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Effect of 3-Amino-1,2,4-triazole on the Formation of Triglyceride in Liver Slice[1]

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The effect of 3-amino-1,2,4-triazole (AT) on the formation of liver triglyceride (TG) was studied by using liver slices. In the liver of rat received AT (1 g/kg, *i.p.*), the incorporation of ^{14}C-acetate into TG decreased about 45 per cent when compared with that of the control, though the incorporation of ^{14}C-palmitate into TG did not differ from that of the control. In addition, the incorporation of ^{14}C-acetate into total fatty acid decreased about 30 per cent in the liver of the received with AT. In *in vitro* experiment, the incorporation of ^{14}C-acetate into TG was inhibited about 40 per cent in the presence of 50 mM AT in incubation mixture and the inhibition was dependent to the concentration of AT. One of the causes of decrease of liver TG level induced by AT may be due to the inhibition of fatty acid synthesis in the liver.

Keywords——aminotriazole; liver slice; triglyceride formation; fatty acid synthesis; incorporation of ^{14}C-acetate

3-Amino-1,2,4-triazole (AT) has been widely used as herbicide. In recent years, it was reported that this compound was able to decrease the lipid contents in leaves.[3] In addition, the material exerted a variety of effects on mammals; catalase in liver and kidney was inhibited[4] and the drug metabolising enzyme in liver is reduced[5,6] by this compound. It was demonstrated by means of histological[7] and biochemical methods[1] that pretreatment of AT prevented the CCl_4-induced necrosis and the accumulation of fat in hepatocyte.

On the other hand, our laboratory has reported that AT markedly decreased triglyceride (TG) level in rat liver[8] and significantly repressed CCl_4-, ethanol- and ethionine-induced fatty livers.[9] Furthermore, it was suggested by *in vivo* experiments that the decrease of TG level is due to a repression of fatty acid synthesis in the liver.[10]

In the present study, we have studied the effect of AT on TG formation and fatty acid synthesis in the liver by using liver slice *in vitro*.

Materials and Methods

Materials——Chemical compounds were obtained from the following companies: 3-amino-1,2,4-triazole (AT) from Tokyo Kasei Co.; sodium acetate-2-^{14}C (42.0 mC/mmol) and palmitate-1-^{14}C (53.2 mC/mmol) from Daiichi Pure Chemicals Co.; and crystallized bovine albumin from Nutritional Biochemical Corp. Albumin-bound palmitate-1-^{14}C was prepared by the method of Milstein and Driscoll.[11]

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Treatment of Animals and Preparation of Liver Slice——Male Wistar rats weighing about 150 g were used throughout these studies. In all cases, rats were fasted for 24 hr prior to the experiment, but were given water *ad. libitum*. AT (1 g/kg), which had been dissolved in physiological saline, was injected intraperitoneally 12 hr before sacrifice; control rats were injected with physiological saline. Livers were removed immediately after the sacrifice and washed with the saline. Liver slices were prepared in the cold with a Stadie-Riggs slicer.

Triglyceride and Fatty acid Formation from ^{14}C-Acetate and ^{14}C-Palmitate in Rat Liver Slice——Randomized liver slices (0.5 g), from the animal were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4), which had gassed for 10 min with 5% CO_2 and 95% O_2. The radioactive substrate in buffer was added to the flask. Total volume of the medium was 5 ml. Incubations were carried out for 1 to 2 hr in an atmosphere of 5% CO_2 and 95% O_2. The reaction was stopped placing the flask on ice and the slices were washed three times with ice-cold distilled water. The slices were minced and the lipids were extracted by the method of Folch, *et al.*[12] The lipids extracted were dissolved in chloroform. An aliquot was taken for TLC and TG was separated as described previously.[10]

Fatty acid were separated by the method of Lieber and Schmid.[13] Lipids extracted in Folch solution were evaporated, the residue was dissolved in 0.5 N KOH–95% ethanol and saponified for over night at 60°. After cooling, ethanol was added to obtaine a final concentration of 50%. The mixture was vigorously shaken with petroleum ether for 10 min to remove nonsaponifiable material. After repeating the extraction with petroleum ether 3 times, ethanol remaining in the aqueous phase was removed by heating on a bath, and the pH was reduced to below 3.0 with 6 N HCl. After cooling, the fatty acids were extracted with 20 ml petroleum ether. The extraction was repeated 3 times. The ether extracts were pooled and evaporated.

Assays——The uptake of radioactive precursors in the liver slices were calculated by determing the radioactivity remained in the incubation medium. Incorporation of radioactive precursors into TG and fatty acid was determined. Radioactivity was determined with a liquid scintillation counter (Aloka LSC-502). TG and fatty acid level were determined according to the method of Van Handel-Kawade[14] and Kushiro, *et al.*,[15] respectively.

Results and Discussion

3-Amino-1,2,4-triazole (AT) markedly decrease the liver triglyceride (TG) level in the rat[8] and repress the hepatotoxines-induced fatty liver.[9] Further, it was demonstrated by the *in vivo* experiment that AT inhibited fatty acid synthesis in the liver.[10] The effect of AT on the formation of liver TG and fatty acid synthesis was also studied *in vitro* by use of liver slice. The formation system of TG in liver is able to divide into two steps; the fatty acid synthesis and esterification of fatty acid into TG. The effect of AT-treatment on overall formation of TG in liver was examined by using ^{14}C-acetate as a precursor (Table I). TG level decreased about 50 per cent in the rat liver by the treatment with AT. Therefore, the incorporation of ^{14}C-acetate into TG was expressed as radioactivity incorporated into TG per g liver. The incorporation of radioactivity into TG decreased about 45 per cent in AT-treated slices when compared with that of the control. This finding may indicate that overall formation of TG in the liver was reduced by AT. The effect of AT-treatment on esterification of fatty acid into TG was shown in Table II. The incorporation of ^{14}C-palmitate into TG was not reduced by AT-treatment when compared with that of the control. This result may suggest that AT did not inhibit the esterification of fatty acid and reduced the fatty acid synthesis in liver in closely agreement with the *in vivo* experiment.[10]

The effect of AT-treatment on total fatty acid synthesis in the liver slices is shown in Table III. In the liver at 12 hr after the AT-injection, total fatty acid decreased about 45 per cent in a similar manner to decrease of TG. Furthermore, the incorporation of radioactivity into fatty acid was inhibited about 30 per cent when compared with that of the control. It may be ascertained from this result that the decrease of TG formation in the rat liver injected with AT was not due to the inhibition of the esterification of fatty acid but to the inhibition of fatty acid synthesis.

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In vitro effect of AT on the incorporation of ^{14}C-acetate into TG in the liver slices of normal rat is shown in Table IV. An addition of 50 mM AT into the incubation mixture decreased 45 per cent the incorporation of acetate into TG when compared with that of the control. This finding suggests that AT inhibits directly on TG formation. When animals were injected with ^{14}C–AT (1 g/kg,b.w.), its concentration in the liver was about 20 mM at maximum value (unpublished data). Therefore, the present result obtained at several AT concentration in this experiment may explain the results from *in vivo* experiment.

TABLE I. Effect of Aminotriazole Treatment on *in Vitro* Incorporation of ^{14}C-Acetate into Liver Triglyceride

	Number of rats	Relative uptake of radioactivity in liver slice (% of addition)	Triglyceride (mg/g liver)	Radioactivity in triglyceride (dpm/g liver)
Control	5	19.6±2.0	3.1±0.2	8000±804
Aminotriazole	4	15.7±2.0	1.6±0.3	4500±997
		$p<0.05^{a)}$	$p<0.01^{a)}$	$p<0.01^{a)}$

The aminotriazole group received aminotriazole (1 g/kg, *i.p.*) 12 hr before sacrifice. The control group received physiological saline at the same time. Liver slices (0.5 g) were incubated for 2 hr in 5 ml Krebs-Ringer bicarbonate buffer (pH 7.4) which contained ^{14}C-acetate (1.07×10^6 dpm) under an atomosphere of 5% CO_2 and 95% O_2. Values are represented as means±S.D.

a) Comparison of treatment with control by *t*-test.

TABLE II. Effect of Aminotriazole Treatment on *in Vitro* Incorporation of ^{14}C-Palmitate into Liver Triglyceride

	Relative uptake of radioactivity in liver slice (% of addition)	Triglyceride (mg/g. liver)	Radioactivity in triglyceride (dpm/g liver)
Control	44.8±2.7	2.5±0.3	39200±2190
Aminotriazole	41.8±0.9	1.8±0.2	36100±4260
	N.S$^{a,b)}$	$p<0.01^{a)}$	N.S$^{a,b)}$

The aminotriazole group received aminotriazole (1 g/kg, *i.p.*) 12 hr before sacrifice. The control group received physiological saline at the same time. Liver slices (0.5 g) were incubated for one hr in 5 ml Krebs-Ringer bicarbonate buffer (pH 7.4) which contained albumin-bound palmitate-^{14}C (4.09×10^5 dpm) under an atmosphere of 5% CO_2 and 95% O_2. Values are represented as means±S.D in 5 animals.

a) Comparison of treatment with control by *t*-test.

b) *p* values larger than 0.05 were considered not significant (N.S).

TABLE III. Effect of Aminotriazole on *in Vitro* Incorporation of ^{14}C-Acetate into Liver Total Fatty Acid

	Relative uptake of radioactivity in liver slice (% of addition)	Triglyceride (mg/g. liver)	Fatty acid (mg/g liver)	Radioactivity in fatty acid (dpm/g liver)
Control	20.9±4.7	5.1±1.8	19.0±4.6	3900±972
Aminotriazole	18.0±4.1	2.8±1.7	10.4±3.4	2730±663
	N.S$^{a,b)}$	$p<0.05^{a)}$	$p<0.01^{a)}$	$p<0.05^{a)}$

The aminotriazole group received aminotriazole (1 g/kg, *i.p.*) 12 hr before sacrifice. The control group received physiological saline at the same time. Liver slices (0.5 g) were incubated for 2 hr in 5 ml Krebs-Ringer bicarbonate buffer (pH 7.4) which contained ^{14}C-acetate (1.05×10^6 dpm) under an atmosphere of 5% CO_2 and 95% O_2. Values are represented as means±S.D in 5 animals.

a) Comparison of treatment with control by *t*-test.

b) *p* values larger than 0.05 were considered not significant (N.S).

Table IV. *In Vitro* Effect of Aminotriazole on Incorporation of ^{14}C-Acetate into Liver Triglyceride

	Relative uptake of radioactivity in liver slice (% of addition)	Triglyceride (mg/g liver)	Radioactivity in triglyceride (dpm/g liver)
Control	52.8±0.5	4.1±0.2	2730±552
Aminotriazole	53.0±1.4	4.5±0.3	1750±263
	N.S[a,b]	N.S[a,b]	$p<0.05$[a]

All animals were fasted for 24 hr before sacrifice. Liver slices were incubated for 2 hr in 5 ml Krebs-Ringer bicarbonate buffer (pH7.4) which contained ^{14}C-acetate (7.56×10^5 dpm) and with or without 50 mM aminotriazole under an atmosphere of 5% CO_2 and 95% O_2. Values are represented as means±S.D in 4 animals.
a) Comparison of aminotriazole group with control by *t*-test.
b) *p* values more than 0.05 were considered not significant (N.S).

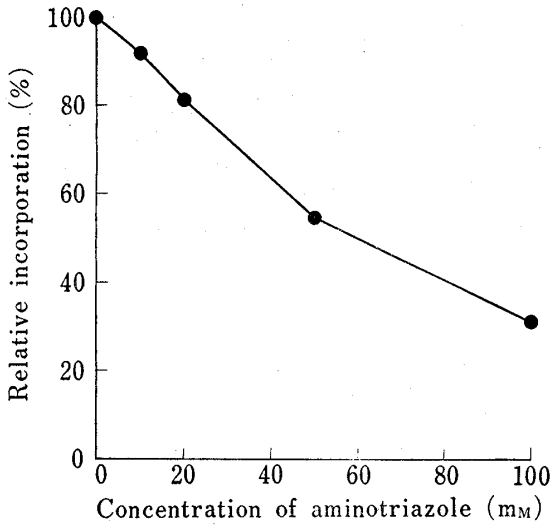

Fig. I. *In Vitro* Effect of Aminotriazole on Incorporation of ^{14}C-Acetate into Liver Total Fatty Acid

Animals were fasted for 24 hr before sacrifice. Liver slices were incubated for 2 hr in 5 ml Krebs-Ringer bicarbonate buffer (pH 7.4) which contained ^{14}C-acetate (1.01×10^6 dpm) and with several concentration of aminotriazole under an atmosphere of 5% CO_2 and 95 % O_2.

The effect of AT on the incorporation of ^{14}C-acetate into total fatty acid in the liver was examined at several concentration (Fig. 1). The incorporation of ^{14}C-acetate into fatty acid decreased significantly at 20 mM AT and the degree of the inhibition depends on the concentrations of AT. This finding agree with the result in the liver slice of the rat received with AT. Thus, it was demonstrated from these experiments that the decrease of liver TG by AT is caused by the inhibition of fatty acid synthesis in the liver. In regard to the inhibition of fatty acid synthesis, the mechanism of AT action to the enzyme systems may be an important problem, be progressing under the investigation now in our laboratory.