

PHYSICAL CHEMICAL STUDIES ON THE
SPECIFIC INTERACTION OF AN
ACRIFLAVINE-PHOSPHOTUNGSTIC ACID COMPLEX
WITH DOUBLE-STRANDED NUCLEIC ACIDS

CHARLES T. LADOULIS and THOMAS J. GILL III

From the Laboratory of Chemical Pathology, Department of Pathology, Harvard Medical School,
Boston, Massachusetts 02115

ABSTRACT

The detailed definition of the structure of DNA in chromosomes and in interphase chromatin is important for correlating the structure of the genetic material with various states of physiological activity. A general approach to developing specific reagents for a variety of such studies in solution and in tissues is to combine a chemically specific organic cation with the electron-opaque phosphotungstic acid (PTA) molecule. The reagent described in this paper was made from the interaction of acriflavine and phosphotungstic acid. The acriflavine-PTA complex (*a*) displays some unique absorption and fluorescence properties, (*b*) binds specifically to DNA and RNA by intercalation of the acriflavine moiety, and (*c*) is electron opaque. In addition, it binds to double-stranded synthetic polynucleotides, but not to a variety of proteins, nucleoproteins, or polysaccharides.

INTRODUCTION

In the course of investigating the physical and chemical structure of mammalian chromosomes, it became apparent that the detailed definition of variations in the structure of the DNA in individual chromosomes and interphase chromatin would be valuable for the identification of genetic material in various states of activity. The general approach to developing a reagent that could be used for such studies was to combine into a single chemical compound a molecule with specific reactivity for native DNA and a molecule which was electron-opaque for visualization in the electron microscope. Many derivatives of acridine interact specifically with DNA and RNA (1-10), primarily by intercalation between adjacent base pairs (9). Of these various derivatives, acriflavine hydrochloride was chosen for several reasons:

(*a*) it has the highest affinity for native DNA of all the acridine dyes (11); (*b*) there is a well-characterized red shift in the absorption spectrum associated with DNA binding (12); (*c*) there is evidence that the reaction of acridine dyes is limited to metabolically active euchromatin, and that they do not interact with dense, supercoiled heterochromatin (13-15); and (*d*) the fixed positive charge of the central ring nitrogen (16) can form a stable ionic bond. Phosphotungstic acid was selected as the electron-opaque moiety, because its cytochemical properties have been well studied; it is a compact, roughly spherical molecule whose structure is precisely known, and it reacts chemically as a strong tribasic anion (17). In addition, the reactivity of the molecule is not limited by directional forces, because the

distribution of the negative charge is symmetrical and undirected (17); thus, it could react with the 3,6-diamino-*N*-methylacridinium ion in such a way as to leave the planar ring system free to intercalate into DNA. Therefore, the acriflavine-PTA complex was prepared and characterized, and its interactions with nucleic acids, polynucleotides, proteins, and polysaccharides were studied *in vitro*. Previous studies (18) explored the utility of the dye-complex in the fluorescent and electron microscopic investigation of nucleic acids in tissue sections.

MATERIALS AND METHODS

Preparation of Dye Complex

Acriflavine hydrochloride (hydrochloride of 3,6-diamino-10-methylacridinium chloride) (Matheson, Coleman, and Bell, Cincinnati, Ohio) was recrystallized according to Albert (19) to give a fine, reddish-orange powder. Purification was assayed by thin-layer chromatography on cellulose acetate (I.T.L.C. Media, Type SG; Gelman Instrument Company, Ann Arbor, Mich.), using an 80:20 (v/v) toluene:methanol solvent mixture. Pure preparations revealed only trace amounts of faster migrating, blue-fluorescent contaminants, which were probably photooxidation products of acriflavine. Phosphotungstic acid was obtained from Fisher Scientific Co., Medford, Mass., and it was used without fur-

ther purification. Ultraviolet absorption spectra of both compounds are shown in Fig. 1.

Method 1

This method was used in earlier studies, including those with fluorescent and electron microscopy (18). Freshly prepared acriflavine, 1.5 ml of a 3.8×10^{-3} M aqueous solution, was quickly mixed with 0.5 ml of a 1.0×10^{-3} M aqueous solution of PTA; rapid mixing was necessary in order to prevent precipitation during the reaction. An insoluble, flocculent, brown precipitate, which was not usable, soon formed under the following conditions: (a) reagent concentrations greater than 10^{-2} M were used; (b) the molar ratio of acriflavine to PTA was excessive; (c) the reagents were mixed slowly; or (d) PTA was added to acriflavine, instead of the reverse. Precipitation eventually occurred after standing for several hours, and it was accelerated by increased temperatures, high concentrations and exposure to light. The precipitation was presumably due to insoluble lattice formation with PTA, to the formation of acriflavine photooxidation products (20), or to both processes. On the basis of this limited solubility, we postulate that acriflavine forms an ionic complex with PTA and that the molar proportions of acriflavine to PTA in the soluble complexes vary, but are less than 3:1, since uncharged, insoluble complexes form at this composition (see below).

The reaction mixtures for these studies were prepared with an excess of acriflavine to ensure that

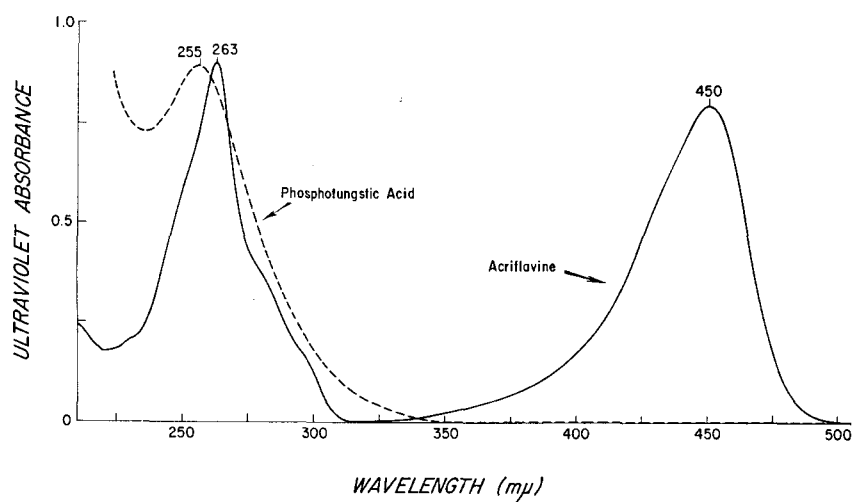


FIGURE 1 The ultraviolet absorption spectra of acriflavine hydrochloride (2.5×10^{-5} M) and phosphotungstic acid (2.5×10^{-5} M) in water. The molar extinction coefficient of phosphotungstic acid at 255 $m\mu$ is 4.0×10^4 . The molar extinction coefficient for acriflavine hydrochloride at 263 $m\mu$ is 4.1×10^4 and at 450 $m\mu$, 3.6×10^4 .

each of the PTA molecules reacted with at least one acriflavine residue. The fresh mixture, which contained the soluble dye-complex, was immediately placed on a 15.0 x 2.0 cm column of Bio-Gel P-2, 40-100 mesh, and eluted with 0.2 M NaCl, pH 6.5; the chromatography yielded two fluorescent fractions. The first, smaller fraction showed a bright blue fluorescence, and the second, larger fraction showed the characteristic fluorescence of acriflavine. The unreacted PTA bound tightly to the polyacrylamide beads of the Bio-Gel column, and it could be eluted only with 0.1 N NaOH. The fluorescent fractions were evaporated and extracted with diethylether to separate the acriflavine-PTA complex from unreacted acriflavine, which is insoluble in diethylether. The ether extracts of both fractions were evaporated by heating under vacuum, and the small gummy residues obtained were extracted with water to give the final product. The preparation from each chromatographic fraction showed one component by thin-layer chromatography. The ultraviolet spectrum of the first fraction demonstrated maxima at 268 and 360 m μ , indicating an acridine derivative; the spectrum of the second fraction had the characteristics of acriflavine. Only the second fraction, which contained the acriflavine moiety, was used. The final product was stored at 2-4° C in the dark, and it was used within 1-2 days of preparation.

Method 2

Acriflavine-PTA prepared in the manner described above varied considerably in the molar ratio of acriflavine:PTA (0.2:0.4), as calculated from the appropriate molar extinction coefficients (Table II). Although the initial experiments demonstrated that a unique acriflavine-PTA complex existed, an alternative method of preparation of acriflavine-PTA was developed because the yield of purified product was not sufficient for chemical analysis and for other studies. The basis of this method was the finding that acriflavine and PTA reacted immediately and stoichiometrically to form a stable dye-complex; therefore, simple mixing of the two components in the desired molar ratio was sufficient. Reagent concentrations from 10⁻⁶ M to 10⁻³ M could be used; at higher concentrations, turbidity developed and the complex gradually precipitated when stored at 4° C for several days. The reaction was immediate, as indicated by (a) the dramatic fall in the molar extinction coefficient of acriflavine at 450 m μ from 3.6 x 10⁴ to 2.0 x 10⁴, (b) the distinctive character of the broad absorption band, and (c) the quenching of acriflavine fluorescence. The effect was the same over the pH range of 5.2-7.1, was unaltered by an increase in ionic strength to 0.5 M NaCl (Fig. 2), and was independent of concentration. In contrast, acriflavine alone did not

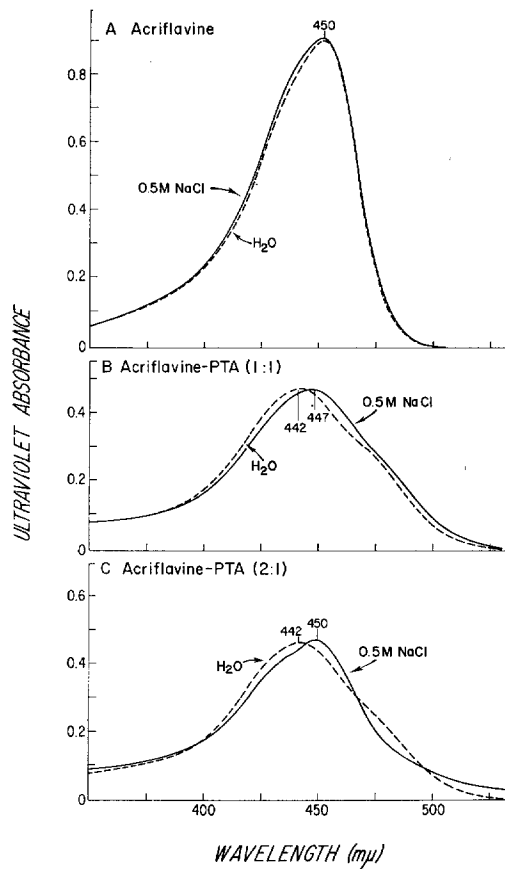


FIGURE 2 The effect of ionic strength on the spectrum of acriflavine and acriflavine-PTA complexes. The final concentration of acriflavine and the dye-complex in each case was 2.5×10^{-5} M, and the solvents are indicated on the various spectra.

behave in a similar manner with comparable variations in ionic strength or pH. A variety of molar ratios of acriflavine to PTA were studied, and at ratios of 3:1 or greater, an insoluble, flocculent, brown precipitate rapidly formed. After repeated washings with water, the precipitate was analyzed for its content of carbon, nitrogen, hydrogen, phosphorus, and tungsten. The results showed an acriflavine:PTA molar ratio of 3:1 (Table I). This ratio is the one which would be expected, since the maximal valence of PTA is 3, and that of acriflavine is 1.

Conductimetric studies of the dye-complex were performed in order to determine the mobility of the complex and to estimate its size. No quantitative conclusions could be drawn, however, because of two complicating factors that were present under the experimental conditions: acriflavine at concentra-

TABLE I
The Calculated Chemical Composition of Acriflavine, Phosphotungstic Acid, and Various Complexes of the Two, and the Experimental Values for the Complex Formed from a 3:1 Acriflavine:PTA Mixture

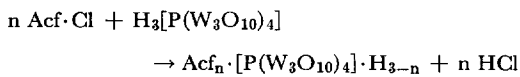
Compound	Formula	Molecular weight	Weight percentage						
			C	N	H	O	P	W	
Acriflavine	$C_{14}H_{14}N_3Cl^*$	260	64.67	16.16	5.44				
Phosphotungstic acid (PTA)	$H_3[P(W_3O_{10})_4]$	2880			0.11	22.22		1.08	76.60
	$H_3[P(W_3O_{10})_4] \cdot 14 H_2O$	3132			0.10	20.43		0.99	70.44
	$H_3[P(W_3O_{10})_4] \cdot 24 H_2O$	3312			0.09	19.32		0.94	66.61
Acriflavine-PTA†	(1:1)	3104	5.42	1.35	0.55	20.62		1.00	71.08
	(2:1)	3328	10.10	2.52	0.94	19.23		0.93	66.29
	(3:1)	3553	14.20	3.55	1.28	18.01		0.87	62.09
Experimental			14.53	3.75	1.34			0.85	60.30

* Chloride = 13.7%.

† Based on the formula for anhydrous PTA.

tions of 10^{-5} M or greater forms dimers, and PTA displays anomalous behavior due to its dissociation into tungstic acid and phosphoric acid (21). Nonetheless, comparison of the conductivity measurements of acriflavine, PTA, and acriflavine-PTA (1:1) at equivalent concentrations revealed that the equivalent conductance of PTA was always greater than that of the complex. This finding provides evidence that the acriflavine and the PTA in the complex are ionically associated.

The acriflavine residues react with the phosphotungstic acid through the quaternary nitrogen atom in the middle ring. A schematic representation of the interaction is shown to scale in Fig. 3. Thus, the chemical reaction between acriflavine and PTA is:



where Acf is the acriflavine residue, and n is 1, 2, or 3. The data on the chemical composition indicate that the reaction is essentially stoichiometric; hence, the proportions of acriflavine and PTA in the complex are governed by the relative proportions of the individual reagents in the reaction mixture.

The preparation of acriflavine-PTA for studies on its interaction with DNA, RNA, proteins, polysaccharides, and synthetic polynucleotides was carried out by mixing equal volumes of each reagent (5×10^{-5} M in distilled water) to give a final concentration of 2.5×10^{-5} M for both components. This concentration was chosen (a) so that the acriflavine absorbance would be sufficient for accurate measurement and comparison of ultraviolet spectral changes, (b) to avoid concentration quenching of fluorescence, which occurs for free acriflavine at approximately 5×10^{-5} M, and (c) so that the macromolecules would be in excess, relative to the amount of dye-complex present, at a final concentration of 1.0 mg/ml, which is convenient for study.

Instrumental Analyses

Ultraviolet absorbance spectra were recorded with a Cary Model 15 spectrophotometer. Fluorescence excitation and emission spectra were recorded with a Farrand Model MK-I spectrofluorimeter equipped with a Hewlett-Packard Model 680 precision strip-chart recorder. The fluorescence spectra were corrected for the spectral energy output of the xenon lamp and for the spectral response of the photomultiplier tube using standard procedures. The polarization of fluorescence measurements were performed with a modified Brice-Phoenix light-scattering instrument equipped with a 436 $m\mu$ band-pass excitation filter and 490 $m\mu$ cut-off emission filter.

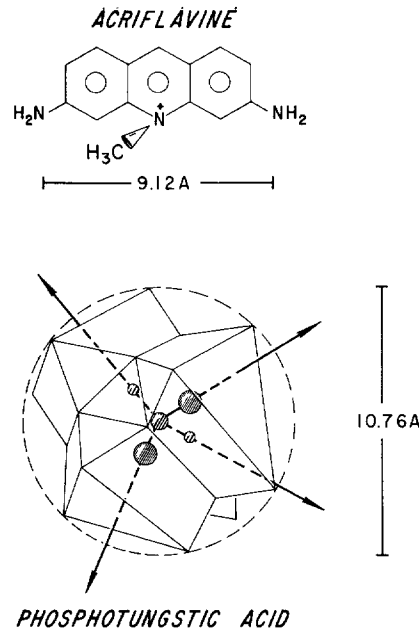


FIGURE 3 Schematic representation of acriflavine and phosphotungstic acid drawn to scale. The dimensions of the acriflavine molecule (9.12 Å long and 3.7 Å thick) were calculated from Pauling (22), and those of phosphotungstic acid (diameter 10.76 Å) were calculated from Keggin (17). In the tribasic phosphotungstate anion, each of the four polyhedra represents the W_3O_{10} group, and they are arranged tetrahedrally around the central phosphorus atom. This spatial arrangement is analogous to that of the oxygen atoms in the PO_4 group (17). The primary amino groups of the acriflavine molecule do not sterically hinder the interaction of the quaternary nitrogen ion with the phosphotungstic acid anion.

Other

The polyribonucleotides, some of the proteins, and the polysaccharides were purchased from Miles Laboratories, Elkhart, Ind. Other proteins were obtained from Calbiochem, Los Angeles, Calif. or Mann Laboratories, New York. The Bio-Gel polyacrylamide beads were manufactured by Bio-Rad Laboratories, Richmond, Calif. The elemental analyses were performed by Dr. Stephen Nagy, Belmont, Mass.

RESULTS

The interaction of the acriflavine-PTA complex with DNA, RNA, a variety of soluble proteins, two polysaccharides, and several synthetic poly-

nucleotides was studied *in vitro* both by observing the characteristic spectral red shift of the 450 m μ absorption maximum of the acriflavine moiety and by measurement of the hydrodynamic prop-

erties of the complex using the polarization of fluorescence technique.

The ultraviolet spectroscopic studies of the interactions between acriflavine-PTA and DNA

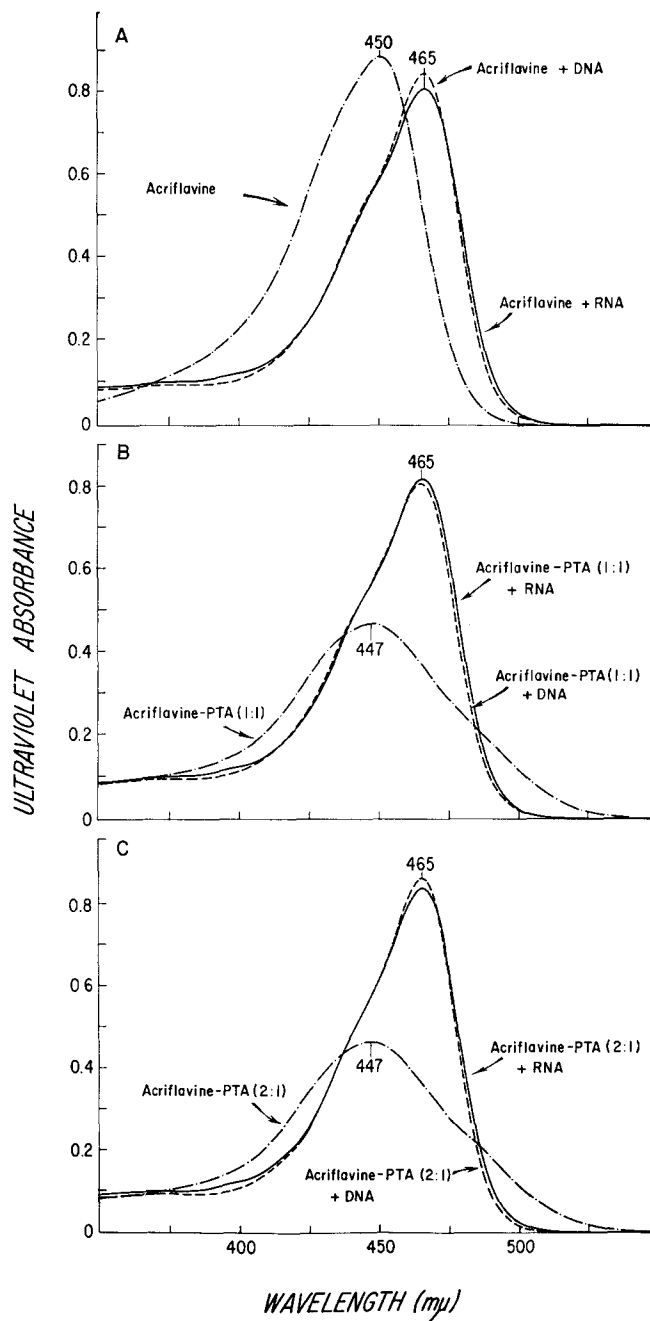


FIGURE 4 The interaction of acriflavine and various acriflavine-PTA complexes with DNA and RNA. In each case, the final concentration of the dye or the dye-complex was 2.5×10^{-5} M and that of the nucleic acids, 1.0 mg/ml. The solvent was 0.082 M saline-citrate, pH 6.4.

or RNA demonstrated the characteristic wavelength shift which was seen when acriflavine interacted with the same macromolecules. The spectra are shown in Fig. 4, and the data are summarized in Table II. When the dye-complex interacted with the nucleic acids, the unique spectrum of acriflavine-PTA reverted to the spectrum typical of free acriflavine when it reacted with DNA or RNA (5, 6), and the molar extinction coefficient for the acriflavine-PTA increased dramatically to the same value as that of bound acriflavine. Interactions with double-stranded polynucleotides (Fig. 5) displayed a spectral red shift similar to that for DNA and RNA,

although the increases in the extinction coefficients were less. There were small and variable spectral changes following the interactions with proteins and with polysaccharides (Table II), but no spectral red shift ever occurred. The polynucleotide homopolymers contain variable amounts of helical structure in solution due to some self-association. This phenomenon occurs to the greatest extent with poly A and with poly I; hence, there is some red shift in the spectrum of the acriflavine-PTA when it interacts with these polynucleotides (Fig. 6). Thus, the experiments indicate that specific intercalation of the acriflavine moiety of the acriflavine-PTA complex occurred only in the re-

TABLE II
*Interaction of Acriflavine-PTA with Various Macromolecules: Ultraviolet Absorption Spectra**

Sample	λ_{\max}	$\Delta\lambda$	$\epsilon \times 10^{-4}$
	m μ	m μ	
Acriflavine	450		3.6
+ calf thymus DNA	465	15	3.4
+ yeast RNA	465	15	3.4
Acriflavine-PTA (1:1)	447 \ddagger		2.0
+ calf thymus DNA	465	18	3.5
+ yeast RNA	465	18	3.5
+ poly A	454	7	2.8
+ poly U	438	-9	2.3
+ poly I	454	7	2.4
+ poly C	448	1	2.3
+ poly (A:U)	462	15	2.5
+ poly (I:C)	464	17	2.8
+ proteins \S	445-447	0-2	2.0-2.8
+ polysaccharides \parallel	445-447	0-2	2.0-2.8

* The solvent was 0.11 M NaCl + 0.04 M phosphate, pH 7.4, for all proteins except protamine sulfate and histone, which were dissolved in 0.1 N HCl, and hemocyanin which was dissolved in 0.2 M borate, pH 8.6. Standard saline-citrate (0.15 M NaCl-0.015 M Na citrate, pH 6.4) was the solvent for the nucleic acids. The solvent for the acriflavine and the acriflavine-PTA (1:1) dye-complex was water; the final salt concentration for the reaction mixtures was one-half that used to dissolve the various macromolecules. The final concentration of acriflavine and acriflavine-PTA in all solutions was 2.5×10^{-5} M (~ 7 $\mu\text{g}/\text{ml}$), and the concentration of all the macromolecules was 1.0 mg/ml. The double-stranded polynucleotides were prepared by mixing the appropriate concentrations of single-stranded polynucleotides at 25°C for 4-16 hr in standard saline-citrate. The presence of double-stranded helices was established by demonstrating hypochromicity at 257 m μ .

\ddagger The absorbance maximum of acriflavine-PTA (1:1) or (2:1) varied with ionic strength: water, 442 m μ ; 0.025 M NaCl, 445 m μ ; 0.05-0.10 M NaCl, 446 m μ ; and ≥ 0.20 M NaCl, 447 m μ . The absorbance at λ_{\max} was always the same, however.

\S Bacitracin, bovine γ -globulin, bovine serum albumin, chymotrypsin, hemocyanin, histone, lysozyme, ovalbumin, paramyosin, pepsin, protamine sulfate, and ribonuclease.

\parallel Heparin and chondroitin sulfate C.

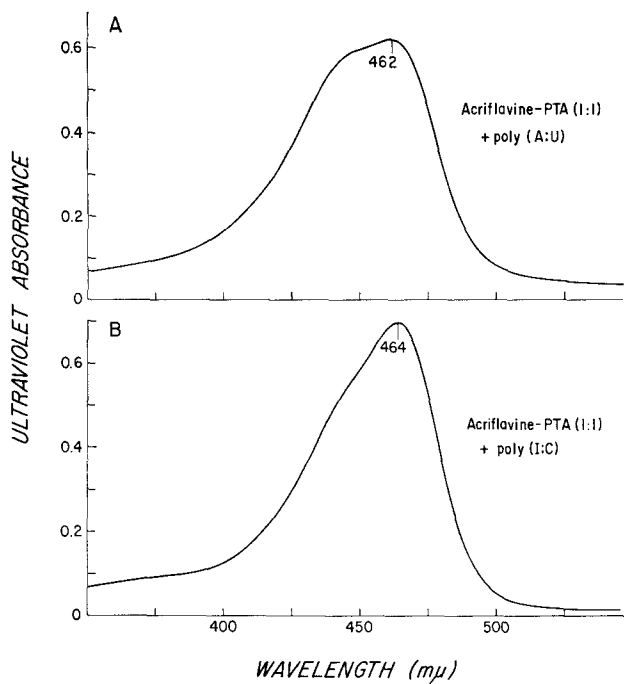


FIGURE 5 The interaction of acriflavine-PTA (1:1) with poly (A:U) and poly (I:C). The final concentration of the dye-complex was 2.5×10^{-5} M and that of the double-stranded polynucleotides, 1.0 mg/ml. The solvent was 0.082 M saline-citrate, pH 6.4.

actions with double-stranded structures: DNA, RNA, and double-stranded synthetic polynucleotides.

Fluorescence spectroscopic studies of the effects of concentration on the properties of the acriflavine-PTA complex and of its interactions with various macromolecules were performed under identical experimental conditions. The effects of concentration on the spectra of acriflavine and acriflavine-PTA (1:1) are shown in Fig. 7. At a concentration of 5×10^{-5} M, acriflavine showed some dimer and oligomer formation, as indicated by the shift in the excitation maximum from 452 to 473 $m\mu$ with increasing concentration. In contrast, acriflavine-PTA displayed no such displacement of the excitation maximum in this concentration range; hence, the dye-complex does not associate under these conditions. This finding provides further evidence for the existence of the dye-complex as a distinct entity.

A comparison of the interactions of acriflavine and of acriflavine-PTA (1:1) with nucleic acids, proteins, and polysaccharides, as studied by fluorescence spectroscopy, is shown in Table III. Both acriflavine and acriflavine-PTA showed the

same kind of shift in the excitation maximum upon interaction with DNA and RNA. The fluorescent emission of the acriflavine-PTA at 498 $m\mu$ was 80% quenched in aqueous solutions at neutral pH, as compared to that of acriflavine under the same conditions. However, the fluorescent emission intensity of the dye-complex was enhanced when it bound to DNA or RNA so that it attained the same level as that seen with bound acriflavine. The most striking feature of the fluorescence spectra was the shift of the excitation maximum with binding. This shift corresponded to that seen with increasing concentration in acriflavine solutions when dimer and oligomer formation occurred (Fig. 7). This interaction is analogous to the intercalation of acriflavine between the base pairs in DNA. In the presence of proteins and polysaccharides, the fluorescent intensity of both acriflavine and acriflavine-PTA varied over a narrow range, and the dye-complex never showed any evidence of enhanced fluorescence following any of the interactions.

In order to measure the interaction of the dye-complex with various macromolecules by a second, different approach, polarization of fluores-

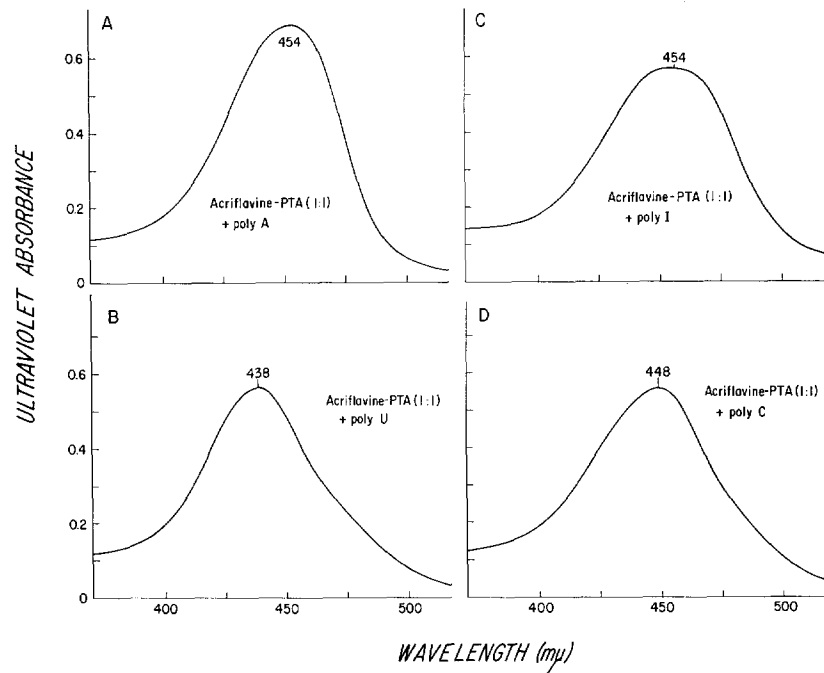


FIGURE 6 The interaction of acriflavine-PTA (1:1) with single-stranded polynucleotides. The final concentration of the dye-complex was 2.5×10^{-5} M and that of the polynucleotides, 1.0 mg/ml. The solvent was 0.082 M saline-citrate, pH 6.4.

cence experiments were carried out. This technique provided a hydrodynamic measurement of the interaction, which was not affected by the spectral shifts that occur on binding. Binding was assessed by the degree of polarization of acriflavine-PTA mixed with various macromolecules compared to that of the dye-complex alone. The rotational Brownian motion of the acriflavine-PTA decreased when it reacted with a macromolecule, and this binding resulted in an increased degree of polarization of the dye-complex. The results are summarized in Table IV. The very small degree of polarization of the dye-complex increased dramatically when it bound to DNA or RNA in solution. In contrast, there was no evidence of binding to either proteins or polysaccharides.

In summary, the specific interaction of the acriflavine-PTA complex with nucleic acids and double-stranded, helical polynucleotides was demonstrated spectroscopically by ultraviolet and fluorescence measurements and hydrodynamically by polarization of fluorescence measurements. Studies by fluorescence microscopy and by electron microscopy (18) also demonstrated

discrete specificity for nucleic acids in tissue sections.

DISCUSSION

The structure postulated for the acriflavine-PTA complex is supported by three lines of evidence: chemical, conductimetric, and spectroscopic. Firstly, the reactivity of PTA as an anion is well documented (23), and this property is the basis for its reaction with polycations in protein solutions and in tissues. The PTA anion is tribasic (21), and, unlike some other heteropolyacids of tungsten, it exists in unimolecular form in aqueous solutions rather than in bimolecular form (24). Acriflavine is unique among the acridine dyes in that the ring nitrogen is virtually always positively charged ($pK_a > 12$) (19). Thus, the chemical structures and properties of acriflavine and PTA allow them to interact to form a stable ion pair. The dye-complex was soluble when the ratio of acriflavine to PTA was 1:1 or 2:1, but it was insoluble when three molecules of acriflavine reacted with one molecule of PTA to form an uncharged complex. The insoluble precipitate of acriflavine-PTA, which formed when there were 3 moles of

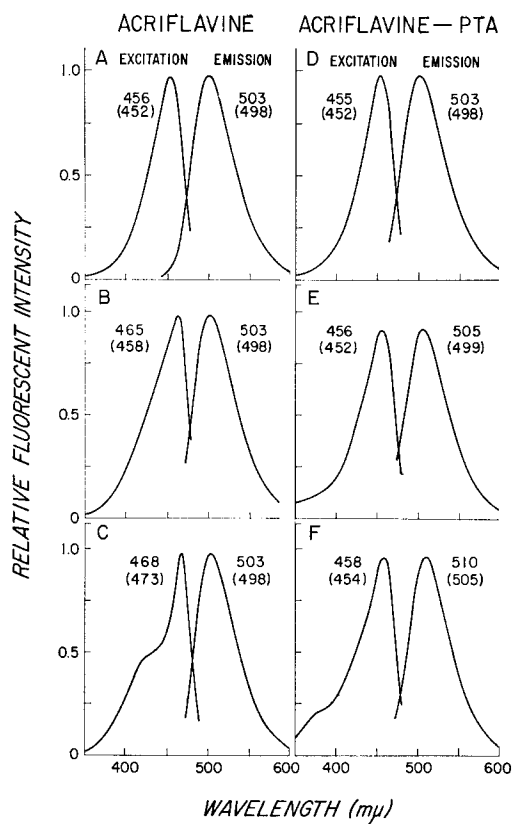


FIGURE 7 Fluorescence spectra of acriflavine and acriflavine-PTA (1:1) as a function of concentration in water. The concentrations of acriflavine in frames A, B, and C were 5×10^{-6} M, 2.5×10^{-5} M, and 5×10^{-5} M, respectively. The concentrations of acriflavine-PTA (1:1) in frames D, E, and F were the same, respectively. The fluorescent spectral intensities were normalized for the purpose of comparison. Experimentally, the fluorescent intensity of the acriflavine-PTA (1:1) was approximately 20% of the intensity of acriflavine at the same concentration (see Table III). The wavelength maximum is indicated near the appropriate spectrum. The value in parentheses is corrected for the spectral energy output of the xenon lamp (excitation) or the spectral response of the photomultiplier tube (emission).

acriflavine per mole of PTA, had the predicted chemical composition for a 3:1 molar ratio of acriflavine to PTA. Secondly, the conductimetric studies demonstrated a decrease in the equivalent conductance of acriflavine-PTA compared to that of PTA. Thus, these measurements suggest that ion-pair formation occurred between the acriflavine and the PTA. Finally, the spectroscopic

measurements showed that acriflavine and acriflavine-PTA behave differently under a number of conditions. The unique absorption spectrum of acriflavine-PTA was virtually unchanged over a wide range of ionic strength and pH, but no comparable effect occurred with acriflavine under the same solvent conditions. The fluorescent excitation spectra showed that self-association of the acriflavine occurred under conditions where there was none with acriflavine-PTA; this finding suggests that the PTA molecule in the acriflavine-PTA complex sterically hinders self-association.

The acriflavine-PTA complex most likely binds to DNA, RNA, and helical polynucleotides by intercalation of the acriflavine moiety between the base pairs of the helix, according to the model proposed by Lerman (7). Two modes of binding have been proposed for proflavine (1), acriflavine (6), and acridine orange (25): intercalation and aggregation outside of the helix. However, both proflavine and acriflavine bind only by intercalation when the ratio of dye to DNA phosphorus (D/p) is very small (26); 0.08 or less for acriflavine (6). In our studies, D/p was 0.02 for the polarization experiments and 0.07 for the ultraviolet absorption and fluorescence spectroscopic studies. Thus, under our experimental conditions, intercalation of the acriflavine moiety is most likely to be the mode of binding.

The absorption spectra observed when acriflavine-PTA interacted with the nucleic acids or with the double-stranded polynucleotides were identical to those seen when acriflavine alone reacted with the same macromolecules. This observation suggests that either the acriflavine-PTA complex dissociates when it binds or the spectral effect due to the interaction of PTA with acriflavine is counteracted by the reaction of the acriflavine moiety with the nucleotide base pairs.

In conclusion, then, the unique reactivity of acriflavine-PTA with DNA, RNA, and double-stranded polynucleotides, which is the basis for its singular cytochemical properties (18), is due to the specific interaction of the acriflavine moiety with helical nucleic acids. The small and variable amounts of interaction with single-stranded polynucleotides are due to the presence of some helical structure formed by partial self-association of the polynucleotides in solution (27). The chemical properties of the electron-opaque moiety, phosphotungstic acid, allow it to react strongly with the quaternary nitrogen atom of the acriflavine

TABLE III
Interactions of Acriflavine-PTA with Various Macromolecules: Fluorescence Spectra*

Sample	Acriflavine			Acriflavine-PTA (1:1)		
	λ_{\max} excitation† (corrected)	$\Delta\lambda$	Relative intensity	λ_{\max} excitation† (corrected)	$\Delta\lambda$	Relative intensity
	$m\mu$	$m\mu$		$m\mu$	$m\mu$	
Alone	458		1.0	452		0.2
+ Calf thymus DNA	473	15	0.3	473	21	0.3
+ Yeast RNA	473	15	0.4	473	21	0.4
+ Proteins§	458	0	0.9	452	0	0.1
+ Polysaccharides§	458	0	0.9	452	0	0.1

* The solvents were the same as those listed in Table II. The final concentration of acriflavine in all experiments was 2.5×10^{-5} M (~ 7 $\mu\text{g/ml}$). The acriflavine-PTA complex was prepared from a 1:1 mixture of the components with a final concentration 2.5×10^{-5} M for each.

† The corrected λ_{\max} emission in all cases was 498 $m\mu$ (503 $m\mu$ uncorrected). The intensity in each case is that relative to acriflavine, 2.5×10^{-5} M in water at pH 5.2-7.1.

§ As in Table II.

TABLE IV
Interaction of Acriflavine-PTA with Various Macromolecules: Polarization of Fluorescence Measurements*

Sample	Final concentration	Degree of polarization
	$\mu\text{g/ml}$	
Acriflavine†	0.6	0.00
Acriflavine-PTA†	0.6	0.01
Acriflavine-PTA	0.2	
+ calf thymus DNA	90	0.13
+ yeast RNA	90	0.08
+ proteins§	90	0.01
+ polysaccharides§	90	0.01

* The solvents were the same as those listed in Table II. The acriflavine-PTA complex used in these studies was prepared by the original method (Method 1) using an acriflavine:PTA ratio of approximately 1:2. The final salt concentration for the reaction mixtures was nine-tenths of that used to dissolve the various macromolecules.

† The concentrations were calculated from the experimentally determined molar extinction coefficient at 450 $m\mu$: $\epsilon = 3.6 \times 10^4$ for free acriflavine and 2.0×10^4 for acriflavine-PTA.

§ As in Table II.

cation without altering the specificity of the latter. The experimental approach underlying the synthesis of the acriflavine-PTA complex can be developed into a general method for producing specific, electron-opaque dye-complexes: the

combination of a chemically specific organic cation with the electron-opaque phosphotungstic acid molecule. For example, complexes with antibiotics or with antimetabolites could be synthesized according to this general principle, and such complexes could be used to identify the sites of action of these biologically important compounds.

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