FUNDAMENTAL STUDIES ON PHYSIOLOGICAL AND PHARMACOLOGICAL ACTIONS OF L-ASCORBATE 2-SULFATE. I. ON THE HYPOLIPIDEMIC EFFECTS

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Summary The effects of L-ascorbate 2-sulfate (AAS) on the lipid metabolism were examined in Triton-induced hyperlipemic mice, hypercholesterolemic and normal rats, the following results being obtained. 1) In Triton-induced hyperlipemic mice, AAS (300 mg/kg) significantly decreased the serum cholesterol level, while L-ascorbate (AA, 175 mg/kg) was found ineffective. 2) In hypercholesterolemic rats fed 0.5% cholesterol diet, the consecutive administration of AAS decreased the level of serum cholesterol and liver triacylglycerols. AA only slightly affected these levels. However, both AAS and AA prevented the unordinal increase in the liver weight caused by cholesterol feeding. 3) In normal rats, the administration of AAS over a 4-week period decreased the level of serum cholesterol and liver triacylglycerols.

It is well known that sulfated polysaccharides have various pharmacological effects, such as being antipeptic (1) and anticoagulant (2) and having lipid lowering actions (3). In the course of the pharmacological study on sulfated monosaccharides, we have found that L-ascorbate 2-sulfate (AAS) possesses an antipeptic (unpublished data) and cholesterol-lowering activity (4). The present paper deals with the effects of AAS on the lipid metabolism in the liver and serum from the Triton-treated mice, cholesterol-fed rats and normal rats.

MATERIALS AND METHODS

Experiment with Triton-induced hyperlipemic mice. Male mice of ddy-strain, weighing 18–22 g, were divided into 7 groups of 10 animals each. Triton WR-1339 (800 mg/kg, in saline) was intravenously administered into the tail vein to all animals, and simultaneously, AAS (300, 150, 75 mg/kg), AA (300, 150 mg/kg),
Table 1. Composition of cholesterol diet containing 0.5% cholesterol.

| Component          | Amount |
|--------------------|--------|
| Cholesterol        | 0.5    |
| Bacto oxgal        | 0.2    |
| Milk casein        | 20     |
| Salt mix*          | 4      |
| Shortening oil     | 10     |
| Choline chloride   | 0.2    |
| PABA               | 0.1    |
| Myo-inositol       | 0.1    |
| Vitamine mix b     |        |
| Sucrose            | 64.9   |
| Total              | 100 g  |

* Salt mix: CaCO₃, 30; K₂HPO₄, 32.5; CaHPO₄·2H₂O, 7.5; MgSO₄·7H₂O, 10.2; NaCl, 16.8; Fe(C₆H₅O₂)₃·6H₂O, 2.8; KI, 0.08; MnSO₄·4H₂O, 0.5; ZnCl₂, 0.025; CuSO₄·5H₂O, 0.03 g.

b Vitamine mix/kg: B₁, 5 mg; B₆, 5 mg; CaP, 20 mg; biotin, 100 μg; B₂, 6 mg; B₁₂, 10 μg; folic acid, 500 μg; niacin, 20 mg; D₂, 3,000 I.U.; A, 30,000 I.U.; K₃, 5 mg.

both dissolved in saline, or clofibrate (100 mg/kg) suspended in 1% CMC was given orally to each group. The administration of these drugs was repeated once 20 hr after the first administration. The control group received saline. Blood specimens (0.5 ml) were taken 40 hr after the Triton treatment. Specimens from the two animals were pooled and kept in a refrigerator for 1 hr. Then, the serum was separated by centrifugation at 8,000 rpm for 15 min. Serum was deproteinized by mixing 0.1 ml of serum with 5.9 ml of isopropyl alcohol and the supernatant was submitted to total cholesterol assay with a Technicon autoanalyzer.

Experiment with hypercholesterolemic rats. Male rats of Wistar-strain, weighing 80–110 g, were divided into 4 groups of 8 animals each, and fed a synthetic diet containing 0.5% cholesterol, shown in Table 1, for 2 weeks, during which AAS, AA and Na₂SO₄ or clofibrate was administered daily, orally or intravenously in the doses specified. The control group received saline. AA and AAS were dissolved in saline for oral administration, and for intravenous administration the solution was sterilized by filtering through a bacterial filter.

Experiment with normal rats. Male rats of Donryu-strain, weighing 90–120 g, were divided into 4 groups of 7 rats each, and maintained on a balanced chow diet (Oriental Fermentation Co., Tokyo). The first to third groups received orally 62.5, 250 and 1,000 mg/kg of AAS in saline for 6 weeks. The last group received saline only.

Analytical procedure. Blood for lipid analysis was withdrawn from the tail vein of rats fasted for 15 hr at the time specified during the experiment, and at the end of the experimental period blood was obtained from the ophthalmic vessels under anesthesia with ether. The liver lipids was extracted by the method of FOLCH et al. (5). For serum and liver lipid analysis the following methods were used: cholesterol—ZAK (6), triacylglycerols—BLOCK and JARRET (7).
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Materials. Sodium L-ascorbate 2-sulfate (AAS), white crystalline powder and odorless, was synthesized in our laboratory. Sodium L-ascorbate (AA) was purchased from Daiichi Chemicals (Tokyo), Triton WR-1339 (Rohm & Haas Co.) from Wako Chemicals (Tokyo). Clofibrate [ethyl 2-(p-chlorophenoxy)-2-methyl propionate] was given by Nissei Kagaku Co. (Tokyo) (Fig. 1).

RESULTS

1. Experiment with Triton-induced hyperlipemic mice

The cholesterol level of the group which received AAS (300 mg/kg) twice was significantly decreased by 26.8% compared to that of the control group. The group treated with the lower doses of AAS (150, 75 mg/kg) had a only slightly lower cholesterol level. In contrast, the AA administration did not affect the cholesterol level. The cholesterol lowering effect of clofibrate (100 mg/kg) approximated that of AAS (300 mg/kg) (Table 2).

Table 2. Hypocholesterolemic effect of AAS in Triton-induced hyperlipemic mice.

| Drug   | Dose (mg/kg) | Rat No. | Mean±S.E. | Decrease (%) |
|--------|--------------|---------|-----------|--------------|
|        |              | 1 2 3 4 5 |           |              |
| AAS    | 300          | 150 251 210 150 255 | 203±23.1 | 26.8a        |
|        | 150          | 315 192 174 270 222 | 235±25.8 | 15.5         |
|        | 75           | 192 210 276 330 210 | 245±25.6 | 11.8         |
| AA     | 300          | 252 255 274 225 342 | 262±20.8 | 5.8          |
|        | 150          | 420 315 174 258 234 | 280±41.6 | -0.9         |
| Clofibrate | 100      | 204 225 198 129 228 | 197±17.9 | 29.0b        |
| Control | none         | 230 282 316 303 255 | 277±15.4 | —            |

a P<0.05, b P<0.01, significant difference from control.

2. Experiment with hypercholesterolemic rats

Table 3 shows the effects of the oral (200 mg/kg) or intravenous administra-
tion (100 mg/kg) of AAS in the rats maintained on a cholesterol diet. The effect of AAS was not evident on the 5th day, whereas on the 10th and 15th day, AAS given by oral administration or by intravenous injection significantly decreased the serum cholesterol level compared to the control rats. The levels of cholesterol and triacylglycerols in the serum and livers at the end of the experimental period are shown in Table 4. The oral or intravenous administration of AAS decreased the level of triacylglycerols both in the liver and serum, while the level of cholesterol was decreased only in the serum. In order to dissociate the effect of AAS from that of AA partly converted from AAS in vivo, another group of rats was set receiving AA and sodium sulfate as one of the controls. Clofibrate was also utilized as a reference compound for cholesterol-lowering agents. The change in the serum cholesterol level during this experimental period is shown in the Table 5. The level of AAS group on the 10th and 15th day was significantly lower by 26% and by 24%, respectively, than that of the control group. The level of AA group slightly decreased but without significance. The clofibrate group had 36.5%, 42.7%, and 51.8% lower level than that of the control on the 5th, 10th and 15th day respectively. The levels of cholesterol and triacylglycerols in the serum and livers at the end of the experiment are shown in Table 6, in which the cholesterol-lowering effect of AAS or clofibrate was evident only in the serum but not in the liver. AA did not affect the level of cholesterol either in the serum or in the liver. The level of triacylglycerols was lowered both in the serum and the liver only by clofibrate.

| Drug                  | Period (day) |
|-----------------------|--------------|
|                       | 0            | 5            | 10           | 15           |
|                       | Serum cholesterol (mg%) |
| AAS (200 mg/kg, p.o.) | 84±2.2       | 186±15.1     | 231±12.5b    | 232±9.3a     |
| AAS (100 mg/kg, i.v.) | 85±2.6       | 188±16.4     | 211±9.5b     | 217±12.0b    |
| Control               | 83±1.7       | 230±15.1     | 303±18.5     | 293±18.4     |

N=8, * P<0.05, b P<0.01, significant difference from control.

| Drug                  | Cholesterol | Triacylglycerols |
|-----------------------|--------------|------------------|
|                       | Serum (mg%)  | Liver (mg/g)     | Serum (mg%)  | Liver (mg/g) |
| AAS (200 mg/kg, p.o.) | 232±9.3a     | 46.4±1.9         | 188±4.5b     | 7.1±0.5a     |
| AAS (100 mg/kg, i.v.) | 217±12.0b    | 46.8±1.3         | 180±3.4b     | 6.7±0.6b     |
| Control               | 293±18.4     | 51.1±2.5         | 210±3.2      | 8.9±0.4      |

N=8, * P<0.05, b P<0.01, significant difference from control.
Table 5. Effects of AAS, AA and clofibrate on serum cholesterol level in hypercholesterolemic rats.

| Drug                  | Period (day) | Serum cholesterol (mg%) |
|-----------------------|--------------|-------------------------|
|                       | 0            | 5                       | 10                      | 15                      |
| AAS (200 mg/kg)       | 97.9±2.52    | 271±18.3                | 245±18.5                | 254±15.1                |
| AA (100 mg/kg)        | 101.1±3.32   | 289±13.6                | 304±21.9                | 281±16.7                |
| Na₂SO₄ (100 mg/kg)    | 91.5±3.02    | 179±16.7                | 189±13.8                | 171±9.2                |
| Clofibrate (150 mg/kg)| 98.3±4.35    | 279±9.2                 | 330±24.0                | 332±24.5                |

N=8, a P<0.05, b P<0.01, significant difference from control.

Table 6. Effects of AAS, AA and clofibrate on cholesterol and triacylglycerols of serum and liver in hypercholesterolemic rats.

| Drug                  | Cholesterol | Triacylglycerols |
|-----------------------|--------------|------------------|
|                       | Serum (mg%)  | Liver (mg/g)     | Serum (mg%)  | Liver (mg/g)     |
| AAS (200 mg/kg)       | 254±15.1     | 36.3±1.25        | 90.6±4.26    | 24.1±1.22        |
| AA (100 mg/kg) + Na₂SO₄ (100 mg/kg) | 281±16.7     | 36.1±1.30        | 85.3±6.34    | 23.4±1.16        |
| Clofibrate (150 mg/kg)| 171±9.2     | 31.1±1.43        | 67.0±5.46    | 11.0±0.60        |
| Control               | 332±24.5     | 34.3±2.54        | 97.0±6.12    | 19.8±0.86        |

N=8, a P<0.05, b P<0.01, significant difference from control.

Table 7. Changes in body weight and liver weight by 2-week administration of AAS, AA or clofibrate in hypercholesterolemic rats.

| Drug                  | Body weight (g) | Liver weight (g) |
|-----------------------|-----------------|------------------|
| AAS (200 mg/kg)       | 140±3.2         | 5.2±0.16        |
| AA (100 mg/kg) + Na₂SO₄ (100 mg/kg) | 139±4.9     | 5.5±0.23        |
| Clofibrate (150 mg/kg)| 130±4.7         | 8.6±0.41        |
| Control               | 140±3.5         | 6.3±0.27        |

N=8, a P<0.05, b P<0.01, significant difference from control.

The body weight of the AAS, AA, clofibrate and control groups, and their liver weight at the end of the experiment are presented in Table 7. As well documented, clofibrate caused severe hepatomegaly. The liver weight of the AA or AAS group was significantly less than that of the control group, indicating a preventive effect of these drugs on a fatty liver caused by cholesterol feeding.

3. Experiment with rats of normolipid level

The effects of AAS in rats of normolipid level are shown in Table 8. Administration of AAS to normal rats decreased serum cholesterol level by 20% in 1,000 mg/kg dose of the 4th week, and more than 20% in every dose at the 6th
Table 8. Hypolipidemic effects of AAS in rats of normolipid level.

| Sex    | Dose (mg/kg) | Serum | Liver | Liver |
|--------|--------------|-------|-------|-------|
|        |              | Cholesterol (mg%) | Triacylglycerols (mg%) | Cholesterol (mg/g) | Triacylglycerols (mg/g) |
|        |              | 0     | 2     | 4     | 6     | 6     | 6     | 6     |
| Male   | Control 62.5 | 101.0±4.19 | 90.6±2.88 | 89.1±2.61 | 91.4±3.96 | 59.8±3.81 | 6.20±0.41 | 27.2±3.12 |
| Male   | 250        | 99.2±3.42 | 78.9±1.32 | 77.8±4.16 | 72.6±2.21b | 43.0±1.93b | 6.50±0.52 | 17.0±1.56a |
| Male   | 1,000      | 99.8±2.73 | 83.7±3.80 | 71.4±4.20b | 66.8±1.72b | 43.1±1.50b | 6.16±0.49 | 15.8±3.13a |
| Female | Control 62.5| 117.0±5.82 | 99.9±6.89 | 106.0±9.50 | 111.0±4.87 | 54.0±3.76 | 5.71±0.28 | 13.9±1.65 |
| Female | 250        | 112.0±3.77 | 92.4±3.99 | 99.1±7.13 | 97.2±2.75 | 41.6±1.87a | 5.73±0.41 | 12.6±1.37 |
| Female | 1,000      | 116.0±2.92 | 86.3±3.26 | 96.8±3.10 | 78.8±2.78b | 39.3±1.87b | 5.16±0.23 | 10.3±0.24a |

*P* < 0.05, *b* P < 0.01, significant difference from control.
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week in male rats. In female rats, 20–30% decrease in serum cholesterol level was observed in 250 and 1,000 mg/kg doses. The serum triacylglycerol level was lowered by 20–30% in both male and female rats at the 6th week in every dose. The liver cholesterol level was not affected by the administration of AAS as observed in the experiment 2, while the liver triacylglycerol level was decreased by 37% (62.5 mg/kg dose) and 60% (1,000 mg/kg dose) in male rats, and 21.5% (250 mg/kg dose) and 30.0% (1,000 mg/kg) in female rats.

In this experiment no difference was observed in the growth rate and the relative liver weight between the treated groups and the control.

DISCUSSION

In our experiments, it has been suggested that AAS is a possible factor in lowering lipid levels in rats and mice (4).

Concerning the effect of AA on the metabolism of serum lipids and atherosclerosis, there are many conflicting reports; some are in favor of its protective effect on hyperlipemia in man and animals, and some argue against these observations. GINTER (8) has recently reviewed this problem.

The occurrence of AAS, a sulfate ester of AA, has been reported in cyst of brine shrimp by MEAD and FINAMORE (9), in human urine and tissues by BAKER et al. (10) and in rat liver by MUMMA and VERLANGIERI (11). In addition, VERLANGIERI and MUMMA (12) observed an increased excretion of cholesteryl sulfate into feces in rats that received AAS by intracardial injection. Then, in what relation do AA and AAS stand in vivo? HORNIG et al. (13) have observed that AA taken orally, is partly converted in intestinal mucosa into AAS, a part of which is in turn excreted into small intestine via liver and portal vein, and deposited in the mucosa, and another part of which is distributed in body via lymphatic vessels and heart. These observations may suggest an interconvertible relation between AA and AAS, but it is not clear whether AAS is merely a deposit form of AA or AAS has specific roles.

In Triton-induced hyperlipemia, the effects of drugs affecting the synthesis of cholesterol and triacylglycerols are commonly consumed to be observed in the first phase, and those of drugs affecting their excretion in the second phase. The marked decrease in the serum cholesterol level in Triton-treated rats by AAS (300 mg/kg) will probably reflect the interaction at the both phases, while that by clofibrate (100 mg/kg) will be attributable to the interaction in the first phase. In the experiment with Triton, AA exerted no effect on serum lipid levels. This result may have an implication for a different action of AAS from that of AA on serum lipid metabolism.

On the basis of the results with hyperlipemic mice, the lipid-lowering effect of AAS was examined with hyperlipemic rats. Unlike quick responses in mice, the appearance of the effect was delayed in rats: a decrease in serum cholesterol level
was observed on the 10th day, and a decrease in serum cholesterol and triacylglycerol level, and liver triacylglycerol level on the 15th day. It seems likely that AAS exerts a rapid lipid-lowering effect in mice and a delayed effect in rats. In a comparative study of AAS with AA, AA and Na₂SO₄ was simultaneously administered in expectation of sulfation because AAS was a sulfate ester of AA. AA did not show any significant effect on lipid levels in this experiment, while AAS gave results similar to that in the experiment described above. According to the report by Hornig et al. (13), AAS is produced from AA in vivo, but how much of AA is converted to AAS remains unknown. The negligible effect on lipid levels of AA observed in our experiment will indicate that AAS and AA differently affect the lipid metabolism.

Using rats of normolipid level, the effect of AAS was also examined in the doses of 62.5, 250 and 1,000 mg/kg. A decrease in serum cholesterol and triacylglycerol level was first observed at the 4th week in all doses. A decrease in the level of liver triacylglycerols at the 6th week in the treated rats was observed but the level of liver cholesterol was unchanged. In rats of the normolipid level, the appearance of the lipid lowering effect of AAS is more delayed than in hyperlipemic rats, and AAS did not seem to affect the level of liver cholesterol as well. In this experiment, neither AA nor AAS affected the liver weight, though they exerted a preventive effect on liver enlargement in rats fed a cholesterol diet.

The results obtained in the experiments with mice and rats suggest that AAS has considerable effects on lipid metabolism, and it is very interesting that physiologically occurring AAS has such specific effects that are different from those of AA.

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