LBL-8623
UC-48

Chemical Biodynamics Division

ANNUAL REPORT 1978

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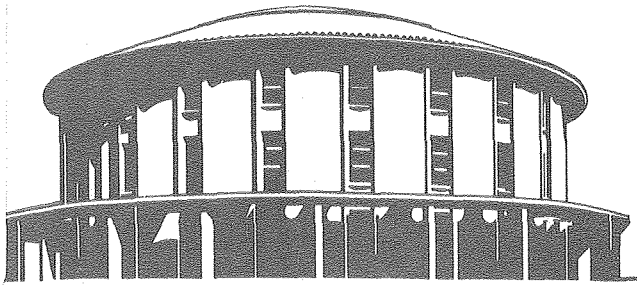

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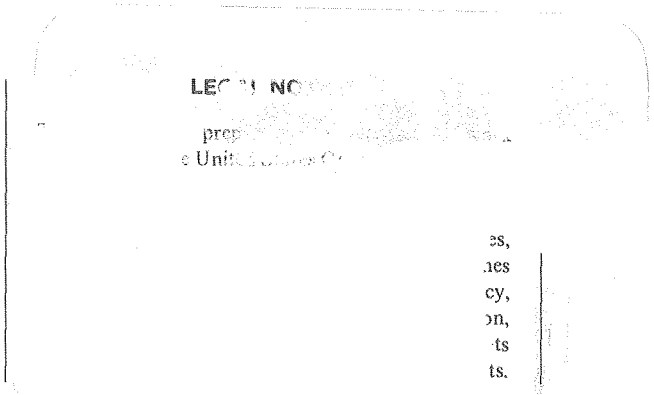

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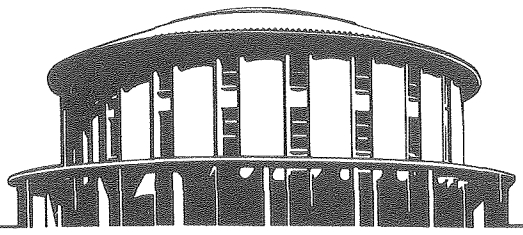

Work performed for the U.S. Department of Energy under Contract W-7405-ENG-48.

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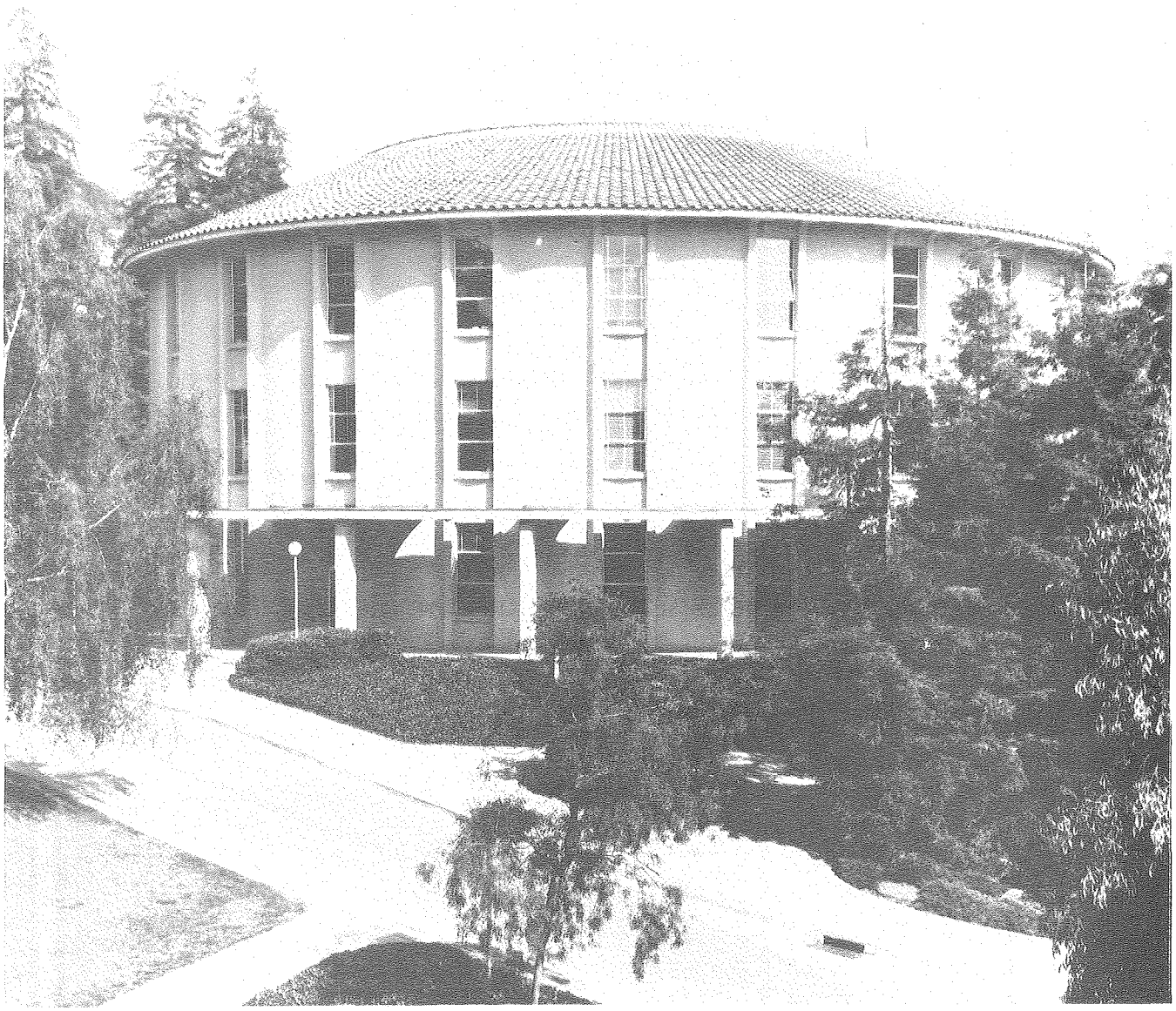

BBC 747-5085

INTRODUCTION

The Chemical Biodynamics Division of the Lawrence Berkeley Laboratory (LBL) has two major objectives which are two aspects of basic research required for the fulfillment of two of the missions of the Department of Energy. One involves the understanding and mitigation of the effects of the use of fossil fuel. The other involves ways and means of economically capturing solar energy particularly by photosynthesis or synthetic processes modeled after it. Beyond this, an effort has been made to carry both of these matters far enough into the applied area so that they may be visible and acceptable to those parts of the agencies whose primary objective(s) is the application of basic research to their missions.

The work of the division still remains focused around two central themes. The first is the environmental theme, represented by the work on chemical carcinogenesis, together with the work on effects of environmental pollutants on plant and animal cells. The second theme is the capture of solar energy by processes of photosynthesis itself, or of physical devices designed on the basis of our growing knowledge of photosynthesis. We also have two additional activities, one peripheral to each of the above major divisions, the first one being research on brain mechanisms in learning (peripheral to the environment problems research) and the second one in the area of chemical evolution (an outgrowth of our interest in the evolution of photosynthesis).

The Chemical Biodynamics Division began 33 years ago as the Bio-Organic Chemistry group of the Radiation Laboratory (now LBL) with special interests in the study of organic reaction mechanisms, animal biochemistry and photosynthesis. We have now grown to a group of over 100 people, with scientists in disciplines from physics to biology, with chemistry as the central science. There is active collaboration with many university departments and groups, together with interaction with various divisions within LBL, to create the diversified interdisciplinary environment which exists.

Interactions among scientific disciplines in the laboratory are many and complex, and the record of our activities in terms of publications, graduate student research, postdoctoral research and teaching, is evidence that the interdisciplinary approach has been highly successful. To my knowledge, there exists no other laboratory anywhere that has this degree of integration of the various scientific disciplines that we have achieved here in the Chemical Biodynamics Division.

The annual report will give a generalized overview of our activities, together with some statistical information on publications, personnel and other matters of interest. This is the first annual report, in addition to the sum of our quarterly reports, that has been prepared by the division for approximately 15 years. Our quarterly reports are usually much more technical than this present document, and we hope that this summary presentation will be of interest to the various scientific and administrative personnel who have occasion to examine the activities of the division as well as LBL.

Melvin Calvin
Division Head

LIGHT INTO CHEMICAL ENERGY VIA PHOTOSYNTHESIS

Kenneth Sauer

Photosynthetically active membranes consist of organized two-dimensional arrays of light-absorbing pigments, excitation trapping sites, electron transporting molecules, and coupling factors. Structural and kinetic evidence suggests that the membranes contain complex photosynthetic units that are separately competent. We have formulated a picture of this assembly as a Pebble Mosaic Model.[1] The pebbles are the individual molecules assembled in the functioning units of photosynthetic activity, and the mosaic results from the repetition of these units in two dimensions throughout the membranes. On a somewhat larger scale the membranes close on themselves, and important differences between inside and outside surfaces are evident. The generation of trans-membrane electric potentials and ion gradients serves as a source of the chemical potential needed for the formation of energy-rich compounds like adenosine triphosphate (ATP).

To determine the detailed nature of the structure of the mosaic, we have begun dissecting it into the component pebbles. Through the use of selective detergents a variety of chlorophyll proteins have been isolated. Some of these are photochemically inactive and represent the components of an antenna of light-absorbing chlorophyll.[1,2] Others exhibit the ability to initiate photoredox steps and are designated reaction center complexes. Spectroscopic studies, especially absorption and circular dichroism, show that the active complexes consist of small aggregates of chlorophylls that are imbedded in protein and have a well-defined geometric arrangement.[3]

We have recently completed a study of one such complex that is the simplest characterized to date.[2] It consists of two bacteriochlorophyll (BChl) molecules in association with two protein subunits of about 10,000 molecular weight. The two BChl are coupled via exciton interactions that are clearly visible in the absorption and circular dichroism spectra, and they constitute a kind of dimer of BChl that is internal to the protein complex. The tight coupling implies rapid delocalization of excitation between the pigment molecules. In an antenna complex from a related organism there is a third BChl molecule and a carotenoid molecule, spheroidene, that are peripherally coupled to the dimer pair. These variations in packaging of the pigments give rise to flexibility in the organism, perhaps in a way that confers advantages in different environments.

Variants of the detergent treatment produce larger segments of the mosaic structure and permit deductions of the juxtaposition of neighboring pebble subunits. By working with plant seedlings that have been grown entirely in the dark, we can study the formation and development of the membranes as they become photosynthetically competent. During this process chlorophyll molecules, newly synthesized from their precursors, become associated with particular segments of protein and are incorporated into the emerging membranes.[4] The structure of the mosaic is now coming into view as a result of studies such as these.

The absorption of light in the photosynthetic membranes produces an excited electronic state of chlorophyll. This excitation energy must be trapped within a nanosecond (10^{-9} sec), lest it reappear as useless radiation or heat. Initial studies have shown that the trapping occurs within 10 picoseconds (10^{-11} sec)--very rapidly indeed. The excitation transfer and trapping process is reflected indirectly by the emission of fluorescent light. We have developed sophisticated instrumentation at the laboratory to measure fluorescence lifetimes and the kinetics of the decay process. Recent studies of the decay of chlorophyll fluorescence from chloroplasts and whole algae demonstrate the sensitivity of the lifetimes to the photochemical state of the traps.[5] When the traps are open and capable of initiating electron transport reactions, the fluorescence decays are single exponential processes with halftimes of 200 to 400 picoseconds. Actinic illumination or inhibitors that block the photochemistry lead to a biphasic decay. The initial relaxation is lengthened about twofold, and an additional slow phase (about 2 nanoseconds) appears. This pattern is interpreted along with fluorescence yield results to indicate that excitation is not localized in single photosynthetic units, but has the capability of being transferred to neighboring sites when nearby photoreaction centers are closed.

In the photochemical reaction center the trapped excitation results in electron transfer from chlorophyll to the first of a sequence of electron acceptors. Subsequently the missing chlorophyll electron is replaced by nearby electron donors. Thus, the oxidation-reduction reactions of electron transport are initiated. Using laser flashes to excite photosynthetic samples and fast detection of optical[6,7] or electron paramagnetic resonance (EPR) transients,[8] we have mapped a sequence of four membrane-bound acceptors associated with chloroplast reaction centers. (Figure 1 shows the instrumentation used in the EPR technique.) The first of these acceptors appears to be a chlorophyll molecule that is part of the core of the reaction center complex that also contains the P700 or special pair of electron donor chlorophylls. It gives rise to a polarization or non-equilibrium distribution of spin states in the radical pair formed as a consequence of the initial charge separa-

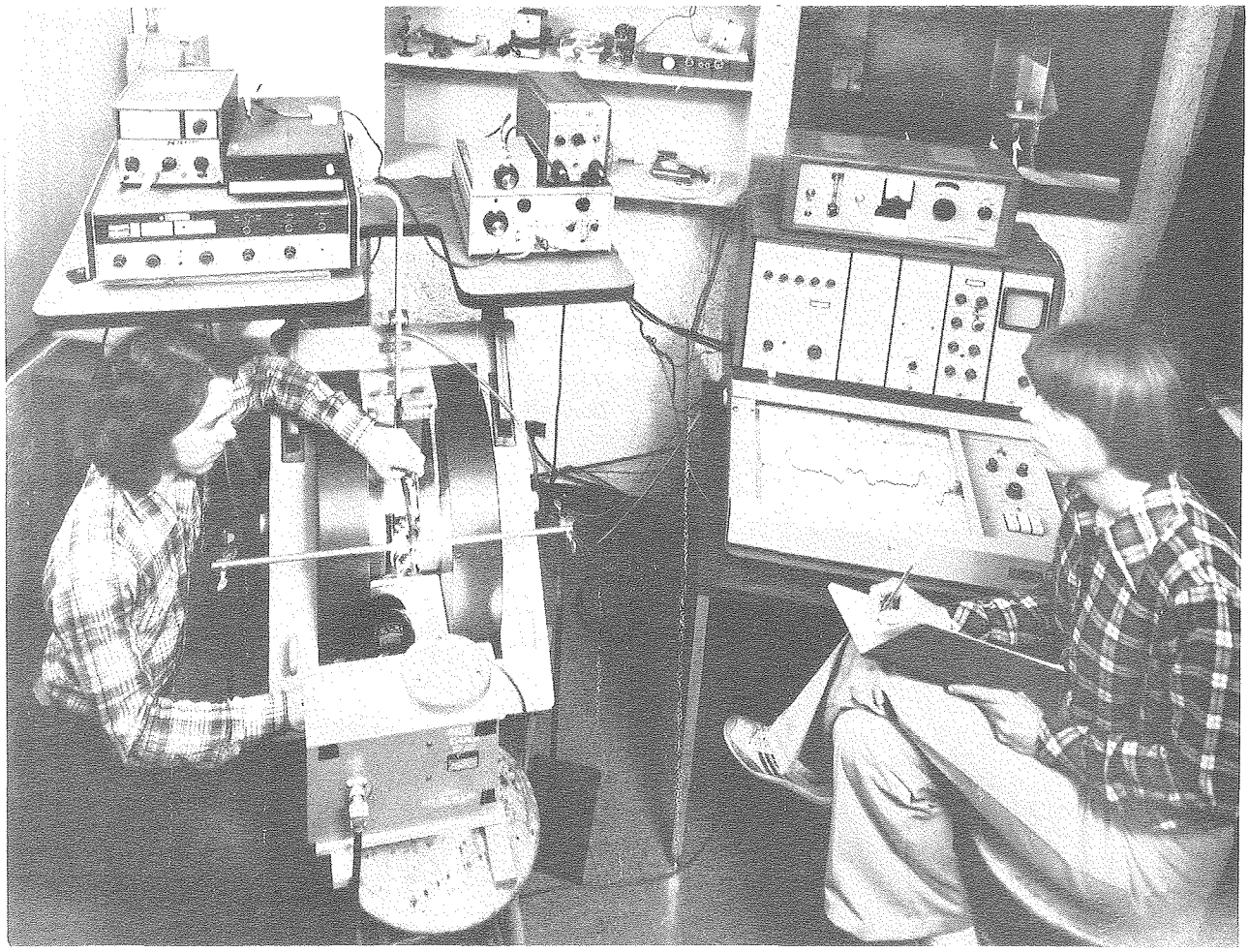

Figure 1. Signals produced upon illumination of photosynthetic bacteria in an EPR spectrometer give information about charge separation in the reaction centers. (CBB 784-4926)

tion.[8] The resulting EPR signals exhibit distinctive features that allow us to follow the dynamics of the charge separation process. In reaction center particles from which subsequent electron acceptors have been removed, the initial charge separation leads only to a rapid back reaction on a microsecond time scale.[9] In studies of this process as a function of temperature, we discovered that the process becomes temperature independent below about 80°K. This indicates that electrons move from the donor to the acceptor species by a process of quantum mechanical tunneling that does not require additional thermal energy.

When the secondary electron acceptors are present, the transfer process proceeds through a rapid sequence of reactions. These were monitored by both optical[6,7] and EPR techniques.[8] Using spatially oriented membranes we showed that several of these intermediates are immobile and ordered in the membrane matrix.[10] They are likely to be iron-sulfur proteins of the Fe_4S_4 type; however, definitive characterization remains to be accomplished. Our current view of the electron transport reactions associated with Photosystem I of higher plants is summarized by the equation

$$\text{Donors} \quad \underset{P700}{(Chl)_2} \xrightarrow{h\nu} Chl \rightarrow (Fe_4S_4)_X P \rightarrow (Fe_4S_4)_B P \rightarrow (Fe_4S_4)_A P \quad .$$

These components are all membrane-bound, and we believe that they span the membrane so as to effect a rapid separation of charge from one surface to the other. The resulting electric field gives rise to ion translocations that couple to the formation of stored chemical potential. This aspect of the primary charge separation of the photosynthetic light reactions provides an important guide for the design of practical solar energy converters to produce useful chemical or electrical power.

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Abstracts

2. BACTERIOCHLOROPHYLL-PROTEIN COMPLEXES FROM THE LIGHT-HARVESTING ANTENNA OF PHOTOSYNTHETIC BACTERIA

K. Sauer and L. A. Austin

Biochemistry 17, 2011-2019 (1978)

Detergent-solubilized bacteriochlorophyll-protein complexes corresponding to the light-harvesting antenna are isolated from Rhodopseudomonas sphaeroides, strain 2.4.1 (wild-type) and R-26 (carotenoidless) mutant, and from Rhodospirillum rubrum, wild-type. Detailed studies of the complex derived from the R-26 mutant indicate that it contains two bacteriochlorophyll (BChl) molecules and two copies of a peptide of 8.5 kdaltons, together with about 20% phospholipid in a unit of about 22 kdaltons. Distinctive absorption and circular dichroism spectra indicate that this unit is present in whole cell chromatophores derived from the bacteria, in large aggregates obtained by fractionation using Triton X-100 and as a monomeric unit in the presence of 0.5% sodium dodecyl sulfate. A similar component derived from the wild-type Rps. sphaeroides contains three BChl, two peptides of about 10 kdaltons, carotenoid, and phospholipid. The additional BChl molecule appears to be associated with an absorption band at 800 nm that is present in addition to the 850-nm band seen in the R-26 mutant complex. A similar complex from Rds. rubrum was studied in less detail. The interactions of the BChl molecules within these complexes are interpreted in terms of intermediate exciton coupling. Additional weaker reactions between the complexes in aggregates or in the intracytoplasmic membranes of the bacteria are invoked to account for fluorescence depolarization and fluorescence yield observations. Excitation transfer within the light-harvesting antenna that leads to photochemical trapping in the reaction centers is probably a direct consequence of these interactions.

*See Abstracts.

3. ABSORPTION AND CIRCULAR DICHROISM SPECTRA OF CHLOROPLAST MEMBRANE FRAGMENTS FROM SPINACH, BARLEY AND A BARLEY MUTANT AT ROOM TEMPERATURE AND LIQUID NITROGEN TEMPERATURE

O. D. Canaani and K. Sauer

Biochim. Biophys. Acta 501, 545-551 (1978)

The absorption and CD spectra of chloroplast fragments from spinach, barley and a barley mutant (chlorophyll b-minus) were studied at temperatures of 23°C and -196°C. The CD spectrum of wild type barley and spinach at -196°C showed troughs at 640, 653, 676 and 695 nm and a maximum at 667 nm. The CD spectrum of the barley mutant at -196°C consisted of a large trough at 684 nm, a small trough at 695 nm and a positive peak at 670 nm. A new feature observed at -196°C but not at 23°C is the trough at 640 nm. This 640 nm CD signal is missing in the CD spectrum of the barley mutant. It is attributable to the light-harvesting chlorophyll a/b protein which appears to be missing in the mutant. Another new feature, the trough at 695 nm, was observed in the CD spectra of spinach, barley and the barley mutant at -196°C. The 695 nm trough appears to be sensitive to detergents and it may be due to a labile chlorophyll a·protein complex. Possible interpretations of these data are discussed.

4. ANALYSIS OF THE SUBUNIT STRUCTURE OF PROTOCHLOROPHYLLIDE HOLOCHROME BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

O. D. Canaani and K. Sauer

Plant Physiol. 60, 422-429 (1977)

The subunit structures of protochlorophyllide holochrome (PCH) and chlorophyllide holochrome (CH) were studied by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. PCH from leaves of dark-grown (Phaseolus vulgaris var. red kidney) is a polymeric pigment-protein complex of approximately 600,000 daltons. It is composed of 12 to 14 polypeptides of 45,000 daltons, when examined prior to and immediately following photoconversion. The protochlorophyllide or chlorophyllide pigment molecules are associated with these polypeptides. Subsequent to photoconversion, the absorption maximum of newly formed chlorophyllide shifts from 678 nm to 674 nm upon standing in darkness. Following the 678 to 674 spectral shift, the chlorophyllide is associated with a polypeptide with a molecular weight of 16,000 daltons. In addition, sucrose gradient centrifugation of PCH and CH under nondenaturing conditions indicates that during the course of the dark spectroscopic shift, the 600,000 dalton CH undergoes dissociation into a small chlorophyllide protein. The dissociation of CH, the change in the molecular weight of the chlorophyllide polypeptide from 45,000 to 16,000 daltons, as well as the dark spectroscopic shift are temperature-dependent and blocked below 0 C. It was also found that each holochrome molecule of 600,000 daltons contains at least four protochlorophyllide pigment molecules.

5. FLUORESCENCE LIFETIMES OF CHLOROPLASTS, SUB-CHLOROPLAST PARTICLES AND CHLORELLA USING SINGLE PHOTON COUNTING

K. Sauer and G. T. Brewington

Photosynthesis 77: Proc. 4th Intern. Cong. Photosynthesis, Reading, 1977, D. Hall et al., eds. (The Biochemical Society, London, 1978), pp. 409-421

Lifetimes of chlorophyll fluorescence from spinach chloroplasts, digitonin subchloroplast particles and Chlorella pyrenoidosa were measured using a single photon counting apparatus capable of detecting lifetimes longer than about 0.1 ns. In the absence of actinic illumination or inhibitors, all of the materials investigated exhibited single exponential decays in the range 0.2 to 0.4 ns. Actinic illumination or inhibitors that block Photosystem 2 produce a biphasic fluorescence decay. The fast decay component becomes several times slower, and it is accompanied by a second, slow decay of about 2 ns. The results are interpreted to support the occurrence of transfer of excitation between Photosystem 2 units.

6. OPTICAL STUDIES OF PHOTOSYSTEM I PARTICLES: EVIDENCE FOR THE PRESENCE OF MULTIPLE ELECTRON ACCEPTORS

K. Sauer, S. Acker, P. Mathis, and A. Van Best

Bioenergetics of Membranes, L. Packer et al., eds. (Elsevier/North-Holland Biomedical Press, Amsterdam, 1977), pp. 351-359

The absorption changes of P700 in chloroplast TSF1 particles enriched in Photosystem I exhibit fast kinetic components under reducing conditions. The addition of dithionite to an anaerobic sample at pH 10 induces a rapid (250 μs) exponential decay of P700 absorption changes following laser pulse excitation. This decay is approximately 100 times faster than the recombination of $P700^+$ with $P430^-$ seen under less reducing conditions. In separate experiments where a good electron donor, neutral red, is added to the anaerobic dithionite-containing samples, a still faster (3 μs) reversal of $P700^+$ is seen when a laser pulse is superimposed on red background illumination. These results are interpreted in terms of two new intermediate electron acceptors that lie between P700 and P430. Preliminary experiments with broken chloroplasts show a similar rapidly decaying component under strongly reducing conditions.

7. ELECTRON ACCEPTORS ASSOCIATED WITH P-700 IN TRITON SOLUBILIZED PHOTOSYSTEM I PARTICLES FROM SPINACH CHLOROPLASTS

K. Sauer, P. Mathis, S. Acker, and J. A. Van Best

Biochim. Biophys. Acta 503, 120-134 (1978)

Flash-induced absorption changes of Triton-solubilized Photosystem I particles from spinach were studied under reducing and/or illumination conditions that serve to alter the state of bound electron acceptors. By monitoring the decay of P-700 following each of a train of flashes, we found that P-430$^-$ or components resembling it can hold 2 equivalents of electrons transferred upon successive illuminations. This requires the presence of a good electron donor, reduced phenazine methosulfate or neutral red, otherwise the back reaction of P-700$^+$ with P-430$^-$ occurs in about 30 ms. If the two P-430 sites, designated Centers A and B, are first reduced by preilluminating flashes or chemically by dithionite under anaerobic conditions, then subsequent laser flashes generate a 250 μs back reaction of P-700$^+$, which we associate with a more primary electron acceptor A_2. In turn, when A_2 is reduced by background (continuous) illumination in presence of neutral red and under strongly reducing conditions, laser flashes then produce a much faster (3 μs) back reaction at wavelengths characteristic of P-700. We associate this with another more primary electron acceptor, A_1, which functions very close to P-700. The organization of these components probably corresponds to the sequence P-700-A_1-A_2-P-430 $\left[\begin{matrix}A\\B\end{matrix}\right]$. The relation of the optical components to acceptor species detected by EPR, by electron-spin polarization or in terms of peptide components of Photosystem I is discussed.

Preliminary experiments with broken chloroplasts suggest that an analogous situation occurs there, as well.

8. ELECTRON SPIN POLARIZATION IN PHOTOSYNTHESIS AND THE MECHANISM OF ELECTRON TRANSFER IN PHOTOSYSTEM I: EXPERIMENTAL OBSERVATIONS

G. C. Dismukes, A. McGuire, R. Blankenship, and K. Sauer

Biophys. J. 21, 239-256 (1978); 22, 521 (1978)

Transient electron paramagnetic resonance (EPR) methods are used to examine the spin populations of the light-induced radicals produced in spinach chloroplasts, photosystem I particles, and Chlorella pyrenoidosa. We observe both emission and enhanced absorption within the hyperfine structure of the EPR spectrum of P700$^+$, the photooxidized reaction-center chlorophyll radical (Signal I). By using flow gradients or magnetic fields to orient the chloroplasts in the Zeeman field, we are able to influence both the magnitude and sign of the spin polarization. Identification of the polarized radical as P700$^+$ is consistent with the effects of inhibitors, excitation light intensity and wavelength, redox potential, and fractionation of the membranes. The EPR signal of the polarized P700$^+$ radical displays a 30% narrower line width than P700$^+$ after spin relaxation. This suggests a magnetic interaction between P700$^+$ and its reduced (paramagnetic) acceptor, which leads to a collapse of the P700$^+$ hyperfine structure. Narrowing of the spectrum is evident only in the spectrum of polarized P700$^+$, because prompt electron transfer rapidly separates the radical pair. Evidence of cross-relaxation between the adjacent radicals suggests the existence of an exchange interaction.

The results indicate that polarization is produced by a radical pair mechanism between P700$^+$ and the reduced primary acceptor of photosystem I. The orientation dependence of the spin polarization of P700$^+$ is due to the g-tensor anisotropy of the acceptor radical to which it is exchange-coupled. The EPR spectrum of P700$^+$ is virtually isotropic once the adjacent acceptor radical has passed the photoionized electron to a later, more remote acceptor molecule. This interpretation implies that the acceptor radical has g-tensor anisotropy significantly greater than the width of the hyperfine field on P700$^+$ and that the acceptor is oriented with its smallest g-tensor axis along the normal to the thylakoid membranes. Both the ferredoxin-like iron-sulfur centers and the X$^-$ species observed directly by EPR at low temperatures have g-tensor anisotropy large enough to produce the observed spin polarization; however, studies on oriented chloroplasts show that the bound ferredoxin centers do not have this orientation of their g-tensors. In contrast, X$^-$ is aligned with its smallest g-tensor axis predominantly normal to the plane of the thylakoid membranes. This is the same orientation predicted for the acceptor radical based on analysis of the spin polarization of P700$^+$, and indicates that the species responsible for the anisotropy of the polarized P700$^+$ spectrum is probably X$^-$.

The dark EPR Signal II is shown to possess anisotropic hyperfine structure (and possibly g-tensor anistropy), which serves as a good indicator of the extent of membrane alignment.

9. RAPIDLY REVERSIBLE FLASH-INDUCED ELECTRON TRANSFER IN A P-700 CHLOROPHYLL-PROTEIN COMPLEX ISOLATED WITH SDS

P. Mathis, K. Sauer, and R. Remy

FEBS Lett. 88, 275-278 (1978)

The reaction center of photosystem I has recently been the subject of very active research. In particular the organization of the chlorophyll-protein complex is better understood. The identification of the electron acceptors has

progressed thanks to the use of optical absorption spectroscopy and of low temperature EPR. Although the field is still rather open, it is reasonable to admit that the bound acceptors include two iron-sulfur proteins (centers A and B) and, preceding them, a chemically unidentified compound X, whose EPR spectrum is known. The photochemical electron transfer from P-700 to centers A or B is irreversible at low temperature, whereas the transfer from P-700 to X (when centers A and B are chemically reduced) is followed by a back-reaction with a half-time of 0.8 s at 6°K.

We report here a much faster reaction (approximately 0.6 ms at 5°K) which occurs in subchloroplast particles and may be indicative of an acceptor more primary than X. A recent study of flash-induced absorption changes in Triton subchloroplast particles led us to propose that P^+-700 can be reduced by a back-reaction either with the terminal-bound acceptors (iron-sulfur centers A or B) in 30-40 ms, with a more primary acceptor A_2 in approximately 250 μs or with a still more primary acceptor A_1 in approximately 3 μs. Observation of the fast phases required the previous reduction of the more remote acceptors. In P-700 chlorophyll a-protein complexes prepared with sodium dodecylsulfate (SDS) it has been shown that the protochemical oxidation of P-700 is absent or very inefficient and also that the iron-sulfur centers are missing. Recent data have shown that SDS subchloroplast particles prepared by electrophoresis retain the ability of P-700 photo-oxidation but with a rather low efficiency. We hypothesized that these particles would give a direct back-reaction between P^+-700 and A_1^- or A_2^-, without the need for a previous reduction of the bound iron-sulfur proteins.

10. THE ORIENTATION OF MEMBRANE-BOUND RADICALS: AN EPR INVESTIGATION OF MAGNETICALLY ORDERED SPINACH CHLOROPLASTS

G. C. Dismukes and K. Sauer

Biochim. Biophys. Acta 504, 431-445 (1978)

The orientation of membrane-bound radicals in spinach chloroplasts is examined by electron paramagnetic resonance (EPR) spectroscopy of chloroplasts oriented by magnetic fields. Several of the membrane-bound radicals which possess g-tensor anisotropy display EPR signals with a marked dependence on the orientation of the membranes relative to the applied EPR field. The fraction of oxidized and reduced plastocyanin, P-700, iron-sulfur proteins A and B, and the X center, an early acceptor of Photosystem I, can be controlled by the light intensity during steady-state illumination and can be trapped by cooling. The X center can be photoreduced and trapped in the absence of strong reductants and high pH, conditions previously found necessary for its detection. These results confirm its role as an early electron acceptor in P-700 photo-oxidation. X is oriented with its smallest principal g-tensor axis (g_x) predominantly parallel to the normal to the thylakoid membrane, the same orientation as was found for an early electron acceptor based on time-resolved electron spin polarization studies. We propose that the X center is the first example of a high potential iron-sulfur protein which functions in electron transfer in its 'superreduced' state. We present evidence which suggests that iron-sulfur proteins A and B are 4Fe-4S clusters in an 8Fe-8S protein. Center B is oriented with g_y predominantly normal to the membrane plane. The spectra of center A and plastocyanin do not show significant changes with sample orientation. In the case of plastocyanin, this may indicate a lack of molecular orientation. The absence of an orientation effect for reduced center A is reconcilable with a 4Fe-4S geometry, provided that the electron obtained upon reduction can be shared between any pair of Fe atoms in the center. Orientation of the 'Rieske' iron-sulfur protein is also observed. It has axial symmetry with $g_{\parallel}$ close to the plane of the membrane. A model is proposed for the organization of these proteins in the thylakoid membrane.

A new EPR signal was observed in oriented chloroplasts. This broad unresolved resonance displays a g value of 3.2 when the membrane normal is parallel to the field. It shifts to $g = 1.9$ when the membrane normal is perpendicular to the field. The signal is sensitive to illumination and to washing of the thylakoid membrane of broken chloroplasts. We suggest that there is a relation between this signal and the water-oxidizing enzyme system.

RESONANCE RAMAN SPECTRA OF METHEMOGLOBIN DERIVATIVES. SELECTIVE ENHANCEMENT OF AXIAL LIGAND VIBRATIONS AND LACK OF AN EFFECT OF INOSITOL HEXAPHOSPHATE

S. A. Asher, L. E. Vickery, T. M. Schuster, and K. Sauer

Biochemistry 16, 5849, (1977)

Resonance Raman spectra have been obtained for the OH^-, N_3^-, and F^- derivatives of methemoglobin by excitation in the 550 to 650-nm region. A selective enhancement with excitation in the charge-transfer bands is observed for peaks at 413 and 497 cm^{-1} and a doublet at 471 and 443 cm^{-1} in the N_3^-, OH^-, and F^- complexes, respectively. These peaks are assigned to Fe-axial ligand stretches on the basis of: (1) a 20-cm^{-1} shift of the 497-cm^{-1} peak of the hydroxide complex to lower energy on isotopic substitution of $^{18}OH^-$ for $^{16}OH^-$; (2) the proximity of the 413-cm^{-1} Raman peak to the 421-cm^{-1} IR peak previously assigned to the Fe-N_3^- stretch in a model heme-azide complex [Ogoshi, H., Watenabe, E., Yoshida, Z., Kincaid, J., and Nakamoto, K. (1973), J. Am. Chem. Soc. 95, 2845]; (3) the selective appearance of the 471- and 443-cm^{-1} peaks in the Raman spectra of the F^- complex. The doublet observed at 471 and 443 cm^{-1} in the F^- derivative may reflect a heterogeneity in the heme cavity due to hydrogen bonding of H_2O to the F^- ligand

in both the α and β subunits, as has been previously suggested based on x-ray diffraction results (Deatherage, J. F., Loe, R. S., and Moffat, K. (1976), J. Mol. Biol. 104, 723). It is suggested that the frequency of the Fe-F^- vibration reflects the out-of-plane distortion of the Fe from the heme plane. The lack of a shift in the frequency of the Fe-F-vibration suggests that there is little or no movement of the iron with respect to the heme plane upon the addition of inositol hexaphosphate, which is thought to alter the allosteric equilibrium between the R and T forms of methemoglobin. This result is consistent with a recent x-ray crystallographic study of an IHP complex of MetHb-F^- (Fermi, G., and Perutz, M. F. (1977), J. Mol. Biol. 114, 421). Excitation profile measurements suggest that the charge-transfer band in methemoglobin OH^- like that in methemoglobin N_3^- is z polarized, while in methemoglobin F^- the charge transfer transition is mixed with a π to π^* transition.

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

Melvin Calvin, Melvin P. Klein, and John W. Otvos

Over the past twenty years considerable progress has been made in understanding the nature of the primary quantum conversion act 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]

PHOTOELECTRON TRANSFER SCHEME

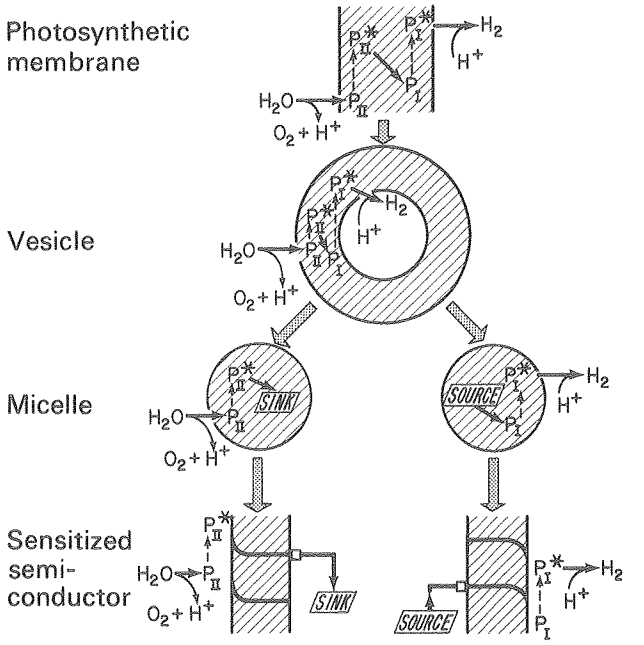

Figure 1. Photoelectron transfer scheme. (XBL 774-4350A)

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 recently promoted an electron (and proton) transfer across the wall to produce both oxidization and reduction products.[2] Previously we had presented evidence for charge migration in intact, photosynthetic organisms.[3] 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.[4] In principle, such half systems can eventually be assembled and coupled on a membrane or vesicle. 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 an n-type semiconductor pictured at the bottom left of Figure 1, and an adsorbed layer of chlorophyll as a sensitizer. Such a cell has been called 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. 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). In our model, the electron transfer is from excited chlorophyll to the zinc oxide crystal. The difficulty with this particular cell is the irreversible destruction of the chlorophyll sensitizer because of chlorophyll oxidation.

Such photoelectrochemical cells can also be constructed involving more stable inorganic sensitizers.[5,6] 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.

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Abstracts

5. ELECTRON TRANSFER AT SENSITIZED TiO_2 ELECTRODES

M. T. Spitler and M. Calvin

J. Chem. Phys. 66, 4294 (1977)

Electron transfer from the excited state of tetra-iodo, tetra-chloro fluorescein (rose bengal) to the conduction band of TiO_2 has been studied through photoelectrochemical techniques. The measured transfer rate was correlated with information from absorbance and adsorption measurements of this dye molecule on the (001) surface of the single crystals used as electrodes. The quantum efficiency for the photoinjection of electrons was determined to be 4.0×10^{-3} independent of the pH of the electrolyte and the dye surface concentration. With these data, an argument is given supporting the existence of excitation transfer among the dye molecules on the surface. A temperature study of the electron transfer efficiency yielded an activation enthalpy of 3.2±0.3 kcal/mole with a pre-exponential factor of 1.0. A heat of adsorption of 7.6±0.5 kcal/mole for this dye on TiO_2 single crystals was derived from analysis of adsorption isotherms measured at 21.5 and 36.5°C. In addition to the photo-oxidation process, a photoreduction was also observed upon cathodic polarization of the semiconductor. Action spectra revealed participation of solution dye molecules in this reaction; the reduction rate is dependent upon oxygen concentration in solution. It was concluded that the oxidized dye in solution accepts an electron from the TiO_2 to generate this current. This photoreduction was found with triphenylmethane and thiazine dyes as well as with other fluorescein derivatives.

6. ADSORPTION AND OXIDATION OF RHODAMINE B AT ZnO ELECTRODES

M. T. Spitler and M. Calvin

J. Chem. Phys. 67, 5193 (1977)

The adsorption of rhodamine B on the (0001) face of ZnO single crystals from an aqueous solution was monitored through measurement of the absorbance of the adsorbed dye. Analysis of these spectra yielded a Langmuir-type adsorption isotherm with a plateau surface concentration of 1.1×10^{-7} mmoles/cm^2. A comparison was made between the absorbance of the adsorbed dye and that of the dye in solution. These data were correlated with electrochemical measurements of the photo-oxidation of this dye at ZnO crystals used as electrodes. The quantum efficiency of photo-oxidation was 0.027, which was approximately constant as a function of surface coverage. A kinetic model was formulated for the time decay of the photocurrent.

HYDROCARBON-PRODUCING PLANTS AND THEIR MANIPULATION

Melvin Calvin, John W. Otvos, and James A Bassham

Solar energy conversion by the process of photosynthesis in green plants supplies virtually all the energy for living cells on earth. Agriculture, which can be defined as controlled photosynthesis to produce food and materials, has been used by man for millenia. Throughout most of history, combustion of photosynthetic products (e.g., wood and straw) has supplied a large part of the energy for cooking and heating, and this is still true in some less developed countries. In 1850, about 91% of the U.S. energy supply came from wood combustion whereas the present U.S. population probably relies on biomass sources for only about 1% of its energy needs.

During the past 150 years, industralized nations have depended increasingly on coal and later on petroleum and natural gas for energy and chemicals. These products of photosynthesis in past ages are being depleted rapidly. Within the next 25 years more than half of the proven world resources of petroleum and gas are expected to be consumed. It may take another century to approach consumption of half the coal reserves, but severe economic and environmental problems will be encountered in using that much coal. The potential use of other forms of fossil fuels such as those found in oil shales appears to be even more difficult.

It has been suggested[1-4] that certain plants rich in polyisoprenes and other hydrocarbon-like materials might be cultivated and grown as renewable sources of highly reduced photosynthetic products. Two distinctly different agricultural methods can be applied. Either we can harvest whole plants from a biomass plantation[5] or we can tap latex-containing plants as is done in the production of natural rubber. Two species, Euphorbia lathyrus and Euphorbia tirucalli, were selected for experimental plantations in 1977 (see Figure 1). The former is an annual with a one-year growth cycle while the tirucalli is a perennial with a two- to three-year growth period to initial harvest. The yields of hydrocarbon-like materials that can be extracted from E. lathyrus are equivalent to about 10 barrels per acre per year. This material consists of a variety of isoprenoids and some glycerides. The average molecular weight of the open chain isoprenoids is of the order of 10,000. The extractables include some cyclic diterpenes and triterpenes as well. In addition to the development of analytical procedures for the determination of the chemical composition of the latex, efforts are under way to devise extraction methods necessary to obtain the products desired. One aspect of the extraction research involves the isolation and determination of the irritant property

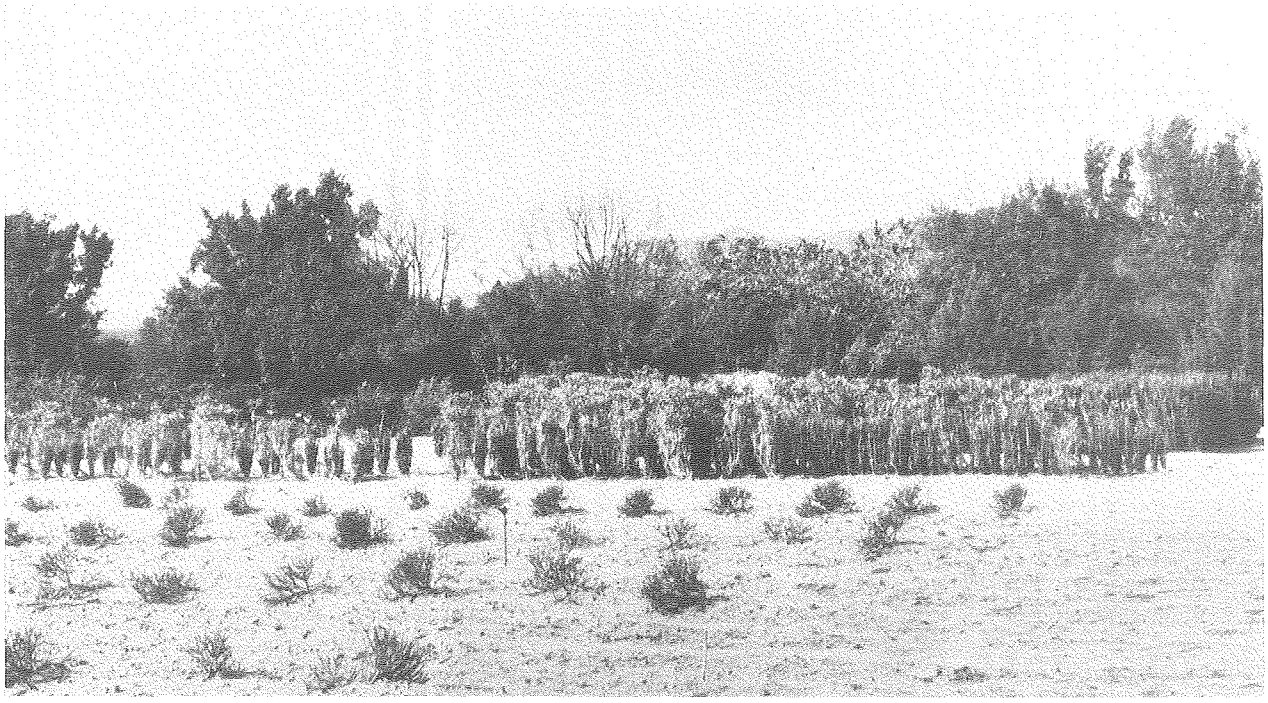

Figure 1. Southern California field station, showing both E. lathyrus and E. tirucalli, July 1978. (CBB 780-12927)

(a phorbol ester) present in the latex so that it can be eliminated by proper chemical treatment.

The yields of hydrocarbon-like materials already obtained from the wild stock appear to be economic[2] in terms of either fuel or chemical raw material production. However, it is obvious that genetic improvement of the plant materials is not only desirable but easily feasible on two levels: by clone and seed selection (conventional plant breeding) and by plant cell culture, cell selection, and plant regeneration. Conventional plant breeding can lead to improved yields of total biomass and of desired products. Other desirable physiological characteristics, such as ability to grow with limited rainfall and poor soil, and disease resistance, may also be sought. Obtaining plants with the full range of desired characteristics could take a long time, using conventional breeding, so it is important to apply methods of plant cell culture to achieve genetic modification. Moreover, such methods may permit rather specific tailoring of plant characteristics through purposeful changes in known metabolic pathways. For example, a better yield of more saturated polyisoprene hydrocarbons might be obtained by specific genetic modification.

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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 this laboratory's existence, 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 radio-autography. 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 nonradioactive (^{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]

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). 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 by the presence of 1 or 2 mM NH_4^+ in the extracellular environment.[2,3]

In parallel with these *in vivo* studies, the mechanisms of metabolic regulation are being studied by investigating the properties of the enzymes that catalyze the regulated steps. For example, it was found that the activity of the carboxylation enzyme, ribulose-1,5-bisphosphate carboxylase (RuBPCase), depends strongly on the order of binding of the substrates, CO_2 and RuBP, and of various other metabolites that act as allosteric effectors.[4] Pyruvate kinase has been isolated from green leaves and purified, and its regulatory properties are now being studied.

As another approach to the study of metabolism and its regulation, both whole 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. For example, we could demonstrate the regulation of glucose-6-phosphate oxidation by NADPH/$NADP^+$ levels and RuBP concentration.

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.[5] 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 (Pi) stays constant throughout the light-dark-light cycle. It was

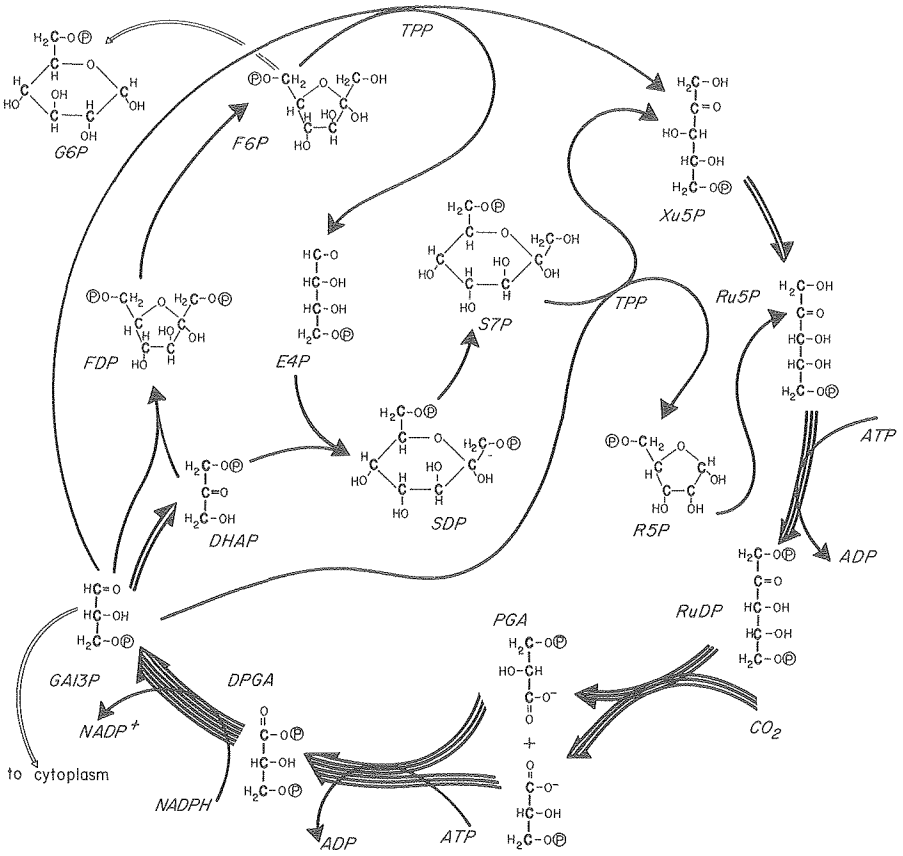

Figure 1. The Reductive Pentose Phosphate Cycle. The heavy lines indicate reactions of the RPP cycle; the faint lines indicate removal of intermediate compounds of the cycle for biosynthesis. The number of heavy lines in each arrow equals the number of times that step in the cycle occurs for one complete 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)

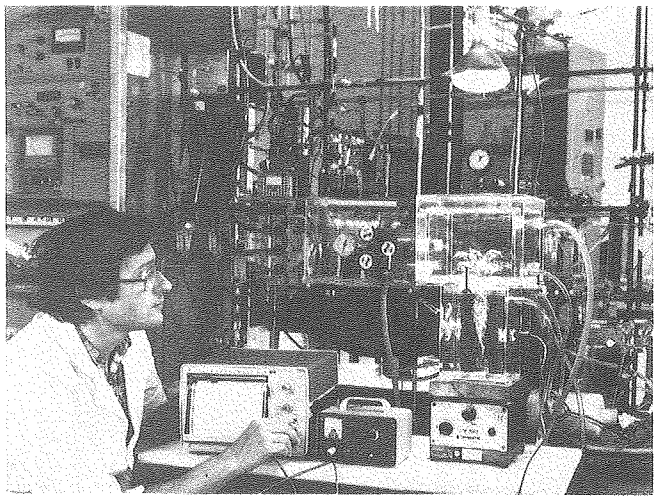

Figure 2. The "steady-state" apparatus--used for detailed investigations of the mechanisms and metabolic dynamics of photosynthesis. (CBB 784-4932)

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, Pi, and hexose monophosphates.

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.[6] 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.

Recently, this laboratory has commenced work on plant cells isolated from leaves.[7] These cells are kept under conditions in which they remain photosynthetically active for several

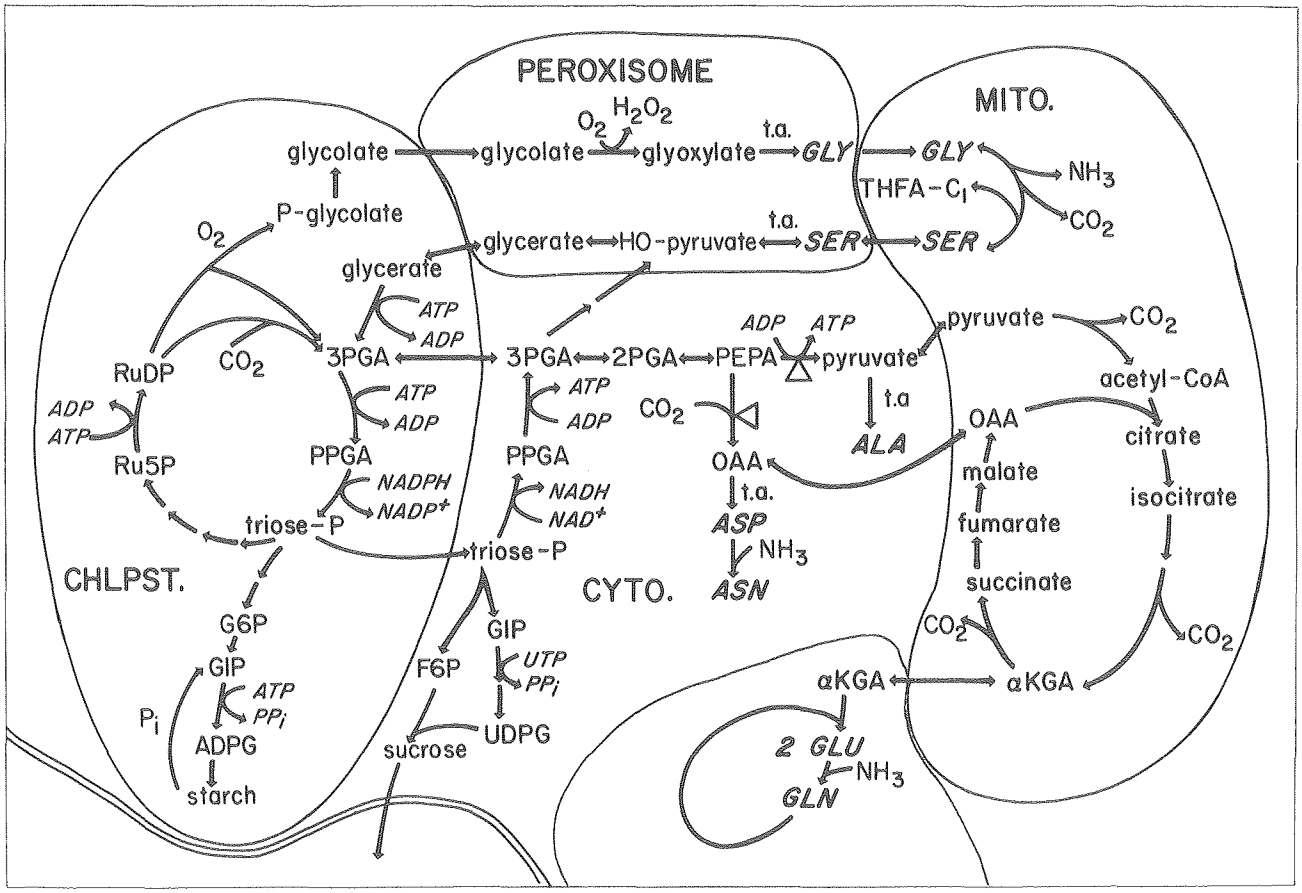

Figure 3. Metabolic pathways among subcellular organelles in green cells. Reduced carbon from photosynthesis in chloroplasts is exported as triose phosphate, PGA and glycolate. These metabolites are subsequently converted to other substances in the cytoplasm, peroxisomes and mitochondria. Abbreviations: 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)

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. As one preliminary finding, we have observed a stimulation of CO_2 incorporation as well as glutamine synthesis when 2,4-D is applied to leaf-free mesophyll cells.[8]

Isolated cells from the poppy have also been used in studies of the effect of sodium bisulfite on photosynthetic and biosynthetic metabolism.[9] Previous work with whole leaves and sulfur dioxide in this laboratory had indicated a complex set of effects of bisulfite and sulfur dioxide on leaf metabolism. Some further effects on chloroplast metabolism in isolated photosynthesizing spinach chloroplasts had also been seen. The advantage in working with the whole cells over leaves is that effects on acidity due to dissolving sulfur dioxide and effects on stomata can be bypassed and the action of bisulfite ion on cellular metabolism determined directly. It was found that short exposures to bisulfite resulted in actual stimulation of photosynthetic CO_2 incorporation.[9] An immediate and pronounced effect was a block in the conversion of glycolate to glyoxylate (evidenced by a rapid rise in glycolate and a rapid drop in glycine which is formed from glyoxylate) as has been previously reported elsewhere. An interesting and new finding was that there was an equally rapid drop in the conversion of hydroxypyruvate to glycerate as evidenced by a sudden rise in the level of serine (formed from hydroxypyruvate) and an equally rapid drop in the level of glycerate (Figure 4). The conversion of hydroxypyruvate to glycerate is another key step in the glycolate pathway thought to be responsible for photorespiration (Figure 3).

Cells from poppy, Papaver somniferum and Papaver brachteatum, have been placed in liquid suspension. These are being used in connection with studies on the biosynthesis of alkaloids (see following section). Callus culture of species of Euphorbia have been prepared, and will be transferred into liquid suspension. We plan to study metabolism of undifferentiated cells and the process of redifferentiation during hormone treatment by using ^{14}C-labeled-sucrose as a substrate for these cells, and by examining metabolic patterns and kinetics analogous to methods used with photosynthetic cells.

Parallel to our studies of the metabolism of whole cells and of subcellular organelles and cytoplasmic enzymes, we plan to examine

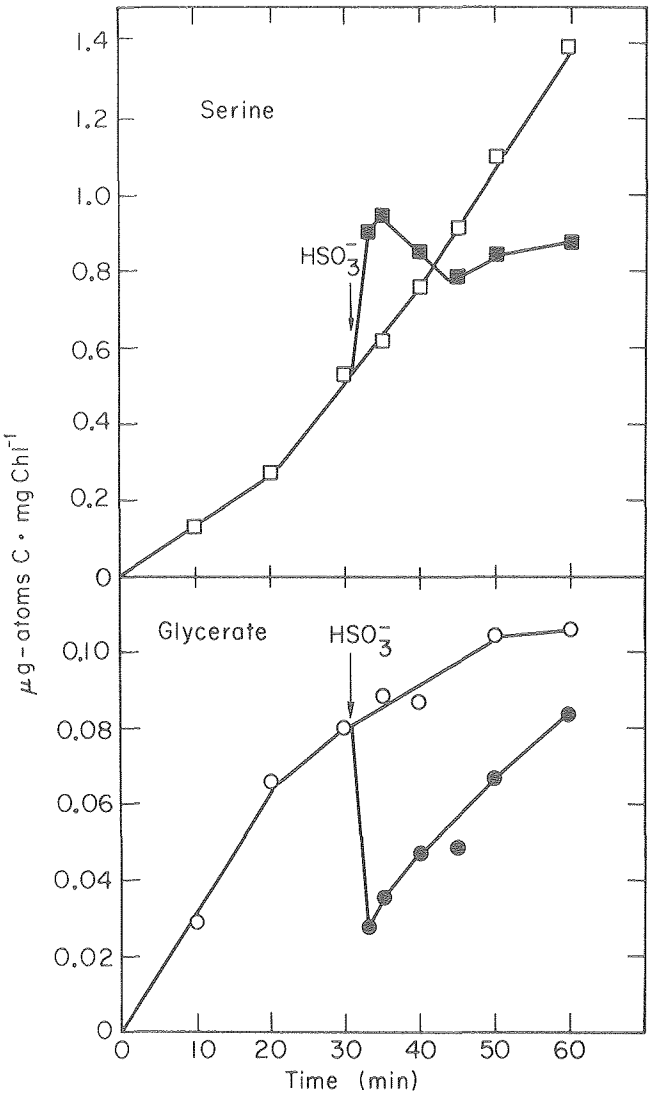

Figure 4. Effect of sulfite (HSO_3^- + $SO_3^=$) on the labeling of serine (■) and glycerate (●) pools. Treatment of the cells was as described in Figure 1, with respective pools being monitored in the presence (solid symbols) and absence (open symbols) of sulfite.

(XBL 7712-4068)

the morphological relationships of subcellular particles by electron microscopy of isolated cells and organelles. We are interested in such questions as proximity of chloroplasts to mitochondria, transport of metabolites between organelles, etc. Also, as experiments on differentiation of cells from tissue culture proceed, we will need to follow morphological changes.

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Abstracts

1. THE REDUCTIVE PHOSPHATE CYCLE AND ITS REGULATION

J. A. Bassham

Encyclopedia of Plant Physiology (New Series), Photosynthesis. Volume II, Regulation of Photosynthetic Carbon Metabolism and Related Processes, M. Gibbs and E. Latzko, eds. (Springer, in press).

The Reductive Pentose Phosphate Cycle (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. AMMONIA REGULATION OF CARBON METABOLISM IN PHOTOSYNTHESIZING LEAF DISCS

S. G. Platt, Z. Plaut, and J. A. Bassham

Plant Physiol. 60, 230-234 (1977)

Alfalfa (Medicago sativa L., var. El Unico) leaf discs, floating on buffer containing NH_4Cl and photosynthesizing with $^{14}CO_2$, produced more labeled amino acid and less sucrose than did control discs (no added NH_4Cl). The level of pyruvate increased and that of phosphoenolpyruvate decreased. These and other changes in levels of labeled compounds led us to conclude that pyruvate kinase was activated by ammonia, resulting in increased transfer of photosynthetically incorporated carbon to synthesis of amino acid skeletons at the expense of sucrose synthesis. Carbon flow through enzymes catalyzing the anaplerotic reactions was apparently stimulated.

*See Abstracts.

3. EFFECTS OF AMMONIA ON CARBON METABOLISM IN PHOTOSYNTHESIZING ISOLATED MESOPHYLL CELLS FROM PAPAVER SOMNIFERUM L.

J. S. Paul, K. L. Cornwell, and J. A. Bassham

Planta 142, 49-54 (1978)

Addition of ammonia to a suspension of photosynthesizing leaf-free mesophyll cells from Papaver somniferum quantitatively alters the pattern of carbon metabolism by increasing rates of certain key rate-limiting steps leading to amino acid synthesis and by decreasing rates of rate-limiting steps in alternative biosynthetic pathways. Of particular importance is the stimulation of reactions mediated by pyruvate kinase and phosphoenolpyruvate carboxylase. The increased rates of these two reactions which result in an increased flow of carbon into the tricarboxylic acid cycle correlate with a rapid rise in glutamine (via glutamine synthetase) which draws carbon off the tricarboxylic acid cycle as α-ketoglutarate. Increased flux of carbon in this direction appears to come mainly at the expense of sucrose synthesis. The net effect of addition of ammonia to mesophyll cells is thus a redistribution of newly fixed carbon away from carbohydrates and into amino acids.

4. IN VIVO CONTROL MECHANISM OF THE CARBOXYLATION REACTION

J. A. Bassham, S. Krohne, and K. Lendzian

Photosynthetic Carbon Assimilation (Ribulose 1,5-Biphosphate Carboxylase/Oxygenase), H. W.Siegelman and G. Hind, eds. (Basic Life Sciences Vol. II, Plenum Press, New York, 1978), pp. 77-93.

That both the synthesis and the activity of the enzyme ribulose 1,5-bisphosphate carboxylase (RuBPCase) are regulated is understandable in terms of the role of this important enzyme in photosynthetic metabolism. This enzyme catalyzes the first step in the incorporation of CO_2 and first reactions are often sites of regulation. The carboxylation reaction is one of four reactions of the Calvin cycle that are unique to that cycle and not found in the oxidative pentose phosphate cycle, which also can operate in the chloroplast in the dark. The rate of the RuBPCase catalyzed reaction must be balanced against the rates of other rate limiting steps in order to maintain acceptable concentrations of Calvin cycle intermediates, triose phosphate and hexose phosphates, which are withdrawn from the cycle for subsequent biosynthesis. A further regulatory requirement is placed on RuBPCase by its oxygenase activity. Thus, under conditions of bright light, the presence of oxygen, high temperatures, and very low levels of CO_2, which would favor the oxygenase activity, levels of RuBP can build up, bind to the enzyme, and convert it to a form that has a higher binding constant for CO_2 as well as oxygen, thus minimizing the effect of oxygenase.

It appears that the activity of the enzyme responds to all of these requirements in one way or another. The principal regulation of the enzyme, particularly between light and dark, appears to be via the combination of an increase in both magnesium ion concentration and pH in the stroma region in the light. Further activation of the enzyme in the light can be accomplished by physiological levels of either 6-phosphogluconic acid or of NADPH. Finally, there is the possibility for inactivation of the enzyme by high levels of RuBP in the absence of CO_2, already mentioned. However, as reported elsewhere in this meeting, the latter inactivation may be due to an impurity that forms when there is free RuBP present. Experiments with reconstituted chloroplasts, lacking their limiting membrane, demonstrate that high levels of photosynthetic CO_2 fixation can be maintained for up to an hour with air levels of CO_2. Thus far it has not been possible to maintain such high levels of CO_2 fixation in the dark by administering conditions of magnesium and pH as well as added NADPH thought to exist under light conditions in intact chloroplasts. Perhaps this is due to inhibition by the amounts of RuBP or of its contaminating inhibitor that have to be added in such experiments.

5. LIGHT-DARK REGULATION OF STARCH METABOLISM IN CHLOROPLASTS, I. LEVELS OF METABOLITES IN CHLOROPLASTS AND MEDIUM DURING LIGHT-DARK TRANSITION

W. M. Kaiser and J. A. Bassham

Plant Physiol., in press

The kinetics of CO_2 fixation, of starch formation and of changes in the levels of metabolites in chloroplasts and the surrounding medium have been investigated during light-dark and dark-light transitions with isolated intact chloroplasts.

The internal level of Pi stays constant throughout a light-dark-light cycle. The concentration of 3-phosphoglycerate in the chloroplasts decreases (from about 4 mM in the light to 1.6 mM in the dark within 3 min), whereas the level of the hexosemonophosphates increases at the same time (from 2.2 mM in the light to about 6 mM in the dark). In the subsequent light period both compounds reach their original levels within 3 min. The chloroplastic concentrations of dihydroxyacetone phosphate, of the pentose monophosphates and of the hexose- and heptose-bisphosphates remain constant at about 0.4 mM throughout the light-dark-light cycle.

In the medium, the concentration of 3-phosphoglycerate increases and dihydroxyacetone phosphate decreases in the dark phase: this is due to an exchange of internal 3-phosphoglycerate for external dihydroxyacetone phosphate. Part of the reimported dihydroxyactone 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 trans-envelope concentration gradients of 3-phosphoglycerate, dihydroxyacetone phosphate and Pi which exist in the light, is completely abolished after 2-3 min 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.

6. 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., in press

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

A change in 3-phosphoglycerate concentration (from 4 mM to 1.4 mM, as in whole chloroplasts during light-dark transition) decreases the rate of ADP-glucose formation 6-7 fold. An increase in hexose monophosphate concentration from 2 mM to 6 mM, which occurs at the same time in whole chloroplasts, stimulates ADP-glucose formation only slightly.

At constant levels of Pi (4 mM) and 3-phosphoglycerate (4 mM), a change in ATP concentration from 0.2 mM to 1 mM causes an immediate 4-5 fold increase in the rate of ADP-glucose formation.

Another significant stimulation of ADP-glucose formation (about 4-6 fold) is obtained after addition of DTT at high concentrations (50 mM).

A simultaneous increase in the concentrations of 3-phosphoglycerate, ATP and DTT, with Pi and Mg^{2+} being constant at 4 mM and 5 mM, respectively, causes a 130-fold increase in the rate of ADP-glucose formation (from 0.042 to 5.49 μg-atoms carbon/mg chlorophyll x h).

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

7. MAINTENANCE OF HIGH PHOTOSYNTHETIC RATES IN MESOPHYLL CELLS ISOLATED FROM PAPAVER SOMNIFERUM

J. S. Paul and J. A. Bassham

Plant Physiol. 60, 775-778 (1977)

The establishment and maintenance of high rates of photosynthetic CO_2 incorporation in mesophyll cells of Papaver somniferum (opium poppy) depend on a regime of dark and light periods immediately following isolation, as well as carefully adjusted conditions of isolation. Analysis of the incorporation pattern of $^{14}CO_2$ by the isolated cells indicates an initial "stress-response" period of approximately 20 hours characterized by increased respiratory-type metabolism and diminished photosynthesis. Under the favorable regime, this period is followed by rapid recovery and the reinstatement of a metabolic state strikingly similar to that of intact leaves in which the initial rate of CO_2 incorporation is between 110 and 175 μ moles CO_2 fixed per mg chlorophyll per hour. The photosynthetic viability of these cells can be maintained for up to 80 hours.

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., in press.

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 stimulation of glutamine synthesis. Evidence is presented that the rise in glutamine is due to increased reduction of nitrate to ammonia.

9. EFFECTS OF SULFITE ON METABOLISM IN ISOLATED MESOPHYLL CELLS FROM PAPAVER SOMNIFERUM

J. S. Paul and J. A. Bassham

Plant Physiol. 62, 210-214 (1978)

Exposure (30 minutes) of leaf-free mesophyll cells from the C-3 plant, Papaver somniferum, to concentrations of sulfite ($SO_2 + HSO_3^- + SO_3^=$) up to 20 millimolar stimulated the rate of CO_2 incorporation as much as 30%. The sulfite rapidly affects the metabolism of newly incorporated CO_2. Ammonia incorporation into glutamine and subsequent transamination reactions were stimulated during the short term exposure periods while glycolate metabolism apparently was inhibited by bisulfite at two points in the pathway. The results further indicate that glycolate is the major precursor of glycine in these cells. Prolonged periods of exposure (24 hours) to sulfite had somewhat different effects on carbon metabolism: the high concentrations (10 to 20 millimolar) severely inhibited all concentrations (1 millimolar) appeared to inhibit ammonia incorporation but stimulated synthesis of sucrose and starch.

BIOSYNTHESIS AND FUNCTION OF ALKALOIDS

Henry Rapoport

Alkaloids are found in about 10% of the plant species investigated. Although many of these alkaloids have found useful application in 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.

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. 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.[1]

In Nicotiana, the biosynthesis of nicotine has been shown to be quite complex, with probably two pathways involved.[2] 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.[3-5]

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Abstracts

3. MORPHINAN ALKALOIDS IN PAPAVER BRACTEATUM: BIOSYNTHESIS AND FATE

C. C. Hodges, J. S. Horn, and H. Rapoport

Phytochemistry 16, 1939-1942 (1977)

The known metabolic pathway for hydrophenanthrene alkaloids in Papaver somniferum has been examined for occurrence in P. bracteatum, a species reported to contain thebaine but no codeine or morphine. 1,2-Dehydroreticulinium-[3-^{14}C] chloride and (±)-reticuline-[3-^{14}C] were fed to P. bracteatum plants and both were incorporated, the former into reticuline and thebaine and the latter into thebaine, suggesting that thebaine biosynthesis is the same in the two species. Studies of the natural abundance of morphinan alkaloids in P. bracteatum and the results from feeding codeinone-[16-^{3}H] and codeine-[16-^{3}H] indicate that this species can reduce codeinone to codeine but can not perform either of the demethylations to produce codeinone or morphine. Fed thebaine-[16-^{3}H] was substantially metabolized but not by pathways that involved demethylations to either oripavine or northebaine.

*See Abstracts.

4. ROLE OF 1,2-DEHYDRORETICULINIUM ION IN THE BIOSYNTHETIC CONVERSION OF RETICULINE TO THEBAINE

P. R. Borkowski, J. S. Horn, and H. Rapoport

J. Am. Chem. Soc. 100, 276 (1978)

The role previously assigned to 1,2-dehydroreticulinium ion as a precursor to the morphinan alkaloids in Papaver somniferum was based on feeding experiments with a synthetic compound of uncertain identity. We have now prepared authentic 1,2-dehydroreticulinium chloride and shown its efficient incorporation into the morphinan alkaloids, supporting the previous hypothesis. Moreover, using a double-label technique and steady-state $^{14}CO_2$ biosynthesis, we have determined that 1,2-dehydroreticulinium ion is a natural product whose native pool size is about one-fifth that of reticuline. These data clearly establish 1,2-dehydroreticulinium ion as an intermediate in morphinan alkaloid biosynthesis.

5. BIOSYNTHETIC CONVERSION OF THEBAINE TO CODEINONE. MECHANISM OF KETONE FORMATION FROM ENOL ETHER IN VIVO

J. S. Horn, A. G. Paul, and H. Rapoport

J. Am. Chem. Soc. 100, 1895 (1978)

Biosynthesis of morphinan alkaloids proceeds by conversion of the enol ether of thebaine to the keto group of neopinone and thence to codeinone. To determine the mechanism of this transformation, [G-^{14}C,6-^{18}O]thebaine was fed to Papaver somniferum and the codeine and morphine were isolated. Comparison of the $^{18}O/^{14}C$ ratios in the codeine and morphine isolated with that of the thebaine fed showed that 34% of the ^{18}O had been retained. Parallel feedings with [G-^{14}C,6-^{18}O]-codeinone demonstrated that the loss was due to nonenzymic exchange. Thus, the mechanism of enol ether cleavage in thebaine is established as cleavage of the 6-O-methyl group with retention of the 6-oxygen in the codeinone.

BIOPHYSICS

Melvin P. Klein

The complex interdependence between structure and function in biological systems provides the impetus for examining the detailed structures and dynamics of molecules and more highly organized assemblies in order to gain a deeper understanding of function. This laboratory employs a wide variety of spectroscopic techniques to obtain information about the structures of biological molecules and about the changes that occur when the molecules participate in their normal function. Model systems are studied extensively; their spectroscopic properties, often amenable to theoretical interpretation, provide the keys for understanding the spectra of the complex biological systems.

During the past several years we have studied extensively the solution conformations of the cyclic hexapeptides, the ferrichromes. These molecules serve as iron-complexing and membrane transporters and have provided fertile examples of the nuclear magnetic resonance features of peptides and proteins. The most recent study[1] shows that the mean inter-proton distances in this class of molecules can be determined from NMR relaxation rates. It also indicates some cautions that must be exercised in interpretations of nuclear Overhauser effects.

Composed primarily of lipids with the addition of proteins and carbohydrates, membranes occupy a central position in a wide variety of cellular functions; hence their structures and related functions comprise an area of active research. The lipid fraction (largely phospholipids) is composed of an assemblage of small molecules that can associate spontaneously to form an organized structure in the form of a bilayer. There is general concurrence that this bilayer formation persists in intact cellular membranes. Because these molecules are not static at physiological temperatures, their structures are largely inaccessible to diffraction methods. Using nuclear magnetic resonance (NMR) methods, however, we have been able to deduct structural and structural dynamic information on model membrane systems. By means of information gained from model systems, together with the development of double resonance methods, these studies are being extended to the membranes of intact cells. Their aim is to probe the differences between the surface properties of normal cells grown in tissue culture and those transformed by oncogenic viruses and chemical carcinogens. The nature of the changes that occur in nerve membranes upon depolarization or propagation of the action potential are also being studied by NMR methods to extend information obtained from optical studies.[2,3]

Most of the world's usable nitrogen is supplied by a variety of nitrogen-fixing microorganisms that convert atmospheric N_2 to reduced forms. The enzyme system responsible, called nitrogenase, is composed of two proteins; the smaller member of the pair contains four non-heme iron atoms while the larger member contains two Mo atoms and about thirty non-heme Fe atoms. It is noteworthy that all known enzymes that act to reduce or oxidize nitrogen contain Mo. In almost all instances the role of these essential Mo atoms is unknown because they are generally inaccessible to observation by conventional spectroscopic methods. Similarly, the world's supply of oxygen is converted by green plant photosynthesis from water to O_2. While a detailed knowledge of the pathway of carbon in CO_2 fixation by plants has been obtained, there is only scant information available on the pathway and mechanisms of oxygen evolution and nitrogen fixation.

It has long been recognized that Mn atoms are essential for photosynthetic oxygen evolution but, again, the role of this element has eluded study by conventional spectroscopies.

This laboratory has recently made significant advancements in devising methods to examine the Mo atoms in nitrogenase and the Mn atoms involved in photosynthetic oxygen evolution. The method is based on analyzing the detailed structure of the x-ray absorption spectrum of the respective elements. These x-ray spectra exhibit features that reflect the formal charge or oxidation state of the element, the symmetry of the ligands, and the distances between the element under study and the atoms in its first, second, and sometimes third coordination shells. The measurements are carried out using the Stanford Synchrotron Radiation Laboratory, a national facility at the Stanford Linear Accelerator Center (SLAC). Measurements of this type have provided the first direct observations of the Mo atoms in the nitrogenase enzyme system and of the Mo atoms in intact nitrifying microorganisms. They suggest no change in the coordination about Mo upon binding N_2.[4] Similarly, these experiments have yielded spectra of Mn atoms in intact leaves and in chloroplasts. There is evidence in these spectra for the presence of Mn atoms in groups of two or more.[5,6]

These experiments will be supplemented using flash-illuminated samples to seek changes in the Mn oxidation states and ligation. In all cases, these experiments were possible because of techniques developed in this laboratory which extended the sensitivity to that required for investigation of *in vivo* biological systems.[7] This new class of spectroscopy is of wide generality and has also been applied to the study of atmospheric pollution and intercalation compounds.[8,9]

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Abstracts

1. AMIDE PROTON SPIN-LATTICE RELAXATION IN POLYPEPTIDES: A FIELD-DEPENDENCE STUDY OF THE PROTON AND NITROGEN DIPOLAR INTERACTIONS IN ALUMICHROME

M. Llinas, M. P. Klein and K. Wüthrich

Biophys. J. 24, 849-862 (1978)

The proton nuclear magnetic resonance (NMR) spin-lattice relaxation of all six amides of deferriferrichrome and of various alumichromes dissolved in hexadeutero-dimethylsulfoxide have been investigated at 100, 220, and 360 MHz. We find that, depending on the type of residue (glycyl or ornithyl), the amide proton relaxation rates are rather uniform in the metal-free cyclohexapeptide. In contrast, the ^1H spin-lattice relaxation times (T_1's) are distinct in the Al^{3+}-coordination derivative. Similar patterns are observed in a number of isomorphic alumichrome homologues that differ in single-site residue substitutions, indicating that the spin-lattice relaxation rate is mainly determined by dipole-dipole interactions within a rigid molecular framework rather than by the specific primary structures. Analysis of the data in terms of ^1H-^1H distances (r) calculated from X-ray coordinates yield a satisfactory linear fit between T_1^{-1} and Σr^{-6} at the three magnetic fields. Considering the very sensitive r-dependence of T_1, the agreement gives confidence, at a quantitative level, both on the fitness of the crystallographic model to represent the alumichromes' solution conformation and on the validity of assuming isotropic rotational motion for the globular metallopeptides. An extra contribution to the amide proton T_1^{-1} is proposed to mainly originate from the ^1H-^{14}N dipolar interaction: this was supported by comparison with measurements on an ^{15}N-enriched peptide. The nitrogen dipolar contribution to the peptide proton relaxation is discussed in the context of $\{^1H$ --$^1H\}$ nuclear Overhauser enhancement (NOE) studies because, especially at high fields, it can be dominant in determining the amide proton relaxation rates and hence result in a decreased effectiveness of the ^1H--^1H dipolar mechanism to cause NOE's. From the slope and intersect values of T_1^{-1} vs. Σr^{-6} linear plots, a number of independent estimates of τ_r, the rotational correlation time, were derived. These and the field-dependence of the T_1's yield a best estimate $\langle\tau_r\rangle \approx 0.37$ ns, in good agreement with $0.38 \text{ ns} \lesssim \langle\tau_r\rangle \lesssim 0.41$ ns, previously determined from ^{13}C and ^{15}N spin-lattice relaxation data.

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*See Abstracts.

2. ^{31}P NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF INTACT CELLULAR MEMBRANE SYSTEMS: INVESTIGATIONS OF GARFISH OLFACTORY NERVE, RAT HEPATOCYTES, AND CHICK EMBRYO FIBROBLASTS,

S. J. Kohler, C. M. Oshiro, and M. P. Klein

Submitted to Biochemistry

Until quite recently the majority of attempts to study biological membranes using nuclear magnetic resonance (NMR) spectroscopy have resulted in spectra with broad, relatively featureless lines, which were in general quite difficult to interpret. The broadness of the lines was primarily due to dipolar interactions, and the recent use of higher magnetic fields, strong decoupling power, and sophisticated multiple pulse techniques has shown it to be possible to remove the dipolar broadening in these systems and reveal the previously hidden chemical shielding information. It is demonstrated that the chemical shielding information in ^{31}P NMR spectra of unsonicated phospholipid bilayers may be interpreted in a straightforward manner to give detailed information about the motion of the phosphate headgroup of the phospholipids. Spectra obtained from whole cells and their membranes resemble those of unsonicated phospholipid multilayers and suggest that in the biological systems the degrees and types of motion are similar to those in the model systems. We find that there are essentially no differences in membrane mobility between the membranes of normal and transformed chick embryo fibroblasts. Preliminary findings suggest that there are differences between the polarized and depolarized membranes in the garfish olfactory nerve.

3. EFFECTS INDUCED BY VARIOUS DIVALENT INORGANIC CATIONS ON THE ^{31}P CHEMICAL SHIELDING ANISOTROPY OF UNSONICATED PHOSPHOLIPID BILAYERS

C. Goulon-Ginet, S. J. Kohler, and M. P. Klein

Submitted to Biochemistry

Qualitative observations of the effects of various divalent cations on the proton-decoupled ^{31}P NMR spectra of unsonicated phospholipid dispersions are reported. Specific interactions between calcium ions and brain extract phosphatydyl serine (PS) dispersions are shown to induce a significant immobilization of the phosphodiester headgroup, a situation that apparently does not occur under similar experimental conditions if the calcium ions are replaced by Mg^{2+}, Sr^{2+}, Ba^{2+} or Cd^{2+}. The diamagnetic uranyl ions are also shown to hinder strongly the motion of the phosphate groups in both brain extract PS and DPPC dispersions. For the latter well-defined system, the temperature and ion concentration dependence of the line broadening are reported. A correlation is suggested between the changes of the observed ^{31}P chemical shielding anisotropy and the corresponding shifts of the phase transition temperature. These results are discussed with respect to the most recent experimental evidence for such ion-phospholipid interactions.

4. MOLYBDENUM X-RAY ABSORPTION SPECTRUM OF NITROGENASE IN THE PRESENCE OF DINITROGEN

J. P. Smith, J. A. Kirby, M. P. Klein, A. S. Robertson, and A. C. Thompson

Submitted to Proc. Natl. Acad. Sci. U.S.A.

The molybdenum x-ray absorption spectra of solutions of the molybdenum-iron protein of nitrogenase equilibrated with argon or dinitrogen as the gas phases have been compared. The Mo in nitrogenase is often thought to function as a site for the coordination of substrates such as dinitrogen. The results presented in this paper show that the presence of dinitrogen does not result in any significant changes in either the absorption edge spectrum or the extended x-ray absorption fine structure (EXAFS) of the Mo in nitrogenase. These results suggest that the presence of substrate does not cause any significant perturbations of the coordination environment of the Mo. Analysis of the EXAFS data by Fourier transformation and curve fitting show substantial agreement with the model for the Mo site in nitrogenase proposed by Cramer et al. [J. Am. Chem. Soc. 100, 3398-3407 (1978)]. Due to the low signal-to-noise ratio, the present EXAFS data are not precise enough to completely rule out the possibility that dinitrogen is entering the coordination environment of Mo under the conditions of these measurements.

5. OBSERVATION OF THE MANGANESE SITES IN THE PHOTOSYNTHETIC APPARATUS BY X-RAY ABSORPTION SPECTROSCOPY USING SYNCHROTRON RADIATION

J. A. Kirby, A. S. Robertson, J. P. Smith, A. C. Thompson, and M. P. Klein

Submitted to Proc. Natl. Acad. Sci. U.S.A.

Manganese atoms have been implicated as essential elements in photosynthetic oxygen evolution. It has been postulated that these atoms occur in clusters and that their wide range of oxidation states permit them to act as loci for the storage of oxidizing equivalents generated by light acting at photosystem II in green plants and algae. Heretofore, direct observation of the Mn in vivo has been precluded by the absence of any spectroscopic features assignable directly and specifically to Mn. Accordingly, nothing is known about the coordination of Mn, the number or numbers of Mn atoms that are involved, nor the oxidation state or states of the metals in either dark adapted or illuminated specimens. This paper reports the first direct observations of the Mn atoms in samples of chloroplasts from Spinacea olcifera by x-ray absorption spectroscopy. Analysis

of the extended x-ray absorption fine structure (EXAFS), based on calibrations against reference compounds, permits a preliminary determination of the coordination of the Mn. If we assume that the first coordination shell contains carbon, nitrogen, or oxygen ligands, the distances lie between 1.73 and 1.86 A. The data are consistent with a second coordination shell containing chlorine or sulfur at 2.20 A. Located at 2.80 A is an element of the first transition series. Identical samples of chloroplasts subjected to tris washing and osmotic rupture to remove the less tightly bound Mn yield EXAFS features whose Fourier transforms show a gross rearrangement of the coordination about the remaining manganese. The intensity of the feature at 2.80 A decreases markedly, suggesting that it originates from another Mn atom. Accordingly, we conclude from the preliminary results that the Mn atoms occur in clusters of two or more. The energy of the K-absorption edge of the tris-treated chloroplasts is 5 eV lower than that of the intact chloroplasts, suggesting that there are differences in the symmetry, oxidation state, or both, of the Mn atoms in the two classes of samples. Alternatively, there may be a distribution of such states in the intact chloroplasts that remains to be determined. These experiments show that additional information concerning the putative involvement of Mn at the oxygen evolving site may be probed by x-ray spectroscopy and such experiments are currently in progress. Specifically, we are seeking changes in the oxidation state or states of the Mn atoms following successive flashes of light while simultaneously seeking to determine changes in ligation that may be attributable to oxygen and chlorine ligands entering and leaving the Mn coordination sphere. The experiments were performed at the Stanford Synchrotron Radiation Laboratory.

6. MIXED VALANCES INTERACTIONS IN DI-μ-OXO BRIDGED MANGANESE COMPLEXES, EPR AND MAGNETIC SUSCEPTIBILITY STUDIES

S. R. Cooper, G. C. Dismukes, M. P. Klein, and M. Calvin

J. Am. Chem. Soc. 100, 7248-7252 (1978)

EPR examination of the class II (deeply trapped) mixed valence complexes $[(bipy)_2MnO_2\ Mn(bipy)_2]^{3+}$[the bipyridl (III,IV) dimer] and its phenanthroline analogue in acetonitrile solution verifies that these complexes possess inequivalent Mn ions at room temperature. Isotropic hyperfine structure for two Mn ions is resolved with $A_1 = 167 \pm 3G$ and $A_2 = 79 \pm 3G$ for both complexes. The hyperfine pattern with $|A_1| \cong 2|A_2|$ and the small g anisotropy are consistent with high spin Mn(III) antiferromagnetically coupled to Mn(IV), producing an S = 1/2 ground state. At room temperature a rate of less than 10^8 sec^{-1} is estimated for the thermally activated intramolecular electron transfer, consistent with an upper limit of 10^6 sec^{-1} calculated from Hush's theory. The magnetic susceptibility of the (III,IV) complexes is characteristic of a strongly antiferromagnetically coupled S = (2,3/2) pair. The temperature dependence of the data was in good agreement with the isotropic Heisenberg exchange Hamiltonian $H = -2JS_1S_2$, yielding $J = -150 \pm 7$ cm^{-1} for the bipyridyl (III,IV) dimer and $J = 134 \pm 5$ cm^{-1} for the phenanthroline analogue.

7. FLUORESCENCE DETECTION OF EXAFS: SENSITIVITY ENHANCEMENT FOR DILUTE SPECIES AND THIN FILMS

J. Jaklevic, J. A. Kirby, M. P. Klein, A. S. Robertson, G. S. Brown, and P. Eisenberger

Solid State Commun. 23, 679-682 (1977)

The fluorescence intensity is used to measure the X-ray absorption cross section and is found to yield essentially the same results as a more conventional transmission experiment. However, the fluorescence method is shown to extend the sensitivity of the EXAFS technique by two or more orders of magnitude, and thus make feasible the study of extremely dilute species.

8. NOVEL SALTS OF GRAPHITE AND A BORON NITRIDE SALT

N. Bartlett, R. N. Biagioni, B. W. McQuillan, A. S. Robertson, and A. C. Thompson

J. Chem. Soc. Chem. Commun., 200-201 (1978)

Graphite is oxidized by $O_2^+AsF_6^-$ and by OsF_6 to give first-stage graphite salts $C_8^+MF_6^-$; $S_2O_6F_2$ oxidizes both graphite and boron nitride to yield the salts $C_{12}^+SO_3F^-$ and $(BN)_4^+SO_3F^-$m,the latter being the first example of a first-stage boron nitride salt.

9. THE SYNTHESIS OF THE FIRST STAGE GRAPHITE SALT $C_8^+AsF_6^-$ AND ITS RELATIONSHIP TO THE FIRST STAGE GRAPHITE/AsF_5 INTERCALATE

N. Bartlett, B. McQuillan, and A. S. Robertson

Mater. Res. Bull. 13 (11), (1978)

Oxidation of graphite with excess $O_2^+AsF_6^-$, in suspension in SO_2ClF, produces the blue first-stage graphite salt of composition C_8AsF_6, which X-ray single crystal photographs show is hexagonal with a = 4.92(2), c = 8.10(2), V = 170 A^3. The blue first-stage material of approximate composition $C_{10}AsF_5$ obtained from graphite and AsF_5 has a related pseudo cell. Arsenic X-ray absorption-edge spectra show that C_8AsF_6 contains AsF_6^- alone, and that the graphite/AsF_5 intercalate contains AsF_6^- and AsF_3 in accord with the AsF_5 reduction:

$$3\ AsF_5 + 2\ e^- \longrightarrow 2\ AsF_6^- + AsF_3\ .$$

Treatment of the graphite/AsF_5 compound with F_2 gas results in conversion of all of the intercalated arsenic to AsF_6^-.

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.

One approach we are using is to attempt to correlate known base sequences in DNA with known functions. The bacteriophage ΦX174 has been completely sequenced and many important sites have been identified. These include the origin of replication, three promoter regions where RNA polymerase starts synthesizing messenger RNA (mRNA), five termination regions where mRNA synthesis stops, and nine ribosome binding sites on the mRNA molecules where protein synthesis starts. We have found simple algorithms that allow a computer search of the 5386 bases in ΦX174 to locate the sites for termination[1] and initiation of mRNA synthesis in this viral DNA.

Termination sequences in ΦX174 and other DNAs are known to contain a G·C rich region and to be followed by a consecutive sequence of thymines. We proposed a simple algorithm (recipe) that predicts five, and only five, termination sequences in ΦX174 (Figure 1). The location of the five termination signals are approximately known in ΦX174 and the location of our sequences are completely consistent. The algorithm is based on our previous work which showed that a good way to understand the structure of DNA is to consider the sequence two base-pairs (a doublet) at a time. The termination sequence that is necessary and sufficient for ΦX174 is: a $(\frac{GG}{CC})$ double-stranded doublet is followed by any base pair and then five of the seven following doublets must be $(\frac{AA}{TT})$. This algorithm has significant implications. It is interesting that $(\frac{GG}{CC})$ is the sequence that provides the most stability for DNA, whereas $(\frac{AA}{TT})$ is the weakest. Thus, the sequence of the DNA strand being copied is not the important factor. It may contain either two C's or two G's followed by a run of either A's or T's or both. The important factor seems to be the stability of the DNA double strand.

The algorithm for the promoter sequences is more difficult to understand in terms of DNA structure. However, by studying other DNA sequences we hope to be able to generalize the algorithms and to understand what factors control RNA synthesis.

Other computer analyses of base sequences involve: (1) attempts to predict likely sites for mutational events such as addition, deletion, inversion and repetition of DNA sequences, (2) identification of active sites for frame-shift mutation, (3) location of sequences that can form cruciforms, (4) prediction of folding in mRNA and transfer RNA, and (5) location of sites where processing of precursor RNAs occurs. The prediction of folding in RNA is based on our earlier thermodynamic studies on RNA-model oligonucleotides. Data have been obtained for base pairing, which favors double-strand formation, and for the effect of unbonded bases in loops or bulges, which reduce double-strand formation.[2-4] The predictions can be tested and our methods improved by direct comparisons with the experimental studies of Professor John Hearst on RNA folding.

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.

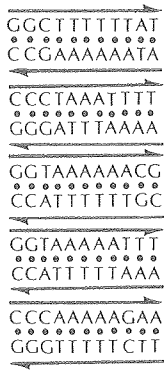

Figure 1. The five proposed termination signals in the DNA of the virus ΦX174. This is where the synthesis of messenger RNA transcribed from the DNA stops. In each sequence the top DNA strand is being copied (as RNA) from left to right. The locations of these terminators are in good agreement with experiment.

At present we are studying the kinetics and thermodynamics of bulge formation using temperature-jump kinetic methods.[5] The rate of formation of a double strand containing a bulge, and the lifetime of a conformation with a bulged base are important factors that affect the probability of frame-shift mutation.

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-N-oxide and mono-nucleotides.[6] Similar studies have shown that ethidium, another mutagen, definitely facilitates the formation of bulges in double strands.

The structures found for complexes between ethidium and model oligonucleotides[7] are shown in Figure 2. They provide a model for the mechanism of frame-shift mutagenesis by molecules that do not react covalently with DNA. Further studies on the sequence specificity of mutagens, and the structures of the complexes, can help provide a mechanism of mutation.

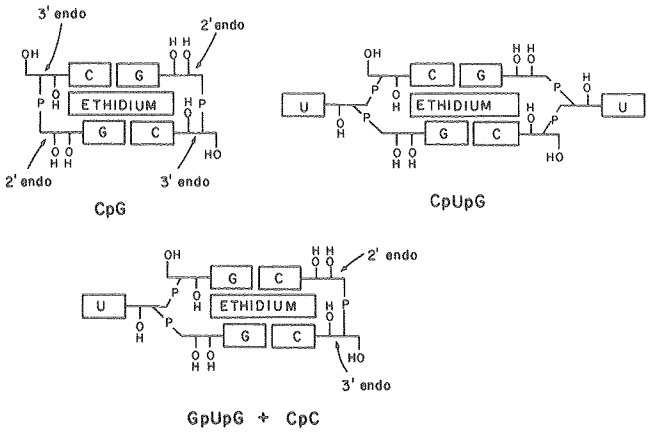

Figure 2. Proposed structures in aqueous solution for complexes containing ethidium. In these complexes the 3' linked sugar of each dinucleoside phosphate is in the 3' endo conformation whereas the 5' linked sugar is in the 2' endo conformation. (XBL 782-7157)

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Abstracts

1. SEQUENCES AND EFFICIENCIES OF PROPOSED mRNA TERMINATORS

J. E. McMahon and I. Tinoco, Jr.

Nature (London) 271 (5642), 275-277 (1978)

The base sequences at the 3' end of the different messenger RNA molecules show a pattern of a GC-rich region followed by five to eight Us. Gilbert's hypothesis that the stability of the template duplex DNA (or of the mRNA-DNA hybrid, or both) determines termination of transcription, implies that the DNA termination sequences, and thus the mRNA sequences at the 3' end, are not unique. Rather, the DNA sequence of the template presumably expresses a certain structural stability, and this pattern of stabilities is responsible for the termination of transcription. Using these ideas we have found five (but only five) sequences in ΦX174 DNA which have a common pattern of stabilities with previous terminator sequences. These sequences are proposed to be responsible for termination of transcription in ΦX174. Three of the five sequences have five or six As following a GC region instead of the Ts present

*See Abstracts.

in known terminators. The lengths of mRNAs predicted on this basis are in excellent agreement with published observations; however, sequence determination of the messengers will be necessary to test our proposal rigorously.

6. INTERACTIONS OF 4-NITROQUINOLINE 1-OXIDE WITH FOUR DEOXYRIBONUCLEOTIDES

S. A. Winkle and I. Tinoco, Jr.

Biochemistry 17, 1352-1356 (1978)

The interactions of 4-nitroquinoline 1-oxide (NQO) with the four 5'-deoxyribonucleotides were probed using absorption spectra of the charge transfer bands and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of nucleotide-NQO mixtures. Spectral data yielded equilibrium constants (K(dpG:NQO) = 16 M^{-1}, K(dpA:NQO) = 12 M^{-1}, K(dpT:NQO) = K(dpC:NQO) = 4 M^{-1}) which 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]. From ^{13}C and ^{1}H NMR data on the mixtures, a structure for the dpG:NQO complex is proposed.

ROLE OF HYPERMODIFIED BASES IN TRANSFER RNA. SOLUTION PROPERTIES OF DINUCLEOSIDE MONOPHOSPHATES

M. T. Watts and I. Tinoco, Jr.

Biochemistry 17, 2455-2463 (1978)

The hypermodified dinucleoside monophosphates, uridylyl(3'-5')-N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl] threonine (Upt^{6}A), adenylyl(3'-5')-N^{6}-(Δ^{2}-isopentenyl)-2-methylthioadenosine (Apms^{2}i^{6}A), and adenylyl(3'-5')-1,N^{6}-etheno-adenosine (ApεA, a synthetic model for adenylyl(3'-5')wybutosine, ApyW), which represent the most common sequences found as the third letter of the anticodon triplet and its adjacent 3' neighbor, have been isolated. Their solution properties have been investigated using ultraviolet absorption, circular dichroism (CD), and high resolution proton magnetic resonance. The properties of these molecules have been compared with those of their unmodified counterparts, uridylyl(3'-5')adenosine (UpA) and adenylyl(3'-5')-adenosine (ApA). These properties measured as a function of temperature have been analyzed employing a two-state intramolecular stacking model. All of the properties show that the stacking of Upt^{6}A is stabilized relative to UpA, while Api^{6}A, Apms^{2}i^{6}A, and ApεA, are slightly destabilized relative to ApA. Thus, Upt^{6}A, Api^{6}A, Apms^{2}i^{6}A, and Ap A have comparable stacking equilibria, indicating that the modifications remove the large difference in stacking between UpA and ApA. Furthermore, cytidylyl(3'-5')adenosine (CpA), which is the most common unmodified sequence in this particular anticodon region, exhibits a stability similar to those of the hypermodified dinucleoside phosphates. Hypermodification therefore seems to keep the flexibility of this crucial part of the tRNA constant. It is proposed that this may result in a more smoothly regulated translation step. Also, it is proposed that the enhanced stacking of Upt^{6}A relative to UpA prevents the incorrect wobble base pairing of this U residue in the tRNA during translation.

7. MUTAGEN-OLIGONUCLEOTIDE COMPLEXES WITH A BULGED BASE. A MODEL FOR FRAMESHIFT MUTATION

C. H. Lee and I. Tinoco, Jr.

Nature (London), 274, 609-610 (1978)

Acridines and ethidium are frameshift mutagens which intercalate into DNA and RNA, and into minihelices made of complementary dinucleotides. However, this intercalation does not provide an explanation of their mutagenic action. Frameshift mutagenesis requires a bulge of one or two bases in either the parent strand (a deletion mutation), or in the newly synthesized strand (an addition mutation). That is, for frameshift mutation to occur, some bases must remain unpaired and outside of the normal double helix during replication. We have prepared minihelices containing such bulged bases stabilized by ethidium. NMR provides strong evidence that a mixture of the oligonucleotides GpUpG plus CpC in the presence of ethidium produces a minihelix containing two G·C base pairs with the uracil base bulged outside the helix and the ethidium intercalated. Similarly, CpUpG forms minihelices in the presence of ethidium containing bulged bases on both strands. These complexes provide models for the mutagenenic action of ethidium.

PHOTOCHEMISTRY AND PHOTOBIOLOGY OF THE PSORALENS

John E. Hearst

The skin-sensitizing activity of the psoralens has been known for more than three thousand years: in early Indian and Egyptian writing the use of certain tropical fruits and seeds, coupled with sunlight, was described as remedy for skin pigmentation disorders such as vitiligo. The photoactive agents in these tropical plants are derivatives of psoralen.

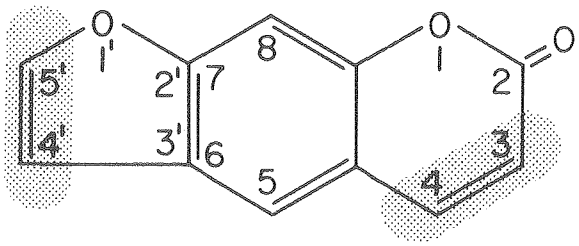
Psoralen and its derivatives photoreact directly with DNA, and elucidation of the mechanism of these reactions both in vitro and in vivo has become our major objective. The reaction occurs in three steps. Because of their planar structure and dimensions, the psoralens readily intercalate between base pairs in the DNA double helix. The various derivatives bind with different affinities depending upon the size and polarity of the substituent groups on the derivative. The second step is the absorption of a photon of 320- to 380-nm light that results in the formation of a cyclobutane bridge between a pyrimidine in the DNA and the psoralen, by reaction of either one of the two double bonds indicated in Figure 1, with the double bond in the pyrimidine. The reaction forms a monoadduct to the DNA which is likely to be mutagenic, resulting in frame-shift mutations. The last of the three possible steps occurs when the remaining reactive double bond in the monoadduct absorbs another photon of 320- to 380-nm light and photoreacts, forming a second cyclobutane bridge to a properly situated pyrimidine on the opposite strand, therefore leaving the DNA photocrosslinked. One such crosslink, if not repaired by a biochemical repair system, is lethal to a cell.

The psoralens are, therefore, light-controlled chemotherapeutic agents and are already being used in the treatment of psoriasis, a disease associated with the rapid division of skin cells. We believe psoralens have great potential in the treatment of cancer, in the production of vaccines, and in the study of basic structural changes in the genetic apparatus associated with replication, transcription, and translation.

We have developed an electron microscopic assay for crosslinks in DNA; and using this assay, we have discovered that not all the DNA of a cell in vivo is equally accessible to crosslinkage by the psoralens.[1] The cross-linking pattern suggests that subunits of histones, called nucleosomes, protect 200 base pair lengths of DNA from crosslinkage in vivo.[2] We are thus left with a pattern in the isolated DNA that relates to the pattern of protein interaction with the DNA in vivo.

Psoralen crosslinkage is also being used to freeze the secondary structure of DNA[3,4] and RNA single strands with the intention of relating these regions of secondary structure to biological function. In the past year this technique has been applied to Simian Virus 40 DNA-histone complexes, to in vitro SV-40 transcription complexes, to the filamentous bacteriophage fd, to SV-40 single-stranded DNA as a model system for messenger RNA processing, and to E. coli 16 ribosomal RNA in order to reveal long-range nucleic acid interactions in these systems.

(a) PSORALEN



(b) TRIOXSALEN

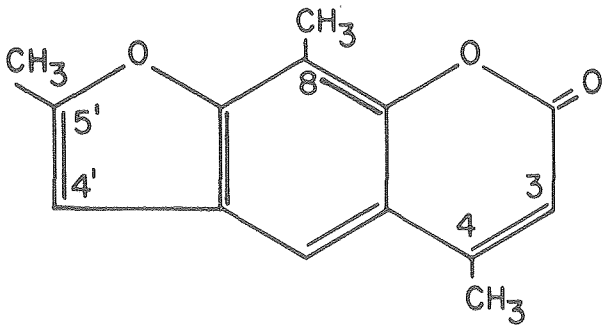


Figure 1. (a) The structure of psoralen; after intercalation in the DNA double helix, psoralens photoreact with pyrimidines, forming cyclobutane bridges at the shaded areas. (b) The structure of 4,5',8-trimethylpsoralen (trioxsalen), one of the derivatives used in these studies.
(XBL 789-11127)

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Abstracts

SYNTHESIS AND CHARACTERIZATION OF NEW PSORALEN DERIVATIVES WITH SUPERIOR PHOTOREACTIVITY WITH DNA AND RNA

S. T. Isaacs, C.-K. J. Shen, J. E. Hearst and H. Rapoport

Biochemistry 16, 1058 (1977)

The synthesis of five new psoralen derivatives is described. Three of these, 4'-hydroxymethyl-4,5',8-trimethylpsoralen, 4'-methoxymethyl-4,5',8-trimethylpsoralen, 4'-methoxymethyl-4,5',8-trimethylpsoralen, and 4'-aminomethyl-4,5',8-trimethylpsoralen hydrochloride, are characterized with respect to their photoreactivity with DNA and RNA. They are found to be greatly superior to 4,5',8-trimethylpsoralen and 8-methoxypsoralen, the two commonly used psoralens, in their abilities to saturate the photoreactive sites on DNA and RNA without repeated addition of reagent. A simplified mechanism for the photoreaction of psoralens with nucleic acids is presented and provides a basis for understanding the superior properties of these compounds. The compounds have superior reactivity not only with isolated DNA and RNA but also in viruses and in cells. Psoralens are shown for the first time to cross-link RNA double helices.

PHOTOCHEMICAL ADDITION OF THE CROSS-LINKING REAGENT 4,5',8-TRIMETHYLPSORALEN (TRIOXSALEN) TO INTRACELLULAR AND VIRAL SIMIAN VIRUS 40 DNA-HISTONE COMPLEXES

L. M. Hallick, H. A. Yokota, J. C. Bartholomew, and J. E. Hearst

J. Virol. 27, 127-135 (July 1978)

We demonstrated here that 4,5',8-trimethylpsoralen (trioxsalen) is a valuable probe for the structure of SV40 DNA-histone complexes. Trioxsalen readily penetrated intact cells and, in the presence of 340- to 380-nm light, covalently cross-linked DNA preferentially at the sites available for micrococcal nuclease digestion. Histograms of the lengths of the regions of SV40 DNA protected from cross-linking, as visualized by electron microscopy, indicated a repeating pattern of base pairs in DNA from both infected cells and virus particles. The ability of the trioxsalen probe to act in vivo and to map the location of protected regions may provide a powerful tool for analyzing the role of nucleosomes in the structure of the virus particle and in intracellular complexes such as transcription templates and replication intermediates.

PHOTOCHEMICAL CROSSLINKING OF TRANSCRIPTION COMPLEXES WITH PSORALEN. I. COVALENT ATTACHMENT OF IN VITRO SV40 NASCENT RNA TO ITS DOUBLE-STRANDED DNA TEMPLATE

C.-K. J. Shen and J. E. Hearst

Nucleic Acids Res. 5 (4) (April 1978)

^{14}C-labeled SV40 DNA has been transcribed with E. coli RNA polymerase using ^{3}H-labeled ribonucleotide triphosphates as precursors. The resulting transcription complexes were then photochemically crosslinked with the psoralen derivative, 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT), at 37°C and analyzed in SDS-sucrose gradients. It was found that the photochemical crosslinking procedure caused the nascent RNA chains to co-sediment with their double-stranded (helical) SV40 templates in the denaturing sucrose gradient. This result and several control experiments suggest that covalent linkages have formed between nascent RNA and helical DNA after the photochemical reaction. The crosslinking phenomenon was observed to be independent of the superhelical state of the DNA used as the template. Prior addition of EDTA to stop the transcription is not required for successful crosslinkage.

A SPECIFIC DNA ORIENTATION IN THE FILAMENTOUS BACTERIOPHAGE FD AS PROBED BY PSORALEN CROSS LINKING AND ELECTRON MICROSCOPY

C.-K. J. Shen, A. Ikoku and J. E. Hearst

J. Mol. Biol., in press.

The molecular structure of the single-stranded fd DNA inside its filamentous virion has been stabilized by the photochemical reaction

with a psoralen derivative and examined in the electron microscope. The results support the notion that the 6389 nucleotide-long DNA molecule is folded back on itself inside the 1μ long protein coat. At one end of the virion, there exists a DNA hairpin region 200 ± 50 base pairs long. This "end hairpin" is mapped on the fd genome to the site of the replication origin. The most stable in vitro hairpin of fd DNA has been mapped previously to this same site. This unique duplex region of fd DNA may play an important role in the formation of specific protein-DNA complexes which are crucial to stages of the fd life cycle: the adsorption of the phage to the bacteria, the initiation of replication of the single-stranded DNA, and the assembly of newly synthesized DNA strands into the filamentous virions.

EVIDENCE FOR A RELATIONSHIP BETWEEN LONG-RANGE BASE PAIRING ON SINGLE-STRANDED DNA AND EUKARYOTIC RNA PROCESSING

C.-K. J. Shen and J. E. Hearst

Anal. Biochem., in press.

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 stablized on denatured SV40 DNA for electron microscope visualization. Six major hairpins were mapped. This study has been extended by crosslinking single-stranded SV40 linear molecules, EcoRI-SV40 and BglI-SV40, in the presence of magnesium ions. After the reaction, in addition to the hairpins detected before, seven DNA hairpins and hairpin loops with sizes from 300 to nearly 2000 nucleotides are observed. These loops are located at specific regions on the SV40 genome; at the EcoRI map positions (0.16±0.02/(0.26±0.03), (0.33±0.03)/(0.39±0.03), (0.51±0.03)/0.57±0.03), (0.72±0.03)/(0.78±0.04), (0.74±0.03)/(0.85±0.03), (0.77±0.05)/(0.94±0.03), and at (0.74±0.04)/(0.09±0.07). 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±0.03), (0.72±0.03)/(0.78±0.04), (0.77±0.05)/(0.94±0.03).

These loop structures are most likely to arise from base pairing between distant regions of the single-stranded DNA. RNA transcribed from the double-stranded DNA template is expected to behave in a similar way, possibly providing recognition sites for processing (splicing) enzymes.

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

Edward L. Bennett

The bases of long-term memory formation remain as one of the most challenging areas of scientific investigation. Knowledge of fundamental properties of the mammalian nervous system is essential in order to evaluate contemporary hypotheses concerning the biochemical and anatomical changes in the nervous system that may account for long-term memory traces. Four projects are concerned with the mammalian brain and its response to environmental stimuli. These are: (I) studies of receptors for neurotransmitters using selected snake toxins as probes, (II) influence of drugs on brain chemistry with emphasis on understanding the neurochemical steps involved in the formation of long-term memory, (III) brain plasticity associated with environmental stimulation and training, and (IV) recovery of function after brain damage.

I. Receptors for Neurotransmitters

Receptors play a key role in neural transmission by modifying ionic permeability at synapses whereby an electrical to chemical energy transduction occurs. The acetylcholine receptor has been one of the most studied, due in part to the fact that high concentrations of this receptor have been available from electric organs of several fish, in part to the availability from several poisonous snakes of naturally occurring toxins with high specificity and affinities for the acetylcholine receptor (see Figure 1), and finally in part to the fact that acetylcholine was historically the first neurotransmitter identified and has been well studied.

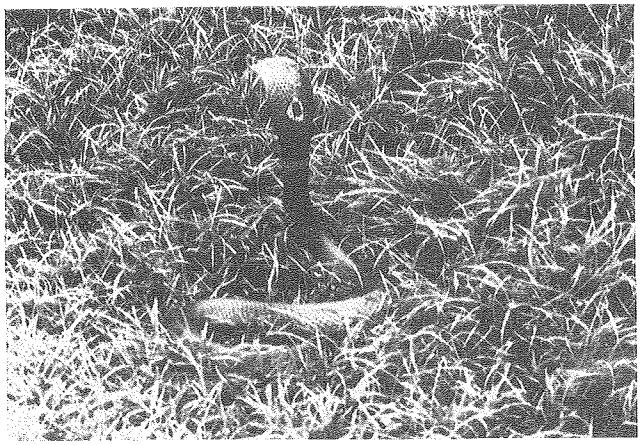

Figure 1. An α-toxin purified from Naja naja is one of the useful probes used to study the properties of the acetylcholine receptor.
(CBB 786-6782)

Methods have been developed to purify α-bungarotoxin from the crude venom of the krait Bungarus multicinctus. This α-toxin has high specificity for the acetylcholine receptor. The purified toxin can be iodinated using iodine monochloride. Methods have been developed to separate the non-iodinated, mono-iodinated, and diiodinated forms on Whatman CM-52. The derivatives can be distinguished from the native toxin on the basis of their ultraviolet absorption and circular dichroism data. The pattern of changes in CD spectra on incorporation of iodine into a single tyrosine residue of α-Bgt and the widespread wavelength distribution of these effects were interpreted as reflecting chemical modification of the tyrosine chromophore as well as vicinal and global secondary structural changes. Native and tritiated α-Bgt were shown to be more effective than iodinated α-Bgt derivatives in competing for specific toxin binding sites on putative nicotinic acetylcholine receptors (nAChR) derived from rat brain reflecting functional perturbation of the modified toxin. In contrast, both membrane-bound and solubilized nAChR from Torpedo californica electroplax displayed little or no specific binding preference for native toxin, nor were there significant differences in lethal potency of α-Bgt derivatives toward mice. These results suggested that peripheral and putative central nAChR may differ in their α-Bgt binding properties and suggested the usefulness of modified toxin in detecting those subtle differences.

The binding of ^{125}I-labeled or [^{3}H]α-bungarotoxin to particulate fractions from rat brain has been studied in depth. The high-affinity, specific binding of radiolabeled α-bungarotoxin to particulate fractions derived from rat brain showed saturability ($B_{max} \simeq 37$ fmole/mg, $K_D^{app} = 1.7$ nM), insensitivity to ionic strength, and was essentially irreversible ($K_{on} = 5 \cdot 10^6$/min mole; $K_{displacement} = 1.9 \cdot 10^{-4}$/min, $\tau_{1/2} = 62$ hrs). Subcellular distribution of specific sites was consistent with their location on synaptic junctional complex and post-synaptic membranes. These membrane-bound binding sites exhibited unique sensitivity to cholinergic ligands; pretreatment of membranes with cholinergic agonists (but not antagonists) induced transformation of α-bungarotoxin binding sites to a high affinity form toward agonist. The effect was most marked for the natural agonist, acetylcholine. This transformation to a high-affinity form toward agonist occurred over a time course of minutes and is consistent with the notion that these sites are authentic central acetylcholine receptors. Agonist inhibition of toxin binding to the high-affinity state was non-competitive, suggesting the existence of discrete toxin-binding and agonist-binding sites

on the central nicotinic acetylcholine receptor. These results have provided a possible explanation of the observed impotency of α-bungarotoxin toward blocking *in vivo* cholinergic responses in the central nervous system.

The 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^{++}, the concentration of acetylcholine (ACh) necessary to prevent half of specific [^{3}H]-labeled α-bungarotoxin binding was ~1 mM for nAChR treated with dithiothreitol (DTT) or N-ethylmaleimide (NEM) (low affinity states), and ~40 μM for nAChR treated with dithio-bis-nitrobenzoic acid (DTNB) or for native nAChR pretreated with ACh (high-affinity states). Addition of Ca^{++} resulted in an increase in effectiveness of ACh toward blocking toxin binding. None of these treatments altered toxin or antagonist binding, nor were there observed differences in Hill numbers for agonist binding. Agonists competitively inhibited toxin binding to low-affinity states, but noncompetitive inhibition was observed for binding to high-affinity states. Values of ACh dissociation constants determined from these data fell within the range of values determined physiologically with nAChR from other systems. The data indicate that the redox state of brain nAChR thio-groups and Ca^{++} may mediate physiologically important changes in receptor state during activation and desensitization.

A homologue of α-bungarotoxin, dendrotoxin 4.7.3, obtained from *Dendroaspis mamba* has been previously shown to differ from α-bungarotoxin in its electrophysiological potency in CNS. We have studied the binding of an ^{125}I-labeled derivative of dendrotoxin to rat brain and *Torpedo californica* electric tissue and compared it to that of [^{3}H]-α-bungarotoxin. There are two ^{125}I-labeled dendrotoxin sites for every [^{3}H]-α-bungarotoxin site in both intact and solubilized rat brain membranes, but the toxin-binding site stoichiometry is one-to-one in intact and solubilized *Torpedo* vesicles. Unlabeled α-bungarotoxin and dendrotoxin quantitatively block specific binding of both the heterologous and homologous radiolabeled toxin in both tissues. Membrane bound toxin-binding sites from rat brain migrate together in a sucrose gradient, co-enrich on solubilization, and have a similar nicotinic pharmacology as judged from cholinergic ligand displacement potencies. These observations indicate that: 1) ^{125}I-labeled dendrotoxin and [^{3}H]-α-bungarotoxin bind to the same receptor in brain; 2) the *Torpedo californica* and rat brain cholinergic receptors are significantly different on the basis of their binding selectivity for closely related protein toxins; 3) the binding to additional toxin sites in brain must be related to dendrotoxin's CNS synaptic-blocking effectiveness, and 4) while α-bungarotoxin recognizes rat brain acetylcholine receptor, it fails to recognize sites that block agonist activation of the receptor. Ultimately, detailed knowledge of the mode of interaction of neurotransmitters with receptors may enable us to understand a possible step in the changes of synaptic function that occur in long-term memory formation and provide an example of one mechanism of synaptic plasticity.

II. Formation of Long-Term Memory

It is generally accepted that macromolecular synthesis is required for long-term memory trace formation. However, the nature of the molecules synthesized, the factors that control the synthesis, and the function of the newly synthesized molecules remain largely unknown. We have found that an inhibitor of protein synthesis, anisomycin, is an effective amnestic agent in mice. It has been particularly useful in studying the role of protein synthesis in long-term memory trace formation. The amnesic effects of anisomycin have been shown to have generality over training and testing procedures (passive or active avoidance). By carefully controlling the conditions of training and testing, it has been possible to obtain semi-quantitative measures of the degree of memory-trace formation.

Many researchers have shown that protein synthesis inhibitors impair the formation of long-term memory, and it is generally accepted that inhibitors of protein synthesis such as acetoxycycloheximide, cycloheximide, and anisomycin (ANI) are effective amnestic agents in a variety of species for a variety of behavioral paradigms. However, less agreement exists concerning the necessity of protein synthesis for long-term memory formation, and numerous alternative suggestions for the amnesic effects of inhibitors of protein synthesis have been advanced. One of the most persistently raised alternative explanations offered to explain the amnesic effect of inhibitors of protein synthesis is that these antibiotic agents also cause marked changes in cerebral tyrosine levels by inhibition of tyrosine hydroxylase and modify the concentrations and rates of accumulation of catecholamines. The same research indicated that inhibition of the synthesis of catecholamine neurotransmitters might be the causal factor responsible for the amnesia induced by protein synthesis inhibitors, rather than the inhibition of protein synthesis. Similarities of the effects of catecholamine depleting drugs and inhibitors of protein synthesis in their amnesic behavioral effects have been pointed out by other investigators.

Amnesic effects of ANI were compared in six experimental paradigms with those of the catecholamine inhibitors (CAIs) diethyldithiocarbamic acid (DDC), α-methyl-p-tyrosine (AMPT), and tetrabenazine (TB). Pretraining administration of any of the these drugs resulted in amnesia for passive avoidance training, but only when training was weak. With stronger training, a series of three injections of ANI (one pre- and two post-training) caused amnesia but similar series of CAI injections did not cause amnesia. Substituting one CAI injection for one of the three ANI injections did not cause amnesia, but substituting cycloheximide (another protein synthesis inhibitor) did produce amnesia.

With active avoidance training, ANI caused amnesia while AMPT did not; d-amphetamine blocked the amnesic effect of ANI but potentiated the effect of AMPT. Whereas ANI lengthened the temporal gradient at which ECS produced amnesia, AMPT or DDC did not. Effects of these drugs on cerebral concentrations of tyrosine and catecholamines and on rate of accumulation of catecholamines were determined. ANI had relatively small effects, whereas the CAIs produced large reductions. When ANI and a CAI were used in combination, the pharmacological effect of the CAI predominated. We conclude that ANI and CAI have distinctly different abilities to produce amnesia and that the mechanisms of their amnesic effects differ. These experiments provide additional support for the hypothesis that protein synthesis is one component necessary for formation of long-term memories.

Another series of experiments has investigated the combined action of anisomycin and colchicine on long-term memory. Colchicine reduces axoplasmic flow in rats shortly after administration. Three successive injections of ANI at 2 hr intervals beginning 15 min prior to training will produce amnesia for training in an active avoidance task. If the second injection of ANI is delayed 90 min beyond the normal inter-injection interval, little amnesia is obtained due to a pulse of protein synthesis that occurs several hours after training. When colchicine (0.06 mg/kg s.c.) is administered 1 hr after the first ANI injection and the second injection of ANI is delayed, mice are amnestic. As the colchicine injection is delayed, the percentage of subjects showing amnesia decreases. It appears that protein synthesized after training must be transported into cell processes to establish long-term memory. Experiments are now in progress to determine both the amount of colchicine taken up by the brain and the colchicine-tubulin ratio.

III. Brain Plasticity and Environmental Stimulation or Training

The demonstration that protein synthesis is required for long-term memory storage does not address the problem of the ultimate changes produced by this protein synthesis. A different set of experiments suggests that an end-product of the biochemical sequences may be long-lasting anatomical changes.

A number of years ago we showed that the brains of rats raised in enriched environments significantly exceeded those of their impoverished littermates in cortical weight and depth, in total acetylcholinesterase and cholinesterase activities, in the ratio of cortex weight to subcortex weight, and in the ratio of glia to neurons. These observations now have been extended to show that the total RNA and the RNA/DNA ratio of the cortex are greater in the rat raised in the enriched environment than the littermate raised in the impoverished environment. We have now completed several extensive series of experiments to evaluate the effects of "social grouping" and "maze training" on brain measures. Social grouping alone is inadequate to explain the cerebral effects of enriched environments and the inanimate stimulus conditions must be taken into account. 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.

IV. Recovery of Function After Brain Damage

Realization that the nervous system is more plastic and responsive to environmental influences than previously accepted not only has important implications for our understanding of memory formation mechanisms, but may also have important practical consequences in re-orienting our concepts on the ability of the brain to recover from injury and other factors that influence this recovery. While recovery of function frequently occurs after brain damage, the mechanisms of such recovery are little understood. Our studies are directed toward the possibility that varied and enriched experience can reduce the impairment of function caused by cerebral injury. In these studies, lesions are made either neonatally or after weaning in the occipital cortex; subsequently the rats are exposed to either enriched or impoverished environments for 30 or more days. At the end of this period, maze performance of the lesioned rats raised in an enriched environment was improved over their counterparts raised in an impoverished environment. Some evidence has also been obtained that a secondary loss of cells occurs in the cortex remote from the site of the lesion. These studies of lesioned animals may be useful as a model to study methods to facilitate recovery from brain lesions arising from a variety of causes including environmental pollutants.

Abstracts

I. Receptors for Neurotransmitters

PROPERTIES OF RADIOLABELED α-BUNGAROTOXIN DERIVATIVES AND THEIR INTERACTION WITH NICOTINIC ACETYLCHOLINE RECEPTORS.

R. J. Lukasiewicz, M. R. Hanley and E. L. Bennett

Biochemistry 17, 2308-2313 (1978).

Column-purified monoiodinated, diiodinated, and tritiated derivatives of α-bungarotoxin (α-Bgt) are distinguished on the basis of their ultraviolet absorption and circular dichroism (CD) spectra. The pattern of changes in CD spectra on incorporation of iodine into a single tyrosine residue of α-Bgt and the widespread wavelength distribution of these effects are interpreted as reflecting primary chemical modification of the tyrosine chromophore as well as vicinal and global secondary structural changes. Native and tritiated α-Bgt are shown to be more effective than iodinated α-Bgt derivatives in competing for specific toxin binding sites on putative nicotinic acetylcholine receptors (nAChR) derived from rat brain reflecting functional perturbation of the modified toxin. In contrast, both membrane-bound and solubilized nAChR from Torpedo californica electroplax display little or no specific binding preference for native toxin, nor are there significant differences in lethal potency of α-Bgt derivatives toward mice. These results suggest that peripheral and putative central nAChR may differ in their α-Bgt binding properties and suggest the usefulness of modified toxin in detecting those subtle differences.

α-BUNGAROTOXIN BINDING PROPERTIES OF A CENTRAL NERVOUS SYSTEM ACETYLCHOLINE RECEPTOR.

R. J. Lukasiewicz and E. L. Bennett.

Biochem. Biophys. Acta 544, 294-308 (1978)

High-affinity, specific binding of radiolabeled α-bungarotoxin to particulate fractions derived from rat brain shows saturability ($B_{max} \approx 37$fmol/mg, $K_D^{app} = 1.7$ nM) and insensitivity to ionic strength, and is essentially irreversible ($K_{on} = 5 \cdot 10^6$ min^{-1}·mol^{-1}; $K_{displacement} = 1.9 \cdot 10^{-4}$ min^{-1}, $\tau_{1/2} = 62$h). Subcellular distribution of specific sites is consistent with their location on synaptic junctional complex and post-synaptic membranes. These membrane-bound binding sites exhibit unique sensitivity to cholinergic ligands; pretreatment of membranes with cholinergic agonists (but not antagonists) induces transformation of α-bungarotoxin binding sites to a high affinity form toward agonist. The effect is most marked for the natural agonist, acetylcholine. These results strongly support the notion that the entity under study is an authentic nicotinic acetylcholine receptor.

CROTOXIN EFFECTS ON TORPEDO CALIFORNICA CHOLINERGIC EXCITABLE VESICLES AND THE ROLE OF ITS PHOSPHOLIPASE A ACTIVITY

M. R. Hanley

Biochim. Biophys. Res. Commun. 82, 392-401 (1978)

The phospholipolytic neurotoxin from Crotalus durissus terrificus, crotoxin, is able to produce a dose- and time-dependent block of carbachol-stimulated ^{22}Na efflux from pre-loaded Torpedo californica excitable vesicles. The blocking activity is dependent on calcium and is abolished by chemical modification with p-bromophenacyl bromide. The isolated basic subunit, crotoxin B, produces an identical block, whereas the isolated acidic subunit, crotoxin A, has no detectable effect. Neither crotoxin nor crotoxin B antagonizes the binding of [^{125}I]-α-bungarotoxin to purified acetylcholine receptor, although at high concentrations they antagonize its binding to acetylcholine receptor-rich membrane fragments. Certain phospholipase A_2 enzymes and the fatty acid products of their digestion can mimic the crotoxin action. It is therefore suggested that, although considered a pre-synaptic neurotoxin, crotoxin can have in vitro post-synaptic effects, possibly mediated by endogenous phospholipase A activity.

BIOCHEMISTRY OF SNAKE VENOM NEUROTOXINS AND THEIR APPLICATION TO THE STUDY OF THE SYNAPSE.

M. R. Hanley

University of California, Ph.D. Thesis, 1978; also, Lawrence Berkeley Laboratory, Report LBL-8422.

Detailed new information has been reported on the structures and functions of pre- and post-synaptic snake neurotoxins. This information has been used to develop new neurobiological applications of the toxins as selective synaptic tools.

II. Formation of Long-Term Memory

MEMORY: MODIFICATION OF ANISOMYCIN-INDUCED AMNESIA BY STIMULANTS AND DEPRESSANTS

J. F. Flood, E. L. Bennett, A. E. Orme, M. R. Rosenzweig, and M. E. Jarvik

Science 199, 324-326 (1978).

Mice were trained in a passive (foot shock) avoidance task. When administered after training, the stimulants caffeine or nicotine blocked amnesia for the task that had been produced by injections of the protein synthesis inhibitor

anisomycin given prior to training. With foot shock at a higher intensity, anisomycin did not produce amnesia by itself, but the administration of the depressants chloral hydrate or sodium phenobarbital after training did cause amnesia. Stimulants and depressants did not have an appreciable influence on the overall degree of protein synthesis inhibition produced by anisomycin. The results support the hypothesis that arousal after training is an important factor in the conversion of short-term to long-term memory.

MEMORY FACILITATING AND ANTI-AMNESIC EFFECTS OF CORTICOSTEROIDS

J. F. Flood, D. Vidal, E. L. Bennett, A. E. Orme, S. Vasquez, and M. E. Jarvik

Pharmacol. Biochem. Behav. 8, 81-87 (1978)

The effects of corticosterone, hydrocortisone and dexamethasone on retention of active and passive avoidance training were studied in male mice. Posttraining administration of any of the hormones facilitated subsequent retention test performance of poorly trained mice when tested one week after training and drug administration. The optimum dose of dexamethasone was 4 mg/kg, while corticosterone and hydrocortisone were effective at 30 and 40 mg/kg, respectively. Dexamethasone significantly facilitated retention when administered up to 150 min but not at 210 min after training. It was further determined that dexamethasone blocked the amnesic effect of two but not four successive injections of anisomycin in both active and passive avoidance tasks. Corticosterone and dexamethasone when administered to anisomycin-injected mice caused only a small, transient increase in the protein synthesis inhibition. In saline-injected control mice, the hormones also caused a small inhibition of protein synthesis which disappeared quickly. Plasma corticosterone levels were measured in mice trained and given anisomycin, cycloheximide or saline. Plasma corticosterone levels were reduced 43% by anisomycin and 89% by cycloheximide. In both cases the corticosterone levels subsequently increased rapidly after the inhibitor injection and were elevated by about 5 times above control levels at 130 min after the inhibitor injection. The results are discussed in terms of the effect of central stimulant action of corticosteroids on memory formation.

RECOVERY AS A FUNCTION OF DEGREE OF AMNESIA DUE TO PROTEIN SYNTHESIS INHIBITION

H. P. Davis, M. R. Rosenzweig, E. L. Bennett, and A. E. Orme

Pharmacol. Biochem. Behav. 8, 701-710 (1978)

Retrograde amnesia following inhibition of cerebral protein synthesis has generally been explained as either a failure of consolidation or impairment of a retrieval mechanism. Major evidence for the retrieval hypothesis is provided by studies which utilize a reminder (usually footshock) to attenuate the effect of the protein inhibitor. To examine this question, mice were injected subcutaneously with anisomycin (1 mg/animal, 7 mg/animal, or 1 mg/animal every 2 hr x 7) and given one training trial in a passive avoidance box. All animals received a single retention test on each of four consecutive days, starting either 1, 7, or 21 days after training. One-half of the mice in each group received a footshock reminder 1 hr after their initial test. The footshock reminder did not attenuate the inhibitor-induced amnesia, but multiple testing did produce partial recovery in animals demonstrating some memory of training (both Saline and Anisomycin animals). Animals injected with anisomycin whose testing began 1 day after training demonstrated partial recovery irrespective of drug dosage level. The extent of amnesia and recovery were dependent upon both drug dosage and training-test interval. Implications for the consolidation and retrieval hypotheses are discussed.

III. Brain Plasticity and Environmental Stimulation of Training

SEQUENCE DIVERSITY STUDIES OF RAT BRAIN RNA: EFFECTS OF ENVIRONMENTAL COMPLEXITY ON RAT BRAIN TOTAL DNA DIVERSITY

L. D. Grouse, B. K. Schrier, E. L. Bennett, M. R. Rosenzweig and P. G. Nelson

J. Neurochem. 30, 191-203 (1978)

The sequence complexities of rat brain RNAs were measured by RNA-driven hybridization reactions with nonrepetitive rat DNA. The total sequence complexity of rat brain HnRNA was estimated to be 6.61×10^8 nucleotides while rat brain poly(A)-mRNA sequence complexity was 1.32×10^8 nucleotides. Up to 33.7% of the total transcribable nonrepetitive DNA was expressed in the nuclear RNA. The nuclear RNAs reacted with complex kinetics over at least 4.5 decades of equivalent R_0t (product of RNA concentration and time), with an apparent division into three major RNA abundance classes. The abundances of average nuclear RNA species in these classes ranged from 2.9×10^9 copies per brain (18 copies per cell) to 2.4×10^5 copies per brain (1.5×10^{-3} copies per cell). Poly(A)-mRNA diversity was sufficient to code for 8.8×10^4 polypeptides of 50,000 daltons. There were also three distinguishable abundance classes of poly(A)-mRNA with frequencies which ranged from 8.9×10^8 copies per brain (5.5 copies per cell) to 3.2×10^5 copies per brain (2×10^{-3} copies per cell). Evidence for compartmentalization of expressed RNA sequences supports the concept that the extensive morphological and physiological specialization evident in brain parallels extensive transcriptional specialization at the cellular level.

Brain and liver RNA diversities were measured under double-blind experimental conditions in three experiments with rats raised in

experientially enriched (EC) or impoverished (IC) environments. Liver RNA diversity of EC animals was not different from that of IC animals. Brain total RNA of EC animals, at equivalent R_0ts of 184,000-212,000, hybridized to 10.6% of rat unique DNA (mean of 11 separate groups of rats). The average hybridization of brain RNA from 11 groups of IC animals in the same range of equivalent R_0t was 8.2% of the unique DNA. The difference was statistically significant at $P < 0.02$. Of 10 groups of 3 littermate pairs (paired across EC and IC groups) brain RNA diversity was greater in EC animals in 8 cases. A least squares fit of the kinetics of hybridization to a pseudo first order reaction showed that, at saturation, the RNA from brains of EC animals was complementary to 16.4% of the unique DNA while that from IC animals was complementary to 9.1%. This difference was found in the least abundant class of rat brain RNA. These changes in sequence diversity reflected either an increase in the number of diverse RNA species present or an increase in the number of copies of certain RNA species in the rats raised in an enriched environment. A change in brain RNA populations of this magnitude may reflect a significant difference in brain function between EC and IC animals.

EXPERIENTIAL INFLUENCES OF BRAIN ANATOMY AND BRAIN DEVELOPMENT IN RODENTS

M. R. Rosenzweig and E. L. Bennett

Studies on the Development of Behavior and the Nervous System, Vol. 4, "Early Influences", G. Gottlieb, ed. (Academic Press, New York, 1978), pp. 289-327.

Research in a number of laboratories has demonstrated that significant changes in several aspects of brain biochemistry and brain anatomy are produced when rodents interact with environments that are somewhat more complex than the usual laboratory colony conditions. Such nonstressful enriched experience causes brain changes in both young and adult animals. Although there are indications that preweaning experience may produce larger changes than postweaning experience, the brain remains plastic in many respects long after ages that various investigators have designated as the end points for cerebral development.

Effects of differential experience should be distinguished from effects of early stressful treatment and also from effects of sensory distortion or restriction. Unlike these other effects, most of those caused by differential experience are not limited to early development, they require direct interaction with the environment, and they do not require extreme conditions for their production.

Among the various mechanisms hypothesized to mediate the cerebral effects, learning and memory storage appear best to encompass the data. Several investigators are therefore using experimental environments in studying the anatomical and biochemical mechanisms of memory storage.

Research on effects of differential experience has been extended to experiential therapy for brain lesions, experimental cretinism, and malnutrition. Work with enriching and impoverishing environments is also being done on development of intelligence in children.

We propose that the effects described here represent a modulation of cerebral values through various types of experience.

SOCIAL GROUPING CANNOT ACCOUNT FOR CEREBRAL OR BEHAVIORAL EFFECTS OF ENRICHED ENVIRONMENTS

M. R. Rosenzweig, E. L. Bennett, M. Hebert, and H. Morimoto

Brain Res. 153, 563-577 (1978)

Several experiments were conducted to test whether, as suggested by Welch et al., mere group living (social stimulation) can account for the significant differences in measures of brain anatomy and brain chemistry that develop between rodents housed in groups in enriched environments and rodents housed singly in restricted environments; the alternative hypothesis was that features of the inanimate environment can significantly affect brain measures of animals living in a social group. Groups of 12 male rats were assigned for 30 days to several types of environment: (a) large cage without stimulus objects, (b) large cage containing varied stimulus objects, (c) large cage containing a maze whose pattern of barriers was changed daily, and (d) a seminatural outdoor environment; in each experiment, littermates of rats in the social conditions were housed in isolation in small colony cages. At the end of the 30-day period, measures were taken of weights of brain regions, RNA and DNA contents of regions of cerebral cortex, and acetylcholinesterase activities of brain regions. Although the number of rats housed together was constant for conditions a-d and cage size was constant for conditions a-c, the magnitudes of the cerebral measures varied significantly as a function of the inanimate stimulus conditions. The differences from isolation-housed littermates was greatest in condition d and smallest in condition a. Thus, social grouping alone is inadequate to explain the cerebral effects of enriched environments and the inanimate stimulus conditions must be taken into account.

MAZE TRAINING ALTERS BRAIN WEIGHTS AND CORTICAL RNA/DNA RATIOS

E. L. Bennett, M. R. Rosenzweig, H. Morimoto, and M. Hebert

Behavioral and Neurol Biology (in press)

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 barrier 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 were 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.

HOW PLASTIC IS THE NERVOUS SYSTEM?

M. R. Rosenzweig and E. L. Bennett

A Comprehensive Handbook of Behavioral Medicine, F. Taylor and J. Ferguson, eds. (Spectrum Publications, New York), in press.

Recent research has demonstrated that the nervous system is plastic not only in ways hypothesized decades ago but also in ways grasped only recently, and further discoveries of this sort are inevitable. Yet this is not the general and almost unlimited plasticity that many assumed during the first four decades of this century. Both the overall layout of the nervous system and many specific connections are determined by genetic instructions. Thus, we now know far more about both plasticity and the limits of plasticity than was true a decade ago.

Many plastic changes in ramifications and connections of neurons have been demonstrated in response to severe or harmful treatments such as depriving young animals of normal sensory stimulation or transecting tracts in the brain. But such harsh treatments are not necessary to induce significant cerebral effects; mild experiences in differential environments and also formal training lead to measurable changes in the biochemistry and anatomy of the brain. Furthermore, many of these effects occur not only in young animals but also in adults. Several alternative hypotheses to account for the cerebral effects of differential experience have been ruled out by direct tests; thus the cerebral effects cannot be attributed to stress, to hormonal mediation, nor to speeded maturation, nor do they follow the model of effects of sensory deprivation or distortion. Recent experiments with improved controls show more clearly than heretofore that training causes significant modification in brain measures.

A catalog of plastic changes in the adult nervous system showed that many possibilities have now been demonstrated. These include the following: (a) Functional changes at existing synapses. (b) Changes between active and quiescent states of neurons or parts of neurons. (c) Anatomical changes in axon terminals, dendrites, dendritic spines, and synapses. (d) Proliferation of glial cells. Although it has been dogma that the production and differentiation of new neurons can occur only early in life, some authors have suggested that even these events can occur to a limited extent in the adult mammalian brain.

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.

SNAP HARNESS FOR BIOTELEMETRY FROM RODENTS

B. Roman and E. L. Bennett

Physiol. Behavior. 20, 349-350 (1978)

A snap harness is described which incorporates a commercially built 5g FM transmitter (FM-110-E3) (Narco Bio-Systems, Inc., Houston, TX) for telemetering biopotentials in rats and other small rodents. The harness allows the stable recording of a variety of biopotentials from unanesthetized freely moving animals for experimental periods of a few weeks. There is the added advantage and flexibility of easily snapping the harness on and off during and after experimental periods, permitting recordings from a number of animals with the same transmitter.

IV. Recovery of Function After Brain Damage

LESIONS IN OCCIPITAL CORTEX OF RAT LEAD TO SECONDARY LOSS OF CELLS IN OTHER CORTICAL REGIONS

M. R. Rosenzweig, E. L. Bennett, H. Morimoto, and M. Hebert

Proceedings, Society for Neuroscience, 8th Annual Meeting, Nov. 1978, p. 478

We reported previously that lesions confined to occipital cortex result in significant loss

of weight and DNA in other intact regions of cerebral cortex (Will et al., J. Comp. Physiol. Psychol. 91, 33-50,1977). In subsequent experiments involving about 400 rats we have investigated how magnitude of remote loss varied with (a) cortical region, (b) size of lesion, (c) time elapsed between lesion and sacrifice, and (d) post-operative environment, either enriched (EC) or impoverished (IC). Somesthetic cortex, although adjacent to the occipital area, showed only about 2% loss of DNA while remaining dorsal cortex and ventral cortex lost 6-8%; hippocampus showed no change. Magnitude of remote loss increased directly with size of lesion. There was not a consistent difference between losses in DNA of EC rats (EC-lesion vs EC-sham) and IC rats (IC-lesion vs IC-sham), so post-lesion enriched experience did not help to protect against secondary loss contrary to a hypothesis suggested in Will et al. The secondary loss was somewhat greater in rats sacrificed 95 days after lesion than in rats sacrificed 35 days post-lesion. This apparent progressive loss is being investigated in further experiments; it may provide a model for studying progressive loss that has been reported in some clinical cases. Although the EC experience did not protect against remote loss of cells, it did improve subsequent learning behavior. Furthermore, social grouping did not have this effect, so inanimate features of the enriched environment are important in recovery of function.

BRAIN PLASTICITY, MEMORY AND AGING, A DISCUSSION

E. L. Bennett and M. R. Rosenzweig

Aging, Vol. 8, "Physiology and Cell Biology of Aging," A. Cherkin et al., 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. However, this lack of explanation of memory does not mean that there is a lack of suggested mechanisms for long-term memory storage. Present theories of memory usually include, first, neurophysiological or electrical events, then a series of chemical events that ultimately leads to long-lasting anatomical changes in the brain. Evidence is increasing for the biochemical and anatomical plasticity of the nervous system and its importance in the normal functioning of the brain. Modification of this plasticity may be an important factor in senescence.

This discussion 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 aging research. A more important applied question is the role of social and intellectual stimulation in influencing aging of the human brain.

CHEMICAL EVOLUTION

Richard M. Lemmon

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, 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 stimulate presumed prebiotic Earth conditions have led to increased understanding of the ways by which biological compounds (such as proteins and nucleic acids) may have been assembled on our planet's surface. This enhanced knowledge of "pre-Darwinian" evolution is also leading to a better understanding of contemporary biological processes.

The following investigations in the area of chemical evolution have taken place during 1978:

(1) We have investigated an interesting imidazole-catalyzed aqueous system that leads to the simultaneous appearance of peptides and oligonucleotides. It is a reasonable presumption that the peptides and oligonucleotides were developed simultaneously on the prebiotic Earth, and that each may have been a catalyst for the formation of the other. Consequently, our imidazole-catalyzed system--which is a simple water solution of an amino acid, ATP, Mg^{2+}, and imidazole--holds considerable interest. At modest temperatures (37°-70°C), it leads to a yield of 1-5% of oligopeptides and a few tenths of a percent of ApA and of other (as yet unidentified) oligonucleotides. This system provides the means to investigate the following questions: (a) Which amino acids give the best yield of peptides? (b) What is the effect of substituting other nucleoside triphosphates for the ATP? (c) Is there a codonic, or anti-codonic, relationship between the various amino acid-nucleoside triphosphate pairs? (d) Is this imidazole-catalyzed system affected if it is carried out in the presence, at the start, of added polypeptides, or polynucleotides, or clay minerals? The answers to these questions will contribute substantially to our understanding of the necessary-for-life chemistry that took place on the prebiotic Earth.

(2) In collaboration with members of the Chemistry Department at Stanford University, we are studying the possible differential radiolysis of optical isomers by polarized electrons (such as β particles from radioactive nuclides, or from electron accelerators).[1-3] These studies are directed toward a possible "prebiological-Earth" explanation of why proteins are constructed (almost exclusively) of L-amino acids, and why the ribose and deoxyribose of the nucleic acids are exclusively of the D configuration. Closely related research involves studies of radiation-induced racemization, a mechanism that may "overwhelm" possible stereo-selective radiolyses.

(3) Recent findings of the radioastronomers have shown that interstellar space contains vast quantities of many molecules that must have played a key role in chemical evolution (see also the following section, "High-Energy

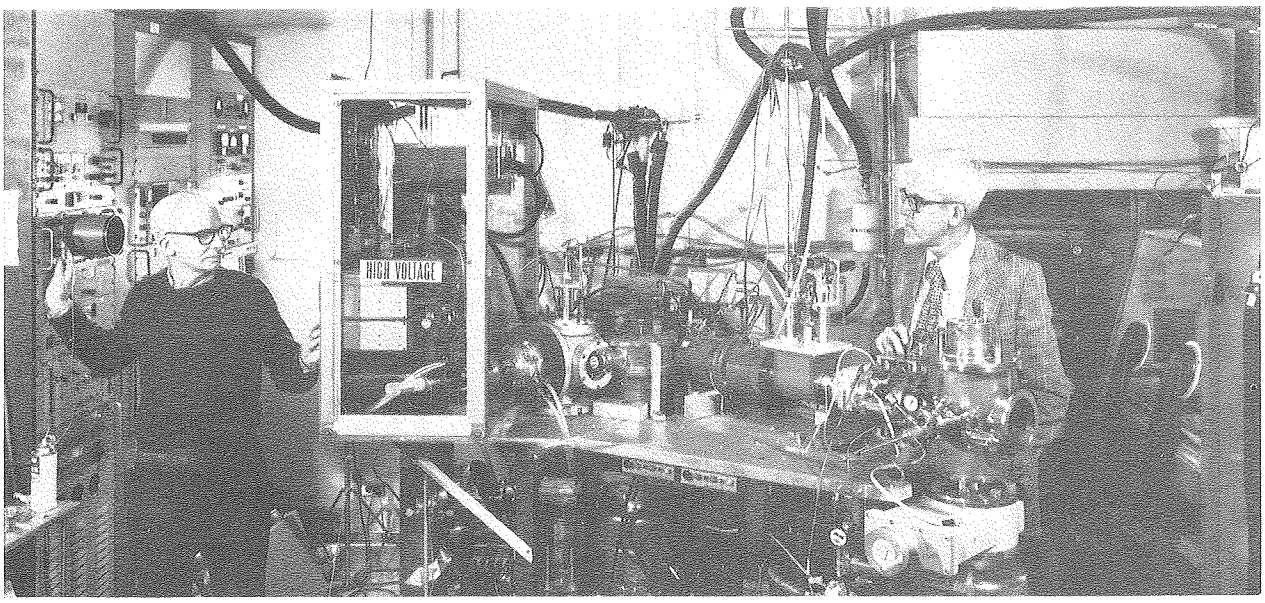

Figure 1. This laboratory's carbon-ion accelerator--used to study high-energy carbon reactions, including the probable carbon chemistry of interstellar space. (CBB 784-4944)

Carbon and Hydrogen Chemistry"). Among such molecules are water, ammonia, formaldehyde, cyanamide, and cyanoacetylene. This laboratory's ion accelerator is now being used to investigate the possible chemistry of interstellar space (Figure 1). We are directing beams of carbon ions at about 1 keV kinetic energy (about the average energy of carbon in the solar and stellar winds) onto frozen targets of water, ammonia, and formaldehyde. Identification of products may prove of value in understanding interstellar chemistry, and in identifying further compounds that should be sought by radioastronomy in interstellar dust clouds.

(4) The blue-green algae are ancient precursors of higher life forms and are probably the first donors of oxygen to the atmosphere. Algal mats provide an excellent opportunity for studies of modern-day examples of early life forms on Earth. Samples of algal mats were collected in Baja California and examined for their distributions of hydrocarbons, fatty acids, amino acids, and sterols.[4,5] All these classes of compounds have chemical and biochemical properties that make them excellent examples of chemical markers or fossils in the probe for the origin of life on Earth.

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Abstracts

1. RADIOLYSIS, RACEMIZATION, AND THE ORIGIN OF OPTICAL ACTIVITY

W. A. Bonner and R. M. Lemmon

Bioorganic Chem. 7, 175-187 (1978)

An investigation has been undertaken to determine whether ionizing radiation might engender racemization (radioracemization) of optically active amino acids along with their well known radiolysis. We have exposed a number of solid and dissolved optically active amino acids to the ionizing radiation from a 3000-Ci ^{60}Co γ-ray source for periods of time which would engender substantial, but not total radiolysis. γ-ray doses which caused 55-68% radiolysis of solid amino acids typically engendered 2-5% racemization. Aqueous solutions of the sodium salts of amino acids which underwent 53-66% radiolysis typically showed 5-11% racemization. The corresponding hydrochloride salts in aqueous solution, however, underwent little or no racemization. In aqueous solution both percentage degradation and percentage racemization were approximately proportional to γ-ray dosage within the range employed (1-36 x 10^6 rads). Mechanisms for the radioracemization of amino acids in the solid state and as dissolved sodium salts are proposed, and the absence of racemization for dissolved hydrochloride salts is rationalized. Implications of these observations with regard to the origin of optical activity by the Vester-Ulbricht β-decay mechanism are discussed, as are their implications regarding the use of diagenetic racemization rates of ancient amino acid samples as criteria for geochronological and geothermometric calculations.

2. RADIOLYSIS, RACEMIZATION AND THE ORIGIN OF MOLECULAR ASYMMETRY IN THE BIOSPHERE

W. A. Bonner and R. M. Lemmon

J. Mol. Evol. 11, 95-99 (1978)

An investigation has been undertaken to determine whether ionizing radiation might engender racemization of optically active amino acids, along with their usual radiolysis. As prototypes, crystalline D- and L-leucine,

*See Abstracts.

as well as aqueous solutions of their sodium salts, were exposed to the radiation from a 3000 Ci ^{60}C γ-ray source. γ-ray doses which caused about 68% radiolysis of solid leucine left a residue which was about 5% racemized, while racemization proved even greater at lower doses for the dissolved sodium salts. In aqueous solution both percent degradation and percent racemization of the sodium salts were proportional to γ-ray dosage within the range employed (1-27 x 10^6 rads). Implications of these observations for the origin of molecular asymmetry by the β-decay parity violation mechanism are discussed.

3. β RADIOLYSIS OF CRYSTALLINE ^{14}C-LABELED AMINO ACIDS

W. A. Bonner, R. M. Lemmon, and H. P. Noyes

J. Organic Chem. 43, 522-524 (1978)

In view of literature reports of alleged slight preferential degradation by β particles of one or the other enantiomorph of racemic amino acids, we have examined the D/L ratio of a number of ^{14}C-labeled DL-amino acids of high specific radioactivity that were prepared 17-25 years ago. The analyses were carried out on gas chromatographic columns that contained optically active substrates. The amino acids were found to have undergone no stereoselective degradations, in spite of self radiolyses as high as 67%.

4. ORGANIC GEOCHEMICAL STUDIES ON KEROGEN PRECURSORS IN RECENTLY DEPOSITED ALGAL MATS AND OOZES

R. P. Philp, M. Calvin, S. Brown, and E. Yang

Chem. Geol. 22, 207-231 (1978)

Kerogen is the most abundant form of organic matter on the earth. In spite of its abundance, relatively little is known of the origin or methods of formation of the various types of kerogen. This paper describes the results from a series of degradation experiments performed on kerogen-like residues isolated from algal mats and oozes at Laguna Mormona, Baja California, and Baffin Bay, Texas. An examination of such residues should provide valuable information on the early-stage reactions and structures of algal-type kerogens.

In the first set of experiments, one residue from Laguna Mormona was subjected to degradation by saponification, alkaline potassium permanganate oxidation, and hydrogen bromide (HBr) treatment. A combination of the results from these three experiments has shown that this residue has a structure which is basically aliphatic with a certain degree of cross-linking. Various components linked as esters to this residue were released by saponification but the HBr treatment gave rise to only one one product, indicating the absence of any large number of ether-linkages readily cleaved by HBr.

In the second set of experiments this residue plus five others, including two isolated from the algal mats at Baffin Bay, Texas, were subjected to pyrolytic degradation. The hydrocarbon fractions were examined by gas chromatography and computerized--gas chromatography--mass spectrometry. The major products included: homologous series of n-alkanes and n-alkenes; pristenes and phytenes; C_{27}, C_{28} and C_{29} sterenes, and hopane-type triterpanes. These experiments again demonstrate the presence of kerogen-like material in these residues of algal origin. However, the differences in distributions of the pyrolysis products from the various residues were not sufficiently significant to distinguish between any environmental or geochemical differences in the original samples used in this particular set of experiments.

5. ISOPRENOID HYDROCARBONS PRODUCED BY THERMAL ALTERATION OF NOSTOC MUSCORUM and RHODOPSEUDOMONAS SPHEROIDES

R. P. Philp, S. Brown and M. Calvin

Geochim. Cosmochim. Acta 42, 63-68 (1978)

Cells of Nostoc muscorum and Rhodopsuedomonas spheroides have been subjected to thermal alteration over varying periods of time. Experiments were conducted using both unextracted and extracted cells in the absence and presence of montmorillonite. The isoprenoid hydrocarbons produced in these experiments have been examined. The major hydrocarbons produced were phytane and five isomeric phytenes. Phytane was observed to form faster from the unextracted cells than from the extracted cells. Montmorillonite increased the amount of phytane formed from the unextracted cells of Nostoc muscorum but not from the cells of Rhodopseudomonas spheroides. No phytadienes, pristane or pristenes were detected in the products of any of these experiments.

HIGH ENERGY CARBON AND HYDROGEN CHEMISTRY

Richard M. Lemmon

Interest in fundamental carbon chemistry has led this laboratory into studies of the interactions of accelerated carbon ions and atoms with organic compounds. Work with the energy-rich atoms leads to a new kind of organic chemistry--a chemistry not accessible in the usual thermal or photo regions of energy. A major impetus for these studies is the recent reports by radioastronomers that interstellar space contains many organic compounds, such as acetaldehyde and cyanoacetylene, which probably played key roles in the processes that led to the appearance of life on our planet. These compounds were formed at higher energies; in fact, most of the universe's chemistry (as distinct from that on our planet's surface) takes place at high energy. Consequently, it is important that we know the chemical mechanisms initiated by high-energy carbon atoms.

This laboratory possesses a unique instrument for the study of carbon of kinetic energies beyond those attainable by thermal means. This carbon-ion accelerator is capable of directing carbon ions and atoms onto targets at any chosen kinetic energy, ranging all the way from several keV down to zero. It is capable of doing the same thing with other accelerated species such as CH^+, CH_2^+, and CH_3^+. The instrument has been used to study the chemical and physical details of the interactions of these ions with solid benzene (and other targets) in the energy range of 5 keV to 2 eV. Carbon-14 labeled products from benzene have been identified as benzene, toluene, cycloheptatriene, phenylacetylene, biphenyl, diphenylmethane, and phenylcycloheptatriene. The yields of these products and the appearance of radioactivity among the various carbon positions have been determined as a function of the beam's kinetic energy over the 5 keV to 2 eV energy range. Such studies have suggested, particularly with respect to the routes to toluene and cycloheptatriene, the charge state (ion or atom), the spin state (singlet or triplet), and the degree of hydrogen bonding (C, CH_2, etc.) of the energetic carbon species as it first interacts "chemically" with the benzene.[1] These studies have been aided by the development of non-destructive high temperature gas radiochromatographic equipment.[2]

Preliminary studies in interstellar-space chemistry have been carried out by irradiating a $HCHO$-NH_3-H_2O mixture (these are all common interstellar molecules) with $^{14}C^+$ ions at 1 keV. This energy is about average for "solar wind" carbon, and, by inference, for "stellar wind" carbon. Identified products were glycolic and oxalic acids, glycine, alanine, and formamidine. Continuation of this research will doubtless provide better understanding of the organic chemistry of interstellar space, and will also be a guide for the kinds of compounds that we may expect to be identified there through the techniques of radioastronomy.

High-energy hydrogen chemistry is being studied as a route to increase the usefulness of tritium as a label in biochemical and biological research. We are working to establish the general applicability of the new system that employs T atoms (tritons) generated from T_2 in a radiofrequency field. We have built up in our laboratory the equipment for such labeling. However, although organic molecules are indeed labeled in this way, little is known about the mechanisms involved, about the selectivity of the thermal tritons for different molecules, or about the sites within a molecule that may become selectively labeled. A fundamental knowledge of the selection rules for the thermal triton reactions is essential for proper use of the resulting labeled (tracer) molecule.

We have determined various products by the microwave-generated tritons reacting with model compounds.[3,4] This research is designed to increase knowledge in, and usefulness of tritium labeling by microwave discharge. The method has considerable potential for the better preparation of high specific activity tritium-labeled compounds for tracer work in biology and chemistry.

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the American Chemical Society, Honolulu, Hawaii, April 1-6, 1979.

*4. C. T. Peng, W. H. Chiu, B. E. Gordon, W. R. Erwin, and R. M. Lemmon, Dehalogenation and Ring Saturation by Tritium Atoms, to be presented at the 177th national meeting of the American Chemical Society, Honolulu, Hawaii, April 1-6, 1979.

Abstracts

1. IRRADIATION OF BENZENE WITH $^{14}CH^+$ AND $^{14}CH_3^+$ IONS

W. R. Erwin, B. E. Gordon, L. D. Spicer, and R. M. Lemmon

J. Phys. Chem. 82, 6 (1978)

Solid benzene at -196°C was irradiated with $^{14}CH^+$ and $^{14}CH_3^+$ ions at 10-eV kinetic energy. Yields were determined for the labeled hydrocarbon products: benzene, toluene, cycloheptatriene, diphenylmethane, biphenyl, and phenylcycloheptatriene. The radioactivity distributions between the ring and the methyl group of the toluene product were also determined. These results have been compared to those previously obtained with $^{14}C^+$ and $^{14}CH_2^+$ ions. The comparisons have provided both insight into the reaction mechanisms and a tentative estimate of the distribution of the species ($^{14}CH_x$) that react with the benzene.

2. NONDESTRUCTIVE HIGH TEMPERATURE GAS RADIOCHROMATOGRAPHY

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

Anal. Chem. 50, 179 (1978)

A proportional flow counter for continuous operation at 300°C has been developed. It serves as the radioactivity detector in a gas radiochromatographic system. With associated electronics it is capable of both qualitative and quantitative analyses of the emerging peaks. The counter is decontaminated in situ by treatment with oxygen at 325-350°C. Backgrounds are low (~75 cpm) and, at the flow rates used, 5 dpm generate 2 counts. Possible quenchers are readily detected by their effect on the count rate produced by an external cobalt-60 source. Frequency of disassembly is low, averaging about once a year over the past three years.

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

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

To be presented at the 177th national meeting of the American Chemical Society, 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 had demonstrated that side reactions generate undesirable by-products of extremely high specific activity. Removal of these is possible with small molecules but 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 -197°C for five minutes to T atoms generated in a microwave plasma at 5 Torr T_2 and 18-20 W. Model compounds included n-hexane, heptene-2, benzene, 3-methylhexane, methylcyclohexane, 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; heptene-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.

4. DEHALOGENATION AND RING SATURATION BY TRITIUM ATOMS

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

To be presented at the 177th national meeting of the American Chemical Society, 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. ^3H-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 labeled products (cyclohexane/benzene). These results indicate a mechanism by which carrier-free ^3H-labeled products including biomacromolecules, etc. can be produced.

CARCINOGENESIS AND THE PHYSIOLOGICAL STATE OF THE CELL

James C. Bartholomew

Current concepts of the mechanisms(s) of chemical carcinogenesis involve the covalent interaction of the carcinogen with DNA. Interest in this particular type of interaction originated from the observation that the phenotypic properties of cancer cells are passed on to progeny cells in a "heritable" fashion, implying an alteration in the genome through mutation. In fact, there is a good correlation between the mutagenicity and carcinogenicity of a compound as well as between the ability of a compound to bind covalently to DNA and its carcinogenic potential. Although the mutation theory of chemical carcinogenesis has been disputed, it remains a central focus of much of the research into the mechanisms of chemical carcinogenesis. The general flow of this research has been to identify compounds that form covalent adducts with DNA, determine the structure of these adducts and how they alter the DNA secondary structure, and search for specificity of reaction that might relate to carcinogenesis. The emphasis of these studies has been principally on the chemistry of the interaction of carcinogens with DNA. It is becoming increasingly evident, however, that the physiological state of the cells 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 physiological states have different susceptibilities. Given that DNA from species to species and cell to cell have similar structures, i.e., made up of the same bases linked through phosphodiester bonds, the chemistry of carcinogen DNA interaction should be similar in all cases. Obviously, the cell is modulating the chemistry of carcinogen DNA interaction and/or the physiological response to these interactions. During the last year we have been investigating how the physiological state of the cell, in particular the position in the cell cycle, may be regulating carcinogenesis.

To understand how the cell cycle position could be altering the response to chemical carcinogens it is necessary to review what is known about the steps involved with a chemical binding to DNA (Figure 1). Chemicals that have been shown to be carcinogenic can be divided into two broad groups. The direct acting carcinogens such as certain alkylating agents react directly with DNA plus many other molecules in the cell whereas the second group of compounds, like the polycyclic aromatic hydrocarbons, require a prior activation via metabolizing enzymes found in the endoplasmic reticulum before they are reactive. The sensitivity of cells to malignant transformation by this second class of carcinogens depends at least in part on the ability of the cells to metabolize the compound.

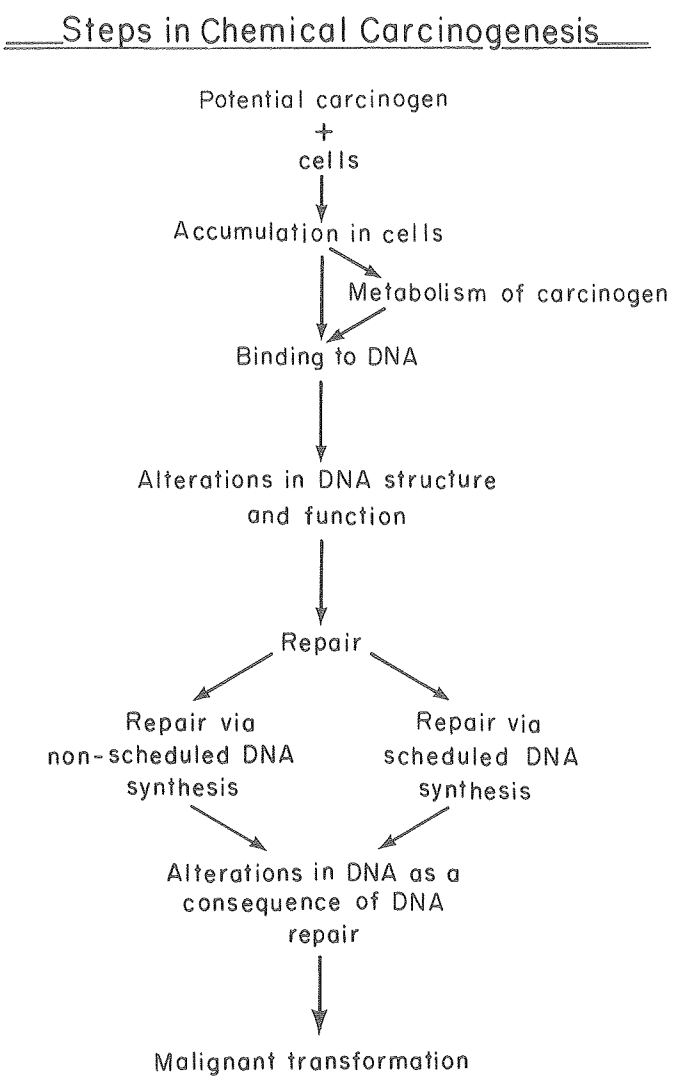

Figure 1. Steps in chemical carcinogenesis. (XBL 7810-4363)

Once the carcinogen is in the cell in an activated form, its binding to the DNA may depend on the condensation state of the DNA. DNA in the cell is complexed with nuclear proteins to give chromatin. The association of these proteins, in particular the histones, condenses the DNA into chromatin and chromosomes. The condensation state of the DNA changes as its functional state changes. For example, when the DNA is serving as a template for RNA synthesis, it is more accessible to enzymes that digest DNA. These alterations in chromatin state appear to be cell-cycle related in that dyes that intercalate into DNA have altered affinities to the DNA in different parts of the cell cycle. These observations suggest

that carcinogen binding may be affected by chromatin state and therefore may be cell-cycle dependent.

Once bound to DNA the damage caused in the structure of DNA is subject to repair by enzymes in the cell. These enzymes may break the DNA strand and excise the damaged nucleotide, or they may remove only the base, and in each case replace an undamaged nucleotide or base. The importance of this repair process in carcinogenesis has been dramatically emphasized in people who have diseases that result in deficiencies of DNA repair. These individuals have greatly increased incidences of skin cancer caused by UV irradiation. It has been shown that repair of damaged DNA is quite faithful in that the correct base or nucleotide is generally substituted for the damaged molecule.

The repair systems may not alway free the DNA of damage prior to replication. If damage is remaining in the DNA at the time of replication, then the replication system must correct the damage or bypass it in some way. It seems likely that this is the point where most of the alterations occur in DNA leading to mutagenesis and carcinogenesis. What these alterations are and how they give rise to malignant transformation are not known.

In the last year we have concentrated on four elements in this pathway to carcinogen-induced alterations in DNA and DNA functions.

1. Diet-Induced Alterations in Carcinogen Metabolism.

It is clear from epidemiological studies that diet has an influence on the incidence of cancer in the human population. Since in most cases it has been shown that the cell metabolizes carcinogens to more active derivatives, it seems reasonable to try to control this metabolism and direct it away from carcinogenic derivatives as one approach to reducing the incidence of cancer. The enzymes involved in this metabolism are membrane bound and therefore their activity should be influenced by the lipid environment of the complex. Alterations in the diet that change this lipid environment should affect not only the activity of the complex, but also the type of products formed from carcinogens.[1-3]

2. Effect of Chromatin Structure on Carcinogen Binding to DNA.

We have been studying the effect of BaP diol-epoxide on the structure and function of SV40 DNA and chromatin. SV40 DNA is a supercoiled circular molecule containing 5375 base pairs. If the supercoiled structure (form I) is nicked in a single strand, the molecule relaxes into an open circular structure called form II. A second nick in the other strand of the DNA opens the circle to give a linear molecule called form III. Normally the SV40 DNA harvested from monkey cells producing the virus is mostly supercoiled with a small amount of form II. When this DNA is reacted with increasing concentrations of BaP diol-epoxide, the supercoiled form is first converted into form II and then into form III structures. At much higher concentrations the DNA is fragmented. The formation of DNA strand breaks was also shown by electron microscopy of the DNA after reaction with BaP diol-epoxide. Using this system of analysis, strand breaks are detected when the DNA is reacted with enough BaP diol-epoxide to give approximately 30 adducts per genome. At lower levels of adduct formation where strand breaks do not occur, an unwinding of the DNA supercoil takes place as detected by polyacrylamide gel electrophoresis. The angle of unwinding per adduct (46^{o}) is much greater than the 26^{o} normally associated with intercalation, suggesting considerable denaturation of the DNA strands. Reaction of SV40 chromatin with different concentrations of diol-epoxide and separation of the chromatin on agarose gels shows little effect on the chromatin structure. However, when the proteins are removed by SDS treatment and the DNA electrophoresed, the fragmentation of the DNA is detected. Preliminary studies on the effect of BaP diol-epoxide on the replication of SV40 DNA in monkey cells suggests that the carcinogen interferes with the initiation of DNA synthesis as well as in some late step in the formation of the two daughter molecules.

3. Repair of DNA Damaged by Benzo[a]pyrene in Human and Mouse Cells Growing in Culture.

Rodent cells are more susceptible to carcinogenesis by polycyclic aromatic hydrocarbons than human cells. Part of this increased sensitivity can be attributed to an increased metabolic capacity of rodent cells for polycyclic aromatic hydrocarbons. We have found, however, that another factor in this resistance to polycyclic aromatic hydrocarbons is the repair capacity of cells for damage to their DNA. When mouse liver cells and human lung cells were compared for their ability to remove benzo[a]pyrene adducts from the DNA, the mouse cells could remove only approximately 20% of the damage in 72 hr whereas the human cells removed nearly 70% of the damage. In both cell strains this level was the maximum removed and there was always a residual amount of damage remaining in the DNA. A common promoter of carcinogenesis by these hydrocarbons is 12-O-tetradecanyl-phorbol-13-acetate (TPA), which had no effect on the repair of DNA damage.

4. Effects of Chemical Carcinogens on DNA Synthesis.

The mechanism of action of chemical carcinogens is not known, but evidence is accumulating that they act by causing mutations. These mutations presumably arise through a direct binding of the carcinogen to DNA, causing errors in the replication of the genome. This binding to the DNA may also cause alterations in the DNA replication rate, which can be detected by analysis of the kinetic parameters of the cell cycle. We have used flow cytometry (FCM) 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 (BaP) and derivatives of BaP on DNA synthesis. The derivatives tested were: 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene(7,8-diol); 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene-9,10-oxide (Diol Epoxide), and 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxybenzo[a]pyrene (tetrol). One cell strain used in this study, NMuLi cl 7, is not highly inducible for the enzyme system Aryl Hydrocarbon Hydroxylase (AHH) that converts the parent compound into the derivatives listed above. The second strain, NMuLi cl 8, is highly inducible for AHH. 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. FCM analysis showed that the Diol-Epoxide increased the number of cells involved in DNA synthesis, but that the rate of DNA synthesis was greatly reduced. BaP and 7,8-Diol had this same effect on NMuLi cl 8, but had no effect on NMuLi cl 7.

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Abstracts

PHOTOCHEMICAL ADDITION OF THE CROSS-LINKING REAGENT 4,5',8-TRIMETHYLPSORALEN (TRIOXSALEN) TO INTRACELLULAR AND VIRAL SIMIAN VIRUS 40 DNA-HISTONE COMPLEXES

L. M. Hallick, H. A. Yokota, J. C. Bartholomew, and J. E. Hearst

J. Virol. 27, 127-135 (1978)

We demonstrated here that 4,5',8-trimethylpsoralen (trioxsalen) is a valuable probe for the structure of SV40 DNA-histone complexes. Trioxsalen readily penetrated intact cells and, in the presence 340- to 380-nm light, covalently cross-linked DNA preferentially at the sites available for micrococcal nuclease digestion. Histograms of the lengths of the regions of SV40 DNA protected from cross-linking, as visualized by electron microscopy, indicated a repeating pattern of base pairs in DNA from both infected cells and virus particles. The ability of the trioxsalen probe to act in vivo and to map the location of protected regions may provide a powerful tool for analyzing the role of nucleosomes in the structure of the virus particle and in intracellular complexes such as transcription templates and replication intermediates.

1. FATTY ACID REQUIREMENTS AND TEMPERATURE DEPENDENCE OF MONOXYGENASE ACTIVITY IN RAT LIVER MICROSOMES

J. F. Becker, T. Meehan and J. C. Bartholomew

Biochim. Biophys. Acta 512, 136-146 (1978)

The effect of variation in the microsomal membrane fatty acid composition on Arrhenius plot phase transition temperatures for p-nitroanisole O-demethylation and benzo[a]pyrene hydroxylation has been investigated. In liver microsomes from normal-dieted rats, p-nitroanisol O-demethylase activity has a break temperature at 24°C, while that of benzo[a]pyrene hydroxylase occurs at 29°C indicating that these two enzymes may exist in different patches of membrane. The microsomal membrane fatty acid composition was altered by starving rats for 48 h and then refeeding them a fat-free diet for 4 or 5 days. In microsomes having diet-altered fatty acid compositions, benzo[a]pyrene hydroxylase has a break temperature at 33°C, a value higher than that observed in normal-dieted rats. This observation correlates with the increase in saturation observed in the diet-altered fatty acid composition and thus may correspond to a phase transition roughly dependent on

*See Abstracts.

the fatty acid melting point. Induced and basal levels of cytochrome P-450 and P-448 in animals having different microsomal fatty acid composition are reported. Phenobarbital-induced levels of p-nitroanisole O-demethylase in normal microsomes were six times higher than those in microsomes having diet-altered composition, whereas 3-methylcholanthrene-induced levels of benzo[a]pyrene hydroxylase were similar regardless of diet. The low level of p-nitroanisole O-demethylase activity in membranes with altered fatty acid compositions suggests that a particular type(s) of fatty acid was not present in sufficient quantity to permit the induction of maximal enzyme activity. Since the induced benzo[a]pyrene hydroxylase activity was the same regardless of diet, there was presumably sufficient quantities of the appropriate fatty acids present in the membrane for induction of this activity. Therefore, particular fatty acids may be necessary for the induction of maximal activity of particular enzymes in the mixed function monooxygenase system.

SELECTIVE FLUORESCENCE QUENCHING OF BENZO[a]PYRENE AND A MUTAGENIC DIOL EXPOXIDE DERIVATIVE IN MOUSE CELLS

C. G. Wade, D. E. Baker, and J. C. Bartholomew

Biochemistry 17, 4332-4337 (1978)

The overlap of the fluorescence spectra of benzo[a]pyrene and its metabolites, including an ultimate carcinogen diol expoxide (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene, impedes the use of fluorescence in studying the impact of these important carcinogens on cell populations. It is shown that in cells certain collisional quenchers can be used to selectively quench the benzo[a]pyrene fluorescence, leaving the diol epoxide emission. Because such quenchers work only with close contact over distances much less than the cell dimensions, the preferential solubilities can be used to probe fluorophore locations. Benzo[a]pyrene and its diol epoxide derivative were added directly to mouse cells in culture: NMuLi (mouse liver epithelial cells), Balb 3T3 A31 HYF (mouse fibroblast cells), and MSV/MLV Balb 3T3 A31 HYF (Moloney sarcoma virus transformed mouse fibroblast cells). Fluorescence lifetimes of the aromatic hydrocarbons were measured, and fluorescence and flow cytometric studies were made on the effects of various quenchers added to suspensions of these cells. The collisional quencher n-octyl iodide, which is lipid soluble, quenches the benzo[a]pyrene emission by a factor of six with little change in the diol epoxide emission. At comparable molarity, the ionic quencher, KI, is relatively ineffective and does not offer selective effects, presumably because the I^- does not penetrate the cell. At comparable molarity, methylene iodide is a relatively effective quencher for both aromatic hydrocarbons, but it offers little selectivity. The results were, in general, independent of the particular type of mouse cell strain. The results provide information on the location of the hydrocarbons when added to the cells. Evidence indicates that the selectivity offered by the n-octyl iodide arises from the fact that collisions with benzo[a]pyrene are much more likely than collisions with the diol epoxide. Consequently, the possible locations of the metabolite under the experimental conditions are: bound in proteins or attached near the polar head groups of lipids in membranes or attached in a nonlipid portion of the cell. The potential for using the relative solubilities of collisional quenchers in separating the fluorescence emissions of polycyclic aromatic hydrocarbons is discussed. Applications of the technique to flow cytometry are demonstrated.

BASE SPECIFICITY IN THE BINDING OF BENZO[a]PYRENE DIOL EPOXIDE TO SIMIAN VIRUS 40 DNA

L. Mengle, H. Gamper, and J. Bartholomew

Cancer Lett. 5, 131-137 (1978)

[^{14}C] Simian virus 40 (SV40) DNA was reacted with [^{3}H]7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene to give 0.60 adducts per genome. The modified DNA was digested to completion with Hind III restriction endonuclease and the 6 fragments isolated by polyacrylamide gel electrophoresis. Hydrocarbon binding to the fragments was proportional to their guanine-cytosine (G-C) content, reflecting selective reaction of the hydrocarbon with deoxyguanosine residues. No sites unusually susceptible to alkylation were detected.

2. BIOCHEMICAL BASIS FOR THE ACQUISITION OF RESISTANCE TO BENZO[a]PYRENE IN CLONES OF MOUSE LIVER CELLS IN CULTURE

J. R. Landolph, J. F. Becker, H. Gamper, J. C. Bartholomew, and M. Calvin

Chem. Biol. Interact. 23, 331-344 (1978)

In a Namru mouse liver epithelial cell strain designated NMuLi, aryl hydrocarbon hydroxylase (AHH) activity peaked at 12 h post-induction with 1 μg/ml of benzo[a]pyrene (BaP) in both confluent and growing cells. Maximal levels of AHH activity were reached on day two post-plating. This induced activity was inhibited in vitro 78% by gassing the incubation mixture with carbon monoxide for 15 s, and inhibited 93% by addition of 40 μg/ml of 7,8-benzoflavone (BF).

Induced AHH levels were higher in epithelial clones that were sensitive to the toxicity of BaP than in resistant clones. The survival fraction of clones from NMuLi and of subclones derived from a sensitive clone of NMuLi after BaP treatment was a negative exponential function of the maximal induced AHH activity in the clones.

One of the clones, NMuLi cl 8, was extremely susceptible to the toxic effects of BaP, the +(trans)-7α, 8β-dihydroxy-7,8-dihydro-BaP (7,8 diol), and the (±)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydro-BaP (diol-epoxide), known metabolites of BaP. The toxicity of BaP and the 7,8 diol to this clone was inhibited by BF, suggesting that these cells possessed an exzyme activity inhibitable by BF that could epoxidize BaP to the 7,8 oxide and then epoxidize the resultant 7,8 diol to the diol-epoxide. Another clone derived from NMuLi, clone 7, was relatively resistant to the toxic effects of BaP and the 7,8 diol, but still extremely susceptible to the toxic effects of the diol-epoxide. The slight toxicity to BaP in this clone was inhibited by BF, but the toxicity of the 7,8 diol to this clone was not inhibited by BF. A typical cytochrome P450 inhibitor, metyrapone, had no effect on the toxicity of BaP, the 7,8 diol, or the diolepoxide to either clone 7 or clone 8.

The results suggest that these liver cells possess two enzymes that play some role in polycyclic hydrocarbon-induced toxicity. Enzyme A, a BaP-inducible enzyme that is inhibitable by BF, efficiently metabolizes BaP to the 7,8 diol and the 7,8 diol to the diol-epoxide. It is responsible for most of the hydrocarbon toxicity. Enzyme B is not inhibitable by BF and metabolizes the 7,8 diol less efficiently to the diol-epoxide or efficiently to other, less toxic products.

3. ARYL HYDROCARBON HYDROXYLASE INDUCTION IN MOUSE LIVER CELLS--RELATION TO POSITION IN THE CELL CYCLE

J. F. Becker and J. C. Bartholomew

Chem. Biol. Interact., in press

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 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 hr. Addition of BaP (1 μg/ml) 8 hr before serum stimulation, at the time of stimulation, or 8 hr 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.

GENE EXPRESSION AND MALIGNANT TRANSFORMATION

Mina J. Bissell

There are two aspects to the process of differentiation. One is the sequential events in development, i.e., the process by which various tissues are derived from a single cell. The second is the mechanism by which tissues retain or modulate their specific functions. Given the fact that all cells contain the same genetic material, the environment of the cell (matrix, other cells, hormonal factors, etc.) must play a critical role in gene regulation.

The focus of our research in this section is to understand the nature of the interaction of the cell with its environment. In the regulation of this two-way process lie the answers to whether cells are normal or diseased, how they become malignant, and how they age. In order to understand how cells become abnormal, it is important to study the regulation of tissue-specific functions in normal cells under defined 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 yet 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 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 differentiated if it elaborates a given (or 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 well established that in vivo the regulation of such metabolism is unique for each cell type and organ. In culture, tissue-specific differences can also be easily demonstrated initially.[1-3] However, because of the trend to define conditions for growth, as time goes on, all cells in culture tend to become similar metabolically.

Our working hypothesis is that the metabolic state of a cell may indeed regulate gene expression. The similarity in metabolic state may explain why most cells in culture are more alike than different, regardless of the tissues of the origin. This laboratory's extensive experience with regulation of intermediary metabolism in both plants and animals makes it an ideal setting to test the above hypothesis. In order to do so, we are establishing metabolic patterns and defining conditions for optimal gene expression in three cell systems: embryonic avian tendon, rat liver, and human (and mouse) mammary epithelial cells (see Figure 1) Development of meaningful cell systems and conditions for expression of tissue-specific functions should enable us, as well as other investigators, to study the mechanism of gene regulation in molecular terms with more relevance to the in vivo situation.

Regulation of Collagen Synthesis 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.[4] The function is modulated by cell density, ascorbic and lactic acids, serum levels and viral transformation.[4-7] We have transformed these cells with temperature-sensitive mutants of Rous sarcoma virus and determined the type of collagen synthesized in culture.[8] Additionally, the tendon cells are shown to synthesize an extensive extracellular matrix composed mainly of collagen.[9] Thus, the cell system is not only an ideal model for studying regulation of collagen synthesis (30% of total protein synthesized is collagen) and mechanism of action of ascorbate, but also for studies of collagen fiber formation. The mechanism(s) by which such varied compounds and the transforming gene of RNA tumor viruses modulate collagen synthesis and the level at which the function is regulated in each case (transcription, translation, post-translation, etc.) is under investigation.

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 epithelia 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 a floating collagen gel where they are in contact in three dimensions, morphological and functional differences become apparent.[10]

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.

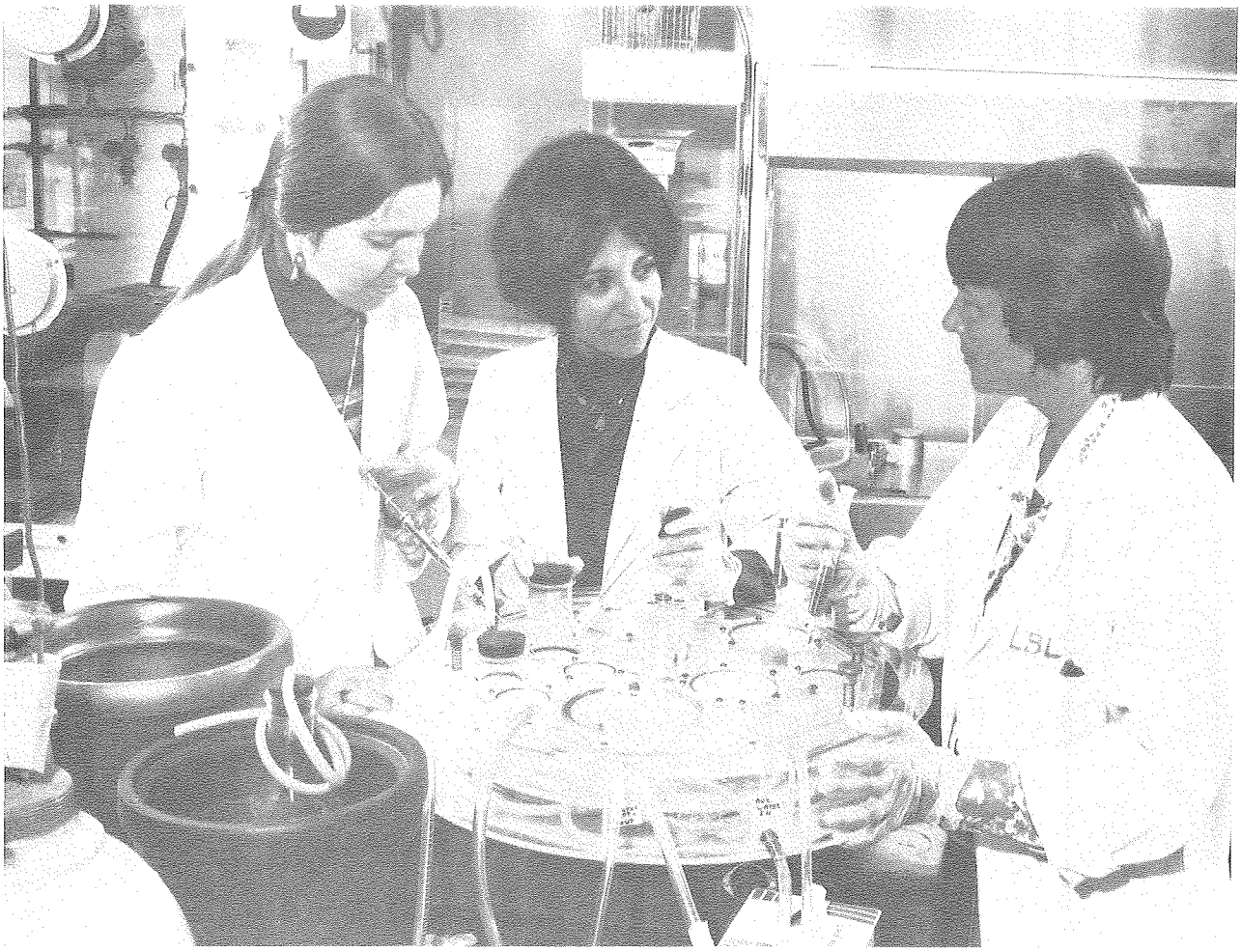

Figure 1. The steady-state apparatus for animal cells in culture was designed and built in this laboratory. Up to 30 replica plates may be kept under constant temperature and pH for kinetic tracer studies. (BBC 765-4658)

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 isolated cells could be demonstrated.[12] 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, Lawrence Berkeley Laboratory.

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.[2,13] We are in the process of correlating maintenance in different carbon sources to expression of liver-specific functions such as the level of cytochrome P-450. These experiments are performed in collaboration with D. M. Bissell of the Department of Medicine, U.C. San Francisco.

Ascorbic Acid and RNA Tumor Virus Replication

Little is known about the mechanism(s) of action of ascorbic acid at the cellular level with regard to animal virus infection. This is despite the widespread belief in the beneficial role of vitamin C against viral disease. While studying the process of transformation in primary avian tendon (PAT) cells, we observed that the presence of the vitamin reduced the rate of transformation of these cells by Rous sarcoma viruses as judged by morphology and the reduction of the rate of sugar uptake. The vitamin had no direct effect on the potency of the virus since pretreatment of virus with ascorbic acid did not reduce infectivity. However, pretreatment of cells for 24-48 hrs at a concentration that was not toxic to the cells (50 μg/ml) greatly

enhanced the retardation of transformation. Most of the treated cells retained normal morphology and their rate of 2-deoxy-D-glucose uptake was close to that of uninfected normal cells. The primary action of ascorbate thus appears to be cellular protection against viral infection, which in turn leads to a reduced level of virus production. This is supported by the fact that the infection of ascorbate-treated cells by a transformation-defective Rous sarcoma virus led to a comparable reduction in the level of reverse transcriptase activity in the medium. However, an additional effect on the process of transformation and/or infectious virus producton cannot be ruled out since the focus forming units of the virus produced in the presence of ascorbate was reduced to a much greater extent than reverse transcriptase activity. Similar results were observed with chick embryo fibroblasts, which synthesize collagen at a much lower level than PAT cells and do not modulate the level of collagen in the presence of ascorbate.[14]

Viral Carcinogenesis and Tumor Promotion

We have recently developed 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 caused by a two-stage process, similar to that proposed for tumor induction on mouse skin by carcinogens and tumor promoters. The product of the transforming gene of viruses (src) is then seen as a tumor promoter rather than the initiator of transformation.[15]

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ANTIVIRAL ACTION OF A RIFAMYCIN DERIVATIVE: FORMATION OF ROUS SARCOMA VIRUS PARTICLES DEFICIENT IN 60 TO 70S RNA

C. Szabo and M. J. Bissell

J. Virol. 25, 944-947 (1978)

Growth of Rous sarcoma virus-transformed cells in the presence of rifazone-8_2, a rifamycin derivative, results in the formation of non-infectious virus particles lacking 60 to 70S RNA.

2. GLUCOSE METABOLISM BY ADULT HEPATOCYTES IN PRIMARY CULTURE AND BY CELL LINES FROM RAT LIVER

D. M. Bissell, G. A. Levine, and M. J. Bissell

Am. J. Physiol. 234(3), C122-C130 (1978)

The metabolic fate of [U-^{14}C] glucose has been examined in detail in adult rat hepatocytes in primary monolayer culture, as well as in two permanent cell lines--Buffalo rat liver (BRL) and transplantable rat hepatoma (HTC) cells--derived from normal rat liver and from rat hepatoma, respectively. Under defined conditions of incubation, at a glucose concentration of 5.5 mM, the three types of cultured liver cells exhibited pronounced differences in glucose metabolism. Primary cultures, like the intact liver, differed from the cell lines in consuming relatively small amounts of glucose and converting approximately 50% of the total metabolized glucose to lactate. By contrast, the permanent cell lines consumed glucose at a 40-fold greater rate than did primary cultures, converting 80-90% of the carbohydrate to lactate. Oxidative metabolism of glucose carbon also differed among the three types of liver culture. Of the total [U-^{14}C] glucose consumed, primary cultures converted approximately 30% to labeled CO_2 per hour, whereas the liver cell lines converted 5-10%. Finally, glucose metabolism in primary culture exhibited adaptation as hepatocytes aged in culture, shifting progressively toward the pattern exhibited by the permanent cell lines. This change occurred over a time course similar to that for other kinds of functional change in hepatocytes in primary culture and thus may be relevant to the general problem of phenotypic alteration in liver cell culture.

INHIBITION OF ADENOCARCINOMA TA3 ASCITES TUMOR GROWTH BY RIFAMYCIN DERIVATIVES

A. M. Hughes, T. S. Tenforde, M. Calvin, M. J. Bissell, A. N. Tischler, and E. L. Bennett

Oncology 35, 76 (1978)

A growth inhibitory effect on adenocarcinoma TA3 ascites tumors in LAF_1/J mice resulted from the repeated IP administration of subtoxic doses of 3 rifamycin derivatives: rifampicin (Rif)[1], dimethylbenzyldesmethylrifampicin (DMB), and rifazone-8_2 (R-8_2). A high-viscosity methylcellulose vehicle was found to be essential for obtaining a uniform drug suspension and a significant antitumor effect by the least water soluble derivatives, DMB and R-8_2. The more hydrophilic derivative, Rif, was found to have a comparable growth inhibitory effect on TA3 cells when prepared in 0.9% NaCl solution with or without added methylcellulose. Oral or SC drug injections did not have an antitumor effect. The results of this study point to the importance of vehicle and route of administration in chemotherapy trials with these compounds.

6. PRIMARY AVIAN TENDON CELLS IN CULTURE--AN IMPROVED SYSTEM FOR UNDERSTANDING MALIGNANT TRANSFORMATION

R. I. Schwarz, D. A. Farson, W.-J. Soo, and M. J. Bissell

J. Cell Biol. 79, 672-679 (1978)

Primary avian tendon (PAT) cells that maintain their differentiated state in culture are rapidly transformed by Rous sarcoma virus. By criteria of morphology, increased rate of 2-deoxyglucose uptake, and loss of density dependent growth control, PAT cells transform as well as their less differentiated counterpart, chick embryo fibroblasts. In addition, the percentage of collagen produced by PAT cells drops on transformation by an order of magnitude, from 23 to 2.5%, but is unaffected by viral replication of a transformation-defective mutant.

The responsiveness of normal and transformed PAT cells to various environmental factors changes dramatically upon transformation. Normal PAT cells respond to the presence of ascorbate and high cell density by raising the level of collagen synthesis from 5 to 23%. Transformed PAT cells are totally unresponsive. These and previously reported results lead us to relate the breakdown in the normal mechanisms used by the cell to maintain the differentiated state to the onset of transformation.

12. A SIMPLE TECHNIQUE FOR DETECTION AND QUANTITATION OF LACTOSE SYNTHESIS AND SECRETION

J. T. Emerman and M. J. Bissell

Anal. Biochem., in press.

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 μg 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 in our ability to study the regulation of mammary-specific functions.

13. CONVERSION OF GLUCOSE TO SORBITOL AND FRUCTOSE BY LIVER-DERIVED CELLS IN CULTURE

G. A. Levine, M. J. Bissell, and D. M. Bissell

J. Biol. Chem. 253, 5985-5989 (1978)

Conversion of glucose to fructose and sorbitol is documented in rat hepatoma-derived cultured cells (HTC cells). After addition of 5.5 mM [U-^{14}C]glucose to incubation medium, labeled sorbitol and fructose accumulated intracellularly at a linear rate over a period of 60 min. The sugars were isolated, identified, and quantitated by paper chromatography, gas-liquid chromatography, and enzymatic phosphorylation of fructose. Primary culture of adult rat hepatocytes was analyzed similarly and demonstrated no significant accumulation of labeled fructose or sorbitol. The basis for this difference between HTC cells and primary hepatocyte culture was examined both in terms of enzyme activities that mediate the formation of sorbitol and fructose and in terms of the catabolism of these sugars. Both types of culture (as well as extracts of intact rat liver) exhibited enzymatic activities catalyzing the conversion of glucose to sorbitol (aldose reductase) and sorbitol to fructose (sorbitol dehydrogenase). However, the cultures differed strikingly with regard to the catabolism of sorbitol and fructose. The conversion of labeled sorbitol to metabolites in HTC cells was negligible; by contrast, hepatocytes in primary culture utilized the sugars at rates comparable to that of glucose, which may account for the lack of their accumulation in primary culture. The findings suggest that the conversion of glucose to sorbitol and fructose by HTC cells may represent a retained normal liver function, one which is amplified by the inability of HTC cells to dispose of these sugars.

RAPID INHIBITION OF COLLAGEN SYNTHESIS AFTER TRANSFORMATION OF AVIAN TENDON CELLS BY A TEMPERATURE-SENSITIVE MUTANT OF ROUS SARCOMA VIRUS

W.-J. Soo, R. I. Schwarz, J. A. Bassham, and M. J. Bissell

J. Biol. Chem., in press.

Transformation of primary avian tendon cells with Rous sarcoma viruses caused the cells to become less differentiated in that the level of collagen synthesis dropped from the in ovo level of 20-30% to 2.5%. By using a temperature-sensitive virus, we were able to study the time course of this change during transformation and reversion to normal phenotype. The reduction in collagen synthesis was found to be an early event during the process of transformation and it was closely associated in time with other early events such as the increase in sugar uptake. During the process of reversion to normal phenotype, however, the increase in collagen synthesis was found to occur much more slowly. Furthermore, unlike what is reported in the literature, neither growth in culture nor the process of transformation caused major changes in the type of collagen produced.

15. IS THE PRODUCT OF THE SRC GENE A PROMOTER?

M. J. Bissell, C. Hatíe, and M. Calvin

Proc. Natl. Acad. Sci. U.S.A. in press (1979).

Addition of a potent promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to primary avian tendon or chick embryo fibroblast cells infected with a temperature-sensitive mutant of Rous sarcoma virus produced a complete transformed phenocopy at the non-permissive temperature by the criteria tested. While normal, uninfected cultures also shifted towards a transformed phenotype after TPA addition, they did not achieve the same degree of morphological and biochemical alterations seen in virus-infected, TPA-treated cells. It is proposed that viral carcinogenesis, despite its rapidity, may occur in two stages: an "initiation" step caused by integration of all or part of the viral genome and a promotion step (itself a multi-step process) caused by the activation of the src gene. The latter could be enhanced or replaced by other promoting agents.

APPENDIX A: DIVISION PUBLICATIONS, 1978

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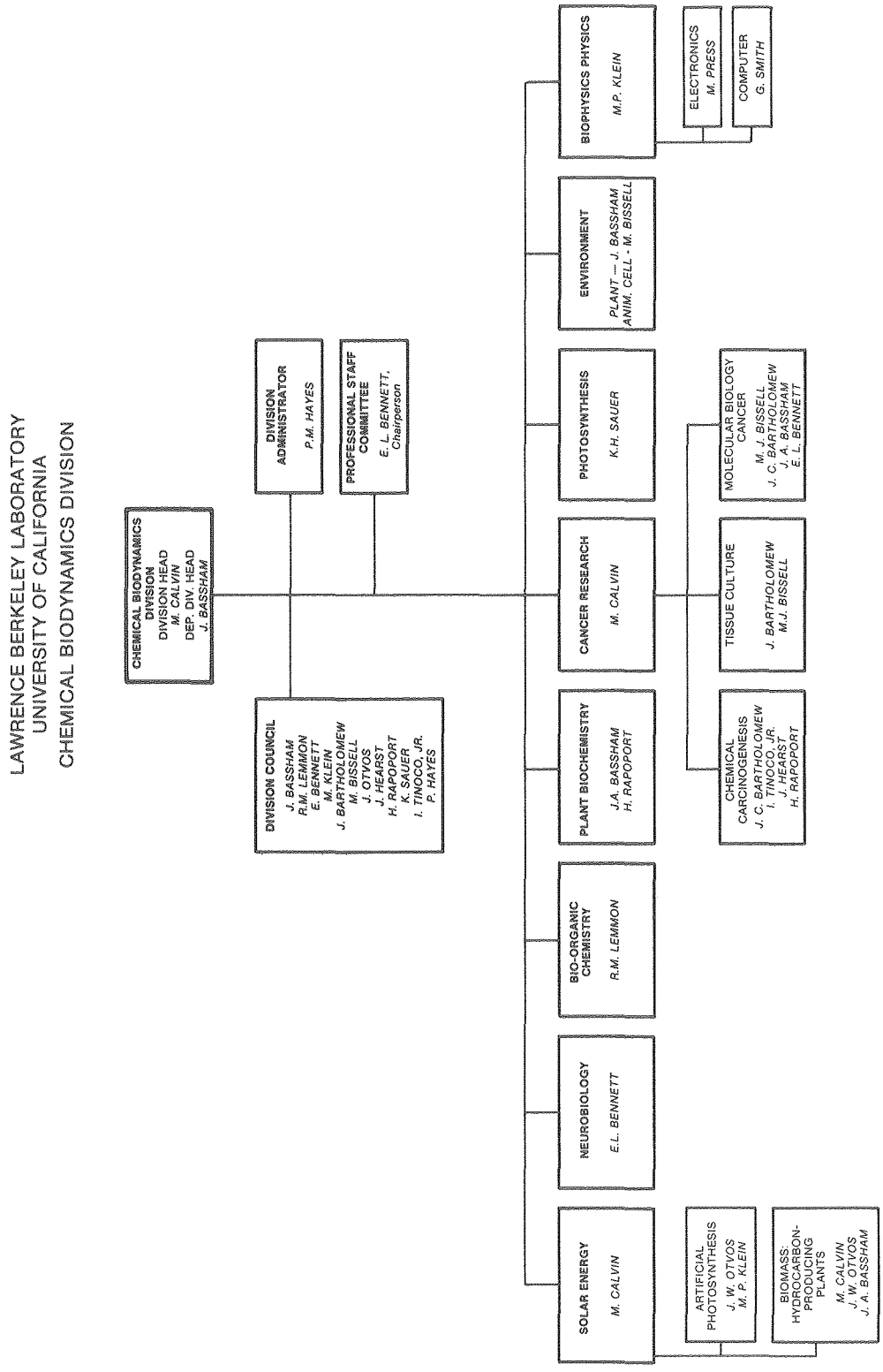
APPENDIX B: ORGANIZATION CHART



APPENDIX C: DIVISION STAFF

Melvin Calvin (University Professor of Chemistry, University of California, Berkeley) -- Division Head, Chemical Biodynamics Division, LBL, and Director, Laboratory of Chemical Biodynamics

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Itamer Willner
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Thomas D. Meehan*

Postdoctoral Visitors

Joseph F. Becker*

Jacques Breton*

Charles G. Edmonds*

G. Charles Dismukes*

Joanne T. Emerman

Arthur J. Frank*

Harry Frank

J. R. Hauske

Bruce B. Johnson*

Ronald J. Lukas

Werner M. Kaiser*

James E. McMahon*

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Francis H. Martin

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Hasker P. Davis
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Stephen A. Winkle

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APPENDIX D: ATTENDANCE AT FOREIGN CONFERENCES, 1978

James C. Bartholomew
6th Engineering Foundation Conference on Automated Cytology. Schloss Elmau, Bavaria, Germany, April 23-29, 1978.

James A. Bassham
World Conference on Future Sources of Organic Raw Materials. Sponsored by Chemical Institute of Canada, American Chemical Society and the International Union of Pure and Applied Chemistry, Chemrawn, Toronto, Canada. July 10-13, 1978.

Caribbean Consultation on Energy and Agriculture, Santo Domingo, Dominican Republic. November 29-December 1, 1978.

Opening of the Desert Institute, Sde Boquer, Israel. December 3-10, 1978.

Edward L. Bennett
UNESCO-WHO Workshop on Basic Knowledge in Neurosciences, Memory and Learning and Neurosciences. Mahidal University and Chulalongkorn University, Bangkok, Thailand. February 21-March 2, 1978.

2nd World Congress of Biological Psychiatry. Barcelona, Spain. August 30-September 6, 1978.

Harden Conference, Wye College, Ashford, England. September 16-22, 1978.

Mina J. Bissell
Third International Gstaad Conference. Gstaad, Switzerland. September 12-15, 1978.

Melvin Calvin
Symposium on Bioenergy. Gottlieb-Duttweiler Institut, Zurich, Switzerland, January 10-12, 1978.

Second Latin-American Botanical Congress. Brasilia, Brazil. January 21-27, 1978.

Centennial Meeting, Chemical Society of Japan. Tokyo, Japan. April 2-5, 1978.

Agricultural Chemical Society, Nagoya, Japan. April 1, 1978.

International Symposium on Chloroplast Development. Spetsai, Greece. July 9-15, 1978.

International Biomass Symposium. University of Ceara, Fortaleza, Brazil. August 15-18, 1978.

International Sugar Congress, Campos, Brazil. August 21-25, 1978.

Arthur J. Frank
Fourth International Photochemistry Conference and Second International Congress on Photochemical Conversion and Storage of Solar Energy. Cambridge, England. August 8-13, 1978.

Michael R. Hanley
International Symposium on Cytopharmacology, Venice, Italy. July 9-13, 1978.

John W. Otvos
Second International Congress on Photochemical Conversion and Storage of Solar Energy. Cambridge, England. August 1-13, 1978.

Gordon A. Parry
European Tissue Culture Meeting. Glasgow, Scotland. July 2-5, 1978.

Andrew Pearlman
Sixth Engineering Foundation Conference on Automated Cytology. Schloss Elmau, Bavaria, Germany. April 23-29, 1978.

Henry Rapoport
The Synthesis of Natural Products, Munich, Germany. September 24-28, 1978.

Ignacio Tinoco, Jr.
NATO Advanced Study Institute on Optical Activity and Chiral Discrimination. "The Optical Activity of Chiral Polymers" and "Fluorescence Detected Circular Dichroism." University of Sussex, Brighton, England.

International Conference on Quantum Chemistry, Biology and Pharmacology. "Circular Dichroism of Large Molecules." Theoretical Physics Institute, Kiev, U.S.S.R. September 17-23, 1978.

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