BREAKING ACTION OF ASCORBIC ACID ON NUCLEIC ACIDS

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Summary The lowering of the viscosity of DNA solution was caused by the action of AsA or EA and facilitated in the presence of Cu²⁺. However, the action of AsA-3-P was very weak. By sucrose density gradient centrifugation, it was observed that single- and double-strand scissions of DNA were provoked by AsA or EA and enhanced with Cu²⁺, while only a single-strand scission was caused by AsA-3-P and Cu²⁺. Similar action of AsA or AsA-3-P was also observed for RNA. Thus, the result indicates that the enediol group of AsA takes an essential part in the breakage of nucleic acids, and Cu²⁺ enhances the action. It was shown that Apu was mainly decomposed by AsA, whereas Apy not, suggesting that some pyrimidine cluster may be one of the regions attacked by AsA. During the reaction with DNA, the reducing activity of AsA decreased first to some extent and then increased, whereas the reducing activity of AsA-3-P was much lower than that of AsA and decreased steadily. The priming activity of DNA for DNA polymerase

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Abbreviations: AsA, ascorbic acid; DAsA, dehydroascorbic acid; DKG, 2,3-diketogulonic acid; MDT, 5-methyl-3,4-dihydroxytetrone; EA, erythorbic acid; DEA, dehydroerythorbic acid; TR, triose reductone; AsA-3-P, ascorbyl-3-phosphate; Apu, apurinic acid; Apy, apyrimidinic acid; PY medium, polypeptone-yeast extract medium; PPO, 2,5-diphenyloxazole; SSC, standard saline citrate (0.15 M sodium chloride + 0.015 M sodium citrate); MNNG, N-methyl-N'N-nitro-N-nitrosoguanidine; Tris, tris (hydroxymethyl)-aminomethane; dATP, deoxyadenosine-5'-triphosphate; dGTP, deoxyguanosine-5'-triphosphate; dCTP, deoxycytidine-5'-triphosphate; dTTP, deoxythymidine-5'-triphosphate.
was changed after treatment with AsA according to the condition. It was enhanced when DNA was treated under mild conditions but decreased with severer action.

The action of several reductones on DNA was investigated at our institute (1-15). It was observed that AsA and its oxidized derivatives (DAsA, DKG, MDT) caused some retardation of the growth of sarcoma-180 implanted in mice and the lowering of the viscosity of DNA solution (1-5). These activities were enhanced with small amounts of Cu^{2+}. The lowering of the viscosity was also observed with TR, 2,3-diketogluconic acid and xylosone, but not with diacetyl and α-ketogluconic acid (4). The phenomenon seems to be caused by breakage of DNA molecules. Indeed, sucrose density gradient centrifugation confirmed that DAsA brings about the single- and double-strand scissions of DNA depending on the concentration of DAsA and Cu^{2+} (2,5). Similar breakages of DNA in vitro as well as in vivo were induced by catechol (6,7), catecholamines such as Dopa, Dopamine, adrenaline and noradrenaline (8-11), and some other reductones such as amino reductones (12-14), TR (14) and MDT (15). The viscosity lowering of DNA with AsA was shown by Conway and Butler (16) or Berneis (17) too. Murata et al. (18-21) demonstrated the degradation of DNA with AsA by the sucrose density gradient centrifugation, in connection with the inactivation of bacteriophages. Similar results were also obtained by Wong et al. (22) on phage R17 RNA. These potencies of AsA may be attributed to enediol or its oxidized group in the molecule. Therefore, in order to confirm it, action on nucleic acids was investigated not only with AsA, but also with EA, D-isomer of AsA containing the enediol structure, and AsA-3-P in which 3-OH group of AsA was esterified with phosphoric acid and the enediol structure was blocked.

MATERIALS AND METHODS

1. Chemicals. Special grade AsA was purchased from Wako Pure Chemicals Co., Ltd; EA and AsA-3-P were supplied by Fujisawa Pharmaceutical Co., Ltd. and Takeda Pharmaceutical Co., Ltd. respectively. DAsA was prepared from AsA by oxidation with o-benzoquinone (5).

2. DNA. DNA was prepared in our institute (23) from calf thymus by the routine SDS-phenol method.

3. RNA. RNA was prepared from mouse liver by the SDS-phenol method and stored at -20°C. The method was modified from that of Kirby et al. (24,25) where SDS was used together with phenol to facilitate the denaturation of proteins and that acetate buffer (pH 5.1) and bentonite (0.5% in the final concentration) were used to prevent the action of RNase contaminated. The preparation consists of ribosomal RNA (18 S and 28 S) and transfer RNA (4 S).

4. Apy and Apu. Apy was prepared by hydrazine treatment according to
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TAKEMURA (26), except that the treatment was elongated. The preparation of Apu was carried out by acid treatment as described by TAMM et al. (27). Complete elimination of pyrimidines in Apy and that of purines in Apu were established by paper chromatography.

5. Viscosity measurement. Time course of the viscosity of the mixture (5 ml) in 0.1 M Tris buffer (pH 7.6) containing 200 μg DNA/ml, 5 mM AsA, EA or AsA-3-P, and 0.3 mM CuSO₄₆ was estimated in an Ostwald's viscometer at 37°C.

6. Sucrose density gradient centrifugation. DNA, AsA (EA or AsA-3-P) and CuSO₄ were dissolved in SSC till a final concentration of 50 μg/ml, 5 mM and 1 mM respectively. The mixture was incubated at 37°C for 4 hr and dialyzed against SSC at 4°C for 15 hr. For comparison, the reaction mixtures in which AsA derivatives and Cu²⁺ or Cu²⁺ alone had not been used, were incubated similarly. An aliquot of 0.2 ml reaction mixture was laid on the top of the linear gradient of sucrose from 5 to 20% in 1 M NaCl-0.01 M Tris buffer (pH 8). After centrifugation with a SW-39 rotor in a Beckman L ultracentrifuge at 30,000 rpm for 4 hr, the solution was fractionated from the bottom of the tube. Each fraction (4 drops) was mixed with 1 ml water and the absorbancy was estimated at 260 nm in a micro-cell (light path: 5 cm) of a Hitachi spectrophotometer, EPU-Type 2. In order to estimate the single-strand scission of DNA, alkaline sucrose density gradient centrifugation was carried out in the same way as for the neutral one except that the sucrose solution in 0.9 M NaCl-0.1 M NaOH was adjusted to pH 11.8 and was employed in place of the neutral solution.

The approximate molecular weight of DNA fractions was roughly calculated from the centrifugal pattern on the equations described by STUDIER (28).

On the other hand, centrifugation of RNA was carried out as follows. RNA (0.3 mg/ml) dissolved in 0.01 M phosphate buffer (pH 7) was incubated with 5 mM AsA or AsA-3-P at 37°C for 2 hr. The reaction mixture was dialyzed against 0.01 M Tris buffer (pH 7.6) at 4°C for 15 hr and centrifuged through a linear gradient of sucrose from 5 to 20% in 0.1 M NaCl-0.01 M acetate buffer (pH 5.1) at 30,000 rpm for 8 hr. Subsequent procedures were the same as those in the case of DNA.

7. Reducing activity. Reducing activity was estimated by the titration method with Tillman reagent which was prepared as follows. Sodium salt of 2,6-dichlorophenolindophenol was dissolved in n-butanol to give a final concentration of 0.1 M, filtered, diluted with 20 volumes of water and was acidified to red by adding a few drops of glacial acetic acid.

8. Gel filtration. One milliliter of 0.15 M AsA was added to 1.5 ml of Apy or Apu solution (1 mg/ml) dissolved in 0.1 M phosphate buffer (pH 7). The mixture was incubated at 37°C for 24 hr, dialyzed against water at 4°C overnight and applied to a 100 × 1.6 cm Sephadex G-50 (medium) column. Apy and Apu were eluted with water at a rate of 5 ml/min. The absorbancy of each fraction (5 ml) was estimated at 256 nm for Apy and at 269 nm for Apu.
9. Assay of DNA polymerase. E. coli B grown on PY medium was harvested at the late stage of the log phase. The cells were mixed with 0.05 M glycylglycine buffer (pH 7), homogenized in a Waring blender and centrifuged at 10,000 xg for 30 min. DNA polymerase preparation was obtained by streptomycin precipitation, autolysis, ammonium sulfate fractionation, DEAE-cellulose chromatography and phosphocellulose chromatography according to RICHARDSON et al. (29), and stored in 50% glycerol at -20°C. The activity of DNA polymerase was assayed by estimating the amount of 3H-TMP incorporated into acid-insoluble material as follows. The reaction mixture shown in Table 1 was incubated at 37°C for 20 min. The 50 μl aliquot was mixed with 3 ml cold 0.5 N perchloric acid and kept in cold for 5 min. Acid-insoluble fraction was collected on glass fiber paper (Whatman GF/C) and washed twice with 3 ml cold 0.5 N perchloric acid, water and 95% alcohol. The filter paper was dried under an infrared lamp. The paper was then put into a vial with 10 ml 0.4% PPO-toluene and counted in a Beckman LS-250 liquid scintillation counter.

RESULTS

1. Viscosity lowering

As shown in Fig. 1, AsA and EA caused the lowering of the viscosity of DNA solution to some extent. No difference was observed in the effect between AsA and EA. The effect was remarkably facilitated by cooperation of Cu²⁺. On the other hand, the lowering of the viscosity was very weak by AsA-3-P and enhanced to the degree of about the same as that of AsA or EA alone after the addition of Cu²⁺. The viscosity was lowered gradually with longer incubation time; the relative viscosity after 20 hr being 1.86 with AsA-3-P and 1.48 with AsA-3-P and Cu²⁺. Of course, Cu²⁺ of the same concentration does not cause the lowering of the viscosity.

2. Breakage of DNA

Since the viscosity-lowering of DNA solution suggests the breakage of DNA,
Fig. 1. Effect of AsA, EA or AsA-3-P on the viscosity of DNA solution. Reaction mixture (5 ml): 1 mg DNA, 5 mM AsA, EA or AsA-3-P, 0.3 mM CuSO₄; 37°C.

DNA was treated with AsA and analyzed by sucrose density gradient centrifugation. The pattern in Fig. 2 indicates that the single- and double-strand breakages were brought about by the action of AsA and stimulated with Cu²⁺. The rough calculation of the molecular weight of DNA fragments indicated that the peak of the original DNA at No. 2 (ca. 2.68 × 10⁸) decreased and the new peaks appeared at No. 14 (ca. 1.14 × 10⁷) with AsA and at No. 19 (ca. 4.8 × 10⁴) with AsA and Cu²⁺, although it is not accurate, after the double-strand breakage shown by the neutral centrifugation. On the other hand, in the single-strand scission shown in alkaline centrifugation, the main peak of DNA at No. 10 (ca. 1.86 × 10⁷) shifted to No. 17 (ca. 9 × 10⁵) by AsA and to No. 19 (ca. 4.6 × 10⁴) by AsA and Cu²⁺. Similar double-strand breakages of DNA by EA with or without Cu²⁺ were also confirmed as indicated in Fig. 3. On the contrary, AsA-3-P induced practically no double-strand breakage in DNA, even with Cu²⁺. However, it was observed that, in the alkaline centrifugation, the peaks at No. 6 (ca. 4.15 × 10⁷) and No. 11 (ca. 1.4 × 10⁷) slightly decreased, whereas the peak at No. 19 (ca. 4.6 × 10⁴) increased, as shown in Fig. 4. When Cu²⁺ was present with AsA-3-P, the peak at No. 19 increased considerably, accompanying the formation of the new peak at No. 10-12. Thus, the results indicates that the degradation of DNA by AsA derivatives is consistent with the viscosity-lowering.
Fig. 2. Sedimentation pattern of calf thymus DNA treated with AsA. Reaction mixture (×—×, 50 µg/ml DNA; ○—○, 50 µg/ml DNA + 5 mM AsA; ●—●, 50 µg/ml DNA + 5 mM AsA + 1 mM CuSO₄) was incubated at 37°C for 4 hr and dialyzed at 4°C for 15 hr. An aliquot of 0.2 ml reaction mixture was subjected to sucrose density gradient centrifugation (5%–20%; pH 8.0 or 11.8) at 30,000 rpm for 4 hr.

3. Breakage of RNA

The single-strand scission of DNA with AsA suggests that AsA decomposes RNA too, since RNA is generally composed of single chain of polynucleotide. Therefore, RNA was treated with AsA or AsA-3-P and analyzed by centrifugation. Figure 5 indicates that mouse liver RNA, in particular 28 S fraction, was almost completely decomposed and smaller fragments were formed by the action of AsA even without Cu²⁺. On the other hand, AsA-3-P did not cause a change of 28 S. However, fractions 18 S and 4 S were moved a little to the side of the lower molecular weight, accompanying a slight decrease of the former and a slight increase of the latter.

4. Degradation of Apy and Apu

Since DNA fragments of relatively high molecular weight are formed after breakage by AsA, it is supposed that some peculiar susceptible region may exist in DNA. Adrenaline decomposes specifically Apu (8). It was also demonstrated that pyrimidine tetra- and pentanucleotides were decomposed by DAsA with
Fig. 3. Sedimentation pattern of calf thymus DNA treated with EA. The same as that of Fig. 2.

Fig. 4. Sedimentation pattern of calf thymus DNA treated with AsA-3-P. The same as that of Fig. 2.
Fig. 5. Sedimentation pattern of mouse liver RNA treated with AsA or AsA-3-P. Reaction mixture (×—×, 0.3 mg/ml RNA; ○—○, 0.3 mg/ml RNA + 5 mM AsA; ⋄—○, 0.3 mg/ml RNA + 5 mM AsA-3-P) was incubated at 37°C for 2 hr. After dialysis at 4°C for 15 hr, the reaction mixture was subjected to sucrose density gradient centrifugation (5%-20%, pH 5.1) at 30,000 rpm for 8 hr.

Cue, while pyrimidine mono-, di- or trinucleotide as well as all purine oligonucleotides were not (3). This suggests that the pyrimidine cluster arranged successively with pyrimidine bases should be susceptible region where adrenaline or DAsA attacks. Therefore, action of AsA on Apy or Apu was examined and shown in Fig. 6. It was confirmed that Apu was mainly decomposed by AsA, whereas Apy not. The result suggests that DNA may be decomposed by AsA in a region of pyrimidine cluster, as in the cases of adrenaline and DAsA.

5. Reducing activity
The breakage of nucleic acids by AsA seems to attribute to its enediol structure. Therefore, the change of the reducing activity of AsA was then examined after treatment on DNA. The reaction mixture (10 ml) containing DNA (100 µg/ml) and 1 mM AsA in 0.02 M acetate buffer (pH 4) was incubated at 37°C. At a
given time, 0.1 ml (0.1 \mu\text{moles AsA}) of the mixture was diluted with 4.9 ml acetate buffer and titrated with Tillman reagent. In the case of AsA-3-P, the reaction mixture (16 ml) containing 5 mM AsA-3-P was incubated and 2 ml (10 \mu\text{moles AsA-3-P}) of the mixture were mixed with 3 ml acetate buffer and titrated, because the reducing activity of AsA-3-P was much weaker than that of AsA. The result is shown in Fig. 7.

Since the assay condition differs so it is difficult to compare the results quantitatively, therefore only the tendency of the change of the reducing activity was compared. It was found that the reducing activity of AsA-3-P was about 1/40 of that of AsA and decreased with incubation time during the reaction. On the other hand, the reducing activity of AsA decreased first until 20 min after incubation with DNA and then increased to a constant level. This change suggests that AsA was oxidized to DAsA which turned to aci-reductones. Indeed, it was observed that the reducing activity increased with reaction time when AsA-3-P was replaced by DAsA.

6. DNA polymerase

DNA serves as a template not only for RNA polymerase, but also for DNA polymerase. When DNA was cleaved, its priming activity for DNA polymerase
Fig. 7. Change of the reducing activity during the reaction of DNA with AsA, AsA-3-P or DAsA. Reaction mixture: 37°C, pH 4. AsA, 100 µg/ml DNA + 1 mM AsA; AsA-3-P, 100 µg/ml DNA + 5 mM AsA-3-P; DAsA, 100 µg/ml DNA + 5 mM DAsA. Titration: Tillman reagent. AsA, 0.1 ml reaction mixture was diluted with 4.9 ml acetate buffer (pH 4); AsA-3-P, 2 ml reaction mixture was diluted with 3 ml acetate buffer (pH 4); DAsA, the same as with AsA-3-P.

Fig. 8. Priming activity of DNA treated with AsA for DNA-polymerase. DNA was treated with AsA of different concentration with or without Cu²⁺ at 37°C for 60 min. After dialysis at 4°C for 48 hr, DNA was employed for the assay of DNA polymerase on the primer template.
would change, leading to a variation of DNA synthesis. Thus, DNA was treated with AsA of different concentration with or without Cu²⁺ at 37°C for 60 min, dialyzed against to 0.1×SSC for 48 hr in cold and employed for DNA polymerase as the primer template. Figure 8 indicates that the priming activity of DNA treated with AsA of 10⁻⁷ to 10⁻⁸ M was almost the same as that of control DNA, and that the activity increased by the treatment with 10⁻⁴ to 10⁻⁶ M but decreased with 10⁻³ M. In the case of coexistence of Cu²⁺ with AsA, the priming activity was enhanced with the increase in the concentration of AsA in the range of 10⁻⁶ to 10⁻⁸ M, but decreased at a higher concentration. This tendency resembles to the case for RNA polymerase with several DNA breaking reagents (12,13,32,33).

DISCUSSION

The results indicate that AsA breaks both DNA and RNA, especially in the presence of Cu²⁺. The enediol group of AsA plays an essential role in the degradation of nucleic acids and the ability is diminished when the enediol group has been blocked. This is supported by the observations that DNA is depolymerized by EA and other enediol compounds, and that the DNA breaking ability of TR or DAsA is diminished when 2-OH of TR has been esterified with phosphoric acid (30) or DAsA has been combined with o-phenylenediamine (5). RNA is also decomposed by AsA, but not by AsA-3-P. In addition, 28 S of RNA is preferentially broken by AsA similar to TR or some amino reductones (14), although 28 S, 18 S and 4 S of RNA are equally decomposed by hydroxylamine, nitrous acid (31), adrenaline, MNNG or mitomycin C (32) in the cooperation of Cu²⁺. Yamafuji et al. (31,34) demonstrated that cytodifferentiation and cytanoenalization such as carcinogenesis, carcinostasis, virogenesis, mutagenesis or hormonal action might be initiated through a suitable scission of chromosomal DNA. Substances inducing these phenomena were actually confirmed to break DNA and are summarized as “nucleic acids breaking reagens” (32). These include the following groups so far examined; carcinogens: 3-hydroxyanthranilic acid, 3-hydroxykynurenine (12,13) and MNNG (32); antitumoric substances: mitomycin C (32), catecholamines (8–11), xylans (35) and some kinds of proteins (36,37); virogens and mutagens: hydroxylamine, nitrous acid, hyponitrite and oximes (31,33); hormones: cortisone, ecdysone (38), adrenaline and noradrenaline (8–11). Thus, AsA derivatives having the reductonic structure (AsA, EA, DAsA, DEA, DKG, MDT etc.) could be included in the group of “nucleic acids breaking reagents.” Although DNA breaking abilities of these substances had been investigated in vitro, the breakage of DNA in vivo by aromatic reductones was recently confirmed (10,11). Therefore, for the simple preliminary survey of carcinogenic, carcinostatic, mutagenic or virogenic substances, it may be useful to examine the nucleic acids breaking ability by the sucrose density gradient centrifugation or by the lowering of the viscosity. Of course, it is difficult to distinguish
the above phenomena only by breakage of nucleic acids. However, some clues may be obtained from the mode of degradation of Apy and Apu or 28 S, 18 S and 4 S of RNA, in addition to breakage of DNA, by "nucleic acids breaking reagents." MURATA (21) demonstrated that inactivation of bacteriophage by AsA was caused by free radicals formed during the autooxidation of AsA. This may be possible in the breakage of DNA. Considering the Cu²⁺-promoted breakage of DNA by "nucleic acids breaking reagents" which do not form free radicals, specificity of degradation of oligonucleotides by DASA (5) or other substances (32), and mode of degradation of Apy, Apu and RNA by AsA cited above, however, another possibility proposed by MURAKAMI and YAMAFUJI (9) through the formation of co-ordinating linkage of the reagents with bases of polynucleotide and Cu²⁺ should be kept in mind, since it is doubtful that free radicals decompose specifically polynucleotides.

Variation of replication of nucleic acids may be an important joining stage between the proper breakage of DNA and cytodifferentiation or cytoanomalization in Yamafuji's hypothesis. It was already observed that appropriately (probably single-strand) scissioned DNA promoted the activity of DNA-dependent RNA polymerase, indicating that this was a better template of the enzyme, while the excessive (probably double-strand) breakage of DNA by severer treatment retarded the activity (12,13,32). Quite similar effects of the breakage of DNA by AsA on the template activity was demonstrated in DNA replication by DNA polymerase too.

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