A Letter from the Director

As many of you know, Gary Holtom recently resigned his position with the RLBL to take up new challenges at the Batelle- Pacific Northwest Laboratory in Richland, Washington. Gary was a critical player in the evolution of this facility and is largely responsible for its success. We will sorely miss him. I know that all of our past and present users and regulars of our Newsletter will want to join me in wishing Gary great success in his new adventures.

One of Gary's great accomplishments here was the development of a user-friendly single photon counting fluorescence lifetime apparatus. Starting from scratch, he assembled and created lasers and associated computer controls and analytical methods that brought our Facility into the cutting edge of the fluorescence field. Routinely achieving a resolution of 19 ps on an electronically controlled instrument is a major accomplishment on its own but to have made this instrument user friendly is nothing short of remarkable. Approximately 200 different projects were carried out on this instrument: it is Gary's legacy to the Facility and we are committed to stay ahead in this area. Accordingly you are all invited to submit proposals to use it!

We are extremely pleased that Mark Phillips has graciously agreed to take over as Head of Laser Operations. This means that Mark will be the new Editor of our Newsletter. I am confident that Mark will do a fine job since I know he is devoted to the goal of providing laser related resources and expertise not otherwise available to biochemical, biophysical and biomedical researchers.

It is our intention in the Spring to bring on board another staff member who will broaden our range of experimental skills into the most needed areas. I would be happy to receive suggestions from any readers on this matter.

Robin M. Hochstrasser
Director, RLBL

Editor's Note

I look forward to the new challenges presented to me as Head of Laser Operations. Two new facility resources are nearing completion. The first is a picosecond transient absorption spectrometer which employs the same state-of-the-art multichannel detection as in the nanosecond transient absorption spectrometer and the second is a general Raman instrument which is to be computer interfaced in order to make it user-friendly. We feel these two systems will greatly enhance the capabilities of the RLBL.

In this Newsletter we present two studies which demonstrate the wide scope of activities performed at the Facility. The first (performed with Mark Chance at Georgetown University) centers around the use of transient absorption techniques to investigate the activation dynamics of coenzyme B\textsubscript{12}, a biochemically important radical initiator. The second describes a series of groundbreaking studies in which the goal is non-invasive tissue imaging using picosecond order light pulses (in conjunction with Britton Chance and the Medical School of the University of Pennsylvania).

Topics to be discussed in the next issue (scheduled for mid-Spring release) will be excited triplet state reaction dynamics in metal-substituted myoglobins and molecular mechanisms of muscle contraction.

Mark Phillips (215) 898-3605.
Nanosecond Transient Absorption Spectroscopy of Coenzyme B$_{12}$: Quantum Yields and Spectral Dynamics.

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The focus of our research is to understand the mechanism of adenosylcobalamin (coenzyme B$_{12}$) activation. Studies on B$_{12}$ dependent enzyme systems such as ethanolamine ammonia-lyase, ribonucleotide reductase and diol dehydrase have shown that a crucial step in activation is the homolytic cleavage of the unusual organometallic Co-C bond, generating an optically detectable Co(II) species and a deoxyadenosyl radical. 1-3 Although much is known about the structure of isolated coenzyme, relatively less is known regarding its interaction with enzymes, the accompanying structural changes upon binding, and the mechanism of homolytic cleavage. The goal of many cobalamin studies are focused on the factors that weaken the Co-C bond and displace the equilibrium of homolysis towards dissociation. There has been an emphasis on the importance of several structural features of coenzyme B$_{12}$ and of steric factors in homolysis of the bond. 4-7

It is known that photolytic cleavage of the Co-C bond, as in enzyme induced cleavage, also results in homolytic cleavage. Since enzymatic cleavage does not involve excited states, as does photolytic cleavage, there are undoubtedly differences in the mechanisms. Determination of a mechanism for photolytic cleavage may help us understand why the two processes give the same products. Our immediate goals are to determine the importance of geminate processes and structural distortion, especially of the corrin ring, on bond lability, and to compare these factors in other cobalamin compounds. At present we have used laser photolysis and a nanosecond transient absorption system with a dual diode array detector to investigate the structure and lability of the cobalt-carbon bond for two cobalamin compounds, coenzyme B$_{12}$ and "base-off" B$_{12}$.

The quantum yield of photolysis for coenzyme B$_{12}$, 0.23 ± 0.04, is fivefold higher than base-off B$_{12}$, 0.045 ± 0.015.

The base-off B$_{12}$ is obtained by acidification of the coenzyme B$_{12}$ which results in cleavage of the Co-N bond of the DMB group, and subsequent

![Figure 1. Structure of adenosylcobalamin.](image)
displacement of the Co atom out of the plane of the equatorial corrin ring ligands towards the adenosyl ligand. The resultant structure has a stabilized Co-C bond, relative to its parent coenzyme B$_{12}$, and is therefore expected to demonstrate a lower tendency for photolysis. This structural prediction is in accordance with our experimental results and demonstrates a correlation between cobalt-carbon bond strength and quantum yield.

Model compound studies and x-ray analyses have demonstrated that the corrin ring in coenzyme B$_{12}$ is highly strained due to the presence of two bulky axial ligands - the dimethylbenzimidazole group and the adenosyl ligand (Figure 1). Photolysis of the Co-C bond presumably results in relaxation of the corrin ring distortion. The changes of the corrin ring from an energetically unfavorable, "excited" structure to an energetically favorable conformation may be one driving force for bond cleavage. Enzymatically, the corrin ring may influence homolytic cleavage through a transfer of strain energy from ring to bond. Since studies have shown that photon absorption is principally due to the corrin ring, it is likely that photolysis is a consequence of a similar transfer of photon energy to the bond. We examined the degree of communication between the ring and bond by determination of the wavelength dependence of the quantum yield. For coenzyme B$_{12}$ the quantum yield at two laser wavelengths showed no significant change, demonstrating 50% or greater efficiency in energy coupling of the ring to the Co-C bond. We expect that for base-off B$_{12}$, whose bond is much harder to photolyze, that the transfer of energy would be less than 50% and that the quantum yield would have greater wavelength dependence.

After the Co-C bond is cleaved, relaxation of the corrin ring is expected to follow. We observed in our data for coenzyme B$_{12}$ reproducible variations between nanosecond and static difference spectra (Figure 2). A possible explanation is the presence of a long lived strained state, which implies that some structural memory of the excited state is retained even after several nanoseconds. We have followed the spectral relaxation from 5ns to 5ms and are currently attempting picosecond transient absorption studies in order to observe a more highly strained conformation. The spectral differences for base-off B$_{12}$ are also being investigated.

A comparison of our results with previous picosecond transient absorption studies suggest that geminate recombination plays a significant role in control of homolytic cleavage. Although previous data is poor it indicates that the extent of geminate recombination for methyl B$_{12}$, which has a CW quantum yield twice that of coenzyme B$_{12}$, is approximately half that of coenzyme B$_{12}$. This suggests that CW quantum yields are controlled by geminate rebinding. If geminate recombination is substantial, a broken Co-C
bond will not lead to separation and generation of radical species. Dynamic structural processes which raise the barrier to geminate recombination allows for radical formation and shows a higher nanosecond quantum yield. Future experiments at the RLBL will involve studies on ultra-fast spectral dynamics in order to understand the role of geminate recombination and the role of energy transmission from the corrin ring in the mechanism of Co-C bond homolysis.

REFERENCES


Applications of Time Correlated Single Photon Counting to Study Photon Migration in Tissues and Model Systems

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In 1987, Chance and coworkers\(^1\) applied Time Correlated Single Photon Counting (TCSPC) techniques to the in vivo study of photon migration in tissue arising from an incident picosecond order pulse of light. These temporal studies revealed the existence of a fast rising component followed by a long decay.

Further investigations showed that the decay of the photon intensity is determined by the quantity of absorbing material which is expressed by the equation:

\[ I(t) \sim I_0 e^{-2.303 k t} \]  

(1).

This is a consequence of the Beer-Lambert-Bougher law:

\[ I(t) = I_0 e^{-2.303 \varepsilon C / l} \]  

(2)

where \(\varepsilon\) is the molar absorptivity, \(C\) is the concentration of absorber, and \(l\) is the effective pathlength. This pathlength is related to \(t_l\), the "time of flight":

\[ l = c t_l / n \]  

(3).
In this equation c is the speed of light and n the refractive index of the bulk media (in our case, water; n =1.33). Using the equations above, a semi-logarithmic relationship describing the temporal decay of the detected light intensity follows:

\[ \mu = -1/ \log \left[ \frac{I(t)}{I_0} \right] = \varepsilon C \]  

(4)

where \( \mu \) is the linear absorption coefficient (in \( \text{cm}^{-1} \)).\(^1\) Therefore the concentration of absorber can be inferred from a measured photon decay rate.

Two theories of photon migration in tissue have since been formulated. The theory of Bonner, et al.\(^2\) describes the migration of photons as a random walk along a discrete scattering lattice. Patterson, et al.\(^3\) describe the migration in terms of a diffusion process. Both theories give very good approximations to our preliminary \textit{in vivo} experiments.

A representative sample of the TCSPC photon migration experiments which have been performed at the RLBL are described in the following four sections; i) model experimental systems in order to test the theories of photon migration, ii) remote \textit{in vivo} sensing of oncogical photosensitizers, iii) hemoglobin saturation in tissue and iv) determining the sensitivity of time resolved technique for the ultimate goal of localization of metabolic heterogeneities such as hemorrhagic brain.

REFERENCES


I. Photon Migration Through Layered Media

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Time-resolved optical spectroscopy is being used to study the pathlength distributions of photons moving through composite media. Model experimental systems have been constructed from polymer gels impregnated with varying amounts of well-defined scatterers and appropriate chromophores as absorbers. These specimens are being examined in order to understand how the sample characteristics and instrument probe geometries affect optical remote sensing of physiological parameters. Computer simulations have indicated strong interdependences on these variables; for example, when the outer layer of a composite medium is much more strongly absorbing than is an underlying substrate, the spacing between delivery and detecting optical fibers determines whether re-emergent photons mostly probe the top-most or bottom-most layers (Fig. 1). Quantitative studies are being carried out to test the predictions of analytical theories, and similar investigations are being made on systems in which bone comprises the superficial layer.
When the fiber separation is sufficiently great, path length characteristics are those of photons which propagate through the underlying low absorption substrate; when the fibers are closely spaced, the detected photons primarily move only within the superficial layer.

![Figure 1. Photon Migration Through Layered Media; expected value of path length vs. distance between delivery and detection fibers, plotted for differing thicknesses of superficial layer (optical absorption in the upper layer is twenty times that of the bottom layer - see reference).](image)

REFERENCES


II. Applications of Time-Resolved Light Scattering Measurements to Photodynamic Therapy Dosimetry

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When tumors are treated with photodynamic therapy, two physical variables of great importance in determining biological response are the distribution of photoactivating light and the concentration of photosensitizer within the tumor. While a number of methods are being investigated to measure these variables, there are currently no generally accepted techniques for noninvasively obtaining this information on individual patients. The advent of picosecond light sources and fast detectors, however, offers the ability to study the time dependence of light scattered by tissues.

Having this time resolution makes possible determination of the absorption and scattering coefficients of tissue; this information can then be used to calculate the temporal variations of light arising from various source/detector geometries. In addition, if a tunable light source is used, the spectral dependence of the absorption and scattering coefficients can be measured. If a photosensitizer is present in the tissue then the magnitude of light absorption and scattering can be used directly to yield the concentration of the photosensitizer.
In preliminary experiments, time resolved photon counting techniques were used to measure the light scattered from feline leg muscle as increasing amounts of the photosensitizer aluminum chloro-sulphonated phthalocyanine (AlSPC) were injected intravenously. The magnitude of photon absorption was found to be directly proportional to injected dose (Fig 2).

This work is supported by the National Cancer Institute of Canada and the Ontario Laser and Lightwave Research Centre.

REFERENCE


III. Hypoxia and Hemodilution in Canine Brain Model

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The altered migration of light at 760 nm during severe hypoxia and hyperemia has been studied. The purpose of this work is to determine the change in the effective light pathlength ($\Delta l$) during severe hypoxia and $\Delta l$ during hemodilution to determine the contribution of brain tissue background absorption at 760 nm; this wavelength corresponds to the absorption maximum of deoxyhemoglobin. This will assist us in creating an in vivo procedure for measuring deoxyhemoglobin levels in the brain.

A venous catheter was applied to an adult male dog. Optical fibers for transmitting and receiving pulsed laser light were placed at 2.5 cm separation on the exposed animal skull. TCSPC spectra were taken both at normoxia and hypoxia (14%, 12%, and 10% $O_2$ in the ventrous mixture). Hemodilution consisted of removing 100 cc of blood and plasma...
and replacing with an equal amount of saline. A spectrum was taken during each hemodilution. After 500 cc of hemodilution, three hypoxic events were measured. The range of hemodilutions is indicated in Fig. 3. The $\Delta l$ during hemodilution was $\sim 6$ cm.

IV. Quantization of Hemoglobin Deoxygenation in Adult and Neonate Brain

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The use of red and near infrared light for spectroscopy of hemoglobin and cytochrome in the brain has been confounded by the inability to quantify the concentration changes due to a lack of knowledge surrounding optical pathlength determinations in highly light scattering tissue. The use of picosecond light pulses in TCSPC enables "time of flight" determinations of the optical path and thus quantization of hemoglobin concentration changes during cerebral hypoxia or other pathological insults.

Figure 3. A) Quantization of hemoglobin concentration by photon-transit through dog brain (760 nm). The initial slope, $\mu \ [= -1/\log (l/\text{effective path length})]$, decreases with Hb deoxygenation (decreased venous $O_2$ percentage [fraction inspired oxygen-$FiO_2$]). B) increasing slope through induced anemia (the numbers indicate cc of blood withdrawn and replaced by equal volumes of saline). Input pulse ($\times 10^{-4}$) solid line, output pulse dotted line.

Figure 4. Derived pathlength during course of hypoxia and hemodilution in canine model (760 nm; ordinate indicates spectrum sequence). Pathlength decrease ($\Delta l \sim 2.9$ cm) during hypoxia due to increase in deoxyhemoglobin and decreases during hemodilution ($\Delta l \sim 5.9$ cm) due to removal of hemoglobin.

Figure 5. Temporal response of scattered 760 nm light on human forehead; 5 cm fiber separation.

Fiber probes are applied to the surface of the head at spacings of several centimeters. Photons propagating directly from input to output largely escape through the surface of the skin; those that successfully migrate from input to output travel along paths as long as 1 meter (at...
23 cm/ns). Fig. 5 illustrates the temporal response of the output light pulse.

If the migrating photons encounter a region of hemorrhagic brain, increased absorption will occur at wavelengths where the molar absorptivity of deoxyhemoglobin (Hb) is greater than that of oxyhemoglobin (600-800 nm). In this case $\mu$ will increase, resulting in a faster observed decay and an overall attenuated photon output (Fig. 6). The deoxyhemoglobin concentration can be calculated from the known molar absorptivity ($\varepsilon$), optical path ($l$), and detected light intensity ($I$) according to the algorithm described in the introductory section.

While the decay characteristics strictly follow an exponential decay in uniform tissue such as normoxic brain (Fig. 5), discontinuities are observed in models of brain bleeding when an absorber such as deoxyhemoglobin is proximal to the input and output points for the light pulses resulting in a steeper initial slope corresponding to short migration pathways (Fig. 6). The value of $\mu$ depends strongly on the relative proximity of the two absorbers in the hemoglobin containing cylinder (Fig. 7), it therefore appears that localization algorithms can be developed from multipoint data acquisition.

**CONFERENCE PRESENTATIONS**

SPIE Technical Symposium on Laser Spectroscopy (14-19 January 1990, Los Angeles);
Conference 1204-Time Resolved Laser Spectroscopy in Biochemistry II

*Artifacts and diagnostics in fast fluorescence measurements*
G.R. Holtom-Univ. of Pennsylvania

*Reactions of excited triplet states of metal-substituted myoglobins*
S.J. Papp, J.M. Vanderkooi, C.M. Phillips-Univ. of Pennsylvania

*Optical properties of adult human brain and skeletal muscle as studied by pulse time and phase modulation techniques*
B. Chance, G.R. Holtom, M.B. Maris, K. McCully-Univ. of Pennsylvania
Picosecond resolution study of intramolecular energy transfer in lumazine protein  
J.W. Lee-Univ. of Georgia  
G.R. Holtom-Univ. of Pennsylvania

COLLABORATIVE PUBLICATIONS


CORE RESEARCH PUBLICATIONS


Solvent effects on intramolecular proton transfer. Y.R. Kim, J.T. Yardley and R.M.

