MODEL STUDIES ON BIOLOGICAL ENERGY CONVERSION MECHANISMS

BY

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ABSTRACT

This thesis is concerned with the design of ATP-producing physical and chemical model systems and the investigation of the mechanisms of ATP formation at the molecular level. The ultimate purpose of this study is to apply the concepts derived from these model systems to more complex biological systems. The mechanisms of energy transduction in living organisms may then be approached at a molecular level.

Based on the study of the model systems, three modes of activation during the ATP-forming chemical steps for either ADP or inorganic phosphate have been identified. They are:

(1) Through a one-electron reduction of ADP into a radical form;

(2) Through the creation of oxidizing agents with redox potentials sufficient to oxidize inorganic phosphate;

(3) Through an activation of phosphate by coordination to a reduced heme-complex, followed by aerobic oxidation of the resultant phosphate-heme complex.

In biological systems, the first mode may involve utilization of a reducing power of an electron transport chain, of a reducing functional group such as thiol in the ATP synthetase. The second mode may involve PS II or generation of delta singlet oxygen by various energy transfer processes. The third mode may involve the coordination of inorganic phosphate to a cytochrome, such as cytochrome-a_3 in an cytochrome-c oxidase complex.

TABLE OF CONTENTS

Table of Contents Cont.

Table of Contents Con.

Page

LIST OF FIGURES

LIST OF FIGURES Cont.

LIST OF TABLES

INTRODUCTION

The essential feature of all living organisms is their ability to capture, store and transform various forms of matter and energy for the purpose of growth, self-regulation and self-replication. Using the language of thermodynamics, living systems are open systems where the continuous exchange of matter and energy with the environment is ongoing. Through these exchange processes, living organisms continually create "order out of order" in the sense of self-replication and "order out of disorder" as a result of metabolism. For a living organism the cessation of these exchange processes, or in another words, artificially turning the "natural" open system into an isolated closed system, will bring death to the organism. Under such artificial conditions, the entropy production of the system is positive as a result of internal chemical processes, the organism will degrade into a random state of equilibrium when the entropy reaches a maximum. The most important consequence of allowing the continuous exchange of matter and energy with the environment for a living organism is to maintain the latter in a quasi-stationary state, a state of good health.

The primary energy source for all living organisms on the earth is sunlight. Photosynthetic organisms, including green plants, algae and some bacteria, can utilize directly the radiant energy from the sun for the conversion of simple molecules into a variety of macromolecules which then become the body constituents of the organisms. Unwanted molecules and some heat produced are released back to the surroundings. Thus the energy of the sun is transformed into storable chemical energy within the body in the form of newly created chemical bonds.

In contrast to the photosynthetic organisms, non-photosynthetic organisms, such as animals and many micro-organisms, obtain their nutrient molecules from the photosynthetic organisms, directly or indirectly. Energy required for maintaining their lives is secured by releasing bond-energies of the nutrient molecules through enzymatic reactions, predominantly by oxidative processes.

It is now known that the energy acquired by all living organisms is conserved in a useful form mainly as molecules of ATP. Whether the energy comes from the sunlight directly or from the oxidation of nutrient molecules, most of it is invested in the synthesis of ATP, which then serves as a "common energy currency" that can be used in powering the majority of functions of the cells within organisms.

Following is a summary of those functions in which ATP is crucially involved:

(A) Development and preservation of integrity in the organization of a living body, as in the synthesis of carbohydrates, proteins, lipids, nucleic acids and other tissue components.

(B) Transport of molecules and ions against concentration gradients across bio-membranes.

(C) Muscular contractions and cilia movements.

(D) Generation of trans-membrane potential differences in nerve systems for the transmission of a nerve impulse.

(E) Bioluminescence in some organisms, such as in luminescent bacteria, protozoa, fungi, worms, crustaceans and fireflies.

One may anticipate that ATP production should be one of the most incessant biochemical reactions for a living organism in order to maintain life. Based on the estimation of Krebs et al.[1], the daily turnover of ATP for an average human body may reach 60 kilograms or more, almost as much as the body weight. Furthermore, the machinary to produce ATP is so efficient that there is very little loss of free energy during the energy transduction process[2-3].

ATP was discovered in 1929 from muscle extracts by Lohmann[4], Fiske and SubbaRow[5]. Its chemical structure was determined in 1935 by Lohmann[6]. For the first several years, investigations related to ATP were mainly concentrated on anaerobic metabolism, such as on glycolysis and fermentation[7]. Soon phosphorylations connected with respiration were discovered in kidney homogenates by Kalckar[8] in 1937 and in muscles by Belitzer and Tsibakowa[9] in 1939, and the currently used term, "oxidative phosphorylation", was adopted by Kalckar[8].

Situations related to the study of energy transduction in biological systems as a whole were dramatically changed by important discoveries made between the late 1940s and early 1950s. In 1949 Lehninger and Kennedy[10], and Schneider and Potter[11] independently showed that the tricarboxylic acid cycle, fatty acid oxidation and oxidative phosphorylation take place in cellular organelles called mitochondria. In 1951 Lehninger further demonstrated that, in mitochondria, the oxidative phosphorylation is associated with the respiratory chain of electron transport to oxygen[12]. Following these events, two equally significant discoveries related to photosynthetic organisms were made. In 1954 Arnon and his collaborators showed that isolated leaf chloroplasts house the complete apparatus for photosynthesis necessary for oxygen evolution, carbon dioxide fixation, photophosphorylation and NADP reduction[13]. In the same year Frenkel found that isolated chromatophores from photosynthetic bacteria have photo-phosphorylative activity[14]. These important findings unambiguously located the so-called "power houses" of living organisms. The new era of "Bioenergetics" was thus founded.

During the past three decades, dynamic research efforts have been carried out in order to clarify this central problem of biological science—how living organisms make ATP within those sub-cellular organelles?

It is now known that the electron transport systems associated with the inner membrane of mitochondria , the thylakoid membrane of chloroplasts and the plasma membrane of photosynthetic and non-photosynthetic bacteria are functionally linked to an ATP synthesizing system by a mechanism fundamentally similar in all living organisms. However, after much endeavour by numerous investigators for many years, we still do not know the molecular mechanism of ATP formation in any living systems. The difficulty in clarifying the mechanism at a molecular level is undoubtedly due to the complexity inherent in the bio-membrane systems which are macro-molecular, multi-component, heterogeneous, structurally and funtionally dynamic, and, in many cases, non-equilibrium in character. The structural arrangement and functional properties of each component in the bio-membranes are still obscure. New components are still being discovered.

Currently there are four major hypotheses concerning the mechanism of ATP formation in the bio-membrane, namely: the Chemiosmotic Hypothesis, the Conformational Hypothesis, the Internal Acidification Hypothesis and the

Chemical Intermediate Hypothesis. Each of the above hypotheses will be described in Chapter I. Each hypothesis is characterized by the difference in defining "energized state" which couples a redox reaction during an electron transport process to the ATP forming reaction. These hypotheses have been modified several times to accomodate newly discovered physiological phenomena induced by changing experimental conditions. These include: addition of electron-transport inhibitors, uncouplers, energy-transfer inhibitors, ATP, ADP, inorganic phosphate, acids, bases, salts, oxidizing or reducing agents. Addition of these chemicals may affect the electron transport process, ATP formation and consumption processes, or the degree of coupling between those processes. However, none of the hypotheses can explain the phenomena observed by using well-defined chemical terms. In fact, because of insufficient knowledge regarding the detailed molecular structure and the physical and chemical properties of membrane components, the hypotheses themselves have not been formulated in clear chemical terms.

When detailed molecular information is lacking, many explanations are possible for any given physiological phenomenon and arguments about possible molecular mechanisms will persist. It is my considered opinion that a rational approach to the problem of energy transduction must involve extensive studies of related model systems in which chemical components are well-defined and their reaction mechanisms can be studied conveniently. One may begin studying a model system in solubilized state, followed by studying the system in more aggregated state such as in an artificial membrane. Theoretically one can improve the models and bring them asymptotically close to natural systems as our knowledge increases regarding the chemical structure of bio-membranes.

It is generally believed that ATP formation in a bio-membrane system would occur only when the membrane structure has not been drastically changed. The most widely supported, namely the chemiosmotic hypothesis, further demands a vesicular structure as a necessary condition for a bio-membrane to have phosphorylative activity. If one finds that a property of a bio-membrane cannot be inferred from the molecular properties of its chemical components in a solubilized state, a possible cause may be the improper selection of the solvent. Most investigators of energy-transducing bio-membranes study their systems in aqueous environments. Failure to produce ATP from a solubilized system in water should not be used by the supporter of any hypothesis to speak for his own view, or against that of others. Logically speaking, negative results can not be used as the basis for argument. The failure to produce ATP in an aqueous medium may well be due to the hydrolysis of an intermediate which otherwise may lead to ATP formation. The requirement of a membrane for ATP formation may be of secondary importance to the mechanism of the reaction. If this be the case, a search for an effective non-aqueous solvent may provide a medium for the phosphorylation reaction to occur in and lead to the clarification of the reaction mechanism. It should be noted that bio-membranes are essentially non-aqueous in nature.

Formation of ATP and inorganic phosphate in all bio-membranes is coupled to a series of redox reactions during the electron-transport process. The redox reactions are energy-donating reactions and the phosphorylation reaction, energy-accepting reaction. The efficiency of the coupling is known to be extremely high. This means that the energy-donating reactions ensure the energy-accepting reaction. The mechanism of the coupling device may involve a direct activation of either ADP or inorganic phosphate by an unidentified oxidant or reductant which may or may not be a member of an electron-transport chain. This possibility has been proposed in the past for the activation of inorganic phosphate, but this concept is no longer popular among the majority of investigators[15]. The reason for neglecting this possibility is that, so far, nobody has ever succeeded in isolating or detecting any so-called "high energy phosphorylated intermediate" during the energy-coupling process. However, the possibility still exists that the intermediate may be labile under ordinary experimental conditions and be difficult to identify. Most investigators believe that the electron transport process and the dehydration process between ADP and inorganic phosphate occur at different domains, and the two processes are mediated by an "energized state" such as an electrochemical proton gradient, the membrane potential, by intra-membrane acidification or by the energized conformational state[15]. ATPase is thought to be directly involved in the dehydration process.

This study presents some experimental results of ATP-forming chemical reactions for key reactants, ADP and inorganic phosphate, from which three different modes of activation are proposed. In the activation processes, several members of the electron transport chain of photosynthetic and respiratory living organisms are used. These include, chlorophylls, plastoquinones, ubiquinones and molecular oxygen. Heme complexes are also used as the model molecules for some natural cytochromes. In order to assist in the elucidation of the reaction mechanisms of the model systems, some reagents totally irrelevant to biological systems are also used.

It will be shown that, for the following over-all phosphorylation reaction to occur:

$$ADP + P_i \longrightarrow ATP + H_2O$$

either ADP or the orthophosphate, P_i, can be activated by one of the three modes, namely,

(i) One-electron reduction of ADP;

(ii) Oxidation of P_i;

(iii) Coordination of P_i to a ferro-heme complex, followed by an aerobic oxidation of the phosphate adduct to its ferric state.

Unlike most chemistry problems in which the molecular species of interest are known before hand, the design of chemical models related to biological energy transduction reactions requires some "guesswork" regarding which molecules to choose. This is because we do not know with certainty what molecular species are directly involved in the reactions. Chapter I is devoted to a description of the current knowledge related to the energy-transducing biological systems. Electron-transport chains of various organisms and four major hypotheses on the mechanism of energy transduction are presented. In Chapter II, the simplest ATP-producing model system, that is, ATP formation controlled potential electrolysis, is discussed. Here ATP is produced from ADP plus inorganic phosphate by electrochemical means, namely, by the cathodic reduction of ADP and by the anodic oxidation of inorganic phosphate. The results provide some basic information for understanding the nature of the activation processes by which ATP is formed from the reactants. In Chapter III, the results obtained from the study in Chapter II are further "cross-checked" by adding simple reductants for the ADP activation, and simple oxidants for the phosphate activation. Particularly noteworthy is the finding that dioxygen in its excited state (delta singlet oxygen), produced either by photochemical processes or by a disproportionation reaction of superoxide anion in the dark, can serve as the strong oxidant for the phosphate to produce ATP. This proposed mechanism is further evaluated by using photochemical model systems containing some naturally occuring components such as chlorophylls and quinones. Here, the delta singlet oxygen is generated from photo-excited chlorophylls and quinones. These energy-transfer processes are described in Chapter IV. A new method of generating the superoxide anion by an aerobic oxidation of some hydroquinone derivatives is also presented in Chapter IV. Evidence is given suggesting that ATP formation associated with this oxidation also involves the generation of singlet oxygen, probably by the same disproportionation process. In Chapter V, a different type of phosphate activation is described. It is shown that a phosphate-co-ordinated ferro-heme complex may become a phosphorylating agent when it is oxidized by dioxygen. This model may be correlated with the energy transduction reaction involving a cytochrome such as the one observed in the cytochrome-c oxidase system. Finally, in Chapter VI, three newly proposed molecular mechanisms based on the model systems are compared with currently available data from the study of biological systems.

It is beyond the scope of this thesis to describe numerous interesting chemical models of ATP formation designed by previous investigators (see references 310 and 329). Only two classes of chemical models pertinent to the present study are described in APPENDIX I.

CHAPTER I

HISTORICAL BACKGROUND

I.1 Electron Transport Chain of Chloroplasts

It is thought by some authors that the earliest plant capable of converting solar energy into chemical energy appeared on the earth more than one billion years ago. Scientific investigation of the photosynthetic process was not carried out actively until Joseph Priestley discovered in 1771 that oxygen was liberated from plants in the sunlight. Several important discoveries have been made towards the understanding of photosynthesis during the past three and half decades. The following milestones are particularly significant:

(a) Experiments with isotopic oxygen by Ruben, Kamen and their associates showed that the oxygen evolved from photosynthesizing plants originated from water[16].

(b) The dark reactions of the carbon dioxide reduction cycle were established through the efforts of Calvin, Benson, Bassham and co-workers employing carbon radio-isotope tracer technique[17-20].

(c) Isolated leaf chloroplasts were shown by Arnon and his collaborators to house the complete apparatus for photosynthesis necessary for oxygen evolution, carbon dioxide fixation, photosynthetic phosphorylation and nicotinamide adenine dinucleotide phosphate ($NADP^+$) reduction.[13]

(d) Isolated chromatophores of photosynthetic bacteria were found by Frenkel to have photosynthetic phosphorylation activity[14].

(e) The discovery of the so-called "chromatic transients" by Blinks[21] and the "enhancement effect" by Emerson et al[22]. in the rate of oxygen evolution laid the basis for the current mechanistic view of two pigment systems (PS I and PS II) cooperating for photosynthesis in plants and algae.

(f) The flash experiment of Emerson and Arnold with algae suggested the presence of a "photosynthetic unit".[23*]

(g) The reaction centers in the chromatophores of purple bacteria and blue-green mutant strains of *Rhodopseudomonas spheroides* were identified respectively as P890 by Duysens[27] and P870 by Clayton[28]. In chloroplasts the reaction centers of PS I and PS II respectively were identified as P700 by Kok[29] and P680 by several investigators.[30-33] The photochemistry at these reaction centers most likely follows one of the three patterns observed by Tollin and his collaborators.[34*]

(h) The widely accepted "Z-scheme" of photosynthesis in chloroplasts was proposed almost simultaneously by several groups of investigators.[35-41] The contributory works include: measurement of the redox potential of chloroplast cytochromes by Hill, Bendall and Davenport;[35-37] spectroscopic confirmation of an antagonistic effect exerted by PS I and PS II on the redox state of electron-transfer components based on the studies of Duysens, Amesz and Kamp,[38] Witt, Müller and Rumberg,[39] Kok and Hoch.[40] Correlation between the change of chlorophyll fluorescence yields and the redox state of a member in the photosynthetic electron transport chain investigated by Kautsky, Appel and Amann.[41]

23* The concept of "photosynthetic unit" has recently been challenged by several investigators.[24-26] The whole bacterial chromatophore and whole thylakoid vesicle of a chloroplast are considered to be the functional units.

34* The three patterns are: (A) photo-production of chlorophyll cation radical, (B) one-electron photo-transfer from bacterio-chlorophyll to quinone and (C) chlorophyll photo-sensitized one-electron transfer from hydroxylic compounds to quinone.

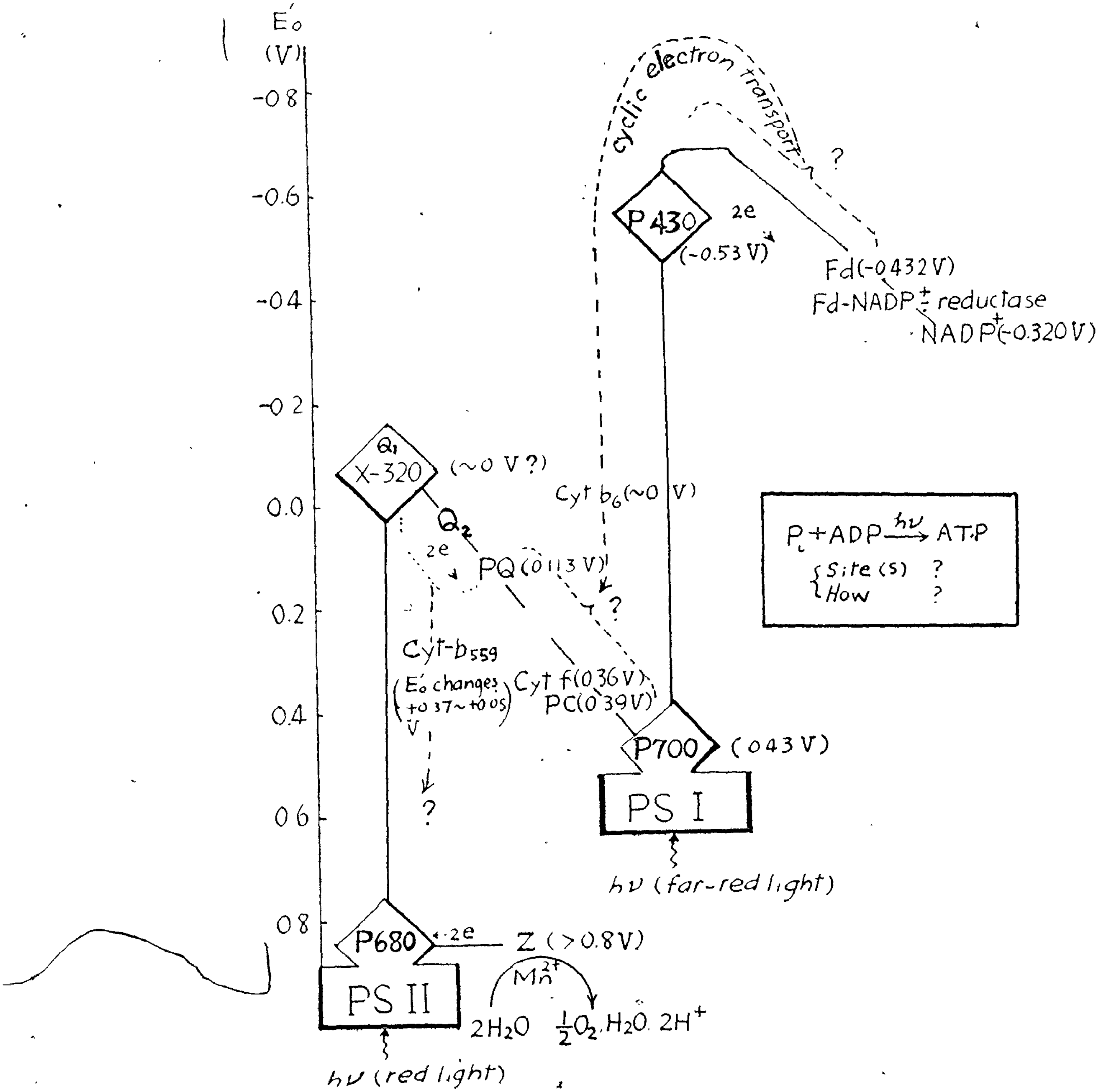
A. Z-Scheme of Chloroplast electron-transfer System

Figure 1 is a representation of the Z-scheme for photosynthesis which has proven adequate to encompass most of the available data from chloroplasts of green plants and algae. The conversion of light energy into useful free energy occurs at the thylakoids* or grana** where the chloroplast pigments and members of the electron transport chain are located. The free energy is stored in the form of ATP, reduced nicotinamide adenine dinucleotide phosphate (NADPH), membrane potential and ion-gradients across the thylakoid membrane. According to the Z-scheme, the reduction of $NADP^+$ is achieved by cooperative redox reactions between the two pigment systems, PS I and PS II. Water is oxidized to oxygen at PS II and reducing equivalents (electrons or hydrogen atoms) are pumped against the redox potential gradient at PS I to generate a strong reductant capable of reducing the $NADP^+$. Formation of ion-gradients is likely preceded by extremely rapid generation of a light-induced electric field across the membrane. Existence of this electric field has been demonstrated unambigously in the laboratories of Kok[43] and Witt.[44-47] Most investigators believe that STP formation is coupled to the dark electron transfer process between PS I and PS II, but the exact site(s) and mechanism of its formation have remained unknown.

* Thylakoid is a sub-chloroplast vesicle having closed flattened sac-like shape which was named by Menke[42].

** Grana are a stack of thylakoids.

Figure 1. Z-Scheme of Photosynthesis in clloroplasts of green plants and algae.



E'o (V)
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
cyclic electron transport
?
P430
2e
(-0.53 V)
Fd(-0.432 V)
Fd-NADP+ reductase
NADP+(-0.320 V)
Q1
X-320
(~0 V?)
Q2
Cyt b6(~0 V)
2e
PQ(0.113 V)
?
Cyt-b559
(E'o changes +0.37~+0.05 V)
Cyt f(0.36 V)
PC(0.39 V)
P700
(0.43 V)
PS I
?
hν (far-red light)
Pi + ADP → ATP
Site(s) ?
How ?
P680
2e
Z (>0.8 V)
Mn2+
PS II
2H2O
1/2 O2, H2O, 2H+
hν (red light)

Figure 1.

Current views of the actual processes represented by the scheme may be described as follows:

Initially, the quanta absorbed by PS II are funnelled by some energy transfer process into the reaction centre, P680, a specialized chlorophyll-a complex. PS II operates with maximum efficiency only at wavelengths less than 680 nm. Reaction of this excited P680 with an unidentified neighbouring molecule generates a compound, Z^+, with a high redox potential ($E_o' = +0.82V$).[48] This is sufficient to oxidize water to molecular oxygen. Four or more manganese atoms are involved in some unknown manner in the oxidation of water.[49] From the oscillations in the yield of oxygen upon illumination of *Chlorella* or chloroplasts with single short flashes, a four quantum process in oxygen evolution was deduced.[50-51] It is reasonable to assume that the oxygen evolving enzyme, "S" is a manganese enzyme and has four oxidation states. When it accumulates four positive charges through four consecutive oxidations by Z^+, the evolution of oxygen starts. Reducing equivalents derived from water oxidation are then received by a primary acceptor denoted as Q,[52] C550[53] or X320.[54] Recent observations suggest that C550 may not be the actual primary acceptor, but rather a membrane-bound chromophore indicator responding to the redox changes of the primary acceptor.[55-56] Probably C550 is β-carotene.[57] Several studies suggested that X320 could be a special bound plastoquinone.[58-60] The midpoint redox potential of the primary acceptor has been reported by several investigators: +0.180V by Kok, Malkin, Owen and Forbush;[58] +0.095 V by Knaff;[59] +0.025 V by Erixon and Butler;[60] +0.035 V and -0.270 V by Cramer and Butler;[61] +0.025 V and -0.270 V at pH 7.6 by Malkin & Barber[62]. This potential was found to be pH dependent.[59,61,63]

The reduced primary acceptor[+] of PS II initiates dark transport of the reducing equivalents along the redox potential gradient to the reaction centre of PS I, P700. Known members in the redox potential gradient between Q (or X320) and P700 are: plastoquinone, cytochrome-f and plastocyanin. Plastoquinone ($E_o' = +0.113$ V at pH 7.0[64]) functions as an "electron pool" (also as Q_1, Q_2-the primary & secondary electron acceptor of PS II[65]). There has been controversy over the relation between cytochrome-f and plastocyanin on the oxidizing side of PS I. Midpoint redox potentials of these two components are so similar that it is inappropriate to use them as a basis to judge the relative position of them in the redox chain.* Recent observations are in favour of assigning plastocyanin as the immediate electron donor for PS I. Gorman and Levine found that mutants lacking in plastocyanin could not reduce $NADP^+$ by PS I light while mutants lacking in cytochrome-f could.[70,71] Siedow, Curtis and San Pietro showed that plastocyanin-depleted chloroplast particles prevented photooxidation of reduced cytochrome-f by PS I[72]. A similar property was observed in systems in which a plastocyanin inhibitor was added.[73,74]. A number of recently introduced plastocyanin inhibitors are: histones and polylysine,[73,75,76] potassium cyanide[77] and a low concentration of mercuric ion.[74,78] More recently, kinetic studies by Wood and Bendall on the photo-oxidation of plastocyanin and cytochrome-f clearly indicated that the rate of the plastocyanin oxidation was much faster.[79]

*Plastocyanin: $E_o' = +0.320$ V[67], +0.390 V.[68]
Cytochrome-f: $E_o' = +0.330$ V[69], +0.365 V[37], +0.375 V[67]

+Recently, there is some evidence that the primary acceptor is pheophytin rather than X-320.[66]

The reaction centre of PS I was termed as P700 by Kok[29]. This is a bound chlorophyll-a dimer,[85] present in a concentration of about 1/400 that of total chlorophyll[29]. The redox potential of P700 was initially reported with a value of +0.430 V[29]. Subsequent studies yielded more positive values[67,80-84]. Knaff and Malkin performed a redox titration of P700 monitored by absorption changes or by electron spin resonance and obtained a value of +0.520 volt in both cases[67]. Operation of PS I is more efficient at wavelengths greater than 700 nm. Photochemistry of P700 probably follows the pattern (A) of Tollin[34] which results in the formation of $P700^+$. Reduction of $P700^+$ is achieved by receipt of an electron derived from the photo-oxidation of water at PS II.

The reducing site of PS I provides a strong reductant which can reduce unspecifically a multitude of added compounds of known redox potential. The estimated midpoint potential of the primary electron acceptor of PS I is around -0.530 volt (-0.5 $\sim$-0.7 V).[85] Two different methods have been used to yield information about the identity of this primary acceptor namely: spectrophotometric and low temperature electron spin resonance (ESR). Hiyama and Ke reported a newly detected abosrption band at 430 nm in PS I chloroplast fragments[86]. Quantum yields and the action spectrum of this absorption are quite similar to those obtained for photo-oxidation of P700, indicating that the absorber (or band) may be associated with the primary electron acceptor. Malkin and Bearden observed in whole chloroplasts already freed of soluble ferredoxin a light indiced ESR spectrum at 25°K[87]. The signal showed a non-axial g tensor with g-values of 1.86, 1.94 and 2.05 and was indicative of the presence of the reduced form of an iron-sulphur protein. Because the reduction occurred at such a low temperature where ordinary diffusion-limited chemical processes could be precluded, Malkin and Bearden suggested that this iron-sulphur protein was acting as the primary electron acceptor. Ke, Beinert and Hansen subsequently presented evidence for the equivalence of the compound absorbing at 430 nm and the bound iron-sulphur protein[88]. They studied the conditions necessary for the appearance of the low-temperature ESR signal when methyl viologen was added to sub-chloroplast fragments. When the sample was illuminated at room temperature, the secondary electron acceptor removed electrons from the reduced primary electron acceptor and the subsequently frozen sample did not cause any accumulation of the ESR signal due to reduced iron-sulphur protein. This ESR signal would appear only when a reductant for $P700^+$ was also added to the system to provide electrons for the reduction of the primary acceptor, or when the illumination was carried out at low temperature to prevent the redox reaction between the primary and the secondary electron acceptors. Kinetic-spectrophotometric measurements of the absorption band at 430 nm showed the compound responsible for the absorption followed the similar reaction pattern described above. Redox titration of the PS I subchloroplast fragments showed that possibly three bound iron-sulphur proteins were present in the reaction centre of PS I whose midpoint potentials were found to be -0.530, -0.580 and $<$ -0.580 V, respectively[88]. Illumination at low temperature of samples previously frozen in the dark led to the reduction of the iron-sulphur protein with highest potential. The primary electron acceptor of PS I is now generally termed as "P430".

Transport of reducing equivalents from the P430 to $NADP^+$ is mediated by ferredoxin and the flavoprotein, ferredoxin-NADP oxidoreductase[89,90]. Ferredoxin is a non-heme multi-iron compound and its concentration in green leaves is low (approximately 1/400 of chlorophyll)[91]. Therefore, its role in transferring reducing equivalents to $NADP^+$ is a catalytic one. Ferredoxin-NADP oxidoreductase plays an important function in this catalytic activity. This flavoprotein is a metal-free enzyme and contains one flavin adenine dinucloeotide (FAD) as its prosthetic group[92]. Although the oxidation and reduction of the FAD (or FMN) moiety in flavoproteins are usually written formally as reactions involving simultaneous transfer of two reducing equivalents, these reactions occur more likely in two separate one-electron steps. From the electron spin resonance study of ferredoxin-NADP oxidoreductase and pure FAD, this author found that the apoenzyme stabilized the radical form of the bound FAD on reduction[93*]. Since chloroplast ferredoxin ($E_0' = -0.43$ V) is a one-electron redox carrier[94**] and $NADP^+$ ($E_0' = -0.32$ V) is a two-electron acceptor, this property may qualify the flavoprotein as an excellent mediator in the electron transport from ferredoxin to $NADP^+$ [93].

* This author used the term "chloroplast $NADP^+$ reductase", but now "ferredoxin-NADP oxidoreductase" is the accepted official name.

** Bacterial ferredoxins are two-electron redox carriers in which only two irons are reduced in the multi-iron proteins.

It is still not quite clear where b-type cytochromes should be placed in the Z-scheme[95]. In chloroplasts, comparatively well studied b-type cytochromes are cytochrome-b_{559} and cytochrome-b_6*. At room temperature, Cramer and Butler showed cytochrome-b_{559} reduction by PS II light and the oxidation by PS I light[96]. On the other hand, Knaff and Arnon observed the oxidation of this cytochrome by PS II light at low temperature[97]. The situation became more uncertain when Bendall found that there were two types of cytochrome-b_{559}: a high potential type (E'_0 = -0.37 V) and a low potential type with a potential approximately 0.3 volt lower[98,99]. The potential may be dependent upon the structural as well as functional elements of the chloroplasts[100-2]. The high potential type was not detected in etiolated leaves but the low potential type was[103-5]. An increasing number of investigators favour the view that the former is associated with PS II in a side path, while the latter, with PS I[103-8]. Cytochrome-b_6 can be photo-oxidized or photo-reduced by PS I light depending on the ambient redox potential provided by added redox carriers[109,110]. From these observations most investigators assume that it is a member of the cyclic electron transport chain associated with PS I. Böhme and Cramer proposed that electrons in the cycle returned to PS I *via* plastoquinone[109]. Recently this point was further substantiated by the work of Biggins[111] and Böhme.[112] The mid-point potential of cytochrome-b_6 is near 0.0 volt[60,110,113-4] or -0.110 volts[108] which is low enough to reduce the plastoquinone.

* Cytochrome-b_6 is now frequently called "cytochrome-b_{563}", because its reduced form has an absorption at 563 nm.

Very recently Arnon et al. proposed a new scheme alternative to the Z-scheme[305]. According to these authors, PS II is activated by two quanta of light. One quantum is used to transfer an electron from water to ferredoxin on the outer side of a thylakoid membrane, a second quantum is used to reduce plastoquinone and enable it to transport protons to the inside of the membrane. In the original Z-scheme photo-reduction of ferredoxin by water can be achieved only under the cooperation between PS II and PS I. The role of PS I in the new scheme is predominantly for the production of ATP by cyclic photophosphorylation. At present this new scheme has not been generally accepted[306].

B. Photosynthetic Phosphorylation of Chloroplasts

In addition to the reduction of $NADP^+$, light absorbed by PS I and PS II contributes to ATP formation. According to the carbon dioxidereduction cycle established by Calvin *et al.*, two moles of NADPH and three moles of ATP are required for the reduction of one mole of carbon dioxide.[17-20] The term "photosynthetic phosphorylation", or more commonly called "photophosphorylation", refers to the ATP formation in photosynthetic organisms. Currently it is believed that the ATP formation is coupled to electron transport *i.e.* coupled to a dark redox reaction analogous to "oxidative phosphorylation" in the mitochondrial respiratory chain. Photochemical reactions related to PS I and PS II serve to maintain electron transport in the phosphorylation process. According to Arnon *et al.*, two types of electron transport processes are involved: non-cyclic and cyclic.[115] The former refers to the electron pathway from water to $NADP^+$ operated through two photochemical pigment systems, PS II and PS I; while the latter, to a cyclic electron pathway operated only by the activation of PS I. Photophosphorylation associated with each electron transport process is operationally defined as: non-cyclic and cyclic photophosphorylation.[115]

At present, it is still not quite certain as to which members are involved in the cyclic pathway. Accumulated data indicate that P700, ferredoxin, cytochrome-b_6, plastoquinone, cytochrome-f and plastocyanin may be involved.[109-112, 116-120] Conflicting opinions exist as to whether ferredoxin-NADP oxidoreductase is also a member of the cyclic pathway.[120-1]

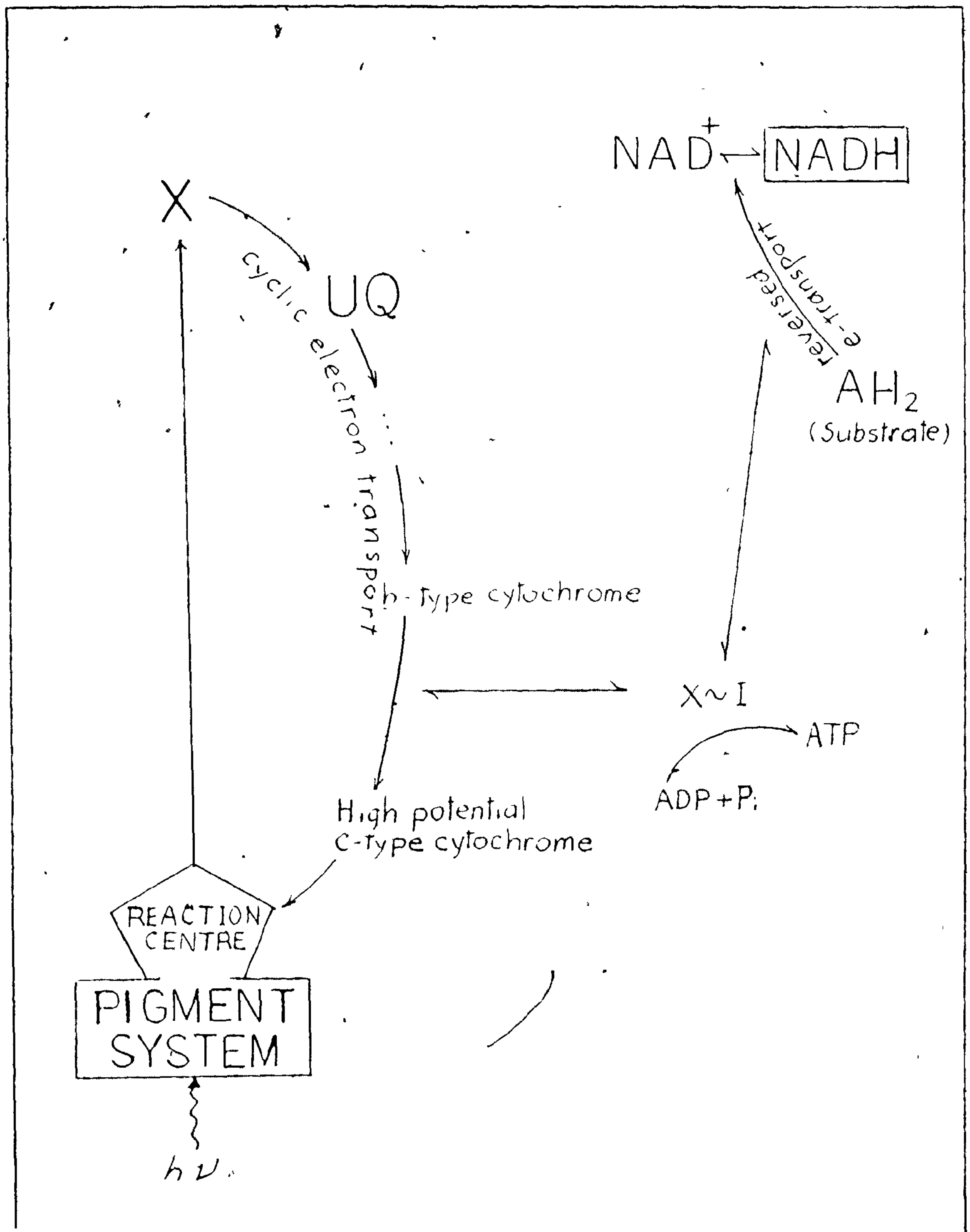
The exact site(s) of both types of photophosphorylation remain unknown. Several investigators suggested that one coupling site might be near PS II before plastoquinone, and another site, between plastoquinone and cytochrome-f.[122-9] The first site does not control the rate of electron transport and operates best at pH 7.3; while the second site does control the rate and operates at an optimum pH 8.0-8.5.[122-5]

Cyclic photophosphorylation is sensitive to the ambient redox potential[118-9] When oxygen is present, this type of photophosphorylation is greatly enhanced.[130-5] The cause of the enhancement is not clear; probably it involves the reduction of oxygen-the so-called Mehler reaction.[136] The cyclic photophosphorylation of this nature is called pseudocyclic photophosphorylation.[137]

I.2 Electron Transport Chain of Photosynthetic Bacteria

For photosynthetic bacteria, it is still not possible to write a simple, unified scheme to account for the numerous observations related to ATP formation and generation of reducing power as reduced nicotinamide adenine dinucleotide(NADH). Photosynthetic bacteria display a remarkable metabolic diversity and versatility which renders such an attempt difficult. It has been suggested frequently that some species (for example, *Chromatium vinosum* and

Figure 2. Hypothetical scheme of one reaction centre for bacterial photosynthesis.



NAD+
NADH
X
UQ
cyclic electron transport
reversed e-transport
AH2
(Substrate)
b-type cytochrome
X~I
ATP
ADP+Pi
High potential
C-type cytochrome
REACTION
CENTRE
PIGMENT
SYSTEM
hν

Figure 2.

Rhodospirillum rubrum) contain more than one type of reaction centre. However, the observations can also be interpreted by a scheme involving only one reaction centre. From a survey of the literature, the different views may be classified into two distinct schemes: a scheme with one reaction centre and a scheme with two reaction centres.

A. Scheme with one reaction centre for bacteria

In the scheme with one reaction centre, a single photo-reaction centre is thought to operate ~~in the~~ the production of ATP and NADH. Figure 2 is conceptual representation of this scheme. ATP formation is associated with a cyclic electron transport process initiated by photons absorbed by the pigment system. The reduction of NAD^+ by a substrate of higher redox potential is considered to be a consequence of reversed electron transport mediated by a high-energy intermediate generated by the cyclic electron transport or ATP. This view is held by Gest[138] and Vernon.[139] Among other significant investigations is the work of Keister and Yike.[140] They demonstrated that in the chromatophores of *Rhodospirillum rubrum*, the reduction of NAD^+ ($E_0' = -0.320$ V) by succinate ($E_0' = +0.031$ V) was inhibited by the addition of electron inhibitors or uncouplers*, but was stimulated by energy-coupling inhibitors** such as oligomycin in the light-induced reaction. In the dark, however, addition of ATP in the presence of an electron transport inhibitor resulted in the succinate-linked NAD^+ reduction. Similar NAD^+ reduction reactions were observed in other bacterial chromatophores by using different substrates.[141-3] These

* An uncoupler disengages the coupling between electron transport and formation of a high energy intermediate (phosphorylated or non-phosphorylated) leading to ATP and stimulates the electron transport.

** An energy-coupling inhibitor inhibits the conversion of a non-phosphorylated high energy intermediate into ATP.

observations demonsteated the possibility that a single reaction centre could produce both ATP and NADH. It should be mentioned that the midpoint redox potential of primary electron acceptors in these chromatophores is higher than that of NAD^+, hence a direct photo-reduction of NAD^+ at the reaction centre is less likely to proceed.*

B. Scheme with two reaction centres for bacteria

The scheme with two reaction centres is represented in Figure 3. In this scheme ATP formation is considered to be a coupled product of cyclic electron transport similar to the scheme described for a single photo-reaction centre. However, the reduction of NAD^+ is thought to be achieved by a photochemical redox reaction at a different reaction centre.[144-6] From the study of *Chromatium* chromatophores, Cusanovich, Bartsch and Kamen proposed the existence of two separate electron transfer systems: one containing P890, cytochrome-C_{555}, cytochrome-cc' and ubiquinone-7 is responsible for cyclic photophosphorylation; the other containing P905, cytochrome-C_{552} is responsible for substrate oxidation and NAD^+ reduction.[147] Sybesma and Fowler succeeded in characterizing some kinetic and spectral properties of the light-induced oxidations of cytochrome-C_2 and of C-428 (a cytochrome-like pigment having an absorption band at 428 nm) in *Rhodospirillum rubrum* without any added substrate.[148] However, the two photo-oxidation reactions were carried out at different light intensities and under different redox conditions. The

* This statement should not be over-emphasized. Because the midpoint redox potential is normally measured at equilibrium in which all associated reactions are complete (formation of primarily reduced species, it's protonation or deprotonation etc.) However, the photo-chemical redox reaction is usually very rapid and the primarily reduced species might have a much lower redox potential than that of the final species.

Figure 3. Hypothetical scheme of two reaction centres for bacterial photosvnthesis.

Fp flavoprotein

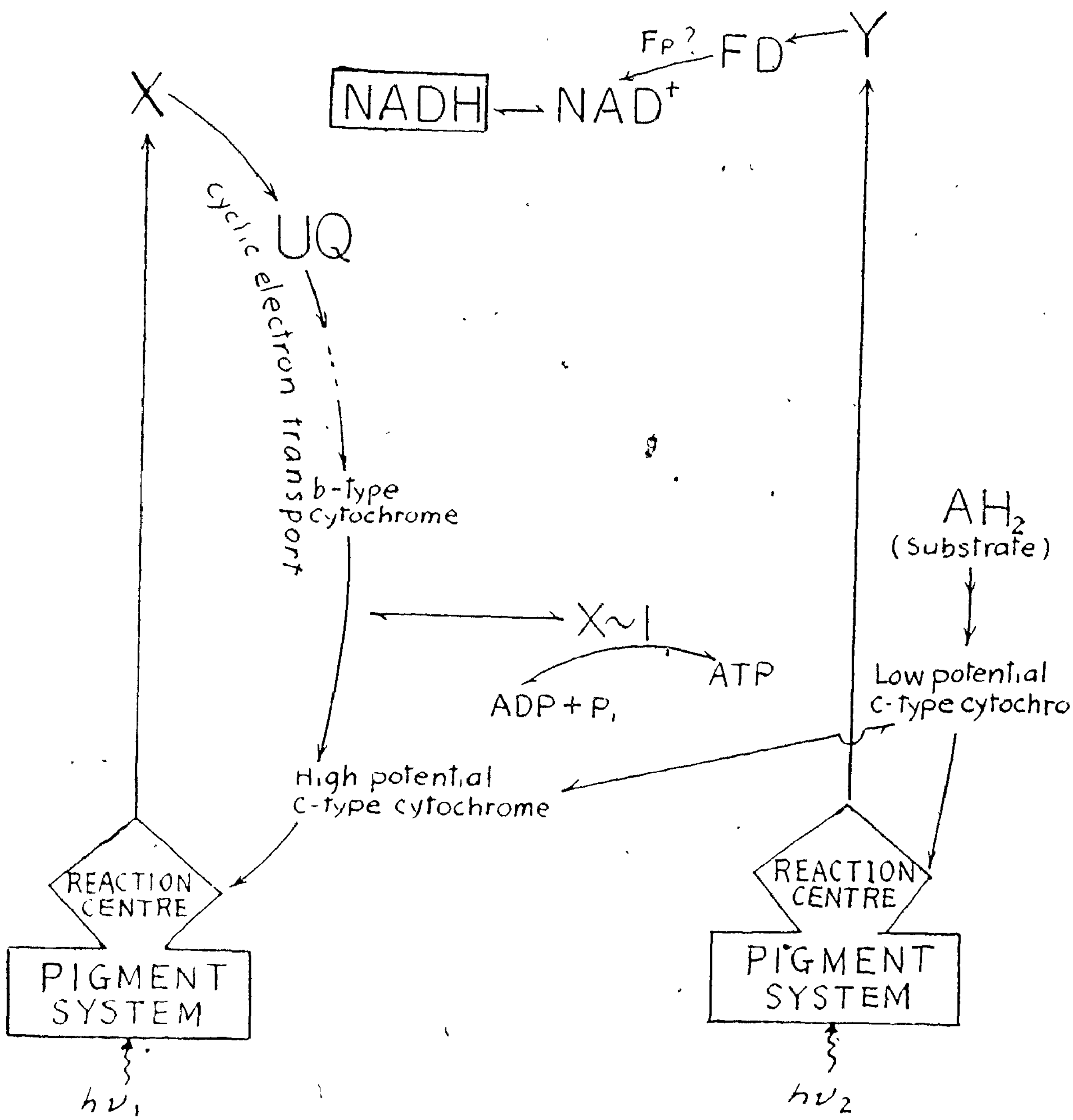


Figure 3.

action spectrum for the cytochrome-c_2 oxidation showed that the reaction was driven by light absorbed at 890 nm (P890) and 805 nm (P800), while that of the C-428 oxidation showed that only the 890 nm light was effective. Based on this difference in the two action spectra, they suggested the existence of two photo-reaction centres. Also based on action spectral studies, Morita has proposed the possible existence of three photo-reaction centres in *Chromatium*.[149]

No definite conclusion can be made as to whether single or multiple photo-reaction centre systems are applicable or if the NAD^+ reduction is photo-chemical in nature or dependent on a high energy intermediate.

Recently several review articles appeared on the subject of bacterial photosynthesis.[150-6,85] Most authors seem to favour the concept of a single photo-reaction centre. The original suggestion of Sybesma and Fowler has been modified in favour of a scheme with a single reaction centre in *Rhodospirillum rubrum*.[157] Thornbar isolated a reaction centre particle containing P890 (plus P800) with two cytochromes from *Chromatium* and found that the reversible oxidations of cytochrome-C_{555} (high potential) and the oxidation of chemically reduced cytochrome-C_{552} (low potential) were both associated with the same photo-reaction centre, namely, P890.[158] More direct evidence for a single reaction centre was produced by Parson and Case in the study of *Chromatium* cell suspensions. By using an actinic flash light together with kinetic spectrophotometry, they found that cytochrome-C_{552} and cytochrome-C_{555} exhibited identical action spectra for photo-oxidation.[159-160] The same primary and secondary electron acceptors appear to be involved in the oxidation of both cytochromes mediated by the same reaction centre.

It should be noted that most photosynthetic bacteria contain a high potential c-type cytochrome involved in cyclic electron transport and a low potential c-type cytochrome is implicated in the transfer of electrons from substrates *via* dehydrogenase enzymes.[159*] The above investigations support the view that these two cytochromes are photo-oxidized by a single reaction centre, not separately by two reaction centres. A one photo-reaction centre system may be disguised as a multiple photo-reaction centre system for several different reasons. Action spectra of different cytochromes are obtained at different redox potentials. There is the possibility that a single reaction centre may exhibit different physical and chemical properties depending on the redox potential imposed by experimental conditions. Some of these conditions may include the aeration of media, the redox potentials of substrates, and the light intensities used. When these conditions are changed, the degree of interaction between the reaction centre and the light-harvesting pigment systems may also be changed. If this is the case, any changes in the latter will manifest themselves in different action spectra. Interpretation of experimental evidence may be further compromised by shifts in the spectral bands of the reaction centre and other pigments as a result of changes in the membrane potential or conformational changes in membrane structure created by photochemical reactions. It is known that an electric field may induce the electrochromic shift of an absorption band[161-4] as in the "carotenoid shift" observed in chloroplasts.

* Among the commonly studied photosynthetic bacteria, only chromatogphores from Rhodopseudomonas spheroides do not have the low potential c-type cytochrome. There are three b-type cytochromes, but their functional roles are unclear.

Whether NAD^+ reduction is photo-chemical or high energy dependent may depend upon the kind of reducing substrate used. Succinate-linked NAD^+ reduction is generally accepted to be a high energy intermediate dependent dark reaction in *Rhodospirillum rubrum*[140,165] and *Rhodopseudomonas capsulta*.[166] Electron transport is reversed from succinate to NAD^+ similar to the earlier mitochondrial observations of Chance and Hollunger.[167] Ascorbate *plus* dichlorophenolindophenol (DPIP) reduces NAD^+ photochemically in both *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata*,[165-166] but Isaev et al. showed definitely that ascorbate *plus* N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) reduced NAD^+ in *Rhodospirillum rubrum* by a high energy intermediate dependent reversed electron transport mechanism.[143] More work will be required for elucidation of the basic mechanism of conversion of light energy into the chemical free energy for NAD^+ reduction and ATP formation.

C. Components of the Bacterial Electron Transport Chain

Electron transport components of photosynthetic bacteria and their functional relationships in the photosynthetic apparatus vary from species to species. Three major techniques have been commonly applied to acquire the knowledge, namely, kinetic spectral analysis, redox titration and electron spin resonance. Although the photosynthetic bacteria reveal their diversity, we now have enough knowledge to show that there are several common points in their Electron Transport Systems. All species that have been examined contain the following six components in a cyclic electron pathway:

(a) A photo-reaction centre of redox potential between 0.4 to 0.5 volt where a rapid photo-oxidation occurs.* Examples: P890 in *Rhodospirillum rubrum* ($E_0' = +0.44$ V)[170] and in *Chromatium vinosum* ($E_0' = +0.49$ V)[147]. P870 (*plus* P800) in *Rhodopseudomonas spheroides* (E'_o = +0.45 V)[171] and in *Rhodopseudomonas capsulta* (E'_o = +0.44 V).[172] P985 (*plus* P830) in *Rhodopseudomonas viridis* (E'_o = +0.48 V).[173] Green photosynthetic bacteria such as *Chlorobium* and *Chloropseudomonas* have a reaction centre P840 with lower redox potential (E'_o = +0.33 V).[174] These potentials are pH independent.

(b) A primary electron acceptor, X, possibly a complex of ubiquinone and iron-protein.[*,85,175,179] The redox potential in each species is: -0.145 V at pH8 for *Rhodospirillum rubrum*,[176] -0.160 V at pH8 for *Chromatium vinosum*,[176] -0.025 V at pH7 for *Rhodopseudomonas capsulata*,[173] -0.095 V at pH7 for *Rhodopseudomonas viridia*,[177] -0.140 V at pH7.4 (77°K) for *Rhodopseudomonas geratinosa*,[178] and -0.130 V at pH8.5 for *Chlorobium thiosulfatophilum*.[174]

(c) A secondary electron acceptor identified as ubiquinone[164,175] with a redox potential between +0.030 V and +0.098 V.[180-3]

(d) A high potential c-type cytochrome (E'_o = +0.30 V to +0.35 V) acts as a primary electron donor to the reaction centre.[151-2, 180] Examples: Cytochrome-c_2 (E'_o = +0.293 V) in *Rhodospirillum rubrum*,[171] cytochrome C_{555} (E'_o = +0.33 V) in *Chromatium vinosum*,[147] cytochrome-c_2 (E'_o = +0.295 V) in *Rhodopseudomonas spheroides*.[171] In *Rhodopseudomonas capsulata* the cytrochrome has a midpoint potential of +0.34 V.[172]

(e) Among various commonly studied photosynthetic bacteria, many species contain a low potential c-type cytochrome (E'_o near 0.0 V) which behaves as a direct electron donor to the reaction centre.[152] The functional role of this cytochrome is not clear, probably mediating the transfer of electrons from substrates *via* dehydrogenase in a non-cyclic pathway,[144,151]

* The centre is probably composed of bacteriochlorophyll dimer.[168-9]

** This term, X, is used merely for historical reasons. The true primary acceptor is bacteriopheophytin.[155]

(f) b-type cytochromes are present in *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides*[171] and *Rhodopseudomonas capsulata*.[172] Some of these are involved in the cyclic electron transport process, donating electrons to the high potential c-type cytochrome. *Chromatium vinusum* does not have b-type cytochrome.[147]

D. Photosynthetic Phosphorylation of Bacteria

For the synthesis of ATP, significant observations have been made concerning the site of its formation for *Rhodospirillum rubrum*.[184-186] Baltscheffsky and Arwidsson used proper combinations of an uncoupler (valinomycin), an artificial redox carrier (phenazine methosulphate, PMS) and an electron transport inhibitor between b- and c-type cytochromes (antimycin A or 2-n-heptyl-4-hydrocyquinoline-N-oxide, HOQNO) in studying their effects on ATP yields and found that there may be two sites for ATP formation. One site was found insensitive to the uncoupler and the inhibitor (PMS-added system) while the other site was sensitive.[184-5] The latter site was located between the b-type and c-type cytochromes by applying the so-called "cross-over theorem" of Chance et al.,[187] where Baltscheffsky observed the reduction of the b-type cytochrome and the oxidation of c-type cytochrome after adding ATP or an inorganic pyrophosphate.[186] The molecular mechanism of the energy transduction at these two sites is not known.

I.3 Electron Transport Chain of Mitochondria

The door to our current knowledge of the mitochondrial electron transport chain was opened in the middle twenties by David Keilin of Cambridge University.[188] In a series of publications, he described a chain of cytochromes by which electrons could be transferred from hydrogen donors at one end to oxygen at the other. He also showed that the electron transport process could be inhibited when reagents such as azide, cyanide and hydrogen sulfide were added. What is admirable is the fact that those important conclusions were derived from work in which only a hand spectroscope was used.

After the electron transport chain and its associated ATP-producing machinery was located in the mitochondrion by Lehninger and Kennedy,[10,12] and also by Schneider and Potter,[11] a clearer picture of the interaction of components within the chain has emerged from the works of many schools around the world. Leading contributors to this field are: B. Chance,[187] M. Klingenberg,[188] E. Slater,[189] L. Ernster,[190] Y. Hatefi,[191] D.E. Green,[192] T.E. King,[193] D.F. Wilson,[194] A.L. Lehninger[195] K. Okunuki,[196] B. Mackler,[197] T.P. Singer[198] T. Ohnishi[199] and H. Beinert.[200]

A. Structure of the Electron Transport Chain

By using kinetic-spectrophotometry, electron spin resonance spectroscopy and potentiometric titration techniques, combined with skillful membrane fragmentation and biochemical methods, the order of a member in the chain has been determined. Furthermore, through a sequence of biochemical purifications after membrane fragmentation, each member has been found to remain in one of four subunits.[191] These subunits are now called: NADH-ubiquinone reductase complex (complex I), succinate-ubiquinone reductase complex (complex II), ubihydroquinone-cytochrome-c reductase complex (complex III) and cytochrome-c oxidase complex (complex IV). Figure 4 is the schematic representation of this electron transport chain. Here, ubiquinone pool and cytochrome-c are not placed within any one of the complexes because both of them, compared with the others, can be easily extracted from the mitochondrial membrane.

Figure 4. Electron transport chain of animal mitochondria.

"*" --Starred components exhibit unusually large shifts in the mid-point potentials when the membrane is energized by adding excess ATP.

Subscripts, "N", "s" and "bc" under the iron-sulfur centres (Fe-S) represent their presence in the NADH dehydrogenase, succinate dehydrogenase and the complex of cytochrome b-c segment, respectively.

Visible and invisible Cu means the copper detectable and non-detectable by the electron spin resonance method.

Redox potentials that appear in figure 4 are cited from references 194,199 and 200.

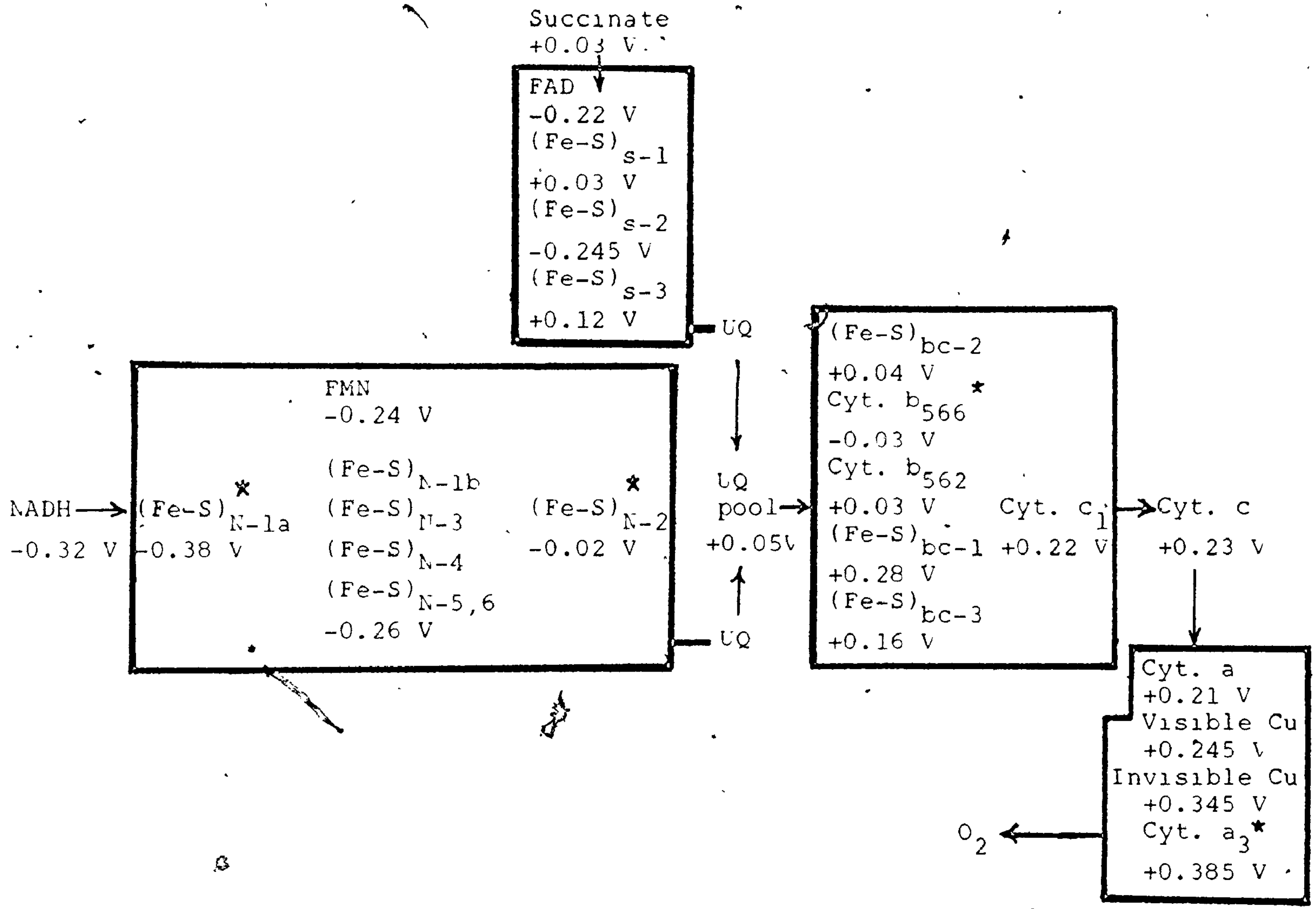

Succinate
+0.03 V
FAD
-0.22 V
$(Fe-S)_{s-1}$
+0.03 V
$(Fe-S)_{s-2}$
-0.245 V
$(Fe-S)_{s-3}$
+0.12 V
UQ
NADH
-0.32 V
$(Fe-S)_{N-1a}$*
-0.38 V
FMN
-0.24 V
$(Fe-S)_{N-1b}$
$(Fe-S)_{N-3}$
$(Fe-S)_{N-4}$
$(Fe-S)_{N-5,6}$
-0.26 V
$(Fe-S)_{N-2}$*
-0.02 V
UQ
UQ
pool
+0.05V
$(Fe-S)_{bc-2}$
+0.04 V
Cyt. b_{566}*
-0.03 V
Cyt. b_{562}
+0.03 V
$(Fe-S)_{bc-1}$
+0.28 V
$(Fe-S)_{bc-3}$
+0.16 V
Cyt. c_1
+0.22 V
Cyt. c
+0.23 V
Cyt. a
+0.21 V
Visible Cu
+0.245 V
Invisible Cu
+0.345 V
Cyt. a_3*
+0.385 V
O_2

Figure 4.

Quinone molecules of the pool are spread widely across the lipid bilayer structure of the membrane.[201] Cytochrome-c, on the other hand, is located outside the membrane because of its hydrophilic nature.[202] It should be noted that some ubiquinone molecules are resistant to the extraction and attached strongly to a protein in the complexes.[193] These quinones are separately depicted as "UQ-" with associated complexes.

The sequence of the electron transport reactions as shown in Figure 4 can be rationalized by the following facts:

(a) By using kinetic-spectrophotometric measurements, Chance demonstrated the time sequence in which the fully reduced members of anaerobic, intact mitochondria went into the oxidized state when oxygen was introduced.[187]

(b) The sequence is consistent with the standard reduction potentials of the individual members in that the potentials become more positive as reducing equivalents pass from substrate to oxygen.[187,194,199]

(c) When a specific inhibitor of the electron transport is added, members before the site of inhibition are reduced by reducing substrates produced from tricarboxylic acid cycle, while members on the oxygen side of the inhibition site are oxidized.[187] Frequently used inhibitors are: rotenone (complex I), antimycin A (complex III, cyt. b-c segment), cyanide (complex IV, cyt. a_3), DBMIB (ubiquinone pool), etc.

(d) Each complex may be used as a building block to reconstitute part or all of the electron transport chain. For example, complex I catalyzes the reduction of ubiquinone by NADH but not the reduction of cytochrome $a+a_3$ without adding complex III and cytochrome-c.[191,203]

B. Oxidative Phosphorylation of Mitochondria

It is recognized that, when NADH is the supplier of electrons towards the terminal acceptor oxygen, ATP yields corresponding to three coupling sites can usually be obtained.[195] If succinate is used instead of NADH, one obtains ATP yields equivalent to the existence of only two coupling sites along the electron pathway.[195] However, it should be mentioned that such estimation of the number of coupling sites is not based on any theoretically rigorous ground. Here, it is assumed that formation of every molecule of ATP needs the passage of two electrons at the coupling site, even the molecular mechanism is unknown.

By reconstitution experiments, Racker and his associates successfully demonstrated ATP formation when complex I or IV was combined with a hydrophobic protein fraction from mitochondria, phospholipids, and coupling factors.[203-4] When the reconstituted vesicles contained complexes III and IV, approximately twice as much ATP could be produced, indicating the complex III was also contributing to the ATP formation. Unfortunately those systems are still too complicated, from a chemists viewpoint, to suggest the participation of any particular member in the complexes in the phosphorylation proper.

Much information was gained with respect to the electron transport system and its mutual interaction with the phosphorylation system during the 1970s. By applying electron spin resonance spectrometry and potentiometry, various iron-sulfur proteins (Fe-S centres) were discovered in complexes I, II and III.[200,198] Combinations of electron spin resonance measurements and potentiometric titrations not only characterized the diversified Fe-S centres by their redox potentials and ESR g-factors, but also suggested the involvement of some of these centres in the energy transduction reaction.[199] The addition of ATP caused an apparent shift in the redox potential by 125 mV higher for the $(Fe\text{-}S)_{N-2}$ centre and 60 mV lower for the $(Fe\text{-}S)_{N-1a}$ centre. For various cytochromes, b- and a-type cytochromes in particular, Wilson and co-workers applied potentiometric titration and spectrophotometric methods to the systems and gained further insight concerning their constitution, property and function.[194] The existence of more than one species of cytochrome-b was recognized: cytochrome-b_{566} with $E_{m7.2}$=-30 mV and cytochrome b_{562} with $E_{m7.2}$= +30 mV. Addition of excess ATP changed the midpoint redox potential of cytochrome b_{566} from -30 mV to +245 mV, suggesting the possible involvement of this species directly in the energy transduction process. Similar behavior was also observed in complex IV for cytochrome-a_3. Addition of ATP reduced the midpoint redox potential of cytochrome-a_3 from +385 mV to +155 mV, while cytochrome a showed only a slight shift from +210 mV to +250 mV. No shift in potential could be observed for two copper proteins in the complex. These results led to the suggestion that cytochrome-a_3 is involved in the energy transduction proper.[194]

It should be noted that the observed changes in the midpoint redox potential of $(Fe\text{-}S)_{N-1a}$, $(Fe\text{-}S)_{N-2}$, cytochrome-b_{566} and cytochrome-a_3 by the addition of ATP may or may not be interpreted as evidence for their direct participation in the phosphorylation reaction. The addition of ATP may create some intermediate energy states or chemical forms not directly associated with any of the members mentioned above. Hinkle and Mitchell proposed that a membrane potential created by the ATP addition may shift the apparent redox potentials.[205-6] Klingenberg, on the other hand, speculated that the changes in the apparent redox potentials may be caused by the reversal of electron transport.[188]

C. The Electron Transport Chain and Oxidative Phosphorylation of Other Systems

Oxidative phosphorylation takes place not only in animal mitrochondria, but also in plant mitochondria and respiratory bacteria such as E. coli. However, these living systems havn't been as widely studied as the others in the past. Generally speaking, there are much resemblance to the animal mitochondrial system in nature, except that their electron transport chains are more branched at quinone pools.[207-9] The molecular mechanism of phosphorylation is presumably similar to the other systems, and therefore, not going to be expounded here.

I.4 Four Hypotheses Concerning the Mechanism of ATP Formation

More than a dozen hypotheses have been proposed during the past twenty years. Currently, there are four major hypotheses concerning the mechanism of energy transduction: the chemical coupling hypothesis, the conformational coupling hypothesis, the chemiosmotic hypothesis, and the internal acidification hypothesis.

A. Chemical Coupling Hypothesis

This hypothesis, modelled on the mechanism of substrate-linked phosphorylation, proposed that the energy released from the redox reaction between two adjacent members of the electron transport chain at the site of ATP formation is conserved in a high energy chemical intermediate composed of one of the products of the redox reaction or of a third compound which is not a member of the electron transport chain. Energy in the intermediate is then transferred through several reaction steps to the formation of a high energy phosphoryl intermediate which then phosphorylates ADP into ATP. This hypothesis may be represented by the following reaction scheme:

(i) $$A_{red} + B_{ox} + I \rightleftharpoons \begin{cases} A_{ox} \sim I + B_{red} \\ \text{or} \\ B_{red} \sim I + A_{ox} \end{cases}$$

(ii) $$\left.\begin{matrix} A_{ox} \sim I \\ \text{or} \\ B_{red} \sim I \end{matrix}\right\} + X \rightleftharpoons X \sim I + \begin{cases} A_{ox} \\ \text{or} \\ B_{red} \end{cases}$$

(iii) $$X \sim I + P_i \rightleftharpoons X \sim P + I$$

(iv) $$X \sim P + ADP \rightleftharpoons X + ATP$$

where A_{ox}, B_{ox}, A_{red} are the oxidized and reduced forms of two members, A and B, in the electron transport chain. I and X are hypothetical energy transfer carriers common to all sites of the ATP formation along the chain. P_i is an inorganic phosphate. The squiggles, "$\sim$", represent "High energy"* chemical bonds of unknown complexes.

In the scheme reaction (i) is denoted as the "energy coupling reaction" and reactions (ii), (iii), (iv), as the "energy transfer reactions."

The earliest scheme was proposed by Lipmann in 1946. The energy coupling reaction implied phosphorylation of one of the redox pair (A_{ox} or B_{red}) in the electron transport chain prior to ATP formation.[210] In 1953 Slater suggested a third compound, I, was involved in the formation of the primary high energy intermediate (see reaction (i)).[211] This modification became necessary after the discovery that the electron transport chain could operate without P_i and ADP under certain conditions, as in the non-phosphorylative

* "High Energy" has been used in a rather undefined manner among biochemists. It is synonymous with "highly negative standard free energy of hydrolysis."

Keilin and Hartree preparation.* Furthermore, in the presence of an uncoupler, 2,4-dinitrophenol (DNP), the rate of electron transport was stimulated without forming ATP from P_i and ADP. The presence of the uncoupler was believed to cause the hydrolysis of either $A_{ox} \sim I$ or $B_{red} \sim I$. In 1964 Ernster and Lee introduced a new hypothetical compound, X, such that a system with multiple sites of ATP formation could have a common high energy intermediate, $X \sim I$, for energy-dependent processes.[212] ATP synthesis, ion translocation, the pyridine nucleotide transhydrogenase reaction,** reversal of electron transport*** and various kinds of exchange reactions**** were claimed to be dependent on $X \sim I$. This concept developed from the effect of addition of DNP or oligomycin to the above processes in a mitochondrial system showing proper electron transport. Oligomycin is considered to be the inhibitor of reaction (iii). When oligomycin or DNP is added the formation of $X \sim I$ was thought to be prevented, and all the above processes ceased to function normally.

No high energy intermediate has ever been isolated, thus the validity of the hypothetical scheme is uncertain. In addition it is unknown whether this scheme derived from the study of mitochondrial systems can be applied to photosynthetic systems in which some photo-excited components are involved.

* Mitochondrial membrane fragments prepared by sonic or mechanical treatment capable of electron transport but not ATP formation.

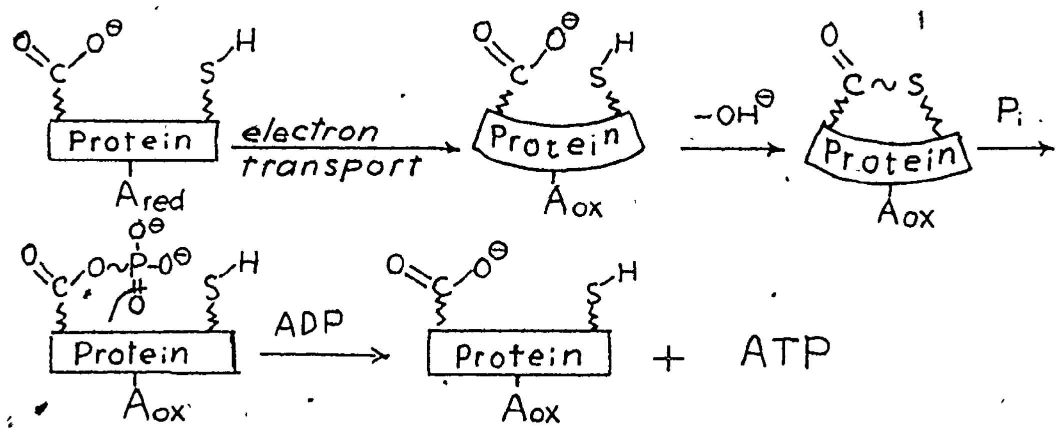
** $NADH + NADP^+ + X \sim I \rightleftharpoons NAD^+ + NADPH + X + I$

*** e.g. succinate $+ NAD^+ + X \sim I \rightleftharpoons$ fumarate $+ NADH + X + I + H^+$

**** ATP-P_i, ATP-ADP and P_i-H_2O oxygen exchanges.

B. Conformational coupling hypothesis

In 1964 Boyer proposed that the energy from the electron transport chain could be conserved as a conformational change in a protein component of the chain and that this change was associated with a phosphorylation reaction.[213] As a possible intermediate reaction, he suggested the formation of an intramolecular high energy S $\sim$ acyl bond:



This conformational coupling hypothesis is formally similar to the chemical coupling hypothesis if the protein component is A or B. The only difference is that the high energy is stored in the folding of the protein component rather than in a "high energy" bond between the member (A or B) and a third molecule, I, or in some other pair, X $\sim$ I.

Since 1973, the conformational coupling hypothesis has been extended to include a radically new concept for the mechanism of energy transduction, owing to a series of interesting findings. These include:

(a) Membrane-bound or isolated ATPase (coupling factor 1, F_1)[203] can tightly bind several nucleotides (ADP, ATP).[214-6]

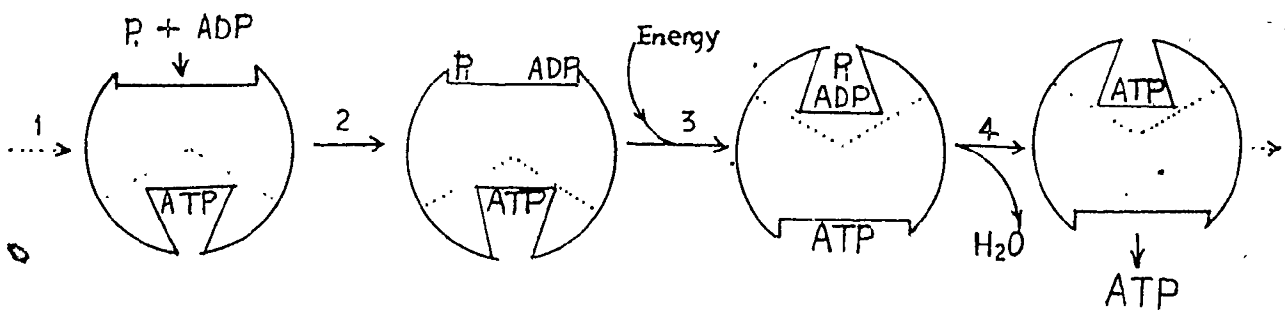
(b) The tightly bound nucleotides can be released to the medium by energizing the membrane.[189]

(c) ^{18}O exchange between the inorganic phosphate and water catalysed by the membrane-bound coupling factor is relatively insensitive to uncouplers.[215]

(d) In addition to releasing the tightly bound nucleotides, energizing the membrane also promotes binding of the inorganic phosphate in a mode favoring bound-ATP formation.[217-8]

These observations have led Boyer[219] and Slater[189] to propose a conformational coupling hypothesis very different from the original one. According to this new hypothesis, the coupling factor 1 forms complexes with ATP, ADP and inorganic phosphate, whose stability depends on the membrane conformational state. In an energized state elicited either by light or by a dark redox reaction in the membrane, ADP and inorganic phosphate are tightly bound to the coupling factor while the corresponding ATP binding becomes weak. In this state, the weakly bound ATP dissociates itself into the medium, and the bound ADP and inorganic phosphate form ATP with very little energy expenditure. In a de-energized state of the membrane, the conformation of the enzyme is changed such that the ATP formed becomes tightly bound, whereas the ADP and the inorganic phosphate become weakly bound. In other words, an energy input to the membrane in the absence of an uncoupler is mainly used in the release of the tightly bound ATP, rather than in its formation at the reaction site of the coupling factor.

In Boyer's laboratory, continual investigations of respiratory and photosynthetic systems by various ^{18}O exchange reactions and radio-isotope labelling techniques led him to develop the new concept into a mechanistic model called the "alternating-dual-catalytic site model"[15,220] as depicted in the following scheme:



How the dehydration proceeds from step 3 to step 4 and how redox carriers interact with the phosphorylation proper have not been explicitly presented in this model.

C. Chemiosmotic Hypothesis

According to Mitchell's chemiosmotic hypothesis, the primary energy-conserving act is the translocation of protons and the formation of membrane potential across a biological membrane. Relaxation of the proton-gradient and/or the membrane potential through the action of so-called "proton-translocating reversible ATPase" or "ATP-synthetase" results in ATP formation.[221-2]

Figure 5(A) illustrates how redox reactions in the mitochondrial electron transport chain initiate proton translocation. According to Mitchell, hydrogen carriers and electron carriers alternate in the chain. Protons are accepted on one side of a membrane by a hydrogen carrier and are released on the opposite side where the hydrogen carrier is oxidized by an electron carrier, thus creating a proton-gradient. In mitochondria protons are translocated from the inner phase of the membrane to the outer phase. At the same time the inward movement of electrons creates an electrical potential difference or membrane potential. Mitchell used the term "proton motive force" to represent the electrochemical potential of protons across the coupling membrane in which both the potential due to the proton concentration gradient and the membrane potential were included.

Figures 5(B) and 5(C) illustrate how the postulated proton motive force operates. Here, ATPase I and II refer to systems in which one and two protons from the outer phase are used for the synthesis of X—I by a dehydration reaction between X^- and IO^- brought electrophoretically from the outer part of the membrane. X—I then moves to the inner surface and is transformed into a high energy compound, $X \sim I$, which then reacts with P_i and ADP, leaving X^- and IO^- in the membrane for another cycle. The driving force for ATP formation may be due to a low proton concentration of X^- and IO^- close to the inner surface of the membrane. These processes can be summarized as:

(i) $2H^+ + X^- + IO^- \rightleftharpoons XH + IOH \rightleftharpoons X{-}I + H_2O$

(ii) $X{-}I \rightleftharpoons X \sim I$

(iii) $X \sim I + H_2PO_4^- + ADP^{3-} \rightleftharpoons X^- + IO^- + 2H^+ + ATP^{4-}$

According to Mitchell, (i), (ii) and (iii) are catalyzed by "X—I hydrolase", "X—I translocase" and "X—I synthetase", respectively.

Figure 5. Chemiosmotic hypothesis of Mitchell.

A. Loops inside the mitochondrial inner membrane to allow the proton translocation.

B. ATPase I of Mitchell effecting the synthesis of ATP in mitochondria. $ATP/H^+=1$.

C. ATPase II of Mitchell effecting the synthesis of ATP in mitochondria $ATP/H^+=2$.

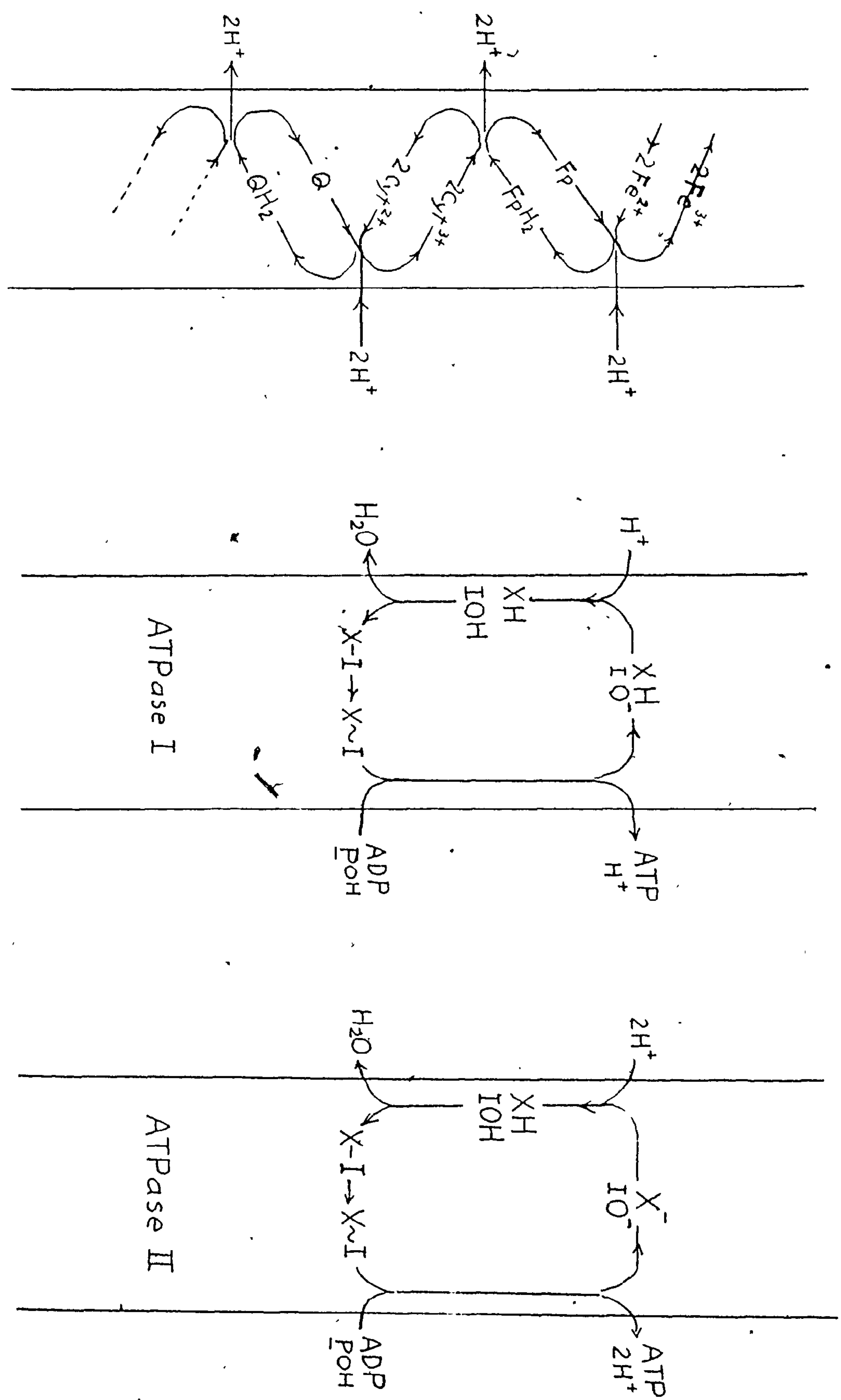

A
B
C
2H+
2H+
2H+
2H+
2Fe3+
2Fe2+
Fp
FpH2
2Cyt3+
2Cyt2+
Q
QH2
H+
H2O
XH
IOH
XH
IO-
X-I→X~I
ATP
H+
ADP
POH
ATPase I
2H+
H2O
XH
IOH
X-
IO-
X-I→X~I
ATP
2H+
ADP
POH
ATPase II

Figure 5.

In photosynthetic systems proton translocation occurs in the light. In chloroplasts and bacterial chromatophores, inward rather than outward proton translocation was observed.[223-4] Witt et al. have demonstrated the existence of a membrane potential across the thylakoid membrane as shown by absorption changes of illuminated chloroplasts.[44-6] This was proved unambiguously by Fowler and Kok.[43] In 1969 Jackson and Crofts observed that an artificial membrane potential (diffusion potential of K^+) across the chromatophore membrane of *Rhodopseudomonas speroides* in the dark induced a carotenoid absorption change.[225] These studies indicate that, in both chloroplasts and chromatophores, the inner surfaces of the membranes are positively charged in the light. A close relationship between the absorption change of pigments (hence the membrane potential) and the high energy state leading to ATP formation has been claimed.[44-6]

However, the most direct evidence for Mitchell's chemiosmotic hypothesis comes from the "acid bath" experiment of Jagendorf and coworkers.[226-8] In the dark and in the presence of P_i, ADP, magnesium ion and an electron transport inhibitor, ATP synthesis was observed if chloroplast fragments were placed in a medium at pH4 containing a weak organic acid and the medium was then rapidly brought up to pH8.5.

Mitchell also discussed the chemiosmotic hypothesis in terms of quantitative relationships between the hydrolysis or formation of ATP, the pH difference across the biological membrane (due to the proton concentration gradient) and the membrane potential. In his ATPase II system, the hydrolysis (or synthesis) of ATP was represented as:

(iv) $$ATP + H_2O + 2H^+_{in} \rightleftharpoons ADP + P_i + 2H^+_{out}$$

The equilibrium constant for the hydrolysis of ATP coupled to the translocation of protons was given by:

(v) $$\frac{[ADP][P_i][H^+_{out}]^2}{[ATP][H_2O][H^+_{in}]^2} = K \quad \text{or} \quad \frac{[ADP][P_i]}{[ATP]} = K[H_2O]\,\frac{[H^+_{in}]^2}{[H^+_{out}]^2}$$

where $K[H_2O]$ is equal to the equilibrium constant for ATP hydrolysis in a homogenous aqueous solution; H^+_{in} and H^+_{out} represent protons in the inner and outer phases separated by the membrane; the brackets represent, according to Mitchell's expression, "electrochemical activities" of the enclosed components. ADP, P_i and ATP all participate in the equilibrium within the same phase. If there is a membrane potential of $\Delta\psi$ millivolts between the two phases, positive at the outer surface (mitochondria), the following relationships are assumed to hold:

(vi) $$\log \frac{[H^+_{out}]}{[H^+_{in}]} = pH_{in} - pH_{out} + \frac{\Delta\psi}{Z}$$

where Z= 2303 RT/F, F is the Faraday; the pH_{in} and pH_{out} are the pH of inner and outer phases, respectively. From equations (v) and (vi), one could easily obtain the following equation:

(vii) $$\log \frac{[ATP]}{[ADP][P_i]} = 2\,\{pH_{in} - pH_{out} + \frac{\Delta\psi}{Z}\} - \log\{K[H_2O]\}$$

When T is 300°K, Z is close to 60 mV and K $[H_2O]$ for ATP is close to 10^5. As the electrochemical activities of ATP, ADP and P_i all refer to the same phase, they can be approximately equated with concentrations. Assuming the concentration of P_i to be 0.01 M, then from equation (vii) one obtains:

$$\text{(viii)} \quad \log \frac{[ATP]}{[ADP]} \doteq \frac{\Delta\psi}{30} + 2\,\{pH_{in} - pH_{out}\} - 7$$

Therefore, the electrochemical potential difference of protons necessary for the ATP/ADP ratio of 1 under these conditions would be:

$$\text{(ix)} \quad \Delta\psi + 60\,\{pH_{in} - pH_{out}\} = 210 \quad \text{(in mV)}$$

Hence, a pH differential of 3.5 or a membrane potential of 210 mV would be sufficient energetically to phosphorylate ADP into ATP.

Although Mitchell derived these relationships from studies of mitochondria, they have been considered to be equally applicable to photosynthetic systems with the direction of the proton translocations and the polarity of the charged membrane during the energization processes being different. In all cases Mitchell's chemiosmotic hypothesis requires:

(a) A high energy state (proton gradient and/or membrane potential) leading to ATP formation in a topologically closed membrane structure.

(b) A membrane relatively impermeable to protons and other ions such that a high electropotential difference is created to drive ATP synthesis.

(c) The presence of an anisotropic reversible ATPase in the membrane which couples proton gradients and/or membrane potential to ATP synthesis.

D. Internal Acidification Hypothesis

This hypothesis was proposed by Williams in 1961.[229] In contrast to Mitchell's chemiosmotic hypothesis in which the energy conversion mechanism involves a transmembrane charge separation and a "chemiosmotic" process, Williams proposed that energization might result from an intra-membrane acidification and a localized charge polarization.[230] According to him the coupling between the redox reactions of an electron transport chain and the acid base condensation between ADP and inorganic phosphate is realized by means of increased proton activities in the hydrophobic environment within the membrane. The changes in proton activity bring about changes in the activity of all bases, including water molecules nearby. According to this hypothesis, the endothermic reaction, $ADP + P_i \longrightarrow ATP + H_2O$, proceeds *via* coupling to the exothermic reaction, $H^+ + H_2O \longrightarrow H_3O^+$.

Williams emphasized the acidification within the membrane, because, if the protons escape into the aqueous medium as is proposed in Mitchell's hypothesis, the resultant hydrated form of the protons (hydronium oil) is not energetically favoured to go back to the hydrophobic surroundings of the coupling site.[231]

Williams' concept seems to be chemically more realistic, but designing a chemically well-defined system for proving it is difficult.

CHAPTER II

ATP FORMATION BY ELECTROLYSIS OF ADP PLUS PHOSPHATE

Both oxidative and photosynthetic phosphorylations are coupled to redox reactions among the members in the electron transport chain. The coupling efficiencies are known to be extremely high. The formation of ATP from ADP and phosphate is an energetically unfavorable, endothermic reaction. The redox reactions are, on the other hand, exothermic reactions, donating their released Gibbs free energy to drive the reaction of ATP formation. How the energy-donating redox reactions ensure the phosphorylation reaction without losing the energy in the form of heat during the coupling steps is the central problem of bioenergetics. The coupling steps must involve energetically unfavored but kinetically favored reactions in which an energized state or high energy intermediate can cause the energy to relax along a definite reaction path leading to ATP formation. One can speculate that the proton-gradients and the membrane potential of the chemiosmotic hypothesis, the acidified membrane of the internal acidification hypothesis, or the high energy form of coupling factors of the conformational coupling hypothesis serve as the energized common intermediate between the redox and phosphorylation reactions. Even if they do, probably they all do, to express how this occurs from the redox reactions and how they lead to ATP formation in well-defined chemical terms is at least as difficult a problem as how to use the redox reactions to product ATP. For these hypotheses, in the strict sense, have not yet been evaluated at the molecular level.

One possible molecular mechanism that has not received much attention is one involving activation of the phosphorylation reactant, the ADP or the inorganic phosphate, by allowing it to participate in a redox reaction. If this is true, the reduction or oxidation centre for the reactants may be a member of the electron transport chain, or located at the active centre of the coupling factor, ATPsynthetase. To examine such a possibility, the most straightforward method is to study whether ATP can be produced by electrolysis in a ststem containing only ADP and inorganic phosphate. Controlled potential electrolysis is most appropriate for this purpose in which oxidation and reduction of the reactants can be studied separately without interfering with each other. This electrochemical method has another advantage in that the controlled electrical energy can be more efficiently converted into chemical energy than by other means. Electrode processes usually have a low energy of activation and hence high efficiency. Methods with high efficiency in activating the reactants would provide us with more informative data and should help in understanding the nature of the reaction.

A. Experimental Method

The device used in the controlled potential electrolysis is depicted in Figure 6. This device can be used to study the cathodic reduction and the anodic oxidation simultaneously. This is not only convenient and time-saving, but also useful for comparing the effect of the oxidation and the reduction on the reactants from the same stock solution under the same conditions.

Figure 6. ATP production from ADP plus phosphate by controlled potential electrolysis.

E------high capacity battery (1.5 to 6.0 volts).

R------variable resistance

AAKCL--agar-agar KCl junction bridge (KCl half-saturated).

Pt-----platinum electrode

M------magnetic stirrer

DM-----high impedance digital multi-meter

SCE----saturated calomel electrode (standard)

A------cell for cathodic reduction of the reactants.

B------cell for anodic oxidation of the reactants.

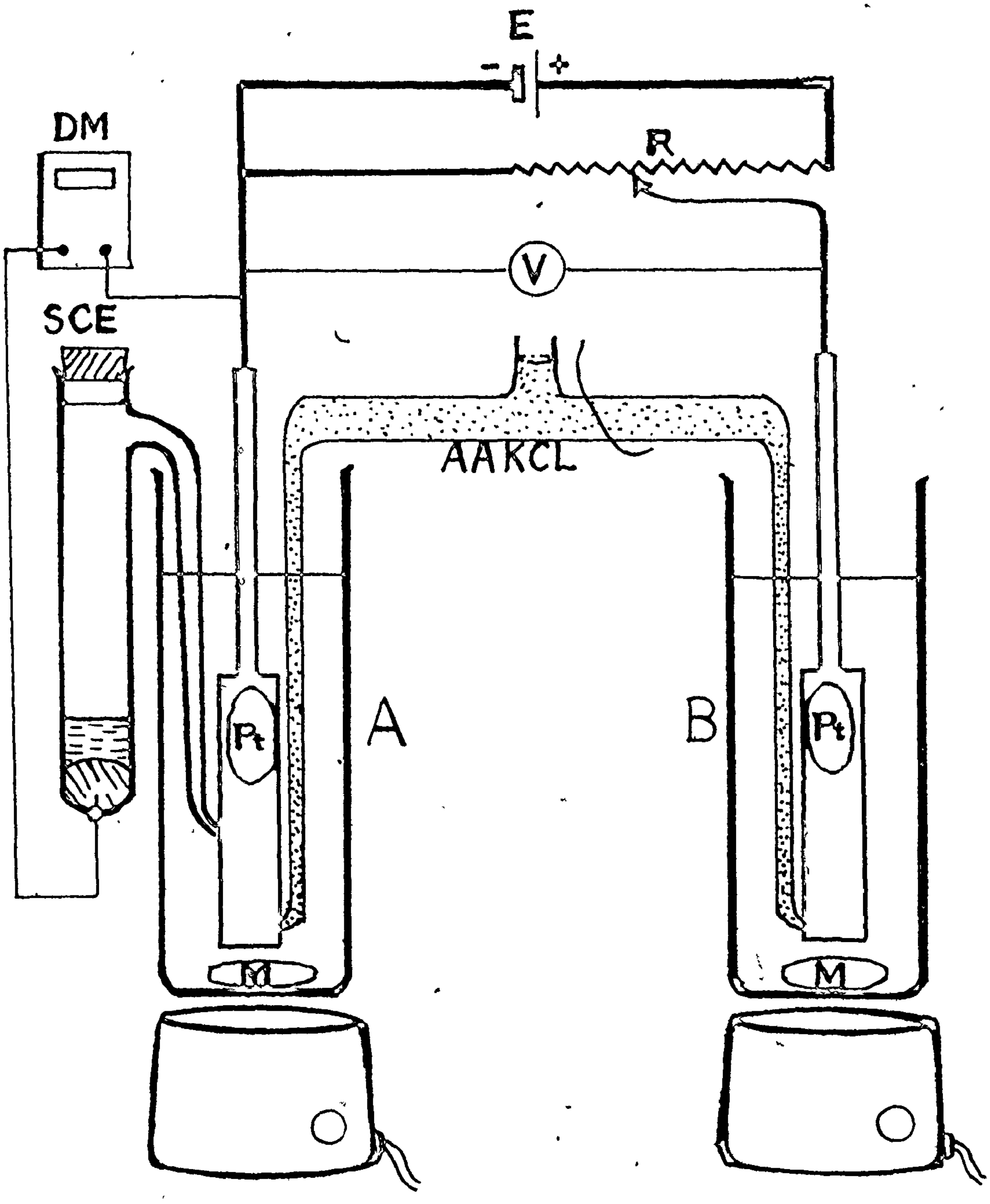


Figure 6.

The main power for the electrolysis is provided by a high capacity battery, E. This high capacity is essential because the time-span of the electrolysis is long, ranging from twenty to seventy hours. The controlled potential is obtained by adjusting the variable resistance R, until the desired potential is registered on the voltmeter, V. An agar-agar potassium chloride bridge is used as the junction, and its two ends are allowed to touch the electrodes. This minimizes the resistance of the medium, hence minimizing the potential drops which would otherwise be large, caused by a higher resistance of the non-aqueous solution. Magnetic stirring is applied to each cell in order to minimize the possible over-potential at the cathode and the anode. Cathodic and anodic potentials are separately measured by using a saturated calomel electrode as the standard. Potential differences between the electrodes and this standard are measured by a high impedance digital multi-meter (20 megohms internal resistance). Readings of the potential difference have been checked by using a Beckmann potentiometer and were found to be accurate within ± 0.002 volts.

In the apparatus described above the voltage applied across the electrodes for a solution of ADP plus phosphate can be given by the expression:

$$V = -(E_{cathode} + E_{o.c.}) + ir + (E_{anode} + E_{o.a.})$$

where $E_{cathode}$ and E_{anode} are the reversible single electrode potentials between the electrodes and the solution; $E_{o.c.}$ and $E_{o.a.}$ are the over-potentials at the cathode and anode, respectively; ir is the potential drop across the junction bridge.

Figure 7. Phosphorylation associated with cathodic reduction of AMP and anodic oxidation of inorganic phosphate.

The electrolytic solution is 1.1×10^{-3}M AMP plus 2.8×10^{-3}M diimidazolyl hydrogen ortho-phosphate in N,N-dimethylformamide. Duration of the controlled potential electrolysis is 20 hours. ADP produced is analyzed by the column ion exchange method of Cohn and Carter.[232]

Solid line---in air; dotted line---in nitrogen atmosphere.

The voltage is expressed with reference to the normal hydrogen electrode.

The "dots" denote experimental results.

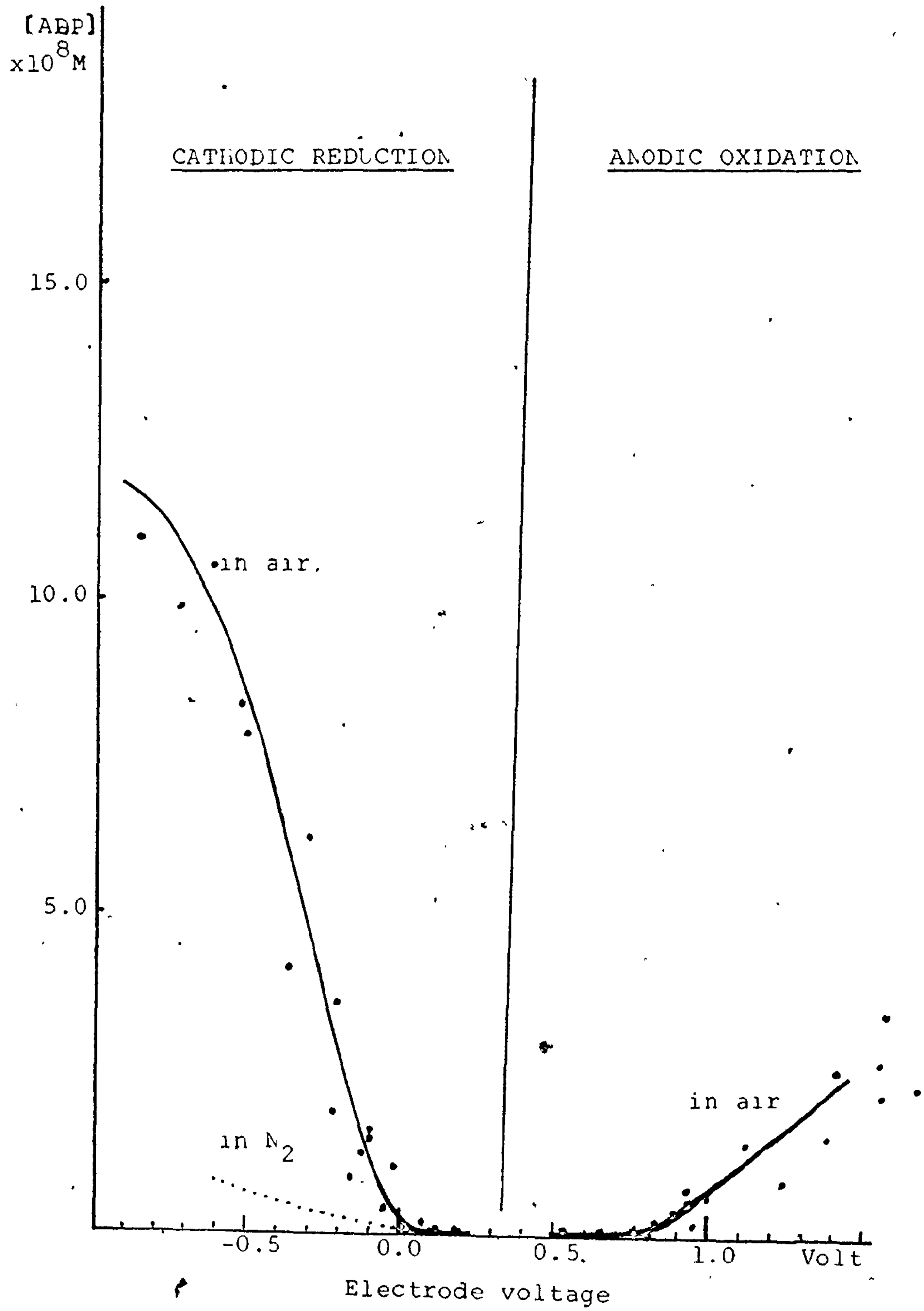

[ABP]
x10^8 M
CATHODIC REDUCTION
ANODIC OXIDATION
15.0
10.0
5.0
in air.
in N2
in air
-0.5
0.0
0.5
1.0
Volt
Electrode voltage

Figure 7.

In the equation, the ir term may be very small, due to the low resistance. Under normal conditions, the current measured is between two and seven microamperes. $E_{o.c.}$ and $E_{o.a.}$ are minimized, but probably not nullified. They may be the main sources of error contributing to the observed values of cathode and anode single electrode potentials.

In actual experiments, when the applied voltage, V, is gradually increased by adjusting the variable resistance, R, one finds that the cathodic potential is correspondingly decreased and the anoidc potential, increased. It is better to start the experiment with low applied voltages. Applied voltages higher than 1.0 volt result in nonreproducible data for the electrode potentials. This may be due to side reactions in the system. The algebraic sum of the apparent potentials of two electrodes is close to the applied voltage, if the latter is lower than 1.0 volt, suggesting that $E_{o.c.}$ and $E_{o.a.}$ may be small under such experimental conditions.

II.2 Phosphorylation Associated With Electrodic Redox Reactions

A. Phosphorylation by Cathodic Reduction of Adenine Nucleotides

When the potential is gradually lowered in the cathodic cell, we can see that phosphorylation reactions start at +0.05 volts (Figure 7). The lower the potential is, the higher the yields observed. In most experiments AMP is used as the adenine nucleotide since the phosphorylation products of electrolysis are ADP and ATP. Simultaneous determination of the concentration of ADP and ATP can provide valuable information for understanding the nature of phosphorylation reactions as will be discussed in the final section of this chapter (see II.3).

It should be emphasized that the phosphorylation is caused by the reduction of AMP, not by that of inorganic phosphate. Analysis of the phosphate by ammonium molybdate method in the absence of AMP in the cathodic cell did not show any changes in its concentration before and after the electrolysis.

B. Phosphorylation by Anodic Oxidation of the Inorganic Phosphate

Also shown in Figure 7 is evidence for the phosphorylation reaction caused by an oxidation process in the anodic cell. Here, it should be mentioned that one cannot apriori render the observed phosphorylation to be due to the anodic oxidation of the phosphate, since both components can be oxidized. However, the following facts strongly suggest that the phosphorylation reaction occurring at anodic potentials above +0.85 volts is caused by a phosphate oxidation:

(a) There are only two components as reactants of the phosphorylation, AMP and phosphate. It has been reported by Dryhurst and Elving that the electrochemical oxidation of adenine results in a series of irreversible reactions leading to the rupture of the heterocyclic ring(s).[321] Probably the adenine ring in AMP may be destroyed by a similar mechansim.

(b) Participation of the solvent used in the phosphorylation reaction is unlikely, since one obtains similar results whether N,N-dimethylacetamide, N,N-dimethylformamide or dimethyl sulfoxide is used.

(c) When only the phosphate is present in N,N-dimethylacetamide solution, the anodic oxidation with potential at +0.90 volts for 20 hours results in the reduction of its concentration.

(d) Oxiaation of AMP does not result in the phosphorylation. Adenine nucleotide analyses show that anodic potentials between +0.50 and 0.80 volts cause the reduction in AMP concentrations but the phosphorylation reaction does not occur within this voltage range.

II.3 Why Nature Uses ADP as the Phosphate Acceptor?

Cathodic reduction of AMP in the presence of a phosphate not only produces ADP, but as the electrolysis continues, the ADP produced can be further phosphorylated into ATP. Furthermore, the concentration of ATP produced gradually surpasses that of ADP, even though the unphosphorylated AMP still represents more than 90% of the total adenine nucleotides. This fact clearly indicates that the phosphorylation of ADP into ATP is much faster than that of AMP into ADP. The free energy of hydrolysis of ATP into ADP at pH7.0 is −7.3 kilocalories per mole, and that of ADP into AMP under the same conditions is about the same,[233] or even numerically less negative.[234] In other words, purely from the energetics point of view, the phosphorylation of AMP into ADP should be easier than that of ADP into ATP. What is the specific factor which makes the phosphorylation of ADP much faster?

The relative rates of phosphorylation for ADP and AMP can be determined easily in the presence of a large excess of phosphate. Under this condition, the series of reactions from AMP to ATP may be treated as a "pseudo first order consecutive reaction":

$$\mathrm{AMP} \xrightarrow[\text{excess } P_i]{k_1} \mathrm{ADP} \xrightarrow[\text{excess } P_i]{k_2} \mathrm{ATP} \qquad \text{(i)}$$

a	o	o	at t=o
a−x	x−y	y	at t=t

It is assumed that at time t=0 there are $\underline{a}$ moles of AMP, and by time t there remain $\underline{a-x}$ moles of AMP, together with the production of $\underline{x-y}$ moles of ADP and $\underline{y}$ moles of ATP. Rate equations for the reaction sequence (i) can be written as:

$$\frac{dx}{dt} = k_1 (\underline{a}-x) \qquad \text{(ii)}$$

$$\frac{d(\underline{x-y})}{dt} = k_1(\underline{a}-x) - k_2(\underline{x-y}) \qquad \text{(iii)}$$

Integration of equation (ii) yields

$$\underline{a-x} = \underline{a}\, e^{-k_1 t} \qquad \text{(iv)}$$

Inserting (iv) into (iii), we get

$$\frac{d(\underline{x-y})}{dt} + k_2(\underline{x-y}) = k_1\underline{a}\, e^{-k_1 t} \qquad \text{(v)}$$

Let $(\underline{x-y}) = z$, then (v) becomes the heterogeneous linear equation which can be solved by Euler's method. From (v)

$$\frac{dz}{dt} + k_2\, z = k_1\underline{a}e^{-k_1 t} \qquad \text{(vi)}$$

Multiplying both sides by an integration factor, $e^{k_2 t}$, we get a solvable differential equation (vii):

$$\frac{d}{dt}(ze^{k_2 t}) = k_1\underline{a}\, e^{(k_2-k_1)t} \qquad \text{(vii)}$$

The solution of (vii) is:

$$ze^{k_2 t} = \frac{k_1\underline{a}}{k_2-k_1}\left[e^{(k_2-k_1)}-1\right]$$

or

$$\underline{x-y} = \underline{a}\frac{k_1}{k_2-k_1}\left(e^{-k_1 t} - e^{-k_2 t}\right) \qquad \text{(viii)}$$

The rate constants, k_1 and k_2 can be obtained from equations (iv) and (viii). From the concentrations of AMP before and ~~after~~ the electrolysis for a duration t,a and a-x, respectively, we can obtain the numerical value of k_1. By substituting this k_1 into the equation (viii), and from the concentration of ADP, x-y, produced after the electrolysis, k_2 can be determined.

The following are results of the cathodic reduction experiments conducted under very different conditions:

Experiment	t(hours)	Cathodic potential(volts)	k_2/k_1
1	40	-0.92	91.2
2	28	-0.21	130.9
3	18	-0.09	73.0

[AMP]=1.1 x 10^{-3}M; imidazolium hydrogen phosphate saturated in DMF.

These results show that ADP is phosphorylated at a rate approximately one hundred times faster than AMP phosphorylation. On the basis of these observations one may conclude that nature adopts reaction (x) rather than reaction (ix) to store chemical energy because of kinetic reasons.

$$AMP + P_i \longrightarrow ADP + H_2O \qquad (ix)$$

$$ADP + P_i \longrightarrow ATP + H_2O \qquad (x)$$

II.4 Mechanism of the phosphorylation of adenine nucleotide.

Reduction of adenine derivatives by electrochemical means has been investigated by many previous workers. Most of the earlier investigators examined the reactions in a strongly acidic medium.[235-6] Under such conditions adenine derivatives give a reduction half-wave potential of approximately -1.0 volt at a dropping mercury electrode. The reduction was accompanied by the evolution of ammonia, opening of the adenine ring and formation of imidazole derivatives as the final products.

More recently the electrochemical reduction reaction was studied at pH values close to neutral.[236-7] The reduction was performed at very low cathode potentials near -1.8 volts. Two products were identified: a single-electron reduction product with anodic half-wave potential of 0.13 volts and an absorption band at 295 nm, and a two-electron reduction product. The single electron reduction species is oxidized readily in the air and the two electron reduction product is resistant to aerobic oxidation.

Studies by Makarov and co-workers on the buffer capacity of the amino group and the phosphate group in AMP and ADP before and after electrolysis provided some significant information concerning the nature of the molecules at different reduction stages.[238] The basicity of the amino groups was increased by the reduction, shifting its pK_a value from 3.95 to 9.35. The increase in the basicity caused the amino group to interact more strongly with the terminal phosphate of the nucleotides or free inorganic phosphate when this was also present in the solution. Aerobic oxidation of the reduced ADP in the presence of free phosphate led to the formation of small quantities of ATP. Because the reduction was conducted at a fixed potential of -1.85 volts, there was no definite conclusion as to which reduced form leads to ATP formation.[238] The low ATP yields might be caused by the hydrolysis of some intermediate as the reaction was carried out in aqueous solution.

Another important work which provides information on the conformational differences among AMP, ADP and ATP was done by studying the optical rotatory dispersion of these molecules. Levedahl and James obtained results which are consistent with the view that the folded structure is most pronounced in the order:[239]

ATP > ADP > AMP

The study also suggests that the amino group in ADP and ATP intereact strongly with the terminal phosphate, while no such interaction was observed in AMP. The data further suggests that, in ATP there is an additional interaction between the adenine ring and the pyrophosphate chain. The folded structure of ATP was also confirmed by X-ray analysis.[241] Quantum mechanical calculations showed a folded structure has a much lower energy than the extended form.[242]

According to Lebedeva et al.,[237] the electrochemical reduction of ADP at the cathode begins at -1.7 volts (normal calomel electrode as the reference). A polarographic study by the same author of the reduction products shows an anodic wave at half-wave potential around 0.13 volts (normal hydrogen electrode as the reference) which is assigned to the redox property of a one-electron reduced species. No explanation of the disparity between those data was given. The medium used in the study was water.

Figure 7, indicates that the phosphorylation reaction starts at the cathodic potential of 0.05 volts in DMF solution. The lower the potential, the higher the yield of the product, ADP. Closeness of this potential (0.05 volts) to the anodic half-wave potential (0.13 volts) obtained by the previous investigators strongly suggests that the phosphorylation reaction may be initiated by one-electron reduction of AMP or ADP. In addition, comparison of the concentration of AMP before and after electrolysis indicated that AMP in

Figure 8. Concentration changes of AMP in the cathodic and Anodic cells before and after electrolysis.

Original Concentration of AMP: 1.1×10^{-3} M

Concentration of diimidazolium hydrogen phosphate: 3.3×10^{-3} M.

Solvent: N,N-dimethylformamide.

(1) before electrolysis.

(2) AMP in the cathodic cell.

(3) AMP in the anodic cell.

Duration of electrolysis: 20 hours

Currents during the electrolysis: 2.0-10.0 microamperes.

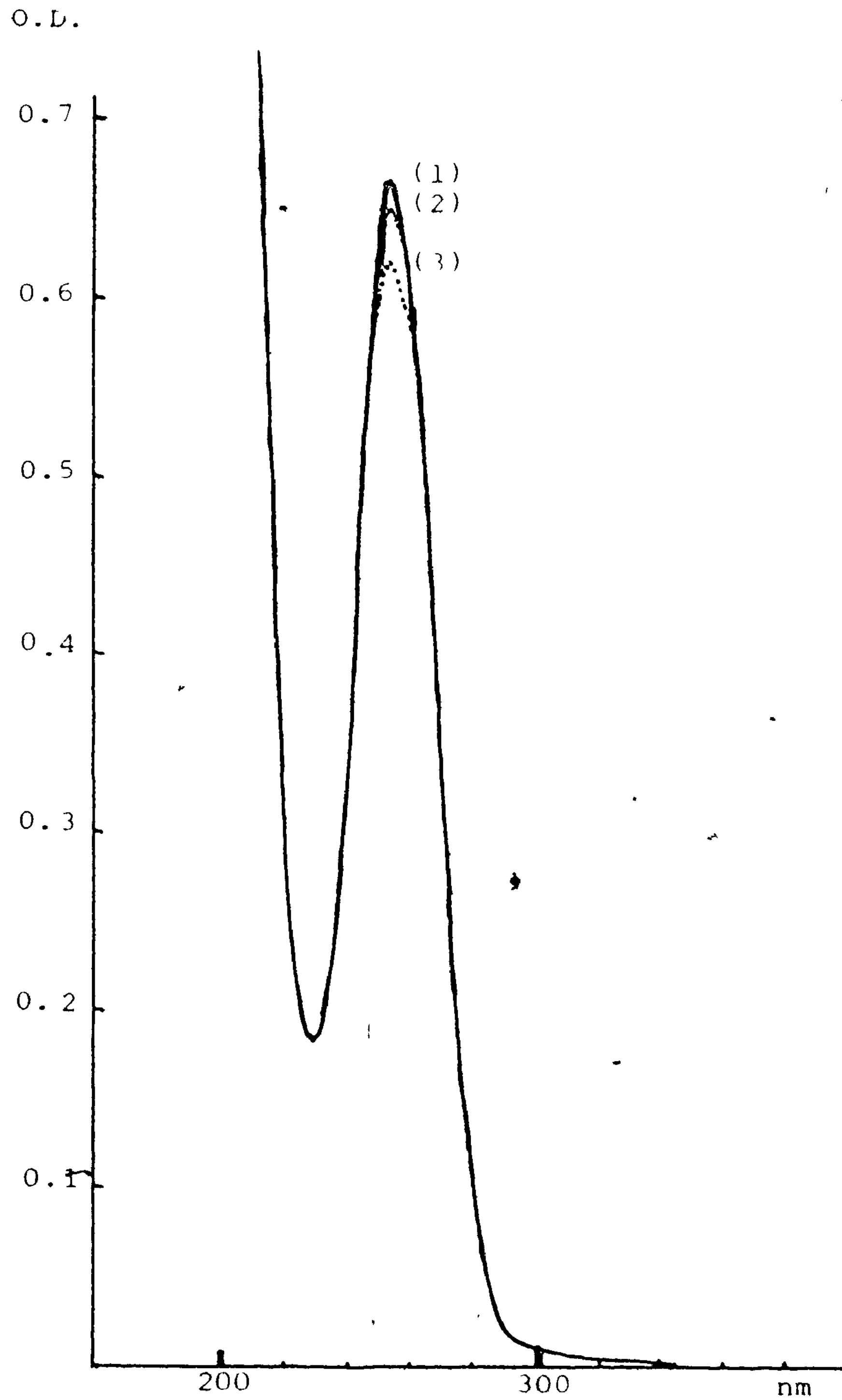


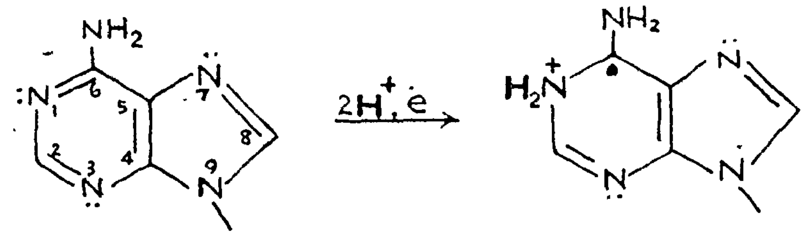
Figure 8

the cathodic cell started to decrease as the potential went below 0.05 volts. The experimental results are depicted in Figure 8.

AMP, ADP and ATP concentrations in DMF or DMAC solutions were determined by a combination of the ion-exchange method of Cohn and Carter[232] and spectrophotometry. For the anion-exchange column AG1-X2 (200-400 mesh from Bio-Rad Lab.) made into a bed-size of 1.5 cm x 1 cm^2 was used. The column was first washed with 100 ml of 0.01 M ammonium chloride. Then 4 ml of a solution containing the mixture of AMP, ADP and ATP was mixed with 50 ml of 0.01 M ammonium chloride and carefully pipetted onto the column which was allowed to drain. The column was then washed with 100 ml of 0.01 M ammonium chloride solution to remove the organic solvent used. Flow rates between 3.0 to 3.5 ml per minute were found adequate for the separation of the three adenine nucleotides. For the elution of AMP 100 ml of 0.003 N HCl was used. For 1.1×10^{-3} M of AMP in 4 ml DMF solution, approximately 60 ml of the eluent was sufficient to remove all AMP form the column. After the AMP was separated ADP and ATP was respectively eluted out by using 100 ml of 0.01 N HCl plus 0.02 M NaCl, and 100 ml of 0.01 N HCl plus 0.2 M NaCl. Approximately 70 ml each of the eluent was required to remove the corresponding nucleotide.

When the adenine of AMP (or ADP) is one-electron reduced, the amino group may increase its basicity owing to the loss of aromatic character in the ring. An ESR study of purine derivatives showed that when they are one-electron reduced to their radical forms, the single electron localizes predominantly at the 6 position of the ring with a large hyperfine splitting constant by which the spin density at this position is estimated to be near

50%[240]. This indicates that the nodal plane, formed by π electrons of the aromatic ring, may have disappeared because of the reduction.



The kinetic advantage in phsophorylating ADP over AMP is likely due to the conformational factor. The bound phosphate at the amino group may easily attach to the terminal phosphate of ADP if the latter is also close to the same group. If the terminal phosphate of AMP is located far from the amino group as was suggested by Levedahl and James,[239] condensation between the bound and the terminal phosphates may be more difficult.

As is shown by Figure 7, the presence of oxygen is necessary for the phosphorylation process. Oxygen may function as the "liberator" of the terminal phosphate of the product from being bound to the amino group. Removal of the unpaired electron from the radical intermediate will bring back the aromaticity of the ring and reduce the basic strength of the amino group.

A proposed mechanism for ATP formation in an anodic cell will be discussed in the following chapter.

In figure 9, the transient intermediate, II, has captured the phosphate in a trigonal bipyramid structure. According to Westheimer, nucleophilic substitution reactions at phosphate centres are generally recognized as proceeding by way of pentavalent transitional intermediates, having a trigonal bipyramidal geometry.

Figure 9. Proposed molecular mechanism of ATP formation from ADP plus inorganic phosphate.

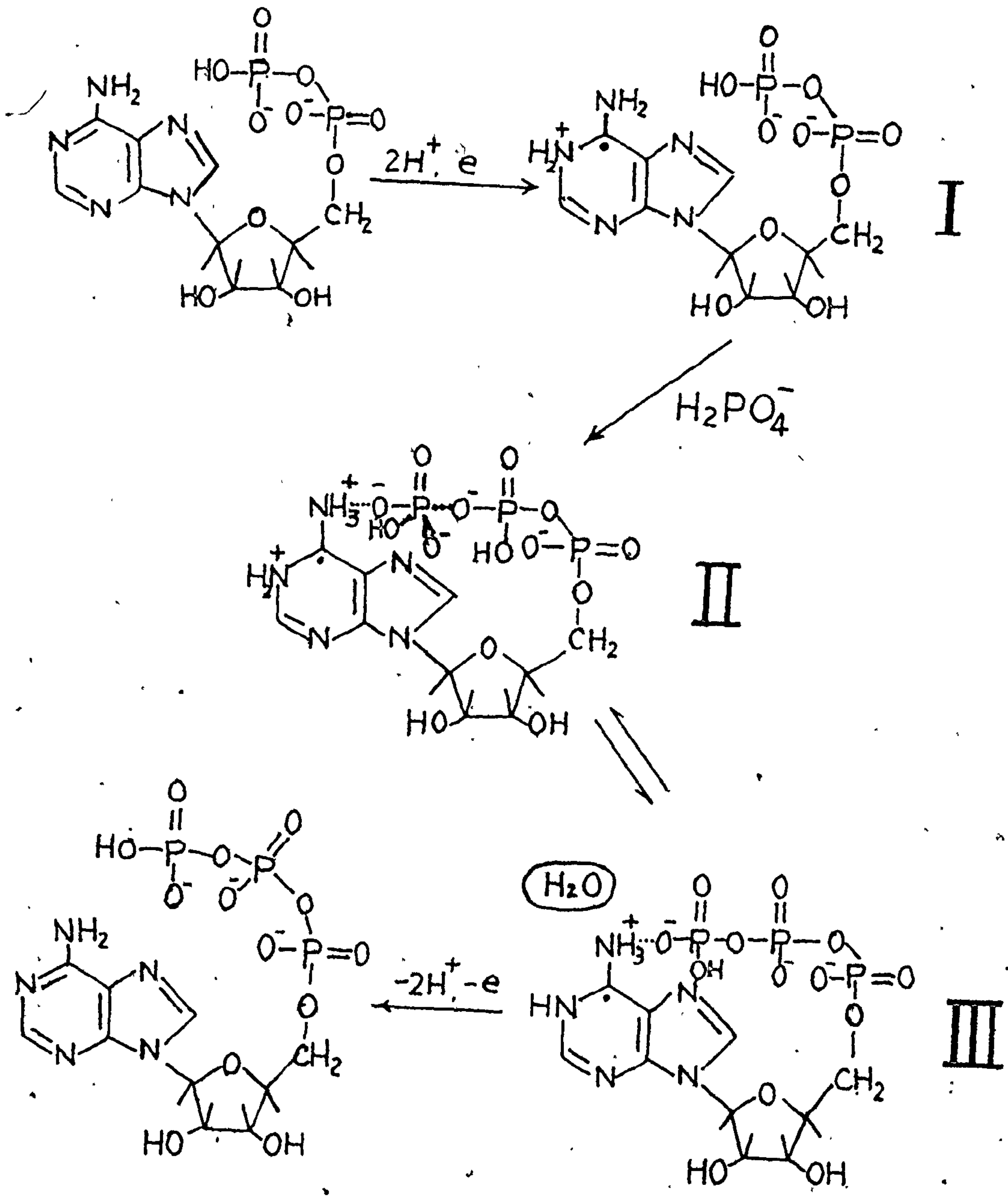


Figure 9.

CHAPTER III

ATP FORMATION BY THE ADDITION OF SIMPLE REDUCTANTS OR OXIDANTS

In the previous chapter, it was shown that the phosphorylation of an adenine nucleotide can be initiated either by reducing the adenine part of the nucleotide or by oxidizing an inorganic phosphate, both electrochemically. In this chapter, it will be shown that the reduction or the oxidation can be caused by chemical means, that is, by adding a proper, simple reductant or an oxidant. In addition a case will be discussed which the oxidant is indirectly generated *in situ* rather than added, such as with the generation of delta singlet oxygen. In biological systems, the existence of this strongly oxidizing excited dioxygen is well documented.[244-6]

III. 1 ATP Formation Associated With Reductants

A. Phosphorylation Involving the Reduction of AMP by Sodium Borohydride

Sodium borohydride is widely used in the reduction of numerous organic compounds. This stable reductant seems to act as a one-electron and a two electron reducing agent. As will be shown in the following two Chapters, it reduces various kinds of ferri-heme complexes and quinones. In aqueous solution the reduction potential of sodium borohydride is dependent on the pH value of the medium. The reduction potential is reported to be -0.43 volts in acid solution and -1.37 volts in basic solution.[247] The reducing power may be greatly enhanced in aprotic solvents.[248]

Evidence of AMP reduction by sodium borohydride is presented in Figure 10. As is expected from the observations of previous investigators,[237] two or more reduced products are formed. One of the products which has an absorption maximum at 310 nm in DMF is readily oxidized in air. This reduced species is oxidized by air back to AMP. The oxidation of this labile species

Figure 10. Reduction of AMP by sodium borohydride in N,N-dimethylformamide.

AMP concentration: 1.2×10^{-3} M.

(1) Before the sodium borohydride is added

(2) 25 seconds after the addition (in air).

(3) 2 minutes after the addition.

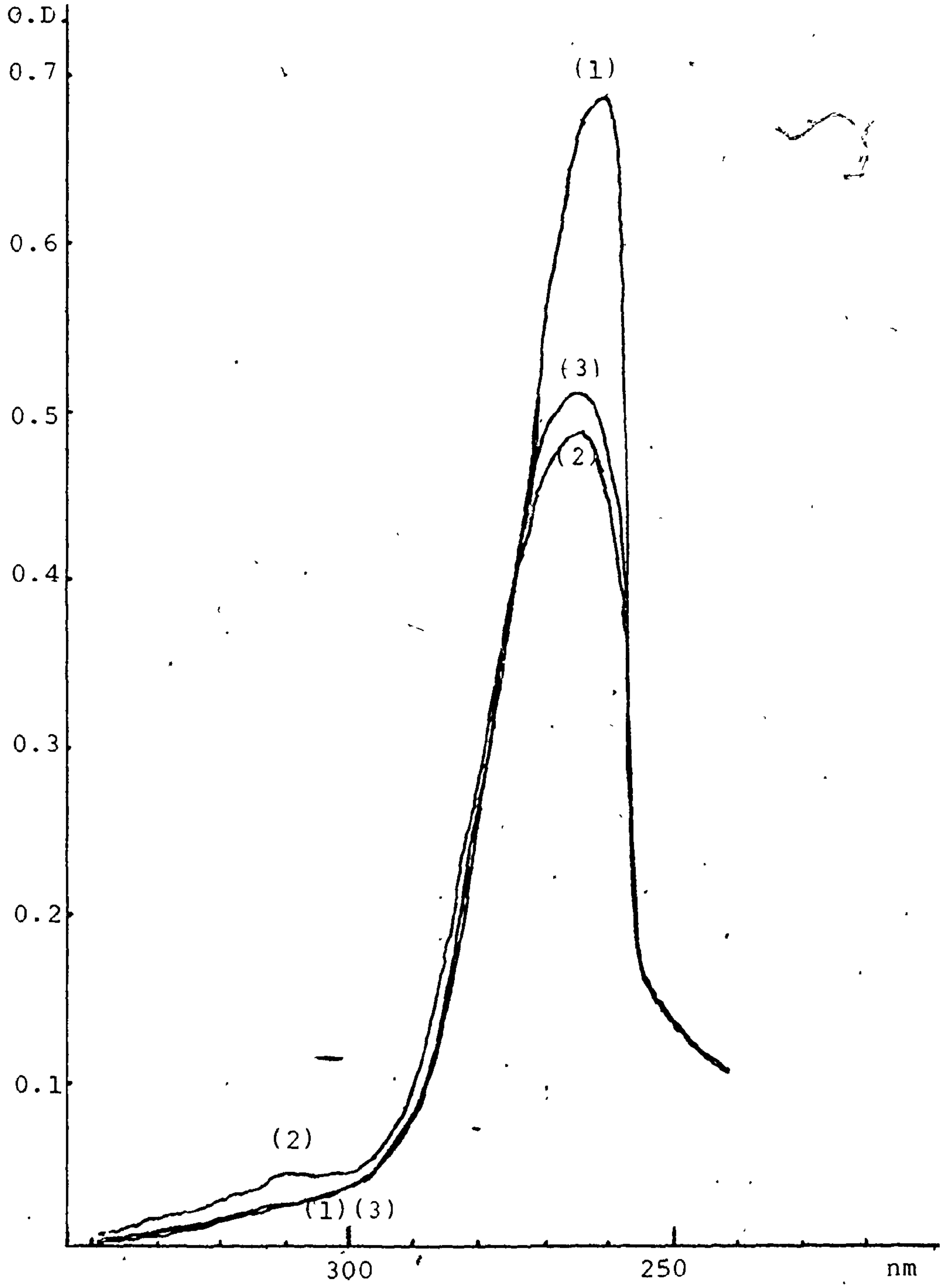


Figure 10

does not result in the total recovery of AMP as is clearly indicated by the bands at 258 nm where AMP absorbs. Even after one hour the band still shows less than half of the total recovery of AMP. This suggests that there must be other product(s) which are resistant to aerobic oxidation.

According to the previous investigators, the labile species from ADP reduction is a one-electron reduced radical ion which shows a broad absorption band at 295 nm.[237,249] The systems they studied were either in an aqueous or a water-ethanol solution. It is reasonable to assume that the species responsible for the band at 310 nm is the corresponding one-electron reduced AMP radical ion by noticing the similar characteristics between the two. From the conclusion given in Chapter II, production of the AMP radical will lead to its phosphorylation into ADP and ATP when an inorganic phosphate is available.

Figure 11 shows some analytical results of the phosphorylation reactions. All nucleotide concentrations were determined spectrophotometrically after separation using the ion-exchange method described in Chapter II. It was clearly shown that, after a reaction time of two hours the yield of ATP was 30% higher than that of ADP, even though the unreacted AMP is the major component present in solution. This observation is similar to one made during cathodic reduction, as was described in Chapter II.

B. <u>Phosphorylation Involving the Reduction of AMP by Metals</u>

It was discovered accidentally that when a nickel-stainless steel spatula or syringe needle was placed into a DMAC solution containing diimidazolium hydrogen phosphate, electron spin resonance signals were observed at low temperatures. The shape of the signals depends on how long the metals

Figure 11. Formation of ADP and ATP by the reduction of AMP with sodium borohydride in the presence of a phosphate in DMF solution.

Starting concentration of AMP: 1.15×10^{-3} M.

Concentrations are analyzed two hours after the reductant is added. (1) Baseline. (2) Concentration of ADP. (3) Concentration of ATP. (4) Concentration of AMP (the actual concentration should be multiplied by 23.

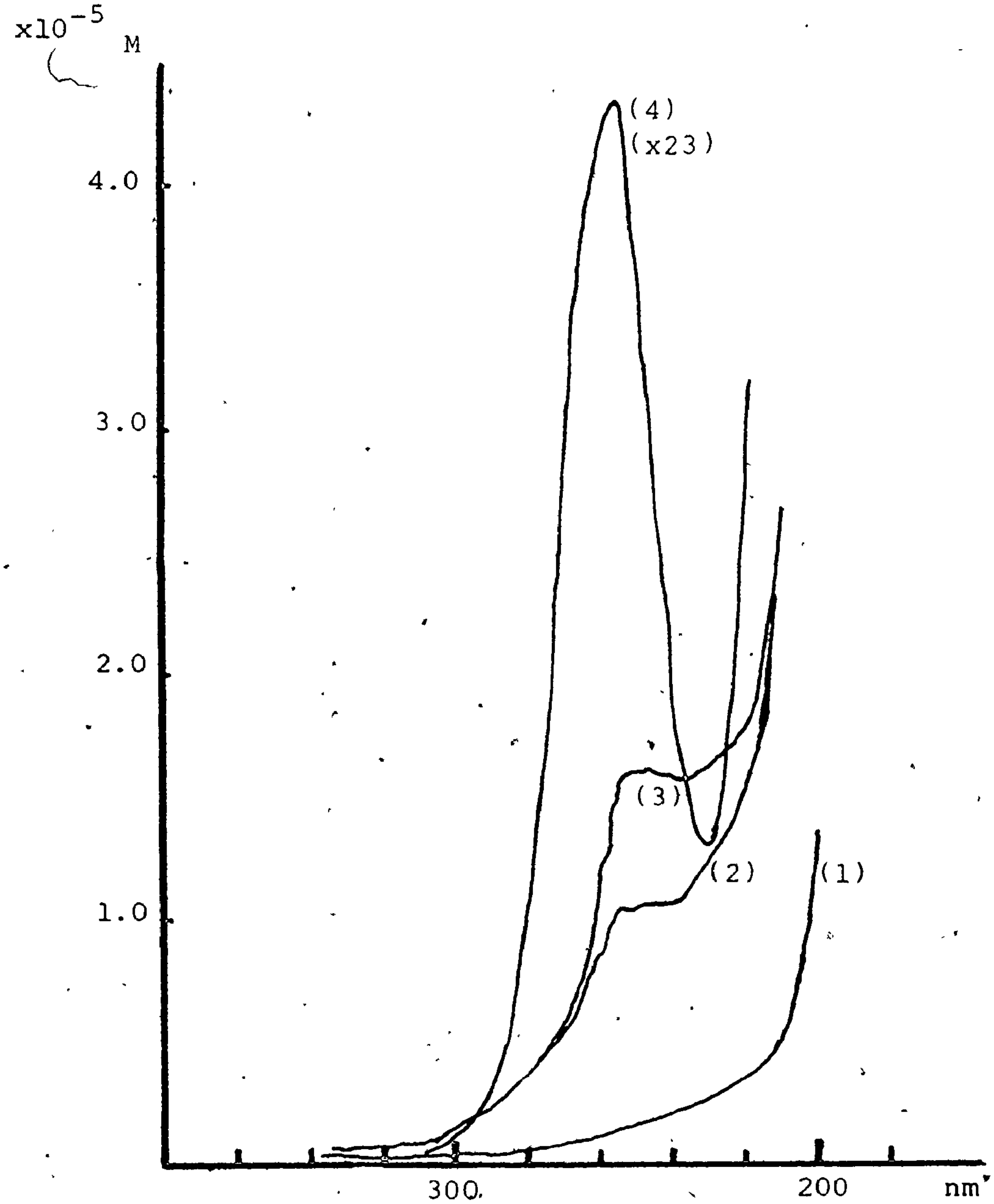


Figure 11

have been kept in the solution. As is shown in Figure 12, the signal obtained from a two day old solution exhibits hyperfine structure probably arising from imidazole nitrogens coordinating to the central metallic ion. The splitting constant is 14.3 gauss. In addition, judging from the shape of the signal of this imidazole-metal complex, the latter may possess a structure with an axial symmetry, with $g_{1} = 2.054$ and $g_{11} = 5.659$. No further study of the detailed structure of the complex was attempted.

The essential point of the aforementioned observation is that the oxidation number of the complexed metal has changed from zero to some positive integer by forming the ESR-detectable complex from the metallic state. This means that the reaction between the metal(s) and the solute involves an oxidation step for the metal.

Even the nature of the reaction is not known, a component in the nickel-stainless steel may serve as a reducing agent in the presence of imidazole. Such an electron-donating system may provide electrons to reduce an adenine nucleotide (AMP or ADP) and produce higher pyrophosphate compounds in the presence of phosphate. The phosphorylation reaction was actually observed.

In a DMAC solution containing 1.1×10^{-3} M AMP and 2.8×10^{-3} M diimidazolyl hydrogen phosphate, a nickel-stainless steel spatula from Fisher Scientific Company was immersed. After two days, the concentrations of AMP, ADP and ATP were analyzed by means of the ion-exchange method described in Chapter II. Final results indicate that, after two days of reaction, the ADP concentration in the DMAC solution was 1.83×10^{-5} M, and the ATP concentration was 4.00×10^{-5}M.

Figure 12. ESR spectra of reaction products between imidazole and nickel-stainless steel in DMAC glass matrix.

(A) Spectrum taken immediately after the reaction.

(B) Spectrum taken 10 hours after the reaction.

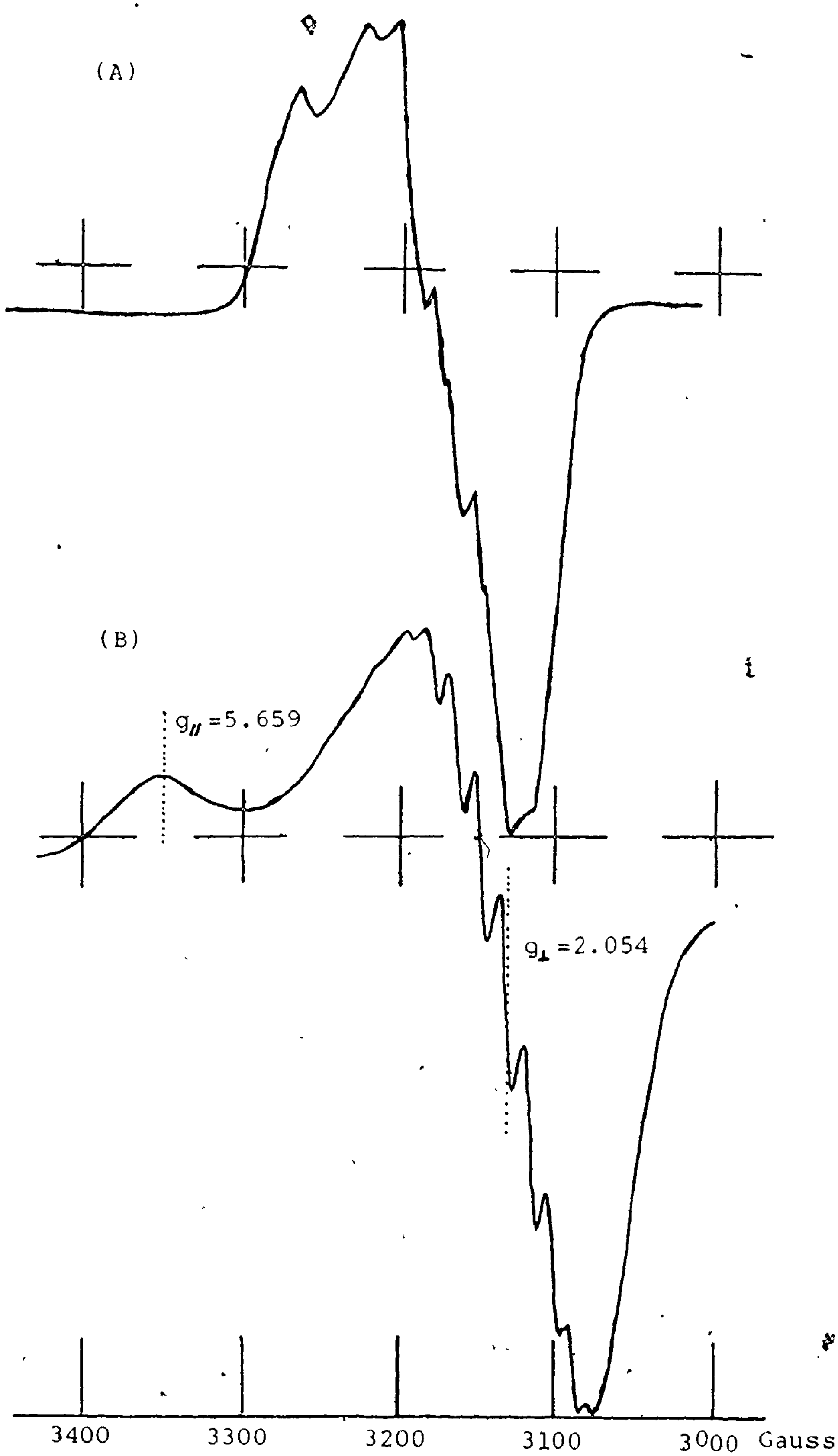


Figure 12

In a separate experiment, pure iron wire was used instead of the nickel-stainless steel spatula. The ADP concentration found in DMAC solution was 1.52×10^{-5} M.

C. Photophosphorylation Catalyzed by Some Semiconductors

Recently, Fan, Chiang and Chien discovered that ATP can be produced by irradiating semiconductors, such as zinc oxide and cadmium sulfide, in an aqueous medium containing ADP and inorganic phosphate.[250-2] No molecular mechanism was presented for the interesting reactions. In view of the fact that NADP can be photoreduced to NADPH by the semiconductors,[250-2] the phosphorylation reaction is likely initiated by photoreduction of ADP, as was proposed in Chapter II. The redox potential of the NADP-NADPH pair is: $E_0' = -0.32$ volts,[253] therefore the photo-activated semiconductors have potentials low enough to act as the reducing agents for ADP.

Photo-activation of a semiconductor involves the excitation of an electron from the valence band to the conduction band of the semiconductor. If the energy level of an electron acceptor is below that of the conduction band, then electron transfer can occur from the semiconductor to the acceptor. Similarly, if the energy level of an electron donor is above that of the valence band, electron transfer to the positive hole can occur. In fact, both photo-catalytic reactions have been demonstrated.[254] According to these authors, the conduction band of zinc oxide and cadmium sulfide has redox potential, -0.4 volts and -0.7 volts, respectively, with reference to the normal hydrogen electrode. Hence, photo-catalytic reduction of ADP is possible by the two semiconductors. Further experimentation is necessary to confirm the reduction of ADP.

III. 2 ATP Formation Associated With Oxidants

A. Phosphorylation Associated with the Oxidation by Sodium Hypochlorite

In Chapter II it was shown that anodic oxidation of the mixture of AMP and inorganic phosphate in DMF or DMAC solution results in the production of ADP, if the anodic potentials are equal to or greater than +0.85 volts. It is proposed that the phosphorylation is initiated by the oxidation of the phosphate, rather than the adenine moiety of AMP.

Sodium hypochlorite is a strong oxidizing agent. Its redox potential in basic and acidic aqueous media is +0.90 volts and +1.49 volts, respectively,[256] Hence this compound was selected to test whether it can be used to initiate the phosphorylation of adenine nucleotides or not.

Phosphorylation reactions were induced by the addition of sodium hypochlorite to a solution of AMP in DMAC (Figure 13- (A) and (B). Here again, phosphorylation products were analyzed by the column ion exchange method, as described in Chapter II.

It should be noted that, when AMP was used as the phosphate acceptor, ADP is the major product. Only very little ATP is produced. Such a distribution of products is very different from the case when the phosphorylation reactions are initiated by the addition of a reductant or by the cathodic reduction. In the latter case the particular conformational factor of ADP causes the rate of its phosphorylation to be much faster than that of AMP through the reduction of the adenine ring, while in the former case, in which the reaction is thought as initiated by the oxidation of the inorganic phosphate, the activated phosphate may react indiscriminately with any major adenine nuclectide present. Hence, in this oxidative process the major phosphorylation product of AMP would be ADP.

Another point which should be mentioned is that if the terminal phosphate of AMP or ADP is also activated by the oxidation, there may be additional phosphorylation products formed, depending on the pairs which constitute the pyrophosphate linkages. For example, products such as, AMP-AMP, ADP-ADP, etc., may also be produced. The column ion exchange method is less specific than the enzymatic method in identifying a chemical species. If products such as AMP-AMP and ADP have similar ion exchange characteristics, they cannot be distinguished by the present method. They are expected to have very similar absorption bands in ultra-violet region, since their chromatophores are at the adenine ring. To determine whether the additional pyrophosphates are also produced, one should cross-check the products by other means, such as enzymatic analysis.

B. Phosphorylation Associated with the Oxidation by Halogens

The reduction potential of Cl_2/Cl^-, Br_2/Br^- and I_2/I^- pairs is: +1.3583 volts, +1.087 volts and +0.535 volts, respectively.[256] Hence halogens should be effective oxidants to test the proposal made in Chapter II that reagents with potentials higher than +0.85 volts may induce a phosphorylation reaction, unless there are some other side reactions to hinder the reaction.

Preliminary tests show that chlorine gas reacts very rapidly with adenine and AMP, ADP, ATP, when the chlorine is passed into DMF solutions of the nucleotides. The characteristic absorption bands around 260 nm due to the presence of the adenine ring diminish rapidly when chlorine is present. However, adenine is much more resistant to bromine and iodine. Therefore, these

Figure 13. Phosphorylation of adenine nucleotides by the addition of sodium hypochlorite.

(A) 4 ml of 1.10×10^{-3} M AMP plus 2.80×10^{-3} M diimidazolium hydrogen phosphate in DMAC solution is treated with 1 ml of 12% NaOCl aqueous solution. Reaction time is one minute.

(B) 4 ml of 2.25×10^{-3} disodium salt of ADP plus 2.01×10^{-2} M disodium hydrogen phosphate in water is treated with 1 ml of 12% NaOCl aqueous solution. Reaction time is five minutes.

Concentrations of the adenine nucleotides are determined by the absorption band intensities at 259 nm, after each component has been separated by the ion exchange method.

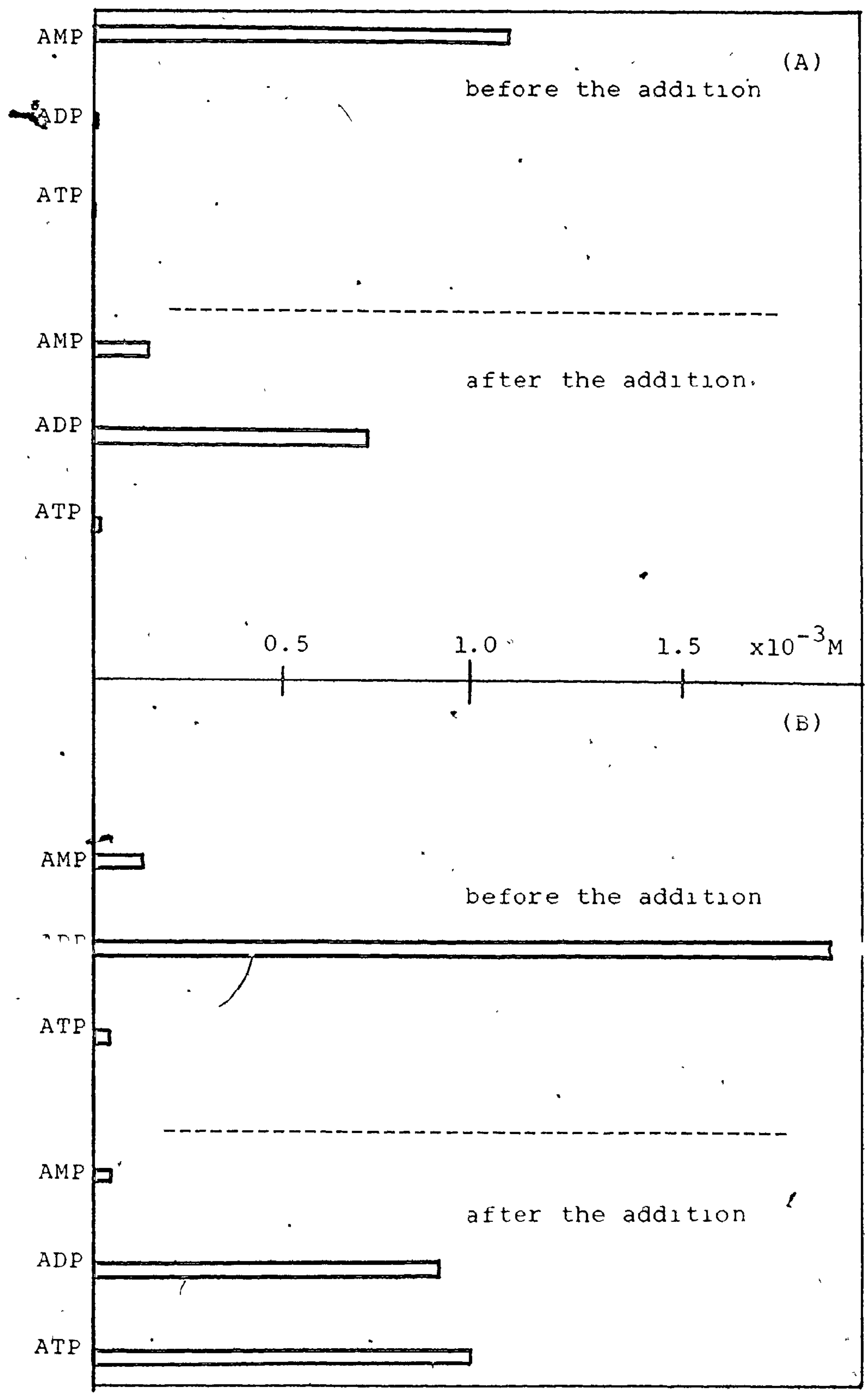

AMP
ADP
ATP
(A)
before the addition
AMP
ADP
ATP
after the addition
0.5
1.0
1.5
x10^{-3}M
(B)
AMP
ADP
ATP
before the addition
AMP
ADP
ATP
after the addition

Figure 13

two halogens were selected as oxidants for the phosphorylation experiments.

As expected, bromine is effective in initiating the phosphorylation reaction whereas iodine is not. Experimental results are summarized in Figure 14.

Previous investigators studied the oxidative phosphorylation reaction of bromine in the presence of some sulfur compounds.[257-9] According to Wieland and Bäuerlein, ATP is produced from ADP plus inorganic phosphate when a simple thiol, such as thioglycolic acid, is oxidized by bromine under anhydrous conditions (dry pyridine was used).[257-8] The following reaction mechanism was suggested:

$$RSH + Br_2 \longrightarrow Br^- + H^+ + RSBr \quad \text{(sulfenylbromide)}$$

$$RSBr + H_2PO_4^- \longrightarrow Br^- + RSOPO_3H_2 \quad \text{(sulfenic phosphoric anhydride)}$$

$$RSOPO_3H_2 + ADP^- \longrightarrow ATP + RSO^- \quad \text{(sulfenate)}$$

$$RSO^- + Br_2 \text{ (excess)} \longrightarrow Br^- + RS{\lessgtr}^{O}_{Br} \quad \text{(sulfinylbromide)}$$

$$RS{\lessgtr}^{O}_{Br} + H_2PO_4^- \longrightarrow Br^- + RS{\lessgtr}^{O}_{OPO_3H_2} \quad \text{(sulfinyl phosphate)}$$

$$RS{\lessgtr}^{O}_{OPO_3H_2} + ADP \longrightarrow ATP + RSO_2^- \quad \text{(sulfinate)}$$

Instead of the thiol compounds, Lambeth and Lardy used thio-ethers.[259] Again in dry pyridine solutions, AMP was phosphorylated into ADP by inorganic phosphate when a thioether and bromine were added. The following reaction mechanism was suggested:

$$\begin{matrix} R' \\ | \\ :S: \\ | \\ R \end{matrix} + Br_2 \longrightarrow Br^- + \begin{matrix} R' \\ | \\ R-S:^{+} \\ | \\ Br \end{matrix} \quad \text{(bromosulfonium ion)}$$

Figure 14. Phosphorylation of AMP by the addition of halogens in the presence of phosphate in DMAC solutions.

1———— 1.1×10^{-3} M AMP plus 2.8×10^{-3} M diimidazolium hydrogen phosphate in DMAC.

2———— 7 mg of liquid bromine was added to 4 ml of solution 1. Analysis of the products was made 40 minutes later.

3———— 11.1 mg of iodine was added to 4 ml of solution 1. Analysis of the products was made 40 minutes later.

Ion exchange method described in Chapter II was used for the analysis of AMP, ADP and ATP.

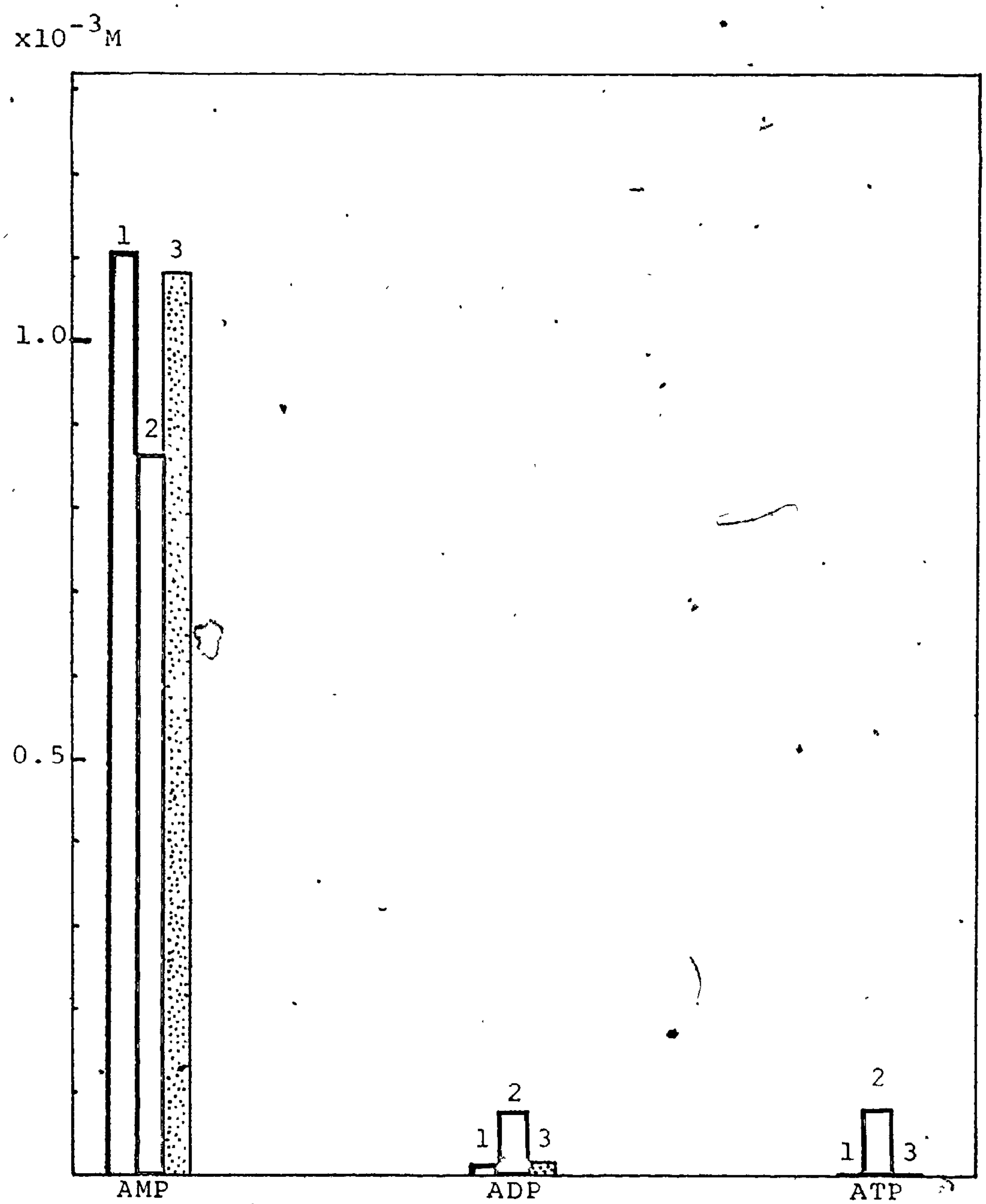
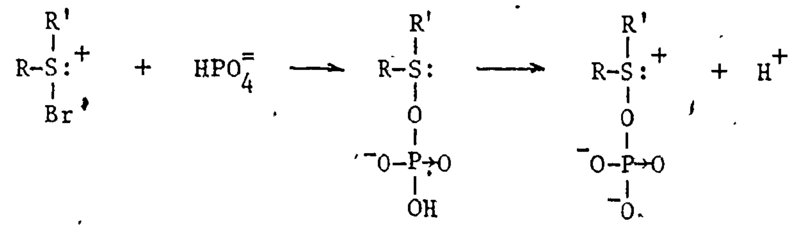
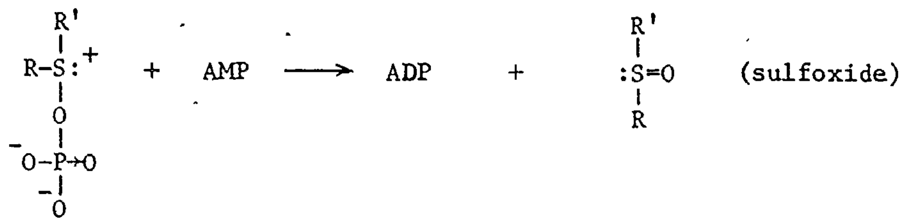


Figure 14





Oxidative phosphorylation reactions can occur in the absence of any sulfur compound as is observed in the present work. In a pyridine solution Wieland and Bäuerlein also observed some ATP production in the absence of the thiol when the bromine concentration was increased[257]. But the mechanisms suggested by the previous investigators only involved oxidized sulfur compounds as the phosphate-activating agents.[257-9] It is probable that the phosphate-activation may also be achieved by a direct oxidation of the phosphate by bromine in pyridine, DMF or DMAC. The phosphate group of AMP may also be oxidized. In this case, various pyrophosphates may be produced. More systematic experiments are necessary to clarify the mechanistic problems.

C. Phosphorylation Associated with the Oxidation by Delta Singlet Oxygen

Oxygen has sixteen electrons. The ground state electronic configuration for O_2 may be represented by:

$$KK(\sigma_g 2s)^2(\sigma_u^* 2s)^2(\sigma_g 2p)^2(\pi_u 2p)^4(\pi_g^* 2p)^2$$

Since the highest occupied orbital does not correspond to a closed shell, several terms are associated with this configuration. These terms correspond to different orbital and spin arrangements of the two π_g^* electrons.

Homonuclear diatomic molecules such as dioxygen belong to the point group, $D_{\infty h}$. Based on the character table of this symmetry group, since the π_g^* orbitals transform as the irreducible representation E_{1g} in the symmetry operations,[260] we obtain the direct product of the E_{1g} representation as follows:

$$E_{1g} \times E_{1g} = A_{1g} + E_{2g} + A_{2g},$$

or, expressed in their corresponding term representations:

$$\pi_g \times \pi_g = \Sigma_g^+ + \Delta_g + \Sigma_g^-.$$

The orbital functions represented by the first two terms are symmetric with respect to the exchange of the two electrons, while that represented by the last term is antisymmetric.[261] Since, by the Pauli principle, the overall function must be antisymmetric with respect to the electron exchange, the correct combinations of orbital and spin functions give $^1\Sigma_g^+$, $^1\Delta_g$ and $^3\Sigma_g^-$.

Those three state terms arise from the same electronic configuration. However, by Hund's rules the triplet has the lowest energy; of the two singlets, the one with the larger value of Λ has the lower energy. The energy level of the states is therefore,

$$^3\Sigma_g^- < {}^1\Delta_g < {}^1\Sigma_g^+$$

with experimental energy separations:[262]

$$E(^1\Delta_g) - E(^3\Sigma_g^-) = 0.98 \text{ eV } (22.6 \text{ Kcal/Mole})$$
$$E(^1\Sigma_g^+) - E(^3\Sigma_g^-) = 1.63 \text{ eV } (37.6 \text{ Kcal/Mole}).$$

Based on the data shown above one expects that the dioxygen in the two singlet excited states, $^1\Delta_g$ and $^1\Sigma_g^+$, will have a redox potential approximately 1.0 volt and 1.6 volts higher respectively than the normal dioxygen. This assumes a thermal equilibrium has been reached during the lifetime of the excited states for the redox pair O_2/O_2^-. Concerning the lifetime, since the transition between these two singlet excited states and the triplet ground state is both symmetry- and spin-forbidden, the two excited states must be long-lived. Experimentally determined lifetimes are 45 minutes for the $^1\Delta_g$ state and 7.1 seconds for the $^1\Sigma_g^+$ state at zero pressure.[263-4] The lifetimes in solutions for the singlet states become drastically shortened, with estimates of 10^{-3} to 10^{-6} seconds for the $^1\Delta_g$ state and 10^{-9} to 10^{-11} seconds for $^1\Sigma_g^+$ state.[264-6] It is generally accepted that the latter singlet state is easily quenched, undergoing a non-radiative transition to give the former singlet state. Hence, reactions involving dioxygen excited states are predominately caused by the $^1\Delta_g$ state.[261,266]

One of the simplest means to generate the singlet dioxygen is by the disproportionation reaction of superoxide anion in the presence of a proton source.[267-9] Khan observed the formation of the singlet dioxygen by adding water to a DMSO solution of potassium superoxide.[267] Mayeda and Bard produced the superoxide anion electrochemically in acetonitrile solution and detected the singlet dioxygen from its reaction products as well as from its fluorescence spectrum.[269]

In the present experiment, sodium or potassium superoxide is used as the singlet dioxygen generating material. Stability and relative solubilities of the superoxides in various non-aqueous solvents were first checked by observing the behavior of the electron spin resonance signal of the superoxide anion radical in those solvents. Under very dry conditions, the ESR signal intensities and their lifetimes indicate that the stability of the superoxide anion radical in the non-aqueous solvents studied can be ranked in the following order:

DMAC >> DMF > DMSO ≈ Acetonitrile.

In dry DMAC solution, the superoxide anion radical can survive for a long time (Figures 15,16). For a potassium superoxide solution, a change in its low temperature ESR signal intensity is not detected even after the sample has been thawed and kept at room temperature for 15 minutes. The stability of the superoxide anion radical is most likely due to the repulsive force operating between anionic charges. Disproportionation reaction of the following kind may thus be prevented:

$$O_2^{-\cdot} + O_2^{-\cdot} \longrightarrow O_2 + O_2^{=} \qquad (1)$$

However, when water or an acid is added to the system, the ESR signal disappears immediately because of the following disproportionation reaction:

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \longrightarrow O_2 + H_2O_2$$

or

$$O_2^{-} + HO_2^{\cdot} + H^+ \longrightarrow O_2 + H_2O_2 \qquad (2)$$

and accroding to previous investigators,[267-9] a part of dioxygen generated in the equation (2) is delta singlet dioxygen.

To test whether the generated, highly oxidizing delta singlet dioxygen can induce a phosphorylation reaction, 0.5 ml of deuterium oxide was injected into 4 ml of DMAC solution containing 1.1×10^{-3} M of AMP, 2.8×10^{-3} M of diimidazolium hydrogen phosphate and 2 mg of potassium superoxide. Repeated experiments under similar conditions have shown that, approximately 2.1 to to 2.4% of the AMP was converted into ADP when the products were analyzed by the ion exchange method described in Chapter II. If β-carotene was added to the reaction mexture, no ADP production was observed. This experiment strongly suggests that the phosphorylation of the AMP is indeed caused by the delta singlet dioxygen generated by the introduction of deuterium oxide to the superoxide containing system. β-Carotene is known as the most efficient quencher for the singlet dioxygen.[266] In all the experiments, deuterium oxide instead of water was injected because it is known that the delta singlet oxygen can servive in deuterium oxide with a lifetime ten times longer than in water.[265] The lifetime in water is known to be the shortest among the solvents studied.[265] The relatively low yields of ADP observed under the present conditions may reflect a low concentration of the delta singlet dioxygen due to the quenching effect of deuterium oxide.

It is still premature to estimate the actual redox potential of the delta singlet dioxygen/superoxide anion pair by using currently available data. The following reasons are given:

(a) Even as a reference for the estimation, the redox potential of normal dioxygen/superoxide anion pair determined by previous investigators ranges from -0.07 volts to -0.59 volts.[270-3]

(b) Most of the redox potentials were determined in aqueous media in which the effect of solvation may be very different from that in non-aqueous solvents.

(c) Singlet dioxygen generated by various methods may form several kinds of dimers (dimols).[261,266] The physical and chemical nature of these dimers has not been studied extensively.

Figure 15. ESR spectrum of superoxide anion radical in anhydrous DMAC solution at -170°C.

Solute: Potassium superoxide.

Solvent: Commercially available DMAC first passed through a combined column of alumina and anhydrous magnesium phosphate, then dried over phosphorous pentoxide and vacuum-distilled into the ESR tube containing the solute.

Modulation amplitude: 0.8 x 10 gauss.

Modulation frequency: 100 Kc per second.

Microwave power: 3.2 milli-watts.

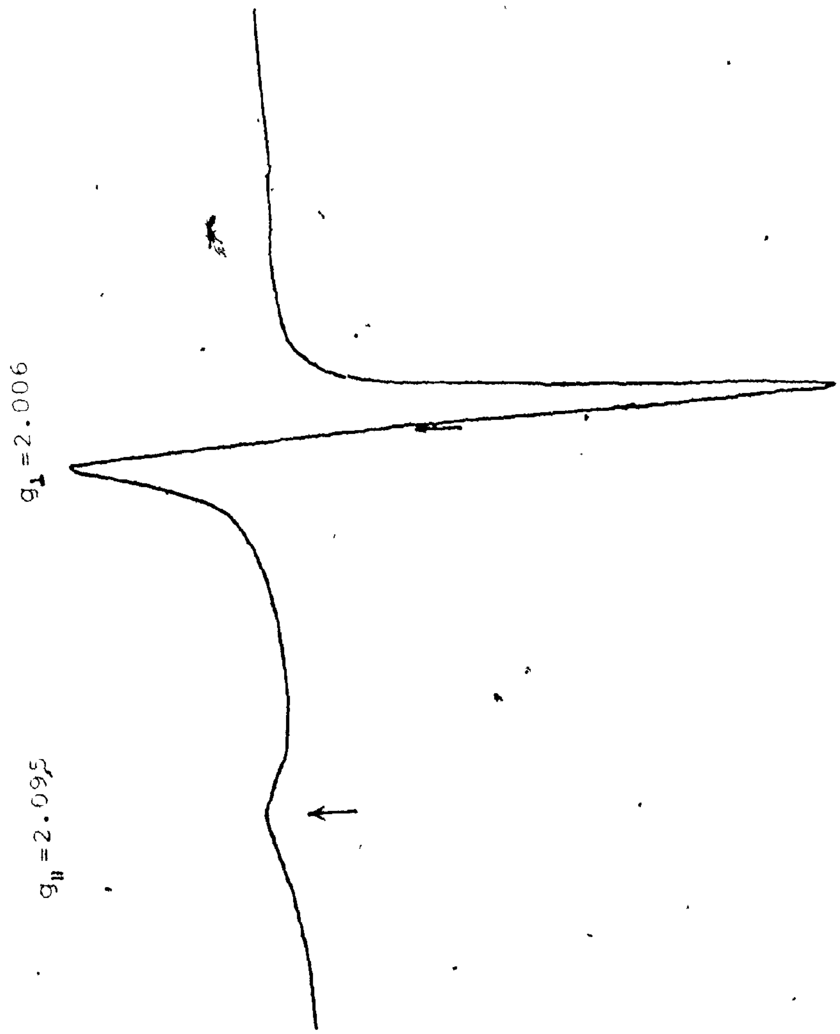


Figure 15

Figure 16. ESR spectrum of ^{17}O-labelled superoxide anion radical in anhydrous DMAC.[310]

Source of the superoxide anion: NaO_2 plus $Na^{17}O^{16}O$ is prepared in a vacuum line system by allowing 21% ^{17}O-labelled dioxygen to react with sodium mirror in a container at a controlled temperature of 10°C. Dry DMAC is vacuum-distilled into the container after the yellowish sodium superoxide is formed. The downward arrows indicate the resonance peaks of $^{16}O^{16}O^{-\cdot}$ for $g_{1}=2.006$ at ten times reduce d scale and for $g_{11}=2.095$ at full scale, respectively. The six upward arrows indicate the g_1 resonance peaks of $^{17}O^{16}O^{-\cdot}$ due to the interaction between the electronic magnetic moment with the nuclear magnetic moment of ^{17}O (nuclear spin=$(5/2)\hbar$; nuclear magnetic moment = -1.893 nuclear magneton). The six hyperfine structure lines are evenly spaced at 74 gauss intervals. The observed large hyperfine splittings indicate appreciable s-character of the unpaired electron in $O_2^{-\cdot}$.

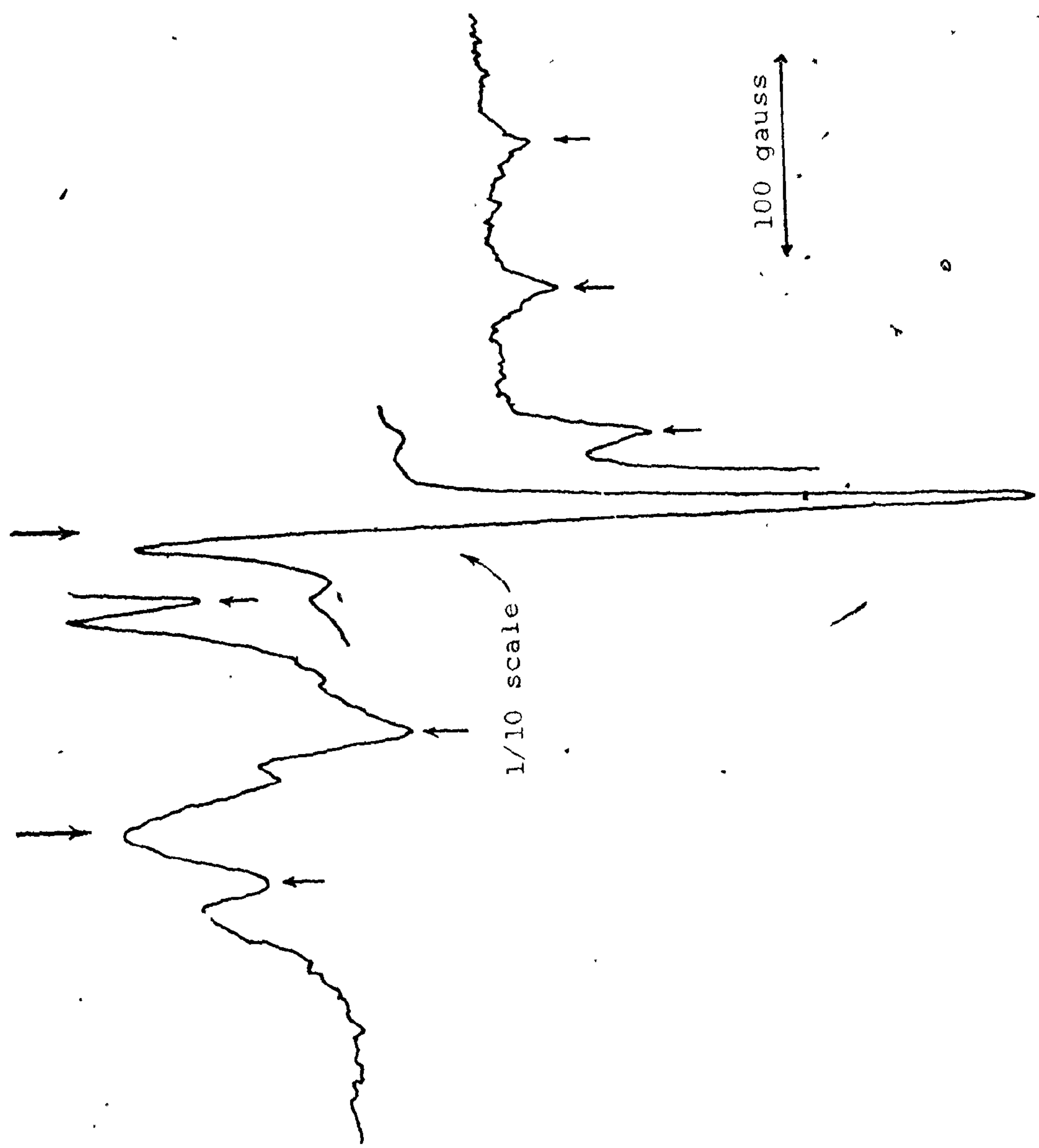


Figure 16

(d) It is not certain whether a thermal equilibrium is reached for the reaction involving the electronically excited state(s). Several possible vibrational excited states may also be involved for the reacting electronically excited species as well as for the product molecules.

At present, what we know about the delta singlet dioxygen is the fact that it possesses highly oxidizing chemical properties and an ever-increasing number of oxidized products generated by it are being discovered. The singlet dioxygen, generated by the superoxide anion disproportionation reaction in DMAC, can serve as a strong oxidant to produce ADP from AMP plus inorganic phosphate.

Generation of the delta singlet dioxygen from biologically-related molecules and its function in the phosphorylation reaction will be presented in the next chapter.

CHAPTER IV

ATP FORMATION IN SYSTEMS CONTAINING CHLOROPHYLLS OR QUINONES

There are numerous methods for the production of singlet dioxygen, including physical methods, chemical methods and photochemical methods.[274] In the last section of Chapter III, the singlet dioxygen production was accomplished by applying one of the chemical methods, namely, by the disproportionation of superoxide anion radical in the presence of a proton source.

Among the various methods mentioned above, perhaps the most common one is through a pigment-sensitized energy transfer to the ground state dioxygen:

$$^1P \xrightarrow{h\nu} {}^1P^* \xrightarrow[\text{crossing}]{\text{intersystem}} {}^3P^* \xrightarrow{^3O_2} {}^1P + {}^1O_2 \qquad (1)$$

where "P" denotes the pigment and "P*", the photo-excited pigment.

Chlorophylls and quinones are the two most abundant classes of pigments in living systems. Although the photochemical properties of chlorophylls (including bacteriochlorophylls) have been extensively studied, much less attention has been paid to the behavior of photo-excited quinones in living systems.

In this Chapter, the possibility of participation of the chlorophylls and quinones in photochemical phosphorylation will be examined. It has been demonstrated that the delta singlet dioxygen possesses an oxidizing capacity strong enough to induce the phosphorylation of AMP into ADP by inorganic phosphate. The earth is constantly exposed to sunlight and is surrounded by abundant dioxygen. According to the generalized photochemical reaction (1), the occurrence of energy transduction can be expected when the pigments are photo-excited. The evaluation of such possibilities are naturally of considerable biological significance and interest.

In addition to photo-chemical studies, a newly discovered, non-photochemical method of phosphorylation, involving the reaction of some less-substituted benzoquinones and nitrogenous bases under aerobic conditions, will be presented. Based on the results of some indirect experiments, the delta singlet dioxygen may also be generated during the course of the reactions. This will be discussed in the latter part of this Chapter.

IV-1. <u>Photophosphorylation Induced by the Excitation of Chlorophylls</u>

It is well-known that chlorophylls absorb strongly in the "blue" and "red" regions of visible spectrum. In the present study, two other pigment components, quinones and β-carotene, absorb light at wave-lengths shorter than 550 nm. Hence, when a light source with wave-length longer than 600 nm is used for the photo-excitation of the reaction system, only the participating chlorophyll is excited. A Kodak-800 slide projector with 300 and 500 Watt tungsten lamps was used as the light source. A proper combination of coloured plastic filters (Edmund Scientific Co., Barrington, New Jersey) and a 2-centimeter long water-filled infrared light absorber is placed between the light source and the sample. The filter and absorber system allow only visible light of wave-length greater than 600 nm to enter the reaction system.

As was shown in Table I, the photochemical reaction systems generally contain a chlorophyll, AMP inorganic phosphate and dioxygen (from the air). In order to elucidate the nature of the reaction, an additional component was added to interact with the reacting component and the yields of ATP were compared. For the study of the reaction system under anaerobic conditions, nitrogen gas was passed through the system for ten minutes. Concentrations of ATP were determined by the enzymatic process, namely, by using luciferin plus luciferase after the reaction mixtures in DMAC solutions had been illuminated for six hours.

For the determination of ATP concentrations in the sample solutions, 20 micro-litres of the latter were mixed with 2 milli-litres of distilled water. Firefly extracts, FLE-50 from Sigma Chemical Company that contain both luciferin and luciferase, were mixed with five milli-litres of ice-cooled distilled water, stirred, and then placed in a centrifuge in order to separate the clear supernatant solution from the residues. After the separation, the clear solution of the enzyme extracts was kept around 0°C in ice. For each determination, 0.1 milli-litres of this solution was injected into the 2 milli-litre aqueous solution of the sample in an appropriate bottle and the intensity of chemiluminescence was measured after ten seconds from the injection. A Beckman LS-230 Liquid Scintillation System was used to detect the chemiluminescence. The intensity was read as the number of counts per six seconds. The absolute concentration of ATP in a sample was determined by comparing the readings with those from standard ATP solutions. Figure 17 represents the relationship between the count readings and the ATP concentrations of the standards. On a logarithmic scale, a clear linear relationship is observed if the concentrations of

ATP are below 2.0 x 10^{-6} M. It is not certain whether the non-linear relationship observed above this concentration is due to the characteristics of the photomultiplier in the Scintillation Counter or due to some unknown chemical feature.

Experimental results listed in Table 1 clearly show that excitation of chlorophyll molecules to the lowest singlet excited state can phosphorylate AMP into ADP, followed by phosphorylation of the latter into ATP under aerobic conditions. Since the singlet excited state of the chlorophyll is short-lived (natural lifetime is about 5.0 x 10^{-9} sec)[275] and the energy is rapidly dissipated in the form of fluorescence or converted into the long-lived lower triplet excited state ($\sim 10^{-3}$ sec)[276] the photochemically active chlorophyll is probably in the triplet excited state.

Under aerobic conditions, the intersystem crossing from the singlet to the triplet excited state has a significant consequence. According to equation (1), singlet dioxygen may be generated. In fact this has been unambiguously demonstrated by Krasnovskii.[277] Phosphorylation occurs in the presence of dioxygen but not in its absence as is shown in the experiments 1 and 6 of Table 1. This supports the previous proposal for the role of singlet dioxygen, namely, to act as a strong oxidant for the reaction. Imidazole is known as a chemical quencher for singlet dioxygen.[278] This may be the reason why ATP yields from experiment 3 were lower than those from experiment 1. Durohydroquinone may also act as a chemical quencher for singlet dioxygen.[278]

Figure 17. Relationship between the count readings and ATP concentration by using luciferin-luciferase method.

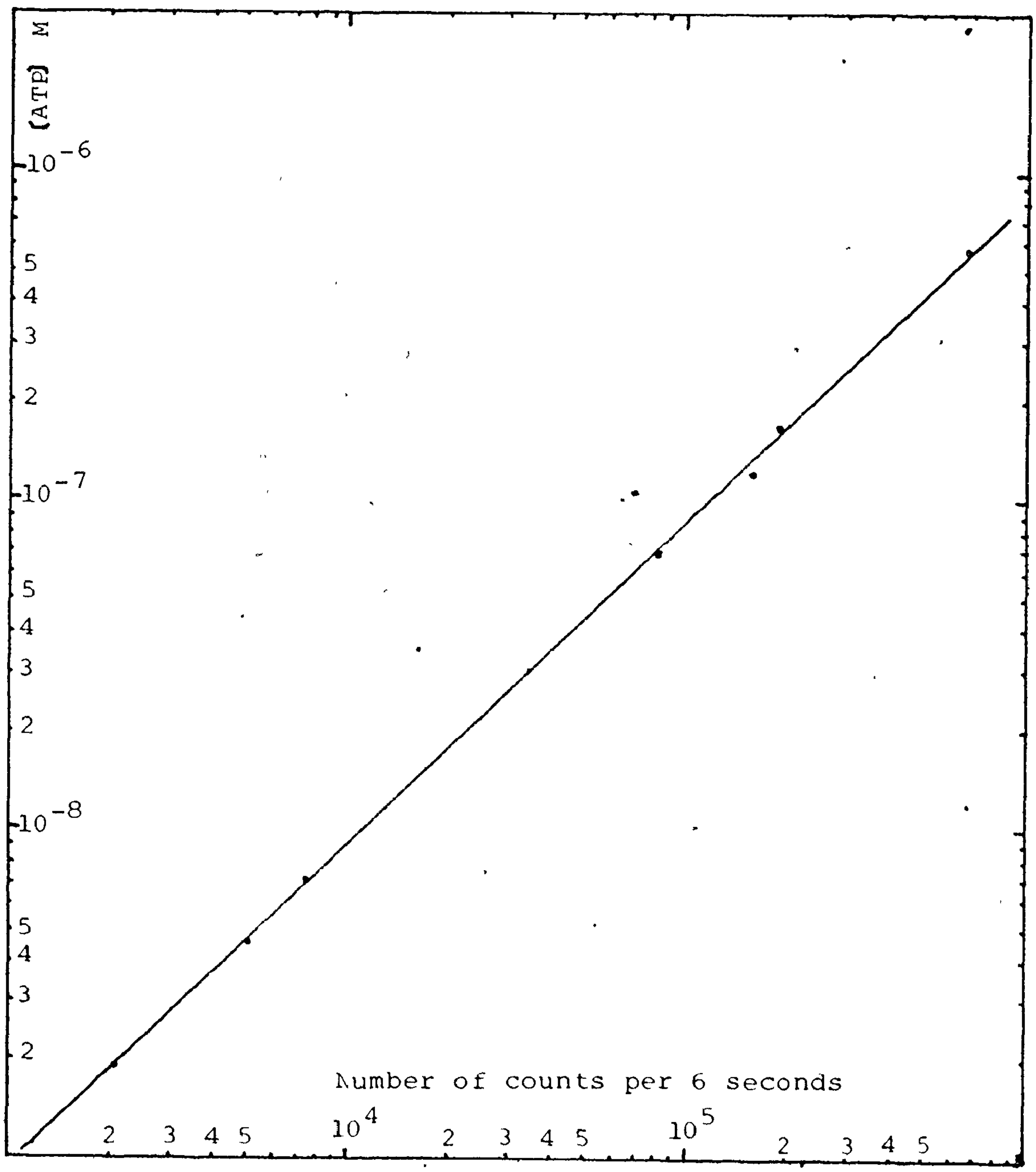

[ATP] M
10^-6
5
4
3
2
10^-7
5
4
3
2
10^-8
5
4
3
2
Number of counts per 6 seconds
2
3
4
5
10^4
2
3
4
5
10^5
2
3
4
5

Figure 17.

Table 1. Photophosphorylation induced by the excitation of chlorophyll-a in DMAC solutions.

Concentrations in moles/litre: AMP: 9.09×10^{-4}, Diimidazolium hydrogen phosphate: 1.98×10^{-3}, chlorophyll-a: 4.32×10^{-5}, pyridine: 5.58×10^{-2}, imidazole: 5.58×10^{-2}, durohydroquinone: 6.17×10^{-3}, β-carotene: saturated in DMAC.

Illumination: red light of wave-length longer than 600 nm from 300 watts tungsten lamp. Duration of illumination is 6 hours.

ATP concentrations are determined by luciferin-luciferase chemiluminescence intensities described in the text.

Expt	Condition	Components in DMAC	No. of Counts	ATP(M)
1	in air	Chl + AMP + ImP_i	131590/6 sec	9.9×10^{-8}
2	in air	Chl + AMP + ImP_i + Py	212740/6 sec	1.8×10^{-7}
3	in air	Chl + AMP + ImP_i + Im	8650/6 sec	7.7×10^{-9}
4	in air	Chl + AMP + ImP_i + DQH_2	150/6 sec	$<< 1 \times 10^{-9}$
5	in air	Chl + AMP + ImP_i + Car	360/6 sec	$<< 1 \times 10^{-9}$
6	in N_2	Chl + AMP + ImP_i	240/6 sec	$<< 1 \times 10^{-9}$

Chl: chlorophyll-s, ImP_i: diimidazolium hydrogen phosphate, Py : pyridine

Im: imidazole, DQH_2: durohydroquinone, Car: β-carotene

This may be the reason why ATP yields from experiment 3 were lower than those from experiment 1. Durohydroquinone may also act as a chemical quencher for singlet dioxygen.[279] Many hydroquinones are used as antioxidants to prevent the so-called "photodynamic effect" in plastics. β-carotene is known as an effective quencher for both the chlorophyll triplet excited state[281] and for singlet dioxygen.[266] When the system was saturated with β-carotene, the phosphorylation reaction was inhibited.

Generation of singlet dioxygen from photo-excited pigments generally has a side-reaction: photodynamic oxidation of the pigments. This photochemical destruction also occurs in chlorophyll-containing systems. When chlorophyll-a alone in DMAC was illuminated by red-light in the presence of dioxygen, decoloration of the pigment was observed in ten minutes. The addition of AMP and inorganic phosphate retarded the decoloration. The addition of pyridine resulted in the further retardation of the side-reaction. Prevention of the photo-bleaching of chlorophyll by pyridine has also been observed by Raman and Tollin.[280] The high ATP yields observed in experiment 2 (Table 1) are very likely due to the protective action of pyridine on chlorophyll.

In a separate experiment when a low concentration (instead of a saturated solution) of β-carotene was used under a stronger red-light (from 500 watt tungsten lamp), it was found that the protective action of β-carotene was similar to the pyridine case and more ATP was produced as compared with the phosphorylation system without any β-carotene.

Experimental results obtained in the present investigation are at variance with those of previous investigators.[307-8] Brinigar et al.[307] reported that when hematophorphysrin was photo-excited in the presence of imidazole, inorganic phosphate and ADP in DMAC with a trace of dioxygen present in the system, ATP was produced. If pyridine or some other nitrogenous bases was used instead of imidazole, the ATP yield was very low. 1-Phosphoimidazole was isolated.[307-8] Imidazole radical was detected and this radical was suggested to be the precursor of 1-phosphoimidazole formation by reacting with inorganic phosphate.[308] As is shown in Table 1. the presence of pyridine not only does not affect the ATP yield, but in many cases stimulates the phosphorylation reaction, while the presence of imidazole is inhibitory to the reaction. The inhibitory action is most likely caused by the chemical quenching of imidazole towards the delta singlet dioxygen produced in the photochemical reaction.[278] Isolation of 1-phosphoimidazole in the work of previous investigators does not guarantee that this is the precursor of ATP formation through a phosphoryl group transfer process to ADP. Nor the detection of imidazole radical in the imidazole-containing system constitutes an evidence for the radical's role as the necessary species to activate the inorganic phosphate for the ATP formation. Phosphorylation of imidazole to 1-phosphoimidazole can be achieved by many other phosphorylating conditions. 1-Phosphoimidazole has been isolated from all the other imidazole-lacking ATP-producing model systems presented in this thesis when imidazole is subsequently added. If imidazole radical is the necessary precursor for the phosphorylation reaction to occur as was suggested by previous investigators,[308] ATP should also occur under a strict anaerobic condition. But no ATP production was observed by this author when the photochemical reaction was carried out under the dinitrogen atmosphere.

IV-2. Photophosphorylation Induced by the Excitation of Quinones

Quinones are ubiquitous in living systems. Various benzoquinone derivatives are known as the obligatory members of an electron transport chain in the membrane of mitochondria, chloroplasts and bacterial chromatophores.[282] In all living systems, the stiochiometric amount of the quinones occurs in a multiple molar excess to other members of the chain.[283] Up to the present, quinones have been considered to participate in the biological process only at their ground state, i.e. to act as carriers of electrons and protons in the "dark reaction" of electron transport.[282]

On the other hand, in the field of Photochemistry, quinones are known as "initiators" of various kinds of redox and addition reactions when they are photo-excited.[284] To what extent these reactions proceed in photosynthetic organisms remains to be explored.

In this section, a new kind of photochemical reaction involving an energy transfer process from the lowest triplet exicted state of the quinones to molecular oxygen is presented. The energy transfer results in the formation of delta singlet dioxygen which, in turn, acts as an oxidant for the phosphorylation reaction similar to the chlorophyll-containing systems.

The absorption spectra of 1,4-benzoquinone derivatives normally show bands at three regions: 240-275 nm, 275-340 nm and 400-450 nm. The first two bands are due to "allowed" and "forbidden" π-π^* transitions with approximate extinction coefficients of 20000 and 350, respectively. The third band is due to a "forbidden" n-π^* lowest singlet transition with an extinction coefficient of approximately 20.[282]

The strongly forbidden n-π^* lowest triplet absorption band can be observed for benzoquinone, toluquinone and 2,3-,2,5-, 2,6-dimethylbenzoquinones in cyclohexane. The bands appear at 539 nm, 528 nm, 521 nm, 519 nm and 537 nm, respectively, with molar extinction coefficients in the range 0.2 to 0.5. Similar data has been obtained by Kuboyama in n-heptane.[285] The lowest triplet energy level of duroquinone has been estimated to be at 518 nm by Hammond et al.[286] and at 526 nm by Herre et al.,[287] though most workers suggest that there is a strong π-π^* character in this triplet excited state.[287-288]

Similar to most carbonyl compounds,[289] quantum yields for generating the lowest triplet excited state from the relaxation of higher excited states are very close to one.[290-2] Since the transition from the triplet excited state to the singlet ground state is strongly forbidden, the lifetime of the triplet state is expected to be long. Progressive introduction of methyl groups into the benzoquinone ring enhances the lifetime of the triplet from less than 10 nanoseconds for the benzoquinone to 9 microseconds for duroquinone in ethanol.[288] It should be noted that, in this solvent, a hydrogen abstraction reaction may shorten the lifetime considerably.[284] In a relatively inert solvent such as DMAC, the lifetime is expected to be much longer.

The aformentioned factors predict a high population of the lowest triplet excited state of benzoquinones in DMAC solutions when the compounds are photo-excited. If dioxygen is present in the quinone solutions, one may expect that relatively high yields of singlet dioxygen can be generated through an energy trnasfer from the quinone triplet excited state similar to the chlorophyll systems. If this is the case, phosphorylation is expected when the benzoquinones are photo-excited.

As shown in Table II, photophosphorylation did indeed take place when the quinones were photo-excited. ATP yields in the presence of imidazole are lower than that of the pyridine-containing system. This is probably due to the quenching effect of imidazole toward the singlet dioxygen produced.

Anthracene is known as an efficient energy-transfer quencher for the lowest triplet excited state of benzoquinone derivatives. The energy level of the lowest triplet excited state of anthracene lies below that of the benzoquinones (176 kJ mol^{-1} vs. 220 kJ mol^{-1}, or 682 nm vs. 539 nm).[288,293-4] In contrast with this, naphthalene has the corresponding triplet state energy level higher than that of benzoquinones, hence it cannot act as an energy-transfer quencher for the quinone triplet state (255 kJ mol^{-1} vs. 220 kJ mol^{-1}). The different effects of anthracene and naphthalene on the quinone triplet state are reflected by the different yields of ATP, as shown in Figure 18. The drastic reduction in ATP yield observed on the addition of anthracene may also be caused by the reaction of singlet dioxygen generated from the electron transfer and anthracene, though this reaction rate is not as fast as the rate of energy transfer from the quinones to the anthracene.[288,293]

Addition of hydroquinones to the system also greatly reduced the ATP yield (Table III). Hydroquinones are chemical quenchers for singlet dioxygen.[279]

Under anaerobic conditions, excitation of the benzoquinones did not result in ATP formation.

The aformentioned observations strongly suggest that photophosphorylation is caused by the generation of singlet dioxygen according to the photochemical reaction (1) in which the benzoquinones serve as the pigments, "P".

Table II. Comparison of ATP yields under the photo-excitation of various quinones.

Samples contain 0.1 M quinones, 0.05 M imidazole or pyridine, 2.04×10^{-2} M H_3PO_4 and 1.3×10^{-3} M AMP. Illuminated under aerobic conditions by an unfiltered 500 watt tungsten lamp for 24 hours, followed by 20 hours storage in the dark. Samples containing histidine are heterogeneous systems because of its limited solubility in DMAC.

Quinones	Base	[ATP] x 10^7 M
2,6-dimethylbenzoquinone	imidazole	2.9
trimethylbenzoquinone	imidazole	2.7
duroquinone	imidazole	1.3
plastoquinone	imidazole	2.5
ubiquinone Q_6	imidazole	0.7
vitamin K_1	imidazole	0.1
anthraquinone	imidazole	0.1
duroquinone	pyridine	6.5
trimethylbenzoquinone	histidine	0.18
duroquinone	histidine	0.17
vitamin K_1	histidine	0.06

ATP concentrations are determined by the firefly extracts.

It should be mentioned that, among the benzoquinones studied, ubiquinone Q_6 is an exception. First, the ATP yield in this system was lower than that from the other benzoquinone systems. Second, the ATP yield was insensitive to the addition of either anthracene or naphthalene. According to Amouyal, Bensasson and Land, 2,3-dimethoxybenzoquinones (including ubiquinones) have a much lower quantum yield (0.09) for the generation of the lowest triplet state, and the energy level of this state is lower than that of anthracene.[294]

Finally, as is shown in Table IV, the presence of a nitrogenous base, such as imidazole or pyridine, greatly increases the yield of ATP. The cause of this enhanced yield remains to be determined.

IV-3. Phosphorylation Associated with the Reaction of Simple Benzoquinones and Nitrogenous Bases.

In the previous section, simple benzoquinones such as p-benzoquinone and toluquinone were excluded from the other quinones in the photophosphorylation study. There is no reason to believe that these two benzoquinones would behave differently than the others under photochemical conditions. However, they are exceptional in the sense that both of them react with imidazole and pyridine derivatives under proper conditions, and oxidation of the reaction products by the parent quinones can induce a phosphorylation reaction in the presence of dioxygen. The other quinones, having two or more methyl- or methoxyl-substituents but without 2- and 3- positions open for the reaction with nitrogenous bases, fail to induce the phosphorylation reaction.

Figure 18. Effect of the addition of anthracene or naphthalene on ATP yields in quinone systems.

Concentrations of components are: plastoquinone 3.15×10^{-2} M, imidazole 1.20×10^{-1} M, diimidazolium hydrogen phosphate 3.42×10^{-3} M and AMP 2.10×10^{-3} M. Irradiated under aerobic conditions by bright sunlight for 2 hours, then kept in darkness for 24 hours before the analysis of ATP yields.

No addition of any quencher

Anthracene added

Naphthalene added

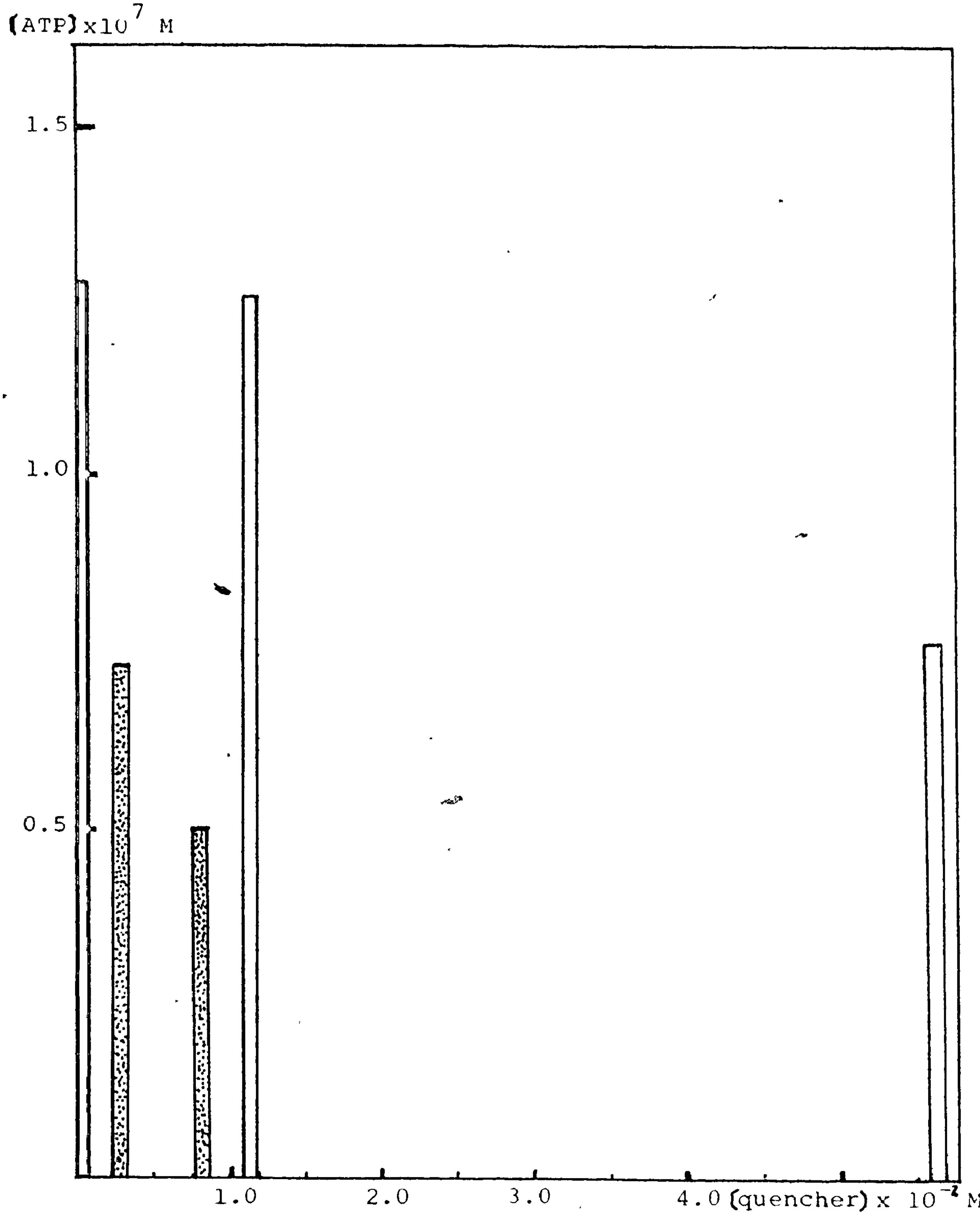


Figure 18.

Table III. Effect of hydroquinones on ATP yields.

Samples contain 3.0×10^{-2} M imidazole, 3.1×10^{-3} M diimidazolium hydrogen phosphate and 1.83×10^{-3}M AMP in DMAC solution. Samples are irradiated for varying times by 450 watt Osram xenon lamp. Relative ATP concentrations are expressed in terms of the number of counts per 0.1 minute and are examined after 16 hours in darkness. 20 micro-liters of sample solution and one milli-liter of firefly extract was used in the analyses.

Quinone + Hydroquinone		Irradiation	ATP
2,6-dimethylbenzoquinone	5.2×10^{-2} M	30 min	332233
2,6-dimethylbenzohydroquinone	0		
2,6-dimethylbenzoquinone	2.6×10^{-2} M	30 min	12
2,6-dimethylbenzohydroquinone	2.6×10^{-2} M		
2,6-dimethylbenzoquinone	0	30 min	3
2,6-dimethylbenzohydroquinone	5.2×10^{-2} M		
Trimethylbenzoquinone	1.5×10^{-2} M	1 min	1008
Trimethylbenzohydroquinone	0		
Trimethylbenzoquinone	1.5×10^{-2} M	1 min	13
Trimethylbenzohydroquinone	1.5×10^{-2} M		

Table IV. Effect of a nitrogenious base on ATP yields.

Samples contain 3.1×10^{-3} M diimidazolium hydrogen phosphate and 1.83×10^{-3} M AMP. Short irradiation was done using a 450 watt Osram Xenon lamp for a one minute exposure time under a nitrogen atmosphere. Longer irradiation was carried out in bright sunlight for ten hours exposure time in the air. ATP concentrations are expressed in number of counts per 0.1 minute by firefly assay, determined after a 20 hour dark period. 20 micro-liters of sample solutions and one milli-litter of firefly extract was used for the analyses.

Trimethylbenzoquinone	Imidazole	Irradiation	ATP yields
1.5×10^{-2} M	3.0×10^{-2} M	1 min	1008 (counts/0.1 min)
1.5×10^{-2} M	0	1 min	7 (counts/0.1 min)
1.0×10^{-1} M	5.0×10^{-2} M	10 hrs	5.6×10^{-7} M
1.0×10^{-1} M	0	10 hrs	5.8×10^{-8} M

Trimethylbenzoquinone	pyridine	Irradiation	ATP yields
1.0×10^{-1} M	5.0×10^{-2} M	10 hrs	3.4×10^{-6} M

Figure 19 shows the time course of ATP production from ADP in systems containing p-benzoquinone, a nitrogenous base (imidazole or pyridine), diimidazolium hydrogen phosphate in DMAC under aerobic conditions. The time course of ATP production depends on how the components are mixed. The following three sets of experiments were selected to clarify the nature of the reactios involved in the phosphorylation reaction:

(1) $(B + Q + P_i) + ADP$

(2) $(B + Q) + P_i + ADP$

(3) $B + Q + P_i + ADP$

where B, Q and P_i stand for a nitrogenous base, p-benzoquinone and an inorganic phosphate, respectively. The parentheses indicate "pre-mixing" of the components in order to generate a certain intermediary product with a higher concentration in the reaction.

In the systems where pyridine was used as the nitrogenous base, 2,4-dinitrophenol was added in the "pre-mixing" stage of the reactions (1) and (2) to act as a proton source so that formation of the product between the base and the quinone was facilitated. This point will be explained in the latter part of this section. ATP in DMAC in the presence of 2,4-dinitrophenol had been examined and found to be stable even after four days.

As is shown in Figure 19, the sigmoid curve of the reaction system (3) suggests the existence of a rate-limiting imtermediary process prior to the phosphorylation of ADP. As the initial rate of the phosphorylation in the reaction system (2) was faster than that of the system (3), the intermediary process must involve the reaction between the base and the quinone. A phosphorylated high-energy imtermediate, such as quinol phosphate or phosphorylimidazole, does not seem to be involved, as the initial rate of reaction

Figure 19. Rate of phosphorylation under various conditions of mixing.

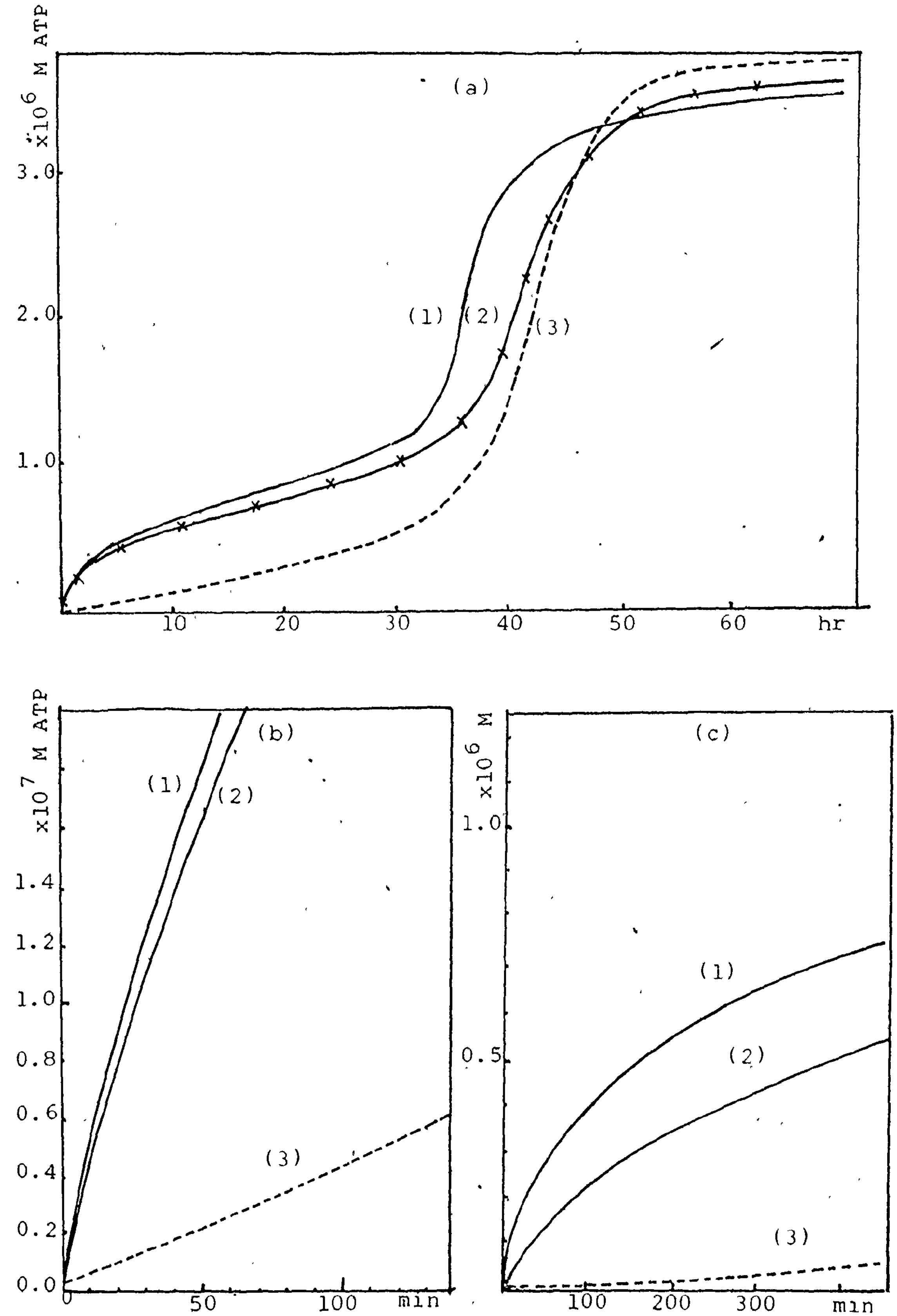
(1) Pre-mixing a nitrogenous base, p-benzoquinone and phosphate, followed by the addition of ADP (————).

(2) Pre-mixing a nitrogenous base and p-benzoquinone, followed by the addition of phosphate and ADP (-*—*-).

(3) All components are added simultaneously (------).

(a), (b) : System containing p-benzoquinone 9.34 x 10^{-2} M imidazole 2.94 x 10^{-2} M. diimidazolium hydrogen phosphate 4.35 x 10^{-3} M and ADP (imidazolium salt) 2.04 x 10^{-4} M. Duration of pre-mixing stage is 6 hours and 50 minutes.

(c) : System containing p-benzoquinone 7.7 x 10^{-2} M, pyridine 3.92 x 10^{-2} M 2,4-dinitrophenol 3.62 x 10^{-2} M, phosphiric acid 7.36 x 10^{-4} M and ADP (imidazolium salt) 1.98 x 10^{-4} M. 2,4-Dinitrophenol is added in all pre-mixing stage. Duration of pre-mixing stage is 1 hour and 50 minutes.



(a)
x10^6 M ATP
3.0
2.0
1.0
(1)
(2)
(3)
10
20
30
40
50
60
hr
(b)
x10^7 M ATP
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(1)
(2)
(3)
0
50
100
min
(c)
x10^6 M
1.0
0.5
(1)
(2)
(3)
100
200
300
min

Figure 19.

systems (1) and (2) is almost the same when imidazole is used as the base.

When pyridine instead of imidazole was used for the nitrogenous base, and orthophosphoric acid instead of diimidazolium hydrogen phosphate was used for the phosphate source, the initial rate of the reaction system (1) was faster than that of reaction system (2), which was faster than reaction system (3). In reaction system (1), in addition to 2,4-dinitrophenol, phosphoric acid may provide the additional protons necessary for the formation of the intermediary product between pyridine and p-benzoquinone, increasing the concentration of this product.

It should be noted that, when "pre-mixing" involves B and P_i, or Q and P_i, the over-all time course of ATP production is similar to that of reaction system (3). Thus there is no product between the two components participating in the phosphorylation reaction.

That the formation of an intermediary product between a nitrogenous base and a benzoquinone is a necessary condition for the phosphorylation to occur can be proven by the following observations:

(i) Among the benzoquinones tested, only p-benzoquinone and toluquinone react with imidazole or pyridine to produce ATP. 2,5-Dimethylbenzoquinone, 2,6-dimethylbenzoquinone, trimethylbenzoquinone and duroquinone do not react with the nitrogenous bases due to a steric hindrance exerted by the methyl substituents.

(ii) Among the imidazole and pyridine derivatives tested, only those bases without a substituent adjacent to the nitrogen atom can react with p-benzoquinone and induce the phosphorylation reaction. This is also due to a steric effect exerted by the substituent.

(iii) If there is no steric hindrance, reaction mixtures containing nitrogenous bases of higher basicity in a homologue produce more ATP. Reaction system using 3.4-lutidine (pKa = 6.61) as the base instead of pyridine (pKa = 5.3) was found to generate twice as much ATP. Similarly, system containing 1-methylimidazole (pKa =7.25) was found to produce more ATP than that containing imidazole (pKa = 6.95). In the same homologue, the higher the basicity, the stronger the nucleophilicity a nitrogenous base may exhibit, and the more addition product between the base and bezoquinone may be produced. (The reason is explained below).

All experimental results are summarized in Figure 20.

What is the reaction product between the p-benzoquinone and the nitrogenous base?

A carbon-carbon double bond conjugated with an electron sink may serve as a good substrate in nucleophilic addition reactions. Quinones are α,β-unsaturated carbonyl compounds. Therefore, they belong to this category. The conjugation in those compounds may be represented by the following structures:

$$\{ \;-C{=}C{-}C{=}O \qquad \overset{\oplus}{-C}{-}C{=}C{-}\overset{\ominus}{O} \;\}$$

and the nitrogenous bases should attack the carbon atom with a positive formal charge. For p-benzoquinone the nucleophilic attack is usually followed by an aromatization process, leading to the formation of a hydroquinone derivative. IN the case of pyridine, the stabilizing aromatization process requires a proton source:

Figure 20. Steric effect of nitrogenous bases in ATP-producing systems.

(a) Concentrations of components are: p-benzoquinone- 9.25×10^{-2} M; nitrogenous bases- 8.82×10^{-2} M; phosphoric acid- 4.90×10^{-3} M; AMP- 1.56×10^{-3} M. ATP concentrations are determined 29 hours after the mixing.

(b) Concentrations of components are: P-benzoquinone- 10.00×10^{-2} M; nitrogenous bases- 5.00×10^{-2} M; phosphoric acid- 1.97×10^{-2} M; AMP- 1.30×10^{-3} M. STP concentrations are determined 47 hours after the mixing.

(c) Concentrations of components are: 1,4-naphthoquinone- 9.20×10^{-2} M; nitrogenous bases- 10.00×10^{-2} M; phosphoric acid- 4.89×10^{-3} M; AMP- 1.59×10^{-3} M. ATP concentrations are determined 71 hours after the mixing.

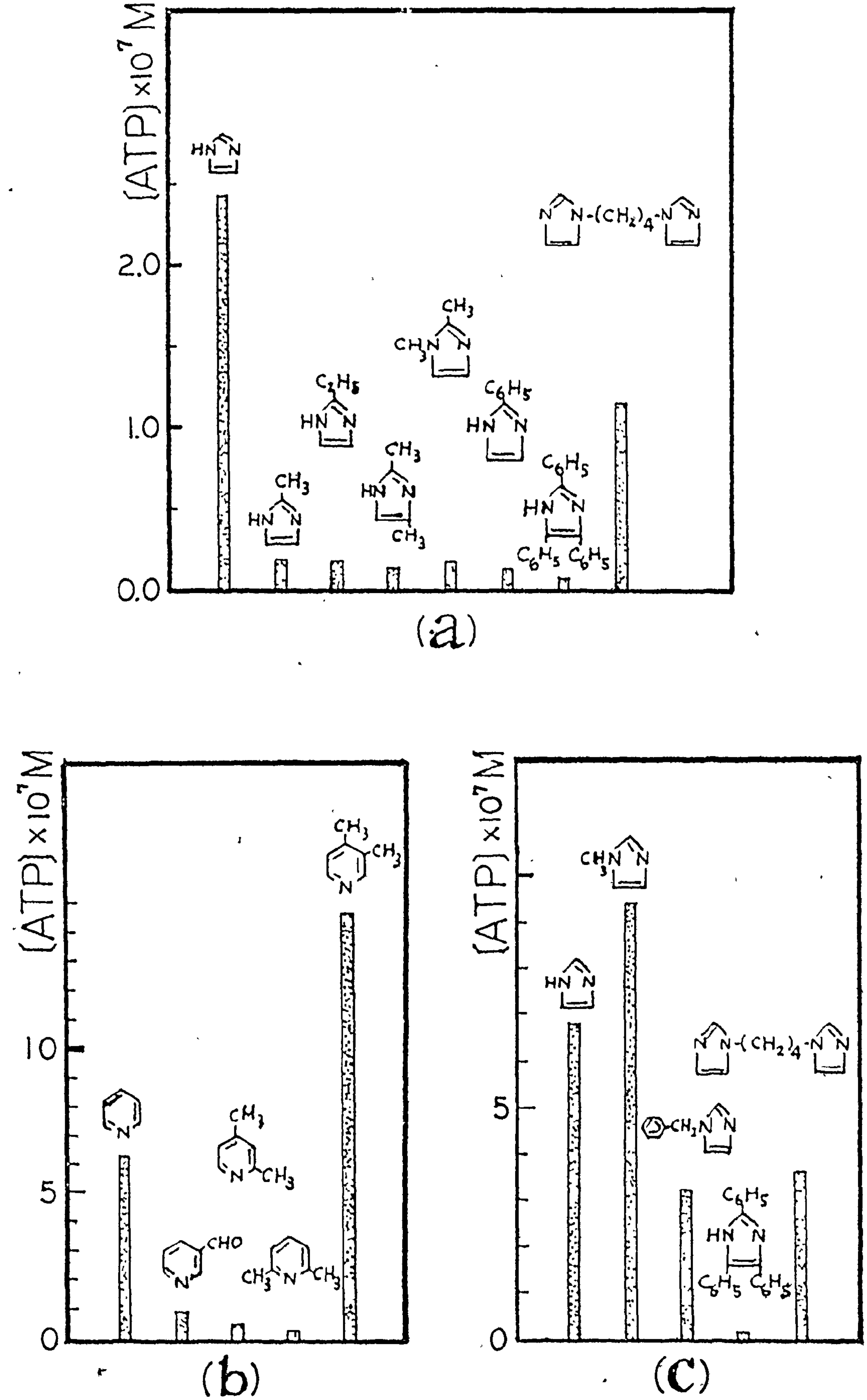
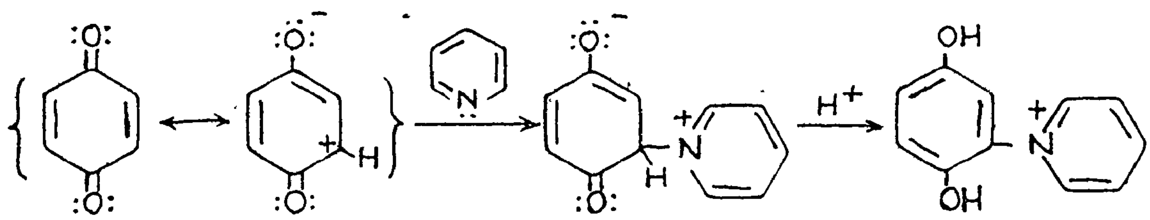
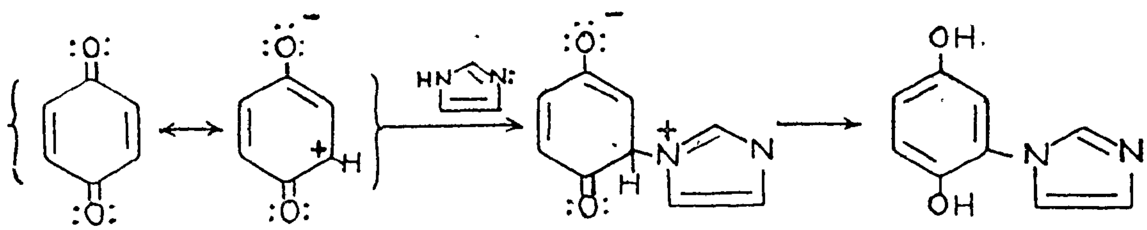


Figure 20.



When imidazole is used as the nitrogenous base, the proton required for the aromatization may come from imidazole itself:



If the imino hydrogen atom of imidazole is replaced by a substituent (for examples, 1-methylimidazole, 1-benzylimidazole), the case becomes similar to that of pyridine, namely, a proton source is necessary for the aromatization to occur.

The addition products can be readily oxidized by the original p-benzoquinone into the corresponding quinones, which in turn, can react with another nitrogenous base, forming di-substituted hydroquinones.

Both mono- and diimidazolylhydroquinones have been isolated as reaction products in tetrahydrofuran under strict anaerobic conditions. The former show an absorption band at 370 nm, and the latter, at 350 nm. In basic aqueous media, they can be oxidized by dioxygen into semiquinone anion forms which can be identified by their ESR spectra (Figures 21, 22 and 23). Both hydroquinones

Figure 21. Electron spin resonance spectrum of 2-imidazolylbenzosemiquinone anion in aqueous solution.

pH of the medium: 8.5

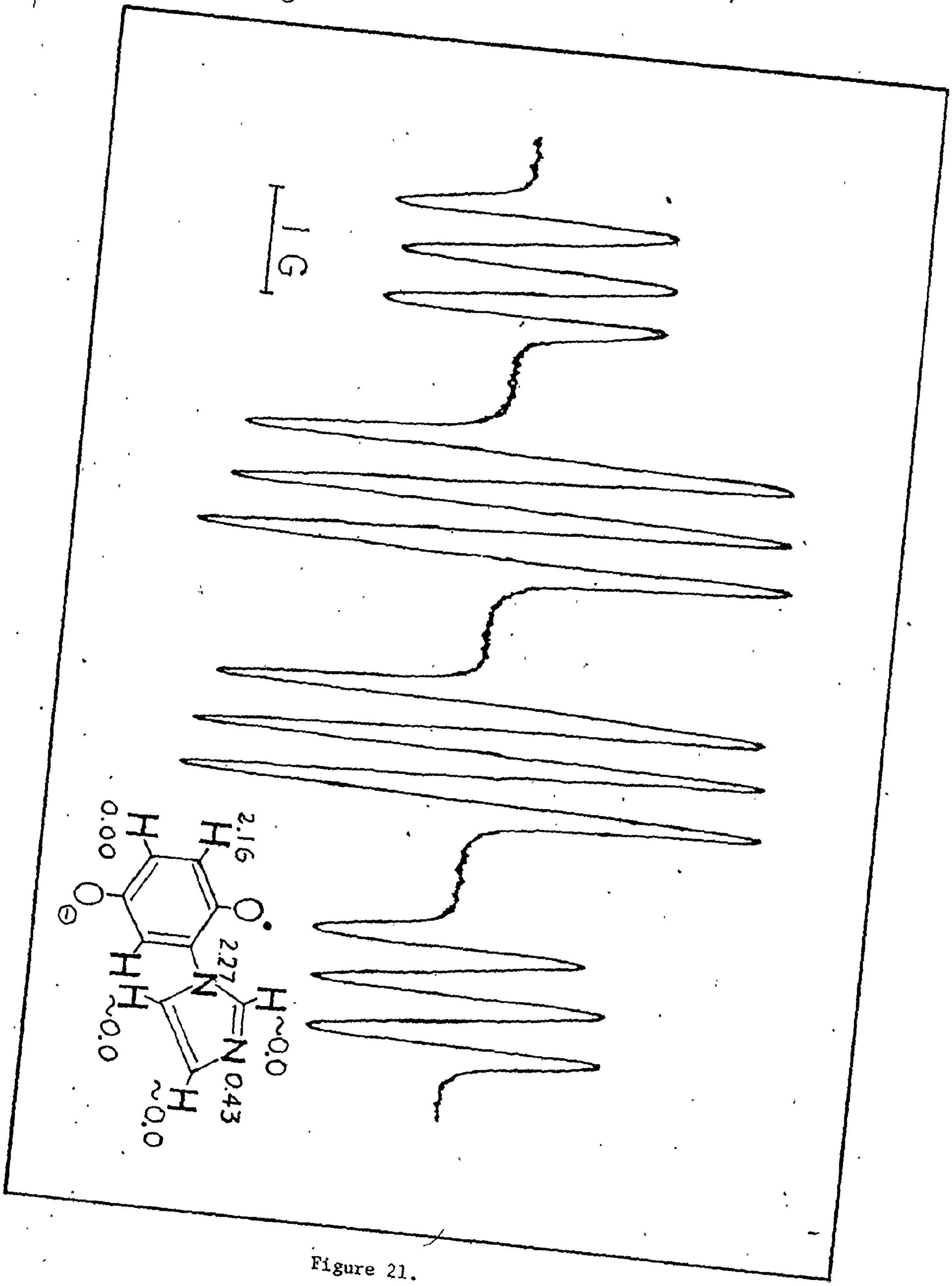


Figure 21.

Figure 22. Electron spin resonance spectrum of 2-imidazolyl-d_4-benzosemiquinone anion in deuterium oxide.

pH of the medium: 8.5

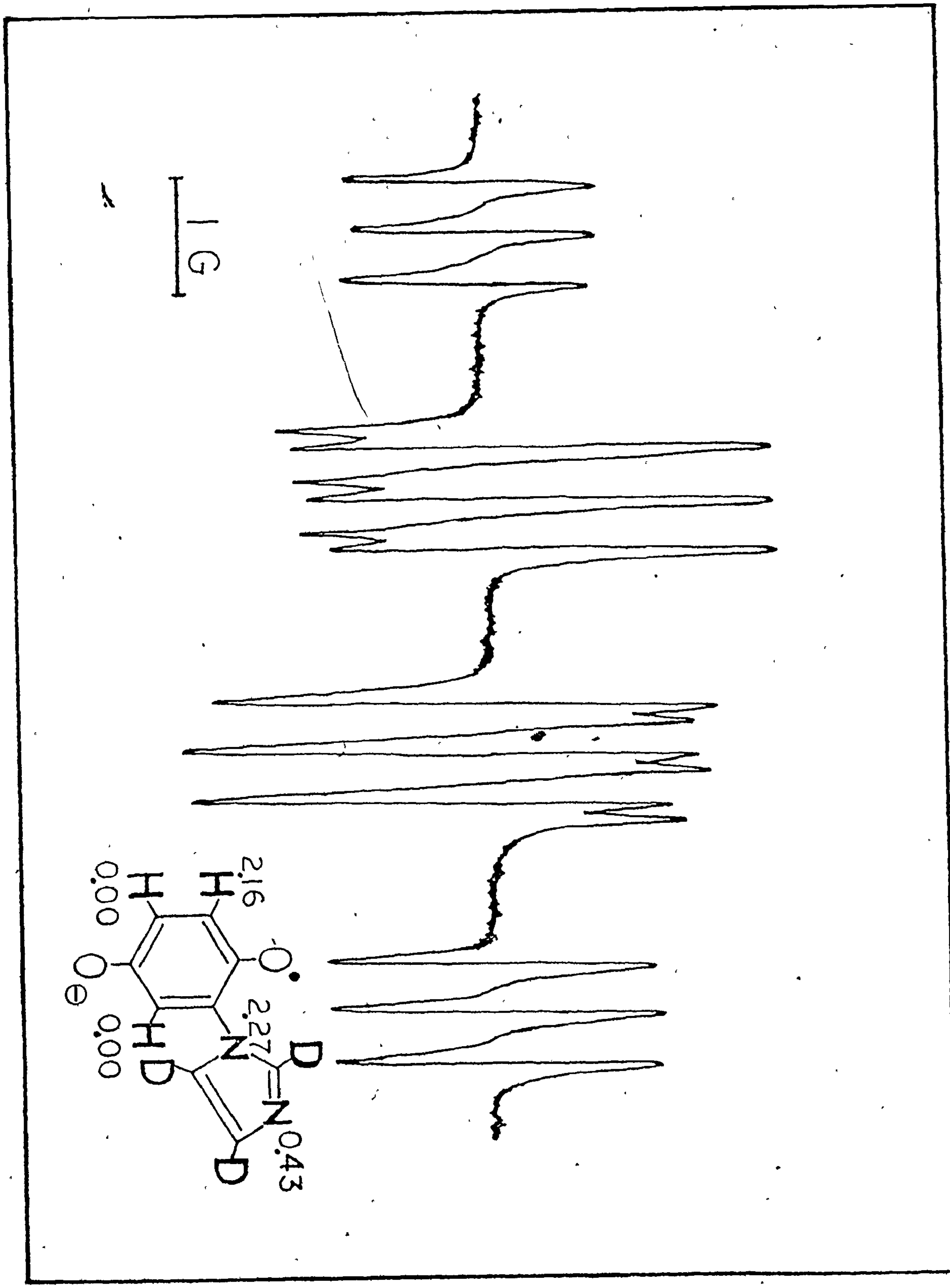


Figure 22.

Figure 23. Electron spin resonance spectrum of diimidazolylbenzosemiquinone in aqueous solution.

pH of the medium: 8.5

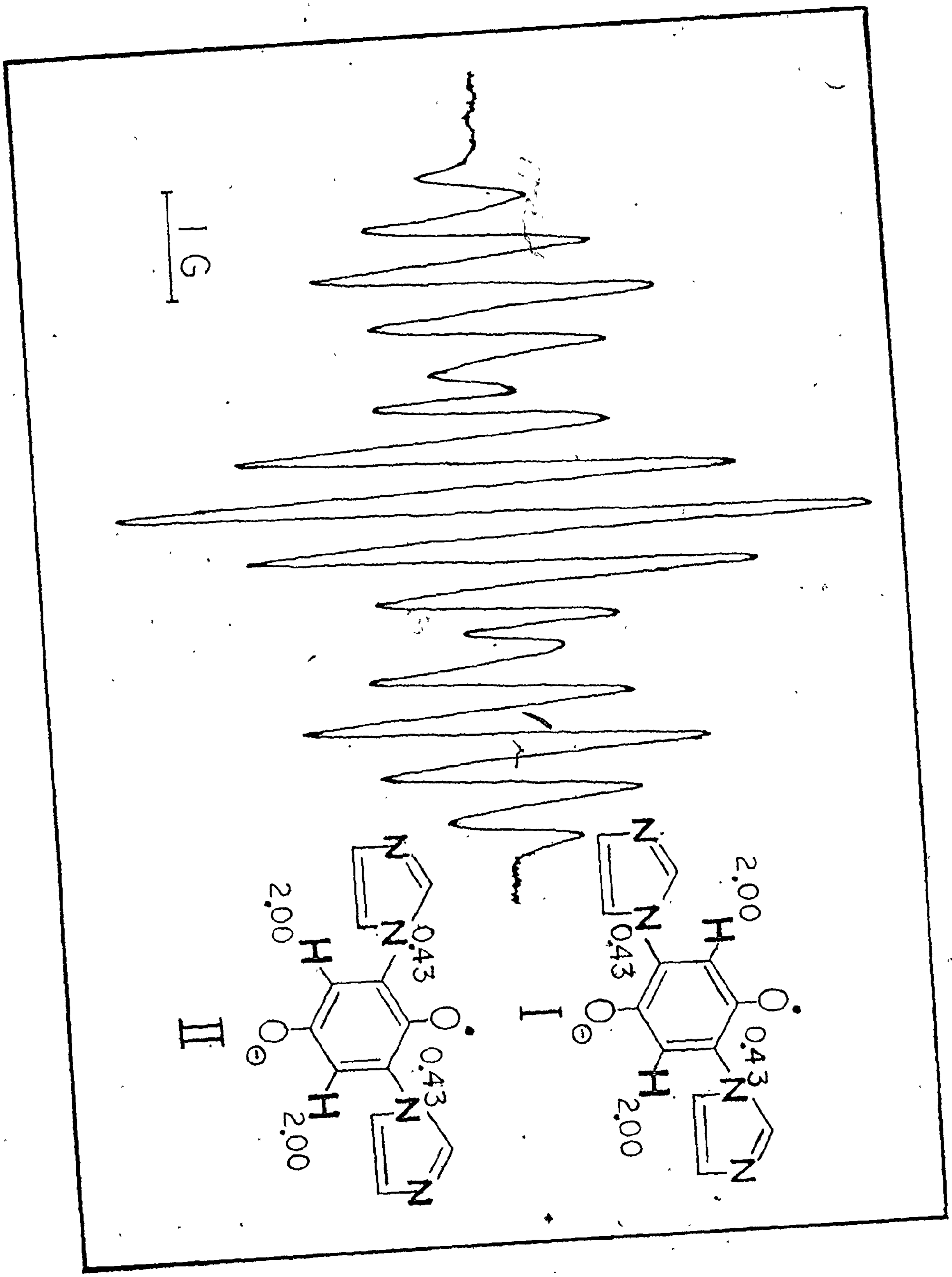


Figure 23.

are auto-oxidizable. 2-Imidazolylbenzoquinone shows an absorption band at 311 nm and diimidazolylhydroquinone, at 305 nm.

2-Imidazolylbenzoquinone can be oxidized by p-benzoquinone through a two-step one-electron process. The intermediate stage of the reaction shows two kinds of semiquinone species: benzosemiquinone and 2-imidazolylsemiquinone. The semiquinones can be detected by the ESR spectrometric method. Figure 24 shows the ESR signals of these two semiquinone species. The ESR signals can also be detected in DMAC solution by using a flat cell, but the resolution of the signals was much lower. 2-Imidazolylsemiquinone shows an absorption band at 468 nm. Under aerobic conditions, this semiquinone is rapidly oxidized by dioxygen. The dioxygen is most likely reduced into superoxide anion radical.

The phosphorylation of ADP observed in the reactions (1), (2) and (3) under aerobic conditions is caused by the generation of delta singlet dioxygen through the disproporation reaction of the superoxide anion radicals formed. This statement is based on two experimental observations:

(a) No ATP formation is found under a strict anaerobic condition.

(b) Under aerobic conditions, addition of an efficient singlet dioxygen quencher, β-carotene, results in the inhibition of the phosphorylation.

Furthermore, chemiluminescence has been observed when semiquinones are treated with dioxygen.[304]

Figure 24. Electron spin resonance spectrum of reaction products from p-benzoquinone and imidazole in aqueoustetrahydrofuran solvent mixture. pH of the medium: approximately 9.

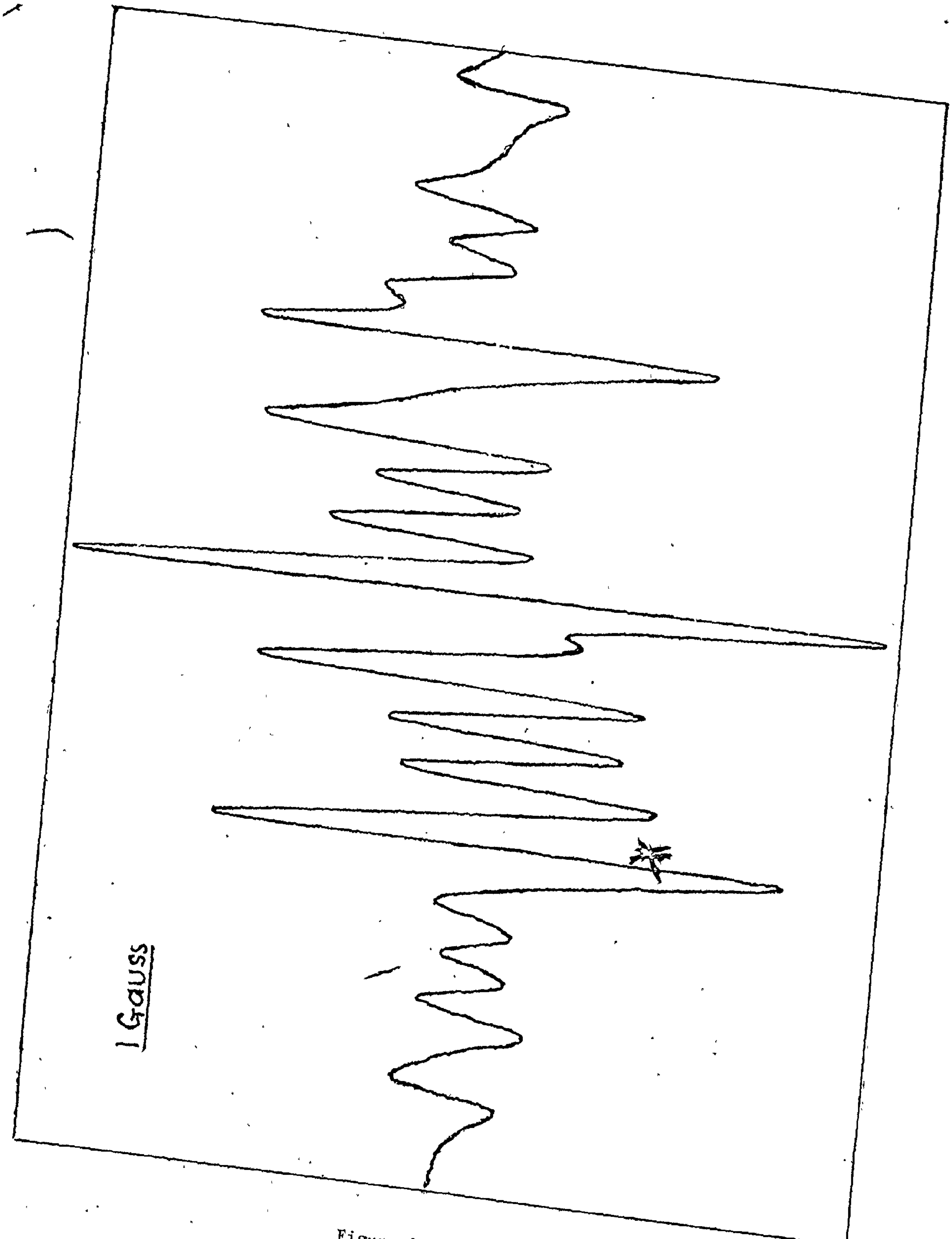


Figure 24.

CHAPTER V

ATP FORMATION COUPLED TO THE REDOX REACTION BETWEEN HYDROQUINONES AND HEME-COMPLEXES.

Haemoproteins are very important chemical species existing in the majority of living systems. However, their chemical structures and exact functions are only partially known. They are known as electron carriers in the electron transport chain of mitochondria, chloroplasts and bacterial chromatophores. Whether they participate directly in the phosphorylation proper or not is still a matter of dispute.[194]

Among the haemoproteins in the biological electron transport chains, cytochrome-c oxidase complex of mitochondria has been comparatively well-studied in relation to the phosphorylation reaction. The following observations by previous investigators are particularly noteworthy:

(a) Wilson et al. found that the half reduction potential of cytochrome a_3 is strongly dependant on the "energy state" of the mitochondria.[194] Addition of excess ATP at pH 7.2 causes a shift in the potential from 0.385 volts to 0.155 volts.

(b) Yong et al. isolated a cytochrome-c oxidase complex which manifested phosphorylation activity as well as "respiratory control" without adding external phospholipids or other coupling factors.[295] If the complex is not contaminated with ATP-producing impurities, this work provides one good example for supporting the concept that the phosphorylation reaction is intimately associated with the members in the electron transport chain.

Unfortunately, this work seems to require more study to confirm the observation, as the results could not be reproduced in other similar system.[296]

(c) Based on the "cross-over theorem" of Chance and Williams,[187] an energy transducing site was located between cytochrome a_3 and dioxygen.[297-8]

These interesting observations mentioned above do not constitute definite proof for the concept that some members of the electron transport chain participate directly in the phosphorylation reaction, since the observations can also be interpreted from another point of view, such as the one based on the chemiosmotic hypothesis.[299] At any rate, there isn't a well-defined molecular mechanism in the existing hypotheses to explain the aforementioned observations.

The study presented in this Chapter is a simple chemical model system in which ATP formation is observed from the reduction of heme-complexes by hydroquinones in the presence of AMP and inorganic phosphate, followed by an aerobic oxidation in DMAC solutions.

V-I. Reduction of Heme-Complexes with Various Hydroquinones

Reduction of several heme-compounds requires extreme care since the reduced hemes may be rapidly oxidized by the air. An apparatus suitable for observing the redox behavior of the heme-complexes is depicted in Figure 25. In this apparatus when the heme-complexes were reduced under oxygen-free conditions, the products remain unchanged even after two days.

Hemin (ferriprotoheme chloride) and dimethylhematin (ferridimethylprotoheme hydroxide) are used as "mother compounds" of the heme-complexes in the present model systems. Both of them are known as high-spin compounds, with square-pyramidal symmetry.[300] When the chloride or hydroxide of the heme

Figure 25. An apparatus used for the study of redox behavior of heme-complexes.

A: Reservoir for a heme-complex in DMAC solution.

B: Side tube for a hydroquinone.

C: Optical cell for observing any chemical changes during the reaction.

D: A tight rubber cap.

E: A hypodermic syringe which shall be pulled out after the air is replaced by dinitrogen gas. The hole remaining was closed by an application of vacuum grease.

Chemicals may be introduced by using spatula and the whole apparatus can be weighed on a balabce.

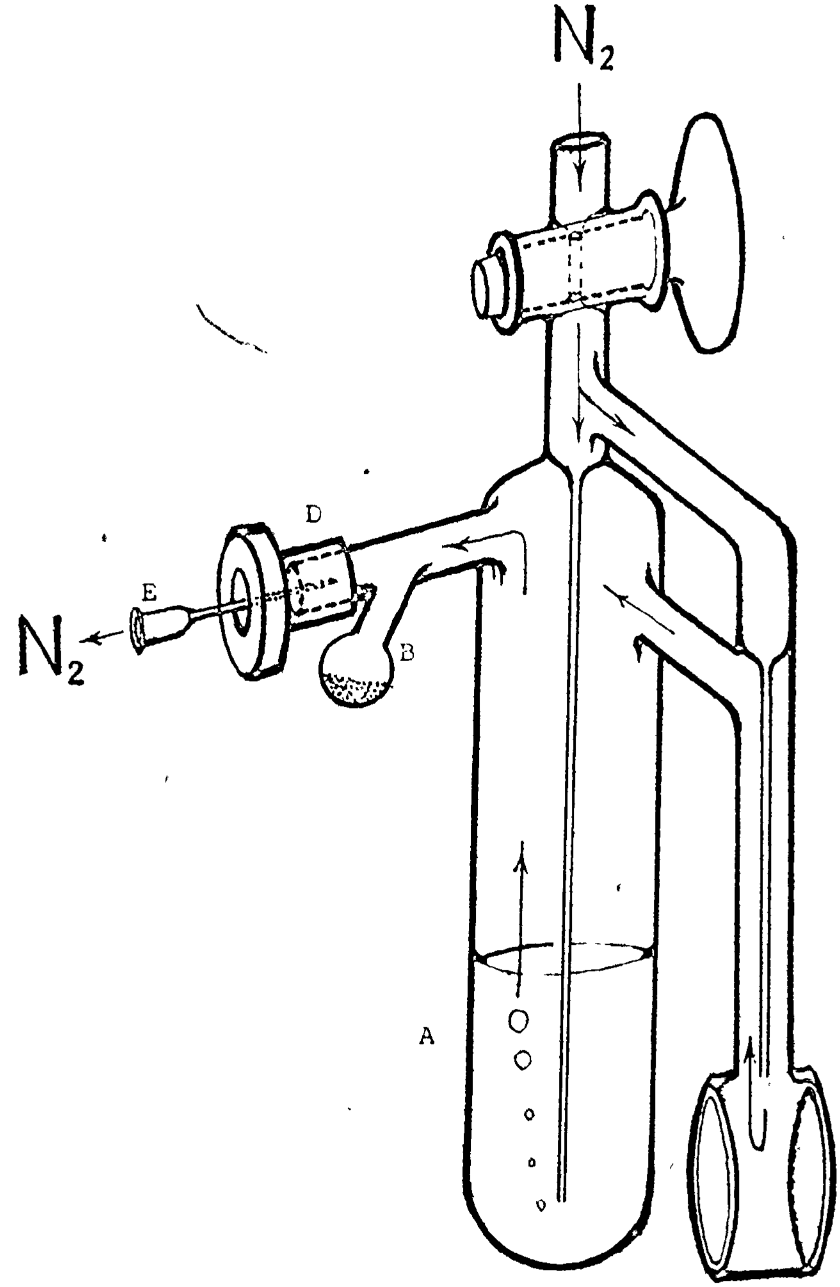
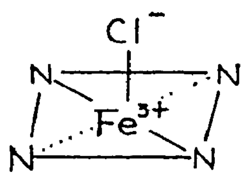
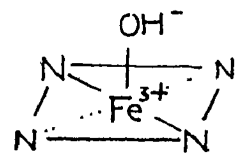


Figure 25.

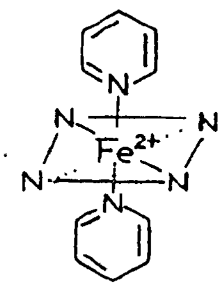
compounds is replaced by a pair of much stronger ligands (imidazole or pyridine), the resultant heme-complexes change from the high-spin state to the low-spin state.[300]



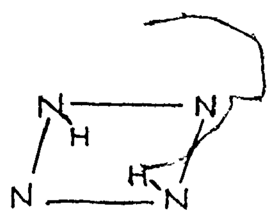
Hemin



Hematin



Hemochrome

Here  represents protoporphyrin.

At pH 7.0, the redox potential of hemin is -0.115 volts,[301] and that of p-benzoquinone, toluquinone, 2,5-dimethylbenzoquinone, trimethylbenzoquinone and duroquinone is, respectively, 0.28, 0.22, 0.18, 0.10 and 0.02 volts.[302]

Hence, if the corresponding hydroquinones were used as the reductants, only durohydroquinone exhibited a partial reduction of hemin, converting it to the ferrous form. Addition of other hydroquinones to hemin or hematin caused slight changes in the shape of the absorption bands of the hemes, without showing any bands of reduced hemes. The changes in band shapes probably reflect the formation of intermolecular complexes between the hemes and the hydroquinones. Figure 26 shows two examples for the reduction of dimethylhematin by hydroquinones under anaerobic conditions.

The situation was very different when a nitrogenous base such as imidazole or pyridine was added to the systems. The heme compounds were readily reduced to the corresponding hemochromes by all hydroquinones mentioned above. A typical example is given in Figure 27. Here, hemin was reduced to dipyridine hemochrome by benzohydroquinone.

Introduction of air to the DMAC solution of dipyridine or diimidazole hemochrome resulted in a gradual oxidation of the latter. The rate of oxidation of the hemochromes was drastically accelerated when inorganic phosphate was added to the system. In addition, the absorption bands characteristic to the dipyridine hemochrome were reduced under anaerobic conditions when the phosphate was also present in the system. These observations suggest that the pyridine ligand in the hemochrome may be replaced by a phosphate, forming a phosphate-coordinated ferroheme complex which can be oxidized by dioxygen with a much higher rate than the original one. For a hemochrome, pyridine is known as a relatively weak ligand ans can be replaced by some other stronger ligand, forming a so-called "mixed ligand" complex.[303]

In contrast to dipyridine hemochrome, diimidazole hemochrome is much more stable. In the presence of excess imidazole, the rate of oxidation of the hemochrome by air was very slow. In this case addition of diimidazolium hydrogen phosphate to the system did not show any acceleration in the rate of oxidation. Presumably imidazole is a much stronger ligand in the hemochrome and is not replaceable by the other weaker ligand, such as phosphate.

Figure 26. Reduction of dimethylhematin ester by hydroquinones in DMAC.

(1) ———————— Dimethylhematin ester only (1.06×10^{-4} M).

(2) — — — — — — Dimethylhematin ester (1.06×10^{-4} M) plus benzohydroquinone (6.74×10^{-3} M).

(3) -·-·-·-·-·-·-· Dimethylhematin ester (1.06×10^{-4} M) plus durohydroquinone (6.74×10^{-3} M).

(4) .... Dimethylhematin ester reduced by $NaBH_4$.

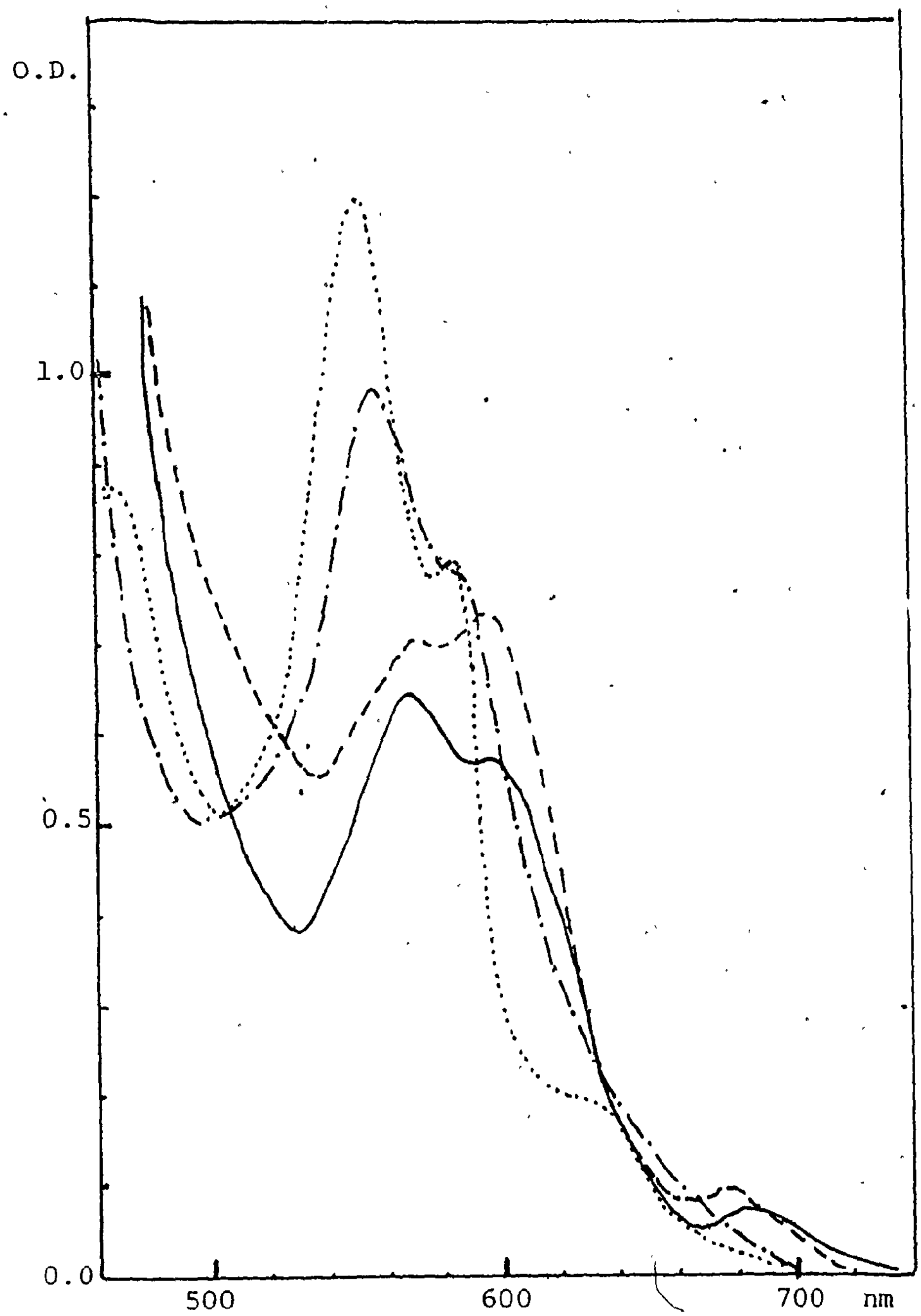


Figure 26.

Figure 27. Reduction of hemin by hydroquinone in the presence of excess pyridine in DMAC.

(1) ———————— Hemin only (1.026×10^{-4} M).

(2) .... Hemin (1.026×10^{-4} M) plus hydroquinone (2.02×10^{-2} M) with excess pyridine (0.362 M).

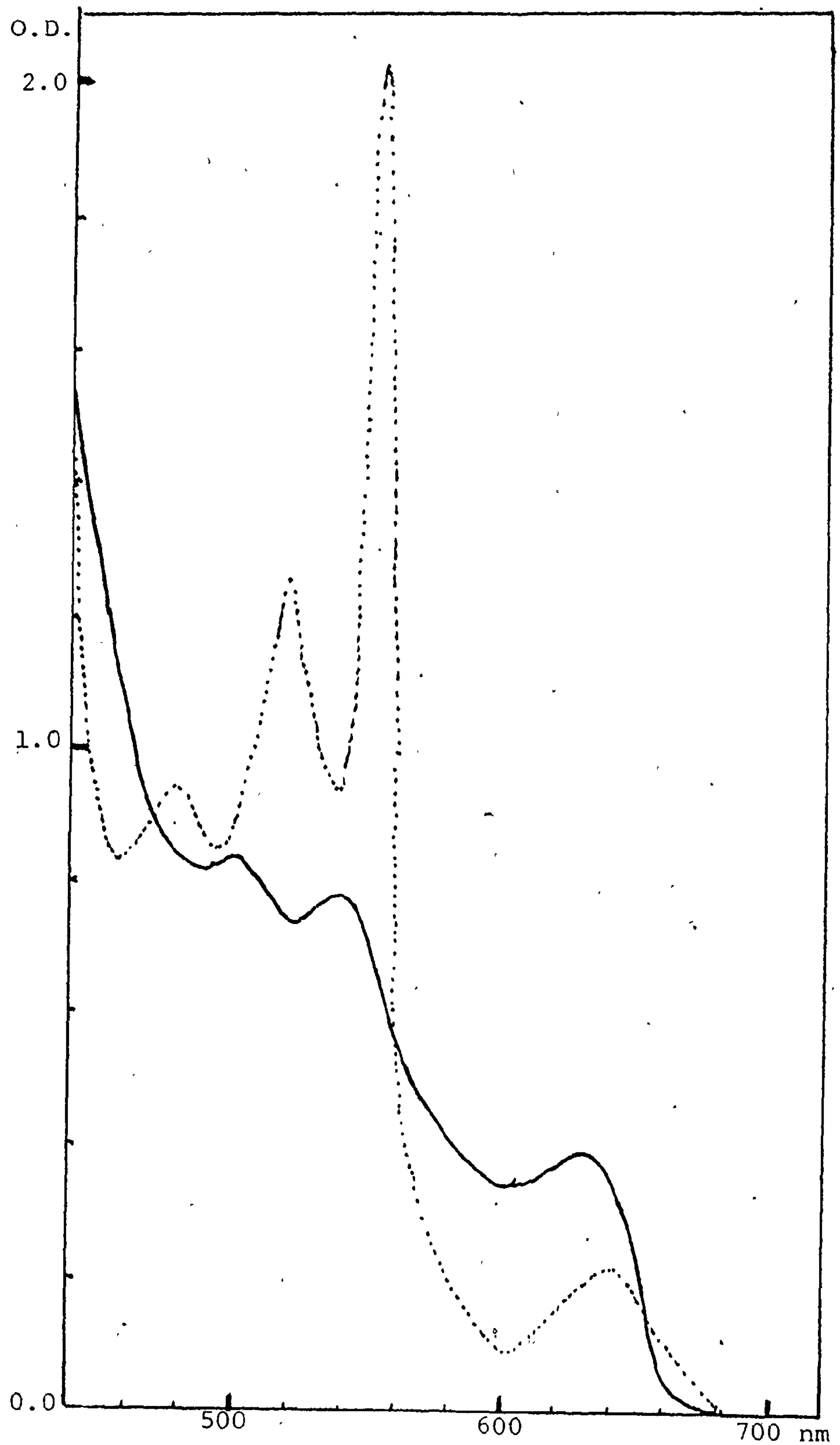


Figure 27.

It should be noted that, generally the aerobic oxidation of dipyridine hemochrome in the presence of phosphate exhibits two phases: a rapid phase and a slow phase. The rate of the oxidation in the rapid phase was found to be linearly proportional to the concentration of the phosphate present.

V-II <u>Oxidative Phosphorylation Coupled to the Redox Reaction Between Hydroquinones and Hemochromes</u>.

Table V shows the results of oxidative phosphorylation associated with the redox reaction between hydroquinones and hemin. Phosphorylation could be observed only under aerobic oxidation of the reduced heme-complex. In dinitrogen atmosphere, ATP was not formed. Thus the phosphorylation is "oxidative" in nature.

Also noteworthy is the fact that, when the other conditions are the same, reduction by durohydroquinone generates much more ATP than hydroquinone. This is understandable from the redox potentials of related reactants. The redox potential of dipyridine hemochrome is 0.137 volts.[300] That of hydroquinone and durohydroquinone is respectively 0.28 and 0.02 volts. More dipyridine hemochrome, thus more phosphate-coordinated ferroheme complex may from in the durohydroquinone system.

Diimidazole hemochrome with escess imidazole did not generate ATP under similar conditions. Provably the imidazole ligand cannot be replaced by phosphate, and the activation of phosphate thus becomes impossible.

Ferrihemin did not show any changes in its absorption spectrum when pyridine was added. Probably in the ferric state, the hemin still possesses a square pyramidal structure with a "high spin" electronic configuration.

Table V. ATP formation coupled to the redox reaction between hydroquinones and hemin.

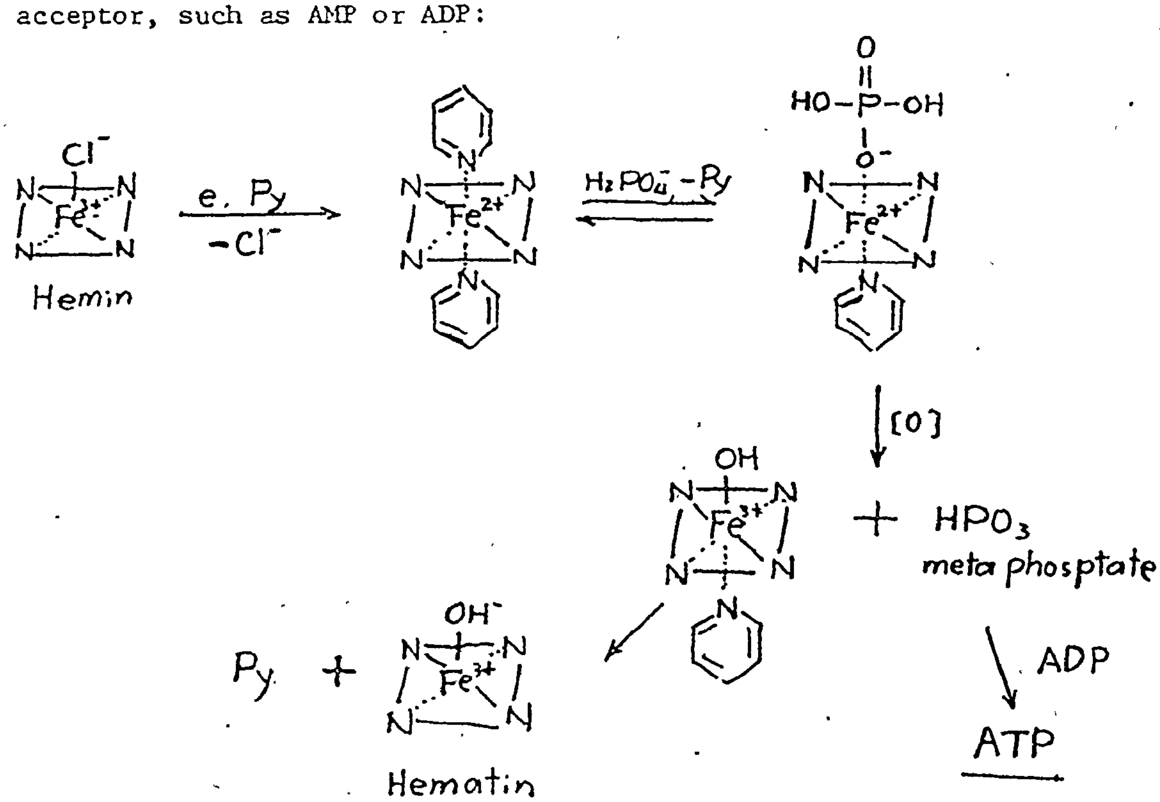
ATP analysis is done by using luciferin-luciferase method described on page 114. Reaction time: 10 hours.

Experiment	Condition	Hemin	Pyridine	ATP-Counts
1	BQH_2, air	2.3×10^{-3}M	0.35 M	860
2	BQH_2, N_2	2.3×10^{-3}M	0.35 M	26
3	BQH_2, air	2.3×10^{-3}M	0.00 M	711
4	DQH_2, air	2.3×10^{-3}M	0.35 M	167840
5	DQH_2, N_2	2.3×10^{-3}M	0.35 M	520
6	DQH_2, air	2.3×10^{-3}M	0.00 M	3260

All systems contain 7.4×10^{-4} M AMP and 8.1×10^{-3} M H_3PO_4. BQH_2: hydroquinone, 2.28×10^{-2} M. DQH_2: durohydroquinone, 2.28×10^{-2} M.

When it is reduced in the presence of pyridine, the complex becomes "low spin" with an octahedral structure. The higher redox potential observed in dipyridine hemochrome in comparison with the potential of hemin is probably caused by the ligand field stabilization energy of dipyridine hemochrome.

Regarding the possible mechanism, the phosphate coordinated to the ferrous atom may be activated by a strengthening of the iron-oxygen bond. This can be achieved by oxidizing the iron to the Ferric state. The iron atom will then be coordinated with a hydroxyl group. When the iron-oxygen bond is strengthened by the oxidation, the adjacent phosphorus-oxygen bond may be weakened. Thus the process may result in transferring the phosphoryl group to an acceptor, such as AMP or ADP:



ATP-forming model system involving the aerobic oxidation of dimidazole hemochrome in the presence of ADP and inorganic phosphate has been studied by Brinigar et al.[307] Based on the observation in a series of subsequent studies,[308-9] a reaction mechanism was postulated.[310] According to the proposed, mechanism, phosphate activation is achieved by reacting with imidazolyl radical which is produced by a 2-electron oxidation of diimidazole ferrohemochrome by dioxygen. The phosphate activation eventually leads to the formation of a "high energy" phosphorylating compound, 1-phosphorylimidazole, capable of phosphorylating ADP into ATP.

Several points should be mentioned on this postulated mechanism:

(i) Imidazolyl radical has not be detected in the system.

(ii) Concentration of imidazole present in the ATP generating system is extremely low. Under such conditions, a part of imidazole ligand may dissociate from the hemochrome. If an excess imidazole is added, ATP yield is drastically reduced.

(iii) Not only imidazole, pyridine may also be used as the ligands in the ferrohemochrome and an aerobic oxidation of the dipyridine ferrohemochrome produces even more ATP from inorganic phosphate and ADP.

The aforementioned observations are difficult to be explained by the mechanism proposed by previous authors. However, they can be readily explained by the mechanism proposed in this Chapter.

CHAPTER VI

CONCLUSION AND PERSPECTIVE

Based on the study of model systems described in the preceeding chapters, the following conclusions are drawn:

(A) ATP formation can be brought about by a one-electron reduction of AMP or ADP in the presence of inorganic phosphate. A weak reducing agent with a redox potential near 0.05 volt or lower is sufficient to initiate the phosphorylation reaction.

(B) ATP formation can also be initiated by an oxidation of inorganic phosphate. A strong oxidizing agent with a redox potential higher than + 0.8 volts is necessary for the reaction to occur. Delta singlet dioxygen has been shown to possess such a high oxidizing power.

(C) If inorganic phosphate is brought to coordinate with the ferrous ion in a heme-complex, aerobic oxidation of the resultant phosphate-heme complex may lead to the activation of the phosphate, which in turn, may lead to the phosphorylation of AMP or ADP.

Since the conclusions are drawn purely from the study of physical and chemical model systems, it is still improper to state whether those reaction mechanisms are operating in living systems. However, the present author believes that all the conditions necessary to bring about the phosphorylation of ADP into ATP by following the aforementioned reaction mechanism do exist in living systems.

Energization of biological membranes always involves a creation of a reducing power. As described in Chapter I, many reductants created in living systems have redox potentials low enough to reduce ADP into the radical form. Even in ATP-synthetase itself, there are several reducing centres such as thiol groups present which have redox potentials capable of reducing ADP.[311] Close contact of an electron transport chain and ATP-synthetase may constantly provide such reducing power in the enzyme. If the ADP is activated by reduction in this manner followed by its aerobic oxidation in the ATP forming process, the ADP may be considered as an electron-transport member in biological systems.

Numerous experiments have shown that proton transport across biological membranes is in some way associated with ATP formation, The strongest experimental support for Mitchell's chemiosmotic hypothesis is the acid-base transition experiment of Jagendorf et al. on isolated chloroplasts.[226-7] However, contents of chemiosmotic hypothesis in the present form is still at physiological level, not at molecular level. We don't know what functional groups are involved and by what molecular mechanism is the proton movement and ATP synthesis connected.

Suppose that an internal acidification of ATP-synthetase causes a conformational change in the enzyme by which some thiol groups are exposed for ADP reduction, the resultant one-electron reduced ADP may lead to the ATP formation. In this respect, it has been reported that changes in pH may result in a change in the conformation of ATP-synthetase in yeast mitochondria.[312] Furthermore, several investigations have indicated that thiols are crucially involved in energy transduction processes.[313-5] Thiol reagents have been shown to be good inhibitors of ATP-related reactions. In addition, by applying titration methods, studies on the behavior of thiol groups in many other enzymes also have shown that exposure of the thiols to the titors is very sensitive to changes in the chemical environment of the enzymes.[322-4] Conformational structures must have changed accordingly.

Regarding the proposed mechanisms involving one-electron reduction of ADP, ESR studies on mitochondria have demonstrated an increase in the concentration of unidentified radical(s) when the mitochondria are energized but before ATP formed.[325-6] The paramagnetic precursor of ATP may be identical to the so-called "non-phosphorylated high energy intermediate," which may in turn be identical to the one-electron reduced ADP radical tightly bound to the ATP-synthetase. Lifetime of the ADP radical may further be prolonged by protonation of the amino group of its adenine moiety so that disproportionation of the radicals can be prevented by the electric repulsion among the protonated radical species.

In effect this newly proposed molecular mechanism of ATP formation catalyzed by ATP-synthetase may serve to unify the currently existing hypotheses of biological energy transduction described in Chapter I. This new concept may be depicted as in the following diagram:

Electron transport

$\downarrow H^{\oplus}$ movement

Internal acidification of a bio-membrane including ATP-synthetase

$\downarrow$

Conformational changes of ATP-synthetase bring about the contact between a SH group and a bound ADP

$\downarrow$

One-electron reduction of ADP by the SH group

$\downarrow$

"Non-phosphorylated high energy intermediate"- a protonated bound ADP radical (see Figure 9, I)

$\downarrow P_i$

"Precursor of Phosphorylated high energy intermediate"- a ADP-P_i radical (see Figure 9, II)

$\downarrow O_2$

"Phosphorylated high energy intermediate"- newly formed bound ATP

$\downarrow$ ADP (medium)

Phosphoryl transfer from a bound ATP

$\downarrow$

ATP (medium)

Up to the present, available experimental data have definitely demonstrated the involvement of proton movements in ATP formation. However, the existing data have not proven that this is the only pathway of biological energy transduction. Therefore, consideration of other possible pathways is still meaningful.

Singlet dioxygen is detected in living systems.[316-9] This species is only known to exert a destructive effect. Can it also contribute in a positive way, for a living system namely in ATP formation? It is known that cyclic photophosphorylation is more efficient when dioxygen is present.[130-5] One should investigate whether singlet dioxygen is directly involved in an energy transduction process.

In plant light absorbed by PS II creates a reaction centre where water is oxidized to dioxygen. To be able to do this, the reaction centre must have a redox potential higher than +0.82 volts.[48] Whether inorganic phosphate can be activated by reacting with this highly oxidative reaction centre remains to be investigated. It is known that one of the photophosphorylation sites is associated with the PS II.[122-9]

Finally, in cytochrome-c oxidase complexes, one of the two axial ligand positions in cytochrom-a_3 is occupied by the imidazole group of histidine. The ligand <u>trans</u> to the imidazole is freely exchangeable with exogenous ligands.[320] Inorganic phosphate may be activated by coordinating at this site. This possibility has not been investigated in living systems. The nature of the energy transduction mechanism within cytochrome-c oxidase complexes is still under dispute.[327-8]

APPENDIX I

ON SOME CHEMICAL MODELS OF ATP FORMATION

We here describe two classes of chemical models for the production of ATP.

A. Phosphorylation Effected by Imidazole Radical Generation

Wang and co-workers have postulated that the activation of inorganic orthophosphate may be initiated by reacting with imidazole radical.[307-310] 1-Phosphorylimidazole is considered as the primary phosphorylating agent produced which then gives rise to ATP *via* phosphoryl transfer to ADP (or AMP). According to these authors, the imidazole radical can be produced photochemically in a mixture containing imidazole and a porphyrin compound;[307-8] or generated non-photochemically by two-electron oxidation of imidazole ferrohemochromes.[307,309,310]

Based on the results reported in this thesis, alternative mechanisms for the above mentioned reactions are proposed. The mechanism for the photochemical model is described and discussed in CHAPTERS III and IV, and that for the non-photochemical model, in CHAPTER V.

B. Phosphorylation Effected by the Generation of Electron-deficient Sulfur Compounds

ATP formation through the formation of a high energy phosphorylated intermediate, derived from an electron-deficient sulfur-containing species, has been reported independently by several investigators.[257-9,330] Molecular bromine, Br_2, is used as the oxidizing agent for the generation of the electron-deficient reaction centre in sulfur compounds.[257-9]

This work provided evidence for the involvement of electron-deficient sulfur compounds in the activation of phosphate. However, one of the papers clearly shows that bromine alone at higher concentrations can also give rise to ATP from ADP and phosphate in dry pyridine solutions in the absence of sulfur-containing compounds.[257]

The work reported in this thesis suggests that bromine may act as an oxidizing agent for the activation of phosphate. This possibility is discussed in CHAPTER III.

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