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THE USE OF A NITROXIDE RADICAL AS A TEST REAGENT
OF THE PRIMARY PROCESSES OF PHOTOSYNTHESIS

Gerald Aloysius Corker
(Ph. D. Thesis)

September 1967

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UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

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ABSTRACT

The presence of photo-induced paramagnetic species in photosynthetic organisms was demonstrated over ten years ago. However, the identification of these species and their connection with the processes of primary quantum conversion remain elusive even though numerous approaches have been applied to this problem. This work reports an attempt at identifying these species using ditertiarybutylnitroxide, a stable free radical, as a test reagent.

Spinach chloroplasts sensitize a photo-reduction of the nitroxide to the corresponding hydroxylamine. Oxygen is produced from H_2O concurrently with the reduction in a ratio of 1 mole of oxygen evolved to ca. 4 moles of the nitroxide reduced. The reduction appears to be coupled with photosystem II in the chloroplasts. Also, the following reagents inhibit (or partially inhibit) the reaction: 1) 3-(3,4-dichlorophenyl)-1, 1-dimethylurea, 2) 2,6-dichlorophenol-indophenol (reduced or oxidized form), 3) salicylaldehyde, and 4) potassium ferricyanide.

The photo-reduction, which is shown to occur with fresh chloroplasts, fresh fragments or aged fragments, is accompanied by a dark reaction which also involves a reduction of the nitroxide.

The reduced form of the nitroxide, di-*tert*-butylhydroxylamine, undergoes a photo-oxidation with spinach chloroplasts. It is proposed that the amount of the hydroxylamine photo-oxidized is controlled by the amount of an indigenous oxidant present in the chloroplasts and that this oxidant, though coupled to electron transport, is not an actual participant and is irreversibly reduced.

A product which could be ascribed to a coupling reaction between the nitroxide and the radical species which gives rise to the photo-induced EPR signal in spinach chloroplasts was not detected, even using radioactive tracer methods. The nitroxide is not suitable as a trapping agent.

The nitroxide is oxidized by potassium ferricyanide to isobutene and 2-methyl-2-nitrosopropane. The reaction is complicated by a photochemical degradation of the nitroso compound.

A reversible photo-reaction between the nitroxide and various quinones appears to involve the formation of a di-peroxide type molecule resulting from the interaction of the nitroxide with the triplet states of the quinones.

Also reported in this work is a discussion of a widely accepted mechanistic view of photosynthesis, a review of the experimental electron paramagnetic resonance observations obtained with photosynthetic organisms, a brief investigation of charge-transfer complexes formed between pentafluorobenzonitrile and hexafluorobenzene with the organic bases, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and dimethylaniline, and a discussion of an improved method for the detection of tritium combining the techniques of thin-layer chromatography, scintillation counting and radioautography.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Melvin Calvin, under whose guidance the work reported in this thesis was conducted. His advice, demanding criticisms and unrestrained scientific curiosity have made my years as a graduate student both fruitful and interesting. I would also like to thank him for making my residence in the Laboratory of Chemical Biodynamics possible.

I shall always be indebted to Dr. Melvin Klein for his continual interest, advice and encouragement. The invaluable discussions with him greatly extended my knowledge of the principles of spectroscopy.

It was a privilege to have associated with Drs. Didier Lafont and Albert Bobst, with whom I collaborated in the studies reported in Chapter III, and with Dr. Thomas Dehner, with whom I collaborated in some of the work described in Chapter IV.

To the staff, visitors and students of the Laboratory of Chemical Biodynamics, and in particular Drs. Kenneth Sauer, David Samuel, Marianne Byrn Caple, and Mrs. Johanna Onffroy, I would like to extend my thanks.

To my family and Mrs. Pam Hulse goes my deep appreciation for their many hours of patient listening and their unwavering encouragement.

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PREFACE

Sufficient experimental data to justify proposing a mechanism for the primary quantum conversion act of photosynthesis existed as early as 1955. At that time, Bradley and Calvin¹ proposed a mechanism involving an ordered array of chlorophyll molecules. Their suggested sequence of events involved the absorption of light, resulting in the formation of chlorophyll singlet states which, by intersystem crossing, were converted into chlorophyll triplet states. With the energy in this form, an electronic ionization occurred leading to trapped electrons and holes--the reductants and oxidants, respectively--required for photosynthesis.

According to this scheme, one would expect to find at least three different types of species exhibiting paramagnetism during photosynthesis--the triplet state of chlorophyll, the trapped electrons and holes, and organic free radicals produced by enzymatic reactions. Although photo-induced paramagnetic species have been detected in photosynthetic organisms by electron paramagnetic resonance (EPR) techniques, little success has been achieved in identifying the species exhibiting this paramagnetism. The present work is an attempt at identifying these species using di-tert-butyl nitroxide (DTBN) as a test reagent.

This work is presented in four chapters. The first of these presents a discussion of the current mechanistic view of photosynthesis, a review of the EPR observations obtained with photosynthetic organisms and a statement and analysis of the approach used in attempting to identify the photo-induced paramagnetic species in

photosynthetic organisms. The view of photosynthesis discussed here was used as a model for some experiments reported in this work.

Chapter II contains the results of brief investigations of the interaction of DTBN with various chemicals, knowledge of which was required for this work. Experiments conducted with DTBN and photosynthetic materials are reported in Chapter III. Included are kinetic experiments which relate a photochemical destruction of DTBN with photosynthetic oxygen production and analysis of the products of this destruction. Conclusions and suggested continued work are presented at the end of Chapter III.

Chapter IV contains the results of investigations of charge transfer complexes and of a method for detecting tritium using thin-layer chromatography, scintillation techniques and radioautography.

The method of preparation and the properties of DTBN (including the preparation of ^{14}C -labeled DTBN), a list of materials, their sources and methods used to prepare and/or purify them, and descriptions of the instruments used for the work, are contained in the appendix. Also included in the appendix is a brief analysis of using EPR technique for kinetic studies.

CONTENTS

	<u>Page</u>
CHAPTER I: INTRODUCTION	1
Photosynthesis	1
Electron Paramagnetic Resonance	16
Statement of Problem	29
CHAPTER II: CHECK OF STABILITY OF DTBN	33
Experimental	33
Results and Discussion	35
a) Reaction with semiquinone	38
b) Reaction with $K_3Fe(CN)_6$	41
c) Reaction with Quinones	47
CHAPTER III: REACTION OF DTBN WITH PHOTOSYNTHETIC ORGANISMS	68
Kinetic Experiments	68
a) Experimental	69
b) Results and Discussion	82
c) Summary: Kinetic experiments	95
Measurement of Oxygen Evolution	96
a) Experimental	97
b) Results and Discussion	99
c) Summary: Measurements of oxygen evolutions	120
Product Analysis	120
a) Experimental	121
b) Results and Discussion	123
c) Summary: Product analysis	140
Discussion, Chapter III	141

	<u>Page</u>
CHAPTER IV: MISCELLANEOUS TOPICS	149
Charge Transfer Complexes	149
a) Introduction	149
b) Experimental	152
c) Results and Discussion	154
d) Summary of CTC	171
Tritium Detection	172
a) Introduction	172
b) Method	173
c) Experimental	173
d) Discussion	176
APPENDIX I	177
Sources of Error in Using EPR for Kinetics	177
APPENDIX II	181
A) Preparation and Properties of DTBN	181
B) Preparation of Radioactive DTBN	190
APPENDIX III	196
A) Equipment	196
B) Chemicals	199
C) List of Abbreviations	201
REFERENCES	203

CHAPTER I

Introduction

This chapter is divided into three sections. The first of these discusses superficially the development of a widely held view on the mechanism of photosynthesis. No attempt at annotation is made. Nor is a complete historical development of this view attempted. Instead, listed in Table I are the investigators who have made major contributions to the development of the current view.

The second section describes the observations obtained with photosynthetic organisms using the technique of electron paramagnetic resonance (EPR) spectroscopy. No discussion of the principles, instruments or applications of EPR is given since numerous publications describe these topics.¹⁻⁵ Knowledge of the principles of EPR required to understand the remainder of this thesis is given in the appendix.

The third section discusses the problem toward which the work in this thesis is addressed, the approach to be used, and the problems anticipated with the approach.

Photosynthesis

An overall view of photosynthesis, the complex process by which plants, algae and certain bacteria convert electromagnetic energy into chemical energy in the form of vital organic materials, is ordinarily abbreviated, using equation 1. This equation, summarizing experiments conducted in the late seventeen and early eighteen hundreds, says nothing about the intricate manner in which these organisms perform this important reaction. However, this equation is based upon observations

Table I

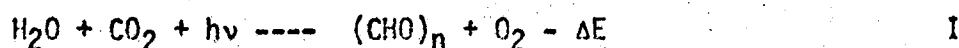
Major Contributions to the Current View of Photosynthetic Electron Transport

Date	Contributor	Contribution	Réference
1777	Priestly, J.	Showed that green plants could change toxic atmosphere to one which supports oxidation	6
1779	Ingen-House, J.	Showed that sunlight is required and O_2 is actually evolved	7
1782	Senebier, J.	Showed that toxic material is actually removed by plant	8
1804	de Saussure, T.	Showed that water is involved in process	9
1845	Mayer, R. M.	Recognized that the process is efficient in energy storage	10
1864	Sachs, J. V.	Demonstrated starch accumulation in leaves during process	11
1881	Engleman, T. W.	Showed process occurs in chloroplasts and chlorophyll is required	12
1883	Engleman, T. W.	Discovered photosynthetic bacteria	13
1887	Winogradsky, S.	Discovered chemosynthetic bacteria	14
1905	Blackman, F. F.	Demonstrated that overall process consists of both light and dark reactions	15
1931	Van Niel, C. B.	Pointed out similarities between bacterial and plant photosynthesis: proposed that photo-act involves H_2O , not CO_2	16
1932	Emerson, R., and Arnold, A.	Separated light and dark reactions in time; introduced concept of photosynthetic unit	17

Table I. (Cont.)

Date	Contributor	Contribution	Reference
1939	Hill, R.	Observed O_2 evolution with isolated chloroplasts	18
1941	Ruben, S. <u>et al.</u>	Demonstrated that O_2 evolved originates from H_2O , not CO_2	19
1943	Emerson, R. and Lewis, C. M.	Observed "red drop phenomenon" of quantum yield	20
1954	Arnon, D. I. <u>et al.</u>	Obtained CO_2 fixation and ATP and $NADPH_2$ formation by isolated chloroplast ²	21
1957	Calvin, M. and Bassham, J. A.	Mapped fixation pathway of CO_2 ; showed that sugar phosphates are initial products from CO_2 ; defined ATP and $NADPH_2$ requirements.	22
1957	Emerson, R. <u>et al.</u>	Observed "enhancement phenomenon"	23
1958	Trebst, A. V. <u>et al.</u>	Showed that formation of ATP and $NADPH_2$ associated with light and membrane of chloroplast; CO_2 fixation associated with soluble enzyme	24
1960	Hill, R. and Bendall, F. F.	Proposed two photo-act model of photosynthetic electron transfer in green plants	25

that green plants when subjected to sunlight assimilate carbon dioxide and water and evolve oxygen. These observations gave rise to a mechanistic view that sunlight splits CO_2 into its component atoms, the oxygen atoms being liberated as O_2 while the carbon and water were used in a synthesis of the organisms' body material.⁷ This view prevailed through the nineteenth century, with the additional observation that the cellular sites of O_2 evolution were the chloroplasts, the chlorophyll-containing organs of the leaves.¹²



The discovery of photosynthetic bacteria,¹³ organisms which also utilize sunlight to assimilate CO_2 or organic substrates but without the accompanied evolution of O_2 , and chemosynthetic bacteria,¹⁴ organisms which also assimilate CO_2 but without the use of sunlight and also without the evolution of O_2 , should have raised doubts about the validity of this mechanistic view. However, it was not until thirty years after the turn of this century, when van Niel¹⁶ using a comparative approach in a study of photosynthetic green and purple-sulfur bacteria proposed that light promoted a photolysis of water and not a splitting of CO_2 , that the first serious objection was raised against the early view.

According to van Niel, sunlight is used both in green plants and bacteria to effect a redox reaction involving water, resulting in the formation of a reductant and an oxidant. The reductant ultimately reduces the carbon source while the oxidant leads to oxygen evolution in green materials but interacts with another moiety in bacteria to

produce water again and another product, for example, sulfur.

Van Niel's prediction that the oxygen liberated during green plant photosynthesis originates from the water and not the CO_2 was substantiated experimentally by Rubin and workers,¹⁹ who conducted experiments with algae and CO_2 and H_2O containing ^{18}O .

The observations of Blackmann¹⁵ and those of Emerson and Arnold¹⁷ are also consistent with van Niel's concept of the photo-act. In a study with green plants, Blackmann, by varying the light intensity, CO_2 concentration and temperature, demonstrated that overall photosynthesis consists of two types of events: photochemical events which are light intensity dependent, but temperature insensitive; and chemical events involving the CO_2 , which are light intensity independent, but temperature sensitive.

Emerson and Arnold measured the yields of photosynthetic oxygen production from algae using saturating light in brief flashes and showed that the two types of events found by Blackmann could be separated in time. They also observed that approximately one CO_2 molecule was assimilated per 2500 chlorophyll a molecules present when saturating light was used, an observation which led them to propose the concept of a photosynthetic unit which will be discussed below.

These observations suggest (as proposed by van Niel) that CO_2 is not involved in the photochemical events but is assimilated by a dark, temperature sensitive reaction involving a product generated in the photochemical reactions. In fact, Calvin and co-workers²² have since elucidated the reactions by which CO_2 is accepted by ribulose diphosphate (RuDP), an organic compound found within green plants and algae,

and is transformed into sugars with regeneration of the initial CO_2 acceptor. Although the CO_2 fixing process consists of dark, enzymic reactions, the reducing power, the reduced form of nicotinamide adenosine dinucleotide phosphate (NADPH_2) and the energy source, adenosine triphosphate (ATP), both of which are required for the assimilation of CO_2 , are generated by or coupled intimately with the photochemical events.

Bacteria display a great diversity both in regard to the carbon source used in the synthesis of their body materials and in regard to the primary source of electrons to reduce the carbon.²⁶ Some strains are known which assimilate CO_2 . However, no strain is known in which the fixation of the carbon source is accompanied by the evolution of O_2 . Instead, one of many organic or inorganic compounds other than water is oxidized. Also, although carbon fixation occurs by dark enzymatic reactions and is accompanied by light dependent formation of ATP,²⁹ the requirement for a light produced reducing agent for utilization in carbon fixation is not a universal requirement.²⁷ Instead, metabolic products are used as reducing agents.

Since the assimilation of the carbon source during photosynthesis appears to involve orthodox biochemistry, many current investigations focus upon the photochemical events, hopefully to explain how ATP, NADPH_2 and O_2 are produced, what chemical products result initially from the photo-act, what role is played by the pigments, and what mechanism enables photosynthetic organisms to transform 35 kcal quanta into chemical energy with an efficiency of 35% or better.²⁸

The investigations of these photochemical events have been possible largely through the use of subcellular organelles of photosynthetic organisms, namely, chloroplasts from green materials and chromatophores from bacterial cells. Although the level of activity, as based upon their chlorophyll content, is generally much lower in these units than that displayed by the organisms from which they are isolated,^{21,29,30} they perform the same photochemical reactions.

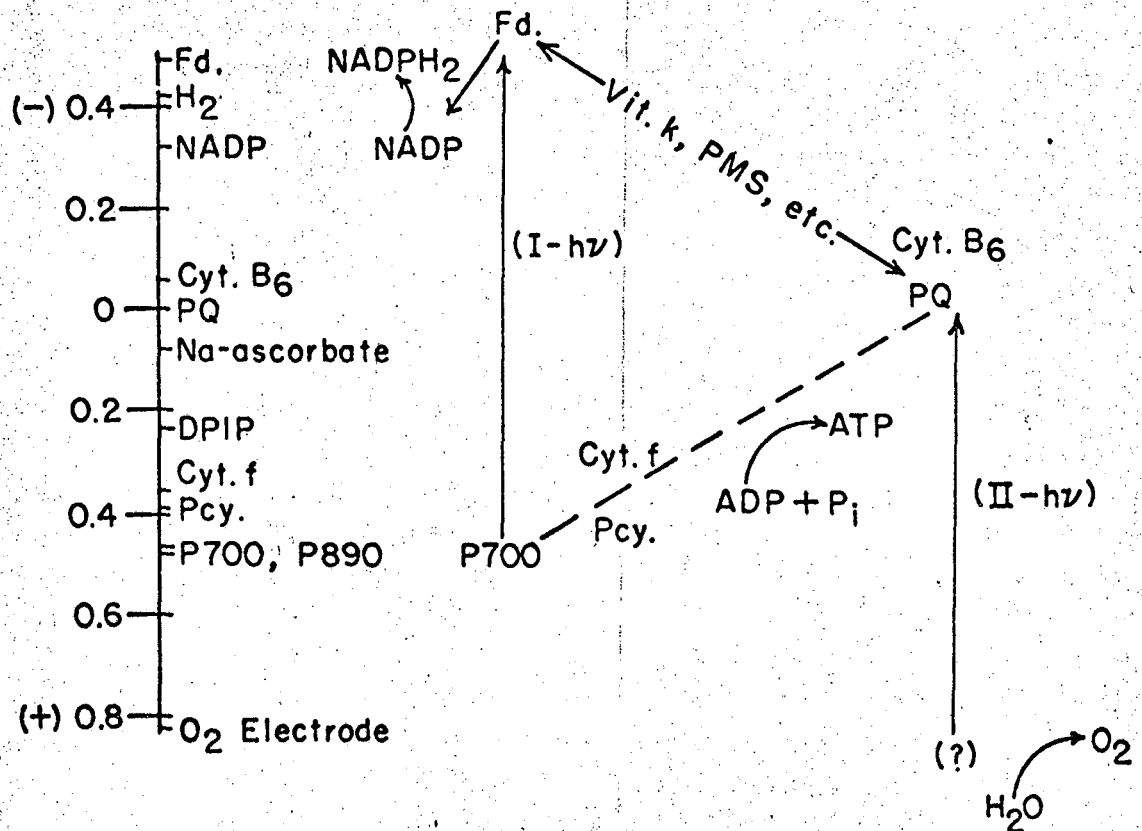
In fact, a major advance in photosynthetic research was made by Hill¹⁸ when he demonstrated that chloroplasts promote the evolution of oxygen if subjected to illumination and supplied with a suitable electron acceptor such as ferric ion. Since then, a variety of artificial oxidants besides the ferric ion have been found to be effective in promoting chloroplasts and light catalyzed O_2 evolution. This process is called the "Hill reaction". Hill was unable to show that CO_2 would function as the oxidant. However, other workers²¹ have since demonstrated that isolated chloroplasts are the sites of carbon fixation and of light dependent formation of the co-factors, $NADPH_2$ and ATP. Thus, chloroplasts are able to reduce NADP, generate ATP, evolve oxygen and assimilate CO_2 .

In order to effect these reactions, electrons from water are raised to a negative redox potential ($E = -0.43$) high enough to reduce ferredoxin.⁴³ The formation of ATP is coupled with this electron flow. The complete process, including the formation of ATP, is commonly referred to as photosynthetic electron transport and occurs in specialized units (photosynthetic units) contained within the chloroplast. Such units exist also in chromatophores isolated from bacterial cells.³²

The photosynthetic unit is a concept first proposed by Emerson and Arnold¹⁷ and since expanded upon by other workers.³³ This unit is an ordered collection of lipid, protein and pigment molecules functioning together to collect and transfer light quanta to specific reaction centers where the quanta promote redox reactions resulting in the formation and storage of a stable chemical potential, a process called primary quantum conversion. The reaction centers are the sites of electron transport and contain chlorophyll molecules complexed in a manner which makes them distinct from the bulk of the chlorophyll present.³⁴⁻³⁷ These chlorophyll complexes are associated with other types of molecules, for example, cytochromes and quinones, which play a role in photosynthetic electron transport.^{25,38,39,36}

Van Niel's concept of the photo-act has been incorporated into a scheme, diagrammed in Figure 1, which represents the most widely accepted view of photosynthetic electron transport in green plants and algae. This scheme resulted from the use of comparative biochemistry because two cytochromes, f and b₆, are found exclusively in the photosynthetic apparatus. The possibility that these play a role in an electron transfer process analogous to the role played by cytochromes in oxidative electron transport,⁴⁰ prompted Hill and Bendall²⁵ to propose a scheme basically similar to the one shown in Figure 1.

The two arrows in Figure 1 represent two light-acts supposedly occurring in two different pigment systems (called photosystems I and II) and driven by separate wavelengths of light. Photosystem II is driven by 650 mμ light which promotes the oxidation of water⁴¹ and the reduction of some unknown, possibly one of the cytochromes or a plastoquinone, which also has been shown to be involved in the electron



XBL 677-4549

Figure 1. Schematic diagram showing the approximate redox relationships of some species involved in green plant photosynthetic electron transport.

transfer process.³⁹ The pigment composition of system II is subject to speculation, however; an accessory pigment, either chlorophyll b or phycobillin, and a part of the chlorophyll a are thought to be involved.

Photosystem I is operated by light of 680 mμ, which brings about the reduction of NADP by a sequence of reactions involving an iron containing protein, called ferredoxin, and a flavoprotein of unknown structure.^{42,43} This 680 mμ light causes also the oxidation of both cytochrome f and a pigment complex contained in this photosystem.³⁴ The pigment contained in this complex (termed P700) is a chlorophyll a molecule. The moiety (or moieties) with which it is complexed is unknown.

The dashed line in the figure represents an electron transport chain connecting the primary oxidant of photosystem II with the primary reductant of photosystem I. Little is known regarding the composition of this chain except that P700, the two cytochromes, plastocyanin and a plastoquinone are involved. At some point along this chain, as the electron drops from the redox level of the primary oxidant of system II to the redox level of P700, the energy loss is used to phosphorylate ADP.

An abundance of experimental data can be explained by this scheme. Most significant among these are the observations of Emerson and co-workers,^{20,23} who in their studies of the wavelength dependence of the quantum yield of photosynthesis observed that the efficiency decreased at excitation wavelengths greater than 670 mμ even though light absorption by the cellular pigments occurs further into the red

(red drop phenomenon) and that the low quantum yield obtained by excitation with light in the long wavelength spectral region could be enhanced if the system was simultaneously exposed to light of shorter wavelength (enhancement). The yield observed by excitation with two wavelengths was greater than the sum of the yields observed by excitation with each wavelength separately. These observations suggest that efficient photosynthetic activity in plants and algae requires the simultaneous excitation of the photosynthetic apparatus by two wavelengths of light as proposed in the scheme depicted in Figure 1.

Also, photo-induced oxidations and reductions of the components shown on the dashed line in Figure 1 are manifested by absorbancy changes. Far red light promotes the oxidation of cytochrome *f*,^{41,44} P700^{34c} and plastoquinone,⁴⁵ while shorter wavelength light causes their reduction. The reductions are inhibited by dichlorophenyl-dimethylurea (DCMU), a potent inhibitor of photosynthetic oxygen evolution. The oxidation of P700 and the reduction of NADP can be effected with far red light with chloroplast poisoned with DCMU.⁴⁶ These observations are all consistent with the scheme shown in Figure 1 and an interpretation that DCMU stops electron flow between H₂O and light-act II.

Two types of photosynthetic phosphorylation (cyclic and non-cyclic) have been demonstrated with chloroplasts.⁴⁷ Non-cyclic phosphorylation is coupled to the reduction of a Hill oxidant or NADP and the oxidation of H₂O. This type of phosphorylation involves both photosystems and the electron transport chain, whereas cyclic phosphorylation, which

requires the presence of an added co-factor such as phenazine methosulfate or vitamin k but proceeds in the absence of oxygen evolution, involves only photosystem I and the transport chain. The added co-factor interacts with the primary oxidants of both photosystems, therefore establishing a cyclic pathway for electrons involving the electron transport chain and one photo-act.

Arnon⁴⁸ proposes by analogy to dark phosphorylation of glycolysis and respiration that photosynthetic phosphorylation is coupled with the electron transport chain connecting the two photosystems, the energy for formation of the pyrophosphate bond being obtained as the electron drops from the redox level of the primary oxidant of system II to the primary reductant of system I.

Although bacterial and green plant photosynthesis occur in different organisms and differ greatly in their synthetic chemistry, these organisms have many features in common, especially within their photosynthetic apparatus. They also exhibit some marked differences. Some of these differences and similarities are enumerated in Table II. The observations listed in Table II suggest that bacterial photosynthetic electron transport is essentially a simplification of the system operating in green plants and algae. Schemes which represent two types of bacterial photosynthesis are presented in Figure 2.

If the mechanisms of photosynthetic electron transport, as depicted in Figures 1 and 2, represent a true picture of the events occurring during photosynthesis, and if the photo-induced redox reactions and accompanying electron transport processes occur via one

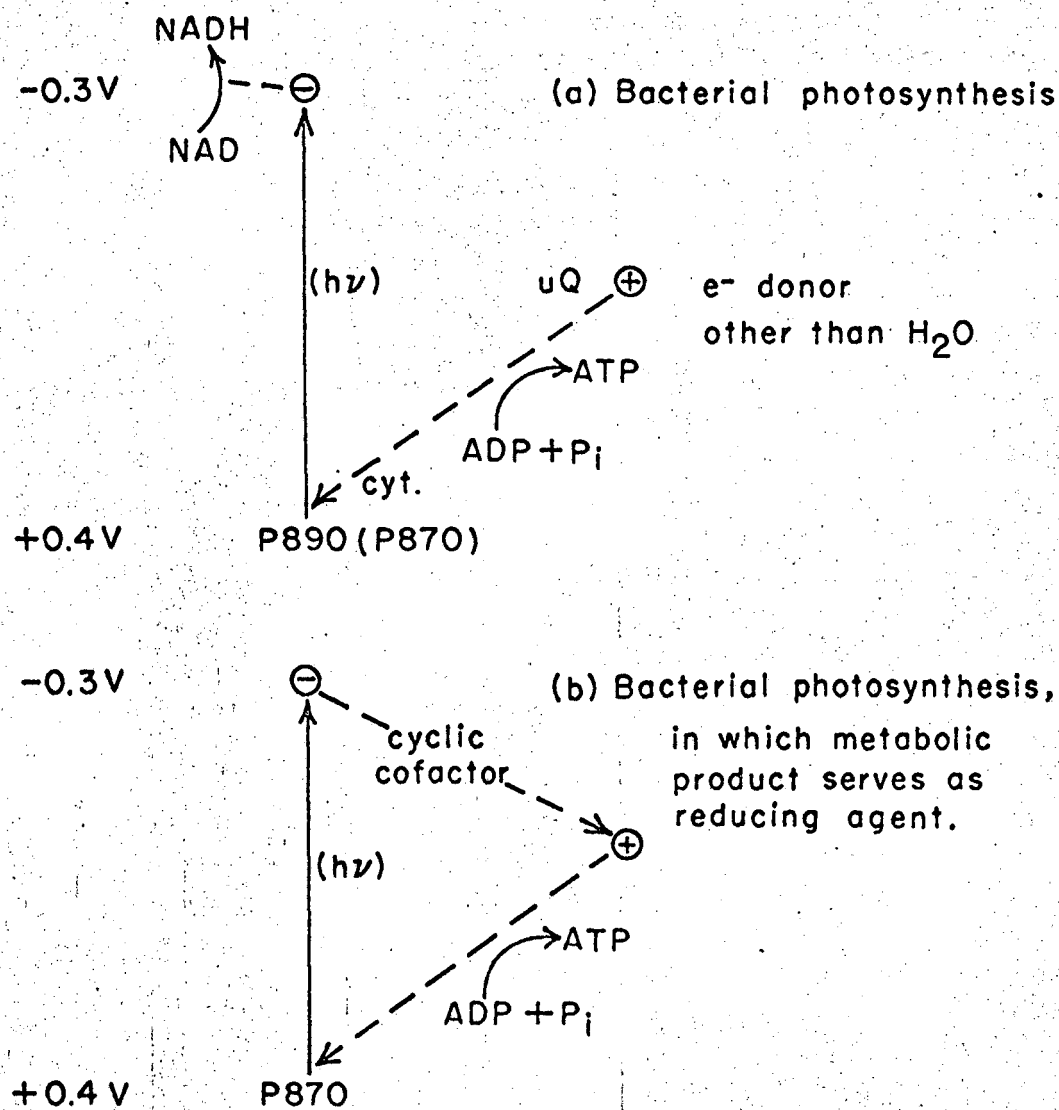
Table II

Comparison of Photosynthetic Bacteria to Plants and Algae

Characteristics in common	Reference	Differences	Reference
1) Photosynthetic units exist in both	17,32,33	1) Plants and algae evolve O_2 whereas bacteria do not	18,26
2) Chlorophyll pigment necessary for photochemical function of organism	56	2) Bacteria display greater variation in their carbon source for synthesis	26,57
3) Chlorophyll pigment exist in different micro environments within organism	34,35, 36,37	3) Chlorophyll pigment in bacteria absorbs at longer wavelength than that found in plants and algae (less energetic quanta required for synthesis)	56
4) Chlorophyll pigment complex undergoes rapid, reversible changes upon illumination or treatment with chemical oxidants; change associated with a cytochrome	34,35 36,37	4) Chlorophyll pigment in bacteria in reduced state compared to pigments in plants and algae	56
5) Contain carotenoids and quinones which although not identical structurally seem functionally the same	39,56,58	5) Green plants and algae exhibit cooperative light effects; bacteria do not	23,55

Table II (Cont.)

Characteristics in common	Reference	Differences	Reference
6) Contain cytochromes, at least two, with different oxidation potentials	25,38,54	6) Two photoinduced EPR signals found in green plants and algae, one in bacteria	67,81
7) Identical or very similar enzymes serve as reducing agents for carbon source	22,57	7) Ratio of photosynthetic units to the amount of chlorophyll pigment present is higher for bacteria than plants and algae	32,34
8) Photophosphorylation of ADP to ATP occurs in both	21,29		
9) Energy is transferred from the accessory pigments to the chlorophyll complexes	37,56		
10) Light promotes EPR signal of similar spectrum characteristics	67,81		



XBL 677-4548

Figure 2. Schematic diagram showing the approximate redox relationships of some species involved in the electron transfer processes of bacterial photosynthesis.

electron transfer step, one would expect photo-induced free radicals to occur as intermediates during photosynthesis. This expectation was substantiated when Commoner⁶⁷ and Sogo and Calvin⁶² demonstrated the presence of photo-induced free spins in photosynthetic material using an electron paramagnetic resonance (EPR) spectrometer. Since these initial observations, numerous reports dealing with the photo-induced EPR signals present in photosynthetic materials have appeared and will be discussed in the next section of this chapter.

Before ending this section, it should be noted, however, that the scheme proposed by Hill and Bendall is not the only one which visualizes the mechanism of photosynthesis to involve the cooperative action of two distinct light reactions connected in series,^{35,41,49,50,51} although these schemes are basically similar to the Hill-Bendall model. In addition, several investigators, especially Gaffron⁵² and Franck,⁵³ take exception to the series formulation and propose a parallel formulation and an alternating formulation, respectively. However, the scheme shown in Figure 1 is the one used for the work in this thesis.

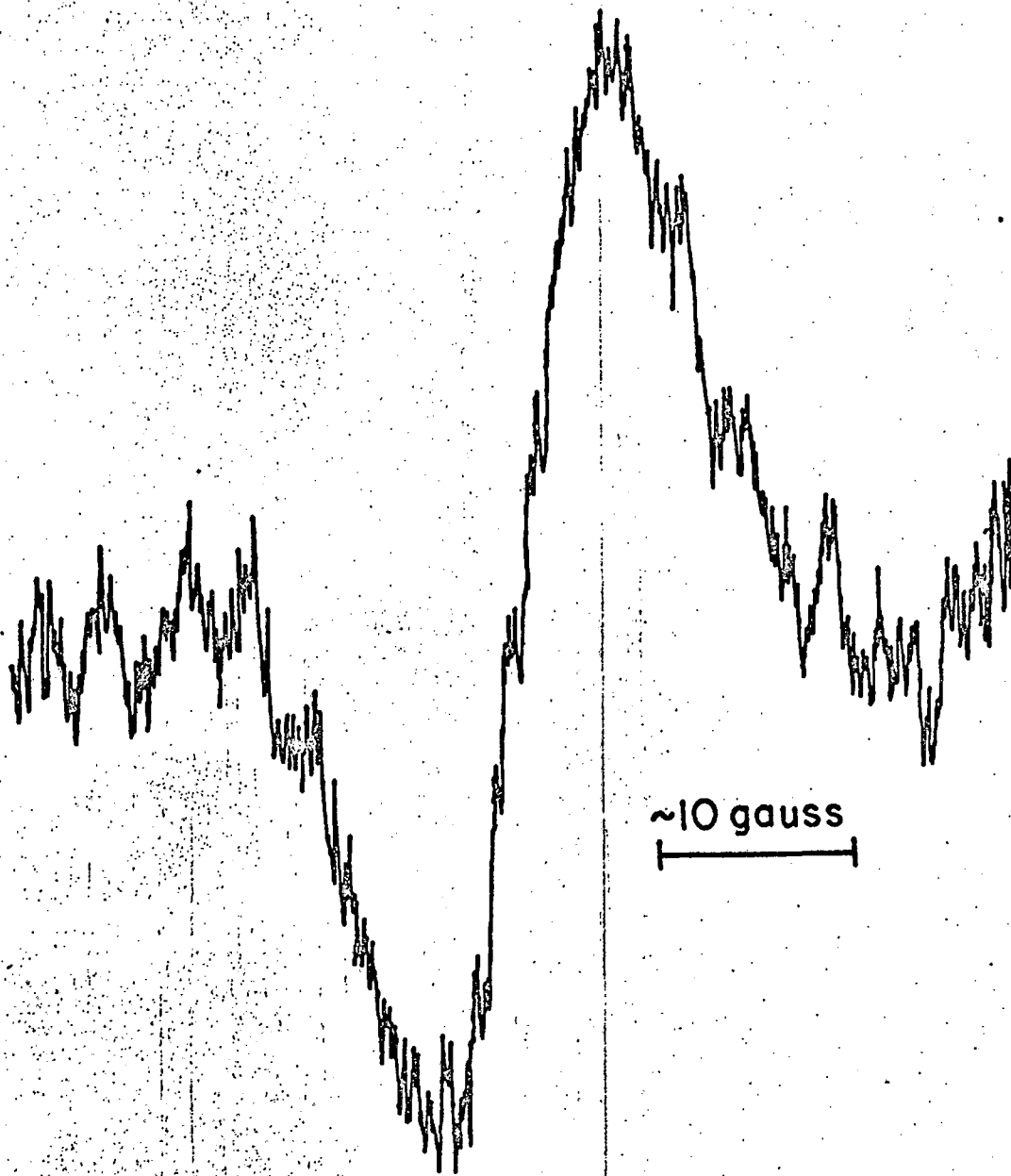
Electron Paramagnetic Resonance

Since Commoner's and Sogo and Calvin's initial observations of the photo-induced EPR signals in photosynthetic material, such signals have been detected in all photosynthetic systems. The samples investigated include intact organisms (green leaves,⁷⁶ whole cell algae⁵⁹ and photosynthetic bacteria⁵⁹), fragments (chloroplasts,⁸⁷ quantasomes⁷⁶ and pigment solutions^{71,79}). Experiments have been conducted both on dry films and on H₂O suspensions of these materials.

Photo-induced EPR signals have been detected in purified chlorophyll a and b.^{95,96,100} Although these observations demonstrate that these compounds could be the species giving rise to the signals found in the photosynthetic organisms, the spectral characteristics of the observed EPR pattern differ from those obtained from the organisms. The work conducted with the purified components will not be included in this discussion.

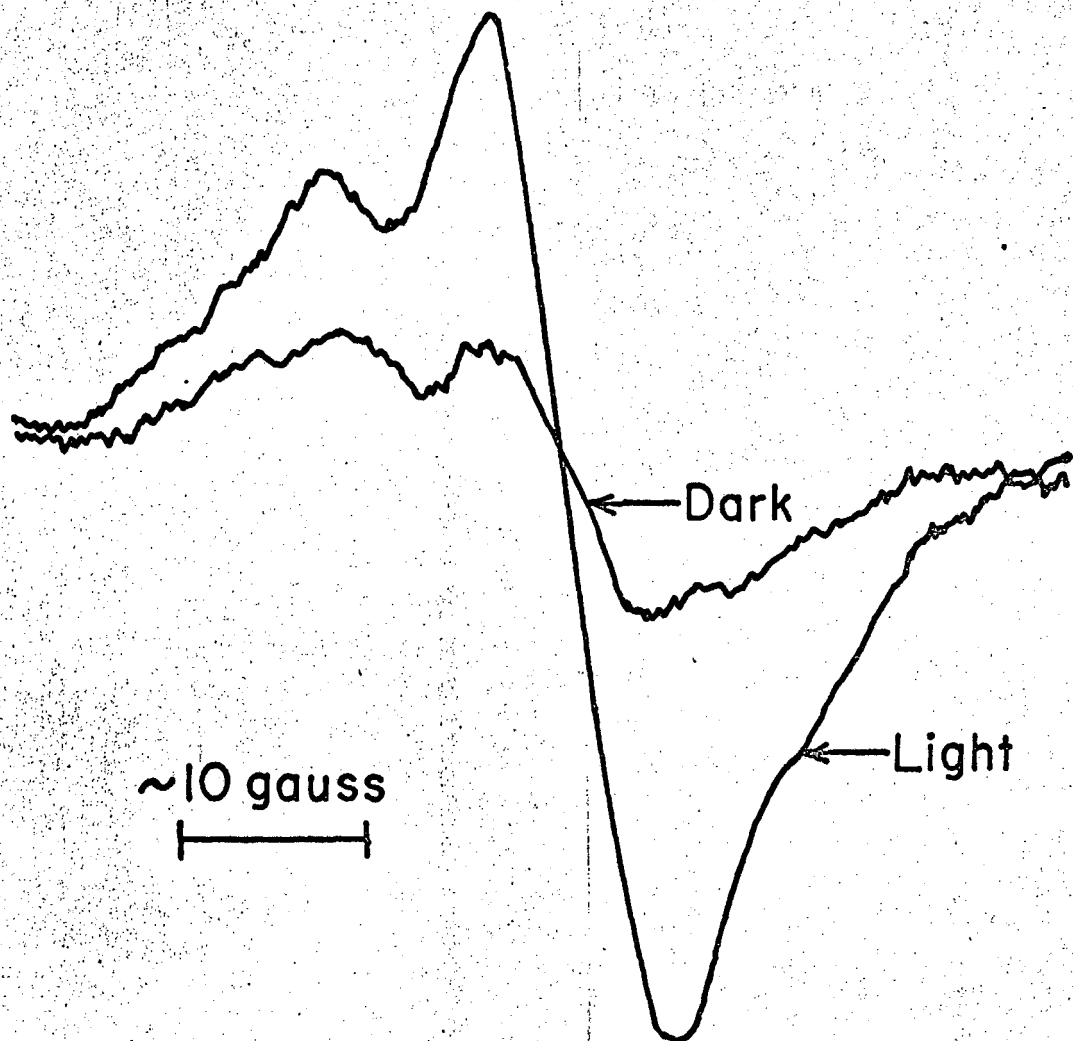
Figure 3 shows a typical light-induced signal obtained with the photosynthetic bacterium Rhodospirillum rubrum. Shown as the first derivative of the absorption versus the strength of the applied magnetic field, the signal consists of a single, non-structured line with a g-value between 2.002 and 2.003 and a linewidth ($\Delta H_{1/2}$) of approximately 11 gauss. A light-induced signal with these same characteristics is found in all photosynthetic bacteria and chromatophores containing bacteriochlorophyll.

The signals found in green plants and algae, which in contrast to photosynthetic bacteria have a capacity to evolve oxygen, can be resolved into two photo-induced signals as shown in Figure 4. The narrower of these two signals, which is generally referred to as signal I, has a g-value, line shape and $\Delta H_{1/2}$ similar to the signal found in bacteria. Not only these spectral characteristics, but the response to light and to temperature of signal I (as is shown in Table III) is so similar to the signal found in bacteria that the two can be equated operationally.



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Figure 3. Photo-induced EPR signal from Rhodospirillum rubrum
illuminated with white light.



(after Calvin and Androes)

XBL 679-6168

Figure 4. Photo-induced and dark signals from spinach chloroplasts illuminated with white light.

The broader signal, which is called signal II, has a g -value between 2.004 and 2.005, a $\Delta H_{1/2}$ between 17-21 gauss and a line shape consisting of a five line hyperfine structure with 5.5 gauss separations⁷⁰ and with intensity ratios of ca. 1:4:6:4:1 indicative of an organic free radical in which the free spin interacts with four chemically equivalent protons.

Unfortunately, the spectral characteristics of these signals yield very little information as to what chemical species give rise to these signals or whether these species play a role in photosynthetic electron transport or primary quantum conversion. These two questions need to be answered if the results of EPR are going to substantiate or distinguish between the various theories put forth to explain the mechanisms of these processes.^{64,102-107} In further attempts to answer these questions, investigators have probed the effect of physical, chemical and biological variations on the signals produced in the various photosynthetic systems. A compilation of the results of many of these investigators is presented in Tables III, IV and V.

Difficulties arise in the analysis of the data in these tables for several reasons. (1) No distinction is made between experiments conducted with intact systems as opposed to those on subunits. However, the bulk of the data were obtained with whole cell algae or bacteria and chloroplasts. Also, the primary differences generally encountered between intact systems and subunits involve the kinetics of formation and decay and therefore the steady-state level of the spins detected. (2) No distinctions are made between the various types of photosynthetic bacteria nor between algae and green plants (and their subunits,

Table III

Effects of Physical Perturbations on EPR Signals of Photosynthetic Materials

Variation	Bacterial Signal	Signal I	Signal II	References
1) Microwave power	1) --	1) Saturates at higher value than signal II	1) Saturates at lower value than signal I	59,60,61
2) Light:				
a) Wavelength	a) Action spectrum corresponds to optical absorption spectra	a) Same as for bacterial signal	a) Same as for signal I	a) 59,60,63-69
b) Intensity	b) Saturates at high intensity	b) Saturates at high intensity	b) Saturates at low intensity comparable to values for photosynthesis	b) 61,70-73
c) Quantum yield	c) Approximately one (1)	c) 0.03	c) --	c) 74,75
3) Temperature:				
a) Above biokinetic range	a) --	a) 60°C (5') enhances signal 100°C (10') destroys signal	a) 60°C (5') destroys signal	a) 73,85
b) 5-30°C	b) Signal increases below 20°C	b) Same	b) Same	b) 71
c) Below 0°C	c) Larger @ -15°C than 25°C Smaller @ -160°	c) Larger @ 25° than @ -150° or -140°	c) Larger @ 25° than @ -140°	c) 59,71,72

Table III (Cont.)

Variation	Bacterial Signal	Signal I	Signal II	References
4) Time:				
a) <u>Room Temp.</u>				
1) Rise	a)1) Fractions of a second	a)1) Fraction of a second	a)1) Fraction of a second	a) 76,72,70,
2) Decay	2) " " " "	" " " "	2) Initial--seconds	71,59,65,
			Complete decay--hours	77,78
b) <u>-150°C</u>				
1) Rise	b)1) " " " "	b)1) Instrument limited	b)1) --	b) 66,71,65,
2) Decay	2) " " " "	2) Irreversible--no decay	2) --	76,77,59

Table IV

Effects of Chemical Perturbations on EPR Signals of Photosynthetic Materials

Variation	Bacterial Signal	Signal I	Signal II	References
1) Substitution of (D) for (H)	1) Causes narrowing of signal--interacting protons not exchangeable	1) Causes signal to decrease in width (H/D ca. 4.3)	1) Causes signal to decrease in width (H/D ca. 2.6)	59,70,79
2) Presence of reduced dyes	2) Increases rate of decay--decreased steady-state concentration	2) Decreased steady-state concentration of signal	2) Decreased steady-state concentration of signal	72,80,81
3) Addition of DCMU	3) --	3) Apparent increase of amplitude--slows rate of decay	3) Effect not discernible	68,82
4) $K_3Fe(CN)_6$ Titration	4) $K_3Fe(CN)_6$ produces signal in dark--redox potential = +0.44 volts	4) $K_3Fe(CN)_6$ produces signal in dark--results suggest two one-electron transfer acts. +0.46.	4) --	66,83
5) Removal of pigments a) -CHL(95%) b) -carotene and quinones	5)a) Signal unchanged b) --	5)a) Signal still present b) No effect on signal	5)a) -- b) --	a) 84,83 b) 85
6) Addition of PMS	6) Destroys signal	6) --	6) --	86

Table V

Effects of Biological Perturbations on EPR Signals of Photosynthetic Materials

Variation	Bacterial Signal	Signal I	Signal II	O ₂ Evolution	References
I) Systems:					
a) Green plants:					
1) Chloroplast	--	Present	Present	Normal	65,70,76,87,88
2) Quantasomes	--	"	Not detectable	Low	59
b) Algae (whole cell)	--	"	Present	Normal	67-71,78,82,87-90
1) Complex P700	--	"	Not detectable	Low	84
2) " P672	--	"	"	"	92
c) Bacteria (whole cell)	Present	--	--	None	59,71,75,78,91
1) Chromatophores	"	--	--	"	59,77,91
II) System Treatments:					
a) Sonication	Present	Present	Not detectable	None	84,92
b) Washing	--	"	Disappears	Decreases	85,87
c) Aging at 0°C	--	"	"	Decreases to 0	85
d) Freezing	Enhanced	Enhanced	Decreased	None	59
III) Mutants:					
a) Lacking green pigments	--	Absent	Absent	None	59,72
b) Lacking CHL. <u>b</u>	--	Present	"	"	60
c) Lacking carotenoids	Present--power saturation diff.	--	--	--	59
d) Etiolated	--	Absent	Absent	None	59,67,88
e) Etiolated exposed to light	--	Increases w/[CHL]	--	Correlates w/[CHL]	59,88
f) No O ₂ evolution but normal pigments	--	Present	Absent	None	89

Table V (Cont.)

Variation	Bacterial Signal	Signal I	Signal II	O ₂ Evolution	References
g) No CO ₂ fixation but normal pigments	—	#8 - absent #17 - small #18 - normal	Present	Normal	89
h) Added cofactors required to reduce NADP	--	Present	Absent	Low	69
i) Mn deficient	--	"	"	Very low	93
j) No photosynthetic growth but normal pigments	Absent	--	--	--	94

chloroplasts). (3) The data were obtained by numerous investigators, and experimental conditions vary considerably. (4) A particular variation may have been attempted with bacteria but not with green plants or algae, or vice versa, so a comparison cannot be made.

(5) The compound giving rise to signal II in green plants and algae is extremely sensitive to mild perturbations, as shown in Table V, so this compound may not be present in order for one to determine the effect of another perturbation on this signal.

Even with these difficulties considered, comparisons of the data in these tables yield some useful information. Taken together, the narrowing of the signal linewidth when the cells are cultured in D₂O (see reference 2), the spacing of the energy levels (g-value) and the hyperfine structure strongly indicate that the compound giving rise to signal II is organic. Furthermore, the slow decay exhibited by this signal, both in whole cells or when leached into water as observed by Calvin and Androes,⁵⁹ indicates that either this organic moiety has a large, resonating structure in order to delocalize the unpaired electron or a large, sterically hindered structure to protect the electron. The effects of reduced dyes upon the decay kinetics suggest that the free spin results from an oxidation of the organic compound, not a reduction.

It is also evident from the tables that this organic moiety is intricately connected with the oxygen evolving mechanism of photosynthesis and requires a specific form of binding in order to take part. This assertion is substantiated by the following: (1) Signal II is

observed only in green plants and algae, not bacteria. (2) Mild perturbations, such as washing, shaking, heating, etc., cause a loss in the magnitude of signal II and a concomitant reduction in the rate of photo-evolved oxygen. (3) Agents which impair oxygen evolution, such as DCMU, reduce signal II. (4) Mutant strains of algae which do not evolve oxygen do not display signal II. (5) The signal saturation to light intensity corresponds to that observed for photosynthesis.

A kinetic analysis of the two signals in whole cell algae, made by Commoner and Heise,⁷⁰ is interpreted to mean that signal II follows signal I in time and that the two signals are connected, signal II resulting from electrons supplied by the component giving rise to signal I. Although the interpretation that the two are connected, signal II being formed after signal I, may be correct, the effects of reduced dyes on the decay rates and the fact that signal I can be produced in the dark by ferricyanide suggest that the species giving rise to the signals are in oxidized states. Therefore, it is more probable that species I causes an oxidation of component II, not a reduction.

The data in Tables III, IV, and V indicate that the component (or components) which gives rise to signal I is also an organic compound (q -value, effect of deuterium substitution for hydrogen), is firmly bound in the photosynthetic apparatus (requires extreme physical treatment to destroy capacity to show signal), and is connected with the capacity to reduce NADP (NAD) or CO_2 (data obtained with mutants).

The line shapes of signal I in algae and the signal in bacteria were compared by Weaver⁶⁸ and Androes^{59c}, respectively, to the two

theoretical possibilities, Lorentzian and Gaussian, and were found to more nearly approximate the Gaussian shape. A Gaussian shape is indicative either of a spin system in which electron-electron interactions of neighboring molecules or electron-nuclear interactions within the same molecule contribute to the linewidth or a system consisting of several distinct radicals in which case the EPR pattern is the resulting envelope of narrower overlapping lines.^{59c}

The fact that a portion of the signals observed at room temperatures can be produced at liquid nitrogen and helium temperatures, suggests that a photo-physical process (photo-induced charge separation) is involved in the production of at least a portion of the spins detected at room temperature (spectrum characteristics suggest that the same signals are detected at both high and low temperatures). This, the rapid response of the bacterial signal and signal I to light, the correspondence between the kinetics of formation and decay of the bacterial signal to those exhibited by the pigment complex P890,⁹⁷ and the presence of signal I in the complex P700 isolated from algae, strongly indicate that signal I and the bacterial signal are intricately associated with the photosynthetic apparatus in these organisms and probably with the primary quantum conversion process. Additional data which substantiate this assertion are that the concentrations of spins produced correspond to the concentration of P700⁸⁴ and P890⁸⁹ in algae and bacteria, respectively, and the redox potentials of the species giving rise to signal I and the bacterial signal, as determined by titration with ferricyanide^{84,66} are equal to that found for P700 and P890 by the same method.^{35,99}

The quantum yields of spin production in bacteria and in chloroplasts have been measured, as shown in Table III; however, the values reported must be accepted with reserve due to the technical difficulties encountered in making the required measurements of the number of spins produced and the quanta of light absorbed.

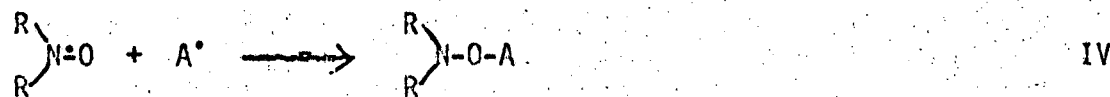
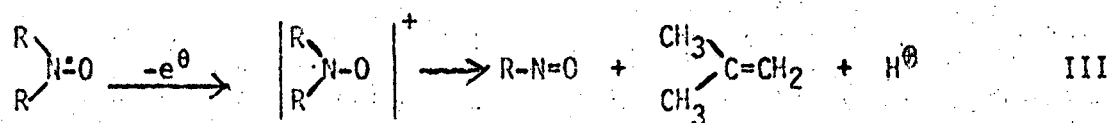
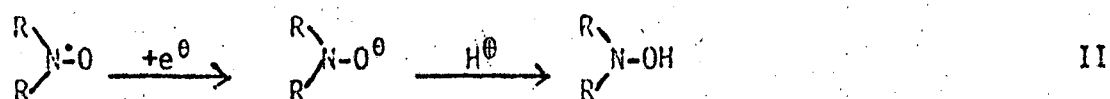
In reviewing the data shown in Tables III, IV and V, and the statements made above, one must conclude that little success has been made in identifying specifically the chemical species responsible for the signals or in relating the signals to the primary quantum conversion process. The data strongly indicate, however, that the signals are connected with some portion of the photosynthetic process.

Statement of Problem

What are the chemical species responsible for the photo-induced EPR signals in photosynthetic systems? Do these species play a role in photosynthetic quantum conversion or electron transport? These are the principal questions which the work described in this thesis sought to answer. More specifically, the work was performed to answer the questions whether di-tertiarybutylnitroxide (a stable free radical) would couple with the photo-induced radicals formed in photosynthetic materials and what product (or products) is formed if a coupling does occur.

Di-tertiarybutylnitroxide (DTBN) is a water-stable, soluble-free radical first prepared by Hoffman¹⁰⁸ and which he described as a "vigorous free radical scavenger". It shows a sharp, well resolved, symmetrical, three-line paramagnetic resonance spectrum that is relatively insensitive to the molecular environment. The chemistry of

DTBN has not been studied extensively. However, four distinct types of interactions can be envisioned for this molecule. It could undergo a one-electron reduction to form a hydroxylamine which can be reduced subsequently to the amine; an oxidative degradation to 2-methyl-2-nitroso-propane and isobutylene; or a coupling with another radical forming either an oxygen-substituted hydroxylamine or a tri-substituted amine oxide. These reactions are represented by equations II through V.



R = tertiary butyl radical; A[•] = unspecified radical

The present work sought to determine whether, in the presence of photosynthetically active organisms and light, the nitroxyl radical would react with the radicals produced in these organisms via reactions IV and V. If so, the use of a ¹⁴C-labeled nitroxide would make it possible to isolate and determine which constituent (or constituents) gives rise to the photo-induced EPR signals.

Several difficulties were anticipated with this approach. One of these is the stability of the nitroxide itself. The steric hindrance provided by the bulky tertiary butyl groups supposedly confers on this radical its stability. Therefore, this hindrance could also keep the

nitroxide from reacting with the photo-induced radicals. However, Hoffman's statement that the nitroxide is a radical scavenger, and the fact that he was able to isolate tri-tertiarybutylhydroxylamine, which according to him was formed by a radical mechanism, show that this steric factor can be overcome.

In addition, radical-radical interactions are not determined by the lifetime of only one of the radicals involved. One must consider the stability of both species as well as the energy gained by the formation of the chemical bond. If both radicals are extremely stable, one would not expect a coupling to occur. However, if one of the radicals is relatively unstable, its instability, aided by the energy gain from the formation of a chemical bond, may supply the driving force to promote the coupling. The radical cannot be too unstable or else it can promote the formation of other radicals by hydrogen abstraction or undergo self-destruction via disproportionation reactions.

The rapid decay exhibited by the EPR signal in photosynthetic organisms when the light is turned off, suggests that the radical giving rise to this signal is unstable and therefore one might trap it with a nitroxide radical. However, if the photo-induced radical (or radicals) is located in the lipid-protein matrix of the photosynthetic apparatus, it may not be accessible to the nitroxide.

The redox potentials found within photosynthetic organisms extend at least over a range between that of the oxygen electrode at +0.8 volts to that of ferredoxin at -0.43 volts. The chance that the

nitroxide would not undergo an oxidation or a reduction within this range of potentials is not too great. Hopefully, however, a coupling reaction would occur in parallel and at a rate to be competitive with these forms of destruction.

Even if a coupling reaction were to occur between the nitroxide and the radicals found in photosynthetic materials, the reported values of the number of spins contributing to the detected signals^{84,98} suggest that the amount of material obtained by this type of reaction would be quite small and therefore insufficient for proper chemical characterization. It must be kept in mind, however, that the signal detected by EPR represents only a steady-state value and not a measure of the amount of material available.

The product which would result from a coupling reaction would have a structure involving an N-O-R linkage (R being an unspecified atom) which is a relatively unstable species. Therefore, it might not be possible to isolate the product.

Even with all these difficulties considered, and since the experiments conducted to date were not successful in identifying the species giving rise to the EPR signals in photosynthetic organisms or in connecting these signals with the act of primary quantum conversion, it seemed worthwhile to conduct the experiments reported in this thesis.

CHAPTER II

CHECK OF STABILITY OF DTBN

The purpose of the work reported in this chapter was to test the stability of DTBN in various chemical environments. The chemicals chosen for these investigations include compounds which are found in photosynthetic organisms, others which possess functional groups or structures similar to those possessed by compounds found in these organisms, and yet others which are known to interact with the photosynthetic processes.

Due to the lack of information on the chemistry of DTBN and the complex mixture of compounds found in photosynthetic organisms, knowledge of the interactions of DTBN in various chemical environments was required in order to facilitate the search for the products of the interactions of DTBN with photosynthetic organisms. In addition, knowledge of the effects of chemicals which interact with the photosynthetic processes on the interaction occurring between DTBN and photosynthetic organisms can be used to relate the latter interaction with the photosynthetic processes. This would require, however, that these chemicals not interact directly with DTBN.

Experimental

Since DTBN exhibits an EPR signal (see appendix II), an EPR spectrometer was used to detect whether DTBN reacted with a chosen chemical. The procedure involved recording the DTBN spectrum, positioning the magnetic field so that a maximum deflection of the recorder pen occurred, and following the amplitude of this deflection in time. Since all these samples were subjected to illumination, a perturbation which could give

rise to an EPR signal in the chosen chemical in the field region of the nitroxide absorption, and since maximum deflections in the DTBN spectrum occur at three different field positions, the field strength was varied between these three positions in different experiments.

Two methods were used to prepare the samples. One of these involved preparing a stock solution of DTBN in an appropriate solvent, placing a weighed amount of the other chemical into a 5 ml volumetric flask, adding a measured amount of the DTBN solution to the flask, and bringing the resulting mixture to volume with the solvent. The second method involved preparing stock solutions of both DTBN and the chosen chemical and mixing measured amounts of these.

The EPR spectra, with the exception of those in solution in acetonitrile, ethanol and water, were taken using cylindrical quartz tubes of approximately 4 mm I.D. These samples were frozen, evacuated to a pressure of less than 5×10^{-5} mm Hg to remove oxygen, and thawed. This procedure was repeated at least three times on each sample. The tubes were sealed through the use of greased vacuum stopcocks. The spectra of the acetonitrile, ethanol and water solutions were obtained using a 5 cm by 1 cm by 0.4 mm rectangular quartz cell. This cell is not strong enough to permit evacuation or freezing of the sample. Therefore, in order to remove oxygen from these samples, a nitrogen purge was used. The sample used for the EPR was transferred under N_2 with a hypodermic syringe into the EPR cell, which was sealed with a rubber septum.

The light sources used for illuminations were a 1000-watt 115-volt GE projection lamp (tungsten filament) or a 150-watt xenon

lamp. The beam of the tungsten lamp was passed through a water bath (1-1/2 in) containing an IR filter and was focused onto the sample through slits in the EPR cavity. The beam from the xeon source was passed through a monochromator and focused onto the sample using a quartz lens. In experiments involving the attenuation of the light intensity, metal screens, the transmittance of which had been determined on a Cary 14 spectrometer, were inserted between the sample and the light source. The energy output of these two light sources was measured using a thermopile which was calibrated against a light source supplied by the National Bureau of Standards. This information is reported in the appendix.

Also reported in the appendix are the method of preparation and properties of DTBN, the sources or methods of preparation and purification of the various solvents and chemicals, and a description of the instrumental equipment used in these investigations.

In two of the systems investigated, as will be discussed below, the products resulting from the interactions were looked for, using optical spectroscopy (a Cary 14 spectrometer), gas manometers (see appendix for description) and vapor phase chromatography (description in appendix).

Results and Discussion

Experimental results are summarized in Table VI. The headings in this table are self-explanatory, with the possible exception of the last two columns. A plus (+) sign is used in these two columns to indicate that a reaction occurs between DTBN and the compound specified in the second column. A minus (-) sign indicates that no reaction

Table VI

Sensitivity of DTBN to Various Chemicals

Concentration of DTBN	Compound(s)	Conc.	Oxygen		Solvent	Results	
			with	without		Dark	Light*
All conc.	(none)	---		x	Cyclohexane	(-)	(-)
1.06×10^{-5} M	N,N-Dimethylaniline	.247 M	"	"	"	(-)	(-)
"	N,N,N',N'-Tetra- methyl-p-phenyl- ene diamine	.207 M	"	"	"	(-)	(-)
"	Hexamethylbenzene	.104 M	"	"	"	(-)	(-)
"	Quinoline	.363 M	"	"	"	(-)	(-)
1.64×10^{-5} M	p-Chloranil	(sat.)	"	"	"	(+)	(-)
8.0×10^{-5} M	o-Chloranil	.001 M	"	"	"	(+)	(-)
3.95×10^{-5} M	Chlorophyll <u>a</u>	(sat.)	"	"	"	(-)	(-)
"	Chlorophyll <u>b</u>	"	"	"	"	(-)	(-)
"	Benzoquinone	.03 M	"	"	"	(-)	(+)
"	"	"	x	"	"	(-)	(-)
2.94×10^{-5} M	Naphthoquinone	.02 M	"	x	"	(-)	(+)
"	Duroquinone	.03 M	"	"	"	(-)	(+)
2.88×10^{-5} M	Hydroquinone	(sat.)	"	"	"	(-)	(-)
6.2×10^{-5} M	Hexafluorobenzene	-	"	"	C ₆ F ₆	(-)	(-)
"	Hexafluorobenzonitrile	-	"	"	C ₆ F ₅ CN	(-)	(-)
2.72×10^{-5} M	(none)	-	x	"	CH ₃ CN	(-)	(-)
"	Tetracyanoethylene	.025 M	"	"	"	(+)	(-)
6.5×10^{-4} M	(none)	-	"	x	CHCl ₃	(-)	(-)
"	s-Trinitrobenzene	.210 M	"	"	"	(-)	(-)
"	m-Dinitrobenzene	.179 M	"	"	"	(-)	(-)
"	p-Nitrobenzonitrile	.199 M	"	"	"	(-)	(-)
"	p-Chloranil	(sat.)	"	"	"	(+)	(-)

Table VI

(Cont'd)

Concentration of DTBN	Compound(s)	Conc.	Oxygen		Solvent	Results	
			with	without		Dark	Light*
6.5x10 ⁻⁵ M	(none)			x	CH ₃ CH ₂ OH	(-)	(-)
"	Chlorophyll <u>a</u>	(sat.)	"	"	"	(-)	(-)
"	Chlorophyll <u>a</u> + 1,4-benzoquinone	(sat.)	"	"	"	(-)	>540 mμ
"	"	(sat.)	"	"	"	(-)	>350 mμ (+)
3.95x10 ⁻⁵ M	(none)	-	"	"	Benzene: Dimethyl- formamide (5:1)	(-)	(-)
"	Lumiflavin	(sat.)	"	"	"	(-)	(-)
"	Lumichrome	(sat.)	"	"	"	(-)	(-)
"	Anthroquinone	.01 M	"	"	Benzene	(-)	(+)
All conc.	(none)	-	x	"	0.5 M sucrose in H ₂ O; pH 6.8 PO ₄ [±]	(-)	(-)
1.9 x10 ⁻⁴ M	KBH ₄		"	"	"	(-)	(-)
"	KCN	.1 M	"	"	"	(-)	(-)
"	DCMU**	1.9x10 ⁻³ M	"	"	"	(-)	(-)
"	NaDCPIP (ox)**	6.0x10 ⁻⁵ M	"	"	"	(-)	(-)
"	" (red)**	"	"	"	"	(-)	(-)
"	Na Ascorbate	5.0x10 ⁻³ M	"	"	"	(+)	(?)
"	K ₃ Fe(CN) ₄	5.0x10 ⁻⁴ M	"	"	"	(-)	(?)
.01 M	"	.01 M	"	"	H ₂ O	(+)	(?)
1.5x10 ⁻⁴ M	Salycylaldoxime	.01 M	"	"	"	(-)	(-)
2.7x10 ⁻⁴ M	Hydroquinone	.03 M	"	"	H ₂ O: pH >7	(+)	(-)

*Visible light, λ greater than approximately 300 mμ.

**DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DCPIP = 2,6-dichloro-phenolindophenol.

takes place. If a plus sign appears in the column labeled "dark" and a minus sign in the column labeled "light", this means that exposure of the sample to visible light had no effect on the decay rate which is observed in the dark.

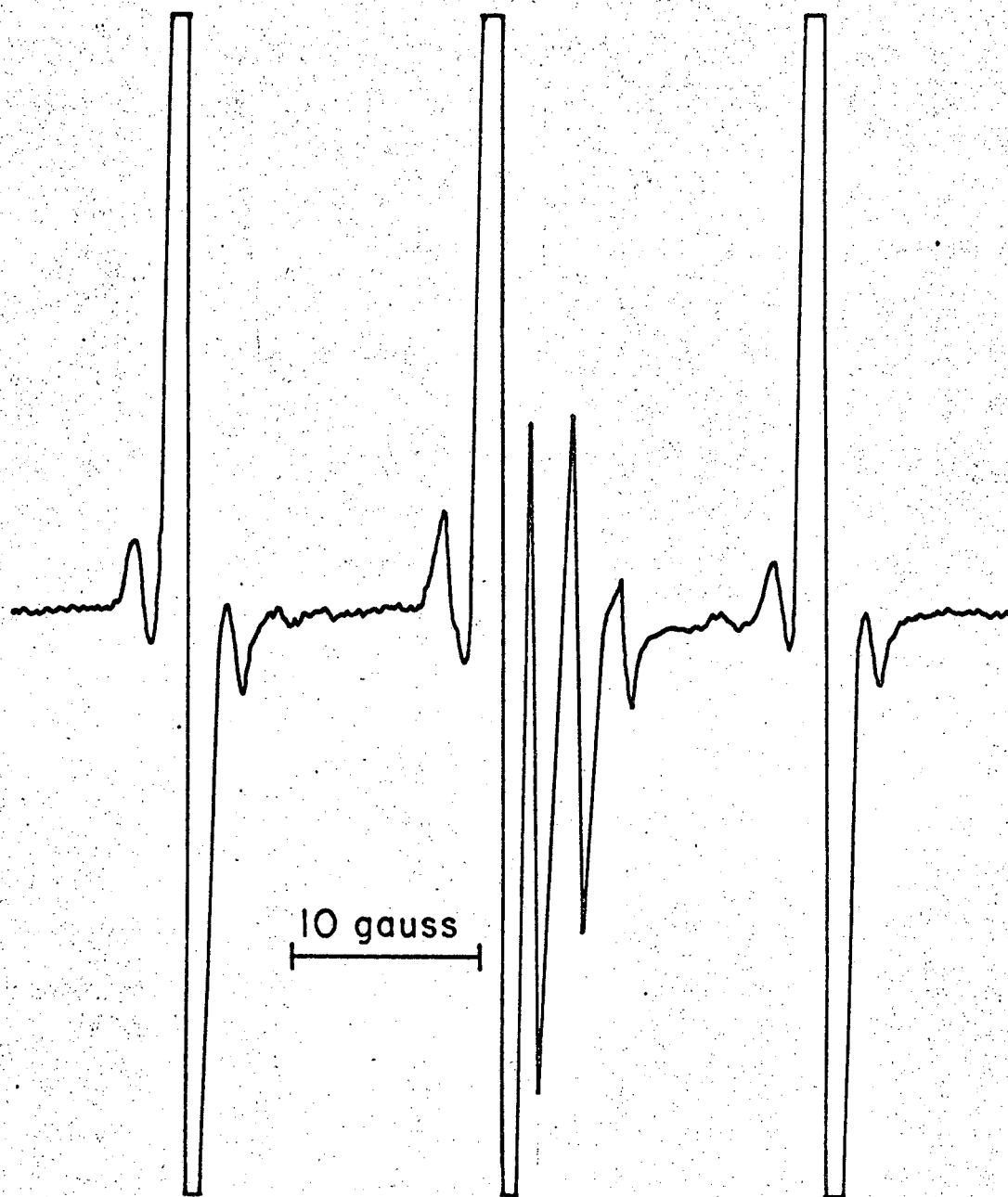
As shown in Table VI, DTBN undergoes reactions in the dark with tetracyanoethylene, *o*-chloranil, *p*-chloranil, sodium ascorbate, the semiquinone of benzoquinone and potassium ferricyanide. With the exception of the last two reactions, these interactions were not studied further except to show that the reactions were not influenced by illumination and the dark reactions were irreversible.

Reaction with Semiquinone

When DTBN is added to a solution of 1,4-benzhydroquinone in water buffered at pH 10 with phosphate, a destruction of the nitroxide occurs. If the sample is removed from the EPR tube and exposed to air with shaking, the nitroxide reappears. As shown in Figure 5, which is an EPR spectrum of a mixture containing hydroquinone and DTBN at pH 10, both the nitroxide radical and the semiquinone of the hydroquinone are present in this sample. Both of these radicals disappear with time.

The nitroxide itself is stable in the buffer used in these experiments. However, at this pH the hydroquinone is oxidized by oxygen to the semiquinone which is unstable and undergoes further reactions. Thus, although the destruction of the nitroxide is associated with the presence of the hydroquinone, it is not known whether it is also connected with the presence and destruction of the semiquinone radical.

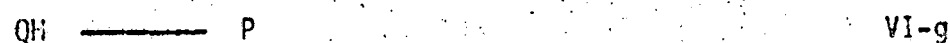
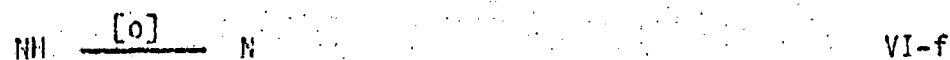
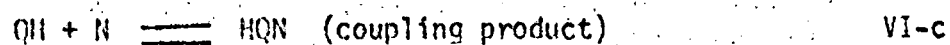
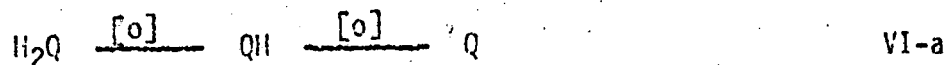
The following equations express the possible reactions which could be occurring in these samples. In these equations M represents the



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Figure 5. EPR spectrum of a mixture containing DTBN (10^{-4} M) and benzhydroquinone (4 mg/ml) in water buffered at pH 10 with phosphate.

nitroxide; NH, ditertiarybutylhydroxyl amine; H₂Q, hydroquinone; QH, semiquinone; Q, benzoquinone; P, unidentified polymer products; QH₂Q, a quinhydrone complex; HQN, a coupling product; and [o], molecular oxygen.



Experiments show that reactions VI-a, b, f and g occur in these samples and the reversible nature of the nitroxide destruction suggests that either reaction VI-d or VI-e is also taking place and that reaction VI-f is unable to compete with reaction VI-a. Thus, the oxidation of the hydroxylamine does not occur until either the hydroquinone is expended or an unlimited supply of oxygen is present (until the sample is removed from cell and is shaken with air). The reversible nature of the nitroxide destruction does not, however, rule out reaction VI-c, especially if the coupling of the radicals results in the formation of a relatively unstable, oxygen-oxygen bond between the nitroxide and the semiquinone.

Whether the nitroxide is destroyed in this system through a coupling with the semiquinone or by a reduction to the hydroxylamine is not known. The answer to this question requires the identification of the product (or products) whose instability so far precludes its isolation.

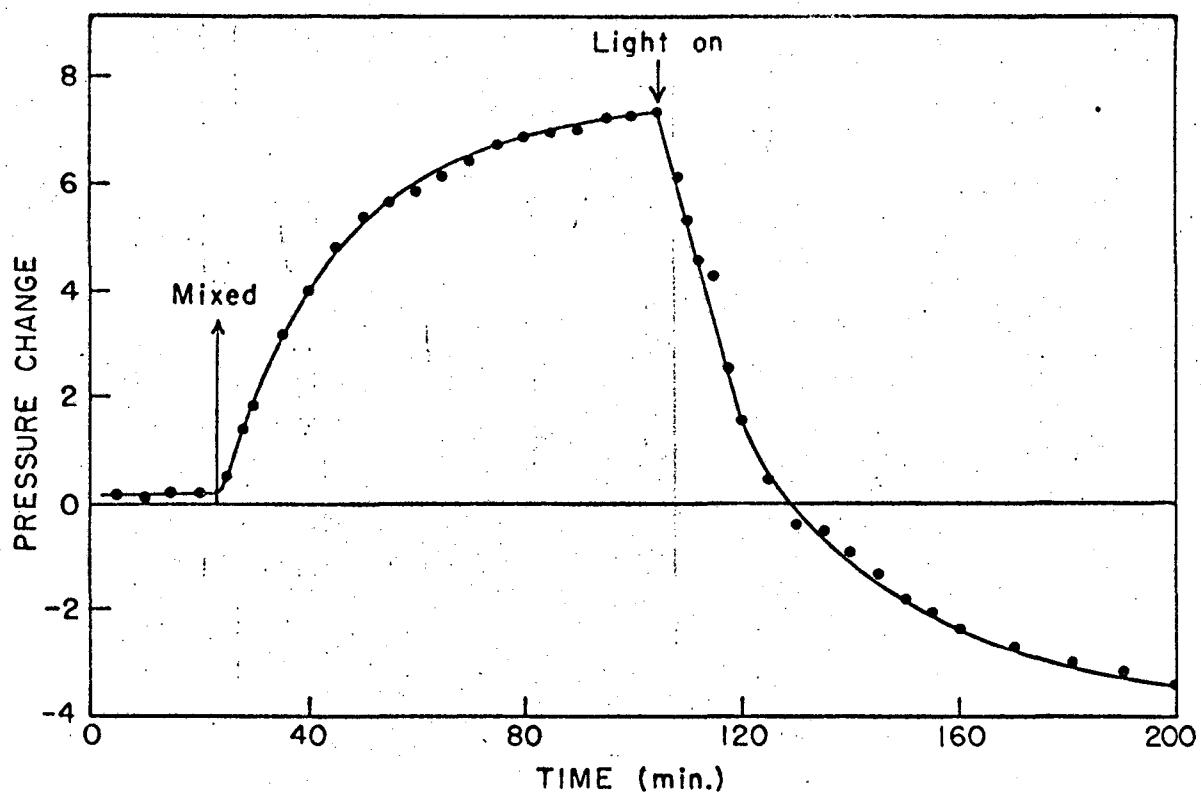
Reaction with $K_3Fe(CN)_6$

The interaction of DTBN with $K_3Fe(CN)_6$ is an oxidation of the nitroxide. If the two chemicals are mixed in the dark in equimolar amounts ($\sim .01$ M) in a closed system and the pressure above the solution is monitored, a pressure increase is observed as shown in Figure 6. If the sample is then exposed to visible light, a rapid decrease in pressure is observed (also shown in Figure 6).

The gas released in the dark reaction was analyzed by vapor phase chromatography and found to contain 2-nitroso-2-methylpropane and isobutene in a molar ratio of 3.6:1. The identities of these two were ascertained by co-chromatography of the atmosphere over the sample with added amounts of the known compounds. The response of the VPC detection system to these two compounds was calibrated by injecting known amounts of these two compounds and plotting the recorded peak area (measured by triangulation method) versus the amount injected. Using the resulting curves and measured peak areas, the molar ratio of the two was determined. A trace from the VPC of the gas atmosphere is shown in Figure 7.

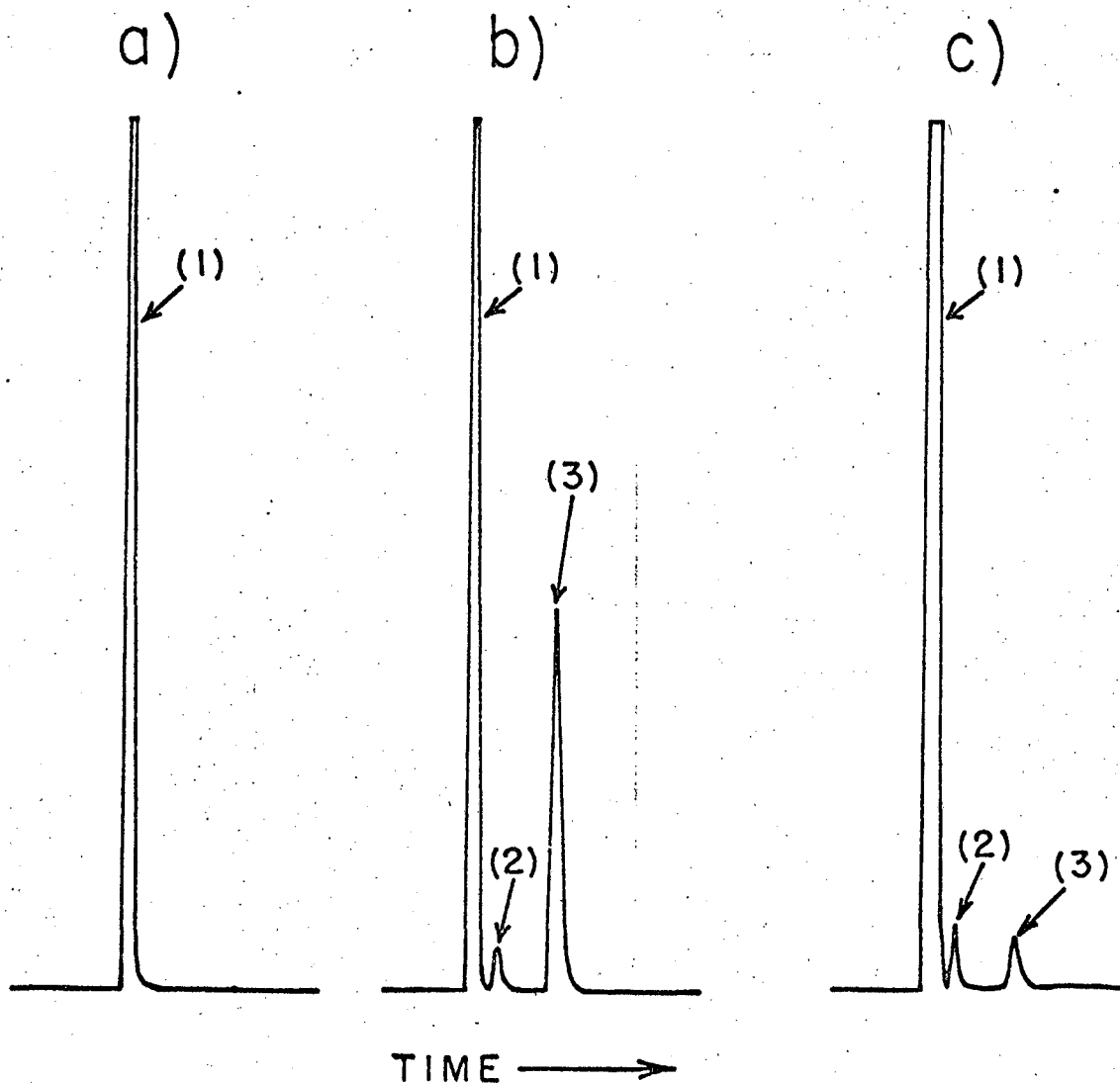
Extraction of a similar reaction mixture by pentane yields a solution which has an optical spectrum as shown in Figure 8. The absorption displayed in the region of 680 m μ is identical to that exhibited by the nitroso compound.¹⁰⁹

Unfortunately, a material balance was not possible due to the heterogeneous nature of the system. However, since both the nitroso compound and isobutene are insoluble in water, the ratio of these two compounds in the gas phase is probably a true reflection of the



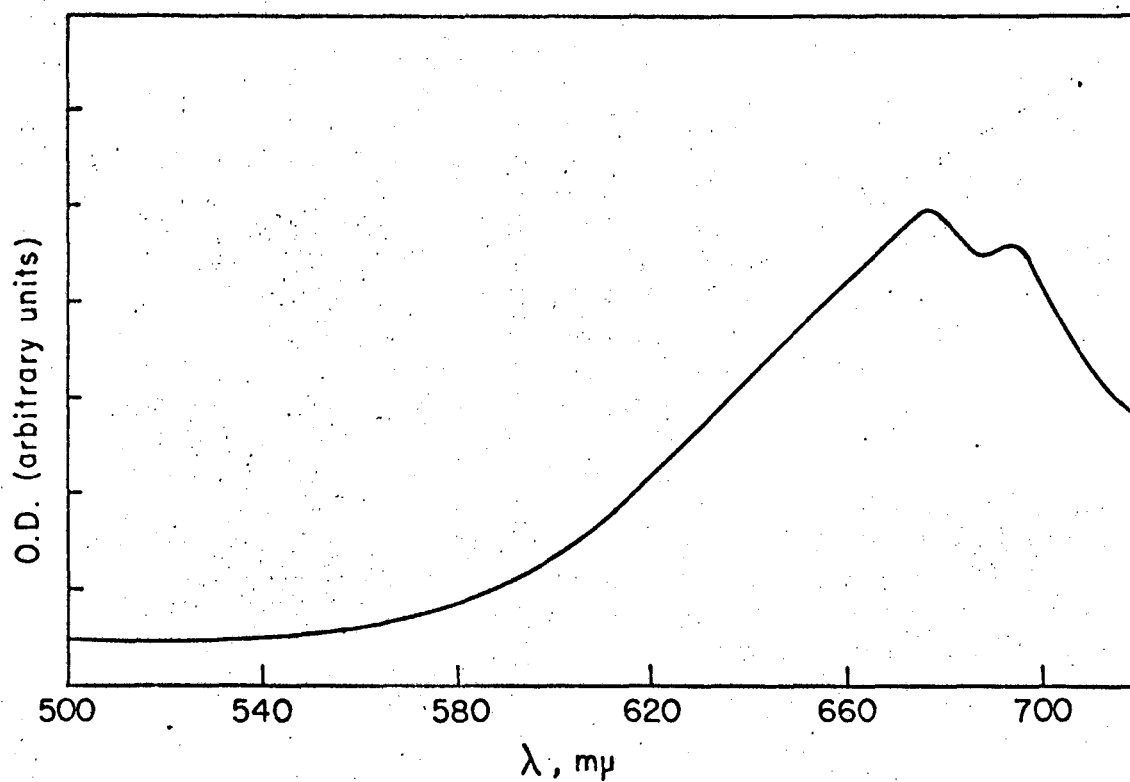
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Figure 6. Pressure changes above a solution containing DTBN and $K_3Fe(CN)_6$; 0.01 M in both.



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Figure 7. VPC traces of the atmosphere above a solution containing 0.01 M DTBN and $K_3Fe(CN)_6$ before mixing (a), in the dark after mixing (b), and in the light after mixing (c). The column was 10% SE-30 on chromosorb W. The column, the injector, and the detector were at 45, 60 and 60 degrees centigrade, respectively. (1) is air. (2) is isobutene. (3) is 2-methyl-2-nitrosopropane.



XBL 678-4582

Figure 8. Optical absorption spectrum of a pentane extract of a reaction mixture containing DTBN and $K_3Fe(CN)_6$; 0.01 M in both, kept in the dark.

ratio formed in the reaction. If anything, one would expect that the ratio of the two formed in the reaction is greater than 3.6 since the nitroso compound is known to form a dimer in aqueous solution¹¹⁰ which is insoluble and precipitates, whereas one would expect to find the isobutene primarily in the gas phase.

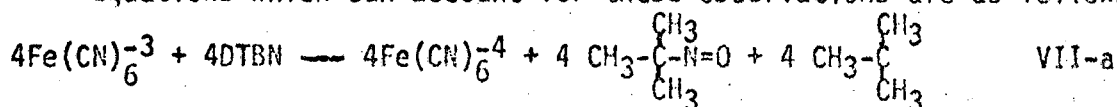
Attempts to detect by VPC additional compounds containing a tertiarybutyl group, such as tertiarybutyl cyanide or tertiarybutyl alcohol, failed due to the tailing of H₂O in the VPC columns. Attempts to extract the water with pentane or diethylether also failed to yield a compound identifiable as a tertiarybutyl derivative.

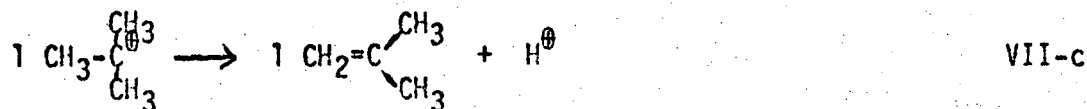
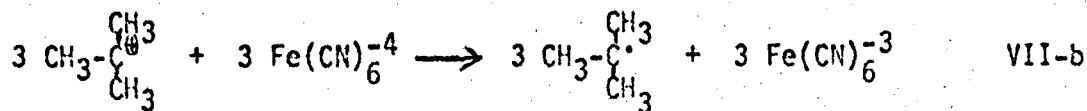
Examination of the water solution by EPR did show that the nitroxide was completely destroyed. However, the optical spectrum of this solution has an absorption maximum at 420 mμ which is due to K₃Fe(CN)₆. The magnitude of the optical density of this peak ($\epsilon = 10^3$) indicates that 3.6 times as much DTBN is destroyed as K₃Fe(CN)₆.

The correspondence between the ratio of DTBN to K₃Fe(CN)₆ destroyed and the ratio of the nitroso compound to isobutene formed, suggests that the ferrocyanide initially formed in the reaction is re-oxidized to ferricyanide and possibly by the tertiarybutyl group which is unaccounted for.

It should also be mentioned that the reaction occurs even if oxygen is excluded from the system. Also, a slight blue precipitate forms which is probably ferro-ferricyanide or ferri-ferrocyanide.

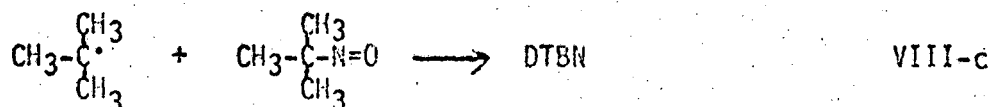
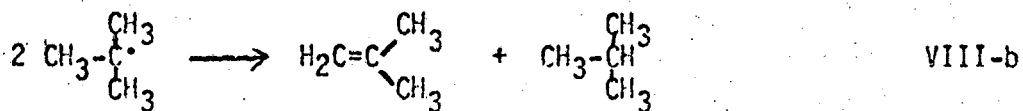
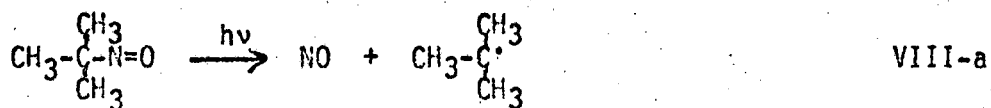
Equations which can account for these observations are as follows:





The fate of the tertiarybutyl radical is probably a coupling with DTBN to form tri-tertiarybutylhydroxylamine, although this compound was not detected. The retention time (r.t.) of this compound is very large with the best conditions found to detect it, as is evident in Figure 64 of appendix II. With such a large r.t., the quantity of this material one would expect to find in these samples would not be detectable due to broadening of the VPC peak with a resulting decrease in the amplitude.

Following the dark reaction, when the sample is illuminated with white light, a rapid decrease in pressure is observed (see Figure 6). Analysis of the gas phase, again by VPC, shows that the ratio of isobutene to the nitroso compound has increased (see Figure 7). This observation is in agreement with the experiments of de Boer,¹¹¹ who observed a photochemical degradation of 2-nitroso-2-methylpropane which suggested the sequence of reactions shown below:



The nitroxide formed by equation VIII-c would then interact with the $\text{Fe}(\text{CN})_6^{-3}$ again producing more nitroso. This would continue until the nitroxide is transformed to NO, isobutene, and some unknown tertiarybutyl derivative. The NO is probably complexed with the ferric and cyanide ions to form $[\text{Fe}(\text{CN})_5(\text{NO})]^{-3}$ or with free ferrous ions, to form $[\text{Fe}(\text{NO})]^{+2}$. However, a search for either of these ions was not conducted.

Although the destruction of DTBN by ferricyanide is quite rapid if substrate quantities of both are used, the rate of this interaction when the concentration of ferricyanide is below 10^{-2} M and the concentration of DTBN is approximately 10^{-4} M is so slow that no noticeable change in the EPR spectrum of the nitroxide is observed over a 30-min period. One would expect a decrease in the rate by a factor of 10^2 just by consideration of the concentration differences, between $(.01 \text{ M}) \times (.01 \text{ M})$ and $(10^{-2} \text{ M}) \times (10^{-4} \text{ M})$ assuming a second order reaction involving one equivalent of each species.

Reaction with Quinones

Since compounds with quinone functional groups are found in plants and photosynthetic bacteria and since I intended to investigate the interaction of DTBN with these latter systems under the influence of visible light, I decided to investigate further the quinone-DTBN photochemical reactions reported in Table VI.

As shown in the table, DTBN does not interact in the dark with 1,4-benzoquinone (BQ), duroquinone (DQ), or 1,4-naphthoquinone (NQ) in cyclohexane nor with 9,10-anthroquinone (AQ) in benzene. However, when these systems are exposed to visible light, a rapid destruction

of the nitroxide occurs. These reactions are inhibited if oxygen has not been removed from the samples. A typical trace of the decay of the nitroxide resonance signal in the benzoquinone-DTBN system is shown in Figure 9.

After the destruction of the nitroxide and the samples are placed in the dark, the nitroxide reappears slowly, as shown in Figure 10. The amount of the nitroxide which reappears varied between 50 and 85 percent of the original starting material in different experiments. Table VII shows the free spin concentrations, both before and after exposure to light, in systems containing originally 2.94×10^{-5} M DTBN with .031 M BQ, .032 M DQ, and .017 M NQ. Also included in a sample containing only the nitroxide.

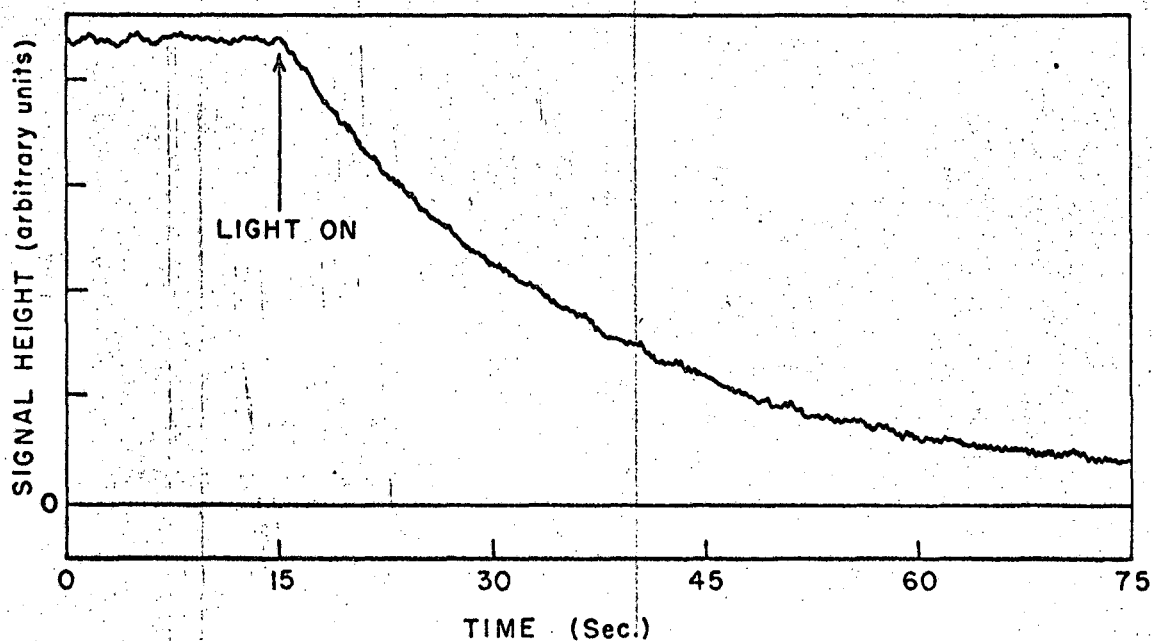
Table VII

Time (hrs)	2.94×10^{-5} M DTBN with			
	2.94×10^5 M	.031 M BQ	.032 M DQ	.017 M NQ
0	2.9×10^{15} spins**	2.7×10^{15} spins	2.4×10^{15} spins	2.7×10^{15} spins
0*	2.9 " "	0	0	0
12	3.0 " "	1.7×10^{15} spins	1.9×10^{15} spins	1.2×10^{15} spins
24	2.9 " "	1.9 " "	1.9 " "	1.9 " "
39	2.8 " "	1.5 " "	1.9 " "	2.1 " "
65	2.8 " "	1.9 " "	1.6 " "	1.9 " "

*After exposure to light

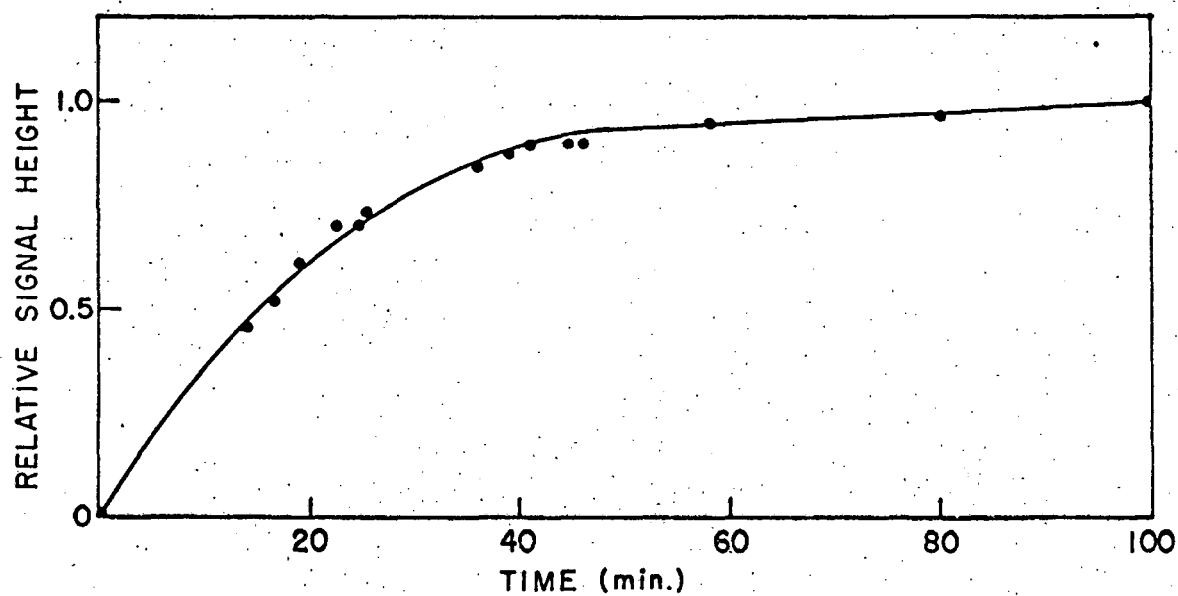
**Calculated from concentration and volume, 3.1×10^{15} spins

$t_{1/2}$ for the destruction of DTBN in these samples was 3.4 sec for BQ, 1.9 sec for NQ and 17.2 sec for DQ.



XBL 677-4547

Figure 9. Time response of DTBN EPR signal to light. The initial reaction mixture contained 0.03 M benzoquinone and 3.5×10^{-5} M DTBN in cyclohexane without air. Light of wavelength $458 \pm 5 \text{ m}\mu$ was used.



XBL 678-4573

Figure 10. Reappearance of DTBN EPR signal in the dark. The initial reaction mixture contained 0.03 M benzoquinone and 2.8×10^{-5} M DTBN in cyclohexane without air.

The procedure followed in obtaining the data shown in Table VII involved measuring the spin concentration immediately after the samples were prepared, exposing the samples to light and then storing the samples in the dark at room temperature; measuring the concentration of spins at the times shown in the table.

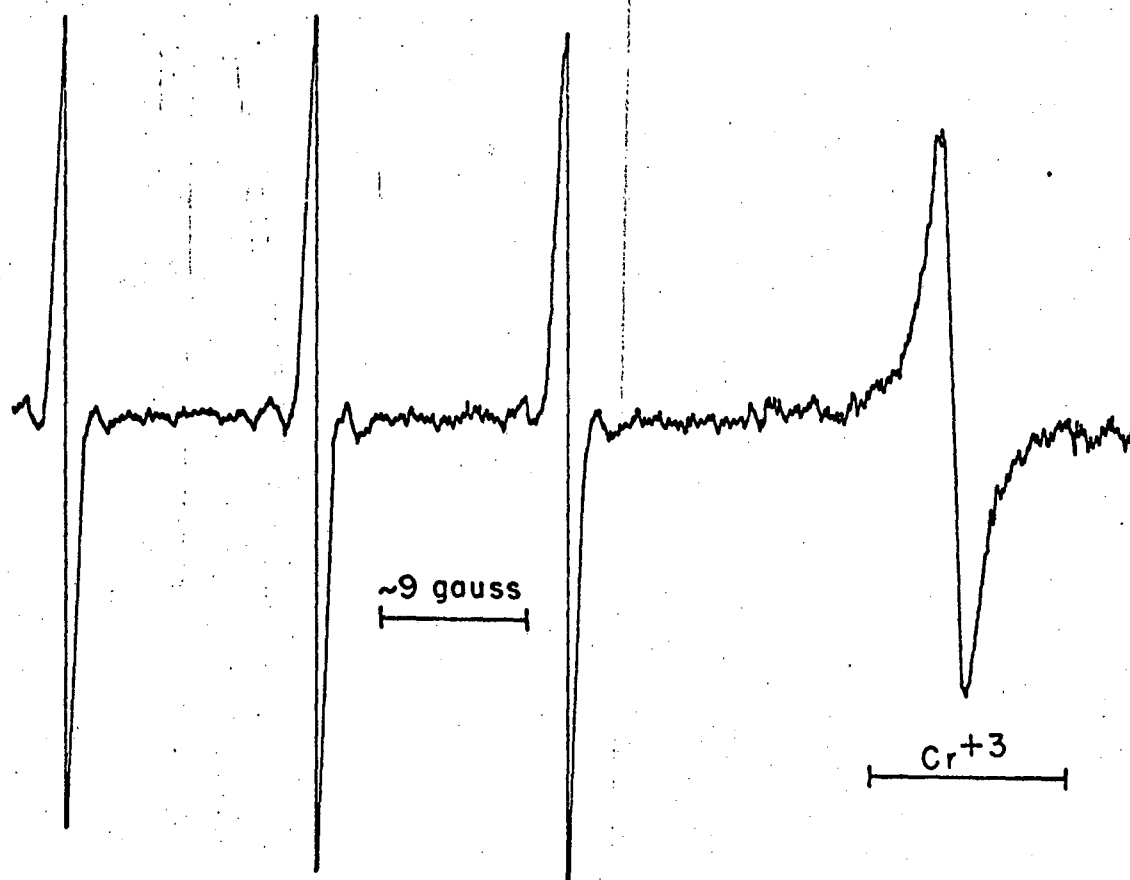
The actual number of spins was determined using the following formula:

$$\# \text{ spin } X = \frac{H_{mS}}{H_{mX}} \frac{\Delta H_X}{\Delta H_S}^2 \frac{A_X}{A_S}^2 \frac{G_S}{G_X} \frac{g_S}{g_X} \# \text{ spins } S \quad \text{IX}$$

where S refers to a standard, X to an unknown, and H_m = modulation amplitude, ΔH = line width, A = the signal amplitude, G = the gain of the complete system, and g = the g-value of the resonance line.

This formula is based upon the assumption that the shape of the EPR absorption spectrum approximates a Lorentzian line (which is true for the DTBN spectrum) and requires the simultaneous measurement of the EPR spectra of a standard containing a known number of spins and a compound with an unknown number of spins.

The standard used in this work was Cr^{+3} (a known quantity) incorporated as a substitutional impurity in MgO which was then dispersed in polyethylene. An EPR spectrum showing the signals due to both DTBN and Cr^{+3} is shown in Figure 11. To record this spectrum, a solid sample containing Cr^{+3} was fixed in the EPR cavity alongside a quartz tube containing DTBN in solution. The data taken from similar spectra were used to calculate the number of spins reported in Table VII. The numbers shown in the DTBN sample compare quite well with the number of spins calculated using the concentration of the DTBN in these samples and the volume used.



XBL 677-4542

Figure 11. EPR spectrum showing the signals from DTBN (10^{-4} M) and Cr^{+3} (10^{16} spins); different gain settings.

Attempts to detect products from the reaction of DTBN with 1,4-benzoquinone using VPC and thin-layer chromatography (TLC) failed. Samples containing equal concentrations of the two species yield peaks in the VP chromatogram due to the nitroxide and the quinone both before and after illumination. An additional compound is present following the illumination; however, it is also formed in a quinone blank after exposure to light.

Since the quinone undergoes a photochemical reaction by itself, an experiment was conducted to see if DTBN was interacting with the product of this reaction. Tubes were constructed with stopcocks isolating two compartments, one above the other. A quinone solution (.03 M) was placed in the lower half of this tube, oxygen was removed, and this section was closed off at a pressure of 10^{-5} mm Hg. A DTBN solution (ca. 10^{-5} M) was then placed in the upper section, oxygen was removed, and an atmosphere of N_2 was introduced.

With the tube in the EPR spectrometer, the quinone was illuminated for 3 min and then left in the dark for 1 min. The DTBN was then allowed into this section, the pressure difference causing a rapid mixing of the two solutions. Comparison of the number of spins in the resulting mixture with the number found in an experiment without the quinone, showed that none of the nitroxide was destroyed. If the two solutions were mixed at the time of turning off the light, approximately 50% of the nitroxide was destroyed, indicating that the species with which the nitroxide is interacting has a long lifetime compared to ordinary singlet state lifetimes.

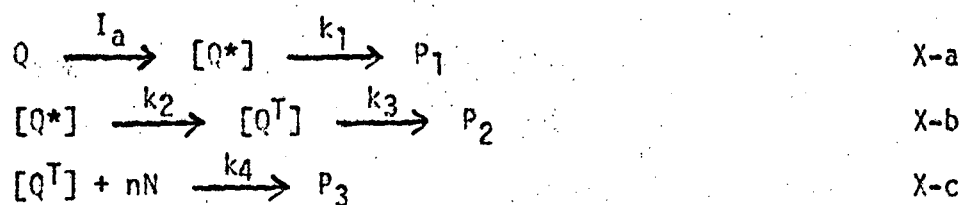
This latter observation and the fact that oxygen inhibits the reaction (see Table VI and page 48) suggest that the nitroxide is

reacting with the triplet state of the quinone although this species was not detected in these samples by EPR at room temperature or at liquid nitrogen temperature.

Additional evidence which substantiates the proposal that the triplet state of the quinone is involved is the lack of a photochemical reaction between DTBN and ortho or para-chloranil. The heavy chlorine atoms are known to quench triplets. This evidence must be accepted with reserve, however, since DTBN reacts with the chloranils in the dark. A photochemical reaction between DTBN and these species might also be occurring but at a rate much slower than the dark reaction rate. Therefore, the photochemical reaction would contribute only slightly to the overall destruction rate and would be undetectable.

The kinetics of the reaction in this system were also investigated with variation of the light intensity (monochromatic light @ $\lambda = 458 \pm 5 \text{ m}\mu$) and the concentration of DTBN.

The simplest sequence of reactions that one would expect to occur in this system is as follows:



where Q represents the quinone; Q^* and Q^T , the singlet and triplet state of the quinone, respectively; P_1 and P_2 , products from the excited states, including the quinone itself; N, the nitroxide; P_3 , the product from the nitroxide and quinone; n, the number of nitroxides involved; k, the rate constants of the specified reaction; and I_a , the amount of light absorbed by the quinone.

The equations which describe the variation of the excited state quinones and the nitroxide concentrations with time are as follows:

$$\frac{\partial Q^*}{\partial t} = \phi I_a - (k_1 + k_2)[Q^*] \quad X-d$$

$$\frac{\partial Q^T}{\partial t} = k_2[Q^*] - k_3[Q^T] - k_4[Q^T][N]^n \quad X-e$$

$$\frac{\partial N}{\partial t} = -k_4[Q^T][N]^n \quad X-f$$

where ϕ represents the primary quantum yield.

Assuming a steady-state level of the excited states of the quinone, their concentrations and the rate of the change of the nitroxide are given by the following:

$$[Q^*] = \frac{\phi I_a}{(k_1 + k_2)} \quad X-g$$

$$[Q^T] = \frac{k_2 \phi I_a}{(k_3 + k_4[N]^n)(k_1 + k_2)} \quad X-h$$

$$\frac{\partial N}{\partial t} = \frac{-k_4 k_2 \phi I_a [N]^n}{(k_1 + k_2)(k_3 + k_4[N]^n)} = \frac{-k' I_a [N]^n}{(k + [N]^n)} \quad X-i$$

where $k' = \frac{k_2 \phi}{k_1 + k_2}$ and $k = \frac{k_3}{k_4}$

The solutions to equation X-i for the cases of $n = 1$ and $n = 2$ in terms of measurable parameters, assuming a direct proportionality between the concentration of nitroxide and the height of the EPR spectral lines, are:

$$(n = 1) \quad k \ln S/S_0 + N_0 [S/S_0 - 1] = -k' I_a t \quad X-j$$

$$(n = 2) \quad S/S_0 = \frac{k' I_a}{N_0} \frac{t}{S_0/S - 1} - \frac{k}{N_0^2} \quad X-k$$

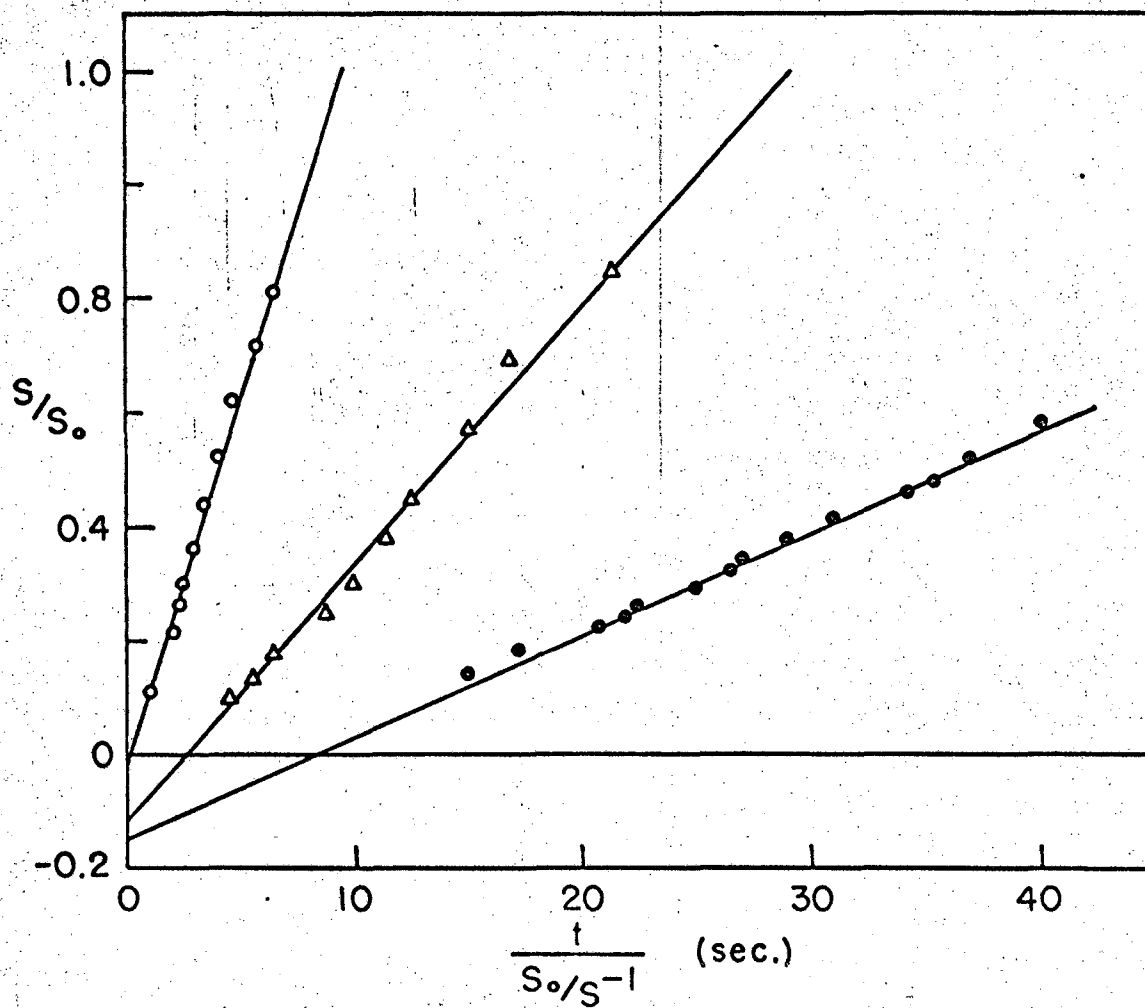
where S_0 and N_0 represent the height of the nitroxide EPR spectral line and the concentration of nitroxide, respectively, at time zero; S and N , these same quantities at another time; t , the time; and k and k' , as shown above.

It should be noted that these equations are valid (even if the mechanism proposed above is correct) only so long as the assumption regarding the direct proportionality between the signal height and the nitroxide concentration is correct. Also, another assumption needs to be considered. This is that the total change in the signal height is due to a change in the nitroxide concentration. This is not an insignificant consideration, as is discussed in the appendix.

As can be seen in Figures 12 and 13, which are plots of experimental data according to equation X-k for a variation of the initial concentration of nitroxide (N_0) and a variation of the light intensity, respectively, the destruction of the nitroxide follows the mechanism shown above in which two equivalents of nitroxide react with one quinone equivalent.

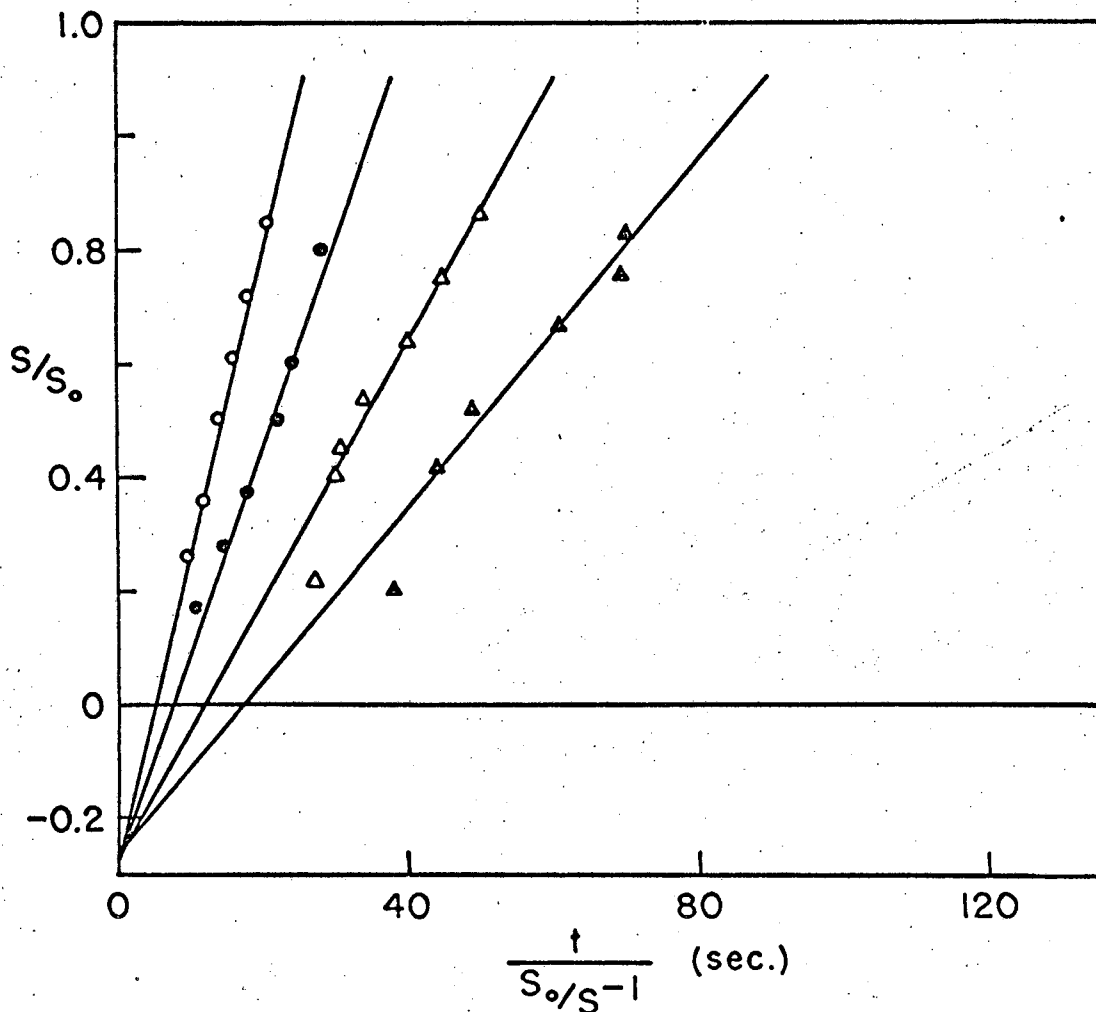
According to equation X-k, the slope of the straight lines in Figures 12 and 13 should vary linearly with the reciprocal of the initial concentration of the nitroxide and the relative light intensity, respectively. The intercepts of the lines in Figure 12 should vary linearly as the square of the reciprocal of the initial concentration. As shown in Figures 14, 15 and 16, these quantities do vary in a linear manner with one another. However, the intercept of the plot of the rate versus the reciprocal of the concentration should be zero. The reason for this inconsistency is unknown, but it is possibly due to the problems considered in appendix I.

The values of the rate constants, k and $k'I_a$, calculated from the data contained in Figures 12 through 16 are: $k'I_a = 2.5 \times 10^{-6}$ and 2.0×10^{-6} moles/l. sec and $k = .42 \times 10^{-10}$ and 3.8×10^{-10} .



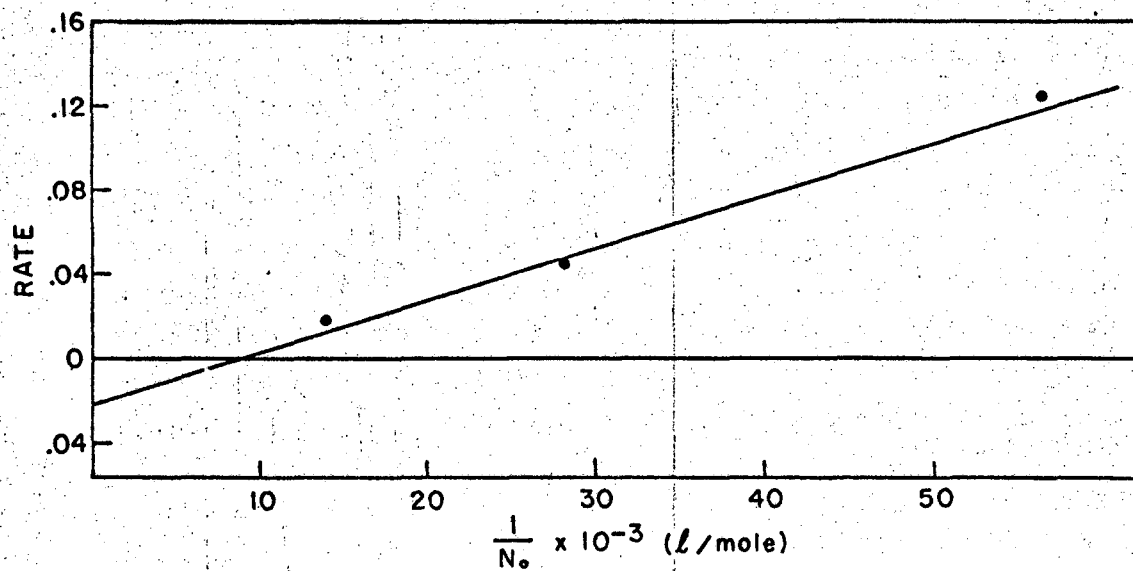
XBL 678-4570

Figure 12. Concentration dependence of DTBN EPR signal photo-induced decay in 0.03 M benzoquinone cyclohexane solution without air; illuminated with light of wavelength 458 ± 5 m μ . The coordinates are defined by equation X-k in Chapter II. The concentrations of DTBN were 1.8×10^{-5} M (open circles), 3.5×10^{-5} M (triangles), and 7.1×10^{-5} M (closed circles).



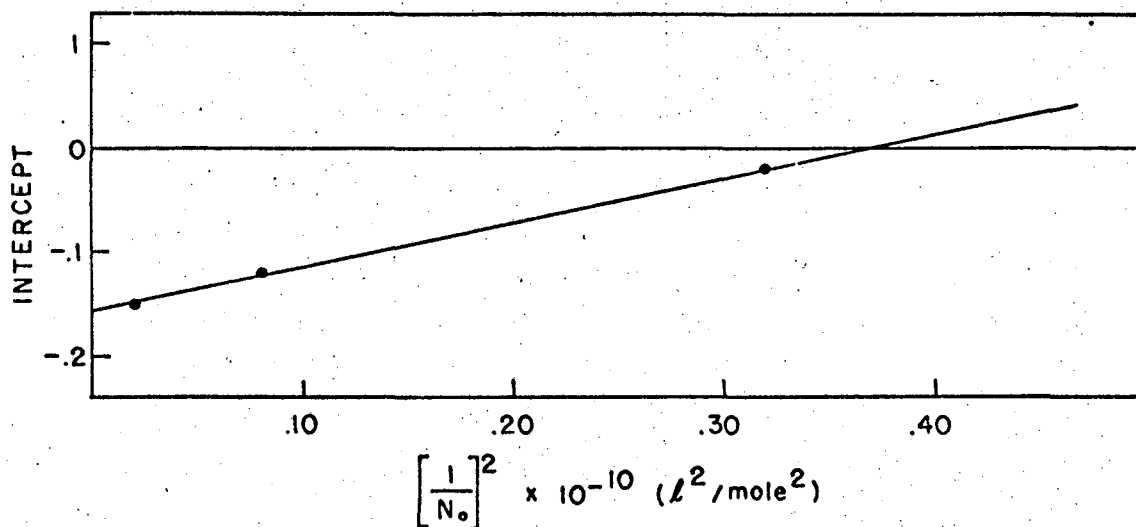
XBL 678-4571

Figure 13. Photo-induced decay of DTBN (3.5×10^{-5} M) EPR signal in a 0.03 M benzoquinone cyclohexane solution without air as a function of relative light intensity. The coordinates are defined by equation X-k in Chapter II. The samples were illuminated with light of wavelength 458 ± 5 m μ (5.0×10^{14} quanta/sec/cm 2). The relative light intensities were 100% (open circles), 60.7% (closed circles), 53.7% (open triangles), and 31.8% (closed triangles).



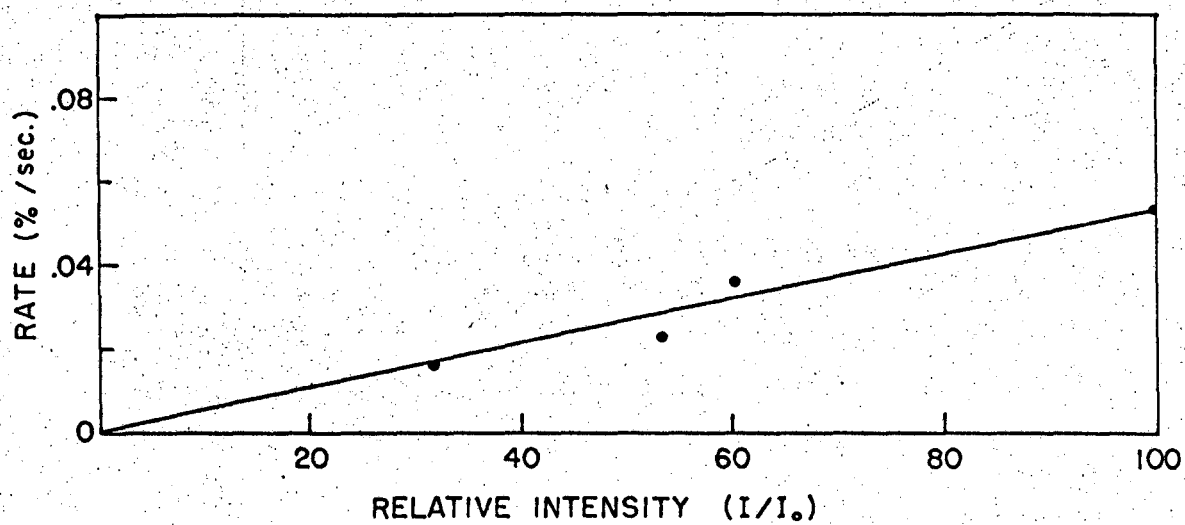
XBL 678-4583

Figure 14. The slope of the lines in Figure 12 plotted versus the reciprocal of the initial concentration of DTBN.



XBL 678-4575

Figure 15. The intercepts of the lines in Figure 12 plotted as a function of the square of the reciprocal of the initial concentration of DTBN.



XBL 678-4574

Figure 16. The slopes of the lines in Figure 13 plotted as a function of the relative light intensity.

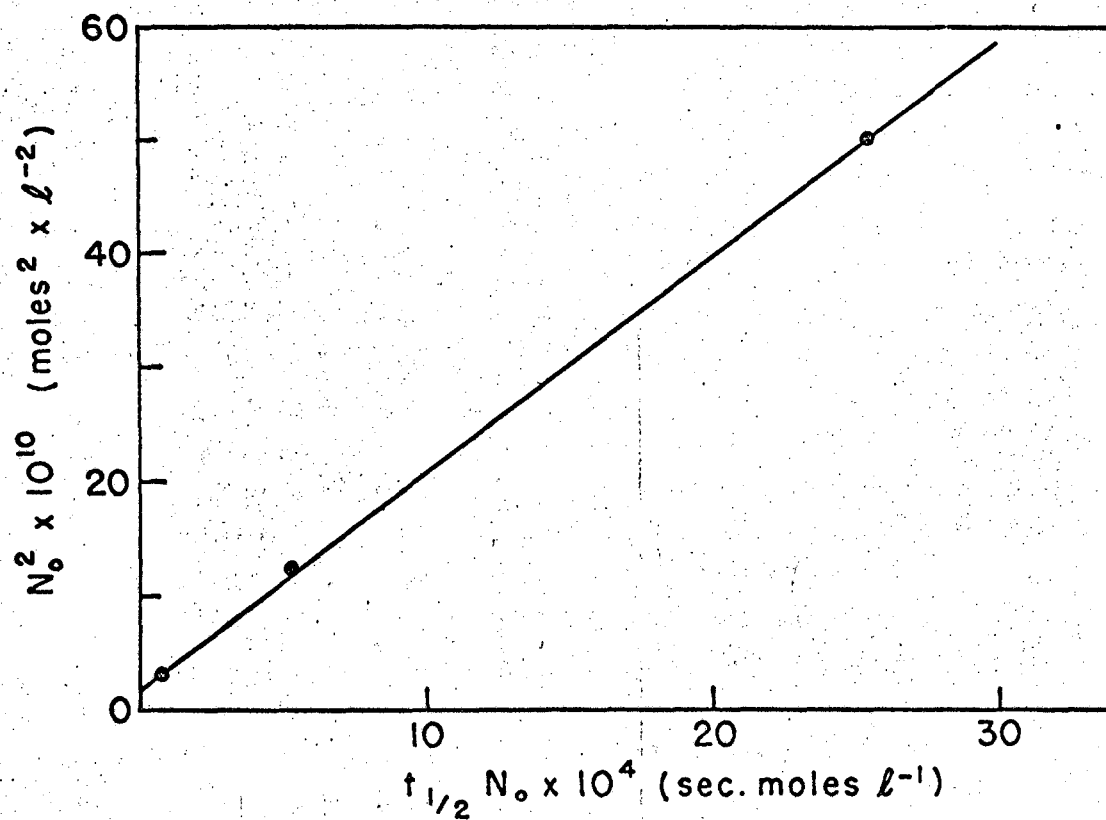
When $S = 1/2 S_0$, equation X-k reduces to the following:

$$N_0^2 = 2 k' I_a (t_{1/2} N_0) - 2 k \quad X-1$$

$$\frac{1}{t_{1/2}} = \frac{2 k'}{(N_0^2 + 2 k)} I_a \quad X-m$$

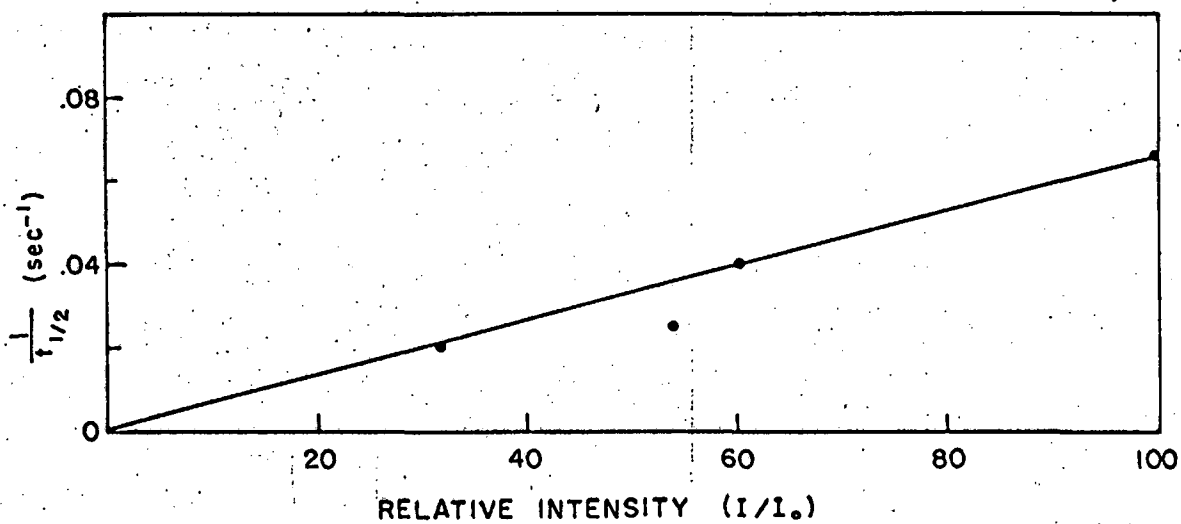
Figures 17 and 18 show the plots of the data (the same experimental data used for Figures 12 and 13) according to equations X-1 and X-m, respectively. From these plots the values of the rate constants, $k' I_a$ and k , are: $k' I_a = 1.0 \times 10^{-6}$ and 1.1×10^{-6} moles/l. sec (or 1.5×10^{-6} depending upon whether $k = .4 \times 10^{-10}$ or 3.8×10^{-10}), and $k = -.8 \times 10^{-10}$. Again the intercept of the plot involving the variation of concentration is not in agreement with the equation. In Figure 17, the intercept should be negative according to equation X-1.

The values of the rate constants ($k' I_a$, ca. 10^{-6} moles/l. sec and k , ca. 10^{-10}) are not unreasonable. The measured light intensity of the source was 5.0×10^{14} quanta/sec/cm². This light was passed through a glass filter (4-96) which transmitted 94.3% of the light at 458 mμ as measured in a Cary 14. Another 57% of the intensity was lost in passing into the EPR cavity (metal with entrance slits--optical density measured in Cary 14). Allowing for another 50% loss due to scattering upon the cylindrical tube used in the EPR experiments, the approximate intensity reaching the sample was 1.0×10^{14} quanta/sec/cm². The length of tubes illuminated was 1 cm (dimension of spot on entrance plate of cavity-diameter 1 cm) and the width of the tube was 0.2 cm. Therefore, the area illuminated was 0.2 cm². Thus, the intensity of light reaching the sample was approximately 2×10^{13} quanta/sec. The concentration of benzoquinone was 0.03 M and the extinction coefficient



XBL 678-4577

Figure 17. The data shown in Figure 12 plotted according to equation X-1 in Chapter II.



XBL 678-4576

Figure 18. The data shown in Figure 13 plotted according to equation X-m in Chapter II.

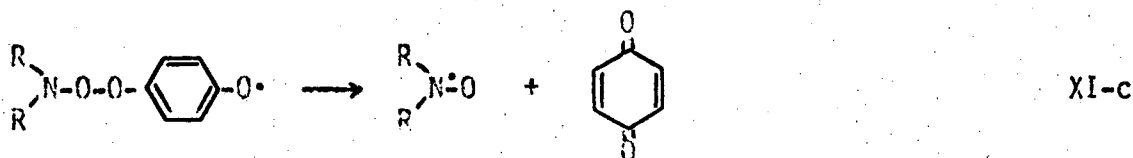
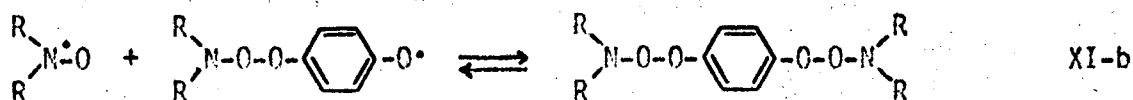
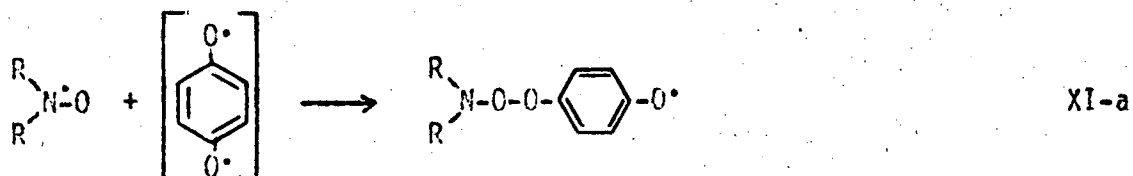
for this quinone at 458 mμ is 21.6 l./mole cm. Therefore, assuming a path length of 0.2 cm (the diameter of the tube), the sample absorbed ca. 10^{12} quanta/sec or ca. 10^{-11} einsteins/sec. The rate constant, $k'I_a$ is ca. 10^{-9} moles/cc/sec. The volume of sample illuminated was approximately 0.03 cc. Therefore, $k'I_a$ is approximately 10^{-11} moles/sec, k' is ca. 1. Since $\phi = 1$, this would mean k_1 is equal to k_2 or within a factor of 10^3 of k_2 considering all the approximations required to reach this conclusion.

Since the absorption band at 458 mμ in benzoquinone is an $n \rightarrow \pi^*$ transition, the probability of this transition is quite low ($\epsilon_{\max} = 21.6$). Therefore, k_1 would be approximately 10^5 to 10^7 corresponding to a lifetime for this state of 10^{-5} to 10^{-7} sec. This is a long lifetime compared to $\pi \rightarrow \pi^*$ states which normally have lifetimes of 10^{-8} to 10^{-10} sec. The long lifetime of the $n \rightarrow \pi^*$ state increases the probability of inter-system crossing (singlet to triplet). Another factor operating in the $n \rightarrow \pi^*$ state to increase the probability of singlet to triplet conversion is the low energy gap between the singlet and triplet states.

Also, the value of k (10^{-10}) means that $k_4 \sim 10^{10} k_3$ (rate constants of the reactions X-b and c). This is also reasonable since k_3 reflects the lifetime of the triplet state which is long (again due to spin forbiddenness) and therefore k_3 is small, whereas k_4 , which represents possibly a coupling of two radicals (the nitroxide and the triplet state of the quinone which can be considered a bi-radical), could be quite large.

The data in Figures 12 through 18 suggest that two nitroxides are destroyed by one quinone, a reasonable ratio, especially if the

quinone triplet state is involved. A possible sequence of reactions consistent with this ratio is as follows:



The fact that the experimental data fit equation X-k means that XI-b is much more rapid than XI-a or XI-c. Also, the slow kinetics displayed in the reappearance of the nitroxide (see Figure 10) suggest that the rate of one of the back reactions depicted in XI-b and XI-c is quite slow, if the scheme shown above is correct.

More evidence which supports the given scheme is the observation that duroquinone, 1,4-naphthoquinone and anthroquinone all photoreact with the nitroxide. This suggests that the two oxygens of their quinoid structures are involved and not the ring positions.

Summary

The data presented in this chapter indicate that DTBN is stable to a number of different chemical environments. The exceptions to this are the chemicals, tetracyanoethylene, chloranil and $\text{K}_3\text{Fe}(\text{CN})_6$, which are normally considered strong oxidants, and also a strong reductant, sodium ascorbate. The reaction between $\text{K}_3\text{Fe}(\text{CN})_6$ and DTBN

was shown to involve an oxidation of DTBN to isobutene and 2-methyl-2-nitrosopropane which was complicated by a photochemical reaction of the nitroso compound.

A photochemical reaction between DTBN and several quinones is also reported. Kinetic experiments involving the photochemical interaction of DTBN with 1,4-benzoquinone suggest that two molecules of DTBN react with the triplet state of the quinone to form an unstable di-peroxide type compound. The instability of the product of the DTBN-quinone reaction makes its isolation impossible.

As shown in Table VI, DTBN is stable in the presence of chlorophyll a and b, both in the dark and in the light. This is pointed out because these two compounds are the primary pigments in the biological system used in the investigations reported in the next chapter.

Chapter III

REACTION OF DTBN WITH PHOTOSYNTHETIC ORGANISMS

In the foregoing, evidence was presented which shows that DTBN is unstable to both strong oxidants and a strong reductant. It also undergoes photochemical reactions with quinones. However, it is stable both in the dark or in the light to chlorophyll a and b, the two principal pigments found in photosynthetic green plants.

As stated in Chapter I, it is conceivable that DTBN, possessing an unpaired electron, could couple with the photoinduced paramagnetic species observed in photosynthetic organisms. This chapter contains the results of an investigation of the photochemical interaction of DTBN with chloroplasts isolated from spinach leaves. These results are presented in four sections. The first of these is concerned with studies conducted to determine the effects of various chemical and physical perturbations upon this interaction. The second discusses experiments which relate the interaction with photosynthetic oxygen production. The third gives an analysis of the products of this interaction. The fourth discusses the conclusions drawn from this work.

Kinetic Experiments

The basic experiment to be discussed in this section is the observation by EPR techniques of the photo-destruction of the nitroxide (DTBN) contained in a water suspension of spinach chloroplast fragments.

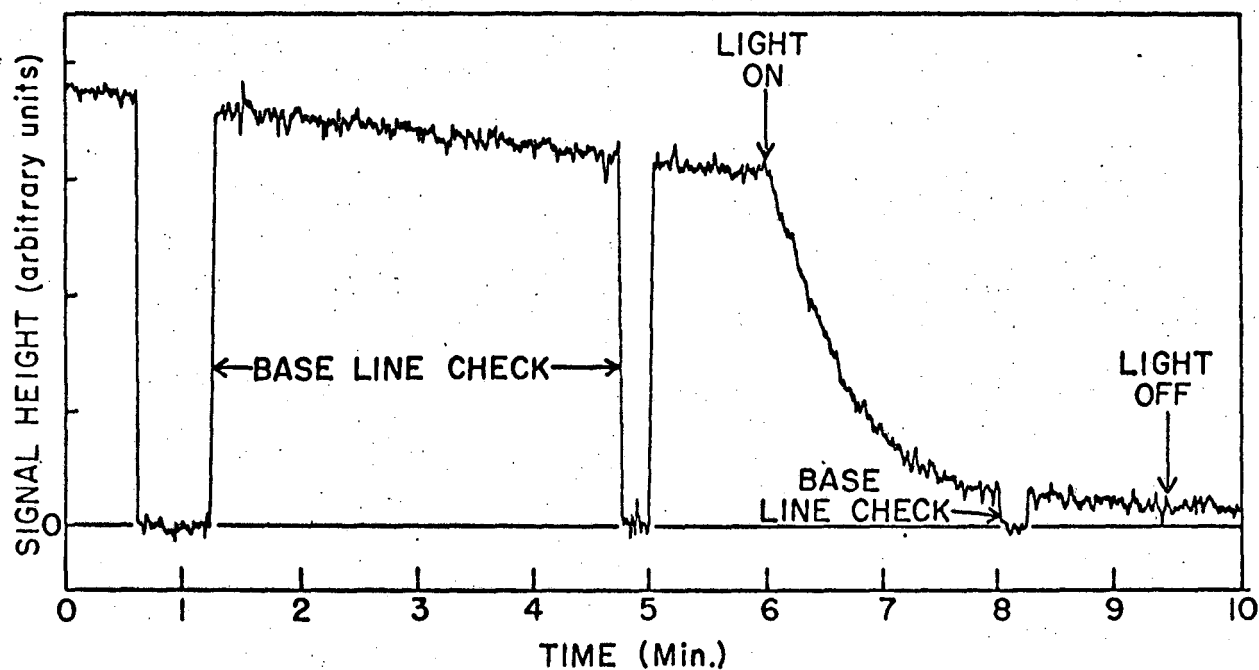
When freshly isolated chloroplasts from spinach leaves are suspended in a 0.5 M sucrose solution buffered at pH 6.8 by phosphate and containing DTBN, a rapid, irreversible destruction of the nitroxide occurs in the dark. The same observation is made with intact Chlorella cells (algae) and the photosynthetic bacterium Rhodospirillum rubrum.

Chromatophores isolated from Rhodospirillum rubrum by sonication of the whole cells also cause a fast, dark destruction of DTBN. When these samples are illuminated, no change in the dark decay is observed. This suggests that either no photo-reaction occurs in this system or else that the photo-decay is not rapid enough to produce a detectable change in the dark destruction.

However, when spinach chloroplasts are washed several times with buffer solution or are allowed to age several days in a cold room in the dark and chloroplast-DTBN mixtures prepared from these chloroplasts are examined in the dark by EPR, only a slow decay of the nitroxide resonance is observed. When such preparations are illuminated with light of wavelengths greater than 5400 Å, the rate of destruction of the radical is increased. The same results are observed with chloroplast fragments obtained by rupturing the chloroplasts in distilled water or in 0.05 M sucrose solution buffered at pH 6.8. Results typical of this effect are shown in Figure 19.

Experimental

The chloroplasts used in these studies were isolated from spinach leaves according to the procedure of Park and Pon.¹¹² The leaves were purchased commercially (Berkeley Market). Following the isolation, the chloroplasts were then either washed three times with buffer solution or else allowed to age in the cold room three days. The results observed using chloroplasts treated in either of these two ways were qualitatively the same. Both treatments yielded chloroplasts which when mixed with DTBN caused only a slow destruction of the nitroxide in the dark. The photo-induced destruction of nitroxide is still observed.



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Figure 19. Time response of DTBN EPR signal to light. The initial reaction mixture contained 1.4 mg chlorophyll (a+b) per ml of suspension, 1.67×10^{-4} M DTBN, 0.05 M sucrose, 0.05 M phosphate buffer, pH 6.8. Light with wavelength between 5400 Å and 9800 Å was used. Base-line check was made by displacing field from resonance value. Sample purged with nitrogen.

Chloroplast fragments were obtained, as stated above, by rupturing chloroplasts either in pure distilled water or in .05 M sucrose solution buffered at pH 6.8, although the latter method is the one generally followed. The dark destruction of the nitroxide still occurs if fragments are used; however, it is much slower than if chloroplasts are used.

In fact, the rates of both the dark and the light-induced decay vary for differing chloroplast preparations (from one isolation to another), for different treatment of the chloroplasts or fragments, and with the age of the chloroplasts or fragments. Therefore, it has been assumed that the same photochemical destruction of the nitroxide is occurring with both the chloroplasts and the fragments, and no distinction is made between the two in the results to follow.

In attempting to ascertain the effect of a particular perturbation or chemical upon the photo-destruction, a control showing the destruction of the nitroxide with the chloroplast (or fragment) preparation in question was compared with a sample which had been subjected to the perturbation of interest or which contained the chemical of interest.

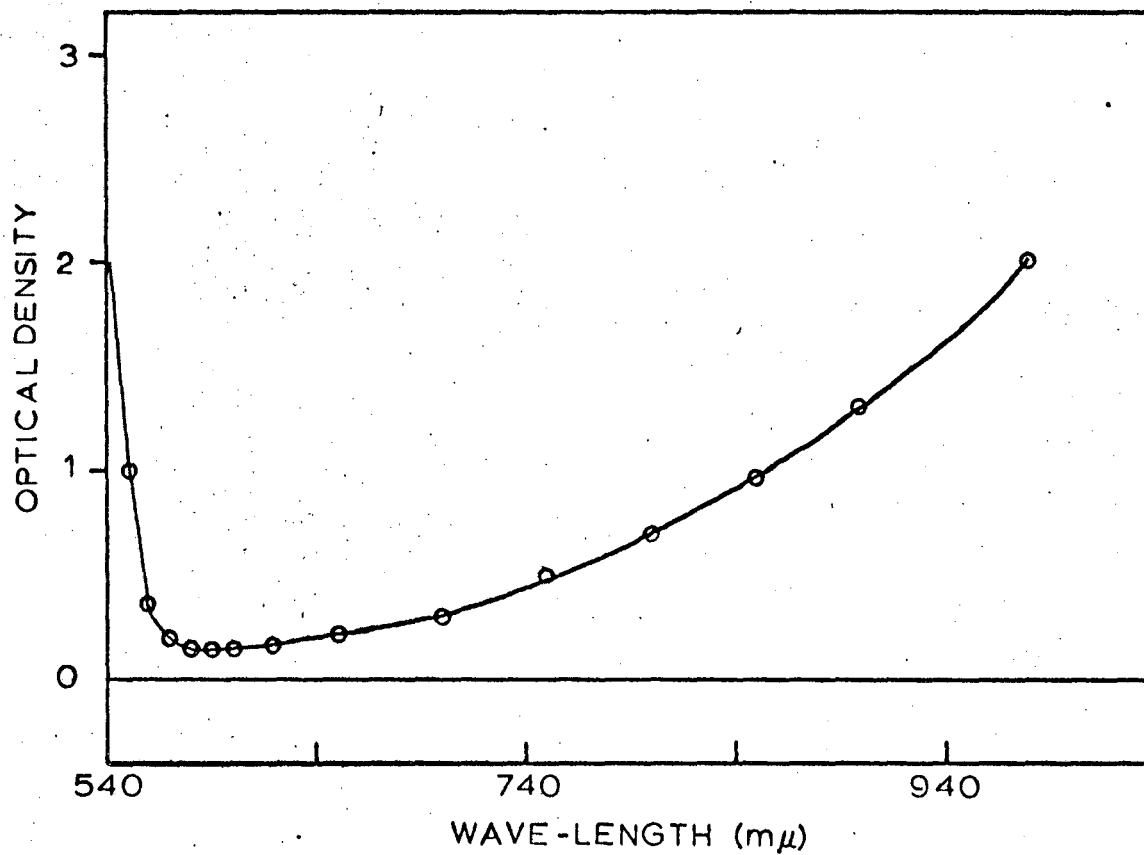
The procedure followed in preparing the samples used in these studies consists in suspending the chloroplasts in the sucrose buffer and using this suspension to bring known amounts of DTBN solutions to a known volume. Stock solutions of DTBN were prepared in 0.5 M sucrose solution buffered at pH 6.8 and their concentration determined assuming the extinction coefficient of the nitroxide in this solution to be the same as it is in water (see Appendix II, Figure 65).

When another chemical was added to the DTBN-chloroplast mixture, a stock solution of this chemical was prepared and a known amount of

this solution was added. An equal amount of buffer solution was then added to the DTBN-chloroplast control in order to maintain equal concentrations of DTBN and chloroplasts in all samples. If the chemical was not soluble in water, it was prepared in a suitable solvent and added to the DTBN-chloroplast suspension using this solvent. In these cases, the same solvent was used for the control sample instead of the sucrose buffer.

The experimental procedure was essentially the same as that employed for the study of the interaction of DTBN with quinones reported in Chapter II. The EPR spectrum of DTBN was recorded. Then the magnetic field of the EPR spectrometer was positioned at one of the three field strengths where maximum deflection of the recorder pen occurs in the nitroxide resonance (see Figure 11, Chapter II). The position of the base line (the field region where the microwave power is not absorbed) was checked through the use of additional coils around the EPR cavity. When current was applied to these coils, the resulting field was superimposed upon the main spectrometer field, causing a displacement from the resonance value of the nitroxide. Thus, the signal height of the particular nitroxide resonance line chosen for observation was followed with time with periodic checks on the base line. Typical results are shown in Figure 19.

The light source used for illumination of the samples was a 1000 watt, 115 volt G.E. projection lamp (tungsten filament). The light from this source after passing through a water bath (water flowing) 1-1/2 inches long, containing an infrared filter, was passed through additional filters (Corning 1-69 and 3-67) and focussed onto the sample through slits in the EPR cavity. Figure 20, the absorption spectrum of



XBL 679-6140

Figure 20. Optical absorption spectrum of Corning filters 1-69 and 3-67 in combination.

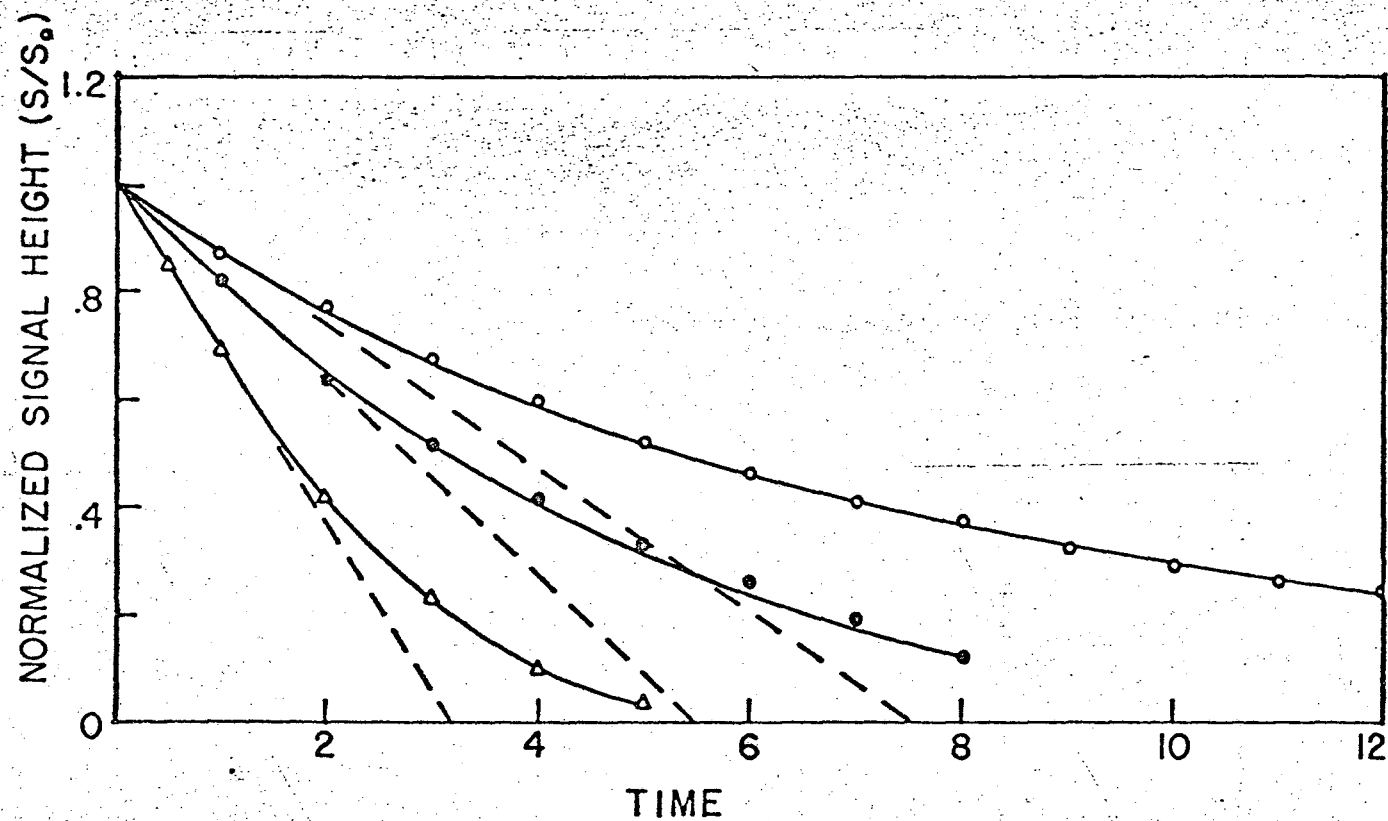
this filter combination, shows that light of wavelengths less than 5400 Å and greater than 9800 Å are removed. This light was excluded since chloroplasts contain quinones which could photo-react with the nitroxide (see Chapter II).

Attempts to use monochromatic light supplied by a 150 watt xenon lamp failed. The light intensity obtained from this source was not strong enough to produce a photo-destruction rate large enough to compete with the dark reaction.

The dark reaction causes another complication. If a stock mixture of the nitroxide and chloroplasts is prepared and samples are taken from this mixture and examined at different times, the results shown in Figure 21 are obtained. However, if each DTBN-chloroplast mixture is made up fresh and examined immediately after mixing, the results shown in Figure 22 are obtained. The data shown in these two figures were obtained with samples placed in the EPR with approximately 45 min between each observation (two different sets of experiments).

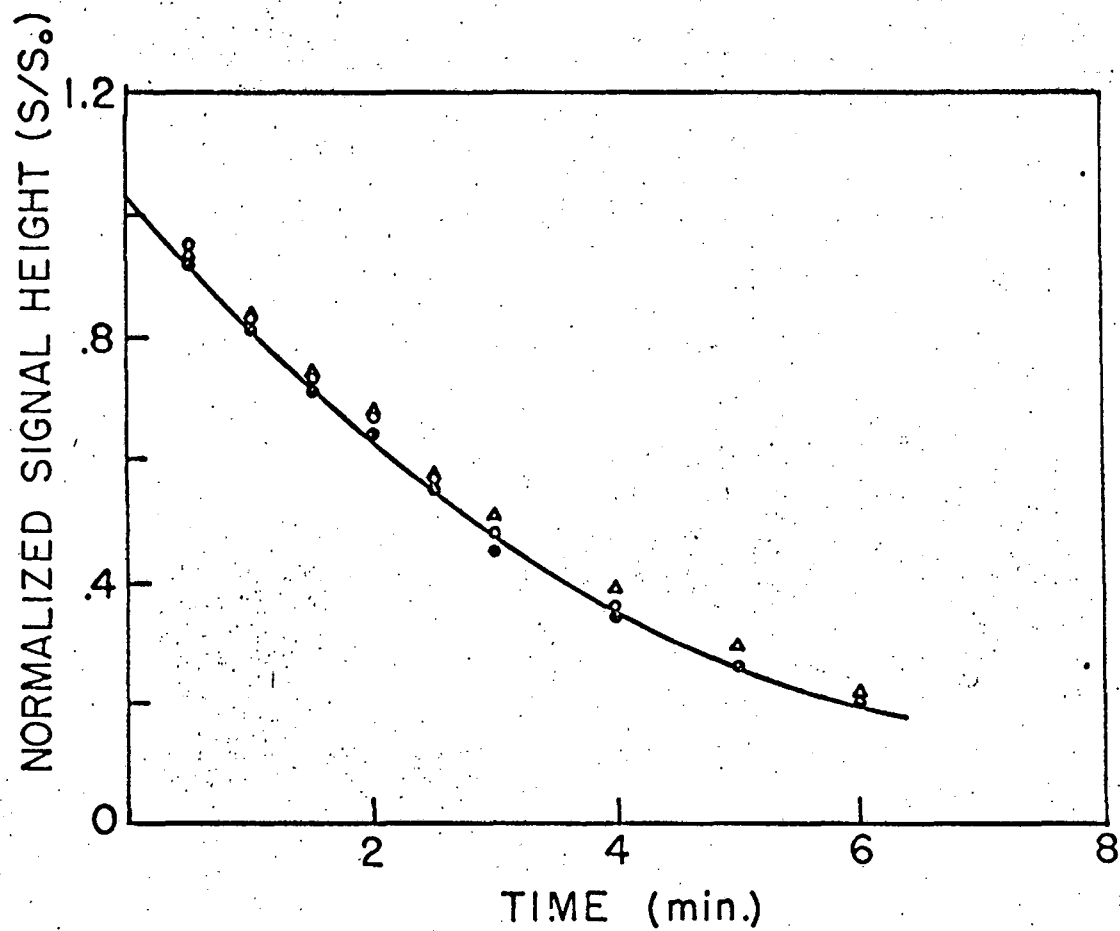
The destruction rate of the nitroxide is dependent upon the initial concentration of the nitroxide, as is shown in Figure 23. These samples were prepared, each separately, from a stock solution of DTBN and a common suspension of chloroplast. These data indicate that the variation in destruction rate shown in Figure 21 results because of variations in the concentration of DTBN due to the dark decay. Therefore, the procedure followed in preparing samples for the experiment reported in this section was that used to obtain the results shown in Figure 22.

The oxygen tension in the sample also affects the destruction rate of the nitroxide, as is shown in Figure 24. In this experiment, the



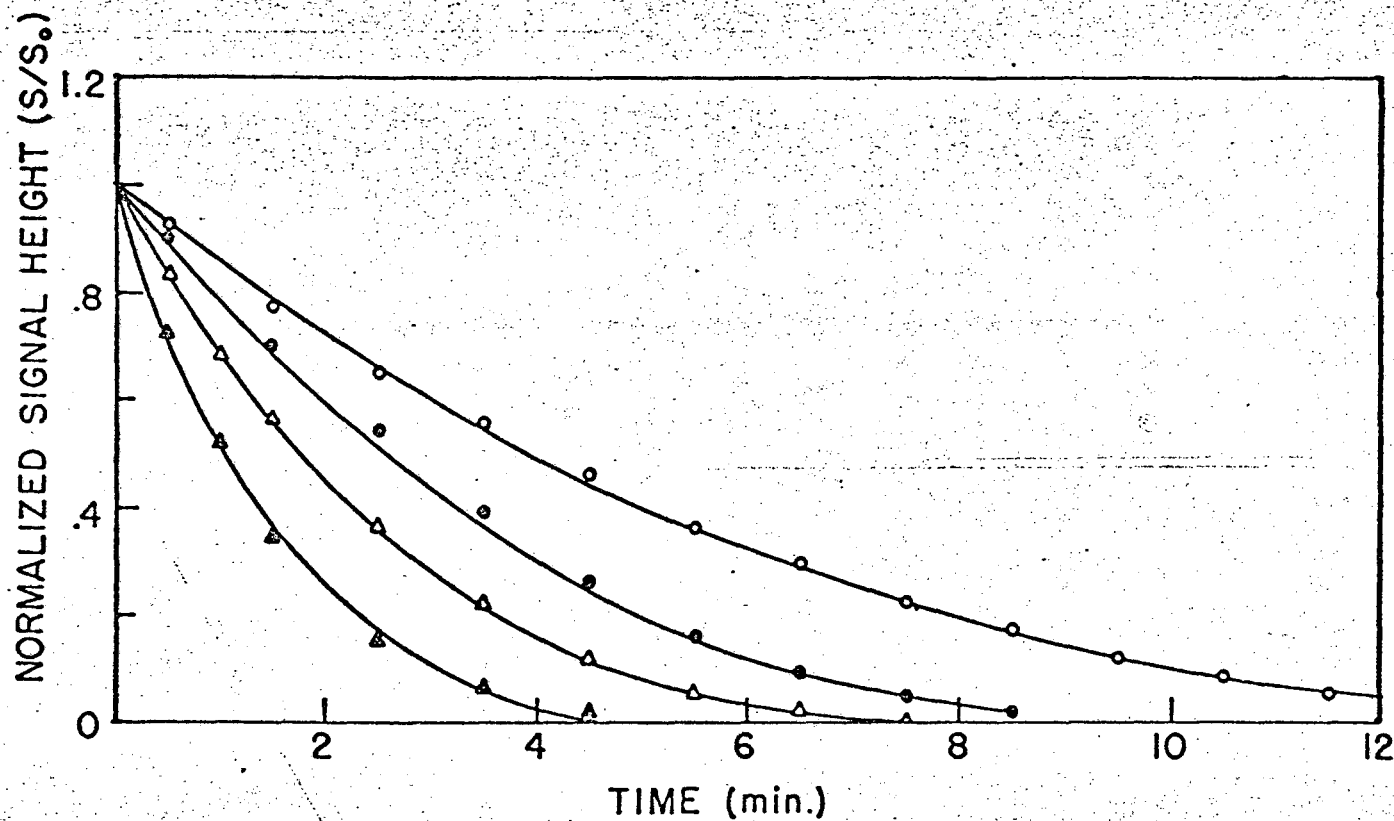
XBL 679-6167

Figure 21. Photo-induced decay of DTBN EPR signal sensitized by chloroplasts as a function of the age of the solution. Approximately 45 min between each experiment.



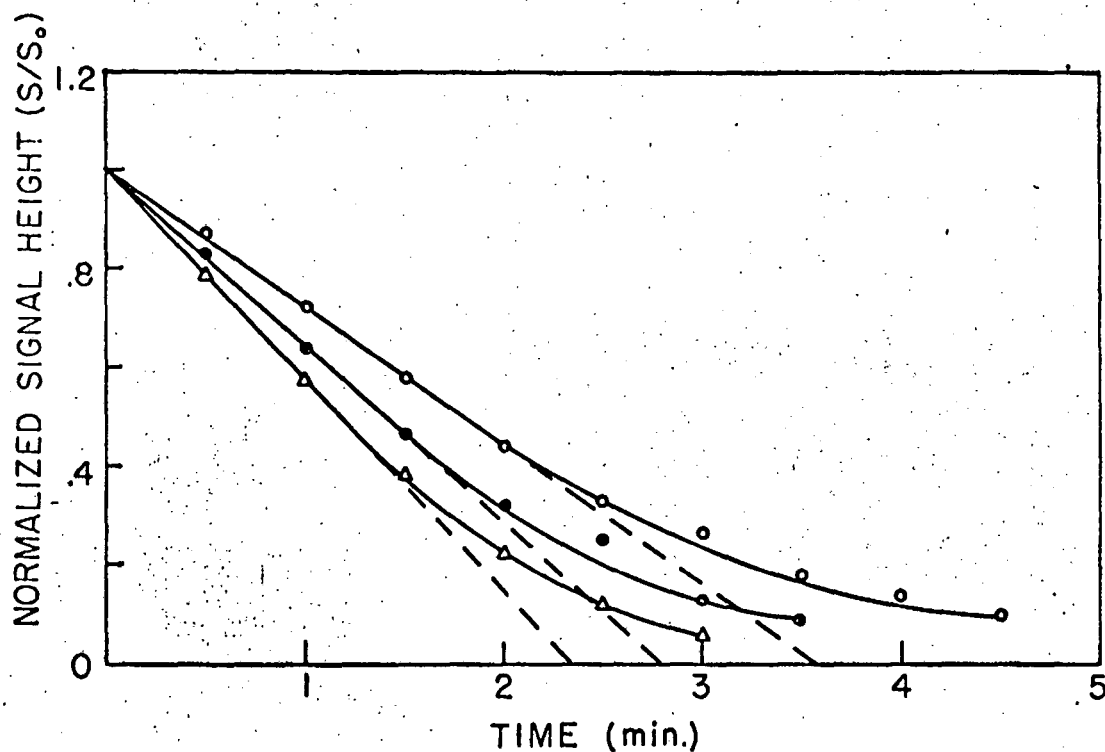
XBL 679-6164

Figure 22. Photo-induced decay of DTBN EPR signal in three identical samples freshly prepared with approximately 45 min between each sample.



XBL 679-6158

Figure 23. Photo-induced decay of DTBN EPR signal sensitized by chloroplasts as a function of the initial concentration of DTBN. The concentrations of DTBN were 1.5×10^{-4} M (closed triangles), 3.1×10^{-4} M (open triangles), 4.6×10^{-4} M (closed circles), and 6.6×10^{-4} M (open circles).



XBL 679-6152

Figure 24. Photo-induced decay of DTBN EPR signal sensitized by chloroplasts showing the effects of O_2 upon the observed decay. One sample was purged with nitrogen (open triangles); one sample was untreated (closed circles); and one sample was purged with oxygen (open circles).

chloroplast suspension was divided into three portions. One portion was purged with nitrogen for 20 min, one was purged with pure oxygen for 20 min, and the third was untreated. Even though the effect of oxygen on the decay rate is small in comparison to the effects of the other chemicals used in these studies, a standard procedure of purging the chloroplast suspension and the nitroxide solution for 20 min each was adopted.

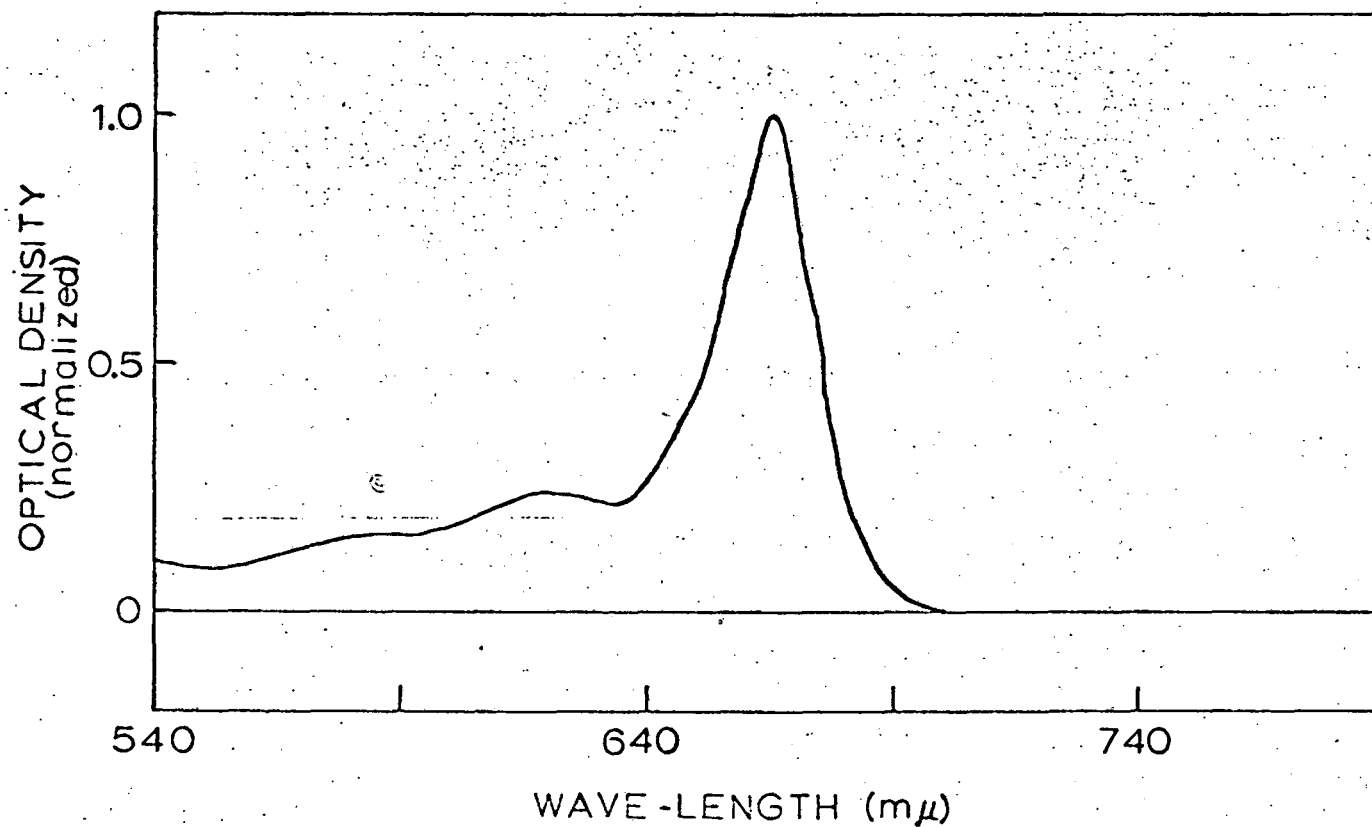
The chlorophyll content of each chloroplast suspension was determined according to a method of Vernon.¹¹³ The pigments were extracted from known volumes of the chloroplast suspensions with 80% acetone. The acetone extracts were brought to known volumes and visible absorption spectra were recorded. A typical spectrum is shown in Figure 25. The following equations were used to determine the chlorophyll content in mg/ml of chloroplast suspension:

$$\text{Chl. } \underline{a} \text{ (mg/l)} = 11.63 (A_{665}) - 2.39 (A_{649}) \quad \text{XII-a}$$

$$\text{Chl. } \underline{b} \text{ (mg/l)} = 20.11 (A_{649}) - 5.18 (A_{665}) \quad \text{XII-b}$$

where A_{665} and A_{649} are the optical density of the acetone extract measured at wavelengths of 665 mμ and 649 mμ, respectively. The total amount of chlorophyll ($\underline{a} + \underline{b}$) contained in the suspensions used in these studies varied between 0.5 mg to 1.5 mg per ml of suspension.

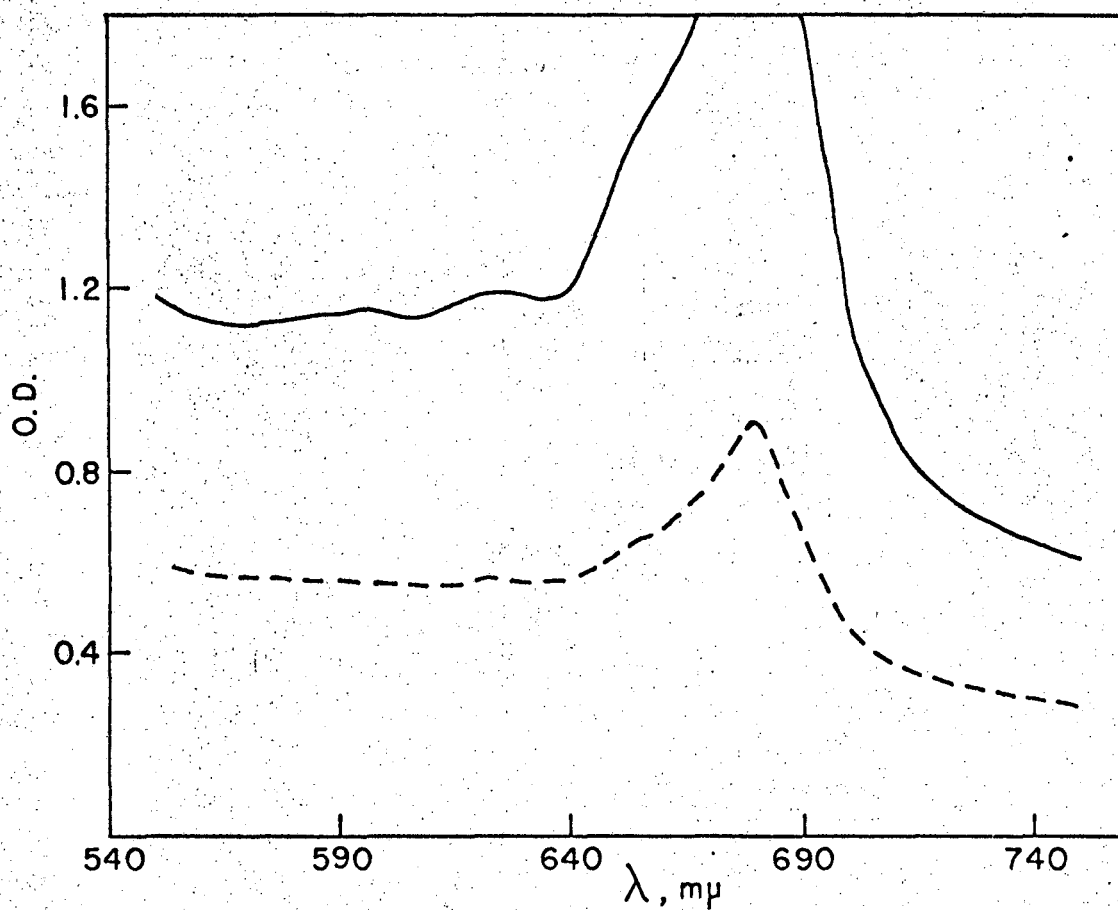
Figure 26 shows the absorption spectrum (solid line) of a typical chloroplast suspension (total chlorophyll = 1.0 mg/ml) recorded using the EPR cell (0.4 mm path length) taken against air. The dashed line in this figure is the spectrum of the same sample diluted approximately one to five. Although these samples were complicated by light scattering by the suspended particles, one can calculate from the chlorophyll concentrations, the extinction coefficients and path length, that the



-08-

XBL 679-6169

Figure 25. Optical absorption spectrum of 80% acetone/water extract of spinach chloroplasts normalized to the optical density at 665 mμ.



XBL 678-4578

Figure 26. Optical absorption spectrum of chloroplast suspension in EPR cell (path length 0.4 mm). For the solid line the suspension contained 1.0 mg chlorophyll (a+b) per ml of suspension; the dashed line is for the same sample diluted approximately 1 to 5.

absorption at 678 m μ should be ca. 3.4 optical density units for the solid line.

This large O.D. value signifies a large, exponential variation in the light intensity through the sample. This is true for all the samples examined in these studies. A rigorous kinetic analysis of the experimental data is precluded because of this non-linear variation of the light intensity and also because non-monochromatic light was used (see above). Therefore, the initial rate of decay of the nitroxide or the half-life of the decay are used to compare the results in the following discussion.

Results and Discussion

As stated above, when DTBN is mixed with chloroplasts and the resulting mixture is illuminated, a rapid irreversible destruction of the nitroxide follows. Since I had hoped that this destruction was a result of a chemical trapping of the nitroxide by the photo-induced paramagnetic species within the chloroplasts, I tried various perturbations which affect the chloroplast EPR signals to see what effect these had on the nitroxide destruction.

Since the EPR spectrum of DTBN mixed with chloroplasts does not exhibit any asymmetry which would indicate that the nitroxide was associating with the chloroplasts or a chloroplast component,¹¹⁴ a mixture of DTBN (10^{-4} M) and chloroplasts in 0.5 M sucrose buffered at pH 6.8 was centrifuged at approximately 100 x g for 5 min, washed twice by resuspending in buffer solution and re-centrifuging at the same speed, and finally resuspended in buffer solution.

The level of nitroxide detected in the resulting solution was lower than the original. However, this is probably due in part to

the washing and in part to the reaction, since it is impossible to exclude light completely during the washing procedure. The DTBN which did remain was still sensitive to light. These results indicate that the nitroxide is soluble in the chloroplasts--a result which was expected because of the affinity of the two tertiarybutyl groups of the nitroxide for the lipid components of the chloroplasts.

As shown in Table III of Chapter I, if a chloroplast suspension is heated at 50°C for 5 min, signal I is enhanced while signal II is no longer detectable. These observations were repeated and confirmed. However, it was also observed that the drastic treatment of heating the chloroplast at 100°C also converts the bulk of the chlorophyll to pheophytin. When the chloroplasts are subjected to this type of treatment, the photo-destruction of the nitroxide is affected as shown in Table VIII. Also shown are data obtained with samples of the chloroplast preparations which were not heated.

Table VIII

Treatment	DTBN Conc.	k_1 (%/min/mg Chl)	$t_{1/2}$
R.T. - 15 min	1.6×10^{-5} <u>M</u>	--	.70
64° - "	"	--	.72

R.T. - "	4.7×10^{-4} <u>M</u>	-24.0	5.04
100° - "	"	- 0.9	--

These results suggest that the photo-destruction of the nitroxide is connected with the capacity of the chloroplasts to exhibit signal I.

Another treatment of the chloroplasts which causes an apparent increase in the level of signal I, is the addition of DCMU to the suspension.⁶⁸ Therefore, the effect of DCMU on the destruction of the nitroxide was investigated with the results shown in Table IX.

Table IX

Concentrations DTBN	DCMU	$t_{1/2}$ (min)	k_1 %/min/mg Chl
$2.08 \times 10^{-4} \text{ M}$	0	1.3	-18.3
"	$2.28 \times 10^{-4} \text{ M}$?	-0.48

$5.0 \times 10^{-5} \text{ M}$	0	1.05	-46.0
"	$4.7 \times 10^{-4} \text{ M}$?	0

$1.2 \times 10^{-5} \text{ M}$	0	7.6	100*
"	$5.0 \times 10^{-5} \text{ M}$	0.68	7.8*

*Chlorophyll content unknown; rate in % min.

The blockade of the photoreaction of DTBN by DCMU, together with the apparent increase of the amplitude of signal I in DCMU-treated materials,⁶⁸ precludes the notion that DTBN is coupling with the species responsible for this particular photo-induced signal. DCMU is a potent inhibitor of photosynthetic oxygen evolution and electron flow in general. Thus, it is conceivable that DCMU blocks the formation of a radical species which contributes only slightly to the EPR pattern obtained with chloroplast suspensions, and it is this radical with which the nitroxide might couple.

The inhibitory effect of DCMU upon the photo-reduction of NADP can be removed through the use of ascorbate and a catalytic amount of DCPIP.¹¹⁵ In addition, during the course of NADP reduction with DCMU-poisoned chloroplasts, using ascorbate and DCPIP, photophosphorylation also occurs.¹¹⁶ These observations, interpreted in terms of the mechanism in Figure 1, suggest that the DCPIP is reduced by the ascorbate, and, in the reduced form, interacts with an intermediate in the electron transport chain connecting the two photo-acts. Thus, photosystem I operates, but photosystem II does not.

Unfortunately, DTBN is not stable to Na-ascorbate. However, it is stable to KBH_4 and the reduced form of DCPIP. Therefore, DCPIP was reduced by KBH_4 in water, and this was added to a DCMU poisoned chloroplast preparation in order to see whether the inhibition caused by DCMU upon the nitroxide reaction could be removed. The results of this experiment are shown in Table X.

Table X

DTBN	Concentrations DCPIP (red.)	DCMU	k_i %/min/mg Chl
$2.08 \times 10^{-4} \text{ M}$	0	0	-18.3
"	$7.4 \times 10^{-5} \text{ M}$	0	- 6.4
"	0	$2.3 \times 10^{-4} \text{ M}$	- 0.48
"	$7.4 \times 10^{-5} \text{ M}$	"	- 0.19

As is evident in the table, the reduced form of DCPIP does not lift the inhibition of DCMU and, in fact, it also causes a partial

inhibition of the nitroxide destruction. The reduced form of DCPIP also causes an increased rate of the dark destruction of the nitroxide. It was also observed that treatment of the chloroplasts with KBH_4 caused an increase in the dark destruction of the nitroxide and a slower light decay. KBH_4 will reduce plastoquinones, NADP and probably other compounds found within the chloroplasts. This is probably true for the reduced dye also. The increased dark destruction of DTBN in these systems suggests that the dark decay is a reduction. The inhibition of the light reaction by these reagents probably is the result of a reduction of a compound in the chloroplasts which is required in its oxidized form in order to obtain efficient electron turn-over. Plastoquinone is one possible candidate, especially if its function in the chloroplasts is to accept electrons from photosystem II.

Since chloroplasts contain plastoquinones which undergo photo-reductions which are inhibited by DCMU,⁴⁵ and since quinones are known to form relatively stable semiquinones, the possibility of an interaction between DTBN and plastoquinone was investigated. Bishop³⁹ has shown that if chloroplasts (or fragments) are extracted with petroleum ether, the quinones are removed from the chloroplasts, causing a reduction in their photosynthetic activity as measured by oxygen evolution or reduction of a Hill reagent. If the petroleum ether extract or a solution of plastoquinone in petroleum ether is evaporated onto the fragments which had been extracted, a recovery of photosynthetic activity is observed.

As shown in Table XI, when chloroplasts are extracted with heptane, the initial rate of photo-destruction of the nitroxide is drastically reduced. Also shown is the effect of adding Plastoquinone-A (PQ-A).

back to the extracted chloroplasts using the evaporation procedure of Bishop. The effects of evaporating pure heptane and the heptane extract are also shown. The addition of PQ-A by this procedure causes a slight recovery of the lost activity. The effect of the heptane extract is understandable. The extracted PQ could have undergone decomposition and the heptane extracted more PQ. However, the effect of pure heptane casts doubts upon the experimental procedure.

Table XI

Concentration DTBN	k_i (%/min/mg Chl)				
	Untreated	Extracted	PQ-A*	Heptane	Extract
4.7×10^{-4} M	24.0	6.0	9.1	--	--
1.5×10^{-4} M	14.0	7.0	9.0	--	--
2.5×10^{-4} M	13.9	5.8	--	--	2.2
2.7×10^{-4} M	--	5.3	5.3	5.3	--
1.7×10^{-4} M	--	11.9	14.1**	13.5	--

*Saturated solution of PQ-A in heptane used.

**Concentration of PQ-A in heptane in 3.6 mg/400 λ .

The method used in these experiments involved extracting wet-packed chloroplasts with heptane (ca. 1 ml heptane per mg wet-pack) at 0°C in the dark. The resulting chloroplasts were suspended in buffered sucrose solution which was divided into several portions. Some of these portions were used to observe the nitroxide destruction rate with extracted chloroplasts. The remainder were centrifuged. The solution to be tested (either heptane, PQ-A in heptane, or extract) was applied

by evaporation under a N_2 stream to the chloroplast pellets resulting from the centrifugations. These chloroplasts, suspended in sucrose buffer, were used to observe the rate of the nitroxide destruction.

The results indicate that the photo-destruction of the nitroxide is connected with the presence of plastoquinones. However, this is not conclusive since it is not known exactly what, in addition to the extraction of the quinone and β -carotene, heptane does to the chloroplasts. Even if the decrease in rate observed with extracted chloroplasts is due to the absence of plastoquinone, this does not distinguish between a radical coupling of the nitroxide with the semiquinone and a reduction or an oxidation of the nitroxide.

If one assumes that the inhibitory effect of DCMU occurs at only one site within the photosynthesizing organism, namely, on the oxygen-evolving side of the mechanism depicted in Figure 1, the data in Table IX (effects of DCMU) suggest that the nitroxide is oxidized or reduced by a species activated by photosystem II (see Figure 1).

A sample containing DTBN ($.01\text{ M}$) and chloroplasts was treated with $K_3Fe(CN)_6$ ($.01\text{ M}$) in the dark in a sealed flask. Examination of the atmosphere in the flask by VPC after 30 min of reaction showed the presence of both isobutene and 2-methyl-2-nitrosopropane. However, if the $K_3Fe(CN)_6$ was excluded and instead the chloroplasts-DTBN mixture was exposed to light, these two products were not detected. Furthermore, no absorption due to 2-methyl-2-nitrosopropane was detected in the UV-vis absorption spectra of samples of chloroplasts in which the nitroxide radical had been destroyed by light. These results rule out the possibility that the photo-destruction of the nitroxide involves an oxidation.

In order to determine whether the DTBN-chloroplast interaction involved a reduction of DTBN to hydroxylamine or to an oxygen substituted hydroxylamine which would result by a coupling of DTBN with a free radical species, the effect of two Hill reagents upon the nitroxide destruction was examined. The Hill reagents used were $K_3Fe(CN)_6$ and DCPIP. The results are contained in Table XII.

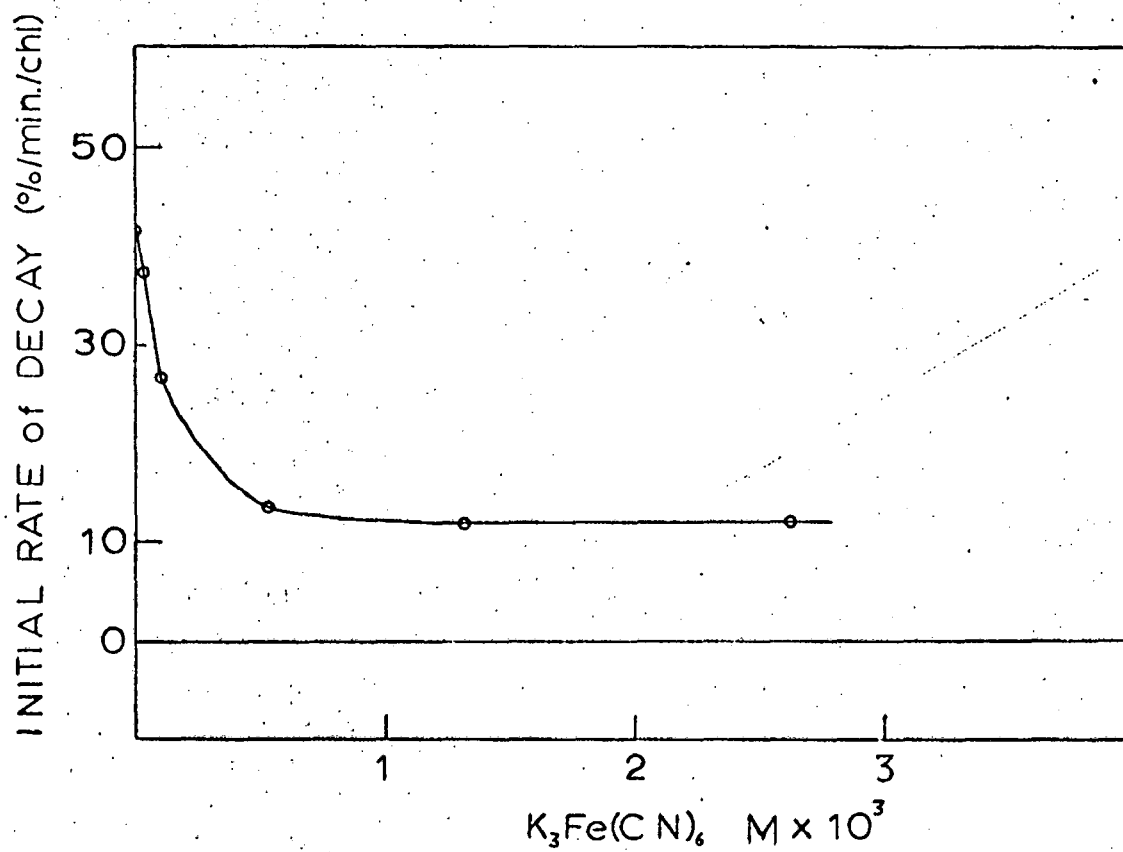
Table XII

DTBN Concentration	Hill Reagent	k_d (%/min/mg Chl)
2.08×10^{-4} M	(none)	-18.3
"	7.4×10^{-5} M DCPIP	-16.1
"	5.9×10^{-4} M $K_3Fe(CN)_6$	-12.9

The inhibitory effect of $K_3Fe(CN)_6$ can be removed by washing the treated chloroplasts with buffer solution. This effect is shown in Table XIII. In addition, the effect of increasing $K_3Fe(CN)_6$ concentration upon the nitroxide decay rate was investigated with the results in Figure 27. These types of experiments were not performed with the DCPIP-treated chloroplasts.

Table XIII

DTBN	Concentrations $K_3Fe(CN)_6$	Before washing k_d (%/min/mg)	After washing k_d (%/min/mg)
1.0×10^{-4} M	0	-47.7	-24.6
"	2.2×10^{-3} M	-11.5	-24.2



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Figure 27. Plot of the initial rate of decay of DTBN EPR signal sensitized by chloroplasts as a function of $K_3Fe(CN)_6$ in the sample.

The partial inhibitory effects caused by the Hill reagents suggest that a competition for electrons exists between DTBN and these reagents. The reversibility of the inhibition caused by $K_3Fe(CN)_6$ shows that ferricyanide does not cause permanent damage to the chloroplasts and supports the proposal that the inhibition stems from a competition between DTBN and $K_3Fe(CN)_6$ for electrons. The leveling of the curve in Figure 27 indicates that the sites of interaction of DTBN and $K_3Fe(CN)_6$ within the chloroplasts are different. Several mechanisms could be proposed to explain the shape of this curve; however, sufficient experimental data to justify any of them are not available.

Salicylaldoxime is thought to remove the copper atoms from plastocyanine (Pcy),¹¹⁷ a copper-containing protein that plays a role in the electron transport mechanism of photosynthesis (see Figure 1), thus terminating electron flow at this point.

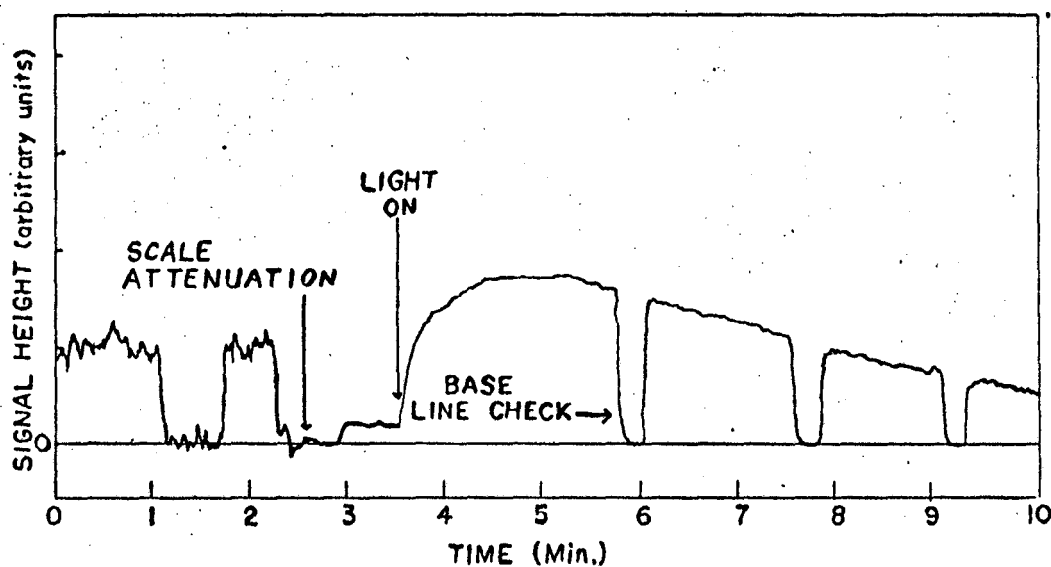
When chloroplasts were incubated in this oxime for 20 min prior to adding DTBN, the initial rate of decay of the nitroxide decreased from 32%/min/mg Chl for a sample containing untreated chloroplasts to 12%/min/mg Chl for the sample with oxime. The concentrations of DTBN and the oxime were 1.5×10^{-4} M and 9.6×10^{-3} M, respectively. This result indicates that the nitroxide reaction is connected with photosynthetic electron transport. However, it yields no information as to the mechanism of the nitroxide destruction.

The results of the experiments reported in this section suggest that the photochemical destruction of DTBN by chloroplasts involves a reduction. However, the product which would result from a reduction of DTBN, ditertiarybutylhydroxylamine (DTBNH), has no physical property

which would allow one to detect it readily in mixtures containing chloroplasts. In addition, this hydroxylamine is extremely sensitive to atmospheric oxygen and one would expect the nitroxide destruction to be reversed in the dark upon exposure to air unless another factor was competing for the oxygen and acting as an anti-oxidant.

To test this proposition, DTBNH was prepared as reported in Appendix II. This reduced form of the nitroxide is not oxidized in the dark in the presence of chloroplast fragments and air, nor with freshly isolated chloroplasts and air. However, if the fragment mixture is illuminated with light of wavelengths greater than 540 mμ, a rapid increase of the nitroxide resonance signal occurs followed by a gradual decay. This effect is shown in Figure 28. This effect is also observed in samples containing DTBN and freshly isolated fragments--preparations in which 80-95 per cent of the nitroxide has disappeared during the period between the preparation of the samples and detection of the nitroxide signal in the EPR spectrometer. The change in signal height is not as large in these latter samples as with the ones containing the deliberately reduced form and fragments. No signal increase is observed with fresh, whole chloroplasts with either DTBN or DTBNH, either in the dark or in the light. However, if the whole chloroplasts are washed with buffer several times, the effect shown in Figure 28 is also observed with them, suggesting that the rate of destruction of DTBN is too rapid with freshly isolated chloroplasts to detect the oxidation of DTBNH.

The increased signal height shown in Figure 28 was shown to be due to an enlarged nitroxide signal and not due to some other photo-generated radical by sweeping through the three-line spectrum of the



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Figure 28. Effect of light on DTBNH EPR signal in the presence of large excesses of DTBNH. Initial mixture contained 1.85 mg chlorophyll (a+b) per ml of suspension, 2.1×10^{-4} M DTBNH, 0.05 M sucrose, 0.05 M phosphate buffer, pH 6.8, N_2 atmosphere, illuminated with light of wavelength of 4500 Å to 9800 Å. Check of base line made by displacing magnetic field from resonance. Nitroxide present initially assumed due to air oxidation of DTBNH during sample preparation. Complete oxidation of DTBNH originally present would result in a signal height of 5.1 units on this scale.

nitroxide before and after illumination. In addition, the decay of the signal amplitude was shown to involve the photochemical degradation of the nitroxide. If the light is turned off, the decay stops and the signal levels off.

Although the samples which were exposed to light were purged with nitrogen prior to each experiment, it is conceivable that the increased signal height could be due to a photo-reaction involving the uptake of oxygen which would cause a narrowing of the width of the nitroxide resonance line and a concomitant increase of the signal height, if the concentration of DTBN remained constant. This is unlikely, however, since the magnitude of the signal change would require a narrowing of the signal by a factor of two or more. This narrowing was not observed. Also, oxygen is produced in this system, as will be shown below.

If a sample of the hydroxylamine with a concentration equal to that of the sample used to obtain the data shown in Figure 28 is oxidized with $K_3Fe(CN)_6$, the signal height of the nitroxide formed is 5.1 units on the scale used in this figure.

A separation of the oxidation of the hydroxylamine from the destruction of the nitroxide was attempted using a DCMU-poisoned chloroplast preparation. However, it was observed that DCMU also poisoned the photo-oxidation of the hydroxylamine.

Since it is assumed that light produces both an intermediate oxidant and an intermediate reductant in spinach chloroplasts, it is possible that both processes, oxidation and reduction, could operate simultaneously on the hydroxylamine-nitroxide system, thus establishing a steady-state ratio of the two. Since this does not occur

(the nitroxide becomes undetectable by EPR), there must be an irreversible drain of material from the chloroplast-nitroxide-hydroxylamine system.

As will be discussed below, the primary mode of destruction of the nitroxide is indeed a reduction to DTBNH. The observations that illuminated chloroplasts reduce the nitroxide to the hydroxylamine (causing a destruction of the nitroxide EPR signal) and oxidize the hydroxylamine to the nitroxide are in contradiction to one another. This disparity could result because of one of the following causes: (1) The species which oxidizes DTBNH is available in only limited quantity and is expended; (2) the ratio of the reduced form to the oxidized form maintained in illuminated chloroplasts is large and, therefore, the level of DTBN is low and undetectable by EPR; or (3) the identification of DTBNH as the reaction product of DTBN-illuminated chloroplasts' reaction is incorrect.

These three proposals will be discussed at greater length following the presentation of the data which show that the photochemical degradation of DTBN involves a reduction.

Summary: Kinetic Experiments

Di-tertiarybutylnitroxide undergoes both a dark and a photo-induced reaction in the presence of chloroplasts and light. The photo-reaction is inhibited by DCMU, an inhibitor of photosynthetic oxygen evolution and photosynthetic electron transport, which is thought to truncate electron flow in the reactions coupling the oxidation of water to photosystem II. This is consistent with the observation that R. rubrum chromatophores do not photo-react with DTBN and suggests that the reaction is connected with photosystem II

(see Figures 1 and 2). DCMU has no effect upon the dark reaction.

The dark reaction is stimulated by the presence of KBH_4 and the reduced form of DCPIP. However, these reagents impair the photo-reaction. The reduced form of DCPIP does not lift the inhibition caused by DCMU.

When plastoquinones are extracted from the chloroplasts with heptane, the photo-reaction is slower than normal, implicating the quinones in the photo-reaction; This conclusion is uncertain since not all the effects of heptane upon chloroplasts are known.

The presence of additional Hill reagents partially impedes the photo-reaction. This is interpreted as competition between DTBN and the Hill reagents for electrons.

Salicylaldoxime also partially inhibits the photo-reaction. This compound is thought to stop photosynthetic electron transport by binding the copper atoms of plastocyanine, a protein involved in the electron transport chain connecting the two photosystems operating in green plants and algae.

No products which would result from an oxidation of DTBN could be detected. In addition, the reduced form of DTBN undergoes a photo-oxidation to DTBN with illuminated chloroplasts. These results suggested that the DTBN reaction was an addition of DTBN to a component of the chloroplasts. This is inconsistent with evidence to be presented below. The oxidation of DTBNH is not in agreement with the rest of the results.

Measurement of Oxygen Evolution

With the exception of the experiments conducted with the reduced form of the nitroxide, the data reported in the previous section are

all consistent with an interpretation that the nitroxide undergoes a reductive degradation with illuminated chloroplasts. This implies that DTBN is functioning as a Hill reagent. Therefore, this system should evolve oxygen. The experiments discussed in this section deal with the measurement of oxygen evolution from the chloroplast-DTBN suspensions.

Experimental

The basic experiment described in this section is one which involves the use of a Warburg apparatus to measure gas exchange between the DTBN-chloroplast suspensions and the atmosphere above these suspensions.

The photosynthetic materials used in these studies include freshly isolated chloroplasts, fresh chloroplast fragments, and aged chloroplast fragments. These were suspended in 0.5 M sucrose solution buffered at pH 6.8, to give suspensions varying in concentration of chlorophyll between 0.5 mg and 1.5 mg per ml of suspension. The chlorophyll content of each suspension was determined according to the method reported in the previous section.

The procedure followed in these experiments involved placing 2 ml of the chloroplast suspension in the main body of the Warburg flask and 1/2 ml of buffer, DTBN or $K_3Fe(CN)_6$ solution in the side arm. The system was then isolated from air and allowed to equilibrate in the dark with shaking at the temperature of the constant temperature bath (normally 21°C).

Once the readings on the micromanometers became constant, the contents of the side arms were added to the chloroplast suspensions. Generally this caused an irreversible change in the pressure levels

in the systems. The mixing was done in a darkened room using a dim flashlight. The samples were allowed to equilibrate again until the micromanometer readings were constant. The lights (bank of photo spots) were turned on and the change in pressure followed with time. A flask containing water was used to measure fluctuations in the barometric pressure of the room and in the temperature of the water bath. The pressure changes recorded for the other systems were corrected for these fluctuations.

The change in pressure in the flasks is measured with micromanometers calibrated in centimeters. These readings can be converted into μ moles of oxygen evolved by the use of the following equation:

$$\mu\text{moles O}_2 \text{ evolved} = \frac{k \cdot h}{22.4}$$

$$\text{where } k = \frac{V_g \frac{273^\circ\text{K}}{T} + V_f \alpha}{P_0}, \quad h = \text{manometer reading in mm, and } V_g \text{ and } V_f$$

are the volumes of the gas phase and liquid phase, respectively; T , the temperature in degrees Kelvin; P_0 , Brodie's manometer fluid constant equal to 10^4 ; α , the solubility of oxygen in the liquid phase.

Most of the data in this section is reported in changes of pressure in centimeters. Where μ moles of oxygen are used, the appropriate flask constant was calculated assuming α equal to the solubility of oxygen in water at the proper temperature (0.031 @ 21°C) and the centimeters were converted into μ moles of O_2 .

The amounts of DTBN destroyed in the dark and in the light were measured using identical samples to those used for measurement of the pressure change. Known volumes of these samples were diluted 1:100 and EPR spectra were taken at time zero and following the illumination

period. The ratio of the signal heights times 100 were taken as the per cent nitroxide destroyed. Using the concentration of nitroxide initially present and this percentage, the μ moles of nitroxide destroyed were calculated.

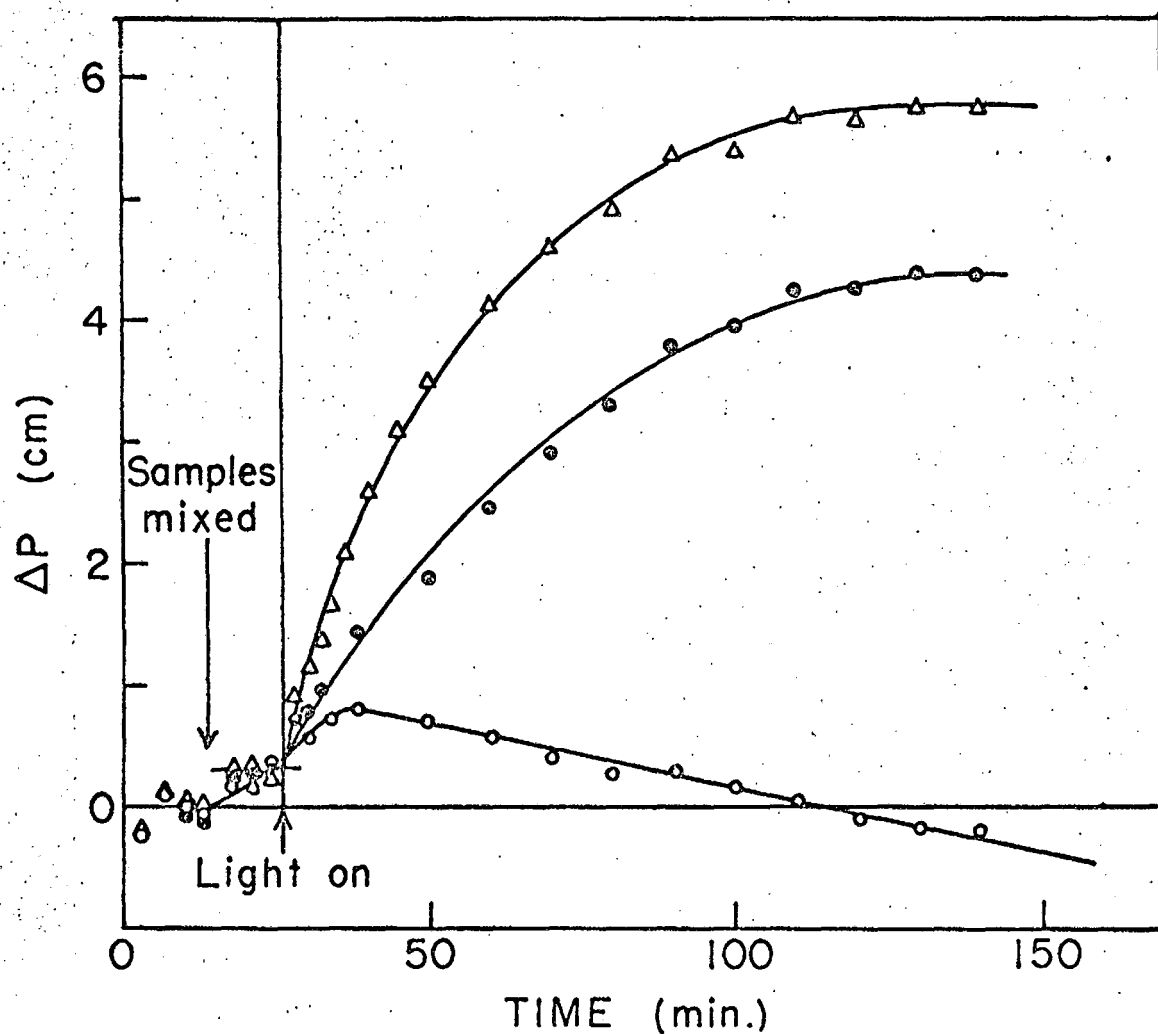
Results and Discussion

When mixtures containing freshly isolated chloroplasts and $.01 \text{ M}$ DTBN are examined in the Warburg apparatus with illumination, the pressure above the suspension increases as shown in Figure 29. Also included in this figure are the pressure variations observed in two systems, one containing the same chloroplast preparation with 0.01 M $\text{K}_3\text{Fe}(\text{CN})_6$, the other the chloroplast suspension and buffer.

Vapor phase chromatography of the gas phase of the samples described above failed to show that the increases of pressure observed in these samples were due to increases of molecular oxygen. The amounts of O_2 initially present in these samples prevented the detection of the small quantity of gas produced in these systems.

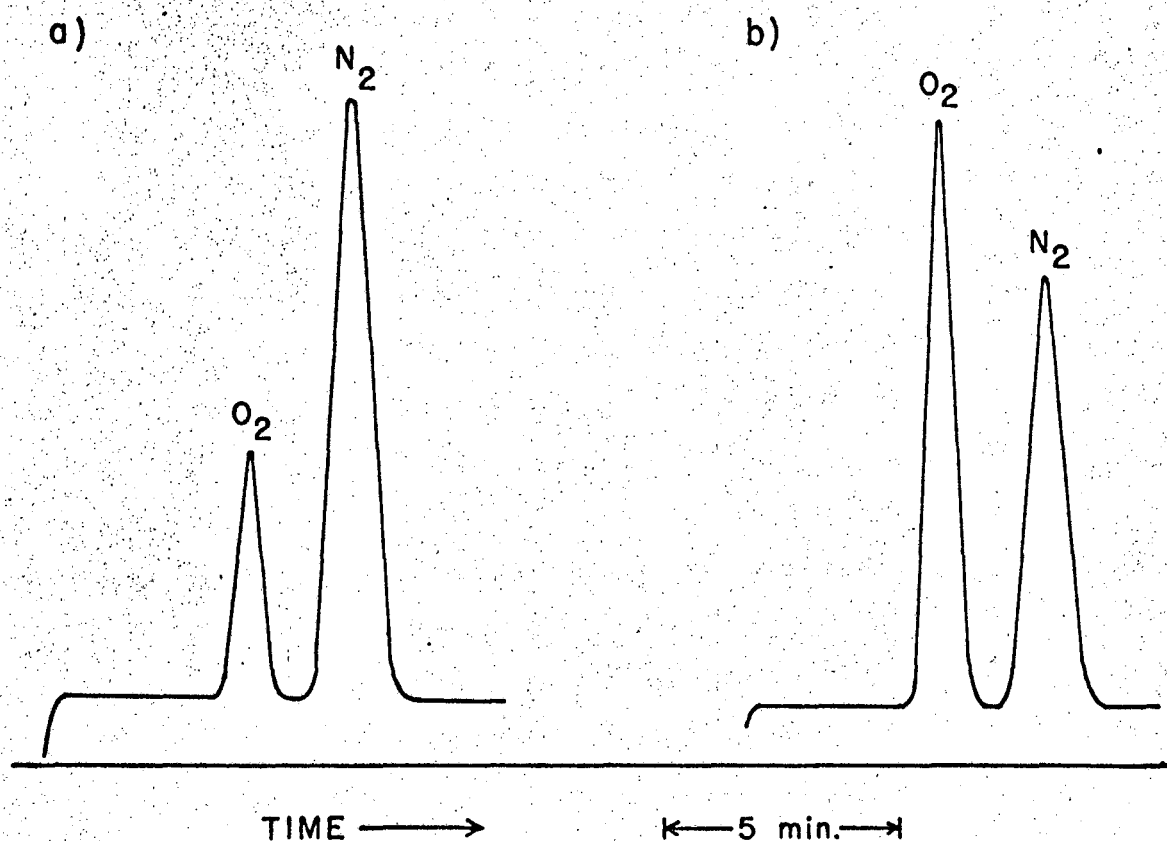
However, when identical samples are prepared in flasks sealed with rubber septums and these flasks are purged with helium (the gas used for the VPC carrier gas) prior to the illumination, most of the air is removed. The results shown in Figures 30 and 31 were obtained when the gases over these samples were analyzed by VPC.

These results conclusively demonstrate that DTBN functions as a Hill reagent. However, DTBN could couple with a free radical produced by the electrons obtained from the water through photosystem II (see Figure 1). If this were the case, oxygen would still be produced and DTBN would be functioning as a Hill reagent--however, not in the normal sense. The reduction would be by another radical



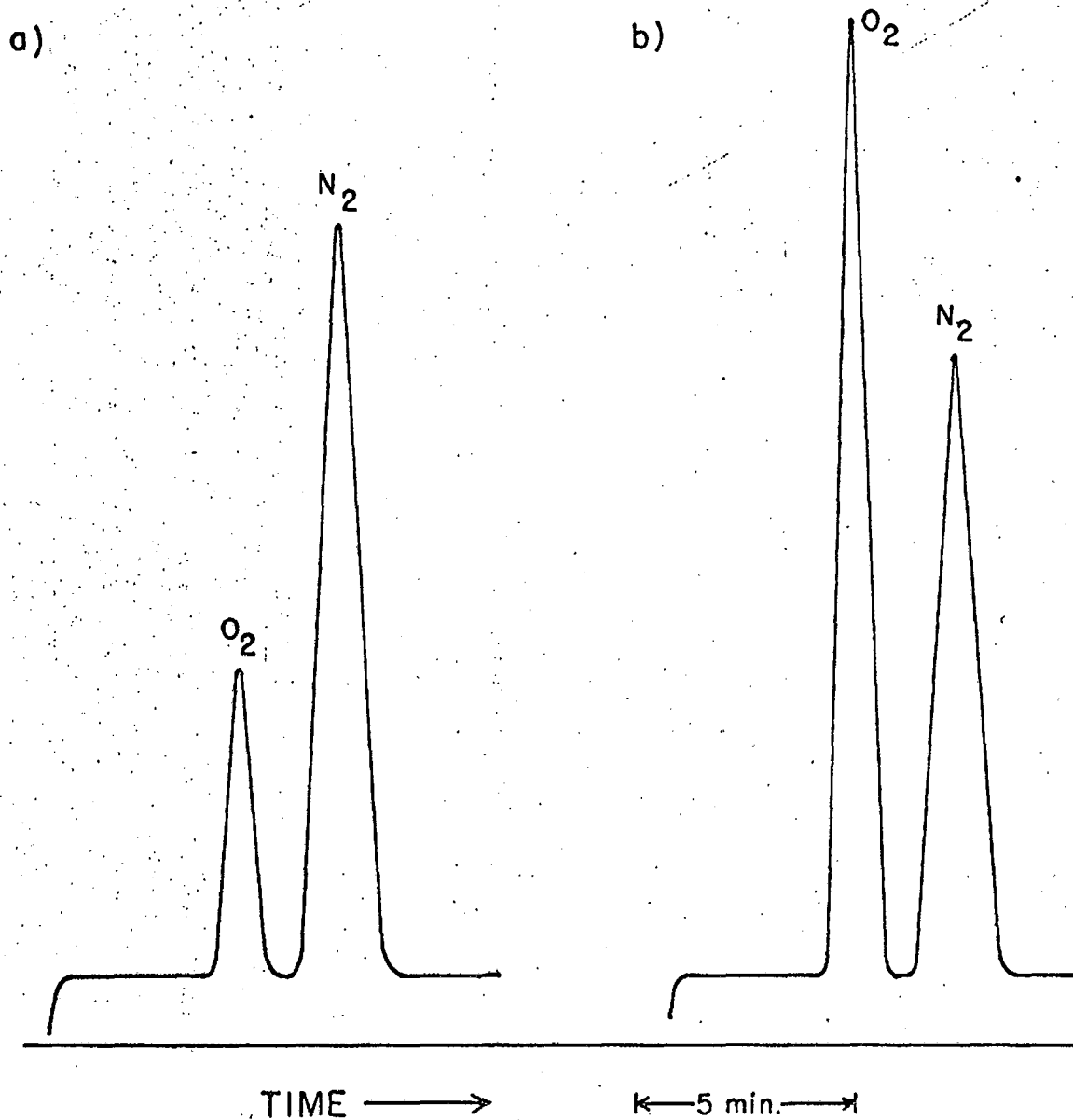
XBL 679-6165

Figure 29. Pressure changes above fresh chloroplast suspensions containing 1.10 mg chlorophyll (a+b) with 0.01 M $K_3Fe(CN)_6$ (open triangles), 0.009 M DTBN (closed circles) and buffer (open circles). Illuminated with white light.



XBL 679-6166

Figure 30. VPC traces of a sample of the atmosphere above a chloroplast suspension containing 0.92 mg chlorophyll (a+b) and 0.003 M DTBN before (a) and after (b) illumination of the sample for 1 hr with white light. The column was Molecular Sieve 5-A. The temperature of the column, injector, and detector were 55°, 180° and 250°C, respectively. The flow was 12 ml/min. The vessel was purged initially with helium.



XBL 679-6146

Figure 31. VPC traces of a sample of the atmosphere above a chloroplast suspension containing 0.92 mg chlorophyll (a+b) and 0.01 M $K_3Fe(CN)_6$ before (a) and after (b) illumination of the sample for 1 hr with white light. The column was Molecular Sieve 5-A. The temperature of the column, injector, and detector were 55°, 180° and 250°C, respectively. The flow was 12 ml/min. The vessel was purged initially with helium.

instead of a hydrogen atom.

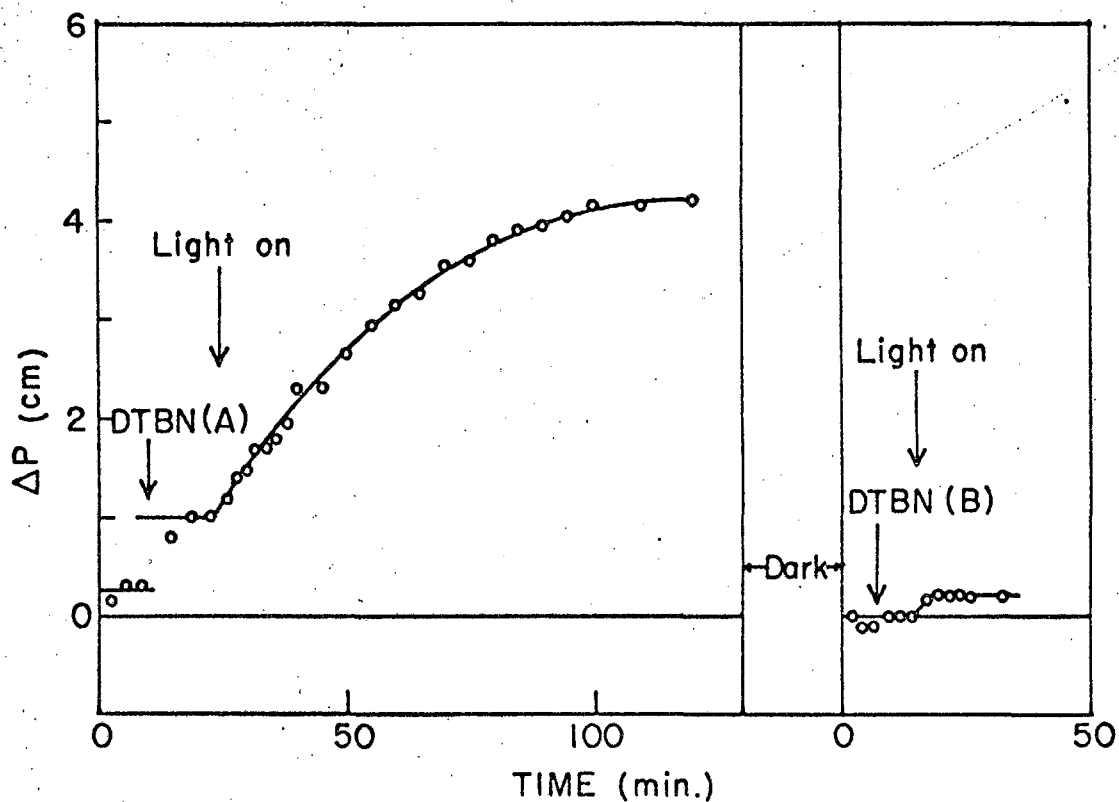
As shown in Figure 29, after approximately 100 min of illumination, oxygen production in these systems stops. This could result in three ways: (1) the Hill reagent is expended; (2) the chloroplasts have become damaged either through aging in suspension with shaking at 21°C or through an irreversible photo-induced change; or (3) the reaction with $K_3Fe(CN)_6$ or the nitroxide removes (or damages) a compound (or compounds) required for photosynthetic electron transport.

Several experiments were conducted seeking to differentiate between these three possibilities. The results are reported in Figures 32 through 35.

Figure 32 shows the data from an experiment which involved using a Warburg flask with two side arms. Equal quantities of DTBN solution were put into these side arms. One of these was added to the chloroplasts, the resulting mixture was illuminated and the pressure changes followed until oxygen production stopped. The vessel was then opened to air, equilibrated, sealed again, and the procedure repeated. This data demonstrates that termination of oxygen production is not caused by a lack of Hill reagent.

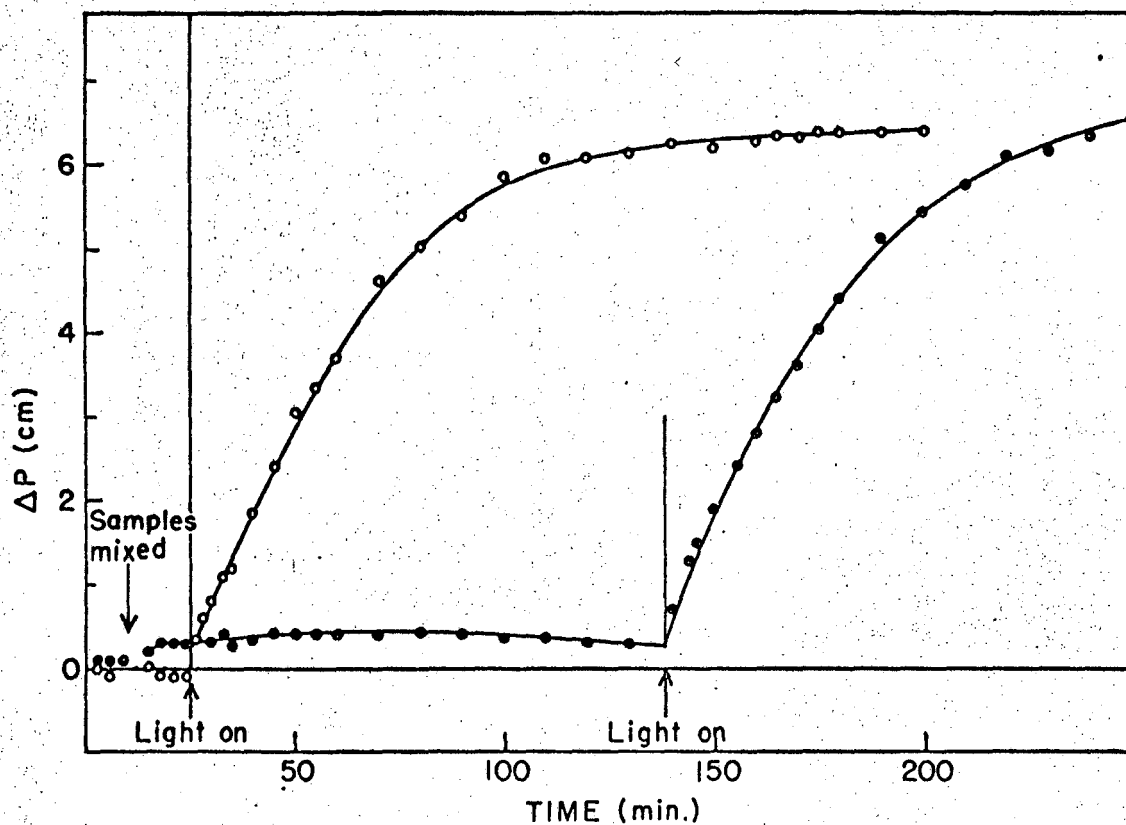
Four separate vessels were used to obtain the data shown in Figures 33 and 34--two containing chloroplasts with DTBN, the other two, chloroplasts with $K_3Fe(CN)_6$. In these experiments the Hill reagents were added to the chloroplast at essentially the same time.

Two samples (one with DTBN, the other with $K_3Fe(CN)_6$) were exposed to light and two were kept in the dark by wrapping them with metal foil. When the production of oxygen had stopped in the samples exposed to light, the other two samples were illuminated. As can be



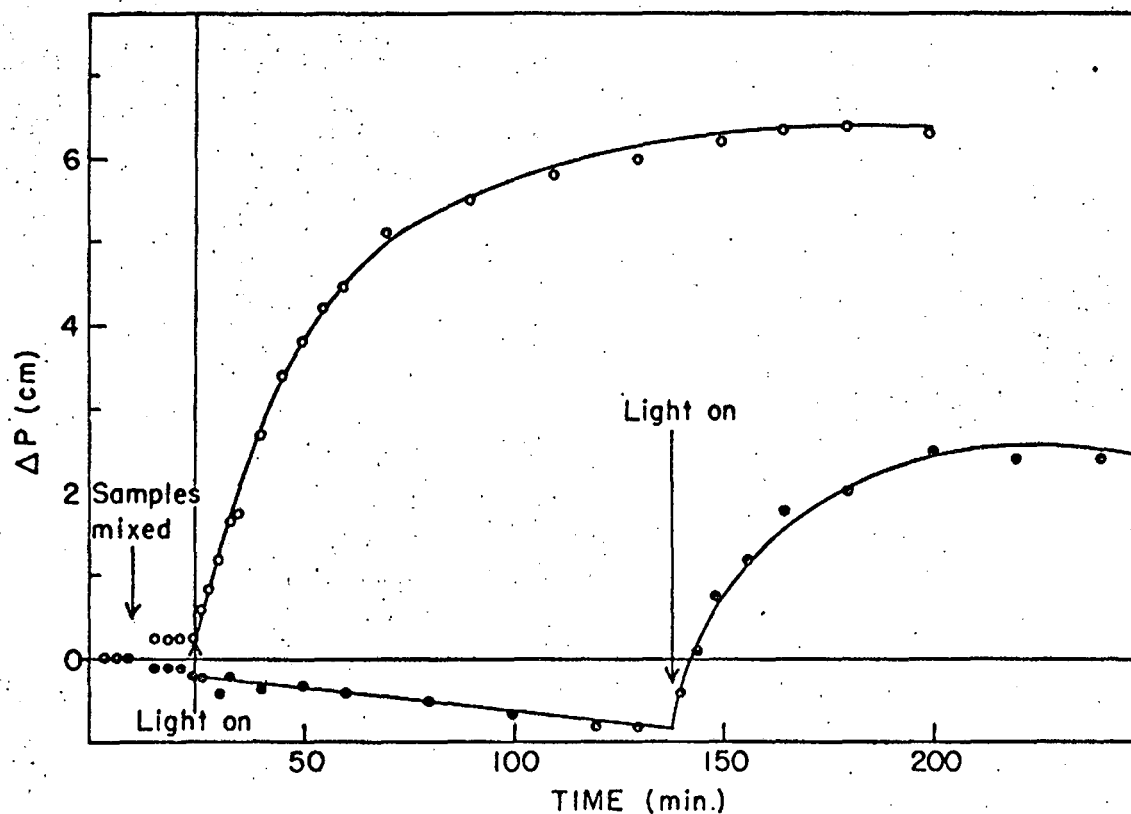
XNL 679-6156

Figure 32. Pressure changes above chloroplast suspension containing 0.013 M DTBN showing that after the evolution of oxygen from a sample stops, the addition of more DTBN does not promote the evolution again.



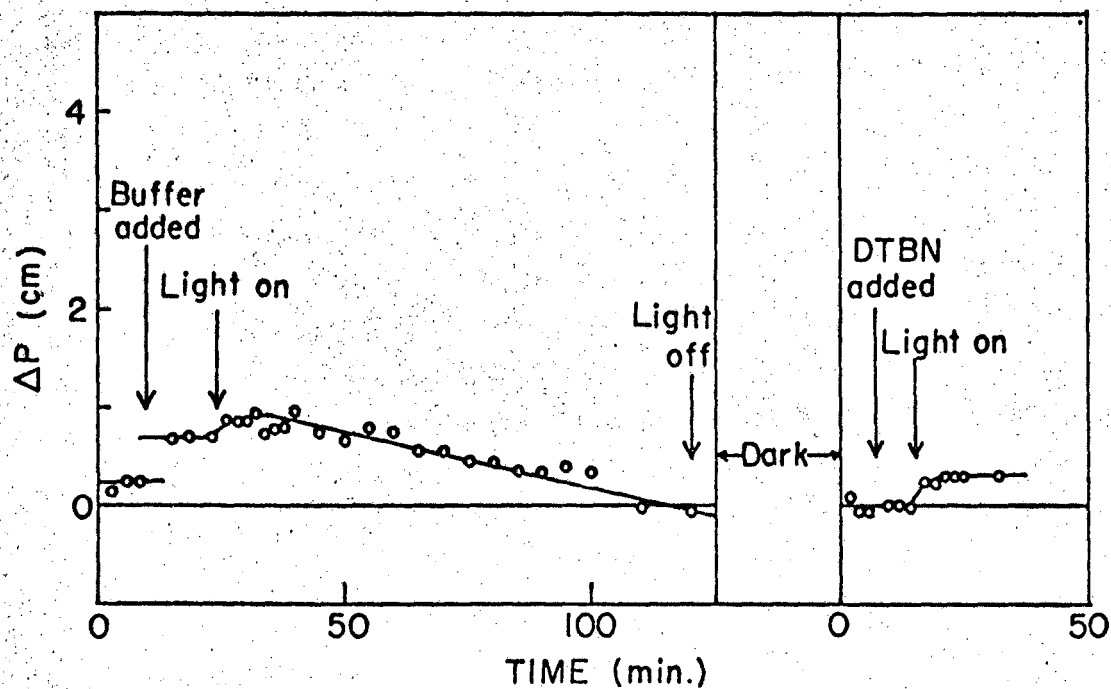
XBL 679-6160

Figure 33. Pressure changes above chloroplast-DTBN mixtures showing the effect of incubating the chloroplasts in the dark at 21°C for 138 min with DTBN. The mixtures contained 1.10 mg of chlorophyll (a+b) and .01 M DTBN.



XBL 679-6159

Figure 34. Pressure changes above chloroplast- $K_3Fe(CN)_6$ mixtures showing the effect of incubating the chloroplasts in the dark at $21^\circ C$ for 138 min with $K_3Fe(CN)_6$. The mixtures contained 1.10 mg chlorophyll (a+b) and $0.01 \text{ M } K_3Fe(CN)_6$.



XBL 679-6147

Figure 35. Pressure changes above chloroplast-DTBN mixture showing the effect of illuminating the chloroplast suspension prior to adding the DTBN. The mixture was 0.013 M in DTBN with 1.21 mg of chlorophyll (a+b).

seen in the figures, incubation of the chloroplasts with the Hill reagents at 21°C and shaking had little effect upon their capacity to evolve oxygen for the sample containing DTBN. The ferricyanide apparently causes damage to the chloroplast in the dark.

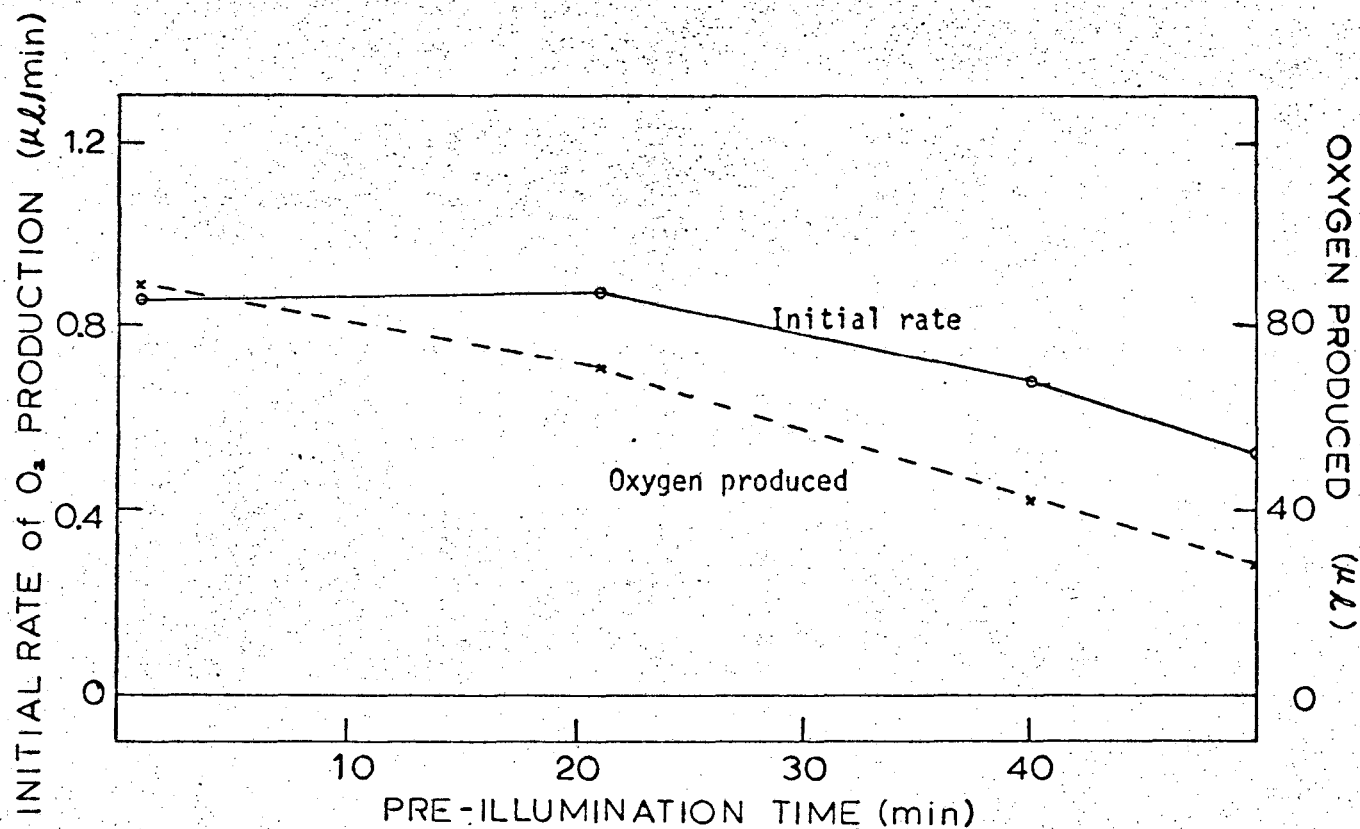
Figure 35 shows the effect of illuminating the chloroplast prior to adding DTBN. The chloroplasts used in this experiment were the same as those used to obtain the data shown in Figure 32. In fact, the data shown in Figures 35 and 32 were obtained simultaneously.

The result in Figure 35 demonstrates that chloroplasts subjected to white light for extended periods of time lose their capacity to evolve oxygen.

This same type of experiment was conducted using shorter periods of illumination before adding the DTBN in order to see how the initial rate of oxygen production is affected. The results are presented in Figure 36. Also included in this figure is a curve showing the variation of total oxygen output in these experiments versus the period of pre-illumination.

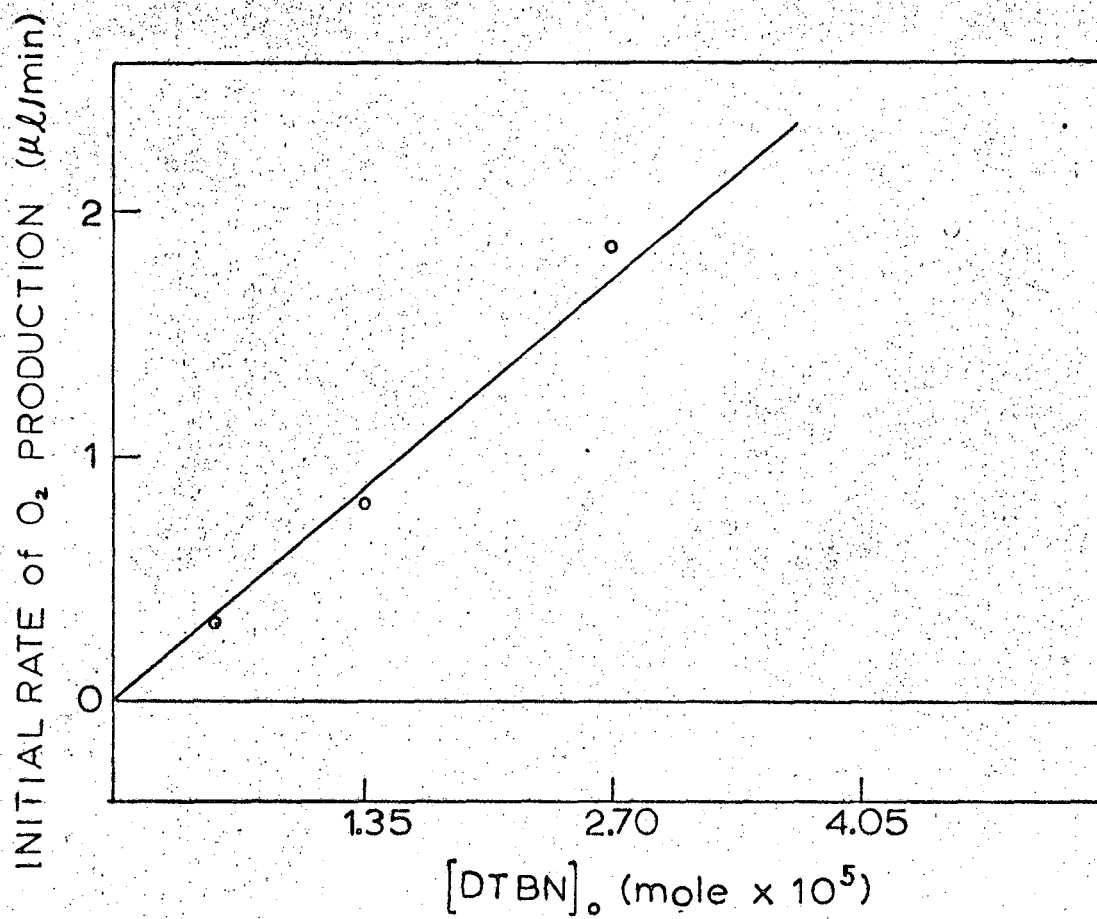
The results of Figures 35 and 36 demonstrate that the termination of oxygen production observed in these studies is due to a photochemical degradation within the chloroplast.

When the initial concentration of nitroxide is varied, the initial rate of oxygen production, the amount of nitroxide destroyed, and the total amount of oxygen produced also vary, and in a linear manner to the initial concentration of DTBN. (See Figures 37, 38 and 39.) The slope of the dashed line in Figure 38 divided by the slope of the line in Figure 39 gives the ratio of the moles of nitroxide



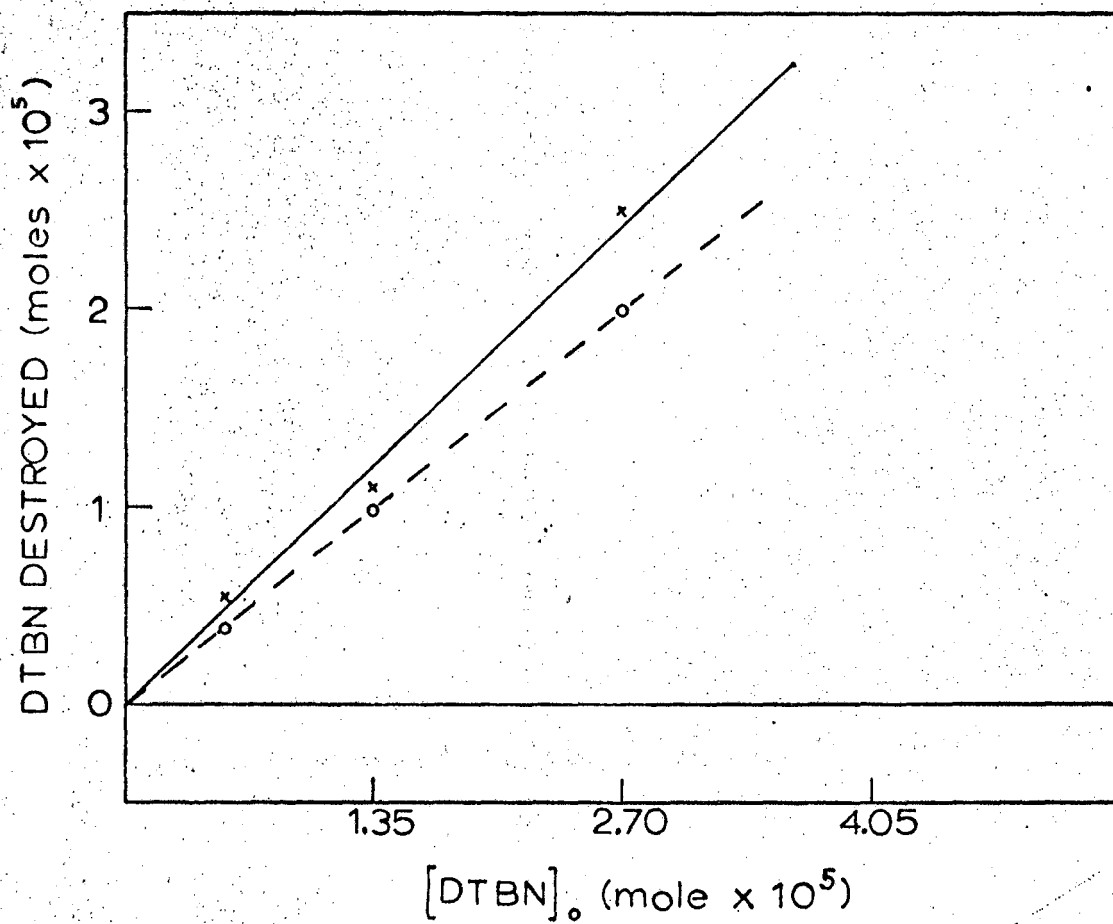
XBL 679-6142

Figure 36. Effects of pre-illumination of the chloroplast suspension prior to adding the DTBN upon the initial rate of oxygen evolution and the maximum quantity of oxygen evolved.



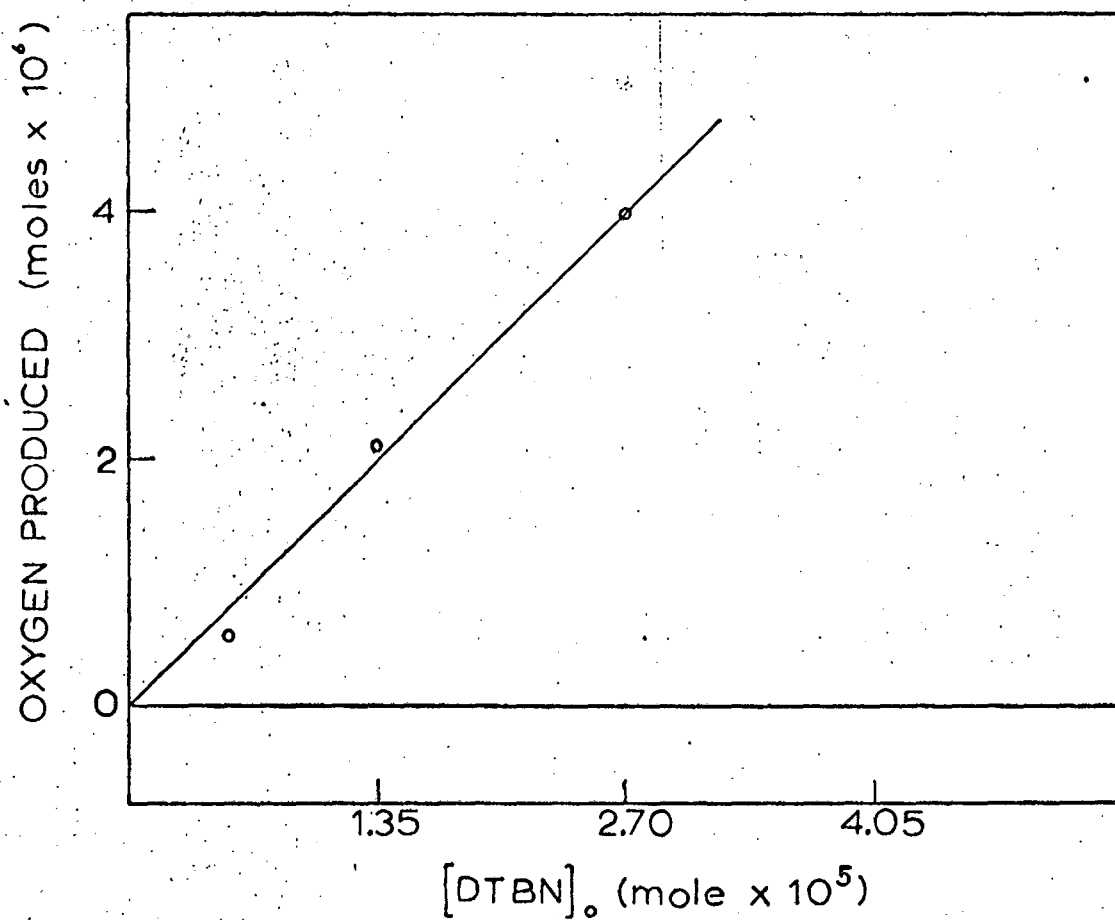
XBL 679-6163

Figure 37. Initial rate of O_2 production as a function of initial DTBN concentration.



XBL 679-6155

Figure 38. Total amount of DTBN destroyed (solid line) and amount photo-destroyed (dashed line) as a function of the initial concentration of DTBN.



XRL 679-6148

Figure 39. Maximum amount of O_2 produced as a function of the initial concentration of DTBN.

destroyed in the light to the moles of molecular oxygen produced. The value of this ratio is 4.6.

Since four electrons must be removed from water in order to yield molecular oxygen, this value is not surprising. The deviation from a value of 4 probably arises because no correction in the data shown in Figure 39 was made to account for the uptake of oxygen by the chloroplasts (see control in Figure 29). If this were done, the ratio of the two slopes would be smaller since the amounts of O_2 produced would be larger for each experiment.

DCMU poisons the photo-production of oxygen and the photo-destruction of the nitroxide. However, it has no effect upon the dark destruction of the nitroxide. These effects are shown in Figure 40 and Table XIV.

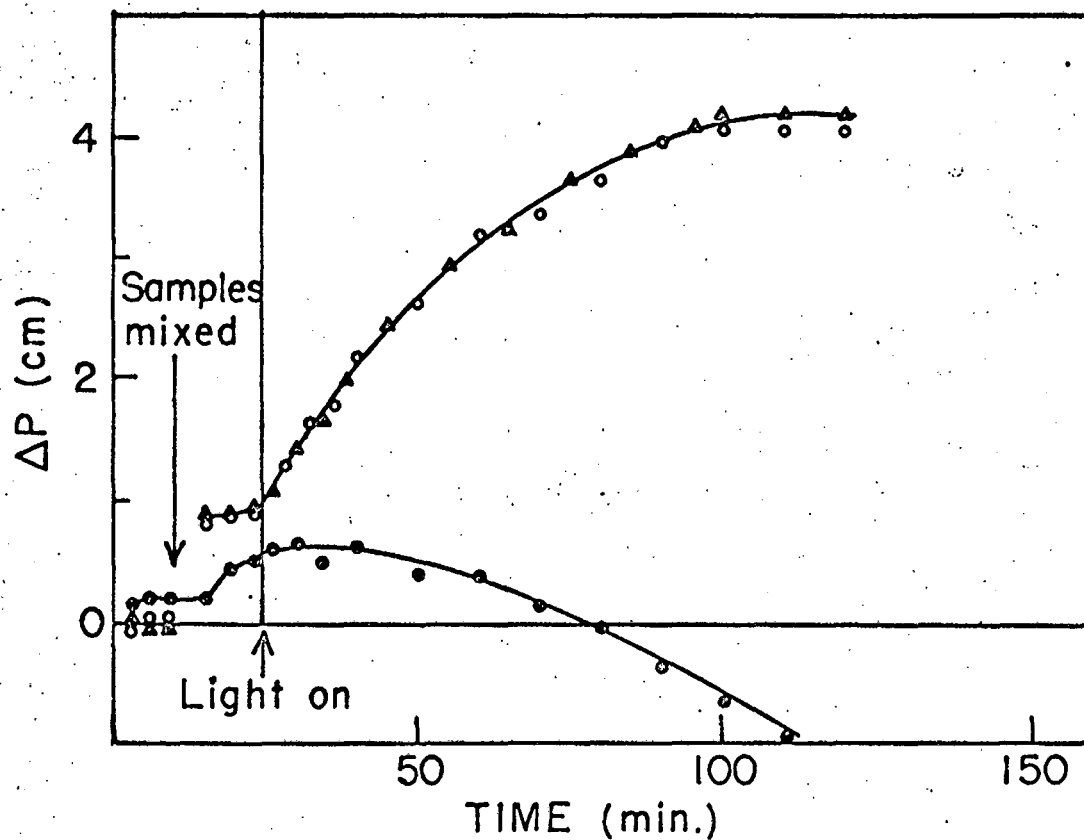
Table XIV

DTBN	Initial Amounts DTBNH	DCMU	Condt.	Oxygen Produced	DTBN Destroyed
3.3×10^{-5}	0	0	Dark	0	1.44×10^{-5}
"	0	0	Light	2.4×10^{-6}	2.82×10^{-5}
"	0	3.0×10^{-7}	Light	0	1.34×10^{-5}
"	3.9×10^{-5}	0	Light	2.4×10^{-6}	2.07×10^{-5}

All quantities reported in moles.

Each sample contained 1.21 mg chlorophyll (a+b).

The data obtained with samples containing both DTBN and DTBNH are also included in Figure 40 and Table XIV. These data show that the



XBL 679-6162

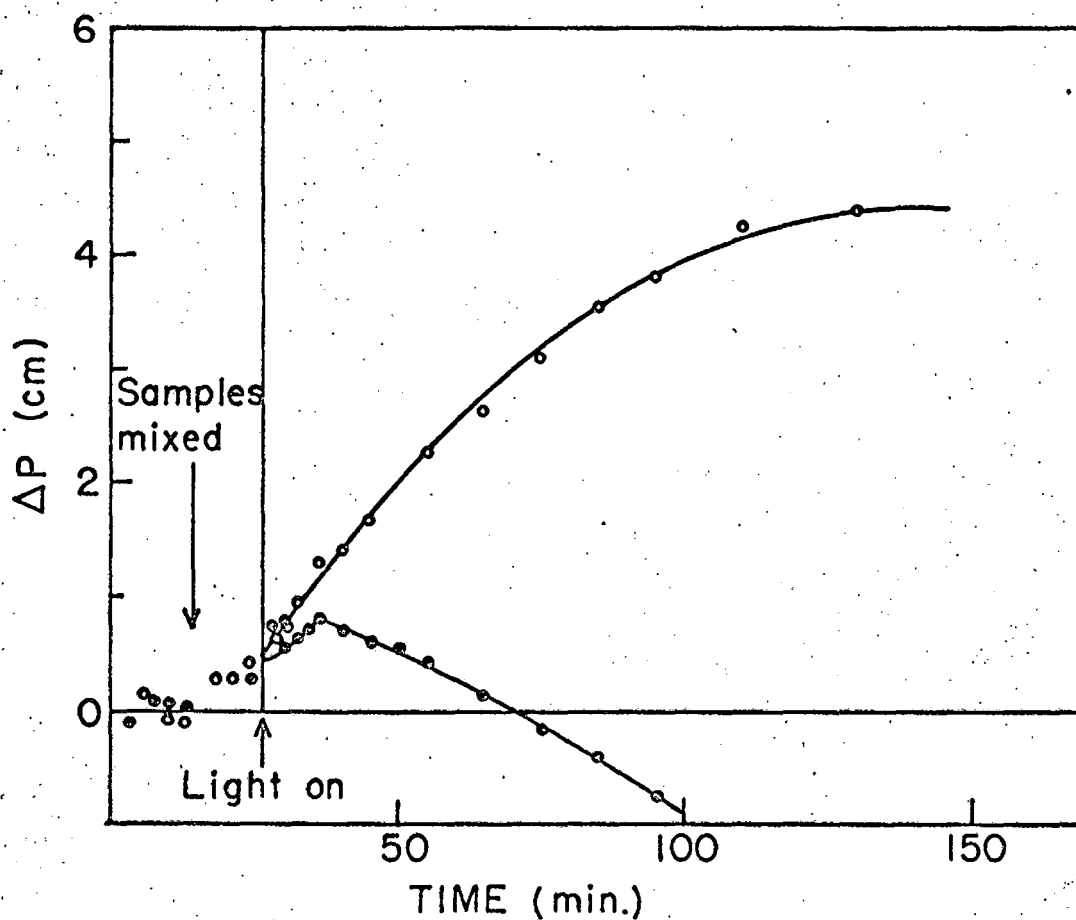
Figure 40. Pressure changes above chloroplast suspensions containing 1.21 mg chlorophyll (a+b) and 0.01 μ DTBN with 7 mg of DTBNH-HCl (open circles) and with 1×10^{-4} μ DCMU (closed circles). Changes for a sample containing only the chloroplasts and DTBN are shown by the solid triangles.

presence of DTBNH has no effect upon the oxygen released during the destruction of the nitroxide. However, Table XIV indicates that the photo-oxidation of DTBNH occurred in this sample (ca. 7.5×10^{-6} moles).

The foregoing results suggested that DTBN was reduced to DTBNH in the light. However, the EPR studies and Table XIV showed that DTBNH is oxidized by light to DTBN. Since the nitroxide was observed to become undetectable in the EPR studies, these observations are in contradiction to one another. A possible explanation of these results was that DTBNH was also photo-reduced irreversibly to ditertiarybutylamine (DTBNH_2) in this system. If this were true, then DTBNH should function as a Hill reagent.

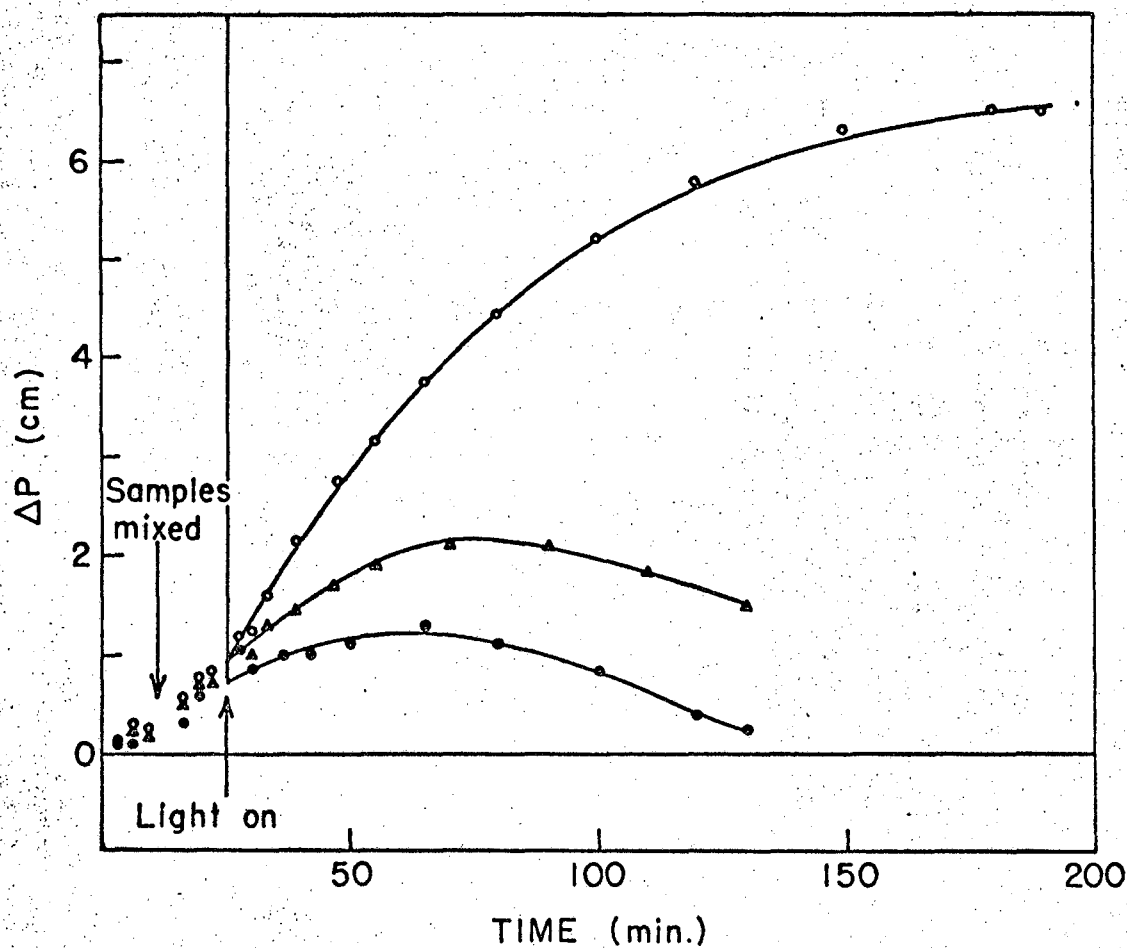
The results of an investigation of this possibility are shown in Figure 41. No oxygen is produced in the system containing chloroplasts and DTBNH. This observation indicates that DTBNH is not further reduced by chloroplasts and light.

Figure 42 shows a comparison of the oxygen production observed using DTBN with freshly isolated chloroplasts, fresh chloroplast fragments and aged fragments (6 days old). Figure 43 shows these same effects using $\text{K}_3\text{Fe}(\text{CN})_6$ instead of DTBN. The decrease in the initial rate of oxygen production and in the maximum obtainable pressure observed in going from freshly isolated chloroplasts to aged fragments is probably a reflection of an increased sensitivity of the fragments to photo-destruction or to a more rapid uptake of oxygen. These two effects are probably related. The amounts of nitroxide destroyed in these samples in the dark and in the light and the amounts of photo-produced oxygen are tabulated in Table XV. The quantities of oxygen produced were not corrected for oxygen uptake.



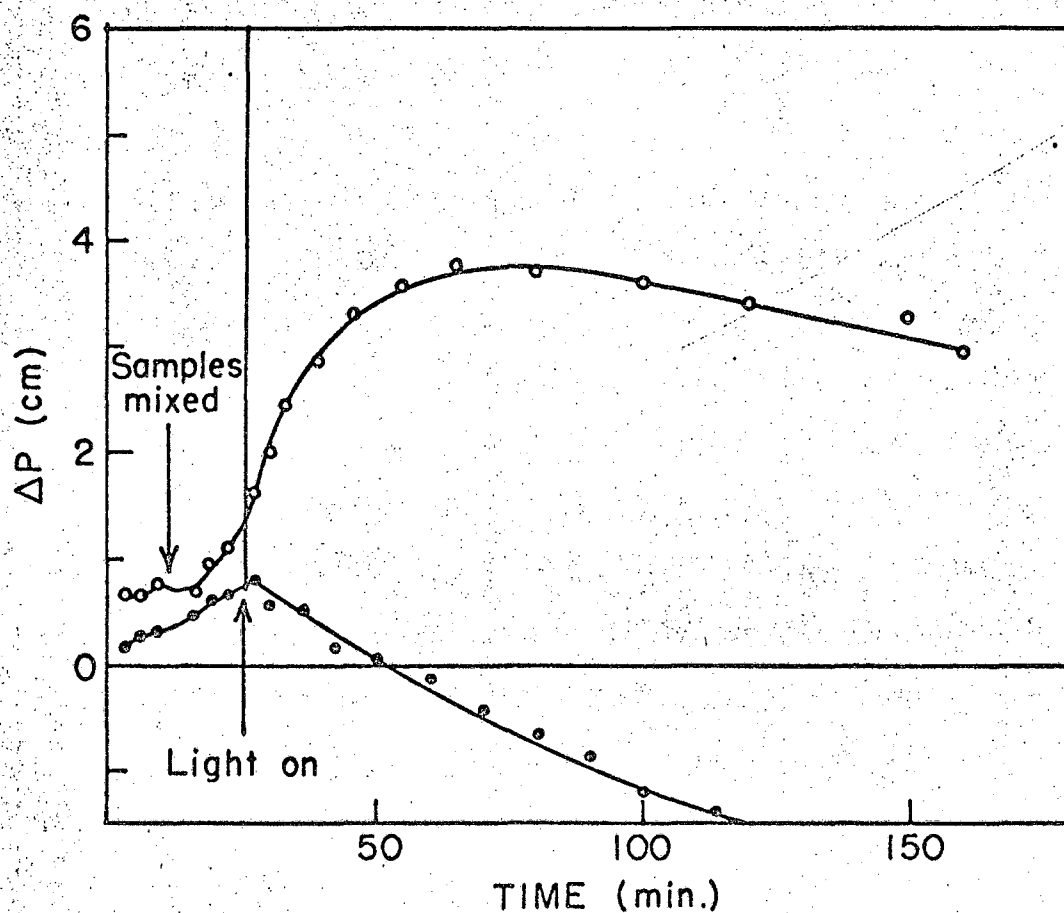
XBL 679-6141

Figure 41. Pressure changes above chloroplast suspensions containing 0.92 mg chlorophyll (a+b) and 0.009 M DTBN (open circles) and 10.7 mg DTBNH-HCl (closed circles).



XBL 679-6157

Figure 42. Pressure changes above solutions of 0.011 M DTBN with fresh chloroplasts (open circles) containing 1.3 mg of chlorophyll (a+b), with fresh fragments (closed triangles) containing 1.3 mg chlorophyll (a+b), and with aged (6 days) fragments (closed circles) containing 1.7 mg of chlorophyll (a+b).



XBL 679-6149

Figure 43. Pressure changes above solutions 0.01 M in $K_3Fe(CN)_6$ with fresh fragments (open circles) containing 1.3 mg of chlorophyll (a+b) and with aged (6 days) fragments containing 1.7 mg of chlorophyll (a+b).

The rate of oxygen production in the aged fragment sample is just great enough to compensate for the oxygen uptake in this sample. Therefore, no quantity of oxygen produced is given in the table for this system.

Table XV

Photosynthetic material	Initial DTBN	DTBN Destroyed Dark	DTBN Destroyed Light	Oxygen produced
Fresh chloroplast	2.7×10^{-5}	0.49×10^{-5}	1.9×10^{-5}	4.8×10^{-6}
Fresh fragments	"	0.27×10^{-5}	1.0×10^{-5}	1.6×10^{-6}
Aged fragments	"	0.02×10^{-5}	0.76×10^{-5}	(?)

All quantities reported in moles.

The data in this table show that photosynthetic oxygen production using DTBN as the Hill reagent is dependent upon the state of the biological material. Although the amount of nitroxide destroyed in the light in the samples containing fragments is not very different, the detectable levels of oxygen are quite different in the two samples. If one assumes that the nitroxide destruction occurs by the same mechanism in both samples (evidence that this is the case will be presented in the next section). The marked differences in detectable oxygen levels in these samples suggest that the aged fragments use oxygen in the light more rapidly than fresh fragments which use it more rapidly than fresh chloroplasts. This suggests that the photochemical degradation occurring within the chloroplasts which causes the termination of oxygen

production is due to a photo-oxidation of a chloroplast component required for photosynthetic electron transport. Evidence will be given below which rules out a degradation of the chloroplast pigments.

It should be mentioned that the ratio of DTBN destroyed to the amount of chlorophyll present in the sample varied from sample to sample. However, ratios as high as 25 were observed.

Summary: Measurement of Oxygen Evolution

The data presented in this section show that the destruction of DTBN by chloroplasts, chloroplast fragments or aged fragments is accompanied by oxygen production. This reaction is inhibited by DCMU but is not affected by DTBNH, although the latter also appears to undergo photo-oxidation with fresh chloroplasts. DTBNH does not function as a Hill reagent with illuminated chloroplasts.

In addition, illumination of chloroplasts destroys their ability to evolve oxygen. Evidence suggests that fresh and aged fragments also experience this photo-destruction even more rapidly than fresh chloroplasts. Also, this destruction appears to involve a photo-oxidation as evident by an increased rate of oxygen uptake.

Product Analysis

Despite the collection of evidence presented in the preceding two sections which indicated that DTBN experienced a reduction to a hydroxylamine, the observation that this hydroxylamine was photo-oxidized to the nitroxide was a strong argument against this conclusion.

Since no product resulting from an oxidation of the nitroxide was detected, and since DTBNH was not reduced further to the di-substituted amine, the remaining alternative was that DTBN addition to a component of the chloroplasts occurred in these systems.

Therefore, a search for the products of the nitroxide reaction was undertaken. This section describes the results of that search.

Experimental

The studies reported in this section were conducted with samples, with and without DTBN, some of which were exposed to light and others which were kept in the dark. The photosynthetic materials included freshly isolated chloroplasts, fresh chloroplast fragments, and aged fragments.

The DTBN-chloroplast (or fragments) mixtures were prepared by mixing 2 ml of the chloroplast suspension with 1/2 ml of DTBN solution in 30 ml flasks sealed with rubber septums. The samples without DTBN contained 2 ml of the suspension and 1/2 ml of buffer solution. All samples were prepared in the dark. The samples which were to remain in the dark were wrapped with two sheets of metal foil. All samples were put into the constant temperature bath of the Warburg apparatus ($T = 21^{\circ}\text{C}$) on a stand which allowed continual shaking of the samples. The light was turned on and the mixtures were allowed to react for at least 100 minutes with shaking.

Oxygen evolution was followed according to the procedure described in the previous section, using samples of the chloroplast preparation used in these studies. This was done to check the activity of the chloroplasts. The destruction of the nitroxide was checked by the EPR spectra of the samples containing DTBN diluted 1:100 both before and after the illumination period.

Following the period of illumination, the samples (2.5 ml) were transferred in the dark to test tubes and centrifuged at $5000 \times g$ for 20 minutes. The supernatant was removed with a pipette. The chloro-

plast pellet was extracted with methanol in the dark in a cold room with the methanol at 0°C until the solid residue was essentially colorless. The amount of methanol used was kept constant in each set of experiments by bringing each extract to a final volume of 10 ml. The methanol extract and the water supernatant (or an ether extract of the water) were analyzed by the method described below.

The primary method of analysis was thin-layer chromatography (TLC). The TLC material was silica gel G supplied by Warner-Chibbett Labs. The solvent system was a mixture of diethylether, petroleum ether and tertiarybutyl alcohol in a ratio of 160:100:5, which was prepared fresh before each chromatogram was developed. The silica gel was applied to the thin-layer glass plates (8 x 8 in) to a thickness of 5 mm from a slurry containing x grams of gel to 2x ml of acetone.

Three methods were used to detect compounds on the developed chromatograms, besides visual inspection. The first of these involved the following three procedures: (1) an inspection with ultraviolet light; (2) locating spots which were fluorescent by the use of a darkened box equipped with an excitation source; and (3) by the use of chemical sprays. The spray primarily used was phosphomolybdic acid (PM), 10% in ethanol. If this spray is used, the chromatogram has to be heated before low concentrations of reducing compounds can be detected. Thus, when this spray was applied, the chromatograms were heated at 60° for approximately 2 minutes.

The other two methods of detection involved the use of radioactive carbon. One of these methods was radioautography using ¹⁴C. The thin-layer chromatogram was placed in direct contact with the

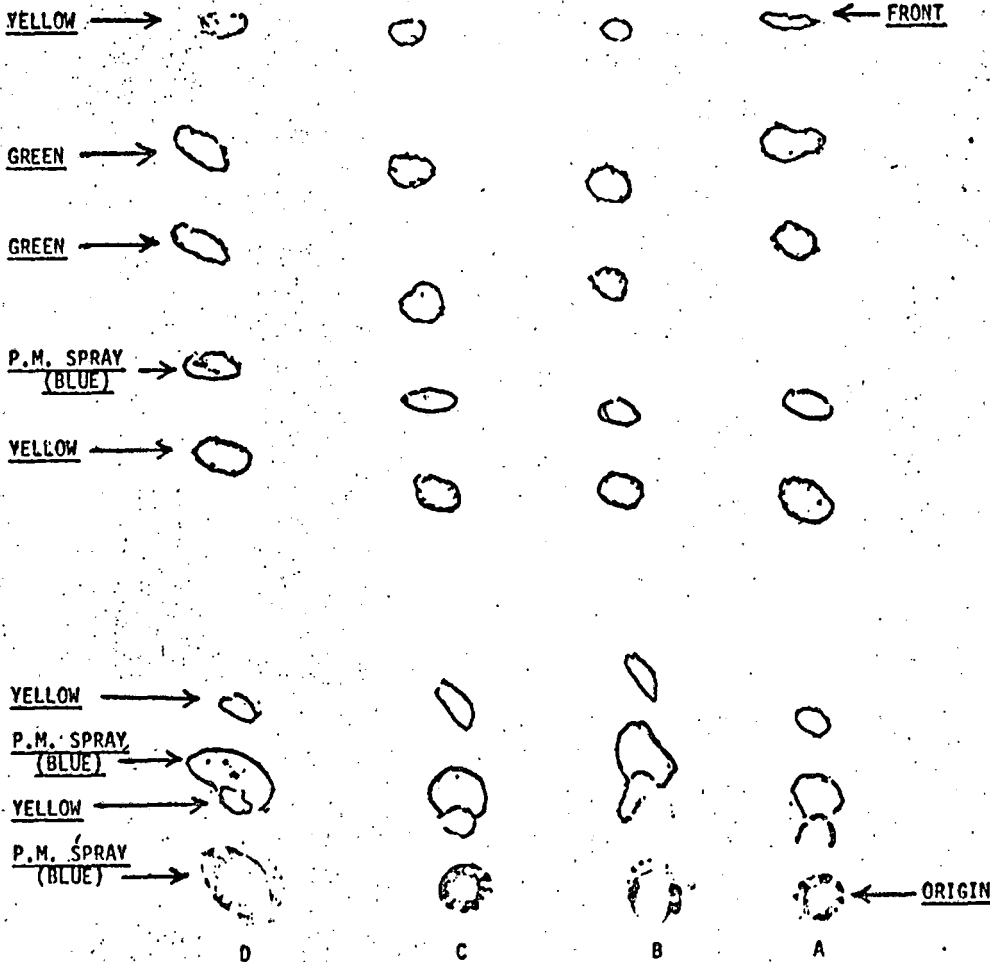
X-ray film (Kodak - no screen). The other method was zonal mapping. This involves spotting the solution to be analyzed the full breadth of the plate, developing it in one direction, scraping every half centimeter into scintillation counting solution, counting the radioactivity in each sample, and plotting the count versus the position the sample was on the plate.

Permanent records of the TLC plates were made either by taking polaroid pictures of them, or else the spots on the chromatogram were circled using a sharp object and Xerox copies were made of them.

Results and Discussion

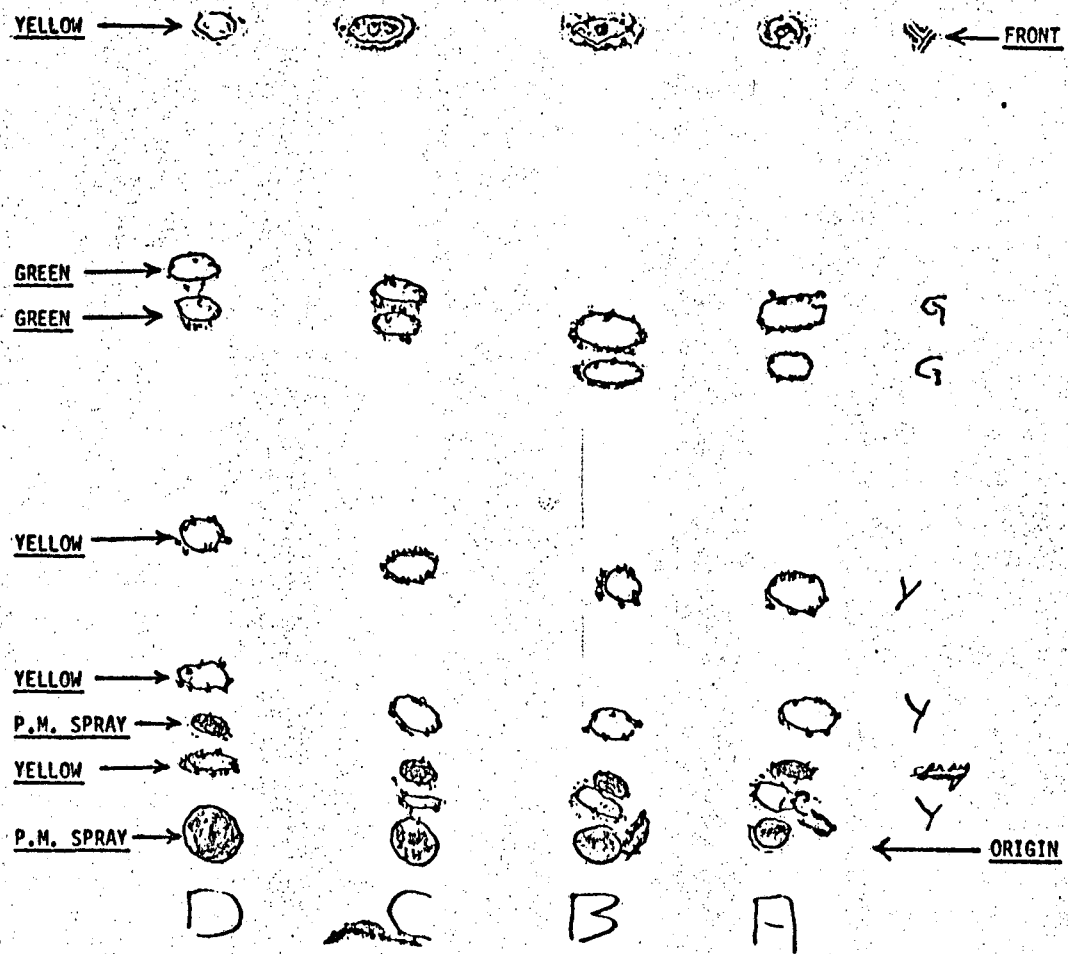
Since the destruction of DTBN involved a photochemical reaction sensitized by chloroplasts which contain several different types of pigments, experiments were conducted to see if changes in pigment composition occurred during the DTBN reaction. Figures 44 and 45 are Xerox duplicates of TLC chromatograms of methanol pigment extracts (50 μ l of 10 ml solution) obtained in experiments with freshly isolated chloroplasts and aged chloroplast fragments (3 days old), respectively. Neither DTBN nor light effected gross changes in the pigments detected. In addition, no material which could be ascribed to a product of DTBN was detected upon these chromatograms.

However, when the water supernatant from the reactions were extracted with diethylether and this extract was analyzed by TLC, the results shown in Figures 46 and 47 were obtained (experiments with fresh chloroplasts and aged fragments, respectively). The large spot with an x through it in column D of Figure 47 was made by my finger during the process of outlining the spot in column C.



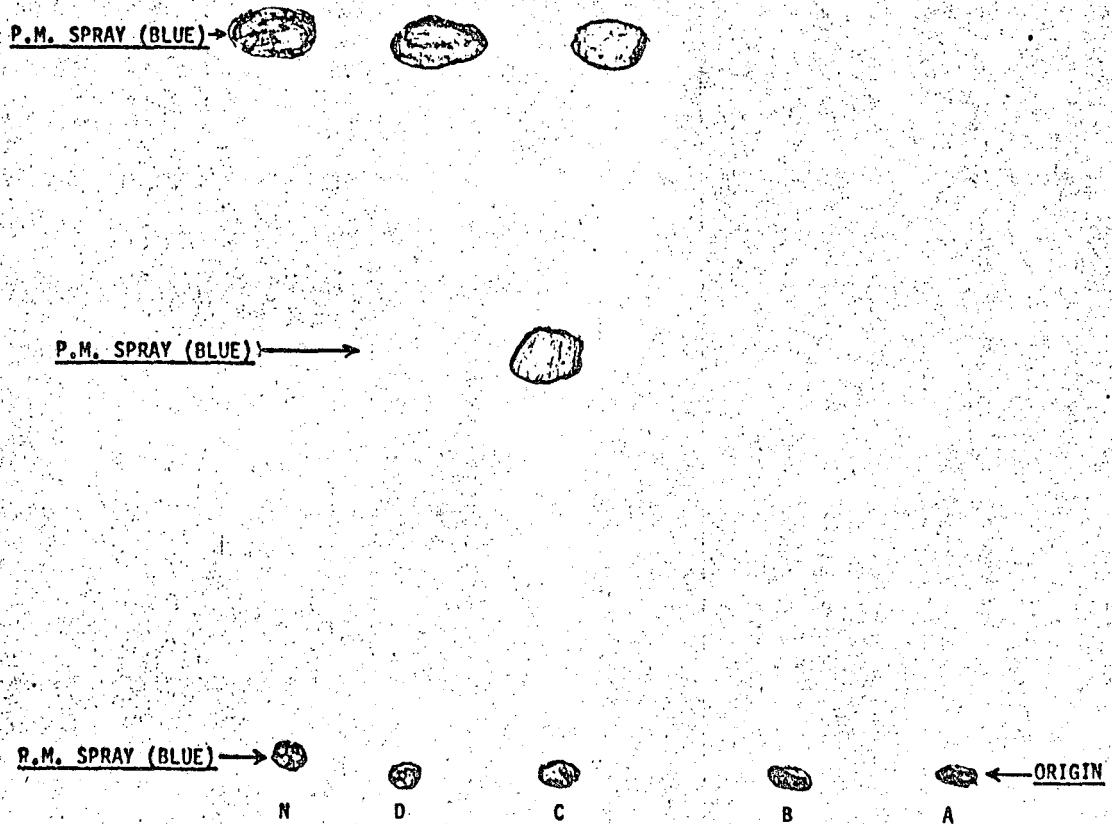
XBL 678-6115

Figure 44. Thin-layer chromatogram of methanol extract of fresh chloroplasts.



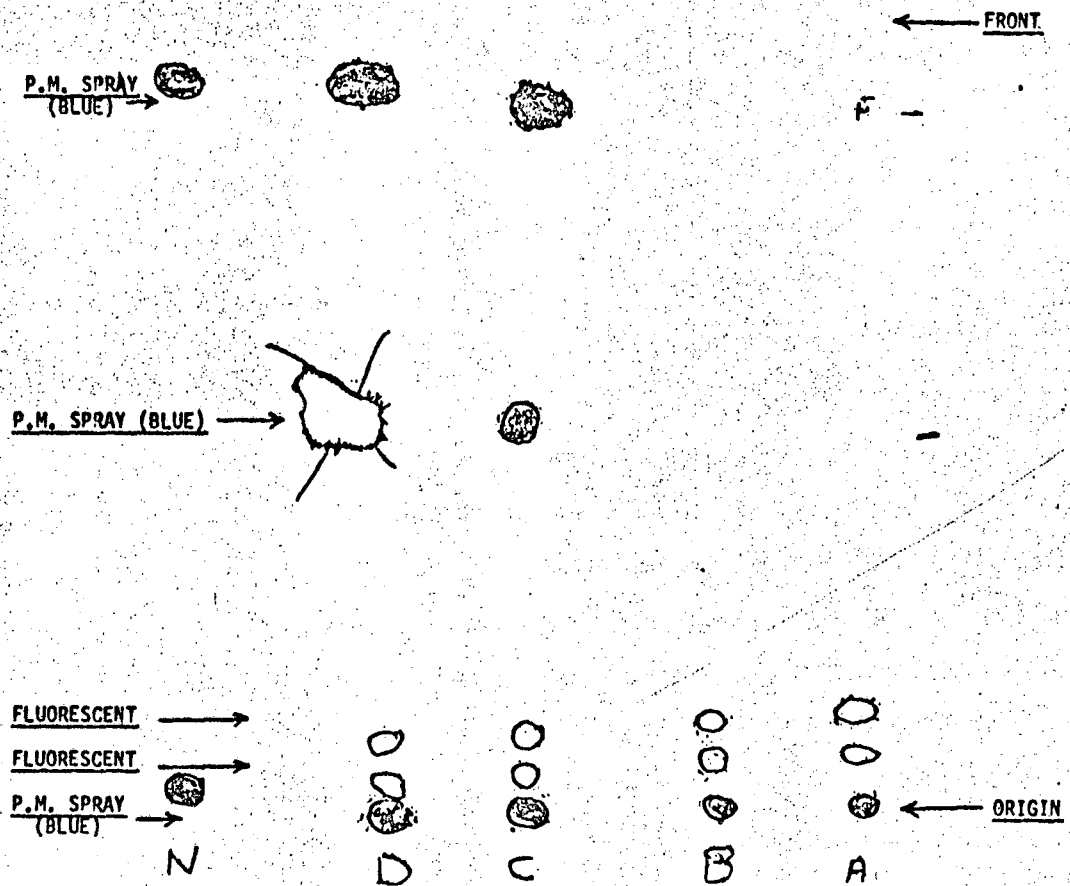
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Figure 45. Thin-layer chromatogram of methanol extract of aged fragments.



XBL 678-6112

Figure 46. Thin-layer chromatogram of ether extract of the water phase from samples containing fresh chloroplasts. The symbols are explained in the text.



XBL 678-6111

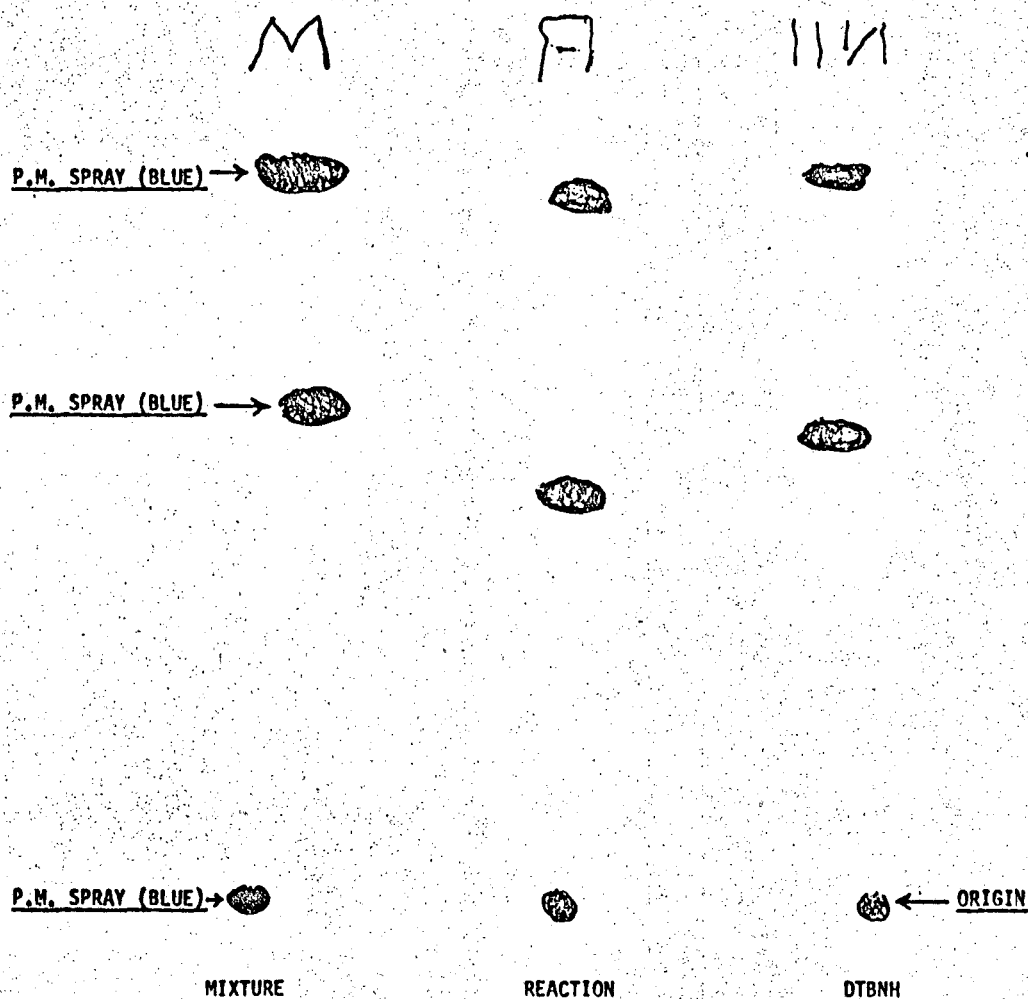
Figure 47. Thin-layer chromatogram of the ether extract of the water phase from samples containing aged fragments. Symbols are explained in the text.

The symbols in these four figures represent extracts obtained from the following: A, a sample of chloroplasts (or fragments) without DTBN exposed to light; B, a sample of chloroplasts without DTBN kept in the dark; C, a sample of chloroplasts with DTBN exposed to light; D, a sample of chloroplasts with DTBN kept in the dark; and N, a sample of DTBN.

The darkened spots in these four figures are spots which turned blue upon treatment with PM spray and heat. The blue color fades rapidly.

The spots in column C of Figures 46 and 47, which are absent in the other columns of these figures, were taken as a product of DTBN. This product was suspected to be di-tertiarybutylhydroxylamine. Therefore, a chromatogram was run to which a sample from a reaction of DTBN with chloroplasts, a sample of the hydroxylamine and a mixture of these two were applied. The mixture contained equal volumes of the other two solutions and twice as much of it was applied to the TLC plate as the other two. The results (see Figure 48) show that the material contained in the new spot is indeed the hydroxylamine. When 1 ml of acetic acid was added to the TLC solvent system, the compounds again exhibited identical r.f. values.

Attempts to remove this material from the TLC chromatogram failed. The reaction mixture was extracted with ether and the whole sample applied to a TLC plate. Development of this plate was followed by masking all but one edge of the resulting chromatogram with another pane of glass. The exposed edge was sprayed to determine the location of the hydroxylamine. This section of the chromatogram corresponding to this location was scraped off and extracted with



XBL 678-6113

Figure 48. Thin-layer chromatogram of ether extract of the water phase from DTBN-chloroplast reaction mixture (exposed to light), ether extract of water solution of DTBNH, and a mixture of these two.

various solvents. The hydroxylamine was not detected by re-chromatography.

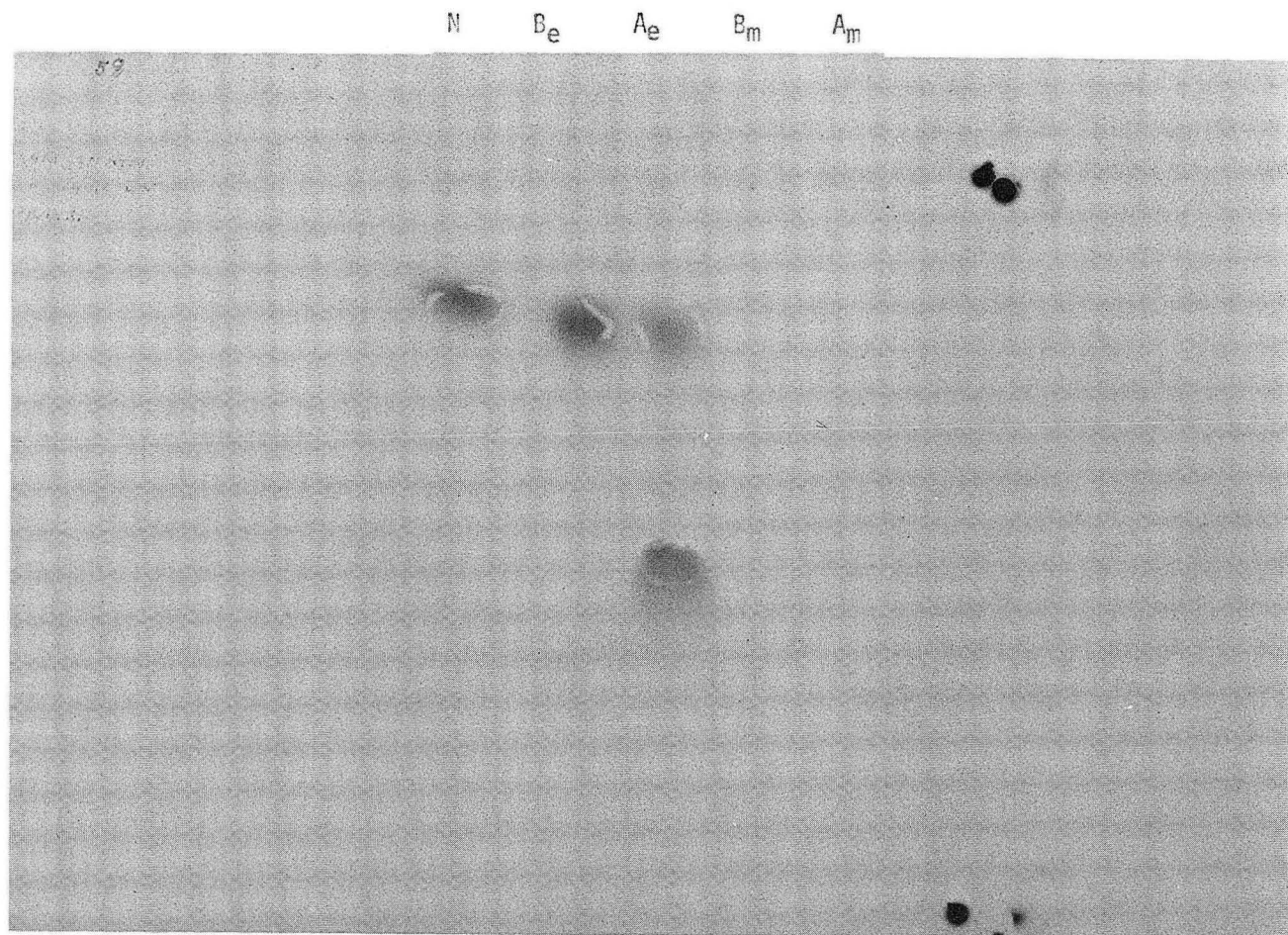
The above procedure was repeated and the TLC powder was put into a flask equipped with an arm which fit into a mass spectrometer. After putting the powder into the flask, the flask was cooled to liquid nitrogen temperature, evacuated and then fitted onto the mass spectrometer. The mass spectral pattern obtained from the atmosphere in this flask was the same as that obtained from a TLC plate without anything on it except the TLC solvent.

A sample of DTBNH gave these same results.

Column chromatography of the DTBN-chloroplast mixture (ether extract of water phase) using silica gel and the same solvent used for TLC failed to yield the product, DTBNH. One hundred and fifty 5 ml samples (20 min/sample) were collected. An aliquot of each sample was spotted on a silica gel TLC plate and sprayed with PM. The only material detected in the 150 samples was DTBN.

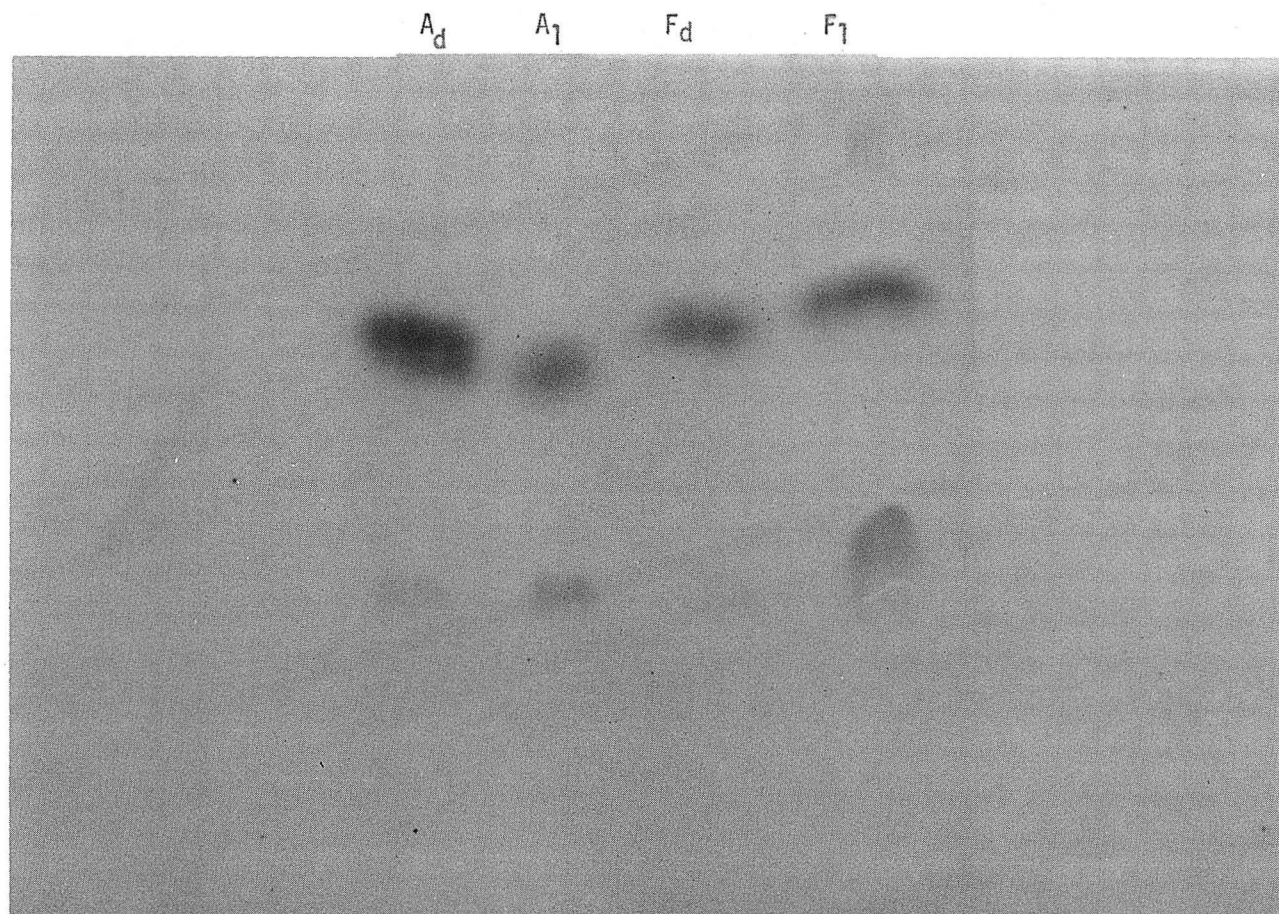
The above results show that one product from the photo-destruction of DTBN sensitized by chloroplasts is DTBNH. However, the possibility of a parallel coupling reaction was not ruled out by this result since a material balance was not done. Furthermore, if the product of such a reaction were insensitive to PM, colorless and non-fluorescent, it would not have been detected.

Therefore, a ^{14}C -labeled DTBN was used. Figures 49 and 50 are radioautograms of thin-layer chromatograms obtained in experiments using fresh chloroplasts and fragments (fresh and aged), respectively. Figure 49 represents an X-ray film exposed for 58 hours to the radioactive chromatogram. In this figure, A stands for the sample exposed



XBB 678-4981

Figure 49. X-ray film exposed for 58 hours to a thin-layer chromatogram containing ^{14}C -DTBN (N), ether extract of the water phase from a ^{14}C -DTBN-chloroplast reaction mixture kept in the dark (B_e) and exposed to light (A_e) and the methanol extract of chloroplasts from the ^{14}C -DTBN-chloroplast reaction mixtures kept in the dark (B_m) and exposed to light (A_m). Small dark spots on the right due to radioactive ink used to align developed film with TLC plate.



XBB 678-4980

Figure 50. X-ray film exposed for two weeks to thin-layer chromatogram containing ether extract of the ^{14}C -DTBN-fresh fragment reaction mixture, water phase, exposed to light (F_1) and kept in the dark (F_d) and ether extract of the water phase of a ^{14}C -DTNB-aged fragment reaction mixture, exposed to light (A_1) and kept in the dark (A_d).

to light; B, the sample kept in the dark; sub m, methanol extract; and sub e, ether extract. Sample N is the radioactive nitroxide. Although both samples A and B contained 1.38×10^6 dpm originally, the only radioactive spot to be detected besides the nitroxide itself is the hydroxylamine (DTBNH) found in the ether extract of the water phase of sample A. This is from the sample which had been exposed to light. Longer exposures (3 weeks) do not yield new spots on the film. A slight darkening in sample B in the region of the chromatogram which would correspond to DTBNH is observed. This could signify that DTBNH is formed in the DTBN-chloroplast dark reaction or else light was not excluded completely.

The X-ray film shown in Figure 50 was exposed for approximately 2 weeks. In this figure, A stands for aged fragments, F for fresh fragments, sub l and d for light and dark, respectively. The chromatogram used to expose this film was one run of the ether extracts of reactions between ^{14}C -DTBN and aged and fresh fragments. No radioactive spots were found in the methanol extracts of the pigments of these systems even with 3 week exposure of the X-ray film. The film obtained with the material from the experiments with fragments has a total of 8 spots corresponding to 8 spots on the chromatograms, 4 of DTBN and 4 of DTBNH. However, the DTBNH spots are less intense in the two dark samples with films obtained with shorter exposure times. Also, when the chromatogram was sprayed with PM, the blue spots observed in the dark samples were much fainter than the spots of the light sample.

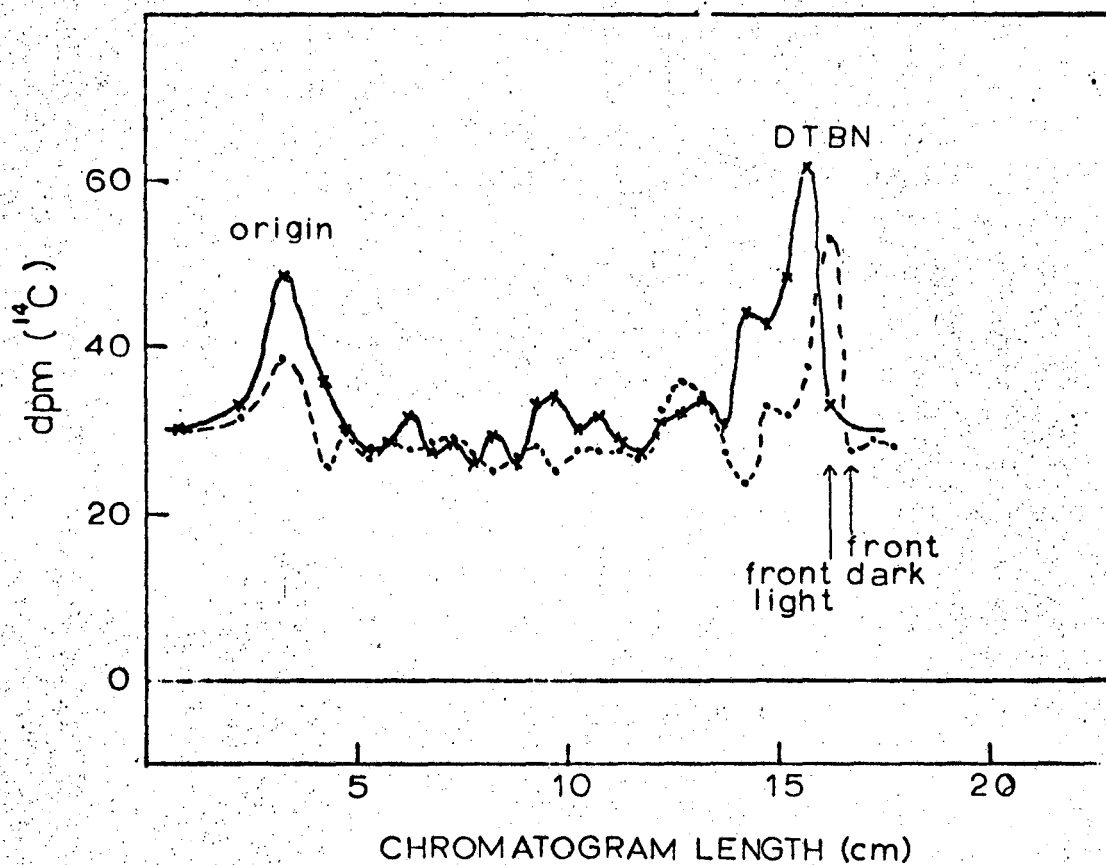
The fact that DTBNH is detected in the dark samples again suggests that the dark reaction is a reduction. The DTBNH could be due

to the failure to exclude light completely. However, extreme care was used to exclude light and because of the low activity of the fragments and also because no product which can be ascribed to the dark reaction was detected in these samples, other than DTBNH, I think the DTBNH detected in the dark samples is due to a dark reduction reaction.

When a methanol extract of the chloroplasts from a ^{14}C -DTBN-chloroplast mixture, an ether extract of the water phase of such a mixture or the water phase itself are applied the full length of a TLC plate and the resulting chromatogram is divided into 0.5 cm samples and the radioactivity is counted in each sample and plotted versus the position of the sample on the chromatogram, the zonal maps corresponding to Figures 51, 52 and 53, respectively, are obtained.

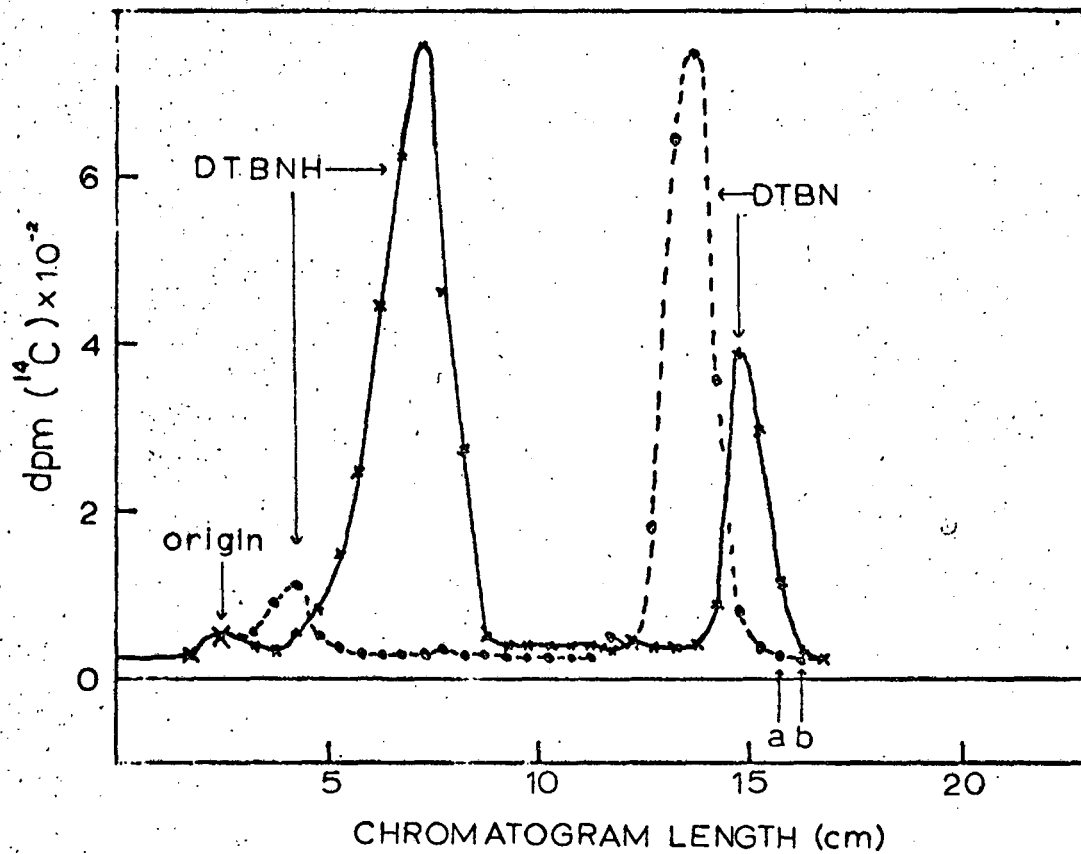
The amounts of material applied to each plate were 600 λ of the methanol extracts, 1 ml of the ether extracts, and 300 λ of the water solutions. These values of the methanol extract and water are the maximum one can use with these systems. When more of the methanol extracts was applied, no resolution of the pigments occurred. Water is difficult to apply to silica gel. Due to the strong interaction which occurs between them, the water does not evaporate from the origin and contributes its influence in the solvent system.

The methanol extracts contained 6.5×10^2 dpm/ml in the light samples and 6.0×10^2 dpm/ml in the dark sample. These values for the water solutions are for the light sample, 4.3×10^3 dpm/ml and for the dark sample, 4.5×10^3 dpm/ml. The counts in the ether extract are unknown.



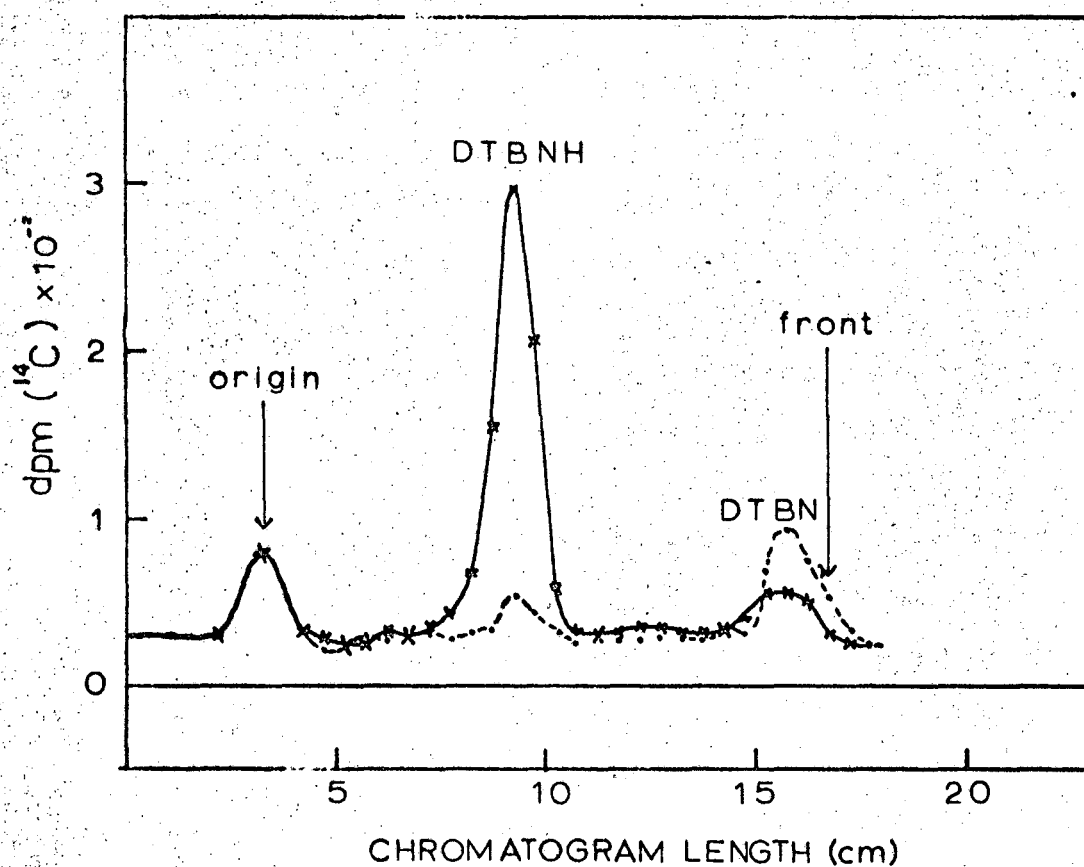
XBL 679-6151

Figure 51. Zonal map of chromatogram of methanol extract of chloroplasts from ^{14}C -DTBN-chloroplast reaction mixtures. Solid line is light sample; dashed line is dark sample.



XBL 679-6143

Figure 52. Zonal map of chromatogram of water phase of ^{14}C -DTBN-chloroplast reaction mixture. Solid line is light sample; dashed line is dark sample.



XBL 679-6145

Figure 53. Zonal map of chromatogram of ether extract of the water phase of ^{14}C -DTBN-chloroplast reaction mixture. Solid line is light sample; dashed line is dark sample.

As is evident in these zonal maps, the major radioactive peaks are those corresponding to DTBN and DTBNH. Radioactivity is detected in the region corresponding to the origins. However, the values in the cases of the water and the ether extract are identical for both the light and dark samples, suggesting that the observed radioactivity may be due to the application of the samples to the silica gel plate. There is a difference in the values detected between the light and dark samples of the methanol extract. However, the background count in these samples is a considerable portion of the counts found in these samples. In addition, due to the amount of pigment applied to these plates, not all the pigment moved from the origin. Traces of green were still visible at the origin. The source of the radioactivity at the origins was not investigated further, since it was assumed due to DTBN or DTBNH which remained at the origin because of an interaction with the silica gel or a compound bound to the silica gel.

Table XVI gives an account of the radioactivity in the water phase and in the methanol extract. The radioactivity in the insoluble protein fraction of the chloroplasts was counted for one experiment and found to contain less than 0.1% of that found in the original mixture. Two trends are obvious in the data in Table XVI: (1) The water phase always contains more radioactivity than the methanol; and (2) the radioactivity found in the dark samples is always less than that found in light samples. The first of these is understandable. The distribution is a reflection of the solubility of the radioactive compounds in chloroplasts and in water. The second implies that either a systematic error was made in the experimental procedure or

Table XVI

Overall Account of Radioactivity for ^{14}C -DTBN-Fresh Chloroplast Reaction

(DTBN)	Cond.	Original Mixture		Water Phase			Methanol Extract			Total: $\text{H}_2\text{O} + \text{CH}_3\text{OH}$		
dpm		dpm	%DTBN	dpm	%Mix.	%Det.	dpm	%Mix.	%Det.	dpm	%DTBN	%Mix.
1.38×10^6	Light	-	-	1.13×10^6	-	97.5	3.0×10^4	-	2.2	1.18×10^6	85.5	-
"	Dark	-	-	1.05×10^6	-	98.2	2.0×10^4	-	1.4	1.08×10^6	78.2	-
2.76×10^5	Light	2.50×10^5	90.5	2.20×10^5	88.0	97.1	6.5×10^3	2.6	2.9	2.26×10^5	81.9	90.4
"	Dark	2.37×10^5	85.9	2.05×10^5	86.4	97.2	6.0×10^3	2.5	2.8	2.12×10^5	76.8	89.4
4.90×10^4	Light	5.15×10^4	105.3	4.46×10^4	86.6	99.3	3.08×10^2	0.59	0.58	4.49×10^4	91.6	87.1
"	Dark	4.72×10^4	96.3	4.32×10^4	91.5	99.7	1.38×10^2	0.29	0.32	4.33×10^4	88.4	91.7
"	Light	5.16×10^4	105.3	4.47×10^4	86.6	99.5	2.21×10^2	0.43	0.49	4.49×10^4	91.6	87.0
"	Dark	4.67×10^4	95.3	4.28×10^4	91.6	99.7	1.18×10^2	0.25	0.28	4.29×10^4	87.6	91.4
"	Light	5.32×10^4	108.6	4.47×10^4	84.0	99.3	2.51×10^2	0.47	0.56	4.50×10^4	91.8	84.5
"	Dark	4.74×10^4	96.7	- *	-	-	-	-	-	-	-	-

(DTBN) is the original dpm calculated from known volumes of DTBN solution used and observed dpm per ml of solution. "% mix." is the percent of the dpm found in original mixture of DTBN and chloroplast.

"% det." is the percent of the total detected in the water phase plus methanol phase.

*Sample lost during centrifugation.

else a volatile compound was formed in the dark which is lost.

Furthermore, Figures 51 and 53 indicate that DTBNH is essentially in the water phase. If DTBN is the only other radioactive compound present and it is higher in concentration in the dark sample than in the light (see Figures 52 and 53), then the level of radioactivity in the methanol extract of the dark sample should be larger than or equal to that found in the light sample. However, this is not the case. This suggests that another compound is formed in the light which is soluble in the chloroplasts. This could be the compound which caused the increased activity of the light sample at the origin or the shoulder on the DTBN peak in Figure 51. This possibility was not investigated further.

Summary: Product Analysis

The only product, besides oxygen, identified in the reaction mixture of DTBN with illuminated chloroplasts (and fragments) was DTBNH, which was the major (>95%) product formed from DTBN. The product was shown to be this compound by co-chromatography with an authentic sample in two different solvent systems. Data which indicate the possibility of another photo-product are given. However, the quantity of this material is extremely low. Therefore, the possibility was not pursued.

Although the experiments with ^{14}C suggest that the dark reaction involves the formation of DTBNH, they also indicate that a volatile compound is formed in the dark, suggesting an oxidation of DTBN to isobutene and 2-methyl-2-nitroso-propane.

The photo-destruction which occurs within the chloroplasts illuminated for lengthy exposure times (see section on oxygen measurement)

does not appear to involve the chromophoric groups of the pigments of fresh chloroplasts or of aged fragments.

Discussion

The studies reported in this chapter show that chloroplasts sensitize a photo-reduction of di-tertiarybutyl nitroxide to the corresponding hydroxylamine. Oxygen is produced concurrently with the reduction in a ratio of 1 mole of oxygen evolved to ca. 4 moles of DTBN destroyed. These results are consistent with an interpretation that the DTBN reduction is coupled with photosynthetic electron transport and that the oxygen evolved originates from water which is oxidized photosynthetically.

The lack of a photo-destruction of DTBN by chromatophores isolated from the photosynthetic bacterium Rhodospirillum rubrum suggests that the reduction of DTBN by spinach chloroplasts occurs by electrons supplied by photosystem II (see Figures 1 and 2), the reduction occurring near the photo-act. It is not known whether the chromatophores used in these studies were photosynthetically active. Therefore, this suggestion needs further substantiation.

Consistent with this suggestion, however, are the following observations: (1) DCMU, which truncates photosynthetic electron flow between water and photosystem II, inhibits the photo-reduction of DTBN; (2) Plastoquinones are thought to be the primary oxidants coupled to photosystem II. When these are partially extracted from chloroplasts the rate of destruction of DTBN is decreased; (3) KBH_4 and the reduced form of DCPIP which will reduce plastoquinones cause a reduction in the destruction rate; (4) The reduced form of DCPIP has been used in DCMU-poisoned chloroplasts to effect the reduction of NADP which is

effected by photosystem I. However, this reagent will not lift the inhibition caused by DCMU upon the photo-destruction of DTBN.

An observation that appears inconsistent with the interpretation that DTBN is photo-reduced by chloroplasts is that when a solution of DTBNH (1.6×10^{-4} M in sucrose buffer) was examined by EPR, the formation of DTBN resulting from the oxidation of DTBNH by atmospheric oxygen was observed. However, this oxidation does not occur in the presence of chloroplasts (.14 mg chlorophyll per sample). The chloroplasts were kept in the dark prior to the addition of DTBNH. Since the chloroplasts assimilate oxygen in the dark, the amount of oxygen in the chloroplast suspensions could be very low. Therefore, the oxidation of DTBNH would be limited by the amount of available oxygen which would have to diffuse across the interface between the liquid phase and air, the surface area of which was only 0.04 cm^2 . Therefore, if the chloroplasts react with the oxygen more rapidly than DTBNH, they could use the oxygen as it diffuses into the suspension, thus effectively inhibiting the DTBNH oxidation.

The data from experiments using a Warburg apparatus show that chloroplasts use oxygen in the dark at a rate of ca. 1.6×10^{-9} moles per sec per mg chlorophyll (assuming total pressure change due to O_2 uptake, no exchange of other gases). Since the Warburg vessels were shaking during the measurement of the pressure changes, this rate is probably the actual rate of oxygen usage by the chloroplasts, not the rate of O_2 diffusion into the suspensions. Using this rate and the amount of chloroplast suspension used in the EPR experiments, one can calculate that the chloroplasts can remove O_2 from the EPR samples in the dark at a rate of 2.6×10^{-11} moles per sec.

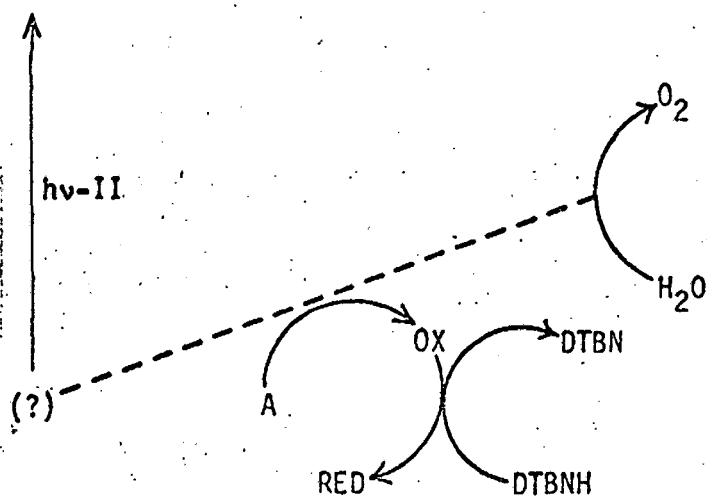
In contrast, in experiments involving only DTBNH in sucrose solution, the initial increase of DTBN corresponded to a rate of formation of DTBN of ca. 6.0×10^{-12} moles per sec. During this period, less than 20% of the original DTBNH was oxidized to DTBN. However, following this period, the rate of formation of DTBN became slower and linear in time, indicating that the oxidation had become limited by a diffusional process--probably the rate at which O_2 could diffuse into the solution. The observed rate was ca. 1.5×10^{-12} moles per sec.

A factor which significantly influences the rate of oxygen uptake by the chloroplasts is their condition, i.e., age, source, extent of fragmentation, etc. Therefore, a strict comparison of the rates stated above cannot be made because these were obtained from experiments performed with differing chloroplast preparations. The numbers do demonstrate that the rate of the chloroplast-oxygen reaction can be faster than that of the DTBN-oxygen reaction.

Another observation that is inconsistent with the interpretation of the photo-destruction of DTBN given above is that chloroplasts when supplied with DTBNH convert it photochemically to DTBN. This implies that a steady state ratio of DTBN to DTBNH should exist in the light. However, the studies with EPR indicate that this is not the case unless the ratio is extremely small, thus rendering the amount of nitroxide undetectable by EPR.

Another possible explanation for this disparity, represented pictorially on the following page, is that the species which oxidizes DTBNH (ox. in scheme) is present in a limited amount and is expended rapidly, thus terminating the photo-oxidation of DTBNH. Since this oxidation was observed in a chloroplast suspension which had not been

supplied with an additional oxidant, and since no oxidation of DTBNH occurred in the dark with chloroplasts, the species which photo-oxidized DTBNH must be one generated in the chloroplasts with light. If this is the case, its concentration is very small compared to the amount of DTBNH used in these studies. Assuming the scheme pictured below, one can calculate, using the data in Figure 28 without correcting for the photo-reduction of DTBN, that the concentration of this oxidant (or species from which it is generated) should be greater than 5×10^{-8} moles per mg chlorophyll. Making a similar calculation with the data shown in Table XIV without correcting for oxidation due to O_2 , one arrives at the conclusion that the concentration of this species should be less than 6×10^{-6} moles per mg chlorophyll. If one could correct for the photo-reduction in the first calculation and the air oxidation in the second, these two numbers would both be adjusted toward 10^{-7} moles per mg chlorophyll. The approximate correspondence of these numbers is surprising, especially since the data used in these calculations were obtained using in one case aged fragments and in the other fresh chloroplasts. If this correspondence is real, it supports the scheme pictured below.



Another observation which supports the picture above is shown in Figure 41. The curve representing the pressure changes occurring in the sample containing substrate amounts of DTBNH (ca. 0.01 M) demonstrates that no O_2 is produced in this sample. If DTBNH were undergoing a continuous photo-oxidation (if an unlimited supply of oxidant were present), the DTBN formed should have functioned as a Hill reagent, thus promoting the evolution of O_2 .

Since the oxidation of DTBNH is photo-induced, the oxidant must be coupled with the photosystems found within the chloroplasts. The inhibitory effect of DCMU signifies that the coupling is with a participant in the sequence of reactions which couple the oxidation of H_2O to photosystem II. However, since the oxidant has to be irreversibly reduced (or removed during the illumination) if it is to be expended rapidly, it itself cannot be a participant in photosynthetic electron transport or the photo-reduction of DTBN should cease.

DTBN was also observed to undergo a reaction with chloroplasts in the dark. In experiments conducted with radioactive DTBN, the only product detected which could be ascribed to the dark reaction was DTBNH. Only low concentrations of DTBNH were present in the dark samples. The source of this DTBNH could have been the light reaction. However, extreme care was taken to exclude light from these samples. In addition, the reduced form of DCPIP and KBH_4 both cause an increase in the rate of the dark reaction. Since these compounds do not react directly with DTBN, their effects upon the DTBN-chloroplast dark reaction suggest that the latter reaction involves a reduction of the radical.

Upon prolonged exposure to light, chloroplasts lose their capacity to evolve oxygen. Analysis of the pigments by TLC gives no indication that this loss of capacity results because of gross changes in the chromophoric groups of the pigments. In addition, the reaction which causes this loss appears to involve molecular oxygen.

A product which could be ascribed to a coupling reaction between the nitroxide and the radical species which gives rise to the photo-induced EPR signal in spinach chloroplasts was not detected. Unfortunately, the most sensitive methods used for detection of the products (zonal mapping and X-ray film) were not calibrated to yield quantitative data. However, in samples containing ca. 7% of the original ^{14}C -DTBN, an intense darkening of the X-ray film caused by this DTBN was observed in 58 hours. As an estimate, I would say that if a coupling product corresponding to at least one per cent of the original ^{14}C -DTBN were formed, it should have exposed the X-ray film.

Although the attempt to trap the species giving rise to the photo-induced EPR signal in photo-synthetic materials was unsuccessful using DTBN, the method should be attempted again but with a more reactive radical (one which is not as stable as DTBN) and preferably a radical which, if it did couple to another radical, would yield a carbon-radical bond. The use of a radical more reactive than DTBN could lead to difficulties in distinguishing between the products which result from the radical through disproportionation or hydrogen abstraction reactions and those which result from a coupling of the radical with the photo-induced radical. However, this difficulty could be overcome by the use of dark controls. One must use a highly reactive radical, however, if a coupling reaction is going to be

competitive with the reduction reactions associated with the redox-potentials found in photosynthetic materials.

In addition, several aspects of the DTBN-chloroplast photo-reaction should be investigated further. If one could conclusively demonstrate that DTBNH is oxidized by an indigenous species of the chloroplasts and a method could be found to remove this species, then the reduction of DTBN could be observed uncomplicated by a back reaction. If this could be accomplished, then the measurement of the action spectrum of the quantum yield of the photo-reduction should be determined. This would show whether the destruction of DTBN is associated with photosystem II as suggested above.

Furthermore, since the reduction of DTBN can be followed by EPR, the use of extremely low levels of DTBN and chloroplasts is possible. Therefore, theoretically one could measure both the rate of DTBN reduction and the rate of light absorption simultaneously. The accuracy in using small concentrations of DTBN, as long as they were still detectable by EPR, would be the observed signal to noise. This could be improved by the use of multiple scan, averaging techniques which would require the use of a flow system. However, the use of low concentrations of DTBN and chloroplasts would reduce several sources of error normally encountered in measuring the quantum yields of the Hill reactions. The major source of error in these types of measurements is the light scattering exhibited by the chloroplasts. This could be reduced by using the EPR cell, which is only 0.4 mm thick, and a low concentration of chloroplasts. Another source of error occurs because one normally determines the rate of the Hill reaction by following changes in the optical absorption

spectra of the Hill reagents, the optical absorption of which occurs in a region of the electromagnetic spectrum where changes in the optical spectrum of the chloroplasts also occur. In addition, the chloroplasts are illuminated by one wavelength of light while changes at another are measured. Both of these could lead to errors in the measurement of the rate of reduction of the Hill reagents. However, the EPR spectrum of DTBN can be observed independently of the constituents of the chloroplasts. Thus, the observation of the rate of reduction lacks the complications encountered in using the optical methods.

Chapter IV

MISCELLANEOUS TOPICS

The investigations of two different topics will be discussed in this chapter. The first of these is concerned with the phenomena of charge transfer complexes (CTC); the second, a method of detection of tritium combining the techniques of thin-layer chromatography and radioautography. These studies relate to the work contained in the first part of this thesis only so far as the latter stems from observations made during the investigations of CTC. The work with the tritium was conducted because of a phenomenon observed during the thin-layer chromatographic analysis of the products of the DTBN-chloroplast reaction. This is an observation that polyethylene powder which can be used for TLC forms a transparent film if heated.

The results from these investigations are presented in separate sections.

CHARGE TRANSFER COMPLEXES

Introduction

In 1959, Tollin, Sogo and Calvin¹¹⁸ proposed a mechanism for the primary act of photosynthesis in which the chemical oxidation and reduction reactions of photosynthesis proceed independently at electron deficient and electron rich sites within the plant. The formation of these sites requires electron donors and acceptors. However, since very little was known about the mechanism of electron transfer between organic species, research was initiated in this laboratory to

investigate a class of chemical entities known as "charge transfer complexes" in hopes of understanding the conditions required to promote or cause electronic ionization and/or electron transfer in organic systems.

For information regarding the definition of a charge transfer complex, the various theories explaining their existence, and the means of detecting them, the reader is referred to the excellent reviews¹¹⁹ and books on these topics.¹²⁰ The results of the work previously conducted in this laboratory by other workers can be found in their theses.¹²¹

The work to be discussed in this section was a continuation of the researches conducted in this laboratory by D. R. Kearns, J. W. Eastman, and D. F. Ilten. Specifically, the work reported here was pursued in order to examine the magnetic and electric properties of charge transfer complexes, the acceptor component of which exists normally as a liquid. The principal interest was to determine whether photo-induced electron migration could be observed in these systems as evidenced by the observation of photo-conductivity or photo-generated radical ions.

Since the physical state of organic compounds which were known to function as the acceptor components in charge complexes at the start of this investigation was the solid state, a search was made to find organic compounds which were liquids under standard conditions and which would function as acceptor molecules with suitable complimentary molecules.

Pentafluorobenzonitrile (C_6F_5CN), hexafluorobenzene (C_6F_6) and ditertiarybutylnitroxide (DTBN) were the liquids investigated in this

study. Since both the cyano group and fluorine atoms are known to be highly electronegative, it was felt that C_6F_5CN and C_6F_6 would function as acceptors. DTBN is a radical and, therefore, one of its electrons is unpaired. The possibility that this electron could be shared with another molecule to form a CTC was examined.

Two different physical methods were used in attempts to detect CTC of these materials with various donor and acceptor molecules. The first method entailed the measurement of the electronic spectra of various mixtures. The presence of CTC are normally manifested in the electronic spectra of solutions containing both components. When CTC are present, a band (or bands) not found in the spectra of either of the compounds is detected in the spectrum of the mixture.¹²⁰

The second method involved the measurement of the electron paramagnetic resonance (EPR) spectra of mixtures containing DTBN. The spectrum of DTBN consists of a resonance split into a symmetrical triplet by the presence of the nitrogen atom. The hyperfine splitting constant (A_n) of the triplet is 15.4 gauss in cyclohexane solution. Theoretically, this splitting constant is proportional to the charge density on the nitrogen atom.¹²² If this is true and if the bonding in an electron donor-acceptor molecular complex takes place by delocalization of electrons over the constituents of the complex,¹²³ then the charge density and, therefore, the splitting constant, should change if DTBN functions as one of the partners in a CTC. If a full electron transfer occurs (either to or from DTBN), the EPR absorption should disappear.

Experimental

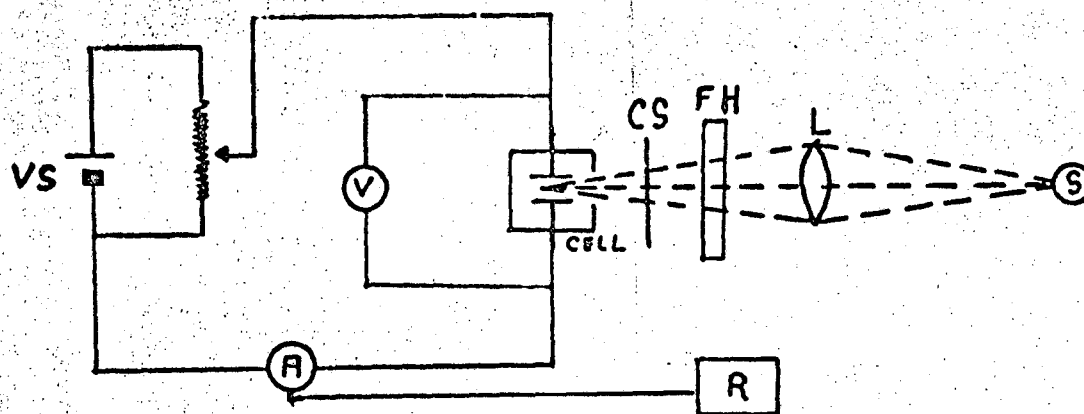
DTBN was prepared as described in appendix II. The solutions containing DTBN were prepared as stated in Chapter II and III. A description of the EPR equipment is given in appendix III.

The C_6F_6 used in this work comes from two sources. Material supplied by Pierce Chemical Company was used after one distillation in the studies involving equilibrium constants, spectra and ESR. Material received from Imperial Smelting Corporation was used in the conductivity experiments. It was twice distilled in a spinning-band column under vacuum. However, after distillation the material exhibited less electrical resistance than that from the source. Therefore, the source material was used without further purification. The material from the two companies have identical spectra in cyclohexane, and both behave identically on a di-2-ethylhexyl sebacate column in a Wilkenson VPC.

C_6F_6 was supplied by Monsanto and was once distilled before use. For the source of the other materials, see appendix III.

The electronic spectra were recorded on a Cary-14 recording spectrophotometer using quartz 1 cm-path length cells. Spectrograde cyclohexane was used in making the solutions.

Electrical measurements using a direct current method were made at a temperature of 33°C using a circuit shown schematically in Figure 54. The conductivity cell consisted of a circular, Pyrex container of approximately 5 ml volume with fixed, platinum electrodes. These were circular discs of one centimeter radius separated by 0.5 centimeter. On one side of the container, a quartz window had been attached using an Epoxy resin. The window was positioned so that



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Figure 54. Experimental arrangement used in direct current conductivity experiments. VS-7.5 volt dry cell; A-Keithley 410 picoammeter; V-Keithley 610A electrometer; R-Mosley 680 chart recorder; C-conductivity coil; Cs-camera shutter; FH-filter holder; L-quartz len; S-super high pressure Hg arc lamp (pek-107).

light passing through the cell passed between the two electrodes. The distance from the outer surface of the window to the center of the electrodes was approximately 1.5 cm. The container was surrounded, except for the quartz window, by a plastic container for circulating a liquid around the cell to maintain a constant temperature in the cell. This assembly, painted black except for the window, was enclosed in a metal container which was used as an electrical shield.

A Keithley Model 610A electrometer with an input impedance of 10^{14} ohms was used to measure the voltage drop across the cell. A Keithley Model 410 micromicroammeter, capable of measuring currents as low as 10^{-13} , was used to measure the current. The output of the ammeter was supplied to a Mosely Model 680 recorder. The light source was a super high pressure mercury arc lamp, type PEK-107. All samples were purged with N_2 prior to each experiment.

The measurements of the electronic spectra were taken on a Cary 11 or 14 recording spectrophotometer or a Perkin-Elmer 221. One (1) cm cells were generally used.

Results and Discussion

1. DTBN. The results of the EPR experiments conducted with DTBN and various donor and acceptor molecules are summarized in Table XVII. Many of the compounds shown in Table VI (Chapter II) should also be included in this table. However, the EPR data on the basis of the splitting constant, all of which are within experimental error, indicate that no charge transfer interactions are occurring between DTBN and the molecules examined in these studies. The same conclusion resulted from an examination of the electronic spectra of DTBN and various donor and acceptor molecules.

Table XVII

Interaction of DTBN with Various Chemicals

Concentration of DTBN	Donor or Acceptor		Concentration Ratio [comp.]/[DTBN]	Solvent	An ⁺ (g)	ΔH_{if}^{\ddagger} (g)	Temp. (C)
	Compound	[Conc.]					
I) 1.06×10^{-5} M	(none)	---	---	Cyclo- hexane	15.4	1	20°
II) "	N,N-Dimethylaniline	.247 M	2.3×10^4	"	15.4	1	20°
III) "	N,N,N'-Tetramethyl- p-phenylene diamine	.207 M	2.0×10^4	"	15.4	1	20°
IV) "	Hexamethylbenzene	.104 M	1.0×10^4	"	15.4	1	20°
V) "	Quinoline	.363 M	3.0×10^4	"	15.4	1	20°
VI) 1.64×10^{-5} M	(none)	---	---	"	15.4	1	R.T.
VII) "	Chloranil	Sat.	(?)	"	15.6	1.3*	R.T.
VIII) 2.72×10^{-5} M	(none)	---	---	Aceto- nitrile	15.7	3.3*	R.T.
IX) "	Tetracyanoethylene	.025 M	9.2×10^2	"	---	---	R.T.
X) 6.5×10^{-4} M	(none)	---	---	CHCl ₃	16.0	.8	22°
XI) "	s-Trinitrobenzene	.210 M	3.2×10^2	"	15.7	.6	22°
XII) "	p-Nitrobenzonitrile	.199 M	3.0×10^2	"	15.8	.6	22°

Table XBII (Cont.)

Concentration of DTBN	Donor or Acceptor		Concentration Ratio [comp.]/[DTBN]	Solvent	An ⁺ (g)	ΔH_{if} [‡] (g)	Temp. (C)
	Compound	[Conc.]					
XIII) 6.5×10^{-4} M	m-Dinitrobenzene	.179 M	2.8×10^2	CHCl ₃	15.8	.6	22°
XIV) "	Chloranil	Sat.	(?)	"	---	---	22°

+) An = hyperfine splitting constant $\pm .2g$

‡) ΔH_{if} = peak width at inflection points of absorption curve

*) Width due to modulation broadening.

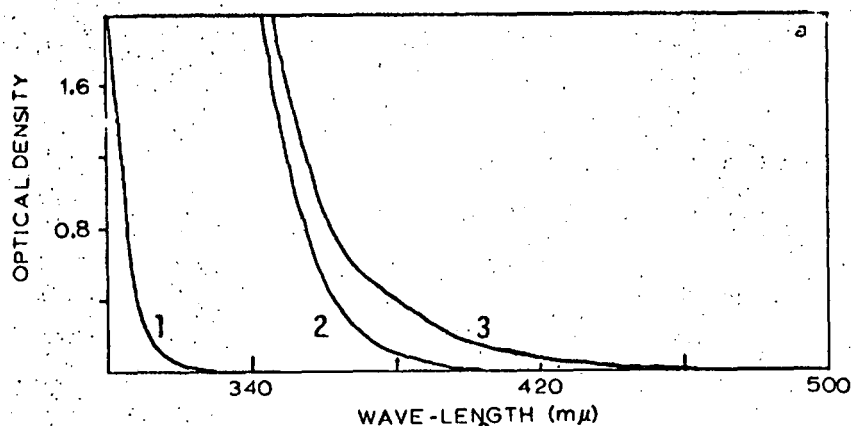
2. $\text{C}_6\text{F}_5\text{CN}$ and C_6F_6 . As indicated by the spectra shown in Figure 55 (the presence of an extended shoulder in the spectra of the mixtures), pentafluorobenzonitrile complexes with N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), with N,N-dimethylaniline (DMA) and with phenothiazine in cyclohexane. In addition, crystalline complexes of $\text{TMPD-C}_6\text{F}_5\text{CN}$ and $\text{DMA-C}_6\text{F}_5\text{CN}$ are obtained when the pure materials are mixed.

These two organic bases also form solid adducts with C_6F_6 when the materials are mixed in the pure state in a ratio of 1:1. However, new absorptions are not detected in the spectra (in cyclohexane solutions) of mixtures of TMPD or DMA with C_6F_6 . When C_6F_6 is used as a solvent for TMPD or DMA, the solutions are visibly yellow. However, with time a reaction occurs, as evident by the formation of a black precipitate.

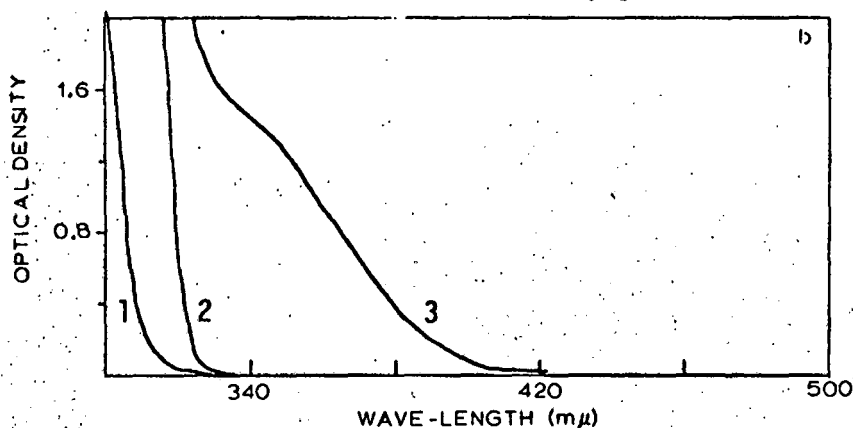
The ultra-violet-visible absorption spectra of $\text{C}_6\text{F}_5\text{CN}$, C_6F_6 , TMPD and DMA (in cyclohexane) are shown in Figures 56 and 57, respectively.

The solid complex formed between $\text{TMPD-C}_6\text{F}_6$ was analysed by a combination extraction and spectroscopic method and by vapor phase chromatography and found to contain an approximate ratio of the two components of 1:1. This result agrees approximately with an elemental analysis of this complex, which was as follows:

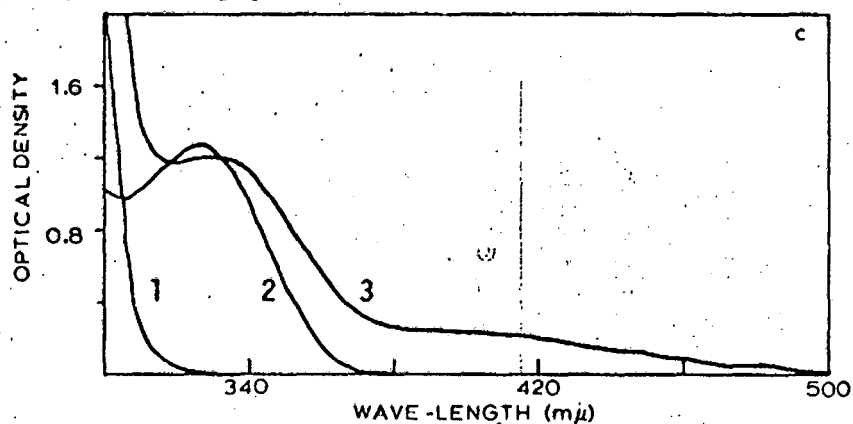
	C	H	N
Calc.	57.13	4.51	11.78
Found	59.87	5.56	12.06



(a) 1. 0.74 M C₆F₅CN; 2. 1.3 x 10⁻³ M phenothiazine; 3. mixture of 1.3 x 10⁻³ M phenothiazine and 0.1T M C₆F₅CN.



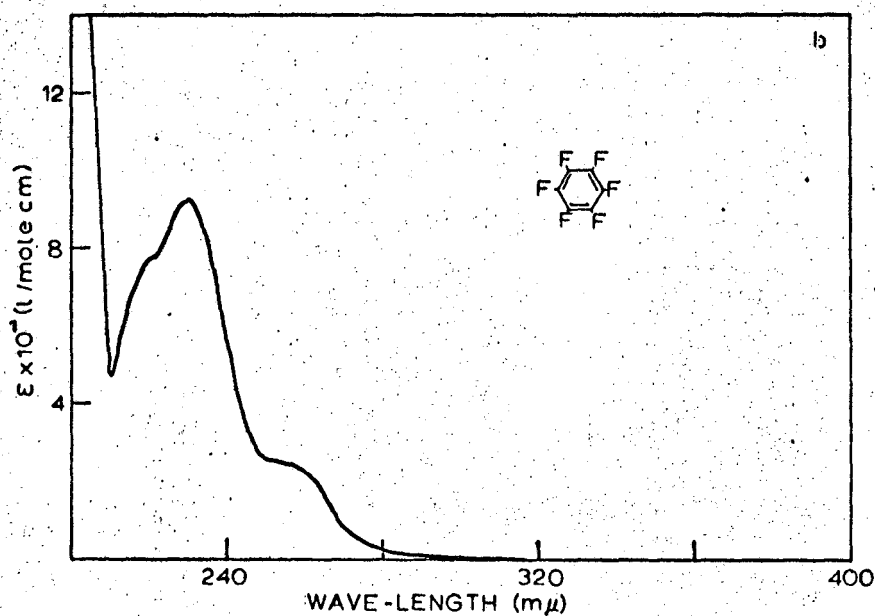
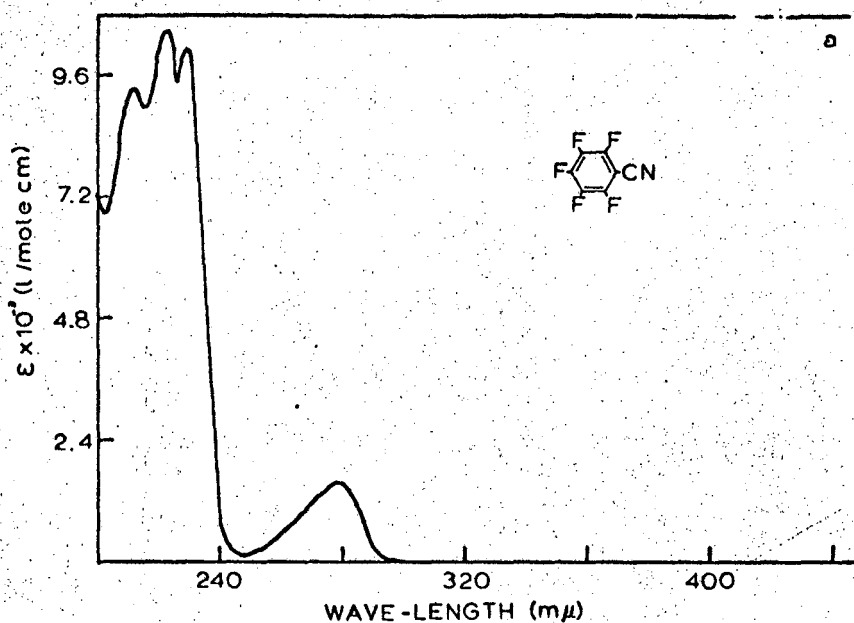
(b) 1. 0.74 M C₆F₅CN; 2. 4.52 x 10⁻³ M DMA; 3. mixture of 4.52 x 10⁻³ M DMA and 0.66 M C₆F₅CN.



(c) 1. 0.07 M C₆F₅CN; 2. 5.06 x 10⁻⁴ M TMPD; 3. mixture of 5.06 x 10⁻⁴ M TMPD and 0.73 M C₆F₅CN.

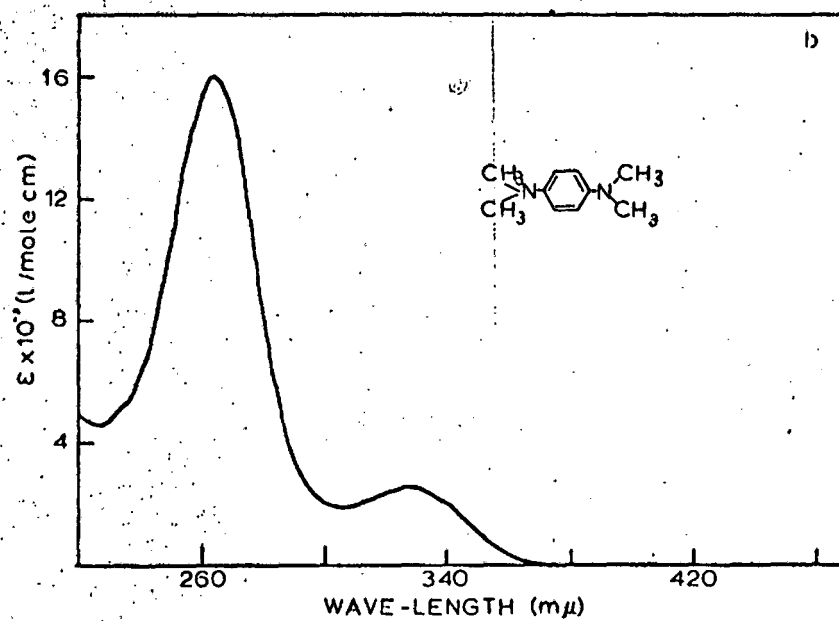
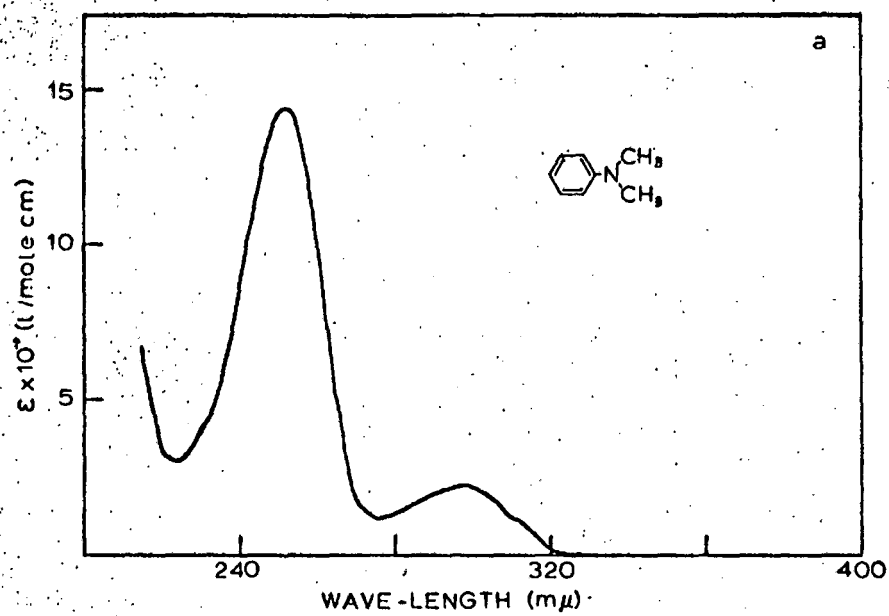
XBL679-6137

Figure 55. Optical spectra in cyclohexane solution at room temperature in 1 cm cells.



XBL 679-6136

Figure 56. Optical spectra of pentafluorobenzonitrile (a), and hexafluorobenzene (b), in cyclohexane.



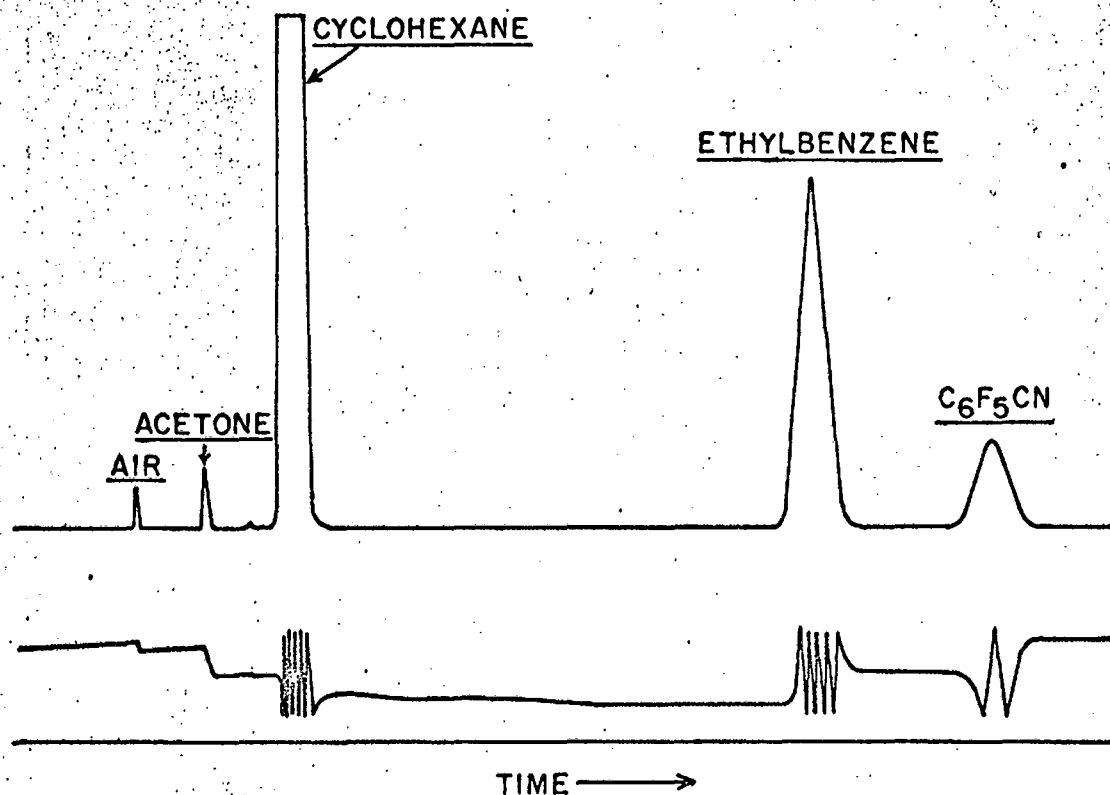
XBL 679-6135

Figure 57. Optical spectra of DMA (a), and TMPD (b), in cyclohexane.

The analysis involving the extraction consists of removing the organic base from a cyclohexane solution containing a weighed amount of the complex. The base was removed with dilute HCl solution. The UV-vis spectrum of the cyclohexane solution exhibited the spectrum of C_6F_5CN (see Figure 56). Using the known extinction coefficient of C_6F_5CN , the quantity in the complex was calculated. The difference in weight between that of the complex used minus the amount of C_6F_5CN found was assumed due to TMPD. The molar ratio of TMPD to C_6F_5CN was calculated to be 1:1. A control to determine the amount of C_6F_5CN extracted into the HCl was performed simultaneously. C_6F_5CN is essentially not soluble in HCl. The TMPD was extracted from the cyclohexane almost totally, as evident from the lack of absorption above 300 m μ (compare Figures 56 and 57).

The analysis by VPC involved the use of an internal standard, ethylbenzene. Unfortunately, conditions were not found by which both C_6F_5CN and TMPD could be detected by VPC simultaneously. Therefore, a known amount of the (TMPD- C_6F_5CN) solid complex was analysed for its C_6F_5CN content and the difference between the amount of the complex used minus the amount of C_6F_5CN detected was assumed due to TMPD.

The procedure involved mixing known amounts of the complex with known amounts of ethylbenzene (EB) and cyclohexane. Known amounts of this solution were injected into the VPC (20% di-(2-ethylhexyl) Sebacate on 60/80 firebrick column. From the resulting chromatogram, the ratio of areas between ethylbenzene and C_6F_5CN were determined using a disk integrator. A typical trace is shown in Figure 58. The detector system was calibrated by injection of mixtures of ethylbenzene and C_6F_5CN in cyclohexane. The ratio of the weight versus



XBL 679-6161

Figure 58. VPC trace of an aliquot of a cyclohexane solution containing 6.8 mg of TMPD-C₆F₅CN charge transfer complex and 7.0 mg of ethylbenzene. The column was 20% di-(2-ethylhexyl) Sebacate on firebrick. The temperatures of the column, the detector, and the injector were 105°, 240° and 230°C, respectively. The flow was 60 ml/min. Lower curve is trace from disk integrator.

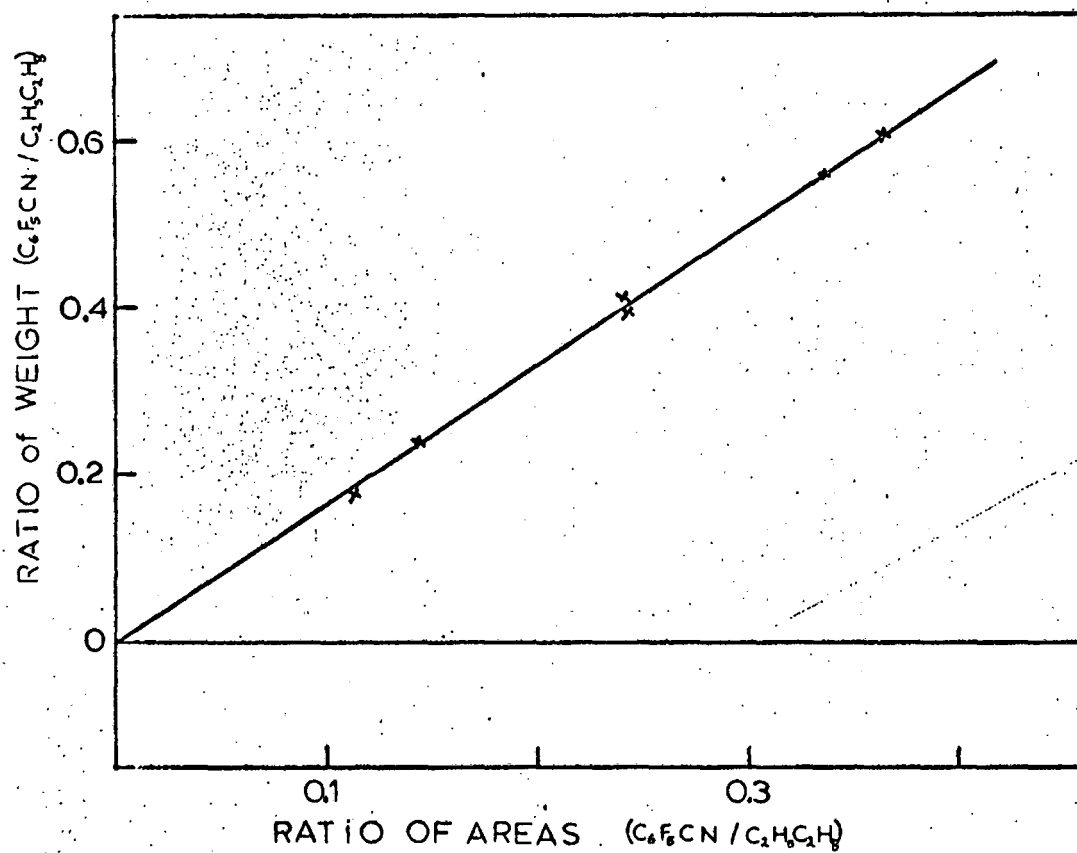
the ratio of detected areas for C_6F_5CN to EB was plotted as shown in Figure 59. This plot was used to obtain the weight of C_6F_5CN in a given weight of the complex using the VPC data obtained with the EB/TMPD- C_6F_5CN complex mixtures. The complex was found to contain, for three different samples, 51%, 51% and 52% by weight C_6F_5CN . If one assumes a 1:1 complex between C_6F_5CN and TMPD, the per cent C_6F_5CN by weight in the complex is calculated to be 51%.

Analysis of DMA- C_6F_6 or DMA- C_6F_5CN was complicated by the presence of excesses of the liquid components on the crystalline complexes. When attempts were made to dry the crystals, the crystals dissociate as the excesses evaporate until the crystals and the two components totally disappear. The complex, C_6F_6 -TMPD, was not analysed. Nor was the C_6F_5CN -phenothiazine.

The association constants for the TMPD- C_6F_5CN and the DMA- C_6F_5CN complexes were determined according to the method of Hildebrand and Benesi.¹²⁴ The equation developed by these workers, assuming one of the components is used in high excess over the other, is as follows:

$$\frac{1}{\epsilon_I} = \frac{1}{[A]K} + \frac{1}{\epsilon_C} + \frac{1}{\epsilon_C} = \frac{O.D.}{l \cdot [D]}$$

where $[D]$ and $[A]$ are the concentrations of the donor and acceptor, respectively; K , the association constant; O.D., the measured optical density at a particular wavelength; l , the path length of the cell; and ϵ_C , the extinction coefficient of the complex, at the wavelength where the O.D. is measured. Another assumption in this equation is that only the complex absorbs at the wavelength employed, to determine the K value.



XRL 679-6144

Figure 59. Calibration plot of VPC detection system.

The data obtained for the C_6F_5CN -TMPD and C_6F_5CN -DMA complexes, plotted according to the above equation, are shown in Figure 60.

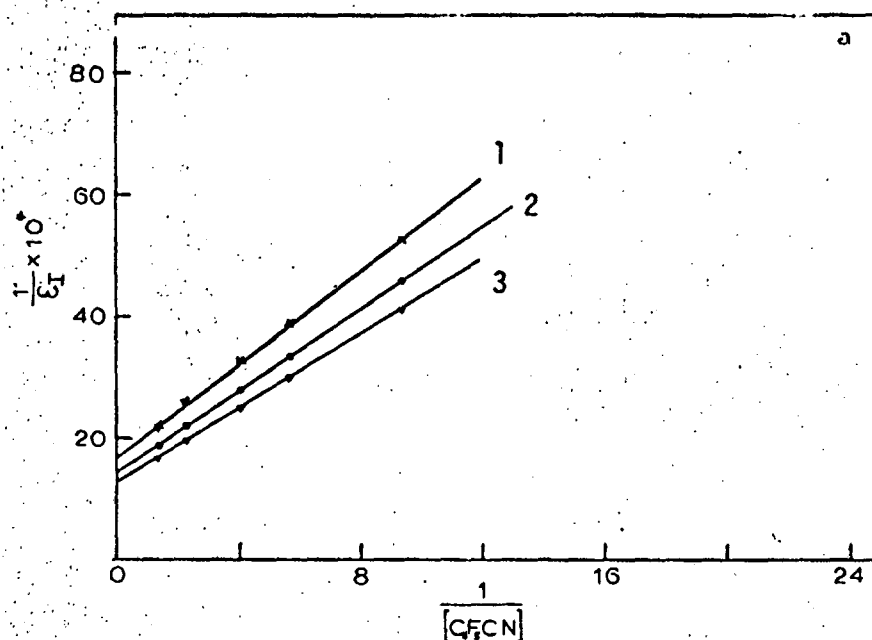
The association constants and extinction coefficients are given in Table XVIII.

Table XVIII

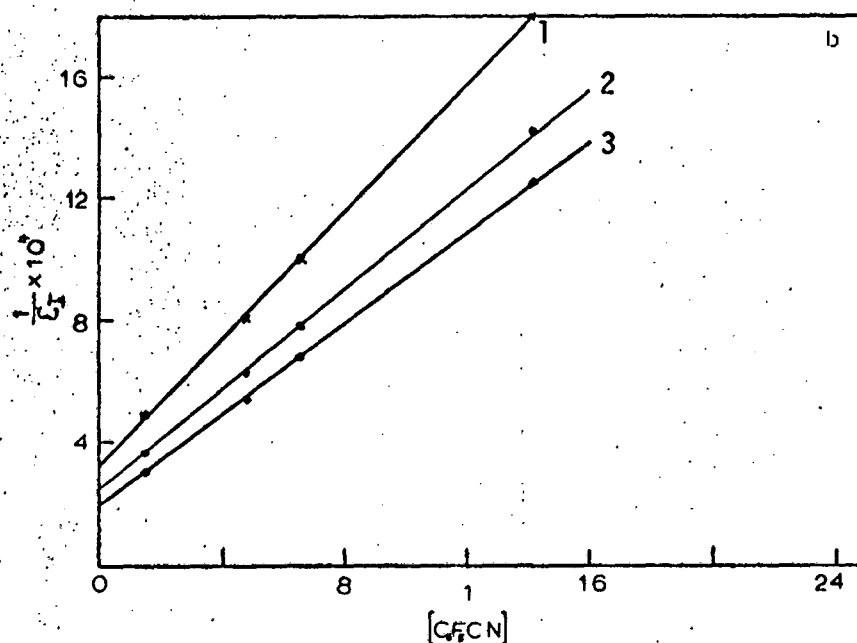
C_6F_5CN	Concentrations TMPD	DMA	D (m)	K moles/l	ϵ_c (l/mole·cm)
0.08 to 0.73 <u>M</u>	5.06×10^{-4} <u>M</u>	---	380	4.0	800
" "	"	---	400	4.1	700
" "	"	---	420	4.3	600
0.07 to 0.66 <u>M</u>	---	4.52×10^{-3} <u>M</u>	340	2.7	500
" "	---	"	350	3.1	400
" "	---	"	360	3.1	310

The path length of the cell was 1 cm in all these experiments.

The nuclear magnetic resonance (NMR) spectra of DMA and TMPD in C_6F_5CN are shown in Figures 61 and 62. Also included in these figures are the NMR spectra of these materials in CCl_4 . The broadened resonance lines of TMPD in C_6F_5CN is believed due to lifetime broadening caused by the paramagnetic cation, $TMPD^+$, although this species was not detected by EPR even with illumination. The lack of broadening in the DMA resonance lines in C_6F_5CN demonstrates that the line broadening observed in the TMPD solution is not connected with the complexing of TMPD.



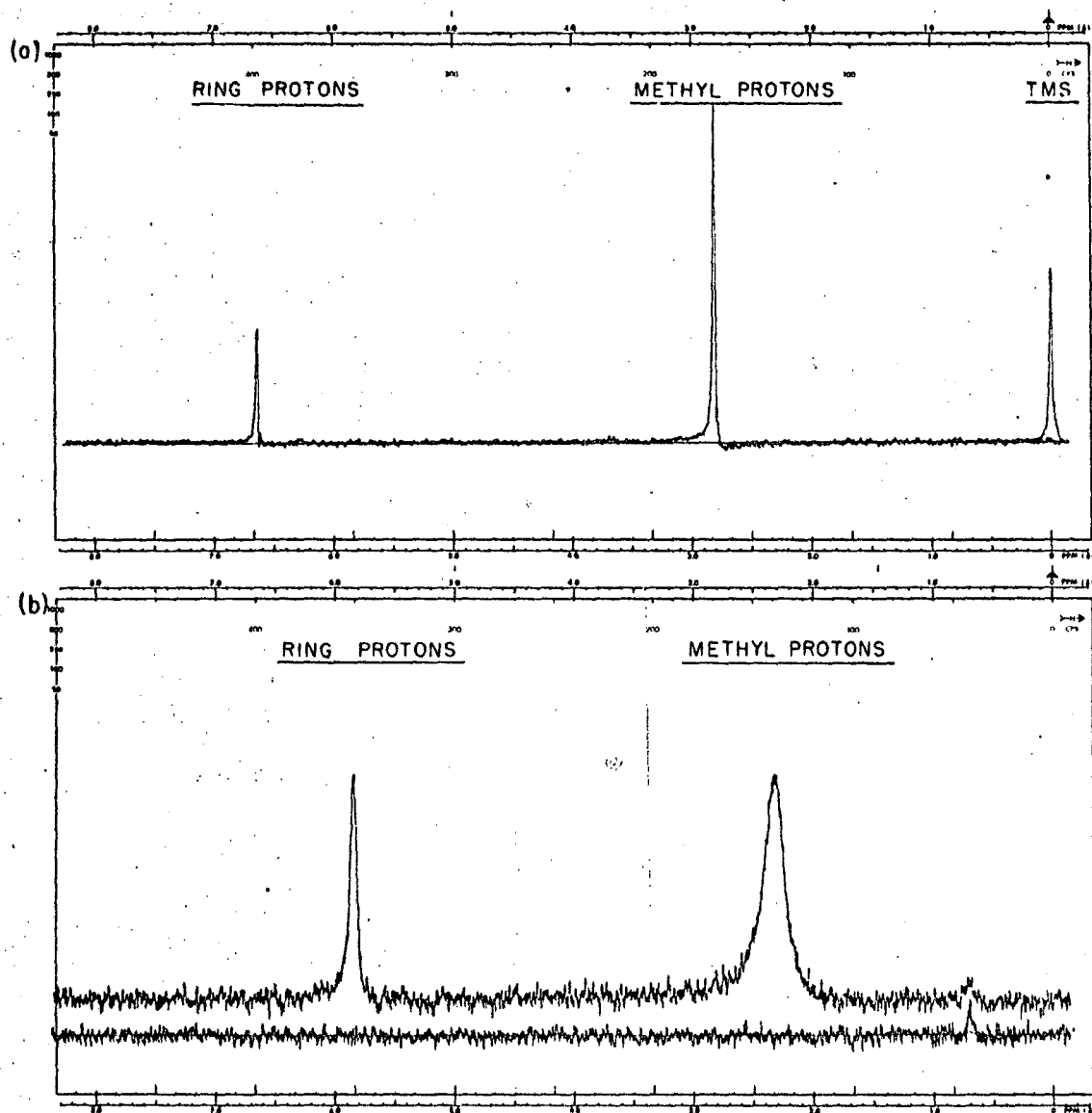
(a) TMPD-C₆F₅ complex at 1) 420 mμ, 2) 400 mμ, and 3) 380 mμ.



(b) DMA-C₆F₅CN complex at 1) 360 mμ, 2) 350 mμ, and 3) 340 mμ.

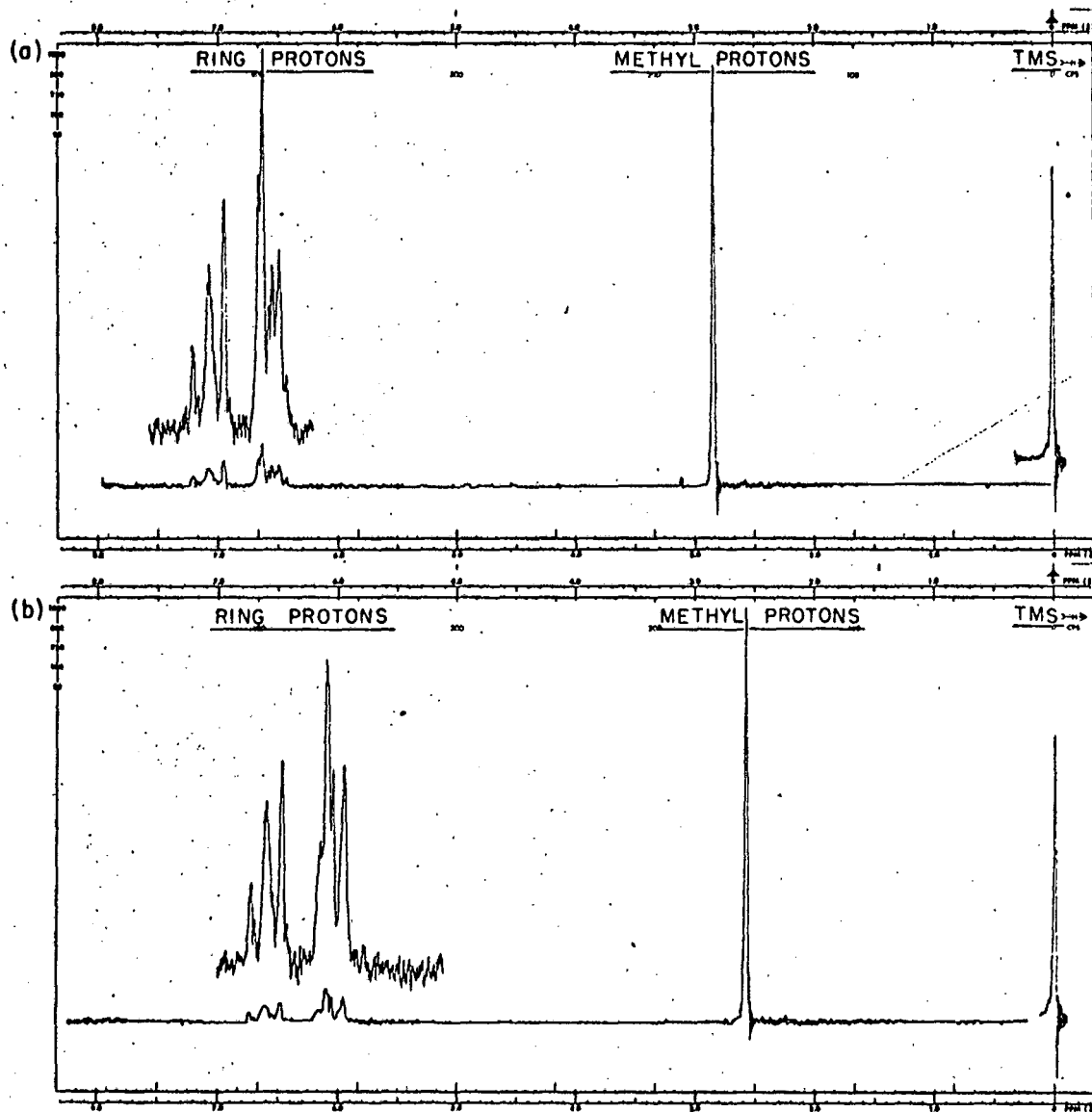
XBL 679-6134

Figure 60. Hildebrand-Benesi plot of charge transfer absorption.



XBL 679-6139

Figure 61. NMR spectra of TMPD in (a) CCl_4 and in (b) $\text{C}_6\text{F}_5\text{CN}$.



XBL 679-6136

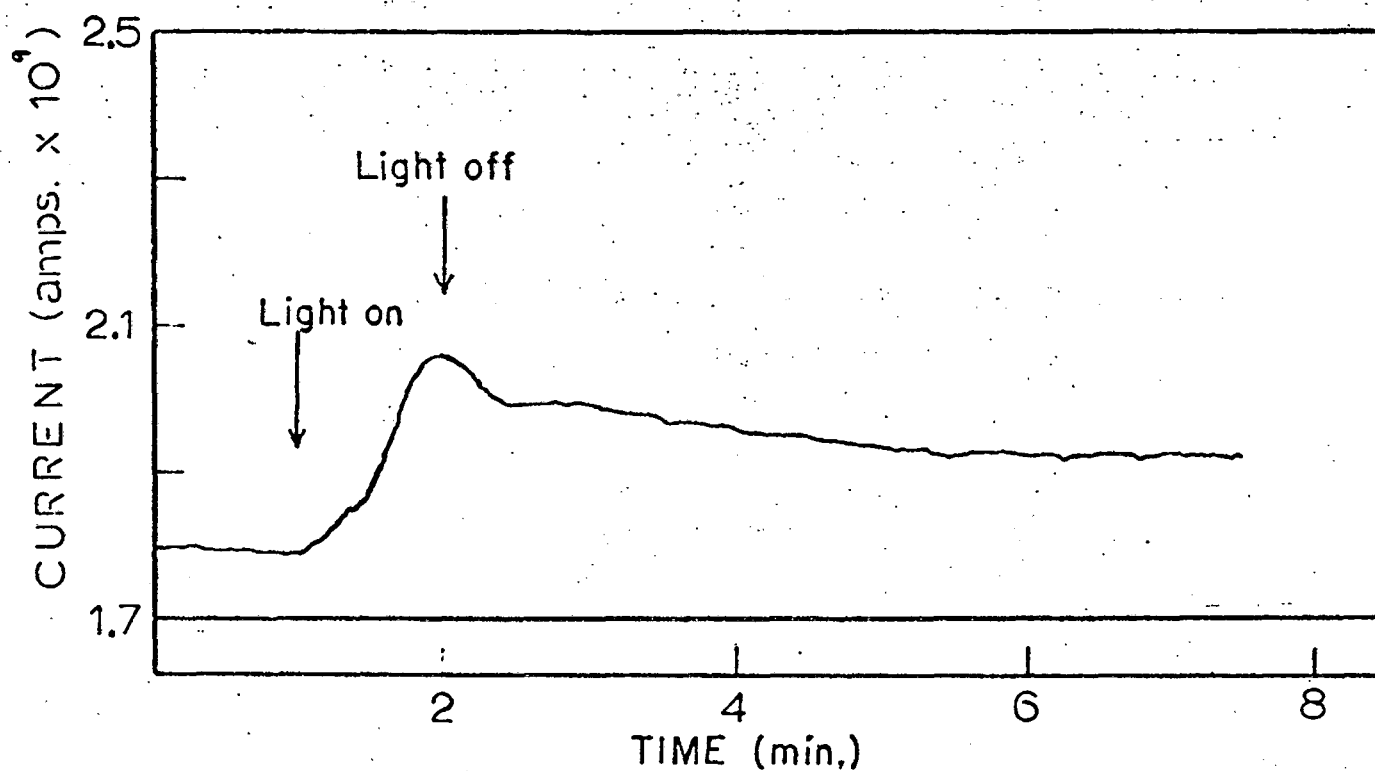
Figure 62. NMR spectra of DMA in (a) CCl_4 and in (b) $\text{C}_6\text{F}_5\text{CN}$.

The shift in the resonance lines to higher fields in C_6F_5CN is also observed for the resonance line of cyclohexane in C_6F_5CN when compared to solution in CCl_4 . The shift is probably due to internal fields caused by the ring currents of the aromatic system of the nitrile and by the magnetic moments of the fluorine atoms.

Although the NMR spectrum of the TMPD- C_6F_5CN complex suggested that free radicals were present in this complex, none could be detected by EPR either in solution (TMPD in C_6F_5CN) nor in the solid complex. Illumination with ultraviolet or visible light did not produce a detectable level of paramagnetic species.

The resistance of a 8.6×10^{-4} M solution of TMPD in C_6F_5CN is ohmic from 0 to 6 volts and has a resistivity of 3.5×10^8 ohm-cm at $33^\circ C$. The solvent, C_6F_5CN , has a resistivity of 8.8×10^8 ohm-cm at this temperature and exhibits no detectable variation of its resistivity when illuminated with ultraviolet or visible light. A 9.0×10^{-4} M solution of TMPD in spectrograde cyclohexane has a resistivity of 8.4×10^{10} ohm-cm at 33° . Light does not produce a resistivity variation in this solution either.

However, the solution of TMPD in C_6F_5CN when subjected to a 0.1 volt potential at $33^\circ C$ and illuminated by light from a mercury arc exhibits a definite variation in resistivity as evident in Figure 63. The curve shown in this figure was found to be non-reproducible (even qualitatively) from day to day unless the TMPD solution was made up fresh and the electrodes were cleaned with H_2SO_4 between each experiment. These observations suggested that photo-chemical reactions were occurring in these systems.



-170-

XBL 679-6150

Figure 63. Photo-current in a 8.6×10^{-4} M solution of TMPD in C_6F_5CN . The temperature was $33^\circ C$ and the applied voltage was 0.1 volt. The dark current was 1.9×10^{-9} amp.

In addition, when a filter was inserted between the light source and the cell so that only light of wavelength greater than 3600 Å passed through the cell, the light did not produce a variation in the resistivity. As this is the region of the electromagnetic spectrum in which the charge transfer band of this complex lies, this result indicates that the observed decrease of resistivity is not connected with the presence of the complex. Since it was necessary that both C_6F_5CN and TMPD be present in order to see the photo-effect, the photochemical reaction probably involves both species. However, a larger quantum of energy than that supplied by the complex absorption is required to produce the reaction.

Summary of CTC

Dimethylaniline and N,N,N',N'-tetramethyl-p-phenylenediamine form charge transfer complexes with both hexafluorobenzene and pentafluorobenzonitrile. Phenothiazine also forms a complex with the nitrile. Analysis of the C_6F_5CN -TMPD complex showed that the ratio of the two components in the complex is 1:1. The association constants for the DMA- C_6F_5CN and TMPD- C_6F_5CN complexes, as determined by the method of Hildebrand and Benesi,¹²⁴ are 3.1 and 4.1, respectively.

The NMR spectra of DMA and TMPD in C_6F_5CN differ from those attained using CCl_4 as the solvent in that the resonance lines are found at higher fields, the lines are closer together, and in the case of TMPD, the line width of the resonance lines are much broader. This latter observation is interpreted as due to the presence of the paramagnetic cation, $TMPD^+$, which causes increased relaxation and, thus, uncertainty broadening.

Illumination of the DMA or TMPD containing complexes with light from a mercury arc does not produce detectable levels (by EPR) of paramagnetic species. In addition, illumination of the TMPD in C_6F_5CN with light absorbed by the complex does not promote a photo-conductance in the solution. When UV light is used, a photo-induced change in the resistance of the solution is observed. However, the change in resistance appears to be due to the generation of ions during a photo-chemical reaction of TMPD.

Since light caused only photo-chemical reactions in these systems, these investigations were suspended and the investigations with DTBN initiated.

Tritium Detection

Introduction

Many common isotopes, important in biological systems, i.e., ^{14}C , ^{35}S , and ^{32}P , can be detected easily by using the combined techniques of thin-layer of paper chromatography and radioautography. If the chromatogram containing the radioactive element is placed in opposition to a film, the β -particles emitted by the isotope penetrate the emulsion leaving a darkened track upon development of the film. This technique has proven very useful for the detection of elements emitting medium or high energy β -particles. Tritium, however, emits a very soft beta (max. energy .018 mev) with a small penetration radius. Therefore, detection of compounds containing low levels of tritium by the above technique has not been too successful due to lack of penetration of the β -particles into the emulsion.

The following is a preliminary report on the development of a process for detecting low levels of tritium utilizing the combined techniques of thin-layer chromatography and scintillation counting.

Method

The small penetration distance of tritium β -particles could possibly be overcome by converting the beta emission into photon emission, in situ, by incorporating a scintillator into the absorbant of a thin-layer chromatogram. With the scintillator in close proximity to tritium, the β -particle causes an excitation of the scintillator, resulting in photon emission. Generally, most of the photon emission would not be detected because of the light scattering nature of the powder of the chromatogram. However, by rendering the powder transparent, more efficient light transmission occurs. Powdered polyethylene has been found suitable as the absorbant material for it forms an almost transparent film by heating at 120° for several minutes.

Experimental

A chemically inert scintillator (approx. 2% by weight), either phenylbiphenyloxadizole-1,2,3 [PBD], 4,4'-bis-(2-butyloctyloxy)-p-quaterphenyl [BOQP], 2,5-diphenyloxazole (PPO) or a mixture, containing manganese activated zinc silicate and silver activated zinc sulfide, was mixed uniformly with powdered polyethylene. The resulting mixture was used to prepare a chromatogram .25 mm thick using an acetone slurry (50 g powder to 200 ml acetone).

The uniformity of the scintillator distribution on the plates was checked by visual inspection of their fluorescent emission in a darkened box containing a UV light source to effect excitation. Each

plate was then developed using an acetone/water (80/20) solvent system, and the uniformity checked again by the same method. No movement of the scintillator was detected. The powdered polyethylene was then fused into a transparent film by heating at 120° for several minutes and the uniformity of fluorescence was checked again by visual inspection.

In order to check the effect of the scintillator on the characteristics of the chromatogram, three plates, one containing PBD, one containing the inorganic mixture, and one without a fluor, were prepared and spotted with phenylalanine hydrochloride. The plates were developed in 80/20 acetone/water and sprayed with ninhydrin in order to locate the amino acid. The amino acid displays approximately the same R_f value on all three plates. This system would not be good for chromatography of this material since the amino acid HCl ran very near the solvent front. However, the important conclusion is that the scintillators did not appear to influence the movement.

Two plates, one with PBD and one with the inorganic materials, were spotted with some tritiated phenylalanine hydrochloride. The test solution contained 1.07×10^6 dpm per lambda (λ). Four spots were applied to each of the plates [10^4 , 10^5 , 10^6 , and 10^7 dpm] and the plates were developed until the amino acid had migrated up a third of the plate. Following fusion of the polyethylene, the chromatograms were placed in opposition to the film for a specific period of time. Three different types of film were used. The films used were Kodak Royal X Pan, Kodak Medical X-ray "no screen", and Kodak Medical X-ray Royal Blue. The spots containing 10^7 and 10^6 dpm

caused an intense darkening of all three films with both scintillators. The spot with 10^5 dpm also caused a detectable darkening of the Royal Blue and the "no screen" X-ray film; however, the X-Pan film was spoiled by an unknown exposure in the area of this spot. The spot containing 10^4 dpm did not expose any of the films. By visual inspection of the darkened areas, the combination of the organic fluor and the Royal Blue X-ray film appear to be the most sensitive combination.

Separate polyethylene plates containing either the organic scintillator BOQP or the inorganic scintillator mentioned above, were prepared. Four different concentrations of each (2, 1.5, 1.0 and 0.5%, and 8, 4, 2 and 1%, respectively, were used. Six spots (10^3 , 5×10^3 , 10^4 , 5×10^4 , 10^5 , and 10^6 dpm) of the tritiated amino acid were applied to each plate. The powder was fused without development of the plates and the resulting chromatograms placed in opposition to the Royal X-Pan film. The film was exposed for 34 hrs and developed with Kodak D-19 developer. The spots containing activity as low as 5×10^3 dpm caused a detectable darkening of the film. The darkening of the film and the level of detectability of the activity appears to be independent of the scintillator concentration in the concentration ranges examined. These experiments need to be repeated to include the development of the chromatogram prior to the fusion. If development is not included, the tritiated compound is found in a ring in the area of application, thus concentrating the tritium.

Discussion

These preliminary results suggest that this method is more sensitive by at least a factor of ten over currently used techniques utilizing autoradiography for the detection of tritium.¹²⁵

In an attempt to increase this sensitivity, the possible use of photomultiplier detection of the photon emission was being investigated. A light-tight box fitted with mounts for a thin-layer plate and a photomultiplier had been constructed. After the photomultiplier output pulse were amplified and suitably shaped, they were collected in a pulse height analyzer. Very preliminary results indicated a detection efficiency of 0.5 to 1.0 per cent.

APPENDIX I

Sources of Error in Using EPR for Kinetics

The procedure normally employed in EPR spectroscopy is to subject the spin system to constant electromagnetic radiation as the externally applied magnetic field is varied. As the resonance condition is traversed (when $h\nu = g\beta H$), power is absorbed from the radiation field causing an electrical imbalance in the spectrometer. Recording this imbalance of the spectrometer as a function of the applied field strength will yield a curve proportional to the absorption. Generally, however, the external magnetic field is modulated with an amplitude smaller than $\Delta H_{1/2}$ and the resulting AC signal is demodulated in a phase-sensitive detector. The imbalance that is then recorded as the field sweeps through the resonance condition is not proportional to the absorption but is proportional to the first derivative of the absorption. This derivative is what is called "signal" in this work.

The shape of the derivative as a function of applied field is dependent upon characteristics of the spectrometer (e.g., the operating behavior of the detector, the frequency at which the field is modulated, etc.), and characteristics of the spin system.

For a voltage sensitive microwave detector and an unsaturated spin system exhibiting a Lorentzian line shape function, the maximum in absorption derivative curve is proportional to the following: 1) a so-called filling factor which is the ratio of the sample volume to the cavity volume times a parameter which depends upon the microwave field distribution in the cavity and over the sample, 2) the quality factor of the EPR cavity which is defined as the ratio of the energy

stored to the energy loss in the cavity, 3) the amplitude of the field modulation (H_m), 4) the resonant frequency of the spin system at a field strength of H_0 , 5) the reciprocal of the temperature of spin system at equilibrium, 6) the square of the reciprocal of the line-width ($\Delta H_{1/2}$) of the absorption curve, 7) the square of the amplitude of the microwave field, and 8) the number of paramagnetic species (N) comprising the spin system (see reference 2-5). The constant of proportionality includes gains, time constants and various numerical constants (i.e., π , B , h , etc.).

In using the EPR for kinetic studies of paramagnetic species, one generally assumes that all the factors listed above are constant with time except N and therefore the maximum of the curve (S_{\max}). During a particular kinetic experiment this assumption is probably valid, the possible exception being that the line-width also varies with changes in N (see Figure 77 in appendix II).

Therefore,

$$\frac{\delta S_{\max}}{\delta t} \propto \frac{1}{(\Delta H_{1/2})^2} \frac{\delta N}{\delta t} - \frac{\delta N}{(\Delta H_{1/2})^3} \frac{\delta \Delta H_{1/2}}{\delta t} \quad (1)$$

$$\text{however, } \frac{\delta \Delta H_{1/2}}{\delta t} = \frac{\delta \Delta H_{1/2}}{\delta N} \times \frac{\delta N}{\delta t} \quad (2)$$

Therefore,

$$\frac{\delta S_{\max}}{\delta t} \propto \frac{1}{(\Delta H_{1/2})^2} \left[1 - \frac{\delta N}{\Delta H_{1/2}} \frac{\delta \Delta H_{1/2}}{\delta N} \right] \frac{\delta N}{\delta t} \quad (3)$$

For the kinetic experiments reported in this work, the second term in the bracket of equation (3) is ca. 0.04 ($N_0 = 10^{-5}$ mole/l;

$\Delta H_{1/2}/\Delta N$ ca. 200 gauss/mole/l and $\Delta H_{1/2}$ ca. 1 gauss). Thus the contributions to the decay of S_{\max} due to $\Delta H/\Delta N$ are slight. The effects of other perturbations which cause changes in the line-width (for example, the generation of other paramagnetic species, saturation, etc.) are also probably very slight.

The major problem encountered in using EPR for kinetic studies is the impossibility of reproducing all the conditions required to obtain an identical proportionality constant from experiment to experiment. This difficulty is generally circumvented by normalizing the data according to the following equation:

$$\frac{S_t}{S_0} = \frac{N_t}{N_0} \quad (4)$$

where S_t and N_t are the signal height and the concentration of the paramagnetic species at time t , and S_0 and N_0 are these same quantities at $t = 0$.

The difficulty in treating the data in this way is that unless the chemical destruction of N is first order in the concentration of N , the rate of decay observed from plots of S_t/S_0 versus t are a function of the value of N_0 . For many systems investigated by EPR, this value is difficult to determine. Fortunately, N_0 could be determined in this work by measurements of optical absorption spectra.

Another source of error in using the EPR for kinetic analysis is the possibility that the rate of change of S_{\max} is not directly proportional to the rate of change of N . This could arise if drift occurs in the spectrometer. Normally in following the decay of S_{\max} , one positions the magnetic field at one field strength (the position where S_{\max} occurs). If the spectrometer drifts from this position

because of variations in the magnet, in the frequency of the klystron, or within the cavity, the rate of change of S_{\max} is then also a function of this drift.

Thus,

$$\frac{\delta S_{\max}}{\delta t} = \frac{\delta N}{\delta t} + \frac{\delta S}{\delta H} \times \frac{\delta H}{\delta t} \quad (5)$$

where $\delta H/\delta t$ represents the drift, and $\delta S/\delta H$ is the slope of the derivative curve which is normally quite large in the region of S_{\max} (see Figure 11, Chapter II). This drift is probably not significant unless lengthy decay times are encountered. Also, the drift is generally not constant nor linear in time. In the experiments reported in this work, this type of drift normally caused a machine limited decrease in S_{\max} which was obviously due to shifts from the selected field position.

Other variations which contribute to the observed decay of S_{\max} are changes in the base line. This is an insignificant source of error unless the changes are extremely sporadic. Normally the drift resulting in these is linear in time and in one direction during one kinetic run. Therefore, the observed decay of S_{\max} can be corrected for this drift. This was done in experiments reported in this work. The position of the base line was checked by the procedure stated in Chapter II.

APPENDIX II

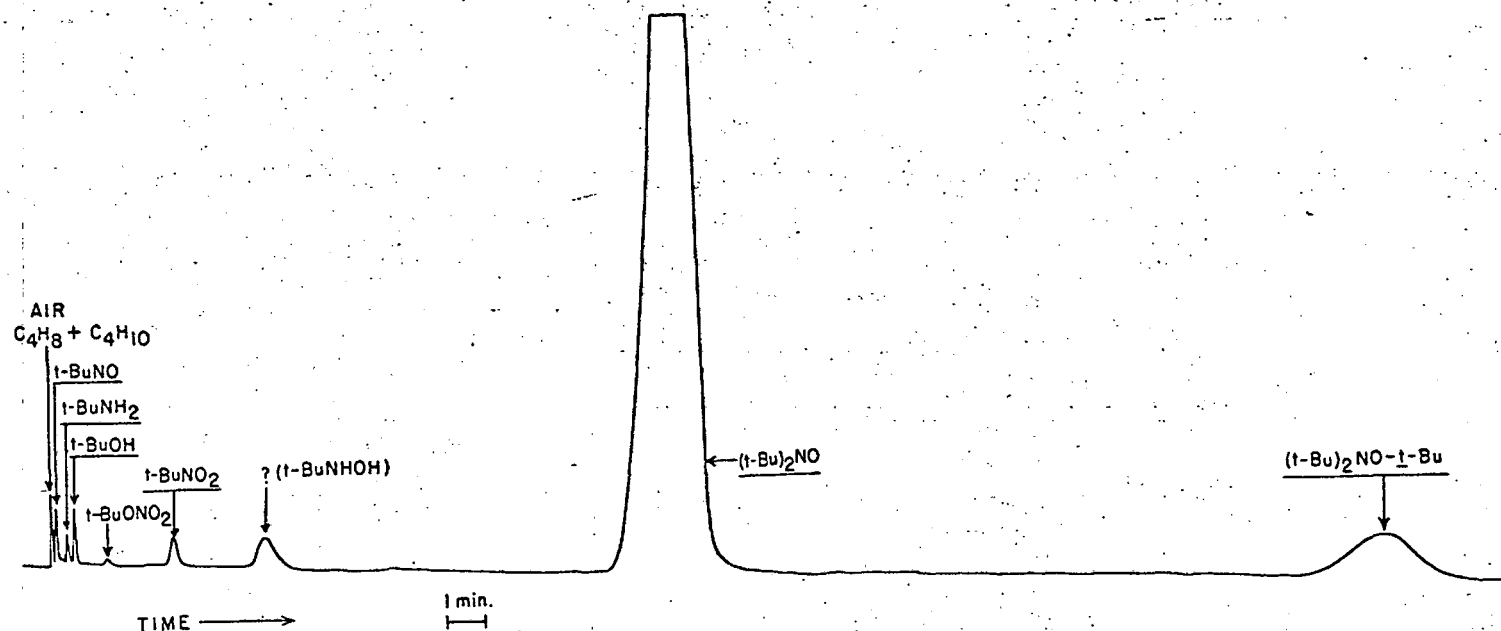
A. Preparation and Properties of DTBN

Ditertiarybutylnitroxide and di-tertiarybutylhydroxylamine were prepared according to the procedures of Hoffman.¹⁰⁸ The nitroxide was purified by spinning band distillation at a pressure of 11 mm Hg at 58°C and stored in a refrigerator. The hydroxylamine was isolated as the hydrochloride and purified by crystallization from an alcohol/diethylether mixture. The starting material, 2-methyl-2-nitropropane, was prepared according to the method of Kornblum¹²⁶ from tertiary-butylamine which was supplied by Matheson, Coleman and Bell.

Analyses of the three products were as follows:

$C_{12}H_{27}NO:$	C-66.60,	H-12.58,	N-9.71	Calc'd
	-66.35	-12.35	-9.76	Found
$C_{12}H_{29}NOCl:$	-52.88	-11.09	-7.71	
	-52.86	-11.40	-7.91	
$C_4H_9NO_2:$	-46.59	- 8.80	-13.78	
	-46.87	- 8.83	-13.35	

The purity of the nitroxide radical, checked by vapor phase chromatography (VPC), was found to be greater than 99%. Figure 64 is a spectrum obtained from the VPC which shows the various impurities found in the nitroxide. The identities of the compounds underlined were assigned by co-chromatography of the radical with known compounds. The remainder were assumed to be those reported by Hoffman.



-182-

XBL 677-4545

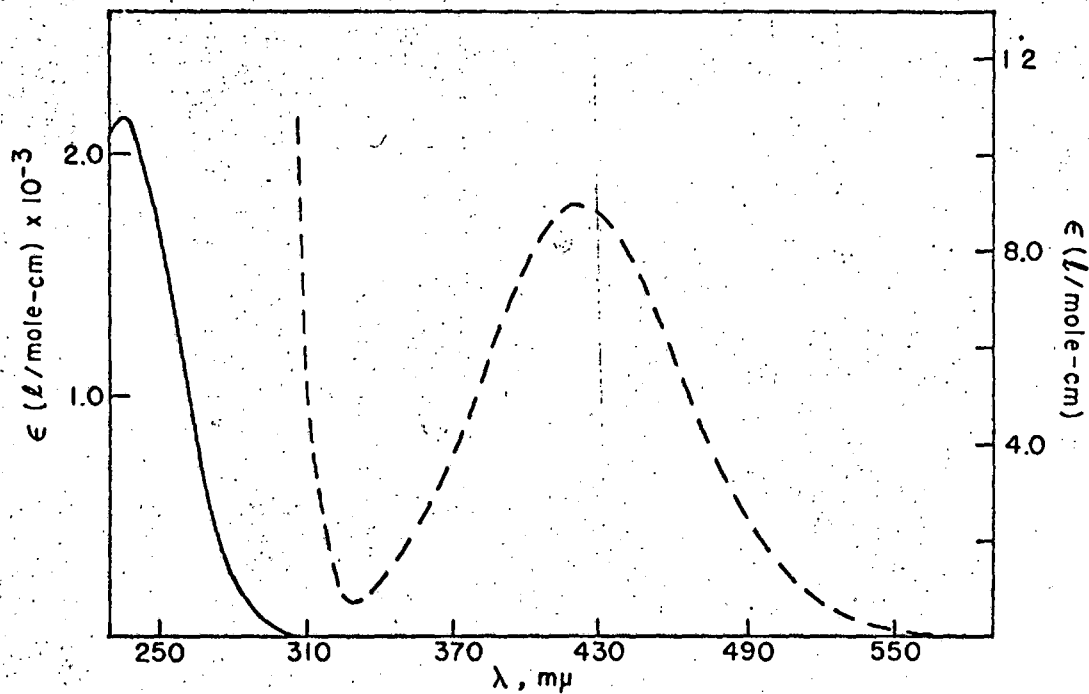
Figure 64. VPC trace showing the impurities in DTBN. The column was 15% diisodecyl phthalate on chromosorb W at 90°C. The temperatures of the detector and the injector were 110° and 105°, respectively. The He flow was approximately 20 ml/min. The peak with the question mark (t-BuNHOH) is also found in t-BuNO₂ prepared by KMnO₄ oxidation of t-BuNH₂.

The ultraviolet-visible spectrum of DTBN in H_2O is shown in Figure 65. The visible absorption in H_2O and in cyclohexane are compared in Figure 66. These two solvents were used as solvents for the nitroxide in much of the work in this thesis. These spectra were obtained using a Cary 14 spectrophotometer and 1 cm cuvettes.

Figures 67 and 68 are the EPR spectra of the same solution of DTBN in cyclohexane. The difference in the two spectra is caused by the presence of oxygen in the sample used for Figure 68. The paramagnetism of oxygen causes lifetime broadening of the nitroxide signal which results in a decreased signal height.

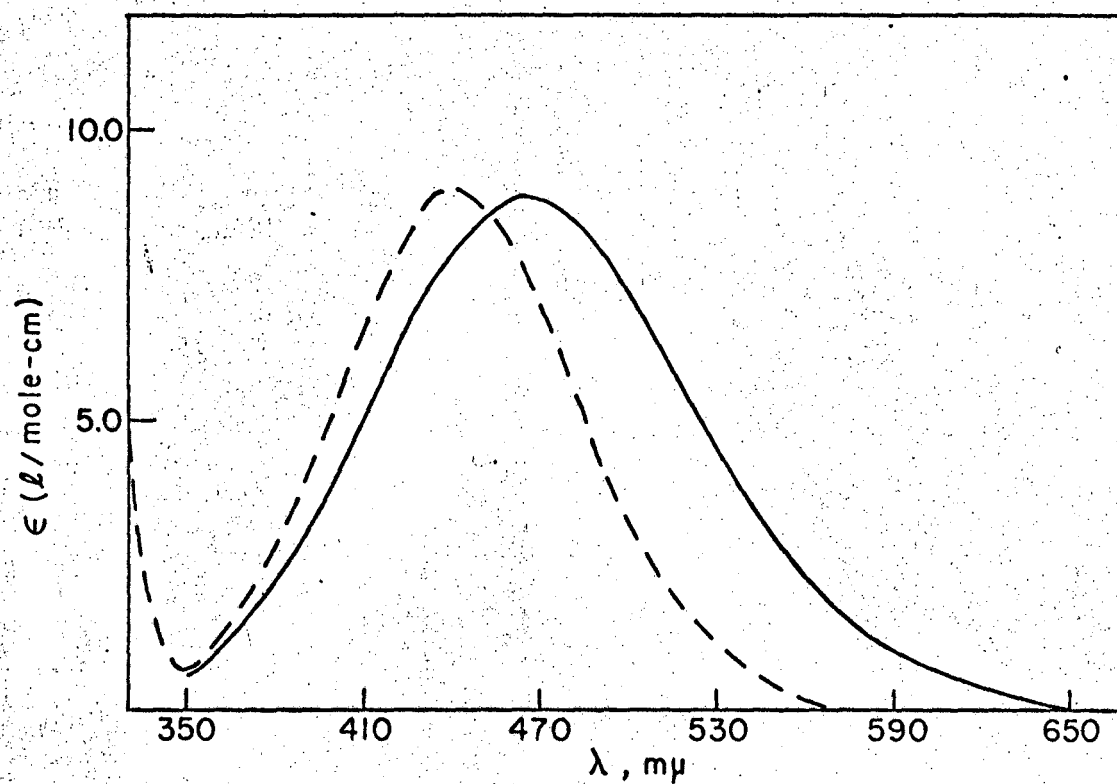
Since the height of the nitroxide EPR signal was to be used to follow the reactions of DTBN, the effects of several mild perturbations upon the nitroxide signal height were investigated with the results shown in Figures 69 through 73. The slope of the line in Figure 69 is $\pm 1.05\%$ per cent change in power setting. This parameter is dependent upon the experimental conditions (the concentration, temperature, etc.) used in measuring the spectra. However, the power value where saturation sets in should not differ greatly for the range of conditions reported in this work. The power settings employed varied between 0.4 to 0.7 of the maximum value available.

Figure 70 shows the variation of the nitroxide EPR spectral line-width with concentration. The small insert in this figure shows the concentration region used in this work. This is the concentration range where three distinct lines are observed for the nitroxide. When the concentration of DTBN is increased above $10^{-2} M$, the three lines become so broadened that they start to overlap. This



XBL 678-4569

Figure 65. Optical spectrum of DTBN in water. The scale on the right applies to the dashed curve; the scale on the left to the solid curve.



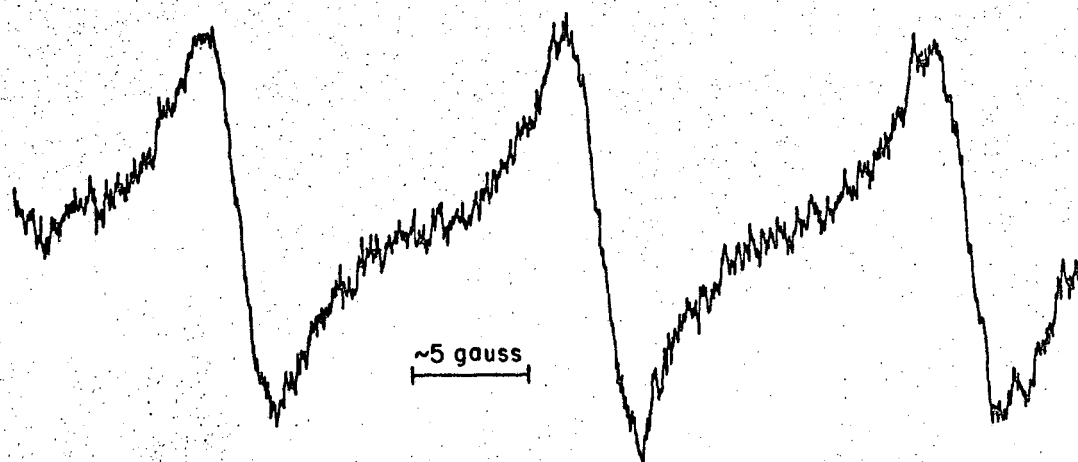
XBL 678-4568

Figure 66. Comparison of the visible absorption spectra of DTBN in water (dashed curve) and in cyclohexane (solid curve).



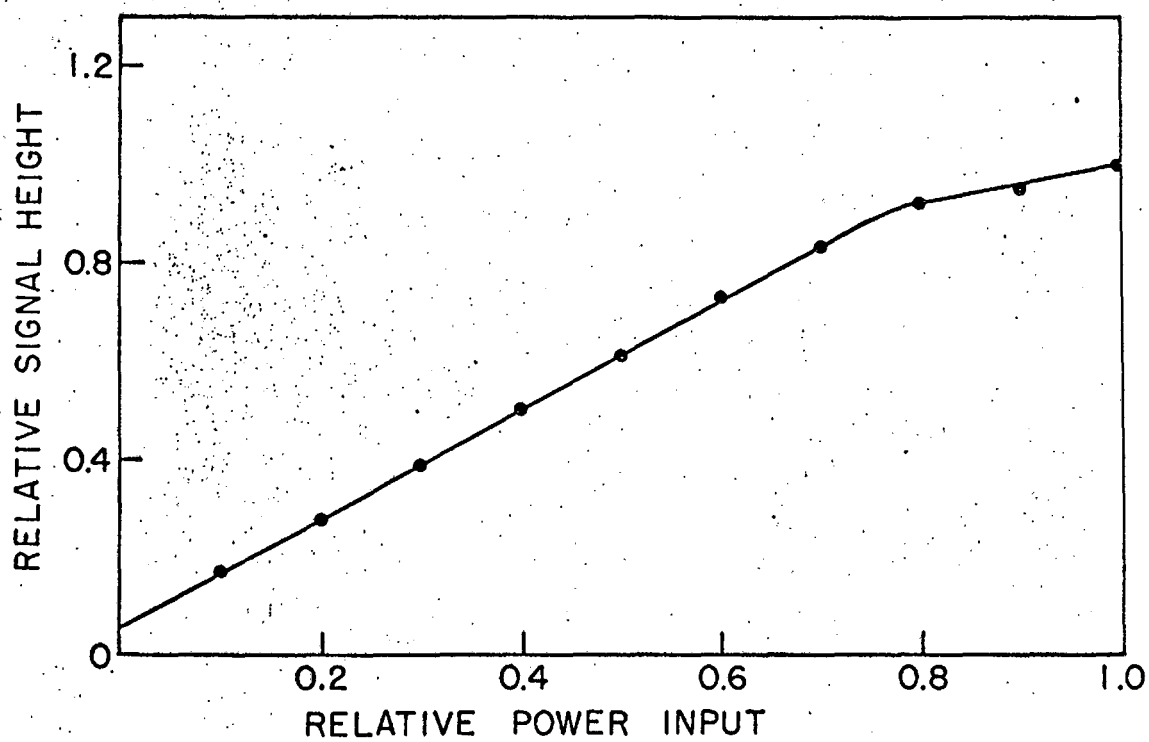
XBL 677-4538

Figure 67. EPR spectrum of 1.06×10^{-5} M DTBN in cyclohexane without air.



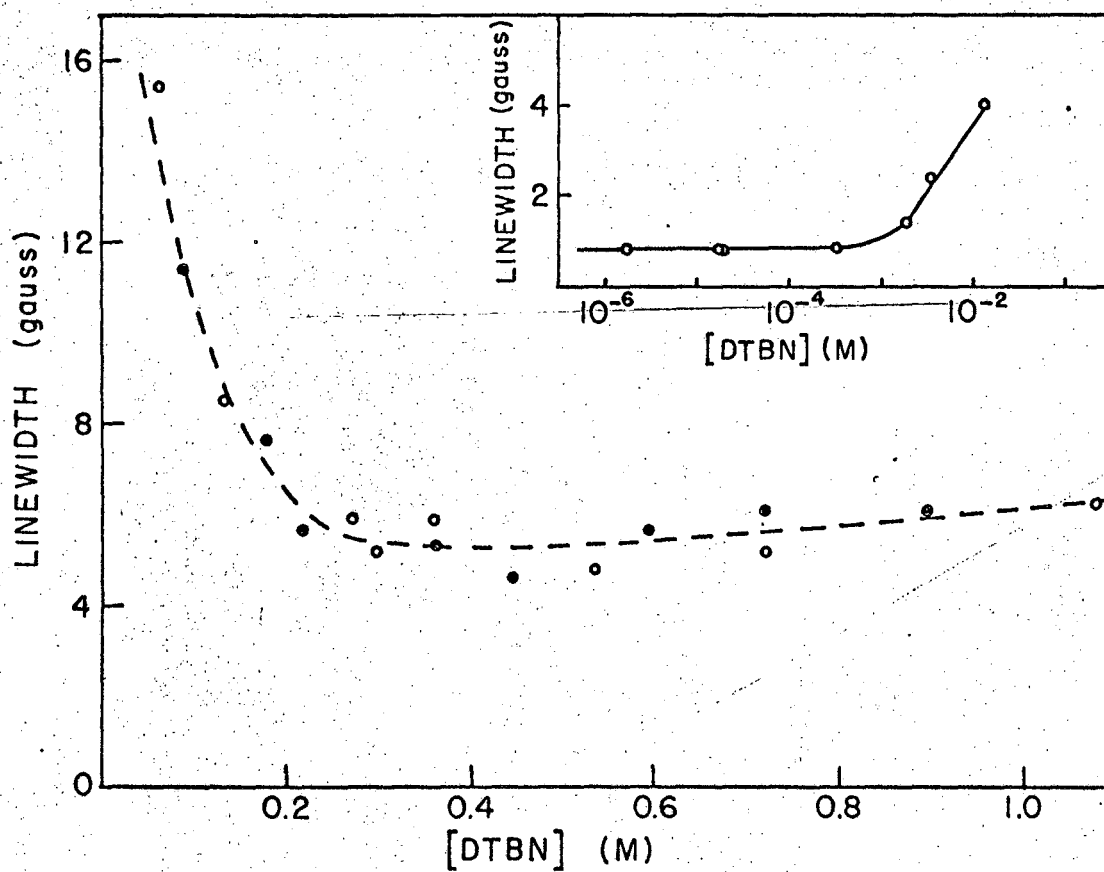
XBL 677-4539

Figure 68. EPR spectrum of 1.06×10^{-5} M DTBN in cyclohexane with air.



XBL 678-4580

Figure 69. Relative signal height of DTBN EPR signal as a function of relative microwave power input. Concentration of DTBN was 9.4×10^{-5} M.



XBL 678-4579

Figure 70. DTBN EPR signal line-width as a function of DTBN concentration in cyclohexane.

effect is shown in Figure 71. Finally, at a concentration around 0.1 M, the hyperfine structure of the nitroxide collapses completely due to rapid electron exchange and only one broad line is observed. Continued increase of concentration causes a narrowing of the line and then a broadening. The concentration range used in this work was around 10^{-4} to 10^{-5} . In this range the rate of change of the line-width with concentrations is ca. 200 gauss/mole/l⁻¹ (see appendix I).

The effect of temperature upon the signal height of a 10^{-4} M solution of DTBN is shown in Figure 72. The rate of change in height with temperature is -0.05%/°C. This rate also will depend upon several instrumental parameters and experimental conditions. However, the conditions used in the experiment to obtain Figure 72 were typical of those employed in this work. When the temperature drops below the freezing point of the solvent, more drastic effects are observed. Figure 73 is a spectra at liquid nitrogen of DTBN ($\sim 10^{-5}$ M) in cyclohexane. For an explanation of this effect, see reference 114.

B. Preparation of Radioactive DTBN

The ¹⁴C-labeled DTBN was synthesized combining the procedures of Ritter and Minieri,¹²⁷ Kornblum et al.,¹²⁶ and Hoffman et al.¹⁰⁸ The starting material, ¹⁴C-tertiarybutyl alcohol, was supplied by New England Nuclear Corp. VPC showed this to be greater than 99% pure in radioactivity. The principle impurity was ¹⁴C-isopropanol.

t-Butyl alcohol (.032 moles) was added to 3.4 mg of ¹⁴C-t-butyl alcohol (0.5 mC) in 10 ml of acetic acid (AcOH) in a 100 ml round bottom flask. NaCN (.048 moles) was added with the flask submerged

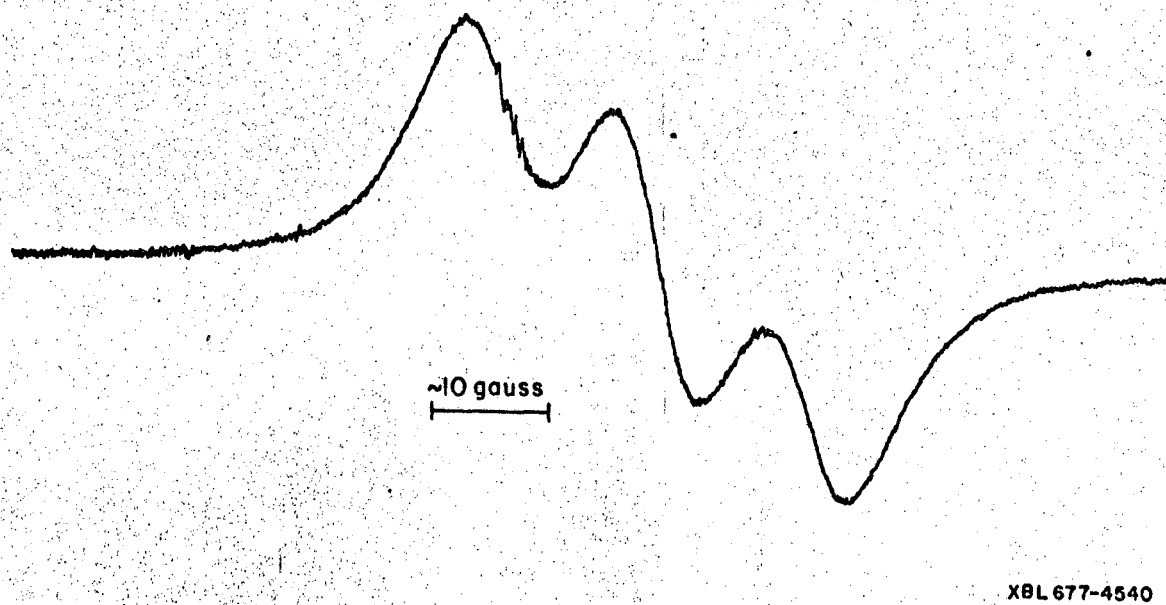
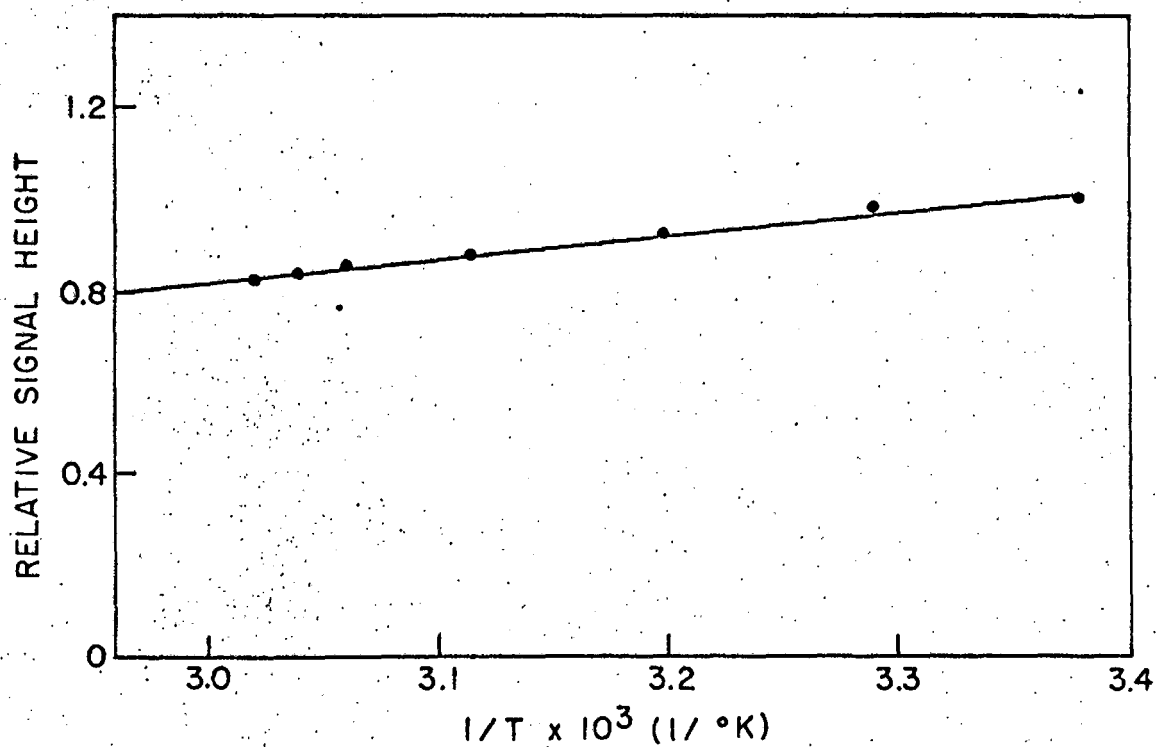
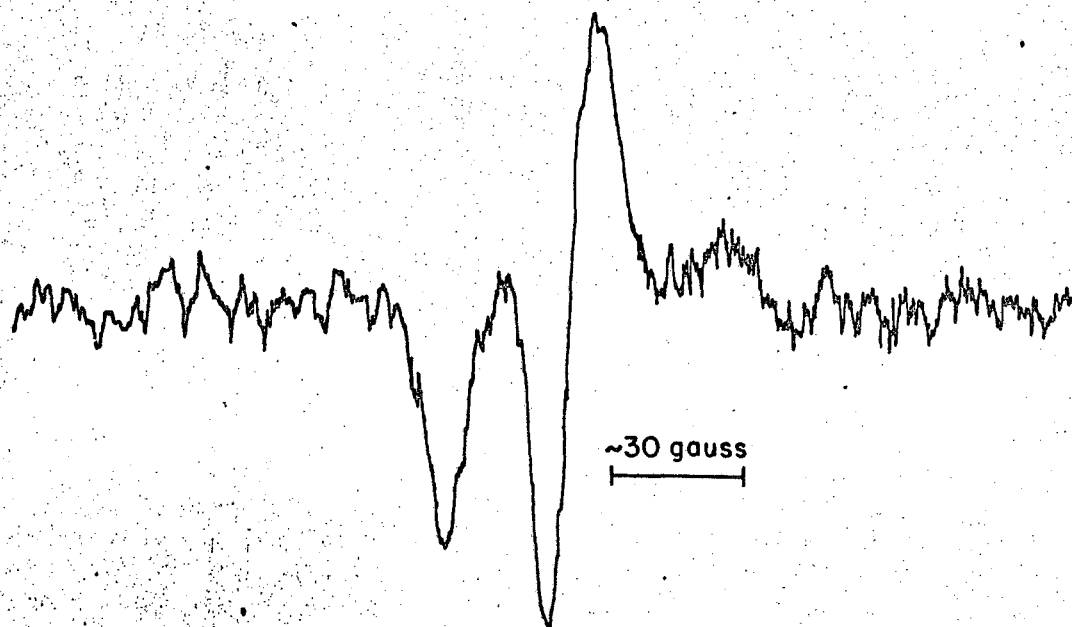


Figure 71. EPR spectrum of a 0.03 M cyclohexane solution of DTBN.



XBL 678-4581

Figure 72. Effect of temperature on the DTBN EPR signal height normalized to the height observed at 22°C. The concentration of DTBN was ca. 10^{-4} M.



XBL 677-4541

Figure 73. EPR spectrum of a 1.0×10^{-4} M cyclohexane solution of DTBN without air at liquid nitrogen temperature.

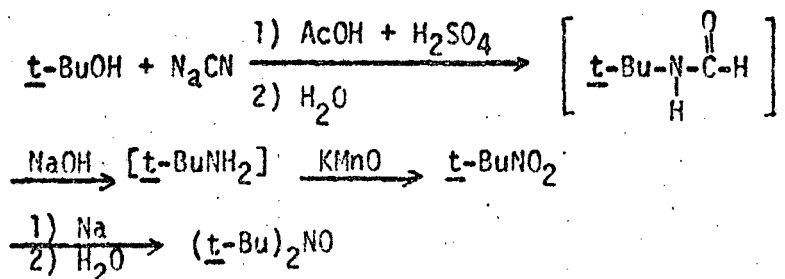
in ice. Then a mixture of 10 ml AcOH with 15 ml of H_2SO_4 was added over a period of 20 minutes to the 100 ml round bottom flask in a closed system. When the acid mixture was introduced, the temperature rose rapidly. The temperature was maintained between 45-55°C. After the addition of the acid mixture, the solution was allowed to stand at room temperature for six hours. The reaction mixture was poured into 50 ml of H_2O , to which was added 100 ml solution of NaOH (0.4 g/ml) with cooling. The resulting basic mixture was refluxed for twelve hours. Then a portion was distilled to another vessel using two condensers (vertical) and an HCl-solution-trap. This 12 hour reflux and distillation was repeated two times. The distillate was collected in a 250 ml round bottom flask. To the distillate, 150 ml of H_2O and 40 g K_2MnO_4 were added and the connection between this flask and the one with the NaOH was removed. The flask containing the K_2MnO_4 solution, fitted with a reflux condensor and magnetic stirrer, was allowed to stand for 24 hours. After this time, the condensor was removed, the flask was fitted with an inlet tube for steam, and the contents were steamed-distilled. The product was collected at 0°C.

The nitro compound obtained at this point was removed from the water layer with ether (an excess). The ether layer was separated from the water in a separatory funnel, dried over K_2CO_3 , and distilled to a volume of approximately 1-2 ml. Glyme was then distilled under N_2 from Na directly into the nitro-ether solution. The glyme was dried by refluxing with LiAlH_4 and distilled from LiAlH_2 under N_2 directly into a flask containing Na. Approximately 0.2 g Na was added to the nitro-glyme solution. During the

addition, a nitrogen purge was used to exclude air. A magnetic stirring bar was added and the flask was sealed with a ground glass stopper. The reaction mixture was allowed to stand at room temperature with stirring until a white slurry formed and most of the Na was expended. The solution exhibited the following color changes: golden, pale blue, dark blue, lavender, and powder white. The solution was filtered to obtain a white precipitate. Sodium bits were removed by hand. Then the precipitate was added to water. Two layers formed. The organic layer was extracted with pentane at 0°C until the pentane became colorless. The pentane was dried over K_2CO_3 and most of the pentane was removed by distillation at 35°C. DTBN was isolated from the pentane extract using an FMN Auto-Prep. The VPC column (.3/8" by 4') contained Dow-11 on firebrick (60/80 mesh). The temperature of the column was 70°, the injector, 60°, the detector, 60°, and the collection trap, 0°C. The helium flow rate was 0.5 on prep flow control.

^{14}C -DTBN was recovered from the VPC trap with pentane to a final volume of 10 ml. The solution in pentane was 0.12 M by its visible absorption spectrum. Two λ were used for counting of the radioactivity (13,400 dpm). The yield of ^{14}C -DTBN was 0.03 mC (6%) and 0.0012 g (6%). The pentane was removed by distillation.

The following equations summarize this sequence of reactions:



The compounds in brackets were not isolated.

APPENDIX III

A. Equipment

EPR. The EPR data were obtained using an x-band spectrometer (9.5 KMc). An x-y recorder was used to record the signal which was recorded as the first derivative of the radical absorption curve. The y-axis was used to record the signal intensity. The x-axis, plotted in gauss, was driven by a sweep generator which was used to vary the magnetic field or else by the output of a Bell "240" Incremental Gaussmeter which was used to determine the width of the sweep in gauss.

The signal intensity was also recorded on a Mosely model 680 chart recorder so that the kinetics of the light-induced changes in the signal intensity could be recorded.

The temperature of the cavity was varied using a Varian low temperature attachment. The temperature was monitored using a copper-constantan thermocouple with an ice water mixture as the reference temperature. The temperature was not determined if temperatures around room temperature were used.

A 0.01 M solution of peroxyamine disulfonate (Fermi Salt, supplied by Alfa Inorganics, Inc.) in water solution saturated with carbonate was used for accurate calibration of the x-axis. The EPR pattern of this salt consists of a symmetrical triplet with a 13.0 gauss splitting constant. Once the splitting constant of DTBN in cyclohexane was determined to be 15.4 gauss, this solution was used for a standard.

NMR. A Varian A-60 NMR instrument was used to obtain the NMR data.

UV-Vis. The optical spectra reported in this work were obtained using either a Cary 11 or 14 recording spectrophotometer or a Perkin-Elmer 221.

Radioactivity. A Packard Tri-Carb, liquid scintillation spectrometer, model 3375, was employed for assaying the amounts of radioactivity in the solution containing ^{14}C -labeled DTBN. The scintillation solution (18 ml in a glass vial) contained 18.0 g 2,5-diphenyloxazole (PPO), 0.4 g 1,4-bis [2-(5-(4-methylphenyl) oxazolyl)-benzene] (Dimethyl POPOP), 200 g naphthalene in 1 liter of ethanol, 1400 ml of toluene and 1600 ml dioxane. The efficiency of counting in this solution was determined by Wallace Erwin using ^{14}C -labeled toluene.

The radioactive purity of ^{14}C -tertiarybutyl alcohol was determined using a proportional tube employing a 1:1 by volume mixture of helium and methane gases. The tube was connected directly to the exit of a VPC. A dual pen recorder was used to record simultaneously the mass peaks and radioactivity.

Gas Pressures. Gas pressures were monitored using a Warburg apparatus which is described elsewhere.¹²⁸

VPC. The VPC instruments used for analytical work include Aerograph VPC models 90A, 350-A, 1520 and the Aerograph Autoprep. For the isolation of ^{14}C -DTBN, a Hewlett-Packard 775 prepmaster was used. The detection system employed with all these instruments was a thermal conductivity bridge. The carrier gas employed was helium.

The columns used in this work, with the exception of the one used

for the isolation of ^{14}C -DTBN which was 3/8 inch in diameter and 4 ft long, were 0.25 inch and either 6 or 10 ft long.

The substrates, their concentration, the support, mesh and the materials separated upon these were as follows: (1) Molecular sieve 5-A, 100%, 30/60 mesh, nitrogen and oxygen; (2) Dow-11, 15%, chromosorb W, 60/80, DTBN, isobutene, 2-methyl-2-nitrosopropane; (3) diisodecyl phthalate, 15%, chromosorb W, 60/80, DTBN, benzoquinone; (4) SE-30, 10% chromosorb W, 60/80, isobutene, 2-methyl-2-nitrosopropane; (5) di-(2-ethylhexyl) sebacate, 20%, firebrick, 60/80, $\text{C}_6\text{F}_5\text{CN}$, $\text{C}_6\text{H}_5\text{C}_2\text{H}_5$.

Light Intensity. The intensity of the light sources used for illuminations in the EPR experiments (1000 watt tungsten and 150 watt Xeon) were determined using a thermopile calibrated with a lamp (#424) supplied by the National Bureau of Standards according to the procedure described in reference 129.

The 1000 watt source has an output of 7×10^5 erg/sec cm^2 after passing through the water bath and IR filter. With filters 1-69 and 3-67 the intensity drops by 61.3%.

The light from the Xeon source was passed through a monochromator and the quanta/sec cm^2 determined for wavelengths from 300 $\text{m}\mu$ to 700 $\text{m}\mu$. The output at 458 $\text{m}\mu$, the wavelength used for the study of the quinone-DTBN reaction, was 5×10^{14} quanta/sec cm^2 .

B. Chemicals

<u>Chemical</u>	<u>Source and Method of Purification</u>
DTBN	See appendix II
DTBNH	" " "
¹⁴ C-DTBN	" " "
¹⁴ C- <u>t</u> -Butyl alcohol	New England Nuclear Corp. - no further treatment
Chlorophyll <u>a</u>	Isolated from spinach by M. Byrn - column chromatography
Chlorophyll <u>b</u>	" " " " " "
Plastoquinone A	" " " " P. Scott " "
<u>t</u> -Butyl amine	Matheson, Coleman and Bell - no further treatment
1,4-Benzoquinone	Eastman Organic Chemical - crystallized
1,4-Benzhydroquinone	" " " "
1,4-Naphthoquinone	" " " "
1,4-Duroquinone	Calbiochem - not further treated
9,10-Anthroquinone	Eastman Organic Chemicals - no further treatment
Tetracyanoethylene	" " " - sublimed (vac.)
<u>s</u> -Trinitrobenzene	" " " - no further treatment
<u>m</u> -Dinitrobenzene	" " " " " "
<u>p</u> -Nitrobenzonitrile	" " " " " "
Hexamethylbenzene	" " " " " "
Quinoline	" " " - distilled (vac.)
<u>p</u> -Chloranil	Source unknown - sublimed (vac.)
<u>o</u> -Chloranil	" " " "
lumiflavin	" " - not purified
lumichrome	" " " "

<u>Chemical</u>	<u>Source and Method of Purification</u>
Hexafluorobenzene	Monsanto - distillation (spinning band)
Pentafluorobenzonitrile	Pierce Chemical & Imperial Smelting - distillation (spinning band)
N,N-Dimethylaniline	Eastman Chemical - distilled (spinning band)
N,N,N',N'-Tetramethyl-p- phenylene diamine	" " - sublimed free base
Ethylbenzene	Matheson, Coleman & Bell - distillation
Isobutene	Matheson Company - no further treatment
2-Methyl-2-nitrosopropane	Prepared according to reference 130
Phenothiazine	Research sample furnished by Smith, Kline and French Labs of Philadelphia - no further treatment
DCMU	duPont de Nemours & Co. - from Kenneth Sauer
DCPIP (ox.)	K & K Laboratories - from Kenneth Sauer
DCPIP (red.)	Prepared from ox. form with KBH_4
Salicylaldehyde	Prepared and purified according to reference 131
Tritiated Phenylalanine	New England Nuclear Corp. - no further treatment
Silica gel G	Warner-Chilcott Labs - no further treatment
Polyethylene	Dow Chemical - no further treatment
Phosphomolybdic acid	Merck - no further treatment

C. List of Abbreviations

(see next page)

C. List of Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
AQ	9,10-anthroquinone
BQ	1,4-benzoquinone
Chl	chlorophyll
CTC	charge transfer complex
Cyt. b ₆	cytochrome b ₆
Cyt. f	cytochrome f
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP or DPIP	2,6-dichlorophenol-indophenol
DMA	N,N-dimethylaniline
dpm	disintegration per minute
DQ	1,4-duroquinone
DTBN	di-tertiarybutylnitroxide
DTBNH	di-tertiarybutylhydroxylamine
DTBNH ₂	di-tertiarybutylamine
EB	ethylbenzene
EPR	electron paramagnetic resonance
Fd.	ferredoxin
$\Delta H_{1/2}$	linewidth at half-maximum of EPR spectral line
NAD	nicotinamide adenosine dinucleotide
NADH	reduced form of NAD
NADP	nicotinamide adenosine dinucleotide phosphate
NADPH ₂	reduced form of NADP

NMR	nuclear magnetic resonance
NQ	1,4-naphthoquinone
O.D.	optical density
P700	pigment complex which absorbs at ca. 700 mμ
P890	" " " " " " 890 mμ
Pcy	plastocyanine
PM	phosphomolybdic acid
PMS	phenazine methosulfate
PQ-A	plastoquinone-A
RuDP	ribulose diphosphate
TLC	thin-layer chromatography
TMPD	N,N,N',N'-tetramethyl-p-phenylene diamine
Vit. k	vitamin k
VPC	vapor phase chromatography

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