Fluorescence Techniques in Cell Biology

Edited by
A. A. Thaer and M. Sernetz

Fluorescence Techniques in Cell Biology

Edited by
A. A. Thaer and M. Sernetz

With 303 Figures

Springer-Verlag Berlin · Heidelberg · New York 1973

Preface

For there to be progress in science, there must first be communication between experts of different disciplines. This is particularly true of modern biology which is becoming more and more of an interdisciplinary field. The present situation in cell biology clearly reflects this development and demonstrates that the application of physical techniques was necessary before this field of biological research could be developed on an objective and quantitative basis. The utilization of optical phenomena as measuring parameters at the microscopic level has provided the basis for the development of quantitative cytochemistry. This rapidly growing extension of conventional cytochemistry and histochemistry is based on the visual oberservation of qualitative chemical criteria in correlation with the microscopically resolved structure of cells and tissues. Furthermore, the introduction into cytochemistry of such optical measuring techniques as absorption photometry, interferometry, and fluorometry, as well as the measurement of optical anisotropy, diffraction and scattered light, has provided the methodological bridge for the exchange of knowledge between cell biology on the one hand and biochemistry, or molecular biology, on the other.

Travelling in one direction across this bridge, the cell biologist in general and the cytochemist in particular profit by the accumulated experience of the biochemist concerning the theoretical basis and the application of these optical measuring techniques and the interpretation of results at the molecular level. The traffic in the opposite direction involves an essential part of the results obtained by biochemistry: unless these data are applied and interpreted in terms of the functional structure and organization of the cell, they will lack most of the desired biological significance. Thus, what cell biologists have learned in this field should be of equal interest and value to the biochemist. There is probably no better example of this fruitful interaction than the application of quantitative fluorescence techniques in both biochemistry and cell biology.

More than any other method, fluorescence techniques are not just a tool for applying quantitative cytochemistry to cellular composition, but must also be seen as providing perhaps the most important basis for "biochemistry at the level of the single intact cell and its structure" and may be expected to make an essential contribution to the understanding of the molecular organization of the cell.

The subject and scope of the conference the proceedings of which are published in this volume should be seen against this background. The organizers of the conference habe tried to construct a scientific program which reflects not only the present stage in the application of quantitative fluorescence techniques in biochemistry and cytochemistry, but also the increasing interaction between these two disciplines. Moreover, the program was intended to pinpoint crucial problems which probably cannot be solved except by such an interaction. They confidently hope they have gone some way toward meeting this goal, despite the sometimes highly divergent views with regard to the material to be investigated, the application of fluorescent techniques for obtaining quantitative information, and the interpretation of the results. There is still much to be done in overcoming "language problems".

The sequence selected for sessions I to V is certainly not the only possible one. However, the methodological aspect of the conference was the dominant consideration. Thus, it seemed reasonable to start with two reviews (Part I) on the utilization of two fluorescence parameters – fluorescence polarization and decay time – that have been succesfully used for the biochemical investigation of solutions, suspensions and homogenates, but have not yet been fully utilized for measuring single cells at the microscopic level.

The papers in Part II deal with the instrumental and standardization requirements for microscope fluorometry and spectrofluorometry on single cells. Not only do they reflect the present state of the art in this field, they also open the way for the systematic introduction of spectrofluorometry on solutions into ultramicroanalysis at the microscopic level. This in turn permits the direct comparison of information obtained for cells and solutions under identical microscopic conditions, which is most welcome because the data can be used for calibration and standardization and also for interpreting the results obtained for single cells. These contributions thus form a bridge to Part III and the papers dedicated to the central subject of quantitative cytochemistry: the chemical composition of cells and cellular compartments. In addition to the progress achieved in the cytofluorometric determination of DNA, RNA, proteins and mucopolysaccharides microscope fluorometry has successfully penetrated new areas: chromosomal analysis, study of biogenic amines, and immunology.

The new techniques of automated cytofluorometry permit the rapid assessment of cytochemical parameters in cell populations and their recording in histogram form. Such techniques open up new horizons for studies of cell population kinetics, for instance in pharmacology and for clinical diagnostics.

The same is true, at least in part, of the application of microscope fluorometry to the measurement of cellular activity at the level of the single cell and its compartments (Part IV). For instance, it has been used to investigate the intracellular turnover of fluorogenic substrates of different cell populations under various influences. A further step towards the ultimate goal of revealing the functional structure of the living intact cell is to apply techniques for the rapid microfluorometric recording of fluorescence intensity and spectrum to the study of intracellular enzyme reactions and transport phenomena and to correlate the findings with the microscopically resolved cellular structure.

Finally, the various papers in Part V deal with the use of fluorescent substances as molecular probes to study the configuration, conformation and reaction sites of biological macromolecules. This session illustrates particularly well the complementary nature of studies on known solutions on the one hand, and those on organized biological material like fractionated cellular compartments and even intact cells on the other.

Models like artificial membranes are becoming increasingly important in the study of such properties of well-defined macromolecular structures. Models can aid in the interpretation of the results obtained by measuring the fluorescence parameters of fluorescent molecular probes bound to cellular structures. There is no doubt that during the next few years this very promising application of fluorescence techniques in cell biology will considerably increase in importance, for it correlates biochemical information and cell structure, i. e. the morphology and chemical topology of the intact cell.

The editors – who were also responsible for the organization of the conference – gratefully acknowledge the cooperation of all authors who contributed to this volume and their understanding of the editors' problems. They also wish to thank the session chairmen, Dr. H NEURATH (I), Dr. S. S. WEST (II), Dr. F. RUCH (III), Dr. B. THORELL (IV) and Dr. G. WEBER (V), for their effective coordination and their determination to make each session a real success.

The organizers' and editors' heartfelt thanks are due to Dr. T. CASPERSSON for taking on the conference chairmanship, and for his valuable advice concerning the preparation of the scientific program, his introductory remarks to the conference, and his own outstanding contribution.

The conference was made possible by the generous support provided by the Battelle Institute, Life Sciences Program. The organizers would like to express their sincere thanks to Dr. H. NEURATH, University of Washington, at that time coordinator of the life sciences activities sponsored by the Battelle Institute, for his deep and permanent interest in the organization of the conference and for his suggestions concerning the preparation of the scientific program.

The help of the staff of the Battelle Seattle Research Center, in particular of Mr. L. BONNEFOND and his staff, before and during the conference is also gratefully acknowledged.

The essential part of the work concerning the preparation of all manuscripts for the delivery to the publisher was performed by Mr. A. W. Roecker, Librarian of the Battelle Seattle Research Center, and by his staff, to whom the editors' thanks are due.

September, 1973

The Editors

Contents

Opening Remarks 1

Part I
Introductory Papers

Polarized Fluorescence. G. WEBER 5
Techniques for Fluorescence Lifetime Measurements and Time-Resolved Emission Spectroscopy. W. R. WARE 16

Part II
Instrumentation and Standardization in Fluorometry and Microscope Fluorometry

Some Aspects of Instrumentation and Methods as Applied to Fluorometry at the Microscale. G. VON SENGBUSCH and A. THAER 31
Microcapillary Fluorometry and Standardization for Microscope Fluorometry. M. SERNETZ and A. THAER 41
The Use of Human Leukocytes as a Standard for the Cytofluorometric Determination of Protein and DNA. F. RUCH 51
A Microspectrofluorometer for Measuring Spectra of Excitation, Emission and Absorption in Cells and Tissues. F. W. D. ROST 57

Part III
Cytofluorometric Determination of Cellular Substances

Absorbance and Fluorescence Cytophotometry of Nuclear Feulgen DNA. A Comparative Study. N. BÖHM, E. SPRENGER, and W. SANDRITTER 67
Automation of Cytofluorometry by Use of the Impulsmicrophotometer. W. GÖHDE 79
Quantitative Determination of DNA and Protein in Single Cells. F. RUCH 89
Fluorescence Properties of the Monoaminoacridines and Some 2-Aminoacridine-Derivatives. D. WITTEKIND 95
Fluorometric Recognition of Chromosomes and Chromosome Regions. T. CASPERSSON 107
Chemical Aspects of the Fluorescence Analysis of Chromosomes. E. J. MODEST and S. K. SENGUPTA 125
Fluorescence Cytochemical Studies of Chromosomes: Quantitative Applications of Fluorescein Mercuric Acetate. R. R. COWDEN and S. K. CURTIS 135
Microfluorimetric Comparison of Chromatin During Cytodifferentiation. B. L. GLEDHILL and G. L. CAMPBELL 151
Immunofluorometric Determinations of Surface Immunoglobulins of Individual Normal and Malignant Human Lymphoid Cells. D. KILLANDER, U. HELLSTRÖM, B. JOHANSSON, E. KLEIN, A. LEVIN, and P. PERLMANN 163
Cytofluorometry of Biogenic Monoamines in the Falck-Hillarp Method. Structural Identification by Spectral Analysis. A. BJÖRKLUND and B. FALCK 171
Microfluorimetric Quantitation of Biogenic Monoamines. E. M. RITZÉN 183

Quantitation of Biogenic Monoamines Demonstrated with the Formaldehyde Fluorescence Method. G. JONSSON 191
The Identification of Arylethylamines by Microspectrofluorometry of Acid- and Aldehyde-Induced Fluorescence. F. W. D. ROST and A. G. E. PEARSE 199

Part IV
Investigation of Enzyme Reactions and Transport Mechanisms

Quantitative Aspects of Rapid Microfluorometry for the Study of Enzyme Reactions and Transport Mechanisms in Single Living Cells. E. KOHEN, C. KOHEN, B. THORELL, and G. WAGENER 207
Rapid Microfluorometry for Biochemistry of the Living Cell in Correlation with Cytomorphology and Transport Phenomena. E. KOHEN, B. THORELL, C. KOHEN, and M. MICHAELIS 219
Newer Fluorometric Methods for the Analysis of Biologically Important Compounds. G. G. GUILBAULT 235
Microfluorometric Investigations on the Intracellular Turnover of Fluorogenic Substrates. M. SERNETZ 243
Changes in the Membrane Permeability of Human Leukocytes Measured by Fluorochromasia in a Rapid Flow Fluorometer. B. ROTMAN 255

Part V
Fluorescent Molecular Probes for Complex Biological Molecules

Probes for Enzyme Conformation. G. K. RADDA 261
The Fluorescence of Bilirubin-Albumin Complexes. R. F. CHEN 273
The Effect of the Excitation Wavelength on the Fluorescence Depolarization of Protein-Dye Complexes or Conjugates. B. WITHOLT and L. BRAND 283
Fluorescence, Absorption, and Optical Rotatory Behavior of Acridine Orange: Poly-L-Glutamic Acid Complexes. B. MYHR and J. FOSS 291
Ethidium Bromide: A Fluorescent Probe of Nucleic Acid Structure and its Potential for *in-vivo* Studies. J.-B. LE PECQ 301
Nanosecond Fluorescence Spectroscopy of Biological Macromolecules and Membranes. J. YGUERABIDE 311
Measurements of Single Molecules of Antibody by Their Ability to Activate a Defective Enzyme. B. ROTMAN 333
Nanosecond Time-Resolved Fluorescence Spectroscopy in Molecular Biology. M. R. LOKEN, J. R. GOHLKE, and L. BRAND 339
Fluorescence Spectroscopy as a Tool for Studying Drug Interactions with Biological Systems. C. F. CHIGNELL 345
Temperature Sensitivity of Fluorescent Probes in the Presence of Model Membranes and Mitochondria. J. VANDERKOOI 359
Conformational Dynamics of Model Membranes. J. YGUERABIDE 367
Atebrin and Related Fluorochromes as Quantitative Probes of Membrane Energization. R. KRAAYENHOF 381
Fluorescent Molecular Probes in Fluorescence Microspectrophotometry and Microspectropolarimetry. S. S. WEST and A. E. LORINCZ 395

List of Attendees 409
Subject Index 413

OPENING REMARKS

by

T. Caspersson

I am very pleased to have been asked to give some opening remarks on this occasion, for I believe that this will be an unusually productive conference, and that it is being held at precisely the right time. Work in the field of biological fluorescence is in a period of almost explosive development, but it is proceeding along so many different lines — biophysical, methodological, organochemical, cell physiological, genetical, etc. — that it is impossible for one man to follow more than part of it. Thus, just now, there exist real information gaps, and this conference is very ably planned to begin bridging these gaps. One of the crucial reasons why development has accelerated so quickly during the last few years is that, at long last, really quantitative methods have been developed and used. It is thus very proper to use the quantitative approach as the common denominator in our discussions this week.

The field of microscopy was the first field in biology to benefit from fluorescence approaches. The great microscopy pioneer, August Köhler, gave the first impetus as early as in 1904, and in 1911 Lehmann built a good instrument and produced the first photographs in 1913. I believe it is correct to say that above all, it was the primary fluorescence of porphyrins that lay behind the great widening of the work in the late twenties and the thirties, even though interesting work was performed using secondary fluorescence on different cell constituents. Acridine orange was even then a very popular substance.

At that time the fluorescence microscopes used carbon arcs or sparks between metal electrodes as light sources and functioned quite well even if their stability was not very good. Optics and filters were of a very satisfactory standard, although the choice was not very wide. Moreover, soon after came the gas discharge lamps which replaced all other light sources. Many different lines developed and many types of biological materials were studied.

Little or nothing was then heard of microscope fluorometry. We tried twice in the early forties to build ultramicrofluorometers at our laboratory. In applying them to biological objects on a microscale, however, fluorescence decay became such a problem that we had to abandon the project.

While optics, filters, and light sources were satisfactory, the photoelectric aspects offered almost insurmountable difficulties until photomultipliers came along after the war. Then suddenly, everything improved. At the same time, however, there were so many other fields for the new sensitive light detection systems that, to my knowledge, little was done on fluorescence until in this last decade, when work was taken up in many places, and really quantitative work on cells was started.

Fluorescence analysis is a complex field. Many physical and chemical factors influence the intensity and the character of the fluorescence of an object. It is most encouraging that during these last years these fields have grown so rapidly and that therefore the amount of information available has also greatly increased. We will be hearing a great deal on this subject during our meeting.

Furthermore, physicochemical studies have shown that fluorescence analysis can offer much more than a means for quantitative chemical determinations in biological materials. Methods such as measurement of fluorescence polarization and depolarization and work on relaxation kinetics have opened new vistas in the study of the macromolecular organization of cellular compounds.

During the last two decades we have received hardly any essentially new basic tools in the field. However, different types of biological problems make very different demands on the instrumentation to be used, both in routine fluorescence measurements and in measurements of more sophisticated kinds. This field is well covered in this symposium.

All in all, this syposium presents surveys of a number of fields, often of very different kinds, but all aimed at really quantitative work on the composition and structure of cellular constituents. Many of these ostensibly rather unrelated fields

are difficult for workers in other areas to penetrate. Thus this symposium is an unusually meaningful convocation of research workers who share a common interest but who are following different lines.

There will also be a series of papers giving examples of applied biological work in several fields, from enzyme kinetics to nerve function, and these, together with the presentation of physical and chemical background information, will, I am sure, stimulate all of us to a further widening of the realm of biological fluorometry.

We are all very grateful to the Battelle organization for this opportunity.

Part I
Introductory Papers

POLARIZED FLUORESCENCE

Gregorio Weber

Roger Adams Laboratory, Department of Biochemistry
University of Illinois, Urbana, Illinois

ORIGIN OF FLUORESCENCE POLARIZATION

The emission from a fluorescent solution is always polarized to some degree. By this we understand that an observer who sees the light emitted in a direction at right angles to the direction of the excitation through a polarizer observes changes in the intensity of the emission as he rotates the polarizer (Figure 1a). This polarization, first discovered by Weigert (1920) has received considerable attention; its origin and significance are well understood in the case of fluorescent solutions. Briefly, the polarization results from the existence of a preferential orientation in the molecules at the time of the emission. The exciting light produces a selection of the orientations because the molecules preferentially excited are those in which the transition moment in absorption is parallel or at a small angle to the electric vector of the polarized excitation. This original orientation is preserved if the molecules undergo negligible motion between excitation and emission, in which case the polarization is a maximum, called the fundamental or limiting polarization P_0.

EFFECT OF MOLECULAR MOTIONS

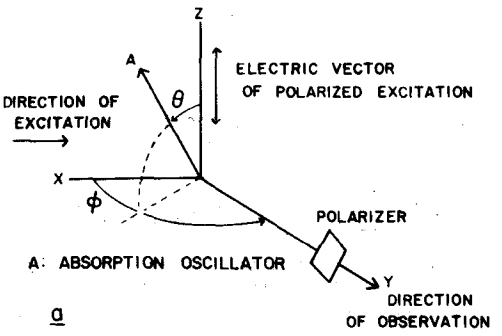
On the other hand, if the brownian motion of the molecules is very lively, the initial orientation is all but lost in the few nanoseconds elapsing between excitation and emission; in this case only a residual polarization of the order of a few parts per thousand to a few parts per hundred is observable. It follows that in dilute solutions the polarization of the fluorescence is a good indicator of the motion of the particles responsible for the fluorescence. In fact, the polarization observed is determined by only three factors: the limiting polarization P_0 which would obtain if no disorientation followed the excitation, the rate of rotation R of the particles, and the interval between excitation and emission, τ. In 1926 Francis Perrin showed that these quantities were linked by the relation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)(1 + 6R\tau) \qquad (1)$$

The simplicity of equation 1 is deceptive. While P, P_0 and τ have a simple physical significance and can be subjected to direct experimental measurement, R can have a very complex origin. If the fluorescent elements are attached to rigid spherical particles, R is the rate of rotation of a sphere, which Einstein in 1906 showed to be equal to:

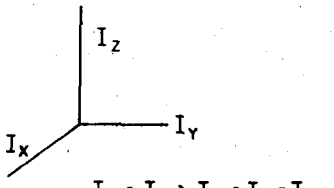
$$R = \frac{1}{6}\frac{kT}{\eta V} \qquad (2)$$

Here k is the Boltzman constant, T the absolute temperature, η the viscosity of the solvent in which the particle moves, and V the volume of the particles. While some simple molecules of weight of a few hundred daltons may be considered to come close to "rigid spheres," the fluorescent molecules of the biological specimens cannot always be assumed to have both of these characteristics. Some particles will be irregular, so that their motion is not characterized by a single rotational rate but by three rates R_1, R_2, and R_3 about a set of cartesian axes attached to the particles. The effects that the rotations about these different axes would produce as regards the polarization of the fluorescence will depend upon the angles that the three axes make with the transition moments in absorption and emission. Both a phenomenological (Weber 1971) and a rigorous diffusion theory (Perrin 1936 ; Belford et al. 1972; Ehrenberg and Rigler 1972) have been developed to account for the rotational motions and the ensuing depolarization of the fluorescence of these irregular particles. We shall not dis-



a

Figure 1a. *Coordinates showing direction of excitation and observation*



b

Figure 1b. *Intensity of the polarized components* I_z, I_y, I_x, *present when the solution is excited with light polarized along* OZ

cuss these here, but will refer the interested readers to reviews and original papers on this subject. Some general conclusions, however, may be stated. If we consider the conditions of the experimental observations, we see (Figure 1) that the excited molecules form a system with an axis of symmetry determined by the electric ve ctor of the polarized excitation. As a result of this symmetry, some molecular rotatory motions do not produce depolarizing effects. It follows that the depolarization reflects the rotations very much like a two-dimensional picture reflects a three-dimensional object. In the latter case, the three-dimensional object may be seen in its true shape by taking pictures from various angles. The analogy here is the observation of the depolarization under excitation by several different wavelengths. The limiting polarization P_0 can be shown in fact to be a function of the exciting wavelength. According to an equation originally due to Levshin (Perrin 1936; Weber 1971)

$$Cos^2\theta = \frac{1 + 3P_0}{3 - P_0} \qquad (3)$$

where θ is the angle between the transition moments in absorption and emission. The emission oscillator is placed in a fixed direction in the molecule, while the absorption oscillator has a different direction for each electronic absorption band.

POLARIZATION IN AROMATIC FLUOROPHORES

Most, if not all, of the fluorophores extensively employed in biochemistry and biology are aromatic molecules in which the absorption transitions as well as the unique fluorescence transition are of typical $\pi \rightarrow \pi^*$ nature. There is general agreement (Platt 1949) — if not extensive experimental proof — to the effect that the transition moments of $\pi \rightarrow \pi^*$ transitions in aromatic molecules are all contained in the plane of the aromatic rings. Consider then an irregular particle, typically a macromolecule carrying an aromatic fluorophore rigidly attached to it in a unique orientation (Figure 2). The rotational motion of the particles takes place about three axes of which one, R_3, is normal to the plane of the aromatic ring. As the absorption falls upon different directions contained in the plane of the ring, different

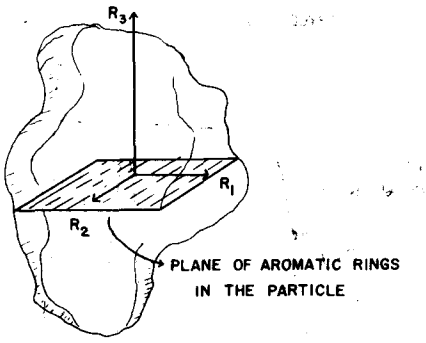


Figure 2. *Phenomenological axes of rotation, when an aromatic fluorophore is rigidly attached to a macromolecule*

weights are assigned to the effects of the rotational axes. When the absorption and emission oscillators are at 45° to each other, that is when P_0, according to equation 3, equals 1/7, the effect of R_3 upon the depolarization becomes null. This results from the fact that under these conditions all orientations obtained by rotation about R_3 are equally represented at the time of excitation. Further rotation about R_3 can only exchange positions; it cannot destroy the original random orientation. In this case therefore, the only active rotations are those that bring the aromatic rings out of the plane that they originally occupied at the time of the excitation (out-of-plane rotations). On the other hand, if the absorption and emission are coincident ($P_0 = 1/2$) the rotations that bring the aromatic ring out of its own plane, and those about R_3 that keep the rings in it (in-plane rotations) have equal weight. This generalization is valid only if the rotations are small, that is, if the values of P observed are not very much smaller than those of P_0. In these cases Perrin's Equation 1 will apply, with R being some weighted average, $\bar{R}$, of R_1, R_2 and R_3. From our description of the motions we can write for this average value $\bar{R}$,

$$\begin{aligned} \bar{R} &= (Rip + Rop)/2 \quad &if\ P_0 = 1/2 \\ \bar{R} &= Rop \quad &if\ P_0 = 1/7 \end{aligned} \tag{4}$$

where Rip = rate of in-plane rotations, Rop = rate of out-of-plane rotations. Therefore, simple observations of the dependence of the polarization at these values of P_0 can tell us whether the in-plane and out-of-plane rotations of the ring are equivalent or otherwise. The figures below show observations of this type for two different fluorophores: perylene (Figure 3 and 4) and 1-naphthylamine (Figures 5 and 6). In both cases, but particularly so in perylene, the out-of-plane rotations are markedly more sluggish than the in-plane rotations.

POLARIZATION OF FLUORESCENCE FROM PROTEIN CONJUGATES

In the case of a protein carrying a fluorophore rigidly attached in a fixed orientation as depicted in Figure 2, the same considerations apply. Witholt and Brand (1970) have studied the effect of changing the wavelength of excitation upon the observed value of $\bar{R}$ in complexes of anilino naphthalene sulfonate (ANS) and bovine serum albumin (BSA). They found that as P_0 decreases the value of $\bar{R}$ also decreases, which, from the

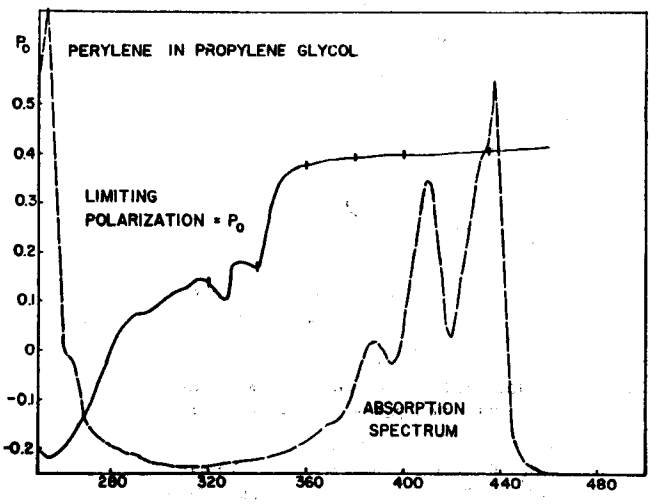


Figure 3. *Absorption and polarization spectra of perylene. The fluorescence polarization is that observed when the whole of the emission at wavelength longer than 450 nm is measured as function of excitation wavelength*

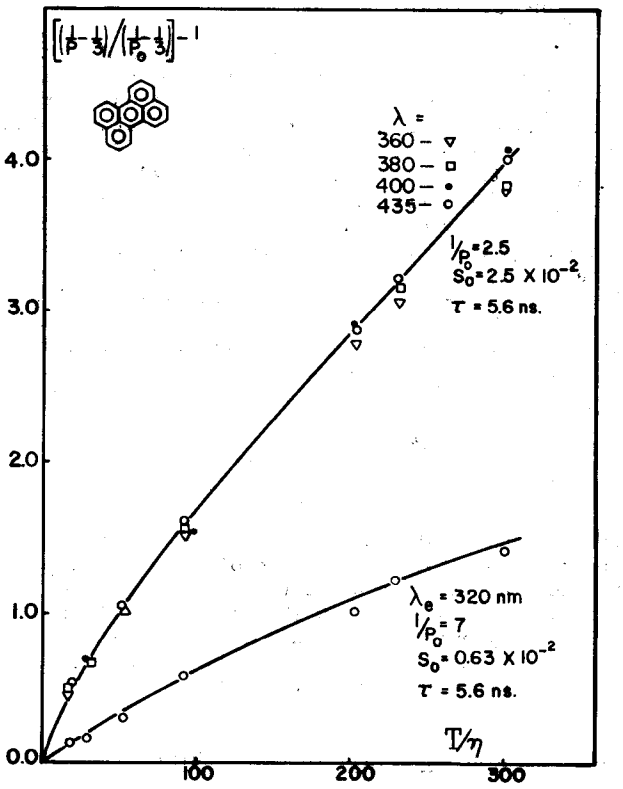


Figure 4. *Plots of relative anisotropy against temperature/viscosity for perylene in propylene glycol. Temperatures varied from -20°C to 25°C. S_0 is the initial slope in the plots. The in-plane rotations are some seven times faster than the out-of-plane rotations*

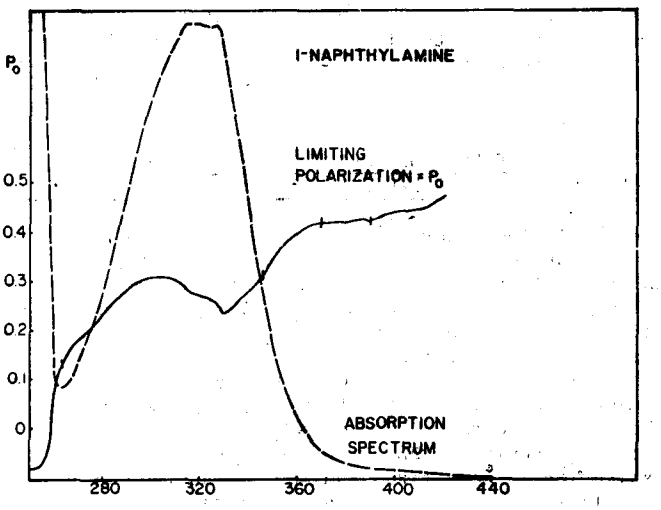


Figure 5. *Absorption and polarization spectra of 1-naphthylamine. The fluorescence at wavelengths longer than 425 nm was observed*

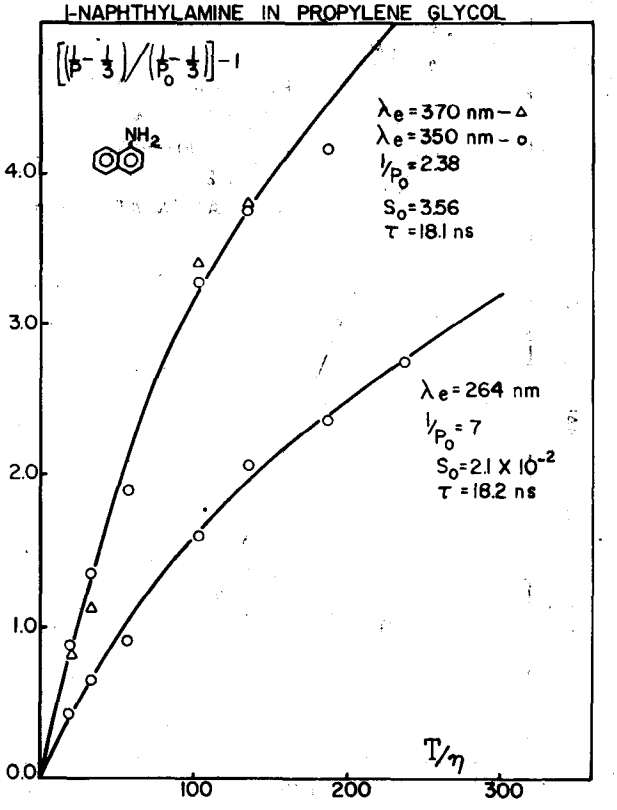


Figure 6. *Plots of relative anisotropy against* T/η, *as in Figure 4. From the initial slopes it follows that in-plane rotations are twice as fast as out-of-plane rotations*

above remarks, is easily interpreted as showing that the *ANS* planar ring system is oriented in the complex so that the in-plane rotations are faster than the out-of-plane rotations, or in other words that R_3 is an axis of fast rotation as compared with R_1 or R_2.

EFFECTS OF ENERGY TRANSFER UPON $\bar{R}$. EQUIVALENCE OF THE DEPOLARIZING EFFECTS OF TRANSFER AND ROTATION

When two identical fluorophores are close by in a rigid macromolecule, and by "close by" we mean 20 or 30 Å units, there is a distinct probability that energy transfer between them will take place, so that the exciting radiation may be absorbed by one and the fluorescence emitted by the other. P_0 is then determined by the angle between the absorption oscillator of one molecule and the emission oscillator of the other, since this is the polarization that would be observed for the motionless particle. If the particle is now allowed to rotate by decreasing the viscosity of the medium, the two mentioned oscillators determine the plane containing R_1 and R_2 in Figure 2, and therefore also the normal direction R_3. Serum albumin is capable of binding four or five molecules of ANS with high fluorescence efficiency. If the wavelength of excitation is kept fixed and the average number $\bar{n}$ of ANS molecules bound per BSA is allowed to increase, $\bar{R}$ is determined increasingly by the absorption and emission oscillators belonging to different ANS molecules. If there were no pre-

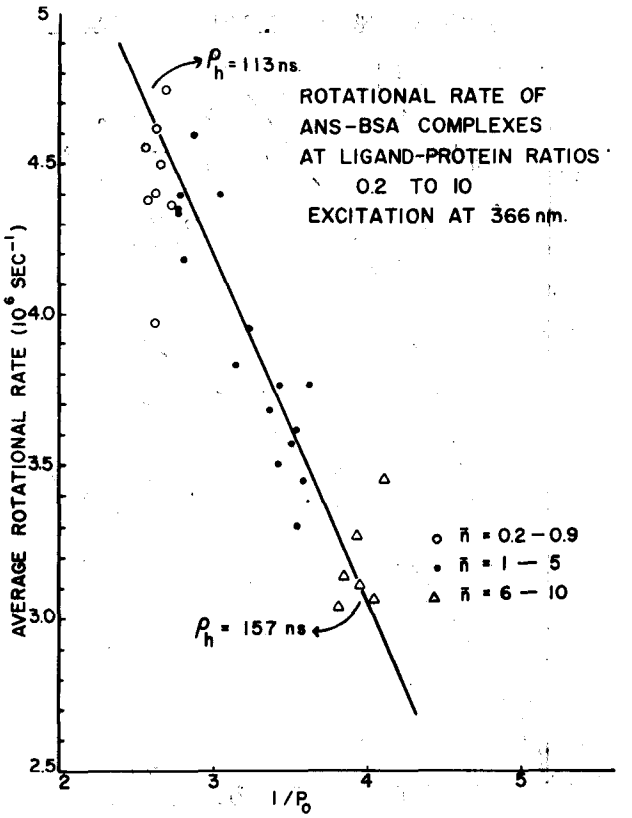


Figure 7. *Data of Weber and Anderson replotted to show the dependence of the apparent rotational rate upon limiting polarization when this varies due to energy transfer among ANS molecules*