

# THE ALKALINE DENATURATION OF DNA

M. AGENO, E. DORE, *and* C. FRONTALI

*From the Physics Laboratory, Istituto Superiore di Sanità, Rome, Italy*

**ABSTRACT** A kinetic study of the alkaline transition of DNA, in clearly defined physico-chemical conditions, is presented, which allows us to identify, within the alkaline transition region, different pH ranges, corresponding to different rate-limiting factors. This analysis brings into consideration three distinct intervals of time which characterize the whole process, namely the time necessary for full hyperchromicity to be reached, the time required for strand separation in the case of a single DNA molecule, and the time for complete denaturation to be reached in the case of a DNA solution.

The results obtained from ultracentrifugal, and spectrophotometric measurements, involving rapid mixing experiments, seem to indicate the following conclusions: whereas, in the lower pH ranges considered within the transition region, the denaturation process is limited by the first time constant, this same constant becomes extremely short at higher pH. On the other hand the fact that, in the higher pH range, the second and third time constants do not coincide (the time to unwind a single T2 DNA molecule being at least one order of magnitude shorter than the time required for bulk denaturation to be reached) suggests that in this pH range the overall denaturation rate is limited by a statistical process governing the initiation of unwinding.

These observations are discussed in terms of a model in which the unwinding energy is given by the electrostatic repulsions which originate in the deprotonated DNA molecule. The model itself suggests some experiment which seem to confirm it.

## INTRODUCTION

Although the physico-chemical properties of DNA molecules have been extensively investigated in recent years, much work has still to be done in order to understand its behaviour from the point of view of its functions in the cell.

In a sense, we might say that in a chemical chain reaction there is always some molecule that duplicates itself. However, no other molecule is yet known which is able to duplicate such a complex pattern carrying chemical information, i.e. which is at the same time the starting point of a very long and complex series of chemical reactions.

Not much is yet known about the duplication process and about the mechanism through which chemical information is copied at the right moment by a molecule of messenger RNA. What is known seems to indicate that both processes involve partial separation of the two complementary strands (1-5).

Denaturation studies *in vitro* are generally carried out either by a suitable variation of the concentration of hydrogen ions, or by raising the temperature of the medium in which the DNA molecule is immersed. Thermal denaturation of DNA has been extensively investigated in recent years (6-9). Less attention has been given to the pH-denaturation process (10-12), in spite of the fact that it is hard to conceive of a physico-chemical condition in the cell in which thermal denaturation occurs, while the other process is likely to give a hint of what happens in the cell.

With this point in mind, we have undertaken a careful investigation of the alkali denaturation process of DNA. Our main purpose was to collect information on the properties of this very important molecule and to work out, if possible, a reasonable model of the partial denaturation process that might control duplication of the molecule and synthesis of messenger-RNA.

The two conditions in which the DNA molecule is found at equilibrium during thermal or alkaline denaturation can be shown to be different, for instance by means of spectrophotometric absorption measurements on the same DNA either at high temperature or at high pH.

In Fig. 1 the hyperchromic effects measured for T2 DNA when the solution is brought either to 88°C, or to pH 12.5 are plotted as functions of the wavelength. (The measurements were performed with a Cary model 15 spectrophotometer (Cary Instruments, Monrovia, Calif.) flushed with dry nitrogen.) The difference is remarkable especially in the short wavelength region.

In both cases the molecules certainly have an extended conformation and no hydrogen bond can survive. But clearly heat-melted DNA is in a different state from that of the same DNA at pH 12.5 as revealed by the strong difference in absorption below 250  $m\mu$ . Such an effect, on the other hand, could be anticipated, given the spectral differences observed in nucleotides at neutral and high pH (13). The dispersion of the hyperchromic effect of heat-melted DNA agrees with data of Felsenfeld and Hirschman (14). The absorption spectra of heat-denatured and alkali-denatured DNA at room temperature and at pH 7 are indistinguishable. (See dashed curve in Fig. 1.)

It is the purpose of this work to discuss the physical aspects of alkaline denaturation. To this end we will first describe some kinetic experiments which allow us to discuss the nature of the rate limiting factors for the denaturation process, at different pH values, over a range covering the alkaline transition. As a consequence of this discussion we will consider three distinct intervals of time which characterize the whole process, namely the time necessary for full hyperchromicity to be reached, the time required for strand separation in the case of a single DNA molecule, and the time for complete denaturation to be reached in the case of a DNA solution.

A series of data obtained by spectrophotometric and ultracentrifugal techniques, and involving rapid mixing experiments will then be presented, this suggesting as a possible interpretation, which explains all of the obtained results, a new dynamic model of the process, whose main features are the following:

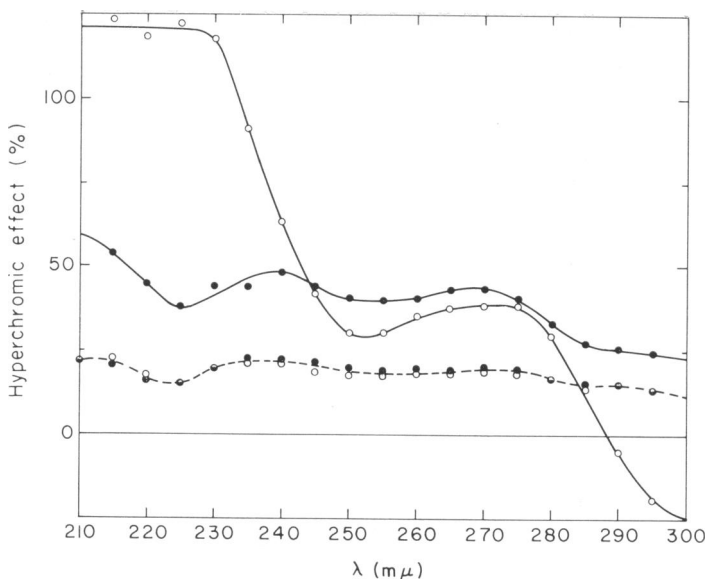


FIGURE 1 — Dispersion of the hyperchromic effect due to alkaline (○) or to heat (●) melting of T2 DNA ---. Dispersion of the residual hyperchromic effect (after rapid removal of the alkaline (○) or heat (●) denaturing conditions). The spectra of native, melted and denatured DNA were recorded with a Cary model 15 Spectrophotometer, and the hyperchromic effects calculated for different wavelengths.

(A) At low pH the process is limited by the first time constant, that is by the time required to break the bonds which are responsible for the hyperchromic effect. This time depends in a characteristic way on the pH in the transition region;

(B) At high pH (well over the transition region) the rupture of the bonds responsible for the hyperchromic effect is almost instantaneous. The time required by a single molecule to unwind is of the order of 1 sec or less (for T2 DNA), but the overall denaturation rate is limited by a statistical process governing the initiation of unwinding. The energy source for the fast rotation of the DNA molecule is found in the electrical repulsions originating in the deprotonated DNA molecule.

Finally an experiment will be described, in which the deprotonation of the DNA molecule is revealed by titrating a sodium hydroxide solution by the addition of DNA. This experiment has been designed for directly testing the proposed model, which may not be the only one which accounts for all the experimental evidence, but certainly is the simplest one can think of.

## MATERIALS AND METHODS

First of all, we studied the kinetics of the alkaline denaturation process. Our presentation of the problem was as follows. Suppose we suddenly move (at time  $t = 0$ ) a sample of DNA from ordinary to alkaline denaturation conditions. How does the hyperchromic effect increase with the time?

Let us consider the conditions which must be fulfilled in order to make the rise-time

measurements meaningful. Some of them concern the measuring technique, others the sample of DNA used, the physico-chemical state of the environment and of the test solution.

As far as the measuring technique is concerned, it is necessary to change the pH of the sample in a time interval as short as possible. The change in pH can be obtained by mixing two solutions (rapid mixing is required). However a compromise must be made between rapid mixing and careful handling of DNA samples in order not to damage the DNA molecules. Two different methods were adopted.

### *Hand-mixing*

1.6 ml of DNA solution at a concentration of about 20  $\mu\text{g}/\text{ml}$  were put in a beaker and 0.7 ml of NaOH solution 0.2 M were added with a graduated pipette. Air bubbles formed by blowing gently down the pipette were sufficient to cause complete mixing in about one second.

In another set of measurements, we took care to avoid that not even a small fraction of the DNA was exposed to a pH appreciably higher than the final one. Thus, the procedure adopted was the following: 4.5 ml of NaOH solution 0.05 M were put in a beaker and 0.5 ml of DNA at a concentration of 300  $\mu\text{g}/\text{ml}$  were added, using Marburg micropipettes (Eppendorf Gerätebau, Hamburg, Germany) with disposable tips, which were cut to a diameter of 2 mm to avoid damaging the DNA molecules. Magnetic stirring was used and in less than one second sufficient mixing occurred. Using dyes as indicators we verified that in a few tenths of a second all the DNA solution reached the pH of denaturation. Temporary pH-overshoot was less than 0.1 units at pH 12.5.

Hand-mixing, however, can be used only when the hyperchromic effect increases very slowly, which happens only in the neighbourhood of the transition pH-value.

### *Automatic Mixing and Spectrophotometric Recording Unit*

When the rise-time of the hyperchromic effect is of the order of 1 sec or less, hand-mixing is no longer suitable. In order to be able to follow the kinetics of denaturation also in these cases, we designed and constructed a simple device for the rapid mixing of an NaOH solution with the solution of DNA in the cuvette of a double-beam spectrophotometer (Beckman model, DB, Beckman Instruments, Inc., Palo Alto, Calif.). We were thus able to observe the hyperchromic effect from the beginning of the mixing process itself.

The device we used is shown in Fig. 2. It consisted of two identical syringes,  $S_1$  and  $S_2$ , whose plungers could be moved in synchronism by a small electric motor  $M$ . The plexiglass block  $B$  rested on the border of the two cuvettes of the spectrophotometer, which contained two identical samples of DNA (1.6 ml of a DNA solution, 20  $\mu\text{g}/\text{ml}$  in 0.1 M NaCl). The syringes were partially immersed in the liquid so as to avoid the formation of air bubbles. The syringe on the sample cuvette contained 0.7 ml of 0.2 M NaOH solution, and the other one, on the reference cuvette, 0.7 ml of an 0.1 M NaCl solution. In this way when the motor was started, the dilution effect was the same on both sides and for this reason no net absorption effect occurred. However, in the first cuvette the pH was brought up to 12.5 in a very short time, while in the second it remained at the initial value of 7.0. The outflow was rapid enough to cause effective mixing, but not so rapid as to damage the DNA molecules. This, however, was a very difficult point and a somewhat critical compromise was at last obtained. In our opinion the chosen compromise was not such as to affect the kinetics appreciably. Two microswitches caused an electronic timer to start and stop at the beginning and at the end of the plunger travel. (The second one also caused the motor to stop.) Thus,

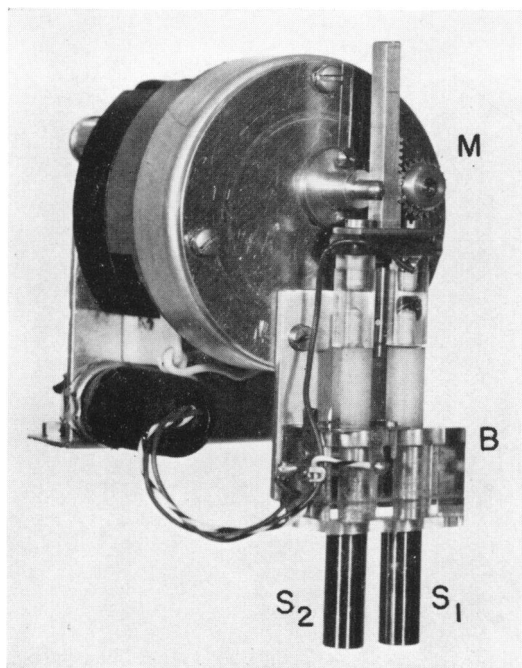


FIGURE 2 Automatic mixing device developed to inject simultaneously different liquids into the two cuvettes of a D. B. Beckman spectrophotometer. The bottom of the two syringes  $S_1$  and  $S_2$  bears three small apertures (0.2 mm in diameter) which allows the outlet of the syringe contents when the motor  $M$  pushes the pistons downwards, but reduce to a minimum the contact between solutions before the moment of injection.

the injection time was known to an accuracy of one hundredth of a second. Its value was, in all our measurements,  $0.40 \pm 0.01$  sec (maximum errors are indicated).

A push-button started the motor and released the sweep of an oscilloscope (Tektronix RM 35 A Oscilloscope, Tektronix, Inc., Beaverton, Ore.), the vertical axis of which was connected to the output of the spectrophotometer. In this way, the vertical deflection of the trace of the oscilloscope was always proportional to the hyperchromic effect of the DNA solution treated with alkali. The oscilloscope was photographed with a DuMont Oscillograph Record Camera (DuMont Laboratories, Clifton, New Jersey), using Polaroid film Pola Pan 200.

Several preliminary experiments were made with this device, in order to test its characteristics and reliability. In particular, the rise-time of the instrument was measured under different conditions. The beams were suddenly interrupted, either singly or both at the same time; one of the beams, previously interrupted, was suddenly freed, and the free beam simultaneously blocked, and so on. Small overshoots were sometimes observed and it was demonstrated that they were instrumental effects due to the amplifier of the spectrophotometer. Since the rise-time of the instrument depended somewhat on the variation in optical density of the two samples, sudden variations in the optical density of the same order as those caused by denaturation of DNA were produced by moving suitable absorbers in the path of the two beams. Under these conditions, the rise-time of the instrument was:  $\tau_o =$

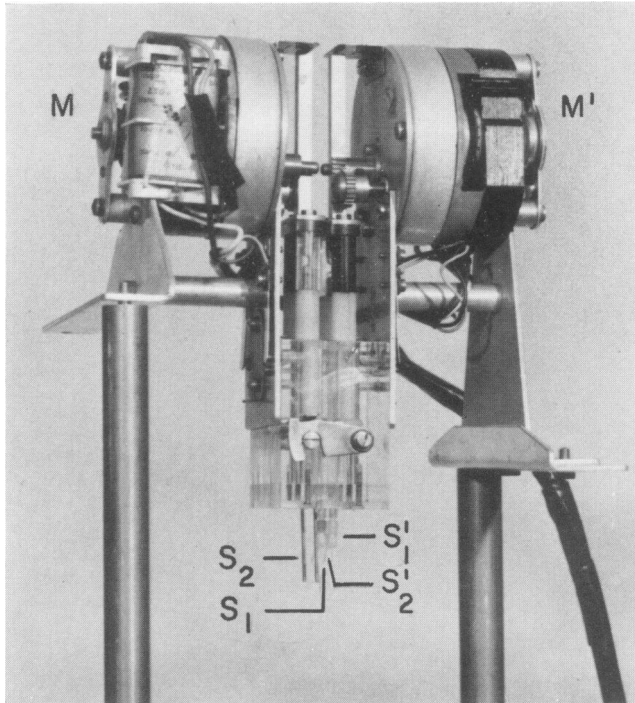


FIGURE 3 Modified mixing device, developed to inject simultaneously into the cuvettes of a D. B. spectrophotometer first two different solutions (syringes  $S_1$  and  $S_2$ , driven by the motor  $M$ ) and, after a predetermined interval of time, again two different solutions (syringes  $S'_1$  and  $S'_2$ , motor  $M'$ ).

0.28 sec, and this value was used in following calculations. Fig. 5 *a* shows one of the photograms obtained.

Later on, this device was improved in order to be able to neutralize the solution in the cuvettes of the spectrophotometer, at a given time after denaturation. Two other syringes were added,  $S'_1$  and  $S'_2$ , whose plungers were moved in synchronism by a second small electric motor  $M'$ . The needle tips touched the liquid surface. The sequence of operations was as follows. (*a*) The push-button started the motor  $M$  and released the sweep of the oscillograph. The syringes  $S_1$  and  $S_2$  injected an 0.2 M NaOH solution into the first cuvette and an 0.1 M NaCl solution into the second. The first DNA sample was thus denatured and the second diluted. Two microswitches started and stopped an electronic timer which measured the injection time. (*b*) The endrun microswitch stopped the motor  $M$  and started an electronic timer, which after  $\tau$  seconds started the motor  $M'$  ( $\tau$  could be varied at will). The syringes  $S'_1$  and  $S'_2$  injected a suitable HCl solution into the first cuvette and an NaCl solution into the second one in order to balance the dilution effect. In this way the pH of the first sample was again brought to 7.0. The second injection time could also be measured, if necessary, by another electronic timer. The second pair of syringes was equipped with multiple needles, in order to aid mixing. In Fig. 3 the two pairs of syringes with their motors are shown.

The DNA used in our experiments was either a bacterial DNA (extracted from *Escherichia*

*coli* and *Bacillus megaterium*) or a phage DNA (from phage T2 or  $\alpha$ ). Most work was done with the T2 phage DNA. The extraction methods adopted were those of Marmur (15) and of Mandell and Hershey (16). The extracted DNA was always tested for integrity and purity, some compromise between the two being usually unavoidable. The integrity tests were made with the Spinco model E Analytical Ultracentrifuge (Spinco Div., Beckman Instruments). The buoyant density of the DNA in CsCl density gradient was first determined. The profile of the band was carefully plotted. Generally, photography of the band and automatic tracing of the optical density of the plate with a Joyce microdensitometer (Joyce, Loebel and Co., Ltd., Gateshead-on-Tyne, England) was used. If necessary, the reading of the densitometer was automatically digitized and the shape of the curve examined with an IBM 7040 electronic computer. A gaussian shape was considered an essential, even if not a sufficient test for the presence of a single well characterized component in the extracted pool of DNA. The density of the maximum and the half-height width were taken as a first rough characterization of its type and state. More careful tests of the integrity of the DNA molecules were performed measuring the sedimentation coefficient of native, melted, and denatured DNA in suitable conditions. For instance, in the case of the T2 DNA the following measurements were usually made:

(a) Sedimentation coefficient of DNA in the native state (extrapolated to zero concentration). The requirement was a result in the 60–65 S range, corresponding to a molecular weight of  $120 \cdot 10^6$ , according of the empirical formula of Doty et al. (17).

(b) Sedimentation coefficient of DNA in the denatured state. A result of about 110 S was required, corresponding to molecular weight of  $\sim 32 \cdot 10^6$ , according to the empirical formula of Studier (11).

(c) Sedimentation coefficient of DNA in the melted state, at high pH, as suggested by R. L. Baldwin (private communication). Two components are to be seen, the first one at 68 S (corrected, for density and viscosity of the solvent) corresponding to the molecular weight of  $60 \cdot 10^6$  which is expected for unbroken strands. The second more diffuse one, at about 57 S corresponds to a mean molecular weight of about half that of the unbroken strands. A ratio of the two components between 30% and 70% was always obtained.

In conclusion, the T2 DNA used in our experiments was mainly made up of unbroken two stranded molecules, and a mean value of no more than one break per strand was tolerated.

The purity of the DNA used was guaranteed by the extraction procedures. Divalent metal ions were eliminated by operating in the presence of EDTA during purification of the phage suspensions, and during DNA extraction. The DNA solution, after treatment with ethyl-ether was dialyzed against an 0.1 M highly purified NaCl (or KCl) solution. The dialysis tube was washed repeatedly with EDTA solution and distilled water, no other treatment proving to be more efficient than this. Finally, the ratio of the absorption coefficient at 260 and 280  $m\mu$  was measured, for which a minimum of 1.80 was required.

## RISE-TIME OF THE HYPERCHROMIC EFFECT

The result obtained measuring the rise-time of the hyperchromic effect with the manual procedure described in section entitled "Hand-mixing" in Materials and Methods, for  $\alpha$  DNA and T2 DNA are plotted in Fig. 4. The pH of the DNA sample was suddenly raised to  $\varphi$  (the abscissa in Fig. 4) by mixing with alkali, and the actual absorption at 260  $m\mu$  measured as a function of time with a spectrophotometer. In the case of the  $\alpha$  DNA a Beckman model D.U. Spectrophotometer:

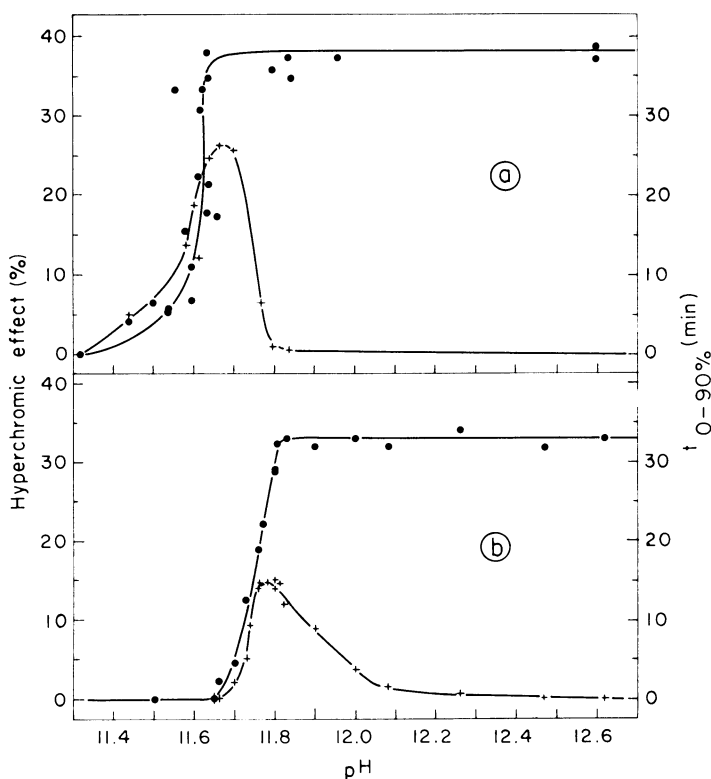


FIGURE 4 ●—● Alkaline transition curves for (a)  $\alpha$ DNA and (b) T2 DNA followed through the increase in optical density at 260  $m\mu$ . Hyperchromic effect, as percentage of native absorbance, is reported on the left scale; +—+ curve representing the time between addition of alkali and attainment of 90% of the hyperchromic effect at each pH. Time scale is on the right.

at room temperature was used, while in the case of T2 DNA a Cary model 15 Recording Spectrophotometer thermally regulated at 20°C was used; pH values have been measured with a Beckman Expandomatic pH-meter equipped with type E2 electrodes. For each  $\varphi$  value, the final hyperchromic effect and the corresponding rise-time (defined as the interval of time in which the hyperchromic effect passes from 0 to 90% of its final value) were read on the graph. The most striking feature of the plot is the rise-time peak corresponding roughly to the upper knee in the curve of the hyperchromic effect. It must be noted that on the left side of the peak the hyperchromic effect does not reach its maximum value and only partial denaturation of the DNA occurs. The upper part of the curve of the hyperchromic effect in the transition region goes up very steeply and it is difficult to decide if it really shows a sharp upper knee, or there is a region of instability. We can only say with certainty that above the pH at which all the DNA molecules of the sample reach complete



TABLE I  
RISE-TIME OF THE HYPERCHROMIC EFFECT

DNA	Observed rise-time T
	<i>sec</i>
T2	0.49
$\alpha$	0.50
<i>E. coli</i>	0.49
<i>B. megatherium</i>	0.49

denaturation, the rise-time decreases very rapidly with increasing pH, and very soon the manual procedure of mixing is no longer suitable.

In order to see how the denaturation process behaves at a higher pH, we have also made some sets of measurements at pH 12.5 with the automatic device previously described. Four different DNAs were tested, two of them from T2 and  $\alpha$  phages and two bacterial DNAs from *E. coli* and *B. megatherium*. The results are given in Table I.

They must be compared with the rise-time of the instrument of 0.28 sec and with the time of mixing which, as already stated, was always  $0.40 \pm 0.01$  sec. These two intervals of time and the actual rise-time  $T_0$  combine quadratically to give the observed rise time  $T$ . Thus we have:

$$\sqrt{(0.28)^2 + (0.40)^2 + T_0^2} = 0.49$$

from which we must conclude that, at pH 12.5,  $T_0$  is always less than 0.1 sec.

In Fig. 5, some photographs obtained with our automatic device are shown. The top value of the hyperchromic effect is always a little lower than 40% and, in all our experiments, is equal to the hyperchromic effect measured in the usual way on a sample of the same DNA after complete denaturation. Thus the observed hyperchromic effects are those corresponding to complete denaturation. However, our results must not be interpreted as meaning that in less than one tenth 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 a tenth of a second at most, and perhaps much shorter) the ordered arrangement of the bases in the two-stranded configuration of a DNA molecule 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, however, or bound by a small number of remaining bonds.

In conclusion, our measurements of the rise-time of the hyperchromic effect of DNA seem to indicate that the investigated pH range must be divided into four intervals in which the DNA shows different behaviours. For clarity, we refer in particular to the case of T2 DNA as shown in Fig. 4 b.

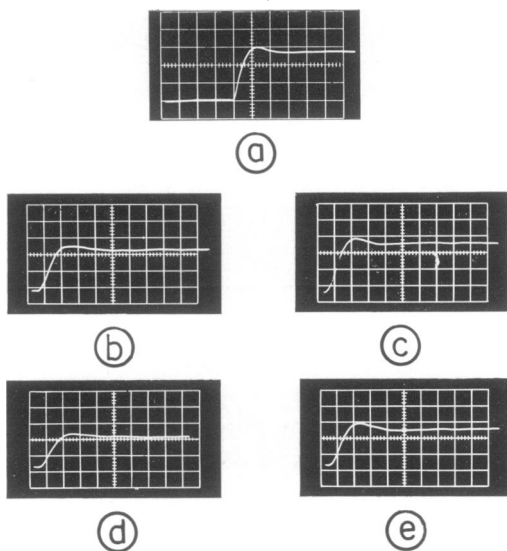


FIGURE 5 Oscillographic traces obtained with the automatic mixing device shown in Fig. 2. Time scale is in every case 0.5 sec/division. (a) Instrumental rise-time, due to the amplifier built into the spectrophotometer, measured by effecting a rapid OD variation of the same order as those of the following images. The small overshoot is also of instrumental origin; (b) rise-time of the hyperchromic effect for T2 DNA; (c) for  $\alpha$  DNA; (d) for *E. coli* and (e) for *B. megatherium* DNA.

(a) Below pH 11.65, no hyperchromic effect can be measured and the DNA remains, at least seemingly, in the native state.

(b) Between pH 11.65 and pH 11.8, the hyperchromic effect does not reach its maximum value. Its value increases with increasing pH, as well as its rise-time.

(c) Above 11.8, the hyperchromic effect reaches the total denaturation value. The rise-time decreases steeply with increasing pH and above 12.1 it can no longer be measured with our techniques. The interval 11.8  $\div$  12.1 seems to be considered an intermediate region.

(d) In the pH range above 12.1, the hyperchromic effect goes up almost instantaneously, as soon as the denaturation conditions are established. However, the unwinding of the two strands of the molecule is likely to be much slower than the breakage of the bonds responsible for the reduced UV-absorption of the bases.

Other experiments are necessary, in order to understand how denaturation proceeds in the different pH ranges here considered.

#### IRREVERSIBILITY OF THE HYPERCHROMIC EFFECT

The main question we want to investigate is the connection between the kinetics of the hyperchromic effect and the unwinding process of the two strands of the DNA

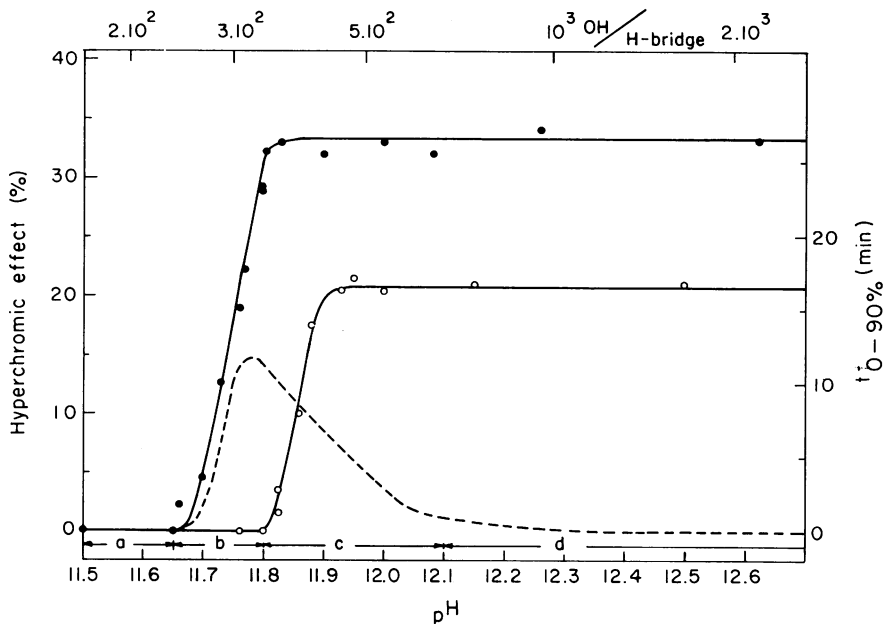


FIGURE 6 Phenomenology of the alkaline transition region for T2 DNA as described by the curves of full (●) and residual (○) hyperchromic effects (scale at left) and by the curve of time required to reach 90% of the full hyperchromic effect (dashed curve, scale at right). Four different regions are identified and indicated with *a*, *b*, *c*, and *d*. The upper scale reports for comparison the number of hydroxylions present in solution per hydrogen bridge in the native DNA molecule.

molecule. First of all we can ask if the hyperchromic effect is always accompanied by irreversible process of denaturation or if it is partially or totally reversible.

To this aim, the pH of a solution of native DNA of phage T2 was suddenly increased to a value  $\varphi$ . After a time  $\tau$ , the solution was brought back to pH 7 and the *residual hyperchromic effect* was determined. The experiment was repeated for different values of  $\varphi$  and  $\tau$  in the various range of pH, previously considered.

The results obtained in the pH region, in which the hyperchromic effect does not reach its maximum value (11.65 ÷ 11.8) and in the transition region (11.8 ÷ 12.1) are plotted in Fig. 6. In this case  $\varphi$  was made variable and  $\tau$  was equal to several minutes, that is long enough to allow the hyperchromic effect to reach its final value for every  $\varphi$ . The absorption of the sample at 260 m $\mu$  was recorded as a function of time with a Cary model 15 spectrophotometer and the absorption of the sample after neutralization was measured with the same instrument. For comparison the *direct hyperchromic effect* and its rise-time are also plotted in Fig. 6.

As is apparent, in the *b* region, the hyperchromic effect is completely reversible, its residual value after neutralization being zero. This result is easily interpreted if we assume that in this region an equilibrium is established between the alkaline

solution and the DNA molecules in which a number of secondary weak bonds between the bases are broken, but this number is not sufficient to cause strand separation. Careful examination of the spectrophotometric graphs showed that, when the pH was suddenly raised to a given value, a very rapid initial increase of the absorbing power of the sample (lasting no more than few seconds) was followed by a much slower variation. It may be that the initial increase was due to random breaks of bonds between neighbouring bases and that the following slower increase was due to an internal rearrangement of each molecule. In fact, the breaks are likely to form clusters, when equilibrium is reached, owing to the free energy of stacking stored between neighbouring bases (18).

In the *c* region (pH 11.8 ÷ 12.1) on the contrary, a residual irreversible hyperchromic effect builds up, and increases with increasing pH. At pH 11.95, the top value, corresponding to complete denaturation of the DNA, is reached, and at higher pH the hyperchromic effect is completely irreversible (*d* region). However, in this region, the adopted procedure gives no information on the denaturation process, owing to the very high value of  $\tau$ .

A new set of measurements was thus obtained in which, at fixed pH (12.5),  $\tau$  was varied between zero and 30 sec or more. In Fig. 7, typical results obtained for T2 DNA are shown. Owing to the manual mixing procedure adopted, the points corresponding to the first two seconds are not very reliable. Moreover,  $\tau$  is the time between the end of mixing with alkali and the injection of the neutralizing solution. This is why at  $\tau = 0$  the residual hyperchromic effect is not zero. The most striking aspect of the curve of Fig. 7 is the rise-time of the residual (irreversible) hyperchromic effect, about two orders of magnitude longer than the rise-time of the total hyperchromicity previously considered. In fact in the case of T2 DNA this rise-time is about twenty seconds and in the case of  $\alpha$  DNA it is nearly four seconds. Our values agree with those obtained by Davison (12).

These results can be easily interpreted if we take into account the double stranded structure of the DNA molecules. When the denaturation conditions at high pH are established suddenly, a first stage of the denaturation process follows in which most of the secondary bonds between the bases have been broken, but the two strands of the molecules are still in their place, so that for some time the process is completely reversible. As time goes on, the two strands separate and the process gradually becomes irreversible.

At high pH, the rupture of the bonds and the strand separation are thus two fairly distinct processes, while in the intermediate (*c*) region, most probably they are largely contemporary. It is likely that near the transition point, the rupture of the bonds determines the overall rate of the denaturation process while at extreme pH the unwinding time predominates.

However, it is not clear whether or not the rise-time of the irreversible hyperchromic effect is to be interpreted as the unwinding time of a single DNA-molecule. The previous results are in fact liable to two different interpretations. We can sup-

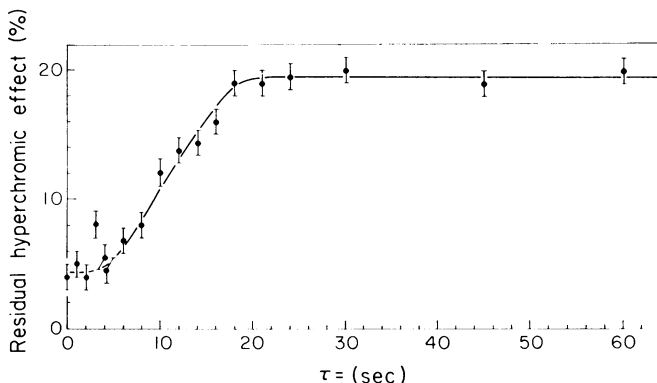


FIGURE 7 Residual hyperchromic effect for T2 DNA as a function of the time of exposure to pH 12.5.

pose that each DNA molecule is gradually unwinding, so that its residual absorption of light is gradually increasing with the time of exposure to the high pH. But we can also suppose that each molecule takes a short time to become denatured when the process of separation of the strands has begun, and that the number of denatured molecules is gradually increasing with the rise-time of the irreversible hyperchromic effect. These two different hypotheses correspond to different shapes of the curve of Fig. 7, but the accuracy of our measurements is not sufficient to differentiate between them and it seems necessary to make use of different techniques to single out the right interpretation. At this point we can only say that the following three characteristic intervals of time have to be considered as regards the process of alkaline denaturation of DNA.

1. The rise-time  $t_1$  of the total hyperchromic effect, which gives a measure of the melting rate of the intramolecular bonds in the actual physicochemical condition of the DNA solution. At high pH its value is less than 0.1 sec, while near the transition point it determines the much slower overall rate of the denaturation process.

2. The interval of time,  $t_2$ , required for strand separation in the case of a single DNA-molecule.

3. The rise-time,  $t_3$ , of the residual (irreversible) hyperchromic effect of the DNA, solution, which, at high pH, is at least two orders of magnitude greater than  $t_1$ .

Our problem was now to determine if  $t_2$  and  $t_3$  do coincide or not.

## STRAND SEPARATION OF THE DNA MOLECULES IN SOLUTION AT HIGH PH

### Results

The technique employed to study the process of strand separation in a DNA solution was the CsCl density gradient method with the analytical ultracentrifuge. The experiments were carried out as follows. A DNA solution was brought suddenly to

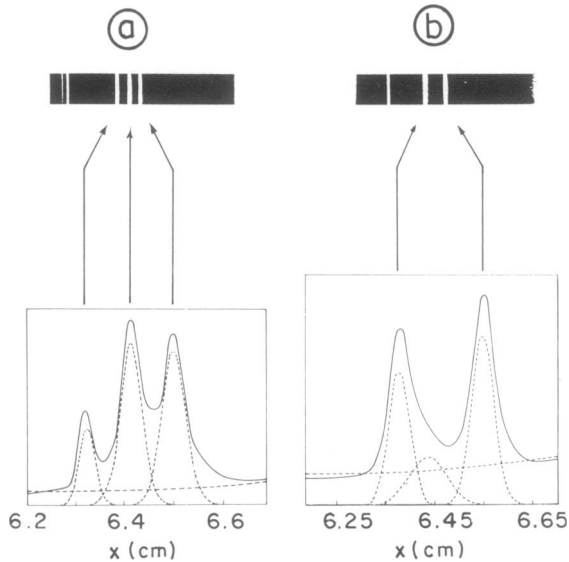


FIGURE 8 CsCl density gradient profile observed for partially denatured DNA (a) from phage  $\alpha$  ( $\tau = 1$  sec); (b) from phage T2 ( $\tau = 10$  sec), together with the results of the analysis in gaussian components. In the case of T2 DNA, a small amount of material of intermediate density is present.

a predetermined pH in the denaturation region at which it was kept for  $\tau$  (a few seconds, and then suddenly neutralized. After that, the buoyant density spectrum of the DNA was examined in a CsCl density gradient, making use of a Spinco model E Analytical Ultracentrifuge (Spinco Div., Beckman Instruments). As is wellknown, the native and the 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 molecules. A partially denatured molecule must therefore have an intermediate density between that of the native and that of the completely denatured DNA and we can easily foresee what kind of patterns are to be observed in the two different hypotheses previously considered. If  $t_2$  and  $t_3$  do coincide, that is if the unwinding time of a DNA molecule is also the rise time of the residual (irreversible) hyperchromic effect, in the CsCl density gradient a single band of DNA has to be observed, and its density must vary from that of the native to that of the denatured DNA, while the denaturing interval of time  $\tau$  is varied from zero to  $t_3$  ( $\sim 20$  sec in the case of T2 DNA). If, on the other hand,  $t_2$  is much shorter than  $t_3$ , that is if the molecules are denatured at random in the interval of time  $t_3$  and for each of them the transition from the native to the denatured state is almost instantaneous, two bands at a time should be observed in the CsCl density gradient. The first one must have the density of the native DNA and its intensity must decrease as  $\tau$  increases between 0 and  $t_3$ . The second one, at the density of the de-

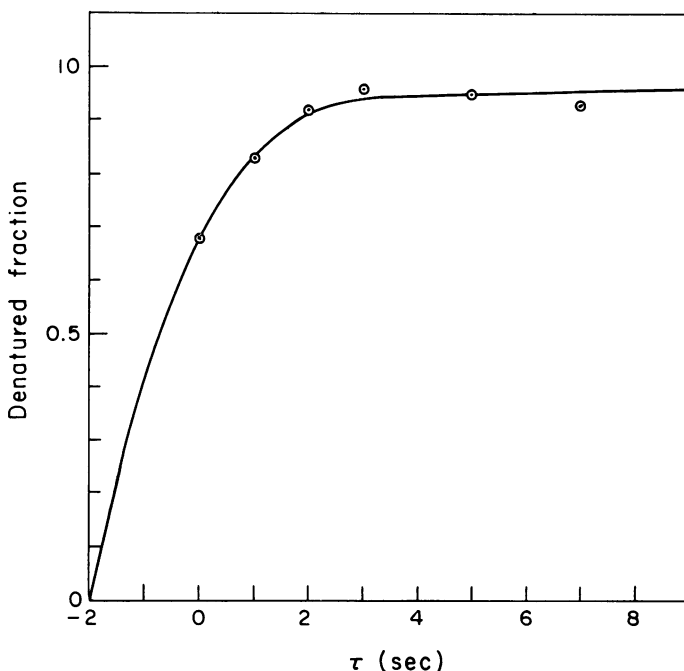


FIGURE 9 Percentage of  $\alpha$  DNA molecules which appear as totally denatured in CsCl density gradient, as a function of the time  $\tau$  of exposure to pH 12.5. The exponential curve fitted through the experimental points crosses the  $\tau$  axis 2 sec before the origin, this time corresponding to the sum of the mixing times.

natured DNA, must increase with increasing  $\tau$ , its intensity being zero at  $\tau = 0$ . A small number of molecules of intermediate density must be found between the two main bands, the total quantity of this material depending on the ratio of  $t_2$  to  $t_3$ .

A first set of experiments was performed at pH 12.5, at which the two processes of the rupture of the secondary intramolecular bonds and of the unwinding of the two strands of each molecule are presumably fairly distinct. The denaturation time  $\tau$  was varied between zero and thirty seconds and three different DNAs were used, one bacterial (*E. coli*) and two phage DNAs (T2 and  $\alpha$ ). The obtained results are shown in Fig. 8 and Figs. 9–11 and can be summarized as follows.

1. We have never observed any variation in the density of the native DNA band, for any value of  $\tau$ . However it becomes weaker and weaker as  $\tau$  increases, until the band disappears completely.

2. Even for the smallest values of  $\tau$ , a new band appears, its density being equal to that of the denatured DNA. This band does not change its position in the gradient either, but its intensity gradually increases with increasing  $\tau$ , until it becomes the only band which can be seen in the UV optical density pattern.

3. No consistent amount of DNA can be observed in our photographic patterns

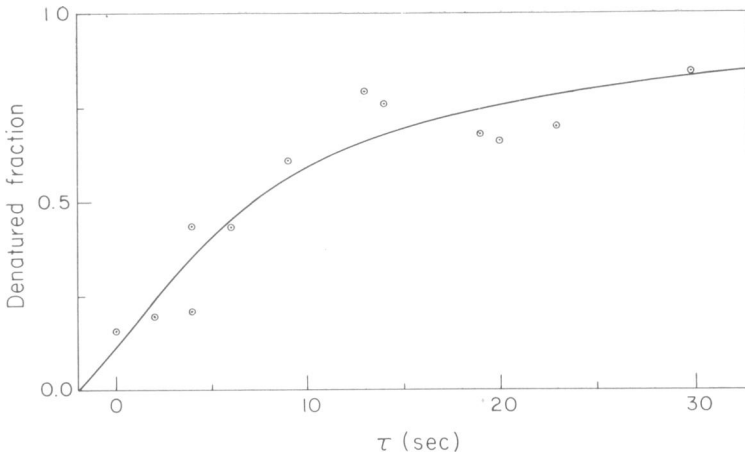


FIGURE 10 Percentage of *E. coli* DNA molecules which appear as totally denatured in CsCl density gradient, as a function of the time  $\tau$  of exposure to pH 12.5. The exponential curve fitted through the experimental points crosses the axis 2 sec before the origin. This value corresponds to the sum of the mixing times.

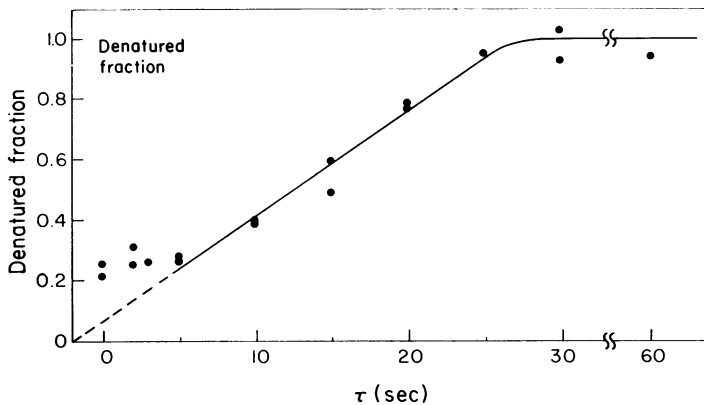


FIGURE 11 Percentage of T2 DNA molecules which appear as totally denatured in CsCl density gradient, as a function of the time  $\tau$  of exposure to pH 12.5. The curve fitted through the experimental points crosses the  $\tau$  axis 2 sec before the origin. This value corresponds to the sum of the mixing times.

of the density gradient between the two bands of the native and of the denatured DNA, in the case of *E. coli* and of phage  $\alpha$  DNA. In the case of phage T2 DNA, on the contrary, the UV optical density pattern can be interpreted by saying that between the two extreme gaussian bands a third weak and not strictly gaussian band is usually present. It is already present for the shortest values of  $\tau$  ( $\tau \simeq 0$ ) its intensity being independent from  $\tau$ . It disappears only after the native band has completely disappeared. Concerning this intermediate band, it is not clear whether, in the case of  $\alpha$  and *E. coli* DNA, it escaped observation, or is actually noticeable



only for a longer DNA. Control mixtures of native and denatured T2 DNA showed no material of intermediate density, so that an artifact due to aggregation of a single and a double stranded molecule should be ruled out.

In Fig. 8 two typical density gradient patterns of two partially denatured DNA samples are shown. The values of  $\tau$  were so selected as to have comparable amounts of DNA in the native and in the denatured states. Fig. 8 *a* refers to the  $\alpha$  DNA, with  $\tau = 1$  sec, Fig. 8 *b* to the T2 DNA with  $\tau = 10$  sec. It must be noted that the first photograph demonstrates the complete separation of the two strands of the molecules that have undergone denaturation during the interval of time  $\tau$ . In fact, the two strands of  $\alpha$  DNA have different bouyant density (19) and two distinct bands are observable in addition to that of the native  $\alpha$  DNA.

In Fig. 8 the corresponding densitometric plots are also shown, together with the results of the electronic computer analysis in gaussian components, a continuous background and an intermediate semidenatured distribution. A modified Orango program (a Montecarlo method followed by an iterative analytical procedure of approximation) was used (20). Finally, for each value of  $\tau$ , the areas of the gaussian bands were measured, and taking into account the difference in absorbance between native and denatured DNA the denatured fraction of molecules was calculated, and the graphs of Figs. 9–11 obtained. Obviously, this measurement is a rather indirect one and the resulting points are somewhat scattered. However, there is no doubt that the rise-time of the number of the totally denatured molecules is of the same order as the rise-time of the residual (irreversible) hyperchromicity and at least two orders of magnitude longer than  $t_1$ .

In conclusion, the experimental results seem to demonstrate that  $t_2$  (the time of unwinding of a single molecule) is much shorter than  $t_3$  (the rise-time of the irreversible hyperchromicity) and that strand separation is a very short random process in the solution, provided the transition pH is exceeded.

## DISCUSSION

This conclusion cannot be accepted without a very careful criticism of the experimental conditions and procedures previously described and a series of alternative explanations of the obtained results must be considered.

First of all, we must ask if the neutralization of the solution actually stops any denaturation or renaturation process of the DNA molecules. Although it is generally assumed that half denatured molecules rewind themselves completely when neutral pH is re-established, no direct demonstration of this phenomenon exists. It seems herefore legitimate to wonder whether under our conditions the effect of a very rapid neutralization is not more likely to be the formation of, imperfectly paired, short sequences. Such an effect would hinder rather than favour ordered rewinding.

One might suspect that after neutralization some half-denatured molecules complete their unwinding and the remaining ones rewind themselves completely. In this

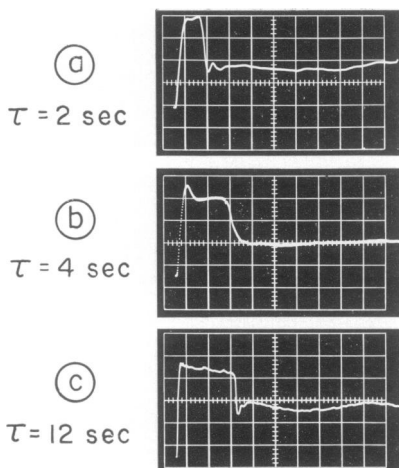


FIGURE 12 Oscillographic traces obtained for T2 DNA with the automatic mixing device shown in Fig. 3. The first increase corresponds to the injection of NaOH. The interval of time  $\tau$  after which an equivalent amount of HCl is added to neutralize, is indicated. Time scales are 2 sec/div. in (a) and (b); 5 sec/div. in (c). The decrease time of the hyperchromic effect is in every case as fast as the initial increase.

case, we cannot find any half-denatured molecule in the CsCl density gradient and the all or none aspect of the strand separation process would be an artifact, with no relation at all to the denaturation process.

This interpretation of the experimental results seems, however, quite artificial. One cannot see why some molecules must unwind and others must rewind themselves, whatever  $\tau$  may be, if during the denaturation time they are all gradually and regularly unwinding themselves. If for instance, for a certain value of  $\tau$ , after neutralization some molecules rewind and the other unwind completely, a small change in  $\tau$  must eliminate this all-or-none effect. In particular for very small value of  $\tau$  no denatured molecules at all should be found. We might suspect, however, that we are dealing with broken DNA molecules of every length and that the all-or-none effect may be due to the fact that the shorter the molecules are, the faster they reach the point at which after neutralization the unwinding process continues till strand separation. In this case the half-width of the bands in the CsCl density gradient would change in a characteristic way with increasing  $\tau$ , but, as we will see later, this is not the case. Moreover, there are other arguments against this interpretation of the experiments. Making use of our four syringe automatic mixing device, we have carefully examined how the absorption of the DNA solution at  $260\text{ m}\mu$  does change from neutralization onwards. In Fig. 12, some photographic records of the trace of the oscillograph are shown in which the absorption of the sample is plotted against the time. The two almost vertical edges of the square wave correspond to the injection of alkali and to the neutralization of the DNA solution. Its width is proportional to the denaturation time and its height to the total hyperchromic effect. The fact that the absorption falls in a time comparable with the rise-time whatever  $\tau$  may be, seems to demonstrate that the neutralization actually stops the unwinding or rewinding of the DNA molecules and that only the bonds between still neighbouring bases are restored almost immediately. From neutralization onwards the

trace is horizontal, apart from some occasional undulation due to imperfect mixing. In the first tens of seconds no variation in the absorption of the solution is detectable.

Actually, observing of the falling edge of the absorption wave is quite difficult. The injection rate of the neutralizing solution is critical and a sharp compromise must be made between imperfect mixing and shear degradation of the DNA molecules. However, even if the residual hyperchromic effect evaluated in this way is not completely reliable, (actually the residual hyperchromic effects measured in this way are higher than those reported in Fig. 7) the steepness of the fall is in any case very meaningful and no spurious effect seems to be able to increase it.

It was also verified experimentally that, when care is taken to work in rigorously controlled conditions, and particularly at constant temperature, the absorption of the DNA sample does not undergo any slow variation during an interval of time of several minutes after neutralization, and this for very different values of  $\tau$ . Thus, it seems difficult to avoid the conclusion that neutralization stops any process of denaturation or renaturation within a few tenths of a second.

At first glance these results seem to contradict what is generally accepted concerning the reversibility of denaturation, on the basis of the experiments carried out by E. P. Geiduschek (21, 22) on DNA thermally denatured. In effect, if the unwinding of the two strands is gradual, and the renaturation of partially denatured molecules easy and fast, we should be able to observe a decrease in the optical density over a period of time at least as long as  $\tau$ . Furthermore, in the CsCl density gradient only native molecules would be present for each value of  $\tau$  shorter than a specific critical value. The same would be true of the residual hyperchromic effect, so that the curves in Figs. 7 and 11 should have a characteristic step shape.

This contrast, however, is only an apparent one, since the experimental conditions here are quite different from those used by Geiduschek (21, 22). In particular, denaturing conditions are removed in a much shorter time than that required for the procedure of ice-quenching (6) as demonstrated in Fig. 12.

A second possible interpretation of our experimental results is that the density spectrum of the DNA sample is an artifact due to its staying in the CsCl solution for at least 24 hr, i.e., the time necessary for the formation of the density gradient in the ultracentrifuge.

To test this interpretation, a search for a very slow variation in the absorbance of a semidenatured DNA sample was made and the effect of the 8 M CsCl solution investigated. To this aim the optical density of partially denatured T2 DNA samples was recorded with a Cary model 15 spectrophotometer during more than 20 hr. CsCl was added to a final concentration 8 M at different times after neutralization, and the optical density further recorded. No variation with time of the hyperchromic effect was observed. The lowering of the hyperchromic effect caused by the addition of CsCl (calculated taking into account the volume variation of the sample) was found to be independent of DNA concentration and of time of addition and is to

be attributed to the more compact shape single stranded molecules assume in high ionic strength (11).

The behaviour of DNA in the CsCl density gradient was also tested directly in the analytical ultracentrifuge. It has already been mentioned that in the case of T2 DNA, a small quantity of material of intermediate density is present between the two bands of native and denatured DNA. A sample of half-denatured T2 DNA ( $\tau = 15$  sec) was examined in a CsCl density gradient and two photographic UV-records of the density spectrum were taken after 23 and after 50 hr of permanence of the sample in the ultracentrifuge. The densitometric readings of the two photographs could be brought to coincide exactly, the quantity of material of intermediate density not being appreciably changed during 27 hr. On the other hand, we have proved that the material of intermediate density can renature very easily, in the proper physico-chemical conditions. As an example, in Fig. 13 two density spectra of partially denatured T2 DNA are shown. Imperfect mixing caused an unusually large quantity of intermediate material, as is shown in (a). A sample of the partially denatured solution was then incubated for 7 min at 45°C. As it is shown in (b), this treatment was sufficient to cause complete disappearance of the partially denatured material. This experiment also shows that the material of intermediate density cannot be interpreted as due to partly double, partly single-stranded molecules resulting from single-strand breakages and partial denaturation. Such molecules would, in effect, renature more slowly than fully denatured ones, instead of being more efficiently renatured, as shown here.

Moreover, it is possible to show that artificially prepared half-denatured molecules can remain for more than 24 hr as such, in the CsCl density gradient. Results obtained by us with partially renatured DNA (23) as well as those reported by Subirana (24) demonstrate that half-renatured molecules (annealed either by thermal treatment, or by exposure to a suitable pH value) do remain as such in the CsCl density gradient. The rate of renaturation measured at room temperature, even in the presence of 8 M CsCl, is too slow to cause noticeable reannealing of this material.

This line of evidence is not really conclusive, since half-renatured material may possess a structure different from that of the half-denatured material yielded in the course of alkaline denaturation. This latter material may be more ordered than the former (although it is likely that on neutralizing so fast wrong complementary sequences—either inter or intrastrand—are formed by chance), and renaturation may then proceed at a much faster rate. The experiment illustrated in Figure 13, however, shows that even in this latter case renaturation is not instantaneous, but requires several minutes under the proper annealing conditions, and that 24 hr in CsCl are by no means sufficient to cause renaturation.

In conclusion, also the CsCl artifact hypothesis seems untenable.

A third possible interpretation of our all-or-none effect starts from the consideration that a single DNA strand is much weaker than a double-stranded molecule.

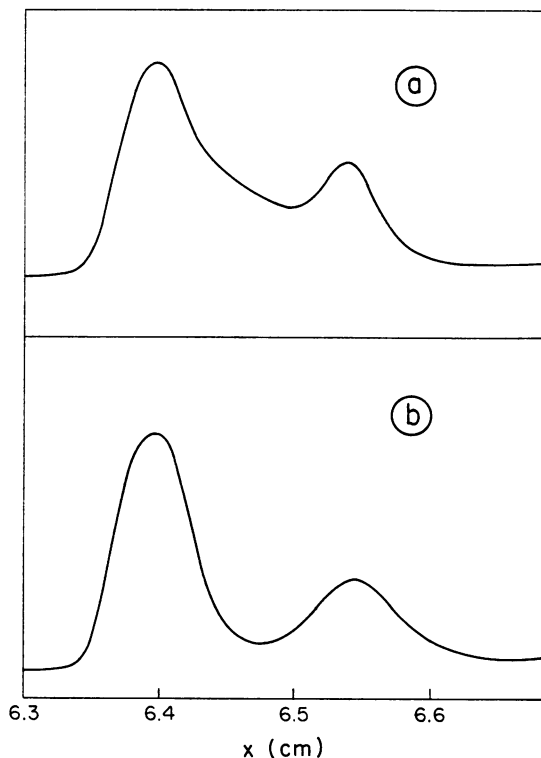


FIGURE 13 (a) Partially denatured T2 DNA. (b) The same after a very mild renaturation treatment. (7 min at 45°C, at 5  $\mu\text{g/ml}$ ). The intermediate material has completely disappeared.

When the DNA solution, after partial denaturation, is neutralized, segments of single-stranded DNA can be wrenched out of partially unwound molecules. For instance, if we assume that the unwinding precedes regularly from the free ends of a molecule, it may be that the branching points are subject to particularly intense stresses and in these points the DNA will most probably break. In this case we should observe in the CsCl density gradient a band in the position of denatured DNA, even if no molecule has effectively reached strand separation. If, moreover, the remaining unwound double-stranded segments would renature quickly, we should observe only another band in the position of the native DNA, and the all-or-none effect would be explained.

At first sight, this interpretation seems most plausible. However, it can be easily tested experimentally. In fact, the mean molecular weight of the native fraction would decrease with increasing  $\tau$  and that of the denatured fraction would increase. Both the half width of the gaussian band in CsCl density gradient, and the sedimentation coefficient depend on the molecular weight.

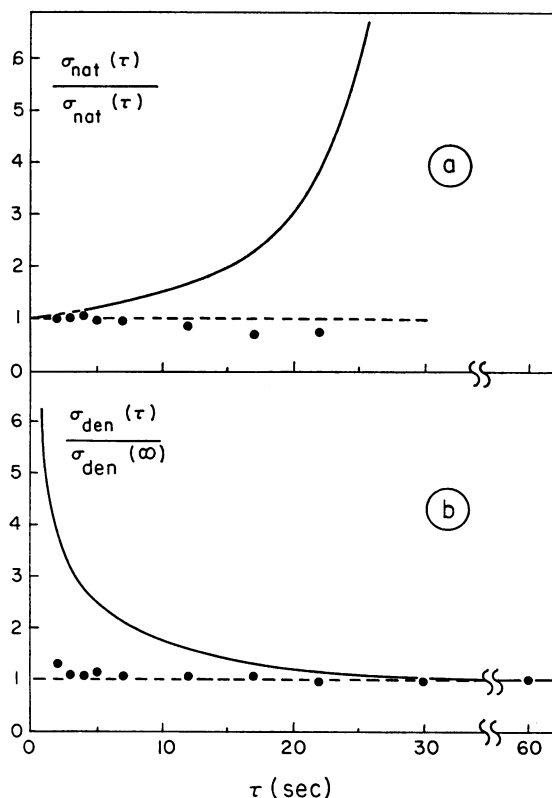


FIGURE 14 ----- standard deviations of the gaussian components obtained by the electronic computer analysis for partially denatured T2 DNA as a function of  $\tau$  (time of exposure to pH 12.5). The width of the native band (a) is relative to the width of undenatured DNA, the width of the denatured band (b) is relative to the width of totally denatured DNA. The continuous curves (—) give the variation of the bandwidths which would be expected under the hypothesis of simultaneous unwinding of the molecules at a constant rate, accompanied by breakage at the bifurcation point, assuming that the molecular weight is inversely proportional to  $\sigma^2$ .

The half widths of the gaussian bands were measured on the relevant densitometric records, and the results plotted in Fig. 14. All the experimental points are compatible with a horizontal straight line, and no indication of the foreseen variation with  $\tau$  was obtained. To give an idea of the disagreement, in each figure a curve calculated for a DNA in which the unwinding proceeds with constant speed and goes to an end in 30 sec was reported. Obviously, this is not a very realistic hypothesis, but it is presented only for comparison.

Since CsCl bandwidths are not very reliable with regard to the molecular weight, the sedimentation coefficient of the native and denatured fractions were also measured as functions of  $\tau$  and the results are plotted in Figure 15. Here also the continuous curves are calculated only for comparison, in the case of a constant unwind-

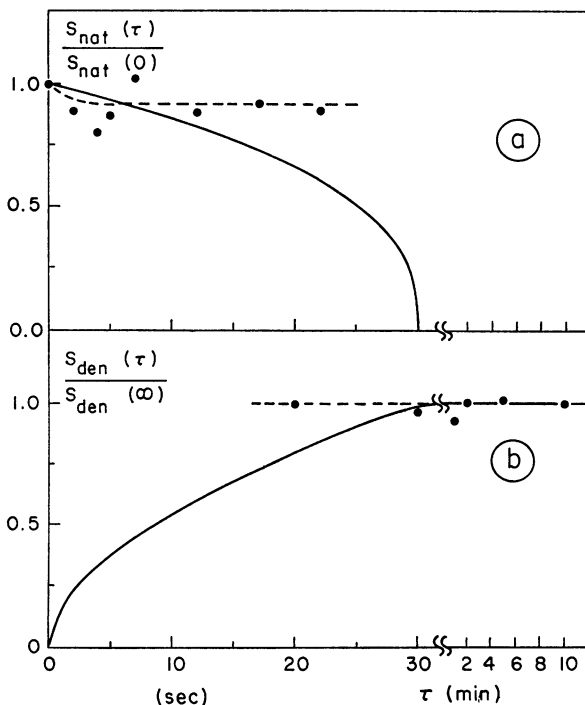


FIGURE 15 ---- sedimentation coefficient of the components (*a*, native; *b*, denatured) observed in partially denatured T2 DNA, as a function of  $\tau$  ( $S_{\text{nat}}(0) = 65 \text{ S}$ ;  $S_{\text{den}}(\infty) = 110 \text{ S}$ ). The continuous curves (—) give the relative variation which should be observed in sedimentation coefficients under the hypothesis of simultaneous unwinding of the molecules at a constant rate, accompanied by breakage at the bifurcation point, assuming that the molecular weight is proportional to  $S^{1/0.37}$  (17).

ing speed and a strand separation time of 30 sec. Also, in this case the disagreement with the fragmentation hypothesis is complete, and, in conclusion this hypothesis also seems to be untenable.

#### STRAND SEPARATION IN THE TRANSITION REGION AND THE DEPENDENCE ON THE IONIC CONCENTRATION

If we assume that the all-or-none effect in the alkaline denaturation of DNA must be interpreted in the sense that the time  $t_2$  required by a single DNA molecule for strand separation is much shorter than the rise time  $t_3$  of the residual (irreversible) hyperchromic effect, we can evaluate  $t_2$  from the fraction of material of intermediate density between that of the native and that of denatured DNA, observed in CsCl density gradient. As we have already said, this fraction is very small even in the case of the T2 DNA, whose molecular weight is as high as  $120 \cdot 10^6$  dalton. Thus we cannot make a measurement of  $t_2$ , but only a very rough guess of its order of mag-

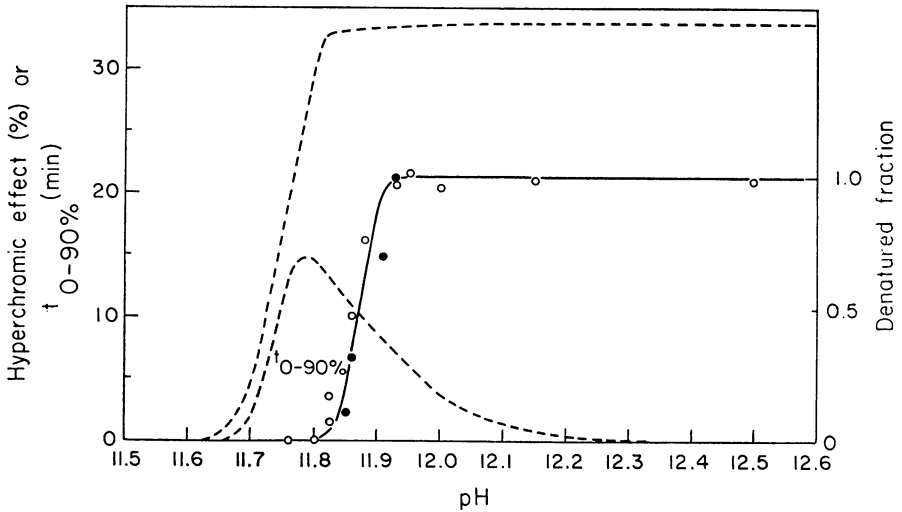


FIGURE 16 On the same graph of Fig. 6 are reported here as black dots (●) the percentages of T2 DNA molecules which appear totally denatured in CsCl density gradient. (Scale at right.)

nitide. In the case of T2, the result is  $t_2 \sim 1$  sec, at pH 12.5, and, as it is obvious, it depends on the correctness of our general interpretation.

Furthermore, the occurrence of single strand breakages, which is almost unavoidable (an average of one break per strand is already initially present, see Materials and Methods) may effect the absolute values of  $t_2$  and  $t_3$ , which are therefore given as rough estimates. Still, we believe that such an effect cannot change the general picture of the process.

Let us now consider what happens in the transition region. Referring to Fig. 6, we have seen that the phenomenology in the transition region of pH is complex. We meet first a region (*b* region) in which the hyperchromic effect and its rise-time are both gradually increasing with increasing pH, the process being totally reversible. In the following region (*c* region), the rise-time of the hyperchromic effect falls and the process becomes gradually irreversible. What about strand separation in these two regions?

The same samples of DNA with which the points in Fig. 6 were obtained, were also examined at the ultracentrifuge in the CsCl density gradient. As expected, no strand separation at all was found in the *b* region, where the process is completely reversible. In the *c* region, on the contrary, the all-or-none effect already studied in the *d* region, is present and the relative amount of totally denatured DNA increases in practically the same way as the irreversible hyperchromic effect. The black points in Fig. 16 represent the denatured fractions deduced in the usual way from the UV-photographic records of the CsCl density gradient.

At this point, we are able to present the following tentative description of the



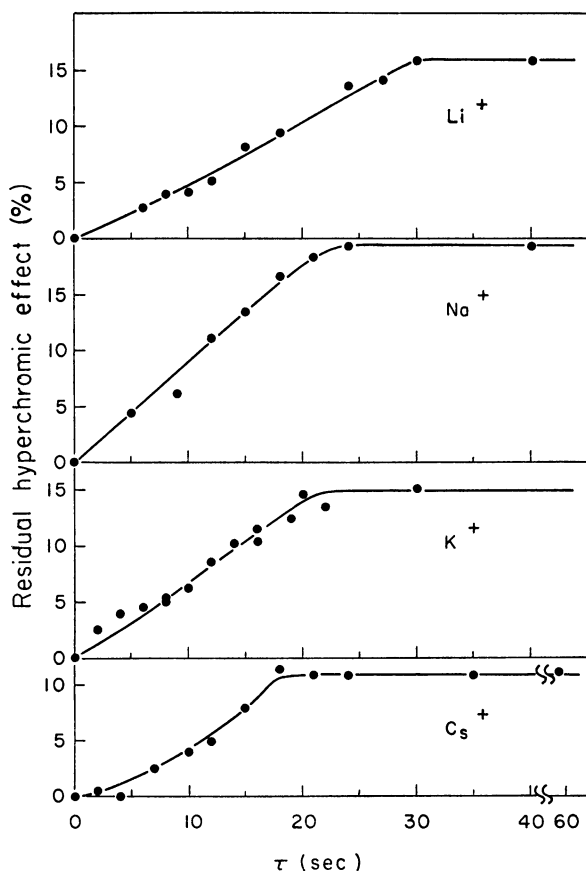


FIGURE 17 The effect of the type of positive ion present in solution (at a concentration of 0.1 M) on the rise-time of the residual hyperchromic effect of T2 DNA.

whole process of alkaline denaturation of DNA. It obviously refers to the particular physico-chemical conditions of our experiments, but the general features are most probably the same in a large number of different environments. If the pH of a diluted DNA solution is gradually increased, there is a fairly defined point at which the absorbance at  $260\text{ m}\mu$  begins to increase and over a small range of pH values it becomes maximum. In this interval, an equilibrium is established in which a fraction of the secondary intramolecular bonds is broken. This fraction increases with increasing pH, as well as the time required to reach the equilibrium. The limiting factor seems to be the number of available  $\text{OH}^-$  ions, which in this region is not sufficient to permit strand separation. If however the pH is further increased, there is a second fairly well defined point at which strand separation becomes possible. It must be remembered that in each DNA molecule the secondary intramolecular bonds are already almost all broken, but strand separation occurs in some mole-

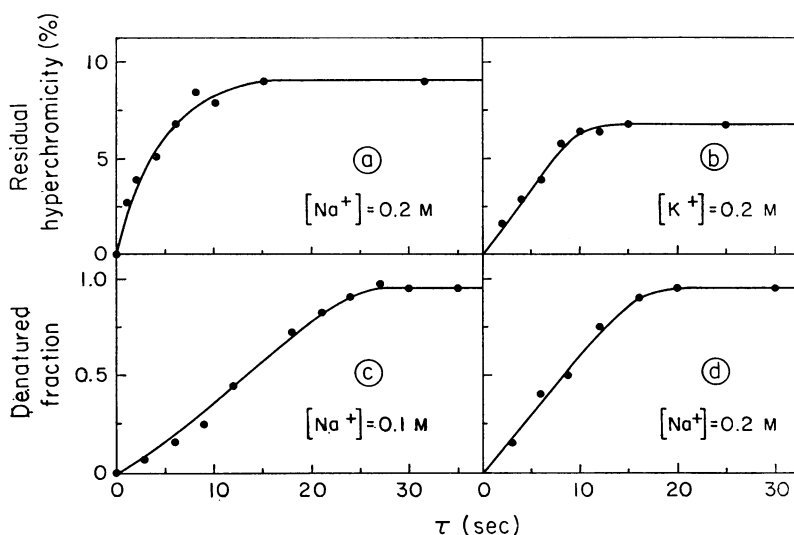


FIGURE 18 The effect of the concentration of the positive ion present in solution on the rise-time of the residual hyperchromic effect of T2 DNA (*a* and *b*), and on the rise-time of the denatured fraction observed in CsCl density gradient (*c* and *d*).

cules, only if the pH exceeds a critical value. Here, too, the limiting factor in the number of molecules which have undergone strand separation seems to be the number of  $\text{OH}^-$  ions above that of the critical value of pH. If the pH is further increased, all the molecules reach the complete denatured state, not all at the same time however, but randomly at a rate which depends on  $\text{OH}^-$  concentration. At very high pH (*d* region) the rise-time of bulk denaturation becomes very short.

The solution which undergoes denaturation contains other ions different from hydroxyl-ions, i.e. positive metal ions, generally  $\text{Na}^+$ , at least equal in number to the  $\text{OH}^-$  ions, and also some negative ions other than  $\text{OH}^-$ , such as  $\text{Cl}^-$ . What is the role of these ions? We must expect that both the nature of the positive ions and their concentration influence the time  $t_8$  of bulk denaturation. Experiments were made to see if this prediction was correct. The residual hyperchromic effect of a solution of the Na-salt of T2 DNA was measured as a function of the denaturation time  $\tau$ , in the usual way, using each of the following bases to prepare denaturing solutions at pH 12.5: LiOH, NaOH, KOH, CsOH. In this way, about 10% of the total number of the positive ions was  $\text{Na}^+$  in every case. The K-salt of the T2 DNA was also prepared and tested with similar results. Thus, we think that the presence of 10% of  $\text{Na}^+$  ions is not very important. Some anomalous results were obtained when the initial DNA concentration, before addition to the alkaline solution was rather high and the ionic force of the latter rather low. The residual hyperchromic effect as a function of the denaturation time showed two well separated steps and we were able to show that this rather unusual aspect of the curve was essentially

due to aggregates of DNA molecules. This phenomenon will be discussed at length elsewhere. In Fig. 17 the results obtained with an initial DNA concentration of 150  $\mu\text{g/ml}$  (to be diluted 10 times in the denaturing solution) are shown, for LiOH, NaOH, KOH and CsOH. The final concentration of positive ions was in the four cases 0.1 M. It is evident that the bigger the positive ion, the steeper the bulk denaturation process. Similar curves with a final concentrations of  $\text{Na}^+$  and  $\text{K}^+$  0.2 M are shown in Fig. 18 *a* and *b*. As the ionic force increases the rise-time of the irreversible hyperchromic effect becomes shorter. In Fig. 18 *c* and *d*, similar results were obtained, measuring in a CsCl density gradient the fraction of denatured molecules as a function of  $\tau$  for  $\text{Na}^+$  concentrations equal to 0.1 and 0.2 M. It must be said that in many cases we have measured a rise-time of the fraction of denatured molecules a little longer than that of the irreversible hyperchromic effect of the same DNA solution. This point however was not sufficiently investigated and we cannot say if the effect is real.

In conclusion, an effect of the positive ions on the bulk denaturation time of a DNA solution was put in evidence, this time becoming shorter as the positive ion concentration was increased and as the ion radius increased.

#### ENERGY CONSIDERATIONS. THE DEPROTONATION MODEL

All the previous results must now be interpreted in the framework of the molecular model of Crick and Watson. At first sight, no difficulty arises from the qualitative point of view, but it does not seem to be easy to understand the mechanics of the denaturation process.

Even Watson and Crick in one of their classical papers on the structure of the DNA molecule, have pointed out the necessity of a very careful examination of the mechanical aspects of strand separation and duplication of the molecule (25). The question is that the unwinding movement in a viscous medium wastes energy, and the total energy dissipation depends on the rate of unwinding. Several years ago, Levinthal and Crane (26) evaluated the total energy requirement, making use of the data then available on the molecular weight of DNA and on the time required for strand separation. The result was not out of keeping with the energy set free by the triphosphates of the bases, during the process of DNA duplication, and the question appeared to be settled.

However, the results discussed in this paper, obtained with DNA of known molecular weight, much higher than that considered by Levinthal and Crane, open the question again. We have found that at pH 12.5 the total unwinding of a T2 DNA molecule requires about one second. If we use the formula of Levinthal and Crane to give a very rough estimation of the energy required by a DNA molecule of  $120 \cdot 10^6$  dalton to unwind in 1 sec, the result is about  $1.2 \cdot 10^{19}$  cal/rev, that is one thousand times higher than the previously considered value.

Now the question arises, "what is the energy source that makes strand separation

energetically possible in the alkaline medium?" Obviously, no energy rich molecule is available in the solution, which contains only water,  $\text{OH}^-$ ,  $\text{Na}^+$  (and some unpredictable impurities). Thus, we have no alternative. Only the recombination of hydroxylions with the hydrogen atoms of the H-bridges between bases to form water, can be considered as a likely energy source. And, in fact, it is so, since the energy set free ( $13.5 \text{ kcal mole}^{-1}$ ) is sufficiently higher than the mean binding energy of one proton of the H-bridges. Assuming that the difference between the two is of the order of  $10 \text{ kcal/mole}$ , and considering that there is an average of 24 H-bridges per turn of the helix in T2 DNA, we can estimate to about  $4 \cdot 10^{-19} \text{ cal/rev}$  the energy available for the rotation of the molecule. This value compares favourably with the energy requirement ( $1.2 \cdot 10^{-19} \text{ cal/rev}$ ).

These energy considerations allow us to discuss one more alternative hypothesis which can be put forward to explain our results. According to this hypothesis the initial rapid melting, occurring in a time  $t_1$ , proceeds by unwinding the double helix. Unwinding therefore starts rapidly in all molecules, but there is one final step, slow and variable, which is responsible for the time  $t_2$  required for irreversible denaturation. The molecules which still possess a nucleus for rewinding, rewind rapidly on re-neutralization.

In order to be compatible with all our results, such a hypothesis requires that both unwinding and rewinding take place in less than  $0.1 \text{ sec}$  (a figure which was given as an upper limit for  $t_1$ ). The energy requirement then increases by more than a factor of ten, and it becomes very difficult to think of a possible energy source.

This conclusion agrees fairly well with the description of the alkaline denaturation process we have given in the previous paragraph. It seems possible that the hyperchromic effect is in part a consequence of deprotonation as suggested by the spectral difference shown in Fig. 1. The high charge density in the interior of the helix will probably prohibit stacking of the bases. The following considerations explain how one can envisage a hyperchromic state in which DNA is not unwound.

When a proton of a H-bridge is extracted from the DNA molecule and a water molecule is formed with a hydroxyl-ion of the solution, a fairly high part of the energy set free remains stored in the DNA molecule, as repulsive electrostatic energy, between the electrons of the shoulders of the destroyed bridge. As the deprotonation proceeds further, the molecule even more resembles a charged spring. However, one must not forget the presence in the solution of the positive ions, in number at least equal to that of the hydroxyl-ions. They tend to shield one deprotonated site from the neighbouring ones, so that the work of extraction of a proton from the molecule does not increase as the deprotonation proceeds. Although they cannot substitute the protons, they lower the electrostatic repulsion between the two halves of the molecule, so that their presence tends to stabilize the DNA. This is a well-known effect, depending on which the melting point increases with increasing ionic strength.

A deprotonated molecule, in which each deprotonated site is surrounded by a

cloud of positive ions, is probably a case of unstable equilibrium. Occasionally, in one place, the two mutually repelling shoulders of the destroyed bridge can be pushed back, and a positive ion can take the place of the dismissed proton. In this case, two consequences follow. The first is that the two relevant bases no longer interact strongly, because the positive ion cannot substitute the proton in the bridge. The second is that, if the ion is much bigger than the proton, the molecule will be highly deformed in the surrounding area. Consequently in the neighbouring deprotonated sites, the two repelling shoulders are pushed back, and a second cation can take the place of the next dismissed proton, and so on. Thus strand separation begins and is more likely to go quickly to completion if the positive ions are heavier. This model seems to be able to explain not only the all-or-none effect we have observed, but also the fact that if the positive ion concentration increases, the rise-time of the irreversible hyperchromic effect is lowered, because the probability that one of them can take the place of a dismissed proton increases, (although different hypotheses are possible, for example an effect of the positive ion on the viscosity of the DNA solution, or on the transition pH).

To further test our model, the deprotonation process was demonstrated directly, by measuring the drop in pH of an NaOH solution due to the addition of a given amount of DNA. In fact, the proton capture must be accompanied by the disappearance of an equal number of hydroxyl-ions, with the formation of water molecule.

In order to measure the drop in pH below the value of the corresponding blank, it is advisable to raise the DNA concentration as much as possible. However, it is necessary to use relatively large volumes of solution ( $\sim 30$  ml) of not too high a viscosity, in order to facilitate mixing, and this, in practice, limits the concentration to about  $100 \mu\text{g/ml}$ .

Under these conditions the drop in pH turned out to be rather small, of the order of a few hundredths of one pH unit in the transition region (between pH 11.8 and 12.0). The measurements were performed at  $20.0^\circ\text{C}$  with a Beckman Research pH-meter, accurate to 0.001 pH units, and equipped with a glass electrode suited to high pH values. The precision limit depends on the manual operations of dilution and mixing, which could be reproduced only within 0.01 pH units.

In order to work at a constant  $\text{Na}^+$  concentration, the denaturing solution for every pH value was prepared by mixing solutions of NaCl 0.1 M and NaOH 0.1 M in suitable proportions. To 26 ml of this solution, 4 ml of a concentrated DNA solution were added, and mixed for 60 sec. Readings were taken at intervals of 3–5 min over a period of about 40 min, and extrapolated back to zero time, in order to compensate for the drift in pH due to the  $\text{CO}_2$  present in the air. The difference in pH between blank and sample can be easily converted into the number of hydroxyl-ions which disappeared during the deprotonation process. The final concentration of DNA was deduced from the optical density of the solution at  $260 \text{ m}\mu$ .

In Fig. 19 the results obtained as a function of pH are given and compared (upper

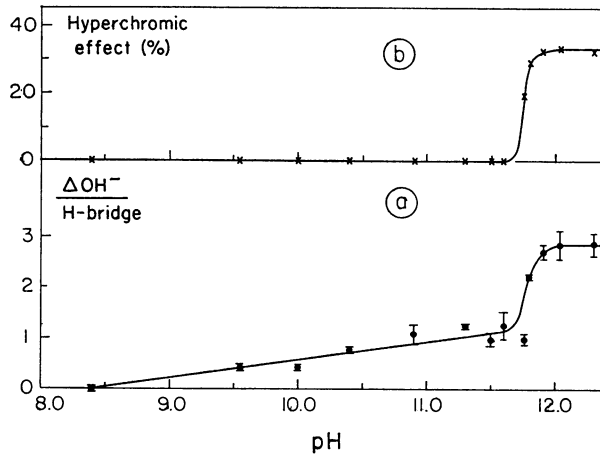


FIGURE 19 (a) Number of  $\text{OH}^-$  ions lost per hydrogen bridge of added DNA, as a function of the pH of the solution, measured before addition. The abrupt increase in  $\text{OH}^-$  trapping corresponds to the alkaline transition, shown in (b), where the hyperchromic effect is plotted vs. the same abscissa.

part of the figure) with the hyperchromic effect of the same solution of DNA at the corresponding pH. Between pH 8.4 and 11.6 there is a slow almost linear increase in the number of hydroxyl-ions lost per H-bridge of the DNA. This regular increase can be thought of as due to the titration of the phosphate groups, and perhaps partially to impurities of the solution. The most interesting feature of the curve however is the abrupt increase in the number of trapped  $\text{OH}^-$  ions, just in the region of the alkaline transition. There is little doubt that this increase corresponds to the capture of the protons bridging complementary strands. The results confirm the well known titration curve of Gulland, Jordan and Taylor (27) obtained with DNA samples and under experimental conditions quite different from those we can obtain today. The total size of the jump (1.6  $\text{OH}^-/\text{H-bridge}$ ) appears to be larger than expected. However the statistical errors are quite large and, furthermore, it is conceivable that, as a result of the opening of the double stranded structure, other hydrogen atoms in the molecule become suddenly accessible and may be captured by the same mechanism.

In conclusion, it seems to us that the deprotonation model here proposed is directly confirmed by experiment and that all our results have a place in the framework of the well known Crick and Watson model of the DNA molecule.

Even if the alkaline denaturation process seems to have no more bearing than thermal denaturation, on what happens in the cell when DNA is duplicated or its information copied, it does seem possible that partial unwinding of the double stranded structure is produced by some proton acceptor molecule present in the cell in the right place, at the right moment. This process would be much more similar

to the alkaline denaturation here studied than to the thermal denaturation process, even if neither high temperature nor extreme value of pH can exist in the cell.

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