# The Relationship of Structure to the Effectiveness of Denaturing Agents for Deoxyribonucleic Acid

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The effectiveness of a number of compounds as denaturing agents toward DNA has been measured with a specific immunochemical technique for the determination of denatured DNA. In contrast to the results obtained with bovine serum albumin, an increase in the number or size of alkyl substituents on amides, ureas, carbamates, and alcohols increases denaturing effectiveness toward DNA. The structural modifications which increase denaturing effectiveness also increase adenine solubility and decrease pyrophosphate solubility in aqueous solutions of the denaturing agents. The effects of variations in time, temperature, *p*H, ionic strength, guanosine-cytosine content of the DNA, and DNA concentration on DNA denaturation are described. It is concluded that the denaturing agents examined exert their effect by stabilizing denatured DNA relative to native DNA by decreasing the ion-solvating power and increasing the hydrophobic character of the solvent.

The experiments reported in the accompanying paper (Gordon and Jencks, 1963) led to the conclusion that the mechanism of denaturation of bovine serum albumin by compounds of the urea class does not involve either simple, monofunctional hydrogen bonding or a hydrophobic interaction with the denaturing agent. With other proteins, such as ovalbumin, hydrophobic interactions appear to play a more important role. The availability of a specific immunologic method (Levine *et al.*, 1960) for the measurement of denatured deoxyribonucleic acid (DNA) prompted us to examine the effectiveness of a number of denaturing agents toward DNA, to make possible a comparison of the structural requirements for the effectiveness of denaturing agents toward DNA and toward proteins.

Urea, guanidinium chloride, salicylate, formamide, dimethyl sulfoxide, aromatic compounds, large anions, and a variety of alcohols are known to be denaturing agents for DNA (Rice and Doty, 1957; Geiduschek and Holtzer, 1958; Mandell and Hershey, 1960; Marmur and Ts'o, 1961; Helmkamp and Ts'o, 1961; Herskovits *et al.*, 1961; Geiduschek and Herskovits, 1961; Duggan, 1961; Ts'o *et al.*, 1962a; Hamaguchi and Geiduschek, 1962). It had generally been supposed that many of these compounds act as denaturing agents by virtue of their hydrogen bonding properties, but it has been suggested in several reports, which appeared after the completion of most of the work reported here, that the mechanism of denaturation of DNA by alcohols, dimethylformamide, large anions, and certain aromatic compounds involves a hydrophobic interaction of the denaturing solvent with DNA (Herskovits *et al.*, 1961; Geiduschek and Herskovits, 1961; Ts'o *et al.*, 1962a; Hamaguchi and Geiduschek, 1962).

A preliminary report of this work has appeared (Gordon *et al.*, 1962).

## Materials and Methods

*DNA.*—Bacteriophage DNA, kindly supplied by Dr. H. Van Vunakis, was prepared by treatment of purified bacteriophage preparations with 0.001 M pyrophosphate buffer at *p*H 9.0 at room temperature (Van Vunakis and Herriott, 1962). Three phenol extractions at room temperature (Gierer and Schramm, 1956) were used to remove protein. The DNA solution was dialyzed at 2–4° against 0.05 M Tris [tris-(hydroxymethyl)aminomethane] buffer in 0.001 M EDTA (ethylenediaminetetraacetic acid) at *p*H 7.6. DNA was isolated from *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Streptomyces viridochromogenes* and *Diplococcus pneumoniae* by the procedure of Marmur (1961) and was kindly supplied by Dr. J. Marmur. DNA from calf thymus and salmon sperm was purchased from the California Corporation for Biochemical Research and was further purified by ethanol and isopropanol precipitation.

*Reagents.*—Compounds which were not of reagent grade or which did not have melting points in agreement with literature values were recrystallized or redistilled before use.

*Antisera.*—Rabbit antiserum to $T_4$ bacteriophage DNA was prepared by intravenous injection of alum-precipitated ruptured bacteriophage (Levine *et al.*, 1960). Lupus erythematosus sera, which contain antibodies to denatured DNA (Stollar and Levine, 1961), were obtained through the cooperation of Drs. Earl Chapman and John Mills of the Massachusetts General Hospital. When tested for activity toward 42 DNA preparations, the antibodies in these lupus erythematosus sera reacted more effectively with denatured than native DNA (Stollar *et al.*, 1962b). The antibodies in one of the lupus sera used are directed toward thymidylic acid sequences (Stollar *et al.*, 1962a).

*Denaturation of DNA* was determined as follows: 0.5 ml of an appropriate dilution of the denaturing agent to be examined in 0.05 M Tris buffer, *p*H 7.6, containing $10^{-3}$ M EDTA (Tris-EDTA), was placed in a 1.0 or 2.0 ml glass-stoppered test tube at room temperature. After addition of 0.05 ml of DNA (160 μg/ml) the tubes were inverted several times and placed in a water bath at 73 ± 0.1°. During incubation the tubes were mixed two or three times. For examination of volatile denaturing agents, the stoppers were carefully greased. Control experiments with methanol, the most volatile of the denaturing agents examined, gave the same results in glass-stoppered tubes and in sealed vials. After incubation for 30 minutes, the samples were rapidly chilled in an ice bath and diluted in cold veronal-serum albumin buffer. The concentration of denatured DNA was then determined by the complement fixation technique of Wasserman and Levine (1961). In all experiments, controls, consisting of DNA which had been incubated in Tris-

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EDTA at 73° in the absence of a denaturing agent, as well as DNA which had been completely denatured by heating at 100° in the same buffer for 10 minutes, were included. In later experiments, DNA standards which had been incubated with 1.0 and 2.0 M urea were also included.

## Results

The denaturation of DNA was followed by an immunologic method which determines only denatured DNA, presumably by reaction of antibody with the collapsed, non-hydrogen-bonded regions of single-strand, denatured DNA, even in the presence of native DNA (Levine *et al.*, 1960; Murakami *et al.*, 1961). After incubation with the denaturing agent, usually for 30 minutes at 73° in Tris-EDTA buffer, *p*H 7.6, the DNA samples were cooled rapidly in ice and the amount of denatured DNA was determined by measurements of complement fixation after 1:400 dilution in cold buffer. This dilution permitted determination of the complement fixation curve from the region of slight antigen excess to that of antibody excess. In later experiments the chilled reaction mixtures were diluted 1:1100 in the same buffer and complement fixation was performed in duplicate at the equivalence point and at one point in the region of antibody excess. The quantity of denatured DNA in each sample was estimated by comparison of the complement fixation curves with a calibration curve obtained with thermally denatured DNA. For complement fixation analyses, the highest concentration of denaturing reagent (after 1:400 or 1:1100 dilution) was analyzed for its effect on complement. No inactivation of complement was found with any of the denaturing reagents used. The immunologic method permits evaluation of denaturing agents which absorb at 260 mμ, and avoids complications from possible solvent effects on the absorbancy of native or denatured DNA.

The results of the complement fixation assay for denatured DNA in a series of DNA samples which had been incubated with various concentrations of hexanamide at 73° are shown in Figure 1. The complement fixation curve for a sample of DNA which had been completely denatured by heating at 100° for 10 minutes is included for comparison. The ratio of the amounts of thermally denatured DNA to hexanamide-treated DNA which give 50% complement fixation in the region of antibody excess is equal to the fraction of denatured DNA in the hexanamide treated sample (Kabat and Mayer, 1961). For example, the data in Figure 1 show that 50% complement fixation was obtained with 2.8 mμg of thermally denatured DNA. The same amount of C′ fixation was obtained with 5.4 mμg of the DNA which had been incubated with 0.19 M hexanamide, indicating that 52% of the DNA had been denatured in this sample.

The effect of temperature on the denaturation of $T_4$ bacteriophage DNA, measured by the immunologic procedure after cooling, is shown in Figure 2. A typical "melting curve" is obtained with a $T_m$ (temperature at which 50% denaturation occurs under the conditions described) of 75.8°. In the presence of 1 M urea the $T_m$ is lowered 2.8° to 73.0°. At 73° there is essentially no denaturation of DNA in the absence of a denaturing agent. This temperature was chosen for evaluation of other denaturing agents because it permits a sensitive assay of denaturing effectiveness at relatively low concentrations of denaturing agent; thus, the activity of compounds which are too insoluble to permit study at lower temperatures can be investigated. Furthermore, under these conditions a small change in the concentration of denaturing agent causes a large change in the amount of DNA denaturation, so that the relative effectiveness of different compounds may be compared by determination of the concentration of denaturing agent which gives 50% denaturation of DNA under the standard conditions.

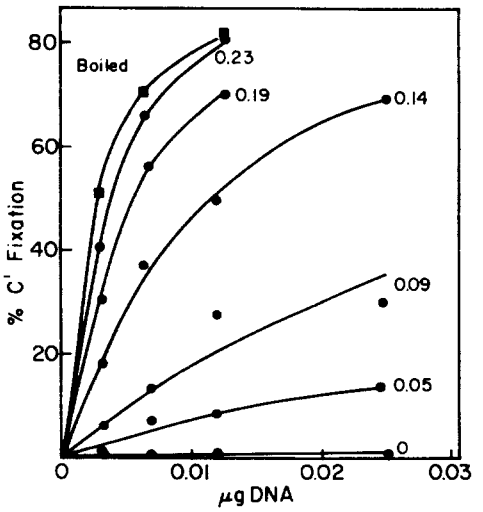


FIG. 1.—Fixation of complement by $T_4$ bacteriophage DNA after incubation at 73° with hexanamide at the indicated concentrations (M) and by DNA denatured by heating at 100° for 10 minutes.

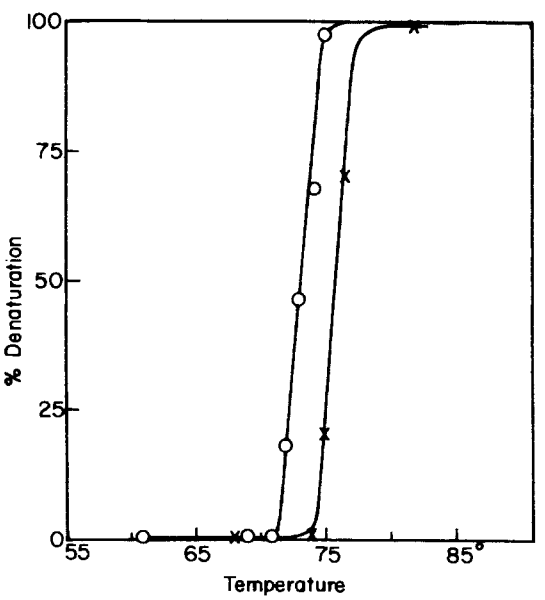


FIG. 2.—Thermal stability of $T_4$ bacteriophage DNA as determined by the immunologic method. DNA was incubated at the indicated temperature for 15 minutes prior to assay, as described in the text. X, in the presence of 0.05 M Tris buffer, 0.001 M EDTA, pH 7.6; O, buffer plus 1.0 M urea.

The effect of time of incubation at 73° on the amount of denaturation of DNA in various concentrations of urea is shown in Figure 3. At any given concentration of urea, the amount of denaturation reaches a constant level in 10 minutes, and does not change over the following 30 minutes.[1]

[1] The absence of a time-dependence for urea denaturation, as well as the very sharp temperature dependence for urea denaturation, indicate that decomposition of urea to cyanate and ammonia, which occurs very slowly at this temperature, is not responsible for the observed denaturation.

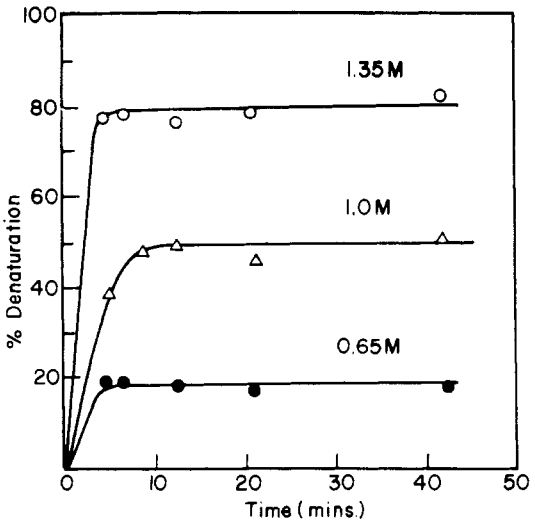


FIG. 3.—The effect of time of incubation at 73° on the amount of denaturation of DNA at three concentrations of urea.

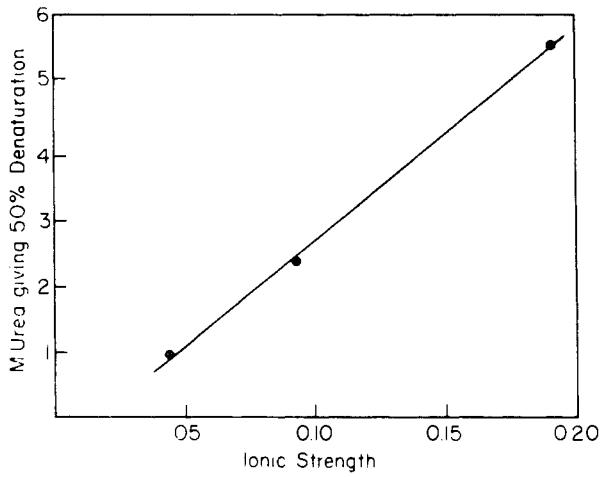


FIG. 4. -The effect of increasing ionic strength on the concentration of urea which gives 50% denaturation of $T_4$ bacteriophage DNA in 0.05 M Tris, $10^{-3}$ M EDTA, *p*H 7.6. Ionic strength increased by the addition of NaCl.

The amount of urea required to give 50% denaturation of DNA at 73° increases with increased ionic strength (Fig. 4). The ionic strength of the Tris-EDTA buffer, 0.043, was selected for further experiments. It is well known that the denaturation of DNA occurs more readily at lower ionic strength (Doty *et al.*, 1959).

The amount of denaturation of DNA by 1.0 M urea at 73° is dependent on the concentration of DNA, with a larger amount of denaturation observed in the more dilute solutions (Table I). We have not attempted to account quantitatively for this concentration dependence. A theoretical treatment by Zimm (1960) indicates that the concentration dependence of the extent of DNA denaturation should be small. The amount of denaturation of DNA in 1 M urea was shown to be independent of *p*H in a series of Tris-EDTA buffers of constant ionic strength, between *p*H 6.9 and 8.4.

TABLE I

EFFECT OF DNA CONCENTRATION ON DENATURATION BY 1.0 M UREA AT 73°[a]

| DNA (μg/ml) | % Denaturation |
|---|---|
| 0.9 | 77 |
| 1.8 | 75 |
| 3.8 | 70 |
| 7.5 | 58 |
| 15.0 | 48 |
| 30.0 | 43 |

[a] In 0.05 M Tris buffer, 0.001 M EDTA, *p*H 7.6, 30 minutes.

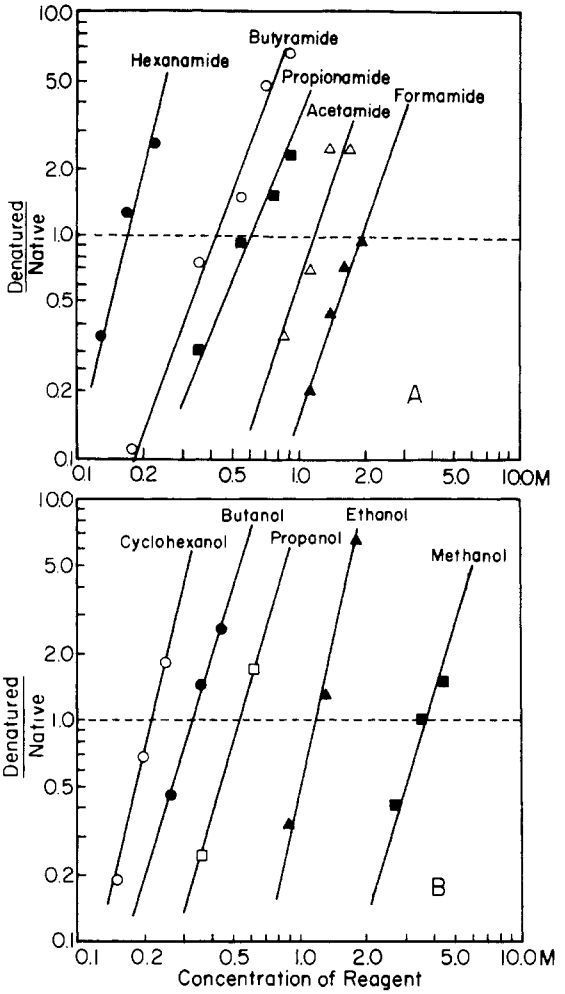


FIG. 5.—Logarithmic plots of the ratio of denatured to native $T_4$ bacteriophage DNA as a function of the concentration of denaturing agents at 73°. The dashed line indicates the concentration of reagent necessary to give 50% denaturation.

With this information available, the following standard conditions were selected for evaluation of the relative effectiveness of different denaturing agents: Incubation for 30 minutes at 73° in 0.05 M Tris buffer, *p*H 7.6, containing $10^{-3}$ M EDTA, with a constant DNA concentration of 16 μg/ml. This concentration of DNA permits dilution of the reaction mixtures to a point at which the denaturing agent does not interfere with the complement fixation assay.

The concentrations of denaturing agents required to give 50% denaturation of DNA under these conditions were determined from linear, logarithmic plots of the ratio of denatured to native DNA against the concentration of denaturing agent. Examples for a series of amides (*A*) and alcohols (*B*) are shown in Figure 5.

The concentrations of different reagents which give 50% denaturation of $T_4$ bacteriophage DNA under the standard assay conditions are summarized in Table II. The tabulation of reagents has been subdivided into

TABLE II

REAGENT CONCENTRATION (M) GIVING 50% DENATURATION OF $T_4$ BACTERIOPHAGE DNA IN AQUEOUS SOLUTION AT 73°, Ionic Strength 0.043

| | M | | M |
|---|---|---|---|
| **Aliphatic alcohols** | | | |
| Methyl alcohol | 3.5 | Isobutyl alcohol | 0.45 |
| Ethyl alcohol | 1.2 | *n*-Butyl alcohol | 0.33 |
| Isopropyl alcohol | 0.90 | *tert*-Amyl alcohol | 0.39 |
| *n*-Propyl alcohol | 0.54 | | |
| Allyl alcohol | 0.50 | Ethylene glycol | 2.2 |
| *sec*-Butyl alcohol | 0.62 | Glycerol | 1.8 |
| *tert*-Butyl alcohol | 0.60 | | |
| **Thio alcohols** | | | |
| Dithioglycol | 2.2 | | |
| **Cyclic alcohols** | | | |
| Cyclohexyl alcohol | 0.22 | Phenol | 0.08 |
| Benzyl alcohol | 0.09[a] | *p*-Methoxyphenol | 0.09 |
| Inositol | 1.5[a] | | |
| **Other cyclic compounds** | | | |
| Aniline | 0.08 | 1,4-Dioxane | 0.64 |
| Pyridine | 0.09 | γ-Butyrolactone | 0.55 |
| Purine | 0.13 | 3-Aminotriazole | 0.42 |
| **Amides** | | | |
| Formamide | 1.9 | Butyramide | 0.46 |
| *N*-Ethylformamide | 1.0 | Hexanamide | 0.17 |
| *N,N*-Dimethylformamide | 0.60 | | |
| Acetamide | 1.1 | Glycolamide | 1.1 |
| *N*-Ethylacetamide | 0.88 | Thioacetamide | 0.32 |
| *N,N*-Dimethylacetamide | 0.60 | δ-Valerolactam | 0.34 |
| Propionamide | 0.62 | | |
| **Ureas** | | | |
| Urea | 1.0 | Ethyleneurea | 0.53 |
| Carbohydrazide | 1.0 | | |
| 1,3-Dimethylurea | 1.0 | Thiourea | 0.41 |
| Ethylurea | 0.60 | Allylthiourea | 0.28 |
| *tert*-Butylurea | 0.22 | Ethylenethiourea | 0.32 |
| **Carbamates** | | | |
| Urethan | 0.50 | *N*-Propylurethan | 0.24 |
| *N*-Methylurethan | 0.38 | | |
| **Other compounds** | | | |
| Cyanoguanidine | 0.21 | Acetonitrile | 1.2 |
| Sulfamide | 1.1 | Tween 40 | >20% |
| Glycine | 2.2 | Triton X-100 | >10% |

[a] Based on a single experimental point.

chemical groups and arranged in approximate order of increasing denaturing effectiveness within each group. Replicate determinations indicate the accuracy of the method to be within approximately ±10%.

In the series of alcohols, effectiveness as a denaturant increases with increasing chain length in the series methyl, ethyl, *n*-propyl, and *n*-butyl alcohol. Branching of the alkyl chain decreases the denaturing effectiveness and increases the solubility in water of the denaturants, as compared to the straight chain isomer. Masking of the hydrocarbon chain in glycol, dithioglycol, and glycerol, as compared to ethyl and propyl alcohol, decreases denaturing effectiveness. Cyclohexyl alcohol gives 50% denaturation at a concentration of 0.22 M compared to 0.33 M for the longest chain aliphatic alcohol studied, *n*-butyl alcohol. This increase in effectiveness is approximately the amount expected for the addition of two carbon atoms to *n*-butyl alcohol. Again, masking of the hydrocarbon chain in cyclohexyl alcohol by hydroxyl substituents, as in inositol, markedly reduces activity.

Phenol, *p*-methoxyphenol, benzyl alcohol, aniline, pyridine, and purine are the most effective denaturants examined. Nonaromatic cyclic and heterocyclic compounds show moderate denaturing activity.

In the amide series, formamide causes 50% denaturation of DNA at a concentration of 1.9 M. As in the alcohol series, lengthening of the alkyl chain in the series formamide, acetamide, propionamide, butyramide, and hexanamide increases denaturing effectiveness. Substitution of methyl or ethyl groups on the nitrogen atom increases the activity, compared to the parent compound. The five-carbon cyclic amide, δ-valerolactam, is more effective than the four-carbon straight chain amide, butyramide, but is less effective than the six-carbon straight chain amide, hexanamide. Thioacetamide, as compared to acetamide, is less soluble in water and is a better denaturant.

In this series, as well as in the other series of compounds examined, denaturation is not due to a decrease in the concentration of water in the solutions containing denaturing agent. Not only is there no correlation between the amount of denaturation and the concentration of water in the solutions, but the concentration of water in solutions of the more effective denaturing agents is considerably higher than in solutions of the less effective compounds.

Urea is more effective than formamide and is as effective as acetamide. Large alkyl substituents on the nitrogen atoms of urea increase effectiveness as a denaturant in the series 1,3-dimethylurea, ethylurea, ethyleneurea, and *t*-butylurea. Amino substitution on both nitrogen atoms of urea (carbohydrazide) does not alter the activity. The sulfur analog of urea, thiourea, is a more effective denaturant than urea and is less soluble in water.

Substitution of a methyl or propyl group on the nitrogen atom of urethan again results in an increase in denaturing effectiveness. Furthermore, the presence of the ethoxy substituent on carbon, in urethan, increases effectiveness of the amide group as compared to a hydrogen or amino substituent, in formamide and urea.

Among the miscellaneous compounds, cyanoguanidine is highly effective. Sulfamide, a structural analog of urea which is inactive as a denaturing agent toward bovine serum albumin, shows an activity similar to that of urea toward DNA. The nonionic detergents, Tween 40 and Triton X-100, are not effective denaturants in the highest concentration tested.

The stability of a series of different DNA molecules toward heat denaturation increases with increasing guanine plus cytosine content of the DNA (Marmur and Doty, 1959). The amounts of urea or pyridine required to give 50% denaturation of DNA isolated from $T_4$ bacteriophage, *Diplococcus pneumoniae*, *Bacillus subtilis*, calf thymus, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Streptomyces viridochromogenes* were determined. Instead of anti-$T_4$ DNA, however, the antibodies present in lupus erythematosus sera were used to estimate denaturation. As shown in Figure 6, the stabilities of these different DNA molecules toward denaturation by urea and by pyridine increases with increasing guanine plus cytosine content of the DNA.

The immunologic method measures the amount of denatured DNA after rapid cooling. The results obtained after rapid cooling are slightly different from those obtained by measurement of the properties of DNA at ambient temperature; *i.e.*, there are two stages in denaturation, one of which is measured at ambient temperature and is reversible on quick cooling, and a second stage, which is irreversible on quick cooling (Doty *et al.*, 1959; Geiduschek, 1961). In order to determine whether the results obtained for the effect of different types of denaturing agents on the second stage of denaturation, measured after rapid cooling, also apply to the first type of denaturation, a series of

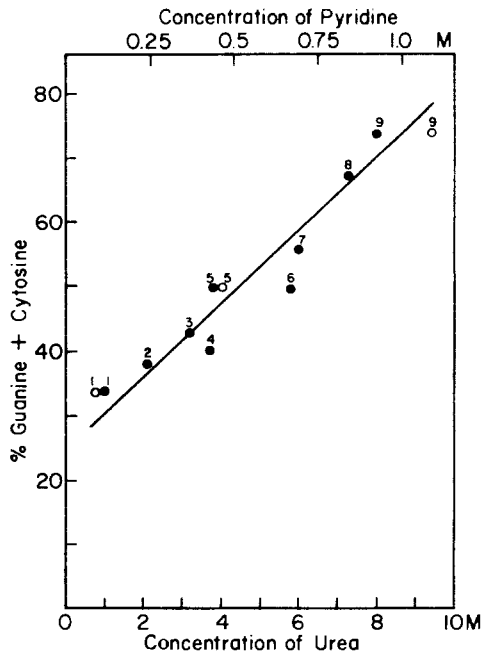


FIG. 6.—The concentrations of urea (lower scale) or pyridine (upper scale) required to give 50% denaturation of DNA preparations which vary in their guanine plus cytosine content. ●, urea as the denaturing agent; ○, pyridine as the denaturing agent. 1, $T_4$ bacteriophage; 2, *Diplococcus pneumoniae;* 3, *B. subtilis;* 4, calf thymus; 5, *E. coli* C; 6, *E. coli* B; 7, *Serratia marcescens;* 8, *Ps. aeruginosa;* 9, *S. viridochromogenes.*

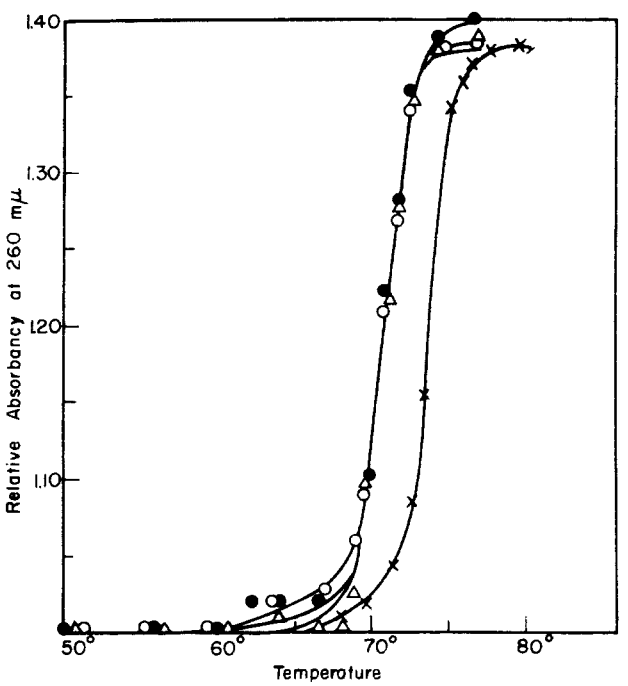


FIG. 7.—Thermal stability of $T_4$ bacteriophage DNA as determined by the relative change of optical density in 0.05 M Tris buffer, 0.001 M EDTA, *p*H 7.6, at 260 mμ at the indicated temperatures, compared to 25°. X, no additions; ●, 0.33 M *n*-butyl alcohol; △, 0.60 M *t*-butyl alcohol; ○, 1.0 M urea.

measurements was carried out in which the change in absorbancy of DNA on denaturation was measured at ambient temperature (Doty *et al.*, 1959). As shown in Figure 7, the $T_m$ for DNA denaturation measured in this way is 73.7°, 2° lower than the $T_m$ observed

TABLE III
RENATURATION OF $T_4$ BACTERIOPHAGE DNA

| Denaturing Agent | Optical Assay: Relative Absorbancy at 260 mμ[a] Ambient, 78° | Renatured, 57°[b] |
|---|---|---|
| None | 1.38 | 1.03 |
| 1.0 M urea | 1.38 | 1.04 |
| 0.60 M *t*-butyl alcohol | 1.39 | 1.03 |
| 0.33 M *n*-butyl alcohol | 1.40 | 1.05 |
| | Immunologic Assay: % Denatured DNA After Incubation for 30 Minutes at 73° | |
| | Quick-Ccoled | Incubated at 55°[d] |
| None[c] (100°) | 100 | 25 |
| 2.0 M urea | 100 | 38 |
| 1.0 M thiourea | 100 | 34 |
| 1.2 M urethan | 100 | 40 |
| 1.2 M thioacetamide | 100 | 42 |
| 1.5 M dimethylformamide | 100 | 38 |

[a] Absorbancy relative to a control DNA solution maintained at 25°; corrected for the volume expansion of water. [b] DNA was heated slowly to 78° in the presence of reagent. Solid NaCl was added to 0.3 M and the solution was allowed to cool slowly to 57° over 45 minutes; the final reading was obtained after 120 minutes. No significant change in optical density occurred immediately after the addition of NaCl. [c] DNA fully denatured in the absence of reagent by boiling for 10 minutes. [d] Denatured samples were dialyzed at 55° for 120 minutes against 100 volumes of a solution containing 0.5 M NaCl plus the original concentration of denaturing agent.

by the immunologic method after quick-cooling (Fig. 2). Three denaturing agents, *n*-butyl alcohol, *t*-butyl alcohol, and urea, were examined at concentrations which give the same amount of denaturation, 50%, in the standard immunologic assay. As shown in Figure 7, these concentrations of denaturants also have identical effects on the lowering of the $T_m$, measured spectrophotometrically at ambient temperature. This suggests that the results obtained by the immunologic assay are similar to those obtained by measurement of denaturation at ambient temperature, except that in the latter case the effects are observed at a temperature which is about 2° lower.

The thermal denaturation of DNA, of both type I and type II described above, may be partly or completely reversed by slow cooling or incubation in the presence of salt at 55° (Marmur and Lane, 1960; Doty *et al.*, 1960; Levine *et al.*, 1960). As shown in Table III, renaturation can also be observed after complete denaturation in the presence of a number of the denaturing agents described above, as measured both by change in absorbancy and by the immunologic assay.

In Table IV are shown the results of a study of the solubility of tetrasodium pyrophosphate and of adenine, in a series of aqueous solutions of denaturants. The concentration of denaturant in the adenine series is 1 M and in the pyrophosphate series the denaturant is 3 M. The solubility of pyrophosphate decreases with increasing chain length or alkyl substitution on the nitrogen or oxygen atoms of the compounds examined. For example, the solubility of pyrophosphate in solutions of alcohols progressively decreases in the series methyl, ethyl, and propyl alcohol. Pyrophosphate solubility is decreased slightly less by *n*-propyl alcohol than by isopropyl alcohol, although *n*-propyl alcohol

TABLE IV

SOLUBILITY OF TETRASODIUM PYROPHOSPHATE AND ADENINE AT 37.0 ± 0.5° IN AQUEOUS SOLUTIONS OF DENATURING AGENTS

| Reagent | Pyrophosphate Solubility[a] (M) | Adenine Solubility[b] (M × $10^2$) |
|---|---|---|
| $H_2O$ control | 0.310[c] | 1.54[d] |
| Urea | 0.215 | 1.77 |
| Carbohydrazide | 0.182 | 2.01 |
| Ethylurea | 0.052 | 2.25 |
| Thiourea | 0.224[e] | |
| Urethan | 0.057 | 2.71 |
| *N*-Methylurethan | 0.048 | |
| *N*-Propylcarbamate | | 3.42 |
| Methyl alcohol | 0.124 | 1.59 |
| Ethyl alcohol | 0.062 | 1.77 |
| Isopropyl alcohol | 0.039 | 2.02 |
| *n*-Propyl alcohol | 0.054 | 2.26 |
| *tert*-Butyl alcohol | 0.031 | 2.18 |
| Isobutyl alcohol | | 2.71 |
| *n*-Butyl alcohol | | 2.84 |
| *t*-Amyl alcohol | | 2.67 |
| Allyl alcohol | 0.064 | |
| Ethylene glycol | 0.166 | 1.68 |
| Glycerol | 0.211 | |
| Formamide | 0.166 | 1.54 |
| *N,N*-Dimethylformamide | 0 043 | 2.77 |
| *N,N*-Diethylformamide | 0.018 | 3.35 |
| Acetamide | 0.177 | 2.06 |
| Propionamide | 0.105 | 2.25 |
| Butyramide | 0.056[f] | 3.01 |
| Pyridine | 0.059 | |
| Dioxane | 0.030 | 2.96 |
| Acetonitrile | | 2.03 |
| 3-Aminotriazole | | 2.79 |
| Glycine | 0.254 | |

[a] The concentration of reagent in aqueous solution was 3 M, except as noted. Tetrasodium pyrophosphate was determined as inorganic phosphate after acid hydrolysis. Equilibrated by vigorous shaking for 72 hours with excess pyrophosphate; 48-hour analyses generally agree to within 2%. Solubilities are reproducible to ±5%. [b] The concentration of reagent in aqueous solution was 1 M. The solubility of adenine was determined spectrophotometrically at 260 mμ, after dilution, utilizing a molar extinction coefficient of 13.3 × $10^3$ (Dawson *et al.*, 1959). Aliquots were analyzed at 48 hours and every 24 succeeding hours until two successive determinations agreed within 1%. The *p*H of a water solution saturated with adenine was 6.49. [c] Experimental value. Interpolated literature value = 0.29 M (Merck Index, 7th ed., Merck and Co., Rahway, New Jersey, 1960, p. 1026). [d] Literature value = 0.665 × $10^{-2}$ M at 25° and 18.5 × $10^{-2}$ M at 100° (Dawson *et al.*, 1959). [e] 2.9 M solution. [f] 2.5 M solution.

is a better denaturant. In different groups of compounds, thiourea, urea, and formamide are better denaturants than methyl alcohol, although the solubility of pyrophosphate in the presence of the former compounds is higher.

The solubility of adenine is increased by alkyl substitution on nitrogen, oxygen, or carbon (extension of the chain) in a given series of compounds. Both the solubility of adenine and the denaturing effectiveness toward DNA increase in the series methyl, ethyl, propyl, and *n*-butyl alcohol. Branched chain alcohols are less effective denaturants and are less effective in solubilizing adenine than their straight-chain isomers.

## DISCUSSION

According to the Watson-Crick model, native DNA has a high density of negative charge on the outside and has its relatively non-polar bases on the inside of a

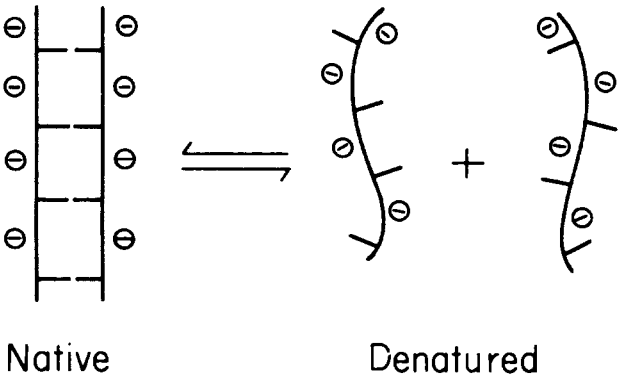


FIGURE 8

double-stranded helix which is held together by hydrogen bonds. Upon denaturation there is a partial or complete separation of two strands (Marmur and Lane, 1960; Doty *et al.*, 1960) with a resulting decrease in the density of negative charge (Ascoli *et al.*, 1961) and an increased exposure of the hydrophobic bases to the solvent. An oversimplified, highly schematic representation of native double strand and of fully denatured single strand DNA is shown in Figure 8.[2] Denaturation will be favored by solvent changes which destabilize high charge density (*i.e.*, solvents of low ion-solvating power, in which compounds of high charge density exhibit a relatively high activity coefficient) and which stabilize the exposed bases (*i.e.*, "hydrophobic" solvents, in which the non-polar DNA bases exhibit a relatively low activity coefficient). These two properties of the solvent may be expected to change in a similar, but not identical, manner with changes in the composition of the solvent. Additional factors which could favor denaturation include such specific interactions as hydrogen bonding of the solvent to the exposed bases and molecular compound formation, which may involve a "charge-transfer" interaction of the π-electron systems of the aromatic bases with the solvent (Weil-Malherbe, 1946; Booth and Boyland, 1953).

Tetrasodium pyrophosphate is a compound of high charge density, and conditions which destabilize this molecule (*i.e.*, increase the activity coefficient) would be expected to destabilize the high charge density of native DNA, compared to denatured DNA. This destabilizing effect was measured by examining the effect of a series of denaturing agents on the solubility of tetrasodium pyrophosphate. Since the activity of

[2] A discussion of the detailed structure of the various forms of partly and fully denatured DNA is beyond the scope of this paper, but may be found in a review by Marmur *et al.* (in press); we are concerned here only with the difference in properties between native DNA and the various forms of denatured DNA, all of which may be expected to exhibit, to a greater or lesser extent, a decreased charge density and increased exposure of hydrophobic bases to the solvent, compared to native DNA. It may be noted that rapidly reversible denaturation (measured at ambient temperature, Fig. 7) and denaturation which is irreversible on rapid cooling exhibit similar sensitivity to solvent changes; these two types of denaturation presumably involve different degrees of strand separation. The temperature range over which denaturation occurs is small for $T_4$ bacteriophage DNA, reflecting the high degree of homogeneity of these preparations; the fact that temperatures exist at which denaturation is only partial may be interpreted in terms of incomplete strand separation due to inhomogeneity of the strands, or in terms of a mobile equilibrium in which, at any moment, a constant fraction of the DNA is denatured. Both of these formulations are consistent with the time-independence of denaturation (Fig. 3).

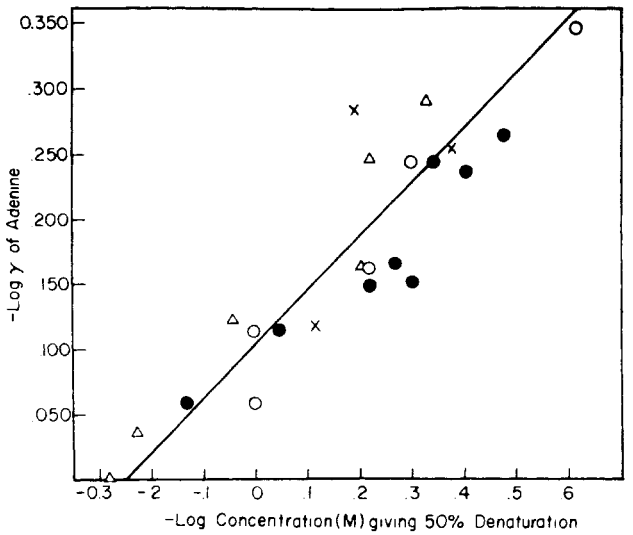


FIG. 9.—Logarithmic plot of the denaturing effectiveness of a series of compounds toward DNA against the effect of 1.0 M solutions of the same compounds on the activity coefficient of adenine, from the solubility data of Table IV. ●, alcohols; ○, ureas and carbamates; △, amides; X, other compounds.

the solid phase is constant, a decrease in solubility corresponds to an increase in activity coefficient, *i.e.*, a "destabilization" of the compound, which may reasonably be ascribed to its highly ionic character. As might be expected, the addition of alkyl substituents or an increase in the chain length of alkyl groups on the compounds examined destabilizes pyrophosphate (*i.e.*, increases its activity coefficient) in aqueous solutions of the compounds. Such structural changes may also, therefore, be expected to decrease the stability of the high charge density of native DNA relative to denatured DNA in aqueous solutions of the compounds. It is of particular interest that urea decreases the ion-solvating ability of water toward pyrophosphate in spite of the fact that urea has a higher dielectric constant than water; this is another example of the frequently observed poor correlation between dielectric constant and ion-solvating ability (Fainberg and Winstein, 1956; Kosower, 1958).

Herskovits *et al.* (1961) have concluded that the electrostatic free energy of DNA is lower in methanol than in water, because of the large amount of ion pairing which occurs in this solvent. However, the molar free energy of ions and ion pairs must be greater in alcohol solutions than in water, because their generally low solubility means that they exhibit increased activity coefficients in alcohol, compared to aqueous, solutions. DNA is no exception to the generalization that the stability of charged compounds is decreased (*i.e.*, the activity coefficient is increased) in alcohol solutions, since denatured DNA is insoluble in alcohol in the presence of a low concentration of salt (Herskovits *et al.*, 1961). Thus, the activity coefficient and molar free energy of DNA are higher in alcohol than in water, in spite of the exposure of non-polar bases to the solvent on denaturation. The solubility is decreased by the addition of salt, which must increase the amount of ion pairing. This behavior indicates that the poor ion-solvating power of alcohol and related solvents decreases the stability of DNA in these solvents, relative to water. In this connection it is of interest that native DNA, with its high charge density, is insoluble, but denatured DNA is soluble in dimethyl sulfoxide (Helmkamp and Ts'o, 1961). It is concluded that the decreased ion-solvating power of the solutions of denaturing agents examined is one factor contributing to their denaturing effect.

The increase in denaturing effectiveness with an increase in the number or size of alkyl substituents in the alcohol, amide, urethan, and urea series of denaturing agents is consistent with a contribution of nonspecific hydrophobic interactions to DNA denaturation by these compounds. This effect arises by a stabilization of denatured DNA, relative to native DNA, because of exposure of the hydrophobic bases to the solvent in denatured DNA. That such an effect contributes to DNA denaturation is indicated by the increased solubility of adenine in solutions of denaturing agents. Increases in the number or size of alkyl substituents increase the solubilizing effectiveness of denaturing agents toward adenine (Table IV) and, in contrast to the situation with tetrasodium pyrophosphate, there is even a moderately good correlation among different groups of compounds between denaturing effectiveness and effect on adenine solubility (Fig. 9). Again it is of interest that urea, in spite of its high dipole moment, increases adenine solubility. Thus, effective denaturing agents increase the stability and decrease the activity coefficient and free energy of adenine and, presumably, of the exposed bases of denatured DNA.

Herskovits *et al.* (1961), Geiduschek and Herskovits (1961), Hamaguchi and Geiduschek (1962), and Ts'o *et al.* (1962a) have independently obtained evidence favoring a hydrophobic mechanism for DNA denaturation by alcohols, dimethylformamide, large anions, and aromatic compounds, and Ts'o *et al.* (1962b) has further shown that a series of aromatic compounds increases the solubility, and therefore the stability, of several DNA bases. Although it is possible that aromatic denaturants are more effective than aliphatic denaturants because of an interaction with the aromatic DNA bases through some sort of $\pi$-electron interaction (Ts'o *et al.*, 1962a), our data do not provide any strong support for such a hypothesis. Although aromatic denaturants are generally somewhat more effective than aliphatic analogs, the differences are not great. Hexanamide, cyclohexyl alcohol and *t*-butylurea are only two- to three-fold less effective than most aromatic compounds and are more effective than 3-aminotriazole. These data do not exclude a special mechanism for the action of aromatic compounds, but they indicate that any such mechanism does not make a very large contribution in addition to that which is expected from the nonspecific hydrophobic character of the aromatic compounds we have examined.

The results of this and other studies provide no evidence that hydrogen bonding between denaturing agent and DNA contributes to the denaturing effectiveness of the compounds examined. The high denaturing activity of compounds containing no acidic protons and the increase in activity upon substitution of an alkyl group for hydrogen rule out hydrogen bonding by proton donation from the denaturing agent. The absence of any correlation between basicity and denaturing effectiveness and the activity of compounds, such as dioxane and acetonitrile, which are considerably weaker bases than water rule out hydrogen bonding in which the denaturing agents act as proton acceptors. Bifunctional hydrogen bonding is ruled out by the activity of compounds which contain no acidic protons and only a single basic site. Furthermore, since the results appear to be adequately accounted for by the decreased ion-solvating properties and the increased hydrophobic character of the solutions of denaturing agents, compared to water, there is no reason to invoke other mechanisms, such as hydrogen bonding.

It should be emphasized that the forces involved in the denaturation of DNA are not necessarily the same as those which are important in maintaining the structure of native DNA.[3] Although it is quite likely that hydrophobic forces contribute to the stability of native DNA (Herskovits *et al.*, 1961), the effectiveness of hydrophobic denaturing agents does not prove this hypothesis and, in particular, the conclusion that hydrogen bonding of DNA to the denaturing agent does not contribute significantly to the denaturing activity of the compounds examined does not imply that hydrogen bonding is not important in maintaining the structure of native DNA.

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[3] See footnote 2 of the accompanying paper (Gordon and Jencks, 1963).


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