effectiveness of the α-tocopherylquinone which indicates that its site of function is closely related to the transfer of electrons to cytochrome *c* through photosynthetic pyridine nucleotide reductase. Added β- and γ-tocopherylquinone can apparently substitute for α-tocopherylquinone at this site.

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[1] M. D. HENNINGER, R. A. DILLEY AND F. L. CRANE, *Biochem. Biophys. Res. Commun.*, 10 (1963) 237.
[2] R. A. DILLEY AND F. L. CRANE, *Biochim. Biophys. Acta*, 75 (1963) 142.
[3] D. L. KEISTER, A. SAN PIETRO AND F. E. STOLZENBACK, *Arch. Biochem. Biophys.*, 98 (1962) 235.

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Appearance of fluorescence on treatment of histidine residues with *N*-bromosuccinimide

It has been observed that treatment of proteins with *N*-bromosuccinimide (NBS) in phosphate buffer, gives rise to visible fluorescence. The proteins that were tested in this way include horse liver alcohol dehydrogenase (EC 1.1.1.1), bovine serum albumin, ovalbumin, chymotrypsin (EC 3.4.4.5) and RNAase (EC 2.7.7.16)*.

In an effort to find which amino residues are involved in this phenomenon we followed the reaction between all the amino acids present in the above proteins and NBS fluorometrically. NBS was added to each of the amino acids in various mole ratios (from 1 to 12 moles/mole). The reactions were carried out in 0.1 M phosphate buffer (pH 7.4). Under these conditions visible fluorescence was observed only with histidine and tryptophan. Tyrosine and tryptophan lost their characteristic ultraviolet fluorescence after being reacted with NBS.

The reaction between NBS and histidine has been studied in detail and some of the results are presented here. Two distinct fluorescent products are formed with different fluorescence excitation and emission spectra. The optimum mole ratio of NBS to histidine for fluorescence appearance is different for these two products. One type (emission maximum at 540 mμ) is formed predominantly when four moles NBS are used. The fluorescence appears within 1–2 min. The other type (emission maximum at 460 mμ) predominates when two moles NBS are used and over 20 min elapse before the maximum fluorescence is formed. The fluorescence excitation and emission spectra of these two products are shown in Fig. 1. It is the second of these products (emission maximum near 460 mμ) which is seen when proteins are reacted with NBS.

Abbreviation: NBS, *N*-bromosuccinimide.

* The finding of visible fluorescence with horse liver alcohol dehydrogenase and NBS confirms unpublished results of L. BRAND AND N. O. KAPLAN.

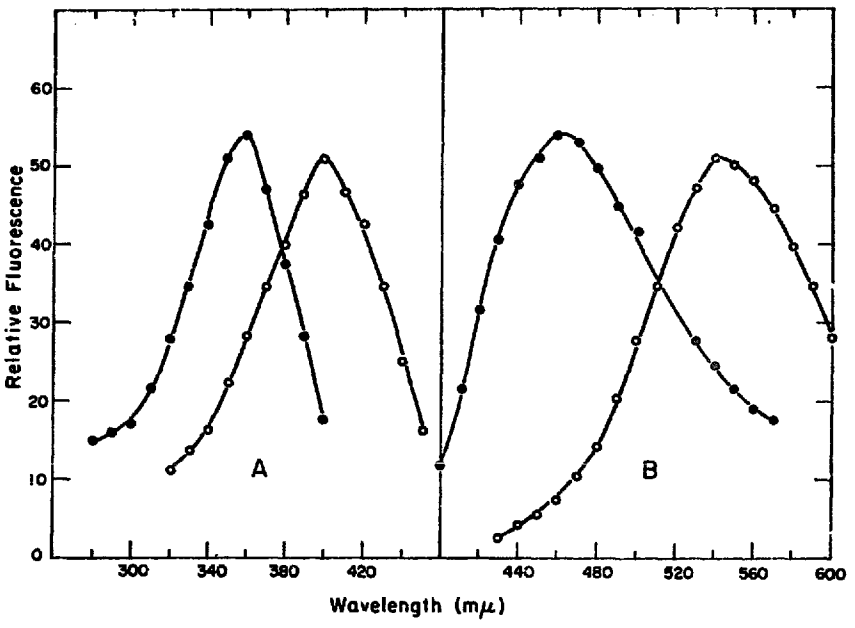

Fig. 1. Fluorescence spectra observed on treatment of histidine with NBS. Excitation spectra (A) were corrected for the non-linearity of the light source[1] and emission spectra (B) were corrected for the non-linear response of the phototube (1P-28) and the non-linear transmission of the grating monochromator[2]. Measurements were made with the Aminco-Keirs spectrophotofluorometer at 21–24°. Reaction mixtures contained 1 μmole of histidine and 2 (●—●) or 4 (○—○) μmoles of NBS in a total volume of 2 ml of 0.1 M phosphate buffer (pH 7.4). The spectra obtained with 4 moles/mole were measured after 2 min and those with 2 moles/mole after 20 min. Fluorescence readings are given in relative numbers and refer to this figure only.

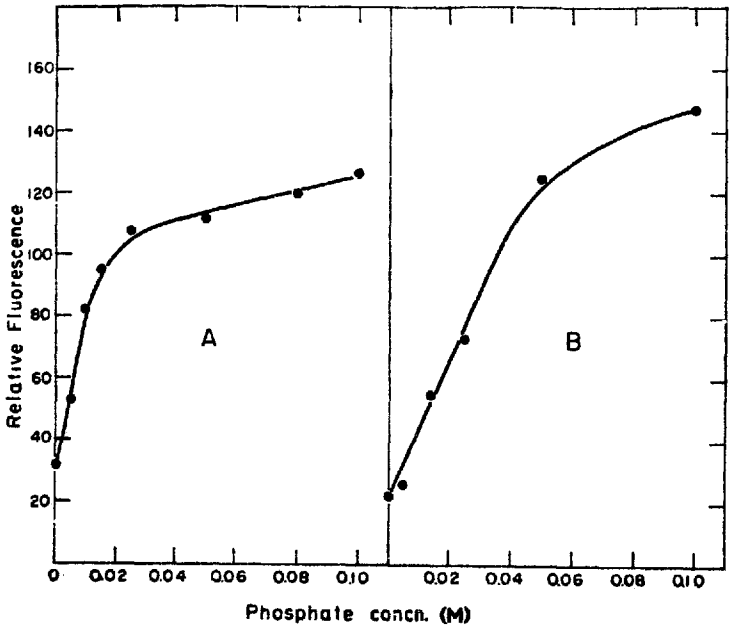

Fig. 2. Effect of phosphate concentration on the amount of fluorescence formed. Curve A was measured after 20 min of reaction time (2 moles NBS/mole histidine; excitation at 365 mμ; emission at 455 mμ) and Curve B after 2 min (4 moles NBS/mole histidine; excitation at 400 mμ; emission at 520 mμ). Reaction mixtures and instrument as described in the legends to Fig. 1. Wavelengths given are uncorrected.

The reaction with histidine leading to both types of fluorescence are pH-dependent with maximum fluorescence appearing at neutral pH. Attempts were made to carry out the reaction in water, 0.1 M Tris (pH 7.4), 0.1 M borate (pH 7.4) as well as in 0.1 M phosphate (pH 7.4). Maximum fluorescence was observed in phosphate buffer. Moreover, when the reaction was performed in various phosphate–borate mixtures keeping the molar-sum constant, the amount of fluorescence formed was a function of the concentration of phosphate (Fig. 2).

The appearance of fluorescence cannot be attributed merely to the imidazole ring. Imidazole itself, histamine, $N_{(\alpha)}$-carbobenzyloxyhistidine and carnosine showed no fluorescence under conditions where histidine does give rise to fluorescence. On the other hand, other models for histidyl residues in proteins such as acetylhistidine amide, $N_{(\alpha)}$-carbobenzyloxyhistidylphenylalanine amide and $N_{(\alpha)}$-carbobenzyloxyhistidylglycine fluoresce when treated with NBS.

The amount of fluorescence formed varies from one histidine model to another (Fig. 3). While acetylhistidine gives no fluorescence, acetylhistidine amide gives more fluorescence than does histidine itself. In other experiments it has been shown that the amount of fluorescence formed from $N_{(\alpha)}$-carbobenzyloxyhistidylglycine is much larger than that of $N_{(\alpha)}$-carbobenzyloxyhistidylphenylalanine amide. These findings indicate that in the case of proteins the amount of fluorescence formed might also depend on structure near the histidines.

In addition to NBS treatment, reaction of proteins or histidine with iodine also leads to fluorescent derivatives.

NBS was recently introduced as a reagent for cleavage of peptide bonds[3–5]. However, under the conditions described above for the formation of fluorescence essentially no cleavage occurs (less than 5 %).

The fluorescent products formed when histidine, peptides containing histidine,

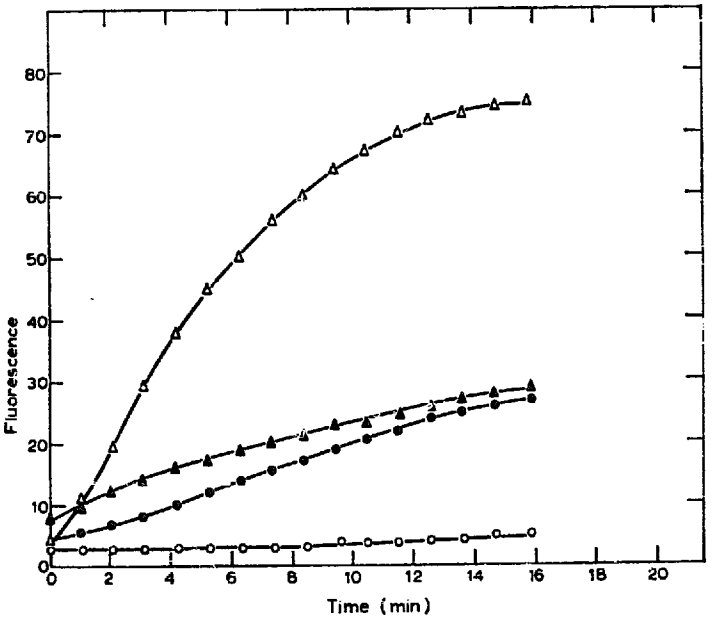

Fig. 3. Relative fluorescence formed on treatment of different histidine models with 2 moles/mole NBS. The following excitation–emission wavelengths (uncorrected) were used: ●—●, histidine, 365–455 mμ; O—O, *N*-acetyl histidine, 365–455 mμ; ▲—▲, histidine amide, 365–455 mμ; Δ—Δ, *N*-acetyl histidine amide, 360–430 mμ. At these wavelengths optimal fluorescence was observed. Reaction mixtures and instrument as described in the legend to Fig. 1.

and proteins are reacted with NBS, or iodine are being further investigated. The preliminary result described here already indicate that the reaction may be influenced by reactive groups near the imidazole ring. The modifications of histidyl residues in proteins to fluorescent derivatives may be of value in studies of active sites of enzymes or other biologically active proteins.

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[1] F. J. W. TEALE AND G. WEBER, *Biochem. J.*, 65 (1957) 476.
[2] C. E. WHITE, M. HO AND E. Q. WEIMER, *Anal. Chem.*, 32 (1960) 438.
[3] A. PATCHORNIK, W. B. LAWSON, E. GROSS AND B. WITKOP, *J. Am. Chem. Soc.*, 82 (1960) 5923.
[4] G. L. SCHMIR, L. A. COHEN AND B. WITKOP, *J. Am. Chem. Soc.*, 81 (1959) 2228.
[5] S. SHALTIEL AND A. PATCHORNIK, *Bull. Res. Council Israel*, 10A (1961) 48.

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