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EFFECT OF 4-(4′-CHLOROBENZYLOXY)BENZYL NICOTINATE (KCD-232) ON TRIGLYCERIDE AND FATTY ACID METABOLISM IN RATS

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Abstract—The effects of KCD-232, a new hypolipidemic agent with a structure of 4-(4′-chlorobenzyloxy) benzyl nicotinate, on triglyceride (TG) and fatty acid (FA) metabolism were studied in rats. KCD-232 dose-dependently reduced both liver and serum TG levels. From *in vivo* and *in vitro* studies, the hypotriglyceridemic action of KCD-232 was shown to be based on the inhibition of hepatic TG synthesis due to both decreased FA synthesis and increased FA oxidation in the liver. Of two metabolites of KCD-232, i.e. 4-(4′-chlorobenzyloxy)benzoic acid (MII) and nicotinic acid, MII was found to be responsible for the decreased synthesis and increased oxidation of FA in the liver, the latter apparently being due to increased mitochondrial oxidation activated by MII. MII was demonstrated to form a xenobiotic TG in which one fatty acid moiety was substituted by MII and to form a thioester with CoA by rat liver microsomes. This thioester, MII-CoA, inhibited fatty acid syntheses from [^{14}C]acetate, [^{14}C] acetyl-CoA and [^{14}C]malonyl-CoA in cell-free enzyme systems from rat liver both with and without an NADPH-generating system, whereas MII as such showed no effect. MII-CoA was therefore considered to be a chemical entity for the inhibition of hepatic fatty acid synthesis by KCD-232 and was suggested to inhibit fatty acid synthetase directly.

In the study of a series of compounds possessing hypolipidemic activity in rats, 4-(4′-chlorobenzyloxy)benzyl nicotinate (KCD-232) has been found to be a new hypolipidemic agent without any appreciable toxicity and has been reported to show a hypocholesterolemic activity in experimental animals [1]. In the present paper, we describe the influence of KCD-232 on serum and liver triglyceride (TG) levels in rats and its effect on hepatic TG synthesis from various labeled precursors either *in vivo* or *in vitro*. Its effect on [^{14}C]palmitic acid oxidation was also studied. During the course of these studies, one metabolite of KCD-232 with a structure of 4-(4′-chlorobenzyloxy)benzoic acid (MII) was found to be esterified to glycerol in the rat liver like other benzoic acids such as 4-benzyloxybenzoic acid [2] and 3-phenoxybenzoic acid [3]. The existence of such a xenobiotic glyceride shows that 4-(4′-chlorobenzyloxy)benzoyl-CoA (MII-CoA) is undoubtedly formed in rats. Usual fatty acyl-CoA thioesters have been reported to inhibit the activities of acetyl-CoA carboxylase [4] and fatty acid synthetase [5], the regulatory enzymes of fatty acid biosynthesis. We studied, therefore, the influence of KCD-232 on hepatic fatty acid synthesis both *in vivo* and *in vitro*, and confirmed the formation of MII-CoA. The effect of MII-CoA on fatty acid synthesis was also studied using cell-free enzyme systems. The structures of KCD-232 and MII are shown in Fig. 1.

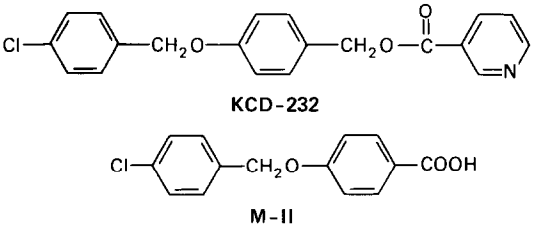

Fig. 1. Structures of KCD-232 and MII.

MATERIALS AND METHODS

Chemicals. Sodium [1-^{14}C]acetate (60.2 mCi/mmole) and [U-^{14}C]glycerol (175 mCi/mmole) were obtained from The Radiochemical Centre, and [1-^{14}C]palmitic acid (50.2 mCi/mmole), [^{3}H]water (100 mCi/ml), [1-^{14}C]acetyl-CoA (52.5 mCi/mmole) and [2-^{14}C]malonyl-CoA (39.2 mCi/mmole) were from the New England Nuclear Corp. In *in vitro* studies, specific radioactivities of these labeled compounds were diluted with corresponding unlabeled compounds, if necessary. KCD-232, sodium salt of MII, [*carboxyl*-^{14}C]MII, clofibrate (ethyl 4-chlorophenoxyisobutyrate) and the sodium salt of clofibric acid (4-chlorophenoxyisobutyric acid) were prepared in this laboratory. Crystallized bovine serum albumin (Fr. V), reduced glutathione (GSH), CoA, NAD, NADP, NADPH, acetyl-CoA and malonyl-CoA were purchased from the Sigma Chemical Co. Other chemicals were of the best grade commercially available.

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Animals and treatment. Male Wistar rats were used throughout these experiments. The animals were preliminarily fed a commercial stock pellet (CE-2, CLEA Japan Inc., Tokyo) and water *ad lib.* in an air-conditioned room until they had reached an average body weight of 200 g, unless otherwise stated. Where indicated, animals were kept under a reversed light and dark cycle. Drugs were suspended in 0.5% carboxymethyl cellulose solution and given to rats by oral intubation (0.5 ml/100 g body weight). Control rats received 0.5% carboxymethyl cellulose alone.

Estimation of triglyceride-lowering effects of KCD-232 and clofibrate. Rats were divided into seven groups and drugs were administered for 10 days. Animals of group 1 were used as control, and groups 2, 3, 4, 5 and 6 received KCD-232 at doses of 10, 20, 40, 80 and 160 mg/kg body weight once a day. Group 7 received clofibrate at a dose of 160 mg/kg body weight once a day. The final administration of drugs was performed at 9:00 a.m., at which time rats were deprived of their diet but allowed free access to water. They were decapitated 4 hr later, and blood was collected in a glass tube and left to clot at room temperature. The liver was quickly removed, washed with cold 0.9% NaCl solution, blotted on filter paper, and weighed. The serum (1 ml) obtained by centrifugation and the liver (*ca.* 0.5 g) were homogenized with chloroform–methanol (2:1, v/v), and total lipids were extracted and purified according to the method of Folch *et al.* [6]. Triglyceride levels of the serum and liver were determined by the colorimetric method of Van Handel [7].

Estimation of triglyceride synthesis in vivo. In an experiment on incorporation of [^{14}C]acetate, rats were kept in a room with a reversed light and dark cycle (8:00 p.m. to 8:00 a.m. light cycle) for 2 weeks. They were then divided into three groups, one of which was the control; the other two received KCD-232 at doses of 100 and 300 mg/kg body weight/day. On day 16, they received the final administration of KCD-232 and an intraperitoneal injection of sodium [^{14}C]acetate (9.7 μCi/100 g body weight) 5.5 hr and 50 min before sacrifice respectively. The rats were allowed free access to their diet and water until killing which was conducted at mid-dark around 2:00 p.m. while the animals were in a lipogenic state. From the liver and serum, total lipids were extraced and purified as described above. Triglycerides were isolated by thin-layer chromatography (TLC) on a Silica gel G (type 60, Merck) plate using a solvent system of petroleum ether–diethyl ether–acetic acid (80:20:1, by vol.) [8]. The plate was exposed to iodine vapor to visualize each lipid fraction. After iodine had sublimed, the TG zone was scraped directly into a counting vial, and radioacivity was measured in a liquid scintillation spectrophotometer (Packard Tri-Carb model C2425) after adding a toluene-based scintillator. Radioactivity in liver total lipids was similarly measured after evaporation of the solvent containing the purified total lipids.

In an experiment on incorporation of [^{14}C]palmitic acid, rats weighing about 260 g maintained under a normal light and dark cycle received KCD-232 (0, 100 and 300 mg/kg body weight/day). The last administration of the drug (9:00 a.m. on day 16) and the injection of [^{14}C]palmitic acid into the jugular vein were performed 5.5 hr and 15 min [9] before sacrifice. Radiolabeled palmitic acid was combined with 4% bovine serum albumin, and the complex was injected at a dose of 5.7 μCi/100 g body weight. Diet and water were freely given to rats until sacrifice.

Measurement of triglyceride synthesis by liver slices. In experiments using rats that had previously received KCD-232, the animals were given KCD-232 (0 and 300 mg/kg body weight/day) orally for 9 days. In fed-state rats the last administration of the drug was performed 17 hr before killing, and diet and water were freely given until sacrified. In fasted rats, they were finally deprived of their diet 18 hr before sacrifice and the last administration of KCD-232 was performed 2 hr prior to killing. Liver slices (0.5 mm thick) were prepared with a tissue slicer (YH-10D, Hotta Rika Co., Ltd., Tokyo). Slices (200 mg) were placed in a plastic tube (18 × 100 mm) containing 2 ml of Krebs–Ringer phosphate buffer (pH 7.4). In experiments observing the effects on TG synthesis of the main metabolites of KCD-232, liver slices (200 mg) obtained from either fed or fasted (18 hr) rats were incubated with 2 mM MII (sodium salt) or 2 mM nicotinic acid in 2 ml of the aforementioned buffer containing 0.5% bovine serum albumin. Precursors used for TG synthesis were 1 μCi of [1-^{14}C]acetate (1 mCi/mmole), 0.3 μCi of albumin-bound [1-^{14}C]palmitic acid (1 mCi/mmole) and 0.4 μCi of [U-^{14}C]glycerol (1 mCi/mmole) per incubation. Each tube was gassed with 100% O_2, sealed with Parafilm (American Can Co.), and incubated at 37° in a metabolic shaker at 120 strokes/min for 2 hr. At the end of the incubation period, the tube was placed in an ice-cold water bath to stop the reaction. The extraction of total lipids from the incubation mixture (slices and medium), isolation, and counting of labeled TG were carried out as described above.

Formation of MII-bound glyceride. Liver slices (100 mg) from fed-state rats were incubated with 0.3 μCi of albumin-bound [1-^{14}C]palmitic acid (1 mCi/mmole) or 0.5 μCi of [U-^{14}C]glycerol (1 mCi/mmole) in a 1 ml of Krebs–Ringer phosphate buffer (pH 7.4) containing 0.5% bovine serum albumin and 2 mM MII (sodium salt) for 1 hr under 100% O_2 phase. When [*carboxyl*-^{14}C]MII (2.5 mCi/mmole, 0.03 μCi/incubation) was used, unlabeled MII (2 mM) was omitted from the incubation medium, but other incubation conditions were identical. Total lipids were extracted from the incubation mixture and purified as already mentioned, and the phase containing purified total lipids was evaporated to dryness. The residue was redissolved in chloroform–methanol (2:1, v/v). Lipids were separated by TLC on a Silica gel 60F_{254} plate (Merck) with petroleum ether–diethyl ether–acetic acid (70:30:1, by vol.) as a developing solvent system. Each fraction was detected under either iodine vapor or an ultraviolet lamp (2536 Å), and radiochromatograms were taken with a scanner (Packard model 7201).

Assay of triglyceride-synthesizing activity. The liver of fed-state rats given KCD-232 (0 and 300 mg/kg body weight/day) for 45 days was homogenized in 50 mM Tris–HCl buffer (pH 7.4) and the homogenate was centrifuged at 500 *g* and 4° for 5 min.

The supernatant fraction was used as the enzyme source. Triglyceride-synthesizing activity was measured by the method of Matsuoka *et al.* [10] using [1-^{14}C]palmitate (1 mCi/mmole). Protein was estimated by the method of Lowry *et al.* [11] after twice washing the enzyme source with 5% trichloroacetic acid. Enzyme activity was expressed as dpm/min/mg protein.

Measurement of fatty acid oxidation by liver slices. In a pretreatment experiment, rats were orally given KCD-232 (0 and 300 mg/kg body weight/day) for 45 days. Finally they were fasted for 18 hr and administered the drug 3 hr before being killed. Liver slices (200 mg) were incubated in 2 ml of Krebs–Ringer phosphate buffer (pH 7.4) in the presence or absence of 2 mM KCN [12, 13]. In another set of experiments to examine the effects of KCD-232 metabolites on fatty acid oxidation, liver slices (200 mg) from either fed or fasted (18 hr) rats were incubated with 2 mM MII (sodium salt) or 2 mM nicotinic acid in 2 ml of the aforementioned buffer containing 0.5% bovine serum albumin. The dose of albumin-bound [1-^{14}C] palmitic acid (1 mCi/mmole) was 0.6 μCi per incubation in both experiments. Each tube was gassed with 100% O_2, connected with a rubber tube (18 mm in i.d.) to a counting vial which held circular filter paper (24 mm in diameter) saturated with 100 μl of Protosol (New England Nuclear), and incubated at 37° in a metabolic shaker at 120 strokes/min for 2 hr. The incubation was terminated with 1 ml of 8% $HClO_4$ via the rubber tube with the aid of a needle, and thereafter each tube was incubated for another hour to adsorb $^{14}CO_2$ to Protosol. The residual layer was extracted twice with petroleum ether to remove traces of [^{14}C]palmitic acid, and an aliquot of the aqueous phase was counted (acid-soluble oxidation products ≅ ketone bodies) [12]. Radioactivity was measured by adding 10 ml of Insta Gel (Packard).

Electron microscopic study. Rats were given KCD-232 and clofibrate at a dose of 100 mg/kg body weight/day for 21 days, and liver cells were observed with an electron microscope (HU-11D, Hitachi Ltd., Tokyo) as described [14].

In vivo *incorporation of* ^{3}H *from* [^{3}H]*water into hepatic fatty acid.* Rats were kept under an 8:00 p.m. to 8:00 a.m. light cycle for 2 weeks. In experiment 1, rats were given one dose of KCD-232 at 300 mg/kg body weight. In experiment 2, they were administered the drug (300 mg/kg body weight/day) for 11 days. Animals were decapitated at mid-dark around 2:00 p.m. An intraperitoneal injection of [^{3}H]water was made exactly 1 hr before sacrifice. Diet and water were given freely until sacrifice. Blood was collected in a glass tube and left to clot at room temperature. The minced liver weighing about 2 g was placed in a screwcap tube and saponified in 2 ml of 15% ethanolic KOH at 85° for 5–6 hr until totally dissolved. Nonsaponifiable lipids were extracted three times with petroleum ether. The residual aqueous layer was acidified to pH 2 with concentrated HCl, and total fatty acids were extracted three times with petroleum ether. Pooled extracts were dried under nitrogen, dissolved in 2 ml of chloroform, and washed twice with water. The chloroform layer was quantitatively transferred to a scintillation vial, dried, and counted after adding toluene-based scintillator. Fifty microlitres of serum obtained by centrifugation was transferred to a scintillation vial, and radioactivity was counted after adding 10 ml of Insta Gel. The specific radioactivity of serum water of each rat was calculated by looking upon the water content as 93%, and μmoles of tritium incorporation into total fatty acids was calculated with the specific radioactivity [15].

Fatty acid synthesis from [^{14}C]*acetate in liver slices.* Rats were kept under a 4:00 p.m. to 4:00 a.m. light cycle for 2 weeks. They were decapitated at mid-dark around 10:00 a.m. As reference, rats maintained under a normal light and dark cycle (8:00 a.m. to 7:00 p.m. light cycle) were also used at the same time. Diet and water were given freely until sacrifice. Liver slices weighing 100–120 mg were placed in a screwcap tube (16 × 100 mm) containing 1 ml of Krebs–Ringer phosphate buffer (pH 7.4) with 0.5% bovine serum albumin and 0.96 μCi of [1-^{14}C]acetate (1 mCi/mmole) in the absence or presence of MII (Na salt) and nicotinic acid. The tube was gassed with 100% O_2, sealed tightly with a screwcap and incubated at 37° in a metabolic shaker at 120 strokes/min for 2 hr. At the end of the incubation period, 1 ml of 15% ethanolic KOH was added to the tube and the mixture was saponified at 75° for 2 hr. After extracting nonsaponifiable lipids, total fatty acids were extracted, washed and counted as mentioned above.

Microsomal formation of MII-CoA. To test the possibility that MII-CoA may be formed in liver, the enzymatic method of Bloomfield and Bloch [16] was applied with a slight modification. Rats (*ca.* 300 g) maintained under a 4:00 p.m. to 4:00 a.m. light cycle for 2 weeks were decapitated at 10:00 a.m. The liver was removed, washed with cold 0.25 M sucrose, minced with scissors, and homogenized in cold 0.25 M sucrose with a Teflon-pestle homogenizer (10% homogenate). The homogenate was centrifuged at 15,000 *g* and 4° for 20 min, and a microsomal pellet was obtained by centrifuging the resultant supernatant fraction at 105,000 *g* and 4° for 1 hr. To a 30-ml Teflon-pestle homogenizer were added 3 ml of 0.1 M ATP, 4 ml of 0.1 M GSH, 10 ml of 0.1 M KF, 3 ml of 0.1 M $MgCl_2$, 2 ml of 1 M potassium phosphate buffer (pH 7.4), 12 mg of CoA and the microsomal pellet obtained from 10 g liver, and the mixture was homogenized. After quantitative transfer of the contents to a 100-ml flask, 5 μCi of [*carboxyl*-^{14}C]MII (0.28 mCi/mmole) suspended by heating in 4 ml of 1% Triton X-100 solution was added, and the mixture was brought to a final volume of 40 ml with distilled water. After incubation for 40 min at 37° under air, 2 ml of 0.1 M ATP was added and incubation was continued for another 20 min. The mixture was chilled in ice, acidified with 1% cold perchloric acid to pH 2, and centrifuged at 10,000 *g* for 5 min. The sediment was washed three times with 20 ml of 1% cold perchloric acid, three times with 20 ml of 80% cold ethanol, and three times with 20 ml of ethanol–ethyl ether (1:1, v/v). The resultant sediment was neutralized (pH 7.4) with diluted ammonium water, and "MII-CoA" was extracted three times with 20 ml of isopropanol–water–ethanol (1:1:1, by vol.). Pooled extracts were evaporated to dryness and dissolved in water.

Purification and identification of MII-CoA were subsequently carried out. The extracted sample was chromatographed on a Silica gel plate $60F_{254}$ (0.25 mm thick, Merck) with chloroform–ethanol–acetic acid (8:1:1, by vol., solvent 1), with which free MII has been known to move near the solvent front (front 1, see Fig. 9a). The plate was dried overnight at room temperature and developed again in a solvent of isopropanol–water–pyridine (1:2:1, by vol., solvent 2) [17] to front 2 (immediately behind front 1, see Fig. 9b). Radiochromatograms were taken before and after developing with solvent 2. Compounds containing MII were also detected with an ultraviolet lamp (2536 Å). The radioactive zone was scraped off and fluxed with isopropranol–water–ethanol (1:1:1, by vol.), and pooled solvents were evaporated. After dissolving in water, an aliquot of the extract was saponified with 50% ethanolic KOH for 1 hr at 65°, acidified with HCl to pH 2, and [^{14}C] MII thus liberated was extracted with diethyl ether, petroleum ether and benzene. Pooled extracts were evaporated to dryness, dissolved in a small amount of ethanol, and chromatographed with solvent 1 on Silica gel plate $60F_{254}$, and the movement of radioactivity was scanned as described above. Other aliquots of the eluate were used for qualitative analyses of the presence of CoA by ribose measurement [18] and the presence of thioester bond by the method of Renkonen [19].

Fatty acid synthesis by cell-free enzyme system from rat liver. Rats (*ca.* 250 g) maintained under a 4:00 p.m. to 4:00 a.m. light cycle for at least 2 weeks were decapitated at 10:00 a.m. The liver was removed, and 20% liver homogenate in 0.25 M sucrose containing 0.01 M $KHCO_3$ was prepared in a Teflon-glass homogenizer. The homogenate was centrifuged at 12,500 *g* and 4° for 30 min, and the supernatant fraction was centrifuged at 105,000 *g* for 1 hr. The latter supernatant (cytosol fraction) was used as enzyme source [20].

In the cell-free system requiring NADPH generation, fatty acid synthetic activity was measured according to the method of Ishii *et al.* [20] and Masoro *et al.* [21]. Fatty acid synthesis from [1-^{14}C] acetate was examined with the incubation system [20] consisting of 1.5 mg of cytosol protein, 40 mM potassium phosphate buffer (pH 7.1), 15 mM $MgCl_2$, 0.3 mM $MnCl_2$, 40 mM DL-isocitrate, 5 mM $KHCO_3$, 2 mM ATP, 1 mM NADP, 2 mM NAD, 0.05 mM CoA, 4 mM L-cysteine, 1 mM [1-^{14}C]acetate (0.3 mCi/mmole) and various concentrations of MII (sodium salt), MII-CoA and clofibric acid (sodium salt). The final volume was 1.0 ml and the system was incubated in air at 37° for 45 min with gentle shaking. The reaction was terminated with 1 ml of 20% ethanolic KOH containing 1 mM palmitic acid, and the system was saponified for 30 min in a boiling-water bath. After acidification with 1 ml of 5 N HCl, fatty acids were extracted three times with 4 ml of petroleum ether. The pooled extracts were evaporated to dryness under nitrogen. The residue was dissolved in 2 ml of chloroform, washed twice with water, and the chloroform layer was quantitatively transferred to a scintillation vial. The chloroform was evaporated, and radioactivity was counted after addition of toluene scintillator. Fatty acid synthesis from [2-^{14}C]malonyl-CoA was determined with the incubation system [20, 21] consisting of 155 μg of cytosol protein, 4.8 mM potassium phosphate buffer (pH 8.8), 2 μM acetyl-CoA, 48 mM KCl, 0.6 mM $MnCl_2$, 40 mM DL-isocitrate, 1.6 mM NADP, 1 μM [2-^{14}C]malonyl-CoA (39.2 mCi/mmole) and various concentrations of MII (sodium salt), MII-CoA and clofibric acid (sodium salt). The final volume was 1.0 ml and the pH 6.8. The reaction was initiated by addition of [2-^{14}C]malonyl-CoA and terminated with 1 ml of 20% KOH in ethanol containing 1 mM palmitic acid after incubation in air for 10 min at 37° with gentle shaking. Saponification, extraction and counting were carried out as described above.

Fatty acid synthetic activity was also determined in the cell-free system containing no NADPH-generating system but supplemented with NADPH. Fatty acid synthesis from [1-^{14}C]acetyl-CoA was measured by a modified version of the procedure described by Matsuhashi [22]. The incubation system consisted of 100 mM potassium phosphate buffer (pH 7.1), 5 mM GSH, 10 mM $MgCl_2$, 10 mM potassium citrate, 2 mM ATP, 10 mM $KHCO_3$, 1 mM NADPH, 120 μM [1-^{14}C]acetyl-CoA (3.3 mCi/mmole), 850 μg cytosol protein and various concentrations of MII (sodium salt), MII-CoA and clofibric acid (sodium salt). The final volume was 0.2 ml. After preincubating for 15 min at 37°, the reaction was initiated by addition of the mixture of ATP, NADPH and acetyl-CoA. The system was incubated for 15 min at 37° with gentle shaking. Fatty acid synthesis from [2-^{14}C]malonyl-CoA was measured by a modification of the assay method described by Hsu *et al.* [23]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.8), 5 mM GSH, 1 mM NADPH, 25 μM acetyl-CoA, 87.5 μM [2-^{14}C] malonyl-CoA (2 mCi/mmole) 46 μg cytosol protein and various concentrations of MII (sodium salt), MII-CoA and clofibric acid (sodium salt). The final volume was 0.2 ml. The enzyme source was added to start the reaction, and the system was incubated for 15 min at 37° with gentle shaking. Both reactions were terminated with 1 ml of 20% ethanolic KOH containing 1 mM palmitic acid. After saponification for 30 min in a boiling-water bath, 1.8 ml of water and 1.0 ml of 5 N HCl were added. Fatty acids were extracted, washed and counted as described above. Cytosol protein was determined by the method of Lowry *et al.* [11]. Results are expressed as dpm/min/mg protein.

Statistical method. Statistical analysis was performed with Student's *t*-test for paired data, and a P value of <0.05 was considered significant.

RESULTS

Effects on serum and liver triglyceride levels. As illustrated in Fig. 2, KCD-232 reduced the TG levels of both serum and liver in a dose-dependent manner. Clofibrate also decreased the serum TG level at a dose of 160 mg/kg/day. It failed, however, to reduce the liver TG level at this dose, while KCD-232 significantly decreased the level. No significant changes were observed in relative liver weight (g/100 g body weight) of KCD-232-treated rats, whereas clofibrate increased it at a dose of 160 mg/kg/day.

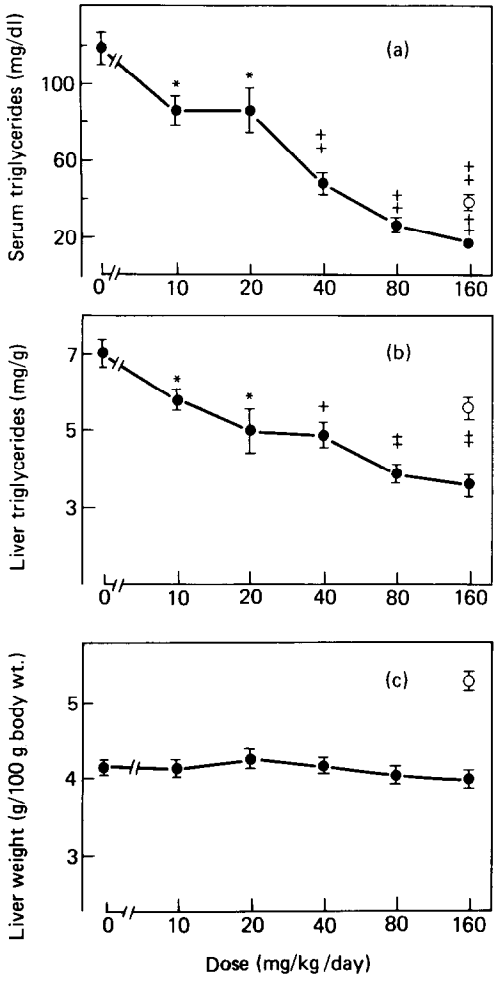


Fig. 2. Effects of KCD-232 and clofibrate on serum and liver triglyceride levels and liver weight. Rats orally received KCD-232 (●) or clofibrate (○) for 10 days at the indicated doses. Serum (a) and liver (b) triglyceride levels were determined as described in Materials and Methods. Liver weight (c) was expressed as the relative weight of liver to 100 g body weight. Each point represents the mean of seven rats. Vertical bars indicate standard errors. Key: (*) $P < 0.05$, (†) $P < 0.01$ and (‡) $P < 0.001$ compared to the control group respectively.

Effects on triglyceride synthesis. Figure 3 shows the effect of KCD-232 on TG synthesis *in vivo* from [1-^{14}C]acetate. The oral administration of the drug significantly inhibited the incorporation of [^{14}C]acetate into the TG fraction of liver and serum total lipids at doses of 100 and 300 mg/kg/day. The inhibitory effect of KCD-232 was greater in the serum TG than in the liver TG: radioactivity in serum TG of KCD-232-treated rats decreased by 74% (100 mg/kg) or 76% (300 mg/kg) in comparison to the control group, whereas that in liver TG decreased by 50% (100 mg/kg) or 64% (300 mg/kg). The [^{14}C]acetate incorporation into liver total lipids also decreased with KCD-232 administration. The *in vivo* incorporation of [^{14}C]palmitic acid into liver and serum TG was also measured (Fig. 4). KCD-232 showed a dose-dependent inhibition on the labeled acid incorporation into liver and serum TG. This inhibitory effect was also more marked in the serum TG than in the liver TG: radioactivity appearing in serum TG of rats given KCD-232 decreased by 63% (100 mg/kg) or 85% (300 mg/kg) as compared to control rats, while that in liver TG decreased by 15% (100 mg/kg) or 53% (300 mg/kg). The incorporation of [^{14}C]

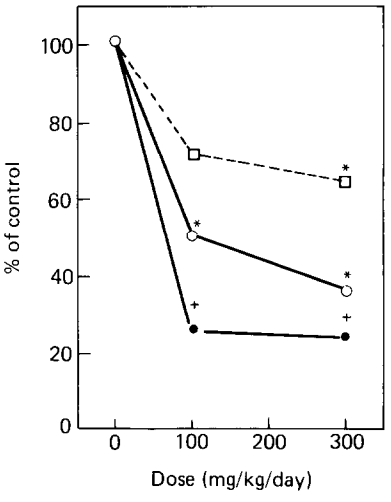


Fig. 3. Incorporation of [1-^{14}C]acetate *in vivo* into liver total lipids and triglycerides of liver and serum in rats given KCD-232 orally. Control values for liver triglycerides (○) and total lipids (□) were 4.95 ± 0.80 ($\times 10^{-3}$) and 3.78 ± 0.34 ($\times 10^{-4}$) dpm/g liver (mean ± S.E.M.) and that for serum triglycerides (●) was 2.13 ± 0.22 ($\times 10^{-3}$) dpm/ml serum (mean ± S.E.M.) respectively. Each point represents the mean of five rats. Key: (*) $P < 0.05$ and (†) $P < 0.005$ compared to the control group respectively.

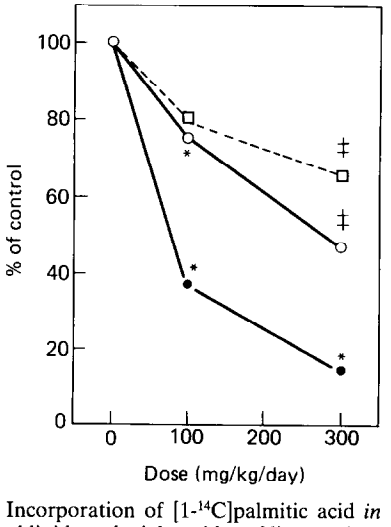


Fig. 4. Incorporation of [1-^{14}C]palmitic acid *in vivo* into liver total lipids and triglycerides of liver and serum in rats given KCD-232 orally. Control values for liver triglycerides (○) and total lipids (□) were 4.80 ± 0.31 ($\times 10^{-5}$) and 1.29 ± 0.06 ($\times 10^{-6}$) dpm/g liver (mean ± S.E.M.) and that for serum triglycerides (●) was 2.94 ± 0.68 ($\times 10^{-4}$) dpm/ml serum (mean ± S.E.M.) respectively. Each point represents the mean of seven rats. Key: (*) $P < 0.05$, (†) $P < 0.01$ and (‡) $P < 0.001$ compared to the control group respectively.

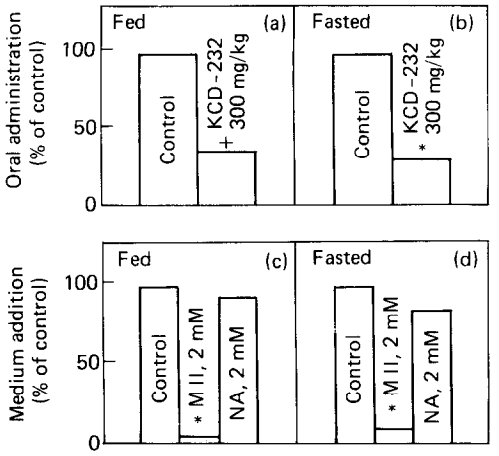


Fig. 5. Incorporation of [1-^{14}C]acetate into triglycerides in liver slices of rats previously given KCD-232 (a, b) or in liver slices supplemented with MII or nicotinic acid (NA) (c, d). Control values for a, b, c and d were $7.07 \pm 1.78 (\times 10^{-5})$, $3.04 \pm 0.41 (\times 10^{-4})$, $5.27 \pm 0.58 (\times 10^{-5})$ and $4.29 \pm 0.46 (\times 10^{-4})$ dpm/g liver (mean of six rats ± S.E.M.) respectively. Key: (*) $P < 0.005$ and (†) $P < 0.05$ compared to the control group.

palmitic acid into liver total lipids was also inhibited by KCD-232 administration in a dose-dependent manner.

As shown in Fig. 5a and b, the [^{14}C]acetate incorporation into TG in liver slices from KCD-232-treated rats was inhibited significantly in both fed and fasted states. This inhibition was based not on nicotinic acid but on MII (Fig. 5c and d). The [^{14}C] palmitic acid esterification to TG in liver slices from KCD-232-treated rats was not inhibited at all in either fed or fasted states (Fig. 6a and b). On the contrary, the esterification of [^{14}C]palmitic acid to TG was inhibited significantly by 75% (fed) or 66% (fasted) when MII was added to the incubation medium (Fig. 6c and d).

During the course of the experiments shown in Fig. 6c and d, an unknown peak which could not be found in the absence of MII was observed on a radiochromatogram of a TLC plate for lipid separation. As illustrated in Fig. 7a, a shoulder-like, unknown peak appeared just ahead of the free fatty acid zone. Under an ultraviolet lamp, a band clearly distinct from free MII was recognized at the position corresponding to this peak. This unknown substance

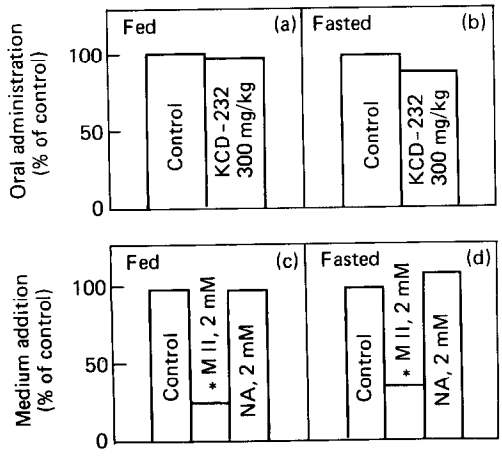


Fig. 6. Incorporation of [1-^{14}C]palmitic acid into triglycerides in liver slices of rats previously given KCD-232 (a, b) or in liver slices supplemented with MII or nicotinic acid (NA) (c, d). Control values for a, b, c and d were 3.73 ± 0.29, 3.43 ± 0.12, 3.11 ± 0.23 and 2.00 ± 0.13 $(\times 10^{-5})$ dpm/g liver (mean of six rats ± S.E.M.) respectively. Key: (*) $P < 0.005$ compared to the control group.

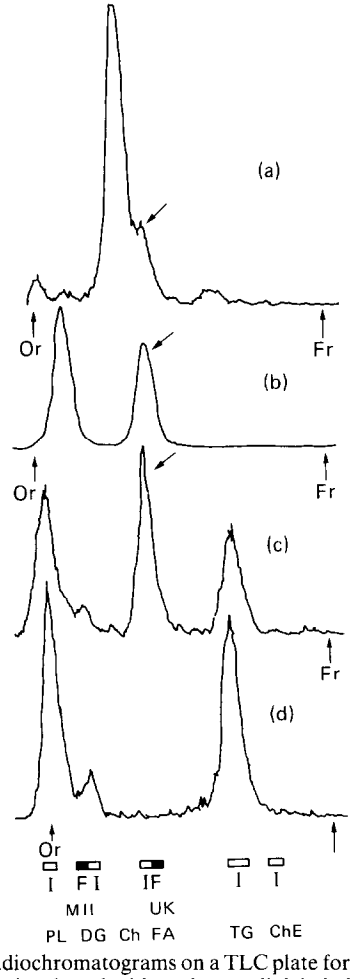


Fig. 7. Radiochromatograms on a TLC plate for lipids of rat liver slices incubated with various radiolabeled substrates. Liver slices weighing 100 mg from fed rats were incubated with 0.3 μCi of [1-^{14}C]palmitic acid (a), 0.03 μCi of [*carboxyl*-^{14}C]MII (b) and 0.5 μCi of [U-^{14}C]glycerol (c, d) in 1 ml of Krebs–Ringer phosphate buffer (pH 7.4) containing bovine serum albumin and 2 mM MII for 1 hr. In experiments b and d, unlabeled MII (2 mM) was omitted from the incubation medium. Total lipids extracted from the incubation contents were purified and separated by TLC on a Silica gel $60F_{254}$ plate using a solvent system of petroleum ether–diethyl ether–acetic acid (70:30:1, by vol.). Each fraction was detected under either iodine vapor or ultraviolet lamp (2536 Å) and with a radiochromatogram scanner. I: visualized with iodine vapor; F: visualized with ultraviolet lamp. Abbreviations: Or, origin; Fr, front; Ch or ChE, free or esterified cholesterol; TG, triglycerides; DG, diglycerides; FA, fatty acids: PL, phospholipids; and UK, unknown.

was considered to originate in MII, since no visible bands were found under the ultraviolet lamp if MII was omitted from the incubation medium. Hence, [^{14}C]MII was incubated with normal rat liver slices, and a radiochromatogram was prepared. As shown in Fig. 7b, two peaks were found: one corresponded to the MII-derived unknown substance and the other to MII itself. It became clear that fatty acid and MII coexisted in the unknown substance. This unknown substance disappeared after hydrolysis, suggesting the presence of an ester bond. The substance was considered not to be the direct ester of fatty acid with MII, because both were carboxylic acids. It was thought to be a kind of lipid containing MII with fatty acid by a bridge of glycerol. Hence, [^{14}C]glycerol was incubated with liver slices in the presence (Fig. 7c) or absence (Fig. 7d) of MII. As was expected, the unknown peak appeared when MII was present but not when MII was absent. These results strongly suggested that this unknown substance was a kind of glyceride. Further analyses showed that two molecules of fatty acids and one molecule of MII were esterified to one molecule of glycerol. Thus, the unknown substance was named "DG-MII".

It was difficult to separate [^{14}C]DG-MII with TLC from labeled fatty acids such as [^{14}C]palmitic acid and [^{14}C]fatty acids synthesized from [^{14}C]acetate for contamination. By contrast, the use of [^{14}C]glycerol as a precursor of glyceride synthesis appeared to enable the [^{14}C]DG-MII separation on TLC without any contamination of radioactivity (see Fig. 7c and d). So experiments on the incorporation of [U-^{14}C] glycerol into TG and DG-MII in liver slices were conducted (Table 1). In liver slices from rats given KCD-232 orally, the [^{14}C]glycerol incorporation into TG was inhibited significantly in slices from fed rats when compared to the control group. The sum total of TG and DG-MII, however, did not show any significant difference between control and KCD-232-treated rats; this tendency was also found in fasted rats. When KCD-232 metabolites, MII and nicotinic acid, were added to the incubation medium, in slices from both fed and fasted rats the [^{14}C]glycerol incorporation into TG was strongly suppressed by MII but not at all by nicotinic acid. Though MII incorporated more [^{14}C]glycerol into DG-MII in both fed and fasted states than into TG, the summation of both TG and DG-MII showed no significant difference from TG formed in the control group. These results suggested that the esterification process of fatty acids including MII to glycerol might not be directly impaired by KCD-232 or MII. Therefore, TG synthesizing activity was directly measured in liver homogenates using [1-^{14}C]palmitic acid. The TG-synthesizing activity in the liver of fed rats was not affected despite the long-term administration of KCD-232 (300 mg/kg/day for 45 days, data not shown).

Effects on fatty acid oxidation. Tables 2 and 3 show fatty acid oxidation activity from [^{14}C]palmitic acid by liver slices. In a preliminary experiment, liver slices from rats given KCD-232 orally for 9 days had been found to produce more $^{14}CO_2$ from [1-^{14}C] palmitic acid than those from control rats. It has been reported that peroxisomal acyl-CoA oxidation is cyanide insensitive [12, 13]. Hence we measured production of acid-soluble oxidation products (ketone bodies) as well as of $^{14}CO_2$ to definitely

Table 1. Effects of oral administration of KCD-232 or medium addition of its metabolites on the incorporation of [U-^{14}C]glycerol into glycerides in liver slices from fed or fasted rats*

Treatment	TG	DG-MII (dpm/g liver) × 10^{-3}	TG + DG-MII
Oral administration of KCD-232			
Fed state			
Control	6.7 ± 0.6		6.7 ± 0.6
KCD-232, 300 mg/kg/day	4.8 ± 0.4†	0.9 ± 0.2	5.6 ± 0.4
Fasted state			
Control	8.2 ± 0.5		8.2 ± 0.5
KCD-232, 300 mg/kg/day	7.6 ± 0.9	3.4 ± 0.5	11.0 ± 1.1
Medium addition of metabolites			
Fed state			
Control (none)	6.8 ± 0.5		6.8 ± 0.5
MII (2 mM)	2.7 ± 0.3‡	3.9 ± 0.3	6.6 ± 0.6
Nicotinic acid (2 mM)	7.1 ± 1.1		7.1 ± 1.1
Fasted state			
Control (none)	5.5 ± 0.7		5.5 ± 0.7
MII (2 mM)	2.6 ± 0.2†	3.2 ± 0.4	5.8 ± 0.5
Nicotinic acid (2 mM)	7.2 ± 0.7		7.2 ± 0.7

* Liver slices (200 mg) prepared from fed or fasted rats were incubated with 0.4 μCi of [U-^{14}C]glycerol (1 mCi/mmole) in 2 ml of Krebs–Ringer phosphate buffer (pH 7.4) for 2 hr. The buffer contained 0.5% bovine serum albumin in the medium addition experiments to attain solubilization of the sodium salt of MII. Triglycerides (TG) and ester of MII with diglycerides (DG-MII) were separated on TLC, and each zone was scraped into scintillation vials. Radioactivity was counted after addition of toluene scintillator. Each value represents the mean of six incubations ± S.E.M.

† $P < 0.05$ compared to the control group.

‡ $P < 0.005$ compared to the control group.

Table 2. Effect of oral administration of KCD-232 on the production of $^{14}CO_2$ and [^{14}C]ketone bodies from [1-^{14}C]palmitic acid in the absence or presence of cyanide in liver slices of rats*

Group	$^{14}CO_2$ (dpm/g liver) × 10^{-3}			[^{14}C]Ketone bodies (dpm/g liver) × 10^{-4}		
	−CN	+CN	Probability (−CN vs +CN)	−CN	+CN	Probability (−CN vs +CN)
Control	42.75 ± 3.05 (100)	2.14 ± 0.34 (100)	$P < 0.001$	28.88 ± 2.39 (100)	2.23 ± 0.21 (100)	$P < 0.001$
KCD-232, 300 mg/kg/day	65.55 ± 3.55 (153)	1.23 ± 0.16 (57.5)	$P < 0.001$	38.53 ± 1.16 (133)	1.74 ± 0.18 (78.0)	$P < 0.001$
Probability (control vs KCD-232)	$P < 0.005$	$P < 0.05$		$P < 0.02$	NS†	

* Rats weighing about 170 g were orally given KCD-232 (300 mg/kg/day) or 0.5% carboxymethyl cellulose (control) for 45 days. Finally, they were fasted for 18 hr and were administered the drug 3 hr before being killed. Liver slices were prepared and incubated with [^{14}C]palmitic acid in the absence or presence of 2 mM KCN. The production of $^{14}CO_2$ and [^{14}C]ketone bodies was estimated as described in Materials and Methods. The final body weight of control or KCD-232-treated rats was 320 ± 12 or 308 ± 9 g. Each value represents the mean of five rats ± S.E.M. Figures in parentheses indicate percent of control.

† Not significant.

determine the increased fatty acid oxidation by the drug. Simultaneously, liver slices from control and KCD-232-treated rats were incubated with and without cyanide to clarify whether or not this activated β-oxidation in the latter was due to increased peroxisomal oxidation. As shown in Table 2, KCD-232 treatment significantly increased the production of $^{14}CO_2$ and ketone bodies, and cyanide severely inhibited the production in liver slices from both control and KCD-232-treated rats. These results suggested that under these conditions the bulk of fatty acid oxidation was not peroxisomal but mitochondrial and that KCD-232 enhanced mitochondrial β-oxidation. This hypothesis was morphologically supported by the electron microscopic study shown in Fig. 8. Significant peroxisome proliferation was not observed in liver parenchymal cells of KCD-232-treated rats (Fig. 8b) but was in those of clofibrate-treated rats (Fig. 8c). Table 3 shows which metabolites of KCD-232, MII or nicotinic acid, enhanced fatty acid oxidation. In both fed and fasted rats, MII significantly increased the production of $^{14}CO_2$ and ketone bodies except for that of ketone bodies in fasted rats. On the other hand, nicotinic acid had no effect on either production at the concentration used. The activation of fatty acid oxidation by KCD-232 treatment is therefore suggested to be due to MII.

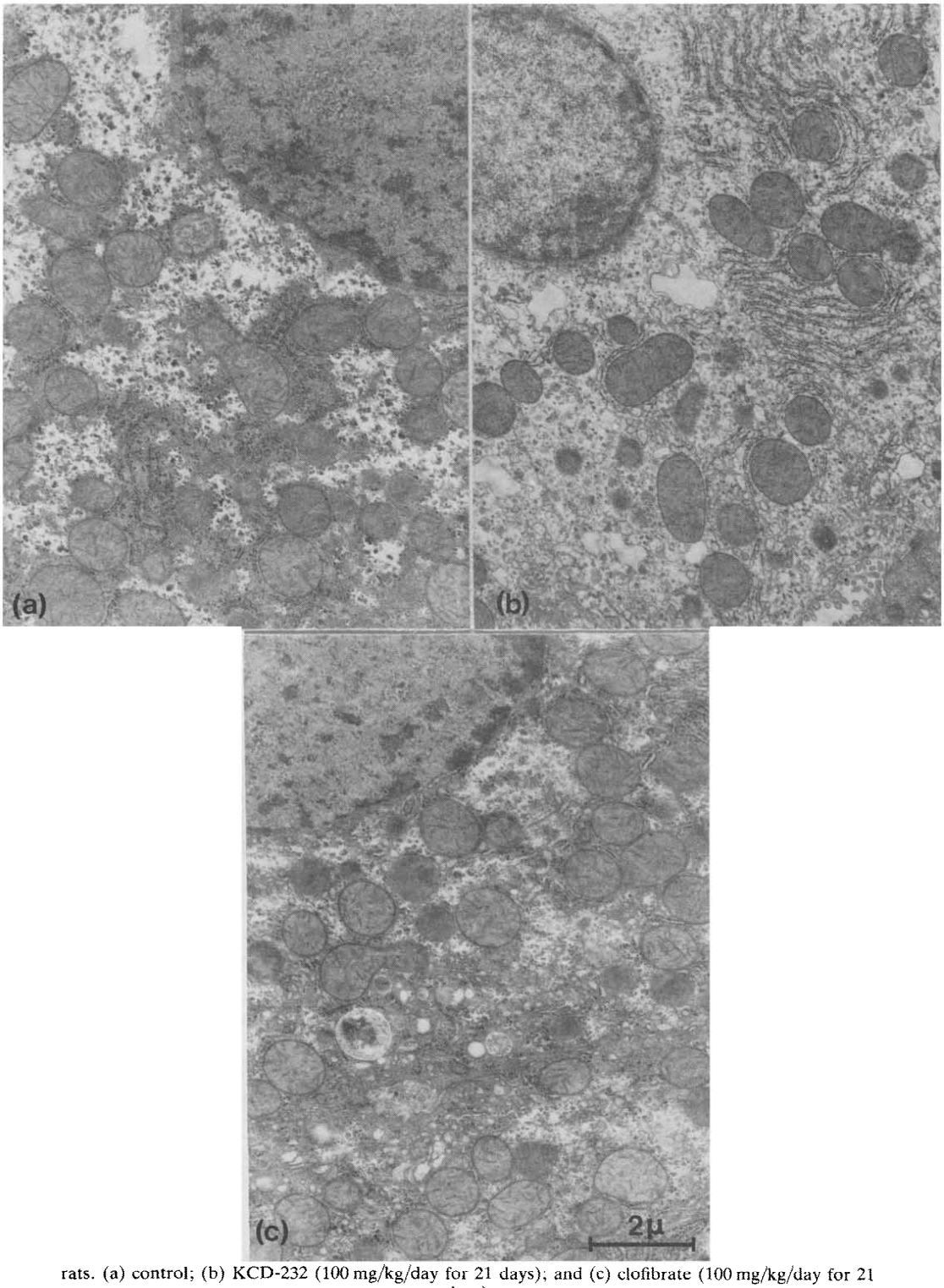
Effects on fatty acid synthesis. The absolute synthetic rate of hepatic fatty acids was measured *in vivo* using [^{3}H]water [15]. The results are shown in Table 4. KCD-232 significantly suppressed hepatic fatty acid synthesis in rats with a single administration (by 36%) as well as in rats with eleven successive administrations (by 43%) at a dose of 300 mg/kg/day. Serum TG concentration was lowered by both single administration and eleven successive administrations of KCD-232. Liver TG level tended to be lowered by the successive administration ($P < 0.1$), while the single administration showed no lowering effect. The relative liver weight showed no significant elevation. To examine which metabolites of KCD-232 possessed the inhibitory effect on hepatic fatty acid synthesis, liver slices were incubated with [^{14}C]

Table 3. Effects of MII and nicotinic acid addition to the incubation medium on the production of $^{14}CO_2$ and [^{14}C]ketone bodies from [1-^{14}C]palmitic acid in liver slices of fed or fasted rats*

Group	Concn (mM)	Fed state		Fasted state	
		$^{14}CO_2$ (dpm/g liver) × 10^{-4}	[^{14}C]Ketone bodies (dpm/g liver) × 10^{-4}	$^{14}CO_2$ (dpm/g liver) × 10^{-4}	[^{14}C]Ketone bodies (dpm/g liver) × 10^{-4}
Control		1.09 ± 0.05 (100)	3.24 ± 0.21 (100)	3.13 ± 0.18 (100)	10.52 ± 0.83 (100)
MII	2	4.25 ± 0.27† (390)	4.80 ± 0.24† (148)	5.54 ± 0.26† (177)	9.50 ± 0.58 (90.3)
Nicotinic acid	2	1.05 ± 0.07 (96.3)	2.92 ± 0.25 (90.1)	3.26 ± 0.29 (104)	10.73 ± 0.62 (102)

* Fed rats were freely given diet and water until they were killed, while fasted rats were deprived of their diet 18 hr before sacrifice. The average body weight of fed or fasted rats was 251 ± 1 or 225 ± 1 g (mean of six rats ± S.E.M.). Liver slices were incubated with [1-^{14}C]palmitic acid in the presence of MII or nicotinic acid. The production of $^{14}CO_2$ and [^{14}C]ketone bodies was estimated as described in Materials and Methods. Each value represents the mean of six incubations ± S.E.M. Figures in parentheses indicate percent of control.

† $P < 0.001$ compared to the control group.



rats. (a) control; (b) KCD-232 (100 mg/kg/day for 21 days); and (c) clofibrate (100 mg/kg/day for 21 days).

acetate in the presence or absence of MII and nicotinic acid (Table 5). Liver slices obtained from rats maintained under a reversed light and dark cycle incorporated 2.6-fold as much [^{14}C]acetate into fatty acids as did those obtained from rats under a normal light and dark cycle. In these lipogenic slices, MII depressed the [^{14}C]acetate incorporation into fatty acids in a dose-dependent manner and inhibited almost completely the incorporation at a concentration of 2 mM where nicotinic acid showed no

Table 4. Effects of one and of eleven successive administrations of KCD-232 on the *in vivo* incorporation of ^3H_2O into total fatty acids in the liver of rats*

Group	No. of rats	Body wt Initial (g)	Body wt Final (g)	Liver wt (g/100 g body wt)	Triglyceride level Serum (mg/dl)	Triglyceride level Liver (mg/g)	^3H_2O incorporation (μmoles H_2O/hr/g liver)	^3H_2O incorporation (% of Control)
				Experiment 1				
Control	6		216 ± 4	4.17 ± 0.05	87.4 ± 7.6	3.64 ± 0.38	7.44 ± 0.80	100.0
KCD-232	6		215 ± 3	4.09 ± 0.11	66.3 ± 2.4†	3.67 ± 0.27	4.74 ± 0.53†	63.7
				Experiment 2				
Control	7	202 ± 5	236 ± 4	3.77 ± 0.03	70.7 ± 6.3	3.36 ± 0.32	8.85 ± 0.85	100.0
KCD-232	7	202 ± 5	231 ± 8	3.93 ± 0.07	46.9 ± 2.5†‡	2.60 ± 0.09	5.04 ± 0.38‡	56.9

* Rats were maintained under an 8:00 p.m. to 8:00 a.m. light cycle for 2 weeks, and then were orally administered KCD-232 once (experiment 1) or eleven times (experiment 2) at a dose of 300 mg/kg/day. The single or final administration of the drug and the intraperitoneal injection of ^3H_2O (2 mCi/rat) were performed 5 hr and 1 hr prior to sacrifice, which was carried out by decapitation at mid-dark around 2:00 p.m. Total fatty acids of the liver were extracted and counted as mentioned in Materials and Methods. Each value represents the mean ± S.E.M.

† $P < 0.05$ compared to the control group.

‡ $P < 0.005$ compared to the control group.

inhibitory effect. Thus, MII was shown to be KCD-232 metabolite which caused the reduction of hepatic fatty acid synthesis.

MII was shown to be incorporated into a triglyceride in which one fatty acid moiety was substituted by MII (Fig. 7). This indicates that MII-CoA is undoubtedly formed in rats. Figure 9 shows evidence for this point. When the sample extracted from the mixture in which [^{14}C]MII was incubated with liver microsomes in the presence of various cofactors was chromatographed with solvent 1, there appeared only a single peak which corresponded to the origin (Fig. 9a). There was no peak near front 1. This indicated that free [^{14}C]MII was completely removed during washing of the incubation mixture, since MII was known to move near the solvent front (front 1) with solvent 1. After drying, the same plate was subsequently rechromatographed using solvent 2 (Fig. 9b). The aforementioned peak moved to approximately the middle part of the plate ($R_f = 0.61$ for solvent 2), which position corresponded to free CoA and could be visualized with ultraviolet light (2536 Å). Aliquots of the extract from this zone were applied to the qualitative methods for ribose and thioester bond and they showed positive responses, indicating the presence of CoA and thioester bond. Another aliquot was hydrolyzed, and liberated acids were extracted and chromatographed with solvent 1 (Fig. 9c). The main peak appeared at the zone corresponding to MII and the minor peak in the midst of the plate. These results clearly show that MII-CoA is actually formed by liver microsomes and hence liver cells.

Table 5. Effects of MII and nicotinic acid on the *in vitro* incorporation of [1-^{14}C]acetate into total fatty acids in rat liver slices*

Group	No. of rats	Concn (mM)	Incorporation (dpm/g liver) × 10^{-6}	% of Control
Control	5		1.81 ± 0.37	100.0
MII	5	0.1	1.13 ± 0.30	62.4
MII	5	0.5	0.14 ± 0.03†	7.7
MII	5	2.0	0.07 ± 0.01†	3.9
Nicotinic acid	5	2.0	1.94 ± 0.32	107.2
Normal§	6		0.68 ± 0.10‡	37.6

*After rats were maintained under a 4:00 p.m. to 4:00 a.m. light cycle for 2 weeks, they were decapitated at mid-dark around 10:00 a.m. They had an average body weight of 253 ± 6 g (mean of five rats ± S.E.M.). From each liver, five groups of slices weighing 100–120 mg each were prepared and incubated with 0.96 μCi of [1-^{14}C]acetate (1 mCi/mmole) in the absence (control, normal) or presence of drugs and analyzed as described in Materials and Methods. Each value represents mean ± S.E.M.

§ Slices of this group were obtained from rats maintained under an 8:00 a.m. to 7:00 p.m. light cycle. Their final body weight was 239 ± 5 g (mean of six rats ± S.E.M.).

† $P < 0.01$, compared to the control group.

‡ $P < 0.05$ compared to the control group.

Usual fatty acyl-CoA thioesters have been shown to inhibit activities of acetyl-CoA carboxylase [4] and fatty acid synthetase [5]. Thus, it was supposed that MII-CoA might be a chemical entity for the inhibitory effect of MII on fatty acid synthesis in liver slices. Fatty acid syntheses from various radiolabeled substrates were therefore estimated in the presence of MII, MII-CoA and clofibric acid using cell-free enzyme systems with or without the NADPH-generating system. As seen in Fig. 10, MII-CoA inhibited fatty acid syntheses from [^{14}C]acetate and [^{14}C]malonyl-CoA at concentrations below 100 μM in the system requiring NADPH generation, whereas MII and clofibric acid had no inhibitory effects at the concentrations used here. In the case of a cell-free enzyme system containing no NADPH-generating system but supplemented with NADPH (Fig. 11), MII-CoA also reduced fatty acid syntheses from [^{14}C]acetyl-CoA and [^{14}C]malonyl-CoA at concentrations below 100 μM, while MII and clofibric acid showed no inhibitory effects at those concentrations. These results show that MII-CoA is an inhibitor of fatty acid synthesis analogous to fatty acyl-CoA thioesters.

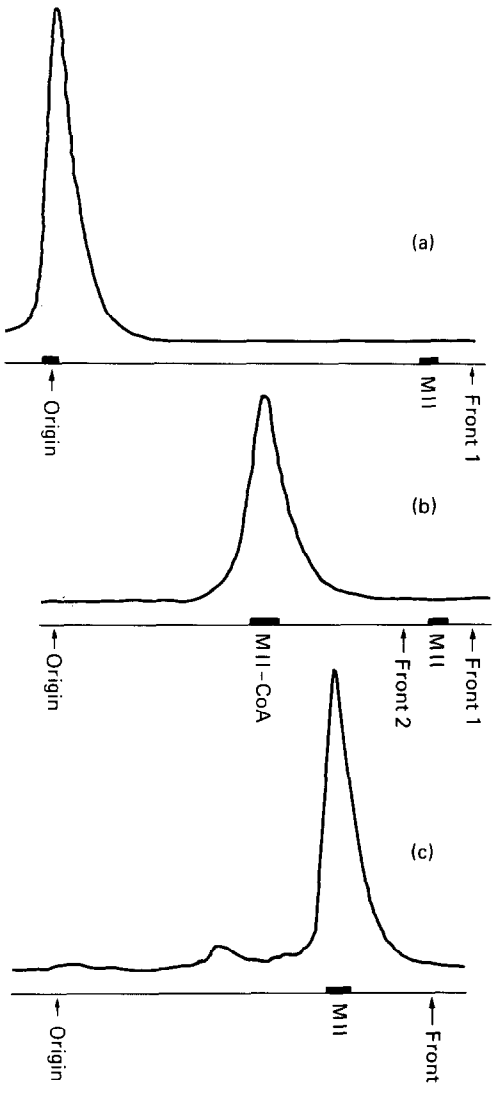


Fig. 9. Radiochromatograms on a TLC plate for [*carboxyl*-^{14}C]MII-CoA. [^{14}C]MII was incubated with rat liver microsomes for 1 hr at 37° in the presence of various cofactors including CoA. The product (MII-CoA) was extracted from the incubation and chromatographed on a Silica gel $60F_{254}$ plate with chloroform–methanol–acetic acid (8:1:1, by vol., solvent 1) to front 1 (a). After drying, the plate was developed again in a solvent of isopropanol–water–pyridine (1:2:1, by vol., solvent 2) to front 2 (b). The zone showing radioacitivity was scraped off and the product was extracted, hydroylzed and acidified. The liberated acids were extracted and chromatographed with solvent 1 (c). Closed rectangles indicate MII or MII-CoA visualized with ultraviolet lamp (2536 Å).

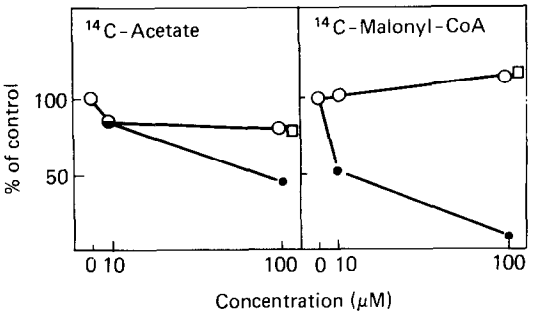


Fig. 10. Effects of MII-CoA, MII and clofibric acid on fatty acid syntheses from [1-^{14}C]acetate and [2-^{14}C]malonyl-CoA by cell-free enzyme systems from rat liver containing an NADPH-generating system. ^{14}C-Labeled substrates were incubated with liver cytosol fraction as enzyme source at 37° in the presence of various cofactors including NADP and MII-CoA (●), MII (○) and clofibric acid (□). Fatty acids synthesized were extracted and counted after incubation for periods as described in Materials and Methods. Each point represents the mean of five (control) or three (others) incubations. Control values for [^{14}C]acetate and [^{14}C]malonyl-CoA were 95 ± 5 and 13,844 ± 43 (mean of five incubations ± S.E.M., dpm/min/mg protein).

DISCUSSION

KCD-232 decreased liver TG content as well as serum TG concentration in fed-state rats (Fig. 2). Further experiments showed that, in rats fasted and refed a fructose diet, both the liver and serum TG levels were markedly elevated as compared to those of rats refed a glucose diet, and that KCD-232 strongly suppressed these fructose-induced rises (T. Irikura *et al.*, unpublished data). Since fasting and refeeding have been reported to lead to enhanced hepatic lipogenesis in comparison to animals fed *ad lib.* [24] and since fructose has been reported to be very lipogenic [25], it is suggested first that the suppression of hepatic TG synthesis may account for the reduction of serum and hepatic TG in KCD-232-treated rats. The *in vivo* incorporation of [^{14}C] acetate into both liver total lipids and TG decreased in KCD-232-treated rats (Fig. 3). The [^{14}C]acetate incorporation into TG in liver slices from KCD-232-treated rats was also inhibited in both fed and fasted states, and this suppression was due to MII, a KCD-232 metabolite (Fig. 5). Though these results strongly

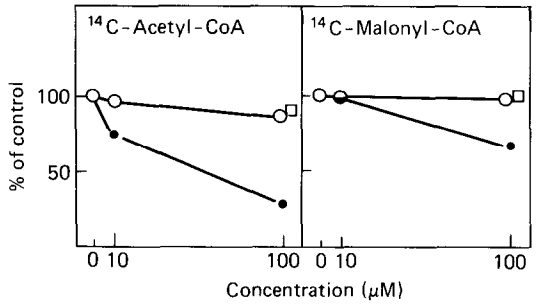


Fig. 11. Effects of MII-CoA, MII and clofibric acid on fatty acid syntheses from [1-^{14}C]acetyl-CoA and [2-^{14}C]malonyl-CoA by cell-free enzyme systems from rat liver containing no NADPH-generating system but supplemented with NADPH. ^{14}C-Labeled substrates were incubated with liver cytosol fraction as enzyme source for 15 min at 37° in the presence of various cofactors including NADPH and MII-CoA (●), MII (○) and clofibric acid (□). Fatty acids synthesized were extracted and counted as described in Materials and Methods. Each point represents the mean of five (control) or three (others) incubations. Control values for [^{14}C]acetyl-CoA and [^{14}C]malonyl-CoA were 286 ± 32 and 18,923 ± 103 (mean of five incubations ± S.E.M., dpm/min/mg protein).

suggest the inhibition of endogenous (*de novo*) fatty acid synthesis in the liver by KCD-232 or MII treatment, it is also highly probable that KCD-232 or MII may interfere with the process of fatty acid esterification to glycerol. Hence, the esterification activity of fatty acids to glycerol was examined by injecting [^{14}C]palmitic acid exogenously under conditions with and without KCD-232 treatment. The *in vivo* incorporation of [^{14}C]palmitic acid into liver TG was significantly lowered in KCD-232-treated rats (Fig. 4). The *in vitro* incorporation of the labeled acid into TG of liver slices also decreased in the presence of MII in the incubation medium (Fig. 6c and d). These results seemingly imply that the esterification process may be interrupted by KCD-232 or MII. However, considering an artificial triglyceride named DG-MII (Fig. 7), it is suggested from results shown in Table 1 that KCD-232 will not interfere directly with the process: the [^{14}]glycerol incorporation into total TG (natural TG + DG-MII) in both liver slices from KCD-232-treated rats and those supplemented with MII to the incubation medium was found to be equivalent to the incorporation into natural TG in control slices. This possibility was confirmed by measuring TG-synthesizing activity using [^{14}C]palmitate (data not shown). DG-MII has a smaller R_f value on the TLC plate than natural TG (Fig. 7). The decreased appearance of natural [^{14}C]TG present in the TG zone by "arresting" the glycerol moiety of TG as DG-MII, therefore, accounts for decreased incorporation of [^{14}C] palmitic acid into the natural TG zone by KCD-232 or MII treatment. No detectable DG-MII was found in the serum of KCD-232-treated rats, suggesting that DG-MII was rarely secreted into the blood stream by the liver (it was found to be secreted into bile). Hence, the hepatic formation of DG-MII appears to contribute partly toward the reduction of natural TG concentration in serum.

KCD-232 suppressed the *in vivo* incorporation of [^{14}C]palmitic acid into liver total lipids as well as that into TG (Fig. 4). This suppression suggests an increased degradation of [^{14}C]palmitic acid, namely, an increase in fatty acid oxidation, since neither the esterification of the labeled acid to total TG (natural TG + DG-MII) nor that into phospholipids (data not shown) was inhibited by the drug administration. In KCD-232-treated rats, [^{14}C]palmitic acid oxidation was indeed accelerated (Table 2), and this acceleration was due to a KCD-232 metabolite, MII (Table 3). Furthermore, MII was considered to increase mitochondrial rather than peroxisomal oxidation (Table 3). Clofibrate, a hypolipidemic drug known to cause marked peroxisomal proliferation, has been reported to increase fatty acid [12] and fatty acyl-CoA oxidation [13] in the rat liver. Recently, a hypothesis which caught our attention was proposed: potent hepatic peroxisome proliferators are hepatocarcinogenic [26]. Although KCD-232 enhanced hepatic fatty acid oxidation, peroxisomal proliferation was thought not to occur in the liver of KCD-232-treated rats as discussed above. Indeed, no significant peroxisome proliferation was observed in electron micrographs of liver cells from KCD-232-treated rats (Fig. 8).

KCD-232 decreased the *in vivo* incorporation of [^{14}C]acetate into liver total lipids and TG (Fig. 3) and did not interfere with the process of fatty acid esterification to glycerol (Table 1). These results imply that hepatic fatty acid synthesis may be suppressed by KCD-232 treatment. The possibility was confirmed by measuring hepatic fatty acid synthesis *in vivo* with tritiated water (Table 4), the use of which has been reported to provide a reliable estimate for the rate of total fatty acid synthesis *in vivo* of liver [15]. Furthermore, hepatic fatty acid synthesis was inhibited by even a single oral dose of KCD-232 (experiment 1 in Table 4). This suggested that KCD-232 inhibited directly the catalytic activity of enzyme(s) associated with fatty acid synthesis rather than decreasing the quantity of enzyme(s) in the liver. This possibility was demonstrated by experiments using cell-free enzyme systems (Figs. 10 and 11). The inhibitory effect of KCD-232 was attributable not to nicotinic acid but to MII (Table 5). MII was thought to be formed by oxidation of 4-(4′-chlorobenzyloxy)benzyl alcohol which was derived from hydrolysis of KCD-232 and could not be detected in the serum. The concentration of MII required for 50% inhibition was 120 μM, which was found to be less than half of the MII level in the liver 3 (0.30 μmole/g $\cong$300 μM) or 7 (0.35 μmole/g $\cong$ 350 μM) hr after a single oral dose of KCD-232 at 100 mg/kg body weight in rats. As was expected from the existence of DG-MII, MII-CoA was actually formed in the liver (Fig. 9). However, no MII was found in the phospholipid fraction on the TLC plate (Fig. 7B), so that MII-CoA was considered to have no effect on cellular or subcellular organelle membrane formation. MII-CoA inhibited fatty acid syntheses from [^{14}C]acetate, [^{14}C]acetyl-CoA and [^{14}C]malonyl-CoA (Figs. 10 and 11), whereas MII as well as clofibric acid, which has been reported to inhibit acetyl-CoA carboxylase [8], showed no effects at concentrations used here. This inhibitory effect of MII-CoA is similar to those of natural fatty acyl-CoA thioesters on acetyl-CoA carboxylase [4] and fatty acid synthetase [5]. Judging from the fact that fatty acid syntheses from the labeled substrates used here were all inhibited by MII-CoA, the site of action of MII-CoA was thought to exist in the steps coming after malonyl-CoA production, i.e. the fatty acid synthetase multienzyme complex. Moreover, MII-CoA inhibited fatty acid synthesis whether or not the NADPH-generating system was added to cell-free systems, suggesting that MII-CoA actually inactivated fatty acid synthetase without affecting NADPH-generating activity. Thus, MII-CoA is shown to be a chemical entity for the inhibition of hepatic fatty acid synthesis by KCD-232. The degree of inhibition of fatty acid synthesis from [^{14}C] malonyl-CoA by MII-CoA in the cell-free system supplemented with NADPH was not as high as that from [^{14}C]acetyl-CoA (Fig. 11). Fatty acyl-CoA thioesters have also been reported to inhibit glucose-6-phosphate dehydrogenase [27] and malate dehydrogenase [28]. Therefore, the possibilities cannot be entirely ruled out that MII-CoA may inhibit acetyl-CoA carboxylase as well as fatty acid synthetase and that it may inhibit other lipogenic enzymes at cellular and whole animal levels. Further intensive studies are needed to clarify these aspects.

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