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Fluorescence Label Studies of the Phase Transitions of T7

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Abstract. Optical density, fluorescence intensity, polarization and lifetime measurements were performed to analyze the temperature-induced phase transitions of phage T7 in several buffers. For labelling the intraphage DNA, ethidium bromide, proflavine and rivanol were applied for the proteins 1,6-diphenyl-1,3,5-hexatriene (DPH). In the temperature range of 20° C–100° C several structural changes of T7 were detected. Making corrections for light scattering by using integrating spheres and analyzing the fluorescence signals, the phase transition of intraphage DNA below 60° C was interpreted as a superhelical relaxation phenomenon. The structural changes found by optical density at higher temperatures could be assigned to a change in the phage DNA or to a change in its protein part on the basis of fluorescence-melting results concerning DNA and protein labels. The effects of ionic strength and environment on the structural changes were studied.

Key words: Phase transitions of phage T7 – Light scattering corrected optical, and fluorescence melting – DPH bound to phage proteins – Acridine dyes – Superhelical structure

Introduction

There are experimental data demonstrating that negatively supercoiled, closed circular DNA has a weakend secondary structure (Burke and Bauer 1980; Lau and Gray 1979; Burke and Bauer 1978; Wang 1974). The existence of superhelical packing was directly connected with a distortion of the DNA double helix by Sussman and Trifonov (1978). A theoretical expression of the superhelix energy as a function of superhelix density and the fraction of open-base pairs was given by Vologodskii and Frank-Kamenetskii (1981). Earlier experimental data on the phase transition of a nucleoprotein, the bacteriophage T7, were also considered to be connected with the superhelical nature of virus DNA. The phase-transition studies using microcalorimetric, optical melting methods

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(Monaselidze et al. 1978), biological-activity melting and small-angle X-ray scattering methods (Fekete et al. 1982), and chiro-optical (Tóth et al. 1983) methods showed that the bacteriophage has several phase-transition steps between room temperature and 100° C, depending on the nature of the buffers applied. Besides the temperature range of the helix-coil transition of DNA (80°–90° C), a low-temperature (early) phase-transition region could also be seen in the range of 50°–60° C. In optical melting studies this early transition was characterized by a decrease of optical density at 260 nm. The circular dichroism studies suggested that a transformation of the intraphage DNA to a conformation with a higher degree of chirality takes place within this transition region since the X-ray scattering data have shown the disappearance of the superhelical order between 50° C and 60° C.

When studying the structural changes of a complex system composed of DNA and proteins, it is a problem to separate the detected signals into those demonstrating the structural transitions of the DNA and those characterizing the structure of the protein part. When optical density is measured at a wavelength corresponding to DNA absorption, the structural changes of proteins may lead to light-scattering changes, thus resulting in a significant optical density change at the wavelength of detection. There are papers presenting optical density results on the early-phase transition of bacteriophages T7 and S_d in several buffers correcting for light scattering with an extrapolation from longer wavelengths (Tikchonenko et al. 1977; Fekete et al. 1982). However, direct light-scattering measurements recently performed in our laboratory (Tóth et al. 1983) have shown that in the case of T7, the scattered light intensity is not a monotonous function of wavelength within the absorption band (similarly to dispersion relations). Thus a direct correction for light scattering and/or other methods differentiating between DNA and protein changes are necessary. The results presented in this paper demonstrate that in the case of the early-phase transition of T7 by using integrating spheres for light-scattering correction, a characterization of superhelical relaxation of intraphage DNA was possible. At higher temperatures the separation of structural changes seen by optical-density-melting experiments was partially solved by using hydrophobic and acridine dyes and by measuring the fluorescence intensity and polarization as a function of temperature. Up to now multiple labellings of DNA and proteins within a complex system have been mostly done for the purposes of cytofluorometry. Eight fluorochrome combinations were applied by Stöhr et al. (1978) and Hutter et al. (1980), and an assay of simultaneous labelling of proteins and DNA was also done by Taylor et al. (1977).

Materials and Methods

Spectroscopically pure samples of bacteriophage T7 and T7 DNA were prepared in the Institute of Biophysics, Budapest (Gáspár et al. 1980) by applying a series of sedimentation to the phage-lysate according to Strauss and Sinsheimer (1963) with a final centrifugation performed in CsCl density gradient. The CsCl salt was then removed by ultrafiltration in an Amicon cell with an XM 300 filter. From the phage lysate the T7 DNA was prepared according to Mandell and Hershey

(1969). The DNA calf thymus was purchased from Sigma. Acridine dyes 3,6-diaminoacridine (proflavine), 6,9-diamino-2-ethoxyacridine (rivanol), and ethidium bromide were obtained from May and Baker (England); 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Serva. The purity of the labels was checked by their absorption and emission spectra.

For acridine dyes and ethidium bromide, the optical densities of the solutions at the excitation wavelengths were set lower than 0.1 to avoid screening and reabsorption effects. A phosphate/dye ratio of > 100 was chosen. The concentration of DPH was adjusted to 10^{-6} mol/l by directly diluting the stock solution of 10^{-3} mol/l (made in tetrahydrofurane) with a phage solution of about 20 μg/ml. The excitation and emission wavelengths used for proflavine were 450 nm and 510 nm, 410 nm and 482 nm for rivanol, 520 nm and 600 nm for ethidium bromide, and 360 nm and 440 nm for DPH. Eventual chemical changes of the dyes during melting were monitored by recording their fluorescence excitation and emission spectra.

The buffers used were as follows:

A_1 = M9: 10^{-3} mol/l $MgSO_4$ + $2 \cdot 10^{-2}$ mol/l NH^4Cl + $4 \cdot 10^{-2}$ mol/l Na_2HPO_4 + $4 \cdot 10^{-2}$ mol/l KH_2PO_4, pH7,

A_2 = 10^{-1} mol/l NaCl + $4 \cdot 10^{-2}$ mol/l tris, pH7,

B = 10^{-2} mol/l NaCl + $2 \cdot 10^{-2}$ mol/l tris, pH7,

C = 10^{-3} mol/l NaCl, pH7.

Buffers A_1 and A_2 were the usual ones in biological experiments maintaining the native state of the bacteriophage. Buffer C was used as a medium of low ionic strength to facilitate the binding of acridine dyes (Bidet and Chambron 1970; Bidet et al. 1971). Buffer B was of intermediate ionic strength.

The optical melting studies were performed by an apparatus equipped with a temperature programmer and derivative unit constructed in the Institut de Physique Biologique, Strasbourg (Rump et al. 1980), which made it possible to record the optical absorption change as well as the first derivative. Fluorescence melting studies and polarization measurements were done with a Spex Fluorolog fluorometer equipped with a temperature programmer and recording unit (precision: ± 0.2° C) constructed in the Institut de Physique Biologique, Strasbourg. In the melting experiments different heating rates were applied in the range of 0.07° C/min to 0.6° C/min. Our preliminary optical melting studies have shown that the melting phenomenon is independent of the heating rate within this range in accordance with earlier microcalorimetric results (Monaselidze et al. 1978).

The optical absorption spectra were measured with a Cary 18 spectrophotometer. Measurements with integrating spheres were performed with a Cary 14 spectrophotometer using the method of Demangeat and Chambron (1978).

Results and Discussion

1. Optical Melting Profiles

A comparative survey of the results of melting experiments can be seen in Fig. 1. The optical (density) melting profiles are represented by the first derivative ($\Delta OD/\Delta T$) of the optical density (OD) measured at 260 nm as a function of

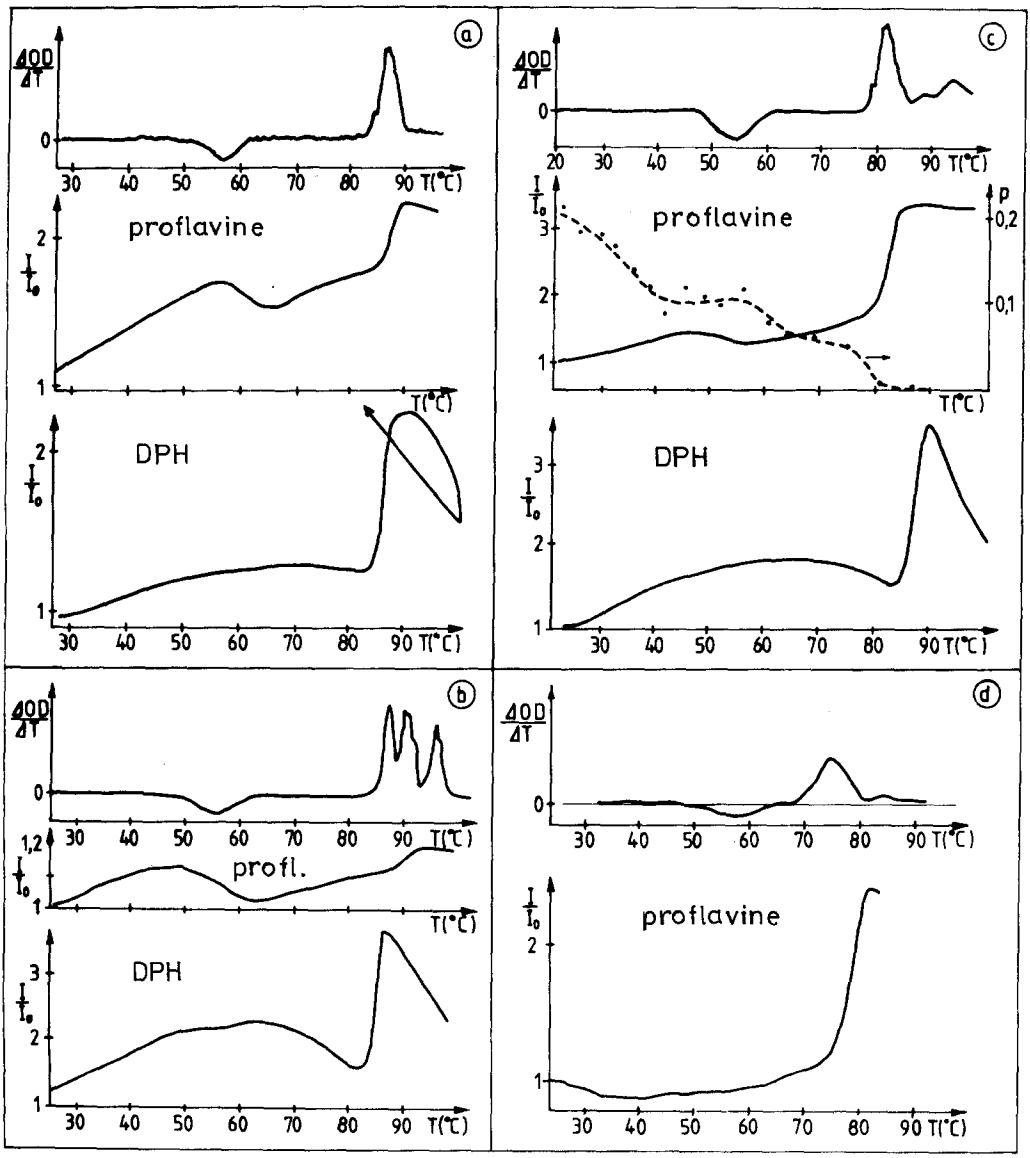

Fig. 1a–d. a First derivatives ($\Delta OD/\Delta T$) of the optical melting curves of T7, fluorescence (I/I_0), and polarization (p) melting curves of dye – T7 complexes measured in buffers M9; **b** 10^{-1} mol/l NaCl + 4.10^{-2} mol/l tris; **c** 10^{-2} 10^{-2} mol/l NaCl + 2.10^{-2} mol/l tris; **d** 10^{-3} mol/l NaCl

temperature. In buffers A_1, A_2, and B, a pronounced low-temperature (early) transition step can be seen which is characterized by a decrease of optical density, e.g., 18% in the case of A_1 and 16% for A_2, within a wide temperature range, while it is not so pronounced in case C. The high-temperature-phase transition range may consist of several steps which are especially well resolved in the case A_2.

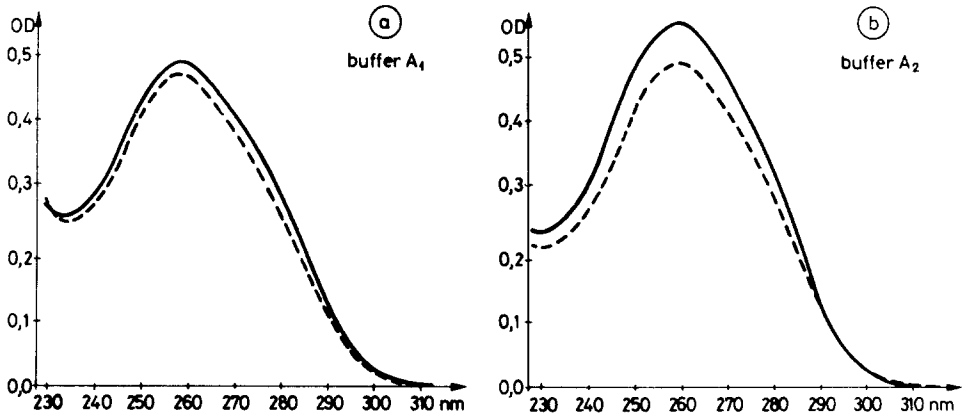

Fig. 2a, b. Absorption spectrum of phage T7 at room temperature in native state and after heating at 65° C for 30 min (*dotted line*) registered by integrating spheres in **a** buffer A_1 (M9) and **b** buffer A_2 (10^{-1} mol/l NaCl 4.10^{-2} mol/l tris)

In our earlier experiments the early-phase transition seemed to be irreversible; thus, the pure hypochromicity of this change could be studied by using integrating spheres at room temperature. Figure 2a and b shows the absorption spectra of T7 without light-scattering contribution (in buffers A_1 and A_2) in native state and after heating at 65° C for 30 min. (Integrating spheres were applied for reference and sample paths, respectively.) In the corresponding spectra measured in the conventional way (not shown), a pronounced decrease of the light-scattering contribution could be seen at wavelengths longer than 300 nm after heating. The results presented in Fig. 2 clearly show that the optical density decrease in Fig. 1. in the region of 50°–60° C cannot be interpreted as a light-scattering change alone in these cases but that it has a definite hypochromic nature as well. A similar technique was applied by Sapin et al. (1973), and a hypochromic change in DNA absorption was found when the protein was removed from the chromatin. In agreement with the results showing that the superhelical structure means a distortion of the normal B secondary structure of double-stranded DNA (Burke and Bauer 1980; Sussman and Trifonov 1978; Vologodskii and Frank-Kamenetskii 1981; Wang 1974) we postulate that the early transition may be a superhelical relaxation leading to a more stack-ordered DNA structure. The hypochromic change in optical density of T7 was found to be very sensitive to the ionic environment, as it was 25% and 75% of the total change at 258 nm in the case of buffer A_1 (M9) and A_2 (10^{-1} mol/l NaCl, $4 \cdot 10^{-2}$ mol/l tris), respectively. This suggests that even in physiological solvents the intraphage DNA may have much different superhelical organizations.

2. *Fluorescence Labels Bound to DNA*

a) Fluorescence Melting Studies. Although the binding of acridine dyes to DNA has been widely studied, it is not yet clearly understood (Bidet and Chambron

1970; Bidet et al. 1971; Pachman and Rigler 1972; Dourlent and Hogrel 1976; Ramstein et al. 1980). The literature data have shown the heterogeneity of the binding (sites): the bound state may be connected with a slight increase of fluorescence quantum yield in the case of A-T sites (emitting sites) and with a strong decrease of fluorescence quantum yield in G-C sites. To follow the binding of the dyes to emitting sites, lifetime studies were also applied (Duportail et al. 1977). In the association of acridine dyes Coulombic forces also play a significant role; thus, the association constant decreases with the increase of the ionic strength.

The fluorescence melting profiles (intensity and polarization) of proflavine – calf thymus DNA complexes in buffers of different ionic strengths were studied by Bidet et al. (1971). The binding of proflavine to synthetic polynucleotides was also studied. It has been shown that in all buffers at room temperature most of the bound dyes are of the quenched type at G-C sites in the case of proflavine-calf thymus DNA complexes. The contribution of emitting sites to the amount of bound dyes is small. The comparison of the fluorescence melting profile of proflavine – calf thymus DNA and proflavine – isolated T7 DNA complexes in buffer B is shown in Fig. 3. The helix-coil transition of T7 DNA is more cooperative and is shifted to higher temperatures. The G-C content of the two types of DNA is 49% in the case of T7 DNA (Woodbury and Record 1975) and 42% in calf thymus DNA (Duportail et al. 1977), leading to a difference of about 3° C in T_m according to Marmur and Doty (1962). The actual difference was about 5° C, which thus can be attributed not to the base content difference alone, but to the different homogeneneities (for the length of DNA) of the two preparations as well. In both cases, the melting profiles are superimposed on a gradual increasing signal originating from the gradual loss of bound dyes, as was found in calf thymus DNA by Bidet et al. (1971).

In the experiments with T7, mainly proflavine and rivanol were applied, although we found ethidium bromide also applicable to our problem. The A-T-specific nonintercalating dye-Hoechst 333258 (Bontemps et al. 1975; Brodie et al. 1975) was also used, but it was found to show a nonspecific behavior in the early-phase transition range of T7. Figure 4 shows typical melting profiles measured in buffer A_1, including proflavine – T7 and ethidium bromide – T7 complexes. The mirror symmetry of the two melting curves confirms the applicability of the two dyes for the problem. The behavior of rivanol is very similar to proflavine; thus, the melting profile for it is not shown.

When we examine the temperature range of the early-phase transition step shown by optical density changes in Fig. 1, we can see that in each case the decrease of optical density is accompanied by a decrease of fluorescence intensity superimposed on a gradual increasing signal (except for buffer C), which corresponds to the loss of the dyes at the applied ionic strengths. According to the references cited above and the results of Bidet et al. (1971), the decrease of proflavine fluorescence intensity at 50°–60° C can be interpreted as a result of the formation of new G-C binding sites within the intraphage DNA by the re-formation of previously distorted hydrogen bonds. This is in accordance with the hypochromic nature of the appropriate optical density changes and data in the literature suggesting that intraphage DNA, having

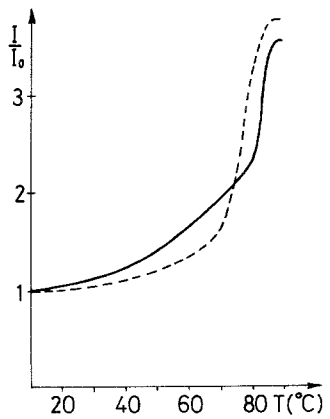

Fig. 3. Proflavine fluorescence intensity (I/I_0) plotted against the temperature in dye – calf thymus DNA (*dotted line*) and dye – isolated T7 DNA complexes in buffer B (10^{-2} mol/l NaCl + 2.10^{-2} mol/l tris)

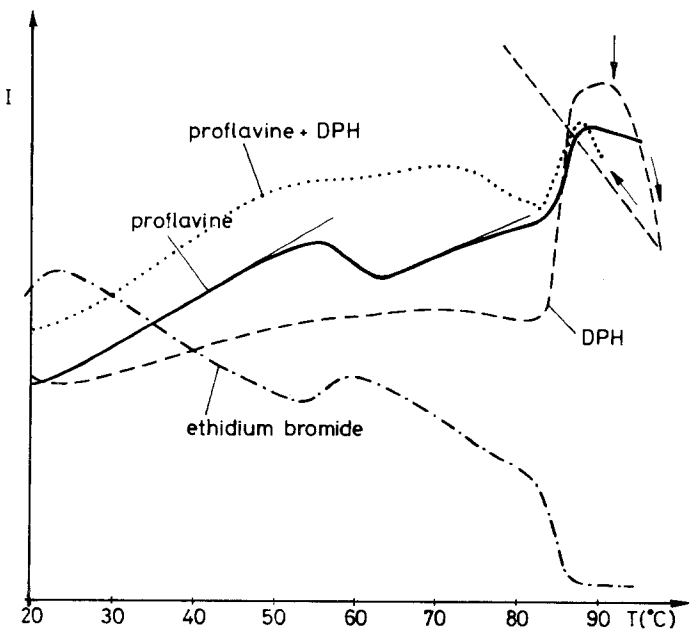

Fig. 4. Change in the fluorescence intensity (I in arbitrary units) of different dyes bound to phage T7 in buffer A_1 (M9) in function of temperature. The heating rate was 0.6° C/min for proflavine and ethidium bromide and 0.4° C/min for DPH. The temperature where polarization of DPH was checked is indicated by a *vertical arrow*

originally a distorted structure, suffers a phase transition to a more ordered conformation in this temperature range. Together with the high light-scattering contribution of this phase transition, the results support the idea that a superhelical relaxation takes place between 50°–60° C, which is probably connected with a strong change in the shape of the scattering particles.

In the range of high-temperature phase transition of bacteriophage T7, an increase of proflavine-fluorescence quantum yield can be seen in Fig. 1 similar to

Table 1. Phase transition temperatures (T_m) of T7 phage determined by optical density melting and fluorescence (proflavine and DPH) melting methods in different buffers: A_1 (M9), A_2 (10^{-1} mol/l NaCl + 4.10^{-2} mol/l *tris*), B (10^{-2} mol/l NaCl + 2.10^{-2} mol/l *tris*), and C (10^{-3} mol/l NaCl)

Buffers	A_1		A_2		B		C
T_m/°C/-optical density	57	87	56	87, 90, 96	54	81, 88, 93	75, 85
T_m/°C/-proflavine	61	87	55.5	90.5	52	82	79
T_m/°C/-DPH		86		85		87.5	ND

the signal of proflavine – DNA complexes. Table 1 compares the T_m values characterizing the change in fluorescence quantum yield with the T_m values shown by the optical density melting experiments. It is especially remarkable that when several phase transitions are shown by optical density changes within the high temperature range, only one of them can be accompanied with a change in proflavine-fluorescence quantum yield (e.g., buffer A_2). This fact suggests that not all of the phase transitions of the complex within this range are connected with helix-coil transitions of the intraphage DNA. Obviously, structural changes of the protein part of the complex can also lead to optical density changes at 260 nm via the change in light-scattering properties of the particles. The fact that the T_m determined by proflavine fluorescence is sometimes a little higher than the corresponding T_m value originating from optical density changes allows us to support the argument of Bidet and Chambron (1970), Bidet et al. (1971), and Duportail et al. (1977). They suggested that when the helix-coil transition of calf thymus DNA takes place, at the beginning a reorganization of the bound dyes occurs from the freshly melted A-T sites to the G-C sites still H-bonded. This reorganization would be followed by a slight decrease of fluorescence quantum yield, which probably can be seen in Fig. 4. If we accept this argument for T7, we can assume that the increase of fluorescence quantum yield shows the end of the helix-coil transition whenever the G-C sites are melted.

The fluorescence polarization of proflavine characterizing the emitting sites could also be measured as a function of temperature. The change in buffer B – supporting the earlier arguments – can be seen in Fig. 1c (dotted curve). The original high value ($p = 0.23$) of polarization is not far from the maximum value ($p = 0.3$) found by Bidet et al. (1971) when all of the dyes were bound. The gradual loss of dyes during melting would result in a gradual decrease; however, a small increase can be seen in the range of the early transition, showing that an ordering of the dyes bound to the emitting A-T sites also takes place. According to Ramstein et al. (1980), proflavine binds by a two-step mechanism to A-T sites. It can be assumed that in the range of the early-phase transition, new places for intercalation of externally bound molecules will be formed by the re-formation of previously distorted H-bonds. At the high-temperature-phase transition range a complete disappearance of the remaining polarization can be detected due to the loss of bound dyes from the emitting sites as well.

3. The Phase Transition Followed by Hydrophobic Labels

We tried to bind two hydrophobic labels in physiological solvents. We found DPH applicable to our problem, but in the case of 1-anilino-naphthalene-8-sulphonate, no binding to the protein coat was obtained.

In Fig. 4 a typical fluorescence melting curve of DPH is also presented for buffer A_1. The hydrophobic DPH dye is highly fluorescent in a hydrophobic environment, but its fluorescence is quenched in a hydrophilic medium. Thus, the first increase of its fluorescence intensity as a function of temperature can be attributed to the binding of originally free dyes or dyes in a hydrophilic medium to more hydrophobic regions – supposedly parts of phage proteins. If the temperature is increased, either gradual binding of the dye or greater accessibility of the dye to more hydrophobic regions due to structural changes of the protein can be assumed. After reaching an equilibrium at a temperature of 70° C, the former change is followed by a decrease in the fluorescence intensity, probably due to the increase of temperature. (The latter assumption is supported by the increase of fluorescence intensity during cooling from 98° C.) In the other buffers the melting profiles are similar, although the changes are more pronounced (see Fig. 1). At higher temperatures (~ 85° C) a sharp increase of fluorescence intensity can be measured, followed by an increase of fluorescence polarization up to a value of 0.37. An abrupt change probably takes place at this temperature in the conformation of the proteins, during which new hydrophobic regions can be reached by the dye (the state of the sample when polarization was determined is indicated by a vertical arrow in Fig. 4). The origin of the fluorescence signal measured at 90° C in buffer A_1 was controlled by the fluorescence excitation and emission spectra (not shown) which were identical with those of DPH. The sharp increase of DPH fluorescence quantum yield within the temperature region of the DNA helix-coil transition supports the theory that the hydrophobic dye is preferentially bound to proteins and not to DNA.

Melting temperatures based on DPH quantum-yield changes could be determined only for the high-temperature-phase transition region. Table 1 compares the results with those of proflavine quantum yield and shows the optical density measurements. It can be seen that by using DNA and protein labels, it is possible to separate the structural changes of the complex to phase transitions of the DNA part and phase transitions characterizing the protein content of it – which cannot be made on the basis of optical density-melting experiments or microcalorimetry alone. It must be mentioned, however, that in buffers A_2, B, and C, where the phase transitions are resolved, the highest-temperature structural change shown by optical density changes is still unidentified. It could be assumed that during this change only light scattering changes take place, but it is clear that the changes are not connected with precipitation phenomena.

Based on the comparison of differently determined T_m values shown in Table 1, it was possible to consider the effect of ionic environment on the helix-coil transition of the DNA part in the T7-complex. Those T_m values of optical density results were considered where the transition was accompanied by proflavine

quantum yield changes, and a logarithic dependence on the Na^+ concentration was found.

4. *Double Labelling*

Energy transfer from bound DPH to proflavine in the case of buffer A_1 was studied by exciting DPH at 360 nm and monitoring the emission of proflavine at 510 nm. This melting profile can also be seen in Fig. 4. Though the shape of the curve can be interpreted by the addition of the melting profiles of DPH T7 and proflavine – T7 complexes, no further information could be obtained in this way. In this case several factors contradict the useful applicaton of this technique. One is the low amount of bound DPH, the other the decrease of association constants due to the temperature rise. Thus, there are too many parameters with considerable influence on the detected intensity changes for the phenomenon to be seen clearly.

5. *Fluorescence of Phage T7*

The fluorescence melting signal (λ_{exc} = 290 nm; λ_{em} = 360 nm) of pure T7 in buffer A_1 was also measured. The emission spectrum excited at 290 nm and the excitation spectra monitored at 340 nm were similar to those of tryptophane. A slight increase with a maximum at about 54° C could be seen, dependent on the temperature superimposed on a nearly continuous decrease of the very low fluorescence intensity.

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