HETEROCYCLES, Vol. 5, 1976

EXAMPLES OF THE USE OF FLUORESCENT HETEROCYCLES IN CHEMISTRY AND BIOLOGY[†]

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The application of fluorescent heterocycles to the study of chemical and biological systems is discussed. The spectroscopic properties of coumarins, pyrrolinones derived from fluorescamine, and nucleic acid base analogs, together with examples of their use, are described in detail.

I. Introduction

The value of a new heterocyclic compound often lies not with the actual synthetic procedures used to prepare it, but with the chemical, physical or biological properties possessed by the heterocycle. While most new heterocycles are characterized by spectral techniques such as infrared, mass

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[†] Dedicated to Dr. Ken'ichi Takeda on the occasion of his seventieth birthday.

spectrometry, nuclear magnetic resonance and ultraviolet spectroscopy, a property that is often neglected is fluorescence spectroscopy. Very briefly, fluorescence is the rapid emission of energy from the lowest excited singlet state upon excitation, usually at the longest wavelength absorption band. According to Stokes law, the emitted fluorescence has a longer wavelength (lower energy) than the absorbed radiation. This implies that the electron, after being raised to a higher energy level by absorption of radiation, falls first to the lowest vibrational level of the excited state, and so emits radiation of lower energy than that used for excitation. The fluorescence of a compound is expressed in terms of the excitation and emission wavelength (λ^{ex} and λ^{em}), the quantum yield of emission (Φ) and the lifetime of the excited state (τ). A detailed treatment of fluorescence theory and technique will not be attempted here as adequate reference works are available. (1-7) Because other mechanisms are accessible for relaxation of the excited to the ground state, such as non-radiative processes and phosphorescence, obviously not all compounds are fluorescent. Accordingly, it has not been possible to predict, *a priori*, that a new heterocyclic system will exhibit fluorescence. Despite this rather serious limitation, many different types of heterocyclic compounds are fluorescent. In many cases this property has been exploited for their detection in natural sources and for studying their behavior in chemical and biological systems.

Fluorescence spectroscopy has proved to be extremely useful in the fields of analytical chemistry and biochemistry. (4-9) In this regard, applications to protein chemistry have been particularly successful and have led to the determination of structural and dynamic properties of macromolecular proteins. (4-8) The sensitivity of fluorescence techniques (in the pmole range in many cases) makes them attractive to analytical and clinical chemists. (3-5)

The initial role of the synthetic heterocyclic chemist lies in the design and synthesis of new fluorescent heterocycles or in the modification of existing fluorescent heterocycles. The approach may follow three different routes: 1) modification of an existing fluorescent heterocycle either to improve its fluorescence characteristics or to provide it with a reactive functionality specific for a particular functional group on a biopolymer, drug, etc.; 2) the design of a non-fluorescent reagent which upon reaction with a particular substrate produces a fluorophore and thus a means for study of that substrate; and 3) the design of a fluorescent analog of a non-fluorescent compound which is usually of biological significance. In order to illustrate these three approaches, we have chosen three different heterocyclic systems: 1) coumarins which are both naturally occurring fluorophores and synthetic fluorogenic reagents; 2) fluorescamine, which is a recently developed, non-fluorescent reagent that reacts with primary amines to generate fluorescent pyrrolinones; and 3) synthetic analogs of the nitrogen bases found in nucleic acids. The emphasis of this review will be on the information that can be obtained through the use of these compounds. Synthetic methodology will not be emphasized, since our chief intention is to illustrate for the heterocyclic chemist the solution of biological problems by means of fluorescence spectroscopy.

There are, of course, many important fluorescent heterocycles that cannot be discussed in a brief review. In most cases, adequate reviews of the properties and applications of these compounds are available. Indole is one of the most widely studied fluorescent heterocycles since it is found in almost all proteins in the form of tryptophan. (10, 11) A second biologically important class of fluorescent nitrogen heterocycles includes the porphyrins and chlorophylls. (11, 12) While a complete listing of all fluorescent

heterocycles is not feasible, some of the more important compounds and their emission ranges are quinoline (385-490 nm), acridine (425-454 nm), methyl-acridine (425-454 nm), xanthone (ultraviolet), carbazole (340-420 nm), alloxazine (violet), and quinine sulfate (410-500 nm). (13) Some organic dyes are highly fluorescent, especially the xanthone group (fluorescein, rhodamine, eosin) and the acridine group (euchrysine, acridine orange, acriflavin). (14) Those that are used extensively in biochemistry include fluorescein isothiocyanate, a labeling reagent for proteins, (15) and acridine orange or ethidium bromide, which fluoresce strongly on binding to nucleic acids. (16)

II. Coumarins

Coumarin (1,2-benzopyran, 1) and its derivatives are distributed widely in nature and have a broad spectrum of biological activity. Several important examples are the skin-photosensitizing psoralens (2), the fungal metabolites, the very toxic aflatoxins (3), and warfarin (4), a rodenticide and anti-coagulant. Although coumarin itself has a very low fluorescence quantum

1

PSORALEN
2

AFLATOXIN B_1
3

WARFARIN
4

yield, many derivatives are highly fluorescent and are accordingly useful both commercially and in research as biological probes. Although the fluorescence depends upon the position and type of substitution, there are some common features. Most coumarins exhibit uv absorption above 330 nm, which allows for excitation outside the range of absorption of proteins and nucleic acids. In addition to having high quantum yields (0.5 and greater), the fluorescence of many substituted coumarins is directly dependent on environmental factors such as pH and solvent polarity. Since many coumarin derivatives with varied fluorescence properties are readily available either commercially or through synthesis, a wide range of fluorometric analyses exists.

The commercial importance of coumarins lies in the dye industry where they have been used extensively as fluorescent brightening agents. The requirements of strong ultraviolet absorbance near 350 nm and blue fluorescence with a high quantum yield are met by derivatives of the 7-hydroxy- and 7-amino-coumarins that contain an aryl or heteroaromatic moiety in the 3-position. (17) Fluorescent brightening agents are used in detergents, paper and textiles to mask yellowing in white materials. A more recent application of coumarin fluorescence is in the field of tunable dye lasers. Among the coumarins evaluated as laser dyes, several are available commercially. (18) Exemplary compounds and their spectral properties are shown in Table 1.

In a study of the photodimerization of coumarin, fluorescence at 357 nm was observed from a concentrated ethanolic solution at room temperature. (19) In subsequent work in dilute solutions, this fluorescence at ambient temperature could not be detected in either polar or non-polar solvents, and a quantum yield of fluorescence of less than 10^{-4} was estimated. (20, 21) In polar solvents at 77°K, fluorescence can be detected at 384 nm although

Table 1

Coumarin Laser Dyes

λ_{max}^{em} (nm)	432	505	466
Tuning Range (nm)	430-480	515-585	465-500

phosphorescence is the dominant process. From low temperature polarization studies and molecular orbital calculations, it was concluded that fluorescence is from the $^1(\pi,\pi^*)$ state partially localized in the pyrone portion of the molecule. (20, 21) When non-polar solvents are used, the fluorescence maximum is red-shifted to 417 nm and becomes more intense in relation to phosphorescence. Dipole moments calculated by molecular orbital methods are in the range of 2.59-4.37 for the $^1(\pi,\pi^*)$ state of coumarin and thus lower than the observed value of 4.61 for the ground state. (20) Non-polar solvents would be expected to stabilize the excited state with respect to the ground state, thereby causing a red shift. Alternatively, this effect has been explained as fluorescence from an exiplex formed in the non-polar solvent. (21)

The tribofluorescence of coumarin (emission from the solid state caused by mechanical stress) has been shown to originate from the $^1(\pi,\pi^*)$ state. (22) Coumarin is the first example of an organic molecule from which tribofluorescence could be detected. The excited states of furocoumarins such as

psoralens (2) have been extensively studied since these compounds are known to photosensitize skin cancer in mice and guinea pigs. (23-26) As in the parent compound, low temperature luminescence studies showed phosphorescence to be the dominant process with a Φ_P/Φ_F ratio of 7.1 . (23-25) The skin sensitizing activity of furocoumarins has been correlated with their photoreactivity toward pyrimidine bases of DNA via cycloaddition. The photoreactivity of the $C_{3,4}$ double bond was shown to be due to energy localization in the $^3(\pi,\pi^*)$ state. (25) Evidence for this was obtained through luminescence studies on 5- and 8-hydroxypsoralens which are inactive as skin photosensitizers. In ionizing solvents the phosphorescence intensity decreases dramatically. This is due to dissociation of the hydroxyl in the excited state effectively competing with singlet to triplet intersystem crossing, thus reducing the photoreactive triplet population. The triplet states of the anions are more delocalized than the neutral psoralens so that they are less reactive towards photocycloaddition with pyrimidine bases of DNA. (26)

The fluorescence of certain substituted coumarins, most notably the 7-hydroxycoumarins (umbelliferones), is markedly dependent on pH. Below pH 2, the 450 nm emission from the 4-methylumbelliferone anion (5a) undergoes a bathochromic shift of 30 nm accompanied by a decrease in intensity. (27) It was speculated that the species responsible for this emission is 5c, formed by a two step phototautomerization of 5b, which emits at 385 nm. (28-32) As the acidity increases below pH 1, the intense emission observed at 415 nm is postulated to result from the protonated species 5d. (28) The nature and formation of the excited states 5a-c were elucidated by gain spectroscopy in which the efficiency of stimulated fluorescence is measured rather than the spontaneous fluorescence over the wavelength range. (29) A similar excited state phototautomerization has been demonstrated for warfarin (4) involving an intra-

	5a	5b	5c	5d
λ^{em} (nm)	450	385	480	415
λ^{ex} (nm)	360	320	320	345

molecular proton transfer in the cation. (33) At high pH, the fluorescence from coumarins shifts to 500 nm, caused by base hydrolysis of the lactone to form o-hydroxycinnamic acids. (27, 34) Since the excitation band of 4-methylumbelliferone shifts with change in pH it has been proposed as a fluorescent pH indicator having a mid-point in its transition interval of 7.6 . (35)

The quantum yields and emission maxima of many substituted coumarins are strongly affected by solvent polarity. For the 7-hydroxy- and 7-amino-coumarins the emission maximum shifts to the blue as the polarity of the solvent decreases. (36-38) This trend is illustrated for the 7-aminocoumarins in Table 2. The shift to higher energy in non-polar solvents is probably due to significant polar character in the excited state from a resonance contributor such as 6. Although the quantum yields vary with solvent polarity, the de-

6

pendence is different for each compound. The fluorescence of aflatoxins shows a similar dependence on solvent polarity. (40-42) The emission band shifts to higher energy and the intensity decreases as the polarity decreases,

Table 2: Fluorescence Properties of Substituted Coumarins

Compound	Solvent	λ_{max}^{ex} (nm)	λ_{max}^{em} (nm)	Φ^a	Relative Intensity	Ref.
	EtOH(95%)	-	357	<0.001	-	19
	PhH	-	364	<0.001	-	19
CH_3, H_2N	H_2O (pH 7)	342	445	0.88	-	36
	EtOH	354	435	0.88	-	36
	Cyclohexane	348	405	0.46	-	36
CH_3, EtHN	H_2O (pH 7)	359	462	0.75	-	36
	EtOH	366	444	0.80	-	36
	Cyclohexane	358	414	0.57	-	36
CH_3, Et_2N	H_2O (pH 7)	383	477	0.058	-	36
	EtOH	373	453	0.064	-	36
	Cyclohexane	-	419	0.52	-	37
CH_3, HO	H_2O (pH 7)	375	470	-	1.00[b]	46
HO	H_2O (pH 7)	376	454	-	0.96[b]	51
CH_3, H_3CO	H_2O (pH 7)	320	415	-	0.10[b]	46

Table 2: (continued)

Compound	Solvent	λ_{max}^{ex} (nm)	λ_{max}^{em} (nm)	Φ^a	Relative Intensity	Ref.
HO, CH_3, O, O	H_2O (pH 7)	343	450	-	0.06^b	46
HO, Ph, O, O	H_2O (pH 7)	420	456	-	2.7^b	51
HO, CN, O, O	H_2O (pH 7)	408	450	-	3.6^b	51
OH, O, O	H_2O (pH 2)	320	400	-	1.00^c	58
OCH_3, O, O	H_2O (pH 2)	318	475	-	0.48^c	58
AFLATOXIN B_1	H_2O (pH 2)	370	435	-	2.5^c	58

[a] Quantum yields are based upon ouinine sulfate $\Phi = 0.55$. We have redetermined $\Phi = 0.70$ for the standard. (39)

[b] The relative intensities are normalized to a value of 1.00 for 7-hydroxy-4-methylcoumarin.

[c] The relative intensities are normalized to a value of 1.00 for 5-hydroxycoumarin.

again indicative of polar character in the excited state. An increase in the viscosity of the medium was shown to increase the quantum yield of a series of 7-dialkylaminocoumarins without shifting the emission band. (36, 37) Fluorescence may be quenched by the interaction of surrounding molecules. Restriction of internal rotation or vibration of an excited coumarin molecule may therefore enhance the quantum yield without changing the energy difference between ground and excited states. (36, 37)

Series of coumarin derivatives have been prepared in order to evaluate the effect of substituents on the fluorescence properties. (36, 38, 43-58) The compounds listed in Table 2 demonstrate the significant substituent effects. Electron-donating groups in the 4-, 5-, 6- or 7-position shift the emission to longer wavelength and increase the intensity. The effect is greatest at the 5- or 7-position where it is possible to have extended conjugation through the molecule as represented by **6**. This is not possible at the 6-position and therefore the shift is much less dramatic. Amino and hydroxy groups are most effective, followed by methoxy and methyl. Electron-attracting groups such as phenyl, cyano or acetyl in the 3-position also cause a red shift and an increase in fluorescence intensity.

Many of the biologically important reactions of coumarins are believed to occur by a photocycloaddition of the 2,3-double bond to a pyrimidine base in DNA. This results in saturation of the pyrone moiety, and the complete loss of fluorescence provides an easy method for following the progress of these reactions. The photocycloaddition of bis-coumarins linked by polymethylene chains of various lengths, (59) the photodimerization of furocoumarins, (60) and the photobiology of benzodipyrones (61) have been studied using this technique. Psoralens (**2**) and related furocoumarins have the potential of forming cross-links between two DNA chains. Photoreaction at the furan double bond

will not destroy the fluorescence, but a blue shift of the emission is observed. (62) The reaction of furocoumarins with DNA can be monitored by the acquired fluorescence of DNA, implying that the furan double bond is the more reactive site. (63, 64) As the reaction proceeds to form a cross-link, the acquired fluorescence of the DNA is lost. (65)

The fluorescence of the highly toxic aflatoxins has been utilized in studying their behavior in biological systems as well as for their quantitative determination. (66) Aflatoxins B_1 and G_1 have been shown to be less fluorescent though more toxic than B_2 and G_2 which are formed by reduction of the terminal furan double bond. (40, 66) As shown in Table 2, aflatoxin B_1 because of the alkoxy substituent at C-7, is five times more fluorescent than the parent 5-methoxycoumarin. (58) The binding of aflatoxin B_1 to DNA has been investigated using fluorescence polarization and quenching techniques. (67) Complete quenching of the aflatoxin fluorescence was observed when bound to DNA, allowing the calculation of 1.58 $l\ mol^{-1}$ for Kn, the product of the equilibrium constant and the number of binding sites per DNA-phosphate. The lack of sensitized fluorescence or phosphorescence from DNA when the aflatoxin is excited and the constancy of the phosphorescence lifetime of aflatoxin in the presence and absence of DNA led to the conclusion that quenching by DNA of the excited aflatoxin singlet leads only to vibrational excitation of the DNA and that there is no reaction of an aflatoxin triplet with DNA.

Among the extensive applications of coumarins in enzymology, 7-hydroxy-4-methylcoumarin (4-methylumbelliferone, 4-MU) has been widely used in fluorometric assays of enzyme activity. These assays rely on the action of an enzyme on a synthetic fluorogenic substrate (usually an ester or ether of 4-MU). By choosing the excitation and emission wavelengths so that the fluores-

cence intensity of 4-MU is much greater than that of the substrates, the enzyme activity may be monitored by observing an increase in intensity. An example of the assay of nucleotide 5'-phosphodiesterase activity from serum samples is shown in Scheme 1. (68) Other examples are: the assay of β-

PHOSPHODIESTERASE

Scheme 1

glucuronidase using 4-MU-glucuronide; (69, 70) the assay of arylsulfatase using 4-MU-sulfate; (71, 72) the assay of acid and alkaline phosphatase with umbelliferone phosphate; (73) the assay of lipase using fatty acyl esters of 4-MU; (74, 75) and the assay of sulfotransferase by observing the decrease in fluorescence during the sulfation of 4-MU. (76) A similar procedure has been used to assay the amidase activity of chymotrypsin using 7-glutarylphenylalaninamido-4-methylcoumarin as the substrate. (77) Fluorescence from umbelliferone has been used to assay the activity of rabbit liver and other tissue homogenates which hydroxylate coumarin. (78) The inherent sensitivity and speed of fluorescence techniques makes these assays especially attractive.

The observation that coumarin fluorescence is dependent on solvent polarity, as mentioned earlier, suggested the use of coumarins as probes for hydrophobic regions on proteins. In particular, the quantum yield and emission maximum of 7-diethylamino-4-methylcoumarin are very sensitive to solvent polarity (see Table 2). Application of this fluorescent probe to proteins indicated the presence of a hydrophobic binding site (or sites) on the molecules of bovine

serum albumin, bovine β-lactalbumin and zinc bovine insulin and the absence of such a site on the molecules of hen egg albumin and hen egg lysozyme. (37) From the magnitude of the blue shift and increase in quantum yield in the presence of these proteins, insulin has the most hydrophobic binding site, followed by serum albumin and β-lactalbumin. A similar probe, 3-benzyl-7-diethylamino-4-methylcoumarin, was used to study the binding sites of chymotrypsin. (79) A large increase of the quantum yield and a blue shift in the emission maximum were observed on addition of α-chymotrypsin, while chymotrypsinogen and inactive forms of chymotrypsin had little effect on the fluorescence. The fluorescence of 7-diethylamino-4-methylcoumarin was less affected by chymotrypsin. From these results it was deduced that the probe is adsorbed on the specificity-determining site of α-chymotrypsin which is aromatic in nature.

Fluorescent reagents specific for functional groups in proteins have yielded much information on the structure and mode of action of proteins. N-(7-Dimethylamino-4-methylcoumarinyl)maleimides (7) have been synthesized as potential fluorescent thiol reagents. (80) 3-Phenylcoumarin-7-isocyanate was proposed as a reagent for use in fluorescence depolarization studies of spherical molecules in the 1,000-60,000 Dalton range. (81)

The observation that 4-bromomethyl-7-methoxycoumarin (BMB, 8) will

react with 4-thiouridine (9) to produce 10 suggested that it would be a valuable probe for RNAs. (82) The reaction of BMB with E. coli tRNA^{fMet} specifically modifies the 4-thiouridine without affecting the methionine acceptor activity. (83) A similar reaction was demonstrated for 5-N-methylaminomethyl-2-thiouridine in E. coli tRNA^{Glu}, and a much slower reaction was observed at N-1 of pseudouridine in E. coli tRNA^{fMet} to produce 11a and b

8 9 10

11a 11b

respectively. (84) Combining the BMB reaction with chemical methods for replacing dihydrouridine and for specifically labeling the 3'-end and 5'-end with fluorescent labels, five tRNAs bearing two different fluorescent probes were prepared for use in singlet-singlet energy transfer determination of intramolecular distances in the tRNAs in aqueous solution. Values could then be calculated for the apparent distances between the 3'- and 5'-end (24 Å),

4-thiouridine to 3'-end (38 Å), pseudouridine to 3'-end (55 Å) and pseudo-uridine to dihydrouridine (36 Å) in E. coli $tRNA^{fMet}$ and between 5-N-methyl-aminomethyl-2-thiouridine in the anticodon and the 3'-end (> 65 Å) of E. coli $tRNA^{Glu}$. (85) As with most distances obtained using fluorescence energy transfer methods, it was assumed that both donor and acceptor are free to rotate rapidly and randomly. If this is not the case, considerable variation in the calculation will occur and caution is therefore advised in the interpretation of this type of measurement. (86)

Coumarins have proven to be versatile fluorophores for the investigation of biological systems. The intrinsic fluorescence of some of the naturally occuring coumarins has also provided a means for studying the biological activity of these compounds. As fluorescent probes, their application to protein structure and function and to nucleic acid conformation has been demonstrated by numerous examples.

III. Fluorescamine

The reaction of ninhydrin with phenylalanine produces a fluorescent compound that forms the basis of an assay for serum phenylalanine used in the diagnosis of phenylketonuria. (87) It was found that the fluorescent ninhydrin reaction is general, so that in the presence of certain aldehydes such as phenylacetaldehyde (formed from phenylalanine in the originally observed reaction), primary amines react with ninhydrin to form ternary products which emit at 475 nm when excited at 390 nm. (88) A procedure was developed to assay peptides and amino acids which is 10-100 times more sensitive than the conventional colorimetric assay. (89) The major fluorescent product from the condensation of ethylamine, phenylacetaldehyde and ninhydrin was shown to be the pyrrolinone, **12**, by chemical and spectroscopic methods. (90) Additional structural evidence was secured through independent synthesis

of the simplified analogs 14 and 15 by treatment of the dimethylaminomethylene derivative 13 with either ethylamine or p-bromobenzylamine. The spectral

12

13

14 R = Et
15 R = $pBrC_6H_4CH_2$

properties of 14 and 15 are quite similar to those of 12, and the structure of 15 was confirmed by X-ray crystallographic analysis. These results were utilized to synthesize two new reagents which replace the fluorescent ninhydrin reaction for the assay of primary amines. These reagents, 4-phenyl-spiro[furan-2(3H),1'-phthalan]-3,3'-dione (fluorescamine, 16) and 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF, 18), react with primary amines to form the fluorescent pyrrolinones 17 and 19. (91) The syntheses of fluorescamine and MDPF have recently been described in detail. (92)

16 17 18 19

Fluorescamine is only sparingly soluble in water so it is usually added to the assay sample dissolved in a water-miscible solvent such as acetone or dioxane. Hydroxylic solvents are not suitable since they can form addition products with the reagents. The pH of the medium is critical to the extent of the fluorogenic reaction. The amine reacts as the free base; accordingly the pH must be high enough that the amine will not be protonated.

If the pH is too high, hydrolysis of the reagents to nonfluorescent 20 and 21 will predominate. A study of a variety of amines (aliphatic amines, peptides

16 $\xrightarrow{H_2O}$ 20 18 $\xrightarrow{H_2O}$ 21

and amino acids) showed that a relatively narrow pH range of 8.0-9.5 is required. (93) Both hydrolysis of the reagents and their reaction with amines are rapid and depend on many factors such as pH, amine used, and the organic co-solvent used. (94) At pH 9.0, the half-time for reaction of fluorescamine with alanine using acetone as the co-solvent is about 1 sec, which is 22 times faster than the hydrolysis reaction. At pH 10, both rates increase, but the ratio of the rate of reaction to rate of hydrolysis drops to 15. Acetone and acetonitrile were shown to have the best properties as co-solvents. From the kinetic results obtained by stopped-flow fluorometry, a mechanism was proposed involving the rapid, reversible addition of a primary amine across the double bond of fluorescamine to form an intermediate 22, which subsequently rearranges in a multistep sequence to the final fluorophore. (94)

16 $\xrightleftharpoons{RNH_2}$ [22] → → 17

22

The fluorophore 17 is a strong emitter at 475-490 nm when excited at 375 nm. While the fluorogenic reaction itself is rather sensitive to pH, the fluorescence intensities of the products are not, remaining fairly constant

in the range between pH 4.5 and 10.5 . (93) The presence of two dissociable acidic functions with pK_a values of 3.8 and 11.6 are shown by the decrease in fluorescence intensity in two distinct steps. The initially high fluorescence intensity of the fluorescamine product in acidic solution is rapidly lost as the fluorophore undergoes structural rearrangements to nonfluorescent lactones 23 and finally tetramic acids 24. (90) A short lived, highly intense emission

17 —pH < 4.0→ 23 → 24

at 520 nm in strong acid has been observed from the fluorescamine adduct with certain indoles having aminoethyl side chains. (95) The species responsible for this emission was not determined. MDPF (18) has been proposed as superior to fluorescamine for the labeling of proteins since the fluorophore produced is stable over a wider pH range. (96) Varying the amine does not have a marked effect on the quantum yield or emission maximum of fluorescence. The quantum yields for the fluorophores from a series of peptides and amines in ethanol ranged from 0.21 for ethylamine to 0.34 for leucylalanine. (93) The quantum yields of the fluorescamine adducts with proteins are generally higher than those from peptides which are higher than those from amino acids. Solvent polarity exhibits an effect on fluorescence lifetime, quantum yield, and emission maximum. As the polarity decreases, the emission shows a slight blue shift along with a small increase in quantum yield. (97) The change in lifetime is more dramatic, ranging from 1.7 nsec in water to 11.7 nsec in acetonitrile for the benzylamine adduct. (93)

Two fluorescent reagents commonly used for the labeling of amine groups

on proteins are dansyl chloride and fluorescein isothiocyanate (FITC). FITC and dansyl chloride attached to bovine gammaglobulin have quantum yields of 0.51 and 0.22 and fluorescence lifetimes of 4.2 nsec and 11.5 nsec respectively. (98) Although fluorescamine attached to various proteins has a lower quantum yield (0.09 - 0.125) than FITC and dansyl chloride and fluorescent lifetimes intermediate between the two (7.5 - 10.3 nsec), it appears to offer some distinct advantages. (97) The reaction with amines is rapid and nearly quantitative. Even when the reagent is not present in large excess, the reaction proceeds to 80-95 percent of theoretical yield. Since fluorescamine or MDPF and their hydrolysis products are non-fluorescent, unlike fluorescein isothiocyanate or dansyl chloride, purification procedures are usually not required. Like the other reagents, the fluorescamine and MDPF fluorophores have excitation maxima outside the normal range of absorption of proteins.

In the several years since its introduction, fluorescamine has been used extensively in protein chemistry. Applications to automated amino acid analysis, determination of peptides in tryptic digests, protein assays, protein labeling for conformational and immunological studies, labeling surface proteins of membranes, and as a spray reagent for thin layer chromatography have been described. (99)

The speed of the fluorescamine reaction, the stability of the fluorophore, the lack of fluorescent impurities and the ability to quantitate the amine concentration by fluorescence intensity direct its application to automated amino acid analysis. (99, 100) Because fluorescamine reacts only with primary amines to give the fluorophore, secondary amino acids such as proline and hydroxyproline cannot be measured directly. It was found, however, that treatment of proline with N-chlorosuccinimide caused an oxidative decarboxylation followed by ring opening to 4-aminobutyraldehyde which then reacts with

fluorescamine. (101) Incorporation of this procedure allowed for automated analysis of all of the natural amino acids. As little as 1 μg of protein has been analyzed with a lower limit of 50 pmole for each amino acid. (102, 103) This gives a sensitivity two orders of magnitude better than machines using colorimetric detection.

Fluorescamine has been used for protein determinations by a semi-automated procedure with sensitivities of 10 ng. (104) The observation that proteins labeled with fluorescamine may be quantitatively collected on membrane filters that pass fluorescamine-labeled low molecular weight compounds forms the basis of an assay in which as little as 0.3 μg of protein can be detected. (105) A major interest in fluorescent protein conjugates stems from the diagnostic usefulness of labeled antibodies for the identification of antigens. MDPF was shown to be well suited to the fluorescent labeling of antigens. (96, 106, 107) Gammaglobulin from several sources has been successfully labeled with both fluorescamine and MDPF to afford intense immunofluorescent staining. The fluorophore to protein ratio was normally between 4 and 14 but could go as high as 20 without loss of activity. A nomograph was constructed for the spectroscopic determination of fluorophore and protein concentrations and fluorophore-to-protein ratio in gammaglobulin conjugates. (106) A spectro-fluorometric method has been developed to determine the accessibility of amino groups in insulin, lysozyme and asparaginase at various pH values using fluorescamine. (108) The compound has also been used to label proteins of plasma membranes (109) and erythrocyte membranes. (110) Only surface proteins are labeled as the reagent is hydrolyzed before penetrating the membrane. In order to solubilize the reagent, a complex with cycloheptaamylose was prepared before treating the membranes. (110) The labeled proteins were subsequently separated and identified by SDS gel electrophoresis. Fluorescamine has been

employed as a histochemical reagent for tissue proteins, (111, 112) for the analysis of zonal centrifuge fractions and cell homogenates after treatment with SDS to disrupt cells and organelles (113) and to identify peptide fragments and amino acids during the sequencing of bacteriophage MS2 protein on a 1 mmol scale. (114) Proteolytic enzyme activity on protein substrates can be measured by following the increase in primary amine groups available to fluorescamine as the protein is cleaved. (115) The ε-amino groups must first be blocked by succinoylation to prevent their reaction with the reagent which would produce a high fluorescence background. A spray reagent containing fluorescamine and triethylamine to stabilize the fluorophore has a lower limit of detection of 500 pmoles of amino acids or peptides (116) and can be quantitated by fluorodensitometry. (117) Using this spray to develop the peptide map of fragment D of fibrinogen showed 35 spots whereas the conventional ninhydrin spray showed only 20. (118) Fluorescamine provides a rapid and sensitive test for the detection of uncoupled products in solid phase peptide synthesis, being used to monitor the coupling reactions for the synthesis of the C-terminal hexadecapeptide of secretin. (119, 120) The reactivity of the α-amino groups of basic proteins is essentially abolished if salt linkages with DNA-phosphate groups are formed. (121) This observation prompted the elaboration of a general assay for the detection of binding parameters for the interaction of proteins and nucleic acids.

The fluorescamine reaction is not limited to protein and its constituents since any primary amine group is potentially reactive. The reagent has been used in clinical toxicology for the rapid detection of drugs of abuse such as amphetamines in biological fluids. (122) A systematic study of the reaction of fluorescamine with a series of drugs containing aromatic and aliphatic amines showed the pH optimum for reaction in the aromatic

series to be 3-4 while it is pH 9.0 in the aliphatic series. (123) This difference makes it possible to detect aromatic amines such as procainamide or sulfadiazine in the presence of aliphatic amines such as histamine or dopa. (124) The sensitivity of the assays could be improved by extraction of the fluorophore at pH 5.0-5.5 into ethyl acetate. (123)

A rapid method has been described for determining sphingosine in the 1-100 mmol range. (125, 126) The long chain amino alcohols are released from the lipid by acid hydrolysis and then allowed to react with fluorescamine. Living mouse L-cells have been labeled with fluorescamine for analysis by fluorescence microscopy. (127)

Hydrazines react with fluorescamine to produce the characteristic fluorophore which had a substantially lower limit of detection than the fluorophore obtained from isomeric phthalaldehydes. (128) Aminophosphonic acids such as the natural product ciliatine (2-aminoethylphosphonic acid) react in the same fashion as the analogous amino carboxylic acids but with a much lower reactivity toward the reagent. (129) The determination of amino groups on soluble synthetic polymers using fluorescamine has been described for two copolymers of 1-vinyl-2-pyrrolidinone with alanine vinyl ester or allylamine. (130) The procedure is limited since a calibration curve must be made for each polymer but would be useful in a series analysis where only one calibration is required.

The numerous examples of the application of fluorescamine and MDPF to biochemical problems demonstrate their usefulness and versatility as fluorescent probes. Because of their reactivity, sensitivity to detection and amenability to automation, they will certainly become indispensible reagents for both researcher and clinician.

IV. Nucleic Acid Base Analogs

A class of fluorescent nitrogen heterocycles that has proven useful in probing biochemical systems is that of imidazo[2,1-*i*]purines (*e.g.* 1,*N*^{6}-ethenoadenosine, εAdo, **25**). Closely related although much less fluorescent are the imidazo[1,2-*c*]pyrimidines (e.g. 3,*N*^{4}-ethenocytidine, εCyd, **26**).

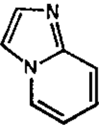
25 26

These etheno-bridged compounds are easily prepared in high yield in aqueous solution at room temperature by the reaction of an α-halocarbonyl compound such as chloroacetaldehyde with 6-aminopurines and 4-amino-1,2-dihydropyrimidin-2-ones. (131-133) The useful fluorescence properties of 1,*N*^{6}-ethenoadenosine have been summarized as: 1) long wavelength UV absorption which allows excitation outside the range of absorption of proteins and nucleic acids; 2) intense fluorescence at 415 nm which allows its detection in the presence of proteins; 3) quantum yield of about 0.6 which allows ready detection at concentrations below 10^{-8} *M*; 4) long fluorescence lifetime (23 nsec for εAMP) which allows for depolarization studies of εAdo fluorescence from nucleotide derivatives bound to molecules as large as 250,000 Daltons; and 5) small structural change in adenosine which allows the biological activity of modified co-enzymes to be preserved to a considerable extent with some enzymes. (133)

Any retention of biological activity implies that the original 1,*N*^{6} region of the adenine nucleus is not required for binding with the enzyme. Where

activity is absent, the 1,$\underline{N}^6$ region *may* be required for binding. Care must be exercised to ensure that the ε-cofactor is very pure so that the activity of a trace amount of unmodified cofactor will not be mistaken for the activity of the ε-cofactor. To illustrate this necessity, the reported (134) incorporation of εAMP into yeast $tRNA^{Phe}$ and $tRNA^{Ser}$ using CCA transferase was found to be an artifact caused by small amounts of ATP in the εATP preparation. (135) A study of the fluorescence lifetimes and emission spectra of εAMP in aqueous solution over the pH range 1.5-12.0 showed that only one emitting species exists, namely, the unprotonated form. (136)

In marked contrast to the imidazo[2,1-$\underline{i}$]purines, the 5,6-dihydro-5-oxoimidazo[1,2-$\underline{c}$]pyrimidines are moderately fluorescent only in acidic media. Protonated 3,$\underline{N}^4$-ethenocytidine is a poor emitter (Φ = 0.003, τ = 30 psec) and thus may not become as useful a fluorescent probe as ε-adenosine types. (137) The fluorescence properties are enhanced, however, due to acetylamino (Φ = 0.8, τ = 4.0 nsec) or phenyl substitution (Φ = 0.6, τ = 3.2 nsec) at C-2 of 5,6-dihydro-6-alkyl-5-oxoimidazo[1,2-$\underline{c}$]pyrimidine. (137-139) A related compound, imidazo[1,2-$\underline{a}$]pyridine (27) exhibits intense fluorescence (Φ = 0.98,



27

τ = 17.2 nsec). (137) The lack of fluorescence from the unprotonated imidazo[1,2-$\underline{c}$]pyrimidin-5-ones is attributed to the lowest energy transition being the $n\rightarrow\pi^*$ of the carbonyl. Upon protonation, the $\pi\rightarrow\pi^*$ level shifts to lower energy with respect to the $n\rightarrow\pi^*$ level so that fluorescence is observed. Phenyl or acetylamino substitution further shifts the $\pi\rightarrow\pi^*$ transition, thereby passing the $n\rightarrow\pi^*$ transition and permitting $\pi^*\rightarrow\pi$ fluorescence emission to

occur. (137)

The 1,$\underline{N}^6$-ethenoadenosine nucleotides (εAMP, εADP, εATP, ε-cAMP) (133), etheno derivatives at the oligo-, poly-, (140-142) and dinucleotide (143-146) levels and chloroacetaldehyde-modified dinucleoside phosphates (147) have been synthesized and characterized. The applications of these fluorescent probes to biological systems were recently reviewed and, very briefly, were as follows (148): 1) the ability of the 1,$\underline{N}^6$-ethenoadenosine nucleotides to act as substrates in enzyme systems was found to depend on the specificity of the enzyme-binding site and varied from no activity in some cases (εATP with firefly luciferase) to full activity in others (εATP with myosin ATPase); 2) binding studies of εADP to pyruvate kinase, H-meromyosin, and mitochondrial ATPase and of εATP to pyruvate kinase, H-meromyosin and aspartate transcarbamylase have exploited the useful fluorescent properties of the 1,$\underline{N}^6$-ethenoadenosine nucleotides in gaining more detailed information concerning these enzyme systems; 3) the chloroacetaldehyde-modified dinucleoside phosphates proved to be resistant to the action of the endonucleases pancreatic ribonuclease A and ribonuclease T_2; 4) the polynucleotides poly(1,$\underline{N}^6$-ethenoadenylic acid) and poly(3,$\underline{N}^4$-ethenocytidylic acid) have been synthesized from εADP and εCDP, respectively, using polynucleotide phosphorylase and both polymers were more resistant to nuclease action than their unmodified counterparts; 5) flavin 1,$\underline{N}^6$-ethenoadenine dinucleotide (εFAD) and nicotinamide 1,$\underline{N}^6$-ethenoadenine dinucleotide (εNAD$^+$) have been tested in enzyme systems and show a reasonable range of activity; 6) determination of fluorescence lifetimes and quantum yields provide data that can be used to calculate the relative proportions of open and intramolecularly complexed conformations, and in the case of εFAD in neutral aqueous solution it was found to exist ca. 90% as an internally complexed or stacked form; and 7) a similar study examined

the degree of internal association for a series of the chloroacetaldehyde-modified dinucleoside phosphates, εApεA (68% complexation), εApG (62%), GpεA (72%), εApεC (58%), εCpεA (15%), εApU (44%) and UpεA (28%).

εADP, εATP and ε-adenylimidodiphosphate (εADP-PNP) have been used to help characterize the nucleotide binding sites on spinach chloroplast coupling factor 1 (CF_1). (149-154) The fluorescence is quenched on binding, which allows for determination of the rates of association. All three nucleotides bind to two identical sites on CF_1 with εADP-PNP binding to a third site as well. The method of fluorescence singlet-singlet energy transfer was used to measure the distance from the nucleotide binding sites to the 7-chloro-4-nitrobenzo-2-oxadiazole (NBD-Cl, 28) sites. (152) With εADP and εADP-PNP used as donors and NBD-Cl specifically bound to either a tyrosine or an amino group of the protein used as the acceptor, a distance of 40 Å from the nucleotide sites to the NBD-Cl sites was determined. The drug quercetin (29) binds to CF_1 at a site which is 40-48 Å from the nucleotide sites. (153) εADP and

Cl
N
O
N
NO_2
28

HO
HO
O
OH
HO
O
OH
29

εADP-PNP were the donors and quercetin the acceptor in this energy transfer experiment. Heat activation of CF_1 exposes two sulfhydryl groups in the γ-subunit. When labeled with N-(4-dimethylamino-3,5-dinitrophenyl)maleimide a distance of greater than 40 Å was estimated from the nucleotide sites labeled with εADP to the sulfhydryl sites. (154)

εATP can be used as a probe of the structure and function of F- and G-actin. G-Actin binds εATP to about the same extent as it does ATP and will

polymerize to form an F-actin-εADP complex. (155) The structure and function of this complex is nearly the same as the F-actin-ADP complex as shown by the Mg-activated ATPase activity of heavy meromyosin. The fluorescence lifetime of the G-actin-εATP complex is 36 nsec with a rotational relaxation time of 60 nsec at 20°. (156, 157) The effect of Ca^{+2} and ATP on the rate of dissociation of the G-actin-εATP complex could be studied by observing the increase in intensity of fluorescence as the εATP was released. (158, 159) The nucleotide dissociates much more easily from Ca^{+2}-free than from Ca^{+2}-bound actin.

E. coli glutamine synthetase can be enzymatically adenylated with εATP. (160) The ε-adenylated glutamine synthetase exhibits similar catalytic and inhibitor properties to those of the naturally adenylated enzyme. The fluorescence of εATP and tryptophan was used to study ligand-induced conformational changes. It was found that Mg^{+2} and Mn^{+2} stabilize different conformational states of the enzyme. Similarly, the ATPase site of a nitrogenase can be labeled with εATP. (161) Distances from the ATPase site to the iron-sulfur cluster of the iron containing protein (30 Å) and the molybdenum-iron protein (10 Å) could be estimated by the resonance energy transfer technique. Also, ε-2',3'-cAMP, εAdo, εApU and εCyd were shown to serve as substrates for hydrolytic and synthetic reactions catalyzed by ribonuclease from *Penicillum brevicompactum*. (162) The divalent metal ions, Cu^{+2}, Co^{+2}, Mn^{+2} and Zn^{+2} form complexes with the etheno nucleotides accompanied by a decrease in fluorescence intensity. (163) The binding is strongest to εATP followed by εADP and εAMP and therefore the quenching of fluorescence is greatest for εATP. This difference allows direct fluorometric measurement of enzymatic reactions in which the ε-nucleotides are active substrates. The method has been tested for pyruvate kinase, phosphofructokinase and alkaline phosphatase.

In a model study of protein-nucleic acid interactions it was found that intramolecular complexation in 1,N^6-etheno-9-[(3-indol-3-yl)propyl]adenine resulted in complete quenching of fluorescence. (164) It is therefore predictable that positioning of the εAdo moiety in close proximity to a tryptophan in a protein will result in complete quenching of the εAdo fluorescence.

Nicotinamide 1,N^6-ethenoadenine dinucleotide (εNAD^+, 30) was shown to be a useful probe for studying the interaction of enzymes with NAD^+. In neutral, aqueous solution at 25°, the quantum efficiency of εNAD^+ is 0.028 and the lifetime is 2.1 nsec. (164) A simultaneous determination of quantum yield and lifetime for εAMP and εNAD^+ allows the calculation of the percentage of stacked and open conformations of the dinucleotide. At 25° in neutral aqueous solution εNAD^+ exists 45±5% in stacked conformations. (164) The binding of εNAD^+ to a series of dehydrogenases was studied, and while the affinity is lower than for NAD^+, the binding characteristics are maintained. In addition to quenching the protein fluorescence, a large enhancement of the εNAD^+ fluorescence and blue shift of the emission are observed, indicating that the open conformation of the dinucleotide is bound. (165-167) The molecular basis

of negative cooperativity in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was studied by the circular polarization of fluorescence of εNAD^+. (168) This technique yields information about the conformation and environment of a molecule in the excited state in much the same way that circular dichroism does in the ground state. The greatest change in the protein tetramer occurs with the binding of the first εNAD^+, with a similar structural change being observed in each nicotinamide subsite. Circular polarization of fluorescence of εNAD^+ was also used to study the nicotinamide binding sites of a large number of dehydrogenases. (169) A structural difference was found for the adenine subsites in cooperative dehydrogenases as opposed to those in non-cooperative dehydrogenases.

Nicotinamide 3,*N*^{4}-ethenocytosine dinucleotide (εNCD^+, **31**) was prepared and studied in several dehydrogenase systems. (170) Although it proved to be a better substrate than εNAD^+ and comparable in many instances with the natural cofactor NAD^+, its fluorescence properties are poor, limiting its utility as a fluorescent probe for the study of enzyme binding and molecular dynamics.

Polyadenylic acid has been modified by chloroacetaldehyde. (171) The modified polymer is inhibitory when unprimed 70S avian myeloblastosis (AMV) RNA is used as the substrate. It is possible that the poly (1,*N*^{6}-ethenoadenylic acid) may be useful as an inhibitor of oncogenic viral polymerases. The chloroacetaldehyde reaction has been extended to naturally occurring 70S AMV RNA. (172) In addition to being an analog inhibitor of 70S RNA the modified RNA is potentially an important probe for studying interactions with the cognate DNA polymerase.

A related group of tricyclic nitrogen heterocycles has been found to exhibit fluorescence and to show potential as probes of biological systems. The naturally occurring "Y bases" (*e.g.*, **32**) are highly substituted guanosine

32

derivatives which, upon excitation at 315 nm, show strong fluorescence emission at 450 nm with a quantum efficiency of 7%.(9) As a result of these fluorescence properties, Y base, which is found in phenylalanine specific tRNAs from several sources, has been exploited in a variety of experiments designed to gain information concerning tRNA tertiary structure. For example, using Y base located in the anticodon loop of yeast $tRNA^{Phe}$ as an energy donor and acriflavin linked to the periodate-oxidized 3'-terminus of yeast $tRNA^{Phe}$ as the energy acceptor, a distance of from 34 to 61 Å between the fluorophores was estimated by resonant energy transfer techniques. (173, 174) Structural elucidation of the Y bases (imidazo[1,2-a]purines) led to the synthesis of other fluorescent tricyclic nucleosides having related structures. From 1-aminoguanosine, 33 and 34 were prepared using $POCl_3$/DMF and biacetyl

R = 1-β-D-RIBOFURANOSYL
R = 1-(2'-DEOXY-β-D-RIBOFURANOSYL)

	33	34	35
λ^{em}	450	540	420
λ^{ex}	285	360	334

respectively. (175) The reaction of guanosine or 2'-deoxyguanosine with glycidaldehyde produces 35 in which the position of the hydroxymethyl group was not established. (176) Substituted malondialdehydes have been shown to give well characterized, fluorescent products when allowed to react with guanine. (177) The product of the reaction of p-methoxyphenylmalondialdehyde (36) and guanine (37) is the tricyclic 38 which emits at 510 nm with a quantum

36 37 38

yield of 0.004 and a lifetime of 0.7 nsec when excited at 320 nm in aqueous solution (pH 6.8). Both the quantum yield and lifetime increase in going from protic to non-protic solvents. For example, 38 has an emission maximum at 500 nm, a quantum yield of 0.031 and a lifetime of 3.6 nsec in ethyl acetate. (177) The fluorescence properties of the products of the reaction of several substituted malondialdehydes with guanine were shown to be quite similar, regardless of substitution. The reaction of 36 with guanosine, 7-methylguanosine, isocytosine, and 5-hydroxyisocytosine has been studied and the products have been identified as 39-42, respectively. (178) The quantum

39 40

41 R=H
42 R=OH

yield dependence on solvent polarity was found to follow that of the parent compound, 38.

A series of "stretched-out" analogs of adenine have been prepared in which the purine ring has been extended by the formal insertion of a benzene ring between the pyrimidine and imidazole rings. (179, 180) The linear version of this series (43 a-h) was found to possess useful fluorescence properties, emitting at 372 nm with quantum yields in the range of 0.44 and lifetimes near 3.7 nsec when excited at 330 nm. (181, 182) The value of these fluorescence properties is closely tied to the biological activity of the compounds. Compounds 43b and 43c are active cytokinins making them potentially useful as probes for detecting sites and modes of cytokinin action. (182) Tests with adenosine deaminase show both 43a and 43d to be substrates with K_m and V_{max} values close to those of the natural substrate. (181) The "stretched-out" versions of the adenine nucleotides 43 e-h were tested with a representative

43a R'=H R=H
b R'= $(H_3C)_2C{=}CHCH_2$ R=H
c R'= $C_6H_5CH_2$ R=H
d R'=H R=HO–(ribose)
e R'=H R=HOP(O)(HO)–O–(ribose)
f R'=H R=HOP(O)(HO)–O–P(O)(HO)–O–(ribose)
g R'=H R=HOP(O)(HO)–O–P(O)(OH)–O–P(O)(OH)–O–(ribose)
h R'=H R= (cyclic 3',5'-phosphate, O=P(OH))

group of kinases which exhibit broad to moderate nucleotide specificity. (183) These nucleotide analogs in general bind strongly and slow somewhat the enzymatic rates. The useful fluorescence properties of the *lin*-benzoadenine nucleotides and their increased π interactions can be directed to a variety of studies of static and dynamic interactions with different moieties, complexation, the nature of enzyme binding sites, and conformational changes induced by surrogate coenzyme/enzyme binding. Moreover, the defined dimensional change, i.e., a lateral stretching of the normal substrates or cofactors by 2.4 Å in compounds **43 a-h**, provides new information concerning the space available at their binding sites with various enzymes.

In conclusion, the many biological and chemical applications of these representative fluorophores demonstrate a useful motivation for the synthesis of fluorescent heterocyclic compounds.

Acknowledgment

Our work has been supported by Research Grant GM-05829 from the National Institutes of Health, United States Public Health Service. We owe special thanks to our colleague, Prof. Gregorio Weber, for his contributions in theory, apparatus and guidance.

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Received, 4th August, 1976