STRAND SEPARATION AND SPECIFIC RECOMBINATION IN DEOXYRIBONUCLEIC ACIDS: BIOLOGICAL STUDIES

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It is clear that the correlation between the structure of deoxyribonucleic acid (DNA) and its function as a genetic determinant could be greatly increased if a means could be found of separating and reforming the two complementary strands. In this and the succeeding paper[1] some success along these lines is reported. This paper will deal with the evidence provided by employing the transforming activity of DNA from *Diplococcus pneumoniae* while the succeeding paper[1] will summarize physical chemical evidence for strand separation and reunion.

Bacterial transformation offers a unique approach to this problem since the activity of DNA isolated from genetically marked strains can be assayed after being subjected to various treatments. We have concentrated on thermal treatment to accomplish our goal for several reasons. First, the accumulated experience in this Laboratory[2–5] has shown that exposure of DNA to carefully controlled temperatures for certain periods of time can denature the DNA molecules with minimal damage to their chemical structure; this is a prerequisite to strand separation and furthermore the ease of precise control of the temperature offers an obvious means of providing nearly reversible conditions which would optimize the chances of reuniting the DNA strands. Moreover, it has been shown in one case that thermal treatment did lead to strand separation[6] and there is considerable evidence that transforming activity falls sharply in the region of thermal denaturation.[5, 7, 8] By utilizing these observations and by giving particular attention to the *rate* of cooling from the elevated temperature we have been able to demonstrate a pattern of inactivation and restoration of biological activity consistent with strand separation and specific reunion. It appears that this reunion can take place between complementary strands or between strands of two closely related molecules from mutant strains of *D. pneumoniae*.

Experimental Details.—The strains of *D. pneumoniae* and the transformation techniques employed have been described previously.[9–11] The isolation of DNA from *D. pneumoniae* and other bacterial species was carried out by a procedure described elsewhere.[12] The samples of pneumococcal DNA had sedimentation constants, $s_{20,w}$ measured in 0.15 *M* NaCl plus 0.015 *M* sodium citrate in the range of 24 to 26 *S* (corresponding to molecular weights of 8–10 million).

In most experiments pneumococcal DNA was dissolved in 0.15 *M* NaCl plus 0.015 *M* sodium citrate (hereafter referred to as standard saline-citrate) and heated in small tubes or flasks by immersion in boiling water. When *fast cooling* was employed, samples were transferred to tubes precooled in ice water. In cases where *slow cooling* was desired, samples were placed in an insulated water bath (six liters) at the elevated temperature, and allowed to cool with the heaters turned off. In a typical experiment the drop in temperature took the following time course: 89°—0 (minutes), 80°—15, 70°—40, 60°—75 and 50°—130. The relationship between concentration and transforming activity of DNA which has been heated at 100°

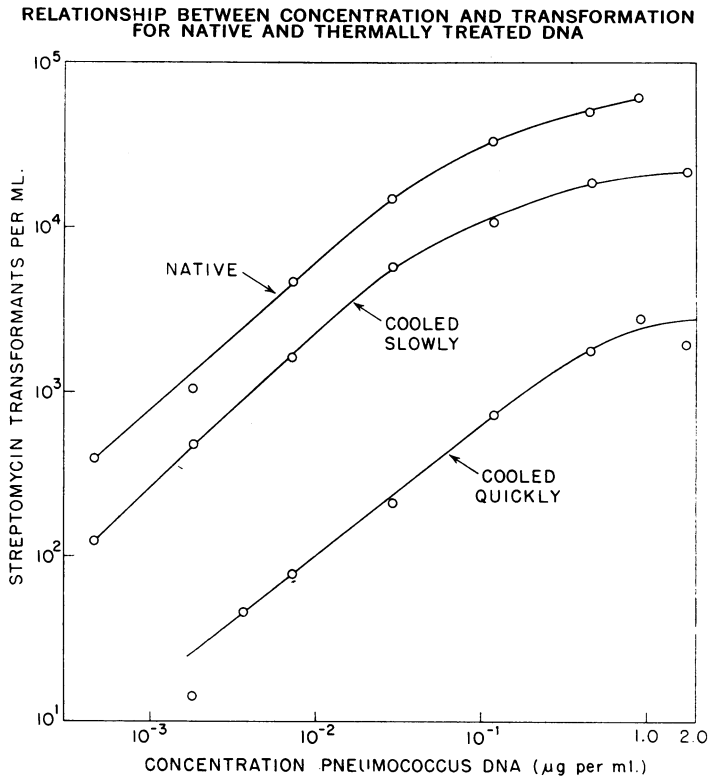

FIG. 1.—Relationship between transformation and concentration for native and thermally denatured DNA (cooled quickly and slowly).

Pneumococcal DNA was heated at 100° for 10 minutes in standard saline-citrate. Hot concentrated saline-citrate was added to a final concentration of 0.3 *M* NaCl and 0.03 *M* Na citrate and a DNA concentration of 20 μg/ml. The mixture was subdivided into two portions, one cooled quickly, the other slowly from 90° to room temperature. The native DNA was an unheated aliquot of the same preparation.

and then cooled quickly and slowly, as well as native unheated DNA is shown in Figure 1. All assays were carried out in the linear portion of the transformation-concentration curve.

Results.—Thermal denaturation and marker inactivation: The thermal denaturation of DNA can be followed by changes in the absorbance at 2600 A. observed either as a function of the temperature of the solution itself or the temperature to which it has been heated prior to cooling to room temperature (25°). The difference between these two temperature profiles results from reformation of base pairs as the temperature is lowered; such reformation has been thought to be nonspecific, involving short regions of chains that are not exactly complementary.[1, 3] The transforming activity, as measured by the ability of the heated pneumococcus DNA to effect transformation of wild-type pneumococcus with respect to three unlinked resistance markers: streptomycin, erythromycin, and bryamycin (which behaves as a high-level micrococcin resistance marker), was followed as a function of temperature. The results together with the absorbance-temperature profiles are shown in Figure 2. From this it is clear that different markers have different ther-

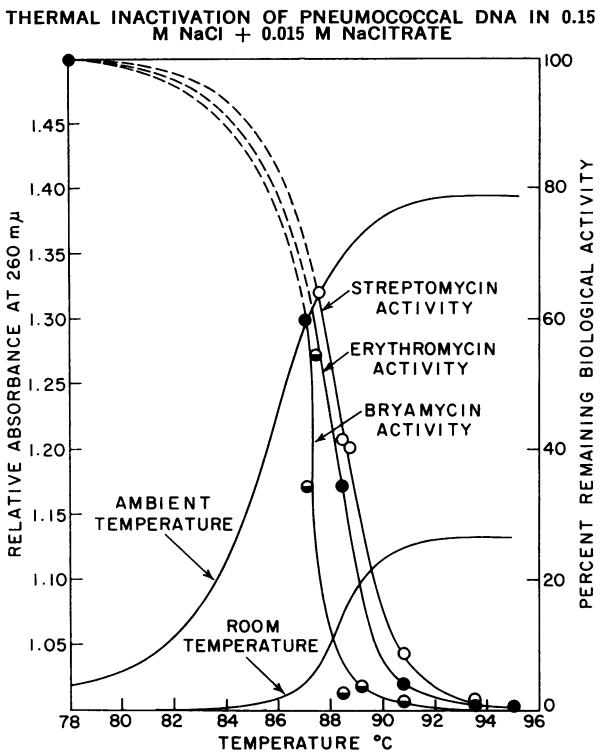

FIG. 2.—Loss in transforming activity and denaturation of DNA as a function of temperature.

Pneumococcal DNA was heated at 20 μg per ml in standard saline-citrate. The absorbance at 260 mμ was recorded for a series of temperatures both at the ambient temperature (after correction for thermal expansion) and after cooling (in air) to room temperature. After each cooling a sample was removed, diluted, and assayed for its ability to transform with respect to streptomycin, erythromycin, and bryamycin.

mal resistance in agreement with the results of others.[7, 8] This difference in sensitivity is most likely related to differences in base composition among the individual DNA molecules in which the markers are located.[5] It is important to note, however, that the thermal inactivations though differing one from another, are much more closely related to the absorbance-temperature curve determined at room temperature than to the one obtained at the ambient temperatures. Hence, the possibility exists that some helical regions melted out at the elevated temperature and have reformed on cooling with return of biological activity.

A stronger suggestion that complementary chains have reunited to a very limited extent is seen in the fact that all three markers retain some residual activity even though all the molecules have been denatured as judged by the optical density increase having reached its maximum. In a separate experiment, transforming DNA was heated at 120° in an autoclave for 10 minutes, cooled in air, and the streptomycin activity assayed: 0.05 per cent of the original activity remained. Since this very elevated temperature exceeded that required to denature even pure guanine-cytosine regions (110°),[4, 5] it is highly improbable that any DNA molecules escaped

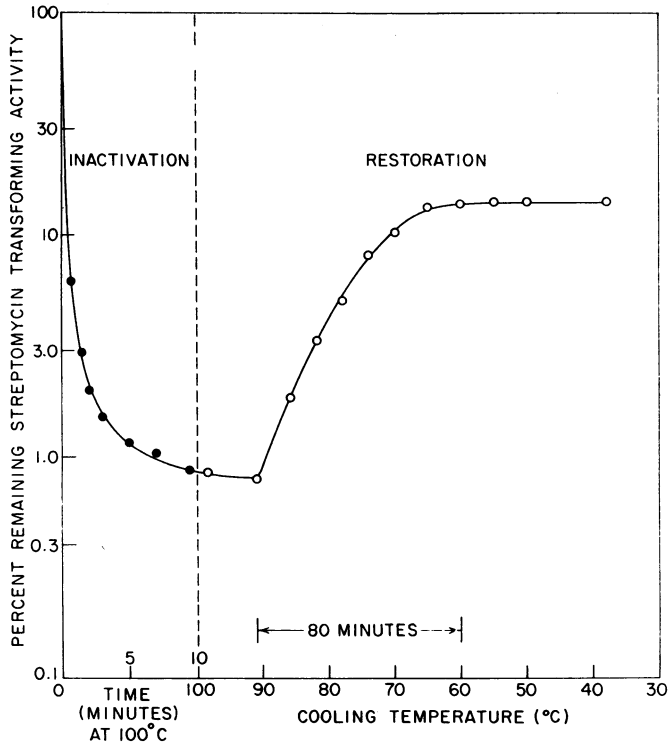

FIG. 3.—Thermal inactivation and restoration of the transforming activity of DNA.

Pneumococcal DNA at 20 μg/ml in standard saline-citrate was preheated for $1^1/_2$ minutes at 85.5° (no loss in biological activity), then transferred to a boiling water bath at 0 minute. At the times shown, samples were removed to an equal volume of 1.5 *M* NaCl plus 0.15 *M* Na citrate in an ice-water bath. After 10 minutes exposure at 100°, an equal volume of hot (100°) 1.5 *M* NaCl plus 0.15 *M* Na citrate was mixed with the DNA solution, the mixture transferred to a large water bath and then cooled slowly. During the gradual descent of the temperature, samples were removed (right of the dashed vertical line) to pre-chilled tubes in an ice-water bath.

denaturation. Thus the residual biological activity must be due to a low level of transformation by denatured DNA molecules or to a small amount of specific recombination to yield two stranded helices. The former possibility is not in accord with the finding that denatured DNA is not taken up by transformable cells.[13] Thus, specific reformation of the separated strands is indicated.

As a consequence a systematic investigation was undertaken of the variables that might affect the recombination so that what has been at best a marginal effect could be maximized. The important variables have been found to be the rate of cooling of the heated solution, the concentration of DNA and the ionic strength.

The effect of slow cooling: The transforming activity of thermally denatured pneumococcal DNA was found to be very dependent on the rate of cooling of the heated solution. Thus when pneumococcal DNA solutions are heated at 100° in standard saline-citrate for various periods of time and a series of aliquots removed, cooled quickly and slowly from 90° to room temperature, it is found that the slow

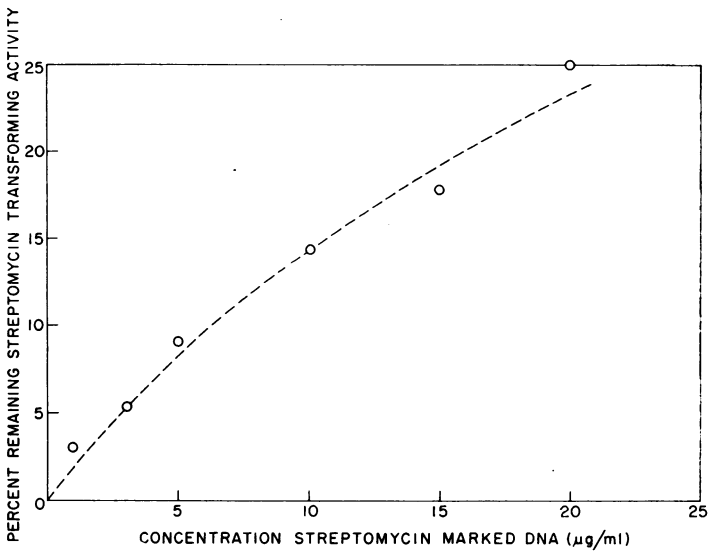

FIG. 4.—Effect of concentration during slow cooling on the recovery of transforming activity of thermally denatured DNA.

Pneumococcal DNA at 20 μg/ml in standard saline-citrate was heated for 10 minutes at 100°, transferred to a large bath at 89° and immediately diluted into pre-heated (89°C) tubes containing standard saline-citrate to give the final concentrations shown on the graph. After slow cooling all samples were diluted to the same concentration for assay. Circles are experimental points; dashed line is theoretical curve.[1]

cooling leads to an activity 35-fold greater than the fast cooling. This ratio is insensitive to the time of exposure at 100° between 5 and 20 minutes.

The transition from the activity characteristic of fast cooling to that characteristic of slow cooling can be seen in Figure 3. The transforming activity is plotted for quickly cooled aliquots taken from a solution during the exposure at 100° and then at various times during the slow cooling (at the right of the vertical dashed line) as the temperature of the bath dropped. Thus it is seen that restoration of the activity begins at about 90° and continues until about 65°. While the level of restored activity is about 15 per cent, this varies from sample to sample and in some cases has been found to be as high as 50 per cent. It is of interest to note that when fast cooled samples resulting in low biological activity are reheated and slowly cooled the transforming activity is restored to almost that of an aliquot that had been slow cooled originally. Thus the low activity of a fast cooled sample represents a repression rather than a destruction of its essential ability to transform. Essentially similar results have been obtained with thermally denatured transforming DNA isolated from *Streptococcus salivarius* and *Bacillus subtilis* on fast and slow cooling (Marmur, Lane, and Levine, unpublished results).

Effect of concentration of DNA during cooling: An important factor in determining the level of stored activity on slow cooling was found to be the concentration of the DNA itself during the cooling period. In contrast, there is practically no dependence of the activity on concentration for fast cooling. To illustrate the former a solution of pneumococcal DNA at a concentration of 20 μg/ml in standard saline-citrate was heated at 100° for 10 minutes and then diluted into hot solvent to give

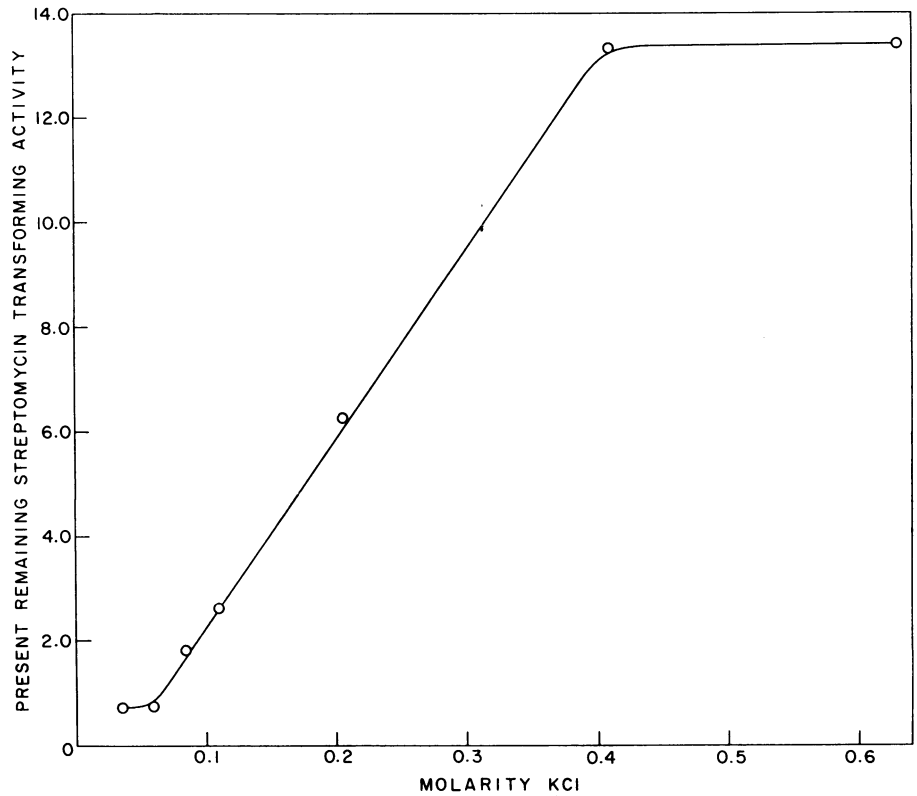

FIG. 5.—Effect of ionic strength on the recovery of transforming activity of slowly cooled denatured DNA.

Pneumococcal DNA at 200 μg/ml in standard saline-citrate was heated at 100° for 10 minutes, diluted 20-fold into KCl of various ionic strength and at 86°. The solutions were cooled slowly (the contribution of saline-citrate to the final ionic strength is included in the graph).

a series of concentration from 1 to 20 μg/ml. When these were slowly cooled the specific activities were found to increase nearly linearly from 3 per cent for 1 μg/ml to 25 per cent at 20 μg/ml (Fig. 4). As shown in the following article, this behavior is consistent with a bimolecular reaction.

Effect of salt concentration: Once the DNA strands have separated, the negatively charged phosphate groups would tend to prevent the reunion of the strands to form the helix. Increasing ionic strength would be expected to favor recombination by repressing this repulsive effect through shielding. This is shown to be the case in Figure 5. DNA was heated at a high concentration in standard saline-citrate at 100° for 10 minutes and then diluted into prewarmed tubes containing various concentrations of KCl. The solutions were cooled slowly, then assayed. There is a linear increase in biological activity between 0.05 *M* KCl and 0.4 *M* KCl. At higher concentrations of KCl (0.4 *M*) the biological activity reaches a maximum representing 14 per cent of the activity of the native material. Essentially similar results are obtained when the cooling is carried out in various concentrations of saline-citrate.

The effect of pH on strand reunion is broad with maximum at neutrality.

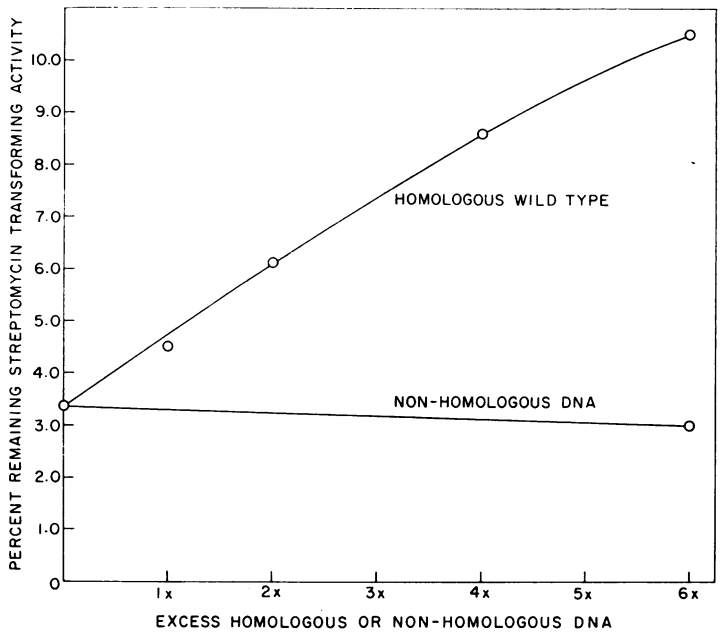

FIG. 6.—The effect of denatured homologous wild-type and heterologous DNA on the transforming activity.

Streptomycin marked pneumococcal DNA at 5 μg/ml was heated for 10 minutes at 97° in standard saline-citrate together with wild-type (homologous) pneumococcal DNA in the relative concentrations shown. Heterologous DNA and streptomycin marked DNA were also heated together. All the mixtures were slow cooled. Circles are experimental points; dashed line is theoretical curve.[1]

Effect of homologous and heterologous DNA: If the two complementary strands of the DNA molecule separate completely, it should be possible, by adding an excess of denatured homologous wild-type DNA during the slow cooling to reform hybrid molecules in which one strand carries a specific genetic marker and the other strand does not carry the marker. DNA isolated from streptomycin resistant pneumococci and various concentrations of DNA isolated from streptomycin sensitive cells were heated together in standard saline-citrate at 100° for 10 minutes and then cooled slowly. The resultant increase in biological activity is shown in Figure 6. Thus, it would appear that hybrid molecules formed in the slow cooling are active in carrying out transformations. Since transformants are selected and counted in a medium containing streptomycin, it is not known at the present time whether a cell transformed by a hybrid molecule results in a pure clone of streptomycin resistant cells or a mixed population of streptomycin sensitive and resistant cells. This question is now being studied using linked markers.

If genetically marked DNA is first denatured and then slow cooled in the presence of homologous DNA in the *native* state there is no increase of the biological activity over that of the control.

If during the slow cooling, denatured heterologous DNA is added at five to six times excess, there is again no increase in transforming activity over that of the control. The heterologous DNA samples tested have been isolated from *Salmonella*

typhimurium (LT-2), *Micrococcus lysodeikticus*, *Micrococcus pyogenes* var. *aureus*, *Streptococcus* D, and calf thymus. These preparations vary in base composition, although that of *Streptococcus* D is identical to the per cent guanine plus cytosine of *D. pneumoniae* (Marmur, unpublished).

Discussion.—Previous studies on the thermal denaturation of DNA have done little to correlate its molecular and biological properties. The relationship between optical density, which is a measure of the helical content, and transforming activity, which is a fine probe of the properties of individual molecules, has led to the discovery that strands of thermally denatured DNA can reunite during slow cooling to form helical structures which are biologically active.

Beyond the demonstration that the biological activity can be restored to denatured DNA by a process of slow cooling which permits restoration of complementary-paired helical regions,[1] the most important observation appears to be the additional increment in biological activity conferred by the presence of homologous wild-type DNA during the cooling process. This is clearly a consequence of forming hybrids between strands of the marked and wild-type DNA and strongly supports the view that the essential genetic information in DNA is carried independently by each strand. Essentially similar conclusions have been arrived at by Pratt and Stent[14] working with bromouracil induced mutants of phage and by Tessman[15] working with nitrous acid mutants of phage and the bacterial virus ϕX 174 which contains single-stranded DNA.[16] The duplicate set of genetic information in the native DNA molecule of the mutant strain is disseminated among a larger number of renatured molecules in which at least one strand carries the information. Closely related DNA from *Streptococcus* D (which can be transformed by pneumococcal DNA[17]) having the same over-all base composition as pneumococcus does not have the same effect as the added homologous denatured pneumococcal DNA during slow cooling. This points to the necessity for a close homology that is required for the interacting strands to form double stranded structures.

The question might arise as to how representative the biological results are of the whole molecular population. It has already been established that a genetic marker is associated with a DNA molecule,[18-20] occupying a small portion of it.[10] The number of DNA molecules in a pneumococcal cell is of the order of several hundred[18] if the weight average molecular weight is 10 million. Since essentially similar results have been obtained with both the streptomycin and bryamycin markers, and since they represent molecules which are relatively rich in guanine-cytosine and adenine-thymine respectively, it is safe to conclude that most of the molecules exhibit the same interactions as the molecules carrying the biological markers being assayed.

Summary.—DNA from *D. pneumoniae* when heated to temperatures where all the molecules are denatured still retains transforming activity. The activity is increased by slowly cooling the denatured DNA as well as by increasing the ionic strength and concentration of DNA during the cooling period. The reunion of the strands is specific and has been shown to occur between populations of denatured molecules of mutant strains of *D. pneumoniae*. The reunion does not occur between the DNA strands of pneumococcus and that from *Streptococcus* D or from organisms with widely varying base ratios. Hybrid molecules in which one strand contains the mutant marker and the other strand derives from wild-

type DNA are active in carrying out transformations with respect to the mutant property.

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The separation and reunification of the complementary molecular strands of DNA, so clearly indicated by the restoration of biological activity,[1] can be demonstrated by physical chemical techniques as well. Such studies permit a more quantitative description of the phenomenon, make possible the inclusion of DNA samples that do not participate in bacterial transformation and lead to a better insight into the controlling features of the reaction. This, in turn, should provide a better basis for understanding the possibilities and limitations that DNA has