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Kinetics of Renaturation of Denatured DNA. II. Products of the Reaction

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Synopsis

The structure of renatured T4 DNA has been studied by CsCl density-gradient centrifugation. It has been found that the products of the reaction differ, depending on the method used for denaturation of the DNA. If denaturation is carried out without taking precautions to prevent chain degradation, for example, by heat, the DNA formed by renaturation shows approximately 70% recovery of the native structure as judged by its density. With long times of annealing, the DNA can recover the native density. This behavior is also observed with bacterial DNA samples. On the other hand, if precautions are taken to prevent chain degradation during denaturation, two products appear as a result of renaturation. One of them is undistinguishable from native T4 DNA, whereas the second one consists of highly aggregated DNA which shows only a partial recovery of the native structure. With long times of annealing, this second species recovers the native density but retains its highly aggregated nature. At higher ionic strengths, renaturation follows a different pattern and a single product is formed. The relevance of all these observations to the kinetic anomalies reported in the previous communication is discussed.

INTRODUCTION

In order to clarify the mechanism of renaturation of denatured DNA, the kinetics of the reaction have been studied as described in the previous communication.[1] It has been concluded that the process can be well represented by an irreversible second-order reaction. However, after long times of reaction, as well as in solvents of high ionic strength, complications arise which make the reaction deviate from such a simple pattern.

The work presented in this paper is aimed at the detailed study of the molecular species formed in the course of renaturation in order to clarify those deviations. The method of study used is density gradient centrifugation,[2] specially suited for our purposes, since it permits separation of native and denatured DNA, which have different densities. This work complements the results obtained with spectrophotometric technique, which only give information on the average extent of renaturation in the reaction mixture.

The DNA employed in this study has been isolated from bacteriophage

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T4. This DNA has been chosen because of its homogeneity and because the experiments described here are meaningful with regard to the problem of a circularly permuted base sequence.[3]

EXPERIMENTAL PROCEDURE

The details have been described in the preceding paper.[1] In the experiments described here, the same stock solutions of denatured DNA were used and aliquots of them were diluted at the proper conditions and annealed in glass vials in a thermostat at the reported temperature $\pm 0.1°C$. The vials were quickly cooled in ice water after the required time of annealing. The T2 DNA was kindly supplied by Mr. R. Sternglanz and Dr. B. Alberts. Unfortunately it was not possible to follow the reaction at concentrations lower than 2 μg./ml. because of the considerable losses of DNA due to adsorption,[4] which are higher at low concentrations, especially for partially denatured species.

It should be noted that density-gradient centrifugation can be safely used as an analytical tool in the experiments reported in this paper. When denatured DNA is stored in concentrated CsCl neither its density nor its optical density shows any significant change. The reactions which take place in this solvent appear only when the DNA is transferred into another solvent of lower ionic strength, as discussed in the previous communication.

RESULTS

Renaturation of Degraded, Denatured DNA

When DNA which has been denatured without taking precautions to prevent degradation is subjected to renaturation, the renatured material usually shows an incomplete recovery of the properties of native DNA, as judged either by its hypochromicity[5] or by its density in a CsCl density gradient.[6]

A typical result obtained with a sample of denatured T4 bacteriophage DNA is shown in Figure 1, where it is easy to see the formation of a renatured band at the expense of the denatured band. Similar results are obtained at lower concentration, although in this case the time scale should be proportionally expanded. This sample of DNA had a sedimentation constant $s_{20,w} = 15$ in $0.015M$ citrate, indicating that it was considerably degraded.

It is interesting to observe that the renatured band which forms in the first hours represents a DNA with approximately 70% of native structure as judged from its density. When this DNA is renatured for a long time it is then possible to recover its native density, as can be seen in Figure 1. However its molecular weight is extremely high, as judged by the small width of the band, as well as by the rate at which it is formed in the CsCl gradient. This result gives support to the hypothesis[6] that the renatured DNA formed in the first hours contains unpaired regions at the ends of the

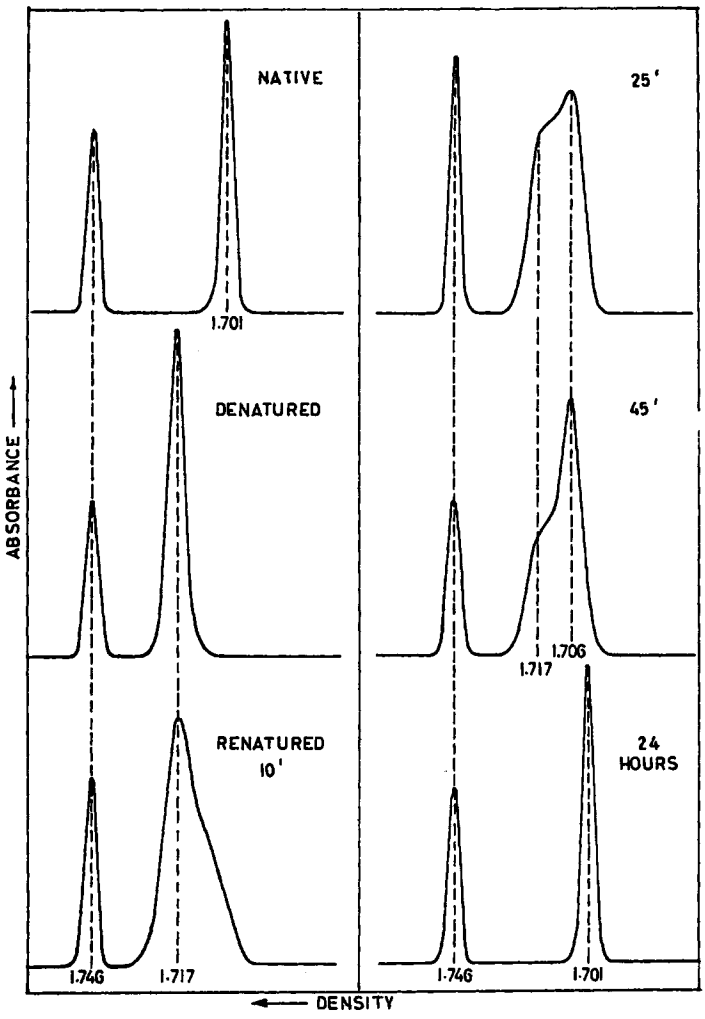

Fig. 1. Renaturation of degraded denatured T4 bacteriophage DNA. A solution of DNA at low ionic strength ($10^{-2}M$ in Na^+) was heated for 10 min. at 100°C. and quenched in ice. The required amount of salt was added in the cold in order to obtain a solution 0.15M in NaCl and 0.015M in Na citrate. The sample was then heated at 56°C. at a DNA concentration of 10 μg./ml. and aliquots were removed after various times. Shown above are the microdensitometer tracings obtained after equilibration in a CsCl density gradient formed by centrifugation at 44770 rev/min. The band at the far left is the standard DNA which was present in each case to provide a reference density.

molecules produced by the unequal length of the two denatured strands which have formed a renatured molecule. In this sense, the heterogeneity in density found in renatured DNA by Rownd et al.[7] is most likely due to the different amounts of native and denatured structures present in the individual molecules. Upon further annealing, the denatured regions remaining in the renatured molecules can interact with corresponding regions in

other molecules. If enough time is allowed, all the denatured regions will disappear, and nativelike molecules formed by many denatured strands adequately paired will result. This product should have a branched nature, which has been actually seen in the electron microscope.[8]

Renaturation of Formamide-Denatured DNA

In the process of thermal denaturation, the length of the denatured molecules is usually reduced because of the effects of thermal degradation. The effect is more noticeable in samples of very high molecular weight.

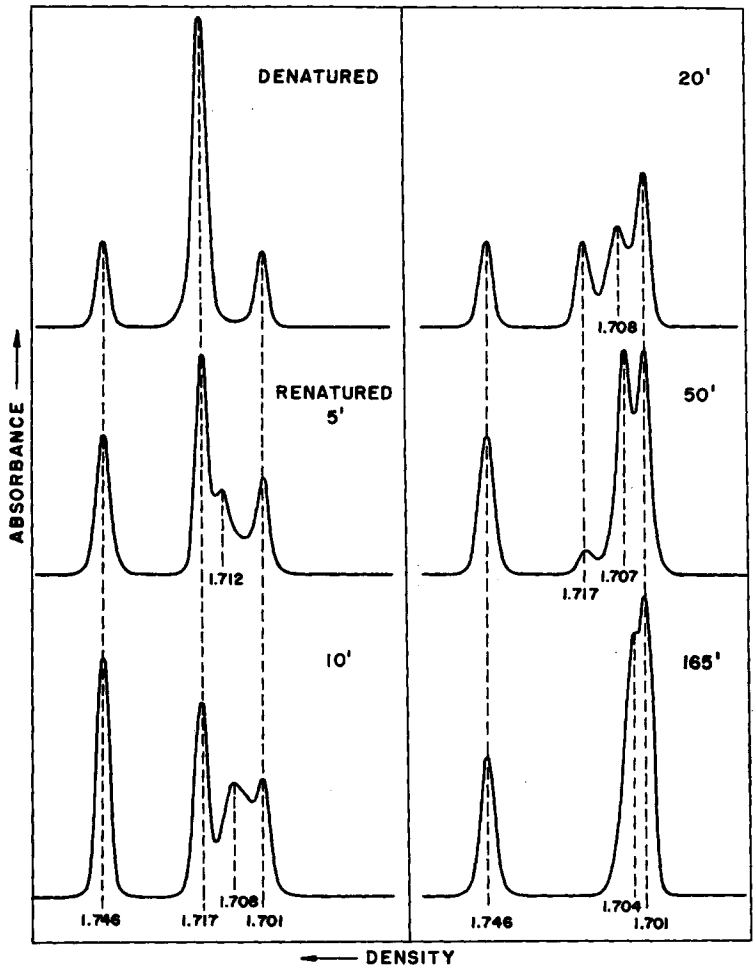

Fig. 2. Denaturation of formamide-denatured T4 bacteriophage DNA. The DNA was denatured by dialysis against formamide as described in the text. Renaturation was carried at 56°C. in a solvent 0.15M in NaCl and 0.015M in Na citrate at a DNA concentration of 10 μg./ml. Aliquots were removed after various times and equilibrated in a CsCl density gradient; the tracings obtained after centrifugation are shown in the figure.

The pattern of renaturation discussed in the previous section is consistent with these properties of the thermally denatured DNA.

If the DNA is treated with a mild denaturing agent such as formamide,[9] it is to be expected that the denatured molecules will be undegraded if there are no pre-existing single strand breaks in the T4 molecules as suggested by Thomas et al.[10] In this way a homogeneous population of denatured molecules will result; all the molecules will have the same length. The base sequence will also be the same, although its order could vary from molecule to molecule if the bases are circularly permutated. This problem is discussed in detail in a following paper.[11]

The results of renaturation experiments with formamide-denatured DNA are presented in Figure 2. It is clear that the process follows a complex pattern. Although approximately 50% of the renatured DNA is undistinguishable from the native, the rest of it has a density which corresponds to a DNA which has approximately 65% native structure (Fig. 2, frame 5). The following remarks are pertinent to the kinetic results shown in Figure 2.

(*1*) The denatured DNA used in this experiment contains approximately 15% of native DNA (Fig. 2, frame 1). This native DNA is resistant to formamide denaturation, and its nature will be discussed in another communication.[4] However, its presence is an advantage, since it provides an intrinsic density standard and shows that the renatured DNA which bands at the lightest density has exactly the same density as native DNA. Results analogous to the ones shown in Figure 2 are obtained when the DNA is originally 100% denatured by using the technique described by Subirana.[4]

(*2*) During the first 10 min. of renaturation only a small amount of renatured DNA appears in the band of native density. Only after 20 min. does a marked increase take place at this position. On the other hand, the amount of DNA present at intermediate densities is larger after 10 min. than at 5 min. or 20 min. These results show that some of the molecules present at intermediate densities react further to end up in the band of native density. In other words, the transition from the denatured to the native density takes a short, but finite time and it is possible to isolate molecules which are in intermediate states of renaturation. These intermediate states have been discussed in detail by Rownd.[12]

(*3*) It could be argued that the two strands of the molecules which appear at the native position at the end of the experiment had never been separated by the process of formamide denaturation. This possibility can be tested by dialyzing the denatured DNA into a low ionic strength solvent at room temperature.[1] If molecules which have stable nuclei are present, they should renature under these conditions. When this experiment is performed, no renaturation is observed, indicating that the process of formamide denaturation has effectively separated the complementary strands of denatured DNA.

(*4*) After 1 hr. of reaction, when all the denatured DNA has disappeared from the system, no further increase in amount of DNA in the band or native density can be detected. At the same time, the band of intermediate

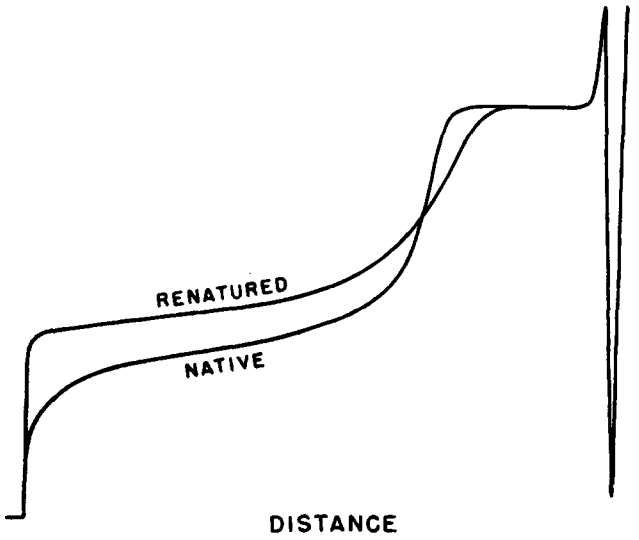

Fig. 3. Sedimentation of native and denatured T4 DNA. Shown are microdensitometer tracings of ultraviolet photographs of samples centrifuged at 29,500 rpm with 0.15*M* NaCl plus 0.015*M* Na citrate as a solvent. The ordinate corresponds to the transparency of the solution, whereas the abscissa gives the distance from the center of rotation. The vertical lines at the right and the left ends of the figure correspond to the meniscus and the bottom of the cell respectively. The duration of centrifugation after attaining the required speed was 20 min. The concentration of DNA was ca. 10 μg./ml. in both cases, so that the microdensitometer tracings for the two samples were the same at the beginning of centrifugation (not shown in the figure).

density becomes very sharp, and with longer time for reaction it slowly moves towards the native density. After 10 hr. of reaction, its density becomes indistinguishable from the native density; at this point all the renatured DNA has the same density as native DNA. However only about 50% of it bands in the CsCl gradient at the same rate as native DNA; the other 50% consists of very high molecular weight molecules which band within the first 2 hr. of centrifugation. From the experiments discussed in the next section it will become clear that this latter component comes from the intermediate band already present after 50 min. and which has shifted slowly in density.

(*5*) It was possible to test whether the renatured molecules had the same sedimentation coefficient as native molecules. This was done in a Spinco ultracentrifuge, Model E, using SSC (0.15*M* NaCl, 0.015*M* Na citrate) as a solvent and sedimenting the solution at 29,500 rpm in a plastic cell (Kel-F) at a DNA contentration of approximately 10 μg./ml. The tracings obtained from the ultraviolet photographs are shown in Figure 3. A reasonably sharp boundary with the same sedimentation constant as native DNA is formed in the renatured sample, showing that its molecular weight does not differ significantly from the one of native DNA. A very heterogeneous fast-sedimenting component is also present (practically all of it has already

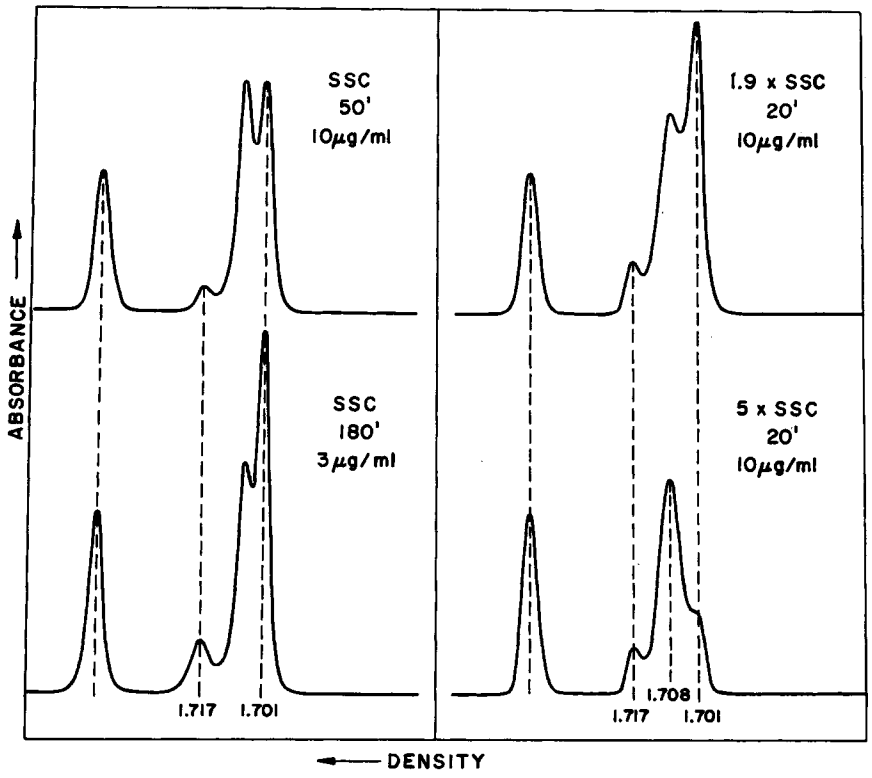

Fig. 4. Influence of various conditions on the renaturation of formamide-denatured T4 bacteriophage DNA. The DNA was denatured by dialysis against formamide as described in the text. Renaturation was carried out at 56°C. at the conditions noted on the figure. Shown are the microdensitometer tracings of samples equilibrated in a CsCl density gradient formed by centrifugation at 44,770 rpm.

sedimented in the figure) corresponding to the renatured molecules of intermediate density in Figure 2. With longer times of renaturation the heterogeneous component shows a somewhat faster rate of sedimentation.

(*6*) The process of renaturation which has been described does not change appreciably with moderate changes in the conditions of renaturation. In Figure 4 one can see that the reaction follows the same pattern in 1.9 × SSC or at lower concentrations in SSC, if the time scale is respectively expanded or reduced. However in the latter case there are relatively less molecules in the intermediate band. Similar results are obtained if the renaturation is performed in SSC at a different temperature (68°C.) or at 4°C. in mixtures of 50% formamide and 50% SSC (also a renaturing solvent). In this case the process is one hundred times slower than the one shown in Figure 2. A sample of DNA from T2 phage behaved in a similar way.

On the other hand, in 5 × SSC the pattern of renaturation is different, as can be seen from the fourth frame in Figure 4; no renatured DNA of native

density has been formed (the small amount present is due to the native DNA originally present). After longer times of annealing, this band of intermediate density slowly moves towards the native position; after 20 hr. its density indicates about 35% native structure. At the same time the rate of band formation in the CsCl gradient increases considerably, indicating that multiple interactions are taking place. The fact that no molecules of native density are formed in this solvent indicates that strong kinetic barriers exist which hinder the completion of renaturation after nucleation between two complementary denatured strands has taken place. At the same time this process facilitates multiple interactions between complementary strands, hindering the formation of nativelike molecules. The nature of the kinetic barriers mentioned above has been discussed by Rownd[12] and in the preceding communication.[1]

Nature of the Intermediate Species Formed on Renaturation

As we have seen, the renaturation of undegraded denatured T4 DNA yields two different molecular species upon renaturation, one of native density and another one of approximately 65% native properties which slowly increases its nativelike characteristics upon long times of annealing. What is the molecular nature of this species?

If the process of band formation in a CsCl gradient is followed, it is observed that the intermediate band forms soon after attaining the required speed of contrifugation. After 50 min. of annealing in SSC at 56°C. its rate of formation (which is equivalent to its sedimentation constant) is approximately two and a half times the similar rate for denatured DNA. This rate of band formation increases with time of reaction until finally, when its density is equal to that of native DNA (after 10 hr. in SSC at 56°C.), it is about five times that for denatured DNA. This high rate of band formation in the ultracentrifuge indicates that this DNA has a large sedimentation constant, i.e., it has a very high molecular weight. This shows that the DNA of intermediate density formed involves many denatured strands. That this is actually so is confirmed by doing an analogous experiment with a mixture of light and heavy labeled DNA samples, which show different densities in the CsCl gradient. The result is shown in Figure 5. When the samples are annealed separately the usual pattern is obtained, i.e., two bands of heavy and light renatured DNA appear, one of native density and another one about 0.005–0.006 density units heavier. On the other hand, when the two denatured DNAs are mixed before renaturation, a different pattern is observed. Two bands appear at the native densities and another one with double amount of DNA as expected is formed at the average hybrid density.[13] A fourth band is formed at a density 0.004 units heavier than the hybrid value, but no similar bands are observed at the equivalent positions near the native densities. This result indicates that many heavy and light denatured strands have interacted to form a partially renatured molecular species. If these partially renatured molecules were formed by

only two or three strands, discrete peaks would appear in the density gradient at the positions corresponding to a heavy and a light strand, two heavy and one light, two light and one heavy, etc., and this result has not been observed. It should be mentioned that to obtain this result (four

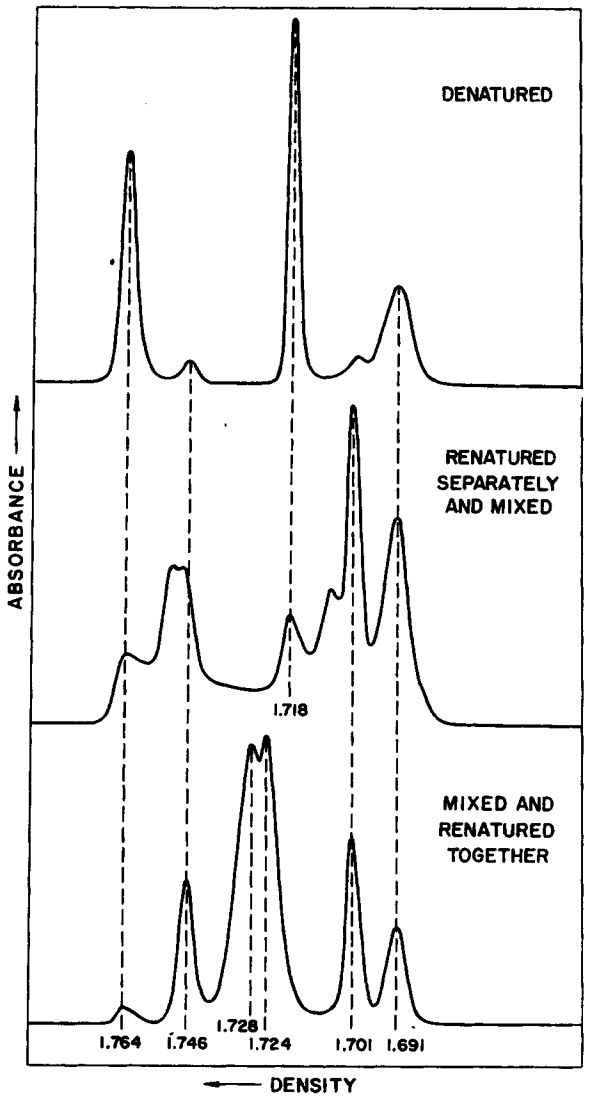

Fig. 5. Renaturation of a mixture of heavy labeled and normal T2 DNA. The two DNAs were denatured by formamide and annealed separately and then mixed for centrifugation (frame 2). In the experiment shown in the lower frame aliquots of the same denatured DNAs were mixed before annealing. In both cases the renaturation was done in SSC at 56°C. and the total DNA concentration was approximately 10 μg./ml. of DNA. The band at the right is the standard DNA which was present in each case to provide a reference density.

renatured bands) it is critical to use a DNA which has been carefully denatured.

If enough time is allowed, these intermediate species further react to recover the native density. How does this process occur? From the last two frames of Figure 2, it is clear that the intermediate band moves as a whole towards lower densities as time goes on. The alternative possibility that molecules progressively leave the intermediate band to increase the amount of DNA in the native band is excluded.

If the renaturation reaction is allowed to proceed at 10 μg./ml. for 50 min. (Fig. 2, frame 5), then diluted 10-fold with the same solvent at the same temperature and the reaction stopped 100 min. after the dilution step, no further reaction is detected. On the other hand, when the reaction is allowed to proceed at 10 μg./ml., a measurable shift in density of the intermediate species is detected (Fig. 2, frame 6). This concentration dependence clearly shows that the further reaction of this intermediate species involves interactions of several molecules. It indicates that the process taking place here is similar to the one observed in the case of degraded denatured DNA; the denatured portions of the molecules interact with complementary regions in other molecules until no more denatured DNA is available. At the same time the molecular weight of this renatured DNA increases, yielding high molecular weight branched structures.

DISCUSSION

The results presented here explain the deviations from second-order behavior in the kinetics of renaturation of denatured DNA which were discussed in the previous paper. It was observed that the second-order constant of the reaction diminished with increased concentrations, the effect being larger in 5 $\times$ SSC than in the lower ionic strength solvents. The second anomaly observed was that after a certain time, the reaction went further than that expected from its initial rate. This latter behavior can be understood from the results presented in Figure 2, where it is clear that the first part of the reaction which obeys second-order kinetics represents the formation of the two renatured bands, whereas the deviations observed after longer times represent the further reaction of the molecules present in the intermediate band to finally yield molecules of native properties. This further interaction involves several of the intermediate molecules, since it is a concentration dependent process as discussed above.

The deviations observed with 5 $\times$ SSC as a solvent as well as the results obtained by Cavalieri et al.[14] using 1M NaCl as a renaturing solvent can be well understood by considering that in this solvent the completion of renaturation after nucleation is a slow process and therefore deviations from second order towards first order behavior are to be expected.

In the case of the analogous reaction of polyriboadenylic acid (poly-A) with polyribouridylic acid (poly-U), deviations from the second-order plot are also observed[15] towards first-order behavior. However, in this

case they occur under different experimental conditions, since the deviations are larger at low polymer concentrations and low salt concentrations,[16] indicating that the reason of this behavior rests on a different physical ground. Probably the first product of the reaction consists of double stranded molecules containing A-U pairs with free ends of poly-A and poly-U. In a later stage rearrangements of these molecules can take place to increase the amount of A-U pairs, thus producing the kinetic anomalies mentioned above.

As was mentioned in the introduction, these experiments are also relevant to the problem of the arrangement of bases in the T4 DNA molecule. This problem will be considered in detail in a following communication,[11] where the renaturation of unbroken DNA extracted from several bacteriophages will be discussed. It is worthwhile here to summarize the results obtained with regard to the mechanism of renaturation of formamide denatured T4 DNA in SSC. As has been shown above the process of renaturation yields two different molecular species, both of them with the density of native DNA. The first one is indistinguishable from native DNA, whereas the second one is a high molecular weight species involving many polynucleotide chains. Both of these species can be isolated in intermediate states of renaturation, but the time required for them to go from the denatured to the native band is very different. In the first case the molecules need less than 10 min., whereas in the second case 10 hr. of renaturation is necessary.

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