STUDIES ON THE STRUCTURE AND OPTICAL BEHAVIOR OF THE ACRIDINE ORANGE COMPLEX WITH POLY-α,L-GLUTAMIC ACID

by

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INTRODUCTION

The biophysics of dye:polymer complexes is still in its infancy, despite the fact that very interesting optical phenomena associated with the attachment of various dyes to polymeric molecules have been observed for many years. Of particular interest has been the combination between the so-called metachromatic dyes and certain polymers, called chromotropes, which may induce the phenomenon of metachromasy under the proper conditions. Metachromasy was first reported by Cornil in 1875 (see Bergeron and Singer, 1958) and may be loosely defined as a hypsochromic, hypochromic shift in the absorption spectra of certain (usually basic) dyes as the concentration in aqueous solution is increased. Chromotropes, when added to dilute solutions of metachromatic dyes, possess the property of being able to induce the spectral changes characteristic of either increasing the dye concentration (metachromasy) or decreasing the concentration of dye (reversal of metachromasy to the orthochromatic spectra of very dilute dye solutions), depending upon the relative concentrations of the two components. The content of this dissertation will be devoted to these metachromatic dye: chromotrope complexes in contradistinction to the many other dye:polymer combinations whose optical properties arise mainly from the union, rather than from interactions between bound dye molecules.

Interest in the molecular structure of the dye:biopolymer complexes was spurred onward several years ago by the discovery of Stryer and Blout (1961) that acridine orange, a metachromatic dye often used in the in vitro staining of nucleic acids and mucopolysaccharides, became strongly optically active upon binding to the right-handed, α-helical conformation of poly-L-glutamic acid. In addition, the induced Cotton effects were reversed in sign by combination with the left-handed helix of poly-D-glutamic acid. Dye bound to the coiled conformation of this synthetic polypeptide was not optically active. These results immediately suggested the possibility of using the optical activity of bound dye as a sensitive indicator of the local conformation, that is, the three-dimensional arrangement, at the binding sites in certain biological macromolecules. Furthermore, the large magnitude of the induced optical activity suggested this new property arose from interactions between adjacently bound dye molecules, not from the binding of single dye molecules to isolated sites in an asymmetric environment, thereby suggesting that polyglutamic acid be classified as a chromotrope. In fact, the two absorption spectra given by Stryer and Blout loc. cit. for the coil and helix complexes appeared similar to those of concentrated acridine orange solutions. Because the degree of optical interaction must depend upon the distance and angle between the adjacent chromophores, it seemed that the absorption and fluorescence

properties of the bound dye might vary with polymer conformation as well and thus, along with the optical activity changes, provide the basis for a new method of investigating polymer structure.

The molecular structure of the acridine orange:polyglutamic acid complex is unknown. Neither is the structure of any other metachromatic dye:chromotrope complex known. It is, in fact, one of the enigmas of research in this field that in spite of the accumulation of such a large body of varied, experimental knowledge, especially for the nucleic acid and polynucleotide complexes, a complete picture of the binding process or satisfactory models of the complex structures have not yet emerged. Unfortunately, these past studies have been hampered or limited by the use of chromotropes which possess chemically different types of binding sites (nucleic acids), chromotropes which possess poorly defined secondary structures in solution (polysaccharides), and chromotropes which seemingly possess no stable secondary structures (such as polyphosphate). Consequently, the relationships between polymer structure (both primary and secondary), the complex structure, and the optical properties of the complex remain poorly understood. On the other hand, the use of polyglutamic acid for a chromotrope offers some promise of elucidating these relationships. This biopolymer appears to possess only one type of chemical binding site (if the end functional groups are ignored) and undergoes a

well-documented conformational transition from a coiled structure to an α-helix over a fairly narrow pH range in aqueous solutions (Doty et al., 1957; Wada, 1960).

The main subject of this dissertation is a systematic investigation of the absorption, fluorescence, and optical rotatory properties of the acridine orange:poly-L-glutamic acid complex in aqueous solutions. This work was prompted by the almost complete lack of absorption and fluorescence data on this system in the literature, easy access to spectroscopic instruments, and the desire to ultimately relate all of the optical properties of the complex in a unified way to its structure. The two immediate goals were to derive as much structural information as possible from relatively simple optical measurements and to discover how the optical behavior of the bound dye is related to the conformation of the polymer. It is in the light of these two goals that the data are presented and discussed. Only when the structure of some model complex and its optical behavior are completely related will the usefulness and limitations of this method of investigating the structures of biological macromolecules be fully appreciated.

REVIEW OF THE LITERATURE

The major portion of this review will be concerned with a fairly detailed presentation of work done since 1959 on the structure and optical behavior of metachromatic dye:polymer complexes. This was the year when optical activity in the absorption bands of bound dye was first reported (Blout and Stryer, 1959) and when the first statistical treatment was given on the distribution of interacting dye molecules bound to a polymer surface (Bradley and Wolf, 1959). No attempt will be made to provide an account of the development of ideas before this period; instead, a brief summary on the concepts of dye:polymer interaction and the general behavior of these complexes is sufficient to introduce the recent work. Excellent reviews from differing viewpoints have already been given by Bergeron and Singer (1958), Kelly (1956), and Schubert and Hamerman (1956).

In reading these reviews and sifting through the earlier literature, especially before 1950, one must bear in mind the following three major reasons why so many conflicting results have been reported:

1. Impure dyes. Commercial biological stains usually contain several and often substantial amounts of dye components other than the stated dye. For instance, British and National Aniline samples of toluidine blue have been found to contain as many as six dye fractions, some of which were blue

with no staining properties, and others which did not exhibit metachromasy (Ball and Jackson, 1953). Commercial thionine often contains a large fraction of a bright red dye which, unfortunately, happens to have the same color as the metachromatic color of thionine itself (Kramer and Windrum, 1955). Early workers rarely purified their dye samples and must have often reported metachromatic staining of cellular or tissue components when all that was occurring was a selective staining process. Another factor that appears to have been largely neglected is the chemical stability of dye solutions. Many dyes are light sensitive and solutions may deteriorate with age, especially in soft glass containers (Epstein, Karush, and Rabinowitch, 1941).

2. Operational definition of metachromasy. Quite aside from the problem of dye impurity, there exist differences of opinion on which dyes are metachromatic. This controversy stems mainly from the dual use of vision and spectrophotometers to detect color changes. Hence, the histologists' and physical chemists' lists of metachromatic dyes often disagree. A rather celebrated case concerns methylene blue, which shows a well-defined spectrophotometric shift in absorption bands but exhibits almost no visual change in color upon binding to chromotropes. Michaelis continued to discredit methylene blue from metachromatic properties as late as the 1940's, then changed his mind and used the dye extensively in later spectrophotometric studies (Michaelis and Granick, 1945;

Michaelis, 1950). Kelly (1956) acknowledged the spectrophotometric metachromasy of methylene blue but still preferred to classify dyes in his review on the basis of their visual behavior. Only one acridine dye, acriflavine, was included in his list of cationic, metachromatic dyes.

3. Distinction between staining and solution behavior. A clear distinction was not always made between the results of test tube experiments on dye:chromotrope systems and the results of staining heterogeneous tissue preparations. While these two systems often show similar behavior, this should not be expected to be the rule. Opportunities for additional binding forces, alteration of chromotrope structure by combination with other cellular components, and the masking or production of additional binding sites are greatly enhanced possibilities in the tissue systems. For instance, the metachromasy obtained in solutions of chromotropes and dyes is always readily reversed by alcohols, but the metachromatic color in cartilage and mast cell sections is not reversible by alcohol treatment. Continued soaking results only in a gradual decoloration (Kramer and Windrum, 1955). Furthermore, the marked lack of any standard technique of metachromatic staining, which is still lacking today, resulted in considerable controversy over which cellular polymers could "rightfully" be called chromotropes.

In general, the spectral changes observed upon mixing some chromotrope with a dilute aqueous solution of metachro-

matic dye were found to be very similar to those occurring upon increasing the concentration of dye alone. But only certain types of dyes and polymers are capable of forming these metachromatic complexes. It seems appropriate, then, to summarize the main points in the older literature under the separate headings of metachromatic dyes, chromotropes, and dye:chromotrope complexes.

Metachromatic Dyes

The majority of dyes reported to exhibit metachromasy can be grouped into eight classes: the acridine, azine, azo, oxazine, thiazine, xanthene, carbocyanine, and triphenylmethane dyes. Specific dyes and their structures are shown in their monocationic forms in the appendix under their respective classes. It was noted that all these dyes are planar, or nearly so, and all contain at least two nitrogen atoms. Furthermore, the permanent charge is intimately associated with the resonance system responsible for the color. Nearly all dyes without these three general characteristics are not metachromatic, yet many dyes possessing these requirements, such as the monoaminoacridines, do not exhibit the phenomenon. It should also be noted in passing that the charge on nearly all anionic dyes is usually localized on one group, such as a sulphonate. Therefore, these anionic dyes do not possess one of the stated requirements for metachromasy and, in fact, are not metachromatic. Only those anionic dyes in which the nega-

tive charge is a part of the resonance system, such as eosin and related dyes, show m cachromasy (Förster and König, 1957).

Various chemical theories, such as the formation of hydrolytic adducts (Lewis et al., 1943) or the existence of tautomeric equilibria (Holmes, 1926; Lison and Fautrez, 1939), failed to satisfactorily explain the metachromasy observed upon increasing the concentration or decreasing the temperature of dye solutions. Instead, the idea of aggregation into dimers and polymers finally gained wide acceptance. Conductance measurements (Robinson, 1935), analysis of spectral changes in terms of association equilibria (Vickerstaff and Lemin, 1946) and a careful study of the fluorescence metachromasy of acridine orange (Zanker, 1952) left little room for doubt that metachromasy was closely related to dye association. This concept easily encompassed all the known classes of metachromatic dyes; their planar nature and extensive conjugation suggested that aggregation occurred by stacking one molecule on top of another. London dispersion forces between the ring systems were held responsible for the aggregation and interaction between the mobile π-electrons resulted in the observed spectral shifts.

However, aggregation per se could not completely account for metachromasy. Dry crystals and films of several carbocyanine dyes showed no evidence of spectral interaction, whereas interaction seemed to appear with increased humidity (Sheppard, 1942). Similarly, desiccated samples of metachro-

matically stained tissue sections (Kramer, 1955) and agar films (Bergeron and Singer, 1958) slowly became orthochromatic, while breathing over these samples immediately restored the metachromatic color. Bergeron and Singer (1958) mention that the solid powders of metachromatic dyes are metachromatic but contain water that is usually difficult to drive off. Methylene blue must be heated to 150°C to remove the water, whereupon it loses its metachromatic color until re-hydrated. Examples of aggregation without metachromasy are also found among the anionic dyes, such as Congo red and Orange G. These dyes were known to exist in aggregates ranging from two to over one hundred molecules in water solution, yet Beer's law is behaved (Schubert and Hamerman, 1956).

The above examples suggest a role for water in the metachromasy of cationic dye solutions, as was suggested, although for obscure reasons, by several authors throughout the history of metachromasy. It was known that organic solvents, e.g., alcohol, destroy the metachromasy of dye solutions, and Sheppard (1942) and Lewis *et al.* (1942) suggested that water was necessary to the dimerization process by acting as an electronic buffer between the charged ions. But a more intimate—in fact, essential—role for water was suggested by Bergeron and Singer (1958) in an idea that has apparently been forgotten by later workers. They suggested that the mobile charge migrates to opposite ends of

two dye molecules as they approach one another. This, in effect, places a nitrogen with a lone pair of electrons on one molecule just opposite a positively charged nitrogen on the other. A co-ordinate bond is then formed on each end of the dimer and serves to hold it together without much influence on the mobility of electrons in either cation. Water, a strong dipole, is then envisioned as orienting between these nitrogen pairs, causing the mobility of electrons to be substantially reduced along the major axes of both dye molecules. Transitions occurring at right angles to this direction will be only slightly affected by dimerization.

The question of whether the new absorption bands produced upon aggregation are new electronic bands characteristic of the aggregate or are the result of a different enhancement of vibrational levels already present in the monomer was never resolved. Sheppard and Geddes (1944), in a survey of a variety of dyes, noted that the dimer (β) band always arises at the site of a shoulder that is present even in alcohol or dilute aqueous solutions. They favored the vibrational hypothesis suggested earlier by Lewis et al. (1943) from a study of the shape of the fluorescence spectrum of methylene blue. But Zanker (1952) preferred to identify the β band of acridine orange with an electronic transition and invoked the weak coupling theory of Förster (1946) to explain the spectral shifts. A more complete account of the possible electronic origins of metachromasy will be deferred

to the Results and Discussion.

Finally, the spectral behavior of the uncharged, basic form of proflavine in organic solvents should be noted. Levshin (1955) and later, Mataga (1957), who also found that acridine orange behaves similarly, discovered that proflavine aggregates very easily in organic solvents, particularly in acetone or pyridine. The spectral changes resemble metachromasy in water, except that a well-defined isosbestic point exists at 417 mμ, even at high concentrations where spectral changes no longer occur. Obviously, only two spectral species exist (monomer and N-mer), which is much different from the cation association in water. On the other hand, the ultraviolet absorption area remains essentially constant while a shoulder on the long wavelength side of an absorption peak at 260 mμ grows at the expense of the peak. Again, the ultraviolet absorption behavior of proflavine in water is different in that there is no redistribution of energy and the total area drops slowly as the concentration is increased. Furthermore, the aggregated proflavine remains highly fluorescent, although the fluorescence maximum is red-shifted, whereas aggregation of cations in water results in quenching. Thus, a change in solvation and charge state of a dye molecule results in different optical behavior that is still described as metachromatic. The rather inclusive and complex nature of the term metachromasy becomes evident. It is also quite possible that different classes of dyes described as

metachromatic under identical solution conditions may exhibit similar spectral shifts but still differ in the sources of these changes.

Chromotropes

All known chromotropes, or substances capable of forming metachromatic complexes with dye, were discovered to be anionic polyelectrolytes of high molecular weight or low molecular weight materials that easily aggregate into micelles. The natural, biological chromotropes are represented almost exclusively by the nucleic acids and polysaccharides, such as heparin, hyaluronate, and chondroitin sulfate. The protein fibrin seems to be the only exception. Examples of biological products are agar, alginate, and dextran, chitin, and pectin sulfates. A couple of inorganic polymers, i.e., silicates (Merrill, Spencer, and Getty, 1948) and polyphosphates (Wiame, 1947), form metachromatic complexes, as do associative, low molecular weight soaps and detergents, such as Duponol, phospholipids, myristate, and sodium laurate. Several synthetic organic polyanions were also reported to be chromotropes: polyvinyl sulfate (Koizumi and Mataga, 1953 and 1954), polyacrylic acids (Pal and Basu, 1958), and polyadenylic acid (Steiner and Beers, 1958).

The likelihood of a relationship between chromophore conformation and its metachromatic properties was suggested by several investigators, but few studies along this line

were reported before 1959. This work will be mentioned in the next section. However, it was realized quite early that structures with a greater number of exposed anionic groups were stronger chromotropes and that the order of increasing effectiveness seemed to be carboxylate, phosphate, and sulfate groups (Bank and Bungenberg de Jong, 1939; Sylvén, 1954).

Dye:chromotrope Complexes

The initial work of Lison (1935) and especially the later study of Michaelis and Granick (1945) clearly established the similarity in spectral behavior caused by increasing the concentration of a metachromatic dye solution or adding a chromotrope to a dilute dye solution. The long wavelength band (α band) is markedly reduced, a new broad peak is formed at a shorter wavelength than the β band, and the entire visible spectrum is broadened so that the absorption actually increases at wavelengths above the α band position. Often the spectral changes are more pronounced in the presence of a chromotrope. Thus, the new absorption maximum in chromotrope metachromasy (μ band) can be displaced to shorter wavelengths than the maximum (γ band) observed in concentrated dye solutions. Michaelis introduced the μ band terminology, but there is no real basis for making a distinction between γ and μ bands. Neither band has a definite position; both shift to shorter wavelengths under conditions

favoring increased dye-dye interaction. Consequently, the term γ band has come to be used in describing the new, blue-shifted maximum produced in both dye and complex solutions.

Recalling that the α, β, and γ bands had been associated, respectively, with the monomer, dimer, and polymer forms of dye molecules, Michaelis and Granick attributed the metachromasy of dye:polyanion solutions to the enhanced formation of dye aggregates in the presence of the polymer. These aggregates were then electrostatically adsorbed to the polymer surface, one dye combining with one acidic side chain to form a salt-like compound. This concept was developed further (Michaelis, 1947) by noting that salt, increased temperature, and the introduction of ethanol destroyed the metachromasy. However, Michaelis (1950) soon seriously questioned this notion for two reasons. When an agar gel was stained with a very dilute solution of toluidine blue or other metachromatic dyes, a distinct γ band was still obtained. The adsorption of dye polymers was considered to be much more unlikely in this case than a monomolecular distribution of dye over the anionic sites of the polymer. Also, nucleic acids, contrary to the other polyanions studied, always seemed to "depolymerize" dye solutions, yielding spectra like those obtained in ethanol. He finally concluded that the similarity of spectral changes observed in dye and dye:chromotrope systems was misleading, that the chromotrope metachromasy was more the result of dye-polymer interaction than dye polymerization.

The spectral behavior of dye solutions clearly illustrated that metachromatic dye energy levels are particularly sensitive to external electrical fields. Different chromotropes were imagined to affect the energy levels of bound dye in different ways, depending upon the particular structure of each chromotrope.

Wiame (1947) and Jaques et al. (1947) introduced a direct method of assessing the metachromatic behavior of dye: chromotrope solutions that became very widely used in later work. A family of absorption curves was obtained for a series of complexes in which the total dye concentration was maintained at a fixed, dilute value, but the amount of added chromotrope was varied. Wiame proceeded to examine the toluidine blue:hexametaphosphate complex by plotting the (γ band/α band) extinction ratio as a function of the chromotrope concentration and discovered that this ratio increased until the phosphate group concentration was about eight times that of the dye. Increasing the polymer concentration beyond this point resulted in a reversal of the spectral shifts until, eventually, the spectrum was nearly the same as the free dye solution. His explanation of this behavior was that the dye behaved as if polymerized when many molecules were bound close together on a molecule of hexametaphosphate. But the addition of excess sites caused the dispersal of these aggregates until each bound dye was located far enough from another that they could no longer interact. The absorption

spectrum then reverted to a shape characteristic of monomeric dye. This idea is still basic to modern theories of dye: chromotrope spectral behavior.

Subsequent work elaborated upon the above idea. Metachromasy and its reversal with the addition of excess chromotrope was noted with the nucleic acids (Lison and Mutsaars, 1950; Weissman et al., 1952), polysaccharides (Levine and Schubert, 1952a), anionic detergents (Levine and Schubert, 1952b), and polyvinyl sulfate (Koizumi and Mataga, 1953). Goldenberg and Goldenberg (1955) also discovered that cholesterol and β-cholestanol sulfates formed highly metachromatic complexes with toluidine blue and methylene blue that started reverting to the normal color with increased amounts of chromotrope. Hardly ever (vide infra) did the visible absorption spectra exhibit isosbestic points in the mole ratio region where metachromatic complexes were formed, indicating the presence of dye aggregates of several different sizes bound to the chromotrope sites. On the other hand, the ultraviolet bands, in the few cases where they were examined (Schubert and Levine, 1953 and 1955; Steiner and Beers, 1959), did not exhibit metachromasy. Often one or more bands are reduced in height as chromotrope is added, reducing the total oscillator strength, but never does one band grow at the expense of another. Fluorescence quenching, and the reappearance of the fluorescence in the presence of excess chromotrope, was also found upon adding silicates to Rhodamine G

(Merrill et al., 1948), native DNA to acriflavine (Heilweil and Van Winkle, 1955), and polyadenylic acid to acridine orange (Steiner and Beers, 1958). Similar quenching also occurs in concentrated dye solutions, but, in addition, the emission maximum is red-shifted.

Protonation of the chromotropes was found to be more effective in reducing metachromasy than salt addition, and the effectiveness of salt increased with the valence of the cations. Peacocke and Skerrett (1956) demonstrated by equilibrium dialysis the high sensitivity of the proflavine:DNA complex to magnesium ion concentration. Even basic proteins, such as protamine and histone sulfates, were found to markedly inhibit the metachromasy of heparin complexes (Kelly, 1955). Similar observations led Weissman et al. (1952) and Sylvén (1954) to conclude that dye:chromotrope interaction involved both polar and nonpolar bonds. The polar, or salt-like, linkages were considered to be formed in the first step of binding, were sensitive to salt and heat, and caused little, if any, changes in the spectrum of monomeric dye. The nonpolar bonds were then formed in the second binding step between the ring systems of adjacently bound dye molecules, and this interaction resulted in the large spectral shifts. These bonds were sensitive to heat and solvation and had no opportunity to form when the number of sites greatly exceeded the number of dye molecules. Sylvén also commented that the formation of an ordered dye aggregate on the polymer

surface would be facilitated if the dyes presented both hydrophilic and hydrophobic regions in the initial binding step. Thus, the possibility of multiple bonding to the polymer was not excluded.

Few relationships between chromotrope secondary structure and metachromasy were discovered or suggested. Walton and Richetts (1954) found that the degree of polymerization of anionic polysaccharides did not influence the amount of binding (and presumably, metachromasy) as long as the polymers contained at least four sugar units. Schubert and Levine (1955) felt that chromotropes existed in solution as globular clusters or micelles of high anionic density. These micelles selectively and reversibly bound polymeric dye cations of the highest charge available from the dye equilibria existing in solution. Metachromasy was depressed with increasing chromotrope concentration because of the concomitant increase in counterion concentration and the increased binding of monomeric dye at widely separated sites. Pal and Basu (1958) pointed out that linear polyelectrolytes, at infinite dilution, will be fully extended. The introduction of salt and especially dye (because of its tendency to aggregate) will reduce the coulombic repulsion and cause the chain to become coiled. He cites the very pronounced reduction in the relative viscosity of a dilute solution of polyacrylate upon binding toluidine blue, compared to the effect of NaCl, as evidence for the enhanced coiling caused by dye

binding. Such coiling could result in the superpositioning of widely separated bound dye, leading to the observed metachromasy. Supposedly, the introduction of more counterions with increased chromotrope concentration would cause further coiling and eventually the steric restraints of tight coiling would not be conducive to dye-dye interactions. Hence, metachromasy disappears.

The study of nucleic acid metachromasy was complicated by the possibility of purine and pyrimidine involvement in the binding. RNA and both double- and single-stranded DNA yielded metachromatic, non-fluorescent complexes, but differences in binding constants were noted that were dependent upon primary and secondary structure. Lawley (1956a, b) found for both meta- and orthochromatic complexes that the binding constant of rosaniline to DNA was reduced upon heat denaturation, as was the competitive effect of sodium cations. He suggested that denaturation allowed the phosphate groups to become involved in intramolecular hydrogen bonding to the pyrimidine and purine bases. Sodium ions were less effective in the denatured state because dye was now apparently binding primarily through nonpolar van der Waals bonds to the exposed, planar bases. However, in an earlier suggestion, Oster (1951) felt that the bases also played a part in the binding of dye to the native, double-stranded DNA. The planar dye molecule might well be intercalated between adjacent bases with the positive charge oriented toward the negative phosphate group.

Further evidence of base involvement was obtained by Steiner and Beers (1958, 1959) from a study of the polyadenylic acid: acridine orange complex. Spectrophotometric titrations revealed that a maximum of one dye molecule could be bound per nucleotide, resulting in a metachromatic, nonfluorescent complex that they called Complex I. The fluorescent, orthochromatic complex formed in the presence of excess chromotrope was called Complex II. This distinction between two types of complexes grew from observations of differences in behavior like the following: treatment of polyadenylic acid with formaldehyde, which converts the 6-amino group of adenine to a Schiff base, destroyed Complex I (loss of metachromasy and return of fluorescence) but did not affect Complex II (no reduction of binding); protonation of adenine competitively disrupts Complex I but does not affect Complex II. Complex I seems to involve the adenine rings and is apparently stabilized by nearest-neighbor interactions. They suggested the possibility that Complex II was entirely a salt linkage, possibly between the dye and the doubly-charged terminal phosphate group.

Precipitation of the dye:chromotrope salts was very often noted, especially at higher dye concentrations, but this phenomenon was not and still has not been well studied. Early workers regarded metachromasy as a property of the precipitate and, in fact, Walton and Richetts (1954), in their study of dye binding by measurement of the amount of

precipitated dye and chromotrope, concluded that metachromasy was dependent upon the solubility characteristics of the complex. But precipitation was not always reported and the relationship between metachromasy and water insolubility remained unclear. The existence of very finely dispersed solid in the highly colored complex solutions could have been easily overlooked in the reporting of soluble complexes. Precipitate formation was, however, utilized in assays for chromotropes. The precipitation of aqueous solutions of heparin:dye complexes was often facilitated by shaking with petroleum ether, causing aggregate formation at the interface. The complex is apparently at least partially soluble in the organic phase, but the amount of heparin was derived from the reduction in dye concentration in the aqueous phase.

Finally, the unique metachromasy of the pyronine G: polyvinyl sulfate (PVS) complex will be cited (Koizumi and Mataga, 1954) to illustrate how a small change in dye structure can result in a complex with quite different spectral behavior. Pyronine G differs from acridine orange only in that the ring nitrogen is replaced by oxygen. Its absorption spectrum in water is in itself rather unique. The α band decreases in height as the β band grows larger, but a well-defined isosbestic point exists over a wide range of concentrations. No hint of a γ band appears. The addition of PVS to a dye solution of concentration less than 2×10^{-4} M (in which the β band is well-developed) results in no spectral

changes, regardless of the amount of PVS added. But with a higher dye concentration, PVS addition causes both the α and β bands to decrease equally while a new γ band appears. An isosbestic point exists between the γ and β bands at all PVS concentrations and remains there as the spectral changes are reversed by excess PVS. This behavior is much different from the gradual peak shifts noted with other systems and other dyes bound to PVS. Koizumi and Mataga suggested that pyronine G does not aggregate beyond the dimer in solution, that both dimer and monomer are adsorbed equally well by PVS, and that a new molecular species, probably a higher aggregate, is formed only on the PVS surface.

Recent Literature

The basic groundwork for recent work on metachromatic complexes was provided by Bradley and Wolf (1959) in their statistical interpretation of the reversal of metachromasy caused by excess chromotrope. Lawley (1956a) had already given a simple treatment for the random distribution of dye among sites when both free and bound dye are present. His data for rosaniline binding to DNA, in fact, fit the random binding model reasonably well. But Bradley and Wolf extended the usefulness of this approach by treating the case where all the dye is bound and non-randomness in dye distribution may occur. Theoretical curves for various degrees of non-random binding were then found to closely fit the experi-

mental points obtained in a study of metachromasy reversal for acridine orange bound to excess DNA, RNA, polynucleotides, heparin, and polyphosphate.

Several assumptions were made in the statistical treatment. A polymer of infinite chain length was assumed that defined a regular, linear arrangement of specific binding sites. Each site could be filled by only one bound dye and dye-dye interactions could take place only between dyes bound to adjacent sites. A bound dye with an empty site for both neighbors was treated as an isolate and called a monomer. Since all the dye in the system was assumed to be bound, only two dye species, both bound, were considered—the monomer and a polymer (stack) of unspecified length. The average fraction, F, of bound monomer (unstacked molecules) could then be computed for a random distribution of dye and related to the ratio, P/D, of polymer sites to dye molecules by the following stacking equation:

$$P/D = (1-F^{1/2})^{-1} \qquad (1)$$

In order to treat the non-random distribution, a stacking coefficient, K, was introduced that quantitatively expresses a preference for binding in stacks. K was defined as a probability ratio, P(1)/P(2), where P(1) is the probability that a dye molecule will occupy a particular site with a neighbor and P(2), the probability that it will occupy a site with no neighbors. This stacking coefficient is independent

of the relative numbers of dye molecules and sites and becomes equal to unity for a random distribution. By assuming that K is a constant for a given system, the following approximate stacking equation for the non-random case was developed by Bradley and co-workers:

$$P/D = (1-F^{1/2})^{-1} + (K-1)F^{1/2}(1+F-F^{1/2})(1-F^{1/2})^{-1} \qquad (2)$$

Later, the statistical problem was solved exactly to yield a simpler equation used in subsequent work (Lamm, Childers, and Wolf, 1965):

$$P/D = (1-F^{1/2})^{-1} + (K-1)F(1-F^{1/2})^{-1} \qquad (3)$$

Statistical equations were also developed for finding the fraction of dye molecules involved in a run of any desired length (Geisser and Bradley, 1962), but such equations are not so amenable to experimental verification. The fraction of unstacked dye molecules, however, is easily estimated from the spectral changes in systems that do not have very large stacking tendencies. Bradley and Wolf chose to measure extinction coefficients at the α band position and estimated F by:

$$F = \frac{\epsilon - \epsilon_{1:1}}{\epsilon_{1:\infty} - \epsilon_{1:1}} \qquad (4)$$

where ϵ, $\epsilon_{1:1}$, and $\epsilon_{1:\infty}$ are, respectively, the extinction coefficients at any $P/D > 1$, at $P/D = 1$, and as $P/D \rightarrow \infty$. Implicit in this equation is the assumption that dimer and

larger aggregates have nearly identical contributions to the absorption at the wavelength where F is being determined. If the choice of wavelength is near the α band position, any unequal contributions will tend to be minimized. Nevertheless, Stone and Bradley (1961) did note that F values varied by several percent when computed at a wavelength 10 mμ longer than the α band. More reproducible results were reported to be obtainable at the band maximum of bound monomer dye.

When the fraction of bound monomer, or unstacked, acridine orange was plotted against P/D for the polymers studied by Bradley and Wolf (1959), curves were obtained that were similar in shape to Equation 1 (for random distribution) but displaced by various amounts to higher P/D values. Hence, the fraction of stacked molecules was always greater than that predicted on a random basis, demonstrating in absolute numerical terms the aggregative tendency of dyes bound to the various polymers. Values of K needed to fit Equation 2 to the experimental points ranged from near 1 for DNA, confirming the earlier nearly-random binding model of Lawley (1956a), to about 800 for heparin and polyphosphate, which reflects the very large excess of chromotrope needed to reverse metachromasy in these two polymers. In addition, other measures of stacking tendency, entirely experimental in nature and based upon the aggregative theory of metachromasy, were found to correlate with the value of K obtained by curve-fitting (Stone and Bradley, 1961). These parameters are the rate,

$d\epsilon/d(P/D)$, at which the extinction at a given wavelength (usually the α band) increases with increasing chromotrope concentration; the rate, $d(\alpha/\beta)/d(P/D)$, at which the ratio of the heights of the α and β bands change; the P/D at which the α and β bands are of equal height, $(P/D)_{\alpha=\beta}$; and the P/D at which the α band has increased to one-half its final height, $(P/D)_{F=1/2}$. Further evidence for the aggregative theory was noted in the poly U and heparin complexes with acridine orange, where α, β, and γ bands may exist simultaneously. The β band appeared as an intermediate stage in the transition from the γ band spectrum to the α band spectrum of bound monomer. This is the expected behavior of a process involving dissociation of aggregates through the dimer stage to monomers.

The ground state free energy of interaction between a pair of neighboring dyes, which determines the degree of nonrandomness in binding and can be found from the relation, $K = \exp(-\Delta F/kT)$, was thought to be a function of the polymer structure. The striking differences in stacking tendencies of a particular dye on a number of different polymers could probably be related, primarily, to the relative orientation of adjacently bound dyes and to the rigidity of site spacing maintained by the polymer structure. It was speculated that flexible polymers should have high stacking coefficients because the binding sites are relatively free to assume optimum positions for dye stacking, whereas the more struc-

tured polymers would more likely have sites held rigidly in positions less than optimal and, therefore, have smaller K values. This speculation seemed to be confirmed experimentally. Thus, for double-stranded DNA, K = 1.16, while heat-denaturation increased the value to K ≅ 3-5 in a manner that exactly paralleled the heat-induced changes in viscosity and ϵ_{260} (Bradley and Felsenfeld, 1959); for RNA, K = 2.9, and the very flexible polyphosphate polymer had K = 827. The stacking coefficient of neutral, single-stranded poly A (K = 161) was found to decrease markedly upon protonation of the adenine (K = 12.3), presumably due to the formation of a rigid, double-stranded structure, as later verified by Rich et al. (1961). Stone and Bradley (1961) also examined the stacking tendencies of acridine orange on 24 DNA samples isolated from various sources by a variety of methods to demonstrate that the stacking coefficient remained uniformly low at 1.25 ± 0.07 for the native DNAs. As expected, heat denaturation always increased the value of K, providing further support for the theory that the stacking tendency depends upon the molecular structure of the polymer.

The experimental stacking curves could not always be fitted well by a single value of K; i.e., at higher P/D values the initial value of K decreases for neutral poly A and increases for heat-denatured DNA. Although some misfit at high P/D is expected from the assumption of infinite polymer length (end effects of finite polymers lead to a

lower degree of stacking than predicted by Equation 3 for a given value of K), these deviations are large enough to raise questions about the possibility of heterogeneity of binding sites and, in particular, the precise roles played by the phosphate groups and the planar bases in the binding of dye. Bradley and co-workers assumed the dye to be bound externally to the DNA surface only by electrostatic forces, but the statistical analysis does not specify and is not dependent upon the chemical nature of the binding sites. The model is, however, essentially a one-site model whose fit to the experimental data does not constitute proof for only one type of binding site. Their data may also be fitted to a two-site model in which the ratio of the binding constants varies continuously over the excess chromotrope region. This possibility is particularly appropriate for the nucleic acid and polynucleotide complexes, as has been demonstrated by the recent work summarized below, but provides only an ad hoc alternative to the one-site model for most other dye:polymer complexes.

Nucleic Acid and Polynucleotide Complexes

Extensive studies by a variety of chemical and physical techniques have been carried out in recent years on the binding of cationic dyes, particularly the aminoacridines, to DNA, RNA, and polynucleotides. Interest has been centered on the one hand upon the optical properties of bound dye in an

effort to deduce the complex structure, thereby providing a basis for understanding its optical behavior, and on the other hand upon the structural implications of their mutagenic action (Steiner and Beers, 1961; Orgel and Brenner, 1961; Crick et al., 1961). A complete account of all this work will not be given; instead, many of the experimental facts are, perhaps, best summarized in the light of the two basic, non-exclusive models of the complex structure currently in favor. One model, derived from the initial work of Lerman (1961, 1963), involves the intercalation of the dye between adjacent bases, whereas the other model is the external, edgewise attachment of the dye to phosphate sites, as suggested by Bradley and Wolf (1959). Both modes of binding may apparently occur with DNA, and the models tend to merge under conditions where results are best explained by partial intercalation, i.e., the involvement of base-dye interactions in binding to single-stranded polymers.

The equilibrium dialysis investigation of Peacocke and Skerrett (1956) had shown that two binding constants were involved in the combination of proflavine with DNA, the stronger binding being saturated at one dye bound per 4 or 5 nucleotides. Dye continued to be bound, then, by a weaker process until a maximum of one dye per nucleotide was achieved. This maximum level of binding was verified later by the spectrophotometric titrations and ultrafiltration work of Stone and Bradley (1961). The strong binding sites were

associated with bound monomers, while the weak sites were held responsible for dye aggregate formation and the resultant metachromasy. More recently, Neville and Davies (1966) attempted a direct method of deducing the structure of the monomer dye:DNA complex by comparing the X-ray diffraction patterns of DNA and DNA:dye fibers. Both acridine orange and proflavine caused an increase in the disorder and layer-line spacing over the DNA pattern, a result that is easily understood by an intercalation of dye between adjacent base pairs. The level of intercalation, as judged from the increase in layer-line spacing, was probably less than 1 dye per 100 nucleotides, corresponding to less than 6% of the total dye bound to the fiber. It was also found, unexpectedly, that a decrease in the hydration caused both the layer-line spacing to decrease toward that of DNA alone and the proportion of bound monomer dye in films to decrease. Again, the intercalation model accounts for this behavior if the dye molecules swing out of the DNA interior when the hydration is decreased and subsequently interact with each other and other dye molecules already attached to the exterior surface. Rosaniline, a triphenylmethane dye, did not alter the layer-line spacing and, therefore, must be entirely bound to the DNA exterior.

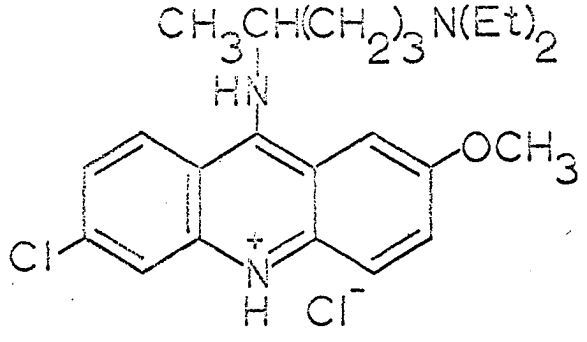
Neville and Davies (1966) also compared their X-ray diffraction patterns with ones obtained from optical transforms of simple masks representing the DNA helix, an interca-

lated dye complex, and an exterior binding model in which the helix rotation was decreased by the bound dye. Both of these models gave patterns which closely resembled the X-ray results, thus making it impossible to decide, on the basis of X-ray patterns alone, which model is appropriate for monomerically bound dye. However, as the authors pointed out, the intercalation model easily accounts for the experimental observations, whereas obscure reasons must be invented to explain how externally bound dye could change the helix rotation by a mechanism dependent upon the degree of hydration. Furthermore, the intercalation model was favored as being consistent with evidence obtained by other workers for the extension of the DNA helix upon binding aminoacridines. The low-angle X-ray scattering studies of Luzzati et al. (1961) showed a loss of mass per unit length for DNA:proflavine which could be explained by intercalation of every dye bound in the range D/P = 0 to 0.25. Cairns (1962) demonstrated DNA extension by autoradiography, and the physical and chemical studies of Lerman argue strongly for an intercalation model in which the dye plane is parallel to the base pair plane and nearly perpendicular to the helix axis.

Lerman (1961) identified the strong binding mode with intercalation on the basis of viscosity, sedimentation, flow dichroism, and flow-polarized fluorescence studies (Lerman, 1963, 1964a). The model suggested involves the local untwisting of the deoxyribose-phosphate backbone to permit a

space for acridine intercalation between previously adjacent base pairs without disturbing their hydrogen bonding. The extent of untwisting possible for the DNA structure and required for intercalation has been estimated by various authors to yield an angle between the two base pairs in the range 45° (Fuller and Waring, 1964) to -9° (a left-handed twist) (Lerman, 1961), although Lerman (1964a) finally decided upon 0°, the value used by Neville and Davies (1966) in their optical transforms. This extension of the helix caused by intercalation results in a local stiffening of the chain and provides a basis for understanding the large increase in viscosity noted upon binding acridine orange and proflavine to DNA. Also, the sedimentation coefficient, which is nearly proportional to the mass per unit length, was discovered to decrease in accordance with the intercalation prediction. At higher dye concentrations the complex sedimentation coefficient became larger than that for DNA, suggesting increasing amounts of external binding. In contrast, the carbocyanine dye, pinacyanol, which supposedly is geometrically unsuitable for intercalation, always caused a decrease in viscosity and an increase in the sedimentation coefficient that could be expected on the basis of external, electrostatic binding. The flow dichroism and flow-polarized fluorescence results on the DNA complex with monomeric quinacrine (or atebrin) (I) required that the acridine dyes and the base pairs be essentially perpendicular to the helix

axis, thus ruling out an external binding model in which the dye causes a stretching of the helix and strong tilting of the base pairs. Additional support for intercalation in the helical DNA polymer was obtained by Weill and Calvin (1963), who demonstrated sensitized dye fluorescence as a result of efficient energy transfer from the electronically excited bases.

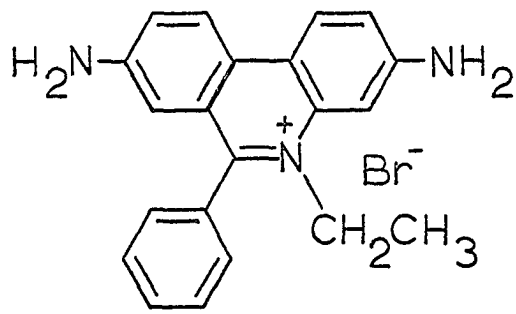


I

The rate constant for the diazotization of proflavine and other aminoacridines was diminished nearly twentyfold in the presence of DNA (Lerman, 1964a, b). Such a rate reduction supports an intercalation model in which the upper and lower faces of the aromatic dyes are out of contact with the solvent. The binding of dyes to other synthetic polyanions, including some containing broad aromatic groups which can, at best, form open-faced sandwiches with dye, never reduced the rate more than 50%. Amino groups that can fall inside the van der Waals limits of the nucleotides are apparently protected from diazotization; both rosaniline and fuchsin, triaminotriphenylmethane dyes, reacted at one-third the rate of free dye and Doebner's violet, the homologous diaminotri-

phenylmethane dye, was as inhibited as proflavine. (Note, however, that Neville and Davies (1966) found no layer-line spacing changes upon binding rosaniline to DNA and concluded it was not intercalated.) Since combination of dye with DNA also liberated no formaldehyde-reactive amino groups, the hydrogen bonding of the base pairs is apparently not greatly disturbed.

More recently, Drummond et al. (1965, 1966) have found for various aminoacridines that the strong binding process is not diminished by denaturation of DNA. Similarly, the strong binding of the metachromatic compound, ethidium bromide (II), which saturates at one molecule per 4 to 5 nucleotides, is not influenced by disruption of the double helix (Waring, 1965, 1966). Since the Lerman intercalation model, in which



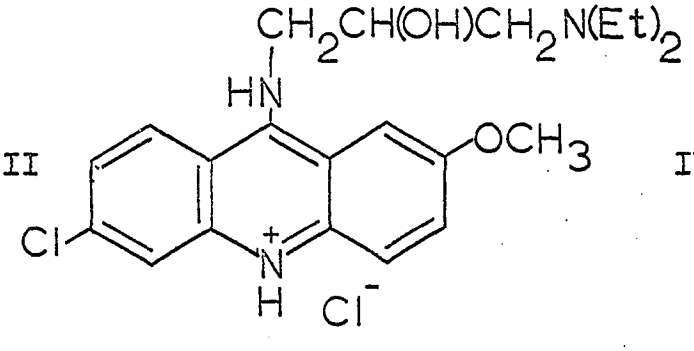
II

the dye is placed across the entire length of a hydrogen-bonded base pair, would predict a loss or large reduction of the strong binding upon strand separation, Pritchard et al. (1966) were led to propose a modified intercalation model, suggested by model building studies, to account for this insensitivity to the DNA structure. They suggested inserting

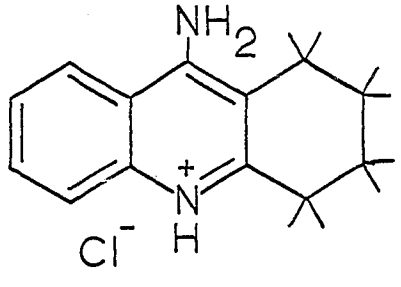
the dye between two adjacent bases on the same polynucleotide chain, rather than across a base pair, thereby placing the positively charged ring nitrogen immediately adjacent to a negatively charged oxygen atom on the phosphate group, which is able to swing into the inside of the chain. The complementary bases on the other polynucleotide chain are therefore not directly involved with the binding of monomeric dye and strand separation would have little effect on the strong binding process. Unlike the Lerman model, this modification easily explains the marked effect of ionic strength upon the strong binding by implicating ionic bonds, as well as the nonpolar ones between the dye and adjacent bases.

Intercalation of acridines with long side chains on the 9-position, as for atebrin (I) and acranil (III), which bind more strongly than proflavine to denatured DNA (Drummond *et al.*, 1965; Kurnick and Radcliffe, 1962) is not hindered in either model. But the modified model also allows intercalation of the aromatic portion of molecules like (IV), which contain a buckled ring system that cannot be fitted into the space between base pairs in Lerman's model.

III



IV



The planar acridine dyes may also become optically active upon binding to nucleic acids. Thus, Neville and Bradley (1961) reported that acridine orange exhibited two Cotton effects in its visible absorption region when bound to native, helical DNA. At P/D larger than 56, no optical activity was observed, but a large positive Cotton effect centered at 504 mμ (α band position) appeared as the P/D ratio was lowered. This Cotton effect reached its maximum height near P/D = 4, at which point it was gradually replaced by a smaller, negative Cotton effect centered near 465 mμ (β band position). This second Cotton effect was largest at P/D = 1, where the positive Cotton effect was completely absent. Since the magnitude of the rotation depended upon P/D, the optical activity was considered to arise from electronic coupling between nearby bound dyes which, considered as a spectroscopic unit, did not possess a plane or center of symmetry. The negative Cotton effect was associated with dye aggregates, but the position of the positive one near the monomer absorption band made its origin unclear. They suggested that bound monomer dye located on alternating sites could have sufficient coupling to cause optical activity and yet not affect the absorption spectrum. The statistical distribution of this type of dye sequence as a function of P/D, in fact, fit the rotation magnitude of the positive Cotton effect quite well when the number of monomers in the run was two or three. Although the positive Cotton

effect is maximal at the P/D limit for the strong binding process for proflavine, no attempt was made to identify the sites. Finally, they noted that the heat denatured complex was optically inactive.

In contrast with Neville and Bradley, Blake and Peacocke (1965) found that proflavine forms optically active complexes with denatured DNA and with RNA as well. They subsequently (1966) reported that acridine orange is also optically active when bound to denatured DNA, although the magnitude of the positive Cotton effect was considerably less than with the native form. Proflavine complexes were studied almost exclusively because this acridine has only one apparent absorption band in the free and bound states. A single Cotton effect was expected, and found, but the trough always remained about twice the size of the peak over a wide range of conditions. As DNA or RNA is added to a solution of proflavine, the absorption maximum at 443 mμ is at first reduced in height and then shifted via an isosbestic point to 460-465 mμ with excess nucleic acid. No shifts in the position of the positive Cotton effect (inflection point at 460 mμ) were reported as P/D or other solution variables were changed.

For native DNA complexes, since the magnitude of the positive Cotton effect is maximal at P/D = 4 or 5, the limit of the strong binding process, the optical activity was associated with intercalated proflavine (Blake and Peacocke,

1966). Most experiments were performed in the P/D range and at ionic strengths where only the strong binding process occurs. As the ionic strength was lowered, increased binding and larger Cotton effects were obtained but not in direct proportion. Plots of the molar rotation of bound dye versus r, the ratio of bound dye to DNA phosphate, were then constructed for the strong binding process and discovered to be essentially independent of the ionic strength. Assuming a random distribution of dye (Proflavine is only weakly aggregative, but preferential binding to certain base pairs may occur.), a statistical equation was developed that related the fraction of bound molecules in groups of n or more to the value of r. The statistical curve fit the experimental rotation curve reasonably well when n = 3 and 4, suggesting that interaction between at least 3 or 4 proflavine molecules is required for optical activity. Similarly, Neville and Bradley's (1961) statistical analysis had fit their data best in the strong binding region when n = 3. Here, however, the acridine orange molecules were placed on every other site in order to preserve their monomeric spectral character.

Upon acid or heat denaturation of DNA, the size of the Cotton effect produced by proflavine binding at neutral pH increases considerably (not entirely attributable to the increase in binding), and its variation with P/D and ionic strength parallels that for RNA. A statistical analysis for both RNA and denatured DNA, analogous to the one performed on

native DNA, now showed the best fit to the molar rotation versus r curves when n = 2 (Blake and Peacocke, 1967). Hence, an increase in the disorganization of nucleic acids seemed to be accompanied by a decrease in the number of interacting dye molecules needed for optical activity.

The optical activity of the native DNA:proflavine complex was found to be independent of the pH down to 3.5, at which point it suddenly disappeared as the DNA became denatured. As the pH of the complex was restored to neutrality, the Cotton effect increased more gradually than it had disappeared until the magnitude at pH 7 was about 1.4 times as large as for native DNA (Blake and Peacocke, 1966). RNA complexes also became optically inactive at low pH; however, the changes with pH were more gradual and were reversible (Blake and Peacocke, 1967). An examination of the spectra showed no changes, indicating that no change in the extent of binding had taken place, but the strong binding process was demonstrated to be nearly absent in the denatured DNA system at low pH. Thus, Blake and Peacocke were led to conclude that base-pairing, which is destroyed at low pH, is essential for optical activity. The involvement of base-pairing <u>per se</u>, as sites for dye intercalation, did not seem plausible in view of the increased optical activity for the denatured DNA complex at neutral pH. Rather, the role of base-pairing seemed to be one of providing a relatively rigid substructure in the macromolecule for holding bound dye in an asymmetric

arrangement. The limited base-pairing present in RNA and denatured DNA must be sufficient to provide the necessary structure for the asymmetric binding of dye. It was further concluded that the modified intercalation model of Pritchard et al. (1966), where dye is placed between base pairs on the same polynucleotide chain, probably best represents the nucleic acid complexes.

Poly U causes no spectral changes when added to a proflavine solution, whereas poly A causes a distinct drop in the absorption (Blake and Peacocke, 1967). Neither polynucleotide induces Cotton effects at neutral pH. But as the pH is lowered below 6, poly A assumes a more rigid, double-stranded structure. A single Cotton effect appears, centered at the absorption maximum, that is opposite in sign to the Cotton effect observed for the native DNA complex. However, poly (A+U), also a double-stranded structure, causes nearly the same spectral shifts and induced Cotton effect behavior as native DNA, and values of the molar rotation of this complex plotted against r fall on the native DNA curve. Apparently, both purine and pyrimidine bases are required for complex formation as it occurs in DNA, but the results with poly A still demonstrated the requirement of a rigid conformation (localized or extended) for the induction of Cotton effects in bound dye.

Recent detailed examinations of the optical rotatory dispersion (ORD) (Yamaoka and Resnik, 1966) and circular

dichroism (CD) (Gardner and Mason, 1967) of acridine orange: DNA complexes have revealed the very complicated nature of the optical interactions. This increased complexity over the proflavine behavior may, in part, be due to the greater aggregative tendency of acridine orange, but it may also belie an over-simplified treatment of the proflavine optical activity. Yamaoka and Resnik resolved the ORD curves of acridine orange:DNA complexes into no less than four components (one positive and three negative Cotton effects), whose positions and magnitudes both depend upon P/D. In spite of this complexity, Gardner and Mason (1967) attempt to assign CD peaks to various configurations of bound dye (by employing only three obvious peaks) and to deduce the complex structure from changes in the CD peak heights resulting from partial orientation by flow (Mason and McCaffery, 1964). In the flow experiments only two CD peaks were differentiated, and the negative peak assigned to a parallel polarized transition was later shown by Yamaoka and Resnik (1966) to be composed of three negative components. Furthermore, as noted by Blake and Peacocke (1966), the CD flow measurements were conducted in the P/D range where both the strong and weak binding processes are operative, thereby shedding more doubt on their conclusion that the dye is bound externally, in the form of a super-helix, with the dye plane oriented at an angle between 90° and 45° to the helix axis.

Gardner and Mason (1967) took cognizance of the two

binding processes and proposed another model from a rather tenuous interpretation of the dependence of the three CD band heights upon P/D and the very complicated, nonreversible CD changes noted upon forward and reverse titrations of the DNA: AO complex. They suggested that monomeric acridine orange is only partially intercalated and forms a left-handed helix with a non-zero radius and four dye molecules per turn. The dye planes are parallel to the base planes, but the intercalation causes the base-pairs to be slightly tilted, forming an angle of 87° with respect to the helix axis. A second mode of binding, the attachment of skewed dimers by ionic bonds to the DNA exterior, was also postulated. These skewed dimers contributed both a negative and a positive band to the CD spectrum.

A dramatic shift in the fluorescence of acridine orange from green to red is often observed in the in vivo staining of nucleic acids. For example, Loeser et al. (1960) reported the development of a 640 mμ fluorescence band upon heavy staining of DNA in ascites cells. Mayor and Diwan (1961) and Mayor and Hill (1961) found that DNA viruses fluoresce green, whereas RNA viruses exhibit a brilliant red fluorescence. This red and green fluorescence has also been employed extensively to locate and distinguish between cellular structures containing RNA or DNA, respectively (Armstrong, 1956). However, the assignment of the type of nucleic acid should be supported by ribonuclease and deoxyribonuclease experiments.

The total dye concentration in the area being examined, which is determined by the stain concentration and membrane permeability, appears to be another important factor in determining the color of fluorescence. Schümmelfeder et al. (1957) suggested that the fluorescence color is also a function of molecular weight, since the DNA in fixed tissues fluoresced red after partial degradation with hydrochloric acid.

Red fluorescence has never been reported for solutions of acridine orange:nucleic acid complexes. Since aggregation in acridine orange solutions is accompanied by quenching and a fluorescence shift from green to red (Zanker, 1952; Zanker et al., 1959), Van Duuren (1963 and 1966) noted that the lack of any shift upon binding to nucleic acids is inconsistent with the Bradley-Wolf model of external dye stacking. Also, the quenching was never more than 80% of the free dye level, much less than the 99% quenching of 10^{-3} M dye solutions compared to the level in 10^{-5} M solutions. Gardner and Mason (1967) stated, however, that the green fluorescence was almost entirely absent from solutions of the DNA:AO complex at P/D less than 4, and Weill and Calvin (1963) reported a nearly zero quantum yield under similar conditions.

As DNA or RNA is added to a solution of acridine orange, the fluorescence near 530 mμ decreases until P/D becomes equal to one. With further chromotrope addition, the fluorescence quickly reappears (Ranadive and Korgaonkar, 1960; Boyle et al., 1962). Curiously enough, the final intensity reached

for the acridine orange:DNA complex was about twice as great as the dye solution alone (3×10^{-6} M), although the closely related acriflavine, 5-aminoacridine, and coriphosphine O dyes did not quite attain their original level. A closer study of the fluorescence quenching as a function of P/D (Van Duuren, 1966) revealed that, for the DNA:AO complex, the fluorescence intensity remained constant from P/D = 1 to 2 and then slowly increased. On the other hand, the absorption at the α band position immediately rises beyond the equivalence point. Since the absorption and fluorescence changes do not parallel each other in the excess chromotrope region, it was suggested that acridine orange binds to DNA in at least two different manners. In contrast, the fluorescence and absorption intensity changes were found to parallel each other over all P/D values for the complexes formed with the flexible polymers, poly A and hexametaphosphate.

Polysaccharide Complexes

Stone et al. (1963) studied the optical behavior of acridine orange, methylene blue, proflavine, and neutral red complexes with several sulfonated polysaccharides, particularly the λ- and κ-carrageenans, in the hope of deriving some structural information about these polymers. The λ-carrageenans appear to be essentially linear chains of galactose units, each unit containing one sulfate group, whereas the κ-carrageenans consist of branched chains of repeating

disaccharide units in which the branches are more highly and irregularly sulfonated. These two polymers characterized two general classes of polysaccharides whose complexes differ sharply in their dye titration behavior. When proflavine or methylene blue solutions were titrated with increasing amounts of polymer, sharp endpoints, corresponding to equal dye and sulfate molarity, were obtained with the λ-carrageenans. The κ-polymers exhibited either no stoichiometric endpoint or, at best, only a moderately sharp break. This behavior was considered to be consistent with the regular site spacing on the λ-carrageenans and the irregular array, nonhomogeneous in intersite distance, on the branched κ-carrageenans. Nevertheless, both groups have large stacking tendencies that are larger for the polymers with higher sulfate content; values for the derived ground state free energy of interaction ranged from -2.8 kcal to -6.0 kcal per mole of dye pairs. It was also noted that the absorption maximum of bound monomer was not red-shifted relative to the free dye, unlike the nucleic acid complexes.

The stacking tendency for dyes bound to a given polymer was in the order acridine orange > methylene blue > proflavine, but the largest changes in absorption properties were exhibited by methylene blue, which also has the largest transition moment length of the dyes used. The degree of metachromasy was considered to increase with larger changes in the position of the absorption maximum, the intensity

ratio of α to β or γ bands, the transition moment length, and the visible oscillator strength. In general, the polymers with a larger sulfate content caused the largest decrease in both the transition moment length for bound dye and the visible oscillator strength (hypochromism), the total visible absorption area decreasing as much as 16% for neutral red complexes to 40% for methylene blue. The transition moments and observed wavelength shifts were also used to compute, according to nearest-neighbor exciton theory (Bradley, Tinoco, and Woody, 1963), the maximum distance between acridine orange and methylene blue molecules bound to λ-carrageenan to be 9.3 Å and 8.9 Å, respectively. But the observed hypochromism shows the involvement of additional dye coupling modes not considered in the simple exciton model.

The additional discovery of complex Cotton effects in the absorption bands of all four dyes upon forming metachromatic complexes with λ-carrageenans strongly suggested the presence of a regular secondary structure that imparts a helical pitch to the binding sites. Later, these complex Cotton effects, similar in shape to that predicted by exciton theory for a helical array of identical chromophores, were found for heparin, kerato sulfate, and chondroitin sulfate metachromatic complexes (Stone, 1964a, b; 1965). The reduction of metachromasy in heparin complexes by the addition of $MgCl_2$ or excess chromotrope was always accompanied by a loss of optical activity, although not in direct proportion; i.e.,

optical activity nearly disappeared in the excess chromotrope region by the time only 30% of the bound dye appeared as bound monomer. Besides the two sulfates per repeat unit, heparin contains 6 or 7 labile sulfate groups whose position in the molecule and structural importance are unknown. Hydrolysis of these groups caused little change in metachromasy, yet the Cotton effects were diminished by 67% for neutral red and 37% for methylene blue complexes. The parallel dependence of the optical activity of helical DNA and poly-L-glutamic acid dye complexes upon metachromasy and structural integrity (Neville and Bradley, 1961; Blout and Stryer, 1959), led Stone (1964b) to conclude that heparin and other polysaccharides that induce optical activity in bound dye possess helical secondary structures. In this respect it is interesting to note that heparin, an α-D-glycosaminidic polymer, and chondroitin sulfate, a β-D-glycosaminidic polymer, exhibited Cotton effects of opposite sign in the ultraviolet and induced rotations of opposite sign in their methylene blue complexes (Stone, 1965). Another interesting observation was that the addition of histamine to heparin complexes with methylene blue and neutral red resulted in a 50% reduction in the degree of metachromasy without affecting the magnitude of the induced rotation. Apparently, histamine preferentially displaces optically inactive arrays of bound dye without disturbing the structure of the optically active regions, thus supporting the view that only localized regions

of rigid, asymmetric structure are sufficient for the induction of the observed Cotton effects (Blake and Peacocke, 1965; Myhr and Foss, 1966). Stone (1964b) suggested the possibility that electronic interactions responsible for the induced optical activity may be operating over a longer range than those required for maximum metachromasy.

Virtually no recent literature exists on the fluorescent behavior of solutions of metachromatic dye:polysaccharide complexes, even though both exciton theory and the behavior of concentrated acridine orange solutions predict that dye stacking on the polymer surface should result in quenching and a bathochromic shift for the emission maximum. Appel and Zanker (1958) stated that the fluorescence color shifted from green to red as increasing amounts of heparin were added to an acridine orange solution, but no spectral data was given. It remains to be established that this fluorescence shift was the result of dye-dye interaction. Harris et al. (1961) found that strandin, a brain lipid containing a polysaccharide side chain, when present in small amounts, quenched the fluorescence of acriflavine, rivanol, acridine orange, and atebrin. Factors which caused the loss of metachromasy also reduced the degree of quenching. In addition, they found that the effectiveness of alcohol in restoring fluorescent intensity was greater with increasing chain length (n-propanol > ethanol > methanol). No shifts in the emission spectrum of acriflavine were observed, while the acridine orange emission

shifted 5 mμ to the red. However, this shift is probably associated with dye-polymer interaction, since under apparently the same conditions, the absorption was larger than that for free dye and was also red-shifted 5 mμ.

Since earlier work had suggested a relationship between metachromasy and precipitation, Pal and Schubert (1961, 1962) examined the sedimentable behavior of a number of polysaccharide:dye complexes. Credit for their discoveries must, however, be given to Szirmai and co-workers, whose earlier and unquoted work was obtained in a nearly identical study (see Szirmai and Balazs, 1958). Centrifugation at 700 x g caused no changes in the metachromatic absorption spectra and yielded no visible sediment, but a rapid sedimentation did occur at 60,000 x g, leaving behind an orthochromatic supernatant. In water solution the amount of material sedimented increased with increasing chromotrope concentration until an equivalent ratio of one was reached. With the addition of increasing amounts of excess chromotrope, the amount of sediment rapidly decreased toward nothing, while no corresponding changes in the extent of metachromasy occurred. The addition of small amounts of NaCl (.005 M) greatly increased the complex insolubility in the excess chromotrope region; further NaCl addition (to 0.1 M) eventually completely destroyed both metachromasy and sedimentation, presumably by destroying complex formation. But a chemical analysis of the pelleted compound revealed that the dye content and chromo-

trope equivalents were always equal, regardless of the equivalent ratio in the original complex solution. Sediments obtained from solutions containing a twentyfold excess of binding sites still contained one dye per site. These experiments demonstrated the marked tendency of the metachromatic dyes to fully saturate a few polysaccharide molecules rather than distribute more or less evenly over all available macromolecules. The fully saturated macromolecular complexes are insoluble in water and aggregate to form sedimentable particles, whereas the very few polyanions that are less than saturated, but still metachromatic, remain in solution.

Ethanol has long been known to destroy metachromasy, but the mechanism by which it does so is still unknown. Pal and Schubert (1962), in studying the effect of ethanol on the sedimentation behavior of polysaccharide:dye complexes, made two new observations: increasing amounts of ethanol caused a progressive drop in the amount of sedimentable material, and the amount of ethanol needed to destroy both metachromasy and sedimentation depended markedly on the particular dye:chromotrope complex. Proceeding on the notion that ethanol caused the dissociation of metachromatic compounds with liberation of free dye, a practical spectroscopic method was devised to measure the relative stability of the various combinations of dyes and chromotropes. Nearly 1:1 complexes were prepared in water and the rise in α band absorption was recorded as a function of the amount of added ethanol. The amount of

ethanol required to increase the absorption to half its final value, called the mid-plateau value, was then considered to be a sensitive measure of the stability of a particular metachromatic compound; the larger the required amount of ethanol, the greater the stability. In this way both dyes and chromotropes could be arranged in an order corresponding to their metachromatic "strength". For instance, the dye order was found to be toluidine blue > acridine orange > methylene blue > crystal violet, except in the case of chondroitin sulfate, where acridine orange formed a slightly stronger complex than toluidine blue. The chromotrope order, regardless of the dye used, was heparin > chondroitin sulfate > hyaluronate. But what relationship this ordering has to differences in complex structure is unknown, since it is not at all clear what forces are being measured by the so-called metachromatic "strength". The complex need not be dissociating, of course, and the fact that urea, on a molar basis, was found to give the same ordering and mid-plateau values suggests that hydrophobic bonds are not being broken. It is quite likely that the ordering is a relative measure of the ease with which water is displaced from the arrays of bound dye, which in turn may depend upon the relative dye orientation and intersite distance.

Another very interesting optical phenomenon was first described by Booij (1958) as strong proof for dye association on a polymer surface. When equal amounts of neutral red and

toluidine blue were added to a solution of chondroitin sulfate, the resulting absorption spectrum was intermediate between the spectrum of each dye complex alone and, furthermore, differed from the sum determined from the separated complexes. This only occurred when dyes of similar configuration were employed and was also observed in mixed dye solutions, in the absence of polymer, when salt was added to induce the formation of dye polymers. Thus, Booij concluded that dye association on the polymer surface must take place, that similar dye structures will easily form mixed polymers, and that the mixed polymer has its own characteristic absorption spectrum. Apparently unaware of Booij's work, Pal and Schubert (1963) also reported this phenomenon, but in greater detail, for a number of dyes and the chromotropes, chondroitin sulfate and λ-carrageenan. The term compound metachromasy was introduced to describe the new absorption spectra of mixed dye arrays. Although similar compound metachromasy should be observable, in principle, with other chromotropes, this original work has not yet been extended beyond the polysaccharides.

Pal and Schubert (1963) employed ten different dyes and their various pairs to illustrate the spectral changes that result from the formation of hetero-aggregates. Difference spectra obtained by subtracting the absorption sum of the single dye complexes from the observed dye pair:chromotrope spectrum very clearly revealed a common pattern. There is

always a positive deviation from absorption additivity at a wavelength near the γ band of the dye whose γ band is at the shorter wavelength and always a negative deviation near the γ band of the other dye component. The same was observed for mixed dye solutions (no chromotrope) at high enough concentrations where the appearance of difference spectra showed the formation of hetero-aggregates. Hence, both homo- and heteropolymerization of metachromatic dyes seem to be characterized by the suppression of a band at a longer wavelength and the emergence of a band at shorter wavelength. Some dye pairs, however, such as acridine orange and crystal violet, never exhibit difference spectra, even though both dyes readily form homo-aggregates. The formation of hetero-aggregates apparently requires structural similarity. The dye pairs acridine orange-methylene blue and crystal violet-rosaniline, for instance, exhibit the difference spectra characteristic of compound metachromasy.

The stoichiometry of the acridine orange:methylene blue: chondroitin sulfate complex was examined by centrifugation of solutions containing an excess, but equal amounts, of both dyes. In all cases the amount of acridine orange removed from solution was about 10% greater than the amount of methylene blue, showing that nearly equal amounts of both dyes are involved in the hetero-aggregate. But nothing is known of the sequential arrangement nor to what extent hetero-aggregation is preferred over the aggregation of like dyes.

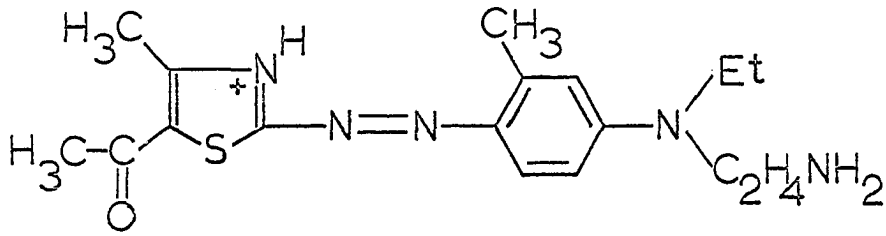
On the basis of the mid-plateau values obtained from destruction of metachromasy by ethanol, it would seem likely that in systems where large difference spectra are obtained, the majority of dye is involved in an hetero-aggregate. For example, acridine orange has a considerably larger mid-plateau value than methylene blue when both dyes are bound separately to hyaluronate, yet they both have the same value, near that for the acridine orange complex, when bound together. The same is true for chondroitin sulfate complexes, except the value for the mixed complex is larger than for either separate system. If either of the two dye components formed significant amounts of homo-aggregates, two mid-plateau values should be observed near those for the separate complexes. This was, in fact, found for the crystal violet:acridine orange:chondroitin sulfate complex for which compound metachromasy was never observed.

Polyglutamic Acid Complexes

Because polyglutamic acid (PGA) undergoes a reversible helix-coil transition as a function of pH in water solution, Stryer and Blout (1959) were led to investigate what effect the polymer conformation may have on the optical rotatory properties of several dye complexes. They consequently discovered that both acriflavine neutral (a commercial mixture of acriflavine and proflavine) and rhodamine 6G became optically active when bound to helical L-PGA but not with the

random form existing at pH above 6. In fact, the induced Cotton effects were stated to exhibit the same pH-dependency as the helical content of the polymer. That both dyes still bind strongly to the random conformation was supposedly shown by unreported equilibrium dialysis and spectral shift studies. Thus, it seemed established that an asymmetric, helical structure was responsible and required for the induced Cotton effects.

Later work with acridine orange, pseudoisocyanine, and a substituted toluidine-thiazolylazo dye (V) confirmed these results and showed that the signs of the induced Cotton effects for all three dyes are dependent upon the sense of the helix (Stryer and Blout, 1961; Stryer, 1962). Cotton



V

effects induced by helical D-PGA (a left-handed α-helix) were found to be quite similar in size and shape, but opposite in sign, to those induced by the right-handed, α-helical L-PGA. But the sign of the Cotton effect is also critically dependent upon the actual complex structure, which, in turn, seems to be greatly influenced by small changes in dye structure. For instance, the very similar Cotton effects of the acrifla-

vine neutral and acridine orange complexes with L-PGA are opposite in sign. Since Chin (1967) discovered that the proflavine complex with helical L-PGA exhibits Cotton effects of the same sign as acridine orange, it is apparently the acriflavine component of acriflavine neutral that yields the Cotton effects of opposite sign. Acriflavine differs from proflavine only in that a methyl group is substituted for the proton on the ring nitrogen. This conclusion is supported by Chin's discovery that a methyl group substitution on the ring nitrogen of acridine orange reverses the sign of the Cotton effects. Even more remarkable and unexplainable was the finding that the ethyl derivative yields Cotton effects of the same sign as acridine orange, while the sign was reversed again by the propyl derivative.

A closer study of the acridine orange:helical L-PGA complex by Stryer and Blout (1961) revealed that either one or two Cotton effects, opposite in sign, may exist, depending upon solution conditions. The magnitude of the negative one near 468 mμ was stated to be independent of pH from 4.3 up to the beginning of the helix-to-coil transition at pH 4.9. It was also invariant to changes in ionic strength (μ) from 0 to 0.18 and did not depend upon the glutamyl residue/dye molar ratio, R/D, over a very wide range (from 10 to 10,000). In contrast, the second Cotton effect, positive in sign, appeared near 510 mμ only when all three conditions of low pH, high ionic strength, and high R/D were satisfied. Its

magnitude was zero at pH 4.9, μ = 0.018, and R/D = 40, but became maximal and about twice the size of the shorter wavelength Cotton effect when the pH = 4.3, μ = 0.18, and R/D = 500. This quite different behavior strongly suggested that the two Cotton effects are associated with two distinct modes of binding of AO to the helical polymer.

Stryer and Blout (1961) proposed three general models to explain how bound dye might acquire optical activity. In Model I monomerically bound dye, randomly distributed on the PGA surface, becomes optically active by interacting with the asymmetric environment of the α-helix. But experimental support for this model was said to be lacking; in particular, the spectral shifts and very large molar rotations, based on the total dye concentration, indicated the presence of dye-dye interactions. For the AO complex, the maximum sizes of the negative and positive Cotton effects were on the order of 100,000 and 200,000, respectively. In Model II, two or more dye molecules were considered to form a head-to-tail super helix around the polypeptide α-helix, the sense of the super helix being dictated by the chirality of the polypeptide. Model III is also a dye helix, but differs from Model II in that it projects away from the polypeptide backbone and involves an overlapping of successive dye planes (like a helical stack of coins). The function of the α-helix is then merely to provide an asymmetric "seeding center" to favor one screw sense of dye clusters over the other. This last model

was deemed especially appropriate for the micellar pseudoisocyanine complex, since this dye had been shown by X-ray diffraction studies to form helical aggregates when concentrated in water solution (Rich and Kasha, unpublished data, quoted by Stryer and Blout, 1961). These micelles exhibit a sharp J-band that remains upon binding to helical PGA and also acquires a very large Cotton effect (molar rotation on the order of 1,000,000). The simplest explanation is that helical PGA favors the formation of tangential dye helices of one chirality over the other. On the other hand, the optically active soluble pseudoisocyanine complex may involve other binding modes, since no J-band nor corresponding Cotton effect was reported.

None of these models explained all the observations on the AO:L-PGA complex. It was originally suggested that the 468 mμ Cotton effect arose from Model III binding, whereas the one at 510 mμ was the result of dye super helix formation described in Model II. This assignment was based on the spectral shifts associated with the chromophore arrangements considered by McRae and Kasha (1958). A head-to-tail ordering of dye transition moments, as in Model II, results in a shift of the monomer absorption to the red, while blue shifts were predicted for planar stacks of transition moments. But the glaring fact remained that the positive Cotton effect at 510 mμ became maximally developed under precisely the most unfavorable conditions for dye-dye interaction. Not only is

a high R/D ratio necessary, but a low pH and high ionic strength are required. Thus, Stryer (1961b) was led to conclude in his Ph.D. thesis that the 510 mμ Cotton effect was associated with monomer dye (Model I) probably bound by hydrogen bonds between its two dimethylamino groups and the side chain carboxyl groups. Hydrophobic bonds between the polymer backbone and the planar surface of the dye were also considered possible. However, the monomer hypothesis does not readily explain the large induced rotations; work apparently still in progress and described by Blout (1964) may soon provide an experimental basis for accepting or rejecting this hypothesis. Poly-γ-benzyl-L-glutamate with the toluidine-thiazolylazo dye (V) covalently attached at one end was found by Dr. K. Yamaoka to exhibit Cotton effects in the dye absorption bands, but no indication of the size or location was given.

Stryer (1961b) also briefly examined the effect of α-helix length upon the magnitudes of the two Cotton effects of the AO:L-PGA complex. Since the incorporation of L-serine into L-PGA disrupts the α-helical conformation, copolymers with different percentages of L-serine were prepared, and the amount of helix relative to L-PGA was determined by the b_o values. As the proportion of helical structure was decreased, the magnitude of the 510 mμ Cotton effect dropped in a nearly parallel manner, but the 468 mμ Cotton effect was hardly affected, remaining within 90% of its original size even when

roughly 75% of the total helix content was destroyed. The same trend was found in experiments with low molecular weight L-PGA. Less than 20% of the 510 mμ Cotton effect remained with AO bound to completely helical PGA with an average length of 45 residues, while 60% of the negative Cotton effect still existed. These experiments again suggested that the 468 mμ Cotton effect is associated with Model III binding. Only a short helical region is needed to determine the handedness of a tangential dye helix, thus accounting for the relative insensitivity of the 468 mμ Cotton effect to α-helical length. But both Models I and II require a longer helical section and readily explain, qualitatively, at least, the much sharper sensitivity of the 510 mμ Cotton effect to changes in the length of helix. Since the dye is about 15 Å long, each monomer could conceivably be hydrogen bonded through its two dimethylamino groups to side chain carboxyls located three or four turns apart. Thus, Stryer regarded the assignment of the 510 mμ Cotton effect to Model I binding as being consistent with his helix length studies.

In contrast to the work of Stryer and Blout, Ballard et al. (1966) mentioned that pseudoisocyanine formed optically active complexes with both the coil and helical conformations of L-PGA. Since this dye also forms optically active self-polymers in the presence of (+)-tartrate (Mason, 1964), the implication became even stronger that the helical structure of PGA was not necessary for the selective formation

of a dye tangential helix of one handedness (Model III). Instead, the asymmetric environment around each α-carbon seemed sufficient for such a function in this particular case.

Ballard et al. also obtained results differing from those of Stryer and Blout in their study of the circular dichroism (CD) of the AO:helical L-PGA complex. Not only was the presence of a third optically active transition revealed near 438 mμ (positive in sign), but all three CD peaks showed the same dependence upon R/D in the range 1000 to 100, becoming nearly twice as large at the latter ratio. Upon lowering the R/D ratio to 20, the long wavelength Cotton effect (positive CD peak near 520 mμ) remained nearly constant in height, while the other two decreased considerably. Since these results were obtained in water solution, there is almost complete disagreement with Stryer and Blout on the effect of R/D and ionic strength on the magnitude of the several Cotton effects. The only point of accord appears to be the relative enhancement of the long wavelength Cotton effect caused by the addition of electrolyte. Having no good reason to associate the observed CD bands with different modes of binding, Ballard et al. preferred a dye super helix model, similar to Model II of Stryer and Blout, in which the Cotton effects arose from electronic transitions polarized parallel and perpendicular to the helix axis.

Ballard et al. (loc. cit.) assigned a parallel polarization to the negative CD band at 468 mμ and a perpendicular

polarization to the positive one located near 438 mμ by examining the changes in the CD spectrum under streaming conditions with light propagated along the flow lines. The intensity of the positive band near 510 mμ (in 0.2 M NaCl) did not change under the orientating conditions used, indicating that its polarization may be mixed. These results, together with the observation of Cotton effects in the UV region and a comparison of the CD and unpolarized absorption spectra, prompted the following inferences to be made about the structure of the dye super helix:

1) The molecular plane of AO is orientated neither parallel nor perpendicular to the α-helix axis. This conclusion is based upon the observation of both parallel- and perpendicularly-polarized CD bands.
2) The long axes of the dye molecules must form a left-handed super helix about the PGA core in order to give the observed signs to the identified CD bands.
3) The large unpolarized absorption near the perpendicularly-polarized transition region, as opposed to the small absorption in the parallel-polarized transition region, requires the long axis of AO to lie at an angle less than 45° to the α-helix axis.
4) The short axis of the dye is probably orientated radially to the α-helix direction. No Cotton effect was observed in the 295 mμ absorption peak assigned by molecular orbital calculations to a transition polarized along the

shorter axis. Supposedly, a radial orientation of this axis allows dye-dye coupling to yield only pitch-dependent rotational strengths that mutually cancel (due to small frequency separation and opposite signs).

On the basis of the above postulates and Drieding models, it was proposed that each AO molecule is attached by three hydrogen bonds (through its nitrogen atoms) to carboxyl groups located in the sequence n, (n+3), and (n+6) along the polypeptide chain. Side chain mobility then allowed the angle between the dye long axis and the α-helix axis to vary between 40° and 5°. The exact position of the next dye in the super helix was not made clear, however, in the diagram shown.

Yamaoka and Resnik (1966) resolved the ORD curves of AO:helical L-PGA complexes obtained for several R/D ratios between 250 and 10,000 into a minimum of four Cotton effects. Then, by use of a modified Kronig-Kramers transform, the component and resultant CD curves were computed for three representative cases; the CD curve at R/D = 500 was noted to agree closely with the experimental data given by Ballard et al. (1966) for R/D = 400. Also, the dependency of the Cotton effect magnitudes upon R/D appeared to follow the same general pattern described by Ballard et al. (1966), but not by Stryer and Blout (1961).

In the case where nearly twenty polymer molecules were present for each AO molecule (R/D = 10,000), substantial

Cotton effects were still observed, prompting Yamaoka and Resnik to believe that monomerically bound dye, rigidly held to unidentified sites, accounted for the optical activity observed near 500 mμ. This conclusion was suggested by the large monomeric character of the absorption spectrum of this complex. Attention was also directed to the possibility that two resolved Cotton effects (opposite in sign) at 495 mμ and 505 mμ be associated with two separate electronic transitions located in the α band region. Zanker et al. (1959) had assigned the major band at 495 mμ to the ^1L_b transition and, even earlier, had noted an isosbestic point at 518 mμ for dilute AO solutions at high ionic strength (Zanker, 1952). Wittwer and Zanker (1959) suggested the band located above 518 mμ be associated with the ^1L_a transition, which is forbidden for the free monomer dye. Since the binding of AO to PGA is always accompanied by an increase in absorption at wavelengths above the α peak position, Yamaoka and Resnik point out that dye-polymer interaction, as well as dye-dye association at lower R/D, may partially lift the forbiddeness of the ^1L_a transition. This transition may be perturbed by the asymmetric environment encountered upon binding to PGA and may therefore contribute considerably to the Cotton effects observed above 500 mμ.

The polarization directions of the bound dye transition moments relative to the α-helix axis can be determined by measuring the Kerr constant dispersion through the absorption

bands of the complex. Powers (1967) used this method for dimethylformamide solutions of AO:helical L-PGA complexes in the R/D range of 1000 to 4100. A positive anomalous dispersion occurs for dye moments orientated along the applied electrical field direction (also the direction in which the α-helix axis is aligned), and a negative dispersion is obtained for transition moments orientated perpendicular to this direction.

Powers discovered three component dispersions: a positive one in the α band region (but red-shifted from the peak), and two negative dispersions in the β and γ spectral regions. The size of the positive dispersion was also found to increase with larger values of R/D in a manner consistent with the notion that it is associated with monomerically bound dye. Since the sign is positive, Powers concluded that AO is bound to helical PGA with its long axis predominantly parallel to the α-helix axis in agreement with the conclusions of Ballard et al. (1966). While this seems to be the case for bound AO monomer, the negative dispersions found in the dimer and aggregated dye spectral region remained poorly understood and suggestive of a nearly perpendicular orientation for the long axis of interacting dye molecules. Powers, however, preferred the super helix model of Ballard et al. in which the monomer units are tilted with respect to the α-helix axis and the moments are split into perpendicular and parallel components. But no mention was made of the failure

to observe a positive Kerr effect, corresponding to the proposed parallel component, in the aggregated dye spectral region.

Hammes and Hubbard (1966) studied the kinetics of AO dimerization and binding to PGA in the hopes of deducing the mechanistic processes involved and, thereby, obtain some information on the nature of the complex. The temperature-jump method was employed, since AO self-association and binding to PGA are very fast, essentially diffusion-controlled processes. Two relaxation processes were observed for AO binding to both the helical and coil conformations of PGA, but both relaxation times were considerably larger for the helical polymer. Presumably, the higher degree of protonation in the helix case and the consequent reduction in electrostatic field accounts for the slower rates. Since the relaxation times for both the coil and helical complexes showed very nearly the same dependence upon R/D and the dye concentration, it was concluded that the overall mechanism of binding is independent of the PGA conformation. But, unfortunately, none of the several proposed mechanisms would quantitatively fit the data. The general mechanism appeared to be an initial, very rapid (probably electrostatic) binding followed by relatively slower intramolecular processes. Whether the two observed relaxation times described the initial reaction followed by a slower step or described two consecutive intramolecular steps (the initial binding being

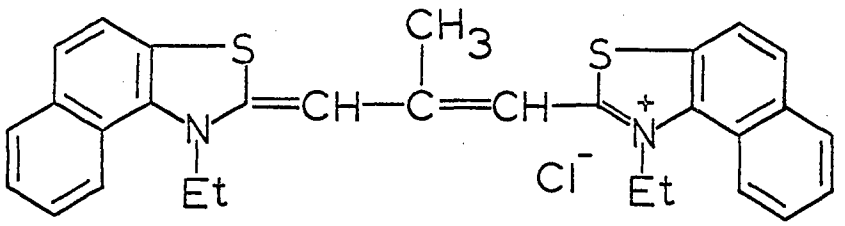
too fast to follow) could not be decided. In any case, at least one intramolecular rearrangement (stacking?) of dye molecules appears to be operative that probably involves the displacement of many solvent molecules and counterions.

Other Polymer Complexes

Very little recent work has been reported on the optical behavior of metachromatic dyes bound to polyelectrolytes other than those already mentioned. Blauer (1961a) briefly studied the pH-dependency of the absorption spectra of acridine orange:polymethacrylic acid (PMA) complexes at a fixed concentration of dye, polymer, and buffer. The usual metachromatic shift from the α band to a well-developed band at 452 mμ (448 mμ in water solution) was observed as the degree of ionization of PMA increased from 10% at pH 5 to over 90% at pH 9. Hence, as expected, dye aggregation on the polymer increases with an increase in the density and number of available binding sites. It was also noted that glutaric acid, which may serve as a dimer model for PMA, caused no spectral shifts under identical solution conditions.

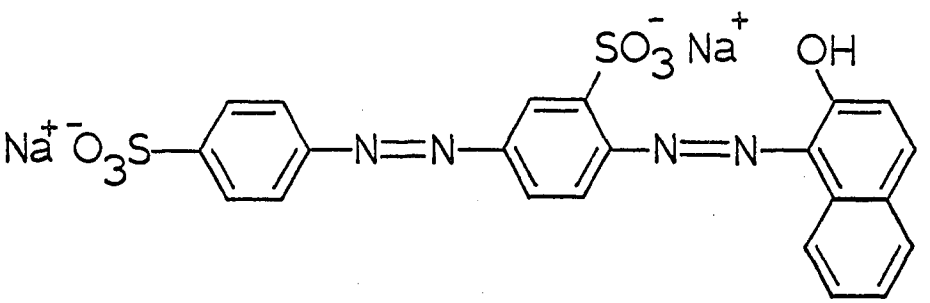
Kay, Walwick, and Gifford (1964a, b) presented an extensive survey of the many spectral changes occurring upon binding a thiacarbocyanine dye (VI) to a number of macromolecules including 32 different proteins. Besides being metachromatic, this dye exhibits a sharp J-band near 650 mμ that is characteristic of high polymer formation (Jelley, 1936, 1937). In

general, the extent of dye aggregation was related to the number of available anionic sites (affected by pH and structural changes), but the importance of the structural arrangement of these sites was indicated by the fact that poly-L-aspartic acid gave rise to only one new absorption peak (associated with dye dimers), while proteins caused the formation of both small and large aggregates.



VI

An inverse system, that is, the metachromatic binding of an anionic dye to polycations, was reported for the first time by Winkelman and Spicer (1963) for Biebrich scarlet (VII) complexes with histone, poly-L-lysine, polyvinylpyridinium bromide (PVP), and spermidine.



VII

This doubly-charged, diazo dye was found to behave Beer's law in aqueous solution over a wide concentration range, suggesting little or no tendency to aggregate, as further indicated

by the direct proportionality between solution conductance and dye concentration. But addition of any one of the polycations to the dye solution resulted in a large hypochromism and a bathochromic shift in the absorption maximum, both effects being reversed in the presence of excess chromotrope. In the case of histone, the maximum absorption was shifted from 505 mμ to 520 mμ; the shoulder at 545 mμ was similarly shifted but not reduced as much in height. Such a red shift upon aggregation on a polymer surface is predicted for certain stacking geometries by the exciton theory, but it has never been observed for the cationic dye:polyanion systems.

That the mode of binding is somewhat different from the cationic dyes is suggested by the stoichiometry at the point of maximum metachromasy, determined by conductance changes and a sharp break in the spectrophotometric titration curve: 2 sites per dye for histone, 2:1 for PVP, 4:1 for poly-L-lysine, and 10:1 for spermidine. Apparently, the dye binds by two salt linkages and requires a definite spacing of cationic sites. Further work with the histone complex (Winkelman and Bradley, 1966) established that the stacking coefficient is small (K = 3), corresponding to a ground state free energy of interaction of -0.7 kcal/mole of dye pairs. The rate of increase in the intensity of the monomer band, $d\epsilon_M/d(P/D)$, was large enough (167) to indicate that histone may possess a secondary structure. The authors specifically suggested a helical conformation, although the complex was

not examined for optical activity. For comparison, it was noted that the stacking coefficient of Biebrich scarlet on the flexible polymer, poly-L-lysine, was considerably larger than for histone. Nevertheless, the suggestion for structure is weak, being based upon the behavior of the quite different polynucleotide:cationic dye system.

Just recently, Winkelman (1967) reported on the fluorescence changes accompanying the binding of another anionic dye, $\alpha, \beta, \gamma, \delta$-tetraphenylporphinesulfonate, to several cationic proteins and poly-L-lysine. The porphyrin becomes maximally quenched near the equivalence point of dye and cationic sites, but the fluorescence reappears with the addition of excess chromotrope in a manner very dependent upon the particular polymer, eventually becoming greatly enhanced in the presence of a large excess of chromotrope. Also, the fluorescence was red-shifted about 9 mμ for bound monomer and about 5 mμ for the bound aggregate. Hence, the fluorescence quenching was ascribed to dye-dye interaction and the shift in emission wavelength to a dye-polymer interaction that is somewhat altered by dye aggregation.

Another system in which chromophore interaction on a polymer surface may possibly be responsible for the large reported changes in optical behavior is the poly-L-lysine: hemin complex. Blauer (1961b) found that the spectrum in the 500 to 650 mμ range for the helical poly-L-lysine complex differed greatly from that for hemin alone and closely

resembled that of the heme portion of ferricytochrome c. On the other hand, both poly-DL-lysine and the coil form of poly-L-lysine induced yet another spectrum that was somewhat similar to hemin alone. The Soret band of hemin was also stated to shift from 385 mμ to 420 mμ for the helical poly-L-lysine complex and to shift back to 388 mμ for the two coiled complexes. Subsequently, Stryer (1961a) found an unsymmetrical, negative Cotton effect in the Soret band at 420 mμ for only the helical complex. No anomalous dispersion was observed in the 500 to 650 mμ range for any of the complexes. The large size of this induced Cotton effect (peak-to-trough molar rotation of 400,000) strongly suggests transition moment interactions between closely bound hemin molecules, although this hypothesis was not advanced by Stryer. Unfortunately, neither Blauer nor Stryer examined the effect of molar ratio on the optical behavior of this complex to determine if a stacking process is operative and responsible for the induced rotation and Soret band shifts.

EXPERIMENTAL

Materials

Acridine orange

Commercial acridine orange (AO), marketed as the zinc double salt, $(AO)_2ZnCl_2$, was purified to remove the zinc and any minor dye components. About 5 gm of AO (Allied Chemical Corp.) was suspended in 100 ml of ice-cold 0.5 M NaOH and shaken for 30 min. After suction filtration, the AO base was resuspended in cold 0.5 M NaOH and shaken for another 30 min. This suspension was then suction filtered, washed with about 100 ml cold 0.5 M NaOH, and scraped into 200 ml of 65% ethanol-water (by volume). Dissolution was effected by heating to near boiling and stirring for 5-10 min. Then the hot solution was suction filtered and the filtrate allowed to cool before being titrated with 1 N NaOH. About 60 ml of base is required before AO crystallization occurs. The crystals were collected and dried *in vacuo* at 45°C. The melting point was 181-182°C (uncorrected), which agreed with Beilstein (180-182°C) and the 181°C value of Moudgill (1922). In addition, the visible spectrum of a 10^{-5} M solution in benzene was identical with that obtained by K. K. Yamaoka (unpublished preprint, 1963).

The AO hydrochloride (AO:HCl) was prepared by dissolving 1 gm of AO base in 100 ml of benzene, filtering, and passing HCl gas through the solution until AO:HCl precipitated and

HCl bubbled off. The dark red material was collected by suction filtration, washed twice with benzene, and air-dried. Then the AO:HCl was dissolved in water, passed through a 1/2-inch long G-25 Sephadex column (available from independent experiments) to remove filter paper lint, and collected by flash evaporation at 80°C. The dye was stored in the dark at room temperature.

Poly-L-glutamic acid

Poly-L-glutamic acid (PGA) was obtained as the sodium salt from Pilot Chemicals, Inc., Watertown, Mass. Although several different lot numbers were used in preliminary work, only one lot (G-76) was used in the majority of spectroscopic work reported here; the use of other lots will be specifically noted. This sample had a stated degree of polymerization of 712, which is a weight average figure obtained from viscosity measurements, and a molecular weight of approximately 100,000.

In order to remove low molecular weight material, the entire G-76 lot was prepared as a 2% solution, dialyzed against water for 48 hours at ca. 4°C, and then lyophilized. Prior to dialysis, the cellophane tubing (LaPine Sci. Co., no. 104-16, 21 mm) was boiled in dilute $NaHCO_3$ solution for about 10 minutes to remove any loose polymeric material and then thoroughly washed. Usually about a 25% loss in weight of the commercial PGA occurs during the dialysis-lyophilization procedure—10% due to water loss and about 15% from

passage of material across the membrane. Since some trouble was experienced with holes developing in the cellophane bags, portions of the lot were subjected to ultrafiltration before being lyophilized. A Diaflo membrane, type UM-2, which passes solute below a 500-600 molecular weight limit, was employed in conjunction with pressure dialysis equipment (Amicon Corp., Model 400, Design No. P-2). The lyophilized PGA was stored in a desiccator over anhydrous $Mg(ClO_4)_2$, since it readily absorbs water from the air to as much as 25% by weight.

Other materials

All other materials used were analytical grade reagents and were not further purified, unless specifically stated in the procedures obtained from the literature. The distilled water used in preparing all solutions was obtained from the AEC distillation system and was found to be consistently low in UV absorption and conductance.

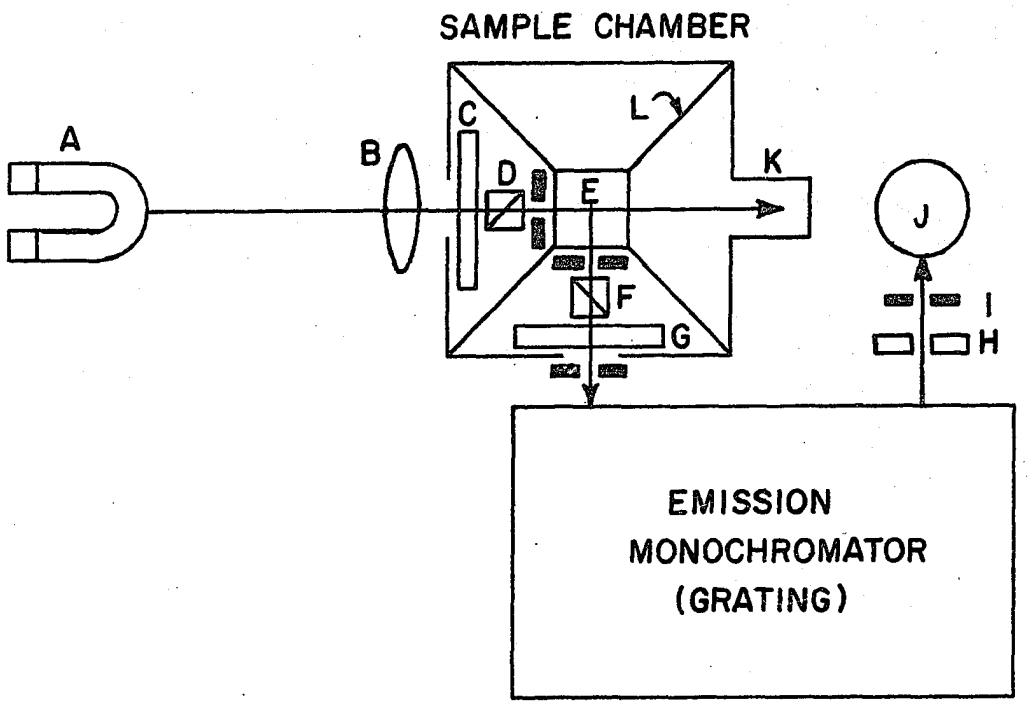
Spectroscopic Instruments

Absorption

All absorption determinations and spectra were obtained at room temperature (20-25°C) on a Cary 15 spectrophotometer. Quartz cuvettes with a 1 cm path length were used, and extinction coefficients are expressed in units of l/mole • cm.

Fluorescence

An Aminco-Kiers spectrophotofluorometer was modified to



LEGEND

A	Hanovia mercury arc	G	Yellow Filter
B	Quartz focusing lens	H	Shutter
C	UV-pass filter	I	Photomultiplier slit
D	Glan-Thompson polarizer	J	Photomultiplier
E	Cuvette holder	K	Light trap
F	Glan-Thompson analyzer	L	Light baffle

Figure 1. Optical schematic for fluorescence measurements

provide the increased light intensity needed for emission studies on the weakly fluorescent AO:PGA complex. The excitation monochromator was removed and the light source replaced by a U-shaped, 100 watt Hanovia quartz lamp, type S-100. Figure 1 shows the optical arrangement employed; light from the source (A) was passed through a quartz focusing lens (B) and a Corning UV-pass filter, C.S. 7-54, before falling on the sample in a 1 x 1 cm quartz cuvette (E). Scattered UV radiation was removed from the fluorescent beam by a yellow filter (G) placed directly in front of the entrance slit to the emission monochromator. This yellow filter has a low, nearly constant absorption throughout the AO emission region and sharply cuts off radiation below 416 mμ (transmission characteristics similar to a C.S. 3-73 Corning filter). An RCA 1P28 photomultiplier tube was used for the detector. With these modifications the sensitivity of the instrument to fluorescence near 535 mμ was increased by a factor of over 500.

The various slit widths employed were the smallest possible ones consistent with a reasonable signal level for a highly quenched complex. Sufficient signal was achieved with 5 mm square slits on the cuvette holder, a 0.5 mm entrance slit on the monochromator, and a 0.5 mm photomultiplier slit (I). This latter slit is located on a rotatable cylinder containing various slit sizes and closed positions. Since the cylinder did not accurately reposition the slit after

returning from a closed position, a camera shutter (H) was placed in the light path and the slit (I) left in position and undisturbed. Much more consistent readings were obtained at low light levels in this manner. Emission intensity readings were taken directly from the Aminco microphotometer and desired scans were recorded on a Moseley X-Y recorder (Autograph), Model 2S. The response of the detector and amplifier system was also checked by placing $KMnO_4$ solutions of known transmittancies between the detector and emission monochromator; the percent transmission versus photometer reading was found to be linear over the range of light intensities encountered in the experimental work.

In order to account for fluctuations in light intensity and instrumental response over the experimental time period and from day-to-day, a highly fluorescent, uranyl glass rod was used to provide a reference signal. The rod fluorescent intensity at 535 mμ was measured immediately after each experimental reading and compared to a prechosen, constant reference value, the resultant proportion then being used to adjust the experimental reading to a common basis. Solvent blank readings were essentially zero.

None of the recorded emission spectra were corrected for reabsorption, monochromator transmission, or photomultiplier sensitivity. Hence, the peak positions are apparent and the distortion at higher wavelength is probably large. For instance, the correction tables of White et al. (1960) and an

experimental measurement of AO emission through a rhodamine B fluorescence integrating solution (see Weber and Teale, 1957, for details) show that the recorded intensity at 600 mμ must be multiplied by about 4 to be directly comparable with the intensity measured at 535 mμ (apparent AO emission maximum). Above 600 mμ the 1P28 tube response falls off more rapidly, making the correction factor relative to 535 mμ much larger. The emission monochromator wavelength reading was compared with the mercury emission lines and found to be accurate within ±2 mμ of the stated value.

Fluorescence polarization was measured by placing two Glan-Thompson polarizing prisms (D and E) in the light path as shown in Figure 1. The prisms, rotatable through 90°, were mounted on the cuvette holder. In order to compensate for the large reduction in light intensity, the photomultiplier slit (I) was increased to 5 mm.

The polarization (P) was calculated from

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \qquad (5)$$

where I_{VV} and I_{VH} are the measured fluorescent intensities at 535 mμ with the excitation beam vertically polarized and the analyzer (F) oriented vertically and horizontally. G is a correction factor needed to account for the depolarizing effect of the monochromator grating and anisotropy in the photomultiplier response. Its value changes with the emission wavelength setting and is given by (I_{HV}/I_{HH}) for any

fluorescent sample (Chen and Bowman, 1965; Azumi and McGlynn, 1962). A value of 0.735 for G was obtained at 535 mμ. When the G correction was applied to a determination of the polarization spectrum of rhodamine B in glycerol (using the original fluorometer), the results agreed very well with the polarization values reported by Chen and Bowman (1965).

All fluorescence readings and spectra were recorded at ambient temperature (20-25°C). The sample chamber was maintained at room temperature by using an air blower and by blocking the intense exciting light between measurements.

Optical rotation

Optical rotation measurements and dispersion curves were obtained at room temperature on a Jasco recording polarimeter, Model ORD/UV-5. Usually a 1 cm Teflon cell with quartz end windows attached by silicone grease was used for the complexes, while a similar cell with a 5 cm path length was employed for PGA solutions. The absorption of the complex solutions rarely exceeded 1.5, normally being much lower, and no measurable artifacts due to stray light, birefringence, or fluorescence were observed. For stable solutions the rotational uncertainty was about ±0.001°.

Molar rotational values were computed either on the basis of total glutamyl residue or total dye concentration from the relation

$$[m] = \frac{10\,\alpha}{l \cdot c} \qquad (6)$$

where [m] is the molar rotation expressed in degrees $\cdot$ l $\cdot$ dm^{-1} $\cdot$ $mole^{-1}$, α is the observed rotation, l is the path length in decimeters, and c is the concentration in molarity. This equation is derived from the usual expressions for molar and specific rotations (Eliel, 1962, pp. 7-9). Dye molar rotations were computed only for the complexes in which nearly all the dye was bound and, as such, represent a lower limit to the true value for the optically active dye transitions.

Circular dichroism

The circular dichroism of complexes in a 1 cm cuvette was measured at room temperature with a modified Roussel-Jouan dichrograph. McCarville (1967) describes in detail the modifications and replacements made to achieve a 15-fold increase in sensitivity. Light from a 450 watt xenon arc was dispersed by a Cary Model 15 double prism monochromator and modulated by a Baird-Atomic crystal of potassium dideuterium phosphate, Model No. JV-1D(UV). A Princeton Applied Research lock-in amplifier, Model JB-4, replaced the original Jouan amplifier, and the modulating frequency was changed from 60 cps to 82 cps to minimize noise pickup. The dichrograph was calibrated by using a value of 0.00369 for ΔA for a 1 mg/ml solution of 5-α-cholestan-3-one in methanol in a 1 cm cell. This value was supplied by Dr. Paul Schatz, University of Virginia.

General Methods

Dye solutions

A 10^{-3} M stock solution of the purified AO was prepared in water and stored in the dark in a Pyrex volumetric flask covered with aluminum foil to help exclude light. The same flask was previously equilibrated with 10^{-3} M dye and, after only rinsing well with distilled water, was used for all subsequent stock solutions. At this concentration the percentage of dye lost by adsorption was very small; absorption spectra obtained from dilute solutions prepared from this stock were identical in shape and essentially so in magnitude after as much as a half year of storage.

The concentration of AO stock was initially established by the dry weight of dye dissolved in the 100 ml volumetric flask. Then the peak absorption of the band near 268 mμ was determined as a function of concentration, known from dilution volumes, over the range 3×10^{-6} M to 4×10^{-5} M. This band was chosen because, contrary to the visible bands, it was found to conform fairly well to Beer's law in aqueous solution over the desired concentration range. Stock solution was dispensed from a previously equilibrated syringe microburet (Micro-Metric Instruments Co., Model SB2, 1 μl per division) directly into a 1 cm cuvette containing a known volume of citrate-phosphate buffer, pH 4.5, 0.1 ionic strength (Colowick and Kaplan, 1955, p. 141). After quickly mixing by a wire attached to an electromagnetic vibrator, the

absorption was immediately and quickly scanned through the band position. An immediate rescan always showed a reduction in absorption (about 0.01 units), attributable to dye adsorption on the cuvette walls, that continues with successive rescans. But consistent readings could be obtained by working quickly after dilution. No adsorption losses in the microburet were noted over the experimental time period.

At least 5 readings (from 5 separate solutions) were averaged for each point on the absorption versus concentration plot. Although the points were fitted somewhat better by three straight line segments, a single straight line fitted by a least squares analysis yielded 47,500 $\pm$ 100 for the extinction coefficient and 0.013 $\pm$ 0.003 absorbance units for the intercept. The concentrations of all subsequent stock solutions were obtained from this plot. Stock solutions were also checked periodically for changes in concentration.

PGA solutions

Stock solutions of PGA were prepared by dissolving about 50 mg of the dialyzed, G-76 PGA in 25 ml distilled water. Any undissolved material was then removed by filtering through a 450 mμ Millipore filter (HA) in a Swinny hypodermic adapter and Leuer-Lok syringe assembly. Stocks were kept refrigerated and used for no longer than 3 months. More concentrated solutions for special experiments were also filtered before use.

Glutamyl residue concentration

Portions of a G-76 PGA stock solution were analyzed for total nitrogen content by the micro-Kjeldahl method. Both the precision and accuracy of this analysis were within 1% (based upon analysis of glutamic acid standards). The residue molar concentrations of earlier stock solutions (other PGA lot nos.) were obtained by sealed-tube, acid hydrolysis at 110°C for 24 hours followed by a ninhydrin color test for α-amino groups (Troll and Cannan, 1953). The ninhydrin color development was standardized against glutamic acid solutions of known concentration. A check on the method by a micro-Dumas nitrogen determination on solid PGA indicated the accuracy was within 2%. The standard deviation of the color test was about 3%.

After obtaining the residue molar concentration, portions of the stock were used to find the rotation at 236 mμ as a function of residue concentration. Such a plot was constructed for each lot of PGA and used to determine the residue concentration of all subsequent stock solutions of that particular lot. Although the dispersion curve trough was at 233 mμ, the additional light available at 236 mμ reduced the noise level considerably. A 5 cm path length was employed and measurements were made at room temperature upon solutions adjusted to 0.2 M in NaCl and to pH 6.0. Under these standard conditions, minor deviations in salt concentration, temperature, or pH caused very slight changes in the

observed rotation. Solutions were prepared by mixing known volumes of water and PGA stock, adding salt from a 2.5 M solution in a microburet, and adjusting the pH to 6.0 with 0.1 N HCl delivered from another microburet. The Jasco polarimeter was also calibrated with a standard sucrose solution before constructing the concentration plot and before making measurements on later stock solutions. In determining the concentration of subsequent stocks, three solutions of different concentrations were prepared and the results averaged. Use of the rotation plot adds about another 1% uncertainty to the precision obtainable by direct chemical analysis.

Complex preparation

Two of the variables discovered to affect the optical behavior of bound dye are the method of complex preparation and the age of the complex. In particular, the induced optical activity is often dramatically dependent upon these two factors. It is therefore very important to state precisely how a complex was prepared and how soon its optical properties were observed. The normal procedure for complex formation and optical measurements is described here, and intentional variations or those required by experimental technique will be noted when appropriate.

The complexes were prepared directly in a 1 x 1 cm cuvette; efficient mixing was accomplished with a wire attached to an electromagnetic vibrator. Desired volumes of

PGA and NaCl solutions were added by microburets to a measured volume of water in the cuvette. Then the pH was measured with a combination glass-calomel electrode and adjusted by the slow addition of 0.1 N HCl or 0.1 N NaOH from another microburet. After the PGA solution was prepared and mixed well, 10^{-3} M AO stock was added quickly from the pre-equilibrated syringe microburet, the solution mixed until just homogeneous, and the optical property immediately measured. Occasionally, the effect of aging on the optical property and solubility was noted; otherwise, the complex was discarded. Any desired spectral scans were obtained as quickly as possible on freshly prepared complexes. In the basic pH region the addition of dye often caused the pH of the PGA solution to drop as much as 0.3 units. The pH values recorded herein are those of the complex measured after the optical reading was obtained.

The normal procedure was not practical for experiments at very high R/D ratios. Instead, the experiment proceeded from high R/D to lower values by diluting the initial complex with dye solution of the same concentration as in the initial complex. A measured volume (3.0 ml) of concentrated PGA in water, previously adjusted to the desired pH and then filtered, was placed in a cuvette. Then 0.012 ml of 10^{-3} M AO stock was added from the equilibrated syringe microburet to prepare the initial complex. Successive complexes were prepared by transferring the preceding complex to a beaker,

adding the desired amount of 4×10^{-6} M AO stock by pipet, and mixing by magnetic stirring and several careful transfers between the cuvette and beaker. When the total complex volume reached about 15 ml, a 3 ml aliquot was placed in another beaker and the dilution continued with more dye stock. Loss of dye by adsorption to glass was negligible, since the dye was completely bound to PGA throughout the experiment. The dilute dye stock (4×10^{-6} M) was kept in a polyethylene container to minimize adsorption losses, and glass pipets were rinsed with dye solution before use. No precipitation of the complex was ever observed, and the absorbance of the excess complex solutions remained constant over the experimental time period. The glutamyl residue concentration of the filtered PGA solution was determined from the rotation standard plot by the usual procedure after first diluting 75 λ from a "to contain" micropipet to 25 ml.

pH measurements

Both Beckman and Sargent combination glass-calomel electrodes attached to a Heath pH meter, Model EUW-301, were used to permit pH measurements directly in a 1 x 1 cm cuvette. This method was found to be particularly advantageous for the optical studies in that small volumes may be used, solution is not lost, and pH changes occurring upon transferring dilute, unbuffered complexes are avoided. But a word of caution is in order. After about a half year of use, the Sargent combination electrode gave incorrect readings in

water solutions even though it operated normally in salt solutions and the standardizing buffers. It is not known whether this anomaly was a peculiar fault of the particular electrode used or whether the accumulation of strongly adsorbed dye is responsible. In any case, combination electrodes should be periodically checked against other electrode systems.

A Beckman Research pH meter and full-sized glass and calomel electrodes were used for more precise pH measurements in determining the percent protonation of PGA in the presence and absence of AO. The pH readings were recorded to the nearest 0.01 unit. Titrations were performed on 25.0 ml of solution in a closed, 50 ml beaker under a continuous supply of nitrogen passed previously through a concentrated KOH solution. Carbonate-free 0.1 N NaOH was used to adjust the initial pH of the solutions and blanks to pH 9, where the PGA was assumed to be 100% ionized. Then enough standardized 0.1 N HCl titrant was added to bring the pH near 5. At this point, the titration was continued with small aliquots of titrant, the solutions being stirred magnetically after each addition for no less than 1 min before taking a pH reading on the undisturbed liquid. Very little electrode drift, much less than 0.01 unit, was noted. The percent protonation of PGA as a function of pH was computed from the volume of added titrant minus the solvent blank volume.

Ultracentrifugal studies

Complexes were sedimented at 90,000 x g (computed for the center of the tube) for 2 hours using a Spinco Model L ultracentrifuge and the SW-39L swinging bucket rotor. Neither dye nor PGA solutions yielded any visible sediment under these conditions. Dye blanks were run along with the complexes in order to account for adsorption losses in the Lusteroid tubes. Immediately after centrifugation, the upper halves of the blank and complex solutions were removed with a polyethylene pipet with a turned-up tip and placed directly into a cuvette for an absorption scan.

The complexes were initially prepared in a polyethylene container by adding PGA to a 4×10^{-5} M dye solution. No aggregation was observed. Absorption scans of the complexes and dye blanks were also obtained before centrifugation.

RESULTS AND DISCUSSION

Initial Binding Studies and Problems

Equilibrium dialysis

The binding of AO to PGA at room temperature was initially examined by several equilibrium dialysis techniques. Solutions of dye and PGA in 0.1 M Tris[a], pH 8.1, and 0.1 M acetate buffer, pH 4.35, were prepared to study binding to the coil and helix, respectively. Usually the dye was dialyzed into a cellophane bag containing PGA solution; alternatively, a complex was dialyzed against free dye. Unfortunately, problems with precipitation of the complex long before the attainment of equilibrium were never solved. Heavy binding of dye to the cellophane membrane so retards the passage of dye molecules (probably electrostatically) that about 90 hours were required for the equalization of dye concentration across the membrane in control experiments and 60 to 70 hours for a close approach to equilibrium. Precipitation of the complex, however, was clearly evident within 24 hours and probably occurred much earlier near the membrane surface. Later, unrelated work revealed that the complexes usually precipitate or form suspensions of aggregated material, particularly in salt solution, within minutes after mixing.

By analogy to the chondroitin sulfate-dye complexes studied by Pal and Schubert (1961), the insoluble complex

[a]Tris(hydroxymethyl)aminomethane

species are probably those PGA molecules which have a high percentage of their sites filled by dye molecules. The extremely large stacking tendency of AO on both conformational forms of PGA (see later) would then seem to seriously limit the usefulness of this method for obtaining binding parameters. A consequence of the large stacking tendency is the predominance of nearly saturated PGA molecules, even at high R/D ratios, over the more soluble, only partially saturated polymers. The use of a very large excess of PGA over dye to avoid precipitation (R/D values on the order of 1000) would then be required, as well as the use of variable amounts of salt to avoid complete binding of all of the available dye.

Although the precipitation difficulties allowed no quantitative analysis, the binding behaviors of the helix and coil at an ionic strength near 0.1 were found to differ. The form of the precipitate was much different, being loose and particulate for the coil complex and a gelatinous slime that settles and clings to the dialysis tubing for the helix case. By lowering the dye concentration, precipitation of the coiled PGA could be avoided, but the extent of binding was also reduced to a value below the experimental error term. In contrast, the helix complexes precipitated under the same concentration conditions, suggesting the dye is more strongly bound to the helix than the coil in the presence of 0.1 M buffer. For the purpose of comparison to later work, the

initial R/D molar ratio for the soluble coil system was about 80 (considering the dye to be evenly distributed across the membrane), while the initial R x D was about 2.3×10^{-8} M^2. At this R x D value in 0.1 M Tris, the extent of binding was less than the detectable limit of 1 dye per 33 glutamyl residue groups. An increase in the initial R x D value to 4.4×10^{-8} M^2 by increasing the dye concentration (initial R/D lowered to about 40) caused considerable precipitation of the complex and increased the binding to about 1 dye per 6 residues. Thus, as soon as the extent of binding became measurable over the error term, the formation of nearly saturated complex species also apparently became so favorable that precipitation occurred.

The largest amount of binding noted in the dialysis experiments was about 1 dye per residue for the coil and 1 dye per 3 to 4 residues for the helix. However, no attempt was made to ascertain the maximum amount of dye that would bind to either conformation.

Inherent problems

It gradually became apparent from initial absorption and optical rotation measurements that the AO:PGA system is a very complicated one characterized by a host of metastable states and hysteresis effects. In addition, adsorption and precipitation problems had to be solved before reproducible spectral results could be obtained. A first step toward understanding this system was made with the development of a

standard and convenient method of complex formation, described in the Experimental section, that gives, at least, reproducible absorption and fluorescence spectra. But it must be understood that the data collected in this manner depends upon the method; nonequilibrium states are being measured and, as will be seen later from optical rotation measurements, there is still a lack of control over the precise structure of the complex. In this section the experimental rationale behind the chosen method will be given to show why it was necessary to work with nonequilibrium states.

The first problem that had to be overcome was the adsorption of dye and complex from solution. Dilute dye solutions cannot be placed in glass containers without incurring considerable losses. For instance, several transfers between a Pyrex glass tube and a quartz cuvette reduced the absorption at 490 mμ of a 4×10^{-5} M solution some 15-20%; polyallomer containers are equally bad. In agreement with Lamm and Neville (1965), polyethylene bottles were found to be quite suitable. Repeated transfers to a quartz cuvette reduced the absorption by only 4%, which could be ascribed to adsorption to the cuvette. Reproducible spectra should then be obtainable for complexes prepared in polyethylene containers were it not for the fact that the complex itself, particularly in salt solutions, often adsorbs to the walls near the meniscus. No such adsorption to Pyrex glass or quartz was observed, so the preparation of the complex directly in a

cuvette appeared to be the most suitable procedure. Dye adsorption losses could be avoided by adding 10^{-3} M AO stock as the last component and taking readings immediately after mixing. No measurable dye adsorption occurs within a half-minute after mixing, but thereafter, the absorption of dilute dye solutions quickly falls some 4 to 5%. Problems with the binding of complex to the platinum or tin-plated wires used for vibration mixing were avoided by the short mixing period and by washing with ethanol after each preparation.

The second major problem was coping with the aggregation and precipitation that so often occurs. Except at very high R/D ratios, the complex solutions are often slightly cloudy and the small particles begin to aggregate soon after mixing, eventually forming a mass of material that settles to the bottom of the container. The rate at which this occurs depends upon solution conditions, such as pH and salt concentration, and may occur within a minute after mixing or require days to form a sediment. Consequently, meaningful optical data either cannot be obtained several minutes after mixing or a large degree of scatter is found for solutions in which no visible changes in the state of aggregation are noted. Reproducible results could be obtained, however, if the mixing of dye and PGA was not extended much beyond that needed for homogeneity and the optical property was immediately measured. Vibration mixing rapidly disperses any aggregated material formed during the initial contact between AO

and PGA. Even under the adverse condition of pH below 4.0, the complex will remain in a dispersed state long enough for a quick absorption or fluorescence measurement.

Early work also pointed out the importance of using a standard technique for complex formation. Thus, the absorption spectra of complexes prepared by dialyzing dye into PGA solutions could not be duplicated by the direct addition of 10^{-3} M AO. Changing the order of mixing of dye and PGA solutions also produced somewhat different absorption spectra and, as will be seen later, may drastically alter the optical rotatory behavior of bound dye. This dependence upon mixing order was not fully investigated and may, in fact, be more related to the relative concentrations of the dye and PGA solutions being mixed. It was also noted that complexes prepared by changing the PGA conformation in the presence of AO did not exhibit the same optical rotatory behavior as those prepared by first changing the PGA conformation and then adding dye. And the fact that the positive CD band near 515 mμ could sometimes be partially and selectively removed by centrifugation suggested that the observed optical properties may be partly characteristic of dye involved in aggregate formation. If the nature and extent of aggregated material depends upon the method of mixing dye and PGA, then the use of several methods might be expected to yield variable optical data.

By using the technique of complex formation described

earlier and taking measurements immediately after mixing, consistent absorption and fluorescence data could be collected. This data provides a starting point for investigating the effects of time, heat, and changes in method. Some of these effects will be described later along with the data presented. That the method is not entirely satisfactory is shown by the variable optical rotatory behavior of the coil complexes. Hence, variables still exist that must be identified and controlled before systematic optical rotatory data can be obtained in the coil pH region.

Ultracentrifugal Binding Analysis

To help provide interpretations of initial optical titration data, another attempt was made to determine the concentration of free dye in equilibrium with the complex. Velick, Hayes, and Harting (1953) described an ultracentrifugal method in which a DPN-enzyme complex was completely sedimented and the upper portion of the supernatant subsequently withdrawn to determine the near-equilibrium concentration of free DPN[a]. By applying this method to the AO:PGA system and making several assumptions, a working value for the average number of binding sites per glutamyl residue could be obtained as well as an association constant for the binding process described below.

For a polymer possessing n binding sites that are identi-

[a]Diphosphopyridine nucleotide

cal and do not interact with one another, regardless of the extent of binding, the association with a smaller molecule or ion, A, is described by the equation (Klotz, 1953)

$$\frac{1}{r} = \frac{1}{nk} \cdot \frac{1}{c} + \frac{1}{n} \qquad (7)$$

where r is the ratio of the moles of bound A to the total moles of polymer in the system, c is the concentration of uncombined A, and k is an association constant for the reaction

$$\text{free site} + A \rightleftharpoons \text{occupied site} \qquad (i)$$

When the reciprocal of r is plotted against the reciprocal of the free concentration of A (a Klotz plot), a straight line is obtained that yields 1/n from the ordinate intercept and the value of k from the slope. A linear Klotz plot, in fact, serves as a test for the assumptions of identical and noninteracting sites under the solution conditions employed. If either or both of these assumptions fail to hold, as is the case for most real systems, a nonlinear plot will be obtained that is impossible to extrapolate with certainty to the ordinate intercept. The intercept still has the same meaning as in the identical, noninteracting case because $r \longrightarrow n$ when $c \longrightarrow \infty$, whatever the nature of the binding sites. However, only a reasonable guess can be made for the extrapolation, and consequently, the working value of n so determined may represent a lower limit.

The application of the ultracentrifugal method toward

determining the free dye concentration depends upon the aggregative nature of the AO:PGA complex, since the molecular weight of the individual polymer complex is not large enough for appreciable sedimentation under the conditions used. This requirement was checked experimentally by noting pellet formation and conversion of the visible absorption spectrum from the initial metachromatic form to the orthochromatic form characteristic of free dye. Also, the assumption was made that the combination of dye with PGA always leads to a spectral shift. As discovered later, this amounts to assuming a large stacking coefficient and requires that the total number of sites never greatly exceeds the total number of dye molecules. The free dye concentration was determined from an absorption measurement at the 268 mμ peak and the amount of bound dye obtained by difference from the dye concentration in the control tube after the ultracentrifugation. In this way the dye adsorption losses could be roughly accounted for.

The dye complex with helical PGA was first investigated in citrate-phosphate buffer, pH 4.5, ionic strength near 0.1, over the R/D range 0.9 to 3. The initial dye concentration of 4×10^{-5} M was reduced to about 3.5×10^{-5} M by adsorption losses over the experimental time period. Hence, experiments were confined to PGA concentrations where the amount of dye bound would result in at least a 1×10^{-5} M reduction in dye concentration. After centrifugation a pellet of material was

always obtained and the absorption spectrum of the supernatant could be matched with a dye solution. Because the spectra of the complexes before centrifugation were clearly different from that for the dye alone, the determination of free dye concentration under these conditions seemed approximately correct. When a Klotz plot was constructed from the data, a straight line was obtained as shown in Figure 2. In order to circumvent the problem of a distribution of molecular weights for PGA, the definition of $\underline{r}$ was changed to the ratio of the moles of bound dye to the total moles of glutamyl residue present; $\underline{n}$ then becomes the average number of binding sites per residue. Extrapolation of a least squares fit of the data yields a value of 3 for 1/n, meaning that, on the average, only one dye is bound for every three glutamyl groups. Since the extrapolation is very long, although this is not readily apparent from the Klotz plot, this value for 1/n would have to be regarded as an upper limit in the absence of any supporting information. Spectroscopic titrations to be described later, however, yield somewhat larger values for 1/n.

The slope of the line yields an association constant of $3.2 \times 10^5\ M^{-1}$, corresponding to a standard free energy change, ΔF°, of -3.4 kcal/mole at 20°C for an equilibrium like that shown in Reaction (i). However, the straight line was a surprising result in view of the literature studies on other, similar dye:polymer complexes. If the sites are assumed to

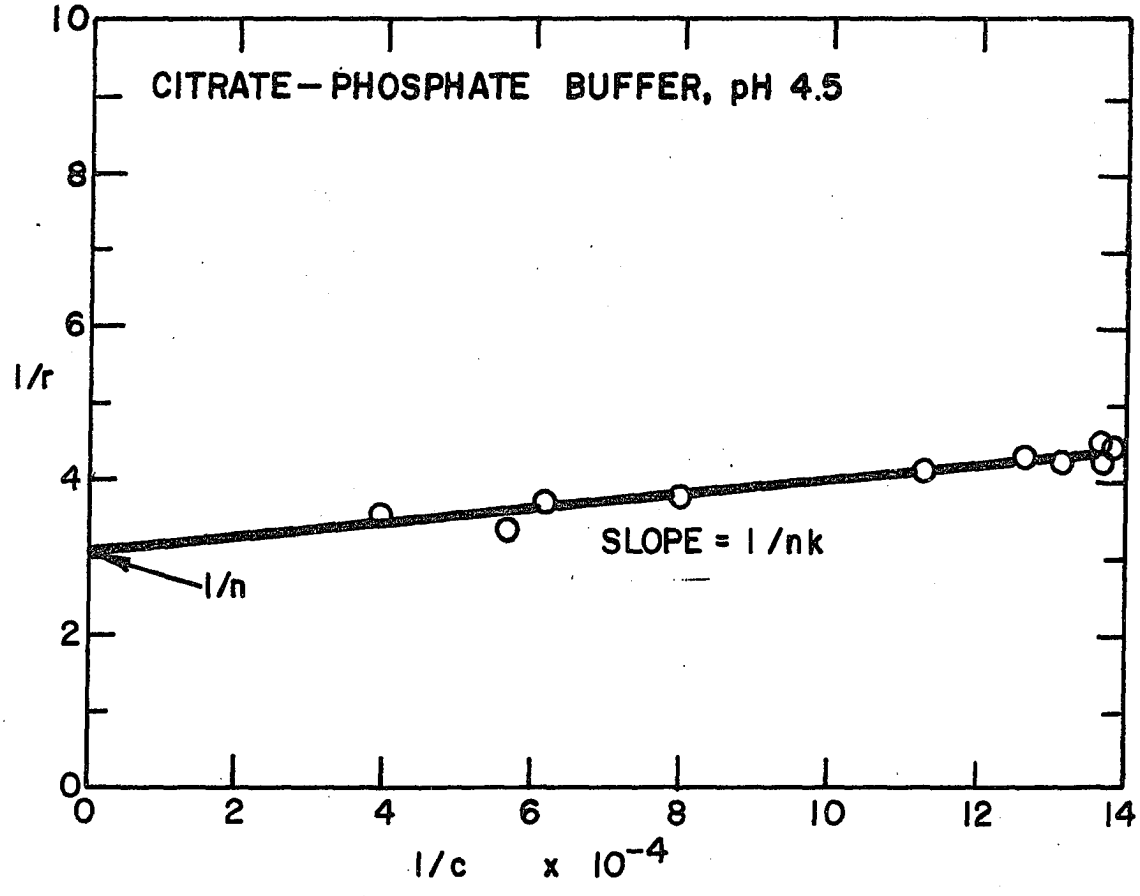
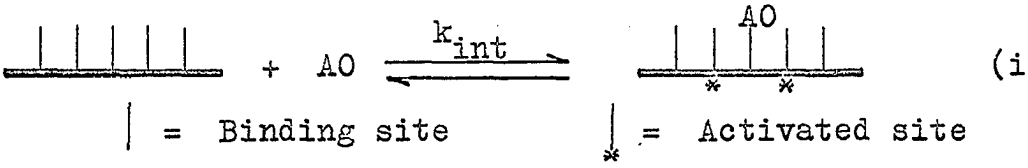


Figure 2. Klotz plot for ultracentrifugal data

be the side chain γ-carboxylate groups, so that ionic bonding is involved, then the identity of sites seems a reasonable possibility. In addition, electrostatic interactions between sites will be reduced considerably at an ionic strength of 0.1. Nevertheless, Bradley and co-workers have clearly shown that the second condition of noninteraction between sites is not satisfied for AO binding to several different polyanions. There is often a pronounced tendency, expressed by the stacking coefficient of Equation 3, for dye to aggregate on the polymer surface rather than to distribute randomly. Interaction between the bound dye molecules leads to the observed metachromasy. Since a very similar metachromasy is exhibited by the AO:PGA complex, it is reasonable to suppose both an interaction between AO molecules bound to PGA and a predominance of associated groups of bound dye over isolated species. The problem, then, is to reconcile this probable picture of the binding process to the linear Klotz plot in Figure 2. It turns out that the assumption of a large stacking coefficient is necessary for this purpose.

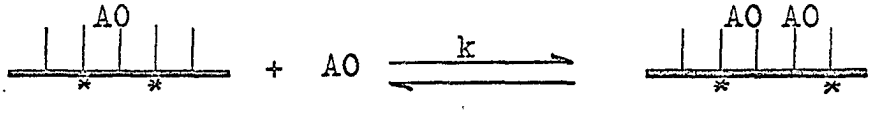
If the stacking concept of metachromasy is correct, the reaction between AO and PGA can be considered to occur in two consecutive steps—an activation step followed by a propagation process (stack formation). When a single dye molecule reversibly binds to any one of a number of identical sites, as represented by Reaction (ii), the binding affinities of both adjacent sites are altered. Two new sites are thereby

created that possess the strong, attractive, van der Waals forces responsible for the considerable dimerization occurring in AO solutions as well as the ionic and any other



(ii)

attractive forces characteristic of the original sites. Thus, the binding of a single dye molecule, described by an intrinsic association constant, k_{int}, may be considered as an activation step for the subsequent binding of more dye. The degree of activation is, of course, directly proportional to Bradley's stacking coefficient, which determines the magnitude of the association constant, $\underline{k}$, describing the propagation process in Reaction (iii). Assuming the magnitude of $\underline{k}$



(iii)

to be independent of the length of the bound dye stack, the stacking coefficient is given by

$$\text{stacking coefficient} = \frac{k}{k_{int}} \qquad (8)$$

Restricting our attention for the moment to the dimer formed in Reaction (iii), it can be seen that it is immaterial which dye was bound first. In fact, either dye may be considered to have been bound according to Reaction (iii). This is equivalent to saying that two dye molecules have combined with two identical, noninteracting, hypothetical

sites by an equilibrium process described by the association constant k. Once dimer formation has occurred, the hypothetical sites become real, and the same association constant describes further additions to the stack. The important condition that must be fulfilled before the system can be described in this manner is that the binding of a single dye molecule is always followed by the adjacent attachment of a second molecule. If the stacking coefficient is large, that is, $k >> k_{int}$, and one imposes the experimental restriction that the number of sites never greatly exceeds the number of dye molecules, this condition will essentially be met. The system can then be regarded as being composed of identical, noninteracting sites as long as the stacking does not proceed beyond the dimer stage. If it does, the additional requirement must be made that the stacking coefficient be virtually independent of the stack length.

Hence, the linear Klotz plot obtained experimentally suggests that helical PGA has a large stacking coefficient. Dye stacking also apparently does not proceed much beyond the dimer stage or else the stacking coefficient is not altered significantly by the stack length. The association constant derived from the slope is identified with k of Reaction (iii), and it allows the standard free energy change for AO dimerization on the PGA surface to be compared with that for dimerization in solution. Thus, Zanker (1952) gives $\Delta F^{o} = -5.7$ kcal/mole for AO dimer formation in citrate-phosphate buffer, pH 6,

while the corresponding value on the PGA surface is approximately 2 x (-3.4) = -6.8 kcal/mole. A value for k_{int} can also be obtained from Equation 8 once the stacking coefficient has been determined from spectroscopic titrations.

Attempts to apply the ultracentrifugal method to coil complexes at pH 7 failed. At this pH the ionic strength of the citrate-phosphate buffer becomes about 0.2, which is apparently large enough to destroy most of the complex. Absorption spectra of the complex solutions differed little from those for the free dye solutions and no sediment was collected upon centrifugation. On the other hand, a distinct γ band was formed in water solutions, but the complex was not completely removed by centrifugation. The absorption of the supernatant was only about half that of the original solution and the spectrum remained unchanged in shape. Unfortunately, it was not realized at the time that the R/D range of 2 to 11 used in these experiments was too large to expect all the complex molecules to aggregate and form sedimentable particles. The PGA molecules are about 90% ionized at pH 7 and it appears that nearly all these charges must be neutralized by dye binding before complete aggregation occurs. Hence, the ultracentrifugal method might still be applied to the coil complex solutions if the R/D ratio is restricted to values near or less than unity.

Optical Titrations

When increasing amounts of PGA are added to a solution of AO, the spectral changes produced are typical of the metachromatic reactions already described. The total visible absorption area is progressively reduced, the absorption maximum shifts to shorter wavelengths, and the fluorescence becomes quenched. These observed changes should continue to be enhanced until enough PGA has been added to bind all of the available dye. The addition of PGA beyond this point can then produce no further optical changes unless a redistribution of dye among the excess sites occurs and the optical behavior of bound dye is sensitive to its distribution. Thus, if some optical parameter can be found that is a linear function of the free dye and complex concentrations, a dye solution may be titrated with PGA to yield the average number of dye molecules bound per glutamyl residue. A break, whose sharpness will depend upon the size of the association constant, will occur in a plot of this optical parameter versus R/D. Judging from the size of the association constant obtained in the ultracentrifugal study, the stoichiometric break should be sharp enough to clearly locate the endpoint.

Titrations with coiled PGA

The effect of various amounts of the coil form of PGA on the absorption spectrum of 4×10^{-5} M AO is shown in Figure 3. These solutions were prepared in water, pH 8.3, by a constant

Figure 3. The visible absorption spectra for acridine orange and several coil complexes in water solution

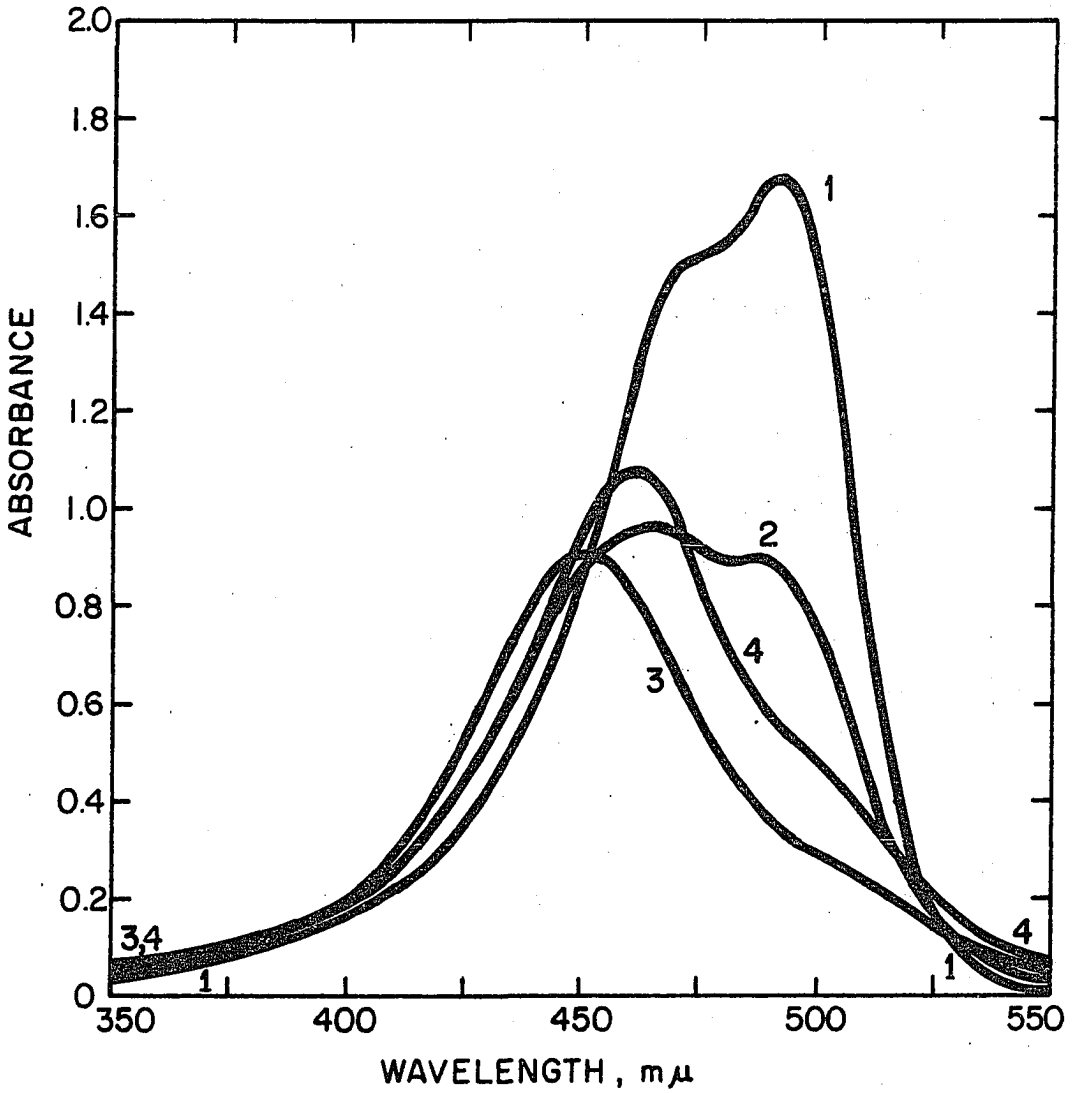
Total dye concentration is 4×10^{-5} M.

Curve 1: R/D = 0, pH 8.5

Curve 2: R/D = 0.58, pH 8.3

Curve 3: R/D = 1.25, pH 8.5

Curve 4: R/D = 56, pH 8.7


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
350
400
450
500
550
WAVELENGTH , mμ
1
2
3
4
3,4
1
1
4

volume titration. At this concentration of dye, a prominent shoulder at 474 mμ (corresponding to the β band) marks the presence of considerable AO dimer formation in the dye solution alone (Curve 1). Upon adding PGA to a molar R/D ratio of 0.68 (Curve 2), the shoulder becomes the absorption maximum and shifts to a shorter wavelength. A large hypochromism is also clearly evident, especially at the α band position (492 mμ). Further addition of PGA to R/D = 1.25 results in the development of a distinct γ band at 450 mμ (Curve 3). When the R/D molar ratio is increased to 56 (Curve 4), it appears that the absorption spectrum has begun to revert back toward that of the AO solution; the absorption maximum has shifted to 462 mμ and the α band absorption has increased.

The spectral changes attending the first two additions of PGA are very similar to those occurring upon increasing the concentration of an AO solution (Zanker, 1952). At a saturated concentration of 9×10^{-2} M, the molar extinction area of AO is markedly reduced from the level in dilute solution and the absorption maximum is shifted to 451 mμ. It is this striking similarity in spectral behavior that led to the theory of chromotropes acting as stacking templates for bound dye (see Review of the Literature). The stacking theory, in turn, predicts that the complex absorption spectrum will revert to the dye monomer spectrum in the presence of a large excess of sites (Bradley, 1961). The

partial return of the complex spectrum at R/D = 56 strongly suggests that the metachromasy of the coil PGA:AO complex has the same origin as in other metachromatic complexes—interaction between closely bound dye molecules.

In the early portion of the titration there are at least three species in solution: free monomer, free dimer, and the completely saturated PGA molecules. Because all three species have different absorption spectra with overlapping bands, no isosbestic points should be observed, and, in fact, none were. After the free dye concentration has been depleted by the addition of more PGA, the proportion of free dimers must decrease significantly. Now, essentially only two species exist, and rough isosbestic points appear as the titration is continued to completion. These points are shown in Figure 3 at ca. 453 mμ and 527 mμ. The existence of another transition above 527 mμ, enhanced in the presence of PGA, is clearly established. Particular attention will be drawn to this transition later.

As the titration is continued into the excess sites region, an unknown number of complex species are formed from the redistribution of bound dye. Thus, Curve 4 does not pass through the two isosbestic points. Isosbestic points were not noted in the excess chromotrope region itself, but few spectral comparisons were made. The absence of these points in this region would signify the presence of more than one type of binding site or the stacking of dye beyond the dimer

stage, each type of dimer or stack of different length having a different absorption spectrum.

Since the largest absorption changes occur at the α band position, the absorption at 490 mμ was chosen to follow the course of the titration. Thus, a decrease in A_{490} means an increase in the concentration of complex. A true titration curve, however, will be obtained only if both the free dye and complex obey Beer's law at this wavelength. Because of the monomer-dimer equilibrium among the free dye molecules, this component does not satisfy the requirement; the extinction coefficient at 490 mμ increases from 47,000 at 4×10^{-5} M in water to ca. 60,000 at infinite dilution. This 28% increase in ϵ_{490} with decreasing free dye concentration will cause the endpoint to be observed at a higher R/D than the true value. On the other hand, the coil complex was assumed to adhere fairly well to Beer's law. Earlier work with the helix complex in C-P buffer, pH 4.5, with the R/D ratio held constant at 8 revealed that this complex obeyed Beer's law at total dye concentrations below 4×10^{-5} M.

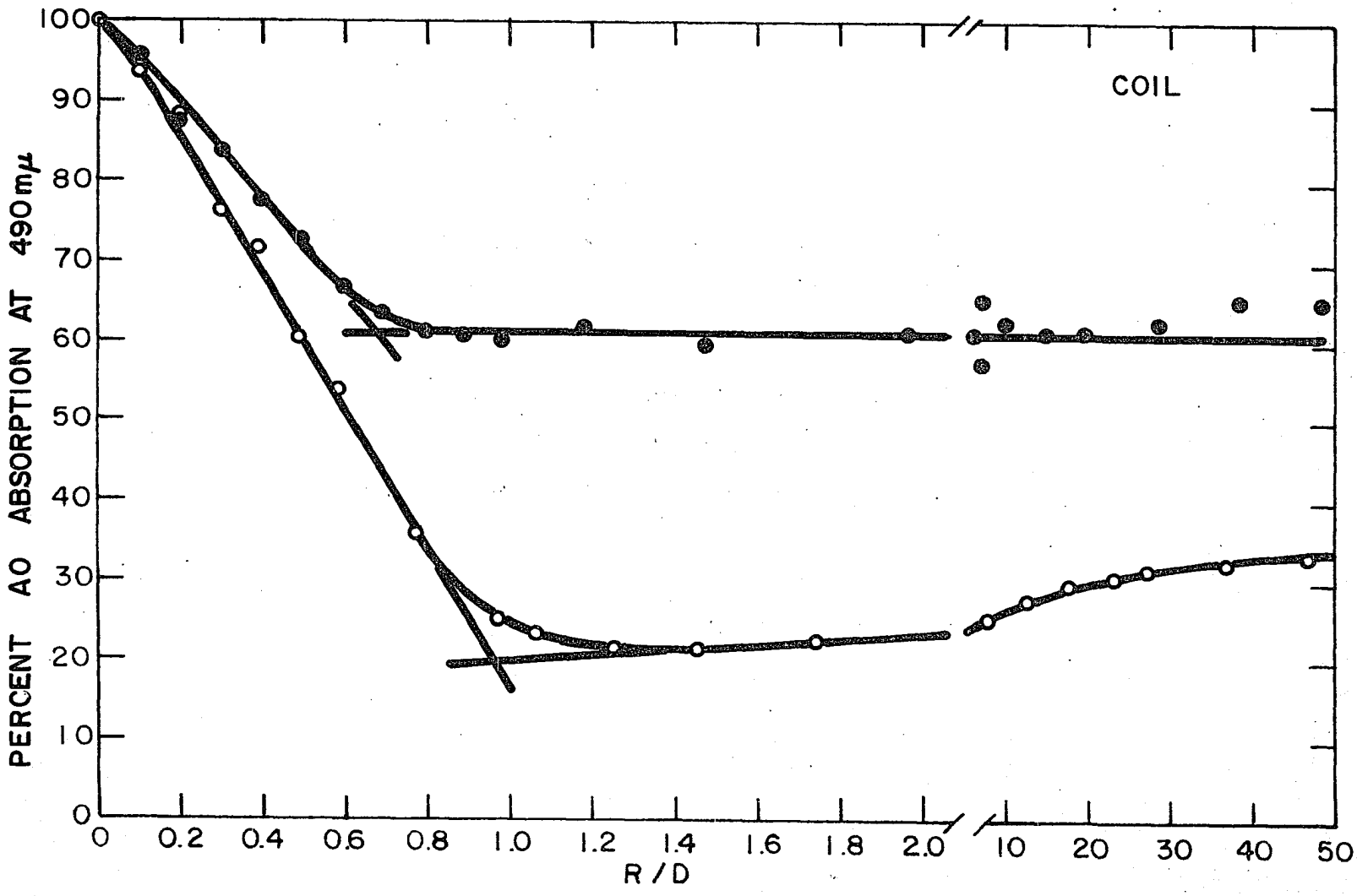
Figure 4 shows the result of titrating 4×10^{-5} M AO with the coil form of PGA. The absorption at 490 mμ is expressed as the percent of the AO absorption in the absence of PGA in order to minimize the effect of small day-to-day fluctuations in the initial absorption due primarily to room temperature changes and small changes in the AO stock concentration. Thus, the initial absorption at 490 mμ was 1.67 ±

Figure 4. The effect of an increasing proportion of the coil conformation of PGA on AO absorption at 490 mμ

Total dye concentration is 4×10^{-5} M.

o—o: Water solution, pH 8.1 to 8.7

●—●: 0.067 M NaCl solution, pH 8.0 to 8.7


COIL
PERCENT AO ABSORPTION AT 490mμ
R/D
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
10
20
30
40
50

0.02 in water and 1.43 ± 0.03 in 0.067 M NaCl. Each of the points shown is an average of the absorption of 2 to 4 separate complex solutions, and unless otherwise stated, the same will be true for subsequent titration plots.

The titration in water is seen to yield a break at a R/D ratio between 0.9 and 1.0. Because the free dye concentration is not linearly related to its absorption, the true break probably occurs close to 1.0, meaning that, on the average, one dye molecule is attached to each glutamyl residue. Evidence will be presented later that strongly suggests a γ-carboxylate group is included in each binding site. In the pH range of this titration the PGA molecule is nearly 100% ionized (Wada, 1960).

Soon after the equivalence point, A_{490} begins to rise, as indicated earlier in Figure 3. A redistribution of dye among the excess sites therefore takes place that results in an increase in the average distance between the bound dyes and a consequent loss in the amount of dye-dye interaction. The absence of isosbestic points in the R/D region of 1.25 to 60 reveals the presence of more than two complex species. If the sites are assumed to be identical, then dye stacks of several different lengths must exist, and a stacking coefficient may be determined by fitting Equation 3 to data obtained from continuing the titration to very large R/D ratios. This was done later for a fluorescence titration of the coil in water.

In 0.067 M NaCl solution, however, the break is observed at 0.68, corresponding to about 1.5 dye molecules per residue. The sharpness of the break and the flat plateau also require an explanation other than a competition of sodium ions for sites for the effect of NaCl on the coil complex. Instead, chloride anions may be involved in the multiple binding of dye to a site. Another possibility, which will receive considerable attention later, is that the sites may be altered by an increase in the polymer flexibility upon going from water to salt solution.

Besides the above absorption changes, the fluorescence of AO at 535 mμ was found to be about 99% quenched when bound to the PGA coil in water. Thus, it appeared that a fluorescence titration could be performed in which the observed emission at 535 mμ would be a function only of the free dye concentration. The result of such a titration in water, as shown in Figure 5, is a break at R/D = 0.93, which agrees closely with the A_{490} break. In 0.1 M NaCl, the coil complex is highly fluorescent and once again, the break occurs at a lower R/D ratio than in water. The break at R/D = 0.5 corresponds to two dye molecules per residue and suggests, after comparison with the absorption break in 0.067 M NaCl, that multiple dye binding to each glutamyl residue increases with the NaCl concentration.

The agreement of the fluorescence breaks with those obtained in the absorption titrations appears to be largely

Figure 5. The effect of an increasing proportion of the coil conformation of PGA on AO fluorescence at 535 mμ

Total dye concentration is 4×10^{-5} M.

o—o: Water solution, pH 7.9 to 8.8

●—o: 0.1 M NaCl solution, pH 8.2 to 8.7

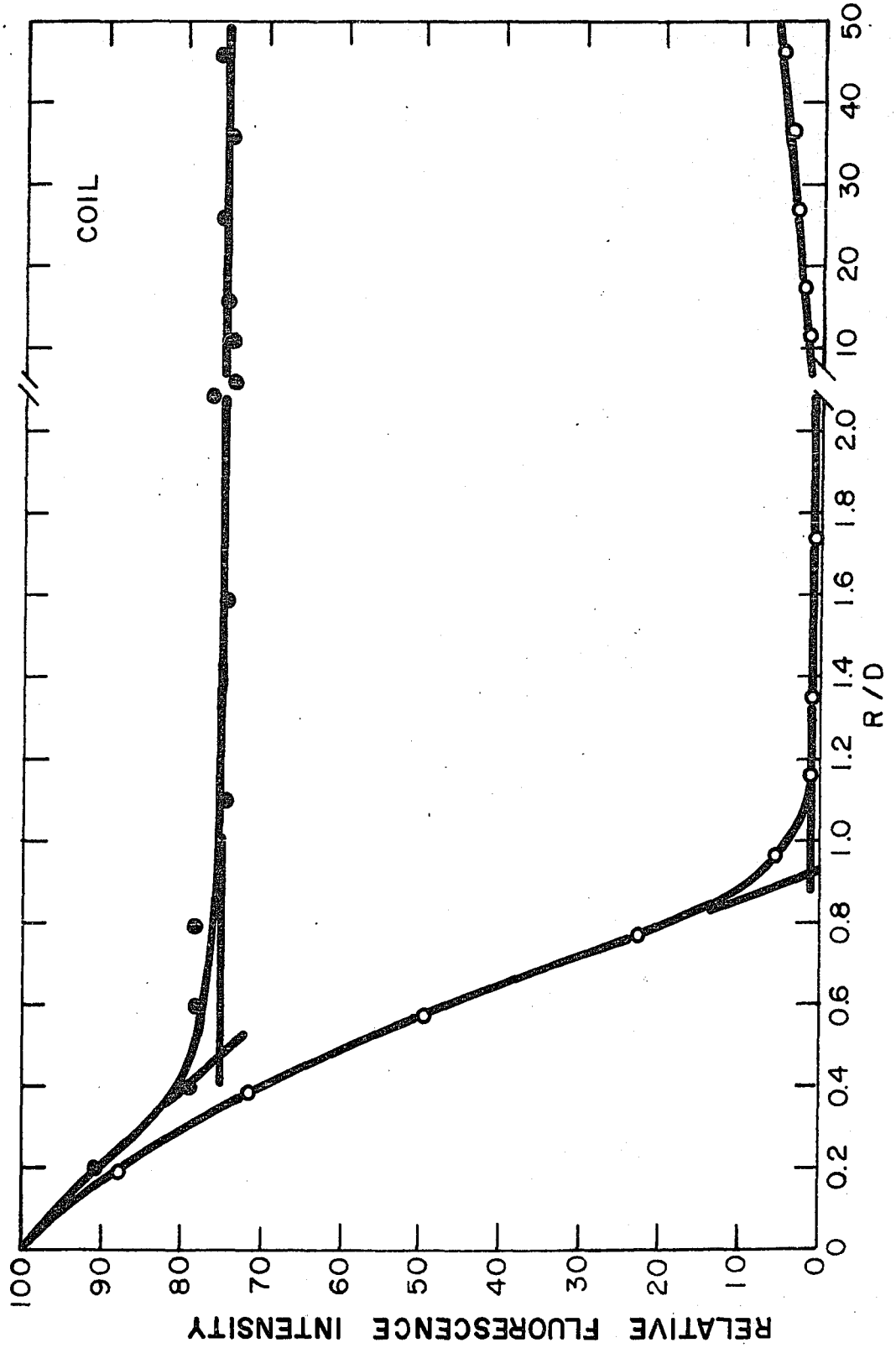
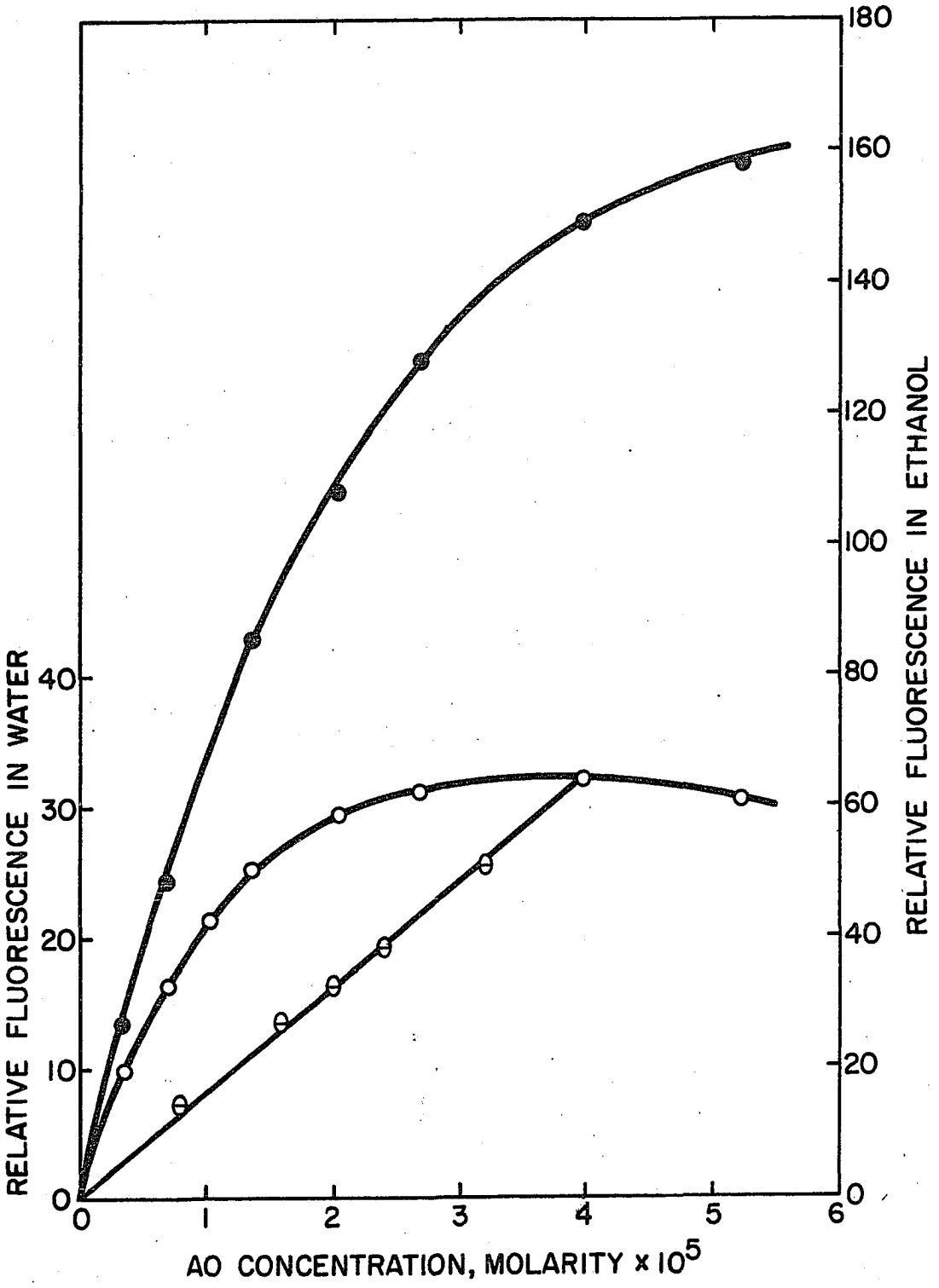

COIL
RELATIVE FLUORESCENCE INTENSITY
R/D
0
10
20
30
40
50
60
70
80
90
100
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
10
20
30
40
50

Figure 6. The relative fluorescence intensity of acridine orange at 535 mμ versus concentration

o—o: In water

⊙—⊙: In ethanol (Water content increases from 0% to 5% with increasing AO concentration.)

θ—θ: In the presence of coil complex during the R/D-titration in Figure 5


RELATIVE FLUORESCENCE IN WATER
RELATIVE FLUORESCENCE IN ETHANOL
AO CONCENTRATION, MOLARITY x 10^5
0
10
20
30
40
0
20
40
60
80
100
120
140
160
180
0
1
2
3
4
5
6

fortuitous, however. When the fluorescence intensity of the dye solution in water (at 535 mμ) was plotted against its concentration, as in Figure 6, a large deviation from linearity was found that has two principal causes. Most of the apparent decrease in fluorescence yield with increasing concentration is due to the formation of dimers, which were shown to be nonfluorescent by Zanker (1952). The magnitude of this loss of fluorescent molecules is clearly illustrated by the curve obtained in ethanol (Figure 6). Dimerization does not take place in ethanol over the concentration range shown (even though some water has been introduced from the AO stock solution), and the emission of a 4×10^{-5} M solution is seen to be almost 5 times as great as in water. The second cause is the inner filter effect exerted by the absorption of exciting radiation by any species in solution. It is this inner filter effect (Udenfriend, 1962, pp. 13-17) which results in the curvature of the plot in ethanol solutions. As the concentration of dye is increased, a larger and larger proportion of the exciting light is absorbed in the front portion of the cuvette. But the fluorescent molecules being observed are those near the center of the container, and these are being excited by less and less of the incident light beam. Thus, an apparent decrease in fluorescence yield is measured, and at sufficiently high concentrations the observed emission will fall to zero. Both dimerization and the inner filter effects of the monomers and nonfluorescent

dimers are responsible for the highly curved plot obtained in water.

A comparison of the water curve in Figure 6 with the fluorescence titration of the coil in water (Figure 5) reveals the fortuitous nature of the titration. Ignoring for the moment the inner filter effect of the nonfluorescent complex, it can be seen that a 10% drop on the titration curve corresponds to a change in free dye concentration from 4×10^{-5} M to about 2×10^{-5} M. This suggests that either half the available dye is bound at R/D = 0.14 (ca. 3.6 dyes per residue) or a complex composed of one dye per residue is quenching free dye. Only the latter alternative is consistent with the absorption titrations.

If the inner filter effect of the complex being formed throughout the titration is now considered, it should be realized that the titration is not equivalent to moving along the water curve of Figure 6 from right to left. Instead, the increasing concentration of complex is absorbing an increasing proportion of the exciting radiation. Within the spectral range passed by the Corning filter (230 mμ to 420 mμ) there are four mercury lines—254 mμ, 265 mμ, 303 mμ, and 313 mμ—that were judged to be of nearly equal importance for the excitation of free dye. Because the relative intensities of the lines were not measured, an estimate of their importance was made by taking relative values from Koller (1952, p. 39) and multiplying by the filter transmittance and the

percent of light absorbed by AO at each wavelength. Next, the absorption of the coil complex at a plateau R/D ratio was determined at each line. The absorption at 254 mμ, the mercury resonance line, was 0.50 for a 0.5 cm path, and the average absorption for all four lines was 0.35. Choosing 0.50 as an upper limit and assuming a 1:1 complex with a large association constant (as supported by the A_{490} titration), the expected free dye fluorescence in the presence of the complex was calculated for several points in the titration. The fluorescence intensity of the free AO concentration at each point was taken from Figure 6 and multiplied by the percent of exciting light transmitted by the complex to the center of the cell. The results of this calculation are plotted in Figure 6. The straight line obtained shows that the measured fluorescence intensity at 535 mμ is, in fact, nearly a linear function of the free dye concentration in the presence of complex. This result is not critically dependent upon the choice of 0.50 for the complex absorption, since the estimated average value of 0.35 gives a curved line close to the one drawn. Hence, the choice of a 4×10^{-5} M total dye concentration and the magnitude of the complex absorption just happen to result in a fluorescence titration curve that is roughly correct.

The above calculations are not sufficiently precise to discount the interesting possibility of the coil complex acting as an "energy trap" for excited free dye. Lavorel

(1957) has presented quantum yield data which strongly suggests the nonfluorescent dimer of thionine may be quenching free monomer. The overlap of the monomer emission band and the new absorption band formed at a longer wavelength than the α band upon dye aggregation makes the possibility of resonance energy transfer worth consideration. By application of the Stern-Volmer equation to a system in which the free dye concentration is held constant while the amount of complex is varied, it could be determined whether or not any quenching occurs. A nonzero slope would establish the quenching action of the complex and provide an estimate of the new lifetime of excited free monomer dye.

Titrations with helical PGA

Absorbance and fluorescence titrations were also performed with helical PGA in order to determine what effect the change in conformation and degree of protonation may have on the average number of dyes that will bind per residue. The spectral changes are much the same as with the coil form but are less pronounced. In Figure 7 are shown the results of an absorption titration at 490 mμ for the helix complex in water and in 0.1 M NaCl. The break in water now corresponds to only one dye per three residues at pH 4.8. In going to the salt solution, the average drops even further to one dye per four residues, suggesting a competition of sodium cations and an ionic nature for the binding sites.

The fluorescence emission spectrum of AO in water is

Figure 7. The effect of an increasing proportion of helical PGA on AO absorption at 490 mμ

Total dye concentration is 4×10^{-5} M.

o—o: Water solution, pH 4.7 to 4.8, initial $A_{490} = 1.684$. The points are single measurements and lot G-44 PGA, DP 475, was used.

●—●: 0.1 M NaCl solution, pH 4.5 to 4.6, initial $A_{490} = 1.450$.

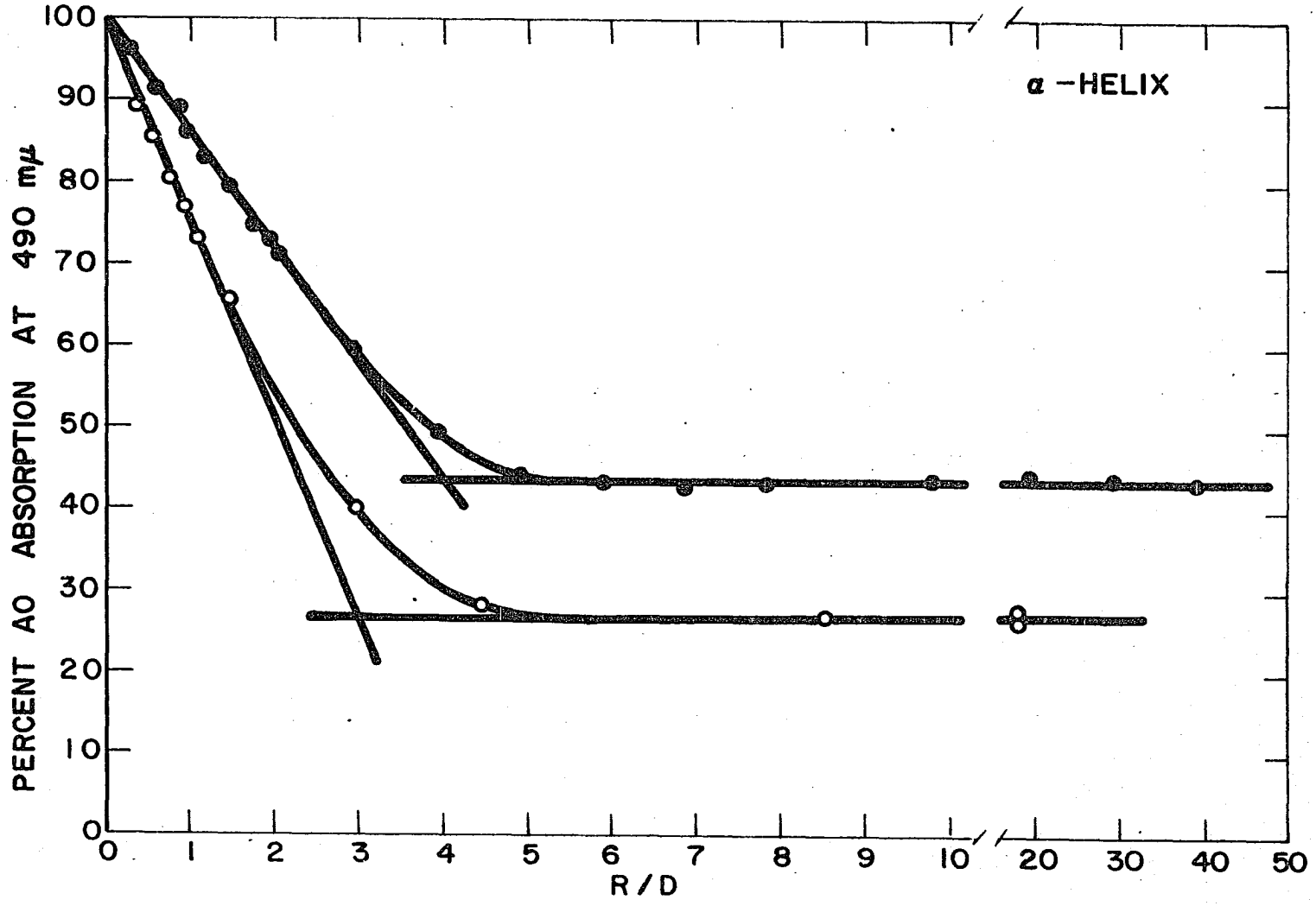

α -HELIX
PERCENT AO ABSORPTION AT 490 mμ
R/D
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
20
30
40
50

Figure 8. The quenching action of helical PGA on the AO fluorescence spectrum in water solution

Total dye concentration is 4×10^{-5} M.

Curve 1: R/D = 0, pH 4.5

Curve 2: R/D = 2.45, pH 4.5

Curve 3: R/D = 8.17, pH 4.5

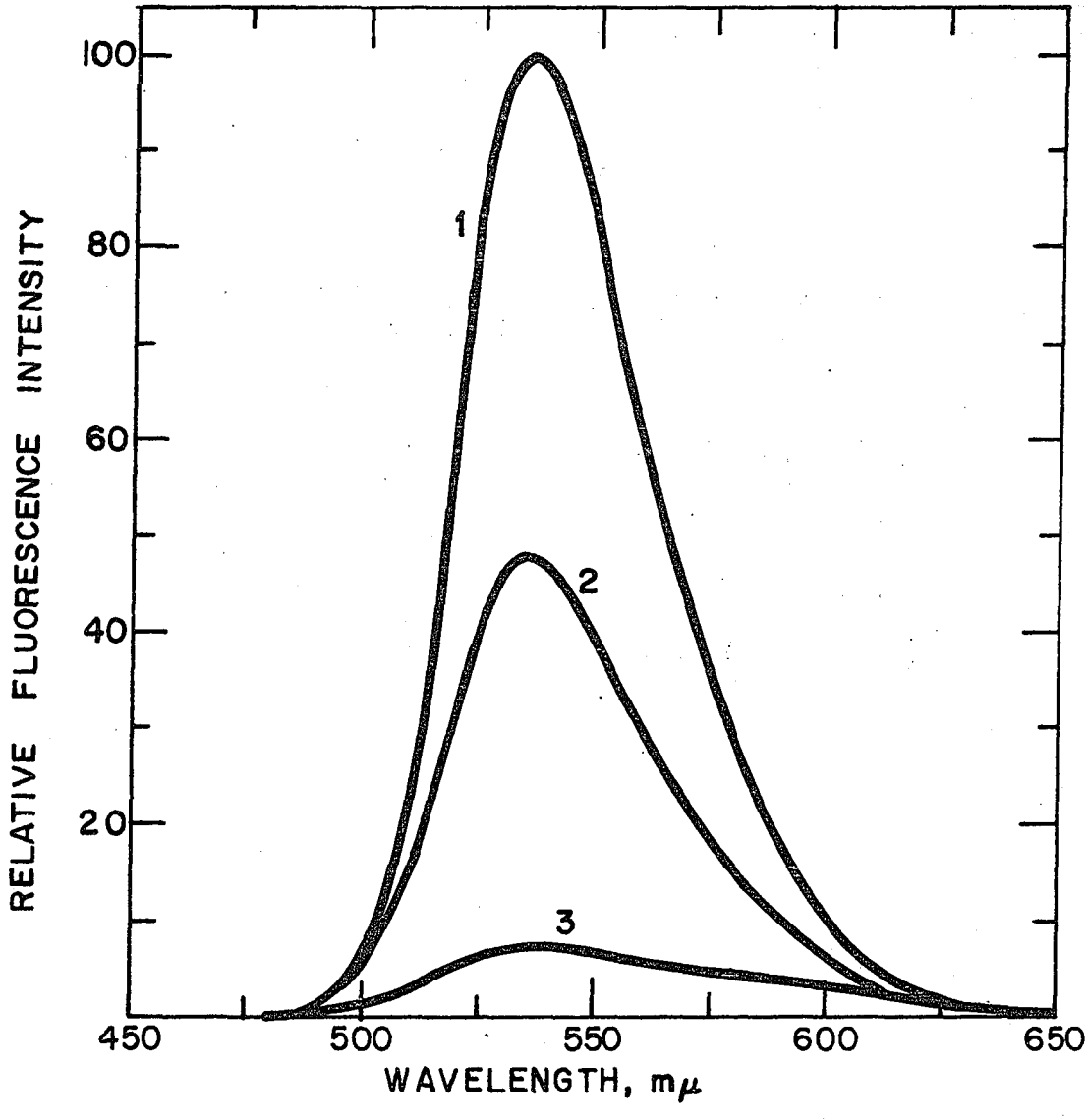
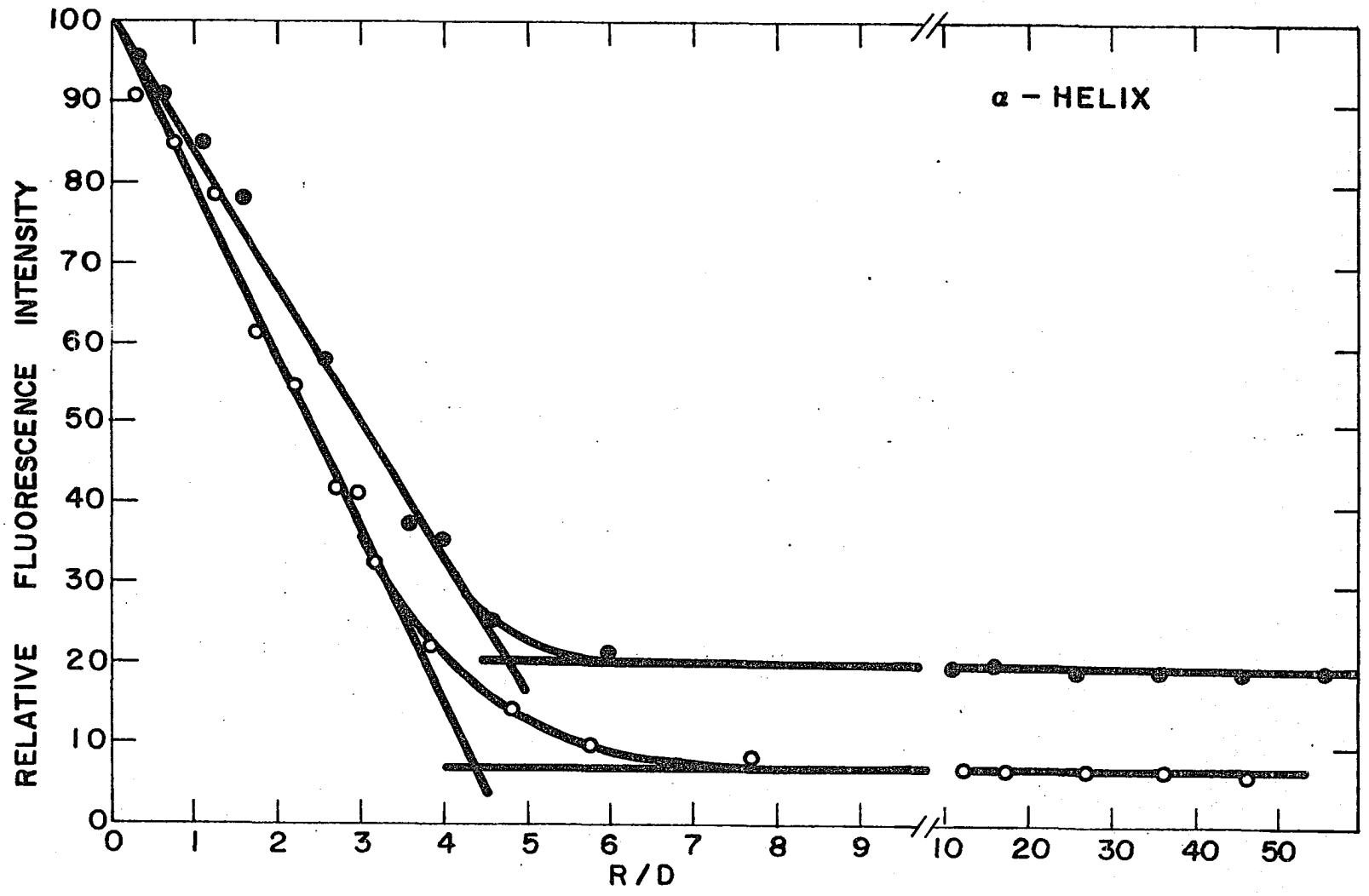

RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
20
1
2
3
450
500
550
600
650
WAVELENGTH, mμ

Figure 9. The effect of an increasing proportion of helical PGA on AO fluorescence at 535 mμ

Total dye concentration is 4×10^{-5} M.

o—o: Water solution, pH 4.4 to 4.6

●—●: 0.1 M NaCl solution, pH 4.4 to 4.6


α – HELIX
RELATIVE FLUORESCENCE INTENSITY
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
20
30
40
50
R/D

shown by Curve 1 in Figure 8 along with the effect of helical PGA on its size and shape. The observed emission at 535 mμ is cut in half at R/D = 2.45 (Curve 2) and to 7% of the initial intensity for the completely bound dye (R/D = 8.17, Curve 3). If the relative intensity at 535 mμ is followed as a function of R/D at pH 4.5, the titration curve shown in Figure 9 is obtained that exhibits a break at R/D = 4.4. In 0.1 M NaCl solution the break again shifts to a larger R/D ratio (4.75). A small amount of evidence suggests that the ultraviolet absorption of the helix complex is nearly independent of the ionic strength at values less than 0.1 and is almost equal to the coil complex absorption in water. (The absorbance of the coil complex in water, pH 8.1, R/D = 1.5, in a 1 cm path was 1.330 at the 264 mμ peak. In citrate-phosphate buffer, pH 4.5, R/D = 15, the absorbance of the helix complex was 1.400. The absorption of the helix complex in water will certainly lie between these two figures. No information exists on the ultraviolet absorption of the coil complex in salt solution.) Therefore, the same arguments presented in showing that the coil fluorescence titration in water was roughly correct will apply to the helix titration in both water and 0.1 M NaCl.

It seemed likely from the positions of the absorption and fluorescence breaks that the degree of protonation is more important than the coil-to-helix conformational change in determining the average number of dyes that will bind per

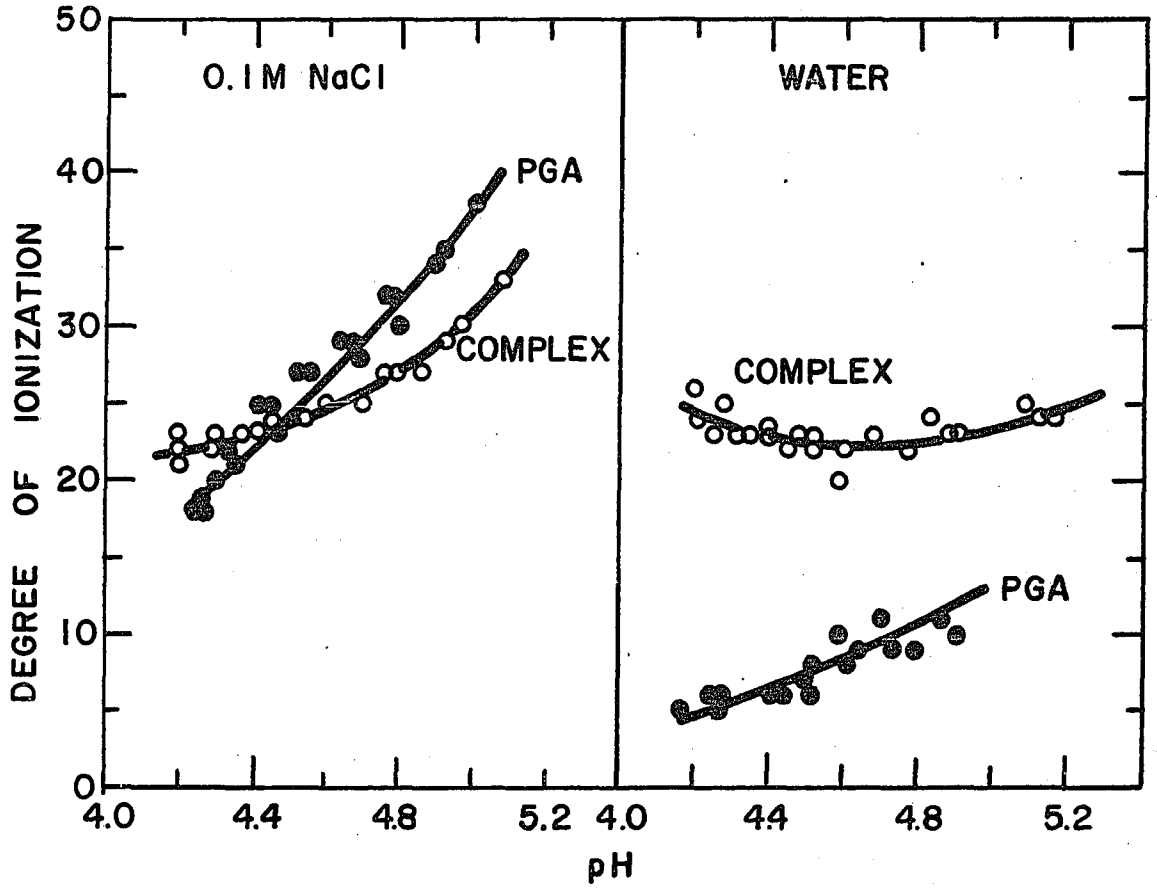
residue. The percent ionization of PGA in the pH range of these titrations is only on the order of 20% to 30% (Wada, 1960; Applequist and Breslow, 1963). Thus, a conversion of the R/D molar ratio to a carboxylate/dye ratio places the breaks close to unity. Before this interpretation may be placed on the titration breaks, however, the effect of dye binding on the degree of protonation of PGA in the helical pH region must be determined.

Assuming 100% ionization at pH 9, the degree of ionization in the helical pH region was found for PGA solutions in water and 0.1 M NaCl. Figure 10 shows these results for PGA solutions of the same order of concentration (0.005% by weight) as those occurring near the optical titration breaks. It is interesting to compare these ionization curves with those in the literature. Wada (1960) obtained a curve for ca. 0.03% PGA in 0.01 M NaCl that falls only slightly below the curve shown in Figure 10 for the 0.1 M NaCl solution. Upon going to 0.2 M NaCl, both Wada loc. cit. and Applequist and Breslow (1963) (who used a 5% PGA solution) obtained ionization values that fall from 53-54% at pH 5.0 to 15-20% at pH 4.0. Thus, it appears that a very dilute solution of PGA yields somewhat smaller percent ionization values than expected by interpolation from curves at higher PGA concentrations. This depression is more dramatic in water. Only Jacobson (1964) has published an ionization curve in water, and her curve for 0.15% PGA drops from 45% at pH 5.0 to 24%

Figure 10. The percent ionization of helical PGA and AO:PGA as a function of pH in water and 0.1 M NaCl solutions

●—●: PGA, $[R] = 31.6 \times 10^{-5}$ M or about 0.005% by weight

o—o: AO:PGA complex, $R/D \cong 4$, $[AO] = 8 \times 10^{-5}$ M


0.1 M NaCl
WATER
PGA
COMPLEX
COMPLEX
PGA
DEGREE OF IONIZATION
pH
0
10
20
30
40
50
4.0
4.4
4.8
5.2
4.0
4.4
4.8
5.2

at pH 4.0. These results lie far above the PGA curve shown in Figure 10, but they are not consistent with Wada's measurements in salt solution. Wada showed very clearly that the percent ionization increases with increasing NaCl concentration at any given pH. This is due in part to the competition of sodium ions for the γ-carboxylate sites and primarily to the decrease in energy required to separate a proton from a carboxyl group as the ionic strength (or electrostatic shielding) is increased. Curiously enough, Jacobson's curve in water is very close to that obtained by Wada in 0.2 M NaCl. Although it would appear that the water curve in Figure 10 should lie considerably lower than the one shown for 0.1 M NaCl, its position still seems disturbingly low. Apropos of the remarks in the Experimental section on the erroneous pH readings obtained in water, it is possible that PGA adsorption on the glass electrode is leading to incorrect results in this case also. And aside from the unknown effect of extreme dilution on the percent ionization values, the different charge states of the electrode surfaces in Jacobson's laboratory may partially account for the large discrepancy noted.

Figure 10 also shows the effect of AO binding on the percent ionization of PGA. To approximate the solution conditions near the optical titration breaks, the total dye concentration was set at 8×10^{-5} M, fixing R/D at 4. It turns out that the dye has little effect around pH 4.5 in

0.1 M NaCl solution, while in water the whole percent ionization curve is displaced to larger values. Thus, near pH 4.5 the number of protons bound to the complex is only slightly larger in water than in 0.1 M NaCl. If percent ionization values are now taken from these plots and used to convert the R/D ratios at the optical titration breaks to carboxylate/dye ratios, the figures shown in Table 1 are obtained.

Table 1. Conversion of the helix complex titration breaks from R/D to R^-/D

Titration	Medium	pH	Percent ionization	R/D	R^-/D
Absorption	water	4.8	23	3.0	0.69
Absorption	0.1 M NaCl	4.5	24	4.0	0.96
Fluorescence	water	4.5	23	4.4	1.01
Fluorescence	0.1 M NaCl	4.5	24	4.75	1.14
				Average	0.95

The resultant average R^-/D of 0.95 supports the notion that a carboxylate group is required for dye binding. And the fact that the titration break shifts to higher R/D values in going from water to salt solution is consistent with this notion. At a 2500-fold excess of sodium ions over AO cations, the sodium apparently competes to a small extent for binding sites. Of course, the average number of bound dyes per carboxylate says nothing about the actual distribution of dye. Because the titrations are performed in the presence of excess dye, one would expect a dye molecule to attach to every possible site. Nevertheless, the possibility cannot be

excluded that about half the carboxylates are sterically blocked by the attachment of dye to the other half, for instance, so that two dyes are bound to each site containing one carboxylate group. The low value of 0.69 obtained for R^-/D in the A_{490} titration in water (Table 1) suggests that some doubling up of dye on a site might occur. However, this titration was performed much earlier than the others and consists of only a few single points. The complexes were first prepared in a small polyethylene container (with pretitrated PGA) before pouring into a 1 cm cuvette for a quick spectral scan. This titration method is less accurate than the titrations performed directly in a cuvette, because the complex often adheres to the container wall.

Finally, some comment should be made about the relative positions of the percent ionization curves for the PGA and AO:PGA solutions. The curves in water solution can be understood from the standpoint that protons are displaced by dye binding over the entire pH range shown. But in 0.1 M NaCl solution, the two curves cross near pH 4.45. Below this pH value protons are released by dye binding, as in the water solution, but above pH 4.45 the degree of protonation actually seems to increase when dye is added. This result is not understood and is suspiciously regarded as evidence for erroneous pH measurements. In absorption experiments it was noted that the complex in salt solutions has a greatly increased tendency to bind to quartz. It is therefore

possible that the complex is partially coating the glass electrode, thereby introducing another junction potential into the pH-measuring circuit. Hence, the true percent ionization curve for the complex in 0.1 M NaCl may actually parallel the PGA curve, probably lying somewhat above it.

Stacking Coefficient Determination

The strongest support for the stacking theory of metachromasy in other dye:chromotrope systems comes from the reversal of the spectral changes at high R/D ratios. The AO:PGA complex is no exception. Figure 11 shows how the fluorescence intensity at 535 mμ, relative to that of free AO at 4×10^{-6} M, reappears as R/D becomes very large. If the dye is becoming distributed as monomer units on the PGA surface and the lifetime of the excited state is not altered by attachment to PGA, the final intensity should exceed that of the free dye solution. A comparison of the intensity in water and ethanol at 4×10^{-6} M (Figure 6) shows this final level would be about 2.8 times as intense. The fact that it is only about 90-95% of the free dye intensity suggests the excited state lifetime is shortened by coupling with the polymer vibrational modes. Only a small portion of this observed decrease in fluorescence yield may be attributed to the UV absorption of PGA in the spectral range passed by the Corning filter.

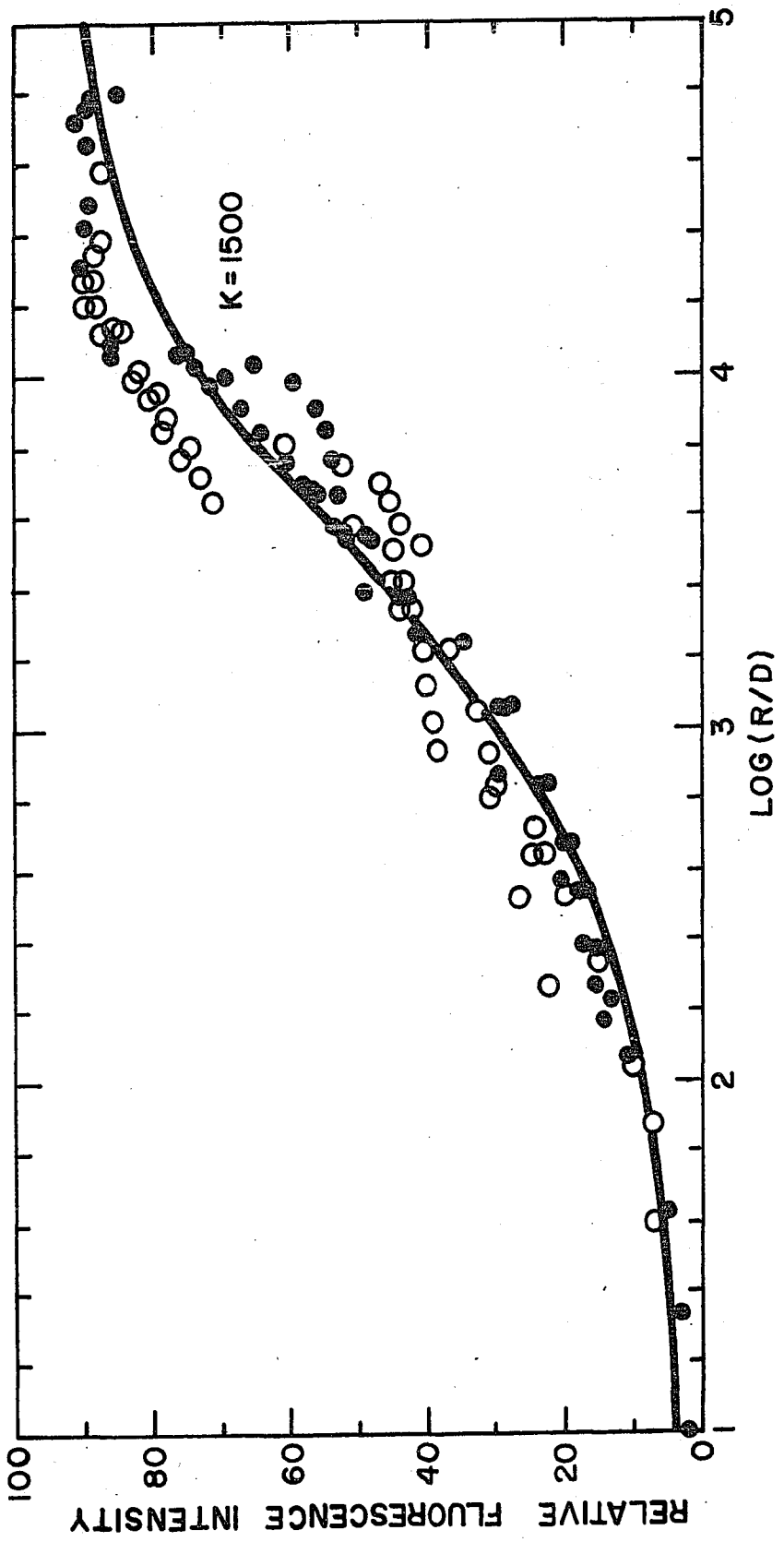
Of more interest is a comparison of the stacking tenden-

Figure 11. The relative fluorescence intensity of AO:PGA at 535 mμ as a function of very large R/D ratios

Total dye concentration is 4×10^{-6} M.

o—o: Helix complex in water, pH 5.2 to 5.5

●—●: Coil complex in water, pH 8.0 to 8.4


K=1500
RELATIVE FLUORESCENCE INTENSITY
LOG(R/D)
0
20
40
60
80
100
2
3
4
5

cies of AO on the helical and coiled conformations of PGA. Bradley and co-workers have shown that the stacking coefficient of AO on nucleic acids and polynucleotides increases markedly upon going from helical to coiled structures. Similarly, the flexible polysaccharide and polyphosphate polymers exhibit very large stacking coefficients (see Review of the Literature). The attractive explanation advanced was that the flexible polymers are free to assume intersite spacings for nearly optimal dye-dye interaction, while rigid polymers are not so free and the sites are likely to be held in a less favorable arrangement, resulting in less interaction energy. Figure 11 shows, however, that within the experimental scatter of points, both the helix and coil conformations of PGA in water solution have the same stacking coefficient for acridine orange. Furthermore, the stacking coefficient, K, is very large. A fit of Bradley's stacking equation, Equation 3, to the fluorescence data yields a value of ca. 1500 for K, corresponding to a free energy of interaction of -4.4 kcal/mole of dye-pairs. This fit was obtained by assuming no monomers at the relative intensity level of 4%, a complete redistribution to monomers at the 92% level, and by visually examining the fit for several values of K. The fit seemed somewhat poorer for K values of 1400 and 1600.

Bradley's empirical generalization on the value of K being related to polymer conformation obviously does not extend to poly-L-glutamic acid. Model building indicates, in

fact, that the spacing of sites on the α-helical conformation of PGA may be close to optimal. Dimers can be placed on this helical polymer in a number of ways that positions the charged ring nitrogens opposite two carboxylate groups and the aromatic rings in van der Waals contact (Rabinowitch and Epstein, 1941). Hence, some doubt is cast on the premise that the intersite spacings on rigid chromotropes will generally not be conducive to a high degree of dye-dye interaction. It is well to be reminded that Bradley's rule has not yet been formally extended beyond the nucleic acids and that the stacking coefficient may be insensitive to conformational changes in a number of dye:chromotrope systems.

The large scatter in Figure 11 is attributed to the experimental difficulties in working over such a wide range of R/D values. A low total dye concentration was used to avoid highly viscous solutions, but the viscosity of the initial complexes was still large enough to create some uncertainty in the initial PGA volume and the assumption of volume additivity throughout the experiment. Some loss of material may also have occurred during the many transfers involved. More precise experiments might point to the presence of breaks in the curve and thereby establish the presence of more than one kind of binding site for dimers. A hint of a break occurs near $\log(R/D) = 3.4$ ($R/D = 2500$), but this may easily be an artifact caused by the experimental technique. Instead, it is more likely that the various modes of binding,

if there are more than one, as suggested by model building, will differ so little in energy that only a smooth curve will be observed.

The absorption at 490 mμ was also followed while obtaining the first stacking curve for the coil complex in water. The visible absorption spectrum of the complex at log(R/D) = 4.8 (R/D = 63,000) appeared identical to that for the dye alone. Although anomalous breaks were obtained in working out the method, the changes in A_{490} and F_{535} closely paralleled each other. This is mentioned because the fluorescence behavior of a substance is, in general, more sensitive to environment than the absorption behavior, and it is possible that more than one empty site around each dye is required to prevent quenching. In this event, the fluorescence stacking curve would yield a larger value for K. A stacking curve should also be obtained from absorption measurements for comparison purposes, but the parallel behavior in the initial trial indicates about the same value for the stacking coefficient would be obtained. It should also be mentioned that the parallel behavior of A_{490} and F_{535} provides evidence against an efficient transfer of energy from an excited bound monomer to another monomer in the ground state.

Knowledge of the stacking coefficient allows an estimation of the intrinsic binding constant described in the Ultracentrifugal section. Using the value of 3.2×10^5 for the apparent association constant, the intrinsic constant is

given by Equation 8 as 2.1×10^2. This corresponds to an apparent pK of 2.3 for the dissociation of bound monomer dye from the α-helix at an ionic strength of 0.1. Since Wada (1960) obtained an intrinsic pK of 4.25 for the dissociation of protons from the helix in 0.2 M NaCl, these results show that AO will not effectively compete with protons for sites in salt solution. This is the same conclusion presented in the discussion of the percent ionization curves shown in Figure 10 for the PGA and AO:PGA solutions in 0.1 M NaCl. The shift in the percent ionization curve obtained in the presence of AO in water solution suggests that the apparent pK for the monomer binding process increases with decreasing ionic strength.

Effect of Salt on the Helix and Coil Complexes

It is obvious from the plateau levels in the absorption and fluorescence titrations that NaCl has a much greater effect on the optical properties of the coil complex than on the helix complex. Experience has suggested that the α band absorption level be regarded as an inverse function of the degree of dye-dye interaction. That is, a decrease in A_{490} has always been accompanied by a shift in the absorption maximum to a shorter wavelength, an increase in the height of the absorption peak, or both. A treatment of interacting chromophores by either the exciton model (Bradley, Tinoco, and Woody, 1963) or a classical model (DeVoe, 1964) shows that

increased dye-dye interaction results in a larger splitting of the first excited electronic state, but the selection rules are determined by the angle between the transition moments or electronic oscillators. Thus, it is not entirely clear that the absorption at the monomer band position should always be sensitive to changes in the dye interaction energy and that a decrease in A_{490} means an increase in this energy. The respective changes certainly will not be inversely proportional, but as a practical matter, the absorption at 490 mμ seems to be the most convenient optical parameter to follow as a function of solution conditions. Whenever sudden changes in A_{490} are encountered, complete spectra can be obtained to see more clearly what is happening.

The absorption and fluorescence of the helix complex increase gradually with the NaCl concentration as shown in Figures 12 and 13. In these figures the R/D ratio is held constant near 18 to insure that all of the available dye is bound. The flat plateaus in the optical titrations show that none of the dye is being displaced by the addition of NaCl to a concentration at least as high as 0.1 molar and that dye stacks larger than dimers probably do not exist. Therefore, the increases in A_{490} and F_{535} are probably not due to a redistribution of dye from stacks to a greater number of dimer configurations. Neither is the α-helical conformation of PGA disrupted by the addition of NaCl nor does it seem likely that the inorganic ions alter the stacking geometry of

Figure 12. The AO:PGA complex absorption relative to free AO at 490 mμ versus NaCl concentration

Total dye concentration is 4×10^{-5} M.

o—o: Coil complex, R/D = 18, pH 8.0 to 8.6

●—●: Helix complex, R/D = 18, pH 4.4 to 4.7

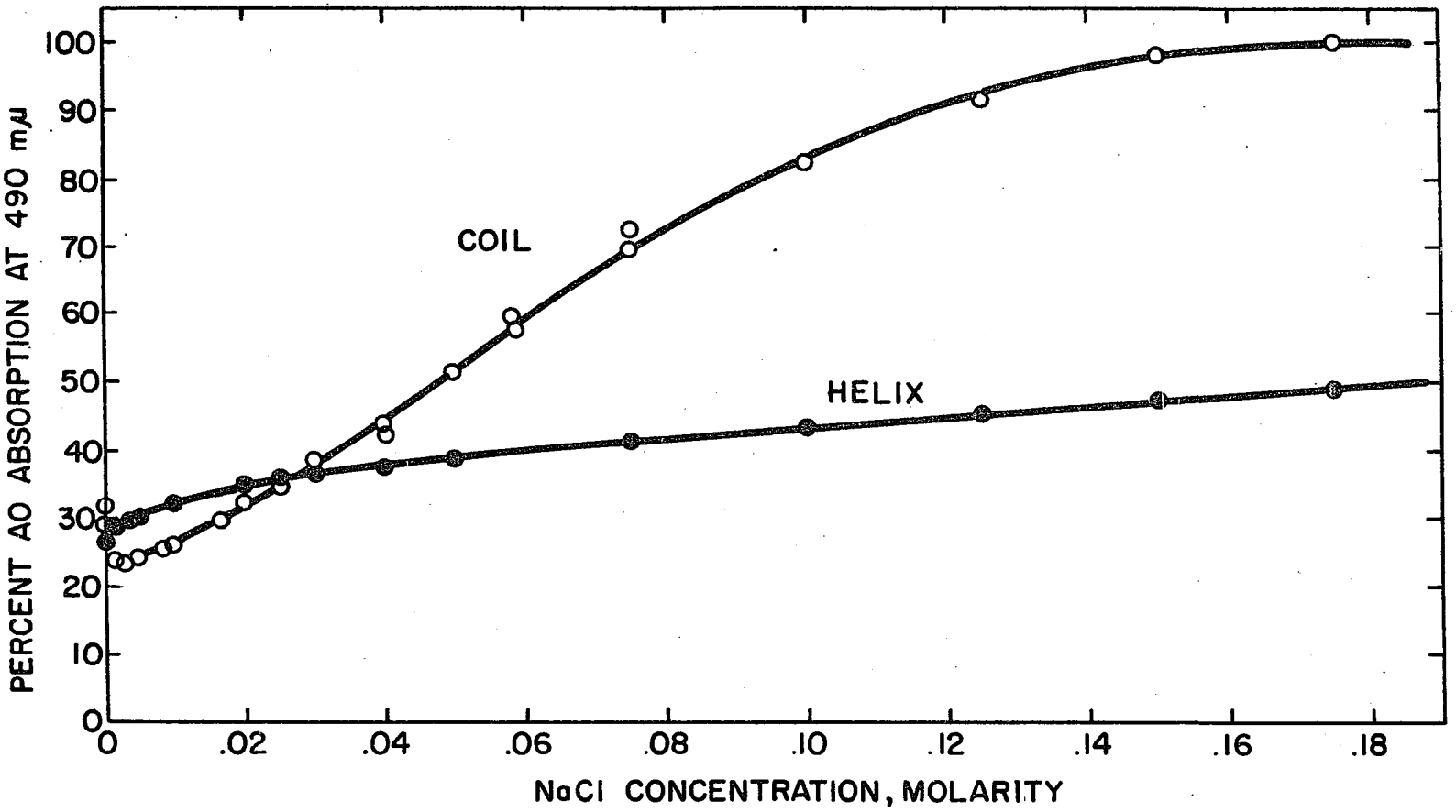
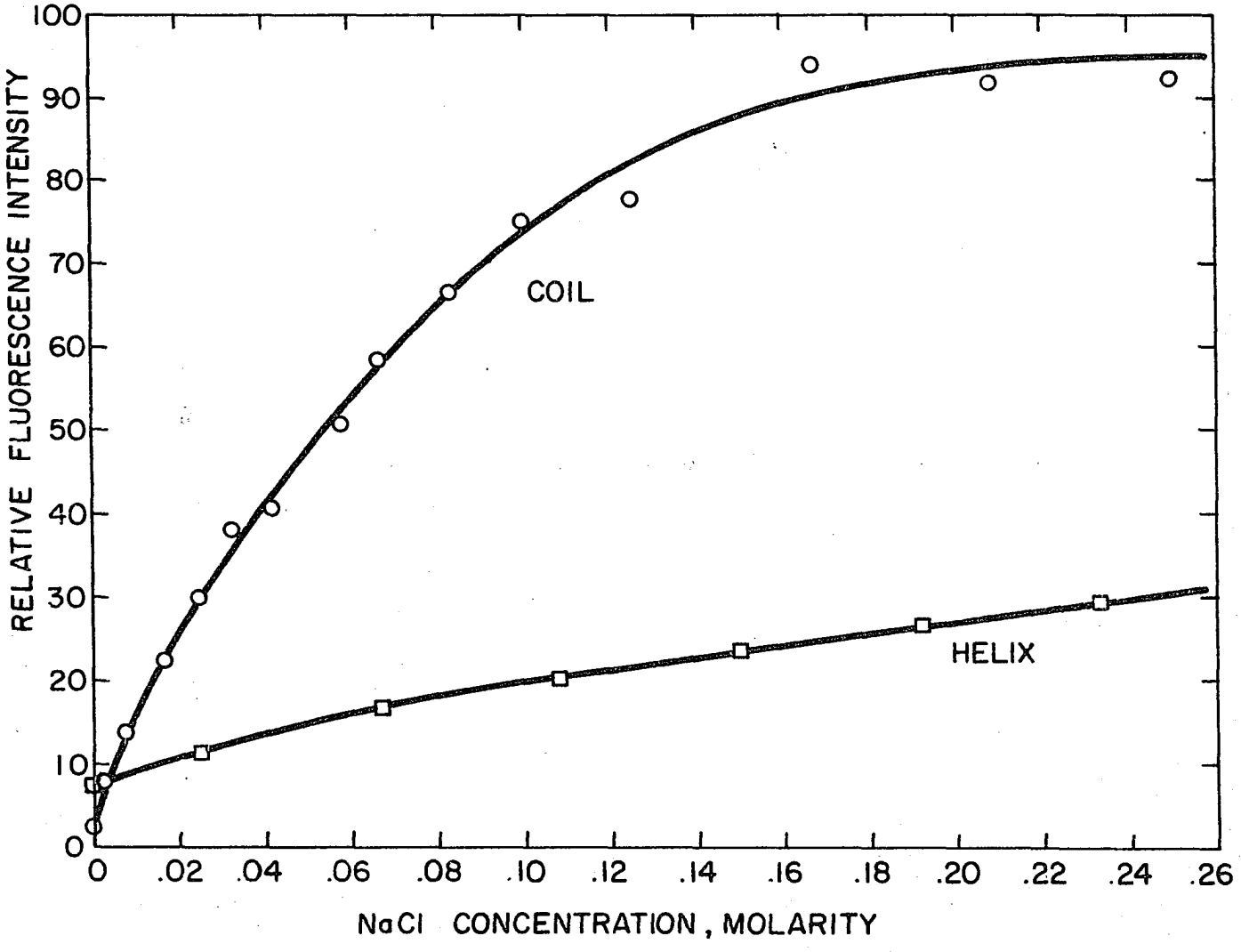

PERCENT AO ABSORPTION AT 490 mμ
100
90
80
70
60
50
40
30
20
10
0
COIL
HELIX
0
.02
.04
.06
.08
.10
.12
.14
.16
.18
NaCl CONCENTRATION, MOLARITY

Figure 13. The AO:PGA complex fluorescence relative to free AO at 535 mμ versus NaCl concentration

o—o: Coil complex, R/D = 17.7, pH 8.6 to 8.9

□—□: Helix complex, R/D = 17.7, pH 4.5 to 4.7


RELATIVE FLUORESCENCE INTENSITY
100
90
80
70
60
50
40
30
20
10
0
COIL
HELIX
0
.02
.04
.06
.08
.10
.12
.14
.16
.18
.20
.22
.24
.26
NaCl CONCENTRATION, MOLARITY

the dimers. It is possible that the competitive binding of sodium ions causes a small increase in the proportion of bound monomers and even more likely that the competition causes a redistribution of dye among several different dimer sites on the helical surface. Evidence for the presence of dimers with different stacking geometries (and, therefore, different A_{490} and F_{535} values) was obtained from the optical activity behavior of the helix complex and will be discussed later. That a competition of the sodium cations for sites is involved is clearly shown by the movement of the titration breaks to larger R/D values. It should be noted, however, that the increases in A_{490} and F_{535} are not quite as large as they appear to be in Figures 12 and 13, because these quantities for the free dye solution decrease in magnitude with increasing NaCl concentration. Thus, A_{490} increases by a factor of 1.4 in going from water to 0.1 M NaCl solution, while the percent values shown in Figure 12 increase by a factor of 1.6 over the same concentration range.

In contrast, the coil complex absorption and fluorescence are greatly altered by NaCl addition. Figure 12 shows first a sharp decrease in A_{490} in going from water to 2×10^{-3} M NaCl and then a rapid rise to the free dye level in 0.18 M NaCl. Again, the flat plateau in the R/D-titrations in salt show that essentially all the dye remains bound up to ionic strengths at least as high as 0.1. That more is involved than a competition of sodium ions for sites is shown

by the increase in the average number of dyes that will bind per residue. The very nature of the complex seems to be changed by the addition of salt.

In Figure 14 representative spectra are given for the coil complexes in water, in 3.3×10^{-3} M NaCl, and in 0.1 M NaCl solutions. The drop in A_{490} is seen to correspond to a shift in the absorption maximum from 457 mμ to 450 mμ and to a decrease in the peak height. Also, the absorption at all wavelengths above the α band position decreases significantly. Unfortunately, an analysis of this spectral behavior in terms of specific structural changes is not yet possible. The loss of absorption above 490 mμ indicates that the transition probabilities to the exciton band levels have been altered, ruling out just a simple change in the distance between adjacent dye molecules. But the distribution of absorption intensity within an exciton band can be altered either by a change in the angle between the stacked dye molecules or by an increase in the length of the dye stacks (Bradley et al., 1963). Since both structural changes may occur simultaneously, along with a change in the distance between the chromophores, the spectral changes per se are not very informative. Some evidence for the formation of larger dye stacks was obtained from the behavior of a new fluorescence band at 590 mμ (see later), but this point might be checked experimentally by looking for the larger stacking coefficient required under the solution conditions for Curve 2 in

Figure 14. The visible absorption spectra of the coil complexes at different NaCl concentrations

Total dye concentration is 4×10^{-5} M, R/D = 18, 1 cm path length.

Curve 1: Water, pH 8.0

Curve 2: 3.3×10^{-3} M NaCl, pH 8.3

Curve 3: 0.1 M NaCl, pH 8.8

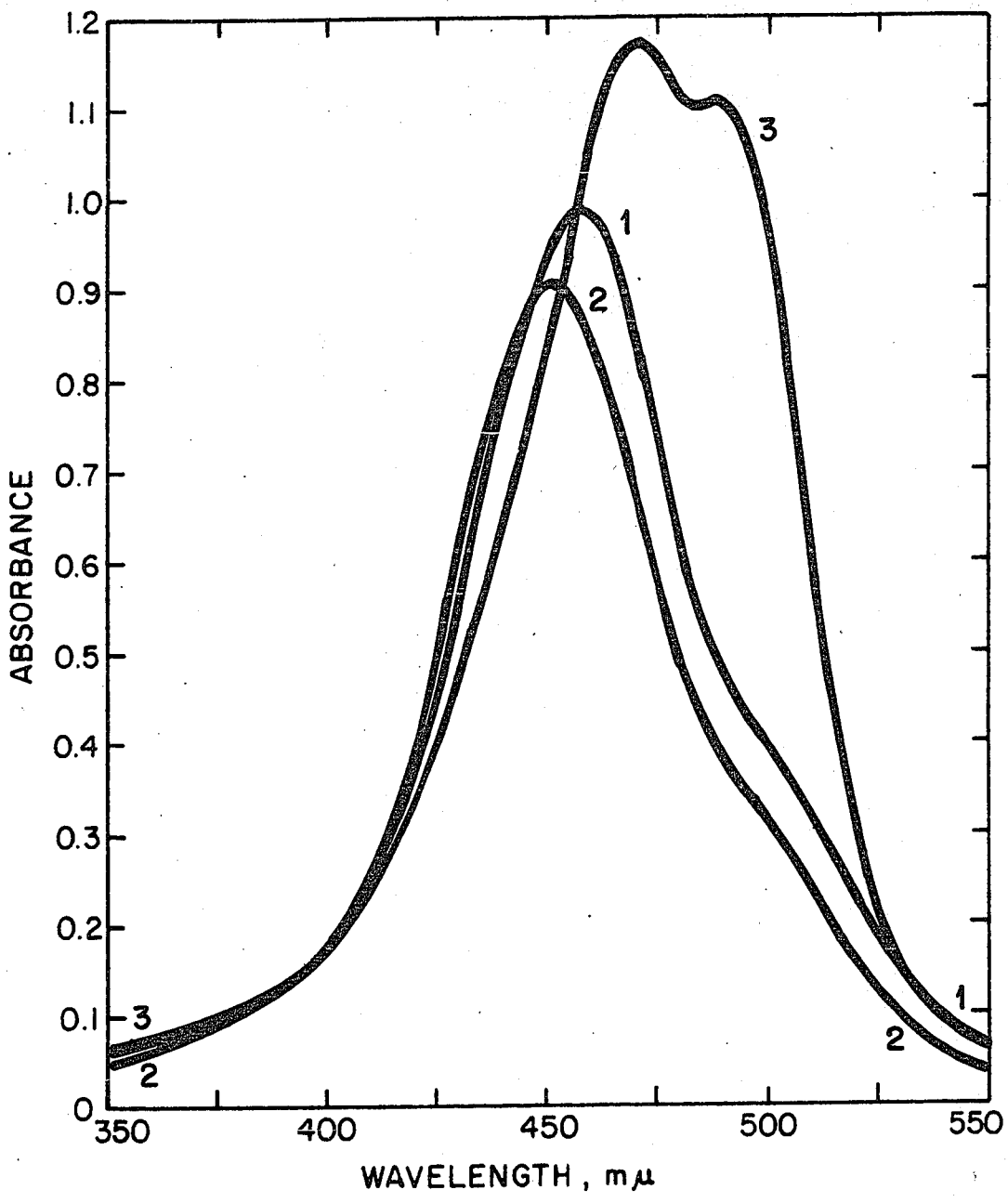

1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
350
400
450
500
550
WAVELENGTH , mμ
1
2
3
1
2
3

Figure 14. That is, NaCl should be added to keep the R/Na ratio constant near 0.2 as A_{490} or F_{535} is followed as a function of R/D at pH 8.3.

In water solution above pH 8, the highly charged PGA probably assumes a relatively rigid, extended conformation with the γ-carboxylate groups spaced as nearly equidistant from each other as possible. As in the case for the α-helix, these carboxylates happen to be properly spaced for a high degree of dye-dye interaction (large spectral changes and stacking coefficient). However, unlike the helix case, nearly all of the carboxyl groups are ionized, making it possible to obtain stacks larger than the dimer if the spatial arrangement of the carboxylates is favorable. It is suggested that the introduction of a small amount of salt reduces the electrostatic repulsion between side chains sufficiently to allow the extended coil to contract and twist into a conformation somewhat more favorable for dye-dye interaction and the formation of larger stacks. Such a conformational change would qualitatively, at least, account for the spectral behavior in Figure 14.

At higher salt concentrations, the increased polymer flexibility apparently destroys the binding sites which give rise to the metachromasy phenomenon (Figure 14, Curve 3). This is especially interesting in view of the fact that the number of dyes which may bind per glutamyl residue actually increases to about two as the NaCl concentration is increased

to 0.1 molar (see Figures 4 and 5). Whether or not this number continues to increase with larger salt concentrations is unknown. Thus, at R/D ratios less than unity, the number of dyes bound to a given length of the PGA chain is larger in 0.1 M NaCl than in water solution, yet the degree of dye-dye interaction is less. The explanation tentatively advanced is that the development of metachromasy depends upon the maintenance of a nearly fixed relative orientation between adjacent binding sites. The relative motions of the γ-carboxylate groups in adjacent sites on the α-helix and the highly charged, extended coil appear to be restricted sufficiently to allow dimer formation between the dyes attached to both sites. Consequently, the optical properties of AO change considerably upon binding to either conformational form of PGA. But the addition of salt reduces the electrostatic repulsion between the side chains and allows a greater freedom of rotation between adjacent glutamyl residues in the coil. In other words, the coil becomes more flexible and the distance and angle between carboxylate groups take on a range of constantly changing values. It is suggested that this situation approaches that for the addition of small anions to a solution of the dye (Zanker, 1952). Dimerization increases to a small extent over that for the dye solution alone, but the effectiveness of the polymer as a template for the formation of regular dye arrays is nearly destroyed. The fact that the maximum number of dyes which may bind per residue

seems to approach two as the NaCl concentration is increased suggests also that dimerization now involves only a single carboxylate group. Because the dimerization of AO in aqueous solution may involve a chloride ion, it appears possible that the addition of flexible PGA to the AO solution essentially results in the substitution of a carboxylate group for the chloride ion. In this respect, it is interesting to note the dimer spectra derived by Lamm and Neville (1965) for the two dimerization models of counterion and no counterion participation. The extinction coefficient at 490 mμ for the counterion model is considerably larger than for the latter model. Thus, the changes in the optical behavior of the coil complex upon the addition of NaCl might be formally regarded as the result of a transition from a dimerization model not involving counterion participation (except as to provide well-defined dimer sites) to a model which does involve a counterion (carboxylate).

The fluorescence intensity of the coil complex at 535 mμ rises steadily in the NaCl concentration range where A_{490} drops (Figure 13). Since the transition probability to the lowest excited state energy level is apparently reduced in 3.3×10^{-3} M NaCl (see Figure 14, Curve 2), this behavior is not understood. However, the fluorescence behavior is particularly difficult to interpret in view of the facts that the exciton model does not seem to be strictly applicable to this system (note the hypochromism) and that a second

electronic transition appears to exist above the α band position. Furthermore, the excited state lifetime of bound dye may depend upon the PGA conformation or flexibility in a manner as yet unknown. Hence, the absorption behavior of the complexes may be sensitive to structural changes not detectable by fluorescence measurements. But since the reverse may also be true, a description of the fluorescence behavior as a function of solution variables is valuable as complementary data to the other optical properties.

Fluorescence Depolarization

Support for the notion that the rotational motion of bound dye is more restricted when bound to the coil conformation in water solution as opposed to salt solutions comes from measurements of the depolarization of fluorescence. The extent to which the plane of polarization of the incident light beam is partially depolarized depends on how far the molecule has rotated during the lifetime of the excited state. If a fluorescent dye is rigidly attached to a stiff polymer and if the dye has a suitable excited state lifetime, the rotational diffusion constant of the polymer may be obtained from measuring the fluorescence depolarization. But the attachment of dye to a flexible polymer leads to different information; the rotational motion of the dye will now not require the entire polymer to rotate, and the depolarization of fluorescence yields some information about local

restraints to rotation (Oster and Nishijima, 1964). Therefore, the use of fluorescent dye:polymer complexes and fluorescence depolarization to measure rotary diffusion constants of macromolecules can never be completely unambiguous; the possibility that the attached fluorescent molecule is capable of independent motion through internal rotational freedom cannot be arbitrarily dismissed. In this case, however, the magnitude of the polarization has no particular meaning, since the AO:PGA complex was excited in a number of its UV absorption bands. The observed polarization must be regarded as being the sum of both positive and negative polarizations. Furthermore, the rotation of a rod-like polymer (such as the extended coil or α-helical conformations) about its long axis at room temperature in low viscosity solvents will, in general, be rapid in comparison to the lifetime of the excited dye (on the order of 10^{-9} sec for AO). Thus, small polarization values were expected for the AO:PGA complexes and attention was focused on a comparison of the polarizations obtained in water and in 0.1 M NaCl solutions.

Figure 15 shows the fluorescence polarization for complexes in water and 0.1 M NaCl over a pH range that includes both the coil and helix conformations. The coil-to-helix transition regions for PGA in both solutions (see Figure 16) are shown by the cross-hatched areas. Because the data for the water solutions was collected at R/D = 8.2, the transition region (Region A) for the complex probably occurs

Figure 15. The fluorescence polarization of the complex in water and 0.1 M NaCl solutions as a function of pH

Total dye concentration is 4×10^{-5} M, emission observed at 535 mμ.

o—o: Water solution, R/D = 8.2

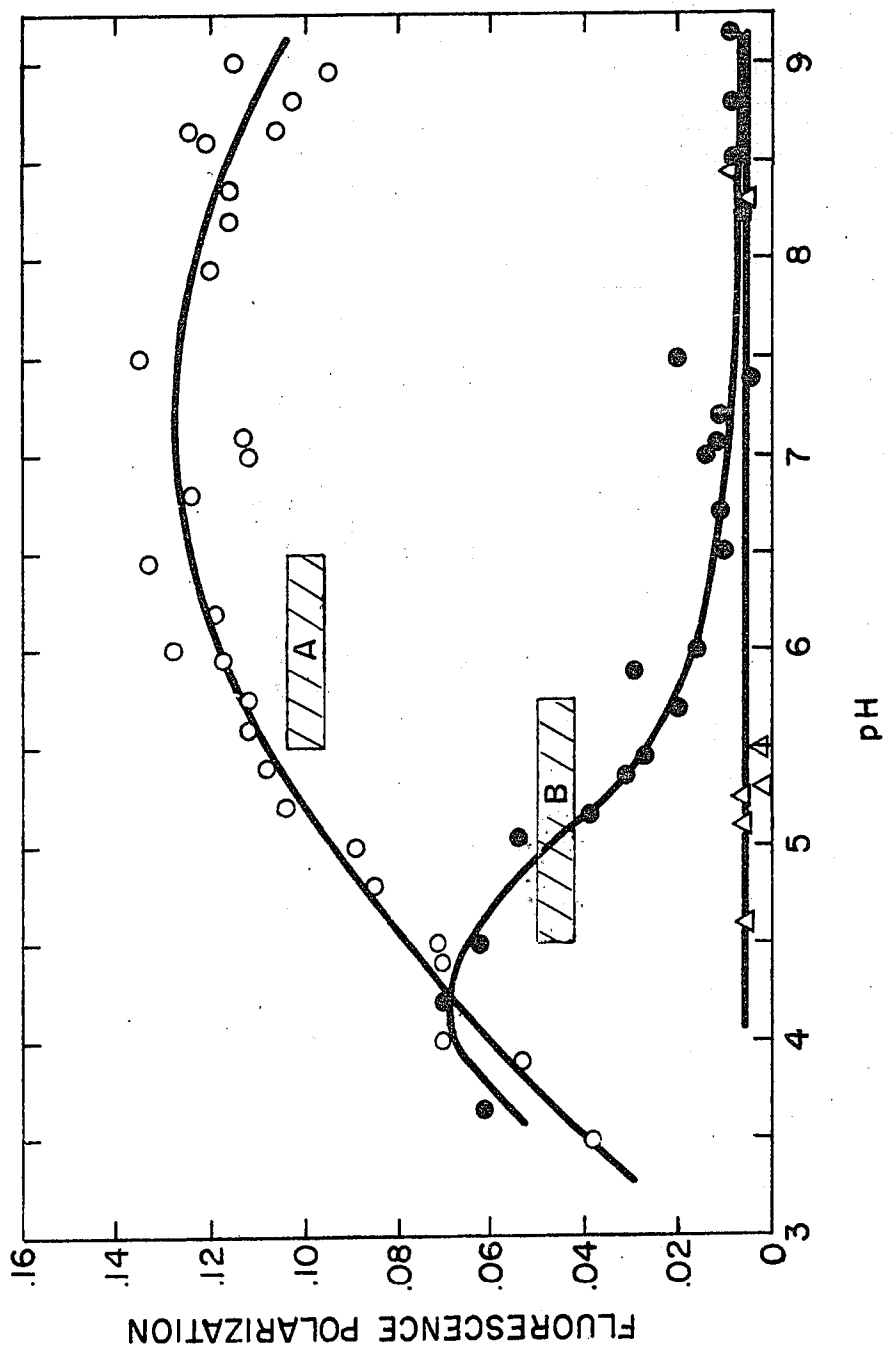
●—●: 0.1 M NaCl solution, R/D = 18.4

Δ—Δ: Free AO in water

Region A: Helix-coil transition region in water

Region B: Helix-coil transition region in 0.1 M NaCl

The transition region positions are those for PGA solutions alone or the complexes at R/D ratios near 18. At R/D = 8, Region A should probably be placed between pH 6 and pH 7 (see later in text).


FLUORESCENCE POLARIZATION
.16
.14
.12
.10
.08
.06
.04
.02
0
3
4
5
6
7
8
9
pH
A
B

between pH 6 and pH 7 (see later). In 0.1 M NaCl the fluorescence polarization of the coil complex is essentially zero, as it is for free dye over the entire pH range. Lowering the pH through the transition region causes an increase in polarization that seems to follow the coil-to-helix conformational change reasonably well. In contrast, the coil complex in water solution exhibits a polarization value significantly different from zero, and its magnitude is not influenced by conversion to the helical complex. Thus, a significant restraint to bound dye rotation is present in the coil complex that is destroyed by the addition of salt. That this restraint in rotational freedom in water solution is probably more than local is suggested by the nearly equal polarization value obtained for the rigid, α-helical complex between pH 5.5 and pH 6.0. The same conclusion is reached by considering the fact that PGA is composed of identical monomeric units, each of which can be involved in the binding of dye. The local restraints to rotary motion shown to exist by the polarization observed in the coil pH region must therefore exist along the entire chain, thereby implying that the entire polymer is more rigid in water solution than in 0.1 M NaCl at pH 8. The low value of 0.12 ± 0.01 is attributed primarily to whatever local mobility is present for both the α-helical and extended coil conformations, to the rapid rotation about the long axes of both rod-like conformations, and to any cancellation in the observed polarization due to

excitation in several absorption bands.

In order to obtain PGA solutions above pH 8, a small amount of NaOH was added, thereby increasing the concentration of sodium ions. Perhaps this increase in ionic strength reduces the rigidity of the coil complex in water sufficiently to account for the slight decrease in fluorescence polarization in this pH region. Of more interest is the polarization behavior in the transition and helical regions. The fact that the polarization remains essentially constant throughout the transition region strongly suggests the rigidity of the polymer is unaffected as the coil is converted into an α-helix. Since one would expect by electrostatic arguments that the coil is extended in water solution, the polarization results also suggest the coil might exist as a twisted structure that simply tightens into an α-helix by a continuous process as the carboxylate groups are protonated. A twisted, rigid coil, in fact, provides a basis for understanding the striking optical activity discovered in the dye absorption bands, although aggregate formation seems a more likely explanation (see later). Nevertheless, the insensitivity of the fluorescence polarization to the conformational transition in water solution suggests the transition mechanism mentioned above should be seriously considered for solutions of PGA in which no electrolyte has been added.

The continuous decrease in polarization as the pH is lowered in the helical region in water is probably due

primarily to the increased conformational mobility of the side chains and, to some extent, an increase in the polymer Brownian motion. Electrostatic restrictions to free motion are being continuously reduced by a higher degree of protonation. Another possibility is energy transfer from one excited dye molecule to another, but there seems to be no reason why the efficiency of this process should increase with the degree of protonation or be greater for the helical complex than for the coil complex.

pH-Induced Optical Transitions

The helix-coil transition of PGA in aqueous solution has attracted much attention because of the opposing interplay of internal hydrogen bond formation and electrostatic repulsion between the charged side chains in determining the polymer conformation. Both Wada (1960) and Jacobson (1964), for instance, have shown that at a constant temperature, the conformational change is dependent upon the degree of ionization, although the relationship is not a linear one. An increase in the ionic strength results in a decrease in the degree of protonation at any given pH because the electrostatic shielding allows protons to be removed with less work. Hence, the transition, followed as a function of pH rather than the degree of ionization, will occur in a lower pH region as the salt concentration is increased. This shift is clearly shown in Figure 16, where the transition is monitored by the

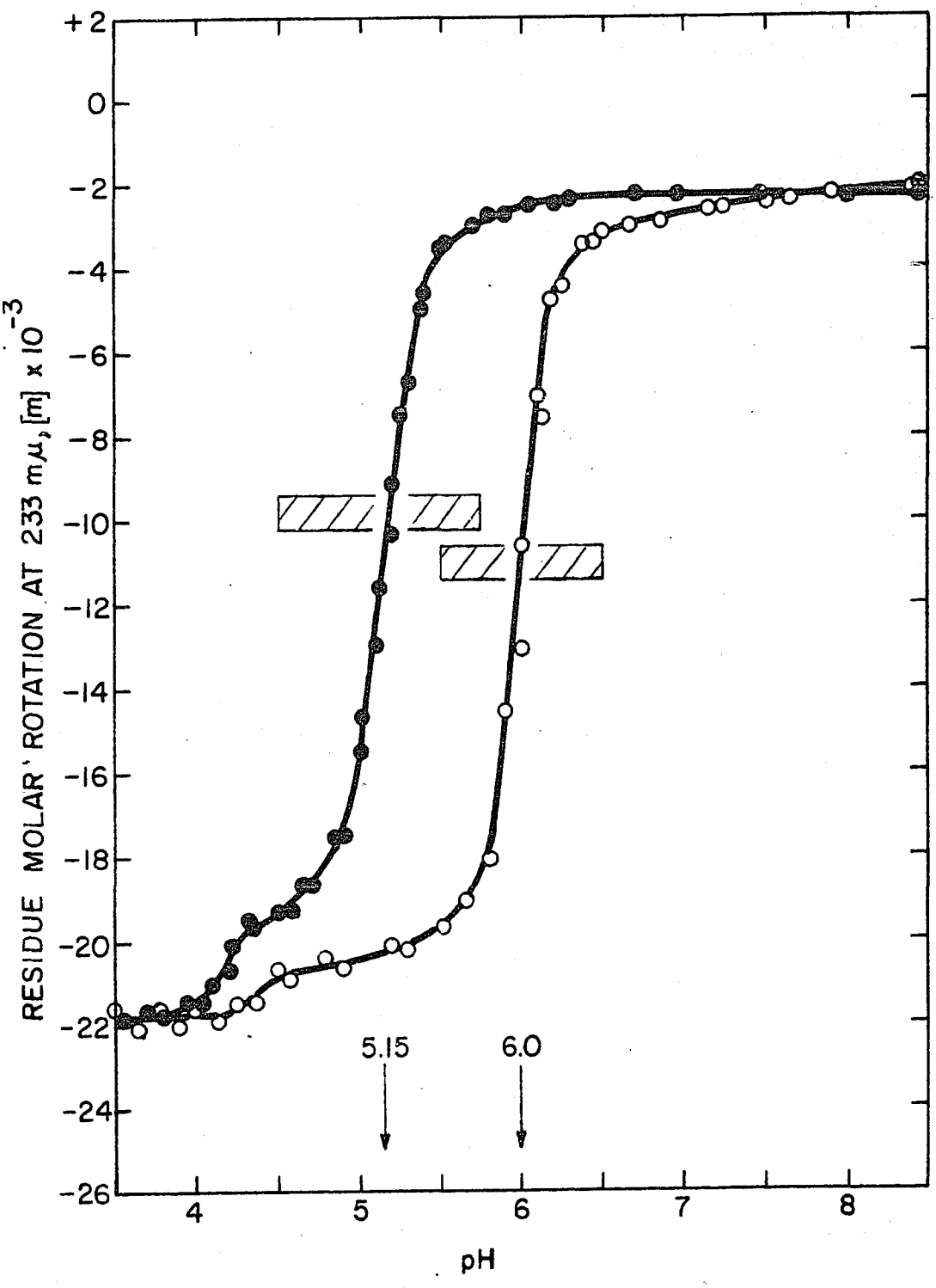
Figure 16. The residue molar rotation of PGA at 233 mμ as a function of pH

$[R] = 1.42 \times 10^{-3}$ M

o—o: In water solution

o—o: In 0.1 M NaCl solution

The two curves define the two cross-hatched regions used to denote the helix-coil transition regions in other figures.


+2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
4
5
6
7
8
pH
5.15
6.0
RESIDUE MOLAR ROTATION AT 233 mμ, [m] x 10^-3

optical rotation at 233 mμ, the helical dispersion trough, for water and 0.1 M NaCl solutions of PGA. The transition midpoint shifts from pH 6.0 in water to pH 5.15 in the salt solution.

Because the optical activity of PGA increases with the number and degree of amide dipole interactions in a helical array (Tinoco, Woody, and Bradley, 1963), the rotation at some fixed wavelength has often been used as a convenient means of following the helix-coil transition. The linear relationship generally assumed between the rotation and α-helical content was shown to be essentially correct by Nagasawa and Holtzer (1964). The helical content was obtained from a Wada plot of potentiometric titration data and compared with determinations of the Moffitt-Yang dispersion constant b_o; a plot of $-b_o$ versus the degree of helix yielded a straight line passing through the origin for aqueous solutions of PGA. Changes in b_o as a function of pH closely parallel the optical rotation changes at a single wavelength (Jacobson, 1964).

The transition midpoints in Figure 16 occur roughly where expected from the midpoints obtained in solutions of various NaCl concentrations by Idelson and Blout (1958), Wada (1960), Applequist and Breslow (1963), Jacobson (1964), and Ptitsyn (1967). Some variations occur in the positions of the midpoints obtained by these investigators that could well be due to differences in the concentration of PGA employed.

Cassim and Taylor (1965), for instance, obtained b_o midpoints considerably removed from the results of those cited: a midpoint pH at 5.38 for water solutions and at pH 4.78 in 0.2 M NaCl (PGA concentration unspecified).

Figure 16 defines the cross-hatched areas used to mark the helix-coil transition in other figures. The centers of these areas are located at the midpoint pH and their lengths include about 90% of the transition regions. A second, small transition was discovered below pH 4.5. This transition is easily missed by not obtaining points sufficiently close together. Earlier titrations with other lots of PGA did not include enough points to determine whether this was some common peculiarity of Pilot lots G-76 and G-72 PGA (see Figure 18), but the recent work of Tomimatsu et al. (1966) shows this break to be characteristic of aggregation. These workers obtained an identical break in a careful study of the molar rotation of PGA in this pH region and found from light scattering measurements that the break is associated with a sharp increase in molecular weight. Three PGA samples—two were gifts from other laboratories and the third was Pilot lot G-53, DP 610—gave essentially identical results. Although there is a considerable variation in the rotation data at low pH as reported by other workers, this transition was apparently missed by not recording enough points. No visible precipitation of the PGA was ever noted above pH 4.

Before the optical properties of bound dye can be

related to the PGA conformation, some assurance is needed that the conformation is not altered significantly by the process of binding dye. Such an alteration will most likely occur when the number of sites and bound dye molecules are approximately equal. In Figure 17 the titration of a coil complex at R/D = 4 is compared with the titration of PGA alone at the same residue concentration. The coil complex was prepared by adding stock PGA at pH 8.3 to a dilute dye solution (8×10^{-5} M), rather than the reverse order of mixing, in order to avoid an optically active coil complex (see later). This complex was then titrated with 0.1 N HCl; the plot in Figure 17 includes the points obtained from two separate complex titrations.

The results in Figure 17 show that the complex undergoes the coil-to-helix transition near pH 7, a full pH unit higher than the free PGA. The small, second transition near pH 5.85 is probably associated with the aggregation phenomenon found for PGA near pH 4.4. Because the dye becomes optically active in the helical region, Cotton effects in the UV region contribute to the rotation observed at 236 mμ, causing it to differ considerably from that for PGA alone. The important thing to observe is that the bound dye does not seem to retard the PGA conformational transition or aggregation, but, instead, either forces or allows both processes to occur at a higher pH than normal. Although the reasons for the early coil-to-helix transition are not yet clear, it does not seem

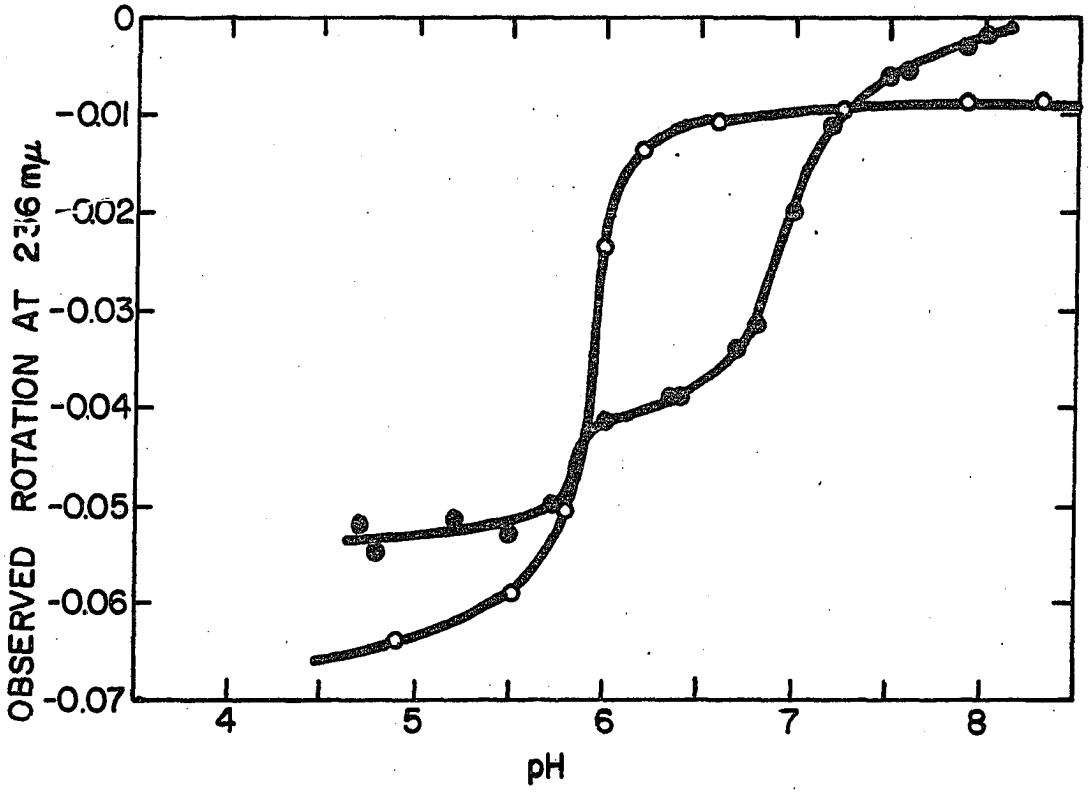


Figure 17. The observed rotation at 236 mμ as a function of pH for water solutions of PGA and AO:PGA complex

1 cm path length

o—o: PGA, $[R] = 3.2 \times 10^{-4}$ M

●—●: AO:PGA complex, R/D = 4, total $[AO] = 8 \times 10^{-5}$ M

likely that the dye is forcing the conformational transition. The nearly equal stacking coefficients obtained for the helix and extended coil complexes (Figure 11) show that the attractive ground state interactions between dye molecules bound to both conformations are essentially equal. Instead, it seems more fruitful to consider what effect the bound dye might have on the energetics of the transition and the electrostatic forces between the carboxylate groups. A couple of exploratory, potentiometric titrations through the coil and transition pH regions indicated that somewhat fewer protons are bound to the complex than to PGA alone at any given pH in water solution. Hence, the complex undergoes the transition at a much higher degree of ionization than does PGA alone. At least three principal reasons for this stabilization of the helix at higher degrees of ionization can be suggested that, together, may account for the shift of a full pH unit in the transition midpoint. It might be argued, for instance, that dye binding reduces the number of conformations available to the extended coil, thereby making the coil-to-helix transition entropically more favorable. Also, the binding of cationic dye reduces the net charge of the polyelectrolyte and hence stabilizes the helical conformation by reducing the electrostatic repulsion between the γ-carboxylate groups. A third contribution, which could outweigh the other two, may involve a disruption of the hydration of the coil upon the attachment of a dye molecule to every fourth residue. The

bulky, hydrophobic ring system of AO may partially exclude water molecules from the vicinity of the polymer. Because the electrostatic shielding provided by the AO ring system is probably much less than that of the dipolar water molecule, the repulsion between carboxylate groups may not be significantly reduced by dye binding. However, the partial exclusion of water from the vicinity of the amide hydrogens would allow an increase in the number of intramolecular hydrogen bonds, resulting in an increased stability for the helical conformation. Some support for this latter idea comes from a comparison of the PGA titration curves obtained by Applequist and Breslow (1963) and Doty et al. (1957). At the midpoint of the helix-coil transition, Applequist and Breslow obtained a degree of ionization of approximately 55% in 0.2 M NaCl, while the curves obtained by Doty et al. in 0.2 M NaCl-dioxane (2:1 by volume) show about 65% ionization at the midpoint. It might also be contended, however, that the electrostatic repulsion is reduced by increased ion-pair formation in the aqueous dioxane solvent.

At a larger R/D ratio, the bound dye has much less influence on the position of the helix-coil transition. In the lower half of Figure 18, the rotation at 233 mμ as a function of pH for PGA alone was again compared with the complex, this time at R/D ≅ 18. Pilot lot G-72 PGA, DP 680, was employed in these experiments, and the coil complex was heated to 85°C after preparation to destroy most of the dye

optical activity (see later). Both a forward titration with 0.1 N HCl and a subsequent reverse titration with 0.1 N NaOH yielded essentially the same curve, illustrating what little effect the dye has on the reversibility of the helix-coil transition. At this R/D ratio the curve appears to be shifted to slightly higher pH values, but the shift is hardly outside the experimental error. Hence, if the R/D ratio is maintained close to 20, the optical properties of the bound dye may be followed as a function of pH and correlated with the helix-coil transition pH regions established by Figures 16 and 18. In Figures 15 and 29, where data is presented at R/D = 8.2, the transition region was estimated to occur between pH 6 and pH 7.

In the upper half of Figure 18 is shown the titration of a helix complex, R/D $\cong$ 19, to the coil pH region. The helix complex was prepared in the normal manner, that is, by addition of 10^{-3} M AO stock to the pretitrated PGA. Instead of following the rotation at 233 mμ, the magnitude of the 446 mμ peak in the Cotton effects induced in the visible absorption bands of the bound dye was followed with the addition of 0.1 N NaOH. In agreement with the statements of Stryer and Blout (1961), the rotation decreases to zero in a manner that roughly parallels the helix-to-coil transition. But the broader curve indicates the dye-dye interactions do not bear quite the same relationship to the PGA conformation as the interactions between the amide groups.

Figure 18. Observed rotations for water solutions of PGA and AO:PGA complex as a function of pH

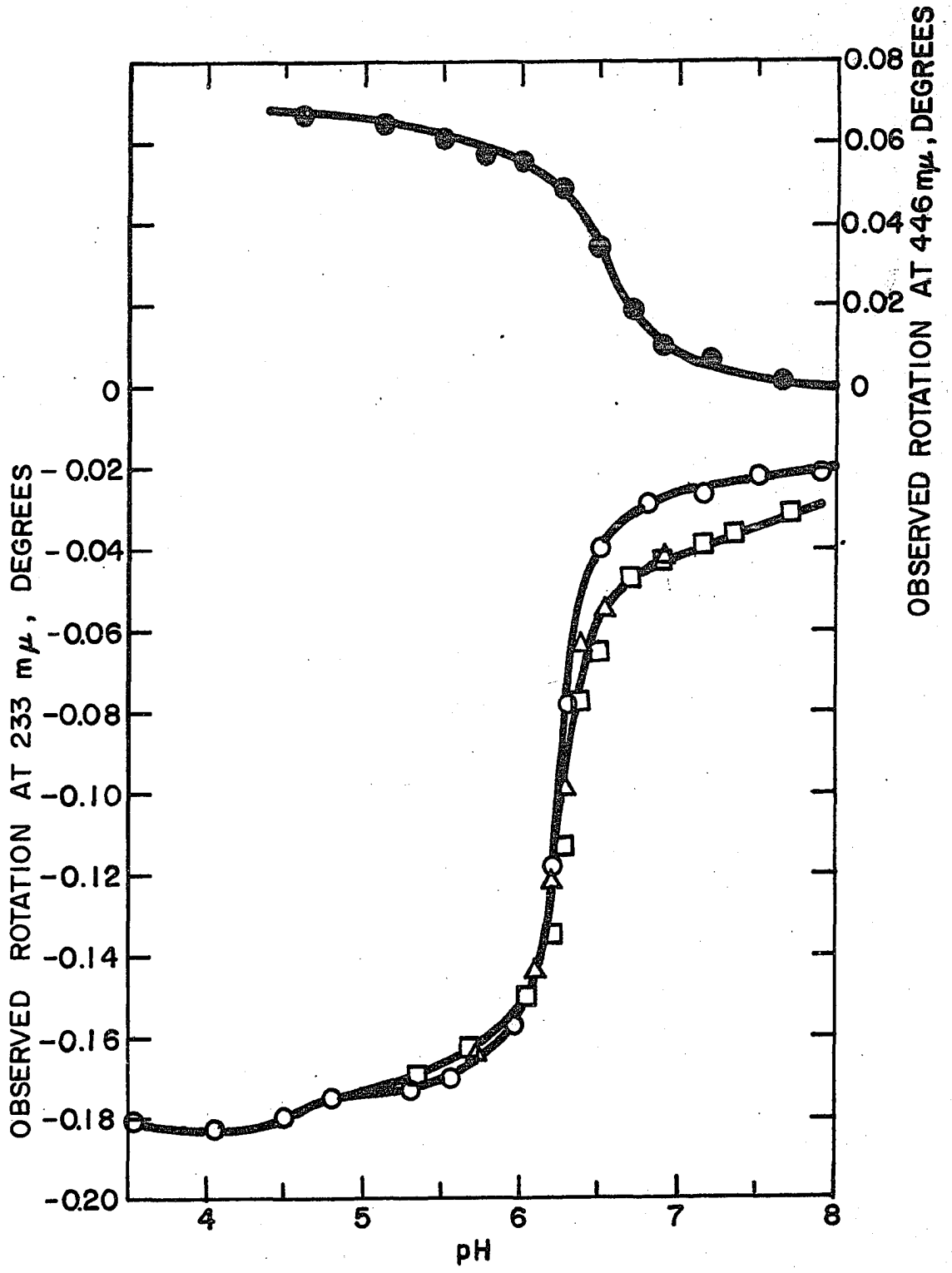
1.07 cm path length, Pilot lot G-72 PGA, DP 680

o—o: PGA, 233 mμ, $[R] = 6.8 \times 10^{-4}$ M

□—□: Forward titration of AO:PGA complex, 233 mμ, R/D ≅ 18, total $[AO] = 4 \times 10^{-5}$ M

Δ—Δ: Reverse titration of the above complex, 233 mμ

⊙—⊙: Reverse titration of helix AO:PGA complex, 446 mμ, R/D ≅ 19, total $[AO] = 4 \times 10^{-5}$ M


OBSERVED ROTATION AT 233 mμ, DEGREES
OBSERVED ROTATION AT 446 mμ, DEGREES
pH
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
-0.16
-0.18
-0.20
4
5
6
7
8

The relationship between the absorption of the complex at 490 mμ and pH is shown in Figure 19 for water and 0.1 M NaCl solutions. Each point corresponds to a separate complex prepared by adding AO to pretitrated PGA. Since the R/D ratio was held between 17 and 18 (total AO concentration held constant at 4×10^{-5} M), the absorption changes may be compared with the helix-coil transition regions established in Figure 16. These regions are shown by the cross-hatched areas. As expected from the optical titration plateau levels, the A_{490} changes in 0.1 M NaCl are more dramatic than those in water, but it is obvious that no simple relationship exists between A_{490} and the backbone conformation in either solution. Significant drops in A_{490} occur long before the coil-to-helix transition is observed from the amide interactions. Further changes occur in the transition and helical pH regions that are as large as those preceding the transition in water solution. In the pH range 4.0 to 4.5, the A_{490} behavior apparently reflects the anomalous transition discovered earlier from the optical rotation measurements on PGA. Below pH 4, of course, the rise in A_{490} may be attributed to a decrease in binding caused by protonation of the γ-carboxylates.

The lack of a simple correlation between A_{490} and the polymer conformation was not entirely unexpected. Interactions between dye molecules are sensitive to the site spacings, which in turn need not be simply related to the

Figure 19. The absorption at 490 mμ of the AO:PGA complex relative to AO alone as a function of pH

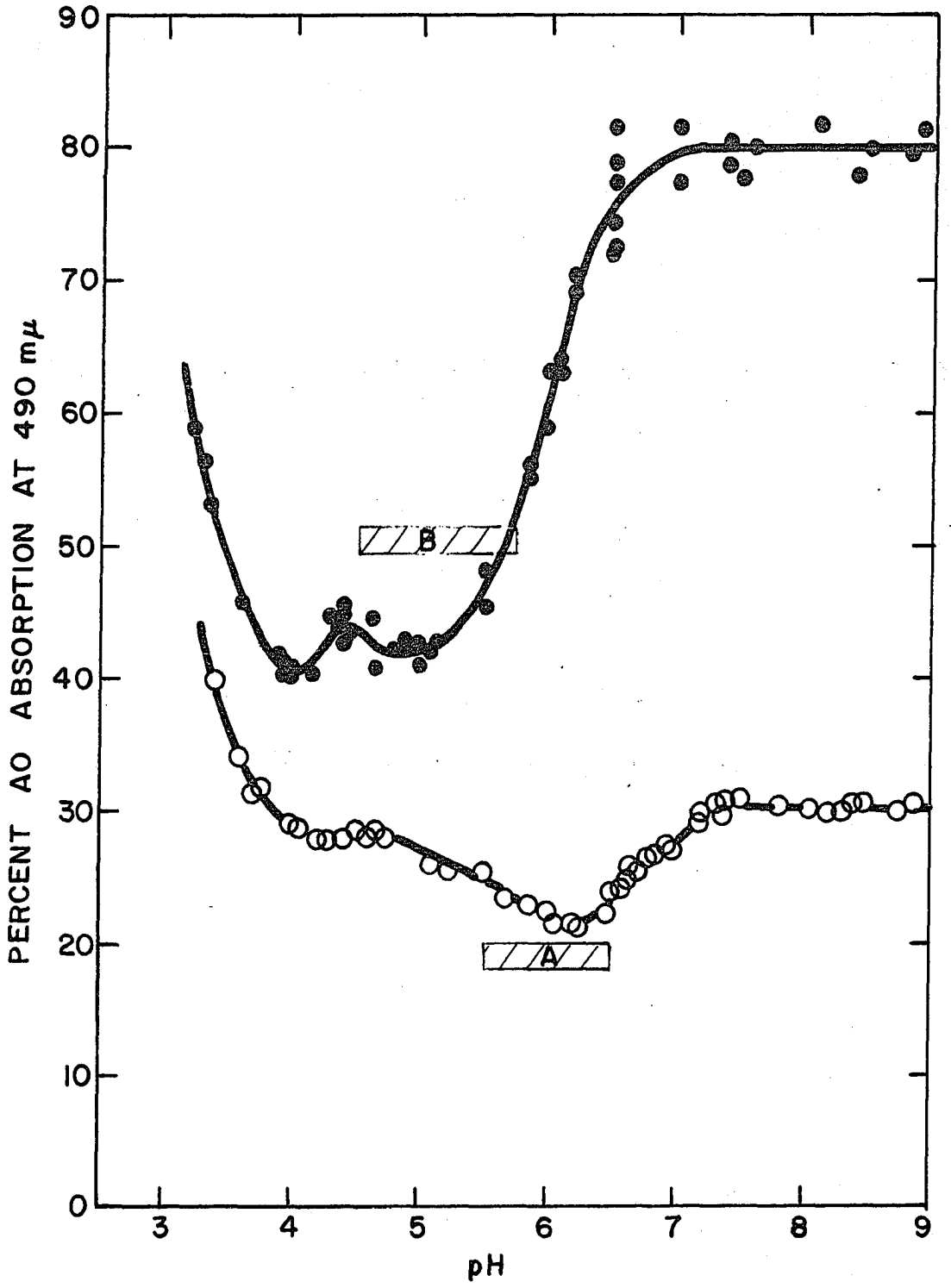
Total dye concentration is 4×10^{-5} M,
R/D = 17 to 18.

o—o: In water

●—●: In 0.1 M NaCl

Region A: Helix-coil transition region in water

Region B: Helix-coil transition region in 0.1 M NaCl


PERCENT AO ABSORPTION AT 490 mμ
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
pH
B
A

amide (or backbone) structural arrangement. But because it was postulated earlier that the development of metachromasy depends upon the carboxylate groups being held more or less rigidly in position, the behavior of A_{490} in 0.1 M NaCl was of special interest. If this hypothesis is correct, the results in Figure 19 show that a significant restriction in the side chain mobility occurs before the helical content reaches 4 or 5%. There is no reason to suspect that the coil is partially returning to the extended state existent in water solution; instead, it seems more likely that the opposite process, an increase in the compactness of the polymer, is responsible. Thus, Doty et al. (1957) found an unexplained, sharp drop in the intrinsic viscosity just prior to the coil-to-helix transition of PGA in 0.2 M NaCl-dioxane (2:1). Because more "metachromatic sites" are being created than can be accounted for by the helical content in this pH region, some mechanism is needed to explain the formation of these sites in the coiled segments of the chain. Qualitatively, an increase in compactness provides a possible answer by implying a loss in the degree of freedom of chain motions. Such a restriction in chain mobility may be sufficient to once again provide sites between proximal carboxylate groups or even between distant groups brought close together by chain folding. It is also possible that hydrogen bonding between the helical segments, analogous to that occurring on an intermolecular level at low pH, may occur, thus providing

some stability to a folded conformation.

At this point it is interesting to note that Ptitsyn (1967) has shown from statistical considerations alone that the root mean square end-to-end distance of PGA passes through a minimum in the early stages of the coil-to-helix transition. In 0.1 M NaCl the measured intrinsic viscosity compared to that of a statistically coiled chain decreases with increasing helical content to a minimum value near 60% helical content. Reference to Figure 19 shows this is approximately the position of the first minimum in A_{490}. The agreement may, of course, be coincidental. For the present, the results of Figure 19 must be regarded as inconclusive about the rigidity requirement for the development of metachromasy. However, it is clear that some structural changes are occurring to which the absorption of bound AO is much more sensitive than the amide interactions.

In Figures 20 and 21 are presented the visible absorption spectra at selected points along the A_{490} curves in Figure 19. These are not very helpful in suggesting structural changes and serve mainly to show the optical changes are more complicated than A_{490} would indicate. For instance, the spectra for helical complexes in 0.1 M NaCl (Figure 20) show that the increase in A_{490} in going from pH 4.9 to pH 4.4 corresponds to both an increase in the absorption maximum and a shift of the peak to a longer wavelength. But at the second minimum at pH 4.0, where A_{490} is the lowest, the

Figure 20. The visible absorption spectra for helix complexes in 0.1 M NaCl at selected pH values

Total dye concentration is 4×10^{-5} M, R/D = 18, 1 cm path length.

Curve 1: pH 4.9

Curve 2: pH 4.4

Curve 3: pH 4.0

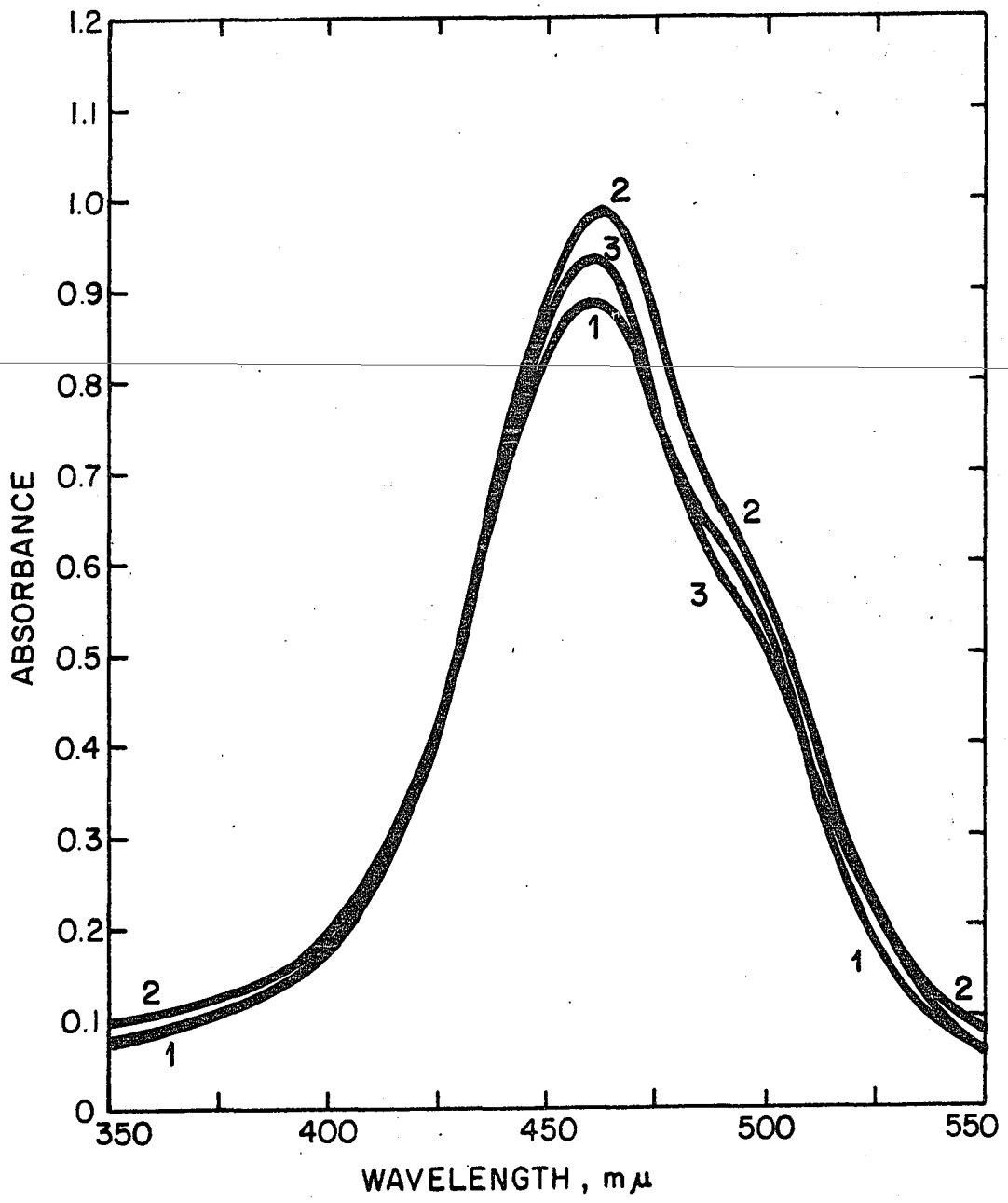

1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
350
400
450
500
550
WAVELENGTH , mμ
1
2
3

Figure 21. The visible absorption spectra for AO:PGA complexes in water at several pH values

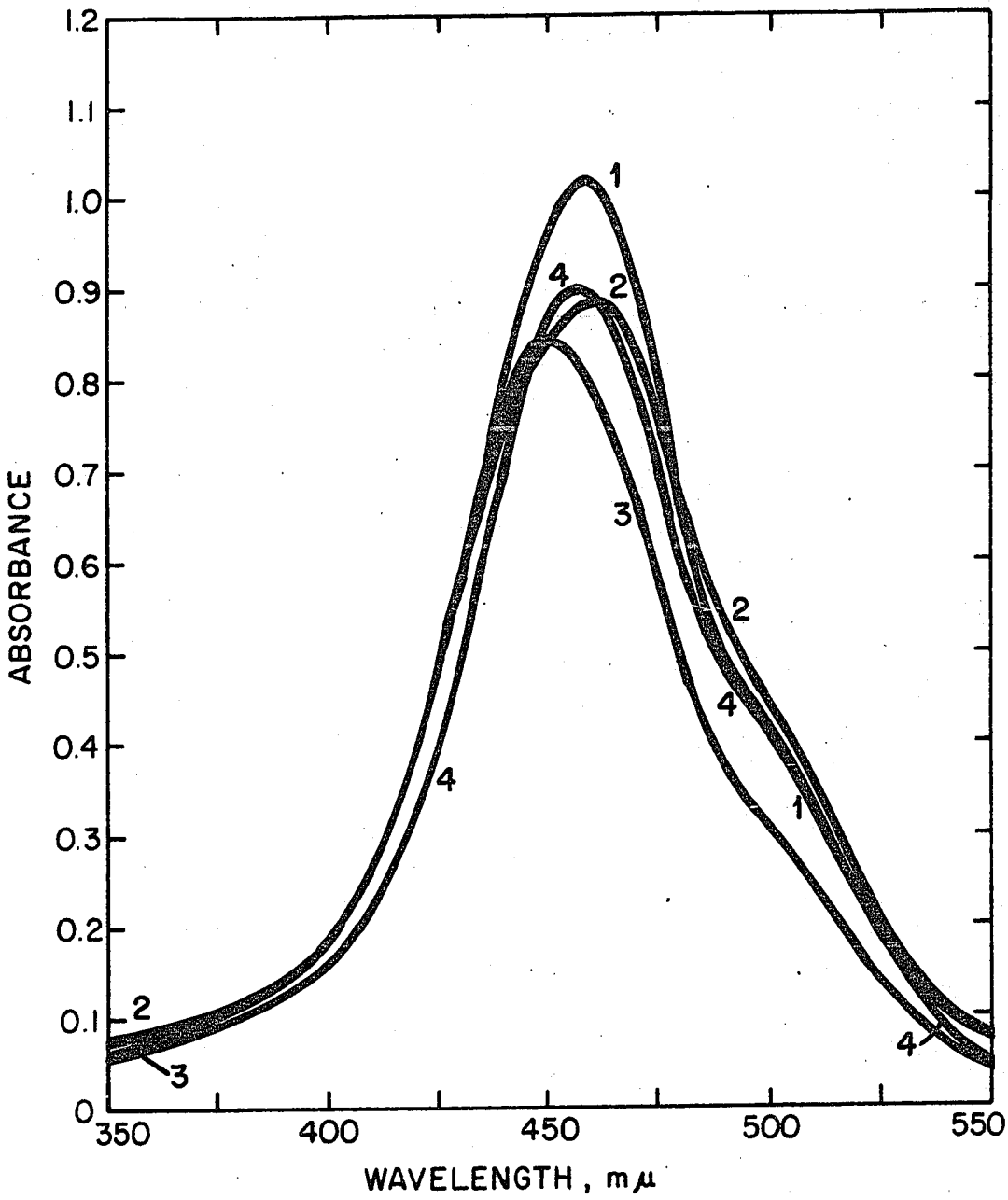
Total dye concentration is 4×10^{-5} M, R/D = 18, 1 cm path length.

Curve 1: pH 8.75

Curve 2: pH 7.50

Curve 3: pH 6.2

Curve 4: pH 4.2


ABSORBANCE
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
350
400
450
500
550
WAVELENGTH , mμ
1
2
3
4

absorption maximum is larger than at pH 4.9 and is located at the same position (460 mμ). The absorption maximum never shifts to as short a wavelength as observed in water solutions of the helix complex (456 mμ).

In water solution a variety of spectra are obtained (Figure 21) in which the position of the absorption maxima are roughly correlated with A_{490}. Thus, at pH 6.2 the maximum is shifted to the lowest position ever observed (449 mμ). The similarity between this absorption spectrum and that obtained at pH 8.3 in 3.3×10^{-3} M NaCl (Figure 14) suggests the drop in A_{490} from pH 7.3 to pH 6.2 corresponds to the same structural changes that occur upon adding a small amount of NaCl to the coil complex at pH 8.3 (Figure 12). In fact, NaCl is formed during the titration of the PGA coil to pH 6.2 before the dye is added. Because the polarization of fluorescence remains essentially constant through this pH region, the spectral changes are probably not the result of changes in the relative motion of adjacent binding sites. It is suggested, instead, that the polyelectrolyte remains essentially extended throughout the coil pH region in water. As the protonation and gradually increasing ionic strength reduces the electrostatic repulsion between the side chains, the distance and angle between the carboxylate groups will change, resulting in different dye stacking geometries and also, perhaps, an increase in the number of dyes in a stack at pH 6.2.

As might be expected from the absorption results, the fluorescence intensity of the complex at 535 mμ also is not simply related to the helix-coil transition. Figure 22 shows the intensity relative to that for the free dye as a function of pH in water and 0.1 M NaCl solutions. Since essentially all the dye remains bound over the pH range shown, the changes in intensity, like those in A_{490}, must be due to changes in the extent of dye-dye interaction. Aside from the small hump near pH 7, which will be magnified and discussed in the next section, the curve in water is rather uninteresting. No hint of the conformational change appears nor does the shape of the curve closely follow the change in degree of ionization. Of course, the fluorescence intensity was expected to be relatively insensitive to changes in the dye stacking geometry, since less than 4% of the original intensity is left to work with. Nevertheless, the lack of any sudden increase in the 535 mμ intensity suggests once again that in water solution a continuous transition from coil to helix occurs during which the nature of the "metachromatic binding site" is hardly affected.

In 0.1 M NaCl the emission intensity at 535 mμ (F_{535}) drops sharply in the pH region 6.5 to 5.75 while less than 4% of the glutamyl residues become arranged in the α-helical structure. The sharpness of the break at pH 6.5 is probably closer to that shown by the curve drawn through the F_{535} results than the A_{490} curve drawn in Figure 19. An averaged

Figure 22. The fluorescence at 535 mμ of the AO:PGA complex relative to AO alone as a function of pH

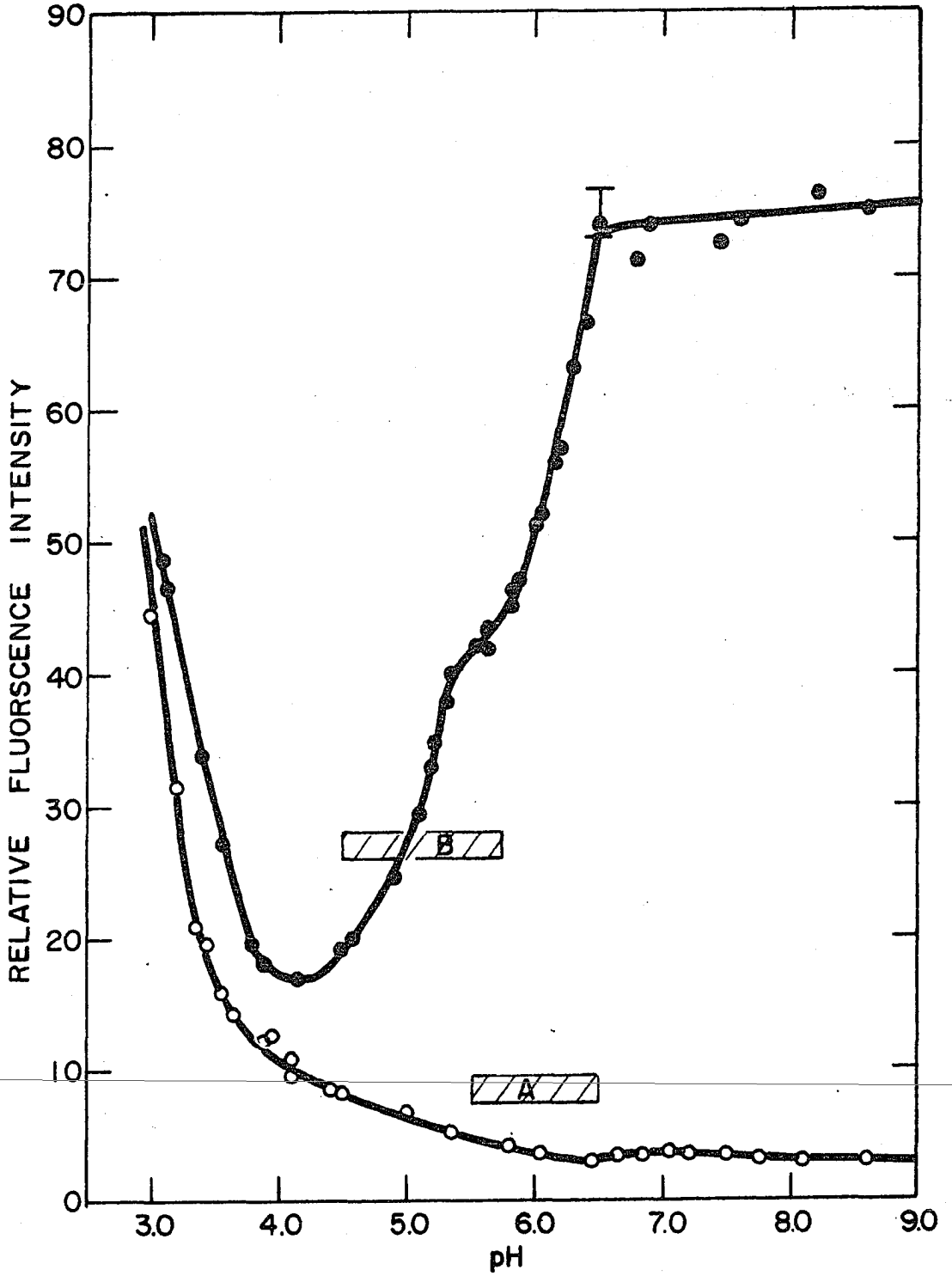
Total dye concentration is 4×10^{-5} M, R/D = 18.

o—o: In water

⊙—⊙: In 0.1 M NaCl

Region A: Helix-coil transition region in water

Region B: Helix-coil transition region in 0.1 M NaCl


RELATIVE FLUORSCENCE INTENSITY
90
80
70
60
50
40
30
20
10
0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH
B
A

point (from 7 measurements) is shown at pH 6.5 with the experimental range of values given by the brackets. More scatter was obtained in the A_{490} measurements (thought to be the result of larger temperature fluctuations) and the points were not averaged. The drops in F_{535} and A_{490} closely parallel each other until pH 5.4 is reached, then F_{535} suddenly drops again to a minimum near pH 4.2. The large drop in F_{535} beginning at pH 6.5 immediately suggests the formation of "metachromatic sites" in the coiled segments of the PGA molecule, while the shoulder in the curve suggests the number of these sites reaches a maximum near pH 5.6, just before the α-helical content begins to increase rapidly. By the term "metachromatic sites" is meant those sites on which sufficient interaction exists between the bound dyes to considerably reduce the observed A_{490} and F_{535} magnitudes. The sites themselves, of course, are not metachromatic. It has already been proposed that these sites are formed when the relative motion between the carboxylate groups is restricted to the point where stable dimers can form from dye molecules bound to adjacent carboxylates.

Not enough data has been collected to explain the interesting differences in behavior between the A_{490} and F_{535} curves in 0.1 M NaCl solution. For instance, the number and identity of the metachromatic coil sites formed below pH 6.5 and the relative sizes of the several binding constants are not known. Neither does any information exist on the magni-

tudes of the changes in F_{535} and A_{490} which would occur if all the dye were bound to the metachromatic coil sites. There is also no good reason to expect the absorption and fluorescence properties of the bound dye to respond similarly to a given structural change, particularly since the fluorescence yield is influenced by dye-polymer interactions. A case in point in which A_{490} and F_{535} change in the opposite direction, for example, was presented earlier in the effect of NaCl on the coil complex near pH 8, but the magnitudes involved were small. Similarly, the differences in shape between the A_{490} and F_{535} curves in Figures 19 and 22 are small compared to the overall change in optical behavior due to the helix-coil transition in 0.1 M NaCl solution. A few speculations on the origin of these small differences can be given, however, that might be useful in suggesting future experiments.

As mentioned earlier, the shoulder in the F_{535} curve at pH 5.6 suggests the number of metachromatic coil sites increases from zero at pH 6.5 to a maximum value in the vicinity of pH 5.6. Thus, as the pH is lowered from pH 6.5, the amount of dye bound to the special coil sites increases and F_{535} drops rapidly until the maximum number of these sites is approached near pH 5.6. At this point, the number of helix sites begins to increase rapidly, causing F_{535} to drop sharply once again to the minimum level at pH 4.2. If dye bound to the helix sites had been highly fluorescent, a

single minimum would have been observed near pH 5.6.

Turning now to the question of why no shoulder was observed in the A_{490} curve, it can be argued that the absorption of dye on the metachromatic coil sites is similar to the absorption behavior of dye bound to the helix, whereas the quenching of fluorescence is much greater on the latter conformation. This argument requires most of the dye molecules to be bound to the metachromatic coil sites near pH 5.6. Lowering the pH below this point then results in a redistribution of dye to the sites on the helix; the number of the coil sites must also decrease, of course, as the PGA becomes completely helical. A break in the A_{490} curve is not observed because the additional decrease in A_{490} is too small. Why A_{490} rises to a small maximum near pH 4.4 is unclear, but this behavior may be related to the PGA aggregation which occurs in this region (Tomimatsu et al., 1966).

On the other hand, it could also be postulated that the metachromatic coil sites are very similar to those on the extended coil in water solution, and therefore, if all the dye were completely bound to these sites, the absorption and fluorescence levels near pH 5.6 would lie near 25% and 5%, respectively. Hence, the observed levels at pH 5.6 in the A_{490} and F_{535} curves predict that only 46-56% of the dye is bound to the metachromatic coil sites. No break in the A_{490} curve is observed because the A_{490} level characteristic of complete binding to helix sites in 0.1 M NaCl is considerably

larger than the postulated level for the metachromatic coil sites and because the observed absorption at pH 5.6 is already near the helix level. In contrast, the fluorescence intensity still has a considerable distance to fall. If about 50% of the dye is bound to the metachromatic coil sites at pH 5.6, then at least 3% of the total residue groups present must be involved in the formation of these sites. The actual percentage is probably much higher in order to overcome competition from the other coil sites for the dye.

So far, it has been assumed that the rotational freedom of some of the carboxylate groups in the coiled segments of the chain has been restricted below pH 6.5, thereby creating coil sites similar to those existing in water solution. It is also possible that just a decrease in the average distance between the bound dye molecules with the increasing protonation is sufficient for the development of metachromasy. Earlier results, such as the flat plateaus in the optical titrations of the coil in salt solution, do not support this idea. However, a distinction between these two explanations of the large drop in F_{535} and A_{490} occurring before a sufficient number of helix sites are formed might be made by performing the experiments in Figures 19 and 22 at a much larger R/D ratio, such as R/D = 100 to 200. Because the average distance between bound dyes would then be larger, a greater reduction in the distance between carboxylate groups would be required to bring the bound dyes close enough for

optical interaction. Hence, if the second alternative is true, the breaks in F_{535} and A_{490} should shift from pH 6.5 to a lower value. On the other hand, the development of rigid coil sites depends only upon the pH or helical content and not upon R/D. The break should therefore either remain at pH 6.5 or shift to a higher value, depending upon the relative sizes of the several binding constants, if the rigidity hypothesis is closer to the truth. Although a greater number of rigid coil sites will be present at pH 6.5, so will the number of other sites be increased. Only if the affinity of dye for the rigid coil sites is very much greater will the break shift to a higher pH. If this should be the case, the F_{535} curve, for example, will drop sharply and a minimum should be observed in the vicinity of pH 5.6. Otherwise, the F_{535} curve at R/D = 100 to 200 should differ little from that shown in Figure 22.

Fluorescence Spectral Behavior

The dye stacking concept and the exciton model for dye-dye interaction predict a bathochromic shift in the emission maximum. Although the main effect of PGA addition to AO is a marked quenching of its fluorescence, it is apparent from Figure 8 that the quenching action is not uniform at all wavelengths. In particular, the emission intensity of AO near 600 mμ is reduced by only two-thirds at R/D = 8.17, while the intensity at 535 mμ has fallen to 7% of

the free dye level. When Curve 3 of Figure 8 is redrawn on the expanded scale of Figure 23 (Curve 2), the presence of a second emission peak near 590 mμ is clearly revealed. The ordinate scale of Figure 23 refers to the free AO emission intensity at 535 mμ. It is not certain from this data alone whether this 590 mμ band is the result of complex formation or whether its presence is simply being revealed by the loss of the intense emission at 535 mμ. Curve 2 shows the maximum development of the 590 mμ shoulder relative to the 535 mμ peak that was observable for the helix complex. An increase in R/D or the salt concentration causes an increase in the 535 mμ emission that obscures the presence of the second band. The emission spectrum in 0.1 M NaCl is shown as an example (Curve 1).

For the coil complex in water solution, however, the new band becomes well-developed compared to the 535 mμ emission, because the quenching of the latter band is more complete. The 590 mμ band intensity is also considerably smaller than for the helix complex and was found to depend critically on the R/D ratio in the vicinity of molar equivalence. Thus, the three representative fluorescence spectra shown in Figure 24 indicate that the maximum development of the 590 mμ band relative to the band at 535 mμ occurs near R/D = 2 to 3. For the reasons stated in the Experimental section, these spectra are highly distorted above 550 mμ; the true height of the new band compared to the observed intensity at 535 mμ is

Figure 23. Fluorescence spectra of the helix complex relative to the free AO emission at 535 mμ

Total dye concentration is 4×10^{-5} M, R/D = 8.17, pH 4.5.

Curve 1: In 0.1 M NaCl solution

Curve 2: In water solution

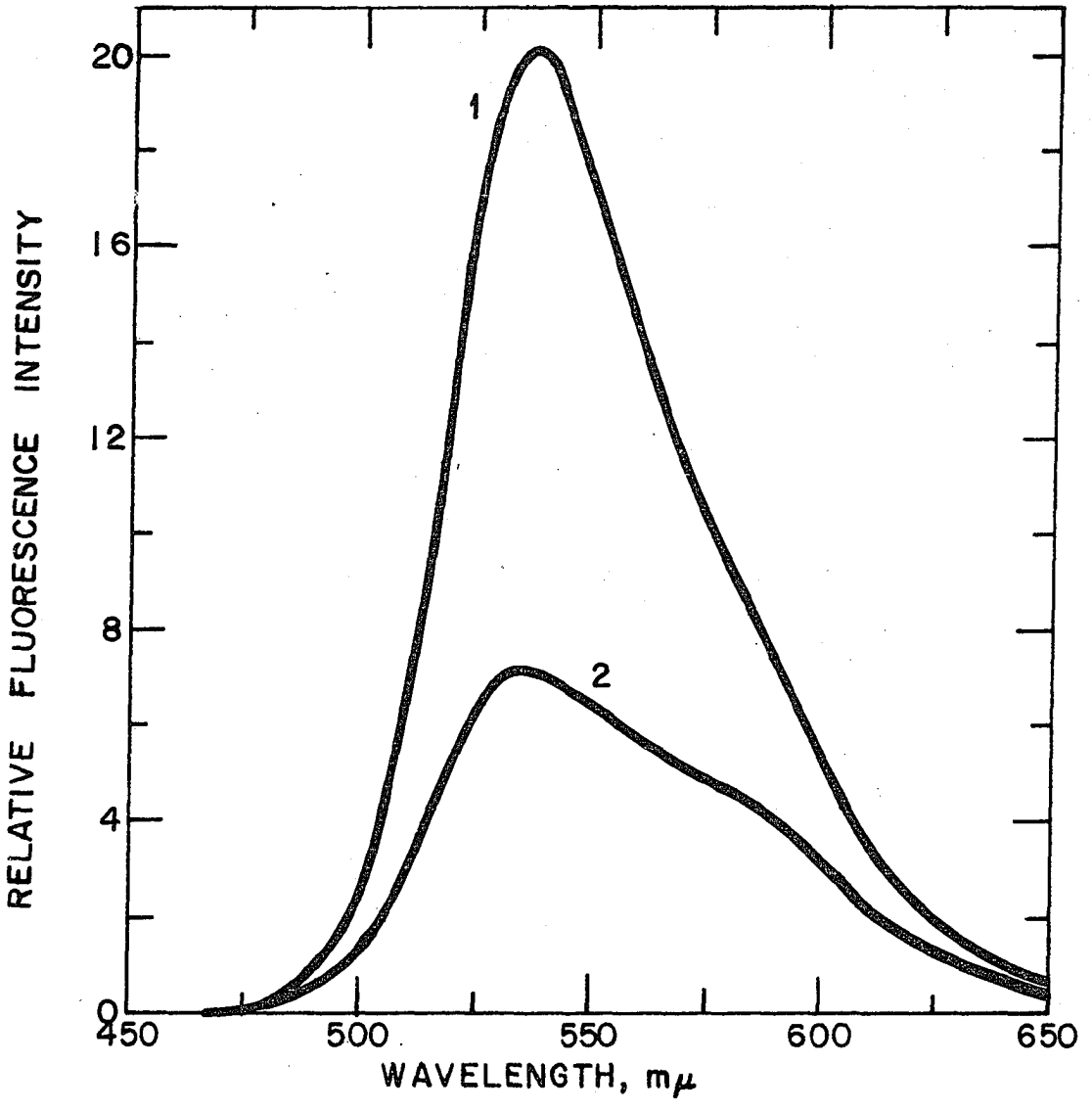

RELATIVE FLUORESCENCE INTENSITY
20
16
12
8
4
0
1
2
450
500
550
600
650
WAVELENGTH, mμ

Figure 24. Fluorescence spectra for several coil complexes in water solution relative to the free AO emission at 535 mμ

Total dye concentration is 4×10^{-5} M, pH 8.3.

Curve 1: R/D = 1.23

Curve 2: R/D = 2.45

Curve 3: R/D = 6.13

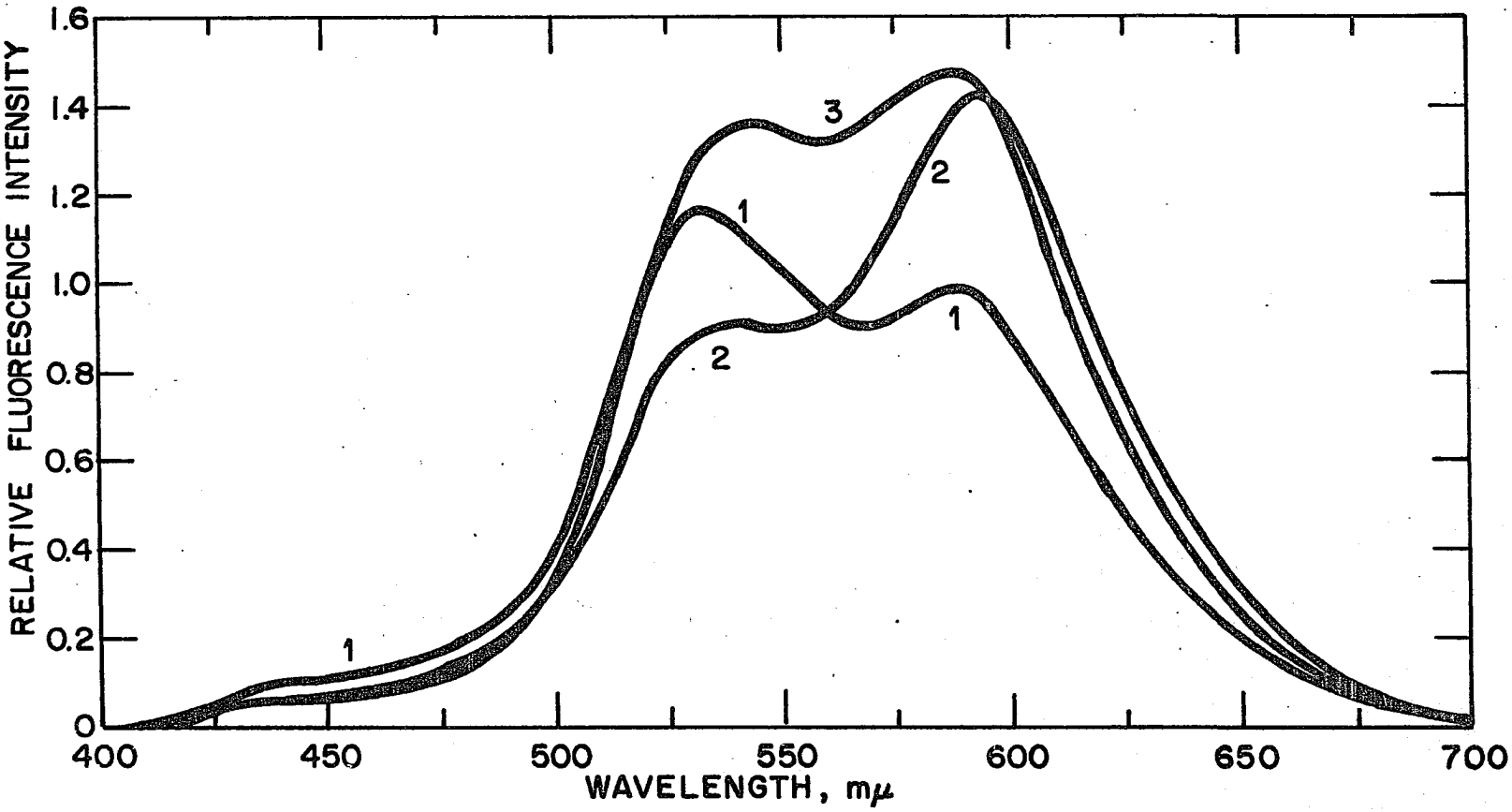

RELATIVE FLUORESCENCE INTENSITY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
400
450
500
550
600
650
700
WAVELENGTH, mμ
1
2
3
1
2
1

Figure 25. The AO:PGA complex fluorescence intensity at 535 mμ and 590 mμ relative to the free AO emission at 535 mμ as a function of R/D in water solution

Total dye concentration is 4×10^{-5} M.

Helix complexes: pH 4.3 to 4.5

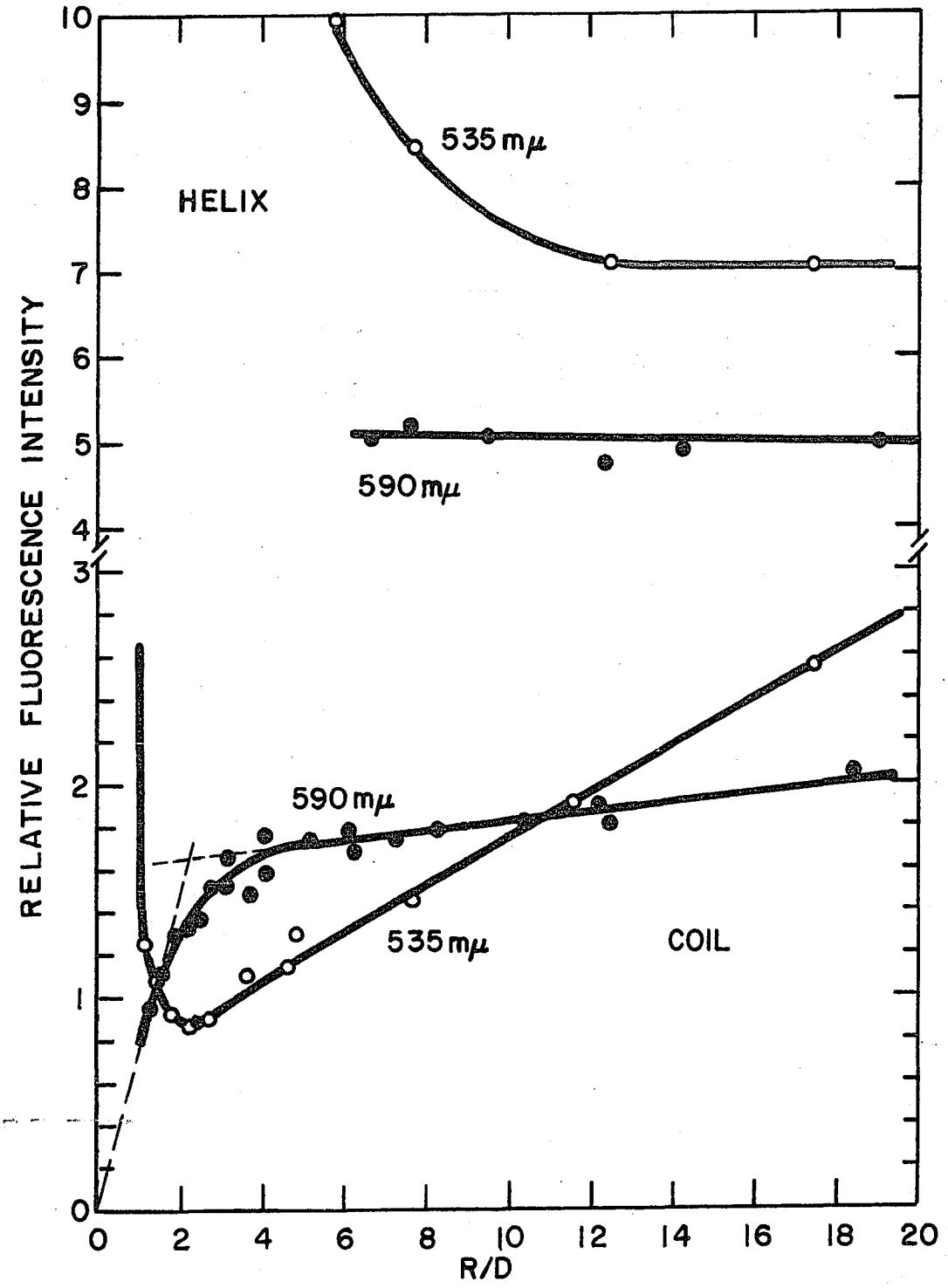
o—o: Intensity at 535 mμ

⊙—⊙: Intensity at 590 mμ

Coil complexes: pH 8.1 to 8.7

o—o: Intensity at 535 mμ

⊙—⊙: Intensity at 590 mμ


RELATIVE FLUORESCENCE INTENSITY
R/D
HELIX
COIL
535 mμ
590 mμ
590 mμ
535 mμ
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12
14
16
18
20

about 4 times larger than shown and its true position lies above 590 mμ. Therefore, only the changes in the shape of the emission spectrum brought about by various experimental conditions will be of interest.

In Figure 25 the fluorescent intensities at 535 mμ and 590 mμ are plotted as a function of R/D for the helix and coil conformations of PGA in water solution. Because only the ratio of the two intensities was actually measured, the 590 mμ points were calculated from this ratio and the optical titration curves at 535 mμ, shown in Figures 4 and 9. The titration curves at 535 mμ were replotted on the expanded ordinate scales of Figure 25 before interpolating and calculating the 590 mμ intensity points shown.

Considering first the helix case, it will be noted that the intensity at 590 mμ (F_{590}) remains essentially constant from R/D = 6 to 20 while the F_{535} curve decreases to a plateau. The F_{590} measurements were not continued to lower R/D ratios because the curve would have risen sharply from contributions from the intense, rapidly increasing 535 mμ band. Hence, the 535 mμ band allows no information to be obtained on the 590 mμ band behavior at low R/D. This band may be present in the AO solution alone and be entirely unaffected by complex formation or it may develop as a consequence of binding and reach a plateau somewhere below R/D = 6. The data is helpful, however, in showing that below a 10% relative intensity level, the 535 mμ band contributes

very little to the measured intensity at 590 mμ.

Luckily, the 535 mμ fluorescence band is so highly quenched by the extended coil form of PGA that the development of the 590 mμ band can be followed down to R/D = 1. The relative intensity of the 535 mμ band remains far below 10% for R/D ratios larger that unity, as shown in the lower half of Figure 25. It is now possible to see that the F_{590} curve falls in the R/D range 4 to 1 and may be extrapolated through the origin, thereby demonstrating the new band to be the result of complex formation. A new, red fluorescence has long been observed in concentrated AO solutions and in the in vitro staining of certain polyanions with dilute AO, but no corresponding fluorescence shift has yet been reported for AO:chromotrope complexes in solution. As Van Duuren (1966) has pointed out, this disturbing lack of fluorescence metachromasy in the complex solutions studied by him presents the major objection yet remaining to the dye stacking concept. Thus, the appearance of the 590 mμ band encourages the concept of AO aggregation on the PGA molecule and the application of the exciton model toward explaining the optical behavior. Because the new band could arise from dye-polymer interactions, however, definite support cannot be provided for the exciton model until it has been demonstrated that the 590 mμ band disappears at high R/D ratios. Unfortunately, the concomitant return of the 535 mμ fluorescence would seem to preclude such an experiment.

The most interesting aspect of the R/D-dependency of the 590 mμ emission is the evidence for the existence of an equal number of two kinds of sites on the extended coil. As shown in Figure 25, the 590 mμ curve breaks near R/D = 2, while the two limbs of the 535 mμ curve cross at R/D = 1. In order to interpret this behavior, it must be realized that the breaks obtained in this kind of titration yield essentially different information, depending on whether the optical parameter being followed is primarily a property of the free dye or of the complex. Because the 535 mμ emission is characteristic of free dye, a titration followed at this wavelength monitors the amount of dye in excess of the number of binding sites, and the break occurs when a sufficient number of sites has been added to bind all of the dye. Hence, this break yields the average total number of sites per residue without saying anything about the identity of these sites. If, on the other hand, the chosen optical parameter is a function only of the amount of complex, a break will occur when a sufficient number of a particular kind of site is present. In the case where all the sites are identical, the break will be observed at the same position as the 535 mμ emission break. The same will be true, of course, if the sites are not identical and the optical parameter being followed is not influenced by the nature of the site. The emission at 590 mμ apparently is not a dye property induced by binding to all of the sites, and, therefore, the break occurs at a larger R/D ratio than the

535 mμ break. The fact that the break occurs at R/D = 2 suggests only half of the dye bound to the fully saturated PGA molecule is emitting at 590 mμ.

Without discounting the possibility that two sites may be present, the more simple, one-site model can be preserved by postulating the 590 mμ fluorescence band to be characteristic of bound dimers. Like the 535 mμ band, it may be suppressed when the number of interacting dyes in a stack is larger than two. Thus, the extrapolation of the F_{590} curve as drawn in Figure 25 would be incorrect. Between R/D = 0 and 1, the slope may be zero or, at least, less than the initial slope observed just beyond R/D = 1. As soon as excess sites are added (beyond R/D = 1) the dye can then redistribute as dimers over the PGA surface. The sharpness of the break in the 590 mμ curve and the small slope above R/D = 4 suggest the formation of "isolated" dimers is greatly preferred over the crowded stacking conditions on the fully saturated, extended coil. Meanwhile, the emission at 535 mμ also slowly increases beyond the equivalence point, presumably due to the breakup of dye stacks into dimer configurations.

Besides the obvious advantage of not having to propose ad hoc reasons for why there should be equal numbers of two kinds of sites on the extended coil, the dimer concept for the origin of the 590 mμ emission easily explains the larger intensity observed for the helix complexes. Most of the

carboxylate groups are protonated at pH 4.5, and the probability of finding sequences of sites necessary for stack formation beyond the dimer stage becomes correspondingly very small. Hence, essentially only dimers are bound to the helix surface, and the distance between these sets of dimers, as shown by the large R/D breaks in the optical titration curves, is considerably greater than that between dimers bound to the extended coil conformation. Because the interactions between the sets of dimers must then be less, both the 535 mμ and 590 mμ emission bands are less suppressed. There are probably additional reasons, of course, for the increase in emission intensity, such as a change in the distance and angle between the pair of dyes in each dimer.

If the one-site model is correct, then the 590 mμ curve should break at the same R/D ratio as the 535 mμ curve for the helix complex. (In the two-site terminology, only "dimer sites" are provided by the helix, whereas both "polymer" and "dimer sites" may exist on the extended coil surface.) And since the 590 mμ band is considered to be a property of the dimer, the emission at this wavelength should fall to zero at very high R/D ratios. Unfortunately, as mentioned earlier, the presence of the intense 535 mμ band does not allow the direct experimental testing of these predictions.

It should also be pointed out that the intensity of the 590 mμ band in the free dye solution is only surmised to be near zero from the extrapolation in Figure 25. Since about

half the dye actually exists as dimers at a concentration of 4×10^{-5} M, some fluorescence at 590 mμ might, in fact, be expected. But there is one essential difference between free and bound dimers that could result in considerably different fluorescence behavior. The NMR studies of Blears and Danyluk (1967) on concentrated AO solutions have confirmed the stacking arrangement suggested earlier by Zanker (1952) in which the charged, ring nitrogens are located on opposite sides of the dimer sandwich. In contrast, the electrostatic binding of AO to PGA requires the ring nitrogens to be placed directly over each other. Thus, Zanker (1952) found the major new band produced by aggregation in water solution to occur near 650 mμ, not near 590 mμ. However, the presence of other, weaker bands closer to 590 mμ and on the order of only 5% of the 535 mμ intensity observed in dilute dye solutions could easily have been buried in the major fluorescence bands.

In reality, the behavior of the 590 mμ emission is much more complicated than the results presented so far would indicate, making the origin of this band even more uncertain. The 590 mμ band is characteristic of some nonequilibrium state of the complex that happens to be kinetically favored by the preparation methods employed. Under the influence of both time and heat, the fluorescence spectra, particularly of the coil complexes, change considerably as the 590 mμ component disappears while the 535 mμ band either increases or decreases in magnitude. Some typical emission scans of the

coil complex at R/D = 2.8 are shown in Figure 26. Curve 1, showing a well-developed 590 mμ band, was obtained immediately after preparing the complex in the usual manner, which results in a slightly cloudy solution. If the complex is prepared by PGA addition to the dilute AO solution, a clear solution is obtained and the 590 mμ band is even slightly better developed. The 590 mμ intensity decreases somewhat immediately after mixing, then increases to a maximum value in about 30 sec. Within the next 2 min, it falls rapidly and, thereafter, at a much slower rate. Curve 2 shows a typical scan obtained 2 min after the complex preparation. The 535 mμ intensity has increased while the 590 mμ band has fallen until both bands are nearly equal in height. These changes continue until at 8 min (Curve 3), a broad, intermediate peak is formed near 550 mμ; this band is usually more intense than the original one at 590 mμ. If either this aged complex or a freshly prepared complex is heated to 90°C for several minutes and then cooled to room temperature, an emission scan like Curve 4 is obtained. The total emissive area is reduced, a single peak is located at 535 mμ, and the emission below 500 mμ becomes larger. All trace of the 590 mμ band is buried in the smooth tail trailing out to 675 mμ.

A somewhat different response to the heat treatment is exhibited by the coil complex at a larger R/D ratio. Curve 1 in Figure 27 shows the fluorescence spectrum of a freshly

Figure 26. The effect of age and heat on the fluorescence spectrum of the coil complex in water at a low R/D ratio

Total dye concentration is 4×10^{-5} M, R/D = 2.8, pH 8.5.

Curve 1: Freshly prepared complex

Curve 2: 2 min later

Curve 3: 8 min later

Curve 4: After heating to 90°C

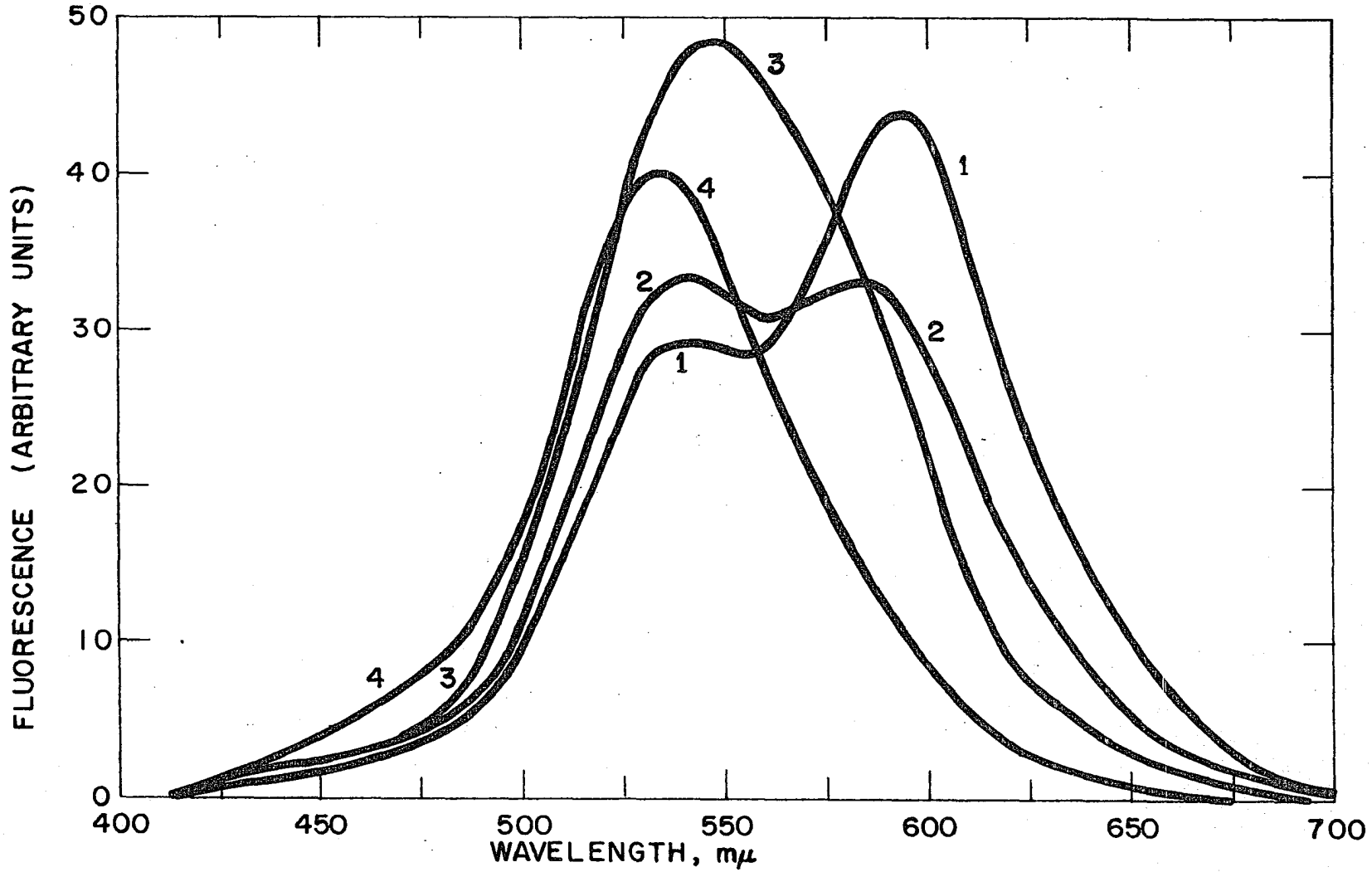

FLUORESCENCE (ARBITRARY UNITS)
50
40
30
20
10
0
400
450
500
550
600
650
700
WAVELENGTH, mμ
1
2
3
4
1
2
3
4

Figure 27. The effect of heat on the fluorescence spectrum of the coil complex in water solution at R/D = 19

Total dye concentration is 4×10^{-5} M, pH 8.5.

Curve 1: Freshly prepared complex

Curve 2: After heating to 90°C

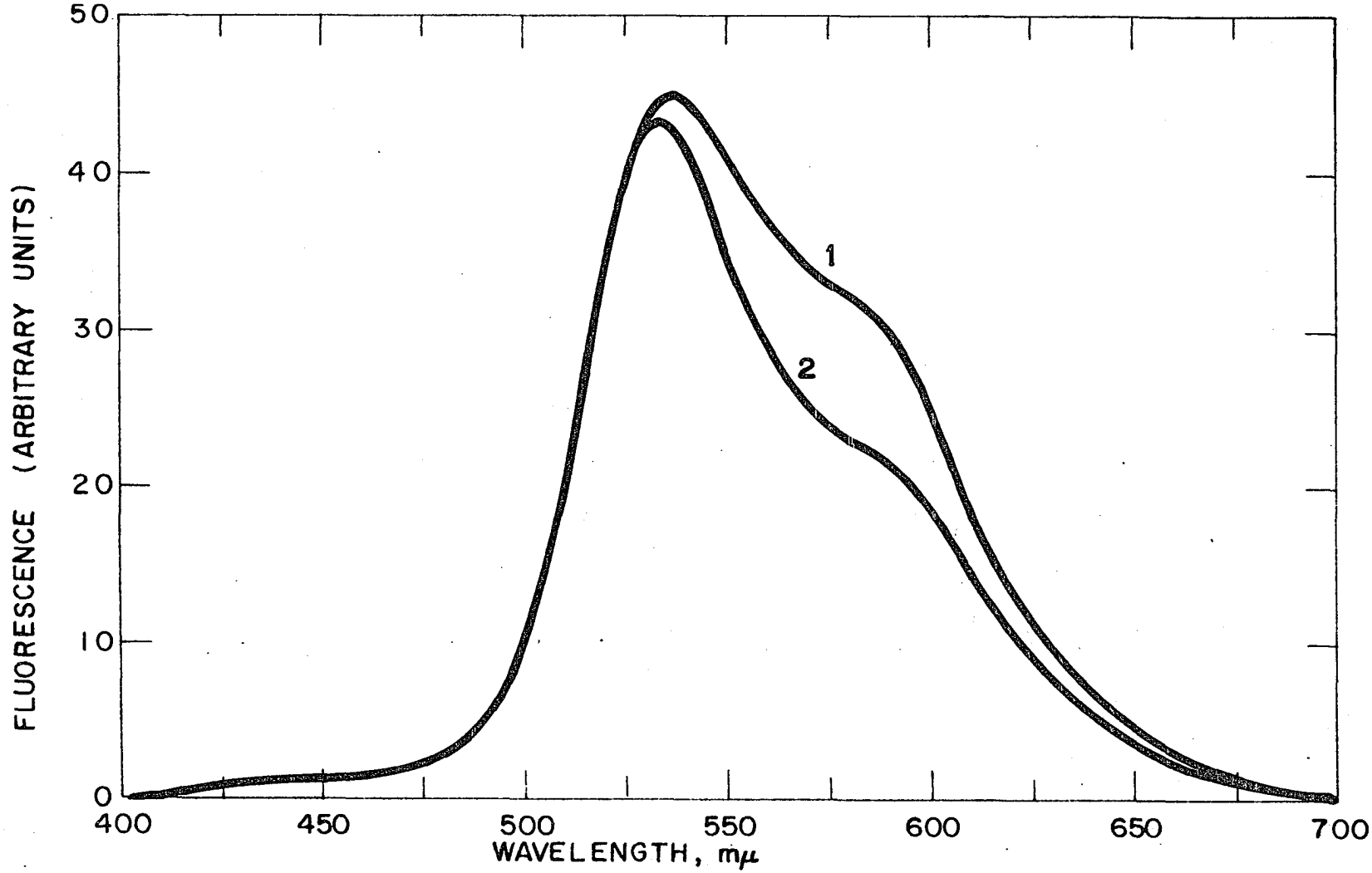

50
40
30
20
10
0
FLUORESCENCE (ARBITRARY UNITS)
400
450
500
550
600
650
700
WAVELENGTH, mμ
1
2

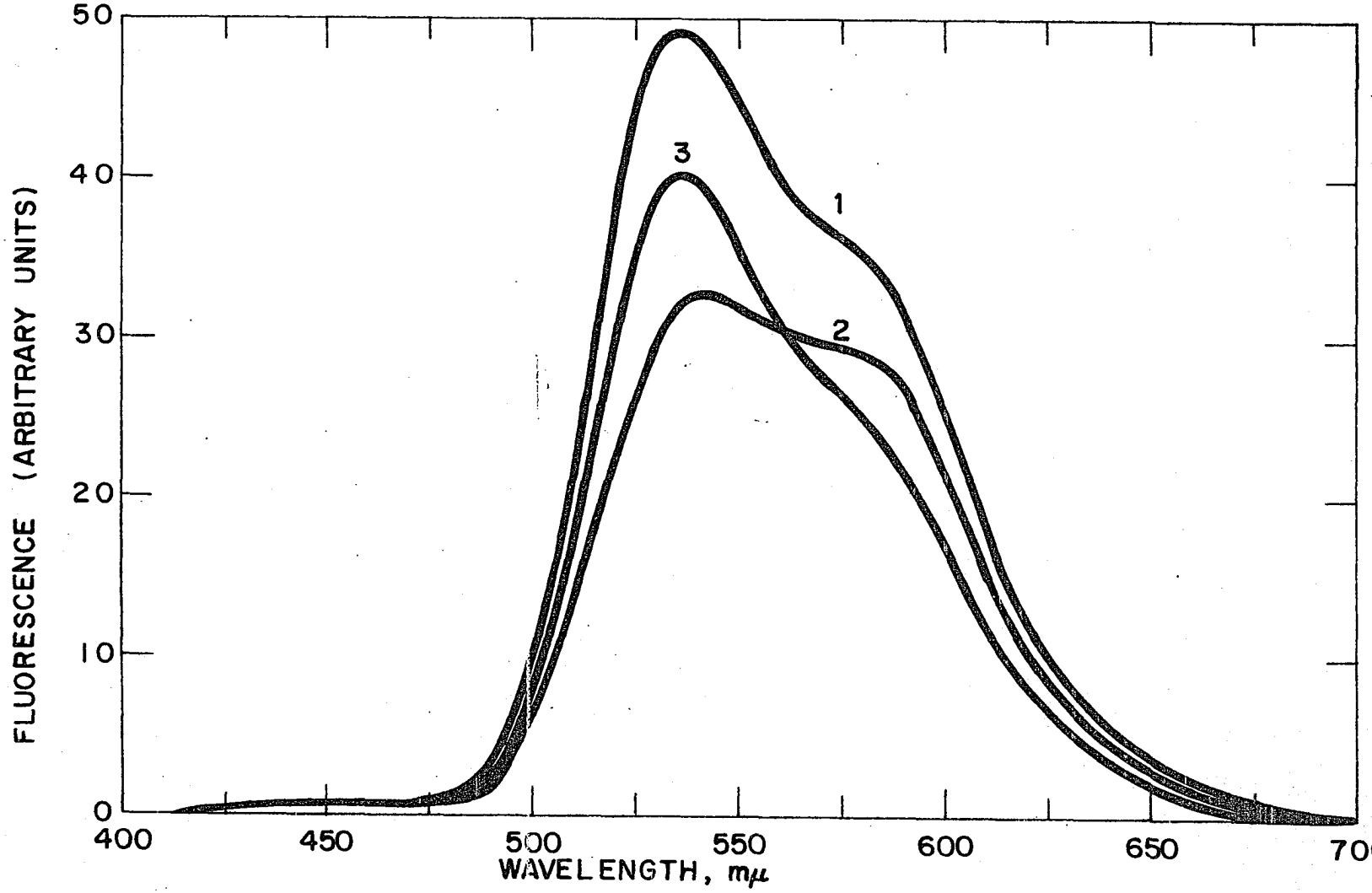
Figure 28. The effect of age and heat on the fluorescence spectrum of the helix complex in water solution

Total dye concentration is 4×10^{-5} M, R/D = 14, pH 4.5.

Curve 1: Freshly prepared complex

Curve 2: 40 min later

Curve 3: After heating to $90^{\circ}C$


FLUORESCENCE (ARBITRARY UNITS)
50
40
30
20
10
0
400
450
500
550
600
650
700
WAVELENGTH, mμ
1
2
3

prepared coil complex at R/D = 19. At this R/D ratio, the 590 mμ band always appears as a prominent shoulder. When this complex is given the 90°C heat treatment, the main effect is now simply a reduction in the height of the 590 mμ band. However, this band still remains as a prominent shoulder (Curve 2). Evidently, the presence of a larger excess of binding sites imparts some stability to the dye ensembles formed in the preparation process.

The fluorescence behavior of the helix complex is similar to that found for the extended coil complex near R/D = 19. In Figure 28, the fluorescence spectrum of a freshly prepared helix complex at R/D = 14 is shown by Curve 1. Some 40 min later (Curve 2), both the bands, especially the one at 535 mμ, have decreased in height to the point where the 535 mμ band is now only slightly larger than the one at 590 mμ. The heat treatment causes the 535 mμ intensity to increase halfway back to its original level while the 590 mμ band falls even farther (Curve 3). Once again, the 590 mμ component is only partially destroyed by heating, but unlike the extended coil complex, the 535 mμ intensity is concurrently reduced by nearly the same amount from its original level.

The fluorescence spectral changes presented in Figures 26, 27, and 28 clearly establish the importance of kinetically controlled processes in determining the precise structure of the complex. Even though these processes are not yet

understood, the fact that the 590 mμ component can be induced to disappear suggests this new band does not arise from dye-polymer interactions. But it is still be no means clear what arrangement of bound dye is responsible for the 590 mμ emission. For example, the new band might partially arise from interactions between dye molecules bound to different PGA chains, and the emission scan changes would then reflect changes in the state of aggregation of the barely soluble complex. It would be more satisfying, however, if these observations could be explained by the dimer hypothesis suggested earlier for the origin of the 590 mμ band. Thus, it could be postulated that dimer formation is greatly favored kinetically over the formation of larger stacks of interacting dyes. In the case of the extended coil, every carboxylate is a potential binding site, and if the dye is aligned properly, it is possible to form long stacks along the length of the chain. But the proper alignment of many dye molecules is likely to be a slower process than the random formation of dimers on the PGA surface. Hence, when a two- or threefold excess of sites are present, the 590 mμ fluorescence band, characteristic of the dimers formed initially, disappears as these dimers reorient or redistribute with time or heating to effectively form larger stacks. Such a process would be entropically favorable only at low R/D ratios; as the R/D ratio is increased to 20 and beyond, very little redistribution of dye to stacks larger than the

dimer would be expected. In the case of the α-helix, the high degree of protonation allows essentially only dimer formation to take place, regardless of how small a R/D ratio is chosen. Thus, the kinetically favored dimer formation is also the most energetically favorable arrangement of the dye. These arguments qualitatively explain why the 590 mμ bands of the helix complex and the extended coil complex at R/D = 19 are not destroyed by heating, while this band for the coil complex at R/D = 3 is very labile. Only the increase in the 535 mμ intensity for the coil complex at R/D = 3 remains to be satisfactorily explained. It has already been noted, however, that the 535 mμ intensity sometimes increases when the degree of dye-dye interaction, as measured by A_{490}, appears to increase.

As a matter of curiosity, the development of the 590 mμ was also followed as a function of pH in water solution. The complexes were prepared in the normal manner and the intensity readings were recorded when the 590 mμ band reached its maximum size (about 30 sec after mixing). Because it was experimentally easier to record the 590 mμ/535 mμ intensity ratio, the results are shown in this form in Part B of Figure 29. The R/D ratio was held constant at 8.2. Unfortunately, it was not realized at the time that the helix-coil transition region might be significantly shifted from its position for PGA alone. The cross-hatched area in Figure 29 locates the transition region at R/D = 18, but this region

Figure 29. The relative fluorescence intensity at 535 mμ and 590 mμ as a function of pH for the coil complex in water solution

Total dye concentration is 4×10^{-5} M.

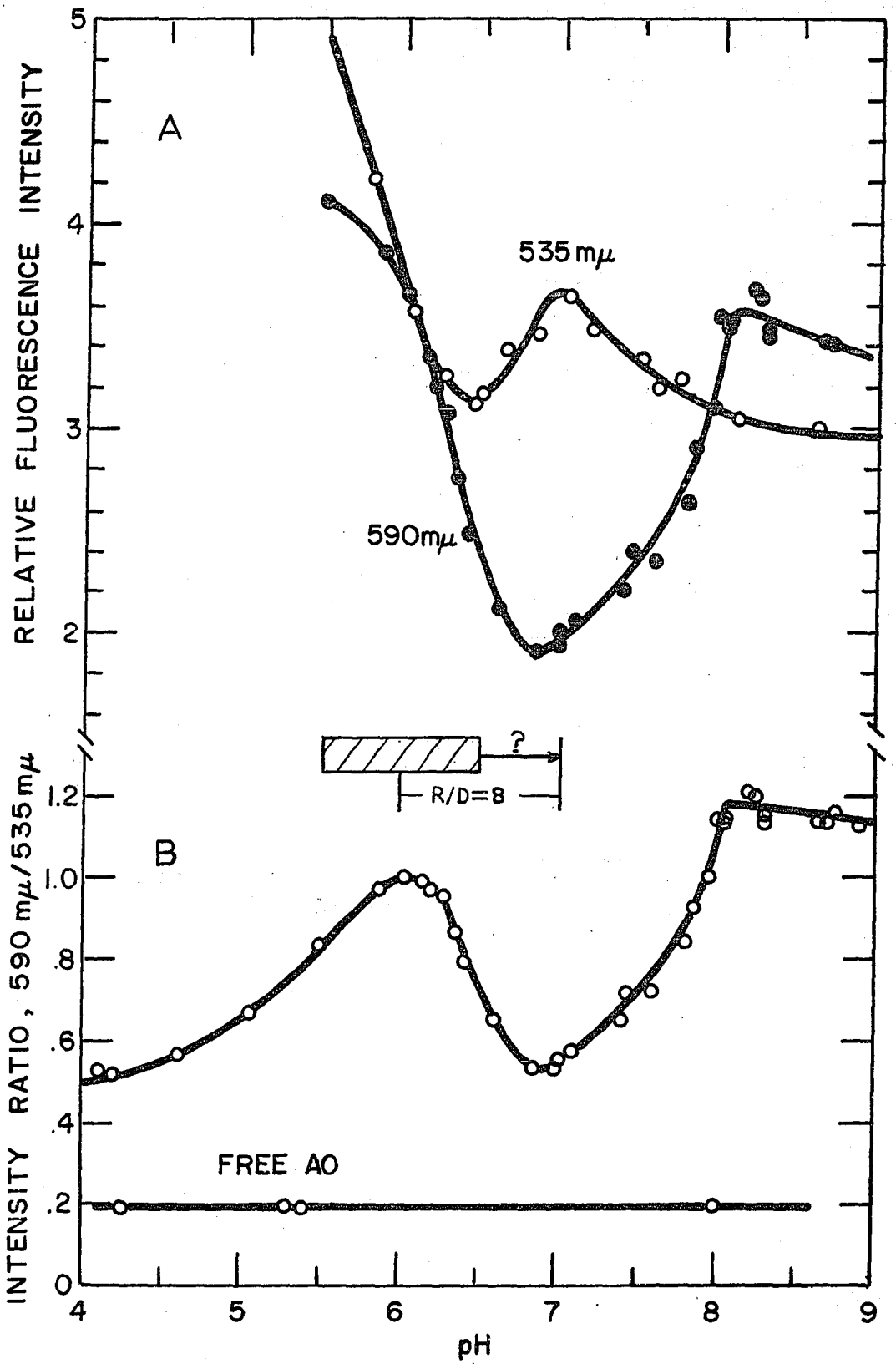
Part A: Intensity relative to free AO emission at 535 mμ for complexes at R/D = 18

o—o: Intensity at 535 mμ

o—o: Intensity at 590 mμ (approximated)

Part B: 590 mμ/535 mμ intensity ratio at R/D = 8.2

Cross-hatched area shows the helix-coil transition region at R/D = 18. The estimated position at R/D = 8 is also indicated (see text).


A
B
535 mμ
590mμ
?
R/D=8
FREE AO
RELATIVE FLUORESCENCE INTENSITY
INTENSITY RATIO, 590 mμ/535 mμ
pH
0
.2
.4
.6
.8
1.0
1.2
2
3
4
5
4
5
6
7
8
9

was shown to be shifted one pH unit upscale at R/D = 4 (Figure 17). Thus, it seems a reasonable guess that the helix-coil transition occurs between pH 6 and pH 7 at R/D = 8.2. If this is true, the 590 mμ/535 mμ ratio responds roughly in the same manner as A_{490} (see Figure 19) to the conformational changes that precede the formation of the α-helix. Both optical parameters abruptly decrease in magnitude about one pH unit above the transition region and reach a minimum near the beginning of the transition. Apparently, changes in the spatial arrangement of the carboxylate groups occur in this pH region that facilitate the formation of dye stacks. The ratio then increases again to a maximum at the completion of the coil-to-helix transition. Below pH 6, the ratio falls because F_{535} has begun to increase rapidly.

The behavior of F_{590} as a function of pH closely parallels the 590 mμ/535 mμ intensity ratio curve above pH 6, since the changes in F_{535} in this pH region are comparatively small. The curves in Part A of Figure 29 were constructed merely to show this to be the case. Relative fluorescence data from Figure 22 was replotted on the more sensitive scale shown in Part A and used to calculate the F_{590} points from the intensity ratio data shown in Part B. Because the F_{535} data was obtained at R/D = 18, this procedure is not strictly correct, yet it serves to provide approximate F_{590} values in the absence of F_{535} data at R/D = 8.2. The errors incurred are essentially twofold: (1) the 535 mμ intensity at

R/D = 8.2 is less than at R/D = 18, thereby causing the calculated F_{590} values to be slightly too large, and (2) the F_{535} curve should probably be shifted to larger pH values by half a pH unit before calculating F_{590} in order to account for the earlier conformational changes induced by dye binding at R/D = 8.2. In spite of these errors, the F_{590} curve does show that the changes in the intensity ratio above pH 6 in Part B are largely reflective of the changes in the emission intensity at 590 mμ.

The fact that F_{590} seems to be related to the PGA conformation in much the same manner as A_{490} provides further support for the idea that this new fluorescence band is characteristic of bound dimers. In the first place, if the new band arose simply from dye-polymer interactions, a curve like that obtained in Figure 29 would not be expected. Essentially no change in F_{590} should be observed above pH 6, where a sufficient number of carboxylates exist to bind all of the dye. Secondly, an increase in the degree of dye-dye interaction, as judged by the decrease in A_{490}, results in a loss of the 590 mμ band intensity. Hence, the concept of the 590 mμ emission being a property of bound dimers that is suppressed by stack formation is consistent with the previous interpretations of the A_{490} data and the parallel behavior of the A_{490} and F_{590} curves as a function of pH. Why stack formation apparently becomes more favorable in the pH region preceding the appearance of α-helix is unknown. Neither is

the more complicated behavior of F_{535} in this pH region understood. As shown in Part A of Figure 29, F_{535} first increases slightly and then quickly drops just before the transition region. It is believed from the polarization of fluorescence results in water solution that these changes in the optical properties of the bound dye are associated with a change in the distance and angle between adjacent carboxylate groups as an extended coil shortens and twists continuously into the α-helical conformation.

Induced Optical Activity

Background

Acridine orange is a planar, symmetrical molecule and, therefore, cannot in itself display any optical activity. But if the dye is rigidly attached to a substrate like PGA, the electronic environment will, in general, be asymmetric, and the consequent perturbation of the electronic transition will result in an induced optical activity. Such environmentally induced Cotton effects are usually much smaller in magnitude than those observed for inherently asymmetric chromophores. Another way in which optical activity may be induced is through the asymmetric coupling of the transition moments of two or more dye molecules. This is a special kind of environmental interaction in which the energy absorbed by one dye molecule may migrate by resonance transfer to another, so that the excitation energy cannot be identified with any

one molecule. Thus, a dimer or stack of dye molecules forms, in a very real sense, a new chromophore whose optical activity behavior depends upon the spatial relationships between the component transition moments. The rotational strengths associated with regular stacks of identical, interacting chromophores can be very large. As many excited state energy levels are produced as there are interacting molecules in the ensemble, and because the rotational strengths associated with excitation of the system to each of the levels must sum to zero, characteristic ORD and CD spectra are obtained (Tinoco, 1964). Tinoco *et al.* (1963) and Bradley *et al.* (1963) have employed exciton theory to derive expressions for the rotational strengths in terms of several helical stacking parameters.

It is instructive to consider what basic operations will produce an optically active, bound dimer. In Figure 30 is shown a representation of a dimer in which the two planes are parallel and stacked directly on top of each other; such a

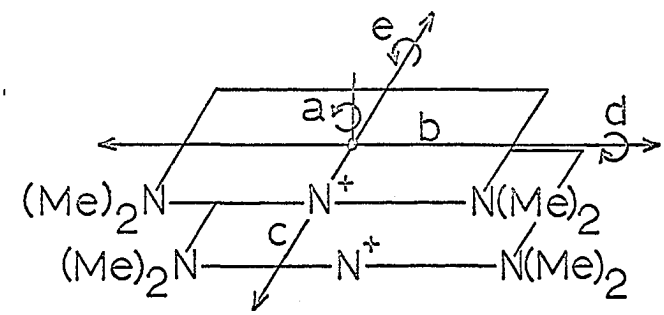


Figure 30. Illustration of the basic ways of altering a straight dimer stack

configuration is optically inactive (two planes of symmetry) and is referred to as a straight stack. The relative orientation of the two planes may be changed by the five basic operations shown, two of which lead to an asymmetric structure. If the stack is twisted by operation a, a rotation around the vertical axis, the resulting stack forms a segment of a right-handed or left-handed helix, depending on the sense of the twist. The other operation leading to an optically active structure is a displacement of the parallel planes along the major axis direction (operation b). The two configurations formed from equal displacements in the opposite directions are nonsuperimposable. All the other operations produce structures with a plane of symmetry: lateral displacement along the minor axis by operation c, and rotation of one of the dye planes about either the major axis (operation d) or about the minor axis (operation e). Of course, any combinations of the "asymmetric operations" with the "symmetric" ones will produce an asymmetric configuration.

So far it has been tacitly assumed that the symmetrical charge distribution on the monomer unit is not altered by dimerization. The very unfavorable positioning of the charged ring nitrogens directly over each other in the straight stack can be alleviated, for instance, by one or more of the operations already given and by the partial neutralization attending ionic bond formation with the γ-carboxylates. However, the alternative possibility of a

shift in the charge distribution, particularly in water solution, should be seriously considered. The positive charges may migrate to a terminal nitrogen on opposite ends of the stack as the two monomers approach each other to form a dimer. Such a shift in the charge distribution would be favored by the strong dipole character of the water molecule, which, in turn, could orient between the two pairs of the terminal dimethylamino groups, as shown in Figure 31. This idea was

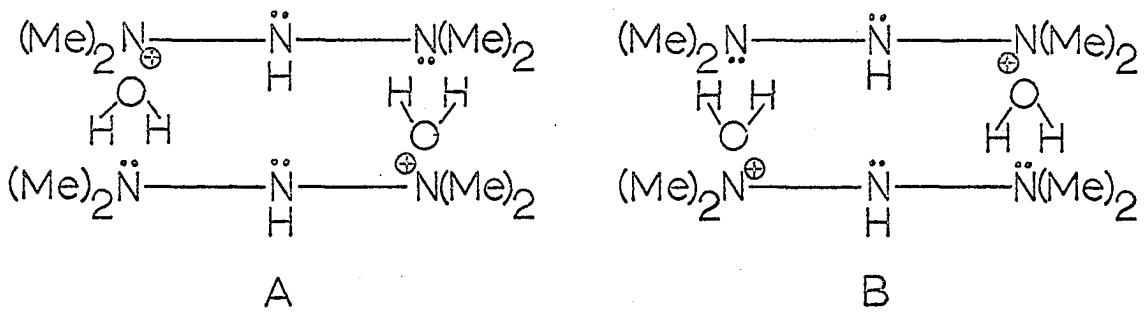


Figure 31. Two charge configurations of the straight stack that are optical isomers

first suggested by Bergeron and Singer (1958) in expressing their conviction that water plays an essential role in the development of metachromasy (see p. 10 in the literature review). The energy required to induce an electronic transition polarized along the major axes of both dye molecules would be increased by the necessity of reorientating the water dipoles. In contrast, the electronic transition moments along the minor axes would be only slightly affected by dimerization. This is an attractive idea in that it readily suggests a reason for the large hypochromism observed

in the visible spectral region for the AO:PGA complex; the transition polarized along the major axis direction would supposedly shift to a position in the UV spectral region. Hypochromism in the exciton band spectral region (in this case, from 450 mμ to 550 mμ), is not a characteristic of the dipole-dipole interactions considered in simple exciton theory. Therefore, coupling with electronic transitions located in the UV region and a lending of absorption intensity must occur if the exciton treatment is applicable to the metachromasy phenomenon. It seems possible that both exciton interactions and suppression of electron mobility by water orientation may be operative in producing the observed spectral changes.

Of further interest is the observation that the two charge configurations shown in Figure 31 are optical isomers. A straight stack may therefore become optically active if there is a charge migration as shown. Since dye solutions would be a racemic mixture of both forms, optical activity would not be observed unless one form was selectively or preferentially removed by binding to a suitable substrate. It may turn out, then, that dimer formation on the PGA surface involves such a charge displacement and the formation of ionic bonds to the dimethylamino groups rather than to the ring nitrogens. But model building suggests no reason why one charge configuration should be favored over the other; both left-handed and right-handed sites exist for the

extended coil and α-helical conformations. Because of rather subtle energy differences or the formation of non-ionic bonds between the dye and polymer, one form might possibly be favored over the other. Hence, the formation of straight stacks with a charge displacement provides a third basic way of achieving an optically active complex. The recognition of this basic mode may be extremely important, for it seems quite likely that some charge displacement will always occur upon dimer formation. If this is true, all bound dimers will be optically active, regardless of the stacking geometry.

Even though the optical activity behavior of AO bound to PGA is potentially a rich source of information about the structure of the complex, it should be apparent by now that this data will be very difficult to interpret unambiguously and quantitatively. The absorption and fluorescence properties are not so sensitive to small changes in the relative orientation of the interacting dyes and, of course, are identical for the optical isomers. But the observed optical activity will be the complex resultant of additions and cancellations between optical isomers and the optical activities of an unknown number of somewhat different stacking modes. Furthermore, the coil complexes, in particular, are not stable. A redistribution of dye often begins immediately after mixing, making it impossible to record undistorted ORD and CD spectra.

Finally, there is the question of how much the electronic

transition located above the α band position contributes to the observed optical activity. This transition, which is supposedly polarized perpendicularly to the one responsible for the α, β, and γ bands (Wittwer and Zanker, 1959), may also be split by exciton interactions and/or be assymmetrically perturbed by binding to the polymer. Yamaoka and Resnik (1966) call attention to this transition and believe it to be the origin of the CD bands observed above 500 mμ for the α-helix complex. However, they feel these CD bands arise entirely from environmental perturbations (even though this is difficult to reconcile with the large rotational strengths observed). It is clear from Figure 3 that the degree of stacking does influence the absorption behavior of this band and, therefore, optical activity might also arise from molecular exciton formation. On the other hand, there is apparently no splitting of this band upon stack formation in concentrated dye solutions. Zanker (1952) found an isosbestic point at 520 mμ over the entire concentration range, indicating that only an enhancement of the transition probability occurs with stack formation.

A study of the ORD and CD behavior of the AO:PGA complex has done little more than reveal the complexity of the system. Nevertheless, some very interesting and peculiar results were obtained that may be useful in planning or suggesting future research. Some typical examples of the variable ORD and CD spectra obtained will be given from which it will become

evident that much remains to be done after a suitable method of complex preparation is discovered.

Effect of PGA conformation

Because Stryer and Blout (1961) had reported that AO exhibits optical activity only when bound to the helical polymer, it was of interest to see how well the induced optical activity was correlated with the helix-coil pH transition region. Complexes were prepared by the addition of 10^{-3} M AO to pretitrated PGA in water solution to a constant R/D ratio of 16.4. Instead of finding a correlation between α-helical content and AO optical activity, four general pH regions characterized by different ORD curves were discovered. Typical ORD curves at pH values within these four regions are shown in Figure 32, where it is immediately obvious that Cotton effects as large as those produced by binding to the α-helix can also be induced in the extended coil pH region. This surprising result was reported in an earlier communication (Myhr and Foss, 1966). The failure of Stryer and Blout to observe optically active coil complexes arises from their different method of complex preparation. Dye was added to helical PGA and the system was then titrated with base to produce the coil complexes. When this method was employed in this laboratory, the Cotton effects always disappeared in the helix-coil transition region in agreement with the results of Stryer and Blout (see the upper half of Figure 18).

The rotatory dispersion shown by Curve 1 of Figure 32 is

Figure 32. Typical visible optical rotatory dispersions of AO:PGA complexes in water solution at several pH values

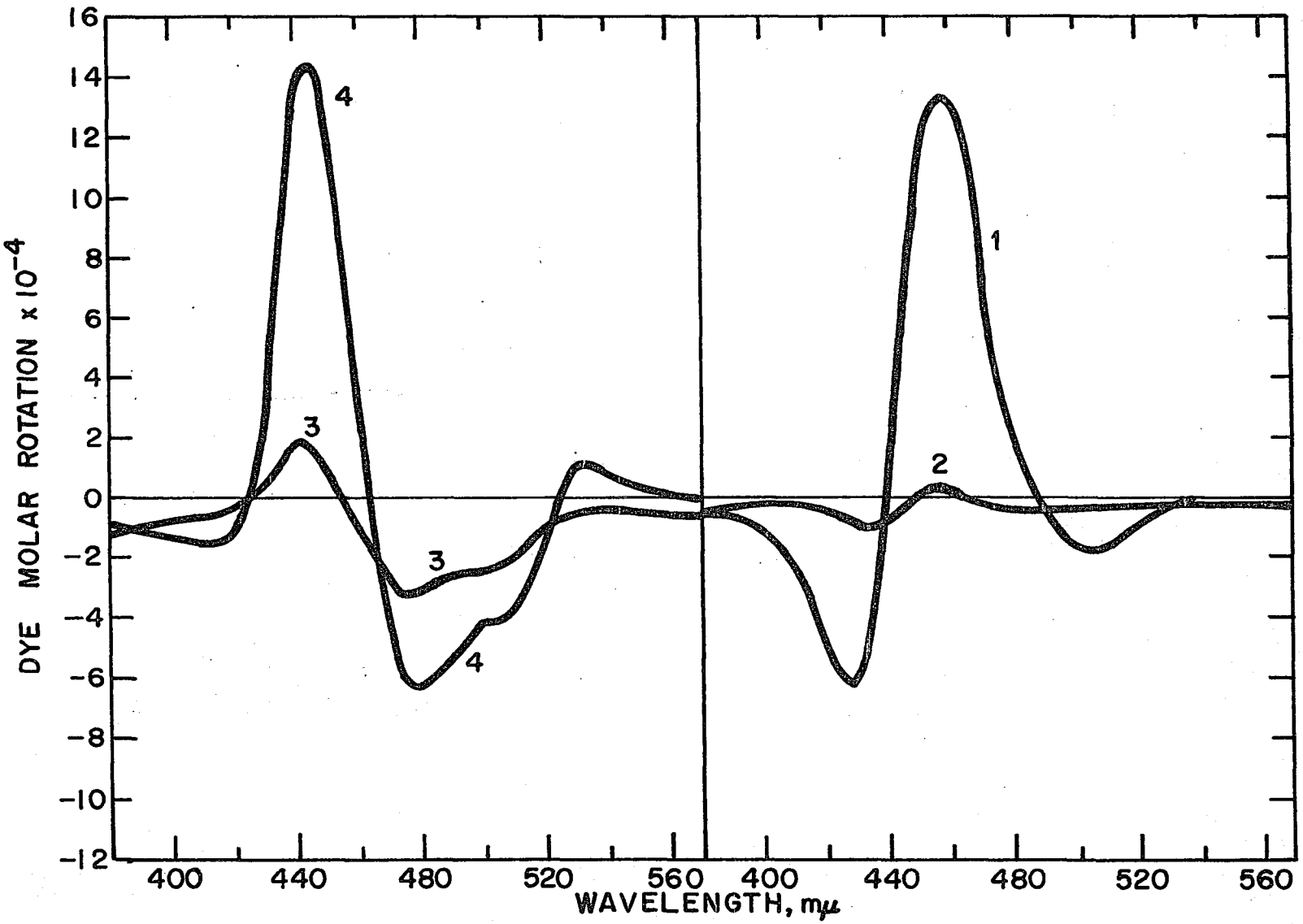
Total dye concentration is 4×10^{-5} M, R/D = 16.4.

Curve 1: pH 8.0

Curve 2: pH 7.0

Curve 3: pH 6.2

Curve 4: pH 4.5


DYE MOLAR ROTATION x 10⁻⁴
16
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
400
440
480
520
560
WAVELENGTH, mμ
1
2
3
3
4
4

representative of the maximum development of the Cotton effects in the pH 7.8-8.6 region. The magnitude of this ORD curve is reproducible to better than 5% only if some rather peculiar preparation requirements are observed. These requirements will be given later. A second general pH region begins around pH 7.6 and extends to the beginning of the coil-to-helix transition region (about pH 6.5). Only very small Cotton effects could be produced in this region by a variety of preparation methods. Curve 2 is a typical ORD obtained at pH 7.0. Even though it appears as if a single, positive Cotton effect is located at 445 mμ, the exact shape is obscured by the noise level, and the shape seems to change from complex to complex. As the pH is lowered through the transition region, dispersion curves similar in shape to that observed for the fully helical complex at pH 4.5 (Curve 4) begin developing. Curve 3 shows the ORD obtained at pH 6.2, where the α-helical content is about 30% and the absorption changes are maximal (Figure 19).

The Cotton effects at pH 4.5 and pH 8.0 are typical of those given by Tinoco (1964) as characteristic of molecular exciton formation. And the very large dye molar rotations observed can only be readily explained by dipole interactions between the bound dyes. Thus, some ordered arrangement of sites (and dyes) must exist for the extended coil conformation as well as for the α-helix. What type of ordered structure is present at pH 8 is by no means clear. As

suggested earlier, it is possible that the fairly rigid, extended coil actually exists as an extended helical structure. Or the act of binding dye may cause the coil to twist in one preferred direction. Local regions of ordering might also exist or be induced by dye binding. Perhaps the most likely possibility is that the optical activity is characteristic of certain AO:PGA aggregates formed during the mixing process. Any PGA aggregates existing before the addition of dye (Schuster, 1965) cannot be responsible, however. When the PGA solution is heated for several minutes at 85°C and then cooled to room temperature before adding dye, the Cotton effects are unaltered in size and shape. Dye and PGA molecules may aggregate during certain mixing processes, however, to produce structures similar to the highly optically active, cholesteric form of liquid crystals (Fergason, 1964). The optically active coil complex solutions are always slightly cloudy.

Optical activity behavior of coil complexes

Before adopting the method of preparing complexes directly in a quartz cuvette, the components were mixed by swirling in a small polyethylene beaker and then poured into an optical cell. The ORD data shown in Figure 32 was obtained in this manner. But relatively stable and reproducible ORD curves at pH 8 could be obtained only if the dye was added ca. 3 min after dilution of the PGA stock. If the 10^{-3} M AO solution was added immediately after mixing the required

volumes of stock PGA and water, the resultant ORD curves were much smaller and they changed in shape during the scan period. This dilution time period requirement is not understood, for no changes in the PGA conformation could be observed by following the rotation at 233 mμ after diluting the PGA stock.

A convenient way of determining the effect of PGA dilution time on the optical activity of the pH 8 complex is to follow the rotation at 458 mμ versus time. The positive peak of the ORD curve occurs at this wavelength for the stable complexes formed after a 3 min dilution period. If the dye is added about 3 min after dilution of the PGA stock, the rotation at 458 mμ is always positive and relatively stable for at least a half-hour. The particular manner in which the AO is added has little influence on the magnitude. But when the dye is added immediately after PGA dilution, the initial rotation at 458 mμ is <u>negative</u> and the absolute magnitude decreases rapidly with time. Often, the rotation changes to a small positive value, most of the change occurring within 5 min after preparing the complex. If the dye is added in short spurts rather than by a continuous delivery from the buret, the initial rotation starts out even more negative and never reaches a positive value. It was not at all uncommon to observe initial dye molar rotations exceeding -250,000, which is far removed from the stable, positive level of 130,000 shown in Figure 32. The addition of dye in spurts also produced considerably more cloudy solutions. These

results are not due to birefringence problems, since stirring and reversing the cuvette did not alter the sign or course of the observed changes.

The circular dichroism behavior of the pH 8 complex is illustrated in Figure 33. Curve 1 was obtained immediately after the addition of AO to a dilute PGA solution that had just been prepared. Its shape is distorted because the complex is not stable during the scan period, as is shown by the trace obtained immediately afterwards (Curve 2). With the elapse of time, the single, positive peak splits into a positive and a negative band. In contrast, the CD spectrum of the complex prepared by adding AO some 3 min after the PGA dilution was stable and could be exactly retraced (Curve 3). This CD spectrum is characteristic of molecular exciton formation and the two bands correlate well with the ORD curve in Figure 32. On the other hand, the positive CD band in Curve 1 does not seem reasonable in view of the large negative rotations observed initially at 458 mμ. Since rotations at 458 mμ were not being examined at the time when this CD curve was recorded, it is possible that another type of unstable complex was produced in these experiments.

During the course of measuring the degree of stacking at very large R/D ratios, the CD spectrum of the pH 8 coil complex was also obtained at R/D = 62,900. Quite surprisingly, a large CD band was observed (Curve 4, Figure 33) that was, in fact, larger than the bands noted at R/D $\cong$ 18. Because

Figure 33. The visible circular dichroism spectra of several coil complexes in water solution

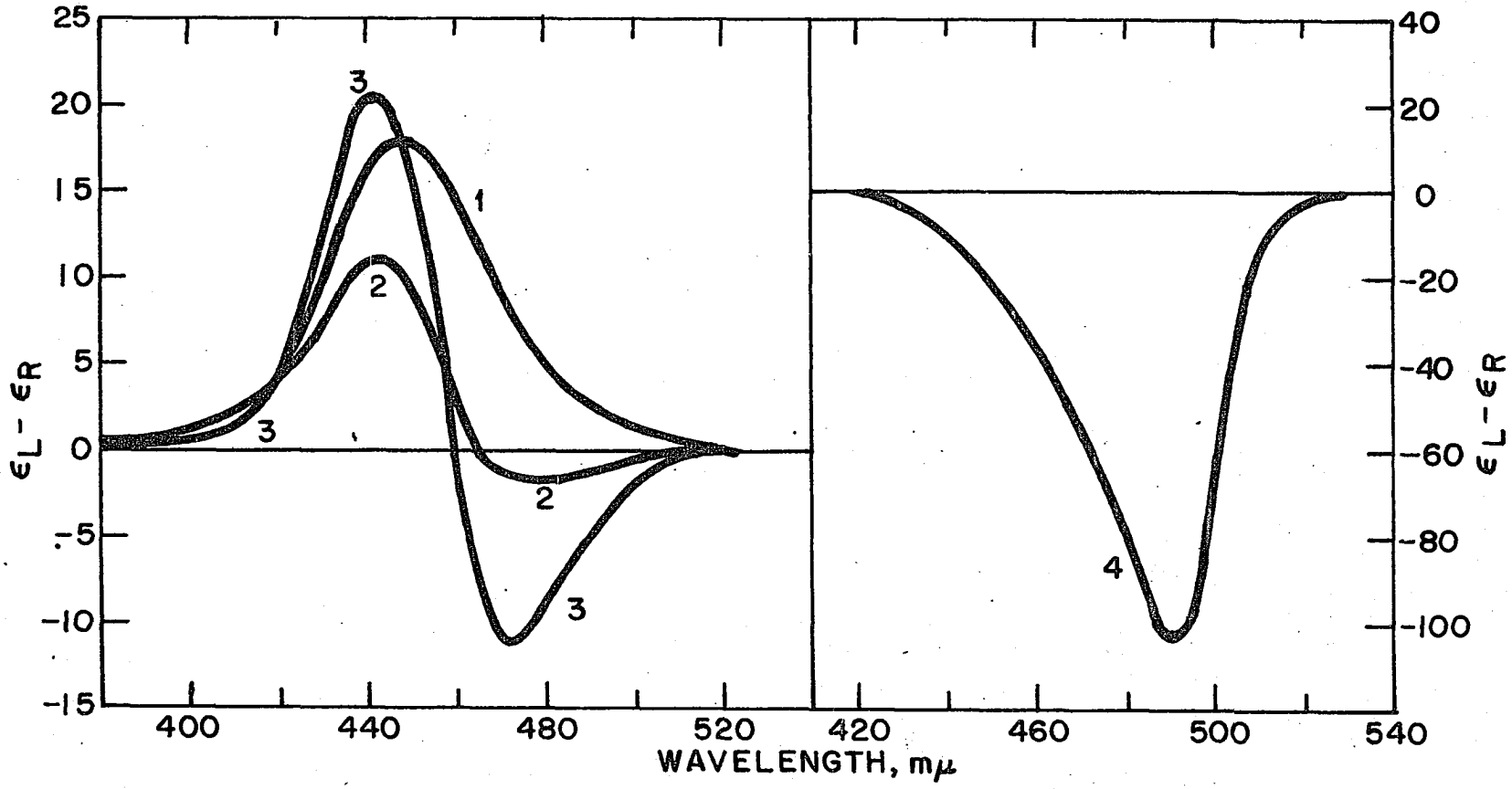
For Curves 1 to 3: Total $[\overline{AO}] = 4 \times 10^{-5}$, R/D $\cong$ 18, pH 8

Curve 1: AO added soon after dilution of stock PGA

Curve 2: An immediate rescan of the above complex (15 min later)

Curve 3: AO added 3 min after dilution of stock PGA

Curve 4: Coil complex at R/D = 62,900, total $[\overline{AO}] = 4 \times 10^{-6}$ M, pH 8.5


25
20
15
10
5
0
-5
-10
-15
40
20
0
-20
-40
-60
-80
-100
$\epsilon_L - \epsilon_R$
$\epsilon_L - \epsilon_R$
1
2
3
3
2
3
4
400
440
480
520
420
460
500
540
WAVELENGTH, mμ

the dye is bound almost completely in its monomeric form, this result immediately suggests that the optical activity arises from binding to an asymmetric environment. And yet, the $\Delta\epsilon$ value of -100 is so much larger than the range expected for environmentally induced Cotton effects ($\Delta\epsilon$ below 10) (Mason, 1967) that it seems this CD band must somehow arise from interactions between the dye chromophores. The coupling between the dye transition moments falls off as $(1/r)^3$, where r is the distance between the moments, so even if sufficient coupling exists to produce Cotton effects without causing much change in the absorption behavior, the rotational strength should not increase at very large R/D ratios. Thus, one is led to suspect that the CD band in Figure 33 might be a property of some aggregate formed as the stock AO is added to the concentrated PGA solution. In fact, this CD band is not stable. When the same complex solution was examined later in the stacking experiment for circular dichroism, the CD band was found to be much smaller at R/D = 54,000 and completely absent at R/D = 37,800. The loss of the band is probably due to the breakup of the aggregate with time rather than a result of the change in R/D.

Returning now to the optical activity behavior of the pH 8 complex at R/D near 18, two other requirements for obtaining the ORD curve in Figure 32 were discovered. For instance, a stable complex cannot be prepared by the standard technique of mixing the components directly in a 1 x 1 cm

cuvette and stirring with the vibrator. The rotation at 458 mμ always starts out negative and rapidly changes toward zero or a positive level. This is essentially the same behavior noted earlier for the addition of AO immediately after PGA dilution in a small beaker. However, the length of time after dilution of the stock PGA now has no effect on the results obtained in the cuvette. Neither do various methods of adding the dye ever result in a stable, positive rotation. If the dye is added in short bursts, the solution again becomes very cloudy and the initial complex becomes even more strongly levorotatory at 458 mμ. A stable complex could never be produced in the cuvette. There is obviously some mixing mode obtainable in the small polyethylene container that cannot be duplicated by the addition and mixing of components by vibration in the cuvette. (The complex was never prepared in a small glass beaker to determine if the container material has any effect on the optical activity behavior.)

The third requirement is that the stock AO must be added to the dilute PGA solution and not vice versa. Whenever the stock PGA is added to a dilute dye solution (4×10^{-5} M), the resulting complex is not optically active (or only slightly so). The complex solutions also appear to be clear, which is in contrast to the slightly cloudy, optically active complex solutions. Although it is desirable to prepare optically clear solutions of the complex, the procedure of adding PGA to AO solutions is very inconvenient when it is desired to

follow an optical property as a function of pH in unbuffered solutions. Hence, another mixing method was found that seems to duplicate the result of adding PGA to AO. The PGA solution is first prepared in a 1 x 1 cm cuvette and adjusted to the desired pH. Then the required volume of 10^{-3} M AO is placed in a square, 1 x 1 cm Teflon well that has four small holes drilled in the bottom. Surface tension prevents the AO from draining out. A long rod attached to the well then allows the well to be plunged into the cuvette and moved up and down to mix the complex. Coil complex solutions prepared at pH 8 in this manner are clear and optically inactive. Thus, it appears that the optical activity is associated with the aggregate responsible for the cloudiness.

If the optically active pH 8 complex is heated to 80°C and then cooled to room temperature, the optical activity is almost completely destroyed. The slightly cloudy solutions are also converted into clear ones. It will be recalled that the fluorescence band at 590 mμ is also quite labile and is destroyed by heat, but the two optical phenomena are not necessarily related. For example, the fluorescence band is slightly enhanced by the addition of PGA to AO and by the use of the Teflon mixer for adding AO, whereas both of these methods produce optically inactive coil complexes. The 590 mμ emission was tentatively ascribed to the formation of an excess number of dimers in the initial combination of AO with PGA. It was of interest, then, to see if the several

forms of optically active coil complexes differed in their degree of dye-dye interaction and whether or not heating always caused an increase in this interaction.

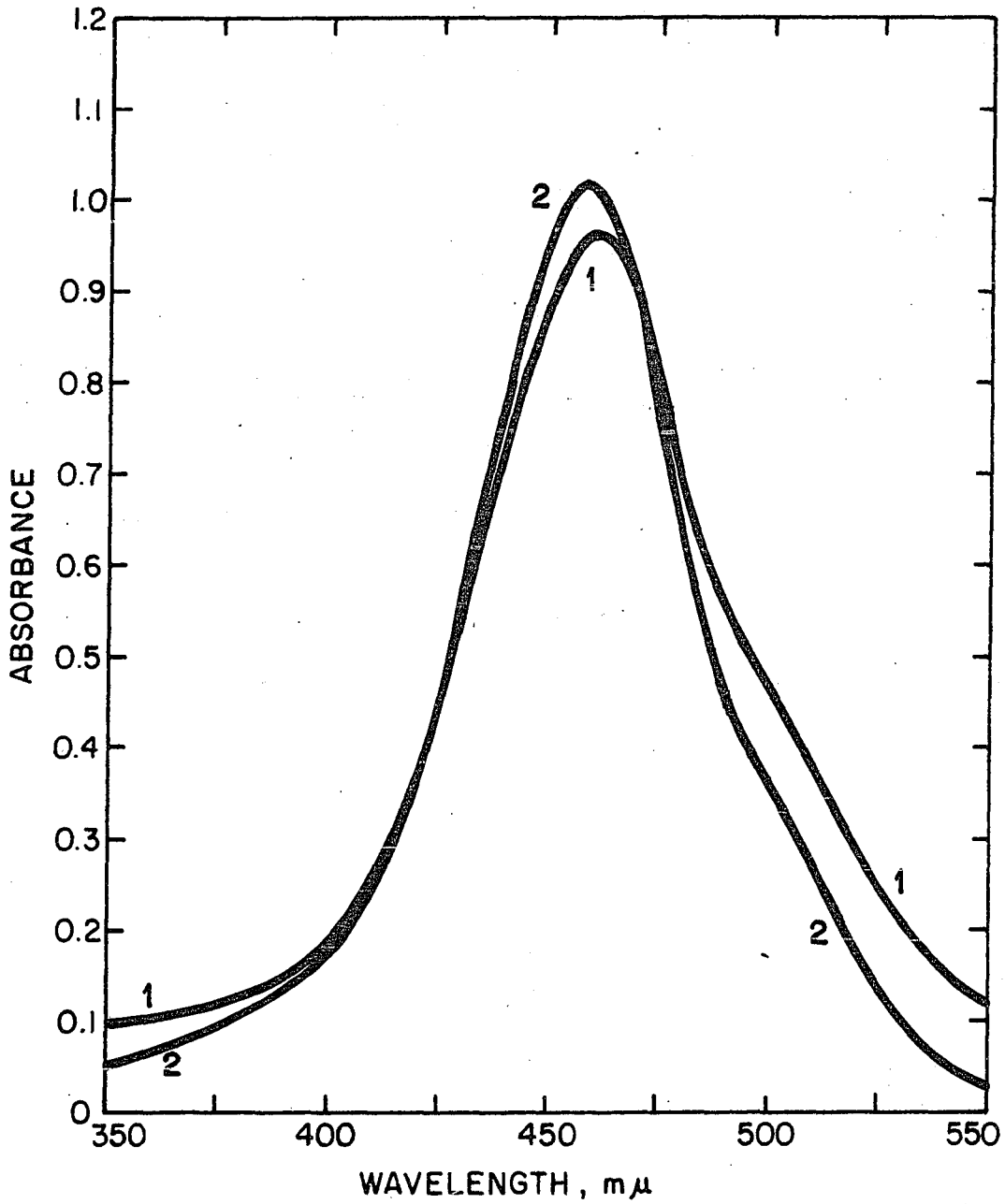
In Figure 34 are shown two visible absorption spectra that represent the various coil complexes at pH 8 and the effect of heat. Curve 1 is the spectrum of the complexes prepared by adding AO from a microburet to the dilute PGA solution, regardless of what method is followed. Both the relatively stable complex which exhibits a large positive rotation at 458 mμ (ORD curve in Figure 32) and the unstable complex, which is strongly levorotatory at 458 mμ, yield this spectrum. When the optical activity is destroyed by heat, Curve 2 is obtained. This second spectrum is also characteristic of the optically inactive complexes prepared by the addition of PGA stock to the 4×10^{-5} M AO solution. Thus, the degree of dye-dye interaction appears to be greater for the optically inactive coil complexes; A_{490} is smaller and the absorption maximum is shifted 2-3 mμ toward shorter wavelengths. It is not yet possible to determine what proportion of this change is due to a redistribution of bound dye and/or a reorientation of the transition moments. But it does seem that the formation of optically inactive complexes does not involve simply an equalization in the number of bound optical isomers. If this were true, Curves 1 and 2 should be nearly identical. Both the positively and the negatively rotating complex species (at 458 mμ) exhibit the same absorption

Figure 34. The visible absorption spectra for the pH 8 coil complex in water for two mixing orders

Total dye concentration is 4×10^{-5} M, R/D $\cong$ 16, 1 cm path length.

Curve 1: Stock AO added to dilute PGA

Curve 2: Stock PGA added to dilute AO


1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
350
400
450
500
550
WAVELENGTH, mμ
1
2
1
2
1
2

spectra. Such an equalization might occur, but only in addition to other changes in the complex structure.

The minimum in the A_{490} versus pH curve in water solution (Figure 19) occurs near pH 6.2. Complexes prepared in the usual manner at this pH are almost clear. When stock AO is added by the Teflon mixer, a clear solution is obtained that exhibits very nearly the same absorption spectrum. The absorption maximum is still located at 448-450 mμ and is the same height; only A_{490} is somewhat smaller than that obtained by adding AO from a microburet. Either mixing method produced essentially the same optical rotatory behavior at 458 mμ. This was a poor wavelength to follow because the dispersion curve shown in Figure 32 passes through zero near this position. Nevertheless, it was learned that the complexes prepared in a cuvette are much less stable than those produced in the polyethylene beaker. The rotation at 458 mμ was not reproducibly negative or positive and usually disappeared completely within a couple minutes after mixing. The few dispersion curves obtained were small and dissimilar to the one shown in Figure 32. Hence, even the presence of ca. 30% α-helical content does not impart a stability to the optically active complexes formed in the cuvette.

A marked difference in the manner in which PGA solutions at pH 8 and pH 6.2 interact with the 10^{-3} M AO stock was noted that may be worthwhile mentioning. Aliquots of either PGA solution were placed on a glass slide and a small amount

of AO stock was added by syringe to form swirls of colored solution. When these swirls in the pH 8 case were examined through a dissecting microscope (magnification X 30-50), a sharp phase boundary was discovered to form immediately around the colored solution. After a second or two, the light scattering of the colored phase suddenly increases considerably. Soon the interface begins taking on a granular appearance, particularly in the more narrow swirls. A closer look at the boundaries revealed that small black bodies begin developing first at the outer edge of the interface and then farther into the colored phase. These bodies appear to be uniform in size and probably appear to be black because of their small size and light interference effects. The narrow swirls eventually become composed entirely of thousands of these small black bodies, and an examination of these regions between crossed Polaroids showed them to be either birefringent or optically active. Gentle shaking does not disrupt the swirls or strings of bodies and mixing yields a homogeneous solution in which the bodies do not coalesce. The solution is extremely cloudy and does not settle immediately.

In contrast, the initial swirls of AO formed in the PGA solution at pH 6.2 are more diffuse and no phase boundary is immediately evident. One soon develops, however, and large, orange-colored particles begin forming throughout the colored region. Gentle shaking easily disrupts the swirls and causes

the particles to coalesce into lumps of aggregated material. Shaking or vibration mixing merely disperses this material more finely. At pH 4.5, where the PGA is fully helical, no boundary ever develops around the AO swirls, which are even more diffuse. Again, gentle shaking of the slide causes aggregation of the complex into large lumps of material. These aggregation observations serve to underline the virtue of mixing the complex by vibration immediately after adding the dye and quickly making any optical measurements.

The small black bodies developed at pH 8 might be worth further investigation. It is likely these aggregates are responsible for the optical activity observed in the coil pH region. Spurious results were obtained in a few initial attempts to measure the optical activity of the very cloudy solutions, however, so it is not known whether these aggregates are highly optically active.

The preceding paragraphs were concerned with salt-free solutions of the coil complexes; in buffered solutions it was not possible to obtain reproducible ORD curves, even in the polyethylene beaker, in the coil pH range. Figure 35 shows typical examples of the wide range of ORD curves obtained. In citric-phosphate (C-P) buffer, ionic strength ca. 0.15, two kinds of ORD curves were observed that are illustrated by Curves 1 and 2. The fact that these two particular dispersions were recorded at different pH values is of no consequence. A positive, bell-shaped curve was always obtained

Figure 35. Some visible optical rotatory dispersions for coil complexes in buffered solutions

Total dye concentration is 4×10^{-5} M, R/D = 14 to 16.

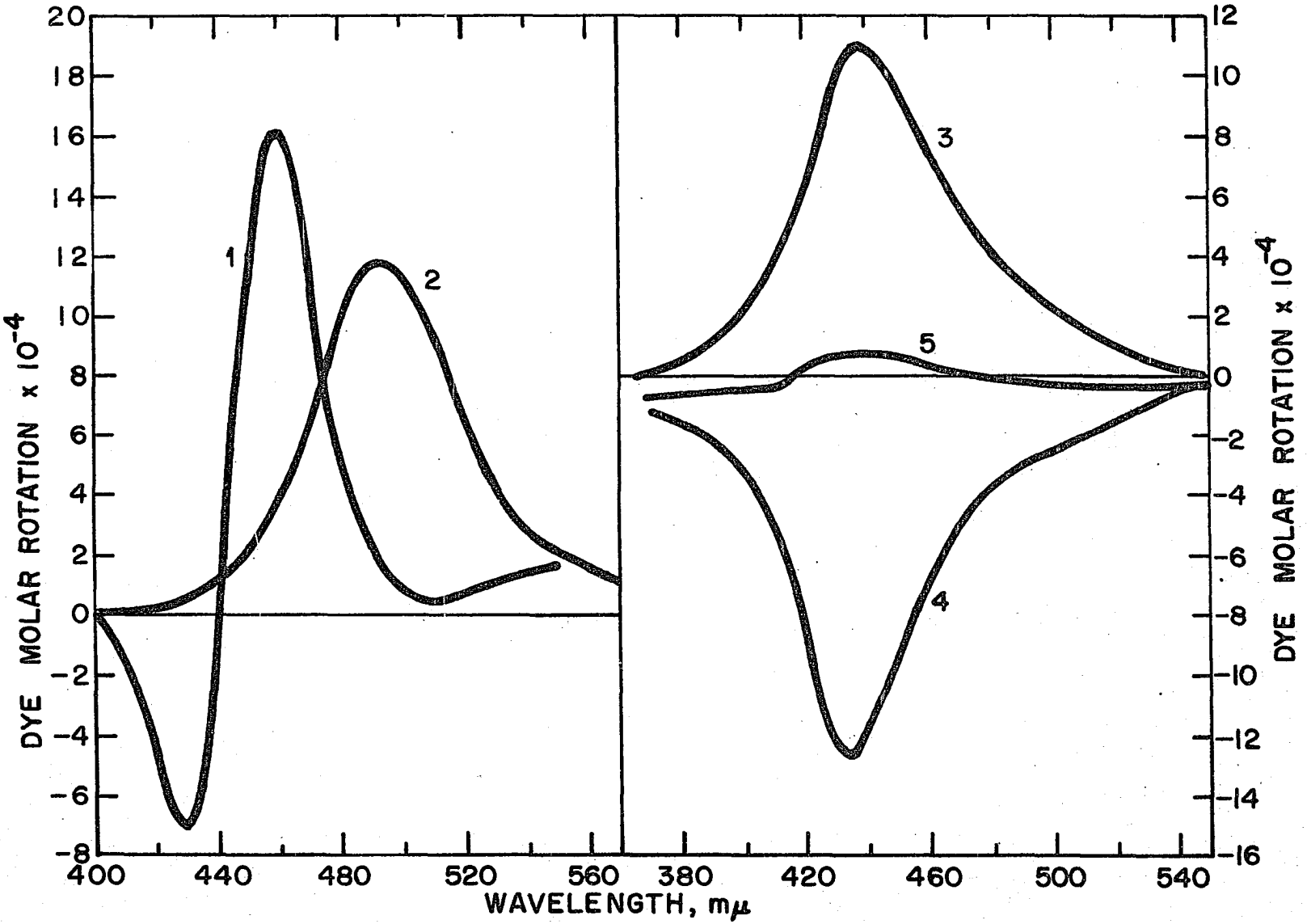
Curve 1: C-P buffer, pH 6.4

Curve 2: C-P buffer, pH 7.3

Curve 3: 0.05 M Tris buffer, pH 8.1, 3 min after PGA dilution

Curve 4: Another complex prepared as in Curve 3

Curve 5: Complexes 3 and 4 some 12 hours later


DYE MOLAR ROTATION x 10^-4
WAVELENGTH, mμ
1
2
3
4
5

throughout the coil pH region when these investigations were begun. The magnitude was variable and would either increase or decrease during the scan time period. Later in the study, the shape of the ORD suddenly switched to that shown by Curve 1, and bell-shaped curves could no longer be obtained. No known changes were made in the preparation method.

The rotatory behavior was more perplexing in 0.05 M Tris buffer, pH 8.1. Only bell-shaped ORD curves were observed that were either negative or positive in over ten successive trials of preparing the complex (Curves 3 and 4). The sign and size of the dispersion curves seemed completely random, not being influenced by PGA dilution time or the manner in which AO is added to the PGA. Of course, the addition of PGA to 4×10^{-5} M AO in either the Tris or C-P buffers produced optically inactive complexes. Also, the optical activity of any of the complexes in either buffer system usually disappeared within a day. Curve 5 shows a typical ORD obtained some 12 hours after preparation of either of the complexes characterized by Curves 3 and 4.

The strange, bell-shaped ORD curves cannot be constructed from theoretically-shaped, component dispersion curves. This suggests they might be artifacts arising from the action of birefringent aggregates on the plane-polarized light beam. However, the solutions could be stirred and the cuvette reversed in the sample holder without causing any changes in the observed rotation. The shape of these ORD curves and the

apparent randomness of their sign remain unexplained. A shift in the instrumental base line was not responsible for the lack of small peaks or troughs on either side of the bell-shaped ORD.

Because of the increased polymer flexibility in buffered solutions, the induced optical activity was not expected. The possible existence of local regions of rigid structure to which asymmetric dimers could be held more or less rigidly in place does not seem likely from the large A_{490} and F_{535} values observed. And larger regions of asymmetric structure on which the dyes could be bound farther apart are rendered very unlikely by the almost complete depolarization of fluorescence observed in 0.1 M NaCl solutions (Figure 15). Yet, optically active complexes are formed in C-P buffer at an ionic strength near 0.15. Thus, the development of optical activity is not understood, unless a small percentage (such as 10%) of the dyes are involved in aggregate formation in which they become extremely optically active. In this case, the molar rotation of the dye molecules involved would be on the order of one million.

The ultraviolet rotatory dispersion curves for the coil and helix complexes in water solution are shown in Figure 36. These curves are continuations of the ones recorded at pH 8.0 and pH 4.5 in Figure 32. Similar Cotton effects seem to occur in both dispersion curves if the coil complex curve is considered to be shifted some 15-20 mμ toward shorter wave-

Figure 36. The UV optical rotatory dispersions of the coil and helix complexes in water solution

Total dye concentration is 4×10^{-5} M, R/D = 16.4.

(---): Coil complex, pH 8.0

(——): Helix complex, pH 4.5

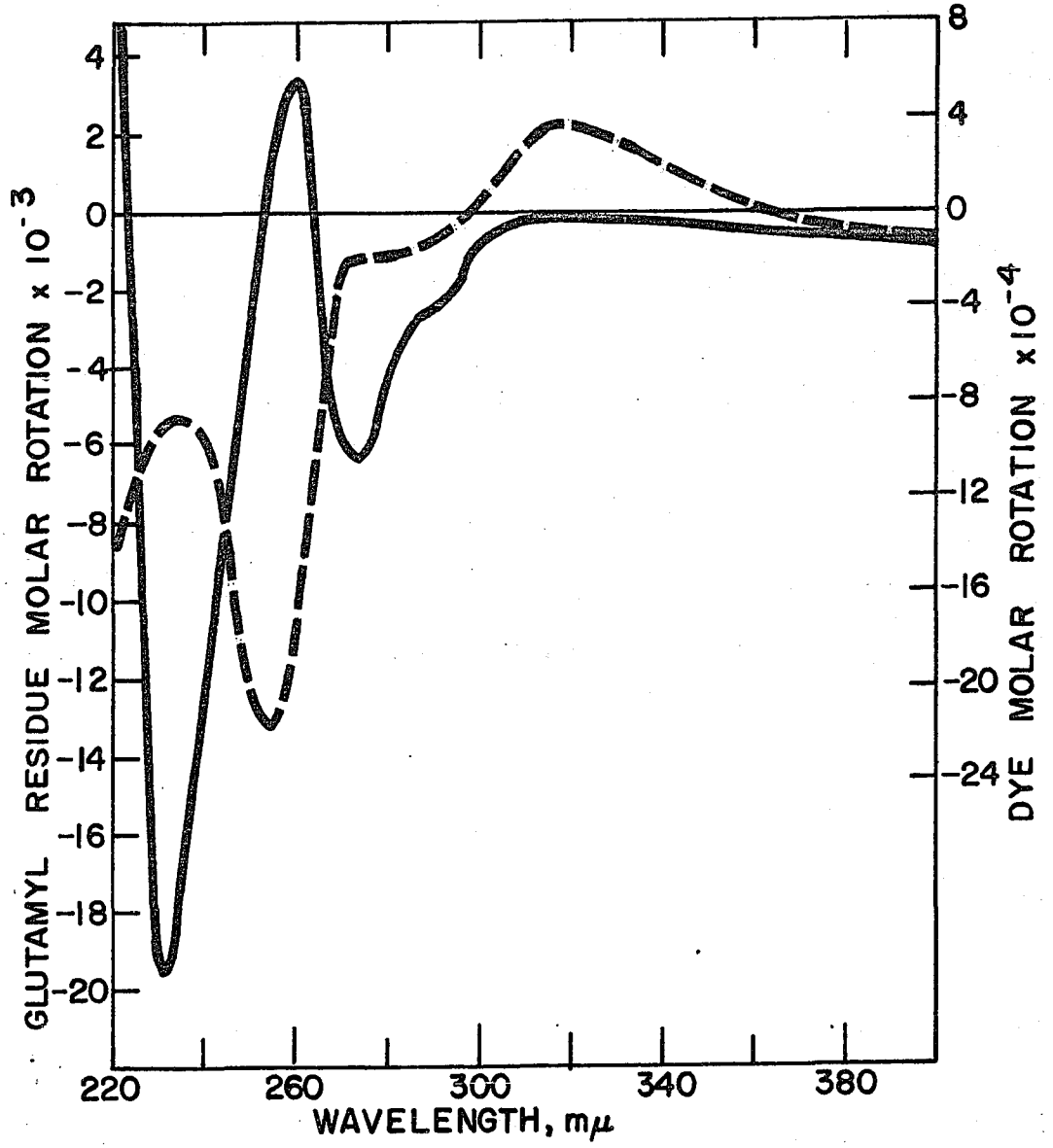
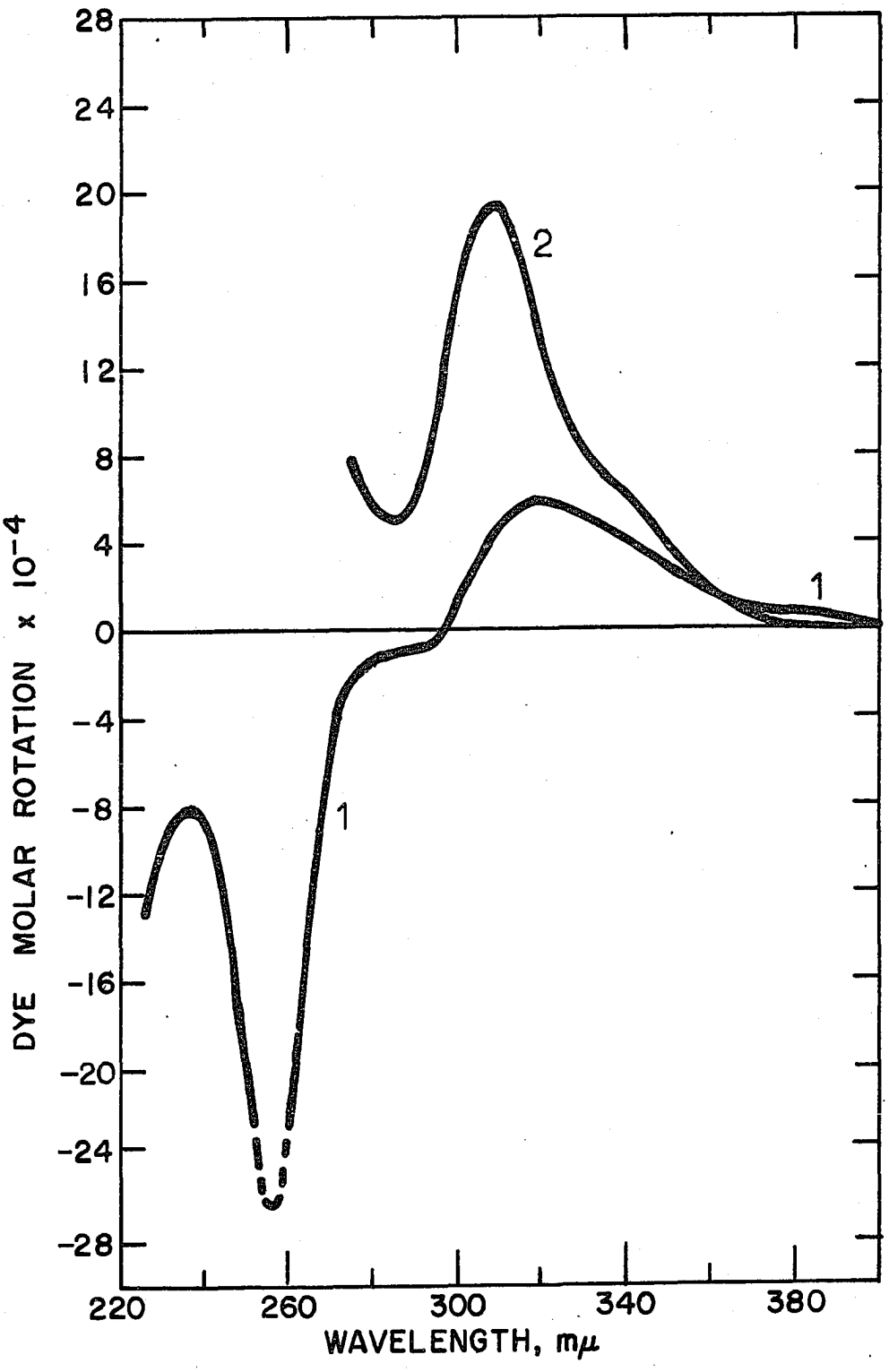

GLUTAMYL RESIDUE MOLAR ROTATION x 10⁻³
DYE MOLAR ROTATION x 10⁻⁴
WAVELENGTH, mμ
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
8
4
0
-4
-8
-12
-16
-20
-24
220
260
300
340
380

Figure 37. The UV optical rotatory dispersions of the coil complex in C-P buffer

Total dye concentration is 4×10^{-5} M, R/D $\cong$ 15.

Curve 1: Obtained with the "normal" visible ORD (Figure 34, Curve 1), pH 6.4; the dashed portion is estimated.

Curve 2: Obtained with the bell-shaped visible ORD (Figure 34, Curve 2), pH 7.3


28
24
20
16
12
8
4
0
-4
-8
-12
-16
-20
-24
-28
DYE MOLAR ROTATION x 10^{-4}
220
260
300
340
380
WAVELENGTH, mμ
1
2
1

lengths than for the helix complex case. Thus, the trough at 255 mμ corresponds to the trough observed at 274 mμ for the helix complex, and this negative Cotton effect is preceded by a smaller, positive, and more broad one in the 300 mμ region. However, the positive peak of the negative Cotton effect for the coil complex is missing, which suggests the presence of a very large negative Cotton effect below 220 mμ. The rotation at 220 mμ is, in fact, starting to go strongly negative. Whether this proposed Cotton effect is associated with the amide or the dye chromophores is unknown. Its trough is certainly much larger than the 233 mμ trough associated with the α-helical arrangement of the peptide groups. If this Cotton effect could be measured and definitely assigned to an amide electronic transition, then strong evidence would be obtained for the induction of a regular structure in the polypeptide coil by the act of binding dye. The coil conformation of PGA exhibits a trough near 204 mμ, but its magnitude is about the same as the α-helical trough at 233 mμ (Blout et al., 1962; Holzwarth et al., 1962).

In Figure 37 are shown the ultraviolet portions of the two types of ORD curves obtained for the coil complexes in the C-P buffer solutions (see Figure 35). Curve 1 corresponds to the "normal" visible ORD curve and is very similar to the trace obtained in water at pH 8 (Figure 36). But the complex characterized by the positive, bell-shaped visible ORD also usually exhibits a substantially different ORD in the

ultraviolet region (Curve 2). This is shown better, perhaps, by Curve 1 in Figure 38, where the results in 0.05 M Tris buffer, pH 7.6, are given. These are the two types of ultraviolet dispersions most often obtained for the coil complexes. Although the helix complex dispersion was nearly repeatable from time to time, some evidence was obtained with the coil complexes that the Cotton effects in the two spectral regions can be independent of each other. Such might be the case, for instance, if the two regions are associated with two different electronic transitions polarized at right angles to each other. It is commonly assumed and was supposedly shown by molecular orbital calculations (Ballard et al., 1966) that the electronic transition in the visible region (α, β, and γ bands) is polarized along the long axis of the dye, while the 295 mμ band, among other UV bands, is short-axis polarized. If the stacking geometry of bound dimers changes in such a manner that the relative angle between one of the pairs of axes is changed more than the other set (by rotation of one dye about one of its axes), then one should observe different Cotton effects in one of the spectral regions not accompanied by significant changes in the other region.

Thus, while different preparation methods caused a change in the sign of rotation at 458 mμ, no sign changes were noted in the peaks and troughs below 350 mμ. Only the magnitudes decreased for the unstable complexes. Occasionally, almost no UV Cotton effects accompanied well-developed,

positive, bell-shaped curves in the visible region. Often the UV dispersion curves of different coil complexes were similar in shape but shifted relative to the baseline, or their magnitudes varied widely for a given magnitude in the visible region. Very little can be said, however, about the differences in behavior in the UV region for the complexes characterized by positive or negative bell-shaped curves. Only one ultraviolet curve was recorded for a complex exhibiting a negative, bell-shaped ORD in the visible region; this particular curve displayed only a small trough near 255 mμ and a negative, rapidly increasing end rotation at 220 mμ. In contrast to the other complexes, no positive rotation was observed. These results suggest that the dye planes in bound dimers are not parallel and that both spectral regions might be studied more carefully to provide information on the relative orientations of the two sets of axes.

The effect of time on the coil complex in 0.05 M Tris buffer, pH 7.6, is shown in Figure 38 to illustrate the interesting way in which the UV Cotton effects often disappear. About 5 hours after preparation, the visible portion has nearly disappeared, while the main change in the UV region is a shift to more negative rotation values. After 4 days, the UV Cotton effects have almost disappeared, but the rotation lies somewhat more negative than that for PGA alone, and a rapidly increasing rotation is clearly evident

Figure 38. The UV optical rotatory dispersion of a coil complex in 0.05 M Tris buffer, pH 7.6, as a function of time

Total dye concentration is 4×10^{-5} M, R/D ≅ 16.

Curve 1: Immediately after preparation

Curve 2: 5 hours later

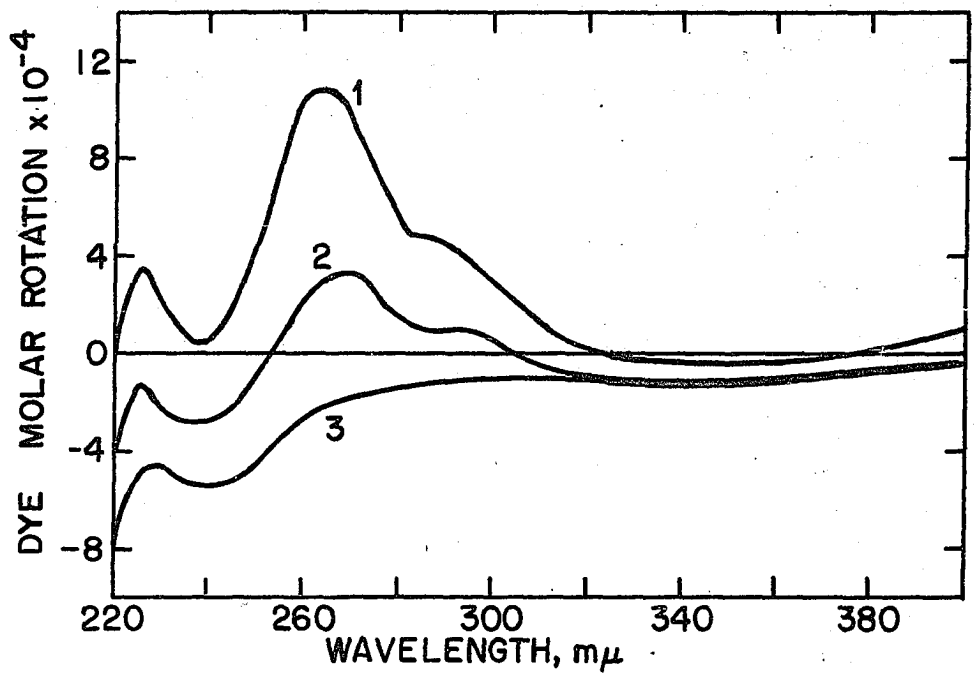
Curve 3: 4 days later

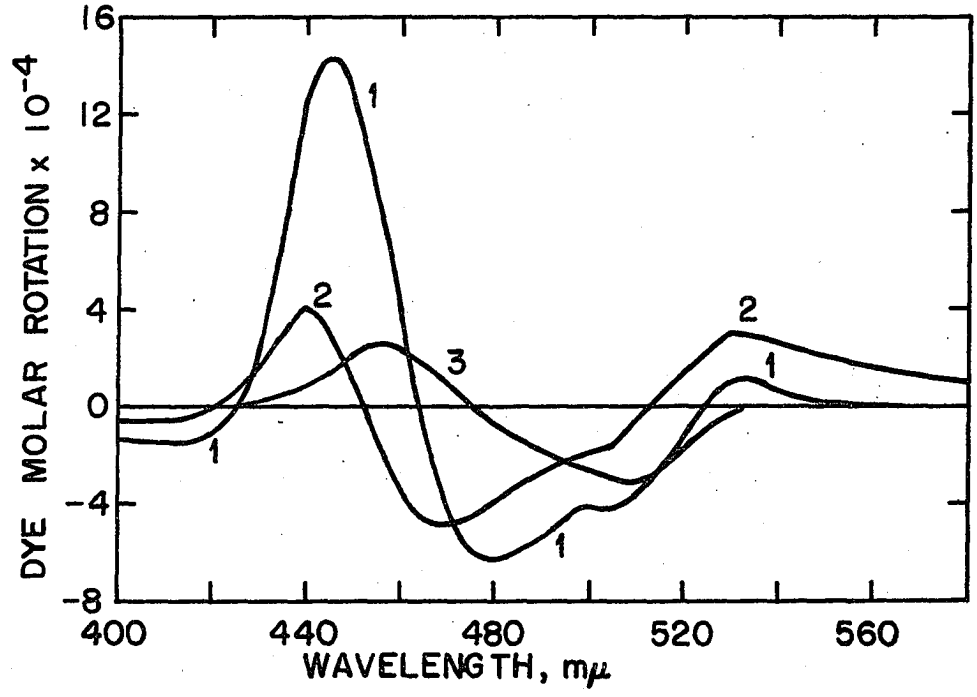
Figure 39. The visible optical rotatory dispersions of the helix complex in several solvents

Curve 1: Water, pH 4.5, R/D ≅ 16, total $[AO] = 4 \times 10^{-5}$ M

Curve 2: C-P buffer, pH 4.5, R/D ≅ 19, total $[AO] = 2 \times 10^{-5}$ M

Curve 3: 0.2 M $MgCl_2$, pH 3.8, R/D ≅ 16, total $[AO] = 4 \times 10^{-5}$ M


DYE MOLAR ROTATION x 10⁻⁴
12
8
4
0
-4
-8
1
2
3
220
260
300
340
380
WAVELENGTH, mμ


DYE MOLAR ROTATION x 10⁻⁴
16
12
8
4
0
-4
-8
1
2
3
400
440
480
520
560
WAVELENGTH, mμ

at 220 mμ. This behavior again suggests the presence of intense Cotton effects below 220 mμ that might be assigned to either the amide or dye chromophores. Interactions between bound dyes may gradually cause the PGA coil to fold into a more ordered structure.

Optical activity behavior of helix complexes

Turning now to a brief description of the visible optical activity of the α-helical complexes, it was noted that more than one type of optically active dimer seems to exist. The relative proportions of the different dimeric structures is dependent upon the ionic strength and the degree of protonation. This conclusion is suggested by the change in shape of the ORD and CD curves upon changing either one of these solution parameters. For comparison purposes, three dispersion curves obtained at three different ionic strengths in unrelated experiments have been collected in Figure 39. The curve in water solution (Curve 1) is the largest and is composed of at least three Cotton effects, the approximate positions being 520 mμ, 465 mμ, and 430 mμ. Upon going to the C-P buffer solution, $\mu \cong 0.1$, the negative Cotton effect near 465 mμ is reduced considerably while the positive one near 520 mμ is enhanced. At an even higher ionic strength of 0.6 in the 0.2 M $MgCl_2$ solution, the dispersion seems to be reduced to a single negative Cotton effect at 480 mμ. In this experiment the pH was set at 3.8, where the minimum in A_{490} occurs as a function of pH; A_{490} at this pH is almost as

low as A_{490} in 0.2 M NaCl.

It will be recalled that all of the dye is bound to the PGA at the R/D values given in Figure 39. Furthermore, the α-helical conformation of the PGA polymer is not disturbed by the change in ionic strength. Therefore, the differences in the ORD curves must be due to differences in the distribution or stacking geometry of the bound dye. Since (1) the α-helical PGA has a very large stacking coefficient for AO, (2) the early plateaus in R/D-titrations are absolutely flat, and (3) most of the γ-carboxylates are protonated, it can be reasonably inferred that the dye is bound almost entirely as dimers at R/D ratios near 20. Hence, the addition of electrolytes must either cause a shift in the structure of the dimer or a different distribution of dimers among several different sites. The latter alternative seems to be the better choice at present, since it is not clear how the geometry of the dimer could be changed by the presence of small ions. Instead, it is known from model building that a number of possible binding sites for dimers are presented by the α-helical conformation. By stacking two dye molecules in van der Waals contact, the dimer can be placed on two γ-carboxyl groups or between two such suitably spaced groups in a variety of ways. It is also obvious that both left-handed and right-handed sites exist. But it is not so obvious which modes of binding are energetically or kinetically favored, nor is it known whether or not the dimethylamino groups form bonds (hydrogen

or ionic) with two other properly spaced γ-carboxyl groups. However, the affinity of dye for the various sites undoubtedly varies to some extent, and the competitive binding of inorganic cations to the various sites may disturb the distribution of dimers existing in water solutions of the complex.

The effect of different degrees of protonation on the optical activity of the helix complex is shown by the CD spectra in Figure 40. These spectra were obtained in C-P buffer solutions at ionic strengths near 0.1. Only very small Cotton effects could be induced in the pH range just preceding the coil-to-helix transition region, but in the transition region three distinct CD bands, alternating in sign, appear and begin increasing in magnitude with the degree of helical content. Thus, the bands in the CD spectrum for the fully helical complex at pH 4.5 (Curve 2) are larger than those obtained at pH 5.0 (Curve 1), where the helical content is about 70%. A fourth band, positive in sign, also appears near 490 mμ for the fully helical complex at pH 4.5. This new band apparently disappears, however, as the pH is lowered to 3.94 (Curve 3). Furthermore, the two bands located near 430-440 mμ and 460-470 mμ continue to increase in magnitude with the increase in degree of protonation, the changes being as large as those accompanying the coil-to-helix conformational transition. Below pH 3.94, the Cotton effects rapidly decrease in size. These CD changes in the pH region where the PGA is fully helical again suggest

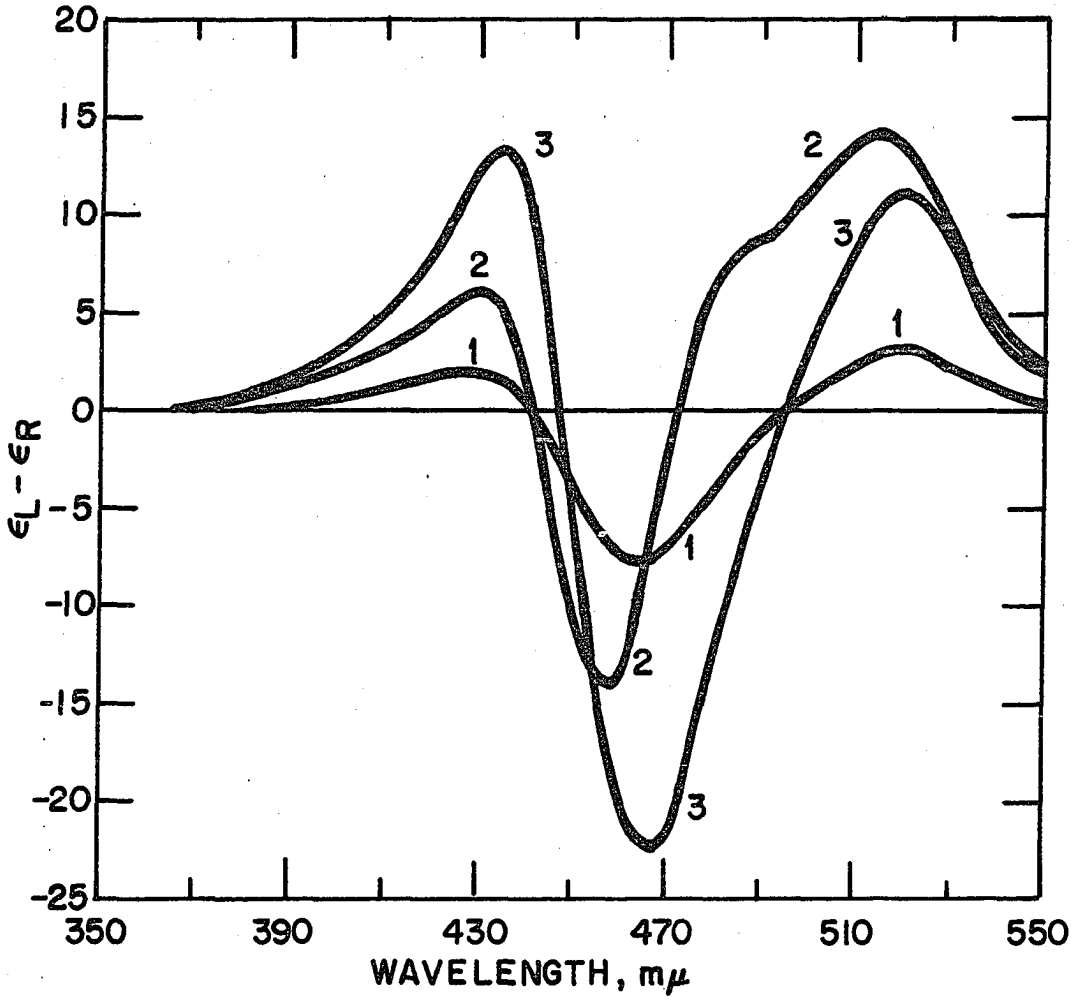


Figure 40. The visible circular dichroism spectra of helix complexes in C-P buffer as a function of pH

Total dye concentration is 4×10^{-5} M, R/D = 15.

Curve 1: pH 5.0 (in the helix-coil transition region)

Curve 2: pH 4.5

Curve 3: pH 3.94

the presence of several dimer sites, each characterized by a somewhat different asymmetric arrangement of the dye molecules. The relative proportions of the several dimer sites are apparently altered by unequal affinities for protons.

Yamaoka and Resnik (1966) showed from an analysis of the ORD curves of several helix complexes at high R/D ratios in water solution that at least four partial Cotton effects are required to reconstruct the experimental ORD curves. The CD data in Figure 40 also show that at least four Cotton effects can be present, but that the number may be dependent upon the pH and ionic strength. That the size, position, and sign of some of the CD bands may also be markedly influenced by R/D ratios beyond that required for complete binding of the dye was clearly shown by the work of Yamaoka and Resnik. For example, the magnitude of the negative CD band near 464 mμ increased from $\Delta\epsilon = -94.4$ to $\Delta\epsilon = -115.8$ as R/D was increased from 250 to 500. Similarly, early CD measurements in this laboratory on helix complexes at R/D ≅ 4000 in 0.1 M acetate buffer, pH 4.5, revealed a negative band at 468 mμ with a magnitude of about -40 for $\Delta\epsilon$. In comparison, the size of the corresponding CD band in Figure 40 is only on the order of $\Delta\epsilon \cong -25$ at R/D ≅ 15 in C-P buffer, pH 4.5. Why the CD bands should do anything but decrease in magnitude with increasing R/D (beyond the point needed for complete binding) is not understood.

Perhaps one of the variables influencing the optical

activity behavior of the helix complexes is the manner in which the dye and PGA molecules are brought into contact with each other, particularly in the preparation of complexes at large R/D ratios. This factor has not been considered by other investigators, yet the experience gained in this laboratory with the coil complexes and data like that presented in Figure 41 suggest this variable may be very important. The possibility that the R/D-induced changes reported by Yamaoka and Resnik (1966) may be due in large part to "mixing artifacts" will be discussed after presenting the results shown in Figure 41.

Rather than titrating a PGA solution to a desired pH before adding AO, a coil complex may be prepared near pH 8 and then titrated with 0.1 N HCl. When this was done, it was learned that complexes prepared by changing the PGA conformation in the presence of AO are not identical to those prepared by first changing the PGA conformation and then adding dye. An optically active complex prepared at pH 8.5 in water solution does not, for instance, lose its optical activity in the pH region preceding the coil-to-helix transition. Instead, the Cotton effects shift continuously in position and size as the complex is titrated through this pretransition pH region. If aggregate formation at pH 8.5 is responsible for the induced Cotton effects, as seems very likely, then this optical rotatory behavior shows that the structure of these aggregates may be changed somewhat by lowering the pH, but

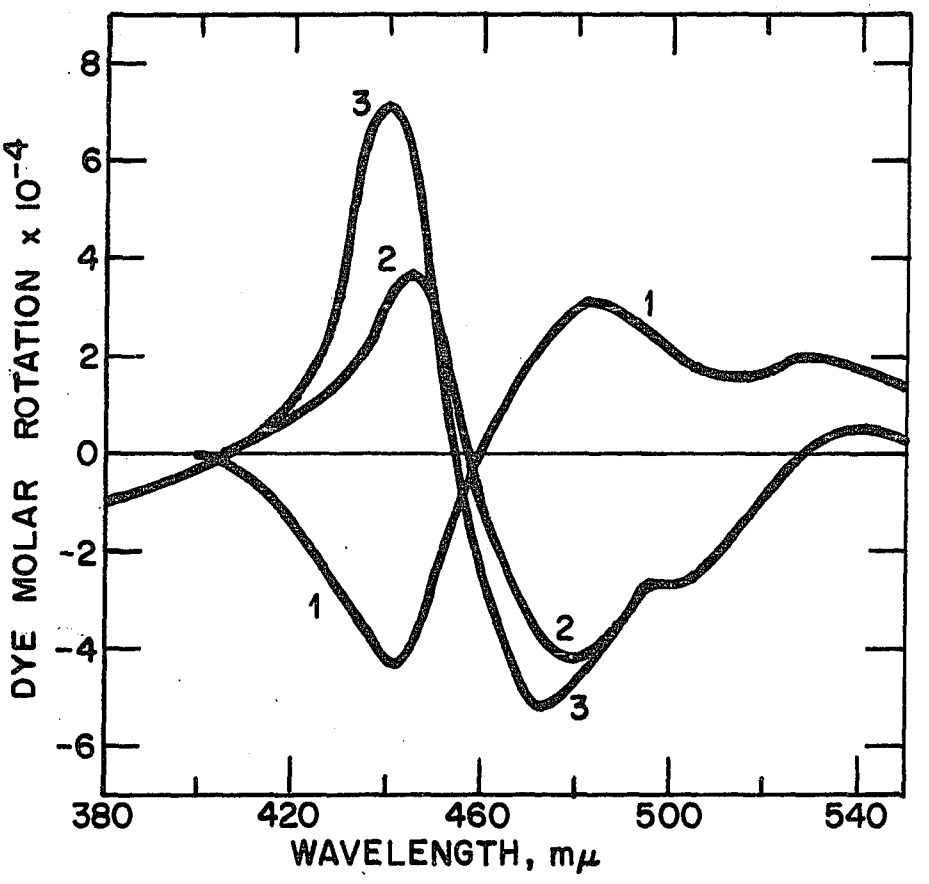


Figure 41. Typical optical rotatory dispersions resulting from the titration of a coil complex in water solution

Total dye concentration is 4 x 10^{-5} M, R/D = 17.

Curve 1: pH 6.8

Curve 2: pH 4.6

Curve 3: After heating the complex at pH 4.6 to 90°C and cooling to room temperature

they are not destroyed. Hence, it seems probable that these aggregates still exist at pH 4.5, where their presence is obscured by the optical activity of dye bound to single, helical PGA molecules. In this connection, it is most interesting to note the ORD curves presented in Figure 41.

Curve 1 shows a typical ORD curve obtained at pH 6.8 after titrating an optically active complex from pH 8.5. The resultant Cotton effects are much larger than those obtained by adding AO to pretitrated PGA (see Figure 32, Curve 2). Upon continuing the titration to pH 4.6, Curve 2 was obtained. These two dispersion curves are only typical of the results obtained, since the magnitudes and shapes of the curves varied somewhat from trial to trial. However, the ORD curves at these two pH values were usually almost exactly opposed in sign and the magnitude of the helix complex Cotton effects were much smaller than those obtained when AO was added to the pretitrated PGA solution (see Figure 32, Curve 4). In fact, the positive peak near 445 mμ was consistently about one-third the size of the peak shown in Figure 32. If, as suggested earlier, aggregates formed in the mixing process at pH 8.5 are still present at pH 4.6 and these aggregates are characterized by an ORD curve similar to Curve 1, then an explanation can readily be given for the small size of the Cotton effects in Curve 2. The optical rotatory behavior of the aggregates partially cancels the optical activity exhibited by the helix complex species at most of the wave-

lengths in the visible spectrum.

A way of testing for the presence of aggregates at pH 4.5 was suggested by the effect of heating on the optical activity of the coil complexes. Since most of the aggregates are apparently destroyed by heat, the optical activity of the helix complex solutions containing aggregates should increase after heating to 90°C. It was discovered that this heat treatment had only a slight effect on the helix complex prepared at pH 4.5 by the addition of AO to the pretitrated PGA; repeated heat treatments reduced the Cotton effect magnitudes slightly each time, and there were no changes in the shape of the ORD curve. But the heating of helix complex solutions prepared by titrating the coil complexes always caused a significant increase in the optical activity, as shown by Curve 3 in Figure 41. This increase was not as large as expected, and only rarely did the positive peak near 445 mμ attain a height as much as 65% of the peak height shown in Figure 32. Repeated heat treatments only caused the Cotton effects to decrease in small, successive increments. Although it might simply appear as if the aggregates are not completely labile to this heat treatment, other data suggests that aggregates may not even be involved.

In the process of heating the helix complex solutions to 90°C, most of the α-helical content is destroyed. Whether or not the dye is also completely dissociated from the PGA at this temperature is unknown. The absorption spectrum of the

complex reverts to a shape characteristic of free dye, but this does not provide an unequivocal answer to the question of binding. If the complex is, in fact, completely dissociated at 90°C, then the fact that the Cotton effects in Curve 3 of Figure 41 are smaller than those obtained for the heated, pretitrated helix complex would establish the presence of some optically active, heat resistant aggregate. But it cannot be certain that the two methods of preparation do not simply lead to two different kinds of helix complexes, one of which is slightly more stable at elevated temperatures. Most of the dye may remain bound at 90°C and the structure of the asymmetric dimers need not be altered by the conformational change. Optical rotation measurements as a function of temperature would be helpful in deciding if any differences in stability exist between the optically active dimers in the two helix complexes.

The possibility that both optically active helix complexes remain essentially intact at 90°C does not seem very likely, yet this alternative is suggested by experiments with heat-treated coil complexes. The aggregates formed in the preparation of the optically active coil complex at pH 8.5 appear to be destroyed by heat. Why these aggregates should be labile to heat at pH 8.5 and not at pH 4.6, however, is not understood. When the heat-treated, optically inactive coil complexes are titrated to pH 4.6, ORD curves similar in shape and magnitude to Curve 2 in Figure 41 are obtained. In

addition, heat treatment of these helix complexes yields the same result described earlier—a small increase in magnitude to the level shown by Curve 3. If the aggregates were destroyed at pH 8.5, this result suggests the helix complex formed by titration is somewhat different from the one formed with pretitrated PGA and that both optically active helix complexes are relatively stable to the heat treatment. But the possibility that optically active aggregates are reformed during the titration of the coil complex cannot be excluded. Obviously, much work remains to be done before the structural differences between the two helix complex solutions are fully understood.

In spite of the fact that the foregoing results cannot be satisfactorily explained, they have important implications for any proposed study of the optical activity of helix complex solutions. Both helix complex solutions discussed above are quite stable at room temperature, which means there is not a rapid interconversion between complex species formed during the preparation process to produce some equilibrium state. Hence, several different complex species or aggregates may be present in solution, depending upon the particular method of preparation, and the presence of these different species may easily go undetected. If care is not taken to always employ the same mixing technique, a far different dependence of the optical activity upon some solution parameter might be obtained than if the preparation method had been carefully

controlled. Even changes in the solution conditions may result in a different mode of contact between the PGA and AO solutions. It is suggested this may be particularly true in studies of the R/D-dependency of the induced optical activity.

At R/D ratios near 15 to 20, the particular manner in which the 10^{-3} M AO stock is added to the dilute PGA solution in water, pH 4.5, has little influence on the absorbance and optical rotatory properties of the complex. The dye may be added by the Teflon mixer or by microburet in short spurts or in a continuous stream, with or without continuous stirring; the optical behavior is hardly affected. But as the PGA concentration is increased in order to obtain optical rotation measurements at large R/D ratios, the exact manner in which the dye and PGA solutions are mixed could well become increasingly important. The only experimentally feasible way to prepare complexes at high R/D is to add a small volume of concentrated (10^{-3} M) dye solution to a much larger volume of viscous PGA solution. Under these conditions, hardly any diffusion of the dye solution takes place; the dye molecules are immediately and completely bound in a small region of the PGA solution as soon as they leave the microburet. Many PGA molecules must initially become completely saturated with dye before the dye redistributes to polymers with no attached dye. Furthermore, the formation of optically active aggregates may be enhanced in small, local regions of high PGA and AO concentrations. It seems clear that the mixing mode in these

solutions is much different from that occurring in the preparation of complexes at low R/D, which raises the question of whether or not it is strictly correct to compare the optical activity behavior of low R/D and high R/D complex solutions prepared in the same manner.

Thus, the increase in the size of the Cotton effects upon going from low R/D to R/D = 500 (Yamaoka and Resnik, 1966) may be largely an artifact brought about by the relative concentrations and volumes of the solutions being mixed. What is needed to make more valid comparisons of optical activity over a wide range of R/D values is a method of mixing in which the dye is distributed evenly throughout the PGA solution volume before becoming bound to the PGA. This, of course, is an ideal mixing mode that cannot be achieved, but perhaps a close approach can be made by performing some operation on the complex solution that first releases the bound dye and then allows it to recombine with the PGA. Heating to nearly 100°C for a certain time period might be sufficient. It would be interesting, for example, to note the effect of a heat treatment on the large Cotton effects observed by Yamaoka and Resnik at R/D = 500.

That the method of mixing may strongly influence the optical activity behavior of helix complex solutions even at very low R/D ratios was indicated by two experiments performed several years apart in this laboratory. In some early work the visible CD spectra of a series of helix complexes in

0.1 M acetate buffer, pH 4.6, were recorded. These complexes were prepared by mixing varying proportions of 6×10^{-5} M solutions of AO and PGA (residue concentration) in acetate buffer. The reagents were delivered from 10 ml burets directly into a cuvette and were mixed by vibration. Later, when the magnitudes of the large negative CD bands were converted to a molar basis (using the total AO concentration) and plotted as a function of R/D, a break in the vicinity of R/D = 0.5 was obtained. The maximum level was achieved at R/D = 3 ($\Delta\epsilon$ = -42) and remained constant up to R/D = 8, the largest ratio examined. Because the absorbance and fluorescence R/D titrations performed later showed the break should occur in the R/D range 4.0 to 4.8, another CD titration was performed. This time, however, the standard technique used in the R/D titrations of Figures 7 and 9 was employed, except the titration was performed in 0.05 M acetate buffer, pH 4.5. The break in the circular dichroism at 454 mμ now occurred at R/D $\simeq$ 4.7 and the value of $\Delta\epsilon$ in the plateau region was -16. Since both of these CD titrations were only single, exploratory experiments, this apparent dependence of the CD behavior of helix complex solutions on the preparation methods given should be studied more thoroughly.

Finally, it should be stressed that visible aggregation does occur in the helix complex solutions and that the effect of this aggregation on the optical activity behavior of the solutions has not been properly considered. Yamaoka and

Resnik (1966) reported that precipitation occurred in the preparation of their helix complexes at high R/D ratios, and the solutions had to be filtered just prior to making ORD measurements. Stryer and Blout (1961) also reported aggregation in their earlier work. In this laboratory aggregation has been dramatically observed in the microscopy experiments mentioned and also noted in solutions at low R/D ratios. In fact, the use of the ultracentrifugal method for determining the free dye concentration depends upon the aggregative nature of the helix complex. Some evidence was also obtained that suggests aggregation may influence the observed CD spectra. In the early phase of this study, the visible CD spectra of helix complex solutions in 0.1 M acetate buffer, pH 4.5, were occasionally examined after a brief centrifugation. Usually the R/D ratio was in the range 200 to 400. Several different results were noted, such as a decrease in magnitude of all the CD bands or a reduction in height of the two positive bands by one-half while the negative band height was unaffected. In another trial the negative CD band was selectively removed. Thus, the removal of aggregated material may affect the shape of the CD spectrum, and a preparation method should be employed that, at the very least, yields finely dispersed material.

Because PGA itself tends to aggregate at pH 4.5 and below, especially at higher concentrations (Schuster, 1965; Tomimatsu et al., 1966), the formation of aggregates in the

helix complex solutions is probably unavoidable. But the extent of aggregation can probably be reduced by not adding 10^{-3} M AO directly to the PGA solution from a microburet. Mixing methods in which small regions of high AO and PGA concentrations are produced should be avoided as much as possible. For instance, the use of a mixing apparatus like the Teflon mixer described earlier is much more desirable than adding the AO from a microburet. On the other hand, the chosen method of mixing should not involve a long time period, because considerable aggregation between initially dispersed complex molecules will occur. Fortunately, sufficiently stable helix complex solutions can be prepared in spite of the aggregation problems. ORD and CD scans can usually be exactly retraced, and reproducible optical behavior can be obtained with the standard mixing technique used in this study.

SUMMARY

1. The absorption, fluorescence, and optical activity behavior of acridine orange (AO) bound to poly-L-glutamic acid (PGA) have been described as functions of the mole ratio of the two components (R/D), pH, and NaCl concentration. This spectral data has been interpreted in terms of structures for the AO:PGA complex and changes in the conformation of the polypeptide.

2. The AO:PGA system was discovered to be very complicated, being characterized by aggregation and an unknown number of metastable states. The optical behavior (and, therefore, the structure) of the complex often depends upon the preparation method. Problems with adsorption losses and precipitation were discussed, and a standard, convenient technique of complex preparation was presented. In order to obtain reproducible spectral results, the majority of the measurements were made on the nonequilibrium state produced immediately after the complexes were prepared.

3. Advantage was taken of the aggregative nature of the helix complexes in determining the free dye concentration by an ultracentrifugal method. A Klotz plot of the data indicated the binding sites could be considered as being identical and noninteracting under the conditions employed in citric-phosphate buffer, $\mu \cong 0.1$. Also, an association constant for the binding was obtained and an estimate of the

average number of binding sites per glutamyl residue was provided and later confirmed by optical titrations.

4. Absorption and fluorescence titrations in which the total dye concentration was held constant while the concentration of PGA was increased yielded sharp breaks at R/D ratios consistent with the notion that one dye molecule may bind to each γ-carboxylate group present in both the coil and α-helical conformations in water solution. Determinations of the degree of ionization of PGA in the presence of AO at R/D = 4 supported this interpretation.

5. The return of the fluorescence intensity at 535 mμ and the absorption at 490 mμ (near the α band position) in the presence of a very large excess of binding sites showed the AO:PGA system to belong to the chromotrope:metachromatic dye class of dye-polymer complexes. Most of the spectral changes are due to interactions between adjacently bound dye molecules and are very similar to those occurring upon increasing the concentration of an AO solution. Besides the usual metachromasy phenomenon, the existence of a transition above 527 mμ (above the α band position) was clearly established and found to be enhanced by dye aggregation on the PGA surface.

6. The stacking tendency of acridine orange on PGA was found to be very large and essentially equal for both the α-helical and extended coil conformations in water solution. The free energy of the dye-dye interaction is about -4.4 kcal/mole of

dye-pairs. Thus, essentially no dye molecules will be bound monomerically (isolated from other bound dyes) on either conformational form of PGA in water solution, unless the number of binding sites is in great excess over the number of dye molecules.

7. The spectral behavior in the mole ratio region below R/D = 50 in water solution suggests the stacking hardly proceeds beyond the dimer stage for the helix complex at pH 4.5, while larger stacks are formed on the extended coil. This result is in harmony with the much higher degree of protonation of the helix complex. The maximum degree of metachromasy (and probably the longest stack lengths) occurs in water solution when the α-helical content is about 30%; at R/D = 18, this occurs at pH 6.2, where the absorption maximum has shifted to 449 mμ.

8. The effect of NaCl on the absorption and fluorescence of the helix complex is small and can be attributed to a weak competition of sodium ions for the binding sites. However, the addition of NaCl to solutions of the coil complex seems to change the very nature of the complex structure. Most of the metachromasy is destroyed in 0.1 M NaCl solution, yet the dye remains completely bound and the average number of sites per glutamyl residue increases to about two. It was suggested that the quenching of fluorescence and the development of metachromasy depends upon a restriction in the relative motions of the γ-carboxylate groups. A sufficient restriction

is provided by the α-helical structure at low pH and by electrostatic repulsion at high pH in water solution. But the addition of NaCl to solutions of the coil form of PGA allows the conformation to change from an extended structure to a more randomly coiled one. This increase in flexibility destroys the effectiveness of PGA as a stacking template and appears to result in dimer formation about separate carboxylate groups (counterion model) rather than between dye molecules bound to adjacent carboxylates held fairly rigidly in position (dimer site model).

9. Depolarization of fluorescence studies showed that a significant restraint to bound dye rotation is present in the extended coil complex and that this restraint is destroyed by the addition of salt. Because the polarization remains essentially constant in water solution as the pH is lowered through the coil-to-helix transition region, it was suggested the extended coil might exist as a twisted structure that simply tightens and shortens into an α-helix by a continuous process as the carboxylates are protonated. The absorption and fluorescence behavior of the complex as a function of pH in water solution are consistent with this suggestion and indicate, at least, that no significant change in the nature of the binding sites occurs as the pH is changed.

10. Bound dye does not prevent or interfere with the coil-to-helix transition; instead, the transition in water solution occurred at a higher degree of ionization at R/D = 4 and at

the same position as PGA alone when R/D = 18. Several possible reasons for the displacement of the transition region by one pH unit at R/D = 4 were discussed. The sharpness and reversibility of the transition were not affected by the bound dye, indicating that no strong bonds are formed between the dye and the polypeptide backbone.

11. A large increase in metachromasy and decrease in fluorescence intensity occurs in 0.1 M NaCl solution when the pH is lowered from the random coil region to the α-helix region. However, no simple relationship exists between either optical property and the conformational transition region. Large drops in the α band absorption and the fluorescence intensity occurred before the helical content reached 4 to 5%, suggesting that the mobility of some of the coiled segments of the chain is restricted, perhaps by an increase in compactness, in the early stages of the coil-to-helix transition. Thus, it was suggested that sites are created in the coiled segments of the chain that are similar to those existing on the extended coil in water solution.

12. A new, weak fluorescence band at 590 mμ (apparent position) was discovered and demonstrated to be the result of complex formation and very likely the result of interactions between bound dye molecules. This is the first report of fluorescence metachromasy in a solution of a dye:chromotrope complex, although this phenomenon has long been observed in concentrated dye solutions and in the in vitro staining of

certain biopolymers. The behavior of the 590 mμ band as functions of R/D and pH in water solution and studies of its lability to heat and time suggest this new band is a property of bound dimers that is suppressed by larger stack formation.

13. The effect of time and heat on the fluorescence spectra of complexes in water solution clearly established the importance of kinetically controlled processes in determining the precise structure of the complex. The high lability of the 590 mμ fluorescence band of the coil complexes at low R/D in water solution suggests that a fast process of dimer formation is followed by a slower redistribution to larger stacks of interacting dyes.

14. In contrast to the results of Stryer and Blout (1961), Cotton effects similar in size and shape to those produced by binding to the α-helix were observed in the dye absorption bands in the coil pH region. This disagreement arises from the use of different complex preparation methods. The preparation method was discovered to be a particularly important variable in determining the optical activity behavior of both the helix and coil complexes. Complexes prepared by changing the PGA conformation in the presence of dye never displayed the same optical rotatory behavior as those prepared by adding dye to pretitrated PGA solutions. Optically active coil complexes could only be prepared by adding dye to a solution of the PGA coil and not by titration of a helix complex. Also, the magnitude of the Cotton effects of the helix complex

prepared by titrating a coil complex were consistently only about one-third the size of those produced by adding AO to helical PGA.

15. Optically active coil complexes could only be prepared by adding dye to a PGA solution and not by the reverse order of mixing. Several peculiar preparation requirements for obtaining reproducible ORD curves in water solution were discussed, and it was noted that heating the coil complex to 80°C for several minutes destroys most of the optical activity. The optically active coil complexes are always slightly cloudy; the heat treatment results in clear solutions, and preparation methods yielding clear solutions do not give optically active complexes. These results and others presented herein strongly suggest the optical activity is associated with aggregates which are formed in the preparation procedures and are responsible for the cloudiness. The observation of a very large, unstable CD band ($\Delta\epsilon \cong -100$) at R/D = 62,900 in the coil pH region was attributed to the formation of extremely optically active aggregates upon adding AO to the viscous PGA solution.

16. Reproducible ORD curves could not be obtained in buffered solutions of the coil complex. Strange, bell-shaped ORD curves were often observed, and the size and sign of these curves seemed to be completely random and unaffected by changes in the preparation method. The bell-shaped ORD curves could not be attributed to birefringence problems.

17. Some evidence was obtained with the coil complexes that the Cotton effects in the visible and ultraviolet spectral regions can be independent of each other. For instance, different preparation methods caused a change in the sign of rotation at 458 mμ, but no sign changes were noted in the peaks and troughs below 350 mμ. These and other differences suggested the dye planes in bound dimers are not parallel and that both spectral regions might be compared more carefully to provide information on the relative orientations of the long and short axes of the dye molecules.

18. As many as four CD bands could be clearly observed for the helix complexes, but the number, position, and size of these bands depends upon the ionic strength and the degree of protonation. The simplest explanation of the large CD changes is that several dimer sites are provided by the α-helical conformation, each characterized by a somewhat different asymmetric arrangement of the attached dye molecules. Model building also suggests the presence of several dimer sites that may impart either a left-handed or right-handed twist to the dimer geometry. The relative proportions of the several sites available for dye binding are apparently altered by unequal affinities for protons and sodium ions.

19. The different optical activity properties of the helix complexes prepared by adding AO to helical PGA or by titrating a coil complex suggested that caution be exercised in studying the dependence of the helix optical activity upon

any solution variable, particularly the molar ratio of PGA and dye. The optical properties may be strongly influenced by aggregates formed during the preparation or by a slow attainment of equilibrium between several complex species formed in the mixing process. Thus, a study of the size of the Cotton effects as a function of R/D may yield results distorted by "mixing artifacts" brought about by the change in the relative concentrations and volumes of the solutions being mixed. The extent to which aggregation and the mixing method influence the optical activity behavior of the helix complex solutions must be carefully determined before any future studies can be made very meaningful.

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APPENDIX

The structural formulas of the dyes quoted in this dissertation and a few other dyes commonly encountered in the literature on metachromatic dyes and their complexes with chromotropes are listed below. For a complementary listing, the reader is referred to Kelly (1956). As far as possible, these dyes have been grouped into their respective classes and are listed in tables beneath the structural formulas of the parent compounds. Most of the dyes below have been reported to be metachromatic to various degrees; those which exhibit spectral shifts or depart from Beer's law only at very high concentrations will be marked SM, for slightly metachromatic, while those dyes which are not considered to be metachromatic are marked NM, for not metachromatic. All of the dyes are shown in their charged forms, since this is the form required for appreciable solubility and metachromasy in aqueous solutions.

Acridine Dyes

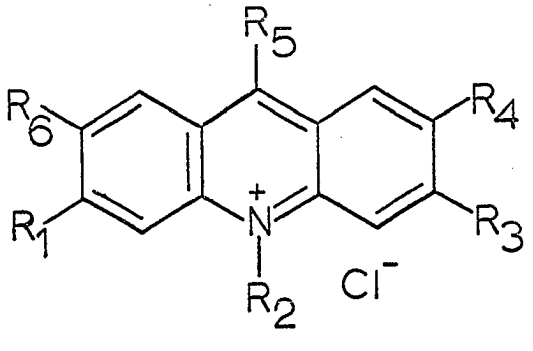


Table 2. Acridine dye structures

Dye name	R_1	R_2	R_3	R_4	R_5	R_6
Acridine orange	$N(Me)_2$[a]	H	$N(Me)_2$	H	H	H
Proflavine	NH_2	H	NH_2	H	H	H
Acriflavine	NH_2	CH_3	NH_2	H	H	H
Acridine yellow	NH_2	H	NH_2	CH_3	H	CH_3
Coriphosphine O	$N(Me)_2$	H	NH_2	CH_3	H	H
Rivanol	H	H	NH_2	H	NH_2	EtO[b]

[a]Me = methyl group

[b]Et = ethyl group

Azine Dyes

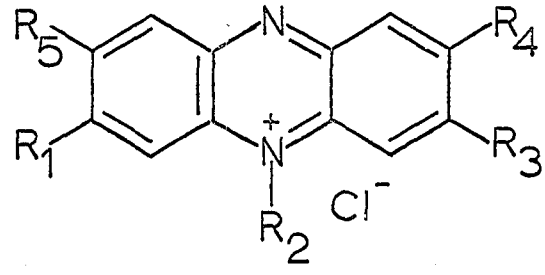


Table 3. Azine dye structures

Dye name	R_1	R_2	R_3	R_4	R_5
Neutral red	$N(Me)_2$	H	NH_2	CH_3	H
Phenosafranine[a]	NH_2	Ph[b]	NH_2	H	H
Safranine O (or T)	NH_2	Ph	NH_2	CH_3	CH_3

[a]Also called Safranine B

[b]Ph = phenyl group

Thiazine Dyes

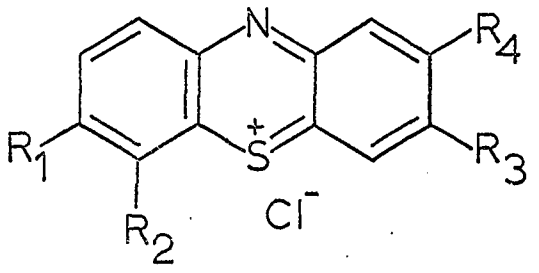


Table 4. Thiazine dye structures

Dye name	R_1	R_2	R_3	R_4
Methylene blue	$N(Me)_2$	H	$N(Me)_2$	H
Toluidine blue	$N(Me)_2$	H	NH_2	CH_3
Azure A	$N(Me)_2$	H	NH_2	H
Azure B	$N(Me)_2$	H	NHMe	H
Azure C	NHMe	H	NH_2	H
Thionine	NH_2	H	NH_2	H
Methylene green[a]	$N(Me)_2$	NO_2	$N(Me)_2$	H

[a] NM

Triphenylmethane Dyes

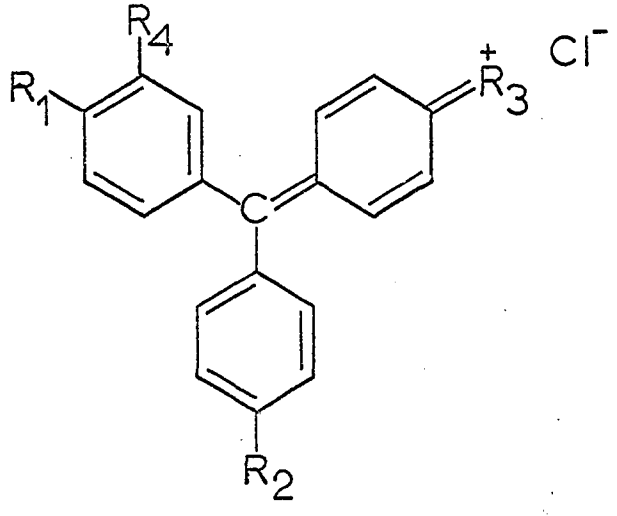


Table 5. Triphenylmethane dye structures

Dye name	R_1	R_2	R_3	R_4
Crystal violet	$N(Me)_2$	$N(Me)_2$	$N(Me)_2$	H
Rosaniline	NH_2	NH_2	NH_2	CH_3
Basic fuchsin	NH_2	NH_2	NH_2	H
Malachite green[a]	$N(Me)_2$	H	NH_2	H
Doebner's violet[a]	NH_2	H	NH_2	H
Methyl green[b]	$N(Me)_2$	$N(Me)_3$	$N(Me)_2$	H

[a] NM

[b] SM; divalent cationic dye

Xanthene Dyes

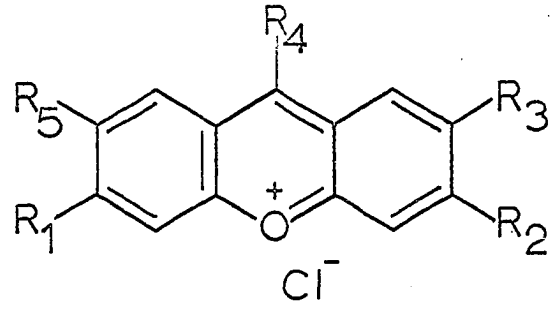


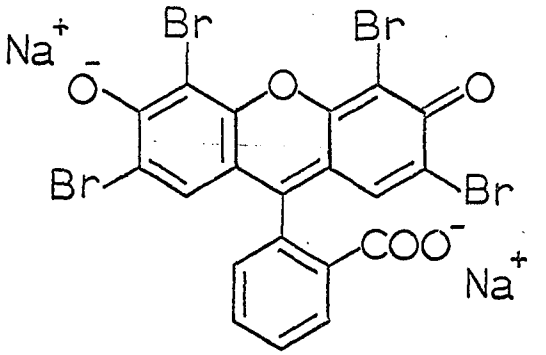
Table 6. Xanthene dye structures

Dye name	R_1	R_2	R_3	R_4	R_5
Pyronine G	$N(Me)_2$	$N(Me)_2$	H	H	H
Rhodamine B	$N(Et)_2$	$N(Et)_2$	H	2-BA[a]	H
Rhodamine 6G (or G)	NHEt	NHEt	CH_3	2-BE[b]	CH_3
Acridine red	NHMe	NHMe	H	H	H

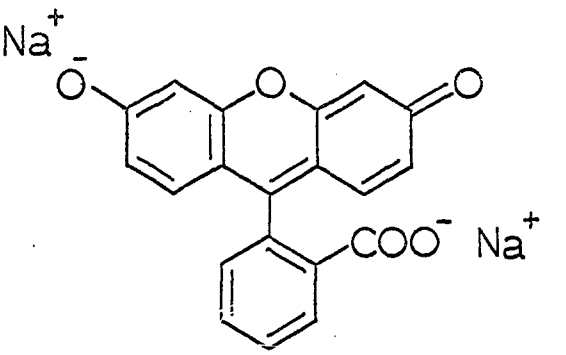
[a] 2-BA = 2-benzoic acid

[b] 2-BE = ethyl ester of 2-benzoic acid

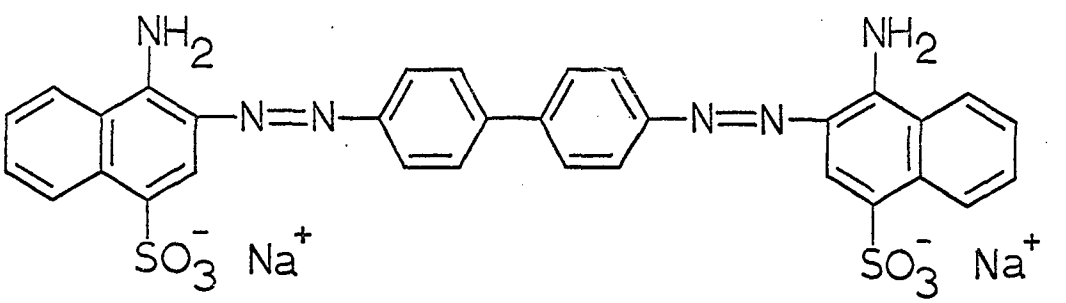
Anionic Dyes



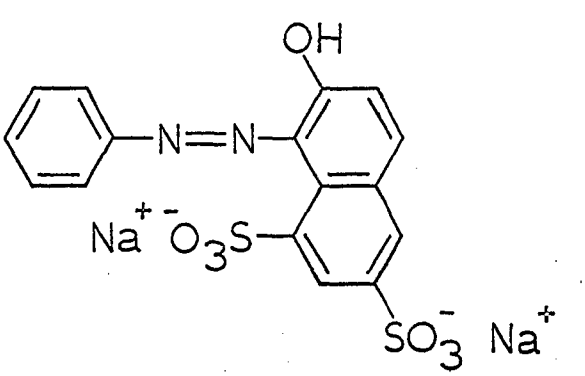
Eosin



Fluorescein

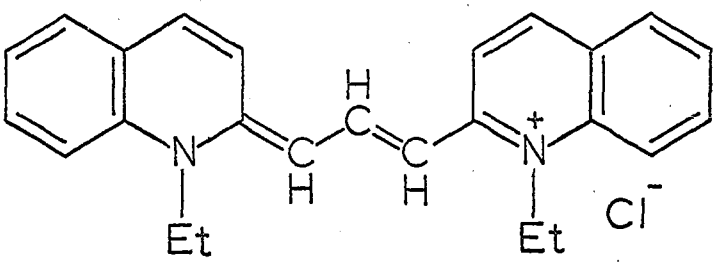


Congo red, NM

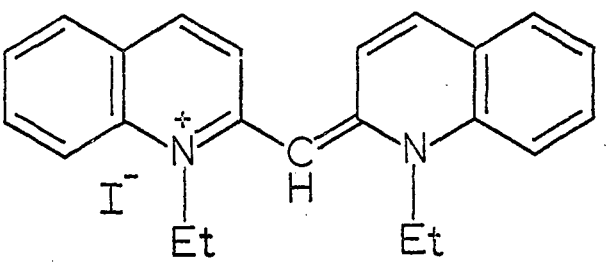


Orange G, NM

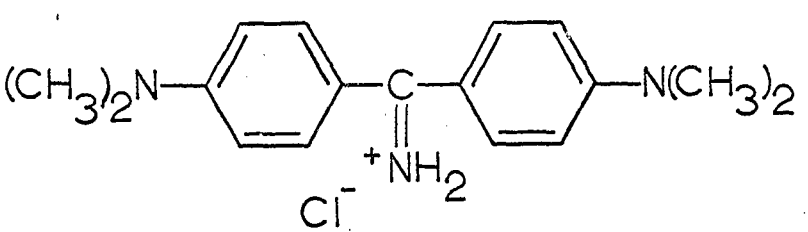
Other Dyes



Pinacyanol



Pseudoisocyanine



Auramine O