CHEMICAL BIODYNAMICS DIVISION

Annual Report

October 1, 1985 – September 30, 1986

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MASTER

ANNUAL REPORT, CHEMICAL BIODYNAMICS DIVISION

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TABLE OF CONTENTS

	Page No.
I. INTRODUCTION	iii
II. ORGANIZATIONAL CHART	iv
III. OVERVIEW OF RESEARCH	1
A. KCO3 - Chemical Sciences	2
1. The Chemistry of Electronically Excited Molecules Professor George C. Pimentel	3
2. Photon Conversion Through Storage of Metastable Molecules Dr. Heinz Frei	5
3. Artificial Photosynthesis Professor Melvin Calvin and Dr. John W. Otvos	7
B. KCO6 - Biological Energy Research	10
1. Photosynthesis Reactions Professor Kenneth Sauer and Dr. Melvin P. Klein	11
2. Regulation of Expression of Genes in <i>Rhodobacter capsulatus</i> by Light and Oxygen Professor John E. Hearst	14
3. Light Regulation of Nuclear and Chloroplast Nucleic Acid Synthesis in <i>Euglena gracilis</i> Dr. James C. Bartholomew	17
4. Tissue Specific Gene Expression in Plants Dr. Francesca Leach and Dr. James C. Bartholomew	18
5. Plant Biochemistry Professor Henry Rapoport	21
6. Hydrocarbons from Plants Professor Melvin Calvin and Dr. John W. Otvos	25
C. HAO2 - Environmental Research and Development	30
1. Biomolecular Structure Analysis by NMR Professor David E. Wemmer	31
2. Biophysical Chemistry Dr. Melvin Klein	33
3. Mutagenesis - Fundamental Chemistry Professor John E. Hearst	34

4. Control of DNA Synthesis in Human Cells	
Dr. James C. Bartholomew	36
5. Structural Biology	
Professor Sung-Hou Kim	40
D. National Tritium Labeling Facility	
Professor Henry Rapoport	47
E. DOE Sponsored University Contract	
Professor Ignacio Tinoco	52
IV. APPENDICES	
A. Ph.D. Theses	53
B. Statistical Data	54

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INTRODUCTION

The Laboratory of Chemical Biodynamics (LCB) was established in 1945 as a Division of the Lawrence Berkeley Laboratory to conduct basic research on the dynamics of living cells and on the interaction of radiant energy with organic matter. The Division has made rich contributions to our understanding of the molecular mechanisms of photosynthesis and of the effects of environmental pollutants on plant and animal cells. Much of its funding is provided by the U.S. Department of Energy and the National Institutes of Health through the Lawrence Berkeley Laboratory. LCB is also an Organized Research Unit (ORU) of the University of California and additional research support comes to the ORU from the National Science Foundation, the National Institute of Health, and from private and industrial grants-in-aid. Chemical Biodynamics draws its M.S. and Ph.D. candidates from over thirteen departments and groups of the University, with the single largest component being in chemistry. The Division attracts research scientists from all over the world; during the current year 11 foreign postdoctoral and visiting faculty personnel from seven countries have conducted research in the Chemical Biodynamics Laboratory.

Four broad aims guide the Division:

- to maintain the strong interdisciplinary character of the laboratory.
- to build upon the expertise of the existing laboratory staff and to exploit the excellent array of facilities in LCB.
- to develop coherent and contemporary research themes that are fundamental in character, forward looking in their broad direction, and that optimize the likelihood of synergistic interaction across the disciplines represented in the laboratory.
- to strengthen collaborative ties to faculty research scientists in the biological sciences.

Two new research themes are being developed that are consistent with these aims. Both are directed at fundamental areas of knowledge essential to the most effective utilization of solar energy. The first theme, **Photon Conversion**, pertains to the fundamental aspects of the interaction of light with matter; the absorption, migration, relaxation, transformation, and storage of energy derived from photons. It will extend from photobiology, already a strong area within the laboratory, through photochemistry to photophysics.

The second theme, **Genetics of Photosynthesis**, is the application of recombinant DNA techniques to photosynthetic organisms. The staff's experience in molecular biology, plant cell culture, and the functional mechanisms of the chloroplast are being exploited as we investigate and attempt to control the genetic material from which the photosynthetic apparatus arises.

George C. Pimentel, Associate Director
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OVERVIEW OF RESEARCH

The Laboratory of Chemical Biodynamics continues to base its research plan on the importance of its role in the application of sophisticated chemical sciences to problems relevant to the mission of the Department of Energy. Our Laboratory is in the unique position that it is staffed mainly by investigators trained in chemistry with an interest in applying these skills to both biological and energy science problems. Our mission is to carry out research that takes advantage of our unique skills, as well as to train young investigators in the fields we so strongly represent.

The research in the Laboratory of Chemical Biodynamics is almost entirely fundamental research. The biological research component is strongly dominated by a long term interest in two main themes which make up our Structural Biology Program. The first interest has to do with understanding the molecular dynamics of photosynthesis. The Laboratory's investigators are studying the various components that make up the photosynthetic reaction center complexes in many different organisms. This work not only involves understanding the kinetics of energy transfer and storage in plants, but also includes studies to work out how photosynthetic cells regulate the expression of genes encoding the photosynthetic apparatus. The second biological theme is a series of investigations into the relationship between structure and function in nucleic acids. Our basic mission in this program is to couple our chemical and biophysical expertise to understand how not only the primary structure of nucleic acids, but also higher levels of structure including interactions with proteins and other nucleic acids regulate the functional activity of genes.

In the chemical sciences work in the Laboratory, our investigators are increasing our understanding of the fundamental chemistry of electronically excited molecules, a critical dimension of every photosynthetic energy storage process. We are developing approaches not only toward the utilization of sophisticated chemistry to store photon energy, but also to develop systems that can emulate the photosynthetic apparatus in the trapping and transfer of photosynthetic energy.

A. KCO3 - CHEMICAL SCIENCES

This program encompasses research directed at a fundamental understanding of electronically excited molecules with special attention to features that relate to the storage of photon energy in the form of high free energy chemical bonds.

- One project focuses on the manganese catalytic function in the oxidation aspect of artificial photosynthesis, the photo-induced reduction of CO_2 into organic products potentially useful as fuels, and charged colloid or polyelectrolyte interfaces for increasing quantum efficiency in photosynthetic processes based on electron transfer.
- A second project spotlights the special chemistry of electronically excited molecules and atoms using infrared spectroscopy as a diagnostic tool and tuned laser excitation to map electronic reaction hypersurfaces, both for unimolecular (photochromic) and biomolecular reactions.
- The third project in this program investigates the indefinite storage of long-lived electronically excited molecules including those that can be initially prepared with near infrared photons, the spectral region in which most of the solar energy is found.

1. THE CHEMISTRY OF ELECTRONICALLY EXCITED MOLECULES (G.C. Pimentel)

Using infrared spectroscopy as our primary diagnostic technique, we are studying chemical changes as they occur on electronic hypersurfaces. In order to map the upper surfaces, photochemistry is initiated using tuned laser excitation. Photochromic systems furnish unimolecular reactions that take place on an electronic hypersurface; we are studying the azirine/ylide system. Bimolecular reactions under study include the reactions of electronically excited Hg, Cd, and Zn atoms with halogenated ethenes and the photoinduced reaction of HX with acetylene.

The research problems all intend to increase our fundamental understanding of the special chemistry of electronically excited molecules, which is inherent in every photosynthetic photon energy storage process. In its excited state, the orbital occupancy, charge distribution, molecular structure, and chemical reactivity of a molecule can differ radically from those of the ground state. It is these factors that we wish to exploit for energy storage.

Electronically Induced Unimolecular Reactions

Phenyl azirine can be photoconverted into its ylide counterpart with ultraviolet light (e.g. 250 nm) and then the ylide can be converted back to the azirine form with longer wavelengths (e.g. 350 nm). With the phenyl azirine suspended in a cryogenic matrix of solid argon, it can be converted quantitatively to the ylide form as shown by the FTIR spectrum. A number of isotopically substituted phenyl azirines have been so studied in order to establish for the first time an unequivocal structure for the ylide. We have shown conclusively that the nitrogen atom occupies the central position in an allene-like zwitterion structure.

We are now attempting to measure the quantum yield as a function of photon energy, using our tuned laser photolysis source, to map the electronic hypersurface on which this photochromic interconversion takes place.

Electronically Induced Biomolecular Reactions

HX-Acetylene. In a matrix, this reaction pair has the very special characteristic that the orientation of the HX molecule is fixed by hydrogen bonding in a T-shaped geometry with the hydrogen atom pointed toward the triple bond.

When the HX molecule is photolyzed, it reaches a state that is dissociative for the isolated molecule, so the hydrogen atom receives translational energy essentially equal to the difference between the photon energy and the bond energy. Whether this will remain true for an HX molecule interacting with a nearby acetylene is a question of some interest. This is especially so when the excess energy becomes small, because then the change from HX to DX implies a considerable change in the momentum at fixed energy.

Our results show that vinyl bromide, if formed, is not responsible for the observed products via secondary photolysis. Whereas photolysis of vinylbromide in a matrix gives predominantly $\text{HBr} \bullet \text{C}_2\text{H}_2$, the photolysis of $\text{HBr} \bullet \text{C}_2\text{H}_2$ in a matrix gives $\text{BrCCH} + \text{H}_2$. When $\text{HBr} \bullet \text{C}_2\text{D}_2$ is photolyzed, $\text{HCCD} + \text{DBr}$, $\text{BrCCD} + \text{HD}$, and $\text{BrCCH} + \text{D}_2$ are all observed. Similar experiments have been conducted with HI and both hydrohalide-acetylene reactions are now being studied as a function of wavelength.

Column 2B Metal Metal-Olefin Reactions

The matrix reactions between Hg, Cd, and Zn atoms excited to the ^3P state with halogenated olefins has been studied and compared to the photochemical products observed in absence of the metal atom with singlet excitation of the double bond. Photoinduced metal atom insertion into a C-Cl bond has been observed for the first time for 2-chloro-1,1-difluoroethene and for both *cis* and *1,1*-dichloroethenes. In addition, the dichloroethenes display Cl_2 elimination but not HCl elimination when reaction is photoinduced on the triplet energy surface defined by the ^3P metal atom. In contrast, HCl elimination occurs but not Cl_2 elimination on the singlet surface reached in absence of the metal atom. The data show that chemistry is different on the triplet surface than on the singlet surface and that the results are not compatible with a simple energy transfer from $\text{M}(^3\text{P})$ to the olefin. It is gratifying that the Cl_2 elimination induced on the triplet surface stores about half of the photon energy, a significant improvement over the 15% stored in HCl elimination on the singlet surface.

2. PHOTON CONVERSION THROUGH STORAGE OF METASTABLE MOLECULES

(H. Frei)

The purpose of this program is to explore photosynthesis with near infrared quanta, with special attention to systems which permit energy storage. The importance of chemistry with such long wavelength photons stems from the fact that more than half of the solar irradiance at ground level lies in the near infrared (wavelengths longer than 700 nm). To illustrate the consequence of the abundance of near infrared quanta in the solar spectral distribution, one may consider the following: If maximum use of solar photon energy were to be attained by two separate photodriven chemical reactions, each with its own spectral sensitivity so as to optimize the combined use of the total solar irradiance, both systems would be required to have their thresholds in the near infrared (around 700 and 1300 nm, respectively). However, photochemical reactions that can be initiated by continuous near infrared light are very sparse. It is the goal of this work to find reactions which permit efficient use of near infrared radiation for chemical synthesis, and to develop the concept of chemical storage of long lived electronically excited molecules (like singlet delta excited O_2) to accomplish storage and conversion into useful energy of these long wavelength solar photons.

We have concentrated our effort over the past few years on near infrared induced chemical reactions of singlet molecular oxygen. Singlet delta oxygen, $O_2(^1\Delta_g)$, carries 23 kcal electronic energy and is a reactive, long lived intermediate which plays a key role in many photooxygenations. $O_2(^1\Delta_g)$ can be chemically stored in the form of aromatic endoperoxides, and we have demonstrated earlier with cryogenic photochemical techniques that the photon induced formation of such endoperoxides from O_2 and parent hydrocarbon may proceed very efficiently with near infrared photons as long in wavelength as 1.3 micron. In the past year, we have focussed on the study of the release of the singlet excited O_2 from substituted naphthalene endoperoxides at room temperature, using detection of the near infrared chemiluminescence of $O_2(^1\Delta_g)$ as a means to monitor the retrieval.

We have studied the dark, thermal backreaction of the singlet oxygen storage systems

in nonpolar and aqueous solutions and observed the $O_2(^1\Delta_g) \rightarrow {}^3\Sigma_g^-$ chemiluminescence at 1.27 micron with a very sensitive Ge photodiode. This constitutes direct evidence for elimination

of singlet excited oxygen from these endoperoxides. FT-IR spectroscopic study of the thermal decomposition of 1,4-dimethylnaphthalene endoperoxide (I) showed that decomposition on the ground state surface regenerates the parent naphthalene and O_2 quantitatively.

In order to study the decay of singlet O_2 expelled from these storage molecules in real time, we have attempted to detect $O_2(^1\Delta_g \rightarrow ^3\Sigma_g^-)$ near infrared chemiluminescence upon electronic excitation of the $S_2 \leftarrow S_0$ transition of 1,4-dimethylnaphthalene endoperoxide (I) and 3,3'-(1,4-naphthylidene) dipropionate endoperoxide (II) with 266 nm pulses of 5 nanosecond duration. Transient 1.27 micron chemiluminescence was observed, which gives the first direct evidence for singlet oxygen elimination from an electronically excited endoperoxide. This allowed us to monitor the time resolved decay of $O_2(^1\Delta_g)$ photochemically eliminated from these two endoperoxides in a number of solvents, including water. While singlet O_2 expelled from the ionic endoperoxide in H_2O , D_2O , and CH_3OH showed lifetimes dictated by solvent collisional quenching (range: 4–60 microsec), $O_2(^1\Delta_g)$ retrieved from 1,4-dimethylnaphthalene endoperoxide (I) exhibited faster decay rates than those characteristic for solvent deactivation, both in polar and nonpolar solvents. Current data suggest that interaction of $O_2(^1\Delta_g)$ with parent naphthalene molecules may contribute to the observed, laser energy dependent lifetime shortening. No near infrared chemiluminescence was observed upon excitation of S_1 of the endoperoxide at 355 nm, consistent with the peroxide $\Pi_{00}^* \rightarrow \sigma_{00}^*$ character of the transition, which leads to O-O bond rupture rather than elimination of O_2 . Initiation of $O_2(^1\Delta_g)$ expulsion through excitation of endoperoxide with 266 nm laser pulses, coupled with detection of its chemiluminescence in solution opens up the study of the excited state redox chemistry of the retrieved O_2 in real time. We will focus on reactions which will permit direct conversion of the $^1\Delta_g$ excitation of the expelled O_2 into electrochemical potential.

In the course of this laser flash-chemiluminescence work, we detected a new emission of photosensitized O_2 at 1.93 micron (5200 cm^{-1}), corresponding to the $^1\Sigma_g^+ \rightarrow ^1\Delta_g$ excited state fluorescence. The lifetime of the emission in the three solvents used, CCl_4 , $CHCl_3$, and C_6F_6 , was shorter than the 24 microsecond response time of the InAs photon detector. This constitutes the first observation of singlet sigma oxygen, $O_2(^1\Sigma_g^+)$, in room temperature solution, which allows us to explore the chemistry of the elusive $O_2(^1\Sigma_g^+)$ in parallel with that of the singlet delta excited species.

In order to determine activation barriers, $O_2(^1\Delta_g)/^3\Sigma_g^-$ branching ratios, and thermodynamics of endoperoxides and other singlet O_2 storing molecules on the electronic ground state surface, we are developing intracavity high overtone-chemiluminescence excitation spectroscopy. This technique uses single photon excitation of high vibrational overtones of the gaseous molecule in the cavity of a cw dye laser to initiate decomposition, which is monitored by detection of the 1.3 micron chemiluminescence of the expelled O_2 molecules. We have constructed a tunable cw dye laser excitation-near infrared detection system and demonstrated its sensitivity by obtaining a rotationally resolved $O_2(^1\Delta_g \rightarrow ^3\Sigma_g^-)$ luminescence excitation spectrum of the very weak $^1\Sigma_g^+, 1 \leftarrow ^3\Sigma_g^-, O$ transition of O_2 at 675 nm with oxygen pressures in the millitorr range. This allows convenient detection of the total yield of O_2 produced upon overtone excitation at very low molecule densities. The most promising singlet oxygen source molecule for study with this technique is methyltetrahydrofuran hydrotrioxide. We have synthesized this molecule and studied its overtone absorption spectrum ($2000\text{--}12000\text{ cm}^{-1}$) in Freon solution by FT-near infrared spectroscopy. Thermolysis in solution gave very strong $O_2(^1\Delta_g, O \rightarrow ^3\Sigma_g^-, O)$ (1.27

micron) and $^1\Delta_g, O \rightarrow ^3\Sigma_g^-, 1$ (1.58 micron) emissions, which makes this system at present our best candidate for intracavity gas phase experiments.

3. ARTIFICIAL PHOTOSYNTHESIS (M. Calvin and J. W. Otvos)

The longterm ultimate solution of our needs for liquid fuels from renewable sources will be the construction of synthetic systems which will enable us to convert solar energy directly into useful chemical species, both as chemicals and for fuels. We are using what we know about the natural process of photosynthesis, insofar as it involves quantum conversion into stable chemical products, to guide us in the design of these totally synthetic systems. The principal factor which enables green plants to convert incoming visible quanta into some stable chemical form long enough to do secondary chemical reactions for storage involves the separation of charge across a phase boundary. The positive charge (hole) is ultimately used to generate oxygen from water and the negative charge is used to reduce carbon dioxide. The phase boundaries under study are: (1) the lipid bilayer walls of phospholipid vesicles which are used to keep separate the initial photoproducts; (2) the surfaces of various polyelectrolytes; and (3) the surfaces of polymeric colloids.

Efforts have been made to find microheterogeneous systems that would involve photosensitizers active in the visible region. These would promote electron transfer from donors to acceptors, which would stabilize the charge separation long enough for suitable catalysts to make use of their separation. The particular systems which have been most useful have involved sensitizers with relatively long-lived triplet states. The two components of such systems can be examined separately by using irreversible electron acceptors on the oxygen-generating side to stabilize the hole and irreversible electron donors on the hydrogen side to inhibit the back reaction of the initial charge separation.

Oxidation Catalysts

We are examining manganese porphyrin species as potential multi-electron oxidation catalyst for oxygen evolution from water and for other oxidation reaction. In photosynthesis some type of manganese complex is involved in the oxygen evolution process. Manganese porphyrin complexes exhibit a rich variety of oxidation states in which the porphyrin macrocycle is resistant to irreversible redox reactions. These properties make them promising oxidation catalysts, and, in addition, it has recently been shown that manganese porphyrin complexes catalyze the oxidation of olefinic hydrocarbons. Our research is directed at characterizing various highly oxidized manganese porphyrin species and studying their chemistry with the view of judging their potential usefulness in the oxidation cycle of an artificial photosynthesis assembly.

The work has proceeded along two parallel pathways. The first is directed at water soluble manganese porphyrins and involves chemical, electrochemical and photochemical studies. However, isolation of intermediate species is frequently easier in organic solvents, and we are also investigating the redox chemistry of manganese tetraphenylporphyrin complexes in organic media. Comparison of similarities and differences in the properties of oxidized manganese porphyrins in aqueous and nonaqueous systems has led to helpful insights and has suggested new experiments.

Electrocatalytic and Photochemical Reduction of CO₂

We have continued to study the electrocatalytic reduction of carbon dioxide with cobalt and nickel macrocycles as catalysts in aqueous solution. We have found that of the various tetra-aza compounds tried as catalysts the ones that have given the best results in electrode reduction of CO₂ have unsubstituted, saturated ring structures. One of these [1,4,8,11-tetra-aza cyclotetradecane nickel (II)] was found to be highly selective in producing CO rather than H₂, as well as giving almost 100% current efficiency and high turnover numbers on Ni(II). After we were able to repeat these results, we selected this catalyst as the basis for a three-component photochemical system for performing the CO₂ reduction. Ruthenium tris-bipyridyl [Ru(bipy)₃²⁺] was the sensitizer and ascorbate buffer the electron donor. Work is now in progress to find the conditions that will maximize the yield of CO.

Polyelectrolytes and Charged Colloids as Interfaces for Increasing Quantum Efficiency

We have continued our studies on the effect of charged interfaces on the photoelectron transfer reaction between the photosensitizer and the electron acceptor. After our initial results with the polyelectrolyte, polystyrene sulfonate (PSS), showed that its presence not only inhibited the back reaction, as does colloidal silica, but also repressed the forward reaction and thus had little effect on the overall quantum yield, we began work with modified silica colloids. However, the usefulness of silica is limited to pH regions above 8. If sodium aluminate is used to react with the surface of the sol, aluminum can be incorporated into the surface. The more acid aluminum sites make the colloid stable and usable down to pH 6 where it still retains all of its effectiveness in preventing back-reaction.

Membranes

In order to achieve photoelectron and hole separation we have proposed to use electron transferring membranes which will keep the oxidizing and reducing agents separated. We have been working with perfluorinated ion exchange membrane (Nafion) obtained from duPont as well as membranes from the Dow Chemical Company. The objective here is to load such a perfluorinated cation exchange membrane with a cationic redox compound and so construct an electron transfer membrane by virtue of electron hopping between the two states of the redox couple bound to the membrane. We have loaded the Nafion membrane with ruthenium trisbipyridyl by electrolyzing the cation through the membrane. Such a fully loaded membrane is placed between an oxidant, such as permanganate anion, and a reductant, such as ferrocyanide anion. Prior tests indicate that such a membrane is impermeable to both of these anions. However, electron transfer does occur between the permanganate and the ferrocyanide anion, producing ferricyanide.

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B. KCO6 - BIOLOGICAL ENERGY RESEARCH

The research in this program is aimed at understanding the unique features of photosynthetic organisms that allow them to collect light energy and store it in the form of chemical energy.

- One project utilizes spectroscopic techniques to map both the components as well as the kinetics of the light reactions. The focus is on excitation transfer and trapping, primary electron transfer, the composition and organization of photosynthetically active membranes.
- We are studying the genetics of the photosynthetic apparatus of *Rhodospseudomonas capsulata* and *Euglena gracilis* with the ultimate aim of using DNA cloning techniques to help understand the mechanisms cells use to capture and utilize light energy.
- Our goal in a third project is to identify the cellular factors which regulate the tissue specific expression of plant genes.
- We are investigating the light regulation of genes encoding components of the photosynthetic apparatus in plant protoplasts as well as components of the carbon metabolic pathways.
- A complete understanding of the chemistry and stereochemistry of phycobiliproteins and phytochrome is sought to facilitate full understanding of the role of light in regulation of gene expression in green plants.
- A sixth program seeks to develop an understanding of the process involved in hydrocarbon production in plants.

1. PHOTOSYNTHESIS REACTIONS (K. Sauer, M.P. Klein)

Photosynthetic light reactions constitute the principal biological energy source derived from sunlight. We are carrying out spectroscopic and other biophysical studies aimed at understanding the mechanism of photon capture and excitation transfer among photosynthetic pigments, the earliest steps in conversion to chemical potential by electron transfer in the reaction centers and the mechanism of water oxidation to O_2 . Both kinetic and structural investigations are carried out using a variety of spectroscopic techniques, including optical absorption and fluorescence, electron paramagnetic resonance (EPR) and X-ray spectroscopy. In related studies we are looking at the composition and organization of the photosynthetic complexes and their arrangement in the thylakoid membranes. We have also investigated model compounds designed to simulate the light-induced electron transfer that lies at the heart of the photosynthetic energy conversion.

Excitation Transfer in Photosynthetic Antenna Pigments

The excited state kinetics of the photosynthetic antenna pigment phycocyanin was monitored using time-resolved fluorescence measurements. The excited state lasts about 1.5 nsec for isolated phycocyanin, but excitation transfer to chlorophyll in the photosynthetic membranes shortens the lifetime nearly one-hundred fold. Phycocyanin occurs as a hexameric species containing 18 chromophores (tetrapyrrole pigments covalently attached to a protein) as well as in trimeric, monomeric and smaller subunits. Fluorescence spectra and polarization measurements showed that excitation transfer occurs rapidly among these chromophores in comparison with the 1.5 ns fluorescence lifetime. Several mechanisms have been proposed for this transfer, including exciton coupling and Förster inductive resonance transfer. Within the past year two reports of detailed molecular structures of C-phycocyanin from two different organisms appeared [Shirmer, *et al*, J. Mol. Biol. 184, 257-277 (1985); 188, 651-676 (1986)]. These were the first photosynthetic antenna pigments to be known sufficiently well to permit detailed calculations of electronic excitation transfer kinetics.

In collaboration with Prof. Hugo Scheer of the University of Munich, we applied the Förster formalism to this structure and obtained excitation transfer rates in agreement with the available experimental data. We conclude that for this particular pigment protein, exciton coupling does not play a significant role in excitation transfer. Other complexes are known where this situation is probably reversed, and new crystallographic data that are now appearing make it worth examining those complexes theoretically as well.

Charge Separation in Photosynthetic Reaction Centers

Primary electron transfer is initiated in the photosynthetic reaction center. In higher plants, algae and cyanobacteria, there are two distinct types of reaction centers, known as Photosystems (PS) I and II. In the recent past we have done detailed investigations of the kinetics of PS II using picosecond to nanosecond time-resolved fluorescence decay, and microsecond and longer EPR transient measurements. We have extended the studies on PS II and have begun analogous investigations of PS I. The use of cyanobacteria affords an opportunity to obtain enriched PS II

reaction center complexes that retain important aspects of the primary electron transfer. These complexes exhibit significantly slower fluorescence decay components (to 20 nsec) compared with those from the higher plant membranes (to 2 nsec) that we had studied previously. The slow components from the cyanobacterial PS II complexes are sensitive to the open/closed state of the reaction centers and, therefore, may reflect charge recombination processes that have been altered by the isolation procedures. These studies will be pursued to map the origin of the several decay components that have been resolved.

A new class of electron transfer inhibitors, including the unsaturated fatty acids linolenic and linoleic, appear to act closer to the PS II reaction centers than any that had been previously observed. In collaboration with Prof. John Golbeck of Portland State University we have investigated the effect of these inhibitors on the fluorescence decay kinetics and the formation of EPR-detectable triplets associated with PS II. The studies are consistent with the model that linolenic acid (LA) blocks electron transport between the primary (pheophytin) and secondary (plastoquinone, Q_A) electron acceptors; however, detailed studies of the effects on absorption and low temperature emission spectra indicate that LA at the low concentrations (50 μ M) used does produce irreversible alterations of the reaction center pigment environments. The EPR studies indicate that the ability to form a radical-pair recombination triplet is retained in the presence of LA, but only when a strong reducing agent like dithionite is also present. The simplest interpretation of these results is that there is an additional electron acceptor in the PS II reaction center that had not previously been detected. The evidence is conflicting, however, and further studies are needed before a satisfactory understanding of the situation will obtain.

Electron donation to the PS II reaction center comes ultimately from water oxidation. The successive storage of oxidizing equivalents in this four-electron transfer process is reflected in the kinetics of intermediate carriers between the water-oxidation complex and PS II. Using kinetic (μ sec) EPR spectroscopy we can monitor Signal II, which we have shown previously to be the primary electron donor to the PS II reaction center chlorophyll. Using a series of up to eight saturating, sub-microsecond flashes, we have monitored the kinetics of rereduction of the Signal II species and have seen oscillations of period 4 that correlate with storage of successive oxidizing equivalents in the water-splitting complex. Of special interest have been further investigations of alterations in this kinetic pattern that occur as the membrane complex is gradually disassembled. The disappearance of the period 4 behavior is a sensitive indicator of the loss of ability to store oxidizing equivalents; however, under many circumstances rapid rereduction of the primary electron donor continues to occur by components that have yet to be identified.

Photosystem I reaction centers behave qualitatively differently from those of PS II. Instead of the large (fourfold) increase in fluorescence yield seen upon closing PS II reaction centers through illumination or chemical reduction of the electron acceptors, it has been difficult to demonstrate any contribution to this variable fluorescence from PS I. Now, using isolated and enriched PS I reaction center preparations we have seen a small (20%) decrease in fluorescence yield associated with closing the reaction centers. By contrast with the behavior of PS II, this variable fluorescence occurs upon oxidation of the primary electron donor chlorophyll, P700. These results for both PS I and PS II are consistent with, but do not prove, a model in which

the increased fluorescence yield comes from the reversal of the primary process leading to the repopulation of the chlorophyll excited singlet state following charge recombination. We are carrying out time-resolved fluorescence decay measurements to try to define the mechanism more precisely.

Model studies were carried out on electron donor/acceptor complexes synthesized by covalent linkage of a photo-excitabile ruthenium complex to a benzoquinone. The linker consisted of oligo peptides containing zero to four prolines. By monitoring the effect of increasing the physical separation of the electron donor (Ru) from the electron acceptor (quinone) using fluorescence yield and time-resolved fluorescence relaxation, we could follow the competing electron transfer in the range from 10^{-9} to 10^{-6} sec. It is clear from these studies, carried out by Miller Research Institute Fellow Dr. Kirk Schanze, that electron transfer can occur rapidly and efficiently across longer non-aromatic bridging groups than had been suspected. In ongoing studies using kinetic EPR spectroscopy we will investigate the lifetime of the charge-separated species (radical pair) that simulates the initial stages of photosynthetic charge separation.

Manganese and Photosynthetic Water Oxidation

Much evidence has now accumulated that the storage of the four oxidizing equivalents needed to produce O_2 from water photosynthetically occurs in a complex containing four manganese atoms. The nature of this complex, which is presumed to involve interaction with a protein environment, is still unknown. Even the kinetics is in dispute. Optical spectroscopic changes have been interpreted to result from three successive one-electron oxidations from Mn^{+3} to Mn^{+4} prior to O_2 release, whereas EPR, X-ray spectroscopy and other approaches favor a mechanism with different oxidation state changes for the three preliminary steps.

We have used X-ray absorption and EXAFS, in conjunction with low temperature EPR measurements to investigate these questions. Absorption edge energy studies on Mn are suggestive that, S_0 , the most reduced state of the complex, has the same oxidation state for Mn as S_1 , where one electron has been removed by the PS II reaction center. Presumably this electron has come from an associated ligand that does not directly involve Mn. The second step, from S_1 to S_2 , definitely involves Mn oxidation, perhaps by two equivalents transferred — one to the reaction center and one to rereduce the ligand. The third step, from S_2 to S_3 , again involves no apparent change in the Mn oxidation state, but the final step S_3 to S_4 and back to S_0 returns the complex back to the fully reduced state. The state S_4 , which should be the most oxidized but is unstable, has not yet been trapped for investigation.

EXAFS measurements give information about the local coordination environment of the Mn in the water splitting complex. Two of the three shells previously identified for S_1 are attributed to O or N atoms, and the third shell to Mn atoms. Further enhancement of signal quality has revealed a fourth shell, apparently also resulting from Mn. A possible model involving a distorted cubic arrangement with Mn and either O or N atoms at alternate corners has been proposed by Brudvig and Crabtree [Proc. Natl. Acad. Sci. USA 83, 4586-4588 (1986)] to account for these and other spectroscopic data. We find that the coordination in S_1 does not differ significantly from that in S_2 , by the EXAFS criteria; however, analogous investigations of S_3 are still under way. Previous proposals that sulfur or chlorine atoms are involved as Mn

ligands are not consistent with the EXAFS data. We have also shown that resolved superhyperfine structure of a low temperature EPR signal associated with S_2 does not result from interactions with Cl nuclei in the immediate coordination environment. Until the successful isolation of the water-splitting complex has been accomplished, further spectroscopic studies such as these will be our principal source of information about the enzyme that accomplishes this important aspect of photosynthesis. The information gained is expected to be valuable for designing biomimetic systems for using light to split water into hydrogen and oxygen as alternative energy sources.

2. REGULATION OF EXPRESSION OF GENES IN *Rhodobacter capsulatus* BY LIGHT AND OXYGEN (John E. Hearst)

The purple nonsulfur photosynthetic bacterium, *Rhodobacter capsulatus*, provides an attractive model for studying the regulation of photosynthesis genes by light, O_2 and other environmental factors. When the concentration of oxygen is lowered, the cell develops an extensive intracytoplasmic membrane (ICM) and induces the biosynthesis of light-harvesting LH-I (B870), LH-II (B800-B850), and reaction center (RC) proteins, as well as bacteriochlorophyll (Bchl) and carotenoids (Crt). Once assembled, the photochemically active complexes harvest and convert light energy into chemical energy. The size and structure of the photosynthetic apparatus is also influenced by light intensity.

The genes for the β (*puf B*) and α (*puf A*) subunits of LH-I complex, as well as the L (*puf L*) and M (*puf M*) subunits of RC complex, are located in the *Bam* HI-C -*Eco* RI-B fragment of the R-prime plasmid pRPS404 isolated from *R. capsulatus*. They are co-transcribed *in vivo* both in *R. capsulatus* and *Rhodobacter sphaeroides*. The gene for the H (*puf H*) subunit of the RC lies in the *Bam* HI-F fragment of the R prime, whereas the genes for the β (*puc B*) and α (*puc A*) subunits of the LH-II are outside of this photosynthetic gene cluster. The structural genes for LH-I, LH-II and RC-L, RC-M, RC-H in *R. capsulatus* have been sequenced. The genes for Bchl and Crt biosynthesis have been mapped by genetic methods and transposon mutagenesis.

It has been shown that lowering the O_2 concentration in cultures of *R. capsulatus* and *R. sphaeroides* increases the levels of mRNA for LH-I, RC and LH-II polypeptides, while the amounts of RNA for pigment biosynthetic enzymes shows only a small increase when the O_2 concentration is reduced. According to one set of experiments, Crt biosynthesis are not directly regulated by O_2 . More recently a sequential and independent expression of LH-I and LH-II genes has been shown in *R. capsulatus* during ICM development.

Recently a great deal of attention has been paid to the regulation of photosynthetic genes in *R. capsulatus* and *R. sphaeroides*. In the present study we have used an improved method for RNA isolation and have probed transcripts with DNA fragments specific to particular genes wherever possible. Northern hybridization technique, in combination with dot blot analysis, was used to measure mRNA quantity and sizes in order to obtain specific information about the regulation of the photosynthetic genes cluster in *R. capsulatus* in response to light and O_2 .

Recent studies of physical structure show that the reaction center subunits L, M and H comprise a single complex in the ICM where the photochemical reaction takes place. Each reaction center is surrounded by approximately 12-18 LH-I (β , α subunits) complexes. The more abundant LH-II (β , α subunits) complexes, located peripherally to LH-I, harvest light energy and transfer exciton energy to LH-I and then to the RC. Bchl and Crt, absorbing light across the visible and near-infrared region, bind to LH-II, LH-I and RC. The location of genes coding for these polypeptides parallels the structural and functional association of these polypeptides. The structural, functional and genetic features of these complexes define the patterns of regulation of the genes coding for these polypeptides. Earlier studies on proteins indicated that LH-II and LH-I/RC complexes were sequentially accumulated during ICM differentiation of *R. capsulatus* and that they were regulated independently by light and O_2 . Here we provide evidence for the independent regulation of the genes for LH-I and LH-II based on the results from a study of mRNA levels. We found that the level of mRNA homologous to the LH-II is more sensitive to change in O_2 concentration, and it varies more than that of LH-I. This result agrees with the measurement of absorption spectra of protein-pigment complexes *in vivo*. In addition, we have evidence suggesting that the mRNA specific for LH-II is degraded much faster than that of LH-I, when the cells are shifted from photosynthetic growth conditions to high O_2 . Since the gene for the third subunit (14 kd) of LH-II has not yet been isolated, we have no information about the regulation of this gene.

In our studies, we found that the Open Reading Frame (ORF) C2397 was transcribed, and the level of its mRNA changed in response to light and O_2 in a similar way to that of the genes for RC-L and RC-M. Since the ORF C2397, 236 bp in size, is located immediately 3' to the gene for RC-M and there is a hairpin structure after the ORF C2397, we suggest that the five structural genes for LH-I (β), LH-I (α), RC-L, RC-M and C2397 comprise a single operon, and that the transcription of this operon stops after the gene for C2397. Whether the gene for C2397 codes for a structural protein, or it functions in the assembly of the LH and RC, or it has some other functional role is not yet known. We assume that it must have some function related to the LH and RC. Although the genes for LH-I (β , α), RC (L, M, H) and C2397 generally showed similar regulation by light and O_2 , some differential regulation was observed: The 2.6 kb mRNA for LH-I (β , α), RC (L,M) and C2397 is more responsive to the change in O_2 concentration than the 0.5 kb mRNA. Such differential expression of these two transcripts was also observed in *R. sphaeroides*.

A close correlation between pigment (Bchl, Crt) and LH/RC biosynthesis has been reported in *R. capsulatus*. We found a general coordinate regulation between genes for Bchl biosynthesis and the genes for LH and RC in response to light and O_2 . Some difference, however, was observed. The mRNAs of *bch* H, K, F (in *Bam* HI-D) and *bch* J, D, G (in *Bam* HI-E) genes increased less than mRNAs for LH and RC when the light intensity decreased. The levels of mRNAs of these *bch* genes decreased not only when the cells were shifted from photosynthetic growth condition to high O_2 but also under steady-state LO_2 condition, indicating that the genes for Bchl biosynthesis respond more stringently to O_2 concentration. The unexpected results were that under steady-state high O_2 the level of mRNA from the *Bam* HI-D fragment even increased. An O_2 shift experiment also showed the induction of an O_2 -activated gene. We believe that there is an O_2 -activated gene(s) located in the *Bam* HI-D fragment.

It is of interest that the genes for Crt biosynthesis are regulated differently from the other photosynthetic genes in response to light intensity and O_2 . We have found that under steady-state high O_2 the level of mRNA from the *Bam* HI-H fragment was relatively high and it increased when the cells were shifted from anaerobic to aerobic conditions. We have evidence suggesting that *crt A* in the *Bam* HI-H is activated by O_2 . This gene is responsible for the oxidation of spheroidene to spheroidenone. Such regulation in response to O_2 may be related to the function of Crt. It has been long known that Crt has two functions: harvesting light and protecting cells including the photosynthetic apparatus from photooxidative damage which only occurs in presence of both high light and O_2 . Activation of *crt A* and other Crt genes by O_2 may be part of the protective mechanism by which Crt scavenges O_2 radicals in the cell. Here we have also found another type of protective mechanism in response to high light. That is, in contrast to the decrease in the levels of mRNA for LH, RC and Bchl biosynthesis, an increase in light intensity raised the levels of mRNA from a number of *crt* genes located in the *Bam* HI-M, *Bam* HI-G and *Bam* HI-H fragments. Although the increase in transcription of *crt* genes in response to high light seems to be a plausible protective mechanism, we do not rule out the possibility that regulation may also occur post-transcriptionally, including the activation of Crt biosynthetic enzymes by light and O_2 .

The 0.4 kb small transcript from the *Bam* HI-J also showed a response to high light in the opposite fashion to the mRNAs for LH and RC. It did not hybridize to either regions genetically mapped as *crt F* or *crt E* (unpublished data, G. A. Armstrong and J. E. Hearst). The response of this transcript to O_2 is very similar to that of mRNAs for LH and RC, but is very different from that of mRNAs for Crt biosynthesis. On the other hand, transcript level increases in response to high light as do the mRNAs for Crt biosynthesis except to a greater extent, but it is very different from that of mRNAs for LH and RC in this respect. The nature and function of this small transcript remains to be characterized.

Other interesting results in this study are the finding of multiple transcripts for the RC-H (1.4 and 1.2 kb) as shown by Northern hybridization. The 1.2 and 1.4 kb transcripts of the RC-H gene, 764 bp in size, are probably initiated from the middle of the ORF F1696. We do not know whether they have different initiation sites, or if the 1.2 kb transcript is the product of processing of the 1.4 kb transcript. The relatively long 5' non-coding region (0.4-0.6 kb) may contain important regulatory sequences.

Assay for transcripts from all of the ORF putative genes near the LH and RC gene clusters in the *Bam* HI-C -EcoRI-B, and *Bam* HI-F fragments resulted in the detection of mRNA from only C2397. We suggest that the putative genes in both fragments are either not expressed under the defined growth conditions, or their mRNAs, if present, are below the limits of detection by our methods.

3. LIGHT REGULATION OF NUCLEAR AND CHLOROPLAST NUCLEIC ACID SYNTHESIS IN *Euglena gracilis* (J. C. Bartholomew)

Our research has been directed towards the understanding of the mechanism(s) controlling the coordinate expression of genes encoding photosynthetic components. While the photosynthetic processes are primarily carried out in the chloroplasts of eucaryotic photosynthetic cells, the genes encoding the components are distributed into both the nuclear and the chloroplast genome. The coordination of expression of these physically separated genomes is the subject of our investigations. We are determining if the chloroplast or its encoded components play a role in the light response of nuclear genes whose products are involved in photosynthesis.

The photosynthetic apparatus is a highly organized and specialized structure made up of many components. The activity of the system is dependent on the activity and arrangement of the individual components. The cell must balance the production of the individual components to optimize the photosynthetic activity of the system. This balancing act is not simply a maintenance of equal numbers of all components since some components in the apparatus are needed in great excess over others. Also, control must be exerted on the production of these components as a function of time. For example, the production of Light Harvesting Chlorophyll Binding Protein (LHCP) must not begin before chlorophyll biosynthesis since the protein is rapidly destroyed in the absence of chlorophyll.

Even though the chloroplasts of photosynthetic organisms contain their own genome, many of the functions carried out in the organelle are encoded in the nucleus. Some of the proteins which function in chloroplast are encoded partially in the chloroplast and partially in the nucleus. The expression of both these genomes is regulated by light. Light stimulates mRNA synthesis as well as DNA synthesis in *Euglena gracilis*. Presumably, this light response is mediated by photopigments in the cells which receive the light and convert it into a signal which control nucleic acid activity in both genome compartments. We are studying a series of bleached mutants of *Euglena* which lack various photopigments as well as have different levels of chloroplast DNA. Using these variants, we hope to determine the involvement of the photopigments and chloroplast DNA in the stimulation of nuclear DNA and RNA synthesis. First, we are characterizing the cell cycle response to light of these various mutants by flow cytometry. Wild type *Euglena* conditioned in the resting medium in the dark can be stimulated to reenter the cell cycle by light. We are comparing the kinetics of reentry into the cell cycle of the bleached mutants with that of the wild type to discern the role of the photopigments and chloroplast supplied functions in regulating the DNA replication response in the nucleus. We are also comparing the light stimulated expression of nuclear encoded chloroplast functions in wild type vs bleached mutants to determine if the chloroplast and/or the missing photopigments play a role in the regulation of the nuclear genes. Can nuclear genes coding for photosynthetic components be regulated by light in the absence of functional chloroplasts? At present we are using heterologous probing to clone the nuclear genes for the light harvesting chlorophyll a/b proteins in *Euglena*. We also have DNA probes for large subunit of ribulose biphosphate carboxylase, the 32 Kdalton herbicide binding protein, and a *Euglena* chloroplast t-RNA^{Leu}. All of these studies are designed to help us understand the interrelationships between the various photopigments and DNA and RNA synthesis in the nuclear and chloroplast compartments.

4. TISSUE SPECIFIC GENE EXPRESSION IN PLANTS

(Dr. Francesca Leach and Dr. James C. Bartholomew)

Nuclear DNA is commonly associated with proteins. These are usually classified as either "structural" proteins or "biologically" active. Proteins of the second class include DNA and RNA polymerases, repressors and inducers for instance. They affect the DNA topology only locally and are responsible for its biological activity.

The project initiated in Sept.85 is designed to study DNA-protein complexes in two different tissues: leaves and roots. The aim is to determine the molecular characteristics of the specific protein-DNA complexes that will explain:

- how the two molecules associate
- how the presence of some of the complexes correlates with root- or leaf-specific transcription.

This study is of direct importance to our understanding of how to generate plants by genetic engineering which express foreign genes in a regulated manner. It is our hypothesis that, in plant cells, as in bacteria and in mammalian cells, DNA-binding proteins play a key role in regulating specific developmental expression. Widening the limited present day understanding of plant transcription mechanisms is a crucial issue in an era where plant genetic engineering is becoming available. Genetic manipulation via one technique or another, from direct protoplast transformation with naked DNA to using Ti plasmid derived vectors, is now possible for many plants. However, it is essential that any foreign gene which is introduced can be expressed in a regulated manner if desired. There is therefore a need on one hand to isolate and characterize the greatest possible number of plant promoters, active in different tissues and with different induction properties, and on the other hand to understand the molecular mechanisms that pertain to the regulation of promoters.

In our experiments, the working hypothesis is that DNA-binding proteins modulate gene expression by acting on regulatory sequences of specific genes. The sequential objectives of the proposed project are as follows:

1. to demonstrate the existence of specific DNA-binding proteins in plants associated with transcription in different tissues.
2. to characterize and isolate root- and leaf- specific transcription proteins.
3. to identify their specific DNA-binding sequences.
4. to clone the genes that encode the tissue-specific DNA-binding proteins and monitor their expression during plant development.
5. to modify the proteins by mutagenesis so as to obtain information on how the DNA-protein complexes associate.
6. to monitor when and where, during plant development, these DNA-binding proteins are expressed.

We have chosen to work with promoters born by the T-DNA of *A.rhizogenes* for the following reasons. First, the pRi T-DNA has been shown to bear promoters that are specific to

leaves and to roots. And second, the pRi T-DNA is a natural vector for plant genetic engineering, compatible with the regeneration of fertile plants; and it can thus be used to introduce modified genetic information into the plant cells studied.

Assay for Specific DNA Binding Proteins

We have been testing conditions that will allow plant protein-DNA complex formation. Tobacco root and leaf protein extracts have been tested for their DNA-binding capacities after separation of the proteins by electrophoresis and transfer to a nitrocellulose membrane. We have seen a number of proteins which show DNA-binding capacities, and have noted large differences in the DNA-binding pattern of extracts from the two tissues. We are presently characterizing the nature of these proteins (histone, HMG, other) and assessing the contribution of chloroplast DNA-binding proteins to the binding pattern observed in the case of the leaf extracts. Even if a major contribution in this difference comes from the leaf chloroplastic proteins, the two smaller MW proteins (7kd and 8kd) present only in the root extract represent a true difference in the DNA binding patterns of the proteins from root and leaf tissue. To test whether this difference is due to an artifact of our extraction procedure, we have tried to denature both extracts with a number of techniques; however, leaf polypeptides never broke down to such small components. This result, although preliminary, is the first evidence of a difference in DNA associated proteins from two plant organs and as such, is worth further investigation.

Isolation of DNA binding proteins from root and leaf tissue

Our technical approach is to isolate proteins which bind specifically with the T-DNA regulatory sequences of the root-specific and the leaf-specific T-DNA genes.

First, proteins are isolated on the basis of their general DNA-binding capacity. To this effect, protein extracts from each plant organ are run on DNA-cellulose affinity columns to fractionate proteins which show general DNA-binding capacities.

The test we use for visualizing DNA-binding proteins is a modified version of the technique published by Bowen et al., 1980: proteins are transferred to nitrocellulose filters and probed with nick-translated labelled DNA. The DNA sequences we are using for these experiments are subclones containing the leaf-specific and the root-specific promoters of the pRiA4 T-DNA. The goal is to show that there is indeed a specificity in the recognition of each of these sequences by the two types of extracts and to identify what proteins are involved.

The difficulty that will be encountered in this part of the project is in defining conditions that will enhance the sequence specificity of the DNA-binding proteins. This has been a major difficulty in the identification of sequence-specific recognition factors. Retarded electrophoretic migration of DNA complexed to proteins has proven to be a useful method in detecting specific over non-specific binding of proteins to isolated sequences: it has recently been used to identify specific transcription factors in SV40 infected cells, and in O₂ induced cytochrome expression in yeast.

We are now testing several approaches in order to determine the general and sequence-specific DNA-binding properties of the Tobacco root and leaf proteins. These include investigating factors influencing the formation of DNA-protein complexes in solution, such as salt conditions, length of reaction and subsequent washes, and competition experiments with random sequence DNA such as calf-thymus DNA. If necessary, the DNA-cellulose column fractionation will be more elaborate to yield protein species in significantly purified form so as to reduce the background of non-specific binding. DNA-protein complex formation will also be tested using smaller probes i.e. purified promoter sequences from the plasmids that bear the leaf and root pRi T-DNA regulated genes. We hope thus to discriminate between sequence specificity and general binding affinities of the proteins now being isolated.

Identification of protein target sites on the DNA

This approach will focus on the other aspect of tissue-specificity i.e. a study of the DNA sequences that interact with cellular factors to control the activity of specialized genes. It is also the most straightforward part of the project proposed. We would like to define what the DNA sequences are that interact with the proteins that are being isolated in 1-. This we will determine by DNA foot-printing experiments : DNA-protein complexes will be formed using purified protein fractions from the root or leaf extracts and purified restriction fragments containing sequences of the pRiA4 T-DNA for the root and leaf-specific promoters. The end-labelled DNA will then be modified for Maxam and Gilbert and sequencing gels run. It will thus be possible to identify the precise positions of the binding-sites of the tissue-specific proteins in the 5'upstream regions of the two genes under consideration.

Footprinting experiments are difficult if the proteins required to complex with the DNA are in low abundance. Hence, this experiment depends on previous work to attain a semi-purification of the proteins required.

A Cellular Approach

The initial aim of this work will be to introduce the plasmids we have from the Ri T-DNA which contain genes expressed preferentially in different tissues, into protoplasts from these tissues. We will study the expression of the genes on these plasmids at the RNA and protein level. The DNA will be introduced into the protoplasts either by transfection or by electroporation, both techniques are presently being used in our lab. The assays in this case will be short term expression assays in which the RNA from the protoplasts which carry the plasmids will be isolated within 48 hr of introduction of the DNA. The RNA encoded by the gene driven from the plasmid promoter will be quantified. To study expression at the level of protein the promoters will be isolated and linked to *in vitro* to the gene coding for the chloramphenicol acetyltransferase enzyme (CAT). CAT activity will be measured after introducing the DNA into protoplasts.

A large part of this work will be directed at maintaining the tissue specific expression of the protoplast by adjusting the growth conditions during the course of the experiment. We will

modulate the culture media by adjusting the ratio of auxin to cytokinin and determine the effect on tissue specific expression. Also, we will investigate the involvement of oligosaccharins in determining the tissue specific expression of these plasmids.

After the transient expression experiments are underway, plants will be regenerated containing these modified Ri T-DNAs. Protoplasts will be isolated from root and leaf tissues of these plants, and nuclei will immediately be tested for transcriptional activity, using the technique of Ackerman et al. CAT activity will be measured in parallel, as it is easily detectable in small amounts of material. By addition to root-nuclei of extracts from leaf tissues and harvesting of the subsequent transcription complexes, it is hoped that it will be possible to activate the silent root-specific promoter (and vice-versa for the leaf-nuclei). This assay will be used to test the factors isolated in the DNA binding work described above for tissue-specific activators.

5. PLANT BIOCHEMISTRY (H. Rapoport)

The objectives of this project are to study the structure and mechanism of action of the non-chlorophyllous plant protein-pigment phytochrome. Phytochrome is the morphogenically active plant pigment that controls all developmental aspects of plant growth. It does this by undergoing a photochemical interconversion upon light excitation to a new, active form.

The specific structural features of this isomerization, as well as some other structural aspects of phytochrome, remains unknown. It is essential that these structural questions be unambiguously established in order to have a full understanding of phytochrome's function. Understanding the underlying mechanism by which phytochrome functions may allow us to control growth, flowering, and fruiting.

The specific aims of this project are:

1. To determine the stereochemistry of the pigment-protein linkage in phytochrome.
2. To determine the structural changes both in pigment and protein accompanying the change of phytochrome from the inactive P_R form to the active P_{FR} form.
3. To establish the relative and absolute stereochemistry of the bilipeptide linkages.
4. To synthesize the specific stereoisomers of S-cysteinylphytochromobilin.
5. To synthesize models of phytochrome consisting of pigment covalently attached to polypeptides of various lengths and composition in order to probe pigment-protein interactions.

Phytochrome is the morphogenically active plant chromoprotein intimately involved in all developmental aspects of plant growth. Because of its complex and sensitive nature, it has also eluded unambiguous structural assignment. However, the data obtained so far indicate it is similar to the phycobilins. We have applied new structural methodology to this extremely important plant pigment as well and have established the structure of the phytochromobilinundecapeptide.

Phytochrome is a reversible biological switch. The action of far red light causes it to change from the inactive P_R form to the active P_{FR} form. The two most reasonable hypotheses for its mode of action in the P_{FR} form are through gene activation or by affecting membrane permeability. However, structural information on exactly what changes accompany the transformation to the P_{FR} form is totally lacking.

We plan to provide information about the P_R to P_{FR} transformation by applying our structural methodology to the P_{FR} form. We also plan to conduct the P_R P_{FR} reaction in a 2H_2O medium and reisolate the P_R form. Detailed NMR analysis, both 1H and ^{13}C , will then reveal if any hydrogen atoms have been exchanged in this process.

Our initial synthetic goal is S-cysteinylphytochromobilin. After this is successfully completed, the synthetic work will be extended to include some small chromopeptides. The synthetic work is essential because it will help us answer some questions about pigment conformation and function. Also, it will answer the final stereochemical question left unanswered in that it will establish the absolute stereochemistry at the thioether linkage.

Our proposed synthesis of S-cysteinylphytochromobilin begins with the protected L-vinylglycine **1** which we have recently prepared from methionine. With HBr in the cold this gives a mixture of about equal parts of the diastereomeric bromides **2** and **2'** which are separable.

The synthesis now proceeds with each separate diastereomer in a displacement with a suitably N and O protected cysteine to **3**. The α -amino group of **3** is now replaced by Br to give **4** and thence with cyanide to **5**. Cyanoacetate **5** is alkylated with α -bromopropionate and the resulting **6** is decarboxylated to a mixture of **7** and **7'** in which the C*'s are of fixed and known stereochemistry. We now proceed individually with **7** and **7'** to **8** (and **8'**). Thus in **8** all three centers are fixed and known, C-2 and C-3' by synthesis and C-3 by the splitting constant of its H with C-2-H.

From **8** we will continue the synthesis to the phytochrome bile pigment **9** and we can then turn to extension of the peptide chain from both the amino and carboxy ends. We shall not present the details of the peptide synthesis methodology here, since we expect to rely heavily on known methods.

Compound **8** has been prepared by two different paths in an optically inactive form as a mixture of various stereoisomers. We are now evaluating and improving these processes in order to select the method best suited for preparing optically active pure compound **8**. We have prepared the compound corresponding to the two central rings (B and C) of compound **9**.

Preliminary synthetic experiments starting with optically active vinylglycine **1** have yielded promising results, in that one isomer of **8** seems to be the major product. If this isomer has the stereochemistry of natural phytochrome, the synthetic prospects are very promising.

At the same time, we have started investigation of an alternative and shorter path via compound **10**, synthesized in its sterically pure form from L-alanine. Initial experiments

demonstrated that suitably protected cysteine derivatives undergo 1,4-addition to **10** to give **8** as a mixture of diastereomers. Furthermore, the same addition reaction occurs with the vinyl analogue of the A-B ring dimer. In the latter case, the diastereomers have been separated and are crystalline.

During the next year we will be synthesizing optically active pure compound **8** and will synthesize ring D of compound **9**. Once these compounds are available we will proceed with the synthesis of compound **9**. Emphasis will be placed on the newly discovered route involving 1,4-addition of a protected cysteine derivative.

Following the completion of synthesis of S-cysteinylphytychromobilin, the extension of the peptide chain from both carboxyl and amino ends will be carried out.

6. HYDROCARBONS FROM PLANTS (M. Calvin and J. W. Otvos)

The overall goal of this program is to develop an understanding of the processes involved in hydrocarbon production in plants. Specifically, we are interested in the mechanisms and control of isoprenoid (terpenoid) biosynthesis. Acquiring this basic information will be necessary before we can genetically manipulate plants to increase hydrocarbon yields.

We use the latex isolated from laticifer cells of the *Euphorbia lathyris* plant for our biosynthetic studies. These cells are the site of both the biosynthesis and the storage of large quantities of sterols (triterpenols). We are currently examining the final steps of sterol synthesis, the epoxidation and cyclization of squalene, to determine if more than one squalene cyclase is involved in sterol synthesis. We are also attempting to identify the organelle involved in the conversion of mevalonic acid (MVA) to sterols.

A secondary interest is the synthesis of sesquiterpenes which are a better candidate for direct use as fuel than are the triterpenoids. The manipulation of a plant towards increased sesquiterpene production at the expense of sterol product is thus a long-term goal of our research. This requires an understanding of the processes involved, specifically the initial cyclization of farnesyl pyrophosphate (FPP), the branch point at which sesquiterpene synthesis diverts from the general isoprenoid biosynthetic pathway.

Environmental Effects on Hydrocarbon Production (Latex Biosynthesis)

It may be possible to increase the hydrocarbon content of plants by changing the environment around these plants. We have found that by increasing the day length from about 12 hours to 16 hours and by maintaining a constant day/night temperature regime, we observed about a 9-fold increase in the activity of 3-hydrox-3-methylglutaryl-Coenzyme A Reductase (HMGR). Since this enzyme plays a key role in the synthesis of isoprenoids, it is possible that hydrocarbon production is sensitive to environmental controls.

Controlled changes in environmental conditions could restrict growth while having only small effects on the photosynthetic rate (per area). If applied when the plant approached maturity, this could result in an increase in assimilates available for partitioning into isoprenoid biosynthesis. We also hope to identify the internal controls of carbon allocation, since they also could be manipulated to increase plant hydrocarbon production. To investigate these possibilities we are observing the effects of three different environmental variables on hydrocarbon production: salinity stress, water stress and nitrogen deficiency.

Stress Studies

After establishing the growth conditions necessary to grow *E. lathyris* hydroponically, we completed the preliminary study of salinity stress. Extractions of the hydrocarbons and sugars will be performed on the water-stressed plants. However, experiments utilizing nutrient stress have not been successful to this point as low levels of nitrogen cause rapid senescence and death.

The effects of salinity on growth were determined from changes in shoot length, total fresh weight and root and shoot dry weights. Changes in the photosynthetic apparatus were determined from chlorophyll content, thylakoid proton gradient formation, and *in vivo* fluorescence patterns. Changes in the levels of energy-rich compounds were determined by heptane and methanol extractions of the dried plant. Only the shoot portion of the plant was used for this extraction as this is what would be available for harvest. The preliminary results indicate that salinities of 50 mM NaCl and greater affect both growth and photosynthesis, though the reduction in growth is sharper than the reduction in photosynthesis. Results of extraction experiments with plant components also indicate that increased salinity causes an increase in carbon allocation to the heptane and methanol fractions, with most of the increase occurring in the carbohydrate-rich methanol fraction. Salinization also caused a 3-fold increase in HMGR activity.

To obtain a more comprehensive understanding of carbon flow through *E. lathyris*, we performed some preliminary carbon-labeling experiments. Now that we have established that it is possible to use isotope labeling to monitor latex production and follow carbon translocation in *E. lathyris*, this technique will be used further in stress experiments.

Regulation of Terpenoid Production in Euphorbia

The regulatory enzyme HMGR has been associated with various organelles in higher plants. Its localization in latex is not yet known, but our results indicate that it is associated with an as yet unidentified organelle. We are studying latex organization not only for the purpose of locating HMGR, but also to investigate other aspects of isoprenoid synthesis. We are already able to separate the site of (MVA) to triterpene (C₃₀) synthesis from the site of HMGR activity. The site of MVA metabolism is also an organelle, but different from the site of HMG-CoA metabolism. By separating the two sites we now have a system where we can study some of the latex stages of isoprenoid production in latex.

Organization of Euphorbia Latex.

Isolation of organelles from *E. lathyris* latex has been hindered by the high lipid content of the latex. Attempts to remove the lipid by lyophilization and/or organic solvents resulted in the loss of sterol biosynthesis. We have partially isolated an organelle responsible for the conversion of mevalonic acid to sterols by a combination of differential and Percoll gradient isopycnic isolation.

We have localized specific organelles in latex using centrifugation and marker enzymes. Enzymes specific for mitochondria, endoplasmic reticulum (microsomes) and vacuoles all have been found in latex. Tests for peroxisomes and rough endoplasmic reticulum have provided negative results. Experiments to identify plastids are still underway. Nucleic acids were also found in latex.

Previous differential centrifugation studies have indicated that the synthesis of sterols from acetate can be physically divided into two separate processes: the conversion of acetate to MVA

and then the conversion of MVA to sterols. These results also indicate that the two processes are compartmentalized in two separate subcellular organelles. Since compartmentalization is one form of biological control, we have attempted to isolate the individual organelles found in *E. lathyris* latex, so that we may study further their role in the control of isoprenoid biosynthesis.

Mechanism of Squalene Cyclization

The major components of *E. lathyris* latex are tetracyclic triterpenoids, which are stored as free triterpenols, and their fatty acid esters. To understand the biosynthetic processes involved in the production of sterols it is necessary to elucidate the structures of these compounds. We have previously identified four of the six major triterpenols as cycloartenol, 24-methylenecycloartenol, lanosterol and 24-methylenelanosterol. With the results obtained from ^3H -NMR, chemical shift reagents, ^{13}C -NMR and optical rotation we have identified the fifth compound as eupha-7,24-dienol. We have made a preliminary identification of the sixth compound as euphol on the basis of its behavior in gas chromatography.

In most plant systems the initial product of squalene cyclization is cycloartenol and not lanosterol as found in animals and fungi. However, since lanosterol is a major component of *E. lathyris* latex, we are attempting to determine if lanosterol is (a) a product of cycloartenol, (b) produced via separate, parallel pathway, or (c) is a precursor of cycloartenol. To determine if one of the first two mechanisms is responsible for squalene cyclization, we have synthesized deuterium-labeled MVA to be used as a substrate to follow its latex-catalyzed conversion to lanosterol.

One of the major difficulties in this analysis is the high level of endogenous (and unlabeled) sterols present in the latex. These make the small amount of newly synthesized deuterated compounds difficult to detect by gas chromatography-mass spectrometry. Attempts at delipidation by extraction with organic solvents such as butanol and diisopropyl ether greatly reduced the level of triterpenols but also destroyed all biosynthetic activity. Purification by rate sedimentation and isopycnic centrifugation has reduced the lipid level to some extent, but we are still trying to find the proper analytical conditions that will allow us to employ the GC-MS to observe the deuterium label.

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C. HAO2 - ENVIRONMENTAL RESEARCH AND DEVELOPMENT

The Chemical Biodynamics programs in the Energy Research and Development section of DOE involve understanding the relationship between nucleic acid structure and function.

- The studies include determining the structure of protein-DNA complexes by X-ray crystallography, NMR, ^3H -NMR, and fluorescence correlations spectroscopy. This work is aimed at determining how these two types of macromolecules find each other and how agents which cause mutations perturb their interactions.
- This program contains work which is directed at determining how specific lesions placed in DNA give rise to mutational events. We are engineering into plasmids a site directed psoralen molecule and transfecting the plasmids into human cells to study the effect of the lesion on replication, recombination, and mutation of the DNA.
- We are studying the regulation of genes during the cell cycle with particular emphasis on the the expression of genes thought to control the commitment to replicate DNA.

Underlying all of these studies is a continuing effort to understand the basic structure of nucleic acids and how secondary structures within the molecules regulation functions of genes.

1. BIOMOLECULAR STRUCTURE ANALYSIS BY NMR (David E. Wemmer)

Our efforts this year have been directed to several problems of protein and oligonucleotide structure, and have used high resolution NMR spectroscopy extensively. The general goals of the projects described are: to understand the solution structure of DNA molecules with a nick or gap in the sugar phosphate backbone as simple models for damaged DNA; to determine the structure of a drug/DNA complex, and determine the microscopic kinetics of the binding steps; and to analyse the structures of several small cystine rich proteins to enhance our understanding of the active parts of the structure. A significant amount of time has gone into implementation of methods for preparing large quantities of specific sequence DNA oligomers for study, and to setting up the necessary software on our VAX computer systems for analysis of NMR data, and the required structure calculations. The results of these efforts are discussed for particular projects below.

Gap DNA structures

By choosing special sequences for synthetic DNA oligomers we have been able to form stable "sticky end" dimers in solution from DNA hairpin with dangling ends. The sequences are chosen with a central section which is not complementary to any other in the molecule, which is flanked by a self-complementary sequence. Such sequences have been shown previously to lead to hairpin formation. We have modified these sequences to include a self complementary dangling tail, which allows two such molecules to associate in solution. We have observed the imino proton spectra from these molecules to confirm that such a dimer is indeed the stable form in solution. The imino protons have been assigned using nuclear Overhauser effect difference spectra, and confirm that the dimer is essentially a single continuous double helix in solution. On each of the backbone strands, however, there is a break and a missing phosphate. We have now collected complete two dimensional NOE data sets (NOESY spectra) of two different sequences. In these spectra, when collected at sufficiently short mixing times, the cross peak intensities reflect the inverse sixth power of distances between protons. By analysing the intensities of many cross peaks in the spectrum, and paying particular attention to those at the site of the gap in the backbone, we have determined that the backbone is relatively undistorted, as compared to a standard "B DNA" even at the site opposite the gap. There does appear to be a slight overwinding of the DNA at this site, giving a slightly greater than average local helix twist angle. We have also used the temperature dependence of the NMR spectrum to examine the melting in these sequences. We find that, as expected, melting occurs first as a separation of the dimer into monomers, followed at higher temperature by opening of the hairpin loop. For sequences with 4 GC base pairs in the overlap region, and six in the stem of the hairpin loop these melting events are separated by about 15°C, with the first transition having slower exchange kinetics (between monomer and dimer) than the latter.

DNA-drug binding

We have previously carried out NMR studies of the binding of the antibiotic distamycin-A to a specific DNA dodecamer. This drug shows a strong preference for binding at AT rich

sequences, AATT in the cases which we have studied in detail. From the NMR data it was possible to position the drug relative to the DNA, and to qualitatively evaluate the degree of distortion of the DNA by the drug. We have now supplemented these measurements by carrying out energy minimization calculations with the AMBER program from UCSF. A general problem with such calculations is that the minimization algorithm cannot find a global minimum of energy in a reasonable time. We find that starting with the drug relatively far from the position determined from NMR, convergence is obtained, but to a drug binding position which is far from that experimentally determined. However, when the calculation is started near the experimentally determined structure the energy obtained upon convergence is lower. The coordinates obtained through such experimentally guided modelling are better than what can be determined from the NMR data alone. Presently we are trying to extend the measurements to other DNA sequences, and to analyse the differences in binding constant and kinetics of drug binding in light of the detailed structural information we now have about the bound state.

Analysis of protein structures

We are interested in the determination of protein structures in solution for several reasons. First, we expect to be able to use cystines at specific residues in proteins to predictably fold the remainder of the sequence. The expectations are based upon the observed folding in several naturally occurring peptides, which have common cystine positions. While we have previously assigned the resonances in two of these peptides, and qualitatively modeled their secondary structure, it is important to carry out a more quantitative analysis. To do this we have collected 2D NOE spectra and integrated cross peak intensities to obtain interproton distances for apamin, a small neurotoxic peptide from honey bee venom. With a relatively large number of such distances we have carried out distance geometry calculations. This approach takes the distance estimates, including estimates for the experimental precision, and computes from these coordinates for structures which are consistent with all of the input data. From the range of structures obtained from repeated calculations we can analyse the precision with which the structure is determined, e.g. its effective "resolution".

In a similar fashion we have begun analysis of the relationship between structure, function and immunogenicity of protein toxins isolated from sea anemones. These again are related through having common cystine positions, and several other conserved residues. However different toxins from this family are active against different receptors, and do not all show antibody cross reactivity. We have now fully assigned resonances of the *Radianthus paumotensis* toxin II, and have established that its only regular secondary structure is beta sheet, with strands connected with a variety of loops and turns. We will now begin structure calculations for this protein, and at the same time have begun assigning a related protein Rp III. We have just obtained the sequence of Rp III through a collaboration with Prof. Ken Walsh at the University of Washington, and have already established that there is a high degree of homology in the structures. From the refined structures for proteins in a related family, such as these, we should be in a good position to look for the common structural features which give rise to the similar folding of the peptide chain, and yet be able to see the differences which lead to different activities, and immunogenicity.

2. BIOPHYSICAL CHEMISTRY (Melvin P. Klein)

In Vivo NMR Spectroscopy

Phosphorus-31 NMR spectroscopy is evolving into an important means for determining the *in vivo* concentrations of phosphorylated metabolites and has definite clinical implications and applications. Our previous contributions to this field demonstrated the feasibility of employing implanted radio frequency coils around organs of laboratory animals to permit eliciting the NMR spectra over long periods to establish base line spectra. Using these devices and techniques we have determined phosphorus exchange reactions in rat hearts and kidneys, *in situ*, and have demonstrated that there are pools of metabolic intermediates that are not directly visible in the NMR spectra. Comparison of the results from NMR spectroscopy with those obtained from radiolabeling studies on Chick Embryo Fibroblasts (CEF) also showed that there are significant pools of phosphorus not visible in the conventional P-31 NMR spectrum. Both sets of studies suggest that compartmentation occurs. The invisibility of these pools is assumed to arise because of immobilization of the molecules by cellular macromolecules or organelles.

The methods of solid state NMR spectroscopy are being applied to render visible these solid-like species. In particular we use the technique of magic angle sample spinning, together with cross polarization for signal enhancement, (MASS/ CP). Application of these methods to a large number of biological phosphorylated molecules, for which crystal structure data are available, has permitted us to correlate the values of the chemical shielding tensor elements with details of chemical bonding within the phosphate moieties. Upon application to lyophilized tissue, we observe phosphorus signals attributable to phospholipid head groups. The proton spectra of lyophilized tissue, elicited with these techniques, are surprisingly rich and exhibit narrow features reminiscent of solution spectra. These narrow features are assigned to hydrocarbon chains of the membrane phospholipids of the tissue. Further support for such an interpretation is provided by the C-13 spectrum of these samples whose features are completely compatible with those of lipid chains.

We interpret these surprising findings to result from the fact that at the temperature of observation, at room temperature, the membrane phospholipids of the tissue are in the liquid-crystal state characteristic of their molecular composition. Normal functioning of the cellular membrane, as exemplified by the fluid-mosaic model, is assumed to require a high degree of dynamic mobility. That we observe such high resolution proton spectra in lyophilized tissue is indeed dramatic support for such a model.

Nuclear Magnetic Resonance Spectroscopy

The Chemical Biodynamics Division of LBL has inaugurated Tritium NMR (TMR) spectroscopy in conjunction with the establishment of the National Tritium Labeling Facility. The potential applications of TMR to problems in structural biology and biophysics are very great. They promise to extend the molecular weight range of molecules that can be profitably studied with NMR by several fold, will permit the study of interactions between enzymes and bound substrates, between receptors and effectors, and between proteins and nucleic acids. This

potential derives from the facts that the intrinsic sensitivity of the triton is some 7% greater than that of the proton, that there will be zero interfering background signals and because the tritium spectrum will be sparse, arising only from those tritons at the sites specifically labeled. Importantly, the abundant protons can be decoupled from the tritons thus reducing their contribution to resonance broadening. We are able to work at the millicurie level of activity.

In a first application of TMR to a biological problem, we have observed the conversion of glucose, tritiated at the C-1 position, to lactate upon incubation with human erythrocytes. During this metabolism, additional resonances appear transiently indicative of metabolic intermediates. The most remarkable aspect of these spectra is the ability to observe the tritiated hydroxyl species at micromolar levels in the presence of 55 molar water without interfering background.

During June of 1986 a new 300 MHz NMR spectrometer, specifically configured for optimum utility with TMR was installed in the laboratory.

3 MUTAGENESIS - FUNDAMENTAL CHEMISTRY (John E. Hearst)

Our objective is to develop a molecular model for chemical mutagenesis from *in vitro* and *in vivo* studies of replication and transcription of chemically modified DNA templates.

Many carcinogenic as well as chemotherapeutic agents cause covalent linkages between complementary strands of DNA. Crosslinked DNA is a block to DNA replication which, if unrepaired, constitutes a lethal lesion to the cell. While the subject of DNA crosslink repair has been an area of intensive study, the molecular events of this process have not been well characterized. Genetic studies of *E. coli* have demonstrated that ABC excision nuclease, coded for by the three unlinked genes *uvr A*, *uvr B*, and *uvr C*, plays a crucial role in DNA crosslink repair.

To study the molecular events of ABC excision nuclease mediated DNA crosslink repair we have engineered a DNA fragment with a psoralen-DNA interstrand crosslink at a defined position, digested this substrate with the pure enzyme, and analyzed the reaction products on DNA sequencing gels. We find that the excision nuclease a) cuts only one of the two strands involved in the crosslink, (b) cuts the crosslink by hydrolyzing the ninth phosphodiester bond 5' and the third phosphodiester bond 3' to the crosslinked furan-side-thymine, and c) does not produce double strand breaks at any significant level.

We have constructed a substrate for the ABC excision from 6 oligomers which were then ligated together to form a 40 bp DNA fragment containing the central 8-mer, TCGT*AGCT, in which the internal thymine is modified on the 3' side by a psoralen derivative 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT). Both pyrone (3,4, and furan (4',5') monoadducts have been isolated, the latter of which (upon the absorption of a photon of 360 nm light) reacts with the thymine in the opposite DNA strand to form a crosslink. The crosslinked 40 bp DNA fragment was then purified on a denaturing polyacrylamide gel.

The crosslinked DNA was terminally labelled at either the 5', 3', of both termini, digested with ABC excision nuclease and the reaction products were analyzed on a DNA sequencing gel.

The cutting pattern observed with crosslink is slightly different from those seen with nucleotide monoadducts and intrastrand diadducts (thymine dimers).

First, the enzyme cuts on both sides (8 bases on the 5' and 2 bases on the 3' side) of the furan side crosslinked thymine.

Second, the cutting sites with regard to furan side-monoadducted thymine are 7 bases on the 5' and 4 bases on the 3' side which are different from those seen with the crosslink. One plausible explanation for the difference in the cutting patterns for these two psoralen adducts may be that the crosslink creates a substantially different helical deformity in DNA than that caused by monoadducts or intrastrand diadducts so that the ABC nuclease complex must "slide" in the 5' direction to accommodate the crosslink. We have quantified the relative amounts of ABC-mediated incision of the monoadducted and crosslinked fragment and found that the crosslink is 2-3 fold less efficiently cleaved. Using model building studies and energy minimization techniques, it has been proposed that a psoralen crosslink produces a kink and local DNA unwinding at the site of the damage. It is possible that the crosslink distorts the local DNA structure to a greater degree than the monoadduct, so that the DNA helix is not in the typical B-form and the two excinuclease incisions occur the same distance in space, but fewer nucleotides away from one another.

Third, ABC excision nuclease performs its dual incisions of the furan side monoadducted DNA in a highly coupled process because bands corresponding to a lone 3' incision on the 5' labelled fragment of a lone 5' incision of the 3' labelled fragments were not seen in significant amounts (less than 1

Finally, in HMT crosslinked DNA in which the thymine is modified on the 3' side, it appears that ABC excision nuclease acts only on the DNA strand which is attached via the furan ring of the crosslink. To confirm that ABC excinuclease does not make incisions on both the pyrone and furan sides of the crosslink, resulting in double strand breaks, the digestion pattern was analyzed on non-denaturing gels where not such double strand breaks were found.

A molecular model for chemical mutagenesis associated with psoralen photoaddition will be achieved. The site and stereo specific placement of psoralens into M13 and SV40 will continue. We anticipate initiation of the *in vivo* mutagenesis studies on both of these viral DNA's in the coming fiscal year. Procedures for making these constructs are nearly completed and the biological experiments can now begin.

The preference by ABC excinuclease for incision on the furan side of the HMT crosslink is highly significant and we believe is indicative of the DNA structure of the crosslink rather than any recognition by the enzyme for the furan moiety. Further studies on ABC excision nuclease - mediated incisions on DNA containing psoralen crosslinks in various orientations will yield information on both the mechanism on the enzyme's action, as well as the conformation of psoralen crosslinked DNA.

4. CONTROL OF DNA SYNTHESIS IN HUMAN CELLS (J. C. Bartholomew)

The genome of human cells contains approximately 10^9 nucleotide pairs organized into a particular sequence. The faithful replication of this amount of information into each daughter cells is obviously a formidable task. A clue as to how the genome sequence is normally maintained in such a highly organized state during DNA replication is to learn something about the factors that destabilize the replication of the genome. Our research program centers its activity on the hypothesis that much of the control of genome replication takes place at the level of initiation of DNA replication within sections of the genome. We are interested in understanding what cellular factors regulate this initiation, how this regulation breaks down in various disease states, and how external environmental stresses can lead to aberrant initiation of DNA replication resulting in increased gene copy number.

Because the human genome is so large and the mechanisms regulating its replication likely to be so complex, we have attempted to develop model systems which can help us understand these processes. One approach we have taken is to study the control of oncogenes thought to be involved with regulating the commitment of cells to DNA synthesis. The second study we have been carrying out is to study the aberrant initiation of DNA synthesis that results during gene amplification, and finally we have been investigating the effect of chemical carcinogens on DNA replication and mutation.

Regulation of *N-myc* Gene Expression

We are investigating the involvement of protooncogene sequences in the regulation of initiation of DNA synthesis in cells growing in culture. Our hypothesis is that these sequences code for components the cells need to traverse the cell cycle and initiate DNA synthesis. We have focussed our attention on a member of the *myc* family of oncogenes - *N-myc*. The *N-myc* oncogene is amplified and/or expressed at a high level in many cell lines derived from neuronal tumors; non-neuronal cells apparently do not express this gene. Y79 cells derived from a retinoblastoma tumor have been shown to have 50 - 100 copies of the *N-myc* gene transcript per cell. We first tested whether *N-myc* is preferentially expressed during specific parts of the Y79 cell cycle. Y79 cells were synchronized with double thymidine blocks at the beginning of S, then released from the block and collected for isolation of total cytoplasmic RNA and for analysis by flow cytometry. *N-myc* DNA probes hybridized with dot blotted RNAs and RNAs from Northern blots of agarose-formaldehyde gels showed no differences in the numbers of transcripts as the cells traversed G₁ and entered the S phase over a period of 12 hrs. Protein synthesis inhibitors added to randomly growing Y79 cells did, however, show a dramatic increase in *N-myc* transcript number over a period of 4 - 6 hrs; control genes showed no such increase. Inhibition of RNA synthesis by actinomycin D treatment led to a rapid decrease in *N-myc* transcript number which was partially prevented by protein synthesis inhibitors. Thus, *N-myc* transcripts have a short half-life whose stability may be increased by inhibition of protein synthesis. These results confirm that similarities between *c-myc* and *N-myc* go beyond their partial sequence homology and suggest that *N-myc* may serve the same (as yet unknown) function in neuronal cells that *c-myc* plays in non-neuronal cells.

Gene Amplification

The gene copy number is normally a highly conserved factor during DNA replication. It is clear that on an evolutionary scale genes have increased in number sometimes forming functional gene copies; however, often the amplification results in the formation of pseudogenes. Recent studies have indicated that the control of gene copy number may break down on a shorter time scale. Numerous examples have been found in which the number of gene copies for a particular selectable marker has increased when cells are grown in the presence of a selecting agent. Generally, after applying a selecting agent these amplified genes appear within a few cell generations in the form of extrachromosomal gene copies and may progress after long exposure to form reintegrated tandemly arranged multiple gene copies. Whether the selecting agent is inducing the amplification of genes or selecting for pre-existing cells with amplified genes is not known. It has been shown, however, that an agent which damages DNA can induce either the stabilization of amplified genes, or the amplification process itself.

As a model system for the study of gene amplification, we have been studying the regulation of copy number of SV40 viral sequences present in human cells after transformation. Our hypothesis is that there are cellular factors that regulate the copy number of these sequences and that we can identify these factors by developing an *in vitro* DNA replication system. We have begun by characterizing the number of copies of SV40 DNA present in human cells transformed by this virus. In our initial studies of two human cell lines we obtained from the American Type Tissue Culture collection that were transformed with wild type SV40 virus we discovered that the cells contained many copies of SV40 that were extrachromosomal elements. The cell lines we are studying are GM637, a human fibroblast cell line from an apparently normal individual, and XP12RO, a line from a *Xeroderma pigmentosum* patient containing a mutation in a DNA repair gene. Both of these cell lines were transformed with wild type SV40 by other investigators. The aim of these studies has been to determine the involvement of DNA repair processes in gene amplification.

The presence of the extrachromosomal copies of SV40 in human cells has been observed by other investigators. The origin of this extrachromosomal DNA and its relationship to the input transforming DNA is not known. To understand the system better we began to characterize the DNA. We found that in an actively growing population of GM637 cells the copy number of SV40 DNA is approximately 400 per cell. The extrachromosomal DNA is also present in XP12RO cells. Other human cells we are carrying in the lab that are not transformed with SV40 virus do not have the extrachromosomal SV40 DNA. On agarose gel electrophoresis, the extrachromosomal DNA from both cell lines has a lower molecular weight than wild type DNA. Restriction enzyme digestion indicates that the lower molecular weight is the result of a deletion in the A-gene coding for the T-antigen. In both cell lines the deletion maps near the boundary of the intervening sequence and the small exon of the T-antigen. The difference between the two DNAs seen so far is that the size of the deletion for the DNA from GM637 is about 300 base pairs and for the DNA from the XP12RO cells is about 250 base pairs. We have cloned the extrachromosomal DNA from GM637 into a pUC vector and then subcloned a Hind III fragment containing the deletion into M13 for sequencing. The sequence indicates that the deletion has eliminated one of the splice sites for the intervening sequence and has stopped just

short of the translation termination site for the small t-antigen. The deletion stops out-of-frame so that presumably the translation machinery reads through the normal termination signal, but encounters another termination signal in the new frame just upstream from the deletion. The prediction from this data is that the mutant DNA encodes a mRNA that is similar in size to the message for wild type T-antigen, but that the premature translation termination signal would result in a protein being made that is just slightly smaller than small t-antigen. We are presently isolating the mRNA and the proteins from the cells to determine what is made from the mutant DNA. At this point we know nothing about the function of this extrachromosomal DNA, or about its origin from the wild type virus used to transform the cells. Its curious that two cell types that originated from separate transformation events give rise to very similar extrachromosomal deleted DNA.

Replication of Carcinogen Modified DNA

We have been developing a system for the study of the replication of carcinogen modified DNA in mammalian cells. For this assay we have constructed a shuttle vector that can replicate both in bacteria and mammalian cells. This vector was constructed by cloning of the SV40 origin for DNA replication into an M13 vector. M13 virus contain a single stranded DNA which replicates efficiently in *E. coli*, and is ideal for sequencing DNA placed in the Messing cloning site. The construct we have made contains the SV40 origin in the I region adjacent to the cloning site and the β -galactosidase gene. The assay for replication in mammalian cells has been to transfect the double stranded replication form into mammalian cells at approximately 50 ng per 10^6 cells. The DNA is harvested at different times after transfection, digested with Dpn I to eliminate the DNA that has not replicated, and then used to transform *E. coli*. The surviving and replicated DNA will form plaques on a lawn of *E. coli*. M13 DNA which has been mutated to block the active production of β -galactosidase can be distinguished from input DNA by a color assay. Our studies so far have indicated that the DNA we have constructed can indeed replicate in Cos 7 cells which provide a functional T-antigen from an integrated copy of SV40. So far we have not been able to demonstrate replication of our vector in human cells; however, we have only tested the GM637 cells which already contain many copies of their own extrachromosomal DNA(see above). We plan to construct a transfection recipient of human cells by utilizing an approach similar to that used to construct the Cos 7 cell line. Wild type SV40 will be inactivated for replication by cutting at the origin with Bgl I followed by treatment with Bal I to digest away the ends. The deletions generated will then be transfected directly into untransformed human fibroblast cells and transformed variants will be isolated. The transformed cells will be tested for extrachromosomal DNA, and only those clones with integrated SV40 will be used as transfection recipients. We intend to carry out this technique with different human cells so that we can have recipients to test the effect of such things as differentiation state of the cells and DNA repair capacity on the ability to replicate transfected DNA both with and without carcinogen modification.

Using the M13 vector containing the SV40 origin we constructed we are testing the effect of carcinogen adducts on the replication of DNA. This project has come out of our extensive work characterizing the effect of benzo[a]pyrene on the replication of SV40 viral DNA. The

conclusions of these studies has been that the carcinogen causes a build up of replicated intermediates which were unable to complete the final steps in separating the daughter molecules into mature completely replicated forms. One difficulty with these studies has been the random location of the adduct and the inability therefore to locate the replication block relative to the adduct. By site specifically placing the adduct we hope to get around this difficulty and to also provide a vector for studying the kind of DNA sequence changes that occur when adducted DNA is replicated.

The vector does replicate in monkey cells containing an active gene for production of T-antigen with a low mutation frequency as judged by the plaque assay on *E. coli* described above. We have treated the DNA with benzo[a]pyrene at a level which reduces the yield of plaques by 50%, and have obtained vector lacking the ability to induce β -galactosidase from DNA replicated in the monkey cells. We are presently characterizing these vectors to determine precisely what mutations have occurred.

Work is progressing in the site-specific placement of a psoralen adduct in the cloning region of M13. This work is being done in collaboration with the Prof. John Hearst's laboratory. In our approach, we have made two M13 vectors containing the SV40 origin. One vector contains a synthetic oligomer in the cloning region, and the second lacks this region. Hybridization of these to single strands creates a double strand DNA except in the region of the cloned synthetic oligomer. The approach is then to place a psoralen adduct on another synthetic oligomer that hybridizes to the oligomer cloned into the vector and then to seal the fragment in place with ligase. We have completed the work to the formation of the gapped intermediate and presently are determining if the synthetic oligomer will hybridize into the gap and be ligated. Our future work will then be to transfect this DNA into cells with different capacity to replicate and/or express the DNA and determine how the adduct affects these processes.

***In Vitro* Replication of Carcinogen Modified DNA's**

Our experience working with DNA transfected back into mammalian cells has shown us that the DNA is greatly modified during the transfection procedure. In order to overcome these difficulties we have begun to develop a system for conducting the DNA synthesis experiments *in vitro*. Until recently it was not possible to get any mammalian cell DNA replication to occur *in vitro*. Recent work in other laboratories have shown that it is possible to replicate SV40 based DNA in complex mixtures of factors from human cell lines. Not only does elongation of preinitiated replication occur, but multiple rounds of initiation are possible. We are setting up this assay in our lab to look at the replication of the psoralen modified vectors described above. This system will also be used to investigate the cellular factors required for DNA synthesis and how carcinogen pretreatment of the cells affects the levels of DNA replication.

5. STRUCTURAL BIOLOGY (S. -H. Kim)

Biological systems have sophisticated and precise means of communicating through specific recognition and interaction between communicating molecules. Our overall objective has been to understand the mechanisms of this biomolecular communication from a structural viewpoint. Also since it is now thought that the molecular recognition is an interactive process involving changes in the structure of the participants, the conformational flexibility of these molecules has been studied as well.

Structural Models for DNA Damaged by Covalent Interaction with Chemicals and/or UV

Chemicals and radiation in the environment often interact adversely with DNA of living cells resulting in abnormal behavior and or death of the cells if the damage is not repaired. To understand how the damaged DNA is recognized and repaired one needs to know, among other things, the structures of the damaged molecules and their intermediates at the molecular level. Two of the most studied damaged DNAs are those containing UV induced thymine dimer and psoralen cross-link.

Combining the structural information from the crystal structures of a double stranded DNA fragment and the psoralen-thymine monadduct (Peckler et al., 1982, *J. Mol. Biol.* **162**, 157) we have recently constructed a molecular model for the psoralen cross-linked DNA. This model was then further refined by the energy minimization technique to remove any stereochemical violations. The final model (Pearlman et al., 1985, *Science* **227**, 1304-1308) revealed that the psoralen cross-linking of DNA introduces a drastic kink and unwinding of the DNA (right, Fig. 1). A similar approach was taken to derive the molecular model of the DNA containing a thymine dimer. Here again we find the similar type of DNA distortion: Kinked and unwound DNA (middle, Fig. 1). These two distorted DNA models have been compared with the DNA duplex model (left, Fig. 1) that has been energy-minimized in exactly the same way as the two damaged DNA models. The comparison showed that the psoralen cross-linked DNA is kinked by 47 degrees and unwound by 88 degrees, while the thymine dimer containing DNA is kinked and unwound by 27 and 20 degrees, respectively. These models further suggest that such a gross structural distortion may be one of the important common features of other damaged DNA as well, and that the DNA repairing enzymes may be recognizing such a structural distortion. The technical details of model construction, energy minimization and structure comparison have been published (Pearlman, Holbrook, Prikle, and Kim, 1986, *Science* **227**, 1304-1308).

In summary, our study presents two detailed atomic models for the DNA damaged by covalent modification, psoralen cross-linked DNA and thymine dimer containing DNA. The models indicate that the magnitude of these changes are quite large, providing possible recognition signals for repair enzymes, while potentially able to cause great harm to cells if not properly repaired. The significant kinking and unwinding in our models suggest that the effects of these types of DNA damage may be long-range, as well as short-range. Although no direct visualization of these damaged DNA by experiments have been reported, various indirect studies of the conformational effects of UV-induced DNA damage have offered evidence that such lesions cause major conformational changes consistent with our models (Denhart and Kato, 1973, *J. Mol. Biol.* **77**, 479; Legerski et al., 1977, *J. Biol. Chem.* **252**, 8740; Wang and

McLaren. 1972, *Biophysik* **8**, 237; Wiesehan and Hearst, 1978, *Proc. Nat. Acad. Sci. USA* **75**, 2703; Ciarrocchi and Pedrini, 1982, *J. Mol. Biol.* **155**, 177).

Changes in DNA Mobility in Non-Covalent Complex between DNA and Daunomycin

Interaction between DNA and protein or other ligands in solution is profoundly affected by the flexibility of the DNA at or near the interaction site, probably because most specific interaction accompanies the conformational changes of DNA. To assess the local flexibility of a given DNA sequence, we use x-ray crystallographic data. X-ray diffraction data in addition to supplying the atomic coordinates of atoms of a molecule in a crystal, contains information about the mobility of all the atoms in the crystal. We have developed a procedure (Segmented Rigid Body Model) which allows us to extract information about both the magnitude and direction of the atomic mobility in macromolecules. We have applied this procedure to the nucleic acids in order to obtain the first experimental data concerning the local conformational flexibility of DNA and RNA molecules.

Our first studies were on several small RNA fragments (single and double stranded) and on the dodecanucleotide duplex of the sequence CGCGAATTCGCG which forms a B-DNA double helix (Holbrook and Kim, 1984, *J. Mol. Biol.* **173**, 361). We have now extended these studies to a Z-DNA hexanucleotide duplex and a hexamer double helix DNA intercalated by the antibiotic daunomycin. Comparison with our earlier results on these studies of DNA duplex alone revealed that the intercalation of daunomycin reduces the flexibility of the DNA considerably. Such reduction in flexibility may be one of the key factors contributing to the antibiotic property of daunomycin.

Expression of Mouse Metallothionein Gene in *E. coli*; Increased Tolerance To Heavy Metals

Metallothioneins (MTs) are a group of small eucaryotic proteins whose level of expression can vary several folds in response to heavy metal exposures. Exposure to heavy metals leads to the rapid and increased production of MTs (Dumam, D.M. and Palmiter, R.D., 1981, *J. Biol. Chem.* **256**, 5712-5716; Enger, M.D., Rali, L.B., and Hildebrand, C.E., 1979, *Nucleic Acids Res.* **7**, 271-288; Karin, M., and Herschman, H. R., 1980, *Eur. J. Biochem.* **107**, 395-401; Maiti, I., Mbikay, M., Marengo, C., and Thirion, J.P., 1982, *J. Cell Physiol.* **112**, 35-41) as a result of enhanced thionein mRNA synthesis and gene amplification (Beach, L. and Palmiter, R., 1981, *Proc. Natl. Acad. Sci.* **78**, 2110-2114). The induction of MTs in response to heavy metals implies a role for these proteins in the metabolism and detoxification of heavy metals (for example, see ref. Garvey, J.S. and Chang, C.C., 1981, *Science* **214**, 805-806). these proteins may also mediate the transport of heavy metal ions in cells and sequester the excess ions into organs such as liver or kidney (Brady, F.O., 1982, *TIBS*, April, 143-145).

MTs bind a wide range of metal ions with different coordination geometry and strength, including silver, copper, cadmium, zinc, nickel, and cobalt (Winge et al., 1975, *Arch. Biochem. Biophys.* **170**, 242; Piotrowski et al., 1978, in *Metallothionein*, Kagi and Nordberg, eds, pp 363, Berkhauser Verlag, Switzerland). The conformational changes within the protein and the degree of the changes in order to accommodate different metals are not understood. One way to look at this problem is to generate mutant MTs with altered metal specificities. For this purpose a homogeneous production of one isoform of MT is desired, preferable in *E. coli*.

We have constructed a bacterial clone containing the cDNA of a mouse MT-1 gene and showed that the unique heavy metal-binding ability of MT is successfully reflected in the bacterial phenotype, i.e., an increased resistance to heavy metals. Figure 2 shows that all cell lines grow in agar plates containing $30\text{ }\mu\text{M}$ $\text{Hg}(\text{CH}_3\text{COO})_2$, but only two clones harboring plasmids 19 and pYM101 survive when the metal concentration is raised to $40\text{ }\mu\text{M}$. We believe the heavy metal resistant phenotype of this clone provides an attractive system for future isolation of mutant metallothioneins with different specificities and binding strengths for various metal ions.

(c)

(b)

(a)

Fig. 1

KEY

Fig. 2

30 μ M $\text{Hg}(\text{CH}_3\text{COO})_2$

40 μ M $\text{Hg}(\text{CH}_3\text{COO})_2$

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D. NATIONAL TRITIUM LABELING FACILITY – (Professor Henry Rapoport)

This laboratory is a national facility both for carrying out research into the labeling of compounds to high specific activity with tritium as well as for providing a tritium labeling service for other investigators throughout the country. A primary direction this laboratory is moving into in the future is the development of compounds and techniques to allow ^3H -NMR studies of biological macromolecules.

The establishment of the National Tritium Labeling Facility at LBL, under the sponsorship of the NIH provides the setting for pursuing exciting new research in structural biology and biophysics. In particular, we propose the use of Tritium to expand the molecular weight range of biomolecules that can be studied by Nuclear Magnetic Resonance methods. Amino acids and nucleic acid bases will be selectively tritiated and then specifically incorporated into respective protein and nucleic acid sequences. As the triton is magnetically distinct from the proton and will always be dilute, the spectrum will be simple, the assignment of spectral features straightforward and the interpretation of the structural and dynamic aspects more site specific than has been possible heretofore. As there is no natural background signal, observation of the ^3H NMR spectra of tritiated substrates, *in vivo*, provides other opportunities for studying dynamic properties of enzymatics, cellular transport and metabolism.

Summary of Research Progress

The resources of the facility are divided between core research and user service functions. The service program provides the biomedical/biochemical community with a completely equipped, strictly supervised, safe laboratory in which scientists can prepare and rigorously purify their own compounds. Core research involves:

1. The study of labeling reactions of the excited species in a microwave generated tritium plasma.
2. The development of tritium NMR methodology to assist in reaction mechanism studies for labeling biomedically significant species while preserving chirality (eg enkephalins).
3. The use of ^3H -NMR as a highly specific probe in binding studies involving large molecules.
4. The isolation of the active components in a plasma discharge to elucidate the various reactivities and mechanisms of each with selected substrates.

With the acquisition of our own ^3H -NMR system the NTLF has become a unique facility, able to provide high level labeling and structural confirmation heretofore extremely difficult or impossible to obtain. Users of the facility can now obtain specific knowledge of the location and relative abundance of tritium in the compounds they have prepared. This allows a high level of confidence in the integrity of these radiolabeled compounds and in their subsequent application to specific research projects.

Core Research

Core research in the area of microwave discharge activation involved amino acid, dipeptide and tripeptide labeling by adsorption of the substrate onto solid catalyst pellets and exposure of these pellets to a tritium plasma. Results showed higher labeling yields with reduced T_2 pressure, with increased microwave power and with increased dispersion on silica-alumina catalyst (with or without dispersed nickel). Activities of curies per mM were achieved with reaction times on the order of 15 minutes. An alternative technique involved exposure of substrate-free pellets to the tritium plasma and subsequent immersion of the activated pellets in the substrate. Several halogenated aminobenzamide derivatives were labeled in this manner and specific dehalogenation yields of 5-10% were observed. In other reactions, mono and poly halogenated benzoic acids gave tritiated benzoic acid and the saturated cyclohexane carboxylic acid.

High resolution ^3H -NMR (on a borrowed instrument) revealed that the catalytic hydrogenation of organic compounds can yield both stereospecific and regiospecific information as well as incorporation rates into each of the sites. In particular, we have observed the unsymmetrical addition of tritium across double bonds and the unexpected tritiation of halogenated precursors by H-T exchange during catalyzed dehalogenation reactions. An understanding of the conditions required to minimize these side-reactions will lead to higher yields of desired products. A systematic study of the catalytic tritiation of 2-acetamidocinnamic acid showed equal amounts of tritium at the α and β positions in the product N-Ac-(2,3)(^3H) phenylalanine with the theoretical specific activity of 58 Ci/mM. However, tritiation of 2-acetamidoacrylic acid under the same conditions showed a tritium ratio of 5:95 at the α and β positions, but a specific activity of ≥ 80 Ci/mM, thereby demonstrating exchange as well as addition. We have also found that ^3H -NMR has been quite valuable for studying the kinetics of isotope exchange reactions to test the theoretical validity of the Swain equation. In collaboration with the University of Toronto (Prof. A. J. Kresge and Dr. C. Arrowsmith), exchange reactions using acetone showed $K_H/K_T = 1.6$, a value far higher than theory predicted. We intend to study the detritiation rate of acetone, chloroform, and N,N dimethylnitroaniline to further test the validity of the Swain equation and its utility in assessing tunneling and secondary isotope effects.

During this year several preliminary experiments were run using glucose-1(^3H) in admixture with human erythrocytes, and the subsequent metabolic reactions were observed *in vitro* in the NMR probe. Initially, the proton-decoupled ^3H -NMR spectra showed only the two peaks arising from the anomers of glucose, but with incubation these resonances decreased and two other major absorptions appeared. These corresponded to the methyl and hydroxyl groups of lactic acid, the product of glycolysis. Furthermore, additional transient resonances were observed prior to conversion to lactate. The significance of this experiment is that we were able to observe OT resonances at the 10 micromolar level in the presence of 55 molar H_2O . These results were presented before three conferences listed in the publications section.

Service and Training

During the past year (10/1/85-5/1/86) the NTLF has helped visitors from 6 institutions label some 23 compounds. These visitors were from both academic and industrial backgrounds. The compounds labeled ranged from insect hormones to biomedically significant enkephalins. In addition 2 graduate students and one undergraduate received training at the facility.

Resource Advisory Committee

For the past year the NTLF Advisory Committee consisted of:

Name	Title	Institution
Thomas Budinger	Professor	LBL, UC Berkeley
Neal Castagnoli	"	UC San Francisco
Richard Ehrenkauer	"	Univ. of Michigan
Arnold Lieberman	Section Chief	Hoffman-LaRoche Inc.
Nicholas Matwiyoff	Staff Sr. Scientist	Los Alamos NL, NM
Leonard Spicer	Professor	Duke University, NC

Objectives for the Coming Year

1. ^3H -NMR

As of yet no rigorous survey of sensitivity and linewidth limitations have been published for NMR observation of tritons. We intend to compare the S/N ratio for tritium detection for aqueous and non-aqueous samples with theoretical considerations and hence establish benchmarks for routine tritium observation.

We intend to develop an NMR cell which is safe from breakage yet places the maximum amount of sample in the detection coil. This should enhance resolution by at least a factor of 4 and reduce the quantity of radioactivity required per analysis. We believe that with this modification, analyses in the sub-millicurie region will be possible - a matter of importance to all ^3H -NMR spectroscopists.

Triton-proton two dimensional NMR spectroscopy will be an active field of development. These techniques are particularly important in structural determinations and of great potential use in our biomolecular studies. To initiate this work we shall specifically tritiate one of the nucleic acid bases and incorporate it into a suitable polynucleotide of length comparable to that which can be resolved only by 400 or 500 MHz NMR spectroscopy. The ^3H -NMR spectrum should be simple with just as many resonances as labeled bases, and with no background signals. Then, by triton-proton two dimensional spectroscopy, we shall determine through-bond and through-space connectivities between the tritons and nearby protons. It is planned to use some of the same duplex 17-mer employed by our colleague, Dr. David Wemmer, in the Chemical

Biodynamics Laboratory. Because of the conceptual and technical simplicity of ^3H -NMR, we shall investigate tritiated enzyme substrates and co-factors that are bound irrotationally to the proteins. These spectra will help in the development of visualizing spectra of macromolecules. In particular, we intend to use labeled N-acetylglucosamine, a substrate analogue of lysozyme. The proton spectrum of lysozyme has been extensively studied at Oxford and we shall have excellent reference proton spectra to compare with our triton-proton spectra.

The availability of the ^3H -NMR on a full time basis will have a profound effect on all of our other research and service. It is planned to routinely obtain ^3H -NMR spectra of all compounds produced through the service function of the NTLF.

2. Excitation Labeling Research

Two modifications to the microwave system will be completed this year. First, a separate tritium storage capsule (UT_3) will be installed to provide researchers with fresh T_2 whenever needed. Experience has shown that T_2 stored in a glass or stainless steel holding tank becomes contaminated in a few days so that labeling experiments yield non-reproducible results. Fresh T_2 causes no such problem. Secondly, the installation of an ion deflector system in the plasma circulating loop will be completed. This modification will allow us to study the reaction mechanisms of hot atom vs ion reactions of selected substrates, with special reference to addition and substitution reactions. In conjunction with this study, a cell for generating thermal (i.e. "cool") tritium atoms from T_2 has been completed and will soon undergo testing.

We intend to study the lifetime and reactivation of the adsorbed tritium species on supported metal catalysts exposed to the microwave generated plasma. We intend to define the adsorbed species and the mechanism of labeling. We will extend the use of the adsorbed tritium method by applying it further to the labeling of peptides and proteins and to study the efficiency of T/H exchange reactions in aqueous vs non-aqueous systems. We shall also study the reaction of $-\text{C}=\text{C}-$ and $-\text{C}=\text{O}$ groups in 4-ketosteroids with Δ^4 adsorbed tritium on the metallic catalysts because the stability and bulkiness of the molecule may reduce degradation and dimerization resulting in much purer labeled product. Since analysis of tritium distribution and concentration is essential for our study of reaction mechanisms, the ^3H -NMR system will be an important adjunct to these studies.

3. Catalytic and Exchange Tritiations

During our investigations of the reduction of vinylic precursors to desired labeled compounds (e.g. amino acids, di, tri, and pentapeptides) we obtained clear evidence that, in some cases of heterogeneous catalytic hydrogenation, considerable H/T exchange took place prior to addition. This exchange occurred with high specificity and without isomerization. Further exploration of the mechanisms of these competing addition and exchange reactions may allow us to manipulate conditions so as to select the desired reaction.

During our 2 years of operation and interaction with scientists from the biomedical/biochemical community several clear needs have emerged. One of these is the need for labeled proteins of ever higher specific activity via the amine-methyl iodide reaction to form N-methyl (^3H) bonds. Practically all of the work has been done with singly labeled $\text{CH}_2\text{T-I}$. However, it would be of significant benefit to use $\text{CHT}_2\text{-I}$ and even $\text{CT}_3\text{-I}$ if these can be efficiently prepared and promptly used (to minimize radiation damage). We intend to study this synthesis via halomethyl benzoates (modified Baeyer-Villiger reaction) and by the reaction of dichloromethyl benzoate generated by the reaction of benzoyl peroxide with chloromethanes.

Publications - NTLF

- 1 Un, S., G.S. Karczmar, G. S., H. Morimoto, and M. P. Klein. Tritium NMR Spectroscopy - Recent Results and Concerns. 19th Experimental NMR Conference, Asilomar, CA April 1985.
- 2 Klein, M. P. New Opportunities with ^3H -NMR in Biology, Chemistry and Physics. Gordon Research Conference on Magnetic Resonance, Wolfboro, NH June 1985.
- 3 Un, S. and M. P. Klein. Tritium NMR Opportunities in Chemistry and Biology. 27th Rocky Mountain Conference, Denver CO July 1985
- 4 Arrowsmith, C. H., L. Baltzer, A. J. Kresge, M. F. Powell, and Y.S. Tang. Tritium Isotope Effects on Carbon-13 NMR Chemical Shifts, *J. Am. Chem. Soc.* **108**, 1356-1357, (1986).
- 5 Peng, C. T. and O. Buchman. Tritium Exchange Labeling of Compounds Containing $-\text{NO}_2$, $-\text{I}$, $-\text{C}=\text{C}$ and $-\text{C}=\text{O}$ Groups, *Tetrahedron Lett.* **26** 1375-1378, (1985).
- 6 Peng, C. T., B. Y. Cao, and O. Buchman. Tritium Labeling by Activated Adsorbed Tritium Species. In Preparation.
- 7 Peng, C. T. "*Radiation Induced Methods of Labeling*". *Isotopes in Physical and Biomedical Sciences* vol. I. Buncl, E. and Jones J.R. (Eds.) (In press).
- 8 Hua, R. L. and C. T. Peng. Experimental Parameters and Radiochemical Yields in Tritium Labeling by Microwave Discharge Activation of T_2 . *Trans. Am. Nuclear Soc.* **50** 48-50 (1985).
- 9 Hua, R. L., Peng, C. T. Relative Efficiencies of Tritium Atoms and Ionic Species in Peptide Labeling. In Preparation.
- 10 Morimoto, H. and B. E. Gordon. History and Current Status of the National Tritium Labeling Facility. Poster Session - 2nd International Symposium on Synthesis and applications of Isotopically Labeled Compounds, Kansas City, MO Sept. 3-6, 1985.
- 11 Tang, Y. S., H. Morimoto, S. Un, and H. Rapoport. Mechanistic Studies of Catalytic Hydrogenation; Gas Tritiation and Deuteration of; 2-Acetamidoacrylic Acid and α -Acetamidocinnamic Acid. *Proc. International Symposium on Synthesis and Application of Isotopically Labeled Compounds*, Kansas City, MO Sept. 3-6, 1985.
- 12 Tang, Y. S., H. Morimoto, H. Rapoport, N.L. Shimizu, I. Ojima, and N. Yoda. Synthesis of Homochiral Tritium Labeled Peptides of High Specific Activity: Synthesis of $[\text{Ac}-(2,3-^3\text{H})\text{Tyr}^1, D-Glu^2, (2,3-^3\text{H})\text{Phe}^4, \text{Leu}^5-\text{OMe}]$. In Preparation.

- 13 Tang, Y. S. and H. Morimoto. Mechanistic Aspects of Hydrogenation and Hydrogenolysis by ^3H -NMR Spectroscopy. Transactions Am. Nuclear Soc., San Francisco, Nov. 10-14, 1986.

E. DOE SPONSORED UNIVERSITY CONTRACT - Prof. Ignacio Tinoco, Jr.

- This project studies heteroduplexes with an extra base on one strand as intermediates in frame-shift mutagenesis. These studies of double helixes perturbed by non-Watson-Crick base-base opposition, and by extra bases will help provide a fundamental understanding of all types of perturbations of a DNA helix; they may eventually lead to an improved ability to control mutations.
- A second part of this project is to develop a microscope to measure images based on the differential response of the object to polarized light. This microscope has been used to the measurement of the orientation of hemoglobin in individual erythrocytes from patients with sickle cell disease.
- Left-Handed Z-RNA -RNA has long been thought to be conformationally rigid in its double-stranded form. We are studying in RNA a transition from right-handed A-form to a left-handed form similar to that of left-handed DNA. This discovery of a left-handed RNA is significant for nucleic acid structure, and its thermodynamics and kinetics of formation are of interest. However, its main importance will depend on whether left-handed RNA has any biological function. These studies are underway.

IV. APPENDICES

A. LABORATORY OF CHEMICAL BIODYNAMICS THESES

1985

- 1 Boska, Michael Douglas. Ph.D. Thesis. Probing Chloroplasts and Algae Using Electron Paramagnetic Resonance. November 1985. (Kenneth Sauer) Chemistry.
- 2 Casti, Thomas E. Ph.D. Thesis. Environmental Effects on Photoinduced Electron Transfer. December 1985 (Melvin Calvin) Chemistry.
- 3 Hoover, Sara Melissa. Masters Thesis. Steady-State and Time-Resolved Fluorescence Emission of Bacteriochlorophyll in a Green Sulfur Photosynthetic Bacterium. November 1985. (Kenneth Sauer) Chemistry.

1986

- 1 Brown, Ward T. Ph.D. Thesis. Excited-State Proton Transfer Studies: Influence of the Solvent on The Reaction Dynamics. September 1986. (George Pimentel) Chemistry.
- 2 Buhse, Lucinda Fisher. Ph.D. Thesis. Picosecond Dynamics of Solvation: Time-Resolved Fluorescence of 4-Aminophthalimide in Solution. August 1986. (George Pimentel) Chemistry.
- 3 Hou, Ya-Ming. Ph.D. Thesis. Expression of Mammalian Metallothionein Genes in *Escherichia coli* and in *Saccharomyes cerevisiae*. August 1986. (Sung-Hou Kim) Chemistry.

B. STATISTICAL DATA

1. Postdoctoral Visitors, Laboratory of Chemical Biodynamics October 1, 1985 through September 30, 1986

Name	Source of Funding	Associate
Chou, Pi-Tai	LBL	Heinz Frei
Consani, Keith	LBL	George Pimentel
DeVos, Abraham	UC	Sung-Hou Kim
Goswami, Kishalay	LBL	Melvin Calvin
* Grant, Janice	LBL	Melvin Calvin
* Graves, Bradford	NIH	Sung-Hou Kim
Hong, Yong-Ki	Korean Science Engineering Foundation	Sung-Hou Kim
Leone, Anthony	LBL	Melvin Calvin
Maliyackel, Anthony	LBL	Melvin Calvin
Mickols, William	UC	Ignacio Tinoco, Jr.
* Nakanishi, Tomoko	Nippon Zeon Co. Ltd.	James Bartholomew
* O'Brien, Philip	LBL	John Hearst
Orton, Edward	LBL	George C. Pimentel
* Schanze, Kirk	Miller Fellow	Kenneth Sauer
* Shimer, George	UC	Ignacio Tinoco, Jr.
* Worland, Stephen	UC	John Hearst
Yachandra, Vittal	UC	Melvin Klein
* Yamagishi, Akihiko	UC	Kenneth Sauer

* Terminated

STATISTICAL DATA - Cont.

2. Graduate Students, Chemical Biodynamics Division October 1, 1985 to September 30, 1986

Name	Department	Research Director
Sam Abrash	Chemistry	George Pimentel
Douglas Anthon	Chemistry	John Clark
* Kazuko Aoyagi	Plant Physiology	James Bassham
Gregory Armstrong	Chemistry	John Hearst
John Bishop	Chemistry	Henry Rapoport
* Michael Boska	Chemistry	Kenneth Sauer
David Britt	Physics	Melvin Klein
Ward Brown	Chemistry	John Clark
* Lucinda Buhse	Chemistry	John Clark
* Harry Cartland	Chemistry	George Pimentel
* Thomas Casti	Chemistry	Melvin Calvin
Chae Joon Cheong	Chemistry	Tinoco
James Cole	Chemistry	Kenneth Sauer
James Corbett	Chemistry	Ignacio Tinoco
David Cook	Chemistry	John Hearst
Victoria DeRose	Chemistry	Kenneth Sauer
Susan Dexheimer	Physics	Melvin Klein
Marc Donsky	Chemistry	Henry Rapoport
Paul Feldman	Chemistry	Henry Rapoport
Ronald Guiles	Chemistry	Melvin Klein
Douglas Hawkins	Chemistry	Melvin Calvin
Carolyn Hoener	Chemistry	George Pimentel
Sara Hoover	Chemistry	Kenneth Sauer
Ya-Ming Hou	Comparative Biochemistry	Sung-Hou Kim
Michael Howard	Chemistry	Henry Rapoport
David Jones	Chemistry	Henry Rapoport
Chul Hee Kang	Biophysics	Sung-Hou Kim
Gary Karp	Chemistry	Henry Rapoport
Hiromi Komiya	Comparative Biochemistry	Sung-Hou Kim
Sandra Laursen	Chemistry	George Pimentel
William Lubell	Chemistry	Henry Rapoport
Catherina Maulbecker	Comparative Biochemistry	James Bartholomew
Patricia Maxson	Chemistry	Kenneth Sauer
Ann McDermott	Chemistry	Kenneth Sauer
Meredith Morgan	Chemistry	George Pimentel
Ishita Mukerji	Chemistry	Kenneth Sauer
Roberta Mulford	Chemistry	George Pimentel

Name	Department	Research Director
* Howard Nathel	Chemistry	John Clark
David O'Brien	Chemistry	John Hearst
Craig Ogata	Chemistry	Sung-Hou Kim
David Pearlman	Chemistry	Sung-Hou Kim
Joseph Pease	Chemistry	David Wemmer
Jeffrey Pelton	Chemistry	David Wemmer
Joseph Puglisi	Chemistry	Ignacio Tinoco
Renee Roemmele	Chemistry	Henry Rapoport
Bethe Scalettar	Biophysics	Melvin Klein
Paul Selvin	Physics	Ignacio Tinoco
Karen Singmaster	Chemistry	George Pimentel
Cynthia Skrukrud	Comparative Biochemistry	Melvin Calvin
Elizabeth Snowdon	Chemistry	David Wemmer
Richard Storrs	Chemistry	Kenneth Sauer
* Steve Switalski	Chemistry	Kenneth Sauer
Sarah Tabbutt	Chemistry	Kenneth Sauer
Milan Tomic	Chemistry	Sung-Hou Kim
Liang Tong	Chemistry	Sung-Hou Kim
Thein V. Truong	Chemistry	Henry Rapoport
Sun Un	Chemistry	Melvin Klein
Milton Werner	Chemistry	David Wemmer
* Steve Worland	Chemistry	Kenneth Sauer
* Li-wen Xu	Chemistry	John Clark
Muh-Ching Yee	Chemistry	James Bartholomew
* Shiela Yeh	Chemistry	John Clark
* Terminated		

STATISTICAL DATA - Cont.

3. Graduate Student Statistics : Laboratory of Chemical Biodynamics 1948-1986

Departments/(Groups) of the University represented (Ph.D. Theses):

	1985-1986	Total to September 30, 1986
Chemistry	4	131
Biophysics		17
Comparative Biochemistry	1	9
Physics		7
Plant Physiology		4
Psychology		3
Biochemistry		3
Molecular Biology		3
Agricultural Chemistry		2
Botany		2
Geology		1
Physiology		1
Mathematics		1

184 Ph.D. degrees awarded 1948-1986

10 M.S. degrees awarded 1948-1986

STATISTICAL DATA - Cont.

4. Laboratory of Chemical Biodynamics Staff October 1, 1985 – September 30, 1986

Faculty Associates (Chemistry Department)

George C. Pimentel, Director
Melvin Calvin
John E. Hearst
Sung-Hou Kim
Henry Rapoport
Kenneth Sauer
Ignacio Tinoco, Jr.
David Wemmer

Senior Scientific Staff

James C. Bartholomew
James A. Bassham*
Edward L. Bennett*
Melvin P. Klein
Richard M. Lemmon*
John W. Otvos

Divisional Fellows

Heinz Frei
Francesca Leach

Administrative Staff

William Shanahan, Administrator*
Lois Soule, Administrator
Mary Bundy
Gloria Goldberg
Beth Klingel
Evangeline Peterson
Marilyn Taylor

Scientific and Technical staff

Marie Alberti
Mohdasslam Ansari
Christopher Amy*
Matt Bann
Al Dorsky
Phil Eggers
Wallace Erwin*
Benjamin Gordon*
Everett Harvey
Stephen Holbrook
Jarmila Jancarik
Rosalind Kim
Ethel Lefall
Hiromi Morimoto
Ann Orme
Jana Reddoch*
Gary Smith
Manouchar Saljoughian
Janet Splitter
Nancy Stover*
Scott Taylor
Philip Williams
Yu-Sheng Zhu

Undergraduates

Stephen Bassett
Paul Carson
Alice Chuck
Michele Haag*
Henry Huey
Shoaib Khan*
Elizabeth Muller*
Patty Murukami
Christine Orme
Hans Peterson

Graduate Students, Postdoctoral Visitors and Faculty listed separately.

* terminated.

5. Visiting Faculty October 1,1985 – September 30, 1986

George A. Brooks
Department of Physical Education
University of California, Berkeley
(James Bartholomew)

Francesco Ciardelli
Department of Chemistry
University of Pisa, Pisa, Italy
(Tinoco)

Kenneth Eiseenthal
Department of Chemistry
Columbia University, New York, NY
(John Clark)

Heinz Fraenkel-Conrat
Department of Virology
University of California, Berkeley
(George C. Pimentel)

Alexander Glazer
Bacteriology and Immunology Dept.
University of California, Berkeley
(Kenneth Sauer)

Alfred Holzwarth
Max-Planck Institute fur Strahlenchemie,
Mulheim, West Germany
(Kenneth Sauer)

Ning Pon
Biochemistry Department
University of California, Riverside
(Henry Rapoport)

Rafael Rafaeloff
Dept. of Pure and Applied Radiation Chemistry
Soreq Nuclear Research Center, Ravine, Israel
(Melvin Calvin)

Michael Saxton
Plant Growth Laboratory
University of California, Davis
(Melvin Klein)

Beatrice Singer
Department of Virology
University of California, Berkeley
(George C. Pimentel)

Lawrence Spreer
Department of Chemistry
University of Pacific, Stockton
(John Otvos)

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