Studies on Azaserine-Induced Fatty Liver in the Rat (34400)

MARIO MARCHETTI, VITTORIA OTTANI AND PAOLO PUDDU
(Introduced by G. Favilli)
Istituto di Chimica Biologica e di Biochimica Applicata dell'Universitá di Bologna, Bologna, Italy

It has been shown that the administration of the antibiotic azaserine (*o*-diazoacetyl-L-serine) to rats produces fatty liver and liver cell necrosis as well (1, 2). The present paper reports the effect of the administration of azaserine on liver lipid composition, on liver lipogenesis *in vitro*, on serum lipoproteins, and on liver content of biotin and nicotinamide nucleotides. Furthermore, the effects of azaserine on liver ATP content and the effects of ATP or adenine administration to azaserine-treated rats also were investigated. In fact, it has been shown that a variety of agents, *i.e.*, ethionine, orotic acid, ethanol, and CCl_4, which are able to produce a fatty liver, leads to a rapid decrease in the hepatic concentration of ATP, while the development of these types of fatty liver is prevented by administration of ATP or adenine (3–8).

Materials and Methods. Azaserine (*o*-diazoacetyl-L-serine) was a generous gift from Dr. H. B. Wood, of National Institutes of Health, Bethesda, Maryland and from Dr. J. R. Dice of Parke, Davis and Co. Research Laboratories, Ann Arbor, Michigan.

Adenine sulphate, adenosine-5′-triphosphate (ATP) disodium, $3H_2O$, crystalline 99-100%, α-ketoglutarate, NADPH, NADPH, 3 phosphoglycerate, glyceraldehyde 3-phosphate dehydrogenase, and phosphoglycerate kinase were obtained from Sigma Chemical Co. St. Louis, Mo. Sodium acetate-1-^{14}C (10.5 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks. All other chemicals used were of the highest purity available commercially.

Female weanling albino rats of the Wistar strain were fed *ad libitum* on a purified diet composed of sucrose (59%), vitamin-free casein (20%), autoclaved egg white (11%), peanut oil (5%) and mineral and vitamin supplements. After 60 days the animals were divided into 4 groups and fasted just before the following treatments: one lot of rats (group 1) was kept as control and injected intraperitoneally with 0.9% NaCl; another lot of animals (group 2) was injected in the same way with azaserine (7.5 mg/100 g of body wt) and the injection was repeated 24 hr later; the third lot of rats (group 3) was treated with azaserine as above plus 300 mg of ATP subcutaneously in three equal doses: 100 mg together with the first injection of azaserine and the other two doses 8 and 16 hr later; the final group of rats (group 4) was treated with azaserine (see group 2) plus 120 mg of adenine sulfate, equimolecular to the ATP, subcutaneously in three equal doses at the same time intervals as ATP (see group 3).

Three hr after the last injection of azaserine venous blood of the rats from all groups was collected. Then, the rats were sacrificed and the livers were removed for analysis.

Blood was allowed to clot at 37° for 30 min and serum was separated by centrifugation at 300 rpm for 15 min. Paper electrophoresis of serum proteins was carried out in Veronal buffer, pH 8.6, and I 0.1 at 7° for 12 hr. After the run, the strips were dried and stained with Sudan black in 55° (v/v) ethanol (9). The density curves of the spots were obtained with a recording photoelectric densitometer and the areas under the density curves were calculated planimetrically.

Total liver lipids were extracted as previously described (6) and measured gravimetrically; cholesterol was determined according to the method of Sperry and Webb (10) and phospholipids were determined by assaying the phosphorus on total lipids (11).

For the ATP determination, the livers were frozen *in situ* with metal blocks previously

TABLE I. Effect of Azaserine and of Azaserine Plus ATP or Adenine on Serum Lipoproteins in Rats.[a]

Group	Treatment	α-Lipoproteins	β-Lipoproteins
1	Control	100	100
2	Azaserine	38	33
3	+ ATP	32	24
4	+ adenine	48	42

[a] The results, mean of six determinations on different animals, are expressed as percentage of controls. All data were calculated from the areas under the density curves of lipoprotein spots (see text).

cooled to low temperature, homogenized with 4 vol of 1 N-$HClO_4$ and centrifuged at 0°. The supernatants were neutralized (pH 6.5) with 0.5 N KOH and the $KClO_4$ centrifuged down. The ATP was then assayed on aliquots of the clear supernatants with phosphoglycerate kinase according to the Adam method (12).

Biotin was assayed microbiologically with *L. arabinosus* 17/5 on samples of liver homogenate autoclaved with 5 N H_2SO_4 at 121° for 1 hr (13). The oxidized and reduced forms of nicotinamide nucleotides were determined by the Lowry *et al.* (14) fluorimetric procedures.

Liver lipogenesis was determined *in vitro* on slices (500–600 mg) incubated at 37° for 1 hr with 5 ml of calcium-free Krebs–Ringer phosphate medium (pH 7.4) containing 5 μmoles of α-ketoglutarate, and 0.5 μmole of sodium acetate-1-^{14}C.

After incubation total lipids were extracted from the slices with 2:1 chloroform-methanol (v/v) mixture. The extracts were purified according to the Folch (15) procedure and evaporated to dryness. The residues were dissolved in light petroleum (bp 40–60°) and then in chloroform. Aliquots were taken and radioactivity measured in a windowless gas-flow counter.

The results are given as means ± SE of the mean. Fisher's p values are given and are considered significant if p is not greater than 0.05.

Results. The results of paper electrophoresis of serum lipoproteins are shown in Table I. The data shows a marked decrease of α and β-lipoprotein in azaserine-treated rats (group 2). A remarkable decrease of serum lipoproteins is also observed in azaserine-treated rats and injected either with ATP (group 3) or with adenine (group 4).

As shown in Table II, liver total lipid content of azaserine-treated rats increases over the control rats ($p<.001$), and the increase was entirely due to neutral fats ($p<.001$). A higher content of neutral fats was also observed in the rats treated with azaserine and ATP (group 3) ($p<.001$) or with azaserine and adenine (group 4) ($p<.001$). As well as the *in vitro* incorporation rate of acetate-^{14}C into total lipids is concerned, azaserine-treated rats (group 3) show a decreased incorporation rate ($p<.001$) and the injection either of ATP or adenine seems not to affect this behavior to any extent (Table I).

Liver ATP content does not show any modification after treatment with azaserine (Table III) whereas it is significantly in-

TABLE II. Effect of Azaserine and of Azaserine plus ATP or Adenine on the Composition of Liver Lipids and on the Incorporation of Acetate-^{14}C into Liver Lipids of Rats.[a]

Group	Treatment	Liver lipids (mg/g of tissue)				Incorporation of acetate-^{14}C (cpm/g of tissue)
		Cholesterol	Phospholipids	Neutral fats	Total lipids	
1	Control	3.3 ± 0.11	31.5 ± 1.0	30.2 ± 2.5	64.9 ± 1.4	6823 ± 622
2	Azaserine	2.7 ± 0.15 (NS)	27.3 ± 1.1 (NS)	92.1 ± 8.8[c]	123.1 ± 9.9[c]	3274 ± 328[b]
3	+ ATP	3.6 ± 0.18 (NS)	28.5 ± 2.6 (NS)	117.3 ± 5.2[c]	146.4 ± 4.7[c]	2831 ± 273[c]
4	+ adenine	3.1 ± 0.27 (NS)	32.6 ± 2.3 (NS)	99.1 ± 5.8[c]	134.8 ± 4.6[c]	1431 ± 76[c]

[a] Each value is given as the mean ± SE of the mean of six determinations on different animals. The significance of differences is designated as follows: NS, no significant difference ($p>.05$); [b] p .01–.001; [c] $p<.001$.

TABLE III. Effect of Azaserine and of Azaserine Plus ATP or Adenine on Hepatic Levels of ATP in the Rat.[a]

Group	Treatment	Liver ATP (mμmoles/g of tissue)
1	Control	922 ± 23
2	Azaserine	977 ± 20 (NS)
3	+ ATP	1270 ± 36[b]
4	+ adenine	1348 ± 48[b]

[a] Each value is given as the mean ± SE of the mean of six determinations on different animals. The significance of differences is designated as follows: NS, no significant difference ($p > .05$); [b] $p < .001$.

creased in the azaserine-treated groups injected either with ATP or adenine ($p < .001$).

As shown in Table IV the administration of azaserine decreases the liver biotin concentration ($p < .01$–$.001$) and the injection of ATP or adenine does not modify this effect. The liver concentration of nicotinamide nucleotides, and in particular of the reduced forms, decreases in azaserine-treated rats ($p < .001$), while the administration of ATP or adenine to azaserine-treated animals leads to a significant increase in both oxidized ($p < .01$–$.001$) and reduced forms ($p < .001$).

Discussion. Administration of azaserine to rats induces a fatty liver which is mainly characterized by increased neutral fat, while cholesterol and phospholipids show no significant changes. In azaserine-treated rats the incorporation rate of acetate-^{14}C into total lipids is decreased, which shows that the development of fatty liver is not secondary to increased lipogenesis. The decreased rate of lipid synthesis might be due to a decreased concentration of biotin-dependent acetyl-CoA carboxylase, as suggested by the lower liver content of protein-bound biotin.

The injection of azaserine decreases also the liver content of the reduced form of nicotinamide nucleotides. Nevertheless, the cellular depletion of nicotinamide nucleotides cannot be related in all instances to the decreased lipid synthesis, since in the animals treated with both azaserine and ATP or adenine, the liver levels of nicotinamide nucleotides are normal and lipogenesis is still reduced.

The administration of azaserine causes a remarkable decrease in serum lipoprotein concentrations. This might suggest that the azaserine-induced fatty liver is determined by an inhibited secretion of lipids by the liver, as it has been shown in liver steatosis induced by other compounds, *i.e.*, orotic acid [6, 7, ethionine (4, 16) and 4-aminopyrazolo-pyrimidine (17, 18)].

The lack of liver phospholipid accumulation, observed in all these experimental steatosis as well as in azaserine-induced fatty liver might be secondary to an alteration of CTP-dependent phospholipid biosynthetic process (6, 8).

As well as ethionine, CCl_4 and 4-aminopyrazolo-pyrimidine, induced fatty livers are concerned, it is generally believed that the decreased secretion of lipids by the liver is secondary to an inhibited synthesis of the protein moiety of lipoprotein, due to

TABLE IV. Effect of Azaserine and of Azaserine Plus ATP or Adenine on Hepatic Levels of Biotin and Nicotinamide Nucleotides in Rats.[a]

Group	Treatment	Liver biotin (mμg/g of tissue)	Liver nicotinamide nucleotides (μg/g of tissue)	
			Oxidized forms	Reduced forms
1	Control	1820 ± 95	231 ± 7	200 ± 21
2	Azaserine	1340 ± 33[b]	105 ± 4[c]	53 ± 1[c]
3	+ ATP	1276 ± 15[b]	139 ± 5[c]	153 ± 4[c]
4	+ adenine	1308 ± 18[b]	205 ± 9 (NS)	127 ± 7[c]

[a] Each value is given as the mean ± SE of the mean of six determinations on different animals. The significance of differences is designated as follows: NS, no significant difference ($p > .05$); [b] p .01–.001; [c] $p < .001$.

deranged ATP metabolism. However, in regard to azaserine treatment, this mechanism does not seem to be taken into consideration. In fact, liver ATP content in azaserine-treated rats is not changed, and the administration of ATP or adenine is unable to prevent the development of fatty liver in spite of the increased ATP concentration. Therefore, from the present results we cannot exclude that azaserine alters protein and hence lipoprotein synthesis by interfering with RNA metabolism. In fact, there has been shown a decreased microsomal and nuclear RNA content in azaserine-treated cells, and a higher uracil incorporation into RNA together with an increased uracil pool (19). The decreased content of RNA and the presence of RNA molecules containing abnormal bases may determine a decreased ability in synthesizing specific apoproteins (20–24).

Summary. The effect of azaserine on liver lipid composition, on lipogenesis *in vitro* in liver slices and on serum lipoproteins, on liver content of ATP, biotin, and nicotinamide nucleotides in the rat is reported. The effect of ATP or adenine in azaserine-treated rats is also presented. The neutral fats in the liver of azaserine-treated rats significantly increased, while the incorporation *in vitro* of acetate-^{14}C into total lipids decreased. In the same experimental conditions the liver concentration of biotin and nicotinamide nucleotides fell. A marked decrease of the levels of serum lipoprotein also was observed. The administration of ATP or adenine to azaserine-treated rats did not prevent either the development of fatty liver, the decrease of liver biotin and of serum lipoprotein, or the decrease of acetate-^{14}C incorporation into liver lipids. On the contrary, the administration of ATP or adenine partially reversed the azaserine effects on the liver content of nicotinamide nucleotides and increased the liver concentration of ATP.

1. Sternberg, S. S., Philips, F. S., and Clarke, D. A., Federation Proc. **13**, 444 (1954).

2. Hyams, D. E. and Isselbacher, K. J., Nature **204**, 1196 (1964).

3. Euler, L. H., Rubin, R. J., and Handshumacher, R. E., J. Biol. Chem. **238**, 2464 (1963).

4. Farber, E., Lombardi, B., and Castillo, A. E., Lab. Invest. **12**, 873 (1963).

5. Marchetti, M., Puddu, P., and Caldarera, C. M., Biochim. Biophys. Acta **61**, 826 (1962).

6. Marchetti, M., Puddu, P., and Caldarera, C. M., Biochem. J. **92**, 46 (1964).

7. Windmueller, H. G., J. Biol. Chem. **239**, 530 (1964).

8. Puddu, P., Caldarera, C. M., and Marchetti, M., Biochem. J. **102**, 163 (1967).

9. Bhargawa, M. M. and Sreenivasan, A., Biochem. J. **100**, 647 (1966).

10. Sperry, W. M. and Webb, M., J. Biol. Chem. **187**, 466 (1959).

11. Bartlett, G. R., J. Biol. Chem. **234**, 466 (1959)

12. Adam, H., *in* "Methods of Enzymatic Analysis" (H. U. Bergmeyer, ed.), p. 539. Academic Press, New York (1963).

13. Wright, L. D. and Skeggs, H. R., Proc. Soc. Exptl. Biol. Med. **56**, 95 (1944).

14. Lowry, O. H., Roberts, N. R., and Kapphahn, J. I., J. Biol. Chem **224**, 1047 (1957).

15. Folch, J., Lees, M., and Sloanes Stanley, G. H., J. Biol. Chem. **226**, 497 (1957).

16. Farber, E., Shull, K H., Villa-Trevino, S., Lombardi, B., and Thomas, M., Nature **203**, 34 (1964).

17. Henderson, J. F., J. Lipid Res. **4**, 68 (1963).

18. Puddu, P., Ottani, V., and Marchetti, M., Proc Soc. Exptl. Biol. Med. **130**, 493 (1969).

19. Adams, D. H., J. Biol. Chem. **89**, 240 (1963)

20. Chantrenne, H. and Dewreux, S., Biochim. Biophys. Acta **41**, 239 (1960).

21. Mandel, H. G. and Markham, R., Biochem. J. **69**, 297 (1958).

22. Kwam, D. C. and Parks, R. E., J. Biol. Chem. **235**, 2893 (1960).

23. Naono, S. and Gros, F., C. R. Acad. Sci. Paris **250**, 3527 (1960).

24. Aaronson, A. S., Biochim. Biophys. Acta **49**, 98 (1961).

Received May 20, 1969. P.S.E.B.M., 1970, Vol. 133.