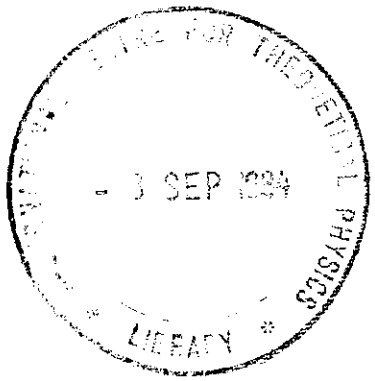

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SECOND SUMMER COLLEGE IN BIOPHYSICS

30 July - 7 September 1984

Lecture 3: Fluorescence and Phosphorescence of DNA Bases and DNA

Lecture 4: Fluorescence of Dye-Nucleic Acids Complexes.

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Lecture 3

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Fluorescence and Phosphorescence of DNA bases and DNA

References: 1) Excited States of Nucleic Acids by Guéron, Eisinger, Lamola in "Basic Principles in Nucleic Acids Chemistry", vol I, Ed: P.O.Tsó Academic Press (1974).

2) Recent Developments in the Fluorescence of DNA bases and DNA at 300°K in "Physico-chemical Properties of Nucleic Acids", vol I, Ed: J. Duchesne Academic Press (1973)

3) "Excited States of Proteins and Nucleic Acids", Ed: R.F. Steiner and I. Weinryb McMillan (1971)

4) "Excited States and Photochemical Reactions in Nucleic Acids" by C. Hélène in "Synchrotron Radiation Applied to Biophysical and Biochemical Research", Eds: A. Castellani, I. Filosofia Plenum (1979)

Fluorescence of DNA bases at 300 K

Large data are available at low temperature (20K in ethylene glycol-water glasses). However in order to understand the properties of DNA excited states in biologically significant conditions are important R.T. measurements. This has been obtained utilizing the technique of digital signal accumulation, that allows the detection of the very low R.T. signal ($\phi \sim 10^{-5} - 10^{-3}$) compared with the luminescence signal at 80°K ($\phi \sim 10^{-1}$). From the comparison between the two sets of data, it can be seen that it is not possible to extrapolate always from low temperature to R.T.

Fluorescence lifetime: $\tau_s \sim 1 - 10$ picoseconds
From the measured fluorescence quantum yelds ranging from $10^{-3} - 10^{-5}$ sec and from the measurement of the area of the first absorption band, which allow the evaluation the radiative lifetime τ_0 (in base), it is possible to estimate $\tau_s = \phi \tau_0 \simeq 10^{-12}$ sec. This determination of the singlet state lifetime of the bases, however, rests on the assumption that the excited state which emits fluorescence is the $\pi \to \pi^*$ transition responsible of the intense absorption band observed, i.e that there are (not) hidden state ($n \to \pi^*$) under the absorption band that are instead responsible of the weak fluorescence (τ will increase, in this case). However the estimated value $\tau_s \sim 10^{-12}$ sec is consistent with the reported fluorescence polarization measurements.

Table 1 : Luminescence characteristics of nucleotides in aqueous solutions at pH 7 at 77 K and 300 K

	77 K				
	$\phi_F^{(a)}$	τ_F (ns)	$\phi_{ISC}^{(b)}$	$\phi_P^{(b)}$	$\tau_P^{(b)}$ (s)
TMP	0.30	3	$<3\times10^{-3}$	8×10^{-3}	0.33
CMP	5×10^{-2}	0.3	0.03	0.01	0.34
AMP	5×10^{-3}	3	0.02	0.015	2.4
GMP	0.17	5	0.15	0.07	1.3

	300 K		
	$\phi_F^{(c)}$ $(\times10^5)$	$\tau_F^{(d)}$ (p sec)	$\phi_{ISC}^{(d)}$
TMP	4	18	8×10^{-3}
CMP	3	3.6	1.5×10^{-3}
AMP	0.3	1.3	4×10^{-4}
GMP	0.2	4.4	4.6×10^{-4}

(a) P.I. Honnas & H.B. Steen (1970) Photochem. Photobiol. 11, 67
(b) references 12 & 4 ; (c) references 4 & 9 ; (d) reference 6 assuming that the rate constant for energy transfer to Eu^{3+} is diffusion controlled : $k_t = 5\times10^9\ M^{-1}.s^{-1}$)

DNA 2.0 ~1.

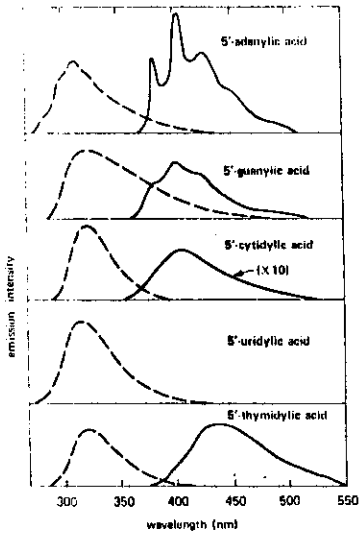


FIGURE 2-11 The fluorescence (dashed lines) and phosphorescence (solid lines) of five mononucleotides in an ethylene glycol–water glass at 80°K. [Data from J. Eisinger, *Photochem. Photobiol.*, 7, 597 (1968).]

Experimentals

The R.T. emission and excitation spectra of DNA bases reported in next figure have been obtained by digital accumulation technique, by Turner Model 210 energy corrected spectrofluorimeter connected to a multichannel analyzer (signal to noise ratio decreases with number of accumulated spectra n as $\sqrt{n}$)

The spectra have been corrected for the Raman scattering of the sample by subtraction through multichannel. Fluorescence spectra of quinine bisulphate standard recorded in part of the multichannel memory has allow the evaluation of corrected yields.

Difference between R.T. and 80°K:

1) Emission spectra - similar in shape, but significant broadening at R.T. (mainly toward the red), due to differences in the dipolar relaxation of the excited state (τ_{rel}).
   - if $\tau_{rel} \gg \tau_s$, the emission occurs before solvent relaxation and the spectrum is a good mirror image of the absorption (this occurs at low T. in rigid matrix).
   - if $\tau_{rel} \ll \tau_s$, complete solvent relaxation of the excited state occurs before emission, that comes from the relaxed state: the spectrum is red shifted.

Fluorescence excitation and emission spectra at R.T.

Comparison with absorption — and with emission at 80K .....

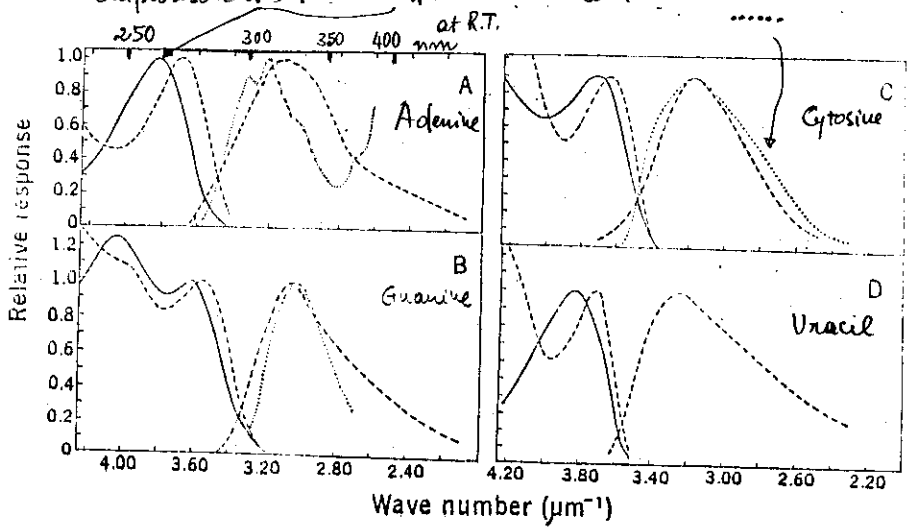


Fig. 1. Corrected fluorescence excitation (dashed line, left) and emission spectra (dashed line, right), absorption spectra (solid line), and emission spectra compared to that in EG:H_2O (1:1, by volume) (dotted line) for four bases. (A) Adenine: two scans (signal-to-noise ratio, > 25); emission compared to that in EG:H_2O at 77°K (8), with fluorescence excited at 3.83 μm^{-1} and emission monitored at 3.13 μm^{-1}. (B) Guanine: two scans (signal-to-noise ratio, > 25); emission compared to that at 195°K (7), with fluorescence excited at 3.63 μm^{-1} and emission monitored at 3.30 μm^{-1}. (C) Cytosine, four scans (signal-to-noise ratio, > 15); emission compared to that at 77°K (9), with fluorescence excited at 3.75 μm^{-1} and emission monitored at 3.13 μm^{-1}. (D) Uracil, four scans (signal-to-noise ratio, > 15); with fluorescence excited at 3.87 μm^{-1} and emission monitored at 3.23 μm^{-1}.

Table 1. Fluorescent properties of the bases at room temperature.

Compound	Concentration ($M \times 10^{-4}$)	pH	Excitation energy (μm^{-1})	Fluorescence quantum yield ($\times 10^4$)	0-0* energy* (μm^{-1})	Singlet lifetimes† ($\times 10^{10}$) (a)	(b)
Adenine	5	7.3	3.83	2.6	3.56	1.0	8.9
Guanine	8	6.3	3.63	3.0	3.34	1.4	3.0
Thymine (5)	5	6.7	3.77	1.02	3.45	0.9	1.5
Cytosine	10	6.5	3.75	0.82	3.49	0.2	0.9
Uracil	8	6.8	3.87	0.45	3.57	0.7	1.4

* Determined by the absorption-emission intersection. † Method of calculation explained in the text.

From M. Daniels and W. Hauswirth, Science 171, 675 (1971)

if $\tau_{rel} \sim \tau_s$ emission occurs both from unrelaxed and from relaxed excited state. Experimentally it is expected: broadening toward the red (due to relaxed emission), but intersection between absorption and emission $\tilde{E}(0\text{-}0)$ at the same energy (determined by the unrelaxed emission) as in low temperature spectra.

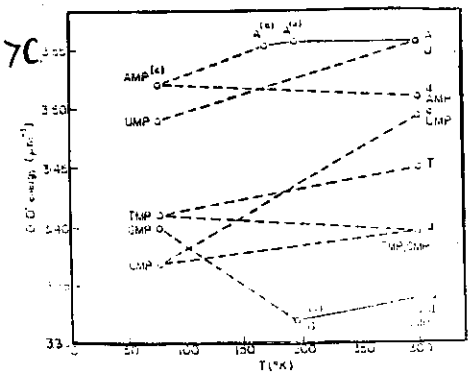
Thymine:	$\tau_{rel} \sim \tau_s$	relaxed + unrelaxed emission
Uracil	$\tau_{rel} \sim \tau_s$	"
Cytosine	$\tau_{rel} > \tau_s$	emission from unrelaxed state
Adenine	$\tau_{rel} \sim \tau_s$	relaxed + unrelaxed emission
Guanine	$\tau_{rel} < \tau_s$	emission from relaxed state

2) Singlet Energy levels $^1E(0\text{-}0)$

It is possible to evaluate them by the energy intersection of the absorption and emission band. This requires careful corrections of the emission data (corrections for the optical response of the systems, for Raman scattering etc.)

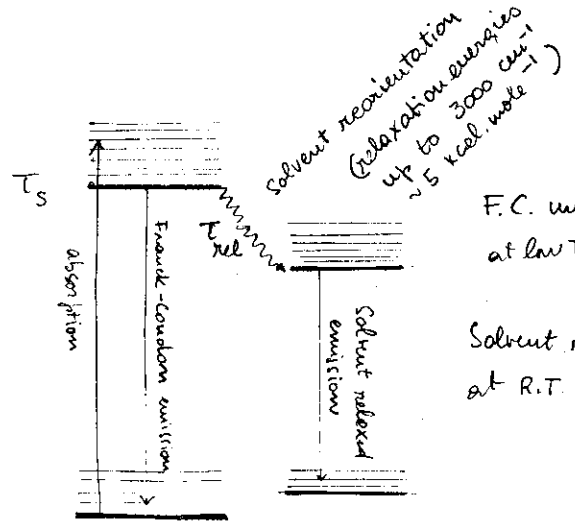
$^1E(0\text{-}0)$ energys are in the order:

$A > U > T \sim G > C$ 77°K.



Lowest singlet energy levels for the bases and nucleotides, all estimated from absorption-emission overlap. a: Data from Callis *et al.* (1964); b: Data from Eastman (1968); c: Data from Gueron *et al.* (1967); d: Data from Vigny (1971b).

$A \simeq U > T > C > G$ 300° K



F.C. unrelaxed emission: at low T. and viscous solvent

Solvent relaxed emission: at R.T. and in fluid medium

3) Excitation Spectra

The excitation spectrum represent the relative probability of emission as a function of the exciting wavelenght.

When the emission band does not change with frequency, the intensity of the emission (observed at constant λ_{em}) varyes with the wavelenght of the exciting light (λ) ($I(\lambda_{em})$ vs λ_{exc} = excitation spectrum) as:

$$I_f \propto \phi_f \cdot I_{abs}(\lambda) = \phi_f \cdot I_0^{\lambda}\left(1 - 10^{-\varepsilon c l}\right)$$

$$\propto \phi_f \cdot I_0^{\lambda}\left[1 - 1 + 2.303 \cdot \varepsilon c l + \cdots (\varepsilon c l)^2 + \cdots\right]$$

$$\propto \phi_f \cdot I_0^{\lambda} \cdot 2.303\ \varepsilon(\lambda) \cdot c \cdot l$$

Therefore when the correction for the wavelenght dependence of the incident exciting light I_0^{λ} is operated (rodhamine correction ...),

$$I_{corr.}(\lambda) = \frac{I_f}{I_0^{\lambda}} \sim \varepsilon(\lambda)$$

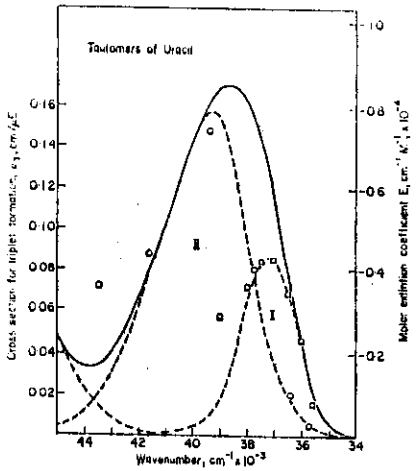
the fluorescence spectrum should overlap the absorption spectrum. Difference between the two spectra can be due to: 1) Instrumental differences (can be checked by measuring absorption and excitation spectra of standard in the same spectral region = quinine bisulphate); 2) Values for Absorbance $= \varepsilon \cdot c \cdot l > 0.05$, since in this case the approximation $I_0(1 - 10^{-\varepsilon c l}) = I_0 \cdot \varepsilon c l$ need to be corrected (taking into account higher terms in the expression).

If difference between absorption and excitation spectra are real (not due to the above effects), as in the case of the DNA bases, two different processes can explain it:

Tautomerism of absorbing species.

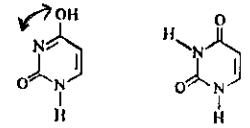
If the absorption spectrum has two components (two possible tautomer of the molecule), then if these two tautomers have different fluorescence yelds their excitation spectrum will mainly coincide with the absorption spectrum of the highly fluorescing tautomer. (the non fluorescing tautomer does not contribute to the total fluorescence)

For uracil and thymine the explanation of the observed difference between excitation and emission spectra is due to tautomerism. For uracil two tautomers are known: enol and diketo form.



Relative fluorescence excitation spectrum (□), and cross-section for triplet formation, (○), fitted to the absorption spectrum of uracil (———). (– – – –) resolved absorption spectra for tautomers I (fluorescent) and II (relatively non-flourescent). *

The emission spectrum excited at 250 nm or 270nm is alike suggesting that only one tautomers has measurable fluorescence.



Enol I Di-Keto II

URACIL TAUTOMERS

* from M. Daniels "Recent Developments in the Fluorescence of DNA bases and DNA at 300°K, in Physicochemical Properties of Nucleic Acids - Vol I. Ed. J. Duchesne (1973) Academic Press.

Two emitting states model.

In general fluorescence is observed from the lowest excited state, and the emission spectrum is constant with the exciting wavelenght. (Kasha rule).
In the case of guanine this is not true: $\lambda_{exc} = 276$ nm gives $\lambda_f(max) \simeq 322$ nm ; $\lambda_{exc} = 240$ nm $\Rightarrow \lambda_f(max) \simeq 240$ nm
Although rare, fluorescence from higher excited singlet states is possible ($S_2 \rightarrow S_0$) and it has been reported for azulene, pyrene, indole.
A dual fluorescences from $S_1 \rightarrow S_0$ and from $S_2 \rightarrow S_0$ can occurr in the case of guanine and adenine, though tautomers fluorescence can also occurr.

Possible tautomers for: G - U - C.

Guanine
$N_1 \leftrightarrow O_6$

Uracil
$N_3 \rightleftarrows O_2$
$\nwarrow O_4$

Cytosine
$N_4 \leftrightarrow O_2$

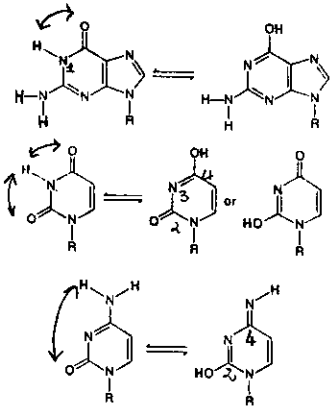
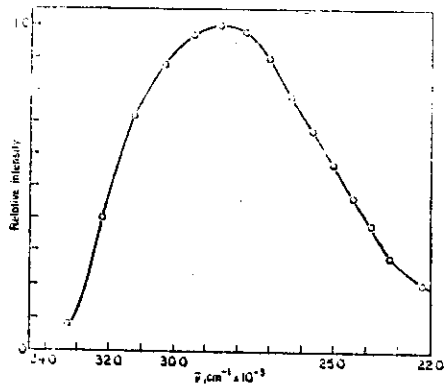


Figure 3-2
Some possible tautomeric equilibria for G, U, and C at pH 7.

Fluorescence of DNA at 300°K.

Relative to the fluorescence of the bases, the spectrum of DNA is broader and red shifted, with $\lambda_{max} \simeq 340 - 350$ nm, with contribution in the range 300 - 330 nm from the bases which is not seen at 77°K.



from M. Daniels in "Physicochemical Properties of nucleic Acids, vol I; Ed. J. Duchesne A. P. (1973)

. Fluorescence emission of DNA at 300°K (Salmon sperm (Calbiochem) in aqueous solution, pH 6·4, A (260) = 0·62). Spectrum obtained from Baird-Atomic SPF-100 spectrofluorimeter, 12 scans into Nuclear Data ND-220A multi-channel analyser; emission bandwidth 6 nm, excitation at 270 nm, 21 nm bandwidth.

Two effects could be responsible for band broadening and red shifted emission: solvent relaxed emission and red-shifted excimer emission.
From $\phi_f = 2 \times 10^{-5}$ and τ_0 calculated from absorption band $\tau_S = \phi_f \cdot \tau_0 = 10^{-12}$ sec at 300 K, whereas $\tau_S = 10^{-9}$ sec at 77°K with $\phi_f \simeq 10^{-1}$.
The fluorescence yeld is much lower than the one observed at 77°K, but it is higher than the average of bases yelds:

DNA	ϕ_f
G	$= 3 \times 10^{-4}$
A	$= 26 \times 10^{-4}$
T	$= 1.0 \times 10^{-4}$ s
C	$= 0.18 \times 10^{-4}$

$\phi_{DNA} \neq \phi_G$

GMP = lowest E(0-0) level.
If energy transfer from other bases to G (= trap) would occurr, then $\phi_{DNA} = \phi_{GMP}$.
$\Rightarrow$ Energy transfer not

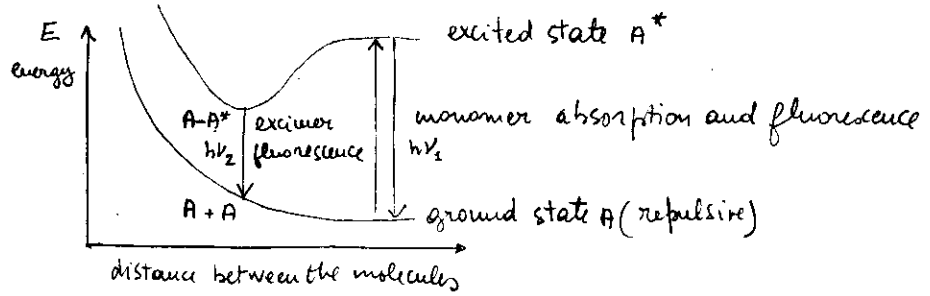
Fluorescence of Adenine, ApA (dinucleotide) and poly A (polynucleotide) at 300K.

Adenine, adenosine, adenosine monophosphate show same spectral shape for the fluorescence emission, although the quantum yield of adenine is one order of magnitude higher than the others. ($\lambda_{max} \sim 300$ nm)

When going to ApA and to poly A a second band appears at 420 nm (ApA) and at 395nm (poly A). This red band can be due to the formation of excimers of A (= excited dimers).

No second band is observed in the absorption spectrum of ApA, poly A when compared with A.

This red band has been observed at 77°K for DNA and polynucleotides and has been assigned to excimer formation:



$A^* + A \rightarrow (A\text{-}A^*) \rightarrow A + A + h\nu_2$

$h\nu_1 \downarrow$

A

dimer of a molecule in its excited state A^* and a molecule in its ground state A

Composé	pH	Rendement quantique (*)	Energie 0-0 en cm^{-1} (**)
Adénine	6	$2{,}6.10^{-4}$	35 650
Adénosine	6,1	$0{,}6.10^{-4}$	35 550
AMP	7	$0{,}5.10^{-4}$	35 550
ApA	7,3	$1{,}4.10^{-4}$	35 350
Poly A	7	3.10^{-4}	34 650

(*) Obtenu en prenant la surface totale du spectre et en prenant pour valeur de référence ϕ_f adénine = $2{,}6.10^{-4}$ (3).

(**) Obtenue en prenant l'intersection des spectres d'absorption et d'émission.

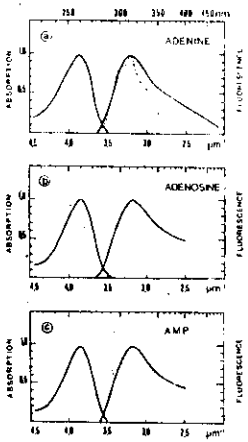


Fig. 1

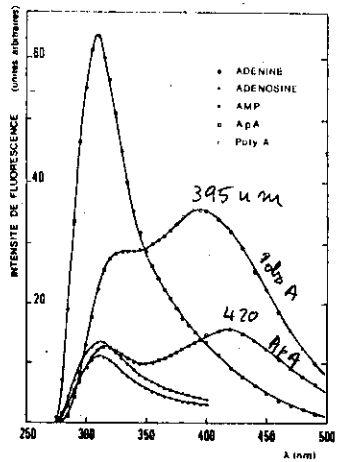


Fig. 2

Fig. 1. — Spectres d'absorption et de fluorescence de l'adénine (*a*), de l'adénosine (*b*) et de l'adénosine 5' monophosphate (*c*) en solution aqueuse et à température ambiante (spectres normalisés à l'unité et portés sur une échelle linéaire en nombre d'onde).

Fig. 2. — Spectres de fluorescence de l'adénine, adénosine, adénosine 5' monophosphate, adénylyl 3'-5' adénosine, et de l'acide polyadénylique en solution aqueuse et à température ambiante (spectres corrigés et normalisés pour une même intensité absorbée. Les rendements quantiques relatifs de fluorescence sont proportionnels aux surfaces des spectres).

from :

M.P. Vigny, C.R. Acad. Sc. Paris, D 277, 1941 (1973)

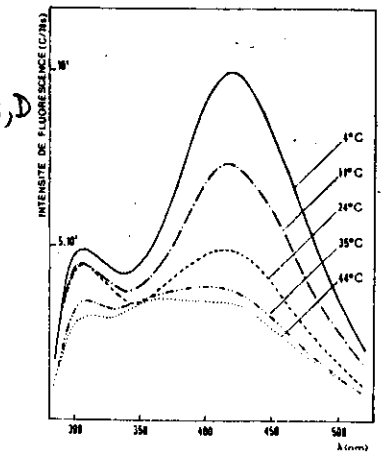


Fig. 3. — Influence de la température sur le spectre de fluorescence de l'adénylyl 3'-5' adénosine

This excited dimer must take place during the life time of the excited state of the molecule, and it is therefore dependent upon temperature and viscosity of the solution. At 77°K when the lifetime of the fluorescence is $\simeq 10^{-9}$ sec, there is ample time for excimer formation to occur. To establish whether this is also possible at 300°K where the lifetime τ_s is reduced to 10^{-12} sec and the situation is less favored, the temperature dependence of the fluorescence spectrum of ApA in the range from 4° to 44°C has been studied.

For dinucleotides it is known from circular dichroism measurements that base stacking is temperature dependent process (favored by lowering T). Since base stacking favores the interaction between the basis, excimer formation is expected to decrease by increasing the temperature of ApA. This is actually observed for the red band at 420 nm.

Excimers can then form also at R.T. and the excimers emission is competitive with that of the monomer. This means that the process of excimer formation is faster or equal to the fluorescence life time $\tau_s = 10^{-12}$ sec.

Intrinsic Polarization of the emission of DNA bases at 300

Intrinsic polarization of fluorescence can be measured if depolarizing effects due to rotational diffusion of a molecule can be eliminated (in crystals or frozen solutions for example).

Actually in the case of the low emission of DNA since the fluorescence life time $\tau_s \sim 10^{-12}$ sec, it is possible that the molecule will emit fluorescence before that they have the time to rotate, and therefore the emission should be polarized also in aqueous solution and at R.T.

From Stokes-Einstein model: $\tau_{rot} \simeq \frac{3\eta V}{kT} \simeq 10^{-10}$ sec

It has been found that for all the bases of DNA with $\Phi_f < 2 \times 10^{-4}$, the degree of polarization of fluorescence

$$P_{em} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} > 0$$

- It decrease when the quantum yield of the bases increases; for guanosine the fluorescence is essentially randomly polarized $P_{em} = 0$ - (request fig 1)

For 5-methylcytosine it is possible to vary the quantum yield of fluorescence by varing the acidity of the solution. Measurements of P_{em} and of Φ allow the evaluation of rotational relaxation times from Perrin relation

$$\frac{1}{P_{em}} + \frac{1}{3} = \left(\frac{1}{P_0} + \frac{1}{3}\right)\left(1 + 3\frac{\tau_f}{\tau_{rot}}\right) \quad \Rightarrow K = \frac{\tau_0}{\tau_{rot}}\left(\frac{3+P_0}{P_0}\right)$$

$$\frac{1}{P_{em}} = \frac{1}{P_0} + K\,\Phi_{em}$$

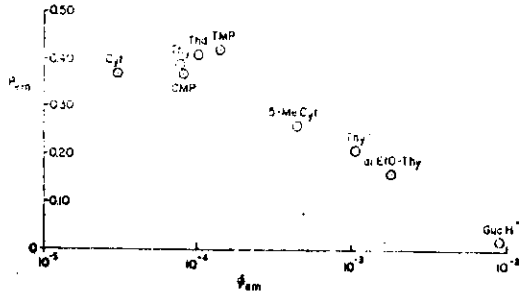


Figure 1. Degree of polarization of fluorescence as a function of quantum yield in aqueous solution, pH 6.8 at 24°C. P_{em} defined as $I_\parallel - I_\perp / I_\parallel + I_\perp$, corrected for spectral sensitivity of apparatus to $\parallel$ and $\perp$ radiation. Quantum yields are relative to ϕ_f (tryptophan) = 0.15 in aqueous solution pH 6.8, consistent with ϕ_f (PPO) = 1.00.

from J.P. Morgan & M. Daniels, Photochemistry and Photobiology 27, 73(1978)

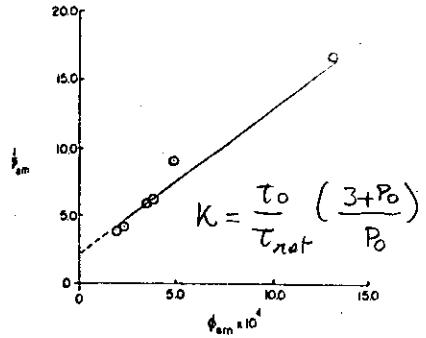


Figure 2. Reciprocal relation between P_{em} (degree of polarization of emission excited by nonpolarized radiation) and quantum yield of emission for aqueous solutions of 5-methylcytosine at 24°C.

From the measured slope, from $\tau_0 = 5$ nsec obtained from the absorption spectrum, and from P_0 = intrinsic degree of polarization = +0.33 for unpolarized excitation it is possible to evaluate:

$$\tau_{rot} \simeq 5 \times 10^{-12} \text{ sec}$$

Intrinsic Polarization of emission of monomer CMP, CpC, and poly C at 30°K.

The corrected emission spectra of CMP, CpC and poly C (when normalized at the peak) displays significant changes: CpC and poly C have components at long wavelenght. Polarization measurements show that the long wavelenght emission is depolarized. This emission has been attributed to excimer fluorescence and to CpC phosphorescence at 460 nm. The emission spectrum of CpC and poly C is therefore composed by: monomer fluorescence ~320 nm - strongly polarized, excimer fluorescence - depolarized ~355 nm and CpC phosphorescence - depolarized (excimer phosphorescence).

Similar results are reported for ApA and poly A. (see M. Daniels and J.P. Morgan, J. of Luminescence 18-19 593 (1979)]

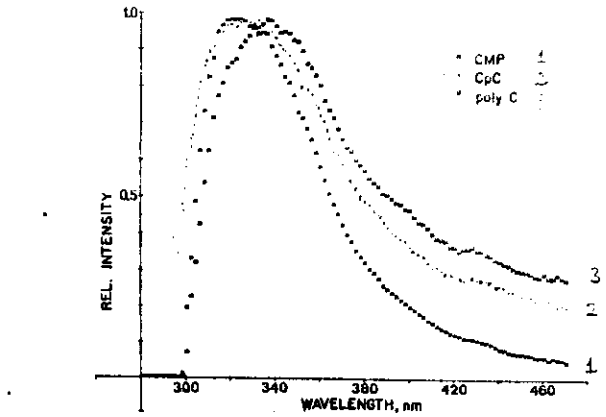


Fig. 1. Normalized corrected emission spectra for CMP, CpC and poly C at pH 6.8, obtained under identical conditions. Excitation wavelength 266 nm.

from M. Daniels and J.P. Morgan, J. of Luminescence 18-19, 593 (1979)

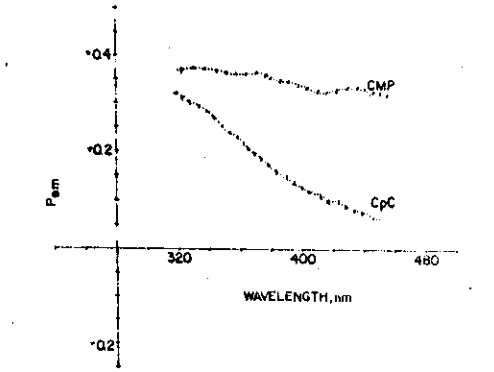


Fig. 2. Degree of polarization of emission spectra of CMP and CpC.

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Fluorescence of Dye - Nucleic Acids Complexes -

Properties of the binding equilibrium of intercalating dyes

Interactions between intercalated dyes and DNA -

Optical response of bound dye and transfer processes dye ↔ DNA -

References: 1) Review article by
G. Löber, Journal of Luminescence 22 (1981) 221

2) "Physical Chemistry of Nucleic Acids"
by V. A. Bloomfield, D. M. Crothers, I. Tinoco.
Academic Press. (1974)

3) "Acridines"
Ed. E. M. Acheson
Interscience N.Y. (1973)

4) "Modern Fluorescence Spectroscopy" vol II
Ed: E. L. Wehry, Plenum Press (1976)

5) Physico Chemical Properties of Nucleic Acids - Vol I. Ed: J. Duchesne.
Academic Press (1973)

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REVIEW ARTICLE

THE FLUORESCENCE OF DYE–NUCLEIC ACID COMPLEXES *

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Contents

Numerous fluorescent compounds, predominantly dyes, are able to form intermolecular complexes with deoxyribonucleic acid (DNA), ribonucleic acid (RNA), nucleoproteins, and various of their synthetic analogues. As a consequence of the binding, fluorescence spectroscopic data, i.e. quantum yield, decay time, and polarization properties, are altered as compared with the free dye. Fluorescence measurements are well suited to gain information on binding processes, steric location of the fluorescent ligands at the nucleic acid template, and energy transfer processes from the nucleic acid bases to complexed compounds. Moreover, conclusions on conformational structures in nucleic acids in vitro and in vivo can be drawn. Finally, they help to study the biological activity of various compounds on a molecular level, including problems involved in chromosomal fluorescence staining.

* Dedicated to my colleague Dr. J. Koudelka, Institute of Biophysics of the Czechoslovak Academy of Sciences, Brno, who died on 24 December 1980.

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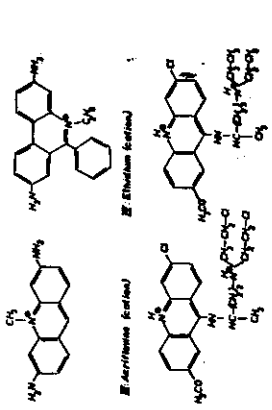
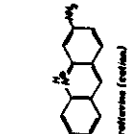


I: Acridine orange (active) II: Ethidium (active) III: Quinacrine (active) IV: Quinacrine mustard (inactive) V: Proflavine (active)

1. Introduction

Several biologically active dyes are capable of forming intermolecular complexes with deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) under free energy changes of less than 10 kcal/mol (42 kJ/mol). These complexes are in the first place investigated on isolated biopolymeric components. Nevertheless, there is good evidence that they are also present in biological systems, where the nucleic acids exist in their natural states [1–6].

In the studies of the biological role of these complexes the dyes of the acridine type are specially important. The nature of the interaction of acridine derivatives with nucleic acids has attracted increasing attention since their earliest use as cellular stains. In experiments with acridine orange (I) and living cells Bukatsch and Haitinger [7] and Strugger [8] found that the emission color of the dye changed from green to red after the death of the cells. Concomitantly the interest has been growing in the reason of the color change occurring in the biological cell. Zanker [9,10] interpreted the change in the fluorescence color as due to the formation of associates, a process which is probably favored in dead cells. De Bruyn et al. [11,12] concluded from their fluorescence staining experiments in vivo that the acridine dye proflavine (II) interacts strongly with the nucleic acids of the cell nucleus. Finally Michaelis [13] put forward the idea that in various cases the binding of flat dye molecules to nucleic acids involves an interaction between those molecules and the heterocyclic nucleic acid bases. Later on it was shown by Oster [14] and others [15,16] that the guanine bases quench the fluorescence of purified acriflavine (III). On the other hand the binding of acridine orange [17–21] or the phenanthridine dye ethidium bromide (IV) [22–24] is accompanied by an enhancement of the fluorescence quantum yield; a fact which implies some unsolved problems.



I: Acridine orange (active) II: Proflavine (active)

At present, a number of fluorescent compounds are known, the reactions of these compounds with nucleic acids were followed by fluorescence measurements. The fluorescence technique, because of its high sensitivity, is a convenient method for measuring binding properties of the dye–nucleic acid interactions. Another motive in the study of such interactions has been to use the small molecule as a probe of differences and changes in the structure of the macromolecule to which it is bound. It is the aim of this paper to review some of the recent results and to discuss the fluorescence effects from the point of view of spectroscopy.

2. Biophysical and biochemical background

2.1. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)

The information determining the amino acid sequences in proteins is stored by long-chain polymeric molecules, the deoxyribonucleic acid (DNA). The most important feature is that the polymeric chains are twisted about each other in the form of a regular double helix. Each chain is a polynucleotide, an arrangement of nucleotides in which the sugar of each nucleotide is linked by a phosphate group to the sugar of the adjacent nucleotide. There are 10 nucleotides on each chain every turn of the helix. There are two pyrimidines, thymine (T) and cytosine (C), and two purines, adenine (A) and guanine (G). Both types of bases are flat, they stack above each other perpendicular to the direction of the helical axis, thus forming the so-called B-helix. The distance of the stacked bases is 0.34 nm. The two polynucleotide chains are connected by

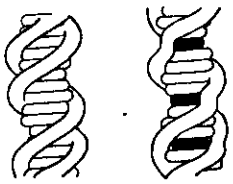


Fig. 2. Sketches demonstrating the helical structure of normal DNA (left) and partially unwound DNA (right) when dye molecules are intercalated (type I_2). Base pairs (white) and intercalated dye molecules (black) appear in edgewise projection (after Lerman [50]).

safranine (VII) [53] (fig. 1). Saturation of type I and type II binding is reached when one dye molecule is bound per four nucleotides ($n = 0.25$) and per one nucleotide ($n = 1$), respectively. Intercalated dye molecules (i.e. type I_2 and type II_1) are capable of unwinding the DNA double helix (fig. 2) [140,141,294] (subsect. 3.5).

2.4. Kinetics and thermodynamics of dye binding

There are various questions which may be asked in biochemistry of dye–nucleic acid complexes to which relaxation kinetics and thermodynamics should provide answers. One of the questions concerns possible intermediates of the intercalation reaction. Kinetic experiments on the binding of proflavine (II) with nucleic acids analogues revealed a fast second-order process (10^6–10^7 M^{-1} s^{-1}), which represents the outside attachment of the cationic dye molecules near negatively charged phosphate residues [296]. The reverse process has a rate constant of 10^3–10^4 s^{-1}, so that a certain dye fraction is permanently placed on the surface giving rise to the occurrence of the type I_1 complex. The rate constant for the subsequent unimolecular rearrangement of surface-bound dye molecules, eventually leading to the insertion of dye between the base pairs (type I_2 complex), is of the order of 10^3 s^{-1}, while the rate constant for the reverse reaction is of the order of 10^2 s^{-1}. The general features of this two-step mechanism were essentially confirmed in related systems [297–302]. A refined model [303], however, assumes that the fast electrostatic binding is followed by an also fast isomerization of this external complex to another form also belonging to type I_1, in which the dye molecules are located in one of the grooves of the nucleic acid helix without intercalation. In agreement with the previous model again the last step corresponds to the intercalation reaction. The kinetics of the daunomycin–DNA interaction determined spectrophotometrically, reflect a single binding step, probably the intercalation process. It's association rate constant evaluated on the basis of a theory which includes the cooperativity and the large size of binding sites, is 9.3×10^6 M^{-1} s^{-1}. The dissociation reaction of the daunomycin-DNA complex is rather slow, 15 s^{-1} [305]. The binding of the ethidium cation (IV) to the synthetic polynucleotide poly(dA–dT), studied with the fluorescence temperature-jump method yielded a single-step reaction from the free to the intercalated form [304]. Since in this case the intercalation reaction is very fast (8.5×10^6 M^{-1} s^{-1}), the kinetics do not discriminate between outside binding and intercalation.

The fluorescence stopped-flow technique was applied to studies on the acridine orange (I)–DNA interaction [92]. From these studies it has become apparent that dimerization of acridine orange on the outside of DNA as well as intercalation are preceded by a rapid binding of dye monomers at the DNA surface. Thus, the observed dimerization is brought about by a rearrangement of randomly bound dye molecules, which may be considered as a one-dimensional movement of dye monomers along the array of DNA-phosphates. This implies that the dimerization on the matrix bears a unimolecular character with a rate constant of about 10^3 s^{-1}.

In the past two decades various attempts have been directed towards the determination of binding constants, which for the binding of the dye in monomeric form usually range from 10^4–10^7 M^{-1} [6,306–313]. However, most of the binding constants are only apparent ones, since they usually represent an average of data which are associated with different binding modes. For proflavine the binding parameters of individual binding steps are known [296–299]. In some cases the apparent binding enthalpies (ΔH_{app}) and binding entropies (ΔS_{app}) have been determined: proflavine (II), $\Delta H_{app} = -28$ to -32 kJ/mol [306]; $\Delta H_{app} = -21$ kJ/mol [307]; ethidium bromide (IV), $\Delta H_{app} = -26$ to -28 kJ/mol and $\Delta S_{app} = 42$ J mol^{-1} K^{-1} [306]; daunomycin, $\Delta H_{app} = -27$ kJ/mol and $\Delta S_{app} = 32$ J mol^{-1} K^{-1} [308], $\Delta H_{app} = -22$ kJ/mol and $\Delta S_{app} = 42$–46 J mol^{-1} K^{-1} [309]; actinomycin, $\Delta H_{app} = 8.4$ kJ/mol and $\Delta S_{app} = 163$ J mol^{-1} K^{-1} [308]. The release of water molecules from the nucleic acid during the binding process could account for the positive entropy changes.

Various evaluation techniques have been described in the literature for the cooperative binding, i.e. the stacking of dye molecules on the matrix [89,90,313,316]. Besides the cooperative binding constant and a parameter, which gives the number of binding sites per phosphate, binding is characterized by a factor measuring the strength of cooperativity. Cooperative binding is favored at low ionic strength, in linear biopolymer model compounds such as polyphosphate or single-stranded polynucleotides, in single-stranded nucleic acids and for dyes with a high stacking tendency.

hydrogen bonds between the bases. Adenine is always paired with thymine (A.T base pair) and guanine with cytosine (G.C base pair).

DNA, however, is not the direct template that determines the amino acid sequences of a protein. These intermediate templates are large polymeric molecules of ribonucleic acid (RNA), which are chemically related to DNA. The sugar of DNA is deoxyribose, whereas RNA contains ribose. Moreover, RNA contains the pyrimidine derivative uracil (U) instead of thymine in DNA. RNA exists to a large extent as a single polynucleotide strand. Before the amino acids line up against the RNA template (mRNA) they are covalently attached to relatively small molecules of transfer RNA (tRNA). Each amino acid has its own tRNA. There is only one way to fold the polynucleotide chain of tRNA under a maximal number of base pairs: all tRNA's form a three-dimensional cloverleaf structure.

2.2. The biological role of the interaction

The number of papers dealing with the biological effects of the complexes of a biogically active compound is so large that only the principal features can be outlined here. The complexes of fluorescent dyes with DNA and RNA in both fixed and living cells differ with respect to the color of their fluorescence. Green fluorescence of acridine orange (I) indicates a double-stranded nucleic acid, e.g. DNA, and red fluorescence indicates single-stranded nucleic acid, e.g. RNA [25–28]. Various acridine derivatives stain the nuclei without killing the cells [7,8,29]. More recently, Caspersson et al. [30] found that each eukaryotic chromosome stained with the fluorescent dye quinacrine mustard (V) showed its own specific fluorescence banding pattern, and visualization and discrimination of metaphase chromosomes (cf. glossary) of a number of organisms have become possible [31,32]. Fluorescence of the Y chromosome stained with quinacrine mustard (V) or quinacrine dihydrochloride (VI) is now routinely used for sex determination [33]. Moreover, the possibility of diagnosing some hereditary diseases by means of the chromosomal fluorescence banding technique has been recognized [34]. It is proposed that various dyes are mutagenic because they stretch the DNA molecule [35,36] or because they stabilize it [37], this means that in both cases distinct conformational changes of the DNA molecule are responsible for the dye-induced mutagenesis. The most investigated mutational effects of dyes on yeast have been those resulting in the formation of strains that produce small colonies ("petite colonie" mutants) [44]. Those appear to be the result of interaction with the mitochondrial nucleic acids [45,46]. Binding of dyes to extrachromosal nucleic acids abolishes the ability of bacteria to transmit resistence to sulfonamides and antibiotics [47]. It has been considered that, at least in some cases, the interaction of inhibitory molecules with nucleic acids is likely to be responsible for the inhibition of both DNA and RNA synthesis [38–40]. Various dyes capable of binding to

nucleic acids show antibacterial action [5,41]. Structure–activity relationships convincingly demonstrated a correlation between the complex forming tendency for nucleic acids and the bacteriostatic action [4,41–43] or the inhibition of multiplication of bacterial viruses (phages) [4,45]. Attempts to correlate the ability of various dyes or aromatic hydrocarbons to bind with nucleic acids and to produce cancer are under active investigation [48].

2.3. The structure of the complexes

Since the fundamental work of Peacocke and Skerrett [49] it seems very likely that the dye binding occurs by two principal types termed as type I and type II (fig. 1). Type I corresponds to the monomer binding which occurs in that the dye is placed in one of the grooves of DNA (type I_1) or between adjacent base pairs (type I_2, intercalation). It depends on the dye structure whether the binding takes place as double-strand [50] or single-strand [51] intercalation. Type II may correspond to an outside stacking binding without base specificity (type II_2). The dye molecules bound by type II_2 can form longer polymeric units. Perhaps as a transitional state between type I_2 and type II_2 the binding type II_1 can be seen where a second dye molecule binds to the exposed part of a dye molecule already partially intercalated. Type II_1 binding has been confirmed for acridine orange (I), proflavine (II) [52] and pheno-

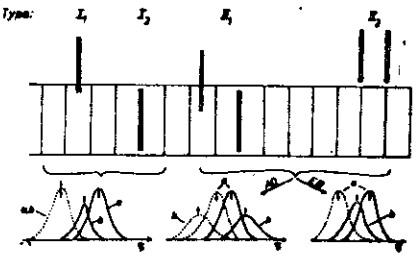


Fig. 1. Schematical representation of various types of dye binding on DNA, black bulks: dye molecules; changes in absorption (———) and fluorescence (.....) spectra are shown; a, free dye; b, bound dye; AO, acridine orange (I); EB, ethidium bromide (IV); arrows indicate the direction of the changes for the individual binding types with degree of binding.

2.5. Glossary and abbreviations

Nucleic acid bases: A, adenine; T, thymine; G, guanine; C, cytosine; U, uracil.
Nucleoside: a base containing either ribose or deoxyribose as sugar residues.
Nucleotide: a base containing a phosphate sugar residue; sugar is either ribose or deoxyribose.
DNA, mRNA, tRNA: see subsect. 2.1; *rRNA*, ribosomal RNA.
Ribosome: small particle in the cell, in which protein synthesis occurs.
Eukaryotic cells: cells which possess nuclei with defined nuclear membranes.
Metaphase chromosomes: phase of the division cycle of eukaryotic cells, in which the chromosomes are arranged in the equator.
Chromosomal fluorescence banding pattern: inhomogeneous distribution of the fluorescence emission of dyes along the metaphase chromosomes upon staining. Regions displaying bright fluorescence are called bands, regions displaying weak fluorescence are called interbands (fluorescence banding may also appear, when different binding types, e.g. type I and type II, give rise to a locally different color of the fluorescence emission on the chromosome).
Chromatin: material isolated from eukaryotic nuclei, which essentially contains deoxyribonucleoprotein (DNP).
Condensation: state in which DNA, DNP or chromatin have become compact by a special spatial arrangement of the macromolecules. The condensed genetic material is usually biologically inactive.
Histones: a group of basic proteins, which are present in the cell nuclei. Histones are of low molecular weight, occur in all eukaryotic organisms and contain 20–30% basic amino acids. They are constituents of chromatin.
Nucleohistone: complex of nucleic acids, preferentially DNA, with histone.
Y chromosome: male sex chromosome; *Y body*: fluorescent Y bodies are detectable in interphase nuclei upon staining with fluorescent basic dyes as brilliantly fluorescing spots. Those spots are identical with the distal part of the long arms of the Y chromosome.
Mutagenic dyes: dyes capable of producing mutations mainly by complexing with DNA.
Poly(G): single-stranded polynucleotide with G as base and ribose as sugar residue; *poly(U)* analogously with U.
Poly(dG): Single-stranded polynucleotide with G as base and deoxyribose as sugar residue.
Poly(dG–dC): single-stranded polynucleotide with alternating sequence of G and C, sugar is deoxyribose; *poly(dA–dT)* analogously. Such polynucleotides form in parts double-stranded regions.
Poly(A,U): Single-stranded polynucleotide with unknown sequence of A and U; sugar is ribose; double-stranded regions by refolding.
Poly(A)–poly(U): double-stranded polynucleotide where one strand is poly(A) and the other strand is poly(U); A and U are hydrogen bonded; sugar is ribose.

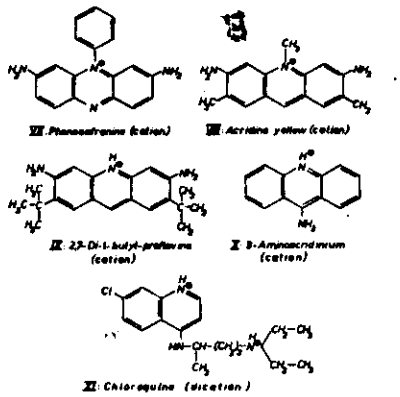
Poly(dA)–poly(dT): Double-stranded polynucleotide where one strand is poly (dA) and the other strand is poly (dT); A and T are hydrogen bonded; sugar is deoxyribose; *poly(dG)–poly(dC)* analogously.

3. Spectral effects caused by the complex formation

3.1. Wavelength shifts

3.1.1. Type I complexes

The most characteristic effect of interaction with nucleic acids on dyes is the displacement of the electronic absorption spectrum to longer wavelengths, when dye molecules are bound by type I_1 or type I_2. The red-shift was originally observed by Michaelis [13] for toluidine blue, phenosafranine (VII), and pyronine and was later confirmed for proflavine (II) [49]. This spectral shift connected with the complex formation was reconfirmed for a great number of substances, not only for dyes [3,4]. Thus, the absorption technique became one of the standard procedures for qualitative and quantitative determination of the dye binding.



Different suggestions have been offered in order to explain this spectral behavior:
(1) The red-shift can be discussed in terms of pure electrostatic interactions.

Thus, on the basis of quantum chemical calculations salt-like bonds between the negatively charged nucleic acid-phosphates and the amino groups of the proflavine cation have been discussed as being responsible for the shift [54]. The binding of a positively charged dye with a negatively charged counterion, can lead to ion pair-like properties, which may cause a band splitting [55]. Depending on the symmetry of the ion-pair complex, only the transition corresponding to the long-wavelength part of the splitted band may be allowed. In apparent agreement with a salt-like bond Lang [56] found that complex formation between the proflavine cation (II) and various mononucleotides in non-aqueous solution produced a red-shift of the long-wavelength absorption band, while no spectral shift was observed when the nucleosides have been used [56]. Not consistent with this assumption is the finding that the binding of uncharged aromatic hydrocarbons [57,58] uncharged chromophores of antibiotics [59,60] with DNA yields the same spectral behavior. Moreover, it seems improbable that the stability of DNA with cationic dyes might predominantly result from ion-pair complex formation of the dye with the DNA, since ion-pair complexes are usually stable in a non-aqueous medium and less stable in water, while the opposite is true for DNA–dye complexes [61,62]. The most serious objection, however, against an electrostatically induced red-shift comes from studies on the binding of acridine orange (I) [63], ethidium bromide (IV) [64], and phenosafranine (VII) [65] to a linear inorganic polyphosphate (Graham salt), which consists of 22–24 phosphate subunits. In an aqueous solution, Graham salt preferentially binds those dyes by process II_2 (stacking complex). At very high ratios of phosphate subunits to dye molecules stacking disappears and the spectral properties of the free dye molecules reappear. Nevertheless, dye molecules are still bound as monomeric units, as the results of equilibrium dialysis show [63]. This clearly indicates than an electrostatic interaction can hardly explain the observed red-shift, thus it seems reasonable to assume that the outside binding of dye monomers, the kinetics of which were spectroscopically measured (subsect. 2.4), does not follow the pattern of pure electrostatic attachment.
(2) A spectral analysis of the shape of the long-wave absorption band of proflavine (II) [19,66], acriflavine (III), and acridine yellow (VIII) [66] shows a narrowing of the band, in particular at the short-wave side (fig. 3). This narrowing can be caused by changes in the vibrational structure of the band, which can also produce a red-shift of the maximum. However, this shift is only minimally related to the hindrance of vibrations by intercalation as was suggested in refs. [19,66]. This conclusion is supported by data obtained for the binding of the quinoline dye pinacyanol [50,67] to DNA and for the binding of the oligopeptide antibiotics netropsin and distamycin A [68] to DNA. None of those ligands are intercalated, but all display a pronounced red-shift upon the binding.
(3) Another interpretation on the origin of the spectral red-shift is based on the

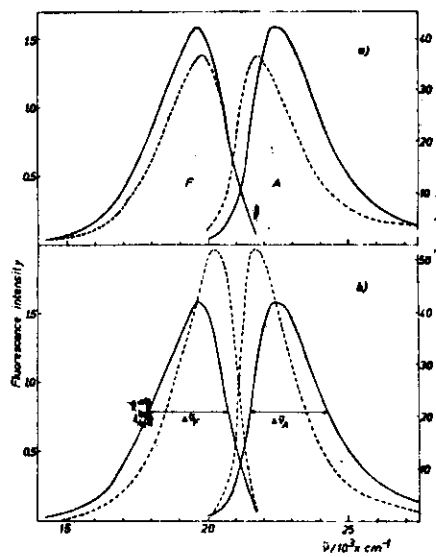


Fig. 3. Absorption (A) and energy corrected fluorescence (F) spectra of proflavine (II); dye concentrations for absorption and fluorescence measurements were 4×10^{-6} M and 2×10^{-6} M, respectively. (a) Spectra in absence (————) and presence (- - -) of DNA in aqueous solution (0.15 M NaCl); molar ratio of DNA phosphate to dye is about 10^3; bandwidths for free dye: $\Delta\bar{\nu}_A \approx \Delta\bar{\nu}_F = 2800$ cm^{-1}; bandwidths for bound dye: $\Delta\bar{\nu}_A = 2400$ cm^{-1}, $\Delta\bar{\nu}_F = 2700$ cm^{-1}. Spectra demonstrate the wavenumber shifts upon binding and the preferential narrowing in the absorption band of bound dye. (b) Spectra in aqueous medium (————) and in ethanol (- - -); band widths in ethanol $\Delta\bar{\nu}_A = 2200$ cm^{-1}, $\Delta\bar{\nu}_F = 2300$ cm^{-1}. Spectra demonstrate wavenumber shifts in passing from an aqueous to an organic environment and the similarity of the latter with the DNA-bound state of the dye; narrowing in absorption and fluorescence spectra is indicated.

intercalation hypothesis which necessarily implies some interaction between the heterocyclic ring system of the ligands with the nucleic acid bases. A π-electron overlap favors the hypochromicity of the long-wave absorption band of the ligand [69,70]. Quantum chemical calculations performed for

aromatic hydrocarbon–DNA complexes yielded an increased polarizability of the ligands in the first excited singlet state [71]. This causes an increase of dispersion forces and a lowering of the excited state level, a fact which could be responsible for the long-wave shift. In agreement with this idea are those findings which show that polarization forces contribute to the stability of the intercalated complexes in the ground state [73] and which may be enhanced in the excited state. This approach, though giving a plausible interpretation for intercalation, excludes some ligands, e.g. netropsin [68], distamycin A [68], pinacyanol [67], and 2,7-di-t-butyl-proflavine (IX) [72], which are not in close proximity with the bases but externally attached to DNA while their spectra are shifted towards red.

(4) An alternative interpretation of the red-shift in the absorption spectra induced by an interaction of bound dye molecules with organic moieties of the nucleic acids comes primarily from the measurements of the solvent effects on the DNA–proflavine complex [61,62,74]. The stability of this complex could only be ensured in an aqueous medium. Recent results obtained for ethidium bromide (IV), phenosafranine (VII) and other similar cationic dyes are in accord with those observed for proflavine (II). The effectiveness of solvents to destabilize the complexes increases in the order: water < glycerol < ethylene glycol < methanol < formamide < ethanol < isopropanol < n-propanol < p-dioxane < dimethylsulfoxide. The complex formation is effectively suppressed by organic solvent concentrations, at which the DNA still preserved its double helical conformation [75] and at which other conformational transitions are largely excluded [76]. It is also obvious that the observed effect cannot be related to the dielectric constant (ε_d) of the solvent added. Even though both ethanol ($\varepsilon_d = 25.8$) and p-dioxane ($\varepsilon_d = 2.24$) have a considerably lower, and formamide ($\varepsilon_d = 110.5$) a considerably higher, dielectric constant compared with water, all decrease the binding of dyes to DNA. Since the order of the solvents substantially coincides with their ability to alterate the water activity [76], we suppose that water activity is important for the stability of dye–DNA complexes.

In order to obtain information on a possible dye–solvent interaction in the absence of DNA, absorption and fluorescence spectra were measured in various ethanol–water mixtures. It can be seen from fig. 4 that, by increasing the organic solvent content, the long-wavelength absorption band of proflavine (II) becomes red-shifted, while the fluorescence spectrum is blue-shifted. Absorption measurements have shown that the solvent-induced bathochromic shift is not very strongly dependent on the kind of organic solvent used. Applying current theories on solvent dependency of electronic spectra [77–79], no measurable dipole moment changes were observed on excitation of the proflavine molecule to its first excited singlet state [80]. This coincides with the shifts found in the absorption and fluorescence spectra as demonstrated in fig. 4 for ethanol–water mixtures. Water gives an absorption maximum at the shortest wavelength. The lack of an isosbestic point in the organic solvent–water mixtures excludes a specific interaction between the solute and solvent molecules. Thus, one inclines to consider that a perturbation of the hydration shell around the solved dye molecules by organic solvent molecules opens a possibility for the latter to interact with the dye. This gives rise to the observed spectral changes which are found for all used solvents as being of approximately the same magnitude. The direction and magnitude of the red-shift in the absorption band by DNA binding agree essentially with the solvent effects found, although the fluorescence shift is not as strongly pronounced for DNA.

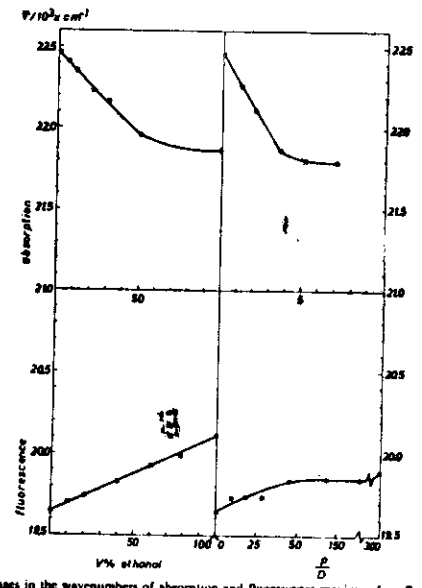


Fig. 4. Changes in the wavenumbers of absorption and fluorescence maxima of proflavine (II) on addition of ethanol or DNA. The figure demonstrates the similarities of both cases. Dye concentration is 2×10^{-5} M; P/D: ratio of DNA phosphate to dye. (The small hypsochromic shift of the fluorescence band goes over a wide P/D range and does not directly correspond to the bathochromic shift of the absorption band.)

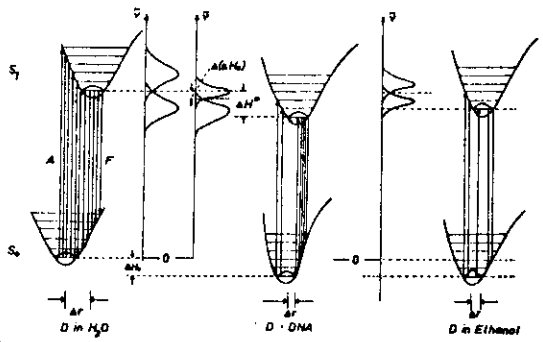


Fig. 5. Tentative scheme of the cross sections of potential hypersurfaces along an (unspecified) nuclear coordinate r responsible for the main progression in the spectra of the dye (D); exemplified in the case of proflavine (II) in H_2O, in ethanol and bound to DNA by type I_1 or type I_2. S_0 is the singlet ground state; S_1 is the first excited singlet state; A is absorption; F is fluorescence; ΔH_0 and ΔH_0^* are the apparent binding enthalpies in S_0 and S_1, respectively; $\Delta(\Delta H_0) = \Delta H_0^* - \Delta H_0$; $\tilde{\nu}$ is wavenumber; r is a nuclear coordinate; the potential curves of D in H_2O and ethanol are of approximately same shape in S_0 and S_1 (Δr smaller in ethanol). The potential curve of D in complex with DNA is steeper in S_0, (Δr being smaller in complex). Those assumptions would explain: (1) the bathochromic shift of the long-wave absorption band and its narrowing in complex with DNA; (2) the hypsochromic shift of the fluorescence band without narrowing in complex with DNA; (3) the bathochromic shift of the long-wave absorption band, the hypsochromic shift of the fluorescence band, and narrowing of both in substituting the aqueous with an organic environment (ethanol); (4) the increased probability of the 0,0 transition, when the dye is bound to DNA or dissolved in an organic solvent, as compared with the aqueous environment.

Thus, one can conclude that the changes of environment, when a dye molecule goes from its hydrated state to the bound state where it is surrounded by purine, pyrimidine, and sugar moieties of nucleic acids, are similar to those accompanying the transition from water to an organic solvent. Birshtein [81] established that hydrophobic interactions, for whose contribution the positive entropy changes are indicative (subsect. 2.4), are mostly pronounced in such molecules which contain both polar groups which are responsible for the solubility in water, and nonpolar groups, which are structuring water in the layer near the hydrocarbon moiety. Both conditions are fullfilled for charged dye molecules capable of interacting with a nucleic acid by formation of stable dye–nucleic acid complexes. Taking as a further basis the kinetic two-step or multistep mechanisms (subsect. 2.4), the first step of the reaction might

represent outside attachment of the dye by binding to the DNA surface in any case, while subsequent steps might then be accompanied by conformational changes of the DNA and rearrangement of water molecules in the hydration shell.

Interpretative energy diagrams and potential curves appropriate to the binding of dye molecules by type I_1 and type I_2 (fig. 5) should be based on the following findings (energy differences between the Franck–Condon state and the equilibrium state are neglected. This seems reasonable, when the dipole moment does not change with excitation, so that reorientation effects are small): (a) ΔH_{app} is mostly negative (except for actinomycin). This results in a lowering of the energy of the ground state in the dye–DNA complex, when compared with dye in water (since for actinomycin the placement of peptide rings in the small groove of the DNA significantly contributes to the binding enthalpy, a correlation of ΔH_{app} with electronic terms of the actinomycin chromophore appears doubtful); (b) the red-shift of the 0,0 transition in going from the free to the bound state of dye is small and corresponds to approximately 2.1 kJ/mol [66]. Thus, the level of the excited single state is only slightly more lowered than the level of the singlet ground state; (c) the long-wave absorption band of a bound dye is red-shifted compared to that of a free dye. Simultaneously, the absorption band is narrowed mainly by the retention of light absorption at the short-wave side. I interpret this as a smaller difference in the interatomic distances between the potential curves of the excited singlet state and the singlet ground state of the complex, whose origin, however, is not clear. Nevertheless, this ad hoc assumption would reflect the only small shift of the fluorescence band towards blue, since the small lowering of the electronic level in the excited singlet state is adjusted and even overcompensated for by the fact that the fluorescence maximum corresponds to emission into a lower lying vibrational level of the ground state (fig. 5).

3.1.2. Type II complexes

Besides the binding of dye monomers with nucleic acids there are indications that various cationic dyes are capable of binding in the form of dye aggregates at a polyanionic matrix (type II_1 and II_2 in fig. 1) or, more precisely, a free dye molecule attaches to a dye molecule already bound [13,49,82–92]. This interpretation is now generally accepted and the spectral properties of dye aggregates can be explained by appropriate theories [9,93,94]. Since there is no significant difference in spectra of type II_1 and type II_2 complexes, both can be considered commonly as type II. The most frequent picture of type II binding which emerges, is that of acridine cations attached approximately edgewise and externally to the polyphosphate chain, with their positive ring nitrogen atoms close to the phosphate groups. Quantum chemical calculations according the LCAO–MO method done for the proflavine cation (II) yielded indeed that the greatest part of the positive charge, about +0.3

charge units, is located at the ring nitrogen atom [95]. The mutual interaction between bound dyes can be sufficiently strong and may be described, e.g. for acridine orange (I), as the binding of dimers or aggregates, which are similar to those which exist in concentrated dye solutions and which give rise to similar spectral shifts [9,10,96]. Acridine orange (I) has its associate bands at shorter wavelengths as compared with dye monomers. Matrix-rank analysis of visible absorption spectra of acridine orange–Graham salt complexes distinguish clearly between the maximal absorption of dye monomers at 492 nm, dimers at 462 nm and longer polymeric units at 447 nm [97]. Similarly to type I complexes it has been found that type II complexes of acridine orange (I) are only stable in a aqueous solution. Addition of organic solvents decreased the stacking ability [63,98].

The existence of dye associates in solution or adsorbed at the nucleic acids induces prominent changes in the fluorescence properties. Thus, the fluorescence of the monomeric acridine orange cation (I) vanishes and a red fluorescence emission typical for stacked associates appears. The type I complex of double-stranded DNA with acridine orange (I) shows fluorescence emission with a maximum at 532 nm and a broad shoulder around 581 nm. After thermal denaturation of DNA, a procedure which changes DNA from a double strand, to single strands, the former emission maximum has disappeared completely and the latter one is shifted into the red region at 637 nm. This indicates that denaturation of DNA enhances the tendency to bind by type II. The behavior of complexes of acridine orange (I) with RNA and Poly(U) is rather similar to that of denatured DNA, due to the single-stranded structure of those polymers. This means that red fluorescence of acridine orange (I) indicating a stacking binding is favored in systems where the structural order of the nucleic acids is significantly lowered [99]. It would be tempting to attribute the change of the emission color of acridine orange-stained biological cells, from green to red after the death of the cells [7,8], to denaturation processes of the cellular nucleic acids.

Type II binding is strongly preferred when an inorganic polyphosphate, e.g. Graham salt, has been used as dye receptor. In fig. 6 the changes in optical density at the absorption maximum and in fluorescence intensity at the fluorescence maximum of acridine orange (I) are plotted as a function of the ratio of Graham salt phosphates (P) to dye (D) molecules (P/D). It is evident that absorption and fluorescence changes essentially correspond to each other. The reappearance of spectral properties of dye monomers at higher P/D ratios is typical for the cooperativity of the binding process. Dye monomers, although electrostatically attached to the polyphosphate chain at a high ratio of P/D, were neither in absorption spectra nor in fluorescence spectra detectably changed as compared to free dye. Similar results were obtained when proflavine (II) [43] or phenosafranine (VII) [65] were used. On the contrary to acridine dyes, the phenanthridine dye ethidium bromide (IV) has its aggregate

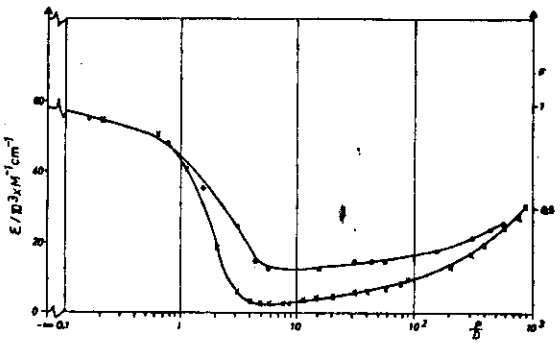


Fig. 6. Changes in the absorption spectrum at 20400 cm^{-1} (.....) and in the fluorescence intensity at 18800 cm^{-1} (×---×) for acridine orange (I) in the dependence on the concentration ratio of Graham salt phosphates to dye, P/D; state of the dye: at $P/D < 0.1$, preferentially free; at $P/D \approx 6$, stacked as associates; at $P/D \approx 10^3$, preferentially bound as monomers.

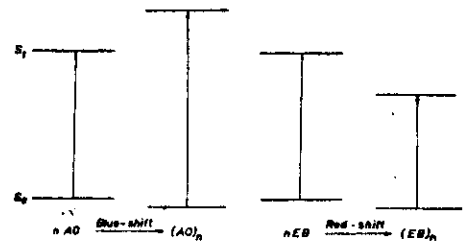


Fig. 7. Simple term schemes of free and associated acridine orange (AO,I) and ethidium bromide (EB,IV) molecules when only electronic terms were considered; $(AO)_n$ and $(EB)_n$ mean associates of AO and EB, respectively; n is the number of subunits forming the associate; the scheme involves the dimers II_1 and II_2 shown in fig. 1 (ground-state levels of associates lowered, since association enthalpies are negative).

spectrum on the long-wave side of the monomer band [64,100,101]. It has been pointed out for other dyes [102,103] that, according to Förster's hypothesis on band splitting [93], the forbidden electronic transition at the long-wave side becomes increasingly allowed, if for electronic or steric reasons an asymmetric aggregate structure is preferred. An approach to interactions, where the dye molecules were treated with a one-dimensional electron gas model for branched electron systems, leads finally to similar results [94,104]. Depending on the steric arrangements of dye molecules in the stacked aggregates, both short and long-wave shifted spectra can again be obtained. The first mainly for parallel stacks, the latter if dye molecules do not lie parallel, i.e. if their planes are displaced towards a head-to-tail binding with only a partial overlap of their π-electron systems. The latter holds for ethidium bromide (IV), since the heterocyclic phenanthridine ring system carries asymmetrically located bulky phenyl and ethyl groups, which produce steric hindrances for the formation of symmetric aggregates [64,101]. In agreement with those ideas is the fact that quantum-chemical calculations of the charge-density distribution in the ethidium cation (IV) yielded an asymmetric electronic structure [105]. The electronic term schemes of both the acridine orange type and the ethidium bromide type of aggregates are presented in fig. 7.

3.2. Fluorescence quenching

For many organic compounds capable of binding to nucleic acids a decrease in the fluorescence intensity was observed, which is primarily caused by a lowering of the fluorescence quantum yield (table 1). Firstly, in 1951 it was

Table 1 (continued)
Fluorescence effects of substances upon complexing with nucleic acids

Binding active compound	Receptor	Fluorescence effects	References
Proflavine (II) [a]	DNA	Quenching	[3,4,19,109,127, 149–155]
	DNA	Polarization	[19,112,127]
	DNA	Excitation transfer	[19,112,143–145]
	RNA	Quenching	[152,194]
	Ribosome	Quenching	[156]
	Chromatin	Quenching	[157]
	Poly(C)	Quenching	[107]
	Poly(dA–dT)	Enhancement	[107,205]
Ring-N-alkylated proflavine, alkyl: methyl (acriflavine, III)	DNA	Quenching	[14–16,20,21,159,162]
	RNA	Quenching	[159]
		Quenching, information on tRNA-folding	[160,194]

Table 1 (continued)
Fluorescence effects of substances upon complexing with nucleic acids

Binding active compound	Receptor	Fluorescence effects	References
	Chromatin	Quenching, A.T specificity of the binding, role of histones	[161]
	Poly(dA–dT) Poly(dG). poly(dC)	Enhancement Quenching	[205]
Alkyl: ethyl n-propyl, n-butyl, n-amyl	DNA	Quenching	[3,4,109,151]
2,7-di-t-butyl-proflavine (IX)	DNA Nucleohistone	Enhancement A.T specificity	[72,209]
Acridine orange (I) [b]	DNA	Enhancement (at high phosphate-to-dye ratio)	[3,4,17–21,66,109, 118,149,151,163–165, 192,195]
	DNA	Quenching (at low phosphate-to-dye ratio)	[18,163,165,166]
	DNA	Excitation transfer	[111,143,192]
	DNA	Binding kinetics by means of fluorescence	[92,167]
	DNA	Polarization	[192]
	DNA	Ratio of native to denatured DNA by means of fluorescence measurements	[116,137,168–170]
	RNA	Ratio of double- to single-stranded RNA by means of fluorescence measurements	[170]
	tRNA	Polarization	[19,128]
	Chromatin	Enhancement	[171,172]
	Chromatin	Decay time	[145]
	Mononucleosides, mononucleotides		[111,152,164,165,173]
Ring-N-alkylated acridine orange; alkyl: methyl, ethyl, n-propyl, benzyl	DNA	Enhancement	[3,4,109,151]

Table 1 (continued)
Fluorescence effects of substances upon complexing with nucleic acids

Binding active compound	Receptor	Fluorescence effects	References
Rivanol [c]	DNA	-Quenching	[3,4,109,151]
Acridine yellow (VIII) [d]	DNA	Quenching Delayed fluorescence	[20,21] [143]
9-aminoacridine (X)	DNA DNA-poly-L-Lysine	Quenching Quenching	[190] [191]
Quinacrine (VI) [e]	DNA	Quenching by G.C Enhancement by A.T	[108] [107]
	Poly(dA–dT)	Enhancement	[107]
	Poly(dA).poly(dT)		[107]
	poly(A).poly(U)		[107]
Diacridines [f]	DNA,RNA	Enhancement by A.T or A.U	[237]
Ethidium bromide (IV) [g]	DNA	Enhancement DNA/DNA determination Decay time	[22–24,113,237] [174,175,211] [121,130]
	RNA	Enhancement Decay time, polarization	[24,176,177,193,210] [121,130,176,178]
	Chromatin	Enhancement	[206–208]
Benzoflavine [h]	DNA	Quenching	[149]
Chloroquine (XI) [i]	DNA	Enhancement	[142]
Thionine [j]	DNA	Quenching	[152]
Thiopyronine [k]	DNA	Quenching, energy transfer	[197]
Methylene blue [l]	DNA	Quenching	[152]
1,9-dimethyl methylene blue, Azure A, Azure B, Azure C	DNA, Chromatin	Quenching	[179]
Phenosafranine (VII) [m]	DNA	Quenching	[180]
LSD [n]	DNA	Quenching	[181]
Berberine [o]	DNA	Enhancement	[182]
Aromatic hydrocarbons	DNA	Quenching Polarization	[57,183–185] [125]

Table 1 (continued)
Fluorescence effects of substances upon complexing with nucleic acids

Binding active compound	Receptor	Fluorescence effects	References
Daunomycin [p]	DNA	Quenching	[60,203]
Violamycin [p]	DNA	Quenching	[179]
Adriamycin [p]	DNA	Quenching	[202]
Actinomycin [q]	DNA	Blue shift, quenching	[286]
Aromatic amino acids	DNA, nucleosides, nucleotides	Quenching, wavelength shift	[187,188]
Oligopeptides	Polynucleotides	Quenching of tryptophan and tyrosine	[201]
Aminoacyl-tRNA synthetase [r]	tRNA	Quenching of tryptophan fluorescence	[158,176,189,196]
Hydroxytilbamidine	DNA	Enhancement, A.T specificity	[204]

[a] 3,6-diaminoacridinium hydrogen sulphate.
[b] 3,6-bis-dimethylaminoacridinium chloride.
[c] 3,9-diamino-7-ethoxyacridinium lactate.
[d] 3,6-diamino-2,7,10-trimethylacridinium chloride.
[e] 9-(4-diethylamino-1-methylbutylamino)-3-chloro-7-methoxy acridinium chloride.
[f] Two acridine rings are linked in position 9 by the chain –NH–R–NH–; R: alkyl chain varying in length.
[g] 3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide.
[h] 3,6-diamino-2,7-dimethyl-9-phenylacridine.
[i] 4-aminoquinoline, antimalarial compound.
[j] 2,7-diamino-phenazthionium chloride.
[k] 3,6-bis-dimethylamino-thioxanthene chloride.
[l] 2,7-bis-dimethylamino-phenazthionium chloride (Methylene blue); 2-methylamino-7-dimethylamino-phenazthionium chloride (Azure B); 2-amino-7-dimethylamino-phenazthionium chloride (Azure A); 2-amino-7-methylamino-phenazthionium chloride (Azure C).
[m] 3,6-diamino-10-phenylphenazinium chloride.
[n] Lysergic acid diethylamide.
[o] Fluorescent alkaloid.
[p] Anthracycline antibiotics.
[q] Enzyme which, in complex with tRNA, is responsible for protein synthesis.
[r] Phenoxazon antibiotic.

supposed by Oster [14] that quenching of the fluorescence of acriflavine (III) by DNA is caused by an interaction of the DNA bases with dye molecules. This result has been confirmed [15]. Since the fluorescence quantum yield of the DNA–acriflavine complex decreases linearly with the G.C content of DNA, a charge transfer process taking place between guanine (which acts as electron donor) and the excited dye molecule (which acts as electron acceptor) was discussed as being responsible for quenching [16]. Quantum chemical calculations indeed yielded better electron donor properties for guanine than for one of the other major bases: adenine, cytosine or thymine [106]. Proflavine (II) and quinacrine (VI) behave similarly to acriflavine (III); the higher the G.C content of the natural DNA's was, the more they quenched the fluorescence of both dyes [107,108]. Quenching was also seen with poly(G) or poly(dG) alone, but not with poly(C) or poly(dC) alone. This result supports the idea that guanine is more involved in the quenching process than cytosine. According to our recent results a one-electron redox mechanism takes place in which preferentially guanine in the ground state becomes oxidized whereas the dye in the excited singlet state becomes reduced. In some cases also adenine is capable in reducing the fluorescence efficiency [123,290].

The ability of several binding active substances, in particular of the dyes, to aggregate, gives rise to another kind of fluorescence changes. Dye aggregates are either non-fluorescent or show a fluorescence emission whose spectral position is quite different from that of monomers [9,10]. In both cases the fluorescence of originally present monomeric dye molecules decreases, e.g. polymerization of acridine orange (I) decreases the intensity of the green fluorescence of monomers and enhances the red fluorescence of dimers or higher aggregates. Fluorescence properties of type II complexes have been thoroughly discussed elsewhere [99].

3.3. Fluorescence enhancement.

Several dyes non- or only weakly fluorescent show an increase in fluorescence intensity when bound to nucleic acids or nucleic acid analogues regardless of the base composition, e.g. acridine orange (I), various derivatives of acridine orange, the alkaloid berberine, the trypanocidal dye ethidium bromide (IV) and others (table 1). The fluorescence increases also on complexing of quinacrine (VI) to polynucleotides which contain no guanine as, e.g. poly(dA–dT) or poly(A).poly(U) [107,108]. In the past various attempts were made to explain the nucleic-acid-induced fluorescence increase as observed for some complexes. The simple interpretation, that aggregates of dyes present in solution, dissociate in favor of a type I binding, e.g. by intercalation, can be excluded since the fluorescence increase of bound acridine orange (I) was observed at a concentration as low as 2×10^{-7} M, where the formation of dye aggregates is negligible [109]. Moreover, ethidium bromide (IV) is well known

for its low aggregation tendency [64], but exhibits a significant fluorescence increase on binding with DNA. Another question that arises is, whether light energy primarily absorbed by nucleic acid bases can be transferred to bound dye molecules giving rise to an enhanced fluorescence. Indeed, it was pointed out that excitation energy is transferable from a nucleic acid triplet to a dye singlet [111], yielding a delayed fluorescence emission of dye molecules [112]. However, it has been shown that fluorescence of bound acridine orange (I) is even enhanced if it was excited with wavelengths longer than 445 nm, where singlet and triplet excitation of nucleic acid bases is not very probable. The investigation of various amino-, monoalkylamino- and dialkylamino-substituted acridines with respect to their fluorescence intensity changes upon DNA binding showed fluorescence quenching so long as both amino hydrogen atoms were not substituted. The dialkylamino dyes are characterized by an enhancement of the fluorescence intensity in the presence of DNA [109]. However, the results on the DNA–ethidium bromide complex do not fit in this scheme: ethidium bromide (IV) with two unsubstituted amino groups yields an increase of the fluorescence intensity upon binding to DNA [113]. More recently it has been suggested that the proton transfer of an amino proton from the excited single state is the process primarily responsible for the low fluorescence yield of ethidium bromide (IV) in water. Enhancement of fluorescence upon intercalation is supposed to be attributed to a reduction in the rate of the excited state proton transfer to solvent molecules [289]. This mechanism, however, is not transferable to acridine orange (I), which shows a strong increase in fluorescence on complexing with DNA but which has no exchangeable amino protons. Even if the ring nitrogen proton became substituted by an alkyl group fluorescence enhancement remained. Thus far, further research is necessary in clarifying this question.

There are indications that the fluorescence of quinacrine (VI) and phenosafranine (VII) enhances if the dyes are bound adjacent to A.T sites while G.C sites quench fluorescence. In the case of natural DNA's, the ability of G.C pairs to quench the fluorescence of both dyes predominates over the tendency of A.T pairs to enhance fluorescence. Thus the overall effect consists in fluorescence quenching by most DNA's. On the other hand, fluorescence of acridine orange (I) is clearly not quenched by guanine but even enhanced, as established by investigating complexes formed with poly(dG).poly(dC), poly(dG) [114] or poly(G) (unpublished result of the author) and the overall effect consists in fluorescence enhancement by most DNA's.

The question arises which other factors except those mentioned above may contribute to the enhanced fluorescence of some dyes upon binding to nucleic acids. In subsect. 3.1.1 it has been pointed out that the environmental change when a dye molecule passes from its free water-solved state to the nucleic acid-bound state can be simulated by investigating the dye spectra in various organic solvent–water mixtures. Striking similarities of the spectroscopic prop-

erties of dyes bound to nucleic acids by type I complexes and of the same dyes dissolved in organic solvents have been shown. The fluorescence intensity of dissolved dyes enhances as a rule with the amount of organic solvent. A plausible hypothesis to explain this phenomenon has been developed [115]. The explanation is based on a different lowering of the singlet and triplet states energies of the dyes in which a small singlet–triplet separation in water facilitates intersystem crossing, thereby lowering the fluorescence quantum yield. Hence, a fluorescence increase is expected when substituting the aqueous surrounding with an organic surrounding as also occurs on the binding to the nucleic acids. The latter suggestion effectively operates in the interpretation of fluorescence banding patterns on chromosomes (subsect. 4.1). But, according to my feeling, various mechanisms are contributing to the fluorescence enhancement effects observed when chemically different dyes become bound.

3.4. Fluorescence decay time

The first measurements on decay times of fluorescent ligands bound to nucleic acids have been reported by Borisova and Tumerman [116]. These authors found an increase in the fluorescence lifetime of bound acridine orange (I). This increase is greater for double-stranded DNA when compared with thermally denatured and therefore partially single-stranded DNA. The absorption and fluorescence characteristics for the monomeric and the dimeric complexes of acridine orange (I) with deoxyribonucleoprotein (DNP) were shown to be identical to those for the complex of the dye with double-stranded DNA. However, quantum yield and lifetime for the dimeric complexes with double-stranded DNA or DNP were only one half of that for the dimeric complex of acridine orange (I) with denatured DNA. This led to the conclusion that the structure of protein-free regions of DNP essentially corresponds to the structure of the double-stranded DNA. However, the difference between the decay times of dimeric complexes formed with native and denatured DNA suggests a different geometry of the complex structure which is not yet known. There is also a distinct difference between single-stranded RNA and single-stranded DNA in the fluorescent behaviour of dimeric complexes with acridine orange (I) which has been discussed on the basis of different complex structures [117].

There are differences in decay times between acridine orange (I) and proflavine (II) bound to DNA, which confirm the differences of the fluorescence quantum yields. The decay time of the monomeric fluorescence of bound acridine orange reflects the increase of the fluorescence intensity so that the ratio of both remains constant, whereas the decay time of the monomeric fluorescence of bound proflavine is only slightly enhanced but the fluorescence intensity is strongly decreased [118]. This supports the assumption that one part of proflavine molecules is attached to binding sites where the fluorescence

is completely quenched (G.C pairs) and another part is located at binding sites which do not quench as effectively as G.C pairs (A.T pairs). Recently, the fluorescence lifetimes and quantum yields of various acridine dyes bound to DNA or polynucleotides have been measured at a high ratio of phosphate to dye, where practically all dye is bound by type I complexes [119]. The main results which are summarized in table 2 indicate that acridine dyes can be classified into three groups: the first group contains substances all binding sites almost completely quench the fluorescence of the dye. Acridines belonging to the second group fluoresce when they are bound to two adjacent A.T pairs; that is, the G.C pair almost completely quenches the fluorescence of the dye. For acridines of the third group the fluorescence properties of the bound dye do not show dependence on the kind of binding sites. Thus far, the

Table 2
Fluorescence lifetimes and quantum yields of acridines (taken from [119], with permission)

	System [d]	τ(ns) [a]	ϕ_F [a]
Group I	10-methylacridinium	54.8	1.00 [b]
	10-methylacridinium + DNA	[c]	< 0.01
	9-aminoacridinium (X)	17.5	0.98
	9-aminoacridinium + DNA	[c]	~ 0.01
Group II	3-aminoacridinium	3.7	0.21
	3-aminoacridinium + DNA	6.3	0.07
	3-aminoacridinium + Poly(dA–dT)	7.5	0.22
	3-aminoacridinium + Poly(dG).poly(dC)	[c]	< 0.01
	3,6-diaminoacridinium (proflavine, II)	5.0	0.44
	3,6-diaminoacridinium + DNA	6.1	0.15
	3,6-diaminoacridinium + Poly(dA–dT)	6.9	0.47
	3,6-diaminoacridinium + Poly(dG).poly(dC)	[c]	~ 0.01
	Quinacrine (VI) [c]	4	~ 0.30
	Quinacrine + DNA	20.7	0.12
	Quinacrine + Poly(A,U)	21.8	0.83
Group III	3,6-bis-dimethylaminoacridinium (acridine orange, I)	1.7	0.25
	3,6-bis-dimethylaminoacridinium + DNA	5.2	0.75
	3,6-bis-dimethylaminoacridinium + Poly(dA–dT)	5.1	0.77
	3,6-bis-dimethylaminoacridinium + Poly(dG).poly(dC)	5.5	0.81
	3,6-bis-diethylaminoacridinium	0.9	0.15
	3,6-bis-diethylaminoacridinium + DNA	4.1	0.63

[a] τ and ϕ_F denote the fluorescence lifetime and the fluorescence quantum yield, respectively.
[b] Taken from ref. [122].
[c] Not measurable because the intensity of fluorescence was very weak.
[d] Experimental conditions: phosphate-to-dye ratio about 200; 5 mM phosphate buffer, pH 6.8; dye concentrations 10^{-6}–10^{-5} M; temperature 25°C.
[e] Taken from ref. [120]; experimental conditions: phosphate-to-dye ratio greater than 100; SSC buffer, ionic strength 0.01; pH 6.8; dye concentration 10^{-5}–10^{-4} M; temperature 20°C.

results of decay-time measurements coincide well with those of previous quenching experiments where it has been suggested that for acridine dyes of the second group, e.g. proflavine (II) or acriflavine (III), the quenching of the fluorescence is mostly attributed to an interaction with the G.C pairs. The well known chromosal stains quinacrine (VI) and quinacrine mustard (V) also belong to the second group. The fluorescence lifetime for the quinacrine–DNA complex increases about 5 times but the quantum yield decreases about 1.5 fold ([120] and table 2). It is concluded that at phosphate-to-dye ratios greater than100 the quinacrine–DNA complex fluoresces with a high quantum yield if there is a sequence of at least three A.T pairs in the binding site of the dye.

As in the case of acridines of the third group, ethidium bromide (IV) shows an enhanced fluorescence quantum yield and an increase in decay time as a consequence of complexing with nucleic acids by process I [121]. The decay time increases with the phosphate-to-dye ratio, reaching a maximum of 23 ns for DNA and of 19.5 ns for RNA (that of the free dye is of the order of 1 ns). The decay time of ethidium is independent of the fact whether excitation took place by the bound dye directly, or by UV light which is absorbed mainly by the nucleic acid bases. This indicates that excitation energy is transferred from the nucleic acids to the intercalated ethidium cation, but the energy transfer process is not rate determining.

3.5. Fluorescence polarization

Investigations of the polarized fluorescence of organic ligands complexed with DNA are based on the fact that fluorescence emitted by the bound ligand is polarized, whereas no polarization is observed in solutions of free ligands. This means that bound ligands are not able to undergo Brownian rotations as effective as in the free state in solution.

Polarization of fluorescence has been used to measure the effective volume, shape, and rigidity of macromolecules, when they are labelled with a fluorescent molecule [126]. Because of the large size of the DNA, however, polarization of fluorescence of the DNA–dye system may yield less information about the DNA as a whole, but may provide some indication of the rigidity of the DNA–dye complex in the region of the bound dye. If, e.g. intercalated proflavine (II) molecules where held rigidly within the DNA helix, and if, in addition, the DNA itself was rigid, the value of the fluorescence polarization would be expected to be perhaps as high as that observed for proflavine (II) in glycerol at low temperature. However, the degree of polarization p is about 0.375, significantly lower than observed for proflavine (II) in glycerol at 4°C, $p = 0.468$. Possible mechanisms for the observed depolarization, including local flexibility in the neighborhood of the dye molecule, and alteration of the position of the dye molecule during the excited state lifetime, are discussed [127]. The binding of proflavine (II) and quinacrine (VI) with

various types of DNA have been studied by fluorescence polarization techniques [128]. Amongst the acridine–polynucleotide complexes the poly(dG–dC) exhibits a significantly low value of p. This might be caused by an increased rotational freedom of either poly(dG–dC) and/or of the bound acridine.

For plane-polarized exciting light, the Perrin equation (1) relates the value of the degree of polarization p to the molecular characteristics provided that the molecule is spherical [131]

$$1/p - 1/3 = (1/p_0 - 1/3)(1 + 3\tau/\rho_0), \quad (1)$$

where p_0 is the limiting value of p when $T/\eta = 0, \rho_0 = 3V\eta/RT$ is the rotational relaxation time of the molecule, R is the gas constant, T is the absolute temperature, τ is the lifetime of fluorescence, η is the viscosity of the solvent and V is the molar volume of the rotating molecule. If the molecule is ellipsoidal in shape one has to substitute ρ_0 in eq. (1) with the harmonic mean $\rho_h = 3V_e\eta/RT$ of the three principal relaxation times, where V_e is the effective hydrodynamic volume of the molecule [126,132,133]. If V and τ are constant eq. (1) predicts a linear relation between $1/p$ and T/η and the rotational relaxation time can be calculated from the slope of such a plot. As has been shown for the complexes of DNA with acridine orange (I) [134], proflavine (II) [127] and other acridine derivatives [135], the $1/p$ vs. T/η curves start with a linear part. However, there is a strong increase in the value of $1/p$ above approximately 60°C. This fact is probably caused by a structural change which takes place in the beginning of the thermal denaturation of the DNA. Since the temperature effects were reversible up to 70°C the structure of the native DNA is not as significantly lost as the polarization of the bound dye is altered. Therefore, fluorescence polarization measurements of bound dyes should be suited to give information on premelting processes occurring in the native DNA that are normally not reflected in the melting profiles obtained from the ultraviolet absorption spectra of the DNA.

Perrin's theory assumes that the decay of the polarized fluorescence components is influenced by the Brownian motion. However, as Jablonski [291] has pointed out, the average lifetimes are different for each of the polarized components (anisotropy decay). The anisotropy decay of the ethidium-bromide DNA-complex has been investigated and it could be confirmed that the depolarization is mainly due to a local deformation motion of the DNA [129]. The excitation energy transfer enhances the anisotropy decay with the number of bound dye molecules per nucleotide [138,139,292]. Taking a statistical distribution of chromophores among equivalent binding sites into consideration, with the condition that two adjacent sites cannot be simultaneously occupied (neighbour exclusion model), and taking an elongation of the DNA of 0.34 nm per intercalated dye molecule, then the angular deformation characterized by the unwinding angle δ can be calculated from the anisotropy decay [138,293]. In the case of ethidium bromide (IV) the best fit was obtained

for $\delta = -(16 \pm 4)°$. However, this unwinding angle is significantly smaller than those got from sedimentation measurements [294], electron microscopy [314] (both yielded $\delta = -26°$) and X-ray crystallography ($\delta = -28°$ [140,141]).

A thorough evaluation of polarization measurements carried out for ethidium bromide (IV) yielded two relaxation times [130]. The longer one (28 ns) is thought to result from the intercalated dye (type I_2 binding), while the shorter one (1 ns) presumably results from the rotation of flexibly attached dye molecules bound by type I_1. Evidence for two classes of attachment of ethidium bromide (IV) to DNA intercalation and outside binding has also been published elsewhere [23].

An analysis of molecular morphology of tRNA's from the rotational relaxation time measured from fluorescence polarization of acridine orange (I) adsorbed on the tRNA molecules has been described [136,137]. A decrease of the degree of fluorescence polarization with increasing temperature was found which indicates a conformational transition below 40°C. It is likely that melting of some parts of the tertiary structure of the tRNA produces a rotational freedom between helical parts of the molecule, since the secondary structure remains practically intact at this temperature. The investigation of the rRNA–ethidium bromide complex yielded an accelerated depolarization above a transition temperature of 32°C, which suggests a shift in the conformational characteristics of the rotating or deforming units of the rRNA molecule [130].

The observation of fluorescence anisotropy on streaming the solution of DNA–dye complexes, where an orientation of the rodlike DNA molecules takes place in the flow direction, implies that the dye molecules are not arranged at random at the DNA matrix. The pattern of intensity changes, observed consistently for binding active polycyclic compounds, i.e. for the cationic acridine derivative, quinacrine (VI) [36,124] and polycyclic hydrocarbons [125] are practically the same. Thus there was an increase with both excitation and emission polarizers transmitting light polarized horizontally, i.e. with the electric vector perpendicular to the flow axis and a corresponding decrease with both polarizers passing vertically polarized light, i.e. with the electric vector parallel to the flow axis (fig. 8). The relevant transitions within the plane of the polycyclic compounds must be close to the horizontal when the ligands are oriented by shear, i.e. nearly perpendicular to the flow axis, and hence to the DNA helix axis. The simplest explanation would be that the molecular planes within which the transitions lie are themselves oriented close to perpendicular to the DNA helix axis. However, the application of a sensitive method for linear dichroism detection of the ethidium bromide–DNA complex yielded an average angle of 60° between the helix axis and the long axis of the ethidium bromide molecule. This value formally contradicts the Watson–Crick model or the intercalation model but may be explained by extension and deformation effects on the chain caused by the flow [317].

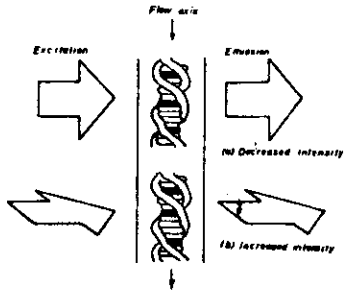


Fig. 8. Scheme showing the relative orientation of DNA molecules under flow conditions and the planes of polarization of exciting and emitted light (after Green and McCarter [125], with permission).

3.6. Excitation energy transfer

The process of singlet–singlet energy transfer between identical molecules causes an energy migration which in turn produces a fluorescence depolarization (subsect. 3.5).

This mainly takes place for smaller phosphate-to-dye ratios where bound dye molecules have a closer spatial proximity. Since fluorescence polarization is different for the complexes of proflavine (II) and acridine orange (I) with DNA, it was demonstrated that, if both dyes are present, nearly 80% of the fluorescence primarily excited in proflavine (II) was emitted from acridine orange (I) [19]. This points to the energy migration from (II) to (I) via a singlet–singlet energy transfer. Additionally, the excitation energy primarily absorbed by the DNA bases can be transferred to the bound dye by a singlet–singlet mechanism [19,23,142].

Indications are available that nucleic acid triplets may also act as energy donors. Thus, complexes of acridine orange (I), proflavine (II), acriflavine (III), quinacrine (IV), and acridine yellow (VIII) with DNA show a delayed fluorescence emission if excitation was performed with ultraviolet light which can be absorbed by both the nucleic acid bases and the dye molecules [112]. The excitation spectrum of the delayed fluorescence does not resemble the absorption spectrum of the dye. The delayed fluorescence cannot, e.g., be activated by absorption in the visible region of the spectrum. The data support the idea that

part of the singlet excitation energy absorbed by the nucleic acid bases is converted to triplet excitation, which migrates along the bases till the neighborhood of a bound dye molecule is reached. Part of the energy may then be transferred to the dye where it is emitted either as dye fluorescence or as dye phosphorescence. A crude estimation showed that the triplet–singlet resonance transfer from a base triplet to an adjacent dye singlet is rather efficient. It should also be realized that a 0.34 nm separation overlap will also assist in the triplet–singlet conversion. An intercalation model (type I_2) makes the triplet to singlet energy migration reasonable and the efficient transfers may be taken as a verification of such a model. The existence of delayed fluorescence in dye–DNA complexes has been confirmed [143–145].

Enhanced ratios of phosphorescence to fluorescence in the complexes of DNA with 9-aminoacridine (X) [146] and chloroquine (XI) [142] when the excitation changed from visible to ultraviolet pointed out that triplet–triplet transfer occurs, a process which probably proceeds through short range interactions [147]. Thus, sensitized phosphorescence implies a close physical proximity of donor (DNA-bases) and acceptor (dye). Hence observation of sensitized phosphorescence measured at 77 K is also compatible with the hypothesis of dye intercalation between adjacent base pairs. The depopulation of the DNA triplet states by the bound dye leads to a slowing down of the photochemical processes with DNA triplets. This concerns mainly the photochemical dimerization of pyrimidines, which goes via the triplet state and which is inhibited by various intercalating dyes [142,146,148]. A dye triplet–triplet annihilation process, taking place predominantly at a low phosphate-to-dye ratio, may also contribute to the occurrence of a delayed dye fluorescence. In the latter case, the delayed fluorescence emission arises from two triplet dye molecules placed very close together and it is tempting to suggest that type II_1- and type II_2-complexes favor the annihilation mechanism.

4. Dye–nucleic acid interaction in higher organized systems

4.1. Fluorescence labelling of chromosomes

At present a very intense application of the fluorescence of dye–nucleic acid complexes is found in chromosome research. Eukaryotic chromosomes contain as major constituents about 15% DNA, 13% RNA and 72% basic and acidic proteins. Physicochemical studies of the interaction of basic dyes with isolated DNP indicate that DNA is the main receptor for cationic dye molecules. Since a considerable part of the binding sites of DNA may be occupied or covered by the proteins, the binding capacity is decreased to about half of that

observed for isolated DNA [198–200,238]. Binding of cationic dyes by chromosomal nucleic acids is probably the most important step in the process of chromosomal staining. Nonstained metaphase chromosomes have been difficult to study because most of them have rather similar morphology so that their identification is not easy. Therefore it must be considered as a real breakthrough when Caspersson et al. [30] found that each chromosome stained with the fluorescent dye quinacrine mustard (V) showed its own specific fluorescence banding pattern. These bands are denoted as Q-bands (Q stands for quinacrine or quinacrine mustard). Positive and negative Q-bands mean chromosomal regions with high and low fluorescence intensities, respectively. Because of the reactivity of mustards with the position N7 of guanine, it was postulated that quinacrine mustard (V) specifically stained chromosomal region rich in G.C pairs [30,31,212]. This assumption is, however, questionable by the finding that treatment of nucleic acids and synthetic polynucleotides with quinacrine mustard (V) resulted in covalently bound fluorescence markers to all natural bases, with adenine and guanine being preferentially attacked [108]. The similarity of the staining pattern obtained with quinacrine mustard (V) and quinacrine dihydrochloride (VI, abbreviated as quinacrine) in chromosomes may indicate common properties of DNA binding involved [213,251]. For the understanding of the processes resulting in chromosomal banding after staining with the appropriate fluorescent dyes it is necessary to know the factors that produce a highly localized brilliant fluorescence as well as a less intense fluorescence along a chromosome (fig. 9). Probably of importance are the spectroscopic process of fluorescence quenching and fluorescence enhancement of dyes as a consequence of dye–DNA interaction with G.C and A.T-rich chromosome regions, respectively [107,114,200,214–216].

Other independent lines of biochemical and cytological research have provided evidence for the existence of longer stretches of DNA that might be highly enriched for A.T pairs and which are strongly fluorescent upon fluorescence staining [214,217–221]. Relatively strong fluorescence has been observed upon staining of the Y chromosome with quinacrine (VI) or quinacrine mustard (V) [222,223], so that a Y body can be visualized even in interphase nuclei [31,32,224]. Fluorescence of the Y chromosome is now routinely used for sex determinations [33,224–226]. It has been shown by DNA denaturation experiments that regions of high quinacrine fluorescence, e.g. the distal half of the Y chromosome, are among the first regions to become denatured by sodium hydroxide [220]. This fact coincides well with idea of a high A.T content since the A.T pairs are well known to become denatured firstly. Drugs with complementary binding specificities, e.g. actinomycin D which binds to G.C sites [227] or netropsin which binds to A.T sites [68] on duplex DNA, were employed in order to obviate the effect of those base selective competitive ligands on the fluorescence of the Y chromosome after quinacrine staining. In the analysis, it was found that only pretreatment of the Y chromosome with

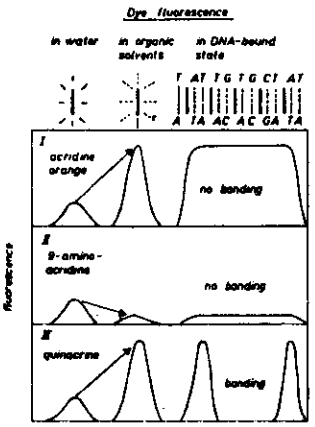


Fig. 9. Model for the dye-induced fluorescence banding patterns in eukaryotic metaphase chromosomes; schematical representation of the fluorescence changes (indicated by the arrow) of different dyes in passing from an aqueous environment to an organic environment (dye dissolved in organic solvent or attached to DNA). Three cases can be distinguished: (case I) no banding, since no base-specific quenching occurs (metachromatic effect left out of consideration), (case II) no banding, since quenching occurs irrespective of the DNA-base composition; (case III) Banding appears, since guanine-specific quenching takes place. The upper part of the figure shows schematically a small chromosomal DNA segment along which the fluorescence in Cases I–III is referred to (taken from [315]).

netropsin, but not with actinomycin D could eliminate quinacrine fluorescence [229]. This means that strongly fluorescing chromosome regions are rich in A.T DNA. Besides the effect of A.T and G.C pairs on the fluorescence of bound dye molecules, the intercalative binding of various acridine dyes exhibits A.T specificity [230–232]. This has been recently confirmed for quinacrine (VI) by observation of an exciton-like splitting between the third excited state of quinacrine (VI) and the lowest excited state of DNA or poly(dA.dT) with a concomitant intensity redistribution in the 260–300 nm region [233–235]. At least it should be mentioned that a study was made on the laser-induced fluorescence properties of the complexes formed by quinacrine mustard (V) bound to DNA, poly(dA).poly(dT) and poly(dG).poly(dC) [236]. In agreement

with other investigations [120] those studies gave a longer fluorescence decay time for the dye bound to A.T sites. The fluorescence decay curves obtained by focusing the laser beam inside or outside a fluorescent band of a chromosome are different. Since the fluorescence decay time measured inside the band regions is close to that known for poly(dA).poly(dT), a direct indication follows that a larger fraction of A.T sequences is present inside the band than outside.

Table 3
Fluorescence labelling of chromosome

Stain		References
Quinacrine (VI) and quinacrine mustard (V) [a]	Chromosome identification	[30–32,34,212,213,221,222,251,257]
	Sex determination	[33,224–226]
	A.T specificity of fluorescence enhancement	[107,108,114,128,200, 214–220,229,239–242, 254–256,267–269]
	Inhibition of fluorescence banding by A.T specific netropsin	[229,243–246]
Hoechst 33258 [b]	Chromosome identification and structure	[255,259–261,263,264]
	A.T specificity of binding and fluorescence enhancement	[110,186,228,256,262,265–267,285]
Berberine [c]	Chromosome identification	[252]
Daunomycin [d]	Chromosome identification	[253]
Adriamycin [d]		[267]
C-15 [d]	Chromosome identification	[254]
Acranil [f]	Mechanism of chromosome banding	[257]
	Inhibition of fluorescence banding by A.T-specifically bound Hg^{++}	[258]

[a] See table 1.
[b] 2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)-benzimidazole-3HCl.
[c] Fluorescent alkaloid.
[d] Anthracycline antibiotics.
[e] 3-bromo-7-methoxy-9(4-diaethylaminobutylamino)-acridine-2HCl.
[f] 3-chloro-9-(3-diethylamino-2-hydroxypropylamino)-7-methoxy-acridine-2HCl.

Arguments against the mechanism of Q-banding in terms of DNA base compositional differences along the lengths of chromosomes have also been presented [247–250]. Those arguments mainly consider that besides base composition also heterogeneous protein distribution along a chromosome may contribute to chromosome banding. Besides quinacrine derivatives various other fluorescent dyes are capable of producing fluorescent bands on chromosomes (table 3).

4.2. Fluorescence staining in living systems

A number of fluorescent compounds which bind specifically with nucleic acids have been studied as probes because their fluorescent characteristics alter in response to complex formation and these changes allow the investigator to look into some of the otherwise inaccessible properties of nucleic acids in living systems. One of the questions concerns the condensation properties of DNP. When, e.g. appropriately fixed and acridine orange-stained cytological cell preparations have been used, fluorescence is most intense in extended DNP and weakest in condensed DNP [99]. This fact has been emphasized by the finding that the uptake of acridine orange (I) in logarithmically growing cells, where the DNP material is believed to exist in a more extended form, was higher than that of the stationary culture [271]. A five-fold increase was observed in the number of binding sites of the chromosomal DNA in going from the resting phase to the dividing phase of yeast cells [272]. A very high green fluorescence intensity of acridine orange (I) has been found in tumor cells of mice when compared with normal cells. Therefore, detection of the presence of tumor cells in a mixed population containing normal cells as well might be accomplished by detecting cells with high relative fluorescence intensity. It is assumed that the high fluorescence intensity observed for those cells could be considered as being indicative of the presence of excess amounts of DNA [275]. This is in keeping with the large excesses in intranuclear DNA found in some members of the tumor cell populations that have been assayed with Feulgen microspectrofluorometry [276]. Studies with acriflavine (III) have also shown a correlation between the presence of tumor cells and an increased uptake of the dye by these cells [277]. By using the fluorescent Schiff reagent of rivanol (see footnote to table 1) evaluations of the relative DNA amounts in cells by cytofluorimetric quantitative analyses have been obtained [278].

Replicating, i.e. dividing cells can also be identified by fluorescence, due to the sensitivity of the fluorescence of the stain 33258 Hoechst (see footnote to table 3) to 5-bromodeoxyuridine incorporation into nuclear DNA. 5-bromodeoxyuridine can be incorporated into DNA instead of thymine. Heavy polarizable atoms such as bromine have been known to quench the luminescence of many organic dyes. This effect can in principle be used for the fluorimetric detection of the DNA synthesis [279].

Ethidium bromide (IV) is taken up rapidly by bovine kidney cells and yeast cells. The fluorescence characteristics of the dye inside the cells, i.e. polarization, excitation spectrum and decay time, are typical of RNA with intercalated ethidium bromide [273]. This statement has been confirmed with other types of living cells [274]. It has been shown earlier that the compact conformation of DNA in bacterial viruses (phages) favors the surface stacking of the dye bound to such a structure (type II_2) [287,288]. Even ethidium bromide (IV) which is known for its rather low stacking tendency forms a stacking complex with phage DNA in situ which yields a decrease of the relative fluorescence intensity with respect to the free dye [64]. Intercalation is significantly diminished in systems which possess DNA in higher compact form.

In agreement with these results we can summarize: nucleic acids in situ show a decreased number of binding sites for dyes as compared to isolated nucleic acids. This is partially due to the fact that proteins which are attached to them can markedly reduce the binding capacity for dyes. Moreover, the condensation state of DNP might influence the ability to bind the dyes. Usually, binding is the greatest in extended DNP and the lowest in condensed DNP. This, however, may be changed when a dye uptake induces the removal of proteins and/or a decompaction of DNA in the DNP complex. This fact would explain that condensed DNP display considerably higher levels of fluorescence than extended DNP in some preparations of cells upon staining with acridine orange (I) [270]. Nevertheless, changes in the nucleic acid–protein interactions or changes in the condensation state of nucleoproteins associated with the transition from resting states to biologically active states, appear to account for most of the alterations in the dye uptake of nucleic acids in situ. Such biochemical modifications of the genetic material can be studied by fluorescence techniques, when the proper fluorescent dyes are used as probes.

4.3. Functioning of tRNA

Most, but not all of the heterocyclic nucleic acid bases, have such low fluorescence quantum yields that they are considered as non-fluorescent at room temperature. However, the Y base at the 3′ site of the anticodon of yeast $tRNA^{Phe}$ (tRNA which is responsible for transfer of phenylalanine in the process of protein synthesis) is fluorescent, but has a rather low absorptivity and fluorescence quantum yield. This base can serve as an internal fluorescent probe for tRNA and ribosome structure [280–283]; the development of sensitive single-photon counting equipments and computerized data handling procedures contributed to an enormous progress in this field of research. The Y base, which has three coplanar rings, can be chemically replaced with various other compounds, where, fortunately, strongly fluorescent and covalently linked proflavine only moderately influences the biochemical function of $tRNA^{Phe}$ (abbreviations used: $tRNA^{Phe}$ is unmodified and $tRNA^{Phe}_{PF}$ is $tRNA^{Phe}$

in which the Y base is replaced by proflavine). Transfer of electronic excitation energy by a non-radiative transition has been observed from the Y base of unmodified $tRNA^{Phe}$ in the donor ribosomal site to the proflavine moiety of modified $tRNA^{Phe}_{PF}$ in the acceptor ribosomal site [283,284]. The results appear to be entirely consistent with the hypothesis that the anticodons of these tRNA's are bound simultaneously to adjacent codons of mRNA. These findings demonstrate clearly the feasibility of fluorescence experiments for investigating ribosome structure and function.

5. Summary

Binding of dyes to nucleic acids occurs by two principal types termed as type I and type II. Type I corresponds to the monomer binding while dyes bound by type II can form dimers and longer polymeric units. The prominent feature of type I-bound fluorescent dyes is that, depending on the structure of the dye the fluorescence becomes either quenched or enhanced. An indication is available according to which quenching occurs by a one-electron redox mechanisms where mostly guanine (second group), but additionally also adenine (first group), in the ground state becomes oxidized, whereas the dye in the excited singlet state becomes reduced. Although the fluorescence of acridine orange, which belongs to the third group, can efficiently be quenched by proper electron donors (dimethylamino- and methoxy-substituted benzenes and naphthalenes [295]), none of the nucleic acid bases is effective in the excited singlet state quenching. Thus, the dominating effect of dyes belonging to the third group is fluorescence enhancement independent of whether the dye molecules are attached to G.C pairs or A.T pairs.

Various mechanisms of fluorescence enhancement upon binding have been presented, among others the energy transfer from an excited nucleic acid base to a bound dye, the environment-induced shifts in the singlet–triplet energy separation leading to a reduction in the singlet-to-triplet intersystem crossing rate of a bound dye and the reduction in the rate of excited state proton transfer to solvent molecules. It has been mentioned that probably none of those mechanism has general validity for all dyes which show enhanced fluorescence upon binding.

Investigations of the polarized fluorescence of organic ligands complexed with nucleic acids yielded information that, in agreement with the intercalation hypothesis, the molecular planes in which the spectroscopic transitions lie are themselves oriented close to perpendicular to the nucleic acid helix. It has become apparent that the fluorescence anisotropy decay of a dye bound to a nucleic acid yields information on the flexibility of the macromolecule and the local motions of the attached dye. Studies of the depolarization by excitation energy transfer provide data on the spatial arrangement of identical chromophores on the matrix. Since these measurements have become promoted, due to the development of the single photon counting technique, the latter is expected to become of increasing importance in systems of biological interest. The possibility of determining the spatial distribution of bound dye molecules by fluorescence methods completes the information obtained by hydrodynamic and nuclear magnetic resonance studies.

Depolarization of the fluorescence of bound substances at a high temperature, but still below the melting region, is related to premelting processes occurring in the native DNA. Thus, the depolarization technique can be seen as a tool for studying small conformational changes as they occur in the course of premelting. Similarly, an analysis of molecular morphology of tRNA's from the rotational relaxation time measured from fluorescence polarization of the dye adsorbed on the tRNA molecule allowed us to get insight into the conformational transitions of the tertiary structure taking place at a moderately high temperature. But, in spite of the temptation to propose a fluorescence technique for detailed investigation of the structural modifications during the biochemical functioning of nucleic acids, such a postulate must await further research.

Although dye–nucleic acid complexes are mainly investigated on the isolated biopolymeric components there is good evidence that they are also present in more complex systems such as nucleoproteins, chromosomes and living cells. Independent lines of research on the production of fluorescence banding patterns in eukaryotic metaphase chromosomes, have provided evidence that strongly fluorescent regions upon staining with quinacrine or quinacrine mustard contain longer stretches of DNA that might be highly enriched in A.T pairs, whereas G.C containing regions display weak fluorescence. A number of fluorescent compounds have been used as probes because their fluorescent characteristics alter in response to complexing and these changes allow us to observe otherwise inaccessible properties of nucleic acids in living systems. This concerns the condensation and the decondensation processes occurring in cellular nucleoproteins, the conformational changes of nucleic acids accompanying the transition from the resting phase to the dividing phase of cells, the differentiation between normal and tumor cells on the nucleic acid level and the feasibility of fluorescence experiments for investigating steps of protein synthesis in ribosomes.

Besides the binding of dye monomers (type I) various cationic dyes are capable of binding in the form of dimers or longer associates (type II). This binding type is accompanied by replacement of the fluorescence of the monomeric dye by a new fluorescence emission which is typical for stacked associates. Denaturation and thus single-strandedness of the nucleic acids enhances the tendency to bind by type II. Usually, the occurrence of a new emission, which accompanies binding by type II, was taken as an indication for single-stranded nucleic acids. It is of interest to note at this point that by using DNA in a more compact form, e.g. DNA inside bacterial viruses, complex formation preferentially takes place by type II. Compact structures of nucleic acids in living systems could therefore be at least partially responsible for binding of dyes as associates.

References

[1] A. Blake and A.R. Peacocke, Biopolymers 6 (1968) 1225.
[2] D.O. Jordan, in Molecular Associations (Academic Press, New York, 1968) p. 221.
[3] G. Löber, Z. Chem. 9 (1969) 252.
[4] G. Löber, Z. Chem. 11 (1971) 92, 135.
[5] A.R. Peacocke, in Acridines, Ed. R.M. Acheson (Interscience, New York, 1973) p. 723.
[6] Progress in Molecular and Subcellular Biology 1, vol. 2, Complexes of Biologically Active Substances with Nucleic Acids and Their Modes of Action (Springer, Berlin 1971).
[7] F. Bukatsch and M. Hastinger, Protoplasma 34 (1940) 515.
[8] S. Strugger, Jenaische Z. Naturwiss. 73 (1941) 97.
[9] V. Zanker, Z. Phys. Chem. 199 (1952) 225.
[10] V. Zanker, Z. Phys. Chem. 200 (1952) 250.
[11] P.P.H. De Bruyn, R.G. Robertson and R.S. Farr, Anatom. Rec. 108 (1950) 279.
[12] P.P.H. De Bruyn, R.S. Farr, H. Banks and F.W. Morthland, Exp. Cell. Res. 4 (1953) 174.
[13] L. Michaelis, Cold Spring Harbor Symp. Quant. Biol. 12 (1947) 131.
[14] G. Oster, Trans. Faraday Soc. 67 (1951) 660.
[15] H.G. Heilweil and Q. Van Winkle, J. Phys. Chem. 59 (1955) 939.
[16] K.R. Tubbs, W.E. Ditmars, Jr. and Q. van Winkle, J. Mol. Biol. 9 (1964) 545.
[17] C.N. Loeser, S.S. West and M.D. Schoenberg, Anatom. Rec. 138 (1960) 163.
[18] R.E. Boyle, S.S. Nelson, F.R. Dollish and M.I. Olsen, Arch. Biochem. Biophys. 96 (1962) 47.
[19] G. Weill and M. Calvin, Biopolymers 1 (1963) 401.
[20] G. Löber, Photochem. Photobiol. 4 (1965) 607.
[21] G. Löber, Monatsber. Dtsch. Akad. Wiss. Berlin 7 (1965) 133.
[22] J.B. Le Pecq and C. Paoletti, Anal. Biochem. 17 (1966) 100.
[23] J.B. Le Pecq and C. Paoletti, J. Mol. Biol. 27 (1967) 87.
[24] I. Sela, Biochim. Biophys. Acta 190 (1969) 216.
[25] A. Krieg, Experientia (Basel) 10 (1954) 172.
[26] J.A. Armstrong, Exp. Cell. Res. 11 (1956) 640.
[27] J. Smiles and E.A.R. Taylor, Nature 179 (1957) 306.
[28] D. Roth and M.L. Manjon, Biopolymers 7 (1969) 695.
[29] P.P.H. De Bruyn, R.C. Robertson and R.S. Farr, J. Biol. Chem. 108 (1951) 279.
[30] T. Caspersson, S. Farber, G.E. Foley, J. Kudynowski, E.J. Modest, E. Simonsson, U. Wagh and L. Zech, Exp. Cell. Res. 49 (1968) 219.
[31] T. Caspersson, L. Zech, C. Johansson, J. Lindsten and M. Thieten, Exp. Cell. Res. 61 (1970) 472.
[32] P.L. Pearson, M. Bobrow and C.G. Vosa, Nature 226 (1970) 78.
[33] V. Beensen, M. Becker and K. Sommer, Biol. Rdsch. 12 (1974) 280.
[34] T. Caspersson and J. Lindsten, Triangel 11 (1972) 73.
[35] S. Brenner, L. Barnett, F.H.C. Crick and A. Orgel, J. Mol. Biol. 3 (1961) 121.
[36] L.S. Lerman, Proc. Natl. Acad. Sci. USA 49 (1963) 94.
[37] G. Streisinger, Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi and M. Inouye, Cold Spring Harbor Symp. Quant. Biol. 31 (1966) 77.
[38] B.H. Nicholson, in Acridines, ed. R.M. Acheson (Interscience New York, 1973) p. 759.

[39] M.J. Waring, Nature 219 (1968) 1320.
[40] M.J. Waring, Symp. Soc. Gen. Microbiol. 16 (1966) 235.
[41] A.C.R. Dean, in Acridines, ed. R.M. Acheson (Interscience, New York, 1973) p. 789.
[42] A. Albert, The Acridines, 2nd ed. (Edward Arnold, London, 1966).
[43] G. Löber, Habilitationsschrift, F. Schiller Universität Jena (1969).
[44] B. Ephrussi, Nucleo-Cytoplasmic Relations in Microorganisms (Clarendon Press, Oxford, 1953).
[45] G. Löber, W. Fleck, H.-E. Jacob and K. Rost in Wirkungsmechanismen von Fungiziden, Antibiotika und Cytostatika (Akademie Verlag, Berlin, 1970) p. 39.
[46] B. Ephrussi, H. Hottinguer, Cold Spring Harbor Symp. Quant. Biol. 16 (1951) 75.
[47] T. Watanabe, Bacteriol. Rev. 27 (1936) 87.
[48] D.B. Clayson, in Acridines, ed. R.M. Acheson (Interscience, New York, 1973) p. 815.
[49] A.R. Peacocke and J.N.H. Skerrett, Trans. Faraday Soc. 52 (1956) 261.
[50] L.S. Lerman, J. Mol. Biol. 3 (1961) 18.
[51] N.J. Pritchard, A. Blake and A.R. Peacocke, Nature 212 (1966) 1360.
[52] R.W. Armstrong, T. Kurucsev and U.P. Strauss, J. Am. Chem. Soc. 92 (1970) 3174.
[53] V. Kleinwächter, Z. Balcarová, J. Koudelka, G. Löber, K.E. Reinert, L.P.G. Wakelin and M.J. Waring, Studia Biophys. 67 (1978) 53.
[54] L.L. Ingraham and H. Johansen, Arch. Biochem. Biophys. 132 (1969) 205.
[55] F. Feichtmayr and J. Schlag, Ber. Bunsenges. Phys. Chem. 68 (1964) 95.
[56] H. Lang, private communication.
[57] E. Boyland, B. Green and S.L. Lim, Biochim. Biophys. Acta 87 (1964) 653.
[58] I. Isenberg and S.L. Baird, Biopolymers 5 (1967) 477.
[59] W. Müller and D.M. Crothers, J. Mol. Biol. 68 (1972) 21.
[60] E. Calendi, A. di Marco, M. Reglani, B.M. Scarpinato and L. Valentini, Biochim. Biophys. Acta 103 (1965) 25.
[61] G. Löber, Studia Biophys. 24/25 (1970) 233.
[62] G. Löber, H. Schütz and V. Kleinwächter, Biopolymers 11 (1972) 2439.
[63] G. Löber, V. Kleinwächter and H. Berg, Studia Biophys. 35 (1973) 29.
[64] G. Löber, J. Koudelka and E. Smětal, Biophys. Chem. 2 (1974) 158.
[65] Z. Balcarová, V. Kleinwächter, J. Koudelka, R. Klarner and G. Löber, Biophys. Chem. 8 (1978) 17.
[66] G. Löber, in Elektrochemische Methoden und Prinzipien in der Molekularbiologie (Akademie Verlag, Berlin, 1966) p. 147.
[67] M. Kodama, Y. Takashira and C. Nagata, Biochim. Biophys. Acta 129 (1966) 638.
[68] Ch. Zimmer, K.E. Reinert, G. Luck, U. Wähnert, G. Löber and H. Thrum, J. Mol. Biol. 58 (1971) 329.
[69] D.S. Drummond, V.F.W. Simpson-Gildemeister and A.R. Peacocke, Biopolymers 3 (1965) 135.
[70] N.F. Gersch and D.O. Jordan, J. Mol. Biol. 13 (1965) 138.
[71] M.J. Matione, Photochem. Photobiol. 17 (1973) 169.
[72] W. Müller, D.M. Crothers and M.J. Waring, Eur. J. Biochem. 39 (1973) 223.
[73] W. Müller, H. Bünemann and N. Dattagupta, Eur. J. Biochem. 54 (1975) 279.
[74] G. Löber, V. Kleinwächter, J. Koudelka and E. Smětal, Studia Biophys. 45 (1974) 91.
[75] T.T. Herskovits, Arch. Biochem. Biophys. 97 (1962) 474.
[76] G. Malenkov, L. Minchenkova, E. Minyat, A. Schyolkina and V. Ivanov, FEBS Lett. 51 (1975) 38.
[77] N. Mataga, Y. Kaifu and M. Koizumi, Bull. Chem. Soc. Japan 29 (1956) 465.
[78] E. Lippert, Z. Elektrochem. Ber. Bunsenges. Physik. Chem. 61 (1957) 962.
[79] W. Liptay, Z. Naturforsch. 20 a (1965) 1441.
[80] H. Lang and G. Löber, Ber. Bunsenges. Physik. Chem. 73 (1969) 710.

[81] T.M. Birshtein, in Water in Biological Systems, ed. L.P. Kayushin, translated by Consultants Bureau (New York, 1969) p. 9.
[82] R.F. Beers, Jr., D.D. Hendley and R.F. Steiner, Nature 182 (1958) 242.
[83] D.F. Bradley and G. Felsenfeld, Nature 184 (1959) 1920.
[84] R.F. Steiner and R.F. Beers, Jr., Arch. Biochem. Biophys. 81 (1959) 75.
[85] D.F. Bradley, Trans. New York Acad. Sci. 24 (1961) 64.
[86] A.L. Stone and D.F. Bradley, J. Am. Chem. Soc. 83 (1961) 3627.
[87] A.L. Stone, Biochim. Biophys. Acta 148 (1967) 193.
[88] A.N. Dey and S.R. Palit, Ind. J. Chem. 6 (1968) 260.
[89] G. Schwarz, Eur. J. Biochem. 12 (1970) 442.
[90] G. Schwarz, S. Klose and W. Balthasar, Eur. J. Biochem. 12 (1970) 454.
[91] B.C. Myhr and J.G. Foss, Biopolymers 10 (1971) 425.
[92] M. Sakoda, K. Hiromi and K. Akasaka, J. Biochem. 71 (1972) 891.
[93] Th. Förster, Naturwissens. 33 (1946) 166.
[94] Y.V. Czikkely, G. Dreizler, H.D. Försterling and H. Kuhn Chem. Phys. Lett. 6 (1970) 207.
[95] H. Lang and G. Löber, Tetrahedron Lett. 46 (1969) 4043.
[96] W. Appel and V. Zanker, Z. Naturforsch. 13 b (1958) 126.
[97] I. Petri, W. Förster and G. Löber, Studia Biophys. 45 (1974) 61.
[98] G. Löber and V. Kleinwächter, Studia Biophys. 33 (1972) 73.
[99] R. Rigler, Acta Physiol. Scand. Suppl. 67 (1966) 267.
[100] V. Crescenzi and F. Quadrifoglio, Eur. Polymer J. 10 (1974) 329.
[101] G. Löber, Studia Biophys. 52 (1975) 25.
[102] G. Scheibe and O. Wörn, Angew. Chem. 78 (1966) 304.
[103] K.K. Rohatgi and A.K. Mukhopadhyay, Photochem. Photobiol. 14 (1971) 551.
[104] Y.V. Czikkely, G. Dreizler, H.D. Försterling, H. Kuhn and J. Sondermann, Z. Naturforsch. 24 a (1969) 1821.
[105] P.O. Giaccononi and M. Le Bret, FEBS Lett. 29 (1973) 277.
[106] B. Pullmann and A. Pullmann, Rev. Mod. Phys. 32 (1960) 428.
[107] B. Weisblum and P.L. De Haseth, Proc. Natl. Acad. Sci. USA 69 (1972) 629.
[108] A.A. Michelson, C. Moony and A. Kovoor, Biochimie 54 (1972) 1129.
[109] G. Löber and G. Achtert, Biopolymers 8 (1969) 595.
[110] T. Chen, Hum. Genet. 36 (1977) 283.
[111] S. Basu and J. Greist, J. Phys. Chem. 67 (1963) 1394.
[112] I. Isenberg, R.B. Leslie, S.L. Baird, Jr., R. Rosenblath and R. Bersohn, Proc. Natl. Acad. Sci. USA 52 (1964) 379.
[113] J.B. Le Pecq, P. Yot and C. Paoletti, C.R. Hebd. Sci. Acad. Sci. 259 (1964) 1786.
[114] B. Weisblum and P.L. De Haseth, Chromosomes Today 4 (1973) 35.
[115] J.J. Selistkar and L. Brand, J. Amer. Chem. Soc. 93 (1971) 5414.
[116] O.F. Borisova and L.A. Tumerman, Biofizika 9 (1964) 537.
[117] S. Ichimura, Biopolymers 14 (1975) 1033.
[118] G. Weill, Biopolymers 3 (1965) 567.
[119] Y. Kubota, Chem. Lett. (1973) 299.
[120] O.F. Borisova, A.P. Razjivin and V.I. Zaregorodtzev, FEBS Lett. 46 (1974) 239.
[121] V.W.F. Burns, Arch. Biochem. Biophys. 133 (1969) 420.
[122] G. Weber and F.W.J. Teale, Trans. Faraday Soc. 53 (1957) 646.
[123] L. Kittler, G. Löber, F.A. Gollnick und H. Berg, Bioelectrochem. Bioenerget. 7 (1980) 503.
[124] L.S. Lerman, J. Cell. Comp. Physiol. (Suppl. 1) 64 (1964) 1.
[125] B. Green and J.A. McCarter, J. Mol. Biol. 29 (1967) 447.
[126] G. Weber, Biochem. J. 51 (1952) 155.
[127] N.F. Ellerton and I. Isenberg, Biopolymers 8 (1969) 767.
[128] U. Pachmann and R. Rigler, Exp. Cell. Res. 72 (1972) 602.
[129] Ph. Wahl, J. Paoletti and J.B. le Pecq, Proc. Natl. Acad. Sci. USA 65 (1970) 417.
[130] V.W.F. Burns, Arch. Biochem. Biophys. 145 (1971) 248.
[131] F. Perrin, J. Phys. Radium 7 (1926) 390; Ann. Phys. (Paris) 12 (1929) 169.
[132] G. Weber, Adv. Protein Chem. 8 (1953) 415.
[133] R.F. Steiner and A.J. McAlister, J. Polym. Sci. 24 (1957) 105.
[134] E.V. Anufrieva, M.V. Volkenstein and T.V. Sheveleva, Biofizika 7 (1962) 554.
[135] Y. Kubota, Bull. Chem. Soc. Japan 46 (1973) 2630.
[136] O.F. Borisova, L. Kisselev, A. Surovaya, L. Tumerman and L. Frolo[illegible]d. Nauk SSSR 159 (1964) 1154.
[137] A. Surovaya, O.F. Borisova, T. Jilyaeva, V. Scheinker and L. Kisselev, FEBS Lett. 8 (1970) 201.
[138] D. Genest and Ph. Wahl, in Dynamic Aspects of Conformation Changes in Biological Macromolecules, ed. C. Sadron (Reidel Dordrecht, 1973) p. 367.
[139] J.L. Tichadou, D. Genest, Ph. Wahl and G. Aubel-Sadron, Biophys. Chem. 3 (1975) 142.
[140] C.C. Tsai, S.C. Jain and H.M. Sobell, J. Mol. Biol. 114 (1977) 301.
[141] S.C. Jain, C.C. Tsai and H.M. Sobell, J. Mol. Biol. 114 (1977) 317.
[142] J.C. Sutherland and B.M. Sutherland, Biochim. Biophys. Acta 190 (1969) 545.
[143] Y. Kubota, Y. Fujisaki and M. Miura, Bull. Chem. Soc. Japan 42 (1969) 853.
[144] Y. Kubota, Bull. Chem. Soc. Japan 43 (1970) 3121.
[145] Y. Kubota, Bull. Chem. Soc. Japan 43 (1970) 3126.
[146] B.M. Sutherland and J.C. Sutherland, Biophys. J. 9 (1969) 292.
[147] W.C. Galley, Biopolymers 6 (1968) 1279.
[148] R. Beukers, Photochem. Photobiol. 4 (1965) 935.
[149] G. Löber, Studia Biophys. 2 (1967) 79.
[150] G. Löber, Studia Biophys. 2 (1967) 99.
[151] G. Löber, Photochem. Photobiol. 8 (1968) 23.
[152] G. Tomita, Z. Naturforsch. 23b (1968) 922.
[153] J.C. Thomas, G. Weill and M. Daune, Biopolymere 8 (1969) 647.
[154] R. Bidet, J. Chambron and G. Weill, Ann. Phys. Biol. et Med. 1 (1970) 1.
[155] G. Strauss, S.R. Broyde and T. Kurucsev, J. Phys. Chem. 75 (1971) 2727.
[156] M. Schoentjes and E. Fredericq, Biopolymers 11 (1972) 361.
[157] J.J. Lawrence and M. Louis, Biochim. Biophys. Acta 272 (1972) 231.
[158] C. Hélène, F. Brun and M. Yaniv, Biochim. Biophys. Res. Commun. 37 (1969) 393.
[159] L.M. Chan and Q. Van Winkle, J. Mol. Biol. 40 (1969) 491.
[160] A. Surovaya and S. Trubitsyn, FEBS Lett. 25 (1972) 349.
[161] R.E. Minyat, O.F. Borisova, M.V. Volkenstein and G.F. Georgiev, Mol. Biol. 4 (1970) 291.
[162] L.M. Chan and J.A. McCarter, Biochim. Biophys. Acta 204 (1970) 252.
[163] G. Tomita, Biophysik 4 (1967) 23.
[164] G. Tomita, Biophysik 4 (1967) 118.
[165] S. Yamabe, Arch. Biochem. Biophys. 130 (1969) 148.
[166] M.K. Pal, Histochemie 5 (1965) 24.
[167] K. Akasaka, M. Sakoda and K. Hiromi, Biochem. Biophys. Res. Commun. 40 (1970) 1239.
[168] M.D. Feddejeva, Cytologija 11 (1969) 56.
[169] S. Ichimura, M. Zama and H. Fujita, Biochim. Biophys. Acta 240 (1971) 485.
[170] L.N. Romanovskaja, A.M. Kulba and I.M. Gabrilovich, Biofizika 17 (1972) 313.
[171] O.F. Borisova and E.E. Minyat, Mol. biol. 3 (1969) 758.
[172] D. Killander and R. Rigler, Exp. Cell. Res. 54 (1969) 163.
[173] O.F. Borisova, P. Horacheck, G.V. Gursky, E.E. Minyat and V.G. Tumanyan, Mol. Biol. 2 (1968) 475.
[174] K. Van Dyke and C. Szustkiewicz, Anal. Biochem. 23 (1968) 109.
[175] J.A. Doukeraloot, S.A. Robrish and M.J. Krichevsky, Appl. Microbiol. 24 (1972) 179.

[176] R. Rigler, E. Cronwall, R. Hirsch, U. Pachmann and H.G. Zachau, FEBS Lett. 11 (1970) 320.
[177] A. Maelicke, Studia Biophys. 24/25 (1970) 343.
[178] R. Bittman, J. Mol. Biol. 46 (1969) 251.
[179] G. Löber and R. Klamer, unpublished results.
[180] Z. Balcarová, V. Kleinwächter, J. Koudelka, G. Löber, K.E. Reinert, L.P.G. Wakelin and M.J. Waring, Biophys. Chem. 8 (1978) 29.
[181] K.L. Yielding and H. Sternglanz, in Progress in Molecular and Subcellular Biology, Vol. 2, ed. F.E. Hahn (Springer Verlag Berlin, 1971) p. 163.
[182] F.E. Hahn and A.K. Krey, in Progress in Molecular and Subcellular Biology, Vol. 2, ed. F.E. Hahn (Springer Verlag Berlin, 1971) p. 134.
[183] E. Boyland and B. Green, Brit. J. Cancer 16 (1962) 507.
[184] E. Boyland and B. Green, J. Mol. Biol. 9 (1964) 589.
[185] A.M. Liquori, B. De Lerma, F. Ascoli, C. Botre and M. Frasciatti, J. Mol. Biol. 5 (1962) 521.
[186] S. Misawa, T. Takino, M. Morita, T. Abe and T. Ashihara, Jap. J. Hum. Genet. 21 (1977) 1.
[187] T. Montenay-Garestier and C. Hélène, Studia Biophys. 24/25 (1970) 359.
[188] C. Hélène, T. Montenay-Garestier and J.L. Dimicoli, Biochim. Biophys. Acta 254 (1971) 349.
[189] C.J. Bruton and B.S. Hartley, J. Mol. Biol. 52 (1970) 165.
[190] P.D. Lawley, Biochim. Biophys. Acta 22 (1956) 451.
[191] H. Hasumi, K. Akasaka, H. Hatano and K. Hiromi, Biochem. Biophys. Res. Commun. 50 (1973) 992.
[192] E. Fredericq and C. Houssier, Biopolymers 11 (1972) 2281.
[193] C. Urbanke, R. Römer and G. Maass, Eur. J. Biochem. 33 (1973) 511.
[194] O.F. Borisova, A.P. Potapov, A.N. Surovaya, S.N. Trubitsyn and M.V. Volkenstein, Mol. Biol. 4 (1973) 509.
[195] S. Yamabe, Arch. Biochem. Biophys. 154 (1973) 19.
[196] M. Ehrenberg, E. Cronwall and R. Rigler, FEBS Lett. 18 (1971) 199.
[197] S. Lalitha and A. Haug, Biochem. 11 (1972) 1590.
[198] D.E. Comings and E. Avelino, Chromosoma (Berlin) 51 (1975) 365.
[199] P. Krejči, V. Kleinwächter, J. Koudelka, Z. Balcarová, J. Filkuka and G. Löber, Folia Biologica (Prague) 22 (1976) 201.
[200] G. Löber, V. Kleinwächter, J. Koudelka, Z. Balcarová, J. Filkuka, P. Krejči, P. Döbel, V. Beensen and R. Rieger, Biol. Zentralbl. 95 (1976) 169.
[201] F. Brun, J.J. Toubiné and C. Hélène, Biochem. 14 (1975) 558.
[202] A. di Marco and F. Arcamone, Arzneim. Forsch. (Drug. Res.) 25 (1975) 368.
[203] F. Zunino, R. Gambetta, A. di Marco, G. Lunol and A. Zaccara, Biochem. Biophys. Res. Commun. 69 (1976) 744.
[204] B. Festy and M. Daune, Biochem. 12 (1973) 4827.
[205] J.P. Schreiber and M. Daune, J. Mol. Biol. 83 (1974) 487.
[206] J.J. Lawrence and M. Louis, FEBS Lett. 40 (1974) 9.
[207] L.M. Angerer, S. Georghiou and E.N. Moudrianakis, Biochem. 13 (1974) 1075.
[208] J.J. Lawrence and M. Daune, Biochem. 15 (1976) 3301.
[209] J. Bontèmps, C. Houssier and E. Fredericq, Biophys. Chem. 2 (1974) 301.
[210] C. Gatti, C. Houssier and E. Fredericq, Biochim. Biophys Acta 407 (1975) 308.
[211] G.J. Boer, Anal. Biochem. 65 (1975) 225.
[212] T. Caspersson, L. Zech and E.J. Modest, Science (Washington) 170 (1970) 762.
[213] C.G. Voss, Chromosoma (Berlin) 30 (1970) 366.
[214] B. Weisblum, Cold Spring Harbor Symp. 38 (1973) 441.
[215] G. Löber, Studia Biophys. 48 (1975) 109.
[216] G. Löber, V. Kleinwächter and J. Koudelka, Studia Biophys. 55 (1976) 49.
[217] T.C. Hsu, Ann. Rev. Genet. 7 (1973) 153.
[218] R. Rigler, Nobel 23 (1973) 335.
[219] J.R. Ellison and H.J. Barr, Chromosoma (Berlin) 36 (1972) 375.
[220] A. de la Chapelle, J. Schröder, R.K. Selander and K. Stenstrand, Chromosoma (Berlin) 42 (1973) 365.
[221] N. Yamasaki, Chromosoma (Berling) 41 (1973) 403.
[222] T. Caspersson, L. Zech and C. Johansson, Exp. Cell. Res. 60 (1970) 315.
[223] K.P. George, Nature 226 (1970) 80.
[224] D.H. Hollander and D.S. Borgaonkar, Acta Cytol. 15 (1971) 452.
[225] K. Sommer, V. Beensen and M. Becker, Z. ärztl. Fortbild. 68 (1974) 861.
[226] V. Beensen and G. Börner, Z. med. Labortech. 16 (1975) 347.
[227] M. Gellert, C.E. Smith, D. Neville and G. Felsenfeld, J. Mol. Biol. 11 (1965) 445.
[228] L.L. Wheeler and L.C. Altenburg, Chromosoma (Berlin) 62 (1977) 351.
[229] G. Löber, V. Beensen, H. Hanschmann, Ch. Zimmer, V. Kleinwächter and J. Koudelka, Abstract to the 5th Int. Congress of Histochemistry and Cytochemistry (Bucharest/Romania, 1976).
[230] V. Kleinwächter and J. Koudelka, Biochim. Biophys. Acta 91 (1964) 539.
[231] V. Kleinwächter, Z. Balcarová and J. Bohàček, Biochim. Biophys. Acta 174 (1969) 188.
[232] J. Ramstein and M. Leng, Biophys. Chem. 3 (1975) 234.
[233] M. Nastasi, R.W. Yip, V.L. Seligy, A.G. Szabo and R.E. Williams, Nature 249 (1974) 248.
[234] A.G. Szabo, V.L. Seligy, M. Nastasi and R.W. Yip, Biochem. Biophys. Res. Commun. 62 (1975) 830.
[235] M. Nastasi, J.M. Morris, D.M. Rayner, V.L. Seligy, A.G. Szabo, D.F. Wilkins, R.E. Williams and R.W. Yip, J. Am. Chem. Soc. 98 (1976) 3979.
[236] A. Andreoni, A. Longini, C.A. Sacchi, O. Svelto and G. Bottiroli, International Conference on Tunable Lasers and Applications (Loen/Norway, 1976).
[237] J.B. Le Pecq, M. Le Bret, J. Barbet and B. Roques, Proc. Natl. Acad. Sci. USA 72 (1975) 2915.
[238] R.K. Selander, Acta Chem. Scand. B 28 (1974) 937.
[239] R.K. Selander, Acta Chem. Scand. B 28 (1974) 45.
[240] R.K. Selander and A. de la Chapelle, Nature New Biol. 245 (1973) 240.
[241] B. Weisblum, Nature 246 (1973) 150.
[242] C.C. Lin, H. van de Sande, W.K. Smink and D.R. Newton, Can. J. Genet. Cytol. 17 (1975) 81.
[243] G. Löber, V. Beensen and H. Hanschmann, Microscopica Acta 80 (1978) 239.
[244] G. Löber, Z. Balcarová, V. Beensen, H. Hanschmann, V. Kleinwächter, J. Koudelka and P. Krejči, Studia Biophys. 67 (1978) 61.
[245] G. Löber, V. Beensen, Ch. Zimmer and H. Hanschmann, Studia Biophys 69 (1978) 237, Microfiche 2/46–59.
[246] J. Schlammadinger, H. Poulsen and M. Mikkelsen, Hum. Gen. 39 (1977) 309.
[247] J. Bostock and S. Christie, Chromosoma (Berlin) 48 (1974) 73.
[248] A.T. Sumner and H.J. Evans, Exp. Cell. Res. 81 (1973) 223.
[249] A.T. Sumner, Chromosomes Today, Vol. 5, Proc. Leiden Chromosome Conference, ed. P.L. Pearson and K.R. Lewis (Wiley, New York, 1976) p. 201.
[250] A.T. Sumner, A.R. Mitchell and J.R. Gosden, Cytobios 13 (1975) 151.
[251] A.T. Natarjan and A. Gropp, Exp. Cell. Res. 74 (1972) 245.
[252] J. Montachen, N. Degraeve and M. Montachen-Dahmen, Cytobiologie 8 (1973) 112.
[253] C.C. Lin and J.H. van de Sande, Science (Washington) 190 (1975) 61.
[254] J. Limon, H. Hübner and A. Ledochowski, Bull. Acad. Pol. Sci. Ser. Sci. Biol. Cl. II, XXI (1973) 17.
[255] K. Simola, R.K. Selander, A. de la Chapelle, G. Corneo and E. Ginelli, Chromosoma (Berlin) 51 (1975) 199.

[256] M. Gatti, S. Pimpinelli and G. Santini, Chromosoma (Berlin) 57 (1976) 351.
[257] K.C. Tsou, B. Giles and G. Kohn, Stain. Techn. 50 (1975) 293.
[258] K. Simola, R.K. Selander and A. de la Chapelle, Chromosoma (Berlin) 51 (1975) 207.
[259] I. Hilwig and A. Gropp, Exp. Cell. Res. 75 (1972) 122.
[260] S.M. Jalal, A. Markvong and T.C. Hsu, Exp. Cell. Res. 90 (1975) 443.
[261] A. Kim, Humangenetik 28 (1975) 57.
[262] B. Weisblum and E. Haenssler, Chromosoma (Berlin) 46 (1974) 255.
[263] G.P. Holmquist and D.E. Comings, Chromosoma (Berlin) 52 (1975) 245.
[264] W.G. Filon, P. MacPherson, D. Blakey, S. Yen and A. Culpeper, Exp. Cell. Res. 99 (1976) 204.
[265] J. Bontemps, C. Houssier and E. Fredericq, Nucleic Acid. Res. 2 (1975) 971.
[266] D.E. Comings, Chromosoma (Berlin) 52 (1975) 229.
[267] D.E. Comings and M.E. Dreta, Chromosoma (Berlin) 56 (1976) 199.
[268] S.A. Latt, S. Brodie and S.H. Munroe, Chromosoma (Berlin) 49 (1974) 17.
[269] D.E. Comings, B.W. Kovacs, E. Avelino and D.C. Harris, Chromosoma (Berlin) 50 (1975) 111.
[270] R.R. Cowden and S.K. Curtis, Histochem. 40 (1974) 305.
[271] T. Ito, Sci. Pap. Coll. Gen. Educ. University of Tokoyo 24 (1974) 37.
[272] T. Ito and K. Kobayashi, Biochim. Biophys. Acta 378 (1975) 125.
[273] V.W.F. Burns, Exp. Cell. Res. 75 (1972) 200.
[274] J.C. Stockert, Naturwissens. 61 (1974) 363.
[275] J.F. Gokden and S.S. West, J. Histochem. Cytochem. 22 (1974) 495.
[276] U. Letman, F. Ruch and P. Strauli, Acta Cytol. 12 (1968) 381.
[277] R.L. Hayes and D. Roth, Oral. Surg. 36 (1973) 517.
[278] E.N. Khachaturow and E.A. Smirnova, Izv. Akad. Nauk USSR Ser. Biol. 6 (1966) 900.
[279] S.A. Latt, J. Cell. Biol. 62 (1974) 546.
[280] D. Beardsley, C.R. Cantor and T. Tao, Biochem. 9 (1970) 3524.
[281] C.R. Cantor and T. Tao, in Procedures in Nucleic Acid Research, ed. G.L. Cantoni and D.R. Davies (Harper and Row, New York, 1971) p. 31.
[282] J. Yguerabide, in Methods in Enzymology, Vol. XXVI, ed. C.H.W. Hirs and S.N. Timasheff (Academic Press, New York, 1972) p. 498.
[283] K. Beardsley and C.R. Cantor, Proc. Natl. Acad. Sci. USA 65 (1970) 39.
[284] O.W. Odom, B. Hardesty, R.A. Gorse, Jr. and J.M. White, in Transaction of the Gulf Coast Molecular Biology Conference, TJS Spec. Publ. No. 1, August 1976, p. 81.
[285] S.A. Latt and J.C. Wohlleb, Chromosoma (Berlin) 52 (1975) 297.
[286] L. Chinsky and P.Y. Turpin, Biochim. Biophys. Acta 475 (1977) 56.
[287] D.B. Duncnberry and R.B. Uretz, Biophys. J. 12 (1972) 1056.
[288] J. Koudelka, A.S. Krivisky and V.V. Esipova, Studia Biophys. 36/37 (1973) 407.
[289] J. Olmsiedt III and D.R. Kearns, Biochem. 16 (1977) 3647.
[290] G. Löber and L. Kittler, Studia Biophys 73 (1978) 25.
[291] A. Jablonski, Z. Phys. 95 (1935) 53, Z. Naturforsch. 16 a (1961) 1.
[292] M. Fayet and Ph. Wahl, Biochim. Biophys. Acta 181 (1969) 373.
[293] D. Genest and Ph. Wahl, Biophys. Chem. 1 (1974) 266.
[294] J.C. Wang, J. Mol. Biol. 89 (1974) 783.
[295] E. Vogelmann, W. Rauscher and H.E.A. Kramer, Proceedings VIIth IUPAC Symposium on Photochemistry (July 24–28, 1978, Heverlee (Leuven), Belgium 349).
[296] H.J. Li and D.M. Crothers, J. Mol. Biol. 39 (1969) 461.
[297] P.J. McCall and V.A. Bloomfield, Biopolymers 15 (1976) 2323.
[298] J. Ramstein and M. Leng, Biophys. Chem. 3 (1975) 214.
[299] D.E.V. Schmechel and D.M. Crothers, Biopolymers 10 (1971) 465.
[300] T.R. Tritton and S.C. Mohr, Biochem. Biophys. Res. Commun. 45 (1971) 1240.

[301] J. Ramstein, M. Dourlent and M. Leng, Biochem. Biophys. Res. Commun. 47 (1972) 874.
[302] M. Sakoda, K. Hiromi and K. Akasaka, J. Biochem. (Tokyo) 71 (1972) 891.
[303] M. Dourlent and J.F. Hogrel, Biochem. 15 (1976) 430.
[304] T. Jovin, in Biochemical Fluorescence: Concepts, Vol. 1, ed. R.F. Chen and H. Edelhoch (Marcel Dekker, New York, 1975) p. 305.
[305] E. Stutter and W. Förster, Studia Biophys. 75 (1979) 199.
[306] F. Quadrifoglio, V. Crescenzi and V. Giancotti, Biophys. Chem. 1 (1974) 319.
[307] J. Chambron, M. Daune and Ch. Sadron, Biochim. Biophys. Acta 123 (1966) 306.
[308] F. Quadrifoglio and V. Crescenzi, Biophys. Chem. 2 (1974) 64.
[309] Y.M. Huang and D.R. Phillips, Biophys. Chem. 6 (1977) 363.
[310] M.J. Waring, J. Mol. Biol. 13 (1965) 269.
[311] W. Müller and D.M. Crothers, J. Mol. Biol. 35 (1968) 251.
[312] V.A. Bloomfield, D.M. Crothers and J. Tinoco, Jr., Physical Chemistry of Nucleic Acids (Harper and Row, New York, 1974).
[313] H. Schütt, P.A. Gottmick and E. Stutter, Studia Biophys. 75 (1979) 147.
[314] L.F. Liu and J.C. Wang, Biochim. Biophys. Acta 395 (1975) 405.
[315] L. Kittler and G. Löber, in Photochemical and Photobiological Reviews, Vol. 2, ed. K.C. Smith (Plenum Press, New York, 1977) p. 39.
[316] J.D. McGhee and P.H. von Hippel, J. Mol. Biol. 86 (1974) 469.
[317] B. Nordén and F. Tjerneld, Biophys. Chem. 6 (1976) 191.