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Effect of Some Reductones on Protein Synthesis *in vitro*[1]

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Reductones such as ascorbic acid and 5-methyl-3,4-dihydroxytetrone (enediol), scorbamic acid (enaminol) and mercapto-hydroxycoumarine (thiolenol) degraded polysomes as well as ribosomal RNA in ribosome particles in ***vitro***. They caused significant changes in amino acid incorporation by polysome and pH 5 enzyme system or liver slices with endogenous RNA or poly U as the messenger. The results suggested the possibility that reductones affect protein synthesis through the breakage of mRNA and rRNA and give some clues elucidating processes between DNA breakage and cytodifferentiation or cytoanomalization demonstrated as Yamafuji's effect.

INTRODUCTION

In the preceding study conducted on the basis of the ability of several reductones to break nucleic acids, it was confirmed that they brought about variation of the priming activity for DNA-primed DNA and RNA polymerases (Tomita et al., 1979). Since it was supposed that protein synthesis may be also influenced through nucleic acids synthesis, effect of reductones on the protein synthesizing system was then investigated in *vitro,* in relation to mutation of bacteria (Omura ***et al.,*** 1978) or repression of growth of tumor cells by them (Yamafuji and Murakami, 1968; Yamafuji ***et*** al, 1970, 1971; Omura et al., 1974, 1975).

MATERIALS AND METHODS

1. Chemicals

Reductones employed include ascorbic acid (AsA), 5-methyl-3,4-dihydroxytetrone (MDT), ascorbate-3-phosphate (AsA-3-P), scorbamic acid (ScA) and mercapto-hydroxycoumarine (HMC). They were same as those employed in the preceding study. ATP. GTP, poly U, phosphoenolpyruvate and pyruvate kina-

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se, type II were purchased from the Sigma Chemicals Co. L-(U-^{14}C)-leucine (324 mCi/mmole), L-(U-^{14}C)-phenylalanine (522 mCi/mmole) and (U-^{14}C)-protein hydrolysate (57 mCi/matom carbon) were obtained from the Radiochemical Center, Amersham.

2. **Ribosomes**

Preparation of ribosome fraction: Preparation of ribosomes was carried out principally according to Muramatsu (1972) with some modifications.

A male ddN mouse (4 to 5 weeks old) was killed by decapitation after starving on water for 24 hrs. The liver was immediately excised and homogenized in 3 ml of 0.25 M sucrose-TKM buffer (50 mM Tris buffer, pH 7.6-25 mM KCl—5 mM $MgCl_2$) per 1 g of liver with 10 strokes of a teflon homogenizer of Potter-Elvehjem type. The homogenate was centrifuged at 13,000 rpm for 10 min to remove mitochondria or other cell debris. The supernatant, post-mitochondria fraction, was further centrifuged at 40, 000 rpm for 60 min to obtain microsome pellet. To the suspension of the pellet in TKM buffer was added sodium deoxycholate to give a final concentration of 1.3 %. The solution was carefully placed on the layer of 0.5 M sucrose-TKM buffer and centrifuged at 40,000 rpm for 2.5 hrs to wash the pellet. The precipitate was suspended in a small volume of TKM buffer and the insoluble part was removed by centrifugation at 4,000 rpm for 5 min. The supernatant was employed as a ribosome fraction for study. The absorption maximum and the minimum of the preparation was 258 nm and 236 nm respectively, and the ratio of the optical density at 280 nm to that at 260 nm (E_{280}/E_{260}) was 0.57.

Treatment of ribosomes with reductones: The sample of the ribosomes (2. Omg /ml) was incubated with 5 mM reductones with or without 50 μM Cu^{2+} in 20 mM Tris buffer, pH 7. 6-10 mM KCl-2 mM $MgCl_2$ at 37°C for 60 min and the ribosomes were recovered by centrifugation at 40, 000 rpm for 2 hrs.

Estimation of centrifugal pattern of ribosome RNA: The ribosomes treated with reductones were suspended in 0.3 M SDS-50 mM acetate buffer, pH 5.7-O. 14M NaCl. The suspension was then mixed with the same volume of 0.1 % 8-hydroxyquinolinc-70 % phenol-10 % *m*-cresol—20 % water. The mixture was vigorously shaken at room temperature for 15 min and centrifuged at 10,000 rpm for 5min to separate aqueous layer from phenolic one. The treatment with phenol was samely repeated. To the aqueous layer were added 2 volumes of 2 % potassium acetate-90 % ethanol-10 % m-cresol. The mixture was stirred and stored at -16°C overnight to precipitate RNA. The RNA was collected by centrifugation at 10, 000 rpm for 10 min, washed with 95 % ethanol 3 times and dissolved in 50mM acetate buffer, pH 5.7.

A 0.2 ml—aliquot of the RNA solution (550 μg/ml) were put on the top of the linear gradient of sucrose from 5 % to 20 % in 0.1 M NaCl—10 mM acetate buffer, pH 5.1 and centrifuged at 40,000 rpm for 2.5 hrs with a Beckman centrifuge, horizontal type 50-l rotor. After centrifugal separation, the sample was continuously introduced into a microflow cell by injecting 50 % sucrose carefully from the bottom of the tube with a peristal pump for microtube (Tokyo Rikaki Co.). Then, the absorbance was recorded automatically by estimating at 260 nm with a spectrophotometer, Hitachi Model 200-10.

3. Polysomes

Preparation of polysomes: Polysomes were prepared by the method of Bloenmendal ***et al.*** (1974) from rat liver with a slight modification as follows.

A WKA male rat (about 150 g body weight), which had been fasted on water for 24 hrs, was killed by decaptation. The liver was excised soon and washed with 0.25 M sucrose-TKM buffer. The liver was homogenized with 2.5 ml per g of the above buffer solution by stroking 8 times of a Potter-Elvehjem teflon homogenizer. The homogenate was centrifuged at 17,300 rpm for 10 min and 1/3 of the upper layer of the supernatant was carefully collected by aboiding the contamination of the topmost fatty layer and precipitated fraction of nuclei and mitochondria. To the postmitochondria fraction was added sodium deoxycholate to give the final concentration of 1.3 %. The solution was stirred for 30min and applied to the top of the double layer of 0.5 M and 2M sucrose containing TKM buffer set in a nitrate tube for a Beckman ultracentrifuge, L type 30 rotor. The tube was centrifuged at 28,000 rpm for 5 hrs and the clear pellet was obtained. The pellet was then washed with a TKM buffer and suspended in a small volume of the buffer. The suspension was centrifuged at 4, 000 rpm for 5min to remove undissolved matters. The supernatant was employed as polysome fraction for the study. This fraction showed an absorption maximum at 259 nm and a minimum at 236 nm. The ratio $E_{280}/_{260}$ was 0.53. Amount of polysomes was expressed as the absorbance at 260 nm (e. g. E_{260}/ml).

*Treatment **of** polysomes with reductones and estimation of centrifugal pattern* : Polysomes (20 E_{260}/ml) were incubated with 2 mM reductones in 40 mM Tris buffer, pH 7.6—20 mM KCl-4mM $MgCl_2$ at 0°C for 30min. They were applied to the sucrose linear gradient from 15 % to 30 % containing TKM buffer and centrifuged at 35,000 rpm for 60min with a Beckman ultracentrifuge, horizontal type 50-1 rotor. After centrifugal separation, the absorbancy of the polysomes was estimated automatically with a microflow cell connected with a peristal pump as above.

4. **Protein synthesizing system**

Preparation of pH 5 fraction: A pH 5 fraction was prepared from the postmitochondria fraction according to Takanami (1968) as follows.

The postmitochondria fraction prepared as above was centrifuged at 105, 000×g for 2 hrs to remove microsomes. To upper 2/3 of the clear supernatant was added 1 N acetic acid under stirring to adjust its pH to 5.0. The mixture was centrifuged after standing for 5min. The precipitate was washed with ice-cold deionized water and suspended in a small volume of water. The suspension was dissolved by increasing the pH value to 8.0 by dropwise addition of 1 N KOH. Tris buffer, pH 7. 6, was additionally added to give a final concentration of 50mM. The concentration of protein in the solution was adjusted to about 1 % and used as "pH 5 fraction".

Assay of protein synthesis: Protein synthesis was assayed by estimating the radioactivity of '"C-amino acid incorporated into acid-insoluble fraction. Reaction mixture was shown in Table 1. The system was incubated at 37°C for 20

Table 1. Components of the protein synthsizing system.

polysomes	1 E_{260} unit
pH 5 fraction	1 mg (protein)
a) ^{14}C-Leu*	10 nmoles (0.32 μCi)
b) poly U	25 μg
b) ^{14}C-Phe**	10 nmoles (0.52 μCi)
ATP	0.5 pmoles
GTP	62.5 nmoles
phosphoenolpyruvate	2.5 μmoles
pyruvate kinase	25 mg
$MgCl_2$	2.5 μmoles
KC1	12.5 pmoles
Tris-HCl (pH 7.6)	12.5 μmoles
Total	250 μl.

* L-(U-^{14}C)-Leu employed: 324 mCi/mmole, The Radiochemical Center, Amersham.
** L-(U-^{14}C)-Phe employed: 522 mCi/mmole, The Radiochemical Center, Amersham.
a) for incorporation of leucine depending on endogenous mRNA.
b) for incorporation of phenylalanine depending on poly U.

min and cooled with ice to terminate the reaction. Subsequently, it was mixed with 2ml 0.5N perchloric acid and left for 30min. Acid-insoluble fraction was collected on glass filter paper (Whatman GF/C), washed twice with 3 ml portion of cold 0.5 N perchloric acid and dried under an infrared lamp. In the case of incorporation of ^{14}C-Phe depending on poly U, the synthesizing system excluded them was preliminary incubated at 37°C for 20min to remove endogenous mRNA from polysomes. After that, poly U and ^{14}C-Phe were added to start the reaction. To stop the reaction, 0.5 ml of phenylalanine (2 mg/ml) were first added and then 3ml cold 0.5 N perchloric acid. Radioactivity was estimated with a Beckman LS 250 liquid scintillation counter, using 0.4% PPO-67% Triton X-100-33% toluene as scintillant. The assay of protein synthesis was carried out duplicatedly and the average value was indicated.

Protein synthesis with liver slice: The test was carried out by the procedure of Gonzalez-Cadavid and Quijáda (1974).

A WKA male rat weighing about 150g was killed by decaptation. The liver was rapidly excised, freed from extraneous tissues, washed with 0.9 % NaCl (20" to 22°C) and cut into rectangular pieces (0.4 to 0.5 g). They were transferred into 4ml culture medium A (a mixture containing 95mM NaCl, 10 mM $CaCl_2$, 10 mM KC1 and 40 mM $NaHCO_3$ was preliminary passed with 5 % CO_2 -95 % air and was then supplemented with 20 mM glucose just before using).

The liver slices in the culture medium A were kept at 37°C in an incubator containing 5 % CO_2—95 % air. The slices were contacted with 0.5 mM reductones for 30 min, followed with 25 μCi "C-amino acid mixture ([U-^{14}C] prntein hydrolysate) for 60 min. Afer the reaction, the slices were put into ice-cold water and freed from the culture medium by centrifugation at 1,000 rpm for 5 min. The slices were homogenized in 0.3M sucrose with a teflon homogenizer and centrifuged at 6, 000 rpm for 10 min to remove cell debris. The cell extract was further centrifuded at 13,000 rpm for 10 min. The supernatant, postmitochondria fraction, was centrifuged at 28, 000 rpm for 150min

to precipitate the microsome fraction. The pellet was dissolved in 0.25 M SDS-50 mM NaCl--5 mM Tris buffer, pH 7.4-O. 5 mM EDTA. An 0. 5 ml-aliquot of the solution was mixed with 10 ml 0.4 % PPO-67 % Triton X-100-33 % toluene and the radioactivity was estimated with a Beckman LS 250 liquid scintillator. Protein content of the solution was also samely determined by Lowry's me- thod (Lowry *et al.*, 1951).

RESULTS

1. Breakage of RNA by treating ribosomes with reductones

Degradation of RNA as well as DNA with reductones was confimed mostly in *vitro*, especially using the purely extracted nucleic acids. Their breaking ability must be further established, although degradation of chromosomal DNA was observed in cultured mammalian cells, RFL and HeLa, even with aroma-

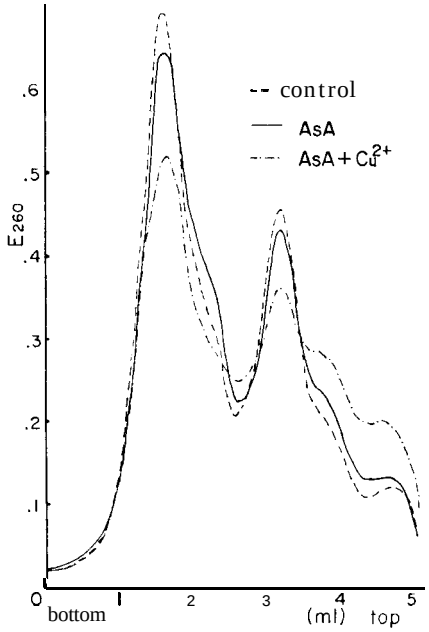

Fig. 1. Sedimentation pattern of rRNA from ribosomes treated with AsA. Ribosomes (2 mg/ml) were treated with 5 mM AsA with or without 50 μM Cu^{2+} at 37°C for 1 hr. After extracting rRNA from the ribosome, the RNA was subjected to the sucrose density gradient centrifugal analysis (5 %–20 %).

tic reductones such as epinephrine, norepinephrine and their derivatives (Murakami *et al.*, 1975, 1978a, b). On the other hand, it was demonstrated that crude DNA preparation containing much protein was more resistant than the purified DNA against the action of reductones, using some amino reductones,

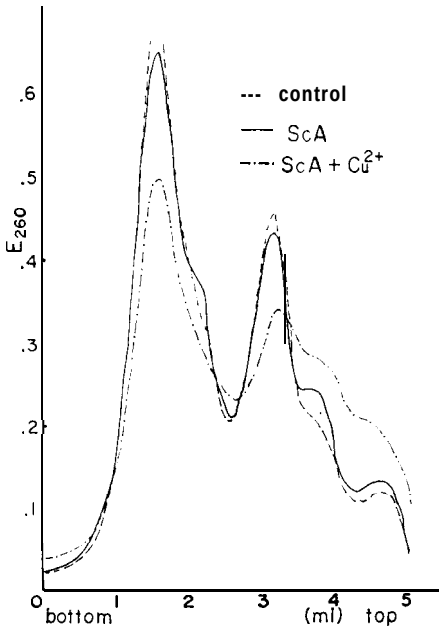

Fig. 2. Sedimentation pattern of rRNA from ribosomes treated with ScA. The same as in Fig. 1.

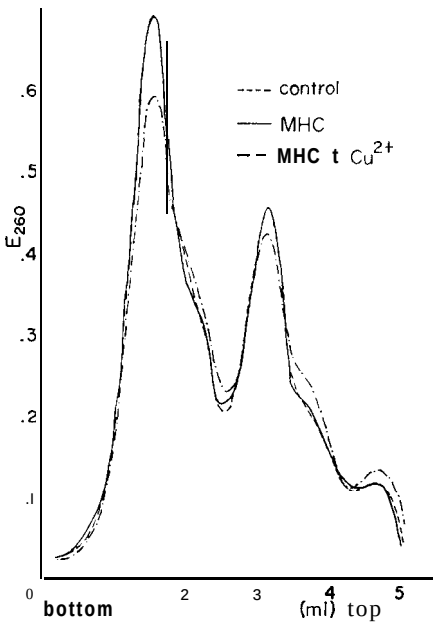

Fig. 3. Sedimentation pattern of rRNA from ribosomes treated with MHC. The same as in Fig. 1.

3-hydroxykynurenine and 3-hydroxyanthranilic acid which are carcinogenic metabolites of tryptophan (Fujii ***et al.,*** 1972). In ribosomes, RNA seems to be coated and/or mingled with ribosomal protein. Therefore, the susceptibility of ribosomal RNA to reductones should be examined in order to estimate some variation of ribosomal functions after treatment with them, if occur. Ribosomes were treated with 5 mM AsA, ScA or MHC with or without 50 μM Cu^{2+} at 37°C for 60min. Ribosomal RNA was extracted from them and examined by the sucrose density gradient centrifugation. The patterns are shown in Figs. l-3. It was thus confirmed that all reductones examined, i.e. enediol-, enaminol- and thiolenol-reductones, are able to break rRNA even in the ribosome particles, similar to their action on the pure RNA preparation ***in vitro.*** In the case of the reductone alone, both the main fractions of rRNA, 28 S and 18 S, were degraded a little by AsA and ScA, but not by MHC. However, Cu^{2+} accelerated the action and higher degradations were observed not only by AsA and ScA, but also by MHC.

2. **Action of reductones on polysomes**

Polysomes take an important part in protein synthesis, in which RNA occupies the central part, especially translation of information in DNA.

Polysomes are separable according to the degree of polymerization by means of the sucrose density gradient centrifugation. Isolated polysomes were treated with 2 mM reductone at 0°C for 30 min and examined their variation of the sedimentation pattern. Since Cu^{2+} disturbed the pattern, the reductones were treated only in the absence of Cu^{2+}. The results are shown in Figs. 4-8.

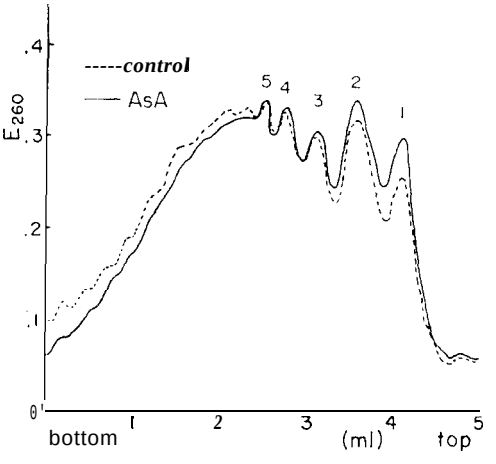

Fig. 4. Degradation of polysomes by AsA. Polysomes (20 E_{260} units/ml) were treated with 2 mM AsA at 0°C for 30 min and applied to the sucrose density gradient centrifugal analysis (15 %–30 %).

AsA, MDT and ScA caused some decrease of fractions of higher degree of polymerization more than pentamer and increase of monomer and dimer.

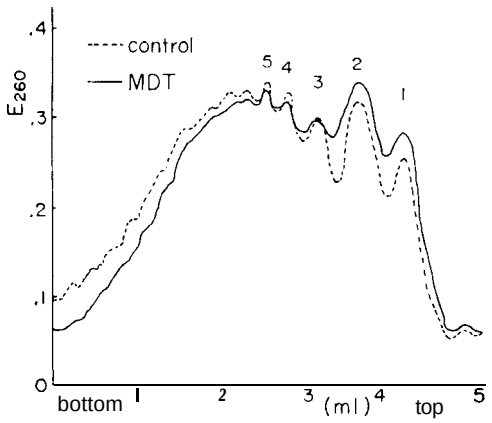

Fig. 5. Degradation of polysomes by MDT. The same as in Fig. 4.

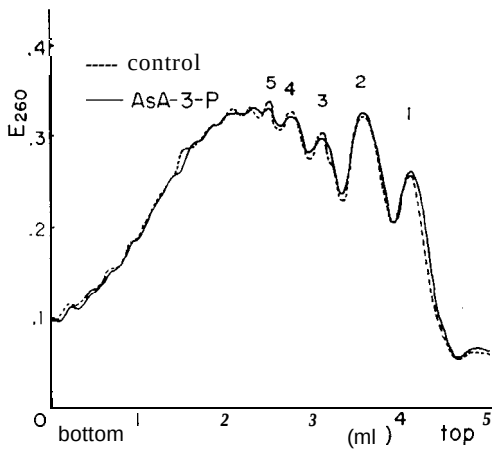

Fig. 6. Sedimentation pattern of polysomes treated with AsA-3-P. The same as in Fig. 4.

The similar variation was observed with MHC, while not so remarkable. However, AsA-3-P had no effect on polysomes, suggesting the importance of the reductonic group in the molecule as case of the breakage of nucleic acids.

3. **Effect of reductones on the protein synthesis**

In order to estimate the effect of reductones on the protein synthesis, the incorporation of ^{14}C-amino acid was first assayed using the system of the polysomes and the pH 5 fraction in the presence of 0.1 mM recdutone at 37°C for 20 min. The assay was carried out duplicatedly and the average value was indicated. In the case of ^{14}C-Leu, the incorporation was estimated depending on the endogenous mRNA in the system. The result is shown in Table 2. The incorporation was retarded to 77% with AsA, 62% with **MDT,**

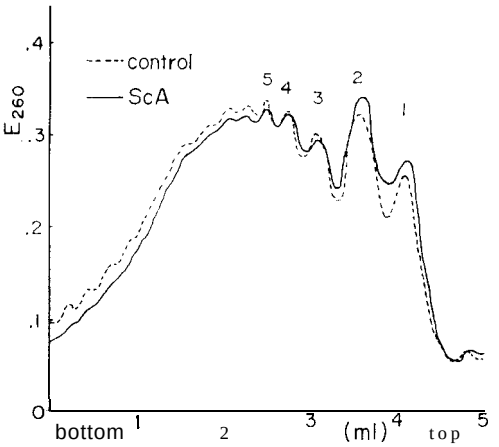

Fig. *7.* Degradation of polysomes by ScA. The same as in Fig. 4.

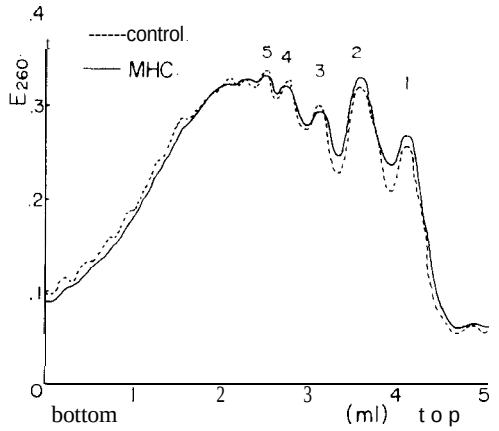

Fig. 8. Degradation of polysomes by MHC. The same as in Fig. 4

'Table 2. Effect of reductones on the protein synthesis depending on endogenous mRNA.

Reductones	Radioactivity incorporated (cpm/mg RNA)	(%)
Control	2.19 × 10^4	100
AsA	1.69 × 10^4	77
MDT	1.36 × 10^4	62
ScA	1.06 x 10^4	49
MHC	1.10 x 10^4	50

49% with ScA and *50%* with MHC. As shown in Table 3, similar effect of reductones was observed regarding the incorporation of ^{14}C-Phe depending on poly U as the messenger in place of the endogenous mRNA. In this case too, the decrease of the incorporation was brought about. By the action of AsA,

Table 3. Effect of reductones on the protein syhthesis depending on poly U.

Reductones	Radioactivity incorporated (cpm/mg RNA)	(%)
Control	3.69 x 10'	100
AsA	3.23 × 10^4	87
MDT	3.36 × 10^4	91
ScA	1.76 x 10"	48
MHC	1.75 x 10^4	47

the rate was repressed to 87 %, to 91% by MDT, to 48% by ScA and to 47 % by MHC. Finally, the protein synthesis was estimated using liver slices with 0.5 mM reductone at 37°C for 20 min. The assays were conducted independently 3 times and the average values of the incorporation of 'Y-protein hydrolysate are shown in Table 4. A weak repression of about 15 to 20 % was observed with AsA, MDT or ScA, but not with AsA–3–P or MHC. Thus, the retardation of the protein synthesis in the polysomes and the pH 5 fraction was induced by the enaminol and thiolenol, although that was not so much by the enediol. On the other hand, compared with this system, the effect of the enediol and enaminol on the liver slices were relatively weak and that of MHC and AsA–3–P was not significant.

Table 4. Effect of reductones on the protein synthesis in liver slices.

Reductones	Radioactivity incorporated (cpm/mg protein)	(%)
Control	2.38 x 10^2	100
		81
AsA–3–P	2.35 × 10^2	99
MDT	2.02 × 10^2	85
ScA	1.94 × 10^2	82
MHC	2.57 × 10^2	108

DISCUSSION

In the case of the purified RNA preparation, the ribosomal RNA was also breaked in the particle by the action of reductones, although to less extent. On the other hand, even by the mild treatment of them at 0°C, the sedimentation profile of the polysome was altered with inducing increase of the lower fractions and decrease of the higher ones. A similar phenomenon was observed when the polysome had been prepared with vigorous procedure or treated with ribonuclease (Wettstein et *al.*, 1963). The variation might be attributed to the scission of mRNA connecting the ribosomes by the reductones. However, as often observed, AsA–3–P had not the ability and the breaking degree of the enediol (AsA, MDT) or the enaminol (ScA) was higher than that of the thiolenol (MHC). These observations indicate that the reductone structure is indispensable for the breakage of the nucleic acids and that the activity is dependent on the kind of them.

The information in DNA is transferred to protein through transcription

and translation, in which mRNA takes an important part. Although role of rRNA is not clarified, some one such as a mere framework mingled or covered with protein might be presumed. Therefore, the damage of mRNA as well as rRNA, if occurred in the polysomes and ribosomes, may cause not only the structural but also the functional variation of the polysomes. In addition, it was shown that the priming activity for the RNA polymerase was brought about by degradation of the template DNA with reductones. This might change the amounts of RNA formed too. These variations might affect on protein synthesis. Actually, the incorporation of amino acids into protein in the protein synthesizing system of the polysome and pH 5 enzyme with endogenous RNA or poly U was inhibited by the reductones. Contrary to the breaking ability on nucleic acids, however, the inhibiting effect was remarkable with the enaminol and the thiolenol. Furthermore, the incorporation of amino acids into rat liver slices was also slightly repressed by the enediol and the enaminol. The discrepancy between the liver slices and the polysome system is complicated, but may be interpreted as being due to some barriers of the metabolisms or permeability in liver slices, for example. At any rate, the results obtained might indicate that all reductones examined affect on the protein synthesis, probably through the degradation of nucleic acids.

On the basis of the many systematical researches in our Institute, Yamafuji *et al.* (1970a, 1971) presented a hypothesis, the Yamafuji's effect, which demonstrates that a suitable scission of chromosomal DNA should be an initiating step in several fundamental biological functions such as cytodifferentiation and cytoanomalization. These phenomena include antibody formation, brain function, hormonal action, mutagenesis, carcinogenesis, carcinostasis and virogenesis. Some of them was confirmed with reductones. However, steps between DNA breakage and these biological phenomena had been remained to be elucidated. Thus, studies carried out along the central dogma provided some clues. Variation of the priming activity was provoked by the action of reductones on the template DNA for DNA and RNA polymerases, which was attributed to the degradation of DNA. This variation might induce the qualitative and quantitative changes of nucleic acids formed. In addition, since RNA in ribosome and polysome was breaked by the action of reductones, protein synthesis was also influenced. Thus, the results may give an additional support to the Yamafuji's effect.

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