

## Cascade Blue Derivatives: Water Soluble, Reactive, Blue Emission Dyes Evaluated as Fluorescent Labels and Tracers

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Fluorescent dyes based on the pyrenyloxytrisulfonic acid (Cascade Blue) structure were prepared and evaluated. The dyes contain functional groups that react with amines, thiols, acids, aldehydes, and ketones, forming covalently bonded, fluorescent derivatives of molecules with broad biological interest. Reactive groups in the Cascade Blue dyes include carboxylic acids and activated esters, amines, hydrazides, alcohols, photoaffinity reagents, acrylamides, and haloacetamides. The dyes exhibited absorption maxima at 374–378 nm and 399–403 nm, with extinction coefficients in the range of  $1.9 \times 10^4$ – $2.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  and  $2.3 \times 10^4$ – $3.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ , respectively. Emission maxima ranged from 422–430 nm. The spectral properties of the fluorescent dyes are sufficiently different from fluorescein to permit simultaneous use of both dyes with minimum spectral interference. The Cascade Blue derivatives have narrower spectral bandwidths and smaller Stokes' shifts than other reactive dyes with similar spectral properties, do not show appreciable sensitivity to pH, have higher solubilities in aqueous solution, and have good to excellent quantum yields. Cascade Blue conjugates of a number of histochemically and biologically useful molecules were prepared, including dextrans, albumins,  $F_c$  receptor binding proteins, antibodies, lectins, membrane receptor binding proteins, and biotin binding proteins, as well as biological particles and bacteria. Cascade Blue conjugates of secondary and tertiary labels yielded specific fluorescence localization in the indirect immunofluorescent staining of human epithelial cell (HEp-2) nuclei. © 1991 Academic Press, Inc.

For many applications that utilize fluorescent dyes as tracers, it is necessary to chemically react the dye with a

biologically active molecule. Biologically significant molecules include proteins, antibodies, enzymes, drugs, hormones, nucleotides, nucleic acids, polysaccharides, lipids, or other biomolecules, as well as cells, tissues, and natural or synthetic polymers. The fluorescent conjugates provide a probe for detection and/or quantification of the biochemical interaction under investigation. Alternatively, the dye may be reacted *in situ* by methods such as fixation or photoaffinity labeling. It is frequently useful to employ more than one fluorescent conjugate simultaneously and to quantify the conjugates independently, which requires selective detection of each fluorescent probe. It was the object of this research to provide improved fluorescent dyes that have high water solubility, high quantum yields, low fluorescence sensitivity to solution pH, and the chemical reactivity necessary for conjugation to the functional groups commonly found in biomolecules. It was also an object to provide fluorescent tracers that can be used in conjunction with fluorescein and other commonly used fluorescent probes.

There is a recognized need for suitable fluorophores for multicolor fluorescence applications such as microscopy (1), flow cytometry (2), immunoassays (3), and DNA sequencing (4). Most of the dyes proposed for these applications have emission maxima at wavelengths longer than for fluorescein. Since fluorescein has essentially no fluorescence below 490 nm, there is a clear opportunity to detect suitable fluorophores that have strong emission below this wavelength. The optimum dyes would have the following properties:

- (i) High fluorescence quantum yield with a narrow emission peak, occurring at wavelengths sufficiently shorter than the emission maximum of fluorescein that the longest wavelength components of the dye emission

have little or no spectral overlap with the fluorescein emission band.

(ii) High absorptivity as measured by extinction coefficient. Preferred are dyes that can be excited with the most intense emission lines of common excitation sources, such as the 365-nm line of the mercury arc lamp. Excitation below 365 nm is less desirable since ultraviolet irradiation can result in cell injury or death in applications where fluorescence measurements are performed on living cells. Autofluorescence of proteins, nucleic acids, and other biomolecules present in cells (such as NADH, which has absorption and emission maxima of 340 nm and 460 nm, respectively) is also increased with shorter wavelength excitation. Additionally, excitation wavelengths longer than 350 nm permit use of less expensive glass optics instead of quartz optics.

(iii) High solubility of the dye and its reactive derivatives in aqueous solution to optimize the utility of the dye for modification of cells, proteins, saccharides, and other biopolymers.

(iv) Low sensitivity of the fluorophore to solution pH so that the measured signal is proportional only to the absolute quantity of dye present and not to the pH of the solution or preparation.

(v) Suitability of the dye for preparation of reactive derivatives of several different types that exhibit selective reactivity toward a variety of chemically reactive sites.

(vi) Intrinsically low toxicity or biological activity of the dye.

A number of fluorophores have been described in the literature that can be excited and detected at wavelengths shorter than 500 nm. Several chemically reactive, fluorescent dyes that can be excited in the near ultraviolet (uv) and short wavelength visible region of the spectrum have also been evaluated. In general, these tracers have been derived from naphthalene derivatives such as 5-dimethylaminonaphthalene-1-sulfonic acid (Dansyl)<sup>1</sup> (5), 3-isothiocyanatonaphthalene-1,5-disulfonic acid (6), and *N*-(4-methylphenyl)-5-isothiocyanato-1,8-naphthalimide (7); pyrene derivatives such as 1-pyrenebutyric acid (PBA) (8), 8-isothiocyanatopyrene-1,3,6-trisulfonic acid (IPTS) (9), and 8-hydroxypyrene-1,3,6-trisulfonyl chloride (10); stilbene derivatives such as 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) (11); or coumarin dyes such as 3-carboxy-7-methoxycoumarin (12), and more re-

cently, 4-methylumbelliferone-3-acetic acid (4-MUA) (13), 7-amino-4-methylcoumarin-3-acetic acid (AMCA) (1), and 7-diethylaminocoumarin (3).

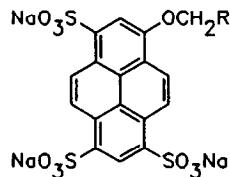
The previously described reactive, blue fluorescent dyes do not possess the combination of high extinction coefficient, high quantum yield, high water solubility, compatibility with common excitation sources, and minimal spectral overlap with fluorescein that is exhibited by the Cascade Blue dyes and their conjugates. The Cascade Blue dyes are readily soluble in aqueous preparations, insensitive to pH, minimally quenched on conjugation with macromolecules, and show low overlap with the emission spectra of commonly used green or yellow fluorescent dyes, such as fluorescein and Lucifer Yellow. The Cascade Blue derivatives that were prepared and evaluated have the general structure shown in Fig. 1. In this structure, R is a carboxylic acid, an activated ester, or a substituted amide that is further modified to provide chemically reactive functional groups. Three sulfonic acids or salts of sulfonic acids are present which confer water solubility. The subsequent modifications of R include chemically reactive derivatives of carboxylic acids such as succinimidyl esters and acyl azides, or other reactive groups that include amine and hydrazine derivatives, haloacetamides, acrylamides, aryl azides, amino acids, and alcohols. Cascade Blue derivatives and conjugates are also suitable for a variety of biological, immunological, and histochemical applications.

## MATERIALS AND METHODS

8-Hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS), 5-dimethylaminonaphthalene-1-sulfonic acid (Dansyl), pyrene-1-butyric acid (PBA), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), 4-methylumbelliferone (4-MUA), 7-amino-4-methylcoumarin-3-acetic acid (AMCA), the succinimidyl ester of AMCA, succinimidyl acrylate, and succinimidyl 4-azidobenzoate were provided by Molecular Probes, Inc. (Eugene, OR). 8-Isothiocyanatopyrene-1,3,6-trisulfonic acid (IPTS) was acquired from Polo Lambda (Norwalk, CT). Solvents and other reagents were of commercial reagent grade and were used without further purification. Fixed human epithelial cells derived from carcinoma of the larynx (HEp-2 cells) and human anti-nuclear antibody solution (NOVA Lite ANA) were purchased from INOVA Diagnostics, Inc. (San Diego, CA). Monoclonal mouse anti-human IgG was obtained from Zymed Laboratories, Inc. (San Francisco, CA). Other antibodies were purchased from Fortron Bio Science, Inc. (St. Marys, PA).

Absorption measurements were performed with an IBM Model 9420 uv/visible spectrophotometer and uncorrected fluorescence data were obtained with a Perkin-Elmer Model 650-40 fluorescence spectrophotometer equipped with a Perkin-Elmer/Hitachi 057 X-Y

<sup>1</sup> Abbreviations used: Dansyl, 5-dimethylaminonaphthalene-1-sulfonic acid; PBA, 1-pyrenebutyric acid; IPTS, 8-isothiocyanatopyrene-1,3,6-trisulfonic acid; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid; 4-MUA, 7-hydroxy-4-methylcoumarin-3-acetic acid; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid.

Compound - R

1	CO <sub>2</sub> CH <sub>3</sub>
2	CO <sub>2</sub> H
3	CONH(CH <sub>2</sub> ) <sub>2</sub> NH <sub>2</sub>
4	CONH(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub>
5	CONH-Dextran-NH <sub>2</sub>
6	CONH(CH <sub>2</sub> ) <sub>2</sub> NHCOC <sub>2</sub> Cl
7	CONH(CH <sub>2</sub> ) <sub>6</sub> NHCOC <sub>2</sub> I
8	CO <sub>2</sub> -succ. <sup>a</sup>
9	CON <sub>3</sub>
10	CONH(CH <sub>2</sub> ) <sub>2</sub> OH
11 <sup>b</sup>	CONHNH <sub>2</sub>
12 <sup>c</sup>	CONHNH <sub>2</sub>

Compound - R

13 <sup>d</sup>	CONHNH <sub>2</sub>
14	CONH(CH <sub>2</sub> ) <sub>2</sub> NHCOCH(CH <sub>2</sub> ) <sub>3</sub> NH-t-BOC
15	CONH(CH <sub>2</sub> ) <sub>6</sub> NHCOCH(CH <sub>2</sub> ) <sub>3</sub> NH-t-BOC
16	CONH(CH <sub>2</sub> ) <sub>6</sub> CN
17	CONH(CH <sub>2</sub> ) <sub>2</sub> NHCOCH(CH <sub>2</sub> ) <sub>3</sub> NH•(CF <sub>3</sub> CO <sub>2</sub> H)
18	CONH(CH <sub>2</sub> ) <sub>6</sub> NHCOCH(CH <sub>2</sub> ) <sub>3</sub> NH•(CF <sub>3</sub> CO <sub>2</sub> H)
19	CONH(CH <sub>2</sub> ) <sub>6</sub> NHCOC <sub>2</sub> CH <sub>2</sub>
20	CONH(CH <sub>2</sub> ) <sub>6</sub> NHCO(C <sub>6</sub> H <sub>4</sub> )N <sub>3</sub>

a. Succ. indicates a succinimidyl ester

b. Free sulfonic acid

c. Sodium salt

d. Ammonium salt

FIG. 1. Structures of Cascade Blue derivatives.

recorder. Stock solutions were prepared by accurately weighing and dissolving 5- to 10-mg samples of the compounds in water. Absorbance solutions were prepared by further dilution of the stock solution with 50 mM potassium phosphate buffer at pH 8.0. Extinction coefficients ( $\epsilon$ ) of the dyes at their absorption maxima ( $\lambda_{\text{max}}^{\text{Abs}}$ ) were determined by standard Beer's Law calculations. Fluorescence maxima ( $\lambda_{\text{max}}^{\text{Em}}$ ) of the pyrenyloxytrisulfonic acid dyes were determined for the reactive dyes, and for their conjugates with model compounds, by dissolving the dye or the conjugate at 1–5  $\mu\text{M}$  in an appropriate solvent, such as water or methanol. Fluorescence could also be observed for the dyes in solution, or on thin-layer chromatography (TLC) plates, by visual inspection with illumination by a suitable source, such as a long wavelength uv (354 nm) handlamp. An estimate of the quantum yields of the dyes relative to quinine sulfate (quantum yield = 0.55 in 1 N sulfuric acid) was obtained by integration of the uncorrected emission spectra of the dyes excited at the same wavelength (360 nm) and optical density as the standard (14). Since the photomultiplier response was found to vary less than 5% as a function of wavelength between 360 and 500 nm, correction of the spectra did not appreciably change the observed emission intensities and integrals. Consequently, uncorrected spectra were employed. In order to compare the quantum efficiency of conjugates of dyes

that exhibit different maxima, extinction coefficients, and bandshapes, the molar emission intensities of conjugates were calculated as

$$I = \epsilon D I^{\text{rel}},$$

where  $I$  is the product of the extinction coefficient ( $\epsilon$ ) of the dye at the absorption maximum, the degree of substitution of the conjugate ( $D$ ), and the emission intensity of the conjugate at  $\lambda_{\text{max}}^{\text{Em}}$  ( $I^{\text{rel}}$ ), relative to a quinine sulfate standard of the same optical density at 360 nm, the emission intensity of which was arbitrarily set at 1.0. It was assumed that the extinction coefficients of the dyes were unchanged by coupling to the protein or other ligand.

The purity of the dyes was determined by HPLC analysis (performed with a Waters system consisting of a Model 600E multisolvent delivery pump, a Model 700 WISP automated injector, and a Maxima 820 chromatography workstation) of 5- $\mu\text{l}$  aliquots of solutions containing 0.33–1.0 mg/ml of the compounds dissolved in 5 mM tetrabutylammonium phosphate buffer (pH 7.5). Samples were eluted from a 150  $\times$  4.6 mm Supelcosil LC-8-DB (5  $\mu\text{m}$ ) column with a gradient of 5 mM tetrabutylammonium phosphate buffer (pH 7.5) and acetonitrile (0–60% acetonitrile in 30 min.) at a flow rate of 2 ml/min and an initial pressure of 1700 psi. Samples

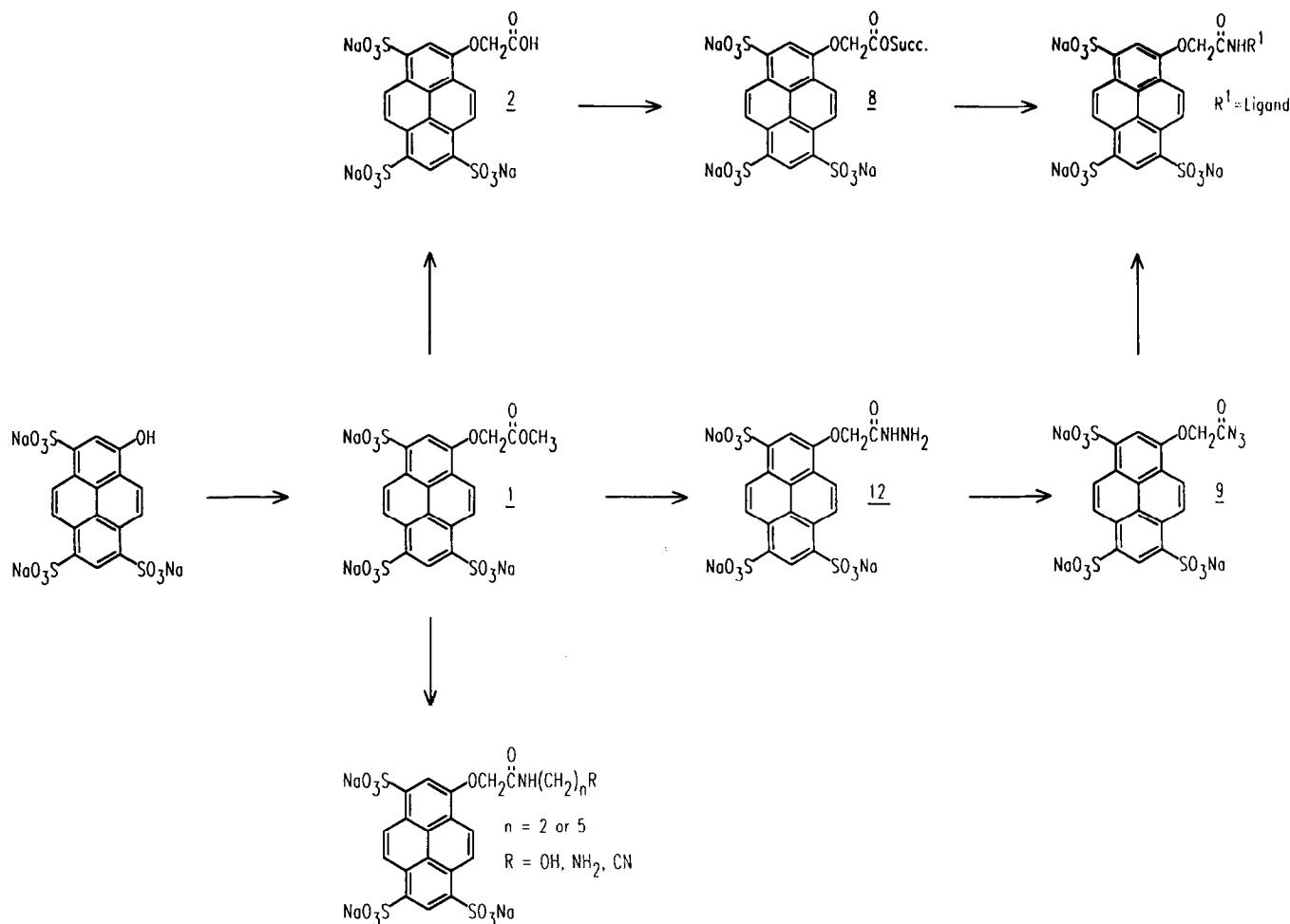


FIG. 2. Synthetic scheme for the preparation of reactive Cascade Blue derivatives.

were detected by absorbance at 400 nm and purity was calculated by integration of this absorbance. Retention times ( $R_t$ ) correspond to times of peak absorbance at 400 nm. The structural identity of the products was determined by 300 MHz  $^1\text{H}$  NMR. The spectroscopic data and chemical reactivity of the Cascade Blue derivatives were consistent with the structural assignments reported here (Fig. 1 and Fig. 2). The chemical reactivity of the Cascade Blue derivatives was determined by incubation of the reactive derivatives with model compounds in aqueous, methanolic, or dimethylsulfoxide solution. Their reactivity was demonstrated by TLC in a solvent that separated the reactive dye from its products with visual detection of the fluorescence emission or by HPLC with photometric detection.

#### Preparation of Methyl 1,3,6-Trisulfo-8-pyrenyloxyacetate, Trisodium salt (1), and Free acid (2)

To a refluxing mixture of 26.2 g 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt in 1.7 l. methanol,

29 g methyl bromoacetate and 18 g diisopropylethylamine were added in three portions over 5 h. After refluxing for an additional 3 h, the mixture was allowed to cool, and was filtered and evaporated under reduced pressure. The resulting slurry was stirred in 500 ml of isopropyl alcohol for 30 min, then the solid was collected by filtration. After drying over potassium hydroxide for 2 days under vacuum, 28.7 g (96% yield) of the ester was obtained as a light yellow powder. A sample of the dye was purified by chromatography over Sephadex LH20 using water for elution.  $^1\text{H}$  NMR: 3.76, s-3H; 5.01, s-2H; 8.06, s-1H; 8.58, d-1H ( $J = 9.6$  Hz); 8.90, d-1H ( $J = 9.9$  Hz); 8.94, d-1H ( $J = 9.9$  Hz); 9.04, d-1H ( $J = 9.9$  Hz); 9.15, s-1H. HPLC:  $R_t = 10.74$  min (90.7%). A solution of 6 g 1 in 20 ml of water containing 0.4 g of sodium hydroxide was warmed to 60°C and stirred for 15 min. The solution was cooled to room temperature and acidified with 0.5 ml concentrated hydrochloric acid. Isopropyl alcohol (30 ml) was added to the resulting slurry and the solid was collected to give 4.6 g (79% yield) yellow powder after air drying. A sample of the dye was purified

by chromatography, as described above for the methyl ester. <sup>1</sup>H NMR: 4.95, s-2H; 8.19, s-1H; 8.90, d-1H (*J* = 9.9 Hz); 8.93, d-1H (*J* = 9.9 Hz); 9.02, d-1H (*J* = 9.9 Hz); 9.10, d-1H (*J* = 9.9 Hz); 9.14, s-1H. HPLC: *R<sub>t</sub>* = 9.32 min. (>97%).

*Preparation of 1-(1,3,6-Trisulfo-8-pyrenyloxyacetamido)-(5-aminopentane), Trisodium Salt (4), and N-(1,3,6-Trisulfo-8-pyrenyloxyacetyl)-N-(4-azidobenzoyl)-1,5-pentanediamine, Trisodium Salt (20)*

To a solution of 1.0 g 1 in 50 ml of methanol, 2 ml of cadaverine was added. The mixture was refluxed for 3 h, cooled, and evaporated under reduced pressure. The residue was stirred for 30 min in 100 ml isopropyl alcohol and the solid was collected. The crude product was purified by chromatography over lipophilic Sephadex in water. Product-containing fractions were combined and lyophilized to give 0.83 g (74% yield) yellow powder. <sup>1</sup>H NMR: 0.61, m-2H; 1.08, m-2H; 1.15, m-2H; 2.28, t-2H (*J* = 7.5 Hz); 2.92, t-2H (*J* = 6.3 Hz); 4.69, s-2H; 7.97, s-1H; 8.46, d-1H (*J* = 9.6 Hz); 8.88, d-1H (*J* = 9.9 Hz); 8.93, d-1H (*J* = 9.9 Hz); 9.01, d-1H (*J* = 9.6 Hz); 9.14, s-1H. HPLC: *R<sub>t</sub>* = 8.77 min. (>97%). Other amides, such as 3, 10, and 16 were prepared from the methyl ester, 1, in an analogous manner.

The photoaffinity reagent, 20, was prepared by addition of 100 mg of succinimidyl 4-azidobenzoate to a solution of 70 mg 4 in 2 ml of dimethyl sulfoxide and 4 drops of diisopropylethylamine. After stirring overnight, the mixture was poured into 20 ml of chloroform and the product was collected by centrifugation. The crude product was purified by chromatography over lipophilic Sephadex in water. Product-containing fractions were combined and lyophilized to give 37 mg (43% yield) of yellow powder. HPLC: *R<sub>t</sub>* = 11.38 min. (>97%). The amine, 4, also reacted with other activated esters, such as succinimidyl *N*-t-BOC-L-proline and succinimidyl acrylate to give 15 and 19, respectively.

*Preparation of 1,3,6-Trisulfo-8-pyrenyloxyacethydrazide, Trisodium Salt (12), and the Acetyl Azide (9)*

To a solution of 3 g 1 in 150 ml of methanol, 2 ml of anhydrous hydrazine was added and the mixture was refluxed overnight. The mixture was cooled to 4°C for 2 h and the solid was collected by filtration. After washing with methanol and drying, 2.3 g (77% yield) of yellow powder was obtained. A sample of the dye was purified by chromatography over lipophilic Sephadex using water for elution. HPLC of a solution of 12 in water and a solution of 12 with one drop of acetone in water showed that 12 reacted to form a new product in the presence of acetone. HPLC: *R<sub>t</sub>* = 8.96 min. (>97%). <sup>1</sup>H NMR: 4.95, s-2H; 7.91, s-1H; 8.32, d-1H (*J* = 9.6 Hz);

8.81, d-2H (*J* = 9.3 Hz); 8.95, d-1H (*J* = 9.9 Hz); 9.08, s-1H. The acetyl azide, 9, was prepared from the hydrazide, 12. A solution of 100 mg of 12 in 10 ml of water was acidified with 2 drops of concentrated hydrochloric acid and cooled in an ice bath. A solution of sodium nitrite in water (1.0 ml, 0.013 g/ml) was added to the cold suspension. After stirring for 30 min, the ice bath was removed and the mixture was allowed to warm to room temperature. After stirring 15 min further, an additional 1.0 ml of sodium nitrite solution was added. The mixture was stirred 10 min longer and then poured into 100 ml of isopropyl alcohol. The precipitate was collected and washed with tetrahydrofuran to give 56 mg (55% yield) 9 as a tan powder.

*Preparation of Conjugates of Cascade Blue*

The dextran conjugate, 5, was prepared by adding 12 mg 9 to a solution of 100 mg aminodextran (average *M<sub>n</sub>* = 70,000 g/mol, 38 amines/mol) in 5 ml of dimethyl sulfoxide (DMSO). After stirring overnight, the mixture was diluted to approximately 50 ml and dialyzed against 3 × 4 liters of 2% sodium sulfate in water then 3 × 4 liters of water. The solution of dextran was lyophilized to give 90 mg of light yellow powder. The degree of substitution was determined by comparing the molar absorptivity of the conjugate to the molar absorptivity of compound 12. The dextran was found to contain approximately 13 mol of dye per 70,000 g of dextran. Bovine serum albumin (BSA) conjugates were prepared by dissolving the protein (10 mg/ml) in 100 mM sodium bicarbonate. Aliquots of 9, AMCA succinimidyl ester, or IPTS in DMSO were added to solutions of protein such that several different levels of loading were obtained. After standing for 2 h at room temperature, an aliquot of hydroxylamine was added to give a final hydroxylamine concentration of 0.15 M. After 1 h, the conjugate was separated from free dye by gel filtration over Cellufine G-25 medium eluting with 30 mM ammonium acetate. The protein-containing fractions were lyophilized. The degree of substitution of the protein conjugates was determined as described for the dextran conjugate above. Other proteins and the Cascade Blue labeled bioparticles (heat or chemically killed *Saccharomyces cerevisiae*, *Escherichia coli*, and *Staphylococcus aureus*) were labeled under comparable conditions. In the case of the biological particles, free dye was removed by extensive washing with buffer.

*Indirect Immunofluorescence Staining*

Fixed human epithelial (HEp-2) cells were incubated with human anti-nuclear antibody solution provided by the manufacturer for 30 min, followed by washing with PBS. This system provides a versatile method of determining the specificity and nonspecific background for secondary detection reagents since localization of the

antibody complex is specific for the nucleus. All of the incubations were performed at ambient room temperature, with no special handling techniques. Following incubation with the anti-nuclear antibody or negative anti-nuclear antibody, the staining was completed by one of the following methods: (i) The cells were incubated with 10 or 20  $\mu\text{g}/\text{ml}$  of Cascade Blue goat anti-human IgG (4.2 dye/protein) for 30 min and washed with PBS. (ii) HEp-2 preparations were incubated with 40  $\mu\text{l}$  of undiluted mouse anti-human IgG solution, as provided by the manufacturer. The cells were then incubated with 40  $\mu\text{l}$  of 10 or 20  $\mu\text{g}/\text{ml}$  Cascade Blue labeled goat anti-mouse IgG (7.8 dye/protein) for 30 min, followed by washing with PBS. (iii) Cell preparations were incubated with 40  $\mu\text{l}$  of 10  $\mu\text{g}/\text{ml}$  biotinylated protein A in PBS for 30 min and washed with PBS. Following incubation with biotinylated second label, the cells were fluorescently stained with 40  $\mu\text{l}$  of 12.5 or 25.0  $\mu\text{g}/\text{ml}$  Cascade Blue labeled avidin (6.7 dye/protein). Subsequent to fluorescent staining, the cells were again washed with PBS. Fluorescence microscopy was performed with a Zeiss Axioplan microscope equipped with a 50 W high pressure mercury arc lamp and with Hoechst/DAPI filters<sup>2</sup> (Zeiss 487702, BP-360 nm  $\pm$  30, LP-420 nm). The black and white film used was Kodak T-Max 3200, exposed and processed at an effective ASA of 1600. Exposure times were bracketed from 15 s to 2 min.

## RESULTS AND DISCUSSION

The base chromophores for the reactive, hydrophilic Cascade Blue tracers are pyrenyloxytrisulfonic acid derivatives that are structurally similar to nonreactive alkoxytrisulfonic acid derivatives described previously (15). The parent hydroxyl compound was 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS, also called pyranine, Pyranine 108, Solvent green 7, or D&C Green No. 8). A general scheme for synthesis of several Cascade Blue derivatives that are or can be modified to have the desired chemical substituents or reactivities is illustrated in Fig. 2. The general method consists of condensation of an alkylating reagent such as an appropriately substituted alkyl halide, in the presence of a base, with a substituted or unsubstituted pyrene sulfonic acid having a hydroxyl on the 1,3,6, or 8 position to give an alkoxy intermediate such as 1, which may be hydrolyzed or reacted directly with hydrazine or aliphatic amines to give derivatives having the necessary reactivity. Derivatives containing terminal amines may be further reacted with activated car-

boxylic acid derivatives, such as succinimidyl esters of compounds possessing additional reactive functional groups.

The spectrophotometric properties of a number of the dyes and intermediates that were prepared are summarized in Table 1. The dyes exhibited absorption maxima at 374–378 nm and 399–403 nm, with extinction coefficients in the range of  $1.9 \times 10^4$ – $2.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  and  $2.3 \times 10^4$ – $3.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ , respectively. Emission maxima ranged from 422–430 nm, with bandwidths of approximately 50 nm. Quantum yields of the dyes in aqueous solution were generally above 0.5 and frequently above 0.9 (relative to a quinine sulfate standard). The residual fluorescence at wavelengths near the emission maximum of fluorescein was typically less than 5% of the emission intensity in the region of 420 to 425 nm. The absorption spectrum of the fixable Cascade Blue hydrazide, 12, is shown in Fig. 3. Absorption spectra of the other derivatives prepared were very similar in terms of the wavelengths of the maxima, bandwidths, and extinction coefficients. Emission spectra for equimolar solutions (at pH 7.0) of 12 versus 4-MUA, AMCA (both free acid derivatives), and SITS (butylamine conjugate) are compared in Fig. 4a. Relative to AMCA, the pyrenyloxytrisulfonic acid dye exhibits over twofold greater fluorescence intensity, significantly narrower emission spectral width, and smaller Stokes' shift. It is particularly noteworthy that the relative quantum yield of 12 (i.e., approximately 0.5) was somewhat lower than many of the other Cascade Blue derivatives prepared (quantum yields above 0.9 in a number of cases). The emission spectra of a number of different Cascade Blue derivatives are included in Fig. 4b for comparison. The Cascade Blue dyes show considerably higher quantum efficiencies, on a molar basis, than the alternative dyes. Additionally, the narrower spectral width makes the dyes particularly useful for applications requiring multiple dyes, such as multicolor fluorescence microscopy and flow cytometry.

Cascade Blue derivatives possess many of the properties desired in short wavelength, hydrophilic fluorophores. The spectral properties and general water solubilities of representative examples of the previously described dyes and compound 12 of the Cascade Blue series are compared in Table 2. Deficient or undesirable properties of these dyes are indicated with an asterisk. The existing, reactive, blue-emitting fluorophores have weaker absorptivity (extinction coefficients of less than  $2.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  at their absorbance maxima versus greater than  $2.5 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  for the pyrenyloxytrisulfonic acid derivatives and  $7.5 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  for fluorescein at its peak near 490 nm), lower quantum yields, or lower solubility in aqueous solutions than the Cascade Blue derivatives. Many of the dyes are not well suited for excitation with the strongest emission lines of the most commonly available sources, such as the 365-

<sup>2</sup> Omega Optics, Inc. (Burlington, VT) has recently developed filter sets (Cascade Blue Quantitative Filter Sets) that are specifically designed for optimum excitation and detection of Cascade Blue derivatives with several common excitation sources.

TABLE 1  
Spectral Properties of Cascade Blue Dyes<sup>a</sup>

Dye	$\lambda_{\text{max}}^{\text{Abs}}$ (nm)	$10^{-3} \times \epsilon^b$ (M <sup>-1</sup> cm <sup>-1</sup> )	$\lambda_{\text{max}}^{\text{Em}}$ (nm)	Bw <sup>c</sup> (nm)	Em <sup>514 d</sup> (%)	Dye	$\lambda_{\text{max}}^{\text{Abs}}$ (nm)	$10^{-3} \times \epsilon^b$ (M <sup>-1</sup> cm <sup>-1</sup> )	$\lambda_{\text{max}}^{\text{Em}}$ (nm)	Bw <sup>c</sup> (nm)	Em <sup>514 d</sup> (%)
2 <sup>e</sup>	403	27.0	430	52	5.8	13	399	28.0	423	50	3.8
	374	19.8					376	23.3			
3	399	26.4	423	50	4.9	14 <sup>e</sup>	399	26.9	422	49	2.8
	378	21.2					378	21.2			
4 <sup>e</sup>	398	27.6	424	50	3.9	15	399	23.0	423	50	3.0
	377	22.8					377	18.4			
5	400	—	423	50	4.5	16	399	30.0	423	49	3.0
	378	—					377	24.3			
10	399	26.6	423	50	3.3	19	399	26.3	423	49	2.9
	377	21.7					378	20.8			
11	399	25.1	423	51	3.8	20	400	24.8	424	50	3.5
	377	20.7					378	18.8			
12 <sup>f</sup>	399	27.5	423	50	3.9						
	377	22.9									

<sup>a</sup> In 50 mM aqueous potassium phosphate buffer at pH 8.0.

<sup>b</sup> Extinction coefficient.

<sup>c</sup> Full bandwidth at half peak height.

<sup>d</sup> Percentage of emission intensity at 514 nm relative to the maximum emission intensity of the dye.

<sup>e</sup> Quantum yield greater than 0.9 in 50 mM phosphate buffer at pH 8.0.

<sup>f</sup> Quantum yield approximately 0.5 in 50 mM phosphate buffer at pH 8.0.

nm line of the mercury arc lamp. Additionally, the fluorescence of several of the dyes, such as IPTS, SITS, and Dansyl, is low in aqueous solution, resulting in low quantum yields. The lower quantum yield decreases the detection sensitivity or requires use of disproportionately larger quantities of the less fluorescent dye to achieve signal intensities of comparable magnitude.

Dyes in the Cascade Blue series show low spectral overlap with fluorescein derivatives. The emission spectra of stilbene, naphthalene, and coumarin derivatives exhibit large bandwidths with very broad long wavelength components that greatly increase the fluorescence background at wavelengths used for detection of other dyes (for instance fluorescein, Lucifer Yellow, and tetramethylrhodamine) in applications such as DNA hybridization, developmental tracers, and flow cytometry that require detection of multiple dyes or dye conjugates. The large Stokes' shifts and wide emission bandwidths of all of these dyes, except PBA, result in significant residual fluorescence from the uv excited dyes at wavelengths used for detection of fluorescein emission (typically 515–525 nm). The Cascade Blue dyes have uniquely low background fluorescence at the wavelengths of the fluorescein emission maximum. The potential utility of these dyes in multicolor applications is illustrated in Fig. 5. This figure shows the normalized emission spectra of compound 12, fluorescein, and Lucifer Yellow CH, excited at their respective maxima. Compound 12 exhibits a narrow emission peak that is well resolved from the emission bands of these widely used dyes. The Cascade Blue dyes and their conjugates,

in general, show very low emission at wavelengths near the fluorescein emission maximum. For the Cascade Blue derivatives, typically less than 5% of the maximum emission intensity of the dyes remains at 514 nm (the emission maximum of fluorescein).

Cascade Blue derivatives have higher quantum efficiencies than other blue-emitting dyes. While having other desirable properties such as a high extinction coefficient and high water solubility, SITS has a very low quantum yield in water and is photolytically isomerized to the nonfluorescent *cis* isomer. Despite the commercial availability of SITS for over 15 years, use of SITS to form fluorescent conjugates has not been widely adopted, in part due to the low fluorescence yield. Another drawback of SITS is the short wavelength absorption maximum (<350 nm). SITS and related sulfonated stilbene derivatives also have been found to have an inhibitory effect on anion transport systems in red blood cells (16) and on microsomal glucose-6-phosphatase (17). In addition to the greater quantum yield of the Cascade Blue dyes, another potential advantage may be the low toxicity of closely related compounds such as HPTS, which has been reported to have an LD<sub>50</sub> in the range of 900 mg/kg in mice (18) to greater than 1000 mg/kg in Swiss-Webster mice (19), although specific mechanisms of toxicity were not examined. HPTS was also found to remain unbound in the presence of blood proteins.

Cascade Blue derivatives are readily soluble in aqueous solution. With the exception of SITS and IPTS, the water solubility of the commonly used reac-

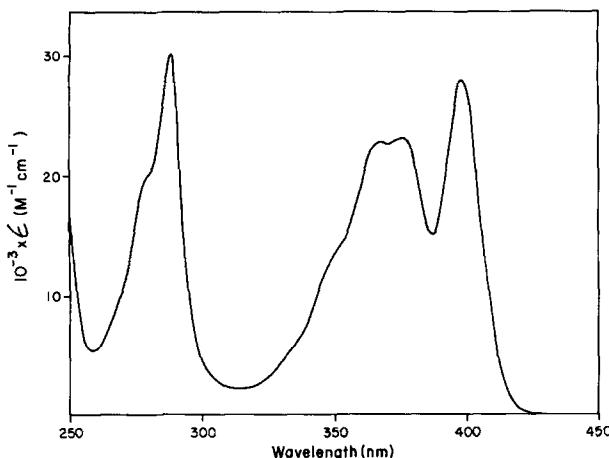


FIG. 3. Absorption spectrum of 12 in aqueous 50 mM potassium phosphate at pH 8.0.

tive forms of the previously reported blue-emitting dyes is low, necessitating use of organic solvent comixtures in forming dye conjugates with many biopolymers. Adequate water solubility is necessary because many important biological applications of fluorescence exist only in aqueous solution. Despite their acceptance as fluorescent tracers, aminocoumarin derivatives suffer from this deficiency, precluding or increasing the difficulty of some useful applications of fluorescent labeling techniques. Reactive dye derivatives such as the succinimidyl ester of AMCA, Dansyl chloride and succinimidyl pyrene-1-butrate are quite lipophilic and not readily soluble in the aqueous solutions that are usually required for fluorescent labeling of proteins, polysac-

charides and other biomolecules. Reactive forms of hydroxycoumarins, such as the succinimidyl ester of 4-MUA, while less hydrophobic than similar derivatives of AMCA, are still sparingly soluble in aqueous solution. The intrinsic polyanionic charge of the pyrenyl-oxytrisulfonic acid fluorophore results in fluorescent derivatives that are highly water soluble. The ionic charge and generally high water solubility of the Cascade Blue derivatives enhance their utility as fluorescent tracers for hydrophilic environments and increase their suitability for use in applications requiring developmental tracers. This property also facilitates the coupling, in aqueous solution, of the fluorescent dye and proteins, drugs, or other ligands of interest.

Fluorescence of a number of the uv-excitable dyes is sensitive to the pH of the medium surrounding the fluorophore, while the Cascade Blue dyes are insensitive to these effects. Typically, hydroxycoumarin derivatives exhibit  $pK_a$  values above 7.0 and show pH-dependent absorption and emission spectra which decrease the fluorescence efficiency of the fluorophore in the physiological pH range. Other phenolic dyes, such as hydroxypyrenetrisulfonyl chloride and 4-MUA, and their conjugates, also have high sensitivity to solution pH in the physiological range (pH 6 to 8). Emission of the pyrenyl-oxytrisulfonic acid fluorophores is essentially unaffected by the pH of the solution, a property that is advantageous in the quantitative determination of fluorescence, where it is desirable to minimize the number of corrections that must be included in the calibration. The Cascade Blue dyes show only minor spectral shifts and intensity changes between water, methanol, and ethanol and have absorption and emission

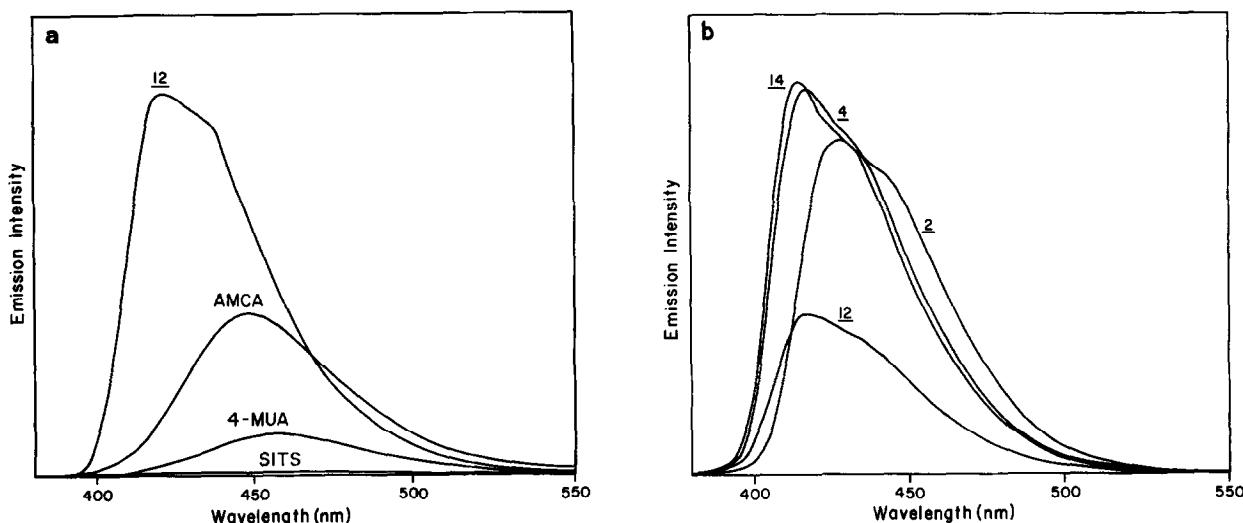


FIG. 4. Uncorrected emission spectra of (a) equimolar solutions of 12, 4-MUA, AMCA, and SITS in aqueous 50 mM potassium phosphate at pH 7.0, excited at their peak absorption wavelengths, and (b) solutions of several Cascade Blue derivatives in aqueous 50 mM potassium phosphate at pH 8.0 (the samples were prepared at the same optical density at 360 nm, the excitation wavelength). The Cascade Blue derivatives show greater quantum efficiencies than other blue-emitting dyes.

TABLE 2  
Properties of Blue-Fluorescent Dyes

Dye	$\lambda_{\text{Abs}}^{\text{Abs}}$ (nm)	$10^{-3} \times \epsilon^a$ (M <sup>-1</sup> cm <sup>-1</sup> )	$\lambda_{\text{max}}^{\text{Em}}$ (nm)	Bandwidth <sup>b</sup> (nm) [cm <sup>-1</sup> ]	$\text{Em}^{514\text{c}}$ (%)	Water Sol'y <sup>d</sup>	Quantum yield <sup>e</sup>
4-MUA	360	19.1*	454	58 [2668]	20*	Low*	Medium
AMCA	354	15.2*	442	57 [2858]	12*	Low*	Medium
SITS	337*	37.4	438	82 [4825]*	19*	High	Low*
Dansyl <sup>f</sup>	340*	4.5*	578*/ <sup>f</sup>	—/ <sup>f</sup>	—/ <sup>f</sup>	Low*	Low*
PBA	341*	45.1	377*	—/ <sup>g</sup>	2	Low*	Low*
IPTS	373	25.6	458	78 [3634]*	38*	High	Low*
<i>l2</i>	399	28.0	423	50 [2168]	3.8	High	High
	376	23.3					

<sup>a</sup> In aqueous 50 mM potassium phosphate at pH 8.0.

<sup>b</sup> Full bandwidth at half peak height.

<sup>c</sup> Percentage of emission intensity at 514 nm relative to the maximum emission intensity of the dye.

<sup>d</sup> Water solubility of the reactive form of the dye.

<sup>e</sup> Approximate quantum yield in aqueous solution.

<sup>f</sup> The spectral properties of dansyl derivatives are very sensitive to environmental effects.

<sup>g</sup> Alkylpyrene derivatives show multiple emission peaks.

\* These are deficient or undesirable properties of the dyes.

spectra that lack significant pH dependence between pH 5.0 and pH 10.0 in aqueous solution, as illustrated in Fig. 6 for 2 relative to 4-MUA. The pyrenyloxytrisulfonic acid dye exhibits fluorescence intensity that is virtually constant over 5 pH units, while 4-MUA is less than 20% as fluorescent in 50 mM phosphate buffer at pH 7.0 as it is at pH 10.0.

Chemically reactive fluorescent reagents, based on the pyrenyloxytrisulfonic acid fluorophore, were developed for modification of a number of the functional groups commonly found in biologically significant molecules under conditions of solvent, temperature, and pH that usually do not destroy the biological activity of the modified biomolecule. Reactive functional groups that are intrinsically present or that can be introduced into ligands of interest include amines, thiols, carboxylic acids, aldehydes, and ketones. Among the most common reactive groups are amines. Amine reactive Cascade Blue derivatives, such as acyl azides, were conjugated successfully with a variety of ligands, including proteins, carbohydrates, and low molecular weight amines. Biopolymers that were labeled with Cascade Blue included polysaccharides such as 10,000 and 70,000 M<sub>w</sub> aminodextran (e.g., 5); albumins such as BSA and lactalbumin; secondary labels such as F<sub>c</sub>-binding protein A and protein G; secondary antibodies such as goat anti-mouse, goat anti-human, and donkey anti-sheep IgG; lectins such as concanavalin A and wheat germ agglutinin; receptor selective toxins such as the acetylcholine receptor binding protein  $\alpha$ -bungarotoxin; and the biotin binding proteins avidin and streptavidin; as well as biological particles, such as heat or chemically killed yeast particles (*S. cerevisiae*), *E. coli*, and *S. aureus*.

Proteins labeled with Cascade Blue were more brightly fluorescent than the analogous conjugates of AMCA or IPTS. In Fig. 7, the emission intensities of bovine serum albumin (BSA) and IgG conjugates of the pyrene isothiocyanate, IPTS, and the succinimidyl ester of AMCA, respectively, are compared to the BSA and IgG conjugates of compound 9. For fluorescently labeled BSA, as shown in Fig. 7a, the Cascade Blue conjugates were approximately 30-fold more fluorescent (per mole of protein) than conjugates of IPTS and 3-fold brighter than AMCA conjugates. Similar results were

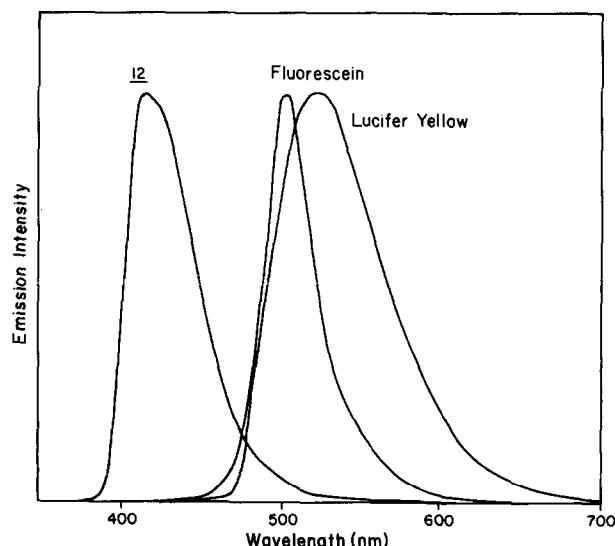


FIG. 5. Uncorrected emission spectra of 12, fluorescein, and Lucifer Yellow CH normalized to the same peak intensity. The Cascade Blue emission is well resolved from these commonly used dyes.

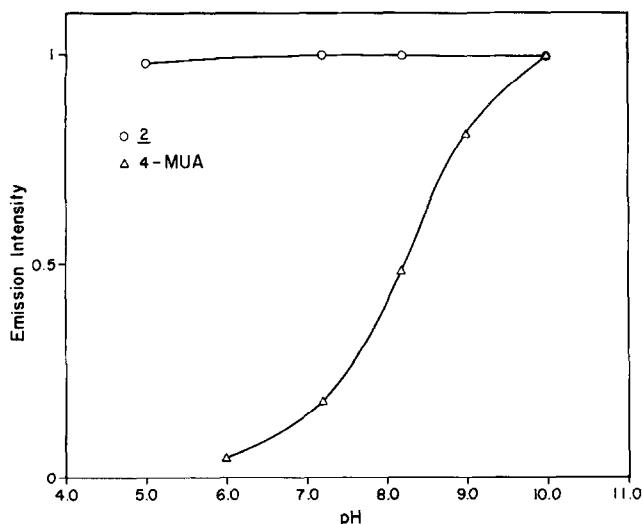


FIG. 6. Emission intensity of 2 and 4-MUA as a function of pH. The emission intensities of the two dyes at pH 10.0 were set equal. While 4-MUA is sensitive to pH in the physiological pH range, the Cascade Blue dye shows emission that is essentially constant over 5 pH units.

obtained for IgG conjugates of Cascade Blue and AMCA, as shown in Fig. 7b. With respect to AMCA, the greater molar emission intensity of Cascade Blue conjugates may be attributed to a reduction in dye-dye and dye-protein interactions, resulting from electrostatic repulsions between dyes and the preference of the polyanionic, hydrophilic dyes for the aqueous medium surrounding the protein relative to the more hydrophobic environment of the protein, in contrast to the elec-

trically neutral AMCA. In the case of IPTS, the greater emission intensity of the Cascade Blue conjugates is due to the intrinsically higher quantum yield of the pyrenyloxytrisulfonic acid fluorophore, relative to the thiourea or dithiocarbamate derivatives of aminopyrenetrifluoromethyl sulfonic acid that result from the reaction of IPTS with amines or thiols, respectively.

Cascade Blue conjugates are suitable for a variety of biological, immunological, and histochemical applications, as shown in Fig. 8. The dye may be conjugated directly to biological particles, such as yeast particles, or conjugated to a second label, such as an antibody or an avidin. The *S. cerevisiae* particles are uniformly labeled, brightly fluorescent, and show virtually no loss of dye into the medium over time, indicating complete removal of the highly soluble free dye. In both of the fluorescently labeled HEp-2 preparations, fluorescent staining with Cascade Blue conjugates of IgG and avidin produced specific localization of the blue emission in the nuclei, with low background levels. The discrete pattern of signal localization demonstrates that the protein conjugates have retained high binding specificity subsequent to covalent modification with Cascade Blue. Similar results were also obtained with HEp-2 cells fluorescently stained with mouse anti-human IgG (not shown). Both the yeast particles and the HEp-2 preparations showed acceptable photostability, although some fading was observed over the time course of the fluorescence observations and photography (15–30 min). Since addition of each dye molecule to an amine on a protein results in replacement of a cationic ammonium group with a trivalent anionic dye, modification of the electrostatic

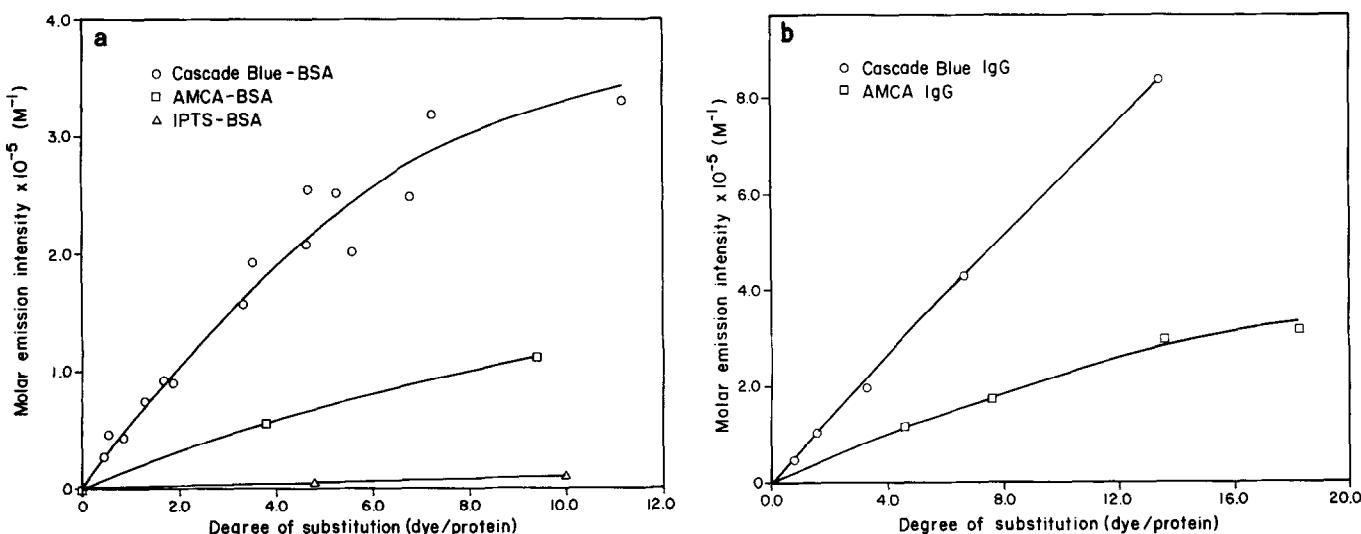
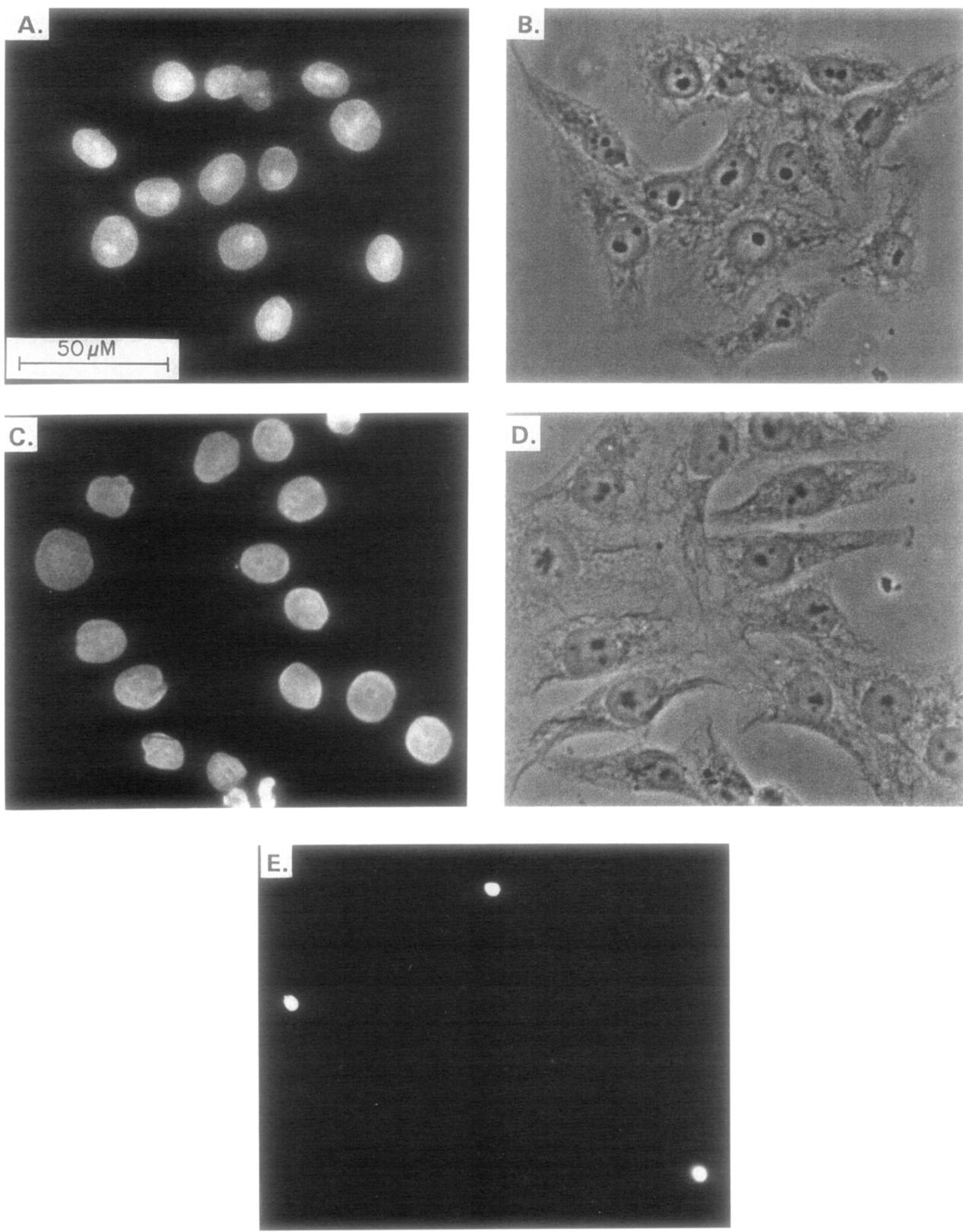


FIG. 7. Emission intensity per mole of protein as a function of degree of substitution for (a) BSA conjugates of 9, AMCA and IPTS, or (b) rabbit IgG conjugates of 9 and AMCA. The molar emission intensity was calculated as the product of the extinction coefficient of the dye, the degree of substitution of the conjugate, and the emission intensity of the conjugate (relative to a quinine sulfate standard of the same optical density at 360 nm, the excitation wavelength). The Cascade Blue conjugates exhibited brighter fluorescence at all loading levels examined.



**FIG. 8.** (A-D) Micrographs of fixed HEp-2 cells incubated with human anti-nuclear antibody, followed by Cascade Blue labeled goat anti-human IgG (viewed with A) fluorescence and (B) phase contrast optics or followed by incubations with biotinylated protein A and then Cascade Blue labeled avidin (viewed with C) fluorescence and (D) phase contrast optics. (E) Fluorescence micrograph of yeast particles (*Saccharomyces cerevisiae*) that were covalently labeled with Cascade Blue. Labeling protocols are described under Materials and Methods. Scale bar indicates 50  $\mu$ m. Cell preparations stained with Cascade Blue conjugates showed specific nuclear staining with low nonspecific background fluorescence.

charge properties of the protein may result in altered function or specificity in some cases. Although there was no indication that the IgG conjugates examined lost specificity, this charge effect may be more significant in lower molecular weight conjugates or macromolecules with a limited number of amines that are crucial to biological activity.

Cascade Blue derivatives with hydrazine and amine functionalities were also investigated. The polar, fixable, aldehyde and ketone reactive hydrazides, 11, 12, and 13, are structurally and functionally similar to Lucifer Yellow CH, a fixable, yellow-emitting tracer that is widely used in developmental and neurological applications. As shown in Fig. 5, the emission bands of Lucifer Yellow CH and Cascade Blue hydrazide are well resolved, with minimal spectral overlap. Additionally, the large Stokes shift and broad absorption bandwidth of Lucifer Yellow CH permit simultaneous excitation of the two dyes in the 400-nm region, although under these conditions, the Lucifer Yellow emission is approximately 25% as intense as the Cascade Blue emission. Counterion effects on the solubility of Cascade Blue dyes were similar to those observed for Lucifer Yellow salts. Thus, solubility of alkali metal salts of Cascade Blue hydrazide in distilled water decreased in the order: Li<sup>+</sup> salt (81 mg/ml) > Na<sup>+</sup> salt (26 mg/ml) > K<sup>+</sup> salt (15 mg/ml).

In addition to carboxylic acid and hydrazide derivatives, Cascade Blue dyes with other reactive groups were prepared and evaluated. Aliphatic amine containing derivatives provided convenient intermediates for further structural modification. Dyes such as 3 and 4 reacted with succinimidyl *N*-t-BOC-L-proline to yield amino acid derivatives such as 14 and 15, and also, after deprotection, 17 and 18, respectively. Thiol reactive haloacetamides, such as 6 and 7, were shown to react with low molecular weight thiols such as 2-mercaptoethanol. More specialized derivatives were also prepared, such as the acrylamide, 19, and the photoaffinity reagent, 20, as well as the alcohol, 10, and the imido ester precursor, 16.

In conclusion, none of the reagents previously described in the chemical or biochemical literature possess the appropriate combination of chemical reactivity, spectral properties, fluorescence yield, high water solubility, and lack of pH sensitivity to make them optimum for simultaneous use with fluorescein in aqueous systems, with excitation and emission wavelengths shorter than 500 nm. In comparison, the Cascade Blue dyes exhibit all of the useful properties described above, namely:

- (i) Small Stokes' shifts, narrow emission bands, and little spectral overlap with fluorescein.
- (ii) High extinction coefficients and quantum yields.
- (iii) High water solubility.
- (iv) Low sensitivity to pH.
- (v) Reactivity with many of the functional groups found in biomolecules.

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