# Fluorescein Hapten: An Immunological Probe

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**Edward W. Voss, Jr., Ph.D.**

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**Edward W. Voss, Jr., Ph.D.**
Professor of Microbiology
Department of Microbiology
University of Illinois
Urbana, Illinois



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# PREFACE

As the solutions to existing problems in immunology demand relatively more sophistication experimentally, development of new probes and systems are required. Because of the extreme diversity of the immune response, various biochemical compounds can serve as immunological probes in the form of haptens. Historically, many hapten systems have proven suitable for the study and characterization of soluble antibody molecules. Although much knowledge has been gained from the study of complex immunogens, such as proteins and polysaccharides, hapten systems have been vital in the elucidation of the molecular basis of immunological specificity.

The biochemical and immunochemical properties of classical monophenyl haptens, such as arsanilic acid and 2,4 dinitrophenyl (Dnp), were important in the synergistic growth of immunoglobulin structure and the chemical nature of the antigen-binding site. The Dnp system proved especially noteworthy since powerful spectral and fluorescence methodology eventually evolved. Upon this awareness of new methodology, immunochemists expanded the haptenic repertoire to include the use of polyaromatic haptens such as 4-aminonaphthalene-1-sulfonate. Measurements of fluorescence enhancement due to the interaction of the naphthalyl ligand with specific antibody provided further information regarding the antibody active site. Recently, relatively large haptens, such as fluorescein, have been utilized as intrinsic haptenic probes. This monograph entitled *Fluorescein Hapten: An Immunological Probe* is intended to comprehensively define the properties of fluorescein. As indicated in the Table of Contents, highly fluorescent haptens such as fluorescein can be used in the study of both soluble protein and cell related systems. The flexibility inherent in a highly fluorescent probe is exemplified in the diversity of experiments performed. The spectrum includes the mechanism of spectral shifts and fluorescence quenching upon specific ligand binding, fluorescence polarization, circular dichroism, solvent perturbation studies, isotope exchange, protein denaturation kinetics, heavy and light chain reassociation studies, idiotypic analyses, molecular basis of affinity including association and dissociation rate constants, and cellular aspects of the immune response.

As editor I am deeply indebted to the authors who have contributed to this monograph. Their expertise with various aspects of the fluorescein system is clearly exemplified in the pertinence and high quality of each chapter.

Edward W. Voss, Jr.

## THE EDITOR

**Edward W. Voss, Jr., Ph.D.,** is Professor of Microbiology in the School of Life Sciences at the University of Illinois at Urbana-Champaign.

Dr. Voss received his A.B. degree from Cornell College, Mt. Vernon, Iowa in 1955. He obtained his M.S. and Ph.D. degrees in 1964 and 1966, respectively, from the Department of Microbiology, Indiana University School of Medicine, Indianapolis. After doing post-doctoral work at Washington University School of Medicine, St. Louis, he was appointed an Assistant Professor of Microbiology at the University of Illinois, Urbana-Champaign in 1967. He became an Associate Professor of Microbiology in 1971, and Professor in 1974.

Dr. Voss is a member of the American Association for the Advancement of Science, American Association of Immunologists, American Society of Biological Chemists, New York Academy of Sciences, Reticuloendothelial Society, Federation of American Scientists, American Chemical Society, and the honorary society Sigma Xi.

He has been the recipient of a National Science Foundation Faculty Fellowship in Science (1975-77), Associate Appointment to the Center for Advanced Study (University of Illinois, 1981-82), Designated Tour Speaker American Chemical Society (1984-85), and the Distinguished Lecturer Award of the School of Life Sciences (University of Illinois, 1983).

Dr. Voss has been the recipient of research grants from the National Institutes of Health, the National Science Foundation, The American Lupus Society and private industry. He has published more than 100 papers. His current research interests are in affinity maturation of the immune response and the autoimmune disease systemic lupus erythematosus.

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# CONTRIBUTORS

**James N. Herron, Ph.D.**
Research Associate
Department of Biology
University of Utah
Salt Lake City, Utah

**David M. Jameson, Ph.D.**
Department of Pharmacology
University of Texas Health Science
Center at Dallas
Dallas, Texas

**Michael R. Jarvis, Ph.D.**
Fellow
School of Medicine
Washington University
St. Louis, Missouri

**David M. Kranz, Ph.D.**
Center for Cancer Research
Massachusetts Institute of Technology
Cambridge, Massachusetts

**Dean A. Oesterle, Ph.D.**
Department of Chemistry
University of Illinois
Urbana, Illinois

**David M. Reinitz**
Department of Microbiology
University of Illinois
Urbana, Illinois

**Robert M. Watt, Ph.D.**
Assistant Professor
Department of Microbiology
Upstate Medical Center
State University of New York
Syracuse, New York

# *Part I*
# *Immunochemistry of Fluorescein as a Hapten*

Chapter 1

# IMMUNOLOGICAL PROPERTIES OF FLUORESCEIN

**Edward W. Voss, Jr.**

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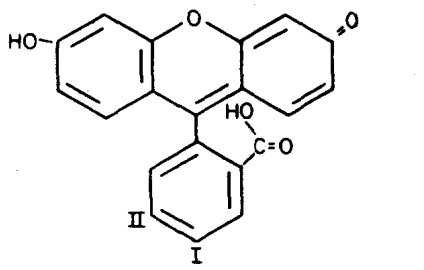
# I. INTRODUCTION

It is generally considered that the immune response is capable of generating $\sim 10^7$ antibodies of different specificity.[1] The diversity of the response has been primarily investigated using immunogenic macromolecules such as proteins and polysaccharides. However, naturally occurring macromolecular antigens are complex and it is difficult to definitively ascertain identity and conformation of their functional groups. Thus, haptens, as defined by Landsteiner,[2] became an indispensible tool of the immunochemist. With haptenic reagents it has been possible to test the specificity of antibodies against almost any defined compound that organic chemists have devised. In this context it is important to appreciate the uniqueness of the immune response. Generally, the protein chemist determines biological specificity in terms of the isolated protein's (e.g., enzyme, receptors, serum-binding proteins, etc.) interaction with a variety of natural substrates or ligands. In contrast, the immunochemist can devise compounds that are not natural to prokaryote or eukaryote systems and elicit specific binding antibodies. Thus, the enormous diversity inherent in the immune response provides a framework within which the chemist can utilize antibodies as models to explore the molecular basis of biological specificity.

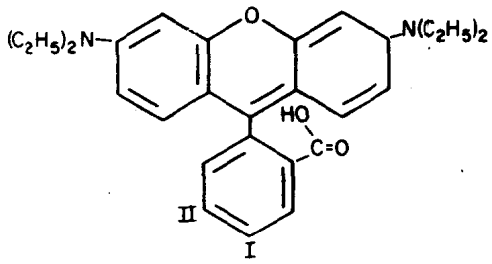
Haptens, by definition, are small compounds of defined structure possessing molecular weights of $<1000$ daltons. They are generally immunologically univalent in that they comprise a single antigenic determinant based on interactions with an antibody active site. Univalency demands that multiple moieties of the hapten must be covalently linked to a suitable macromolecular carrier in order to stimulate the immune response. Thus, covalent linkage to appropriate groups on the carrier serves to convert univalent haptens to the polyvalent state. The same principle is employed in synthesizing reactive test antigens. If the hapten is reacted with a specific antibody population as a univalent moiety it is generically termed a ligand. As a freely diffusible ligand, the hapten can be used to study antigen-antibody reactions on the basis of kinetic or equilibrium measurements. In the polyvalent state, extensive lattice formation results in such visible antigen-antibody reactions as precipitation, agglutination, and lysis. Although the latter are important, they can complicate certain types of studies.

Historically, the chemical nature of haptens has evolved from chemical modifications of known antigens, such as halogenation of proteins, to the almost preferential use of aromatic determinants. In response to demands for increased sensitivity, accuracy, and reliability, an interesting trend occurred in that there was a progression from monophenyl to polyaromatic compounds. Implicit in this trend was a significant change in spectral assays as noted in the advancement from absorption to fluorescence measurements. It is now apparent that other changes occurred in the qualitative and quantitative nature of the immune response. These changes will be more fully discussed in the description of the fluorescein system. Thus, environmentally sensitive systems could be employed through the concept of haptens, and spectroscopic measurements exploited. Different spectral measurements were employed with the various hapten systems. With the use of the important 2,4-dinitrophenyl (Dnp) system, investigators utilized a nonfluorescent haptenic dye with appropriate spectral properties to develop a fluorescence quenching assay.[3] The assay was based on the principle that when Dnp ligands were bound to the active site of specific anti-Dnp antibodies, the fluorescence emitted by excited tryptophanyl residues within the immunoglobulin molecule was quenched. Bound Dnp ligand also exhibited a bathochromic spectral shift in the $\lambda_{max}$.[4] Perhaps one of the disadvantages of the Dnp system was that the intrinsic fluorescent probe (i.e., tryptophan residues) was heterogeneous when the measurements were applied to serum derived antibody populations. This concern was alleviated to some degree with the advent of specifically binding homogeneous myeloma and hybridoma immunoglobulins.

Naphthalenic haptens were effectively employed as antigenic probes because of their




Fluorescein




Rhodamine B

FIGURE 1. The structure of fluorescein (top) and rhodamine B (bottom). I and II represent the isomers of both dyes. Generally, the substituents found in position I or II are the isothiocyanate, amine, or cyanuric acid derivative.

unique fluorescent properties.[5] In general, compounds such as 1-dimethylaminonaphthalene-5-sulfamido (DANS) exhibit a low quantum yield of fluorescence when excited in an aqueous solution. However, when specifically bound to the antibody active site, the naphthalyl ligand shows a significant increase in fluorescence quantum yield ($\Phi$) upon excitation. The sensitivity of the biphenyl hapten to certain environments and electronic states provided additional information about the nature of the antibody active site.

Consideration of relatively large polyaromatic haptens found a theoretical basis in the considerations of Karush.[6] In a discussion of immunological specificity and molecular structure, Karush emphasized the importance of high-affinity antibodies in fully understanding the physicochemical basis of specificity. It was hypothesized that the use of relatively large haptenic groups would provide important advantages in eliciting high-affinity populations and to ascertain the energetic upper limits of antigen-antibody interactions. However, in applying these considerations, one must consider other requisites than molecular weight, such as ionic nature, spectral properties, availability of an activated derivative for covalent conjugation, and immunogenic potential. The latter, of course, is usually determined empirically.

## II. FLUORESCEIN AS A HAPTEN

Upon consideration of all of the above parameters, it was apparent that the fluorescein-rhodamine series of highly fluorescent dyes was worthy of investigation. Fluorescein was selected in the initial studies since it was a dianion at neutral pH (Figure 1). In general, anions have proven to be relatively better immunogens than neutral or cationic determinants. Fluorescein is relatively large in terms of the hapten scale possessing a molecular weight of ~380 daltons. In addition, fluorescein possesses maximum absorption properties in the visible

range ($\lambda_{max}$ = 493 nm) with a suitable extinction coefficient of 72,000 $M^{-1}$ cm $^{-1}$. Upon excitation at the $\lambda_{max}$, fluorescein emits fluorescence at 525 nm with an efficient quantum yield of 0.92. Both the excitation and emission maxima are at wavelengths where the protein is invisible. As will be described later in more detail, the spectral and fluorescence properties of fluorescein are important in measuring ligand-antibody interactions, active site microenvironments and solvent effects. Further, the isothiocyanate derivative of fluorescein is readily available for conjugation reactions, facilitating its use.

After extensive research, the fluorescein hapten system[7-9] is now established as a suitable immunological model system for a number of reasons. Fluorescein has proven to be a potent immunogen upon covalent conjugation to an appropriate foreign carrier (e.g., keyhole limpet hemocyanin or bovine gamma globulin). The immunogenic potency is supported by the fact that 3 to 5 mg/mℓ of purified antifluorescyl antibody can be obtained from rabbit antiserum and 1 to 2 mg/mℓ from murine serum or ascites fluid. Voss et al.,[10] demonstrated that, largely due to the size and shape of the fluorescyl ligand, it approximates a space- or site-filling antigenic moiety.

To support this conclusion, it was shown that there are no significant side-group effects in the binding properties of the fluorescyl ligand.[10] For example, in the monophenyl systems, such as Dnp, measurable side-group effects can be determined. Thus, in the interaction of a series of Dnp ligands with heterogeneous anti-Dnp antibody populations, one generally observes a dependency of affinity on the structure of the ligand.[11] In terms of order of reactivity DnpOH < $DnpNH_2$ < Dnp-gly < Dnp-ϵ-lysine, it is evident that as the ligand structurally simulates the complete antigenic determinant within the immunogen affinity increases. Such experimental observations suggest that anti-Dnp antibodies recognize the Dnp determinant (immunodominant group) and in addition some portion of the carrier. One interpretation of this binding pattern is that the Dnp ligand is not site-filling and therefore the antibody recognizes that portion of the carrier that satisfies the space-filling state. As the size of the hapten increases, the contribution of the carrier-effect should concomitantly decrease. Characteristically, no carrier effect has been demonstrated in the antifluorescein system,[10] consistent with the concept of a site-filling determinant. This advantageous property also minimizes anomalous effects arising from heterogeneous linkages of the hapten to various groups within a protein carrier.[9] Similarly, IgG antifluorescyl antibodies have been reported to possess relatively high binding affinities for the homologous ligand.[12-15] The observed high affinities are consistent with the size, aromaticity and dianionic nature of the fluorophore.

Watt and Voss[16] investigated the complex mechanism by which antibody-bound fluorescein undergoes a red spectral shift and its fluorescence is quenched. When fluorescein is bound to heterogeneous rabbit or murine antifluorescyl antibody populations, the bound ligand possesses a $\lambda_{max}$ at longer wavelengths relative to 493 nm for the ligand free in neutral solution. Thus far, the magnitude of the bathochromic shift has ranged from 10 to 25 nm for antibody bound ligand. The extent of the shift seems to correlate with the average affinity of heterogeneous antibody populations. Thus, low affinity primary antifluorescyl antibodies show a minimal shift of 10 nm, while hyperimmune antibodies exhibit significantly greater shifts. This correlation has not been verified with murine monoclonal antifluorescein antibodies of either the IgG or IgM classes.

The relative reduction in the quantum yield of fluorescence of antibody-bound fluorophore relative to free (unbound) fluorescein has been consistently >90%. Figure 2 shows the typical fluorescence quenching observed with heterogeneous antifluorescein antibody populations. The degree of fluorescence quenching is not constant and appears to be dependent on many factors. Screening of individual L-amino acids for the ability to quench the fluorescence of fluorescein resulted in the finding that L-tryptophan, and to a lesser extent L-tyrosine, were effective quenchers.[16] Stern-Volmer analyses and fluorescence lifetime ($\tau$)

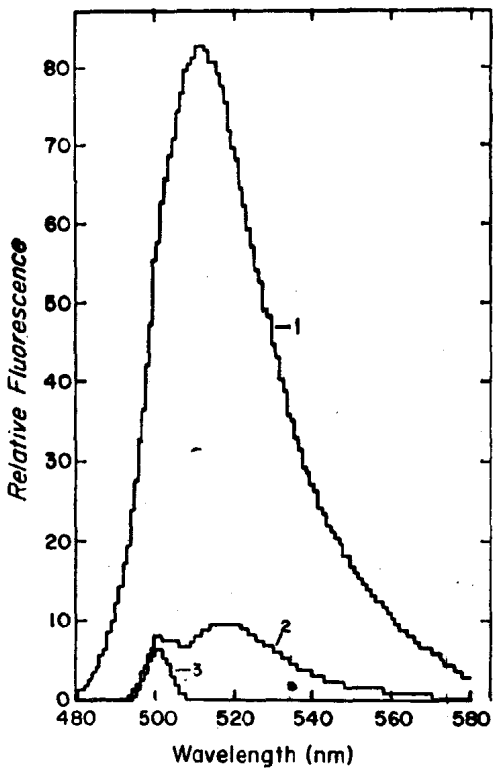



FIGURE 2. Fluorescence emission spectra for the determination of the quantum yield of fluorescein bound to antifluorescyl IgG antibodies. The line labeled 1 is the technical emission spectra of free fluorescein; 2 is the technical emission spectrum of antibody-bound fluorescein; and 3 is the solvent scatter. All spectra were recorded at 20°C. The optical density at 500 nm of both ligand preparations was 0.1. Excitation was at the isosbestic wavelength.

measurements of tryptophan quenching revealed complex fluorescein-tryptophan dynamics (involving both static and dynamic components). In addition, titration of antifluorescein IgG antibody with fluorescyl ligand showed that fluorescence emission of excited tryptophanyl residues within the antibody active site was quenched upon fluorophore binding. The latter is similar to the model anti-Dnp quenching system previously described.[3] Collectively, these experimental results suggest that tryptophan is indeed an interactive component within the antifluorescyl active site.

Derivatives of fluorescein can be readily obtained as two steric isomers, designated I and II (Figure 1). Isomer I is the 5-derivative of fluorescein, usually involving amine, triazinyl, or isothiocyanate substituents. Isomer II is the 6-derivative of fluorescein. Due to the existence of these steric isomers of fluorescein (I and II), it has been possible to elicit antibodies separately to resolved isomers and study the relative effect of steric positioning, immunogenicity and reactivity.[17] Historically, steric orientation has not been studied extensively in terms of immunological specificity, and is a little understood phenomenon. For example, visible absorption spectra of fluorophore bound to the active site of purified antibody (affinity chromatography) elicited to the two isomers (I and II) revealed distinct spectral shifts suggesting that the active sites of the two antibodies (i.e., anti-I and anti-II) were different.

Difference spectroscopy of fluorescein bound to the two purified antibody preparations supported this interpretation.[15]

## III. ACTIVE SITE SOLVENT PERTURBATION STUDIES

Solvent perturbation studies have been used to further characterize the antifluorescyl active site. Deuterium oxide, iodide, and oxygen have been employed in perturbation studies.[18-20] The relative fluorescence quantum yield of fluorescein bound to specifically purified high affinity "liganded" rabbit and chicken IgG antibody was significantly increased in deuterium oxide ($D_2O$) relative to the complex in $H_2O$. The degree of fluorescence enhancement correlated with the average intrinsic affinity of the antibody for the ligand. Fluorescence enhancement in 95% $D_2O$ was identified for ligand bound to 7S IgG and $F(ab)'_2$ fragments derived from the same molecule. The isotope effect was not due to ligand dissociation as shown by a comparison of difference spectroscopy and equilibrium dialysis results in $D_2O$ and $H_2O$, and by similar enhancement of fluorescence with affinity-labeled antibody.[18] Identical extrinsic circular dichroism of antibody-bound fluorophore in $D_2O$ and $H_2O$, coupled with the absence of a measureable isotope effect on the quenching of fluorescein by L-tryptophan, supported the contention that the $D_2O$-effect was an excited state phenomenon.[18]

Comparative kinetic studies of ligand dissociation and $D_2O$ enhancement of fluorescence were performed with both heterogeneous and homogeneous antifluorescyl IgG antibodies.[20] Heterogeneous rabbit and homogeneous murine (monoclonal) antibody preparations were purified by affinity chromatography and found to be pure IgG by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoelectrophoresis. Relatively high affinities of all liganded antibody preparations were determined by dissociation rate studies, exhibiting comparatively long lifetimes for the dissociation of bound fluorescein. Rabbit antifluorescyl preparations were found to display extensive heterogeneity of off-rates, while murine monoclonal antifluorescyl preparations were characterized by a single off-rate indicating homogeneity. Deuterium oxide fluorescence enhancement studies showed that heterogeneous kinetics were observed with both heterogeneous and homogeneous antibody active sites. Temperature studies of ligand $D_2O$ enhancement and dissociation rates using homogeneous antifluorescyl antibodies revealed similar, yet different activation energies (22.7 ± 0.8 cal and 20.2 ± 0.3 cal, respectively) for both phenomena. The studies demonstrated that the antifluorescein antibody active site consists of both solvent accessible and relatively inaccessible components, and that the binding of ligand involves both exchangeable hydrogen atoms and other as yet unresolved interactions.

Differential accessibility of liganded, high affinity rabbit antifluorescyl IgG antibody combining sites to the aqueous milieu has been investigated by solvent perturbation of the extrinsic fluorescence of bound fluorophore.[19] Iodide, a dynamic quencher of fluorescein was selected for use in these studies after examination of a number of water-soluble fluorescence quenchers. Quenching of antibody-bound fluorophore by iodide was measured with a number of liganded antifluorescyl IgG preparations, demonstrating partial solvent exposure of the fluorophore as well as heterogeneity of the high affinity antibody populations. Fluorescence quenching lifetime, and absorption spectroscopy provided evidence that the antibody-bound fluorophore quenched by iodide interacted with it directly, and that anomalous binding of the anion to the surface of the protein, resulting in ground state perturbations of the immunoglobulin, could not explain the observed results.

Oxygen quenching studies with various monoclonal antifluorescyl IgG antibodies have revealed that the accessibility of bound ligand is a constant and independent of affinity.[21]

## IV. AFFINITY MATURATION IN THE ANTIFLUORESCYL RESPONSE

One of the most important characteristics of the antifluorescein IgG antibody system is

the extensive change in affinity (maturation) that occurs following the initial administration of fluorescyl conjugated immunogens.[14] Antifluorescyl IgG antibodies purified from early primary antisera (rabbit or murine) exhibit average intrinsic affinities (Keq values) of $\sim 10^5$ $M^{-1}$, while antibodies purified from late hyperimmune sera characteristically show average Keq values $> 10^9$ $M^{-1}$. However, resolution of these heterogeneous populations into subpopulations on the basis of affinity revealed antibody fractions in the range of $10^4$ to $> 10^{12}$ $M^{-1}$. Dissociation rate measurements[15] have even indicated heterogeneous antifluorescyl antibody subpopulations with Keq values of $\geq 10^{14}$ $M^{-1}$.

The extensive range in affinities exhibited by antifluorescyl IgG antibodies is significant because it provides a foundation upon which one can study (1) high affinity interactions in terms of biological specificity, (2) the cellular and genetic mechanisms involved in affinity maturation, and (3) affinity ranges within the different Ig classes and subclasses. For example, although high affinity sites have been almost solely confined to IgG antibodies, one report showed that high affinity IgM rabbit antibodies were elicited using the fluorescyl hapten system.[22] Comparative analyses of IgM and IgG antifluorescyl antibody active sites substantiated the observation that affinity purified IgM preparations possessed high affinity active sites. Dissociation rate data confirmed that some IgM molecules within the purified antibody population possessed Keq of $> 10^{10}$ $M^{-1}$.

In terms of the potential to understand the molecular basis for affinity and biological specificity, it is becoming more apparent that large ligands like fluorescein provide certain advantages over the monophenyl hapten systems. Although the difference in interactions between low and high affinity antifluorescyl antibodies is not known at this time, it is evident that the characteristic affinities observed can be attributed to various properties of fluoreseein. The relatively large size of fluorescein and related site-filling capacity infers that a multiplicity of interactions accounts for the range of affinities observed with various antifluorescyl antibody populations. These multiple interactions may stem from the direct participation of hypervariable regions from both the heavy and light chains comprising the active site. The nature of the interactions can be inferred from some of the results presented above. The extent of the bathochromic shift exhibited by bound ligand infers van der Waals' forces. The deuterium oxide studies suggest the importance of hydrogen bonding between the antibody protein and bound ligand. Finally, studies by Kranz et al.[23] infer that salt bridges may also play a role in the binding of fluorescein.[23] Energetically, these forces contribute collectively to a significant negative free energy ($\Delta G^\circ$) and the formation of a relatively stable complex.

## V. MONOCLONAL ANTIFLUORESCYL ANTIBODIES

The antifluorescyl repertoire in BALB/c mice has been examined by producing monoclonal (hybridomas) antibodies with fluorescein specificity.[24] Using fluorescyl conjugated keyhole limpet hemocyanin (KLH) as the immunogen, monoclonal antifluorescyl antibodies were obtained that contained kappa light chains and gamma heavy chains (IgG1 or IgG2). Isoelectric focusing profiles of reduced and alkylated Ig preparations demonstrated restricted, yet relatively different spectrotypes. Collectively, the hybridomas provided a diverse range of antibody affinities as determined by several methods, including dissociation rate and ligand inhibition studies. Homogeneity of purified preparations was confirmed by dissociation rate experiments (see Chapters 4 and 5), which showed that each monoclonal antifluorescyl preparation possessed a single first order off-rate. Absorption spectra of bound fluorescein revealed distinct relative differences within the active sites of the molecules studied and suggested the lack of any apparent correlation between affinity and $\lambda_{max}$.[24]

The monoclonal antifluorescyl antibodies have been valuable reagents to study other important immunological phenomena. For example, in order to determine if the heavy and

light chains derived from clones of different affinities for the fluorescyl ligand can be recombined in various ways to better understand their relative contributions, the clones have been important. These studies are reported in depth in Chapter 8.

Monoclonal antibodies are also important in studies to define the mechanism involved in binding of the fluorescyl ligand. Such studies are extremely difficult with heterogeneous antibody populations, facilitating the use of the homogeneous sites. Relative quenching of fluorescence of bound fluorescein, deuterium oxide-induced enhancement of fluorescence, and the effects of pH on binding kinetics have been measured for various clones.[24] Individual antifluorescyl hybridomal proteins exhibited significant differences in the relative contribution of various forces (hydrophobicity, hydrogen bonding, and ionic interactions) to bonding and hence affinity. An analysis of the extent of such variations in bonding mechanisms between monoclonal antibodies specific for the same moiety is indicative of the functional diversity of active sites. In some cases, a particular clone possesses unique properties which permit extensive studies. For example, clone 20-20-3 was significantly different from other clones in the absorption profile of bound ligand.[24] Absorption spectra of fluorescyl ligand bound by purified 7S IgG antibody, Fab fragments, and reassociated heavy and light chains indicated that protonation of the fluorescyl ligand by a residue within the antibody active site contributed to the binding free energy. Comparative dissociation rates of fluorescein and a structural analogue, rhodamine 110, were used to quantitatively substantiate the contribution of the interaction. Association and dissociation rate studies with fluorescein and antibody indicated that: (1) the antibody active site appeared to undergo a conformational change upon ligand binding, and (2) neither intact interchain disulfide bonds nor intersite cooperativity affected the dissociation rate of bound ligand.[24]

All of the antifluorescyl hybridomas discussed in this section were a result of the fusion of immune splenocytes stimulated with fluorescein covalently conjugated to the carrier KLH. Recent studies have indicated that when fluorescein is conjugated to aminoethyl-Ficoll® (AE-Ficoll®) a significantly different series of hybridoma proteins is obtained.[25] Antifluorescyl clones obtained with the Ficoll® carrier have been predominantly IgM or $IgG_3$ with either kappa or lambda light chains. Similar results have been obtained with the Dnp-Ficoll® system.[26] These results may be explained on the T-independent nature of Ficoll®. Such carriers probably obviate Ig class switch and proposed somatic mutational events.[27-29] It is conceivable that antifluorescyl hybridomas obtained upon immunization with fluorescyl-derivatized AE-Ficoll® may represent germ line Ig products.

## VI. FLUORESCYL ANALOGUES

The successful use of fluorescein as a potent antigenic determinant raises the question of other compounds in the fluorescein-rhodamine series. Certain observations and trends can be cited which cannot now be fully explained.

First, the isothiocyanate derivative of fluorescein amine (I or II) is currently the most suitable analogue for covalent conjugation to a carrier in the construction of immunogens. If either 5-(4,6-dichloro-s-triazin-2-yl) aminofluorescein (5-DCTAF) or 6-(4,6-dichloro-s-triazin-2-yl) aminofluorescein (6-DCTAF) are used in the conjugation reaction to KLH, little or no antifluorescein immune response is observed. Dichlorotriazines generally react faster than isothiocyanates. They also react with SH, histidine, and tyrosyl residues. Such cyanuric acid derivatives are very stable. However, it is not certain as to why the triazinyl derivative so alters the immunogenic properties of fluorescein. Fluorescyl test antigens constructed with 5-DCTAF or 6-DCTAF are equally reactive with antifluorescyl antibodies as the isothiocyanate conjugates.

Second, certain structural modifications of fluorescein can be tolerated at the immunogen level. Antibodies have been elicited to dichlorofluorescein and the fluorescence quenching reaction studied upon reaction of ligand and antibody.[5]