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Inhibition and Stimulation of the Biosynthesis of Protein and Nucleic Acid. III.[1] Interaction of Aminoquinone Derivatives with Nucleic Acid, and Their Effects on the Biosynthesis of Nucleic Acid and Protein in Ehrlich Mouse Ascites Tumor Cells *in Vitro*

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On several aminoquinone derivatives the interaction with nucleic acid and the effects on DNA, RNA, and protein synthesis in Ehrlich mouse ascites tumor cells were investigated in relation to the findings that aminoquinone structures in such antibiotics as actinomycins and mitomycins played an important role on their biological effects as a functional group. The results are as follows:

1. Among the aminoquinone derivatives used for this investigation the compounds of aminoquinone imine or aminophenoxazone type showed to interact with nucleic acid by the method of difference spectrum. Besides, the former interacted with both of DNA (calf thymus DNA) and RNA (yeast tRNA).

2. The type of spectral shifts of aminoquinone imines was found to be different from that of aminophenoxazones, and the interaction of these compounds with nucleic acid was become weak by the addition of Mg^{2+}.

3. All the compounds which interacted with nucleic acid inhibited the synthesis of nucleic acid and protein in Ehrlich mouse ascites tumor cells *in vitro*, and the order of inhibition in each case was as follows; DNA>protein>RNA.

4. Among these compounds 2-amino-1,4-naphthoquinone imine–HCl (ANQI) was remarkable for its intensive inhibition on DNA biosynthesis, that is, the incorporation of thymidine-2-^{14}C into DNA was inhibited almost perfectly at the concentrations of ANQI higher than 5×10^{-6} M, and was depressed as much as 33% even at 5×10^{-8} M.

5. These compounds did not stimulate the degradation of DNA, while that of RNA was somewhat stimulated by ANQI.

Some groups of antibiotics have been shown as inhibitors of nucleic acid biosynthesis, resulting an antineoplastic activity to some extent.[3] Among these antibiotics, it has been reported that actinomycins,[4] mitomycins and porfiromycin,[5] streptonigrin,[6] phleomycin,[7] daunomycin,[8,9] cinebrubin,[8] chromomycins,[8,10] streptothricin,[11] nogalamycin,[12] plura-

1) Part II: O. Tamemasa, S. Okada, S. Iwasaki, and E. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **16**, 672 (1968).
2) Location; *Oshika, Shizuoka.*
3) B.A. Newton, *Ann. Rev. Microbiol.*, **19**, 209 (1965); N, Tanaka, *Kagaku To Seibutsu*, **4**, 170 (1966); *etc.*
4) E. Reich and I.H. Goldberg, "Progress in Nucleic Acid Research and Molecular Biology," Vol. 3, ed. by J.N. Davidson and W.E. Cohn, Academic Press, New York, N.Y. and London, 1964, pp. 184—234; *etc.*
5) V.N. Iyer and W. Szybalski, *Science*, **145**, 55 (1964); M.N. Lipsett and A. Weissbach, *Biochemistry*, **4**, 206 (1965).
6) N.S. Mizuno, *Biochim. Biophys. Acta*, **108**, 394 (1965).
7) A. Falaschi and A. Kornberg, *Federation Proc.*, **23**, 940 (1964).
8) W. Kersten and H. Kersten, *Biochem. Z.*, **341**, 174 (1965).
9) E. Calendi, A. DiMarco, M. Reggiani, B. Scarpinato, and L. Valentini, *Biochim. Biophys. Acta*, **103**, 25 (1965).
10) W. Kersten, H. Kersten, and W. Szybalski, *Biochemistry*, **5**, 236 (1966).
11) Ch. Zimmer, H. Triebel, and H. Thrum, *Biochim. Biophys. Acta*, **145**, 742 (1967).
12) B.K. Bhuyan and C.G. Smith, *Proc. Natl. Acad. Sci. U.S.*, **54**, 566 (1965).

mycin,[13] and anthramycin[14] interact with DNA.[15] Actinomycins, mitomycins, porfiromycin, and streptonigrin, among these DNA-interacting antibiotics, possess a common functional group, that is, aminoquinone moiety, in their molecules. As for actinomycins Reich, *et al.* have shown from experimental results of difference spectra and X-ray diffraction that the aminoquinone moiety interacts with guanine residues of DNA.[4] Rao, *et al.*[16] proposed, in connection with such an observation, the biological significance of aminoquinone structure as a functional group for carcinostatic activity. The investigations of simple aminoquinone compounds, however, on the interaction with nucleic acid and on the effects upon nucleic acid biosynthesis have not been performed beyond the studies on actinomycin chromophore (2-amino-4,6-dimethyl-3-oxo-3H-phenoxazine-1,9-dicarboxylic acid).[4,17]

The present investigation was undertaken to see what kinds of simple aminoquinone derivatives could interact with nucleic acid and influence the biosynthesis of nucleic acid or protein. It was found as a result that the compounds being able to bind to nucleic acid, especially to DNA, belonged to the type of aminoquinone imine or aminophenoxazone. It was further observed that these compounds suppressed the synthesis of nucleic acid and protein in Ehrlich mouse ascites tumor cells *in vitro*. Among these compounds 2-amino-1,4-naphthoquinone imine-HCl was noted regarding its intensive inhibition on the incorporation of thymidine into DNA of the tumor cells.

Experimental

Materials——2,5-Diamino-1,4-benzoquinone, 2-amino-1,4-naphthoquinone, 2-amino-1,4-benzoquinone imine-diHCl, and 2-amino-1,4-naphthoquinone 3-imine-HCl were synthesized in the author's laboratory according to the procedure of Fieser.[18] 2-Amino-3H-phenoxazin-3-one was also synthesized by oxidation of 2-aminophenol. Actinomycin S_3 was kindly furnished by Professor J. Kawamata of Research Institute for Microbial Deseases, Osaka University.

Calf thymus DNA (highly polymerized) and yeast tRNA[15] were obtained from Sigma Chemical Co.

Thymidine-2-^{14}C (TdR-2-^{14}C) and L-phenylalanine-U-^{14}C (L-Phe-U-^{14}C) were purchased from Daiichi Pure Chemicals Co., Ltd., and uridine-2-^{14}C (UR-2-^{14}C) was from The Radiochemical Centre. These radioactive compounds were diluted with their stable isomers to 0.5—1 mCi/mmole of a specific activity.

Ehrlich Mouse Ascites Tumor Cells——The intact tumor cells were used, which were obtained as described in detail in the previous paper.[19]

Absorption Spectra and Difference Spectra——These were measured by a Hitachi Recording Spectrophotometer, Model EPS-2U.

Incubation and Fractionation Procedures——Incorporation of a precursor into nucleic acid or protein was performed by incubation of the tumor cells, suspended in Ca^{2+}-free modified Krebs-Ringer phosphate buffer (pH 7.2),[20] with a radioactive precursor in the presence or absence of an aminoquinone derivative at 37° for 1 hr. The fractionation of DNA and RNA was carried out according to the method of Schmidt, Thannhauser, and Schneider,[21] and of protein was performed as described earlier.[19]

Procedure of Degradation of Nucleic Acid——The degradation rate of nucleic acid was measured as follows: The tumor cells after being incubated with TdR-2-^{14}C or UR-2-^{14}C at 37° for 1 hr were washed and re-incubated with an aminoquinone derivative at 37° for 1 hr, then the released radioactivities in the cold 5% trichloroacetic acid-soluble fraction (acid sol. fr.) were counted.

Assay for Radioactivity and Incorporation Rate of Precursor——DNA, RNA, and acid sol. fractions were washed with ether for removing trichloroacetic acid, plated on alminum disks, dried and counted for radio-

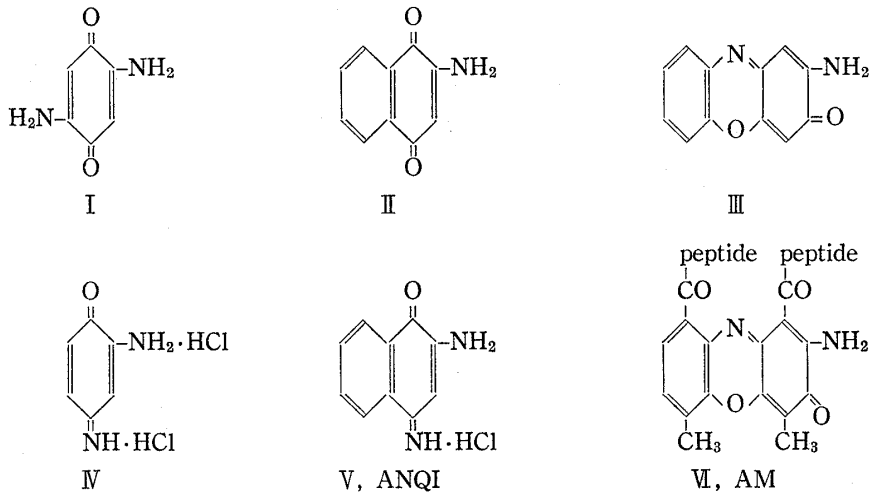
13) N. Tanaka, "Cancer Chemotherapy," (Japanese Cancer Association, GANN Monograph 2), ed. by A. Goldin, K. Kaziwara, R. Kinosita, and Y. Yamamura, Maruzen Co., Ltd., Tokyo, 1967, pp. 59—69.
14) K.W. Kohn, V.H. Bono, Jr., and H.E. Kann, Jr., *Biochim. Biophys. Acta*, **155**, 121 (1968).
15) Abbreviations; DNA (deoxyribonucleic acid), RNA (ribonucleic acid), tRNA (transfer ribonucleic acid).
16) K.V. Rao, K. Biemann, and R.B. Woodward, *J. Am. Chem. Soc.*, **85**, 2532 (1963).
17) W. Müller, *Naturwissenschaften*, **49**, 156 (1962).
18) L.F. Fieser, "Experiments in Organic Chemistry," 3rd ed., D.C. Heath & Co., Boston, 1957, (reprinted by Maruzen Co., Ltd., Tokyo, 1958), pp. 234—238.
19) O. Tamemasa, S. Okada, and Y. Wakita, *Chem. Pharm. Bull.* (Tokyo), **13**, 1193 (1965).
20) M. Rabinovitz, M.E. Olson, and D.M. Greenberg, *J. Biol. Chem.*, **210**, 837 (1954).
21) G. Schmidt and S.J. Thannhauser, *J. Biol. Chem.*, **161**, 83 (1945); W.C. Schneider, *ibid.*, **161**, 293 (1945); W.C. Schneider, *ibid.*, **164**, 747 (1946); S. Mizuno, *Kagaku To Seibutsu*, **3**, 148 (1965).

activities in a windowless 2π-gas-flow counter. The radioactivity in protein was assayed as described earlier,[19] and the correction for self-absorption was also made in a manner to the experiment described earlier.[19] The amount of incorporated precursors was calculated from the radioactivity found in each fraction, concerning with the specific radioactivity of precursor and the counting efficiency of 2π-gas-flow counter. On the other hand, the total amount of DNA or RNA in each fraction was assayed by measuring the absorbance at 260 mμ by a Hitachi Spectrophotometer, Model 101, and of protein was obtained by weighing. The incorporation rate was represented as mμ moles of incorporated precursor per mg of DNA, RNA, or protein, and the inhibition rate by an aminoquinone derivative was further calculated from the incorporation rates of precursor in the presence and absence of an aminoquinone.

Results

Interaction of Aminoquinone Derivatives with Calf Thymus DNA and Yeast tRNA

Aminoquinone derivatives, investigated for the present experiment, were 2,4-diamino-1,4-benzoquinone (I), 2-amino-1,4-naphthoquinone (II), 2-amino-3H-phenoxazin-3-one (III), 2-amino-1,4-benzoquinone imine-diHCl (IV), 2-amino-1,4-naphthoquinone imine-HCl (V, ANQI), and actinomycin S_3 (VI, AM).



As shown in Fig. 1, these compounds except I displayed major absorption peaks in the visible region at 420—490 mμ in a neutral buffer (0.01 M tris–0.01 M NaCl, pH 7.4). IV and V were easily soluble in the buffer, while I—III and VI were a little.

Fig. 2 and 3 present the difference spectra of aminoquinone derivatives and calf thymus DNA or yeast tRNA against aminoquinone derivatives in 0.01 M tris–0.01 M NaCl (pH 7.4). As shown in Fig. 2, the spectral changes were produced in III—VI solutions by calf thymus DNA. Each of the compounds III—VI has aminoquinone imine structure, while the compounds I and II, of which spectra were not shifted by the DNA, do not possess imine residue. The spectral shifts found in III—VI solutions were not of the same type, that is, the shifts found in the absorption of aminoquinone imines (IV and V) were of a type different from that of amino-

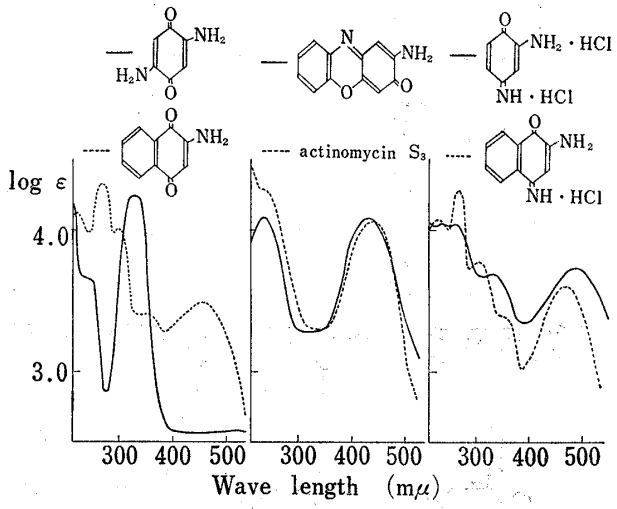


Fig. 1. Ultraviolet–Visible Spectra of Aminoquinone Derivatives in 0.01 M Tris–0.01 M NaCl (pH 7.4)

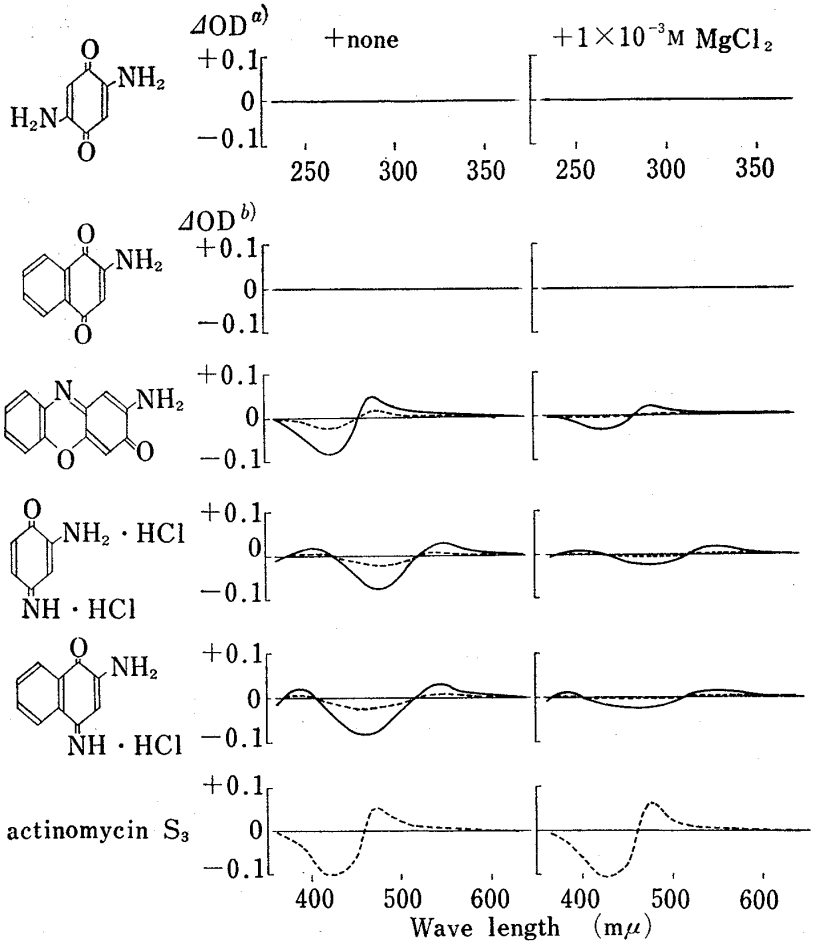


Fig. 2. Difference Spectra of Aminoquinone Derivatives and Calf Thymus DNA against Aminoquinone Derivatives in 0.01 M Tris–0.01 M NaCl (pH7.4)

concentrations of aminoquinones; —— 1×10^{-4} M, ------ 2×10^{-5} M
concentration of DNA-P; 1×10^{-3} M

a) $\Delta OD = \frac{OD_{aminoquinone+DNA}}{OD_{aminoquinone}+OD_{DNA}}$ *b*) $\Delta OD = \frac{OD_{aminoquinone+DNA}}{OD_{aminoquinone}}$

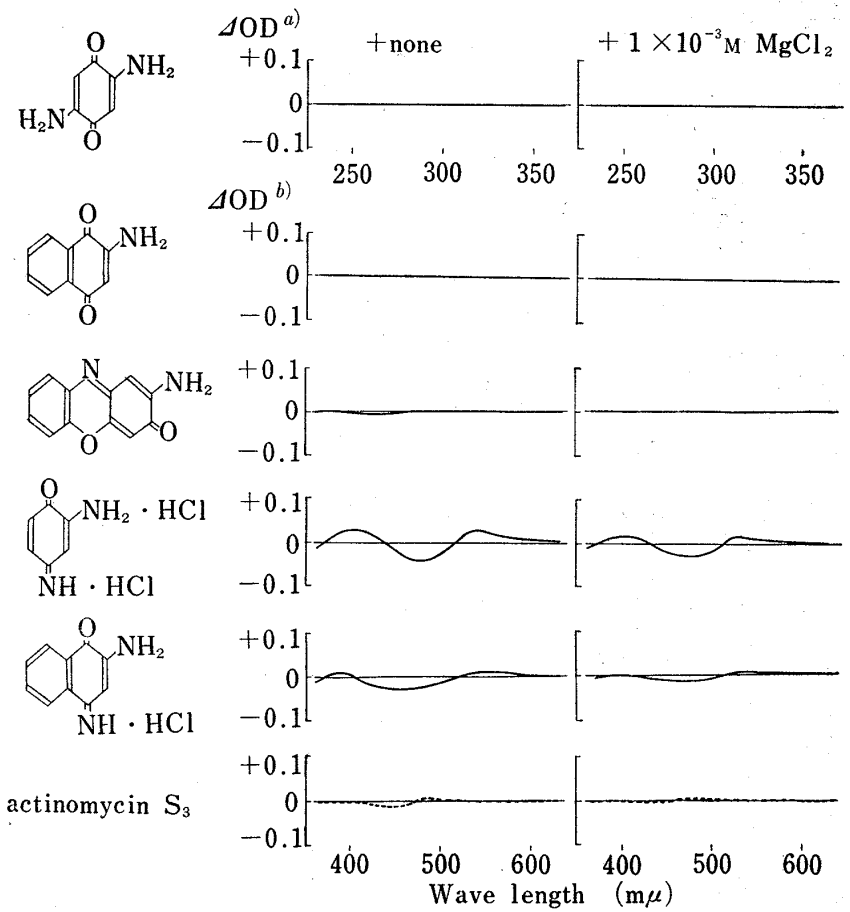


Fig. 3. Difference Spectra of Aminoquinone Derivatives and Yeast tRNA against Aminoquinone Derivatives in 0.01 M Tris–0.01 M NaCl (pH 7.4)

concentrations of aminoquinones; —— 1×10^{-4} M, ------ 2×10^{-5} M
concentration of RNA-P; 1×10^{-3} M

a) $\Delta OD = \frac{OD_{aminoquinone+DNA}}{OD_{aminoquinone}+OD_{DNA}}$ *b*) $\Delta OD = \frac{OD_{aminoquinone+DNA}}{OD_{aminoquinone}}$

phenoxazones (III and VI). In the presence of Mg^{2+} the degrees of spectral shifts were markedly decreased in the cases of III—V. This phenomenon suggests the participation of Mg^{2+} in the interaction of these compounds with DNA, being different from the case of AM.

On the other hand, as shown in Fig. 3, yeast tRNA also caused the spectral changes in IV and V solutions. The mode of interaction of IV and V with nucleic acid is considered, in this respect, to be different from that of AM. The ΔOD's of IV and V solutions were decreased in the presence of Mg^{2+} equally in the cases of DNA.

Inhibition of DNA and RNA Synthesis by Aminoquinone Derivatives in Ehrlich Mouse Ascites Tumor Cells *in Vitro*

When the tumor cells were incubated at 37° for 1 hr with TdR-2-^{14}C or UR-2-^{14}C (1×10^{-4}M, each) in the presence of an aminoquinone derivative (2×10^{-5}M), as shown in Fig. 4, the incorporation of TdR-2-^{14}C into DNA was almost perfectly inhibited by ANQI, and somewhat by III, IV, and AM, while the inhibition by I and II was slight.

On the other hand, the incorporation of UR-2-^{14}C into RNA, which was almost completely inhibited by AM, was also depressed by ANQI to a considerable extent, while the others depressed only a little. The extents of inhibition by III—V on the incorporation of TdR-2-^{14}C into DNA were greater than that of UR-2-^{14}C into RNA, in contrast to the case of AM.

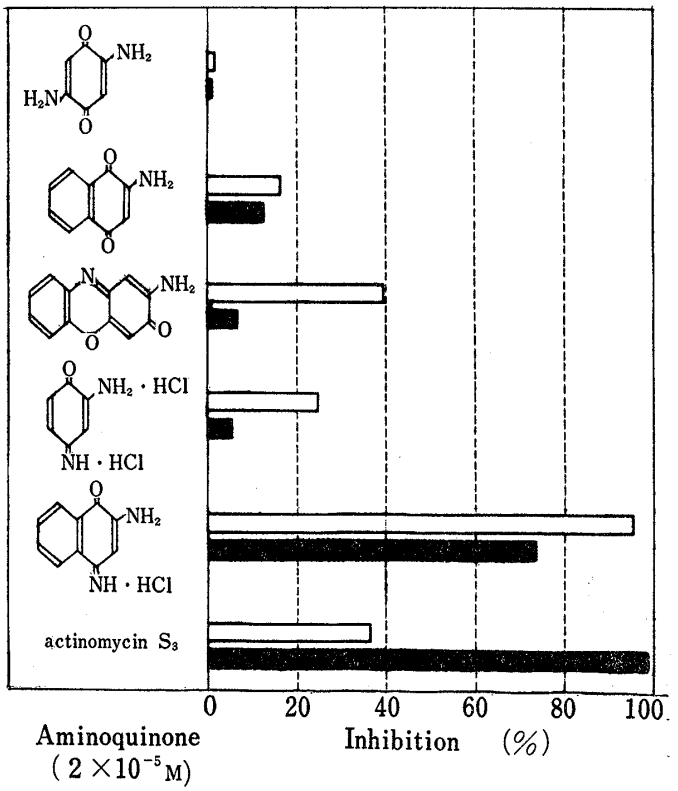


Fig. 4. Inhibition of Thymidine and Uridine Incorporation into DNA and RNA by Aminoquinone Derivatives in Ehrlich Ascites Tumor Cells *in Vitro*

□ inhibition of thymidine-2-^{14}C (1×10^{-4}M) incorporation
■ inhibition of uridine-2-^{14}C (1×10^{-4}M) incorporation

Effects of Aminoquinone Derivatives on DNA, RNA, and Protein Synthesis in Ehrlich Mouse Ascites Tumor Cells *in Vitro* with a Variation of Their Concentrations

Fig. 5 presents the inhibition on the incorporation of TdR-2-^{14}C, UR-2-^{14}C, and L-Phe-U-^{14}C into DNA, RNA, and protein, respectively, in the tumor cells in the presence of III, IV, or ANQI at the concentration range from 2×10^{-5}M to 2×10^{-6}M or 5×10^{-8}M. In this experiment the concentration of each precursor and the condition of incubation were the same as before.

As a result the incorporation of the precursors was inhibited in the order of TdR-2-^{14}C, L-Phe-U-^{14}C, and UR-2-^{14}C in all the cases of the presence of three aminoquinones mentioned above. Among these three compounds ANQI exerted a remarkable inhibition especially on TdR-2-^{14}C incorporation, which was inhibited almost perfectly by ANQI at concentrations higher than 5×10^{-6}M, and was depressed as much as 33% even at 5×10^{-8}M. The extent of inhibition on DNA biosynthesis by ANQI was comparable to that of mitomycins which had been known as one of the most intensive inhibitors on DNA biosynthesis and as an excellent antineoplastic agent.[22]

22) W. Szybalski and V.N. Iyer, *Federation Proc.*, 23, 946 (1964).

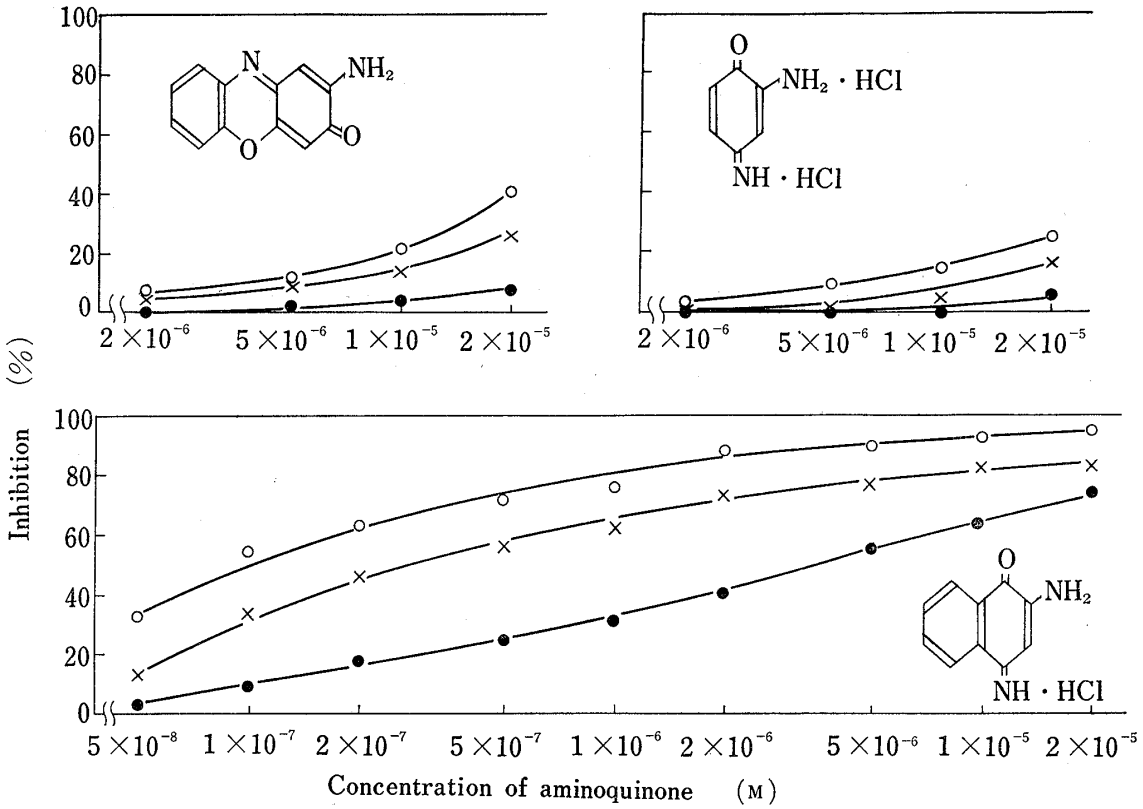


Fig. 5. Inhibition of Thymidine, Uridine, and L-Phenylalanine Incorporation into DNA, RNA, and Protein by Aminoquinone Derivatives at Several Concentrations in Ehrlich Ascites Tumor Cells *in Vitro*

—○— inhibition of thymidine-2-^{14}C (1×10^{-4} M) incorporation
—●— inhibition of uridine-2-^{14}C (1×10^{-4} M) incorporation
—×— inhibition of L-phenylalanine-U-^{14}C (1×10^{-4} M) incorporation

Effects of Aminoquinone Derivatives on DNA and RNA Degradation in Ehrlich Mouse Ascites Tumor Cells *in Vitro*

It has been known that the inhibition of RNA or DNA biosynthesis by actinomycins or mitomycins is accompanied by degradation of RNA or DNA and by resulting accumulation of precursors in the pool.[23,24] In order to see the effects on DNA and RNA degradation, the tumor cells, of which nucleic acid had been labeled by TdR-2-^{14}C or UR-2-^{14}C, were incubated with an aminoquinone, then the radioactivity released into the pool (acid sol. fr.) in the cells was counted. As shown in Fig. 6, II and ANQI were somewhat stimulative to the breakdown of RNA, but the degrees were less than that of AM. On the other hand, all the compounds were not stimulative to the degradation of DNA.

Discussion

Among the aminoquinone derivatives used for the present investigation the compounds of aminoquinone imine and aminophenoxazone types could interact with nucleic acid, while the others, having no imine residue, could not interact. This fact suggests that the type of interaction between the aminoquinone derivatives except III and nucleic acid is somewhat different from the cases of actinomycins,[4,25] mitomycins,[5] porfiromycin,[5] and streptonigrin,[6] to whose interaction with DNA the imine residue makes no direct contribution.

23) C. Levinthal, A. Keynan, and A. Higa, *Proc. Natl. Acad. Sci. U. S.*, **48**, 1631 (1962).
24) H. Kersten, *Biochim. Biophys. Acta*, **55**, 558 (1962); E. Reich, A. J. Shatkin, and E.L. Tatum, *ibid.*, **53**, 132 (1961); I. Smith-Kielland, *ibid.*, **138**, 542 (1967).
25) L.D. Hamilton, W. Fuller, and E. Reich, *Nature*, **198**, 538 (1963).

Although the details of interaction of ANQI with nucleic acid will be reported in the next paper,[26] the aminoquinone imine compounds (ANQI and V) are noticeable regarding their interaction with both of DNA and RNA, being different from the case of actinomycins which interact only with DNA.[4] The interaction of aminoquinone imines with DNA and RNA was affected by Mg^{2+} in such a manner as the cases of several antibiotics and dyes; *e. g.*, chromomycins,[27] daunomycin,[9] and acridine oranges.[28] Such "metal effect" suggests the participation of Mg^{2+} to the interaction of aminoquinone imines.

The incorporation of precursors into DNA, RNA, and protein was depressed most intensively by ANQI. This compound exerted its inhibitory action on the biosynthesis of DNA at the highest degree, which was comparable to that of the known inhibitors of DNA biosynthesis such as mitomycins.[22] Besides, the action of ANQI on DNA turnover was contributed by the inhibition of synthesis, not by the stimulation of degradation.

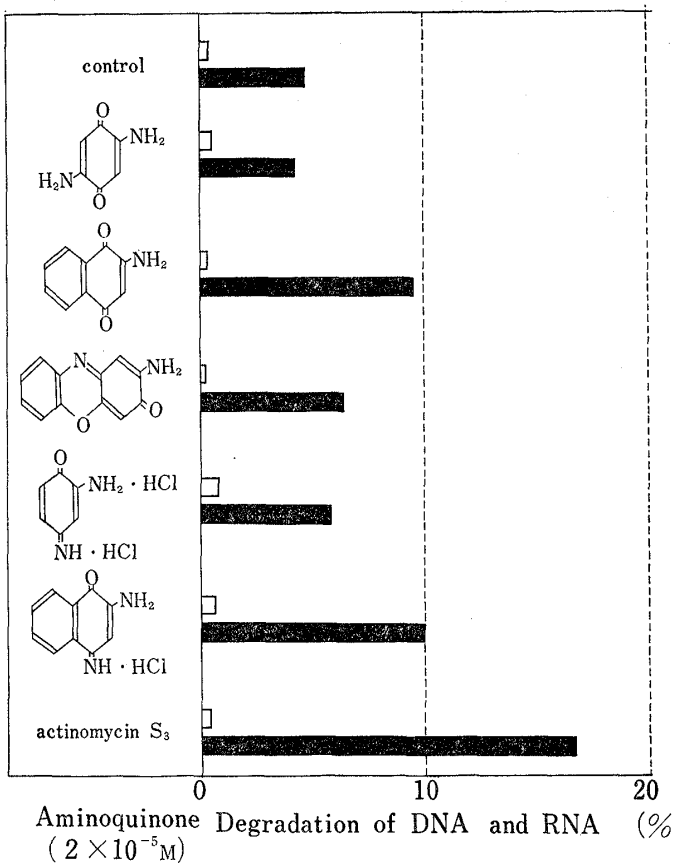


Fig. 6. Effects of Aminoquinone Derivatives on DNA and RNA Degradation in Ehrlich Ascites Tumor Cells *in Vitro*

□ DNA degradation ■ RNA degradation

a) degradation (%)
$$= \frac{\text{radioactivity in acid sol. fr. after addition of aminoquinone}}{\text{radioactivity in DNA or RNA before addition of aminoquinone}} \times 100$$

It may be assumed that the interaction with DNA *in vitro* is responsible for the inhibition of DNA biosynthesis by ANQI, though the mechanisms of the inhibition will be reported later. This assumption may be probable in connection with the cases of some antibiotics,[4–14] acridine dyes,[28,29] and alkylating agents.[30] It is necessary to confirm, however, whether ANQI can interact with DNA in living cells.

Although it is unknown whether the inhibition of RNA and protein biosynthesis by ANQI is a result of the inhibitory action on DNA biosynthesis, there are some possibilities of existence of another function than the inhibition of DNA biosynthesis, for the incorporation of L-Phe-U-^{14}C into protein was suppressed much higher than that of UR-2-^{14}C into RNA.

The inhibition of nucleic acid and protein biosynthesis by IV were slight, in spite of its interacting activity with nucleic acid which was comparable to that of ANQI. An assumption may be probable that the slight effect on the biosynthesis is a result of its instability in aqueous or neutral buffer solution, because IV is rather rapidly oxidized in the solution,

26) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 113 (1969).
27) D.C. Ward, E. Reich, and I.H. Goldberg, *Science*, **149**, 1259 (1965).
28) L. Lerman, *J. Mol. Biol.*, **3**, 18 (1961).
29) H. Nakamura, *Kagaku To Seibutsu*, **4**, 514 (1966).
30) P. Brookes and P.D. Lawley, *Biochem. J.*, **80**, 496 (1961); P.D. Lawley and P. Brookes, *ibid.*, **89**, 127 (1963).

followed by the formation of dark brown precipitates, while ANQI is reasonably stable in the solution,.

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