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INTERACTION OF PHENOSAFRANINE WITH NUCLEIC ACIDS AND MODEL POLYPHOSPHATES.

I. SELF–AGGREGATION AND COMPLEX FORMATION WITH INORGANIC POLYPHOSPHATES

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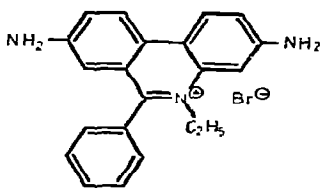
Aggregation of phenosafranine in concentrated aqueous solutions and its interaction with polyphosphates was studied by absorption and fluorescence spectroscopy. At concentrations > 10^{-3} M phenosafranine forms dimers ($K_d = 3.8 \times 10^2$ l.mole^{-1}), which are characterized by a hypsochromic shift of the visible and near ultraviolet absorption maxima accompanied by a hypochromic effect. No fluorescence could be detected from phenosafranine dimers. Analogous spectral changes were observed when a polyphosphate was titrated with phenosafranine, which indicated that with increasing saturation of the polyphosphate binding sites phenosafranine gradually became bound in the aggregated form. Full saturation of the polyphosphate binding sites with phenosafranine was reached only when an excess of free dye was present. The cooperative binding of phenosafranine to a polyphosphate could be evaluated by means of a theory proposed by Schwarz et al. At the zero ionic strength and at 25°C the binding was characterized by cooperative binding constant $K = 6.2 \times 10^5$ l.mole^{-1}, number of binding sites per monomeric phosphate residue $g = 0.4$, and cooperativity parameter $q \approx 30$. Spectroscopic properties of phenosafranine in the aggregated and polyphosphate-bound states were compared with those of ethidium bromide.

1. Introduction

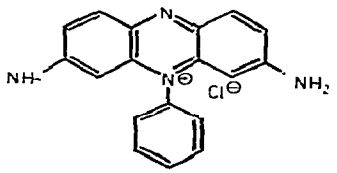
Various organic ligands, e.g. cationic dyes can form stable complexes with synthetic polynucleotides and nucleic acids [4–6]. The dye can be bound by two types of binding [7]. Type I‡ corresponds to a monomeric binding of dye molecules which intercalate between neighbouring base pairs (or bases) of polynucleotides. In a number of papers, using absorption spectrophotometry as a tool in analyzing the complexes of dyes with nucleic acids and polynucleotides, it was established that this type of binding is accompanied by a red shift of the long-wavelength absorption band. Spectroscopic behaviour is discussed on the basis of the interaction of the bound dye molecules with the less polar environment (as compared with the aqueous medium), i.e. purine, pyrimidine and sugar moieties of polynucleotides [8,9].

On the other hand, type II‡ binding corresponds to the binding of dye molecules on the surface of polynucleotide molecules [10–12]. Depending on the dye properties and on the degree of saturation of the polynucleotide binding sites, dye molecules can be bound in stacked aggregates. A new blue-shifted absorption band is often typical for this type of complexes as it was demonstrated e.g. for acridine orange [11,13] methylene blue and toluidine blue [10]. However, this does not hold true without exception. Thus, the phenanthridine dye ethidium bromide (I), well known as an intercalating agent, forms vertically stacked aggregates in concentrated solutions [14] as well as complexes with inorganic polyphosphates [15,16] or maleic acid copolymers [17] characterized by a red shift of the long-wavelength absorption band.

‡ Binding types I and II refer only to the geometry of binding.



I.



II.

It was suggested that this red shift could be due to the asymmetric structure of ethidium. Therefore we chose for our present study a phenazine dye phenosafranine (PS) (II), which has similar, but more symmetric structure.

In the present and following papers we would like to report results on the spectroscopic properties of PS and its interaction with nucleic acids and model polyphosphates. First indications of the complex formation of PS with deoxyribonucleic acid resulted from polarographic investigations which were directed towards the analysis of adduct isotherms and of the inhibition of DNA-unwinding process by the bound dye [18,19]. However, no conclusions can be drawn whether or not both above described types of binding are involved in these processes. Binding of PS, probably by the intercalation mechanism, is decreased for chemically methylated DNA [20]. On investigating the spectroscopic properties of the PS-DNA complex it was found that the observed red shift of the long wavelength absorption peak is due to the interaction of the dye with a less polar environment [8]. From this point of view PS is strongly similar to other dyes capable of intercalation like, e.g., proflavine, purified trypaflavine or ethidium bromide. On the other hand, spectroscopic studies of thermal stability of the PS-DNA complexes [21] did not show unequivocally to what extent the PS binding by type II participates in the interaction with DNA.

The first part of our investigation deals mainly with PS aggregation in aqueous solutions as a function of concentration and attempts to elucidate the properties of PS bound to a polyanion by the type II binding. We chose for this purpose polyphosphates, which can bind cationic dyes only in the form of surface complexes that correspond to the type II binding with DNA.

2. Materials and methods

2.1. Chemicals

Phenosafranine (3,6-diamino-10-phenyl phenazinium chloride, (II)) was a product of Bayer (Leverkusen) and was used without further purification. The long wavelength absorption maximum of PS solution in water was at 19150 cm^{-1} (522 nm) with molar extinction coefficient 4.2×10^4 $l.mole^{-1}.cm^{-1}$, which agreed well with values given for PS picrate by Corbett [22]. Sodium polyphosphates of different molecular weight (18–20 and 128 monomer units) were a kind gift of Dr. Schülke from the Institut für anorganische Chemie der Akademie der Wissenschaften der DDR in Berlin. The total phosphorus content in hydrolyzed polyphosphate solutions was determined spectrophotometrically by the methods of Hesse and Geller [23] or Martin and Doty [24]. The orthophosphate content in polyphosphate samples did not exceed 1% as determined by the method of Eibl and Lands [25].

2.2. Instruments

Absorption spectra were recorded by means of spectrophotometers Unicam SP 700 (Cambridge) and Ultrascan (Hilger & Watts, London). Fluorescence measurements were performed with a fluorescence spectrometer SF 100 E Baird Atomic (The Hague, The Netherlands) and with an apparatus constructed by Smékal [26].

2.3 Methods

All measurements were carried out in nonbuffered solutions prepared from bidistilled water. Stock solutions of 10^{-2}M PS and 1% polyphosphates (1% solution corresponds to 9.8×10^{-2}M solution expressed in phosphate units) were used for preparing the com-

plexes polyphosphate – dye. The titration of polyphosphates under a constant dye concentration was carried out [12] and the complexes were characterized by values p, which represent the ratio of the total phosphorus content to the total amount of dye present in the mixture. In most experiments titration curves were obtained by recording absorption and fluorescence spectra parallelly. In order to minimize errors which could be caused by adsorption of the dye on glass or silica surface, we used always freshly prepared solutions in glassware and cuvettes pretreated with identical dye or complex solutions.

The stability of several complexes of the high molecular weight polyphosphates was checked by equilibrium dialysis. Small volumes (5 ml) separated by a dialysis membrane (Union Carbide Corp., Chicago, Ill.) were equilibrated for 72 hours at 25°C. Before use the membranes were boiled in 5% Na_2CO_3, then in water, and subsequently thoroughly washed; their area was approximately 1 cm^2 so that dye adsorption on the membranes should be minimized. Identical results were obtained when complexes were equilibrated against water or when corresponding concentrations of PS and polyphosphate were equilibrated against each other, provided the amount of PS adsorbed on the membrane was taken into account.

3. Results and discussion

3.1. Spectroscopic properties of phenosafranine

An information was obtained on the dependence of absorption and fluorescence spectra of PS on concentration so that it might be possible to evaluate spectral changes observed upon the binding of PS to a polyphosphate.

The absorption spectral data are summarized in figs. 1 and 2. At concentrations higher than 4×10^{-4}M changes are observed that correspond to formation of PS aggregates: With increasing concentration the positions of both visible and near UV maxima are gradually shifted to higher energies with a concomitant decrease of molar extinction coefficients.

Fluorescence of monomeric PS solutions was represented by a smooth peak with a maximum at 17090 cm^{-1} (585 nm), when excited at the wavelength corresponding to the monomer absorption band (at

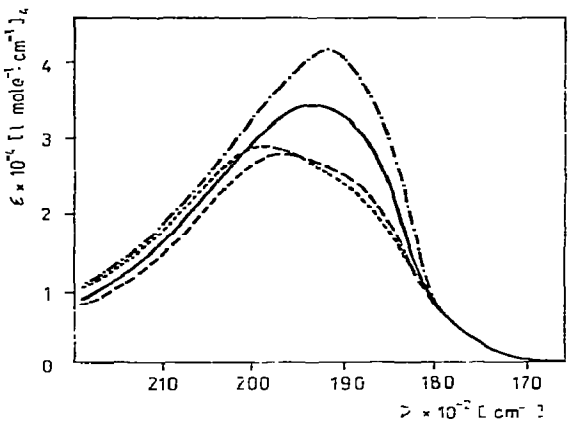


Fig. 1. Visible spectra of different concentrations of phenosafranine in aqueous solutions at 25°C (–·–) 1×10^{-5}M; (——) 1×10^{-3}M; (– – –) 5×10^{-3}M; (· · ·) 1×10^{-2}M.

19150 cm^{-1} (522 nm)). At concentrations higher than 5×10^{-4}M only quenching of this emission was observed. No fluorescence originating in the aggregated PS could be detected, even when wavelength corresponding to aggregate absorption maximum was used for excitation.

The observed changes in absorption spectra resemble those found for symmetric acridine derivatives, e.g. acridine orange, proflavine [4–6], and especially for N-benzyl acridine orange [27], and can be similarly explained as a consequence of formation of vertically stacked PS aggregates. According to criteria of Simpson and Peterson [28] (table 1) PS belongs to the group of molecules representing weak coupling cases.

By extrapolating the values of molar extinction coefficient of the maximum corresponding to monomeric PS to conditions yielding PS present totally in the monomeric form (infinite dilution) on the one hand, and PS totally aggregated on the other hand, concentrations of these two forms were calculated in the investigated range of total PS concentrations [30]. Using the procedure described by Zanker [30], it was found from the slope of the linear dependence of equilibrium monomer vs. aggregate concentrations that PS formed dimers in the investigated concentration range. The mean value of the dimerization constant at 25°C, $K_d = 3.8 \times 10^2$ l.mole^{-1}, was estimated according to Schwarz et al. [2] from the plot shown in the insert in fig. 2b. The value

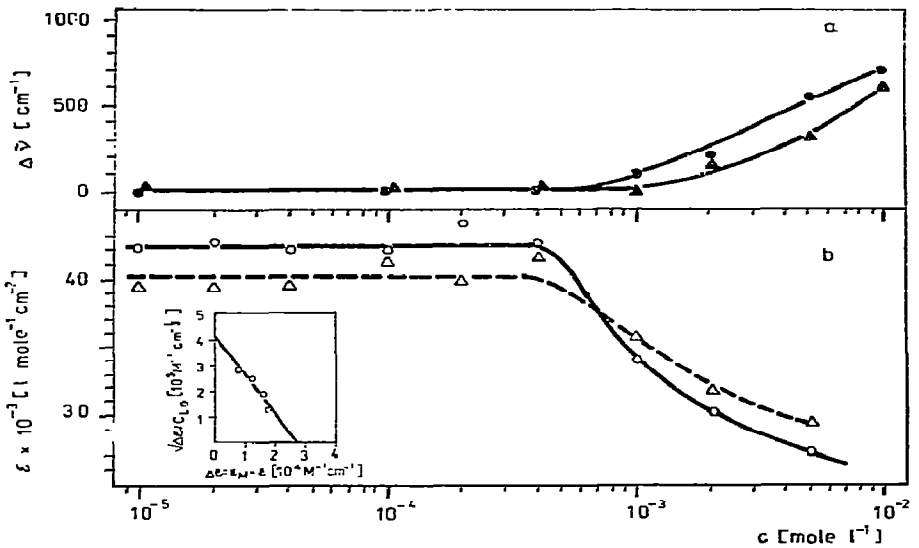


Fig. 2. Changes of positions (a) and molar extinction coefficients (b) of phenosafranine visible (○) and near ultraviolet (△) maxima with concentration in aqueous solutions at 25°C. The insert shows an extrapolation of the dependence $\Delta\epsilon = \epsilon_M - \epsilon$ versus $\sqrt{\Delta\epsilon/c_{L,0}}$ (here ϵ_M is the molar extinction coefficient of monomeric phenosafranine at 19150 cm^{-1}, 42000 $l.mole^{-1}\ cm^{-1}$, and ϵ corresponds to measured absorbance values at phenosafranine concentration $c_{L,0}$). The intercept with x-axis yields the value of the molar extinction coefficient of aggregated phenosafranine, $\Delta\epsilon' = \epsilon_M - \epsilon_D$, the intercept with y-axis corresponds to $\sqrt{2K_d\Delta\epsilon'}$ [2].

of molar extinction coefficient corresponding to dimerized PS at 19150 cm^{-1} obtained by linear extrapolation from this plot (15000 $l.mole^{-1}\ cm^{-1}$) was lower than the value used for calculation of K_d in the preliminary report [21] and corresponded closely to the molar extinction coefficient of PS aggregated on polyphosphates. Consequently, the presently reported value of K_d is lower.

Table 1
Dimerization constants (K_d) and coupling characteristics of different cationic dyes measured in aqueous solutions at 25°C

Compound	$\frac{\Delta w}{s}$ (a)	K_d $l.mole^{-1}$
Proflavine	0.44 (b)	5×10^2 (e)
		4.77×10^2 (f)
Acridine orange	1.5 (b)	1.3×10^4 (c)
N-benzyl acridine orange	1.2 (c)	5.4×10^3 (c)
Phenosafranine	0.6	3.8×10^2
Ethidium bromide	0.4 (d)	41 (f)

(a) Ratio of the monomer bandwidth (Δw) to the value of Davydov splitting (s), in cm^{-1} [28].
(b) Ref. [29].
(c) Taken from spectroscopic data [27].
(d) Calculated from spectroscopic data [15].
(e) Spectroscopic determination [2].
(f) Calorimetric determination [31].

This value is compared with dimerization constants for several acridine derivatives and ethidium bromide in table 1. Even if K_d of PS is lower than that of geometrically similar N-benzyl acridine orange and is close to K_d of proflavine, its value is by one order of magnitude higher than K_d of ethidium bromide. The difference in the strengths of stacking of PS and ethidium bromide in solution might reflect differences in geometrical arrangement of these two dyes in vertical stacks, for which a spectroscopic evidence already exists. In contrast to spectral changes observed with increasing PS concentration, the hypochromic effect in the visible region of the absorption spectrum of ethidium bromide was accompanied by a red shift from 20650 cm^{-1} (484 nm) observed for concentrations 5×10^{-2}M and lower to 19900 cm^{-1} (502 nm) for 5×10^{-2}M solution [15]; for completely dimerized ethidium bromide

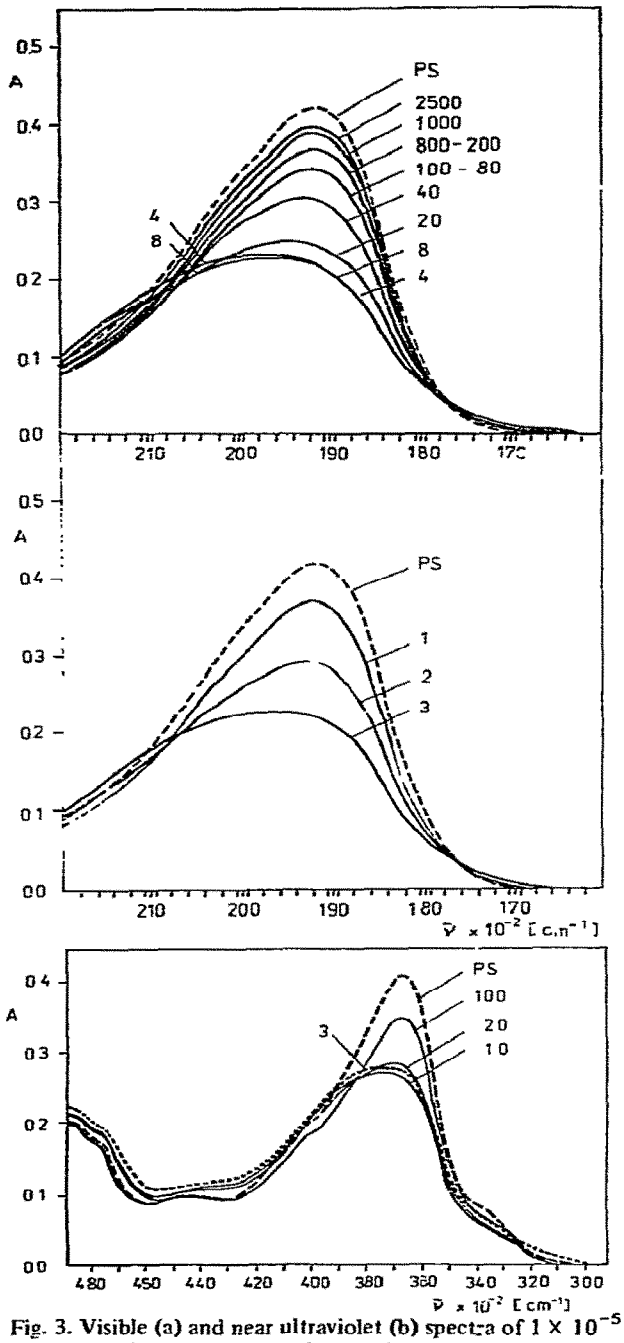


Fig. 3. Visible (a) and near ultraviolet (b) spectra of 1×10^{-5}M phenosafranine bound to a polyphosphate (128 monomer units) in aqueous solutions at 25°C. Values p are given in the figures; (- - -) free 10^{-5}M phenosafranine.

the maximum shifts up to 19300 cm^{-1} (518 nm) as estimated by Crescenzi and Quadrifoglio [17].

3.2. Complexes of phenosafranine with polyphosphates

The changes in PS absorption spectra when titrated with different polyphosphates are illustrated in figs. 3 and 4. It can be seen that at high p spectra of the complexes are similar to the spectrum of free PS; with decreasing p the hypochromic effect becomes prominent and a shoulder appears on the short-wavelength side of the maximum. This means that with decreasing p a complex, in which the dye is bound in the monomeric form, is gradually transformed into a complex with the dye molecules bound in an aggregated form. The correspondence between the positions of absorption maxima, both visible and ultraviolet, of aggregated PS in solution (figs. 1 and 2) and the spectra of the complexes with $p < 10$ (fig. 3) indicates that eventually most of the dye molecules are bound as aggregates.

The titration of the longer polyphosphate (~128 monomer units) was evaluated using the model of cooperative dye binding to linear polymers [1–3]. The dependence of the fraction of free PS γ_L^* (calculated under assumption that all dye is bound in the aggregated form) as a function of low p values is shown in fig. 5 for different dye concentrations. The intercept of the common straight part with the p axis yields the reciprocal value of the number of binding sites per monomer unit, $g^{-1} = 2.5$, which is rather close to p values corresponding to minima on the titration curves (fig. 4). The value of $g = 0.4$ for PS is considerably lower than for proflavine interacting with a polyphosphate (~500 monomer units), $g = 0.8$ [3]. It is most probable that the fact that practically only every second phosphate monomeric unit can be occupied by PS is due to the presence of the bulky phenyl group.

The cooperative binding constant, K, was evaluated from the interception of the curves γ_L^* versus p with the straight line of half the negative slope of the previous line. The intercept corresponds to γ_L^* value of $1/B$. Since the binding strength parameter B is defined as $B = Kc_{L,0}$ ($c_{L,0}$ is the total weighing-in concentration of the dye), $K = B/c_{L,0}$ [1,3]. The mean value of K calculated for the three PS concentrations was 6.2×10^5 l.mole^{-1}, which is comparable to $K = 4.5 \times 10^5$ l.mole^{-1} for proflavine binding to a polyphosphate [3].

The cooperativity parameter q was evaluated from

the fraction of the dye bound as monomers β_L at high p values. Equilibrium dialysis was used to check whether in the broad region of high p values all PS molecules were totally bound to the polyphosphate. Irrespective of the arrangement, i.e. if the equilibrium was reached between a complex solution and water, or between PS on one side of the membrane and polyphosphate on the other one, total binding of PS to polyphosphate was always observed in the region of $p = 50-1000$. Thus, β_L can be easily evaluated from the changes of molar extinction coefficient, ϵ, at 19150 cm^{-1}, using the values of ϵ_L (corresponding to monomeric free or bound PS) and ϵ_D (corresponding to aggregated PS) 4.2×10^4 and 1.5×10^4 $l.mole^{-1}$ cm^{-1}, respectively. From the theory [1] it follows that

$$q = gp(1 - \sqrt{\beta_L})/\beta_L .$$

The mean value of q calculated from data in the range of $p = 50-5 \times 10^3$ was relatively low, $q \doteq 30$. Since by definition $q = K/K^*$ [1], we obtained for the binding constant corresponding to the nucleation of an aggregate of bound ligands $K^* \doteq 2 \times 10^4$.

The positions of minima on the titration curves depend on the degree of polymerization of a polyphosphate: with decreasing chain length they become shifted to higher p. Similar results were obtained with other dyes, e.g. crystal violet and malachite green [32,33] or acridine orange [34]. Unfortunately the scatter of binding data for the shorter polyphosphate was so high that their quantitative evaluation was not possible.

For fluorescence measurements we used complexes polyphosphate-PS that had been prepared either by the absorption spectroscopic titration or by direct titration in the fluorescence cuvette. The radiation of 21320 cm^{-1} (366 nm) or 19150 cm^{-1} (522 nm) (the latter one corresponding to the PS monomer absorption maximum) was used for excitation. The fluorescence maximum of PS bound to polyphosphates was broad and structureless and had the same energy as fluorescence of free monomeric PS, i.e. 17090 cm^{-1} (585 nm). Even if the complexes with low p were excited at 19900 cm^{-1} (502 nm), which corresponds to the maximum of the aggregated form of PS, no shift of the position of the fluorescence maximum was observed.

The course of the fluorescence titration curves (fig. 4c) is in accord with the interpretation which was suggested for the absorption spectral changes. At very high p values the relative intensity of fluorescence, which originates in monomeric bound PS, is practically the same as that of free PS. With decreasing p, which is accompanied by increasing stacking of the dye molecules, the fluorescence intensity decreases reaching at the minimum a value ranging from 30% (for longer polyphosphates) to 45% (for the short polyphosphate) of the free PS fluorescence intensity. This dependence as well measurements carried out with $5 \times 10^{-3}-10^{-2}$M PS solutions indicate that PS in the aggregated form exhibits no detectable emission at 17090 cm^{-1} (585 nm) or longer wavelengths.

Due to the identity of the absorption as well as fluorescence spectra of free PS on the one hand and PS bound to polyphosphates in the monomeric form on the other hand, it is impossible to decide whether the residual fluorescence intensity in the minimum of the titration curves is attributable to the presence of free PS only or whether some contribution comes from PS molecules which are still bound as monomers. The low value of q seems to indicate that in the vicinity of $p \doteq 3$ a small fraction of PS remains to be bound as non-interacting monomers. This can explain the relatively high residual fluorescence intensity in this region as well as the decrease of K observed with decreasing $c_{L,0}$ (fig. 5).

3.3. Comparison of spectroscopic properties of phenosafranine and ethidium bromide

The results obtained demonstrate that at concentration higher than 5×10^{-4}M as well as upon binding to polyphosphates PS can form dimers, eventually in the latter case higher aggregates, containing vertically stacked parallel molecules, in spite of the presence of the bulky phenyl group at the heterocyclic ring nitrogen. The strength of coupling of PS molecules in dimer is weak, as classified according to criteria of Simpson and Peterson [28] (see table 1). Consequently, the tendency of PS to aggregate is also relatively weak, as it is manifested by low value of the dimerization constant ($K_d = 3.8 \times 10^2$ $l.mole^{-1}$) (table 1) and by parameters characterizing its binding to a polyphosphate ($K = 6.2 \times 10^5$ $l.mole^{-1}$, $g = 0.4$, $q \doteq 30$); the binding parameters are reflected by the presence of a relatively narrow plateau at the minimum on the titration curves (fig. 4). On the contrary, strongly aggregating dyes pro-

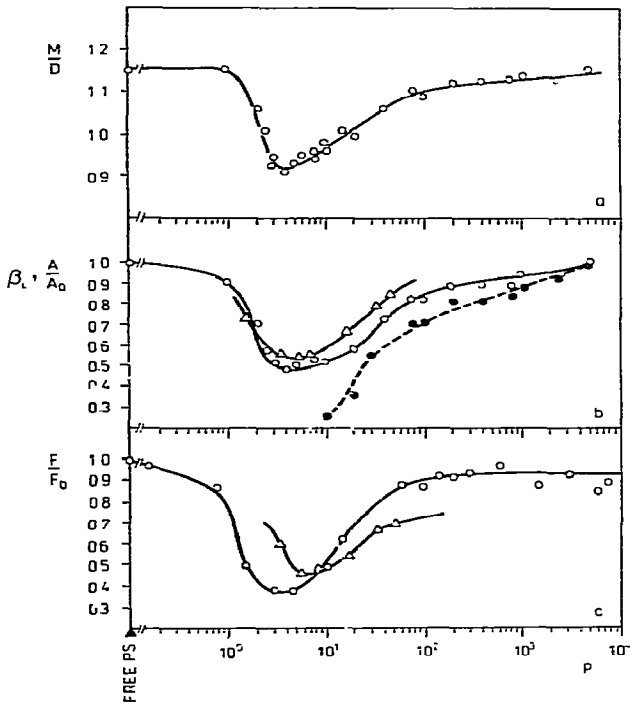


Fig. 4. Titration curves of a short polyphosphate (18–20 monomer units) (Δ) and a long polyphosphate (128 monomer units) (○) with 10^{-5}M phenosafranine in water at 25°C. a) Dependence of the ratio of absorbance in phenosafranine monomer maximum (M, 19150 cm^{-1}) and dimer maximum (D, 19900 cm^{-1}) on p. b) Dependence of relative absorbances at 19150 cm^{-1} on p (A_0 is absorbance of free 10^{-5}M phenosafranine) and the fraction of phenosafranine bound as isolated monomers β_I to the long polyphosphate (●) calculated from A values at high p, where practically all phenosafranine molecules are bound to the polyphosphate. c) Dependence of relative fluorescence intensities at 17090 cm^{-1} on p; the excitation wavelength was 19150 cm^{-1}; F_0 is fluorescence intensity of free 10^{-5}M phenosafranine.

duce usually a much broader plateau sometimes extending over several orders of magnitude of p for polyphosphates of high molecular weight [33,34].

Molecular aggregation has been a subject of numerous investigations. The absorption maximum of vertically stacked parallel molecules is usually located at the short-wavelength side of the transition characterizing the monomer. The most common interpretation of this fact corresponds to the Davydov splitting as a consequence of the intermolecular interaction [35]. (i) For vertically stacked parallel molecules ("card pack" aggregates) the long-wavelength transition is symmetry-forbidden, while the short-wavelength transition is allowed and gives rise to the blue shift in the aggregate spectrum [36].

On the other hand, other orientations of chromophores in an aggregate result in different spectral changes. Thus, (ii) an asymmetric oblique orientation of the corresponding transition dipole moments of the interacting molecules imposes no symmetry limitations and both transitions corresponding to the Davydov splitting can be observed; (iii) for a chain-like arrangement of molecules in an aggregate leading to the head-to-tail interactions of the particular transition dipole moments, the short-wavelength transition is symmetry-forbidden and the long-wavelength one allowed [36].

The latter case evidently corresponds to the shifts in the visible absorption spectrum of ethidium bromide aggregates. Since there exists evidence from NMR spectra [14,37] that ethidium bromide forms stacked dimers, it was suggested as one possibility of the explanation that ethidium cations are mutually shifted in the stacked aggregate [16] due to their asymmetric structure and, consequently, asymmetric distribution of electron density [38]. In such aggregates π-electron clouds overlap only partially and the transition dipole moments corresponding to the lowest absorption maximum at 20650 cm^{-1} are oriented so that the head-to-tail orientation prevails, which gives rise to the red shift of the band. The arrangement of ethidium cations would be thus necessarily different from that in models of dimer structure suggested earlier [37,38]; the mutual orientation of ethidium moieties will be most probably similar to the "shifted" stacking found in ethidium bromide crystals by X-ray analysis [39].

The comparison with PS shows that the bulky phenyl group itself does not interfere with the formation of the "card-pack" dimers of weakly coupled molecules; the observed differences in K_d values and spectral shifts of PS and ethidium bromide should be attributed to the lack of symmetry in the latter molecule. The fact that ethidium bromide aggregates are present mostly as dimers [31] makes less probable the alternative possibility suggested for explaining the red shift in ethidium bromide visible spectrum connected with the aggregation [16], viz. that the shift could be caused by substituting the aqueous environment by a less polar organic one [8] i.e. when an absorbing ethidium cation is surrounded by

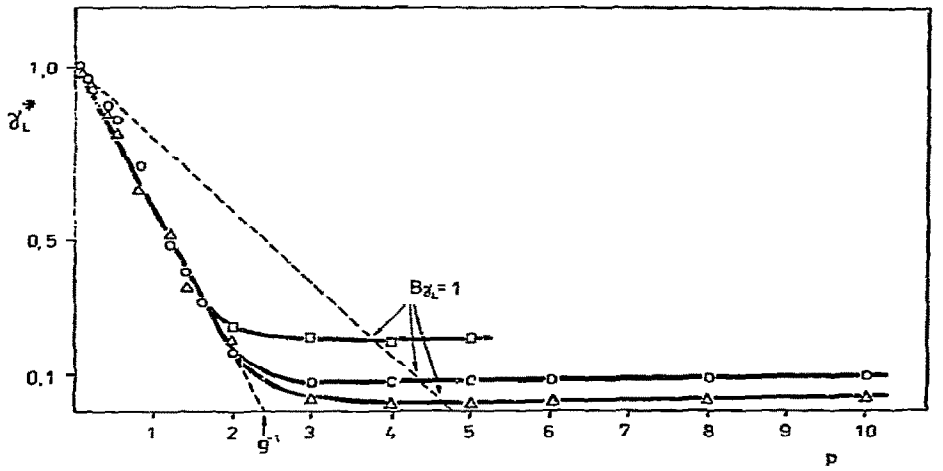


Fig. 5. The fraction of unbound phenosafranine corrected for the free ligand association, $\gamma_L^* = \gamma_L(1 + 2K_d\, c_{L,0}\, \gamma_L)$, as a function of p. The system contained the polyphosphate (128 monomer units) and phenosafranine in concentrations ($c_{L,0}$) 10^{-5}M (□), 2×10^{-4}M (○) and 4.25×10^{-5}M (△). The straight line identical with the initial parts of the curves was used for determining the number of binding sites per monomer unit g, the second straight line having half the slope of the first one yielded the values of cooperative binding constant K (see text).

other ethidium moieties upon the dye molecules became stacked. The dye dimers remain still extensively hydrated and thus the environment of an ethidium moiety in a dimer differs from that of the dye intercalated in DNA, even though spectral shifts of the same magnitude were observed in both cases [8].

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