Residual Activity of Thermally Denatured Transforming Deoxyribonucleic Acid from *Haemophilus influenzae*

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Received for publication 28 December 1964

ABSTRACT

BARNHART, BENJAMIN J. (Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md.). Residual activity of thermally denatured transforming deoxyribonucleic acid from *Haemophilus influenzae*. *J. Bacteriol.* **89**:1271-1279. 1965.—The level of residual transforming activity of heated deoxyribonucleic acid (DNA) (i.e., 1 to a few per cent of native DNA-transforming activity) was found to be independent of the heating and quenching temperatures and less susceptible than native or renatured DNA to heat inactivation upon prolonged heating above or below the critical melting temperature. Similar dose-response curves were obtained for inactivation by formamide of native and renatured DNA, but the residual-active material was much more resistant. Heating DNA above the T_m in the presence of 1% formaldehyde resulted in a level of residual activity 4 logs lower than that obtained without formaldehyde. Residual-active material was not inactivated by *Escherichia coli* phosphodiesterase, but it was susceptible to snake venom phosphodiesterase. A new genetic marker was induced in heated-quenched DNA but not in purified residual-active material following nitrous acid treatment. Residual activity was found to be less susceptible to ultraviolet inactivation and to band at a higher density region in CsCl than native DNA. In conclusion, it is suggested that the residual-active material is a structure formed by intrastrand hydrogen bonding of the separated units of heated-quenched DNA. Such a configuration would result in at least a partially double-stranded structure, which is probably the essential characteristic of the residual-active material endowing it with biological activity.

Heating an aqueous solution of native deoxyribonucleic acid (DNA) to 100 C usually results in a separation of the strands (Doty et al., 1960; Berns and Thomas, 1961; Herriott, 1963). This separation of strands results in a large loss of capacity to be taken up by competent cells and to induce genetic changes in them (Lerman and Tolmach, 1957, 1959; Barnhart and Herriott, 1963). The discovery by Marmur and Lane (1960) that slow cooling of transforming DNA heated to critical temperatures yielded higher recovery of biological activity led some workers to assume that the low activity in samples cooled rapidly (quenched) represented either a small fraction of initially undenatured material or a renatured fraction. This notion changed with the reports of Guild (1961), Rownd, Lanyi, and Doty (1961), and Guild and Robison (1963) that the residual biological activity (i.e., a few per cent of native activity) banded in a CsCl gradient at

a higher density than the original native material and close to that of denatured single-stranded DNA. Preliminary results of Barnhart and Herriott (1962) showed that the residual-active fraction of *Haemophilus influenzae* DNA was not destroyed by the action of *Escherichia coli* phosphodiesterase [Lehman's (1960) exonuclease I] and was, therefore, not representative of the bulk of denatured DNA. The present report contains similar and more extensive studies executed in an attempt to elicit the molecular nature of the units carrying this residual activity in heated and quenched *Haemophilus*-transforming DNA.

MATERIALS AND METHODS

General methodology. The method of Goodgal and Herriott (1961) was followed for the preparation of DNA from *H. influenzae*. Competent cells were prepared according to the procedure described by Barnhart and Herriott (1963). Most of the laboratory techniques have been described previously in these two publications.

DNA. In most of the experiments, the DNA

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carrying the streptomycin-resistance marker was used. This marker, which was first identified by Alexander and Leidy (1953), is the $\text{Sm}^{2,000}$ marker described by Hsu and Herriott (1961); 250 $\mu\text{g}/\text{ml}$ of streptomycin were used to screen for this marker designated Sm^{250} in the present experiments. Heated-quenched DNA was obtained by heating at 100 C in 0.15 M NaCl-0.015 M Na_3 citrate (CS) and quenching the samples in ice water according to Marmur and Lane (1960). In this solvent the T_m was 88 C. Renatured DNA (Marmur and Lane, 1960) was prepared by raising the salt concentration of a heated-quenched DNA preparation to 0.3 M NaCl-0.015 M Na_3 citrate. The DNA in this solvent was incubated in a 4-liter water bath at 66 ± 1 C for 2 hr and was then placed in a beaker containing 1 liter of water at 66 ± 1 C and allowed to cool to room temperature. This treatment generally resulted in restoration of biological activity to $30 \pm 5\%$ of the initial transforming activity (Herriott, 1961). P^{32} -labeled DNA was prepared according to Barnhart and Herriott (1963).

In the cesium chloride centrifugation and nitrous acid experiments, the pour-plate method was used to assay transformants (Goodgal and Herriott, 1961). The uptake studies using P^{32} -labeled DNA were performed according to Barnhart and Herriott (1963).

Transformation and plating procedures. The assay procedure was originally described by Goodgal and Herriott (1961) and modified by Barnhart and Herriott (1963) for optimal DNA uptake and transformation. An amount (0.1 ml) of competent cells and 0.1 ml of DNA were diluted into 2.8 ml of 0.1 M NaCl containing 0.01 M phosphate buffer (pH 6.8). Unless otherwise stated, the DNA was assayed in the linear region of the dose-response curve. After incubation with continuous shaking at 36 ± 1 C, the cells were diluted and plated for transformants with use of the overlay method.

Digestion with *E. coli* phosphodiesterase. Native or heated-quenched P^{32} -labeled DNA was diluted to a final concentration of 4.0 $\mu\text{g}/\text{ml}$ in the enzyme reaction mixture described by Lehman (1960), who generously supplied us with the enzyme preparations used in these experiments. The mixture was incubated at 37 C, and samples were removed and diluted 100-fold in ice-cold CS to stop the reaction and were then counted in a Nuclear-Chicago gas-flow counting chamber attached to a model 95 ultrascaler of the same make.

To determine P^{32} -activity soluble in perchloric acid, 0.5 ml of the enzyme reaction mixture was diluted into 0.3 ml of cold calf thymus DNA (500 $\mu\text{g}/\text{ml}$) to serve as a carrier for precipitation. Ice-cold 0.5 N perchloric acid was added to the mixture, which was then centrifuged in the cold at $10,000 \times g$. An amount (1 ml) of the supernatant fluid was dried on aluminum planchets for counting.

Treatment with snake venom phosphodiesterase. Heated-quenched Sm^{250} DNA labeled with P^{32}

was treated with purified snake venom phosphodiesterase (Worthington Biochemical Corp., Freehold, N. J.) according to Williams, Sung, and Laskowski (1961). An amount (5 $\mu\text{g}/\text{ml}$) of DNA was incubated with 0.05 or 0.15 unit per milliliter of enzyme at 37 C, and samples were removed at various times for trichloroacetic acid-soluble P^{32} -activity and transforming activity.

Formamide treatment. Native, heated-quenched, or renatured Sm^{250} DNA was diluted into 0.02 M NaCl-0.002 M Na_3 citrate containing various concentrations (v/v) of formamide (Matheson, Coleman, and Bell, East Rutherford, N.J.) according to Marmur and T'So (1961). After 20 min of incubation at 37 C, the samples were diluted 100-fold for biological assay.

Formaldehyde treatment. Native, renatured, or heated-quenched Sm^{250} DNA was diluted into saline with or without 1% (w/v) formaldehyde. Some samples remained at room temperature (22 C), whereas duplicate samples in glass screw-capped tubes were heated for 5 min in a boiling-water bath and immediately quenched in ice water. All samples were diluted 100-fold and were assayed for biological activity.

Prolonged heating at 79 and 100 C. Samples (1 ml) of 5 $\mu\text{g}/\text{ml}$ of native, heated-quenched, renatured (2 hr at 67 C), or partially renatured (20 min at 67 C) Sm^{250} DNA in 0.15 M NaCl-0.01 M phosphate buffer (pH 6.8) were heated for varying periods of time in screw-capped glass tubes in 79 or 100 C water baths. The samples were cooled quickly in ice water and diluted for biological assay.

Ultraviolet irradiation. Native, heated-quenched, or renatured Sm^{250} DNA was diluted in CS to a final concentration of 5 $\mu\text{g}/\text{ml}$. Samples (1 ml) were irradiated in topless glass petri dishes (100 by 20 mm), placed on a rotator (Arthur H. Thomas Co.) to ensure adequate agitation of the solution. A 15-w ultraviolet germicidal lamp (General Electric Co.) was used at a dose rate of approximately 30 ergs per mm^2 per sec. The DNA samples were diluted 500-fold for biological assay.

Nitrous acid treatment. The method of Horn and Herriott (1962) was followed. To 1 volume of a mixture of 0.1 M acetate buffer and 2 M NaNO_2 in 0.15 M NaCl (pH 4.7) was added 1 volume of native or heated-quenched DNA bearing a cathomycin-resistance marker isolated from bacteria resistant to 2.5 $\mu\text{g}/\text{ml}$ of cathomycin, C^{2-5} DNA, at a concentration of 50 $\mu\text{g}/\text{ml}$, or residual-active material carrying the same marker. The latter was prepared by treating heated-quenched C^{2-5} DNA with *E. coli* phosphodiesterase (as described in this paper), deproteinizing according to Sevag, Lackman, and Smolens (1938), and then dialyzing to remove the products of digestion. Over 90% of the DNA was rendered soluble in cold 0.5 N perchloric acid. The mixtures were incubated at 37 C, and at various times samples were removed and neutralized to pH 6.8 by diluting 10-fold into 0.1 M phosphate buffer containing 0.3 M NaCl. The DNA (native or heated-quenched,

2.5 $\mu\text{g/ml}$; residual-active material at less than 0.25 $\mu\text{g/ml}$) was annealed according to Marmur and Lane (1960) and was then diluted 30-fold for biological assay. After the transformation reaction (described in this paper), the cells were appropriately diluted and pour-plated with 5 $\mu\text{g/ml}$ of streptomycin to screen for mutants or with 2.5 $\mu\text{g/ml}$ of cathomycin to assay for the native marker.

Cesium chloride (CsCl) density gradient centrifugation. Purified preparations of native $\text{C}^{2.5}$ DNA and heated-quenched Sm^{250} DNA were treated with *E. coli* phosphodiesterase with conditions as described in this paper. The DNA was then deproteinized with chloroform-octanol (Sevag et al., 1938) and dialyzed to remove the products of digestion. An amount of 12 $\mu\text{g/ml}$ of $\text{C}^{2.5}$ DNA and approximately 0.012 $\mu\text{g/ml}$ of Sm^{250} residual-active material were mixed in a solution of CsCl in 0.01 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 7.2). The CsCl was an optical-grade salt (Harshaw Chemical Co., Cleveland, Ohio) which had an optical density for a standard solution at 20 C of less than 0.03 for a 1-cm light path at 260 m μ as measured in a Beckman Model DU spectrophotometer. The salt-DNA solution was adjusted to a refractive index (n_D^{25}) of 1.4002 with Tris buffer as measured in an Abbey refractometer which was standardized with a sucrose standard having the desired n_D^{25} . Calculations according to Ifft, Voet, and Vinograd (1961) showed that, after the 72-hr centrifugation at 35,000 rev/min in the Spinco Model L ultracentrifuge, the range of sample densities was approximately 1.57 to 1.85 g/cm³. Fractions were collected with the apparatus described by Szybalski (1960).

The fractions were not diluted prior to biological assay (as described in Materials and Methods) for the residual-active Sm^{250} marker, but were diluted 10,000-fold before assaying for the $\text{C}^{2.5}$ marker residing on native DNA. After 2 hr at 36 ± 1 C in liquid medium, the transformation mixtures were pour-plated directly into agar containing the antibiotics.

RESULTS

No simple direct means of evaluating the molecular nature of the heat-stable residual activity could be devised so that it was necessary to resort to indirect studies. These are shown below and include determining the effects on the residual activity of several enzymes that attack specific structures or of a denaturing agent on the residual activity, blocking renaturation at elevated temperatures with formaldehyde, and comparing the effect of ultraviolet inactivation and nitrous acid mutagenesis on the residual activity with that on native DNA.

Effect of E. coli phosphodiesterase or exonuclease I (Lehman's enzyme) on the residual activity. This enzyme was reported to digest de-

natured DNA (Lehman, 1960). If the residual activity is denatured single-stranded DNA, as has been suggested, then the transforming activity should fall in proportion to the digestion. The results presented in Table 1 show that 200 units per milliliter of this enzyme digested nearly all the DNA to an acid-soluble form (column 2) but that this treatment had only a slight effect on the residual transforming activity (column 5) and on the fraction of the P^{32} -labeled material adsorbed and irreversibly bound to the bacteria. These results confirm those reported earlier by Barnhart and Herriott (1962). Lehman and Nussbaum (1964) recently showed that if a phosphate resides on the 3' position of the terminal base, the polydeoxyribonucleotide strand is refractory to digestion by exonuclease I. This may explain the observed resistance of residual-active material to digestion.

Digestion with snake venom phosphodiesterase. This enzyme digests DNA by attacking the 5' phosphorylated end groups, releasing 5' mononucleotides (Razzel and Khorana, 1959). Digestion by this enzyme would imply that the molecule is an open structure with free end groups rather than a ring with no terminal bases. The results presented in Fig. 1 show that heated-quenched DNA was rapidly digested by this enzyme. The residual activity was also destroyed. At the lower enzyme concentration, the loss of biological activity exhibited a shoulder which is interpreted to mean that digestion from the ends of the units did not produce an immediate or "one hit" inactivation curve, as would be expected if an endonuclease contaminant were responsible for the loss in activity.

Effect of formamide on the residual activity. Marmur, Schildkraut, and Doty (1962) showed

TABLE 1. Resistance of residual activity to digestion by *Escherichia coli* phosphodiesterase

DNA sample	(1) Enzyme concn (units/ml)	(2) Perchloric acid solubility (% of total)*	(3) Bacterial adsorption of DNA (% of total)*	(4) Bacterial irreversible binding of DNA (% of total)*	(5) Bacterial transformations (% of native control)†
Native	—	9	15	9.1	100
	200	19	9.5	6.8	76
Heated (100 C) and quenched	—	11	2.4	1.3	1.8
	40	89	3.2	1.2	1.4
	200	97	2.0	1.2	1.5

* Calculated on the basis of total P^{32} activity per milliliter of reaction mixture.

† Calculated on the basis of transformations per milliliter of the native control.

FIG. 1. Inactivation of the streptomycin resistance marker of residual-active material by snake venom phosphodiesterase.

FIG. 2. Effect of formamide on the streptomycin resistance marker of native, renatured, and heated-quenched DNA. The solvent was 0.02 M NaCl-0.002 M Na₂ citrate in addition to the indicated formamide.

that the transforming activity of pneumococcal DNA is greatly reduced by exposure to formamide. Since this is a mild method of denaturing, it was of interest to compare its action on native, renatured, and the residual activity of a heated-quenched preparation. The experimental results plotted in Fig. 2 indicate that the transforming activity of native and renatured *Haemophilus* DNA falls sharply with increasing formamide, whereas the residual activity of heat-denatured DNA is much more refractory.

Effect of formaldehyde before and after quick cooling of heated-quenched DNA. The work of Stollar and Grossman (1962) suggests that formaldehyde prevents not only interstrand but also intrastrand hydrogen bonding of single-stranded DNA. If formaldehyde fails to reduce the level of residual activity even at 100 C, at which point the strands are separated (Herriott, 1963), this would eliminate the model of a structure which is endowed with biological activity as a result of hydrogen bond formation. The data in Table 2 show that 1% formaldehyde at 100 C reduced the level of residual-transforming activity far below the level in the control. The formaldehyde had no effect on the residual activity when added after cooling, even if incubated at temperatures up to 60 C. If the action at 100 C is not just an effect of increased rate of reaction at the higher temperature, it suggests that at 100 C the residual-active material has groups available to interact with formaldehyde which are not available immediately after quick cooling to lower temperatures.

Heat inactivation. The results of heating (100 C) native, renatured, and heated-quenched DNA

TABLE 2. Effect of formaldehyde (HCHO) on biological activity of native, renatured, and heated-quenched DNA

Sample	Heated (100 C)	1% HCHO	Transformations per ml	Per cent of native control*
Native	-	-	1.1×10^6	100
	+	-	4.1×10^4	3.7
	-	+	8.7×10^5	79
	+	+	1.0×10^5	<0.0001
Renatured	-	-	2.2×10^5	20
	+	-	1.1×10^4	1.0
	-	+	1.4×10^5	14
	+	+	1.0×10^5	<0.0001
Heated (100 C) and quenched	-	-	4.9×10^4	4.5
	+	-	2.0×10^4	1.8
	-	+	2.9×10^4	2.6
	+	+	3.0×10^5	<0.0003

* Calculated on the basis of transformations per milliliter of the native control.

FIG. 3. Effect of heating at 100 C on the streptomycin resistance marker of native, renatured, and heated-quenched DNA.

are compared in Fig. 3. These curves show that native and extensively renatured (30% of native activity) DNA were rapidly inactivated to 1 to 2% of their respective unheated activities. DNA renatured to 12% of native activity was less susceptible to inactivation, and heated-quenched DNA (initially 1% of native activity) displayed only a gradual decrease in biological activity. Marmur et al. (1962) reported that partially renatured pneumococcal DNA (restored to 7% of native activity) was abnormally resistant to thermal inactivation at a temperature above the T_m . Our data suggest that the inactivation of residual-active material parallels the gradual inactivation of native DNA after the initial rapid inactivation due to collapse of the double helix.

It is interesting that heating for 10 min at 120 C, followed by rapid cooling at 0, -15, or -80 C, did not appreciably reduce the level of residual activity (i.e., less than a 40% decrease).

Curves showing surviving biological activities after heating DNA at the subcritical temperature of 79 C are shown in Fig. 4. It can be seen from these data that the residual activity is more refractory than either native or renatured DNA in its susceptibility to inactivation at subcritical temperatures. If thermal inactivation at subcritical temperatures proceeds at those base-pairs that are open as a result of thermal excitation (Ginoza and Zimm, 1961), resulting in depurination, it appears that a double-stranded helix is a

FIG. 4. Effect of heating at 79 C on the streptomycin resistance marker of native, renatured, and heated-quenched DNA.

more favorable condition for this process and that the residual-active fraction of heated-quenched DNA is particularly resistant to this type of thermal inactivation.

Ultraviolet irradiation. Curves for surviving biological activities of irradiated DNA are shown in Fig. 5. These curves show that residual activity was less susceptible to inactivation than was native DNA for a given ultraviolet dose. The real significance of this observation, also reported by Goodgal (1961), is not apparent at the present time. However, when $\sqrt{T_0/T}$ was plotted against ultraviolet dose according to Rupert and Goodgal (1960), straight-line graphs were obtained for both native and residual-active DNA. The slope of the residual-activity curve was one-third that of the native curve. According to the theory set forth by Rupert and Goodgal (1960), these results would indicate that the marker on residual-active material has a lower probability of being inactivated as a result of molecular breaks or "hits" which would prevent genetic incorporation of the marker assayed. This might mean that the available target area of residual-active material is smaller than that of native DNA.

Another possible explanation for the observed difference in ultraviolet inactivation of the DNA is the following. Harm and Rupert (1963) found a dark repair system for ultraviolet-damaged DNA in *H. influenzae*. It may be that this repair system operates more favorably on a structure such as that of the residual-active DNA.

FIG. 5. Ultraviolet light inactivation of the streptomycin resistance marker of native and heated-quenched DNA.

Nitrous acid treatment. It has been reported by Horn and Herriott (1962) that new genetic markers could be detected in heated-quenched, but not in native, *H. influenzae* DNA when the nucleic acid was treated with nitrous acid and then annealed before biological assay. The experiments reported in this paper were performed to determine whether a new genetic marker, Sm^5 , could be detected in residual-active material after treatment with nitrous acid. (The samples were annealed before biological assay.)

Results showing the effect of nitrous acid on single-stranded and on residual-active $C^{2.5}$ DNA (heated-quenched, exonuclease I-digested and dialyzed) are presented in Table 3. The characteristic decrease which was seen in the original marker ($C^{2.5}$) activity of the heated and quenched material also occurred when residual-active material was treated with nitrous acid. However, the increase in the induced Sm^5 marker of heated and quenched DNA was not observed in the residual-active material. It should be mentioned that no new markers were found in native *H. influenzae* DNA after nitrous acid treatment. These results support the idea that the structure of residual-active material is different from that of the majority of heated-quenched molecules; while this does not mean that the residual activity resides in a structure which must be like that of native DNA, it is quite possible that a partially double-stranded structure is involved.

Cesium chloride (CsCl) density gradient centrifugation. Results showing the separation in a CsCl density gradient (Meselson, Stahl, and Vinograd, 1957) of residual transforming activity from the activity of native DNA are shown in Fig. 6. Before centrifugation, the residual-active material was separated from the bulk of single-

TABLE 3. Effect of nitrous acid on residual-active $C^{2.5}$ DNA

Genetic marker	DNA sample	HNO ₂ reaction time	Titer (transformations per milliliter)	Titer minus background
Original $C^{2.5}$	Heated-quenched	min		
		0	1.0×10^5	—
		10	3.5×10^4	—
	Heated-quenched and phosphodiesterase-digested	20	2.3×10^4	—
		0	1.2×10^5	—
		10	6.0×10^4	—
Induced Sm^5	Heated-quenched	20	4.6×10^4	—
		0	5.4×10^3	0.5×10^3
		10	1.0×10^4	5.1×10^3
	Heated-quenched and phosphodiesterase-digested	20	9.2×10^3	4.3×10^3
		0	4.9×10^3	0
		10	4.3×10^3	0
Sm^5 background (untransformed bacteria)	—	20	4.4×10^3	0
		—	4.9×10^3	0

FIG. 6. Cesium chloride density-gradient centrifugation of native and residual-active DNA bearing the streptomycin resistance marker.

stranded DNA by treating the heated-quenched preparation with exonuclease I (Lehman, 1959), followed by dialysis to remove digestion products. While densities of the fractions have not been determined, it is clear that the residual activity is due to a structure with a greater buoyant density than that of native material.

DISCUSSION

Even though a definite structure for the residual activity of heated-quenched DNA has not been proven, we have presented evidence that the structure is neither characteristic of native nor of the usual denatured (single-stranded) DNA molecule. In some instances, it was found to be dissimilar to renatured DNA. Its susceptibility to snake venom phosphodiesterase indicates that the residual activity resides on a structure which

has free 5'-phosphate end groups and is, therefore, not a closed cyclic structure such as that proposed for ϕ X174 DNA by Fiers and Sinsheimer (1962).

Treatment with formamide, ultraviolet irradiation, or heat inactivation indicated that residual activity was not due to a double-stranded helical structure like that of native DNA. A similarity to native DNA was found when residual-active material was inactivated over 4 logs by heating above the critical melting temperature in the presence of 1% formaldehyde. This indicated that hydrogen bonding was important for biological activity. Such bonds could easily be formed in single-stranded units subsequent to denaturation attempts even in dilute solutions, and the formaldehyde-DNA complexes would prevent intrastrand hydrogen bonding. The relative resistance of residual activity to *E. coli* phosphodiesterase (exonuclease I) may mean that the molecule lacks a free 3'-OH end group or that such a terminal group has been masked by preferential folding-back and intrastrand hydrogen bond formation. If a fraction of the residual-active structures is susceptible to the exonuclease I reaction, as suggested by the 15 to 20% loss in activity after incubation with the enzyme, it could be concluded that the genetic marker is unprotected in some cases by its location on a nonhydrogen-bonded region of the structure (i.e., random formation of hydrogen bonds may not be included in the immediate region of the marker being assayed, thereby leaving it on a single-stranded "tail" subject to destruction by the enzymatic reaction). It is also possible that the slight inactivation of both native and residual transforming activities was due to some endonuclease activity in the exonuclease I preparation.

Banding in a CsCl density gradient revealed that the residual activity had a greater buoyant density than native DNA. This is in keeping with the results of Rownd et al. (1961), Guild (1961), and others who reported that the residual activity banded at a density characteristic of single-stranded material. Based on more recent CsCl density-gradient centrifugation studies, Round, Green, and Doty (1963) concluded that the major fraction of residual biological activity of heated *Bacillus subtilis* transforming DNA (i.e., residual activity less than 0.1%) is due to molecular species which have not undergone complete strand separation. In the same report these authors indicated that DNA banding in the density region characteristic of single strands with a biological activity of approximately 0.001% of unheated DNA can be increased by a factor of 10^3 by a very rapid concentration-independent, heat-dependent reaction probably

resulting in intramolecular rearrangement of hydrogen bonding to produce a biologically active molecular configuration. The latter fraction of activity may be structurally similar to the residual activity of *Haemophilus* DNA.

In our experiment, the residual-active material was isolated from the bulk of single-stranded DNA by treating the denatured preparation with exonuclease I, followed by dialysis to remove digestion products. Material treated in this manner has been observed to band with a sharp peak at higher density. In another experiment we found no change of position in the CsCl density gradient relative to native DNA of heated-quenched or heated-quenched-exonuclease I-digested DNA. This indicated that the presence of the bulk of inactive material did not influence the banding properties of the residual-active fraction, nor did the possible digestion of a portion of the residual-active structure significantly alter its buoyant density.

In view of our findings and the work reported by Guild (1961), Guild and Robison (1963), Ginoza and Zimm (1961), Roger and Hotchkiss (1961), and others previously cited, the residual-active component of *Haemophilus*, and possibly pneumococcal, DNA appears to be characteristic of a structure formed by intrastrand hydrogen bonding of single strands resulting in a structure acceptable to the bacteria, as proposed earlier (Barnhart and Herriott, 1962), rather than of a linear single-stranded or a double-stranded structure like that of renatured or native DNA. It should be noted, however, that Luzatti et al. (1964) characterized by X-ray diffraction studies an intermediate form of DNA which appeared at elevated temperatures. They suggested that this structure could be a nonhelical two-stranded configuration lacking hydrogen bonds but remaining double-stranded by intercalation of the base planes. Although the residual-active material was found to involve hydrogen bonding, it is not inconceivable that some connection exists between the intermediate form and the residual-active structure described in this paper.

From the work of Guild and Robison (1963), it appears that single-stranded pneumococcal DNA is capable of penetrating competent pneumococci (although less efficiently than native DNA) and initiating transformation to drug resistance. While it is possible, in view of the work of Lacks (1962), that pneumococci are receptive to the bulk of ordinary single strands of DNA, it would be helpful in comparing the mechanism of transformation of the two systems to know if a structurally select fraction of heated and quenched pneumococcal DNA is responsible for transformation.

ACKNOWLEDGMENTS

I am indebted to Roger M. Herriott for his advice and encouragement.

This investigation was supported by Public Health Service training grant 5T1 GM 73 from the National Institute of General Medical Sciences, and Public Health Service research grant AI 01218 from the National Institute of Allergy and Infectious Diseases. The author was a Public Health Service trainee and research fellow.

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