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Fluorescent Probes and the Conformation of Proteins

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Fluorescent probes are compounds which undergo changes in one or more of their fluorescent properties as a result of noncovalent interaction with proteins or other macromolecules. Several spectral characteristics of the probes, such as the quantum yield of emission, are strongly influenced by solvent conditions and by the local environment of the macromolecule. For these reasons, probes may be used to detect conformational changes of proteins. Experiments are now described in which the probe TNS (2-toluidinylnaphthalene-6-sulfonate) was used to detect protein denaturation, zymogen activation, and subtle changes in conformation which accompany enzyme–ligand interaction. TNS is nonfluorescent in water but becomes strongly fluorescent when bound to proteins. Solvent studies suggest that it is a probe for hydrophobic regions of proteins. Since TNS may be covalently coupled to protein molecules, it is possible to localize and characterize the binding sites. A short description and review of the applications of fluorescent probes other than TNS are given in the concluding portion of this paper. The concept of probes which combine chemical specificity with the inherent sensitivity of optical responses (chemo-optical probes) has been found useful in absorption spectroscopy, nuclear magnetic resonance spectroscopy, and electron spin resonance spectroscopy, as well as in fluorescence spectroscopy. The combination of techniques promises to yield new information on the structure and function of proteins.

Proteins are among the most complex and diverse substances known in biochemistry. Their complexity is related to a great range of biological functions, including enzymic activity, hormonal and other regulatory activity, immunological activity, and a predominant role in the structure and morphology of organisms. The diversity of proteins is a reflection of the enormous variety of sequences which result when molecules of high molecular weight are formed by joining 20 different amino acids in a linear series of peptide bonds. Interactions of the amino acid side chains with solvent molecules, with one another, and with the polyamide backbone result in the formation of an intricate three-dimensional or tertiary structure. In general, hydrophobic amino acid residues are clustered on the inside of the folded protein; hydrophilic side chains are present on the surface of the folded protein and favor interaction with the aqueous solvents in which proteins function. One of the major aims of protein chemists is to determine the complete structure of a sufficient number of proteins in order to define the relationship between their structure and function.

Recent successes in amino acid sequence analysis and X-ray crystallography of proteins have established the complete structures of myoglobin, hemoglobin, lysozyme, ribonuclease, and chymotrypsin. Ideally, one might hope to approach all proteins by these means. Unfortunately, this is impractical for a number of reasons, and optical methods other than crystallography still occupy a central position in protein chemistry. Even where X-ray analysis is feasible, methods such as absorption spectroscopy, optical rotatory dispersion, light scattering, nuclear magnetic resonance spectroscopy, and relaxation spectroscopy will be useful in analyzing the subtle conformational changes which proteins undergo in solution.

Fluorescence spectroscopy is one of the most sensitive and versatile of the optical techniques for studying protein structure. Most proteins contain the aromatic amino acids tryptophan and tyrosine, and they will fluoresce when excited by ultraviolet light. This *intrinsic fluorescence* may change in intensity, polarization, or wavelength with changes in protein structure that may result from interaction with small molecules or with other proteins. Following the original work of Weber and Teale,[1] intrinsic fluorescence has been studied extensively by many workers. The number and location of tryptophan and tyrosine residues within the folded polypeptide chain are usually unknown. Moreover, the fluorescence of these residues depends upon a great number of parameters. These are difficult to analyze, and, thus far, no general theory of protein fluorescence has emerged.

Because of these difficulties, attempts have been made to use fluorescent compounds of known properties as indicators or probes of protein structure.[2–4]

(1) (a) G. Weber, *Biochem. J.*, **75**, 335 (1960); (b) G. Weber, *ibid.*, **75**, 345 (1960); (c) F. J. W. Teale, *ibid.*, **76**, 381 (1960); F. J. W. Teale, *ibid.*, **80**, 14p (1961).

(2) (a) G. Weber, *Advan. Protein Chem.*, **8**, 415 (1953); (b) J. A. Gally and G. M. Edelman, *Biochim. Biophys. Acta*, **94**, 175 (1965).

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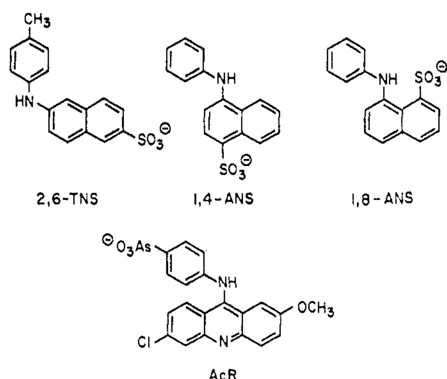

Figure 1. Structures of several compounds used as fluorescent probes: 2,6-TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; 1,4-ANS, 1-anilinonaphthalene-4-sulfonate; 1,8-ANS, 1-anilinonaphthalene-8-sulfonate; AcR, 5-(4′-arsonoanilino)-2-chloro-7-methoxyacridine.

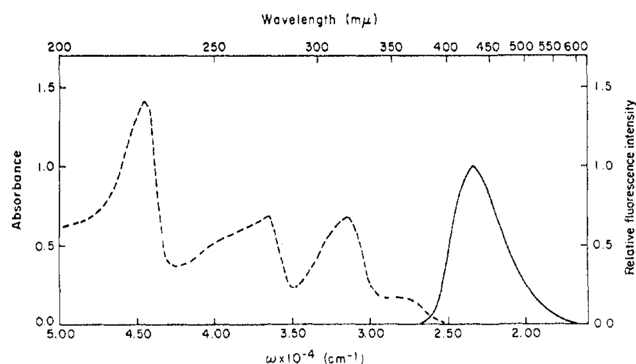

Figure 2. Absorption(- - -) and emission(——) spectra of TNS dissolved in ethanol. The emission spectrum has been normalized to 1.0 at its maximum. See ref 4 for experimental details.

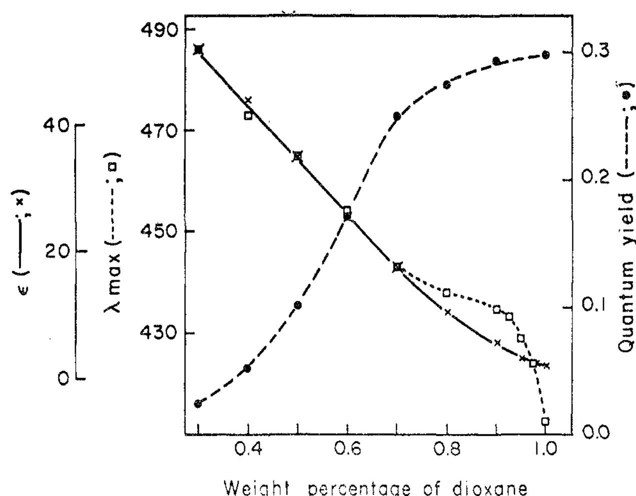

Figure 3. The effect on TNS fluorescence of varying the solvent dielectric constant by altering the composition of dioxane–water mixtures. Data represent changes in TNS quantum yield (●) and wavelength of maximal emission (□). The dielectric constants of the solutions (×) have been superimposed upon the λ_{max} data. See ref 4 for experimental details.

Fluorescent probes may be defined as small molecules which undergo changes in one or more of their fluorescence properties as a result of noncovalent interaction with a protein or other macromolecule. Fluorescent probes are similar to adsorption indicators, and their interactions with proteins may be described in terms of the stoichiometry and affinity of binding. The structure of a probe may be altered to favor interaction with regions of proteins possessing particular properties. By analyzing the fluorescence of suitably chosen probes, one may study regions of protein molecules of particular interest, such as the active sites of enzymes. To interpret the data, it is usually necessary to have information concerning the mechanisms of quenching of the fluorescent probe.

It is the aim of this brief article to describe some experimental applications of fluorescent probes. For the sake of convenience and clarity in this limited space we have emphasized our own experiments, but have made an effort to call the reader's attention to the work of earlier and current workers.

Some examples of molecules which have been used as fluorescent probes (Figure 1) are 2,6-TNS,[5] two positional isomers of ANS, and an acridine derivative AcR.[6] These compounds are practically nonfluorescent in water but fluoresce strongly when dissolved in organic solvents or when bound to proteins.[7] This property makes them particularly useful inasmuch as the background fluorescence of unbound probe molecules is minimal in aqueous solutions.

The mechanism by which the fluorescence of these molecules is changed must be clarified before using them as probes for protein structure. Although the mechanism is not fully understood, some insight is provided by the finding that TNS and related compounds fluoresce more strongly in nonpolar solvents than in polar solvents.[3,4,7] As shown by the spectra in Figure 2, TNS fluoresces blue-green when it is dissolved in alcohol and excited with ultraviolet light. The quantum yield and wavelength of maximal emission (λ_{max}) of TNS fluorescence both change monotonically as a function of changing dielectric constant in dioxane–water mixtures (Figure 3). The quantum yield increases from a value near zero in water to 0.3 in 100% dioxane, and at the same time the wavelength of maximal emission decreases to a limiting value of 412 mμ in 100% dioxane. The most striking feature is the close parallelism between the λ_{max} values and the decreasing dielectric constant of the solvent. The anomalous changes which occur above 70% dioxane (Figure 3) are probably the result of sharp changes in the solvent structure of dioxane–water mixtures in this concentration range.[8] As shown in Table I, the enhancement of quantum yield and shift to shorter wavelengths of maximal emission which occur when solvent

(5) The abbreviations used are: 2,6-TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; 1,4-ANS and 1,8-ANS, 1-anilinonaphthalene-4-sulfonate and the 1,8 isomer, respectively; AcR, 5-(4′-arsonoanilino)-2-chloro-7-methoxyacridine; ethidium bromide, 2,7-diamino-9-phenyl-10-ethylphenanthridium bromide; DNS, 1-dimethylaminonaphthalene-5-sulfonyl.

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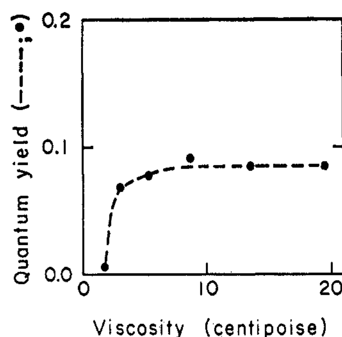

Figure 4. The effect of varying the solvent viscosity on the quantum yield of TNS fluorescence. Solvents of invariant dielectric constant were prepared using mixtures of sucrose and Ficoll, a high molecular weight polymer of sucrose. See ref 4 for experimental details.

Table I
Fluorescence Properties of TNS in Selected Solvents[a]

Solvent	Solvent dielectric constant	Quantum yield	Wavelength of maximal emission, mμ
Water	81.0	0.0008	500
Alcohols			
Methyl	33.2	0.34	443
Ethyl	26.0	0.52	429
n-Propyl	21.3	0.57	424
n-Butyl	19.1	0.57	423
Acids			
Acetic	6.2	0.18	444
Propionic	3.3	0.23	431
n-Butyric	3.0	0.25	425

[a] Data selected from ref 4.

polarity is decreased are also seen in other solvents. Increases in solvent viscosity will also lead to increases in the quantum yield (Figure 4); in general, the effect of viscosity is smaller than that produced by decreasing the dielectric constant.

These findings suggest that TNS is a hydrophobic probe, *i.e.*, the fluorescence is primarily responsive to the polarity of the solvent environment. A mechanism which may account for the enhancement or quenching of TNS fluorescence can be formulated from the data on solvent effects. It can be calculated[9] that the dipole moment of the first singlet excited state of TNS is approximately 10 D greater than that of the ground state. When TNS is excited in the absorbance band of longest wavelength (350–370 mμ; Figure 2), a π electron is promoted to an antibonding π orbital (π–π^* transition). Because the excited state is more polar than the ground state, excited molecules would interact more strongly with polar solvents than would molecules in the ground state. This would lower the energy of the first singlet excited state and, in more polar solvents, would result in a shift to longer wavelengths of emission. At the same time, the probability of nonradiative transitions to the ground state would be increased by interaction of the excited state with solvent. This would result in a decrease in the quantum yield of fluorescence of TNS in polar solvents. The viscosity and temperature of the solvent would also affect the amount of collisional quenching and would be reflected by changes in the quantum yield (Figure 4).

Work with proteins has provided confirmatory evidence that TNS is a hydrophobic probe. Denatured proteins, in which the hydrophobic side chains have been exposed to the solvent, enhance TNS fluorescence to a greater extent than their native forms.[2b,7] Bovine serum albumin, which is known to have hydrophobic binding sites, also enhances TNS fluorescence to a greater degree than other proteins, such as lysozyme (see Table II). Even more striking is the demonstration by Stryer[3] that 1,8-ANS is bound within the heme crevice of hemoglobin and myoglobin with an increase in quantum yield of fluorescence to a value near unity. Chemical and X-ray structural analyses of hemoglobin and myoglobin have shown that the heme crevice is extremely hydrophobic.

Various kinds of conformational changes can be detected with hydrophobic fluorescent probes. One of the most dramatic changes occurs during protein denaturation. As we remarked earlier, the native protein structure is stabilized by a variety of side-chain interactions, including hydrogen bonds, hydrophobic interactions, and electrostatic interactions. Disruption of the side-chain interactions by surface forces, solvent changes, or thermal energy denatures the protein and alters its tertiary structure. Concomitantly, some or all of the hydrophobic groups which were originally in the interior of the molecule are exposed to the solvent. As illustrated in Figure 5, fluorescent probes may be used as highly sensitive indicators of denaturation.[2b] In this experiment, normal light chains, isolated from human γG-immunoglobulin, were denatured by heat while their intrinsic fluorescence was measured. At the thermal transition point the intrinsic fluorescence increased sharply (in some other proteins the intrinsic fluorescence decreases). In a similar experiment, 1,4-ANS was added to a solution of the native protein, and the probe fluorescence was used to follow the unfolding. The thermal transition was accompanied by a large increase in fluorescence at the wavelength of emission of 1,4-ANS (Figure 5).

The changes observed during denaturation are gross alterations in conformation which are often irreversible

Table II
Fluorescence Enhancement in Aqueous Protein Solutions

Protein	Probe: TNS[a]	DNS-tryptophan[b]	DNS-glycine[b]
Bovine serum albumin	0.34	55.5	68.0
Chymotrypsin	0.18	2.6	0.2
Chymotrypsinogen	0.07	0.6	0.2
Ovalbumin	0.06	1.9	2.7
Lysozyme	0.04	0.2	0.6

[a] Quantum yields; see ref 4 for experimental details. [b] Relative fluorescence intensities; data from R. F. Chen, *Arch. Biochem. Biophys.*, **120**, 609 (1967).

(9) E. Lippert, *Z. Elektrochem.*, **61**, 962 (1957).

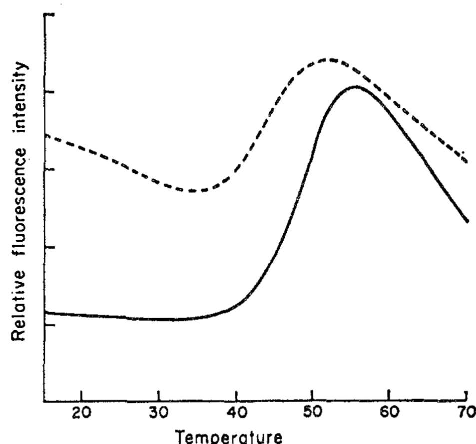

Figure 5. Thermal denaturation of normal light chains from human γG-immunoglobulin. Light chains were heated in the absence (- - -) and presence (——) of 1,4-ANS. The fluorescence intensity of tryptophan residues in the protein (- - -), or of ANS (——), respectively, was measured using appropriate conditions. See ref 2b for experimental details.

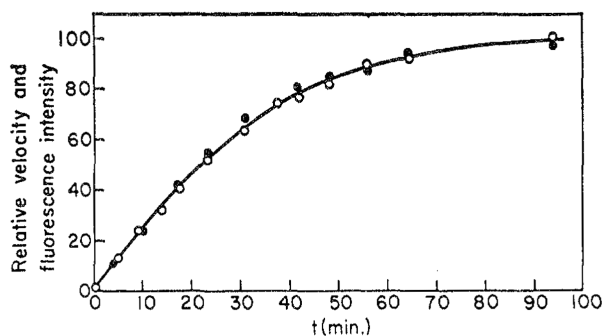

Figure 6. The activation of chymotrypsinogen as measured by the formation of active chymotrypsin (●) and by the increase in fluorescence of TNS incorporated in the reaction mixture (O). Data are presented as the per cent change between observed initial and final values. See ref 15 for experimental details.

and are accompanied by loss of biological activity. We would like to use fluorescent probes to detect smaller reversible changes in the native state of an active protein such as an enzyme. For this purpose it is useful to know the stoichiometry of binding and binding affinity of the probe as well as the influence of different protein binding sites on probe fluorescence.

Only a limited number of studies have been carried out to determine the number of probe molecules bound to a particular protein molecule. Inasmuch as most of the surface of a globular protein is hydrophilic, one would expect that relatively few probe molecules would be adsorbed. Fluorescence titrations, equilibrium dialysis, and gel filtration experiments indicate that each molecule of chymotrypsin binds only a single molecule of TNS.[10] Liver alcohol dehydrogenase binds two molecules of 1,8-ANS[11] and bovine serum albumin binds five molecules of the same compound.[12] Apohemoglobin presumably binds one molecule of 1,8-ANS in each heme crevice, giving a total of four probe molecules per molecule of protein.[3] In general the binding affinities of ANS and TNS for proteins lie between 10^2 M^{-1} and about $3 \times 10^6 M^{-1}$.

Variations in the size, shape, or composition of the hydrophobic binding sites of different proteins should affect their interaction with probes and thus result in differences in probe fluorescence. As listed in Table II, the fluorescence properties of TNS and some α-N-DNS-amino acids do show marked differences when measured in solutions of different proteins. It is also apparent from these data that a given protein can distinguish between probes. For example, chymotrypsin and chymotrypsinogen enhance the fluorescence of α-N-DNS-glycine to the same extent, but the fluorescence of TNS is quite different in solutions of the two proteins.

This observation suggests that TNS may be used to detect the subtle change in conformation which occurs during the conversion of chymotrypsinogen to chymotrypsin. Chymotrypsinogen is a protein which is an enzyme precursor or zymogen. The inactive zymogen is converted to chymotrypsin, an active proteolytic enzyme, after cleavage by trypsin of a single peptide bond between arginine at position 15 and isoleucine at position 16. The activation of chymotrypsinogen is usually followed by measuring the appearance of hydrolytic activity against a synthetic substrate. Recent X-ray evidence suggests that the gross conformations of chymotrypsin and chymotrypsinogen are very similar,[13] although it is known that a conformational change must follow activation of the zymogen.[14] The conformational change may be followed quite accurately[15] by observing the increase in fluorescence of TNS incorporated in an activation mixture (Figure 6). This increase exactly parallels the appearance of enzymatic activity, and thus the probe is responding to the small refolding of a polypeptide chain which accompanies the formation of an active site.

The details and the dynamics of the refolding could be specified more clearly if one understood more about the locus of attachment of TNS on the enzyme and its relation to the active site. With this in mind, studies have been carried out on the binding of TNS to chymotrypsin. By means of equilibrium dialysis and gel filtration it was confirmed that each molecule of the enzyme binds one TNS molecule with a dissociation constant of 2×10^{-4} M. TNS is not bound in the active site of the enzyme, however, but attaches to the protein at a second site. The existence of a separate TNS binding region can be inferred from the fact that the binding of TNS to chymotrypsin is noncompetitively inhibited by compounds which are known to bind at the active site of the enzyme. If the two compounds were bound to the same site, the kinetic and equilibrium data would show competitive binding.[10] This finding is

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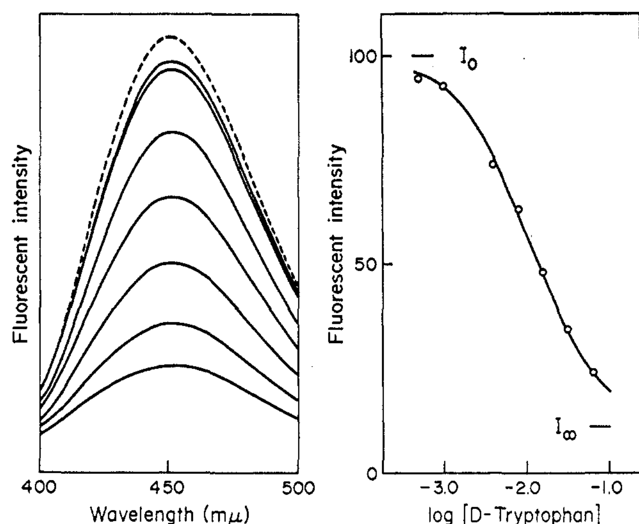

Figure 7. The effect of added D-tryptophan on the fluorescence of TNS in solutions containing chymotrypsin. Left-hand figure: emission spectra of TNS in solutions of chymotrypsin. The upper (dashed) spectrum was obtained in the absence of inhibitor. The lower curves are spectra obtained in the presence of increasing concentrations of D-tryptophan. The maximal intensities of the observed spectra are related to the D-tryptophan concentration in the right-hand figure. The line through the points is a theoretical curve and indicates that fluorescence intensity decreases from a control level observed in the absence of inhibitor (I_0) to a limiting value predicted for a system saturated with inhibitor (I_∞).[10]

somewhat surprising, for it has been shown that the active site of chymotrypsin has a hydrophobic region.[16] Apparently the affinity of the active site for TNS is considerably less than that of a second hydrophobic region.

Despite the fact that substrates and TNS are bound to spatially distinct sites, binding of substrate analogs in the active site produces changes in the fluorescence of the probe. As shown in Figure 7, the fluorescence of TNS in solutions of chymotrypsin is diminished by the addition of D-tryptophan, a competitive inhibitor of the enzymic activity. Additional experiments indicate that this is due to a release of TNS from the enzyme as well as to a change in the environment of attached TNS. We surmise that binding of substrate or substrate analogs to the active site results in a change in the conformation of the enzyme.

The nature of this change is currently being studied. One approach is to prepare a TNS derivative which is capable of forming a covalent bond with the side chains of amino acids in its vicinity.[17] The sulfonyl chloride of TNS (TNS-Cl) appears to have just these properties. After noncovalent association of TNS-Cl and a protein, a covalent bond is formed and the region of attachment is labeled. The labeled portion of the polypeptide chain may be cleaved from the protein and its amino acid sequence determined. Comparison of this sequence with that of the whole molecule and with the X-ray structure should make it possible to locate the TNS binding site with respect to the enzyme active site. With this knowledge, it should be possible to study in detail the dynamics of conformational changes occurring during zymogen activation and enzymic catalysis.

The examples given above illustrate only a few of the uses of fluorescent probes. Although the literature on probes is relatively small, it is expanding rapidly. A brief mention of some salient papers may be useful. One of the earliest applications of TNS was in a study of the action of the antibiotic polymyxin on bacterial cell walls.[18] In 1952, Laurence[19] studied the fluorescence enhancement produced by binding various substituted naphthalenesulfonates to bovine serum albumin. Subsequently, 1,8-ANS was used by Weber and coworkers in binding titrations of bovine serum albumin[12] and in studies of the molecular expansion of this protein which occurs at acid pH.[20] Laurence[21] has recently used this probe in the characterization of histone fractions from calf thymus. A number of other studies on enzymes have yielded new information. The DNS group has been used by Millar, *et al.*,[22] to study the interaction of trypsin and a specific protein inhibitor of trypsin. Using TNS, Kingdon[23] has observed changes in the conformation of glutamine synthetase produced by binding of metal ions.

Because of their sensitivity, probes may be particularly valuable in studies of antigen–antibody interactions. Winkler[24] studied the interactions between TNS and antibodies to naphthionic acid. Parker and his colleagues have used both ANS[25] and ε-N-DNS-lysine[26] to study the homogeneity and binding properties of isolated immunoglobulins in the immune response. 1,4-ANS has been used by Yoo, *et al.*,[27] to study the binding properties of the isolated light chains of purified specific antibodies. The extreme sensitivity of the probe technique permitted these workers to show the presence of weak binding activity that was specific for the ANS molecule.

Molecules which are structurally unrelated to the naphthalenesulfonates may also function as fluorescent probes. Rose bengal, a halogenated derivative of fluorescein, has been used by Brand and coworkers[28] to study the DPN binding sites of alcohol dehydrogenase. As mentioned previously, acridine derivatives have also been employed.[6,7] Chen[29] has examined the

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properties of DNS-amino acids and found them suitable as probes (see Table II). A search for probes capable of binding to regions of proteins other than hydrophobic sites should be extremely rewarding.

Although this review has emphasized proteins, it should be pointed out that fluorescent probes can be used in the study of other macromolecules. LePecq and Paoletti[30] have shown that the trypanocidal dye, ethidium bromide (2,7-diamino-9-phenyl-10-ethylphenanthridinium bromide), behaves as a fluorescent probe with helical polynucleotides. The interaction appears to be specific for base-paired regions in DNA or RNA and the dye appears to be intercalated between base pairs.[30b,31] LePecq and Paoletti[30b] suggest that ethidium bromide is a hydrophobic probe, and its behavior is consistent with that described for TNS.[4]

The concepts applied to fluorescent probes can be extended to optical properties of small molecules other than fluorescence. For example, probes sensitive to changes in absorption spectra,[32] nuclear magnetic resonance spectra,[33] and electron spin resonance spectra[34] have been used to study macromolecular structures. Sigman and Blout[35] have demonstrated the existence of intramolecular charge-transfer complexes between chymotrypsin and a nitroacetophenone derivative which was covalently bound to the protein. These authors use the term "chemical-optical probe" for this class of compounds. Based on their usage, we wish to suggest "chemo-optical probe" as a general term.

Several new chemical and biological applications may emerge from the exploration of chemo-optical probes. For example, the hydrophobic fluorescent probes discussed here might be useful in the study of hydrocarbons and lipids and their interactions. The detection of minute traces of oils in aqueous media can be carried out with great sensitivity and precision, and fluorescent probes might be sensitive indicators of the state of heterogeneous systems such as suspensions of colloid micelles. It is conceivable that optical probes may be used to examine macromolecular interactions in living cells and tissues. All of these approaches offer exciting new possibilities for exploring the conformation and function of proteins and other biologically interesting macromolecules.

Experiments by the authors of this review were supported in part by National Science Foundation Grant No. GB 6546 and National Institutes of Health Grant No. AM-04256.

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Pseudo-Rotation in the Hydrolysis of Phosphate Esters

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Five-membered esters of phosphoric acid are strained. They hydrolyze millions of times faster than their acyclic analogs, with ring opening and with retention of the ring. However, the phostonate **14** hydrolyzes rapidly but almost exclusively with ring opening, and cyclic phosphinates hydrolyze slowly. These facts have been correlated with the hypothesis that hydrolysis external to the ring proceeds through intermediates that undergo "pseudo-rotation," as shown, for example, in eq 5. Pseudo-rotation occurs subject to the constraints that (a) alkyl groups preferentially occupy equatorial positions and (b) five-membered rings span one equatorial and one apical position in trigonal bipyramids. Thus, the intermediate from a cyclic phosphinate should not form readily, and the phostonate **14** should hydrolyze only with ring opening, as observed. Further, the constraints lead to the prediction as to which oxyphosphoranes can, and which cannot, undergo pseudo-rotation; these predictions have been verified by nmr studies of the appropriate alkyloxyphosphoranes.

I. Introduction

The hydrolyses in acid or base of five-membered cyclic esters of phosphoric acid proceed millions of times faster than those of their acyclic analogs.[1–4] A key finding[5] concerning these processes is that the hydrolysis of hydrogen ethylene phosphate is accompanied by rapid oxygen exchange into unreacted hydrogen ethylene phosphate (eq 1 and 2; the label is of course distributed among the oxygen atoms of the phosphoryl and hydroxyl groups).

Similarly, the hydrolysis of methyl ethylene phos-

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