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STRONG AND SPECIFIC INTERACTIONS OF SOME INCAPACITATING PHENOTH--ETC(U)
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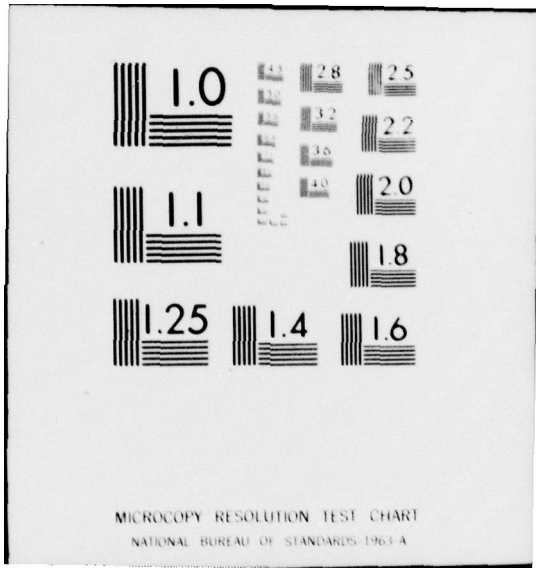
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(6) Strong and Specific Interactions of Some Incapacitating Phenothiazines with Nucleic Acids

(10) Charles E. Williamson Ph.D.
Chemical Systems Laboratory
Aberdeen Proving Ground, MD 21010

(11) JUN 1978

In recent years the anti-psychotic and other neurological effects of the tranquilizing phenothiazines have been well described. As a class, they serve a variety of needs and their potent tranquilizing activities render them exceedingly valuable in the treatment of mental disorders. Widespread research[1] has provided an excellent data base for further neuroleptic studies. At Edgewood, such studies have been directed toward the discovery of new and potent neurological incapacitating agents to temporarily render soldiers mentally incapable of waging warfare. Several phenothiazines such as 2-nitro-10-{3-[4-(2-hydroxyethyl)-1-piperazinyl] propyl} phenothiazine dimaleate (I) were found to be sufficiently potent to produce these effects. Their incapacitating activities are described elsewhere[2].

In searching for new biologically active compounds an understanding of the mechanism of biological action is most important, for such information can lead directly to the design of drugs and agents of superior activity. Furthermore, a knowledge of in vivo interactions of such compounds with tissues is imperative when considering them for human use. Such is the case for the studies described herein where strong and specific interactions are observed between incapacitating phenothiazines and tissue components.

Binding of phenothiazines to nuclear components is generally, but not universally recognized. For example, phenothiazine dyes such as methylene blue, thionin and toluidine blue have long been used as histological stains[3] for cell nuclei, and the common staining patterns are reported to arise from dye-nucleic acid complexes, but no biological responses have been ascribed to these interactions.

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More recently it has been reported that compounds such as chloropromazine, a tranquilizing phenothiazine, do [4-6] and do not[7,8] bind to DNA. A summary of the available literature, however, provides adequate assurance that such binding does, indeed, occur.

The nature of the binding of such compounds to nucleic acids, although partially described for some phenothiazine-type compounds, is still vague and the biological implications of such binding processes are virtually unknown. Numerous interactions of phenothiazines with cellular membranes have been reported to occur and many attempts have been made to relate their tranquilizing activities to these interactions. It is likely that some of the responses elicited by these drugs do arise as a result of such interactions.

In the present study, it is noted that the strongest and most obvious interactions between incapacitating phenothiazines and human cells growing in culture apparently occur with unbound RNA, such as messenger, ribosomal and transfer RNA. Accordingly, such incapacitating phenothiazines might be expected to interfere with the communicative processes that are mediated by these substances. In view of the reports[9] that the quality and quantity of such RNA varies during nervous transmission following stimulation of the cell, interference of these processes might also be expected after treatment with incapacitating phenothiazines.

EXPERIMENTAL

2-Nitro-10{3-[4-(2-hydroxyethyl)-1-piperazinyl] propyl} phenothiazine dimaleate, I; 10-{3-[4- (2-hydroxyethyl)-1-piperazinyl] propyl} phenothiazine-2-carbonitrile dimaleate, II; 2-(2-dicyanoethenyl)-10-{3-[4-(2-hydroxyethyl)-1-piperazinyl] propyl} phenothiazine dimaleate, III; and 2-(2-nitroethenyl)-10{3-[4-(2-hydroxyethyl)-1-piperazinyl] propyl} phenothiazine dimaleate, IV were synthesized in our laboratories. Their synthesis and properties are described by Bossle et al.[2]. All nucleic acids were purchased from Miles Laboratories, Kankakee, IL 60901. All polymers were purchased from Schwartz/Mann, Orangeburg, NY 10962. Nucleic acid and polymer concentrations are expressed as molarity of phosphate groups.

Human dipoid fibroblasts (derived from embryonic skin) were purchased from Microbiological Associates, Inc., Bethesda, MD 20014 after the fourth through the twentieth subcultivations. No primary cultures were performed in our Laboratory. The cells were received in plastic tissue culture bottles and released by the addition of a 0.25% solution of trypsin (Microbiological Associates, Inc., 1:250 trypsin conc.) in Earle's minimum essential medium (MEM) for further

culture. The trypsinized cells were diluted in Hank's MEM with 10% fetal calf serum (Microbiological Associates, Inc.) to approximately 10^5 cells/ml and replanted in 25 cc plastic bottles. The cells were incubated at 37°C. After 2 days and thereafter twice weekly, the medium was replaced with Earle's MEM containing 10% fetal calf serum. In 5 to 7 days the cells were confluent. During culture the pH was monitored by visual observation of the phenol red indicator in the medium.

To all Hank's or Earle's MEM was added 1% of a 200 mM solution of L-glutamine (100 x concentrated) and 1% of a penicillin-streptomycin mixture containing 5000 units each per ml (Microbiological Associates, Inc.).

The association constants between I to IV and salmon sperm DNA were measured by spectroscopy and equilibrium dialysis. Because of the tendency of these compounds to slowly precipitate in the preence of phosphate buffer, equilibrium dialysis experiments were performed in water. The pH in the dialysis cells was approximately 6.3 In these experiments the phenothiazine concentrations were varied from 2.5×10^{-4}M to 10^{-5}M and the concentration of DNA was maintained at 10^{-3}M. The concentration of I in the dialysis cell (reagent side) was monitored by its 325 nm band (E_{max} = 7,000), and II at 320 nm (E_{max} = 2,500). Suitable blanks were employed. Water solutions of compounds I to IV obeyed Beer's Law. Much difficulty was encountered in these measurements, however, because of compound interactions with the dialysis cells and membranes.

To measure association constants of compounds I to IV spectrally, a series of spectra were obtained in various concentrations of the particular nucleic acid or polymer under study. The compounds were dissolved in 0.05 M, pH 7.4 sodium phosphate buffer containing 10^{-3} M salmon sperm DNA and the spectra were determined. Subsequent spectra at lower DNA concentrations were obtained by serially diluting with aqueous solutions of the phenothiazines.

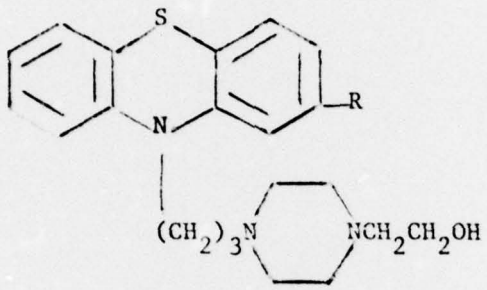
RESULTS

1. Interactions with Nucleic Acids.

The absorption spectra of I in 0.05 M, pH 7.4 sodium phosphate buffer containing various quantities of salmon sperm DNA are illustrated in Fig. 1. The 435 nm band gradually disappears with increasing DNA concentration is is replaced by a new broad band that approaches 525 nm. The latter absorption imparts a bright purple color to the solution.

Essentially the same types of absorption spectra were observed for II, III, and IV. The spectral data together with appropriate association constants are summarized in Table I.

Table I



Compound Number	R	λ max nm (Buffer),	λ max nm (DNA),	DNA Association* Constant, 1/mole
I	$-NO_2$	435	525	550
II	-CN	-	358, 430	170
III	$-CH=C(CN)_2$	450	570	3000
IV	$-CH=CHNO_2$	430	530	3100

*Determined by measurement of the DNA-phenothiazine charge-transfer absorption maximum.

In addition to the spectral measurements, association constants were also obtained by equilibrium dialysis measurements. The values for compounds I to IV were all in the range of 3000 1/mole and are considered approximate owing to the experimental difficulties described.

Upon the introduction of sodium chloride (0.1 M to 0.5 M) to solutions of I, II, III, or IV containing DNA or RNA, the λ max (DNA) bands progressively decreased in intensity and were replaced by the λ max (Buffer) bands which correspond to the unbound forms (Table I). Essentially the same behavior was observed when the phenothiazine-nucleic acid solutions were heated above 60°C; the λ max (DNA) bands

progressively disappeared and were replaced by the λ max (Buffer) bands corresponding to the unbound forms.

The λ max values recorded in Table I were also observed when these phenothiazines were treated with a 16s + 23s ribosomal RNA mixture or transfer RNA, both isolated from E. coli K-12 MO (Miles Laboratories, Kankakee, IL). Attempts to reproduce similar bands by the addition of numerous substances to these phenothiazine solutions failed. They were only seen in the presence of double-stranded nucleic acids or double-stranded synthetic polymers.

2. Interactions with Double-Stranded Polymers.

The interaction of I with the following polymers were examined: polyadenylic acid (poly A), polycytidylic acid (poly C), polyguanylic acid (poly G) and polyuridylic acid (poly U). Equimolar quantities (3×10^{-3} M) of poly A and poly U were dissolved in 0.05 M, pH 7.4 sodium phosphate buffer. A similar solution was prepared using poly G and poly C. The solutions were heated to 60°C for 10 min. and then gradually cooled to room temperature to form the respective poly A - poly U and poly G - poly C hybrids. The spectrum of I (2×10^{-4}M) was then measured in polymer solutions ranging from 8.7×10^{-4} M to 7.5×10^{-3} M. In the presence of poly G - poly C, I exhibits bathochromic and hypochromic changes. Although it retained the characteristic yellow color, its absorption maximum shifted from 435 nm to 453 nm with a decrease in molar absorptivity. In the presence of poly A - poly U, however, solutions of I were purple and its spectrum exhibited the same 525 nm band as observed in the presence of nucleic acids (Fig 1). Measurement of absorbance as a function of polymer concentration allowed the calculation of an association constant for this interaction. A value of approximately 330 1/mole was obtained.

Compounds I and II bind strongly to both poly A - poly U and poly G - poly C as evidenced by the spectral changes of both, and by the formation of immediate and intense precipitates when added to either of the double-stranded polymers in buffer.

3. Interactions With Single-Stranded Polymers.

Table 2 contains spectral data of 5×10^{-4} M of I in the presence of 5×10^{-3} M poly A, poly G, and 10^{-3} M poly U and poly C in water, and 2×10^{-4} M of the double-stranded polymers poly A - poly U and poly G - poly C in 0.05 M sodium phosphate buffer. It is noteworthy that the interactions of these polymers with I result in bathochromic

shifts in each instance. None was shifted sufficiently, however, to produce the purple color seen with I in poly A - poly U solutions. Addition of sodium chloride (0.1 M to 0.5 M) to the aqueous (I) - polymer solutions caused precipitation of the phenothiazine complexes with poly A, poly G and poly U. Precipitation did not occur when sodium chloride was added to the (I)-poly C solution and the spectral changes of I caused by poly C were reversed. The spectral changes of I in both poly G - poly C and poly A - poly U were readily reversed by the addition of 0.1 M to 0.5 M sodium chloride.

Table 2

ABSORPTION MAXIMA OF I[a] IN POLYMER SOLUTIONS

Polymer	λ max, nm
Sodium Phosphate Buffer[b]	435
Poly A[c]	455
Poly G[c]	460
Poly U[d]	445
Poly C[d]	460
Poly A - Poly U[e]	525
Poly G - Poly C[e]	453

[a]Conc. of I was 5×10^{-4} M in all experiments; [b]0.05 M, pH 7.4; [c]5×10^{-3} M; [d]10^{-3} M; [e]2×10^{-4} M.

4. Interactions with Human Fibroblasts Growing In Vitro.

Various concentrations of I and II were added to fresh sub-cultivations of human fibroblasts in plastic cell culture bottles and the cells were allowed to divide at 37°C for 3 days before observation. At this time, the tops were removed from the culture bottles and the microscope objective was immersed directly into the nutrient medium. Growth was normal at concentrations of both at and below 5×10^{-6} M, but abnormal at 10^{-5} M (Plate 1).

Compound I could not be visually detected at concentrations lower than 10^{-6} M. As viewed by light microscopy, cells grown in 10^{-5} M of I were weakly stained in granules within the cytoplasm but not within the nucleus. By fluorescence microscopy only a dim

orange field could be seen, nevertheless, extensive uptake of I was observed within the nucleoli. Because of its poor staining properties and low quantum yield, I was not an effective reagent to study the pattern of phenothiazine uptake by fibroblasts in culture.

Upon examination of II by fluorescence microscopy an intense uptake of reagent in granular bodies outside the nuclei was seen. In living cells, II was not observed inside the nuclei. The nucleoli, loci of high RNA concentration , however, were intensely stained. Destruction of the living cells by lysis or drying caused the nuclei to become stained more strongly than any other cell structures.

In aqueous phosphate buffer II fluoresces slightly (λ max = 520 nm). In the presence of 5×10^{-3} M salmon sperm DNA an enhanced yellow-green fluorescence was observed at the same emission maximum. The fluroescent yield of II was neither enhanced nor quenched by the addition of 10^{-2} M poly A - poly U, however, an equivalent quantity of DNA did produce enhancement. Fluorimetric measurement of the increase in fluorescent yield of II in various concentrations of salmon sperm DNA in 0.005 M, pH 7.4 sodium phosphate buffer produced an association constant of approximately 550 l/mole.

In addition to the spectral measurements an association constant between II and salmon sperm DNA was also obtained by equilibrium dialysis. This value, 3000 l/mole at pH 6, 25° C, is considered approximate owing to experimental difficulties described herein.

DISCUSSION

When solutions of nucleic acids are treated with I, a striking purple color forms concurrently with strong and specific binding. This coloration apparently arises from a charge-transfer complex between I and the double-stranded regions of nucleic acids and is readily measured by a newly formed absorption maximum at 525 nm. Corresponding bands arose from II, III, and IV upon interaction with DNA or RNA including microsomal and transfer RNA. In each instance the charge-transfer bands disappeared when the nucleic acids were melted by heating to 60°C or by the addition of organic solvents.

The charge-transfer bands were also produced when I and II interacted with double-stranded poly A - poly U, but not with other combinations studied. Strong interactions were also observed between these compounds and poly G - poly C but without the charge-transfer phenomena. When the double-stranded polymers were melted by heat or by the addition of organic solvents the charge-transfer bands also

disappeared. Throughout these studies such bands were observed only in association with the double-strandedness of nucleic acids or polymer models.

The mechanism by which these phenothiazines bind to nucleic acids and polymers appears similar to that described for several nucleic acid bound acridines and other phenothiazines. Noteworthy are descriptions of the complexes of chloropromazine [4], quinacrine [10], proflavin [11], acriflavin [12], ethidium bromide [8, 13] and others. Two types of proflavin binding have been observed using bromine atoms covalently attached to poly dA - poly dU as spin orbital probes [14]; 1) a strong internal interaction via intercalation involving overlap of the orbitals of the pyrimidine-purine base pairs with those of the reagent, and 2) a weaker external, partially intercalated interaction involving some orbital overlap, and having electrostatic interactions with a nearby phosphate group [13].

Both modes of binding do not appear to operate between these incapacitating phenothiazines and nucleic acids. In each instance, increases in the ionic strength of the media by the addition of sodium chloride resulted in the disappearance of the charge-transfer bands suggesting that electrostatic interactions (probably between the positively charged phenothiazine 10- position side chain and a phosphate group of the nucleic acid) as well as partial intercalation are the main binding forces (type 2 above). Failure to achieve complete intercalation is understandable in view of the bond angle of approximately 139^{o} between the benzene ring planes in phenothiazines of this type[15].

The same behavior was observed when I or II was treated with poly A - poly U, demonstrating that these interactions occur specifically, and in all likelihood arise in DNA from the double-stranded regions rich in dA-dT and in the corresponding A-U rich regions of RNA.

Measurement of the 525 nm complex band of I in various concentrations of salmon sperm DNA yielded an association constant of approximately 550 l/mole. By equilibrium dialysis, however, a value of approximately 3000 l/mole was obtained demonstrating that the 525 nm binding mode contributes only about one-fifth to the overall I - DNA interaction. Other interactions of I with DNA that contribute to the overall association constant are probably similar to those observed between I and various synthetic polymers. Among these, strong interactions were observed between the incapacitating phenothiazines

and poly G - poly C. Binding to poly A, poly G, poly C and poly U was also seen and can contribute to overall association constants. Charge transfer complexes were associated, however, only with interactions of these compounds with double-stranded A-U or dA-dT moieties. The data are recorded in Tables 1 and 2. The large DNA association constants recorded in this table for compounds III and IV are noteworthy. The larger values of these constants correspond to increased conjugation at the 2- positions of the phenothiazine rings.

The large differences in binding strengths at specific sites as reflected by the data in Table I permits speculation with respect to the biological importance of this mode of binding. The cataleptic activities of compounds I to IV together with binding data are presented in Table 3. It is noted that the cataleptic potency in mice is inversely proportional to the apparent binding strength to the dA-dT base pairs of DNA (K_{DNA}). Furthermore, the activity essentially disappears when this binding strength is large as in III and IV.

Table 3

CATALEPTOGENIC POTENCY OF SELECTED PHENOTHIAZINES[a]
CD50 in Mice[b,c]

Compound	μ mol/kg	K_{DNA}, l/mol
I	2.2 2.3	550
II	0.99 0.41	170
III	28	3000
IV	>57	3100

[a]Data reprinted from ref. (2) by permission of the authors;
[b]CD_{50} dosage to induce 50% of the maximum possible catalepsy score in a group of 6 mice. [c]The cataleptic potency of I was reported to be greater than II in rats(2)

When considering that the overall association constants of these compounds in DNA are approximately equivalent, this data suggests that the cataleptic potency parallels the extent of binding at the dG-dC or G-C base pairs in nucleic acids.

The uptake patterns of the incapacitating phenothiazines in living cells are illustrated in Plate I where human fibroblasts were

grown in sub-lethal concentrations of II. In these dividing cells II was observed to concentrate in granular bodies outside the nuclei in loci corresponding to microsome-rich areas. Uptake of II by the nucleoli is usually seen at higher reagent concentrations. However, detectable uptake by nuclei and cytoplasm did not occur. At cell death (drying or hypotonic lysis) nuclear uptake of II occurred rapidly with intense staining. Since these compounds penetrated the nuclear envelope in living cells to stain the nucleoli, their inability to interact with living nuclear DNA is probably related to the protection afforded by the strong protein-nucleic acid associations in native chromatin.

The patterns of the uptake of II by living cells are compatible with the chemical observations described herein. The strongest interactions apparently occur between these incapacitating phenothiazines and sites associated with microsomal activity - areas of active protein and nucleic acid synthesis in dividing cells and thereby the loci of concentrated unbound RNA.

In view of the observations that unbound nucleic acids bind tightly to these incapacitating phenothiazines in vivo, interference with associated metabolic processes such as protein and nucleic acid synthesis is expected. Simarily, such interference could result in the synthesis of faulty proteins or nucleic acids through strong binding to RNA. Likewise, these compounds should bind strongly to derepressed areas of nuclear DNA, a process known to result in frame-shift mutations.

Neuronal stimulation during nervous transmission is known to alter the nature of the RNA which is produced during this process(9). Incapacitating phenothiazines such as those described in this report would be expected to interact strongly and specifically with these substances and interfere with the nervous processes with which they are associated. The extent of such interference, although unknown, could be sufficient to elicit some of the responses attributed to these compounds.

REFERENCES:

1. Zinkle, C.L. and Kaiser, C., Antipsychotic Agents in Medicinal Chemistry, Burger, A., Ed., p 1410-1469 Wiley - Interscience New York, 1970.

2. Bossle, P.C., Ferguson, C.P., Sultan, W.E., Lennox, W.J., Dudley, G.E., Rea, T.H., and Miller, J.I., J. Med. Chem. 19, 370-373 (1976).

3. Gurr, E. Staining, Practical and Theoretical. p 63. The Williams and Wilkins Co., Baltimore, MD (1962).

4. Kantesaria, P. and Marfey, P., Physiol. Chem. and Physics, 7, 53-67 (1975).

5. Sapin, R., Leterrier, F. and Chambron, J., Biochimie. 57, 127-129 (1975).

6. Legator, M. and Zimmering, Ann. Rev. Pharmacol. 15, 387-408 (1975).

7. Waring, M.J., Humangenetik. 9, 234 (1970).

8. Waring, M.J., J. Mol. Biol. 54, 247 (1970).

9. Hyden, H., Endeavour. 21 144-155 (1962).

10. Weisblum, B. and de Haseth, P.L., Proc. Nat. Acad. Sci. USA 69, 629-632 (1972).

11. Finkestein, T. Weinstein, I.B., J. Biol. Chem. 242, 3763-3768 (1967).

12. Tubbs, R.K., Ditmars, W.E., Jr. and Van Winkle, Q., J. Mol. Biol. 9, 545-557 (1964).

13. Le Pecq, J.B. and Paeletti, C.J., Mol. Biol. 27, 87-106 (1976).

14. Galley- W.C. and Purkey, R.M., Proc. Nat. Acad. Sci. USA, 69, 2198-2202 (1972).

15. McDowell, J.J.H., The Molecular Structures of Phenothiazine Derivatives, p 33-54 in Phenothiazines and Structurally Related Drugs, Forrest, I.S., Carr, C.J. and Usdin, E., Editors, Raven Press, New York, NY, (1974)

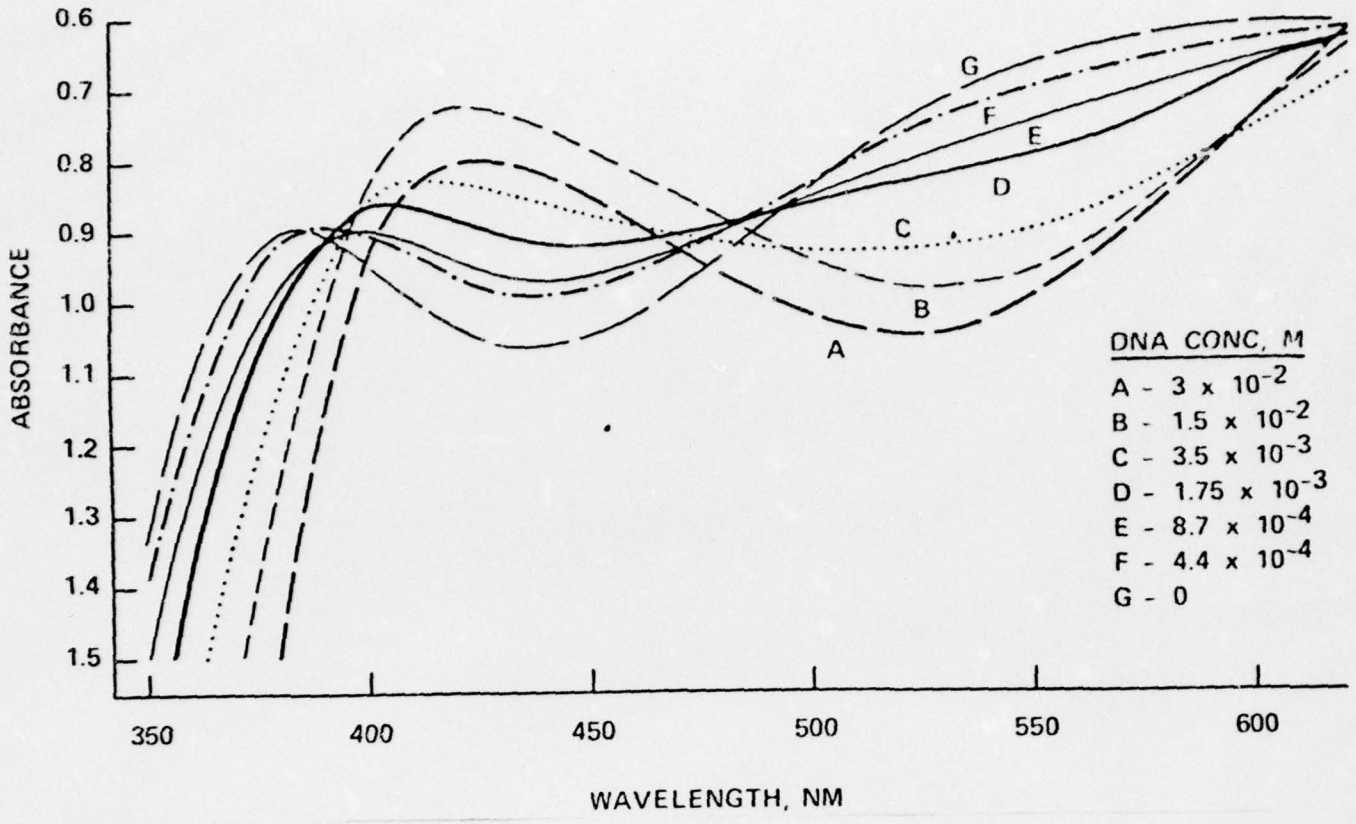


Figure 1. Absorption spectra of 3×10^{-4}M 2-Nitro-10- 3- 4-(2-hydroxy-ethyl)-1-piperazinyl propyl phenothiazine dimaleate, I, in 0.05M, pH 7.4 sodium phosphate buffer containing various quantities of salmon sperm DNA, 25°C.

a

b

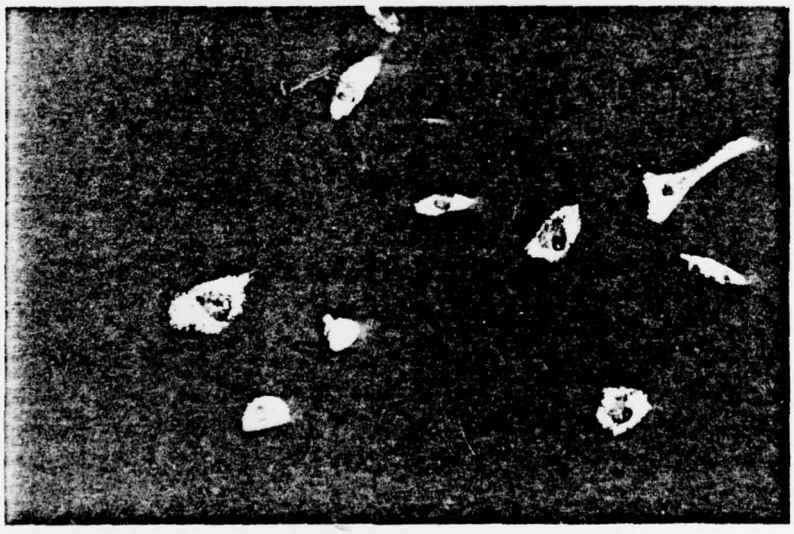


Plate 1. Human fibroblasts growing in 2-Cyano-10-{3-[4-(2-hydroxyethyl)-1-piperazinyl] propyl} phenothiazine dimethanesulfonate, II, three days after treatment. a) 5×10^{-6}M of II, b) 10^{-5}M of II. 160x by fluorescence microscopy.