were anesthetized under pentobarbital anesthesia (40 mg/kg). Then a polyethylene tube was inserted into the portal vein during infusion of the perfusing solution (Ca²⁺ free Hank’s 10 mM Hepes, 0.5 mM EDTA, pH 7.2), and the inferior vena cava below the liver was immediately cut for exsanguination. Under these conditions, the perfusion was continued at a rate of 40 ml/min for 10 min. Then the perfusion was continued for about 13 more min with a perfusing solution consisting of 0.05% collagenase (Type IA, Sigma Chemical Co., St. Louis, U.S.A.), 0.05 mg/ml trypsin inhibitor (Type II-S, Sigma Chemical Co., St. Louis, U.S.A.) and Mg²⁺-free Hank’s 10 mM Hepes, pH 7.5, at a rate of 30 ml/min. The liver was then removed, and about 20 ml of DME culture medium (Dulbecco’s modified eagle medium; Gibco, NY, U.S.A.) was added. After light pipetting with a wide tipped pipette that would disperse the cells sufficiently, the suspension was filtered through a cell filter made of multiple layers of gauze (150 mesh, Ikemoto Rika Co., Ltd., Tokyo, Japan). One hundred fifty ml each of this cell suspension was placed in plastic centrifuge tubes (Falcon 2070, Becton, Co., U.S.A.) and centrifuged at 50×g for 1 min to precipitate the hepatic parenchymal cells. Sixty ml of DME culture medium was added to the precipitate, and the mixture was pipetted lightly to disperse the cells. Then, the suspension was again centrifuged under
the same conditions. After this procedure was repeated twice, 30 ml of DMF culture medium (subsequently abbreviated as 2% FCS-DME-AID) containing 2% FCS (Fetal calf serum; Gibco, NY, U.S.A.), 100 units/ml penicillin G (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika Co., Ltd., Tokyo, Japan), 10^{-9} M insulin (Sigma Chemical Co., St. Louis, U.S.A.) and 10^{-6} M dexamethasone (Sigma Chemical Co., St. Louis, U.S.A.) were added to disperse the cells. The suspension was then centrifuged to prepare the hepatic parenchymal cells. The cell number was counted in a Coulter Counter-ZBI (Coulter Electronic, Inc., U.S.A.) and adjusted to 5x10^5 cells/ml by adding 2% FCS-DME-AID culture medium. Four ml of this suspension was then inoculated on a 60 mm dish (Falcon 3002, Becton Co., U.S.A.) and cultured at 37°C for 3 hr in 5% CO₂-95% air. The unsettled cells were removed, then 3 ml of 5% FCS-DME-AID culture medium was added, and the culture was continued for a further 18 hr to expand the cell layer.

Cholesterol biosynthesis in cultured hepatocytes: The hepatocytes were cultured in the 5% FCS-DME-AID medium for 18 hr, then this culture medium was exchanged with 3 ml of medium containing various concentrations of malotilate. The culture was continued for 24 hr. A solution of 14C-acetic acid (5 μCi/3 ml, 54.9 mCi/mmol; New England Nuclear, Boston, U.S.A.) was added to each medium containing malotilate, and the culture was further incubated for 2 more hr to measure the cholesterol biosynthesis' ability. After the culture medium was recovered, the hepatocytes attached to the wall were washed with 2 ml Dalbecco's PBS (-) (Nissui Seiyaku Co., Ltd., Tokyo, Japan), twice. Cells were collected using a treatment of 0.25% trypsin. The hepatocytes were dissolved in 0.5 ml of 0.1 N NaOH. Lipid was extracted from 0.25 ml of this solution with 5 ml of a chloroform-methanol (2:1) mixture and then concentrated. The whole residue was applied on a silica gel 60F254 TLC plate (Merck & Co., Inc., NJ, U.S.A.) with n-hexane: diethyl ether: acetic acid (73:25:2) as the developing solvent. After the lipid staining under iodine vapor, spots corresponding to free cholesterol and cholesterol ester were scraped off and the radioactivity was measured after the addition of a toluene based scintillator. The cholesterol content in the hepatocytes was measured by an enzymatic method (Dai-Test CHO, Daiichi pure Chemicals Co., Ltd., Tokyo, Japan) after neutralization of samples dissolved by 0.1 N NaOH.

Preparation of drug solution: Malotilate was dissolved in propylene glycol (Kishida Chemicals Co., Ltd., Osaka, Japan) to prepare a solution of 0.1 mg/ml. This solution was diluted with DME-AID culture medium to adjust the concentration to 0.25–2 μg/ml, and propylene glycol was further added to adjust the concentration of the propylene glycol to 0.2%.

Results

Normalization of serum cholesterol in rats with CCl₄-induced liver damage: The serum cholesterol in rats given CCl₄ for 3 weeks fell to 40% of the level in control rats. Such a condition of hypocholesterolemia persisted for 4 days after the administration of CCl₄. Serum cholesterol values in rats treated with malotilate showed a tendency to increase 24 hr after the initial dose of malotilate and achieved to normal levels after an oral dose for 3 days (Table 1). The cholesterol content of serum lipoprotein fractions in rats sacrificed 24 hr after the final dose of malotilate are shown in Table 2. Malotilate administration increased the total serum cholesterol by about 40 mg/dl, VLDL-cholesterol by 15.6 mg/dl and HDL-cholesterol by 18.0 mg/dl. At this stage, cholesterol values of each lipoprotein fraction had almost returned to their normal levels.

Normalization of hepatic triglyceride secretion and serum apolipoprotein A₁ value: The hepatic secretion of triglyceride was studied as one of the indices of liver function. As shown in Table 3, the triglyceride secretion from livers in rats given CCl₄ was inhibited to about 40% of the level in the control rats. However, this inhibition of the triglyceride secretion was completely normalized in response to malotilate administration for 3 days. Apolipoprotein A₁ was immuno-
chemically quantified in the serum samples obtained prior to Triton WR-1339 injection. As shown in Table 3, the apolipoprotein A level in the serum was markedly decreased by CCl₄ administration. Malotilate administration for 3 days significantly increased its serum levels.

**Effect of malotilate on the histopathological findings of the liver:** Hepatocytes in the central and the intermediate zones of the lobule showed marked fatty and hydropic degeneration in the 4th week of CCl₄ injury. Those in the perportal zone showed milder changes with occasional mitotic figures. In the liver of rats treated with malotilate, remission of fatty degeneration as well as obvious hydropic degeneration was noted. In addition to the reduced number of hepatocytes, the size of fatty droplets in them was also definitely smaller.

### Table 1. Effect of malotilate on serum cholesterol levels in rats with fatty liver

|                | Serum cholesterol (mg/dl) | Time after malotilate treatment |
|----------------|----------------------------|---------------------------------|
|                | n                          | 1                              | 24     | 48    | 70 hr |
| CCl₄ + vehicle | 5                          | 29.1±3.7^▲▲                  | 35.9±5.0 | 34.4±1.3 | 32.1±2.1 |
| CCl₄ + malotilate | 5                  | 37.7±0.9^▲▲^△△               | 44.7±4.8 | 68.4±8.9^△△ | 71.8±1.6^▲△△ |
| Normal + vehicle | 5                      | 79.2±2.3^▲△△                 |        |       |       |

*P<0.05, **P<0.01 versus CCl₄ + vehicle at each time. Mean ± S.E. ▲▲P<0.01 versus Normal + vehicle. The rats received subcutaneous doses of CCl₄ twice a week for 3 weeks and then received a daily oral dose of malotilate (100 mg/kg) or vehicle for 3 days.

### Table 2. Effect of malotilate on lipoprotein cholesterol levels in rats with fatty liver

|                | Cholesterol (mg/dl) | Serum | VLDL | LDL | HDL |
|----------------|---------------------|-------|------|-----|-----|
|                | n                   |       |      |     |     |
| CCl₄ + vehicle | 5                   | 32.1±2.1^▲▲                  | 6.8±1.3^▲▲ | 3.3±0.9^▲ | 24.1±2.0^▲▲ |
| CCl₄ + malotilate | 5                  | 71.8±1.8^▲▲                  | 22.4±3.3^**  | 8.6±1.5^*    | 42.1±4.0^**   |
| Normal + vehicle | 5                    | 79.2±2.3^▲△△                | 24.0±3.5^**  | 8.0±1.3^*    | 47.2±1.2^***  |

*P<0.05, **P<0.01, ***P<0.001 versus CCl₄ + vehicle. Mean ± S.E. ▲▲P<0.05, ▲▲P<0.01, ▲▲▲P<0.001 versus Normal + vehicle. Serum samples were obtained 24 hr after the third dose of malotilate (100 mg/kg).

### Table 3. Effect of malotilate on triglycerides secretion and serum apolipoprotein A₁ level in rats with fatty liver

|                | Serum triglycerides (mg/dl) | Serum apolipoprotein A₁ (unit/5 ml serum) |
|----------------|-----------------------------|------------------------------------------|
|                | n                           | before | after | difference | before | after | difference |
| CCl₄ + vehicle | 5                           | 140.5±4.6^△△                 | 473.2±128.6^△△  | 332.8±130.6^△△   | 97.5±11.8^△△ |
| CCl₄ + malotilate | 4                       | 166.8±6.8^*†                 | 996.7±27.0***  | 829.9±23.0***   | 200.0±24.9^*  |
| Normal + vehicle | 5                          | 238.3±9.3^***                 | 1014.3±45.1**** | 803.0±47.6****  | 314.0±30.3**  |

*P<0.05, **P<0.01, ***P<0.001 versus CCl₄ + vehicle. Mean ± S.E. ▲▲P<0.05, ▲▲P<0.01, ▲▲▲P<0.001 versus Normal + vehicle. The rats received subcutaneous doses of CCl₄ twice a week for 3 weeks and then received a daily oral dose of malotilate (100 mg/kg) or vehicle for 3 days. Triton WR 1339 was intravenously injected 24 hr after the last dose of malotilate. Serum samples were obtained before and 1 hr after the dose of Triton WR 1339. One unit was arbitrarily defined as the height of the immunoprecipitin zone for 5 ml of 64-fold diluted standard serum.

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**Effect of malotilate on the histopathological findings of the liver:** Hepatocytes in the central and the intermediate zones of the lobule showed marked fatty and hydropic degeneration in the 4th week of CCl₄ injury. Those in the perportal zone showed milder changes with occasional mitotic figures. In the liver of rats treated with malotilate, remission of fatty degeneration as well as obvious hydropic degeneration was noted. In addition to the reduced number of hepatocytes, the size of fatty droplets in them was also definitely smaller.

**Effect of malotilate on cholesterol biosynthesis:** As shown in Table 4, serum cholesterol values in rats with liver damage was significantly lower than in the control rats, and the cholesterol content of the liver was significantly increased. This increase was not due to liver hypertrophy. Inversely, the cholesterol content of the intestine was
significantly decreased. Malotilate was administered to these rats with fatty liver, and 14C-acetic acid was intravenously injected 6 hr later to study the cholesterol synthesis in the liver and intestine. As shown in Table 5, hepatic cholesterol synthesis in rats given CC14 was significantly suppressed as compared with the control rats. The cholesterol synthesis in the liver of rats treated with malotilate at this stage showed no difference from that in rats given CC14 alone. However, cholesterol synthesis appeared to be definitely facilitated in the liver of rats injected intravenously with 14C-acetic acid 24 hr after the first administration of malotilate (malotilate was administered again 22 hr after the initial administration). However, no such facilitating effect on cholesterol synthesis was noted in the intestine. Even though the cholesterol synthesis in the liver was stimulated, no change was seen in the serum cholesterol value.

Malotilate was administered in rats with liver damage produced under the same conditions for 3 days. 14C-acetic acid was intravenously injected 6 hr later to study the cholesterol synthesis in the liver and intestine. As shown in Table 6, malotilate administration for 3 days caused a significant increase in the serum cholesterol which approached almost to the normal values. Cholesterol values in the intestine showed a significant decrease. The weight of the liver tended to increase in response to the administration of malotilate for 3 days, but the cholesterol content of the liver tended to decrease. The results of cholesterol biosynthesis in each organ of rats treated with malotilate for 3 days are summarized in Table 7. The specific activity of cholesterol in the serum, liver and intestine was not influenced by malotilate administration, but the total cholesterol radioactivity in serum was significantly increased as compared with the control group. The cholesterol level had a slight tendency to fall in the liver, and a significant decrease was noted in the intestine.

**Effect of malotilate on cholesterol catabolism:** This experiment was done in order to follow the changes of radioactivity in the liver in conjunction with organ distribution and excretion into feces of the radioactivity. 3H-Cholesterol was given intravenously 5 hr after the initial administration of malotilate in rats given CC14 for 3 weeks. Malotilate was administered at 24 hr intervals, but no more CC14 was given thereafter. Therefore, the serum cholesterol value in the group given CC14 alone continued to fall up to about 96 hr, and this was followed by a gradual recovery. Serum cholesterol values in the group treated with malotilate rapidly returned to the normal level following administration of malotilate for 3 days. Radioactivity in the blood showed a biphasic decay curve with a turning point about 70 hr after the intravenous injection of 3H-cholesterol. Table 8 shows the half-life of 3H-cholesterol and Fractional Catabolic Rate (FCR) in 3 groups. The half-life of the first phase in the

| Treatment          | N  | Serum (mg/dl) | Liver (mg/g of liver) | Intestine (mg/g of intestine) | Liver (g) | Intestine (g) |
|--------------------|----|---------------|-----------------------|------------------------------|-----------|---------------|
| CCl4 + vehicle     | 5  | 43.7±4.4a     | 7.4±0.7a              | 3.3±0.1a                     | 11.0±0.4  | 4.7±0.3a      |
| CCl4 + malotilate  | 6 hr| 38.6±4.0b     | 8.5±0.4b              | 3.3±0.2                      | 11.1±0.3  | 5.4±0.3       |
|                    | 24 hr| 42.5±2.4c     | 7.8±0.3c              | 3.2±0.2                      | 11.8±0.9  | 5.5±0.3       |
| Normal + vehicle   | 5  | 65.5±6.4*     | 4.9±0.2*              | 3.1±0.2                      | 10.2±0.6  | 6.3±0.2*      |

*P<0.05 versus CCl4 + vehicle. Mean±S.E. a,b,c = P<0.05, d,e,f = P<0.01 versus Normal + vehicle. The rats received subcutaneous doses of CCl4 twice a week for 3 weeks and then received the first oral dose of malotilate (100 mg/kg) or vehicle. The rats received the second dose of malotilate (100 mg/kg) 22 hr after the first dose. Serum and tissue samples were obtained 1 hr after the injection of the labeled precursor.
Table 5. Effect of malatilate on incorporation of $[^{14}C]$-acetate into cholesterol in rats with fatty liver

|                  | Total radioactivity (dpm x 10^-3/dl or g of tissue) | Specific activity (dpm/mg cholesterol) |
|------------------|---------------------------------------------------|----------------------------------------|
|                  | Serum     | Liver   | Intestine | Serum   | Liver   | Intestine |
| CCl4 + vehicle   | 5         |         |           |         |         |           |
| 6 hr             | 14.9±4.7  | 2.18±0.21 | 15.7±2.0  | 348.6±120.2 | 309.9±46.1 | 4744.1±614.7 |
| 24 hr            | 9.7±1.7  | 2.73±0.60 | 11.4±0.8  | 260.8±45.5  | 300.0±62.8  | 3421.7±125.0 |
| Normal + vehicle | 5         |         |           |         |         |           |
|                  | 10.3±1.3  | 5.14±1.51 | 14.0±2.8  | 220.5±26.6  | 663.7±201.5 | 4357.5±722.2 |

*P<0.05 versus CCl4 + vehicle. Mean±S.E. △P<0.05, △△P<0.01 versus Normal + vehicle. The rats received subcutaneous doses of CCl4 twice a week for 3 weeks and then received the first oral dose of malatilate (100 mg/kg) or vehicle. $[^{14}C]$acetate was intravenously injected 6 hr or 24 hr after the first dose. Incorporation of $[^{14}C]$acetate into cholesterol was determined 1 hr after the injection of the labeled precursor. The rats received the second dose of malatilate (100 mg/kg) 22 hr after the first dose.

Table 6. Effect of malatilate on cholesterol levels in serum, liver and intestine in rats with fatty liver (Treatment with malatilate for 3 days)

|                  | Total cholesterol (mg/dl or g of tissue) | Organ weight (g) | Liver/Body weight x 100 |
|------------------|------------------------------------------|------------------|-------------------------|
|                  | Serum     | Liver   | Intestine | Serum   | Liver   | Intestine |
| CCl4 + vehicle   | 5         |         |           |         |         |           |
|                  | 37.2±5.3  | 5.2±0.3  | 2.5±0.1  | 12.1±0.7  | 6.1±0.2 | 4.7±0.1   |
| CCl4 + malatilate| 5         | 64.6±3.1 | 3.9±0.2  | 2.8±0.4  | 13.7±0.4 | 5.7±2.5  | 5.4±0.3   |
| Normal + vehicle | 5         | 72.8±5.1 | 2.5±0.1  | 2.1±0.1  | 12.7±0.5 | 6.5±0.4  | 4.0±0.1   |

*P<0.05, **P<0.01 versus CCl4 + vehicle. Mean±S.E. △P<0.05, △△P<0.01 versus Normal + vehicle. The rats received subcutaneous doses of CCl4 twice a week for 3 weeks and then received a daily oral dose of malatilate (100 mg/kg) or vehicle for 3 days. Serum and tissue samples were obtained 24 hr after the last dose of malatilate.

Table 7. Effect of malatilate on incorporation of $[^{14}C]$-acetate into cholesterol in rats with fatty liver (Treatment with malatilate for 3 days)

|                  | Total radioactivity (dpm x 10^-3/dl or g of tissue) | Specific activity (dpm/mg cholesterol) |
|------------------|---------------------------------------------------|----------------------------------------|
|                  | Serum     | Liver   | Intestine | Serum   | Liver   | Intestine |
| CCl4 + vehicle   | 5         |         |           |         |         |           |
|                  | 4.82±0.79 | 1.37±0.22 | 9.10±0.98 | 1.32±0.20 | 270.2±23.9 | 3589.8±831 |
| CCl4 + malatilate| 5         | 9.08±0.71 | 1.02±0.23 | 7.73±0.45 | 1.42±0.63 | 222.3±47.1 |
| Normal + vehicle | 5         | 2.72±0.47 | 1.60±0.35 | 16.93±6.98 | 3.84±0.76 | 635.8±126.3 |

*P<0.05, **P<0.01, ***P<0.001 versus CCl4 + vehicle. Mean±S.E. △P<0.05, △△P<0.01, △△△P<0.001 versus Normal + vehicle. The rats received subcutaneous doses of CCl4 twice a week for 3 weeks and then received a daily oral dose of malatilate (100 mg/kg) or vehicle for 3 days. $[^{14}C]$acetate was intravenously injected 24 hr after the last dose of malatilate. Incorporation of $[^{14}C]$acetate into cholesterol was determined 1 hr after the injection of the labeled precursor.
group given CCl₄ alone was significantly shortened as compared with the control group, but the half-life of the second phase was significantly prolonged. The FCR appeared to be definitely smaller than that in the control rat with a tendency to decrease in the cholesterol catabolism and excretion. The half-life of the first phase in the group treated with malotilate also showed a definite tendency to shorten as compared with the normal control group. However, the half-life of the second phase was definitely shortened and normalized as compared with the group given CCl₄ alone. Consequently, the FCR value was improved.

The cholesterol content and total incorporated radioactivity in the organs are shown in Table 9. The amount of cholesterol accumulated in liver and the total radioactivity incorporated in the liver of animals given CCl₄ decreased significantly in response to malotilate administration. The cholesterol content and radioactivity in the intestine, kidney and lung were not influenced by malotilate administration (not shown in the Table).

The excreted total radioactivity into the feces was then measured over a period of 6 days. As shown in Table 10, the total fecal excretion of radioactivity in the group given CCl₄ alone was significantly lower than that of the control group. This indicated a sup-

| Table 8. Effect of malotilate on kinetic parameters of ³H-labeled cholesterol metabolism in rats with fatty liver |
|---|---|---|---|
| &nbsp; | t1/2 (hr) | FCR (% × 10⁻³/hr) |
| &nbsp; | first phase | second phase |
| CCl₄+vehicle | 5 | 9.7±1.3** | 134.2±16.5 |
| CCl₄+malotilate | 5 | 10.8±1.4** | 97.0±14.3 |
| Normal+vehicle | 5 | 18.0±1.7** | 74.1±8.4 |

*P<0.05, **P<0.01 versus CCl₄+vehicle. Mean±S.E. \*P<0.05, \*\*P<0.01 versus Normal+vehicle. Experimental conditions were described in the legend of Fig. 2.

| Table 9. Effect of malotilate on tissue cholesterol distribution in rats with fatty liver |
|---|---|---|---|---|
| &nbsp; | Cholesterol (mg or dpm × 10⁻⁴/g of tissue) | Intestine | Kidney |
| &nbsp; | &nbsp; | Cholesterol | Radioactivity | Cholesterol | Radioactivity |
| CCl₄+vehicle | 9.3±1.2** | 57.9±5.4** | 2.8±0.6* | 5.0±0.2 |
| N=5 | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| CCl₄+malotilate | 7.1±0.5** | 33.0±3.1** | 2.9±0.6* | 6.0±0.3 |
| N=5 | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |
| Normal+vehicle | 3.3±0.2** | 10.0±1.5*** | 4.7±0.6* | 6.5±0.6 |
| N=5 | &nbsp; | &nbsp; | &nbsp; | &nbsp; | &nbsp; |

*P<0.05, **P<0.01, ***P<0.001 versus CCl₄+vehicle. Mean±S.E. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 versus Normal+vehicle. Experimental conditions were described in the legend of Fig. 2. Tissue samples were obtained 24 hr after the last dose of malotilate.

| Table 10. Effect of malotilate on fecal excretion of ³H-labeled cholesterol in rats with fatty liver |
|---|---|---|
| &nbsp; | Radioactivity (dpm × 10⁻⁶/g of tissue) |
| &nbsp; | &nbsp; | &nbsp; |
| CCl₄+vehicle | 5 | 0.87±0.08**|
| CCl₄+malotilate | 5 | 1.20±0.08*** |
| Normal+vehicle | 5 | 1.20±0.10*** |

***P<0.001 versus CCl₄+vehicle. Mean±S.E. **P<0.01 versus Normal+vehicle. Experimental conditions were described in the legend of Fig. 2. Feces were collected for 6 days after intravenous injection of ³H-labeled cholesterol.
pression of cholesterol catabolism and excretion in the group given CCl₄ alone. However, the administration of malotilate improved the total fecal excretion of radioactivity and restored it to the level of the control group.

**Effect of malotilate on cholesterol biosynthesis in cultured hepatocytes:** As shown in Table 11, malotilate stimulated the cholesterol biosynthesis in cultured hepatocytes at the concentration range of 0.5–2 μg/ml. An optimal concentration of 0.5 μg/ml was found to induce the highest activity. The specific activity of the intracellular cholesterol was 1.5 times higher than the control value at a concentration range of 0.5–1 μg/ml. The specific activity of cholesterol in the culture medium increased in the same range of malotilate concentration. These findings show that malotilate stimulates cholesterol biosynthesis in the hepatocytes and also facilitates cholesterol secretion from the cells at certain concentration.

### Table 11. Effect of malotilate on cholesterol biosynthesis in primary cultures of rat hepatocytes

|                | Cell          | Medium        | Total         |
|----------------|---------------|---------------|---------------|
| Control        | 65.8±8.0      | 16.4±4.7      | 82.3±7.8      |
| Malotilate 2 μg/ml | 95.9±8.1*   | 10.5±1.2      | 106.4±8.6*    |
| Malotilate 1 μg/ml | 95.9±8.7*   | 16.2±2.9      | 112.1±8.2*    |
| Malotilate 0.5 μg/ml | 95.6±6.0*   | 36.5±4.8*     | 132.1±7.9**   |
| Malotilate 0.25 μg/ml | 62.5±8.1    | 25.1±13.5     | 87.7±19.2     |

Mean±S.E. from 5 dishes. *P<0.05, **P<0.01 versus control. †Control medium includes 0.2% propylenglycol.

The clinical study of malotilate on patients with chronic hepatitis and liver cirrhosis indicated that this drug improves significantly cholesterol, albumin levels and cholinesterase activity (1). This study was focussed on the effect of malotilate in the conditions of hypocholesterolemia. Rats with CCl₄-induced liver damage were used to evaluate the effect of this drug on cholesterol metabolism along with the action of improving liver function.

CCl₄ administration for 3 weeks produced fat accumulations in the hepatic parenchymal cells from the center to the intermediate zone of liver lobules, resulting in fatty or hydropic degeneration. Most of the fat thus accumulated within the liver is triglycerides (11). Such triglycerides accumulation is probably due to the delay of VLDL secretion secondary to the inhibition of apolipoprotein biosynthesis in the liver. Consequently, serum cholesterol was definitely lower in the rats with fatty liver than in the normal rats. Cholesterol content in the liver was significantly high as compared with the various tested organs, so that the secretion of cholesterol from the liver also appeared to be suppressed. After malotilate was administered to these rats with fatty liver at a dose of 100 mg/kg once daily, the serum cholesterol values rapidly improved and returned to their normal levels 3 days later. The values of each lipoprotein cholesterol at the time of recovery also returned to the normal levels. The evaluation of VLDL secretion as a useful index of liver function was based on the difference of serum triglyceride values between before and after the administration of Triton WR-1339. The decreased triglyceride secretion in the group given CCl₄ alone was found to return to the normal level in response to malotilate administration. Thus the normalization of serum cholesterol was shown to occur in a parallel fashion with the normalization of VLDL secretion from the liver. The blood level of apolipoprotein A₁ in HDL occupying about 2/3 of the lipoprotein in rats was definitely lower in rats given CCl₄ alone than in normal rats. This decreased level almost recovered to its normal level in response to malotilate administration. The normalization of serum cholesterol along with improvement in the histopathological findings showed high degrees of correlation with the normalization of liver function indices such as serum albumin value, GOT and GPT.
activities (12). Therefore, normalization of
the cholesterol content in response to
malotilate administration may be considered
as an expression of the functional improve-
ment of hepatocytes. However, the detailed
mechanisms of malotilate’s therapeutic
effects or its ability to improve CCl₄-induced
liver damage has not been sufficiently
elucidated. Malotilate is known to inhibit
20–30% of the binding of the CCl₄-radical,
which is considered to be the essence of CCl₄
toxicity, to a phospholipid in the liver micro-
some (M. Katoh et al., unpublished obser-
vation). The induction of glutathione re-
ductase and glutathione peroxidase probably
participating in the stimulation of hepatic
regeneration and inhibition of lipid per-
oxidation was also demonstrated (M. Katoh
et al., unpublished observation). Imaizumi
et al. (13) reported the recovery of hepatic
protein synthesis, which was reduced in
CCl₄-induced fatty liver, by malotilate admin-
istration. In addition to activation of normal
hepatocytes, this drug has an action of
improving the decreased liver function. These
actions of malotilate are considered to
participate in the improvement of histopa-
thological findings, liver function and serum
cholesterol level which were observed in
this study.

The mechanism of action in the nor-
malization of serum cholesterol was studied
from the viewpoint of cholesterol biosyn-
thesis, catabolism and excretion using fatty
liver in rats. One dose of 100 mg/kg of
malotilate was administered and the cho-
lesterol biosynthesis from ¹⁴C-acetic acid
was studied 6 and 24 hr (malotilate was
again administered 22 hr after the first dose)
after malotilate administration in rats with low
activity of hepatic cholesterol synthesis. Even
though 6 hr after the first malotilate adminis-
tration when malotilate and its metabolites
reached at peak concentration in liver (M.
Katoh et al., unpublished observation), the
ability of hepatic cholesterol synthesis did
not change at all. However, hepatic cho-
lesterol synthesis was definitely enhanced
24 hr after the first dose. Since no changes
were found in the serum and liver cholesterol
contents of the rats at this stage, the enhance-
ment of hepatic cholesterol synthesis was
probably not due to a release in the inhibition
of cholesterol synthesis by the accumulated
cholesterol in the liver. It was more likely due
to a direct action of malotilate or its metabolite
on cholesterol synthesis in hepatocytes. In
fact, malotilate significantly enhanced the
cholesterol biosynthesis of rat hepatocytes in
the primary culture, along with a promotion
of cholesterol secretion from the hepatocytes
to the culture medium at certain concen-
trations. Therefore, these results strongly
suggest that the action of malotilate to
increase the serum cholesterol level in normal
rats and its action to increase serum cho-
lesterol in a healthy man within the normal
range observed in the phase I trial are due to
its action of enhancing cholesterol and protein
synthesis. The ability of cholesterol synthesis
in the liver of rats given malotilate for 3 days
was apparently not different from that in the
group given CCl₄ alone. However, the specific
activity of serum cholesterol was significantly
higher than that in the group given CCl₄
alone, indicating a tendency of the liver
cholesterol content to decrease. The catabolism of cholesterol from the blood at
this stage was not suppressed as compared
with that in the group given CCl₄ alone.
Consequently, the synthesized cholesterol in
the liver was rapidly released into the blood
and for this reason the specific activity in the
liver seems to be not different. This finding
suggests that the stimulation of cholesterol
synthesis in the liver and cholesterol secretion
from the liver is participating in the nor-
malization of serum cholesterol level at this
stage. If cholesterol secretion from the liver is
controlled by the normalization of apoli-
poprotein synthesis, this normalization of
serum cholesterol level is probably due to the
facilitation of lipoprotein secretion from the
liver.

The action of malotilate on cholesterol
catabolism, another factor participating in the
control of serum cholesterol level, was
studied using the same model of rats with
fatty livers. FCR of ³H-cholesterol in the
group given CCl₄ alone was significantly
suppressed as compared with normal rats.
Malotilate administration definitely showed
the tendency to improve it. This was again
confirmed by a marked increase in fecal
radioactivity over a period of 6 days following an intravenous administration of $^3$H-cholesterol. Since the liver was the only major organ that demonstrated a decrease in the cholesterol content, the action of malotilate is probably exerted only on the liver. Malotilate or its metabolite probably acts on the hepatocytes to enhance cholesterol synthesis, normalizing apolipoprotein synthesis, and facilitating cholesterol secretion from the liver to the blood. Serum cholesterol is rapidly catabolized and excreted into the feces by a known action of malotilate to stimulate bile secretion and cholic acid synthesis (14).

References

1 Oda, T. and Tystrup, N.: Hepatotrophic agent malotilate. In Proceedings of a Symposium on Malotilate held at the 7th World Congress of Gastroenterology, p. 54–76, Excerpta Medica, Amsterdam, Princeton, Geneva and Tokyo (1983)

2 Imaizumi, Y., Katoh, M., Sugimoto, T. and Kasai, T.: Effect of malotilate (diisopropyl 1,3-dithiol-2-yldenemalonate) on the protein synthesis in rat liver. Japan. J. Pharmacol. 32, 369–375 (1982)

3 Katoh, M., Kitada, M., Satoh, T., Kitagawa, H., Sugimoto, T. and Kasai, T.: Effect of diisopropyl 1,3-dithiol-2-yldenemalonate on microsomal electron transport system in rat liver. J. Pharmacobiodyn. 3, 261–263 (1980)

4 Katoh, M., Kitada, M., Satoh, T., Kitagawa, H., Sugimoto, T. and Kasai, T.: Further studies on the in vivo effect of diisopropyl 1,3-dithiol-2-yldenemalonate (NKK-105) on the liver microsomal drug oxidation system in rats. Biochem. Pharmacol. 30, 2759–2765 (1981)

5 Tomikawa, M., Nakayasu, T., Tawara, K., Kameda, K. and Abiko, Y.: Effect of pantethin on lipoprotein profiles and HDL subtraction in experimentally hypercholesterolemic rabbits. Atherosclerosis 41, 267–274 (1982)

6 Notari, R.E.: Biopharmaceutics and Pharmacokinetics—An Introduction. Marcel Dekker, New York (1971)

7 Laurell, C.B.: Electroimmunoassay. Scand. J. Clin. Lab. Invest. 29, Supp. 124, 21–37 (1972)

8 Zak, B.: Simple rapid microtechnique for serum total cholesterol. Am. J. Clin. Pathol. 27, 583–586 (1957)

9 Fletcher, M.J.: Acolorimetric method for estimation of serum triglycerides. Clin. Chim. Acta 22, 393–397 (1968)

10 Nakamura, T., Yoshimoto, K., Aoyama, K. and Ichihara, A.: Hormonal regulations of glucose-6-phosphate dehydrogenase and lipogenesis in primary cultures of rat hepatocytes. J. Biochem. 91, 681–693 (1982)

11 Gravela, E., Albano, E., Dianzani, M.U., Poli, G. and Slater, T.F.: Effects of carbon tetrachloride on isolated rat hepatocytes inhibition of protein and lipoprotein secretion. Biochem. J. 178, 509–512 (1979)

12 Nokata, M., Katoh, M. and Sugimoto, T.: Protective effect of malotilate (diisopropyl 1,3-dithiol-2-yldenemalonate) against CCl4-induced liver injury in mice and rats. J. Toxicol. Sci. (in press)

13 Imaizumi, Y., Katoh, M. and Sugimoto, T.: Effect of diisopropyl 1,3-dithiol-2-yldenemalonate (NKK-105) on fatty liver induced by carbon tetrachloride. Japan. J. Pharmacol. 31, 15–21 (1981)

14 Nakayama, S. and Sakamoto, K.: Pharmacological study of diisopropyl 1,3-dithiol-2-yldenemalonate (NKK-105), a new drug for liver diseases—No. 2. Effect on bile secretion and bile components. Showa Med. J. 38, 513–523 (1978)