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# A Model for the Chloroplast: A Study of the Photochemical and Spectral Properties of Pheophytin A Adsorbed to the Surface of Small Particles

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# A MODEL FOR THE CHLOROPLAST: A STUDY OF THE PHOTOCHEMICAL AND SPECTRAL PROPERTIES OF PHEOPHYTIN A ADSORBED TO THE SURFACE OF SMALL PARTICLES

A thesis submitted to the Faculty of The Rockefeller Institute
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

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Approved for Publication
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120132

Photosynthesis is not all sweetness and light.

# PREFACE

My first introduction to the mysteries of photosynthesis came from a study of the photoconductivity of dried chloroplast preparations. The stimulus for that research came from similar studies of Arnold and from the solar battery theory of photosynthesis proposed by Calvin. It became clear, however, from the experimental studies of a number of workers, the most elegant work being that of Witt and coworkers, that the photosynthetic process includes photochemical reactions of chlorophyll. That is, chlorophyll molecules are chemically involved in the process, as opposed to acting physically as electronic conductors in a crystalline lattice.

Through the guidance of Dr. David Mauzerall, I was introduced to porphyrin and, specifically, chlorophyll photochemistry. I still felt that the chloroplast structure, in which the chlorophyll molecules are embedded, has a significant role in controlling the nature of the photoreactions of the active chlorophyll molecules. It was this rationale which led to the studies of the particle systems described in this thesis.

Throughout the course of this work I have benefitted from the guidance and comments of many of the faculty and students of the Rockefeller Institute on the biological, chemical, physical, and mathematical aspects of the problem. I am grateful for the opportunity of working in such a stimulating environment.

I would like to thank Dr. Philip M. Seeman for assistance in taking the electron micrograph of the polystyrene particles shown in figure 8.

The polyethylene used for the chromatography of the chlorophyll and pheophytin was a gift of the Koppers Co., Plastics Division.

I would like especially to thank Dr. Sam Granick for his guidance and his warm hospitality in making available the many resources of his laboratory.

This work would be much the poorer if it were not for the patient tutelage of Dr. Mauzerall. In addition to teaching the tangible, such as organic and photochemistry, and the "semi-tangible", such as the art and science of designing and performing experiments, he has also demonstrated the great importance of the intangible, critical scientific thinking.

## ABSTRACT

One of the major problems in the study of photosynthesis is understanding the role which the structure of the photosynthetic apparatus has in influencing the nature and efficiency of the photochemical electron-transfer reactions of chlorophyll. This research has involved the study of a simple model for the chloroplast with the purpose of gaining some insight into this problem.

It is known that chlorophyll and related molecules will photosensitize oxidation-reduction reactions both in solution and in the adsorbed or colloidal state. The model chosen as being most closely related to chlorophyll in vivo has the pigment adsorbed to the surface of small particles. Pheophytin was used in these studies because chlorophyll was sometimes degraded by the particle surface. A method was developed to study quantitatively the photosensitized reduction of an azo dye in the highly scattering particle suspensions. Measurements of the absorption and fluorescence properties of the coated particles were made to learn more about the state of the pheophytin on the particle surface. The effect of the surface was differentiated from the effect of interactions among the pigment molecules by varying both the type of surface and the concentration of the pheophytin on the particle surface.

At less than 0.01 surface coverage of pheophytin on nonpolar polystyrene particles, the quantum yield of photosensitization is the same as that with the pigment in 90% methanol solution, within a factor of three. The quantum yield falls to 1/2 this maximum value only at 0.4 coverage. In this region the absorption spectra are broadened toward longer wavelengths, indicating aggregation of the pheophytin. The fluorescence also decreases with increasing surface concentration and is 50% quenched at 0.03 coverage. The quenching of monomer fluorescence is accounted for by energy transfer to the very weakly fluorescent aggregates, the number of which is estimated by a one-dimensional Ising model. Analysis of these results shows that small aggregates are photoreactive, but probably not as active as the monomers.

On a polar surface (zeolite particles) the absorption spectra are always broadened and the fluorescence yield is very low for all surface concentrations of pheophytin. The photochemical activity increases

with increasing coverage, but at best only reaches the low yield of the 1.0 coverage polystyrene. The type of surface thus has a profound effect on the adsorbed pigment.

This model shows that adsorption to an interface does not necessarily affect the photochemical activity of the adsorbed pigment and that photochemical reactions can occur at relatively high pigment concentrations. Comparison shows that the particle model has some properties which are strikingly similar to those of the photosynthetic apparatus. Further studies of this model, e.g., the stepwise adsorption of additional reactants known to be present in the chloroplast, should lead to a greater understanding of the photochemical reactions in photosynthesis.

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# CHAPTER I

## INTRODUCTION

"Photosynthesis is a series of processes in which electromagnetic energy is converted to chemical free energy which can be used for biosynthesis" (Kamen, 1963). This definition is quite different from the ones usually encountered in textbooks, such as

$$\text{light} + 6\ CO_2 + 6\ H_2O \xrightarrow{\text{chlorophyll}} C_6H_{12}O_6 + 6\ O_2\ . \qquad \text{(I)}$$

However, it implicitly recognizes the photosynthetic bacteria, which do not produce oxygen, as do algae and higher plants, but are able to use the energy in sunlight to manufacture biological materials. Photosynthetic processes can be divided into the "light reactions," the highly efficient photochemical reactions of chlorophyll and bacteriochlorophyll, and the "dark reactions," those that proceed in the absence of light. Kamen's definition emphasizes that the photochemical reactions are the unique property of photosynthetic organisms. Most of the dark reactions are at least similar to the biochemical processes found in all living things.

Electron microscopical studies have revealed that the photosynthetic apparatus has a very complex structure made up of membranes or lamellae. The pigments such as chlorophyll are embedded in the lipid and protein membranes of this structure. While photochemical reactions of chlorophyll and bacteriochlorophyll have been studied extensively in solution, no photochemical reaction has been found which will convert light energy to useful chemical energy with the efficiency attained by the plant. One of the major reasons for this must be the structural complexity of the photosynthetic apparatus. The object of this research has been to develop a model in which the effect of organization on the photochemistry can be studied.

To put the studies to be described into the proper perspective, it will be useful to review briefly what is know about the steps in the photosynthetic process. There are many excellent reviews and collections of papers which discuss photosynthesis in more detail. Among these are the volumes edited by Ruhland (1960), McElroy and Glass (1961), and Gest,

San Pietro and Vernon (1963), the symposia sponsored by the National Academy of Sciences (1963) and the Centre National de la Recherche Scientifique (1963), the article of Gaffron (1960), the books by Bassham and Calvin (1957) and Calvin and Bassham (1962) and the great treatise by Rabinowitch (1945, 1951, 1956). The discussion will be limited primarily to the photosynthesis of algae and higher plants.*

In higher plants, the entire photosynthetic process, as defined in reaction I, occurs in the chloroplasts, the small chlorophyll-containing bodies in the plant cells (Arnon, 1960). Photosynthesis can be divided into the carbon-dioxide fixation reactions (Bassham and Calvin, 1957)

$$12\ NADPH_2 + 18\ ATP + 6\ CO_2 \xrightarrow{(dark)}$$

$$C_6H_{12}O_6 + 6\ H_2O + 18\ (ADP + P_i) + 12\ NADP, \qquad (II)$$

and the reactions which produce the reducing agent, $NADPH_2$, and the energy source, ATP, (Arnon, 1960).

$$light + n\ ADP + n\ P_i \xrightarrow{chlorophyll} n\ ATP \qquad (IIIa)$$

$$light + 2\ ADP + 2\ P_i + 2\ NADP + 4\ H_2O$$

$$\xrightarrow{chlorophyll} 2\ ATP + O_2 + 2\ NADPH_2 + 2\ H_2O. \qquad (IIIb)$$

In the above reactions NADP and $NADPH_2$ are the oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide phosphate, ATP is adenosine triphosphate, ADP, adenosine diphosphate, and $P_i$, inorganic phosphate ($PO_4^{3-}$). In summary, the light-requiring reactions, IIIa and IIIb, provide the electrons and the energy necessary for the conversion of carbon dioxide into carbohydrates and other cellular materials.

The reactions IIIa and IIIb, which have been called cyclic and non-cyclic photophosphorylation, respectively, by Arnon, make up the electron-transport system of the chloroplast. The most widely accepted scheme for this system is diagrammed in figure 1. It has been reviewed by

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* An entirely different view of the photosynthetic process from that described here is held by Warburg (1958, 1963; see also Good, 1965; Vennesland, 1964; and Bladergroen, 1960).

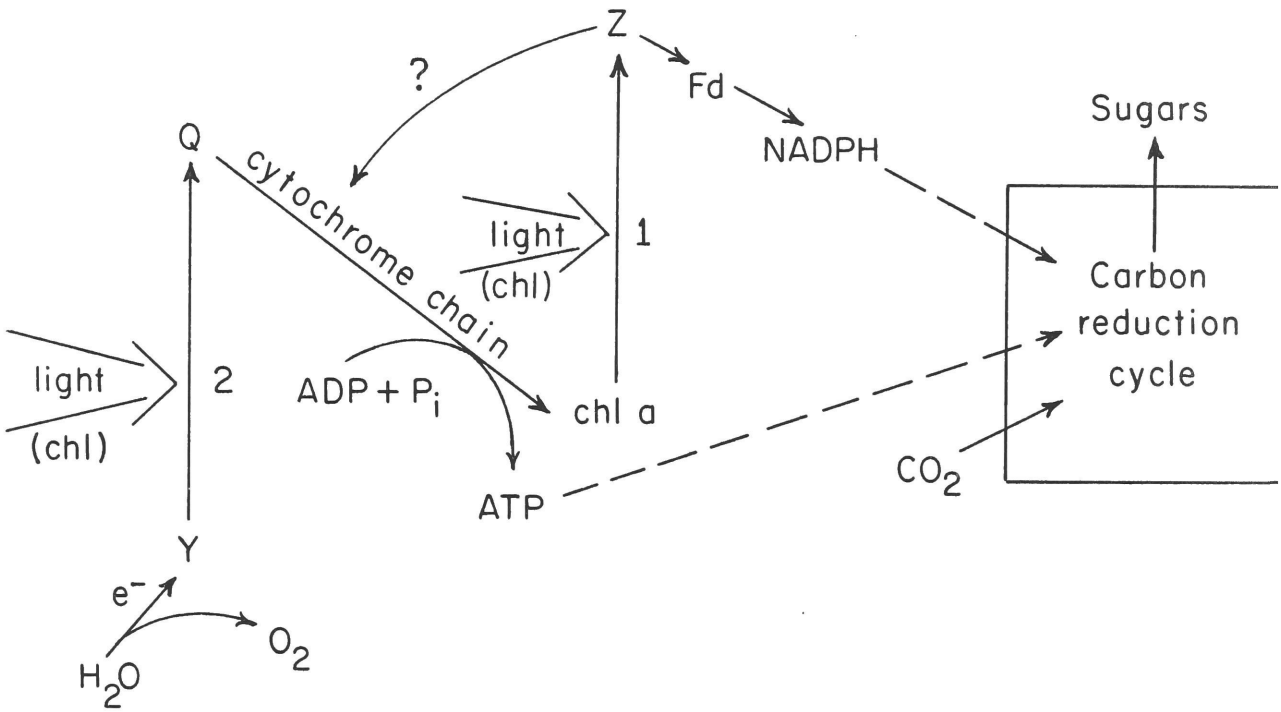



Figure 1. Schematic diagram of green plant photosynthesis. The electron-transport chain, including light reactions 1 and 2, produces the $NADPH_2$ and the ATP used in the fixation of carbon dioxide. The process indicated by the question mark may involve an additional phosphorylation (Arnon, 1963).

Clayton (1963), who has also considered its relationship to bacterial photosynthetic electron transport. The diagram is similar to that originally presented by Hill and Bendall (1960) and modified by Arnon (see Arnon, 1963).

The electrons are transferred from molecule to molecule in this system through a series of oxidation-reduction reactions. While not all the components of the electron-transport system have been defined, they are thought to include plastoquinone (Q), cytochromes $b_6$ and f, which are unique to the chloroplast, chlorophyll a and b (see Rumberg, 1964b, for evidence of chlorophyll b participation), and ferredoxin (Fd; for a review of the biochemistry of bacterial ferredoxins see Valentine, 1964). The light reactions 1 and 2 in figure 1 are chlorophyll-photosensitized oxidation-reduction reactions, i.e., reactions in which the energy of the electronically excited chlorophyll is used to transfer electrons against a chemical free energy gradient. The reactions of the cytochrome chain, including the phosphorylation reactions, are probably similar to those in the electron-transport chain of mitochondria (see Lehninger, 1964).

A variety of evidence suggests that not every chlorophyll molecule is part of an electron-transport chain. Kok and Hoch (1961) have evidence for a special form of chlorophyll, called P700 because of its absorption maximum at about 700 mμ, which they feel is the photochemically active pigment in light reaction 1. The work of Witt and his coworkers is in agreement with this conclusion. Kok and Hoch estimate that there is one molecule of this P700 for every 300-400 chlorophyll molecules in the chloroplast. Park and Pon (1963) have examined the chemical composition of chloroplast lamellae fragments which are able to perform at least part of the photosynthetic reactions, i.e., the Hill reaction with ferricyanide as reductant (see below). They found that for each atom of manganese there were six atoms of iron and 115 molecules of chlorophyll. The manganese is thought to be involved in the oxygen-producing reactions. There would thus appear to be 115 chlorophyll molecules (or some multiple of this depending on the amount of manganese associated with a reaction site) associated with one electron-transport pathway. The iron is present in the cytochromes and in ferredoxin. Gross, Becker and Shefner (1964), by centrifugal analysis, have estimated that the smallest particles able to reduce ferricyanide in the Hill reaction contain about 600 chlorophyll molecules.

The Hill reaction (Hill, 1937) may best be defined as the production of oxygen by isolated chloroplasts (or chloroplast fragments) in the light in the presence of added (external) electron acceptors. Reaction IIIb might be considered to be a variant of the Hill reaction, but the latter is generally studied under conditions in which the phosphorylation is decoupled and with nonphysiological oxidants such as ferricyanide or dichlorophenol-indophenol (DCIP). There is some question as to where these added acceptors accept electrons from the electron-transport pathway (Biggins and Sauer, 1964). This question is important in considering how intact the photosynthetic apparatus is in experiments such as those of Park and Pon and of Gross et al. While Witt and coworkers (Witt, Muller, and Rumberg, 1963; Rumberg, 1964a) claim that ferricyanide can accept electrons from an intermediate, **Z**, reduced in light reaction 1, Biggins and Sauer claim it is reduced by a substance between reactions 2 and 1. I do not feel that the evidence of either of these groups unequivocally establishes their claim. Thus, there is some uncertainty as to how much of the photosynthetic electron-transport system is associated with the 400-600 molecules of chlorophyll determined in the above experiments.

Emerson and Arnold (1932) originally introduced the concept of a photosynthetic unit. They defined "one unit arbitrarily as the mechanism which must undergo the photochemical reaction to reduce one molecule of carbon dioxide." Emerson and Arnold estimated that this unit contained about 2500 chlorophyll molecules. They illuminated a Chlorella suspension with brief ($10^{-5}$ sec), intense flashes of light followed by a dark period of duration sufficient for completion of the subsequent enzymatic dark reactions. The ratio of the number of chlorophyll molecules in the suspension to the number of molecules of oxygen produced per flash at saturating intensities was taken as the size of the unit. Similar results have been obtained by other workers (see Rabinowitch, 1956, p. 1274; Kok, 1956). Since two molecules of $NADPH_2$ and three of ATP are required for the reduction of a molecule of carbon dioxide, there must be at least two or three electron-transport chains in each photosynthetic unit as defined above. Thus there would be 800-1200 chlorophyll molecules per electron-transport chain. This number is the same order of magnitude as

the values quoted above. Because of the uncertainties in these measurements, especially in estimating the chlorophyll in small particles, the values are probably correct only within a factor of two or three. But it does appear safe to conclude that somewhere between 100 and 500 chlorophyll molecules cooperate to facilitate one or the other of the two photochemical reactions. The number in the photosynthetic bacteria appears to be in the lower range.

The photosynthetic unit of Emerson and Arnold is a functional unit and does not necessarily have structural significance. Park and Pon (1961; see also Park, 1962) have observed small particles associated with the membranes of the chloroplast. These particles contain chlorophyll, and aggregates of a few particles are capable of photochemical activity. The particles were initially called quantasomes, but the definition of this term was later revised to include the membranes to which the particles are attached (Park and Pon, 1963). The quantasomes are probably similar to the particles studied by Gross et al., (1964). In contrast to the photosynthetic unit, the quantasome is essentially a structural unit. However, the quantasome may also be a functional unit, which includes part or all of the electron-transport chain. Boardman and Anderson (1964) have recently reported the isolation of two different types of particles from spinach chloroplasts. It appears that light reaction 1 is associated with one type of particle and reaction 2 with the second. Thus, two or three quantasomes of each type, with the water-soluble carbon dioxide fixation enzymes, would combine to fulfill the functions of a photosynthetic unit.

I have used the concept of photosynthetic unit strictly in the sense of Emerson and Arnold's original definition. More recently the term has been applied to the set of molecules around a photochemical reaction center, including the 100-500 chlorophyll molecules mentioned above (Clayton, 1963). By this definition the quantasome might have the functions of one (small) "photosynthetic unit" (or two different units, each associated with one of the two light reactions 1 and 2). Some clarification of terminology would be useful here. For the purpose of the following discussion, I will avoid the use of the term photosynthetic unit except in the sense as originally meant by Emerson and Arnold. The term quantasome will be reserved for the structural unit or particle.

The functional units defined by the photosynthetic electron-transport system and the associated pigment molecules will be described in these terms, though it will also be useful to describe the two photochemical reactions as occuring at reaction centers. I hope in this way to avoid introducing any new terminology until the above confusion is resolved.

What is the nature of the cooperation among the chlorophyll molecules associated with a given photochemical reaction center? The experiments of Kok and Hoch, Witt and coworkers, and others have suggested that not all the chlorophyll is photochemically active. It is generally accepted that the light energy absorbed by the unreactive chlorophyll molecules as well as that absorbed by the accessory pigments, such as carotenoids and phycobilins, is transferred to the reactive ones. That energy transfer does occur in algae and in the photosynthetic bacteria has been demonstrated by French and Young (1952) and by Duysens (1952), using the fluorescene of chlorophyll as the criterion of transfer. Additional evidence comes from the studies of Haxo and Blinks (1950) on the action spectrum of photosynthesis and of Myers and French (1960) and Blinks (1960) on the action spectra of enhancement (Emerson effect) and chromatic transients (Blinks effect) in algae. In addition to demonstrating energy transfer, these latter studies show that the light absorbed by the accessory pigments and chlorophyll b is not only used in photosynthesis, it is required for photosynthesis to proceed at maximum efficiency. French and Young and Duysens, especially, attempted to explain the transfer by using the resonance-transfer theories of Förster (1960) (see chapter III). Other explanations have been in terms of electronic conduction (semi-conduction) and exciton migration (Katz, 1949; Clayton, 1963; Bay and Pearlstein, 1963). For the present purposes it is sufficient to note that, within the chloroplast, there is a reaction center associated with every 100 to 500 chlorophyll molecules. The majority of the chlorophyll molecules and the accessory pigments act as "antennae" for the chlorophyll at the reaction site. That is, they absorb the light and transfer the electronic excitation energy to a site where the conversion of light to chemical energy takes place. This energy conversion occurs in either of the two photochemical light reactions of chlorophyll, 1 or 2 in figure 1, in the photosynthetic electron-transport chain. Some of the biggest gaps in our understanding of the photosynthetic

process are the mechanisms of these highly efficient photochemical reactions.

Many workers have studies the photochemistry of chlorophyll in dilute solution (Livingston, 1960) in order to get some clue to the mechanism of the photosynthetic reactions. Most of these studies have been on chlorophyll-photosensitized oxidation-reduction reactions and the direct photoreduction of chlorophyll itself. Pigments related to chlorophyll, such as pheophytin, have also been used in these studies. The reactions are listed in table I. A most important feature of these reactions is their low energy-conversion efficiency, i.e., how much light energy is converted into chemical energy. Some reactions have high quantum efficiencies, i.e., the number of molecules reacted per quantum of light absorbed, but the reactions do not store energy. The light only helps to get over the activation energy barrier. Some reactions do store energy, but these generally have very low quantum efficiencies.

How similar the mechanisms of the sensitization reactions are to the photosynthetic reactions is unknown. This is because the mechanisms of these reactions have not been unequivocally determined (see chapter III). Moreover, the steps involved in the two photosynthetic light reactions are unknown, although there is some evidence that light reaction 1 involves a photo-oxidation of P700,

$$\text{Light} + \text{chlorophyll} + Z \longrightarrow \text{chlorophyll}^{+} + Z^{-} \qquad \text{(IV)}$$

followed by reduction of the oxidized chlorophyll by a cytochrome,

$$\text{chlorophyll}^{+} + Fe^{II}\text{Cyt.} \longrightarrow \text{chlorophyll} + Fe^{III}\text{Cyt.} \qquad \text{(V)}$$

(Clayton, 1963).

While further studies on the mechanisms of both the in vivo and the in vitro reactions will be useful, there is a major problem in comparing them. Chlorophyll in the plant is not in dilute solution, as it has been in the in vitro studies. Rabinowitch (1945, p. 412) has estimated that the chlorophyll concentration in the chloroplast is about 0.06-2 M. Moreover, it is associated with the lipids, accessory pigments, proteins, etc., of the quantasomes and the chloroplast membranes. Thus, although reactions in dilute solution are relatively easy to study, they may have no relation to biological reality.

TABLE I

MAJOR TYPES OF CHLOROPHYLL PHOTOREACTIONS IN VITRO

| | | Reductants | Oxidants | Light absorber | Properties |
|---|---|---|---|---|---|
| I) | Sensitized autooxidations | allylthiourea<br>ascorbic acid<br>benzidine<br>dihydrobenzyl-nicotinamide | oxygen | chlorophyll<br>pheophytin | no energy storage<br>quantum yield about 1 |
| II) | Sensitized dye reductions | ascorbic acid<br>phenylhydrazine<br>dihydrobenzyl-nicotinamide | azo dyes (e.g.,methyl red)<br>safranin<br>benzyl-nicotinamide<br>NAD | chlorophyll<br>pheophytin | can store energy<br>lower quantum yields (0,2)<br>need anaerobic conditions |
| III) | Direct photoreduction | ascorbic acid<br>phenylhydrazine<br>dihydrobenzyl-nicotinamide | chlorophyll<br>pheophytin | chlorophyll<br>pheophytin | can store energy<br>very low quantum yields<br>need anaerobic conditions |

Attempts to approximate more closely the state of chlorophyll *in vivo* have led to studies of colloidal suspensions (Evstigneev and Gavrilova, 1959; Bannister, 1963) and concentrated solutions (Brody and Brody, 1963; Broyde and Brody, 1964). Many colloids show little or no photochemical activity (Krasnovskii and Brin, 1948; Bannister, 1963) though there are a few that are relatively active.

Vernon (1961) has prepared unstable colloidal suspensions by absorbing chlorophyll to powdered sugar and immediately dissolving the sugar in a buffered aqueous solution. These preparations are able to sensitize the reduction of methyl red by ascorbic acid at rates about one-half those of chloroplast preparations. The rates should be compared with caution, however, because of the different scattering properties (and therefore different light absorbing properties) of the two preparations. Krasnovskii and Brin (1948) found that a dispersion of chlorophyll in aqueous solutions containing detergents was fluorescent and photochemically active. In a more quantitative study, Bannister and Bernardini (1963) found that such a colloidal dispersion (using Tween 20 as the detergent) was able to sensitize the autoxidation of 2,5-toluene diamine hydrochloride with a quantum yield of 0.16. They were unable to compare this with the value in organic solvents because different products were formed in the two cases. However, the quantum yield of photo-oxidation of chlorophyll itself was about the same in the detergent dispersions and in acetone and alcohol solutions (quantum yield about $2 \times 10^{-4}$).

Although these studies are of interest, it is difficult to characterize the state of the pigment in these systems (i.e., the degree of aggregation in concentrated solutions or the structure and size of the colloidal particles). Thus, it is difficult to tell how well these colloids mirror the photosynthetic apparatus.

A more realistic model for the photochemical reaction centers in the plant would be chlorophyll adsorbed to a known interface. Studies of such model systems should reveal the ways the environment (e.g., the adsorbent surface) can influence the properties of the adsorbed chlorophyll. Another feature of these systems is that much can be learned about the state of the pigment at the interface.

One obvious version of such a model is chlorophyll at air/water interfaces. There have been a number of studies of such monolayers

(Langmuir and Schaeffer, 1937; Hanson, 1937, 1939; Jacobs, Holt and Rabinowitch, 1954; Trurnit and Colmano, 1959; Colmano, 1961, 1962). The most elegant work has been that of Bellamy, Gaines, and Tweet (1963), who have taken care to prevent the destruction of the chlorophyll in the monolayer (see Colmano, 1961). These studies have revealed much about the structure and physical properties of chlorophyll in the monolayers, but photochemical studies would be inordinately difficult, because of the large areas required.

I have chosen to study a model which combines the structural features of the surface-film technique with the simplicity of photochemistry in solution. In this model the pigment molecules are adsorbed to the surface of small particles. The specific pigment which I have used is pheophytin a (figure 2), chlorophyll a without its central magnesium atom. Chlorophyll was not used because it was degraded either on adsorption or in the photochemical reactions (see chapter III). I have studied the effect of different interfaces on the photochemistry of the adsorbed pheophytin by using different types of particles, polystyrene and zeolite. Such freedom in the choice of surface is lacking in the monolayer technique. Interactions among the pheophytin molecules, such as might be expected to occur with chlorophyll in vivo, have been studied by varying the surface concentrations of the adsorbed pigment. (This can be done more easily on particles than with surface films.) Optical studies, such as the determination of the absorption and fluorescence spectra, were performed to learn more about the state of the pheophytin on the particle surface. (It should be noted that polarization experiments, however, can be done more easily on monolayer films; Tweet, Gaines and Bellamy, 1964b.)

I have used the quantum yield of the sensitized dye reduction reaction (type II in table I) as a measure of the photochemical activity of the pheophytin-coated particles. The dye used in most of the studies was the azo dye, amido naphthol red 6B (ANR, figure 3). In one series of measurements, the safranin dye, fuchsia (figure 4) was used. The reducing agents used in these studies were N'-benzyl-1,4-dihydronicotinamide (BNH, figure 5,a), N'-carboxamidomethyl-1,4-dihydronicotinamide (CNH, figure 5,b) and ascorbic acid (figure 6). Thus, in a typical reaction, pheophytin adsorbed to polystyrene particles photosensitized (i.e., photocatalyzed) the reduction of ANR by BNH (reaction VI):

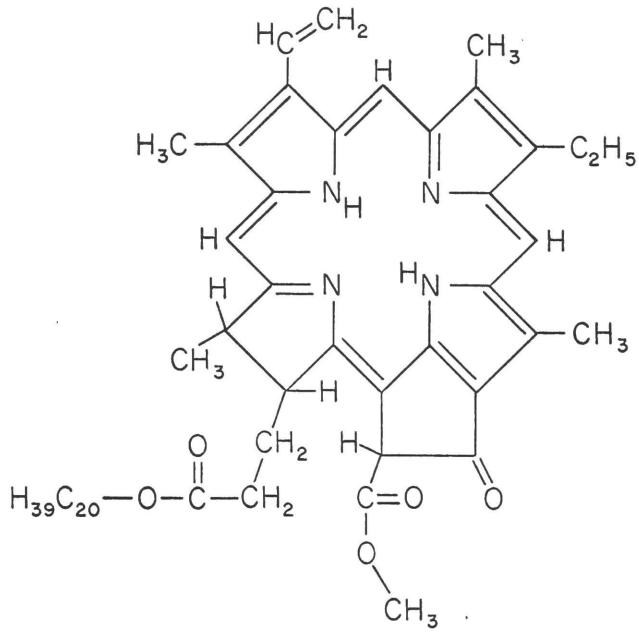



Figure 2. Pheophytin a.

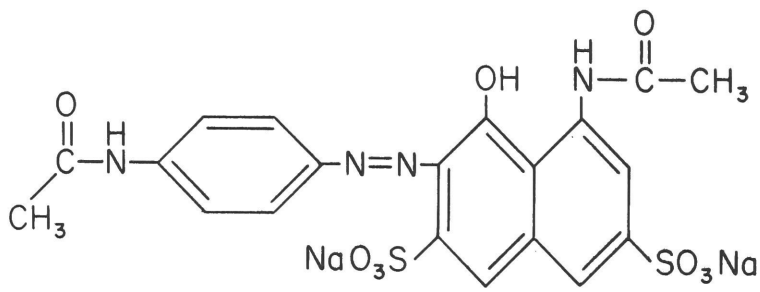



Figure 3. Amido naphthol red 6B (C.I. no. 18055).

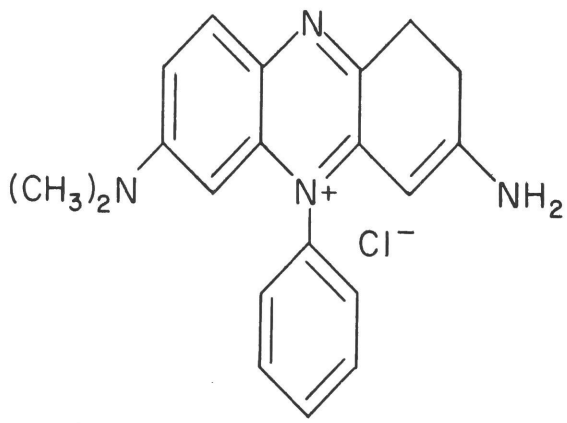



Figure 4. Fuchsia (C.I. no. 50205).

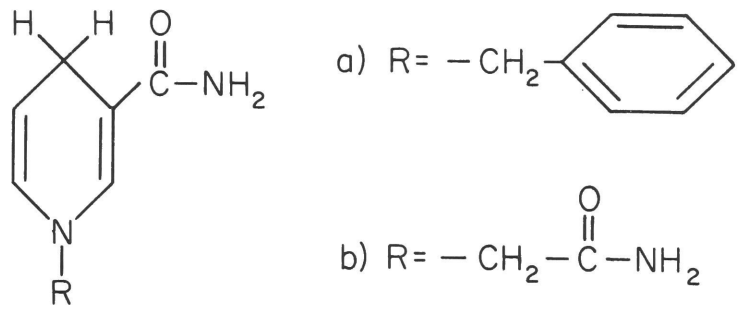



Figure 5. a) N'-benzyl-1,4-dihydronicotinamide; b) N'-carboxamidomethyl-1,4-dihydronicotinamide.

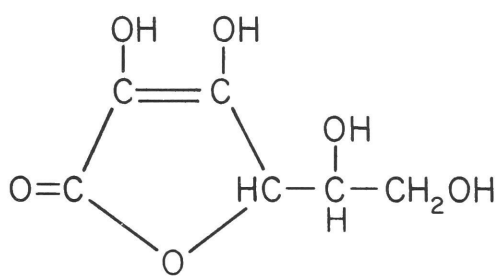
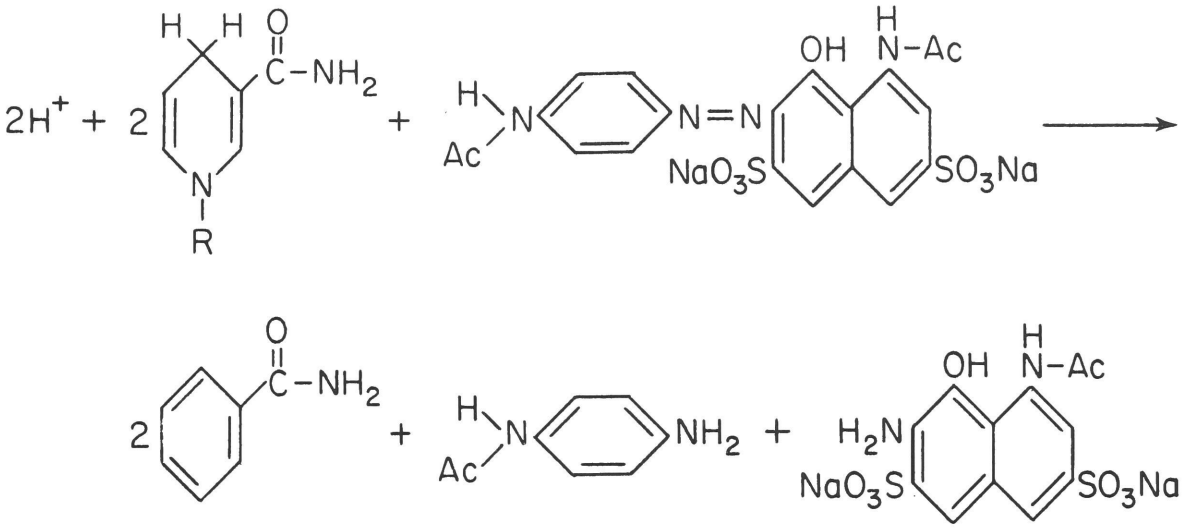



Figure 6. Ascorbic acid.



( VI )

Estigneev and Gavrilova (1960) were the first to show that chlorophyll and related pigments will sensitize the reduction of methyl red by ascorbic acid even when adsorbed to the surface of materials such as powdered alumina. No comparison was made between the rates of the reactions for the pigments on the adsorbent and dissolved in solution, although Evstigneev and Gavrilova do remark that no reduction was observed in the particle systems when safranin T was used instead of methyl red. The latter reaction, which results in some storage of energy in contrast to the reduction of methyl red, which does not, does proceed when chlorophyll is in solution. Evstigneev and Gavrilova did not make extensive studies of the state of the adsorbed chlorophyll, though they did note that its red absorption maximum was shifted only 5 to 10 mμ from its location in solution (about 662 mμ). Since crystalline chlorophyll has a red absorption maximum in the region 720-740 mμ, they concluded that "the layers of adsorbed pigment do not have [a] high degree of orderly aggregation," and that such aggregation was not necessary for photochemical activity.

After the work described in this thesis was completed, it was discovered that the Russian workers had extended their studies somewhat. Komissarov et al (1963) measured the effect of varying the surface concentration of chlorophyll adsorbed to powdered "caprone" (Nylon 6) on the photosensitized reduction of methyl red. As the amount of chlorophyll on the surfact was increased, the amount of methyl red reduced increased until the surface was covered (one complete layer). The amount of reduction did not increase further as additional chlorophyll was adsorbed to

the nylon powder. It appears (though it was not explicitly stated) that the experiment was performed with a constant amount of adsorbent and different amounts of chlorophyll in the reaction mixtures, and the data were not corrected for the variations in the amount of light absorbed by these mixtures. Komissarov et al. manage to conclude, however, that the chlorophyll was most effective as a sensitizer at surface concentrations which were less than one layer and thus "in the adsorbed state the individual molecule serves as an active unit sensitizer, and the presence of an aggregate is not a necessary condition for the appearance of its sensitizing action." Again, no comparison was made between the activity of chlorophyll on the nylon powder and in solution. Thus, the conclusion from this second study is identical to the first.

My experiments have shown that the photochemical properties, the absorption spectra, fluorescence spectra, and fluorescence yields of pheophytin adsorbed to the surface of small particles vary markedly with different surface concentrations of the pigment. With the spectral studies and a theoretical model for the distribution of the pheophytin on the particle surface (as monomers and aggregates), I have been able to demonstrate that a great deal can be learned about the properties of the monomers and aggregates from these particle systems. By comparing the particle systems with pheophytin in solution, I have been able to show how adsorption to a surface can affect the behavior of the pheophytin. These studies are described in chapter III. In the final chapter, the significance of these studies and the relation between the model system and the photosynthetic apparatus will be discussed.

# CHAPTER II

## MATERIALS AND METHODS

### 1. Materials:

All solvents were reagent grade. For the adsorption of pheophytin to the particles and for the photoreactions, the solvents were redistilled from either phosphorous pentoxide or Drierite. Spectroquality dioxane was used for analyzing the polystyrene particles. The distilled water was either twice redistilled, once from alkaline permanganate and a final all-glass distillation, or once redistilled from alkaline permanganate through a 70 cm long distillation column packed with glass coils.

Reagent grade ascorbic acid was recrystallized from ethanol. N'-benzyl- and N'-carboxamidomethyl-1,4-dihydronicotinamide were crystalline preparations obtained from Dr. David Mauzerall (See Mauzerall and Westheimer, 1955). The amido naphthol red 6B (ANR, C.I. Acid Violet 7, C.I. no. 18055; see Soc. of Dyers and Colourists, 1956) was purified by Soxhlet extraction with methanol and chromatographed on alumina. The absorption spectrum of the ANR in distilled water is shown in figure 7. The molar absorptivity in water is $3.08 \times 10^4$ liters $mole^{-1}$ $cm^{-1}$ at the absorption maximum (525 mμ). Fuchsia (C.I. no. 50205) was once recrystallized from an ethanol-toluene mixture. Its molar absorptivity in water was $4.52 \times 10^4$ liters $mole^{-1}$ $cm^{-1}$ at the absorption maximum (553 mμ). Methyl red (C.I. no. 13020) was recrystallized from toluene (Clarke and Kirner, 1922). All other dyes were used directly from the laboratory stocks of Dr.S. Granick without further purification. Most were commercial preparations.

Several methods were used for the preparation of chlorophyll a and pheophytin a. The most satisfactory one was based on the procedure of Anderson and Calvin (1962) using chromatography on powdered polyethylene. I found it convenient to filter the initial acetone extract of the leaves on polyethylene. This removed lipids which tended to precipitate when the acetone was diluted with water. This precipitate complicated the polyethylene chromatography when the initial filtration was not done. The following is the procedure most recently used and was found to be the best one:

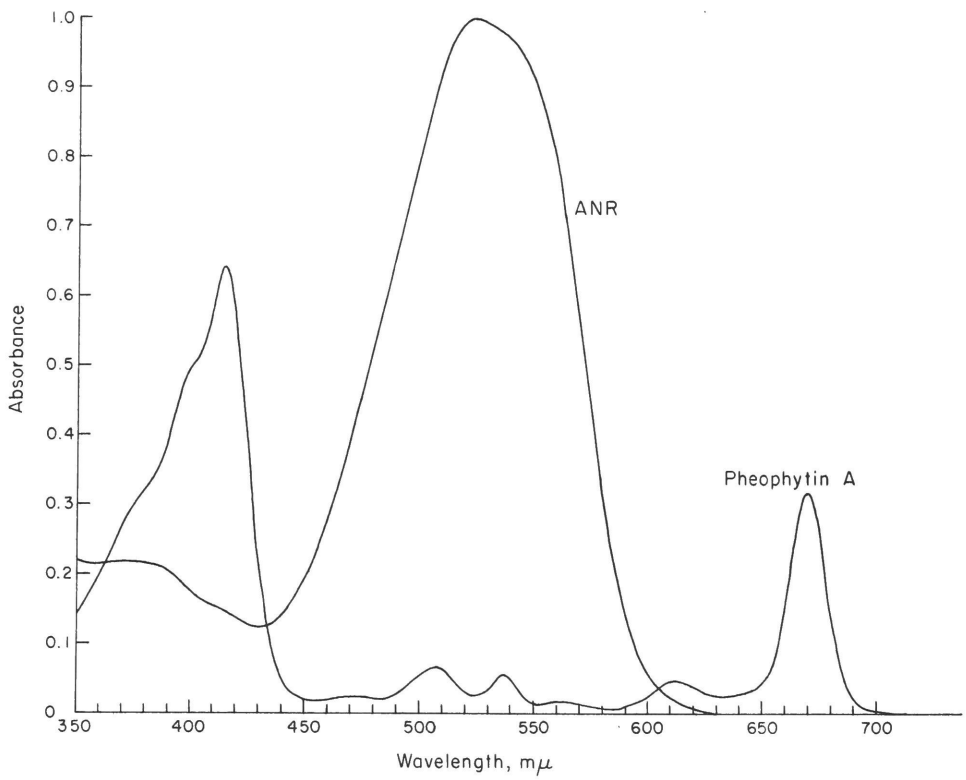



Figure 7. Absorption spectra of $3.22 \times 10^{-5}$ M amido naphthol red 6B (ANR) in water and of $6.5 \times 10^{-6}$ M pheophytin a in benzene (1 cm cell).

Two hundred grams of fresh leaves (spinach or pokeweed) were washed and the petioles removed. They were ground in a Waring blendor with 500 ml of absolute acetone. (Lyophilized leaves, which have been stored in a freezer, can also be used. In this case they should be extracted with 500 ml of 80-85% acetone/water per 200 grams original net weight.) The resulting mash was filtered through a thin pad of powdered polyethylene (Super Dylan PE 6008 Powder, Lot 29A2-14A-1, Melt Index 0.95, Density 0.953, Koppers Co., Plastics Division) on a Buchner funnel. The resulting solution was diluted to 70% acetone with distilled water. The pigments were then adsorbed onto a 4.5 cm diam. x 40 cm long column of the powdered polyethylene. The column was developed with 70% acetone/water. The xanthophylls eluted from the column first, preceding the chlorophylls and the pheophytins. Those carotenes which were not removed in the first filtration on polyethylene remained at the top of the column. After the yellow xanthophylls had been eluted the chlorophylls were washed from the column with 85% acetone. They were transferred to petroleum ether (30-60$^{o}$ bp) and the acetone removed by thorough washing (about 6 times) with water in a separatory funnel. The petroleum ether solution was dried with sodium sulfate and concentrated to about 100 ml under vacuum. The solution can be stored overnight at this stage.

Next, the pigments were adsorbed onto a similar column of dry confectioners sugar (Domino, 10X Powdered). 10% ether in petroleum ether was then passed through the column to remove a small trace of xanthophyll (Perkins and Roberts, 1962). The chlorophylls did not move in this solvent. When the faint yellow xanthophyll band was 1.5-2 cm below the chlorophyll band, the developing solvent was changed to 0.5% isopropanol in petroleum ether. In addition to the blue-green chlorophyll a band followed by the green chlorophyll b band, the respective pheophytins a and b and chlorophylls a' and b' (see Rabinowitch, 1956, p. 1771) were seen as small bands preceding the main bands. When the chlorophyll a was clearly separated from the preceding and following bands, the sugar was dug out of the column; care was taken not to mix the various pigmented bands. The center portions of the chlorophyll a and b bands were saved and the remaining sugar discarded. The chlorophylls were eluted from the sugar with acetone. If the chlorophylls are the desired final product, they can be transferred to petroleum ether as before and chromatographed

separately a second time on powdered sugar. Each chlorophyll should chromatograph as a single band, though chlorophyll b may require a third chromatography to remove chlorophyll a completely.

For pheophytin a, the acetone solution of chlorophyll a from the first powdered sugar column was treated with 10% by volume of 0.5 M oxalic acid in water, as suggested by Bellamy and Lynch (1963), and left in the dark at room temperature for about 30 minutes. By this time the chlorophyll was converted to pheophytin. The reaction was followed by observing the absorbance at 665 mμ which decreases to about 50% of its original value and then remains constant. The pheophytin was then transferred to petroleum ether as before. This solution was washed exhaustively with water to remove the acetone and oxalic acid. The petroleum ether solution was then dried with sodium sulfate and the pheophytin a adsorbed onto a dry-packed powdered sugar column as before. The column was developed with 0.25% isopropanol in petroleum ether and only a single olive green band was seen.

During the final chromatography, the main pigment band was allowed to move about halfway down the column, a sufficient distance to detect and separate other bands if present. The band was then dug out of the column and the pigment eluted from the sugar using a small amount of ether. The chlorophylls and pheophytins can be crystallized as described by Jacobs, Vatter and Holt (1954). We have found it more convenient to transfer the pheophytin to n-propanol by adding the propanol to the ether solution and removing the ether under vacuum. The pheophytin was then precipitated by diluting the propanol to about 25% with 0.005 M phosphate buffer, pH 6.5. The pheophytin was collected by centrifugation and stored wet in 50% methanol/phosphate buffer in the freezer. Such preparations appear to be stable for a period of several months.

The absorption spectrum of pheophytin a in benzene is shown in figure 7, along with that of the ANR. The locations of the absorption maxima and minima, as well as the ratios of the absorbancies at these wavelengths, are shown in table II and compared with the data of Bellamy and Lynch (1963). The agreement between my data and those of the latter workers is very good.

The molar absorptivity of pheophytin a in 80% acetone (by volume) was taken as $4.94 \times 10^4$ liters $mole^{-1}$ $cm^{-1}$ at the absorption maximum,

TABLE II

SPECTRAL PROPERTIES OF PHEOPHYTIN A IN BENZENE

| | This work | Bellamy & Lynch (1963) |
|---|---|---|
| | **ABSORPTION MAXIMA** | |
| R | 670 mμ (half width = 18.3 mμ) | 669 mμ (half width = 19.0 mμ) |
| | 612 mμ | |
| | 537 mμ | |
| Y | 508 mμ | 508 mμ |
| B | 414 mμ | 414 mμ |
| | **ABSORPTION MINIMA** | |
| Absolute minimum ($M_1$) | 583 mμ | 582 mμ |
| Relative minimum ($M_2$) | 455 mμ | 456 mμ |
| | **ABSORBANCE RATIOS** | |
| B/R | 2.02 | 2.1 |
| R/Y | 4.7 | 4.7 |
| $R/M_1$ | 31.8 | 31. |
| $M_1/M_2$ | 1.7-1.8 | 1.9 |

666 mμ (Vernon, 1960). The absorptivity in other solvents was determined by comparison of the absorbance of pheophytin in these solvents with an 80% acetone solution of equal concentration. The values at the red absorption maximum were $5.28 \times 10^4$ liters $mole^{-1}$ $cm^{-1}$ (665 mμ) in absolute acetone, $5.42 \times 10^4$ liters $mole^{-1}$ $cm^{-1}$ (667.5 mμ) in chloroform, and $4.98 \times 10^4$ liters $mole^{-1}$ $cm^{-1}$ (668 mμ) in dioxane.

In agreement with Perkins and Roberts (1964) and contradictory to the original report of Anderson and Calvin (1962), I have found the ratio of the absorbances of the blue and red absorption maxima of chlorophyll a, as prepared by the above method, to be 1.29 in ether. The error of the latter workers was caused by a solvent effect on the spectra.

The polystyrene particles were prepared from a polystyrene emulsion obtained from the Borden Co., Monomer Polymer Division (Lot no. MP 600-135). The particles were precipitated from the suspension by adding an arbitrary amount of magnesium sulfate to a volume of the diluted emulsion. The yellow supernatant was discarded. The particles were then washed successively with 0.1 N HCl in 50% w/w methanol/water, 50% methanol, 0.1 N NaOH + 0.001 M EDTA in 50% methanol, several times with 50% methanol and then either methanol or n-proponal. The particles were collected by centrifugation at 10,000-15,000 x g between washes. The supernatants were colorless. Those particles which had been given a final washing in methanol were dried and resuspended by homogenization as needed. The polystyrene particles which had been finally washed in propanol were resuspended in a small volume of propanol and thoroughly dispersed. The concentration of the polysytrene was determined by dissolving a small aliquot of the suspension in chloroform and determining the absorbance at 260 mμ with a Beckman model DU Spectrophotometer. The absorptivity of polystyrene at 260 mμ was found to be 2.2 liters $gm^{-1}$ $cm^{-1}$ by dissolving a weighed amount of polystyrene (as prepared in methanol) into a volume of chloroform. The necessary volume of the propanol suspension was then taken for the various experiments.

The manufacturer specified the particle size of the polystyrene as 0.05 μ. The particles are not of uniform size, however, but range from 0.012 to 0.13 μ in radius, as determined from the electron micrograph of the polystyrene particles shown in figure 8. The particle-size distribution is broad, as shown in figure 9. The average radius of 353 particles in the micrograph is 0.051 μ. (The sampling interval was 0.005 μ.) This

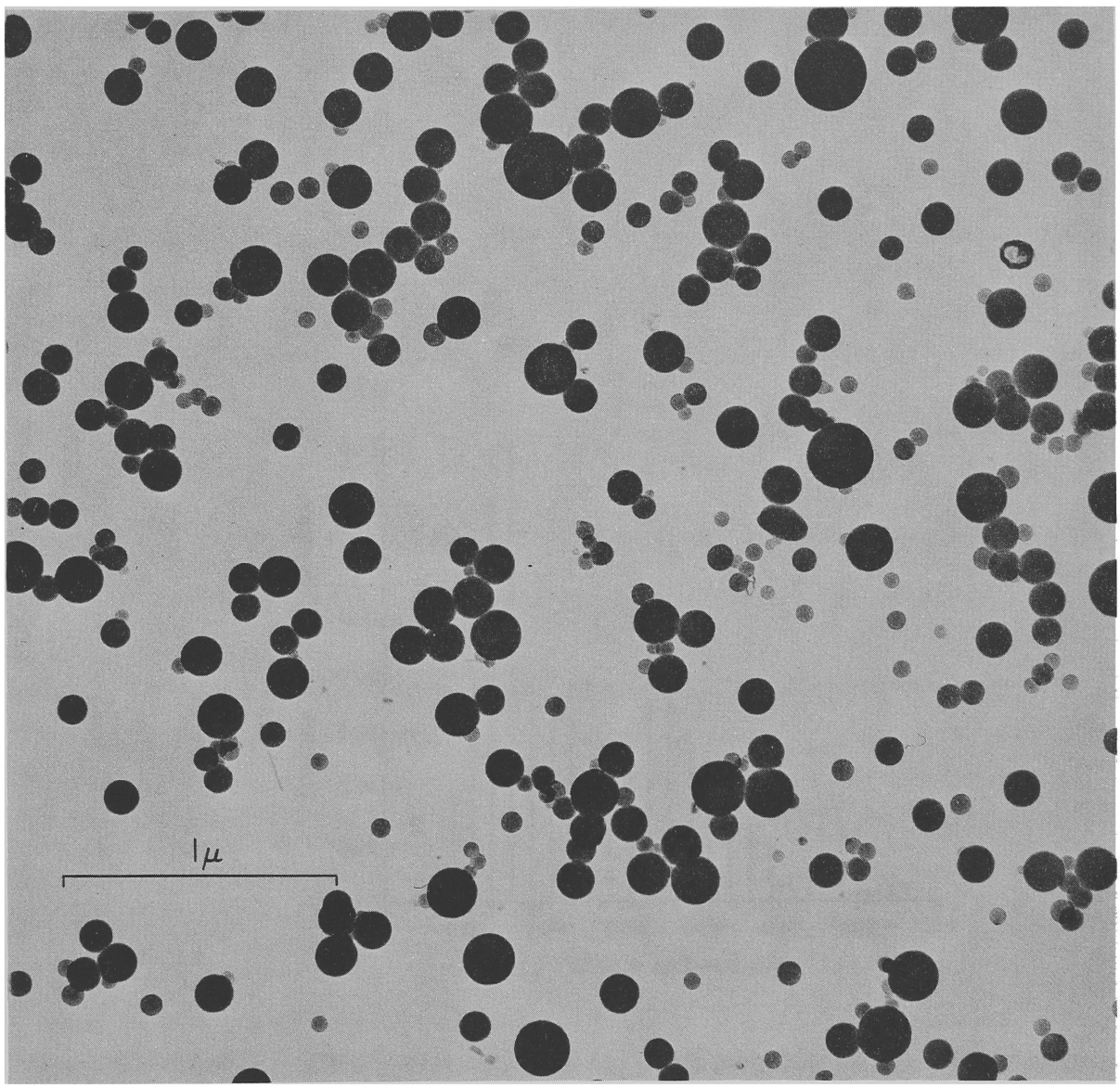



Figure 8. Electron micrograph of washed Bordens polystyrene particles.

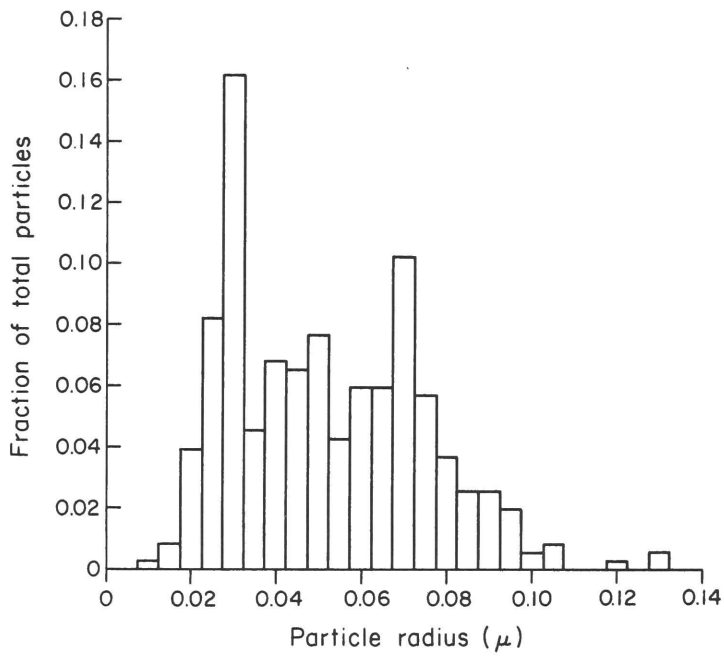



Figure 9. Histogram of particle size distribution of polystyrene latex particles from figure 8. The sampling interval was 0.005 μ.

presumably is the value quoted by the manufacturer as "particle size." The surface average particle radius is 0.070 μ. The surface average particle radius (R) is defined as (see Lloyd, 1959)

$$R = \frac{\sum_i n_i r_i^3}{\sum_i n_i r_i^2} \quad , \tag{1}$$

where $n_i$ is the number of particles of radius $r_i \pm \Delta r$ ($2\Delta r$ is the sampling interval). The sum extends over all values of the radius $r_i$. The specific surface area of the polystyrene, that is, the area per unit weight of particles, calculated from

$$A_S = \frac{\sum_i n_i \, 4\pi r_i^2}{\sum_i n_i \, (4/3)\pi r_i^3 \, \rho} = 3/\rho R \quad , \tag{2}$$

where $\rho$ is the density of the polystyrene (1.05 gms/cm$^3$), is 41 m$^2$/gm.

The zeolite particles used were Linde Molecular Sieve type 4A, powder (Zeolite A, $Na_{12}\left[(AlO_2)_{12}(SiO_2)_{12}\right] \cdot 27H_2O$, $\rho = 1.33$ gm/cm; see Breck, 1964). These particles have 4 A pores which can hold small molecules. The pheophytin was too large to fit into these pores and was presumably adsorbed on the external surface. Due to the negative surface charge on the zeolite, the ANR was not adsorbed to the particles. A test showed that even in aqueous suspension the N'-benzyl-dihydronicotinamide was not adsorbed to the zeolite.

The zeolite particles were prepared as follows: 100 gm of the powder were suspended in 300 ml of $1.4 \times 10^{-3}$ M EDTA. After a few minutes, the larger particles settled to the bottom and the remaining suspension was poured off. The large particles were discarded. A concentrated sodium hydroxide solution was added to the suspension to give a final concentration of 0.01 N NaOH. The suspension was left in the NaOH overnight. The particles were then collected by centrifugation and washed several times with distilled water and then phosphate buffer. The pH of the supernatant from these washes stabilized at about 10. The pH of the aqueous suspension of the zeolite was adjusted to about 7 with orthophosphoric acid. Finally, the particles were washed once with 50% methanol/water and dried at 80° overnight.

From microscopical observation the diameter of the zeolite particles ranged from 3 to 10 μ. The surface area of the particles was determined by the adsorption of a double layer of cetyl pyridinium chloride (average coverage, 27 $A^2$ per adsorbed molecule, Greenland and Quirk, 1963, 1964). The specific surface was about 20 $m^2$/gm, from which one can calculate a surface average particle radius of 0.11 μ. The large area (compared to that expected from the actual particle size) is probably due to the complex contours of the particle surface, since the cetyl pyridinium ions will not fit into the 4A holes.

In the preliminary experiments, two other types of particles were used: Linde alumina abrasives types A (0.3 μ particle size) and B (0.05 μ) and aluminum silicate particles (obtained from Minerals and Chemicals Philip, Corp.),especially types ASP 101, ASP 106, and ASP 170, which differed somewhat in the surface treatments. These particles were found to be unsatisfactory because of degradation of chlorophyll and pheophytin on the particle surface, as discussed in the following chapter. Dow polystyrene latexes were not used, due to difficulties in obtaining sufficient quantities, and also to evidence of contamination of the particle surfaces with the emulsifiers used to prevent clumping during the polymerization process (see Vanderhoff et al., 1956). Other polystyrene particles were too large to be useful.

II. Methods:

The preparation and analysis of the coated particles and the preparation of the reaction mixtures were carried out in dim green light to minimize the possibility of photodegradation of the pheophytin.

A) Preparation of coated particles:

The particles were coated by suspending them in a solvent in which the pheophytin was only slightly soluble or in which the pheophytin could be made insoluble by adding another component. It was found that adsorption to polar particles was effected most easily from nonpolar solvents and, conversely, adsorption to nonpolar particles was easiest from polar solvents. The surface concentration of pheophytin was varied by varying the ratio of pheophytin to the amount of particles in the original coating suspension. Because not all the pigment in the coating

solution adsorbed to the particle surface at high surface concentrations, the coated particles were analyzed for the relative amounts of pigment and particle.

Two methods were used for the adsorption of pheophytin to polystyrene:

I. Methanol procedure: The appropriate amount of polystyrene particles (dry) was weighed out and thoroughly homogenized in 5.8 ml methanol in a glass homogenizer. This served to disperse the particles in the solvent. 100 µl of a $2.5 \times 10^{-3}$ M acetone solution of pheophytin was added. Finally, 5.5 ml of $5 \times 10^{-3}$ phosphate buffer, pH 6.6, were added rapidly with homogenization to give a final mixture of 50% w/w methanol/water. Addition of the buffer to the methanol drives the pheophytin out of solution and onto the particles. The particles were centrifuged, washed with 50% methanol/phosphate buffer, and resuspended in a known volume of 50% methanol.

II. Propanol procedure: The appropriate amount of the n-propanol suspension of polystyrene (see previous section) was diluted to 3.8 ml with propanol and stirred vigorously with a motor-driven glass stirring rod. 100 µl of a $5 \times 10^{-3}$ M acetone solution of pheophytin was added. Finally, 3.0 ml of $5 \times 10^{-3}$ M phosphate buffer, pH 6.4, was rapidly injected from a syringe into the stirred suspension, which gave a final concentration of 50% w/w propanol/water. Again, the pheophytin was driven out of solution and onto the particles. (For higher surface concentrations of pheophytin it was found that more adsorption would occur by diluting with 6 ml instead of 3 ml of buffer.) The particles were collected, washed, and resuspended in 50% methanol/phosphate buffer as before. It was noted that the high-coverage particles tended to aggregate much more readily than the low-coverage ones.

The propanol method was tried because we observed that, at high surface concentrations, the pheophytin tended to form aggregates in the 50% methanol upon dilution, rather than adsorbing onto the particles. The pheophytin is more soluble in the propanol and comes out of the 50% propanol more slowly than out of the 50% methanol. In the 50% propanol, the pheophytin adsorbed onto the particles and did not form separate aggregates in the solvent. The properties of the sets of particles

obtained by the two methods were slightly different (chapter III). This was probably due to the difference in solubility of pheophytin in the two alcohols.

The polar zeolite particles were coated as follows:

An appropriate amount of particles (dry) was suspended in 4 to 8 ml of petroleum ether (bp. 30-60°). While the suspension was stirred, 100 µl of a $2.5 \times 10^{-3}$ M acetone solution of pheophytin was added. The pheophytin is only slightly soluble in the petroleum ether. It adsorbed immediately to the zeolite in those suspensions with a large amount of particles (giving relatively low surface concentrations). At high surface concentrations, a limit was reached in the amount of pigment adsorbed to the particles. Thus, a classical adsorption isotherm was observed. After adsorption (the suspensions were stirred for about 1 minute after adding the pheophytin), the coated particles were sedimented by centrifugation in a clinical centrifuge and the supernatant poured off. Any remaining solvent was allowed to evaporate in air.

I feel that more pheophytin can be adsorbed to the particles than the maximum obtained in the procedure described above. I have observed that the degree of adsorption is dependent on the small amount of acetone in the petroleum ether. Though pheophytin will precipate from the petroleum ether in the absence of zeolite particles at lower acetone concentrations, this did not occur at the conditions of 2.5% acetone and approximately $6 \times 10^{-5}$ M pheophytin.

B) Analyses of the coated particles:

I will define the surface coverage of the particles as the fraction of the particle surface covered with pheophytin:

$$\text{Coverage} = \frac{N_{ph}\, a_p}{A} = \frac{n\, N_o\, a_p}{A_S} = \frac{1}{3}\, n\, N_o\, a_p \rho\, R \qquad (3)$$

where $a_p$ is the surface area covered by one pheophytin molecule, $N_{ph}$ is the number of molecules on the surface of a single particle with surface area A. More practically, the average coverage can be calculated if we know n, the number of moles of pheophytin per gram of particles and $A_S$, the specific surface area of the particles ($A_S = 3/\rho R$, where R is the surface average particle radius). $N_o$ is Avogadro's number.

We estimate $a_p$ as follows: The area of the plane of ethyl chlorophyllide (chlorophyll with the phytol chain replaced by an ethyl group) is about 242 $A^2$ as determined by X-ray crystallography (see Rabinowitch, 1945, p.448). The phytol chain could add as much as 50 to 100 $A^2$ to this. However, studies of pheophytin monolayers at an air/water interface (Bellamy, Gaines, and Tweet, 1963) indicate that the surface area occupied by pheophytin is only 80 - 100 $A^2$, depending on the surface pressure. I would expect an increased attraction energy between the phytol and the polystyrene surface as compared to that between the phytol and the aqueous interface. Thus there might be a larger contribution of the phytol to the area covered by the pheophytin on polystyrene particles. On these considerations I have estimated the surface area covered by one pheophytin molecule on the polystyrene to be about 100 $A^2$. I have used a slightly different approach with the zeolite particles, as will be seen below.

The value of 100 $A^2$ implies that the pigment molecules are sitting with the plane of the chlorin ring at an angle, not lying flat on the surfaces. The validity of this assumption will be discussed when considering the experimental results. It should be noted that the coverage values resulting from the above assumption would be as much as a factor of 3 too small (if the area were closer to 300 $A^2$ with the molecules flat on the surface) or as much as a factor of 2 too large (if the area covered was about 50 to 75 $A^2$ with the molecular planes perpendicular to the surface).

The specific surface of the polystyrene particles is 41 $m^2$/gram, as discussed earlier. To determine the ratio of pheophytin to polystyrene, a sample of the coated particles was dissolved in either dioxane or chloroform (the dioxane was found to be more satisfactory because the small amount of water from the particle suspensions was miscible with the dioxane), and the absorbance of the solution determined at the absorption maxima of pheophytin (668 mμ) and polystyrene (259, 262, 265, 269 mμ). The absorptivities of the pheophytin and polystyrene at these wavelengths (table III) were determined with solutions of known concentration. From this data, n was calculated.

On the basis of this analysis, the surface coverage of the polystyrene particles varied from 0.001 to 3.1, that is, from a value of 0.1% of the surface covered to effectively more than 3 layers of pheophytin

TABLE III

ABSORPTIVITIES OF PHEOPHYTIN AND POLYSTYRENE IN DIOXANE

| Wavelength | Pheophytin | Polystyrene |
|---|---|---|
| 668 mμ | $4.98 \times 10^4$ l/m cm | 0 |
| 269 mμ | $1.37 \times 10^4$ | 1.72 l/g cm |
| 265 mμ | $1.28 \times 10^4$ | $1.73_5$ |
| 262 mμ | $1.25 \times 10^4$ | 2.20 |
| 259 mμ | $1.23 \times 10^4$ | 2.14 |

on the surface. In the following discussions, "0.001 PS" will refer to those polystyrene particles coated with pheophytin at a surface coverage of 0.001 as defined above. A similar notation will be used for other surface coverage values.

The zeolite particles were analyzed by extraction of the pheophytin from a weighed amount of the particles. The pheophytin was extracted in a known volume of 80% acetone. (Absolute acetone does not extract the pigment from the particles completely.) The amount of pheophytin was estimated from the absorbance at 665 mμ. This was then used to calculate the ratio of pheophytin to zeolite. On the basis of the value of 20 $m^2$/gm for the specific surface of the zeolite, determined from the cetyl pyridinium chloride adsorption, the maximum coverage reached on the zeolite was 0.25, according to equation 3. However, the cetyl pyridinium chloride is a smaller molecule (surface coverage 54 $A^2$ per molecule; Greenland and Quirk, 1963) than the pheophytin. Because of irregularities, more of the surface may be available to the pyridinium ring than to the pheophytin. For this reason, I have chosen the highest coverage obtained as the 1.0 coverage zeolite (1.0 Z), and I have normalized all the remaining coverage values to this.

C) Photochemical studies:

All reactions were done with the reducing agent and the ANR (or other dye) in solution. For the reactions with the pheophytin in solution, 90% w/w methanol/water was the solvent. This solvent was chosen because meaningful pH measurements could be made (see below). The reactions with the coated zeolite particles were done in aqueous solutions. The photoreactions with the coated polystyrene particles were done in 50% w/w methanol/water. This solvent was used in preference to pure water because of the hydrophobic properties of the polystyrene. For example, when an aqueous suspension of polystyrene particles was bubbled with argon, the particles tended to collect at the surface. The polystyrene dispersed well in the 50% methanol, however. A small amount of pheophytin was extracted from the higher coverage polystyrene particles by the 50% methanol. About 0.25% of the pigment was extracted from the 0.72 PS particles and about 1% from the 3.1 PS particles. Only in the latter case could this extracted pigment be significant for the photochemistry (see chapter III).

I tested for loss of pheophytin from the polystyrene by suspending the particles in the solvent, centrifuging at 15,000 g, and extracting the supernatant with ether. Presence of pheophytin was tested for by visual observation of any red fluorescence in ultraviolet light. For comparison, the pheophytin was extracted from the sedimented polystyrene particles with a volume of ether equal to that used to extract the supernatant. The fluorescence intensity of the supernatant extract was compared with that of serial dilutions of the particle extract to obtain an estimate of what proportion of the pigment had been extracted by the 50% methanol. The sensitivity of the method was such that 0.1% of the pheophytin on the particles could have been detected if it had been extracted. The 50% methanol supernatants had no detectable fluorescence even when the ether extracts did. It should also be noted that the values for the amount of pheophytin extracted would be too high if some of the small polystyrene particles were not completely removed by the centrifugation.

Most of the reactions were performed at about pH 7 in each solvent, though a few experiments were done to test the effect of variation in pH. The pH measurements were made with a Beckman no. 39183 Combination pH electrode and a Beckman model G pH Meter. Beckman buffers were used to standardize the electrode for the aqueous determinations. A 0.01 M succinate buffer was used to standardize the electrode in 90% methanol (de Ligny et al., 1960). A 0.1 M succinate buffer of pH about 4.3 was prepared from succinic acid and sodium hydroxide in distilled water, and its pH was determined vs. Beckman pH 4 standard buffer. This was then diluted to 90% w/w methanol. The pH of this solution, which was used as the standard in 90% methanol, was assumed to be equal to

$$6.73 + (pH_{aq} - 4.12) \quad ,$$

where $pH_{aq}$ is the pH of the 0.1 M aqueous solution and 6.73 and 4.12 are the pH values of equimolar solutions containing 0.01 M succinic acid and 0.01 M lithium hydrogen succinate in 90% methanol and in pure water, respectively (de Lingy et al., 1960). It was assumed that there would be no significant difference between lithium and sodium as cation for our purposes. The linear correction was used because the Henderson-Hasselbach equation,

$$pH = pK_a + \log \frac{Salt}{Acid} \quad , \qquad (4)$$

was assumed to hold in both water and 90% methanol. Since the liquid junction potential between water and 50% methanol is small, the aqueous buffers were also used to standardize the electrode for routine determinations in 50% methanol. This introduced an error of about 0.1 pH unit (de Ligny and Rehbach, 1960). For titrations in 50% methanol the electrode was standardized using a succinate buffer prepared in a manner similar to that for the 90% methanol standard.

The following pK's were determined: Ascorbic acid in 90% methanol at 30° and 0.1 ionic strength: $pK_1 = 6.72 \pm 0.05$; in 90% methanol saturated with KCl at 30°: $pK_1 = 6.50 \pm 0.05$; in 50% methanol at 30° and 0.1 ionic strength: $pK_1 = 4.95 \pm 0.05$. (These values may be compared with the value of $pK_1 = 4.21$ at 30° and 0.1 ionic strength in water; Kortüm et al., 1961, p. 510.)

Orthophosphoric acid in 50% methanol at 27.5°, about 0.12 ionic strength: $pK_2 = 7.95 \pm 0.1$.

Phosphate was used as buffer in the reactions done in water and in 50% methanol. Succinate or ascorbic acid was used to buffer the solutions in 90% methanol.

Since the dye reduction is inhibited by oxygen (due to the sensitized autoxidation of the reducing agent, at least in part), the reaction mixtures were bubbled for 10 to 20 minutes with argon (Matheson, containing 1-4 ppm oxygen according to the manufacturer). The argon was passed through a gas-washing apparatus to equilibrate it with the solvent used for the reaction. It was then fed into the reaction cell through a length of thin teflon tubing. The teflon was permeable to oxygen, however, and sufficient oxygen diffused into the argon to inhibit the reaction to a small extent. The reactions with N'-benzyl-dihydronicotinamide as reductant seemed to be much more sensitive to oxygen than were those with ascorbic acid. A coaxial tube was built, with argon flowing through both the outer and inner tubes. Only the inner tube led into the reaction mixture. In this way, oxygen was effectively eliminated from the reaction.

Ascorbic acid solutions were adjusted to the desired pH by adding a solution of sodium hydroxide. Since ascorbic acid is rapidly oxidized by oxygen under basic conditions, these solutions were thoroughly deoxygenated with argon before mixing. If any yellowness was observed in the

final solution (characteristic of the oxidized ascorbate), the solution was discarded. Although the dihydronicotinamides are not easily autoxidized, their solutions were bubbled with argon during their preparation.

The photoreaction was performed by illuminating the reaction mixture with a beam of red light, absorbed only by the pheophytin. The light sensitized the reduction of the ANR. This particular azo dye was chosen because, among other reasons, its absorption maximum fell at a wavelength (525 mμ) in between the absorption maxima of pheophytin (see figure 7). Since the dye is bleached upon reduction, it was thus possible to follow the reaction photometrically during the period of illumination. A monitoring light beam at 525 mμ passed through the solution or suspension and was detected with a phototube. Changes in the current of the phototube reflected the changes in the ANR concentration in the solution. Because the absorbance of pheophytin is relatively low at 525 mμ, the monitoring beam did not sensitize the photoreduction. Moreover, the ANR was not directly photoreduced by the monitoring light. (Direct photoreduction did occur when other dyes were studied, see chapter III).

A diagram of the experimental apparatus is shown in figure 10. The actinic (photochemical) light source was a 1000 watt slide projector (P). The power supply (PS) for the projector consisted of a 2 KVA Sola voltage regulating transformer and a Powerstat (1.6 KVA) which was used to vary the lamp voltage. The light was filtered through 20 cm of water or water/methanol (W) to remove infra-red and reduce heating in the reaction mixture. Colored glass filters (F), such as Corning 2-59 and, in some reactions, a Bausch and Lomb (B & L) interference filter, were used to obtain light of the desired wavelength distribution. The light intensity was varied in known steps by inserting either neutral density interference filters or calibrated screens in the light beam. Illumination of the reaction cell (C) was controlled with a shutter (S).

The monitoring beam passed through the reaction cell at right angles to the actinic beam. The monitoring lamp (M) (G.E. 1493 or G.E. 1615) was powered (MS) by a microscope lamp transformer in combination with the Sola transformer or by a Harrison model 809A Regulated DC Power Supply. The 1615 lamp powered by the Harrison supply was the most satisfactory arrangement in terms of available intensity and low noise level. The monitoring beam passed through two sets of blocking and interference

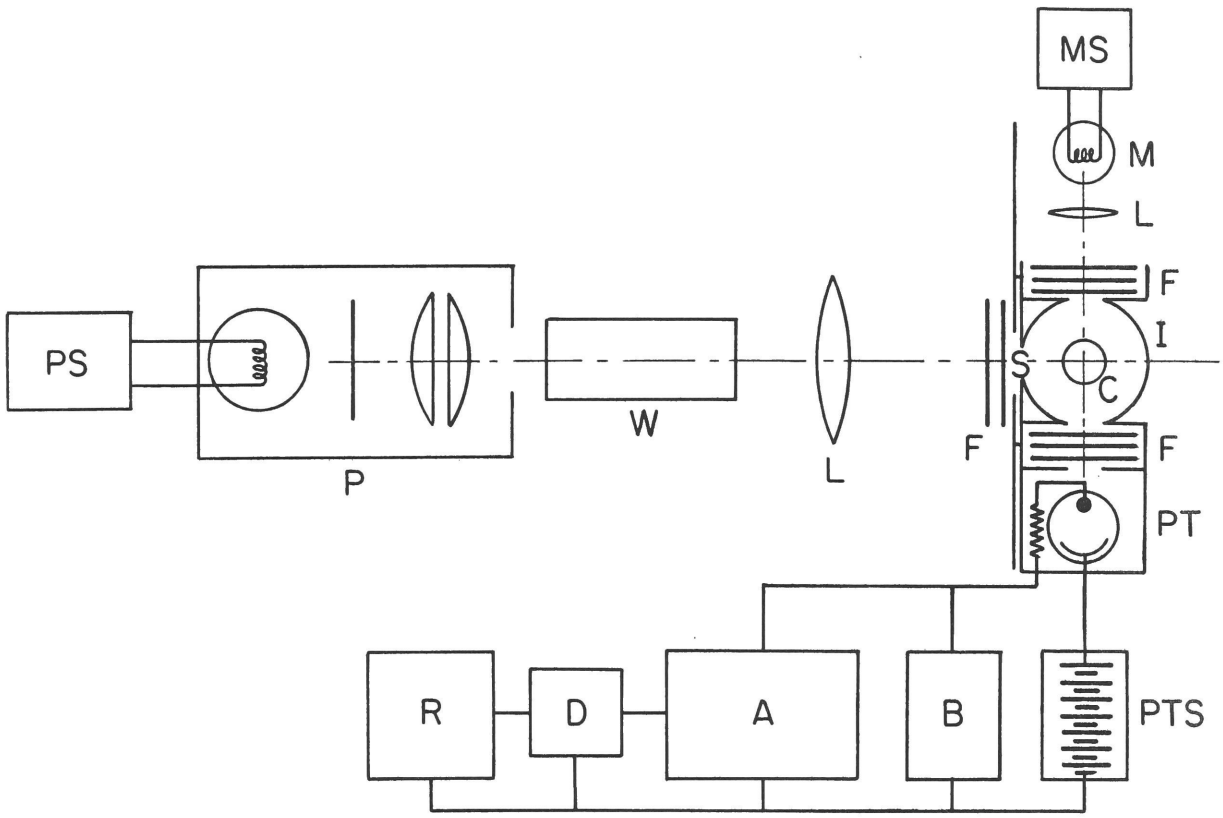



Figure 10. Diagram of experimental apparatus. See text and figures 11 and 12 for details.

filters (F) before (Corning 3-70 and 4-96, B & L 525 mμ interference) and after (Corning 4-72, B & L 522 mμ interference ) the reaction cell. These served to isolate the light at about 525 mμ and to prevent the scattered actinic light from reaching the phototube (PT). The monitoring beam was detected with an RCA 926 vacuum phototube (S-3 response) or an RCA 5582 gas phototube (S-4 response), at a potential of 90 V supplied by batteries (PTS). The current from the phototube was determined with a Kiethly model 414 Micro-microammeter (A) and a Varian model G-10 recorder (R). A bucking circuit (B) (circuit diagram shown in figure 11), was used to increase the sensitivity by suppressing the major portion of the photocurrent. Between the ammeter and the recorder was a circuit (D in figure 10, see also figure 12) which was designed to filter out transients, to reduce the output voltage of the ammeter to match the recorder input limits, and to provide a small spike on the recorder chart when the shutter was opened or closed. A microswitch ($S_2$) was attached to the cable release of the shutter.

The light intensity at the location of the reaction cuvette was measured periodically with a small selenium photocell (Edmund Scientific, no. 30,411). The current from the photocell was determined with the Micro-microammeter. The cell was calibrated approximately by comparing its readings with those of a calibrated Epply thermopile.

I have used the quantum yield of ANR reduction as a measure of the photoreactivity of a given pheophytin preparation. The quantum yield, defined as the number of molecules of dye reduced per quantum of light absorbed, is also equal to the ratio of the rate of dye reduction to the rate of light absorption.

The initial experiments in solution were followed with the 926 vacuum phototube. Using neutral density interference filters, I determined that the photocurrent was linear with respect to the intensity of the monitoring beam incident on the tube. The reaction cells were square cuvettes and Beer's law was used to calculate the rate of the reaction from the change in photocurrent for the first 30 to 60 seconds:

Since $i = K I$, where i is the photocurrent and I is the light intensity reaching the phototube, and since the concentration of ANR in the solution is given by $C = 1/(\epsilon d) \log (I_o/I)$, where $\epsilon$ is the molar

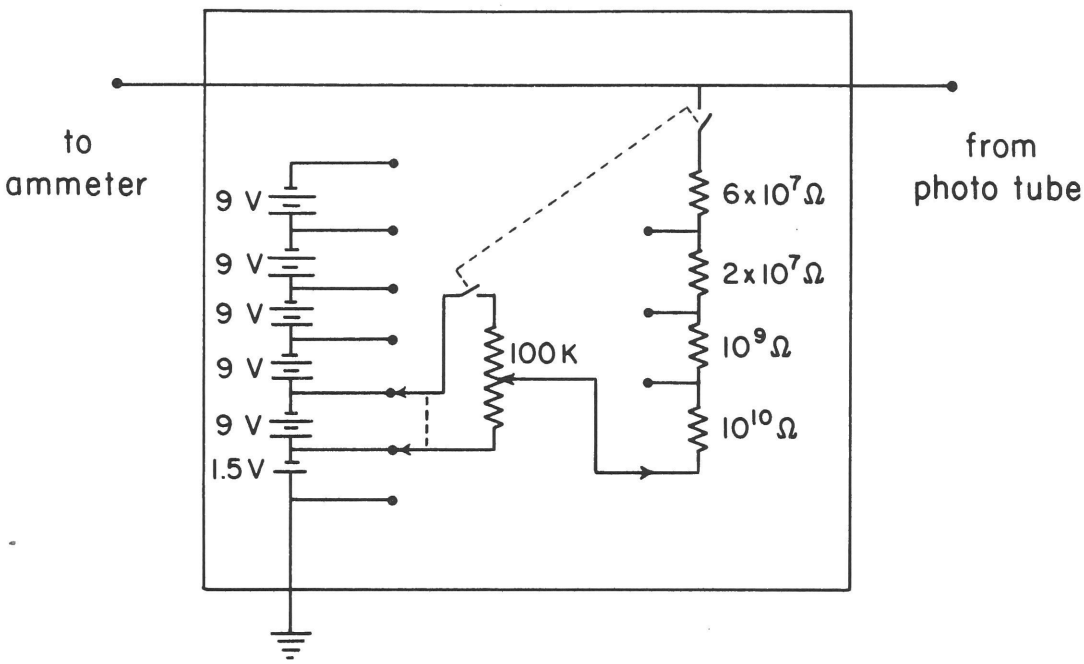



Figure 11. Current bucking circuit (B in figure 10).

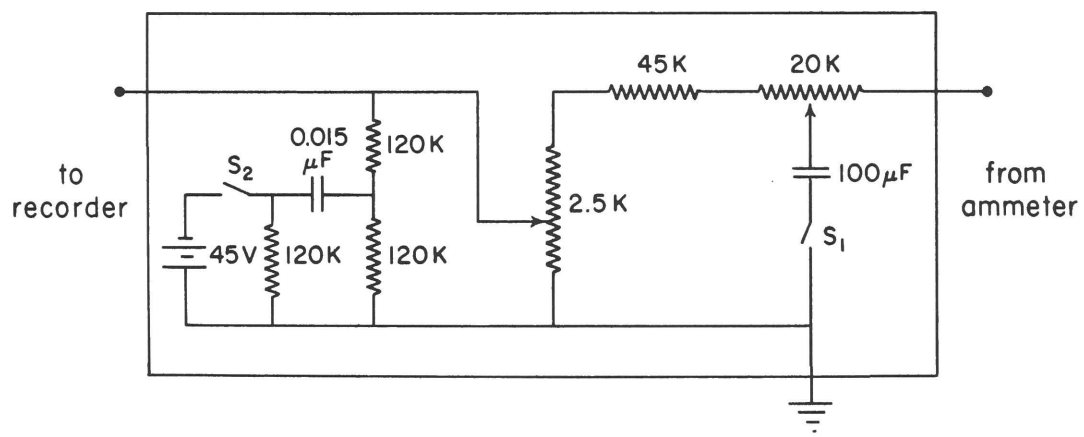



Figure 12. Filter, voltage divider and marking circuit (D in figure 10).

absorptivity of the ANR, d is the path length through the solution and $I_o$ is the light intensity with no dye in the solution, the rate of the photoreaction,

$$R = \frac{C_{t_2} - C_{t_1}}{t_2 - t_1} V = \frac{V}{\epsilon\, d\, \Delta t} (\log I_o/I_{t_2} - \log I_o/I_{t_1}).$$

Substituting i/K for I, and noting that $I_o$ is constant with time, we obtain

$$R = \frac{V}{\epsilon\, d\, \Delta t} \log (i_{t_1} / i_{t_2}), \qquad (5)$$

where V is the volume of the solution, and $\Delta t = t_2 - t_1$ is the time over which the change in current is measured. If the actinic light intensity and the pheophytin concentration are kept constant during a series of experiments, the rate of light absorption will be constant and the ratio of the rates R will be a measure of the relative quantum yields. This procedure of determining relative yields avoids the difficulties inherent in determining the number of quanta absorbed absolutely.

For the particle suspensions a cylindrical geometry was used with the hope of maximizing absorption of the actinic light. The reaction tube (a round test tube) was placed in the center of an "integrating cylinder" (I) (figure 10), a can painted white on the inside so that light scattered by the particle suspension would be reflected back into the suspension. Openings were cut in the can for the actinic and monitoring beams. Because of the cylindrical geometry and the high scatter of the suspensions, the rate of reaction could not be calculated in the same way as in the solution. By suppressing the photocurrent with the bucking circuit (figure 11) and using the more sensitive scales of the Micromicroammeter, I was able to measure changes in dye concentration with greater accuracy. The 5582 gas phototube was used in these experiments because of its greater sensitivity. With a syringe microburet (Micro-Metric Instruments, model SB-2) small aliquots of dye could be added to the reaction mixture and this served as an internal calibration. The sensitivity of the apparatus was such that a ½% change in the total dye concentration (equivalent to the reduction of $5 \times 10^{-10}$ moles of ANR) could easily be detected (see figure 13 for a sample experimental record). This arrangement had the additional advantage that several different experiments could

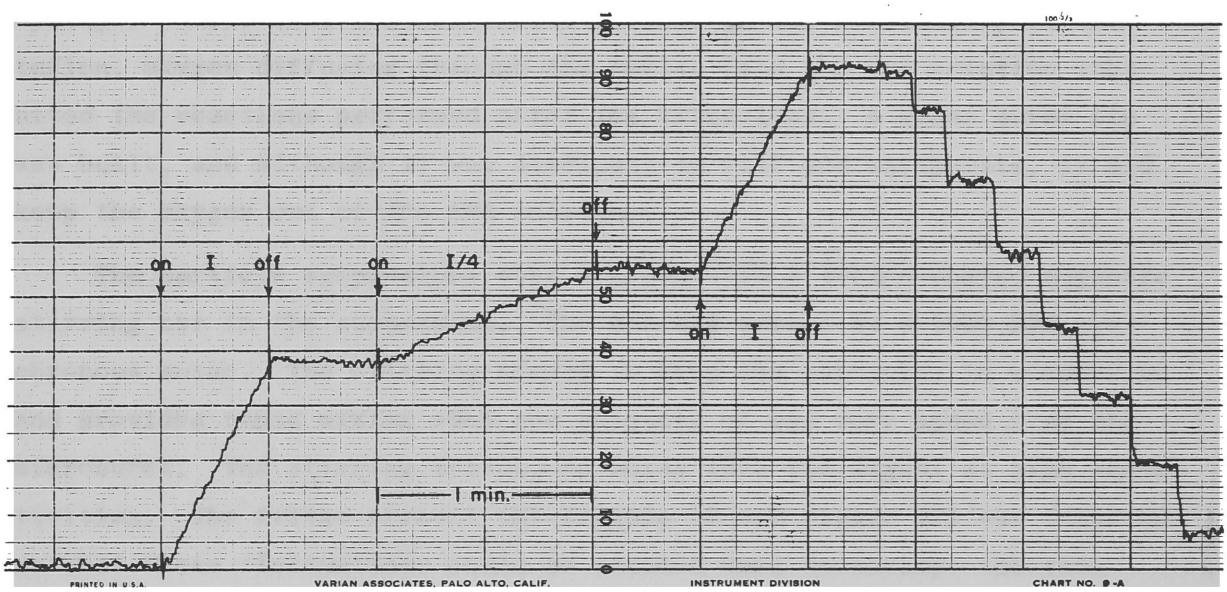



Figure 13. Sample experimental record showing the photoreduction of ANR by pheophytin in 90% methanol solution with added uncoated polystyrene particles. The abcissa is the expanded phototube current (3 x $10^{-10}$ amp full scale, total current is 7.5 x $10^{-9}$ amp). The ordinate is time. There is no reduction of the dye in the dark and a flat base line is obtained. When the shutter is opened, the light is absorbed by the pheophytin and the dye is reduced, which results in an increase in the phototube current. In the dark, the reaction stops abruptly. It can be repeated at different light intensities (for example, at $\frac{1}{4}$ the intensity). The scale is calibrated in terms of moles ANR reduced by titrating in aliquots of dye; each step, except the first, represents 2 x $10^{-9}$ moles of ANR added to the suspension. Several more repetitions can be made in the same reaction solution. Reaction conditions: 3.6 x $10^{-6}$ M pheophytin a (1.1 x $10^{-8}$ moles in 3.0 ml); 3.0 x $10^{-5}$ M ANR; 1.0 x $10^{-3}$ M BNH; 50 mg uncoated polystyrene particles; 0.016 M succinate buffer, pH 7. Illumination through Corning 2-59 and B & L 662 mμ filters, without and with 0.25 transmittance screen. The approximate incident intensity without screen was 2 x $10^{-8}$ einst. $cm^{-2}$ $sec^{-1}$.

be done with the same particle suspension starting with the same dye concentration. Moreover, the calculation of the rate of the reaction could be made directly from the slope of the recorder curve, since the recorder current was linear with dye concentration at the sensitivity used.

The dye solutions were originally titrated into the reaction mixture from a teflon "needle" (Hamilton Syringe Co.) attached to the microburet syringe. While this was convenient because of the flexibility of the teflon, oxygen diffusing through the teflon into the dye solution inhibited the reactions performed after the titrations. A glass buret tip was built, and although it was difficult to fill and manipulate, it did keep the oxygen out of the ANR solution.

The particle suspensions were stirred with a teflon-coated magnetic stirring bar in the suspension. The bar was driven by a 1000 RPM synchronous motor. The stirring served to keep the particles in suspension and provided rapid mixing when the aliquots of dye were added from the microburet. The stirring introduced noise into the signal at high sensitivities. The filter circuit (D) (figure 10 and figure 12) was inserted so that these fluctuations could be reduced when necessary; the time constant of the circuit could be varied as desired.

In the particle suspensions the problem of obtaining a measure of the amount of light absorbed was again solved by performing all the reactions in a given series under what were estimated to be equivalent conditions, that is, the same amount of light was absorbed in each reaction vessel. In some cases, the data were corrected for deviations from this condition with empirically derived relationships. The relative quantum yield was then taken as the ratio of the rates of dye reduction at constant light absorption.

The conditions for having the same amount of light absorbed in each reaction vessel were: 1) that there be the same total amount of pheophytin in the reaction vessel; 2) that the incident intensity be constant within a given series; and 3) that the same amount of polystyrene particles be in each reaction cell. The necessity for the last condition is seen upon consideration of figure 14. The rate of photosensitized reduction of the ANR at a given light intensity and total pheophytin in solution is a function of the total amount of polystyrene particles in the suspension.

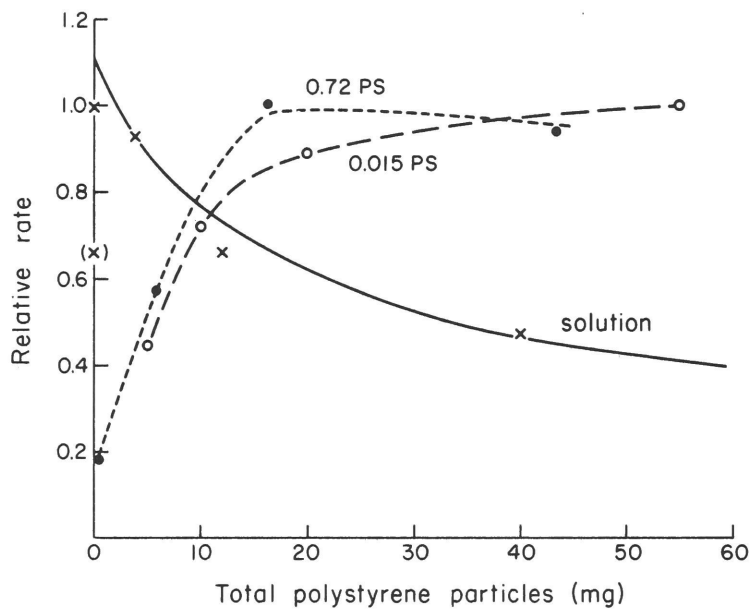



Figure 14. The relative rate of ANR reduction, sensitized by pheophytin a in solution or adsorbed to polystyrene particles, as a function of the total amount of polystyrene in suspension. The solid line is a theoretical curve (see text). The data have been normalized to the maxium rate observed for each pheophytin preparation. Reaction conditions: Solution: as described in figure 13 except $1.0 \times 10^{-3}$ M CNH as reductant, light intensity about $4 \times 10^{-8}$ einst. $cm^{-2}$ $sec^{-1}$ (interference filter); total particles as shown. Coated particles: solvent, 50% methanol, $2.3 \times 10^{-3}$ M phosphate buffer, pH 7; $3 \times 10^{-5}$ M ANR; $1.0 \times 10^{-3}$ M BNH; 5 mg 0.015 PS ($5 \times 10^{-9}$ moles pheophytin in 3.0 ml) or 0.36 mg 0.72 PS ($1.8 \times 10^{-8}$ moles pheophytin) plus uncoated polystyrene particles to give total amount shown; illumination through Corning 2-59 filter only.

This experiment was done by adding additional uncoated polystyrene particles to a suspension of coated particles. With pheophytin in solution, the relative rate decreases with increasing scatter, while adding uncoated particles to a suspension of coated particles tends to increase the rate of reaction. In each case, the rate tends to level off at about 40-50 mg of particles in the suspension.

The addition of highly scattering particles to a solution of pigment should have two effects: 1) some light will be reflected by the surface of the suspension (back scatter) and 2) the optical path of light that does go into the suspension will be increased due to multiple scatter; for an example, see the work of Butler (1962). The net result of these effects, as seen from the graph, is that the amount of light absorbed by the pigment drops sharply with the introduction of scattering materials but tends to level off as the scatter is increased. Reasonable agreement with experiment can be obtained from the theory of Kubelka (1948) for the optical properties of scattering media. Kubelka gives formulae for the transmittance and reflectance of a scattering and absorbing suspension. The suspension is assumed to have planar geometry and to be illuminated uniformly and diffusely on one face. Edge effects are neglected. By subtraction of the fraction of the light which is reflected and transmitted from 1, we obtain the fraction of the incident light which is absorbed,

$$A_f = \frac{\sinh \mu Kx + \mu[(\cosh \mu Kx) - 1]}{\frac{1}{2}(\mu^2 + 1)\sinh \mu Kx + \mu \cosh \mu Kx} \quad , \qquad (6)$$

where $\mu = (1 + 2S/K)^{\frac{1}{2}}$, S and K are, respectively, the scattering and absorption coefficients (to the base e) under diffuse illumination, and x is the depth of the suspension. It is easily shown that as S goes to 0, $A_f$ goes to $(1 - e^{-Kx})$, as would be expected for a solution with no scatter. The rate of photoreduction is directly proportional to the light absorbed (chapter III), so a plot of $A_f$ as a function of scatter should follow the decrease in the rate as the number of scattering particles in a pheophytin solution is increased. That this is so is shown by the solid curve in figure 14. I have taken the value of Kx corresponding to the absorbancy of the solution used for the experiments: Kx = 2 x 2.3 A where A = 0.18. The factor 2.3 comes from the conversion from base 10 to base e. The factor 2 is introduced because the absorption coefficient

under diffuse illumination (which was assumed to hold for the scattering suspensions) is twice that for parallel illumination (Kubelka, 1948). I have assumed that the scattering coefficient, S, is directly proportional to the amount of polystyrene in suspension. I have chosen the proportionality constant and the normalization factor between $A_f$ and the rate so that the theoretical curve passes through the experimental points at 4 mg and 40 mg of polystyrene particles. I did not normalize the theoretical curve to a rate of 1.0 at 0 particles because of uncertainty as to whether the diffuse or parallel illumination absorption coefficient should be used. The theoretical curve extrapolates to the predicted rate for diffuse illumination. The (x) at 0.66 at 0 particles gives the predicted rate for parallel illumination. Within experimental error (10%) the datum agrees with the value for diffuse illumination. It appears that even with no particles present, the integrating cylinder and the cylindrical geometry of the apparatus result in diffuse illumination. The consistency between the experimental data and the theory, in spite of the fact that the experimental conditions are quite different from those to which the theory applies, suggests that our analysis is basically correct.

The scattering properties of a suspension are quite different when the light absorbing pigment is adsorbed to the surface of the particles rather than being in solution. The coated particle is a "packet" of pigment, i.e., the pheophytin is not distributed homogeneously throughout the suspension. In addition, I have observed that the coated particles tend to aggregate, especially at high coverages. Observation with the light microscope indicated that these aggregates were as large as 3.0 μ in diameter (containing about 30,000 particles) for the 0.72 PS particles. Smaller aggregates (0.5 - 1 μ, 300-1000 particles) were noted in the 0.015 PS and uncoated polystyrene particle suspensions in 50% methanol. The aggregates may have been slightly smaller in the case of the uncoated particles. It was difficult to make accurate measurements of the size of the aggregates because of the small size of the individual particles, which diffracted the light. The adsorbed pheophytin seems to act as a "glue" to hold the particles together.

Duysens (1956) and Gledhill and Julian (1963) have shown that, in the absence of scatter, if the light absorbing pigment is in packets, the amount of light absorbed is less than if the pigment were dispersed uniformly throughout the solution. The ratio of the absorbance of the suspension

of pigmented particles to that of the homogeneous solution decreases as the light absorbed by a single particle increases. (This means the absorption maximum is "flattened" in the suspensions; Duysens, 1956). Even at high coverages on the polystyrene particles, the light absorbed by a single (unclumped) particle is not sufficient to explain the observed decrease in rate. In an aggregate of particles, however, the light absorbed would be larger, not only due to the increase in "particle" diameter (Absorbance = $\epsilon$ cd) but also because of the multiple scatter which would occur in the aggregate (Butler, 1962). The path length of light in the aggregate of high-coverage polystyrene particles would have to be increased 100-200 times over that of a single particle to explain the observed rate with no additional particles. Even larger aggregates of the 0.015 PS particles must be assumed to explain the low rate observed in the absence of uncoated particles. Since these aggregates were not observed, it is not clear that the above analysis will account completely for the observed behavior.

A second effect of aggregation would be to bury some of the particles in the aggregate so that the ANR or the reductant could not get to them to react. If only pheophytin adsorbed to the particles on the outer layer of the aggregate could react, the rate of reduction would be about 1/5 the rate in the case that all particles were available, for the 0.72 PS. For the 0.015 PS particles, the reduction in rate due to this second effect would be about 1/2 to 1/3.

The lowered rate for the 0.72 PS particles in the absence of uncoated particles is probably due to a combination of both results of particle aggregation. For the 0.015 PS the second effect is probably the most important. The addition of uncoated particles serves to break up the aggregates, both dispersing the pigmented particles more evenly throughout the suspension and yielding smaller aggregates. Thus more light is absorbed and more particles become available for reaction. The reaction saturates with increasing particles as the aggregates approach the size of the aggregates of uncoated particles. The latter are probably less tightly packed than the aggregates of coated particles, allowing the inside particles to react also.

Since the zeolite particles were much larger, their light scattering properties were different from those of polystyrene. Kortüm, Braun and Herzog (1963) have shown that the scattering coefficient (S) is inversely

proportional to the particle diameter for sizes greater than the wavelength of the light. Thus, the scattering coefficient for the zeolite particles is about 1/100 that of the polystyrene. Addition of uncoated zeolite particles to either a solution of pheophytin or a suspension of coated particles did not significantly affect the rate of reduction (table IV), which suggests that the amount of light absorbed is the same in each case. The result with the coated particles is consistent with the observation that the polar zeolite particles dispersed well in the aqueous buffer used for the experiments even at high coverages. The absorption spectra of the zeolite particles indicate some aggregation of the pheophytin on the particles (as opposed to aggregation or clumping of the particles) is occurring at low coverage. As discussed above, this aggregation can lead to inhomogeneous light absorption. We may estimate the maximum effect of the aggregation on the amount of light absorbed by assuming that all of the pheophytin on a zeolite particle is in a single packet. For a particle about 5 μ diameter, there are about $16 \times 10^6$ molecules on the surface at 0.0094 surface coverage, The absorbance of this packet at the red absorption maximum is about 2. This means that only about 1/2 as much light would be absorbed by this suspension as would be if the pigment were in solution, as estimated from the curves of Gledhill and Julian (1963). Therefore, this effect could not explain the twenty-five times lower activity of the 0.0094 **Z** particles (chapter III).

In order to determine the wavelength dependence of the quantum yield of the reaction, it was necessary to determine the amount of light absorbed by pheophytin at the various wavelengths used. The relative incident intensities were determined with an uncalibrated thermocouple (Charles M. Reeder Co., model Rx-9). The fraction of the incident light absorbed by the pheophytin was determined from the percent transmission spectra of solutions of ANR and ANR plus pheophytin at the concentrations used in the experiment. It was necessary to determine both spectra since the ANR also absorbs light below 600 mμ (figure 7). In the region in which both compounds absorbed, the fraction absorbed by pheophytin, $A_{f;P}$, was determined from the following equation:

$$A_{f;P} = A_{f;P+ANR}\left[A_P / (A_P + A_{ANR})\right] . \qquad (7)$$

TABLE IV

EFFECT OF SCATTER BY ZEOLITE PARTICLES ON ANR REDUCTION SENSITIZED BY PHEOPHYTIN A

I). Pheophytin in solution in 90% methanol

| Additional particles | Rate of ANR reduction |
|---|---|
| 0 mg | 0.153 ± 0.015 nm/sec |
| 10 | 0.139 |
| 100 | 0.160 |

II). Pheophytin adsorbed to 0.22 coverage zeolite particles

| Total particles | Rate of ANR reduction |
|---|---|
| 4 mg coated + uncoated | 0.038 ± 0.005 nm/sec |
| 4 mg coated + 100 mg uncoated | 0.042 |

Reaction conditions: Solution: as described in figure 14 except for additional zeolite particles as indicated. Particle preparation: solvent, water, $5 \times 10^{-3}$ M phosphate buffer, pH 7; $3 \times 10^{-5}$ M ANR; $1.0 \times 10^{-3}$ M CNH; $1 \times 10^{-8}$ mole pheophytin in 3.0 ml. Illumination through Corning 2-59 filter only.

Here $A_{f;P+ANR}$ is the fraction of the light absorbed by the solution with both compounds in it, and $A_P$ and $A_{ANR}$ are the absorbances (or optical densities; $A = \epsilon d c$, where $\epsilon$ is the absorptivity, d is the path length and c is the concentration) of the pheophytin and ANR respectively. This was derived from the assumption that the fraction of incident light absorbed by each compound in a thin layer (dx) of the solution is proportional to its absorbance ($dA = \epsilon c dx$).

The values for the fraction of light absorbed were taken at the wavelength of maximum transmission of the interference filter used, except at 662 mμ. At this wavelength, correction was made for the finite band pass of the filter (assuming a triangular distribution with 10 mμ half width). This was necessary due to the large change of slope of the absorption spectrum at the absorption maximum. At other wavelengths, the slope was approximately constant over the interval.

D) Spectral measurements:

The absorption spectra of the coated particles were determined on a Cary model 14 MR spectrophotometer using a technique similar to that described by Thomas and Govindjee (1960) for determining the absorption spectra of algae. The particles to be measured were suspended in a small volume of buffer and deposited on a 13 mm diameter Millipore filter, held in a Swinny Adapter (Millipore Filter Corp.), by forcing the liquid through the filter. This resulted in a reasonably uniform distribution of particles over an area about 10 mm in diameter on the filter. The filter was then placed in a cuvette and placed in the cell compartment so that the spectrophotometer beam passed through the particles. A second filter, with uncoated particles deposited on it, was used for a reference. With an equal number of particles on the two filters, a reasonably flat base line was obtained. In order to reduce the distorting effects of multiple scatter, (Butler, 1962) the samples were prepared so that the measured absorbance was less than 0.1 and the 0.1 slide wire of the Cary was used. A Hamamatsu R-136 red sensitive photomultiplier tube was used to obtain relatively small slit widths in the red. Over the range of interest, the spectral band pass of the spectrophotometer varied from 2-5 mμ. Because the thickness of the layer of particles varied from filter to filter, a quantitative comparison of the absorbances of the different particles was not possible. The spectra shown in figures 27-29, 39 and 40 (chapter III) have been traced from the original curves and

corrected for variations in the base line. Because of the difficulties of reproducing the conditions for a given measurement, it was not always possible to determine the base line exactly. For this reason there could be an error of 0.0025 - 0.005 in the absorbance across any individual spectrum.

The absorption spectra of the particles were also determined on an apparatus built by Dr. W. L. Butler (Butler, 1962) specifically designed to measure the absorption spectra of highly scattering materials. The results obtained with this instrument were similar to those obtained with the method described above. However, we feel that the spectra taken on the Cary with the filter technique are more reliable because the total absorbance of the sample was quite a bit lower than with Butler's instrument. At the higher absorbances, the absorption peaks are flattened due to the multiple scatter and reabsorption (Bulter, 1962).

The fluorescence spectra of the coated particles were determined with an EMI 9558B photomultiplier and a Jena Glaswerke interference wedge filter (Veril S-200) combination. The effective bandwidth was about 12 mμ, and unless noted, the spectra are not corrected for the wavelength response of the photomultiplier (S 20) and wedge. A long-wave hand UV lamp (maximum wavelength 355 mμ, halfwidth about 30 mμ) was used as the exciting source. The particles generally were in dilute suspension to reduce the effects of scatter as much as possible.

The intensity of the fluorescence was determined for several dilutions of the particle suspensions; and in the range spanned, for all coverages of polystyrene particles, the fluorescence intensity was directly proportional to the amount of pheophytin in the suspension. This held over at least a thirty-fold variation in concentration. The relative fluorescence yields were determined by dividing the observed fluorescence signal at a given wavelength by the total amount of pheophytin in the suspension, and normalizing to the value for the 0.0014 coverage polystyrene.

Fluorescence excitation spectra were obtained using the Cary 14 monochrometer with reversed light path as the exciting source and detecting the fluorescence with the photomultiplier-wedge combination set at the desired wavelength. To achieve the required sensitivity a phase-sensitive amplifier locked on to the Cary light-chopping system was used. The spectra have been corrected for the variation in quantum output of the monochromator

as determined with the thermopile. For the fluorescence excitation spectra, the particles were coated as a paste onto a glass coverslip and covered with a second coverslip to reduce evaporation of the solvent. The fluorescence was measured from the illuminated face of this "cell."

# CHAPTER III

## EXPERIMENTAL RESULTS AND DISCUSSION

In this chapter the results of the photochemical and spectral studies on the pheophytin-coated polystyrene and zeolite particles are described. I have used the quantum yield of the photosensitized reduction of the azo dye, amido naphthol red 6B (ANR), or the safranin dye, fuchsia, as a measure of the photoactivity of the coated particles. The spectral studies include the determination of absorption, fluorescence emission and fluorescence excitation spectra, as well as a determination of the relative quantum yields of fluorescence of the coated particles. These results are analyzed in terms of a model for the distribution of pheophytin molecules, as monomers and aggregates, on the particle surface.

### I. Preliminary studies of the photosensitization reaction.

I first studied the photochemical properties of chlorophyll in solution to define the range of variables (such as concentration, pH, etc.) for the photosensitization reaction. This work included the search for an appropriate solvent, reducing agent and electron acceptor (dye), as well as the development of the measuring system described in the previous chapter. I discovered sometime after most of these results had been obtained that many of the experiments had been done not with chlorophyll a but with a degraded form of the pigment as the sensitizer. I am including these studies because they show some of the general properties of the photoreaction. The results are probably applicable to chlorophyll and pheophytin, though Livingston (1960, p.869) cautions that different pigments may have different mechanisms for the same overall reaction.

I concluded that the sensitizer used in these experiments was not chlorophyll a because its blue absorption maximum occurs at 420 mμ in 90% methanol, while that of chlorophyll a occurs at 433 mμ. The red absorption maxima of the degraded and pure chlorophylls are at 663 and 665 mμ, respectively; the lack of any major shift in the red absorption maximum mislead me to assume that there had been no degradation of the pigment. The degraded pigment was not further characterized, but it is probably an oxidation product of chlorophyll which had formed in the concentrated stock solutions in acetone. In the later work with particles, solutions were made up fresh from a slurry of precipitated pheophytin (see chapter II).



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The product of autoxidation of chlorophyll in methanol solutions is usually referred to as "allomerized chlorophyll" (Rabinowitch, 1945, p.459; 1956, p. 1773). Hill (1963) has used this term for the oxidation product formed in any solvent, but I will use it in the former, more restricted sense. I will refer to the product from the acetone solutions as "degraded chlorophyll."

The initial reactions were done with the azo dye, methyl red (figure 15), as the electron acceptor, since it had been used by many previous workers (see Livingston, 1960). One disadvantage of the methyl red is that its color goes from red to yellow above pH 5. This complicates the study of the reaction as a function of pH, since one is dealing with different species of the molecule with different reactivities (Livingston and Pariser, 1948). In addition, the Soret (blue) absorption maximum of chlorophyll interferes with the photometric analysis of the yellow form of the dye (which has its absorption maximum at 430 mμ, as does chlorophyll a) above pH 5. A search through the laboratory dye stocks turned up the amido naphthol red 6B (ANR; figure 3) with absorption maximum at 525 mμ (figure 7) and which showed no changes with pH. The ANR was used in all subsequent studies except as noted.

The choice of ANR was advantageous from another point of view. When an azo dye is reduced, the N-N double bond is split and the products are the two amines (Zollinger, 1961):

$$\text{Ar-N=N-Ar}' \xrightarrow{\text{reduction}} \text{Ar-NH}_2 + \text{Ar}'\text{-NH}_2. \qquad \text{(VII)}$$

In some azo dyes, such as methyl red, there is an amino group para to the azo linkage, and upon reduction paraphenylene diamine is produced. Livingston and Pariser (1956a), studying the pheophytin-sensitized reduction of butter yellow (p-dimethylaminoazobenzene; figure 16), found that the N,N-dimethyl-p-phenylenediamine produced by the reduction acted as a catalyst for the reaction. This made the reaction autocatalytic. I have noted a similar effect with methyl red, and especially for lanacyl violet (figure 17) which yields 1,4-naphthalenediamine as a reduction product (figure 18). The N-acetyl-p-phenylenediamine produced by the reduction of ANR does not act as a catalyst for the reaction, presumably because the acetyl group withdraws electrons from the nitrogen. This destabilizes the diamine cation,

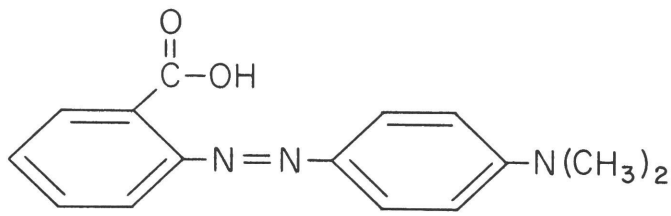


Figure 15. Methyl Red (C.I. no. 13020).

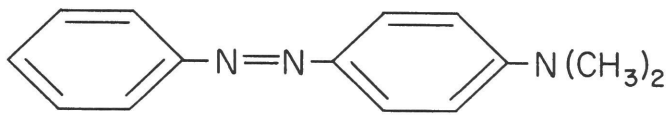


Figure 16. Butter Yellow (C.I. no. 11020).

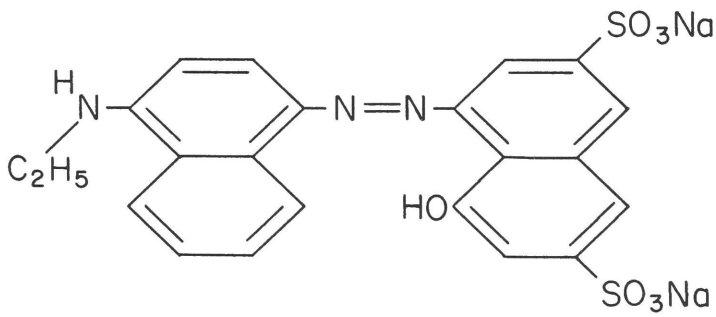


Figure 17. Lanacyl Violet BF (C.I. no. 13375).

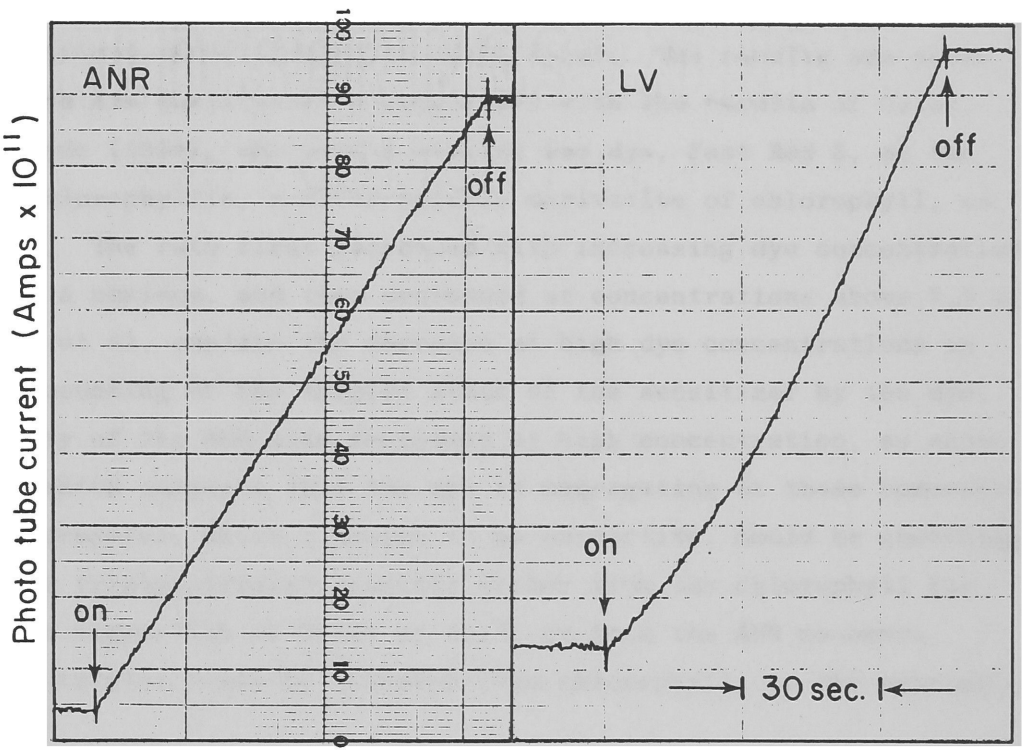


Figure 18. Comparison of the time course of the pheophytin-sensitized reduction of amido naphthol red (ANR) and lanacyl violet (LV) by BNH, showing the autocatalysis of LV reduction. In each case, full scale represents about 3.5% of the total dye concentration (ca. $3 \times 10^{-5}$ M for ANR and $4\text{-}6 \times 10^{-5}$ M for LV), and the ordinate is linear in dye concentration within 3.5% for the ANR and 2% for the LV. The increase in slope with time for the LV indicates the quantum yield is increasing with time. Reaction conditions: as described in table V below. Illumination through Corning 2-59 and B & L 662 mμ filters. Incident intensity $2.5 \times 10^{-9}$ einst. $cm^{-2}$ $sec^{-1}$ for the ANR, $2 \times 10^{-8}$ einst. $cm^{-2}$ $sec^{-1}$ for the LV.

which is likely involved in the catalysis as an electron shuttle. Thus the ANR reaction is not autocatalytic, and under optimal conditions, the reduction rate is constant for at least the first ten percent dye reduced.*

The ANR concentration used in these experiments ($3 \times 10^{-5}$ M) was originally chosen so that the absorbance of the reaction solution was about 1 at the absorption maximum of the ANR, 525 mμ. In an experiment designed to test the effect of varying the dye concentration on the rate of photosensitization, it was found that the chosen concentration gave rates very close to the optimum, other components being equal. The results are shown in figure 19 and are qualitatively consistent with the results of Oster, Bellin and Broyde (1964), who used a similar azo dye, Fast Red S, as the acceptor and chlorophyllin, a water-soluble derivative of chlorophyll, as the sensitizer. The rate first increased with increasing dye concentration, passed through a maximum, and then decreased at concentrations above $7.5 \times 10^{-5}$ M. Oster et al. explain the decrease at high dye concentrations in terms of the quenching of the triplet state of the sensitizer by the dye. The absorptivity of the ANR also decreased at high concentration, as shown in figure 19, which suggests that the dye is aggregating at these concentrations. The aggregates, which I assume to be unreactive, could be quenching the reaction by triplet-triplet transfer either from the chlorophyll (in accord with the suggestion of Oster et al.), or from the ANR monomers, excited to the triplet state by transfer from chlorophyll, to the aggregates (see below).

I have qualitatively compared several different types of dyes as electron acceptors in the pheophytin-sensitized reduction reaction. These are listed in table V with the experimental results. There are some dyes, such as the azo dyes ANR and lanacyl violet, which are not directly photoreduced, but whose reduction can be sensitized by pheophytin; there are some, such as oxonine (oxazine type) and fuchsia (safranin type) which are both directly photoreduced and reduced in the sensitized reaction; of particular interest

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* Livingston and Pariser (1956b) reported that they did not observe the autocatalytic effect in sensitization reactions other than the pheophytin-butter yellow-ascorbic acid system (including those with allomerized chlorophyll as sensitizer); on this result Livingston based his aforementioned remark regarding the possible difference in mechanisms of apparently similar reactions. However, as noted above, I have noted the autocatalytic effect for reactions sensitized by chlorophyll (or degraded chlorophyll). The reasons for this discrepancy are not clear.

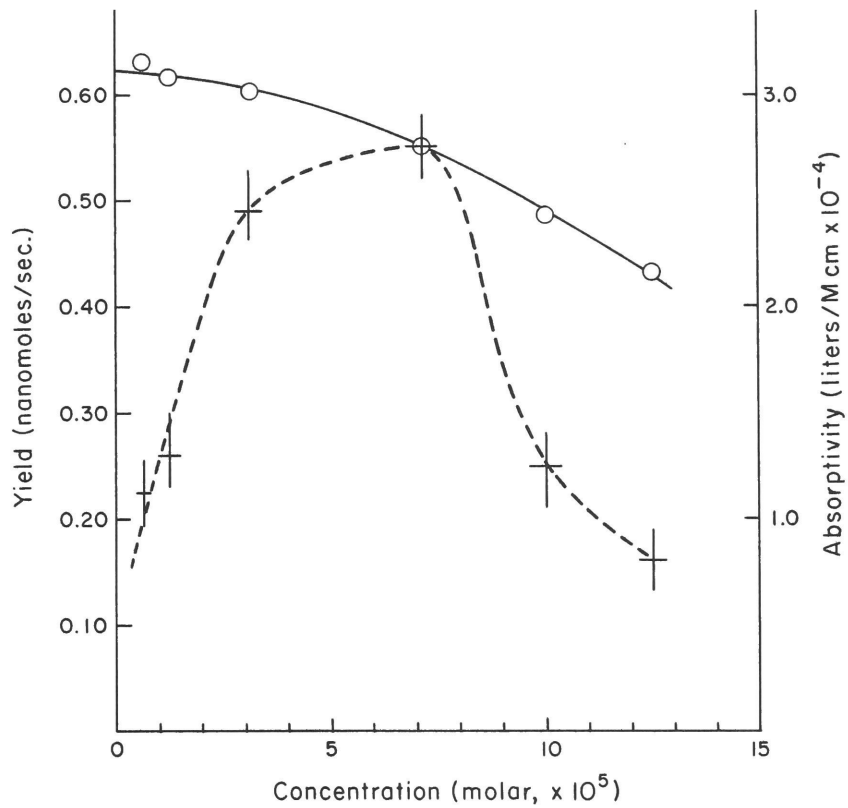


Figure 19. Dependence of the yield of sensitized ANR reduction (-------) and of ANR absorptivity (————) on ANR concentration. Reaction conditions: 90% methanol as solvent, 0.016 M succinate buffer, pH 7; 0.012 M ascorbic acid; $6 \times 10^{-6}$ M chlorophyll a. Illumination through Corning 2-59 and B & L 662 mμ filters. Incident intensity about $1.5 \times 10^{-8}$ einst. $cm^{-2}$ $sec^{-1}$.

TABLE V

COMPARISON OF DIFFERENT DYES AS ELECTRON ACCEPTORS

| Dye | Dark Reduction | Direct Photoreduction | Sensitized Photoreduction | Notes |
|---|---|---|---|---|
| Amidonaphthol Red | - | - | + | |
| Lanacyl Violet | - | - | + | Autocatalytic |
| Naphthol Sulfonate-Indophenol | + | | + | |
| Neutral Red | - | - | + | |
| Oxonine | + | + | (+) | Sensitized at pH 11; dark & direct reductions too fast at pH 7 to test sensitization |
| Eosin | - | + | - | |
| Pararosanalin HCl | - | - | + | Absorbance change goes through maximum; reaches steady state with trace of $O_2$; reversible in dark. |
| Safranin | + | | + | Does not reach steady state with BNH; add'n of $O_2$ restores dye. |
| Fuchsia | - | + | + | Behaves as Safranin |
| Fuchsia (Ascorbic Acid as reductant) | - | - | + | Reaches steady state, amount reduced in steady state $\propto$ I; reverses in dark without $O_2$ |

All reactions done in 90% methanol/succinate buffer with pheophytin ($10^{-8}$ moles in 3.0 ml) as sensitizer and 1 x $10^{-3}$ M dihydrobenzylnicotinamide as reductant (except as noted). Dye concentrations were adjusted to give equal absorbance at 525 mμ (about 1 for a 1 cm cell). Direct photoreduction was tested for by noting whether shutting off the monitoring beam changed the rate of dye reduction observed in the absence of actinic light. If there was no change in dye concentration in the abscence of red light, both dark reduction and direct photoreduction were assumed to be absent. In two cases, naphthol sulphonate indophenol and safranin, dark and direct photoreduction were not distinguished.

is eosin (a fluoran dye), which was directly photoreduced, but was not reduced in the photosensitized reaction. I believe this last result has some implications for the mechansim of the photosensitized reduction reaction.

There have been several mechanisms proposed for these photosensitized reactions (it should be noted that all of these propose the lowest triplet state of the sensitizer as the photochemically active state): 1) Since the same reducing agents are effective in the direct photoreduction of chlorophyll as in the sensitized reactions, Krasnovskii (see Krasnovskii, 1960) has suggested that the sensitized reaction proceeds through a reduced form of the sensitizer (i.e., chlorophyll is reduced by, say, the ascorbic acid, and the reduced chlorophyll reduces the dye); 2) Evstigneev, Gavrilova and Savkina (1963) have recently acknowledged that the reaction could also proceed via an electron transfer from the chlorophyll to the dye, with subsequent reduction of the oxidized chlorophyll by the reducing agent; 3) Livingston (1960, p. 873) suggests two possible mechanisms involving a termolecular reaction of chlorophyll with the dye and reducing agent simultaneously; the difference between the two mechanisms being whether the dye or reductant initially forms a complex with the chlorophyll, with the complex reacting with the remaining component; 4) On the basis of an analysis of the photosensitized reduction of safranin by ascorbic acid, Bannister (1963b) has recently revived the possibility of a triplet-triplet transfer mechanism, that is, a direct energy transfer from chlorophyll in its lowest triplet state to excite the safranin to its triplet state, with the excited safranin being reduced by the ascorbic acid.

While I cannot agree with the reasoning which lead Bannister to suggest the triplet-triplet mechanism,* I believe that the lack of sensitized reduction of eosin can be explained most easily by this mechanism.

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* In his analysis, Bannister assumes that oxidation products of ascorbic acid do not affect the kinetics. We have observed that the reducing agent does influence the kinetics of the reaction (compare fuchsia with ascorbic acid and benzyl-dihydronicotinamide as reductants, table V); moreover, the first order dark reoxidation of safranin observed by Bannister can be easily explained by the presence of as little as 1% oxidized ascorbate in the solution. Other parts of his analysis are open to similar criticism. In addition, where Bannister claims that the steady state level of safranin reduction is proportional to the square root of the intensity, I observed that it was directly proportional to the intensity in the case of fuchsia being reduced by ascorbic acid. The reason for this discrepancy is not clear.

Efficient electronic energy transfer between molecules will occur only if the excited state of the acceptor is of equal or slightly lower energy than that of the donor (Hammond, Turro, and Leermakers, 1962). Long-lived luminescence (phosphorescence ?) has been observed from chlorophyll a and b only at wavelengths longer than 750 mμ (Fernandez and Becker, 1959; Singh and Becker, 1960) and eosin phosphorescence occurs at about 650 mμ (Pringsheim, 1949). Thus the chlorophyll triplet level lies at most 1.67 ev above the ground state (and pheophytin would be expected to be very similar to chlorophyll) and the eosin triplet level is 1.92 ev above the ground state. Thus, there would be no transfer from triplet pheophytin to triplet eosin, and assuming that the sensitized reaction proceeds by triplet-triplet transfer, there would be no sensitized reduction of eosin.

At two different times, I compared the effectiveness of different reducing agents for the sensitized dye reduction. The first group, tested with degraded chlorophyll as the sensitizer, included thiols and ethylene diamines (known to be active in the photoreduction of the porphyrins, Mauzerall, 1960). None of these was as effective a reductant as ascorbic acid, which was at least 20 times more reactive than thioglycolic acid, the next most effective reductant tried (table VI).

Somewhat later, I tested the effectiveness of some reduced nicotinamide-adenine dinucleotide (NADH) analogues, as well as NADH itself, as reductants. These were tried because of the ease of oxidation of the ascorbic acid made preparation of the reaction solutions difficult, and because of difficulties in obtaining high rates with ascorbic acid in aqueous solution (see below). The most effective of these analogues (table VII) was N'-benzyl-1,4-dihydronicotinamide (BNH), and it was about as effective as ascorbic acid at the concentration tried ($5 \times 10^{-3}$ M). The BNH was not susceptible to autoxidation, but the photoreaction with it as reductant seemed more sensitive to oxygen than with ascorbic acid (probably because the latter can react with and eliminate the oxygen while the BNH cannot). The BNH also reduced ANR in the dark, about 10 times more rapidly in water than in 50% methanol and 100 times more rapidly in water than in 90% methanol at a given concentration; in 90% methanol the dark reaction was significant only at the highest concentrations used (0.01 M). The N'-carboxamidomethyl-1,4-dihydronicotinamide (CNH) was somewhat less photoreactive than the BHN (by a factor of 3), but it was distinctly less reactive with the ANR in the dark (by a factor of 50).

TABLE VI

COMPARISON OF DIFFERENT REDUCTANTS

| Reductant | Relative Yield |
|---|---|
| 0.012 M Ascorbic Acid | 1.0 |
| 0.025 M Thioglycolic Acid | 0.043 |
| 0.012 M Mercaptosuccinic Acid | 0.019 |
| 0.015 M EDTA, tetramethyl ester | 0.018 |
| 0.025 M Dimethylaminoacetaldehyde, dimethyl acetal | 0.012 |
| 0.12 M Carboxymethylmercaptosuccinic Acid, trimethyl ester | 0.0080 |
| 0.12 M Allylthiourea | 0.0078 |
| 0.025 M Dimethylaminoacetonitrile | 0.0062 |
| 0.9 M Diethylphenylmalonate | 0.0035 |
| 0.012 M Tetramethylethylenediamine | 0.0015 |

Reaction conditions: Solvent, 90% methanol, 0.016 M succinate buffer, pH 7; degraded chlorophyll a as sensitizer (absorbance about 0.5 at 662 mμ); $3 \times 10^{-5}$ M ANR. Illumination through Corning 2-59 filter only.

TABLE VII

COMPARISON OF DIFFERENT REDUCTANTS

| Reductant | Relative Yield |
|---|---|
| Ascorbic Acid | 1.0 |
| N'-benzyl-1,4-dihydronicotinamide | 1.0 |
| N'-carboxamidomethyl-1,4-dihydronicotinamide | 0.33 |
| Diethyl-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate | 0.20 |
| NADH | 0.03 |
| Diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate | 0.005 |

Reaction conditions: Solvent, 90% methanol, 0.016 M succinate buffer, pH 7; $1.7 \times 10^{-8}$ moles (in 3.0 ml) pheophytin a as sensitizer; $3 \times 10^{-5}$ M ANR; reductant concentration was $5 \times 10^{-3}$ M in all cases. Illumination through Corning 2-59 and B & L 662 mμ filters; incident intensity about $2 \times 10^{-8}$ einst. $cm^{-2}$ $sec^{-1}$.

We have used ascorbic acid and both BNH and CNH in the photosensitization reactions, using the CNH in situations where the BNH gave too large a dark reaction (mainly those experiments done in water). From table VII, it is interesting to note that the addition of a methyl group at the 4 position of the reduced pyridine ring remarkably reduces the effectiveness of the compound as a reducing agent in the sensitized reaction.

The variation in the rate of the photosensitized reduction of ANR as a function of BNH concentration is shown in figure 20, for pheophytin both in solution and adsorbed to polystyrene particles. Similar behavior, approaching saturation at high reductant concentration, was observed with ascorbic acid as reductant and oxidized chlorophyll as the sensitizer. On a reciprocal plot (1/rate vs. 1/concentration) the data fall along a straight line. Thus they follow the relation

$$R = R_o \frac{A}{A + k} , \qquad (8)$$

where A is the reductant concentration and $R_o$ and k are constants. Equation 8 is consistent with various theories for the mechanism of the reaction, as discussed by Livingston (1960). The values of $R_o$ (the saturation rate) and k (the concentration at half saturation) are functions of the rate constants of the steps in the reaction and of the concentrations of the other reactants. For pheophytin in solution (90% methanol), $R_o$ is $6.2 \times 10^{-9}$ moles/sec and k is $6.4 \times 10^{-3}$ M under the conditions described in figure 20. For the 0.015 PS particles (50% methanol) under the same conditions, $R_o$ and k are $1.7 \times 10^{-9}$ moles/sec and $2.0 \times 10^{-3}$ M, respectively. (The point at the lowest BNH concentration for the 0.015 PS particles does not lie on the straight line and was excluded in the above calculation.) Thus the reaction with pheophytin on the polystyrene particles saturates at lower BNH concentration, but has a lower maximum rate. This lower rate may be due, in part, to the effect of aggregation of the coated particles as discussed in chapter II. Another factor may be the ten times larger dark rate in the 50% methanol solutions, especially at the high reductant concentrations. I have noticed that the rate of reduction in 50% methanol at high BNH concentration decreases with time. For example, in one case the observed rate decreased about 50% in six minutes with 0.01 M BNH, and the reaction mixtures were frequently deoxygenated for periods longer than this before the first illumination. The decreased rate is probably due to

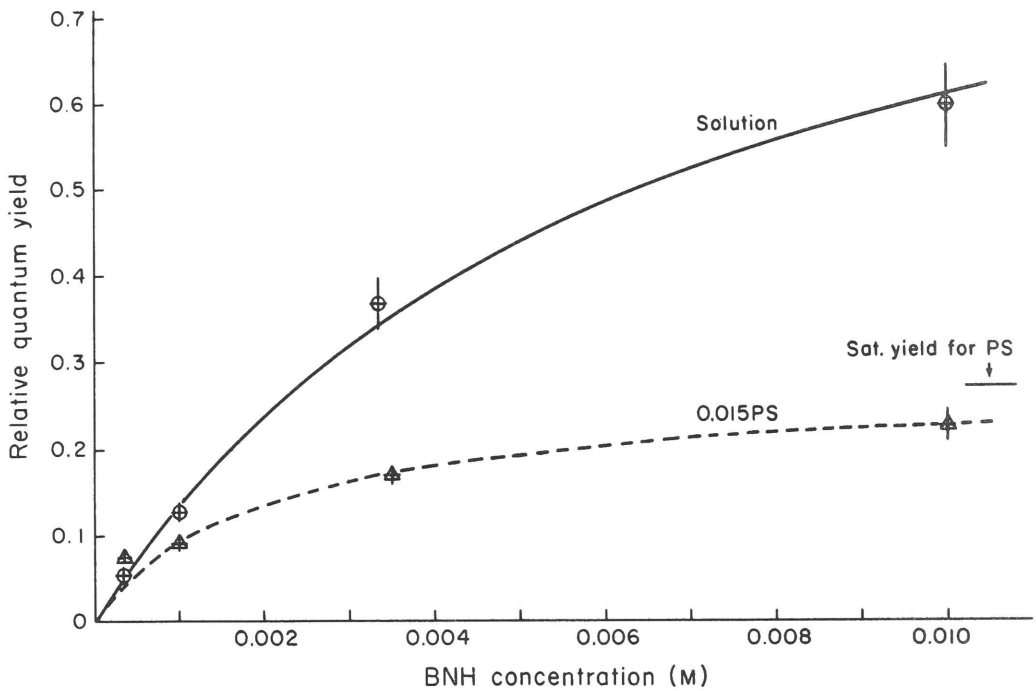



Figure 20. The dependence of the quantum yield of ANR reduction on reductant (BNH) concentration, with pheophytin a in solution and adsorbed onto polystyrene particles as sensitizer. The lines are theoretical curves based on equation 8. The data have been normalized to the saturation rate ($R_o$) for the solution reaction. This rate is $6.2 \times 10^{-9}$ moles $sec^{-1}$. Reaction conditions: Solution: solvent, 90% methanol, 0.016 M succinate buffer, pH 7; $3 \times 10^{-5}$ M ANR; $5 \times 10^{-9}$ mole pheophytin in 3.0 ml; 40 mg uncoated polystyrene particles. Particle system: solvent, 50% methanol, $2.3 \times 10^{-3}$ M phosphate buffer, pH 7; $3 \times 10^{-5}$ M ANR; 20 mg coated particles ($2.2 \times 10^{-8}$ mole pheophytin in 3.0 ml) plus 22 mg uncoated particles; the experimental data have been multiplied by 0.55 to correct to $5 \times 10^{-9}$ mole pheophytin (the factor 0.55 was determined by comparison of the quantum yield for $5 \times 10^{-9}$ mole and $2 \times 10^{-8}$ mole pheophytin adsorbed to 0.04 PS particles with 40 mg polystyrene in suspension and 0.001 M BNH as reductant). The incident light was passed through a Corning 2-59 filter, only, with the same intensity for the two sets of data.

inhibition by products of the dark reaction. This inhibition, which would be BNH-concentration dependent, could also lower the value of k.

The quantum yield of the reaction with the dihydronicotinamide compounds as reductants was constant with light intensity (i.e., the rate of reduction was linearly dependent on the incident light intensity) in both the particle and solution reactions, as long as oxygen was successfully eliminated from the suspension. This did not appear to be the case with ascorbic acid as reductant (figure 21) as the rate of reaction seemed to follow the equation,

$$R = R_o \, (I/I_o)^{0.8} \quad . \tag{9}$$

The reason for the dependence of the quantum yield of the photoreaction on light intensity is not clear. In contrast to these results, Oster, Bellin and Broyde (1964) found that the quantum yield of a similar reaction sensitized by chlorophyllin (water-soluble derivative of chlorophyll) was independent of light intensity over a one-hundred fold range.

The photosensitized reduction reaction saturates, or achieves a broad maximum, with increasing pheophytin concentration (figure 22). I have plotted the rate of photoreduction, rather than the quantum yield, because the amount of light absorbed is different at the different sensitizer concentrations. However, if the rate of the reaction is only a function of the light absorbed, then one should be able to fit the data with the equation,

$$R = R_{max} \, (1 - 10^{-\epsilon dc})^{0.8} \quad , \tag{10}$$

where $\epsilon$ is the molar absorptivity, d is the path length in the solution and c is the concentration of the sensitizer. Equation 10 is derived by assuming Beer's law holds. The exponent 0.8 appears because ascorbic acid was used as the reducing agent for the experiments of figure 22.

The data for pheophytin in solution agree well with the theoretical curve as shown in figure 22. Only the value of $R_{max}$, $0.57 \times 10^{-9}$ mole/sec, has been assumed in order to fit the observed data. The agreement between theory and experiment means that the quantum yield of the photochemical reaction is independent of pheophytin concentration, at least up to $2 \times 10^{-5}$ M. This is in agreement with the results of Livingston and Pariser (1956a).

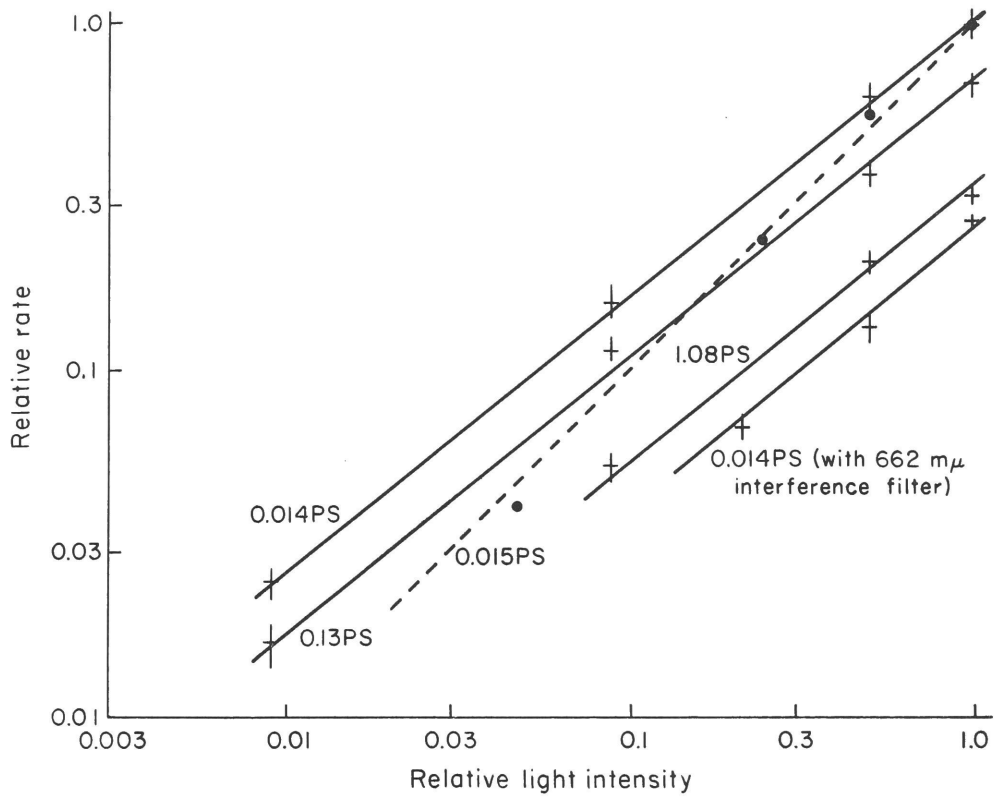



Figure 21. The dependence of pheophytin-sensitized reduction of ANR on the incident light intensity. The data points with the crosses are with ascorbic acid as reductant. The solid lines have a slope of 0.8 to fit equation 9. The data with the circles were obtained with CNH as the reductant and the dashed line has a slope of 1.0. The ascorbic acid data were obtained using the Corning 2-59 filter only except for the one curve with the B & L 662 mμ filter in addition as noted. With CNH the illumination was through the Corning 2-59 and B & L 675 mμ filters. Light intensity was varied with neutral density screens.

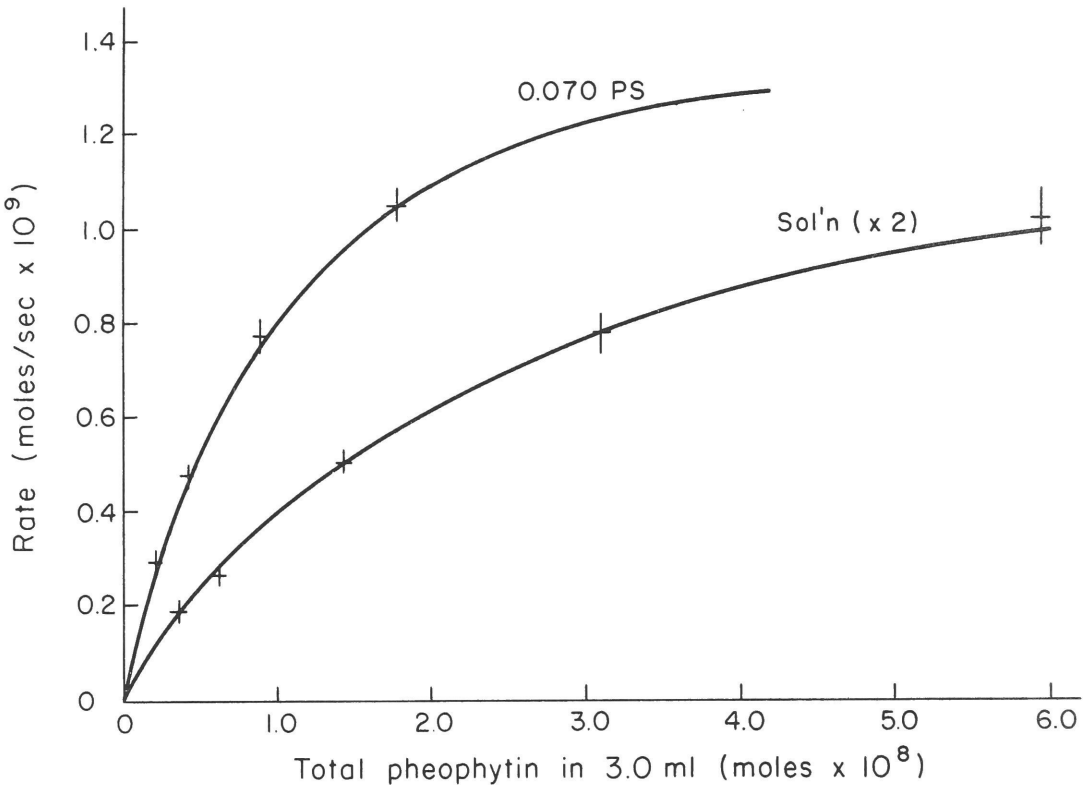



Figure 22. Rate of reduction of ANR sensitized by pheophytin in solution or adsorbed to polystyrene particles, with ascorbic acid as reductant, as a function of the amount of sensitizer in the reaction. The amount of polystyrene was kept constant at 10 mg in the latter experiment. The curves are theoretical based on equation 10, as described in the text. Reaction conditions: Solution: square cuvette, solvent 90% methanol, 0.012 M ascorbic acid neutralized to pH 6.7 with NaOH; $3 \times 10^{-5}$ M ANR; illumination through Corning 2-59 and B & L 662 mμ filters; incident intensity about $3 \times 10^{-9}$ einst. $cm^{-2}$ $sec^{-1}$. Particle system: solvent, 50% methanol, $2.3 \times 10^{-3}$ M phosphate buffer, pH 7; 0.167 M ascorbic acid; $3 \times 10^{-5}$ M ANR; illumination through Corning 2-59 filter only (particle reaction done in cylindrical geometry).

The experimental data for the pheophytin on the polystyrene particles can also be fit with a curve of the above form. However, the absorbance values ($A = \epsilon dc$) required are larger than those estimated for the amount of pheophytin in the suspension. This is not due to concentration quenching, since the pigment is bound to the particles. However, the effective path length of light in the suspension (d) is increased due to the light scatter by the particles (Butler, 1962). In order to fit the data, d must be 5.65 cm, while the internal diameter of the cylindrical reaction cell was only 1.1 cm. (I have used $1.75 \times 10^4$ liter $mole^{-1}$ $cm^{-1}$ for the molar absorptivity of the pheophytin in the above calculation. This is the average value of the absorptivity over the range in which light was absorbed, 640 to 700 mμ.) Butler (1962) gives a formula for the increase in path length due to scatter in a suspension. Using the value of the scattering coefficient for 10 mg of polystyrene from the analysis in chapter II, I have calculated that the light path length would be increased by a factor of 4.0 at about $1 \times 10^{-8}$ moles of pheophytin. This means that the path length "in the absence of scatter" would be 5.65/4.0 or 1.4 cm. This value is in good agreement with the measured distance of 1.1 cm, considering the cylindrical geometry.

Evstigneev and Gavrilova (1960) have reported that the sensitization reaction, with the sensitizer adsorbed to different surfaces, would occur in the presence of oxygen. In contrast to this, I have found that oxygen must be eliminated from the particle suspension before a reaction will occur. The most sensitive test for the presence of oxygen in the suspension was the presence of a lag period between the start of the illumination and the onset of dye reduction. Since oxygen is rapidly eliminated by a sensitized autoxidation of ascorbic acid, the methods used by Evstigneev and Gavrilova were probably not sensitive enough to detect the short lag period which occurred. They acknowledge that a possible explanation for their observations is the decreased solubility of oxygen in water compared to pyridine, thus resulting in a smaller amount of oxygen initially present. They attempted to test this hypothesis by comparing the hematoporphyrin-sensitized reduction of methyl red in water in the presence and absence of oxygen; hematoporphyrin is water soluble. They found that removal of oxygen resulted in "some acceleration in the reaction." They did not consider that the difficulties in measuring the reaction in the scattering suspensions, as compared to measurements in solution, would make the determination of small changes in rate difficult.

Some of the initial studies of the reaction with the sensitizer adsorbed to particles indicated that undissociated ascorbic acid (or ascorbate anion plus $H^+$) was the active form of the reducing agent. These studies were done in aqueous suspensions and, at first, the rates obtained appeared to be considerably lower than the rates observed in solution. However, the ascorbic acid concentration in the aqueous solutions had been kept at the value used in the 90% methanol for the experiments in solution. The pK of ascorbic acid is 2.5 pH units larger in 90% methanol than in water. This means that at equal concentrations at pH 7, there is 210 times more undissociated ascorbic acid in 90% methanol than in water. While increasing the total ascorbic acid concentration above 0.01 M did not measurably increase the rate of the sensitization reaction in 90% methanol, at pH 7 in aqueous solution, the rate was still increasing at 1 M ascorbic acid (total), the highest concentration tried (undissociated ascorbic acid concentration about $1.5 \times 10^{-3}$ M).

As additional evidence that the undissociated acid (or ascorbate anion plus $H^+$) is the active reductant, I did experiments at two different pH's using chlorophyll-coated polystyrene. In part of the experiment, tetramethylethylenediamine (TMED) was used as the buffer, and since it is also a reducing agent (table VI), its contribution to the reaction was considered. At pH 7.0, with 0.0125 M ascorbic acid as reductant (phosphate buffer), the rate was 0.0206 (arbitrary units). With 0.0125 M TMED plus 0.0125 M ascorbic acid at pH 7.0, the rate was 0.0473; thus the contribution of TMED to the rate was 0.0267. At pH 6.35, the rate was 0.108 with 0.0112 M TMED and 0.0125 M ascorbic acid. The pH difference of 0.65 means a factor of 4.45 in undissociated ascorbic acid concentration (or in hydrogen ion concentration). This would increase the rate of the ascorbic acid reaction from 0.0206 at pH 7 to 0.0916 at pH 6.35. To this must be added the amount of reaction due to the TMED, corrected for the difference in concentrations used and the pH difference, assuming that the doubly protonated form is unreactive (Mauzerall, 1960) and we are in a linear range. The pK of the second protonation is 5.9. The corrected rate for the TMED is 0.0196 and when added to 0.0916 gives a rate of 0.111, very close to the observed 0.108, giving additional support to the hypothesis.

The BNH did not show these solvent or pH effects. This is to be expected since its pK is less than 6 (reaction with the solvent occurs rapidly in acid conditions) and the pK's of amines do not shift a great

deal in different methanol/water mixtures (de Ligny, 1960). In figure 20 it was seen that there was only a small difference (a factor of three) in the concentration at half saturation between 50% methanol and 90% methanol. This may be compared with ascorbic acid, which has a half saturating concentration (k in equation 8) of $0.9 \times 10^{-3}$ M at pH 7 in 90% methanol, but in water, has a rate which is increasing linearly with concentration even at 0.95 M (undissociated ascorbic acid concentration about $1.2 \times 10^{-3}$ M). I have also studied the effect of pH on the reaction with BNH as reductant in 50% methanol. Over a pH range of 3 full pH units (1000 fold difference in hydrogen ion concentration), the quantum yield changed by a factor of less than three. (The differences observed may have been due to secondary effects of the amine buffers used to obtain the desired pH's.)

One major problem encountered with many types of particles used in the initial experiments was degradation of the chlorophyll or pheophytin upon adsorption to the particle surface. For example, figure 23 shows a series of absorption spectra of pheophytin after adsorption and re-extraction from aluminum silicate particles (ASP) of different surface coverages. At low coverages, there was a marked change in the Soret (blue) absorption peak, while at higher coverages the spectra look quite similar to the standard absorption spectrum of pheophytin. Inspection of spectra of the extracts from other coated particles indicated that there was also some pheophytinization of chlorophyll on the particle surface.

A significant feature of the spectra of the degraded pheophytin is the absence of the shoulder on the short-wavelength side of the Soret maximum. The absence of this "satellite band" also has been noted in the spectra of allomerized (oxidized) chlorophyll (Rabinowitch, 1956, p. 1773, 1804). Holt (1958) has isolated three different components from allomerized chlorophyll, and those in which the isocyclic ring has been opened or oxidized do not have the satellite band. Following Granick (1950), I broke open the isocyclic ring on pheophytin with 30% HCl in dry methanol to yield chlorin $e_6$- trimethyl ester (figure 25). The chlorin is not an oxidation product, but its red absorption maximum occurs around 665 mμ (as does that of the degraded pheophytin) in contrast to 640-645 mμ for allomerized chlorophyll. The spectra of the degraded

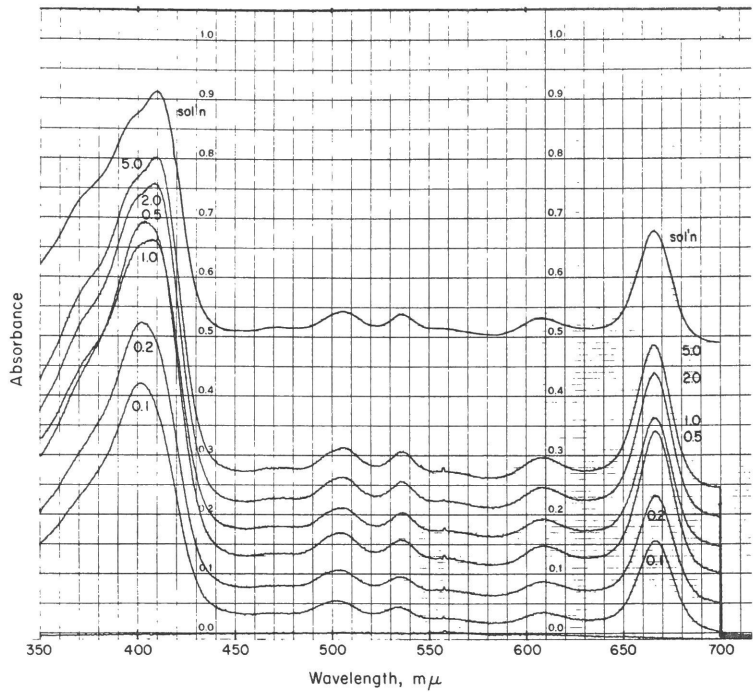



Figure 23. Absorption spectra of pheophytin extracted from coated aluminum silicate particles (ASP 170) of different surface coverages and compared with a standard (untreated) sample of pheophytin in the same solvent (80% acetone).

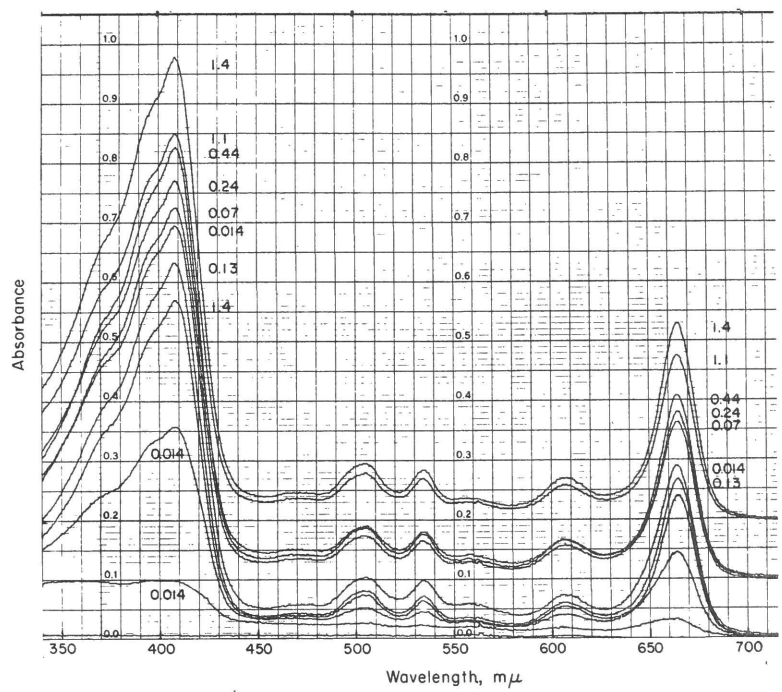



Figure 24. Absorption spectra of pheophytin extracted from coated polystyrene particles of different surface coverages, after the photosensitization reaction. The three curves for the 0.014 particles are for different amounts of material. The solvent is acetone.

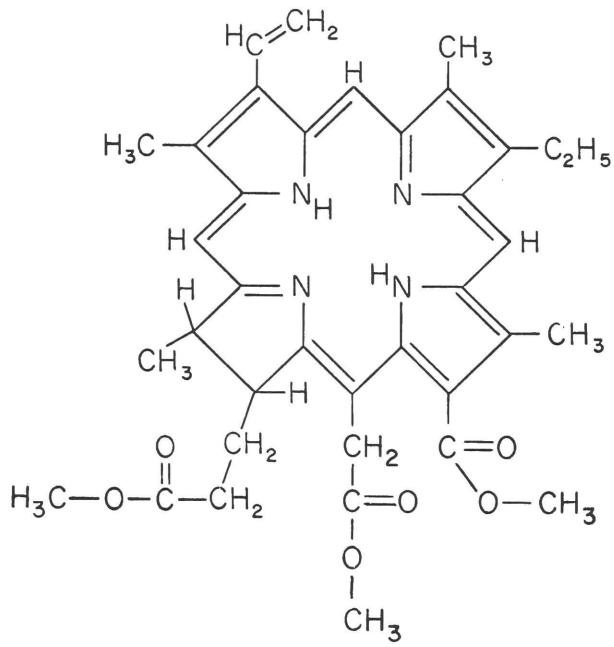


Figure 25. Chlorin $e_6$-trimethyl ester.

pheophytin are very similar to that of the crude preparation of the chlorin $e_6$ (not shown - I did not attempt to purify the chlorin after the acid treatment). The chlorin spectrum had a Soret maximum at 400 mμ (not 410) and there was no satellite band on the short-wavelength side of this maximum. The agreement is not exact (see table IX, below) but does give an indication of the nature of the degradation process. The structure of the degraded pheophytin was not investigated further.

This degradation severely limited the choice of particles for the final experiments, since it was necessary to have no degradation of the sensitizer during the adsorption process or during the photochemical reaction. I found only three types of particles that did not degrade the pheophytin (as evidenced by no difference in the absorption spectra of solutions before and after adsorption and reextraction from the particles), two samples of polystyrene and the synthetic zeolites (see table VIII). The particle size of one of the polystyrene samples was too large to be useful. I have used pheophytin as the sensitizing pigment because there was some evidence of degradation of chlorophyll on the polystyrene.

The absorption spectra of extracts from the polystyrene and zeolite particles were checked immediately after adsorption and after the completion of a series of reactions (figure 24). In all cases the spectra indicated that the pheophytin had not been degraded (i.e., the Soret peak coincided with the maximum for pheophytin for that solvent and the satellite band on the Soret peak was present). A useful quantitative test for the degradation of pheophytin on the particles is the ratio of the absorbances of the blue and red absorption maxima in addition to the location of the blue maximum of the extracted pigment. A tabulation of these values for the spectra shown in figures 23 and 24 are given in table IX, along with values for the zeolite particles. On the basis of these ratios and any shift in the blue absorption maximum, degradation of 5-10% of the pheophytin should be detectable. In some cases however, the observed blue absorbance values may be higher than the actual values due to a small amount of scatter by small particles not removed by centrifuging the extracts. The values for the polystyrene particles in table IX have been corrected for scatter, estimated at about 0.05 absorbance units at 408 mμ from the spectrum of the 0.014 PS particles at the bottom of figure 24, which contained very little pheophytin. The scatter of the ASP extracts

TABLE VIII

SURFACE PROPERTIES OF VARIOUS PARTICLES

| Particle | Dye Adsorption | | Pheophytin Degradation |
|---|---|---|---|
| | Methylene Blue (Basic) | DDNA* (Acid) | |
| Alumina | - | ++ | + |
| Aluminum Silicate | | | |
| # 101 | ++ (purple) | - | + |
| # 106 | + (blue) | - | + |
| # 170 | ++ (blue) | - | + |
| Molecular Sieve 4A (zeolite) | ++ (blue) | - | - |
| Polystyrene Beads | | | |
| Koppers | - | - | - |
| Dow | ++ | - | not tested |
| Polystyrene Latex | | | |
| Bordens | + | - | - |
| Dow | ++ | - | not tested |

*DDNA is 8-(1,8-dihydroxy-3,6-disulpho-2-naphthylazo)-1-naphthol-3,6-disulphonic acid, tetrasodium salt.

TABLE IX

ABSORBANCE RATIOS OF PHEOPHYTIN EXTRACTED FROM COATED PARTICLES

A. Extracts from ASP particles in 80% acetone (figure 23).

| Coverage | $A_{410}/A_{666}$* | Soret max.(S) | $A_S/A_{666}$* |
|---|---|---|---|
| (solution) | 2.21 | 410 mμ | 2.21 |
| 5.0 | 2.31 | 410 | 2.31 |
| 2.0 | 2.31 | 409 | 2.32 |
| 1.0 | 2.38 | 408 | 2.39 |
| 0.5 | 2.37 | 403 | 2.46 |
| 0.2 | 2.43 | 402 | 2.60 |
| 0.1 | 2.42 | 402 | 2.72 |
| chlorin $e_6$ | 2.18 | 400 | 2.45 |

*The values for the particle extracts may be as much as 5% high due to scatter; see text.

B. Extracts from polystyrene particles in acetone (figure 24).

| Coverage | $A_{408}/A_{665}$** |
|---|---|
| 1.40 | 2.20 |
| 1.08 | 2.18 |
| 0.44 | 2.21 |
| 0.24 | 2.21 |
| 0.13 | 2.19 |
| 0.07 | 2.20 |
| 0.014 | 2.20 |

**All Soret maxima occurred at 408 mμ. The absorbance values have been corrected for scatter.

C. Extracts from zeolite particles, before and after photosensitization reaction.

| Coverage | $A_{410}/A_{666}$# before reaction | $A_{408}/A_{665}$## after reaction |
|---|---|---|
| 1.0 | 2.20 | 2.26 ± 0.04 |
| 0.53 | 2.24 | 2.35 ± 0.05 |
| 0.22 | 2.22 | 2.23 ± 0.02 |
| 0.070 | 2.22 | 2.25 ± 0.03 |
| 0.022 | 2.23 | 2.25 ± 0.03 |
| 0.0094 | 2.24 | 2.27 ± 0.07 |

# All Soret maxima occurred at 410 mμ (solvent: 80% acetone). Values have not been corrected for scatter, which was small.
## All Soret maxima occurred at 408 mμ (solvent: acetone). Values have been corrected for scatter.

cannot be estimated as easily, but comparison of figures 23 and 24 suggests that the error due to scatter could be 0.025, an error of about 5%. The effect of the scatter is to reduce the sensitivity of the method for detecting degradation of the pheophytin.

It is of interest that in most cases of degradation noted with the particles, there was no significant change in the location of the red absorption maximum upon degradation of the pigment, though it may have been reduced in intensity. The red absorption maximum of chlorophyll and pheophytin should not be used by itself as a measure of the integrity of the pigment; moreover, the standard methods of determining the amount of pigment in plant preparations, which involve the use of the red absorption maximum of chlorophyll (e.g., Vernon, 1960), should be used with great caution.

The various types of particles which I have been able to obtain are listed in table VIII, along with some of their surface properties. By testing whether they adsorbed a basic or acidic dye or neither I obtained information about the surface polarity of the particles: adsorption of a dye with four sulphonic acid groups (DDNA) indicates the presence of positive charges on the surface, while adsorption of the basic dye, methylene blue, indicates negative charges. A small amount of adsorption of the methylene blue to the nominally nonpolar particles, such as some of the polystyrene, may be due to nonelectrostatic forces. The last column indicates whether pheophytin was degraded upon adsorption to the particles, as discussed above. Most of those listed also degraded chlorophyll. The final studies have been done with the Borden's polystyrene particles, coated with pheophytin. I have also used the zeolite particles, in order to compare the effect of polar and nonpolar surfaces.

II. Pheophytin-coated polystyrene particles.

One of the major objectives of this research is the comparison of the photochemical activity of pheophytin absorbed to a surface at different concentrations. In figure 26 are summarized the results of four series of experiments which show that as the fraction of the polystyrene particle surface covered with pheophytin is increased, the quantum yield of the photosensitized reaction decreased. These experiments have been done with different particle preparations, different reducing agents and electron acceptors (dyes) and with different amounts of scatter and incident light.

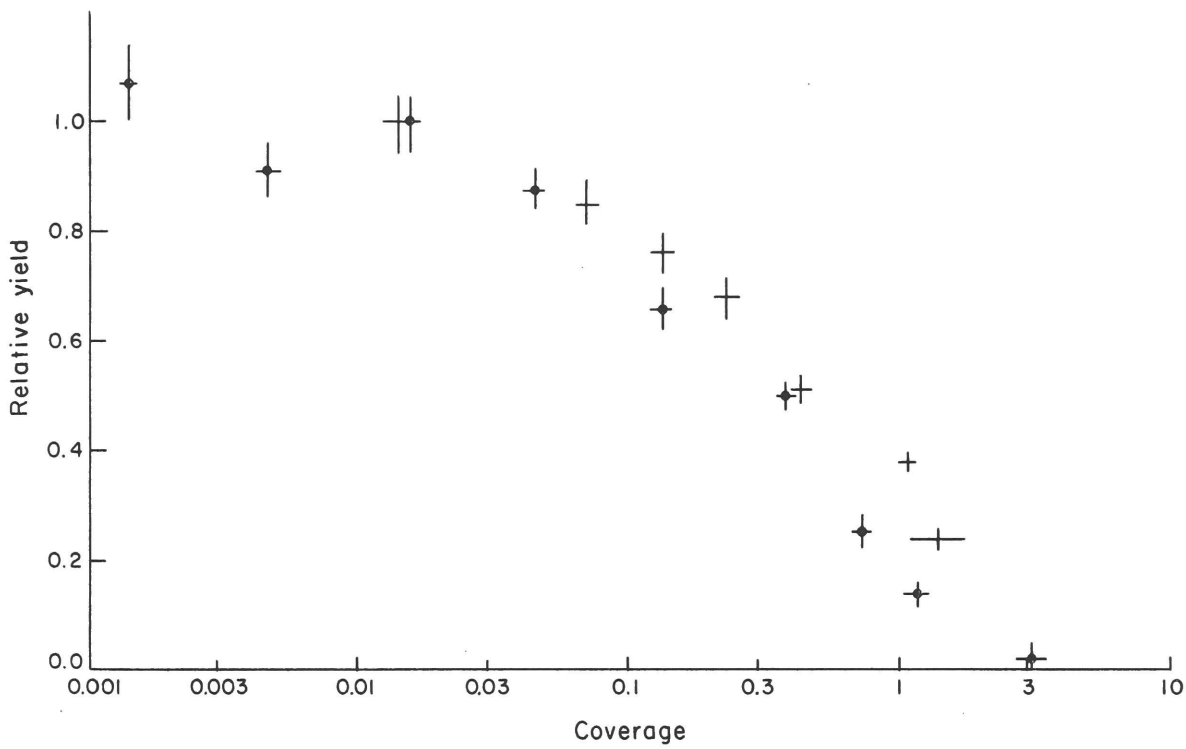



Figure 26. The relative quantum yield of ANR or fuchsia reduction, sensitized by pheophytin a adsorbed to polystyrene particles at various surface coverages. The data points with dots ( ● ) are for particles coated from propanol (method II); those without dots are for particles coated from methanol (method I). The length of the lines is a measure of the possible error in the data, estimated to be about 10%. The reaction conditions are described in the text. See also table X.

While exact agreement was not achieved in all reactions, the data show that there was a consistent decrease in quantum yield with increasing surface coverage (the fraction of the particle surface covered by pheophytin; see chapter II). The different reaction conditions are listed below and the relative quantum yields for each series are listed in table X. The data have been normalized to the yield for the 0.014-0.015 PS.

A) 0.2 M ascorbic acid, $3 \times 10^{-5}$ M ANR, $1.5 \times 10^{-8}$ moles pheophytin in 3.0 ml., with no additional uncoated particles. The data were corrected to a constant scatter of 5 mg polystyrene per tube by using empirical curves similar to those in figure 14 (chapter II). The incident light was filtered through both the Corning 2-59 and B & L 662 mμ filters, and the incident intensity was approximately $1.6 \times 10^{-8}$ einstein $cm^{-2}$ $sec^{-1}$. Rate for the 0.014 particles was $0.18 \times 10^{-9}$ moles/sec.

B,C) 0.2 M ascorbic acid, $3 \times 10^{-5}$ M ANR, $1.6 \times 10^{-8}$ moles pheophytin in 3.0 ml. with approximately 10 mg total particles in the reaction vessel. The data have been corrected using the empirical curves for small differences in the amount of particles in the suspension. The incident light was filtered through the Corning 2-59 filter alone (B) or in combination with the B & L 662 mμ filter (C). The light intensity through the interference filter was approximately $1.3 \times 10^{-8}$ einstein $cm^{-2}$ $sec^{-1}$. The rate for the 0.014 particles was $0.269 \times 10^{-9}$ moles/sec with the interference filter, $1.0 \times 10^{-9}$ moles/sec without it. The particles were coated from methanol/water (method I).

D,E) $1.0 \times 10^{-3}$ M BNH, $3 \times 10^{-5}$ M ANR, $0.8 \times 10^{-8}$ moles pheophytin in 3.0 ml. with 30 mg (D) and 40 mg (E) of polystyrene particles in the suspension (except for the 0.0014 PS, with 100 mg of polystyrene). The indicent light was filtered only through the Corning 2-59 filter, (light intensity same as in C). The rates for the 0.0046 particles were 0.317 and $0.312 \times 10^{-9}$ moles/sec at 30 and 40 mg, respectively. The particles were coated from propanol/water (method II).

F) $1.0 \times 10^{-3}$ M BNH, $3 \times 10^{-5}$ M fuchsia, $1 \times 10^{-8}$ moles pheophytin in 3.0 ml with 40 mg of polystyrene particles in the suspension. The light was filtered through both a Corning 2-61 cutoff filter and a B & L 675 mμ interference filter, with the incident intensity about $8 \times 10^{-9}$ einstein $cm^{-2}$ $sec^{-1}$. The rates were corrected for the photoreaction due to the monitoring lamp beam (which was about $0.03 \times 10^{-9}$ moles/sec). The corrected rate for the 0.015 particles was $0.236 \times 10^{-9}$ moles/sec. The particles were coated from propanol/water (method II).

TABLE X

RELATIVE QUANTUM YIELD OF PHEOPHYTIN-SENSITIZED REACTIONS WITH PHEOPHYTIN ADSORBED TO POLYSTYRENE PARTICLES

| Surface coverage[a] | | Relative quantum yield[b] A | B | C | D | E | F | avg. |
|---|---|---|---|---|---|---|---|---|
| II | 0.0014 | | | | 1.07[c] | | | 1.07 |
| II | 0.0046 | | | | 1.0 | 0.99 | 0.75 | 0.91 |
| I | 0.014 | 1.0 | 1.0 | 1.0 | | | | 1.00 |
| II | 0.0155 | | | | -- | --[d] | 1.0 | 1.00 |
| II | 0.045 | | | | 0.75 | 1.02 | 0.89 | 0.89 |
| I | 0.070 | 0.83 | 0.89 | 0.84 | | | | 0.85 |
| I | 0.133 | 0.72 | 0.83 | 0.74 | | | | 0.76 |
| II | 0.133 | | | | 0.59 | 0.85 | 0.53 | 0.66 |
| I | 0.240 | 0.61 | 0.66 | 0.77 | | | | 0.68 |
| II | 0.386 | | | | 0.44 | 0.66 | 0.39 | 0.50 |
| I | 0.435 | 0.54 | 0.53 | 0.50 | | | | 0.52 |
| II | 0.72 | | | | | | | 0.25[e] |
| I | 1.08 | 0.39 | 0.39 | 0.35 | | | | 0.38 |
| II | 1.16 | | | | 0.16 | 0.11 | 0.14 | 0.14 |
| I | 1.40 | -- | 0.25 | 0.23 | | | | 0.24 |
| II | 3.08 | | | | 0.02 | -- | -- | 0.02 |

Notes: a) The Roman numeral refers to the method of coating the particles described in chapter II. The surface coverage was also defined in chapter II. b) The letter at the top of each column refers to the reaction conditions as listed in the text. c) 100 mg of coated particles used for this reaction, only. d) This set of particles was skipped in these series. The standard for normalization in sets D and E was taken as the 0.0046 PS particles. Because the yields of the 0.0046 and 0.045 particles were the same at saturating amounts of total particles (set E), the 0.015 PS was assumed to have a similar yield. e) This represents a third particle preparation, in a reaction with conditions similar to E.

All reactions were done in 50% methanol with 0.001 - 0.002 M phosphate buffer at pH 7.4 - 7.8.

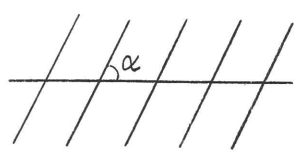
Since the quantum yield tends toward a constant (maximum) value at low surface coverages where the molecules are relatively far apart on the surface, it is of interest to compare the maximum quantum yield observed on the particles with the yield of the photoreaction in solution. The dependence of the quantum yield of the photoreaction as a function of reductant concentration was shown in figure 20. The reactions with pheophytin in solution and adsorbed to the 0.015 polystyrene particles were done under the same conditions, i.e., the same incident light intensity and the same total amount of particles in the suspension; furthermore, the amount of polystyrene particles was 40 mg. in the region of total light absorption (see chapter II).

Under these conditions, the quantum yield at saturating BNH concentrations is about 3.6 times larger for pheophytin in 90% methanol than for pheophytin adsorbed to the polystyrene particles in 50% methanol. I have used BNH for these comparison studies because there is little apparent difference in its behavior in the two different solvents. Comparison at saturating concentration would tend to minimize any solvent effects which were present, except for the problem of the different rates of the dark reaction in 50% and 90% methanol as discussed previously. In the absence of the inhibitory effects of the dark reaction at high BNH concentration in 50% methanol (p.40-41) and of the aggregation of the coated particles (p.29-30), as was also noted in the earlier discussion of these results, the quantum yield of the photoreaction of the low coverage pheophytin-coated particles would be even closer to that of pheophytin in solution. In fact the two quantum yields may be equal, since I have suggested that each of the two inhibitory effects could have reduced the yield of the coated polystyrene particles by a factor of one-half. Thus adsorption to the surface of the polystyrene particles does not, in itself, appear to affect significantly the photochemical activity of the pheophytin. In fact if one compares the quantum yields at low BNH concentration, the lower half-saturation value (k, equation 8) for the particles makes the observed yields equal, within 10%.

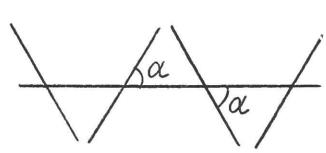
At high surface coverages, the pheophytin molecules are relatively close together. This suggests that the decrease in quantum yield is due to interaction between the pigment molecules. An indication of the state of the pheophytin on the polystyrene surface is given by the absorption

spectra of the coated particles. The absorption spectra of the pheophytin-coated polystyrene particles are shown in figures 27-29. At low surface coverages (see esp. figure 29), the absorption spectrum of the coated particles is very similar to that of phoeophtin in toluene. (I have chosen toluene for the comparison, since it is structurally similar to the polystyrene subunits.) As the amount of pheophytin on the particle surface is increased, the width of the absorption band increases. This broadening, at high coverage, is asymmetric, occurring only toward longer wavelengths (table XI). A measure of the broadness of the band is the "half-width" or the width of the band where the absorbance is one-half the maximum. The half-widths of the absorption bands of the coated polystyrene particles are shown in figure 30. They are also listed in table XI. As the surface coverage of the particles increases, the half-width of the absorption band increases, and the increase in half-width is correlated with the decrease in the quantum yield of the photochemical reaction.

The broadening of the particle spectra at high coverages is undoubtedly due to the formation of aggregates of the pheophytin molecules on the particle surface. For example, Jacobs (see Rabinowitch, 1956, p. 1820; also Jacobs, Holt, Kromhout, and Rabinowitch, 1957) found that "small microcrystals" of ethyl pheophorbide a (pheophytin without the phytol) had an absorption maximum at 690 mμ. This shifted to 715 mμ as the crystals grew into "large microcrystals." McRae and Kasha (1958, 1964) have calculated the changes which would be expected to occur in the absorption spectra of molecules upon aggregation in linear chians. Since the pheophytin molecules are thought to form "sandwich-type" dimers in some solvents (Closs et al., 1963), it is not unreasonable to assume that the pheophytin forms linear aggregates, with the main axis of the aggregate passing through the planar chlorin ring. According to McRae and Kasha, the spectrum of such aggregates will shift only to longer wavelengths if the molecules are "parallel" and inclined at an angle of $54.7^{\circ}$ or less with the line connecting the molecular centers (I). If the angle, $\alpha$, is greater than $54.7^{\circ}$ the spectrum will




I




II

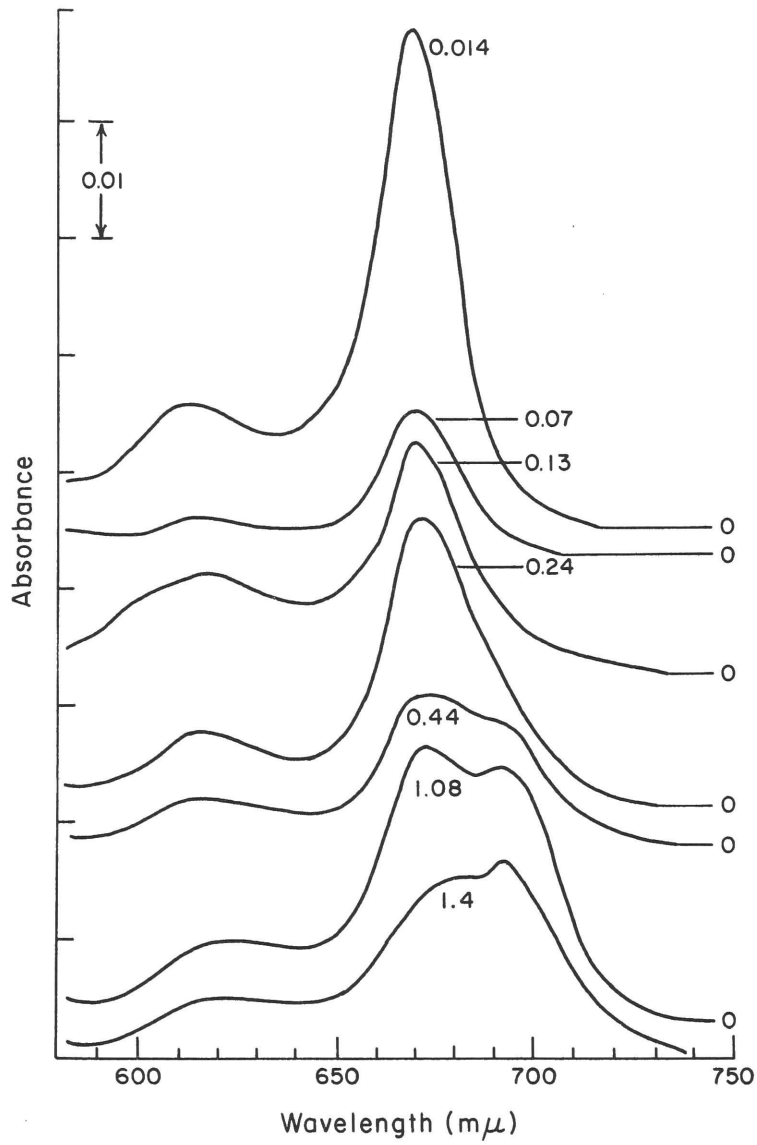



Figure 27. Absorption spectra of pheophytin-coated polystyrene particles of various surface coverages, as noted. The particles were coated from methanol/water (method I).

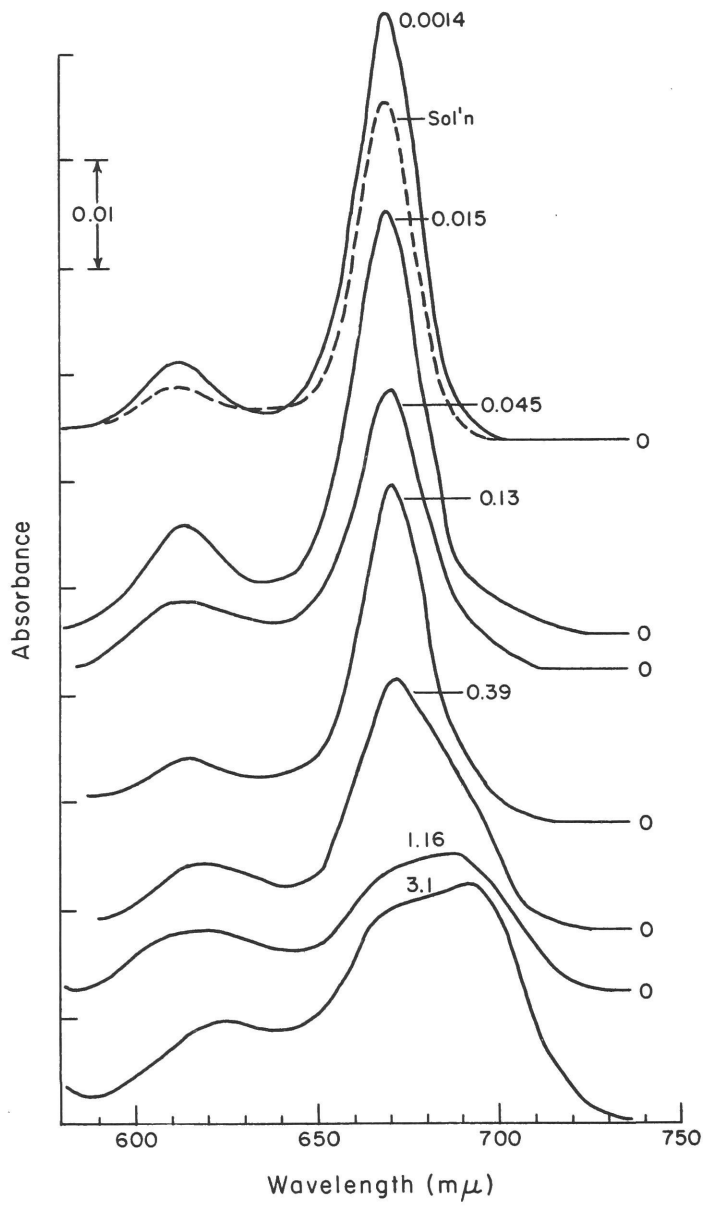



Figure 28. Absorption spectra of pheophytin-coated polystyrene particles of various surface coverages, as noted, and compared with the spectrum of pheophytin in toluene. The particles were coated from propanol/water (method II).

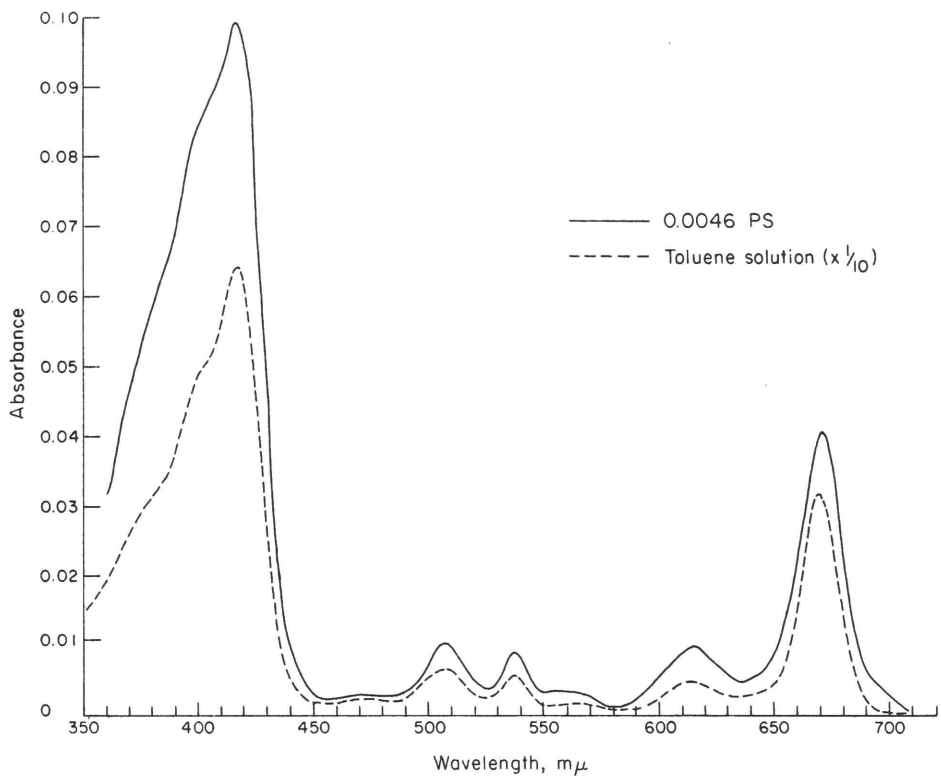



Figure 29. The absorption spectrum of the 0.0046 PS particles compared with the spectrum of $6.5 \times 10^{-6}$ M pheophytin in toluene solution.

TABLE XI

HALF-WIDTHS OF THE RED ABSORPTION AND FLUORESCENCE BANDS OF PHEOPHYTIN-COATED POLYSTYRENE PARTICLES

| Surface coverage[a] | | Absorption half-width | $\lambda_{\frac{1}{2}}$[b] | Fluorescence half-width |
|---|---|---|---|---|
| | (solution) | 19 ± 1 mμ | 661 mμ | 23 ± 1 mμ |
| II | 0.0014 | 20 | 660 | 26 |
| II | 0.0046 | 22 | 659 | 25 |
| I | 0.014 | 23 | 658 | - |
| II | 0.0155 | 22 | 659 | 26 |
| II | 0.045 | 24 | 659 | 31 |
| I | 0.070 | 23 | 659 | - |
| I | 0.133 | 27 | 658 | - |
| II | 0.133 | 24 | 660 | 30 |
| I | 0.240 | 30 | 660 | - |
| II | 0.386 | 35 | 659 | 32 |
| I | 0.435 | 42 | 660 | - |
| I | 1.08 | 48 | 658 | - |
| II | 1.16 | 49 | 656 | 30 |
| I | 1.40 | 47 | 661 | - |
| II | 3.08 | 55 | 656 | 50 |

Notes: a) The Roman numeral preceding the particle coverage denotes the method of coating the particles, either from methanol (I) or propanol (II). b) Wavelength on the short-wavelength side of the absorption maximum for which the absorbance is 1/2 the maximum absorbance.

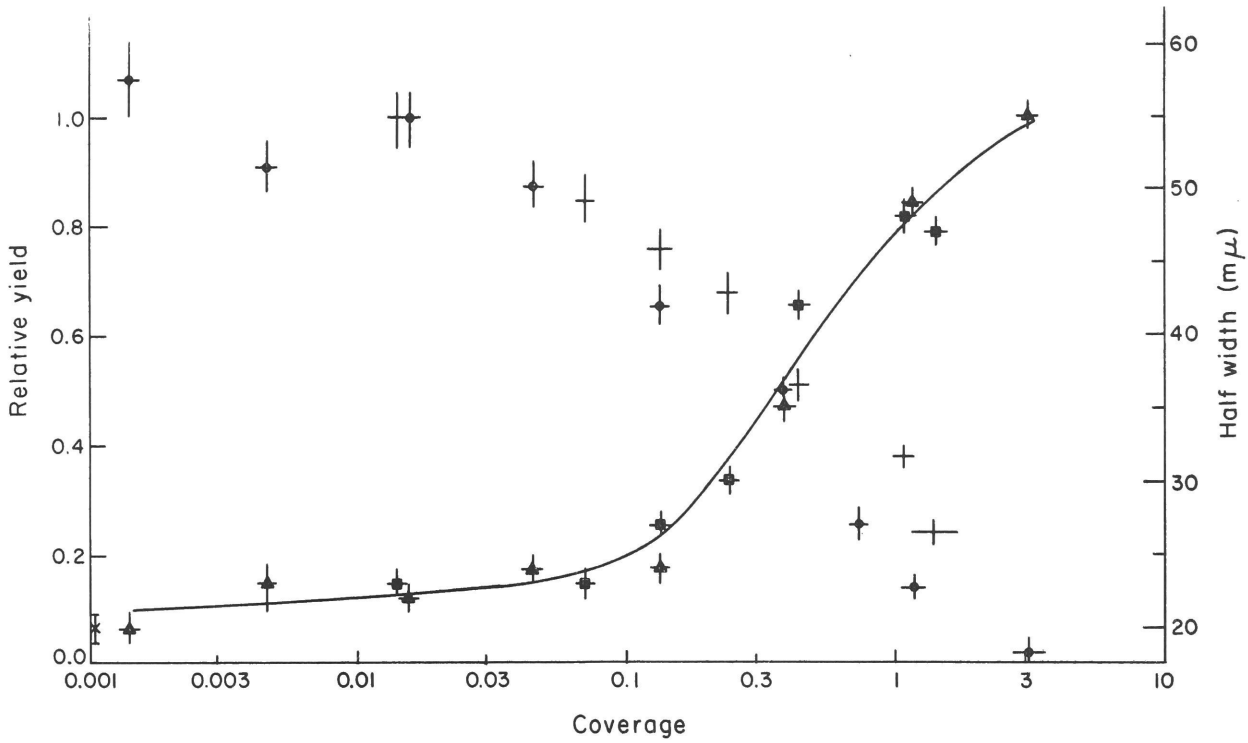



Figure 30. The half-width of the red absorption band of the pheophytin-coated polystyrene particles as a function of the surface coverage of the polystyrene and compared with the relative quantum yield of the photochemical reaction. The cross ( x ) in the extreme lower left-hand corner shows the half-width of pheophytin in toluene solution. The data with the squares ( ■ ) and triangles ( ▲ ) are for the particles prepared by adsorption from methanol (method I) and propanol (method II), respectively. The data for the photochemical quantum yield are as described for figure 26.

shift toward shorter wavelengths, and if the molecules alternate, as in II, the band will split into two components, one at shorter wavelengths and the other at longer wavelengths than the original band. Thus, the broadening of the absorption spectra at high surface coverages indicates that aggregates of pheophytin do form on the polystyrene particle surfaces, and that the molecules are lying at an angle of less than $54.7^{\circ}$ with particle surface, if we assume that the aggregates are linear chains lying on the surface.

The magnitude of the shift of the absorption band depends on the number of molecules in the aggregate (N), the intermolecular distance (r), the angle connecting the molecular centers ($\alpha$); and the transition moment for the monomer transition (m):

$$\Delta E = 2\frac{N-1}{N}\frac{m^2}{r^3}\left|(1 - 3\cos^2\alpha)\right| . \qquad (11)$$

This equation was obtained with the assumption of simple dipole-dipole interaction only. Vibronic coupling was not considered. It holds strictly only for dimers and infinitely long aggregates. Assuming the distance between molecules and the angle $\alpha$ remain constant, the shift approaches a maximum value as the size of the aggregates increases. The absorption spectra are consistent with this; at high coverages, where one would expect to find aggregates containing many molecules, a new maximum appears at about 693 mμ.

While it is not possible to make any conclusions concerning the exact orientation of the pheophytin molecules on the polystyrene surface, from the absorption spectra and equation 11, we can put some limits on $\alpha$ and r, at least for the larger aggregates. We assume that the large aggregates have an absorption maximum at 693 mμ, a shift of 23 mμ from the monomer maximum. For large N, $(N - 1)/N \cong 1$ and $\Delta E = hc\left(\frac{1}{\lambda_2} - \frac{1}{\lambda_1}\right) = hc\,\frac{\lambda_1 - \lambda_2}{\lambda_1 \lambda_2}$ .

The transition dipole moment, m, is related to the oscillator strength of the transition by the relationship (Kauzmann, 1957)

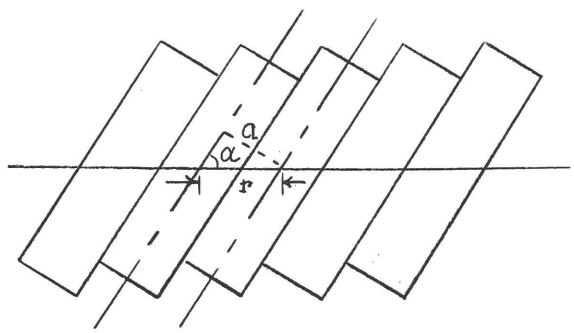
$$f = \frac{8\pi^2 M c m^2}{3 e^2 h \lambda_o} , \qquad (12)$$

where f is the oscillator strength, M and e are the mass and charge, respectively, of the electron, h is Planck's constant, c is the velocity of light and $\lambda_o$ is the wavelength of the electronic transition in the isolated molecule. Jacobs et al, (1957) determined the oscillator strength of pheo-

phytin to be 0.31 and used a value of 652 mμ for $\lambda_0$. From equation 12, m is calculated to be 6.2 debye. From equation 11, a set of values of r and $\alpha$ can be calculated which are consistent with the observed spectral shift. These are shown in figure 31, calculated from the relation

$$r = \frac{\lambda_1 \lambda_2 \, 2 \, m^2}{(\lambda_1 - \lambda_2) hc} \left[ (3 \cos^2 \alpha - 1) \right]^{\frac{1}{3}} . \qquad (13)$$

Since the pheophytin has a finite thickness, some values of r are not attainable. At an angle $\alpha$, the minimum r must be a/sin$\alpha$, where a




is the thickness of the porphyrin ring. The van der Waal's thickness of an aromatic ring is about 3.7 A (Ketelaar, 1958, p. 201). These minimum values for r have also been plotted in figure 31. It is thus seen that r must be between 4.7 and 10.8 A, with $\alpha$ between 52.0° and 20° respectively. From the diagram above, it is seen that the surface area covered by the porphyrin ring of one molecule is r times the width of the ring. This neglects the overhang of the last molecule (a relatively small effect for large aggregates), and the area covered by the phytol has not been included. In chapter II, the area of the chlorophyllide ring was given as 242 $A^2$ or about 15.5 A on each side. From this value, we can calculate that at r = 4.7 A, A = 73 $A^2$ and at r = 10.8 A, A = 168 $A^2$.

At high surface coverages, where the packing of the molecules would be expected to be "tightest," the absorption data suggest that the molecules are about 4.7 A apart at an angle of 52° from the horizontal. The area of the phytol (on the order of 50 $A^2$) must be added to the area covered by the planes of the pheophytin to give a surface coverage of about 125 $A^2$ per molecule, very close to our assumed value of 100 $A^2$. If the main interaction among the pheophytin molecules in the aggregate is "nearest neighbor," i.e., the interaction energy between molecules separated by one or more additional

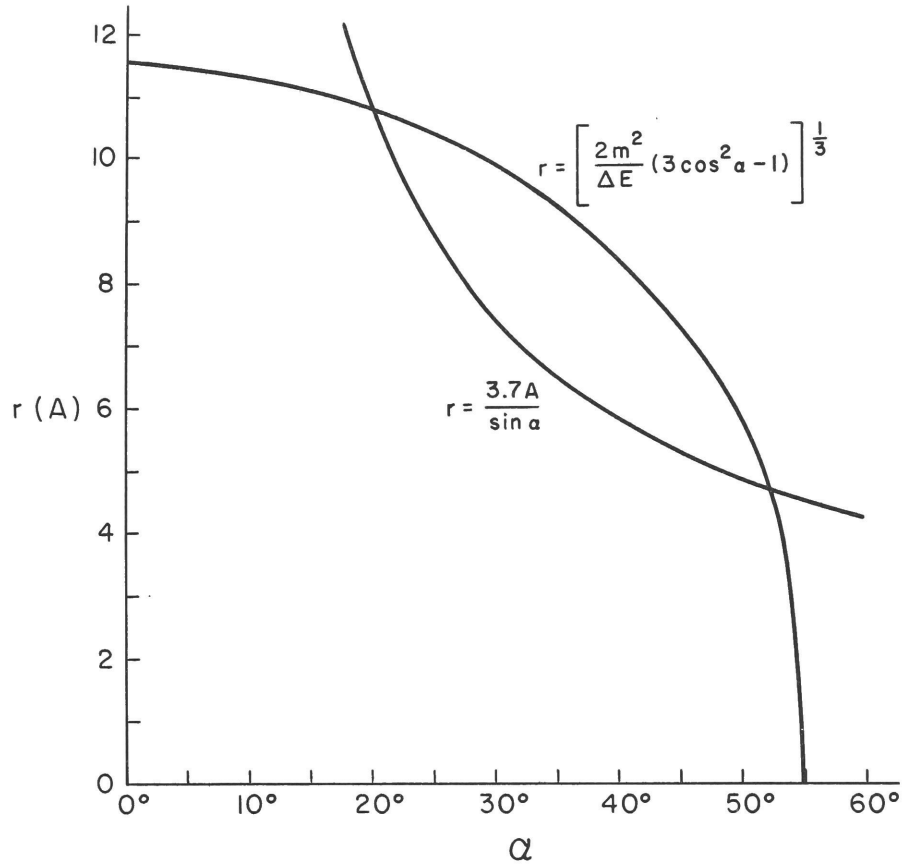



Figure 31. The values of r and $\alpha$ consistent with the observed shift in the absorption spectra of the pheophytin-coated polystyrene particles. See text.

molecules is comparatively small, then molecules in the small aggregates would be oriented approximately as above. If the "nonnearest neighbor" interaction is large, the molecules in smaller aggregates might be bound less tightly, and lie at a shallower angle. Because of the similarity between the low-coverage particle spectra and the spectrum of pheophytin in toluene, it would appear that the pheophytin is adsorbed primarily as monomers at the low coverages (less than 0.01) and is relatively tightly bound to the surface. Thus the plane of the porphyrin ring may even be lying flat on the surface, and the area covered per molecule will be larger than the 100 $A^2$ which I assumed earlier. For the model to be discussed below, the significant area is that covered by a molecule in an aggregate.

As the surface coverage of the polystyrene particles is increased, the quantum yield of the photoreaction decreases and the pheophytin aggregates on the particle surface. It thus appears that the decrease in the photochemical activity can be explained if we assume that the aggregates are unreactive or, at least, less reactive than the monomers. I have attempted to determine the number of aggregates which are present at a given surface coverage. If the aggregates are unreactive, then the number of monomers on the surface should be a measure of the activity.

I have used a one-dimensional Ising model, as developed by Reiss (1964) in another context, to estimate the amount of aggregation on the polystyrene surface. The pheophytin molecules are assumed to be adsorbed at distinct sites on a linear lattice, with an energy of interaction between molecules on adjacent sites (nearset-neighbor interaction only). The distribution of molecular aggregates can be calculated in closed form with the one-dimensional model. For two and three dimensions, this is not possible. Moreover, the one-dimensional mathematical model seems a reasonable approach in view of the linear nature of the pheophytin aggregates as discussed earlier. The interaction forces are thus assumed to occur along the aggregate chains and to be zero perpendicular to the aggregate axis.

From the statistical analysis of Reiss, one can calculate the number of monomers and the number of aggregates containing 2, 3, 4, etc. molecules on the lattice as a function of the total number of sites that are filled and of the interaction energy of the molecules. In figure 32 is plotted the fraction of the total number of adsorbed molecules which are present

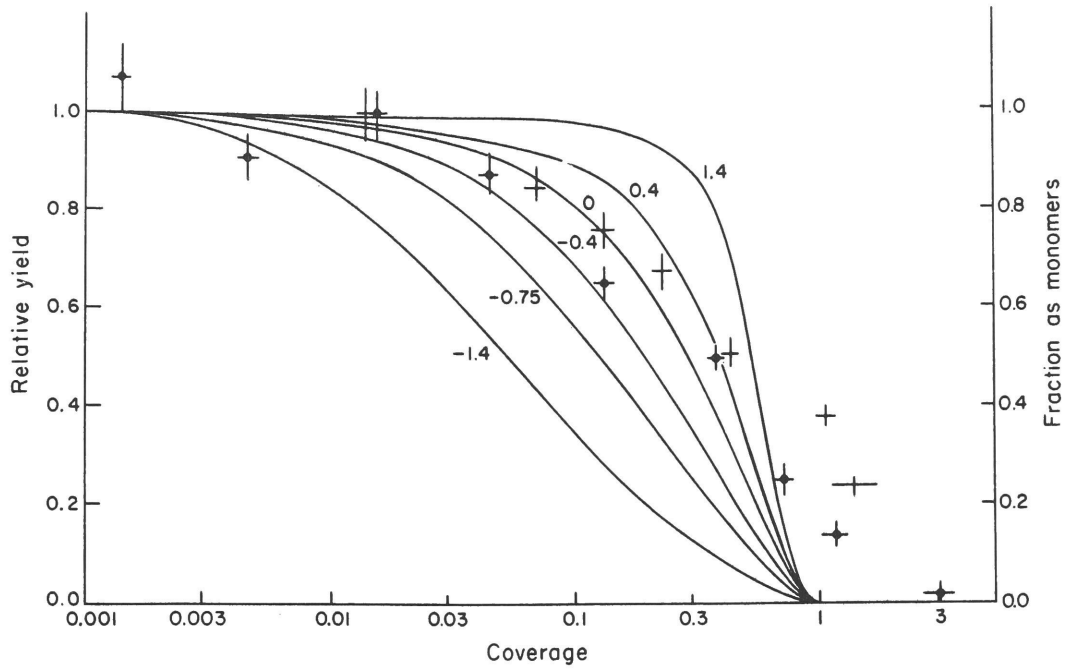



Figure 32. The fraction of total molecules adsorbed as monomers at 300° K, as a function of the surface coverage, according to the one-dimensional Ising model. The curves have been plotted for different values of the interaction energy in kcal/mole, positive for repulsive interaction, negative for attractive. The data points are the relative quantum yields of the photochemical reaction as shown in figure 26.

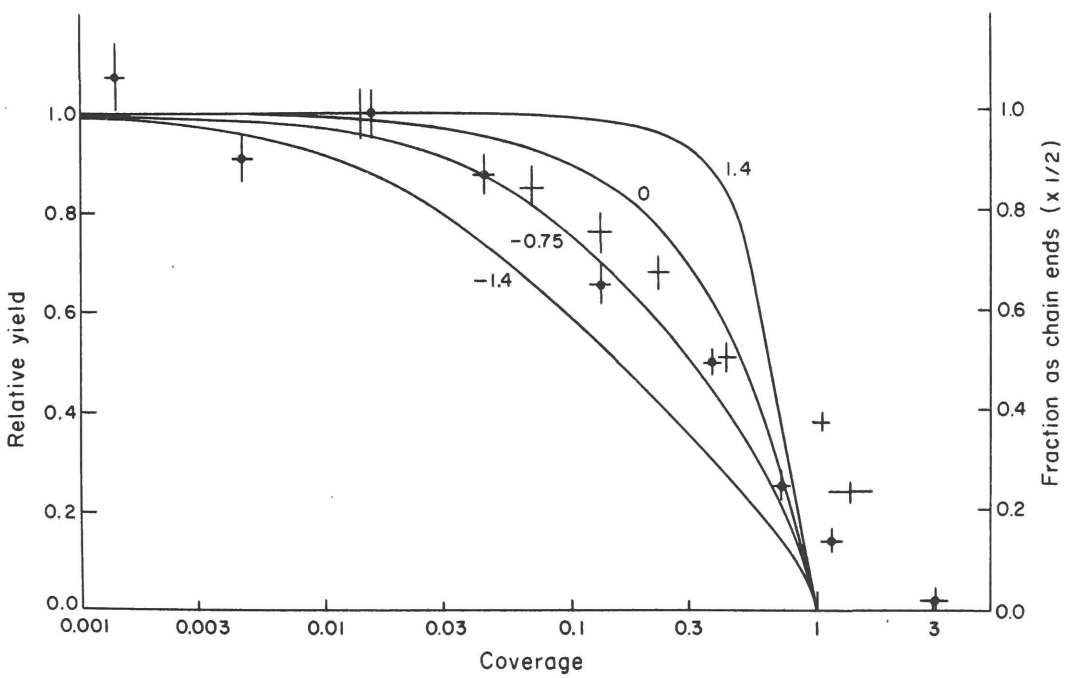



Figure 33. The ratio of the total number of aggregates (or $\frac{1}{2}$ the number of chain ends) to the total number of adsorbed molecules, at 300° K, as a function of surface coverage, according to the one-dimensional Ising model, as in figure 32.

as monomers as a function of the fraction of sites occupied (which we have taken as equivalent to the fraction of the surface covered, i.e., the surface coverage) for various values of the interaction energy. Since McRae and Kasha (1958, 1964) indicated that the total light absorbed (oscillator strength) does not change on aggregation, if only the monomers are photoreactive, the decrease in quantum yield should follow the decrease in the number of monomers exactly, using either broad band illumination or total light absorbing conditions.

For comparison, I have plotted the ratio of the total number of aggregates (or one-half the number of chain ends) to the total number of adsorbed molecules in figure 33. This ratio would be a measure of the photochemical activity if we assumed that each aggregate had the same activity as any other aggregate, independent of the number of molecules in the aggregate. This condition is equivalent to assuming that in an aggregate of n molecules, each molecule has 1/n the photochemical activity of an isolated monomer.

The decrease in the number of monomers with increasing surface coverage does seem to follow the decrease in the photochemical yield, especially around 0 interaction energy. Similarly, the aggregate curve for 0.75 kcal/mole attractive energy between adjacent molecules fits the photochemical data reasonably well. In each case, however, the theoretical curves do not agree with the data at high surface coverage.

It should be noted that there are two degrees of freedom in comparing the theory with the data. The first is the energy of interaction between molecules, compared with the interaction between the molecules and the surface, and the second is the actual surface coverage; i.e., what ratio of pheophytin to polystyrene actually corresponds to one layer.

The net interaction energy between the adjacent pheophytin molecules on the polystyrene surface is actually the difference between the intermolecular attraction of two pheophytin molecules and the interaction between the pheophytin and the aromatic benzene units on the polystyrene surfaces. An additional factor which must be considered is the effect of the solvent during the adsorption process; I noted that the pheophytin tended to aggregate in the 50% methanol and the formation of aggregates may have been influenced by the solvent from which adsorption occurred

rather than by the polystyrene. For these reasons, it is difficult to make an estimate of the interaction energy. But, in molecular crystals, the binding energy is on the order of a few kcal/mole, and the significant value for our considerations is the difference between the adsorbate-adsorbate energy and the adsorbate-adsorbent energy. It is thus not unreasonable to estimate the energy as 0.5 kcal/mole or less. This is consistent with the facts that pheophytin is soluble in toluene, and in 50% propanol pheophytin stays in solution at room temperature but precipitates out of solution at $0^{o}$ C, as I have observed.

We have seen that the main error in the determination of the surface coverage of the polystyrene particles is in the estimation of the area covered by one pheophytin molecule. The analysis above, based on the absorption spectra, suggests that our original choice of 100 $A^2$ is a minimum value and the actual area could be two times larger, although for the aggregate, where it is important, the area is probably only 25% larger. A value greater than 100 $A^2$ per molecule would increase the values for the surface coverage of the polystyrene particles and thus increase the discrepancy between the theoretical curves and the experimental data. Any roughness of the polystyrene surface would increase the specific surface area of the particles, perhaps by a factor of two to three. This would decrease the surface coverage values of the particles.

The one-dimensional model predicts that there would be no monomers on the particles at full coverage. It must be considered, however, that in actuality aggregates could build up from the surface before the surface is totally covered, thus leaving some of the particle surface exposed. In this case, there could be some monomeric pheophytin on the surface.

Evidence for the presence of monomers at high coverage has been obtained from the fluorescence excitation spectra of the polystyrene particles. These spectra, which show the relative effectiveness of quanta of different wavelengths in exciting the fluorescence, give an indication of the molecules responsible for the fluorescence. For many molecules, including chlorophyll and pheophytin, Weber and Teale (1958) found that the fluorescence excitation spectrum was identical with the absorption spectrum within the experimental error of about 6%. I have measured the excitation spectra for the fluorescence both at the wavelength of maximum fluorescence emission, 680 mμ, and

at 750 mμ, on the long-wavelength tail of the emission (see figure 34). These spectra are shown in figure 35, along with the absorption spectrum of pheophytin in toluene. At low surface coverages, the excitation spectra for both the 680 mμ and the 750 mμ fluorescence follow the absorption spectrum quite well. At high coverages, however, there is a difference between the excitation spectra for the fluorescence at the two wavelengths. The much broader spectrum of the 750 mμ fluorescence of the 3.1 PS particles can be considered to be due to the fluorescence of the aggregates. This is consistent with the broader absorption spectra of the highly coated particles, which we attributed to aggregation, and with the increase with the 750 mμ fluorescence intensity compared with the 680 mμ intensity with increasing surface coverage (compare the fluorescence emission spectra of the 0.0046 PS and 3.1 PS particles in figure 34). The excitation spectrum for the fluorescence emitted at 680 mμ, however, resembles very closely the excitation spectra for the low coverage particles and the absorption spectrum of the pheophytin in toluene solution. This similarity suggests that the 680 mμ fluorescence from the high coverage polystyrene is due to unaggregated pheophytin, i.e., that there is a small amount of pheophytin monomer at high surface coverages. The photochemical activity of these molecules would then explain the observed reaction at coverages greater than one. A small amount of the activity of the 3.1 PS could also be due to the 1% of the pheophytin which was extracted from the particles by the 50% methanol (see chapter II). Since this material was not fluorescent in the 50% methanol (at least 10 times less so than in ether), it was probably aggregated and not responsible for much of the activity.

An alternative explanation for the above data is that some pheophytin has dissolved (i.e., diffused) in the polystyrene. Since this diffusion process would continue as the particles "aged," it would explain the decrease in photochemical activity of the particles with time, which I have observed, if it is assumed that these dissolved molecules are inactive. It does not explain the residual activity at high coverages, however.

With this analysis of the discrepancy between the theoretical curves for the fraction of monomers on the surface and the experimental data for the photochemical reaction at high coverages, and the agreement, within the limits discussed above, of the theoretical curves with the data at low coverages, it is tempting to conclude that the pheophytin monomers are the major active species on the polystyrene particles, and that the aggregates are inactive or, at least, less active.

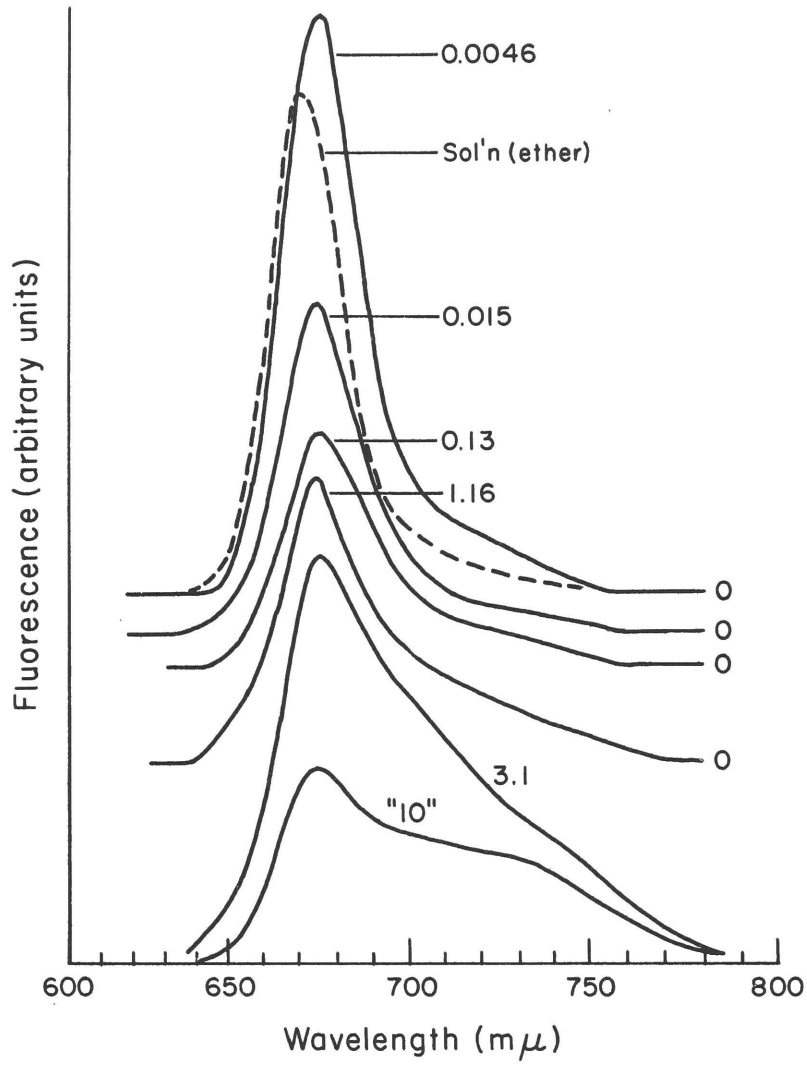



Figure 34. Fluorescence emission spectra of the pheophytin-coated polystyrene particles, suspended in 50% methanol buffer, compared with the spectrum in ether solution.

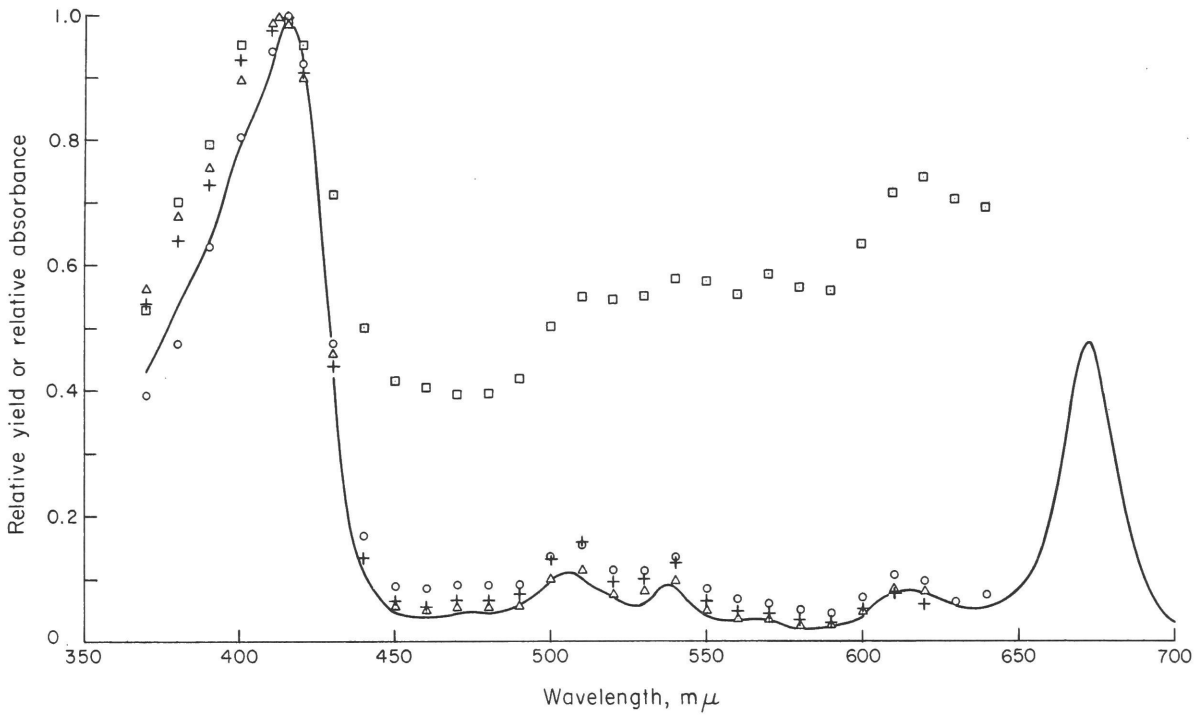



Figure 35. Fluorescence excitation spectra of pheophytin-coated polystyrene particles compared with the absorption spectrum of pheophytin in toluene at comparable spectral band-widths. ( Δ ) 680 mμ fluorescence of 0.0014 PS; ( o ) 750 mμ fluorescence of 0.0014 PS. ( + ) 680 mμ fluorescence of 3.1 PS; ( □ ) 750 mμ fluorescence of 3.1 PS. (————) absorption spectrum of 4.8 x $10^{-6}$ M pheophytin in toluene. The spectra have been normalized to the value of the fluorescence yield or absorbance at the Soret maximum.

However, there is one piece of data that has yet to be discussed. The fluorescence emission spectra shown in figure 34 have been presented without any consideration of the relative fluorescence intensity of the various particle preparations. Since I have attributed the 680 mμ fluorescence to the pheophytin monomers on the basis of the fluorescence excitation spectrum, then we would expect that the quantum yield of the fluorescence at 680 mμ would be a measure of the relative number of monomers on the particles. The relative quantum yield of the 680 mμ fluorescence of the pheophytin-coated polystyrene particles is shown in figure 36 and table XII. By relative quantum yield, I mean the relative number of quanta emitted as fluorescence at 680 mμ per quantum of light absorbed. The data have been normalized to the maximum observed yield, that for the 0.0014 PS. I have compared the data with the decrease in fluorescence yield of chlorophyll at an air/water interface as observed by Tweet, Gaines and Bellamy (1964a), and, as before, with the data for the quantum yield of the photochemical reaction.

The quantum yield of fluorescence decreases with increasing coverage much faster than does the yield of the photochemical reaction and the theoretical estimate of the fraction of monomers on the particles. The very low quantum yields of the fluorescence at high coverages is consistent with fact that aggregates have formed at the high coverage since it is known that crystals and colloidal preparations of chlorophyll are very weakly fluorescent, if at all (see Rabinowitch, 1951, p.775; 1956, p.1824). I have also observed that diluting a methanol solution of pheophytin with several volumes of water markedly quenches the fluorescence. In addition, from figure 34 and table XI it can bee seen that the fluorescence emission spectra do not broaden as rapidly, with increasing surface coverage, as do the absorption spectra. This is because the quantum efficiency of aggregate fluorescence (which occurs at longer wavelengths) is much lower than that of the monomers, and thus the emission spectrum is heavily weighted by monomer fluorescence.

The decrease in fluorescence yield on the coated polystyrene particles can be explained without changing our estimate of the number of monomers on the particle surface. It is now well established that excitation energy can be transferred between molecules up to 50 to 100 A apart. If some of the excitation energy of the monomers is transferred to dimers and higher

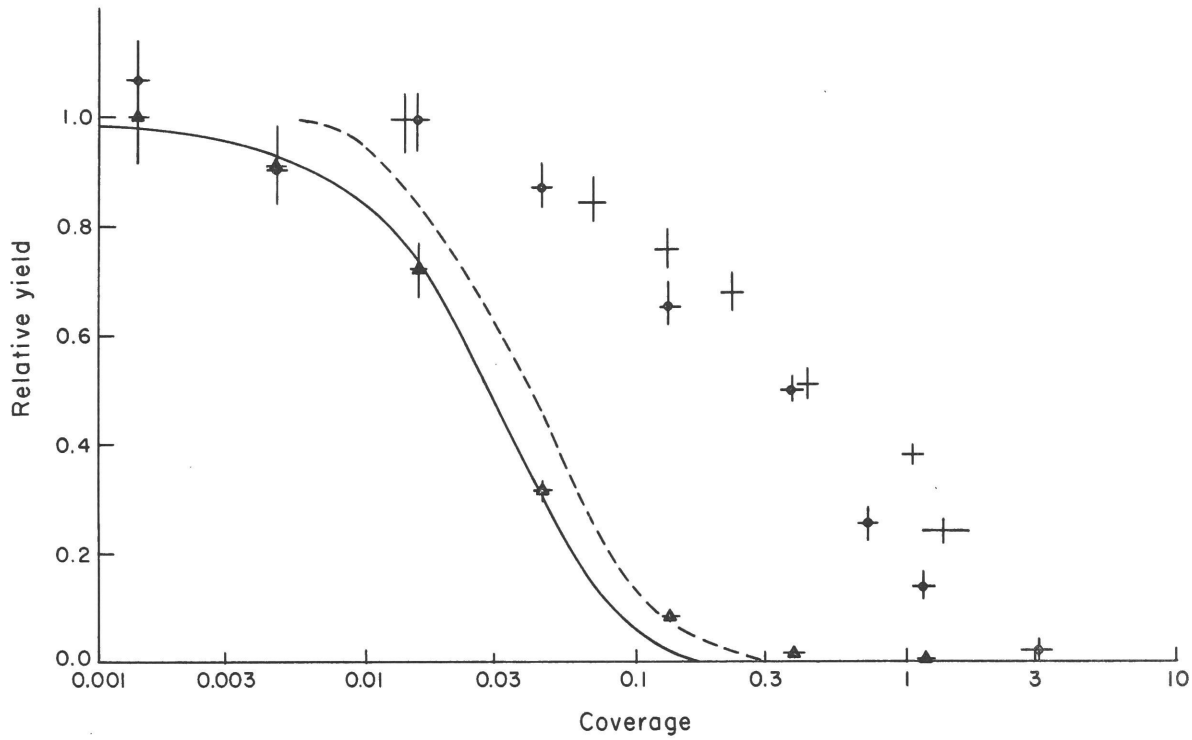



Figure 36. Relative quantum yield of fluorescence at 680 mμ of pheophytin-coated polystyrene particles ( ▲ ). The solid line is a theoretical curve (see text). The dashed line shows the quenching of chlorophyll fluorescence in mixed chlorophyll/oleyl alcohol monolayers at an air/water interface, as a function of the fraction of the area of the layer occupied by chlorophyll (after Tweet, Gaines, and Bellamy, 1964a). The additional data points show the decrease in quantum yield of the photochemical reaction as in figure 26.

TABLE XII

RELATIVE QUANTUM YIELDS OF FLUORESCENCE OF PHEOPHYTIN-COATED POLYSTYRENE PARTICLES AT 680 mμ

| Surface coverage[a] | Relative quantum yield |
|---|---|
| 0.0014 | 1.00 |
| 0.0046 | 0.92 |
| 0.0155 | 0.72 |
| 0.045 | 0.32 |
| 0.133 | 0.086 |
| 0.386 | 0.020 |
| 1.16 | 0.0086 |

Note: a) All particles were coated from propanol/water (method II).

aggregates, which are, at best, very weakly fluorescent, then we would expect the fluorescence to decrease as aggregates formed on the particle surface.

At a surface coverage of 0.1 the fluorescence yield is about 10% of the maximum value observed. If we assume no interaction energy between the pheophytin molecules, there is approximately one aggregate for every 9 monomers. At 0.1 coverage the molecules are about 36A apart, on the average. (To each molecule or aggregate we may associate a circle of radius R, within which there is no other molecule. The area of this circle is $\pi R^2$ and the 100 $A^2$ occupied by the molecule covers about 10% of this area. Since $0.1\pi R^2 = 100\ A^2$, R = 17.8 A and the average distance between molecules $\cong 2R = 36$ A.) Similarly, the monomers are at most 53 A from the nearest aggregate. Thus, if transfer can occur with reasonable efficiency between molecules 36 A apart and we allow one monomer-monomer transfer before a transfer from a monomer to a non-fluorescent aggregate (dimer), then the observed quenching can be explained.

We may obtain a quantitative estimate of the amount of quenching by combining the energy transfer theories of Förster (see, for example, Förster, 1949, 1960; Dexter, 1953), as modified by Tweet, Bellamy and Gaines (1964) for a two-dimensional surface, with our estimate of the number of monomers and aggregates on the surface from the one-dimensional model. The energy is transferred through a resonance interaction between the donor and the acceptor. The classical model is that of coupled oscillators, each with the same frequency of vibration. If one is oscillating at a given time, at some later time the second begins to oscillate. The essential condition for this transfer is that the frequencies of the two oscillators are equal. In the case of molecules, the transfer occurs because of the mutual interaction of the transition dipoles. The "resonance condition" is that the fluorescence emission spectrum of the donor must overlap the absorption spectrum of the acceptor to some degree. The dipole-dipole nature of the interaction leads to an $R^{-6}$ dependence for the transfer probability (R is the distance between the donor and acceptor). Thus, the amount of transfer is a function of the relative concentrations of the donor and acceptor.

Tweet, Bellamy and Gaines (1964) have calculated the effect that transfer to a nonfluorescent acceptor will have on the fluorescence intensity of donor molecules as a function of surface concentration. They

specifically note that the treatment is different depending on whether the donor or the acceptor (quencher) is in excess. (Förster, 1949, has calculated the fluorescence quenching in three dimensions only for the case that the quencher is much more concentrated than the donor.) According to Tweet et al., on a surface the ratio of the fluorescence yields in the presence and absence of a quencher, with quencher concentrations much lower than donor concentrations is

$$\phi(Q)/\phi_o = \int_0^\infty \exp\left[-\left(x - q\,x^{1/3}\right)\right] dx , \qquad (14)$$

where*

$$q = \Gamma(2/3)\ \pi R_{oQ}^{2}\ n_f^{2/3}\ n_Q^{1/3} . \qquad (15)$$

Here $n_f$ and $n_Q$ are the surface concentrations (molecules per unit area) of the fluorescing and quenching molecules, respectively, and $R_{oQ}$ is the critical transfer distance for transfer between the fluorescing molecule and the quencher. The critical transfer distance is the distance between the two molecules at which the rate of transfer is equal to the rate of fluorescence, i.e., at a distance of $R_{oQ}$ between the fluorescing molecule and the quencher, the probability of transfer equals the probability of fluorescence. This distance may be calculated from the expression (Förster, 1960)

$$R_{oQ}^{6} = \frac{9000\ \ln 10\ \langle \kappa^2 \rangle\ \phi_o}{128\ \pi^6\ n^4\ N_o} \int_0^\infty f(\tilde{\nu})\ \epsilon_Q(\tilde{\nu})\ \frac{d\tilde{\nu}}{\tilde{\nu}^4} , \qquad (16)$$

---

* This expression for q differs slightly from the corresponding expression which follows equation 7 in the paper of Tweet, Bellamy and Gaines (1964), which can be written

$$q = \Gamma(2/3)\ \frac{\pi R_{oQ}^{2}}{a_Q}\ K_f^{2/3}\ K_Q^{1/3} , \qquad (15a)$$

where $K_f$ and $K_Q$ are the fractions of the surface covered by the fluorescing and quenching molecules, respectively, and $a_Q$ is the area covered by one quencher molecule on the surface. In checking their calculations, I discovered Tweet et al. had assumed that the area covered by a quencher is equal to the area covered by a fluorescing molecule, though they do not explicitly state this. This assumption does not hold for our conditions, since the quenching aggregates contain at least two pheophytin molecules. (On p. 2073 of the paper of Tweet et al. the gamma function should be $\Gamma(2/3)$ not $\Gamma(2/3)^{\frac{1}{2}}$.)

where $\langle \mathfrak{K}^2 \rangle$ is an orientation factor (5/4 for molecules with their transition dipoles parallel to a surface, but otherwise randomly oriented on the surface), n is the refractive index of the medium, $N_o$ is Avogadro's number, $\epsilon_Q(\tilde{\nu})$ is the molar absorptivity of the quencher, $\phi_o$ is the fluorescence yield in the absence of quencher (as in equation 14), $f(\tilde{\nu})$ is the fluorescence output of the fluorescing molecule, normalized so that

$$\int_0^\infty f(\tilde{\nu}) \, d\tilde{\nu} = 1, \tag{17}$$

and $\tilde{\nu}$ is the wave number. Instead of calculating $R_{oQ}$, I have chosen it so that the fluorescence yield is 31% at 0.045 coverage. I have estimated $N_f$ and $N_Q$ with the one-dimensional Ising model, assuming only the monomers are fluorescent. Since the total number of monomers is decreasing with increasing coverage, I have multiplied the $\phi(Q)/\phi_o$ obtained from equation 14 by the estimated fraction of monomers obtained from the model. This should give us the relative quantum yield of fluorescence of the particles. I have estimated the values for the integral in equation 14 from figure 5 in the paper of Tweet, Bellamy and Gaines, (1964). The theoretical estimate of the relative fluorescent yield of the polystyrene particles, calculated by the above method is shown by the solid line in figure 36, where the value of 45 A was chosen for $R_{oQ}$.

This value of $R_{oQ}$ is not unreasonable. Tweet et al. calculate $R_o$ for transfer between two chlorophyll molecules to be 56 A. However, pheophytin has a lower value for the maximum quantum yield of fluorescence than chlorophyll (0.18 vs. 0.3; Weber and Teale, 1957) and the index of refraction of the polystyrene interface is probably greater than that of the air/water interface. If we follow Tweet et al. and use the root mean square of the refractive indexes of polystyrene and water/methanol for the refractive index of the polystyrene interface (1.46), the 56 A corrects to about 43 A by the above equation. I have so far assumed the overlap integral,

$$\int_0^\infty f(\tilde{\nu}) \, \epsilon_Q(\tilde{\nu}) \, \frac{d\tilde{\nu}}{\tilde{\nu}^4} \quad ,$$

is the same for pheophytin and chlorophyll. This is approximately correct. Jacobs et al. (1957) give 0.31 and 0.38 as the oscillator strengths (a measure of the integrated absorption intensity) of pheophytin and chlorophyll, respectively. This means that $R_o$ is slightly less than 43 A for the pheophytin

monomer-monomer transfer. However, the absorption maximum of the pheophytin aggregates is shifted to longer wavelengths and will overlap the monomer fluorescence spectrum even better than does the monomer absorption. In addition the absorptivity of an n-fold aggregate is n times that of a monomer. Since the aggregates are primarily dimers, this will introduce a factor of $2^{1/6}$ in R. Thus, the $R_{oQ}$ for transfer from the pheophytin monomers to aggregates, calculated from equation 16, is slightly larger than the 45 A obtained by the application of equation 14 to the experimental data.

The above analysis has shown that the decrease in the quantum yield of fluorescence with increasing particle coverage can be explained by assuming that energy is transferred from the fluorescent pheophytin monomers to the very weakly fluorescent aggregates. In point of fact, I assumed that the aggregates were nonfluorescent. If they are weakly fluorescent, the discrepancy between the theoretical quenching curve and the data at high coverages (greater than 0.1 PS) can be explained.

While our estimate of the number of monomers and aggregates on the polystyrene particle surface is consistent with the behavior of the fluorescence yield, a further conclusion from the above data is that the excited singlet state of the monomers decays more rapidly than does the quantum yield of the photoreaction with increasing surface coverage. This implies that there is a photoreactive molecular species which is neither the pheophytin monomer in the fluorescent state (the lowest vibrational level of the first excited singlet state) nor any species derived from this fluorescent state.

I have noted above that it is generally accepted that the photoreactive species is the sensitizer (pheophytin) molecule in its triplet state. It has also generally been assumed that the triplet state is derived from the fluorescent state (Livingston, 1960, p. 841; Parker, 1964, p. 310), though Parker does mention that in some cases there is evidence that "crossing-over" from the higher vibrational levels of the singlet state to the triplet state does occur. In order to explain the results of the photochemical experiments on the pheophytin-coated polystyrene particles, we must make one of the following two assumptions (though they are not mutually exclusive):

1) Small aggregates of the pheophytin must be photoreactive and have an activity similar to that of the monomer; because the quantum

yield of the photoreaction is low at high surface coverages, the larger aggregates must have a very low activity.

2) The photoreactive species on the polystyrene particle is the pheophytin monomer in its triplet state, and this triplet must be derived mostly from higher vibrational levels of the excited singlet state and not from the fluorescent state.

If the aggregates are active, it is possible to obtain an estimate of their activity compared to the monomer from the one-dimensional Ising model. I will make the following assumptions: 1) At 0.0014 PS, the fluorescence yield and the quantum yield of the reaction are those for the monomers only. 2) The fraction of light absorbed directly by the $x_n$ n-mers (n = 1 means monomers, 2, dimers, etc.) is given by the fractional number of molecules in the n-mers, i.e., is $n\, x_n/N$ where N is the total number of molecules on the surface, $N = \sum_{n=1}^{\infty} n\, x_n$. 3) The difference between the fraction of light absorbed by the monomers and the fluorescence yield of the particles is the fraction of incident light transferred to the aggregates. 4) This energy is distributed among the aggregates uniformly; if there are P aggregates ($P = \sum_{n=2}^{\infty} x_n$) then the fraction of the energy transferred to the $x_n$ n-mers is $x_n/P$. 5) The activity of the aggregates is proportional to the amount of excitation energy reaching them, there being no difference between the energy received by transfer and by direct absorption.

From the one-dimensional model (assuming no interaction energy) it can be shown that 0.2 coverage, 64% of the molecules are monomers, 25.6% are in dimers and the rest are in higher aggregates. 80% of the aggregates are dimers. The fluorescence yield of the 0.2 PS is about 0.03 that of the 0.0014 PS. The fraction of the absorbed light energy which gets to the dimers is 0.744, while the total activity of the particles is only 0.6 - 0.65. This means that the dimers cannot be as active as the monomers. If only dimers are active, their activity must be about 90% of the monomers. Further calculation shows that the assumption that the photochemical activity of the aggregates decreases with the number of molecules in the aggregates (either as $1/n$ or $1/n^{\frac{1}{2}}$) does not fit the experimental data. A remarkable fit is obtained by the assumption that dimers are 90% active and trimers and larger aggregates are inactive (table XIII). The error in the experimental data precludes testing for a small activity (less than 10%) of the larger aggregates.

TABLE XIII

PREDICTED RELATIVE QUANTUM YIELDS OF THE PHOTOREACTION ASSUMING DIMERS ARE 90% AS ACTIVE AS MONOMERS

| Surface coverage | Predicted yield | Experimental yield |
|---|---|---|
| 0.010 | 0.99 | |
| 0.015 | 0.97 | 1.00 |
| 0.030 | 0.93 | |
| 0.045 | 0.90 | 0.89 |
| 0.060 | 0.87 | |
| 0.070 | 0.86 | 0.85 |
| 0.10 | 0.81 | |
| 0.133 | 0.77 | 0.71 |
| 0.20 | 0.70 | |
| 0.24 | 0.64 | 0.68 |
| 0.30 | 0.57 | |
| 0.386 | 0.47 | 0.50 |
| 0.60 | 0.23 | |

If the photoreactive triplet state of pheophytin is derived from the higher vibrational levels of the excited singlet state, the quantum yield of the photoreaction should be a function of the wavelength of the exciting light. Specifically, the quantum yield should decrease on the long-wavelength side of the absorption maximum (where there is insufficient energy to reach the higher vibrational levels.) Similarly, the quantum yield of fluorescence of pheophytin should also be a function of wavelength, increasing at long-wavelengths. Weber and Teale (1958), as I have noted above, found that the fluorescence excitation spectrum of pheophytin in hexane followed the absorption spectrum closely, which means the fluorescence yield was not a function of wavelength. But Weber and Teale's measurements did not eliminate the possibility that as much as 6% of the excited singlet molecules could cross over to the triplet state from higher levels, since their experimental error was 6%. A more sensitive test, that of measuring the quantum yield of the formation of the triplet state as a function of wavelength, does not appear to have been made.

To test the first possibility, I have determined the wavelength dependence of the quantum yield of the pheophytin-sensitized reduction of ANR by ascorbic acid in 90% methanol, i.e., with pheophytin in solution. Since I have shown that the pheophytin monomers on the polystyrene surface are approximately as photoreactive as the molecules in solution, the results should be applicable to the polystyrene particles. The results are tabulated in table XIV and show that the quantum yield is relatively constant at wavelengths shorter than about 675 mμ, and increases at longer wavelengths, in direct contradiction to our conclusion that it would fall if crossing over occurred from the higher vibrational excited singlet levels. Because of the very low absorbances of the pheophytin solution at the longer wavelengths (0.07 at 689 mμ and 0.01 at 705 mμ), the error in the relative yields at these wavelengths is probably closer to 50% than to the 20% estimated for the remaining values. However, this does not entirely explain the relatively high yields at 689 and 705 mμ. While there was no evidence for aggregation in the absorption spectrum of the pheophytin in the 90% methanol after about 1 minute's illumination with white light, the higher yields could be due to the presence of aggregates, which would be expected to have greater absorption than the monomer at longer wavelengths, and which would have been formed during the illumination

TABLE XIV

WAVELENGTH DEPENDENCE OF THE QUANTUM YIELD OF THE PHOTOSENSITIZED REDUCTION OF ANR BY ASCORBIC ACID

| Wavelength | Relative quantum yield[a] | Fraction incident light absorbed by pheophytin[b] | Relative number quanta absorbed[c] | Filter[d] |
|---|---|---|---|---|
| 395 mμ | 0.79 ± 0.20 | 0.935 | 1.30 | 5-61 |
| 411 | 0.93 | 0.950 | 1.69 | 5-61 |
| 430 | 1.02 | 0.725 | 1.74 | 5-61 |
| 444 | 0.98 | 0.356 | 0.69 | 5-61 |
| 650 | 0.97 | 0.588 | 1.10 | 2-61 |
| 662 | 1.00 | 0.842 | 1.00 | 2-59 |
| 675 | 0.94 | 0.814 | 0.55 | 2-59 |
| 689 | 1.9 ± 50% | 0.150 | 0.14 | 2-59 |
| 705 | 2.4 | 0.025 | 0.024 | 2-64 |

Reaction conditions: square cuvette without integrating cylinder. Solvent, 90% methanol; $3 \times 10^{-5}$ M ANR, $2.6 \times 10^{-5}$ M pheophytin a ($7.8 \times 10^{-8}$ mole in 3.0 ml); 0.012 M ascorbic acid; 50% of the ascorbic acid was neutralized with NaOH to adjust pH to 6.7.

Notes: a) The amount of ANR reduced per quantum absorbed, relative to the value at 662 mμ. The values have been corrected for the 0.8 power dependence on light intensity of the ascorbic acid reaction (equation 9). b) Determined from the absorption spectrum of the solution as described in chapter II. c) Determined from the fraction absorbed and the incident light intensity. At 411 mμ, the light absorbed was approximately $2.2 \times 10^{-9}$ einsteins per second. d) Corning filters used in addition to the B & L interference filter at the given wavelength (the wavelength of maximum transmission of the filter).

for the reaction at other wavelengths. A more likely alternative explanation is that enough light was transmitted by the interference filters used for the reaction at 689 and 705 mμ in the region of the absorption maximum of the pheophytin to increase the rate of reaction. For example, at 670 mμ, the transmission of the 689 mμ interference filter is 2%, versus about 30% at the peak. Since only 7% of the incident light was absorbed at 689 mμ, while about 90% was absorbed at 670 mμ, approximately twice as much light could have been absorbed as was calculated for the wavelength of maximum transmission of the filter alone.

In connection with the last experiment, I estimated the absolute quantum yield of the pheophytin-sensitized ANR reduction by ascorbic acid to be 0.10 at 411 mμ, the Soret absorption maximum. This implies that the quantum yield of formation of the triplet state of pheophytin must be at least 0.1. If the triplet were formed from higher vibrational levels of the excited singlet state, then a 10% difference in the quantum yield of fluorescence might be expected to appear between short and long wavelengths. This is outside the experimental error of the experiments of Weber and Teale (1958) and gives additional support to the conclusion that the triplet is not formed exclusively from higher vibrational levels of the excited singlet state.

The final conclusion from these studies on the pheophytin-coated polystyrene particles must be that small aggregates of pheophytin (e.g., dimers), as well as monomers, are effective in sensitizing the reduction of ANR. There is little or no intrinsic effect of the polystyrene surface on the photochemical properties of the molecules; the decrease in yield with increasing surface coverage must be due to the decreased activity of the large aggregates which form at the high concentrations of pheophytin on the polystyrene surface. The significance of these conclusions will be discussed in the final chapter.

One other result should be mentioned. As shown in figure 26, the quantum yields for the sets of particles prepared by the two different methods described in chapter II are slightly different. The reason for this difference has not been investigated, but it is probably caused by different amounts of aggregate formation in the two solvents. As was noted in chapter II, pheophytin tends to aggregate more in methanol than in propanol. The half-width of the absorption band of the 0.133 PS prepared from methanol is slightly larger than that of the particles prepared

from propanol (27 vs. 24 mμ). It is not clear that this aggregation will explain the difference in quantum yields, since those for the "methanol" particles are higher than those for the "propanol" particles. However, a possible explanation for this effect is that, in methanol, only small aggregates (i.e.,dimers) are present and adsorb to the surface as such, before further aggregation occurs. On the basis of the Ising model, a dimer takes up one less lattice site than two monomers, because it is necessary to have an empty site separating the two monomers. Thus there will be more sites available to the monomers and dimers on the particles coated in methanol and large aggregates will form at higher coverages on these particles than on the particles coated from propanol. I have previously concluded that the small aggregates are photochemically active. Thus the methanol particles with more small, active aggregates and fewer large, inactive aggregates would have a higher photoreactivity.

III. Pheophytin-coated zeolite particles.

The work described in the preceding section was concerned solely with the behavior of the pheophytin on the nonpolar surface of the polystyrene particles. I have also made some studies of pheophytin adsorbed to the polar surface of synthetic zeolite particles. The results of the studies on these particles show that the nature of the surface can affect the photochemical properties of the adsorbed pigment.

In figure 37 is shown the relative quantum yield of the pheophytin-sensitized reduction of ANR by N'-carboxamidomethyl-1,4-dihydronicotinamide in water as a function of the surface coverage of pheophytin on the zeolite (Molecular Sieve 4A) particles. The data have been normalized to the yield for the 1.0 coverage particles. As mentioned in chapter II, this was the maximum coverage reached on these particles. The maximum quantum yield observed, for the 1.0 Z particles, is about 0.11 - 0.16 the yield with pheophytin in 90% methanol solution.

Preliminary experiments with the aluminum silicate particles suggest that if the surface coverage of the zeolite particles could be increased above the 1.0 maximum reached in the above experiments, then the quantum yield would begin to decrease with increasing coverage (figure 38). Since the aluminum silicate particles tended to degrade the pheophytin and chlorophyll at low coverages, the yields at low coverages of the aluminum silicate are probably not reliable (though they do behave similarly to the zeolite).

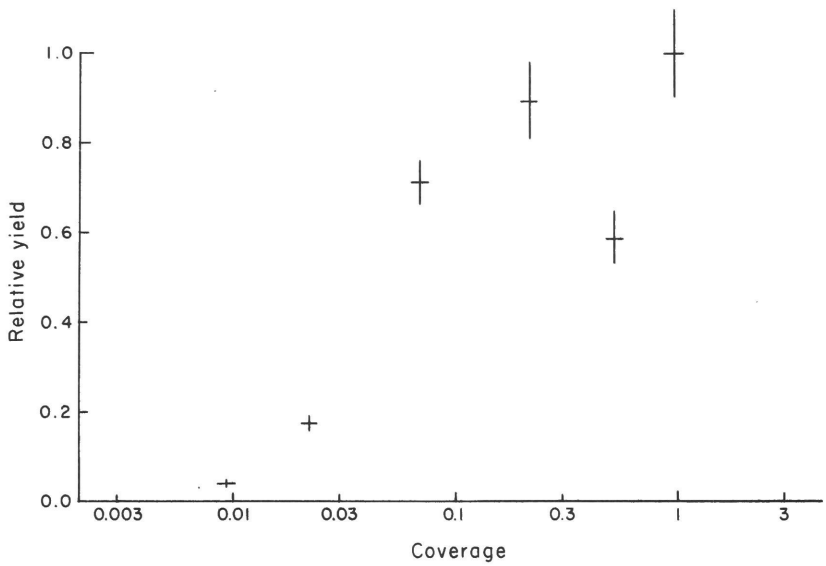



Figure 37. Relative quantum yield of ANR reduction sensitized by pheophytin adsorbed to zeolite particles as a function of surface coverage. Reaction conditions: Solvent, water, 5 x $10^{-3}$ M phosphate buffer, pH 7; 3 x $10^{-5}$ M ANR; 1.0 x $10^{-3}$ M CNH as reductant; 2 x $10^{-8}$ moles pheophytin in 3.0 ml, with a constant 100 mg of particles in the suspension. Illumination through Corning 2-59 filter only. The data have been normalized to the rate for the 1.0 particles, which was 0.137 x $10^{-9}$ mole ANR reduced per second.

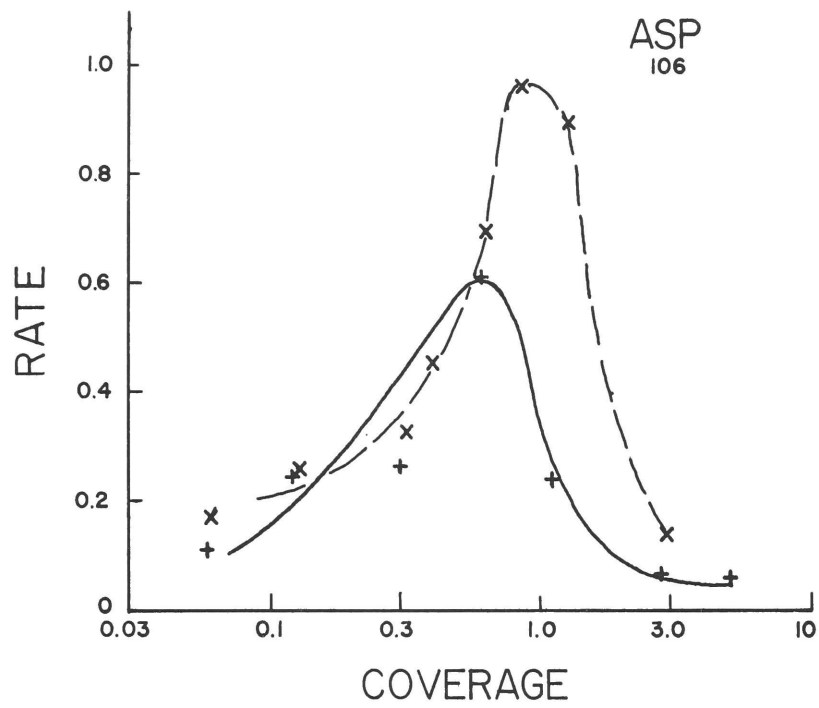



Figure 38. Rate of ANR reduction sensitized by chlorophyll adsorbed to aluminum silicate particles in water at pH 7. Reaction conditions: $3 \times 10^{-5}$ M ANR, 0.75 M ascorbic acid as the reductant; total pigment was about $2 \times 10^{-8}$ moles in 4.0 ml. The two curves are for different particle preparations. The ordinate is $10^{-9}$ mole/sec.

At higher coverages, there was much less degradation of the absorbed pigment (figure 23, table IX) and the results are more reliable. The reason for the difference between the two sets of particles has not been investigated.

The absorption spectra of the pheophytin-coated zeolite particles are shown in figure 39, and the half-widths of the absorption band are tabulated in table XV. Even at very low coverages, the spectrum is broader than that in solution, indicating that there has been some aggregation of the pheophytin on the zeolite surface. With increasing coverage, the broadening increases as in the polystyrene coated particles.

The absorption maxima on the zeolite particles are very close to the maximum observed for pheophytin at air/water (phosphate buffer) interfaces, 675 mμ (figure 40). The upper part of figure 40 has been drawn from the work of Bellamy, Gaines and Tweet (1963). In stable monolayers, the absorption spectrum of pheophytin is similar to the spectra of the 0.022 and 0.070 zeolite particles and of intermediate coverage polystyrene particles (except for a difference in the absorption maximum in the latter case). The absorption maximum of the monolayers and the zeolite particles is shifted 5 to 10 mμ toward the red compared with the maximum in organic solvents (665 mμ in acetone, 669-670 mμ in toluene). This peak position is probably due to the polarity of the surroundings (or a "solvent-shift" as noted by Bellamy et al.) Similar to the coated particles, the stable monolayer has a broader absorption band (half-width about 29 mμ) than in solution, which suggests that there is some aggregation of the pheophytin in the monolayer. Bellamy et al. did not consider the broadness of the absorption band in their discussion.

When the pheophytin film is subjected to increased surface pressure, above 11 dynes/cm, the film is unstable and has a spectrum as shown in the upper half of figure 40. As we have attributed the long wavelength absorption in the coated particle spectra to aggregates on the surface, Bellamy et al., have attributed the long wavelength peak on the unstable monolayer to "tighter packing" of the pheophytin molecules or to formation of a layer more than one molecule thick.

It is interesting to note that there is a difference in the absorption spectrum of the wet and dry 1.0 Z particles. This effect is reversible; if one determines the spectra of the wet particles, dries and rewets them, the absorption spectrum of the rewet particles is very similar to the

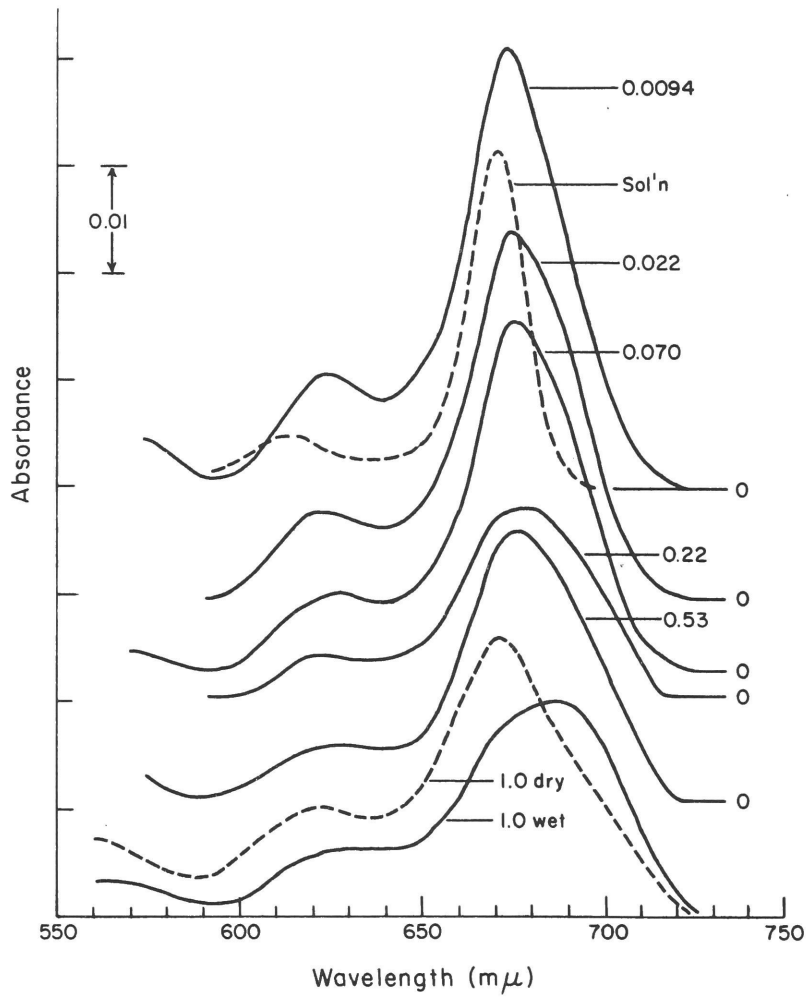



Figure 39. Absorption spectra of pheophytin-coated zeolite particles, compared with pheophytin in toluene solution. All particles are wet except as noted.

TABLE XV

ABSORPTION MAXIMA AND HALF-WIDTHS OF THE PHEOPHYTIN-COATED ZEOLITE PARTICLES

| Surface coverage[a] | Absorption maximum | Half-width |
|---|---|---|
| (toluene soltuion) | 670 mμ | 19 ± 1 mμ |
| 0.0094 | 674 ± 2 mμ | 32 ± 2 mμ |
| 0.022 | 675 | 35 |
| 0.070 | 676 | 35 |
| 0.22 | 677 | 45 |
| 0.53 | 678 | 40 |
| 1.0 (wet) | 690 | 51 |
| 1.0 (dry) | 672 | 43 |

Note: a) All particle preparations wet except as noted.

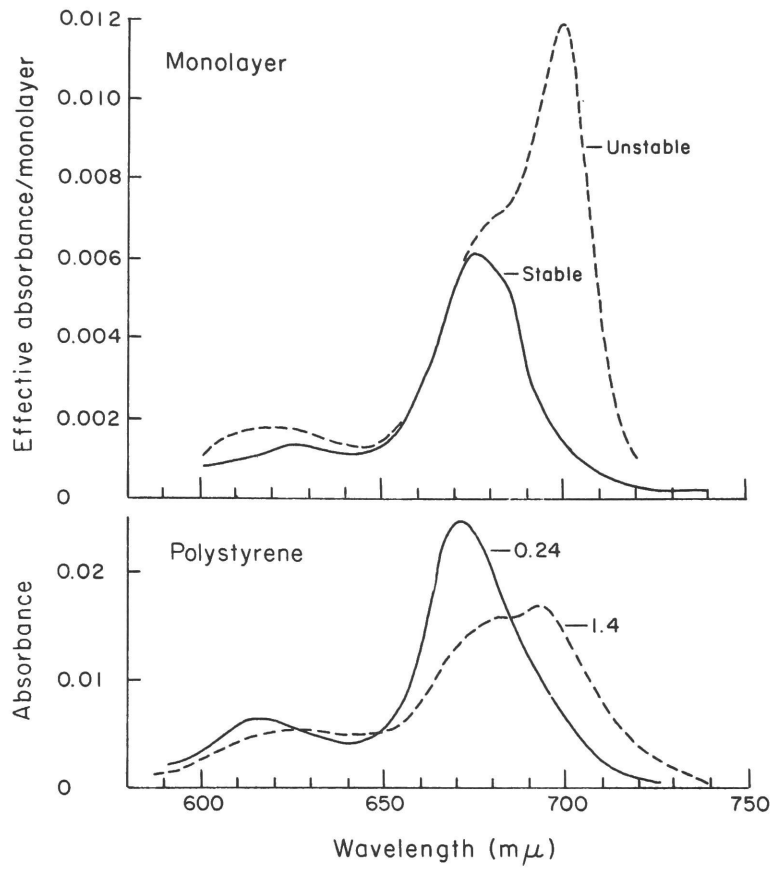



Figure 40. Comparison of the absorption spectra of the pheophytin-coated polystyrene particles with the absorption spectra of pheophytin a in monolayers as determined by Bellamy, Gaines, and Tweet (1963).

original. This shift indicates that the pheophytin is not bound very tightly to the zeolite; the water appears to force the pigment off the surface into larger aggregates. There was no such difference between the wet and dry spectra of the polystyrene particles (see figure 41 for fluorescence spectra) and the 0.0094 zeolite particles.

The 0.53 zeolite particles have a yield which is not consistent with the other zeolite particles. As seen in table XV the half-width of the absorption spectrum is also smaller than would be expected. There did not appear to be any pheophytin aggregates (without particles) in a suspension of the 0.53 Z particles, from microscopic examination. Although the ratio of the blue and red absorbances indicated that there had been no degradation of the pheophytin during the adsorption process (table IX), a check of this ratio for the pigment extracted from the particles in acetone after the reaction indicated that it was 2.35 $\pm$ 0.05. This is larger than the standard ratio of 2.21, and indicates some degradation on these particles. The position of the blue absorption maximum in the extract of the 0.53 **Z** particles was at 408 mμ, identical with untreated pigment.

The fluorescence emission spectra of the zeolite particles (figure 42) broaden with increasing surface coverage as do the absorption spectra. Again there is a shift between the wet and dry 1.0 **Z** particles. While I have not made a thorough study of the fluorescence intensity of the zeolite particles, the quantum yield of fluorescence does decrease with increasing surface coverage. However, in all cases the fluorescence is much weaker on the zeolite than on the polystyrene; the yield of the 0.0094 particles is about 1% that of the 0.001 PS; the difference between the 1.0 zeolite and the 1.0 polystyrene is not quite as much, with the zeolite fluorescence about 10% of the polystyrene.

The absorption spectra of the 0.0094 **Z** and the 0.015 PS particles are quite different (i.e., the half-widths are 32 mμ and 22 mμ respectively). However, the fluorescence emission spectra are remarkably similar in shape (half-widths of 23 mμ and 26 mμ, respectively; the difference is probably not significant), though the intensity of the zeolite particle fluorescence is 1% that of the polystyrene particles. This suggests that there are only a few fluorescent monomers on the zeolite particles, and that the aggregates are nonfluorescent.

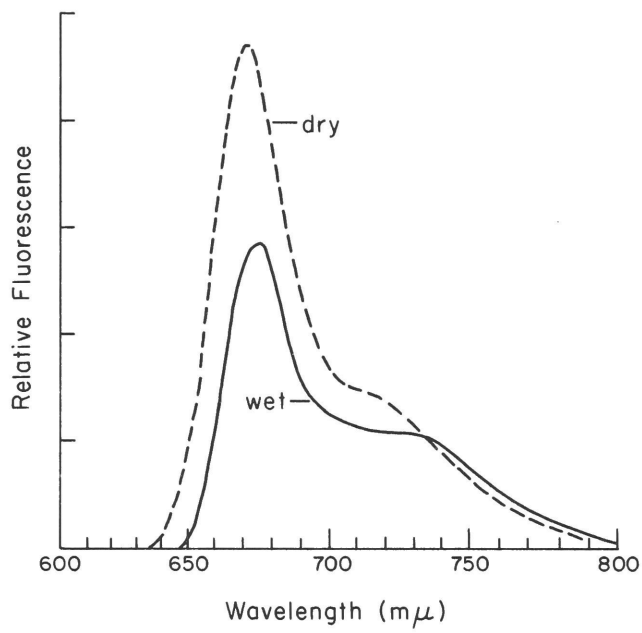



Figure 41. Fluorescence emission spectra of pheophytin-coated 3.1 PS particles on a Millipore filter.

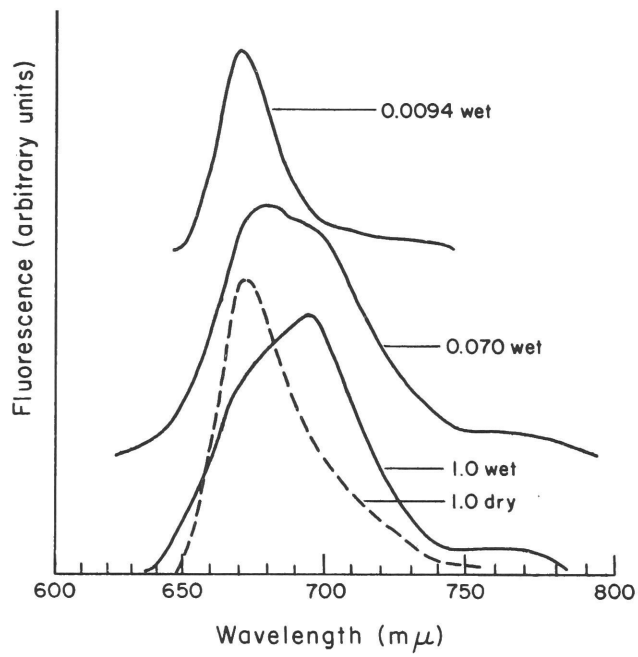



Figure 42. Fluorescence emission spectra of pheophytin-coated zeolite particles.

The lack of fluorescence from the aggregates on the low coverage zeolite particles may be used to explain the very low quantum yield of the photoreaction on these particles. Due to the nature of the interaction with the zeolite surface (which may be either a distinct pheophytin-zeolite interaction or a secondary effect, in which the pheophytin molecules form a different type of aggregate on the zeolite as compared with the pheophytin), the excited singlet state of the pheophytin may be quenched before crossing-over to the triplet can occur, thus quenching the photochemical activity. It is also possible that the interaction rapidly quenches the triplet state, before it has a chance to react, in addition to quenching the singlet.

While the wet 1.0 Z absorption spectrum is still broader than the corresponding polystyrene particle spectrum, the two spectra do have similar shapes and, as noted above, the reactivities of the two particles are similar. This suggests that the structure of the pheophytin aggregates on the high coverage zeolite and polystyrene are similar. The aggregates at high coverages on the zeolite could be formed by the addition of molecules to the nonfluorescent small aggregates in an orientation more like that of the molecules in the aggregates on the polystyrene. These aggregates would then be at least somewhat reactive. A second explanation is that there are a few sites on the zeolites (occupying 1 - 10% of the surface), to which pheophytin is preferentially adsorbed (presumably as aggregates according to the absorption spectrum) and which quench the excited state of the molecules. As these sites (or traps) are filled up, the pheophytin then adsorbs to the remaining surface, most likely as aggregates, since the binding is relatively weak (as evidenced by the shifts in the spectra at the high coverages on drying the particles). The pheophytin not in the traps is similar to that on the polystyrene and thus has a similar reactivity.

It must be remembered that the surface of the zeolite particles has negative charges on it, since it strongly adsorbed methylene blue. The ANR, with the two sulfonic acid groups, would be repelled by these negative charges and would not react with pheophytin adsorbed at or near them. These charges could be the traps discussed above.

Thus, from the studies of the pheophytin-coated zeolite particles we may conclude that the type of surface to which the pheophytin is adsorbed can influence its photochemical properties. It is of interest to note that at higher surface concentrations, the nature of the surface becomes less important, and the interaction between the adsorbed molecules more important.

# CHAPTER IV

## CONCLUSION

The experimental results described in the previous chapter show that much can be learned from a study of the particle systems as a model for the chloroplast. It will be useful to summarize these results and the conclusions from them, before discussing their significance. I will show that this simple model has some important properties which are similar to those of the photosynthetic apparatus. For the purposes of the following discussion, I will assume that there is no significant difference between the properties of chlorophyll and pheophytin.

The quantitative conclusions of this study depend on the care taken to control the difficulties inherent in optical studies in light scattering systems. The methods I have developed for the study of the photochemistry and of the absorption and emission spectra are generally useful for any such scattering system, including photosynthetic materials.

On the polystyrene particles at low surface coverages, the absorption spectrum of pheophytin was identical to that in toluene solution. The photochemical activity of the individual molecules was, within a factor of three, the same as that in 90% methanol solution. As the surface concentration increased, the absorption spectra became broader and the absorption maximum shifted toward longer wavelengths. These effects were interpreted as being due to the formation of aggregates. From the magnitude and the nature of the changes in the absorption spectra, it was concluded that the molecules in the aggregates were in a "card-pack" structure, lying at an angle of about $52^{o}$ above the polystyrene surface. An estimate of the amount of aggregation on the surface was made from a one-dimensional Ising model.

The quantum yield of fluorescence decreased with increasing surface concentrations of the pheophytin on the polystyrene. The fluorescence quenching was quantitatively explained on the assumption that the aggregates were nonfluorescent traps. The number of aggregates was calculated with the model and the mechanism of the energy transfer was assumed to be that of resonance transfer in two dimensions. The critical transfer distance between monomers and aggregates for 50% quenching was estimated

to be 45 A. The good agreement with the experimental data supports the model assumed for the aggregates on the surface.

The quantum yield of the sensitized dye reduction reaction also decreased with increasing surface concentrations of the pheophytin on the polystyrene particles, but at ten times higher surface concentrations than did the fluorescence yield. This striking difference can be explained either by a special pathway to the triplet state or by photochemical activity of some aggregates.

The quantum yield of the photochemical reaction in solution is independent of wavelength; therefore, the photoreactive (triplet) state is most likely derived from the vibrationally equilibrated excited singlet state of the sensitizer. From this, it was concluded that small aggregates, such as dimers, are photochemically active, although the larger aggregates are much less active. One possible interpretation on the basis of the one-dimensional model indicated that the dimers are about 90% as active as the monomers and all higher aggregates are inactive.

The studies with the zeolite particles have shown that the balance between pigment-pigment and pigment-surface interactions is of great importance. The absorption spectra on these polar particles indicated that aggregation had occurred even at very low surface concentrations of pheophytin. The low quantum yields of fluorescence and photochemical activity indicated that the excited states of the adsorbed molecules were rapidly quenched, either because of the particular aggregation or because of a more specific interaction with the surface.

The fluorescence quenching on the polystyrene particles is remarkably similar to that observed in mixed monomolecular films of chlorophyll and oleyl alcohol (Tweet, Gaines, and Bellamy, 1964a). We can thus explain this film data on exactly the same grounds that were used for the polystyrene surface. Tweet et al. were able to explain quantitatively the quenching in their films only by adding a nonfluorescent, extraneous trap, copper pheophytin (Tweet, Bellamy and Gaines, 1964).

The basis for our explanation of the fluorescence quenching was the calculation of the number of aggregates with the one-dimensional model. This model is clearly applicable to many other pigment aggregates. It is far less arbitrary than the usual explanations given in terms of dimers and trimers.

What comparisons can be made between the particles and the photosynthetic apparatus? It will be of interest to compare the pigment concentrations, the absorption spectra, the fluorescence yield and the energy transfer properties of these two systems.

In vivo, the chlorophyll concentration was estimated to be about 0.06 to 0.2 M by Rabinowitch (1945, p. 412). On the basis of the average distance between molecules, this is equivalent to a surface concentration of $1 - 2 \times 10^5$ molecules/$\mu^2$. Thomas and co-workers (as reported by Gaffron, 1960, p.32), on the basis of electron microscopical measurements of the surface area of chloroplast lamellae, estimated the in vivo surface concentration to be $3 - 12 \times 10^5$ molecules/$\mu^2$. On the basis of 100 $A^2$ per molecule it can be calculated that the maximum number of molecules which can fit on a $\mu^2$ of monolayer is only $10 \times 10^5$. The range of surface concentrations quoted above corresponds to surface coverages of 0.1 to 1.2, on the same basis. It will be of particular interest, therefore, to compare the properties of the coated particles at 0.2 - 0.3 coverage with those of the chloroplast.

The first similarity between the particles and the plant is that in both cases the pigment can be concentrated into a small volume and yet remain photochemically active. (The equivalent concentration of the 0.2 PS particles is about 0.2 M, calculated on the basis of intermolecular distance.) In contrast, solutions of the pigments are limited by their solubility and, more important, by self-quenching of the photoexcited states at high pigment concentrations. A possible explanation of this different photochemical behavior is that the pigments are restricted to the particle surface or to the lamellae, which prevents collisions of the pigment molecules.

The chlorophyll in the green plant is in different "forms" as evidenced by the occurrence of at least three different absorption maxima (French, 1961). There are major amounts of chlorophyll with red absorption maxima at about 673 mμ and 684 mμ. I have previously mentioned in chapter I that there is an apparently photochemically active form of chlorophyll in vivo which absorbs around 700 mμ (P700). In all these cases, the absorption maximum is shifted from the position in solution (about 662 mμ in ether). These shifts can be the result of solvent-shifts, aggregation, or specific complexes with molecules other than chlorophyll,

or any combination of these. Solvent effects cannot explain the observed shifts completely; for example, the maximum shift observed is to 672 mμ in carbon bisulfide (Rabinowitch, 1951, p. 640).

Studies on various model systems, including chlorophyll monolayers (Trurnit and Colmano, 1959; Bellamy, Gaines and Tweet, 1963), colloids (Bannister and Bernardini, 1963; Krasnovskii and Brin, 1948), coated particles (Evstigneev and Gavrilova, 1960; the present work), and concentrated solutions (Brody and Brody, 1963), have produced spectra that are similar to the absorption spectra in vivo. On the polystyrene particles, for example, at a coverage of 0.2, the half-maximum absorption occurs at about 18-20 mμ longer wavelengths than the absorption maximum, just as it does in the absorption spectra of chlorella taken under similar conditions (Mr. Arthur Burr, personal communication). (I have considered only the long-wavelength side of the absorption band, because of the complicating effects of chlorophyll b on the short-wavelength side of the in vivo spectra.) The equivalent width on the high coverage polystyrene is much greater, 35 to 40 mμ. This suggests that there is no high degree of aggregation of chlorophyll in the plant. However, an unknown amount of the spectral effects in vivo may be due to association with other chloroplast components. Thus no strong conclusions can be drawn from the absorption spectra until such components are incorporated into the particle model.

Additional evidence that there is no high degree of aggregation in the chloroplast comes from comparison of the quantum yields of the fluorescence of the polystyrene particles with that in the plant. The quantum yield of fluorescence in vivo is about 0.01 (Rabinowitch, 1956, p. 1967). If we assume that the quantum yield of pheophytin fluorescence on the 0.0014 PS particles is the same as in benzene, 0.175 (Weber and Teale, 1947), then the yield on the 0.2 PS would be 0.007 and on the 1.16 PS would be only 0.0015. Thus at a pigment concentration similar to that in the chloroplast, the coated particles have a similar fluorescence yield, but fully coated particles with a larger proportion of aggregates on their surface have a much lower fluorescence yield.

It is possible that the pigment distribution in vivo is more structured than the random distribution on the coated particles. The other components of the chloroplast may contribute to this structure by dispersing

the pigment molecules more uniformly. Under these conditions, there would be fewer aggregates at a given surface concentration than is obtained on the particles.

The studies of the fluorescence quenching of the polystyrene particles with increasing surface concentration of pheophytin are particularly interesting when compared with studies of the changes of *in vivo* fluorescence during greening (Goedheer, 1961a). Goedheer found that the quantum yield of fluorescence of etiolated bean leaves immediately after being placed in the light was about that of chlorophyll in methanol solution, i.e., there was no quenching of the fluorescence at very low *in vivo* concentrations. After about 6 hours in the light, the fluorescence yield had dropped to about 10% of the initial value, although the chlorophyll concentration had risen to only 10% of the level in the green leaf. Further increases in chlorophyll concentration produced very little decrease in the fluorescence yield.

Goedheer proposed that the above described behavior was explained by a decrease in the interchlorophyll distance during the greening process (i.e., a filling in of the spaces between the chlorophyll molecules by more molecules) rather than by a production of more groups of molecules, there being a few of these groups present a low amounts of chlorophyll. He acknowledged that a combination of these two processes could occur, but apparently did not think his data provided evidence that such was the case.

The behavior at low chlorophyll concentrations is similar to the decay of the fluorescence on the low coverage polystyrene particles with increasing pheophytin concentration. However, the data on the polystyrene shows that fluorescence continues to be quenched as larger aggregates are formed. In order to maintain the fluorescence at 10% of the maximum, the traps or quenchers, *in vivo*, would also have to be somewhat fluorescent. If there werea number of relatively large aggregates present in the plant, the fluorescence might be quenched before the energy could reach the traps and this would reduce the yield even more. This suggests that there might be a limit on the maximum concentration in the plant, and that Goedheer's results might be better explained by a combination of his two hypotheses.

It is tempting to consider the pheophytin dimers on the polystyrene particles as models or analogues for the photochemical reaction sites in

the plant. In addition, the 0.2 PS particles seem to be a particularly good model for the energy-collecting system of the chloroplast (as described in the introduction), since they have both reaction sites (the dimers) and the associated nonreacting, energy-absorbing pigment molecules (monomers). These particles are weakly fluorescent, indicating that the absorbed energy is transferred with very high efficiency, and they are relatively active. The fact that we can observe significant amounts of energy transfer on the particles at surface concentrations of pigment similar to those in the plant and that this transfer can be explained by the resonance transfer theory gives support to the argument that energy transfer in the plant occurs by a similar mechanism, rather than migration through a crystalline pigment lattice.

Brody and Brody (1961) have proposed that one of the reactive forms of chlorophyll *in vivo* is present as a dimer. This hypothesis was based on a comparison of fluorescence spectra of concentrated chlorophyll solutions at low temperatures, in which aggregation occurs, with fluorescence spectra of plant material (mainly algae). They have attributed a fluorescence component occurring at 720 mμ *in vivo* as being due to the dimer, primarily because it occurs at wavelengths longer than most of the chlorophyll, which they assume to be monomeric. Butler (1961) has also observed this fluorescence and associated it with a component which absorbs at about 705 mμ. (This, in fact, is probably the P700 of Kok and Hoch, 1961.) Butler proposed that this long-wavelength absorbing form was complexed with cytochrome and acts as a reaction center in the plant. The suggestions of Butler and Brody and Brody are not incompatible, since I suggested above that the photoreactive species would be closely associated (complexes) with other reactants at the reaction site. Additional support that the component responsible for the 720 mμ fluorescence is necessary in photosynthesis was provided by Goedheer (1961b) who showed that increase in photosynthetic activity during greening paralleled the increase in the 720 mμ fluorescence component rather than the increase in total chlorophyll concentration.

Since the photoreactive chlorophyll molecules are associated with other molecules at the reaction site, it is not necessary, *a priori*, to assume that they are also present as dimers. However, one property of the pheophytin dimers appears particularly attractive, and suggests that the dimers could have a specific role. Although the dimers are relatively

nonfluorescent, they are still quite active. I have noted in chapter III that the photoreactive state is the triplet state and that it is most likely derived from the vibrationally-equilibrated excited singlet state or fluorescent state. The weak fluorescence of the dimers suggests that they are relatively short-lived, i.e., the energy of the excited singlet is degraded by other processes before it can be emitted as fluorescence. It would thus be expected that the fraction of molecules converted to the triplet state would be lower than in the monomers, since the excited singlet state does not last as long. Either the singlet-triplet crossing-over is enhanced in the dimers (which would be one cause of the lowered fluorescence) or the triplet state of the dimer is more reactive than the monomer. In the first case the observed dimer activity is somewhat low because although more energy gets into the triplet state, the dimer cannot use the energy as effectively as the monomer. In the second case, the opposite holds: the energy can be used more effectively by the dimer, but net activity is lower because of the smaller number of molecules that reach the triplet state. If, in the first case, the surrounding reactants can react more effectively with the dimer triplet than in the model reaction, or in the second case, interaction with the surroundings would increase the singlet-triplet crossing in the dimers, these molecules would be much more effective in the photosynthetic reactions than the monomers. This possibility indicates that more studies should be performed on the photochemical properties of the dimers.

The above studies have concentrated on the polystyrene particles because of their similarity to the photosynthetic apparatus. Aside from noting the specific effect of the surface, I do not feel that the zeolite particles are a useful tool for studying the chemistry of the photosynthetic pigments. The relatively low fluorescence on the zeolite particles suggests that these pigments cannot be associated with a purely polar environment for maximal efficiency. Specifically, it may mean that the bulk of the chlorophyll in the plant is associated, at least to some extent, with the chloroplast lipids as well as proteins.

As suggested above, the polystyrene particles appear to be a good model for at least a portion of the photosynthetic apparatus. In summary, the two most important properties of the model are that studies can be made at concentrations similar to that in the plant and that the absorbed light energy can be transferred to a photochemical reaction center.

Continued studies of these particle systems should lead to further understanding of the mechanisms of the photosynthetic reactions. The major objective of such studies should be a photochemical reaction of chlorophyll which is able to store energy with high quantum efficiency. I have frequently mentioned in this discussion that the photoreactive molecules *in vivo* are associated with other chloroplast components. Thus, it would appear that some of the first experiments to try involve the adsorption of small amounts of these materials onto the particle surface along with the pheophytin. (It should be noted that by more careful manipulation during the adsorption process, specifically the removal of oxygen and control of pH, one should be able to adsorb chlorophyll to the polystyrene particles without degradation.) Among the different materials are those thought to be associated with the photoreactive chlorophyll *in vivo*: cytochromes (Butler, 1961), plastoquinone (Weikard, Muller and Witt, 1963), and carotenoids. In addition, some of the other known photochemical reactions of chlorophyll in solution (Livingston, 1960) could be studied with the coated particles.

It would also appear fruitful to explore other types of particles as potential adsorbents. Here, semiconducting particles would be of great interest in view of current theories on semiconductor processes in photosynthesis (see Calvin, 1961). Nelson (1957) has observed photoconductivity in crystals of methyl chlorophyllide. He notes, however, that the phytol on chlorophyll itself interferes with the ordering of the molecules and makes interpretation of similar experiments on chlorophyll difficult. In addition, the lack of evidence for large aggregates of chlorophyll *in vivo* argues against any semiconductor effects of chlorophyll, per se, in the plant. But if the chlorophyll were associated with a surface which was capable of donating or accepting electrons, some of its photochemical reactions might become more efficient. Other particle types which could be tried would be those which were of polarity in between the non-polar polystyrene and the ionic zeolite particles. For example, one might use polymer particles made up of water-soluble but nonionic subunits. Such studies would give more information as to the role of surface polarity in pigment aggregation as well as photochemistry.

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