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A New Europium Chelate for Protein Labelling and Time-Resolved Fluorometric Applications

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Synthetic procedures are presented for a new chelator that forms stable and highly fluorescent complexes with Eu^{3+}. This chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) is synthesized in a high-yield three-step procedure. BCPDA can be covalently incorporated into proteins under relatively mild conditions, and when complexed with Eu^{3+} forms a fluorescent product that has a lifetime in the range of 0.4 to 0.7 ms. Thus, it is useful for time-resolved fluorescence immunoassay applications.

KEY WORDS: Nonisotopic immunoassay; time-resolved fluorescence; europium complexes; fluorescence lifetime; fluorescent labels.

Europium complexes have recently received attention in clinical biochemistry because of their potential application as markers in immunoassays (1,2). These fluorescent complexes form attractive labels because: (a) They have exceptionally large Stokes shifts (250–350 nm as compared to ~28 nm for fluorescein, a conventional fluorescent label). (b) The emission spectrum consists of narrow lines characteristic of metal ion emission whose bandwidth at 50% emission is less than 10 nm. (c) Their fluorescence is long-lived, with lifetimes on the order of 100 to 1000 μs as compared to 1 to 100 ns for most organic probes. In these complexes, the excitation radiation is absorbed by the ligand and then is transferred to the europium ion by an internal energy transfer process (3,4), resulting in europium ion emission.

The three characteristics of the europium complexes mentioned above can be used to devise analytical systems with high sensitivity. The nonspecific background fluorescence arising from the sample (e.g., patient serum), the cuvettes, the optics, and the reagents used can be effectively eliminated by using a narrow band pass interference filter in the vicinity of 615 ± 5 nm to isolate one of the specific Eu^{3+} fluorescence lines. In this wavelength region, the background fluorescence from biological samples is minimal. Further discrimination against background fluorescence is obtained by utilizing a time-resolved detection system in which the sample is irradiated using a pulsed excitation light source and the long-lived probe fluorescence is monitored after a preselected time interval during which any short-lived fluorescence has decayed (5,6).

A number of investigators have studied the possibilities of using the favourable characteristics of europium complexes for labelling biological molecules, especially antibodies for immunoassay applications (7–10). In one such analytical system, Eu^{3+} has been introduced as the label into the immunoreactants by using a covalently bound ethylenediaminetetraacetic acid (EDTA) derivative as a carrier. The 1:1 europium-EDTA complex has a high stability constant ($10^{17.3}$ M^{-1}) (11) but is not very fluorescent in aqueous solution (6). Thus, after all incubations and washings have been completed, it is necessary in a typical heterogeneous immunoassay configuration, to release Eu^{3+} using low pH. A highly fluorescent species is subsequently formed by complexing Eu^{3+} in a micellar solution with 2-naphthoyltrifluoroacetone (NTA) and trioctylphosphine oxide (TOPO) (6). This complex (7) is then quantitated in solution using the "time-resolved fluorescence" technique.

Wieder proposed an alternate assay configuration (12) in which the chelator is introduced into the immunoreactants and excess Eu^{3+} is used to form the fluorescent complex. He claimed that quantitation of the chelate can be performed directly. However, for this approach to be practical, a suitable chelator with the following desirable characteristics is needed: (a) It should form stable complexes with Eu^{3+}. (b) The complex should be highly fluorescent, that is, the ligand should have a high extinction coefficient at the excitation wavelength and the fluorescence quantum yield of the complex should be appreciable. (c) It should have a structure that can readily be incorporated in a covalent manner into proteins. (d) It should have minimum nonspecific binding with the serum components. (e) The necessary derivative used for labelling should be easily synthesized.

The europium chelators of the diketone type do not fulfill criterion (a) and attempts to use direct labelling

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have been unsuccessful (6). The chelators of the EDTA type fulfill criterion (a) but not (b). The problems of complex stability and water solubility are also present in the chelators proposed by Wieder as candidate labels for immunoassays (12).

In this article, we report on a new europium chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), which is suitable as a fluorescent labelling agent for proteins. BCPDA can be prepared in a high-yield (88% overall) three-step synthesis starting from 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine) (**I, Figure 1**). A complex with Eu^{3+} is formed that is fluorescent both in aqueous solution and when adsorbed to a solid phase in a heterogeneous immunoassay configuration. The chelate is conveniently excited by conventional UV light sources or by a pulsed nitrogen laser. Incorporation of this label into proteins involves a simple one-step conjugation procedure. In addition to the description of the synthesis of BCPDA and its use in protein labelling, some observations on the fluorescence of labelled protein are also reported. More extensive investigations on the spectroscopy of this chelate are in progress.

Materials and methods

SYNTHETIC PROCEDURES

The preparation of 4,7-diphenyl-1,10-phenanthroline-2,9-dicarboxylic acid (**III**) is similar to the synthetic route followed by Newkome and co-workers for dimethyl 1,10-phenanthroline-2,9-dicarboxylate (13).

The nuclear magnetic resonance experiments were performed at 500.13 MHz on a Bruker AM-500 spectrometer at McMaster University, Hamilton, Ontario. The spectra were recorded over a spectral width of 6 kHz (2.7 s acquisition time) in 32 K data points. Spectra were acquired in 40 scans. Chemical shifts are in parts per million (ppm) downfield from internal tetramethylsilane (0.00 ppm). Splitting patterns are designated as s, singlet; m, multiplet.

Infrared spectra were recorded on a Perkin-Elmer 781 infrared spectrophotometer. Elemental analyses were performed at Guelph Chemical Laboratories, Guelph, Ontario.

2,9-BIS(TRICHLOROMETHYL)-4,7-DIPHENYL-1,10-PHENANTHROLINE (II)

A mixture composed of 4 g (11.1 mmols) bathocuproine (**I**) (Sigma, St. Louis, MO 63178 USA), 9.8 g (73.1 mmols) N-chlorosuccinimide (Sigma), 0.011 g benzoyl peroxide (BDH Chemicals, Toronto, Canada M8Z1K5) in 100 mL carbon tetrachloride was refluxed with stirring for 6 h. The cooled reaction mixture was filtered to remove succinimide. The solvent was removed from the filtrate by vacuum evaporation. The residue was dissolved in 100 mL chloroform. The organic layer was washed once with 100 mL saturated aqueous Na_2CO_3 and dried over anhydrous $MgSO_4$. The product was obtained in 95% yield after removal of solvent by vacuum evaporation. Melting point (Mp) 268 to 273°C. Infrared spectroscopy (IR) KBr disk 1620, 1570, 1550, 1490, 1445, 1405, 1360, 1090, 930, 895, 800, 780, 735, 700, and 620 cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) in deuterated trichloromethane ($CDCl_3$) δ 7.56 (m, 10H), 7.98 (s, 2H), and 8.27 (s, 2H). Analysis calculated for $C_{26}H_{14}Cl_6N_2$:55.06% C, 2.49% H, 4.94% N,

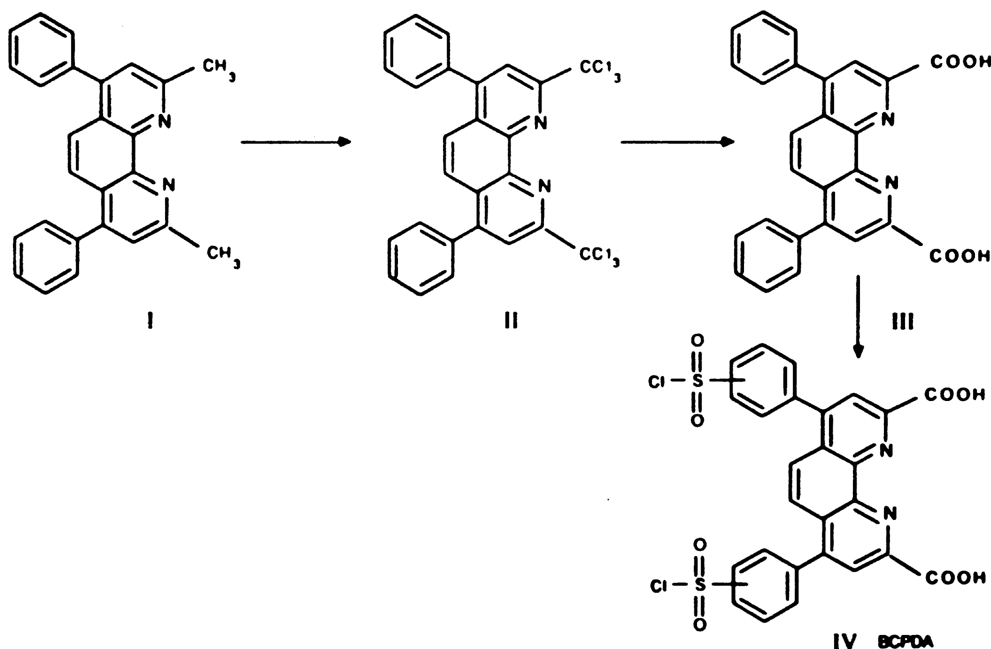

Figure 1—Schematic presentation of the synthesis of BCPDA from bathocuproine. (Details given in Materials and methods).

37.51% Cl. Found 54.81% C, 2.48% H, 4.71% N, 37.50% Cl.

4,7-Diphenyl-1,10-phenanthroline-2,9-dicarboxylic acid (III)

A mixture composed of 1.5 g (2.6 mmols) **II** and 6.5 mL concentrated sulfuric acid was heated with stirring in an oil bath at 80°C for 2 h (fume hood). The mixture was then cooled in an ice bath and 3.2 mL water was slowly added with stirring. Heating with stirring at 80°C was continued for 1 h. The cooled reaction mixture was then added slowly to stirred 100 mL ice water to precipitate the product. The light yellow solid was collected by vacuum filtration, washed thoroughly with distilled water and dried in vacuum. Mp 202 to 208°C. Thin layer chromatography of the product on reverse phase C_{18} plates (Merck HPTLC RP-18F_{254} s) with methanol as eluting solvent indicates that a small amount of impurity (<5%) with higher R_f was present. The yield of the crude product was 99% of the theoretical. This material was used for the preparation of **IV**.

An analytical sample of **III** was prepared by converting **II** to the dimethyl ester (2,9-bis-(methoxycarbonyl)-4,7-diphenyl-1,10-phenanthroline) and hydrolyzing the ester to the diacid as described in the following procedure.

A mixture of 0.6 g (1.06 mmol) **II** in 2.6 mL concentrated sulfuric acid was stirred for 2 h at 80°C in an oil bath (fume hood). The mixture was then cooled in an ice bath and 6.3 mL of dry methanol was added slowly with stirring. The mixture was heated for 1 h at 80°C with stirring. After cooling to room temperature, 75 mL of dichloromethane was added. The organic layer was washed twice with cold saturated aqueous $NaHCO_3$. Carbon dioxide evolved vigorously during the first washing. The organic layer was washed twice with saturated aqueous NaCl, dried over anhydrous Na_2SO_4 and concentrated in vacuum to produce a light yellow solid. The compound was recrystallized from hot methanol-ethyl acetate-dichloromethane. The yield of purified product was 0.22 g (47%). Mp 286 to 290°C. IR KBr disk 2800 to 3100, 1750, 1715, 1590, 1550, 1490, 1450, 1380, 1250, 1130, 770, and 700 cm^{-1}. ^1H NMR in $CDCl_3$ δ 4.16 (s, 6H), 7.55 (m, 10H), 8.02 (s, 2H), 8.46 (s, 2H). Analysis calculated for $C_{28}H_{20}N_2O_4$: 74.99% C, 4.49% H, 6.25% N. Found 74.95% C, 4.77% H, 6.33% N.

The disodium carboxylate salt of **III** was prepared by stirring a suspension of the dimethyl ester in 25 mL aqueous NaOH, 1 mol/L, and 25 mL methanol at 60°C for 3 h. The insoluble white solid was collected by vacuum filtration and dried in vacuum. Mp > 300°C. IR KBr disk 2900 to 3600, 1620, 1550, 1485, 1450, 1400, 1370, 1330, 800, and 700 cm^{-1}.

The pure diacid **III** was prepared by stirring a suspension of the disodium salt in 50 mL 2 M HCl for 3 days. The insoluble light yellow solid **III** was collected by vacuum filtration and dried in vacuum. The yield was 0.92 g. Mp 204 to 210°C. Thin layer chromatography on a reverse phase C_{18} plate reveals only one product. IR KBr disk 2800 to 3600, 2500 (shoulder), 1740, 1620, 1590, 1550, 1490, 1450, 1495, 1360, 1210–1310, 1130, 770, 700, and 610 cm^{-1}. ^1H NMR in deuterated dimethylsulfoxide $(CD_3)_2SO$ δ 7.63 (m, 10H), 7.99 (s, 2H), 8.25 (s, 2H). Analysis calculated for $C_{26}H_{18}N_2O_5$ (monohydrate): 71.23% C, 4.14% H, 6.39% N. Found 71.73% C, 4.49% H, 6.29% N.

4,7-Bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) (IV)

4,7-Diphenyl-1,10-phenanthroline-2,9-dicarboxylic acid (**III**) (0.42 g, 1.0 mmol) was added in small portions to 2.1 mL of continuously stirred cold 97% chlorosulfonic acid (BDH). The mixture was then stirred for 4 h in a 80°C oil bath. After cooling to room temperature, the mixture was added *cautiously* (fume hood) to 80 mL stirred ice water that was cooled externally by a large ice bath. The light yellow product precipitated immediately. The product was quickly collected on a sintered glass funnel by vacuum filtration, washed with cold distilled water, and dried in vacuum (0.1 torr) for 12 h. The product was stored under argon in small vials in a dessicated jar at −20°C. The yield was 0.58 g (94%). Only one spot was observed in thin layer chromatography of hydrolyzed BCPDA on reverse phase C_{18} plates with 1:1 methanol-nitromethane as eluting solvent. Mp > 300°C. IR KBr disk 2800 to 3600, 2500 (shoulder), 1730, 1620, 1595, 1510, 1480, 1450, 1310, 1220, 1180, 1030, 895, 840, and 695 cm^{-1}. The NMR spectrum indicates that the product is a mixture of isomers with varying positions of the sulfonyl chloride groups on the 4,7-phenyl rings. ^1H NMR in $(CD_3)_2SO$ δ 7.61 to 7.67 (m, 4H), 7.83 to 7.87 (m, 4H), 8.02 to 8.13 (m, 2H), 8.30 to 8.33 (m, 2H). Analysis Calculated for $C_{26}H_{14}Cl_2N_2S_2O_8$: 50.58% C, 2.28% H, 4.54% N, 11.48% Cl, 10.38% S. Found 50.27% C, 2.39% H, 4.26% N, 11.67% Cl, 10.51% S.

Procedure for protein labelling with BCPDA

Bovine serum albumin (BSA) was dissolved in carbonate buffer, 100 mmol/L, pH 9.2 at a concentration of 5 g/L. A freshly prepared BCPDA solution in ethanol was then added in four portions over a 2-min period, under continuous vortexing at room temperature and the mixture was incubated for 30 min. The volume of ethanol added was less than 10% of the total reaction volume and the molar excess of BCPDA was five-fold with respect to the total lysyl amino group concentration of BSA (59 NH_2 groups per BSA molecule (14)).

The labelled protein was then separated from unreacted hydrolyzed BCPDA by applying the reaction mixture to a Sephadex G-50 column (1.0 × 17 cm) and eluting with NH_4HCO_3, 50 mmol/L, pH 8.0. The absorbance was monitored at 280 nm (Figure 2). The labelled protein was stored in a dark bottle at 4°C. For spectroscopic studies, buffer salts were removed by lyophilization.

Spectroscopic studies

Spectroscopic studies were performed on a SPEX Fluorolog 212 photon-counting fluorometer with 450 W

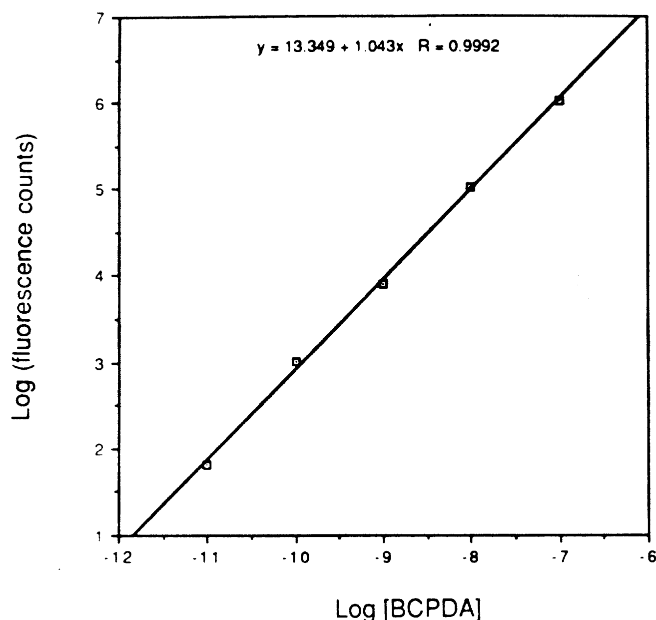

Figure 2—Log fluorescence counts vs log streptavidin-bound BCPDA concentration in the presence of excess Eu^{3+}(1 μmol/L). The concentration on the x-axis refers to that of BCPDA. The molar ratio of BCPDA:streptavidin is 15. Buffer solution used was 0.1 mol/L Tris of pH 7.80. Measurements were carried out on the 1230 ARCUS fluorometer.

Xenon lamp as source. The instrument is equipped with monochromators for both excitation and emission. Lifetime measurements were carried out using the phosphorimeter attachment that uses a Xenon flash lamp for excitation at a repetition rate of 1 kHz (pulse width < 50 μs). Spectra were recorded with slits adjusted to give half band widths of 14 nm (excitation) and 0.2 nm (emission). For excitation spectra, the emission wavelength was 613 nm; for emission spectra, the excitation wavelength was 337 nm. Excitation spectra were corrected for lamp output variation using a rhodamine B quantum counter. Spectra were corrected for variation of detection sensitivity. A Schott KV-418 filter passing only wavelengths greater than 418 nm was placed in the emission path to prevent transmission of second harmonic wavelengths. Lifetime measurements were performed with excitation and emission wavelengths of 337 and 613 nm, respectively (14 nm half-bandwidth for both). Absorption spectra were recorded on an HP8450A diode array spectrophotometer.

For spectroscopic studies labelled protein stock solutions were 0.1 mmol/L in BCPDA, in water. Eu^{3+} stock solution was 1 mmol/L dissolved in 10 mmol/L HCl (prepared from $EuCl_3$ hexahydrate (99.99%, Aldrich)). For the measurements, all samples were 1 μmol/L in BCPDA conjugated to BSA and 20 μmol/L in Eu^{3+}, unless otherwise stated.

Time-resolved fluorometric measurements of solutions were performed on the 1230 Arcus fluorometer (LKB Wallac, Turku, Finland).

Results and discussion

The chelating site of the new fluorescent label BCPDA (Figure 1, **IV**) consists of two carboxyl groups and two heteroaromatic nitrogens. The binding site combines groups that are present in the strong chelators of the polycarboxylic acid type (e.g., EDTA), and the phenanthroline molecule (15). Pyridine-2,6-dicarboxylate is a known chelator with a similar metal-binding site (16). In practice, we have verified that provided the BCPDA labelled protein is immobilized on a solid-surface (either by passive adsorption or through an immunoreaction) in the presence of excess Eu^{3+}, extensive washing with a 0.05% Tween 20 saline solution does not remove Eu^{3+} from the label. This is of fundamental importance since it makes possible the use of the label in immunoassay systems with the final measurement performed directly on the solid-phase. This type of measurement proved to be unsuccessful in previous systems, which used labels of the diketone type (6).

BCPDA can be hydrolyzed by heating a water suspension at 50°C for about 1 h. The water soluble hydrolyzed molecule binds Eu^{3+} as well. The logarithmic plot of fluorescence counts versus concentration of BCPDA bound to streptavidin in the presence of excess Eu^{3+} (1 μmol/L) is shown in Figure 2. The linear dynamic range is at least four orders of magnitude. Under such conditions only the 1:1 Eu^{3+}: BCPDA complex forms. In the presence of excess BCPDA, complexes of Eu^{3+} with more than one BCPDA are also formed. The limit of sensitivity of measuring BCPDA in excess Eu^{3+}, is about the same for both hydrolyzed and protein bound BCPDA (10 pmol/L).

BCPDA has two sulfonyl chloride groups through which it can be covalently attached to proteins. Other fluorescent labelling reagents utilize the same functional group for protein conjugation (17,18). The SO_2Cl groups react primarily with amino groups of the protein to be labelled, under relatively mild conditions. For immunoassay applications, the new label can be used in a number of ways. A universal analytical detection system has been described recently (19,20) and includes BCPDA labelled streptavidin and biotinylated antibodies as complementary reagents for the assessment of alpha-fetoprotein (20). A number of other analytes can also be measured by using the same detection system.

In the case of the typical labelling procedure described, the unreacted hydrolyzed BCPDA is separated easily by using gel filtration (Figure 3). The labelled protein preparation was characterized by measuring the absorbance at 325 nm of the protein-containing fractions. At this wavelength only BCPDA absorbs (extinction coefficient $\epsilon = 1.52 \times 10^4 \ mol^{-1} \cdot L \cdot cm^{-1}$). If it is assumed that the value of ϵ is the same for the covalently bound and the hydrolyzed BCPDA molecule used as a standard and that all protein applied elutes from the column quantitatively, a molar ratio of ~40 BCPDA:BSA can be calculated for the labelled product. Different molar ratios can be obtained by varying the excess of BCPDA used and the protein concentration.

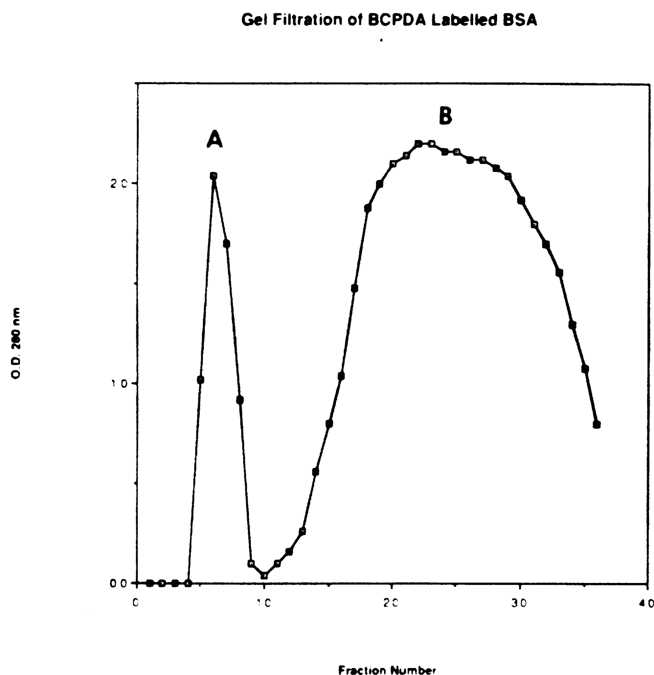

Figure 3—Separation of labelled BSA (peak A) from unreacted hydrolyzed BCPDA (peak B) by gel filtration chromatography. Mobile phase NH_4HCO_3, 50 mmol/L, pH 8.0; flow-rate 0.5 mL/min. Fractions collected were 1 mL each.

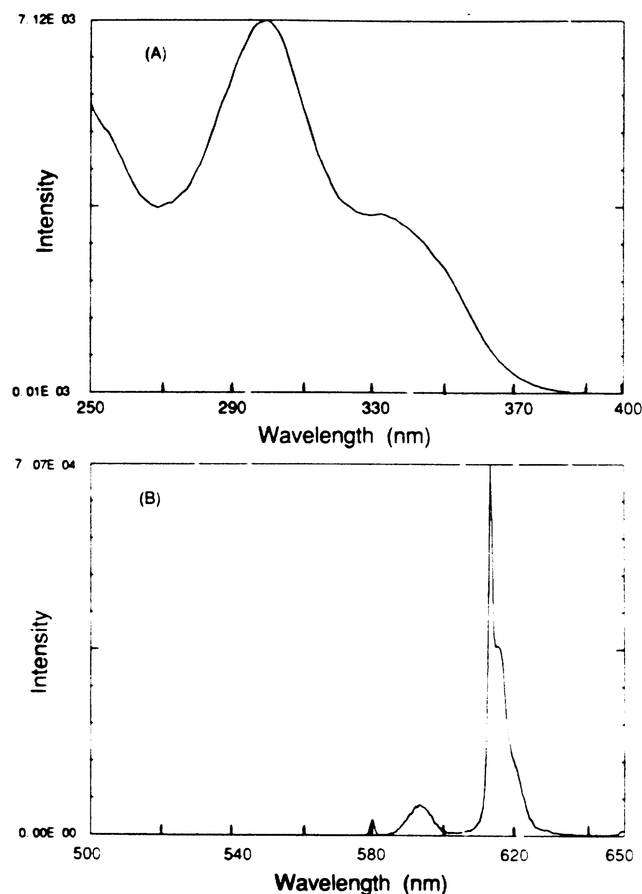

Figure 4—Excitation (A) and emission (B) spectra of BCPDA-labelled BSA in the presence of excess Eu^{3+} (20 μmol/L). The concentration of BCPDA is about 1 μmol/L. The molar ratio of BCPDA/BSA was 13. Buffer was carbonate, 0.1 mol/L, pH 9.5. (Details given in Spectroscopic studies).

Other proteins can also be labelled with similar procedures.

BCPDA bears two sulfonyl chloride groups and this may lead to the formation of polymers during labelling because of protein-protein crosslinking. However, working with BSA as the model molecule, we have observed by gel filtration chromatography that polymer formation did not occur, the only labelled product being BSA $(BCPDA)_n$ where $1 < n < 40$ (data not shown).

For the preliminary spectroscopic studies we have used two labelled BSA preparations containing either 13:1 or 39:1 BCPDA:BSA. In Figure 4 the corrected excitation and emission spectra of the 13:1 conjugate are presented. The spectra were similar to those of the 39:1 conjugate. The spectra presented were taken at pH 9.5 in the presence of 0.1 mol/L carbonate buffer. There is a sharp emission band at 613 nm that is due to the $^5D_0 \rightarrow {}^7F_2$ transition. The emission spectrum is affected by the pH and the nature of the buffering species present. This is presumably due to the fact that BCPDA bears four ionizable groups and at different pH values there are different ionic species present. Also, it is possible that in the presence of buffering agents, mixed complexes containing Eu^{3+}, BCPDA, and buffering species are also formed. These possibilities are under investigation.

The fluorescence lifetime τ of the BCPDA · Eu^{3+} complexes was studied by the pulse technique. After pulsed excitation, the fluorescence decay of a single population of fluors follows first-order kinetics. The plot of ln $I(t)$ versus time, where $I(t)$ is the fluorescence intensity at any time t, is a straight line with a slope of $^{-1}/\tau$ (19). The fluorescence lifetime was in the 0.44 to 0.76 ms range in either unbuffered or in 0.1 mol/L buffered (carbonate pH 9.5, acetate pH 5.8, phosphate pH 7.9, Tris pH 7.9) solutions. Such lifetimes are typical of fluorescent Eu^{3+} complexes and are suitable for time-resolved fluoroimmunoassay applications. For solution measurements, fluorescence was optimal in the pH range 7 to 10 buffered by Tris, borate or carbonate. Phosphate strongly quenches the fluorescence, while dissolved oxygen does not noticeably affect it.

In conclusion, we have presented synthetic procedures for a new chelator that forms stable and highly fluorescent complexes with Eu^{3+}. The chelator can be incorporated covalently into proteins with relatively easy procedures. The lifetimes of the resulting complexes with Eu^{3+} are very long and thus suitable for time-resolved fluorometric measurements. This label has been used in the development of new immunofluorometric and fluoroimmunoassay procedures for analytes of biological importance. The principles of such procedures are described elsewhere (19). An immunofluorometric assay of alpha-fetoprotein has already been described (20). Assays for other analytes of biological importance are under development.

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