FLUORESCENT LABELING OF PROTEINS. A NEW METHODOLOGY

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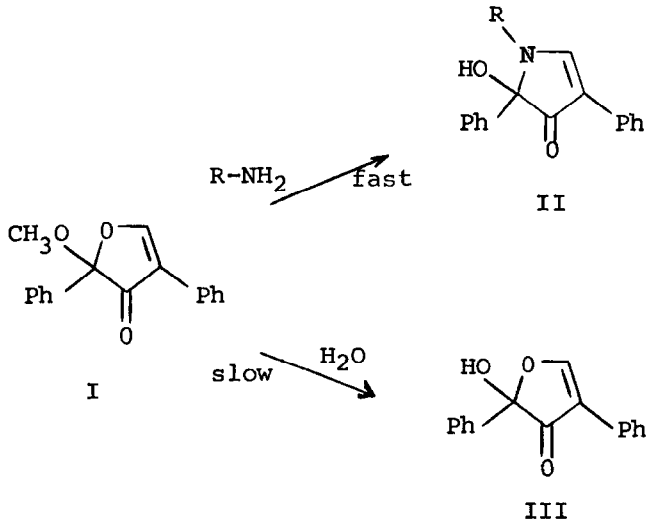
Summary: A new reagent, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) has been utilized for the fluorescent labeling of proteins. MDPF, which is nonfluorescent, reacts with primary amino groups to form fluorescent N-substituted 3,5-diphenyl-5-hydroxy-2-pyrrolin-4-ones. Antibodies labeled with MDPF afford intense immunofluorescent staining.

Recent studies in our laboratories have uncovered the unique propensity of 2-oxysubstituted 3(2H)-furanones to produce highly fluorescent substances upon reaction with primary amines (1). This finding provided the basis for the development of fluorescamine (1,2), a new, sensitive reagent for the fluorometric assay of biologically important amines, including amino acids (3,4), peptides, and proteins (5) It has now been found that another compound of this class, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) I (1), is eminently suited for the fluorescent labeling of proteins.

The major interest in fluorescent protein conjugates stems from the diagnostic usefulness of labeled antibodies in the identification of microorganisms and other antigens (6). Since the pioneering work of A. Coons et al. (7), the preferred labeling technique has relied upon functionalized fluorophors, such as fluorescein isothiocyanate, which will covalently bind to proteins. In spite of its long

history, this methodology has remained rather cumbersome. A particular disadvantage is the need to rigorously purify the resulting conjugates from unreacted fluorescent reagent, which can cause nonspecific staining in immunoassays.

In comparison, MDPF (I) possesses several features which make it distinctly superior to labeling agents currently in use. Most importantly, MDPF itself is nonfluorescent. Fluorophoric 3,5-diphenyl-5-hydroxy-2-pyrrolin-4-one moieties (II) are formed when MDPF reacts with primary amino groups of the proteins ($R-NH_2$) to be labeled. Under tl labeling conditions, excess MDPF is concomitantly hydrolyzed (at a slower rate) to the nonfluorogenic hydroxyfuranone III, thus obviating the need for additional purification (8).



MDPF is only sparingly soluble in water. Efficient reaction with aqueous protein solutions is achieved by adding the reagent in a water miscible organic solvent. Moreover, MDPF lends itself particularly well to the technique of Rinderknecht (9), wherein it is applied as an adsorbate on diatomaceous earth.

The rate of the fluorogenic reaction is strongly pH-dependent as shown in Fig. 1. At pH 9.5 the reaction is complete within 2

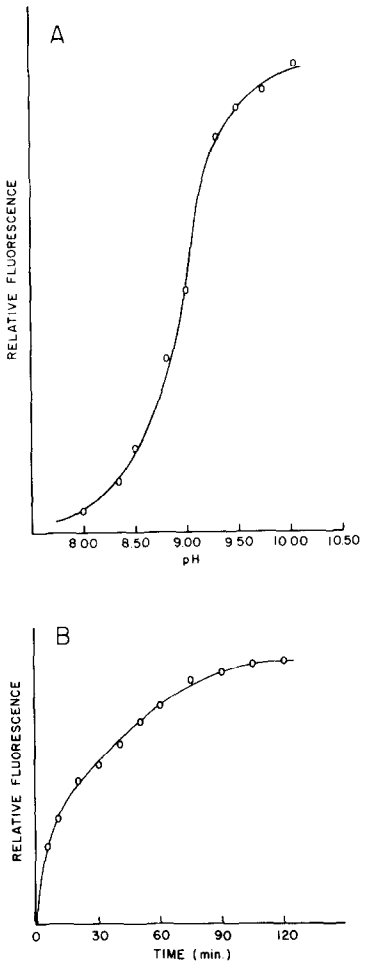


Fig. 1 (A) pH-Dependence of the fluorogenic reaction of MDPF with proteins: 5 ml aliquots of 1% w/v horse γ-globulin in the appropriate 0.05M borate buffer were stirred for 15 minutes with 20 mg of 2% MDPF on Celite. After removal of excess reagent by centrifugation, the fluorescence of the supernatant was measured.
(B) Rate of the reaction of MDPF with proteins at pH 9.5: 20 ml of 1.5% w/v bovine γ-globulin in 0.05M borate buffered saline, pH 9.5, was stirred with 200 mg 2% MDPF on Celite. Samples were taken at time intervals, neutralized, centrifuged, and their fluorescence was measured.

hours. On the other hand, the fluorescence spectra of the resulting conjugates are independent of the pH over a wide range. A characteristic spectrum is given in Fig. 2.

The following labeling procedures are applicable to proteins in general and antisera (or globulin fractions thereof) in particular.

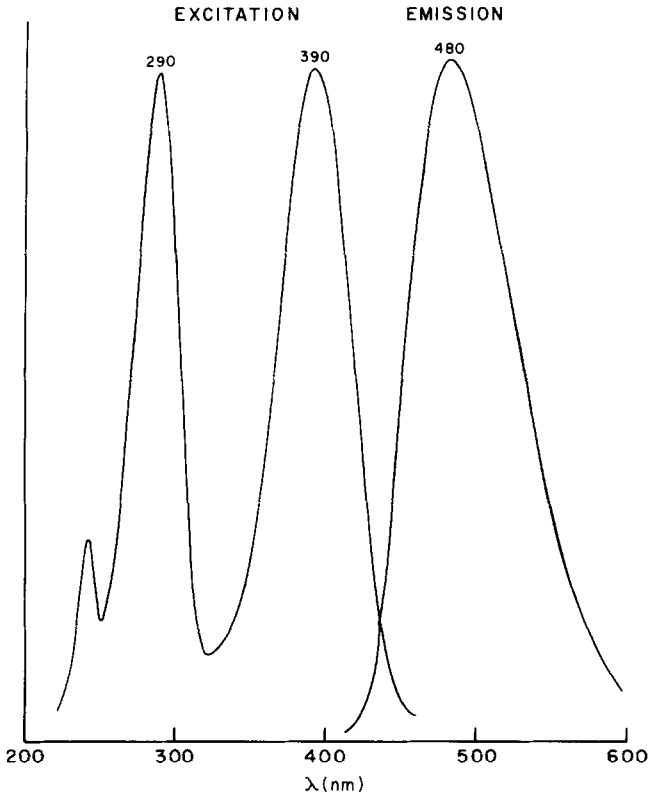


Fig. 2 Fluorescence excitation and emission spectra of a conjugate prepared from horse γ-globulin with MDPF.

(A) Using a reagent solution: 5 ml. of a solution containing 30-50 mg/ml protein is diluted with 5 ml of 0.1M borate buffered saline, pH 9.5. The mixture is cooled in ice/water and 2-4 mg of MDPF, dissolved in 1 ml of acetone is added over a period of 60 minutes. After addition is complete, stirring is continued for another 60 minutes. The resulting fluorescent solution is dialyzed for 4 hours at 4° against a hundred-fold volume of 0.025M phosphate buffered saline, pH 6.85, followed by a second dialysis against fresh buffer for an additional 12 hours.

(B) Using a reagent adsorbate: To 10 g of Celite (10) (which had been washed with methanol and chloroform and then dried in vacuo at 100°) is added a solution of 200 mg MDPF in 75 ml of methylene

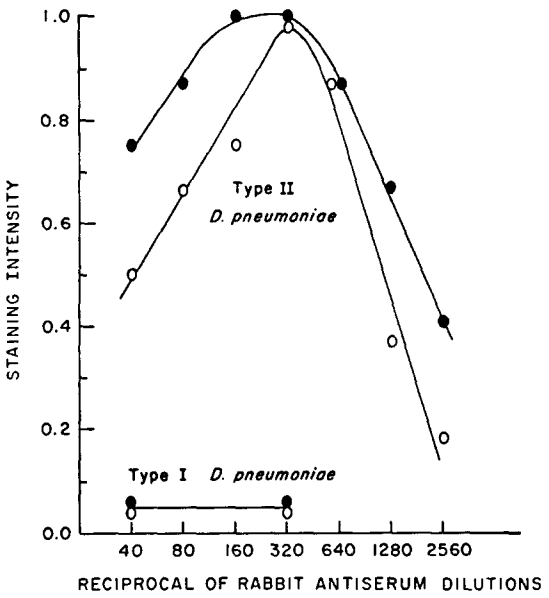


Fig. 3 Indirect immunofluorescent staining of D. pneumoniae. The bacteria were first exposed to serial dilutions of anti-Type II D.pneumoniae rabbit antiserum and then to anti-rabbit globulin goat globulin (final dilution 1:10), labeled by method A (-o-) or method B (-●-). Staining intensities were estimated by fluorescence microscopy.

chloride. The resulting suspension is evaporated under reduced pressure at 25° by means of a rotary evaporator. The adsorbate is then dried for 24 hours under high vacuum at 25°. One ml of a solution containing 30-50 mg protein is diluted with 1 ml of 0.1M borate buffered saline, pH 9.5. To this mixture is added in small portions, at room temperature with magnetic stirring, 20-40 mg of the above reagent adsorbate containing 2% MDPF. After completed addition, stirring is continued at room temperature for another 60 minutes. The reaction mixture is then neutralized (pH 6.9-7.0) by careful addition of 1N hydrochloric acid, and the Celite is removed by centrifugation. The supernatant, when intended for immunological staining, can be used without further purification.

The ultraviolet absorption spectra of a variety of low

molecular weight pyrrolinones of the structural type II have been determined previously (11). On the basis of their molecular extinction coefficients (ϵ ~6,500 at 385 nm), it is estimated that the molar fluorophor/protein ratio in globulin conjugates prepared by these procedures is between five and eight.

The applicability of both methods for fluorescent labeling of antibodies is illustrated by the following experiments.

Indirect staining technique: Saline suspensions of Type I and Type II Diplococcus pneumoniae prepared from overnight broth cultures were applied to standard microscope slides and allowed to air dry. Rabbit antiserum to Type II D. pneumoniae was serially diluted in 0.01M phosphate buffered saline, pH 7.2 (PBS) and each dilution added to separate slides to cover the bacteria for 20 minutes at ambient temperature. Excess antiserum was removed by three washes with PBS and the slides were blotted dry. A commercial globulin fraction (12)(containing 36 mg protein/ml), prepared from anti-rabbit globulin goat antiserum was labeled with MDPF by both the A and B methods. The labeled antibody preparations were diluted with PBS. The appropriate dilution was added to cover the bacteria treated with the rabbit antiserum and allowed to react for 20 minutes at ambient temperature. Excess labeled goat globulin was removed by washing three times with deionized water and the slides were blotted dry.

A fluorescence microscope with a dark-field condenser was used to determine staining. The exciter filter was Corning 5840, 2 mm thick and the barrier filter Schott GG-9. Staining intensity was judged on the basis of an arbitrary scale from 0 to a maximum of 1.0.

Shown in Fig. 3 are the staining results when the rabbit antiserum to Type II D.pneumoniae was serially diluted and the MDPF

labeled anti-rabbit globulin goat immunoglobulin was held at a fixed dilution. Staining intensity was generally better with the material labeled by Method B. However, at 1:320 and 1:640 dilutions of rabbit antiserum the fluorescence observed using globulin labeled by both methods was equivalent. Only a slight background fluorescence was seen when Type I D. pneumoniae was used as the antigen, or with rabbit serum from unimmunized animals. Holding the anti-Type II D.pneumoniae antiserum at a fixed dilution (1:320) and serially diluting two-fold the MDPF labeled immunoglobulin (labeled by Method B), excellent fluorescence was observed out to a dilution of 1:40 and satisfactory staining at a dilution of 1:80.

Direct staining technique: Rabbit antiserum to Type II D.pneumoniae was labeled by method B and applied to slides containing air dried Type I or II pneumococci for 10-30 minutes at room temperature. Minimal background fluorescence was observed when Type I organisms were the antigen, while intense staining occurred with Type II pneumococci. The titer of the MDPF labeled antiserum as measured by the Quellung reaction was not significantly different from that obtained with unlabeled serum. The fluorescent staining capacity of the labeled antiserum was unaltered after 3 months at 4°.

While direct comparisons of staining are difficult, the same lot of commercial anti-rabbit globulin goat immunoglobulins labeled with fluorescein isothiocyanate (FITC) by the manufacturer (12) was also tested by the indirect technique in the pneumococcal system. Staining intensity with MDPF labeled globulin was at least equivalent to that obtained with FITC labeled material.

It is apparent that MDPF is a unique reagent for the introduction of stable fluorescent tags into proteins. The utility of MDPF in the field of immunofluorescence is evident. Other biological applications may deserve to be explored.

References and Notes

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(8) Fluorescamine gives analogous reactions (1). While fluorescamine is superior to MDPF for fluorometric assays, the fluorophors arising from its reaction with primary amines are only stable under optimal assay conditions (pH 8-9). In contrast, MDPF affords fluorophors which are extremely stable over a wide pH-range.
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