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Inhibition by 3-Amino-1,2,4-triazole of Fatty Acid Synthesis in Cell Free System[1]

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In this paper, the mechanism of inhibition by aminotriazole (3-amino-1,2,4-triazole) of fatty acid synthesis was studied with cell free system of the rat liver. The concentration of this compound in the liver was 20 mM at 30 min after the injection of the compound (100 mg/100 g, *i.p.*). About 80% of this compound in the liver cell was recovered in the supernatant fraction (cytoplasm) by subcellular fractionation of the liver homogenate. In the experiment of cell free system, the incorporations of ^{14}C-acetate and ^{14}C-acetyl-CoA into fatty acid were inhibited about 50% by the addition of 20 mM aminotriazole into the system and this inhibition depended on the concentration of the compound. However, the incorporation of ^{14}C-malonyl-CoA into fatty acid was not inhibited by aminotriazole. Aminotriazole repressed the activation and stabilization of acetyl-CoA carboxylase in the rat liver by citrate and inhibited this enzyme non-competitively.

Keywords——aminotriazole; triglyceride; fatty acid synthesis; cell free system; acetyl-CoA carboxylase

Aminotriazole (3-amino-1,2,4-triazole) has been widely used as herbicide, and it was reported recently that this compound was able to decrease the lipid contents in leaves.[3] In addition, this compound exerted a variety of effects on mammals; catalase activities in the liver and kidney were inhibited[4] and the activity of enzyme that metabolized drug in the liver was reduced[5] by this compound. It was demonstrated by histological and biochemical methods that the necrosis and the accumulation of fat in hepatocyte caused by the injection of CCl_4 are prevented by pretreatment with aminotriazole.[6] It was also reported that the lability of lysosomal membrane in the liver by the injection of CCl_4 is prevented by the injection of aminotriazole.[6] We reported previously that aminotriazole decreases markedly triglyceride level in the rat liver[7] and significantly represses the production of CCl_4-, ethanol- and ethionine-induced fatty liver.[8] Furthermore, *in vivo*[9] and *in vitro*[1] experiments demonstrated that the decrease in triglyceride level by aminotriazole is due to the inhibition of fatty acid synthesis in the liver.

In order to clarify the mechanism of inhibition by aminotriazole of fatty acid synthesis in the liver, we studied the effect of aminotriazole on the synthesis in cell free system.

Materials and Methods

Materials——ATP, NADP, NAD, GSH, acetyl-CoA and malonyl-CoA were purchased from Sigma Co. Acetyl-CoA-1-^{14}C, malonyl-CoA-2-^{14}C and ^{14}C-$KHCO_3$ were purchased from Nuclear New England. Sodium-

1) Previous paper: H. Ishii, T. Suga, S. Niinobe, and Y. Inatsu, *Chem. Pharm. Bull.* (Tokyo), **25**, 2283 (1977).
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3) M. Vivekanandan and A. Gnanan, *Plant Physiol.*, **55**, 526 (1975).
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5) R.J. Stenger and E.A. Johnson, *Exp. Mol. Pathol.*, **16**, 147 (1972); R. Kato, *Jap. J. Pharmacol.*, **17**, 56 (1967).
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2-^{14}C-acetate was purchased from Daiichi Pure Chemical Co. 3-Amino-1,2,4-triazole-5-^{14}C was synthesized from 10 mC sodium formate and aminoguanidine sulfate according to the method of Allen and Bell,[10] and identified by comparing its mp, IR and *Rf* value on TLC with those of purified aminotriazole. Specific radioactivity of the product was 0.9 mC/m mol.

Preparation of Liver Extract——A male Wistar rat weighing about 150 g was sacrificed by decapitation, and the liver was removed and 20% liver homogenate in 0.25 M sucrose containing 0.01 M $KHCO_3$ was prepared in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at $600\times g$ for 10 min and the supernatant was centrifuged at $105000\times g$ for 60 min. The supernatant obtained by the latter centrifugation was used as liver extract.

Fatty Acid Synthesis from 2-^{14}C-Acetate——The incubation system consisted of 0.1 ml liver extract (2 mg protein), 40 μmol potassium phosphate buffer (pH 7.1), 15 μmol $MgCl_2$, 0.3 μmol $MnCl_2$, 40 μmol isocitrate, 5 μmol $KHCO_3$, 2 μmol ATP, 1 μmol NADP, 2 μmol NAD, 0.05 μmol CoA, 4 μmol 1-cystein, 1.07 μmol 2-^{14}C-acetate (0.3 μC) and various concentrations of aminotriazole. The final volume was 1.0 ml. The system was incubated at 37° for 45 min without shaking and the reaction was stopped by the addition of 1 ml of 20% KOH in ethanol. The system was saponified for 30 min in a boiling-water bath. After acidification with 1 ml of 5 N HCl, the system was extracted three times with 5 ml of petroleum ether. The petroleum ether extracts were pooled and evaporated to remove petroleum ether. The residue was dissolved in 5 ml of *n*-hexane. An aliquot of the hexane solute was transferred into vial vessel and the radioactivity of this solute was determined with liquid scintillation counter.

Fatty Acid Synthesis from 1-^{14}C-Acetyl-CoA——Incorporation of ^{14}C-acetyl-CoA into fatty acid in cell free system of the rat liver was determined according to the method of Masoro *et al.*[11] The incubation system consisted of 0.2 ml liver extract (3 mg protein), 0.42 μmol ^{14}C-acetyl-CoA (0.05 μC), 48 μmol KCl, 0.6 μmol $MnCl_2$, 40 μmol D,L-isocitrate, 2 μmol NADP, 12 μmol NAD, 1.5 μmol ATP, 4.8 μmol potassium phosphate buffer (pH 8.8), 12 μmol $KHCO_3$ and various concentration of aminotriazole. The final volume was 1.0 ml. The system was incubated at 37° for 30 min. Saponification, extraction and determination were carried out according to the method of the fatty acid synthesis from 2-^{14}C-acetate.

Fatty Acid Synthesis from 2-^{14}C-Malonyl-CoA[11]——The incubation system consisted of 0.1 ml liver extract (0.1 mg protein), 1.5 nmol ^{14}C-malonyl-CoA (0.04 μC), 2 nmol acetyl-CoA, 48 μmol KCl, 0.6 μmol $MnCl_2$, 40 μmol D,L-isocitrate, 1.6 μmol NADP, 4.8 μmol potassium phosphate buffer (pH 8.8) and various concentrations of aminotriazole. The final volume was 1.0 ml. The system was incubated at 37° for 10 min. Saponification, extraction and determination were carried out as described above.

Partial Purification of Acetyl-CoA Carboxylase——Acetyl-CoA carboxylase of the rat liver was partially purified according to the method of Hashimoto and Numa.[12] Wistar rats were fasted for 48 hr and subsequently refed with a fat free diet for 48 hr before removing the livers. The animals were killed by decapitation and the livers were quickly removed. All phosphate buffers employed in this section were potassium phosphate buffer pH 7.5 containing 5 mM 2-mercaptoethanol and 1 mM EDTA. The livers (160 g) were homogenized in 2 volumes of 0.25 M sucrose containing 0.01 M phosphate buffer. The homogenate was centrifuged at $35000\times g$ for 120 min. The supernatant fluid was collected and filtered though cheesecloth. The filtrate was brought to 30% saturation by the addition of solid ammonium sulfate. After stirring for 20 min, the resulting precipitate was collected by centrifugation at $23000\times g$ for 20 min and dissolved in 10 mM phosphate buffer. The solution thus obtained was stirred with calcium phosphate gel suspension (17.0 mg/ml) (protein: gel=1:1.8). After stirring for 20 min, the gel was collected by centrifugation at $2600\times g$ for 10 min and washed 3 times with 33 mM phosphate buffer. The enzyme adsorbed on the gel was then eluted twice with 0.2 M phosphate buffer. The eluate was brought to 30% saturation by the addition of solid ammonium sulfate. After stirring for 30 min, the resulting precipitate was collected by centrifugation at $23000\times g$ for 20 min and dissolved in 0.1 M phosphate buffer. The solution was used as partially purified enzyme and stored at −20°. The specific activity of this enzyme in the solution increased to 21 fold compared with that of the supernatant fluid from liver homogenate.

Subcellular Fractionation——Subcellular fractionation of the rat liver was carried out according to the method of de Duve *et al.*[13]. After rats were killed, the livers were perfused with ice-cold saline and 10% liver homogenate in 0.25 M sucrose was prepared in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at $600\times g$ for 10 min, the pellet was designated nuclear fraction (N). The post-nuclear supernatant was centrifuged at $3300\times g$ for 10 min, the pellet was designated heavy mitochondrial fraction (M). The supernatant of fraction M was centrifuged at $12500\times g$ for 20 min, the pellet was designated light mitochondrial fraction (L). The supernatant of fraction L was centrifuged at $105000\times g$ for 60 min, the resulting pellet was designated microsomal fraction (P). The resulting supernatant was used as supernatant fraction (S).

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11) E.J. Masoro, H.M. Korchak, and E. Porter, *Biochim. Biophys. Acta*, **58**, 407 (1962).
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13) C. de Duve, B.C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans, *Biochem. J.*, **60**, 604 (1955).

Assays——The activity of acetyl-CoA carboxylase was determined by $H^{14}CO_3^-$ fixation assay according to the method of Hashimoto and Numa.[12] The $H^{14}CO_3^-$ fixation assay follows the rate of acetyl-CoA-dependent incorporation of $H^{14}CO_3^-$ into acid-stable material, *i.e.* malonyl-CoA. Acid phosphatase activity was determined by using β-glycerophosphate as substrate, and the inorganic phosphate liberated was measured according to the method of Lindberg and Ernster.[14] Protein content was determined by the method of Lowry *et al.*[15] with bovine serum albumin as a standard.

Results and Discussion

Results of the experiments *in vitro*[1] by liver slice and *in vivo*[6] suggested that aminotriazole inhibits fatty acid synthesis in the liver. In order to clarify the mechanism of this inhibition by aminotriazole of fatty acid synthesis, the time-course of the content of aminotriazole in the liver was first investigated (Fig. 1). At 30 min after the injection relatively high amounts of aminotriazole were found in the liver, about 20 mmol per g. liver. Then its content decreased rapidly in the time between 30 min and 2 hr after the injection. However, after 2 hr the content decreased very slowly and even at 6 hr after the injection the value was still 5 mmoles per g. liver. Thus, it was indicated that aminotriazole is incorporated into the liver soon after the injection. Therefore, its subcellular distribution in the liver was examined. Fig. 2 shows the distribution at 30 min after the injection. A few percent of aminotriazole uptaken into the liver was found in light mitochondrial fraction (L frac-

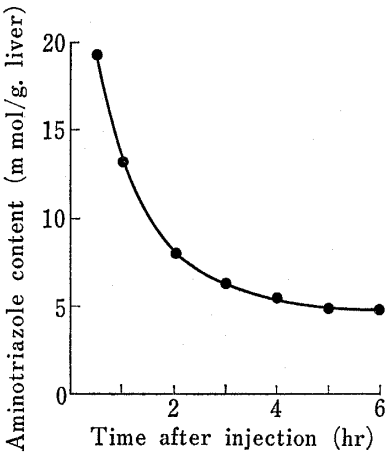

Fig. 1. Changes in Aminotriazole Content in Liver after intraperitoneal Injection of Aminotriazole

Animals were received with ^{14}C-aminotriazole (specific radioactivity; 9.9×10^4 dpm/mg, 100 mg/100 g. b.w., *i.p.*). The livers were removed at each time and washed with 0.25 M sucrose. 10% liver homogenate was prepared with the sucrose. The radioactivity of 1 ml of the homogenate was measured by liquid scintillation counter.

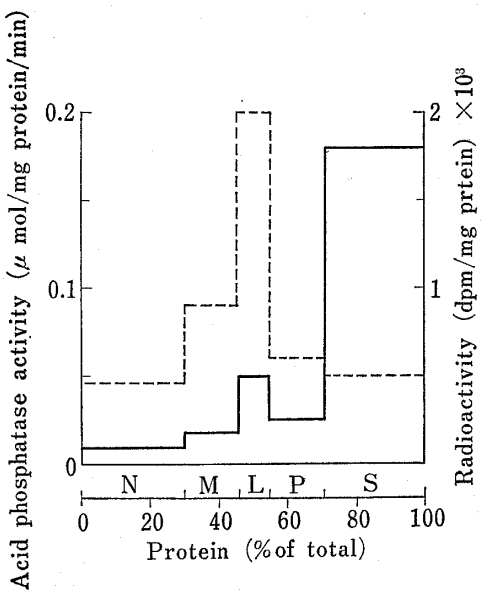

Fig. 2. Subcellular Distribution of Radioactivity in Liver after the Injection of ^{14}C-Aminotriazole

Animals were received with ^{14}C-aminotriazole (100 mg/100 g, *i.p.*). The liver was removed at 30 min after the injection. 10% liver homogenate was prepared with 0.25 M sucrose. Subcellular fractionation was carried out according to the method of de Duve *et al.*[13]
[——]; radioactivity,
[- - -]; acid phosphatase activity.
N; nuclear fraction,
M; heavy mitochondrial fraction,
L; light mitochondrial fraction,
P; microsomal fraction,
S; supernatant fraction.

14) O. Lindberg and L. Ernster, "Methods of Biochemical Analysis," Ed. D. Glick, Vol. III, Interscience, New York, 1956, p. 7.
15) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randolf, *J. Biol. Chem.*, 193, 265 (1951).

tion), although most of this material was distributed in the supernatant fraction (S fraction, cytoplasm). Recovery of aminotriazole in the supernatant fraction was about 80% of the total material in the cell. The same result was also observed at 6 hr and 24 hr after the injection. This subcellular fractionation is justified proper since the activity of acid phosphatase was the highest in light mitochondrial fraction as indicated by de Duve *et al.*[13] Thus, most of aminotriazole uptaken into the liver was distributed in the cytoplasm of the cell, the site of fatty acid synthesis.

The enzymes relating to fatty acid synthesis in the mammalian cell is divided into two enzymes, acetyl-CoA carboxylase and fatty acid synthetase (multi-enzyme complex), both of which are distributed in the supernatant fraction of liver cell.[16,17] In order to study the mechanism of inhibition by aminotriazole of these enzymes in fatty acid synthesis, effect of aminotriazole on the synthesis of fatty acid from ^{14}C-acetate, ^{14}C-acetyl-CoA and ^{14}C-malonyl-CoA was studied in a cell free system of the liver (Fig. 3). Since the concentration of aminotriazole in the liver was 20 mM at 30 min after the injection (Fig. 1) and most of aminotriazole was found in cytoplasm (Fig. 2), the experiment was carried out with the concentration of aminotriazole from 0 to 20 mM. Incorporation of ^{14}C-acetate into fatty acid was inhibited with an increase in the concentration of aminotriazole. The degree of this inhibition depends on the concentration of aminotriazole and reached 50% of control at 20 mM of aminotriazole. These results indicated that aminotriazole inhibits the over all reaction of fatty acid synthesis in cell free system. Similarly the incorporation of ^{14}C-acetyl-CoA into fatty acid was inhibited by aminotriazole and the degree of the inhibition depends on the concentration of this material. In this case the incorporation into fatty acid was inhibited about 40% at 20 mM of aminotriazole. However, the incorporation of ^{14}C-malonyl-CoA into fatty acid was not inhibited by aminotriazole and was not effected even with an increase in the concentration of aminotriazole. These findings indicate that aminotriazole inhibits acetyl-CoA carboxylase but does not inhibit fatty acid synthesis (multi-enzyme complex) in the liver.

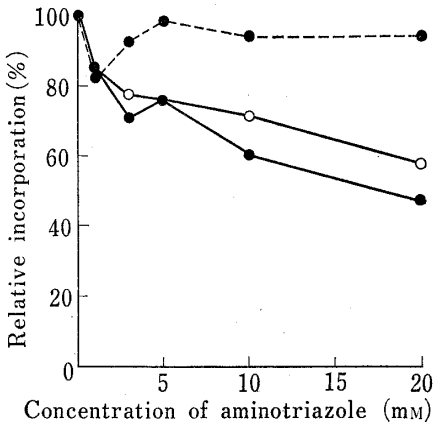

Fig. 3. Effect of Aminotriazole on the Incorporation of Acetate, Acetyl-CoA and Malonyl-CoA into Fatty Acid in the Cell Free System of Rat Liver

The procedures were described in "Materials and Methods".
●—●; acetate, ○—○; acetyl-CoA, ●---●; malonyl-CoA.

Consequently, effect of aminotriazole on partially purified acetyl-CoA carboxylase was examined. The enzyme occurs in two forms, an inactive protomer and an active polymer. Citrate, the positive allosteric modulator, is known to stimulate the enzyme activity by aggregating protomer to form polymer.[16] Fig. 4 shows an influence of aminotriazole on the stimulatory effect of citrate. The enzyme was activated by preincubation for 30 min with 10 mM citrate. However, the activation of this enzyme by citrate in this system was completely inhibited by the addition of aminotriazole. On the other hand, the enzyme activity was markedly decreased by the preincubation without citrate. This repression of the enzyme activity was further enhanced by the addition of aminotriazole into the system. These findings indicate that aminotriazole inhibits the stimulatory effect of citrate. Thus the inhibition by aminotriazole of the enzyme activity may be due to the interference in the formation of the polymerized enzyme. The mode of inhibition by aminotriazole of the

16) S. Numa, *Ergeb. Physiol. Biol. Chem. Exp. Pharmacol.*, **69**, 53 (1974).
17) F. Lynen, *Biochem. J.*, **102**, 381 (1967).

enzyme was further studied by the method of Dixon plot (Fig. 5). The straight line in each concentration of substrate ($KHCO_3$) crossed in one point on the abscissa. This result indicates that aminotriazole inhibits non-competitively the enzyme. The *ki* value of aminotriazole on the enzyme was 33 mM.

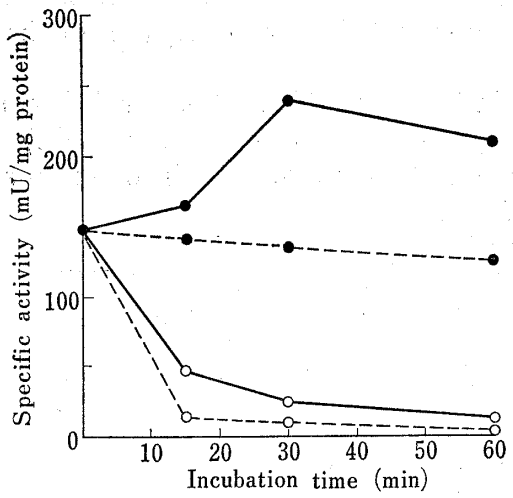

Fig. 4. Effect of Aminotriazole on the Effect of Citrate on Acetyl-CoA Carboxylase

The enzymes were preincubated with each mixture of final volume of 1 ml for various times, and then 0.1 ml of preincubation mixture was added to the reaction mixture containing ^{14}C-$KHCO_3$ and the reaction was carried out for 10 min at 37°.
●—●; The enzyme was preincubated with 10 mM citrate.
●---●; The enzyme was preincubated with 10 mM citrate and 50 mM aminotriazole.
○—○; The enzyme was preincubated without citrate and aminotriazole.
○---○; The enzyme was preincubated without citrate with 50 mM aminotriazole.

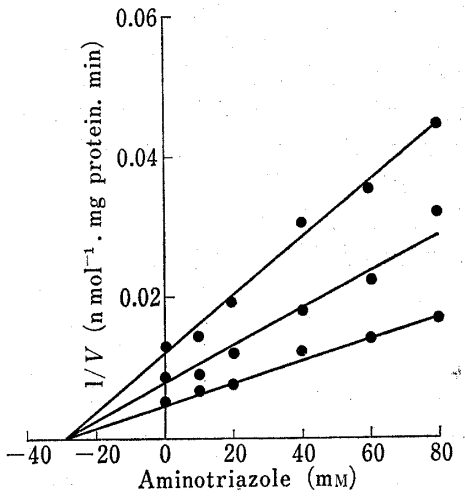

Fig. 5. Dixon Plot of Inhibition of Aminotriazole on Acetyl-CoA Carboxylase

Aminotriazole was added to various final concentrations in the preincubation and reaction mixture. The reaction was carried out for 10 min at 37° with 2, 5 and 10 μmol ^{14}C-$KHCO_3$ as substrate.

It was demonstrated in this paper by the experiment of cell free system that the decrease in liver triglyceride level caused by the injection of aminotriazole is due to the inhibition of fatty acid synthesis. Furthermore, it was established that the relatively high amounts of aminotriazole are incorporated into the liver rapidly after the injection, and that most of the material is distributed in cytoplasm, the site of fatty acid synthesis. The inhibition of the synthesis by aminotriazole was proved to be due to the inhibition of acetyl-CoA carboxylase in cytoplasm, the rate-limiting enzyme in fatty acid synthesis.