METHODS OF BIOCHEMICAL ANALYSIS

Volume 30

METHODS OF BIOCHEMICAL ANALYSIS

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned, not only with the results in the developing fields, but also with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, applier, the teacher, and the student.

It is particularly important that review services of this nature should have included the area of methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore, an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carboydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate, these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evalua-

tion of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analysis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details, a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will be always welcome.

DAVID GLICK

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METHODS OF BIOCHEMICAL ANALYSIS VOLUME 30

The pH Jump: Probing of Macromolecules and Solutions by a Laser-Induced, Ultrashort Proton Pulse—Theory and Applications in Biochemistry

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I. INTRODUCTION

The chemiosmotic hypothesis, alias the Mitchell theory, was accepted in biochemistry with a whole glossary of new terms: proton-motive force, proteicity, proton well, local pH, proton-driven reaction, protogenic site, proton symport, etc. All these terms were intended to describe a specific thermodynamic parameter, or chemical mechanism, with the assumption that their meaning is well defined in the *Biophysical-Biochemical Dictionary*. Although these terms are made up of familiar explicit words, the interpretation of some composite terms is only vaguely implicit. Presently, it is generally accepted that the free energy released by proton transfer between phases of different electrochemical proton potential is converted into other forms of chemical energy (ATP synthesis, active transport, redox reaction). Still, the identity of these phases is not agreed upon. The phases are identified with the whole aqueous bulk, a thin nearly

unimolecular layer on the surface of a membrane, a single proton trapped in an active site, or even an anhydrous hydrochloric acid in the dry lipid interior of a membrane. The various models describing proton transfer through a transmembranal protein consider an array of hydrophilic semirigid carriers spanning the protein, a water molecule channel, an ice-like microthread, or even approximate the proton channel by $Al(OH)_3$ crystal at 300°C.

Proton transfer in biochemical systems is measured, in most cases, as an outcome of external force (ATP, redox potential, etc.) mediated by an enzyme. Enzymic turnover is a million to a billion times slower than the basic events of proton transfer. Because of this huge difference in time scale, enzyme-driven proton transfer is blurred by the noncoherent catalysis. By the time the first turnover is completed, the proton had ample time to equilibrate with the whole bulk of the solution.

Because of these reasons, my colleagues and I initiated a few years ago a detailed study of proton transfer in an aqueous system, where the event is synchronized by a laser pulse. This technique, using signal averaging, retains the temporal parameters of the event and allows the evaluation of the probabilities of finding a proton in putative environments assigned for it by the different bioenergetic models.

During these studies, it became apparent that proton transfer is an extremely sharp instrument for gauging the water in the immediate environment surrounding the site of dissociation. It turned out that the general biological solvent, the water, acquires different properties at the site where biochemical reaction takes place—the surface of the enzyme. These local properties of the water can be measured through the technique of the laser-induced proton pulse, free of perturbation caused by the huge mass of the bulk water.

In this chapter, I shall describe the basic methodology of the laser-induced proton pulse. Starting with the initial event of a synchronous proton dissociation, going through the reaction of a proton with other solutes in a true solution, and ending with the complex multiphasic system of protons, macromolecules, and interfaces associated with the real life of biochemical reaction. In each level of complexity, I shall point out the pertinent information available for interpretation and the mode of mathematical and physical analysis. In some cases, I shall also reflect the conclusions on current hypotheses of biochemical proton transfer.

II. METHODOLOGY AND INSTRUMENTATION

The experiments described in this chapter can be carried out in any laser laboratory equipped for monitoring fast photochemical reactions.

1. Dynamics of Proton Dissociation from Excited Molecules

This reaction is observed through time-resolved fluorescence measurements. The sample is excited by a short laser pulse and the fluorescence intensity at the proper wavelength is followed with time.

The lifetime of the measured events varies between 100 psec to ~20 nsec. The time constant of the measured reaction limits the duration of the excitation pulse. Unless the pulse is shorter than 10% of the lifetime of the measured reaction, the observed signal must be deconvoluted to correct for the time profile of the perturbing event.

The intensity of the excitation pulse is not critical, yet it is advisable to use low-energy density. High-energy flux enhances the probability of undesired two-photon effects.

The light source for time-resolved fluorescence can be a nanosecond pulse of Blum-line nitrogen laser, triple harmonics of yttrium-aluminum-garnet (YAG) laser, second harmonics of mode-locked dye, or gas laser.

The fluorescence decay can be measured with a streak camera, a very fast photomultiplier tube-like Hamamatsu 1294U attached to Tektronix transient digitizer, a box car integrator, or photon counting.

2. Dynamics of Protonation of Ground-State Compounds

The reaction is followed through transient absorbance measurements. The sample is excited by intensive laser pulse (0.2–2 MW/cm^2) and absorbance changes in the irradiated volume (usually 0.05–0.1 ml) are monitored by a probing light beam at the proper wavelength (see Figure 1).

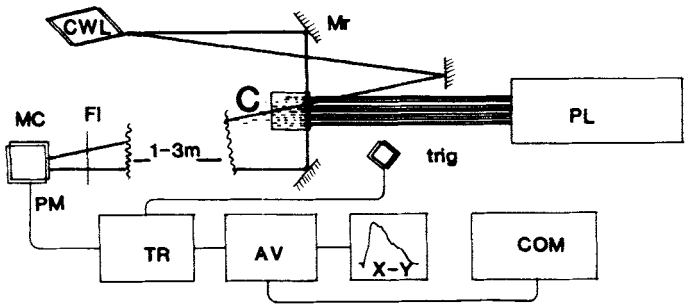

Figure 1. Optical arrangement and elements needed for transient absorbance measurements. C, observation cell; PL, pulse laser; CWL, CW laser; Mr, mirror; Fl, filter; MC, monochromator; PM photo multiplier; Trig, triggering photo diode; TR, transient recorder; AV, signal averager; COM, computer; X-Y, XY recorder.

A. EXCITATION PULSE

The excitation pulse should be less than 10% of the fastest time constant that is measured. A pulse of suitable duration (1–10 nsec) and energy (0.5–10 mJ) can be obtained by many commercially available nitrogen or excimer lasers.

High-energy input into the solution (more than 10 mJ) can lead to rapid accumulation of undesired photoproducts. Thus, it is better to use a short (1–2 nsec), less intensive pulse (0.5–2 mJ) than massive (5–50 mJ) longer ones (5–20 nsec).

The length of the proton cycle is 10–300 μsec. Thus, even at high repetition rate (100–400 Hz) available with some gas lasers, the system will relax to its prepulse state before the next pulse. Thus, high repetition rate cannot compensate for low peak power of the laser. I found it practically impossible to use a peak power of less than 50 KW.

B. MONITORING LIGHT

The monitoring light should fulfill the following requirement: Its energy-density modulation at the entrance slit of the monochromator should be higher than the energy of the fluorescence emitted from the observation cell. About 1–10% of the MW excitation pulse is emitted as fluorescence over a wide spectral range. Even if 0.1% of the fluorescence falls at the wavelength of the monitoring beam, it amounts to 10–100 W of light energy. To prevent it from saturating the photomultiplier, it must be damped below the energy of the oncoming signal. The simplest way to reduce the fluorescent light is to keep the monochromator far from the reaction cell, 1–3 m. The monitoring beam should probe only the irradiated volume, that is, not more than 1–1.5 mm in diameter, and all of its energy should reach the entrance slit of the monochromator—without the assistance of a lens. A lens will focus both monitoring and fluorescent light and no advantage is gained.

The fluorescence spans a wide spectral range, thus the narrower is the wavelength of the probing source, the lesser will be the incremental energy of the fluorescence. Thus the monitoring light should be highly monochromatic collimated intensive beam, that is, the output of a cw laser.

C. MEASURING EQUIPMENT

The measuring equipment needed to follow transient absorbance can be as simple as a fast oscilloscope and a Polaroid camera. But a transient recorder coupled to signal averager, *xy* recorder, and computer have a certain advantage.