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Invited Article
Liquid crystalline dispersions of complexes formed by chitosan with double-stranded nucleic acids†

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Double-stranded molecules of nucleic acids (NAs) were shown to interact with chitosans to form under certain conditions (chitosan molecular mass, content of amino groups, distance between amino groups, pH of solution, etc.) multiple types of liquid crystalline dispersions. The dispersions formed are different in their spatial structures, and hence in the sense and magnitude of the abnormal optical activity. The physicochemical properties of these dispersions were investigated. Time- and temperature-stabilization of dispersions that possess abnormal optical activity were achieved by chemical crosslinking of chitosan molecules in the liquid crystalline dispersions formed from NA–chitosan complexes. The accessibility of these 'NA–liquid crystalline elastomers' with respect to enzyme and drug action was tested. The multiplicity of liquid crystalline forms of DNA–chitosan complexes was possibly explained by the influence of the character of the dipole distribution over the surface DNA molecules on the sense of the spatial twist of the cholesteric liquid crystalline dispersions resulting from these complexes.

1. Introduction

Molecular design based on double-stranded nucleic acids (NAs), fixed in the spatial structure of particles of their cholesteric liquid crystalline dispersions (LCDs) (i.e. the creation of three-dimensional constructions with adjustable physicochemical properties), is a topic of current theoretical and experimental interest and of significant practical importance [1, 2].

A version of molecular design based on the crosslinking of neighbouring NA molecules by polymeric chelate bridges consisting of alternating molecules of anthracycline antibiotics and copper ions was described recently [3]. The crosslinks between NA molecules arise in any direction and their formation is accompanied by the disappearance of 'flow' of the neighbouring NA molecules, i.e. after crosslinking the LCD particles possess a fixed three-dimensional structure. However, the abnormal optical activity of the LCD particles is maintained. The investigation of these LCDs is interesting because the behaviour of the two 'subsystems' (in this case, one is the cholesteric structure formed by the NA molecules, and the second is the cholesteric structure of crosslinks) should be different. Theoretical studies in this field have been initiated [4].

The main limitation on the realization of molecular constructions of this type for fabrication of practical devices (for instance, biosensing units [5]) is their relative instability. As the crosslinking of neighbouring NA molecules in the structure of the LCD particles is carried out by building flat bridges between them, there is an opportunity to increase the stability of the LCD formed. This opportunity is based on the introduction of additional charged groups on the NA surface as a result of complex formation between NA and other polymeric compounds capable of bearing these groups.

Among polymeric compounds, a biodegradable polymer, chitosan (a copolymer, consisting of β-(1→4)-2-amino-2-deoxy-D-glucopyranose and β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose residues) attracts great attention now as a good candidate for building bridges. Indeed, the chemical and spatial structures of chitosan molecules determine their ability to form stable complexes with various compounds [6]. In fact, data obtained recently show that NA molecules might exist in a liquid crystalline state as a result of NA–chitosan complex formation [7]. It should be noted, however, that the properties of the complexes formed by chitosan and NA molecules remain poorly studied [8–10].

The efficiency of molecular constructions based on liquid crystalline dispersions of NA–chitosan complexes

† **In fond memory** of Dr. A.T.Dembo, one of the best known Russian professionals in the field of X-ray analysis of biopolymeric molecules.

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for applications will need to be defined by a panel of properties (such as stability, mobility of structure, mode of packing, etc.) of practical interest. In the present paper the results of investigation of the properties of complexes formed of DNA and RNA molecules with various chitosans, obtained by researchers from the Institute of Molecular Biology of the Russian Academy of Sciences (RAS), the Center of Bioengineering of the RAS, and other Institutes during 1999–2002 are given. These results unequivocally testify that the molecules of the NA–chitosan complex do exist in liquid crystalline state and the properties of the dispersions formed are controlled by many factors that open a way in which to use the liquid crystalline dispersions of NA–chitosan complexes as a background for future molecular constructions.

2. The properties of liquid crystalline dispersions of NA–chitosan complexes

2.1. *The formation of dispersions of NA–chitosan complexes*

DNA–chitosan complexes were prepared by addition of small volumes of an initial aqueous solution of chitosan preparation to a water–salt solution of DNA with stirring. During titration, the absorption spectra of the DNA–chitosan complexes were obtained using a Specord M-40 spectrophotometer within the wavelength region 230–450 nm. Figure 1 shows the change in the absorption spectrum of the water–salt DNA solution ($V=2$ ml, $C_{DNA}=15.5\,\mu g\,ml$) upon titration with small portions of chitosan solution ($V=1$–$4\,\mu l$ of chitosan solution, $C_{chitosan}=2.5$ mg ml). It is seen that, when a 'critical' concentration is achieved, the absorption at $\lambda\sim260$ nm increases and absorption appears in the spectral region $\lambda>320$ nm where neither DNA nor chitosan absorb (an 'apparent' optical density, A_{app},). Such alterations in the shape of the absorption spectrum reflect the formation of a dispersion phase of DNA–chitosan complex whose particles scatter UV light. Similar changes were earlier detected in the case of dispersions resulting from the DNA interaction with synthetic polyamines [11].

The 'critical' concentration of any of the chitosans used, C_{crit} (i.e. the chitosan concentration that is necessary for the formation of dispersions of DNA–chitosan complexes), was determined from the variation in the shape of the absorption spectra. The inset in figure 1 shows the dependence of the 'apparent' optical density measured at $\lambda=340$ nm (A_{340}) on the chitosan concentration in the solution used for the determination of the C_{crit} value, which characterizes the efficiency of DNA–chitosan binding.

Similar alterations in the absorption spectra of double-stranded polydeoxyribonucleotides poly(dA-dT)xpoly(dA-dT) and poly(dG-dC)xpoly(dG-dC), and

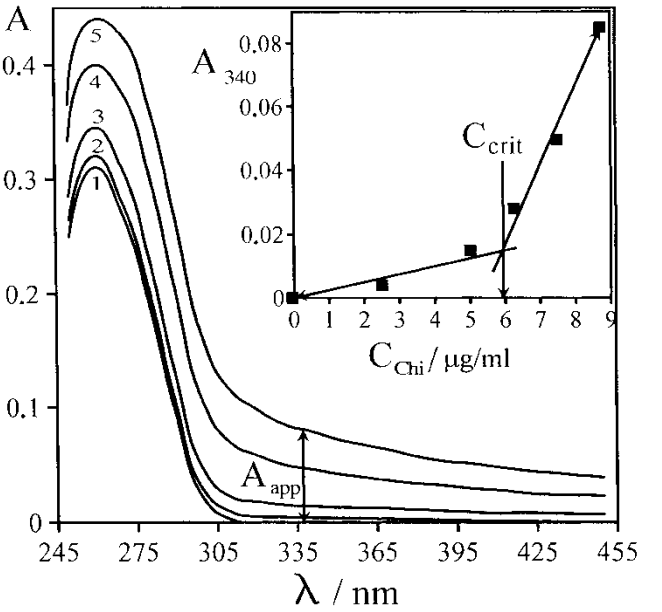

Figure 1. The DNA absorption spectra in the absence (curve 1) and in the presence (curves 2–5) of chitosan in solution. Chitosan concentration in solution: 1–0, 2–2.5, 3–5.0, 4–7.5, 5–8.75 $\mu g\,ml^{-1}$ (chitosan: 85% of amino groups; molecular mass 19 kDa). $C_{DNA}=15.5\,\mu g\,ml^{-1}$, 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Inset: the dependence of the 'apparent' optical density at $\lambda=340$ nm upon chitosan concentration in solution.

of polyribonucleotide poly(I)x poly(C) (used as an analogue of double-stranded RNA), indicating the formation of dispersions of complexes of these polynucleotides with chitosan, are observed when these polynucleotides are titrated with chitosan solution (data not shown).

The dependence of the C_{crit} value on DNA concentration used for preparing the complex is linear [12] . The existence of a linear dependence between the polyamine concentration necessary for DNA aggregation and the total DNA concentration in solution was noted earlier in [13, 14]. The linear character of this dependence indicates that the sizes of separate particles of the dispersion and their refractive index remain constant under the conditions used.

Figure 2 compares the CD spectra of the initial DNA (curve 1) and of dispersions of DNA-chitosan complexes (curves 2–6). It is seen that an intense *positive* band appears in the DNA absorption region ($\lambda\sim270$ nm) when the 'critical concentration' of chitosan is achieved (inset in figure 2). Comparison of figures 1 and 2 shows that the values of C_{crit} determined by different methods virtually coincide. This means that titration of the DNA with chitosan is accompanied not only by the formation of dispersions of DNA–chitosan

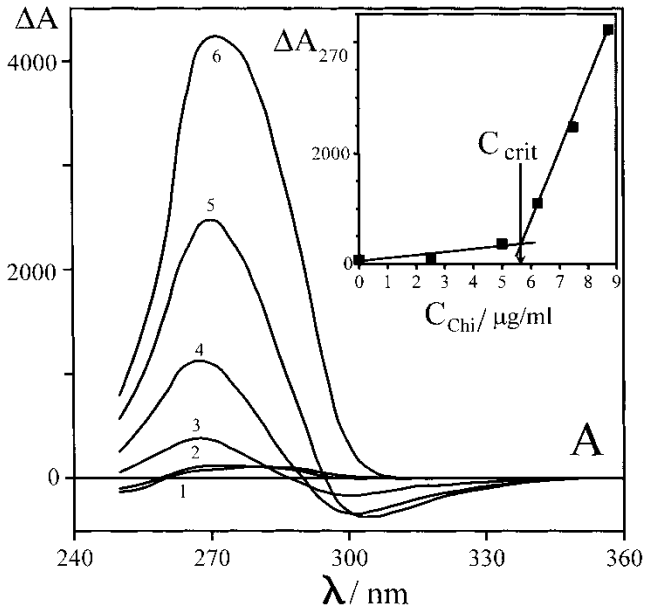

Figure 2. The CD spectra of DNA in the absence (curve 1) and in the presence (curves 2–6) of chitosan in solution. Chitosan concentration in solution: 1–0, 2–2.5, 3–5.0, 4–6.25, 5–7.5, 6–8.75 $\mu g\,ml^{-1}$ (chitosan: 85% of amino groups; molecular mass 19 kDa). $C_{DNA} = 15.5\,\mu g\,ml^{-1}$, 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Inset: the dependence of the amplitude of the band in the CD spectra of the DNA–chitosan liquid crystalline dispersions at $\lambda = 270$ nm upon chitosan concentration in solution. Scale for ΔA: 10^{-6} optical units.

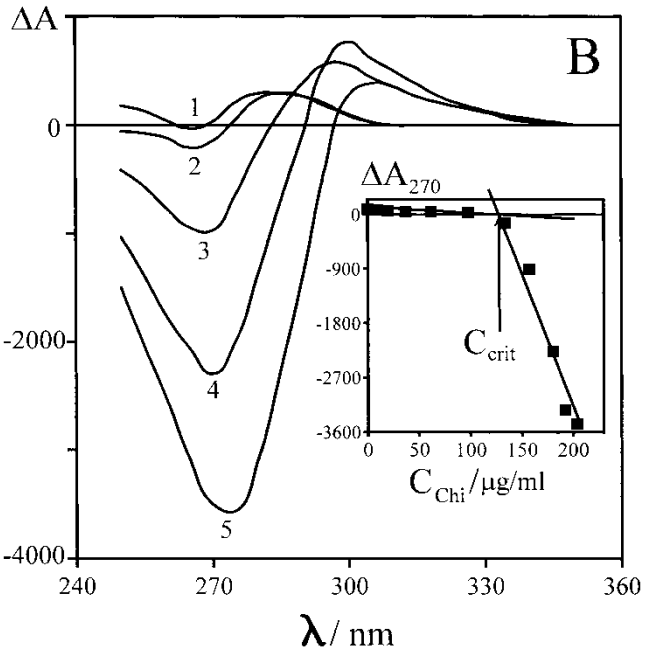

Figure 3. The CD spectra of poly(I) x poly(C) in the absence (curve 1) and in the presence (curves 2–5) of chitosan in solution. Chitosan concentration in solution: 1–0, 2–134.0 3–157.0, 4–181.0, 5–204.0 $\mu g\,ml^{-1}$ (chitosan: 85% of amino groups; molecular mass 2 kDa). $C_{poly(I)xpoly(C)} = 18.0\,\mu g\,ml^{-1}$, 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Inset: The dependence of the amplitude of the band in the CD spectra of the poly(I)xpoly(C) – chitosan liquid crystalline dispersions at $\lambda = 270$ nm upon chitosan concentration in solution. Scale for ΔA: 10^{-6} optical units.

complexes that scatter UV-irradiation, but also by the appearance of the intense *positive* band in the CD spectrum. These two processes take place simultaneously only above the threshold of chitosan binding with DNA.

The formation of dispersions due to the interaction of poly(dA-dT)xpoly(dA-dT) and poly(dG-dC)xpoly(dG-dC) with chitosan, similarly to the case of DNA, results in the emergence of *positive* rather than *negative* bands in the CD spectra [15]. The feature of the CD spectra obtained is that the amplitude of the *positive* band for the poly(dG-dC)xpoly(dG-dC)–chitosan complex is approximately twice that for the poly(dA-dT)xpoly(dA-dT)–chitosan complex. This result may indicate that chitosan molecules have a higher 'affinity' for the secondary structure of a (dG-dC)- rather than a (dA-dT)-containing polynucleotide.

Figure 3 shows the CD spectra of the liquid crystalline dispersion of poly(I)xpoly(C)–chitosan complexes. An intense *negative* band appears in the CD spectrum as chitosan binds to poly(I)xpoly(C). As in the case of the dispersions of DNA–chitosan complexes, the *negative* band appears only after a 'critical' extent of chitosan binding to poly(I)xpoly(C) has been achieved (inset in figure 3).

Theoretical calculations of CD spectra of NA dispersions [16] show that the abnormal band in the CD spectrum located in the absorption region of the nitrogen bases reflects the character of the spatial packing of NA molecules in the particles of the resulting liquid crystalline cholesteric dispersion. In our case, the abnormal band in the CD spectrum reflects the appearance of a spatially twisted (cholesteric) structure of the NA–chitosan complexes in particles of the liquid crystalline dispersion. However, in contrast to 'classical' DNA cholesterics [17], which possess abnormal *negative* bands in the CD spectra and hence relate to *left-handed* twisting of the spatial cholesteric structure, the cholesterics of DNA–chitosan or (polydeoxyribonucleotide–chitosan) complexes are characterized by *right-handed* twisting. In addition, the liquid crystalline dispersion of poly(I)xpoly(C)–chitosan complexes has a *negative* band in the CD spectrum, in contrast to the 'classical' cholesterics of poly(I)xpoly(C)

formed by phase exclusion and having a *positive* band in CD spectrum.

There is a question about the magnitude of the abnormal optical activity observed for the liquid crystalline dispersions of NA–chitosan complexes. Usually, the abnormal optical activity of liquid crystalline dispersions of DNA-chitosan and poly(I)xpoly(C)–chitosan complexes is smaller than the activity of 'classical' cholesterics, but it is comparable.

2.2. *The dependence of formation efficiency and optical activity for the liquid crystalline dispersions of NA–chitosan complexes on chitosan molecular mass*

Figure 4 shows the dependence of the C_{crit} value on the molecular mass of the chitosan samples used for preparing dispersions of poly(I)xpoly(C) (curve 1) or DNA (curve 2). It is seen that the efficiency of NA–chitosan complex formation sharply increases with increase in chitosan molecular mass. This is accompanied by a decrease in the chitosan concentration necessary for NA condensation. However, starting from a molecular mass of chitosan about 4 000 Da, i.e. at a certain chain length of the chitosan molecule, the efficiency of this process is practically independent of molecular mass. One can consider the dependence in figure 4 as an indication of the cooperative binding of chitosan to double-stranded NA molecules. Assuming the 'average' molecular mass of a chitosan monomeric unit to be about 200, one may say that starting from a length of chitosan equivalent to (4 000/200 = 20 monomeric units, i.e. a length of about 100 Å [18, 19], chitosan begins to interact cooperatively with NA molecules. This evaluation ignores the fact that, under the selected conditions, only 50% of chitosan amino groups can interact electrostatically with NA phosphate groups and, furthermore, that due to features of the spatial structure of chitosan molecules [18, 19], amino groups could interact with negatively charged phosphate groups of NA in a 'next-but-one' manner. The result above means, for instance, that in the case of double-stranded DNA the minimal fragment capable of forming an 'insoluble' complex with chitosan should involve 100 Å/3.4 Å, i.e. about 30 base pairs; this agrees well with the assessment (15–20 base pairs) of other authors [8] based on the precipitation of DNA molecules under the action of chitosan. Thus, there is a definite limit in the length of chitosan molecules that triggers the cooperative formation of NA–chitosan complexes.

The abnormal optical activity of dispersions of DNA–chitosan and poly(I)xpoly(C)–chitosan complexes shows different dependences on chitosan molecular mass (figure 5). It is seen that the abnormal optical activity of the dispersions of DNA–chitosan complexes occurs within a wide range of chitosan

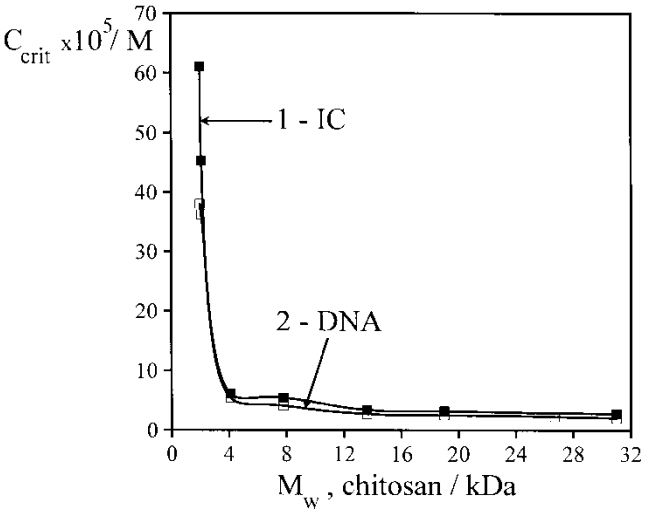

Figure 4. The dependence of critical concentration of the chitosan monomeric units on molecular mass of chitosan (85% of amino groups) preparations for the poly(I)xpoly(C)–chitosan (curve 1) and DNA–chitosan (curve 2) complexes. $C_{DNA} = 15.5\,\mu g\,ml^{-1}$, $C_{poly(I)xpoly(C)} = 15.8\,\mu g\,ml^{-1}$; 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85. C_{crit} : see figure 2 (in the case shown, the C_{crit} value is expressed as molar concentration of chitosan repeating units — sugar residues; the unit mean molecular mass is 220.5 Da).

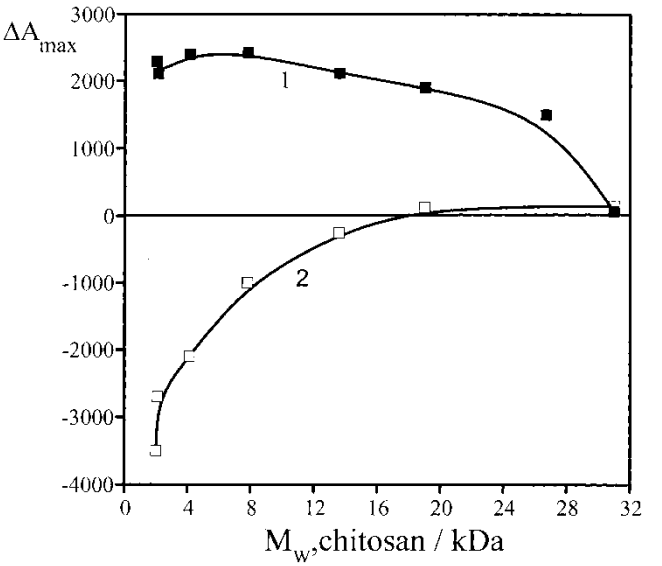

Figure 5. The dependence of maximum amplitude in the CD spectrum at $\lambda = 270$ on molecular mass of chitosan (85% of amino groups) preparations for the DNA–chitosan (curve 1) and poly(I) x poly(C)–chitosan (curve 2) complexes. $C_{DNA} = 15.5\,\mu g\,ml^{-1}$, $C_{poly(I)xpoly(C)} = 18.0\,\mu g\,ml^{-1}$; 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Scale for ΔA: 10^{-6} optical units.

molecular masses (curve 1), whereas, for poly(I)xpoly(C)–chitosan complexes, the region of occurrence of dispersions with abnormal optical activity is much narrower (curve 2). In addition, at a molecular mass of chitosans greater than 30 kDa, both types of NA are capable of forming dispersions with no abnormal optical activity. Thus, depending on chitosan molecular mass, one could produce dispersions of NA–chitosan complexes both possessing abnormal optical activity and having no such activity.

2.3. *The pH dependence of the formation of DNA–chitosan liquid crystalline dispersions*

The apparent pK value of chitosan amino groups is about 6.5, this value being dependent on the extent of deacetylation of the chitosan (content of amino groups in the chitosan molecule) [20]. First, it was of interest to determine the range of pH values in which chitosan could form optically active complexes with DNA [12]. Figure 6 shows the dependence of C_{crit} and $\Delta\varepsilon_{max}$ values for DNA–chitosan complexes on the pH of solutions used for the preparation of the liquid crystalline dispersions. (The value of $\Delta\varepsilon_{max}$ characterizes the molar circular dichroism corresponding to the maximal amplitude of the positive band in the CD spectrum ($\Delta\varepsilon_{270}$), and reflects the molecular optical activity of the nitrogen bases in the DNA molecules involved in the dispersions. The amplitude of the abnormal band in the CD spectrum of DNA–chitosan complexes expressed as $\Delta\varepsilon_{270}$ ($\sim$85 units) exceeds substantially the value of $\Delta\varepsilon_{270}$ ($\sim$2 units) for free DNA molecules. Such a difference in the $\Delta\varepsilon_{max}$ values confirms that the particles of DNA–chitosan complexes have an ordered spatial structure.

It should be noted that the maximum amplitude in the CD spectrum is observed in solutions with pH $\sim$ 6.8, which corresponds to the pK value of chitosan in a water–salt solution. Since the ionization extent of chitosan is close to 50% under these conditions [20], one may suppose that the presence of a certain number of charged groups in these molecules is necessary for the optimum cholesteric packing of the DNA–chitosan complexes. It is also seen that there is a relation between the ionization degree of the chitosan used for preparing the particles of the liquid crystalline dispersion and the abnormal optical properties of these particles.

'Critical' concentrations of chitosan necessary for the formation of DNA–chitosan complexes remain virtually constant in the pH range <7, but a sharp increase is observed at pH >7. At pH $\sim$ 8, i.e. under conditions involving low ionization of chitosan amino groups, the changes in the absorption spectra indicate (data not shown) the formation of light-scattering particles of DNA–chitosan complex, whereas no alterations are observed in the CD spectra ($\Delta\varepsilon_{270} \sim 2$ units).

This result indicates again that there are at least two families of particles in liquid crystalline dispersions of DNA–chitosan complexes differing in the spatial packing in character: the particles of the first family have an intense band in the CD spectrum, whereas the formation of the particles of the second family causes no changes in the spectrum. It may also be noted that there is a correlation between the ionization extent of the chitosan molecules and the ability of DNA–chitosan complexes to produce cholesteric liquid crystalline dispersions.

2.4. *The dependence of the formation of the DNA–chitosan liquid crystalline dispersions on solution ionic strength*

As noted above, the C_{crit} value depends on DNA concentration. However, as in the case of other polyamines producing complexes with DNA, there is a complex dependence for the formation efficiency of DNA–chitosan complexes on the properties of both the chitosan molecules and the solvent (figure 6). Similarly to poly(amino acids) and polyamines whose chemical structure affects C_{crit}, the value of C_{crit} for chitosan also depends on its chemical structure or, more precisely, it depends on the amino group content in the chitosan

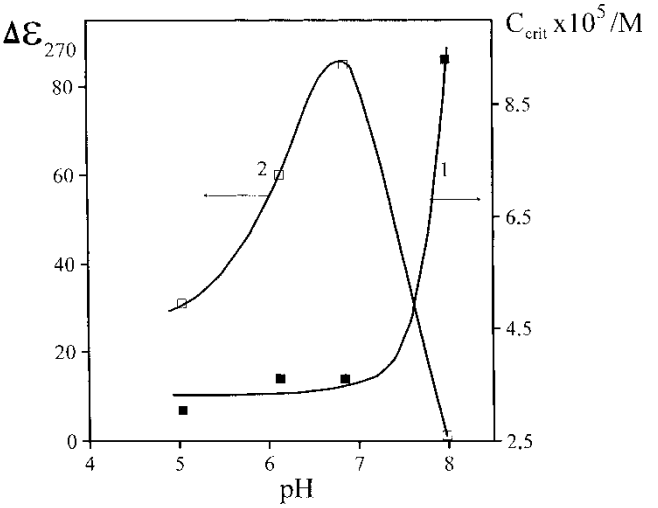

Figure 6. pH dependence of chitosan critical concentration (C_{crit}) necessary for the formation of liquid crystalline dispersions (curve 1, right-hand ordinate) and molar dichroism value ($\Delta\varepsilon_{max}$) at $\lambda = 270$ nm in the CD spectra of the DNA-chitosan liquid crystalline dispersions of DNA-chitosan complexes (curve 2, left-hand ordinate). Chitosan: 85% of amino groups, molecular weight 19 kDa. $C_{DNA} = 15.5\,\mu g\,ml^{-1}$, 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.86.

preparation (chitosan MW = 14.6 kDa). Each chitosan preparation has a definite range of solution ionic strength necessary for the formation of DNA–chitosan complexes with an abnormal band in the CD spectrum. At high ionic strength, light-scattering liquid crystalline dispersions of DNA–chitosan complex do not form at all (right-hand portions of curves 1, 2). On the other hand, dispersions without abnormal optical activity occur at very low ionic strength. In particular, a chitosan preparation (85% of amino groups) produces a light-scattering dispersion of DNA–chitosan complex in a solution with ionic strength lower than 0.02 M. However, no changes in the CD spectrum are observed. A similar situation is observed for a chitosan preparation (46% of amino groups) producing dispersions at 0.1 M ionic strength.

The dependence shown in figure 7 reflects the polyelectrolyte nature of DNA–chitosan complex formation. Since the C_{crit} value indicating 'critical' chitosan concentration necessary for the formation of DNA–chitosan complexes is associated with the equilibrium constant of DNA–chitosan complex formation, one would expect a linear dependence of $\log C_{crit}$ on $\log$[NaCl] [21]. Indeed, considering that points A–F measured under various conditions belong to a 'common' curve, such a dependence is observed for chitosan preparations containing 66, 75, and 85% of amino groups (at ionic strength up to 0.5 M). However, the straight line obtained does not include points related to the chitosan sample containing 46% of amino groups. This indicates that the conformation of the chitosan main chain in combination with the concentration (position) of ionized amino groups is an important factor for the formation of polyelectrolyte complexes of this amino sugar with DNA.

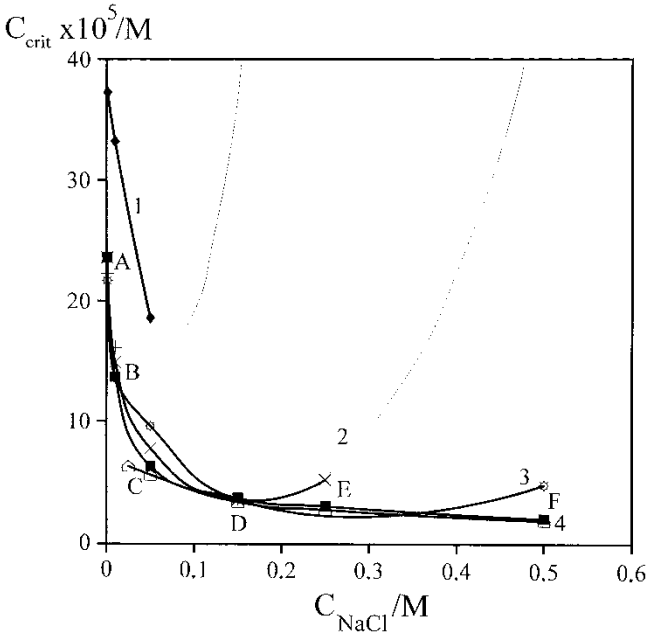

Figure 7. The dependence of the critical chitosan concentration on concentration of NaCl in solution. 1–46, 2–66, 3–75 and 4–85% NH_2 groups in the chitosan molecule; mean molecular masses of the chitosan monomeric units: 212.66, 216.86, 218.75, and 220.85 Da, consecutively. Points A, B,C,D, E and F are average values of C_{crit} at specific NaCl concentrations for the chitosan samples containing 66–85% NH_2 groups. Chitosan molecular mass: 14.6 kDa. $C_{DNA} = 15.5\,\mu g\,ml^{-1}$, 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85.

2.5. *The dependence of the abnormal optical activity of liquid crystalline dispersions of NA–chitosan complexes on the distance between amino groups in the chitosan molecules*

Figures 8 and 9 compare the dependences of the abnormal optical activity of DNA–chitosan and poly(I)xpoly(C)–chitosan complexes (ΔA_{max}) in solutions of various ionic strength upon the distance between amino groups in the chitosan molecules used for preparing dispersions [12]. First, one can see that for both types of NA–chitosan dispersion the abnormal optical activity is a complex function of the distance between amino groups in the chitosan molecules.In the case of dispersions of DNA–chitosan complexes (curves 1–3, figure 8) the amino group content determines the possibility of producing two types of cholesteric liquid crystalline dispersion having either negative or positive abnormal bands in the CD spectra. Furthermore, definite contents of these amino groups can result in dispersions having no abnormal optical activity, the ΔA_{max} value becoming zero at both long and short distances between amino groups in the chitosan molecules. Second, the abnormal optical activity of DNA–chitosan dispersions is a function of ionic strength. The higher the ionic strength, the lower the amplitude of the abnormal band in the CD spectra (compare curves 1, 4, 5) at fixed content of amino groups and molecular mass of the chitosan molecules. Finally, in the case of DNA–chitosan dispersions, the decrease in chitosan molecular mass from 8–14 to 5 kDa results in displacement of the dependences obtained to the region of lower amino group content (compare curves 1,2 and 3 in figure 8).

In the case of dispersions of poly(I)xpoly(C)–chitosan complexes, one can produce cholesteric liquid crystalline dispersions having only negative abnormal bands in the CD spectra irrespective of the molecular mass of the chitosan molecules (figure 9). In addition, at definite contents of these amino groups, one can form dispersions

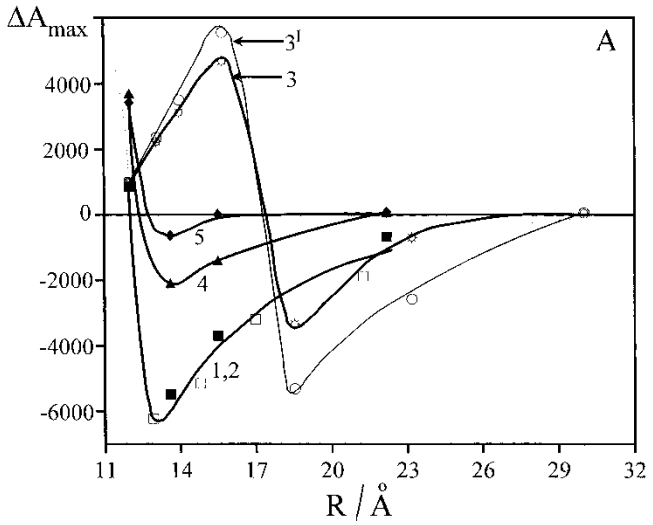

Figure 8. The dependence of the maximum amplitude of the band ($\lambda = 270$ nm) in the CD spectra of the DNA–chitosan liquid crystalline dispersions upon the average distance R between amino groups in the chitosan molecules. The average distance R between amino groups in chitosan molecules was estimated from R (Å) $= 2 \times (5.15 \times 100\%)/[NH_2]$, where 5.15Å is the distance between two neighbouring repeating chitosan units, coefficient 2 takes into account the fact that, due to steric restrictions, neighbouring chitosan amino groups participate in the neutralization of the negative charges of phosphate groups of the DNA in an alternating mode; $[NH_2]$ is the percentage of amino groups in the chitosan sample. Curve 1 – (filled squares) molecular mass of chitosan = 14.6 kDa; curve 2 – (squares) molecular mass of chitosan = 8.4 kDa; curve 3 – molecular mass of chitosan = 5.0 kDa, curve 3^I – takes into account the concentration of 'free' (i.e. non-complexed with chitosan) DNA molecules; curve 4 – molecular mass of chitosan = 14.6 kDa (0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.86); curve 5 – molecular mass of chitosan = 14.6 kDa (0.5M NaCl + 0.001M sodium phosphate buffer, pH 6.86). Curves 1–5 are based on total DNA concentration in the solutions; $C_{DNA} = 15.5\ \mu g\ ml^{-1}$, 0.05M NaCl + 0.001M sodium phosphate buffer, pH 6.85.

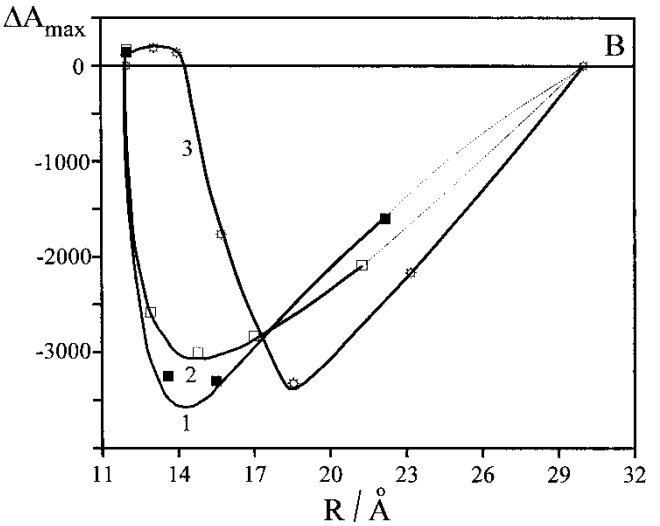

Figure 9. The dependence of the maximum amplitude of the band ($\lambda = 270$ nm) in the CD spectra of the poly(I) x poly(C)–chitosan liquid crystalline dispersions upon the average distance R between amino groups in the chitosan molecules. Curve 1 – molecular mass of chitosan = 14.6 kDa; curve 2 – molecular mass of chitosan = 8.4 kDa; curve 3 – molecular mass of chitosan = 5.0 kDa, All these curves are based on total DNA concentration in the solutions. $C_{poly(I)xpoly(C)} = 15.5\ \mu g\ ml^{-1}$, 0.05M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Scale for ΔA: 10^{-6} optical units.

having no abnormal optical activity. As in the case of DNA–chitosan dispersions, the decrease in molecular mass of chitosan to 5 kDa is accompanied by displacement of the dependences to the region of lower amino group content (compare curves 1, 2 and 3). Thus, the liquid crystalline dispersions of NA–chitosan complexes have multiple spatial forms depending on specific preparation conditions.

The difference in the shapes of curves specific to DNA- and poly(I)xpoly (C)–chitosan complexes (figures 8 and 9) permits the following suggestion to be made. The details of the local structure of NA–chitosan complexes depend on (i) the peculiarities of helical grooves on the surface of the NA molecules, where the negatively charged phosphate groups are located, and probably, (ii) the peculiarities of the helical structure of the chitosan molecules, such as the sense of helical structure, that provide the most efficient steric contact with NA–molecules. Chitosan interacts with NA in such a manner that amino groups of the chitosan sugar residues not only neutralize the negative charges of the NA phosphate groups, but also create a particular distribution of positively charged amino groups in proximity to the NA surface [22]. Thus the orientation of the chitosan molecules and the distribution of amino groups of chitosan sugar residues on the surface of the NA molecules determine the character of the interaction of neighbouring, approaching NA molecules and, hence, the direction of the spatial twist of these molecules in the resulting liquid crystalline dispersions. One may suppose that not only the chemical, but also the spatial structure of chitosan affects the character of the packing of NA–chitosan complexes in the particles of the liquid crystalline dispersions.

Thus, the character of the spatial packing for the molecules of DNA–chitosan and poly(I)xpoly(C)–chitosan complexes in liquid crystalline dispersions depends on the parameters of the secondary structure of the NA molecules and on the mode of distribution of

the chitosan molecules in proximity to the surface of the NA molecules. Hence, in contrast to 'classical' cholesterics, the distribution of positive charges (distance between charges, chitosan conformation, etc.) in the chitosan molecule interacting with NA is the determining factor for the spatial structure of the resulting dispersions. Such an interaction provides not only the neutralization of the negative charges of NA phosphate groups, but also makes it possible to 'introduce' additional positive charge into the surface structure of the NA molecules that, in turn, determines the value of the anisotropic contribution to the free energy of interaction of neighbouring NA molecules. This means that the change in the distribution of positive charges in chitosan molecules interacting with NA can influence the mode of spatial twist of the NA–chitosan complexes.

2.6. *The time- and temperature-stability of DNA–chitosan liquid crystalline dispersions*

Figure 10 shows the dependence of the amplitude of the intense band in the CD spectrum of the DNA–chitosan dispersion (chitosan MW = 5 kDa) upon time (curve 1) and temperature (curve 2). The time-stability

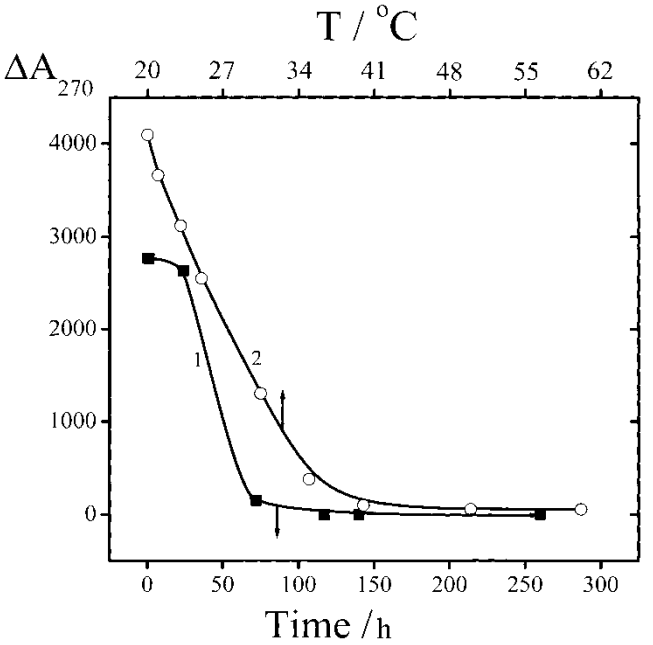

Figure 10. The dependence of the amplitude of the band in the CD spectra of the DNA–chitosan liquid crystalline dispersions at $\lambda = 270$ nm on time (curve 1) and temperature (curve 2). Chitosan concentration = 17.4 μg ml^{-1} (chitosan: 65% of amino groups; molecular mass 5.0 kDa). $C_{DNA} = 15.0$ μg ml^{-1}, 0.05M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Scale for ΔA: 10^{-6} optical units.

of this dispersion can reach 25 h. The relative time-stability of the DNA–chitosan dispersion reflects a tendency of particles to aggregate. As a result, the real concentration of particles begins to drop and, hence, the measured abnormal optical activity is diminished. The possibility is not excluded that a tendency of chitosan molecules, bound in complex with DNA, to form additional 'physical bonds' with free chitosan molecules in solution or in neighbouring particles plays a role in the process of aggregation. Temperature increase is accompanied by a drop in abnormal optical activity of the DNA–chitosan dispersion until this disappears at a temperature of about 40°C. Also, increase in temperature results in the disappearance of 'apparent' optical density in the absorption spectra (data not shown).

In contrast to 'classical' cholesteric liquid crystalline DNA dispersions, it is interesting to note a very low extent of restoration of abnormal optical activity after cooling the 'heated' DNA–chitosan particles. This means that temperature increase is accompanied by two processes. First, dissosiation of the DNA–chitosan complexes can take place; as a result, the disintegration of the spatial structure of the particles of the DNA–chitosan liquid crystalline dispersion occurs. Second, during the heating the reordering of chitosan molecules on the surface of DNA molecules or the change in spatial structure of adjacent chitosan molecules due to formation of thermal crosslinks takes place [23]. This prevents adoption of the proper spatial orientation of the molecules of the DNA–chitosan complexes and, hence, the restoration of a high degree of abnormal optical activity of the DNA–chitosan dispersions after cooling. Thus, the DNA–chitosan dispersion has only a limited time- and temperature-stability.

The above data pose the question of the existence of possible chitosan crosslinks in the DNA particles, i.e. a question about the 'fluidity' of the DNA–chitosan molecules in particles of the dispersions.

2.7. *The accessibility of DNA–chitosan liquid crystalline dispersions to enzymes*

In order to detect the enzymatic degradation of chitosan molecules complexed with DNA, in DNA–chitosan liquid crystalline particles, we have not used the optimum conditions (pH and temperature) necessary for enzyme action, but paid attention to stability and disintegration of the particles. We have tested the degradation of chitosan by various enzymes with established chitosanolytic activities [24], including lysozyme and an enzyme complex produced by Streptomyces kurrssanovii [25]. The effect of treatment of DNA–chitosan liquid crystalline dispersions (chitosan

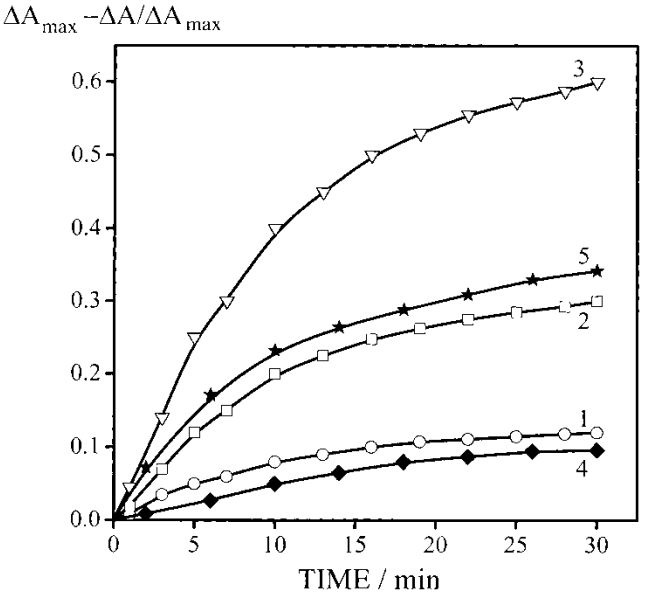

Figure 11. The relative change in the amplitude of the band at $\lambda = 270$ nm in the CD spectra of the DNA–chitosan liquid crystalline dispersions upon time of treatment by lysozyme (Sigma) (curves 1–3) and chitinase (Sigma) (curves 4,5). 1–1, 2–2.5, 3–5 $\mu g\,ml^{-1}$ of lysozyme, 4–2, 5–10 $\mu g\,ml^{-1}$ of chitinase. Chitosan: 85% of amino groups; molecular mass 19 kDa. $C_{DNA} = 16.5\,\mu g\,ml^{-1}$, 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85, 30°C.

MW = 19 kDa, 85% of amino groups) with various concentrations of enzymes was monitored using the reduction in the amplitude of the positive band in the CD spectrum specific to these dispersions.

Figure 11 shows, as an example, the dependence of optical activity upon time of treatment for lysozyme (curves 1–3) and chitinase obtained from Sigma (curves 4,5). One can see that the action of the enzymes on the chitosan results in a rapid decrease in abnormal optical activity of the dispersion. This means that the enzymatic splitting of the chitosan molecules causes full disintegration of the dispersions.

It should be noted that the efficiency of disruption of DNA–chitosan liquid crystalline dispersions is directly proportional to the concentration of enzymes used. Calibration curves allow one to detect concentations of enzymes as low as 0.5–1 μg ml (data not shown). Taking into account various modes of chitosan splitting by enzymes, our data show that different sites in the polymeric chain of the chitosan forming complexes with DNA are accessible to enzyme action.

Comparison of the curve of chitosan degradation in the DNA–chitosan liquid crystalline dispersions by lysozyme with a reported curve obtained for free chitosan [26], reveals their similarities. This means that a large number of chitosan molecules is accesible for enzymatic degradation in the liquid crystalline system. Hence, the number of possible 'physical' crosslinks between chitosan molecules, that can influence the degradation rate, is relatively low, i.e. the spatial structure of the dispersion has a relatively high degree of 'fluidity'.

2.8. *The accessibility of the base pairs of DNA molecules in the content of DNA–chitosan liquid crystalline dispersions to drugs*

Figure 12 shows the CD spectrum of a dispersion of DNA–chitosan complex in water–salt solution (curve 1) and the spectra of this dispersion after treatment with an anticancer anthraquinone, mitoxantrone (MX) (curves 2–6), capable of intercalating between base pairs of DNA molecules. It is seen that the amplitude of the positive band in the UV region of the CD

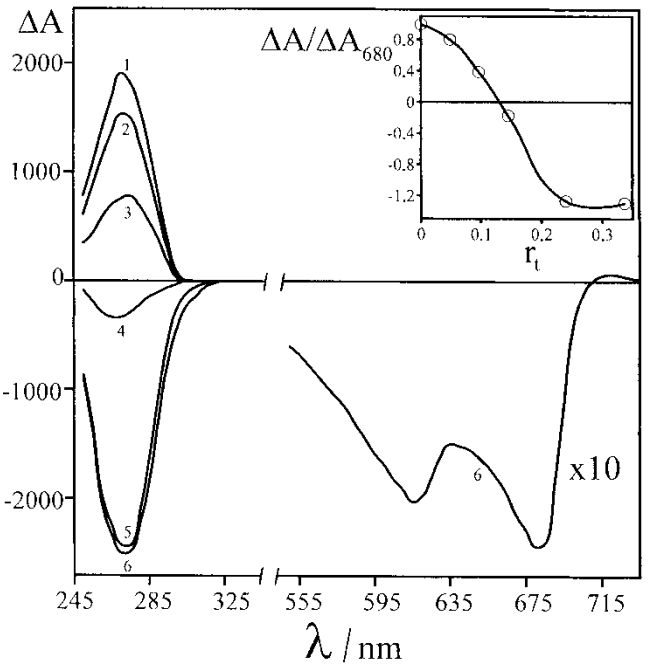

Figure 12. Circular dichroism spectra of liquid crystalline dispersions of the DNA–chitosan complex in the absence (curve 1) and in the presence (curves 2–6) of MX in solution. 1–0, 2–2.2×10^{-6}, 3–4.4×10^{-6}, 4–6.58×10^{-6}, 5–10.9×10^{-6}, 6–15.3×10^{-6} M MX in solution. Chitosan concentration: 9.98 $\mu g\,ml^{-1}$ (chitosan: 85% of amino groups; molecular mass 19 kDa). $C_{DNA} = 15.0\,\mu g\,ml^{-1}$, 0.15M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Inset: The dependence of the relative change in the amplitude of the band at $\lambda = 270$ nm in the CD spectrum of the liquid crystalline dispersion of the DNA–chitosan complex on the r_t value (r_t = relation of molar concentration of MX to molar concentration of the DNA bases). Scale for ΔA: 10^{-6} optical units.

spectrum begins to decrease as the MX concentration increases and the positive band changes its sign to negative at an extent of MX binding to DNA in the chitosan complex characterized by the value of $r_t > 0.15$ (inset in figure 12).

An additional negative band arises in the absorption region of MX after treatment of the dispersion of DNA–chitosan complex with MX. The emergence of two negative bands (figure 12) located in different regions of the CD spectrum could be explained only in terms of insertion of intercalator molecules between the base pairs of DNA molecules involved in the liquid crystalline dispersion of the DNA–chitosan complex. These two bands confirm that the molecules of the DNA–chitosan complex form the cholesteric liquid crystalline dispersion. Similar results indicating the 'inversion' of the sign of the CD band of liquid crystalline dispersions of the DNA–chitosan complex were obtained when these dispersions were treated with intercalators such as the anthracycline antibiotics daunomycin (DAU) or aclacinomycin A, as well as compounds such as PicoGreen, and ethidium bromide.

Here, one can stress that insertion of drug molecules between base pairs of preformed particles of the liquid crystalline dispersion of initial DNA molecules indeed results in the appearance of an additional band in the CD spectra in the drug absorption region, but it is never accompanied by inversion of the sense of the abnormal optical activity under standard conditions. In agreement with data shown in figure 10, the intense bands in the CD spectra of liquid crystalline dispersions of DNA–chitosan complexes treated with intercalators are observed for a long time (longer than 60 h).

When liquid crystalline dispersions of the DNA–chitosan complex were treated with compounds of distamycin type, which locate ('embed') in one of the helical grooves on the surface of the DNA molecule rather than intercalate between base pairs, only minor changes in the shape of CD spectra were detected. Most likely, this fact reflects the impossibility of anisotropic 'embedding' of the molecules or its absence for steric reasons. Consequently, the character of spatial packing of molecules of the DNA–chitosan complex in the particles of liquid crystalline dispersion could be altered only through insertion of intercalator molecules between DNA base pairs.

To reveal how the secondary structure of the nucleic acid and its nucleotide sequence can affect the efficiency of the change in spatial structure of molecules of DNA–chitosan complex under the action of intercalators, we compared the CD spectra of liquid crystalline dispersions of different polynucleotides. Treatment of dispersions of poly(dG-dC)xpoly(dG-dC)–chitosan and poly(dA-dT)xpoly(dA-dT)–chitosan with DAU results, similarly to the case of DNA, in virtually 100% inversion of the intense positive bands of these dispersions and the appearance of negative bands in the CD spectra [15]. Treatment of the liquid crystalline dispersion of poly(I)xpoly(C)–chitosan complex with daunomycin leads to no marked changes in the CD spectrum of this dispersion. This can be predicted, because DAU molecules do not intercalate between base pairs of poly(I)xpoly(C) and hence cannot arrange anisotropically in the structure of the particles of this dispersion.

One can add that the relationship between the amplitudes of the intense bands located, for instance, in the DNA and MX absorption regions (i.e. 1/5 in the case of 'classical' liquid crystalline DNA dispersions) is only 1/10 in the case of particles formed of DNA–chitosan complexes. This difference may reflect the fact, that the chitosan molecules are so tightly bound to DNA that this leads to a decreased rate of permeation of drug molecules throughout the chitosan shell. On the other hand, this fact suggests the presense of a definite degree of physical crosslinks between chitosan functional groups that influences the penetration of MX through the chitosan shell on the surface of the DNA molecules. However, both suggestions speak in favour of a relatively low number of chitosan 'bonds' inside the particles.

Thus, the structure of natural molecules of deoxyribonucleic acid and synthetic polydeoxyribonucleotides in the particles of liquid crystalline dispersions containing chitosan is accessible to interaction with intercalators. The intercalation is accompanied by a change in the sign of the spatial twisting of the NA–chitosan cholesterics. This effect is absent in the case of 'classical' cholesterics produced from NA molecules via phase exclusion, i.e. the initial cholesteric structure of the NA–chitosan complexes is relatively 'fluid'. Hence, the number of possible crosslinks in the DNA–chitosan particles is small and cannot influence strongly the 'fluidity' of neighbouring molecules of DNA–chitosan complexes.

2.9. *X-ray scattering parameters of the phases of nucleic acid – chitosan complexes*

The samples of phases of NA–chitosan complexes for X-ray diffraction studies were obtained by low speed centrifugation (5 000 rpm, 40 min, 4°C, K23 centrifuge, Germany) of liquid crystalline dispersions of NA–chitosan complexes obtained in a large volume ($V = 100$ ml) after measurement of their absorption and CD spectra. The precipitate obtained by centrifugation, i.e. a phase of NA–chitosan complex, was placed with a small amount of solvent in a thin-walled quartz capillary (0.5 mm in

diameter) and X-ray diffraction analysis was performed. We used an Amur-K small-angle X-ray diffractometer manufactured in the Shubnikov Institute of Crystallography of the Russian Academy of Sciences. The radiation source used was a BSV-29 acute-focal X-ray tube with a copper anode; the CuK_α radiation wavelength is 1.54 Å. Scattered radiation was registered with a linear position-sensitive proportional detector manufactured in the Institute of Nuclear Physics, Siberian Division of the Russian Academy of Sciences, Novosibirsk, Russia). Measurements were made in angle range from 0.2° to 7.4°. X-ray parameters commonly used for describing scattering curves, such as size of 'crystallites' L, 'interaction radius' r_m, and 'disorder' parameter Δ/d were estimated in [27].

Figure 13 shows, as an example, the X-ray scattering curves for the phase prepared from DNA–chitosan complex at 20°C (curve 1), for the same phase heated to 80°C (curve 2) and then cooled to 20°C (curve 3) [28]. The scattering curves for the phase formed by this procedure and having an abnormal optical activity of positive sign show one distinct Bragg reflection. The absence of highest orders of the small angle reflection in the X-ray scattering patterns points to the absence of a regular three dimensional order in the arrangement of the DNA–chitosan complexes, i.e. the phase has no ideal crystalline structure. This means that only 'short-range' orientational order, typical of liquid crystalline phases prepared from DNA molecules, can exist in the arrangement of neighbouring molecules of the DNA–chitosan complexes. The magnitude of this reflection and the X-ray scattering parameters of the resulting phase are given in table 1.

To determine the parameters of the secondary structure of the DNA forming the complex with chitosan, the X-ray scattering curves within the angles $2.5° < 2\theta < 20°$ were measured. The experimental wide range reflections for the phase of the DNA–chitosan complex confirm that the DNA molecules hold the initial B-conformation.

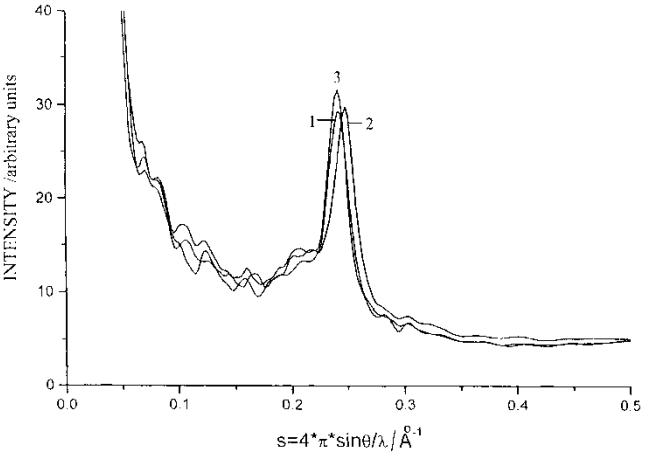

Figure 13. Small-angle X-ray scattering curves of the liquide crystalline phase of the DNA-chitosan complex at different temperatures. 1–20°C, 2–80°C, 3 – specimen 2 after its cooling to 20°C. Chitosan concentration: 20.4 μg ml^{-1} (chitosan: 85% of amino groups; molecular mass 19 kDa), $C_{DNA} = 15.0$ μg/ml^{-1}, 0.15M NaCl + 0.001 M sodium phosphate buffer, pH 6.85.

The data for the phases made of poly(I)xpoly(C) complexes with chitosans of different molecular masses are interesting. The magnitude of the reflections and the X-ray scattering parameters of the phases are listed in table 2. It is shown that the Bragg reflection is expressed extremely weakly for the phase of the poly(I) xpoly(C)–chitosan complex where the chitosan has a molecular mass 19 kDa [28]. In this case the phase formed has no abnormal bands in the CD spectrum (figure 4). At the same time, this reflection is present for the phase of

Table 1. X-ray scattering parameters for the phase formed by the DNA–chitosan complex.

Sample	T °C	d_{Bragg} Å	β_S rad	L, Å	r_m Å	Δ/d_{Bragg}
Phase of DNA–chitosan complex	20	26.060	0.00584	264.115	416.891	0.100[a]
		26.579	0.00989	155.975	246.203	0.131[b]
	40	25.804	0.00525	293.738	463.645	0.094[a]
		25.323	0.01048	147.210	232.364	0.135[b]
	60	25.552	0.00523	295.119	465.821	0.094[a]
		26.060	0.0093	165.419	261.105	0.126[b]
	70	25.315	0.00523	295.122	465.821	0.093[a]
		26.060	0.01050	146.867	231.822	0.134[b]
	80	25.315	0.00582	265.236	418.650	0.098[a]
		25.804	0.00991	155.593	245.593	0.130[b]
Cooled sample	20	26.060	0.00525	293.735	463.645	0.095[a]
		26.579	0.00930	165.852	261.793	0.127[b]

[a]X-ray beam collimation taken into account.
[b]X-ray collimation ignored.

poly(I) xpoly(C)–chitosan complex where the chitosan has a molecular mass 2 kDa and the phase has abnormal optical activity of negative sign.

Analysis of the data presented in tables 1 and 2 underlines a number of important facts. First, the distance between neighbouring NA molecules in the resultant phase ($D_{inter} = 2 \times d_{Bragg}/3^{1/2}$) is about 30 Å. Second, this distance decreases only slightly with temperature change from 20 to 80°C. Third, the value of the small angle reflection is completely restored upon cooling to 20°C. Finally, the NA molecules are ordered in the phases irrespective of the presence or the absence of abnormal optical activity. Comparion of data given in tables 1 and 2 shows that, in spite of the differences in sign of the abnormal optical activity, the X-ray scattering parameters of the phases of DNA–chitosan and poly(I)xpoly(C)–chitosan complexes are very similar. Hence, the sense of the spatial twist of the neighbouring NA–chitosan complexes in the dispersions does not affect the value of the Bragg reflection and its temperature variation.

The above results indicate that the X-ray properties of the phases of NA–chitosan complexs are of interest. These properties differ from those of 'classical' liquid crystalline cholesteric phases produced by phase exclusion of DNA molecules in PEG-containing solutions. First, the distance between DNA molecules in ''classical' cholesteric phases is within 30 to 51 Å [29]. Taking into account that the distance between neighbouring DNA molecules in the phase of DNA–chitosan complexes is about 30 Å, one can conclude that the extent of ordering of adjacent DNA–chitosan complexes in the phase is higher than that of DNA molecules in the classical cholesteric phase. Second, in the case of 'classical' cholesteric phases of initial double–stranded DNA, change in temperature is accompanied by a marked (3–6 Å depending on conditions) increase in the distance between DNA molecules [30] and in the pitch of cholesteric structure. This is not the case for the phases of NA–chitosan complexes.

A very small change in the Bragg reflection (table 1) upon temperature increase allows one to suppose that the molecules of the NA–chitosan complexes in the phases formed as a result of sedimentation of preformed liquid crystalline dispersions are not as 'fluid' as the NA–chitosan molecules in dispersions and pure DNA molecules in cholesteric phases. Additional confirmation comes from the fact that the Bragg reflections coincide with the d values specific to the transition area between the cholesteric and hexagonal phases of DNA molecules [29].

In contrast, figure 10 demonstrates the flexibility of the structure of the DNA–chitosan complexes. Hence, there is a discrepancy between the 'stability' of the Bragg reflection and the alteration of abnormal optical activity as the temperature increases. Taking into account data on the properties of chitosan molecules, it may be proposed that chitosans play a slightly different role in the case of dispersions and sedimented phases of NA molecules. In the case of separated particles of NA dispersions, the chitosan molecules are providing, first, an efficient neutralization of the NA molecules and, hence, a dense packing of neighbouring NA molecules in the microscopic particles of dispersions formed in very dilute solutions. Second, they play the role of the 'medium' affecting the character and the efficiency of interaction of neigbouring NA molecules in particles of dispersions. Of course, due to the tendency of chitosan molecules to form 'bonds' with adjacent chitosan molecules, there is the probabilty of crosslinking both neighbouring NA–chitosan complexes and particles of NA–chitosan complexes. This is confirmed, in part, by the relative instability of NA–chitosan dispersions (figure 10). However, the number of such crosslinks inside NA–chitosan dispersions is small and NA molecules in particles of the liquid crystalline dispersion retain their 'fluidity'. This is confirmed by the change in the sense of abnormal optical activity of the DNA–chitosan particles on treatment by intercalating drugs, in particular, MX (see above, figure 12). The response of the spatial structure of the DNA liquid crystalline dispersion to MX action confirms a low number of chitosan 'bonds' inside the particles. Hence, the layers of molecules of NA–chitosan complexes inside the individual particles of their liquid crystalline dispersions are capable of moving, i.e. they are 'fluid'.

A different situation occurs in the case of sedimented phases. Here, it is necessary to take into account two additional cirsumstances. First, the tendency of chitosan molecules to form networks in solution [23] will

Table 2. X-ray scattering parameters for the phases formed by poly(I)xpoly(C)–chitosan complexes.

Chitosan Mw/kDA	T °C	d_{Bragg} Å	β_S rad	L Å	r_m Å	Δ/d_{Bragg}
19	20	27.441	0.00592	260.481	411.172	0.103[a]
2	20	28.595	0.01362	113.230	178.741	0.160[a]

[a]X-ray beam collimation taken into a account.

provide for formation of bonds (crosslinks) between approaching NA molecules on their concentration during sedimentation, i.e. on formation of macroscopic NA–chitosan phases. Second, additional crosslinking can be formed by heating [23], i.e. by the thermal treatment (table 1) of the concentrated phase used for detection of the Bragg reflection. As a result, the formed (and heated) phases possessing the network of chitosan molecules are capable of not only fixing the spatial position of the NA molecules, but also providing a very low fluidity of NA–chitosan molecules. Hence, the properties of NA–chitosan phases are closer to the properties of so-called liquid crystalline elastomers [31] than those of 'classical' cholesterics. Thus, in contrast to 'classical' cholesteric dispersions and phases formed of 'pure' NA molecules, the behaviour of NA–chitosan dispersions will be different from that of phases with reference to temperature change (compare figures 10 and 13).

Thus, the data obtained indicate that the optical properties of liquid crystalline dispersions of NA–chitosan complexes, as well as the X-ray scattering properties of the phases formed from these dispersions, do not exactly coincide with those of 'classical' cholesterics. This statement is based on the possibility of formation of physical crosslinks between chitosan molecules during concentration of the NA–chitosan liquid crystalline dispersions.

2.10. *Chemical crosslinking of chitosan molecules in DNA–chitosan liquid crystalline dispersions*

Taking into account the results of the X-ray studies on the phases formed by NA–chitosan complexes one can expect high temperature- and time-stability of NA–chitosan liquid crystalline dispersions after chemical crosslinking of the chitosan molecules in the dispersions. We have obtained chitosan crosslinking by using of glutaraldehyde, the most common crosslinking agent [23, 32]. The crosslinking mechanism is explained, mainly, by reaction of the aqueous glutaraldehyde with the amino groups of chitosan to produce imino bonds. The formation of crosslinks between chitosan functional groups leads to decreased hydrophilicity, thus slowing the rate of drug diffusion via the chitosan shell. It was shown that the glutaraldehyde treatment is accompanied by only a small decrease in the amplitude of the abnormal band in the CD spectrum of the liquid crystalline dispersion.

Figure 14 shows temperature (curve 1) and time (curve 2) stability of a DNA–chitosan liquid crystalline dispersion treated by glutaraldehyde. One can see that the dispersion retains its abnormal optical activity for a long time (in contrast to the data presented in figure 10). This unequivocally confirms a substantial increase in stability of the dispersion. The conservation of abnormal optical activity specific to the initial DNA–chitosan liquid crystalline dispersion means that crosslinking occurred in such a way that the spatial position of the DNA molecules is fixed, but unchanged. To check this, the treatment of crosslinked dispersions with MX (see above) was examined.

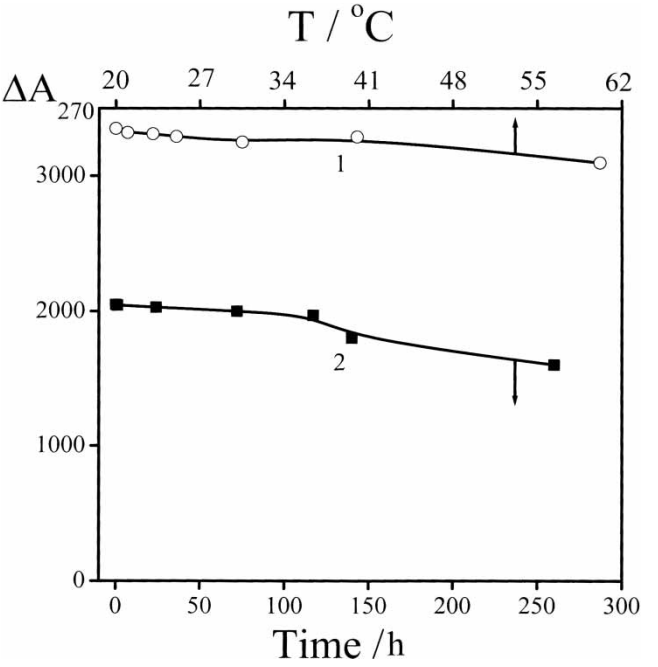

Figure 14. The dependence at $\lambda = 270$ nm of the amplitude of the band in the CD spectra of the DNA–chitosan liquid crystalline dispersions that had been treated with glutaraldehyde on temperature (curve 1) and time (curve 2). Chitosan concentration: 17.4 μg ml^{-1} (chitosan: 65% of amino groups; molecular mass 5.0 kDa), $C_{DNA} =$ 15.0 μg ml^{-1}, $C_{glutaraldehyde} = 1.25 \times 10^{-3}$ M, 0.05M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Scale for ΔA: 10^{-6} optical units.

Figure 15 presents the results of MX addition (curves 1–4) to DNA–chitosan liquid crystalline dispersions treated by glutaraldehyde. The extent of MX binding to DNA in the DNA–chitosan liquid crystalline dispersion is characterized by r_t values (inset in figure 15). The appearance of the intense band in the CD spectrum located in the region of MX absorption shows that intercalation of MX molecules between the base pairs of the DNA molecules takes place, i.e. DNA base pairs are sterically accessible for intercalation. However, despite the intercalation, both intense bands in the CD spectrum have positive signs. Because the sign of the intense band reflects the sense of twist of the spatial structure of the DNA–chitosan liquid crystalline dispersions, this means that, in contrast to

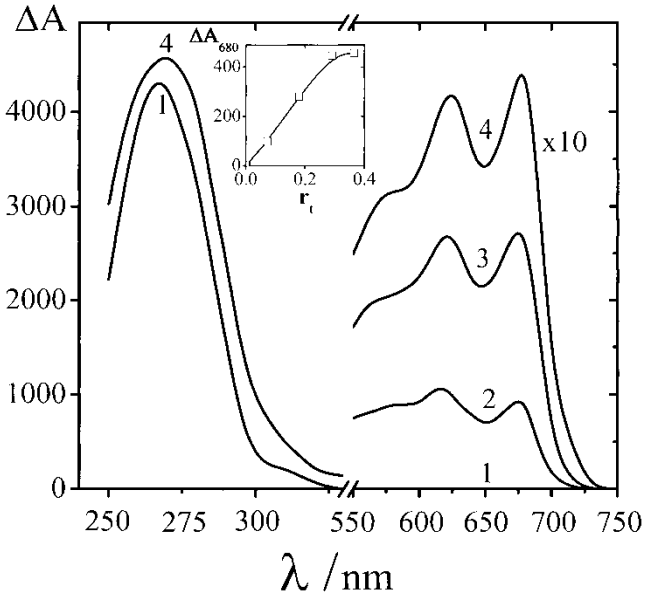

Figure 15. The circular dichroism spectra of liquid crystalline dispersion of the DNA–chitosan complex treated with glutaraldehyde in the absence (curve 1) and in the presence (curves 2–4) of MX in solution. 1–0, 2–3.8×10^{-6}, 3–9.5×10^{-6}, 4–18.9×10^{-6} M MX in solution. Chitosan concentration: 23.7 μg ml^{-1} (chitosan: 65% of amino groups; molecular mass 5.0 kDa). $C_{DNA}=17.1$ μg ml^{-1}, $C_{glutaraldehyde}=1.25\times10^{-3}$ M, 0.05M NaCl + 0.001M sodium phosphate buffer, pH 6.85. Scale for ΔA: 10^{-6} optical units. Inset: The dependence of the relative change in the amplitude of the band at $\lambda=680$ nm in the CD spectrum of the liquid crystalline dispersion of the DNA–chitosan complex treated with glutaraldehyde on the r_t value (r_t = relation of molar concentration of MX to molar concentration of DNA bases).

the corresponding non-crosslinked dispersions (see figure 12), the structure of the dispersion treated by glutaraldehyde does not change the sense of the spatial twist. In other words, the motions which require a macroscopic reorientation of the layers of DNA–chitosan molecules are strongly slowed down after chitosan crosslinking, i.e. the neighbouring molecules of DNA–chitosan complexes are 'non-fluid'.

Hence, crosslinking of chitosan molecules in the DNA–chitosan liquid crystalline dispersions results in preservation of the spatially twisted structure of the dispersions. Therefore, we are dealing now not with a 'fluid' cholesteric liquid crystalline DNA dispersion, but with a 'non-fluid' structure that possesses high abnormal optical activity. In this case, it is better to use the term 'DNA liquid crystalline elastomer' [31] to signify a new structure formed as a result of crosslinking of chitosan molecules in the DNA–chitosan liquid crystalline dispersions. This result is of high theoretical and practical importance.

3. An attempt at a theoretical description of the interactions occurring in the DNA–chitosan complexes and resulting in the formation of liquid crystals with different optical properties

According to latest theoretical notions [33], the ordered arrangement of electrical dipoles along the long axes of helical polymeric molecules can affect the properties of the liquid crystalline phase (dispersion) produced by phase exclusion of these polymers. It is implied that the dipoles are perpendicular to the polymer molecular axis and smoothly rotate around it to form their own helical structure. Polymeric molecules are assumed to involve short range steric repulsions which favour nematic ordering of neighbouring molecules in the nascent phase, and dipole–dipole interactions which produce the perturbations resulting in the helical twisting of the phase, i.e. the formation of the cholesteric phase. Depending on the direction of the spatial twist of the helical structure in the nascent phase, there results an abnormal optical activity of different sign.

Condensation of complexes resulting from the interaction of double-stranded, rigid, linear DNA molecules with polypeptides of different chemical structure and carrying positively charged amino groups, is known to give rise to the formation of cholesteric liquid crystalline phases (dispersions) having different signs of the abnormal optical activity [34]. In the liquid crystalline dispersions of DNA, the direction of helical twist of their cholesteric structure changes with temperature or the dielectric properties of the solvent [35], and this could be explained in theoretical terms by the disbalance of the forces of steric repulsion and dispersion interaction of the helical segments of the nucleic acid. The spatial twist direction for the cholesteric structure of particles of liquid crystalline dispersions of DNA also changes when the DNA molecules are preliminarily bound in complexes with antibiotics of the anthracycline group used for the condensation [36]. The authors of [37] suggested a possible mechanism for this phenomenon. When the planar daunomycin molecule intercalates between DNA base pairs, the charged amino groups of the sugar residues of daunomycin 'remain' on DNA surface. Therefore a helical distribution of efficient lateral dipoles arises along the long axis of the DNA molecule. This provides an additional contribution to the chiral interaction between DNA molecules. At a defined average distance between neighbouring positive charges generated by the presence of daunomycin molecules, an additional arising contribution leads to change in the

helical twist of the cholesteric structure formed by the DNA–antibiotic complex.

A phenomenological expression for the free energy of a cholesteric liquid crystal is the following [33, 35]:

$$F_d = (1/2)K_{22}(n \operatorname{rot} n)^2 + \lambda(n \operatorname{rot} n) \quad (1)$$

where the unit vector $\mathbf{n}(r)$ is a director that defines the average orientation of the long axes of molecules producing the liquid crystal, and K_{22} is the twist elastic constant. The pseudoscalar parameter λ determines the cholesteric twist and depends only on the chiral portion of the intermolecular interaction potential.

In the case of cholesteric twisting, $n_x = \cos\ \theta\ (z)$, $n_y = \sin\ \theta\ (z)$, $n_z = 0$ and the free energy minimum corresponds to the helical distribution of the director $\boldsymbol{n}(\boldsymbol{r})$ with pitch ($\boldsymbol{p}$):

$$2\pi/p = \lambda/K_{22}. \quad (2)$$

It was shown recently [37] that, for DNA molecules bound to anthracycline antibiotics, electrostatic interaction between two charged helical structures of the DNA–antibiotic complexes provides the following contribution for parameter λ:

$$\lambda_d = -(9/16)(d^4\rho\sigma^2 L/kT)\exp(-2xR)R^{-6}\sin(2qb) \quad (3)$$

where $R = (R_0{}^2 + b^2)^{1/2}$, $d = r_0 e$, $q = 2\pi/p_s$, and p_s- is the pitch of the helical structure of the B-form of the DNA molecule. In this case r_0 is the radius of a DNA molecule, L is the length of a DNA molecule, ρ is the number of DNA molecules per unit volume, σ is the number of closest neighbours, R_0 is the average distance between DNA molecules in the arising phase, and b is the average distance between neighbouring positive charges of antibiotic molecules. The exponential member in the expression for λ_d equation (3) corresponds to the electrostatic shielding of negative charges of DNA phosphate groups by counterions present in solution. In equation (3), $\mathbf{x}$ means the Debye–Huckel screening parameter $\mathbf{x} = (8\pi e^2/100\varepsilon_r kT)^{1/2}\mathrm{I}^{1/2}$ where I is the solution ionic strength, ε_r the mediem relative and dielectric constant and the other parameters have the standard meanings.

The pitch of the cholesteric structure of the DNA liquid crystal is determined by two factors: parameter λ_d, depending on bound antibiotic concentration, which affects the value of b, and the contribution of all other chiral interactions between DNA chains, λ_0:

$$2\pi/p = (\lambda_0 - \lambda_d)/K_{22} \quad (4)$$

where p is the pitch of the macroscopic helix of the cholesteric structure formed by the DNA molecules.

At particular magnitudes of b, the value $\lambda_0 - \lambda_d$ becomes zero, which causes disappearance of the helical twist of the cholesteric phase (dispersion) of DNA–antibiotic complex and, hence, of the abnormal optical activity of the liquid crystalline phase (dispersion). Moreover, a change in the sign of the initial abnormal optical activity of the cholesteric is possible at certain values of λ_0 and λ_d. The authors of [38] noted an alteration in band sign upon condensation of DNA molecules preliminarily complexed with increasing concentrations of anthracycline antibiotics. (It should be noted that an increase in antibiotic concentration is equivalent to a decrease in the distance between the positive charges on the DNA surface.)

One may suppose that a similar mechanism also takes place in the DNA–chitosan system. Indeed, helical chitosan molecules contain amino groups carrying partial positive charges at pH $\sim$ 7. However, due to the spatial features of the chitosan molecules, the amino groups could interact with the phosphate groups of DNA in an alternating mode [18]. This provides not only the orientation of chitosan molecules along the DNA long axis upon complexation, but also the formation of a helical array of electric dipoles. At a certain distance b between the charged amino groups of chitosan arranged along the DNA, the corresponding dipole–dipole interaction may compensate the contribution of the dispersive chiral interaction between DNA molecules. In this case, the direction of the spatial twist for the helical structure of the cholesteric phase (dispersion) of the DNA–chitosan complex may change, and this in turn will result in a modification in the sign of the abnormal optical activity of this phase (dispersion).

Taking into account that molecular mass of the DNA used in the chitosan complexation experiments was about 7×10^5 Da, this corresponds to molecular length $L = 3.5 \times 10^{-7}$ m, while the R_0 value estimated from the X-ray diffraction study was 30×10^{-10} m; the calculation of λ_d versus b, i.e. the distance between the amino group charges, was performed for different values of parameter $\mathbf{x}$ (defined by solution ionic strength) at the following magnitudes of other parameters, see equation (3): $\rho = 10^{25}$ м$^{-3}$; $r_0 = 10 \times 10^{-10}$ м; $\sigma = 6$; $T = 25^\circ$C.

Figure 16 shows a few theoretical curves calculated for the cholesteric DNA–chitosan dispersions under different conditions. Although both the theory used in [37] and the calculations performed are approximate and ignore parameters such as the polymeric nature of the chitosan molecules, possible conformational change of the chitosan molecules in solutions of different ionic strength, etc., the above calculations have several consequences: (1) the value of λ_d changes on decreasing the distance between charged groups in the chitosan molecule and becomes zero at specific distances between them; (2) parameter λ_d increases when the

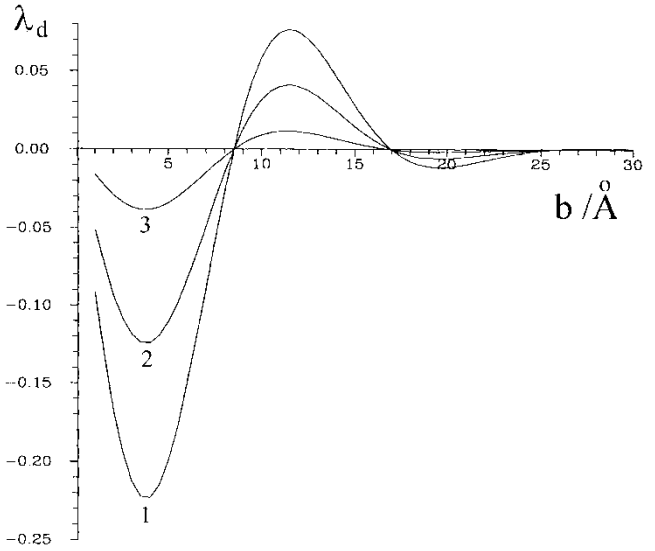

Figure 16. Theoretical curves of the variation of the λ_d parameter calculated for cholesteric dispersions of DNA–chitosan complexes depending on the average distance b between positively charged amino groups in the chitosan molecules [12]. Curves 1–3 correspond to the values of x parameter calculated from the solution ionic strength: 0.263 (curve 1), 0.3 (curve 2), 0.38 (curve 3).

ionic strength of solutions producing the DNA–chitosan cholesteric phase decreases.

Assuming that parameter λ_d is associated with the pitch of the cholesteric helical structure according to equation (4), the decrease in the pitch of the cholesteric structure of DNA–chitosan dispersions indicates that the twist of the cholesteric structure of the DNA–chitosan liquid crystal could occur as the solution ionic strength decreases. In this case one may expect an increase in the abnormal optical activity of this structure. It should be noted that, from equation (4), the pitch of the cholesteric structure is associated not only with λ_d, but also with λ_0. Therefore, it is obvious that the theory is not yet sufficiently perfect to predict the signs of abnormal optical activity of the cholesterics resulting from molecules of DNA–chitosan complexes. However, the theory does allow one to predict the tendency of changes in abnormal optical activity upon alteration in the b value or solution ionic strength. This opens up an opportunity for experimental assessment of the above consequences of the theory.

Figure 8 shows data describing the abnormal band in the CD spectra of DNA–chitosan cholesteric dispersions obtained under different conditions. Comparing these curves, we can note the following:

1. The sign of the abnormal band in the CD spectrum of the cholesteric phase of the DNA–chitosan complex is changed when the b value, i.e. the distance between charged amino groups in the chitosan molecule, decreases. The data from [39] indirectly testify to the alteration in the band sign, i.e. the direction of the helical twist of the DNA–chitosan cholesteric, upon alteration in the distance between the charged groups on the DNA surface. The acetylation of ethyl cellulose followed by the formation of liquid crystals from such molecules is known to produce cholesterics whose physical properties, in particular the sign of helical twisting, changes from left-handed to right-handed when the acetylation degree becomes 0.2.
2. At a constant distance between amino groups, in particular at $b = 5.0$ or 12 Å, the decrease in ionic strength of the solution where the DNA–chitosan cholesteric phase arises is indeed accompanied by an increase in the amplitude of the negative CD band of this phase (compare curves 5 and 1 in figure 8). This result agrees well with experimental data from [38], which showed that the shorter the pitch of the cholesteric helical structure for the particles of liquid crystalline DNA dispersions, the higher was the amplitude of the anomalous band in the CD spectrum.
3. Under certain conditions, DNA–chitosan complexes could produce phases having no abnormal band in the CD spectrum.

Indeed, the formation of dispersions from DNA–chitosan complexes at pH ~ 8 (see figure 6), in spite of the onset of an 'apparent' optical density, is not accompanied by changes in the shape of the CD band characteristic of the initial B-form of DNA and the emergence of an abnormal CD band under these conditions. This fact is of interest from two points of view. First, it indicates that scattering itself does not cause the emergence of the abnormal band in the CD spectrum. Second, it indicates that the character of the spatial packing for the DNA–chitosan complexes in dispersions is associated with a strict equilibrium of the different types of interaction forces operating between these molecules.

Thus, in spite of the limitations of the calculations performed, the main consequences of the theory [37] agree well with the experimental results obtained for the DNA–chitosan liquid crystalline dispersions. However, the theory in its current state is not capable of describing the differences between expetimental results obtained for the the liquid crystalline dispersions formed by DNA–chitosan and poly(I)xpoly(C)–chitosan complexes. This discrepancy beween theory and experimental data holds also for the dispersions formed by chitosan molecules of various molecular masses

(compare curves 1,2 and 3 in figure 8). This means that the fine details of the structure of NA molecules or, probably, chitosan, need to be included in theoretical considerations.

4. Summary

In conclusion, considering the possibility of using chitosan molecules as candidates for molecular design, one can stress a few circumstances. Data obtained in this work indicate not only the existence of numerous types of packing for the molecules of NA–chitosan complexes in liquid crystalline dispersions, but also show that the physicochemical properties of these dispersions readily change upon alteration in the properties of both the chitosan molecules and the solution used for their preparation. Hence, chitosan molecules can provide the formation of numerous molecular constructions with easily regulated properties. In addition, part of the NH_2 groups of the sugar residues of chitosan really appears to be exposed to the solvent. In combination with OH groups of sugar residues, both types of group are indeed additional binding sites on the surface of NA molecules. For instance, the distance between OH and NH_2 groups of the sugar residues of chitosan is adequate to provide the formation of a very stable chelate complex with transition metal ions. (The constant of complex formation of chitosan with divalent copper ions is very high and is estimated to be $10^{14}{}_{\text{M}}{}^{-1}$ [22]. This complex has a planar structure [40], i.e. the type of structure that is necessary for building polymeric chelate bridges.) Hence, OH and NH_2 groups can serve as sites for generating bridges that can link, in this case not NA molecules, but the neighbouring molecules of 'alien' polymeric compounds, i.e. chitosan.

Preparation of molecular constructions of the type considered above is now in progress.

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