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Rise-time and Reversibility of the Hyperchromic Effect in the Alkaline Denaturation of DNA in vitro

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Biofisica. — *Rise-time and Reversibility of the Hyperchromic Effect in the Alkaline Denaturation of* DNA in vitro (*). Nota di MARIO AGENO (**), ELISABETTA DORE (**) e CLARA FRONTALI (**), presentata (***) dal Corrisp. M. AGENO.

RIASSUNTO. — Si mettono in rilievo le difficoltà che il classico modello della molecola di DNA proposto da Crick a Watson presenta in relazione alla conservazione del momento della quantità di moto e l'opportunità di sottoporre a diretto esame sperimentale alcune caratteristiche secondarie di tale modello. Si riferiscono quindi i risultati ottenuti in due diverse serie di esperimenti in cui il processo di denaturazione alcalina del DNA è seguito nel tempo attraverso l'osservazione dell'effetto ipercromico. Le esperienze dimostrano che, in quattro DNA di diversa origine l'effetto ipercromico ha già raggiunto il suo valore massimo un centesimo di secondo dopo che le condizioni di denaturazione siano state realizzate. L'effetto è tuttavia inizialmente reversibile e la irreversibilità s'instaura assai più lentamente, con un tempo di salita di ~ 18 secondi nel caso del DNA di T 2. Questo fatto è suscettibile di due diverse interpretazioni, tra le quali il solo studio dell'effetto ipercromico non permette di decidere. Sull'esperienze eseguite in proposito con altre tecniche verrà riferito in una Nota successiva.

It is well-known that the Crick and Watson [1] model for the DNA molecule is based on the X–ray diffraction analysis and represents fairly well the experimental results obtained to date in genetics and molecular biology. However, some difficulties appear when the model is examined from the physical point of view.

Let us consider, for instance, the replication of T 2 phage DNA in *E. coli*. This molecule is made up of around two hundred thousand nucleotide pairs and is about thirty times as long as the host cell in which the replication process takes place. Hence it must be wound up with at least fifteen turns. In spite of this, the molecule reproduces itself in a very short time, probably shorter than one minute. The two strands separate from one another during reproduction while, at the same time, the intact tail of the molecule must make twenty thousand turns on itself. (Its turning speed equals that of a centrifuge!). Owing to the very small diameter of the molecule, this does not imply any difficulty from the energetic point of view [2]. However it is hard to understand the origin and nature of the torque [3], and how the angular momentum is conserved.

Considerations of this type suggest a direct experimental test of those details of the Crick and Watson model which are not involved in its essential features of order and complementarity of the bases, which are obviously correct.

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In particular, one may look to the experiment, for the direct proof that the two strands are really twisted together.

With this aim in view, we decided to study, by several different physico-chemical techniques, the process of alkaline denaturation of DNA *in vitro*. This process was preferred to heat denaturation, since it allows much more rapid changes in the experimental conditions and a more detailed study of the kinetics of the reaction.

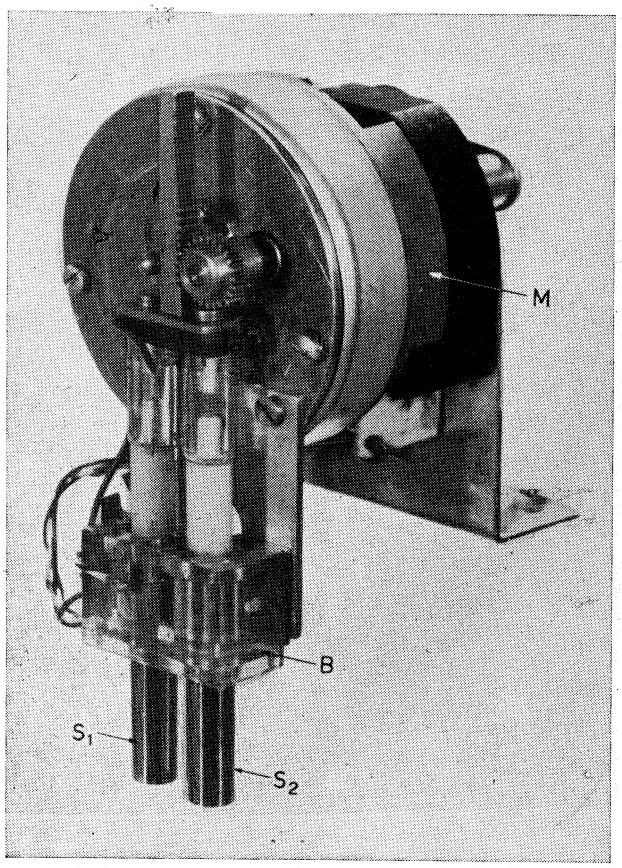

Fig. 1.

The present paper deals with the results obtained following, in a spectrophotometer, the hyperchromic effect of a solution of DNA during the first seconds after denaturation conditions have suddenly been established. In subsequent papers, we shall report the results obtained using the other techniques and the problem will then be much more clarified. However, the measurement of the hyperchromic effect at the very beginning of the reaction allows definite conclusions to be reached and thus can be separately discussed.

Since a preliminary set of experiments has shown that a few seconds after the establishment of the denaturation conditions most of the DNA is

already denatured, we have designed and constructed a simple device for the rapid mixing of a NaOH solution with the solution of DNA in the same cuvette of the double-beam spectrophotometer (Beckman DB). This was done in order to be able to observe the hyperchromic effect from the beginning of the mixing process itself.

Our device is shown in fig. 1. It consists of two syringes, S_1 and S_2, of the same volume, whose pistons can be moved in synchronism by a little motor M. The plexiglass block B rests on the border of the two cuvettes which contain the same DNA solution (1.6 cc of a DNA solution, 20 γ/ml in 0.1 M NaCl). The syringes plunge into the liquids so as to avoid formation

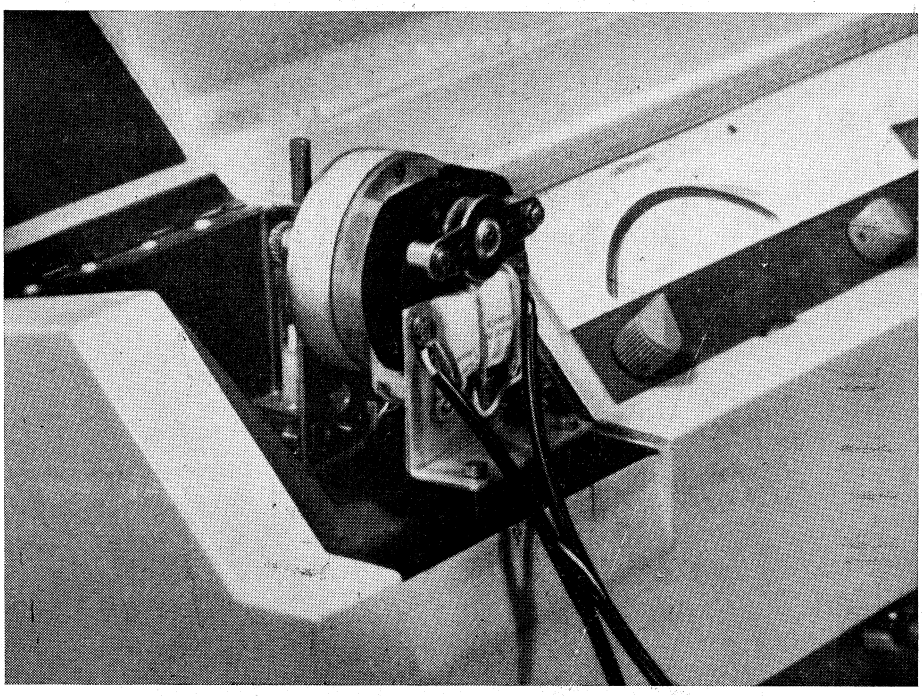

Fig. 2.

of bubbles. The outflow is rapid enough to cause effective mixing, but not so high as to damage the DNA molecules in the cuvettes. One of the syringes contains 0.7 cc of a 0.1 M NaCl solution and the other 0.7 cc of a 0.2 M NaOH solution. In this way, the dilution effect is the same in the two cuvettes, but in the first one the pH remains at the initial value of 7.0 while in the second it is, in a very short time, brought to 12.4. Two microswitchs cause a timer (Autocontatore el. decimale S 12, Italelettronica) to start and to stop, at the beginning and at the end of the piston run. Thus the injection time is known with the accuracy of one hundreth of a second. Its value was, in all our measurements, 0.40 ± 0.01 s.

A push-button starts the motor and releases the sweep of an oscilloscope (Tektronix RM35A Oscilloscope), the vertical axis of which is connected to the output of the spectrophotometer. In this way, the vertical deflection of the trace of the oscilloscope is always proportional to the hyperchromic effect

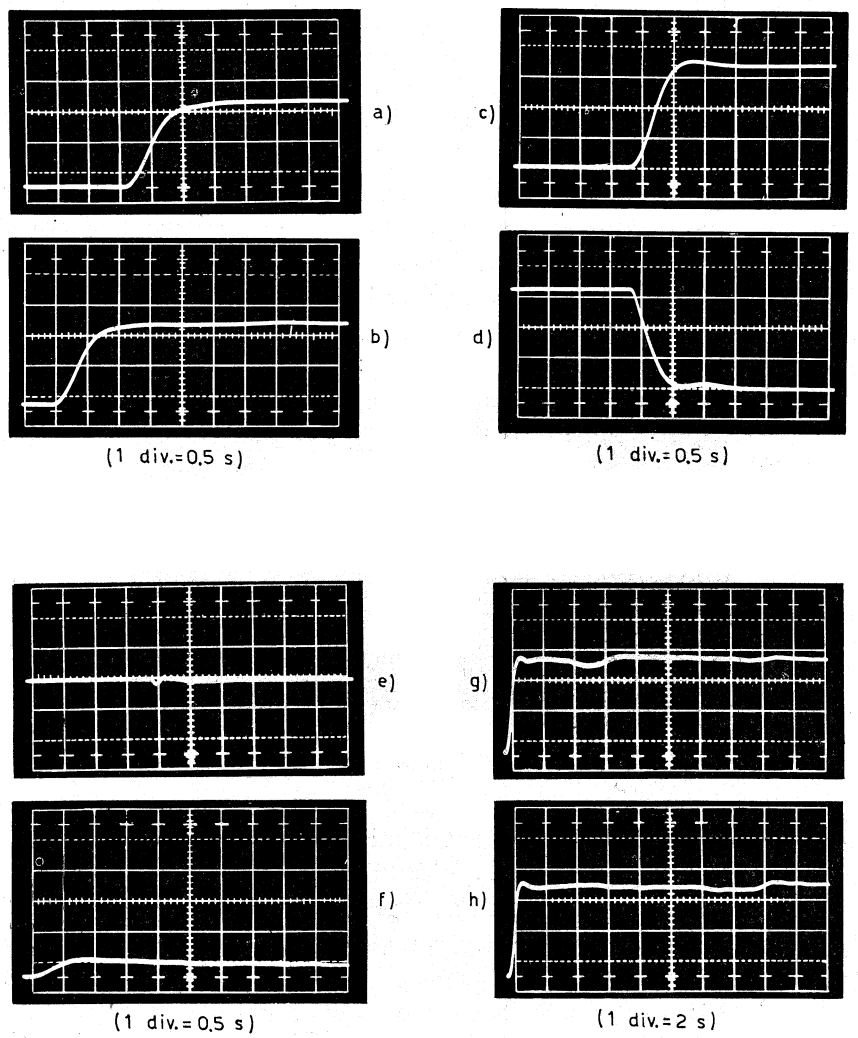

Fig. 3.

of the solution of DNA treated with NaOH. The oscilloscope is photographed with a Du Mond Oscillograph Record Camera, using Polaroid film Pola Pan 200.

Fig. 2 shows our automatic device in place over the cuvettes of the DB Spectrophotometer. To avoid complications caused by diffuse light in the room, the opening is closed by a panel of a convenient shape, in place of the usual one, when measurements are to be made.

The results of some preliminary experiments are shown in fig. 3. In *a*, *b*, *c*, and *d*, the rise-time of the instruments was measured under different conditions. In *a* and *b*, only one of the beams was interrupted suddenly and the measured rise-time (from 10 % to 90 % of the maximum excursion, overshoot excluded) was about 0.68 sec. In *c* and *d*, one of the two beams was interrupted in the initial condition of the apparatus. The situation was then inverted suddenly, the free beam interrupted and the interrupted beam opened. The rise-time was now somewhat shorter than before, only about 0.48 s. In *e*, both beams interrupted suddenly and practically no transient signal was observed.

The last three experiments were mixing experiments done to study the response of the apparatus under conditions somewhat similar to those of the real experiments with DNA. In *f*, the two cuvettes contained 0.6 cc of 0.1 M NaCl solution. The first one was diluted with 0.7 cc of the same solution while the second was diluted with 0.7 cc of 0.2 M NaOH solution. A small effect

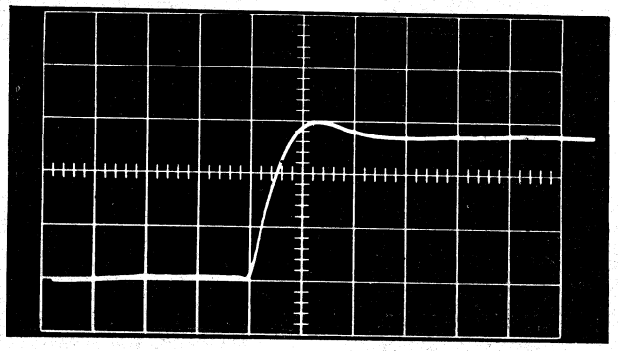

Fig. 4.

of mixing is evident, which must be taken into account in the real experiment when calculating the hyperchromic effect from the total vertical deflection of the trace of the oscilloscope. In *g* and *h*, the hyperchromic effect was simulated by injecting into the distilled water contained in one of the two cuvettes a convenient solution of Methylene blue.

The overshoots observed in certain conditions are instrumental effects due to the amplifier of the spectrophotometer.

Owing to the fact that the rise-time τ_0 of the instrument depends on the variations in the optical density of the two specimens, we have made another measurement of this quantity producing sudden variations in the optical density, of the same order as those caused by the denaturation of DNA, by moving absorbers in the paths of the two beams. In this way, we have found $\tau_0 = 0.28$ s (fig. 4).

Next, denaturation experiments were performed using four different types of DNA, extracted from phages T 2 and α and from *E. coli* and *B. megatherium*. The results obtained are shown in fig. 5.

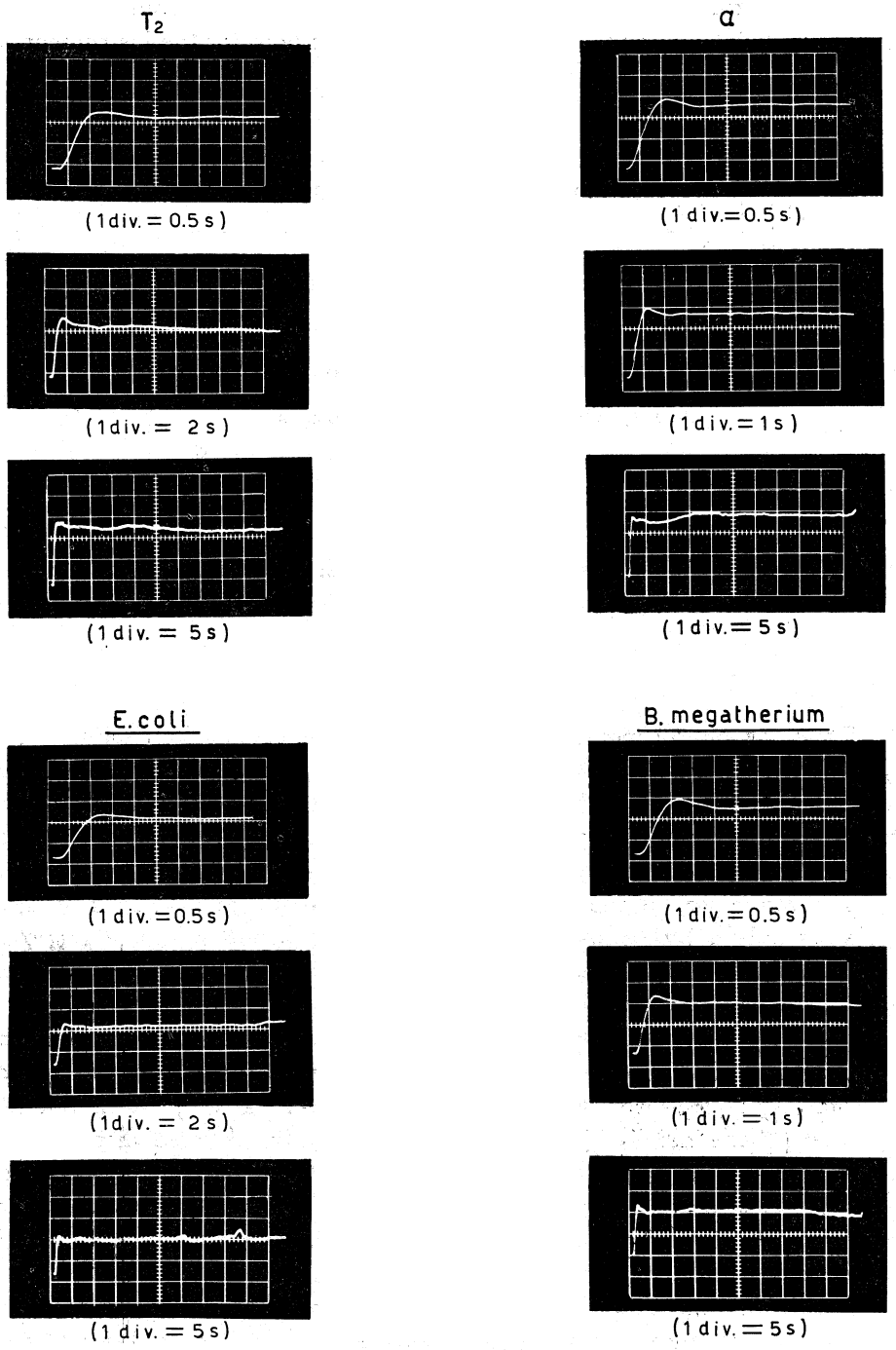
T2
(1 div. = 0.5 s)
(1 div. = 2 s)
(1 div. = 5 s)
α
(1 div. = 0.5 s)
(1 div. = 1 s)
(1 div. = 5 s)
E. coli
(1 div. = 0.5 s)
(1 div. = 2 s)
(1 div. = 5 s)
B. megatherium
(1 div. = 0.5 s)
(1 div. = 1 s)
(1 div. = 5 s)

Fig. 5.

The rise-times evaluated from the photographs are given in Table I. They must be compared with the previously given ones for the instrument, τ_0.

TABLE I.

DNA	Observed Rise-time
T 2	0.49 s
α	0.50 s
E. coli	0.49 s
B. megatherium . . .	0.49 s

However, it is also necessary to take into account the time of mixing, which, as already said, was always 0.40 ± 0.01 s. The two times, the time of mixing and the rise-time of the instrument, combine quadratically and we have:

$$\sqrt{(0.4)^2 + (0.28)^2} = 0.49 \text{ s}.$$

Thus, we must conclude that in all our experiments the risetime of the hyperchromic effect is much shorter than the instrumental rise-time. Moreover, the close agreement between the four results in Table I shows that the statistical error in our measurements is certainly less than 0.005 s. The rise-time of the hyperchromic effect is, thus, less than one hundreth of a second.

The maximum of the hyperchromic effect in the photographs of fig. 5 is always only a little lower than 40 % and, in all our experiments, equals the hyperchromic effect measured in the usual way on a sample of the same preparation of DNA, after complete denaturation. However, our results must not be interpreted in the sense that in one hundreth of a second the denaturation is complete. We can only say that, when the proper physico-chemical environmental conditions are reached suddenly, in a very short time (of the order of one hundreth of a second, at most) the ordered disposition of the bases in the two-helix configuration of the molecule of DNA is completely lost, which implies that the major part of the hydrogen-bonds between complementary bases must have been broken. The two strands can still be twisted together, or bound by a number of remaining bonds.

In order to understand what has happened in the first stage of the denaturation process, another set of experiments was made using a different technique. A sample of a DNA solution of an optical density of 0.4 was manually diluted in 1 second with a 0.2 M NaOH solution, in such a way as to bring the pH from 7.0 to 12.4, and the denaturation conditions for the DNA were reached. After τ seconds had elapsed, the sample was again manually diluted

in 1 second with a 0.2 M NaCl solution, so as to bring the pH again to the initial value of 7.0. The optical density was then measured and the residual hyperchromic effect calculated, taking into account the initial optical density of the sample and the dilution effect. The experiment was repeated with various values of τ from 0 to 60 seconds.

Fig. 6 shows the results obtained with samples of phage T 2 DNA. The plateau value is the residual hyperchromic effect of a sample of the same preparation of DNA, after complete denaturation. Now, it must be remembered that, before neutralization, the hyperchromic effect has already reached its maximum value for every value of τ. Thus, it is necessary to conclude that when the environmental denaturation conditions are reached suddenly, the hyperchromic effect rises to its maximum value in a very short time, as

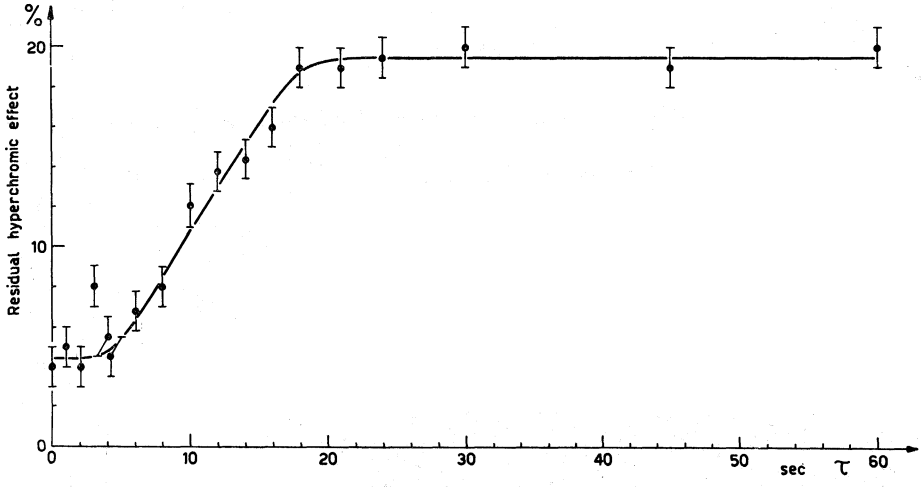

Fig. 6.

demonstrated by the first set of experiments. However, it remains almost completely reversible for some time, and irreversibility is reached only gradually with a much longer rise-time than that of the hyperchromic effect itself. This rise-time can be evaluated from fig. 6 to be $\sim$ 18 s.

When the denaturation conditions are established suddenly, there follows a first stage of the denaturation process in which the major part of the hydrogen-bonds between complementary bases has been broken, but the two strands of DNA remain in place, so that for some time the process is completely reversible. As time passes, however, the two strands separate and the process gradually becomes irreversible.

Obviously, these results can be interpreted in two different ways. We may suppose that each molecule of DNA is gradually unfolding so that its absorption of light is gradually increasing. But we may also suppose that each molecule takes a short time to become denatured when the process of separation of the strands has begun, but the number of denatured molecules is only gradually increasing, with the characteristic time of $\sim$ 18 s.

These two hypotheses correspond to different shapes of the curve in fig. 6, but the accuracy of our measurements is not sufficient to discriminate between them. Thus, we cannot choose the right interpretation only on the basis of measurements of the hyperchromic effect. In a subsequent paper we shall describe the results obtained, trying to solve this problem with different techniques.

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

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