The Effect of Metabolites of 2-Naphthylamine and the Mutagen Hydroxylamine on the Thermal Stability of DNA and Polyribonucleotides*

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SUMMARY

The melting properties of salmon sperm deoxyribonucleic acid (DNA) were used as a criterion for the study of the in vitro reactions of hydroxylamine, a phage mutagen, and aromatic amines related to the bladder carcinogen 2-naphthylamine. The mutagen hydroxylamine and the tissue carcinogens, 2-amino-1-naphthol and 1-amino-2-naphthol, lowered the transition midpoint (Tm) of salmon sperm DNA irreversibly and had similar effects on synthetic polyribonucleotides. The reaction appeared to be specific, since a number of metabolically related noncarcinogens had no effect on the Tm. The carcinogen 2-amino-1-naphthol was bound to the DNA in proportion to its ability to lower Tm, whereas chemically similar compounds without action on the Tm were not bound. The parallel action of a mutagen and carcinogen on DNA is of interest to the understanding of the mechanism of carcinogenesis.

Recently it has become apparent that an increasing number of carcinogens are capable of interacting with nucleic acids (10, 14, 23). These observations may be causally related to the mechanism of carcinogenesis as being a special case of mutagenesis. The chemical reactions of some mutagenic agents such as nitrous acid, hydroxylamine, and alkylating agents with DNA have been studied with the view of explaining their mutagenic action.

A need exists, however, for a physical method, sensitive to small chemical alterations in DNA, which, according to this hypothesis, could provide the means to predict mutagenic action.

The use of the "melting" behavior of DNA appeared promising from observations of modifications of this property as a result of ultraviolet radiation (16) and reactions with nitrous acid or mustard gas (12). This approach is explored in the present paper. In a group of chemically related aromatic amine derivatives, only the two ortho-aminophenols, known to be active carcinogens at the tissue level, caused an irreversible alteration in the secondary structure of salmon sperm DNA. Inorganic hydroxylamine, a known phage mutagen (11), was also shown to have a similar effect on the melting behavior of this DNA.

These changes become apparent through a significantly altered thermal stability, measured by hyperchromicity as a function of temperature. This technic presumably measures the tenacity of attachment between two strands of DNA, which in turn is largely determined by the complementarity of the bases guanine to cytosine and adenine to thymine. That this is indeed the mechanism for hyperchromicity was first clearly demonstrated by the experiment of Warner (27) showing a lowering of ultraviolet absorption when poly U

1 The abbreviations used are: poly A, polyadenylic acid; poly U, polyuridylic acid; poly I, polyinosinic acid; poly C, polycytidylic acid.
and poly A were allowed to combine. The lost ultraviolet absorption reappeared when the A+U polymer was heated, and the strands of poly A and poly U separated. The hyperchromic effect with heating has been observed with many different types of DNA (13, 15, 17). The midpoint of this rise of ultraviolet absorption has been called the transition midpoint (Tm) or "melting point." A clear relationship of guanine-cytosine content to the lowering of Tm can be readily rationalized, because the cytosine in the polymer has been chemically modified, thus reducing the complementariness of the two strands. The relative sensitivity of the melting point depression was indicated by the fact that the ultraviolet spectrum of DNA treated with hydroxylamine was unchanged, whereas the Tm was greatly lowered. We have made similar observations with synthetic polynucleotides which offered the additional opportunity of confirming that it was indeed cytosine and uracil that were attacked in the polymer in the reaction leading to a lower Tm.

The carcinogen used in our study was 2-amino-1-naphthol, the metabolic product of 2-naphthal, presumably responsible for bladder cancer in man and dog (5). This carcinogen appears to interact with nucleic acids through an unidentified oxidation product. When oxidation is prevented by addition of a reducing agent or by esterification of the phenolic group by sulfate, both interaction with DNA and tissue carcinogenicity are abolished (5).

MATERIALS AND METHODS

A sample of 2-naphthalmin, labeled in the 8-position with C14, was obtained commercially. This material was converted to 2-amino-1-naphthyl sulfate by the method of Boyland (8), and this was converted to 2-amino-1-naphthol by hydrolysis at 100° C. for 3 hours with 6 N HCl containing stannous chloride at 2 times the molar concentration of 2-amino-1-naphthyl sulfate. The 2-amino-1-naphthol crystallized from this mixture and was washed with 6 N HCl containing stannous chloride. Radioactivity was measured in a gas-flow counter and corrected for self-absorption. The concentrations of 2-naphthalmin and 2-amino-1-naphthyl sulfate were determined colorimetrically by converting them to naphthoquinone derivatives by reaction with 1, 2-naphthoquinone sulfonate (24). The concentration of 2-amino-1-naphthol was measured colorimetrically by conversion to the 1, 2-naphthoquinone with ferric chloride and coupling with aniline to form the 4-anilino-1, 2-naphthoquinone (25).

The oxidation products of 2-amino-1-naphthol were prepared by shaking 25 mg. 2-amino-1-naphthol hydrochloride in 25 ml. 0.1 M phosphate buffer, pH 7.5, and allowing the mixture to oxidize for 4 days at room temperature. A purple precipitate formed, and no free 2-amino-1-naphthol could be demonstrated by the colorimetric test involving the conversion to the 1, 2-naphthoquinone (25).

The 2-amino-1-naphthylglucosiduronic acid (9) and 2-amino-1-naphthol were prepared as described previously (25), and the 2-amino-1-naphthyl sulfate (8) and 2-amino-1-naphthylphosphate were prepared according to Boyland and Manson (6). Salmon sperm DNA was obtained commercially and had the ultraviolet absorption characteristics reported in the literature. The polynucleotides poly A, poly U, poly C, and poly I were obtained commercially from Miles Laboratories.

Reaction of DNA with chemicals.—Salmon sperm DNA, 250 µg/ml, was dissolved in 0.1 M phosphate 0.001 citrate buffer, pH 7.5, and allowed to react with the chemicals tested. Hydroxylamine was removed by 3X repeated dialysis against 100X its volume of 0.01 M phosphate, 0.001 M citrate, pH 7.5 (standard buffer). The aromatic amine compounds were removed by extraction with an equal volume of water-saturated phenol, followed by three extractions with water-saturated n-amyl alcohol. Alternatively the DNA was precipitated with indium chloride (1), and the precipitate was washed with ethanol until the wash solution was free of color or fluorescence. The precipitate was then dissolved in the standard buffer with addition of 0.1 M ethylenediaminetetraacetic acid. After either procedure the salt concentration was adjusted to that of the standard buffer by dilution or dialysis. The solutions were diluted to 10 ± 40 µg DNA/ml for the determination of the Tm.

Determination of Tm.—A Beckman spectrophotometer was modified by inserting a heating tape below the cell carriage. Tap water was circulated through the thermo spacers. One cuvette was fitted with a thermometer or glass-tipped thermistor, and absorbancy was determined in 1-cm. cuvettes with a capacity of 8 ml at 1°C temperature intervals. The temperature rise was kept at approximately 1°C/min. Identical curves were obtained when heating was carried out at either twice or half this rate. The Tm was the temperature of the median absorbancy increment. The slope was arbitrarily taken as the average absorbancy rise per degree
RESULTS

Hydroxylamine.—Hydroxylamine HCl, brought to pH 7.5 and allowed to react with DNA over an 8-day period at 37°, lowered both the Tm and slope. At 4° the effect on the melting profile was much smaller (Chart 1). This temperature effect was observed with all the active agents used and may represent the consequences of a slightly modified secondary structure of the DNA at higher temperatures. When cytosine was allowed to react with hydroxylamine following the procedure of Freese (11), the ultraviolet changes which he described were observed to appear slowly over an 8-day period. The absorption spectrum of the DNA solutions after 8 days of reaction with hydroxylamine was identical, however, with that of the control. The relatively lower reactivity of the cytosine in the DNA as compared with the free compound may be the basis for this finding.

The lowering of the Tm is apparently a more sensitive indicator of nucleic acid alteration than the ultraviolet absorption spectrum.

Similar findings were made with the synthetic polyribonucleotides. When poly U was exposed to hydroxylamine and then allowed to interact with poly A, the melting point was strikingly lowered compared with the control A + U polymer. On the other hand, when poly A was allowed to react with hydroxylamine and then treated with poly U, the Tm was identical to that of the control (Chart 2). This was the result to be expected from the known reaction of hydroxylamine with uridine and lack of reaction with adenosine (11) (Table 1). Similar experiments with

CHART 1.—Salmon sperm DNA (250 μg/ml) in 0.1 M sodium phosphate, 0.001 M sodium citrate buffer was treated with 1 M hydroxylamine adjusted to pH 7.5 at 37° C. Samples were removed at different time periods and dialyzed for 48 hours against 0.01 M sodium phosphate, 0.001 M sodium citrate, pH 7.5. Optical density measurements were performed from 35° C. to 65° C. at 1° intervals.

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polyinosinic acid and polycytidyllic acid revealed that both reactions resulted in a lowered Tm in the double-stranded I + C polymer. Chemical modification of the cytosine-containing compounds has been described (11). The reaction of hydroxylamine with inosine, however, will require investigation.

Naphthylamine and related compounds.—The effect of interaction of 2-naphthylamine and several related compounds with DNA is summarized in Table 2. All compounds were allowed to react

CHART 2.—The reaction of hydroxylamine with poly A and poly U. Conditions as described in Table 1.
for 8 days at 37° C. Significant lowering of Tm was observed only with 2-amino-1-naphthol and 1-amino-2-naphthol. The effect of reaction of 2-amino-1-naphthol over a 12-day period with DNA is shown in Chart 3. These results may be correlated with the biological activity of these compounds (Table 2). The aminonaphthols (1-amino-2-naphthol and 2-amino-1-naphthol) are active carcinogens in the mouse bladder test, whereas 2-naphthylamine and 2-amino-1-naphthyl sulfate are inactive (2, 5). The carcinogenicity of the glucosiduronate and phosphate conjugates of 2-amino-1-naphthol is probably due to the aminonaphthol formed by enzymatic hydrolysis. Urine and bladder mucosa contain very active phosphatases which have been shown to hydrolyze 2-amino-1-naphthyl phosphate. Much weaker glucuronidase activity has also been observed. Sulfatases, however, although present in urine and

TABLE 1

THE EFFECT OF THE REACTION OF HYDROXYLAMINE AND 2-AMINO-1-NAPHTHOL ON THE TM OF POLYRIBONUCLEOTIDES

Stock solutions of poly A, poly U, poly I, and poly C (0.5 mg polyribonucleic acid/ml 0.1 M phosphate, 0.001 M citrate buffer, pH 7.5) were treated for 3 days at 37° C. with 5 mg 2-amino-1-naphthol/ml polyribonucleic acid or for 5 days at 37° C. with 1 M hydroxylamine adjusted to pH 7.5. The 2-amino-1-naphthol-treated samples were extracted 3 times with water-saturated n-amyl alcohol and adjusted to 0.45 M sodium chloride, 0.01 M sodium phosphate, 0.001 M sodium citrate. The hydroxylamine-treated samples were adjusted to 0.45 M sodium chloride, 0.01 M sodium phosphate, 0.001 M sodium citrate with a G-25 Sephadex column. The reacted samples were treated with unreacted polyribonucleic acids in the same buffer solution.

<table>
<thead>
<tr>
<th>A+U polymers</th>
<th>Tm degrees centigrade</th>
<th>I+C polymers</th>
<th>Tm degrees centigrade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly A+U</td>
<td>69.5</td>
<td>Poly I+C</td>
<td>73</td>
</tr>
<tr>
<td>Poly A+U+U†</td>
<td>69.5</td>
<td>Poly I+I+U</td>
<td>65</td>
</tr>
<tr>
<td>Poly A+U</td>
<td>58.0</td>
<td>Poly I+I+U</td>
<td>58</td>
</tr>
<tr>
<td>Poly A+U</td>
<td>58.0</td>
<td>Poly I+I+U</td>
<td>51</td>
</tr>
<tr>
<td>Poly A+I</td>
<td>68.0</td>
<td>Poly I+I+U</td>
<td>69</td>
</tr>
<tr>
<td>Poly A+U</td>
<td>68.0</td>
<td>Poly I+I+U</td>
<td>71</td>
</tr>
<tr>
<td>Poly A+U</td>
<td>66.5</td>
<td>Poly I+I+U</td>
<td>65</td>
</tr>
</tbody>
</table>

* The samples were placed on a Sephadex-G25 column equilibrated with the 0.45 M sodium chloride buffer and eluted in the outside volume with the same buffer.
† Subscript h-polymer reacted with hydroxylamine at 37° C. 5 days.
‡ Subscript an-polymer reacted with 2-amino-1-naphthol at 37° C. 5 days.

TABLE 2

THE EFFECT OF NAPHTHYLAMINE METABOLITES ON THE MELTING OF SALMON SPERM DNA

Salmon sperm DNA, 250 μg/ml of 0.1 M phosphate, 0.001 M citrate, pH 7.5, was treated with metabolites of naphthylamine for 8 days. The solutions were freed of the metabolite by thorough extraction with phenol, n-amyl alcohol, and dialysis against 0.01 M sodium phosphate, 0.001 M sodium citrate, pH 7.5.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Conc. (mg/ml)</th>
<th>Tm</th>
<th>Slope</th>
<th>Cancer in situ</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Naphthylamine</td>
<td>5</td>
<td>71-74</td>
<td>3.3</td>
<td>–</td>
<td>(5)</td>
</tr>
<tr>
<td>2-Amino-1-naphthol</td>
<td>2</td>
<td>74</td>
<td>3.3</td>
<td>–</td>
<td>(2, 5)</td>
</tr>
<tr>
<td>2-Amino-1-naphthol+sodium dithionite</td>
<td>2</td>
<td>60</td>
<td>0.6</td>
<td>+</td>
<td>(+SnCl₂, 5)</td>
</tr>
<tr>
<td>1,3-Naphthoquinone</td>
<td>5</td>
<td>73</td>
<td>2.9</td>
<td>-</td>
<td>(4)</td>
</tr>
<tr>
<td>2-Amino-1-naphthol</td>
<td>3</td>
<td>71</td>
<td>2.7</td>
<td>+</td>
<td>(4)</td>
</tr>
<tr>
<td>2-Amino-1-naphthol phosphate</td>
<td>5</td>
<td>60</td>
<td>0.8</td>
<td>+</td>
<td>(4)</td>
</tr>
<tr>
<td>2-Amino-1-naphthol glucoseiduronide</td>
<td>5</td>
<td>73</td>
<td>3.0</td>
<td>+</td>
<td>(4)</td>
</tr>
<tr>
<td>2-Amino-1-naphthol sulfate</td>
<td>2</td>
<td>71</td>
<td>2.8</td>
<td>+</td>
<td>(4)</td>
</tr>
<tr>
<td>2-Hydroxylamino naphthalene</td>
<td>2</td>
<td>72</td>
<td>2.0</td>
<td>–</td>
<td>(2, 5)</td>
</tr>
<tr>
<td>Oxidized products of 2-amino-1-naphthol</td>
<td>0.5</td>
<td>63</td>
<td>1.3</td>
<td>–</td>
<td>Footnote 3</td>
</tr>
</tbody>
</table>

* These compounds are not soluble to this extent and were used in suspension.
tissue, are unable to hydrolyze 2-amino-1-naphthyl sulfate (2). The conversion of 2-naphthylhydroxylamine, which is also carcinogenic, to 2-amino-1-naphthol by tissue enzymes is a possibility, since the analogous N-hydroxy-2-acetyl aminofluorene is converted to ortho-hydroxy metabolites (18).

The presence of a reducing agent such as sodium dithionite prevents the reaction with the DNA. This parallels the observation that the presence of stannous ion in 2-amino-1-naphthol abolished the carcinogenicity of this compound (5). The major oxidation product of 2-amino-1-naphthol in tissues is 1, 2-naphthoquinone, which reacts with the amino group of lysine in proteins (4). The 1, 2-naphthoquinone is, however, inactive with DNA. Other oxidation products which are possible candidates for reaction with DNA are the intermediate-free radical, the iminoquinone, or polymers formed between oxidized products. The latter view was supported by the observation that aminonaphthol oxidation products did lower the Tm of the DNA (Table 2).

A definite relationship was observed between the lowering of Tm and the quantity of radioactivity bound to the DNA when radioactive 2-amino-1-naphthol was used as a reactant (Chart 4). Reaction of 0.4 μM 2-amino-1-naphthol/m mole DNA phosphorus lowered the Tm by 1°C. The radioactivity bound to the DNA was not removed by amyl alcohol extraction or dialysis. The quantity bound was a direct function of time during the first 8 hours, probably representing the rate of formation of the active oxidized intermediate. The melting point lowering follows the same pattern with time as the increase in bound material. No significant binding of radioactivity was observed when 2-naphthylamine or 2-amino-1-naphthol in the presence of dithionite was used in the reaction with DNA. This was ascertained by incubating 2 mg. of C14-labeled aromatic amines, containing 4.5 X 10^7 counts/min/m mole

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**Chart 3.**—Salmon sperm DNA (250 μg/ml) treated at 37°C for different time intervals with 2-amino-1-naphthol (2 mg/ml). Solutions were dialyzed for 48 hours against 0.01 M sodium phosphate, 0.001 M sodium citrate. Optical density measurements were performed from 95°C to 90°C at 1°C intervals.

**Chart 4.**—Salmon sperm DNA (250 μg/ml) in 0.1 M sodium phosphate, 0.001 M sodium citrate buffer, pH 7.5, was treated with C14-labeled 2-amino-1-naphthol (2 mg/ml) at 37°C for various times. The solutions were extracted 3 times with water-saturated n-amyl alcohol and dialyzed for 48 hours against 0.01 M phosphate, 0.001 M citrate buffer, pH 7.5. Tm was obtained as described in the experimental part, and aliquots of each solution were used for measurement of radioactivity in a gas-flow counter.

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2-amino-1-naphthol, in the presence of 10 mg. sodium dithionite, with 250 μg. DNA in standard buffer for 24 hours at 37°C, and observing no significant radioactivity remaining in the DNA after solvent extraction and dialysis as described above. The lack of effect on the Tm under the same reaction conditions supports a relationship between binding and effect on Tm shown in Chart 4 for 2-amino-1-naphthol.

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no significant Tm changes were observed before the 7-day incubation (Chart 5). The lowering of Tm observed after 7 days of incubation may be due to the interaction of purines or pyrimidines chemically modified by 2-amino-1-naphthol.

No decisions as to which purine or pyrimidine is most decisively affected by 2-amino-1-naphthol could be made by studying the interaction with synthetic polyribonucleotides. It was apparent, however, that interaction with the carcinogen had a somewhat greater effect on the Tm if either poly I or poly C, rather than poly U or poly A, was reacted (Table 1). Further work is clearly indicated to learn the precise nature of the reaction of the carcinogen with DNA.

**DISCUSSION**

Although there appears little doubt that combination with a tissue constituent must precede the modification of normal cells to malignant ones, the nature of this constituent has remained obscure. Many investigators have stressed the importance of protein combination with carcinogens, pointing in some cases to a relationship of protein binding to carcinogenesis (21). Indeed, in work reported from our laboratory concerning the combination of 2-amino-1-naphthol with bladder tissue, protein combination with the oxidation product 1, 2-naphthoquinone was observed to be quantitatively the most extensive binding (4).

On the other hand, progress in the understanding of the biochemical genetics, as well as the clear demonstration that viruses containing RNA and DNA are causative agents of certain cancers, have made nucleic acids an attractive object for carcinogen reactions. The main difficulty with the hypothesis that nucleic acids are modified by carcinogens has been a failure of clear demonstration of reaction (21). Yet recent observations of incorporation of radioactive N-hydroxy-N'2-ace
tylaminofluorene, after feeding, into liver DNA, as well as the demonstration that DNA binds dimethylnitrosamine, have given encouragement to the somatic mutation theory of carcinogenesis (10, 14). The present paper, concerning the in vitro DNA reaction with carcinogens, demonstrates a high selectivity among compounds for reaction. The carcinogens used in this study are related to a group of bladder carcinogens—i.e., aromatic amines with the para position blocked, such as 2-naphthylamine, benzidine, and 4-amino-biphenyl. The view that these compounds cause bladder cancer in man by virtue of their ortho-hydroxy metabolites has long been expounded, and only recently has this view found a competitor in the hydroxylamine metabolite described by Cramer, Miller, and Miller (9) from the analogous acetylanilinofluorene. The corresponding 2-hydroxylaminonaphthalene has been observed as a metabolite of 2-naphthylamine in man and dog (7, 26), and this compound has recently been shown to be carcinogenic in the mouse bladder test.3 Our in vitro work with DNA demonstrates that 2-amino-1-naphthol does react with DNA and that 2-hydroxylaminonaphthalene does not. Although the final decision as to the precise nature of the proximal carcinogen cannot be made from the available data, the ortho-aminophenol, or more precisely an unidentified oxidation product of this compound, appears to be a strong candidate for the following reasons: (a) It serves to explain the importance of the blocked para position of these carcinogens; the block making orthohydroxylation imperative. This is also true for the aminofluorene compounds since the 4-acetylanilinofluorene is inactive as a carcinogen (28). (b) The rearrangement of hydroxylamino compounds is a known chemical reaction (20). Indeed, the in vitro biological conversion of N-hydroxy-2-acetylanilinofluorene to 1-hydroxy-2-acetylanilinofluorene has been demon-
The mechanism proposed for this conversion involves deacetylation, which would lead to the ortho-aminophenol as an intermediate (20). Although the lack of carcinogenic activity of the 1-hydroxy-2-acetylaminofluorene on producing mammary cancer on intraperitoneal injections (19) and the failure of 1- and 3-hydroxy-2-acetylaminofluorene on feeding to produce liver cancer (92) point out the unusual position of the acetyl-N-hydroxy compound in these experimental cancers, it does not exclude the possible final role of an ortho-hydroxy amine in the production of these tumors. No evidence that the acetylated amines were hydrolyzed before conjugation of the hydroxyl group to sulfate and glucuronide was obtained, and it is difficult to speculate about the proximal carcinogen from experiments involving many metabolic steps such as feeding or intraperitoneal injections at a site removed from the occurrence of the tumor (19, 92). The greater carcinogenicity of N-hydroxy-2-acetylaminofluorene may in fact be due to its capacity to pass through cell membranes and then rearrange to an ortho-hydroxylation product. The reverse reaction, the conversion of the ortho-aminophenol to a hydroxyaminofluorene, is highly improbable. This would tend to put the ortho-aminophenol at a later stage in metabolism than the 2-hydroxyaminonaphthalene.

ACKNOWLEDGMENTS

The continued interest and advice of Dr. Norton Nelson are gratefully acknowledged.

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