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Interaction of 2-Amino-1,4-naphthoquinone Imine with Nucleic Acid

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On 2-amino-1,4-naphthoquinone imine-HCl (ANQI), which had previously been reported to interact with nucleic acid and to inhibit the synthesis of nucleic acid and protein, especially of DNA, in Ehrlich mouse ascites tumor cells *in vitro*, the details of the interaction with nucleic acid were investigated by the methods of melting experiment for DNA, difference spectrum, equilibrium dialysis, and flow dichroism. The results are as follows:

1. The sites in DNA for attachment of ANQI were the purine base moieties, *i.e.*, both of adenine and guanine. ANQI interacted with a highly polymerized native DNA at the highest degree and hardly interacted with such monomers as bases, nucleosides, and nucleotides.

2. ANQI was found to be able to bind to calf thymus DNA up to the ratio of 2 moles per 4 nucleotides by the method of equilibrium dialysis. This means that ANQI can attach with all the purine bases in DNA.

3. Such bivalent metal ions as Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Ba^{2+} interfered with the interaction between ANQI and DNA. On the other hand, Hg^{2+} increased the extent of interaction.

4. It was found from the measurement of flow dichroism on DNA-bound ANQI that the orientation of ANQI was nearly parallel with the planes of DNA bases.

In the previous paper[2)] it was reported that among several aminoquinone derivatives 2-amino-1,4-naphthoquinone 4-imine-HCl (ANQI) interacted with both of DNA[3)] and RNA,[3)] and inhibited nucleic acid and protein, especially DNA, synthesis in Ehrlich mouse ascites tumor cells *in vitro*. An assumption was further made that the interaction of ANQI with DNA was responsible for its intensive inhibition of DNA biosynthesis. The extent of this inhibition was the highest among the aminoquinones investigated in the previous study.[2)] Further investigation was therefore undertaken on the mechanisms of inhibition on DNA biosynthesis by ANQI.

The biological effects of some antibiotics,[4)] acridine dyes,[5)] alkylating agents,[6)] carcinogenic substances,[7,8)] *etc.* have been considered to be related with their interacting activities with nucleic acid, and the mode of interaction of these compounds with nucleic acid has been investigated and clarified by many workers.[4–8)]

This paper describes the mode of interaction of ANQI with nucleic acid from this point of view. It may be concluded from the results of the present investigation that ANQI interacts with both moieties of adenine and guanine bases in nucleic acid at the orientation in nearly parallel with the planes of the bases.

1) Location; *Oshika, Shizuoka.*
2) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 105 (1969).
3) Abbreviations; DNA (deoxyribonucleic acid), RNA (ribonucleic acid), tRNA (transfer ribonucleic acid).
4) B.A. Newton, *Ann. Rev. Microbiol.*, **19**, 209 (1965); N. Tanaka, *Kagaku To Seibutsu*, **4**, 170 (1966); *etc.*
5) L. Lerman, *J. Mol. Biol.*, **3**, 18 (1961); H. Nakamura, *Kagaku To Seibutsu*, **4**, 514 (1966); *etc.*
6) P. Brookes and P.D. Lawley, *Biochem. J.*, **80**, 496 (1961); P.D. Lawley and P. Brookes, *ibid.*, **89**, 127 (1963).
7) C. Nagata, M. Kodama, Y. Tagashira, and A. Imamura, *Biopolymers*, **4**, 409 (1966); C. Nagata, M. Kodama, A. Imamura, and Y. Tagashira, *Gann*, **57**, 75 (1966).
8) T. Okano and K. Uekama, *Chem. Pharm. Bull.* (Tokyo), **15**, 1812 (1967).

Experimental

Materials——ANQI was synthesized in the author's laboratory according to the procedure of Fieser.[9] Calf thymus DNA (highly polymerized) and yeast tRNA[3] were obtained from Sigma Chemical Co. Apurinic and apyrimidinic calf thymus DNA were prepared in the author's laboratory in the manners described by Tamm, *et al.*[10] and by Takemura,[11] respectively. Heat denatured calf thymus DNA was prepared by heating the DNA solution in a neutral buffer (0.01 M tris–0.01 M NaCl, pH 7.4) at 100° for 15 min and then by cooling it rapidly to 0°. Poly A[12] and polyU[12] were purchased from Sigma Chemical Co. Nucleotides, nucleosides, and bases of nucleic acid were the products of Sigma Chemical Co. and Nutritional Biochemicals Corp.

Thermal Denaturation Curves for DNA——The curves for calf thymus DNA in the presence and absence of ANQI were obtained in 0.001 M tris–0.001 M NaCl (pH 7.4) by the apparatus of Dr. H. Hashizume of Faculty of Science, Shizuoka University, by his courtesy.

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

Equilibrium Dialysis of DNA-ANQI Solution[13]——Visking dialysis tubing No. 8/32 was used for the experiment, which had been washed with 1% EDTA–2Na, boiling distilled water, hot 5% $NaHCO_3$, and 90% ethanol prior to dialysis. Each tubing contained 0.5 ml portion of buffer solution (0.01M tris–0.01M NaCl, pH 7.4) of calf thymus DNA was inserted in a glass stoppered tube which contained 5 ml portion of the same buffer solution of ANQI. The tubes were then allowed to stand at 0—5° for 72 hr in dark with occasionally shaking. An equilibrium was well-established after 72 hr dialysis, for it had been found by a preliminary experiment that the equilibrium was almost established after 24 hr and that ANQI was stable under this condition for 72 hr. The amount of ANQI interacted with DNA was calculated from the concentrations of ANQI outside the tubing after and before dialysis. The concentrations were determined, in this experiment, from optical densities at 470 mμ, *i.e.*, $\lambda_{max}$of ANQI,[2] by subtracting the adsorbed amount of ANQI on the tubing which was calculated from DNA-free experiment.

Flow Dichroism Measurement——The measurement was carried out by the apparatus of Dr. C. Nagata of National Cancer Center Research Institute by his courtesy. The details of the principle and the measuring procedures have been described by Wada, *et al.*[14] and Nagata, *et al.*[7]

Results

Effect of ANQI on Thermal Denaturation Curve for Calf Thymus DNA

As stated in the previous paper,[2] the visible absorption band of ANQI was slightly shifted to longer wave length by calf thymus DNA (Fig. 1). The extent of spectral shift was 5 mμ at the concentrations of 1×10^{-4}M AMQI and 1×10^{-3}M DNA–P in 0.01M tris–0.01M NaCl (pH 7.4). For confirming that the spectral shift was a result of interaction between them, a melting experiment was carried out on calf thymus DNA in 0.001M tris–0.001M NaCl (pH 7.4) in the presence of ANQI. The reason for the lower concentrations of tris and NaCl in this experiment than that in the experiments of difference spectra is to reduce the error in measurement at high temperature.

Fig. 2 shows the melting curves of 1×10^{-4}M (as nucleotide) of calf thymus DNA in the presence and absence of 2×10^{-5}M of ANQI. ANQI caused an apparent rise, 7.7° higher than that of DNA alone, of melting temperature (Tm). The existence of the interaction between ANQI and DNA was further confirmed by the findings that ANQI also caused an appreci-

9) 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.
10) C. Tamm, M.E. Hodes, and E. Chargaff, *J. Biol. Chem.*, **195**, 49 (1952).
11) S. Takemura, *Bull. Chem. Soc. Japan*, **32**, 920 (1959).
12) Abbreviations; poly A (polyadenylic acid), poly U (polyuridylic acid).
13) A.R. Peacocke and J.N.H. Skerrett, *Trans. Faraday Soc.*, **52**, 261 (1956); L.F. Cavalieri and A. Angelos, *J. Am. Chem. Soc.*, **72**, 4686 (1950); P.O.P. Ts'o and P. Lu, *Proc. Natl. Acad. Sci. U. S.*, **51**, 17 (1964); K. Yamagami, E. Moriguchi, H. Fujita, K. Suzuki, and A. Wada, *Hoshasen Igaku Sogo Kenkyusho Nempo*, **38** (*in Showa*), 68 (1963); T. Ito, H. Fujita, S. Imamura, and M. Zama, *Tampakushitsu Kakusan Koso*, **12**, 555 (1967).
14) A. Wada and S. Kozawa, *J. Polymer Sci.*, **2**, 853 (1964); A. Wada, *Tampakushitsu Kakusan Koso*, **10**, 1173 (1965).

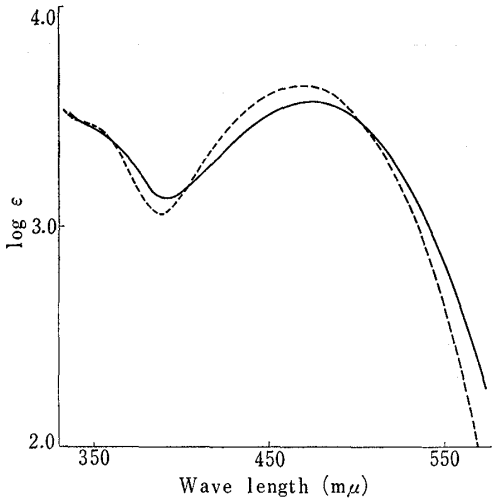

Fig. 1. Absorption Spectra of ANQI in the Presence and Absence of Calf Thymus DNA

Concentrations of ANQI and DNA-P are 1×10^{-4} M and 1×10^{-3} M, respectively, in 0.01 M tris–0.01 M NaCl, pH 7.4.

—— ANQI + DNA $\lambda_{max}=475$ mμ
------ ANQI $\lambda_{max}=470$ mμ

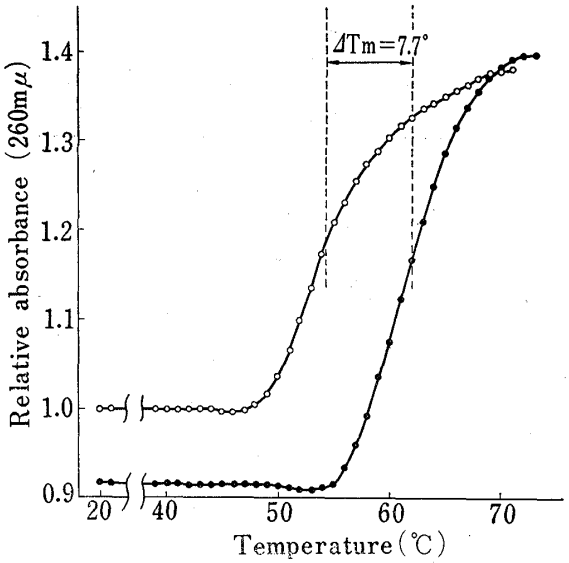

Fig. 2. Thermal Denaturation Profiles of Calf Thymus DNA in the Presence and Absence of ANQI

Concentrations of DNA-P and ANQI are 1×10^{-4} M and 2×10^{-5} M, respectively, in 0.001 M tris-0.001 M NaCl, pH 7.4.

—○—○— DNA Tm=54.3°
—●—●— DNA + ANQI Tm=62.0°

able decrease in absorbance of DNA at 260 mμ at low temperature and a considerable increase in hyperchromicity of DNA with a rise in temperature, compared with the case of DNA alone.

Interaction with Bases, Nucleosides, and Nucleotides[15]

As it had become evident that ANQI interacted with nucleic acid, especially with DNA, the interaction with bases, nucleosides, and nucleotides was investigated by the method of difference spectrum in order to clarify which moiety in DNA ANQI interacted with. Fig. 3 presents the difference spectra of ANQI (1×10^{-4}M) and each of bases, nucleosides, and nucleotides (1×10^{-3}M, each) against ANQI (1×10^{-4}M) in 0.01M tris–0.01M NaCl (pH 7.4), under this condition ANQI–calf thymus DNA had shown an apparent spectral shift (ref. 2 and Fig. 4). As shown in Fig. 3, any of these bases, nucleosides, and nucleotides showed no spectral change under this condition. This observation suggests a poor interacting activity of these monomers with ANQI, though AMP and GMP in high concentrations could interact with ANQI as stated in later (Fig. 5).

Interaction with Polynucleotides and Nucleic Acids

Since the monomers had produced no spectral change in ANQI solution, it was investigated what kinds of the polymers, *i. e.*, homopolymers, modified and native calf thymus DNA, and yeast tRNA, caused spectral shifts. As shown in Fig. 4, the visible spectrum of ANQI was appreciablly shifted by such polymers having purine bases as poly A, apyrimidinic, heat denatured, and native calf thymus DNA, and yeast tRNA under the same condition as in the cases of monomers, while it was not changed by the others, that is, poly U and apurinic calf thymus DNA, which had no purine base. It was, furthermore, observed that the shift caused by native calf thymus DNA was the most prominant in the polymers mentioned above.

15) Abbreviations: AMP (adenosine-5′-monophosphate), GMP (guanosine-5′-monophosphate), CMP (cytidine-5′-monophosphate), UMP (uridine-5′-monophosphate), dTMP (deoxythymidine-5′-monophosphate), ATP (adenosine-5′-triphosphate), GTP (guanosine-5′-triphosphate), CTP (cytidine-5′-triphosphate), UTP (uridine-5′-triphosphate).

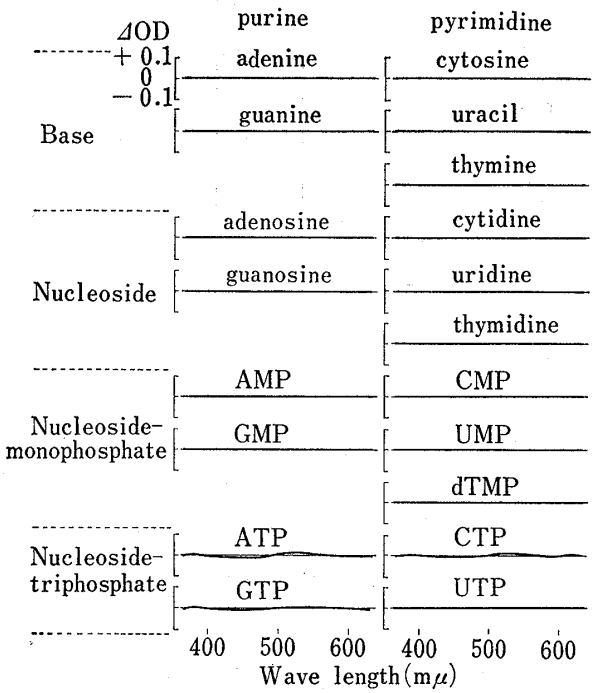

Fig. 3. Difference Spectra of ANQI and Bases, Nucleosides, and Nucleotides against ANQI

Concentrations of ANQI and constituents of nucleic acid, except guanine (saturated solution), are 1×10^{-4} M and 1×10^{-3} M, respectively, in 0.01 M tris–0.01 M NaCl, pH 7.4.

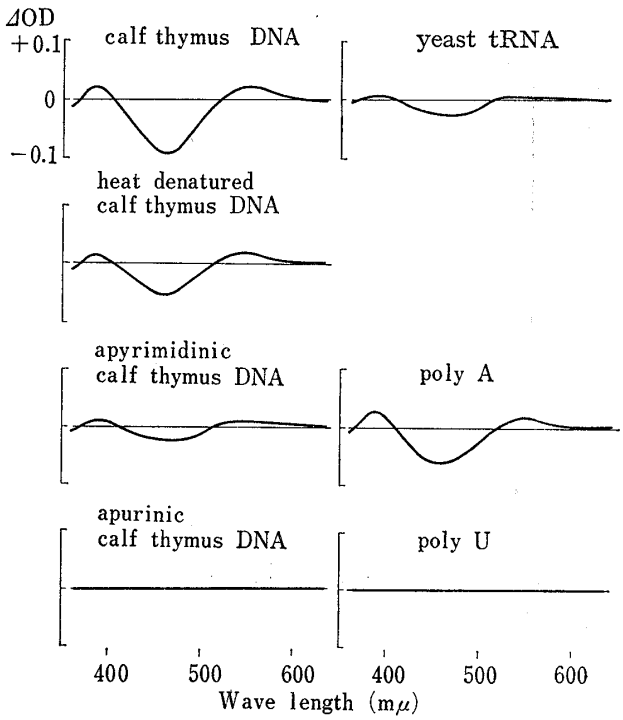

Fig. 4. Difference Spectra of ANQI and Nucleic Acid Derivatives against ANQI

Concentrations of ANQI and polymers-P are 1×10^{-4} M and 1×10^{-3} M, respectively, in 0.01 M tris-0.01 M NaCl, pH 7.4.

It may be considered from these observations that ANQI attaches to the purine base moieties in the polymers, not to the moieties of pyrimidine bases, sugars, and phosphates, and that the longer the molecular chain of polymer is, the more ANQI attaches with it, because the chain of native calf thymus DNA is considered to be longer than that of the other polymers having purine bases. It was, however, not clear in this experimental step whether both of the two purine bases, adenine and guanine, interacted with ANQI or only one of the two bases did.

Interaction with Nucleotides in High Concentrations

As an approach to the problem mentioned above the difference spectra of 1×10^{-4} M ANQI and 1×10^{-2} M nucleotides were measured. The concentration of the latter was 10 times higher than that in the usual experiments. As shown in Fig. 5, the visible absorption band of ANQI was apparently shifted by such a high concentration of AMP and GMP, while the shift was not appreciable in the presence of pyrimidine nucleotides. This finding suggests the possibility that both of the purines in nucleic acid act as sites for attachment of ANQI.

Binding Rate of ANQI to Nucleotides of DNA

As another approach to determine the sites of nucleic acid for interaction with ANQI than the spectral approach, the binding ratio of ANQI to nucleotides of DNA was investigated by the method of equilibrium dialysis. The amount of ANQI attached to calf thymus DNA was estimated from measurements of the absorbance at 470 mμ of ANQI outside the dialysis tubings containing DNA solution after and before dialysis. The parameters of interaction, *i. e.*, the number of binding sites per nucleotide n and the binding constant K, were derived from the relation[13]

$$\frac{1}{r}=\frac{1}{nK}\cdot\frac{1}{c}+\frac{1}{n} \qquad \text{(Eq. 1)}$$

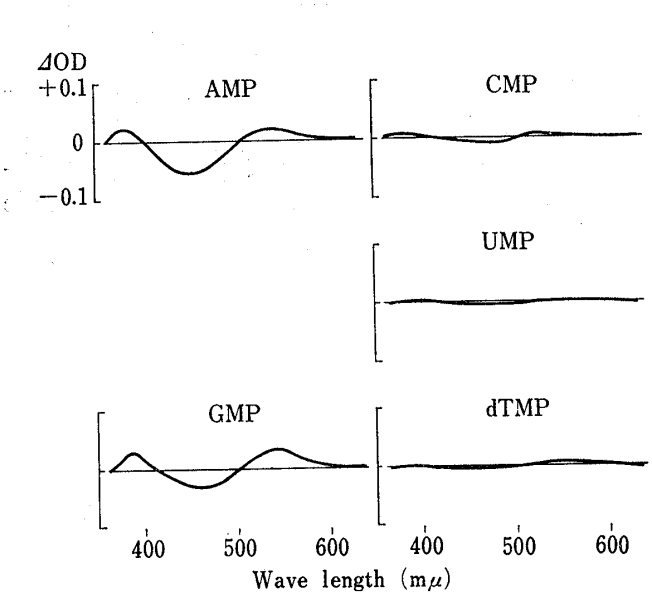

Fig. 5. Difference Spectra of ANQI and Nucleotides in High Concentration against ANQI

Concentrations of ANQI and nucleotides are 1×10^{-4} M and 1×10^{-2} M, respectively, in 0.01 M tris-0.01 M NaCl, pH 7.4.

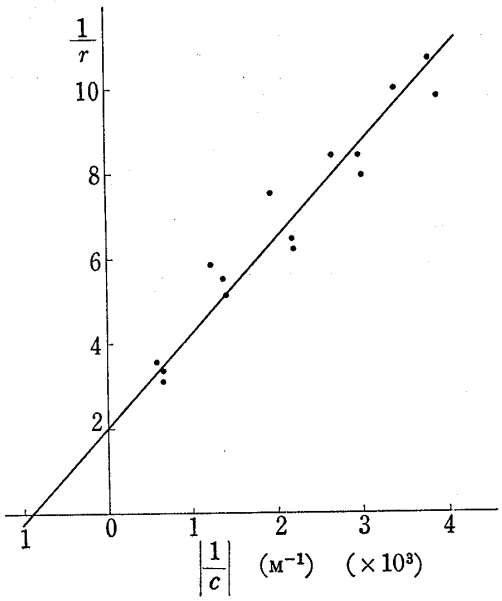

Fig. 6. Binding Curve of ANQI to Calf Thymus DNA

The experimental condition and the parameters should be referred to the text.

where r is the number of ANQI molecules bound per nucleotide and c is the molar concentration of free ANQI.

The experiment was carried out over a 5-fold concentration range (4×10^{-4}M—2×10^{-3}M) of ANQI but at one concentration level (1×10^{-2}M, as nucleotide) of DNA. Fig. 6 presents the result of equilibrium dialysis performed at 0—5°. It was found that each plot obtained was arranged on a straight line (Eq. 1), and that it crossed the axis of ordinates ($1/r$) at the point of 2 and crossed that of abscisses ($1/c$) at the point of $|9\times10^{2}|$. This result leads to a conclusion that the binding of ANQI to DNA corresponds to the simple equation (Eq. 1) at least in the concentration range of the present experiment, and that ANQI can bind to calf thymus DNA up to a ratio of 2 moles per 4 nucleotides at a binding constant of 9×10^{2} M^{-1}. It can be further confirmed in connection with the spectral data that ANQI interacts with both moieties of adenine and guanine bases in DNA concerning with the fact that the molar ratio of purine and pyrimidine bases in DNA is generally 1:1.

It was further found that ANQI bound to DNA was rapidly released into H_2O when the tubing contained ANQI-bound DNA solution was re-dialyzed against H_2O. The bond is therefore considered to be weak, at least not covalent, from this observation.

The experiment at 37° was unsuccessful, because the stability of ANQI in the buffer solution at 37° was not so enough for the period to reach an equilibrium.

Influence of Metal Ions on the Interaction

Since it had been reported that the interaction between nucleic acid and several antibiotics, dyes, and chemotherapeutic agents, *e. g.*, chromomycins,[16] daunomycin,[17] acridine oranges,[5,18] luteoskyrin,[19] ethidium bromide,[20] chloroquine,[21] *etc.* was affected by some

16) D.C. Ward, E. Reich, and I.H. Goldberg, *Science*, **149**, 1259 (1965).
17) E. Calendi, A. DiMarco, M. Reggiani, B. Scarpinato, and L. Valentini, *Biochim. Biophys. Acta*, **103**, 25 (1965).
18) B.L. Gittelson and I.O. Walker, *Biochim. Biophys. Acta*, **138**, 619 (1967).
19) Y. Ueno, A. Platel, and P. Fromageot, *Biochim. Biophys. Acta*, **134**, 27 (1967).
20) M.J. Waring, *J. Mol. Biol.*, **13**, 269 (1965).
21) S.N. Cohen and K.L. Yielding, *J. Biol. Chem.*, 240, 3123 (1965).

metal ions, the influence of some salts on the visible spectrum of ANQI–calf thymus DNA solution was investigated.

As shown in Fig. 7, the degree of shift in ANQI spectrum caused by the DNA was decreased by the addition of such salts as $MgCl_2$, $CaCl_2$, $MnCl_2$, $CuSO_4$, $ZnCl_2$, and $BaCl_2$ at a final concentration of 1×10^{-3} M which was equal to the concentration of DNA nucleotides, while by $HgCl_2$ a marked increase was observed. On the other hand, any appreciable change could not be found in the visible absorption band of ANQI by the addition of these salts without DNA. These phenomena suggest the participation of the bivalent metal ions in the interaction between ANQI and DNA.

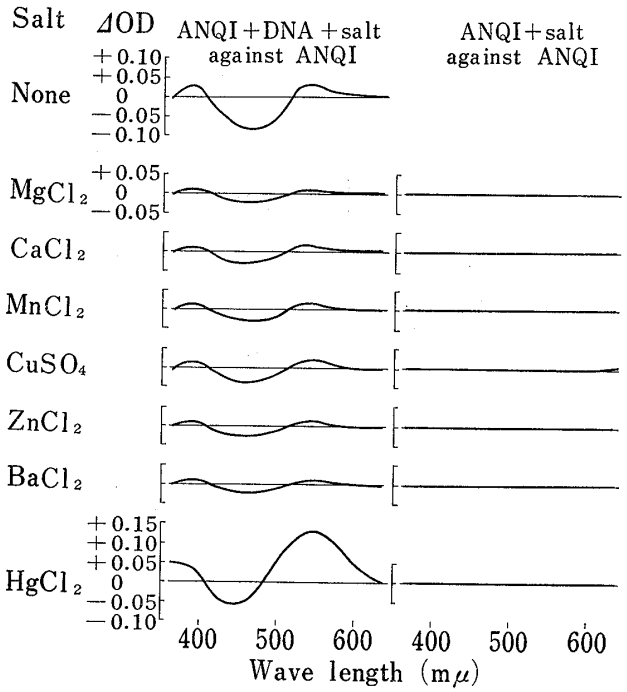

Fig. 7. Influence of Some Bivalent Metal Salts on the Interaction between ANQI and Calf Thymus DNA

Difference spectra of ANQI+DNA+salt against ANQI (left) and of ANQI+salt against ANQI (right) were measured at the concentrations of 1×10^{-4} M ANQI, 1×10^{-3} M DNA-P, and 1×10^{-3} M salts in 0.01 M tris–0.01 M NaCl, pH 7.4.

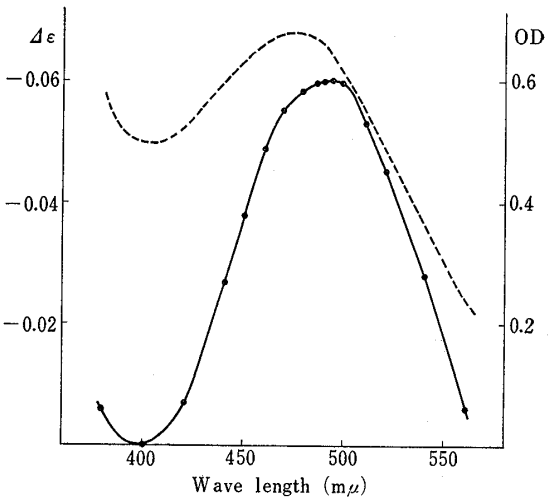

Fig. 8. Dichroism and Absorption Spectra of ANQI in Calf Thymus DNA Solution

—●— Dichorism spectrum ($\Delta\varepsilon$) of 1.5×10^{-3} M ANQI and 1.5×10^{-2}M DNA-P in 0.05 M borate buffer, pH 7.0, thickness of solution in light path is 0.1 cm.

--------- Absorption spectrum (OD) of 1.5×10^{-4}M ANQI and 1.5×10^{-3}M DNA-P in the same buffer, thickness of solution is 1.0 cm.

Orientation of ANQI to the Planes of DNA Bases

ANQI–DNA complex was prepared for the dichroism measurement by mixing equal volume of 3×10^{-3} M ANQI in 0.05 M borate buffer, pH 7.0, and 3×10^{-2} M (as nucleotide) calf thymus DNA of the same buffer solution which had previously been stirred for 12 hr at 4° and dissolved. The final concentrations were 1.5×10^{-3} M ANQI and 1.5×10^{-2} M DNA–P in 0.05 M borate buffer, and this concentration of ANQI was corresponded to its absorbance of about 0.7 at 470 mμ in a cell of 0.1 cm light path. The measurement of flow dichroism was carried out by rotating coaxial cylinders at a velocity of approximately 1300 rpm for the production of velocity gradient, and by entering the monochromic light through the gap between the outer and inner cylinders made of quartz. The width of the gap and the thickness of solution in the light path were 0.05 and 0.1 cm, respectively.

As shown in Fig. 8, the complex of ANQI–DNA represented a dichroism, which showed a negative value. This result indicates an evidence that ANQI enters into direct interaction with DNA, and suggests the orientation of ANQI to the planes of DNA bases to be nearly parallel.

Any apparent dichroism spectrum, however, could not be observed at lower concentrations of DNA than that stated above, and it remained ambiguous why $|\Delta\varepsilon|$ value was so small than that in the cases of acridine oranges and several carcinogenic substances.[7]

Discussion

It was confirmed from the results of melting experiment on DNA, difference spectra, and equilibrium dialysis, that ANQI interacted with nucleic acid, especially with DNA, *in vitro*, and that both of the purine bases (adenine and guanine) in nucleic acid acted as the binding sites for ANQI. Such a mode of interaction is similar to that of mitomycins,[22] nogalamycin,[23] ethidium bromide,[20,24] chloroquine,[21,25] 4-nitroquinoline-1-oxide,[7] *etc.* However, it remains obscure on the mechanism of affinity to purine bases, likewise with the cases of the substances mentioned above.

The interaction of ANQI with DNA was interfered by common bivalent metal salts except $HgCl_2$ as was seen in difference spectra (Fig. 7). This phenomenon has been also observed in the cases of chromomycins,[16] daunomycin,[17] acridine oranges,[5,18] ethidium bromide,[20] and chloroquine,[21] and is opposite one to the case of luteoskyrin which interacts with DNA only when Mg^{2+} exists.[19] It is unlikely to consider that the bivalent metal ions tested in the present study bind directly to ANQI, as they caused no spectral change without DNA (Fig. 7). Therefore, the action of such metal ions is considered to shift the equilibrium in ANQI–DNA interaction to the dissociation side. It may be accordingly assumed that, when ANQI interacts with DNA in living cells, the extent of attachment *in vivo* is lower than that *in vitro* because of the presence of metal ions such as Mg^{2+} in living cells.

The presence of Hg^{2+}, on the other hand, caused a marked shift in the spectrum of ANQI–DNA. Although the interaction of Hg^{2+} itself with DNA has already been reported,[26] a consideration may be possible that, besides the binding of Hg^{2+} itself to DNA, this ion increased the extent of interaction between ANQI and DNA in the present study. Furthermore, some influences of Hg^{2+} is expected on the biological action of ANQI to inhibit nucleic acid and protein biosynthesis.[2]

The result of equilibrium dialysis leads to a conclusion that the binding of ANQI–DNA is not a covalent but an electrostatic or a hydrogen bond. On the other hand, it remains unclear whether the location of ANQI in DNA is between two helixes of DNA (intercalation) or outside (stacking), though the orientation to DNA was found to be nearly parallel with the planes of DNA bases from the result of flow dichroism.

The mechanism of inhibitory action of ANQI on the synthesis of nucleic acid and protein, especially of DNA, in Ehrlich mouse ascites tumor cells *in vitro*[2] may be for the most part explained as a result of interaction between ANQI and nucleic acid, if ANQI actually could interact with nucleic acid in living cells. In order to clarify this problem, further studies are in progress on the interaction in living cells.

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22) M. Kodama, *J. Biochem.* (Tokyo), **61**, 162 (1967).
23) B.K. Bhuyan and C.G. Smith, *Proc. Natl. Acad. Sci. U.S.*, **54**, 566 (1965).
24) M.J. Waring, *Biochim. Biophys. Acta*, **114**, 234 (1966).
25) R.L. O'Brien, J.G. Olenick, and F.E. Hahn, *Proc. Natl. Acad. Sci. U.S.*, **55**, 1511 (1966).
26) S. Katz, *J. Am. Chem. Soc.*, **74**, 2238 (1952); C.A. Thomas, *ibid.*, **76**, 6032 (1954); T. Yamane and N. Davidson, *ibid.*, **83**, 2599 (1960); M. Matsuda and E. Takeuchi, *J. Biochem.* (Tokyo), **61**, 523 (1967).