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Chemical Biodynamics Division

ANNUAL REPORT 1979

August 1980

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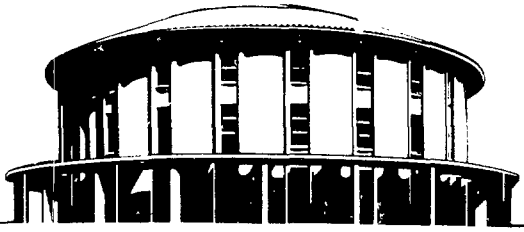

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CBB 747-5084

INTRODUCTION

Melvin Calvin

The Chemical Biodynamics Division of LBL continues to conduct basic research on the dynamics of living cells and on the interaction of radiant energy with organic matter. Many aspects of this basic research are related to problems of environmental and health effects of fossil fuel combustion, solar energy conversion and chemical, viral carcinogenesis. The division began in 1945, and its interactions with various departments on the Berkeley campus of the University of California are enhanced by its physical location. We have had graduate students from the departments of chemistry, physics, botany, molecular biology, psychology, and geology, and graduate groups such as biophysics, plant physiology, and comparative biochemistry.

From the very beginning, the research of this division has involved extensive interdisciplinary collaboration. This is fostered by unfettered discussions, strong efforts to communicate across disciplinary lines and, most important, a cooperative process for planning and administration.

As mentioned in the last annual report, the record of the division--both in its publications and in the activities of its students, postdoctoral visitors, and visiting faculty--is the principal evidence that this interdisciplinary approach has been highly successful over the years. It is this quality of the laboratory that we consider to be its unique contribution to the university community, not only in the state of California but in the United States as a whole. There exists no other laboratory, to my knowledge, that has this degree of integration of the various scientific disciplines that we have achieved here in the Chemical Biodynamics Division.

During the past year, we have begun research in an entirely new scientific area, x-ray crystallography, with the arrival of Professor Sung-Hou Kim and his research group. We look forward to the contributions that this new group will make to our activities. A second event in the development of our scientific capability has been the completion of the Animal Cell Tissue Culture Facility on the Hill which will be occupied in the spring of 1980. The research in animal cell tissue culture, virology and carcinogenesis will be moved into the new facility, vacating space in the roof area of the present building which will be renovated and used for research in plant cell tissue culture. This latter effort is very central to the research on hydrocarbon-producing plants, as plant cell tissue culture is one of the most important ways of modifying plants so that they can produce more of certain types of chemicals which are alternate sources of fuel and materials.

Finally, it should be mentioned that in 1980 the directorship of the Chemical Biodynamics Division will pass into new hands, those of Dr. George C. Pimentel, a faculty member of the Department of Chemistry of the University of California, Berkeley, and at present Deputy Director of the National Science Foundation. Under his leadership we envision the development of new research areas as well as the preservation and continuation of ongoing research programs.

Melvin Calvin,
Division Head

PHOTOSYNTHETIC LIGHT REACTIONS

Kenneth Sauer

Light absorbed by photosynthetic pigments results in excitation transfer among a large number of light-harvesting or antenna chlorophylls prior to reaching the photosynthetic reaction centers. In the reaction centers charge separation occurs, and electrons are transferred rapidly from the primary donor to bound electron acceptors. Secondary donors replenish the electrons to the oxidized reaction center components. In higher plants there results a net transport of electrons from water to $NADP^+$ and ultimately to CO_2. This complex process occurs in membranes, where the components are organized in a mosaic pattern that facilitates electronic communication and charge translocation.[1]

We have used detergents to dissect these membranes and to study the individual pigment-protein complexes. The simplest of these is a bacteriochlorophyll (BChl)-protein that contains 2 BChl together with two peptides in a complex of 20 kdaltons.[2] Absorption and circular dichroism spectra are consistent with the isolated complex having essentially a native configuration. We have used linear dichroism studies of stretched films containing the complex to characterize the geometric arrangement of the BChl molecules and to help to resolve the spectral exciton components that result from their interaction with one another.[3] In the intact intracytoplasmic membranes (chromatophores) these BChl-proteins are closely associated with the reaction centers. Photooxidation using strong light causes a pronounced bleaching of the light harvesting pigment absorption and the attendant loss of the strong dimeric exciton interaction.[4] At the same time the pronounced red-shift of the *in vivo* spectra remains, indicating that this shift results from the environment of the BChl in the protein and not from the exciton coupling itself, as had previously been assumed.

The cells of certain types of bacteria, such as *Rhodopseudomonas viridis* or *Rps. palustris* can be aligned readily in an external magnetic field of about 20 kG. When such samples are frozen in the field, the alignment is preserved, and orientation-dependent properties can be measured. In one such study we investigated the orientation-dependent triplet EPR signals associated with light-induced charge separation in photosynthetic bacteria. In the bacterial reaction centers,[5] as well as in the photosystem II reaction centers,[6] we have found that selection of triplets to populate the zero-field sublevels of the triplet spin tensor in the directions of the reaction center optical transitions.[6] From studies like these we can begin to provide detailed information about the organization of the photosynthetic membranes.

The charge separation in the reaction centers of Photosystem I of higher plants occurs on the time scale of a few picoseconds. We have found that this process can occur reversibly even at very low temperatures, 4 K, and the temperature independence of the back reaction suggests that electron tunneling is involved.[7] The charge recombination in the reaction center produces a chlorophyll triplet state that can be detected by EPR techniques, and the polarization of this spectrum is diagnostic for a radical-pair mechanism as its origin.[8] When the reaction centers are closed, a different triplet is observed by EPR; its spectrum is quite distinct from that of chlorophyll or similar molecules.[9] Subsequent studies have shown that this triplet resides on β-carotene in the reaction center.[10] We have now produced triplets in a variety of carotenoids in photosynthetic bacteria and in vitro and observe their triplet EPR signals, which apparently have not been reported previously. We hope to extend these investigations to a variety of other systems that incorporate carotenoids, including the visual pigment rhodopsin.

We have continued to investigate charge separation in Photosystem I, using measurements of electron spin polarization and rapid relaxation. Theoretical modeling of the observations points to the existence of two distinct reduced electron acceptors, with different g-values and spin tensor anisotropies, that couple with the unpaired electron on the oxidized primary donor.[11] The first acceptor has the properties of a chlorophyll molecule and the second appears to be a species, X, which has an orientation-dependent, anisotropic spin tensor. The rapid transfer of the electron through a set of four membrane-bound acceptors results in an effective charge separation across the membrane--presumably the origin of the generated electrochemical potential.[12]

One of the least understood areas of plant photosynthesis is the oxidation of water to produce molecular oxygen. Although there have been reports for many years implicating manganese in this process, no direct evidence of its participation was forthcoming. We have for the first time been able to show directly that Mn changes its oxidation state in reactions that accompany O_2 evolution.[13] Furthermore, these oxidation-state

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This work was supported by the Division of Biomedical and Environmental Research, Office of Environmental Research and Development, and the Division of Biological Energy Conversion and Conservation, Office of Basic Energy Sciences, U.S. Department of Energy, under Contract No. W-7405-ENG-48; and by the National Science Foundation, Grant No. PCM 7812121.

changes occur in a periodic fashion in flashing-light experiments and in a fashion that links them to the intermediate species participating in water oxidation. Our evidence suggests that the water-splitting complex contains two cooperating Mn atoms. These undergo changes involving the states Mn(II), Mn(III), and, transiently, Mn(IV), and the stage prior to O_2 release involves the partial oxidation of water by a process of transferring electrons to the Mn centers (Figure 1). A recent report of the isolation of a water-splitting complex involving 2 Mn in 65 Kd of protein[14] indicates the possibility that there will soon be a breakthrough in our understanding of solar energy conversion based on the photosynthetic model.

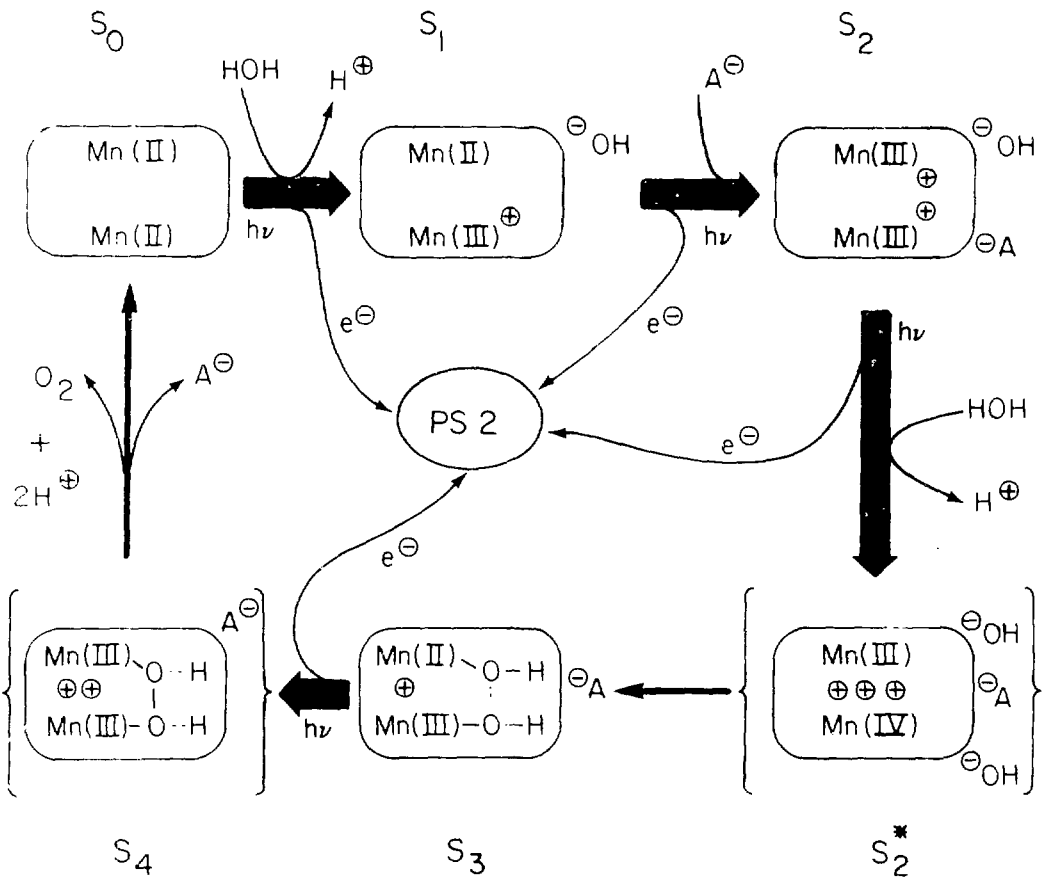

Figure 1. Changes in the oxidation state of Mn upon illumination by a series of flashes are used to deduce a plausible model for the role of 2 Mn in the water-splitting reaction of photosynthesis. Successive removal of 2 electrons from the Mn-protein complex results in a progressive increase in the oxidation state of Mn from S_0 to S_2, whereupon the next electron removed causes a partial oxidation of water and a state S_3 with decreased Mn oxidation state. The last electron removed results in the release of O_2 and restores the complex to its initial state.
(CBB 799-12351)

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Abstracts

3. LINEAR DICHROISM OF LIGHT HARVESTING BACTERIOCHLOROPHYLL PROTEINS FROM Rhodopseudomonas sphaeroides IN STRETCHED POLYVINYL ALCOHOL FILMS

John Bolt and Kenneth Sauer

Biochim. Biophys. Acta 546, 54-63 (1979)

Light-harvesting bacteriochlorophyll-protein complexes from Rhodopseudomonas sphaeroides 2.4.1 and R-26 mutant are solubilized in sodium dodecyl sulfate and imbedded in polyvinyl alcohol. Stretching induces orientation, and the linear dichroism of visible and near infrared absorption is analyzed. Based on a simple model, angles

*See Abstracts.

between the particle axis and the transition dipole moments are found. In the near infrared absorption band of the R-26 light-harvesting protein the dichroic ratio varies from 1.30 to 1.57. Using the absorption curves the band is resolved into two exciton components. In the visible absorption band the dichroic ratio has a constant value of 0.43 for the R-26 protein but varies with wavelength for the wild type light-harvesting protein. This variation is attributed to an additional bacteriochlorophyll not present in the R-26 protein.

4. PHOTOOXIDATION OF ANTENNA BACTERIOCHLOROPHYLL IN CHROMATOPHORES FROM MICROTENOIDLESS MUTANT *Rhodopseudomonas sphaeroides* AND THE ATTENDANT LOSS OF DIMERIC EXCITON INTERACTION

C. N. Rafferty, J. Bolt, K. Sauer, and R. K. Clayton

Proc. Natl. Acad. Sci. U.S.A. 76, 4429-4432 (1979)

Intense continuous illumination of purified chromatophores from carotenoidless mutant *Rhodopseudomonas sphaeroides* produces results in progressive photooxidation [illegible] of the near infrared absorption band near [illegible] nm assigned to antenna bacteriochlorophyll. The quantum yield of this reaction is low, approximately [illegible] $\times 10^{5}$. The loss in near infrared absorption is accompanied by a proportional shift in the absorption maximum to shorter wavelengths. The double circular dichroism feature in the near infrared decreases at a faster rate than does the absorbance. These results are explained by a model in which the antenna bacteriochlorophyll, initially associated as dimers (λmax = [illegible] nm), is progressively converted to the monomeric state (λmax = [illegible] nm). The wavelength shift is attributed to disruption of exciton coupling in the dimer. Acetone-methanol extraction indicates that the [illegible] extinction coefficients of the dimer and monomer differ by more than 4%. The [illegible] absorption maximum at 853 nm for monomeric bacteriochlorophyll in a protein complex demonstrates that it is not necessary to invoke aggregation of the chromophores as the origin of the [illegible] in typical organic solvents.

5. THE [illegible] OF THE PRIMARY DONOR IN BACTERIAL [illegible]

Harry A. Frank, [illegible] Friesner, John A. Nairn, G. Charles [illegible] and Kenneth Sauer

Biochim. Biophys. Acta 547, 484-501 (1979)

The triplet state EPR spectra of magnetically aligned whole cells of *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* display a marked dependence on the orientation of the static EPR field with respect to the alignment field direction. This observation implies that the primary donor species on which the triplets are localized are ordered within the membranes. We have developed a theoretical model for the system to enable calculation of the orientation of the magnetic axes of the primary donor species with respect to the membranes in which they reside. The triplet state spectra are generated by an ensemble of partially ordered magnetic systems and a computer simulation of the experimental results. The triplet orientation is very similar for the two organisms studied, where one axis lies predominantly in the plane of the membrane and the other two axes have approximately equal projections onto the normal to the membrane.

6. MAGNETOPHOTOSELECTION OF THE TRIPLET STATE OF REACTION CENTERS FROM *Rhodopseudomonas sphaeroides* R-26

Harry A. Frank, John Bolt, Richard Friesner and Kenneth Sauer

Biochim. Biophys. Acta 547, 502-511 (1979)

Reaction centers of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26, give rise to large triplet state EPR signals upon illumination at low temperature (11 K). Utilizing monochromatic polarized light to generate the EPR spectra (magnetophotoselection) we have shown that the intensities of the observed triplet signals are strongly dependent upon the wavelength and polarization direction of the excitation. These data can be used to calculate the orientations of the excited transition moments with respect to each other and with respect to the triplet state principal magnetic axes system. Our quantitative approach is to follow the procedure outlined in a previous publication (see Ref. 5 abstract above), where computer simulations of the observed triplet state spectra were employed.

The results presented in the present work indicate that the transition moment at 870 nm which is associated with the bacteriochlorophyll 'special pair' lies almost entirely along one of the principal magnetic axes of the triplet state. Also, the 870 nm transition moment makes an angle of approx. 60° with the 546 nm transition moment which is associated with a bacteriopheophytin. This latter result is in agreement with previous photoselection studies on the same bacterial species (Vermeglio, A., Breton, J., Paillotin, G. and Coggdell, R. (1978) Biochim. Biophys. Acta 501, 514-530).

7. PHOTOSYNTHESIS--THE LIGHT REACTIONS

Kenneth Sauer

Ann. Rev. Phys. Chem. 30, 155-178 (1979)

Recent developments are reviewed in the absorption, transfer and trapping of photon excitation in photosynthetic membranes of plants and bacteria. The primary events of electron transfer in reaction centers of Photosystems 1 and 2 of higher plants are presented and compared with those of photosynthetic bacteria. Electron donors and acceptors are characterized, and the dynamics of the electron transfer process are described from the picosecond time regime onwards. Over 200 references to the recent literature are included.

8. ABSORPTION CHANGES OF P-700 REVERSIBLE IN MILLISECONDS AT LOW TEMPERATURE IN TRITON SOLUBILIZED PHOTOSYSTEM I PARTICLES

Kenneth Sauer, Paul Mathis, Suzanne Acker and J. A. Van Best

Biochim. Biophys Acta 545, 466-472 (1979)

Triton-solubilized Photosystem I particles from spinach chloroplasts exhibit largely reversible P-700 absorption changes over the temperature range from 4.2 K to room temperature. For anaerobic samples treated with dithionite and neutral red at pH 10 and illuminated during cooling, a brief (1 μs) saturating flash produces absorption changes in the long wavelength region that decay in 0.95 ± 0.2 ms from 4.2 to 50 K. Above 80 K a faster (100 ± 30 μs) component dominates in the decay process, but this disappears again above about 180 K. The major decay at temperatures above 200 K occurs in about 1 ms. The difference spectrum of these absorption changes between 500 and 900 nm closely resembles that of P-700. Using ascorbate and 2, 6-dichlorophenolindophenol as the reducing system with a sample of Photosystem I particles cooled in darkness to 4.2 K, a fully reversible signal is seen upon both the first and subsequent flashes. The decay time in this case is 0.9 ± 0.3 ms.

9. TRIPLET STATES IN PHOTOSYSTEM I OF SPINACH CHLOROPLASTS AND SUBCHLOROPLAST PARTICLES

Harry A. Frank, Mary B. McLean, and Kenneth Sauer

Proc. Natl. Acad. Sci. U.S.A. 76, 5124-5128 (1979)

We report light-induced electron paramagnetic resonance triplet spectra from samples of chloroplasts or digitonin photosystem I particles that depend upon the dark redox state of the bound acceptors of photosystem I. If the reaction centers are prepared in the redox state P-700 A $X^-Fd_B^-Fd_A^-$, then upon illumination at 11 K we observe a polarized chlorophyll triplet species which we interpret as arising from radical pair recombination between $P\text{-}700^+$ and A^-. This chlorophyll triplet is apparently the analog of the P_R state of photosynthetic bacteria [Parson, W. W. & Cogdell, R. J. (1975) Biochim. Biophys. Acta 416, 105-149]. If the reaction centers are prepared in the dark redox state P-700 A X $Fd_B^-Fd_A^-$, then upon illumination at 11 K we observe a different triplet species of uncertain origin, possibly pheophytin or carotenoid. This species is closely associated with the photosystem I reaction center and it traps excitation when P-700 is oxidized.

11. DEVELOPMENT OF ELECTRON SPIN POLARIZATION IN PHOTOSYNTHETIC ELECTRON TRANSFER BY THE RADICAL PAIR MECHANISM

Richard Friesner, G. Charles Dismukes, and Kenneth Sauer

Biophys. J. 25, 277-294 (1979)

We have extended the radical pair theory to treat systems of membrane-bound radicals with g tensor anisotropy. Analysis of the polarized electron paramagnetic resonance (EPR) signals of $P\text{-}700^+$, originating from photosystem I of higher plants, in terms of the radical pair mechanism provides information about the sequence of early electron acceptors. To account for the orientation dependence of the line shape and integrated area of this polarized signal, we propose the electron transfer sequence to be $P\text{-}700 \rightarrow A_1 \rightarrow X \rightarrow Fd(A,B)$, where A_1 is a small organic molecule (possibly chlorophyll), X is the acceptor species observed recently in low-temperature EPR studies, and Fd(A,B) are the ferrodoxin iron-sulfur centers A and B. Our calculations provide information about the lifetimes of A_1^-, and X^-, and their exchange interactions with $P\text{-}700^+$. We also find supporting evidence for the orientation of X^- in the thylakoid membrane reported recently by G. C. Dismukes and K. Sauer (Biochim. Biophys. Acta. 504:431-445.)

13. PERIODIC CHANGES IN THE OXIDATION STATE OF MANGANESE IN PHOTOSYNTHETIC OXYGEN EVOLUTION UPON ILLUMINATION WITH FLASHES

T. Wydrzynski and K. Sauer

Biochim. Biophys. Acta 389, 56-70 (1980)

The pattern of manganese released from chloroplast membranes by a rapid temperature shock after various illumination regimes indicates that changes in the oxidation state of bound manganese occur during photosynthesis. Continuous illumination decreases by 35-40% the amount of Mn(II) released in the presence of $K_3Fe(CN)_6$ compared with a dark-adapted control. Following illumination and heat treatment, the addition of the reductant H_2O_2 to the samples causes an increase in the level of electron paramagnetic resonance (EPR)-detectable manganese. The pH dependence of the H_2O_2 reduction indicates that the non-EPR-detectable manganese present in the heated sample after illumination is in the form of higher oxidation state compounds, e.g. MnO_2. The light-induced Mn(II) decrease is reversible in the dark with $t_{1/2}$ approximately 40 s and can be prevented by the presence of the Photosystem II inhibitors 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea or fluorocarbonylcyanide phenylhydrazone during the illumination period.

After a series of brief flashes of light the Mn(II) released by heat treatment oscillates over periods of four flashes. The pattern is similar to the O_2 yield flash pattern and suggests that a cycling of manganese oxidation states is involved in the O_2 evolution mechanism. The oscillations in the Mn(II) release are analyzed in

terms of the current four-step model for O_2 evolution. The analysis suggests that manganese is successively oxidized in the first two steps, but undergoes a partial reduction on the third step. This result is consistent with the concept that water undergoes a partial oxidation prior to the release of O_2 from the water-splitting complex.

DIRECT CALCULATION OF THE ORIENTATIONAL DISTRIBUTION FUNCTION FROM PARTIALLY ORDERED ENSEMBLES FROM THE EPR LINE SHAPE

Richard Friesner, John A. Nairn, and Kenneth Sauer

J. Chem. Phys. 71, 2[illegible]-2[illegible] (1979)

We have developed a method for the determination of the orientational distribution function of partially ordered systems from the EPR line shape that is an alternative to the usual one of expansion in the elements of the Wigner rotation matrices. The method can be applied to distribution functions whose expansion in the rotation matrix elements are slowly convergent or where the EPR intensity cannot be computed analytically. It can also be used to extract geometrical information relating the principal magnetic axis system to the preferred direction of orientation. An application to a partially ordered ensemble of triplets observed in the photosynthetic bacterium Rps. viridis is given, and it is suggested that our technique may prove particularly valuable in analysis of biological systems.

A GENERAL THEORY OF THE SPECTROSCOPIC PROPERTIES OF PARTIALLY ORDERED ENSEMBLES. I. ONE VECTOR PROBLEMS

Richard Friesner, John A. Nairn, and Kenneth Sauer

J. Chem. Phys. 7[illegible], 221-230 (1980)

We have developed a method for determining the response of a static, partially ordered ensemble of molecules to various types of electromagnetic probes. In our formalism a physical model is constructed for the orientational distribution function, which is represented as a "[illegible]" weighted by a set of weighting factors. Procedures are given for obtaining geometrical and statistical parameters from analysis of the observed line shapes and intensities. Explicit treatments of Zeeman spectroscopy, ESR, NMR, and magnetic resonance are developed as well as detailed mathematical techniques for calculating the orientation function.

ELECTRON SPIN POLARIZATION FROM THE PHOTOSYNTHETIC LIGHT REACTION: PHOTOSYSTEM I

Charles Dismukes, Anne McGuire, Richard Friesner, and Kenneth Sauer

Reviews of Chemical Intermediates, 3, 99-[illegible] (1980)

This review summarizes our experimental and theoretical studies of electron spin polarization associated with the Photosystem I light reaction of green plants and algae. Populations of the electron spin sublevels of the oxidized reaction center-electron donor complex, which are not in thermal equilibrium with their surroundings, can be observed promptly after formation by photooxidation. This polarization serves as a rich source of information about the magnitude of the coupling between neighboring electron transport cofactors, their organization within the photosynthetic membranes, and the kinetics of electron transfer.

Following a brief account of the current knowledge of the sequence of electron transfer reactions in Photosystem I, the unique contributions provided by studies of electron spin polarization are presented in detail.

DIRECT CONVERSION OF SOLAR ENERGY INTO ELECTRICAL AND CHEMICAL ENERGY VIA PHOTOSYNTHETIC MODELS

Melvin Calvin and John Otvos

Over the past [illegible] years, considerable progress has been made in understanding the nature of the primary quantum conversion in photosynthesis, although it is certainly not yet understood enough to be reconstructed in the laboratory. A number of fruitful suggestions have generated an interest in model systems that are being constructed and studied.[1-4] In the photosynthetic membrane model at the top of Figure 1 there are two quanta absorbed, one near each side of the membrane. Electron and proton transport stimulated by the photoexcited pigments produces oxidation on the left and reduction on the right. An analogue to the membrane system is the vesicle shown below it, which also divides the system into two parts. Using an alkylated $[Ru(bipy)_3]^{+2}$ complex as a sensitizer that localizes in the phospholipid vesicle wall, we have been able to promote an electron (and proton) transfer across the wall to produce both oxidation and reduction products.[5,6] Previously, we had presented evidence for charge migration in intact, photosynthetic organisms.[7] Half of the complete system, having only a single interface like a micelle or an emulsion, is also being studied to build up knowledge about electron transfer reactions at interfaces. We have shown that fluorescence quenching by electron transfer can be greatly enhanced in the presence of an interface where the reactants are held.[8] In principle, such half systems can eventually be assembled and coupled on a membrane or vesicle. With water-in-oil microemulsions, we have also produced photoinduced electron transfer from one phase to the other across the interface.[9,10] Different charged interfaces are expected to affect the interaction of acceptors and donors and thereby modify their undesired recombination reactions. The oxidizing and reducing parts of the system should be kept apart and, in general, each one requires a catalyst in the final step of hydrogen and oxygen production. The encapsulation of these catalysts into vesicles and microemulsions is now under investigation.

The sensitized semiconductor-electrolyte interface is another single interface that has attracted our attention. Here, an exciton conversion into charge carriers occurs by electron transfer into orbitals, or an orbital band, which will allow charge to move in a semiconductor. A model for this has been constructed using zinc oxide as a n-type semiconductor pictured at the bottom left of Figure 1, and an adsorbed layer of dye as a sensitizer.[11] Such a cell has been [illegible] a "photoelectrochemical" cell because it directly generates a photovoltaic effect depending upon the relative energies of the sensitizer orbitals and the bands of the semiconductor. This effect, then, can lead to electron transfer from the excited sensitizer into the semiconductor with the production of chemical changes in the electrolyte. Or the reverse process may take place; that is, the electron may transfer from the filled orbital of a p-type semiconductor into the lower vacant orbital of the excited sensitizer (bottom right of Figure 1).

The search for such systems, which could permit a relatively efficient direct conversion of solar energy, goes forward. In particular, interactions between the semiconductor and sensitizer dyes are being examined in detail to seek out those characteristics that optimize the efficiency of electron transfer and at the same time protect both the dye and the semiconductor from decomposition. Ideally, this would involve a detailed knowledge of the relative and absolute energy levels of the dyes and substrate orbitals. Finally, by combining an n-type and p-type semiconductor and connecting the electron sink of one to the electron source of the other, the entire oxidation-reduction system analogous to the photosynthetic membrane could again be produced.

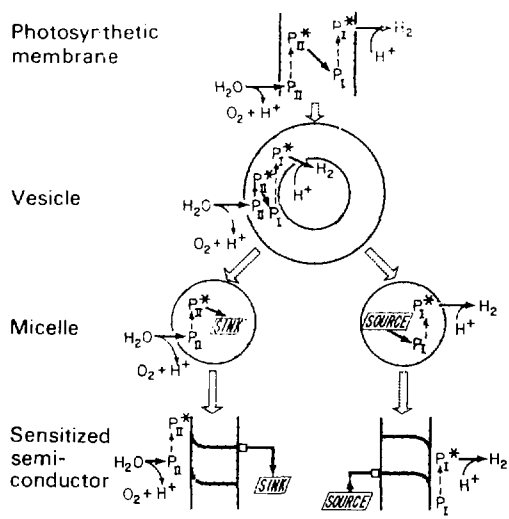

Figure 1. Photoelectron transfer scheme. (XBL 774-4350A).

This work was supported by the Division of Chemical Sciences, Office of Basic Energy Sciences, U.S. Department of Energy, under contract no. W-7405-ENG-48.

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Abstracts

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W. [illegible]

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The principal function of the chloroplast is to capture [illegible] energy and to store them in some stable form. We are in the process of trying to construct a totally synthetic system that would simulate some of the reactions of the two photosystems which occur in natural chloroplasts. These synthetic chloroplasts will not resemble the natural entity in detailed construction, as they will contain no protein.

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*See Abstracts.

b. PHOTOSENSITIZED ELECTRON TRANSPORT ACROSS LIPID VESICLE WALLS: QUANTUM YIELD DEPENDENCE ON SENSITIZER CONCENTRATION

W. L. Ford, J. W. Otvos, and M. Calvin

Proc. Natl. Acad. Sci. 76, 3590 (1979).

An amphiphilic tris(2,2'-bipyridine) ruthenium(2+) derivative that is incorporated into the walls of phosphatidylcholine vesicles photosensitizes the irreversible oxidation of ethylenediaminetetraacetate(3-) dissolved in the inner aqueous compartments of the vesicle suspension and the one-electron reduction of heptylviologen(2+) dissolved in the continuous aqueous phase. The quantum yield of viologen radical production depends on the phospholipid-to-ruthenium complex mole ratio. A kinetic model is used to derive an order-of-magnitude estimate for the rate constant of electron transport across the vesicle walls. The results are inconsistent with a diffusional mechanism for electron transport and are interpreted in terms of electron exchange.

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[illegible] bulk solution are 3.0×10^4 and 1.6×10^4 M^{-1}, respectively. The quenching efficiency of [illegible] latex is [illegible] higher than that in the methanol solution. The fluorescence lifetime of rhodamine [illegible] is 4 ns in methanol. The fluorescence quantum yields in methanol [illegible] and water [illegible] were also determined. The implications of these results to photochemical storage reactions are discussed.

9. PROTON-INDUCED ELECTRON TRANSFER ACROSS A WATER-OIL BOUNDARY AS A MODEL FOR REDOX REACTION SEPARATION

Itamar Willner, William E. Ford, John W. Otvos and Melvin Calvin

Nature 280, 823-824 (1979)

The creation of artificial solar energy conversion and storage systems that mimic the photosynthetic pathway has involved, among other approaches, the photolysis of water involving the mediation of two photosystems in the generation of reduced and oxidized species which are the active components in the decomposition of water. Homogeneous (aqueous) solutions of these components suffer from the basic limitation that the reducing and oxidizing agents can react with each other and thus no reaction can be observed. Several types of synthetic "photosynthetic membranes" have been suggested as a means of separating the two redox units and overcoming these fundamental difficulties. Recently, photosensitized electron transfer across vesicle walls has been suggested as a means for oxidizing and reducing agents in separate water compartments. This report involves the photochemical electron transfer across the interface of a water-in-toluene microemulsion, and this system is proposed as a model for the separation of the oxidized and reduced species.

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[illegible] dissolved in the continuous aqueous phase. Intentionally added membrane-bound electron and proton carriers are not required for electron transport across the vesicle walls. Mechanisms for electron transport are considered.

The photoinduced decomposition of water by using suitable redox catalysts needs further development. The reduction of water to produce H_2 is established; however, the complementary oxidation of water to O_2 is more difficult due to the need for a multistep electron transfer process. A cycle that involves the partial oxidation of water with the concomitant production of H_2 is presented. In this cycle, the oxidation of water is photosensitized by a manganese complex. The possibilities of utilizing the cycle for production of useful chemicals or oxygen in secondary reactions are described.

HYDROCARBON-PRODUCING PLANTS AND THEIR MANIPULATION

Melvin Calvin, John W. Otvos, and James Bassham

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[illegible]

Figure 1. Southern California field station, showing both E. lathyrus and E. tirucalli, July 1978. (CBB 780-12927)

This work was supported by the Division of Biomass, Office of Solar Technology, U.S. Department of Energy, under Contract No. W-7405-ENG-48.

initial harvest. Considerable success has been achieved with [illegible] by the Japanese on Okinawa, while most of our efforts have been with lathyris. In an early experiment, a yield of total extractables [illegible] barrels of oil per acre-year was achieved. With the knowledge gained, this yield should go up to 10 barrels or more. The extract consists of a complex mixture of tetra and pentacyclic triterpenoids functionalized as ketones, [illegible], and fatty acid esters, together with [illegible] and some straight chain hydrocarbons. In addition to the development of analytical compositions of the extract, efforts are under way to devise practical methods of removing the materials from the rest of the plant to obtain the products desired. One aspect of the extraction research involves the isolation and determination of the resistant property of a phenolic ester present in the latex, so that it can be eliminated by proper chemical treatment.

[illegible] matter [illegible] pretty engage [illegible] future techniques for metal [illegible] stations [illegible] improve the [illegible] [illegible].

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Abstracts

6. ANALYSIS OF EXTRACTABLES FROM ONE EUPHORBIA

E. K. Nemethy, J. W. Otvos, and M. Calvin

J. Amer. Oil Chemists' Soc. 56, 957 (1979).

Chemical analyses have been made of the heptane extractable material of Euphorbia lathyris, a plant which has been proposed as an "energy farm" candidate. The heptane extract is 4-5% of the dry plant weight and has a heat value of $\sim 18 \times 10^3$ BTU/lb. This reduced photosynthetic material consists almost entirely of polycyclic triterpenoids.

*See Abstracts.

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[illegible] renewable resources for materials and energy. The production of fermentation alcohol from sugar [illegible] as a major source of materials for chemical feedstocks [illegible] as well as the direct photosynthetic production of hydrocarbons from known plant sources. Experiments are underway to analyze the hydrocarbons from Euphorbias and other hydrocarbon containing plants with a view toward determining their various chemical components. In addition, experimental plantings of several species of Euphorbias have begun to obtain data on which species would be most successful. There are indications that Euphorbia lathyris may yield approximately 10 barrels of hydrocarbon material per acre in a seven-month growing period on semiarid land.

PETROLEUM PLANTATIONS AND SYNTHETIC CHLOROPLASTS

M. Calvin

Energy 4, 851 (1979).

In considering the subject of this conference it seemed proper to examine the idea of how best to use the knowledge acquired through basic

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With the natural green plant as a model, [illegible] to construct a purely synthetic system capable of capturing solar quanta [illegible] storing them in some stable form. This synthetic system, called "synthetic chloroplasts," would not resemble the natural entity in detailed construction but would be capable of a similar function, that is, it would mimic the way the green plant captures solar quanta to generate oxygen on one side of a membrane, reducing power on the other. The potential reactants could be separated and stored and brought together later in a suitably constructed physical system for energy recovery. These synthetic systems could thus capture the quanta and convert them to some other energy form and store that energy for indefinitely long periods with the possibility of future recovery at will in some convenient form. These artificial systems could be used to generate hydrogen, for example, which could be used in chemical processing, or as an energy source, and thus release some of the petroleum products now used in this way for other purposes.

METABOLIC REGULATION AND PATHWAYS IN GREEN PLANT CELLS AND LEAVES

James A. Bassham

Radioactive isotopes have made possible the charting of complex biosynthetic paths in living cells. Ultimately, all such paths begin with the photosynthetic carbon-reduction cycle, in which carbon dioxide is converted by green plants to sugar phosphates and finally to starch and sucrose.

During the first years of work on photosynthetic metabolism, Melvin Calvin and co-workers used radiocarbon and radiophosphorous as labeled atoms to follow the path of carbon dioxide uptake and reduction in photosynthesis (Calvin cycle). Given $^{14}CO_2$ and ^{32}P-labeled phosphate, green plants in light made radioactive metabolites. These substances were separated and identified from extracts of the plant material by two-dimensional paper chromatography and radioautography. Analysis of the kinetics of appearance of these labeled compounds as a function of time of photosynthesis revealed their sequence along the pathway from CO_2 to carbohydrates (Figure 1).

From that early work, precise quantitative methods were developed for following the flow of tracers through metabolic paths during constant physiological states, such as steady-state photosynthesis or glycolysis of green plants in the dark (see Figure 2 for example of steady-state apparatus). These methods also permit study of transient changes in levels of labeled metabolic pools during transition from one state to another. Both radioactive (^{14}C, ^{32}P, and ^{3}H) and non-radioactive (^{15}N) tracers have been used. The level of specific activity of these isotopes is maintained constant for the duration of an experiment. Aliquot samples of the biological material taken and killed during the course of the experiment are analyzed. Then the tracer content of each compound, divided by the known specific activity of the tracer used, gives a measure of pool sizes and rates of turnover.

These methods have found numerous applications in the study of photosynthetic and respiratory metabolism in green algae, blue-green algae, and green tissue from leaves of higher plants. Such studies have led to the discovery of many aspects of the way in which metabolism is controlled in these plant cells. The basic reductive pentose phosphate cycle of photosynthesis has been found to be regulated at several key reactions, including the initial carboxylation reaction and the fructose-1,6-bisphosphatase-mediated steps.[1,2]

Other sites of regulation on biosynthetic pathways leading from the basic cycle have been identified. Sugar phosphates, once formed via the Calvin cycle, can be either converted to starch in the chloroplasts, or exported from the chloroplasts for subsequent conversion to sucrose or to proteins, lipids, and other materials in the green cells (Figure 3).[3] Sucrose in higher plants is commonly translocated to other plant tissues where it may be converted to many products of secondary biosynthesis. Among such products, hydrocarbons and resins are of particular interest in this laboratory because of their potential as liquid fuels and chemical feedstocks. Within the green cell, the distribution of sugar phosphates to sucrose as compared to protein and fat synthesis is controlled to a large extent by the activities of several rate-limiting enzymes, including pyruvate kinase and phosphoenolpyruvate carboxylase, both of which are considerably activated during photosynthesis by the presence of 1 or 2 mM NH_4^+ in the extracellular environment. In the dark with isolated poppy leaf cells (see below) activation of phosphoenolpyruvate carboxylase only was seen,[3] in contrast to earlier studies with the unicellular algae, Chlorella pyrenoidosa, where both enzymes were activated in light or dark.

As another approach to the study of metabolism and its regulation, both whole and isolated chloroplasts and broken and reconstituted chloroplasts are examined. Comparison of metabolism of the reconstituted (in vitro photosynthesis) system with whole isolated chloroplasts makes it possible to distinguish regulation mechanisms related to transport through the outer chloroplast membrane from other mechanisms. The reconstituted system is also very useful for studying the effects of added soluble cofactors, metabolites, and inhibitors that might not penetrate the outer membrane of intact chloroplasts.

During the past year, the kinetics of CO_2 fixation, of starch formation and of changes in the levels of metabolites in chloroplasts and the surrounding medium were investigated during light-dark and dark-light transitions with isolated intact chloroplasts.[4] Of particular importance were changes in the levels of 3-phosphoglycerate and hexose monophosphates between light and dark, together with changes in ATP, which have been previously studied in this laboratory with isolated chloroplasts. Also of interest was the fact that the level of inorganic phosphate (P_i) stays constant throughout the light-dark cycle. It was previously reported that the activity of the enzyme ADPG pyrophosphorylase, which catalyzes a rate-limiting step in the conversion of hexose monophosphates to starch, is affected by 3-phosphoglycerate, P_i, and hexose monophosphates.

This work was supported by the Division of Biological Energy Conversion and Conservation, Office of Basic Energy Sciences, U.S. Department of Energy, under Contract No. W-7405-ENG-48.

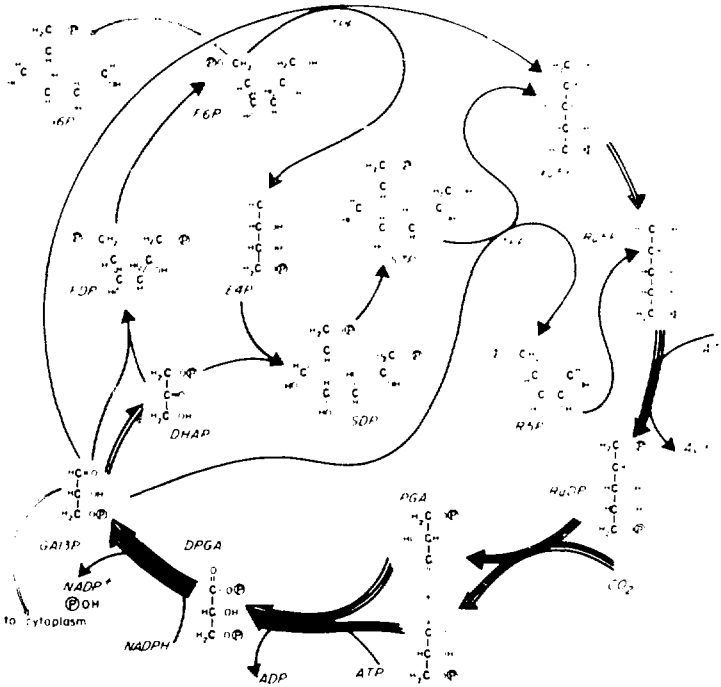

Figure 1. The Reductive Pentose Phosphate Cycle. The heavy lines indicate reactions of the RPP cycle; the faint lines indicate removal of intermediate compounds of the cycle for biosynthesis. The number of heavy lines in each arrow equals the number of times that step in the cycle occurs for one complete turn of the cycle, in which three molecules of CO_2 are converted to one molecule of GA13P. Abbreviations: RuDP, Ribulose 1,5-diphosphate; PGA, 3-phosphoglycerate; DPGA, 1,3-diphosphoglycerate; NADPH and $NADP^+$, reduced and oxidized nicotinamide-adenine dinucleotide phosphate, respectively; GA13P, 3-phosphoglyceraldehyde; DHAP, dihydroxyacetone phosphate; FDP, fructose 1,6-phosphate; G6P, glucose 6-phosphate; E4P, erythrose 4-phosphate; SDP, sedoheptulose 1,7-diphosphate; S7P, sedoheptulose 7-phosphate; Xu5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; and TPP, thiamine pyrophosphate. (XBL 7611-9660A)

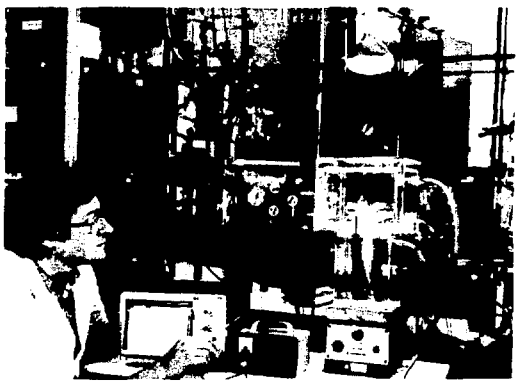

Figure 2. The "steady-state" apparatus--used for detailed investigations of the mechanisms and metabolic dynamics of photosynthesis. (CBB 784-4932)

The observed changes in metabolite levels from the whole chloroplast experiments were then applied to studies of reconstituted chloroplasts and consequent rates of ADPG glucose formation in such preparations in the dark.[4,5] With the reconstituted system, changes in 3-phosphoglycerate concentration and other changes known to occur between light and dark resulted in a 130-fold increase in the rate of ADPG glucose formation in the light as compared with the dark. Thus it is possible to explain the complete regulation of starch formation between light and dark in terms of metabolite concentrations known to change in whole chloroplasts.

In isolated chloroplasts in the dark, both the oxidative pentose phosphate (OPP) cycle and glycolysis provide a pathway from glucose-6- phospate (formed during starch breakdown) and other sugar phosphates.[6] In isolated chloroplasts, the OPP cycle stops at pentose phosphates, which are not converted to PGA. The PGA formed from ^{14}C-G6P apparently came via the glycolytic pathway. In vivo, and especially under conditions of rapid

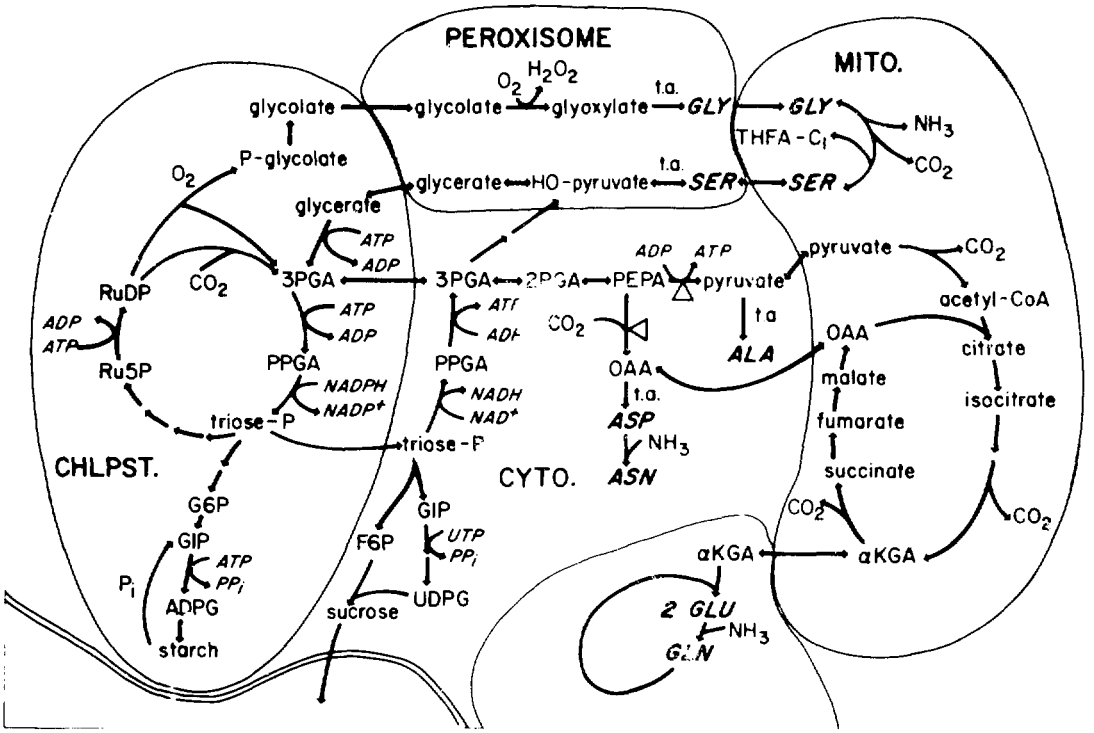

Figure 3. Metabolic pathways among subcellular organelles in green cells. Reduced carbon from photosynthesis in chloroplasts is exported as triose phosphate, PGA and glycolate. These metabolites are subsequently converted to other substances in the cytoplasm, peroxisomes and mitochondria. Abbreviations: PEPA, phosphoenolpyruvic acid; OAA, oxalacetic acid; ASP, aspartic acid; ASN, asparagine; GLY, glycine; SER, serine; GLU, glutamic acid; GLN, glutamine; THFA-C, tetrahydrofolic acid bound to a one-carbon moiety; G1P, glucose-1-phosphate. For other abbreviations, see Figure 1. (XBL 7710-4660)

cell growth requiring chloroplast growth and division at night, the OPP cycle could be expected to operate to produce triose phosphates while supplying NADPH for lipid synthesis.

Another mechanism involved in the inhibition of the reductive pentose phosphate cycle and activation of the oxidative pentose phosphate cycle in the dark was found to be H_2O_2 formation.[7] H_2O_2 oxidizes the reduced factors involved in light activation.

During the past three years, much work has been done with plant cells isolated from leaves.[3,8,9] These cells are kept under conditions in which they remain photosynthetically active for several days. Such cells are used in metabolic studies with labeled substrates. In addition to observing regulatory effects of externally applied NH_4 (mentioned above) we have initiated studies on the effects of plant hormones on the pattern of carbon flow from photosynthesis into biosynthesis. It is expected that one of the effects of plant hormones will be to influence the synthesis of enzymes that catalyze key rate-limiting reactions in the metabolic pathways. For example, we observed a stimulation of CO_2 incorporation as well as glutamine synthesis when 2,4-D is applied to leaf-free mesophyll cells.[8]

A factor in pine needles which prevents photosynthetic $^{14}CO_2$ uptake by either isolated pine chloroplasts or isolated spinach chloroplasts contaminated with pine chloroplasts was investigated. It was found to specifically inhibit fructose and sedoheptulose bisphosphatases in the reductive pentose phosphate (RPP) cycle. It was isolated in small amounts, and was found to be lipid soluble, but extractable into alkaline aqueous solutions.[10]

Isolated mesophyll cells from poppy were compared with similar cells in intact leaves in their ability to export ^{14}C-labeled photosynthates.[9] It was concluded that export of photosynthate from the mesophyll requires intact symplastic connections. Although poppy cells (Papaver somniferum) could be isolated and maintained in an active state for several days, and were used for several studies, alternative material was needed. During the past year, much effort was spent in developing methods to isolate and maintain cells from spinach (Spinacea oleracea) leaves. Spinach plants require no special license to grow, and have the advantage that much more work has been done on their photosynthetic carbon metabolism due to the ease with which spinach chloroplasts can be isolated. Unfortunately, methods used for poppy cells didn't work well for spinach, and a great many variations had to be tested and new conditions chosen. We are now able to maintain isolated spinach cells in a photosynthetically active form for several days, and they are being used for studies of amino acid biosynthesis and of photosynthetic and photorespiratory metabolism.

Also during the past year, the steady-state photosynthesis apparatus has been improved to permit more experimentation with controlled artificial atmospheres, such as those with enriched CO_2 or reduced CO_2 levels. Improvements have been made in $^{14}CO_2$ gas storage, handling, and recovery to insure that $^{14}CO_2$ release to the atmosphere is reduced to zero.

Steady state experiments with whole evergreen ash (Fraxinus uhdei) saplings were carried out to determine the translocation of labeled sucrose to and out of a wound. Sealing by callose formation was effectively prevented by ethylene diamine tetraacetic acid, thus allowing sucrose exudation for as long as 48 hours. Further studies during the past year of photochemical H_2 evolution by the photosynthetic bacteria, Rhodopseudomonas sphaeroides were performed with both a wild type and a mutant capable of quantitative conversion of glucose to H_2 and CO_2. The mutant apparently has been freed of a block in the conversion of gluconate, which accumulates in the wild type.[13]

For several years, we have been advocating the use of controlled-environment agriculture (CEA) in the future.[12,13,14] CEA applications could increase crop yields through CO_2 enrichment with its concomitant greatly increased N_2 fixation in legumes, year-round growth, and for other reasons, in areas with high solar energy but limited water (the U.S. southwest, for example). CEA could become an important factor in water conservation. An essential aspect of the research required to implement a CEA system in the future is an understanding of the response of green plants to elevated CO_2 pressure (such as 0.1% CO_2 in air). Even though photosynthesis increases in rate with short exposures to elevated CO_2, long-term growth does not necessarily increase, due to natural regulatory processes. A high light intensity plant growth chamber has been installed and is now nearly equipped with gas control and monitoring systems. Alfalfa, the initial test plant, is being grown hydroponically in the chamber and will be subjected to different controlled levels of CO_2, for subsequent evaluation of regulatory mechanisms.

Several research projects on plant cell tissue culture have been initiated. These include (1) plant cell culture of Euphorbia lathyrus, a "hydrocarbon" producing plant, (2) callus culture of tobacco and examination of metabolism of ^{14}C-sucrose during redifferentiation induced by hormones, and (3) metabolism of photoautotrophic tobacco cells in tissue culture.

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Abstracts

1. THE REDUCTIVE PENTOSE PHOSPHATE CYCLE AND ITS REGULATION

James A. Bassham

"Photosynthesis II, Photosynthetic Carbon Metabolism and Related Processes," Encyclopedia of Plant Physiology, New Series, Vol. 6, M. Gibbs and E. Latzko, eds. (Springer-Verlag, Berlin, Heidelberg, New York, 1979)

The reductive pentose phosphate (RPP) cycle is the basic biochemical pathway whereby carbon dioxide is converted to sugar phosphates during the process of photosynthesis. This pathway is apparently ubiquitous in all photoautotrophic green plants. In some higher plants, there is an additional pathway (C_4 cycle) via which CO_2 is first incorporated into four-carbon acids or amino acids and is later released to be refixed via the RPP cycle. This C_4 cycle is not an alternative to the RPP cycle, but rather functions to utilize ATP from photochemical reactions to bring carbon into the chloroplasts where the RPP cycle is operating. Information about the regulation of the RPP cycle came from studies of pool sizes of intermediate metabolites, and from measurement of oxygen evolution, phosphorylation, etc., in leaves, whole cells, isolated chloroplasts, and reconstituted chloroplasts. Much additional knowledge came from studies of the individual enzymes in both crude extracts and isolated form. This article describes the RPP cycle, its mapping, the requirements for regulation, and the kinetic evidence, based on measurements of labeled metabolites in cells, chloroplasts, etc., for regulation in vivo.

2. REGULATION OF PHOTOSYNTHETIC CARBON METABOLISM AND PARTITIONING OF PHOTOSYNTHATE

James A. Bassham

ICN-UCLA Symposium on Molecular and Cellular Biology, Vol. 13, Modulation of Protein Function, D. Atkinson and C. Fox, eds. (Academic Press, New York, 1979), pp. 139-159.

The rate-limiting steps in the reductive pentose phosphate cycle (RPP Cycle) were identified

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*See Abstracts.

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[illegible] of these two types of carbon [illegible] from the cycle can vary, a fine tuning of the [illegible] of FBPase and SBPase compared to [illegible] required. With isolated chloroplasts, [illegible] phosphate export increases with external [illegible] phosphate (P_i) concentration, and this may be an important regulatory mechanism in vivo as well. In developing cells, exported triose phosphate is mainly converted via phosphoenolpyruvate (PEPA) to amino acids, fatty acids, and other substances required for cell growth. In more mature cells, the triose phosphate is largely converted to sucrose, which is translocated to other parts of the plant. Rate-limiting steps for these branching pathways include pyruvate kinase and PEPA carboxylase as well as sucrose phosphate synthetase.

4. STIMULATION OF DARK CO_2 FIXATION BY AMMONIA IN ISOLATED MESOPHYLL CELLS OF Papaver somniferum L.

K. E. Hammel, K. L. Cornwell and J. A. Bassham

Plant and Cell Physiol. 20(8), 1523 (1979)

Addition of 2 millimolar ammonium ion to isolated mesophyll cells of Papaver somniferum resulted in a 3-fold or greater increase in their rate of dark ^{14}C fixation, while even 0.1 mM NH_4^+ nearly doubled that rate. The most rapid increase in the labeling of a metabolite occurred in aspartate, and was accompanied by a decrease in the steady-state level of labeled phosphoenolpyruvate. No change in labeled pyruvate level occurred, and alanine labeling declined. Ammonium ion addition had no effect on the pyruvate kinase reaction in the dark. Our results are compared with earlier findings, and the possible regulatory function of ammonia is discussed.

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In the [illegible], the [illegible] of 3-phosphoglycerate [illegible] phosphate [illegible] in the dark period. This is due to an exchange of internal 3-phosphoglycerate for external dihydroxyacetone phosphate. Part of the reimported dihydroxyacetone phosphate is converted into hexose monophosphates via aldolase and fructose bisphosphatase during the first minutes of darkness. Due to the observed exchange transport reactions, the large difference between the transenvelope concentration gradients of 3-phosphoglycerate, dihydroxyacetone phosphate, and orthophosphate which exist in the light, is completely abolished after 1 to 3 minutes in the dark.

The kinetics and the magnitudes of the changes of metabolite concentrations during the light-dark-light cycle are compared to the kinetics of starch formation, and their relevance for a possible light-dark regulation of starch synthesis is discussed.

5. LIGHT-DARK REGULATION OF STARCH METABOLISM IN CHLOROPLASTS: II. EFFECT OF CHLOROPLASTIC METABOLITE LEVELS ON THE FORMATION OF ADP-GLUCOSE BY CHLOROPLAST EXTRACTS

W. M. Kaiser and J. A. Bassham

Plant Physiol. 63, 109-113 (1979)

The rate of ADP-glucose formation from [^{14}C] glucose 6-phosphate and of ATP formation by the soluble fraction of lysed chloroplasts is studied as a function of the levels of metabolites (3-phosphoglycerate, orthophosphate, hexose monophosphate, and ATP) as determined in whole chloroplasts of Spinacia oleracea in light and dark.

A change of orthophosphate concentration [illegible] chloroplasts during light-dark transition increases the rate of ADP-glucose formation [illegible]. An increase in orthophosphate concentration from [illegible] to 6 mM, which occurs at the same time in whole chloroplasts, stimulates ADP-glucose formation only slightly.

At constant levels of orthophosphate (4 mM) and 3-phosphoglycerate (4 mM), a change in ATP concentration from [illegible] causes an immediate 4- to [illegible]-fold increase in the rate of ADP-glucose formation. Another significant stimulation of ADP-glucose formation (about 4- to 6-fold) is attained after addition of dithiothreitol at high concentrations (50 mM). A simultaneous increase in the concentrations of 3-phosphoglycerate, ATP, and dithiothreitol, with orthophosphate and Mg^{2+} being constant at 4 and 5 mM, respectively, causes a 130-fold increase in the rate of ADP-glucose formation (from 0.047 to 5.49 microgram atoms carbon per milligram chlorophyll per hour).

The role of these and other factors is discussed with respect to light-dark regulation of starch formation in intact chloroplasts.

6. CARBON METABOLISM OF CHLOROPLASTS IN THE DARK: OXIDATIVE PENTOSE CYCLE VERSUS GLYCOLYTIC PATHWAY

W. M. Kaiser and J. A. Bassham

Planta 144, 193-200 (1979)

The conversion of U-labeled [^{14}C] glucose-6-phosphate into other products by a soluble fraction of lysed spinach chloroplasts has been studied. It was found that both an oxidative pentose phosphate cycle and a glycolytic reaction sequence occur in this fraction. The formation of bisphosphates and of triose phosphates was ATP-dependent and occurred mainly via a glycolytic reaction sequence including a phosphofructokinase step. The conversion of glucose-6-phosphate via the oxidative pentose phosphate cycle stopped with the formation of pentose monophosphates. This was found not to be because of a lack in transaldolase (or transketolase) activity, but because of the high concentration ratios of hexose monophosphate/pentose monophosphate used in our experiments for simulating the conditions in whole chloroplasts in the dark. Some regulatory properties of both the oxidative pentose phosphate cycle and of the glycolytic pathway were studied.

7. REVERSIBLE INHIBITION OF THE CALVIN CYCLE AND ACTIVATION OF OXIDATIVE PENTOSE PHOSPHATE CYCLE IN ISOLATED INTACT CHLOROPLASTS BY HYDROGEN PEROXIDE

Werner M. Kaiser

Planta 145, 377-382 (1979)

Hydrogen peroxide (6×10^{-4} M) causes a 90% inhibition of CO_2-fixation in isolated intact chloroplasts. The inhibition is reversed by adding catalase (2500 U/ml) or DTT (10 mM). If [illegible] intact chloroplasts in the light, the incorporation of carbon into hexose and heptulose bisphosphates and into hexose monophosphates is significantly increased, whereas carbon incorporation into hexose bisphosphates and ribulose 1,5-bisphosphate is decreased. At the same time formation of triose phosphates is dramatically stimulated, and the level of ATP is increased. All these changes induced by H_2O_2 are reversed by addition of catalase or DTT. Additionally, the [illegible] of [^{14}C] glucose-6-phosphate into different metabolites in intact chloroplasts in the dark has been studied. In presence of H_2O_2 or peroxide, formation of ribulose 1,5-bisphosphate is inhibited, whereas formation of triose bisphosphates, of triose phosphates, and of monophosphates is stimulated. Again, DTT has the opposite effect. The release of [^{14}C] from added [^{14}C] glucose-6-phosphate by the soluble fraction of lysed chloroplasts with the reactions of oxidative pentose phosphate cycle is completely inhibited by DTT (2.5 mM) and re-activated by appropriate concentrations of hydrogen peroxide.

8. STIMULATION OF CO_2 INCORPORATION AND GLUTAMINE SYNTHESIS BY 2,4-D IN PHOTOSYNTHESIZING LEAF-FREE MESOPHYLL CELLS.

J. S. Paul, S. D. Krohne and J. A. Bassham

Plant Sci. Lett. 15, 17-24 (1979)

Addition of 2,4-D to mesophyll cells isolated from leaves of Papaver somniferum resulted in a significant increase in photosynthetic CO_2 incorporation as well as a stimulation of glutamine synthesis. Evidence is presented that the rise in glutamine is due to increased reduction of nitrate to ammonia.

9. RELEASE OF PHOTOSYNTHATES FROM MESOPHYLL CELLS *in vitro* AND *in vivo*

W. M. Kaiser, J. S. Paul and J. A. Bassham

Z. Pflanzenphysiol. Bd. 94, 377-385 (1979)

Isolated mesophyll cells from *Papaver somniferum* and mesophyll cells in intact leaves from both *Papaver somniferum* and *Spinacia oleracea* were compared for their ability to export ^{14}C-labeled photosynthates.

It was found that under isotonic conditions isolated mesophyll cells release only 0.75% of the total fixed carbon during a 1 h light period. Amino acids seemed to be released faster than all other compounds.

In a detached whole leaf at least 6% of the carbon fixed during a 1 h light period in a small illuminated leaf area was exported into the surrounding tissue, which was kept in the dark. The main exported products were the free sugars

[illegible] Exported photosynthates were [illegible] from the [illegible] ones.

[illegible] photosynthate [illegible]

10. [illegible] FIXATION BY PINE NEEDLE [illegible]

[illegible] Macler and J. A. Bassham

[illegible]

Chloroplasts isolated from pine needles were found to be inactive with respect to CO_2 fixation. [illegible] it was suspected that pine needles may contain substances inhibitory to photosynthesis. Studies were carried out using photosynthetically active isolated spinach chloroplasts and chloroplasts isolated from pine needles. When isolated pine chloroplasts were suspended in buffer and were added to isolated spinach chloroplasts they inhibited photosynthetic CO_2 fixation. When the pine chloroplasts were separated from the medium by centrifugation, the separated pine chloroplasts severely inhibited CO_2 fixation by isolated spinach chloroplasts, but the supernatant solution from the pine chloroplasts was not inhibitory. As little as 5% pine chloroplasts (based on chlorophyll content) produced 50% inhibition of CO_2 fixation by the spinach chloroplasts.

Studies of fixation of ^{14}C-labeled CO_2 by spinach chloroplasts were carried out in which after 5 min photosynthesis the pine chloroplasts were added. It was found that the subsequent inhibition of spinach CO_2 fixation was due neither to any effect on the rate of export of photosynthetic metabolites from the chloroplasts to the medium, nor to a direct effect on the RUBP carboxylase reaction. The principal effect was found to be an inhibition of the conversion of fructose-1,6-bisphosphate and sedoheptulose-1,7-bisphosphate to the respective monophosphates and inorganic phosphate. From this finding it was concluded that a principal effect of the inhibition by pine chloroplasts is probably an inhibition either directly or indirectly of the bisphosphatase enzymes in the spinach chloroplasts. From its distribution between organic and aqueous acidic or neutral solutions, the inhibitory factor of the pine chloroplasts must be lipophilic. Most of the factor could be transferred to an aqueous phase in a strongly alkaline solution. Following subsequent acidification of the aqueous phase the activity could be completely transferred back into the organic phase. This procedure allowed for separation of the inhibitory factor from most of the pigments and other lipophilic substances present in the pine chloroplasts and yielded a preparation which could be subsequently fractionated by thin layer chromatography. UV absorption was found in two fast-moving spots and at the origin. The fast-moving [illegible] from the [illegible] was found to be the [illegible] activity.

11. HYDROGEN FORMATION IN NEARLY STOICHIOMETRIC AMOUNTS FROM GLUCOSE BY A RHODOPSEUDOMONAS SPHAEROIDES MUTANT

B. A. Macler, R. A. Pelroy, and J. A. Bassham

J. Bacteriol. [illegible], 446-45[illegible].

Rhodopseudomonas sphaeroides produces molecular H_2 and CO_2 from reduced organic compounds which serve as electron sources and from light which provides energy in the form of adenosine triphosphate. This process is mediated by a nitrogenase enzyme. A mutant has been found that, unlike the wild type, will quantitatively convert glucose to H_2 and CO_2. Techniques for isolating other strains capable of utilizing other unusual electron sources are presented. Metabolism of glucose by the wild strain leads to an accumulation of gluconate. The isolated mutant strain does not appear to accumulate gluconate.

12. ENERGY CROPS (ENERGY FARMING)

James A. Bassham

In: Biochemical and Photosynthetic Aspects of Energy Production, A. San Pietro, ed. (Academic Press, New York, in press)

The purposeful growing of plants for energy ("energy farming") offers certain advantages, such as ease of transfer of existing technology and storage of product, but is limited by solar conversion efficiencies. A practical upper limit to energy conversion efficiency is 5 to 6%, whereas most agricultural crops show a 0.1 to 2% efficiency on an annual basis. A variety of energy crops may prove to be practical, depending on terrain, water availability, and climate, as well as competition with food crops. In semi-arid or arid regions with high solar energy, covered agriculture may someday be the method of choice, but many practical problems remain. Currently being considered for energy crops are silviculture, marine kelp production, biomass residue from agriculture and forestry, algae, and fresh water plants such as water hyacinths.

13. FEED AND FOOD FROM DESERT ENVIRONMENTS

J. A. Bassham

In: The Biosaline Concept, An Approach to the Utilization of Underexploited Resources, A. Hollaender, J. Aller, E. Epstein, A. San Peitro, O. Zaborsky, eds. (Plenum Press, New York and London, 1979), pp. 17-45.

The biosaline concept has been extended to encompass "the elements of research, development, demonstration, and utilization to apply modern biological sciences and technologies for deriving essential resource materials in an environmentally harmonious manner from marine and arid land mass

[illegible] are the [illegible] of plants adapted [illegible] the environment [illegible] Agricultural [illegible] 14 [illegible] range [illegible] be grown in the [illegible] water supplies [illegible] brackish water. This water [illegible] the sea, from [illegible] water treatment.

14. [illegible] AN [illegible] FARMER [illegible] CHEMICAL

J. A. Bassham

Future Sources of Organic Raw Materials, Chemrawn [illegible] L. St. Pierre and G. E. Brown, eds. Pergamon Press, Oxford, New York, and Toronto, 1969, pp. 601-61[illegible].

Green plants offer a great variety of opportunities for development of renewable resources. Some principles to be kept in mind in developing use of plants include the following: 1) We should seek optimum use of all parts of the plant as [illegible] plants [illegible] with [illegible] chemicals [illegible] study of the regulation of photosynthetic allocation [illegible] green cell, where [illegible] either for new [illegible] parts of the plant [illegible] mechanisms [illegible]

PLANT NATURAL PRODUCTS

Henry Rapoport

A. Biosynthesis and Function of Alkaloids

Alkaloids are found in about 10% of the plant species investigated. Although many of these alkaloids have found medical application as medicine, their role in the plant's economy still remains obscure. This laboratory has under way a detailed study, using radioactive carbon dioxide, of the formation and function of alkaloids in the opium poppy and Nicotiana species.[1]

These studies have dispelled the notion that the alkaloids are merely dead-end storage products. For example, de novo synthesis of thebaine occurs in less than 30 minutes--nicotine in 15 minutes. The biosynthetic sequence to morphine has now been established back five steps, viz., reticuline→thebaine→neopinone→codeinone→codeine→morphine.[2,3] Also, the nonspecificity of one of the enzyme systems has been demonstrated by the biosynthetic conversion of codeine methyl ether, an unnatural compound, to codeine. By use of biosynthetically synthesized compounds (e.g., morphine), the turnover and metabolic fate of these alkaloids are being studied. Morphine, long thought to be the final product in the sequence, is itself metabolized to normorphine, among other products.

In Nicotiana, the biosynthesis of nicotine has been shown to be quite complex, with probably two pathways involved. Further understanding of these pathways is being pursued by short-term kinetic studies with seedlings and with cell-free systems. Nonspecificity of the alkaloid-synthesizing enzyme system has been demonstrated again by formation of nicotine analogues from unnatural precursors of the pyrrolidine ring.

Current emphasis is on aberrant alkaloid biosynthesis, metabolic fate of the alkaloids in the plant, and alkaloid biosynthesis with cell-free systems.[4]

B. Plant Bile Pigments

Phycobilins. Algae contain major auxiliary light-harvesting pigments known as phycobilins. These pigments, which are essential for efficient photosynthesis in the algae, consist of bile-pigment-like chromophores covalently bonded to protein.

[illegible] the structure of the [illegible] synthetic [illegible] the [illegible] the [illegible] link to the [illegible] protein.

We are investigating this problem using an entirely new approach. By selective degradative methodology, using both chemical and enzyme methods, these phycobilins can be degraded to small chromopeptides. We can then synthesize the peptide part. A detailed NMR comparison of synthetic peptide and natural chromopeptide, may give unambiguous structural assignment to the pigment as it exists covalently bound. This has already been done for one of the prosthetic groups of C-phycocyanin, the γ-unit,[5] and we plan to apply this method also to the α and β units. Similar structural studies will be done with the phycoerythrins.

In addition to this structural data, we are also seeking stereochemical data on the covalent pigment-peptide bond. With this information we shall then undertake synthetic work to reconstitute the chromopeptide and define the minimum structural features required for these light-harvesting pigments.

Phytochrome. This morphogenically active plant chromoprotein is 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 our new structural methodology to this extremely important plant pigment as well and have established the structure of the phytochromoundecapeptide.[6]

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 this information by applying our structural methodology to the P_{FR} form.

This work was supported by the Division of Biological Energy Conversion and Conservation, Office of Basic Energy Sciences, U.S. Department of Energy, under Contract No. W-7405-ENG-48.

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Abstracts

4. ENZYMIC REDUCTION OF CODEINONE IN VITRO: CELL-FREE SYSTEMS FROM PAPAVER SOMNIFERUM AND P. BRACTEATUM

C.C. Hodges and H. Rapoport

Phytochemistry 19, in press (1980)

Cell-free extracts have been prepared from Papaver somniferum which perform the reduction of [16-^{3}H] codeinone to codeine. Methodology of quantitating this conversion has allowed conditions for preparing the enzyme extracts to be explored. From P. bracteatum a cell-free system was also prepared which reduced codeinone to codeine, both of which are alkaloids foreign to this species.

5. CHROMOPEPTIDES FROM C-PHYCOCYANIN. STRUCTURE AND LINKAGE OF A PHYCOCYANOBILIN BOUND TO THE β-SUBUNIT

J.C. Lagarias, A.N. Glazer, and H. Rapoport

J. Amer. Chem. Soc. 101, 5030 (1979)

The smallest cyanogen bromide fragment derived from the β-subunit of Synechococcus sp. 6301 C-phycocyanin, the blue heptapeptide 2, has been investigated by 360-MHz ^{1}H NMR spectroscopy. The peptide portion, heptapeptide 3, was synthesized independently and used in comparative spectroscopic analysis. These studies have led to complete assignment of the structure of the peptide-linked phycocyanobilin and elucidation of the nature of the thioether chromophore-peptide linkage.

6. CHROMOPEPTIDES FROM PHYTOCHROME. THE STRUCTURE AND LINKAGE OF THE P_R FORM OF THE PHYTOCHROME CHROMOPHORE

J.C. Lagarias and H. Rapoport

J. Amer. Chem. Soc. 102, in press (1980)

The isolation and chromographic purification of chromophore-containing peptides from the P_R form of phytochrome treated with pepsin and thermolysin are described. From the amino acid sequence and ^{1}H NMR spectral analysis of phytochromobiliundecapeptide (2), the structure of the P_R phytochrome chromophore and the nature of the thioether linkage joining pigment to peptide have been established. Confirmatory evidence was obtained from similar analysis of phytochromobilioctapeptide (3). The implications of this structural assignment with respect to the mechanism of the P_R to P_{FR} phototransformation is considered.

*See Abstracts.

BIOPHYSICS

Melvin P. Klein

Certain microorganisms can reduce atmospheric dinitrogen (N_2) into ammonia in a process known as nitrogen fixation. In fact, biological nitrogen fixation is responsible for 72% of the fixed nitrogen input to the agricultural soils of the earth. It therefore plays a central role in the global nitrogen cycle. Catalyzed by the enzyme system nitrogenase, nitrogen fixation provides a pool of ammonia which is used for the synthesis of biochemically vital nitrogen-containing compounds. In addition to reducing nitrogen, the nitrogenase system evolves hydrogen. When coupled with photosynthesis, as often occurs in nature, this remarkable system uses solar energy to fix nitrogen, produce hydrogen, evolve oxygen, and fix carbon. A deeper understanding of this system promises to offer suggestions on how solar energy may be converted into fuel, food, and fertilizer.

Nitrogenase is composed of two iron-sulfur proteins. The larger contains 1-2 molybdenum atoms and approximately 30 iron and 30 acid labile sulfur atoms per 220,000 daltons. It is known as the Mo-Fe protein. The smaller protein, known as the Fe component, contains 4 iron and 4 acid labile sulfurs per 60,000 daltons. Both proteins, ATP, a divalent metal atom, and a suitable reductant must be present for substrate reduction.

Two unsolved questions are the objectives of our research. The first concerns the role of the Mo atom, essential not only for nitrogenase but for all enzymes which oxidize or reduce nitrogen. The second question is directed toward an elucidation of the intermediates which may be postulated to occur on the pathway between N_2 and NH_3. As none of them are observed in active enzyme preparations, they must remain bound to the enzyme until the reduction is complete.

We have been studying the molybdenum sites in nitrogenase by x-ray spectroscopy using the synchrotron radiation emanating from the SPEAR storage ring at SLAC, at the Stanford Synchrotron Radiation Laboratory. X-ray spectroscopy provides information on the oxidation states and on ligand symmetry and strengths from the precise energy and features at the K-edge of the atom under study, in this case Mo. The oscillatory modulation of the x-ray absorption coefficient at higher energies, the EXAFS, can be Fourier-transformed to yield a radial distribution function giving the apparent distances to the ligand. A more detailed analysis can provide accurate distances, and the numbers and identities of such ligands can be frequently inferred.

We have determined that the Mo in the Mo-Fe component of nitrogenase has about three S atoms at 2.37 Å and three Fe atoms at 2.77 Å. We sought changes in coordination about Mo by exposing the Mo-Fe component to N_2, but to within our detection limits found little change.[1,2] Since it is not known if the isolated Mo-Fe component binds substrate, this finding is not surprising.

This question, as well as that of the nature of intermediates, is being addressed by NMR spectroscopy. In addition to the natural substrate, N_2, it has been found that many other triply bonded compounds are reduced by nitrogenase. C_2H_2 is reduced to a C_2H_4 and forms the basis for enzyme assay. We are using high resolution NMR in the liquid state to determine the binding constants of gaseous substrates, and in the solid state to probe the identity of intermediates.

For these purposes we have recently completed a multinuclear NMR spectrometer using a 63 kilogauss superconducting magnet.[3] This spectrometer incorporates a number of features specifically designed to render it applicable to the study of biological materials. It operates with high sensitivity in the Fourier transform mode and is capable of performing double resonance and multiple-pulse experiments, all under computer control. The dedicated NMR computer is also interconnected to the Laboratory's central computer system, where very large interactive computations can be executed. Of particular interest has been the development of two-dimensional NMR spectroscopy which is capable of providing NMR spectra with higher information content.[3]

Photosynthesis provides a model for the utilization of solar energy to split water to provide reduced substances, useful as fuel or food, and molecular oxygen. This process is poorly understood at the molecular level. In the watersplitting reaction it is known that metastable intermediates are produced following light absorption; manganese and chloride ion are implicated as essential catalytic cofactors. We are using several new biophysical techniques and approaches to explore the role of these and other elements that participate in the water-splitting reaction. In particular, the Mn sites, heretofore invisible, are being studied by x-ray absorption spectroscopy.

This work was supported by the Division of General Life Sciences, Office of Basic Sciences, and the Division of Biological Energy Conversion and Conservation, Office of Basic Energy Sciences, U.S. Department of Energy, under Contract No. W-7405-ENG-48; and by the National Science Foundation, Grant No. PCM 7812121.

Using methods [illegible] have [illegible] the sensitivity required to detect these [illegible] species, we have [illegible] the first [illegible] mation of their [illegible] states and [illegible] environment.[4,5] [illegible] of Mn [illegible] water-splitting reactions, and [illegible] of manganese [illegible] There are two [illegible] of 2.7 Å, which the distance separating the manganese atoms [illegible] Å. The remaining ligands are [illegible] and the EXAFS studies do not yet permit us to distinguish among them. The oxidation states [illegible] appear to be [illegible] complex with [illegible] structure, capable of existing in several oxidation states, is in complete accord with that required for the storage of the four oxidizing equivalents required to oxidize water to molecular oxygen.

Future studies will be directed toward determining the changes in ligation and oxidation [illegible] the four successive flashes of light which are required to supply the required energy.

One of the most useful aspects of x-ray spectroscopy is its ability to provide structural and electronic information on non-ordered materials, be they gaseous, liquid, or solid. We have studied a number of systems, simpler than the biological ones that are our main interest, in order to refine the experimental, theoretical, and computational tools required for interpreting the x-ray spectra, while concurrently addressing important unsolved questions.

In gaseous AsF_3 and AsF_5 we have been able to determine the distances between As and F atoms to a precision rivaling that obtained by electron diffraction.[6] When graphite is exposed to these and other fluorinated complexes, marked changes in the electronic properties of the intercalated graphite occur. Thus it has been determined that when graphite is exposed to AsF_5 the graphite is oxidized and AsF_3 and AsF_6 are the intercalant species.[6]

It has long been known that when alkali and alkaline earth metals are dissolved in NH_3, the solutions become metal-like in their electronic conductivities. The nature of the changes in the [illegible] of the metal [illegible] and the [illegible] between [illegible] [illegible] oxidation state of the metal atoms.

The [illegible] study of cellular components [illegible] membranes, has been a topic of [illegible] for many years. We have [illegible] NMR [illegible] both the liquid and [illegible] states to [illegible] the organization and dynamics of the phospholipid components of model and [illegible] membranes.[9,10] The inherent weakness of NMR [illegible] searches for methods with higher sensitivity. In recent years we have [illegible] fluorescence correlation spectroscopy [illegible] to probe the mobility of membranes and [illegible] of single cells. In this method, the fluctuations in fluorescent light intensity are analyzed to yield kinetic and dynamic properties. The self-diffusion coefficients, of very small concentrations, e.g., [illegible] M, of fluorescent molecules can be determined. Measurements of this type on the nuclei of single mammalian cells grown in tissue culture have permitted us to infer the viscosity of nuclear material and how the viscosity changes when the cells leave the resting state and enter the growth cycle.[11,12,13] Such information can be obtained from as few as 5000 molecules. Thus, this method promises to be a means for probing many cellular processes in individual cells.

In support of the many instrumental and computational needs of the laboratory, we have installed a new computer system. This system is based on a VAX 11/780 computer with disc drives, magnetic tape drive, programming and interactive graphics terminals, line-printer, and graphics plotter. The computer system has been interfaced with the flow cytometer, with the fluorescence lifetimes apparatus, and with the superconducting NMR spectrometer. One of the most useful features of the old computer system was archival storage and retrieval of data acquired on many of the laboratory's instruments. The new system has been programmed to retain this unique capability, giving access to all the previously acquired data.

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Abstracts

3. I. CONFORMATIONAL ANALYSIS OF CYCLIC PEPTIDES II. MULTINUCLEAR NMR SPECTROMETER FOR THE STUDY OF BIOLOGICAL SYSTEMS

Willy Chao-Wei Shih

Ph.D. Thesis, University of California (1979), LBL-10097

A systematic approach to the employment of ^{1}H NMR data to the analysis of the solution conformations of small polypeptides is outlined. Two-dimensional homonuclear J-spectra along with the corresponding 45° projections and contour maps simplify the task of homonuclear decoupling experiments, and the assignments of lines is made very straightforward. The vicinal couplings that are likely to vary as the conformation changes are then examined. Spectrum simulations coupled with x-ray data for model systems allow the formulation of Karplus relationships for the vicinal couplings, and solution conformations may then be inferred.

This approach to conformational analysis was applied to an examination of the metal affinities of cyclo-[3-(4-β-aminoethyl)-phenyloxypropannyl L-prolyl] and cyclo-[3-(4-β-N-methylaminoethyl)-phenyloxypropanoyl-L-prolyl]. The NMR data revealed that the metal affinity of the former is probably a result of the orientation of both carbonyl groups towards the same face of the ring; the sharply lower affinity of the N-methyl cyclopeptide for divalent cations is likely a result of the carbonyl facing opposite sides of the ring.

Part II of this thesis describes the design approach, major construction details, and the test and characterization procedures associated with the construction of a broadband multinuclear NMR

*See Abstracts.

spectrometer system. The design emphasis was on incorporating features that would be useful for biological experiments. [illegible] designs are [illegible] with schematic [illegible].

5. OPTIMIZATION OF EXPERIMENTAL PARAMETERS FOR MEASUREMENTS OF EXTENDED X-RAY ABSORPTION FINE STRUCTURE (EXAFS)

[illegible]

[illegible]

[illegible]

6. X-RAY ABSORPTION [illegible] GRAPHITE INTERCALATION AND METAL-AMMONIA SOLUTIONS

[illegible]

Ph.D. Thesis [illegible] LBL-[illegible]

X-ray absorption spectroscopy (XAS) has been used to study two different systems in this work, namely (1) the [illegible] complexes, including the AsF_5 and AsF_6^- intercalates of graphite, and [illegible] metal-ammonia solutions.

The features in the region near the onset of the K-shell absorption edge, the analysis of which has been termed x-ray absorption edge spectroscopy (XAES), are transitions into the unoccupied molecular orbitals or metallic bands of the element being studied. On the high energy side of the absorption edge, the oscillatory portion of the absorption coefficient, which has been labeled extended x-ray absorption fine structure (EXAFS), can be analyzed to provide a local radial distribution function around the element being studied. XAES and EXAFS data provided information about both systems studied in this work.

The As-F distances obtained for AsF_3 and AsF_5 gas are both in excellent agreement with electron diffraction data (within 0.004 Å). A superior measurement that is significantly shorter than the accepted value of the bond distance in an undistorted AsF_6^- octahedron is reported. Both the XAES and ESAFS data support the hypo-

[illegible]

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[illegible]

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[illegible]

[illegible] the K-edge shifts with temperature and the [illegible] state of M in metal ammonia solutions (MAS); (ii) the Rb-N distance in MAS [illegible] with temperature and concentration in the range t = [illegible]°-60°C and mole ratio R = $[NH_3]/[Rb]$ = 8 to 160; (iii) the metal number density ascertained spectroscopically shows a temperature dependence similar to that measured gravimetrically; and (iv) the effects of phase separation have been detected (R = 25, T = 197 K) in the density as well as in the Rb-N distance as a spread of K-edge values for the two coexisting liquid phases. Transitions (1) appear to be very sensitive to the potential in the neighborhood of the adsorber. These are absent in the pure metals and increase in intensity near the MI transition suggesting that the final state levels for (1) are broadened in the metal. These transitions could be used in the future to develop theoretical models for the potential in the neighborhood of the adsorber near the MI transition, but all models must be consistent with a solvated species where the metal-nitrogen distance is constant.

[illegible] OXIDATION STATE OF RUBIDIUM COMPOUNDS FROM XAS

[illegible] Mathewar, [illegible] Bouldin, [illegible] and M. [illegible]

[illegible] Phys. Rev., in press

The x-ray absorption spectra (XAS) of Rb, [illegible], [illegible], and RbBr near the Rb [illegible] absorption measured to ascertain that the oxidation state of rubidium dissolved in ammonia [illegible] the lower compound is [illegible]. The calculated edge absorptance curves agree with the data. The observed edge shifts with temperature are quantitatively can be explained in terms of the population of band states with the probability [illegible] where [illegible] is the Fermi distribution function for the [illegible] level.

[illegible] IN SITU BY FLUORESCENCE CORRELATION SPECTROSCOPY

[illegible], Mary [illegible]

Ph.D. Thesis, University of California 1979, [illegible]

All systems in thermodynamic equilibrium are subject to spontaneous fluctuations from equilibrium. For very small systems, the fluctuations become apparent, and can be used to study the behavior of the system without introducing any external perturbations. The mean squared fluctuation amplitude contains information about the absolute size of the system. The characteristic time of the fluctuation autocorrelation function contains kinetic information.

The fluorescent dye ethidium bromide is especially useful as a probe of DNA because the changes in the fluorescence properties of the dye upon binding to DNA greatly enhance the effect of spontaneous fluctuations in the binding equilibrium. An autocorrelation function is obtained from as few as 5000 dye molecules. This sensitivity makes small regions of individual cell nuclei appropriate objects for study.

All the measurements described employ a laser beam focussed to dimensions of the order of one micron. Knowledge of the beam radius is critical, and a new method for its determination <u>in situ</u> is reported.

Experiments are described employing well-characterized DNA preparations, including calf thymus DNA, SV40 DNA, and calf thymus nucleohistone particles. Measurements made in small regions of isolated cell nuclei and on nuclei <u>in vivo</u> are described. These data indicate that the strength of dye binding increases in nuclei isolated from green monkey kidney cells when the cells have been stimulated to enter the cell growth cycle. Data are used to infer that the viscosity of nuclear material is between one and two orders of magnitude greater than that of water. It is shown that the viscosity decreases as the cells leave the resting state, and enter the cell growth cycle. Washing the nuclei also lowers the viscosity.

These experiments demonstrate that fluorescence correlation spectroscopy can provide information at the subnuclear level that is otherwise inaccessible.

[illegible] PROFILE OF A FOCUSSED LOW-INTENSITY LASER BEAM NEAR THE FOCAL MINIMUM CHARACTERIZED BY FLUORESCENCE CORRELATION SPECTROSCOPY

Stanley M. [illegible] and Melvin P. Klein

Rev. Sci. Instrum. 50, 111-115 (1979)

Central to the application of fluorescence correlation spectroscopy, to measure the self-diffusion coefficients and average concentration of fluorescent molecules in a volume determined by a focussed laser beam, is the determination of the focal spot size. As the focal spot size in the sample plane is varied by displacing either the focusing lens or sample position along the beam axis, the diffusion time and average number of molecules vary in a parabolic manner. Analysis of the parameters of the parabola leads to estimates of the beam radius at the waist. The results agree with theoretical predictions and provide an independent measurement of the beam profile.

13. THE USE OF FLUORESCENCE CORRELATION SPECTROSCOPY TO PROBE CHROMATIN IN THE CELL NUCLEUS

Stanley M. Sorscher, James C. Bartholomew, and Melvin P. Klein

<u>Biochem. Biophys. Acta.</u>, in press

All systems in thermodynamic equilibrium are subject to spontaneous fluctuations from equilibrium. For very small systems, the fluctuations can be made apparent, and can be used to study the behavior of the system without introducing any external perturbations. The mean squared amplitude of these fluctuations contains information about the absolute size of the system. The characteristic time of the fluctuation autocorrelation function contains kinetic information.

In the experiments reported here, these concepts are applied to the binding equilibrium between ethidium bromide and DNA, a system where the fluorescence properties of the dye greatly enhance the effect of spontaneous fluctuations in the binding equilibrium.

Preliminary experiments employ well-characterized DNA preparations, including calf thymus DNA, SV40 DNA, and calf thymus nucleohistone particles. Additional measurements are described which have been made in small regions of individual nuclei, isolated from green monkey kidney cells, observing as few as 5000 dye molecules. The data indicate that the strength of dye binding increases in nuclei isolated from cells which have been stimulated to enter the cell growth cycle. The viscosity of nuclear material is inferred to be between one and two orders of magnitude greater than that of water, and increases as the cells leave the resting state, and enter the cell growth cycle. Washing the nuclei also lowers the viscosity.

These experiments demonstrate that fluorescence correlation spectroscopy can provide information at the subnuclear level that is otherwise unavailable.

THREE-DIMENSIONAL STRUCTURE OF PROTEIN AND NUCLEIC ACIDS

[illegible]

[illegible]

Nucleic Acid-Protein Recognition

[illegible] cases, interactions between nucleic acids and proteins control the expression of genetic information in cellular replication, regulation, and development. There are two classes of nucleic acid-protein interactions: one in which a protein recognizes a specific nucleotide sequence [illegible] the other without a sequence specificity.

One example of sequence-specific interaction is the restriction-modification system: E. coli RI (EcoRI) DNA restriction and modification enzymes are responsible for the host specificity of the E. coli strains harboring a drug resistance factor. The enzymes recognize unique sites on DNA with a common, two-fold symmetrical sequence of six base pairs:

d(G-A-Ȧ-T-T-C)

d(C-T-T-A-Ạ-G)

EcoRI restriction-modification system is chosen for this category. We have prepared 30 mg of the enzyme. DNA sequence GAATTC has been provided by Dr. E. Jay at the University of New Brunswick. EcoRI endonuclease has been crystallized[1,2] but DNA complexes have not been, as yet. We are currently engaged in the search for the crystalliza-

[illegible] DNA [illegible] between EcoRI [illegible]

[illegible]

[illegible] that these classes [illegible] stranded DNA [illegible] pA com- [illegible] frame shift [illegible]

An [illegible] interest is that [illegible] compounds, especially [illegible] are known for their [illegible] and antibacterial properties. These properties are attributed not [illegible] the intercalation of these compounds into DNA, but to their complexation with tRNA. Recent NMR studies[8] have indicated that the possible interaction site of ethidium bromide is in the amino acid stem of tRNA; x-ray studies indicate otherwise.[9] The detailed structures of these complexes should provide solid ground for understanding the molecular and atomic mechanism of interaction of these molecules with varieties of nucleic acids.

Nucleic Acid-Metal Ion Interaction

It has been known for a long time that the nucleic acid conformation is sensitive to the kinds of metal ions present in the solution. One of the earliest known examples is the interconversion between B-DNA to Aor C-DNA by varying relative humidity or counter-ion types in DNA fiber or gel. There are three classes of metal ion that interact with nucleic acid: phosphate-neutralizing, base-specific, and structure-specific metal ions.

Nucleic acid-metal ion interactions are important in stabilizing secondary and tertiary structures of nucleic acids. The evidence for the conformation-specific metal ion binding in tRNA is overwhelming. However, neither the specific detail of the binding, nor unambiguous binding sites are known. The only example of this class

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known by x-ray crystallography is the determination of the binding sites for metal ions in a tRNA.[10,11,12]

Another aspect of nucleic acid-metal ion interactions is environmental: the toxicity of heavy metal ions on living organisms has been known for some time and has been a major concern. Some toxic metal ions bind tightly to nucleic acids. Our knowledge of the structural details, however, is very weak and limited mostly to the nucleotide level; it needs to be extended to the polymer level. We plan to study heavy atom-tRNA interactions.

DNA Fragments

It has been 28 years since the Watson-Crick DNA model was proposed. Although the model describes the overall symmetry and geometrical features of double-stranded DNA, conformational details such as extent of irregularity of backbone structure dependent on base sequence, G•C content, and effect of metal ions on conformations are not known. These conformational details are essential for understanding DNA functions at different stages of its role. There is an intense effort to reexamine "known" structural features such as the number of base-pairs per turn[13] and DNA conformation as a function of environment. At the present time, the only way one can get a detailed atomic structure of DNA is to determine 3-D structure of short DNA fragments 5-10 base pairs long by single-crystal x-ray diffraction. The value of this method was dramatically illustrated by the crystal structure of the $(CG\ CG\ CG)_2$ double helix,[13] which revealed a totally different structure from that of Watson-Crick, suggesting strongly that base sequence can dictate the local DNA conformation. To test this hypothesis, we plan to determine the crystal structure of several double-helical DNA fragments.

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Abstracts

SOLVENT-ACCESSIBLE SURFACES OF NUCLEIC ACIDS

Charles J. Alden and Sung-Hou Kim

J. Mol. Biol. 132, 411-434 (1979)

Static solvent-accessible surface areas were calculated for DNA and RNA double helices of varied conformation, composition and sequence, for the single helix of poly(rC), and for a transfer RNA. The results show that for DNA and RNA double helices, two thirds of the water-accessible surface area becomes buried on double helix formation; phosphate oxygens retain near maximal exposure while the bases are 80% buried. Transfer RNA exposes slightly less surface per residue than does double-helical RNA, despite the presence of several additional "modified" groups, all of which are exposed significantly.

When a probe corresponding to a single water molecule is used, both the total and atom-type exposures are very similar for A-DNA and B-DNA, although marked differences appear in the major and minor groove exposures between the two conformations. For a given base-pair, the accessible surface area buried upon double-helical stacking is nearly constant (within 5%) for different sequence of neighboring base-pairs.

For probes larger than single water molecules, there exist considerable differences in the total and atom type exposures of A-DNA and B-DNA. Conformational transitions between the A-DNA and B-DNA helical forms can thus be related to differences in the accessible areas for "structured" water, or a secondary hydration shell, rather than to interactions with individual water molecules of the primary hydration shell (Figure 1). The base-composition dependence of DNA helical conformation can be explained in terms of the opposing effects of thymine methyl groups of A•T base-pairs and the amino groups of G•C base-pairs upon the solvent within the grooves.

The area calculations show that primarily the major groove of B-DNA and the minor groove of A-DNA have sufficient accessible surface area to be recognized by a probe size corresponding to the side-chains of amino acids (Figure 2).

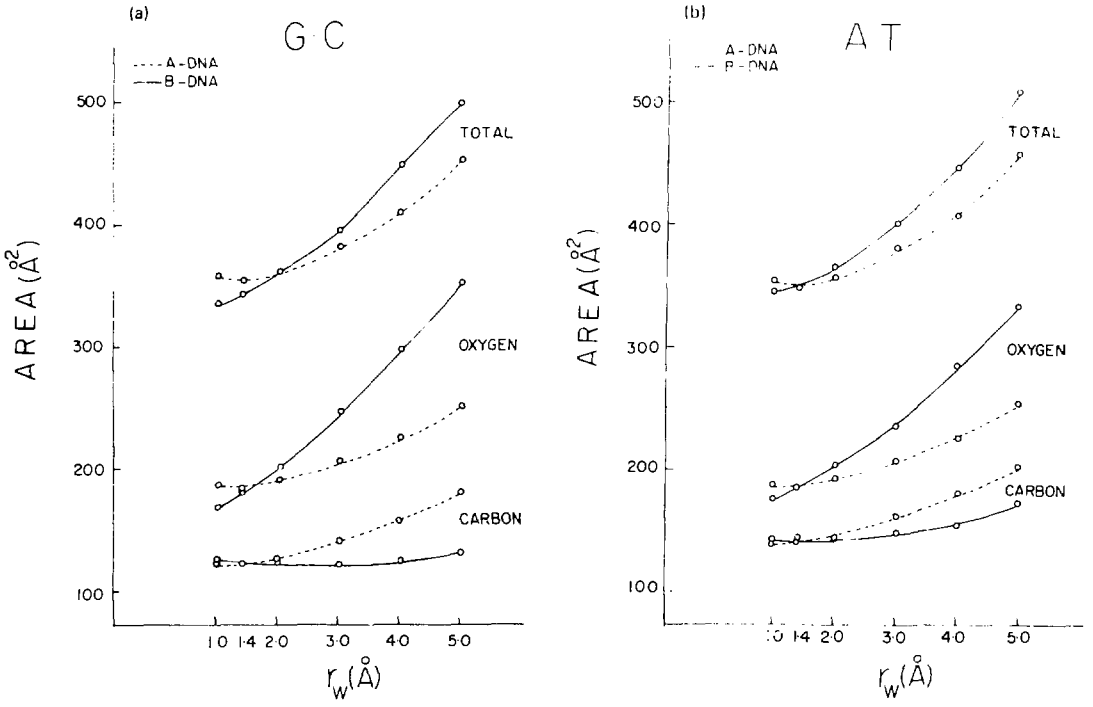

Figure 1. Accessible surface areas for 2 base-paired residues of (a) poly(dG)•poly(dC) and (b) poly(dA)•poly(dT) in the A-and B-helical forms, calculated varying the probe radius r_w. For both polymers, the B-DNA form entails relatively greater total exposure, and particularly a greater exposure of phosphate oxygens, for large probe radii. This result is consistent with the experimentally observed result that high water activity encourages adoption of the B-DNA conformation. (-----) A-DNA; (———) B-DNA.
(XBL 806-10240)

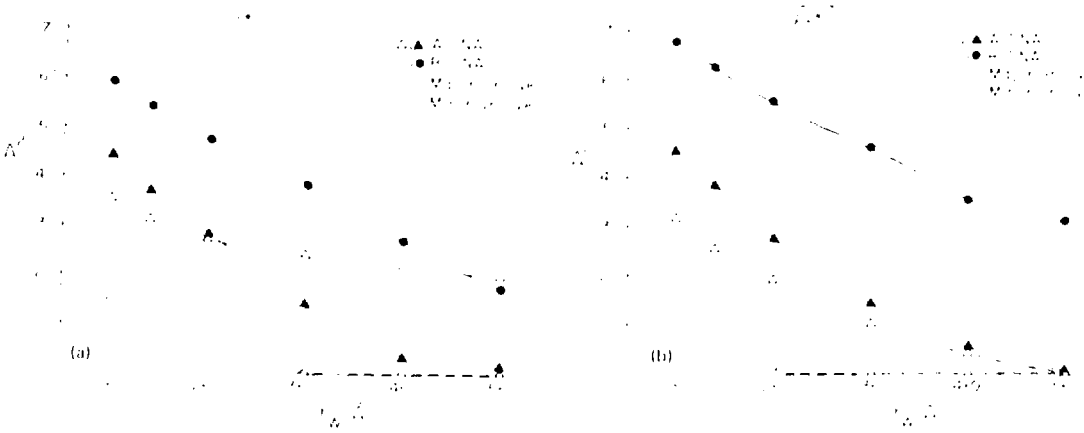

Figure 2. Accessible surface areas for DNA base atoms of the minor and major grooves of A- and B-DNA for (a) G-C base pairs and (b) A-T base pairs, calculated varying the probe radius r_w. As r_w increases to large values, corresponding to extended water complexes or amino acid side chains, the only groups with significant exposures are the methyl groups of T and the amino groups of C and G. (▲, ▲) A-DNA; (○, ●) B-DNA; ([illegible] minor groove; ([illegible] major groove. (XBL 806-10241)

INTERCALATION CONFORMATIONS IN SINGLE- AND DOUBLE-STRANDED NUCLEIC ACIDS

S. R. Holbrook and S.-H. Kim

Internat. J. of Macromolecules 1, 233 (1979)

Two regions in the crystal structure of yeast phenylalanine tRNA, where single-stranded loops interact by intercalation, have been examined in detail. There are four examples of a nucleotide base from one loop intercalating between two sequential bases of another loop in these two regions (Figure 3). These four dinucleoside phosphate conformations serve as models for intercalation in single-stranded nucleic acids. Double-stranded DNA and RNA polymers were constructed by computer model building methods, which incorporated the dinucleoside phosphate conformations found in these single-stranded, intercalation regions in otherwise standard double helices. The results suggest that it is unlikely that there is a unique intercalation geometry for either single or double-stranded nucleic acids, but that nucleic acids may assume one of a variety of intercalating geometries which will best accommodate a particular intercalating agent for a particular base sequence (see Table I).

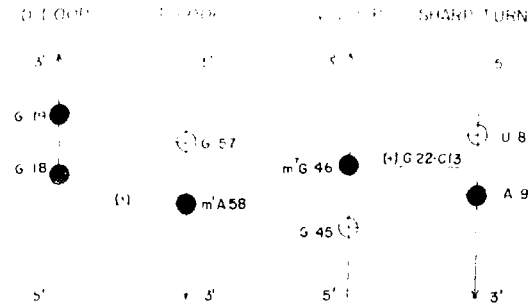

Figure 3. Schematic representation of two regions found in the crystal structure of yeast phenylalanine tRNA where single-stranded loops interact by a process of mutual intercalation. Lines with arrows indicate the progress of the ribose-phosphate backbone, with the bases represented by lines perpendicular to the backbone. Open circles signify a ribose which has a C3' endo pucker while darkened circles denote sugars in the C2' endo conformation. A 'plus' sign indicates a base carrying a net positive charge. (XBL 806-10242)

Table 1. Structural features of DNA and RNA models.

	T[a]	B[b]	DH[c]	AVGD[d]
Models allowing intercalation				
DNA (18-19)	40	37	3.4	7.0
DNA (45-46)	3	1	1.6	7.2
DNA (57-58)	23	11	1.1	6.4
RNA (45-46)	3	7	1.9	7.2
RNA (57-58)	21	7	0.2	6.4
DNA-B-proflavin	23	0	0	6.8
DNA-B-EtBr	10	8	1.0	6.8
Standard nucleic acid models				
DNA-B	36	0	0	3.4
RNA-A	33	0	0	3.4

[a] T - turn angle in degrees, calculated as the angle between the vectors connecting the C1' atoms of each base pair involved in intercalation projected on the average plane between the base pairs around intercalation site.

[b] B - a 'bend' angle in degrees between helical axes formed by base pairs above and below intercalation site.

[c] DH - Angstrom displacement in helix axis at average plane between base pairs above and below intercalation site.

[d] AVGD - average distance in Angstroms between base planes around intercalation site.

SEQUENTIAL FOLDING OF A TRANSFER RNA: (I) AN NMR STUDY OF SUCCESSIVELY LONGER tRNA FRAGMENTS WITH A COMMON 5' END

John Boyle, George Robillard and Sung-Hou Kim

J. Mol. Biol. (in press)

Most folding studies on proteins and nucleic acids have been addressed to the transition between folded and unfolded states of an intact molecule, where an entire residue sequence is present during the folding event. However, since these polymers are synthesized sequentially from one terminus to the other in vivo, their folding pathways may not be greatly influenced by the sequential appearance of the residues as a function of time.

The three-dimensional structure of yeast $tRNA^{Phe}$ in the crystalline state is correlated with 360 MH_z proton NMR resonances from three fragments plus an intact molecule of the tRNA that share a common 5' end (Figure 4) and are in a solution condition similar to that of the crystal structure. This has allowed identification of folded structures present in the fragments and presumably present in the growing tRNA molecule as it is being synthesized from 5' end. The experiments show that only the correct stems are formed in the fragments; no additional or competing helical regions are produced. This suggests that in the biosynthesis of this tRNA, correct folding of helical stems occurs before the entire molecule is formed. Further, some of the tertiary interactions (hydrogen bonds) found in the crystal structure are also probably present before the synthesis is completed (Figure 5). These findings are generalized to consider the precursor of the tRNA as well as other tRNA's.

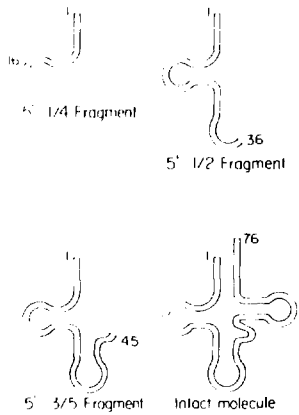

Figure 4. Fragments from yeast $tRNA^{Phe}$ used in this work. (XBL 7911-5094A)

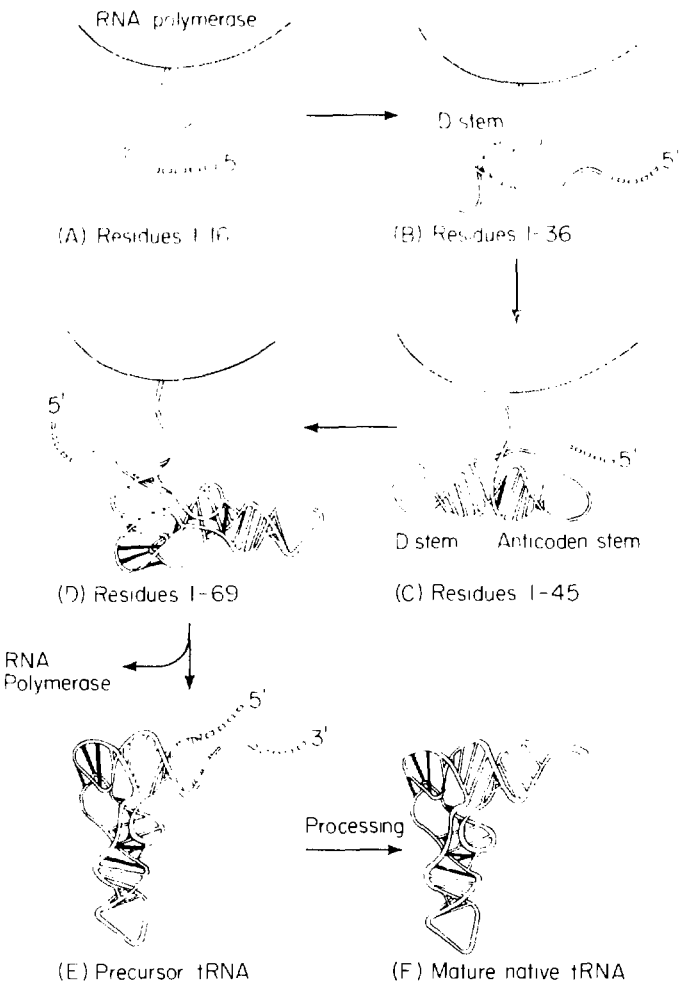

Figure 5. Schematic representation of the sequential folding of yeast $tRNA^{phe}$. (XBL 7911-5095)

STRUCTURES AND FUNCTIONS OF NUCLEIC ACIDS

Ignacio Tinoco, Jr.

The base sequence of a nucleic acid contains the information that determines its biological functions and characterizes its conformations in different environments. We want to be able to interpret a base sequence in terms of function and to be able to predict the conformations that can occur. That is, we are studying how the primary structure (base sequence) of a nucleic acid determines its secondary and tertiary structure (double-strand formation and folding). The primary, secondary and tertiary structures in turn determine and control the biological functions.

We have used semi-empirical potential energy calculations to find possible conformations of dinucleoside phosphates.[1] The three conformations shown in Figure 1 represent the most stable

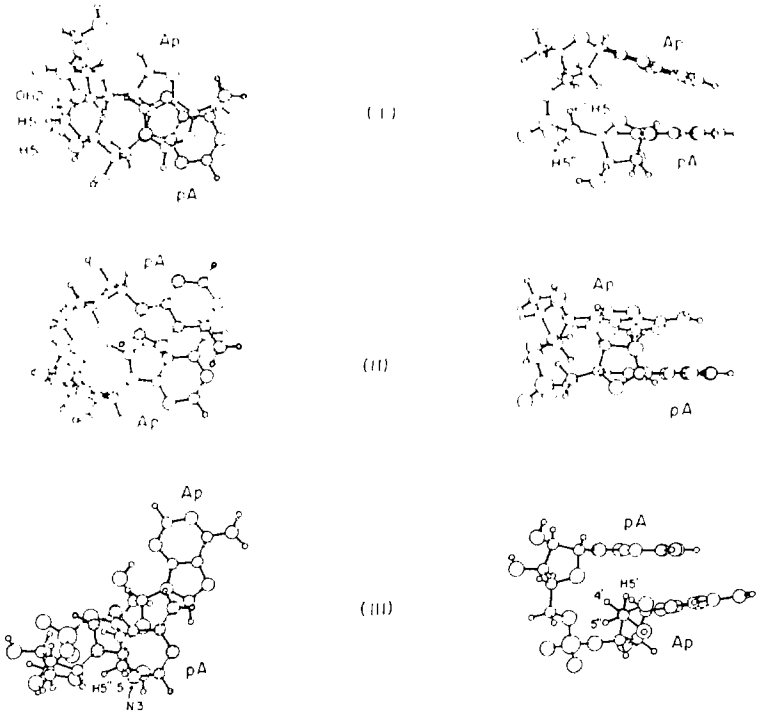

Figure 1. Three minimum-energy conformations of the dinucleoside phosphate, adenylyl-3'-5'-adenosine (ApA). (XBL 806-10243)

This work was supported by the Division of Biomedical and Environmental Research, Office of Environmental Research and Development, U.S. Department of Energy, under Contract No. W-7405-ENG-48.

species with stacked bases. In addition, many other species have unstacked bases. The kinetics and thermodynamics[2] of the transition between stacked and unstacked species in dinucleoside phosphates was studied using spectroscopic methods. The data indicate that the transition does not occur by a simple two-state model.

We have recently started to apply our knowledge of DNA structure and stability to frame-shift mutagenesis. Frame-shift mutations are particularly harmful because they cause the misreading of many words of the genetic message. Other mutations usually cause only one word of the message to be affected. A bulge of one or two unbonded bases in a replicating DNA can cause a frame-shift mutation. If the bulge occurs in the parent strand a -1 frame shift results; if the bulge is in the new strand a +1 frame shift is produced.

Planar aromatic molecules are known frame-shift mutagens. We are studying the interaction of such molecules with oligonucleotides. A combination of ^{13}C and ^{1}H nuclear magnetic resonance and optical spectroscopic studies has provided structures for the complexes between the mutagen 4-nitroquinoline-1-oxide and dinucleoside phosphates (see Figure 2).[3] Similar studies have shown that ethidium, another mutagen, definitely facilitates the formation of bulges in double strands.

Theoretical work on the circular dichroism[4,5] of molecules that are large compared to the wavelength of light has shown that new effects can occur for macromolecules. In particular, the preferential scattering of circularly polarized light, an optical phenomenon related to circular dichroism, can be used to characterize large helices.[6] These methods should be useful in characterizing the DNA in chromosomes, viruses, and cells.

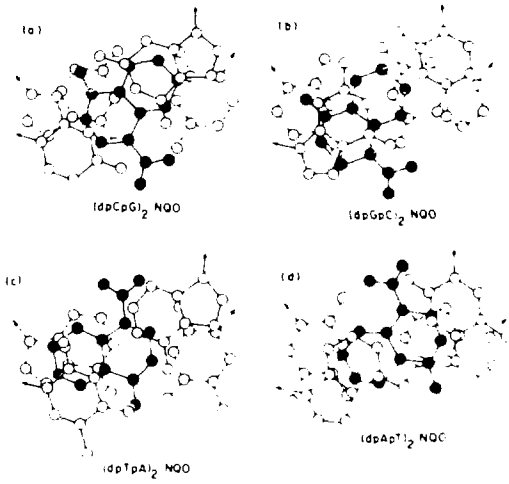

Figure 2. Proposed structures of self-complementary dimer:NQO 2:1 complexes. (a) $(dpCpG)_2$:NQO; (b) $(dpGpC)_2$:NQO; (c) $(dpTpA)_2$:NQO; (d) $(dpApT)_2$:NQO. Only base atoms (excluding hydrogens) are shown for the dimers. Bottom base pair (o--o); top base pair (o o); NQO (● ●). These are approximate representations of the complex structures. (XBL 806-10244)

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*See Abstracts.

Abstracts

1. CONFORMATIONS OF DINUCLEOSIDE PHOSPHATES IN AQUEOUS SOLUTION

Che-Hung Lee, Elliot Charney and Ignacio Tinoco, Jr.

Biochemistry 18, 5636-5641 (1979)

The conformations of dinucleoside phosphates have been re-examined by semi-empirical potential energy calculations. Conformations I, II and III, proposed by Lee and Tinoco (1977), are possible species after refinement of their structures by potential energy minimization. These three conformers can represent three types of dinucleoside phosphate species in solution. Dhingra et al. (1978) had concluded that conformations of type II and III were unlikely or impossible. They favored conformations g^-g^- (equivalent to I), g^+g^+, g^+t and tg^+; the last three conformations have little stacking and are calculated to be energetically less favorable by more than 5 kcal/mole. Common structures of the types I, II and III are found for dinucleoside phosphates with different purine-pyrimidine sequence. The sequence-dependence of the potential energy of these three conformers has been calculated. The experimental NMR data of dinucleoside phosphates are consistent with these three conformations.

2. FLUORESCENCE DETECTED CIRCULAR DICHROISM OF DINUCLEOSIDE PHOSPHATES. A STUDY OF SOLUTION CONFORMATIONS AND THE TWO-STATE MODEL

Charles Reich and Ignacio Tinoco, Jr.

Biochemistry 19, 833 (1980)

Three fluorescent dinucleoside phosphates containing 1,N^6- ethenoadenosine (εA), $\varepsilon A_p \varepsilon A$, $\varepsilon A_p \varepsilon C$, and $\varepsilon A_p U$ were studied using fluorescence detected circular dichroism (FDCD), circular dichroism (CD) and absorption measurements. The FDCD data indicate that $\varepsilon A_p \varepsilon C$ and $\varepsilon A_p U$ can be described as two-state systems consisting of a fluorescent species and a stacked, non-fluorescent species. Thermodynamic stacking parameters are calculated for these molecules using the van't Hoff equation. $\varepsilon A_p \varepsilon A$ is found to be a more complicated system with a fluorescent CD which is different in shape, but comparable in magnitude, to the conventional CD of the dimer. This molecule, unlike the other two dinucleoside phosphates, cannot be characterized as a two-state system; it is described as consisting of at least three states at temperatures above 35°C.

The CD data were subjected to a linear analysis in order to determine the minimum number of states present. In agreement with the FDCD data, $\varepsilon A_p \varepsilon C$ and $\varepsilon A_p U$ are found to consist of a minimum of two states, while $\varepsilon A_p \varepsilon A$ is indicated to have at least three. The more complicated behavior of the latter dimer is also indicated by the values of the unstacked CD obtained in the van't Hoff analysis.

3. INTERACTIONS OF 4-NITROQUINOLINE 1-OXIDE WITH DEOXYRIBODINUCLEOTIDES

Stephen A. Winkle and Ignacio Tinoco, Jr.

Biochemistry 18, 3833 (1979)

The interactions of 4-nitroquinoline 1-oxide (NQO), a potent mutagen and carcinogen, with several self-and non-self-complementary deoxydinucleotides were probed by using absorption spectra of the charge transfer bands and ^1H and ^{13}C NMR spectra. Absorption spectra were analyzed by using Benesi-Hildebrand-type equations to yield stoichiometries and equilibrium constants of complex formation. Non-self-complementary dimers form weak 1:1 complexes [dpTpG:NQO,$\underline{K}$(25°C) = 22 M^{-1}] while self-complementary dimers form strong 2:1 complexes [$(dpCpG)_2$:NQO,$\underline{K}$(25°C) = 2.2 x 10^4 M^{-2}]. A mixture of dpTpG and dpCpA with NQO gives a 2:1 complex [dpTpG:NQO:dpCpA,$\underline{K}$(25°C) = 8.6 x 10^3 M^{-2}]. Analyses of the changes in ^{13}C and ^1H NMR chemical shifts with complex formation gave approximate orientations for the intercalation of NQO with self-complementary dimer minihelixes. In the $(dpCpG)_2$:NQO and $(dpGpC)_2$:NQO complexes, the NO_2 group of NQO probably lies in the major groove and the NO_2, NO containing NQO ring is stacked near the purine imidazole ring. In the $(dpTpA)_2$:NQO and $(dpApT)_2$NQO complexes, the NO_2 seems to project into the minor groove and the NQO benzenoid ring is over the purine imidazole ring.

4. CIRCULAR DICHROISM OF LARGE MOLECULES

Ignacio Tinoco, Jr.

Int. J. Quantum Chem. 16, 111-117 (1979)

The circular dichroism of molecules that are large compared to the wavelength of light is considered. Explicit expressions are obtained for the circular dichroism and absorption of an exciton dimer and of a free particle on a helix. The dimensions are described for which the dipole approximation for the optical properties fails.

5. THE CIRCULAR DICHROISM OF LARGE HELICES. A FREE PARTICLE ON A HELIX.

Dexter Moore and Ignacio Tinoco, Jr.

J. Chem. Phys. 82, 3396 (1980)

Equations are derived for the circular dichroism and optical rotation of light by a free electron on a helix which is of arbitrary size

relative to the wavelength of light. The equations were found to give results in good agreement with measured rotation of microwaves by oriented wire helices. The derivation shows when the usual Rosenfeld approximation for optical activity becomes inadequate.

6. THE OPTICAL ACTIVITY OF NUCLEIC ACIDS AND THEIR AGGREGATES

Ignacio Tinoco, Jr., Carlos Bustamente and Marcos F. Maestre

Ann. Rev. Biophys. Bioengineering 9, 107 (1980)

This review includes experimental and theoretical work on absorption and scattering of light by chiral macromolecules; experimental applications are limited to nucleic acids. Theoretical methods that relate the absorption and circular dichroism of a polymer or aggregate to the optical properties of its constituent parts are discussed. A new experimental method, one that may become increasingly important in characterizing chiral systems, is the circular intensity differential of scattered light. The preferential scattering of right or left circularly polarized light by chiral systems is discussed in terms of a simple, model helix.

CIRCULAR DICHROISM AND FLUORESCENCE DETECTED CIRCULAR DICHROISM OF MACROMOLECULES

I. Tinoco, Jr.

Optical Activity and Chiral Discrimination, Stephen F. Mason, ed. (D. Reidel Publishing Co., Dordrecht, Holland, 1979), pp. 57-85

Experimental and theoretical methods that are pertinent mainly to polymers and macromolecules are discussed. The optical properties of polymers are determined by the interactions between two or more similar chromophores. Therefore, to calculate the circular dichroism of a polymer we need the properties of the individual chromophores (the monomers) and we need to evaluate their interactions. The crucial assumption that has been made in calculations of circular dichroism of polymers is that electrons are localized in each chromophore. Each chromophore is represented by a charge distribution which interacts with the other chromophores in the presence of the light. Any electron exchange or electron transfer between chromophores is assumed to have a negligible effect on the circular dichroism. Calculations of circular dicroism of ploypeptides and polynucleotides have been reasonably successful using these methods.

CIRCULAR DICHROISM OF POLYMERS; THEORY AND PRACTICE

Ignacio Tinoco, Jr.

Optically Active Polymers, Eric Sélégny, ed. (D. Reidel Publishing Co., Dordrecht, Holland, (1979), pp. 1-13

The goal of understanding the circular dichroism (CD) of a polymer is to be able to interpret the measured spectrum in terms of the conformation of the polymer. We want to know if the polymer is rigid or flexible. Is it a helix? What are the helical parameters? To deduce a polymer conformation from a measured CD we must calculate the CD for different assumed conformations. When we get agreement between calculation and experiment, we say this is a possible conformation. There may be other conformations which are also consistent.

Applications to polypeptides and nucleic acids will be discussed.

A new method that is more specific than circular dichroism is fluorescence detected circular dichroism (FDCD). In this method the intensity of light emitted by a fluorescent chromophore excited by left and right circularly polarized light is used to measure the circular dichroism. The FDCD method thus gives information about the conformation of a polymer in the immediate neighborhood of fluorescent groups.

Phenomena related to circular dichroism which may be useful for studying conformations in polymers are the circular intensity differential (CID) for scattered light. The difference in scattered intensity is measured for left and right circularly polarized incident light. At this time these effects have only been reported for small molecules. In principle, however, they may be very useful for the study of polymers.

KINETICS AND THERMODYNAMICS OF DOUBLE STRAND FORMATION IN SELECTED DEOXYOLIGONUCLEOTIDES

Adrienne E. Drobnies

Ph.D. Thesis, University of California, Berkeley, September, 1979

The thermodynamics and relaxation kinetics for the double strand-single strand transition in three deoxyoligonucleotide double helices was investigated. The $\Delta H°$, $\Delta S°$ for double helix formation and the rate constants for helix association and dissociation were measured for dATGCAT, dA_3GCT_3, and $dA_8 + dT_8$ in an effort to compare DNA double helices with similar RNA helices.

From the variation of melting temperature for each oligomer with strand concentration, the $\Delta H°$, $\Delta S°$ for helix formation are:

	$\Delta H°$ (kcal/mol)	$\Delta S°$ (lcal/mol deg)
dA_3GCT_3	-36	-0.100
dATGCAT	-48	-0.147
$dA_8 + dT_8$	-39	-0.115

No trends in these parameters due to base sequence or helix length were observed; however, from a calculation of the free energies for helix formation, it can be seen that double-strand formation is less favored for each DNA oligomer than

for its analogous RNA oligomer. This agrees with earlier measurements of melting temperatures for DNA and RNA single stranded polymers.

The temperature jump technique was applied to the study of the relaxation kinetics of the deoxyoligomers. At 1°C the rate constants are:

	association ($M^{-1}sec^{-1}$)	dissociation (sec^{-1})
$dA_2GC^*_2$	5.6×10^6	13
$dATGCAT^*$	1.9×10^6	110
$dA_8 + dT_8$	20×10^6	990

The association rates are the same as for the comparable RNAs in the case of the two oligomers containing GC base pairs. The association is an order of magnitude faster for $dA_8 + dT_8$ than for $rA_8 + rU_8$.

In contrast to RNA oligomers where those with GC base pairs have positive activation energies for association and those with only AU base pairs have negative activation energies, the deoxyoligomers all have small negative activation energies for helix association.

This is indicative of a mechanistic change between DNA and RNA double strand formation. The formation of one GC base pair is rate-limiting in RNA oligomer association. If no GC base pairs are possible, the formation of a third AU base pair is rate-limiting. In DNA, helix propagation is favored after the formation of two adjacent base pairs. Dissociation activation energies are similar for both DNA and RNA helices.

CONFORMATION STUDIES OF 13 TRINUCLEOSIDE DIPHOSPHATES BY 360 MHz SPECTROSCOPY. A BULGED BASE CONFORMATION. I. BASE PROTONS AND H1' PROTONS

Che-Hung Lee and Ignacio Tinoco, Jr.

Biophys. Chem., 11, 283 (1980)

The 360 MHz NMR spectra of the base protons and the H1' protons of thirteen trinucleoside diphosphates have been analyzed. The sequences chosen represent all purine-pyrimidine sequences. The chemical shifts of the base protons give evidence for strong next-nearest-neighbor effects in some oligonucleotides. Although increasing chain length usually increases nearest-neighbor base-base stacking, it is not always so. Comparing ApCpG, ApUpG and GpUpG to their component dimers, one finds a decrease in stacking of the center pyrimidine with the purine on either side. The coupling constants J1'2' also show that these three trimers show less stacking for their terminal residues than expected from their component dimers. We conclude that the sequence Pu-Py-Pu favors a conformation in which the pyrimidine is bulged out and the two purines stack on each other.

THE INTERACTION WITH 4-NITROQUINOLINE-1-OXIDE WITH NUCLEIC ACIDS

Stephen A. Winkle

Ph.D. Thesis, University of California, August 1979 (B.-966)

The interactions of the potent mutagen and carcinogen, 4-nitroquinoline-1-oxide (NQO) with deoxyribomononucleotides, deoxyribodinucleotides and DNAs were studied. The non-covalent complexes formed by NQO and either the four 5'-deoxyribomononucleotides or the deoxyribodinucleotides were studied using absorption spectra of the charge transfer bands (circa 395-410 nm) and ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra of NQO-nucleic acid mixtures. Absorption spectra for the monomer-NQO and dimer-NQO systems were analyzed using Benesi-Hildebrand type equations to yield stoichiometries and equilibrium constants of complex formation. The equilibrium constants for the formation of the 1:1 monomer:NQO complexes at 25°C (K(dpG:NQO) = 16 M^{-1}, K(dpA:NQO) = 1.7 M^{-1}, K(dpT:NQO) = K(dpC:NQO) = 4 M^{-1}) suggest the preference of NQO for the guanine residue in a DNA. This is in agreement with the data of Okano, T. et al. [(1969) Gann 60, 295]. Non-self-complementary dimers form weak 1:1 complexes (dpTpG:NQO, K(25°) = 22 M^{-1}) while self-complementary dimers form strong 2:1 complexes ($(dpCpG)_2$:NQO, K(25°C) = 2.2×10^4 M^{-2}). A mixture of dpTpG and dpCpA with NQO gives a "2:1" complex (dpTpG:NQO:dpCpA, K(25°C) = 8.6×10^3 M^{-2}). The dinucleotides capable of forming DNA-like minihelices gave similar values of K for NQO complex formation. From ^{13}C and ^1H NMR data, it is proposed that in the dpG:NQO complex the (NO_2), (NO) containing NQO is stacked over the guanine imidazole ring while in the dpG:NQO complex the NQO benzenoid ring is over the adenine imidazole ring. Analyses of the changes in ^{13}C and ^1H NMR chemical shifts with complex formation for dimer-NQO mixtures gave approximate orientations for the intercalation of NQO with self-complementary dimer minihelices. In the $(dpCpG)_2$:NQO and $(dpGpC)_2$:NQO complexes, the (NO_2) group of NQO probably lies in the major groove and the (NO_2), (NO) containing NQO ring is stacked near the purine imidazole ring. In the $(dpTpA)_2$:NQO and $(dpApT)_2$:NQO complexes, the (NO_2) seems to project into the minor groove and the NQO benzenoid ring is over the purine imidazole ring.

Irradiation of NQO-polynucleotide (poly(rA), poly(rU), poly(rC)) mixtures and NQO-DNA mixtures using either a Hg lamp source (340-420 nm) or a dye laser (400-415 nm) produced photoadducts. With the polymers, poly(rC) gave the greatest number of adducts per residue. Poly(rC) and DNA also reacted with NQO in the dark. A dpC-NQO photo-adduct was produced.

DNA-NQO equilibria were studied using analyses of the complex absorption bands (415 nm) and using the phase partition technique. Two NQO molecules bind strongly and cooperatively to a small number of sites on the DNA. The equilibrium constants and binding ratios at 25°C ranged from 4.3×10^9 M^{-2} and 100 base pairs

(bp)/site for calf thymus DNA to 4.3×10^{14} M^{-1} and 4600 bp/site for superhelical SV40 DNA. The binding ratio for calf thymus DNA was temperature dependent, e.g., 7°C, 900 bp/site; 25°C, 1[illegible]0 bp/site; 37°C, 840 bp/site. The equilibrium constants dropped when supercoiled DNA was converted to the linear form (pBR 322, [illegible] $\times 10^{13}$ to 6.9×10^{11} M^{-1}; SV40, 4.0×10^{14} to 1.3×10^{11} M^{-1}). NQO inhibits the cutting [illegible] superhelical SV40 DNA and pBR 322 by S_1 endonuclease which cuts "denatured regions" [illegible] superhelical DNA's (Berg, et al. [illegible]). [illegible] enzyme digests of pBR [illegible] chemically with ^{3}[illegible]-NQO [illegible] pBR 322 [illegible] an S_1 cutting site. The DNA containing [illegible] covalently bound NQO was 62% per NQO [illegible] site and 12% NQO 150% site [illegible] covalent, bound NQO [illegible] chemical addition. Based on these findings [illegible] the covalent binding [illegible] DNA, it is suggested that NQO [illegible] [illegible] site, [illegible] the DNA.

THE PHOTOREACTION OF PSORALENS WITH NUCLEIC ACIDS

John E. Hearst

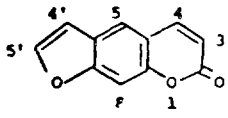
A. Structural Characterization of Psoralen-Nucleic Acid Adducts

Psoralen (Scheme I) and its derivatives have attracted widespread interest as chemical probes of nucleic acid structure. These compounds are furocoumarin-based bifunctional intercalating agents which upon irradiation with long wavelength ultraviolet light (365 nm) become covalently bound to the nucleic acids. The products of irradiation include both mono- and di-adducts, which result in interstrand crosslinks. Model studies on nucleoside monomers suggest that addition occurs via formation of a cyclobutane-type bond between the 5,6-bond of pyrimidines and the 3,4 or 4',5'-bonds of the psoralen. There have been no reports of isolated material obtained from a DNA polymer, however. A rigorous structural analysis of the products obtained from psoralen crosslinked high molecular weight DNA would aid in explaining the specificity of substituted psoralens, and might enable one to design more specific (with respect to either DNA sequence or conformation) crosslinking reagents. Accordingly, we have conducted a series of experiments designed to isolate sub-

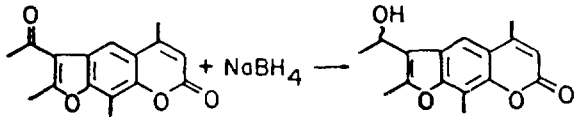
[illegible] Beta II, HMC [illegible] after [illegible] the [illegible] DNA, the [illegible] were [illegible] for [illegible] high resolution [illegible] spectrometry, field desorption [illegible] and [illegible] spectroscopy [illegible] high field [illegible] NMR experiments are also in progress. The results to date [illegible] the identification of at least four monomer HMC-thymidine adducts and [illegible] crosslinks involving [illegible]. The analytical techniques that we have developed will permit the eventual isolation and characterization of virtually all of the products formed in the photoreaction of substituted psoralens with DNA or RNA.

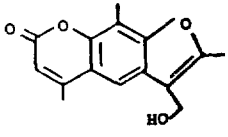
B. Synthesis of Isotopically Labelled Psoralen Derivatives

The [illegible] derivatives which have been synthesized [illegible] the hydroxymethyl, ethyl, and

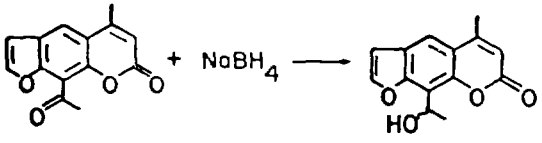


Scheme I





Scheme II



Scheme III

This work has been supported in part by U.S. Public Health Service Grants GM 11180 and GM 25151, by American Cancer Society Grant NP 185, by National Science Foundation Grants PCM-7819544 and GP 10065, and in part by the Division of Biomedical and Environmental Research and Development, U.S. Department of Energy, under Contract No. W-7405-ENG-48.

Abstracts

[illegible] coli RIBOSOMA[illegible]

Pallavi Thurmond, [illegible], Carter, Paul [illegible], Wollenzien, and [illegible]

J. Mol. Biol. [illegible] (1979).

The location and frequency of RNA crosslinks induced by photoreaction of hydroxymethyltrimethylpsoralen with 30 S Escherichia coli ribosomal subunits have been determined by electron microscopy. At least seven distinct crosslinks between regions distant in the 16 S rRNA primary structure are seen in the inactive conformation of the 30 S particle. All correspond to crosslinked features seen when the free 16 S rRNA is treated with hydroxymethyltrimethylpsoralen. The most frequently observed crosslink occurs between residues near one end of the molecule and residues about 600 nucleotides away to generate a loop of [illegible] orientation of this feature [illegible] corresponds to the crosslinked [illegible] located at the 3' end of free 16 S rRNA.

When [illegible] particles are crosslinked in [illegible], [illegible] the seven features seen in the inactive [illegible] particle can still be detected. However, the frequency of several of the features, and particularly the 570-base loop feature, is dramatically decreased. This suggests that the long-range contacts that lead to these crosslinks are either absent or inaccessible in the active conformation. Crosslinking results in some loss of functional activities of the 30 S particle. This is consistent with the notion that the presence of the crosslink that generates the 570-base loop traps the subunit in an inactive form, which cannot associate with 50 S particles.

The arrangement of the interacting regions crosslinked by hydroxymethyltrimethylpsoralen suggests that the RNA may be organized into three

[illegible]

[illegible] RNA [illegible]

[illegible]

[illegible]

[illegible] have been introduced [illegible] in the ribosomal RNA in [illegible] the presence of [illegible]. When the cross-linked RNA is denatured and examined in the electron [illegible] the most striking features are a variety of large open loops. In addition, because the crosslinked molecules are shortened compared to noncrosslinked molecules, there are likely to be small hairpins not resolved by the present technique. The sizes and positions of 11 loop [illegible] have been determined and oriented on the [illegible]. The frequency of occurrence of the different [illegible] of loops depends on the cross-linking conditions. When the crosslinking is done in solutions containing Mg^{+}, at least four of the loop [illegible] appear with greater frequency than they do in 3-5 mM-NaCl. The loops presumably arise because complementary sequences separated by [illegible]; intervening regions are being cross-linked. These base-pairing interactions between residues distant in the primary structure appear to be prominent features of the secondary structure of rRNA in solution.

DETERMINING THE POLARITY OF THE MAP OF CROSSLINKED INTERACTIONS IN Escherichia coli 16 S RIBOSOMAL RNA

[illegible] Morrienzien, John E. Hearst, Catherine Squires, and Craig Squires

J. Mol. Biol. 135, 285-291 (1979)

Hydroxymethyltrimethylpsoralen crosslinked 16 S rRNA from Escherichia coli has been R loop hybridized to two plasmid DNAs containing different sections of the 16 S ribosomal gene. It is possible to identify crosslinked features in the part of the RNA that is not complementary to the DNA. Crosslinked features can be aligned into a relative map of interactions. Crosslinked loops that correspond to features located, originally arbitrarily, in the left part of this map are seen in the 5' half of the 16 S rRNA in one hybrid and loops that correspond to features in the right part of the map are seen in the 3' two-thirds of the 16 S rRNA in the other hybrid. These results confirm the relative orientations of the cross-linked loops and establish that the left end of the map corresponds to the 5' end of the molecule.

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

BINDING OF 4'-AMINOMETHYL 4,5',8-TRIMETHYL PSORALEN TO DNA, RNA, AND PROTEIN IN HeLa CELLS AND DROSOPHILA CELLS

Sune Frederiksen and John E. Hearst

Biochim. Biophys. Acta, in press.

In Drosophila cells and HeLa cells treated with 4'-aminomethyl trioxsalen and ultraviolet light, this compound binds covalently to DNA and RNA. The maximum number of molecules bound to 10^3 base pairs in DNA is 60 and in RNA it is 20. In nuclei treated likewise the number of molecules bound to 10^3 base pairs in DNA can be as high as 376. When cells are irradiated in the frozen state the number of 4'-aminomethyl trioxsalen molecules bound per 10^3 base pairs in DNA is [illegible] and in RNA about 20.

DNA molecules from cells or nuclei treated with 4'-aminomethyl trioxsalen and ultraviolet light are highly crosslinked and appear as loops interspersed by double-stranded regions when analyzed in the electron microscope under denaturing conditions. The loop sizes are heterogeneous and the fraction of double-stranded regions increases to almost complete double-strandedness at high degrees of reaction.

No secondary structures could be found in ribosomal RNA from Drosophila cells or HeLa cells after treatment with 4'-aminomethyl trioxsalen and ultraviolet light.

In cells treated with 4'-aminomethyl trioxsalen and ultraviolet light the RNAase activity is increased considerably, suggesting a release of lysosomal enzymes.

4'-aminomethyl trioxsalen and its photodecomposition products bind strongly to cellular proteins.

A TECHNIQUE FOR RELATING LONG-RANGE BASE PAIRING ON SINGLE-STRANDED DNA AND EUKARYOTIC RNA PROCESSING

Che-Kun James Shen and John E. Hearst

Anal. Biochem. [illegible], [illegible] (19[illegible]).

Our previous results have shown that after photochemical crosslinking with psoralens in solutions of different concentrations of NaCl, short (100-300 base pairs) hairpins can be stabilized on denatured simian virus 40 (SV40) DNA for electron microscope visualization. Six major hairpins were mapped. This study has been extended by crosslinking single-stranded SV40 [illegible] molecules, EcoRI-SV40 and BglI-SV40, in the [illegible]ence of magnesium ions. After the reaction, [illegible] addition to the hairpins detected before, sever[illegible] DNA hairpins, and hairpin loops with sizes fr[illegible] 300 to nearly 2000 nucleotides are observed. These loops are located at specific regions of the SV40 genome; at the EcoRI map positions (0.1[illegible] 0.02)/(0.26 + 0.03), (0.3[illegible] + 0.03)/(0.39 + 0.[illegible]), (0.51 + 0.03)/(0.57 + 0.0[illegible]), (0.72 + 0.03)/(0.[illegible] + 0.04), (0.[illegible] + 0.03)/(0.[illegible] + 0.0[illegible]), (0.77 + 0.[illegible])/(0.94 + 0.[illegible]) and at (0.[illegible] + 0.04)/(0.69 + 0.[illegible]). At least three of these loops are found in regions thought to be involved in splicing of the early and late SV40 transcripts: (0.51 + 0.03)/(0.57 [illegible].03), (0.72 + 0.03)/(0.78 + 0.04), (0.77 + 0.[illegible] (0.94 + 0.03). These loop structures are most likely to arise from base pairing between distant regi[illegible] of the single-stranded DNA. RNA trans-[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible] from [illegible] fragment [illegible] complementary [illegible] near the [illegible] end of [illegible] rRNA has been used to prime reverse transcriptase [illegible] virus RNA-directed DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7). In addition to transcripts that were extended to the 5' end of the RNA, two major transcription intermediates were observed. These discrete-sized cDNA intermediates are the result of a kinetic barrier imposed by monomethylation of the amino group on guanine that participates in base-pairing. Both major transcription intermediates correspond to attenuation at the known positions of N-methylguanine ([illegible]) in the rRNA sequence. The relaxation time for elongation of the cDNA through [illegible] is approximately 3 min. No other major kinetic pauses were observed in the 1340 bases transcribed.

MOLECULAR BIOLOGY OF NERVE CELL FUNCTION: RESPONSE OF THE NERVOUS SYSTEM TO ENVIRONMENT AND CHEMICAL STIMULI

Edward L. Bennett

[illegible] and other animals [illegible] with their environment, and with changes in the environment, depends to a large extent upon [illegible] of these abilities and of their biological [illegible] is therefore important in attempting to [illegible] the impact of the environment on the [illegible] of species of interest. A common theme of [illegible] research projects is the neural bases [illegible] memory formation, and their relations to [illegible] factors.

.. [illegible] of receptors for neurotransmitters

Receptors play a key role in many biological [illegible] processes. Neurotransmitters react with the appropriate receptor at the synapse with a resultant change in ionic permeability. Knowledge of the interaction of neurotransmitters with receptors is essential for us to understand changes of synaptic function associated with neuronal damage from environmental factors. Changes of synaptic function may be one mechanism of plasticity important for long-term memory formation. The objective of this research project is to elucidate fundamental properties of one or more synaptic receptors with the ultimate aim of defining the structure-function relationship of receptors and their associated neurotransmitters. Our aim is to characterize biochemical and biophysical properties of the nicotinic-like acetylcholine receptor (nAChR) from the mammalian central nervous system.

A number of factors have contributed to our focus on the acetylcholine receptor. These include the key role that the acetylcholine system plays in the central nervous system, and the availability of the unique ligands derived from snake venoms which bind to the nicotinic acetylcholine receptor with high specificity. Our strategy is to compare the interaction of selected toxins derived from several venomous snakes with the putative acetylcholine receptor from mammalian brain and other sources. The α-toxins of interest are purified by chromatographic methods from the crude venoms and radiolabeled. The influence of selected agonists, antagonists, and affinity reagents for the acetylcholine receptor on toxin binding is determined using receptor from rat brain and electric ray, torpedo californica, electroplax. [illegible] their methods, similarities and differences between the receptors from different sources [illegible].

.. Neurochemical Steps of Memory Formation.

Knowledge of the steps involved in memory formation is essential in order to predict, evaluate, and overcome damage from environmental factors to higher brain function. Alternative theories of memory formation are being tested with emphasis on a basic understanding of the respective roles that neurotransmitters and neuromodulators, macromolecules (such as RNA, protein, and carbohydrates) and axonal transport play in memory formation. Our studies have focused on long-term memory formation. Using anisomycin, an effective inhibitor of protein synthesis, and a variety of experimental paradigms, results are consistent with the interpretation that protein synthesis during the period immediately following training is essential for long-term memory. Numerous drugs, acting by different mechanisms, can modulate the formation of long-term memory at the same time that the necessary synthesis of protein occurs. Of special significance is the observation that colchicine, in combination with anisomycin, is an effective amnestic agent. The interpretation is that the synthesis and subsequent transport of proteins from the nucleus to the axonal and dendritic endings is necessary for long-term memory formation. However, it has been objected that the blood-brain barrier is generally believed to be impermeable to colchicine. Our recent studies have shown that extremely small amounts do appear to penetrate the blood-brain barrier. Future experiments will attempt to determine if these small amounts of colchicine can indeed modify axonal transport. Other experiments are planned to investigate the time parameters of short- and intermediate-term memory formation in rodents.[8-10]

3. Evolution of Brain Plasticity, Environmental Stimulation, and Brain Changes

Measurable biochemical and anatomical changes can be demonstrated in brain as a result of exposure to various degrees of environmental stimulation. This plasticity of brain may be an important factor that is related to the ability of a species to respond to environmental changes including those which are stressful. In a series of experiments, we and others have shown that the brain of laboratory rodents responds to formal training and differential experience by increasing

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This work was supported by the Division of General Life Sciences, Office of Basic Sciences, U.S. Department of Energy, under Contract No. W-7405-ENG-48.

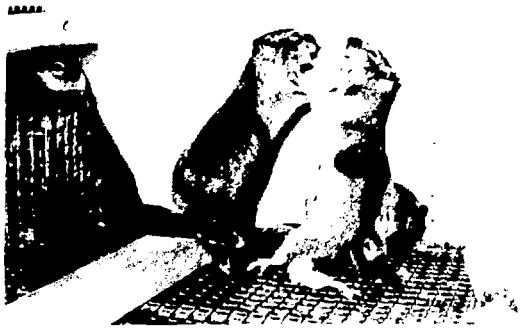

Figure 1. Cerebral plasticity is being studied in the Belding's Ground Squirrel. In the enriched condition, laboratory-raised squirrels exhibit many behaviors seen in the field. (CBB #06-7213)

weight and thickness of the cerebral cortex, the RNA/DNA ratio of cortical tissue, branching of dendrites, numbers of dendrite spines and size and/or density of synaptic contacts.[8,11,12] We have demonstrated that learning is a main cause of cerebral effects. We are now extending these investigations of the cerebral effects of differential environments from the laboratory rodent to several feral species, including the ground squirrel (Figure 1). Preliminary results indicate that juvenile Belding's Ground Squirrels develop differences in brain weights and nucleic acids rather like those previously found in laboratory rats. A related area of study concerns the brain changes that occur during hibernation. Juvenile squirrels that remained awake during the winter gained in both brain and body weight, while those that were placed in a cold room and went into hibernation maintained brain weight and lost body weight.

4. Effects of Brain Damage on Brain Biochemical and Anatomical Measures, on Behavior, and on Recovery of Function

Environmental factors are a frequent cause of brain damage. It is important to understand the long term effect of brain damage on brain composition and function, as well as factors which may improve [illegible] that [illegible] to [illegible] of the [illegible] cells [illegible] 10 not [illegible] A [illegible] cells [illegible] within [illegible] discussed.

[illegible] differences between the [illegible] effects of [illegible] in the [illegible] and those in the [illegible] impairment, [illegible] the radial arms maze, caused by [illegible] in the [illegible] can be partially [illegible] exposure to an enriched environment, whereas the behavioral impairment caused by lesions in the motor or sensorimotor [illegible] not reversed by an enriched environment. This indicates that the ability to modify recovery from brain lesions may be critically dependent on the type and extent of brain damage. Future behavioral and biochemical experiments will employ substantial lesions in selected areas such as hippocampus.

5. Behavioral Effects of Direct and Alternating Current Magnetic Fields

The purpose of these studies is to provide necessary information which will aid in the establishment of safety guidelines for humans by determining the effects of magnetic fields on the behavior of animals. These studies should provide data on a critical issue--can magnetic fields effect spontaneous behavior, brain excitability, or even the processes concerned with long-term memory formation and storage? The general procedure is to train mice on a one-trial step-through passive avoidance task and then, immediately after training, to place the mice in a 15,000 gauss dc magnetic field for one or more days. Subsequently, the mice are tested for memory of the task. In preliminary experiments, no effects of the magnetic field on memory retention have been detected. Future experiments are planned using an ac magnetic field.

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Abstracts

1. EFFECTS OF THIOL MODIFICATION AND CA^{++} ON AGONIST-SPECIFIC STATE TRANSITIONS OF NICOTINIC ACETYLCHOLINE RECEPTOR FROM TORPEDO CALIFORNICA ELECTROPLAX

Jeffrey V. Miller, Ronald J. Lukas and Edward L. Bennett

Life Sciences 24, 1893-1900 (1979)

The agonist binding affinity of nicotinic acetylcholine receptor (nAChR) from Torpedo californica electroplax, as inferred from ability of agonist to inhibit specific curaremimetic neurotoxin binding to nAChR, is sensitive to the duration of exposure to agonist. The concentration of carbachol necessary to prevent one-half of toxin binding over a 30 min incubation with nAChR (K_{30}) is 10 μM when toxin and carbachol are simultaneously added to membrane-bound nAChR, and 3 μM when nAChR are pretreated with carbachol for 30 min prior to the addition of toxin. These alterations in agonist affinity may be mimicked by modification of nAChR thiol groups. Affinity of nAChR for carbachol is decreased following treatment with dithiothreitol (DTT). Dithio-bisnitrobenzoic acid treatment of DTT-reduced membranes yields K_{30} values of 5 μM for carbachol, while N-ethylmaleimide treatment of DTT-reduced nAChR produces nAChR with reduced affinity for carbachol, reflected in K_{30} values of about 400 μM. In the absence of Ca^{++}, K_{30} values for carbachol binding to native and DTT-reduced nAChR are diminished 3-6 fold. These affinity alterations are not observed with d-tubocurarine (antagonist) binding to nAChR. Thus, Ca^{++} and the oxidation state of nAChR thiols appear to affect the affinity of nAChR for agonists (but not antagonists), and may therefore be related to agonist-mediated events in receptor activation and/or desensitization.

2. EFFECTS OF THIO-GROUP MODIFICATION AND CA^{2+} ON AGONIST-SPECIFIC STATE TRANSITIONS OF A CENTRAL NICOTINIC ACETYLCHOLINE RECEPTOR

Ronald J. Lukas, Hiromi Morimoto, and Edward L. Bennett

Biochemistry 18, 2384-2395 (1979).

Agonist-binding affinities of central nervous system nicotinic acetylcholine receptors (nAcChR) are sensitive to the duration of exposure to agonist. These agonist-induced changes in receptor state may be mimicked by appropriate modification of receptor thio groups and/or by manipulation of solvent ionic composition. In the absence of Ca^{2+}, the concentration of acetylcholine (AcCh) necessary to prevent half of specific ^{3}H-labeled α-bungarotoxin binding is ~1 mM for nAcChR treated with dithiothreitol (DDT) or DDT-N-ethylmaleimide (low-affinity states) and ~40 μM for nAcChR treated with DTT-5,5'-dithiobis (2-nitrobenzoic acid) or for native nAcChR pre-

treated with ACh [illegible] states. [illegible] tion of Ca^{2+} results in an increase in the effectiveness of ACh [illegible] ing. None of these treatment, after [illegible] antagonist binding [illegible] ences in [illegible] Agonists competitively [illegible] low-affinity states, but [illegible] is observed for binding to high-affinity [illegible] Values of ACh [illegible] from these data [illegible] determined physiologically [illegible] systems. [illegible] of brain [illegible] physiologically [illegible] state during [illegible]

3. AGONIST-INDUCED [illegible] NICOTINIC ACETYLCHOLINE RECEPTOR [illegible]

Ronald J. [illegible] and Edward L. Bennett

J. Neurochemistry 33, [illegible] (1979)

The [illegible] the specific interaction [illegible] with membrane-bound sites. [illegible] is enhanced when membranes are pretreated with agonist. Thus, pretreatment of [illegible] with agonist but not antagonist [illegible] formation of sites to a high-affinity, [illegible] agonist. This change in receptor state [illegible] with a half-time on the order of minutes, and is fully reversible on dilution of agonist. The results are consistent with the existence of α-Bgt binding sites in the central nicotinic acetylcholine receptors. Furthermore, this agonist-induced alteration in receptor state may represent an in vitro correlate of physiological desensitization. As determined from the effects of agonist on toxin binding isotherms, and on the rate of toxin binding to specific sites, agonist inhibition of toxin binding to the high-affinity state is non-competitive. This result suggests that there may exist [illegible] toxin-binding and agonist-binding sites on central toxin receptors.

4. INTERACTION OF NICOTINIC RECEPTOR AFFINITY REAGENTS WITH CENTRAL NERVOUS SYSTEM α-BUNGAROTOXIN BINDING SITES

Ronald J. Lukas and Edward L. Bennett

Molecular Pharmacology (1980), 17, 149-155 (1980)

Membrane-bound α-bungarotoxin-binding entities derived from rat brain are found to interact specifically with the affinity reagents maleimidobenzyltrimethylammonium (MBTA) and bromoacetylcholine (BAC), originally designed to label nicotinic acetylcholine receptors from electroplax and skeletal muscle. Following treatment of membranes with dithiothreitol, all specific toxin binding sites are irreversibly blocked by reaction with MBTA or BAC. Affinity reagent labeling of dithiothreitol-reduced membranes is prevented (toxin binding sites are not blocked) by prior alkylation with N-ethylmaleimide, by prior oxidation with dithio-bis-(2-nitrobenzoic acid), or by incubation [illegible] sulfhydryl modification on agonist binding affinity appear to be attributable to the [illegible] of the covalent modification rather than [illegible] steric alteration at the receptor active site brought about by chemical modification. Protection of receptor site disulfides [illegible] and consequent preservation of receptor low-affinity state after subsequent alkylation with N-ethylmaleimide) is specific [illegible] in the presence of cholinergic agonist. Taken together, the results of sulfhydryl/disulfide modification and reactivity studies are consistent with involvement of α-bungarotoxin binding site sulfhydryls/disulfides in affinity state transitions, which are tentatively correlated with physiological activation and/or desensitization of functional central nervous system nicotinic acetylcholine receptors.

5. EFFECTS OF LOCAL ANESTHETICS ON CHOLINERGIC AGONIST BINDING AFFINITY OF CENTRAL NERVOUS SYSTEM α-BUNGAROTOXIN RECEPTORS

Ronald J. Lukas and Edward L. Bennett

FEBS Letters 108, 356-358 (1979).

It was suggested that if central nervous system α-bungarotoxin receptors are true nicotinic acetylcholine receptors coupled to ion channels, local anesthetics might be expected to cause bio-

chemically measurable increases in α-bungarotoxin receptor affinity for cholinergic agonists. The rate of transformation of α-bungarotoxin receptor to the high-affinity state was found to be accelerated after the toxin receptor had been treated with local anesthetics at low concentration. It was, therefore, concluded that the α-bungarotoxin receptor in rat brain is coupled to an entity that possesses sites for high affinity interaction with local anesthetics, and that local anesthetic occupation of those sites influences α-bungarotoxin affinity for cholinergic agonists. The implication is that the CNS α-bungarotoxin receptor-local anesthetic binding site complex is a physiologically functional ion-conductance regulator, which displays the pharmacological specificity of an acetylcholine receptor-ionophore complex.

7. CONFORMATION OF THE NEUROTOXIN CROTOXIN COMPLEX AND ITS SUBUNITS

Michael R. Hanley

Biochemistry 18, 1681-1688 (1979)

Crotoxin, the neurotoxin from *Crotalus durissus terrificus* venom, and its two subunits, crotoxin A and crotoxin B, have been examined by fluorescence and circular dichroism techniques. Model spectra of crotoxin, generated from the expected contributions of the individual subunits, agreed with experimental results only at pH 2, where crotoxin was fully dissociated. From pH 4 to 10, crotoxin spectra could not be simulated by subunit contributions. Comparison of the results at pH values favoring either association or dissociation indicated that complex formation was accompanied by (1) a 70% decrease in fluorescent emission intensity and a 7-nm blue shift of the emission maximum, (2) an increase in the far-ultraviolet circular dichroism, and (3) a marked intensity enhancement of the near-ultraviolet circular dichroism spectrum.

These observations were taken as evidence that tryptophan residues were masked by complex formation and that the crotoxin complex contained an increased proportion of ordered secondary structure, particularly β sheets. The results did not distinguish between conformational changes with subunit interaction or creation of ordered structure only at the subunit binding interface. Structural flexibility of the subunits was suggested from their ability to undergo reversible state changes by exposure to acid pH or guanidine hydrochloride. Examination of the concentration dependence of guanidine hydrochloride denaturation indicated that complex formation gave increased stability, although the subunits were individually quite resistant to guanidine hydrochloride denaturation. It is concluded that the ability of crotoxin and its subunits to exist in several reversible states is related to the control of the association-dissociation equilibrium of the complex and that conformational interconversion may be necessary for crotoxin to act on different target membranes.

8. BRAIN PLASTICITY, MEMORY, AND AGING

Edward L. Bennett and Mark R. Rosenzweig

Physiology and Cell Biology of Aging, A. Cherkin, C. E. Finch, N. Kharasch, T. Makinodan, F. L. Scott and B. S. Strehler, eds. (Raven Press, New York, 1979), pp. 141-150

It is generally assumed that memory faculties decline with age. A discussion of the relationship of memory and aging and the possibility of retarding the potential decline is hampered by the fact that no satisfactory explanation of memory is available in either molecular or anatomical terms.

This paper reports experiments that indicate that protein synthesis and anatomical changes may be involved in long-term memory storage. Environmental influences can produce quantitative differences in brain anatomy and in behavior. In experimental animals, enriched environments lead to more complex anatomical patterns than do colony or impoverished environments. This raises fundamental questions about the adequacy of the isolated animal, which is frequently being used as a model for research on aging. A more important applied question is the role of social and intellectual stimulation in influencing aging of the human brain.

9. INHIBITION OF CEREBRAL PROTEIN SYNTHESIS: DISSOCIATION OF NONSPECIFIC EFFECTS AND AMNESIC EFFECTS

Hasker P. Davis, M. R. Rosenzweig, Edward L. Bennett, and Larry R. Squire

Behavioral and Neural Biology 28, 99-104 (1980)

Injection of 210 mg/kg of anisomycin 5 hr prior to training produced more nonspecific behavioral side effects at the time of training than did a low dosage (30 mg/kg) given 20 min prior to training. Yet the low dosage 20 min pre-training produced greater protein synthesis inhibition at training and greater impairment of retention of passive avoidance training than did the high dosage 5 hr pre-training. These results demonstrate that the level of protein synthesis inhibition at or near the time of training is the critical factor for inducing amnesia, and not nonspecific side effects of a protein synthesis inhibiting drug. Various alternative hypotheses would also predict greater amnesia after the high dosage of anisomycin given 5 hr prior to training than after the amnestic low dose given 20 min prior to training. Thus, these results provide further support for the hypothesis that brain protein synthesis is required for long-term memory formation.

10. ROLE OF NEUROTRANSMITTERS AND PROTEIN SYNTHESIS IN SHORT- AND LONG-TERM MEMORY

Edward L. Bennett, Mark R. Rosenzweig, and James F. Flood

Proceedings of the Second World Congress of Biological Psychiatry, Barcelona, 1978. In: Biological Psychiatry Today, P. Obiols, C. Ballús, E. Gonzáles Monclús, and J. Pujol, eds. Elsevier/North Holland Biomedical Press, Amsterdam (1979) pp. 211-[illegible]

Anisomycin is an effective inhibitor of cerebral protein synthesis in mice and is also an effective amnestic agent for both passive and active behavioral tasks. From use of anisomycin in combination with a variety of stimulant and depressant drugs, we conclude that the level of arousal following acquisition plays an important role in determining the duration and the rate of the biosynthetic phase of memory formation. While we have interpreted the experiments with anisomycin as evidence for an essential role of protein in memory storage, others have suggested that side effects of inhibitors of protein synthesis on catecholamine metabolism are the main cause of amnesia. Several experiments were therefore done to compare the effects of anisomycin and catecholamine inhibitors on memory. We conclude that anisomycin's principal amnestic mechanism does not involve inhibition of the catecholamine system. The results strengthen our conclusion that protein synthesis is an essential component for long-term memory trace formation. Also, it is suggested that proteins synthesized in the neuronal cell body are used, in conjunction with other molecules, to produce permanent and semi-permanent anatomical changes.

11. MAZE TRAINING ALTERS BRAIN WEIGHTS AND CORTICAL RNA/DNA RATIONS

Edward L. Bennett, Mark R. Rosenzweig, Hiromi Morimoto, and Marie Hebert

Behavioral and Neural Biology 26, 1-22 (1979)

In order to test whether training leads to anatomical and chemical changes in the brain, individual rats were given self-paced trials in mazes, traversing the maze in order to get from a food station to a water station. In 30 days of this training, during which they had no social interaction, the rats developed significant increases in weight and RNA/DNA of standard samples of cerebral cortex, as compared with littermate rats in either of two control conditions: (a) rats confined to small individual cages (N = 70 per condition); (b) rats that traversed the empty maze box with no maze barriers present (N = 29 per condition). Whereas the rats who faced maze problems decreased average transit times through the maze on successive trials, the rats that traversed the empty box showed no regular trend in running times over trials. The cerebral effects of maze experience versus control conditions were similar in pattern but smaller in magnitude than effects of experience in a social group in a multisensory complex environment. This clear evidence of cerebral changes as consequences of maze training adds further support to the indications that similar cerebral changes resulting from enriched experience are due to learning rather than to other factors. The changes that follow training or enriched experience can be linked with other evidence concerning the roles of RNA and of protein synthesis in the formation of long-term memory traces.

12. HOW PLASTIC IS THE NERVOUS SYSTEM?

Mark R. Rosenzweig and Edward L. Bennett

A Comprehensive Handbook of Behavioral Medicine, Vol. 2, N. Taylor and J. Ferguson, eds. (Spectrum Publishers, 1980), in press

This review is intended to be partly speculative. Besides reviewing research, extrapolations are made. Some of these extrapolations are suggestions for further research and others predict possible applications of current and future research.

Most of the material in this review comes from research with animal subjects rather than from observations on human beings. Most investigators of neural mechanisms take the use of animal models largely for granted. Much justification, both practical and theoretical, can be supplied for extrapolating from animals to human beings where brain-behavior relations are concerned, but it is well to remember that not all such extrapolations are found to work out.

Some of the main topics and questions considered are these:

a) To what extent are neural connections predetermined by neredity and to what extent can they be altered by postnatal determinants? Effects of several factors are considered, including damage to other parts of the system, sensory deprivation or distorted sensory input, and training and experience.

b) Can new connections be formed in the adult mammalian nervous system? It has been shown in some brain regions that when one input tract to the region is removed, the endings from other inputs then sprout and occupy the vacated synaptic sites. Changes in neural connections have also been demonstrated when animals are given enriched experience or formal training.

(c) Does recovery of function after brain damage involve formation of new compensatory connections or only learning ways of using residual capacities to make up for the loss?

Although our knowledge of the forms, extent, and limitations of plasticity is still far from complete, we can envisage possible applications to many conditions and problems of individual and social importance. Among these are the following: (a) Enhancing intellectual ability. (b) Preventing or alleviating some kinds of mental retardation and some kinds of learning disabilities. (c) Alleviating or delaying senile decline in intellectual abilities. (d) Promoting recovery of function after damage to the nervous system. Much can be accomplished along these lines by behavioral techniques, in some cases alone, and in other cases in conjunction with physiological techniques.

RADIATION CHEMISTRY AND CHEMICAL EVOLUTION

Richard M. Lemmon

[illegible] interest at the laboratory [illegible] biologically important compound choline [illegible], which, in one crystalline form [illegible] decomposed with extremely high efficiency [illegible] radiation. In a second crystalline form [illegible] shows only a very little behavior toward [illegible] radiation. During the past year we have shown that the [illegible]'s radiation sensitivity increases rapidly above 50°, but reaches a maximum around 65°, well below the temperature 78°C of the [illegible] crystal phase transition.[1] We are also using the techniques of solid-state NMR, [illegible] ^{2}H, ^{13}C, and ^{15}N labeled cholines to determine the bond lengths of a number of choline analogs, including those of choline chloride, choline bromide, and choline iodide. This work is supplementing past and present x-ray crystallographic studies designed to uncover the unique facet of choline chloride crystals that result in its remarkable radiolysis behavior.

Research on chemical evolution is increasing our understanding of the chemical events that took place on the prebiotic Earth, about 3.5 to 4.5 billion years ago, events that ultimately led to the appearance of the first living cells. Most scientists regard the appearance of life as an inevitable result of the intrinsic physical and chemical properties of matter. Recent laboratory experiments that simulate presumed prebiotic Earth [illegible] conditions such as [illegible] have been assembled [illegible] "primordial soup" [illegible] temperatures [illegible] years.

During 1979, in collaboration with members of the Chemistry Department at Stanford University, we pursued research into the phenomena of racemization, [illegible] the racemization of such chiral compounds as isoleucine, a amino acids, under the effects of gamma rays.[2] This research is important for interpretations of the degree of optical rotatory power of amino acids that are found in meteorites. Since the degree of amino acid racemization in fossils is often used to measure the fossils' ages, this work also has implications for geochronology.[3]

In recent years, collaboration with members of the LBL Physics Division has contributed to our understanding of mechanisms which, on the prebiological Earth, may have contributed to a selective advantage of L-amino acids over D-amino acids.[5] That collaboration has continued during 1979 by preliminary experiments designed to show whether highly polarized, accelerated protons exhibit any selectivity in their reactions with L- or D-amino acids.

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*See Abstracts.

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Abstracts

1. [illegible] AND RADIATION SENSITIVITY OF [illegible] CHLORIDE, BROMIDE AND IODIDE

Vasilios Petrovlos, Amar Nath, and Richard M. Lemmon

Rad. Phys. Chem., in press, 1980

The organic salt choline chloride exists in two crystalline polymorphs. One (α-form), which is present at ambient temperature, is extraordinarily sensitive to ionizing radiation. The other (β-form), which exists above 75°C, is radiation stable. The present work shows that radiation sensitivity increases rapidly above 75°C, but reaches a maximum around 65°, well below the α- to β-crystal phase transition. This behavior is correlated with the onset of various rotational motions that are indicated by recent NMR studies. We have also found that H atom irradiations of the β-form, and of the radiation stable choline iodide, produce as much decomposition as in the α-form, a result that is in accord with previously published theories of the mechanism of the α-form's unique radiolysis.

2. THE RADIORACEMIZATION OF ISOVALINE. COSMOCHEMICAL IMPLICATIONS

William A. Bonner, Neal E. Blair, and Richard M. Lemmon

J. Amer. Chem. Soc. 101, 1049 (1979)

The optically pure D- and L-enantiomers of isovaline (1), which cannot be racemized by ordinary chemical mechanisms involving α-hydrogen removal, and which has been isolated in apparently racemic form from the Murchison meteorite, have been subjected to partial radiolysis by the ionizing radiation from a 3000 Ci ^{60}Co γ-ray source. Both in the anhydrous and hydrated solid states and as solid sodium or hydrochloride salts each enantiomer suffered significant radioracemization of the undestroyed residue during its partial radiolysis. The sodium salt of isovaline in 0.1 M aqueous solution suffered extensive radiolysis with relatively small radiation doses, but showed no detectable radioracemization. The significance of these observations with respect to the primordial enantiomeric [illegible] isovaline and other amino [illegible] of meteorites is discussed.

3. RADIORACEMIZATION OF AMINO ACIDS

R. M. Lemmon and W. A. Bonner

Origins of Optical Activity in Nature, D. C. Walker, ed. (Elsevier, Amsterdam, 1979), pp. 47-5[illegible]

We have shown that optically active amino acids, both as solids and in solution, are racemized when exposed to γ radiation. Quantitative values for this radioracemization as a function of ionizing-radiation dosage have been obtained. These values cast additional doubt on the validity of the Vester-Ulbricht hypothesis for the origin of optical selectivity in biomolecules. It was also found that the radiation doses to which meteoritic material has been exposed in outer space is enough to cause appreciable racemization of the amino acids. These findings render less secure previous conclusions concerning the presumed abiogenic origin of meteoritic amino acids.

4. THE RADIORACEMIZATION OF AMINO ACIDS BY IONIZING RADIATION: GEOCHEMICAL AND COSMOCHEMICAL IMPLICATIONS

William A. Bonner, Neal E. Blair, and Richard M. Lemmon

Origins of Life 9, 279 (1979)

A number of optically active amino acids, both in the solid state and as sodium or hydrochloride salts in aqueous solution, have been exposed to ionizing radiation from a 3000 Ci ^{60}Co γ ray source to see if radioracemization might accompany their well-known radiolysis. γ-Ray doses causing 55-68% radiolysis of solid amino acids typically engendered 2-5% racemization, while aqueous solutions of the sodium salts of amino acids which underwent 53-66% radiolysis showed 5-11% racemization. Amino acid hydrochloride salts in aqueous solution, on the other hand, showed little or no radioracemization accompanying their radiolysis. Both radiolysis and radioracemization were roughly proportional to γ-ray dose in the

range studied (1-36 x 10^6 rads). Mechanisms for the radioracemization of amino acids in the solid state and as aqueous sodium salts are discussed, and the absence of radioracemization for aqueous hydrochloride salts is rationalized. Isovaline, a non-protein amino acid which has been isolated from the Murchison meteorite, contains no α-hydrogen atom and is therefore incapable of racemization via the chemical mechanisms by which ordinary amino acids racemize. Nevertheless, isovaline pattern radioracemization in the solid state to an extent comparable to that shown by ordinary amino acids, as do its sodium and hydrochloride salts in the solid state. The sodium salt of isovaline in aqueous solution, however, fails to racemize during its radiolysis. Several implications of the newly described phenomena of radioracemization are pointed out in the fields of geochemistry and cosmochemistry.

TRITIUM LABELING FOR BIO-MED RESEARCH

Richard M. Lemmon

A very large fraction of what we know about the chemical pathways in the living cell has resulted from the use of radioactively-labeled tracer compounds; the use of tritium-labeled compounds has been particularly important. As research in biochemistry and biology has progressed the need has arisen to label compounds of higher specific activity and of increasing molecular complexity--for example, oligonucleotides, polypeptides, hormones, enzymes. Our laboratory, in concert with the LBL Department of Environmental Health and Safety, has gradually developed special facilities for handling tritium at the kilocurie level. These facilities have already proven extremely valuable in producing labeled compounds that are not available from commercial sources. The principal ways employed for compound labeling are (1) microwave discharge labeling, (2) catalytic tritio-hydrogenation, (3) catalytic exchange with T_2O, and (4) replacement of halogen atoms by T.

Along with the development of the tritium-labeling facility has come a need to better understand the mechanisms by which tritium is incorporated into organic compounds. A better understanding will mean that we can (a) maximize specific activities, (b) minimize radiation induced destruction, and (c) control the sites in the molecule that become labeled.

The microwave discharge method (see Figure 1) is particularly promising for the introduction of tritium into complex molecules, and it has been successfully used to label enzymes, for example, insulin. The essential feature of the method is to pass T through the cavity of a microwave generator where the tritium molecules are partially decomposed into tritium atoms. These are swept past the organic target, which is maintained at -196°C. After an exposure time of about five minutes, the target is removed, warmed to ambient temperatures, and dissolved in an appropriate solvent for subsequent purification and radioactivity counting procedures.

Although the procedure is simple and leads to high specific radioactivity (Ci/mmole), there are problems, and these are under investigation. For example, we have found that exposure of styrene to T atoms leads to labeled styrene, but it also produces polymerization, vinyl saturation, and ring hydrogenation.[1] Other compounds studied as models for T atom effects were n-hexane, heptene-2, benzene, 3-methylhexane, and methylcyclohexane.[1] Studies have also been carried out on tritiation by the replacement of halogen atoms with T atoms. These results indicate that carrier-free tritium-labeled products, including biomacromolecules, can be produced in this way.[2]

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*2. C. T. Peng, W. J. Chiu, B. E. Gordon, W. R. Erwin, and R. M. Lemmon, Dehalogenation and Ring Saturation by Tritium Atoms, Abstract, American Chemical Society's 177th National Meeting, Honolulu, Hawaii, April 1-6, 1979.

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*See Abstracts.

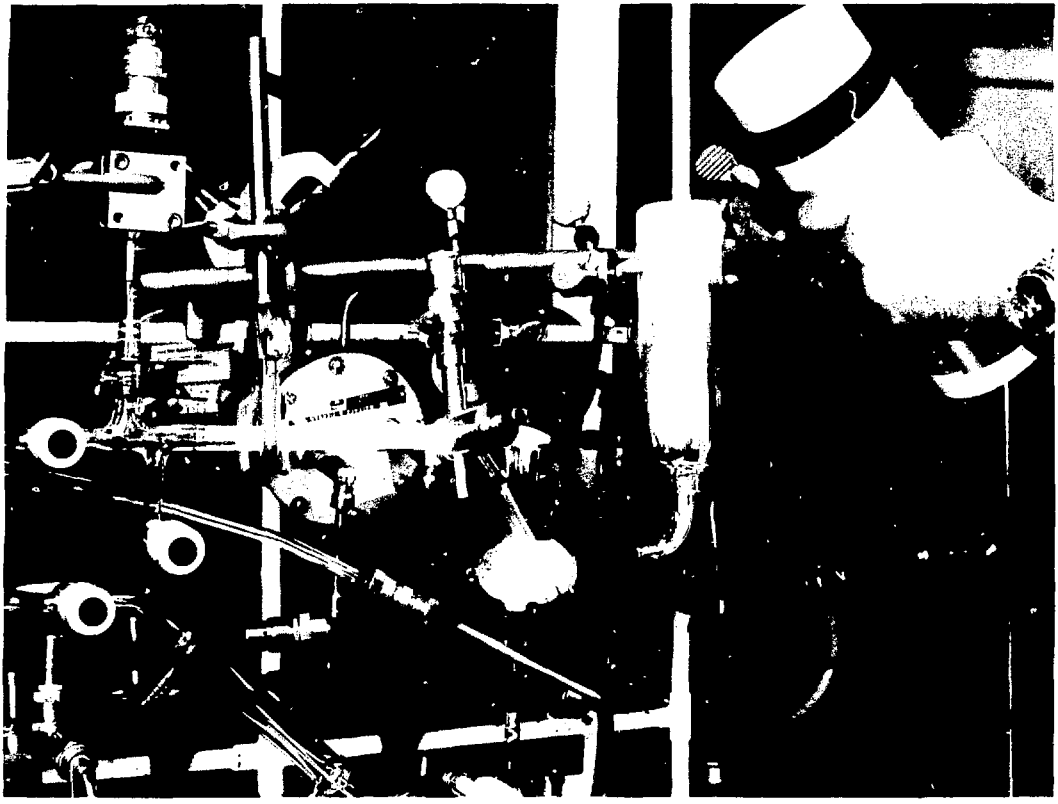

Figure 1. The microwave discharge apparatus. The radiofrequency generator and the glow of the excited T_2 and T atoms are seen in the center. The sample being labeled is held in the liquid nitrogen-cooled receptacle below and to the right of the rf generator. T_2 is admitted from the left through the line with the attached vacuum gauge (upper left). The circulating pump for the T_2 is out of view at the bottom. (CBB 758-6479)

Abstracts

1. EXCITATION LABELING WITH TRITIUM--STUDIES OF SIDE REACTIONS WITH MODEL COMPOUNDS

B. E. Gordon, W. R. Erwin, and R. M. Lemmon

ACS National Meeting, Honolulu, Hawaii, April 1-6, 1979

Labeling organic and bio-organic molecules with tritium atoms produced from T_2 in a radio-frequency plasma is a potentially useful technique. Early work in this field has demonstrated that side reactions generate undesirable by-products of extremely high specific activity. Removal of these, while possible with small molecules, poses serious problems with the large molecules encountered in biochemical studies. Therefore we have begun to study the side reactions associated with this type of tritiation in order to obtain necessary information to ascertain

the suitability of the method for labeling specific molecules and also to gain information for devising purification methods. Samples were exposed at -196°C for five minutes to T atoms generated in a microwave plasma at 5 Torr T_2 and 18-20 watts. Model compounds included n-hexane, heptene-2, benzene, 3-methyl hexane, methyl cyclohexane, and styrene. After the removal of exchangeable tritium, the samples were analyzed by gas liquid radiochromatography. Under these conditions, n-hexane yielded no labeled products; heptane-2 and benzene yielded saturated tritiated heptane and cyclohexane, respectively, and 3-methyl hexane and methylcyclohexane yielded the labeled substrates. Labeling appeared to be selective with tritium incorporation heavily favored by the presence of methyne hydrogen. Parent compound labeling, except for tertiary hydrogens, is a minor reaction. Labeling is not limited to tritium atoms from the plasma alone but may also be caused by long-lived radicals. The latter can attack susceptible solvents used to dissolve the labeled products.

2. DEHALOGENATION AND RING SATURATION BY TRITIUM ATOMS

C. T. Peng, W. H. Chiu, B. E. Gordon, W. R. Erwin, and R. M. Lemmon

ACS National Meeting, Honolulu, Hawaii, April 1-6, 1979

Iodohexane, iodocyclohexane, fluorobenzene, iodobenzene, m-bromotoluene, m-iodotoluene and other compounds were briefly treated with tritium atoms, generated by microwave discharge activation of tritium gas. The purified samples were analyzed by radiogas chromatography on Carbowax and SE-30 columns using temperature programming. Radioactivity was measured by proportional counting of the column effluent and also by liquid scintillation counting of the collected fractions. ^{3}H-labeled hexane and cyclohexane were formed exclusively from iodohexane and iodocyclohexane, respectively. Labeled benzene was formed from fluorobenzene and iodobenzene, and labeled toluene from m-iodotoluene. Also formed were labeled cyclohexane from iodobenzene and labeled methylcyclohexane from m-bromotoluene and m-iodotoluene. Dehalogenation and ring saturation by tritium atoms were apparently efficient under these conditions, but small amounts of labeled halogenated compounds were also formed. The presence of PdO_2, Pd(10%) charcoal, iodine and n-hexane in iodobenzene during reaction altered the extent of tritium incorporation and the activity distribution between the labeled products (cyclohexane/benzene). These results indicate a mechanism by which carrier-free ^{3}H-labeled products, including biomacromolecules, can be produced.

CHEMICAL CARCINOGENESIS AND THE PHYSIOLOGICAL STATE OF THE CELL

James C. Bartholomew

Cancer is a statistical disease. The probability of getting cancer after having been exposed to a carcinogen is not one. The popular press is filled with stories of people who have smoked all their lives and still do not show a sign of lung cancer. But if we analyze the population as a whole, we know that smoking increases the likelihood that an individual will get cancer. These simple observations tell us that carcinogenesis is a complex process that is probably made up of many steps.

It has been estimated that approximately 90% of all cancers are due to both natural and man-made chemicals. In experimental animals the involvement of chemicals in cancer has been well established. In certain human cases, epidemiological techniques have linked chemicals to specific forms of cancer. Although exposure to certain chemicals is likely to increase an individual's risk of getting cancer, it is clear that different individuals have different susceptibilities to these chemical agents. Even though the exact mechanism by which chemicals cause cancer is not known, a great deal has been learned in the last few years to allow us to speculate on some of the elements that play a role in determining susceptibility to cancer-causing chemicals.

1. Modulation of the Metabolism of Polycyclic Aromatic Hydrocarbons

Many carcinogens must be metabolized before they are active. In most cases this metabolism takes place by an inducible enzyme system called aryl hydrocarbon monooxygenase (AHM). For polycyclic aromatic hydrocarbons (PAH) like benzo(a)pyrene (BAP) there is evidence that the important metabolism for carcinogenesis is the formation of epoxides. The epoxide of BAP that is most active in mutagenesis and may be the ultimate carcinogenic form is r-7,t-8- dihydroxy-t-9, 10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BAP diol-epoxide I). The formation of BAP diolepoxide I from BAP involves the conversion to the (-)-t-7, 8-dihydroxy-7,8-dihydrobenzo(a)pyrene via the enzymatic action of AHM and epoxide hydrase(EH) followed by a second epoxidation in the 9,10-position to give the BAP diol-epoxide. Presumably, it is principally this diol-epoxide that reacts with cellular macromolecules in the initiation step of carcinogenesis by BAP.

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Many other cellular reactions involve detoxification of epoxides of PAH. The major detoxification reactions involve conjugation of the epoxides with glutathione catalyzed by glutathione S-epoxide transferase, formation of conjugates of glucuronic acid, and sulfation of the phenols. The importance of these reactions to chemical carcinogenesis is pointed out by the observation that in tissues with a low sensitivity to carcinogenesis by PAH the efficiency of these detoxification reactions is considerably higher than in tissues with a high sensitivity.

Studies have shown that the ability of cells to metabolize PAH is influenced by genetic factors, and the locus determining the inducibility of AHM in mice has been identified. A positive association of this locus with the binding of BAP to DNA and the susceptibility of mice to chemical carcinogenesis has been shown. In humans, the involvement of genetic factors in AHM induction and chemical carcinogenesis has not been clearly established. AHM has been studied in monocytes from monozygotic and dizygotic twins, and considerable variation was found even when cells were harvested from the same individual at different times. Other studies with human lymphocytes in culture have also shown that great individual variability in the metabolism of BAP exists and that persons with lung cancer can more actively metabolize the carcinogen than persons without lung cancer.

Nutrition has also been implicated as a determining factor in the response of cells to chemical carcinogens. Current interest in the role of nutrition in carcinogenesis comes from an analysis of epidemiological data on cancer incidence in human populations and the increasing indications that diet may play a role in human carcinogenesis. Differences in the incidence of cancers among different countries, among population groups within each country, and between migrant and native populations point to environmental factors as explaining such variances. Evidence suggests that nutrition, as used in the broad sense, affects the incidence of a large portion of human cancers, perhaps as much as 50% of all cancers in women and one third of all cancers in men.

Epidemological data on human populations in different countries show a strong positive correlation between dietary fat intake and mortality from breast cancer and leukemia. Similar positive correlations have been found between fat intake and mortality from cancer at such other sites in the body as the prostate, intestine, ovary, skin, and pancreas. Animal data suggest that the effect of lipid diets in enhancing chemical carcino-

genesis may be related to the metabolic activation of carcinogen in the target tissue. However, the mechanism of the lipid effect remains unknown. Experiments indicate that the fatty acid composition of the phospholipids may regulate the structure of the endoplasmic membrane by influencing hydrophobic bonds between membrane proteins and phospholipids, and this may in turn effect the activation of chemical carcinogens.

The effects of dietary lipid variation on monooxygenase activity and induction has been investigated in our laboratory. Phase transition temperature measurements for paranitroanisole(PNA) O-demethylase and BAP hydroxylase indicate that the phase transition temperatures (and membrane fatty acid composition) are altered by dietary lipid changes. In addition, results indicate that particular fatty acids may be necessary for induction of maximal activity of particular enzymes in the monooxygenase, since phenobarbitol-induced PNA O-demethylase activity was lower in diet-altered microsomes than in controls. Thus, changes in dietary lipid composition are capable of causing changes in carcinogen metabolism and the ratio of activation/deactivation products.

Our experiments with the effect of lipids on the metabolism of BAP in C3H 10 T 1/2 mouse embryo fibroblasts has shown that the culture conditions of the cells can affect the quantity and kinds of metabolic products produced. The cells have been cultured in Minimal Essential Medium (MEM) and Basal Medium Eagles (BME) supplemented with either fetal calf serum (FCS) or delipidated fetal calf serum (DFCS). Conditions for equal growth have been established for use in metabolism studies. BAP metabolism has been measured by fluorescence emission spectra and radiometric analysis of tritium labeled metabolites separated by HPLC. Fluorescence emission data indicate that BAP is metabolized to different products in cells cultured with MEM and DFCS compared to cells cultured in MEM and FCS. Fluorescence data also indicate that BAP diol-epoxide is formed when cells are cultured in MEM, while there is no evidence of BAP diol-epoxide formation when cells are cultured in BME. Radiometric analysis of individual metabolites from cells cultured in BME indicated that cells cultured in BME and FCS produced more water-soluble metabolites than cells cultured in BME and DFCS. Cells cultured with FCS also exhibited more overall metabolism of BAP than cells cultured with DFCS. In conclusion, both serum composition and media selection have been shown to effect BPA metabolism.

2. Chromatin Structure and the Distribution of Carcinogen-DNA Adducts

Once activated derivatives of PAH are formed in the cell, they must presumably react with encoded information involved with growth control to exert their carcinogenic effect. The information necessary for the regulation of growth of eucaryotic cells is contained within the genome. Presumably, carcinogens react with this DNA coded information to alter its content or its expression. According to this scheme, the availability of this critical locus or loci for reaction with the activated carcinogen would, in part, determine the susceptibility of cells to carcinogenesis.

In eucaryotic cells DNA is complexed with histones and nonhistone proteins to form chromatin. In recent years, [illegible] have been made into [illegible] chromatin structure. The first [illegible] is a fraction of the genome [illegible] and [illegible] are not [illegible] base pairs of DNA. These structures allow for [illegible] separability [illegible] of DNA at various sites. The bridge DNA is associated with [illegible] which in turn may be further associated to give a [illegible] structure to the nucleosomal arrays. This higher order coiling of nucleosomes results in the bridge DNA being forced to the outside of the array and may account for some of the relatively higher sensitivity of this DNA to endonucleolytic cleavage. Digestion of chromatin with nucleases has been one of the primary tools for probing chromatin structure. These enzymes have not only been useful in dissecting nucleosomal arrays, but have identified regions of the chromatin actively involved in transcription. Deoxyribonuclease I has increased sensitivity to transcription related DNA. Staphylococcal nuclease lacks specificity for transcribing DNA, but digests bridge DNA at a faster rate than nucleosomal DNA. The differential sensitivity of deoxyribonuclease I to chromatin appears to involve the increased acetylation of histones. Histone acetylation increases when the chromatin is involved with transcription, and therefore, may explain why deoxyribonuclease I preferentially digests transcribing DNA. Acetylation does not appear to alter the rate or extent of digestion of chromatin by staphylococcal nuclease; however, acetylated nucleosomes are released from chromatin at a faster rate than non-acetylated nucleosomes. Acetylation, therefore, seems to alter the availability of sections of chromatin to enzymes like nucleases and RNA polymerase, and may also affect the binding of small molecules like carcinogens.

The differential sensitivity of endonucleases to sections of the DNA in chromatin have been used to probe the distribution of carcinogen nucleotide adducts along the DNA. The rationale behind this approach is that if more carcinogen is located in the transcribing genes, then the rate of release of carcinogen from the DNA by deoxyribonuclease I would parallel the digestion rate of transcribing DNA, and that the proportion of carcinogen released would be greater than the proportion of the DNA digested. If the carcinogen is concentrated in the bridge DNA, then staphylococcal nuclease would release the carcinogen as rapidly as bridge DNA is digested. A number of investigators have used this approach; and in general, the evidence supports a larger fraction of the carcinogen binding to bridge DNA. In one case, it has been reported that the distribution of BAP diol-epoxide in the chromatin of human lung cells did show a predominance in the bridge DNA when cells were treated with BAP diol-epoxide, but when these cells were exposed to BAP and allowed to metabolize the compound the resulting DNA adducts were randomly distributed throughout the DNA. This random distribution was attributed to the mobility of the histones during the long exposure to BAP.

The non-random distribution of carcinogen adducts within the genome suggest the following concepts:

1. DNA actively involved with transcription and presumably replication is more available for carcinogen binding than quiescent DNA.

2. Bridge DNA, which has been proposed as the site where DNA-rich fragments begin and possibly also as the binding site for regulatory proteins, has a higher binding efficiency than nucleosomal DNA.

3. Elements of the genome that are involved with regulation (reiterated sequences) bind more carcinogens than other sections of the genome.

In general, the regulatory elements of the genome are more likely to be altered by carcinogens than non-regulatory elements and may indicate why carcinogenesis by chemicals is more efficient than mutagenesis. It also suggests that as the structural elements of the chromatin are altered during changes in the physiological state of the cell, the susceptibility of a given locus to carcinogen-induced damage will also be altered.

We have been developing the simian virus 40 (SV40) "minichromosome" system for studying the effect of chromatin structure on carcinogen binding to DNA. The DNA of SV40 is associated with histones in much the same way as in cellular chromatin; however, the histones of SV40 are more acetylated than cellular histones. Replication of SV40 utilizes cellular enzymes and presumably proceeds in a manner reflective of cellular DNA synthesis. The utility of SV40 in studying the effect of chromatin structure on carcinogen binding comes from the ability to make changes in the chromatin composition and monitor the alterations in structural organization by sedimentation analysis or electron microscopy. Extraction of H_1 converts the chromatin into a more extended form. Similar conversions were observed at extremely low ionic strength in chromatin containing H_1 and in chromatin involved with transcription.

SV40 minichromosomes were reacted with low levels of BAP diol-epoxide to give less than 5 DNA adducts/minichromosome. This level of adducts did not detectably alter the structure of the minichromosomes, but high levels of modification did lead to extensive fragmentation. Relative to naked SV40 DNA, BAP diol-epoxide induced alkylation was reduced and strand scission of minichromosomal DNA enhanced by factors of 1.5 and 2.0, respectively. The reduction in covalent binding was attributed to the presence of histones, which competed with DNA for the hydrocarbon and reduced the probability of BAP diol-epoxide intercalation by tightening the helix. The enhancement of strand scission was probably due to the catalytic effect of histones on the rate of β-elimination at apurinic sites, although an altered adduct profile or the presence of a repair endonuclease were not excluded. Staphylococcal nuclease digestion indicated that BAP diol-epoxide randomly alkylated the minichromosomal DNA. This is in contrast to studies with cellular chromatin where internucleosomal DNA was preferentially modified. Differences in the minichromosomal protein complement were responsible for this altered susceptibility.

4. Replication of Damaged DNA in Mammalian Cells and Model Systems

In many cases chemicals that initiate carcinogenesis have been shown to bind to DNA. These compounds inhibit DNA synthesis with an effectiveness that correlates with their carcinogenicity. While the involvement of DNA synthesis inhibition in carcinogenesis has not been shown, it is reasonable to assume that errors made in the processing of damaged DNA lead to deleterious effects on cellular functions, some of which may lead to cancer. Understanding how cells replicate damaged DNA may help to understanding carcinogenesis by chemicals, and certainly will aid in understanding basic cellular mechanisms of DNA synthesis.

Replication of eucaryotic DNA takes place in a highly complex fashion. Evidence has been presented that there is both a temporal and spatial organization of DNA synthesis throughout the S phase of the cell cycle. Sections of the genome, termed replicons, are the basic unit of replication in that within a replicon DNA synthesis is initiated, elongated, and terminated. Experiments with DNA fiber autoradiography have indicated that replicons are organized in domains such that each replicon in a domain initiates DNA synthesis at approximately the same time within S. Recent analysis of the maturation of newly synthesized DNA fragments confirms the domain concept of replicon organization, and shows that the rate-limiting step in DNA synthesis is the maturation of replicons into large molecular weight DNA.

SV40 replication in monkey kidney cells offers an excellent model system to study the alterations in eucaryotic DNA synthesis caused by chemical carcinogens. The replication of SV40 utilizes an enzymatic apparatus supplied by the cell. The SV40 replicon can be considered as an example of a eucaryotic replicon, but probably lacks the constraints imposed by involvement in a domain. Replication involves initiation at a specific locus on the parent molecule followed by bidirectional synthesis until the growing forks approach each other 180° away from the initiation site. Termination of synthesis is rate limiting in SV40 DNA synthesis as in cellular DNA synthesis and requires separation of daughter molecules containing gaps. The replication forms of SV40 DNA have been partially separated by both sucrose density gradient centrifugation and agarose gel electrophoresis. These techniques give resolution of the supercoiled form I DNA from relaxed circular form II DNA and other replication intermediates. The form II DNA is a mixture of molecules containing gaps in the process of terminating normal replication and molecules with random single strand breaks arising from form I DNA during harvesting.

During the past year our studies on the effect of BAP diol-epoxide on the replication of SV40 have centered on characterizing the gap in the form II molecules that accumulate when SV40 replicates in the presence of BAP diol-epoxide. These form II structures do contain BAP diol-epoxide as indicated by the radioactivity detected in the isolated molecules when replication is carried out in the presence of tritiated BAP diol-epoxide. Eco RI restriction of the form II molecules followed by alkaline sedimentation analysis of the restriction fragments shows that the gap is located randomly around the SV40 DNA. Attempts to fill the gap by adding nucleotide triphosphates with reverse transcriptase have failed, indicating that polymerases are blocked from moving across the gap. These experiments are presently being done with polymerase I from E. coli that has some "proofreading" capacity to see if this type of polymerase can incorporate nucleotide triphosphates into the gap. We have been following the fate of these form II structures over long periods of time to see if the cells can repair the lesions and allow the form II structures to be converted into form I molecules. These studies indicate that the form II and the adducts on the form II are stable for at least 16 hr, and that the cells are not repairing the lesions in the DNA.

Another approach we have taken during the past year to study the structure of the SV40 DNA products that accumulate after treatment with BAP diol-epoxide is to produce antibodies to the BAP diol-epoxide. We have obtained antibodies that do precipitate BAP diol-epoxide labeled DNA specifically, and will use these antibodies to map the position of the BAP diol-epoxide on the SV40 DNA relative to the gap. These antibodies will also be useful in isolating all forms of DNA containing BAP diol-epoxide.

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Abstracts

1. ARYL HYDROCARBON HYDROXYLASE INDUCTION IN MOUSE LIVER CELLS--RELATIONSHIP TO POSITION IN THE CELL CYCLE

J. F. Becker and J. C. Bartholomew

Chem. Biol. Interactions, 26, 257-266 (1979)

The inducibility of aryl hydrocarbon hydroxylase (AHH) by benzo[a]pyrene (BAP) has been studied in synchronously grown cultures of mouse liver cells. These cells (NMuLi cl 8) have low basal levels of AHH which can be induced greater than 100-fold by BAP. Cells were synchronized in $G_1(G_0)$ by serum starvation and in S by release

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*See Abstracts.

from serum starvation in combination with excess thymidine. When released from G_1 (G_0) by replating at lower cell density in fresh medium with 20% serum, cells began entering S with a lag of 12 h. Addition of BAP (1 μg/ml) 6 h before serum stimulation, at the time of stimulation, or 7.5 h after stimulation all gave similar induction kinetics: the AHH activity peaked as the cells began entering S regardless of when the BAP was added. Cells blocked in various parts of S by excess thymidine were inducible for AHH activity as efficiently as cells moving through S and into G_2. These results indicate that the inducibility of AHH is greater when cells are actively proliferating and may be a contributing factor to why growing cells are more sensitive to mutagenesis and transformation than quiescent cells.

2. SPECTRA OF CELLS IN FLOW CYTOMETRY USING A VIDICON DETECTOR

Charles G. Wade, Richard H. Rhyne, Jr., William H. Woodruff, David P. Bloch and James C. Bartholomew

J. Histochem. Cytochem., 27, 1049-1052 (1979).

A TV-type vidicon detector was interfaced to a flow cytometer (FCM) to obtain spectra of fluorophores in cells during flow. The normal operations of the FCM are undisturbed. A spectrograph spreads 320 nm of the fluorophore fluorescence emission across the 500 channels of the detector. Spectra of fluorescamine (a surface labeling agent) and of propidium iodide (a nuclear stain) were obtained from Balb 3T3 cells, and the chlorophyll and phycobilin peaks were resolved from flowing blue-green algae in the FCM. Under typical flow conditions, operation of the vidicon in the continuous mode gives for these fluorophores a S/N of several hundred to one in approximately 3 sec. The vidicon was also gated to obtain spectra of single cells and of cells in selected portions of the cell cycle. For example, the spectrum of fluorescamine was obtained from cells in the G_1 phase of the growth cycle by using as a gate trigger the FCM discriminator output derived from the propidium iodide signal.

3. MODULATION OF THE CELL CYCLE OF CULTURED MOUSE LIVER CELLS BY BENZO[a]PYRENE AND ITS DERIVATIVES

James C. Bartholomew, Andrew L. Pearlman, Joseph R. Landolph and Kenneth Straub

Cancer Res. 39, 2538-2543 (1979)

We have used flow cytometry to monitor the alterations in cell cycle distributions caused by chemical carcinogens. Two closely derived mouse liver cell strains growing in culture have been studied with regard to the effect of benzo[a]pyrene and derivatives of benzo[a]pyrene on DNA synthesis. The derivatives tested were (±)-trans-7α,8β-dihydroxy-7,8-dihydrobenzo[a]pyrene; (±)-7α,8β-dihydroxy-9β, 10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (diol-epoxide); and 7,8,9,10-tetrahydroxy-7,8,9, 10-tetrahydrobenzo[a]pyrene. One cell strain used in this study, NMuLi clone 7, is not highly inducible for the enzyme system (aryl hydrocarbon monooxygenase) that converts the parent compound to the derivatives listed above. The second strain, NMuLi clone 1, is highly inducible for aryl hydrocarbon monooxygenase. Correlated with the high level of metabolic activity is an increased sensitivity to the cytotoxicity of the parent compound. However, both strains were equally sensitive to the diol-epoxide. Flow cytometry analysis and measurements of [^{3}H]-thymidine incorporation into DNA showed that the diol-epoxide increased the number of cells involved in DNA synthesis but that the rate of DNA synthesis was greatly reduced. Benzo[a]pyrene and the 7α,8β-dihydroxy-7,8-dihydrobenzo[a]pyrene had this same effect on NMuLi clone 7 but not on NMuLi clone 1. The perturbations in cell cycle progression took place within one cell cycle, and the cells did not traverse a second cycle. Kinetic model studies indicated that the cell cycle perturbations caused by diol-epoxide are consistent, with the rate of traverse of S being slower than other phases of the cell cycle, and that as cells move through S their rate of DNA synthesis decreases.

4. CHEMICAL CARCINOGENESIS AND THE PHYSIOLOGICAL STATE OF THE CELL

James C. Bartholomew, Howard B. Gamper and Hisao A. Yoneda

Molecular Basis of Environmental Toxicity, K. S. Bhatnagar, ed. (Ann Arbor Science Pub., Inc., 1980), pp. 223-328

It is becoming increasingly evident that the biochemistry of the cell plays an important role in defining susceptibility to carcinogenesis by chemicals. Not all species are equally susceptible to a given chemical carcinogen, nor are all tissues from a given organism malignantly transformed with similar efficiencies. In fact, the same cells in different parts of the cell cycle are transformed with different efficiencies. Current concepts of the mechanism(s) of chemical carcinogenesis involve the covalent interaction of activated chemicals with cellular macromolecules, in particular DNA. In some cases, chemicals are metabolically activated by the cells themselves before covalent binding is possible. This review article presents evidence that the cell susceptibility to chemical carcinogenesis is modulated in three ways. First, metabolism of a carcinogen either to detoxification products or to compounds more active in carcinogenesis varies among cell types and among similar cells exposed to different dietary regimes. Second, alterations in chromatin structure can regulate the availability of critical site(s) for binding a carcinogen as well as the repair efficiency along the DNA. Third, damage remaining in the DNA at the time of replication is most detrimental to the cell, and different cell types have different capacities to replicate DNA in the presence of this damage.

5. DNA ALKYLATION AND UNWINDING INDUCED BY BENZO[a]PYRENE DIOL EPOXIDE: MODULATION BY IONIC STRENGTH AND SUPERHELICITY

Howard B. Gamper, Kenneth Straub, Melvin Calvin and James C. Bartholomew

Proc. Natl. Acad. Sci. USA 77, 2000-2004 (1980)

Superhelical and partially relaxed SV40 DNA were reacted in vitro with (+)7R,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BaP diol epoxide). The modified DNA contained N^2 guanine and N^6 adenine hydrocarbon adducts in the ratio 86:14. Superhelical SV40 DNA was approximately 6% more susceptible to modification than partially relaxed viral DNA. Counterions inhibited DNA alkylation by up to 90%, Mg^{2+} being 50-fold more effective than Na^+. The sensitivity of covalent binding to helix stability is consistent with a reaction complex in which BaP diol epoxide is intercalated.

The superhelical density of the modified DNA substrates was determined electrophoretically relative to partially relaxed standards and an unwinding angle for the hydrocarbon adducts was calculated. The angle was dependent upon the superhelicity of the DNA molecule and ranged from 130° to 30°. This data indicates that the modified base pairs are disrupted and, in the presence of torsional strain, act as centers for the further denaturation of up to 8 adjacent base pairs. In the absence of such strain the alkylation sites have an ordered structure with the attached hydrocarbon probably oriented in the minor or major groove of the helix.

ROLE OF G_0-G_1 ARREST IN THE INHIBITION OF TUMOR CELL GROWTH BY INTERFERON

Abla A. Creasey, James C. Bartholomew and Thomas C. Merigan

Proc. Natl. Acad. Sci. USA 77, 1471-1475 (1980)

We report that human leukocyte interferon preparations are capable of influencing the transition of human melanoma cells from the "A" state to the "B" phase. Human melanoma cells that enter a quiescent stage at high cell density are more sensitive to the cytostatic action of interferon than those that continue to proliferate under similar conditions. Cell cycle perturbations caused by interferon in these cells include a decreased transition rate out of G_0-G_1 ("A" state) into S ("B" phase) and a prolongation of S. These findings support the idea that some metabolic event is required for progress through the G_0-G_1 phase of the cell cycle and is susceptible to interferon action.

GENE EXPRESSION AND MALIGNANT TRANSFORMATION

Mina J. Bissell

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[illegible] cells share the same genetic material, the environment of the cell (cellular matrix, other cells, hormonal factors, etc.) must play a critical role in tissue specificity. The focus of our research is to understand the nature of the interaction of the cell with its environment. In order to understand how cells become diseased, it is important to study the regulation of tissue-specific functions in normal cells under different conditions. Cell culture is the obvious system of choice. However, when eukaryotic cells are removed from the organism and placed in culture, they rapidly lose their ability to remain differentiated and their metabolism becomes increasingly glycolytic as the cells adapt to culture conditions. The relation of this altered metabolism to loss of specific functions is poorly understood and has not been systematically investigated.

Much of the literature on carcinogenesis uses systems that are indeed abnormal or unstable. The answers derived from such studies are at best inaccurate quantitatively and, at worst, meaningless in terms of the mechanisms of initiation of malignant state.

It is our thesis that this situation could improve if more attention were paid to the physiological state and the metabolic makeup of the cell when it is placed in culture. Under the assumption that all cells share common "non-differentiated" functions, a cell has been defined as differentiated if it elaborates a given (or a set of) tissue-specific macromolecule(s). This is an erroneous notion--one that indeed has led investigators to ignore the differentiated nature of metabolic control. Despite the fact that intermediary metabolism is common to all cells, it is [illegible]

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There are two [illegible] within my group. The first is the [illegible] of environmental factors to the regulation of gene expression, with emphasis [illegible] collagen. We are at present establishing [illegible] for optimal gene expression in three cell [illegible] embryonic [illegible], rat liver, and mouse mammary epithelial cells. [illegible] development of meaningful cell systems and conditions for expression of tissue-specific [illegible] is, and other investigators, to study the mechanisms of gene regulation [illegible] with more relevance to the [illegible] situation. The second area is the mechanism of tumor promotion and its relation to viral carcinogenesis. Both are discussed in more detail below.

I. Relation of Environmental Factors to Regulation of Gene Expression

A. Regulation of Collagen Synthesis and Fibronectin in Normal and Virus-Transformed Cells

We have established culture conditions for primary avian tendon where one important tissue-specific function (collagen synthesis) is kept stable at the in vivo level for an extended period in culture--a first accomplishment of its kind.[5] The function is modulated by cell density, ascorbic and lactic acids, serum levels, and viral transformation.[5-8] We have transformed these cells with temperature-sensitive (ts) mutants of Rous sarcoma virus (RSV) and determined the type of collagen synthesized in culture.[9] Thus, the cell system is an ideal model for studying regulation of collagen synthesis (30% of total protein synthesized is collagen), mechanism of action of ascorbate, and cell-virus interactions. The

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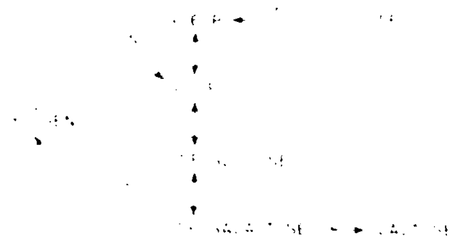
[illegible] the synthesis of fibronectin in the cells is unaltered after transformation [illegible]. We are currently investigating the nature of [illegible] collagen-cell interaction and the importance of matrix in regulation of collagen levels.

B. Normal and Malignant Mammary Cells

We have begun to define conditions under which normal mammary cells could retain their tissue-specific functions in culture. This is especially important for mammary epithelium since no morphological or biochemical differences between normal and malignant cells are observed under usual conditions in culture. However, if cells are grown on special substrates such as floating collagen gel where they are in contact in three dimensions, morphological and functional differences become apparent.

We have defined growth patterns, hexose transport systems and the glucose metabolic patterns of two human mammary cells that are reported to retain some specific functions. The two cell lines (HBL-100 and MCF-7) were similar to each other (and to chick fibroblasts) in many of their characteristics.[11] Furthermore, they were very insensitive to their culture conditions and did not synthesize lactose (a milk component) in response to lactogenic hormones. Under the same conditions, clear differences between lactating and non-lactating mouse cells could be demonstrated.[4] We are therefore in the process of characterizing fresh tissues from human sources in order to provide an in vivo reference point for subsequent culture studies. These experiments are performed in collaboration with scientists at Peralta Cancer Institute in Oakland, a part of the Biology and Medicine Division of LBL.

We have recently discovered an important aspect of lactose synthesis and secretion by mammary cells. *We have shown that mammary epithelial* cells are capable of synthesizing high levels of glycogen during pregnancy. The enzymes of glycogen synthesis and breakdown are shown to be activated and involved at various stages of mammary gland development.[12] We have thus proposed that



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[illegible]

C. The Metabolic State of Rat Hepatocytes in Primary Culture

We have shown that rat liver cell lines derived both from normal and malignant livers have distinct differences with primary hepatocytes in their glucose metabolism.[13] We are in the process of correlating maintenance in different carbon sources to expression of liver-specified functions such as the level of cytochrome P-450.[14] These experiments are performed in collaboration with D. M. Bissell of the Department of Medicine, U.C. San Francisco.

II. Mechanism of Tumor Promotion

A. Viral Carcinogenesis and Tumor Promotion

We have recently proposed a model for viral carcinogenesis that should open up exciting possibilities for new experiments and a better understanding of the available data in the literature. We also hope that the model would make it possible to understand and pinpoint the role of viruses in the etiology of human cancer. The model states that viral carcinogenesis may be a multistage process, similar to that proposed for tumor induction on mouse skin by carcinogens and tumor promoters. The product of the transforming gene of RSV (src) is then seen as a "transformation" promoter rather than as the initiator of transformation.[15] Since our paper appeared last year, others have proposed similar models for DNA virus transformation. The concept that the viral protein kinase ($PP60^{src}$) may be necessary but not sufficient for transformation also has gained additional support. At present we are investigating the similarities of a potent tumor promoter, TPA, to that of the active src protein kinase, using 2-dimensional gels for separation of proteins, metabolite patterns, collagen synthesis and other transformation parameters. Initial studies

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Abstracts

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[illegible] If there is one generalization that can be made from all the tissue and cell culture studies with respect to the differentiated state, it is this: since all functions are changed in culture, quantitatively and/or qualitatively, there is little or no "constitutive" regulation in higher organisms; i.e., the differentiated state in normal cells is unstable and the environment regulates gene expression.

4. A SIMPLE TECHNIQUE FOR DETECTION AND QUANTITATION OF LACTOSE SYSTHESIS AND SECRETION

Joanne T. Emerman and Mina J. Bissell

Analyt. Biochem. 94, 340-345 (1979)

A combination of high specific activity [^{14}C] glucose, two-dimensional chromatography, and autoradiography can be utilized to follow the flow of glucose carbon into lactose and various intermediary metabolites of the mammary gland. The technique described requires a minimum amount of tissue manipulation, is quantitative, utilizes microgram quantities of sample, and can be used for tissue pieces, single cell suspensions, and cells in culture. Clean separation of lactose from intermediates of glucose metabolism in a single chromatogram will aid the study of the regulation of mammary-specific functions.

[illegible] lost upon transformation. We thus examined whether their synthesis was coordinately regulated in Rous sarcoma virus-infected cells. It was found that while the synthesis of both pro α1 and pro α2 peptides was reduced upon transformation, the synthesis of fibronectin was not altered. Nevertheless, long term radiolabeling demonstrated that fibronectin levels were reduced in transformed cells. It is concluded that the reduction in levels of these components at the surface is brought about by different mechanisms, collagen levels being regulated by procollagen synthesis and fibronectin levels by degradation and/or release into the culture medium. The possibility is discussed that fibronectin is lost from the cell periphery of primary avian tendon cells as a consequence of decreased levels of anchoring collagen molecules.

10. REQUIREMENTS FOR MAINTAINING THE EMBRYONIC STATE OF AVIAN TENDON CELLS IN CULTURE

Richard I. Schwarz, Deborah A. Farson, and Mina J. Bissell

In Vitro 15, 941-948 (1979)

Primary avian tendon cells (PAT) maintain their embryonic state when cultured in medium F-12 with

*See Abstracts.

[illegible]

The quantitative loss of function has long been considered to be a cell culture artifact; however, we propose that this drop in collagen synthesis is a reflection of the developmental programming of these cells. In separate series of experiments using organ cultures, we show that tendon tissue from the embryo makes over 30% collagen, whereas "young" tendons make 1[illegible]% and "older" tendons from the adult make less than 1%. Therefore, a quantitative drop in collagen synthesis would be expected if normal development were to occur in culture. Our data are consistent with the idea that cultures of embryonic tendon cells are triggered to mature by a mechanism that correlates with high cell density.

14. FRUCTOSE UTILIZATION AND ALTERED CYTOCHROME P-450 IN CULTURED ADULT RAT HEPATOCYTES

M. Vessal, M. [illegible]. Chowr, M. J. Bissell and D. M. Bissell

Biochim. Biophys. Acta, in press (1980)

Cultured adult rat hepatocytes exhibit increased levels of cytochrome P-450 when incubated in media containing fructose, relative to cells incubated with equimolar glucose, and the effect of fructose varies directly with its concentration between 2 and 10 mM. For investigating the mechanism of the effect of fructose on cytochrome P-450 in cultured cells, [U-^{14}C] fructose or [U-^{14}C] glucose were added to the incubation medium, and their uptake and utilization were compared. While the uptake kinetics of the two hexoses were similar, the rate of phosphorylation of fructose was more than 10-fold that of glucose. The appearance of fructose carbon in metabolic pools, as well as its conversion to CO_2 and cellular glycerolipid, similarly was increased. The latter finding suggested that fructose might alter cytochrome P-450 by stimulating glycerolipid synthesis, since the stability of the cytochrome is lipid-dependent. However, the changes in glycerolipid formation failed to parallel changes in the level of cytochrome P-450 in fructose-treated cells. Moreover, the relative distribution of ^{14}C into specific lipids was similar for both hexoses, suggesting that an increased carbon flux in cells incubated with fructose did not directly impose a qualitative change in cellular lipid synthesis. We conclude that the fructose-mediated alteration of cytochrome P-450 in cultured rat hepatocytes

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16. A[illegible] INFECTIVITY OF AVIAN RNA TUMOR VI[illegible]

M. J. Bissell, [illegible], D. [illegible], R. I. Schwarz, and W. [illegible]

Proc. Natl. Acad. Sci. [illegible], 2711-2715 (1980)

Ascorbic acid, at nontoxic concentrations, causes a substantial reduction in the ability of avian tumor viruses to replicate in both primary avian tendon cells and chick embryo fibroblasts. The virus-infected cultures appear to be less transformed in the presence of ascorbic acid by the criteria of morphology, reduced glucose uptake and increased collagen synthesis. The vitamin does not act by altering the susceptibility of the cells to initial infection and transformation, but instead appears to interfere with the spread of infection through reduction in virus replication and virus infectivity. The effect is reversible and requires the continuous presence of the vitamin in the culture medium.

CULTURE SYSTEMS FOR STUDYING MALIGNANCY

M. J. Bissell, D. C. Bartholomew, J. Folkman, H. Smith and M. Stampfer

Cancer Res. 39, 4293-4295 (1979)

A symposium organized by the above investigators explored the new and more relevant culture systems for studying malignancy and differentiation. The summary of the symposium report makes these general conclusions:

- Both mutational events and changes in gene expression by themselves or in combination

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APPENDIX A. DIVISION PUBLICATIONS, 1979

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APPENDIX B. LABORATORY OF CHEMICAL BIODYNAMICS Ph.D. THESES, 1979

APPENDIX C. DIVISION STAFF

[illegible]

[illegible]
[illegible], Berkeley

[illegible]
[illegible] Institute of Technology, [illegible],
[illegible]

Peder [illegible]
Royal Agri[illegible] and Veterinary University,
Copenhagen, [illegible]

Howard [illegible]
Youngstown State University

[illegible]

Barbara [illegible]*

Karen [illegible]

Krishnakali Das

Wallace [illegible]

Deborah [illegible]

Benjamin Gordon

Carol Hatie

Craig Hodges

Libby Holbrook
Biochemistry, Duke University

Ninon Kafka

Joseph Kao

David Kanne

Greg Karczmar

*Terminated from Chemical Biodynamics Division

*Assigned from other Divisions

APPENDIX D. ATTENDANCE AT NATIONAL AND FOREIGN CONFERENCES, 1979

[illegible]

Twenty-third International Edison Birthday Celebration, Orlando, Florida, February 11, 1979.

AIC Gold Medal Award Address, Philadelphia, Pennsylvania, May 5, 1979.

Sugar Growers Congress, Cooperative Fluminense dos Productores de Acucar e Alcool, Otd. (COPERFLU), Campos, Rio de Janeiro, Brazil, August 24, 1979.

Presidential Plenary Session, American Chemical Society National Meeting, Washington, D.C., September 10, 1979.

Licensing Executive Society, Vancouver, British Columbia, September 18, 1979.

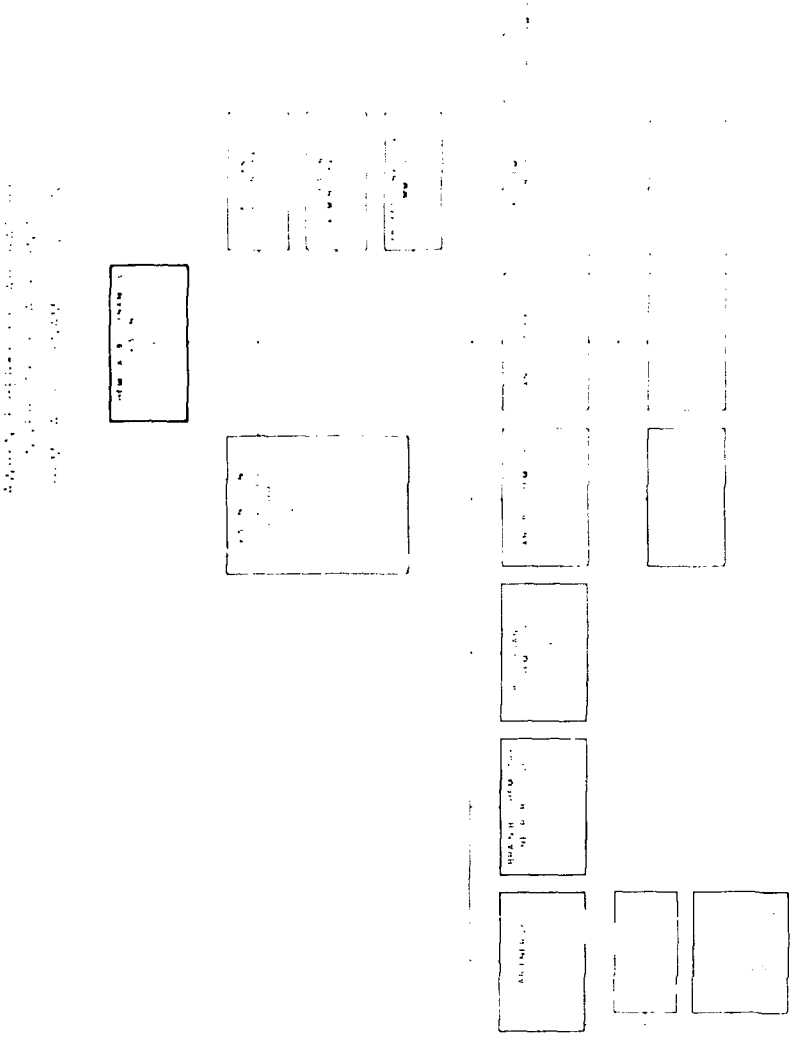
[illegible]

[illegible] International [illegible] Energy: [illegible] Alternative Strategies, Kyungpook National University, Taegu, Korea, October [illegible], 1979.

[illegible] Colloid and Surface Science Symposium, University of Missouri-Rolla, June 11-13, 1979.

Kenneth [illegible]
American Society for Photobiology National Meeting, Asilomar, California, June 24-28, 1979.

Itamar Willner
U.S.-Australia Seminar on Bioelectrochemistry, Pasadena, California, July 9-12, 1979.

Thomas Wydrzynski
American Society for Photobiology National Meeting, Asilomar, California, June 24-28, 1979.

APPENDIX E. ORGANIZATIONAL CHART