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A STUDY OF POTENTIAL LABELS FOR FLUORESCENT DERIVATISATION REACTIONS WITH PROTEINS

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The suitability of the newly synthesised polycylic aromatic dialdehydes as labels to form fluorescent derivatives has been examined. The fluorescence properties of the derivatives such as the reaction time, stability, antigen-antibody reaction and inner filter effect have been investigated. The change in fluorescence properties of the labelled protein forms the basis of a fluorescence method for the determination of biotin.

Key words: Fluorescent derivatives, Potential labels, Polycyclic aromatic dialdehydes.

Introduction

Fluorescence methods have been very useful to study and investigate peptides [1] and proteins including rare proteins for gene cloning in genetic manipulation, to measure amino acid content of tissue, tissue extracts and physiological fluids [2] (e.g. blood and urine) and to analyse biogenic amines [3].

Fluorescence detection is sensitive [4] and has a number of merits, the most important of these being selectivity [5]. Most proteins absorb in the region 270-300nm and show some degree of fluorescence due to mainly aromatic amino acid residues, such as tyrosine and tryptophan. However, other fluorescent structures bound to the protein other than by peptide linkages may also contribute to fluorescence. Proteins can also be combined chemically with the fluorigenic reagents to yield fluorescent protein derivatives. In the combined form the fluorescence properties of the proteins change considerably and this provides information about size and shape of macromolecules.

In the present investigation an effort has been made to investigate the fluorescence properties such as intensity, the excitation and fluorescence spectra, stability and ease of formation using new polyaromatic dialdehyde labels, synthesised in the author's laboratory according to Cook, *et al.* [6]. These labels are naphthalene-2,3-dicarboxaldehyde (NDA),1-phenylnaphthalene-2,3-dicarboxaldehyde (ϕNDA) and anthracene-2, 3-dicarboxaldehyde (ADA). Of these three fluorescent labels, ϕNDA and ADA are new and for the first time being used for labelling proteins, and hopefully these will not only be useful for information on peptides and proteins but will also be of much interest for other areas, e.g., pharmacology, medicine, neurobiology, diagnosis, quality control and environment science.

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Experimental

Chemicals. Cytochrome C (Sigma), myoglobin (Sigma), ATP, adenosine 5'-triphosphate disodium salt (Sigma), albumin, human serum (Calbiochem), human albumin economy (DAKO-immunoglobulins), d-biotin, vit.H (Aldrich) were used. All other reagents and buffer salts used were of AnalaR or equivalent grade.

Apparatus. Fluorescence emissions were made on Perkin-Elmer MPF-44B (with bandpass 10 nm) equipped with differential corrected spectra unit (DSCU-2) and a R928 photomultiplier. Quantum yields were measured using Perkin-Elmer LS-5 luminescence spectrometer. This instruments was fitted with a R928 red sensitive photomultiplier and data were recorded using PECLS II software and a Perkin-Elmer model 3600 data station, which employs a Xenon-pulsed (10 μs half-width, 50 Hz) excitation source.

FLUORESCENT LABELLING OF PROTEINS AND THEIR PURIFICATION.

Fluorescent labelling of proteins. Procedure (Lables: NDA, ϕNDA and ADA). A dilute solution of the sample (0.2-0.3ml) was treated with 1ml of 10.9 mM B-cyclodextrin (BCD). To this were then added 0.6 ml of borax-sodium hydroxide buffer (pH 10) and 0.1ml aqueous thiol solution (0.1% v/v), followed by the addition of a suitable fluorogenic reagent (label) in slight excess over the sample. The final volume was then made up to 5 ml with distilled water.

For inner filter effect studies, absorbers such as cytochrome C (0.006mM), myoglobin (2mg%) and ATP (2mg%) were used unless stated otherwise. For biotin determination, avidin (4mg%) was labelled with NDA according to the procedure described above. The fluorescent labelled avidin was then treated with a gradually increasing amount of d-biotin (10^{-3}M) and fluorescence intensities were measured.

This and all other fluorescence studies were taken at room temperature.

Purification of fluorescent derivatives of proteins. A Sephadex G-25 column was first equilibrated with 25 ml of borate buffer (pH 10.0). 2.5ml of the fluorescent derivative was then loaded at the top of the column and the column eluted with borate buffer (3.5-4.5 ml) in an aliquot of 1 ml of the buffer for each collection.

Results and Discussion

Reaction time. The success of fluorescent labelling method is due mainly to the case of reaction and the time required to give a fluorescent product of measurable and reproducible intensity. In this study the fluorogenic reagents NDA, ϕNDA and ADA have been employed to label proteins in order to determine the reaction time for successful labelling. Fig. 1 is a plot of fluorescence intensity *vs.* time and shows the fluorescence intensities of these derivatives at their maximum within 3-9 min. The reaction times were 3 min for ϕNDA-antibody, 6 min for NDA-antibody and 8 min for ADA-antibody derivatives. The reaction time with proteins seemed to be comparable to that of the simple amino acid reactions.

Fluorescence emission intensities were measured at 520, 520 and 640 nm for NDA, ϕNDA and ADA derivatives respectively (Ex = 462, 462 and 470 nm).

Stability. The NDA, ϕNDA and ADA fluorescent protein derivatives have been studied to determine their stability. The fluorescence intensities of the derivatives were measured at regular time intervals. Fig. 2 indicated that the fluorescence decay of the NDA, ϕNDA and ADA-antibody was found to extend over a period of 10, 4 and 8 hr respectively. The stability of protein derivatives in comparison with simple amino acid derivatives in a separate study was found to be a little less the fluorescence intensity was reduced to its minimum in less than half of the time of simple amino acid derivatives. The increase in instability may be due to unfolding of the labelled protein leading to conformational alteration of protein macromolecule.

Inner filter effects. An insignificant decrease in the fluorescence intensity of fluorescent species may result by the addition of an absorbing molecule to it. The absorption of light by the molecule at the wavelength of emission is called secondary absorption and this inner effect needs to be compensated for before carrying out a quantitative analysis of the results. The present study attempts to investigate such an effect on the NDA- human albumin (NDA-HAlb) fluorophores using cytochrome C, myoglobin and ATP as absorbers.

In fig. 3, the inner filter effect resulting from the addition of cytochrome C, myoglobin and ATP to the NDA-HAlb fluorophores is shown by the curves. These curves represented the uncorrected fluorescence which may be corrected by applying the equation of Geren and Millett [7]

$$F_c = F_o \times \text{antilog}_e\,(A_1 + A_2/2)$$

where F_c is the corrected fluorescence, F_o is the observed fluorescence corrected for dilution and A_1 and A_2 are the primary and secondary absorbencies respectively.

The derivatives of HAlb formed with other fluorogenic reagents ϕNDA and ADA showed a quenching of fluorescence (Fig. 3) by cytochrome C.

For an addition of 0.4ml of absorbers, the fluorescence was quenched roughly by 70, 24 and 58% for addition of

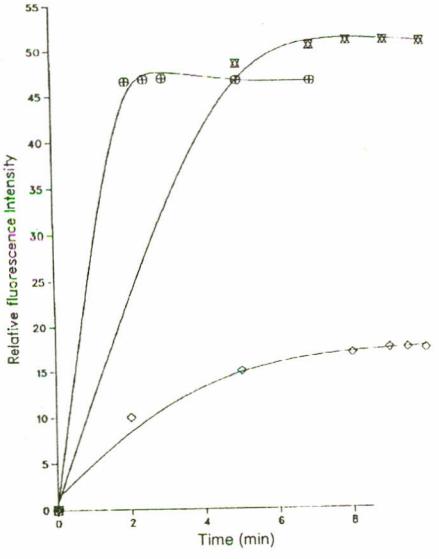

Fig. 1. Reaction time, NDA, ϕNDA and ADA-antibody derivatives.
Legend: XX = ϕ-NDA-antibody ⊕ = NDA-antibody ◇ = ADA-antibody

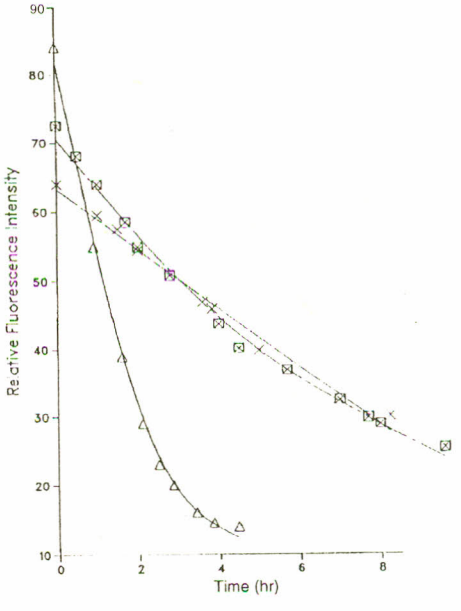

Fig. 2. Stability curves of NDA, ϕNDA and ADA-antibody.
Legend: Δ = ϕ-NDA-antibody. x = ADA-antibody. ⊠ = NDA-antibody

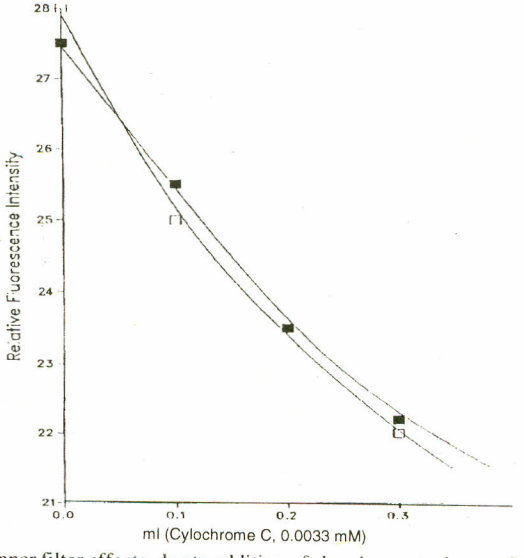

Fig. 3. Inner filter effects, due to addition of absorber, cytochrome C, to ϕNDA and ADA-human albumin fluorophores.
Legend: ■ = ϕ-NDA-HAIb. □ = ADA-HAIb.

cytochrome C, myoglobin and ATP respectively (Fig.3). However, the inner filter effect in the case of ϕNDA and ADA by cytochrome C was found to be small.

The selection of these absorbers for the study was due to the fact that all these are non-fluorescent. Cytochrome C and myoglobin are note worthy in that inspite of these being proteins they are non-fluorescent due to the quenching effect of the heme group present [8].

d-Biotin determination. Biotin- induced changes in the fluorescence [9] properties of avidin form the basis of biotin determination. Fig. 4 showed that the fluorescence of NDA - labelled avidin was quenched upon the addition of biotin. The fluorescence was reduced by half on the addition of 4×10^{-6} mol/ml biotin to the system. The relatively large change in NDA-labelled avidin fluorescence was unexpected since biotin on binding brings no gross change [10] in the structure of avidin. However, a reduction in the intensity occurs upto 40% occurs when biotin binds to densyl avidin [10]. The biotin - induced decrease in fluorescence of NDA groups associated to some sites on avidin may be explained on the basis of local displacement of the NDA groups into a more aqueous environment where they have greater rotational freedom and less interaction with the protein structure, as described applicable to densyl groups [11].

The results indicate that NDA label can be employed for a sensitive detection of d-biotin (vit. H) on a quantitative basis in a complex sample.

Conclusion

The NDA, ϕNDA and ADA labels form stable fluorescent derivatives with proteins. The wagelength of emission

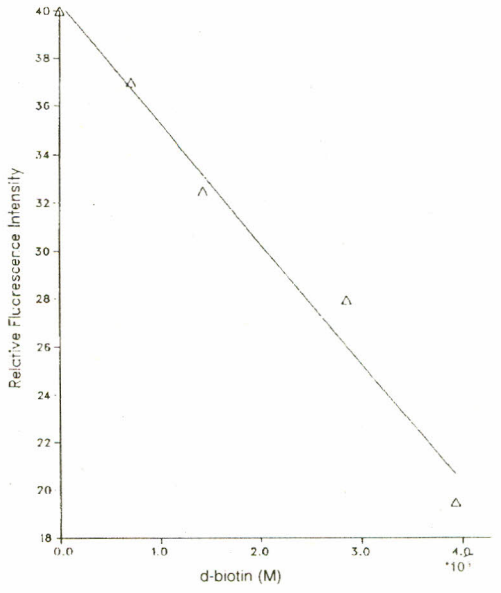

Fig. 4. Curve showing the effect of biotin on the fluorescence intensity of NDA-labelled avidin.

(520nm) of their derivatives is larger than OPA-derivative Em = 450nm. Biological samples may, thus, safely be handled without much background interference in that region. Besides, these fluorogenic reagents may enter into a modified form of reaction by replacing thiol with sodium cyanide in the fluorescent derivatisation reaction.

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