Section 3
ADVANCED TECHNOLOGY DEVELOPMENTS

3.A Picosecond Fluorescence of Biomolecules

Accurate determinations of biomolecular structures which participate in fundamental photoreactions are generally difficult to obtain by standard methods. Chemical analysis, x-ray diffraction, and electron microscopy all require reasonably large samples, and, in the case of x rays, the samples must be crystalline. Fundamental photoreactive structures usually contain a low density of interesting sites. In the case of photosynthetic membranes, for example, there exists only one "trap" (a site where a local energy minimum occurs) for each 300 chlorophyll molecules. The chlorophylls are usually bound into water-insoluble proteins which cannot be crystallized. Finally, the first reactions initiated by light are extremely rapid, often involving photolabile molecules, so that normal time-independent or time-averaged results are of no interest. These are the facts underlying the need for short-pulse excitation and very rapid detection in primary photobiology.

Fluorescence and related processes in a typical biomolecule are illustrated in Fig. 29 by a standard configuration coordinate diagram. The horizontal axis represents displacement along some important structural degree of freedom. Fluorescence begins with an excitation by radiation (A→B). Process B→C is an excited-state relaxation which occurs typically in a few picoseconds or less. The discussion in this article centers largely on the observation of state C by means of fluorescence emitted in the decay C→D. Relaxation to highly distorted or ionized states may also take place (B→E) on a picosecond time

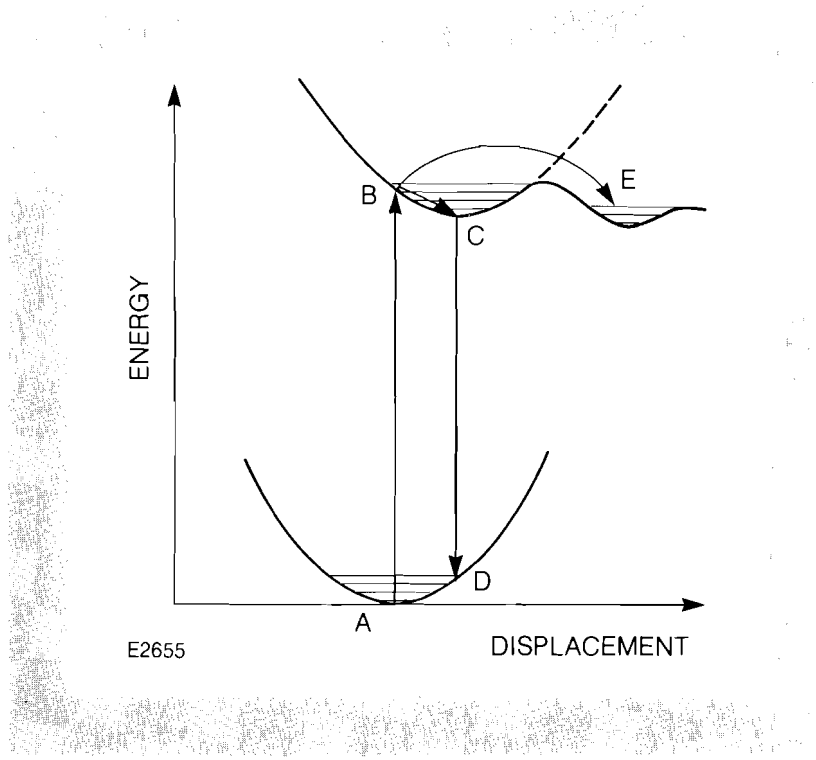

Fig. 29
The states of a molecule initially excited by radiation (A→B) can be sketched on a standard configuration coordinate diagram. The horizontal axis represents the displacement in some important structural mode. Process B→C is excited-state relaxation. Process B→E, where E may involve a highly distorted molecule or an ionized state, also may take place. State C is observed by means of fluorescence emitted in process C→D.

scale. In the experiments described below on primary processes in photosynthesis, the intensity of the fluorescent emission from a given transition C→D is used as a measure of the population of state C. In the second set of experiments on rapid changes in large-scale molecular structure, changes in the polarization of the fluorescent emission, relative to the polarization of the stimulus radiation, are used as a measure of the change in orientation of fluorescing sites on a macromolecule.

The experimental setup shown in Fig. 30 consists basically of a pulsed excitation source, sample, and streak-camera detector capable of 1-ps time resolution.[1,2] The pulse source is an actively and passively mode-locked Nd:YAG laser from which a single 30-ps, 1060-nm pulse is selected (by two Pockels cells in series) and amplified. This pulse triggers the jitter-free, signal-averaging streak camera and, after frequency conversion in crystals (2ω and 4ω), excites the sample. Fluorescence emission by the sample is filtered and imaged onto the streak tube. The streak image is intensified and the data is recorded by an optical multichannel analyzer (OMA). The 4ω crystal, the polarization rotator [Babinet-Soleil (B-S) compensator], and the polarizers are used when the anisotropy of the fluorescence is being measured.

The most relevant photophysical processes occurring in the systems currently under study by users at the LLE picosecond facility are excited-state relaxations within the original manifold of states (B→C in Fig. 29), intermolecular excitation transfer at very small distances such as 1.5 nm, primary electron transfer, and rapid rotation of small fluorescing chromophores (those segments of molecules which absorb

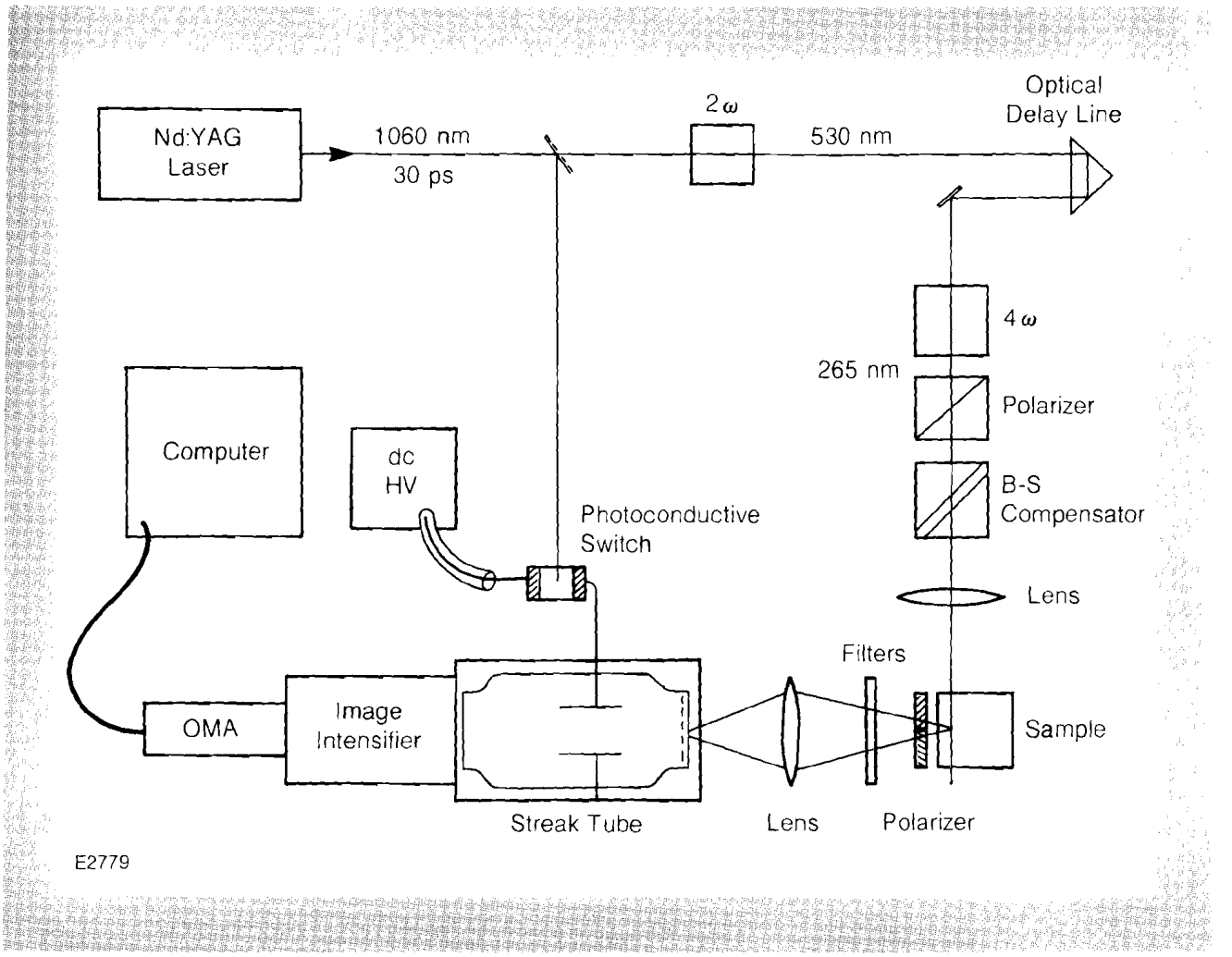

Fig. 30
Schematic of low-jitter, signal-averaging streak camera. A single 1060-nm laser pulse triggers the streak camera, and, after frequency conversion in crystals (2ω and 4ω), excites the sample. Fluorescence emission from the sample is filtered and imaged onto the streak tube. The streak image is intensified and recorded by an optical multichannel analyzer (OMA). The 4ω crystal, polarization rotator [Babinet-Soleil (B-S) compensator], and polarizers are used when the polarization anisotropy of the fluorescence is being measured.

and emit light efficiently) in large macromolecules. The first two of these are known to occur in times of the order of or shorter than 1 ps, while the latter two are in the 1- to 1000-ps range. We have also been studying multiple-excitation transfers on time scales of up to 100 ps, which may be thought of as a diffusion of excitation in a "pool" of chromophores.

While photophysical studies of primary photobiological function may be used to infer structures, it must be noted that such inferences are indirect and in most cases will lead only to the relative orientations of the chromophores involved. However, a more complete picture may emerge when these results are combined with those of more conventional studies of structures.

Research on the Primary Processes of Photosynthesis

The short-pulse detection systems at LLE have been employed to examine the fluorescence from chlorophyll-proteins[3], spinach chloroplasts, and algae. The object of the present research has been to elucidate the physical pathways by which excitation energy is delivered to reaction centers. The various types and functions of these centers are described in Fig. 31. It is now well-established that the

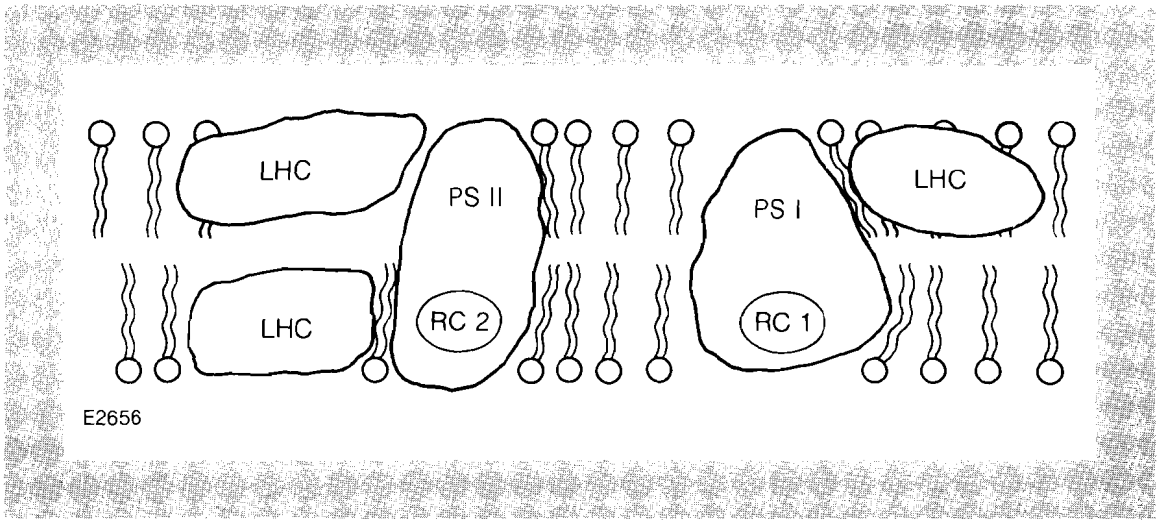

Fig. 31
Schematic cross section of the photosynthetic membrane. Chlorophyll protein LHC (light-harvesting chlorophyll) acts as a photon gatherer; protein complexes PS I and PS II (photosystems I and II) act both as light-gatherers and as loci of photochemical reactions. The small molecules with "tails" represent lipids arranged in a bilayer of thickness about 10 nm. Excitation is funneled to the reaction centers (RC1 and RC2), which energize a long sequence of oxidation-reduction reactions involving numerous intermediates (not shown). In some theoretical models of the primary process, LHC acts also as a medium for transferring excitation energy from PS II to PS I, and in some models, the LHC may move about in the membrane and assist either PS I or PS II in gathering energy.

chlorophyll molecules participating in the primary processes are organized by binding into proteins which are, in turn, imbedded in lipid-bilayer membranes.[4] The details of the chemical steps subsequent to delivery of the excitation energy to the reaction centers are beyond the scope of this review, but we may note that the principal results of the illumination of the structure are the development of a proton gradient across the membrane, oxygen production on one side, and the production of high-energy reductants on the other. The latter ultimately reduces carbon dioxide to starches and sugars in green plants.

After excitation by a visible light pulse, any of the chlorophyll-containing materials mentioned above emits in the region of 685 nm. At liquid nitrogen temperature, a new emission band apparently originating in PS I appears at 735 nm in leaves and chloroplasts (see, e.g., Ref. 5). These emission bands have long been considered as indicators of excitation present in PS II and LHC (685 nm) or in PS I (735 nm).

The research reported here[6] links several earlier approaches. By single-photon counting techniques the low-intensity excitation regime ($\lesssim 10^{12}$ photons cm^{-2} per excitation pulse) has been explored (see, e.g., Ref. 7). Experimentation in this regime has the advantage of being closest to true physiological conditions, but it cannot make use of fluorescence quenching by exciton collisions as a probe, as has been done in the much higher-intensity regime ($\gtrsim 10^{14}$ in the same units).[5] Research in this regime, however, has been limited to studies of total fluorescence yields as a function of intensity and to single-shot or crudely averaged time-course measurements.

Using 30-ps pulses of 530-nm light obtained by frequency-doubling the output of the Nd^{3+}:YAG laser, we have made a long series of measurements of the time-resolved fluorescent response of spinach chloroplasts. Figs. 32(a) through 32(c) summarize the principal results that demonstrate the indirect excitation of PS I and the fluorescence quenching of LHC and PS II at high excitation intensity. The fluorescence time courses have been analyzed in terms of a kinetic scheme

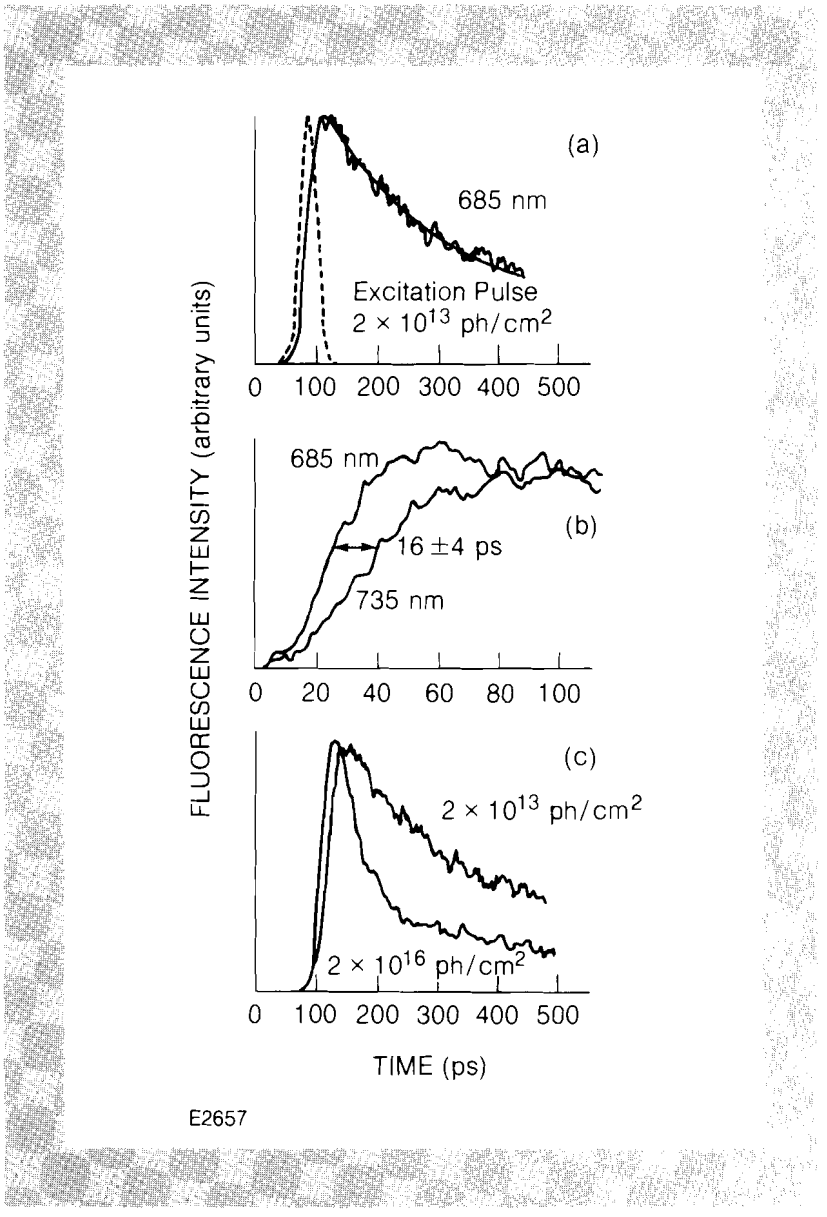

Fig. 32
Fluorescence response of chloroplasts at 77K to a light pulse.

a) Emission of light by spinach chloroplasts in the far-red (685 nm), upper curves, is the result of excitation by a 530-nm pulse, shown by the lower-left curve. The smooth curve is the response expected on the basis of photon-counting results. The 200-shot average response measured by streak camera, the other upper curve, is in close agreement despite high-level excitation at 2X10[13] photons/cm[2] per pulse.

b) The fluorescence at 735 nm, arising from PS I, is seen to be slightly delayed with respect to the 685-nm fluorescence. This fact, along with its rather biphasic rise, indicates that the 735-nm-emitting species receives its excitation indirectly. The excitation of the system was the same as in (a).

c) The 685-nm fluorescence decay acquires a more rapid decay in the 50-ps region when the excitation, otherwise the same as in (a), is made 1000 times more intense. The more rapid decay indicates the premature disappearance of excitons as a result of their colliding with each other at high densities (the process of exciton annihilation).

which accounts for exciton collision effects and corrects for other important nonlinear phenomena. The principal successes of this research are the repeatability of the experiments, the observation of nonexponential portions of the decays, the connections made with previous work, and the possible resolution of some conflicts in the literature.

Work on the photosynthetic apparatus will continue. With both the samples and the detection system now well-characterized, our emphasis will switch to greater spectral resolution and to determining the effects of ion concentrations and different degrees of membrane phosphorylation (chemical addition of phosphate groups to membrane components). The known effects of these physiological variables on membrane configurations can be correlated with their effects on the fluorescence and used for more detailed tracing of excitation pathways.

The study of the primary photoreactions of photosynthesis just described makes use of a direct measurement of the populations of various electronically excited states of chlorophylls from their fluorescent emission. In the work described below, we are interested in fluctuations and motions of the conformation (structure) of large biological macromolecules (polymers). In this case, the fluorescence of small chromophores located in various parts of the macromolecule indicates more indirectly the quantities of interest, namely how fast and by how much the conformation fluctuates. The amplitude and decay rate of the anisotropy (or polarization) of the chromophore fluorescence gives us both quantities. The chromophore's electronic excited-state population, of little interest in itself, serves as a probe of the mechanical-conformational states of the macromolecule.

Probes of Rapid Motion in Biological Macromolecules

Typical macromolecules found in nature can exist in an enormous number of conformational states of roughly the same energy. The two most common examples are the large, polymeric molecules called proteins (polypeptides) and DNA and RNA (polynucleotides). A polypeptide or polynucleotide with N units has roughly e^N such states.[8,9] If $N \approx 200$ (typical polypeptide), there are 10^{90} states; if $N \approx 10^4$ (typical polynucleotide), there are $10^{4,000}$ states. Transition from one state to another can occur when fluctuations cause one unit to move from or rotate about its previous position. Correlated motions of many units can also occur because of the relatively small number (on a macroscopic scale) of atoms involved. The correlated motions can alter enough of the macromolecule to allow water and other physiologically important molecules to move through a previously blocked section of the macromolecule, for example. Measured time scales for motions (fluctuations) range from less than one nanosecond for elementary motions to milliseconds to hours for major conformational changes. The fluorescence depolarization work described below was undertaken to clarify the extent and rate of the fast elementary motions and their dependence upon environmental factors such as temperature, solvent, pH, etc., which alter the biological activity of the macromolecule.

Motional depolarization of fluorescence emission from small chromophores can occur in less than 10 ps, whether the emitter is rotating in solution or executing restricted rotation at a site within a larger macromolecule such as a protein. Measurements on proteins[10] and polynucleotides,[11,12] limited in time resolution to 100-200 ps, have demonstrated motions of bound chromophores as fast as 100 ps, and suggested processes of about 10 ps. To achieve better time resolution we have employed the low-jitter signal-averaging streak-camera system described above which allows simultaneous recording of both emission polarizations in order to achieve good signal-to-noise ratios in determining the fluorescence anisotropy. Both the 2ω and 4ω crystals shown in Fig. 30 were used to convert the laser's fundamental wavelength, 1060 nm, to 265 nm. In one data-collection mode the emission polarizer was cut into two parts. One was oriented horizontally and collected emission from the half of the sample where the laser beam entered. The other was oriented vertically and collected emission

from the other half of the sample. The emission collection lens imaged both halves of the cuvette onto the streak-camera photocathode. The fluorescence anisotropy, defined as

$$r(t) = \frac{I_V(t) - I_H(t)}{I_V(t) + 2I_H(t)}, \quad (1)$$

where $I_V(t)$ is the vertically polarized emission and $I_H(t)$ is horizontally polarized emission (with vertically polarized excitation), decays when the initial preferentially vertical orientation of the excited molecules begins to randomize rotationally.

Human serum albumin (HSA) is a common protein of molecular weight 69,000. It is made up of about 600 amino-acid units. One of these amino-acid units is tryptophan, which fluoresces when excited with ultraviolet light. In Fig. 33 we use this fluorescence to measure the motion of this tryptophan as a function of temperature and solvent. One might expect more and faster motion at higher temperatures. A change in tryptophan's freedom to move might also be expected when a chemical agent such as guanidine hydrochloride is added to unwind the polypeptide chain. In Fig. 33, we find clear time resolution of a fast anisotropy-decay component which develops in HSA at high temperature (320K). This motion is not observed at room temperature (295K). The decay time of the fast motion is about 150 ps; the rotational angle corresponding to the amplitude is about 30°.[2] The slower component decays in at least 3 ns. This is similar to Munro's findings.[10] In the bottom of Fig. 33 we show that addition of guanidine hydrochloride to HSA does not affect r(t) in the same way as high temperature. The value of r(0) is lower, and a fast-decaying component does not appear to be present. The effect of the guanidine is probably due to changes induced in the electronic structure of the tyrosines and tryptophans in HSA. This can be checked by spectral measurements of absorption and fluorescence excitation.

Ethidium bromide (EB) is a dye molecule which binds between base pairs on a DNA chain. If the DNA undergoes torsional motion, the EB will rotate and the fluorescence anisotropy of the dye will decay. In this case we are adding our own chromophore to the macromolecule in order to monitor its motion. Figure 34 shows plotted a typical anisotropy-decay curve for EB bound to calf thymus DNA, together with the excitation light pulse. A fast decay component, roughly 100 ps, is apparent in our data. The theoretical curve was derived using the parameterization of Millar *et al.*[12] (see also Ref. 11) for the Barkley-Zimm[13] elastic-continuum model for torsional motion of DNA. Our data reveal the time course of an additional fast component not contained in the theory. Similar data were found for salmon sperm DNA, for DNA with varying ratios of EB to DNA, and for EB/DNA complexes in solvents of varying viscosities. This fast component may be due to the wobbling of EB within its binding site between two base pairs and not directly connected to the torsions of the DNA chain.

The ultimate importance of rapid internal motions to macromolecular biological activity remains to be determined. The question of the

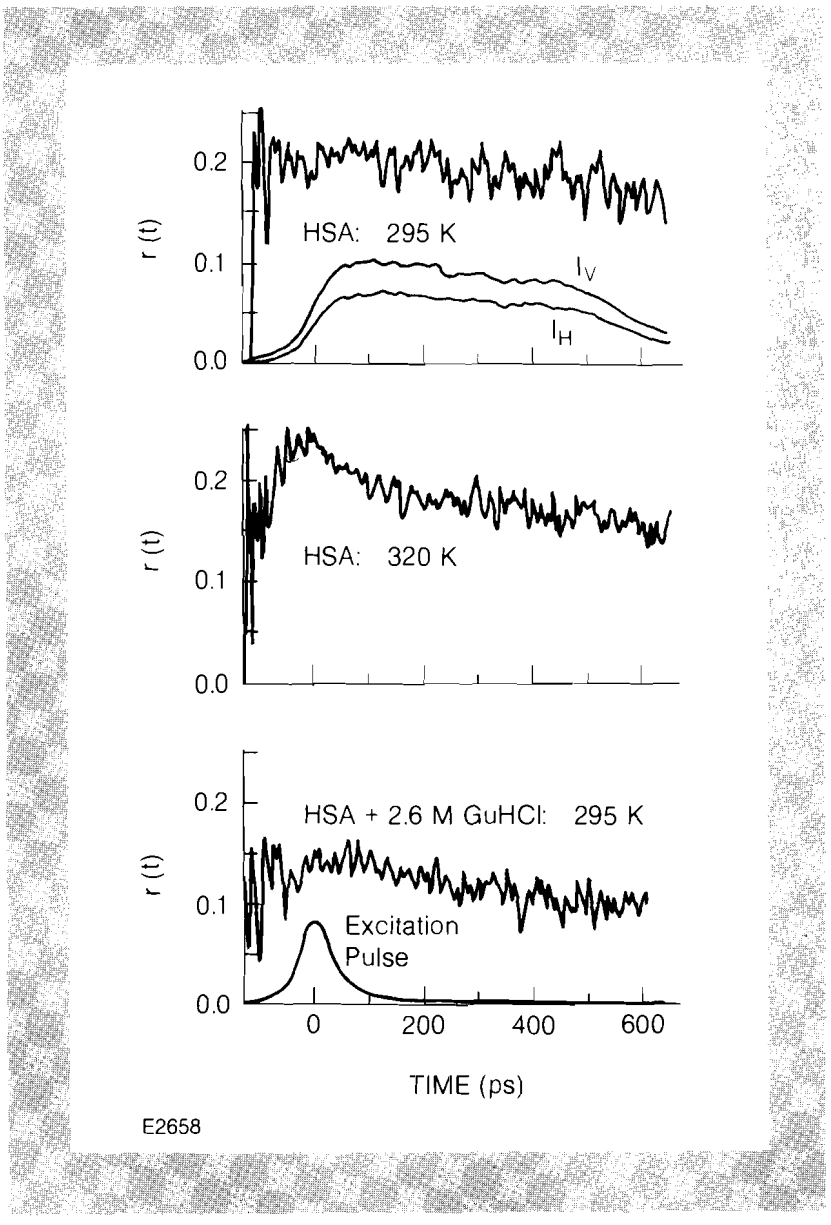

Fig. 33
Fluorescence polarization anisotropy decay from human serum albumin (HSA). The top figure shows the anisotropy at room temperature (295K), together with the vertically and horizontally polarized components of the fluorescence (arbitrary units). There is no fast motion. At 320K, there is fast motion (decay time about 150 ps) which may correspond to a restricted rotation through an angle of 30° (middle). Guanidine hydrochloride (GuHCl, bottom) unwinds the HSA protein chain. One naively expects more rapid, free motion. This is not observed.

correlation of rapid motions to those slower conformational changes known to be biologically significant (inhibitor-induced conformational changes in enzymes which alter reactivity, for example) must be thoroughly studied. It is clear, at least in the case of proteins, that fluctuations of a nanosecond time scale or less are involved in the binding of small molecules to certain proteins (oxygen to myoglobin, for example[9]; see also Refs. 14 and 15). The finding that the active sites of myoglobin and lysozyme contain mobile regions also provides a strong inducement to study the relation of enzymatic activity to fast internal motion. The elastic parameters of DNA (torsional rigidity and fluctuations in torsion angle) are important to know since they reflect base-pairing interactions which affect replication, transcription, and repair of the polynucleotide chain. The binding of mutagenic dyes, histones, and nonhistone proteins to DNA, which are involved in base-pair insertion or deletion, packing of DNA into compact packets, and

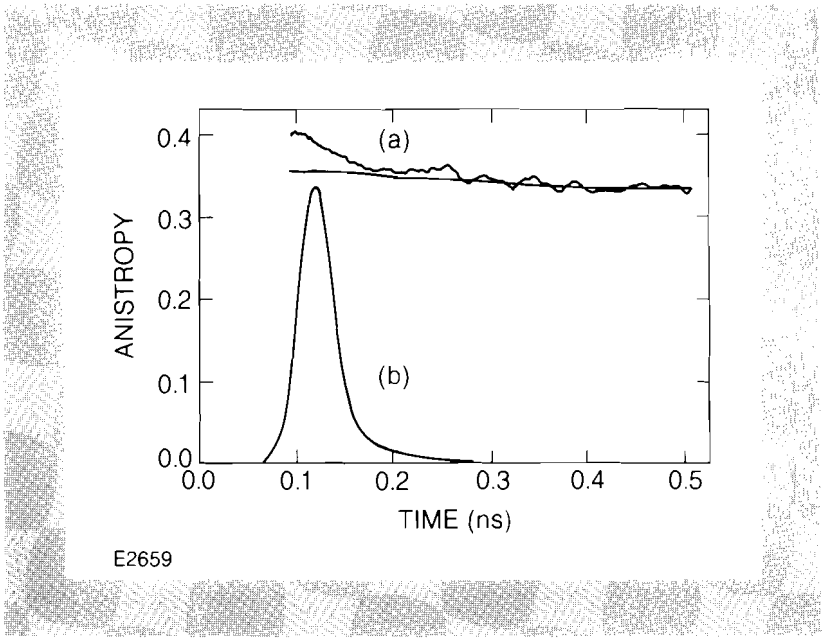

Fig. 34
Fluorescence polarization anisotropy results.
a) The polarization anisotropy decay of emission from ethidium bromide bound to DNA together with a smooth curve whose elastic-continuum-theory parameters have been chosen from a fit to earlier nanosecond data, convolved with instrument response.
b) Instrument response to exciting light (arbitrary units). The time origin is arbitrary.

control of gene function, respectively, must strongly affect the elastic properties of DNA. Finally, it must be kept in mind that, as is always the case with extrinsic fluorescent labels, fast motions may be indicative of label motion independent of the polynucleotide.

Summary

The observations of direct and indirect excitations within the photosynthetic membrane and rapid motion in macromolecules described here represent a sampling of the current picosecond photobiological research at LLE. They typify the application of picosecond techniques to a wide variety of biological questions. The results are being published in more complete form elsewhere.[2,6,16]

ACKNOWLEDGMENTS

The work reported in this article has been initiated and largely performed by users of the LLE picosecond facility and was supported in part by NSF grants which funded the establishment of a subpicosecond biological physics facility. University of Rochester users directly involved in this report are Professor Robert S. Knox and Mr. Bruce P. Wittmershaus, Department of Physics and Astronomy, and Professor Thomas M. Nordlund, of that Department and also of the Department of Radiation Biology and Biophysics. Outside users represented include Professors J. Breton (CEN-Saclay), N. E. Geacintov (New York University), and D. Magde (University of California at San Diego).