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Mario Ageno, Elisabetta Dore, Clara Frontali

Density Gradient Studies of the Alkaline Denaturation of DNA

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

RIASSUNTO. — La cinetica della denaturazione alcalina del DNA di alcuni batteri e fagi è stata studiata per mezzo dell'ultracentrifuga analitica in gradiente di densità. I risultati mostrano che la denaturazione che si ottiene portando temporaneamente il pH della soluzione ad un valore estremo (pH 12,4) è un processo catastrofico nel senso che per brevi esposizioni al pH elevato, tali da causare solo una parziale denaturazione, non si trovano molecole parzialmente denaturate, ma piuttosto una frazione delle molecole già completamente denaturata e una frazione ancora nello stato nativo.

Questi risultati abbassano drasticamente il tempo che generalmente si ritiene sia necessario per separare materialmente le due eliche del DNA ed aumentano le note difficoltà per la comprensione della meccanica del processo.

In a preceding paper [1] the kinetics of the alkaline denaturation of DNA was studied, measuring the hyperchromic effect by means of a differential automatic device, with which rapid mixing of two solutions and the almost instantaneous adjustment of pH to a predetermined value was possible. The main result was that when an extreme value of pH (pH 12.4) was suddenly attained, the hyperchromic effect always reached its maximum value in less than one hundredth of a second. An interpretation of this can be that almost all the hydrogen-bonds between complementary bases in DNA have been broken. The fusion of the bonds, however, is a reversible one at the beginning of the process. If the pH is brought back to a normal value (pH 7.0) after an interval of the order of one second, for instance, no residual hyperchromic effect can be measured and the DNA remains in a state that cannot be distinguished from the native state. As time goes on, an increasing residual hyperchromic effect appears. In the case of T2 DNA, the effect reaches its maximum in about eighteen seconds and the DNA is then completely denatured.

These facts can be easily explained if we take into consideration the classical Watson-Crick model for DNA [3]. The high pH causes the hydrogen-bonds to break, but cannot separate the complementary bases, because the two strands of the molecule are intertwined. Thus, at the beginning the fusion is reversible. Afterwards, however, the two strands, no longer held together by the hydrogen-bonds, twist around each other and gradually separate and the hyperchromic effect becomes irreversible.

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(**) Laboratori di Fisica, Istituto Superiore di Sanità, Roma.

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But this is not the only way in which our results can be interpreted. Thus, we considered it worth-while to verify directly that in the interval of time in which the residual hyperchromic effect is increasing, all the DNA molecules of the solution are partially denatured. In effect it must be remembered that the DNA molecule of phage T 2 is composed of about two hundred pairs of nucleotides. If it takes the two strands about twenty seconds to separate, they must turn at 1000 r.p.s., which is the maximum speed of a Spinco ultra-

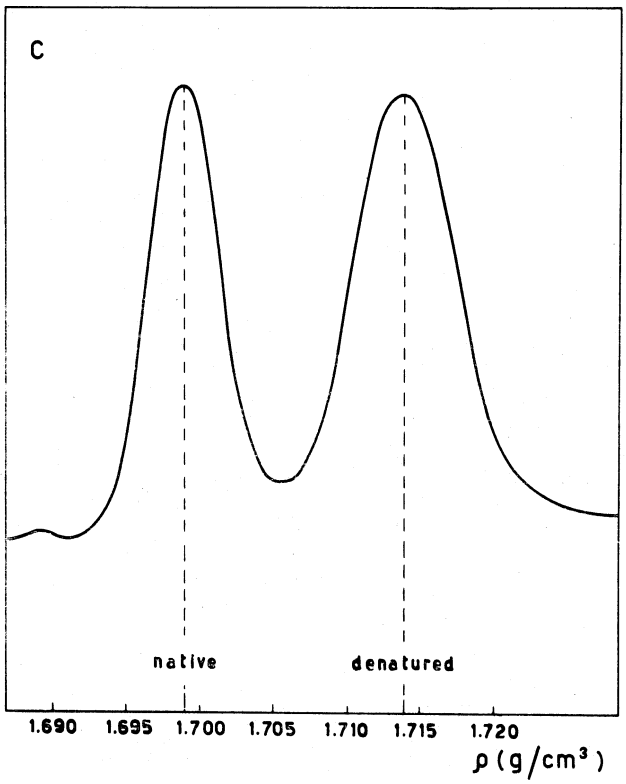

Fig. 1.

centrifuge! Before trying to explain the difficulties involved, from the point of view of classical mechanics, it seemed to us advisable to make sure that this was really so.

We thus planned a very simple experiment which consisted of examining in the analytical ultracentrifuge in a CsCl density gradient the state of a solution of DNA, which had been kept for a few seconds (between zero and about thirty seconds) at an extreme pH (pH 12.4). First, it was experimentally verified that from the instant in which the pH was suddenly restored to 7, the hyperchromic effect did not vary any more. Thus, we could be sure that the state of the DNA molecules in the solution would not change in the time necessary for the formation of the density gradient. In effect we had to make

sure that during this time a partially denatured molecule, kept at pH 7, would not rewind, thus returning to a state similar to the native one, or that it would not continue to unwind, until, in the end, its two strands were completely separated.

As is well-known, native and denatured DNA form two distinct bands in a CsCl density gradient, the single strands having a buoyant density considerably higher than that of the double stranded molecules. A partially dena-

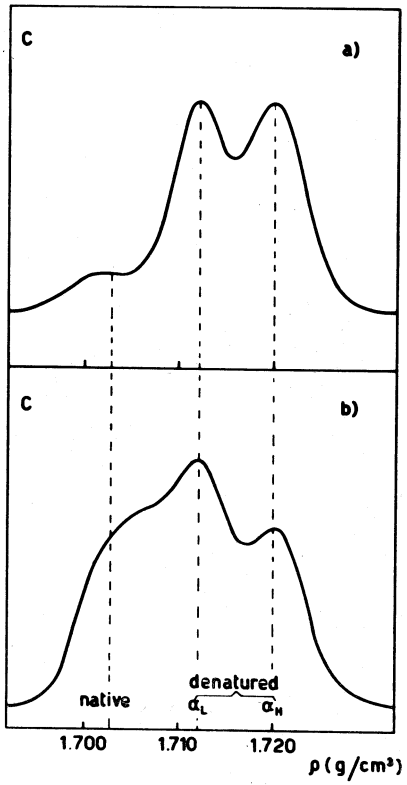

Fig. 2.

tured molecule, must, therefore, have a value which is intermediate between that of the native and that of the completely denatured DNA, and its equilibrium position in the density gradient must be intermediate between the two bands in question. As an example, we show in Figure 1 the UV optical density pattern of a CsCl density gradient of a mixture of a native DNA and of the same DNA, after complete denaturation. The two gaussian bands are evident and are not overlapping.

The behaviour of the partially denatured molecules of DNA in a CsCl density gradient is shown in Fig. 2. Here, a solution of DNA of phage α (active on *B. megatherium*), after complete denaturation at pH 12.4, was kept for three hours at pH 10.8 and then examined in the ultracentrifuge. The two strands of the α DNA have different buoyant densities [4] and the

band of the denatured DNA is represented by two partially overlapping gaussians (fig. 2 *a*). Being kept at pH 10.8 causes a partial renaturation. Many pairs of complementary strands join together and a number of bonds between them are restored. Thus, we observe (fig. 2 *b*) in the UV optical density pattern a continuous distribution of DNA molecules, from the native to the lighter of the two bands of the denatured DNA.

It is then obvious what should be expected from our experiment. According to the Watson-Crick model, the unwinding time of the two strands of the molecule must be the time required for the increase in the irreversibility of the hyperchromic effect. All the molecules begin to unwind at about the same instant when the pH is suddenly raised to 12.4 and all must reach the totally denatured state at almost the same time (when the irreversibility of the hyperchromic effect is complete).

Thus, if the DNA solution is kept at pH 12.4 only for an interval of τ seconds, and we repeat the experiment for different values of τ, between zero an thirty seconds, for instance, we should observe in the UV optical density pattern of the CsCl density gradient, only one gaussian band. This band should move gradually from the density of the native DNA ($\tau = 0$) to the density of the denatured DNA. (A more complex situation would be observed, however in the case of a DNA like that of α, whose two strands have different buoyant densities in CsCl solutions. We limit ourselves to the more general simple case).

The experiment was repeated many times with three different DNAs, one bacterial DNA (*E. coli*) and two phage DNAs (T 2 and α) and the results each time were completely different from those we expected on the basis of the Watson-Crick model.

Our observations can be summarized in the following way:

(1) we have never observed any variation in the density of the native DNA band for any value of τ. Its intensity decreases as τ increases, until the band disappears completely. Only in the case of T 2, which is the heaviest of the three DNAs examined, the band, from the beginning becomes a little skew on the right side, but the position of its maximum remains unaltered.

(2) For the smallest valued of τ, a new band begins to form, with a density equal to that of denatured DNA. This band also does not change its position in the gradient, but its intensity gradually increases with τ, until it becomes the only band which can be seen in the UV optical density pattern.

(3) No consistent amount of DNA is observable, in our patterns, between the two extreme densities of the native and of the denatured DNA, in the case of *E. coli*, and of phage α DNA. In the case of phage T2 DNA on the contrary, the UV optical density pattern, analyzed into gaussian components, can be interpreted by saying that a third intermediate weak gaussian band is formed. This band, however, once formed, never changes its intensity or position, until it completely disappears. Furthermore, it appears before the denatured band becomes observable, and disappears after the native band has completely disappeared.

These results are illustrated in figs. 3–7. Some of the photographs obtained in the ultracentrifuge are shown in fig. 3, where *a*) refers to α DNA, denatured with $\tau = 0$ and *b*) to T 2 DNA denatured with $\tau = 10$ s. It should be noted that the mixing procedure necessary to adjust the pH to the required value was standardized so that it took one second to bring the pH from 7.0 to 12.4 and one second to bring it back to 7.0.

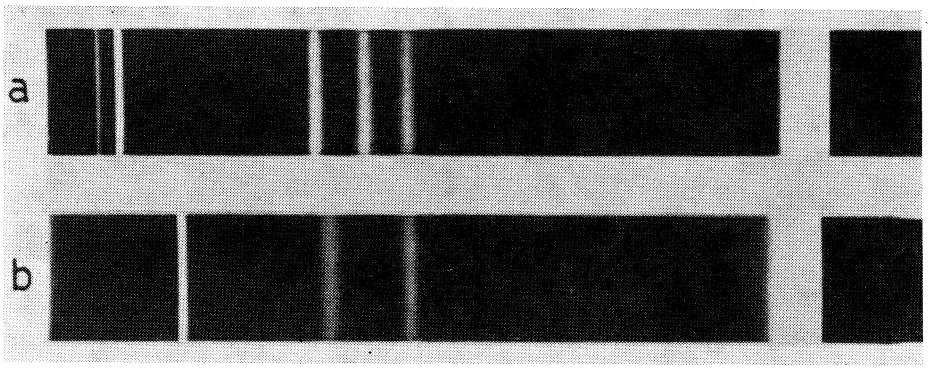

Fig. 3.

Figs. 4 and 5 represent two typical density patterns obtained in different experiments with T 2 DNA and α DNA. Fig. 4 represents the pattern given by α DNA denatured with $\tau = 0$, i.e. in a total time of 2 s. Fig. 5 gives the

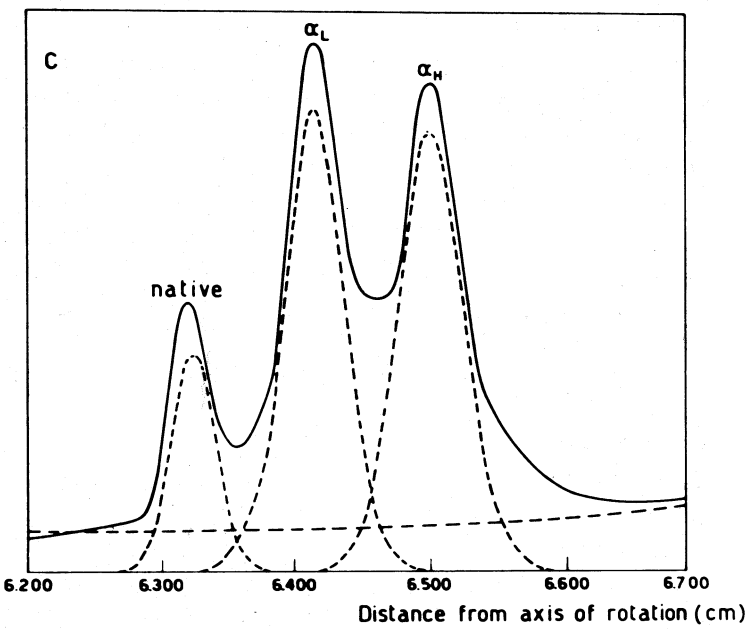

Fig. 4.

profile for T 2 DNA denatured with $\tau = 15$ s, i.e. a total denaturation time equal to 17 s. The graphs in figs. 6 and 7 show how the percentage of denatured DNA (measured by evaluating the areas of the gaussian bands) depend on the total denaturation time in the case of *E. coli* and α DNA respectively.

Obviously we consider our results correct but we cannot completely exclude that a series of strange cases may have influenced our experiments. They were, however, repeated many times under somewhat different conditions and with different samples of DNA and the reported characteristics

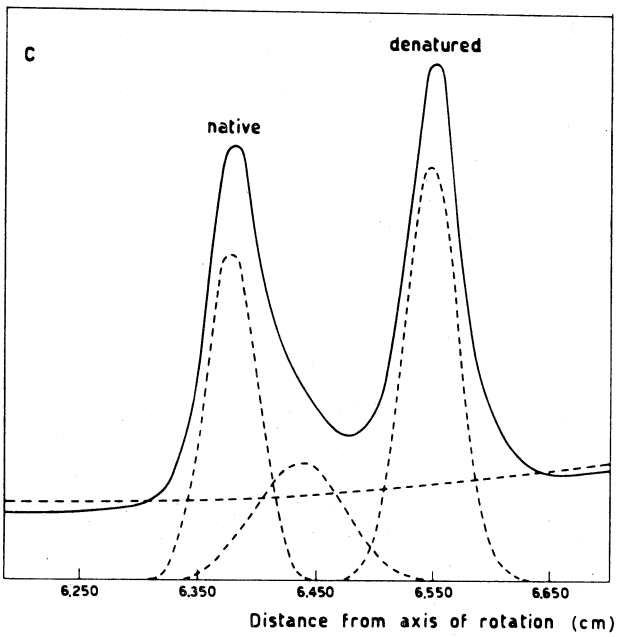

Fig. 5.

were always observed. Other experiments were done in order to control the results and other tecniques were used. Some of these are still in progress. These will be described in a subsequent paper.

We shall now try to draw some conclusions from the results of the experiment described, which should be necessarily valid if the experiment itself is reliable, as we are convinced. The main observation is that the separation of the two strands does not begin at the same instant for all the molecules in the solution and takes a much shorter time than that required by the hyperchromic effect to become totally irreversible. Thus our kinetic results of the hyperchromic effect should be interpreted in the sense that, the fraction that is irreversible is not a measure of the mean degree of partial denaturation of each DNA molecule in the solution, but represents the fraction of molecules that are denatured.

We are thus led to conclusion that, when all the hydrogen-bonds between the complementary base pairs are broken, the two strands are prevented from

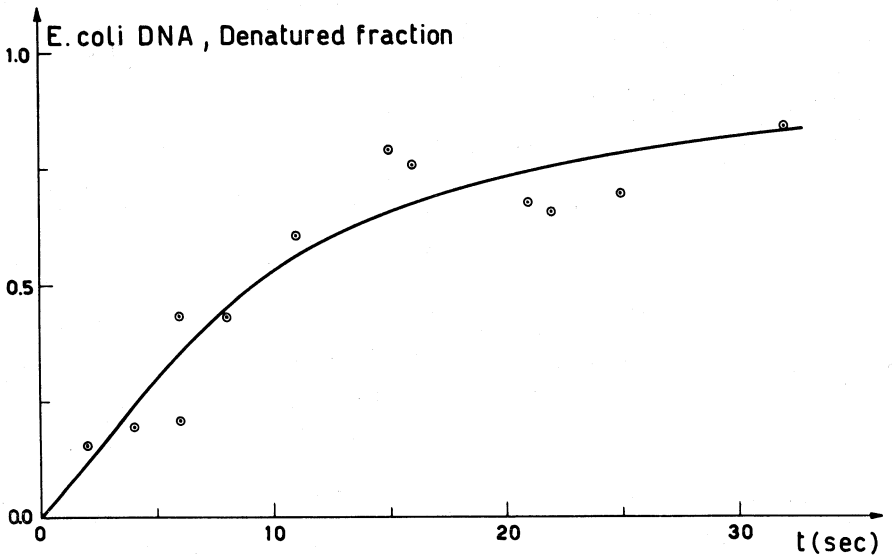

Fig. 6.

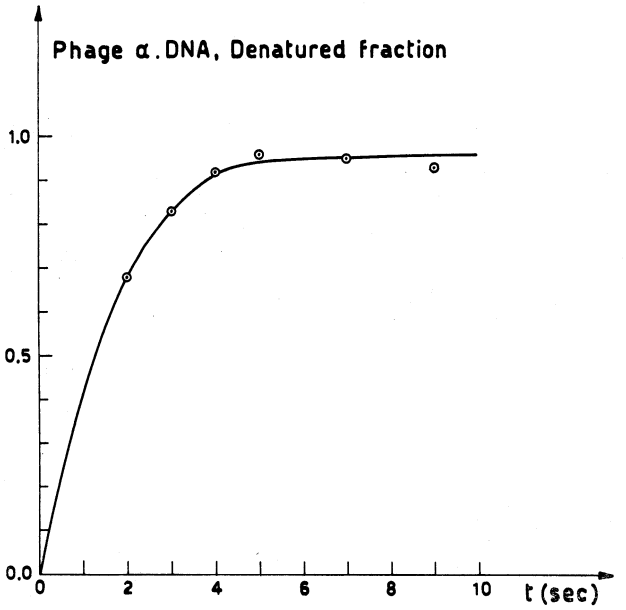

Fig. 7.

separating immediately only by something that can collapse rapidly, but which does not, in general, collapse immediately. We can easily imagine some " hard " bonds distributed along the strands that do not significantly contribute to the hyperchromic effect, but it seems very difficult to explain the experimental result considering that the two strands are intertwined.

Thus, the difficulties presented by the Watson-Crick model for the DNA molecule are in no way reduced.

We wish the express our gratitude to the technicians of our Laboratories Mr. A. Araco, Mr. M. Araco, Mr. A. Flamini, Mr. G. Notargiacomo, Mr. A. Rosati and Mr. P. Savini for invaluable help in preparing and performing the measurements.

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