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## CHEMICAL BIODYNAMICS ANNUAL REPORT, 1965

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# CHEMICAL BIODYNAMICS ANNUAL REPORT, 1965

M. Calvin, Director, Laboratory of Chemical Biodynamics

March 1966

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# CHEMICAL BIODYNAMICS ANNUAL REPORT, 1965

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## FOREWORD

Most of the work of the Chemical Biodynamics Group stems from two primary concerns: First, the development of radioactive isotopes and their application principally to chemical and biological systems, and, second, the interaction of biological and chemical systems with radiation. In this annual report we are continuing our custom of selecting a few of our activities for description in order to indicate the scope of the Group's productivity. Many of the particular projects involve the interaction of radiation with both chemical and biological systems as well as the use of the tracer method to define the nature of those interactions.

Our studies in photosynthesis and radiation chemistry have continued on an expanding frontier that includes both the metabolic behavior, the physical-chemical structure, and the energy conversion process, a few aspects of which are described in this report. It is perhaps worth mentioning specifically that the radiation chemistry and the fundamental problems of information transfer in biology have led us into the problems of chemical evolution as well as into research on brain mechanisms and learning.

Melvin Calvin, Director
Chemical Biodynamics Group
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# CHEMICAL BIODYNAMICS ANNUAL REPORT, 1965

M. Calvin, Director

Lawrence Radiation Laboratory
University of California
Berkeley, California

March 1966

## 1. ORGANIZATION AND FUNCTION OF CHLOROPHYLL PIGMENTS IN PLANT CHLOROPLASTS

Kenneth Sauer

Chlorophyll plays a unique role in nature as the essential catalyst for storing light energy in chemical products of high potential available for photosynthesis. The detailed mechanism of the role of chlorophyll in this process is largely unknown, but it is thought to depend critically on the specific environment and arrangement of the pigment molecules in the chloroplast. Our studies aim at learning the nature of the phenomenon through kinetic measurements of the light reactions associated with photosynthesis and through physical measurements which will clarify the spatial organization of the molecules.

We find that the photoreduction of the endogenous cofactor NADP (nicotinamide adenine dinucleotide phosphate) by chloroplasts occurs with a requirement of 2 to 4 quanta per electron transferred to NADP at wavelengths from 550 to 730 mμ in a system in which oxygen evolution is inhibited and electrons are furnished by reduced $DPIPH_2$ (2, 6-dichlorophenolindophenol).[1] The chloroplast-catalyzed photoreduction of cytochrome c, with reduced trimethyl-p-benzoquinone used as electron donor, occurs with a similar action spectrum but with requirements of only 2 quanta per electron at wavelengths from 620 to 680 mμ and 1 quantum per electron at longer wavelengths (700 to 740 mμ).[2] On the other hand, reactions using water as electron donor and giving rise to molecular oxygen show different action spectra. Quantum requirements are similar (2 to 4 quanta per electron) in the 620- to 680-mμ region, but rise to values in excess of 20 at wavelengths longer than 700 mμ. This is true whether the electron acceptor is the endogenous NADP[1] or Hill oxidants such as ferricyanide or oxidized DPIP.[3] The results are interpreted as reflecting the participation of two pigment systems and two light reactions in the normal reduction of NADP using water and generating molecular oxygen. The two light reactions apparently interact only at the chemical level of photosynthetic electron transport, and the two pigment systems do not seem to transfer significant amounts of electronic excitation from one to the other.[2] Figure 1-1 shows a model of a cross section of the chloroplast lamellar system including several identical quantasome units, in which the two pigment systems and the products of their photoreactions are postulated to be on opposite faces of the lamellar layers. We are continuing these investigations with the objective of learning more about the manner in which the two pigment systems are coupled to the various steps in the electron transport pathway and the way in which they, in turn, are connected to the site of photosynthetic phosphorylation. This latter process provides an additional essential source of high-energy intermediates for driving the carbon-fixing reactions of the chloroplasts.

Optical studies on the chloroplast lamellae continue to produce evidence confirming the ordered nature of the pigment molecules. Measurements of the dichroism spectrum (absorption using polarized light) of quantasome aggregates oriented in a hydrodynamic or flow gradient[4] precisely confirm earlier dichroism measurements in which a pulsed electric field was used to orient the lamellar fragments.[5] Although the bulk (95%) of the pigment molecules gives evidence of no net orientation with respect to the quantasome particle axes, a pigment component (presumably chlorophyll a in a special environment) absorbing at

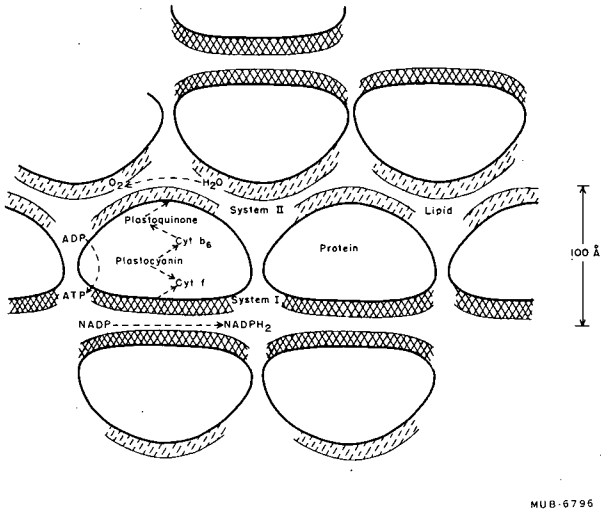


Fig. 1-1. Model of a cross section of the chloroplast lamellar system including several identical quantasome units.

wavelengths longer than 680 mμ does exhibit a small but distinct dichroism. The absorption oscillator for this long-wavelength absorption lies parallel to the streamlines of the flowing sample, which is assumed to be the direction in which the planes of the chloroplast lamellae are oriented. This long-wavelength oriented chlorophyll component may include the photochemically active molecules which appear to participate in the initial oxidation-reduction reactions following the absorption of light.

Further evidence bearing on the aggregated nature of the chlorophyll molecules in quantasomes is found from their optical rotatory dispersion (ORD) spectrum (i.e., the wavelength dependence of the rotation of the plane of polarized light incident upon the sample).[6] Quantasome suspensions exhibit a number of strong dispersion effects (Cotton effects) associated with chlorophyll absorption bands in the red and blue regions of the spectrum. In the ORD spectrum of a pigment extract in acetone these Cotton effects are strongly reduced and reversed in sign. Purified chlorophyll a in solutions in carbon tetrachloride, an aggregating solvent, exhibits profound changes in its ORD spectrum with changing concentration. Large Cotton effects are present at high concentrations at which chlorophyll dimers are known to be stable. The observed dispersions in quantasomes undoubtedly arise from strong interactions among the pigment molecules in their lipoprotein environment, although the chlorophyll molecules do not appear to be present as dimers in the quantasomes. Further studies of the origin of chlorophyll aggregation in solution and of the ORD spectra of photosynthetic bacteria should greatly aid in interpreting these remarkable findings.

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## 2. THE ROLE OF BACTERIOCHLOROPHYLL IN PHOTOSYNTHETIC HYDROGEN TRANSFER

Marianne Byrn and Melvin Calvin

The participation of chlorophyll as a hydrogen transfer agent in the water-splitting reaction of photosynthesis has been the subject of considerable speculation. Some of the chemical mechanisms that have been proposed for green plant photosynthesis involve a reversible photochemical oxidation-reduction cycle of the following type for either one- or two-electron transfers:[1]

chlorophyll a ⟶

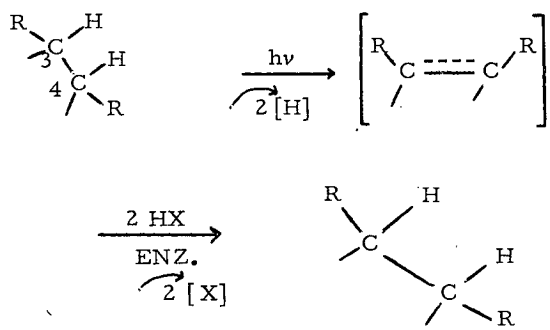
dehydrochlorophyll a + [H],

dehydrochlorophyll a + HOH ⟶

chlorophyll a + 1/2 $O_2$.

Proposals of this type have been centered on the hydrogens in the 7, 8 positions, the δ hydrogen, and the $C_{10}$ hydrogen of chlorophyll a. Investigations designed to evaluate such mechanisms, using tritium as a hydrogen tracer, have been inconclusive; the results show small amounts of incorporation, but these cannot be considered as evidence for or against a hydrogen donor mechanism.[2-4] Deuterium experiments have eliminated δ hydrogen participation, but not the $C_{10}$, $C_7$, or $C_8$ positions.[5]

We would like to introduce the notion that the 3, 4 positions of ring II are photosynthetically important. If this is true, then in the oxygen-producing green plants and algae, which contain the chlorin type of pigments, it is possible that chlorophyll is reduced and then oxidized in the 3, 4 positions. In the anaerobic photosynthetic organisms, such as the purple bacteria, which contain reduced chlorin pigments, the bacteriochlorophyll could be oxidized and then reduced in the 3, 4 positions as shown in the following scheme for 2 H transfer:




The immediate impetus for such a proposal came from the work of Holt et al.[6] and Olson and Romano,[7] who demonstrated the presence of both chlorin and dihydro chlorin pigments in the green photosynthetic bacteria, Chlorobium. This proposal cannot be tested on the green algae, since the chlorophyll is isolated in the oxidized state and would not contain the photosynthetically important hydrogens. However, with anaerobic purple bacteria, the bacteriochlorophyll is isolated in the reduced state and changes in the 3, 4 positions can be evaluated. The recent discovery of the specific oxidation of bacteriochlorophyll[8] in the 3, 4 positions with 2, 3-dichloro-5, 6-dicyanobenzoquinone (converting the dihydro chlorin to the chlorin 2-desvinyl-2-acetyl chlorophyll a) makes it possible for us to determine how much of the incorporated tritium is in the 3, 4 positions.

In order to obtain a sufficient amount of bacteriochlorophyll for measurement, the experiment was performed with an extremely dense suspension (0.5 ml packed cells suspended in 3 ml medium) of 4-day-old Rhodospirillum rubrum. Tritiated water (0.92 curie) was added to give a molar specific activity of $1.09 \times 10^{13}$ dpm/mole; the suspension was placed in a flat cell with a path length of 5 mm. It was then illuminated from both sides with 23 000 foot candles of white light for 15 min. The bacteriochlorophyll was isolated by acetone extraction, purified on powdered polyethylene TLC, and counted. The bacteriochlorophyll was then oxidized to give the 3, 4 dehydro compound and purified on mannitol TLC, and its radioactivity was again determined.

From the specific activity of the initial bacteriochlorophyll it was determined that approximately 2% of the bacteriochlorophyll molecules had incorporated tritium, and that from 62 to 96% of the incorporated radioactivity was in the 3, 4 positions. Since we have no independent way of determining the rate of photosynthesis, we cannot yet relate this number to an electron turnover rate. However, because of the age of the cells and the extremely high density of the suspension in which the experiment was performed, we believe that, for the most part, this does not represent new bacteriochlorophyll synthesis. Under optimal growth conditions for dilute suspensions (1/20 those of the experiment) the rate of bacteriochlorophyll increase is 1.7% for a 15-min period.

In addition to this, a tritium isotope effect has not been included in the calculation of the reported 2% incorporation. It seems probable that this incorporation does indeed represent photoinduced electron turnover in the bacteriochlorophyll. However, the experiments necessary to independently determine photosynthetic electron turnover rate, as well as new chlorophyll synthesis, have yet to be performed. Beyond this, a double isotope tracer experiment (with both deuterium and tritium) is required to estimate the isotope effect both for magnitude and direction. Finally, suitable tests to completely eliminate possible ambiguity introduced by cochromatography of highly labeled contaminant must be accomplished.

In view of the time required to complete these various operations, it seems wise to make known these preliminary results.

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## 3. STRUCTURES OF RIBONUCLEIC ACIDS

I. Tinoco, Jr., C. A. Bush, C. R. Cantor, S. R. Jaskunas, and M. M. Warshaw

The measurements of the optical rotation and absorption of six dinucleoside phosphates[1] and seven trinucleoside diphosphates[2] were described in this annual report last year. All 16 dinucleoside phosphates containing adenine, cytosine, guanine, and uracil have now been studied. Calculations have been made of the optical rotatory dispersion (ORD) to be expected for the dinucleoside phosphates in a stacked conformation--that is, the flat bases are stacked so as to begin to form a helix. Agreement between the calculated ORD and the measured values were generally good for a stack beginning a right-handed helix. We therefore concluded that the conformations of all 16 dinucleosides were very similar. However, from the magnitudes of the rotation and absorption of light it was clear that the amount of stacking was different for each one. Dinucleosides containing uracil seemed to have the least amount of stacking. An alternative explanation is that the uracil dinucleosides are much more flexible. They are stacked, but by torsional oscillation they switch back and forth rapidly from a right-handed helix to a left-handed one. Whatever the conformation of the dinucleoside, it was very encouraging to find that the trinucleoside diphosphate had a similar conformation.[2] The next step was to see if the bases in a polynucleotide also had the same conformation.

The absorption and ORD of polyadenylic acid[3] at neutral pH were those expected from adenylyl (3' - 5') adenosine. We thus concluded that polyadenylic acid is a single-strand stacked helix under these conditions. At acid pH the polynucleotide becomes a double-stranded helix and the optical rotation is quite different from that of the dinucleoside.

Similar studies of the ribonucleic acid from tobacco mosaic virus were made. It was found that in the absence of salt and in neutral pH, the ORD of the nucleic acid could be predicted quite well from the constituent dinucleosides. This indicates that studies of the structures and properties of the dinucleosides can be directly used to learn about the nucleic acids. It is not sufficient to study the mononucleotides; one needs the interaction between the bases, which is first manifested in the dinucleosides. When salt is added the optical properties of the nucleic acid change markedly and they are no longer like those of the dinucleosides. Apparently the nucleic acid curls up when the phosphate charge repulsion has been decreased by the salt. This allows interactions between bases not adjacent on the chain and thus gives effects not seen in the dinucleosides. The usual explanation is that Watson-Crick pairing between complementary bases has occurred. We are presently testing this hypothesis by studying complementary oligonucleotides and polynucleotides of known structure.

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## 4. APPLICATIONS OF ESR, MÖSSBAUER, ELECTRONIC, AND OPTICAL ROTATORY SPECTRA TO PROBLEMS IN BIOLOGY

Melvin P. Klein

A variety of spectroscopic techniques is being applied to the study of intact biological systems, to component parts of such systems, to individual molecules, and to model systems. The overall aim of these investigations is to relate structural parameters and details to biological function.

At the cellular level we are studying the electron spin resonance signals that occur when photosynthetic materials are illuminated by light that is conducive to photosynthetic activity. In green algae and photosynthetic bacteria, the action spectrum for the production of ESR signals roughly parallels that for photosynthesis. This is presumptive evidence that these signals reflect products directly associated with photosynthesis. The detailed nature of these products has heretofore been highly speculative, but recent experiments tend to implicate a semiquinone as one such compound. Tertiary-butyl nitroxide is a stable free radical which exhibits a characteristic three-line ESR spectrum. When this compound is mixed with one of a variety of quinones and irradiated in the absorption band of the quinone, the ESR signal of the nitroxide disappears within a short time (seconds). The algae and bacteria easily tolerate low concentrations of this nitroxide without adverse effects. When cells are allowed to equilibrate with $10^{-5}$ molar nitroxide, and are then centrifuged and washed repeatedly, it is observed that a small quantity of the nitroxide remains bound to the cells. Upon illumination of the treated cells, the nitroxide ESR signals decay just as they do in the quinone solutions. Again, the action spectrum for the disappearance of the signal parallels the photosynthesis action spectrum. When a nitroxide-treated sample of cells is treated additionally with $10^{-4}$ M DCMU, a compound which inhibits the Hill reaction, there is no light effect on the nitroxide signals. We interpret these results as indicating a semiquinone to be formed along the electron transport pathway leading to the evolution of oxygen. The center line of the nitroxide spectrum overlaps the g = 2 position, thus masking the light-induced signal, so that we have not yet determined the effect of the nitroxide on the basic signals. t-Butyl nitroxide containing $^{15}N$ is being synthesized; this compound will produce a two-line spectrum with no overlap in the region of g = 2, thus permitting a determination of the effects, if any, on these signals. The kinetics of disappearance are complex and strongly dependent on the intensity of actinic light. It is planned to extract the quinones from the cells in order to definitely ascertain if they are responsible for the basic ESR signal, as well as for the light-induced destruction of the nitroxide radical.

When the ESR signals of Rhodospirillum rubrum are examined at liquid helium temperatures, it is observed that there is a very much smaller light-induced signal, with kinetic behavior much slower than at room temperature. This is taken as further evidence that the photoinduced ESR signals reflect biochemical intermediates rather than some metastable excited state directly associated with the first photoexcited species. In addition to the free radical signal occurring at g = 2, signals attributable to $Fe^{3+}$ and $Mn^{2+}$ are observed at low temperatures, but they exhibit no light effects in the time domain usually employed for the light-induced free radicals (0.01 to 10 seconds). Manganese is known to be an essential constituent of photosynthetic organisms, and, although it is postulated to participate in the electron transport pathway, its precise function is obscure. It is known that an electron is transferred to a cytochrome within $10^{-6}$ sec after pulsed illumination of a photosynthetic sample. This implies that the first photoexcited species, irrespective of its exact nature, is available for study for times of $10^{-6}$ sec or less. We are currently designing equipment that will permit observation of ESR changes in this short time period.

Complexes between porphyrins and metal ions occur widely in biological materials and serve a variety of functions. To further our understanding of these important complexes we are examining a number of their properties. In some cases they exhibit ESR spectra. When single crystals are available it is usually possible to deduce directly from the observed spectrum the appropriate theoretical parameters that describe the spectrum. When powders, solutions, or random aggregates are studied, the spectra are not easily interpretable although the powder spectra are calculable. Computer programs have been written which permit us to calculate the ESR spectra of transition metal complexes. These programs are sufficiently versatile to allow all the necessary parameters to be independently adjusted in order to achieve the requisite degree of agreement between the computed and measured spectrum. From these parameters we are able to deduce

information about the site symmetry, strength of crystal field interactions and, often, the type of ligands.

We are continuing work on the Mössbauer spectra of iron-containing materials. Improvements in instrumentation and computation techniques have extended the precision of measurements. The Mössbauer spectrum of ferrichrome A has been measured at 0.98 °K, about 1.5°K lower than previously was possible. The effects of an externally applied magnetic field were also measured. This combination has permitted a further refinement of the theory of paramagnetic hyperfine structure in Mössbauer spectra and has also permitted further correlations between the Mössbauer and ESR spectra. Nearly all previous Mössbauer studies have employed concentrated samples, with the result that the metal-metal interactions completely masked many of the details. The use of dilute samples, where the iron-iron distances may be made as large as 20 Å, can increase the resolution by an order of magnitude.

The electron spectroscopic method for determining atomic energy levels has been developed to high accuracy in recent years. The experiments are carried out by irradiating a sample with monochromatic x rays whose energy exceeds the binding energy of the electrons under study. Under these conditions the electrons are expelled from the sample with kinetic energy equal to the difference between the x-ray energy and the binding energy. The kinetic energy, or more properly the momentum, is measured in a double-focusing electron, or β-ray, spectrometer. As each element that is irradiated by monochromatic x rays emits a unique and characteristic photoelectron spectrum, the electron spectroscopic method is capable of measuring the elementary composition of a sample from the positions of the lines, and the resolution is sufficient so that adjacent elements in the periodic table are widely separated. The relative intensities of the photoelectron lines correspond to the relative abundances of the elements in the sample. Only microgram quantities are required, as only those electrons which originate within a few angstroms of the surface escape with the characteristic energy. Perturbations of the electron orbits, including the inner ones, of an atom arise when the atom is a constituent of a larger aggregate, such as a chemical compound. As a result of the chemical bond perturbations, the energies of all the atomic levels are shifted and become different for the same element in different chemical states.

In fact, it is indeed possible to observe the effect of chemical environment on the energies of the photoelectron lines. Work at The Institute for Physics at Uppsala, Sweden, has shown that all of the six valence states of sulfur are distinguishable. It was this observation which aroused our interest in this method, for it affords the possibility of distinguishing the valence states of a large number of atoms contained in molecules of biological interest. The method is applicable to all atoms from carbon upward since it measures, in effect, the Coulombic energy of the electrons. All other spectroscopic methods that can be used to determine valence states depend on interactions of much lesser energy. These, in turn, reflect a propertyor parameter that may disappear in some valence states. Sulfur is an atom that does not lend itself to ready spectroscopic analysis. It is paramagnetic only in special circumstances; it is an unreliable chromophore for optical detection; and its only isotope with a nuclear magnetic moment occurs in low natural abundance.

We have begun studying the electron spectrum of sulfur in a number of proteins, as well as in inorganic and organic compounds. We are also studying the spectra of iron and manganese compounds in various oxidation states. The method holds great promise for learning some details of sulfur bonding in proteins and, since much of the structural integrity of proteins derives from their sulfur content, these data will be of great use.

Optical rotatory dispersion, circular dichroism, and their respective magnetic counterparts have received continuing attention, both theoretically and experimentally. The ORD and MORD of a number of aromatic hydrocarbons have been measured, both at room temperature and at temperatures down to 200 °K. The observed spectra have been compared with those derived theoretically from molecular orbital calculations; in some cases the agreement is sufficient to permit a correlation between molecular parameters and the observed spectra. The spectra, in general, sharpen significantly upon lowering of the temperature.

The circular dichroism and magnetic circular dichroism of a number of metal-free and metal-containing porphyrins have been studied; some heme-proteins have also been studied. An extremely simple theoretical model, based on the Zeeman effect, can account for many of the observed features, both qualitatively and quantitatively. On the basis of this model it is now possible to assign the angular momenta of several excited states of porphyrins, and it appears equally possible to determine some of the properties of the metal ions which are conventionally deduced from ESR measurements. Computer programs

have been written which permit analysis of many more data than could be done manually; these have made the results possible. The spectrometers have been equipped with digital output equipment, making possible least-square fitting programs that adjust the relevant parameters to obtain the best agreement between theory and experiment.

We have designed and constructed equipment that will permit observations of rotation and dichroism spectra at liquid He temperatures. In addition to providing sharper lines, this technique will also permit us to distinguish ground-state splittings from excited-state splitting.

# 5. THE EFFECTS OF DAY LENGTH ON QUANTASOME STRUCTURE AND CHLOROPLAST PHOTOCHEMISTRY IN SPINACIA OLERACEA L. VAR. EARLY HYBRID #7

Roderic B. Park and Susan Drury

## Introduction

Morphological studies of chloroplast fine structure show that to a first approximation the internal structure of a chloroplast consists of two phases. One phase is the internal membrane system and the second phase is the embedding matrix or stroma material of the chloroplast. The chemical processes of photosynthesis are distributed between these two phases.[1,2] The light reactions and associated electron transport reactions are located in the internal membrane system. Here light is absorbed and converted to chemical potential. The chemical potential brings about electron transport from water to ferredoxin and is accompanied by photosynthetic phosphorylation. The $CO_2$ fixation enzymes of the carbon cycle, along with ribosomes and other soluble enzymes, are located in the stroma regions of the chloroplast. A simplified scheme demonstrating the role of these two phases in the photosynthetic process is given in Fig. 5-1.

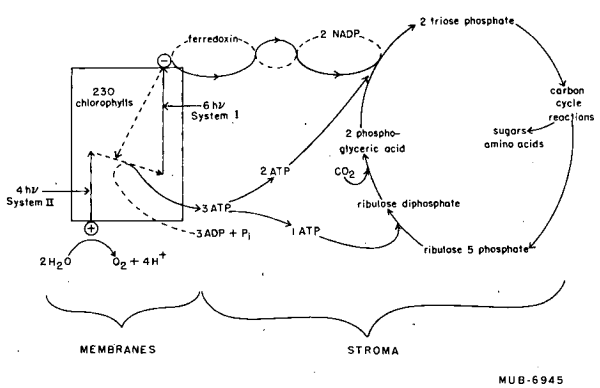



Fig. 5-1. A scheme of photosynthesis showing the distribution of functions between the membrane and stroma phase of the chloroplast.

Over the past several years our efforts have been directed towards study of the internal membrane system of higher plant chloroplasts. We have studied these membranes with respect to chemical composition,[3] membrane surface morphology,[4] and, more recently, metabolic efficiency as measured by quantum requirements for various Hill reactions.[5] It became evident during the course of these studies that the membrane morphology and efficiency were related to the time of year at which the experiment was performed. Further, it appeared that the most efficient membranes that occurred during winter months were also those with the most elaborate surface structure. These observations on field-grown spinach led us to try to reproduce some of these effects under controlled conditions in growth chambers. In the search for an environmental factor that might account for these differences, photoperiod was one of the most obvious candidates. In this paper we discuss some of our preliminary results of the effects of photoperiod on the efficiency and internal structure of chloroplast membranes.

## Materials and Methods

### Growth of spinach plants

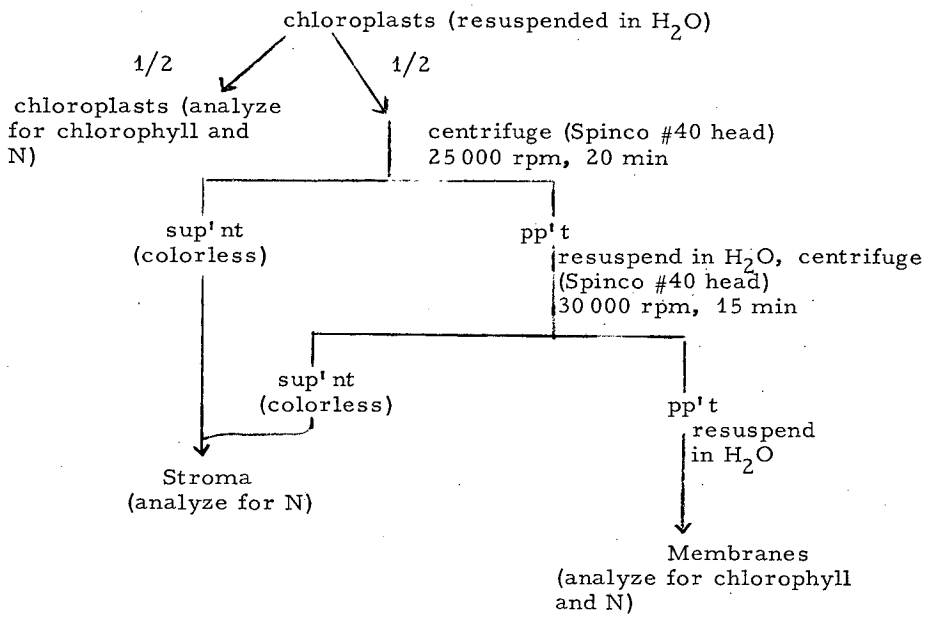
Seeds of Spinacia oleracea (Early Hybrid #7) were planted in a 2:1 soil:vermiculite mixture. The flats containing the seeds were then placed in growth chambers under either short-day (10 hr) or long-day (16 hr) photoperiods. Day temperatures were 20°C and night tempertures 9°C. Light intensity at the level of the plants was about 500 foot candles. Plants were watered daily and fed with a medium enriched in $PO_4^{\equiv}$ and $NO_3^-$ every other day. Under these conditions plants grown under long days began to bolt and flower at about 6 weeks, while plants grown under short days did not flower after periods of 3 to 4 months, at which time they were discarded. Plants 4 to 5 weeks old were used in the experiments reported here.

### Chloroplast isolation

Once-washed chloroplasts were prepared in buffered sucrose,[2] though for quantum requirement measurements they were resuspended in grinding buffer to eliminate starch, especially from the long-day spinach preparation. For chemical analysis they were resuspended in distilled water. The preparation of membranes for analysis then proceeded according to the method on the following page.

### Chemical analyses

Chlorophyll was determined by the method of Arnon.[6] Total nitrogen content of the aliquots was measured by using the micro Kjeldahl method as described by Park and Pon.[3]




Quantum requirements

Quantum requirements for the Hill reaction with 2,6-dichlorophenol-indophenol (DCPIP) as oxidant were measured as a function of light intensity. Reduction of DCPIP was followed at 580 mμ by use of a Cary 14 spectrophotometer equipped with a scatter transmission attachment and modified for simultaneous side illumination as described by Sauer and Biggins.[7] Quantum requirements were determined and calculations were made according to the methods of Sauer and Park.[5] A typical reaction mixture contained (in μmoles/ml) sucrose, 440; potassium phosphate, pH 7.4, 90; methyl amine, 10.0; DCPIP, 0.03 in a reaction volume of 2 ml plus a concentration of chloroplasts to give $OD_{680}$ mμ 0.3 to 0.5. Quantum requirements were measured by using an actinic beam of 650 or 680 mμ supplied by a Bausch and Lomb red blaze grating monochromator with 3-mm slits and an appropriate red cutoff filter. The inner filter effect was taken into account for calculation of quantum requirements for the DCPIP Hill reaction.

Membrane morphology

Sections for electron microscopy were obtained from leaves fixed with 2% $KMnO_4$ for 20 min and embedded in Epon. Membrane preparations were shadowed with Ni-Cr on Formvar-covered grids. Freeze etching was carried out following the methods described by Moor et al.[8] Samples were observed in a Siemens Elmiskop I.

Results

1. Quantum requirement for DCPIP reduction by long- and short-day chloroplasts

The results of a typical quantum requirement experiment are presented in Fig. 5-2, in which quanta absorbed per electron transferred is plotted against light intensity. The chloroplasts used in this experiment were from 33-day-old plants. The most meaningful data are obtained by extrapolating the quantum requirement values to 0 light intensity. In three experiments, the quantum requirement for long-day chloroplasts was 20 to 100% greater than that for short-day chloroplats at both actinic wavelengths. The loss of activity during an experiment was negligible (< 10%), indicating that the difference in quantum requirement is not due to accelerated loss of activity by long-day chloroplasts after isolation. We have not yet established how quantum requirements vary with plant age. It may be that the long-day chloroplasts are senescing more rapidly than those of short-day plants, or perhaps they never are as efficient. A quantum requirement 2 to 3 per electron at 680 mμ is consistent with the Hill-Bendall[9] scheme of electron transport as shown in Fig. 5-1 and discussed by Sauer and Park.[5]

2. Chlorophyll/N ratios for chloroplast fractions

Chloroplasts from long- and short-day

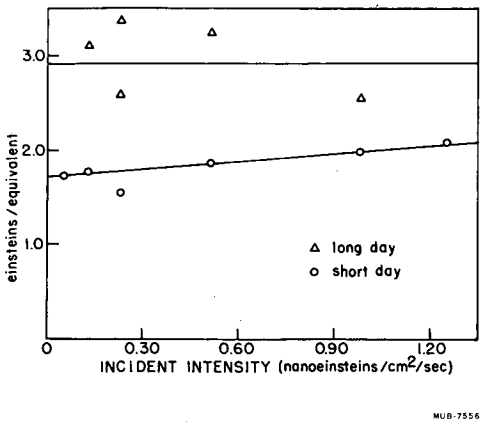



Fig. 5-2. Quantum requirement for the DCPIP Hill reaction as a function of light intensity for long-day and short-day chloroplasts.

plants, prepared and fractionated as described under Methods, were analyzed for chlorophyll and nitrogen. Results of such an experiment are presented in Table 5-I. Data from the first line (Total N) show that a material balance was obtained. They also show that the stroma nitrogen is lower than that reported by Park and Pon.[3] This is probably due to the large number of ruptured plastids (Class II chloroplasts[10]) in the preparation. While this might be expected to reduce the $CO_2$-fixation capacity of the preparation, it would not affect the Hill reaction capacity (see Fig. 5-1). Since Kjeldhal determinations also give chlorophyll N, this amount of N has been subtracted from the total N to give nonchlorophyll N. The interesting part of the data is that the chlorophyll/N weight ratio is the same for both membrane preparations despite the fact that they differ in quantum conversion efficiency and, as will be seen, in morphology. It is interesting to note that the short-day leaves had more isolatable chlorophyll per gram dry weight than did long-day leaves. Long-day leaves contained more starch than short-day leaves, as seen both in thin section and during the isolation procedure.

## Morphology

At an age of 28 to 35 days the morphology and color of spinach leaves are different depending on whether they are grown under long or short days. This is shown in Fig. 5-3. Long days produce a flatter blade, which is deeper green in color and has a waxier surface. The chloroplasts from these leaves differ considerably when viewed in thin section by electron microscopy. Figures 5-4 and 5-5 depict short-day and long-day chloroplasts respectively. The major differences are:

(a) the large starch grains in long-day chloroplasts,
(b) the absence of well-defined stroma lamellae in long-day chloroplasts.

The membrane surfaces of the two kinds of chloroplasts were studied by two techniques. The first was heavy-metal shadowing and the second was freeze etching.

Heavy-metal shadowing of long-day membranes indicated practically no surface structure. Shadowing of the short-day membranes showed more structure and occasional paracrystalline quantasome arrays, as shown in Fig. 5-6. The frequency of these arrays was not nearly so great as that observed in field-grown winter spinach (see Fig. 5-7). Thus we have not yet achieved the growth conditions occurring in the field which produce a large percentage of paracrystalline arrays.

Freeze etching on both preparations yielded

Table 5-I. Chlorophyll/nitrogen weight ratios for chloroplast fractions.

| | Short-day spinach | | | Long-day spinach | | |
|---|---|---|---|---|---|---|
| | chloroplasts | membranes | stroma | chloroplasts | membranes | stroma |
| Total N (mg) | 3.14 | 2.58 | 0.45 | 1.14 | 1.00 | 0.16 |
| Total N (mg/ml) | 0.314 | 0.258 | -- | 0.114 | 0.100 | -- |
| Chlorophyll (mg/ml) | 0.318 | 0.318 | -- | 0.123 | 0.123 | -- |
| Chlorophyll N (mg/ml) | 0.020 | 0.020 | -- | 0.008 | 0.008 | -- |
| Nonchlorophyll N (mg/ml) | 0.294 | 0.238 | -- | 0.106 | 0.092 | -- |
| Chlorophyll-to-nonchlorophyll N | 1.08 | 1.34 | -- | 1.16 | 1.34 | -- |

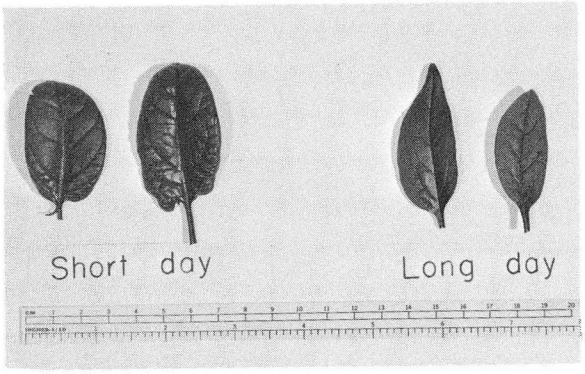



Fig. 5-3. Comparison of 30-day-old long-day and short-day spinach leaves (var. Early Hybrid #7).

comparable results. An example of this similarity is shown in Fig. 5-8. This result is interesting because the air-dried heavy-metal-shadowed preparations were distinguishable from one another. This may be because only in the heavy-metal shadowing is one looking at the exterior of the membrane. Freeze etching, on the other hand, yields primarily interior views of the membrane. A difference in membrane organization between the long- and short-day material may account for not only the difference in external appearance upon drying, but also the difference in quantum conversion efficiency. Such a structural difference is, however, not readily apparent with the freeze-etch technique.

## Discussion and Conclusions

Chloroplasts isolated from 4- to 5-week-old short-day spinach plants are 20 to 100%

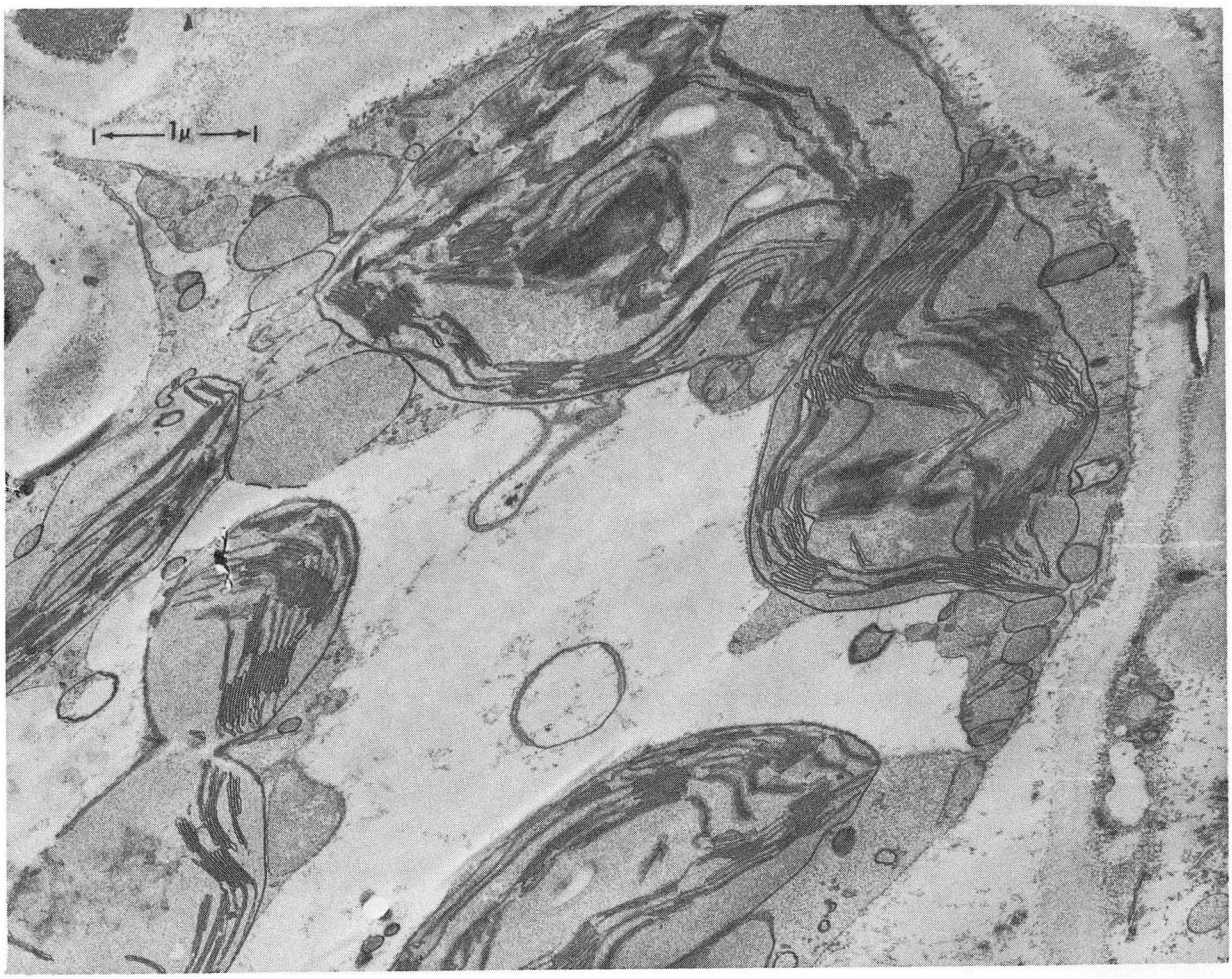



Fig. 5-4. $KMnO_4$-stained mesophyll of short-day spinach.

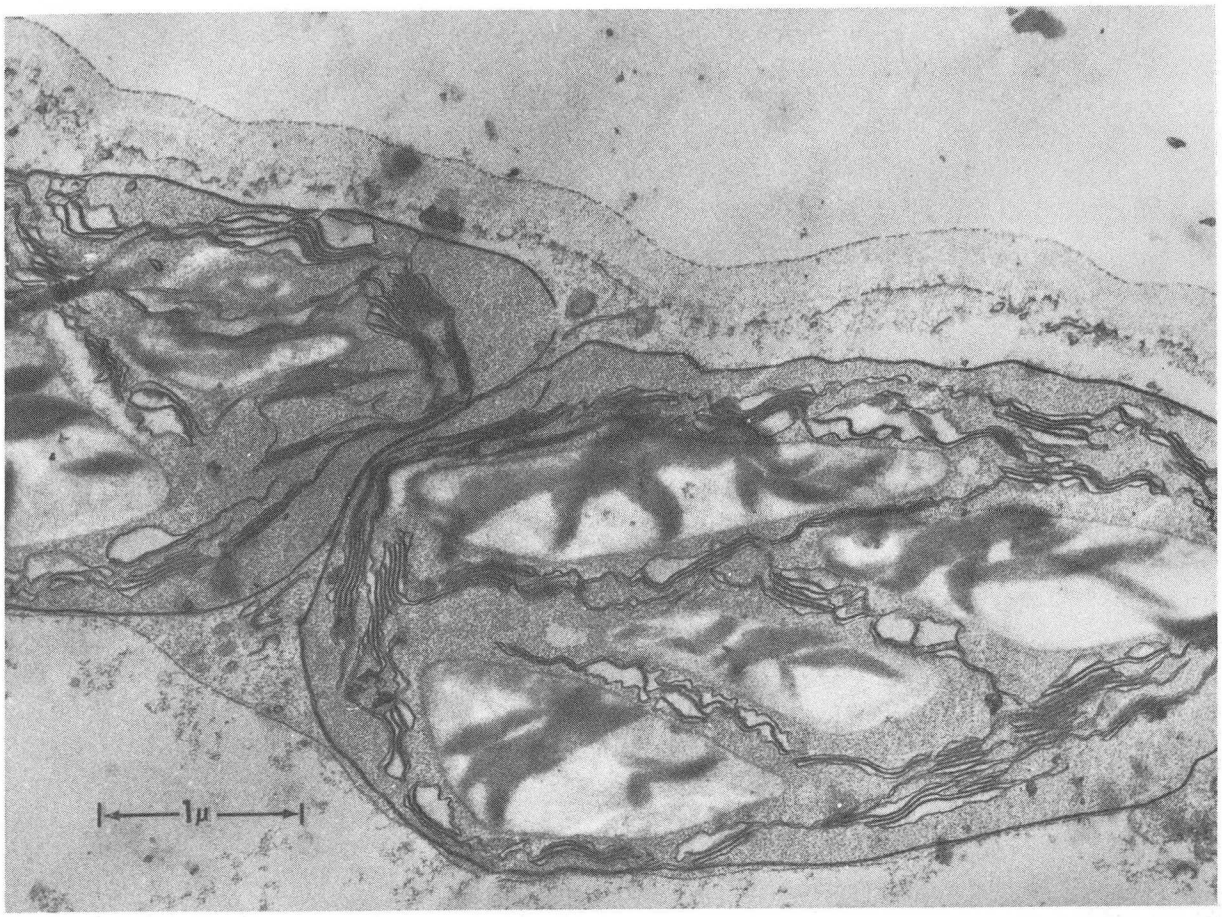



Fig. 5-5. $KMnO_4$-stained mesophyll of long-day spinach.

more efficient than long-day chloroplasts as assayed by Hill reaction activity. This difference in efficiency is correlated with differences in the internal membrane system of the chloroplast as seen both in thin section and in shadowed material. Long-day chloroplasts in thin section contain fewer stroma lamellae and more starch than short-day chloroplasts. When the membrane surfaces are viewed in shadowed material, the short-day membranes are more highly structured than the long-day membranes, occasionally showing paracrystalline quantasome arrays. However, the abundance of these arrays is not so great as observed in field-grown winter spinach. The chlorophyll/N ratio of both long- and short-day membranes is the same, which to a first approximation indicates that the difference in efficiency between the two membranes may be largely a difference in structure rather than a difference in chemical composition.

These preliminary results beg certain intriguing questions. Among them are whether the long-day plastid we have studied is distinguishable from a much older senescing short-day plastid, whether there are variations in compounds other than chlorophyll and N in the membranes, and, finally, what factors yield a high abundance of paracrystalline quantasome arrays found in field-grown spinach.

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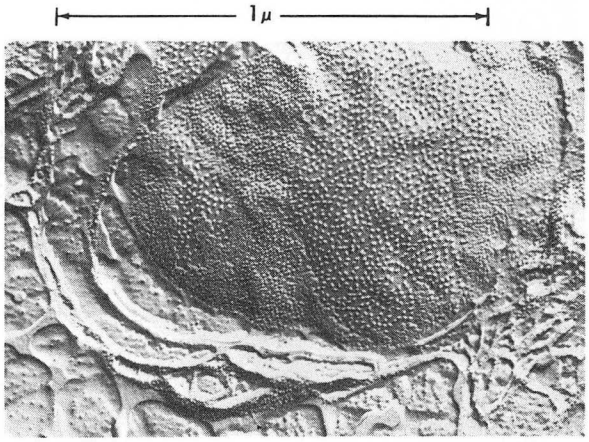


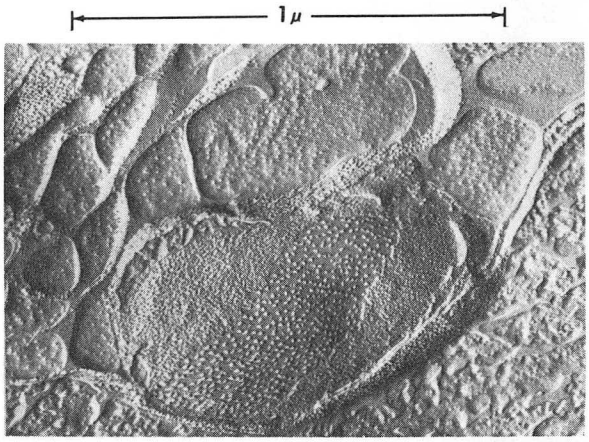




Fig. 5-8 a. (above) Freeze-etched short-day spinach chloroplast.

Fig. 5-8 b. (above) Freeze-etched long-day spinach chloroplast.

Fig. 5-6. (upper left) A paracrystalline quantasome array from short-day *S. oleracea* var. Early Hybrid #7.

Fig. 5-7. (lower left) Paracrystalline arrays from field-grown winter spinach.

# 6. LOCALIZATION OF CHLOROPHYLL IN SPINACH CHLOROPLAST LAMELLAE BY FLUORESCENCE AND ELECTRON MICROSCOPY

P. Lintilhac and Roderic B. Park

## Introduction

Chlorophyll in green plants is generally thought to reside in the internal membranes or lamellae of chloroplasts. These membranes are observable by electron microscopy, and can be roughly grouped on the basis of size and arrangement into grana and stroma lamellae. These lamellae exist as closed, flattened sacs or thylakoids.[1] Although the chlorophyll molecule itself is not demonstrable by electron microscopy, it is readily detected by fluorescence microscopy.[2-4] The deep red fluorescence of chlorophyll, first observed by Sir David Brewster in 1833, was used to advantage by Francis E. Lloyd in 1924,[5] who found the chloroplasts of higher plants to be the source of the fluorescence; and again in 1962 by Spencer and Wildman,[6] who further restricted the red fluorescence to the grana.

The technique described here permits observation of the same specimen by fluorescence and electron microscopy, yielding precise information on the distribution of chlorophyll in the membranes through a direct comparison of ultrastructure and fluorescence.

## Materials and Methods

### Preparation of chloroplast material

Store spinach was ground in a cold Waring Blendor for thirty seconds in a grinding medium composed of 0.5 M sucrose-0.1 M (K)$PO_4$ pH 7.5. This slurry was squeezed through eight layers of cheesecloth into 250-ml centrifuge tubes, and centrifuged at 470×g for 5 min in an International refrigerated centrifuge. The precipitate was discarded, and the supernatant centrifuged at 900×g for 15 min. This precipitate was resuspended in grinding buffer and centrifuged again at 900×g. Osmotic rupture was accomplished by resuspending this pellet in distilled water, followed by two washings in water at 15 000×g.

### Preparation of electron microscope grids

One-hundred-mesh copper specimen screens made by Perforated Products and distributed by Ernest Fullam Inc. were locally mutilated with a pair of needle tweezers to permit rapid and accurate location of material. The mutilated grids were then coated with a Formvar film. A drop of the chloroplast material, diluted to a pale green color with distilled water, was placed on a prepared grid and dried down after removal of excess liquid with an absorbent paper. The grid was then removed to a nitrogen-filled dry-box, allowed to equilibrate with the nitrogen atmosphere, and carefully placed, coated surface downwards, in a drop of deoxygenated water on a glass coverslip which was then upturned on a microscope slide. The wet, covered grids were then quickly transferred to the fluorescence microscope and photographed.

### Photography

Actinic light was provided by a Zeiss fluorescence source, with an Osram HBO 200 high-pressure mercury vapor lamp. Blue and purple cutoff filters mounted in front of the source permitted only light of less than 500 mμ to reach the specimen. The microscope used was a Tiyoda trinocular research microscope equipped with an oil-immersion objective and a Bessler Topcon 35-mm camera back. A deep red cutoff filter, passing light of greater than 630 mμ only (Corning #2-58), was mounted in the tube of the microscope. In order to prevent fluorescence of the filter itself a second filter was mounted below the first, removing all light of less than 520 mμ (Optical Coating Labs., Inc., dielectric rejection filter). Fluorescence was recorded on Kodak High Speed Infrared Film HIR 417.

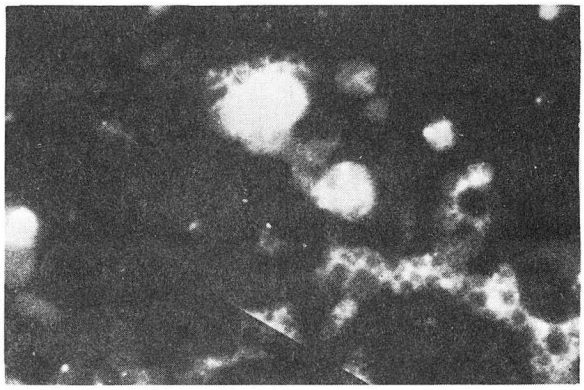
After a chosen area of the grid was photographed, the cover slip was carefully floated off with distilled water and the grid was removed, dried, and shadowed with chromium-nickel in a Mikros vacuum evaporator. Electron microscopy was performed in a Siemens Elmiskop I operating at 40 kV accelerating voltage. Photographs of identical areas obtained by electron and fluorescence microscopy were then enlarged and compared.

The success of the technique depends on the brilliance and duration of the fluorescence, the focus of the light microscope, and the ability of the chloroplast fragments to adhere to the Formvar film throughout the procedure. Adequate fluorescence may be insured by excluding all oxygen from the specimen, and by working quickly to keep diffusion of air under the cover slip to a minimum. Thorough drying of the grids before microscopy will prevent loss of material during manipulation. Under these conditions, exposures of between 2 and 3 sec at a magnification of 2 000 ×

suffices to record the chlorophyll fluorescence of a single membrane thickness.

## Results and Discussion

Figure 6-1 (upper) is a fluorescence micrograph of chloroplast fragments spread on a Formvar film. Figure 6-1 (lower) is an electron micrograph of the same fragments after drying and shadowing. These illustrations correspond exactly, and show that all the membranes visible in the electron micrograph contain chlorophyll.



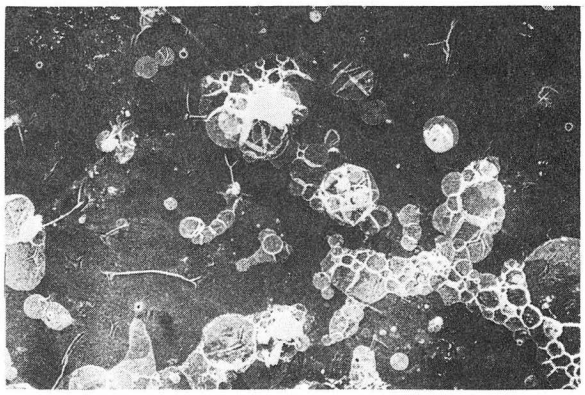


Fig. 6-1 (upper). Fluorescence micrograph of chloroplast fragments spread on a Formvar film.
(lower) Electron micrograph of same fragments after drying and shadowing.

Careful examination shows that the intensity of fluorescence is fairly constant, and variations can usually be correlated with variations in the number of superposed membranes. In some cases a single thylakoid, or membrane sac, is ruptured in such a way that only a single membrane thickness remains; and these areas show a corresponding decrease in intensity of fluorescence, verifying the observation that the chlorophyll is in fact membrane-bound and not merely held captive within the thylakoid.

Since the isolation procedure can be shown to faithfully isolate whole chloroplasts with their inner limiting membrane, and since all membranes observed by this technique contain chlorophyll, it may be concluded that certainly the grana and stroma lamellae, and probably the innermost limiting membrane, all contain chlorophyll.

The discrepancy between these conclusions and those of Spencer and Wildman can be explained by the fact that this technique is capable of detecting the fluorescence of a single chlorophyll-containing membrane, and effectively avoids the light-scattering problems that would tend to obscure any fluorescence of stroma lamellae in whole chloroplasts.

With the possible exception of the highly ephemeral outer limiting membrane, then, chloroplast membranes all contain chlorophyll in similar concentrations.

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# 7. THE EFFECT OF HYDROLYTIC ENZYMES ON THE PHOTOSYNTHETIC EFFICIENCY AND MORPHOLOGY OF CHLOROPLASTS

Elchanan S. Bamberger and Roderic B. Park

Evidence that the entire photosynthetic process is located in higher plant chloroplasts[1] was soon followed by assignment of partial reactions of photosynthesis to chloroplast structures. These studies showed that the light reactions and associated electron transport reactions leading from water oxidation to ferredoxin reduction are located in the internal membranes or thylakoids of the chloroplast, whereas the $CO_2$ fixation and other synthetic enzymes are associated with the stroma portions of the chloroplasts.[2,3] Thylakoids, when observed in heavy-metal-shadowed preparations by electron microscopy, demonstrate a particulate structure.[2,4] This structure, 150 to 200 Å in diameter, making up the membrane, can exist in highly ordered arrays,[5] and was termed in this Laboratory "quantasome".[6,7] Aggregates of small numbers of quantasomes yield Hill reaction rates comparable to those of whole chloroplasts.[2] The chemical composition of these structures has been a subject of several studies at this Laboratory.[5,8,9] The membranes are about 50% protein and 50% lipid.

To further evaluate the relationship between energy conversion processes and thylakoid structure, one would like to employ methods that alter in specific ways both the physiological and morphological characteristics of the membranes. Detergents have often been used for such purposes.[10] However, recent studies have indicated that hydrolytic enzymes may also serve as a useful tool for this kind of investigation.[11,12,13]

This paper reports the effects of two hydrolytic enzymes on the structure and function of isolated thylakoids. The enzymes used were a crude preparation from runner bean leaves (containing galactolipases and galactosidases) and the proteolytic enzyme pronase. Physiological activity was assayed by measuring Hill reaction efficiencies[14,15] extrapolated to zero light intensities. This measure of activity produces considerably more information as to the hydrolytic enzyme effect than does measurement of saturation rate. Structural changes were followed both spectrally and morphologically. Morphological changes within the membranes were followed by use of the freeze-etch technique.[16] A detailed examination of untreated thylakoids by use of this technique has been carried out by Branton and Park.[17]

## Materials and Methods

### Preparation of chloroplasts

Intact chloroplasts and chloroplast fragments were prepared from spinach leaves obtained from local stores. In general, 125 g of leaves was homogenized for 30 sec in 250 ml grinding medium (0.35 M NaCl, 0.02 M tris-HCl, pH = 7.4, and 0.01 M EDTA) in a Waring Blendor. The homogenate was strained through eight layers of cheesecloth and centrifuged according to Park and Pon[2] to isolate once-washed chloroplasts or chloroplast fragments. For the spectrophotometric measurements, concentrated buffered aqueous suspensions of chloroplast fragments were diluted with deionized water in order to achieve a final absorbance between 0.3 and 1.0 at 678 mμ. Chlorophyll was determined by the method of Arnon.[18]

### Enzyme preparation from runner bean leaves

The runner bean leaf enzyme preparation was prepared according to the ammonium sulfate precipitation procedure of Sastry and Kates.[13] Fresh primary leaves (50 g) from 3- to 4-week-old runner bean plants were homogenized with 100 ml of deionized water for 2 min in a Waring Blendor. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15 000 × g for 20 min at 0°. Seventy ml of the supernatant was centrifuged further at 100 000 × g for 60 min at 0°. The resulting supernatant (70 ml) was dialyzed overnight against 10 liters 0.05 M phosphate buffer (pH = 7.0). Solid ammonium sulfate was added to the dialyzate. The fraction precipitating at up to 70% saturation with ammonium sulfate was centrifuged at 15 000 × g for 20 min and dissolved in 0.05 M phosphate buffer (pH = 7.0), dialyzed overnight against 10 liters of the same buffer, and diluted with buffer to 28 ml; protein concentration, 9.24 mg/ml. This preparation (RBE) was kept at -15°.

### Enzymatic degradation of chloroplasts and chloroplast fragments

Concentrated buffered solutions of chloroplasts and chloroplast membranes were treated with the RBE preparation or pronase (*Streptomyces griseus* proteose; B grade; 45 000 PUK units/g Calbiochem.). (PUK: unit

of proteolitic activity of pronase measured by the Kaken Chemical Company.) The reactions were run at 30° under nitrogen and terminated by washing the chloroplasts three times in the same media in which they were initially suspended. Samples were diluted for the spectrophotometric assays.

Release of free galactose by RBE from the galactolipid fraction of chloroplast membranes was assayed with galactose oxidase (Galactostat, Worthington Biochemical Corp., Freehold, New Jersey). Protein nitrogen was determined by the Kjeldahl method except that $SeOCl_2$ was used as a catalyst instead of $HgSO_4$.

Hill Reaction

The DCPIP (2,6-dichlorophenol indophenol) Hill reaction activity was assayed by the spectrophotometric method described by Sauer and Biggins.[14] Experiments were carried out with the Cary Model 14 spectrophotometer, modified so that the absorbance of the Hill oxidant could be monitored continuously at 580 mμ ($E_{580}$ = 19 800 l-mole$^{-1}$ at pH = 7.4)[19] while the sample was being irradiated from the side with longer-wavelength light (650 mμ).

The cuvettes used had four clear sides and a rectangular internal cross section 3×10 mm. The monochromatic measuring beam passed through the 10-mm path length, and the actinic light incident at right angles on the sample cuvette traversed a 3-mm light path in the reaction mixture. Light intensity was measured with a calibrated silicon solar cell (Hoffman, Type 120 cg) placed at the site of the cuvette; corrections for reflection losses were applied.

A stock solution of DCPIP (K & K Laboratories, Jamaica, N. Y.) was always prepared freshly on the day of the experiment. The stock DCPIP solution was prepared in 0.1 dilution of the grinding media, pH = 7.4, and DCPIP concentration was confirmed by absorbance measurements after the undissolved dye had been removed by centrifugation. The reaction mixture contained, in μmoles/ml, tris-HCl, pH = 7.4, 20; NaCl, 350; EDTA, 10; methylamine hydrochloride, 10; DCPIP, 0.03 to 0.05. Once-washed chloroplasts were added in the dark at the start of each measurement to give an absorbance of 0.3 to 1.0 at 678 mμ (10-mm path). Each experiment was performed with 2 ml of reaction mixture, which was divided between the sample cuvette and the reference cuvette in the spectrophotometer.

Optical Rotatory Dispersion (ORD) of chloroplast fragments

Chloroplast fragments were prepared from sonicated spinach chloroplasts according to the method of Park and Pon.[2] The supernatant of a fraction sedimenting at 100 000×g (20 min) in a Spinco Model L ultracentrifuge was recentrifuged, and the final precipitate at 150 000×g (60 min) was resuspended in 0.02 M tris-HCl buffer, pH = 7.4. ORD measurements were made at room temperature by use of a Cary Model 60 spectropolarimeter with a cell of 1.0 cm path length. Absorption and difference spectra of chloroplast fragments were measured by using a Cary Model 14 spectrophotometer.

Freeze-etching technique

The Bendix-Balzers freeze-etch device and methods described by Moor[16] were used in these experiments. Once-washed chloroplasts were suspended in 10% or 20% glycerol in the grinding media and then centrifuged at 2000 g for 10 min. Samples from the precipitate were frozen and etched for 2 min, after which carbon platinum replica was deposited. Micrographs were taken with a Siemens Elmiskop I.

## Results

Effect of enzymes on membrane efficiency

The effect of pronase and the RBE preparation on the biological activity of spinach chloroplasts was estimated by measuring the quantum requirement (einsteins/equivalent) for the DCPIP Hill reaction of treated chloroplasts. As shown in Fig. 7-1, the quantum requirement of the control experiment varied between 2 and 5. The presence of $10^{-3}$ M methylamine in the reaction mixture greatly reduces the dependence of the quantum requirement on light intensity.[15] However, pretreatment of the chloroplasts with either pronase or the RBE preparation yields a linear increase in intensity dependence of the quantum requirement. This increase in quantum requirement is dependent on the enzyme concentration and period of incubation.

Effect of the hydrolytic enzymes on chemical composition

The runner-bean-leaves enzyme preparation contains specific enzymes which catalyze the hydrolysis of monogalactosyldilinolenin and digalactosyldilinolenin to the corresponding free linolenic acid, free galactose, and

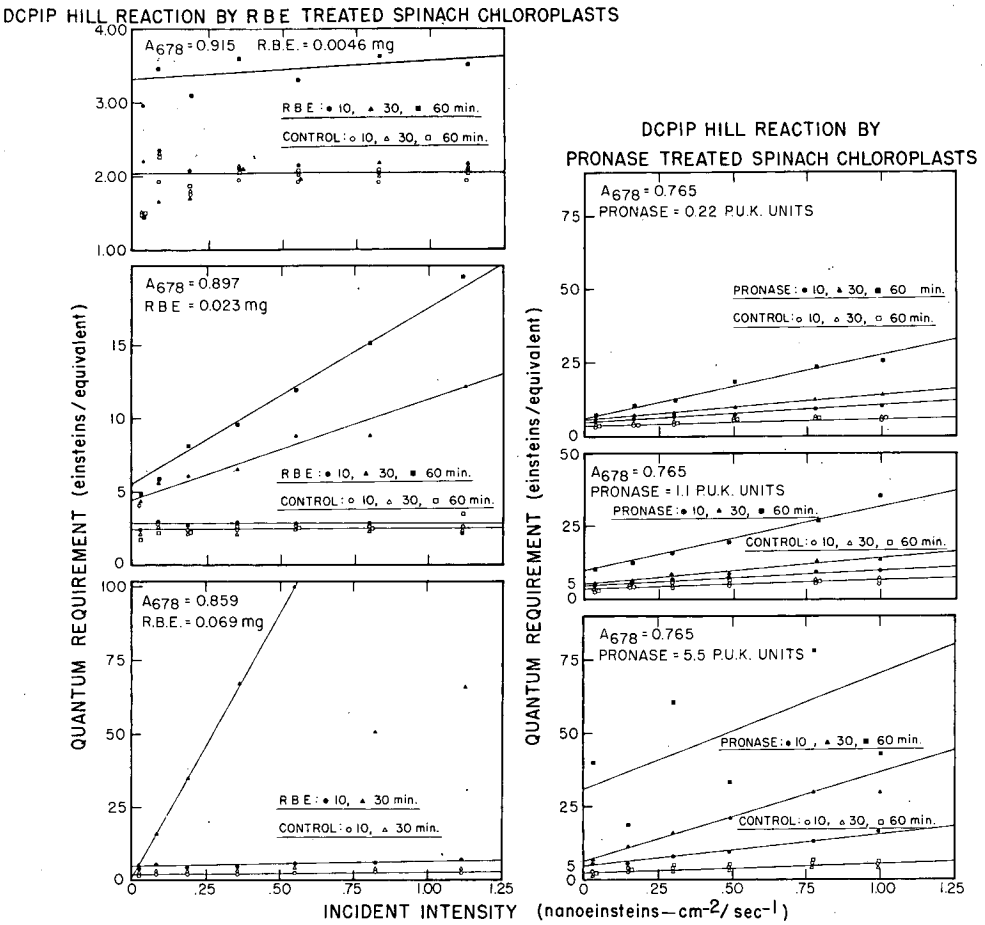



Fig. 7-1. Quantum requirement for the DCPIP Hill reaction at 650 mμ by spinach chloroplasts treated with RBE (left) or pronase (right).

glycerol.[13] The rate of release of free galactose is therefore a measure of the rate of complete hydrolysis of galactolipids by the RBE preparation.

When chloroplast fragments were incubated with the RBE preparation (Fig. 7-2) the rate of release of galactose was about the same at pH 7.0 (optimum pH for the activity of the monogalactosyldilinolenin enzyme) and at pH 5.6 (optimum pH for the activity of the digalactosyldilinolenin enzyme). After 4 hr of incubation at 30°, 50% of the galactose of the chloroplasts' galactolipid fraction was released into solution as free galactose. Only half of this amount was actually released by the added RBE enzyme. The second half was apparently released by endogenous enzymes present in the chloroplast preparation (see control curve, Fig. 7-2). The control level of galactose is initially higher than that of the RBE-treated material. This observation is discussed later.

The data in Table 7-I show that in the RBE-treated chloroplast preparation the ratio of chlorophyll to nitrogen (1.37) decreased with respect to the control preparation (1.78). In the pronase-treated chloroplast preparation the chlorophyll-to-nitrogen ratio increased (2.80) over the control value. This increase represents the amount of protein digested away by pronase from the chloroplasts.

## The effect of the hydrolytic enzymes on spectral properties

The effect of pronase on the chlorophyll red-absorption maximum is shown in Fig. 7-3. The 678.5 ± 0.5-mμ peak of an aqueous

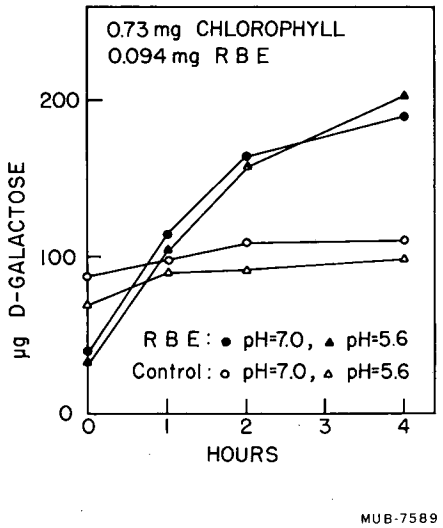



Fig. 7-2. Release of free D-galactose from spinach chloroplast membranes treated with RBE.

Table 7-I. The effect of 3-hour incubation of once-washed chloroplasts with RBE or with pronase on the chlorophyll-to-nitrogen ratio.

| | nitrogen aliquot (mg) | chlorophyll aliquot (mg) | chlorophyll / nitrogen (mg) |
|---|---|---|---|
| Control | 0.100 | 0.178 | 1.78 |
| +5.5 PUK units pronase | 0.075 | 0.210 | 2.80 |
| +0.069 mg RBE | 0.115 | 0.158 | 1.37 |

chloroplast fragment suspension undergoes a blue shift after 30 min incubation of the chloroplast fragments with pronase. The magnitude of the shift was not further increased by longer incubation times nor by higher enzyme concentrations. The blue shift of the red absorption maximum was not observed in chloroplast fragments treated with the RBE preparation.

The complex ORD spectrum of suspension of chloroplast fragments found in our experiments (Fig. 7-4) is similar to the one observed by Sauer.[20] Cotton effects are present throughout the visible and ultraviolet regions,

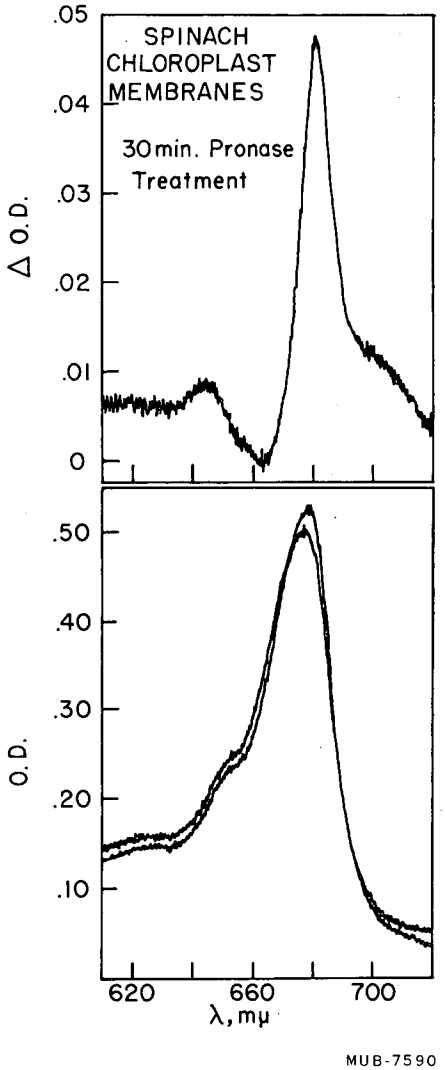



Fig. 7-3. Lower: Spinach chloroplast membranes absorption spectra from 600 to 720 mμ (upper curve), and absorption spectra of pronase-treated membranes (lower curve).
Upper: Difference spectrum control minus pronase-treated membranes.

which are regions of strong absorption of chlorophyll a and b and carotenoids. The pronounced trough at 700 mμ in the ORD spectra of the untreated chloroplast fragments is completely missing from the ORD spectrum of the pronase-treated chloroplast fragments; the rest of the ORD spectrum is similar to the control spectra. The ORD spectrum of the RBE-treated chloroplast fragments is essentially the same as the control spectrum.

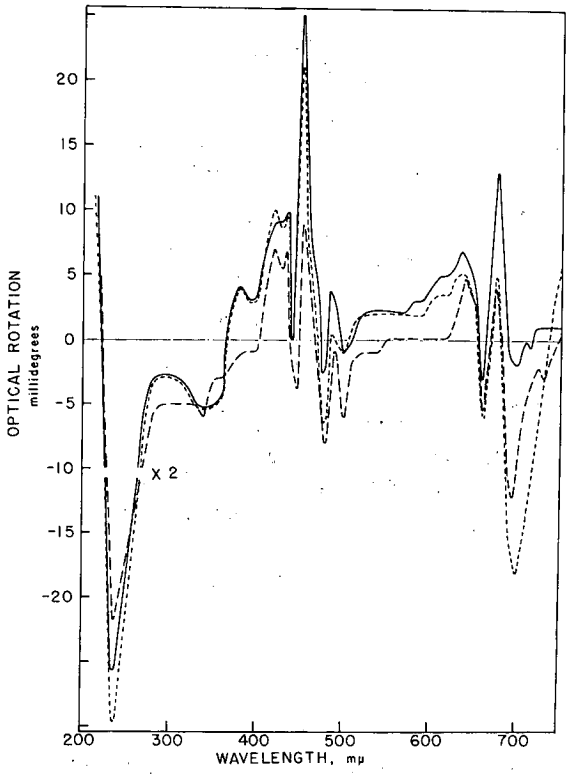



Fig. 7-4. ORD spectrum of buffered aqueous suspensions of sonicated spinach chloroplasts: ----- control; ——— pronase-treated (1.1 PUK units for 60 min); — — — RBE-treated (0.023 mg for 60 min).

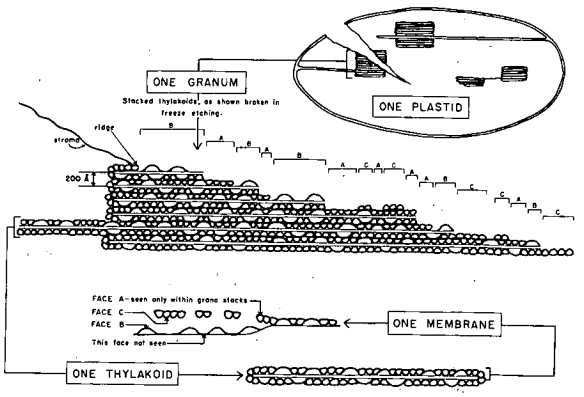


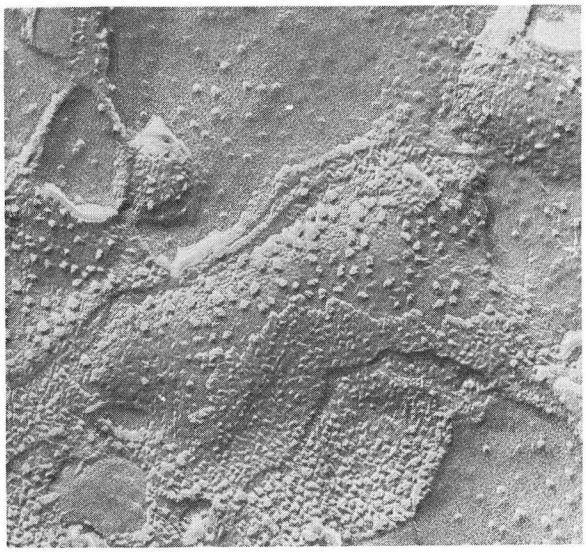

Fig. 7-5. A model for the morphology of spinach chloroplast membranes based on freeze-etch studies by Branton and Park (Ref. 17).

Effects of the hydrolytic enzymes on the morphology of chloroplast membranes

Branton and Park[5] have described the freeze-etch morphology of untreated in vivo and isolated spinach chloroplasts. A model of a single thylakoid derived from these studies is presented in Fig. 7-5. The model consists of a smooth inner layer on which there are large (150 Å) particles. This surface is designated as B. Surface A is a view seen only within grana stacks. It represents the outer portion of the thylakoid and consists of the large particles in an embedding matrix. Surface C is the matching surface for surface B. In these experiments we have attempted to find how the thylakoid membrane, as decribed in Fig. 7-5, is modified by pronase and RBE.

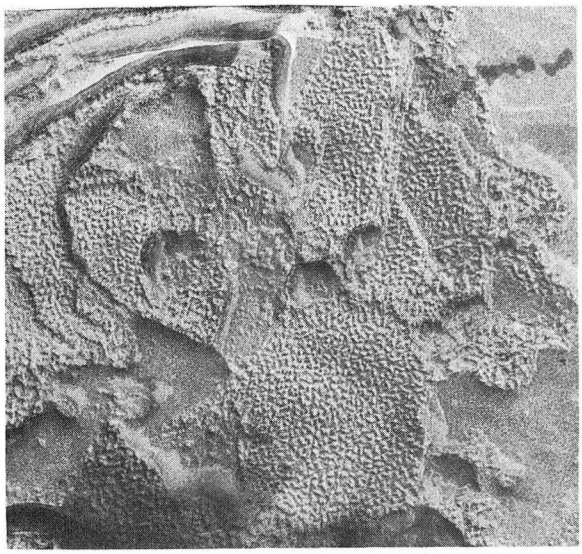
Control membranes were treated in the same way as the samples except that the hydrolytic enzymes were excluded from the reaction mixture. A freeze-etch picture of such a control is presented in Fig. 7-6. Surfaces A, B, and C are marked, with C occurring in a lower thylakoid. Figures 7-7 and 7-8 are typical examples of membranes treated with pronase and RBE respectively. The data may be summarized as follows: Both enzyme treatments lead to decrease both in number and in size of the large (150 Å) particles seen on surface B in Fig. 7-6. Moor[16] has suggested that these particles correspond to the quantasome. In pronase-treated preparations (see Fig. 7-7) surface B is still evident, but fewer particles are attached to it and the particles themselves are reduced in size compared with the control. Also, the A surface is more particulate than in the control. In RBE-treated preparations (see Fig. 7-8) the smooth layer of surface B is no longer observed and appears to be completely removed. The view in Fig. 7-8 is representative, and probably corresponds to modified C and A surfaces.

Some experiments were done in which chloroplasts were isolated from leaves and fixed for 45 min in 5% glutaraldehyde. Control chloplasts appeared similar to the pronase-treated preparations in these experiments. The RBE-treated chloroplasts from fixed leaves were greatly modified, however. The membrane no longer broke along discrete boundaries and appeared as layers of particles suspended in the glycerol freezing solution. The smooth layer of surface B in most RBE-treated plastids was completely absent. A comparison of control and RBE-treated glutaraldehyde-fixed chloroplasts is shown in Fig. 7-9 (control) and Fig. 7-10 (RBE-treated).



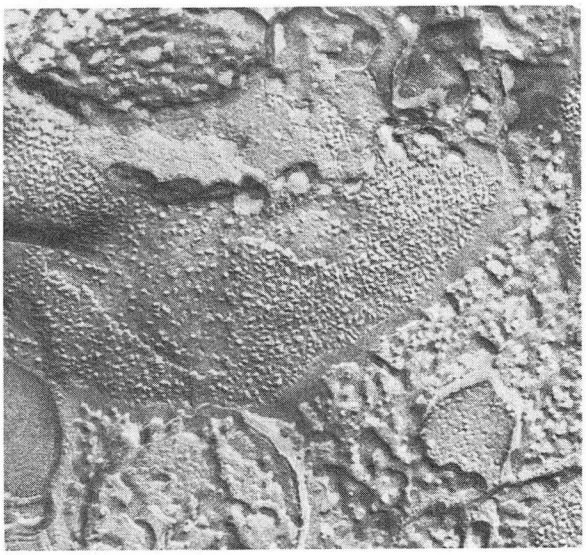
0.5 μ

Fig. 7-6. Freeze-etched control thylakoid. Surfaces A, B, and C correspond to the model in Fig. 7-5.



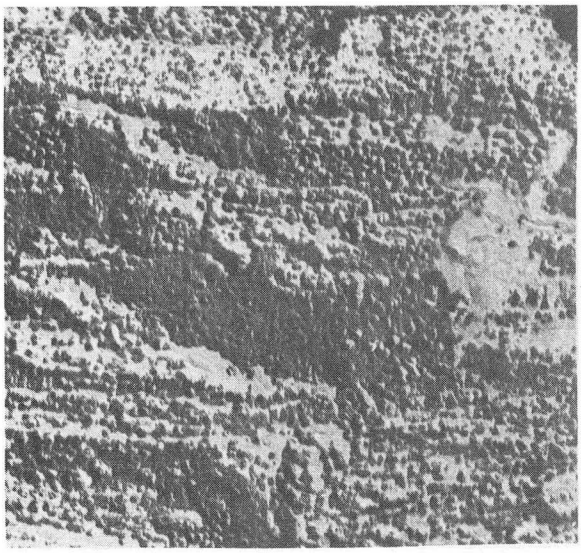
0.5 μ

Fig. 7-8. Freeze-etched RBE-treated thylakoids.



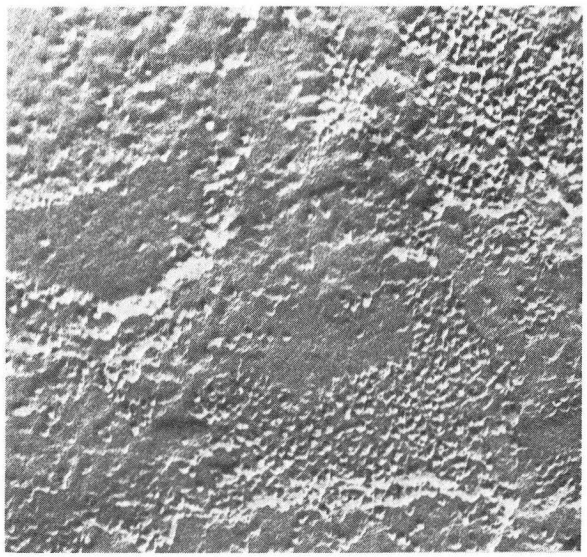
0.5 μ

Fig. 7-7. Freeze-etched pronase-treated thylakoids.



0.5 μ

Fig. 7-9. Freeze-etched chloroplast from 5% glutaraldehyde-fixed spinach leaves.



0.5 μ

Fig. 7-10. Freeze-etched chloroplast from 5% glutaraldehyde-fixed spinach leaves after RBE treatment.

## Discussion

Galactolipids constitute a major class of lipids in photosynthetic tissues.[21] In red clover they account for at least 23% of the total lipids,[22] and were found to be high in linolenic acid. Chloroplast membranes are 50% protein and 50% lipids, and the contribution of the galactolipids (in moles per quantasome) is about 40%.[5] The structure of galactolipids isolated from runner bean leaves was determined[23] and shown to be 2, 3-di-o-linolenoyl-1-o-β-D-galactopyranosyl-D-glycerol and 2, 3-di-o-linolenoyl-1-o-(6-o-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol.

Sastry and Kates[13] reported that runner bean leaves are rich in enzymes necessary for the complete breakdown of galactolipids to fatty acids, glycerol, and galactose. Linolenic acid released by these enzymes was responsible for the changes in biochemical properties of spinach chloroplasts aged at pH = 6 or treated with runner-bean-leaf homogenates.[12]

In confirmation of McCarty and Jagendorf's results,[12] in our experiments the RBE preparation hydrolyzed about 25% of the galactolipids present in chloroplast fragments in a period of 4 hr (Fig. 7-2). The initial concentration of free galactose in the supernatant of the control experiment (no RBE added) was higher than the initial concentration of free galactose found in the supernatant of the treated chloroplast membranes. This might be explained by assuming absorbance of free galactose by the added RBE preparation. Low concentration of RBE preparation yielded a linear increase in intensity dependence of the quantum requirement of the DCPIP Hill reaction by chloroplasts (Fig. 7-1). The order of magnitude of the quantum requirement at very low light intensities remains between 2 and 5 in all three concentrations of RBE preparation added. This suggests that one of the dark reactions in electron transport becomes rate-limiting after short treatment of chloroplasts with the RBE preparation. The dependence of the quantum requirement on light intensity was observed after 30 min treatment of the chloroplast with the RBE preparation. In this time interval only a very small amount of galactolipids was completely hydrolyzed (Fig. 7-2), suggesting that the physiological activity was altered before large chemical changes took place. In contrast to the effect of pronase, the absorbance spectra of chloroplast suspensions treated with RBE were not changed and only slight changes in the ORD spectra were observed (Fig. 7-4).

The Streptomyces griseus protease-pronase has a very broad substrate specificity and is capable of hydrolyzing various kinds of peptide bonds in protein until the majority of amino acids constructing the protein are liberated as individual amino acids. As a result, the extent of hydrolysis of proteins by this protease was estimated to reach 70 to 90%.[24] The increase of the chlorophyll-to-nitrogen ratio (Table 7-I) over the control value of 1.78 to 2.80 for the pronase-treated chloroplasts represents the loss of about 40% protein after 3 hr of incubation. The efficiency of the DCPIP Hill reaction is affected after only 10 min of incubation of the chloroplasts with pronase (Fig. 7-1). After treatment with pronase, the quantum-requirement dependence on light intensity increases. As with the case of chloroplasts treated with RBE, the quantum requirement at very low light intensities after 10 and 30 min of pronase treatment remains in the order of 2 to 5. However, after 1 hour's incubation with pronase (1.1 and 5.5 PUK units) the extrapolated quantum requirement at zero light intensity is 10 and 30, respectively. This change in intercept indicates that the efficiency of the chloroplasts is decreased even at the lowest light intensities. Thus uncoupling of light absorption from electron transport or partial loss of a component of the electron transport pathway must be invoked. After longer incubation periods with pronase no detectable DCPIP reduction was observed.

Treatment of chloroplast membranes with pronase caused a slight blue shift of the absorbance peak at 678 mμ. A difference spectra between the control membranes and the pronase-treated membranes shows a peak at

681 mμ and a satellite peak at 645 mμ. By integration of the area under the absorbance spectra and the difference spectra, the amount of bleaching was calculated at 5%. Pronase apparently causes a destruction of a long-wavelength component of in vivo chlorophyll a.[25] Otherwise this component contributes to the peak at 678 mμ (Fig. 7-3). The disappearance of a single Cotton effect (centered around 682 mμ) in the ORD spectra of pronase-treated membranes (Fig. 7-4) is in agreement with the postulation of a destruction of a long-wavelength chlorophyll a component by pronase. Evidence for the coexistence of two or more forms of chlorophyll a in green plants was given by Krasnovsky and his co-workers.[25, 26]

The fact that the underlying layer of surface B is quite smooth and that it disappears upon treatment with the RBE preparation suggests that the underlying layer is a galactolipid. The fact that the chlorophyll absorption and ORD spectra are not greatly affected by removal of this layer would indicate that chlorophyll is not associated with it. Chlorophyll is apparently associated with the large 150-Å particles and their embedding matrix which make up the A surface. This is supported by large spectral shifts and ORD changes brought about by pronase treatment. The large particles lying on the smooth galactolipid surface appear to contain both lipid and protein, since they are reduced in size but not removed by pronase treatment. The A surface appears to contain both lipid and protein, since it is attacked by both enzyme preparations. The thylakoid as shown in Fig. 7-5 appears to be built of a galactolipid layer on which large particles (150 Å) in an embedding matrix are located. Chlorophyll appears to be associated with the large particles and embedding matrix and not with the galactolipid layer. The large particles protruding from the embedding matrix may correspond to the quantasome seen in shadowed preparations.[27] The small-particle layer on the interior of the thylakoid of Pharbitis, reported by Park,[27] may correspond to the smooth underlying layer of the B surface which has undergone micelle formation during drying.

## Summary

1. Both lipase from runner beans and a protease (pronase) initially cause increased intensity dependence of the DCPIP Hill reaction of spinach chloroplasts. This is followed by an increase in the extrapolated zero-intensity quantum requirement.

2. Pronase treatment of the chloroplasts causes rapid changes in absorption and ORD spectra, whereas the effect of lipase treatment is much less pronounced.

3. Long treatments of the thylakoids with the lipase and protease cause unique morphological changes within the membrane. These changes are used as evidence for assigning chemical compositions to some of the structures revealed by freeze-etching.

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# 8. PHOTOSYNTHESIS OF CARBON COMPOUNDS

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## Introduction

Our general interest in the photosynthetic pathways by which carbon dioxide, phosphate, and other inorganic compounds are converted to organic compounds continues. At present we are studying the mechanisms of the transfer of electrons and phosphate groups to the carbon compounds, and the effects of light quality (wavelength and intensity) on the products of photosynthesis and glycolysis in the photosynthetic apparatus in vivo and in isolated chloroplasts.

Studies reported last year in which inhibitors were used to block specific reactions of the photosynthetic carbon-reduction cycle were continued. These investigations increased markedly in effectiveness during the past year through the simultaneous use of $^{32}P$-labeled phosphate and $^{14}C$-labeled $CO_2$ in detailed kinetic studies of the behavior of photosynthetic carbon cycle intermediate compounds and photosynthetic cofactors, during steady-state photosynthesis and following addition of inhibitors. The results of such experiments with fatty acids and methyl octanoate are summarized as follows:[1]

Photosynthesis in Chlorella pyrenoidosa is quickly inhibited by the addition of $3\times10^{-4}$ to $6\times10^{-4}$ M lipoic acid, octanoic acid, or methyl octanoate. These inhibitions are wholly or partially reversible. Studies with $^{32}P$-labeled phosphate and $^{14}C$-labeled carbon dioxide of transient changes in the levels of photosynthetic intermediate compounds during the time following addition of inhibitor indicate blocking of (a) the carboxylation reaction of the photosynthetic carbon reduction cycle, (b) photophosphorylation (formation of ATP in the light), and (c) conversion of fructose-1,6-diphosphate to fructose-6-phosphate and of sedoheptulose-1,7-diphosphate to sedoheptulose-7-phosphate. Blocking of photophosphorylation results in decreased rates of conversion of ribulose-5-phosphate to ribulose-1,5-diphosphate, of glucose-6-phosphate to polysaccharides and sucrose, and other reactions. These metabolic effects, together with other published results, lead us to conclude that at least several key steps in the photosynthetic carbon reduction cycle may be mediated by enzymes in some organized system associated with the lamellae and photophosphorylation.

Partly because of similarities between the effects of these inhibitors and observed effects with the light-dark transient, further experiments on the light-dark transient with both $^{32}P$ and $^{14}C$ were performed. These studies yielded several interesting results,[2] given in the following summary:

The labeling of intermediate compounds and photosynthetic cofactors during photosynthesis and periods of darkness by Chlorella pyrenoidosa in the presence of $^{32}P$-labeled phosphate and $^{14}CO_2$ have been investigated. Algae adapted to photosynthesis in air were used, and the level of carbon dioxide was maintained at approximately 0.04% and at constant specific radioactivity during the course of the experiments.

The transient changes that occur in the levels of labeled fructose-1,6-diphosphate and in sedoheptulose-1,7-diphosphate, and in the corresponding monophosphates when the light is turned off, suggest a light activation of the diphosphatase enzymes which decays after about 2 min of darkness. It is suggested that a light-dark switch in enzymic activities permits photosynthesis and glycolysis to occur in light and in dark respectively with the same enzymic apparatus.

The greatly diminished rate of disappearance of the carboxylation substrate, ribulose-1,5-diphosphate, after about 2 min suggests that there is also a light activation of the carboxylation reaction in vivo. Large transient changes in the level of pyrophosphate between light and dark indicate that there may be an unstable cofactor which decomposes to give pyrophosphate during or after killing of the algal cells. The possibility that this cofactor is involved in an activation of carbon dioxide for the carboxylation reaction in vivo is suggested.

Light-dark transient changes in labeling of other compounds of the photosynthetic carbon reduction cycle and related compounds were determined, and possible significance of these changes is discussed.

## Recent In Vivo Light-Dark-Light Transient Studies

In the first 2 min of darkness following a period of photosynthesis, there is a transient period during which enzymic activities of the photosynthetic carbon cycle apparently changes. There is a decrease in the activity of the

enzyme that catalyzes the conversion of fructose diphosphate and sedoheptulose diphosphate to their respective monophosphates. At the same time, there appears to be an activation of a phosphofructokinase activity which results in the formation of diphosphates, presumably from monophosphates. Also there is a decrease in the activity of the carboxylation reaction that converts the ribulose diphosphate and carbon dioxide to 3-phosphoglyceric acid (PGA).

The inactivation of the diphosphatase reaction (a photosynthetic reaction) and the activation of the phosphofructokinase activity (representative of glycolysis) could occur at separate points, metabolically isolated from each other. Alternatively, both the photosynthetic reactions and the glycolytic reactions might occur at the same point, utilizing the same enzymic machinery. In this case, the simultaneous activation of phosphofructokinase and inactivation of diphosphatase would be a critical necessity. These two enzymes should not exist simultaneously in active forms at the same site, since they would together function as an ATPase.

Evidence in favor of the occurrence of both glycolysis and photosynthetic carbon reduction at the same site was seen in the double labeling curve for PGA. It was found that PGA was saturated with both $^{14}C$ and $^{32}P$ in the light during the permitted time of photosynthesis with tracers. This time was 34 min with $^{32}P$-labeled phosphate, and 14 min with $^{14}CO_2$. It will be helpful in the following discussion to remember that once the $^{32}P$ label of any compound is saturated, it will remain a measure of the amount of compound (its concentration) for all the remainder of the experiment. This is true because formation of intermediates of either photosynthesis or glycolysis incorporates labeled phosphate exclusively in the presence of $^{32}P$-labeled inorganic phosphate.

When the light was turned off, both the $^{32}P$ label and the $^{14}C$ label of PGA rose rapidly and then began to fall. The $^{14}C$ label continued to fall all during the time of the dark period, as has been seen on many previous light-dark studies. In contrast, the $^{32}P$ label in PGA, after an initial drop, increased and stabilized at a higher steady-state level than in the light. This changing ratio of $^{32}P$ to $^{14}C$ in PGA means that PGA is being synthesized by glycolysis in the dark from endogenous, non-$^{14}C$-labeled compounds.

When the light was turned on again, the $^{32}P$ label dropped very much more than the $^{14}C$ label, as photosynthetic reduction of PGA was initiated. This large drop in $^{32}P$ and small drop in $^{14}C$ strongly suggests that the PGA that had been formed by glycolysis in the dark was being immediately and rapidly reduced by light. This finding supports the idea that photosynthesis and glycolysis occur at a single site.

Since this conclusion is of considerable importance, a repeat experiment under essentially the same conditions has been performed. In this later experiment an analysis of both $^{14}C$ and $^{32}P$ has been made, not only in PGA but also in other intermediates of the carbon reduction cycle. The conditions of this experiment were in all respects similar to those used in the experiment described in Ref. 1.

The $^{14}C$ and $^{32}P$ labeling of PGA in this experiment is shown in Fig. 8-1. The result is essentially the same as in the previously described experiment. The $^{14}C$ and $^{32}P$ labeling of a dihydroxyacetone phosphate (DHAP), the reduction product of PGA, is shown in Fig. 8-2. This compound was saturated with $^{32}P$ and $^{14}C$ in the light. It is noteworthy that although the molecular ratio of carbon to phosphorus is 3:1, in both PGA and DHAP the $^{14}C$ to $^{32}P$ ratio is less at "saturation" in the light for PGA than for DHAP. It was found some years ago[3] that there is a pool (or pools) of PGA that becomes labeled very slowly with $^{14}C$ in algae photosynthesizing in $^{14}CO_2$. Quite likely, there is some labeling of such pools with $^{32}P$ due to glycolytic reactions away from the chloroplasts.

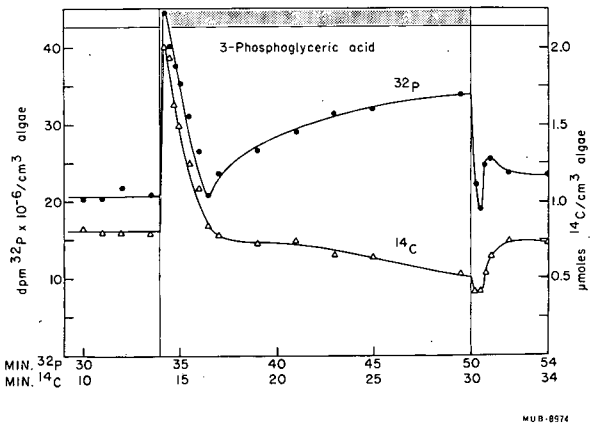


Fig. 8-1. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{32}P$ and $^{14}C$ labeling of PGA.

When the light was turned off, both $^{14}C$ and $^{32}P$ fell rapidly to a very much lower level. This is expected, since, in the dark,

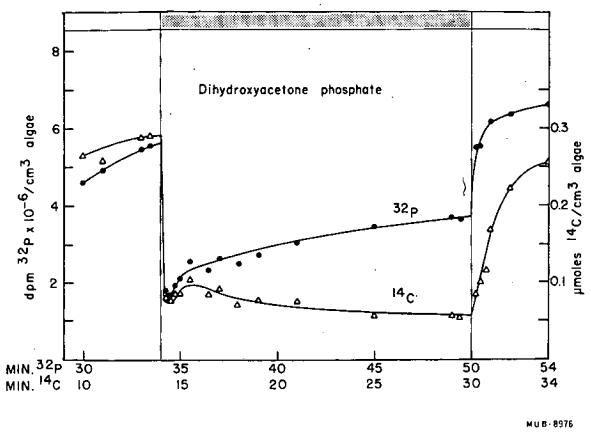


Fig. 8-2. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{32}P$ and $^{14}C$ labeling of DHAP.

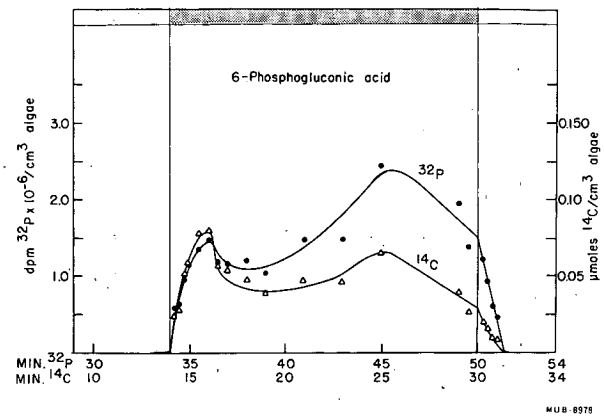


Fig. 8-3. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{32}P$ and $^{14}C$ labeling of 6-phosphogluconic acid.

there is no longer reduction of PGA to DHAP, but the DHAP is converted to other compounds of the carbon cycle by reactions not requiring reduced coenzymes made in the light.

Subsequently, in the dark, the level of $^{32}P$-DHAP rises, while the level of $^{14}C$ in the same compound declines. This is indicative of the formation of new DHAP from endogenous sources not labeled with $^{14}C$. When the light is turned on again, the $^{32}P$ level in DHAP rises very rapidly, indicating the immediate reduction of PGA to DHAP. The $^{14}C$ also rises and comes back to the steady-state level. However, there is a significant difference in the rate at which it rises. It is apparent that the $^{14}C$ in DHAP rises much more slowly during the first minute of light than does the $^{32}P$. This is another indication that there is but a single pool of PGA involved in these transient changes. Since this pool has had its $^{14}C$ diluted by respiratory formation of PGA in the dark, the rise in $^{14}C$ of the reduction product, DHAP, is slower in the light than the increase in quantity of compound indicated by the $^{32}P$ level.

Figure 8-3 shows the behavior of labeled 6-phosphogluconic acid. No measurable amount of labeled 6-phosphogluconic acid appears until the light is turned off, at which point there is a rapid formation of this labeled compound. As time passes in the dark, the ratio of $^{32}P$ to $^{14}C$ in the 6-phosphogluconic acid increases. This again suggests the entry into the pool of sugar phosphates of unlabeled carbon from an endogenous source. When the light is turned on again the 6-phosphogluconic acid disappears as a consequence of its being metabolized but no longer formed. Earlier studies of the effects of inhibitors[4] showed that under conditions such that NADPH is converted to $NADP^+$, 6-phosphogluconic acid appears, even in the light.

The labels in ribulose-1, 5-diphosphate (RuDP) are seen in Fig. 8-4. As was found in earlier studies with light-dark transients and low-$CO_2$ Chlorella, the level of RuDP does not go to zero in the dark. This fact shows that some of the RuDP, after about 2 min in the dark, is no longer available to an active carboxylation enzyme. It may mean that the carboxylation enzyme becomes inactivated in the dark, but it could also mean that some relatively small amount of RuDP is synthesized or is located at a site that is no longer photosynthetically active (such as dead cells). It is of interest, therefore, to see that the ratio of $^{32}P$ to $^{14}C$ increases in the RuDP in the dark. However, this only tells us that this RuDP is, or was, made from partly un-$^{14}C$-labeled sources--it does not tell us when it was made. The information is thus not so useful as in the case of PGA, for which there is a clear synthesis of $^{32}P$-labeled compound at the same time (in the dark) as the disappearance of the $^{14}C$ label.

When the light is turned on again, there is a rapid synthesis of RuDP, preferentially $^{32}P$-labeled, as would be expected if carbon from endogenous sources, not $^{14}C$-labeled, had entered the common pools of photosynthetic and glycolytic intermediate compounds, as already seen with PGA and DHAP. This synthesis of new RuDP cannot be at a metabolically inactive site, and the fact that it is greatly stimulated by light suggests that it is occurring at the site of the photosynthetic apparatus.

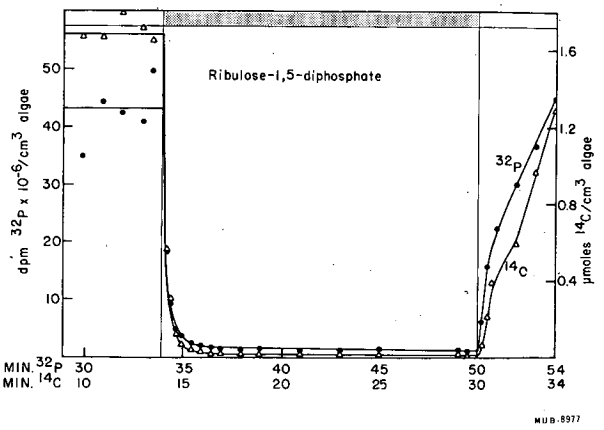


Fig. 8-4. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{32}P$ and $^{14}C$ labeling of RuDP.

Figure 8-5 shows the labeling with $^{32}P$ of ATP, ADP, and UTP. The result is similar to that observed in earlier experiments.[2] The re-establishment of the ATP level in the dark, suggestive of glycolysis, is again seen. The recovery in ATP level in the dark, coupled with the postulated activation of phosphofructokinase and inactivation of diphosphatase, leads to a steady state of glycolysis at the photosynthetic site after several minutes of darkness.

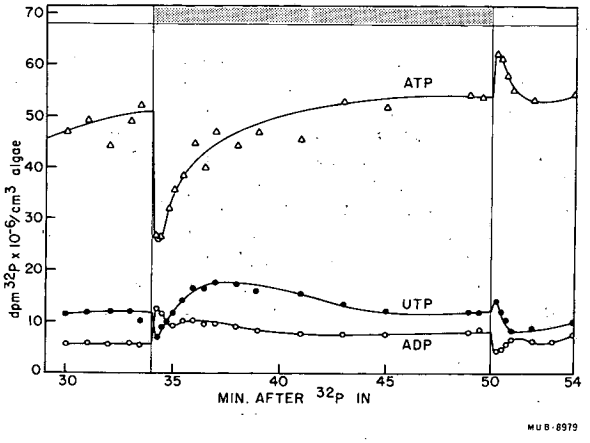


Fig. 8-5. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{32}P$ labeling of ATP, ADP, and UTP.

Figure 8-6 gives the $^{32}P$ and $^{14}C$ labeling for the sum of fructose and sedoheptulose diphosphates. The formation of these sugars

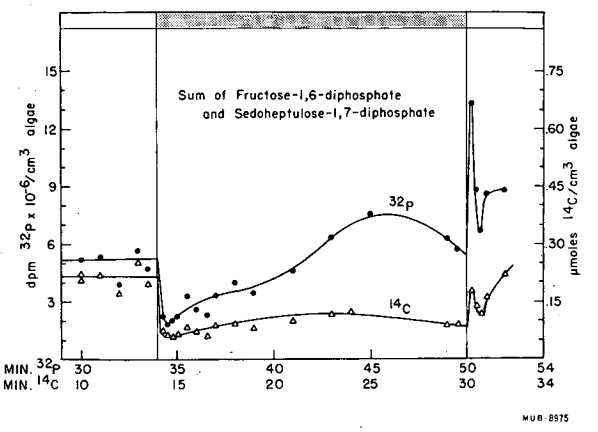


Fig. 8-6. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{32}P$ and $^{14}C$ labeling of the sum of FDP and SDP.

from non-$^{14}C$-labeled precursors in the dark is quite apparent. Although the rise in levels of FDP and SDP in the dark is not so dramatic as in some previous experiments,[1] their formation is clear evidence for the activity of kinases. As already mentioned, such enzymic activity cannot exist at the same locus as diphosphatase activity, but could occur simultaneously if metabolically isolated. Thus, if we can demonstrate that the same pools of metabolic intermediate compounds are subject to one of these enzymes in the light and the other in the dark, we can conclude that light-dark control of these activities occurs.

The rapid formation of additional amounts of these compounds when the light is again turned on is directly related to the rapid formation of DHAP, which was seen in Fig. 8-2. The situation in the diphosphates is complicated by the fact that we can examine only the sum of the two compounds, if we wish to observe both $^{32}P$ and $^{14}C$ labeling, since they do not separate in our chromatographic system.

However, Fig. 8-7 shows the $^{14}C$ content of these sugar phosphates, obtained by treatment with phosphatase and rechromatography. The rapid initial rise in levels of these sugar diphosphates when the light is turned on results from the condensation of the rapidly formed DHAP (Fig. 8-2) with 3-phosphoglyceraldehyde and with erythrose-4-phosphate to give FDP and SDP respectively. Neither of these two aldehydes is ever seen as "spots" on our chromatograms, and it is thought that their concentrations are always very small. However, 3-phosphoglyceraldehyde comes directly from the reduction of PGA, while

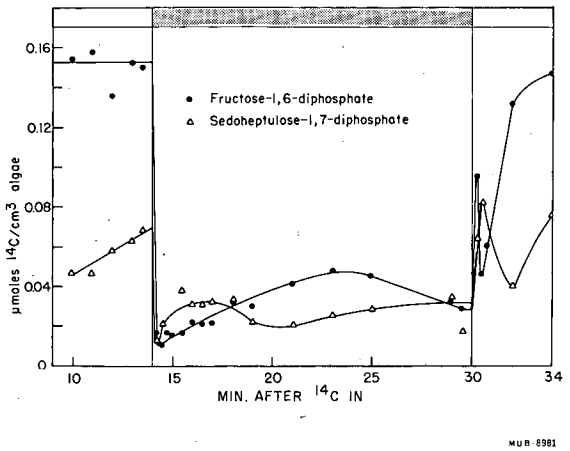


Fig. 8-7. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{14}C$ labeling of FDP and SDP.

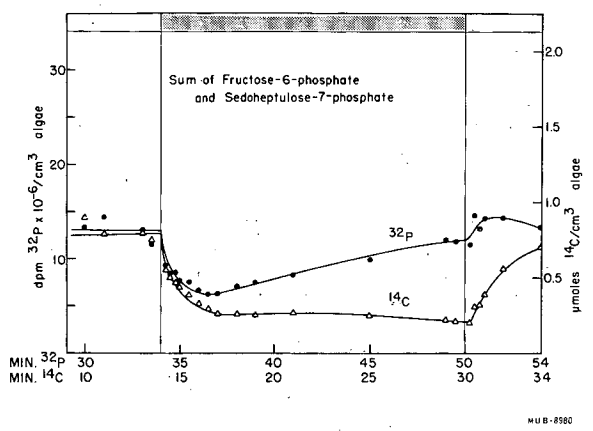


Fig. 8-8. Effects of light-dark and dark-light transition on labeling of photosynthetic intermediates in Chlorella. $^{32}P$ and $^{14}C$ labeling of the sum of F6P and S7P.

erythrose-4-phosphate comes from a transketolase reaction on fructose-6-phosphate, derived in turn from FDP. Consequently, the initial rise is greater for FDP than for SDP.

The fall in the FDP and SDP labels following the initial peak is interpreted as an indication of the activation in the light of the diphosphatase enzymes. Apparently this activation in the light is somewhat faster (in the order of 30 sec) than the inactivation of the same enzymes in the dark (of the order of 2 min[2]). These times can be at present only very rough estimates.

In Fig. 8-8, the $^{14}C$ and $^{32}P$ labeling of the sum of fructose and sedoheptulose monophosphates are shown. Dark synthesis from endogenous sources is again demonstrated. When the light is turned on, no great changes in total concentration ($^{32}P$) occur, since these compounds are not close to points of light action (such as PGA reduction or RuDP formation). It is thus possible to see the "washing out" of unlabeled carbon by $^{14}C$ in the period after the light is again turned on.

In summary, further evidence in the form of $^{32}P$ and $^{14}C$ labeling of intermediates of photosynthetic carbon reduction during light-dark and dark-light transient periods is presented, and leads to the following conclusions:

1. There are, as previously thought, pools of such metabolic intermediate compounds as PGA at sites where they are not in rapid isotopic equilibrium with the intermediate compounds of the carbon reduction cycle.
2. Pools of intermediate compounds of the carbon reduction cycle which are in rapid contact with the photosynthetic apparatus are subject to photosynthetic reactions in the light and to glycolytic reaction in the dark. In the dark, precursors of these pools are endogenous and (in our experiments) largely unlabeled carbon sources.
3. Triose phosphate dehydrogenase and other enzymes common to photosynthesis and glycolysis are used for photosynthesis in the light and for respiration in the dark.
4. Metabolic control is exerted not only by the supply or absence of reduced NADP, but, more precisely, by the enzymes at the FDP to F6P and SDP to S7P reactions. In the light, diphosphatase enzymes are activated and phosphofructokinase is inactivated. In the dark, the activations are reversed. The light activation reactions seem to be somewhat faster than the dark activations.

## Studies with Isolated Chloroplasts

Since kinetic studies in vivo are always subject to the ambiguity of possible mixing of metabolites from different compartments, it would be desirable to repeat many of the above described experiments with isolated chloroplasts. However, until recently, rates of $CO_2$ fixation with isolated chloroplasts have been only of the order of 1 to 5% of "normal" in vivo rates. Thus, we have spent considerable effort developing chloroplast isolation and incubation methods to give good $CO_2$ fixation rates.

Recently, our studies with isolated spinach chloroplasts have produced rates of $CO_2$ fixation as high as 20% of normal in vivo rates.

The limitation in assimilation rates with the isolated chloroplasts seems to occur at the diphosphate reaction and possibly at the carboxylation reaction. Both the loss of $CO_2$ assimilation ability of chloroplasts upon isolation, and the reversible inhibition caused by fatty acids, are interpreted as resulting from physical-chemical effects on the lamellar systems of the chloroplasts. These effects may to some extent mimic effects produced by darkness and may be related to reversal of light-induced chloroplast shrinkage.

Because of the limited diphosphatase reaction, large amounts of sugar diphosphates and triose phosphates are seen. A new spot on the chromatograms of photosynthetic products was identified as 3-phosphoglyceraldehyde. This triose phosphate has long been postulated to be an intermediate in the carbon reduction cycle of photosynthesis, but was not previously seen in photosynthesis experiments.

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## 9. BIOSYNTHESIS OF OPIUM AND TOBACCO ALKALOIDS

H. Rapoport

### Opium Alkaloids

During this year work in this area has concentrated on two aspects of opium alkaloid biochemistry. One has been the role of reticuline as a precursor, and the other has been thebaine's role in the plant's metabolism. Stereochemically, morphine is related to (-) reticuline. However, in seedlings we have found[1] that the natural form of reticuline is (±). In mature plants and in the seed capsule, however, (+) reticuline predominates over (-) by about six to one. A possible explanation for this difference might be the rapid conversion of (-) reticuline into morphine alkaloids in flowering plants as compared with a slower metabolism of the (+) isomer, which, stereochemically, is related to the narcotine group of alkaloids. In seedlings, both isomers appear to be metabolized at an equal rapid rate.

In anticipation of back-feeding experiments, thebaine has been prepared with a specific label of carbon-14 in the $O^6$-methyl group.

### Tobacco Alkaloids

Continuation of studies on nicotine biosynthesis with short-term (2- to 6-hr) exposure to $^{14}CO_2$ has revealed that the pyrrolidine ring does not have a symmetrical labeling pattern.[2] For example, C-2', C-3', and C-5' each contains about 2% of the total nicotine radioactivity, whereas C-4' contains from five to six times as much. Therefore, the symmetrical-glutamate intermediate scheme for the biosynthesis of the pyrrolidine ring, deduced from ornithine feeding, must be a minor or aberrant pathway. This contradiction emphasizes the importance of the $^{14}CO_2$ approach in alkaloid biosynthesis studies.

Biosyntheses from $^{14}CO_2$ carried out with both intact Nicotiana glutinosa plants and seedlings have demonstrated that nornicotine is not the precursor of nicotine in either the root or the aerial portions of the plant. This conclusion contrasts with that reached on the basis of $^3H$ incorporations into the alkaloids of Nicotiana rustica. The most probable relationship, based on $^{14}CO_2$ biosyntheses and supporting some previous conclusions, is that nornicotine arises by demethylation of nicotine in both the root and aerial portions. The relative specific activities of anabasine and anatabine after the $^{14}CO_2$ biosyntheses indicated that anabasine is not the precursor of anatabine. For nicotine, nornicotine, anatabine and anabasine, higher specific activities were noted in the samples isolated from the root than in those isolated from the aerial portions. This shows that these four alkaloids all can be formed in the Nicotiana root, and furthermore, since the $^{14}C$ in these experiments first enters into the metabolism of the aerial portion of the plants, these results indicate that N. glutinosa root is the most active site of formation of each of these alkaloids. Finally, a comparison of the specific activities of anabasine, anatabine, and nicotine suggests that if a precursor-product relationship exists among these alkaloids it must be nicotine → anatabine or anabasine, or both.[3]

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## 10. CATABOLITE REPRESSION OF ENZYME SYNTHESIS

V. Moses and C. Prevost

It has been known for a long time that the synthesis of certain catabolic enzymes in bacteria is controlled by balanced factors. On the one hand, synthesis of the enzymes requires the presence in the bacterial environment of specific inducing substances, identical with or closely related chemically to the enzyme substrate. However, even in the presence of such inducers the enzymes are not formed when the cells are growing on "luxury" sources of carbon and energy, such as glucose and other sugars, or when the cells are laboring under a number of metabolic disabilities. The latter might include withholding a specific amino acid required for growth, or including in the medium a low concentration of an inhibitor of protein synthesis, so that the cells are able to grow only comparatively slowly. In such circumstances, the differential rates of synthesis of the inducible catabolic enzymes are reduced compared with total protein synthesis.

A convenient system for study is the formation of β-galactosidase in Escherichia coli, and its repression by glucose. The natural inducer, and substrate, for this enzyme is probably lactose. When E. coli cells are grown on a mixture of lactose and glucose, no β-galactosidase is made until all the glucose has been exhausted. There is thus very efficient repression of the formation of the enzyme by glucose even though the inducer is present. More powerful inducers than lactose can be made artificially. There are the thio-galactosides, one of which, isopropyl-thio-galactoside (IPTG), is particularly potent. If glucose is added to glycerol-grown cells induced with IPTG, it is found that the repression of enzyme synthesis is not permanent as it is when the cells are induced with lactose. Rather, there is a period of intense repression shortly after the addition of glucose, during which time the rate of enzyme synthesis may fall to 10% or less of the rate before glucose was added. Sometime later the rate of enzyme synthesis increases to perhaps 60 to 70% of the original rate, even though the glucose concentration in the medium is effectively unchanged. The time required for recovery varies greatly among various strains. The shortest time observed is only about 4 min, while the longest is nearly 3 hr. Recovery commonly occurs in 20 to 40 min.

Glucose is not the only compound to give this behavior pattern of temporary severe repression followed by a greater or lesser degree of recovery. Galactose, ribose, uridine, and other compounds show similar effects. Indeed, the repression seems to be associated mostly with the shift of cellular metabolism from growth on glycerol (or other relatively poor substrate) to more rapid growth on glucose or other "luxury" carbohydrate. The added sugar itself does not appear to be directly responsible for the repression. The repression is a consequence of metabolism of the sugar: sugars which are not metabolized rapidly (e.g., galactose in gal$^-$ strains) do not cause repression. Thus, repression is believed to be the result of some catabolic product of metabolism and hence is termed "catabolite repression."

The task of identifying the repressing catabolite of glucose metabolism is facilitated by the temporary kinetic nature of at least some of the repression. Investigation of the kinetics of the concentrations of catabolic products following the introduction of glucose to the bacterial culture permits a correlation to be made with the kinetics of the repression of enzyme synthesis. With use of cells grown entirely in medium in which all the phosphate is labeled with $^{32}P$, all the phosphorylated compounds are radioactive. The pool sizes of such compounds following glucose addition may then be studied by sampling the culture at intervals, killing the cells, extracting the phosphate esters, and analyzing them by paper chromatography. Two strains of E. coli, in particular, have been studied in this way. In one of these strains, Q-22, a typical transient repression of β-galactosidase synthesis is observed when glucose is added. In another closely related strain, W-8, glucose has no effect on enzyme synthesis. Metabolic analysis of these strains showed that in Q-22 addition of glucose results in a temporary rise in the concentrations of glucose-6-phosphate and 6-phosphogluconic acid; these concentration changes were not observed with W-8. This suggested that the glucose repression was in some way related to the operation of the pentose phosphate cycle, since these two phosphate esters are the first two intermediary compounds of that cycle.

Support for this conclusion was obtained by studying the release of $^{14}CO_2$ from [$^{14}C_1$] glucose and [$^{14}C_6$] glucose. In glucose metabolism via the pentose phosphate cycle, $CO_2$ is released preferentially from the $C_1$ of glucose. In glycolytic metabolism, followed by oxidation of the glycolysis

products via the tricarboxylic acid cycle, $CO_2$ is released equally from $C_1$ and $C_6$. Thus, the extent of preferential release of $C_1$ as $CO_2$ may be used as a measure of pentose phosphate cycle metabolism compared with glycolysis. In strain Q-22 there is indeed an initial preferential oxidation of $C_1$ from glucose. After about 30 min this preference becomes less important and a greater proportion of glucose is metabolized via glycolysis than is the case immediately after glucose is first added. With strain W-8, on the other hand, there is no preferential utilization of the pentose phosphate cycle in glucose metabolism. This represents strong support for involvement of the cycle in the period of intense transient repression.

It is not yet known whether glucose-6-phosphate and 6-phosphogluconate are themselves the active catabolic products which cause repression. The fact that varying degrees of repression may be caused by a wide variety of added substrates, some of which would be unlikely to cause much change in glucose-6-phosphate and 6-phosphogluconate, would indicate that some other product or products of the cycle are immediately concerned with repression.

The main products of pentose phosphate cycle metabolism are $CO_2$, ATP, and reduced pyridine nucleotides. Carbon dioxide is unlikely to be the repressing compound; it is always formed by metabolism, including anaerobic metabolism, yet catabolite repression is largely prevented by anaerobiosis. Chromatographic analysis of $^{32}P$-labeled cells failed to show changes in ATP concentration. Although the pyridine nucleotides would also have been labeled in the $^{32}P$ experiments, technical considerations in the performance of the experiments prevented the making of measurements of the concentrations of these compounds, and further attempts to measure them are currently being carried out by use of appropriate modifications of technique.

Although it may eventually be possible to correlate changes in reduced pyridine nucleotide concentrations with β-galactosidase repression, the repression of other enzymes by glucose may be mediated by other metabolites. It is known, for example, that both D-serine deaminase and L-tryptophanase are temporarily repressed by glucose in E. coli, although the duration of the repression is longer for both of these enzymes than it is for β-galactosidase. The repressing catabolites for these enzymes may not be reduced pyridine nucleotides, and the kinetics of their concentration changes may be quite different. Yet, we must also recognize the possibility that in these cases, too, pyridine nucleotides are involved, but the effective concentrations necessary to repress serine deaminase and trypotophanase may be lower than for β-galactosidase. If adding glucose causes a rise in reduced pyridine nucleotide concentration, with a subsequent fall more or less to the initial level, one might expect recovery first in the synthesis of those enzymes which are least sensitive to the elevated concentrations, with later recovery for the more sensitive ones. It is hoped eventually to make a detailed study of the repression mechanisms for a number of these enzymes as a means of increasing our knowledge of the extent and operation of basic control processes that probably have widespread significance among living systems.

# 11. HEREDITY, ENVIRONMENT, LEARNING, AND THE BRAIN

Edward L. Bennett, Marie Hebert, Hiromi Morimoto, and Ann Orme

(In collaboration with Professors David Krech and Mark R. Rosenzweig, Psychology Department, and Marian C. Diamond, Physiology-Anatomy Department, University of California)

For a number of years we have been studying heredity, environment, learning, and the brain, in an attempt to determine some of the changes which occur in brain during learning, and the effects these changes may have on the subsequent problem-solving ability of rats. Genetic differences in brain chemistry have been studied as an additional means of relating brain chemistry and problem-solving ability. The areas of our investigations are schematically shown in Fig. 11-1.

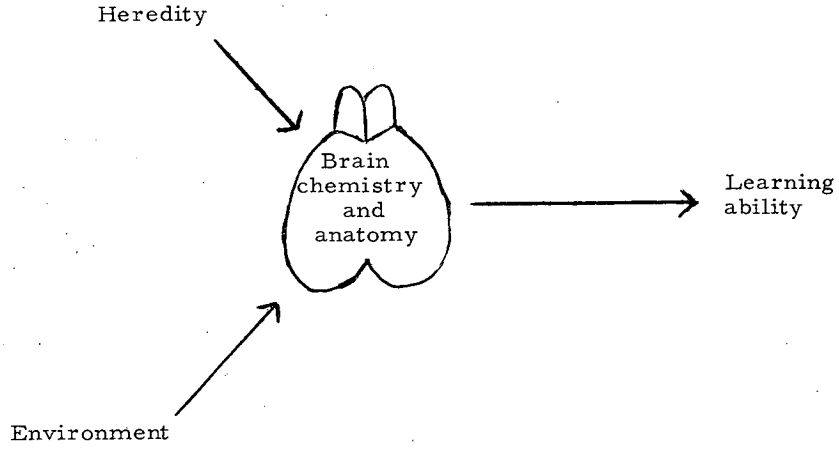


Fig. 11-1. Schematic representation of proposed interrelationships of heredity and environment and learning ability mediated through brain chemistry and anatomy.

In this report, three areas of our research will be summarized which show that:

(a) A rat raised in an enriched behavioral environment has a significantly different brain in terms of chemistry and neuroanatomy than his isolated littermate brother raised in an isolated or impoverished condition. This aspect has been treated more fully in an article in Science.[1]

(b) A rat raised in an enriched environment is significantly better at problem-solving than his isolated brother.

(c) Different strains of rats which differ in problem-solving ability show the same characteristic differences in brain chemistry and brain weight as do the enriched and impoverished animals.

In our experiments to compare the effects of a behaviorally enriched environment with those of a behaviorally impoverished environment, one of each pair of littermate male rats is placed in a small rat cage with three sheet metal sides (so they cannot see or touch another animal), and they are raised in a quiet colony room with the other isolated rats. The rat is handled only at weekly intervals for weighing. This is the IC or Impoverished Condition. Meanwhile their behaviorally enriched brothers are living with 10 or 11 other rats of the same age and strain in a large cage equipped with all the toys a rat should have, and during their life are handled daily and run through a series of behavioral tests. These are the ECT (Environmental Complexity and Training) rats. At 105 days of age, the rats are sacrificed and appropriate analyses are made.

The first measures we get are the weights of the cortex and subcortex. In 13 experiments carried out over a 6-year period and involving 141 littermate pairs of $S_1$ male rats, we have found the cortex of the ECT to be an average of 4% heavier than that of his IC littermate. A positive difference was found in 79% of the littermate comparisons (Fig. 11-2). If we look at the subcortex, we find a small 1% difference in the other

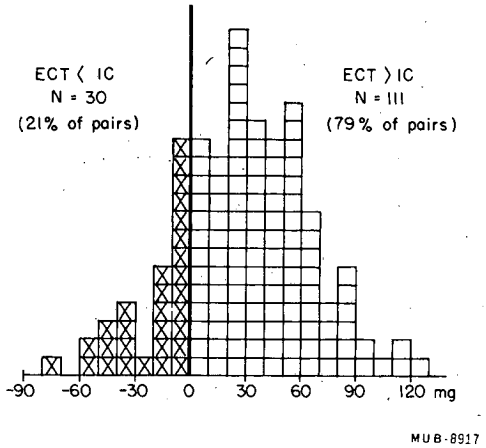


Fig. 11-2. Differences between littermates in weight of total cortex; 13 experiments with 141 pairs of $S_1$ rats.

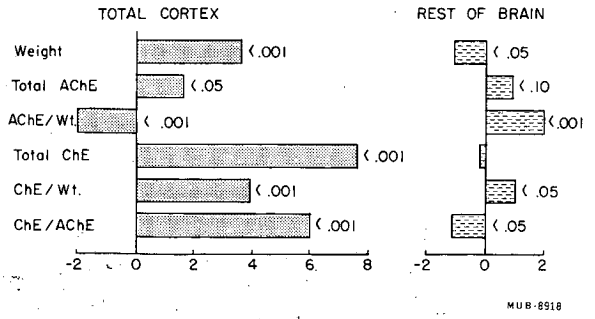


Fig. 11-3. Percentage differences, ECT minus IC, 9 experiments with $S_1$ rats (98 littermate pairs). (Significance of differences is given to the right of each bar.)

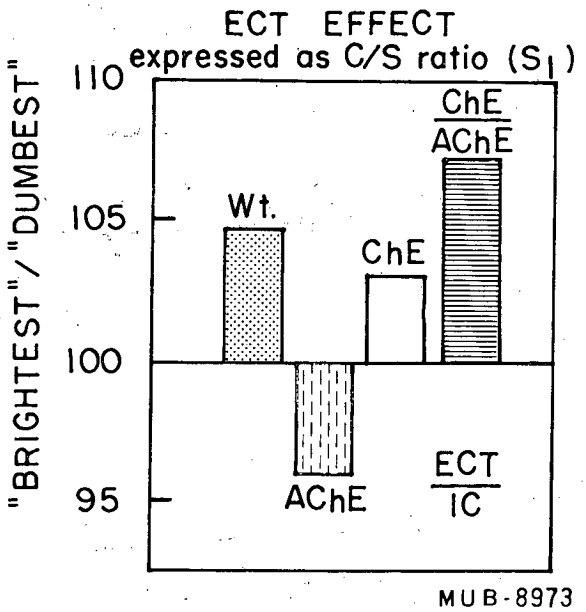


Fig. 11-4. ECT effect, expressed as C/S ratio ($S_1$).

direction (significant at the 0.05 level)--that is, the IC animals have a slightly heavier subcortex than do their more active ECT brothers.

The next measures we normally obtain are the activities of two important cerebral enzymes, acetylcholinesterase (AChE) and cholinesterase (ChE). AChE exists in high concentrations at synaptic endings and hydrolyzes with great rapidity and high specificity the neurotransmitter substance, acetylcholine. ChE is a less specific enzyme which hydrolyzes higher choline esters such as butyrylcholine. ChE exists in comparatively high concentration in glial cells, but its function in brain is not known.

In Fig. 11-3 we have summarized the chemical data, and we can see that in addition to having heavier cortices, the ECT rats have increased total cortical activity of AChE and ChE, and an increased ratio of ChE to AChE. In the subcortex we see a different picture--AChE increased, while ChE changed very little, and the ratio of ChE/AChE decreased slightly. We have termed these effects the "ECT effects," and they are expressed in a slightly different way in Fig. 11-4, where the values are expressed in terms of cortical/subcortical ratios.

In the majority of our experiments, the cortex has been subdivided into four areas--the visual cortex, somesthetic cortex, dorsal cortex, and ventral cortex. Although the direction of changes with respect to weight and chemistry is the same in each cortical area, the magnitude varies, the two extremes being found in the V and S areas, which are adjacent (Fig. 11-5). Thus, the ECT effect has a specificity in different areas of the cortex; also, we have shown, in other experiments, that it varies with the behavioral treatment used.

Anatomical measures taken (by Dr. Diamond and her associates) include cortical depth and the ratio of neurons to glia cells.[2] The increase in weight of cortex has been equated with an actual increase in depth, in four different experiments summarized in Table 11-I.

Although the absolute depths determined in frozen and celloidin sections are quite different,

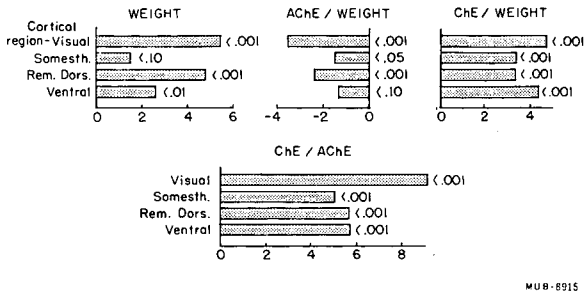


Fig. 11-5. Percentage differences, ECT minus IC, by cortical region, in 9 $S_1$ experiments.

Dr. Diamond's group to obtain a high degree of reproducibility, cells in the same sections used for cortical depth measurements were classified as neurons or glia (Table 11-II). We conclude that brain "exercise" leads to an increase in glial cells.

The significance of these cerebral effects would be enhanced if we could demonstrate differences in learning ability between rats raised in an enriched and in an impoverished environment. The proper demonstration of differences in learning ability between groups of rats such as these is more difficult than would appear at first glance, but we will not take the space here to describe some of the problems. We finally settled on a Visual Reversal Discrimination Test (VRD test) in which a rat first learns to choose lighted alleys as correct to obtain a food reward, and then after it has reached criterion on this task, the cue is reversed to dark. This alternation is repeated as each rat reaches criterion (of 19 correct out of 20 consecutive choices) on one problem. The test continues 18 days--the rat making 40 choices per day.

The learning ability can be expressed in

Table 11-I. Depths (μ) of visual cortex from enriched and impoverished rats (excluding layer I).

| Exp. | N (pairs) | ECT $\bar{X}$ | ECT S.D. | IC $\bar{X}$ | IC S.D. | ECT/IC | p | No. Pairs ECT>IC |
|---|---|---|---|---|---|---|---|---|
| | | | | Frozen sections (25 μ) | | | | |
| I | 11 | 1332 | 56 | 1271 | 73 | 1.048 | <0.001 | 7/11 |
| II | 9 | 1404 | 87 | 1298 | 81 | 1.082 | <0.001 | 9/9 |
| I & II | 20 | 1364 | 72 | 1284 | 76 | 1.062 | <0.001 | 16/20 |
| | | | | Celloidin sections (10 μ) | | | | |
| III | 12 | 813 | 35 | 779 | 38 | 1.044 | <0.05 | 11/12 |
| IV | 9 | 844 | 35 | 772 | 36 | 1.093 | <0.001 | 9/9 |
| III & IV | 21 | 826 | 38 | 776 | 36 | 1.064 | <0.001 | 20/21 |

Table 11-II. Differential cell counts of visual cortex from enriched and impoverished rats. Exp III & IV (17 pairs)

| Cells | ECT $\bar{X}$ | IC $\bar{X}$ | ECT>IC | % diff. | p |
|---|---|---|---|---|---|
| Neurons | 485.8 | 500.0 | 6/17 | -3.0 | NS |
| Astrocytes | 87.0 | 83.0 | 9/17 | 4.7 | NS |
| Oligodendrocytes | 75.2 | 62.5 | 13/17 | 20.4 | < 0.20 |
| A or O | 23.4 | 17.2 | 14/17 | 35.5 | < 0.01 |
| Σ glia | 185.6 | 162.8 | 13/17 | 14.0 | < 0.01 |
| G/N | 0.385 | 0.332 | 12/17 | 15.9 | < 0.02 |

by both methods a 6% increase in cortical depth of the visual area examined was found, and the cortex of the ECT animal was thicker than that of its IC littermate in 36 out of 41 pairs examined.

It is thus established that an ECT rat has both a thicker and a heavier cortex than his isolated littermate--the next question is, what is the nature of this increased material?

Using special techniques developed by

numerous ways, but, as shown in Table 11-III, animals given EC conditions for 30 days do better than their IC littermates, according to a variety of methods of scoring. These EC conditions, unlike ECT, do not include any formal training.

Table 11-III. Comparison of problem-solving ability of $EC_{30}$ vs $IC_{30}$ rats. (41 littermate pairs; 7 experiments. Data $S_1$ and $S_3$ strains combined.)

| | $EC_{30}$ | $IC_{30}$ | $IC_{30}/EC_{30}$ |
|---|---|---|---|
| No. reversals solved | 8.34 | 6.24 | 0.75 |
| Errors/reversal | 26.6 | 39.7 | 1.49 |
| Choices/reversal | 95 | 132 | 1.40 |
| Irrelevant spatial hypotheses/reversal | 0.16 | 0.41 | 2.56 |

We conclude, therefore, that in addition to producing chemical and anatomical effects in brain, EC improves the learning ability of rats. At this point we would like to pass from an area of our research in which we feel quite confident of our results to an area in which we are presently working and in which we frankly need more experiments and more time to examine the data we already have, even though these experiments have taken more than a year and used about 240 rats from 10 different strains.

In our Visual Reversal Discrimination Test, we have compared the performance of 10 strains of rats in 6 experiments taking two or more strains in each experiment, as shown in Table 11-IV. If within each group we take the chemistry of the "brightest" and compare it with that of the "dullest," we can see the comparative "weight and chemical profiles" are similar to those obtained when the ECT rat is compared with the IC rat (Figs. 11-6, 11-7, and 11-8).

Table 11-IV. Grouping of strains to relate biochemistry and behavior

| Strain | Date of Sacrifice: Original | Date of Sacrifice: Replication |
|---|---|---|
| RDH, RDL; RCH, RCL | Feb. 1965 | Aug. 1965 |
| HG, LG, HK, LK | Dec. 1964 | Sept. 1965 |
| $S_1$, $S_3$ | May 1965 | Dec. 1965 |

Figure 11-6 presents data obtained from four strains selected a number of years ago by Roderick to differ in cortical AChE activity.[3]

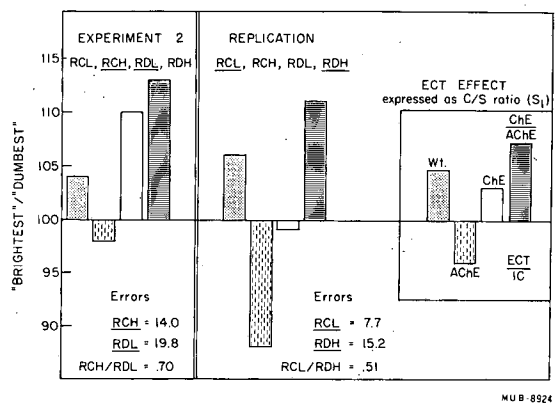


Fig. 11-6. Comparison of C/S ratio of weight, AChE activity, ChE activity, and ChE/AChE of "brightest" and "dumbest" Roderick strains in the light-dark reversal discrimination test to the same measures for the "ECT" effect.

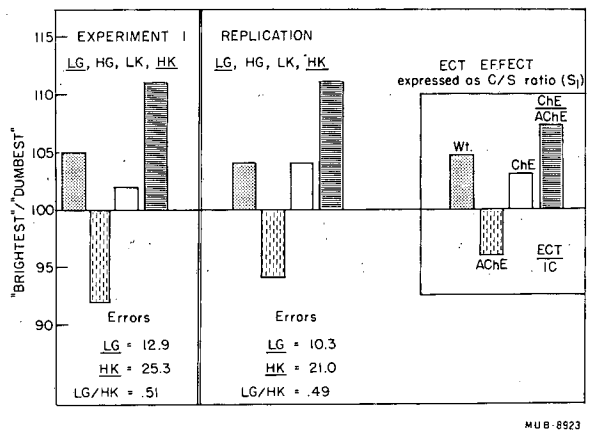


Fig. 11-7. Comparison of C/S ratio of weight, AChE activity, ChE activity, and ChE/AChE of "brightest" and "dumbest" acetylcholine lines in the light-dark reversal discrimination test to the same measures for the "ECT" effect.

In Fig. 11-7 are compared the data obtained from four strains selected to differ in acetylcholine activity.[4] This is perhaps the most striking of the comparisons with the "ECT effect."

And, finally, in Fig. 11-8 is shown the

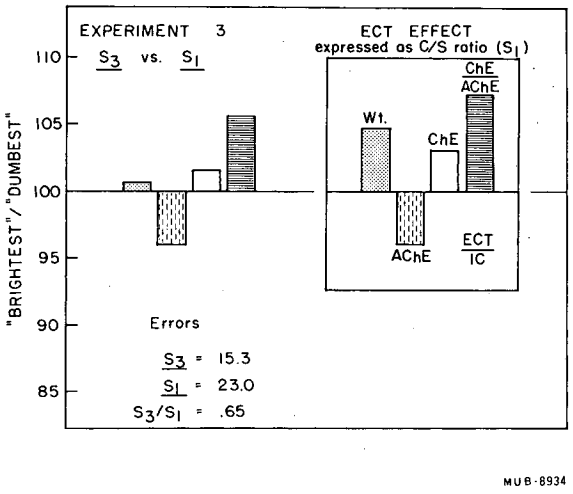


Fig. 11-8. Comparison of C/S ratio of weight, AChE activity, ChE activity, and ChE/AChE of "brightest" and "dumbest" $S_1$ and $S_3$ strains in the light-dark reversal discrimination test to the same measures for the "ECT" effect.

comparison of the $S_1$ versus $S_3$ rats. This has just been replicated for weight and behavior.

In summary, anatomical and chemical characteristics of a rat's brain can be changed by the behavioral environment. The ECT rat has a heavier cortex, lower AChE activity/mg, and higher ChE activity/mg. The ECT rat has a higher cortex-to-subcortex weight ratio and a higher cortical-to-subcortical ratio of ChE to AChE. The ECT rat also has a thicker cortex, a higher glia-neuron ratio, and perhaps greater dendritic branching than his IC littermate. Similar environmental enrichment enhances learning ability in a Visual Reversal Discrimination Test. The characteristic weight and chemical pattern found when ECT rats are compared with IC rats is also found in comparisons between "bright" and "dumb" strains. Taken together, all of these results suggest a chemical and anatomical basis for the learning ability of the rat.

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## 12. INVESTIGATIONS OF THE MECHANISM OF RADIOPROTECTION BY AMINOTHIOLS

Philip E. Brown

Abstract. Cystamine, one of the compounds most effective in protecting living material against ionizing radiation, has been shown to bind reversibly to DNA. The binding results in stabilization of the DNA helix, as indicated by the large increase in the melting temperature of the DNA. The dose of x or β rays at which destruction of the helix occurs is about 20 times as great for DNA bound with cystamine as for DNA alone. This protective effect is not due to "scavenging" by the excess cystamine in solution, but is associated with the bound cystamine. This in vitro protection may be due to removal of free radicals generated on the DNA, but it is suspected that a biological mechanism is more important for in vivo protection.

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Although numerous papers have appeared dealing with the mechanism of action of chemical protective agents against ionizing radiation, there is still no single acceptable theory explaining the intimate mechanism of the prophylactic action.[1] Consideration of the structural requirements for activity in the aminothiol series, $H_2N-(CH_2)_n-SH$, suggested that a steric factor was important, and this in turn suggested that the protective substance needed to be localized on, or bound to, the target molecule. This idea has been investigated for proteins,[2] but not for DNA. Since DNA appears to be the cellular component responsible for the effects of radiation damage, it was decided to investigate the binding of aminothiols to DNA.

It is known that binding of substances to DNA can result in a change in the melting profile of the helix-coil transition, and it was thought that binding to the DNA could be measured and followed by determination of the temperature of the transition midpoint, $T_m$. The hyperchromism associated with the transition was followed by use of a Gilford spectrometer fitted with a water-heated cell compartment. The temperature of the circulating water was raised steadily at about 25°C per hr by a mechanically driven thermoregulator. This apparatus allowed the optical densities of four samples, together with the temperature, to be recorded simultaneously, and $T_m$ values were obtained with a reproducibility of ± 0.2°C per hr.

Several factors suggested that, contrary to the view most often expressed in the literature, the active compounds would be not the aminothiols themselves, but the corresponding disulfides; thus, aliphatic diamines are known to bind to DNA, a free -SH group (required for disulfide formation) is essential for prophylactic activity, and thiols are very readily oxidized by air in warm aqueous solution.

Measurement of $T_m$ values for DNA in the presence of various concentrations of cystamine dihydrochloride ($Cl^-H_3N^+-CH_2CH_2-S-S-CH_2CH_2NH_3^+Cl^-$), cysteamine hydrochloride ($HS-CH_2CH_2-NH_3^+Cl^-$), or ethylamine hydrochloride (used throughout as a blank affecting only the ionic strength of the solution) resulted in the curve shown in Fig. 12-1. The curve shows that (i) cysteamine and cystamine behave identically--i.e., cysteamine is rapidly oxidized to cystamine, the active compound; and (ii) the binding is reversible.

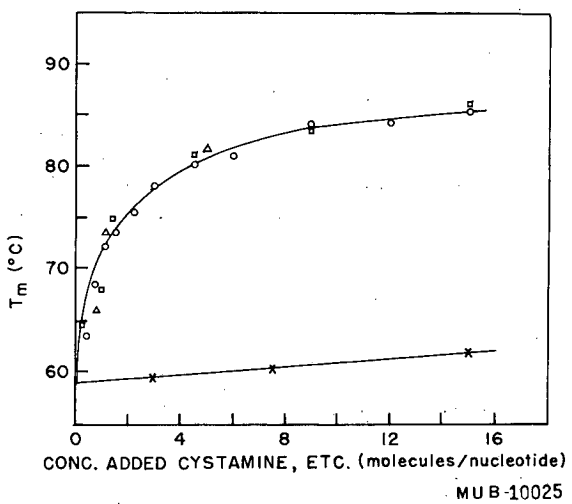


Fig. 12-1. Dependence of $T_m$ on concentration of added cystamine, etc. DNA (77 mg/liter) in 0.001 M phosphate buffer (pH 6.85) containing 0.001 M NaCl.
O - cystamine; □ - cysteamine; × - ethylamine; △ - dilutions from 15 molecules cystamine per nucleotide.

A plot of $1/T_m$ vs 1/concentration of cystamine, or equivalent, gave a good straight line (cf Langmuir isotherm for binding to a number of equivalent sites), showing that probably only one type of site is involved (see Fig. 12-2). A search for intermolecular associations between the nucleotides and disulfides revealed no measurable interactions, the uv spectrum of DNA in the presence of

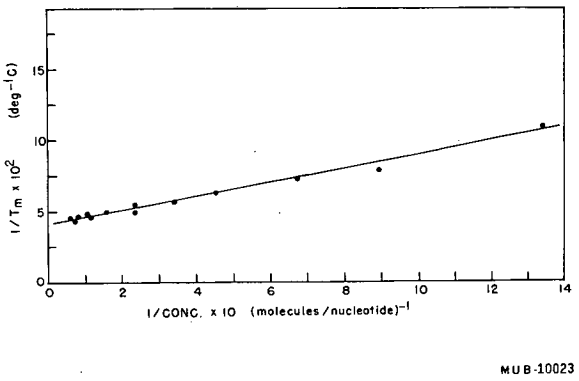


Fig. 12-2. Graph of $1/T_m$ vs 1/concentration of cystamine.

cystamine being identical with the sum of the spectra of the separate components. Soon after these experiments were completed the same conclusions were reached independently.[3]

In order to find a suitable blank for the subsequent irradiation experiments, the effects of various aliphatic diamines on the $T_m$ of the DNA was measured; the results were similar to those of Mahler and Mehrotra,[4] and in consequence, 1,4-diaminobutane dihydrochloride was chosen as a suitable reference compound.

The effect of irradiation by x or β rays on the $T_m$ of the DNA is shown in Fig. 12-3. No effect is observed with DNA alone (blanked with ethylamine hydrochloride) until about $10^5$ rads have been absorbed, whereas the lethal dose for most living organisms is of the order of $10^3$ rads. However, since the effect observed here appears to be complete destruction of the helix, it is felt that the difference in doses reflects only the amount of damage required to give observable physical, as opposed to biological, effects.

Figure 12-3 shows that in the absence of any binding agent (assuming ethylamine hydrochloride, 30 molecules per nucleotide, affects only the ionic strength) the DNA helix is rapidly destroyed at a dose of $5 \times 10^4$ rads, whereas in the presence of 15 molecules of cystamine per nucleotide destruction does not occur until $10^6$ rads have been absorbed. A slight protective effect was observed with diaminobutane (15 molecules per nucleotide), since a dose of $10^5$ rads was required for destruction of the helix. In other words, binding of an aliphatic diamine to DNA results in some protection (a factor of 2), but cystamine affords a far greater degree of protection (a factor of 20).

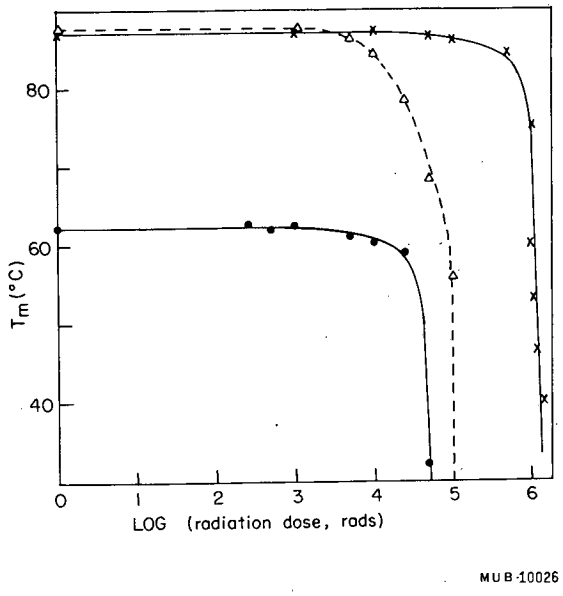


Fig. 12-3. Effect of x or β rays on $T_m$ of DNA.
Legend: —●—●— - ethylamine
– – – △ – – – – △ – – diaminobutane
—×——×— - cystamine
The relative concentrations of diaminobutane and of cystamine were 15 molecules per nucleotide; that of ethylamine was 30 molecules per nucleotide.

Two questions which arise from these observations are: (i) Is the actual amount of damage done to the DNA the same for equal doses received by protected and nonprotected DNA? (ii) Is this protective effect due to scavenging by the excess disulfide in solution?

The first question was investigated by irradiating DNA alone and then adding cystamine when the $T_m$ was that observed for DNA irradiated alone, suggesting that cystamine is not able to disguise damaged DNA. A further experiment is in progress to answer this question. It consists of irradiating DNA in the presence of cystamine, removing the amine by dialysis, and observing the $T_m$.

The second question was investigated by irradiating DNA in the presence of various concentrations of cystamine, at all of which the DNA was effectively saturated with the binding agent, so that the only variable (apart from ionic strength) was the concentration of excess cystamine in solution. The dose at which the $T_m$ had fallen by 30°C was chosen as an arbitrary standard of damage, and this

dose was plotted against the concentration of cystamine to give Fig. 12-4. The result is slightly ambiguous, probably because at the lowest concentration of cystamine the DNA was still incompletely bound. However, the three higher concentrations of cystamine gave essentially constant doses, indicating that protection is not due to the cystamine in solution but to that actually bound to the DNA. It would appear that experiments of this type are not sufficiently sensitive to answer the second question completely unambiguously, and consequently a further experiment is in progress. This consists of using diaminobutane to bind to the DNA and a nonbinding soluble disulfide (dithiodiglycol, $HO\text{-}CH_2CH_2\text{-}S\text{-}S\text{-}CH_2CH_2\text{-}OH$) to test the protective capacity of disulfides in solution alone.

The most likely mechanism by which bound disulfides protect DNA against damage is by the removal of the free radicals generated on the DNA by irradiation. This is a mechanism which has often been advanced by different workers,[4] but for several reasons this is not an entirely satisfactory explanation for in vivo protection. A more biological mechanism, which takes into account the importance of repair mechanisms, is under consideration.

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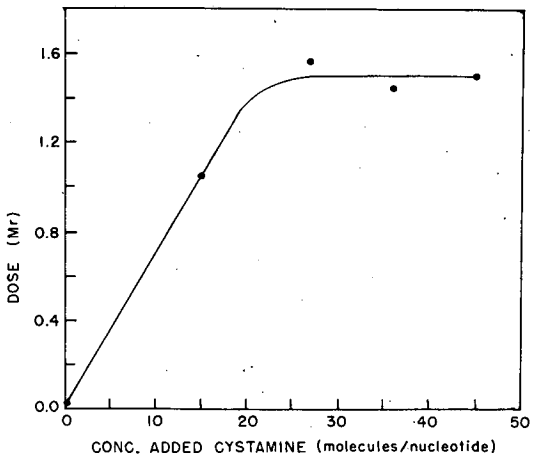


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Fig. 12-4. Dose required to decrease $T_m$ by 30°C as a function of concentration of cystamine.

## 13. PRODUCTION OF CYSTEIC ACID, TAURINE, AND CYSTAMINE UNDER PRIMITIVE EARTH CONDITIONS

Ahmed S. U. Choughuley and Richard M. Lemmon

It is now well established that most of the simple, biologically important "monomers" (such as amino acids, pentoses, purines, and fatty acids) are formed when various high-energy sources are applied to samples of the primitive Earth's atmosphere.[1,2] This atmosphere is assumed to consist mainly of $CH_4$, $H_2O$, $NH_3$, $N_2$, and $H_2$ (the last decreasing rapidly with increased age of the Earth).[3] Although the amino acids are among the most prominent products of the irradiations of "primitive-Earth atmospheres," no report has yet appeared in the literature of the appearance of the sulfur-containing amino acids. This has presented no problem, since it can be safely assumed that the primitive atmosphere contained at least traces of $H_2S$. However, it is always good to have laboratory experimentation to back up assumptions.

Primitive-Earth atmospheres, in the presence of added $H_2S$, have been studied by Heyns, Walter, and Meyer.[4] These authors passed a spark through the mixture and found essentially no effect of the $H_2S$ on the syntheses of amino acids (non-S-containing). They tentatively identified one S-containing product, ammonium thiocyanate. However, a search for S-containing amino acids (by the Stein and Moore method[5]) was unsuccessful, although the authors did report the presence on their chromatograms of six ninhydrin-positive, but unidentified, products. These amino acids may have been sulfur-containing, but their yield was too small to permit identification.

It seemed to us to be worthwhile to search for the "primitive-Earth" synthesis of S-containing amino acids by using $H_2{}^{35}S$ as one of the reactants, thus greatly increasing the detection sensitivity for an amino acid product. In this way we have been able to establish the appearance of cysteic acid, $HO_3SCH_2CH(NH_2)CO_2H$, and the closely related compounds taurine, $HO_3SCH_2CH_2NH_2$, and cystamine, $(SCH_2CH_2NH_2)_2$. Cysteine and cystine probably were formed (there was evidence for them in our early chromatograms), but they appeared to be oxidized to cysteic acid during subsequent chromatography. We found no evidence for methionine production.

In each of the three experiments reported here, 20 ml of 2 N $NH_4OH$, 200 mm of $CH_4$, and 100 mm of $H_2{}^{35}S$ (containing 5 mcurie) were placed in a Pyrex tube and irradiated, with an electron beam, in the manner previously described.[6] No air was present during the irradiations. The electron beam was used for no reason other than that it was a convenient source of ionizing radiation. The radiation dose in each case was about $10^9$ rads. This is a very high dose; however, our products, as they were formed, were removed from the electron beam (in the condensate dripping off a condenser--see Ref. 6) and thus protected from radiation decomposition. The $10^9$ rads was delivered during 45-min exposures to the electron beam.

After the addition of chromatographic quantities of unlabeled carriers (cystine, taurine, etc.), the nonvolatile contents and washings from the irradiation chamber were transferred to a round-bottom flask and evaporated to a small volume at room temperature. Some polymeric material (in part, elementary sulfur) was removed by centrifugation. An aliquot portion (usually 0.1) of the concentrated solution was then placed on Whatman No. 1 chromatographic paper. The solvent in the first direction was n-butanol:acetic acid:water (100:22:50, v/v/v) and, in the second, isopropanol:methanol:water (7:1:2, v/v/v). Positions and shapes of radioactive spots were determined by autoradiography (Kodak "Blue Sensitive" medical x-ray film). Radioactive spots with $R_f$ values similar to those of added carrier compounds were cut out, eluted, and rechromatographed in the following solvent systems: (a) sec. butanol:formic acid (88-90%):water (15:3:2, v/v/v); (b) sec. butanol:tert. butanol:water (43:48.4:8.6, v/v/v). On the second chromatograms the positions and shapes of the radioactive spots were compared to those of the colored spots revealed by spraying the chromatogram with ninhydrin solution. The amount of radioactivity in a given spot was determined either (a) directly on the paper by means of a G-M counter, or (b) after elution, by liquid scintillation counting. This radioactivity figure could then be compared with the total amount of $^{35}S$ (as $H_2{}^{35}S$) that was originally present in the irradiation flask. In this way, the yields of products from the $H_2S$ could be determined (due allowance being made for the half-life of $^{35}S$).

The following $^{35}S$-labeled products (yield from $H_2{}^{35}S$ given parenthetically) were positively identified: cysteic acid (0.01%), cystamine (0.003%), and taurine (0.01%). We expected to find, and therefore intensively searched for, cysteine, cystine, and

methionine; however, if these amino acids are formed, their yields must be well below 0.001%. As was mentioned, cysteine and cystine are probably formed but then disappear by oxidation to cysteic acid. Other compounds for which we searched, but could find no evidence of their appearance, were homocysteine and homocysteic acid.

It is pleasing to be able to record that at least one of the S-containing α-amino acids (cysteic acid) is formed under "primitive-Earth conditions." We believe that others are also formed (for example, the cysteic acid is probably formed by attack of the • OH radical on cysteine). Probably, small changes in the conditions of the "primitive-Earth" experiments (e.g., longer times of irradiation at lower dose rates, presence of catalytic surfaces) will lead to the identification of all the biologically important S-containing amino acids.

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# 14. THE MECHANISM AND PROTOBIOCHEMICAL RELEVANCE OF DICYANAMIDE-MEDIATED PEPTIDE SYNTHESIS

Gary Steinman, Dean H. Kenyon, and Melvin Calvin


## Summary

The mechanism by which dicyanamide (DCA) promotes the synthesis of diglycine from glycine in aqueous solution has been investigated in detail using carbon-14 and tritium-labeled compounds. It was found that protonation of both dicyanamide anion and the glycine carboxylate group are required for dipeptide synthesis. Peptide bond formation is apparently initiated by reaction of the glycine carboxyl group with dicyanamide to form an unstable intermediate which undergoes nucleophilic attack by the amino group of a second glycine molecule, resulting in the production of diglycine and cyanurea. N-cyano-N'-glycyl-urea occurs in the reaction mixture as a prominent side product, suggesting that DCA induces peptide bond formation via an intermediate similar to that postulated in the case of the dialkylcarbodiimides.

The limiting factor in DCA-mediated diglycine synthesis is the amine-catalyzed dimerization of DCA to N, N, N'-tricyanoguanidine, a compound inactive in peptide bond formation. The primary intermediate involved in this reaction is the amine adduct of glycine and dicyanamide--i. e., N-cyano-N'-carboxymethylguanidine.

Enhanced yields of diglycine, as well as appreciable yields of triglycine and tetraglycine, were obtained by slowly adding DCA to reaction mixtures initially containing glycine and HCl.

The possible role of DCA-mediated polypeptide synthesis in prebiological chemical evolution is discussed.


## Introduction

In recent years it has been demonstrated in a number of laboratories that the major types of biochemical monomers can be produced under a variety of simulated primitive-Earth conditions. These monomers include amino acids,[1-8] sugars,[9] purines,[5,10,11] pyrimidines,[12] fatty acids,[1,5,13,14] and the like. Nonenzymatic methods for the condensation of some of these monomers to high-molecular-weight polymers under what, in each case, were presumed to be primitive-Earth conditions have been reported, but special reaction conditions involving the absence or deficiency of water are usually required. For example, polypeptides have been produced from anhydrous, molten mixtures of free amino acids.[15,16] Polypeptides have also been produced from free amino acids with the aid of polymetaphosphate esters in nonaqueous media.[17] Although the special conditions required for these syntheses may have occurred to some extent on the primeval, sterile Earth, a mode of synthesis which could operate in dilute aqueous media would be more consistent with the notion that much of prebiological chemical evolution took place in the primitive oceans,[18,19] and with the related fact that contemporary metabolism occurs in the presence of water.

Since dehydration condensation is the characteristic reaction occurring in the formation of inter-residue linkages in all classes of biopolymer[20] (with the exception of the melanic pigments[21]), the thermodynamic problem encountered in dilute aqueous solution is apparent. In the context of chemical evolution, one solution of this problem would be provided by a simple condensing agent which could reasonably be expected to have been present in the primitive oceans and which could supply the necessary free energy for dehydration condensation. The appearance of nitriles in simulated primitive atmosphere experiments,[22] the relative resistance of carbon-nitrogen multiple bonds to hydrolysis, the synthesis of peptides from free amino acids in heated aqueous ammonium cyanide solutions,[5] and the central role of carbon-ntirogen multiple bonds in dialkylcarbodiimide-mediated peptide bond formation[23] suggestedthat HCN, or some simple derivative of HCN, might have acted as an effective condensing agent during chemical evolution.[24] Cyanamide seemed to be a likely HCN derivative for initial investigation, since it can tautomerize to carbodiimide (HN=C=NH), the parent compound of the dialkylcarbodiimides. Since cyanamide readily dimerizes in aqueous solution to form dicyandiamide (DCDA),[25] condensation experiments were performed with the latter compound. That this compound could have been present on the primitive Earth was demonstrated with electron-beam irradiation of methane, ammonia, and water vapor, and by ultraviolet irradiation of aqueous ammonium cyanide.[26] It was shown the DCDA does promote a variety of dehydration condensations, including the coupling of amino acids to peptides.[27,28] However, the maximum yields of condensed compounds were typically of the order of 1%.

In an attempt to find other simple cyanogen compounds, which might act as more effective condensing agents than DCDA, the use of dicyanamide (NC-NH-CN) was considered, since this compound can take up 2 moles of water, resulting in the formation of biuret,[29] while DCDA takes up only 1 mole of water to form guanylurea.[30] It was shown that dicyanamide is considerably more effective than DCDA in promoting the condensation of free alanine to dialanine.[30] In a system initially containing glycine (0.12 M), triglycine (0.12 M), sodium dicyanamide (0.12 M) (the sodium salt of dicyanamide was used, since free dicyanamide is not readily available), and HCl (0.12 M), both diglycine and tetraglycine were detected, in yields of 2.1% and 6.3%, respectively.[30] These observations suggested that dicyanamide might have played a role in the formation of polypeptides in the prebiotic hydrosphere, and prompted the present detailed investigation of the mechanism of diglycine synthesis in the model system consisting of glycine, dicyanamide, and HCl.

## Materials

Glycine-2-$^{14}$C (specific activity 11.8 μcurie/mg), and glycine-1-$^{14}$C (27 μcurie/mg) were prepared by the methods of Kleerup and Adams.[31-34] Both compounds were purified by paper chromatography. Tritium-labeled glycine (specific activity, 2.7 mcurie/mg) was obtained from New England Nuclear Corp. Sodium dicyanamide (unlabeled) was obtained from Eastman Organic Chemicals (analysis: carbon, 26.94%; nitrogen, 47.4%), and used without further purification. Carbon-14-labeled sodium dicyanamide (specific activity, 31.8 μcurie/mg) was prepared from $Na_2N^{14}CN$ by the method of Madelung and Kern[35] and purified by paper chromatography. The $Na_2N^{14}CN$ was synthesized from $BaN^{14}CN$ (Volk Radiochemical Co.) by treatment with $Na_2SO_4$. Unlabeled diglycine, triglycine, and tetraglycine used as electrophoretic and paper chromatographic standards were obtained from Mann Research Laboratories, Inc., and used without further purification.

## Methods

### Reaction conditions

Reactions were run in stoppered Pyrex vessels at room temperature. The total volume of the solutions was 0.1 to 1.0 ml. In separate experiments the initial concentrations of glycine, sodium dicyanamide (NaDCA), and HCl were varied independently from 0.01 M to 0.2 M. The total amount of radioactivity in a given solution was 0.5 to 5 μcurie.

### Analytical methods

The low-voltage horizontal paper electrophoretic method of Carnegie and Synge[36] was the principal analytical procedure for separating the products of the reaction, although in some cases paper chromatography was also used. Radioactive products were located by means of x-ray film autoradiography,[37] with exposure times of 24 to 48 hr, which permitted the detection of yields ≥0.001%. Glycine peptides were identified by coincidence of the suspected radioactive bands with the ninhydrin color spot for standard, unlabeled peptide added to the reaction mixture before electrophoresis. The identifications were confirmed by elution of the appropriate radioactive bands from paper strips containing no added standard compound, followed by two-dimensional cochromatography of the eluted material with standard carrier peptide. Further confirmation was obtained by paper-electrophoretically demonstrating recovery of radioactive glycine after hydrolysis of the eluted materials with 6 N HCl at 100° C for 10 hr. Yields of radioactive products were determined by counting the activity associated with each band and comparing these activities with the amount of radioactive glycine initially present. Special methods used for the identification of products derived from dicyanamide are described in detail below.

## Results and Discussion

The autoradiogram reproduced in Fig. 14-1 shows the electrophoretic distribution of radioactive products obtained from a system initially containing glycine-2-$^{14}$C (0.1 M), sodium dicyanamide (unlabeled, 0.1 M), and HCl (0.14 N) after a reaction time of 10 min. The corresponding autoradiogram for a system containing no NaDCA is included for comparison. It is apparent that dicyanamide is required for diglycine synthesis.

### Dependence of diglycine yield on pH

Since it is known that dicyandiamide (DCDA) most effectively promotes the formation of peptide bonds at pH ≤ 2,[28] the first step in the investigation of dicyanamide-mediated dipeptide synthesis consisted of determining the yield of diglycine as a function of the initial pH of the reaction mixture after a reaction time of 10 min. (This reaction time was chosen as a basis for comparison, since preliminary observations indicated that the reaction stopped within 10 min.) The results for a system in which the initial concentrations of glycine and NaDCA are 0.01 M are shown in Fig. 14-2. The sharply increasing yield of diglycine with

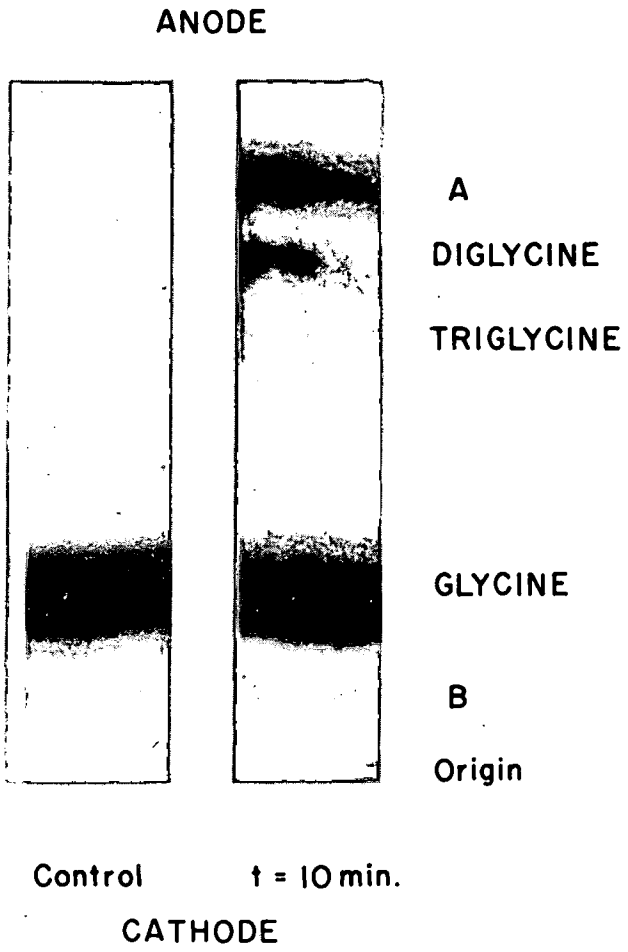



Fig. 14-1. Autoradiogram of an electrophoretic separation of products obtained after 10 min for a reaction mixture initially containing glycine-2-$^{14}$C (0.1 M), sodium dicyanamide (unlabeled, 0.1 M), and HCl (0.14 N). The corresponding "control" autoradiogram for a solution lacking NaDCA is included for comparison. The separation was carried out on Whatman No. 1 paper impregnated with borate buffer at pH 9.2, using a voltage gradient of 6 volts/cm and a running time of 8.5 hr. Compounds A and B are N-cyano-N'-carboxymethylguanidine and N-cyano-N'-glycylurea, respectively. For evidence of these identifications, see details in the text.

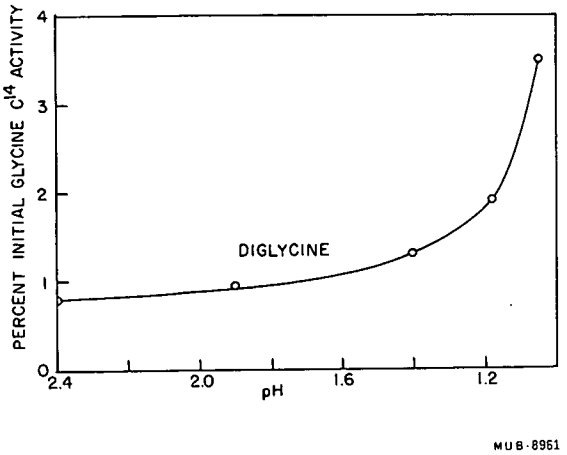



Fig. 14-2. Yield of diglycine versus pH of the initial reaction mixture for solutions initially containing glycine-2-$^{14}$C (0.01 M), NaDCA (0.01 M), and enough HCl to obtain the appropriate pH. Solutions were analyzed by paper electrophoresis (conditions given in Fig. 14-1) after a reaction time of 10 min.

g pH suggests that protonation of yanamide anion (NC-N-CN) or the rboxylate group (or both) is required de synthesis. Since the glycine carboxyl group has a $pK_a$ value of 2.34,[38] whereas the $pK_a$ value for dicyanamide is 0.8 (determined by spectrophotmetric titration[39] with HCl at 211 mμ), it is likely that the sharp increase in diglycine yield beginning at about pH 1.2 reflects the necessary protonation of dicyanamide anion. Evidence that protonation of the glycine carboxylate group is also required for diglycine synthesis was obtained in the following experiment: Two pairs of reaction mixtures were prepared, each containing glycine (0.01 M), diglycine (0.01 M), and NaDCA (0.1 M). The HCl concentration of one of the two solutions in each pair was such that the pH was 1.4, while for the other solution in each pair the HCl concentration was such that the pH was 2.6. These two pH's were selected, since calculation using the known $pK_a$ values of glycine ($pK_a$ = 2.34) and diglycine ($pK_a$ = 3.06) showed that although at pH 1.4 both compounds are nearly completely protonated, at pH 2.6 there is a marked difference in the degree of protonation. The first pair of

solutions contained radioactive glycine whereas the second pair contained radioactive diglycine (prepared from glycine-2-$^{14}$C by means of NaDCA). After 5 min of reaction the solutions were analyzed by paper electrophoresis and the yields of labeled diglycine and tetraglycine determined. The results are summarized in Table 14-I. It is apparent that the percent changes in diglycine and tetraglycine yields at the two pH values parallel the percent differences in degrees of protonation of glycine and diglycine.

Fate of dicyanamide

The rate of diglycine accumulation is shown in Fig. 14-3 for a system initially containing glycine (0.01 M), NaDCA (0.03 M), and HCl (0.1 N). The results show that diglycine synthesis stops within 3 min of initiation of the reaction. At the peak of the diglycine curve the concentration of glycine has fallen to 95% of its initial value and undergoes no further change. If the initial concentration of NaDCA is 0.1 M, dipeptide synthesis stops within 1 min. These data suggest that dicyanamide is rapidly converted to a compound inactive in peptide bond formation, and probably by a process that is higher than first order in DCA.

In a separate experiment it was shown that a flocculent, presumably polymeric[35] precipitate is formed in aqueous solutions of NaDCA containing HCl at concentrations equal to or greater than 0.3 N. It is also known that solutions of free dicyanamide give rise to an insoluble polymeride upon evaporation of the solvent.[35] Therefore it seemed plausible that the explanation for the rapid shutting off of dipeptide synthesis represented in Fig. 14-3 might be in the conversion of dicyanamide to some type of inactive polymer.

If polymerization were the primary fate of the dicyanamide, one would expect to see a larger decrease of solute molality than would correspond to the peptide reaction alone. In order to test this hypothesis, vapor pressure osmometry was used to measure changes in the total solute molality during dipeptide synthesis. An aqueous solution was prepared containing NaDCA (0.4 M) and HCl (0.1 N). Aliquots were withdrawn at various times following mixing and were neutralized immediately with NaOH. The total solute molalities of the samples were measured with a Mekro Labs vapor pressure osmometer. This procedure was repeated with glycine (0.04 M) in the reaction mixture.

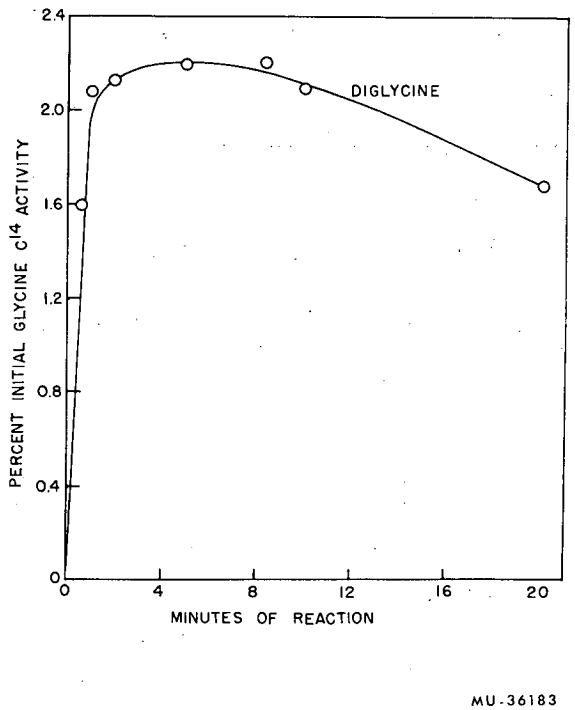


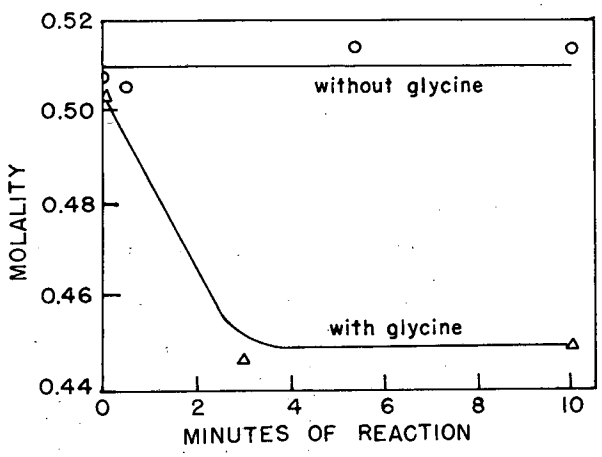

Fig. 14-3. Rate of diglycine accumulation in a solution initially containing glycine-2-$^{14}$C (0.01 M), NaDCA (0.03 M), and HCl (0.1 N). Aliquots were withdrawn from the reaction mixture and subjected to electrophoresis (see Fig. 14-1) followed by x-ray-film autoradiography. Yields were determined by comparing the radioactivity in the diglycine bands with the initial glycine activity.

In order to show the contribution which reactions of dicyanamide make to the observed changes in solute molality, the experimental molality values for the system lacking glycine were corrected for the absent amino acid and for the amount of diglycine known to be produced under the given conditions. Also, the diglycine correction was applied to the t = 0 value obtained for the system containing glycine. The results are shown in Fig. 14-4. The experiment was repeated several times with nearly identical results. Because of the large molar excess of DCA over glycine it can be shown that the magnitude of the observed molality drop cannot be accounted for by assuming that all of the glycine was

Table 14-I. Relative yields of diglycine and tetraglycine at pH 1.4 and 2.6.

| Reactants | Solution | pH | Peptide yields | Percent change in yields | % Change in degree of protonation of glycine and diglycine* |
|---|---|---|---|---|---|
| | | | Diglycine | | Glycine |
| Glycine-2-$^{14}$C, Diglycine, and NaDCA | 1a | 1.4 | 8.9% | 57 | 61.1 |
| | 1b | 2.6 | 3.8% | | |
| | | | Tetraglycine | | Diglycine |
| Glycine, Diglycine-$^{14}$C, and NaDCA | 2a | 1.4 | 13.9% | 29.6 | 24.5 |
| | 2b | 2.6 | 9.8% | | |

*Calculated using the following $pK_a$ values: Glycine, 2.34; diglycine, 3.06 (Ref. 34).




MU-36466

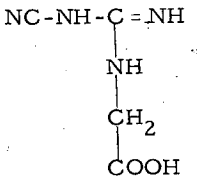
Fig. 14-4. Molality change due to reactions of dicyanamide for a solution initially containing glycine (0.04 M), NaDCA (0.4 M), and HCl (0.1 N) (lower curve). The upper curve corresponds to a similar solution lacking glycine. Total solute molalities were determined by vapor pressure osmometry (see text). The experimental values for the system lacking glycine were corrected for the absent amino acid and for the amount of diglycine which would have been produced under the given conditions had the glycine been present. The diglycine correction was also applied to the t=0 value for the glycine-containing solution.

incorporated into an addition product with dicyanamide. If this were the case, the decrease in solute molality would be only about 7%. However, if one-half of the DCA dimerized, the molality would decrease by about 20%. The change in molality due to peptide synthesis is less than 0.5%. The actual drop is about 13%. If the initial concentration of glycine is increased by a factor of two, the magnitude of the observed effect remains the same. These facts suggested that glycine acted as a catalyst for the polymerization of dicyanamide.

In order to determine whether the observed molality drop was due to the amino group or the carboxyl group of glycine, a series of experiments using model compounds was carried out. When either formic acid or acetic acid was substituted for glycine in the experiment described above, no change in molality was observed in the first 10 min after mixing of the reactants. On the other hand, when methylamine replaced the glycine, the same molality drop was observed as had been observed when the amino acid was present.

One way in which a compound containing an amino group might induce this type of response would be to form an amine adduct with dicyanamide. The resulting addition compound could undergo nucleophilic displacement by another DCA molecule (DCA anion), releasing the amine. Identification of compound A (see Fig. 14-1) by the methods described below showed that the adduct of glycine and dicyanamide is, in fact, formed during DCA-mediated peptide synthesis. In a diglycine synthesis experiment using $^{3}$H-labeled glycine and $^{14}$C-labeled NaDCA, it was shown by scintillation counting that in compound A the molar ratio of the dicyanamide and glycine moieties is 1:1. Several milligrams of A were isolated and purified

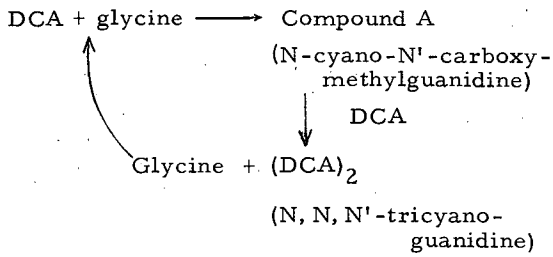
by means of preparative paper chromatography with Whatman 3MM paper followed by recrystallization from 95% ethanol. Analysis of the material by infrared and ultraviolet spectrophotometry revealed the presence of the following groups: carboxylate (1610 $cm^{-1}$), imine (1650 $cm^{-1}$), carboxyl (1700 $cm^{-1}$), cyano (2180 $cm^{-1}$), amino (3160, 3280, 3345, and 3440 $cm^{-1}$), and cyanoguanidino ($\lambda_{max}$ = 217.5 mμ). Hydrogenation of the compound by means of metallic sodium and ethanol resulted in the production of guanidoacetic acid, as determined by cochromatography with an authentic sample of this substance. From these results it was concluded that compound A is N-cyano-N'-carboxymethyl-guanidine,




Compound A.

Analogous amino addition compounds are known in cyanamide[40] and carbodiimide[41] chemistry.

That compound A is probably the primary intermediate in glycine-catalyzed DCA polymerization was shown in the following way: Compound A was prepared from $^{14}C$-labeled NaDCA and $^{3}H$-labeled glycine, and isolated by paper electrophoresis. The double-labeled material was then added to an aqueous solution of unlabeled NaDCA and HCl. Paper chromatographic analysis of the resulting reaction mixture revealed the presence of free labeled glycine and one additional (unknown) radioactive compound. This compound was prepared by paper chromatography followed by elution and lyophilization from a reaction mixture containing $^{14}C$-labeled NaDCA, unlabeled glycine, and HCl. (More than 80% of the NaDCA was converted to the unknown compound within 5 min.) Analysis of the material by infrared spectrophotometry revealed the presence of the following groups: secondary and tertiary amine (1345 and 1385 $cm^{-1}$), imine (1645 $cm^{-1}$), and cyano (2180, 2230, and 2300 $cm^{-1}$). Quantitative comparison of the ir spectra of the unknown compound and cyanoguanidine--i.e., DCDA, NC-NH-C(=NH)-NH2)--showed that the ratio of cyano groups (2230 $cm^{-1}$) to imine groups (1645-1650 $cm^{-1}$) in the unknown is 3:1. Partial hydrogenation (Na and ethanol) of the compound resulted in the production of cyanoguanidine. From these data it was concluded that the unknown compound is the dimer of DCA--i.e., N, N, N'-tricyanoguanidine (NC-NH-C(=NH)-N(CN)-CN). Therefore, the limiting factor in dicyanamide-mediated diglycine synthesis is apparently the rapid, glycine-catalyzed dimerization of DCA, as shown below:




Identification and role of urea byproducts

It has been postulated that the intermediate involved in peptide bond formation using dialkylcarbodiimides is an acylisourea of the form R-NH-C(=O)-NH-R. [42] Such a compound, R'-C(=O)-O, however, is highly unstable and has not been isolated except in one very special case.[43] Its occurrence is inferred from kinetic data and from the appearance in the reaction mixture of both the urea of the carbodiimide, R-NH-C(=O)-NH-R (as a byproduct of peptide synthesis), and an acylurea of the form R-NH-C(=O)-NR-C(=O)-R' resulting from the rearrangement of the proposed intermediate.[23] If the hypothesis is correct that dicyanamide promotes diglycine synthesis by a mechanism similar to that of the dialkylcarbodiimides, then both the urea and the acylurea of dicyanamide should occur as reaction products. This prediction was confirmed by the identification of compound B (see Fig. 14-1) as N-cyano-N'-glycylurea (NC-NH-C(=O)-NH-C(=O)-$CH_2NH_2$) and by the identification of cyanurea-$^{14}C$ (NC-NH-C(=O)-$NH_2$) in reaction mixtures containing NaDCA-$^{14}C$.

A sample of a reaction mixture containing glycine-2-$^{14}C$, NaDCA, and HCl was subjected to electrophoresis in one dimension and paper chromatography in the second direction, along with an authentic sample of N-cyano-N'-glycylurea, which was synthesized from cyanurea and N-carbobenzoxyglycine-p-nitrophenyl ester. Using x-ray-film autoradiography to locate the $^{14}C$-labeled compound, and nitroprusside reagent to locate the unlabeled standard compound, exact coincidence of the unknown and standard spots was observed. Next, a sample of a reaction mixture containing NaDCA-$^{14}C$ was cochromatographed in one dimension with an authentic sample of cyanurea

prepared from cyanamide and potassium cyanate.[44] The $R_f$ value of one of the unknown radioactive products matched that of cyanurea (located by means of p-dimethylaminobenzaldehyde[45]). Cyanurea was shown to be inactive in peptide bond formation in acid medium. Confirmation of the structures of the two standard compounds was obtained by means of ir spectrophotometry and elemental analysis, and, in the case of N-cyano-N'-glycylurea, by paper chromatographic demonstration of glycinamide as a product of hydrolysis in 2 N HCl at 95° C for 3 hr. In the case of the cyanurea standard, it was shown by paper chromatography that biuret is produced in a solution of the compound subjected to 6 N HCl at 100° C for 10 min. The major reactions occurring during diglycine synthesis in aqueous solutions of sodium dicyanamide, glycine, and HCl are summarized in Fig. 14-5.

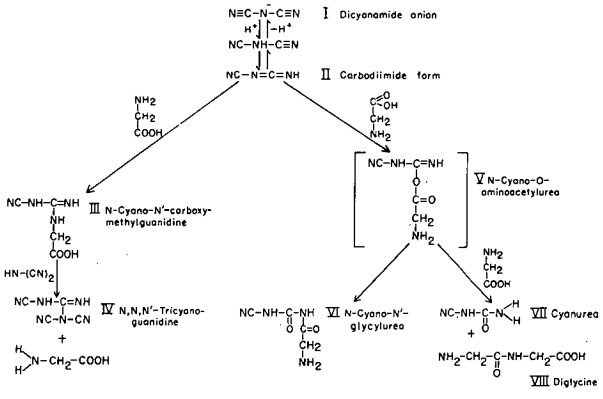



Fig. 14-5. Summary of the reactions occurring during diglycine synthesis in aqueous solutions of glycine, sodium dicyanamide, and HCl. Full details are given in the text.

## Enhancement of yields of glycine peptides

Since the limiting factor in dicyanamide-mediated peptide synthesis is the amine-catalyzed dimerization of dicyanamide, it seemed plausible that the yields of peptides could be increased by slowly adding the condensing agent to the reaction mixture, rather than all at once. This procedure would minimize the amount of DCA available for dimerization (higher than first order), thus favoring the condensation reaction (first order in DCA). In a preliminary experiment two solutions were prepared, each containing glycine-2-$^{14}$C (0.01 M) and HCl (0.1 N). Enough NaDCA was added to one of the solutions to bring its concentration to 0.1 M. The same amount of NaDCA was added to the second solution in two equal aliquots, one at t = 0 and the other at t = 5 min. After 10 min total reaction time the products were separated by electrophoresis. The resultant yields of diglycine were 13.9% for the first solution and 29.9% for the second, thus confirming the expected enhancement.

In an attempt to achieve complete utilization of glycine and to produce higher glycine peptides, the following experiment was performed: A 3-ml solution containing glycine-2-$^{14}$C (0.005 M) and HCl (0.1 N) was prepared. A 30-fold molar excess of sodium dicyanamide was added to the reaction mixture by means of a dropping buret at the rate of 0.68 mg/min over a period of 1 hr. Samples, 0.1 ml each, were withdrawn at 5-min intervals and analyzed by paper electrophoresis. The resulting electropherograms revealed the presence of triglycine and tetraglycine in addition to diglycine. The rates of glycine utilization and peptide accumulation are shown in Fig. 14-6.

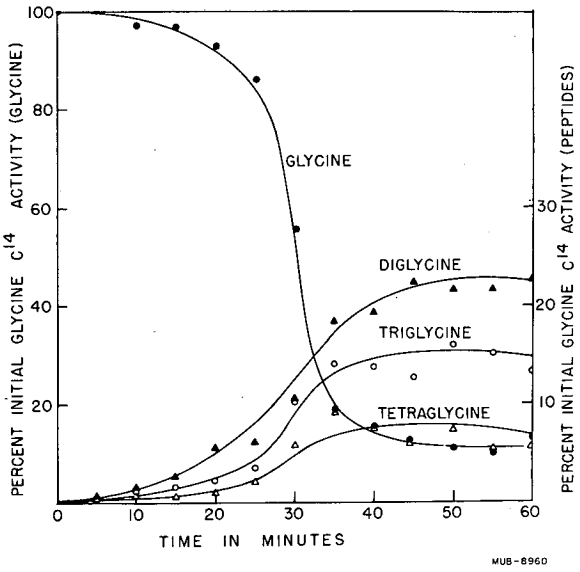



Fig. 14-6. Rates of glycine utilization and polypeptide accumulation in a solution initially containing glycine-2-$^{14}$C (0.005 M) and HCl (0.1 N). Sodium dicyanamide (in 0.1 M aqueous solution) was added to the reaction mixture by means of a dropping buret at the rate of 0.68 mg/min for 1 hr. Samples were withdrawn periodically and analyzed by paper electrophoresis (conditions given in Fig. 14-1).

It is apparent that most of the glycine has reacted within 40 min and that nearly one-half of the initial glycine radioactivity has been incorporated into glycine peptides. These results were confirmed in a similar experiment using glycine-1-$^{14}C$.

## Possible role of dicyanamide-mediated polypeptide synthesis in prebiological chemical evolution

In view of the fundamental and ubiquitous roles of proteins in contemporary metabolism, it is plausible that an accumulation of substantial amounts of polypeptides on the prebiotic earth was required for the subsequent development of living systems. The accumulation of small polypeptides in aqueous glycine solutions continuously supplied with fresh NaDCA, as shown in Fig. 14-6, together with the plausible assumption that DCA and related cyanogen compounds were continuously produced in the primitive atmosphere,[26] suggest that DCA-mediated polypeptide synthesis may have been a key process by which polypeptides were produced in the primitive hydrosphere. It is likely that if, in addition to NaDCA, fresh amino acid were also supplied to the reaction mixture in a continuous way, larger polypeptides would have been produced. If different amino acids were supplied, DCA-mediated copolymerization could lead to the production of a great variety of polypeptides differing in both composition and sequence. Preliminary experiments in our Laboratory have demonstrated that DCA does promote the synthesis of mixed peptides in solutions containing two different amino acids, with some degree of selectivity.[46]

Since DCA-mediated peptide synthesis occurs most efficiently at pH $\leq 2$ (see Fig. 14-2), the question might be raised whether such acidic conditions are likely to have occurred to any significant extent in the primitive hydrosphere. Although the average acidity of the primitive oceans is difficult to estimate, the question nevertheless deserves careful consideration in view of the suggested abundance of ammonia in the primitive atmosphere.[47] Evidence that gases were present in the primitive atmosphere which would have tended to depress the pH of the hydrosphere is provided by the fact that volcanic exhalations (which reflect to some extent the composition of the primitive atmosphere) contain appreciable amounts of juvenile HCl, HF, $H_2S$, and the like,[48] although the quantities of these gases in the primitive atmosphere cannot be estimated in the present state of knowledge. At any rate, repetition of the dipeptide synthesis experiment with a system containing NaDCA (0.1 M) and glycine (0.01 M) at neutral pH resulted in the production of an easily detectable yield of diglycine (i.e., 1%) after 10 min of reaction. Therefore, it is possible that, given enough time, substantial yields of peptides can be obtained, even under neutral conditions.

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## 15. SOME ANALYTICAL RESULTS IN ORGANIC GEOCHEMISTRY

Theodore Belsky, Eugene D. McCarthy, and William Van Hoeven

We have felt that isoprenoid alkanes could serve as "Biological Markers" in our quest for evidence of life among geologically well-characterized ancient shales and oils. Preliminary studies on the oil shale from the Green River formation (Eocene Age) have been reported earlier.[1,2] We have now isolated and identified the $C_{27}$-, $C_{28}$-, and $C_{29}$-steranes, and a $C_{30}$-pentacyclic triterpane from the branched cyclic alkane fraction of the Green River Shale. An analogous investigation of the branched cyclic alkane fraction from the Soudan Shale (2.7 billion years old) yielded a fraction containing the $C_{27}$-, $C_{28}$-, and $C_{29}$-steranes. The occurrence of steranes (and probably pentacyclic triterpanes) in the Soudan Shale provides further evidence for the presence of life processes sufficiently complex to require an enzymatic template and in vivo polyisoprenoid cyclizations at the 2-billion-year mark in terrestrial chronology.

We have commenced an analysis of the Pierre Shale (Late Cretaceous Period); the organic extract from the shale is as high as 8%. This shale is of considerable interest because, although it was laid down under marine conditions (as evidenced by the fossil record), it is thought to contain terrestrial detritus. A preliminary analysis of the hydrocarbon distribution pattern reveals a bimodal distribution pattern not unlike that of the Green River Shale, a fresh water deposition. From a 100-gram sample we obtain approximately 36 mg of saturated alkanes. The normals extend from $C_{13}$ to $C_{31}$ with maxima at $C_{17}$ and again at $C_{25}$. The branched cyclic alkane fraction also shows a bimodal distribution pattern with the first maximum at the $C_{19}$ isoprenoid alkane region, and the second maximum corresponding approximately to the retention time of cholestane. Outcrops of this shale are readily available at various places in the midwestern states, and it will prove of considerable interest to observe how the hydrocarbon pattern varies with marine environment. A clear understanding of the diagenetic processes responsible for the hydrocarbons obtained is essential if we are to have any criteria in extending our research to meteoritic and lunar samples.

We have also started an analysis of modern organic deposits from Mud Lake, Florida. These are post-Pleistocene to Recent organic analogs of the rich oil shale from the Green River formation. A preliminary analysis of the alkanes reveals the dominance of the normal $C_{25}$, $C_{27}$, $C_{29}$, and $C_{31}$ hydrocarbons. There is a close resemblance of the three normal hydrocarbons, $C_{27}$, $C_{29}$, $C_{31}$, to the sequence as it appeared in the purified normal hydrocarbons of the Green River. The absence of normal $C_{17}$ from the Florida Mud, together with its presence in large amounts in the Green River, presents a difference not clearly understood. Another difference is the absence of phytane and pristane from the Florida Mud, and their presence in large amounts in the Green River. This would be in accord with our postulate that chlorophyll is the precursor of the phytane and pristane; in the Florida Mud the phytane--i.e., phytyl group--is still bound as part of the intact chlorophyll molecule.

There is also good evidence for the presence of carotenoids and porphyrins in the Florida Mud. In particular, we have isolated a pigment whose visible spectrum is virtually identical with that of pheophytin a. Another pigment has been isolated whose visible spectra closely resemble those of β-carotene (the spectral shifts in the solvents heptane, benzene, and $CS_2$ correspond to those of pure β-carotene). The exact chemical state of these compounds will be of considerable interest in elucidating the diagenetic processes that take place in sediments.

Finally, an attempt has been made to separate the optical isomers of pristane on a GLC instrument (capillary column: 150 ft×0.01 in.) with a dihydrophytol adipate substrate. This might be of considerable significance in ascertaining biogenic origin of the isoprenoid alkanes. However, no separation was observed. At the present time, various other optically active substrates are being considered.

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## 16. STUDY OF THE RING DEGRADATION OF TOLUENE-1-$^{14}$C

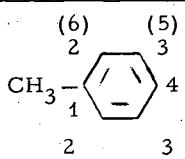
Tz-Hong Lin and Richard M. Lemmon

We have been studying the chemical degradation of toluene-1-$^{14}$C in order to degrade the labeled toluene obtained from the reaction of accelerated $^{14}$C ions with benzene. The degradation was carried out according to the procedure used by Moore and Wolf for the degradation of ethylbenzene. The results are shown in Table I.

acid in 50% sulfuric acid gives an almost-quantitative yield of acetic acid. Acetic acid is further degraded by a Schmidt reaction to $CO_2$ ($C_1$) and methylamine (methyl carbon), which are converted to phenethylammonium phenethylcarbamate and N-methyl-N'-phenylthiourea, respectively. The results from a test degradation of toluene-1-$^{14}$C are shown in Table II.

Table I. Degradation of toluene-1-$^{14}$C.

| Active compounds | Carbon atom[a] in toluene | Activity[b] dpm/μmole |
|---|---|---|
| Toluene-1-$^{14}$C | $C_{Me}$, $C_{1-6}$ | 6.66 ± 0.07 |
| 1-Methyl-1-cyclohexene-1-$^{14}$C | $C_{Me}$, $C_{1-6}$ | 6.51 ± 0.07 |
| 6-Ketoheptanoic acid-6-$^{14}$C semicarbazone | $C_{Me}$, $C_{1-6}$ | 6.55 ± 0.07 |
| Heptanoic acid-6-$^{14}$C | $C_{Me}$, $C_{1-6}$ | 6.66 ± 0.07 |
| Neopentyl-α-bromoheptanoate-6-$^{14}$C | $C_{Me}$, $C_{1-6}$ | 6.50 ± 0.08 |
| Neopentyl heptenoate-6-$^{14}$C | $C_{Me}$, $C_{1-6}$ | 7.68 (impure) |
| Baleric acid-4-$^{14}$C | $C_{Me}$, $C_{1-6}$ | 6.44 ± 0.06 |
| N-n-Butyl-3-$^{14}$C-N'-phenylthiourea | $C_{Me}$, $C_{1-3}$ | 6.57 ± 0.07 |
| Phenethylammonium phenethylcarbamate | $C_4$ | 0 |
| Acetic acid | $C_2$, $C_3$ | 0 |

a. Toluene is numbered here as 


b. Above background

Our previous work showed that for the degradation of a toluene sample, a total activity of $10^6$ dpm would be needed if the method of Steinberg and Sixma were used.[1] Less activity (about $3 \times 10^5$ dpm) is required in the Moore and Wolf method.

We are investigating a modification of the Moore and Wolf procedure as represented in Fig. 16-1.

The methyl carbon on toluene could be removed via 6-ketoheptanoic acid, which can be converted to its oxime. Subsequent Beckmann rearrangement[2] of the oxime gives 5-acetamidovaleric acid, m.p. 53-4°, in very high yield. The hydrolysis of 5-acetamidovaleric

Further degradations can be carried out from the 5-aminovaleric acid; this can easily by oxidized to glutaric acid in ≈70% yield. The glutaric acid could be converted to 1,3-propanediamine via the Schmidt reaction, or to the malonic acid by a Barbier-Wieland degradation. The production of the diamine seems unsatisfactory, since all efforts to oxidize 1,3-propanediamine to malonic acid, to β-alanine, or even to acetic acid failed. Although this amine could be oxidized to a mixture of acetone (10% yield) and propionaldehyde (3% yield) by treatment with nitrous acid,[3] the poor yield and possible rearrangement of the carbons prevent our adopting this sequence. The second method (Barbier-Wieland degradation) appears promising and is being investigated.

Table II. Degradation of toluene-1-$^{14}C$.[a]

| Active compounds | Carbon atom in toluene | Activity[c] dpm/μmole |
|---|---|---|
| Toluene[b] | -- | -- |
| Methyl cyclohexene | $C_{Me}$, $C_{1-6}$ | 11.01 ± 0.11 |
| 6-Ketoheptanoic acid-6-$^{14}C$ oxime | $C_{Me}$, $C_{1-6}$ | 11.13 ± 0.11 |
| Acetic acid-1-$^{14}C$ (phenethylammonium-phenethylcarbamate) | $C_1$ | 10.55 ± 0.11 |
| Acetic acid (N-methyl-N'-phenylthiourea) | $C_{Me}$ | 0.26 ± 0.03 |
| Glutaric acid | $C_{2-4}$ | 0.36 ± 0.04 |

a. Since the sample used in Table I was used up, another batch of toluene-1-$^{14}C$ was used.

b. The starting toluene-$^{14}C$ was diluted with inactive methyl cyclohexene after the reduction.

c. Above background.

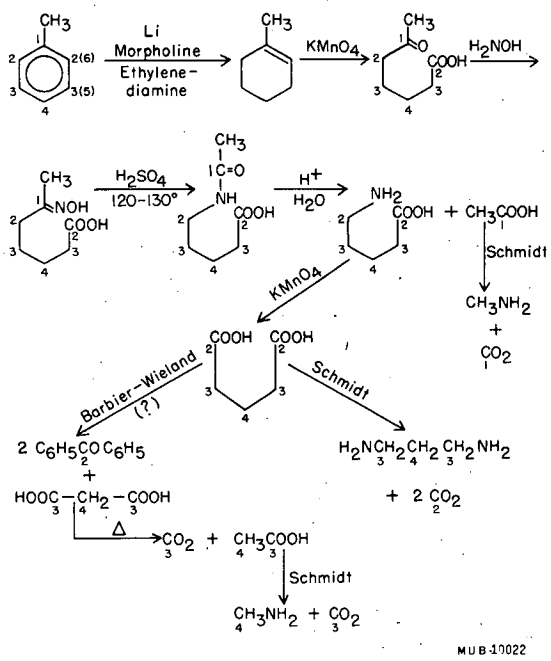



Fig. 16-1. Modification of Moore and Wolf procedure for chemical degradation of toluene-1-$^{14}C$.

A. Degradation via Wolf's Method

1-Methylcyclohexene-1-$^{14}C$. Twenty grams of toluene-1-$^{14}C$ (6.66 ± 0.07 dpm/μmole) was reduced with 6 g of Li in 300 ml of a mixture of ethylenediamine and morpholine (1:2 by volume); 18 g of an organic fraction was obtained. The gas-liquid chromatography (GLC) (on butanediol succinate; Craig) showed the presence of 65.4% of 1-methyl-1-cyclohexene. The yield of 1-methyl-1-cyclohexene was 56.5%. About 50 λ of pure compound was obtained by GLC and the activity counted. The specific activity was 6.51 ± 0.07 dpm/μmole.

6-Ketoheptanoic acid-6-$^{14}C$. Eleven g of the crude 1-methyl-1-cyclohexene-1-$^{14}C$ was oxidized with 44.5 g of $KMnO_4$ in 300 ml water. The keto acid was isolated as its semicarbazone; 7 g of semicarbazone (m.p. 143-45°, 41.8% yield) was obtained. Specific activity: 6.55 ± 0.07 dpm/μmole.

Anal. Calcd. for $C_8H_{15}N_3O_3$: C, 47.76; H, 7.46; N, 20.89
Found: C, 47.79; H, 7.74; N, 21.62

Heptanoic acid-6-$^{14}C$. Seven g of above semicarbazone was converted to heptanoic acid-6-$^{14}C$ by heating with 8.4 g of KOH in 42 g of diethylene glycol; 3.5 g of heptanoic acid-6-$^{14}C$, b.p. 222-5°, was obtained. The yield was 77.2%. Specific activity: 6.66 ± 0.07 dpm/μmole.

Neopentyl-α-bromoheptanoic acid-6-$^{14}C$. Three and a half g of heptanoic acid-6-$^{14}C$ was converted to an α-bromo ester by the Hell-Volhard-Zelinsky reaction; 4 g of neopentyl α-bromo ester, b.p. 86-7°/350 μ, 53.3% yield, was obtained. Specific activity: 6.50 ± 0.07 dpm/μmole.

Anal. Calcd. for $C_{12}H_{23}O_2Br$: C, 51.61; H, 8.24; Br, 28.67
Found: C, 51.51; H, 8.30; Br, 28.96

Neopentyl-heptenoate-6-$^{14}C$. Four g of the

α-bromo ester was dehydrobrominated by refluxing with 7 g of diethylaniline; 1.8 g (63.4% yield) of heptenoate, boiling at 107-110°/17 mm Hg, was obtained. The specific activity was 7.68 dpm/μmole. The activity indicated the presence of impurities; however, no further purification was attempted.

Anal. Calcd. for $C_{12}H_{22}O_2$: C, 72.73; H, 11.11
Found: C, 71.70; H, 11.67

Alkali fusion of heptenoic acid-6-$^{14}$C. Neopentylheptenoate-6-$^{14}$C (0.415 g) was fused with 0.5 of KOH at 300-350°; 61.5 mg of valeric acid-4-$^{14}$C and 90 mg of acetic acid were obtained from the separation by GLC on "FFAP" column, and were titrated with standard NaOH solution with phenolphthalein as indicator. No activity was found in the acetic acid. The activity for the valeric acid was 6.44 ± 0.06 dpm/μmole.

Degradation of valeric acid-4-$^{14}$C. (In this procedure we followed the general directions of Phares.[4]) The titrated solution of valeric acid-$^{14}$C was evaporated under vacuum to dryness in the reaction flask. After the flask was cooled in an ice bath, 1 ml of conc $H_2SO_4$ was carefully added. The sodium valerate was dissolved by gentle warming and shaking. After the flask was again cooled in ice bath, 0.1 g of sodium azide was added, and the mixture was allowed to warm up until the azide was nearly dissolved. The flask was connected to the traps and placed in a bath at 35°, and the temperature was raised over a period of 1 hr to 60 to 70°. The temperature was kept at 60 to 70° for about 30 min and the $CO_2$ was swept out with a nitrogen stream for 10 min. The $CO_2$ was trapped in a spiral bubbler containing 50 ml of anhydrous ether and 3 g of phenethylamine. The precipitated phenethylammonium phenethylcarbamate[5] was filtered in a nitrogen chamber, and washed several times with anhydrous ether. Fifty mg of carbamate (about 29% recovery), m.p. 92-5°, was obtained. No activity was found in this compound.

To the residue of the above reaction, NaOH was added until the solution was strongly basic. The solution was distilled and the butylamine was collected in a test tube immersed in a dry ice–acetone bath. To the collected liquid (about 0.5 g), 0.5 g of phenylisothiocyanate was added, and the mixture allowed to stand at room temperature for 2 hr. The crystallized solid was filtered, washed with petroleum ether, and then washed with water. After several recrystallizations from alcohol, 40 mg of N-butyl-N'-phenylthiourea, m.p. 68°, was obtained. The specific activity was 6.57 ± 0.07 dpm/μmole.

B. Degradation via Beckmann Rearrangement

We were short of the sample used in Section A. A different sample of toluene-1-$^{14}$C (New England Nuclear Co.) was used in this experiment. All the activities were counted in dioxane scintillation solution.

1-Methyl-1-cyclohexene-1-$^{14}$C. Eighteen g of crude 1-methyl-1-cyclohexene-1-$^{14}$C was obtained from reduction of 20 g toluene-1-$^{14}$C (32.01 dpm/μmole) with 12 g Li in a mixture of 100 g each of ethylamine and dimethylamine. After rough purification by GLC on butanediol succinate (Craig) column, 12.3 g of 1-methyl-1-cyclohexene-1-$^{14}$C (I) (31.54 dpm/μmole, GLC showed the presence of 14.4% impurity) was collected. The yield was 50.2%.

6-Ketoheptanoic acid-6-$^{14}$C. A mixture of 4 g 1-methyl-1-cyclohexene-1-$^{14}$C (I) and 7 g of unlabeled 1-methyl-1-cyclohexene (supplied by Aldrich Chemical Co., Inc.) was oxidized with 44.5 g of $KMnO_4$; 13 g of crude keto acid was obtained. The yield was 78.8%.

Two g of the crude keto acids was heated on the steam bath for 30 min with 5 g of $NH_2OH \cdot HCl$, 5 g NaOH, and 45 ml of water. The mixture was cooled, acidified with sulfuric acid, and extracted with ethyl acetate. The solvent was evaporated to 10 ml and was cooled in an ice bath. The crystallized solid was filtered. After several recrystallizations from ethyl acetate, 1.1 g of oxime was obtained, m.p. 94-5°. The yield was 50%. Specific activity, 11.13 ± 0.11 dpm/μmole.

Anal. Calcd. for $C_7H_{13}NO_3$: C, 52.83; H, 8.18; N, 8.81
Found: C, 52.51; H, 8.21; N, 8.65

5-Acetamidovaleric acid. (In this procedure we followed the general directions of Donaruma and Heldt.[2]) One g of the oxime of 6-ketoheptanoic acid (obtained in the same manner as described above) was added in small portions to 2 g of conc sulfuric acid; the temperature of the solution was held below 25° by external cooling. When all the oxime had dissolved, the solution was added dropwise to 5 ml of conc sulfuric acid at 120 to 130°. The solution was kept at this temperature for 5 min and then brought down to room temperature; 28% aqueous ammonium hydroxide solution was then added to bring the pH of the solution to 6. The reaction mixture was then extracted with ethyl acetate. The ethyl acetate extract was evaporated to 5 ml, and was cooled in an ice bath. The crystals formed were collected and recrystallized twice from ethyl acetate; 1 g of colorless crystals, m.p. 53-4°, was obtained. The yield was quantitative.

Anal. Calcd. for $C_7H_{13}NO_3$: C, 52.83; H, 8.18; N, 8.81
Found: C, 53.06; H, 8.24; N, 8.92

Glutaric and acetic acid. (In this procedure we followed the general directions of Donaruma and Heldt.[2]) One g of 5-acetamidovaleric acid was refluxed with 50 ml of 50% sulfuric acid for 3 hr. The mixture was then diluted with 200 ml of water and steam distilled. About 800 ml of distillate was collected and titrated with standard 0.5 N NaOH solution (phenolphthalein as indicator); 377 mg (6.29 mmole) of acetic acid was obtained (the entity of the acetic acid was proved by GLC on FFAP column). The yield was 98.6%.

To the remaining residue of the steam distillation, conc NaOH solution was added until the pH reached 9; 70 ml of 5% $KMnO_4$ solution was added and the mixture heated at boiling for 15 min. The solution was cooled and the excess $KMnO_4$ decomposed by adding 37% formalin solution. The $MnO_2$ was filtered, and the filtrate concentrated to 100 ml. This was acidified with sulfuric acid and extracted several times with ether. The ether extracts were combined and dried over anhydrous sodium sulfate. The ether was evaporated, and the residue recrystallized from petroleum ether; 0.58 g (69.9% yield) of glutaric acid, m.p. 95-6°, was obtained. The melting point was not depressed by mixing with known glutaric acid.

Anal. Calcd. for $C_5H_8O_4$: C, 45.45; H, 6.06
Found: C, 45.07; H, 6.32

Beckmann rearrangement of 6-ketoheptanoic acid oxime-6-$^{14}C$ and the subsequent hydrolysis. One and one-tenth g (6.92 mmole) of the oxime (11.13 ± 0.11 dpm/μmole) was added in small portions to 5 ml conc sulfuric acid. This solution was added dropwise to 20 ml of conc sulfuric acid at 120 to 130°. The mixture was heated at this temperature for another 5 min; 25 ml of water was added and the solution refluxed for 2 hr. Two hundred ml of water was then added and the solution was steam distilled. The distillate was titrated with 0.5 N NaOH solution; 6.35 mmole of acetic acid was obtained (91.7% yield). Identity of the acetic acid was checked by GLC (FFAP column).

The sodium acetate solution from the titration was evaporated under vacuum. The Schmidt reaction was carried out as described in Section A; 0.662 g (36.6% recovery) of phenethylammonium phenethylcarbamate, m.p. 93-4°, was obtained. The melting point was not depressed by mixing with known carbamate. The specific acitvity was 10.55 ± 0.11 dpm/μmole.

Anal. Calcd. for $C_{17}H_{22}N_2O_2$: C, 71.33; H, 7.69; N, 9.79
Found: C, 70.76; H, 7.31; N, 9.62

To the residue of the Schmidt reaction, 10 g of NaOH in 50 ml water was added; the methylamine was distilled off by warming the solution to 90°. The amine was collected in a trap immersed in a liquid nitrogen bath; 1 g of phenylisothiocyanate was then added and the mixture allowed to react at room temperature for 2 hr. The precipitated solid was filtered and recrystallized six times from dilute alcohol; 0.4 g (40% recovery) of N-methyl-N'-phenylthiourea, m.p. 113-5°, was obtained. The specific activity was 0.26 ± 0.03 dpm/μmole.

Anal. Calcd. for $C_8H_{10}N_2S$: C, 57.83; H, 6.02; N, 16.87
Found: C, 57.53; H, 4.69; N, 16.87.

To the residual solution from the steam distillation, conc NaOH solution was added until the solution was basic; 70 ml of 5% $KMnO_4$ was added and the mixture refluxed for 15 min. The $MnO_2$ was filtered, and the filtrate concentrated to 100 ml. This was acidified with conc sulfuric acid and extracted with ether. The crystals, obtained by evaporation of the ether, were recrystallized from ethyl acetate; 0.58 g (69.9% yield) of glutaric acid, m.p. 95-6°, was obtained. The melting point was not depressed by mixed fusion with authentic glutaric acid. The specific activity was 0.36 ± 0.04 dpm/μmole.

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# 17. FURTHER IMPROVEMENTS ON THE 10-kV ION ACCELERATOR

Helmut M. Pohlit and Wallace R. Erwin

## A. Arc Conditions and Filament Lifetime

We have been attempting to obtain a particle beam of very low energy --i.e., less than 100 electron volts (eV) on our carbon-ion accelerator. One problem is the energy spread due to periodic (60-cycle) energy fluctuations of the ions emitted from the arc plasma. The main cause for this periodic fluctuation was voltage fluctuation of the arc power supplies. When the accelerating, arc-anode, and arc-chamber wall voltages were stabilized and the ripple decreased by increasing LC filtering to less than 0.5 V peak to peak (ptp), potential fluctuations up to 30 V ptp still were found. These were due to large periodic (120-cycle) temperature changes of the heated arc filament. (As is well known from the hot-filament electric lamp, the intensity of light emitted from it follows the periodic fluctuations of the ac power delivered to the filament, i.e., it fluctuates in a 120-Hz rhythm. In our case of a filament burning at a temperature sufficient for thermionic emission in a gas at 10 to 50 μ pressure these fluctuations should be even more pronounced because of the more efficient conduction of heat away from the filament during the low-power phase of the ac cycle. The thermionic emission is therefore periodic, with 120 Hz.) The resultant fluctuation of electron emission caused the conductivity of the arc plasma to vary drastically, presenting a nonconstant load to the power supplies. All voltages were found to be time-averaged over nonsinusoidal voltages, and the arc behaved as though it were operated by ac rather than dc power supplies.

We have therefore changed the ac filament power supply to a dc one with a ripple of less than 0.15 V ptp. With the resultant constant-conductivity arc plasma, new typical "dc" arc operating data are:

| | |
|---|---|
| Anode voltage | 60-100 V |
| Wall voltage | 0 V |
| Filament current | 25 A |
| Arc current | 500 mA |
| Pressure | 20 μ |

Usually all parameters can now be adjusted over a wider range than before. Potential fluctuations inside the arc now amount to less than 1 V ptp.

It was also hoped that constant temperature resulting from the dc power supply would increase the lifetime of the filament, because it was assumed that the maximum temperature of the ac-heated filament was much higher than the constant temperature with dc heating. However, filaments burned out after arcs had been struck only a few times. Large current peaks accompanied the striking, which had not been observed with ac-heated filaments. Limiting the arc current now by a series resistor of about 500 Ω between the last capacitor of anode and wall voltage supplies and the arc proper removed the current peaks, and the filament lifetime increased from about 4 hr to about 15 hr. Slow evaporation of rhenium is apparently the cause for this new lifetime limit.

The above-mentioned observations suggest, as reason for the relatively fast burnout of the filament under ac heating, that the filament temperature changes within one-half ac cycle so strongly that the arc is virtually struck again every 1/120 second at or near the maximum of the alternating current, when thermionic emission is high. A different kind of electron-multiplying mechanism must be effective during the low-temperature phase when no thermionic emission from the cathode can supply electrons to maintain the arc. The arc conditions therefore vary between the two extremes of the self-sustaining and non-self-sustaining type. Each periodic new striking of the arc must have caused rather high momentary currents which led to locally limited vaporization of the cathode material. The shapes of the two edges where fracture occurred are consistent with the above explanation. Crescent-shaped edges within relatively thick rhenium material suggest the formation of a small hole in the filament due to local metal evaporation. Consequently, the electrical resistance at this location is increased, which leads to increased heat development there, and eventually the filament burns out.

It is also understandable now why maintaining or striking an arc under these alternating conditions was more difficult, and why arc parameters appeared more critical.

## B. Arc-Disrupting Discharges

A continuing nuisance has been discharges occurring between the arc chamber, which is on high positive potential (5 to 10 kV), and the beam-extraction electrode, which is on ground potential. These discharges usually led to extinction of the arc.

The following modification corrected this problem. The narrow front section of the funnel-shaped extraction electrode was insulated from the larger remaining part of the

electrode, which houses the focus electrode, and which remains on ground potential. This insulated front piece was then connected to ground through a 5-MΩ resistor (Fig. 17-1). The potential of this piece depends therefore on the total current flowing from it to ground.

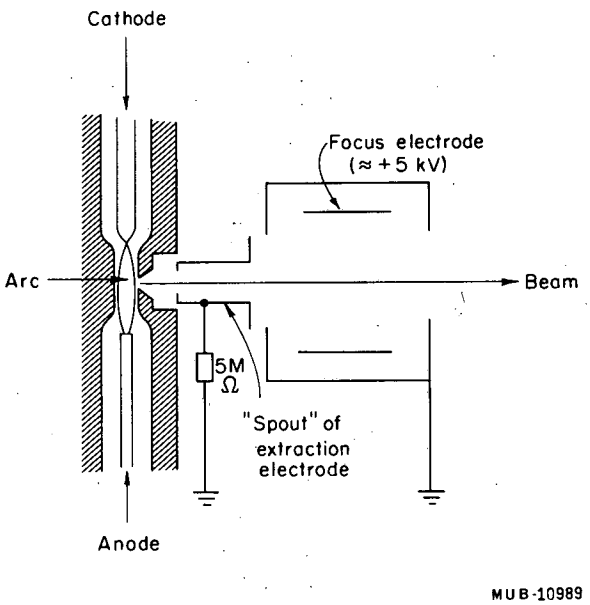


Fig. 17-1. Beam extraction system (see Part B).

Under normal conditions only a few ions strike this piece, setting up a current of less than 1 μA and leaving its potential very close to ground potential. A discharge occurring with larger currents between the arc chamber and this part instantaneously raises the potential to such a high value that the "macro-discharge" cannot develop. The capacitance of this funnel spout being rather small, it was found that 5 MΩ presented a very good compromise between the following two requirements:

(a) Low-time-constant RC, requiring a small R so that the potential jump would be sufficiently fast; also, that the system return quickly to normal after the discharge ends.

(b) Sufficiently high potential jump to decrease the potential difference between arc chamber and spout below breakdown voltage. This condition requires a large R.

With this setup the primary discharges did not impair the operation of arc and beam; only occasionally (about once per hr) would the arc now extinguish due to a discharge.

## C. Efficiency of Ion Production

### 1. Effect of extraction voltage

Impressing different potentials upon this front funnel spout allows one to change the extracting potential without changing the energy of the ions, which is determined by the potential of the arc chamber with respect to ground. Thus one can always optimize the beam output with respect to the extraction voltage. This is particularly important if one intends to decrease the initial energy of the ions in order to facilitate further deceleration to a desired low energy before entering the target area. (Usually the output decreases sharply below 2 kV.) The optimum extraction voltage for $C^+$ from $CO_2$ seems to be about 6 kV. However, for $H^+$ from $H_2$, the $H^+$ current increases by a factor of 2 upon increase of the extraction potential from 5 to 12 kV.

This dependence of the beam intensity on extraction voltage is due to an influence of the extracting field upon the shape of the plasma sheath from which ions are emitted. This plasma sheath determines the initial divergence of the beam.

### 2. Admixtures of other gases with $CO_2$

It has been reported[1] that adding He to $CO_2$ in a ratio of 1:1 increases the $C^+$ current by a factor of 2. This could not be confirmed. It was found, however, that diluting $CO_2$ with He by factors up to 8 would not appreciably decrease the $C^+$ ion current. Thus, the efficiency of $C^+$ ion production is increased by a factor of 8, since the arc pressure, which is directly related to the gas flow, is kept constant.

At higher dilutions, the arc becomes increasingly more difficult to strike and maintain; to maintain an arc in pure He requires very high He pressure.

Argon, a gas that facilitates arc operation, does not exhibit the favorable dilution effect shown by He. Rather it decreases the $C^+$ current by approximately the factor of dilution.

Neon was found to decrease the $C^+$ current, though by somewhat less than the ratio of dilution. It also exhibited the adverse effects of He, but in not so pronounced a manner.

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32. Oxaziridines. I. The Irradiation Products of Several Nitrones. Janet S. Splitter and M. Calvin. J. Org. Chem. 30, 3427 (1965).

33. Coordination Chemistry of Manganese and Porphyrins. Melvin Calvin. Rev. Pure Appl. Chem. 15, 1 (1965).

34. Study of the Radical Termination Mechanisms in the Radiolysis of Crystalline Choline Chloride. M. A. Smith and R. M. Lemmon. J. Phys. Chem. 69, 3370 (1965).

Chemical Evolution and Organic Geochemistry

35. Formation of Cyanamide under "Primitive Earth" Conditions. A. Schimpl, R. M. Lemmon, and M. Calvin. Science 147, 148 (1965).

36. Dicyandiamide: Possible Role in Peptide Synthesis During Chemical Evolution. G. Steinman, R. M. Lemmon, and M. Calvin. Science 147, 1574 (1965).

37. Evidence for Life Processes in a Sediment Two and a Half Billion Years Old. T. Belsky, R. B. Johns, E. D. McCarthy, A. L. Burlingame, W. Richter, and M. Calvin. Nature 206, 446 (1965).

38. Dehydration Condensation in Aqueous Solution. G. Steinman, D. H. Kenyon, and M. Calvin. Nature 206, 707 (1965).

39. Chemical Evolution. Melvin Calvin. In Proceedings of the Xth International Botanical Congress, Edinburgh, August 1964 (printed 1965), 41-56.

40. Chemical Evolution. Melvin Calvin. Proc. Roy. Soc. 288A, 441 (1965).

41. Occurrence of Biogenic Steranes and Penta-Cyclic Triterpanes in an Eocene Shale (52 Million Years) and in an Early Precambrian Shale (2.7 Billion Years): A Preliminary Report. A. L. Burlingame, Pat Haug, Theodore Belsky, and M. Calvin. Proc. Natl. Acad. Sci. 54, 1406 (1965).

## THESES

Work for the following Ph. D. theses was completed during 1965:

1. Theodore Belsky, Organic Geochemistry and Chemical Evolution, UCRL-16605, Jan. 1966.

2. C. Allen Bush, Dispersion of Optical Rotation and Birefringence of Biopolymers. I. Theory of Faraday Rotation in Polymers. II. Electrical Birefringence of Tobacco Mosaic Virus. III. Optical Rotation of Dinucleoside Phosphates, UCRL-16278, July 1965.

3. Stephen L. Davis, Optical Properties of Adenylic Acid and Its Oligomers, UCRL-16409, Aug. 1965.

4. Irwin Douglas Kuntz, Jr., Spectroscopic Studies of Photosynthesis, UCRL-16207, June 1965.

5. Joan Friedman Newmark, Some Aspects of the Photochemistry and Radiation Chemistry of Thymine, UCRL-16353, Aug. 1965.

6. Gary David Steinman, Protobiochemistry: Dehydration Condensation in Aqueous Solution, UCRL-16566, Jan. 1966.

## SCIENTIFIC LECTURES BY THE SENIOR STAFF, 1965

Melvin Calvin

1. Chemical Evolution. Biophysical Society Meeting, San Francisco, California, Feb. 1965.

2. Point in Time. American Association of University Women, Midland, Michigan, March 1965.

3. Chemical Evolution (Graham Young Lecture). Glasgow University, Glasgow, Scotland, April 1965.

4. Chemical Evolution. Lombard Academy of Medicine, Milan, Italy, April 1965.

5. Chemical Evolution (The Bakerian Lecture of The Royal Society). London, June 1965.

J. A. Bassham

1. Effects of Fatty Acids and Fatty Acid Esters on Photosynthesis. A. I. B. S. Meeting, Urbana, Illinois, August 1965.

2. Regulation of Carbon Compound Photosynthesis. Pennsylvania State University, University Park, Pa., September 1965.

E. L. Bennett

1. Evidence for Increased Glia Cells in Rat Cortex Resulting from Environmental Complexity and Training. Federation Meeting, Atlantic City, N. J., April 1965.

2. Genetic Selection of Rats Differing in Brain Biochemistry. Second International Neurochemical Symposium, Oxford, England, July 1965.

3. Heredity, Environment, Learning, and the Brain. American Association for the Advancement of Science Meeting, Berkeley, December 1965.

4. Postnatal Effects of an Enriched Behavioral Environment on Brain Chemistry and Anatomy, and Behavior. Symposium on Plasticity of the Developing Brain, Berkeley, December 1965.

5. Relations of Brain Biochemistry and Behavior. American Physiological Society Meeting, Los Angeles, California, August 1965.

6. The Chemistry and Anatomy of Rat Brain and Relation to Behavior. Seminar at the University of California, Santa Barbara, March 1965.

7. Relations of Brain Biochemistry to Learning. Seminar, University of North Carolina, Chapel Hill, April 1965.

M. P. Klein

1. Applications of Magnetic Resonance to Problems in Biology. Atomic Physics Seminar, Physics Department, University of California, Berkeley, April 1965.

2. Mössbauer Spectroscopy of Biological Compounds. Lockheed Missiles and Space Laboratory, March 1965.

3. The Applications of Physics to Biological Research. National Science Foundation Summer Institute for High School Teachers, University of California, Berkeley, July 1965.

Richard M. Lemmon

1. Series of eight seminar lectures on the general topic, "Radiation Chemistry."

Department of Radiochemistry, University of Helsinki, Helsinki, Finland, February-April 1965

2. Hot Atom Chemistry. Department of Radiochemistry, University of Helsinki, Helsinki, Finland, March 1965.

3. Chemical Evolution. Finnish Biochemistry Society, Helsinki, Finland, May 1965.

4. Radiation Chemistry of Crystalline Choline Chloride. International Union of Pure and Applied Chemistry, Moscow, U.S.S.R., July 1965.

V. Moses

1. The Study of Metabolic Compartmentalization. Australian Biochemical Society, University of New South Wales, Sydney, N.S.W., and Australian National University, Canberra, A.C.T., August 1965.

2. Glucose Repression of β-Galactosidase Synthesis in *Escherichia coli*. University of Adelaide, Adelaide, South Australia, August 1965.

3. Problems in the Isolation of the Repressor of the Lactose Operon in *Escherichia coli*. University of Melbourne, Melbourne, Australia, August 1965.

4. A Model for Catabolite Repression. Monash University, Melbourne, Australia, August 1965.

5. Recent Studies on the Regulation of Protein Synthesis in *E. coli*. Australian Biochemical Society General Meeting, Monash University, Melbourne, Australia, August 1965.

Roderic B. Park

1. Structure in Photosynthesis. Seminar, University of California, Riverside, April 1965.

2. Two weeks of lectures in Plant Physiology. Tuskegee Institute, Tuskegee, Alabama, Spring 1965.

3. Invited symposium talk for California Society of Electron Microscopists, Berkeley, California, May 1965.

4. Lecture on photosynthesis for "PARTICLE." Sponsored by the undergraduate Mathematics-Science Society, University of California, Berkeley, April 1965.

5. Quantum Efficiencies of Chloroplasts from Spinach Plants Grown under Long and Short Days. Pacific Division, AAAS, University of California, Riverside, June 1965.

6. The Effect of Daylength on Quantasome Structure and Chloroplast Photochemistry. "Chloroplast Development and Senescence" Symposium, Gorsem, Belgium, September 1965.

7. Chloroplast Structure. Second Western Europe Meeting on Photosynthesis, Woudschoten, The Netherlands, September 1965.

H. Rapoport

1. Symposium Lecture, Third International Symposium on Biochemistry and Physiology of Alkaloids, Halle, East Germany, June 1965.

K. Sauer.

1. Molecular Orientation in Quantasomes. Biophysical Society, San Francisco, California, February 1965.

2. The Hill Reaction in Chloroplasts. Botany Department, University of California, Berkeley, April 1965.

3. The Optical Rotatory Dispersion of Chlorophyll. Physical Chemistry Seminar, Chemistry Department, University of California, Berkeley, March 1965.

4. The Intermolecular Associations of Chlorophylls. Ibid., November 1965.

5. Ultrastructure of the Photosynthetic Apparatus: Quantasomes, American Chemical Society, Western Regional Meeting, Los Angeles, California, November 1965.

I. Tinoco, Jr.

1. RNA: Structures and Optical Properties. California Section, American Chemical Society Meeting, Berkeley, California, May 1965.

2. Structures and Optical Properties of Ribonucleic Acids. American Association for the Advancement of Science, Berkeley, California, December 1965.

# PERSONNEL LIST

## Senior Staff

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## Research Staff

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