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A Tentative Description of the Process of Alkaline Denaturation of DNA

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Biofisica. — *A Tentative Description of the Process of Alkaline Denaturation of* DNA (*). Nota di MARIO AGENO (**) ELISABETTA DORE (**) e CLARA FRONTALI (**), presentata (***) dal Corrisp. M. AGENO.

RIASSUNTO. — I risultati di studi cinetici precedenti sulla denaturazione alcalina del DNA sollevano notevoli difficoltà dal punto di vista della dinamica del processo, soprattutto per quel che riguarda la sorgente di energia, e la ragione per cui le molecole non iniziano simultaneamente la denaturazione. Nel presente lavoro vengono descritti i risultati di uno studio condotto in un intervallo di pH intorno alla zona di transizione. Essi possono così riassumersi: *a*) A pH compreso tra 11,7 e 12,2, il tempo di salita dell'effetto ipercromico è dell'ordine di alcuni minuti. Esso decresce regolarmente all'aumentare del pH. *b*) La larghezza della transizione rivelata dall'effetto ipercromico è misurabile, mentre assai più ristretta è la larghezza di transizione per l'effetto ipercromico residuo. I risultati dell'analisi in gradiente di CsCl confermano che anche in questo caso il processo appare di tipo quasi catastrofico.

Confrontando i nuovi dati con i precedenti si individuano due intervalli di pH in cui fenomeni diversi limitano la velocità del processo. Sulla base di queste osservazioni viene presentato un modello della denaturazione alcalina capace di rendere conto della dinamica del processo, e di fornire previsioni controllabili sperimentalmente.

Earlier experiments on the alkaline denaturation of DNA *in vitro* [1–4] show that when a solution of native DNA is suddenly put into a physico-chemical condition (pH 12.4 ÷ 12.6) in which total denaturation (i.e. physical separation of the two strands of each molecule) takes place, the hyperchromic effect rises to its maximum value in much less than 0.1 s. This does not mean, however, that the two strands separate in such a short time. If the solution is changed back to pH 7 after a time τ, the hyperchromic effect can be shown to be almost entirely reversible, so long as τ is small. It becomes irreversible only gradually, with a much longer rise-time, which depends on the length of the molecules involved, being usually of the order of several seconds.

Solutions of semi-denatured DNA were also examined in an analytical ultracentrifuge, for various values of τ, using the caesium chloride density gradient technique. The results obtained were discussed in detail and confirmed by separate suitable tests [4]. It was concluded that the time required for the separation of the two strands of a single molecule is much shorter than the rise-time of the irreversible hyperchromic effect of the solution in bulk. Hardly any partially denatured molecules were found in the caesium chloride density gradient, the percentage of irreversibility of the hyperchromic effect being a measure of the fraction of molecules that at the end of the interval of time τ have undergone *total* denaturation. In other words, when denatu-

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ration conditions are suddenly brought about, a chemical reaction begins which transforms the native DNA molecule into the denatured one and the reaction rate controls the increase of the irreversible hyperchromic effect. Some molecules are almost immediately denatured while others remain unchanged for a period of the same order as the rise-time of the irreversible hyperchromic effect. One can quote, as an example, the case of the phage T2 DNA, whose M.W. is 120 millions. The rise-time of the irreversible hyperchromic effect (and of the percentage of totally denatured molecules in the solution) is 20 s, while the time required for the separation of the two strands of a single molecule can be evaluated by the fraction of semi-denatured molecules in the density gradient, being of the order of 1s.

The results of some of our experiments [1–2] compare well with the results obtained by Davison [5] for the alkaline denaturation of the DNA from phage α and T2. The interpretation of these results, however, is different since according to Davison the denaturation time of the bulk solution is taken to be a measure of the denaturation time of a single molecule. If our interpretation is correct, there is no evident reason why the total denaturation time of the solution should depend on the square of the molecular weight. It certainly depends on the molecular weight, but it could equally depend on other properties and conditions, as for instance the difference between the pH used for denaturation and that at which denaturation begins. A simple comparison between DNAs from different sources could then be meaningless.

At first sight, it does not seem easy to fit our results into the framework of the well-grounded model of the DNA molecule proposed in the 1953 by Watson and Crick. Two main points have to be explained. The first concerns the source of the energy required to overcome the viscous drag in unwinding the two strands of each molecule. Levinthal and Crane [6] evaluated this energy and concluded that it is not an important fraction of the energy required for the duplication of the molecule and that it can be supplied in the usual way by energy-rich bonds in some enzymatic process. This conclusion, however, was based on the data concerning the molecular weight and the rate of unwinding of the molecule available at that time and now obsolete. With our data relative to the DNA of phage T2, for instance, the energy required is about one thousand times greater and the question about its source when no enzymatic process occurs, as in our experiments *in vitro*, becomes important.

The second point that must be clarified is the reason why the unwinding of all the molecules does not begin at the same instant when the denaturation conditions are suddenly brought about, and why the reaction rate is not determined by the rate of unwinding.

In this paper, the experimental investigation of the denaturation process will be extended to physico-chemical conditions not previously considered and a tentative general description of the process itself will be presented.

The pH of a solution of native DNA of phage T2 was suddenly increased to a value φ and the hyperchromic effect, as well as its rise time, were spectrophotometrically determined; after a time τ, long enough to obtain the com-

plete hyperchromic effect, the solution was brought back to pH 7. The residual hyperchromic effect, and the buoyant density pattern in the caesium chloride density gradient were observed as previously described [1–2]. The experiments were repeated for different values of φ, starting from the point at which denaturation begins.

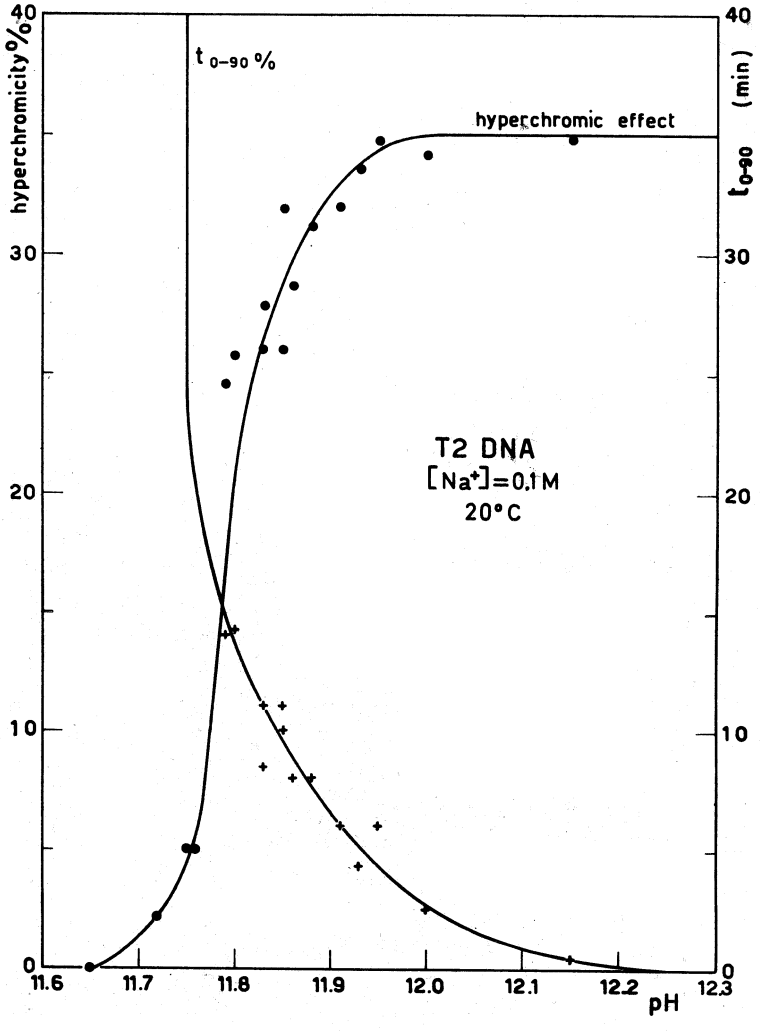

Fig. 1.

The hyperchromic effect was recorded as a function of time with a Cary mod. 15 spectrophotometer and its rise-time and final value were measured on the graph. The results obtained are summarized in fig. 1 which shows that below pH 11.95 the hypercromic effect does not reach its maximum value and its rise-time increases very rapidly with decreasing pH. It seems that in this pH range, the factor limiting the unwinding of the DNA molecules is the breakage of the hydrogen bridges between the pairs of complementary bases.

In fig. 2, the irreversible fraction of the hyperchromic effect (residual hyperchromic effect after neutralization of the solution) and the percentage of totally denatured molecules measured with an analytical ultracentrifuge, using the caesium chloride density gradient method, are shown as a function of the pH of denaturation. The total hyperchromic effect at the pH used for

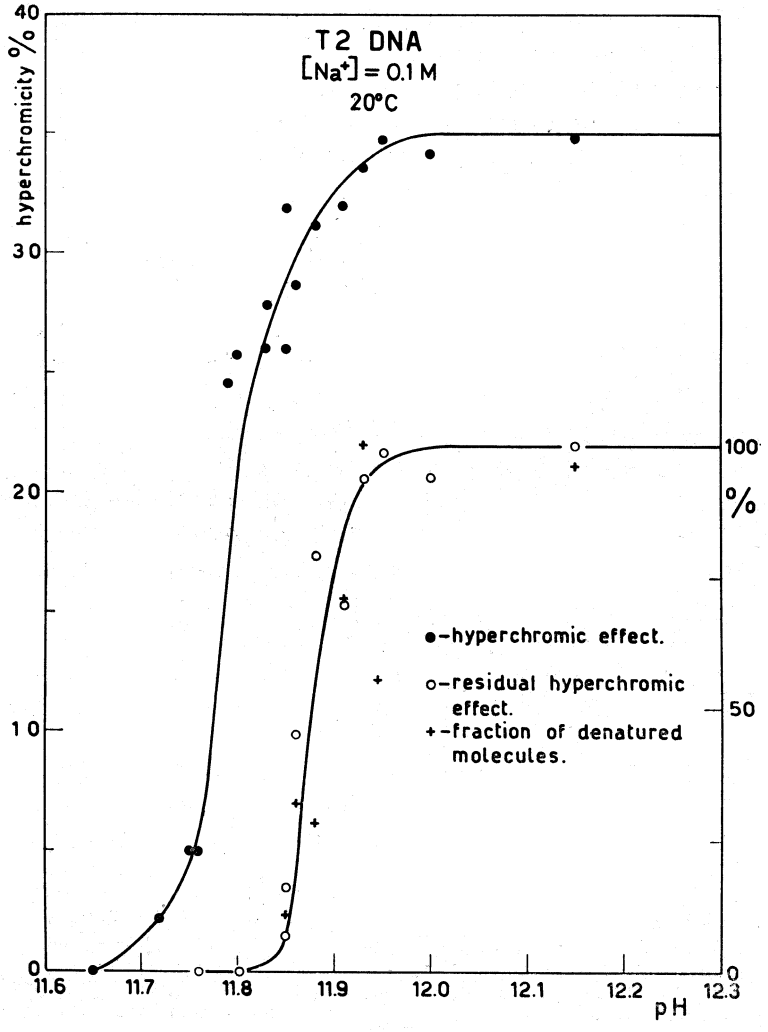

Fig. 2.

denaturation is also given for comparison. It will be seen that the density gradient data fit very well with those of the residual hyperchromic effect thus confirming that below pH 11.95 only a fraction of molecules are denatured and that the remainder is still in the native state.

A tentative description of the process of alkaline denaturation of DNA can be obtained by the separate consideration of two typical ranges of pH. In the higher one (12.4–12.6), the DNA molecules are surrounded by a very

high concentration of hydroxyl ions and deprotonation of the hydrogen bridges existing between the pairs of complementary bases, is very likely to occur. Since the formation of a water molecule sets free 13.5 Kcal mol^{-1} this process is certainly exoenergetic. However, one must not suppose that the energy released is equal to the difference between this figure and the energy required to break one hydrogen bridge, or the binding energy of the relevant proton in a pair of nucleotides. In our case the two nucleotides are firmly bound in the DNA molecule and cannot withdraw when the proton is captured. The energy necessary to extract the proton is thus much higher (it must however be lower than 13.5 Kcal mol^{-1}) and a large part of it remains inside the DNA molecule as repulsive electrostatic energy between the two pairs of electrons of the Oxygen and Nitrogen orbitals previously bridged by the proton.

According to our model of the hydrogen bond [7–10] it is not necessary to remove all the protons of the bridges between each pair of complementary bases in order to break the bonds between the two strands of the molecule. For instance, the two H–bridges between adenine and thymine are correlated since they belong to the same ring and form only one bond between the two bases. When one proton is removed from this ring the bond is broken. This model is strongly supported by the fact that polymethyluracil does not form any hydrogen bonds with polyadenine [11], notwithstanding that *one* conventional hydrogen bridge between methyluracil and adenine is still possible.

When the hydrogen bridges of the DNA molecule are deprotonated, the molecule is subject to a strong internal tension and the repulsion between each pair of nucleotides generates a couple tending to unwind the two strands. Adjacent couples, however, oppose each other so that only the two couples at the free ends of the molecule are effective, and the molecule unwinds itself almost catastrophically, like a loaded spring suddenly released, starting from the ends.

The rise-time of the reversible hyperchromic effect shows however that in this range (pH 12.4–12.6), the deprotonation is completed in less than 0.1 s, even if the different DNA molecules in the solution unwind at different times, so that the spring remains charged until a particular event discharges it. We have shown that this event is not an intrinsic one of the molecule, i.e. a transition from one state to another, because the rise-time of the residual hyperchromic effect depends on the viscosity of the medium [3]. Ions are however present in the solution at a fairly high concentration, in our case Na^+ and Cl^-, 0.1 N. They shield the phosphates of the DNA molecule, so their repulsion cannot cause the unwinding. The shielding effect of the ions must be much lower in the case of the electrons of the deprotonated H–bridges, because the distance between the charged particles is much less. Positive ions would tend to replace the protons of the bridges, but they are not so small as protons and cannot restore the bond. The waiting-time of a molecule, before the quasi-catastrophic unwinding, must then depend on the fluctuations of the local positive ion concentration. It must also depend on the ionic radius, the smaller the radius the longer the waiting-time.

Let us now consider the phenomena in the other typical pH range, that near the point at which denaturation begins (11.7 ÷ 12). Deprotonation of H-bridges is less probable and a relatively long time is required before a

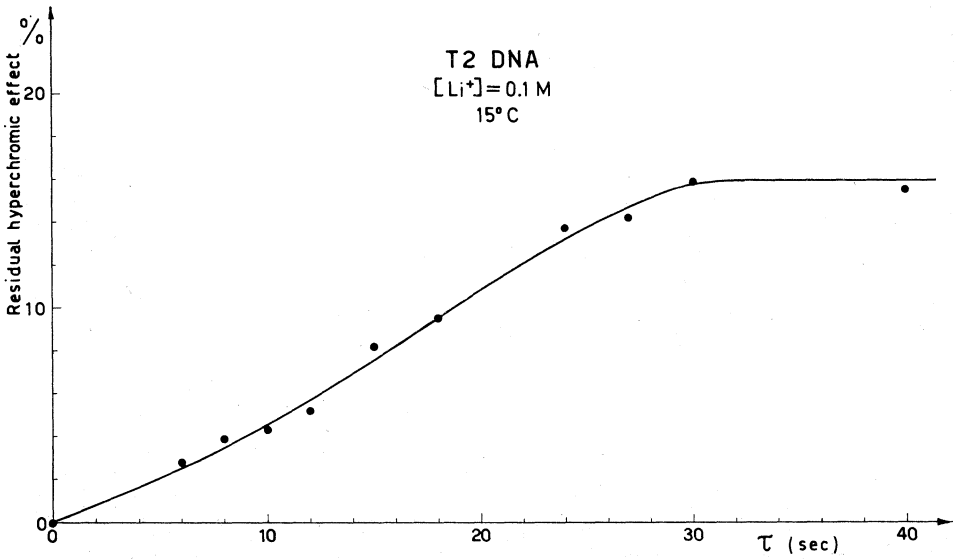

Fig. 3.

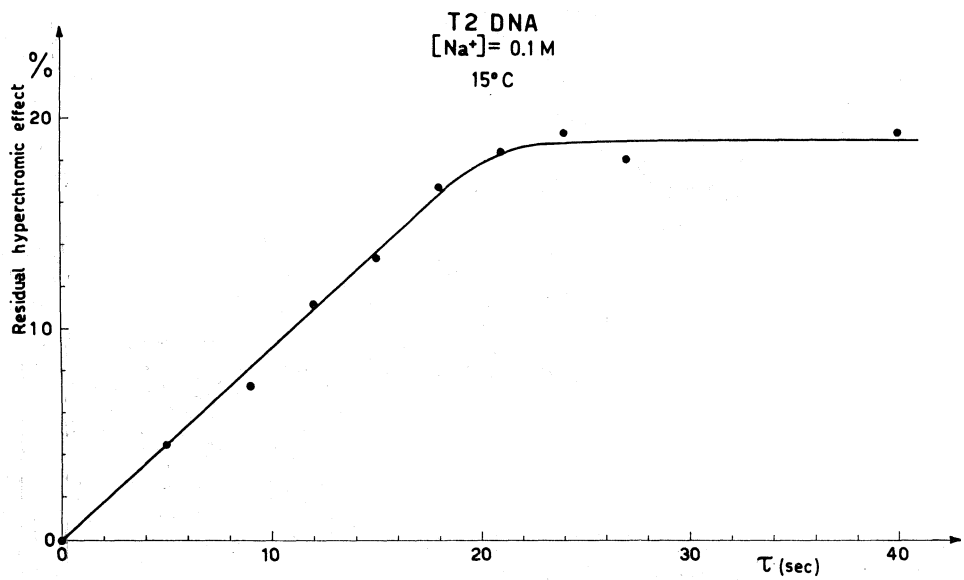

Fig. 4.

molecule reaches a state where the separation of the two strands is an unavoidable event. Fig. 2 shows that in the pH range between 11.65 and 11.85, the hyperchromic effect is completely reversible. Even if it reaches almost two thirds of its maximum value, no denatured molecules can be found after

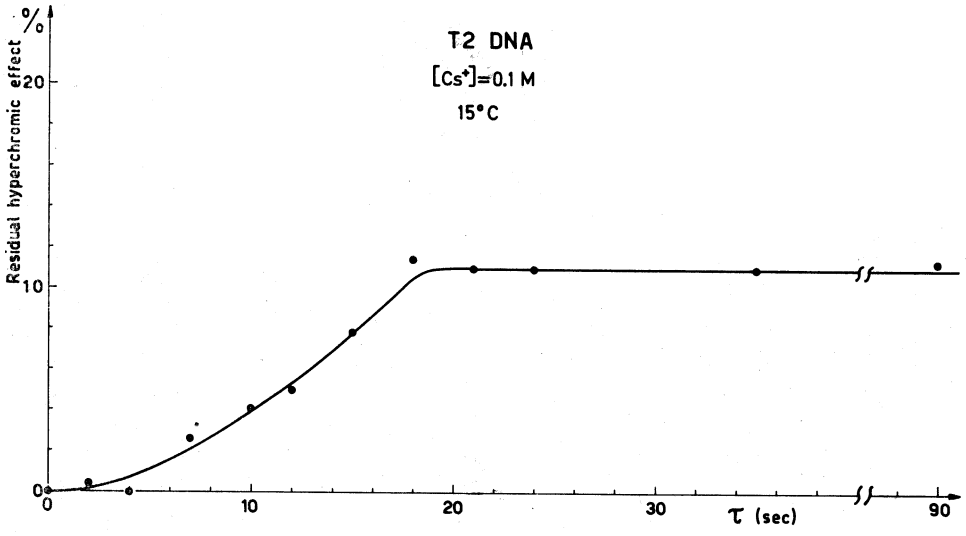
%
Residual hyperchromic effect
20
10
0
T2 DNA
[Cs+]=0.1 M
15°C
0
10
20
30
τ (sec)
90

Fig. 5.

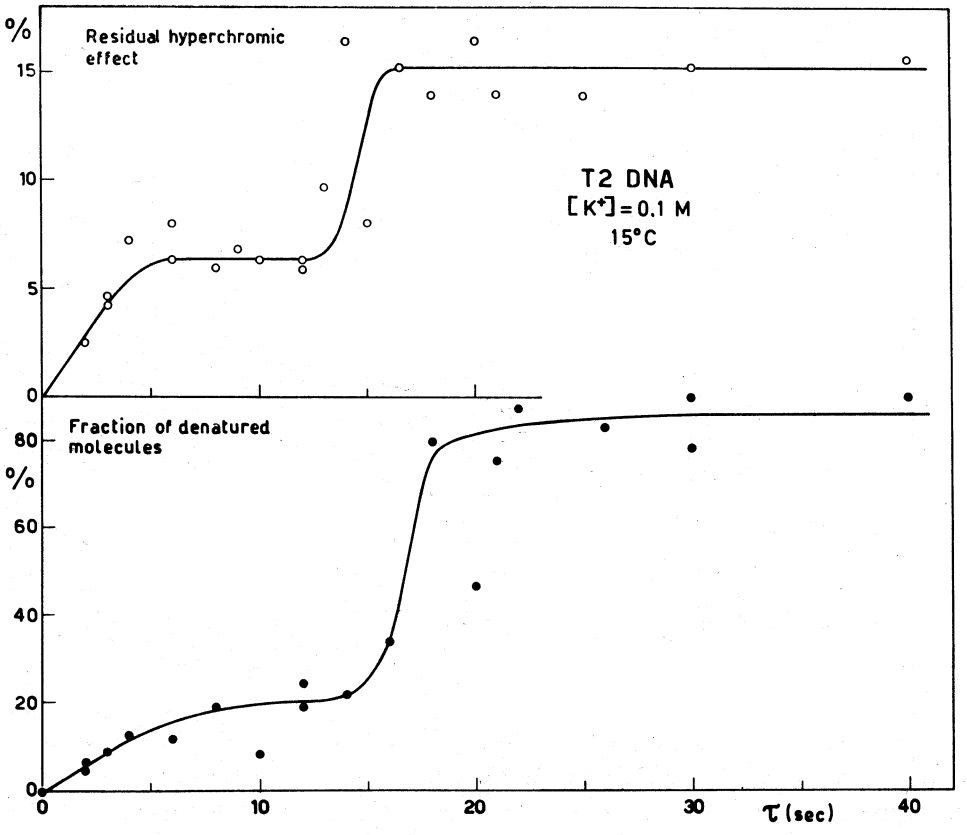
%
Residual hyperchromic effect
15
10
5
0
T2 DNA
[K+]=0.1 M
15°C
Fraction of denatured molecules
80
%
60
40
20
0
0
10
20
30
τ (sec)
40

Fig. 6.

neutralization of the solution. This means that in this range each molecule is partially deprotonated but not sufficiently to unwind completely. Perhaps the CG pairs of nucleotides require more energy to separate than the AT pairs, and the hydroxyl ion concentration here is not high enough to lower the mean potential around the protons of the bridges so as to make deprotonation an energetically favoured process. After a certain time a state of equilibrium is reached in which each molecule is only partially unwound.

It is remarkable that the width in which residual hyperchromic effect rises to its maximum value is much smaller than that of the reversible hyperchromic effect. We cannot exclude that the observed residual width is instrumental in origin. An intrinsic width could be due to renaturation, since, as complete separation of the two strands may depend on local fluctuation in the concentration of the hydroxyl ions, as soon as the fluctuation is over two separated strands still near to each other could have a fairly high probability of joining again.

It seems that this tentative description of the process of alkaline denaturation of DNA can explain all our experimental results. As further confirmation, we are investigating whether the rise-time of the residual hyperchromic effect depends, as foreseen, on the radius of the positive ions. Preliminary results, shown in figs. 3–6, were obtained using Li^+, Na^+, Cs^+ and K^+ as positive ions in the DNA solution at the same molar concentration (0.1 M). For Li^+ Na^+ and Cs^+ the expected trend was observed, the rise-time becoming longer as the radius of the ion decreases. The anomalous results obtained with K^+ ions (fig. 6) were confirmed by measuring the percentage of denatured molecules in an analytical ultracentrifuge using the caesium chloride density gradient technique, and it is now under consideration.

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