Methods for Assaying Complexes between Acridine Orange and Deoxyribonucleic Acid

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SUMMARY

In acridine orange–DNA complexes (AO-DNA), the weak (external) complex can be distinguished from the strong (intercalated) one by hot dialysis and by fluorescence spectra. It has been found that the weak complex dissociates at low temperatures ($<T_m$) whereas the strong complex dissociates only during DNA denaturation. Hot dialysis was carried out at temperatures T such that $50° < T < T^*$ (T^* = temperature at which the melting starts), thus retaining only the intercalated complex. Subsequent T_m determinations of the strong complex yielded a value of (bound dye/nucleotide) saturation ratio (r_{sat}) of about 0.11.

Fluorescence spectra, excited by UV, of AO-DNA (native) and AO-DNA (denatured) complexes indicate that the peak at 515 mμ is due to the strong complex and the peak at 620 mμ to the weak complex. The intensity of the 515 mμ peak reaches a maximum at a value of r_{sat} of about 0.11.

INTRODUCTION

It is general opinion that acridines form two kinds of complexes with DNA: a strong complex and a weak one. According to the model proposed by Lerman (1–4), the strong complex is thought to be due to the intercalation of dye molecules in sandwich fashion between adjacent base pairs, thus deforming the DNA double helix structure (insofar as the dye molecule increases the distance between base pairs by a further 3.4 Å) and causing local unwinding. The weak complex, formed at higher (bound dye:nucleotide) ratios (= r), is probably due to electrostatic interaction of the dye molecules with the phosphate residues. Denatured DNA forms almost exclusively the weak complex with acridine orange (AO).

It is of interest to have independent and reliable methods to distinguish between these complexes, in order to study the mechanism of the mutagenic action of acridines. We have devised two new techniques to identify and assay the strong complex between AO and DNA. We found acridine orange to be very suitable for the fluorescence measurements that we made, because the emission maxima for the two kinds of complexes are very easily distinguished. The first technique consists in dissociating the weak complex and removing the liberated AO by hot dialysis. Subsequently, the strong complex can be assayed by measuring changes in its melting temperature (T_m) with respect to uncomplexed DNA (5). The second technique consists in identifying and assaying the strong complex, in the presence of the weak one, by its fluorescence spectrum under UV excitation. The two techniques yield comparable results.

MATERIALS AND METHODS

DNA, purified by the method of Marmur (6), was extracted from *Streptomyces*

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parvus PSA 145, *Escherichia coli* K12, and *Staphylococcus aureus* NCTC 4136. Commercial acridine orange (B.D.H. Corp.) was purified by recrystallization from ether. AO concentrations were determined as described by Stone and Bradley (7).

AO-DNA complexes were formed by mixing AO and DNA solutions in different proportions to obtain the desired r ratios. Experiments were carried out at concentrations of DNA between 5 and 20 μg/ml and at concentrations of AO $\leqslant$ 2.5 μg/ml, where Beer's law was obeyed. All measurements were made in 5.0 mM NaCl, 0.5 mM sodium citrate, pH 7. Under these conditions the reaction (AO + DNA $\rightleftharpoons$ AO-DNA) is completely displaced on the side of the complex. Absorbancy measurements were made with a thermoregulated Beckman DU spectrophotometer. Samples were contained in hermetically closed quartz cuvettes, and T_m determinations were carried out at 260 mμ. The appropriate corrections were made to take into account the changes of absorbancy of the dye at 260 mμ.

DNA was denatured by heating solutions in a 5.0 mM NaCl, 0.5 mM sodium citrate, pH 7, containing 5–20 μg/ml of DNA, at 95° for 10 min and then cooling rapidly in ice. Fluorescence measurements were made with an apparatus of our own construction: the illuminating unit was a General Electric germicidal lamp equipped with a filter to cut off the radiation of wavelength greater than 300 mμ; samples in quartz cuvettes were contained in a light-proof box; a prism monochromator (Zeiss) was used to analyze the fluorescence; intensity measurements were made with a photomultiplier (E.M.I. Instruments, Inc.) connected to a micro-microammeter.

Hot dialysis was carried out using cellophane membranes (Kalle) of pore size of about 20 Å.

RESULTS

Absorbancy Studies

(a) The stabilizing effect of AO on the double helix structure of DNA [already observed by Freifelder *et al.* (8)] was studied with DNA from three different organisms: *Streptomyces parvus*, *Escherichia coli*, and *Staphylococcus aureus* by measuring the increase in T_m of the complexes as a function of r. The base composition of DNA of these organisms is widely different [percentage of G + C, determined

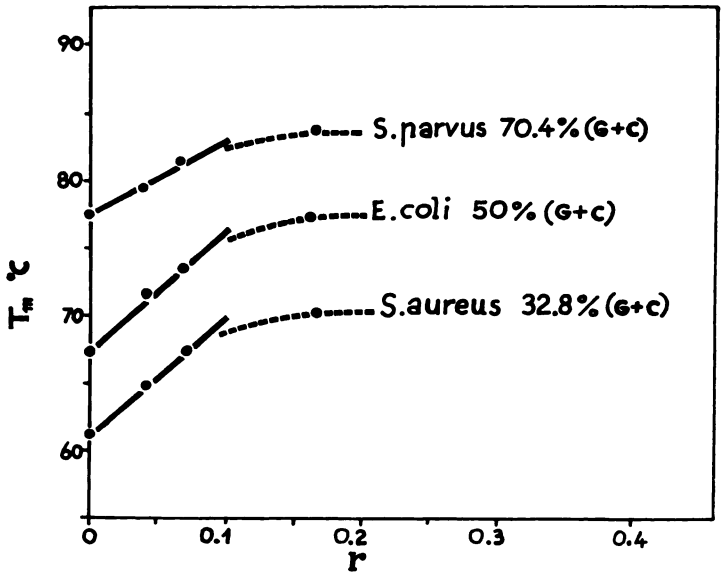

FIG. 1. *T_m of the AO-DNA complexes as a function of r for DNA of various G + C content*
DNA concentration: 10 μg/ml.

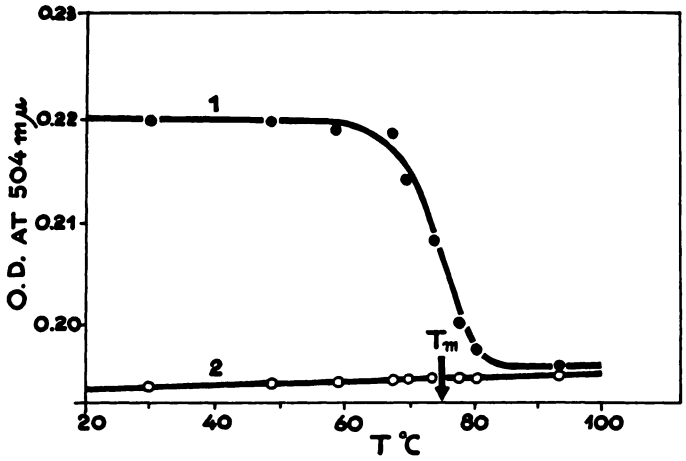

FIG. 2. *The dissociation of the strong complex by temperature*

Curve *1*: AO-DNA (native). DNA from *E. coli*, 18.5 μg/ml; AO, 1.55 μg/ml; $r = 0.1$. Curve *2*: Free AO at the same concentration as for curve *1*. The arrow indicates the T_m of the sample.

from T_m according to Marmur and Doty (9), =70.4 in *Streptomyces parvus*, 50.0 in *Escherichia coli* K12, 32.8 in *Staphylococcus aureus*]. Results are plotted in Fig. 1 as T_m *versus* increasing r values. In each case, the T_m seems to increase linearly up to a certain value of r, and then the curve deviates from linearity. If the initial linear increase in T_m is attributed to the intercalated complex, an approximate estimation of r_{sat} for the strong interaction is between 0.08 and 0.12. These data, on the other hand, offer no evidence that the linear portion of the curve represents only the strong binding; a proof for this could be obtained by using other techniques, as shown in the next paragraphs.

(b) Bradley and Wolf (10) have shown

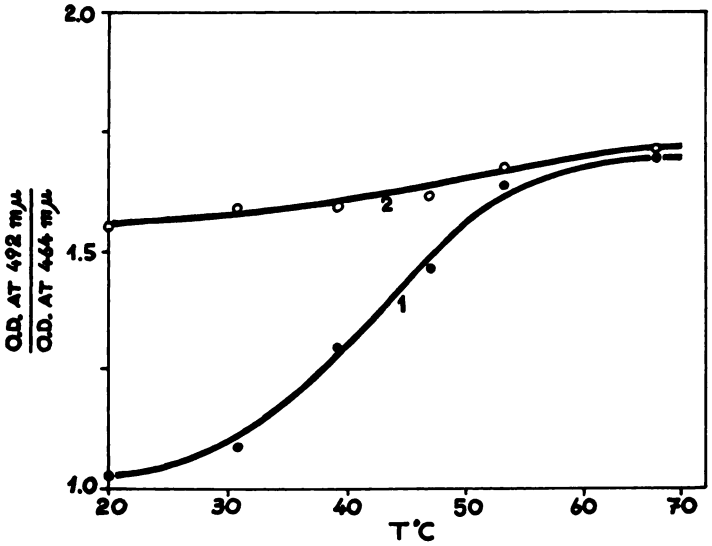

FIG. 3. *The dissociation of the weak complex by temperature*

Curve *1*: AO-DNA (denatured). DNA from *E. coli*, 12.6 μg/ml; AO, 2.2 μg/ml; $r = 0.2$. Curve *2*: Free AO at the same concentration as for curve *1*.

that the spectrum of AO bound to native DNA shifts to a maximum at 504 mμ, whereas, when bound to denatured DNA (corresponding to the formation of only the weak complex), the maximum is at 492 mμ and a new shoulder appears at 464 mμ. This offers a method to determine conditions of dissociation of either complex; it was found that: (i) the strong complex is destroyed simultaneously with the denaturation of DNA where the midpoint of the sharp transition corresponds to the T_m, as shown in Fig. 2; (ii) the weak complex, followed by measuring the spectra of the AO-DNA (denatured) complexes, is progressively dissociated at temperatures lower than T_m (see Fig. 3). In this way we obtained a temperature T^*, at which denaturation would barely begin and in which only the strong complex could be present.

(c) The strong complex was isolated by dialysis at temperature T^* (67.5° in the case of AO-$DNA_{E.\ coli}$ complex, with $r = 1$) with several changes of the solvent. After 48 hours dialysis at T^*, removal of the AO liberated by dissociation of the weak complex was complete as shown by lack of absorption at the wavelength specific to free AO in the last fractions of the dialyzate. The melting curve of the remaining strong complex yielded a T_m of 74.5°, which, from Fig. 1, corresponds to a value of r about 0.1. No higher T_m value could be obtained in this way, an observation indicating that only the linear portion of the curves in Fig. 1 could be attributed to the strong complex.

Fluorescence Studies

The fluorescence emission spectra (excited at 254 mμ) of AO-DNA (native) and AO-DNA (denatured) complexes are shown together with that of free AO in Fig. 4. Free AO in the concentrations used in this experiment exhibits a weak emission band at 515 mμ. AO bound to DNA with the strong complex exhibits a very strong emission peak at 515 mμ whereas AO bound to denatured DNA fluoresces mainly at 620 mμ. We can therefore attribute the maximum of emission at 515 mμ and at 620 mμ to the strong and to the weak complex, respectively. This conclusion is also supported by the following observations: (i) the 620 mμ peak almost totally disappeared on heating at T^*; (ii) the 515 mμ peak was considerably lowered only at $T > T_m$; (iii) at equal r values, the height of the 620 mμ peak of AO-DNA (denatured) complexes was twice that of AO-DNA (native) complexes.

In Fig. 5 the intensity of the 515 mμ peak

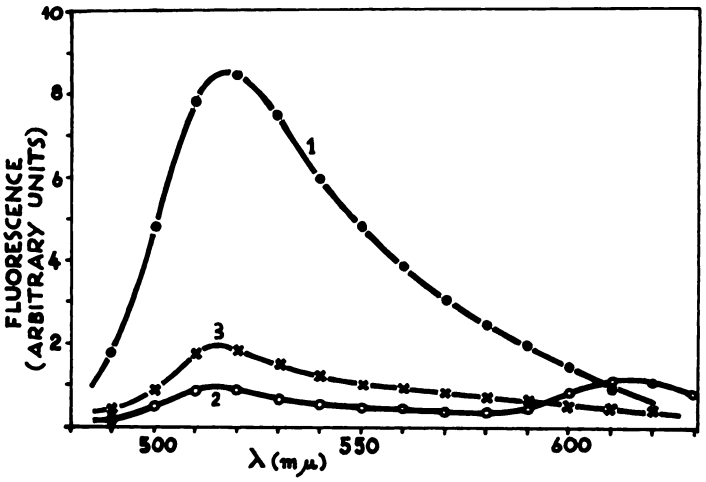

FIG. 4. *Fluorescence spectra of the complexes*

Curve *1*: AO-DNA (native). Curve *2*: AO-DNA (denatured). Curve *3*: Free AO. DNA from *E. coli*, 12.8 μg/ml; AO, 1.15 μg/ml; $r = 0.11$.

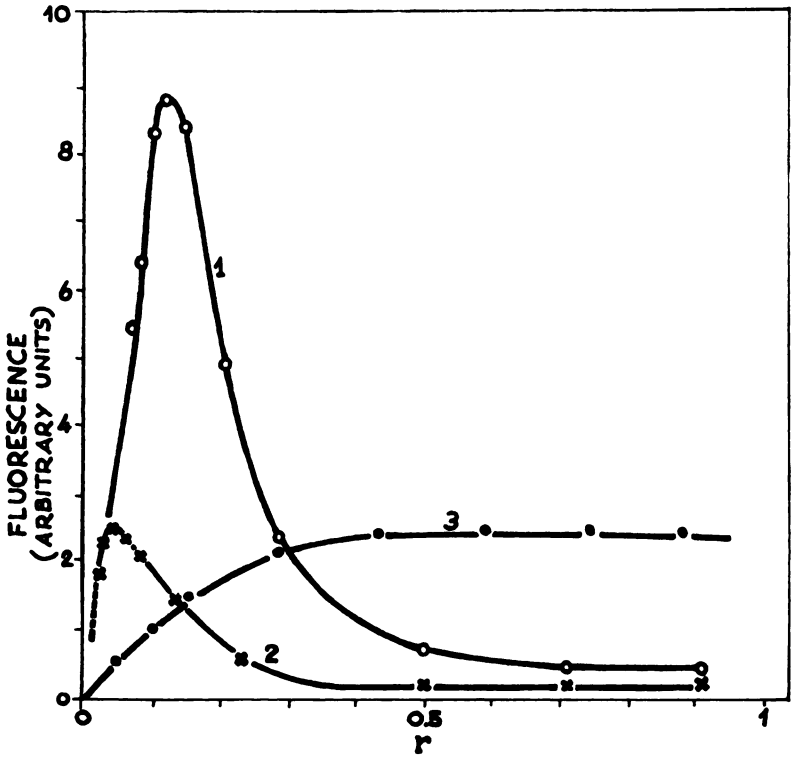

FIG. 5. *Intensity of fluorescence at 515 mμ as a function of r*

Curve *1*: AO-DNA (native). Curve *2*: AO-DNA(denatured). Curve *3*: Free AO at the same absolute concentration (for every *r* value) as for curves *1* and *2*. DNA from *E. coli*, 12.8 μg/ml.

is plotted as a function of *r* for both AO-DNA (native) and AO-DNA (denatured) complexes and for free AO. It can be noticed that, with excitation at 254 mμ, the fluorescence efficiency at 515 mμ of intercalated AO is much greater than that of free AO (curve *3*). This fact can be explained by the observation by Isenberg *et al.* (11) that, in the case of the strong complex, part of the exciting UV radiation absorbed by DNA is reemitted as delayed fluorescence of the dye. The AO-DNA (native) complex shows an intensity maximum at a value of *r* about 0.11. This maximum may be considered the visualization of the phenomenon of saturation of the intercalated complex, and the sharp decrease in fluorescence intensity of the 515 mμ peak at $r > 0.11$ can be interpreted as due to the fact that acridines externally bound to DNA (with the weak complex) absorb a large fraction of the radiation exciting DNA, thus causing a quenching of the fluorescence of the intercalated acridines. The r_{sat} value for the strong complex, according to this interpretation, is about 0.11, in good agreement with the value obtained by hot dialysis. It can be seen in Fig. 5 (curve *2*) that the corresponding curve for the AO-DNA (denatured) complex peaks at a lower *r* value. This is probably due to the residual helicity present in the denatured DNA.

DISCUSSION

The measurements of T_m increase, the hot dialysis experiments, and the fluorescence variations are new and independent complementary tools for studying the extent of the complexes between acridines and DNA. The first technique allows one to eliminate completely the weak complex and any free acridine while maintaining intact only the strong one; by the second tool one may

study either complex independently in the presence of the other.

The simultaneous destruction of the strong complex with the helix-coil transition, and the marked stabilization of the double helix, can be interpreted by assuming that the acridine molecules become part of the secondary structure of DNA, and participate as "supplementary bases" to the cooperative melting (12). It is to be noticed that the intercalation of the acridines does not broaden the sharpness of the helix-coil transition.

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