PURIFICATION AND PROPERTIES OF ACRIFLAVINE, PROFLAVINE AND RELATED COMPOUNDS

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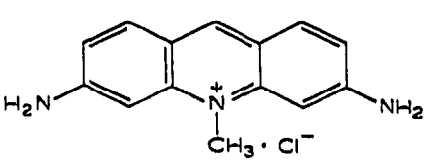
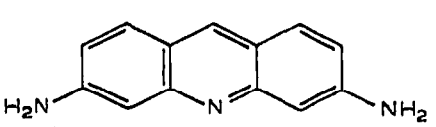
Diaminoacridines, particularly acriflavine and proflavine, are an interesting group of biologically active compounds. In addition to their classical bacteriostatic and bactericidal properties[1,2], they alter drug resistance in some bacteria[3], are mutagenic to some viruses[4], bind DNA and RNA both *in vivo* and *in vitro*[5] and alter the physical shape of DNA[6]. Acridine orange forms complexes with polynucleotides[7], and has been used for staining cytologic preparations in the diagnosis of maglignant neoplasms[8]. Acriflavine hydrochloride N.F., given intravenously during primary immunogenesis, induces a prolonged degree of immune paralysis to bovine serum albumin in rabbit[9], whereas commercial proflavine did not elicit this immunosuppressant effect[10].

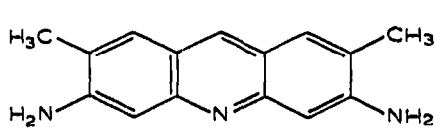
Commercially available acriflavine hydrochloride N.F. is almost always a mixture of acriflavine and proflavine; the latter may comprise as much as 30% of the total. In most of the earlier investigations, cited above, there was no indication of the purity of the acridines employed. It seemed desirable, therefore, to devise chromatographic procedures for purifying commercial samples of acriflavine ((I), 2,8-diamino-10-methylacridinium chloride) and proflavine ((II) 2,8-diaminoacridine). The closely related analogues acridine yellow ((III) 2,8-diamino-3,7-dimethylacridine)



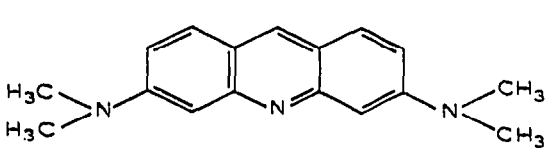
Acriflavine (I)



Proflavine (II)



Acridine yellow (III)



Acridine orange (IV)

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and acridine orange ((IV) 2,8-bis(dimethylamino)acridine) have also been purified. These compounds have been characterized by elemental analysis, absorption spectra, paper chromatography and paper electrophoresis.

MATERIALS AND METHODS

Acriflavine hydrochloride N.F., acridine yellow, and proflavine dihydrochloride were obtained from Allied Chemical Corporation. Purified reference samples of acriflavine, acridine yellow and acridine orange were kindly supplied by Dr. P. BRIAN STEWART, Pharma-Research Canada Ltd. Containers and columns containing diaminoacridines were covered with aluminum foil in order to prevent photochemical decomposition. DEAE-cellulose, obtained from Schleicher and Schuell, Inc., was purified by the method of MATHEWS AND HUENNEKENS[11] and stored in water. Anion exchange resin, chloride form (AG 1-X2, X4 and X8; 200–400 mesh), was purchased from Bio-Rad Laboratories. Reagent grade organic solvents were used without further purification.

Descending paper chromatography was performed at 30° on 20 × 50 cm sheets of Whatman No. 3 MM paper. Chromatograms were equilibrated with the developing solvent for 4 h and then developed for 16–20 h using the following solvents: (a) *tert.*-butanol–methyl ethyl ketone–1 *M* NH_4OH–water (40:30:10:20); (b) *tert.*-butanol–methyl ethyl ketone–methyl alcohol–1 *M* NH_4OH–water (30:10:30:10:20); (c) *sec.*-butanol–methyl alcohol–acetone–1 *M* NH_4OH–water (30:20:10:20:10). Compounds were located by examination of the papers under a Mineralite ultraviolet light.

Paper electrophoresis was performed in a Durrum Cell (Beckman Model R) with Whatman No. 1 paper strips using 0.5 *M* acetic acid (pH 2.5) at 10 V per centimeter for 24 h in the dark. A (+) sign indicates movement toward the anode. Mobility (in cm) is corrected for electroosmosis (measured by movement of cyanocobalamin which is uncharged at this pH). R_F values and electrophoretic mobility values are given in Table I.

Melting points were determined using a Fischer-Johns apparatus. Microanalyses on samples dried at 150° for 2–4 h at 1–2 mm were performed by Spang Microanalytical Laboratories, Ann Arbor, Michigan. Measurements of absorbancy at single

TABLE I

PROPERTIES OF PURE ACRIDINES

Compound	R_F (× 100) *in solvent*			*Mobility upon paper electrophoresis*	*Absorption maxima in ethanol*	
	(*a*)	(*b*)	(*c*)	(*cm*)	γ_{max} ($m\mu$)	$\varepsilon \times 10^{-3}$ ($M^{-1}\,cm^{-1}$)
Acriflavine (I)	22	23	34	+ 5.0	261, 465	62.7, 71.8
Proflavine (II)	56	47	55	+ 5.3	262, 460	58.3, 32.3
Acridine yellow (III)	43	30	43	+ 1.1	263, 459	62.7, 31.4
Acridine orange (IV)	70	55	68	+ 1.5	270, 298, 308, 431, 490	48.0, 35.7, 34.6, 24.5, 25.1

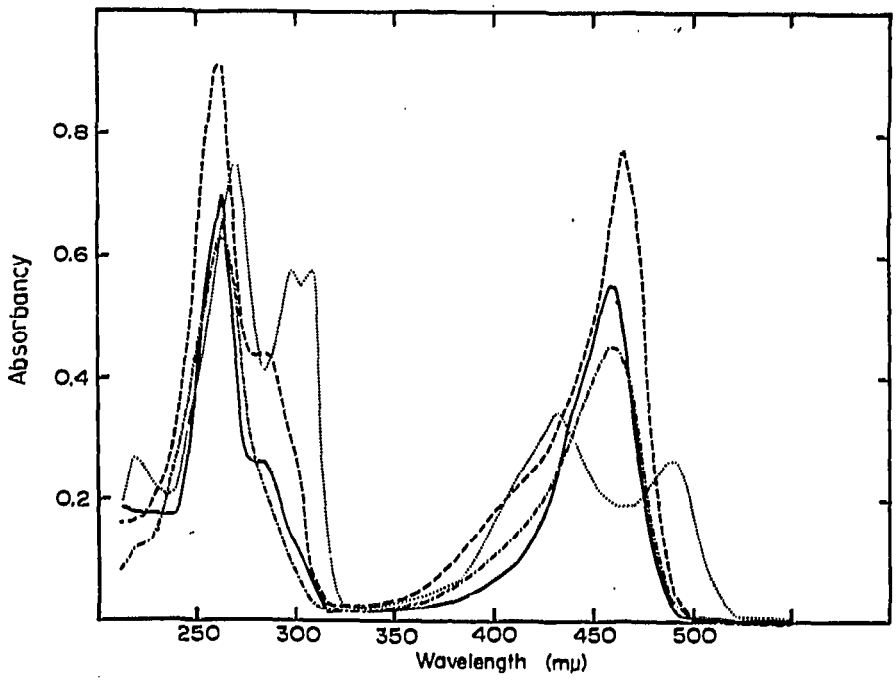

Fig. 1. Absorption spectra of acriflavine (I) (– – –), proflavine (II) (——), acridine yellow (III) (–·–·–) and acridine orange (IV) (· · · ·), in absolute ethanol.

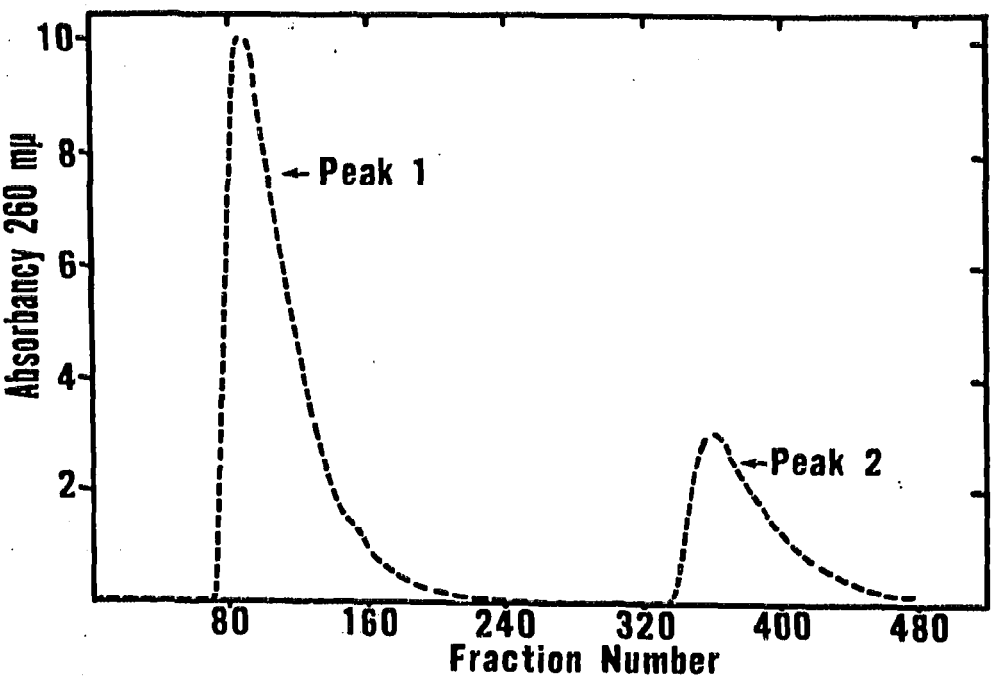

Fig. 2. Chromatography of acriflavine hydrochloride N.F. on a DEAE-cellulose column. Acriflavine = peak 1; proflavine = peak 2.

wavelength were done on a Beckman DU Spectrophotometer, while a Cary recording spectrophotometer. Model 14, was used to measure complete spectra. The absorption spectra of compounds (I), (II), (III) and (IV) are shown in Fig. 1; the absorption maxima and extinction coefficients are presented in Table I.

RESULTS AND DISCUSSION

Separation of acriflavine(I) and proflavine(II) by anion exchange chromatography

Acriflavine hydrochloride N.F. (30 mg) was dissolved in 10 ml of 0.01 *M* Na_2CO_3 and chromatographed on a DEAE-cellulose column (2.5 × 35 cm) that had been washed previously and equilibrated with 10^{-3} *M* Na_2CO_3. The column was eluted with 10^{-2} *M* Na_2CO_3 and 2 ml fractions were collected. The elution profile is shown in Fig. 2. The position of each compound was established by chromatographing reference samples of (I) and (II). The material in peaks 1 and 2 showed absorption maxima at 453 mμ and 395 mμ, respectively, at pH 12.

Since a large volume of buffer was required to elute these compounds from the column whose capacity was limited, the above procedure was modified as follows: AG 1-X2, Cl^--form (500 g) was equilibrated overnight with 4 l of 1 *M* NaOH, followed by two additional changes of 1 *M* NaOH. The resin was placed in a column (3.8 × 40 cm), washed with several liters of water until the effluent was almost neutral and finally washed with 2 l of 10^{-3} *M* NH_4OH. Acriflavine hydrochloride N.F. (1 g) was dissolved in 20 ml of water, adjusted to pH 7.0, and layered on the column. The column was eluted with 0.01 *M* NH_4OH; 25 ml fractions were collected and stored in the dark. Fractions 5 to 20 having an absorbancy at 262 mμ greater than 1.0 were pooled (total volume about 400 ml) and concentrated by lyophilization. The red powder was reconstituted in 25 ml of water, brought to pH 7.0 with *N* HCl and evaporated to dryness under suction on a water bath (500 mg). The yield of pure acriflavine (I) was usually 80–90 % of the amount originally present in commercial acriflavine hydrochloride N.F. (I) moved as a single spot in several solvent systems (Table I). The material was approximately 98 % pure as judged by absorption spectra. After being crystallized twice from methanol–water (2:1) and dried at 120°, (I) was obtained as brick-red microcrystals; m.p. 295–298° (darkening at 285°); Calc. for $C_{14}H_{14}NCl$: C, 64.74; H, 5.43; N, 16.18 and Cl, 13.65, Found: C, 64.69; H, 5.82; N, 16.14; and Cl, 13.68.

Similar results were obtained with more highly cross-linked resins, such as AG 1-X8, except that the volume required for complete elution of acriflavine was greater and yields were lower. Proflavine and additional minor impurities were very tightly bound to this resin at the point of application on the column, and after all acriflavine had been eluted, these contaminants were best removed by taking the top 10 cm of resin. The column was washed with water, an additional 10 cm of fresh resin added, and after equilibration was used repeatedly (at least 4 times) for purification of acriflavine (1 g) each time without any significant loss in recovery.

Chromatography of proflavine (II) on DEAE-cellulose

Chromatographic examination of commercially available proflavine in solvent system (a) revealed three spots in addition to the main yellow band at R_F 0.56. Proflavine dihydrochloride (500 mg) was dissolved in 20 ml of water, adjusted to

pH 7.0 and chromatographed on a DEAE-cellulose column (3.8 × 40 cm). Elution was carried out with 0.1 *M* ammonium acetate, pH 6.9. Twenty ml fractions were collected. The contents of tubes 9 to 25 (400 ml) showing an absorbancy > 2.0 at 260 mμ, and having maxima at 262 and 445 mμ at pH 7.0, were pooled and lyophilized (225 mg). Minor components that appeared after the proflavine had been eluted were not characterized further.

Proflavine also was purified by a slight modification of the procedure of ALBERT[12]. Proflavine dihydrochloride (10 g) was dissolved in 100 ml of water and adjusted to pH 7.0 using 1 *M* NH_4OH. Any insoluble residue was removed by centrifugation. Pellets of NaOH were added to the supernatant until no more proflavine precipitated and the solution was cooled overnight at 4°. The precipitate was collected by centrifugation resuspended in hot water, and treated with acetone until the solution became clear. The solution was brought to boiling, and filtered through Whatman No. 1 paper after treatment with activated charcoal. After refrigeration overnight, (II) was isolated as dark yellowish needles (3.5 g). (II) was homogeneous by paper chromatography and paper electrophoresis (Table I). After two recrystallizations from acetone–water and drying at 120°, (II) was obtained as fine yellow needles; m.p. 277°. Calc. for $C_{13}H_{13}N_3$: C, 74.62; H, 5.30; N, 20.08. Found: C, 74.58; H, 5.26; N, 20.13.

Commercial acridine yellow was purified by the following procedure: Acridine yellow (1 g) was dissolved in 100 ml of boiling water and allowed to cool overnight at 4°. After centrifugation, the dark yellow supernatant was discarded and the residue was crystallized from 175 ml of ethanol after treatment with charcoal. After cooling overnight, acridine yellow (III) was obtained as a yellow crystalline material (150 mg), (III) deteriorates rapidly in organic solvents in the presence of light (solutions turn reddish-brown upon standing) and crystallization should be carried out in dim light. The product was homogeneous by paper chromatography and paper electrophoresis (Table I). After two recrystallizations from ethanol, (III) was obtained as fine yellow needles, m.p., 336–338°; Calc. for $C_{15}H_{15}N_3$: C, 75.92; H, 6.37; N, 17.71. Found: C, 75.40; H, 6.3; N, 17.90.

Purified acridine orange, kindly supplied by Dr. P. BRIAN STEWART, was recrystallized by the same procedure as described for (II). (IV) was obtained as orange needles; m.p. 188–189°. Calc. for $C_{17}H_{19}N_3$: C, 76.95; H, 7.22; N, 15.84. Found: C, 76.3; H, 4.76; N, 15.94.

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SUMMARY

Column chromatographic methods are described for the isolation in the pure state and with high yields of acriflavine and proflavine from commercial samples of

acriflavine hydrochloride N.F. and proflavine dihydrochloride, respectively. A simplified procedure for the crystallization of acridine yellow also is described.

The pure compounds have been characterized with respect to elemental analysis, absorption spectra and paper chromatography.

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