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Strand Breaks in Deoxyribonucleic Acid of Ehrlich Ascites Carcinoma Cells induced by Some Aminoquinone Compounds

Shoji Okada,[1] Nobuo Mochizuki,[1a] and Osamu Tamemasa[1]

Shizuoka College of Pharmacy[1]

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The three aminoquinone compounds, 2-amino-1,4-naphthoquinone imine (ANQI), 2,5-diamino-1,4-naphthoquinone imine (DANQI) and 2-hydroxyamino-1,4-naphthoquinone (HANQ), which had been reported to interact with deoxyribonucleic acid (DNA) *in vitro* and to inhibit DNA synthesis in Ehrlich ascites carcinoma cells, caused strand breaks in DNA of the carcinoma cells.

ANQI and DANQI, when incubated with the carcinoma cells, induced the single strand breaks producing the fragments of homogeneous size. These two aminoquinones, however, did not cause the strand breaks in the carcinoma DNA when *i.p.* injected into the carcinoma-bearing mice. HANQ, on the other hand, induced both the single and double strand breaks in the DNA when applied on either the cells or the carcinoma-bearing mice. Since both types of the breaks occurred at the same concentration or dose of HANQ, the double strand scission might be its dominant action.

From these results, the relationship between the strand scission and the inhibition of the DNA synthesis or the antitumor activity was discussed.

Keywords——drug action mechanism; aminoquinone compounds; Ehrlich ascites carcinoma; strand scission of DNA, single and double; sucrose density gradient centrifugation

It has been reported by the authors that, among several aminoquinone derivatives, 2-amino-1,4-naphthoquinone imine (ANQI),[2-4] 2,5-diamino-1,4-naphthoquinone imine (DANQI)[5] and 2-hydroxyamino-1,4-naphthoquinone (HANQ)[6] (Chart 1) interacted with DNA[7] *in vitro* and potentially inhibited DNA synthesis in Ehrlich ascites carcinoma cells. The preceding paper[8] has described that, to correlate the *in vitro* interaction with the inhibition, these aminoquinones inhibited DNA synthesis catalyzed by *E. coli*[7] DNA polymerase I. The inhibition in this cell-free system, however, was too low when compared with

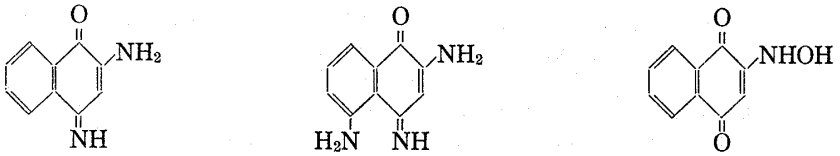

Chart 1

1) Location: *2-2-1 Oshika, Shizuoka*; *a*) Present address: *Bio-Medical Research Division, Nisso Institute for Life Science, Nippon Soda Co., Ltd., Oiso, Kanagawa.*
2) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 105 (1969).
3) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 113 (1969).
4) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 1057 (1969).
5) N. Mochizuki, S. Okada, and O. Tamemasa, *Yakugaku Zasshi*, **97**, 968 (1977).
6) N. Mochizuki, S. Okada, and O. Tamemasa, *Chem. Pharm. Bull.* (Tokyo), **23**, 1077 (1975).
7) Abbreviations: DNA=deoxyribonucleic acid, RNA=ribonucleic acid, *E. coli*=*Escherichia coli*, Ca^{2+}-free KRP=Ca^{2+}-free Krebs-Ringer phosphate buffer; 0.122 M NaCl, 0.013 M KCl, 0.65 mM $MgSO_4$ and 0.01 M phosphate buffer (pH 7.2), tris=tris-(hydroxymethyl)aminomethane, EDTA=ethylenediamine tetraacetic acid, PBS=phosphate-buffered saline; 0.14 M NaCl, 0.03 M KCl and 0.01 M phosphate buffer (pH 7.4).

that of cellular DNA synthesis of Ehrlich ascites carcinoma.[4-6] It was therefore suggested that there might be inhibition mechanisms other than the binding to the template DNA.[8]

The present study was intended to find out another mechanism of the inhibition. Since, among a number of DNA-interacting substances, some antibiotics and carcinogens such as bleomycin,[9] neocarzinostatin,[10] 4-nitroquinoline-1-oxide (4NQO) and its proximate carcinogen, 4-hydroxyaminoquinoline-1-oxide (4HAQO),[11,12] had been known to induce strand breaks in DNA, it seemed to be an effective approach to see if the aminoquinones caused the strand breaks. As a result, ANQI and DANQI induced single strand breaks in DNA of Ehrlich ascites carcinoma cells. On the other hand, HANQ caused double strand breaks, as well as single strand ones, in the carcinoma DNA when the cells were treated either *in vitro* or *in vivo*. The agreement in the aminoquinone concentrations between the inhibition of the cellular DNA synthesis and the strand scission suggests that the latter is the main mechanism for the former.

Experimental

Materials——ANQI,[2,13] DANQI[5] and HANQ[6] were prepared as reported previously.

Treatment of Ehrlich Ascites Carcinoma Cells with the Aminoquinones——The washed carcinoma cells which were obtained from ddY mice (♂, *ca.* 20 g) transplanted intraperitoneally (*i.p.*) with the carcinoma cells 7 days before were suspended in Ca^{2+}-free KRP[7] at a concentration of 0.1 g (wet weight) per ml and incubated at 37° for 60 min with the aminoquinones. In case of *in vivo* treatment, the aminoquinones in physiological saline were *i.p.* injected into ddY mice (♂, *ca.* 20 g) which had been transplanted with the carcinoma cells 7 days in advance. Then, 1 hr later, the cells were harvested and washed with Ca^{2+}-free KRP.

Alkaline Sucrose Density Gradient Centrifugation——The single strand breaks in DNA were analyzed by alkaline sucrose density gradient centrifugation according to, in principle, the method of McGrath and Williams.[14] The carcinoma cells suspended in PBS[7] (1×10^6 cells/0.05 ml) were loaded on a three-layered alkaline sucrose gradient in a Hitachi RPS-40 tube; the gradient composed of 0.2 ml of 1 N NaOH layered on 4.7 ml of linear 5—20% sucrose gradient containing 0.3 N NaOH, 0.7 M NaCl and 0.001 M EDTA[7] with 0.2 ml of 80% sucrose underneath the gradient. After leaving the tubes for 15 min at 4° for lysis of the cells, the tubes were centrifuged at 30000 rpm for 90 min at 4° in an RPS-40 Rotor of Hitachi 40-P Ultracentrifuge. The fractions of 12 drops each were collected from the bottom of the tubes, and their absorbances at 260 nm were measured in a Hitachi Spectrophotometer, Model 101.

Neutral Sucrose Density Gradient Centrifugation——The sedimentation behavior of the carcinoma DNA was analyzed by the procedure of Terasima and Tsuboi.[15] The cell suspension (1×10^6 cells/0.05 ml PBS) was applied to the top of a neutral sucrose gradient in a tube which consisted, from the bottom to the top, of 0.2 ml of 80% sucrose in 0.01 M tris-HCl (pH 7.4), 4.7 ml of linear 5—20% sucrose gradient containing 0.1 M NaCl, 0.01 M EDTA and 0.01 M tris-HCl (pH 7.4), and 0.2 ml of 2% sodium dodecylsulfate. After the cell lysis by keeping at 25° for 20 min, followed by chilling to 4°, the tubes were centrifuged at 30000 rpm for 60 min at 4° in an RPS-40 Rotor. The fractions were collected and treated as described above.

Assay for ^{3}H-labeled DNA of Ehrlich Ascites Carcinoma Cells——Thymidine-6-^{3}H (Radiochemical Centre, 0.1 mCi/0.5 ml physiological saline/mouse) was *i.p.* injected into ddY mouse 6 days after transplantation of the carcinoma. The labeled cells were harvested 24 hr later and washed. To analyze the sedimentation

8) N. Mochizuki, S. Okada, and O. Tamemasa, *Chem. Pharm. Bull.* (Tokyo), **25**, 2856 (1977).
9) H. Umezawa, "Antibiotics," Vol. III, ed. by J.W. Corcoran and F.E. Hahn, Springer-Verlag, New York-Heidelberg-Berlin, 1975, pp. 21—33.
10) Y. Ono, Y. Watanabe, and N. Ishida, *Biochim. Biophys. Acta*, **119**, 46 (1966); Y. Ono, Y. Ito, H. Maeda, and N. Ishida, *Biochim. Biophys. Acta*, **155**, 616 (1968); K. Tatsumi, T. Nakamura, and G. Wakisaka, *Gann*, **65**, 459 (1974); T.A. Beerman and I.H. Goldberg, *Biochem. Biophys. Res. Commun.*, **59**, 1254 (1974); T.A. Beerman and I.H. Goldberg, *Biochim. Biophys. Acta*, **475**, 281 (1977).
11) T. Sugimura, H. Otake, and T. Matsushima, *Nature* (London), **218**, 392 (1968); M. Horikawa, O. Nikaido, T. Tanaka, and T. Sugawara, *Exptl. Cell Res.*, **59**, 147 (1970).
12) T. Andoh and T. Ide, *Cancer Res.*, **32**, 1230 (1972).
13) L.F. Fieser, "Experiments in Organic Chemistry," 3rd ed., D.C. Heath and Co., Boston, 1957, pp. 234—238.
14) R.A. McGrath and R.W. Williams, *Nature* (London), **212**, 534 (1966).
15) T. Terasima and A. Tsuboi, *Biochim. Biophys. Acta*, **174**, 309 (1969).

behavior of the ^3H-labeled DNA, an aliquot (1×10^6 cells/0.05 ml PBS) of the cells was applied on the alkaline or neutral sucrose density gradients as described above. After the gradients were centrifuged and then fractionated also as mentioned above, the absorbance at 260 nm and the radioactivity in each fraction were determined; the latter was performed in a liquid scintillation spectrometer (Aloka LSC-602) by dissolving the fraction in a scintillation mixture of methyl cellosolve system.[16]

***In Vitro* Treatment of Calf Thymus DNA with the Aminoquinones**——Calf thymus DNA (Sigma, Type I, "highly polymerized") was dissolved in 0.01 M NaCl–0.01 M tris-HCl (pH 7.4) at a concentration of 2 mg/ml and incubated with the aminoquinones at 37° for 60 min. The mixture was, then, dialyzed against the same buffer for 12 hr at 4°. The sedimentation behavior of the DNA in the alkaline sucrose density gradients was analyzed in the same manner as stated on Ehrlich ascites carcinoma DNA except that the centrifugation was done for 6 hr.

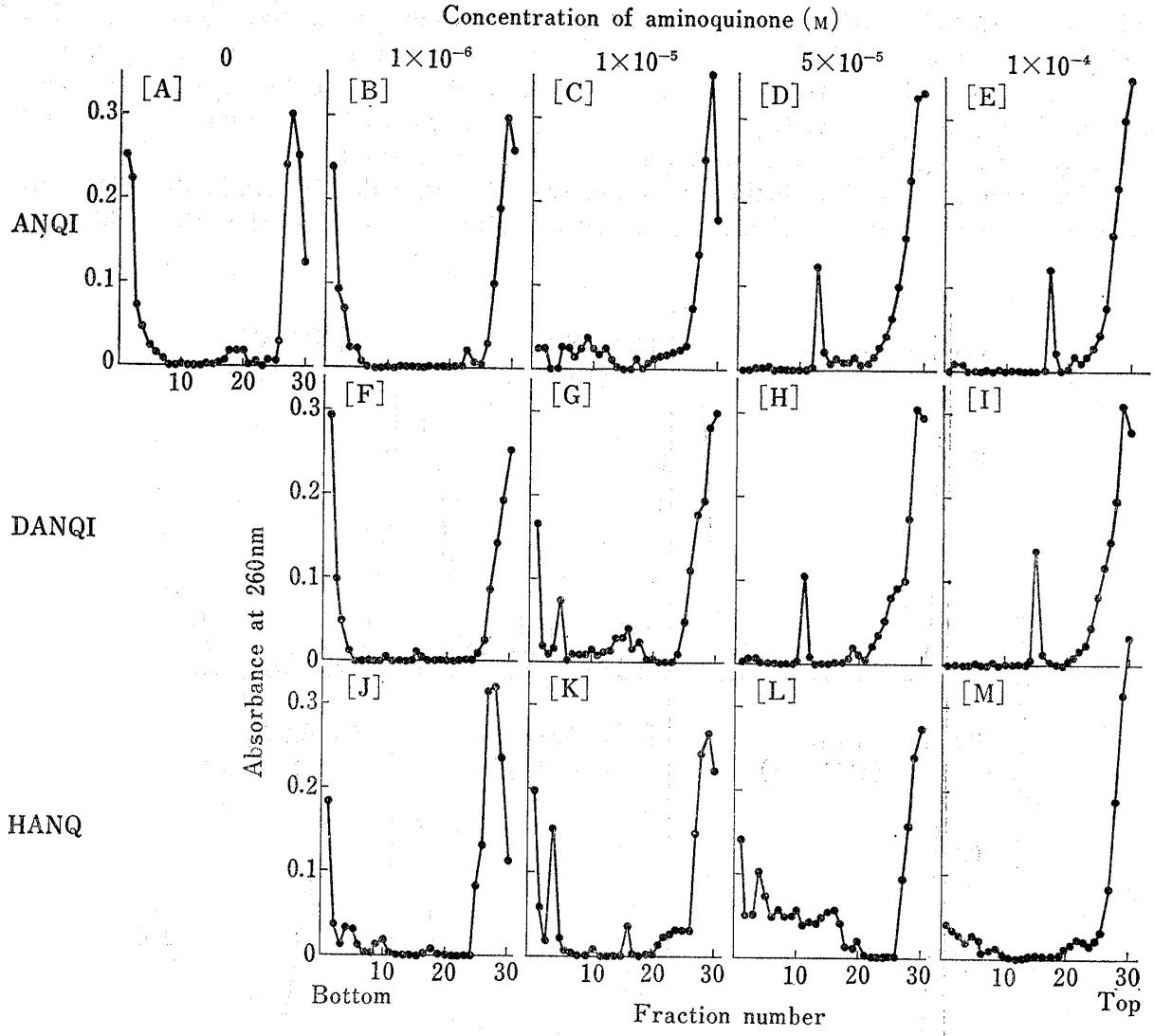

Fig. 1. Alkaline Sucrose Density Gradient Profiles of the DNA in Ehrlich Ascites Carcinoma Cells Incubated with the Aminoquinones

The cells (0.1 g/ml Ca^{2+}-free KRP) were incubated with the aminoquinones at 37° for 60 min. An aliquot (1×10^6 cells) was lyzed at 4° for 15 min in 0.2 ml of 1 N NaOH layered on 4.7 ml of 5—20% alkaline sucrose gradient and centrifuged at 30000 rpm for 90 min at 4° in an RPS-40 rotor. The fractions of 12 drops each were collected from the bottom.

16) The scintillation mixture composed of 2,5-diphenyloxazole (DPO) 5 g, 1,4-bis-2-(5-phenyloxazolyl-) benzene (POPOP) 0.5 g, naphthalene 80 g, methyl cellosolve 428 ml, dioxane 428 ml and toluene 140 ml.

Results

Strand Breaks in the Carcinoma DNA when the Cells were *in Vitro* Treated with the Aminoquinones

As shown in Fig. 1, in alkaline sucrose density gradients all the three aminoquinones caused apparent changes in the profiles of absorbance at 260 nm of the cellular constituents of Ehrlich ascites carcinoma. Since the absorbance peak at the position other than the top of the gradient indicates the location of DNA, as mentioned later (Fig. 3), its shift to the position of lower density refers to strand scission of the DNA. When the cells were incubated with 5×10^{-5}—1×10^{-4} M ANQI or DANQI, the DNA of full chain length, which should be detected at the bottom of the gradients, disappeared and some portion of the DNA was detected as a sharp peak at the intermediate position of the gradients (Fig. 1D, 1E, 1H, 1I). The sharp peak reflects a group of the single-stranded DNA fragments of similar chain length which became shorter with increasing concentration of the aminoquinones. On the other hand, HANQ shortened the DNA chain in a somewhat different manner; 5×10^{-5} M HANQ produced the fragments of heterogeneous size which distributed in approximately 2/3 region from the bottom of the gradient (Fig. 1L), and 1×10^{-4} M HANQ scissored a large portion of the DNA into very short fragments detected at almost the top of the gradient.

Fig. 2 shows the sedimentation profiles in neutral sucrose density gradients of DNA from the aminoquinone-treated carcinoma cells. Double strand scissions of the DNA, which could

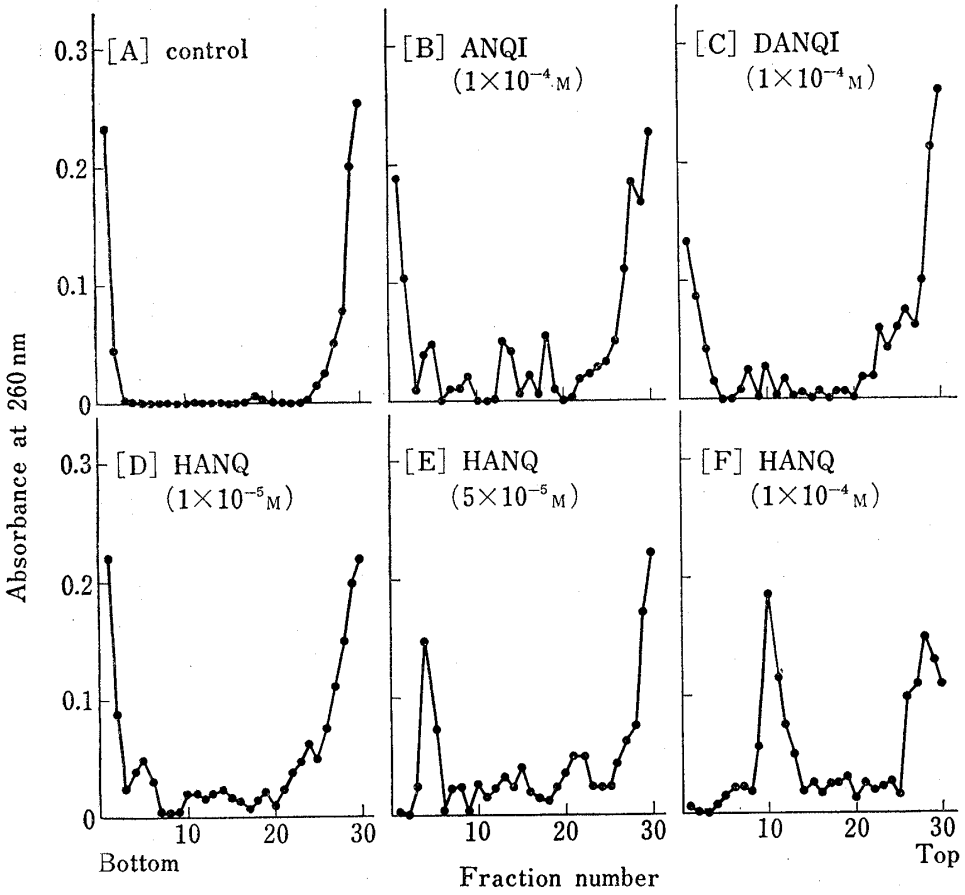

Fig. 2. Neutral Sucrose Density Gradient Profiles of the DNA in Ehrlich Ascites Carcinoma Cells Incubated with the Aminoquinones

The cells (0.1 g/ml Ca^{2+}-free KRP) were incubated with the aminoquinones at 37° for 60 min. An aliquot (1×10^6 cells) was lyzed at 25° for 20 min in 0.2 ml of 2% sodium dodecylsulfate layered on 4.7 ml of 5—20% neutral sucrose gradient (pH 7.4) and centrifuged at 30000 rpm for 60 min at 4° in an RPS-40 rotor. The fractions of 12 drops each were collected from the bottom.

be detected in this system, were not so remarkable by ANQI or DANQI at 1×10^{-4} M (Fig. 2B, 2C), a concentration enough to cause single strand breaks (Fig. 1). HANQ at 5×10^{-5} M or higher, on the other hand, induced the double strand breaks (Fig. 2E, 2F). The double-stranded DNA fragments produced were rather homogeneous in the chain length in contrast with the heterogeneous size of the single-stranded fragments detected by alkaline sucrose density gradient analysis (Fig. 1). The result that HANQ induced both single and double strand breaks in the DNA at the same concentration, 5×10^{-5} M or higher, suggests that the double strand scission is the dominant action of HANQ, although the single strand scission also can not be ruled out.

To confirm that the absorbance peak was really of DNA, an experiment using ^{3}H-thymidine-labeled cells was carried out on typical samples. Fig. 3 represents that the peak of absorbance coincided with that of radioactivity except top parts of the gradients which might contain substances showing absorbance at 260 nm, *e. g.*, RNA,[7] some materials of low molecular weight and a lot of proteins, other than DNA. This coincidence was observed in any of the gradients; either alkaline (Fig. 3A, 3B) or neutral (Fig. 3C, 3D) and both in the presence (Fig. 3B, 3D) and absence (Fig. 3A, 3C) of the aminoquinone. This result, which may be suggested by the data on nondividing brain cells by Chetsanga, *et al.*,[17] indicates that, in the present experiments, the absorbance peak at 260 nm reflects the position of cellular DNA.

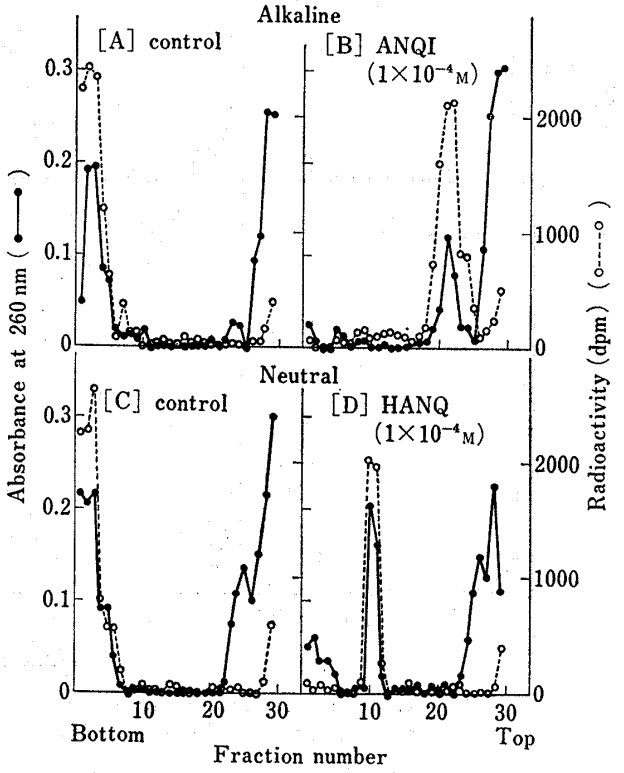

Fig. 3. Alkaline and Neutral Sucrose Density Gradient Profiles of the ^{3}H-Labeled DNA of Ehrlich Ascites Carcinoma Cells

The carcinoma cells were *in vivo* labeled with thymidine-6-^{3}H (0.1 mCi/mouse, *i.p.*) for 24 hr (6th to 7th day). The cells harvested were incubated with or without the aminoquinones and analyzed as described in Fig. 1 and 2.
Sucrose density gradients: [A], [B]—alkaline, [C], [D]—neutral
—●—: Absorbance at 260 nm, ---○---: Radioactivity (dpm).

Strand Breaks in the Carcinoma DNA when the Carcinoma-Bearing Mice were Administered with the Aminoquinones

Fig. 4 represents the sedimentation profiles in alkaline sucrose density gradients of DNA from Ehrlich ascites carcinoma of mice which were *i. p.* injected with the aminoquinones 1 hr before. HANQ at doses higher than 50 mg/kg, *i. e.* approximately 1/6 dose as much as the LD_{50} (318 mg/kg),[6] induced single strand breaks, while ANQI (3.5 mg/kg) or DANQI (13 mg/kg), *ca.* 2/3 dose as much as the LD_{50} (ANQI—5.45 mg/kg,[18] DANQI—19.4 mg/kg[5]), caused little change in the sedimentation of the DNA. The chain size of the single-stranded DNA fragments produced by HANQ became shorter with increasing dose of HANQ (Fig. 4D—4G).

Fig. 5 shows the sedimentation behavior in neutral sucrose density gradients of the DNA *in vivo* treated with HANQ in the same manner as described above. HANQ at doses of 50 mg/kg and higher, *i. e.* the doses to cause single strand breaks (Fig. 4), induced double strand

17) C.J. Chetsanga, M. Tuttle, A. Jacobini, and C. Johnson, *Biochim. Biophys. Acta.*, **474**, 180 (1977).
18) S. Okada and O. Tamemasa, *Chem. Pharm. Bull.* (Tokyo), **17**, 1432 (1969).

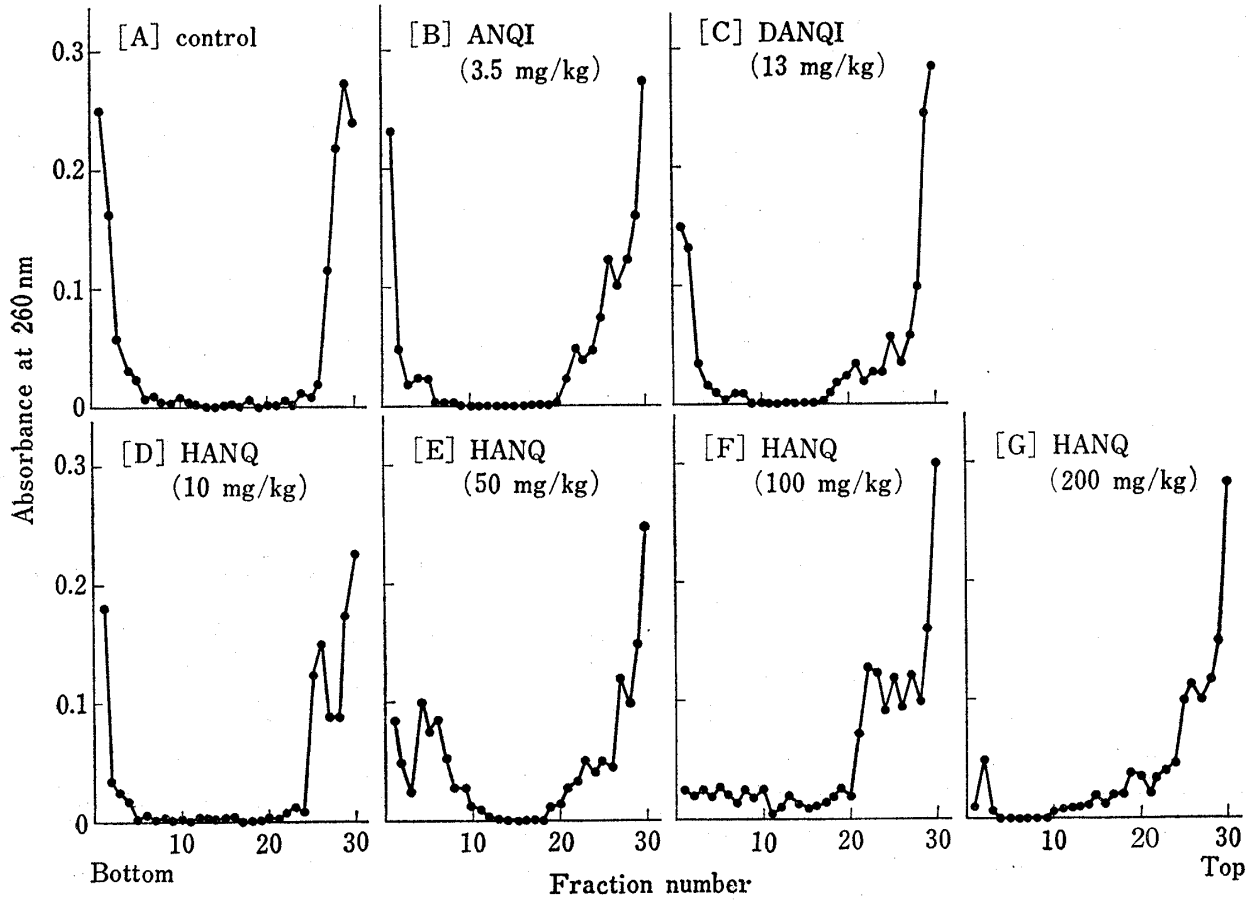

Fig. 4. Alkaline Sucrose Density Gradient Profiles of the DNA in Ehrlich Ascites Carcinoma *in Vivo* Treated with the Aminoquinones

The aminoquinones in physiological saline were *i.p.* injected into ddY mice bearing Ehrlich ascites carcinoma. The carcinoma cells obtained 1 hr after the administration were lyzed and centrifuged as described in Fig. 1.

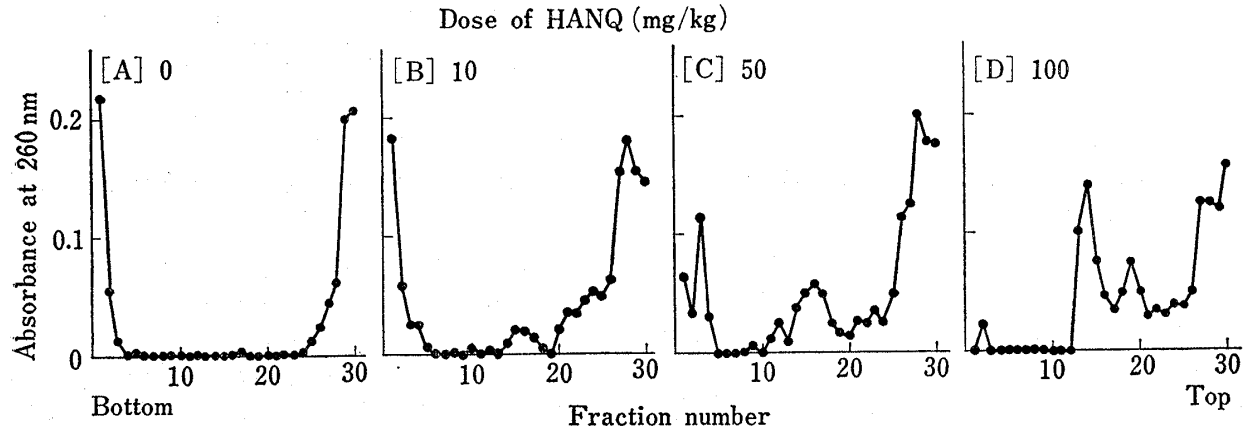

Fig. 5. Neutral Sucrose Density Gradient Profiles of the DNA in Ehrlich Ascites Carcinoma *in Vivo* Treated with HANQ

HANQ in physiological saline was *i.p.* injected into ddY mice bearing Ehrlich ascites carcinoma. The carcinoma cells obtained 1 hr after the administration were lyzed and centrifuged as described in Fig. 2.

breaks in the DNA, too. These results, indicated in Fig. 4 and 5, suggest that the double strand scission was dominant also in case of *in vivo* treatment with HANQ. It was further observed that the dose inducing the strand breaks was in good agreement with that showing antitumor activity on some of experimental tumor of mouse,[6] as discussed later.

In Vitro Effect of the Aminoquinones on the Sedimentation Behavior of Calf Thymus DNA

To see if the aminoquinones directly cause chemical scission on DNA strand without living cells, calf thymus DNA was incubated with the aminoquinones and analyzed by alkaline sucrose density gradient centrifugation. As shown in Fig. 6, no single strand scission, which also meant no double strand scission, was observed by the aminoquinones at a concentration of 1×10^{-4} M.

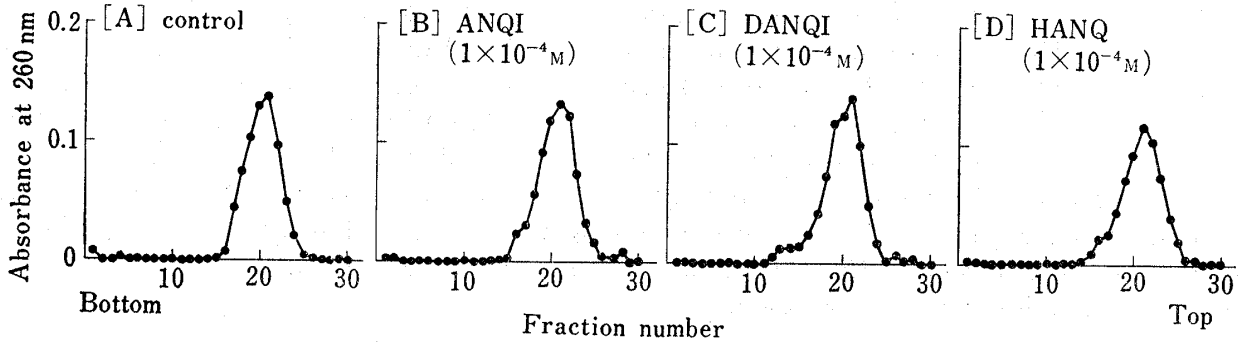

Fig. 6. Alkaline Sucrose Density Gradient Profiles of Calf Thymus DNA *in Vitro* Incubated with the Aminoquinones

Calf thymus DNA (2 mg/ml 0.01 M NaCl-0.01 M tris-HCl, pH 7.4) was incubated with 1×10^{-4}M aminoquinone at 37° for 60 min. After the dialysis against the same buffer, the DNA was alkali-denatured at 4° for 15 min in 0.2 ml of 1 N NaOH layered on 4.7 ml of 5—20% alkaline sucrose density gradient and centrifuged at 30000 rpm for 6 hr at 4° in an RPS-40 rotor. The fractions of 12 drops each were collected from the bottom.

Discussion

The three aminoquinone compounds, ANQI, DANQI and HANQ, have been known as more potent inhibitors of DNA synthesis than mitomycin C in Ehrlich ascites carcinoma cells.[2,4–6] Although the inhibition might be partially due to the interaction with the cellular DNA,[8] the mechanism of the inhibition has not yet been fully clarified. One of the aims of the present study was, therefore, to find out the mechanism.

ANQI and DANQI caused single strand breaks in the DNA when the carcinoma cells were incubated with them (Fig. 1B—1I). The concentrations of ANQI and DANQI to cause the strand breaks corresponded to those to inhibit the cellular DNA synthesis; the strand scission started to be detectable at 1×10^{-5} M aminoquinone (Fig. 1C, 1G) and became remarkable at 5×10^{-5} M (Fig. 1D, 1H) while the inhibition percentages were 70—90% at 1×10^{-5} M and 90—95% at 5×10^{-5} M.[4,5] It is, therefore, possible to assume that the inhibition by ANQI and DANQI mainly resulted from single strand scission of the cellular DNA. On the other hand, that these two aminoquinones have shown no antitumor activity *in vivo*[5,18] is consistent with the present data that the strand breaks were not obvious when the carcinoma-bearing mice were administered with these two aminoquinones (Fig. 4B, 4C). This ineffectiveness may be attributed at least in part to the unstability of these aminoquinones in the ascitic fluid, since ANQI was known to be readily metabolized to its inactive derivative, 2-amino-1,4-naphthoquinone.[18]

HANQ induced both single and double strand breaks in the carcinoma DNA by the treatment either *in vitro* or *in vivo* (Fig. 1—5). In *in vitro* experiments, the concentration to induce appreciable breaks of the double strand (5×10^{-5} M, Fig. 2E), which should be very lethal, was nearly consistent with that for the single strand breaks (Fig. 1L) and also with that for the inhibition of the cellular DNA synthesis (*ca.* 80% inhibition at 5×10^{-5} M).[6] An analogous relationship was found in the dose between the strand scission *in vivo* and the antitumor activity. HANQ was carcinostatic against Ehrlich ascites carcinoma, ascites saroma-180 and L-1210 leukemia at a dose of 100 mg/kg (*i. p.*),[6] while it evidently caused the

double strand breaks as well as the single at 100 mg/kg (Fig. 4F, 5D). Since a part, at least, of the single strand breaks refers to the double strand ones in this experiment, these phenomena suggest that the antitumor activity of HANQ depends primarily on the double strand scission of the tumor DNA.

The modes of the strand scission between the amino- (ANQI and DANQI) and the hydroxyamino- (HANQ) quinones were not the same. The former chiefly caused the single strand breaks producing the fragments of a similar size (Fig. 1D, 1E, 1H, 1I). HANQ, on the other hand, made both the single and double strand breaks although the latter might be dominant. The double-stranded fragments produced by HANQ were rather homogeneous in the chain length (Fig. 2E, 2F), whereas the single-stranded were heterogeneous (Fig. 1L, 1M). This type of the scission is similar to that by 4NQO or 4HAQO.[11,12] As it has been discussed on these carcinogens that some enzymes were possibly involved in the strand scission of DNA at the carcinogen-bound sites,[12,19] it may be reasonable to consider that the breaks may not be caused by the direct action of the aminoquinones (Fig. 6) but by the participation of nucleases which recognize the aminoquinone-bound sites in DNA, although further experiments are required for this discussion.

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19) T. Ide and T. Andoh, *Cancer Res.*, **32**, 1236 (1972).