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THE ROLE OF CHLOROPHYLL AS A CHEMICAL INTERMEDIATE IN PHOTOSYNTHESIS

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UCRL-16946

Research and Development

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

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Marianne Byrn

(Ph. D. Thesis)

June 1966

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THE ROLE OF CHLOROPHYLL AS A CHEMICAL INTERMEDIATE IN PHOTOSYNTHESIS

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ABSTRACT

For several decades the central question of photosynthesis has been that of the mechanism of the primary quantum conversion; or, to phrase the question differently, what is the mechanism of chlorophyll participation in converting electromagnetic energy into chemical energy? The old problem of chemical versus purely "physical" sensitizing action for chlorophyll participation in photosynthetic energy conversion remains an elusive one. The earliest proposals identified chlorophyll as a chemical intermediate in which the molecule acts as a reducing agent (hydrogen donor) in the separation of the primary oxidant and primary reductant. Lack of supporting evidence for such chemical theories led to the more recent proposals for chlorophyll as an energy transfer agent in which the molecule remains chemically unchanged. The work in this thesis evaluates several chemical mechanisms proposed in the literature using isotope tracers, and a new mechanism is proposed and experimental evidence in its favor is presented.

Deuterium and NMR are used to determine the lability of the delta and the 10 position of chlorophyll a. Another investigator meanwhile found the delta position not to be important in the photosynthetic scheme.

Oxygen 18 and infrared were the means of evaluating proposals involving the carbonyl group of the isocyclic ring of chlorophylls a and b. Model compounds relating to the structure of chlorophyll (cyclic ketones, aromatic aldehydes and beta-ketoesters) were studied to determine the lability of carbonyl groups to hydration. Chlorophylls and chlorophyll derivatives were then examined and their exchange properties are discussed in relation to the model compounds.

Oxygen 18 biological experiments were carried out using the green algae Chlorella. The chlorophylls were extracted and analyzed with infrared to determine the amount of incorporation. It was found that the carbonyl functions do not participate as chemical intermediates in either the water splitting reaction or in oxidative photophosphorylation.

Finally, a mechanism is proposed and experimental evidence presented for the role of chlorophyll as a chemical intermediate in photosynthesis. The notion is that the 3,4 positionsof ring II are involved in a reversible oxidation-reduction cycle. This proposal cannot be tested on the green algae, since the chlorophylls a and b are isolated in the oxidized state and would not contain the photosynthetically important hydrogens. The experiments are carried out on R. rubrum, a photosynthetic bacteria, which is allowed to photosynthesize in tritiated water, the bacteriochlorophyll is isolated, and the amount of tritium incorporation is determined. A selective oxidation procedure requiring 2,3-dichloro-5,6-dicyanobenzoquinone is found to remove 60-90% of the incorporated tritium, thus demonstrating that the 3,4 positions are involved in the photosynthetic scheme. Investigations were carried out to determine that the incorporation was not biosynthetic in origin, by attempting to determine the rate

of biosynthesis and rate of photosynthesis, and by doing double illumination experiments in which the tritiated bacteria are allowed to photosynthesize in non-tritiated media for an equal period of illumination. The question of biosynthetic versus photosynthetic incorporation is discussed at great length. A mechanism is proposed for the involvement of the 3,4 positions of bacteriochlorophyll in the photosynthetic scheme. This includes a suggestion for "migratory active sites" in which a small percentage of the bacteriochlorophyll carries out the oxidation-reduction cycle and these molecules are in equilibrium with the larger bacteriochlorophyll pool, as distinguished from being "fixed" active sites.

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ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Professor Melvin Calvin for his advice and encouragement during the accomplishment of this thesis.

To Melvin Klein, David Samuel, John Lindsay Smith and, of course, Gerald Corker, my kindest regards for many helpful hints, thought provoking questions, and answers.

CHAPTER I. INTRODUCTION

For several decades the central question of photosynthesis has been that of the mechanism of the primary quantum conversion; or, to phrase the question differently, what is the mechanism of chlorophyll participation in converting electromagnetic energy into chemical energy? The old problem of chemical versus purely "physical" sensitizing action for chlorophyll participation in photosynthetic energy conversion remains an elusive one. The earliest proposals identified chlorophyll as a chemical intermediate in which the molecule acts as a reducing agent (hydrogen donor) in the separation of the primary oxidant and primary reductant. Lack of supporting evidence for such chemical theories led to the more recent proposals for chlorophyll as an energy transfer agent (resonance transfer and/or electron-hole migration) in which the molecule remains chemically unchanged. The absorbed electromagnetic energy is ultimately transferred to acceptor molecules which then undergo the sequence of chemical reactions which ultimately reduce carbon dioxide and liberate oxygen from water. Despite the collection of evidence which has increased the popularity of the "physical" interpretation of the primary quantum conversion act, there is a persistent gnawing in the minds of some photosynthesis research workers that chlorophyll would, should, or could perform as a hydrogen transfer agent in the water splitting reaction. It is with the belief that new techniques might finally offer evidence for the chemical participation of chlorophyll in the photosynthetic mechanism that the work described in this thesis was undertaken.

Proposed Chemical Mechanisms

One of the earliest proposals for a reversible oxidation-reduction mechanism was that of Dixon and Ball,[1] who suggested that chlorophyll a ($Chl\text{-}H_2$) is oxidized to chlorophyll b ($Chl\text{-}O$).

$$Chl\text{-}H_2 + CO_2 + \text{light} \longrightarrow Chl\text{-}O + 1/n(H_2CO)n$$

and b in turn is reduced to a as follows:

$$Chl\text{-}O + H_2O + \text{light} \longrightarrow Chl\text{-}H_2 + 1/2\ O_2 .$$

Conant et al.[2] proposed that the pigment might act as a two electron reducing agent in a dark reaction

$$12\ Chl\text{-}H_2 + 6\ CO_2 \xrightarrow{\text{enzyme}} 12\ Chl + C_6H_{12}O_6 + 6\ H_2O$$

with the chlorophyll being regenerated by a photochemical process

$$12\ Chl + 12\ H_2O + \text{light} \longrightarrow 12\ Chl\text{-}H_2 + 6\ O_2 .$$

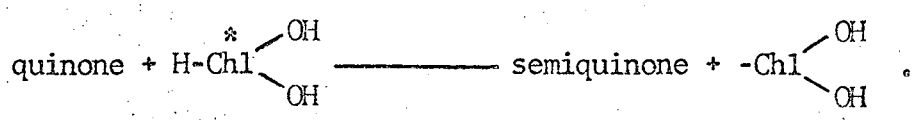
Stoll[3] and Willstätter[4] considered chlorophyll to function as a hydrogen donor in a photochemical reaction producing monodehydrochlorophyll ($Chl\text{-}H$). This free radical then reverts to the original pigment via another photochemical process

$$Chl\text{-}H + H_2O + \text{light} \longrightarrow Chl\text{-}H_2 + OH .$$

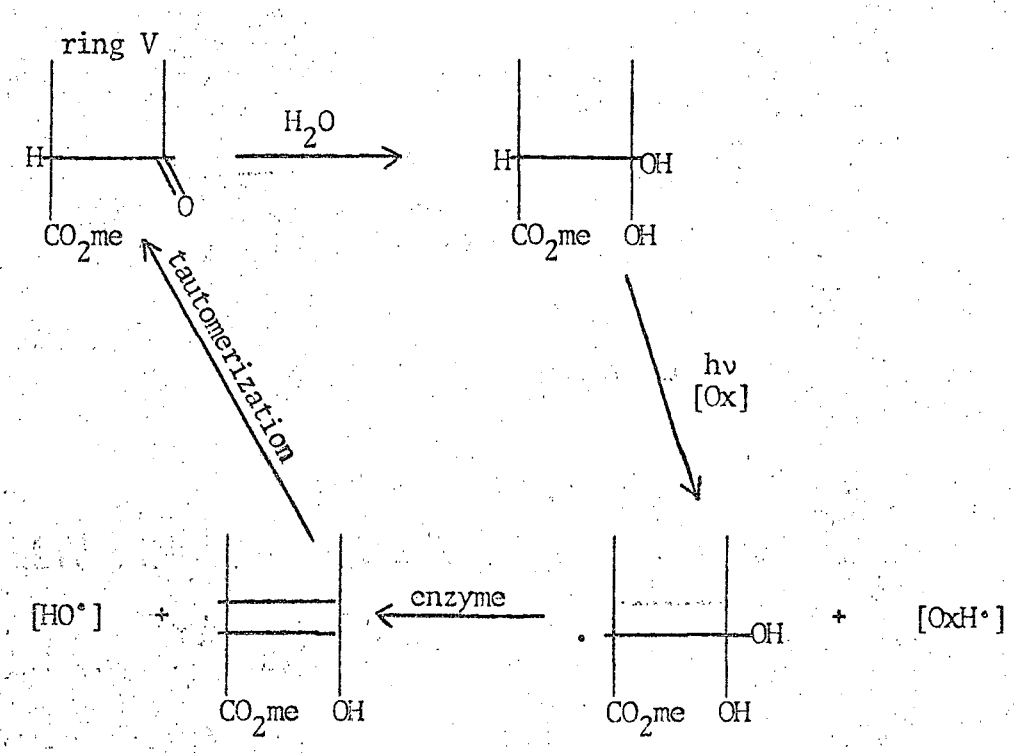
Franck[5,6] has proposed a somewhat similar scheme, but has suggested the following chlorophyll regeneration in which $R'OH$ is different from water.

$$Chl\text{-}H + R'OH + \text{light} \longrightarrow Chl\text{-}H_2 + R'O.$$

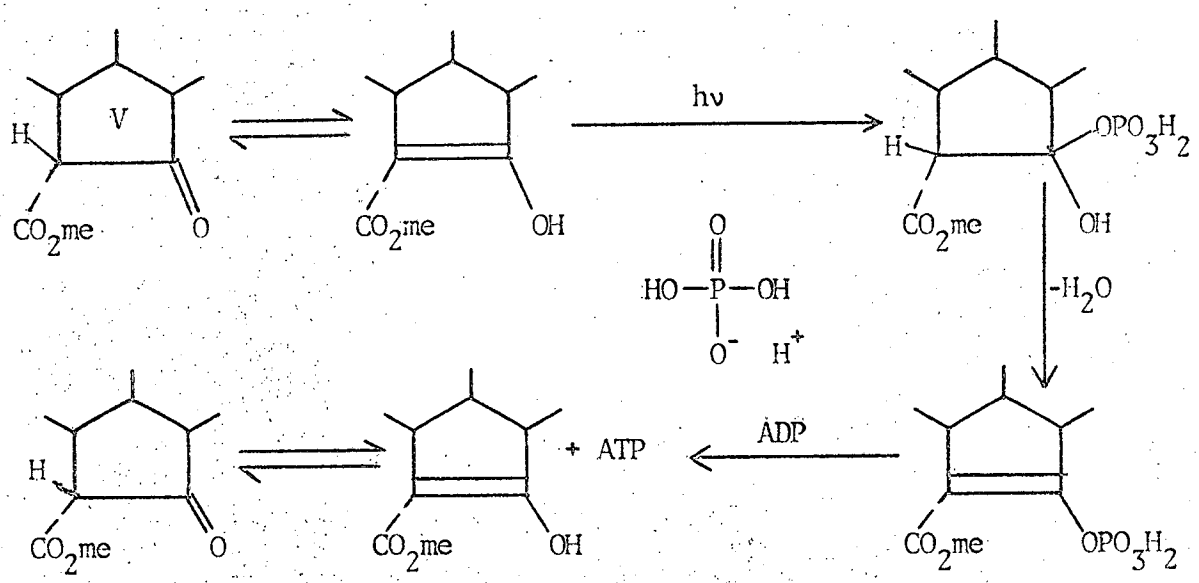
About a decade later, Franck[7] made an even more specific proposal, by identifying the ring V beta-ketoester as the sight of hydrogen donation. The previous proposals for reversible oxidation-reduction cycles were primarily centered on the 7,8 positions of ring V, the reduced pyrrole moiety. Franck's proposal of 1955 suggested that the C_9 carbonyl group would hydrate, H-Chl(OH)(OH), then light light absorption would excite to the first excited state and energetically permit the reaction



One of the hydroxyls of this radical could be removed by an enzyme and utilized for oxygen evolution in a reaction which should be slightly exothermic. This concept was described more definitively in 1957, and can be expressed in the following form:[8]



A similar proposal is one by Calvin,[9] which introduces the possibility that the site of oxidative photophosphorylation is the ring V of chlorophyll. If the enol form of chlorophyll were to add orthophosphate in a light reaction, an enol phosphate could be produced upon dehydration, which presumably would be capable of phosphorylating ADP to make the required ATP.



The most recent suggestion has been that the delta methine position of the macrocyclic ring might be the site of photosynthetic hydrogen transfer. This proposal came about from the discovery by Woodward of the lability of the delta position in chlorins (dihydroporphyrins).[10]

Experimental Examination of Proposed Chemical Mechanisms

Norris, Ruben and Allen[11] carried out the first experiments to test the proposed chemical mechanisms stipulating chlorophyll as a hydrogen transfer agent. They surmised that if photosynthesis is allowed to proceed for a sufficiently long time in water containing HTO, that chlorophyll containing tritium should be formed if the ideas suggested above

are correct. They used a suspension of Chlorella pyrenoidosa, and allowed it to photosynthesize for 3 hours in labeled water. They concluded from several experiments that less than 5% of the theoretical radioactivity expected for complete exchange of one hydrogen was incorporated.

However, before they concluded that chlorophyll does not act as a hydrogen donor they had to consider two possible objections: (1) loss of tritium from chlorophyll by thermal exchange during the extraction process, and (2) an isotope effect--that is, tritium is not an ideal tracer for hydrogen of mass one. They investigated the possibility that chlorophyll contained labile hydrogen which can undergo photochemical or thermal exchange. They concluded that there is no thermal exchange observed between purified chlorophyll and 80% methanol containing HTO (an experimental conclusion which is later shown to be incorrect). Thus, they concluded that any incorporation which may have taken place could not have been lost during the purification procedure. The question of isotope separation is more difficult to analyze. The low amounts of incorporation do not exclude the possible existence of a cycle in which a photoactivated chlorophyll Chl^*H_2 acts as a donor of hydrogen to some unknown substance (A),

$$Chl\text{-}H_2 + light + \longrightarrow Chl^*H_2 + A \longrightarrow Chl\text{-}H + HA$$

and the chlorophyll free radical (Chl-H) formed in this process is reduced to native chlorophyll by a thermal reaction such as

$$Chl\text{-}H + ROH \longrightarrow Chl\text{-}H_2 + RO\ .$$

This second process very likely has a lower activation energy than the analogous reaction involving an oxygen-tritium bond

$$Chl\text{-}H + ROT \longrightarrow Chl\text{-}HT + RO\ .$$

The difference in energies of activation could be of the order of 2 kcal, and consequently the normal specific reaction rate should be 20 times greater than that for the tritium isotope. In the photochemical reaction, the absorption of a light quantum would raise the photoactivated chlorophyll to an energy level well over the top of the energy barrier, and therefore the specific reaction velocities would be the same for tritium and hydrogen and no isotope separation would occur. However, if the donation of the proton from chlorophyll to substance A is a thermal reaction, then the isotope separation would result in which Chl-HT would tend to accumulate over Chl-H_2. Moreover, if the second step is photochemical, no isotope separation would occur.

This discussion simply points out the difficulties in evaluating the type of isotope effect to expect in a complex biological system. The means of evaluating the isotope effect were not available, although the rate of photosynthesis in D_2O was known relative to the rate in water. However, it is possible that photosynthesis involves several thermal reactions in which isotope separations may occur; thus in the tritium experiments, where there is competition between ROH and ROT, the isotope separation may be greater or less than one would expect from a simple consideration of the rate in pure deuterium oxide.

Similar experiments were carried out by Calvin and Aronoff,[12] using deuterated water. In this way the isotope effects could be reduced, although the difficulty with re-exchanging labile hydrogen could not be avoided. Therefore, their experiments were applicable only to non-labile hydrogen. The combustion method of analyzing for deuterium showed that there was no deuterium incorporation into chlorophyll due to photosynthetic hydrogen transfer. Thus, the possibility that the 7,8 positions,

or any other non-labile hydrogens of chlorophyll a, are involved in the photosynthetic scheme is eliminated.

A decade later, Vishniac and Rose[13] again attempted a series of tritium experiments using both Chlorella whole cells and chloroplasts. They detected a small amount of light induced tritium incorporation in the chlorophyll, which they presumed to be in the C_{10} position, even though the radioactivity remained intact under acid treatment and was lost under treatment with alkali. The amount of incorporation attained was only a small fraction of the activity expected for complete equilibration of one position with the hydrogen of water. In 1963, Colman and Vishniac[14] reported further work on the chloroplast experiments, but no definite conclusions could be reached.

There is, in addition, an important consideration which should be discussed. Emerson and Arnold[15] proposed from saturation effects in flashing light experiments that there is a photosynthetic unit in which only a small fraction of the chlorophyll acts as an active site, while the remainder of the chlorophyll serves as a funnel for electromagnetic energy. The most recent summary of this proposal has been made by Clayton,[16] in which he suggests that one of every 200 molecules (1/2%) of chlorophyll a are active sites. If this proposal is to be accepted, than an inherent limitation is introduced into any experiments designed to evaluate the participation of chlorophyll as a hydrogen transfer agent at the active site. In the experiments described previously, only 1/2% of the theoretical amount of incorporation could be expected on the theory of active sites, rather than 100% on the basis of exchange of 1 mole of hydrogen--an incorporation far below the limit of detection in the experiments described. This is a problem which the reader should bear in

mind; and one which will be handled in detail in the final two chapters.

This, then, is the totality of the experimental work carried out to evaluate the chemical mechanisms in which chlorophyll is proposed as a hydrogen transfer agent. The conclusions of these experiments neither support nor refute the proposals, because of the difficulties mentioned: isotope effect and re-exchange of labile protons. The unexplained small amounts of incorporation (less than that to account for 1 mole of hydrogen for complete exchange) have continued to entice speculation and research in this field.

Thesis Proposal

It is the purpose of this thesis to further evaluate the mechanisms that have been suggested for the chemical participation of chlorophyll in the photosynthetic scheme. Three isotopes of the constituents of water will be used: deuterium, tritium, and oxygen 18. The three approaches to this problem will be treated separately in the following four chapters, including both chemical and biological studies.

CHAPTER II. DEUTERIUM EXCHANGE REACTIONS OF CHLOROPHYLL a

The development of nuclear magnetic resonance spectroscopy spurred a new wave of interest in the chemistry of photosynthesis. It was immediately apparent that with this technique of isotope analysis the isotope effect problem in the tritium experiments could be considerably reduced with the use of deuterium rather than tritium. The availability of solvents with high isotopic purity and the lower deuterium bond energies contribute to reducing isotopic selectivity. A further advantage of using deuterium and analyzing by NMR is the possibility that it may not be necessary to obtain the pigments in an extremely pure state, thus partially eliminating the serious drawback of re-exchanging labile hydrogens. Thus, the aim of this research is to follow a biological exchange reaction in D_2O with the green algae *Chlorella*. The pigments will be extracted and the extent of deuterium incorporation will be determined by NMR. Prior to the biological experiments, it is necessary to understand the type of exchange behavior that could be expected in the biological experiments and in the extraction procedure; therefore, the exchange reactions of pure chlorophyll *a* in a variety of organic solvent systems was undertaken. The previous literature on this subject was incomplete and inconsistent, and is reviewed in the following section.

Exchange Reactions of Chlorophyll *a*

Norris, Ruben and Allen[11] concluded in 1942 that a mixture of chlorophyll *a* and *b* did not possess exchangeable hydrogen after a 30 minute exposure to tritium in 80% aqueous ethanol. In 1952, Weigl and

Livingston[17] made a detailed search for exchangeable hydrogen in chlorophyll using neutral organic solvents containing an excess of deuterium oxide. A reaction time of 2 hours was employed and the deuterium was analyzed by infrared after the chlorophyll had been combusted. Weigl and Livingston also concluded that no exchangeable hydrogen was present in the chlorophyll.

The first investigator that did not exclude the possibility that chlorophyll possessed exchangeable hydrogen was Kutyrin.[18] In 1960, Kutyrin found a small amount of hydrogen exchange between chlorophyll and aqueous acetone in a 3 hour period. The first definitive evidence for exchangeable hydrogen came from Katz et al.,[19] in 1961. After 48 hours in a solution of methanol in carbon tetrachloride, both chlorophylls a and b showed one hydrogen atom exchanged. Although the infrared analysis could not designate a particular position for exchange, the investigators implied that from the structural formula it can be anticipated that the hydrogen atom at position 10 is in a favorable position for enolization and should, therefore, be both labile and exchangeable. At the outset of this project, the most recent work by Katz et al.[20] stated that when chlorophyll is treated with methanol in carbon tetrachloride, the NMR results demonstrate unequivocally that only one hydrogen atom undergoes exchange and that the labile hydrogen is located on the porphyrin ring at the delta position. Their results, relating the observations by Woodward and Skaric[10] on the exchange behavior of chlorins to include the chlorophylls, had profound influence in furthering investigations on the mechanism of photosynthesis. (Investigations reported during the progress of this research indicate that both the 10 and delta positions are labile.[21,22])

The solvents (CCl_4, $CHCl_3$, and CH_3OH) chosen by other researchers for experiments designating chlorophyll as possessing labile hydrogens are not those solvents used in the pigment extraction procedure (CH_3COCH_3 and H_2O). Thus, it appears imperative to further establish the lability of chlorophyll in aqueous acetone and to further investigate the exchangeability of the 10 and delta position.

Experimental

Chlorophyll a was isolated from spinach using powdered polyethylene* and sugar chromatography according to the method of Calvin and Anderson.[23] The eluate from the sugar column was evaporated and transferred from an acetone solution to isooctane. This was an additional precaution to remove colorless impurities derived from the final column. The absence of other pigments from the chlorophyll preparations was determined by visible absorption spectra. The absence of colorless impurities was determined by elemental analysis.

Deuterated solvents were obtained commercially except for the heavy water, which was obtained from Livermore Radiation Laboratory.

The NMR spectra to determine the deuterium exchange were taken on a Varian A-60 spectrometer, supplemented by a Mnemotron Computer of Average Transients. This made it possible to use low concentrations (2×10^{-2} M) of chlorophyll in some binary solvents, and to check the completion of the exchange reaction. The peak assignments for chloro-

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*Powdered polyethylene: Dow has discontinued manufacturing this form of polyethylene of melt index <2, and has given its remaining stock of six 60 lb. drums to Prof. H. Rapoport. U.S.I. polyethylene powder, designated "Microthene FN-500", can be substituted for the Dow product. Not all powdered polyethylene will separate the xanthophylls from the chlorophylls, and examination of several lots is generally recommended.

phyll were made by Anderson, and more recently have been supported by the work of Katz.[25] All NMR samples were prepared on the vacuum line. The binary solvents, usually half a milliliter in volume, were degassed and distilled into the NMR tubes which were then sealed under N_2. The exchange reactions were then followed with time using NMR.

At the completion of the reaction, all samples were checked by visible absorption (400-700 mμ) to ensure that degradation had not taken place.

Dark experiments were carried out by covering the NMR probe and darkening the room. The samples were prepared on the vacuum line in a dark room. The effect of light was then tested by placing the tube in a water temperature bath between two photoflood lamps. Table II-1 lists all the experiments which have been completed. In the following discussion, the experiments are referred to by the number given in Table II-1.

TABLE II-1
Chlorophyll a-Deuterium Exchange Reactions

	Solvent mixture		NMR tube		C_{10}			Delta-Hydrogen			Final state
	Solvent	Exc. media	Gas	Sealed	temp.	t-1/2	complete exchange	temp.	t-1/2	complete exchange	
1.	acetone	10% D_2O	N_2	Yes	32°	3 h	13 h	25°	None	in 9 mos.	No oxid.
2.	"	"	air	No	"	"	"	"	"		Some oxid.
3.	"	" + DOAC, 0.01 M	N_2	Yes	"	rapid	1 h	"	4 mos.		
4.	"	" + DOAC, 0.04 M	N_2	Yes	"	"	1/2 h	75°	11 h	24 h	No oxid.
5.	acetone	30% CH_3OD	N_2	Yes	75°	rapid	--	75°	>193 h	None	No oxid.
6.[21]	$CDCl_3$	10% CH_3OD	--	--	38°	6.4 h	--	38°	100 h	--	
7.	"	"	N_2	Yes	32°	24 h	--	32°	150 h	--	
8.[22]	"	30% CH_3OD	"	Yes	75°	--	--	75°	16 h	--	No oxid.
9.	"	"	"	Yes	"	rapid	1 h	"	12 h	22 h	No oxid.
10.	"	"	O_2	Yes	"	"	"	"	"	18 h	Oxid.
11.	"	"	CO_2	Yes	"	"	"	"	"	46 h	No oxid.
12.	"	"	room air	No	32°	76 h	--	32°	1/2 h	2-8 h	
13.	"	30% CH_3OH	"	No	"	"		"	"	1-1/2 h	Slight oxid.
14.	"	"	air	No	"	None	None	"	None	None	No oxid.
15.	"	30% CH_3OD + DOAC, 0.01 M	N_2	Yes	75°	rapid	1 h	75°	4 h	12 h	No oxid.
16.	"	" + D_2O, trace	"	Yes	"	"	"	"	10 h	45 h	No oxid.
17.	$CDCl_3$	10% D_2O	N_2	Yes	75°	rapid	1 h	75°	13 h	>46 h	No oxid.

Results

Acetone-D_2O was the first solvent system in which the exchange reaction was studied. The choice of this system was made because of its applicability to the later biological reactions in which the exchange media will be D_2O and the extraction of the chlorophyll will be carried out using acetone. Thus, it is of importance to be aware of the hydrogen lability to be expected in the isolation of the pigments. Results showed (#1) that the C_{10} proton exchanged with a half life of 3 hours at the NMR probe temperature, 32°. There is no evidence that the delta position exchanges after nine months.

Rapoport[22] (#8) and then Katz[21] (#6) reported exchange reactions using the solvent system $CDCl_3$-CH_3OD, in which they found very slow delta exchange at 38° and at 75°. Because of the general discrepancy in our results between the solvent systems ($CDCl_3$-MeOH shows delta exchange while acetone-D_2O does not), their experiments were repeated in this laboratory to make certain that there was not an error in technique. The results were comparable (#7, 9), thus leaving the problem of explaining the qualitative difference in the behavior of chlorophyll in these two solvent systems.

It is proposed that the more acidic nature of the chloroform system is causing the delta exchange; or, conversely, that the more basic nature of the acetone carbonyl group inhibits the delta exchange. This proposal is based on the finding that when traces of acetic acid are added to either solvent system, a rate enhancement is found for the delta exchange (#3, 4, 15). Rapoport[22] suggested that the delta exchange could be explained by an eneamine tautomerization, which is acid catalyzed; there is other evidence of methine position exchange of chlorophyll

derivatives in acetic acid.[10] Electron density calculations show that the methine position which is adjacent to a reduced pyrrole ring should be subject to electrophilic attack.[26]

Other solvent systems used in this study are acetone-CH_3OD, and $CDCl_3$-D_2O. As would be expected from the acid catalysis theory, no delta exchange was found in the acetone system (#5); but the $CDCl_3$-D_2O showed exchange with a half life comparable to that in the $CDCl_3$-CH_3OD system (#17).

In the course of trying to explain the difference between the exchange behavior in various solvents, an extremely interesting rapid exchange of the delta position was found (#12). If the NMR sample was not prepared and sealed on the vacuum line but simply mixed using $CDCl_3$-CH_3OD in the open air, the half life of the delta position at room temperature is half an hour. This is extremely rapid, in comparison to a half life of 12 hours at 75° when the tube is sealed under N_2. This phenomena does not occur with the acetone solvent system.

It was first shown that the rapid exchange reaction is not a simple oxidation which selectively removes the delta proton. This was accomplished by looking at a sample in $CDCl_3$-MeOH (#14). If it was a simple oxidation, then the delta peak would have been removed from the NMR spectrum in this experiment. Further evidence against oxidation is found in the reversibility of the reaction (#12, 13). When the exchange is complete at the delta position, the solvent is evaporated, and $CDCl_3$-MeOH is added. The delta peak returns with a half life of half an hour. Thus, the loss of the delta peak under these conditions is in fact an accelerated exchange reaction.

Because of the potential biological significance of a rapid hydrogen transfer reaction, the investigation was continued, to try to understand this phenomena. Samples prepared on the vacuum line under oxygen and carbon dioxide failed to show a rapid exchange. The possibility that water vapor in the air could be the cofactor was eliminated, since no rate enhancement was found (#16) by adding D_2O to the system.

It had been assumed that the "air" in some fashion catalyzes this reaction, but it was also possible that the catalyst was a volatile impurity in the chloroform which is removed to some extent during the degassing of the solvents. Thus, the exchange reactions take place only very slowly even when air is admitted to the vacuum line tube before sealing. To test the possibility of a volatile impurity, a tube was prepared as usual on the vacuum line and sealed under nitrogen. The delta position was found to be completely intact. The tube was then opened to the air and shaken thoroughly; no delta exchange was found in two hours following which slight decomposition took place. Thus, it appears that the solvents could have a volatile impurity which is catalyzing the reaction.

At this point it was also found that the immediate exchange, which had been consistently reproducible in the past, did not occur in some of the other samples of deutero-chloroform in the laboratory. Since it was known that the exchange reaction can be acid catalyzed in the acetone-D_2O system, and that the immediate exchange could be catalyzed by such volatile impurities as HCl, acetic acid (0.01 M) was added and the immediate exchange was carried out. It was found that in the samples of chloroform in which the immediate exchange reaction would not take

place, the reaction proceeded normally (maximum half life of 30 minutes) when acetic acid was added.

Thus, it seems that not only can the results of Rapoport[22] and Katz[21] be attributed to acid catalysis, but that the immediate exchange found in this laboratory is also a consequence of acid catalysis. Both Rapoport and Katz state that their exchange reaction vessels and solvents had been carefully purged with nitrogen, so that it is reasonable that their rates of reaction would be slow in comparison to the immediate exchange, but comparable to the vacuum line experiments.

The conclusion of the study of the organic solvent exchange reactions is that acetone is the best solvent for the extraction and purification of the pigments, since the delta position does not exchange under these conditions. It should also be noted that there was no rate enhancement due to light.

Biological Experiments

With the knowledge that the delta position would not re-exchange during the isolation procedure of the biological experiments, the proposed work was started. During the course of the experiments, Katz *et al.*[27] published results for the proposed biological experiments. Their findings show that there is no delta incorporation and no evidence of incorporation in the reduced pyrrole ring in the 7,8 positions, although these signals would be very broad and difficult to detect. Their work went one step further than our plans: they did the reverse experiment using completely deuterated *Chlorella* and photosynthesizing in H_2O. In this manner, detection of exchangeable hydrogen should be easier because the remainder of the hydrogen atoms do not appear in the spectrum.

There is no evidence concerning the 10 position, because this proton would re-exchange during their purification procedure. Katz completely purified the pigments in order to take the spectrum, although this would not be necessary. A spectrum of the algal extract shows the delta position clearly; the 10 position is not identifiable, being hidden under a number of other signals from extractable material.

Because of the difficulty in evaluating the role of the 10 position in photosynthesis using hydrogen isotope tracers, the next chapter will consider the role of the 10 position using oxygen isotope tracers in conjunction with the enolization of the beta-ketoester.

CHAPTER III. OXYGEN 18 EXCHANGE REACTIONS: ALDEHYDES, KETONES AND CHLOROPHYLLS*

This investigation of the exchange reactions of aldehydes and ketones with O^{18} enriched water has been undertaken in order to be able to examine the participation of chlorophyll carbonyl groups as chemical intermediates in the oxidation of water during photosynthesis. Photosynthetic mechanisms, presented in Chapter I, have been proposed by Calvin[9] and Franck[8] in which the separation of oxidant and reductant, required for oxygen evolution and carbon dioxide fixation, is a photocatalytical chlorophyll reaction. Essential to these mechanisms are the ability of a chlorophyll carbonyl group to undergo hydration, and the enolization of the 10 position. The present work evaluates the exchange ability of chlorophyll in comparison with simple ketones, aldehydes, and beta-ketoesters.

The literature on the O^{18} exchange reactions of carbonyl functions indicates that aldehydes exchange very rapidly in comparison to ketones. Acetaldehyde exchanges completely at room temperature in neutral solution within 24 hours,[28] while acetone exchange is incomplete after 24 hours at 100°.[29] A comprehensive survey of the literature by Samuel and Silver[30] gives exchange rates in various solvents, acidic and basic, for acetone, acetaldehyde, acetoacetate, para-substituted benzophenones, methyl cyclohexanones, and substituted benzaldehydes.

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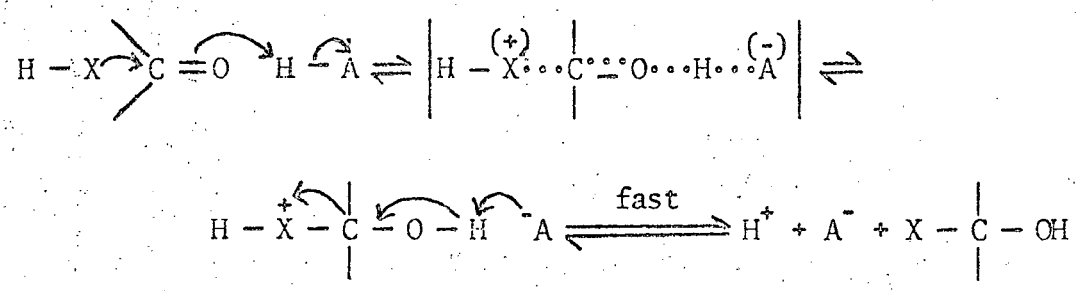
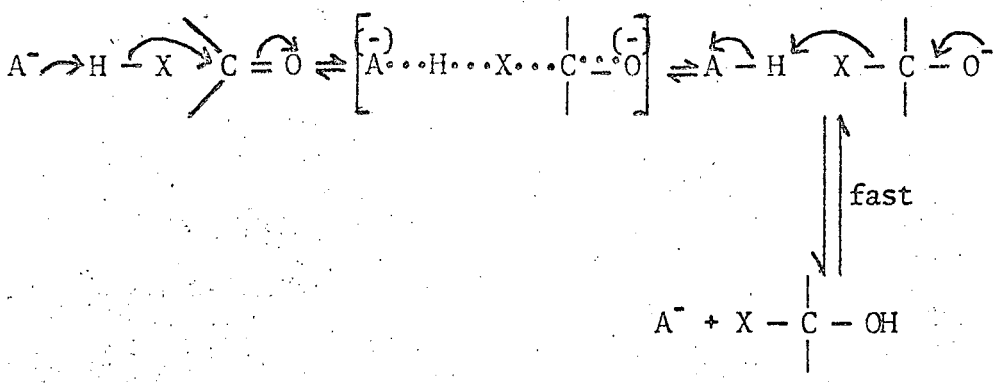
*The contents of this chapter appear elsewhere: Marianne Byrn and Melvin Calvin, J. Am. Chem. Soc. 88, 1916 (1966).



(1)

General base catalysis involves the concerted removal of a proton from the attacking reagent, to facilitate attack at the carbonyl group, as shown in Equation 2.



(2)

In the back reactions, the roles of the acid and base catalysts are reversed, thus completing the O^{18} exchange.[35]

Experimental

Tetrahydrofuran (THF), which is freshly distilled from lithium aluminumhydride, was found to be the only suitable solvent for the hydrochloric acid catalyzed exchange reactions. It is miscible with water, transparent in the infrared from 1500-1800 cm^{-1}, and dissolves chlorophyll, chlorophyll derivatives, ketones, aldehydes and beta-ketoesters. Piperidine was used as the solvent and catalyst for the basic reactions. Pyridine was the only other basic solvent examined which did not possess

interfering absorption in the infrared, but it was not even strong enough a base to catalyze the exchange reaction for acetone. In all cases, 10 μl or .01 g of the substrate, and 10 μl of 60.7% D_2O^{18} (analysis by Weizmann Institute) were used. A high deuterium content water was used because normalized water absorbs in the infrared at 1650 cm^{-1}. A control sample using D_2O^{16} was run for each compound, to ensure that spectral changes were not due to chemical change or deuterium exchange. The molar excess of O^{18} in the water over exchangeable O^{16} was of the order of 25- to 50-fold for the ketones, and 100-fold for the aldehydes. For the ketones, 50 μl of THF or piperidine were used, whereas the aldehydes were less soluble and required 150 μl of solvent.

Infrared cells (0.025 mm path length) with IR-tran windows were used as the reaction vessels, since these windows are resistant to aqueous solutions, both acidic and basic. This enabled the exchange to be followed using a Beckman IR-7 spectrometer as the reaction proceeded at room temperature. The first few minutes of reaction time are spent in order to fill the cell and obtain the first spectrum, thus making it impossible to obtain a "zero time" reading. Because as much as 5 min may have elapsed, in several instances the reaction was complete by the time the first spectrum was obtained. In such cases, the designation in the following tables is for an immediate reaction (Imm.). For the remainder of the cases the time is stated for the earliest spectrum which shows no subsequent change in the carbonyl bands, this being the time required for the substrate and O^{18} enriched water to have come to equilibrium within the sensitivity of the observations. The type of spectra obtained is illustrated in

Figure III-2, depicting a mixture of benzaldehyde in .001 N HCl in THF. The exchange time of 20 min is in the optimum range for observational accuracy using this technique. As the exchange times become longer, they are more difficult to determine because the kinetics are exponential in O^{16} remaining in the carbonyl and O^{18} being incorporated, and as the end point is approached the spectroscopic changes become very small. If the exchange time is greater than an hour, spectra taken every 10-15 min show little change after the first few observations.

High temperature exchange reactions were accomplished in sealed tubes in an oil bath. At the completion of the reaction, the solutions were evaporated and the materials purified using thin layer chromatography.

Using this infrared technique, it is impossible to determine the exact extent of incorporation; the molar extinction coefficient for the heavy isotope band has been shown in previous research not to be the same as for the O^{16} carbonyl band, and the extinction coefficients are not predictable.[32-34] A rough comparison of the peak sizes indicates the exchange approaches 100% of the theoretically possible O^{18} incorporation, but in some cases the equilibrium appears to be at about 60-80% exchange. This could be a solvent effect on the extinction coefficient. However, the extent of incorporation of O^{18} in several of the compounds was determined using low voltage on the CEC Mass Spectrometer 21-130. Ten μl of cyclopentanone, cyclohexanone, and benzaldehyde were respectively mixed in 100 μl of acidic THF and 10 μl of D_2O^{18}, and allowed to stand until completion of the exchange. Fifty μl were used for an IR spectrum, and 50 μl were taken for a mass spectrum. The tetrahydrofuran had to be evaporated in order not to drown out the car-

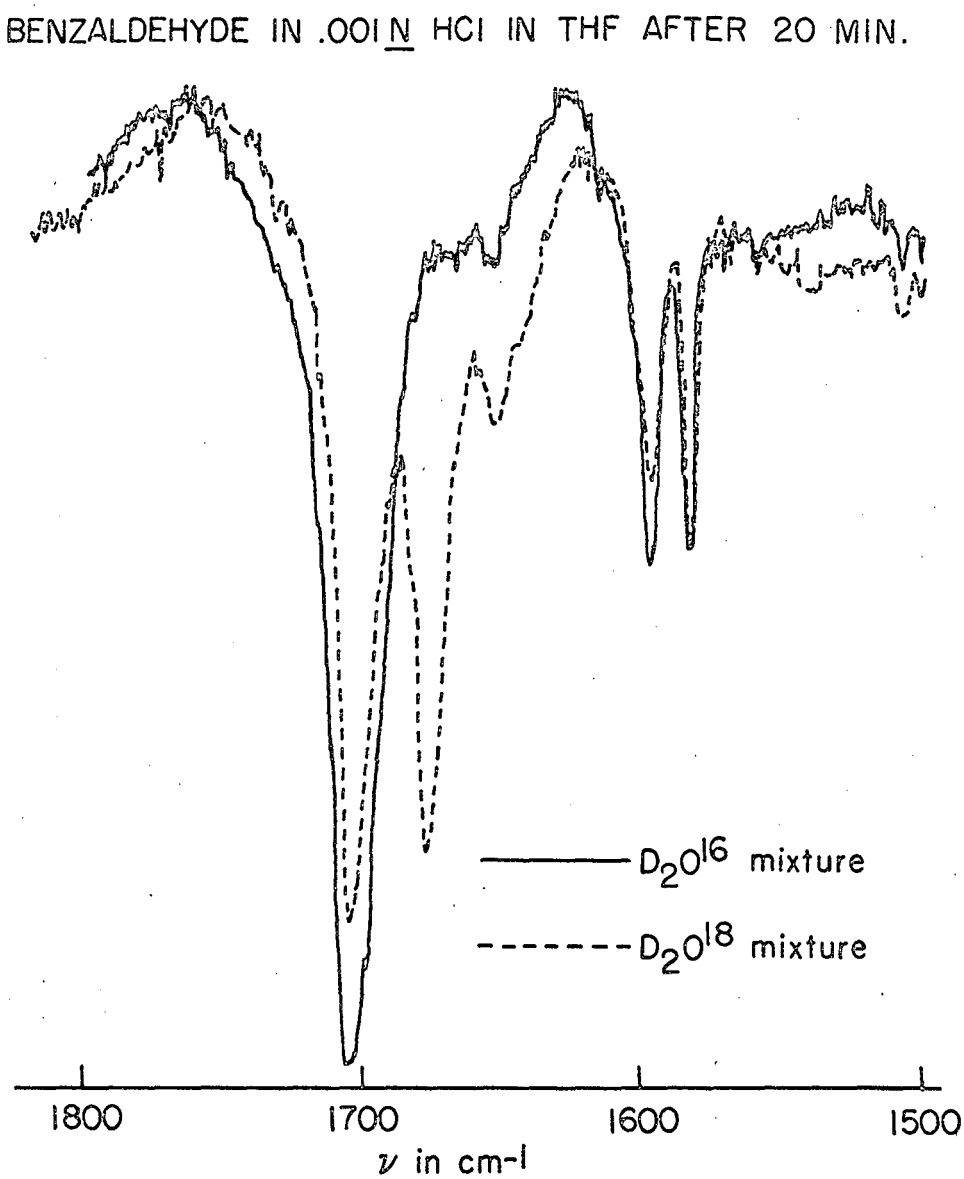


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Figure III-2. Benzaldehyde in .001 N HCl in THF after 20 min.

bonyl compound mass spectrum. From the ratio of the peak heights, the extent of O^{18} incorporation was determined:

$$\% \, O^{18} = \frac{(M+2) \; 100}{M + (M+2)}$$

The limit of detection of $C{=}O^{18}$ by infrared was evaluated by preparing benzaldehyde samples using successively smaller amounts of O^{18} and determining the extent of incorporation by mass spectroscopy. It was found that a 2% O^{18} incorporation is barely detectable above the noise level, assuming the spectrum is intense and the location of the isotope absorption is known. For less ideal conditions, 5-10% incorporation can easily be detected.

The assignments for the bands of the beta-ketoesters were taken from the work by Rhoades et al.[36] The simple ketones and aldehydes showed single bands in the carbonyl region, and presented no difficulty. Cyclopentanone is an exception to this fact in some aqueous solvent systems, in which case two peaks are found. Two peaks are also found for several other compounds, as noted on the tables. The infrared spectrum of chlorophyll has been recently studied by Anderson[24] and Katz,[37] and their assignments are in agreement. The carbonyl region is clear in polar solvents, and allows for relatively easy analysis of an isotope shift of 30 cm^{-1}.

The ketones, aldehydes and beta-ketoesters were obtained from commercial sources and used without further purification. The chlorophylls were prepared by the method of Calvin and Anderson.[23] The Pyro compounds were prepared by the method of Pennington et al.,[38] although a single homogeneous product was not obtained as their procedure stated. Separation from starting materials using thin layer chromatography

yielded the pure products possessing the correct spectral properties.

Results and Discussion

An unfortunate limitation in determining exchange time with this infrared technique is the very small range (5 to 60 min) which can be determined with appreciable accuracy, thus making it necessary to change acid concentrations in order to bring the exchange time into a measurable region.

Since the $C{=}O^{18}$ molar extinction coefficient is not known, nor is the equilibrium constant for the exchange known, I was unable to determine the absolute kinetic constants for each compound as would have been desirable in order to compare reactions of different compounds at various acid concentrations. Presumably the rate law in acid might be expected to obey the relation $\frac{dO^{16}}{dt} = - k\ [H^+]\ [O^{16}]$. For two compounds, indanone and anthraldehyde, the acid concentration was varied in an empirical fashion, keeping the exchange times within the region mentioned above, and then expressing the apparent time for equilibrium as some function of the hydrogen ion concentration. The equilibrium time observation appears to have been made in such a manner as to give the empirical relation shown in Figure III-3. This was used to determine relative exchange rates when necessary.

Aldehydes

The results of the acid catalyzed reactions of a series of aldehydes are summarized in Table III-1. For the series of aromatic aldehydes which exchange in 0.001 N HCl, the results are consistent with the mechanism discussed earlier. The effect of the protonation of the oxygen is to increase the electrophilicity of the carbonyl carbon and make it more

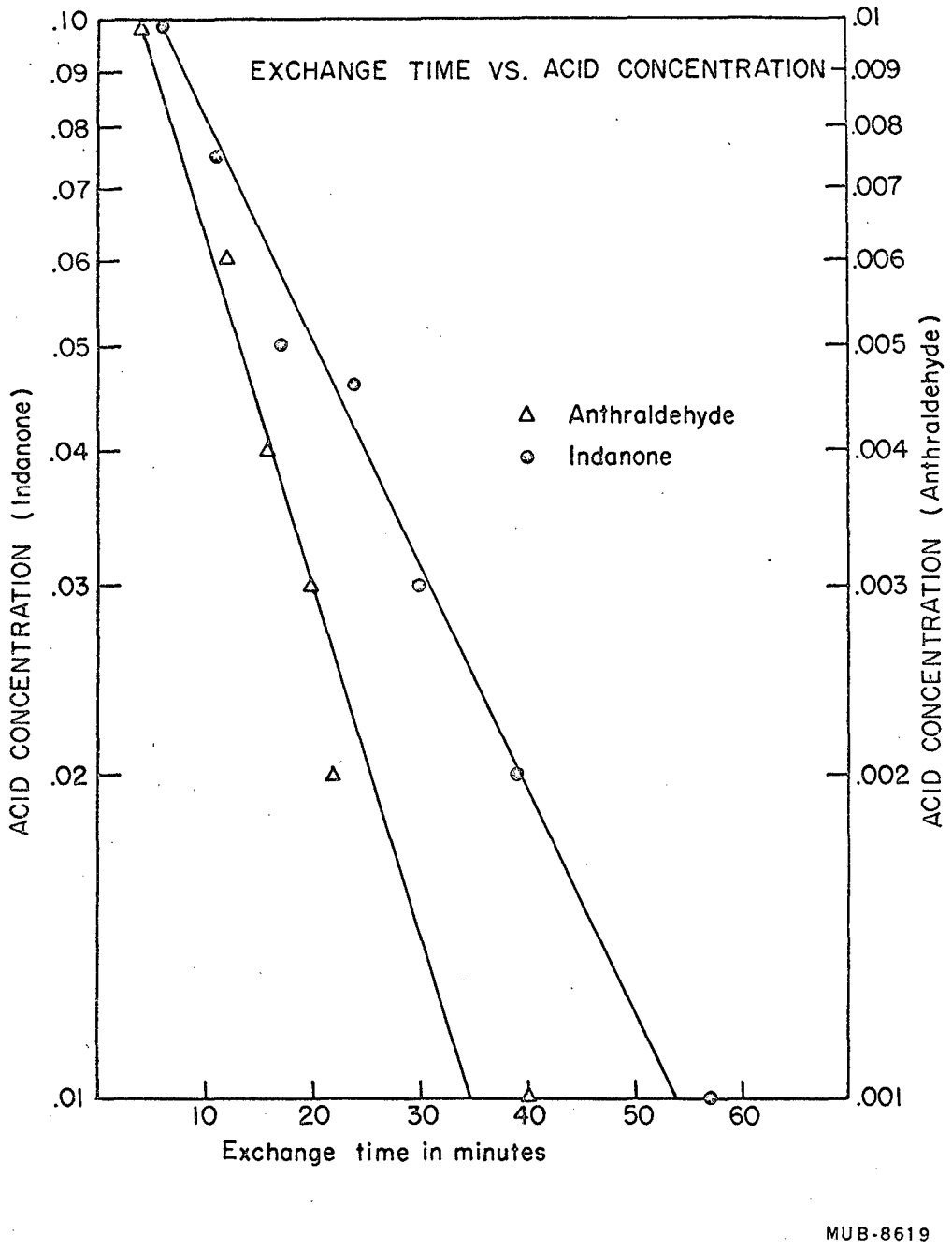
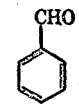
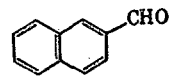
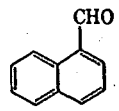
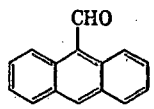
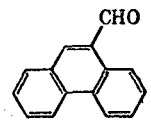
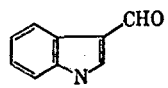


Figure III-3. Exchange time versus acid concentration for indanone and anthraldehyde.

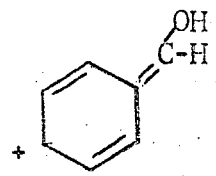
TABLE III-1

Acid Catalyzed Exchange Reactions of Aldehydes in Tetrahydrofuran

Compound		Concn of HCl, *N*	Time, min	ν, cm⁻¹ C=O¹⁶	ν, cm⁻¹ C=O¹⁸	Empirical resonance energy
Acetaldehyde[a]	CH_3CHO	0.001	Imm	1720	1692	0
Benzaldehyde[b]		0.001	20	1703	1675	36.0
2-Naphthaldehyde		0.001	25	1697	1668	61.0
1-Naphthaldehyde		0.001	35	1692	1664	61.0
9-Anthraldehyde		0.001	45	1676	1650	83.5
9-Phenanthraldehyde		0.001	55	1691	1661	91.3
3-Indole aldehyde		0.005	20	1667	1641	...
Chlorophyll b[c]		0.005	20	1665	1637	...
Pheophytin b[d]		0.005	20	1663	1636	...

[a] Decomposes rapidly. [b] 47% O^{18} determined by mass spectroscopy. [c] Purification of $C{=}O^{18}$ compound on mannitol tlc using 2% methanol in isooctane. [d] Purification of $C{=}O^{18}$ compound on mannitol tlc using 13% acetone in isooctane. Frequently, a considerable amount of the labeling was lost during purification.

reactive toward addition reactions. Opposing this increased electrophilicity is the effect of the extensive aromatic system, which acts as an electron donor to delocalize the positive charge on the carbonyl carbon, and hence, reduce the reactivity of the group in addition reactions, as shown in the following resonance form:



This ability of an aromatic system to delocalize charge can be measured as the empirical resonance energy,[39] which is directly related to the HMO delocalization energy and corresponds to the resonance hybrid.[40]

For the exchange reactions of these aromatic aldehydes, a good correlation exists between the empirical resonance energy, the time of the exchange reaction, and the frequency of the carbonyl absorption, both for $C{=}O^{16}$ and $C{=}O^{18}$ (see Fig. III-4). The relationship between the frequency of absorption and the rate of reaction is a reflection of the well known effect of conjugation on carbonyl stretching frequencies.

The three remaining heterocyclic aldehydes, indolealdehyde, chlorophyll b and pheophytin b, have identical exchange times. For indolealdehyde this exchange time, which is three times slower than benzaldehyde, is not caused by the aromatic ring since it is not directly conjugated with the carbonyl group. The slow rate can be attributed to a very stable resonance form involving the nitrogen atom, which would inhibit carbonyl addition reactions.

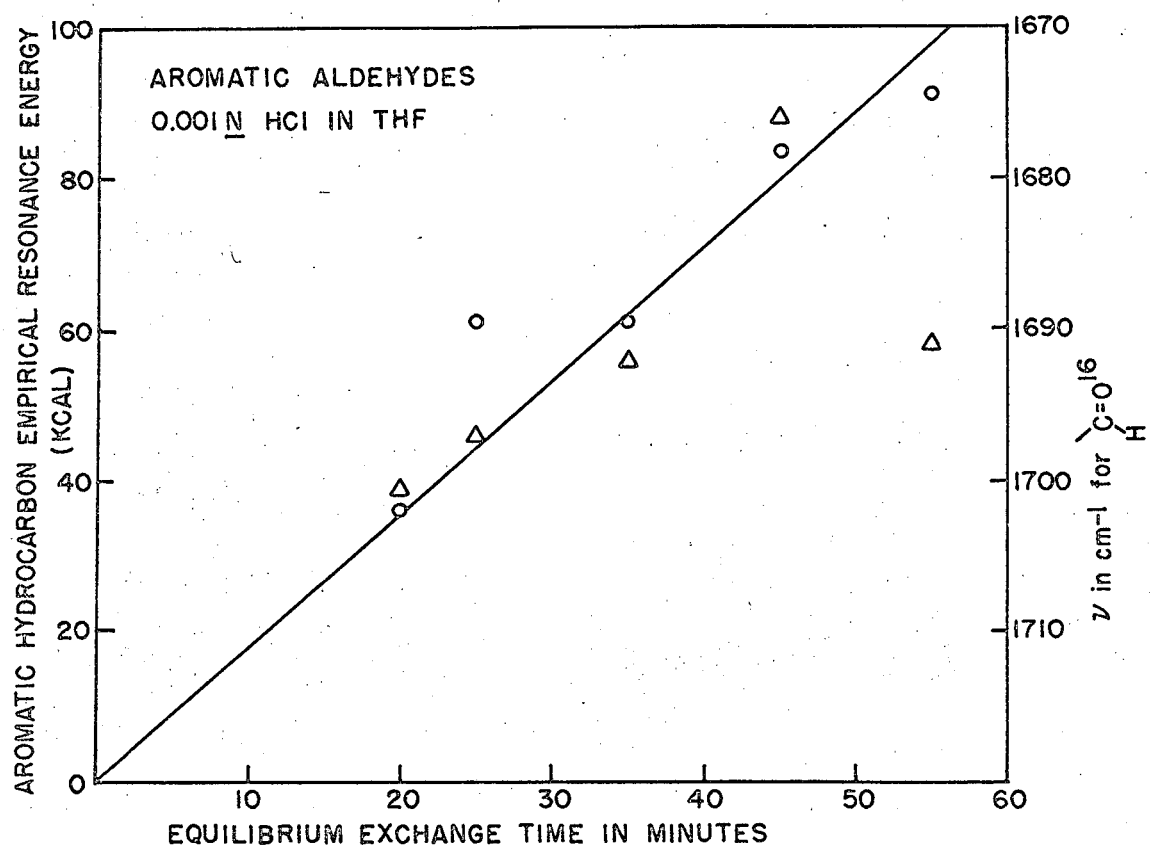


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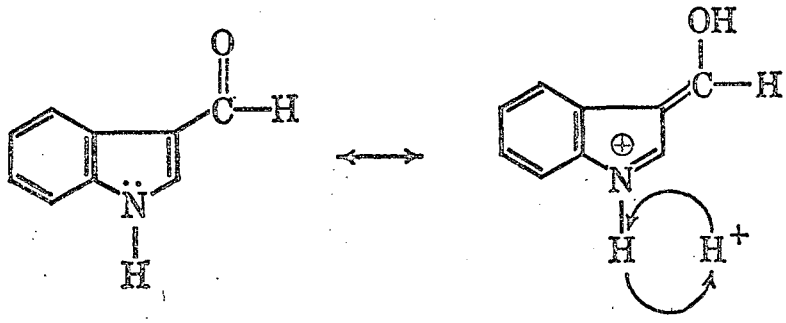
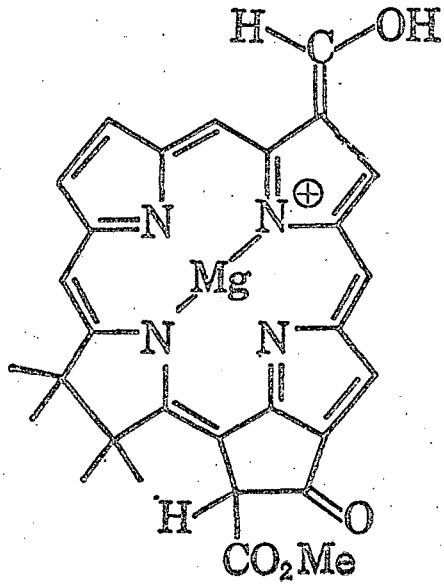
Figure III-4. Relation of equilibrium exchange time to the aromatic hydrocarbon empirical resonance energy and stretching frequency for aromatic aldehydes.



The same type of resonance structure can be drawn for both chlorophyll b and pheophytin b. However, such contributors are much less stable than the one for indolealdehyde because of the electrostatic repulsion of the chelated magnesium and the positively charged nitrogen. With indolealde-



hyde the hydrogen can be easily removed from the positively charged nitrogen, while this is not true of the magnesium. It follows then that the hydrogens which replace the magnesium in pheophytin b must also be unable to freely leave the heterocyclic nucleus since the exchange times for the two compounds

are identical. Thus the slow exchange time for chlorophyll b and pheophytin b is due to the extensive chlorin aromatic system, whereas indole aldehyde exchange is inhibited by the presence of the nitrogen atom.

Base catalyzed aldehyde exchange reactions were attempted using two different solvents, pyridine and piperidine. Pyridine was not sufficiently basic to catalyze the reactions at a rate which can be detected using the infrared technique. Piperidine was found to be so strongly basic that it formed an addition product, removing the carbonyl absorption band. Inorganic hydroxides were not used because they are known to allomerize chlorophyll.

Ketones

The results of a series of base catalyzed ketone exchange reactions and several beta-ketoester exchange reactions are summarized in Table III-2. There are no direct correlations between the exchange times, the stretching frequencies, or any other readily measurable quantity. Cook[41] has found a relationship between the XX'C=O bond angle, the ionization potential and the carbonyl stretching frequency; but these relationships cannot be extended to encompass the exchange times. However, these results can be qualitatively analyzed according to the mechanism presented above, on the basis of both electronic and steric considerations.

Acetone has the fastest exchange time, having neither steric interference nor electron donating groups to reduce the electrophilicity of the carbonyl carbon. Ninhydrin (1,2,3-triketohydrindene) is also extremely rapid, as could be expected with the electron withdrawing effects of the opposed keto groups and the lack of any steric interference.

The series cyclohexanone, cyclobutanone, cyclopentanone can be

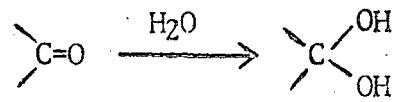
TABLE III-2

Base Catalyzed Exchange Reactions of Ketones in Piperidine

Compound		Time	ν, cm^{-1} $C{=}O^{16}$	ν, cm^{-1} $C{=}O^{18}$
Acetone	$CH_3-C(=O)-CH_3$	1mm	1708	1678
Ninhydrin		1mm	1714[a] 1739	1692
Cyclohexanone		10 min	1707	1678
Cyclobutanone		10 min	1780	1747
Cyclopentenone		15 min	1739	1708
Cyclopentanone		1 hr	1735	1702
Fluorenone		1 hr	1716	1685
Indanone		3.5 hr	1713	1683
Ethyl acetoacetate	$CH_3-C(=O)-CH_2-C(=O)-O-Et$	10 min	1711	1684
Carbethoxycyclopentanone[b]		...	1735	

[a] Two carbonyl frequencies are observed for ninhydrin, as they often are for anhydrides. [b] The ester carbonyl and keto carbonyl stretching frequencies are both in the same region and overlap; therefore, it is impossible to determine a rate for the reaction.

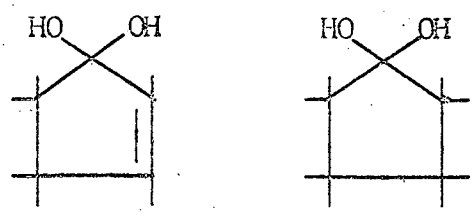
evaluated by considering the amount of ring strain which is lost when the sp^2 carbonyl bond is hydrated to form an sp^3 tetrahedral carbon in the intermediate,



The angles are 117°, 94°, and 108° respectively, for the carbonyl bond, whereas the bond formed for the hydrate is 110°. Thus, cyclopentanone is the slowest because it loses the least amount of ring strain. The relationship between acetone, cyclohexanone, and cyclopentanone can be seen in other carbonyl addition reactions such as semicarbazone formation.[35] Additional views are those of Price and Hammett[42] who note the increased reactivity of cyclohexanone compared to acetone as almost entirely due to a lower heat of activation. Brown, Fletcher, and Johannessen[43] have pointed out that a cyclohexane ring in which all of the carbon atoms are tetrahedral may exist in the particularly stable chair form, in which all of the valences are staggered, but that when one of the carbon atoms is trigonal, as in cyclohexanone, this stable configuration is impossible. Since the rate-controlling step involves the transformation of a trigonal carbon atom to a tetrahedral configuration, the reaction occurs particularly easily with cyclohexanone. In the case of cyclopentanone, the valences are more easily staggered in the ketone than in the reactive intermediate, and a decrease in reactivity is observed.

In the remainder of the compounds, cyclopentenone, fluorenone, and indanone, the ring strain is approximately the same since cyclopentanone rings are the fundamental units involved. The exchange times can be considered on the basis of the number of hydrogens which are eclipsed in the

intermediate and the effect of aromatic rings in reducing the electrophilicity of the carbonyl carbon. Cyclopentenone is faster than cyclopentanone, although a decrease in reactivity could be expected from the additional unsaturated bond of cyclopentenone. However, the unsaturated compound has one less hydrogen to sterically interfere in the intermediate hydrate.



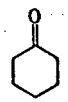
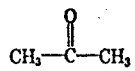
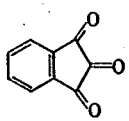
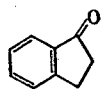
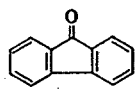
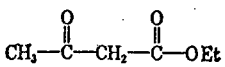
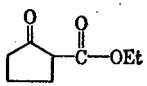
Another example of the predominance of steric interference over electronic inhibition is the difference in exchange times between indanone and fluorenone. Fluorenone, with two aromatic rings adjacent to the cyclopentanone ring, is 3.5 times faster than indanone, which has one adjacent aromatic ring, but two hydrogens which interfere.

The results of the acid catalyzed exchange reactions of ketones and several beta-ketoesters are summarized in Table III-3. It is immediately obvious that the sequence of compounds from the fastest to the slowest acid catalyzed reaction is quite different from the base catalyzed reactions. This reversal of order on going from acid to base catalysis was also noted by Menon[44] when studying p-substituted benzophenones.

The inversion of cyclobutanone and cyclopentanone can be explained on the basis of the basicities of the ketones. The pK_{BH^+} for cyclohexanone, acetone, cyclopentanone and cyclobutanone are -6.8, -7.2, -7.5, and -9.5 respectively.[45] The rate of exchange correlates with

TABLE III-3

Acid Catalyzed Exchange Reactions of Ketones in Tetrahydrofuran

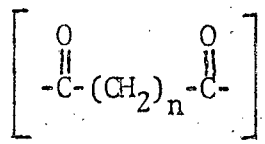
Compound		Concn of HCl, *N*	Time	ν, cm^{-1} C=O^{16}	ν, cm^{-1} C=O^{18}	Relative rates
Cyclohexanone[c]		0.001	Imm	1705	1682	<0.3
Acetone		0.001	Imm	1710	1680	<0.3
Cyclopentanone[c]		0.005	10 min	1745[a] 1728	1706	1
Cyclobutanone[e]		0.01	Imm	1782	1749	1.2
Ninhydrin		0.01	1.5 hr	1730[a] 1757	1704	11
Cyclopentenone		0.1	10 min	1664[a] 1703	1647 1682	12
Indanone		0.1	15 min	1713	1686	19
Fluorenone[d]		0.01	2.5 hr	1719	1686	>76
Ethyl acetoacetate		0.005	20 min	1719	1688	
Carbethoxy-cyclopentanone[b]		0.1	Imm	1750	...	

[a] Shows two ketone peaks in some solvents. [b] The keto peak of carbethoxycyclopentanone is a shoulder of the larger ester carbonyl peak, making it difficult to follow the exchange. The reaction time was determined from the disappearance of the O^{16} shoulder. [c] 46% O^{18} determined by mass spectroscopy. [d] 65°; at room temperature in acid concentration of 1 *N* some exchange takes place immediately, but the spectrum is very broad and difficult to interpret. [e] Decomposes rapidly.

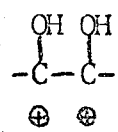
the increasing basicity of the ketones; the least basic, cyclobutanone, is least stable in the hydrated form and thus has the slowest exchange rate.

Campbell and Edward[45] showed that the basicity of cyclic ketones paralleled changes in stretching frequency; this correlation can be made with exchange rates for the first four compounds, although for the remainder of the compounds there is no relationship between stretching frequency, exchange rates, and basicity.

Ninhydrin is quite slow, and could be compared in basicity to a diketone. For diketones, basicity decreases as n decreases



and when n=0, basicity is less than typical ketones--that is, the form:



is very unlikely.[42] Since ninhydrin has three consecutive keto groups (2-hydrate), it follows that the reaction rate should be slower than the simple ketones. The fact that ninhydrin is faster than indanone reflects the electron withdrawing effect of the additional keto group to increase the reactivity of the opposed carbonyl group.

For the remainder of the acid catalyzed ketone exchange reactions, the balance between electronic and steric effects are the reverse of those for the base catalyzed reactions. Cyclopentenone is considerably slower than cyclopentanone and reflects the importance of the additional unsaturated bond and the relative unimportance of the steric

effects of the additional hydrogen. [Dahn[46] found the rate of cholestanone (cyclohexanone) to be 10^3 times faster than cholestenone (2-cyclohexenone), which again demonstrates the effect of one alpha-beta unsaturated bond.] Another instance of the predominant effect of electron donating functions is the very slow fluorenone exchange time relative to indanone. The aromatic ring hinders the reaction more than the additional hydrogens on the indanone moiety. This predominate effect of electron donating aromatic rings over sterically interfering hydrogen atoms in the acid catalyzed reactions is reasonable, since the stability of the conjugate acid towards the formation of the hydrate will be dependent on the electronic effects.

Beta-ketoesters

Cohn and Urey[29] showed that the exchange reaction of acetone does not follow the path of enolization, but is faster than enolization. The enol form is not subject to electrophilic attack and would decrease the rate of exchange. This is demonstrated with both ethylacetoacetate and carboethoxycyclopentanone, which are considerably slower than acetone and cyclopentanone.

Biological model compounds

Table III-4 summarizes the exchange reactions that were attempted with chlorophyll and chlorophyll derivatives.

The exchange reactions that were attempted with chlorophyll a and chlorophyll derivatives generally were unsuccessful. The pigments are unstable in basic solution and the magnesium is removed in acid solution. However, those compounds for which there were stable exchange conditions were quite resistant to hydration and exchange. The reactions were carried out under much more vigorous conditions than the model compounds,

TABLE III-4

Exchange Reactions of Biological Model Compounds

Compound	Acid	Base
Chlorophyll a	Pheophytinization in HCl. No incorporation in 10% HOAC, 66 hr.	Decomposition
Pyrochlorophyll a	Pheophytinization	No incorporation in piperidine 1 day; 65°
Pheophorbide a	No incorporation in .1 N HCl in THF; 1 day at 65°.	Decomposition
Pyropheophorbide a	Incorporation in .01 N HCl in THF; 1 day at 65°.	Decomposition

using temperatures of 65°. For the acid exchange reactions it appears that the large aromatic ring of the porphyrin nucleus has considerably decreased the reactivity of the C_9 carbonyl beyond that of any of the model compounds. The large difference in rate of hydration between chlorophyll a and carboethoxycyclopentanone is further evidence that cyclic beta-ketoesters are extremely poor models for the ring V structure. This disparity has been noted previously in the keto-enol equilibrium,[37,47] in the rate of exchange of the hydrogen in the 10 position,[19] in the inversion of the infrared stretching frequencies for the carbonyl functions,[36,48] and in the difference in solvent effects.[37]

From the results of the ketone exchange reactions in basic solution, it was expected that the simple ketone derivative of chlorophyll a, pyrochlorophyll, would show some exchange reaction under these strong conditions. Steric hindrance of the two C_{10} hydrogens is no greater than for indanone, although the electronic effects are considerably greater. Nevertheless, there is no obvious reason for such complete lack of reactivity of the carbonyl group. It is, however, clear that the isocyclic carbonyl oxygen atom would be stable to exchange during the course of any normal isolation procedure from the biological material..

CHAPTER IV. OXYGEN 18 EXPERIMENTS WITH CHLORELLA

This report is concerned with a series of *in vivo* experiments in which the green algae *Chlorella pyrenoidosa* is allowed to photosynthesize in oxygen 18 enriched water in a Warburg apparatus such that oxygen evolution can be measured, the gas analyzed for O^{18}, and then the chlorophyll extracted from the algae and also analyzed for oxygen 18 content. Extensive work on the exchangeability of the carbonyl functions of chlorophyll put forth evidence to show that during the extraction procedures to be presented here there will be no re-exchange of any oxygen 18 which may be incorporated.

Methods of Analysis

The methods of analysis reviewed previously in Chapter III indicated that the 30 cm^{-1} isotope shift in the infrared for oxygen 18 labeled carbonyl functions was the most suitable technique for the model compound study. However, it was thought that a second method, which was sensitive to small amounts of isotope and which was quantitative, would be more valuable for the biological experiments in which it is necessary to know absolute amounts of incorporation. For the model compound experiments, the infrared technique was satisfactory because the O^{18} enrichment was very high (~50%) and quantitative results were not necessary; although, in several cases where it was feasible, results that could be examined with mass spectroscopy were analyzed and found to be in agreement with an infrared estimation of the amount of incorporation. Assuming the $C = O^{18}$ molar extinction

coefficient is the same as that for $C = O^{16}$, it was found that the compounds were labeled to nearly the same extent as the water.

Mass spectroscopy was also tried with chlorophyll b which had been labeled in the C_2 aldehyde position. It was assumed, on the basis of the previous model compound work, that chlorophyll b would also label to the same extent as the water; thus, chlorophyll b labeled in the aldehyde position with 95%, 50%, 10% and 5% oxygen 18 enriched water were examined in the infrared and found to contain approximately that amount of oxygen 18. These labeled compounds were then analyzed by various mass spectroscopic techniques.

Several attempts were carried out to make a direct analysis of the chlorophylls in the high resolution instrument with a direct inlet system.* However, the results were useless. As other investigators had found,[49,50] an interpretable spectrum cannot be obtained with the means available. Thus it was necessary to attempt several pyrolysis techniques converting chlorophyll to CO_2, which can be readily analyzed.

For the purpose of chlorophyll analysis, pyrolysis introduces an inherent limitation, because chlorophyll a contains 5 oxygen atoms, and chlorophyll b contains 6 oxygen atoms; thus, you cannot specify the position of the incorporation, as in infrared, and there is a large dilution factor which considerably reduces an ability to analyze extremely small amounts of incorporation. That is, if there is 0.5 atom % incorporation in one carbonyl function, the CO_2 which is analyzed will have only an incorporation of 0.083 atom % in excess of

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*Analyses attempted by A. Burlingame in the Space Sciences Laboratory.

the natural abundance of .204%. Thus, this method begins with an important limitation in case the incorporation is small. This becomes a significant problem because in the proposal of the photosynthetic unit[15,16] it has been postulated that only one of every two hundred chlorophyll molecules participates as an active site. In this case, the maximum amount of incorporation to be expected would be 0.5 atom % if the experiments were carried out in pure H_2O^{18}. Despite these initial limitations, a series of analyses were completed.

General methods for the analysis of organic and inorganic compounds include those of Rittenberg and Ponticorvo,[51] and Anbar and Guttman.[52] The procedure of Rittenberg and Ponticorvo requires that the sample be pyrolyzed for several hours at 400°C in a sealed tube containing mercuric chloride. The tube is then opened on the vacuum line and the hydrogen chloride is removed by distilling the gas mixture through a column of 5,6-dibenzoquinoline into another tube immersed in liquid nitrogen. This tube is sealed and then opened on the mass spec inlet system. Anbar and Guttman used the additional reagent mercuric cyanide (which is necessary as a carbon source when water is analyzed) and instead of the quinoline column, the gas mixture was distilled into a tube containing a zinc-mercury amalgum. This tube is sealed and then heated at 200°C for two hours. The contents are then analyzed on the mass spec. Variations of these general methods were also carried out by changing the source of reagents, amounts of reagent, and sample size. It was also possible to eliminate the vacuum line step in which the HCl is removed from the pyrolyzed gases by opening the sealed ampoule in a glass tube containing silver sulfate which is attached to the mass spectrometer. This not only saves time

but also reduces the possible loss of sample in the vacuum line transfer. It appears to be equally as reliable as the other methods, as seen from water analyses. In general, there was no improvement in the results with any particular method. The results for chlorophyll b were routinely 60 to 30% low and the scatter was extremely large. The best results obtained are illustrated in Figure IV-1 for two series of analyses in which various chlorophyll b sample sizes were used. The methods for the two series are slightly different; one is with $HgCl_2$ and the other is with $HgCl_2 + HgCN_2$. It was concluded, after 30-40 samples had been analyzed, that these pyrolysis techniques were not applicable to chlorophyll analysis. Similar difficulties have been encountered with phenol[53] and with quinones, such as vitamin K.[54] For some unknown reason these few compounds do not show accurate results when analyzed by pyrolysis. The oxygen 18 content of vitamin K was finally determined by cracking and analysis of the CO released. However, this is impossible with chlorophyll because the large amount of nitrogen interferes with the carbon monoxide peak.

The unsuccessful attempt at a quantitative analysis through mass spectroscopy necessitated improvement in the detection of oxygen 18 via infrared, at least an improvement in sensitivity, even though the method could not be quantitative. This was first attempted by taking the difference spectra between labeled and unlabeled chlorophyll b with variable path length cells. This was unsuccessful because the cells do not hold organic solvents for a sufficient period of time (10-15 minutes are needed). A more sensitive analysis was accomplished by using a Beckman IR-9, which has a scale expansion unit (the intrument belonging to Dick Diamond, LRL, Bldg. 70) rather than the Beckman IR-7.

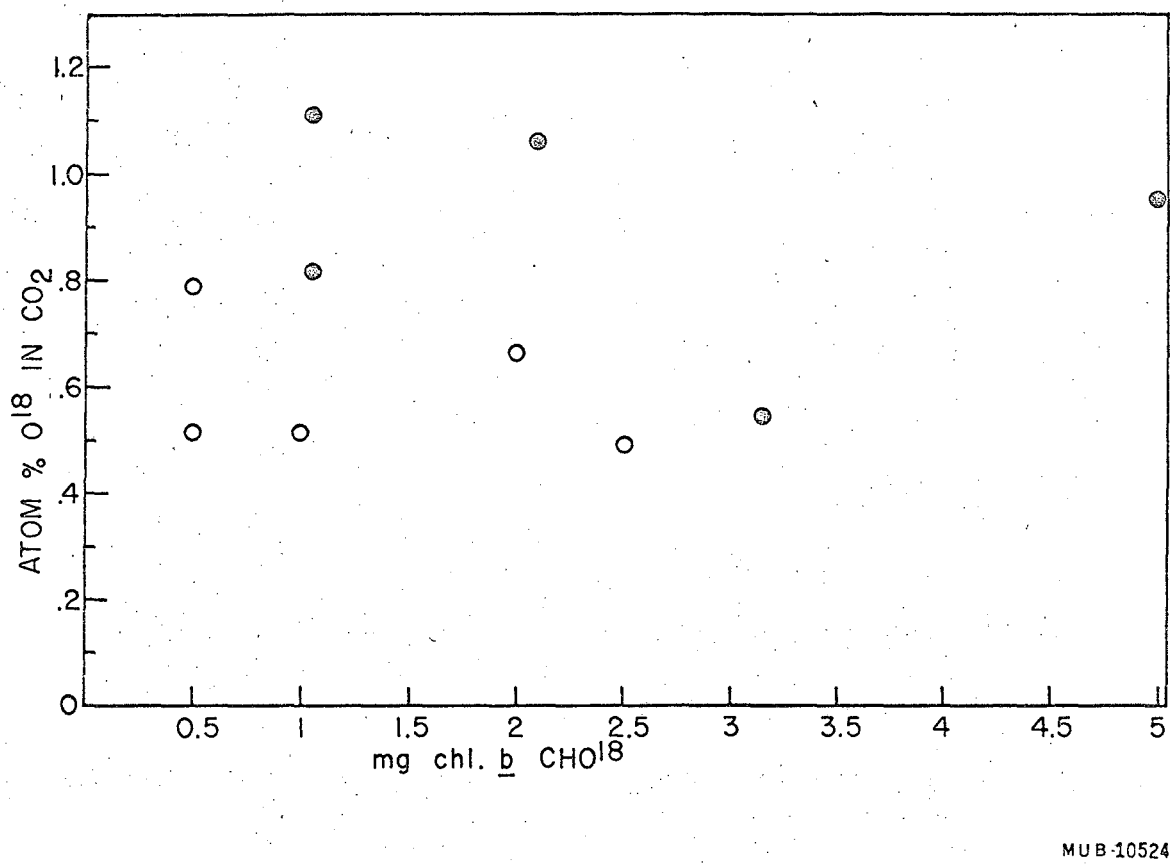

Figure IV-1. Pyrolysis of chlorophyll b CHO^{18} and mass spec. analysis vs. sample size.

o, $HgCl_2$; ⊗, $HgCl_2$ + $HgCN_2$.

This scale expansion unit allows any part of the absorbance spectrum to be expanded approximately ten-fold. With this attachment to the infrared, it became possible to detect as little as 1/2% oxygen 18 in a peak at 1635 cm^{-1}, which is the area where the $C = O^{18}$ stretching frequency of chlorophyll a would be expected (see Figure IV-2). Since it was never possible to label chlorophyll a in the carbonyl group of the isocyclic ring, a test of the limit of detection of O^{18} was carried out using a mixture of chlorophylls a and b. The aldehyde function of chlorophyll b absorbs at 1635 cm^{-1}, which is where the $C = O^{18}$ of chlorophyll a would be expected. In Figure IV-2, the peak at 1635 cm^{-1} is 1/2% of the peak of the chlorophyll a isocyclic ring carbonyl. This allows an approximate analysis of a very small amount of oxygen 18 and the position of incorporation, although an exact amount cannot be determined since the molar extinction coefficient cannot be determined. In the model compound study it was assumed that the extinction coefficient is the same for oxygen 16 and oxygen 18, although previous discussion has made it clear that this is not necessarily true.

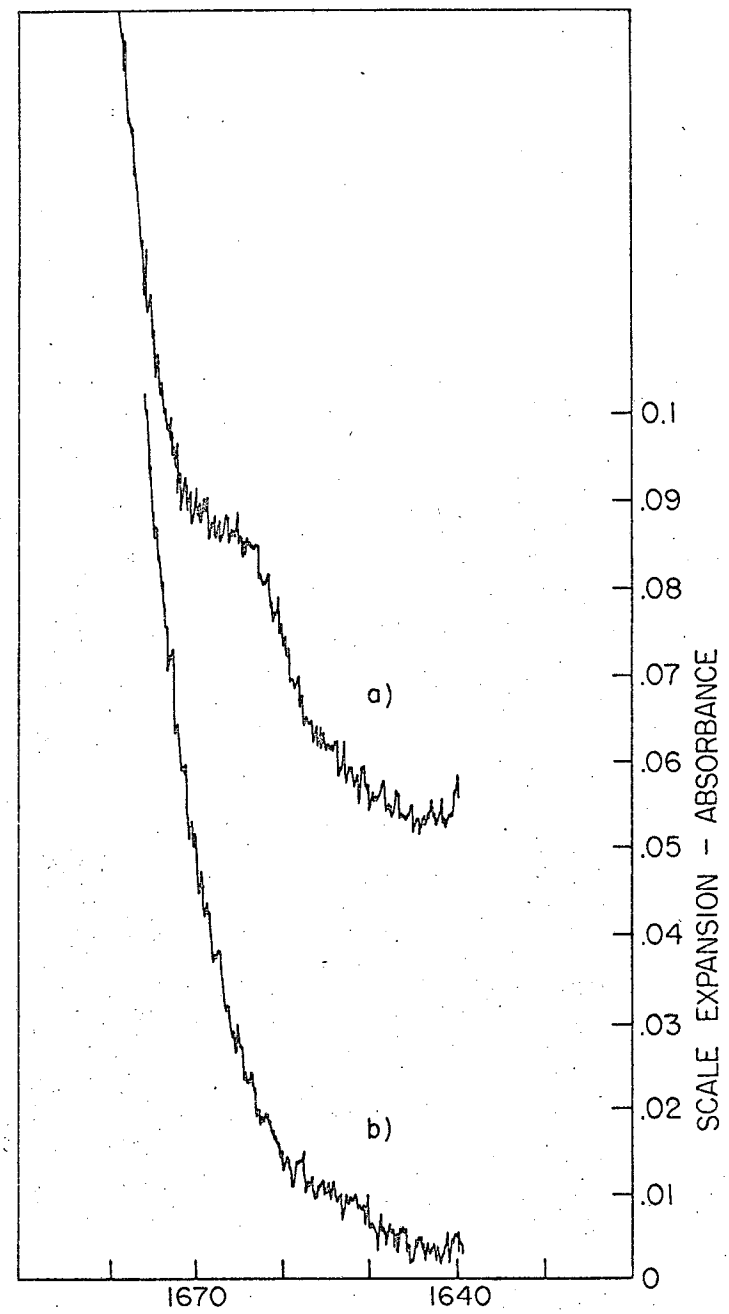

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Figure IV-2. Limit of infrared detection of oxygen 18 in the C_9 carbonyl of chlorophyll a. a) chlorophyll a plus 1/2% chlorophyll b; b) chlorophyll a extracted from Chlorella photosynthesized for 45 min in 60% H_2O^{18}.

Experimental

Warburg Procedure

Three hundred cc of the green algae Chlorella pyrenoidosa were taken from a tube in which they were grown under low light intensity. The algae were centrifuged in the International Centrifuge for 10 minutes at 1700 rpm, and then resuspended in 10 ml of bicarbonate media[5] (5 ml 0.1 M Na_2CO_3 and 95 ml 0.1 M $NaHCO_3$, pH 8.7). This tube is centrifuged for 10 minutes at 3000 rpm, yielding 1-1.2 ml packed cells.

The algae are then resuspended in 5 ml of oxygen 18 enriched media. The media was prepared 12 hours preceding the experiment by weighing out 2.6 mg Na_2CO_3 and 40 mg $NaHCO_3$ into a serum bottle. To this is added 5 ml of oxygen enriched water for which the high deuterium content has been normalized to less than 0.1% D. The medium is prepared ahead of time so that the carbonate may become labeled to the same extent as the water, such that if any oxygen 18 is incorporated in the chlorophyll, the amount of enrichment in the water would not be diluted by a slow exchange with the carbonate of the medium.

The algae suspension is then quickly transferred to a specially designed Warburg flask, to which is attached an evacuated gas tube with a stopcock connection, such that at the end of the experiment a sample of gas may be admitted to the evacuated tube, and this sample analyzed in the mass spectrometer to determine the amount of oxygen 18 evolved during the experiment. The Warburg flask is round, with an area of 63 cm^2, such that the algal suspension, which has a very high optical density, can be spread very thin (0.1 cm). The flask is

allowed to equilibrate in the 20°C water bath for 15 minutes until the manometer has reached an equilibrium value and the algae are respiring at a very slow rate. The lights are turned on to an intensity of 4,000 footcandles and the algae allowed to photosynthesize for 45 minutes. The manometers are read every 5 minutes (see data in Figure IV-3). The lights are turned off, a sample of the gas admitted to the evacuated tube, the manometers and flasks rapidly removed from the water bath, and the algal suspension pipetted into a freeze-dry flask immersed in liquid nitrogen. This procedure takes approximately 2 minutes. The flask is placed on a small freeze-dry apparatus attached to a high vacuum line, in order to recover the enriched water.

Pigment Extraction and Purification

The water-free algae are then placed in a Vertis blendor flask to which is added 5 cc of finely powdered glass, 5 cc of glass microspheres (210-297 microns), and 50 cc of acetone. The Vertis "45" blendor was operated at a speed of 20, sufficient to keep the algae and added material grinding for 1-1/2 hours.

This procedure was the only method found suitable for extracting pigments from lyophilized cells. *Chlorella* pigments can be extracted by lysing with organic solvents if water is present, but the pigments are not freed in non-aqueous solvents. The addition of water to the lyophilized cells was of only a little assistance in freeing the pigments.

The acetone solution of extracted pigments was filtered and evaporated. The pigments were redissolved in 4 cc of acetone and 1 cc of water, applied to a powdered polyethylene column, and purified according to the method of Calvin and Anderson.[23] The eluent containing the

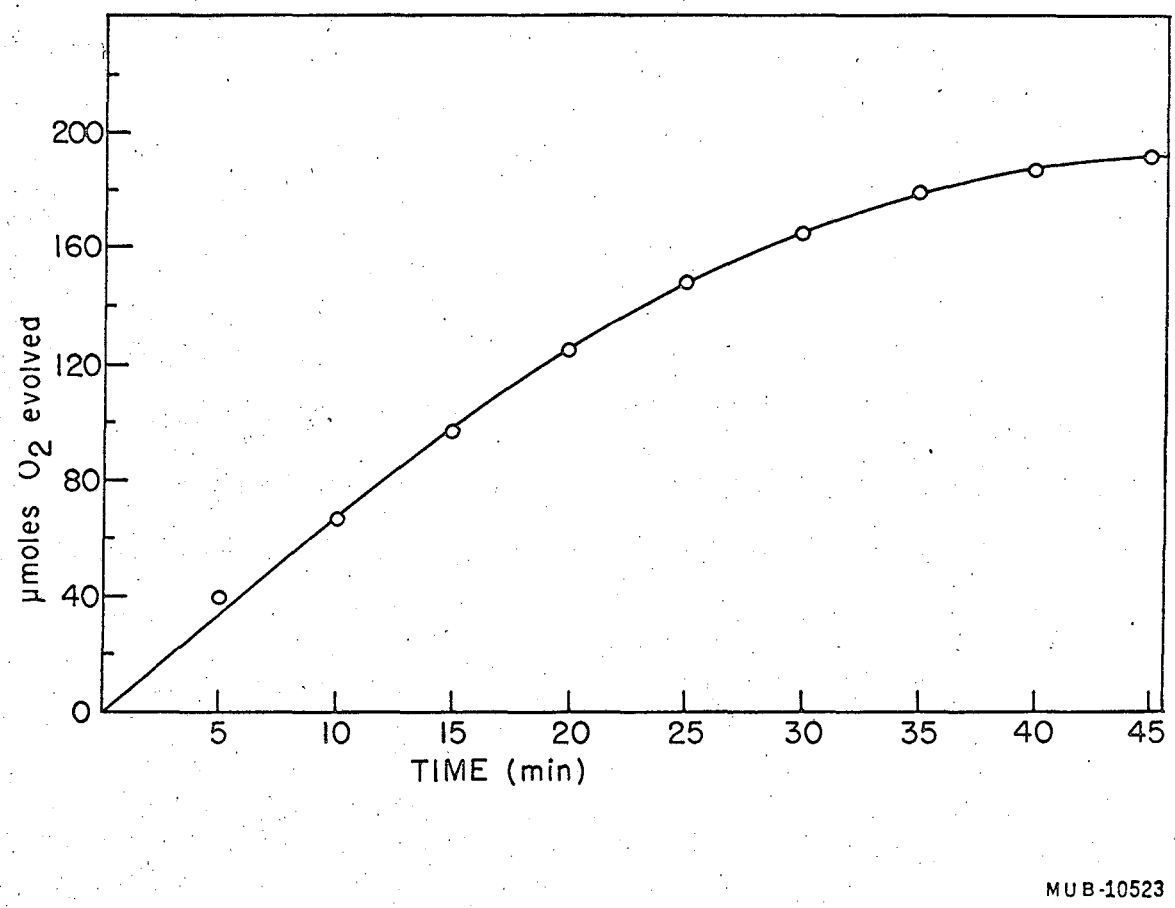


Figure IV-3. Evolution of oxygen during biological experiment with 59% H_2O^{18}.

chlorophylls is evaporated, redissolved in 200 μl of acetone and 2 mls of isooctane (2,2,4-trimethyl pentane), applied to a powdered sugar column, and the purification completed according to the above mentioned procedure. Very little chlorophyll was obtained, and attempts at recrystallization proved that too much valuable pigment was lost. Thus, the pigments were carefully water washed while in an ether-acetone solution, and the ether evaporated. A visible spectrum was then taken and the amount of pigment (ether: Chl a $\varepsilon_{428} = 8 \times 10^4$; Chl b $\varepsilon_{453} = 15 \times 10^4$) was determined by extinction coefficient. The pigment was dissolved in freshly distilled tetrahydrofuran and the amount of oxygen 18 incorporated was analyzed on the Beckman IR-9 in a 1.0 mm path-length barium floride cell.

Oxygen 18 Enriched Water Analysis

Water analyses followed the method of Anbar and Guttman.[52] Sample tubes with a constricted neck were made and dried at 200°C overnight to ensure that all the moisture was removed. A 10 μl sample of water, 100 mg mercuric cyanide and 100 mg mercuric chloride (which had been sublimed and stored in a dessicator) were placed in an ampoule which was evacuated and sealed. The sealed tube is heated for 2 hours at 400°C, and then opened on a vacuum line and the volatile contents condensed by cooling with liquid nitrogen into a second tube containing 2 grams of saturated zinc amalgum. After pumping off the non-condensable gases, the second tube is sealed and heated for 2 hours at 200°C. This treatment removes impurities including HCl, HCN and cyanogen, leaving purified CO_2 for analysis in the mass spectrometer.

The zinc amalgum obtained from commercial sources was found to release considerable quantities of CO_2 during the procedure, and was not

useful for these analyses. The zinc amalgum can be made by refluxing mercury with zinc granules for approximately 12 hours. A liquid paste will be formed and can be pipetted into the constricted tube.

The atom % of oxygen 18 incorporated is calculated from the following equations:[56]

$$\text{to } 5\% = \frac{96}{2\left[\frac{(44)+(45)}{(46)} + 1\right]}$$

$$A = \frac{(46)}{(44)} - \left(0.011 + \frac{B}{4}\right) B$$

$$B = \frac{(45)}{(44)} - .011$$

$$6 \text{ to } 50\% = \frac{A}{2.08 + 1.02\,B + A}$$

$$D = \frac{(46)}{(48)} - .011\left[\frac{(45)}{(48)} - .011\,\frac{(44)}{(48)}\right]$$

$$51 \text{ to } 99\% = 100\left(1 - \frac{D}{1.925 + .98\,E + D} - \frac{E}{1.96 + E + 1.02\,D}\right)$$

$$E = \frac{(47)}{(48)} - .011\,\frac{(46)}{(48)}$$

Analysis of Oxygen 18 Mixture

The change in gas pressure in the Warburg vessels is measured with the micromanometer; these micromanometer readings then can be converted into μmoles of oxygen released, after correction for changes in the atmospheric pressure measured according to the thermobarometer flask. The flask volumes were measured according to the mercury method recommended by Umbriet, and the flask constants calculated:[55]

Vg = volume of gas = 242.7 cc

Vg' = 1238.6 cc

Vf = volume of fluid = 6 ml

Po = Brodie's manometer fluid constant = 10^4

$T = 20°C$

$\alpha = 0.031$ for O_2 in Ringer's solution

$$k_O^2 = \frac{Vg \frac{273°K}{T} + Vf\ (\alpha)}{Po}$$

k_O^2 = (for algae flask) = 22.638

k_O^2 = (for thermobarometer) = 22.259

μmoles oxygen evolved = h (manometer reading) x k_O^2 / 22.4

μmoles oxygen evolved in algae flask = h x 1.01

From these equations the amount of oxygen given off during the period of photosynthesis can be calculated. The amount of the gas which contains oxygen 18 is determined from the mass spectrum, according to the following equations: (brackets designate the peak height of that mass number):[56]

$$\text{concentrations up to 50 atom \%} = \frac{100}{2} \cdot \frac{2(36) + (35) + (34) - 0.25\frac{(33)^2}{(32)}}{(32) + (33) + (34) + (35) + (36)}$$

$$\text{concentrations of 51 to 99\%} = \frac{100}{} \cdot \frac{2(36) + (35) + (34) - 0.25\frac{(35)^2}{(36)}}{(32) + (33) + (34) + (35) + (36)}$$

These calculations will not directly lead to the amount of oxygen 18 released by the algae, since the gas mixture which is analyzed contains both the air in the flask before photosynthesis and that oxygen which is released. Thus, a dilution of the photosynthetic oxygen occurs which is accounted for in the following way. The total gas volume of the flask is known, and the number of moles of oxygen evolved is calculated from the micromanometer data. The amount of oxygen in the total gas sample

analyzed is determined from the mass spectrum by simply taking the oxygen peaks as a percentage of the total spectrum. This can be done for the algae flask and the thermobarometer, such that the values obtained for the thermobarometer gas mixture are assumed to be the values for the algae flask minus the photosynthetic oxygen. Thus, the amount of oxygen in the flask before photosynthesis can be calculated, added to the total amount evolved, and the atom percent oxygen 18 which is evolved can be calculated:

$$\text{atom \% } O^{18} \text{ (gas mixture)} = \frac{O^{18}}{\text{total } O} = \frac{(O^{18}\ \text{unknown evolved}) + \text{natural } O^{18} \text{ abundance}}{O^{16} + O^{18}}$$

Culture of Chlorella

The chlorella was grown in modified Meyers medium[57] at pH 5.3, with carbon dioxide constantly bubbled through. The density was controlled at 0.6% v/v.

Because a large amount of chlorophyll is required for the infrared analysis, and it was not desireable to increase the volume of enriched water for the algae suspension used in these experiments, it was necessary to increase the chlorophyll content of the Chlorella. It has been shown that the plant compensates for a low light intensity by generating more chlorophyll.[58] Normally the Chlorella is cultured in long cylindrical tubes illuminated by fluorescent lamps. The lamps were removed one at a time, the algae given an opportunity to adjust to the reduced light intensity, and then another lamp was removed. This process was continued until the Chlorella would no longer respond to the reduced light intensity and would not maintain the usual turnover rate of approximately 16 hours. It was found that with 4 lamps the algae showed a good

turnover rate. The chlorophyll content was almost double that with 8 lamps. The amount of chlorophyll extracted from 300 cc of culture was sufficient for the infrared analysis.

Results

The results of the biological experiments using highly enriched water are summarized in Table IV-1. Numerous experiments were carried out with non-enriched and slightly enriched water, in order to perfect the procedure. Since there was no significant difference in O_2 evolution, amount of chlorophyll extracted, or gas analysis between the highly-enriched and slightly-enriched water experiments, they are not reported in detail.

It is apparent that sufficient water was oxidized, such that, if the carbonyl functions participated in the water splitting reaction, they would have become highly labeled.

The infrared spectra in Figure IV-2 demonstrate that no oxygen is incorporated in the C_9 carbonyl of chlorophyll a. Both in the absence and presence of phosphate, the infrared spectra did not show any evidence for either chlorophyll a or chlorophyll b involvement in the water splitting reaction or oxidative photophosphorylation.

TABLE IV-1

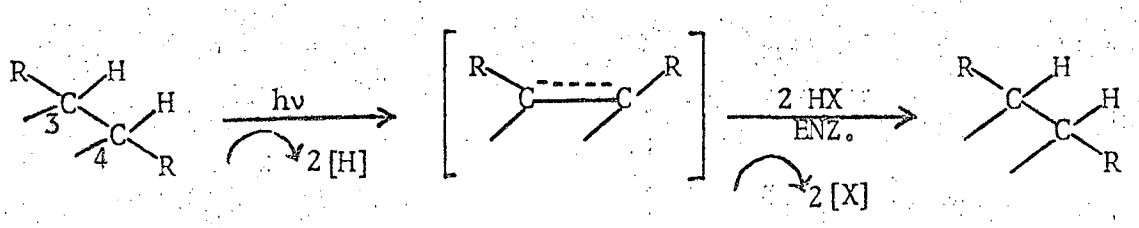
Results of O^{18} Biological Experiments

		I $[H_2O^{18}]$	II $[H_2O^{18} + KHPO_4{}^{18}]$
1.	Cell pack, mls	1.3	1.15
2.	H_2O^{18}, atom % O^{18}	59%	47%
3.	$KHPO_4$, 95 atom % O^{18}	None	0.02 M
4.	Total μmoles evolved	191 (see Fig. IV-3)	204
5.	Gas Analysis:		
	Algae flask: % O_2	18.6	18.9
	Atom % O^{18}	7.4	6.3
	Thermobarometric % O_2	17.0	17.2
6.	Total μmoles $O_2{}^{18}$	147	125
7.	μmoles H_2O^{18} oxidized	294	249
8.	Chlorophyll extracted:	17.7 mg	14.4 mg
	Chlorophyll a	15	12
	Chlorophyll b	2.7	2.4
9.	moles of H_2O^{18} oxidized / moles of chlorophyll extracted	14.8	15.5

CHAPTER V. TRITIUM STUDIES WITH BACTERIOCHLOROPHYLL

In the previous chapters, evidence has been given for a lack of chlorophyll a participation as a hydrogen transfer (or a water splitting) agent. In these studies, the chemical mechanism involved a reversible oxidation and reduction cycle, centered on the 7,8 positions, the delta hydrogen, the ring V C_{10} hydrogen and the C_9 carbonyl, and the C_2 carbonyl of chlorophyll b.

In this chapter, the notion is introduced 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 (see Figure V-1) and then reduced in the 3,4 positions as shown in the following scheme for a 2 hydrogen transfer:



The immediate impetus for such a proposal came from the work of Holt, et al.[59] and Olson and Romano,[60] who demonstrated the presence of both chlorin and dihydrochlorin pigments in the green photosynthetic bacteria,

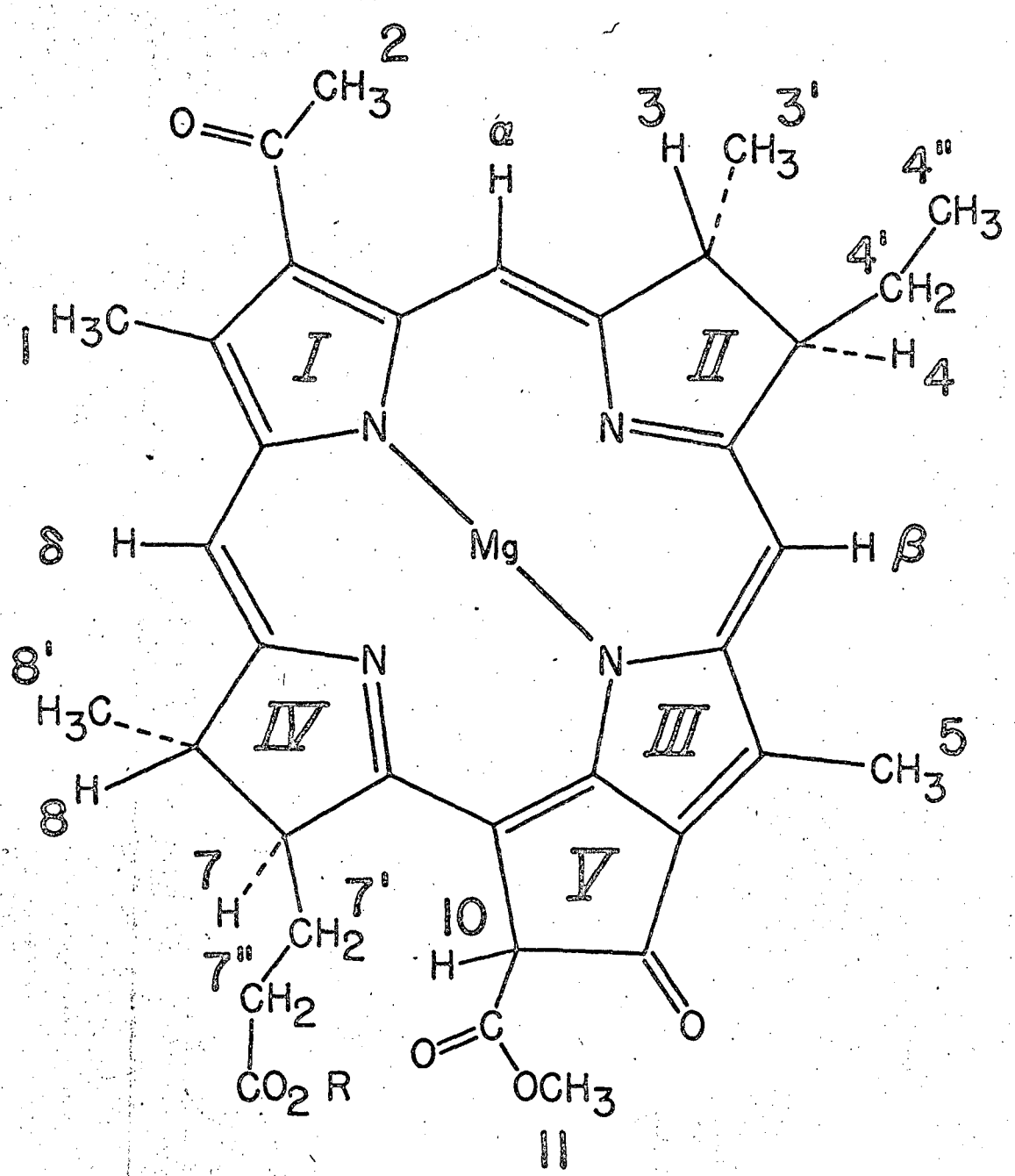


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Figure V-1. Structure of Bacteriochlorophyll and Oxidation Products.

		3 & 4	2
A.	Bacteriochlorophyll	+	+
B.	2-Desvinyl-2-acetyl chlorophyll a	-	+
C.	2-Desvinyl-2-hydroxyethyl chlorophyll a	-	- [-CH(OH)CH_3]

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[61] in the 3,4 positions using 2,3-dichloro-5,6-dicyanobenzoquinone, converting the dihydrochlorin to the chlorin, makes it possible for us to determine how much of the incorporated tritium is in the 3,4 position.

In the present investigation, Rhodospirillum rubrum is allowed to photosynthesize in tritiated media for 15 minutes. The bacteriochlorophyll (BChl) is extracted, purified, counted and then selectively oxidized in the 3,4 positions to form 2-desvinyl-2-acetyl chlorophyll a (OxBChl) (see Figure V-1).[61] The oxidized pigment is purified and counted, and in this manner it can be determined whether or not the radioactivity is specifically incorporated in the 3,4 positions. An attempt was made to distinguish photosynthetic and biosynthetic incorporation by taking bacteria which had incorporated tritium and resuspending them in non-tritiated media for an equal period of illumination. The question of biosynthetic versus photosynthetic incorporation is discussed at great length. A mechanism is proposed for the involvement of the 3,4 positions of bacteriochlorophyll in the photosynthetic decomposition of water.

Experimental

Materials

The acetone, methanol and ether were Baker and Adamson reagent grade, and were used without further purification. The isooctane (2,2,4-trimethylpentane) was Phillips 76 high grade. Polyethylene used for chromatography was a low melt index (M.I. 0.444) and the mannitol was Baker and Adamson reagent grade. 2,3-dichloro-5,6-benzoquinone from K&K laboratories was recrystallized.[61]

Thin layer chromatography

A suspension of 25 g of powdered polyethylene in 125 ml of acetone were mixed in a blender, spread without binder 0.25 mm thick, and allowed to dry at room temperature. The mannitol plates[62] were prepared in an identical manner using 65 g in 100 ml of acetone and 1 cc of a solution of 5 g of corn starch in 10 cc of water.

Procedure

Rhodospirillum rubrum (R.r.) was cultivated anaerobically at 31°C in small batches of 125-500 ml using modified Hutner's media.[63] After a designated period of growth, the bacteria were centrifuged in the International Centrifuge at 3,000 rpm for 20 minutes, resuspended in 12 ml of medium and centrifuged. The wet pack of 1.0 to 1.2 ml was suspended in medium to make a total of 6 ml of suspension. For each single experiment, 0.5 to 0.6 ml wet pack in 3 ml of suspension were placed in a small lollipop (flat cell of 5 mm path length), to which was added 150 or 200 µl of THO, such that the final suspension contained 1.64 or 1.52 x 10^{-2} mole % T, which is equivalent to a molar specific activity of 1.09 x 10^{13} or 1.02 x 10^{13} dpm/mole, (0.92 or

0.86 curies; the second figure pertains only to the final experiment in which a fresh THO sample had to be used). In double experiments, 6 ml of suspension were used and placed in a larger lollipop of the same thickness as the small one (5 mm), with the appropriate amount of tritium added. Nitrogen was bubbled through the solution to stir the mixture, and the lollipop was illuminated from both sides with a total intensity of 23,000 foot candles of white light at the surface. The bacteria were allowed to photosynthesize for 15 minutes.

In the first two experiments the solution was run into a bacterial filter attached to a vacuum line. The lollipop was thoroughly washed with water and the bacterial filter rinsed and sucked dry. For the remainder of the experiments the bacteria were separated by centrifugation for 10 minutes in an International Clinical Centrifuge. The wet pack was resuspended in media twice more and centrifuged. By the third wash, the supernatant contained a maximum of 0.2% of the original tritium. For experiments 4b, 5b, 7c, and 8c, the wet pack was resuspended in 3 ml of media and photosynthesized for an additional 15 minutes in the absence of tritium. The work-up procedure was then the same as for the other sample. During the experiments in which there were three parts (a, b, and c) care was taken that the same time intervals elapsed between centrifugation and photosynthesis. This ensures that any physiological effect of the separation procedure and the period in darkness would be the same.

The extraction of the pigments, and all further work, was carried out in a dark room under orange light from a 15 watt bulb. The asbestos mat filter was removed from the bacterial filter holder, torn apart, suspended in acetone, and the solution filtered. The mat was washed

until it no longer contained any pink coloring. The bacteria obtained from centrifugation were suspended in acetone and allowed to stand for a few minutes. The cell debris was filtered off and washed with several aliquots of acetone.

The acetone extracts were transferred into ether and washed with water several times to remove all readily exchangeable tritium. The pigment extract was then evaporated and dissolved in a small amount of acetone for spotting on powdered polyethylene thin layer chromatograms. The blue spot, which is bacteriochlorophyll, (R_f of 0.27 in 70:30 acetone:water) was scraped from the plate, the pigment dissolved in acetone, the solvent evaporated and the bacteriochlorophyll redissolved in 1 ml acetone. An aliquot was taken for a visible spectrum on a Cary 14 spectrophotometer, and the bacteriochlorophyll concentration was determined from the absorptivity at 770 mμ ($\varepsilon_{770} = 6.8 \times 10^4$ l/mole/cm). Two aliquots were taken for counting on a Packard liquid scintillation counter.

The remainder of the material was oxidized using 2,3-dichloro-5,6-dicyanobenzoquinone, according to the procedure developed by Lindsay Smith.[61] The oxidized pigment was purified on mannitol TLC with 2% methanol in isooctane. The main green band, 2-desvinyl-2-acetyl chlorophyll a, has an R_f of 0.40 and is occasionally followed by two small bands of lower R_f. The main spot was removed and eluted with ether, the solvent evaporated, and the oxidized pigment redissolved in 0.6 ml of acetone, from which aliquots were taken for a spectrum ($\varepsilon_{678} = 6.4 \times 10^4$ l/mole/cm) and counting.

Results

The results of seven experiments are summarized in Table V-1. These experiments indicate that the tritium which is incorporated is largely located in the 3,4 positions of bacteriochlorophyll (last column, Table V-1), and that for 4 day old bacteria, from 1 to 2.7% of the bacteriochlorophyll have been labeled (column 7 of Table V-1). A dark control experiment showed less than 0.03% incorporation. Since it has been proposed by Jones[10] that the final steps in the biosynthesis of bacteriochlorophyll involve reduction of the II pyrrole ring in the 3,4 positions, the question then is whether this photo induced tritium incorporation arises from a cyclic reaction as a part of the photosynthetic function or is due to unidirectional biosynthesis of new bacteriochlorophyll.

Rate of Photosynthesis and Bacteriochlorophyll Synthesis

The possibility of determining the rate of photosynthesis or growth rate of *R. rubrum* is complicated by the fact that there is no gas exchange for a ready measure of the evolution of photoproducts as in the oxygen 18 experiments. Double labeling experiments are precarious, since little is known about the metabolism of the substrates upon which the bacteria grow.[65] The method employed in this investigation involved measuring the increase in the spectroscopic maximum at 880 mμ of *in vivo* bacteriochlorophyll with respect to time (see Figure V-2); this was achieved by removing aliquots from a dense suspension of 4 day old photosynthesizing bacteria and taking difference spectra with a sample kept in the dark. The *in vivo* extinction coefficient ($\varepsilon_{880} = 1.88 \times 10^5$ 1/mole/cm) was found from a comparison of

TABLE V-1. Incorporation of Tritium in *Rhodospirillum rubrum*

Exp. No.	Water	R.r. age Days	BChl dpm/mole	Non-tritiated retention	Effect of centrifugation	% of theor. incorp.[a]	Ox. BChl dpm/mole	% T lost on oxidation
2	THO	4-6	2.0×10^{11}			1.8	1.5×10^{10}[b]	90[b]
3	THO	4-6	1.7×10^{11}			1.6	3.2×10^{10}	115[d]
4a	THO	2	2.9×10^{12}	87.8%		27	7.1×10^{12}	--[c]
4b	H_2O	2	2.6×10^{12}			24	3.3×10^{12}	--[c]
5a	THO	4	3.0×10^{11}	79.8%		2.7	5.5×10^{10}	81.6
5b	H_2O	4	2.4×10^{11}			2.2	5.9×10^{10}	75.2
6	H_2O	4	---			--	---	--
	THO	4	7.8×10^{10}		26% of 5a 30% of 5b	.71	3.1×10^{9}	96.0
7a	H_2O	4	---			--	---	--
	THO	4	1.41×10^{11}		127% of 7b	1.29	2.0×10^{10}	85.8
7b	THO	4	1.11×10^{11}	184%[c]		1.02	5.13×10^{9}	95.4
7c	H_2O	4	2.04×10^{11}			1.87	1.27×10^{10}	106.0[d]
8a	H_2O	3	---			--	---	--
	THO	3	8.57×10^{11}		123% of 8b	8.4[a']	3.14×10^{11}	62.4
8b	THO	3	6.95×10^{11}	75.8%		6.7[a']	5.05×10^{10}	106.7[d]
8c	H_2O	3	5.26×10^{11}			5.2[a']	8.74×10^{10}	83.4

[a] Ratio of specific activity of BChl to specific activity of water. Assuming all 3,4 hydrogens are labeled to the same extent as the water, the BChl would contain 1.09×10^{13} dpm/mole.

[a'] 1.02×10^{13} dpm/mole.

[b] The BChl was over oxidized and gave several green pigments. This figure represents the total for all green pigments.

[c] The amount of tritium in the second step is greater than the amount started with, and is probably due to contamination or a volumetric error.

[d] Bacteriochlorophyll was not completely oxidized, and samples had to be corrected for counts due to BChl.

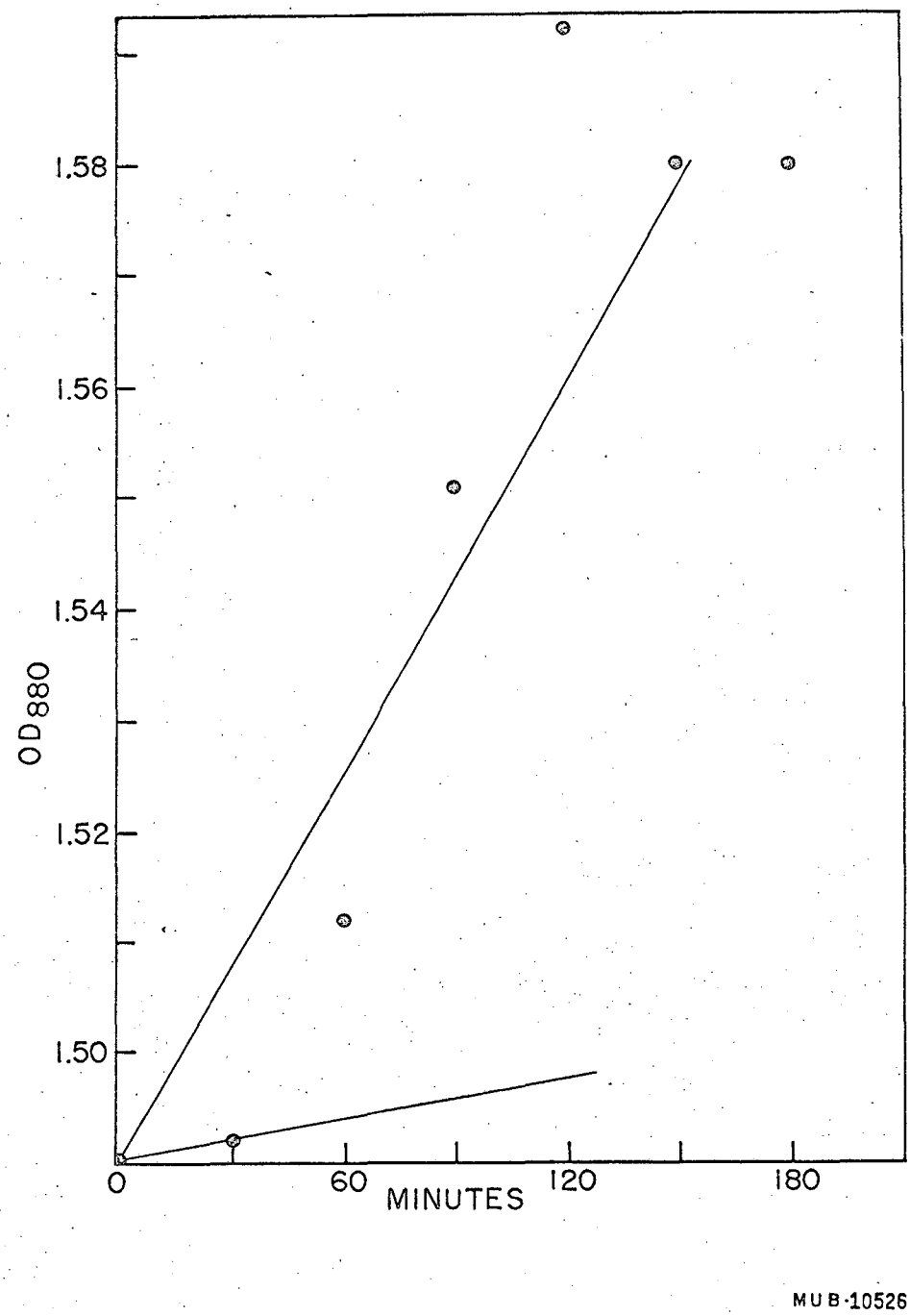


Figure V-2. Rate of increase in bacteriochlorophyll in R. rubrum.

the absorption of in vivo bacteriochlorophyll at 880 mμ with that of an equivalent acetone extract at 770 mμ. From the rate of increase in the bacteriochlorophyll absorption maximum it was calculated that new BChl was synthesized at the rate of 6.0×10^{-9} moles/l (dense suspension)/sec; that is, for the 15 minutes of illumination, an increase of 1.3 moles of bacteriochlorophyll in a suspension containing a total of 4.14×10^{-4} moles/l. From the rate of synthesis of new bacteriochlorophyll, the rate of photosynthesis was calculated. The ratio of bacteriochlorophyll to dry weight of bacteria was determined to be 1.6×10^{-7} g BChl per g dry weight bacteria, a value which remains quite constant as seen by the parallel increase in turbidity (λ 650) and BChl absorption during normal growth (Figure V-3); the dry weight of the cellular material was then expressed as moles of CH_2O by using an average molecular weight of 30. Since the ratio of BChl to dry weight of bacteria (CH_2O) remains constant, the increase in new bacteriochlorophyll is equivalent to an increase of 1.3×10^{-2} molecules of CH_2O/sec/molecule of BChl. For the 15 minute period of illumination, this is equivalent to 11 two-hydrogen reductions accomplished per molecule of bacteriochlorophyll, starting from CO_2.

These calculations are not intended to be a rigorous description of the physiology of the organisms under scrutiny since, as stated previously, the means are not presently developed for such purpose. However, the intention was to attempt a gross approximation of the cellular processes. As can be seen in Figure V-2, the data from which these calculations were made show considerable scatter, and the total change in O.D. is only 1%. A 1% error could easily be expected considering the small volumes used (150 μl) and the fact that difference

spectra were taken on a 0 to 0.1 slide wire on the Cary 14 spectrophotometer. These results appeared more doubtful since the slope of the line which was drawn, an increase of 1.3%, was comparable to an increase of 1.7% obtained by direct spectroscopic measurements following the growth of a batch of R. rubrum in a dilute suspension, over a period of days (Fig. V-3). It is unreasonable that the growth of a much older (4 days), extremely dense, optically strongly absorbing suspension should show a growth rate nearly as fast as that of a culture which is dilute, young, and optically transparent. This is especially important since it was found that the initial doubling time of R. rubrum cultures became longer when the suspensions were more dense[66] (see Table V-2). Thus it could possibly be that Figure V-2 represents an unknown artifact which is being measured or is simply a larger experimental error and not true bacteriochlorophyll synthesis. Although it

TABLE V-2

Rate of Growth of R. rubrum as a Function of Cell Density

Density	Doubling Time in Exponential Growth (hrs)
1	12
$\frac{1}{10}$	7.5
$\frac{1}{100}$	5.5

is questionable, the shape of the curve could be interpreted as a slow induction for building metabolic intermediates and then a short spurt of near normal maximum growth tapering off when the substrates are consumed. If this interpretation is correct, then the information of

interest is the slope of the initial growth in the first 30 minutes, as seen in Figure V-2. This represents a growth increase of 0.06% in bacteriochlorophyll, and is equivalent to 0.5 two electron reductions of CO_2 per molecule of bacteriochlorophyll during the 15 minutes of photosynthesis.

From this discussion it appears that even the spectroscopic method of determining the rate of photosynthesis or bacteriochlorophyll synthesis is of no use. The best interpretation that can be made is to say that the rate must be considerably less than a 1.7% increase in bacteriochlorophyll, and is probably no more than .1%.

Biosynthetic Incorporation

Assuming all the tritium incorporated is biosynthetic in origin, the increase in bacteriochlorophyll by synthesis, calculated from the amount of incorporation, is 1.6 mole % (for experiment #3 on 4 day old bacteria), which is considerably larger than the increase in bacteriochlorophyll by synthesis discussed earlier, as obtained from spectroscopic measurements. Thus, from this comparison it appears that the tritium incorporation is not biosynthetic, and that, although the last step in the biosynthesis of bacteriochlorophyll may be reduction of the 3,4 positions, this is probably only slightly reflected in these experiments. However, this conclusion is tenuous because it rests on the probablistic argument that the rate of biosynthesis in the dense suspension is less than 0.1%, whereas this figure is in fact an unproven experimental quantity.

The second argument against the incorporation being biosynthetic in origin rests on the absence of biological precursors. If the increase in bacteriochlorophyll is 1.6 mole % as determined by the tritium

in the 3,4 positions, one would expect to find that amount of 3,4-dehydrobacteriochlorophyll (OxBChl) as precursor present in the bacteria. During the separation procedure there was no indication of the presence of additional pigments, in contrast to the Chlorobium[59,60] case where there is a detectable amount of a dehydro precursor. An isolation of such precursors from Rhodopseudomonas spheroides was accomplished by Jones[64] with the aid of inhibitors to the biosynthesis of bacteriochlorophyll. Our attempts to identify the presence of a precursor as 2-desvinyl-2-acetylchlorophyll a by spectroscopic measurements on crude acetone extracts of bacteria were unsuccessful. The precursor (OxBChl) has an absorption at 678 mμ, which is in a relatively clear region of the spectrum of the crude extracts, although it is on the slope of the much larger absorption of the bacteriochlorophyll (λ_{max} = 770). A possible explanation for our inability to detect the precursor as OxBChl is that the larger precursor pool exists as the 2-hydroxyethyl pigment (see Figure V-1) rather than the 2-acetyl compound. The 2-hydroxyethyl pigment is the precursor identified by Jones, and could be converted to bacteriochlorophyll with the addition of hydrogen occuring only in the 3,4 positions. The absorption maximum of this compound is at 660 mμ, which is not a clear region in the spectrum of the crude extract of the bacteria and thus this earlier precursor cannot be readily detected.

From this discussion, it appears that the absence of evidence for the presence of acetyl precursor, and the inability to determine the presence of hydroxyethyl precursor in crude extracts as well as its failure to appear on chromatography, is further negative evidence on the possibility that the tritium incorporated is biosynthetic in origin.

Thirdly, the conclusion that the tritium incorporation is biosynthetic in origin can be questioned, since all tritium isotope effects have been neglected. Generally, the isotope effect from tritium in an organic reaction is in the range of 6 to 20, and a more typical value is 10.[67] There are several examples of enzymatic reactions for which isotope effects have been demonstrated. Succinic dehydrogenase involves a deuterium isotope effect,[68] and uracil hydrogenase, which is a NADH linked enzyme, shows a considerable isotope effect in tritium. Deuterium isotope effects have also been obtained with other NADH linked enzymes.[69] For this reason, one would expect the rate of bacteriochlorophyll synthesis calculated directly from tritium incorporation studies to be considerably smaller than the actual rate; that is, 2% tritium incorporation represents a 20% increase in bacteriochlorophyll. This consideration further strengthens the argument that the source of the tritium is not biosynthetic, because there can be no doubt that 20% of a pigment precursor would be visually detected, and that the rate of biosynthesis even under the most ideal conditions never reaches this high a value.

Photosynthetic Incorporation

On the other hand, if the tritium in bacteriochlorophyll is photosynthetic in origin, the cause of the low tritium incorporation of 1 to 2.7% could be due to the fact that there is a tritium isotope effect and that the actual rate of electron turnover is 20% and/or only a small percentage of the bacteriochlorophyll is involved in hydrogen transfer reactions. With the younger bacteria (experiment #4) there was considerably more tritium incorporated, as much as 20%. It is probable that some of this is biosynthetic in origin; but, in addition,

that the younger cells are in a growing phase rather than in a declining phase (see Figure V-3), and that the rate of photosynthesis would be expected to be much greater for the younger cells than the older ones. Certainly, this incorporation could not be biosynthetic at this high a value, which would represent an enormous increase in new bacteriochlorophyll even excluding an isotope effect.

Thus, the arguments put forth all indicate that the tritium incorporation is not biosynthetic, but rather photosynthetic in origin. Moreover, additional experiments involving re-exchange of hydrogens were carried out in an attempt to clarify the mechanism which allows for hydrogen exchange during photosynthesis.

In four experiments, 4ab, 5ab, 7bc, and 8bc, the bacteria which had incorporated tritium were resuspended in non-tritiated media and allowed to photosynthesize for an additional 15 minutes. To ensure that the bacteria had not undergone some physiological change due to the centrifugation and period in darkness between the photosynthetic periods, (experiments 6, 7a, and 8a) controls were run in which the bacteria were illuminated in non-tritiated media for 15 minutes, isolated by centrifugation in the same manner as the tritiated bacteria, and then illuminated for 15 minutes in tritiated media. Experiment #6 indicates that the incorporation of tritium is only 25% of the amount incorporated before centrifugation; however, this experiment could not be repeated since two additional experiments, 7bc and 8bc, demonstrate that the tritium uptake is 125% after centrifugation, in experiments 7b and 8b. These results indicate that there is no deleterious effect on the bacteria from the procedure followed during the double illumination experiments, and that the physiological condition of the bacteria is similar before and after centrifugation.

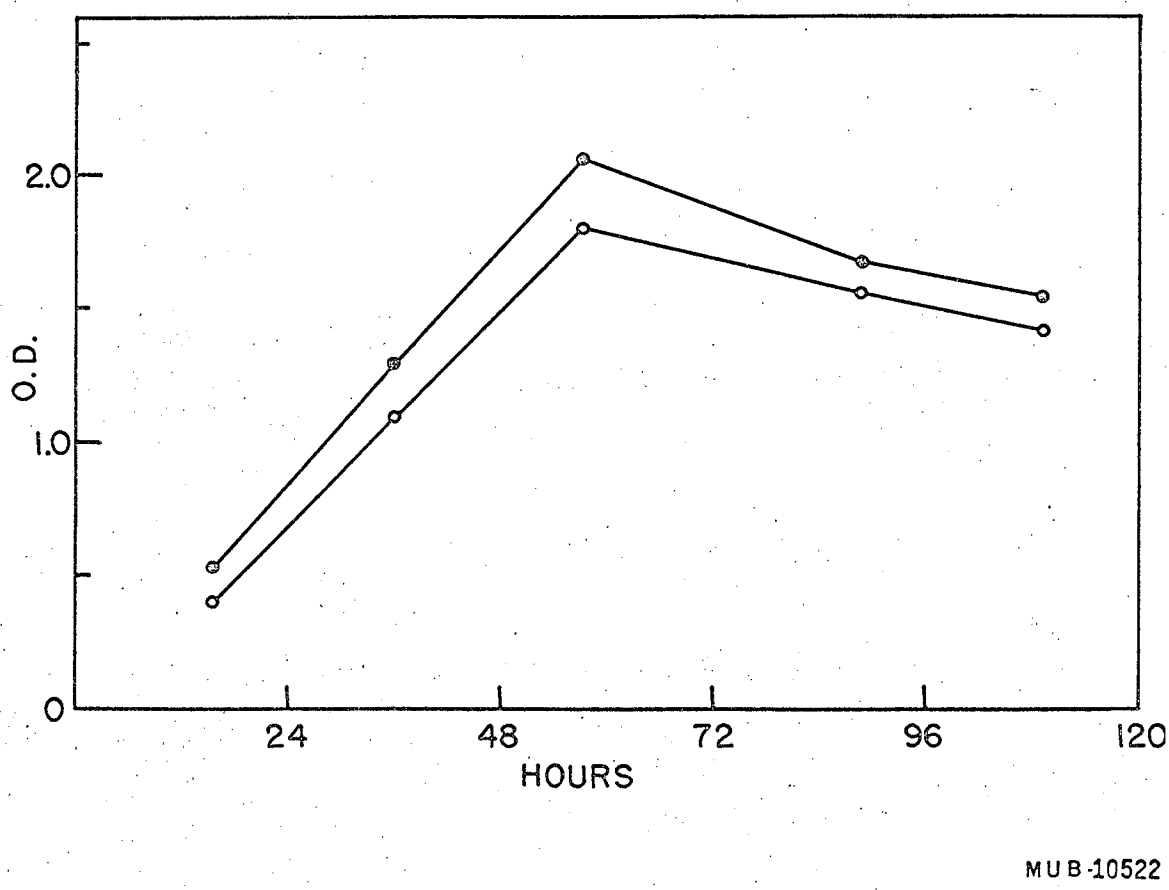


Figure V-3. Growth rate of normal density of R. rubrum.

⊙, 650 mμ (turbidity)

o, 880 mμ (BChl)

The interpretation of the double illumination experiments has been summarized in Table V-3. The three possible mechanisms to account for the tritium incorporation -- biosynthesis, a two electron cyclic process, and a one electron cyclic process -- have been considered in terms of the amount of incorporation to be expected, and the amount of tritium which would be retained when the tritiated bacteria are illuminated in non-tritiated media when only hydrogen atoms are available.

For biosynthesis, only .1% incorporation would be expected from comparison with the value obtained in the spectroscopic measurements for the rate of biosynthesis. If there is an isotope effect, then only .01% incorporation would be expected. In the case of the biosynthesis, all of the tritium should be retained during the second period of illumination, since the rate of bacteriochlorophyll turnover is very small. These values do not compare favorably with those obtained experimentally. The double illumination experiment carried out on 4 day bacteria indicates 2.7% incorporation and only 80% retention in non-tritiated media. In addition, the two experiments on younger bacteria do not at any time show a retention of 100%, which would be expected, especially considering that the increase in incorporation (as great as 20%) may be somewhat due to increased biosynthesis. (An approximation of the amount of tritium to be attributed to biosynthesis for the younger bacteria is unknown; and for that reason, only the 4 day experiment is discussed with reference to Table V-3). Thus, the very large discrepancy between the values expected and those experimentally determined for both incorporation and retention indicate that the tritium uptake cannot be interpreted in terms of biosynthesis.

TABLE V-3

Analysis of Tritium Incorporation and Isotope Effects for Biosynthetic and Photosynthetic Mechanisms Proposed for 4 day old *R. rubrum*

The values expected for various mechanisms proposed and isotope effects.

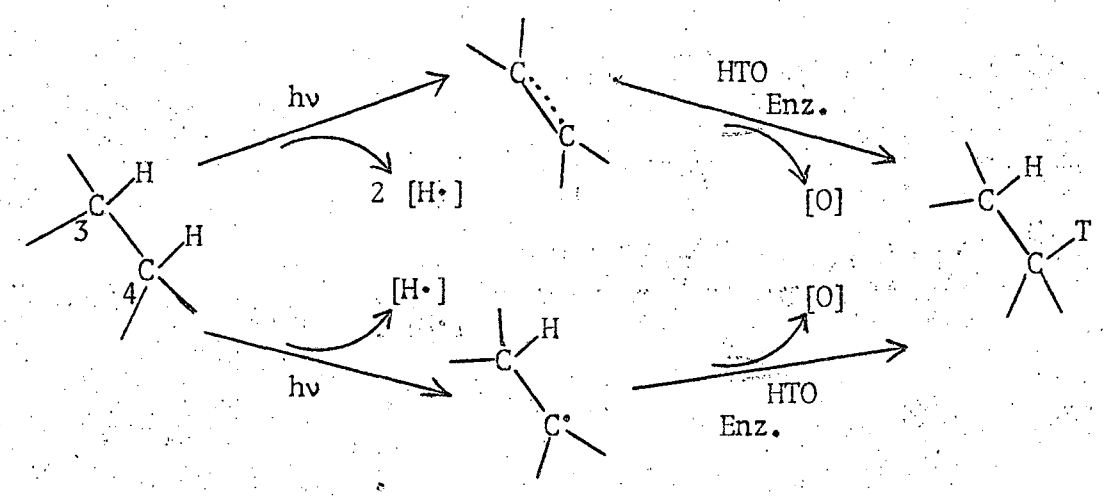
$$\% \text{ incorporation} = \frac{\text{specific activity of Bchl}}{\text{specific activity of water}} \times 100$$

	Biosynthesis: $OxBchl \xrightarrow[Enz]{HTO} Bchl\langle^{H}_{T}$		Photosynthesis:[a] two-electron red. $Bchl\langle^{H}_{H} \xrightarrow{h\nu} [R\text{-}C{=}C\text{-}R] \xrightarrow[Enz]{HTO} Bchl\langle^{H}_{T}$		Photosynthesis:[a] one-electron red. $Bchl\langle^{H}_{H} \xrightarrow{h\nu} [R\text{-}C(H)\text{-}C\cdot\text{-}R] \xrightarrow[Enz]{HTO} Bchl\langle^{H}_{T}$	
Alternative Assumptions	% Incorporation	Incorporated T Retained in H_2O	% Incorporation	Incorporated T Retained in H_2O	% Incorporation	Incorporated T Retained in H_2O
1. No tritium discrimination	.1[b]	100	100	0	200	0
2. Normal isotope effect for organic reactions (10)	.01	100	10	0	20	0
3. 2.5% active sites - Fixed	---	---	2.5	0	5	0
4. 2.5% active sites - Fixed With isotope effect	---	---	0.25	0	0.5	0
5. 2.5% active sites - Complete equilibration	---	---	100	0	200	0
6. 2.5% active sites - Complete equilibration With isotope effect	---	---	10	0	20	0
7. 2.5% active sites - Partial equilibration	---	---	2.5 - 100	97.5 - 0	5 - 200	97.5 - 0
8. 2.5% active sites - Partial equilibration With isotope effect	---	---	0.25 - 10	97.5 - 0	0.5 - 20	97.5 - 0
9. Values obtained for 4 day bacteria: Expt. #5	1.0 - 2.7	80	1.0 - 2.7	80	1.0 - 2.7	80

a There is at least one two-electron reduction accomplished per molecule of bacteriochlorophyll in the period of photosynthesis, and for 2.5% active sites there are at least four turnovers per active site.

b Value assigned on the basis of a spectroscopic determination of the synthesis of new bacteriochlorophyll, which is .1% for 4 day old *R. rubrum*.

The alternative explanation for the tritium incorporation is that of photosynthesis. Two related mechanisms are suggested by which the bacteriochlorophyll may function in either a one electron or two electron cyclic process.



Pertinent to this discussion is the number of two-hydrogen reductions accomplished per molecule of bacteriochlorophyll. From the calculations presented earlier, it was noted that a 1.3% increase in bacteriochlorophyll is equivalent to at least eleven two-electron reductions per molecule of CO_2; for the more reasonable value of a 0.1% increase in bacteriochlorophyll, there are calculated to be at least one two-electron reduction per molecule of bacteriochlorophyll. It should be noted that these are approximations, and represent a minimum value.

For the two electron process, 100% incorporation should be obtained if each molecule of bacteriochlorophyll is involved in the process, since there is at least one two-hydrogen reduction accomplished per molecule of bacteriochlorophyll during the period of photosynthesis. All of the incorporation should be lost when the bacteria are illuminated in non-tritiated media, since both hydrogens

are presumed removed in a light reaction and the incremental energy of activation for tritium is easily overcome by the photon energy.[11] For the one electron process, in which both the 3 and 4 position can randomly participate (assuming a sufficient number of cycles, at least two), then each position should become labeled to the same extent as the water, and the bacteriochlorophyll will show a total of 200% incorporation. In the non-tritiated illumination, all of the tritium should again be lost, since the 3 and 4 positions can each again participate randomly and there are a sufficient number of cycles so that all the tritium would be removed. If the 3 and 4 positions do not participate equally in the one electron reduction, but there is a specificity towards one position, then the values obtained would be the same as for the two electron reduction process. In any case, the experimental values are not consistent with either alternative, even including the isotope effect, if each molecule of bacteriochlorophyll participated as a hydrogen transfer agent.

A further alternative to be considered is that only a small portion of the bacteriochlorophyll molecules participate as "active sites" in the cyclic process. Clayton[16] has concluded from flashing light experiments that only one out of 40 bacteriochlorophyll molecules is involved in the electron transfer process, and perhaps the remaining molecules act as energy funnels to the active site. If this is true then an incorporation of 2.5% tritium would be reasonable, since only 2.5% of the bacteriochlorophyll acts as hydrogen transfer agents. However, one must again consider the isotope effect, which could make the incorporation only 0.25%, and the amount of tritium retained during the second period of illumination would be 0%. However, to consider

the active sites as completely fixed is unlikely since there is a small but definite turnover of bacteriochlorophyll during the lifetime of the organism and there is no reason to assume that particular molecules are exempt from this dynamic system. This brings us to the possibility that there is a migration of the active sites throughout the bacteriochlorophyll pool. The extreme point of view would be that there is a complete equilibration of the active sites with the bacteriochlorophyll pool, such that each molecule participates as an active site during the 15 minutes of illumination. If this is true, then the same values would be obtained as for the first case mentioned in Table V-2. There is reason to believe that the equilibration of the active sites is not that rapid; the original postulation of a photosynthetic unit[15] from saturation effects in flashing light experiments on green plants also means that the active sites are not regenerating rapidly enough to accomodate all the light energy absorbed. Therefore, the most ligical point of view is that there is an equilibration between the 2.5% of active sites and a portion of the bacteriochlorophyll pool. Suppose that the 2.5% active sites can exchange with 25% of the bacteriochlorophyll pool during the period of illumination. Then you would expect an incorporation of 25%, or, 2.5% considering isotope effects. During the interval (90 minutes) between the two periods of illumination, the 25% equilibrated pool becomes homogenized with the bacteriochlorophyll pool such that during the second period of illumination in non-tritiated media the tritium lost is only 25% because that is the fraction of equilibration between the active sites and the pool. Then, the retention is 75%.

Conclusion

The conclusion of this investigation is that there is a definite photo induced incorporation of tritium in the 3,4 positions of bacteriochlorophyll which cannot be accounted for in terms of biosynthesis. The experimental data imply that there is an involvement of the 3,4 positions of bacteriochlorophyll in a cyclic process during photosynthesis. The results do not allow a distinction between a one and two electron reduction, although photo induced ESR signals would suggest a one electron process.[70]

The arguments presented are by no means rigorous; nor are they an exclusive solution to an interpretation of the data. There may be other explanations, such as an unknown enzymatic exchange, which would completely re-open the issue. It is not impossible that a highly labeled contaminant could be co-chromatographed during the purification, although this is improbable because highly labeled contaminants are unlikely to be present in the double illumination experiments carried out in non-tritiated medium. Our conclusions rest on the belief that the isotope effect must be taken into consideration and that there is a partial equilibration between the active sites and the bacteriochlorophyll pool. Separate means of answering these questions are being sought.

One of the greatest problems in this work is understanding and interpreting the physiology of the living organism during the experiments. In the oxygen 18 work, considerable knowledge is readily obtained from a few simple experimental measurements. For this work, it would be advantageous to develop steady state bacteria, as for the Chlorella, such that each experiment could be carried out on organisms

in the same physiological state. In addition, methods are necessary to measure the rate of bacteriochlorophyll synthesis and the rate of electron turnover. It would also be best if dilute, rather than extremely dense suspensions, could be used. These could later be pooled with steady state bacteria so that there would be enough bacteriochlorophyll to work with and yet avoid the problems of using such dense solutions. In principle, the isotope question could be answered by studying the relationship of the rate of incorporation of tritium and deuterium, and from this knowledge determine the absolute tritium isotope effect.[68] A very preliminary report by Katz and Crespi[71] indicates that deuterium labeling in bacteriochlorophyll is under investigation, although the 3,4 positions are not mentioned. An NMR investigation of the deuterium labeled compound would ease the problem of the isotope effect in the tritium experiments. It may be possible to determine the degree of equilibration of the active sites by doing a kinetic study of the percent of incorporation versus time. A change in slope with time might reveal the point at which the rate of equilibration becomes faster or slower than the rate of incorporation. With this knowledge, a more complete analysis of the experimental data might provide a more definitive concept of the role of bacteriochlorophyll during photosynthesis.

CHAPTER VI. A SUMMARY OF THE ROLE OF CHLOROPHYLL AS A CHEMICAL INTERMEDIATE IN PHOTOSYNTHESIS

Although chlorophyll has been shown to have two labile hydrogens, the delta and 10, experiments have shown that neither of these positions nor the 7,8 positions of chlorophyll act as chemical intermediates during photosynthesis. From the earliest tritium experiments in 1942 to the most recent deuterium experiments, the results have consistently shown no isotope incorporation which could be attributed to a photosynthetic mechanism. In those cases in which a small amount of incorporation was found, there was no investigation to determine that it could be excluded on the basis of new chlorophyll synthesis, nor was there any evidence for the position of incorporation except in NMR experiments.

It is important to recognize that although it is extremely doubtful that chlorophyll acts as a chemical intermediate via the delta, 10, or 7,8 positions, one cannot make an unequivocal statement. The experiments reported in the literature were not designed to evaluate the chlorophyll participation at the "active site"; their conclusions simply state that there was "less than 5% incorporation"[11] and other similar remarkds, such that if only one out of 200 molecules (1/2%) are active sites, then 1/2% incorporation is the expected theoretical value. A 1/2% incorporation is far below the experimental error expressed by the investigators in the experiments described in Chapters I and II.

The oxygen 18 investigation with Chlorella clearly shows that the carbonyl function of the isocyclic ring does not participate in the water splitting reaction, nor is it the site of oxidative photophosphorylation. The evidence is quite clear that this is also true for the active site theory; the oxygen 18 incorporation did not approach 1/2%. Because the evidence is quite clear with regard to the carbonyl function, this implicates the 10 position as also being excluded from the photosynthetic scheme. Certainly, any mechanism which included the 10 position could not do so on the basis of hydration of the carbonyl followed by enolization. Thus, the proposals by both Franck and by Calvin are incorrect descriptions of the photosynthetic scheme.

The first concrete evidence for the participation of chlorophyll as a chemical intermediate is that put forth in the preceding chapter, in which bacteriochlorophyll shows 2% tritium incorporation when R. rubrum is allowed to photosynthesize in tritiated water. The tritium incorporation and subsequent bacteriochlorophyll oxidation shows that the 3,4 positions are involved in the photosynthetic mechanism, and that only a very small percentage of the bacteriochlorophyll acts as active sites. This is consistent with Clayton's conclusion that most of the chlorophyll "funnels" the electromagnetic energy to the position of energy conversion which takes place in 2-1/2% (one out of 40) bacteriochlorophyll molecules, and 1/2% (one out of 200) chlorophyll a molecules in green plants.

The idea of a "migratory active site" was presented in order to account for a normal tritium isotope effect, and to account for the fact that there was only a partial loss of the incorporated tritium when the cells were resuspended in non-tritiated medium. A 2% incor-

poration reflects a 20% hydrogen exchange when an isotope effect of 10 is taken into consideration. This 20% incorporation is less than that expected for one mole of exchange, and greater than the 2-1/2% incorporation expected on the basis of 2-1/2% active sites. Thus the suggestion that the 2-1/2% of active sites are in equilibrium with the larger pool of bacteriochlorophyll molecules.

This proposal of "migratory active sites" should be considered not only in terms of these isotope experiments, but should be extended to experiments with green algae photosynthetic systems. Perhaps the action spectrum measurements, flashing light experiments, and other techniques in use in the study of photosynthetic systems may in the future contribute added evidence for this proposal.

The proposal of a migratory active site presents a new interpretation to the earlier tritium and deuterium experiments on Chlorella relating to the chemical mechanism of photosynthesis. That is, one would expect a greater amount of incorporation than that assumed on the basis of 1/2% active sites. This is of little assistance in terms of the tritium experiments, because one still must contend with a large isotope effect and lack of knowledge on the site of incorporation; however, with the deuterium experiments, there was no incorporation of reasonable magnitude in the delta position to be consistent with the proposal of a "migratory active site". If further experiments do bear out this suggestion of a migratory active site, the proposal for the chemical mechanism involving an oxidation and reduction cycle at the 3,4 positions is strenghened. Unfortunately, the mechanism cannot be tested for the green algae

since chlorophylls a and b isolated in the natural state do not contain the hydrogens at the 3,4 positions. However, it is nevertheless possible that these systems undergo reduction and oxidation, at the 3,4 positions, to carry out the water splitting reaction of photosynthesis.

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