Volume 9 Number 16 1981 Nucleic Acids Research

A study of the reversibility of helix-coil transition in DNA

M.P.Perelroyzen*, V.I.Lyamichev, Yu.A.Kalambet, Yu.L.Lyubchenko and A.V.Vologodskii+

Institute of Molecular Genetics, USSR Academy of Sciences, Kurchatov Sq. 46, Moscow 123182, and *Novosibirsk Institute of Organic Chemistry, USSR Academy of Sciences (Siberian Branch), Prospect Nauki 9, Novosibirsk-90, 630090, USSR

Received 1 July 1981

ABSTRACT

The reversibility of DNA melting has been thoroughly investigated at different ionic strengths. We concentrated on those stages of the process that do not involve a complete separation of the strands of the double helix. The differential melting curves of pBR 322 DNA and a fragment of T7 phage DNA in a buffer containing 0.02M Na^+ have been shown to differ substantially from the differential curves of renaturation. Electron-microscopic mapping of pBR 322 DNA at different degrees of unwinding (by a previously elaborated technique) has shown that the irreversibility of melting under real experimental conditions is connected with the stage of forming new helical regions during renaturation. In a buffer containing 0.2M Na^+ the melting curves of the DNAs used (pBR 322, a fragment of T7 phage DNA, a fragment of phage λ DNA, a fragment of ØX174 phage DNA) coincide with the renaturation curves, i.e. the process is equilibrium. We have carried out a semi-quantitative analysis of the emergence of irreversibility in the melting of a double helix. The problem of comparing theoretical and experimental melting curves is discussed.

INTRODUCTION

Recent progress in DNA sequencing has stimulated a detailed investigation of the helix-coil transitions in DNA molecules. The first comparisons of experimental and theoretical differential melting curves of DNA (1-3) demonstrated a reasonable agreement between theory and experiment in a number of cases. With some DNA fragments, however, the theoretical and experimental profiles of the differential melting curves were essentially distinct. It was hypothesized in (2) that the distinction might be due to the heterogeneity of the stacking interaction of base pairs in the double helix. Special analysis showed that this effect, though small, could

cause appreciable changes in the profile of the differential melting curves. Meanwhile the heterogeneity of a stacking interaction could hardly account for the findings of Vizard et al. (4). These authors compared the differential melting curves of ØX174 phage DNA at two ionic strengths. The comparison showed that a change of ionic strength could considerably affect the experimental melting curves. By contrast, the effect of the change on the parameters of the theoretical model was negligible, so that the theoretical melting curves were practically identical in the two cases. This led the authors to doubt the adequacy of the theoretical model. On the other hand, the situation demanded that all the requirements underlying the theoretical model of the helix-coil transition should be met in the experiment. The most important of these requirements was that the process should be equilibrium. The criterion of equilibrium in a real experiment could be the concurrence of the differential curves of DNA melting and renaturation, provided the temperature changed at the same rate in both cases. There was no such control of reversibility in the quoted experiments.

Kinetic studies of DNA reassociation /e.g.(5)/show that this is an extremely slow process in the melting range. Therefore the melting of the entire double helix involving a complete separation of the complementary strands is not reversible under real experimental conditions (5). For long enough DNA fragments ($\gtrsim 10^3$bp), however, the major part of the melting process does not involve a complete separation of the strands, and here one may expect the process to be reversible. It is this particular reversibility of intramolecular melting that will be discussed below.

The differential curves of DNA melting were shown in the early studies of Yabuki et al. (6) and Michel (7) to differ from the renaturation curves at a low ionic strength. However this difference was, at least partly, due to the accumulation of single-stranded breaks in DNA after prolonged incubation at a high temperature. Naturally the breaks may lead to an early dissociation of the damaged fragments and cause the melting process to be non-equilibrium. Since a DNA molecule accumulates single-stranded breaks at a rate that is at least propor-

tional to its length, it is desirable that the reversibility of melting should be investigated in fragments no longer than several thousand base pairs. Furthermore, DNA melting may be made irreversible by a number of other factors that defy control. On the other hand, a demonstration of reversibility at least for a part of the melting process is a good control of the sample's quality. Such a demonstration was performed in (8) for a sample of ColE1 DNA at an ionic strength of 0.02M Na^+ . Wada et al. have shown that only the initial part of the melting process, corresponding to the first peaks in the differential curve of ColE1 DNA melting, is reversible. Unfortunately the paper presents no data for other DNAs or ionic conditions, while other people have found the time of equilibration in DNA melting to depend on the ionic strength (5,9).

A qualitative model for the emergence of irreversibility was proposed by Hoff and Roos (9) and discussed at length by Michel (7). According to this model, irreversibility may emerge when a new helical region is formed in the process of renaturation. This is diagrammatically shown in Figure 1. Renaturation is in this case analogous to reassociation of the strands and has a low rate constant because of the powerful electrostatic

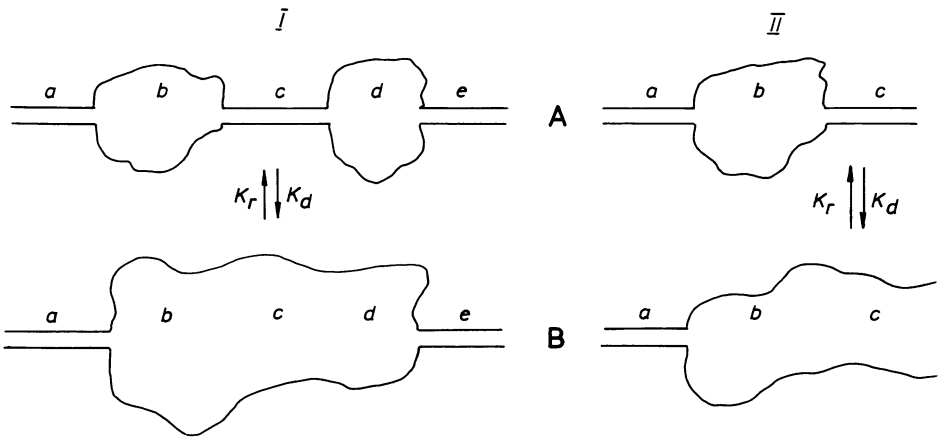

Fig. 1. Diagram of the renaturation stage at which a new helical region is formed. A helical region may form between two open regions (I) as well as at the end of the molecule (II)

repulsion of the strands at a low ionic strength. If renaturation does not involve forming additional helical regions but occurs through extending for existing ones, the melting process should be reversible.

As early as 1969 Spatz and Crothers (10) investigated the kinetics of DNA unwinding and discovered a process with a long relaxation time. Their analysis led them to suppose that the process involved co-operative melting out of regions flanked by melted areas. Hoff and Roos's model (9) for the emergence of irreversibility in DNA melting was largely based on these results. It was supported by the studies (8) and (11). Wada et al. (8) investigated the reversibility of ColE1 DNA melting in a 0.1xSSC buffer and Borovik et al. (11) obtained electron-microscopic maps of denaturation corresponding to different stages in the melting of that DNA.

Thus, over the last decade increasing evidence has been obtained to the effect that under some conditions the melting process may be irreversible even without the complete separation of strands. However the available data are disultory. This study is an attempt at a systematic investigation of the problem using thoroughly characterized DNA fragments and a recently proposed method (11) for visualizing the melting process. Our objective was also to find the conditions under which the process is equilibrium, i.e. the conditions which would allow a comparison of theory and experiment.

MATERIALS AND METHODS

1.DNA. The replicative form of ØX174 DNA was obtained by the method described in (2) with some modifications. A clarified lysate was treated with RNAase, the subjected to triple phenol deproteinization. Short RNA fragments were removed from the CsCl fraction of supercoiled DNA by gel-filtration on Sepharose 2B(Pharmacia, Sweden).

pBR 322 DNA was obtained by the method described in (11). A 1287-bp-long EcoR1 fragment of λ phage DNA containing P_R, P_o and P_{rm} promoters (12) was kindly donated by Dr.E.D. Sverdlov.

A 1459-bp-long BspI fragment of T7 phage DNA containing

A_0,A_1,A_2 and A_3 promoters (13) was kindly donated by Dr.E.F. Zaichikov.

The number of single-stranded breaks was determined by means of an electron microscope. For this purpose DNA denatured in 0.5M KOH was spread and deposited on grids directly after neutralizing the alkali to avoid renaturation, and the average size of the fragments was determined. A fragment of T7 DNA contained about 0.3 breaks in each strand of the double helix; a fragment of λ DNA contained less than 0.2 breaks per strand.

2. Enzymes. pBR 322 DNA was converted into the linear form by restriction endonuclease Pst I under the following conditions: 40 mM Tris-HCl (pH 7), 6 mM 2-mercaptoethanol, 6 mM $MgCl_2$, 6 mM NaCl, 37°C, 1 hour, then the enzyme was removed by phenol deproteinization. The linear form of pBR322 DNA contained about 0.5 single-stranded breaks per strand.

The replicative form of ØX174 DNA was elevated by the enzyme Msp I in a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 0.15M NaCl at 37°C for 1 hour.

3. Restriction fragments of DNA. Preparative separation of Msp I fragments of hydrolyzed replicative form of ØX174 DNA was performed by slab (200x200x6MM)-gel electrophoresis in 3.5% polyacrylamide gel (Serva, FRG), field voltage 5 v/cm, for 48 hours. UV-visualized EtBr-stained fractions of DNA were cut out along with the gel, then electroeluted in an apparatus like the one described in (14) but made of glass in Dr.M.A.Grachev's laboratory at the Novosibirsk Institute of Organic Chemistry, USSR Academy of Sciences (Siberian Branch). The preparations were transferred into solutions: 1xSSC (150 mM NaCl + 15 mM Cit•Na) and 0.1xSSC by gel-filtration on Sephadex G-50 (Pharmacia, Sweden). Analysis of the resulting DNA samples showed them to have no more than a 5% content of other DNA fragments. The fragment used was 1695 bp long. It contained about 0.3 single-stranded breaks per strand.

4. DNA melting and renaturation was carried out on a micro-spectrophotometer (15) and a Cary 219 spectrophotometer with the temperature changing continuously at a rate of 0.15 ± 0.2°/min.

5. Differentiation of the melting curves was performed according to the algorithm described in (11) using an HP 9825 computer (Hewlett-Packard, USA) and a program written by Dr. E.I. Golovanov, as well as by a Minsk-32 computer according to the same algorithm.

6. Electron microscopic denaturation maps were obtained by the technique presented in (11). The only difference was that the melted state was fixed at a temperature corresponding to the peak's maximum but not its end. In the course of fixation the degree of denaturation increased up to the value corresponding to the end of the peak concerned and this degree of denaturation was in good agreement with its electron microscopic value.

RESULTS

Figure 2 shows the complete differential curve for the melting of pBR 322 plasmid DNA in a 0.1xSSC buffer. To investigate the reversibility of the melting process we let it reach different degrees of denaturation. The process proved to be

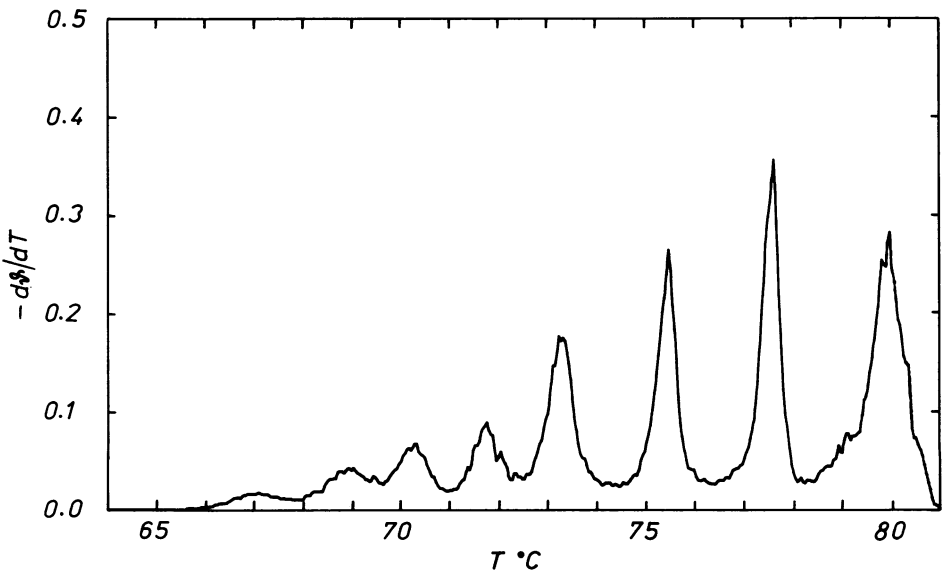

Fig. 2. Complete differential melting curve of pBR 322 DNA in 0.1xSSC buffer.

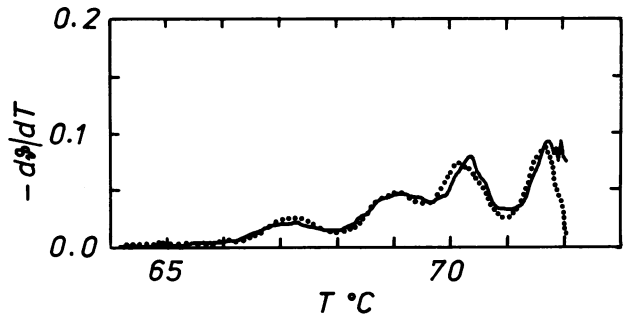

Fig. 3. Differential melting curve of pBR 322 DNA in 0.1xSSC buffer corresponding to 20% denaturation. Denaturation ——— , renaturation ····· .

reversible up to a temperature of 72°, which corresponds to 20% denaturation of this DNA (see Figure 3). The picture is different above 73°. The fine structure of the differential curve of renaturation on cooling from a partly melted state at 74° (see Figure 4) is essentially distinct from the corresponding section of the differential melting curve.

Thus the first sections of the double helix melt out within a reversible process. However the melting out of one or several sections at about 73° makes the subsequent process irreversible. According to Hoff and Roos's model (9) for the emergence of irreversibility, melting should pass through a stage of decreasing the number of helical regions (see Figure 1). To test the

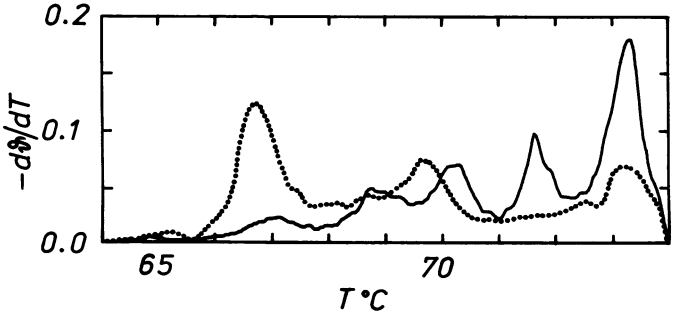

Fig. 4. Differential melting curve of pBR 322 DNA in 0.1xSSC buffer corresponding to 35% denaturation. Denaturation ——— , renaturation ····· .

model we used mild fixation of the unwound regions with glyoxal with subsequent electron-microscopic mapping of denaturation (11). Figure 5 shows maps of partial denaturation of pBR 322 DNA in a 0.1xSSC buffer. Map a) corresponds to the first stage of melting when the process is reversible. Maps b) show that section 0 ÷ I0, which is flanked by the left end of the molecule and the already melted region, melts out within the interval 73-74°C. Figures 3 and 4 show that it is here that the process becomes irreversible.

We may therefore conclude that at a low ionic strength ($\leq$ 0.02M Na^+) the melting stage presented in Figure 1 is the threshold of irreversibility for the subsequent process.

If the low rate constant of the reaction forming a new helical region is due to electrostatic repulsion of the strands, one should expect this rate constant to be strongly dependent on the ionic strength of the solution. Ionic strength

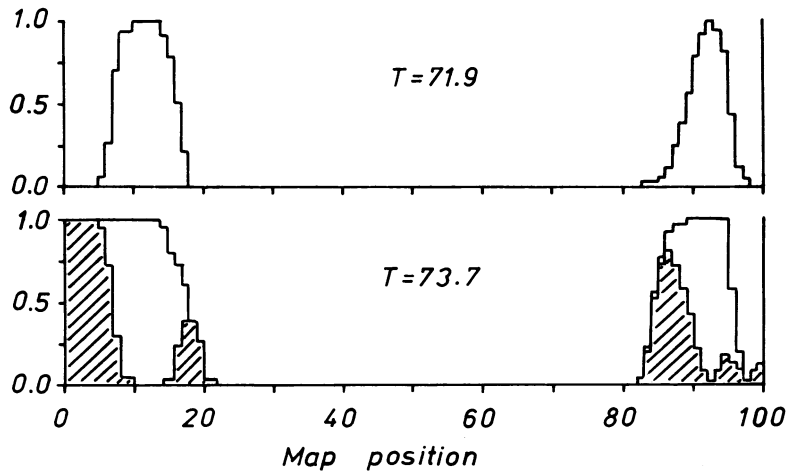

Fig. 5. Electron-microscopic maps of partial unwinding of pBR 322 DNA in 0.1xSSC buffer. The ordinate is the probability of a given section being unwound. The map's origin corresponds to the restriction site for Pst I, the direction is 5' ⟶ 3' in the L-chain. The dashed areas are the melted regions which appeared in the second map but had been absent from the first one. The temperature indicated corresponds to the electron microscopic degree of denaturation and is 0.2-0.3° higher than the fixation temperature.

is known to have a substantial effect on the rate of DNA renaturation (5). Hence it is natural to expect the deviation from equilibrium to be larger at lower ionic strengths. By contrast, a rise in ionic strength should bring the process closer to equilibrium. We investigated the reversibility of DNA melting in a 1xSSC buffer (0.195M Na^+). Figure 6 shows the complete differential curve for the melting of pBR 322 DNA under these conditions. Figure 7 presents the electron-microscopic denaturation maps of this DNA in the 1xSSC buffer; they show that by 90° melting passes through the stage of decreasing the number of helical regions. Indeed, in the temperature range of 88°-90° we observe the melting out of section 0 ÷ I0 which is flanked by the left end of the molecule and the already melted region. Nevertheless, the data in Figure 6 demonstrate that the reversibility of the melting process is not disturbed at this ionic strength even after the stage of decreasing the number of helical regions. Moreover, melting is reversible up to the onset of complete separation of strands.

The degree of reversibility of melting in 0.1xSSC and 1xSSC buffers was also studied for a restriction fragment of T7 phage DNA.

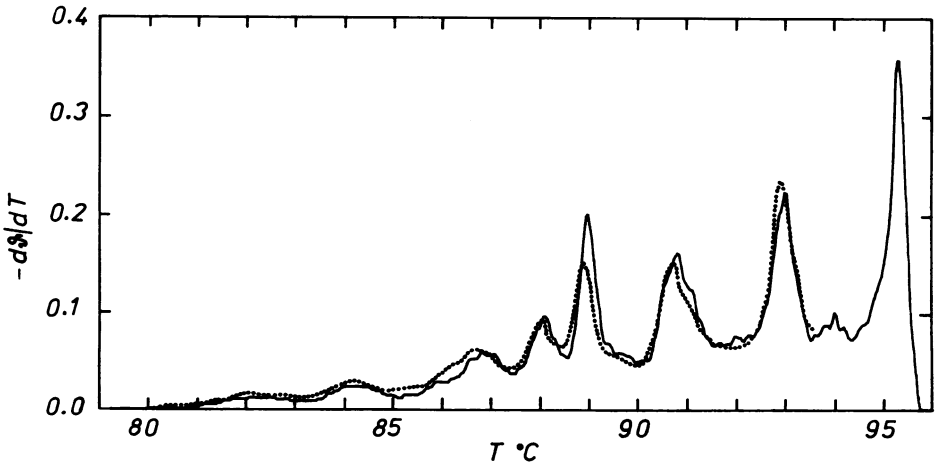

Fig. 6. Complete differential melting curve for pBR 322 DNA in 1xSSC buffer ——— . The renaturation curve ····· was obtained after denaturation of this DNA up to 72%.

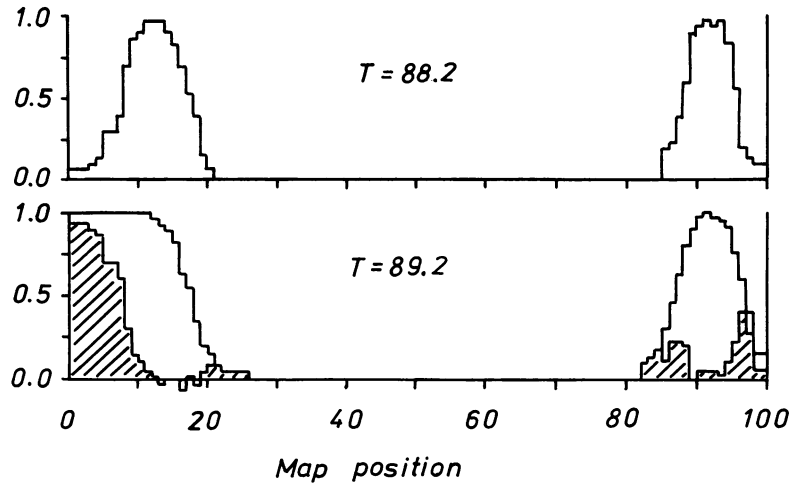

Fig. 7. The same maps as in Fig.5 but for 1xSSC buffer.

Complete differential melting curves of this fragment are presented in Figures 8 and 9. Both curves (for different ionic strengths) show four clear-cut peaks. Since the temperature range of the last peak corresponds to a complete separation of strands, we investigated the reversibility of melting up to temperatures of 70.5° and 88.3° respectively. The extent of unwinding was in both cases about 40%. Figure 8 shows the fragment's melting in the 0.1xSSC buffer to be patently irreversible. Melting to the same degree of denaturation in the 1xSSC buffer (Figure 9) is an equilibrium process.

Thus, for both kinds of DNA studied, melting irreversibility that was observed in 0.1xSSC disappeared in 1xSSC.

We looked at the reversibility of melting at a high ionic strength for two more restriction fragments of λ and ØX174 phage DNA. Figures 10 and 11 show the complete differential curves for the melting of these fragments and for their renaturation. Renaturation was in both cases started at the temperature corresponding to the beginning of the last peak. A comparison of these curves demonstrates that here again melting is reversible at the ionic strength of 0.2M Na^+.

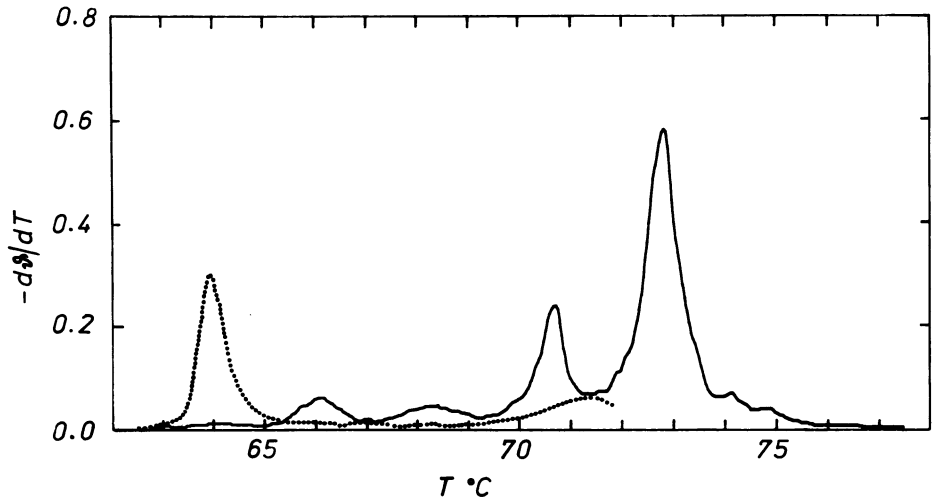

Fig. 8. Complete differential melting curve of a fragment of T7 phage DNA in 0.1xSSC buffer. The renaturation curve ..... was obtained after denaturation of this DNA up to 40%.

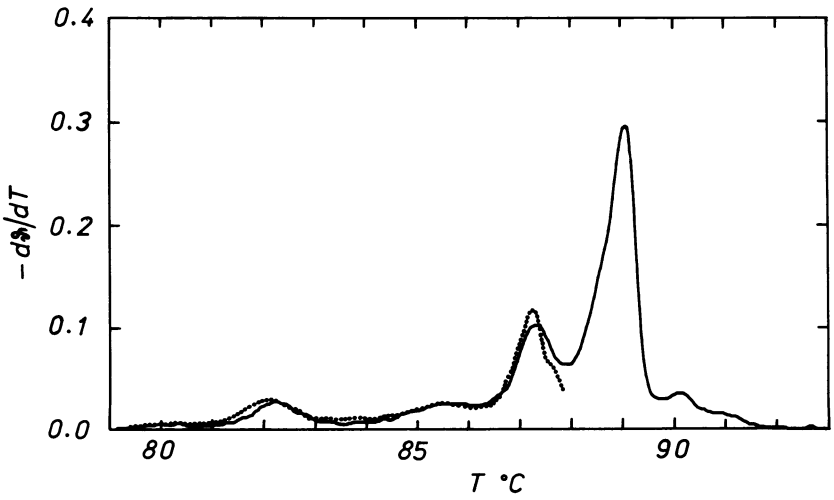

Fig. 9. Complete differential melting curve of a fragment of T7 phage DNA in 1xSSC buffer. The renaturation curve ..... was obtained after denaturation of this DNA up to 40%.

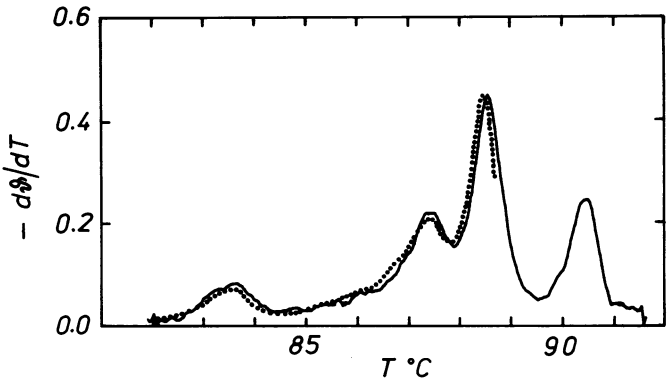

Fig.10. Complete differential melting curve of a fragment of λ phage DNA in 1xSSC buffer ——— and the differential curve of renaturation from the temperature corresponding to 68% denaturation of the fragment ·····.

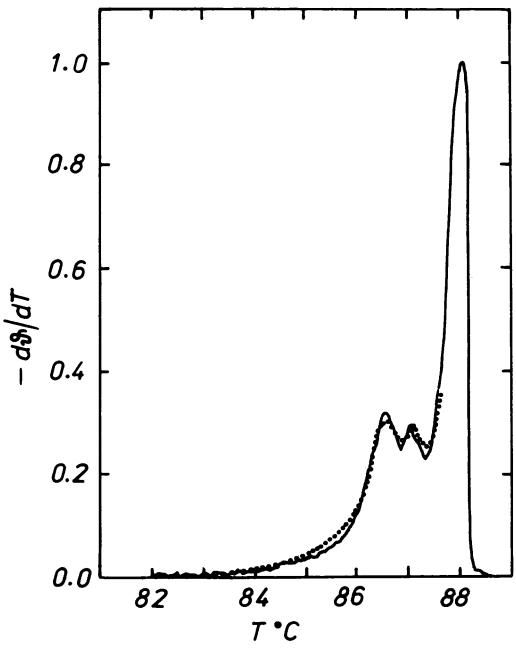

Fig.11. Complete differential melting curve of a fragment of ØX174 phage DNA in 1xSSC buffer ——— and the differential curve of renaturation from the temperature corresponding to 60% denaturation of the fragment ·····.

DISCUSSION

The above results show that DNA melting is usually non-equilibrium at a low ionic strength. Analysis of electron-microscopic maps of DNA unwinding demonstrates that irreversibility springs from the fact that an equilibrium process of renaturation must pass through the stage of forming a new helical section, as was first suggested by Hoff and Roos (9). Since there is a strong electrostatic repulsion between single-stranded sections at a low ionic strength, the rate constant of forming a new helical section is very low indeed.

We shall discuss the matter at some length.

The process presented in Figure 1 is described by a simple kinetic scheme:

$$A \underset{K_r}{\overset{K_d}{\rightleftharpoons}} B,$$

where A stands for the helical state of section c (Figure 1), B stands for its denatured state, K_d and K_r are, respectively, the rate constants of denaturation and renaturation. We assume for the sake of simplicity that section c melts co-operatively. Relaxation time τ is for this process

$$\tau = \frac{1}{K_d + K_r}$$

The value of K_r is small at a low ionic strength and only weakly depends on temperature (see Figure 12). The value of K_d is determined by the difference between the experimental temperature and the melting temperature T_m^{∞} of an infinite double helix whose thermostability corresponds to section c. If $T < T_m^{\infty}$ the value of K_d is highly temperature-dependent. In this temperature range (that of equilibrium melting) the process is similar to the melting of self-complementary oligonucleotides A_nU_n investigated by Pörschke and Eigen (16) and the temperature dependences of K_r and K_d are those found in (16). A more detailed analysis of the matter will be pub-

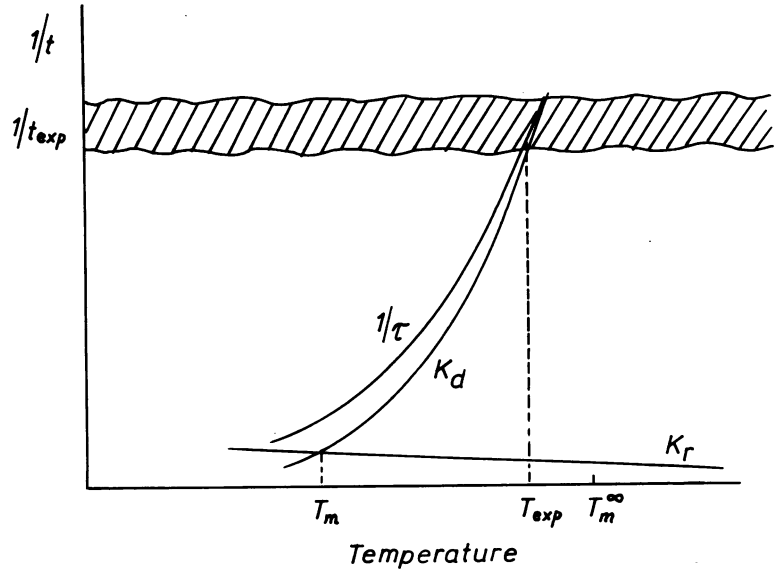

Fig.12. Schematic representation of the temperature dependence of the rate constants of denaturation K_d and renaturation K_r for a section flanked by melted regions (section c in Figure 1). The shaded band corresponds to the characteristic times of the experiment.

lished elsewhere.

The concentrations of states A and B must be the same in the middle of the equilibrium transition. Since for an equilibrium process

$$\frac{[A]}{[B]} = \frac{K_r}{K_d}$$

midway in the equilibrium transition (i.e. at temperature T_m) $K_r=K_d(T_m)$.

Hence a decrease in ionic strength, which brings down the value of K_r, increases the difference between the equilibrium melting temperature T_m of section c and T_m^∞. The time of relaxation to the equilibrium state at T_m obviously equals

$$\tau = \frac{1}{2K_d(T_m)} = \frac{1}{2K_r}$$

and is at low K_r far larger than the characteristic time of changing temperature t_{exp} during the experiment (with continuous heating at a rate of $\frac{dT}{dt}$, $t_{exp} \approx \frac{\Delta T}{dT/dt}$, where ΔT is the width of an individual peak in the differential melting curve, i.e. $\Delta T \approx 0.4^{\circ}$). Now what will the process be like at a higher temperature, other things being equal? Since at T_m $\tau \gg t_{exp}$, section c does not have the time to melt out at this temperature. However in the temperature range of $T < T_m^{\infty}$ the K_d value grows dramatically with temperature (the effective activation energy is about 1000 kcal/mol (10,16)) and at $T=T_m^{\infty}$ relaxation time τ becomes much smaller than experimental time t_{exp}. The unwinding of a helical region proceeds from its ends through random walk until the ends meet ever other. At $T < T_m^{\infty}$ the mean number of steps in the random walk is a sharp function of temperature. At $T=T_m^{\infty}$ the random walk of the ends becomes symmetric and the τ value may be estimated by the simple equation $\tau = \tau_0 n^2$. Here τ_0 is the time of elongation of the helical region by one base pair, n is the number of base pairs in the c region. Provided that $\tau_0 \approx 10^{-6}$ sec (16), n=300, we obtain that at $T=T_m^{\infty}$ $\tau \approx 10^{-1}$ sec. So the c region melts at a temperature within the interval $[T_m, T_m^{\infty}]$. This temperature, T_{exp}, is determined by the equation:

$$\tau = (K_d(T_{exp}) + K_r)^{-1} \approx t_{exp},$$

i.e. the transition will shift from its equilibrium temperature T_m towards higher temperatures T_{exp}, the more so the lower the ionic strength.

A fall in temperature from the range $T > T_{exp}$ will cause no changes at T_{exp}, as at this temperature the equilibrium is shifted towards state B. When we approach the temperature T_m the relaxation time will again become too large for the transition to occur in the time t_{exp}. Since τ will further grow with falling temperature, the transition will not occur at lower temperature either, until we reach the melting temperature for one of regions b or d of Fig.1. The relaxation time is small for these regions, for their renaturation does not require nucleation. But as soon as one of these regions rena-

tures, the renaturation of section c will not require nucleation either and will occur fairly quickly.

Growing ionic strength increases K_r and elevates the intersection of curves $K_d(T)$ and K_r, so that at some ionic strength the relaxation time at T_m diminishes to the characteristic time of the experiment and the process becomes equilibrium. It should be emphasized that when ionic strength grows from a low value to ~ 0.2M Na^+ K_r changes by several orders of magnitude. Nucleation is the limiting stage in the formation of a new helical region as in the case of homopolymer renaturation. Therefore one may expect the relevant rate constants to depend on ionic strength in the same way. According to Blake and Fresco (17), the rate of renaturation of the homopolymer polyA • polyU changes 4.10^4 times when ionic strength grows from 0.01M Na^+ to 0.2M Na^+ .

Thus, the regions that melt out in a non-equilibrium fashion unwind at a higher temperature, which may lead to a considerable distortion of the equilibrium profile of the differential melting curve. Therefore the experimental differential melting curves may be highly different at different ionic strength, especially when these curves are composed to overlapping peaks, as observed by Vizard et al. (4). Naturally such changes in the differential melting profiles cannot be obtained within a theoretical model that treats the process as equilibrium.

Our results show that there are experimental conditions under which the melting process is strictly equilibrium up to the temperature point where the strands start separating. It is only for these conditions, which correspond to a high ionic strength (~ 0.2M Na^+), that one is entitled to compare the experimental results with calculations based on the equilibrium theory of helix-coil transition. Formerly, however, comparisons were made largely for a low ionic strength (0.1xSSC). The natural question is how different the comparison of theory and experiment will be if the experimental curves reflect an equilibrium situation. Preliminary results show that in this case the agreement between theory and experiment is somewhat better than in (1,2). Thus the discrepancy between theory and

experiment in studies (1,2) is partly due to the non-equilibrium nature of melting at a low ionic strength. However the discrepancies remaining in the equilibrium situation still considerably exceed the experimental errors. These discrepancies are probably due to the heterogeneity of stacking interaction among base pairs in the double helix /see(2)/.

ACKNOWLEDGEMENTS

The authors are indebted to Drs. S.A.Grachev, E.F.Zaichikov, B.A.Rebentish, E.D.Sverdlov, L.V.Neumyvakin for the gift of various preparations, to Prof. M.D.Frank-Kamenetskii for fruitful discussions and to Prof. Yu.S.Lazurkin and Dr.M.A.Grachev for unfailing attention and support.

+To whom all correspondence should be addressed.

REFERENCES

1. Lyubchenko, Yu.L., Vologodskii, A.V. and Frank-Kamenetskii, M.D. (1978) Nature 271, 29-31
2. Vologodskii, A.V. and Frank-Kamenetskii, M.D. (1978) Nucleic Acids Res. 5, 2547-2556
3. Benight, A.S., Wartell, R.M., Howell, D.K. (1981) Nature 289, 203-205
4. Vizard, D.L., White, R.A, and Ansevin, A.T. (1978) Nature 275, 250-251
5. Studier, F.W. (1969) J.Mol.Biol. 41, 199-209
6. Yabuki, S., Fuke, M. and Wada, A. (1971) J.Biochem. 69, 191-207
7. Michel, F. (1974) J.Mol.Biol. 89, 305-326
8. Wada, A., Yabuki, S. and Husimi, Y.(1980) Crit.Rev. Biochem. 9, 87-144
9. Hoff, A.J. and Roos, A.L. (1972) Biopolymers 11, 1289-1294
10. Spatz, H.-CH. and Crothers, D.M. (1969) J.Mol.Biol. 42, 191-219
11. Borovik, A.S., Kalambet, Yu.A., Lyubchenko, Yu.L., Shitov, V.T. and Golovanov, Eu.I. (1980) Nucleic Acids Res. 8, 4165-4184
12. Ovchinnikov, Yu.A., Guryev, S.O., Krayev, A.S., Monastyrskaya, G.S., Skryabin, K.G., Sverdlov, E.D., Zakharyev, V.M. and Bayev, A.A. (1979) Gene 6, 235-249
13. Grachev, M.A., Zaichikov, E.F., Kravchenko, V.V. and Pletnev, A.G. (1978) Dokl.Acad.Sci. USSR 239, 475-478
14. Allington, V., Cordy, A., McGalow, M.D., Nelson, J.(1978) Analit. Biochem. 85, 188-196
15. Grachev, M.A. and Perelroyzen, M.P. (1978) Nucleic Acids Res. 5, 2557-2564
16. Porschke, D. and Eigen, M. (1971) J.Mol.Biol. 62, 361-381
17. Blake, R.D. and Fresco, J.R. (1966) J.Mol.Biol. 19, 145-160