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Restructuring space ordering of (DNA–protamine) complexes in liquid crystalline dispersions under proteolytic enzyme treatment

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Complexes of DNA with the protamines stellin A and stellin B, in polymer-containing solutions, form both liquid crystalline phases and liquid crystalline dispersions. The 'non-specific' organization of the (DNA–protamine) phase is determined by the presence of protamine 'cross links' between the DNA molecules and not by the inherent anisotropy (cholesteric) double-stranded DNA molecules. Elimination of these 'cross links' by proteolytic enzyme action causes an increase in the distance between the DNA molecules which results in the appearance of an intense band in the CD spectrum and a 'fingerprint' (cholesteric) texture.

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1. Introduction

Double-stranded linear DNA molecules of relatively low molecular mass (less than 10^6 Da) form lyotropic liquid crystals and liquid crystalline dispersions in water–salt solutions under appropriate conditions [1, 2]. Due to the anisotropic properties of the double-stranded DNA molecules, one would expect the formation of a cholesteric packing of the DNA molecules in the liquid crystals and liquid crystalline dispersions [3]. This is the case, and the right-handed double-stranded DNA molecules actually form left-handed cholesterics. These phases show abnormal optical activity which is displayed as an intense band in the CD or optical rotation spectra in the region of absorption by nitrogen-containing bases [5, 6]. Because the helicoidal pitch tends to be 1 μm or larger, the optical textures have a characteristic 'fingerprint' appearance.

The cholesteric packing of DNA in lyotropic liquid crystalline dispersions is a 'mobile one' [2, 7]. The structural parameters of packing (pitch, intermolecular separation) can vary with changes in the properties of the solvent [2, 8], as well as in the parameters of the DNA secondary structure [2]. In particular, biologically active compounds, which intercalate between the nitrogen-containing bases of DNA, change not only the pitch of the cholesteric helix, but sometimes also the sense of the helix. This is accompanied by a change in the sign of the abnormal optical activity [2, 9].

It is known that when molecules of basic proteins or polypeptides interact with DNA in water–salt solutions the solubility is lowered and dispersions form. However, such dispersions do not usually possess the abnormal optical activity characteristic of cholesteric phases [10]. The DNA concentration in the particles of these dispersions can reach $\sim 100\,\mathrm{mg\,ml^{-1}}$, i.e. values comparable to those at which the cholesteric phase is formed [8]. This does not occur, however, for two reasons. Firstly, molecules of basic proteins can play the role of a 'medium', the properties of which can influence the mode of packing of adjacent DNA molecules [1]; secondly, the geometry of the mutual arrangement of the DNA molecules is determined by protein 'cross links' between the molecules, i.e. the mode of packing of the DNA molecules in particles of dispersions is determined by the presence of 'cross links', and depends on the chemical nature and structure of these 'cross links'. This implies that cleavage of protein 'cross links' in dispersions (DNA–basic protein) or (DNA–basic polypeptide)—(under conditions which ensure conservation of a high local DNA concentration) should be able not only to affect the mode of spatial ordering of adjacent DNA molecules, but also to allow their cholesteric packing to reform with characteristic abnormal optical properties.

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This paper describes the main features of restructuring the spatial organization from optically 'inactive' to optically 'active' dispersions by proteolytic enzyme action on (DNA–protamine) complexes.

2. Materials and methods

Low molecular mass ($0{\cdot}5$–$0{\cdot}7 \times 10^6$ Da) chicken blood DNA ('Reanal', Hungary) was used after additional purification and depolymerization. The DNA concentration in water–salt solutions was determined from the optical density of these solutions measured spectrophotometrically—'Specord M 40' (Germany).

Polyethyleneglycol (PEG, molecular mass 4000, 'Ferak', Germany) was used without additional purification.

Protamines—stellin A and stellin B—from gonads of *Acipencer stellatus* were isolated using previously published techniques [11, 12]. The protamine concentration in the initial water–salt solutions ($0{\cdot}15$ M NaCl + 10^{-2} M phosphate buffer, pH 7·2) was 1 mg ml^{-1}.

The (DNA–protamine) complexes were obtained by adding small volumes of the initial water–salt solution of stellin A or stellin B to samples of the DNA solutions ($C_{DNA} \sim 40\,\mu g\,ml^{-1}$, $0{\cdot}15$ M NaCl + 10^{-2} M phosphate buffer, pH 7·2) with constant mixing. The complexes obtained were characterized according to their r values, i.e. the ratio of the molar concentration of positively charged amino acid residues in the protamine to the molar concentration of DNA nucleotides (which is the same as the molar concentration of the negatively charged phosphate groups). The r values of the samples investigated ranged from 0 to 0·8.

Figure 1 illustrates (i) the formation of the (DNA–protamine) dispersions (stage *A*), (ii) the stabilization of (DNA–protamine) dispersions in PEG-containing solution (stage *B*).

Preparations of trypsin, α-chymotrypsin, papain, pronase P and thrombin ('Sigma', U.S.A.) were used without additional purification.

DNA liquid crystalline cholesteric dispersions, used as controls, were obtained by mixing equal volumes of DNA and PEG water–salt solutions [1, 2]. After 10–15 h, the formation of a DNA dispersion was registered by the appearance of an intense negative band in the CD spectrum in the region of the absorption characteristic of DNA bases ($\lambda \sim 270$ nm); a 1 cm cell and a 'Jobin-Yvon, Mark III' dichrograph (France) were used.

Samples of both cholesteric DNA liquid crystal and the liquid crystal phase formed by the (DNA–protamine) complexes used in the X-ray analysis and polarizing optical microscopy were prepared by previously described techniques [13].

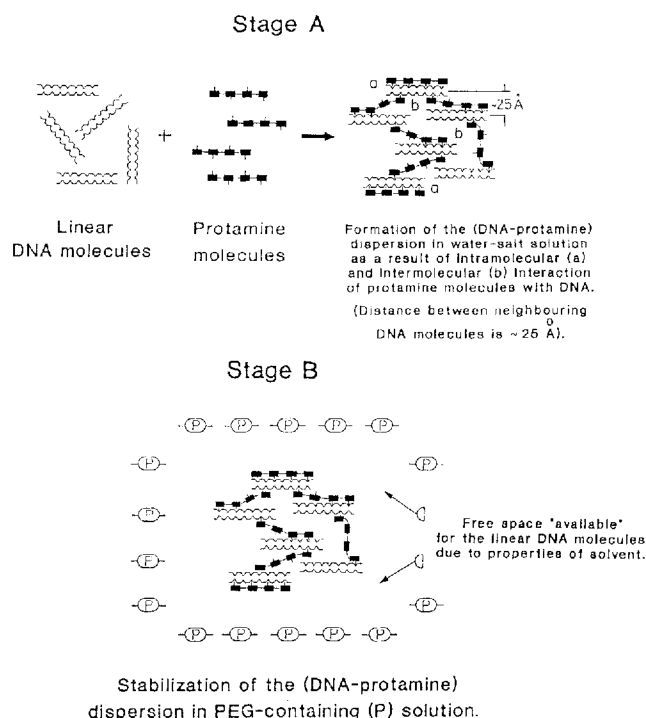

Figure 1. A general scheme of formation of (DNA–protamine) dispersions and their stabilization in polymer-containing solution.

3. Results and discussion

3.1. *The absorption spectra of DNA water–salt solutions containing stellin A or stellin B*

Figure 2 shows absorption spectra of DNA water–salt solutions measured at different stellin A/DNA ratios. As the stellin A concentration increases, the absorption

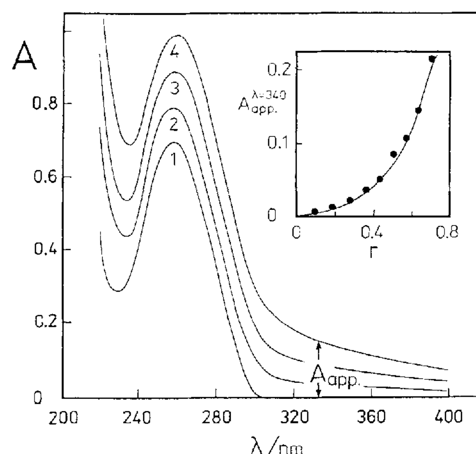

Figure 2. Absorption spectra of the DNA water–salt solutions ($0{\cdot}15$ M NaCl + 10^{-2} M phosphate buffer, pH 7·2) containing stellin A: (1), $r = 0$; (2), $r = 0{\cdot}355$; (3) $r = 0{\cdot}503$; (4), $r = 0{\cdot}636$. Inset—the dependence of an 'apparent' optical density ($A_{app}^{\lambda = 340\,nm}$) versus r.

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spectra (curves 2–4) begin to diverge from that of the initial DNA water–salt solution (curve 1). The absorption band at $\lambda = 258$ nm grows, there is a small 'red shift' (2–3 nm) of its maximum and an absorption appears at $\lambda > 320$ nm, where neither DNA nor protamine alone absorbs. The appearance of an 'apparent' optical density (A_{app}) points to the formation of (DNA–stellin A) dispersions scattering the UV radiation. The dependence of the value of A_{app} (at $\lambda = 340$ nm) on r (see, inset in figure 2) shows that the formation of dispersions from (DNA–stellin A) complexes begins only above the threshold of protamine binding with DNA. From the graph of A_{app} versus wavelength in the region of 320–440 nm, it is possible to evaluate the coefficients K and n, using a well known formula [14] which relates the n value to the diameter of particles forming dispersions. The value obtained for $n = 3{\cdot}4$ at $C_{DNA} \sim 35\ \mu g\ ml^{-1}$ corresponds to a particle diameter of 6400 Å.

The optical properties of DNA–polyamino acid dispersions have been investigated previously [15–17]. The n values (estimated by the same approach) were 2·4–2·9 for polylysine, 3·2 for polyarginine and 3·3 for polyornithine. These values, describing the sizes of 'complex coacervates', are similar to those obtained here. This agreement shows that the diameters of particles of low molecular mass DNA dispersions formed under different conditions are similar. This implies that the size of the particles is determined mainly by features intrinsic to the DNA molecules, rather than by the physical chemistry of the partition between the phases.

The formation of a dispersion as a result of DNA interaction with protamines is the result of a reduction in the solubility of the DNA in the water–salt solution. This is accompanied by the assembly of these complexes to give an arrangement which ensures minimal contact area with the solvent. Taking into account the chemical nature of protamines, one can expect a structural form which is a dispersion of DNA in which the charges on the phosphate groups are not only neutralized, but are also effectively 'cross linked' by the protamine molecules.

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3.2. *X-ray analysis of the phase formed from (DNA–stellin B) complexes*

Figure 3 compares X-ray diffraction curves for the phase obtained as a result of centrifugation of a dispersion prepared from a (DNA–stellin B) complex (curve 1), with that of the cholesteric liquid crystalline phase formed from the initial DNA (curve 2). The position of the maximum on X-ray scattering curve 1 differs appreciably from that of curve 2, which is specific for the DNA cholesteric liquid crystals.

The analysis of the X-ray scattering curves (see figure 3) shows (see also the table) that the interaction of

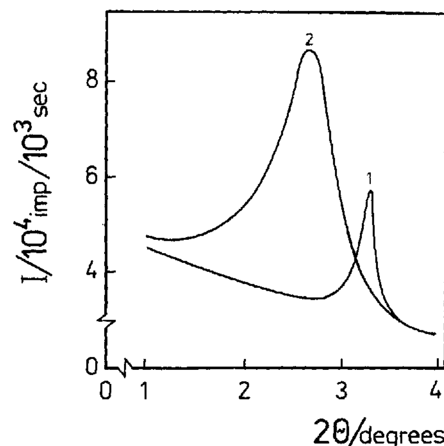

Figure 3. X-ray diffraction curves ($\lambda = 1{\cdot}54$ Å) for both a phase prepared from (DNA–stellin B) complex (curve 1) and cholesteric liquid crystals prepared from linear double-stranded DNA molecules (curve 2). Curve (1), $r = 0{\cdot}6$; 0·15 M NaCl + 10^{-2} M phosphate buffer, curve (2), $C_{PEG} = 170\ mg\ ml^{-1}$; 0·3 M NaCl + 10^{-2} M phosphate buffer.

stellin B with DNA molecules is accompanied by a change in the physical parameters describing the ordered arrangement of DNA molecules in the cholesteric liquid phase: the size of crystallites (L) and the radius of interaction (r_m) are increased, and the parameter of disordering ($\Delta/\bar{a}$) is decreased.

The main conclusion from the data in the table is the following: the extent of ordering of adjacent DNA molecules in a phase formed from a (DNA–stellin B) complex is higher than that of DNA molecules in a DNA cholesteric phase.

It should be noted that the absence of 'high order' reflections on curve 1 indicates that there is no regular three-dimensional ordering, and that there is only so-called 'one-dimensional order' [1] of the DNA molecules in the phase prepared from (DNA–stellin B) complexes.

Table. X-ray parameters for the phase prepared from (DNA–stellin B) complex (1) and DNA cholesteric liquid crystals (2).

No.	$\bar{a}$/Å	β_s/rad	L/Å	$\Delta/\bar{a}$	r_m/Å
1	25·07	0·0042	367·82	0·0831	580·56
2	32·93	0·0115	133·73	0·1580	211·11

$\bar{a} = \lambda/2\sin\theta$: average distance between the axes of adjacent DNA molecules; λ: wavelength of X-rays (here, $\lambda = 1{\cdot}54$ Å); θ: half scattering angle of the X-rays; β_s: integral half width of the maximum; $L = (\lambda/\cos\beta_s$: size of ordered regions (crystallites); $\Delta/\bar{a} = (1/\pi)(\beta_s\bar{a}/\lambda)^{-1/2}$: disorder parameter, Δ: semi-quadratic deviation from $\bar{a}$; $r_m = (\pi/2{\cdot}5)^2(\lambda/\beta_s)$: interaction radius.

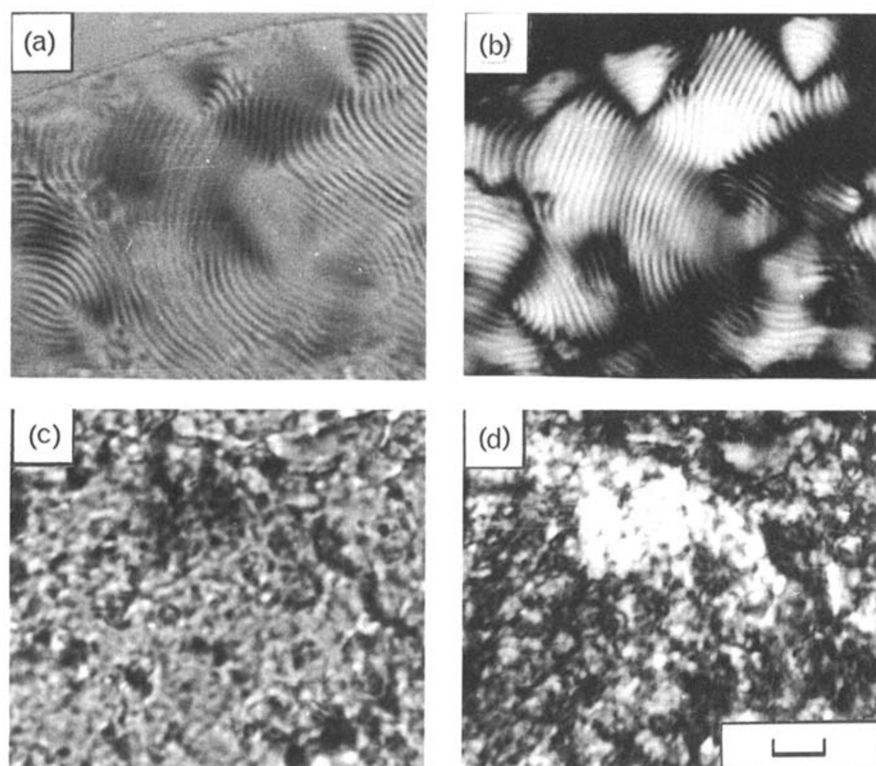

Figure 4. Optical textures of cholesteric liquid crystals prepared from linear double-stranded DNA molecules (*a*), (*b*) and a phase prepared from (DNA–stellin B) complex (*c*), (*d*). (*a*), (*c*): natural light; (*b*), (*d*): polarized light (crossed polarizers). (*a*), (*b*): $C_{PEG} = 170\,mg\,ml^{-1}$; 0·3 M NaCl + 10^{-2} M phosphate buffer. (*c*), (*d*): $r = 0{\cdot}6$; 0·15 M NaCl + 10^{-2} M phosphate buffer. Bar: 10 μm.

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3.3. *Optical textures of the phase of (DNA–stellin B) complexes*

Optical textures of thin films of the phase of a (DNA–stellin B) complex are shown in figure 4.

These textures can be observed both with parallel and crossed polarizers (see figure 4 (*c*) and (*d*)). Similar textures have been obtained by a number of authors, who investigated the properties of phases formed from DNA or polyribonucleotide molecules in concentrated water–salt solutions [8, 18–20]. The textures presented in figure 4 (*c*) and (*d*) differ from the classical 'fingerprint' textures typical of DNA cholesteric liquid crystals (see figure 4 (*a*) and (*b*)).

The X-ray analysis and the polarizing optical microscopy indicate one-dimensional order and optical anisotropy in the phase formed from the (DNA–stellin B) complex.

The 'non-specific' textures presented in figure 4 (*c*) and (*d*) are not strongly characteristic of any mesophase type [3].

The data presented testify that the interaction of protamine and DNA molecules in water–salt solutions of moderate ionic strength results in the formation of a liquid crystalline phase. The structural organization of this phase differs from that typical of DNA cholesteric liquid crystals. Taking into account the high local concentration of DNA in the phase formed ($>100\,mg\,ml^{-1}$), which follows from the X-ray data, one can conclude that the 'non-specific' organization of the (DNA–protamine) phase is determined by the presence of 'cross links' between the DNA molecules (but not by the anisotropy of the DNA itself). This means that one would expect similar 'non-specific' DNA packing in all cases of formation of phases from DNA when using additional polypeptides, polyamines, etc. which neutralize the DNA phosphate groups.

3.4. *The CD spectra of DNA water–salt solutions containing stellin A or stellin B*

Figure 5 represents the CD spectra of DNA water–salt solutions at different stellin A concentrations. Note that the intense band in the region of absorption by DNA nitrogen bases is absent.

Thus, the CD spectra demonstrate that double-stranded DNA molecules interacting with protamines in water–salt solutions of moderate ionic strength form liquid crystalline dispersions without abnormal optical activity.

3.5. *Restructuring of space organization of liquid crystals of (DNA–stellin B) complexes in polymer-containing solution under action of trypsin*

A sample of the liquid crystalline dispersion of the (DNA–stellin B) complex in water–salt solution was transferred to a PEG-containing solution and treated with trypsin. Figure 6 compares the CD spectra of this dispersion before (curve 1) and after (curve 2) addition of

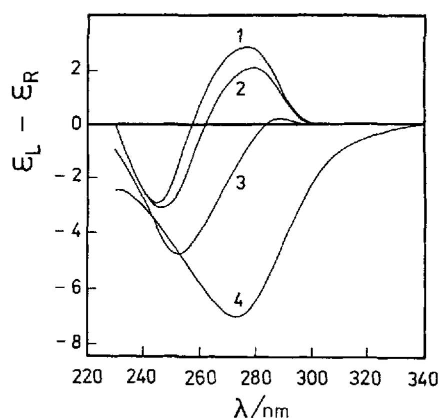

Figure 5. The CD spectra of the DNA water–salt solutions (0·15 M NaCl + 10^{-2} M phosphate buffer, pH 7·2) containing stellin A: (1), $r = 0$; (2) $r = 0{\cdot}355$; (3), $r = 0{\cdot}503$; (4), $r = 0{\cdot}636$.

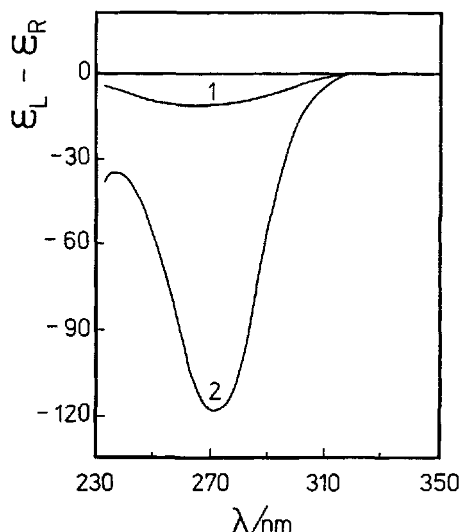

Figure 6. The CD spectra of liquid crystalline dispersions formed from the (DNA–stellin B) complex before (curve 1) and after (curve 2) trypsin treatment. $C_{PEG} = 170\,mg\,ml^{-1}$; $r = 0{\cdot}6$; 0·225 M NaCl + 5×10^{-3} M phosphate buffer, pH 7·2.

enzyme. As in the case of a water–salt solution (see figure 5), the liquid crystalline dispersion of (DNA–stellin B) complexes in PEG-containing solution does not show an intense band. (However, in principle, the physico-chemical properties of a PEG-containing solution are able to create within the particles of the dispersion a spatial organization of DNA molecules typical of DNA cholestric liquid crystalline dispersions. These dispersions are known to possess an intense band in the CD spectrum [1, 2].) The absence of such a band in the CD spectrum of liquid crystalline dispersions of (DNA–stellin B) complexes in PEG-containing solution may be due to a number of factors [21, 22]. In particular, the intense band in the CD spectrum may be absent in the case where the particles of the cholesteric liquid crystalline dispersion have a very small diameter (~ 500 Å [22]). However, the data presented above show that the particles of a dispersion of (DNA–protamine) complexes are considerably larger than this. The size of the particles in a dispersion of (DNA–protamine) complexes in PEG-containing water–salt solution was not determined in our work; nevertheless from general considerations of the phase exclusion of polymeric molecules [23], it is possible to expect only a small change in their diameters. Because of the high local density of packing of the DNA molecules, it can be supposed that the most probable reason for the absence of an intense band in the CD spectrum is the absence of the macroscopic helical structure of the cholesteric phase. This is caused by the protamine 'cross links' in the complexes. Taking the above statement about the properties of PEG-containing solutions into account, one must acknowledge that in such solutions (although the potential exists for neighbouring linear DNA molecules to move away) this is prevented by the presence of the protamine 'cross links'. Cleavage of these 'cross links' due to enzyme action should be accompanied not only by an increase in the distance between adjacent DNA molecules, but also result in mutual orientation which unequivocally depends on the properties of the solvent. Figure 7 illustrates a general scheme of the enzyme treatment of the (DNA–protamine) dispersions and their restructuring.

Indeed, after trypsin treatment of a liquid crystalline dispersion of the (DNA–stellin B) complex, the intense negative band in the CD spectrum develops (see figure 6, curve 2). This band directly reflects a change in the mode

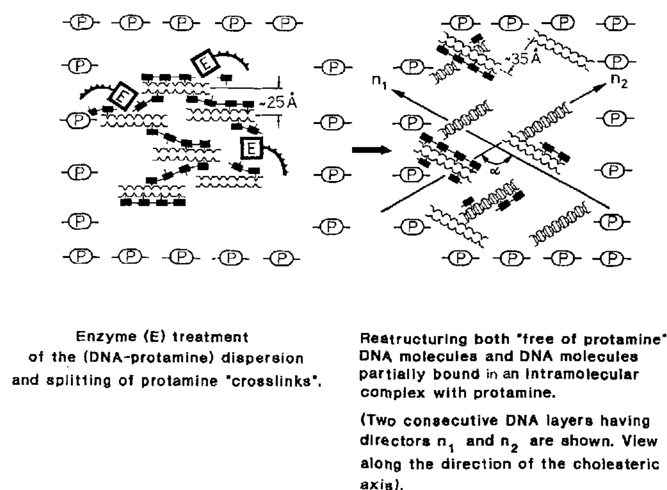

Figure 7. A general scheme of enzymatic treatment of the (DNA–protamine) dispersions in PEG-containing solution and restructuring of the spatial organization of the DNA molecules after splitting of protamine 'cross links'.

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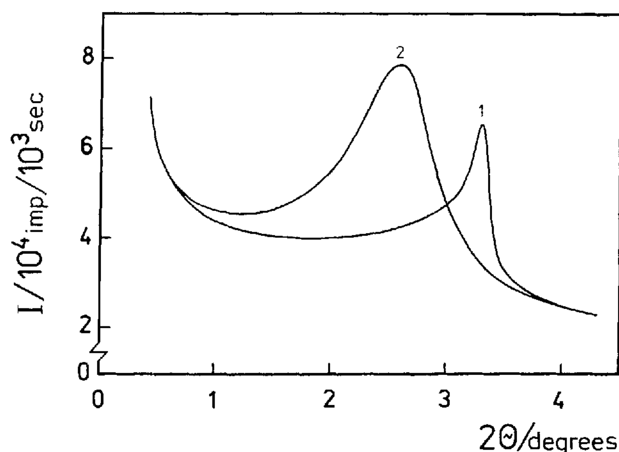

Figure 8. X-ray diffraction curves for the liquid crystalline phase prepared from the (DNA–stellin B) complex before (curve 1) and after (curve 2) trypsin treatment. $C_{PEG} = 170\,mg\,ml^{-1}$; $r = 0{\cdot}6$; $0{\cdot}225\,M$ NaCl $+ 5 \times 10^{-3}\,M$ phosphate buffer, pH 7·2.

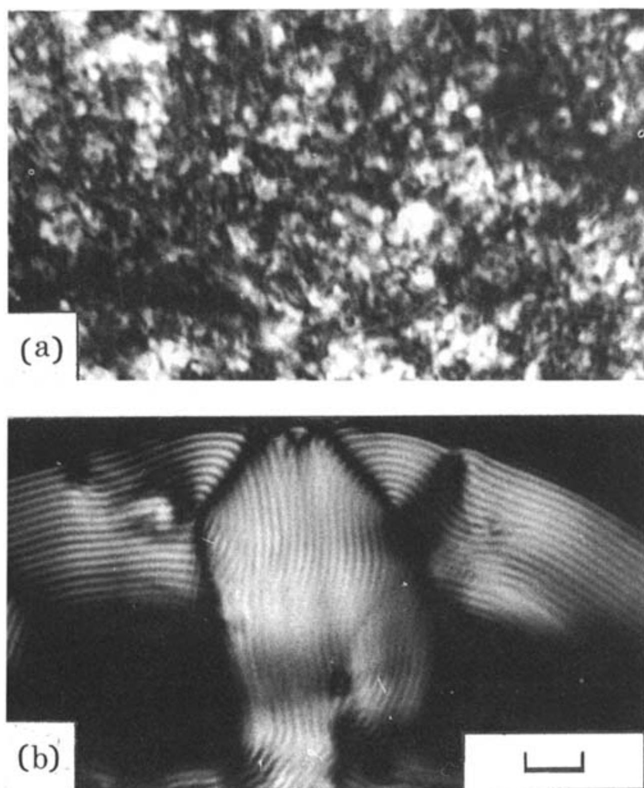

Figure 9. Optical textures of a liquid crystalline phase formed from the (DNA–stellin B) complex before (*a*) and after (*b*) trypsin treatment (crossed polarizers). $C_{PEG} = 170\,mg\,ml^{-1}$; $r = 0{\cdot}6$; $0{\cdot}225\,M$ NaCl$+5 \times 10^{-3}\,M$ phosphate buffer, pH 7·2. Bar: 10 μm.

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of packing of adjacent DNA molecules due to enzyme digestion of the protamine 'cross links', and the appearance of helical twisting between adjacent layers of DNA molecules in the particles of this dispersion. This means, that enzymatic hydrolysis of protamine 'cross links' permits the DNA molecules to adopt a spatial orientation such that it is characteristic of DNA cholesteric dispersions formed at $C_{PEG} = 170\,mg\,ml^{-1}$. Hence, as a result of trypsin digestion of stellin B molecules, the spatial structure of the liquid crystalline dispersion of the (DNA–protamine) complex is restructured. It is significant that addition of the trypsin inhibitor, di-isopropyl fluorophosphate, completely suppresses the above change in the CD spectrum. The intense negative band in the CD spectrum of the liquid crystalline dispersion of a (DNA–stellin B) complex after trypsin treatment also shows that neither the densities of packing of the DNA molecules in the dispersion nor the presence of PEG in the solution inhibits the enzymatic activity of trypsin.

Comparison of X-ray scattering curves (see figure 8) shows that trypsin treatment is accompanied by a displacement of the position of the peak on the X-ray curve, as well as by a peak broadening. This means, that despite the dense packing of adjacent DNA molecules, the distance between these molecules (and, hence, mobility) is increased.

The optical microscopy textures of phases formed from a (DNA–stellin B) complex before and after trypsin treatment (see figure 9) confirm restructuring of the spatial organization of both liquid crystals and liquid crystalline dispersions. Figure 9(*a*) shows that the phase of a (DNA–protamine) complex has a 'non-specific' texture in PEG-containing water–salt solution. Trypsin treatment results in the appearance of a cholesteric phase with a typical 'fingerprint' texture (see figure 9(*b*)).

Hence, trypsin treatment of liquid crystalline dispersions formed from (DNA–protamine) complexes is accompanied by digestion of protamine molecules and results in restructuring of the spatial packing of DNA molecules.

3.6. *Restructuring space organization of liquid crystals of (DNA–stellin B) complexes following the action of various proteases*

The features of restructuring DNA molecules in dispersions through the action of proteases such as trypsin, α-chymotrypsin, pronase P, papain and thrombin on protamine 'cross links' were compared in this work.

Figures 10–12 show the progressive changes in the CD spectra during 3 different enzyme treatments of (DNA–stellin B) liquid crystalline dispersions. Comparing these results permits a number of conclusions to be drawn. Firstly, in all cases enzyme treatment is accompanied by the appearance of an intense negative band in the CD

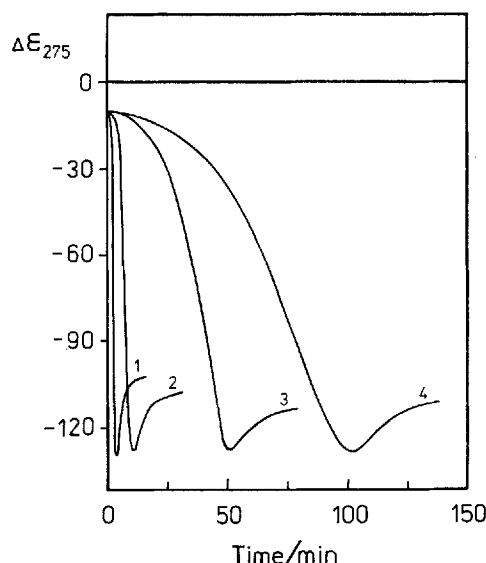

Figure 10. Dependence of the amplitude of the band in the CD spectra ($\lambda = 275$ nm) of liquid crystalline dispersions formed from (DNA–stellin B) complex versus time of trypsin treatment. (1), $C_{trypsin} = 10^{-11}$ M; (2), $C_{trypsin} = 10^{-12}$ M; (3), $C_{trypsin} = 10^{-13}$ M; (4), $C_{trypsin} = 10^{-14}$ M; $C_{PEG} = 170$ mg ml^{-1}; $r = 0{\cdot}6$; 0·225 M NaCl + 5×10^{-3} M phosphate buffer, pH 7·2.

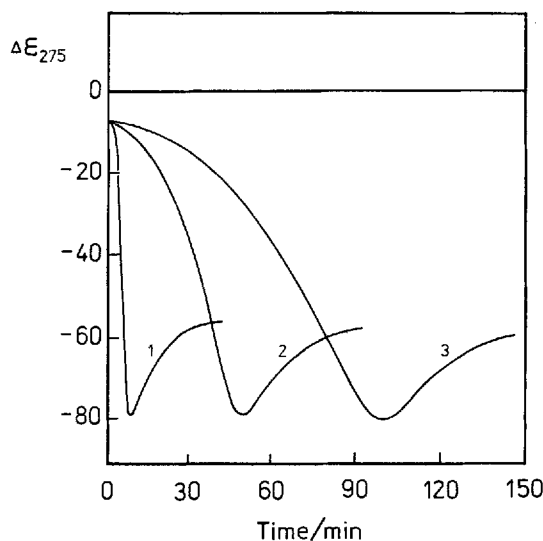

Figure 11. Dependence of the amplitude of the band in the CD spectra (DNA–stellin B) complex versus time of thrombin treatment. (1), $C_{thrombin} = 10^{-7}$ M; (2), $C_{thrombin} = 10^{-8}$ M; (3), $C_{thrombin} = 10^{-9}$ M. $C_{PEG} = 170$ mg ml^{-1}; $r = 0{\cdot}6$; 0·225 M NaCl + 5×10^{-3} M phosphate buffer, pH 7·2.

spectra. Secondly, the rate of reaction depends on the enzyme used. Thirdly, a small decrease in the amplitude of the negative band indicates not only that enzyme action removes the bridging action of the protamine, but also that the cationic protamine molecules, as well as Na^+ ions,

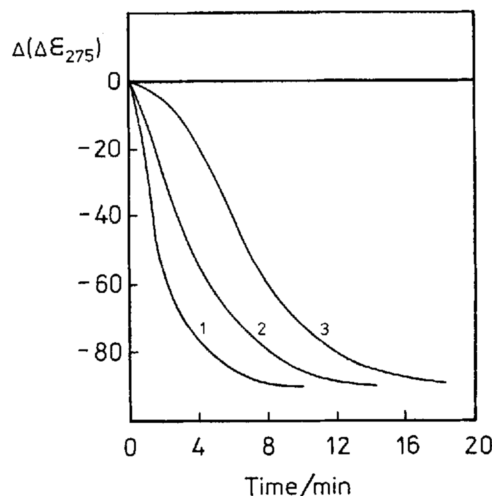

Figure 12. Dependence of the increase in amplitude of the band in the CD spectra ($\lambda = 275$ nm) of liquid crystalline dispersions formed from (DNA–stellin B) complex versus time of pronase P treatment. (1) $C_{pronase\,P} = 2 \times 10^{-6}$ M; (2), $C_{pronase\,P} = 1 \times 10^{-6}$ M; (3), $C_{pronase\,P} = 2 \times 10^{-7}$ M. $C_{PEG} = 170$ mg ml^{-1}; $r = 0{\cdot}6$; 0·225 M NaCl + 5×10^{-3} M phosphate buffer, pH 7·2.

neutralize the negative charges of the DNA phosphate groups. Finally, the hydrolytic digestion and restructuring of the spatial organization of DNA molecules in a liquid crystalline dispersion requires only small concentrations of enzyme; for example, a trypsin concentration of about 10^{-14} M is sufficient.

4. Conclusions

In summary, it is possible to draw a number of conclusions, which are of biological importance. (i) Liquid crystalline dispersions of (DNA–protamine) complexes do not hinder the action of hydrolytic enzymes. (ii) Digestion of the protamine 'cross links' between the DNA molecules (irrespective of the chemical nature of the enzyme) causes a change in the mode of spatial packing of adjacent DNA molecules and the formation of a cholesteric DNA dispersion. (iii) The question of the kinetics of digestion of protamine 'cross links' and the reasons for possible specificity of the action of hydrolytic enzymes under the conditions of formation of DNA liquid crystalline dispersions remain open.

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