THE INHIBITION OF DEOXYRIBONUCLEASE I BY HYDROXYBIPHENYLS


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Running Title: HYDROXYBIPHENYLS AS INHIBITORS OF DNAase I

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above address.
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SUMMARY

DNA extracted with certain commercial brands of phenol is resistant to hydrolysis by the endonuclease DNAase I, while DNA extracted with other brands, or prepared by sodium chloride extraction, is susceptible to hydrolysis. The agent responsible for inhibition has been shown to be an oxidation product produced in some phenols. The inhibitor has been separated from other impurities in phenol by paper chromatography, and, by means of infrared and ultraviolet spectroscopy, it has been identified as o-hydroxyphenylvinyl. The kinetics of inhibition have been studied, and it was found that inhibition arises from direct action on the DNA rather than on the enzyme. Several hydroxybiphenvls and

Abbreviations: sDNA, DNA prepared by NaCl extraction; nDNA, DNA prepared by phenol extraction; Pu, an unidentified purine; Py, an unidentified pyrimidine; CD, circular dichroism; SSC, saline

sodium citrate; PVP, polyvinyl pyrrolidone.

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related compounds have been tested for inhibition, and a theory of molecular structure versus inhibitory effectiveness is suggested from this data. From studies on the chemical reversal of inhibition, as well as from ultraviolet spectral studies (in both absorption and circular dichroism), it appears that the mode of action of the inhibitors is hydrogen bonding to, and intercalation between, the bases of the nucleic acid.

INTRODUCTION

Deoxyribonuclease I (deoxyribonucleate oligonucleotide hydrolase, EC 3.1.4.5) is an endonuclease of molecular weight 30 700 that preferentially hydrolyzes linkages of polydeoxyribonucleotides of the type Pu-3′-D-5′-Py. Hydrolysis occurs between the 5′-phosphate and the 3′-hydroxyl to yield oligonucleotides terminating with phosphoryl groups in the 5′ position. Bivalent cations such as Co²⁺, Zn²⁺, Mn²⁺, Mg²⁺, and Ca²⁺ are required for enzymic activity. These metals are required by the enzyme to maintain disulfide linkages in their oxidized form; reduction of these linkages is accompanied by a loss of activity. Anions such as fluoride, citrate, arsenate, borate, and selenite, which can react with the bivalent cations, are known to inhibit the action of the enzyme. Several non-activating cations can displace the activating bivalent cations and cause inhibition. These are Fe²⁺, Fe³⁺, Cr²⁺, and
Ni$^{2+}$ (ref. 1). FESTY et al.\(^4\) have shown that Fe$^{2+}$ can inhibit DNAase by the formation of a DNA-Fe complex.

LINDEBERG\(^5\) has reported the isolation from beef pancreas of two proteins that inhibit DNAase. An inhibitor-enzyme complex of molecular weight 81,600 has been isolated\(^6\).

DNAase is inhibited by such drugs as ethidium bromide and actinomycin D\(^7\). These compounds exert their action on the DNA; one drug molecule can protect 40 to 50 base pairs from the action of the nuclease. WARRING\(^8\) has established the nature of the ethidium bromide interaction with DNA as intercalation. WAGNER\(^9\) has presented CD evidence for the intercalation of ethidium bromide into DNA.

HOFMANN-OSTENHOF and FRISCH-NIEGEMEYER\(^10\) have reported that two- and three-ring quinones, such as α- and β-naphthoquinone, anthraquinone, and phenanthraquinone, act as inhibitors of DNAase, while such one-ring quinones as benzocaquinone, toluquinone, 5-methyl-toluquinone, and thymoquinone, serve as activators of the enzyme. Furthermore, such phenols as pyrocatechol, hydroquinone, and 1,2,4-benzenetriol, are also activators of the nuclease. These authors suggest that the action of these compounds is on the enzyme; however, no experimental data has been presented to support this.

We have found that DNA extracted with J. T. Baker Liquefied Phenol is resistant to enzymatic hydrolysis, while DNA extracted with Mallinkrodt Liquefied Phenol, or DNA prepared by sodium chloride extraction is susceptible to hydrolysis by the nuclease.
Evidence will be presented that the agent responsible for inhibition acts directly upon the DNA substrate, rather than on the enzyme. We report the isolation and identification of the inhibitor from J. T. Baker phenol, and the testing of related compounds. Spectral studies and investigations on the reversal of inhibition have revealed a possible mode of action of the inhibitors.

MATERIALS AND METHODS

Biochemicals and chemicals

DNA was the A Grade, highly polymerized, sodium salt product from herring sperm or calf thymus obtained from Calbiochem, Los Angeles, Calif. The deoxvribonucleosides were also products of Calbiochem. Lysozyme (muramidase, EC 3.2.1.17) was purchased from the Worthington Biochemical Corp., Freehold, New Jersey. The DNAase used throughout this work was Worthington endonuclease I from beef pancreas. Poly d(A-T), an alternating copolymer of deoxvadenvlate-thymidylate, and dried cells of Micrococcus lysodeikticus (control no. 6162) were products of Miles Laboratories, Elkhart, Indiana. Polyvinyl pyrrolidone was purchased from the General Aniline and Film Corp., New York, N. Y. Tryptamine [3-(2-aminoethyl)-indole] was purchased from the Aldrich Chemical Co., Milwaukee, Wis. All of the hydroxvbinhenyls and related compounds were obtained from the Dow Chemical Co., Midland, Mich. Liquefied phenol was purchased from the J. T. Baker Chemical Co., Phillipsburg, New Jersey, and from the Mallinckrodt Chemical Works, St. Louis, Missouri. All other chemicals were of reagent grade.
Bacterial strains and growth conditions

All strains of *Escherichia coli* used as sources of DNA were derived from wild-type K-12. All cultures were grown with aeration at 37° on Difco-Bacto Nutrient Broth (8.0g/l).

**Extraction of DNA from bacteria**

A modified version of the extraction technique of **MAPMUR** was used for obtaining DNA from strains of *E. coli*. A 500-ml overnight culture was used as the inoculum for a 4-liter culture. Cells were grown for 5 h and harvested by centrifugation at 0° for 20 min at 16 300 X g. The cells were suspended in 50 ml saline-EDTA (0.15 M Na₂EDTA, pH 8.0). The cells were then treated with 20 mg of lysozyme for 15 min at 37°. Sodium dodecyl sulfate (10 ml of a 10% (w/v) solution) was added, and the suspension was allowed to stand for an additional 15 min at 37°. To insure complete cell lysis, the mixture was frozen in a solid CO₂-isopropanol bath, and thawed. Freezing and thawing was repeated twice more. The lysed-cell suspension was combined with an equal vol. of liquefied phenol saturated with Na₂EDTA (titrated to pH 7.0). After gentle agitation by hand for 10 min, the mixture was centrifuged at 32 800 X g for 20 min at 0°. The aqueous phase was removed, the DNA precipitated with an equal vol. of cold 95% ethanol, and collected on a glass stirring rod. The DNA was then transferred to 0.1 X SSC (1.0 X SSC is 0.15 M NaCl plus 0.015 M Na₃Citrate) and the SSC concentration was brought to 1.0 X by the addition of 0.1 vol. of 10.0 X SSC.
Sodium chloride-extracted DNA from both _E. coli_ and _M. lysodeikticus_ was prepared by the method of Zamenhof et al.\(^\text{12}\). We wish to thank Prof. I. Tinoco of the Department of Chemistry, U. C., Berkeley, for the gift of dried cells of _M. lysodeikticus_.

**DNase assays**

The spectrophotometric assay technique of KUNITZ\(^\text{13}\) was the procedure used most frequently in this work. DNase was used at a final concentration of 5 µg/ml (except where noted differently). Two assay buffers were used: (a) 0.01 M MgSO\(_4\) plus 0.05 M Na acetate, pH 6.5; (b) 0.008 M MgSO\(_4\), 0.002 M CaCl\(_2\), and 0.02 M Tris, pH 6.7. Absorbance at 260 nm was measured on a Cary Model 14 Recording Spectrophotometer. The rates of hydrolysis with buffer (a) were somewhat lower than with buffer (b).

We have also measured the hydrolysis of DNA by the nuclease with a pH-stat, using an instrument similar to the one described by Pensky and Szucs\(^\text{14}\). This assay relies on the liberation of one mole of acid for every mole of phosphodiester bonds broken. Thus, by recording the amount of base of known normality required to maintain constant pH during the course of the reaction, and knowing the total amount of DNA in the reaction mixture, the percent hydrolysis can be calculated. The assay was carried out in 0.01 M MgSO\(_4\) at pH 7.0, with a final DNase concentration of 25 µg/ml. All solutions added to the reaction vessel during the course of hydrolysis were previously adjusted to pH 7.0. A gentle stream of nitrogen gas was passed through the reaction vessel to dissipate carbon dioxide. Sensitivity of the instrument was such
that 0.35 ml of 2.0 mM NaOH (prepared fresh every few days) delivered by the micrometer syringe gave full scale deflection.

Oxidation of phenol

One liter of liquefied phenol plus 2.0 g of anhydrous V$_2$O$_5$ was refluxed for 18 h. During reflux, a steady stream of air was passed through the reflux condenser. After refluxing, the phenol was removed by simple distillation, and the residue was taken up in CCl$_4$.

Chromatography

The inhibitor was separated from the other oxidation products of phenol by chromatography on Whatman 3 MM paper with a solvent (to be referred to as the "standard solvent") containing acetic acid - methanol - water (2:25:73, by vol.).

Physical methods

All ultraviolet spectra were taken with the Cary Model 14, and infrared spectra with the Perkin-Elmer Model 127 Sodium Chloride Spectrophotometer. DNA melting profiles were followed by increase in absorption at 260 mp using a Beckman Model DU Spectrophotometer equipped with a Gilford Model 2000 Multiple Sample Absorbance Recorder and a Haake programmed temperature bath. Circular dichroism measurements were made with a Cary Model 60 Spectropolarimeter equipped with a Cary Model 6001 CD attachment. Magnetic circular dichroism measurements were made with an instrument previously described by DRATZ. The path length for all ultraviolet measurements (absorption and CD) was one cm. Mass spectra were taken using an A. E. I. MS-12 mass spectrometer.
Spectra were determined at an ionizing voltage of 70 eV and an ionizing current of 50 μA. The temperature of the ion source was varied from 150° to 200°. Each spectrum was scanned in 7 sec and recorded on an oscillograph recorder.

RESULTS
Effect of DNAase on the DNA preparations

It was found that DNA extracted with J. T. Baker Liquefied Phenol (Baker pDNA) was resistant to the action of DNAase I, while DNA extracted with Mallinckrodt Liquefied Phenol (Mallinckrodt nDNA) was susceptible to hydrolysis. Furthermore, both commercial sodium-salt DNA and DNA prepared by NaCl extraction (sDNA) were readily susceptible to the action of the nuclease. After dialysis of the Baker pDNA at 4° for three 24 h periods, each against 1 liter of 1.0 X SSC, the DNA was still resistant to DNAase hydrolysis. Table I summarizes the results of these preliminary DNAase assays. We note that the relationship between hyperchromicity and percent hydrolysis (as determined by liberated acid) is that approximately twice the percent increase in the absorbance at 260 μν equals the percent hydrolysis.

Under no conditions of temperature, pH, or ionic strength of the assay buffer could the Baker pDNA be hydrolyzed with DNAase; in addition, we knew that this DNA was highly polymerized (and had not been hydrolyzed during the extraction procedure) since it was susceptible to thermal hydrolysis (accompanied by 25% hyperchromicity) and hydrolysis by prolonged exposure to the nuclease. Furthermore, the commercial or E. coli sDNAs could be made resistant to
hydrolysis by treating them before the start of the assay with Baker
phenol (DNA at a final concentration of 20 μg/ml and phenol at a final
concentration of 1 mg/ml for complete inhibition). As long as the pH
of the assay medium was held constant, at no concentration did Mallin-
ckrodt phenol afford protection of susceptible DNAs to hydrolysis.
Freshly-distilled Baker phenol did not inhibit the hydrolysis of sDNA.
However, if the distilled Baker phenol was exposed to air and light
for a period of a few weeks, the inhibitor was found once again to be
present.

In addition to phenol purchased from J. T. Baker, phenol manufac-
tured by the chlorobenzene process, and obtained from the Dow
Chemical Co., contains oxidation products that inhibit DNAase. However,
Dow phenol made by the benzoic acid process does not produce the
inhibitory oxidation products as readily as does the chlorobenzene
phenol.

We conclude that the inhibitor acts directly upon the DNA and
not the enzyme from the following experiment: To one quartz cuvette
was added Baker pDNA and DNAase (20 μg/ml DNA and 5 μg/ml enzvne), and
to a second cuvette was added only DNAase. After 10 min there was no
significant change in the A260 μm in either cuvette. Commercial sDNA
was then added to both cuvettes (to a final concentration of 20 μg/ml).
After incubation for an additional 10 min, identical increases in A260 μm
were observed in both cuvettes, indicating that the enzyme was still
active after exposure to Baker pDNA. Similar results were obtained
with this experiment using the pH-stat technique. Fig. 1 illustrates
percent hydrolysis plotted against time for the pH-stat assay.
Isolation and identification of the inhibitor

Small quantities of inhibitor were obtained from Baker nDNA by means of Bio-Gel P-10 column chromatography. Since the nDNA can be hydrolyzed if it is exposed to the enzyme for a period of several hours (see Fig. 6), the inhibitor must exist in some form of equilibrium with both DNA and an inhibitor-DNA complex. Although this theory is sound, and the three fractions were obtained from column chromatography, insufficient quantities were recovered for investigation by either infrared spectroscopy or nuclear magnetic resonance. In order to produce more inhibitor than normally found in air- and light-oxidized Baker phenol, the latter was subjected to a vanadium pentoxide-catalyzed oxidation.

Isolation of the inhibitor was accomplished with paper chromatography. The UV-absorbing and fluorescing spots were cut out from the dried developed chromatogram, eluted with methanol, and the latter removed by evaporation under reduced pressure at room temperature. The residues were dissolved in 0.01 M NaOH, adjusted to pH 7 with dilute HCl, and tested for enzyme inhibition. Table II gives the $P_P$ of each of the spots tested, as well as the percent inhibition (or activation) from a hypochromic effect assay. Also included in this Table are the major ultraviolet spectral characteristics of each of the spots at pH 7. For these assays, percent inhibition is calculated from the total hyperchromicity 10 min after the addition of the enzyme.

The activators of DNAase found at $P_P$ 0.69 - 0.77 were identified as follows: A comparison was made of the ultraviolet spectra of the
phenolic activators mentioned by HOFTMAN-OSTENHOF AND FRISCH-NICCE-MEYER and that of spot 12. Fig. 2 shows that the \( \lambda_{\text{max}} \) for spot 12 was 277 mp, while that for pyrocatechol was 275 mp. Furthermore, with the standard solvent pyrocatechol showed an \( R_p \) of 0.69 while the \( R_p \) of spot 12 was 0.73. Hydroquinone, with a UV \( \lambda_{\text{max}} \) of 288 mp, showed an \( R_p \) of 0.66 - 0.77 with the standard solvent. Both hydroquinone and pyrocatechol, as well as spot 12, fluoresced on Whatman paper with UV light. From this data, it appeared that spot 12 was a mixture of hydroquinone and pyrocatechol.

Of utmost importance was the inhibitor spot at \( R_p \) 0.55 - 0.61 (spots 9 and 10). In order to identify the inhibitor, UV, IR, and mass spectra have been taken. The mass spectrum indicated that the most abundant parent ion had an m/e value of 186. This could be ascribed either to a dihydroxybiphenyl or to a monohydroxydiphenyl ether. An m/e 278 parent ion was also found; however, the ratio of abundance of m/e 186 dimer (C\(_{12}\)H\(_{10}\)O\(_2\)) to m/e 278 trimer (C\(_{18}\)H\(_{14}\)O\(_3\)) was 50:1. The m/e 170 value was also present; this could correspond to the monohydroxybiphenyls. The ratio of abundance of the m/e 186 parent ion to the m/e 170 parent ion was 10:1.

When spot 9 was rechromatographed with standard solvent, it gave rise to spots 9 through 12. If once again eluted and chromatographed, spot 9 gave the same pattern as before. Thus, even after chromatography, the compound was not pure. Spots 11 and 12 were possibly degradation products of the compounds at spots 9 and 10.
Several hydroxybiphenyls were chromatographed on paper with the standard solvent; one compound, o-hydroxybiphenyl, had an $R_f$ of 0.54 (compared to 0.55 for spot 9). Fig. 3 shows the UV spectra of o-hydroxybiphenyl and spot 9 at various pH values. Fig. 4 compares the IR spectrum of o-hydroxybiphenyl with that of spot 9. This data, coupled with the fact that o-hydroxybiphenyl inhibits DNAase, leaves little room for doubt that o-hydroxybiphenyl is a major component of spot 9.

Studies on the inhibition of DNAase

The preparation of larger amounts of inhibitor by paper chromatography (500 mg per batch) permitted further investigation of its properties. Fig. 5(a) illustrates the inhibitory effect versus concentration of inhibitor, using the standard hyperchromic effect assay. Untreated DNA was used as the standard for zero percent inhibition. Fig. 5(a) shows a typical saturation curve.

From mass spectral data, we take 186 as the average molecular weight for the mixed inhibitor contained in spot 9. From this value, the ratio (moles of inhibitor/mole of nucleotides) may be calculated. Fig. 5(a) is also a plot of percent inhibition against such a ratio. A plot of the inverse of percent inhibition against the inverse of this ratio (now moles of nucleotides/mole of inhibitor) results in two straight lines. The point of intersection of these lines yields a value for the number of moles of inhibitor/mole of nucleotides needed for complete inhibition. Percent inhibition may be calculated from total hyperchromicity up to a given time (10 min in these assays) after the addition of the enzyme, or from initial
slopes. For the inhibitor from Baker phenol the value for moles of inhibitor/mole of nucleotides needed for complete inhibition is 3.5 (Fig. 5(b)). This ratio will be used as a comparative measure of the effectiveness of different inhibitors.

In the usual assay procedure, the inhibitor was incubated with DNA for 10 min prior to the addition of the enzyme. However, it was found that maximal inhibition was attained after only 2 min of incubation of inhibitor (o-hydroxybiphenyl) and DNA before addition of DNAase.

Fig. 6 illustrates kinetics of hydrolysis using inhibited and non-inhibited samples. The initial slope of the non-inhibited sample was 2.5 times that of the inhibited sample. At 11 min after the start of hydrolysis, there was a change in the slopes in both samples. This suggested that all the readily hydrolyzable DNA had by then been hydrolyzed. At this point the extent of inhibition was 35%. From 11 min to the end of the experiment at 50 min, the slope of the inhibited sample was 2.3 times greater than that of the non-inhibited sample. The final period of hydrolysis in the non-inhibited sample probably indicated the hydrolysis of non-Pu - Py linkages. In the inhibited sample, however, the hydrolysis of the unprotected Pu - Py linkages as well as non-Pu - Py hydrolysis probably occurred during this period. The greater slope in the inhibited sample might indicate that as soon as Pu - Py linkages became unprotected, they were hydrolyzed by the enzyme. This would necessitate an appreciable rate of dissociation of inhibitor-DNA complexes. A reasonable model to explain
such a phenomenon might be one in which the inhibitor was complexed to the DNA by means of hydrogen bonding (perhaps to the bases).

If the inhibitor does indeed act directly upon the DNA, one would expect that the amount of inhibition would decrease as the DNA concentration is increased while the inhibitor and DNase concentrations are maintained at constant levels. This is indeed the case (Fig. 7).

Molecular structure versus inhibitory effectiveness

Fig. 8 illustrates percent inhibition versus concentration for three hydroxylbinenyls: α-hydroxybinenyl, p-hydroxybinenyl, and 2,5-dihydroxybinenyl. Table III lists several inhibitors and related compounds which do not act as inhibitors. A value is given for the relative inhibitory effectiveness of each of the compounds (see previous section and Fig. 5).

The results show that a free hydroxyl is needed for inhibition since both biphenyl and p-ethoxybinenyl did not inhibit at any concentration tested. The most effective inhibitors are those with a hydroxyl group α- to the phenyl group; 3,4-dihydroxybinenyl and 3α-hydroxybinenyl are the best inhibitors tested to date. The 2,5- and 2,5'-dihydroxybinenyls were somewhat poorer. The α-hydroxybinenyls were much poorer inhibitors than the corresponding p-derivatives. Furthermore, the p-hydroxybinenyls were either the poorest inhibitors found, or were totally non-inhibitory. A p-hydroxyl group placed on an originally unsubstituted phenyl group rendered the original α- or p-hydroxybinenyl non-inhibitory. Little or no inhibitory activity was observed with the cyclohexyl phenols,
and, similarly, no activity was seen with 4-t-butylpyrocatechol. This demonstrates that a second phenyl group is required for the pyrocatechols and hydroquinones to be inhibitors of DNAase I.

Table III also gives data for the diphenyl ethers as inhibitors of DNAase. Only the 2,2'-dihydroxydiphenyl ether is a strong inhibitor, and should be compared with the marked inhibitory effectiveness of 2,2'-dihydroxybiphenyl. On the whole, however, the biphenyls are more effective inhibitors than the diphenyl ethers with hydroxyl groups in identical positions. This is true for \textit{n}-hydroxybiphenyl versus \textit{m}-phenoxyphenol, as well as for 3,4-dihydroxybiphenyl versus 2-hydroxy-4-phenoxyphenol.

\textbf{Mode of dissolution of hydroxybiphenyls}

The method of dissolving the hydroxybiphenyls is crucial to inhibitory effectiveness. For best results, we first dissolved the compounds in dilute alkali (0.02 N NaOH), and immediately adjusted the pH to 7.0 with dilute acid (0.1 N HCl), and finally diluted to the desired concentration with assay buffer. We found that if we first dissolved the hydroxybiphenyls in organic solvents (e.g., acetone) and then diluted with buffer, we saw a marked decrease in inhibitory effectiveness. Table IV summarizes these results for 3,4-dihydroxybiphenyl.

We suggest that the solvents react with the hydroxybiphenyls to form derivatives that are non-inhibitory. To test this hypothesis, we dissolved 3,4-dihydroxybiphenyl in acetone and then diluted with assay buffer. We then tried to extract the derivatives from aqueous
solution with diethyl ether. After extraction, we evaporated the ether, dissolved the residue in acetone, and ran mass spectra. Parent ions with m/e values greater than 186 were found in these spectra. The m/e values of 238, 250, and 265 were the most predominant. None of these parent peaks are found in the mass spectrum of 3,4-dihydroxybiphenyl. These data support the concept that solvent and biphenyl reacted to form a non-inhibitory derivative.

Dissociation constant for the inhibitor-DNA complex

Based on measurements of initial rates of hydrolysis of sDNA by DNAase at several inhibitor concentrations, Lineweaver-Burk plots were made of the reciprocal of (uninhibited rate minus inhibited rate) versus the reciprocal of the inhibitor concentration for a number of inhibitors. Table V list the dissociation constants for inhibitor-DNA complexes for several inhibitors. Treatment of the experimental data by the algebraic method of WEBB16 gave similar values for the dissociation constants.

Base composition of the DNA substrate and the efficiency of inhibition of hydrolysis

The relative effectiveness of none of the inhibitors changed consistently when the mean base composition of the DNA substrate was varied. A titration of each of the major inhibitors was performed on each of three DNAs of different mean base composition: M. lysodeikticus sodium chloride-extracted DNA, 29% (A+T); commercial herring sperm sodium salt DNA, 55% (A+T); and potassium salt poly d(A-T). Table VI records for o- and m-hydroxybiphenyl and for 2,5- and 3,4-dihydroxybiphenyl
the moles of inhibitor/mole of nucleotides required for complete inhibition. If the inhibitors act by complexing with the bases of DNA, we conclude that the compounds complex with guanine and cytosine pairs just as well as with adenine and thymine pairs.

Reversal of inhibition

By studying the chemical reversal of inhibition, we hoped to gain some insight into the mode of action of the hydroxybiphenyls. We studied reversal of inhibition by the polymer polyvinyl pyrrolidone (PVP), a substance known to absorb hydrogen bonding material \(^{17}\). A series of assays was performed using DNA (25 \(\mu\)g/ml), DNAase (5 \(\mu\)g/ml), and m-hydroxybiphenyl (3 \(\times\) 10\(^{-5}\) M); this concentration of inhibitor gave approximately 40% inhibition. All assays were run spectrophotometrically in acetate buffer (pH 6.5). Two controls were run: a normal hydrolysis (non-inhibited) of DNA by DNAase, and a normal inhibited hydrolysis. DNA was incubated with m-hydroxybiphenyl (3 ml total vol. in all assays) for 10 min at room temperature. PVP (1 g) was then added, and the solution was mixed thoroughly. The milky suspension was clarified by centrifugation, and the supernatant was assayed with DNAase: normal (37%) inhibition was found. In another experiment, the inhibitor was first treated with PVP (1 g in 2 ml) in the absence of DNA, and the solution centrifuged as before.. DNA was added to the supernatant at room temperature, followed 10 min later by DNAase: no inhibition of hydrolysis was seen. When DNAase and inhibitor were first incubated together for 16 min at room temperature, the solution treated with PVP and centrifuged, and DNA
then added to the supernate, no inhibition was observed. These results clearly indicate that the inhibitor interacts directly with DNA and does not affect the enzyme itself. They also show that the inhibitor can either be inactivated or removed from solutions by the action of PVP. If we assume that PVP reverses inhibition by absorbing the inhibitor by the formation of hydrogen bonds, we may infer that in the absence of PVP the inhibitor forms hydrogen bonds with the nucleic acid and thus protects it from hydrolysis. Table VII summarizes the results of these assays.

In order to determine to which part of the DNA molecule the inhibitors bind an experiment was performed in which pyrophosphate, deoxyribose, and the deoxyribonucleosides were used to reverse inhibition. Neither pyrophosphate nor deoxyribose reversed inhibition produced by any of the five inhibitors listed in Table VIII. However, the nucleosides markedly reversed inhibition (Table VIII). The assays were performed as follows: Three controls were included: (a) DNA was hydrolyzed with DNAase to obtain a normal non-inhibited rate; (b) DNA was exposed to DNAase after 10 min pre-incubation with an inhibitor; this gave a normal inhibited rate for that concentration of particular inhibitor; (c) DNA was exposed to a nucleoside for 10 min prior to assay with the nuclease. These controls were performed for each nucleoside and for each inhibitor listed in Table VIII. The last products produced by the action of DNAase (mono-, di-, and trinucleotides) inhibit the action of the enzyme; however, we found no inhibition by
the monodeoxyribonucleosides. Two types of experimental assays were carried out: (1) DNA was incubated with inhibitor for 10 min prior to the addition of a nucleoside. The samples were assayed immediately after addition of a nucleoside; (2) the inhibitor was incubated with the nucleoside for 10 min before the addition of DNA. Again the DNAase assay was begun immediately after the DNA was added. Table VIII shows the results of assays of the second type for five inhibitors and four nucleosides.

In general, the second assay procedure produced more reversal of inhibition than the first. This might suggest reversal of inhibition by the formation of an inhibitor-nucleoside complex; however, no physical evidence could be found for the existence of such a complex. UV and IR spectra of inhibitors and nucleosides in equimolar concentrations were the sums of the spectra for the different compounds taken individually. In support of the contention that nucleoside-DNA complexes are formed, we have demonstrated by means of circular dichroism that the nucleosides do complex with DNA. Fig. 9 compares the CD spectrum of herring sperm DNA alone with that of herring sperm DNA plus deoxyadenosine (approximately 1 mole of deoxyadenosine per mole of DNA nucleotides). The narrowing and decrease in intensity of the positive Cotton band, together with the widening and increase in intensity of the negative Cotton band, is typical of DNA complexed with a planar molecule which has intercalated between the bases of the nucleic acid. Similar CD spectra are seen with intercalating compounds such as ethidium bromide.
or lysergic acid diethylamide\textsuperscript{9}. We recall that ethidium bromide inhibits DNAase\textsuperscript{7} by intercalation\textsuperscript{8-9}. If we may conclude that the nucleosides reverse inhibition by displacing the inhibitors from their site of action, we may then infer that the inhibitors also intercalate between the bases of DNA.

Using molecular models of DNA and several hydroxybiphenyls we have found the structures compatible with such a mechanism for the inhibitors. From the models, it appears that the unsubstituted phenyl group could intercalate between the nucleic acid bases, and that the hydroxyls on the other phenyl group could form hydrogen bonds with the bases. It seems most likely that a \(\text{m}\)-hydroxyl would form a hydrogen bond with the carbonyl of thymine, while an \(\text{o}\)-hydroxyl would form a hydrogen bond with one of the ring nitrogens of a purine. If a \(\text{p}\)-hydroxyl were to form hydrogen bonds, intercalation by the unsubstituted phenyl group would be impossible.

**Physical evidence for the existence of a DNA-inhibitor complex**

(i) DNA melting profiles: The \(T_m\) of Baker pDNA from \textit{E. coli} (in 0.02 M Tris, pH 6.7) is 75.5\(^\circ\), while that for sDNA from \textit{E. coli} (also in Tris) is 70\(^\circ\). When 3,4-dihydroxybiphenyl is added to the sDNA to a concentration that gives 100% inhibition of DNAase, the \(T_m\) increases to 75\(^\circ\). The slope of the melting curve for the pDNA is 0.005 (\(\Delta\text{A}_{260\text{mu}}/\Delta^\circ\)) at the \(T_m\) while the slope at the \(T_m\) for sDNA is 0.035. When inhibitor is added to the sDNA, the slope drops to 0.019. Lerman\textsuperscript{18} has shown that the binding of cationic acridines to DNA effects an increase in the
$T_m$ of the nucleic acid. A 20° increase in the $T_m$ is reported for 9-aminoacridine and streptomycin. The intercalation of acridines into DNA has long been established.

(ii) Ultraviolet absorption spectra: When inhibitors were incubated with commercial DNA, only slight changes in the UV-absorption spectrum of the DNA were observed. When $m$-hydroxybiphenyl or 3,4-dihydroxybiphenyl was incubated with synthetic poly d(A-T), a marked change in the UV-absorption spectrum of the polynucleotide was observed. The $\lambda_{\text{max.}}$ for poly d(A-T) is 263 nm; in the presence of $m$-hydroxybiphenyl (3.7 moles of biphenyl/mole of nucleotides) there was a hypsochromic shift of 60 Å. Fig. 10 illustrates the UV-absorption spectra for poly d(A-T) in the presence of $m$-hydroxybiphenyl. All spectra are corrected for biphenyl absorption. In general, for both $m$-hydroxybiphenyl and for 3,4-dihydroxybiphenyl treated poly d(A-T), we observed an increase in the ratio (absorbance at $\lambda_{\text{max.}}$/absorbance at $\lambda_{\text{min.}}$) and a decrease in the ratio (absorbance at $\lambda_{\text{max.}}$/absorbance at 280 nm).

(iii) Circular dichroism and magnetic circular dichroism: Fig. 11 illustrates the CD spectra for M. lysodeikticus sDNA and for M. lysodeikticus sDNA in the presence of $m$-hydroxybiphenyl (one mole of biphenyl/mole of nucleotides), as well as their difference spectrum. We observed a slight decrease in the positive Cotton band accompanied by a complete loss of the negative Cotton band. Fig. 12 illustrates the CD spectra for E. coli Baker pDNA, sDNA, and sDNA in the presence of 3,4-dihydroxybiphenyl (0.23 mole of biphenyl/mole of nucleotides). Here, upon the
addition of inhibitor to sDNA, we observed a one-third decrease in the strength of the positive Cotton band and a marked increase in the negative Cotton band. The difference spectrum for the M. lysodeikticus sDNA and sDNA plus inhibitor is complex; two positive and two negative bands are found. The major positive band is the mirror image of the negative Cotton band of the sDNA. A negative band peaks at the same wavelength as the positive Cotton band of the sDNA (266 μm). The two other bands peak at 252 μm (for the negative) and 278 μm (for the positive), respectively. The difference spectrum for the E. coli sDNA and sDNA plus inhibitor shows only a singlet peaking at the same wavelength (and with the same sign) as the negative Cotton band of the DNA.

DNA in the presence of lysergic acid diethylamide exhibits the same changes in CD as does E. coli sDNA in the presence of 3,4-dihydroxybiphenyl. The negative Cotton band of DNA is also strengthened by the presence of ethidium bromide. We have pointed out earlier that both of these compounds are known to intercalate between the bases of DNA.

The mono- and dihydroxybiphenyls absorb UV in the same region as DNA; however, in pure solution the biphenyls have no apparent CD activity. On the other hand, in the presence of the nucleic acid, we cannot determine whether changes in the CD upon addition of inhibitor to DNA are due to real changes in the CD of the nucleic acid or to the addition of a DNA-induced biphenyl circular dichroism to the CD of the DNA. Free hydroxybiphenyls have no point of assymetry and, therefore, no CD; if our model of inhibitor action is correct, we
would expect that the unsubstituted phenyl group of the hydroxybiphenyls would be subject to hindered rotation upon intercalation. This hindered rotation would bring about an asymmetry that would be accompanied by CD activity. To give us some idea of the possible magnitude of this DNA-induced CD, we looked at the magnetic circular dichroism of 3,4-dihydroxybiphenyl. We found only a weak positive CD band below 260 μm with the external magnetic field. This magnitude of CD activity could not account for the differences between the CD of DNA and that of DNA plus inhibitors; we therefore conclude that the observed changes in CD are real changes in the spectrum of the nucleic acid. This data, in addition to the reported CD spectra of DNA plus intercalating compounds, lends support to our theory of inhibitor action.

We have also looked at changes in the circular dichroism spectrum of DNAase in the presence of DNA and DNA complexed with an inhibitor. DNAase has a strong negative CD band between 210 and 220 μm. Fig. 13 illustrates the CD spectra of DNAase in the presence of DNA, and in the presence of DNA complexed with 3,4-dihydroxybiphenyl, and the sum of the CD spectra of DNAase and DNA taken individually. All of these spectra were taken in the absence of activating bivalent cations. In the presence of DNA, the strong CD band of the enzyme is reduced by one-third. On the other hand, if the DNA had been treated with an inhibitor before the addition of the enzyme, we did not observe any loss in the CD activity of the DNAase. If the DNA had been complexed with a poor inhibitor (such as ω-hydroxybiphenyl), however, we still
would have observed a reduction in the CD band of the enzyme. This strongly suggests that both inhibitor and enzyme compete for the same site on the substrate, and in the presence of inhibitor, the enzyme is blocked from this site.

**Tryptamine as an inhibitor of DNAase**

We found that tryptamine inhibited DNAase while tryptophan did not. Fig. 14 is a plot of percent inhibition versus the ratio (moles of tryptamine/mole of nucleotides). By holding the concentrations of tryptamine (5·10^-5 M) and DNAase (5 μg/ml) constant, and varying the concentration of DNA (from 5 to 40 μg/ml), we found that the amount of inhibition increased inversely with the DNA concentration. This indicated that inhibition arose by action of tryptamine on the nucleic acid.

We must conclude that the presence of the carboxyl group in tryptophan prevents the amino acid from acting as an inhibitor of DNAase. The negatively charged phosphates of the nucleic acid might repel the acidic group of tryptophan. In the case of tryptamine, however, the phosphates might attract the amine and stabilize a tryptamine-DNA complex.

We found that the CD spectra of herring sperm DNA and that of herring sperm DNA in the presence of tryptophan (one mole of tryptophan/mole of nucleotides) were identical. We have been unable to record the CD spectrum for herring sperm DNA in the presence of tryptamine (at a concentration of amine that would exhibit enzyme inhibition) due to the high extinction coefficient of the drug in the UV-region.

**DISCUSSION**

It is common practice to distill phenol before using it to
extract nucleic acids from micro-organisms. We have found that liquefied phenol, as supplied by certain manufacturers, contains impurities which render extracted DNA resistant to hydrolysis by DNAase I. The variety of impurities present in a particular brand appears to depend on the manufacturing process used.

At least one group of inhibitors, the mono- and dihydroxybiphenyls, has been found in liquefied phenol produced by J. T. Baker Chemical Co. These inhibitors clearly act by complexing with the DNA substrate, and do not directly affect the enzyme. Our evidence suggests that the hydroxybiphenyls act by forming hydrogen bonds with the nucleic acid bases, accompanied by intercalation of the unsubstituted phenyl group between them. Polyvinyl pyrrolidone, which is known to absorb hydrogen-bonding material, can prevent inhibition, presumably by sequestering the inhibitors more effectively than DNA. Deoxyribonucleosides can also prevent and reverse inhibition, probably by competing with the inhibitors for intercalation sites on the DNA. Circular dichroism measurements have shown that both deoxyribonucleosides and hydroxybiphenyls intercalate into DNA.

The requirements for inhibition among the hydroxybiphenyls include both a free hydroxyl group and an unsubstituted (or o-substituted) phenyl group. Thus, neither biphenyl itself, nor p-ethoxybiphenyl, exhibit inhibitory action. The presence of certain bulky groups precludes inhibition, probably by preventing intercalation as a result of steric hindrance. For this reason neither 4-t-butylpyrocatechol nor cyclohexylphenols show inhibition. Cyclohexylphenols, in addition, do
not possess the same electronic structure as hydroxybiphenyls. Pi-cloud interactions between the nucleic acid bases and the unsubstituted phenyl groups of the hydroxybiphenyls are apparently required for inhibition.

The intramolecular position of the hydroxyl group determines inhibitory effectiveness. Hydroxyl at the m-position is the most effective, followed by those at the o- and p- positions, in that order. Experiments with space-filling molecular models explained this effect. A m-hydroxyl can readily form hydrogen bonds with the carbonyl of thymine, or with the ring nitrogens of a purine. This is more difficult to accomplish with an o-hydroxyl, and intercalation proves to be impossible when the hydroxyl group is p- to the unsubstituted phenyl group.

Measurements of enzyme kinetics, and the characteristics of circular dichroism spectra, have shown that the enzyme and inhibitor compete for the same site on the substrate. The active sites of both DNAase and RNAase are known to contain histidine$^{20,21}$. We note, in addition, that four tryptophan residues are present in DNAase$^{22}$, but none is found in RNAase$^{23}$. Tryptophan is essential to the enzyme activity of DNAase: N-bromosuccinimide, which destroys tryptophan residues, strongly inhibits DNAase$^1$. Further, the hydroxybiphenyls which protect DNA from hydrolysis by DNAase are without effect in protecting RNA from RNAase. This data, in addition to our belief that it is the indole nucleus of ergot alkaloids (e.g., lysergic acid diethylamide) which intercalates between the bases of DNA,
leads us to conclude that the tryptophan residues of DNAase intercalate and act as anchor points which hold the enzyme to the substrate. It seems likely that the hydroxybiphenyls, ethidium bromide and actinomycin D are able to inhibit DNAase by preventing this intercalation into DNA of the tryptophan residues of DNAase, thereby obviating the anchoring effect. Tryptamine has been found to inhibit DNAase, and it does so by action on the substrate. Although this model of DNAase action is for the moment unproven, such evidence as we have so far obtained is entirely in its favor.

ACKNOWLEDGMENTS

The work reported in this paper was supported in part by the United States Atomic Energy Commission, and in part by the National Aeronautics and Space Administration (NSG 101-61). We gratefully acknowledge the assistance of Dr. Ronald Cape, Dr. Donald Grav, Mr. Jerry Han, Dr. John Sutherland, and Professor I. Tinoco, Jr. All the hydroxybiphenyls and related compounds were generously given to us by the Dow Chemical Company.
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TABLE I

EFFECT OF DEOXYRIBONUCLEASE I ON THE DNA PREPARATIONS

Both the pH-stat and hyperchromic effect assays were performed as described in MATERIALS AND METHODS. Data from both assay systems were taken 10 min after addition of the enzyme.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Hyperchromic effect assay</th>
<th>pH-stat assay</th>
</tr>
</thead>
</table>
|                            | Increase in $A_{260}$ \%
upon hydrolysis (%)         | Hydrolysis (%)          |
| E. coli sDNA               | 19                        | *             |
| Commercial herring sperm   | 18                        | *             |
| sDNA                       | 18                        | 37            |
| Baker pDNA                 | 0                         | 0             |
| Dialyzed Baker pDNA        | 0                         | 0             |
| Mallinckrodt pDNA          | 19                        | 38            |

* Values for all assays are ±10% (standard error for individual trials).
TABLE II

OXIDATION PRODUCTS OF PHENOL ISOLATED BY PAPER CHROMATOGRAPHY

Inhibitions measured in a DNAase assay using 0.1 ml of a 1:10 dilution of each of the chromatogram spots (eluted and dissolved in water) in 2.0 ml of reaction mixture containing DNA (20 μg/ml) and DNAase (5.0 μg/ml) in buffer system (a).

<table>
<thead>
<tr>
<th>Spot</th>
<th>R_f</th>
<th>Inhibition (%)</th>
<th>Ultraviolet spectra (pH 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>λ_max. (nm)</td>
</tr>
<tr>
<td>Origin</td>
<td>0.00</td>
<td>15.7</td>
<td>276, 282</td>
</tr>
<tr>
<td>1</td>
<td>0.02</td>
<td>23.2</td>
<td>259, 270</td>
</tr>
<tr>
<td>3</td>
<td>0.20</td>
<td>31.5</td>
<td>262</td>
</tr>
<tr>
<td>5</td>
<td>0.36</td>
<td>17.6</td>
<td>252</td>
</tr>
<tr>
<td>7</td>
<td>0.42</td>
<td>25.1</td>
<td>258</td>
</tr>
<tr>
<td>8</td>
<td>0.48</td>
<td>15.7</td>
<td>246</td>
</tr>
<tr>
<td>9</td>
<td>0.55</td>
<td>40.9</td>
<td>247, 283</td>
</tr>
<tr>
<td>10</td>
<td>0.61</td>
<td>100.0</td>
<td>247, 282</td>
</tr>
<tr>
<td>11</td>
<td>0.69</td>
<td>+2.5*</td>
<td>278</td>
</tr>
<tr>
<td>12</td>
<td>0.73</td>
<td>+10.1*</td>
<td>277</td>
</tr>
<tr>
<td>13</td>
<td>0.77</td>
<td>+23.3*</td>
<td>277</td>
</tr>
</tbody>
</table>

* Activation (%).
TABLE III

MOLECULAR STRUCTURE VERSUS EFFECTIVENESS AS INHIBITORS OF DNA HYDROLYSIS BY DNase I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ratio, moles of inhibitor to moles of nucleotides, needed for 100 percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhydroxybiphenyls</td>
<td></td>
</tr>
<tr>
<td>n-</td>
<td>0.69</td>
</tr>
<tr>
<td>o-</td>
<td>1.46</td>
</tr>
<tr>
<td>p-</td>
<td>47.7</td>
</tr>
<tr>
<td>Dihydroxybiphenyls</td>
<td></td>
</tr>
<tr>
<td>3,4-</td>
<td>0.65</td>
</tr>
<tr>
<td>2,5-</td>
<td>0.96</td>
</tr>
<tr>
<td>2,5'-</td>
<td>3.5</td>
</tr>
<tr>
<td>2,2'-</td>
<td>3.5</td>
</tr>
<tr>
<td>3,4'-</td>
<td>non-inhibitory</td>
</tr>
<tr>
<td>4,4'-</td>
<td>non-inhibitory</td>
</tr>
<tr>
<td>Other biphenyls and related compounds</td>
<td></td>
</tr>
<tr>
<td>biphenyl</td>
<td>non-inhibitory</td>
</tr>
<tr>
<td>p-ethoxybiphenyl</td>
<td>non-inhibitory</td>
</tr>
<tr>
<td>4-t-butylpyrocatechol</td>
<td>non-inhibitory</td>
</tr>
<tr>
<td>4-phenylpyridinol</td>
<td>45</td>
</tr>
<tr>
<td>Cyclohexylphenols</td>
<td></td>
</tr>
<tr>
<td>o-cyclohexylphenol</td>
<td>15.6</td>
</tr>
<tr>
<td>4-cyclohexylpyrocatechol</td>
<td>non-inhibitory</td>
</tr>
<tr>
<td>3-cyclohexylpyrocatechol</td>
<td>non-inhibitory</td>
</tr>
<tr>
<td>4-cyclohexylresorcinol</td>
<td>non-inhibitory</td>
</tr>
</tbody>
</table>
TABLE III (Cont'd.)

Hydroxydiphenyl ethers

<table>
<thead>
<tr>
<th>Compound</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2'-dihydroxydiphenyl ether</td>
<td>2.6</td>
</tr>
<tr>
<td>m-phenoxypheol</td>
<td>10</td>
</tr>
<tr>
<td>2-hydroxy-4-phenoxypheol</td>
<td>non-inhibitory</td>
</tr>
</tbody>
</table>
TABLE IV

MODE OF DISSOLUTION OF THE HYDROXYBIPHENYLS

All assays performed spectrophotometrically in assay buffer (b). The concentration of herring sperm ssDNA was 50 µg/ml in all assays. 3,4-dihydroxybiphenyl was 5·10^{-5} M in all inhibited trials. The final concentration of solvent for both inhibited and non-inhibited assays was: 6.6·10^{-2} M dimethylsulfoxide (DMSO); 8.8·10^{-2} M dimethylformamide (DMF); 8.5·10^{-2} M acetone.

<table>
<thead>
<tr>
<th>Mode of dissolution</th>
<th>Rate of hydrolysis $A_{260}$ μM/min</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Inhibited</td>
</tr>
<tr>
<td>Alkali</td>
<td>0.440</td>
<td>0.020</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.645</td>
<td>0.540</td>
</tr>
<tr>
<td>DMF</td>
<td>0.402</td>
<td>0.380</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.380</td>
<td>0.325</td>
</tr>
</tbody>
</table>
TABLE V

DISSOCIATION CONSTANT FOR THE INHIBITOR-DNA COMPLEX

Dissociation constants calculated from Lineweaver-Burk plots of (rate of uninhibited DNA hydrolysis - rate of inhibited DNA hydrolysis)⁻¹ vs. (inhibitor concentration)⁻¹.

<table>
<thead>
<tr>
<th>Inhibitor isolated from Baker phenol (i.e. spot 10)</th>
<th>K_d (moles/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-dihydroxybiphenyl</td>
<td>3.4×10⁻⁶</td>
</tr>
<tr>
<td>m-hydroxybiphenyl</td>
<td>5.9×10⁻⁵</td>
</tr>
<tr>
<td>o-hydroxybiphenyl</td>
<td>1.3×10⁻³</td>
</tr>
<tr>
<td>p-hydroxybiphenyl</td>
<td>2.5×10⁻³</td>
</tr>
</tbody>
</table>
TABLE VI

MEAN BASE COMPOSITION OF THE DNA VERSUS EFFECTIVENESS OF THE MAJOR INHIBITORS OF HYDROLYSIS BY DNAase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>M. lysodeikticus DNA [29% (A+T)]</th>
<th>Herring sperm DNA [55% (A+T)]</th>
<th>Poly d(A-T) [100% (A+T)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-dihydroxybiphenyl</td>
<td>1.1</td>
<td>0.65</td>
<td>0.94</td>
</tr>
<tr>
<td>m-hydroxybiphenyl</td>
<td>0.87</td>
<td>0.69</td>
<td>0.63</td>
</tr>
<tr>
<td>2,5-dihydroxybiphenyl</td>
<td>1.34</td>
<td>0.96</td>
<td>0.85</td>
</tr>
<tr>
<td>o-hydroxybiphenyl</td>
<td>1.7</td>
<td>1.46</td>
<td>1.76</td>
</tr>
</tbody>
</table>
TABLE VII

REVERSAL OF INHIBITION BY POLYVINYL PYRROLIDONE (PVP)

Assays performed as described in text.

<table>
<thead>
<tr>
<th>Mode of assay</th>
<th>Initial Rate of Hydrolysis (A260 μg/min)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DNA + DNAase</td>
<td>0.217</td>
<td>0</td>
</tr>
<tr>
<td>2. DNA + inhibitor incubated 10 min; DNAase then added</td>
<td>0.124</td>
<td>43</td>
</tr>
<tr>
<td>3. DNA + inhibitor incubated 10 min; PVP added, the mixture centrifuged and DNAase added to supernatant</td>
<td>0.136</td>
<td>37</td>
</tr>
<tr>
<td>4. Inhibitor + PVP incubated 10 min; the mixture centrifuged and DNA added to supernatant; 10 min later DNAase added</td>
<td>0.209</td>
<td>0</td>
</tr>
<tr>
<td>5. Inhibitor + DNAse incubated 10 min; PVP added, the mixture centrifuged and DNA added to supernatant</td>
<td>0.226</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE VIII

REVERSAL OF INHIBITION BY THE NUCLEOSIDES

All assay mixtures contained sDNA (20 μg/ml), DNase (5 μg/ml), and acetate buffer, pH 6.5. Concentrations of all nucleosides were $3 \times 10^{-5}$ M. Percent reversal of inhibition is defined as

$$100 \times \frac{\% \text{ Inhibition} - \% \text{ Inhibition with nucleosides}}{\% \text{ Inhibition}}.$$ 

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>dC</th>
<th>dC</th>
<th>dA</th>
<th>dU</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-dihydroxybiphenyl</td>
<td>94</td>
<td>0</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>2,5-dihydroxybiphenyl</td>
<td>97</td>
<td>2</td>
<td>100</td>
<td>68</td>
</tr>
<tr>
<td>m-hydroxybiphenyl</td>
<td>16</td>
<td>0</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>o-hydroxybiphenyl</td>
<td>100</td>
<td>70</td>
<td>56</td>
<td>100</td>
</tr>
<tr>
<td>p-hydroxybiphenyl</td>
<td>37</td>
<td>10</td>
<td>29</td>
<td>55</td>
</tr>
</tbody>
</table>
CAPTIONS TO FIGURES

Fig. 1. Experiment to show that DNAase was still active after exposure to J. T. Baker phenol-extracted DNA. At time zero, pDNA was added to the reaction vessel at a final concentration of 105 μg/ml, and DNAase to a final concentration of 25 μg/ml. After 24 min, commercial sDNA was added to a final concentration of 30.3 μg/ml. Extent of hydrolysis was determined with the pH-stat. After 48 min, 37% of the sDNA had been hydrolyzed while, in a separate control, only 0.2% of the pDNA had been hydrolyzed by 48 min. In another control, the extent of hydrolysis of sDNA alone after 24 min of exposure to DNAase was 37%. % hydrolysis pDNA alone (- -), and % hydrolysis of sDNA in the presence of pDNA (---).

Fig. 2. Ultraviolet absorption spectra of paper chromatogram spot 12 (---) and pyrocatechol (--), in water at pH 7.

Fig. 3. Effect of pH on the ultraviolet absorption spectra of o-hydroxybiphenyl (---) and paper chromatogram spot 9 (--), in water at (a) pH 1; (b) pH 7; (c) pH 14.

Fig. 4. Infrared spectra of o-hydroxybiphenyl (---) and chromatogram spot 9 (--) in CCl₄, with a CCl₄ blank. Spectra taken with cells of 3 mm thickness and a slit setting of 50.
CAPTIONS TO FIGURES (2)

Fig. 5(a). Percent inhibition plotted against the concentration of inhibitor from paper chromatogram spot 9, and against the ratio (moles of inhibitor/mole of nucleotides). 5(b). [Percent inhibition]^{-1} plotted against [moles of inhibitor/mole of nucleotides]^{-1}. For details, see text.

Fig. 6. Kinetics of hydrolysis using inhibited (●) and non-inhibited (-○-) samples. DNA concentration of 20 μg/ml and DNAase concentration of 5 μg/ml in both cases. Chromatographically purified inhibitor at 1.0 μg/ml. At 10 min, the degree of inhibition was 35%, while at 50 min it was 17%, and at 4 h it was only 3.5%. Initial slope of the non-inhibited sample was 2.5 times that of the inhibited sample. Terminal slope of the inhibited sample was 2.3 times that of the non-inhibited sample. Slopes given on graph.

Fig. 7. Concentration of sDNA plotted against percent inhibition for an experiment where inhibitor and DNAase concentrations were maintained at 25 μg/ml in all trials. Assays were performed at pH 7 using the pH-stat. Inhibition values calculated for 10 min after addition of the enzyme.
CAPTIONS TO FIGURES (3)

Fig. 8. α-Hydroxybiphenyl (○), p-hydroxybiphenyl (○), and 2,5-dihydroxybiphenyl (●) as inhibitors of DNAase. Spectrophotometric assays run at a DNA concentration of 20 μg/ml and a DNAase concentration of 5 μg/ml, in buffer (a). Inhibition measured 10 min after addition of the enzyme.

Fig. 9. Circular dichroism spectra for herring sperm sDNA (40 μg/ml) alone (--) and in the presence of 0.1 mM deoxyadenosine (—). Spectra taken in 0.02 M Tris, pH 6.7, and reported in terms of ellipticity (δ). Signal to noise ratio averages better than 9:1. Spectra corrected for nucleoside rotation in pure solution.

Fig. 10. Ultraviolet absorption spectra for poly d(A-T) alone (--); poly d(A-T) with 6.0·10⁻⁶ M α-hydroxybiphenyl (-----); and poly d(A-T) with 1.0·10⁻⁵ M p-hydroxybiphenyl (---). Spectra taken in 0.1 M Na acetate plus 0.01 M MgSO₄, pH 6.5, and corrected for biphenyl absorption. Poly d(A-T) was 8.8 μg/ml, or 2.7·10⁻⁵ M in nucleotides in all samples.

Fig. 11. Circular dichroism spectra for M. lysodeikticus sDNA alone (--), and in the presence of 7.5·10⁻⁵ M p-hydroxybiphenyl (-----), and their difference spectrum (---). M. lysodeikticus sDNA was 25 μg/ml, or 7.5·10⁻⁵ M in nucleotides. Spectra taken in 0.1 M Na acetate plus
CAPTIONS TO FIGURES (4)

0.01 M $\text{MgSO}_4$, pH 6.5, and corrected for biphenyl rotation in pure solution. Signal to noise ratio averages better than 12:1.

Fig. 12. Circular dichroism spectra for *E. coli* Baker phenol-extracted DNA (---), for *E. coli* sodium chloride-extracted DNA alone (---), and in the presence of $2.4\times10^{-5}$ M 3,4-dihydroxybiphenyl (---). Concentration of DNA in all samples was approximately 33 $\mu$g/ml, or $1.1\times10^{-4}$ M in nucleotides. Spectra taken in 0.02 M Tris, pH 6.7, and reported in terms of ellipticity ($\theta$). Spectra corrected for biphenyl rotation in pure solution. Signal to noise ratio averages better than 10:1.

Fig. 13. CD spectrum of herring sperm ssDNA plus DNAase in the absence of bivalent cations (---), and the sum of the spectra of ssDNA and DNAase taken individually (---). Spectrum of ssDNA pretreated with $4.75\times10^{-5}$ M 3,4-dihydroxybiphenyl in the presence of DNAase (---). DNA in all samples was 25 $\mu$g/ml, or $7.6\times10^{-5}$ M in nucleotides; DNAase in all samples was 100 $\mu$g/ml, or $3.3\times10^{-6}$ M. Spectra taken in 0.02 M Tris, pH 6.8. The biphenyl showed no CD activity in pure solution; however, in the presence of the biphenyl, the CD spectrum of the nucleic acid showed a one-third decrease in both the positive and negative Cotton bands. Spectra reported in terms of ellipticity ($\theta$). Above 210 mp, the signal to noise ratio averaged better than 10:1.
Fig. 14. Trypotamine as an inhibitor of DNAase. Plot of percent inhibition versus the ratio (moles of tryptamine/mole of nucleotides). Percent inhibition calculated from initial rates in a spectrophotometric assay performed in buffer system (b).
Time of addition of 5' DNA

% HYDROLYSIS

0  8  16  24  32  40  48

TIME (min)
Fig 3a

PH 1

Absorbance

Wavelength (nm)
% Inhibition vs DNA concentration (μg/ml)
% Inhibition

moles of tryptamine / moles of nucleotides