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Further Experiments on the Alkaline Denaturation of DNA

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Biofisica. — *Further Experiments on the Alkaline Denaturation of* DNA (*). Nota di MARIO AGENO (**), ELISABETTA DORE (**) e CLARA FRONTALI (**), presentata (***) dal Corrisp. M. AGENO.

SOMMARIO. — Dopo aver riassunto i risultati precedentemente ottenuti nello studio del processo di denaturazione alcalina del DNA, vengono esposti i risultati di due nuove esperienze. Nella prima si fa vedere come il tempo di salita della frazione irreversibile dell'effetto ipercromico dipenda linearmente dalla viscosità del mezzo. Con la seconda si dimostra, mediante prove di rinaturazione di soluzioni di DNA sottoposte a denaturazione parziale, che la frazione di DNA che ha assunto la densità del denaturato è effettivamente costituita da eliche completamente separate, mentre il materiale di densità intermedia è costituito da eliche ancora collegate tra loro. Si discute il complesso dei risultati finora ottenuti.

In two previous papers [1, 2], some experiments on the alkaline denaturation of DNA in vitro were reported. They were planned in order to test some characteristics of the Watson and Crick model for the DNA molecule, which at first sight seem difficult to explain from the point of view of classical mechanics. The results obtained can be summarized as follows. When a solution of native DNA is suddenly brought to physico-chemical condition (pH 12.4) in which the DNA is completely denatured, the hyperchromic effect rises to its maximum value in less than one hundreth of a second. This does not mean, however, that the two strands separate in such a short time. In reality, if the solution is brought back to normal physico-chemical conditions (pH 7) after a time τ has elapsed, the hyperchromic effect is almost completely reversible if τ is small. It becomes irreversible only with a much longer rise-time, which depends on the length of the molecules involved and lasts about eighteen seconds for T2 DNA (MW about 120 million).

The two sets of experiments could be interpreted according to the Watson and Crick model of DNA. When the denaturation conditions are suddenly reached, all the hydrogen-bonds between the complementary bases are broken in a very short time. The two strands of the molecule, however, remain intertwined and unwind only gradually taking a much longer time to separate.

Our third set of experiments shows that this is not the case. DNA solutions exposed to denaturation conditions for a time τ were examined in the analytical ultracentrifuge, in CsCl density gradients, for different values of τ ranging between zero and thirty seconds. It was found that the time required for the two strands of a particular molecule to separate is much shorter than the rise-time of the irreversible hyperchromic effect of the solution, so that almost no partially denatured molecules could be found in the CsCl density gradient.

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As τ increases from zero to thirty seconds, the density of the peak of the native DNA remains constant. This peak, however, gradually decreases and the peak of denatured DNA correspondingly increases gradually. This shows that each molecule remains double-stranded until a sort of collapse occurs which brings the molecule from the double-stranded to the denatured state in a time of the order of only one second or even less.

As we have already pointed out, these experimental results increase the difficulties of the Watson and Crick model, from the point of view of classical mechanics. Hence, we have done two further experiments in order to confirm and elucidate our previous findings.

The first experiment is based on the assumption that the time required by any molecule to unwind or to diffuse over a certain distance in the solution must be proportional to the viscosity of the liquid medium. If the viscosity of the medium is changed, the rise-time of the irreversible hyperchromic effect must also change if the collapse of the DNA molecules is dependent upon the motion of the molecules themselves or upon the collisions with some other molecules diffusing through the medium. If, on the contrary, the collapse depends only on the transitions between the internal states of the DNA molecule itself, the rise-time of the irreversible hyperchromic effect must be almost independent of the viscosity of the medium, owing to the fact that the unfolding time of a single molecule is much shorter than the rise-time of the irreversible hyperchromic effect of the solution.

One could suppose however that, while exposed to denaturation conditions, each molecule begins to unwind and when the normal conditions are restored it has a probability of quickly returning to the native state and a probability of passing to the denatured state. That this is not the case is shown by the fact that a very small quantity of partially denatured material can indeed be observed in the CsCl density gradient and that the reset-time of the reversible fraction of the hyperchromic effect is as short as is its rise-time at the beginning of the denaturation process.

T2 DNA prepared by the phenol method [3] was used in this experiment. Its sedimentation coefficient was 51 S when measured at 15 μg/ml. In order to change the viscosity of the medium, four different solutions were prepared: *a*) 0.1 M NaCl, *b*) 9% glycerol in water, *c*) 30% methanol in water and *d*) 50% methanol in water. First, a sample of each solution was brought to conditions in which DNA is totally denatured (pH 12.4), by adding a suitable quantity of 0.2 M NaOH, and the viscosity coefficient was measured. All measurements were performed in a Ubbelohde capillary viscosimeter, whose running-time for water at 20°C was 191.5 s. The temperature was kept constant to within 0,01°C by using a thermostatic bath. The viscosity coefficient was calculated from the formula:

$$\eta = k \rho t$$

where k is the constant of the instrument, ρ the density of the solution and t the measured running-time.

The DNA was dissolved in the solutions, the optical density was measured and samples of each solution were exposed to denaturation conditions for a time τ. After the time τ had elapsed, the solution was rapidly neutralized by adding a suitable quantity of 0.2 M HCl. The irreversible hyperchromic effect was then determined. The measurement was repeated for various values of τ, from zero to sixty seconds.

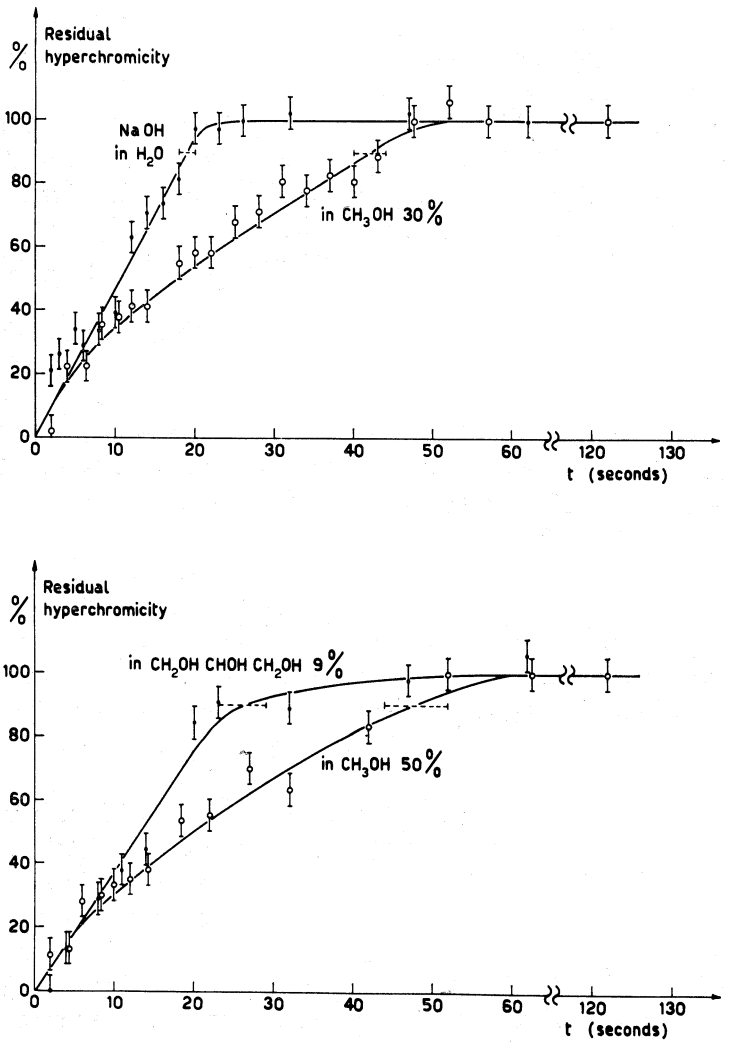

Fig. 1.

The results obtained are summarized in fig. 1. Fig. 2 shows that the rise-time (0–90%) of the irreversible hyperchromic effects depends linearly on the viscosity of the medium. Thus, we may conclude that the collapse of the molecule of DNA does not depend only on the transitions between the internal states of the molecule itself. However, it is difficult to understand

how this result should be interpreted. Further experiments are necessary for this purpose. We can only say, for the moment, that when the solution is suddenly brought to denaturation conditions a process begins, for each DNA molecule, in which some movement in the solution is involved and which ends with the rapid separation of the two strands. We must, however, exclude any process which makes the restoration of the hydrogen-bonds impossible or changes the buoyant density of the molecule. Furthermore, the difficulties encountered in interpreting this experiment are increased by the fact that it is not at all clear how the macroscopic viscosity coefficient is related to the movements of single macromolecules in the solution.

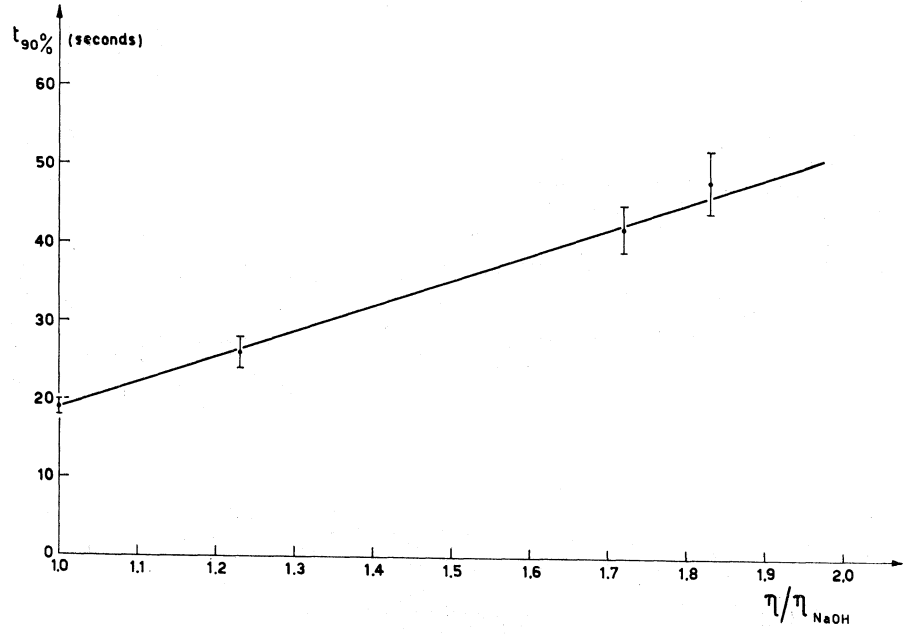

Fig. 2.

The second experiment was planned to verify wheter the native or totally denatured molecules were the only ones present in a solution exposed for a time τ to denaturation conditions. If the solution is maintained for a convenient fixed time under conditions in which renaturation of the DNA molecules occurs, we can determine how the quantity of the renatured material depends on the DNA concentration. If the two strands of the molecules that have undergone denaturation are not completely separated, then this quantity must be independent of the concentration. If, however, the two strands are separated, two complementary strands must collide for renaturation to take place. In this case, the quantity of the material renatured in a fixed time must be proportional to the square of the concentration, as is the number of collisions per unit time, and the process must be much longer than in the previously considered case.

T 2 DNA was tested first. Two DNA solutions at concentrations of 20 γ/ml and 40 γ/ml were exposed to denaturation conditions for a time 10 s after which about half of the hyperchromic effect is irreversible. A sample of each solution was then brought to conditions in which renaturation occurs (pH 10.8 and kept under these conditions for six hours at room temperature. After neutralization, each sample and the corresponding non-renatured solution were examined in the analytical ultracentrifuge in CsCl density gradients.

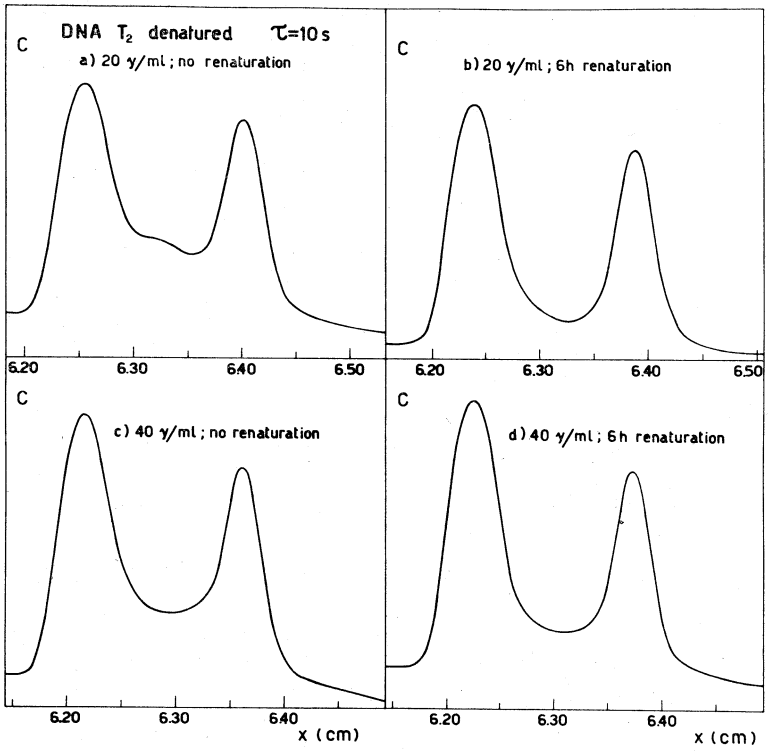

Fig. 3.

The results obtained are shown in fig. 3. As can be seen, all the material between the peaks of the native and of the totally denatured DNA has disappeared after renaturation. The peak of the native DNA has increased a little. The peak of the denatured DNA, however, remained unchanged. We must conclude that, in this experiment, the material whose buoyant density is that of the denatured DNA behaves very differently from the material of intermediate buoyant density. While the latter is completely renatured, the former is not renatured at all. We can only interpret this result by saying that the two strands of the molecules of the intermediate band are only partially separated (as suggested by the intermediate density), while the two strands in the band that has the buoyant density of the denatured DNA are completely separated.

This experiment was repeated with α DNA as a control. In this case, the two separated strands have different buoyant densities in CsCl solutions and in a number of molecules, they are evidently separated after partial denaturation. As before, a solution of α DNA was exposed to denaturation conditions for a time sufficient to raise the irreversible hyperchromic effect to half its maximum value. Three samples of the solution were diluted to 10 γ/ml, 20 γ/ml and 40 γ/ml and kept for six hours under conditions in which renaturation occurs (pH 10.8). The samples were then examined in the analytical ultracentrifuge in CsCl density gradients, together with a similar solution which had not been exposed to renaturation.

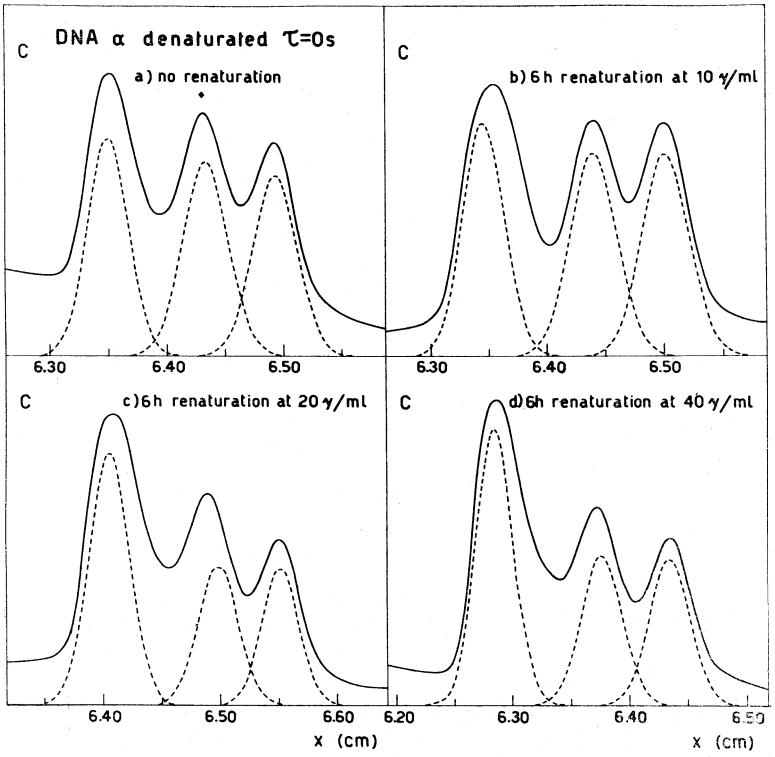

Fig. 4.

The results are shown in fig. 4. The dependence of the quantity of renatured material on the concentration is evident and is roughly quadratic, as expected (fig. 5).

The fact that T 2 DNA is much more difficult to renature than α DNA, can be explained if it is remembered that the former is about four times as heavy as the latter. Thermal motions are then less efficient in causing collisions between complementary strands and favouring complete alignment of the two strands.

Let us now make some concluding remarks. In this, and in the two previous papers, we have reported several experiments whose aim was to test the Watson and Crick model for the DNA molecule. The questionable point was the relative position of the two strands, which in the Watson and Crick model are intertwined. When a denaturation process takes place, the two strands must unwind and rotate with a very high angular speed and this produces some difficulties from the point of view of classical mechanics. At this point we must briefly examine the main conclusions that can be drawn from our research.

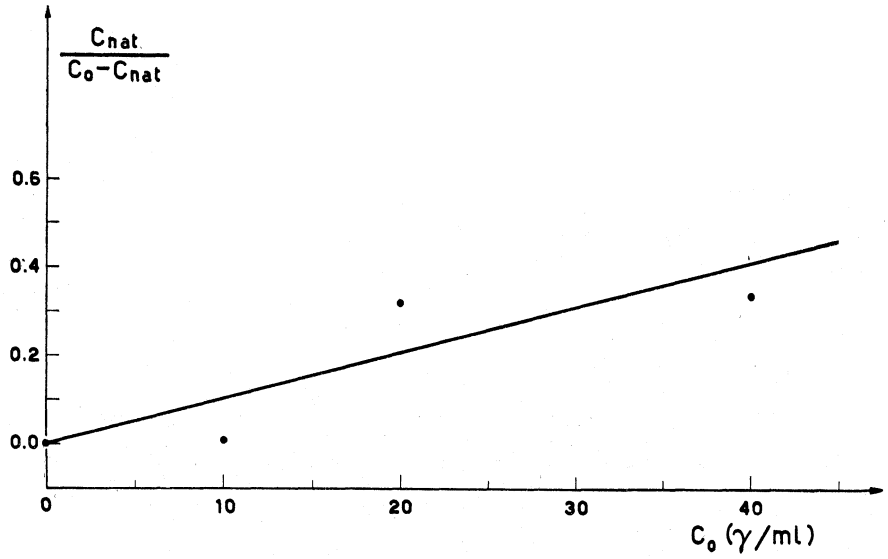

Fig. 5.

Some of our findings can be easily interpreted within the framework of the Watson and Crick model. These are the results obtained on the reversible and the irreversible hyperchromic effect, and the dependence of the rise-time of the irreversible hyperchromic effect on the viscosity of the liquid medium. At first sight, all this seems to indicate clearly that when denaturation conditions are suddenly reached, all the hydrogen-bonds between complementary bases are broken in a very short time and all the molecules begin to unwind and gradually reach the denatured state in a few seconds.

Other results, however, seem to contradict this apparently obvious interpretation. These are the results of the experiments done in the analytical ultracentrifuge, with the CsCl density gradients. The rise-time of the curve, which represents the increase of the peak of the denatured DNA with denaturation time, agrees fairly well with the rise-time of the irreversible hyperchromic effect. However, the density gradient patterns do not show the expected gradual increase, with denaturation time, of the buoyant density of the DNA. What is observed is a gradual decrease of the peak of the native DNA, a

gradual increase of the peak of the denatured DNA and a very small quantity of material of intermediate density.

All of this must be interpreted in the light of the previous results. One might think that when the normal pH is suddenly restored the partially denatured molecules rewind or unwind completely with various probabilities. This is not the case, however, because: 1° the reset-time of the reversible fraction of the hyperchromic effect seems to be no longer than its rise-time at the beginning of the experiment; 2° the hyperchromic effect does not change any more in the time that follows and 3° some material of intermediate density can be observed in the density gradient pattern and renaturation experiments show that it is made up of partially denatured molecules. Thus is seems that the density gradient experiments should be interpreted by saying that the separation of the two strands of the molecule is a process which takes a much shorter time than the rise-time of the irreversible hyperchromic effect, a time of the order of one second in the case of T 2 DNA. It seems that after the rupture of the hydrogen-bonds, a molecule remains in the two stranded state until a sort of collapse occurs and the two strands separate rapidly.

This interpretation, however, presents other difficulties. First, the dependence of the rise-time of the irreversible hyperchromic effect on the viscosity of the medium becomes difficult to understand. Secondly, one may ask what causes the collapse of the DNA molecule. In the third place, if the unwinding of the molecule takes so short a time, the mechanical difficulties of the Watson and Crick model are increased.

Levinthal and Crane [4] have calculated the energy required per revolution to overcome the viscous drag. Using their formula, in the case of T 2 DNA (20,000 turns in the molecule, 1 second required for the denaturation) it can be calculated that the energy wasted in the viscous drag is of the same order as the total energy of the phosphate bonds in the molecule. This figure is a thousand times greater than that obtained by Levinthal and Crane. It seems, however, more difficult to understand how angular momentum is conserved in alternate denaturation and renaturation processes.

In conclusion, further experiments are necessary in order to be able to provide answers to the questions asked above.

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