Studies on the Formation of Phthalanilide-Deoxyribonucleic Acid Complexes and Their Relationship to Chemotherapeutic Activity

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SUMMARY

Complexes between DNA and substituted iso- and terephthalanilides occur in dilute aqueous solution and are measured by spectrophotometric methods. There appears to be no specificity among DNA preparations from different sources or different phthalanilides with regard to the stoichiometry of the interaction. The DNA- phthalanilide complex is less affected by heating and cooling than DNA alone. Most of the phthalanilide-DNA complexes examined are partially dissociable at moderate (0.01 m) salt concentrations and completely dissociable in 8 m urea. Poly-L-lysine also causes a dissociation of the complex. However, 1 congener, 4',4''-bis(N'-methylamidino)tereophthalanilide, forms a complex with DNA which is stable in 0.1 m CaCl₂ or 0.155 m NaCl.

In general, the phthalanilide-DNA complex appears to be the result of weak ionic forces, with the exception of 4',4''-bis(N'-methylamidino)tereophthalanilide. Hydrogen bonding and perhaps hydrophobic bonding are also thought to play a lesser role in the formation of phthalanilide-DNA complexes. The disposition of DNA as a helix or random coil may also affect the interaction.

The interaction between DNA and the phthalanilides in vitro does not appear to be correlated with the therapeutic efficacy of this class of compounds in the treatment of mouse leukemia, although it has not been ruled out as a possible primary site of action in vivo.

The relationships between DNA and the histones of the nucleus are thought to be critical to the function of DNA as a coding template for messenger RNA synthesis (1, 3, 13, 30). Oligoamines and diamines have been reported to interact with DNA to raise the temperature at which the helix → coil transition occurs (10, 16—19, 31). Diamines, such as cadaverine, also protect against the dark inactivation of DNA in the presence of proflavine (10). The association of aromatic amidines with DNA is suggested by the prevention of the antimicrobial action of stilbamidine by the addition of DNA to the growth medium (2). Thus, the finding that the polybasic substituted phthalanilides, which are potent chemotherapeutic agents against rodent leukemias (27), bind to DNA (28) has prompted an investigation into the nature of this interaction to determine its possible relationship to the chemotherapeutic effect of the drugs.

This report deals with the specificity of the interaction with regard to both drug structure and source of DNA. The stability of the DNA-phthalanilide complex to heat and in urea and salt solutions also was studied in order to define partially the forces involved in the binding of phthalanilides to DNA.

MATERIALS AND METHODS

The interaction between DNA and phthalanilides was characterized by spectral measurements, which were made at room temperature with a Zeiss spectrophotometer model PM QII. Complex formation was measured as the absorbance of the DNA-phthalanilide solution at 325 mμ minus the absorbance of the phthalanilide solution at 325 mμ; the contribution of DNA at 325 mμ was negligible (ΔA₁₉ = 0.002 − 0.005). The changes in spectra were observed immediately on mixing of the DNA and phthalanilide solutions. Although the spectra were constant up to 24 hr at room temperature (28), measurements were generally made within 30 min of the time the solutions were prepared. It was determined previously that the hyperchromic maximum of the difference spectrum occurred at 325 mμ and that the magnitude of the absorbance at 325 mμ increased in proportion to increased DNA con-

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3 The following abbreviations are used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

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centration at constant phthalanilide concentration. The spectra of 2-chloro-4',4''-di(2-imidazolin-2-yl)terephthalanilide and salmon sperm DNA and the stoichiometry of their interaction in water have been published (28). Based on the average molecular weight of a base pair of DNA, which is 630, 1 molecule of the phthalanilide formed a complex with 1 base pair equivalent.

The Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health supplied the phthalanilides, vincristine, and mitomycin C used in this study.

Poly-L-lysine-HBr was obtained from The New England Nuclear Corporation; it had a molecular weight of 190,000 based on viscosity measurements. Prior to use, a solution of the polymer (10 mg/10 ml) was dialyzed against 2 liters of distilled water for 3 days at 1°—4°C with changes of water each day.

The procedures described by Marmur (20) were employed to obtain DNA preparations from P388 cells that were either sensitive or resistant to NSC 60339. The ratio of absorbances at 230, 260, and 280 μm for DNA from sensitive cells was 0.49:1.00:0.58, and from resistant cells, 0.42:1.00:0.53. These values agree with the ones reported by Marmur as being representative of a typical DNA preparation. Highly polymerized salmon sperm DNA was obtained from Calbiochem.

The L1210 test system used for detecting antileukemic activity is described elsewhere (27). The compounds were tested over a wide range of doses. The optimal daily dose is defined as that which provides the maximum increase in mean survival time of the treated animals with respect to control animals in replicate experiments. A 25% increase in mean survival time is necessary for statistical significance.

RESULTS

STOICHIOMETRY AND SPECIFICITY OF PHTHALANILIDE-DNA INTERACTION

Many phthalanilide derivatives were examined for their ability to bind to DNA, and without exception all the compounds tested formed complexes. The variety of terephthalanilides capable of interaction with nucleic acids is shown in Table 1.

The formation of a phthalanilide-DNA complex in dilute aqueous solution was measured by the hyperchromicity at 320–330 μm of a difference spectrum taken between phthalanilide alone and phthalanilide in the presence of DNA. The absorbance of DNA at these wave lengths was negligible. The formation of the complex was proportional to phthalanilide concentration until all binding sites on DNA were occupied (Chart 1). Previously, we had determined, by varying DNA concentration at constant phthalanilide concentration, that the amount of DNA required for maximal complex formation with 3 representative compounds ranged between 1.1 and 1.4 μg of DNA/μg of phthalanilide (28). Titration of DNA with NSC 60339 resulted in a value of 1.25 μg of DNA/μg of NSC 60339, as compared to a value of 1.20 obtained by the inverse titration. Further, the capacity of different DNA preparations to interact with NSC 60339 was identical. Salmon sperm DNA, as well as DNA preparations isolated from P388 tumor cells, which were either sensitive or resistant to NSC 60339, were equivalent in their ability to form complexes (Chart 1). NSC 38278, a phthalanilide that was inactive against mouse leukemia, responded similarly. The low absorbance of the complex formed with NSC 38278 compared to the absorbance of the DNA-NSC 60339 complex is most likely due to the marked difference in their extinction coefficients at 325 μm. The molar absorbance of NSC 38278 at 365 μm is only about 25% of the molar absorbance of NSC 60339.

Since the DNA-phthalanilide interaction did not appear to show any specificity for the DNA preparations that were examined, it was of interest to determine if some drug specificity existed by comparing the interactions with DNA of 2 pairs of closely related phthalanilides. One compound of each pair had antileukemic activity and one was inactive. It is apparent from the data in Chart 2 that the active and inactive compound of each pair interacted with salmon sperm DNA to the extent of 1.0 to 1.5 μg of DNA/μg of phthalanilide and that there appears to be no significant difference between these active and inactive phthalanilides in the extent to which they form complexes with DNA in vitro.

In addition to the terephthalanilides listed in Table 1, 4',4''-di(2-imidazolin-2-yl)isophthalanilide (NSC 53212) formed a complex with DNA in a similar manner. Vincristine (NSC 67574) and mitomycin C (NSC 26980) were examined for their ability to form complexes with DNA, since certain mouse leukemias have been found to be cross resistant to vincristine and the phthalanilides (4, 5, 15), and mitomycin has been implicated as an inhibitor of DNA synthesis (24). The absorption spectra of these compounds in the presence of DNA gave no evidence of the existence of a complex. The failure to find evidence of complex formation with mitomycin in these experiments is consistent with the report of Iyer and Szybalski (14) that mitomycin reacts with DNA only after reduction and loss of a methoxy group.

The predominant structural features common to the molecules which have been found to interact with DNA are terminal amidino groups (often as imidazolin-2-yl) with various substituents and the phthalanilides either as the meta or para derivative. Molecular models of these compounds reveal that they can assume a variety of shapes from planar to horseshoe-like (Fig. 1) and that in the planar form, the nitrogens of the amide and amidine or imidazolynyl groups correspond rather closely to the spacing of the negatively charged phosphate groups of the DNA backbone. In an attempt to determine whether the phthalanilide-drug interaction was a simple ionic phenomenon or was more complex, the interaction was studied under various conditions.

HEAT DENATURATION OF DNA AND COMPLEX FORMATION

The heating and rapid cooling of DNA in solution is known to result in preparations containing a significant proportion of denatured DNA (6, 21—23). In order to develop some information bearing on the nature of the interaction between DNA and the phthalanilides, the effect of heating on the drug-DNA complex was examined.
<table>
<thead>
<tr>
<th>CCNSC No.</th>
<th>Terephthalanilide substituents</th>
<th>Antileukemic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Dose range used (mg/kg)</td>
</tr>
<tr>
<td>35843</td>
<td>4',4''-di(2-imidazolin-2-yl)-</td>
<td>2-128</td>
</tr>
<tr>
<td>53313</td>
<td>4',4''-bis(N'-methylamidino)-</td>
<td>0.38-120</td>
</tr>
<tr>
<td>64902</td>
<td>4',4''-bis(trimethylamidino)-</td>
<td>0.94-120</td>
</tr>
<tr>
<td>64907</td>
<td>4',4''-bis(N'-3-methoxypropyl)-amidino)-</td>
<td>0.75-120</td>
</tr>
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<td>53306</td>
<td>4',4''-bis(2-imidazolin-2-ylamino)-</td>
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<td>2-chloro-4',4''-di(2-imidazolin-2-yl)-</td>
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<td>38278</td>
<td>2-chloro-3',3''-di(2-imidazolin-2-yl)-</td>
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<td>63664</td>
<td>2-chloro-4',4''-bis(N'-methylamidino)-</td>
<td>1.88-120</td>
</tr>
<tr>
<td>62762</td>
<td>2-chloro-4',4''-bis(N',N''-dimethylamidino)-</td>
<td>1.25-120</td>
</tr>
</tbody>
</table>

* NSC 60339 is the free base; all other compounds are dihydrochlorides. NSC 64902 and NSC 63664 are monohydrates.

**CCNSC screening data.**

**Solutions of salmon sperm DNA (25 µg/ml)** and NSC 60339 and NSC 38278. The absorbance at 325 µm due to DNA alone is negligible at 325 µm.

**Chart 1.** Stoichiometry of DNA interaction with NSC 60339 and NSC 38278. The absorbance at 325 µm due to DNA alone is negligible at 325 µm.

**Chart 2.** Titration of the binding capacity of DNA for phthalanilides. The absorbance at 325 µm due to DNA alone is negligible at 325 µm.
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CHART 3.—The effect of heating and quenching on DNA in the presence and absence of NSC 60339.

wave length of absorption maximum or in the general shape of the absorption spectrum of NSC 60339, no decomposition is indicated. Nevertheless, when drug and DNA were heated together and quenched, the amount of absorbance at 320 m\(\mu\) due to the drug-DNA complex was found to be essentially the same as before heating (Chart 4). These data suggest that the presence of phthalanilide protects the DNA against heat denaturation. That this might be the case is also suggested by the data shown in Chart 3. The presence of NSC 60339 in DNA solution reduces by half the hyperchromicity at 260 m\(\mu\) caused by heating and rapid cooling in the absence of the phthalanilide, suggesting that the protective action of the phthalanilides on the secondary structure of DNA might be similar to that of polyamines such as spermine (19).

EFFECT OF UREA ON COMPLEX FORMATION

In 8 M urea, the spectra of DNA and NSC 60339 exhibited a hypochromicity of 12\% at the absorption maximum of each of the compounds. Further, the formation of a DNA-phthalanilide complex was prevented in 8 M urea as determined by the difference spectrum between drug alone and drug in the presence of DNA (Chart 5). In 4 M urea, the DNA-phthalanilide complex was unaffected.

EFFECT OF COUNTERIONS ON COMPLEX FORMATION

Since inorganic cations have been reported to bind to both double- and single-stranded nucleic acid polymers

CHART 4.—The effect of heating and quenching on the NSC 60339-DNA complex. The conditions were as stated in Chart 3.

CHART 5.—The effect of 8 M urea and 0.155 M NaCl on the NSC 60339-DNA complex. The control contains salmon sperm DNA (10 \(\mu\)g/ml) and NSC 60339 (8 \(\mu\)g/ml) in water.
(7–9), the influence of cations on the formation of phthalanilide-DNA complexes was examined. The formation of the DNA-phthalanilide complex was attempted in 0.155 M NaCl with the use of NSC 60339. The results showed clearly that the hyperchromicity at 320–330 m\textmu, which was due to complex formation, was completely eliminated. However, some reduction in absorbance in the region from 250 to 300 m\textmu remained, indicating that an interaction, other than a cation-dependent one, between DNA and NSC 60339 remained (Chart 5). In addition, the absorption spectrum of DNA alone showed a hypochromicity of about 10\% at 260 m\textmu in 0.155 M NaCl as compared to the spectrum of DNA in water. Phthalanilide spectra were unaffected by these changes in ionic environment. These results suggest that complex formation may also depend on the amount of DNA present in helical or random coil form.

In a similar set of experiments with NSC 53313, 8 m\text{urea} caused a complete dissociation of the complex, whereas in 0.155 M NaCl, the DNA-NSC 53313 interaction was not disturbed, suggesting that the ionic forces in this interaction were markedly stronger than those observed with other phthalanilides.

The finding that the NSC 60339-DNA complex was partly dissociable in 0.155 M NaCl led to a study of the effect of concentration of DNA and various cations on the interaction of DNA with several phthalanilides, some active and some inactive as chemotherapeutic agents in mouse leukemia, to obtain an estimate of the degree of complex formation which might occur in vivo.

Chart 6 presents a typical set of determinations, which show that the magnitude of ion-dependent DNA-NSC 60339 complex formation, as measured by absorbance differences at 325 m\textmu, is related to both Ca\textsuperscript{++} and DNA concentration.

Of all the phthalanilides examined, the only one forming a complex with DNA that appeared to be stable to high salt concentrations was NSC 53313. All of the other phthalanilides were almost completely dissociated from DNA in 10\textsuperscript{-2} M Ca\textsuperscript{++}, except for NSC 35843, whose complex with DNA was only 0.1 as sensitive to cation concentration as the others studied in this system (Chart 7).

Poly-L-lysine also was examined for its ability to prevent the formation of NSC 60339-DNA complex and was found to be quite effective (Chart 8). Furthermore, the addition of poly-L-lysine to a solution in which a complex was demonstrable caused a dissociation of the portion of the

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**Chart 6.**—The effect of calcium chloride and DNA concentration on the NSC 60339-DNA complex. The absorbance at 325 m\textmu due to DNA alone is negligible at 325 m\textmu.  

**Chart 7.**—The effect of calcium chloride on DNA-phthalanilide complexes.

**Chart 8.**—The effect of poly-L-lysine on the NSC 60339-DNA complex.
interaction which was sensitive to cation concentration. As in the case of complex dissociation in the presence of Na\(^+\) or Ca\(^{2+}\), a residual hypochromicity was found in the region from 250 to 300 nm of the difference spectrum.

**DISCUSSION**

The interaction of imidazolinyl- and amidino-substituted phthalanilides with nucleic acids appears to be a general phenomenon characteristic of a variety of polybasic compounds. Although the exact nature of this interaction cannot be defined completely with the information presently available, some properties of the mode of binding of these compounds to nucleic acids can be described.

**SPECIFICITY**

Since DNA preparations from different vertebrate sources (calf thymus, salmon sperm, mouse L cells) appear to contain a similar proportion of guanine + cytidine residues, as reflected by the melting temperatures (17, 21, 22, 24), the lack of specificity of the phthalanilide-DNA interaction with the 3 samples of DNA studied is not surprising. Furthermore, if the interaction is dependent largely on the ionic binding between the terminal nitrogen of the substituted phthalanilides and the phosphates of the DNA chains, the interaction would not be specific for any particular type of nucleic acid. In fact, yeast RNA and polyadenylic acid do interact with phthalanilides, albeit somewhat differently than DNA (28).

Spacing of the nitrogen atoms of the phthalanilides does not appear to be a critical factor, since the flexibility of the phthalanilide molecules could allow for the formation of complexes with DNA regardless of spacing. Also, the stoichiometry of the reaction reveals that the capacity for binding approximates 1 mole of phthalanilide/mole of DNA base pair. Thus, the phthalanilide, at concentrations which saturate the nucleic acid-binding sites, probably does not lie along the phosphate backbone of the helix, but rather, it may protrude from a single binding site and possibly cross-link to another DNA chain or assume a horseshoe configuration and bind to adjacent or nonadjacent phosphates of the nucleic acid.

Since compounds which are therapeutically active as well as inactive against mouse leukemia react to the same extent with DNA, specificity of interaction cannot be correlated with therapeutic efficacy. Thus, although the DNA-drug interaction may be ultimately the critical one, other physiologic reactions apparently must occur before tumor inhibition is manifest. The results of recent experiments have suggested that cell permeability to the phthalanilides may be one of the most important factors responsible for the efficacy of some phthalanilides and the ineffectiveness of others that are structurally similar.

**BINDING CHARACTERISTICS**

The influence of ionic strength, 8 M urea, and heat on the properties of the DNA-phthalanilide interactions has revealed that the reaction is not simply an ionic attraction. The ionic interaction is most likely the primary one, and, in many respects, it is similar in character to the interactions of diamines with DNA reported by Mahler and Mehrotra (17). Except for NSC 53313, increases in ionic strength result in concomitant decreases of the extent of the DNA-phthalanilide interactions. Nevertheless, the interaction is not disrupted completely by the presence of counterions to DNA, suggesting that other forces are operating in the formation of the complexes. Possibly, the majority of the phthalanilides tested react with the coiled form of DNA, whereas those that form stable complexes in the presence of cations may react with both helical and coiled forms of DNA. The influence of 8 M urea, which completely disrupts the interaction, indicates that hydrogen bonding and hydrophobic interactions may play a significant role in the stability of the complex. The protection of DNA by a phthalanilide against the denaturing effects of high temperature is another property which is comparable to the types of reactions observed with spermine and the diamines on the melting temperature of DNA (10, 16–19).

If the binding of phthalanilides to DNA or RNA has any physiologic importance, certain biochemical reactions directly related to the function of nucleic acids might be inhibited. Although gross RNA, DNA, protein, and lipid syntheses were apparently inhibited to some degree by exposure of mouse leukemia cells to a phthalanilide (NSC 60339) in vivo (26), no definitive primary biochemical lesion has been associated with drug exposure either in vivo or in vitro (4, 5, 11, 15, 26, 29, 32–35).

**ADDENDUM**

After this manuscript was submitted, Ochoa and co-workers (25) reported the inhibition of amino acid incorporation into polypeptide by microsomes from NSC 60339-treated L1210 cells. This microsomal inhibition may be the locus of phthalanilide effect on protein synthesis; however, these results do not explain our reports on the effects on nucleic acid synthesis (26) or lipid synthesis as amplified by Gellhorn et al. (12, 26).

**REFERENCES**


Fig. 1.—Planar and horseshoe configurations of NSC 38280.
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