Trans. Nat. Acad. Science & Tech. (Phils.)
1988: 10: 33-42

STUDYING NATURAL AND SYNTHETIC MACROMOLECULES BY FLUORESCENCE

Herbert Morawetz
Polytechnic University, Brooklyn, NY 11201

ABSTRACT

Fluorescence techniques have been used to characterize the flexibility of antibodies, to study the folding of newly synthesized proteins and to sort chromosomes. With synthetic polymers, fluorescence phenomena have illuminated the mechanism by which chain molecules change their shape and the rate at which such changes take place. The miscibility of polymers in bulk and the interpenetration of similar chain molecules in solution has also been studied by fluorescence techniques. Finally, flourescence has been employed to follow kinetically the exchange of counterions between chain molecules in solutions of nonpolar polymers carrying a small number of ionized substituents.

Introduction

The measurement of fluorescence constitutes a method for the study of a wide variety of phenomena. With steady illumination, the parameters of interest are the emission intensity, the spectral distribution of the emitted light and the polarization of fluorescence. More recently, instrumentation has become available which allows us to follow the decay of the emission intensity and the decay of the polarization after a flash irradiation by "time resolved fluorimetry" and this has greatly expanded the information obtainable from flourescence.

The application of fluorescence techniques for the study of macromolecules was pioneered by Weber almost 40 years ago. He first showed[1] how the polarization of the emitted light can be used to estimate the size of a globular protein molecule to which a fluorecent label is rigidly attached. This is so because polarization decreases with the extent of rotational motion of the fluorescent species during the time interval between the excitation and emission, and this motion will be slowed down with an increase of the size of the protein to which the label is attached. Specifically, if we define a polarization by $P = (I_{ii} - I_i)/(I_{ii} + I_i)$ where I_{ii} and I_i are the intensities of emitted light polarized parallel and perpendiular to the polarization of the exicting beam, then we obtain for spherical protein molecules of volume V in a solution with a viscosity η .

$$\frac{P^{-1} - (1/3)}{P_o^{-1} - (1/3)} = 1 + \frac{\tau_e kt}{2V\eta} \qquad (1)$$

where P_o is thepolarization in a medium of high viscosity, so that no rotation is possible, τ_e is the lifetime of the excited fluorophore (usually of the order of 10^{-8} seconds), k is Boltzmann's constant, T the absolute temperature and η the viscosity of the solvent.

With the introduction of time-resolved fluorimetry, more complicated motions could be analyzed. This is exemplified on a recent study[2] of an immunoglobulin whose molecule is known to contain three globular subunits as shown in Figure 1. The two antigen-binding fragments F_{ab} are attached by a flexible bond to the F_c fragment. The course of fluorescence decay of the label attached to F_{ab} has been interpreted as suggesting two motions, an oscillation of the angle between the axes of the two F_{ab} fragments and a six times slower rotation of the F_{ab} around its axis. Such data are of interest since the biological action of the immunoglobulin is influenced by its flexibility.

Another early contribution from Weber's laboratory was the discovery of dyes which fluoresce up to two orders of magnitude more strongly in organic solvents than in water[3]. When such dyes are attached to specific sites on a protein molecule, they act as "reporters" of the accessibility of these sites to the aqueous environment; this is, for instance, an important factor in the binding of substrates to enzymes. Very small changes in protein structure, which are difficult to detect by other spectroscopic techniques, may produce dramatic changes in the emission intensity of the reporter[4].

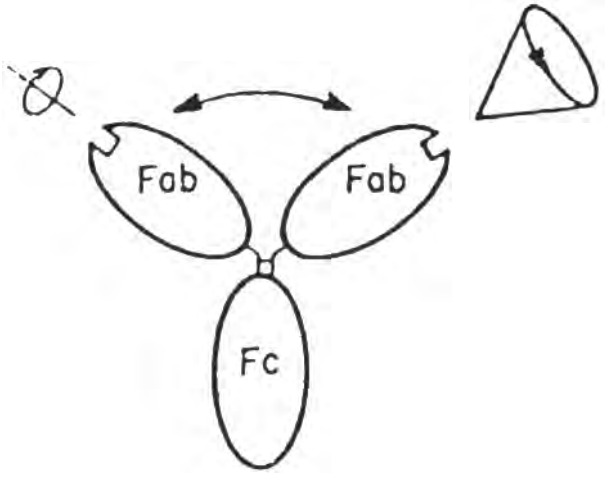


Figure 1. Schematic representation of the motions in immunoglobulin IgG (ref. 2).

When a macromolecule carries two labels, such that the emission of one ("the donor") overlaps the absorption spectrum of the other ("the acceptor"), excitation energy absorbed by the donor can be transmitted over relatively long distances (up to 50A) to the acceptor by a "nonradiative energy transfer" (NET). According to Forster's analysis[5] the efficiency of such transfer is given by

$$\text{Eff} \cdot = [1 + (r/R_o)^6]^{-1} \tag{2}$$

where r is the distance between the two chromophores and R_o is a characteristic distance depending mostly on the overlap between the donor emission and the acceptor absorption spectra. If the acceptor fluoresces, radiant energy absorbed by the donor will lead to the characteristic acceptor emission; if the acceptor does not fluoresce, NET will only lead to a decrease in the donor fluorescence intensity. The function (2) is such is such that Eff is very sensitive to r for r/R_o not too far from unity and NET has been extensively used by biochemists to "measure" distances between two labels attached to specific protein sites. For instance, we should like to know the pathway by which a newly synthesized protein folds up to the unique flobular structure and this problem may be appproached by studying the reverse process, the intermediate stages of the unfolding of a globular protein under denaturation conditions. If the protein is labeled in two specific sites, this unfolding will result in their gradual separation which can be followed by NET[6].

In recent years the use of fluorescent labels has also led to a spectacular development of an instrument which can sort chromosomes at a rate of 20,000 per second[7-9]. The procedure takes advantage of the fact that two fluorescent dyes, Hoechst 33258 (H) and Chromomycin A3 (C), which differ in the wavelength at which they are excited and at which they emit, bind preferentially to different bases of DNA. The small difference in the DNA composition of different human chromosomes is sufficient to allow the apparatus, shown diagramatically in Figure 2, to separate all of the chromosomes except those numbered 9-12. (A modification of the standard procedure allows also these four chromosomes to be separated from each other). This efficient method of chromosome separation will undoubtedly play a crucial role in the most ambitious goal of contemporary biochemistry, the sequencing of the entire human genome, that is, the description of the hereditary message of the human species in chemical terms. A similar procedure has also been used for the separation of different types of cells[10].

Studies of synthetic polymers by fluorescence utilize some of the techniques which were established in the protein investigations and some additional phenomena. Here we can only cite a few typical examples of a very active field of research.

Unlike the molecules of globular proteins, in which the macromolecular chains are folded in a precisely defined way, the molecules of most synthetic polymers are flexible chains whose shape in solution is subject to rapid change. Two

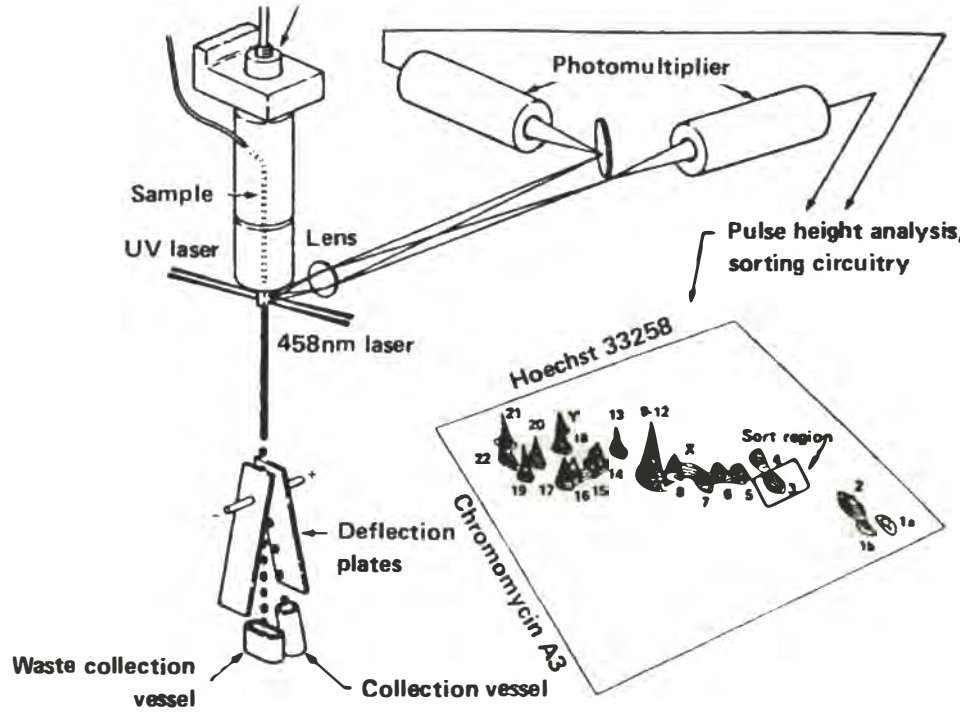


Figure 2. Diagram of the apparatus for the sorting of chromosomes. A stream of a dilute chromosome suspension is exposed to a UV laser exciting the H label and a 458 nm laser exciting the C label. After the stream breaks up into droplets, those containing a chromosome with the desired H and C fluorescence are electrically charged and deflected into a separate container. The insert shows the fluorescence intensities of the two labels characteristic of the various human chromosomes (ref. 8).

questions then arise: (1) How rapidly do such flexible chains change their shape under equilibrium conditons? (2) How rapidly do such molecular chains readjust their shape to changing conditions?

One aspect of the first question is illustrated in Figure 3. If a change in the shape of a polymer chain had to involve a series of rotations around a single bond in the chain backbone, then each of these rotations would force a large section of the chain to swing through the viscous solvent, a process which would require a prohibitive expenditure of energy. It was concluded, therefore, that molecular chains could not change their shape in this way and it was suggested that this difficulty could be circumvented by having two rotations take place simultaneously so that only a small segment of the chain would have to move in what was described as a "crankshaft-like motion". But since the rotation around a covalent bond involves passage over an energy barrier, such a "crankshaft-like motion" in which two energy barriers would have to be surmounted would be about a hundred times slower than an analogous process in a small molecule with a single hindered rotation. It was, therefore, of interest to see whether such a difference can be demonstrated.

A fluorescence phenomenon provided a powerful method for studying this question. In a molecule containing two phenyl residues separated by four bonds,

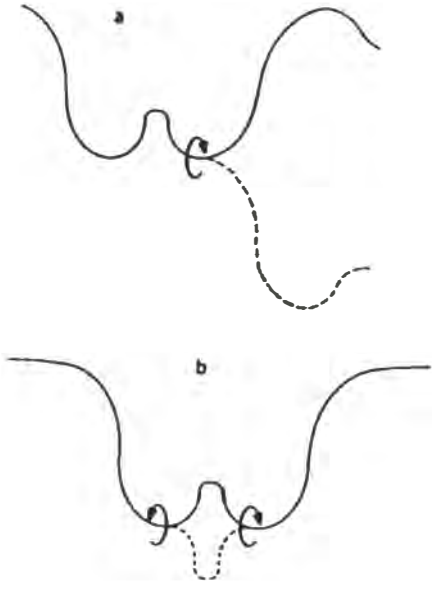


Figure 3. Schematic representation of a flexible polymer chain undergoing (a) a single hindered rotation, (b) two correlated rotations in a "crankshaft-like motion."

such as the di(p-methyl benzyl)acetamide in Figure 4, the excitation of one of the phenyls leads to two emission bands with maxima at 284 nm and 330 nm. The 284 nm emission is characteristic of compounds with a single phenyl group, whereas the 330 nm emission arises from the association complex of an excited and a non-excited phenyl group lying face to face to each other, a so-called "excimer" (with a

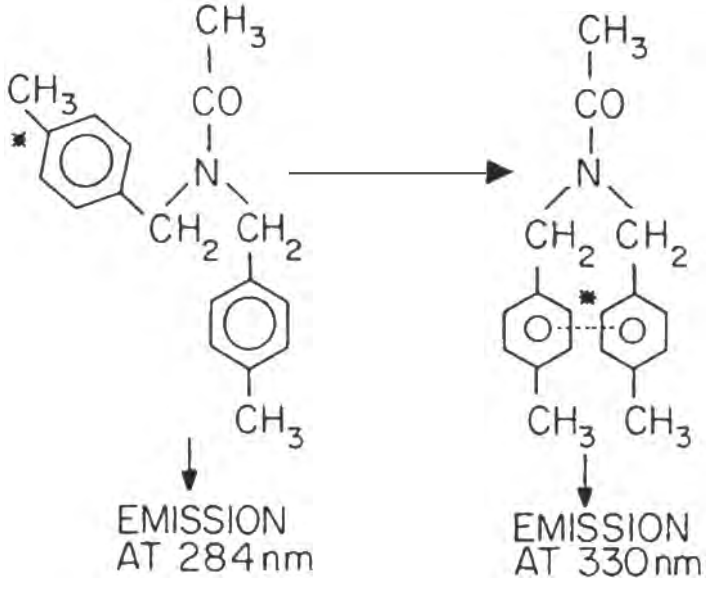
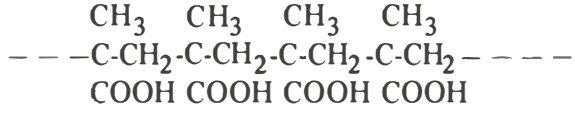


Figure 4. Formation of an excimer by a hindered rotation of di(p-methyl benzyl)acetamide.

molecular geometry which is unstable in the absence of excitation). The relative intensity of the 330 nm and 284 nm emission bands is, therefore, a measure of the probability that a bond rotation produces the excimer geometry during the lifetime of the excited state of the phenyl group. The observation that this relative intensity remains virtually unchanged when the p-methyl groups are replaced by long polymer chains[11] demonstrates that the hindered rotation by which the excimer is formed is essentially as easy when the dibenzyl acetamide is inserted into the middle of a long polymer chain as in the small analog and this rules out the concept of "crankshaft-like motion".

The change in the shape of a flexible polymer chain when the nature of the solvent is changed is studied most easily with a polycarboxylic acid, where the addition of a base leads to a large expansion of the molecule as a result of the electrostatic repulsion between the ionized carboxyls. We have studied the rate of this expansion on poly(methacrylic acid) (PMA) using two techniques: In the first, PMA was labeled with a dye which fluoresces strongly in organic media but very weakly in water and the chain expansion was followed by the decay in emission intensity as the label was increasingly exposed to the aqueous environment[12]. In the second approach, the PMA carried both a donor and an acceptor label and the expansion was reflected by a decreasing energy transfer as the distance between the labels increased[13]. It was satisfying that the two methods yielded essentially identical results.



PMA

By contrast with low molecular weight organic liquids which are almost always miscible with each other, miscibility of long chain polymers is much more restricted. For those unfamiliar with the behavior of polymers it will come as a surprise that even such small differences between two polymers as the substitution of methyl by ethyl side chains leads to complete immiscibility of the two substances. If two polymers are labeled with a donor and acceptor fluorophore, then the emission spectrum of their blend will furnish a sensitive measure of their miscibility, since the NET will be much more efficient if the two polymers mix at the molecular level than if they are segregated into two phases. This is illustrated in Figure 5 on blends of poly(methyl methacrylate) with copolymers of methyl methacrylate with ethyl or butyl methacrylate[14]. The ratio of the emission intensity of the naphthalene donor to that of the anthracene acceptor first increases gradually, reflecting decreasing energy transfer with decreasing mixing, and reaches a plateau when the difference between the polymers is such as to preclude any interpenetration of the two species.

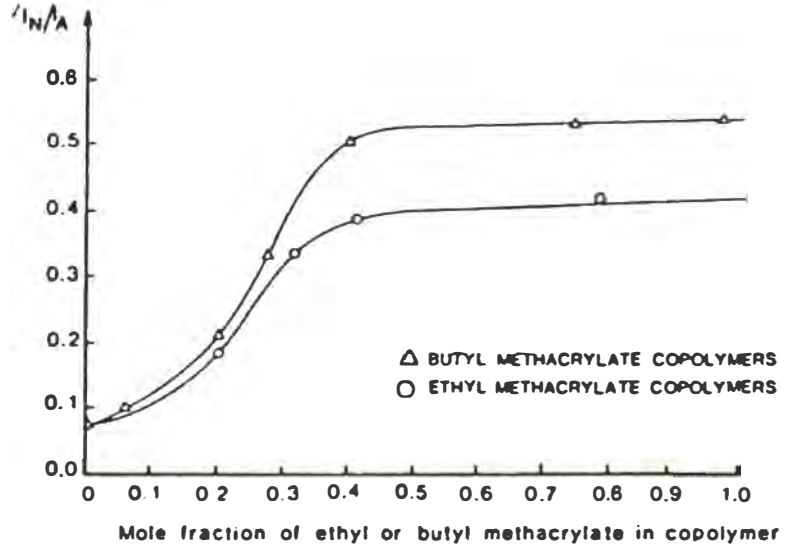


Figure 5. Ratio of emission intensities at 336 nm from a naphtyl-donor-labeled methyl methacrylate copolymer and at 408 nm from poly(methyl methacrylate) labeled with an anthracene acceptor (ref. 14).

In dilute solution, even identical polymer chains resist strongly mutual entanglement. However, as the solution concentration is increased, a point is eventually reached at which the swollen coils occupy all of the available space and beyond this critical concentration c* chains must become entangled. (It should be understood that a randomly coiled chain molecule extends over a much larger volume than the volume of the "dry" molecule, so that c* may be reached at a low polymer concentration). In trying to obtain the fraction of polymer chains entangled with other chains we proceeded as follows[15]: A solution of a polymer, half of which was tagged with a donor and half with an acceptor label, was frozen within a fraction of a second. The frozen solvent was removed by sublimation, the resulting powder was pressed into a pellet and the fluorescence spectrum of the pellet was recorded. Since the extent of chain entanglement in the original solution could not change during the rapid freezing, the pellets, all with the identical composition, differed greatly in their emission spectra, reflecting the varying efficiency of energy transfer. As a standard, a film was prepared by slow evaporation of a solution of mixed donor and acceptor labeled polymers, obtaining a sample in which the two chains were randomly mixed. From the comparison of the ratio of the acceptor and donor emission intensities in the freeze-dried and slowly evaporated sample the fraction of chain molecules entangled with other chain molecules could be estimated. Figure 6 plots typical data for polystyrene in three solvents, Up to c* the energy transfer is minimal and independent of the original solvent. Beyond this, the entanglement increases with solution concentration at a rate which is largest for benzene solutions, in which the polymer chains are most expanded, and smallest in cyclohexane, a poor solvent in which polystyrene is most contracted.

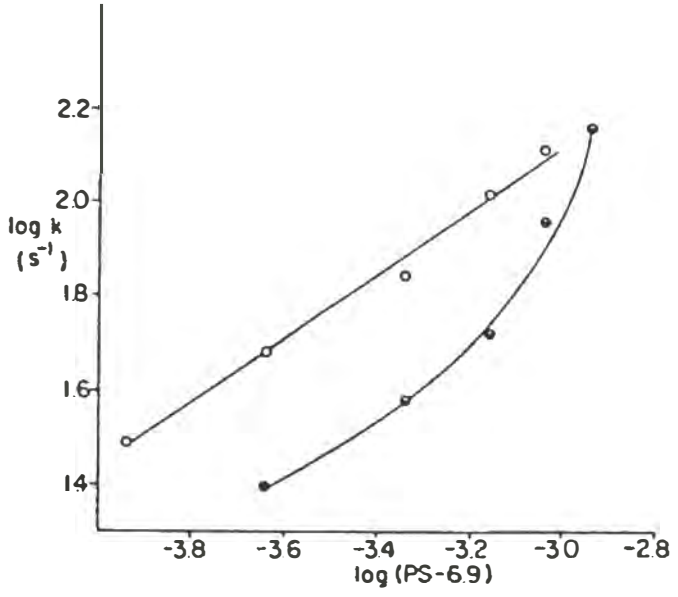
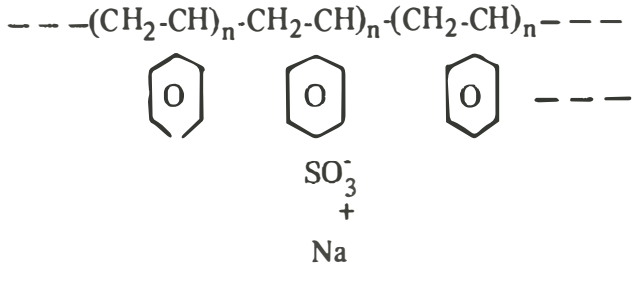


Figure 6. Relation between the ratio of the emission intensities of the anthracene acceptor label and the carbazole donor label in a freeze-dried sample, I_A/I_C, and in a slowly dried film, $(I_A/I_C)_f$, for blends of equal weights of donor and acceptor labeled polystyrene (molecular weight 400,000), as a function of the original solution concentration. Original solution in benezene (Δ), dioxane (□) and cylohexane (○). (Ref. 15).

In recent years a considerable effort has been devoted to the study of polymer chains carrying a small number of acid groups which are neutralized with base. Such substances are referred to as "ionomers"; a typical example is slightly sulfonated polystyrene in which the sulfonic acid groups were converted to their alkali salts:



Since the anionic groups and their counter-ions are imbedded in a medium of low dielectric constant, they are associated to ion-pairs; moreover, these ion-pairs attract each other strongly and this leads to a pronounced increase in viscosity both in bulk and in solutions of moderate concentration. It has been pointed out[16] that that the viscosity of systems containing chain molecules carrying a small number of associating groups should depend not only on the association equilibrium, but also

on the rate at which these groups associate and dissociate. This provides one of the motivations for an attempt to study the rate at which the cations are exchanged between polymer chains such as the one depicted above.

In our approach to this problem[17] we used lightly sulfonated polystyrene with two fluorescent counterions, one containing the naphtalene donor, the other the anthracene acceptor moiety. When solutions of the two ionomers were mixed, energy transfer was negligible as long as the two fluorescent counter-ions were associated with different polymer chains. However, as the counter-ions were exchanged between the polymers, the the donor and acceptor chromophore become associated with the same chain, so that their spacing is sufficiently short for efficient energy transfer. Thus a change in the emission spectrum can be used to follow the counter-ion exchange.

Results of experiments using this principle are represented in Figure 7. In dioxane solution, the rate constant for the counter-ion exchange is proportional to the 0.7 power of the ionomer concentration. In a 1:1 mixture of dioxane and cyclohexane, the rates are lower in highly dilute solution, as would be expected since the nonpolar co-solvent stabilizes the ion-pairs. At higher concentration in this medium, the rate of counter-ion exchange increases rapidly since the solubility limit is approached leading to association of the polymer molecules.

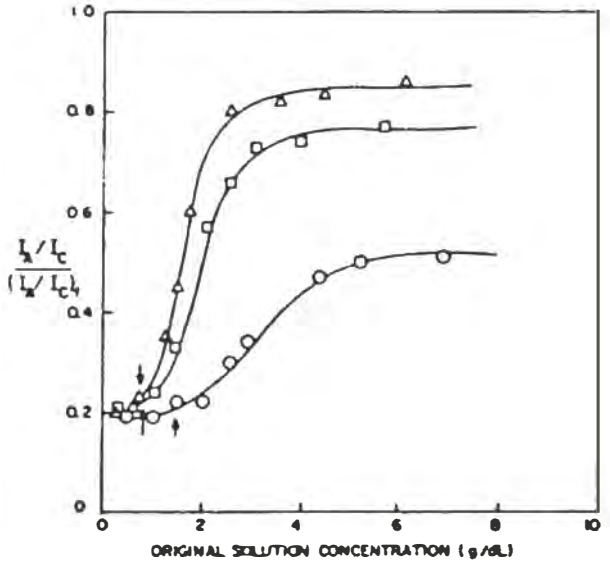


Figure 7. Rate constants for the counter-ion interchange between polystyrene (number average molecular weight 105,000) with 6.9% of the monomer residues sulfonated and 32% of the sulfonic acid groups neutralized with 2-(aminomethyl)naphtalene donor and 9-(aminomethyl)anthracene acceptor, respectively, as a function of the molar concentration of the monomer residues (PS-6.9). Dioxane solutions (○); solutions in a 1:1 mixture of dioxane and cyclohexane (○). (Ref. 17).

In this brief account I have been able to allude only to a few studies of biological and synthetic macromolecules using fluorescence techniques. An extensive account of studies of biological macromolecules by these techniques is contained in a monograph edited by Chen and Edelhoch[18]. Synthetic polymer studies employing fluorescence are covered in monographs by Philips[19] and Guillet[20] and in a symposium volume edited by Winnik[21].

Acknowledgment

Financial support for some of the studies reported in this article and for the writing of this review was provided by the National Science Foundation, Polymers Program, by their Grant DMR-85-00712.

References

1. Weber, G. 1952. *Biochem. J.*, 51: 145, 155.
2. Hanson, D.C., J. Iguerabide and V.N. Shumaker. 1981. *Biochemistry*, 20: 6842.
3. Weber, G., and J.R. Laurence. 1954. *Biochem. J.*, 56: XXXI.
4. McClure, W.O. and G.M. Edleman. 1967. *Biochemistry*, 6: 559, 567.
5. Forster, T. 1959. *Discuss. Faraday Soc.*, 27: 7.
6. Mc Wharten, C.A., E. Haas, A.R. Leed and H.A. Scheraga. 1986. *Biochemistry*, 25: 1951.
7. Gray, J.W. *et al.* 1979. *Chromosoma*, 73: 9.
8. Gray, J.W. *et al.* 1986. *Cold Spring Habor Symposia on Quantitative Biology*, 51: 141.
9. Gray, J.W. *et al.* 1987. *Science*, 238: 323.
10. Horen, T.K. and L.L. Wheeless. 1977. *Science*, 198: 149.
11. Liao, T.P. and H. Morawetz. 1980. *Macromolecules*, 13: 1228.
12. Bednar, B., H. Morawetz and J.A. Shafer. 1985. *Macromolecules*, 18: 1940.
13. Horskey, J. and H. Morawetz. *Makromol. Chem.*, in print.
14. Amrani, F., J.M. Hung and H. Morawetz. 1980. *Macromolecules*, 13: 649.
15. Chang, L.P. and H. Morawetz. 1987. *Macromolecules*, 20: 328.
16. Worsfold, D.J. and S. Bywater. 1972. *Macromolecules*, 5: 393; Worsfold. 1982. *J. Polym. Sci., Polym. Phys. Ed.*, 20: 99.
17. Morawetz, H. and Y. Wang. 1988. *Macromolecules*, 21: 107.
18. Chen, R.F. and H. Edelhoch. 1976. eds., "Biochemical Fluorescence", Marcel Dekker, New York, Vol. 1, 1975, Vol. 2.
19. Philipps, D. 1985. ed. "Polymer Photophysics", Chapman and Hall, London, 1985.
20. Guillet, J.E. 1985. "Polymer Photophysics and Photochemistry", Cambridge University Press, Cambridge, UK.
21. Winnik. 1986. ed., "Photophysical and Photochemical Tools in Polymer Science", NATO ASI Science Series, Ser. V, Vol. 182, D. Reidel, Dordrecht, Holland.