3419

NATIONAL LIBRARY

OTTAWA

CANADA

BIBLIOTHÈQUE NATIONALE

OTTAWA

NAME OF AUTHOR....JOHN RANDAL VOSE.....

TITLE OF THESIS...ENERGY DEPENDENT REACTIONS IN CHLOROPLASTS

.....

.....

UNIVERSITY....UNIVERSITY OF ALBERTA, EDMONTON, ALBERTA.....

DEGREE...Ph.D.....YEAR GRANTED....1968.....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed)...J. R. Vose.....

PERMANENT ADDRESS:

.51,.Delamere.Road,..

.Gatley,.Cheshire,...

.ENGLAND.....

DATED.February.20th,.1969

NL-91

THE UNIVERSITY OF ALBERTA

STUDIES ON ENERGY DEPENDENT REACTIONS IN ISOLATED CHLOROPLASTS

by

© JOHN RANDAL VOSE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

May 1968

ABSTRACT

This investigation was undertaken to study certain energy requiring reactions in isolated spinach chloroplasts. The major reaction studied was CO_2 fixation. By utilization of recent advances in chloroplast isolation techniques, it was found possible to isolate chloroplasts with a CO_2 fixation rate near that of the *in vivo* rate. These advances are based mainly on a rapid isolation method combined with the use of HEPES buffer, and the inclusion of inorganic pyrophosphate.

The role of inorganic pyrophosphate in certain endergonic reactions was investigated. It was shown that the addition of inorganic pyrophosphate enhanced both CO_2 fixation and calcium ion uptake. This effect was the same as that shown by addition of adenosine triphosphate. The enhancements of CO_2 fixation by adenosine triphosphate or inorganic pyrophosphate were equally affected by changes in pH and magnesium ion concentration. The energy transfer inhibitor, phloridzin, prevented utilization of these high energy compounds for CO_2 fixation. The main difference in the two reactions was that adenosine triphosphate could enhance CO_2 fixation in a dark reaction, while inorganic pyrophosphate could not. It was concluded that pyrophosphate may be utilized in a light dependent reaction with the associated enhancement of CO_2 fixation.

The mechanism of ATP synthesis was studied, and preliminary evidence for the presence of two reactions of adenosine triphosphate formation was produced. The major difference lay in their response to the inhibitor, iodoacetamide.

Finally, the effects of two uncouplers of electron transport from phosphorylation, phloridzin and quinacrine, on adenosine triphosphate synthesis and CO_2 fixation were tested. At a concentration severely inhibitory to adenosine triphosphate synthesis, there was only a slight inhibition of CO_2 fixation. This was especially clear when the initial kinetics of adenosine triphosphate synthesis and CO_2 fixation were studied.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Mary Spencer for her encouragement, advice and interest given throughout this work.

Thanks are given to my colleagues, Dr. Ming Ho Yu, Miss Enid Davidson, and Messrs. Ron Knight, Robert Stinson and Hugh White for their helpful criticisms and suggestions. The assistance of Mr. Peter Shargool of the Botany Department is also acknowledged, as is that of Mr. Gordon Heaps of the University Greenhouse Staff.

I am indebted to Dr. Hiruki and Dr. Milligan for use of their Scintillation Counters during part of this investigation.

I am grateful to the National Research Council of Canada for a Scholarship, and the University of Alberta for a Dissertation Fellowship.

I thank Mrs. Doreen Simpson for typing the manuscript. Finally, I wish to acknowledge the assistance and moral support given by my wife, Nancy.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP: Adenosine triphosphate

ADP: Adenosine diphosphate

PPi: Inorganic pyrophosphate

Pi: Inorganic orthophosphate

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)

cpm: Counts per minute

HEPES: N-2-hydroxy ethyl piperazine-N'-2 ethane sulphonic acid

TES: N-tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid

MES: 2-(N-morpholino)ethane sulphonic acid

TRIS: Tris(hydroxymethyl)aminomethane

TRICINE: N-tris(hydroxymethyl)ethane sulphonic acid

PPO: 2,5-diphenyloxazole

POPOP: p-bis 2-(5-phenyloxazaloyl)-benzene

EDTA: Disodium ethylene diamine tetra-acetic acid

IBB: Isobutanol-benzene reagent

PMS: Phenazine methosulphate

PSP: Photophosphorylation

DTT: Dithiothreitol

R5P: Ribose 5-phosphate

INTRODUCTION

General remarks on the problem

At present, knowledge of the photosynthetic process is of an extremely diverse and complex nature. Many aspects of photosynthesis have been studied in great detail, but it has been only recently that chloroplasts studied *in vitro* could react in a manner akin to that observed in life. In this thesis, chloroplasts retaining many of their "normal" characteristics were isolated, and used to study the mechanism by which energy is utilized for endergonic reactions of photosynthesis. Chloroplasts were studied both with and without non-physiological catalysts of electron transport. It is hoped that the results obtained may have a close bearing upon the naturally occurring process of photosynthesis and present some new aspects of this old problem.

The last few years have seen a great leap forward in our knowledge about photosynthesis. In a review article in 1954 (1) it was noted that the chloroplast was a much simpler system than that required for photosynthesis, and was the site of only the light-absorbing and water-splitting reactions of the overall photosynthetic process. Photosynthesis was regarded at that time, as was fermentation in the days of Pasteur, as a process that could not be separated from the structural and functional complexity of whole cells.

That the locale of carbon dioxide fixation was actually within the chloroplasts was discovered with the aid of radioactive carbon and with newly developed chromatographic techniques by Arnon's group (2) in the mid-1950s. The same group later demonstrated (3) that chloroplasts isolated from spinach leaves could metabolize $^{14}CO_2$ to carbohydrates, including starch, with a simultaneous evolution of oxygen. The detailed pathway of CO_2 fixation and reduction was investigated by Calvin, Benson, Bassham and their coworkers (4). They identified 3-phosphoglyceric acid and other intermediates of glycolysis, as being among the early products of photosynthesis. Bassham and Calvin (4) proposed a photosynthetic carbon dioxide reduction cycle - a sequence of reactions for the assimilation of CO_2 in which the only reductive step was the conversion of 1,3-diphosphoglyceric acid to glyceraldehyde 3-phosphate. This conversion is believed to utilize ATP as an energy source, and NADPH as the reducing agent.

During this period, the light-induced phosphorylation of ADP by chlorophyll-containing cellular organelles at the expense of radiant energy (photophosphorylation, PSP) was discovered independently by Arnon's group (2), and by Frenkel (5).

This phosphorylation was capable of coupling with a light-driven transfer of electrons from water to NADP (or to a non-physiological electron acceptor, such as ferricyanide) with a concomitant evolution of oxygen (6). This reaction was termed non-cyclic photophosphorylation.

Since then, studies on photosynthesis have produced a prodigious amount of knowledge about many of these reactions. It is apparent that those aspects of this problem that are readily studied by means of standard biochemical techniques have received most attention. On the other hand, the more involved and intricate reactions, e.g. the mechanisms involved in the photolysis of water, have received far less attention. It is not surprising that one of the most studied aspects of photosynthesis, namely PSP, is at present the site of much controversy. There is so much known that clarity has tended to become obscured by the wealth of information covering many aspects of this problem.

There have been a number of review articles covering many aspects of photosynthesis published in recent years. These have taken the form of books, compiled papers from conferences, and articles in review publications. A list is given of some of the more useful ones (7-15).

The central problem of the last few years has been the elucidation of those photo-chemical reactions that provide

the energy necessary for the endergonic fixation of carbon dioxide into cellular constituents. In this thesis, some of the diversified studies on PSP, ion uptake, conformational changes on chloroplasts and CO_2 fixation will be reviewed. The object of this study is to attempt to pull some of these studies together into a unified position. Data will be presented that shed new light on the function and nature of the high energy intermediates produced prior to, or alongside, ATP synthesis. It is hoped that this work will clarify some of the steps involved in the utilization of energy in the photosynthesis of higher plants. Finally some studies that are relevant to the evolutionary development of PSP will be considered.

The Mechanism of Energy Conservation

The mechanism that links the redox reactions of electron transport in either chloroplasts or mitochondria with the synthesis of ATP is at presently only partially understood. In spite of the tremendous amount of both time and money spent over the last few years in the investigation of this problem, the solution still appears to be a long way off. Hypotheses regarding the operation of this mechanism have been proposed by a number of workers, and the controversy resulting from these has been both vociferous and lively. The main argument at present rests on the validities of the traditional chemical theory (16,17), and the chemi-osmotic theory proposed by Dr. P.

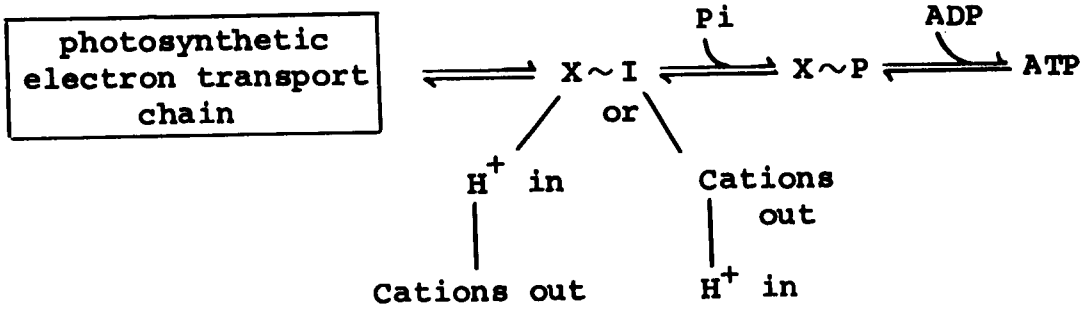
Mitchell in Britain (18,19). Figure I illustrates the essential features of these two theories, and points out the main differences between them. Each of these theories has received wide support, and the present view appears to suggest that both these hypotheses may hold part, but not all, of the answer to this problem. Both theories were originally developed to explain the mechanism of energy conservation in the cytochrome chain of mitochondria, and are also considered to account for the process by which energy is conserved in photosynthetic systems.

The Chemical Intermediate Theory

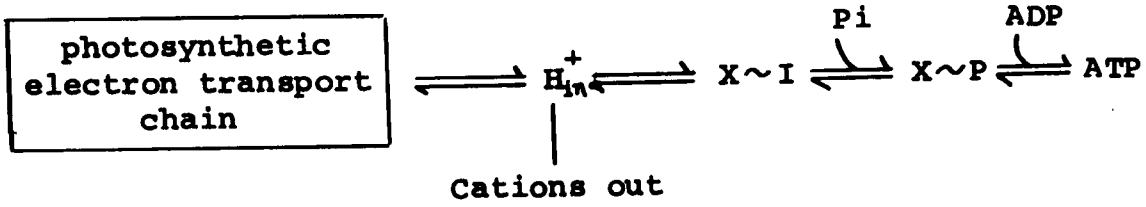
The basic concept of the traditional theory is the formation of a chemical intermediate. Electron transport along a chain of oxidation-reduction intermediates is thought to lead to the formation of an intermediate that is generally termed X~I. The "squiggle" indicates a "high-energy linkage". This is shown diagrammatically in Figure Ia. The generally recognized function of X~I is to lead to the phosphorylation of ADP to give ATP. In the chemical intermediate theory another function of X~I is to activate a primary movement of protons, coupled to a secondary counter-movement of cations; or a primary movement of cations coupled to a secondary counter movement of protons. In the chemical mechanism, the proton gradient is a result of X~I, while in the chemi-osmotic theory the proton

Figure I. Diagrammatic representation of the Chemical Intermediate and the Proton Gradient theories of Energy Conservation

(a). Chemical Intermediate:



(b). Chemi-osmotic Theory:



"In" and "Out" refer to movements of protons or cations into or out of the chloroplast.

gradient itself is coupled to electron transport to yield a high energy intermediate (Figure Ib).

The sequence of events proposed by the chemical theory for the mechanism of membrane-bound, coupled PSP is shown in Figure II. By some means not understood, a "high energy bond" is created upon the reduction of component C by the BH_2-I complex. The energy stored in the B$\sim$I is utilized for ADP phosphorylation, through the formation of a P$\sim$I compound, and B is then released for further electron transport.

Support for this hypothesis would come from isolation and identification of even one of these "high-energy intermediates". Unfortunately for the proponents of this theory, no such evidence has been forthcoming, although numerous attempts have been made (see e.g. reference 20 for a list of proposed high energy intermediates of oxidative phosphorylation, and reference 21 for a proposed intermediate in PSP, a phospho-histidine).

The work of Kahn and Jagendorf (22) has pointed to the formation of a high energy phosphorylated intermediate capable of phosphorylating ADP to form ATP in the dark. The nature of this intermediate is unknown, but it is thought to be neither a protein-bound phospho-histidine nor protein-bound phospho-serine (23).

Figure II. The sequence of events in a typical chemical theory of photophosphorylation

electron transport:	AH_2	+	B	$\longrightarrow$ BH_2	+	A
	BH_2	+	I	$\longrightarrow$ BH_2-I		
	BH_2-I	+	C	$\longrightarrow$ B$\sim$I	+	CH_2
phosphorylation:	B$\sim$I	+	P	$\longrightarrow$ P$\sim$I	+	B
	P$\sim$I	+	ADP	$\longrightarrow$ ATP	+	I
uncoupling:	B$\sim$I	+	H_2O	$\longrightarrow$ B	+	I

taken from ref. (8), p. 70

A, B and C represent compounds of the electron transport chain, for example, plastoquinone, cytochrome b_6 and cytochrome f. I is termed a "coupling factor" and P refers to inorganic phosphate.

The existence of a high energy, non-phosphorylated compound or condition has been proposed independently by Shen and Shen (24), and Hind and Jagendorf (25). This was termed "X_e", the "X" for unknown and the "e" for energetic! (26), and was presumed to be active in the formation of the phosphorylated intermediate mentioned above. It can be synthesized in the light in the absence of added ADP, inorganic phosphate (Pi), or Mg^{2+}, and if these are then added in the dark, ATP is formed. This intermediate was found to be very labile, with a half-life of 0.5 seconds at pH8 and room temperature, or two seconds at $3^{o}C$ (25). Since redox dyes such as reduced pyocyanine were necessary in the light phase for formation of the X_e, it is presumably formed concomitantly with, rather than before, electron flow. It has been suggested that X_e may itself be a proton gradient, and is able to induce synthesis of phosphorylated intermediates (26). Also, it is possible that the X_e may represent a "side-pool" of energy storage able to drive the phosphorylation mechanism but not essential for its functioning (26). The initial rate of X_e accumulation was found to be only 20% of the rate of ATP formation, while large amounts of X_e (measured by its capacity to initiate ATP synthesis in the dark when the phosphorylation intermediates are added) were still formed in the chloroplasts (26). If the X_e represents a sidepool of extra energy, its major significance

may lie in ion transport (27), induction of reversed electron transport (28), light-induced conformational changes of chloroplasts (29), or even of the initial steps of CO_2 fixation itself. These points will be developed later on in this thesis.

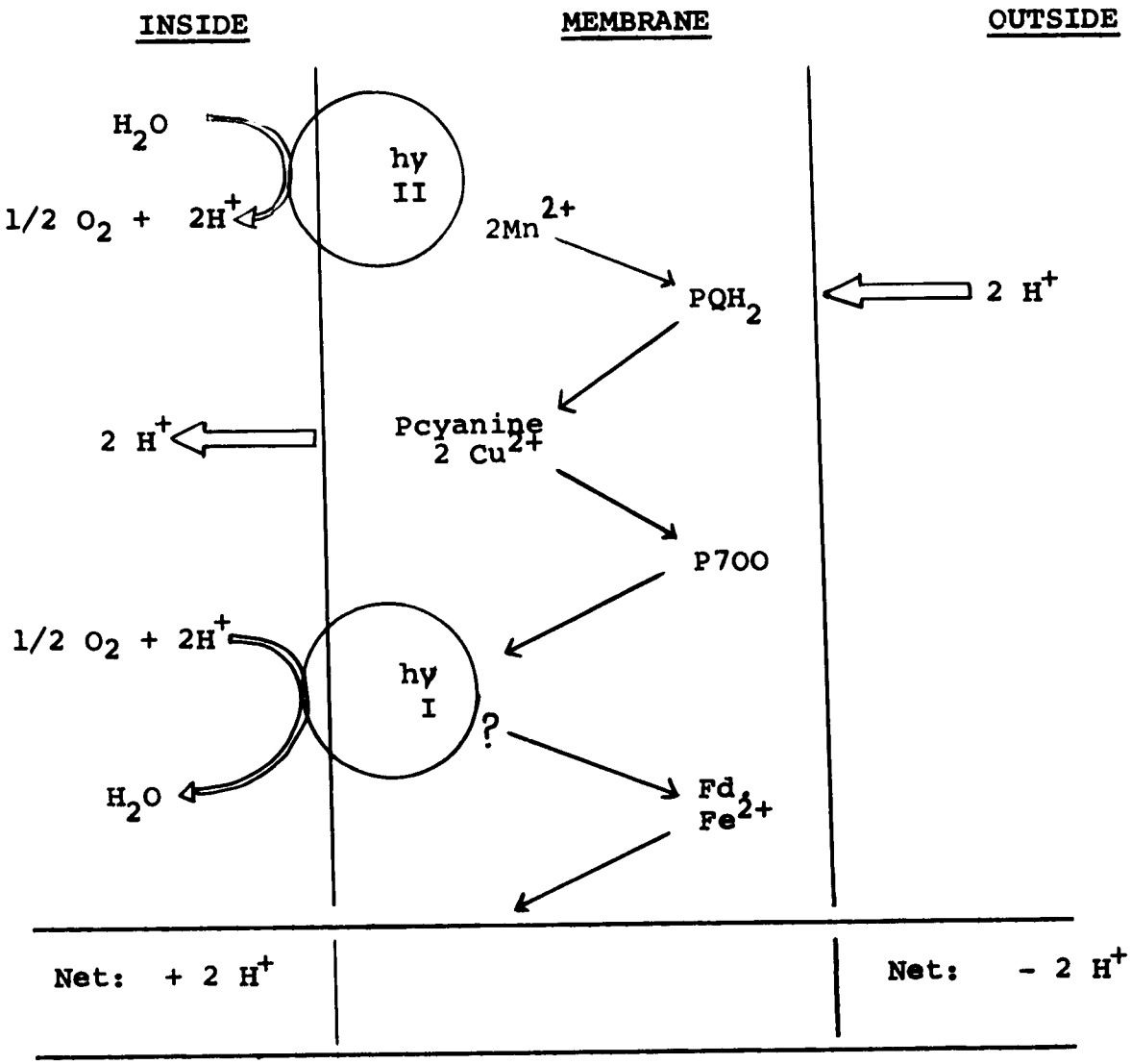
The Chemi-osmotic Theory

It should be emphasized that the identity of the X_e is not known, and this difficulty in identification of an intermediate of a "high-energy" nature led Mitchell to develop his elegant yet controversial chemi-osmotic theory. This theory has no requirement for high energy intermediates until just prior to the synthesis of ATP, and as mentioned earlier defines X_e as an unstable proton gradient within the granal membrane. The essence of the Mitchell theory has been summarized and criticized by Slater (16). There is a requirement for

(1) a proton-translocating reversible ATPase system
(2) a proton-translocating oxido-reduction chain
(3) an exchange diffusion system coupling proton-translocation to that of anions and cations
(4) a membrane that is impermeable to the passive movement of ions, in which points 1-3 reside

Jagendorf (30) proposed a scheme for photosynthetic electron transport as applied to the chemi-osmotic hypothesis, and the elements of this are illustrated in Figure III. It should be noted that the chemi-osmotic theory as it stands at

Figure III. Diagrammatic Scheme of the Chemi-osmotic Hypothesis



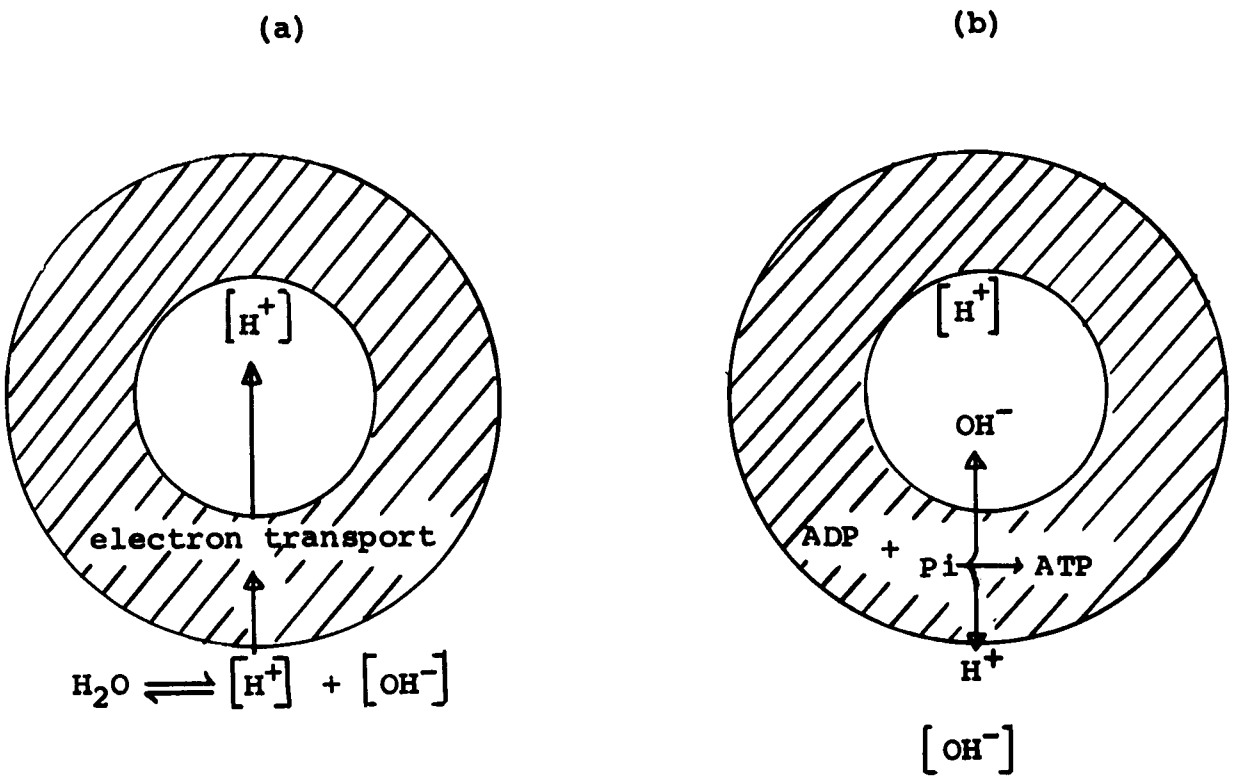
Key: hy = photosystem
PQH_2 = reduced plastoquinone
Pcyanine = plastocyanine (copper containing intermediate)
P700 = chlorophyll a "P700"
Fd = ferredoxin (containing a non-heme iron)

(Adapted from reference (8), p. 71)

present, depends largely on conjecture regarding the spatial orientation of the intermediates of electron transport in a membrane impermeable to protons. It is envisaged that the primary event of energy coupling is the flow of protons and electrons through the photosynthetic chain. There then follows a mandatory separation of the proton and electron, resulting in a vectorial movement of protons across the semi-permeable membrane. A secondary event is the conversion of this proton gradient into an electron motive force gradient (20). It is important to remember that this theory still requires the formation of chemical intermediates (see Figure I) in order to phosphorylate ADP. No model system has yet been set up to support the Mitchell theory, and he has relied strongly on postulates, backed by some experimentation. For this theory to be tenable, it is essential that the electron transport components are in the "correct" order (as shown in Fig. III), and "folded" with just the right geometry. The end result of this process is a translocation of hydrogen ions in a definite direction as a direct result of electron transport through the membrane-bound electron carriers. Energy is stored in the creation of this concentration difference. That the medium becomes less acid upon illumination of a chloroplast system has been observed many times (31,32). This rise is taken to signify an uptake of hydrogen ions by the chloroplasts, with a

consequential increase in the proportion of hydroxyl ions outside the chloroplasts. This work has been interpreted as favouring the Mitchell theory. Extra support has come from the work of Dilley and Vernon (33) who demonstrated that chloroplasts excrete potassium and magnesium ions at the same time that they are apparently picking up protons. These changes in pH and the ion translocations will be discussed in greater depth later in the thesis. However, this brief comment serves to introduce the interesting work of Jagendorf and Uribe (34). They postulated that if the Mitchell theory held they should be able to induce synthesis of ATP by artificially producing a proton gradient in dark incubated chloroplasts. They "loaded" the "inside" of the chloroplasts with protons by incubating them in an acid-medium (at pH 4.0). This would simulate formation of a high energy state or pH gradient. Then after 60 seconds, the chloroplasts were injected into a basic medium (pH 8.0) (see Figure IVa) containing ADP, inorganic phosphate, $MgCl_2$ and enough NaOH to neutralize the buffer used in the acid stage. Presumably this alkaline pH was necessary for inducing the enzymic phosphorylation reaction. ATP was indeed synthesized, and this synthesis was demonstrated to be absolutely dependent on achieving a pH below 7 in the acid stage, and on added phosphate and ADP in the alkaline stage. The energetic condition produced during this acid-base transition, and that was found to be capable of forming

Figure IV. Diagrammatic representation of PSP



(from Jagendorf (9), p. 73)

ATP, was shown to completely decay after 8 seconds, with a half-life of 1-2 seconds. This dark ATP formation could be severely inhibited by a number of known uncouplers of electron transport from phosphorylation including quinacrine (atebrin), NH_4Cl and phenyl mercuric acetate. This work has since been repeated and verified by McCarty and Racker (35) who were able to inhibit the reaction with the uncoupler Dio 9, and also with an antibody to part of the ATPase system. The "acid-stage" of this reaction, presumably induces the formation of a pH gradient or membrane potential, that is then able to generate ATP at an alkaline pH. The mechanism by which an "acid-bath" can induce "active" transport of protons across the "ion-impermeable" membrane (16) is not yet known.

These results were considered to be consistent with the chemi-osmotic theory (20), and of special interest is the fact that the acid-base induced state has several features in common with the X_e of Hind and Jagendorf (25). However, a new factor in the pH-jump experiments was the enhancement noted when certain organic acids, especially succinic acid, were added in the reaction buffer at the acid-stage. It was considered (34) that the added organic acids could penetrate into the chloroplasts in their undissociated forms (without the need to expel cations) and provide an internal reservoir of dissociable protons thereby accounting for the higher yields of ATP. Later

work (36) by Uribe and Jagendorf showed that succinate could penetrate quite rapidly at pH 4.0, but at pH 6.5 where succinate is fully dissociated, there was little or no penetration, or augmentation of ATP production. Also (37) they demonstrated that of the various organic acids studied for their effectiveness in this process, dicarboxylic acids of 4 and 5 carbon atoms with one pKa in the range of 4.2 - 4.4 and the second at 5.3 - 5.5 were the most effective in increasing the yields of ATP in their acid-base transition experiments. The diversity of organic acids having some effect made it seem unlikely that they were simply serving as substrates for enzyme systems in the chloroplasts.

The high internal reservoir of protons is thought to drive ATP synthesis by promoting the dehydration reaction of phosphorylation. This is shown diagrammatically in Figure IVb.

Recently Kaplan, Uribe and Jagendorf (38) demonstrated that the acid-base transition technique could also induce the operation an ATP hydrolysis reaction, if a sulphydryl-protectant reagent, for example, dithiothreitol, and magnesium ions were present. This acid-base induced ATPase was susceptible to the same inhibitors as the light-triggered ATPase (39,40). The amount of ATP hydrolyzed was dependent on the size of the organic pool used, 10mM succinate being optimal (the same as for ATP synthesis (34)). It was concluded that despite some

differences, acid-base induced ATP hydrolysis was the counterpart of acid-base induced ATP synthesis. However, the actual mechanism by which a proton flux could induce ATP hydrolysis was not clear from this work.

Utilizing this acid-base transition mechanism, Mayne and Clayton (41) were able to induce spinach chloroplasts to emit a flash of light having the spectrum of chlorophyll fluorescence. This chemi-luminescence was either abolished or inhibited by several inhibitors or uncouplers of photosynthetic phosphorylation, including ammonia, methylamine, CCCP (m-chloro carbonyl cyanide phenylhydrazone) and desaspidin. There was also a requirement for certain anions, for example, succinate, malate and fumarate during the acid-phase. Thus it appeared that the state produced by acid-base transition could act as a starting point, not only for ATP synthesis (34) and ATP hydrolysis (38), but also for a sequence of back-reactions culminating in the excitation of chlorophyll and consequent luminescence.

Later, Reid, Moyle and Mitchell (42) attempted to use similar techniques to those of Jagendorf and Uribe (34) for the synthesis of ATP in rat liver mitochondria, utilizing the knowledge that in oxidative phosphorylation, protons are ejected and not taken up from the medium, as is the case with chloroplasts. They immersed the mitochondria in alkali (pH 9) for 2 minutes,

and then into acid at pH 5. Small amounts of ATP were synthesized, and this synthesis was abolished by oligomycin. They concluded that this proton-driven ATP synthesis was more transient than that observed in chloroplasts. This may be ascribed to the relatively vigorous ATPase activity in mitochondria, which unlike that of chloroplasts, does not require thiol or light-activation. The reaction was conducted in the presence of both potassium ions and the antibiotic valinomycin (which induces potassium efflux). This may have some bearing on their results as will be noted later.

Chance, Lee and Mela (20) noted that pH jump experiments in membraneous systems caused large changes of the redox states of the respiratory (and also the photosynthetic) electron carriers. They pointed out that this must be taken into consideration in the calculation of ATP formation in these systems.

Cockrell, Harris and Pressman (43) also using rat liver mitochondria, indicated from their results that neither potassium efflux, nor hydrogen ion accumulation per se was necessarily linked to the formation of ATP. They showed that valinomycin would increase access of potassium to an energy-linked cation transport system tightly coupled to ATP synthesis. They were able to induce a net synthesis of ATP presumably driven by the stimulation of potassium efflux by valinomycin. Based on the

premise of Chance and Mela (44) that the mitochondrial membrane pH gradient is only a fraction of a pH unit, Cockrell et al (43) deemed it unlikely that the movement of protons down a pH-gradient could supply the energy necessary for driving ATP synthesis.

Mitchell and Moyle (45) have reported that protons were ejected from rat liver mitochondria when ATP was being hydrolyzed. Their conclusions have recently been questioned by Rossi, Carafoli, Bielawski and Lehninger (46) who suggested that these exchanges are a result of the presence of endogenous bivalent cations, and considered that movements of hydrogen ions associated with calcium accumulation by mitochondria were the consequence rather than the cause of calcium uptake. Calcium uptake was considered to be a result of activity by a hypothetical high energy intermediate.

Lynn and Brown (47) demonstrated that protons could temporarily inhibit the rate of phosphorylation. This fact was consistent with the Mitchell theory because a rapid increase in the external proton pressure would require the consumption of energy to redistribute the increased proton pressure against the proposed anisotropic membrane before phosphorylation could again proceed. However, they also showed (48) that hydrogen ions, phosphate, and ammonium ions all competed for chloroplast phosphorylation sites suggesting that protons competitively inhibited phosphorylation by displacing phosphate from electron-

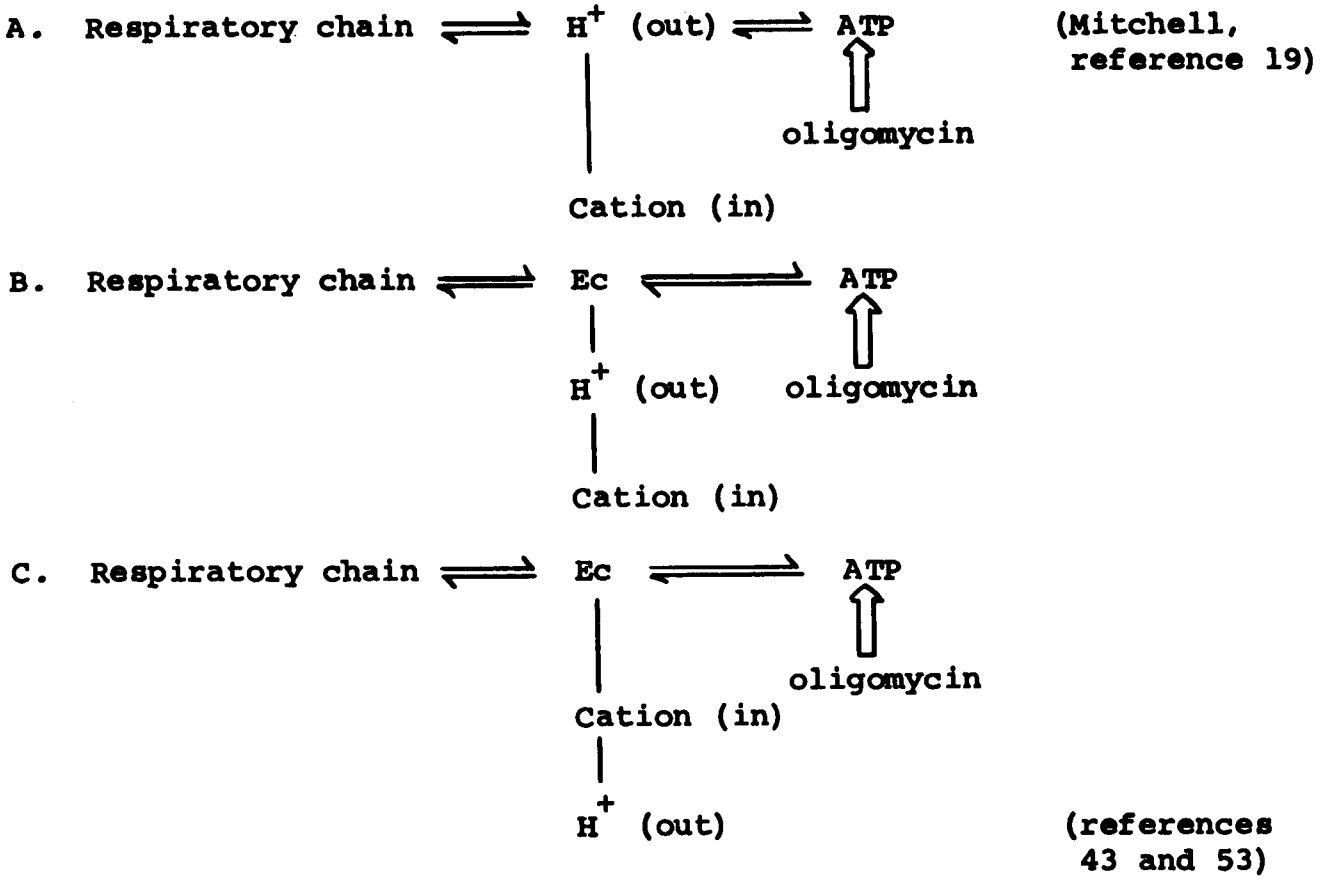
activated phosphorylation sites. The fact that protons inhibited electron transport also suggested that they uncouple phosphorylation not by creating an energetic drain on the system (19), but rather by binding of the protons with the phosphorylative site. They proposed that the initial energy-requiring step in the phosphorylation reaction was the removal (or "activation") of the proton from bound-magnesium phosphate by the passage of an electron through a semi-conducting anionic lattice, possibly acidic lipids.

Chance, Nishimura, Avron and Baltscheffsky (49) took advantage of the fact that the Mitchell theory requires that every time an electron moves and leaves an oxidized hydrogen carrier, a proton must be anisotropically separated from its hydrogen ion. Therefore upon illumination of the chloroplasts, a "jump" in the internal proton concentration should occur. They used the bromothymol blue technique (50) that has been successfully used with mitochondria, and is found to bind tightly to the inside of the mitochondrial membrane, with a preparation of bacterial chromatophores. They were able to measure a uptake of protons in this system, equivalent to only about 1 proton per 700 chlorophylls. This was judged to be far less than would be expected if the Mitchell theory was valid. The pH changes generally observed by other groups of workers were considered (49) to result from the movement of cations from the intra-

vesicular space through the membrane to the solution surrounding the vesicles. The cation would leave a proton within the vesicle, and carry an OH^- to the outside. Baum (51) has, however, drawn attention to the fact that the chemi-osmotic hypothesis does not actually require a significant pH difference across the membrane system (also see reference 19), and he concluded that the experimental facts may well be consistent with the driving of ATP synthesis by a proton current that flows through the reversible ATPase system in the membrane under a mainly electrical proton motive force, not a pH differential. He queried, though, how the small number of free protons in the inner aqueous phase could permit the flow of a effective proton current. This was explained by Mitchell (52) by noting the buffering capacity within submitochondrial vesicles, that would limit the pH change of the inner aqueous phase to less than 0.1 pH units when 2 protons were translocated into or out of the resicle.

Slater (16) represented the possible mechanisms of energy coupling in mitochondria as shown in Figure V. He pointed out that an important test for the three schemes as presented here, is whether or not permeant cations are necessary for H^+ extrusion by mitochondria or H^+ uptake by chloroplasts, driven by respiration, photosynthesis, or by ATP. According to Figure VA and B_1 cations may not be necessary, while according to Figure VC, the high energy intermediate actively drives cations

Figure V. Possible mechanisms of energy coupling in mitochondria



Ec - high energy intermediate

"in" and "out" refer to movements of protons and cations into and out of the mitochondrial elementary particles.

into the mitochondrion, and as a result protons are extruded from the organelle in order to retain ionic equilibrium with the medium. Chance and Mela (53) demonstrated that although a proton gradient can be seen when respiration is linked to calcium uptake, no gradient was found in the absence of a permeant cation. Thus Slater supported the situation given in C, Figure V. This work applied to mitochondria, and it is not known whether or not the same situation can be applied to chloroplasts.

Recently more doubt has been cast on the validity of the chemi-osmotic hypothesis by Karlish and Avron (54). They proposed two critical tests of the Mitchell theory. One was that the ratio of protons taken up, to electrons transported should equal the number of sites of energy conservation in the electron transport pathway. They found the ratio of protons to electrons varied with pH, reaching a maximum of six at pH 6.0. The presence of six coupling sites is most unlikely, when considered in the light of all the other work on this aspect. Secondly, they pointed out that for the Mitchell theory to hold, a fall in the steady state extent of the proton gradient should be observed during phosphorylation, as a result of the stoichiometric utilization of one or two protons per ATP synthesized. However, they found that in the presence of a complete phosphorylating system, the steady state extent of the proton uptake by chloroplasts was increased! Another surprising result was that

in the presence of ADP and Mg^{2+}, arsenate would uncouple PSP, but unlike other uncouplers was found to either have no effect or to increase proton uptake. Because of these anomolous results, Karlish and Avron were led to propose a model in which the prime function of the proton pump was to enable co-transport into the chloroplasts of a negatively charged complex of HPO_4^{2-}, Mg^{2+}, and ADP^{3-}. Arsenate could substitute for phosphate in the complex.

In conclusion it can be seen that there is a considerable amount of data available both supporting and questioning the validity of the Mitchell hypothesis. Most of the work has been repeated many times in different laboratories, and the results given above are undoubtedly accurate for the particular in vitro conditions under which the experiments were conducted. Thus it is the interpretation of these facts which is the real problem, a task that has been accentuated by attempting to correlate results obtained in vitro to conditions occurring in vivo. To determine whether or not the proton gradient or membrane potential is the cause rather than an effect of ATP synthesis, more data are required. Also, these data should be derived from experiments designed to tackle the problem from a fresh angle, for example, intensive studies of fractionated mitochondrial or chloroplast membrane systems, reconstitution studies, isolation and identification of coupling factors and isolation and purification

of the ATPase are aspects of this problem that only now are beginning to receive attention.

The proposals of Mitchell have stimulated interest and research in this field. Out of this assortment of ideas, the answer to the precise mechanisms behind both oxidative and photosynthetic phosphorylations may well be found in the not too distant future. In the following sections some of the recent studies pertaining to ATP synthesis and hydrolysis mechanisms of chloroplasts will be discussed.

Stoichiometry of Energy Conservation

The energy of light quanta is utilized by chloroplasts to drive the synthesis of ATP and NADPH (2,5). These compounds are widely found in nature, and are known to drive a number of endergonic reactions. In the preceding pages, the initial steps by which electron flow may be coupled to the phosphorylation of ADP have been discussed. ATP is thought to be required for the phosphorylation of ribulose-5-phosphate, and for the conversion of 3-phosphoglyceric acid to 1,3-diphosphoglyceric acid. According to the scheme proposed by Bassham and Calvin (4), three molecules of ATP, and two molecules of NADPH are necessary for the assimilation of one molecule of CO_2. Other schemes for CO_2 fixation have been proposed by Warburg and Krippahl (55), Stiller (56), and Hatch and Slack (57), but in all four schemes the total energetic requirements are the same. The stoichiometry of

PSP has therefore received a lot of attention. When noncyclic PSP was first described by Arnon et al (58), the ratio of ATP formed, to electrons transported, was found to be unity. These data were confirmed many times in vitro (59-62), and in all cases the ATP/$2e^-$ ratio has been one or less. This ratio does not however, agree with the expected value obtained from the postulated Calvin CO_2 fixation mechanism. Attempts have been made to obtain higher values, mainly by altering the method of calculation. Values greater than one are obtained (62) if the Pi-enhanced reduction rate is corrected for the reduction occurring in the absence of Pi, and dividing this corrected value into the rate of phosphorylation. This practice has been queried by Punnett (63). He suggested that if a "correction" is to be made, it should be taken from the reduction rate found in the absence of ADP, Mg^{2+} and HPO_4^{2-} rather than just in the absence of ADP (also see reference 61). If this is done, the ATP/$2e^-$ ratio still lies from 0.80 - 1.0. Fewson, Black and Gibbs (64) were able to obtain ratios as high as 3.5, but only in the presence of high concentrations of NaCl (more than 0.2M); under most other conditions studied they found ratios of about one. Recently Lynn and Brown (65) observed P/$2e^-$ ratios of about 3 in isolated spinach chloroplasts with ferricyanide and benzoquinone as the terminal electron acceptors. However, they could not observe this high value when they used NADP (as the electron acceptor)

and spinach ferridoxin. It is most apparent that care must be taken in the interpretation of results obtained from *in vitro* experiments with nonphysiological catalysts. From this body of evidence, it would appear that for every NADP molecule reduced, one molecule of ATP is synthesized. However, it is possible that each site involved in ATP synthesis may require a different reaction condition for its operation. In this case, experiments utilizing only one set of conditions may select for the activity of one of the ATP generation sites, and another may be inhibited.

The number of phosphorylation sites along the electron transport pathway is also unknown. Fewson *et al* (64) concluded that there was only one site of PSP associated with NADP reduction (also see (14), p. 287), although Good (62) disagreed with this conclusion. Punnett (63) noted that both cyclic and noncyclic ATP production have the same number of sites, but whether this is one or more is not known. A new approach to this problem is necessary. Perhaps isolation and fractionation studies of the chloroplasts electron transport chain, utilizing Chance's cross-over theorem may be one way to resolve this problem.

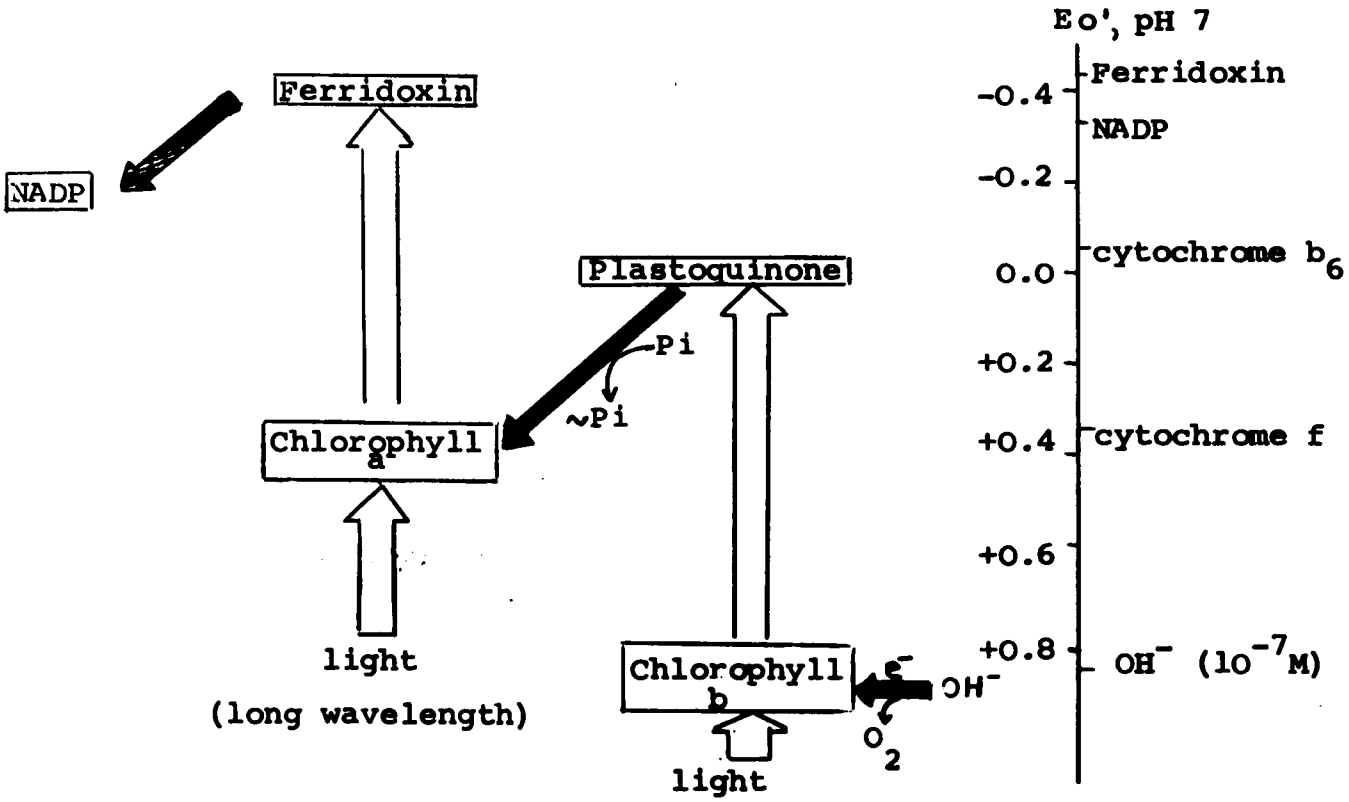
Photophosphorylation

The nature of PSP has been intensively studied by Arnon and his coworkers. Much of this research is summarized

in reference 11. The diagram in Figure VI gives a simplified scheme for electron transport and PSP. A variety of redox dyes can substitute for ferridoxin, perhaps the most common being phenazine methosulphate (PMS) (67). Also, the NADP may be replaced by other terminal electron acceptors e.g. ferricyanide. The two photosystems shown in Figure VI may be separated by use of particular reagents, e.g. H_2O can be prevented from donating electrons by the inhibitor CMU, (p-chlorophenyl-1,1-dimethyl urea) and by addition of an ascorbate/dye couple (68). Photosystem II can therefore be distinguished as a partial reaction by abstracting electrons from the electron transport chain. No ATP was formed, but oxygen evolution did occur. These two photosystems have been thoroughly studied (for review see reference 69), and although much is known of the sequence of intermediates and their nature (70), there is still a dearth of knowledge regarding the mechanisms of water photolysis. Recent work has centered on attempts to physically separate the discrete photosystems I and II, and some success has been achieved (71,72). Perhaps endeavour in this direction will shed some more light on this perplexing reaction of water photolysis.

In contrast there is a vast amount of literature covering the conditions necessary to observe ATP formation and the mechanism involved in the terminal stages of its synthesis. This has involved studies of exchange reactions, ATPases, possible

Figure VI. A scheme for the electron transport system in photosynthesis of higher plants



(taken from Whatley (66))

intermediates, coupling factors and PSP itself, in both bacterial chromatophores and the chloroplasts of higher plants.

Exchange Reactions

The exchange of ^{14}C-ADP with ATP has been studied by Kahn and Jagendorf (73). They explained this exchange reaction by postulating that the final step in photosynthetic ATP formation was $X\sim P + ADP \rightleftharpoons ATP + X$ (also see reference 22). The exchange activity was about one third of the photophosphorylative capacity of the chloroplasts. Kahn (74) demonstrated ADP $\rightleftharpoons$ ATP exchange activity in the supernatant fluid from preparations of spinach chloroplasts. Repeated washing and fragmentation of the chloroplasts reduced the exchange activity to 0.4% of that of unwashed chloroplasts, while PSP activity was largely unaltered. The relationship of this reaction to PSP is not yet understood.

There has also been much work on ATP $\rightleftharpoons$ ^{32}P exchange reactions (75-78) in both chromatophores and chloroplasts. Earlier work had suggested that this exchange did not occur at appreciable rates in the light or dark in cell-free extracts of photosynthetic organisms. Indeed Avron and Jagendorf (79) pointed out that isolated chloroplasts that were capable of catalyzing ATP formation at high rates, lacked both ATPase and ATP $\rightleftharpoons$ ^{32}Pi exchange capacity, Recently, chloroplasts receiving a short light period in the presence of dithiol reagents were shown to catalyze an ATP $\rightleftharpoons$ ^{32}Pi exchange reaction in a following dark

reaction (75-77). This light-triggered ATP $\rightleftharpoons$ ^{32}Pi exchange was deemed to be related to PSP, and to the light-triggered ATPase (40), by the similarity it exhibited in its requirements for magnesium ions, phenazine methosulphate, and flavin mononucleotide, and the inhibition by uncouplers and electron transport inhibitors. The ATP $\rightleftharpoons$ ^{32}Pi exchange reaction showed substrate specificity for ATP and was competitively inhibited by ADP. It is of significance to the results to be submitted later in this thesis that the energy transfer inhibitor phloridzin, and the uncoupler quinacrine, inhibited only the dark phase of this exchange reaction. This adds weight to the supposition (80,81) that these inhibitors act at the terminal stage of PSP. The work of Rienits (77) also suggested that the ATP $\rightleftharpoons$ ^{32}Pi exchange and ATPase are elements of the same mechanism, and that this is probably part of the terminal enzyme system of PSP. This exchange activity can be explained by either of the following models:

$$\text{(a)}\quad X \sim {}^{32}P + ADP \rightleftharpoons {}^{32}P\text{-}ATP + X \quad (22)$$

$$\text{or}\quad \text{(b)}\quad X \sim ADP + {}^{32}Pi \rightleftharpoons {}^{32}P\text{-}ATP + X \quad (79)$$

There is evidence from both chromatophores and chloroplasts supporting each of these views; possibly different plants and micro-organisms have different mechanisms in the terminal stages of PSP. McCarty and Racker (82) also found an ATP $\rightleftharpoons$ ^{32}Pi exchange in chloroplasts under conditions able to induce the magnesium-dependent ATPase activity. In Tris buffer, light and

dithiothreitol were necessary for the induction of both reactions, while with Tricine, the dithiothreitol activated the reaction just in the dark. Fewson et al (64) noted that Tris may act as an uncoupler of ATP synthesis under certain conditions, and may be displaying a similar effect here. McCarty and Racker proposed that the dithiothreitol and light altered the properties of a coupling factor, and thus allowed reversal of PSP, as measured by ATPase and ATP $\rightleftharpoons$ ^{32}Pi exchange, to take place.

Schultz and Boyer (83) observed in spinach chloroplasts that an oxygen atom was lost from inorganic phosphate during ATP synthesis, and the terminal "bridge" oxygen atom of the ATP was derived from the added ADP. However, more recent studies by this group (84) have complicated the issue by demonstrating that the oxygen atom of water appeared in ATP. There is still a lack of knowledge about this apparent ATP $\rightleftharpoons$ HOH exchange. This exchange was also found to accompany ATP synthesis in the dark resulting from an acid-base transition of the chloroplasts (cf. ref. 34). Reversal of the water formation reaction incurred in ATP synthesis could possibly give rise to this ATP $\rightleftharpoons$ HOH exchange (as well as to the Pi $\rightleftharpoons$ HOH and Pi $\rightleftharpoons$ ATP exchanges also noted).

ATP hydrolysis

Finally in this section the ATPase reaction of chloroplasts will be briefly considered, having been discussed in full elsewhere (10,14). This reaction is considered to be of

importance as many workers have theorized that ATP hydrolysis could represent a reversal of a reaction in the mechanism of ATP synthesis. A number of different mechanisms have been demonstrated for ATP hydrolysis. First, there is a dark ATPase, that has an extremely low activity. Second, there is a light activated ATPase that may well be relevant to the photoproduction of ATP, because several factors are common to both. Two types of light-activated ATPase have been noted. One required a sulphydryl protectant, magnesium and a catalyst of PSP, e.g. phenazine methosulphate or flavin mononucleotide, for activation. Light was required to trigger the reaction, which could then continue in the dark (40). The other also required a catalyst of PSP, but was stimulated by calcium ions (and not magnesium) and was completely dependent upon light (39). The relations of these two types of ATPase reactions both to one another, and to the PSP process as a whole, are not yet understood. They do however, appear to have a number of features in common with PSP, and hopefully their place in the general scheme of ATP synthesis will soon be fully elucidated, perhaps by the study of soluble systems with methods successfully used on mitochondrial fractions. The dark ATPase, although shown to possess low activity, may itself hold the key to future knowledge regarding this problem. It would be interesting to see if carefully isolated intact

chloroplasts would possess the low levels of dark ATPase activity noted for the previously studied broken preparations.

It can be concluded from the previous discussion that although several aspects of ATP synthesis have been thoroughly studied there still are many problems to be solved. In order to attain a greater depth of knowledge regarding the conservation and utilization of energy in photosynthesizing systems, a number of different approaches have been used, other than those mentioned above. These include cation and anion movements in chromatophores and chloroplasts, conformational changes, and pH changes. Some recent studies of these photosynthetic characteristics have yielded interesting data of use in interpreting the general picture of energy conservation in photosynthesis. This work is discussed in the following section.

Chloroplast conformational changes

Changes in volume induced by a light reaction have been noted on many occasions in both isolated chloroplasts and chromatophores (14,86,87) and in intact leaves (85). The illumination of a chloroplast suspension in vitro results in two types of volume changes. One is a light-induced swelling that occurs slowly and is not reversible when the light is off. It is not influenced by conditions conducive to either ATP hydrolysis or synthesis, or indeed by the presence of uncoupling agents. The

second type is a light dependent shrinkage that is both rapid and reversible. The reaction is enhanced by conditions necessary for either ATP hydrolysis or synthesis. When light and ATP are both furnishing energy for shrinkage, high energy intermediates are thought to accumulate in the membrane-associated system (29). In the absence of appreciable ATP hydrolysis, light itself is still able to support 60% of the full shrinkage response (36). It is of interest that Dilley and Vernon (29) found that the maximum light-induced absorption change (shrinkage) occurred in their system at pH 5-6, and it became very low at pH 8.0. This pH dependence is similar to that described for the formation of X_e by Hind and Jagendorf (25). Another significant feature of this work (29) is the effect noted with the uncoupler quinacrine. At a concentration inhibitory to the formation of ATP, quinacrine had a slightly stimulatory effect on the light-induced shrinkage of chloroplasts. The less ATP formed by their system, the greater was the conformational change. They concluded that the energy for this shrinkage was derived from the high energy intermediate formed prior to ATP synthesis. Quinacrine has since been shown to act at a point following X_e synthesis (81). These conclusions were later verified by Hind and Jagendorf (89). However, they pointed out that the degree of volume change could not itself be used as a physical measurement of the high energy state. Pyocyanine doubled the total yield of X_e, but only

increased the rate, not the total amount of the shrinkage. Also X_e was noted to possess faster kinetics than those of the shrinkage reaction (although this could be a result of the different experimental techniques used to measure these parameters). It was concluded by Hind and Jagendorf (89) that this absorbance change was secondary to, but probably dependent on, the high energy state X_e. They also favoured the concept of X_e being a trans-membrane pH gradient, or membrane potential (31) rather than a distinct chemical entity containing a high energy bond.

The grana have been shown to be the primary units that undergo shrinkage, and have recently been intensively studied by Gross and Packer (90). This shrinkage was accompanied by a net efflux of potassium and magnesium ions, and a net influx of protons, whereas the re-swelling in the dark was accompanied by the reversal of these processes - suggesting a cation-exchange system (33).

The mechanism by which these volume changes occur is not understood. Ohnishi (91) reported the isolation of a contractile protein from chloroplasts, corresponding to striated muscle myosin and actin-like compounds. These conclusions were later questioned by Young and Packer (92).

Ion transport in photosynthesis

There is much evidence supporting the relationship

between ion movements and these chloroplast volume changes. This work may or may not support the view that X_e is actively associated with these changes, depending on whether X_e formation is dependent on, or linked with, ion movements (see Fig. V).

The contraction of isolated pea chloroplasts was found to be promoted by bivalent cations (93). Similar associations have also been noted by other workers (33,94,95). Crofts, Deamer and Packer (94) concluded that the light-induced volume changes resulted from an increased concentration of protons inside the chloroplasts. The volume changes were then necessary to regain osmotic equilibrium.

The energy-dependent uptake of ions by chloroplasts has received a thorough investigation, e.g. potassium (94), sodium (96), calcium (96,97) and ammonium ions (98). The uptake of calcium ions has been most studied, and was demonstrated to be dependent upon both light and ATP (96). Magnesium ions, a thiol reagent, e.g. reduced lipoic acid, and a catalyst of electron flow, e.g. phenazine methosulphate, were all necessary for the optimal uptake of calcium ions. The magnitude of the light-induced shrinkage of chloroplasts was increased by calcium ions, and it has been concluded that volume changes and ion translocation are processes that may be intimately related to one another (86).

Ion uptake is usually studied by either centrifuging down the chloroplasts after the reaction is over, or by filtering them with a Millipore filter, and counting the activity in either supernatant or pellet. That the ions were actually taken up within the chloroplasts, and not simply adsorbed to the outside came from the studies of Nobel, Muraki and Takamiya (98). By means of electron microscopic evidence, they showed that both strontium and calcium ions were deposited internally on the chloroplast lamellae. Nobel later studied calcium ion uptake in relation to ATPase and PSP activity of chloroplasts (96). He was able to demonstrate only a slight inhibition of calcium ion uptake by the energy transfer inhibitors phloridzin (99) and Dio 9 at concentrations that were shown to be extremely inhibitory to PSP. Here is yet another example of an endergonic reaction in chloroplasts being driven by a high energy intermediate, and not directly by ATP itself. This conclusion is supported by a recent report by Packer (100). He found that the disappearance of the light-dependent proton gradient was accompanied by the loss of light-induced conformation changes, inhibition of PSP, and of the light-triggered hydrolysis of ATP. He suggested that the monovalent cation transport in illuminated chloroplasts competes by dissipation of proton gradients for the energy required for PSP. He proposed that under normal circumstances, the principal monovalent cation accumulated by

chloroplasts is the proton, with only a small proportion of the ion uptake being other monovalent cations. Crofts (97) observed that phloridzin at a concentration that completely inhibited PSP, had little effect on the rate or extent of proton uptake by spinach chloroplasts. These reports gave added weight to the Mitchell hypothesis (18,19), but did not clarify whether proton uptake is the primary energetic event, or if it is merely coupled to some other reaction e.g. the formation of a high energy compound as proposed by the traditional chemical theory (16,17). Another explanation of these results is that the ion transport observed by many workers may be a non-physiological artifact induced by abnormal conditions. This is an important point to remember in all photosynthetic studies; most of the conclusions on PSP have been made with broken chloroplasts, utilizing non-physiological catalysts. Endogenous ATP synthesis is far lower than the optimal achieved with PMS, but may be a more realistic figure. In this regard, a recent paper by Nobel (101) is of interest, as it elucidates a method by which endogenous PSP may be measured in a preparation of largely intact chloroplasts.

Light-induced pH changes

Finally in this section some of the more recent features of light-induced pH changes in chloroplasts will be considered. This work has generally been taken to support the

concepts of Mitchell. However, some recent work has questioned this conclusion. The work of Karlish and Avron (54) has been mentioned earlier in the discussion of the chemi-osmotic theory (see p. 18), and these results still have to be explained by the proponents of the Mitchell theory. Secondly, von Stedingk (102) considered that the slow initial rate of the light-induced pH rise as compared with the rate for PSP was inconsistent with the concept of a proton-driven synthesis of ATP. However, this may be a reflection of the inefficiency of the glass electrode to measure rapid pH changes (104). Izawa and Hind (104) used a constant flow method applied to a conventional glass electrode pH-measurement system, which they claimed would accurately resolve the initial kinetics of pH changes in illuminated chloroplasts. Their results revealed that the kinetics of X_e are similar to those of the pH changes, and concluded that their data were compatible with the theory of chemi-osmotic coupling as applied to the mechanisms of PSP.

Photosynthetic carbon dioxide fixation

The previous sections of the introduction have dealt with some of the characteristics of chloroplasts and chromatophores that have aided in the elucidation of the mechanism by which energy is conserved and utilized in photosynthesis. The most important reason for this mechanism has not yet been mentioned, namely CO_2 fixation. There is a

vast literature covering many aspects of CO_2 fixation in photosynthesis and this has been reviewed in a number of publications e.g. refs. 4,7,8,13). However, in the last two years some important developments have taken place that shed new light on this old problem. It has been the ambition of many biochemists to obtain complete photosynthesis in isolated chloroplasts. For many years, attempts to achieve this end met with virtual failure. The low levels of CO_2 fixation obtained were only a fraction of the fixation ability of the tissue in vivo. The levels of CO_2 fixation achieved by different workers are shown in Table I.

Chloroplast isolation techniques

The method of isolation of the chloroplasts has been found to be extremely important if the ability to fix CO_2 is to be retained. Walker's method (107) involved isolation of the chloroplasts in isotonic sorbitol or sucrose, and not NaCl, and reduced to a minimum the time taken to obtain the chloroplast fraction. Speed in the isolation procedure has also been found by others to be crucial (109). Jensen and Bassham (108) improved upon the Walker technique by the inclusion of inorganic pyrophosphate (PPi) in the reaction buffer, and the use of HEPES, one of the hydrogen ion buffers introduced by Good, Winget, Winter, Connelly, Izawa and Singh (110). The mechanism by which

Table I. Levels of *in vitro* CO_2 fixation by isolated chloroplasts

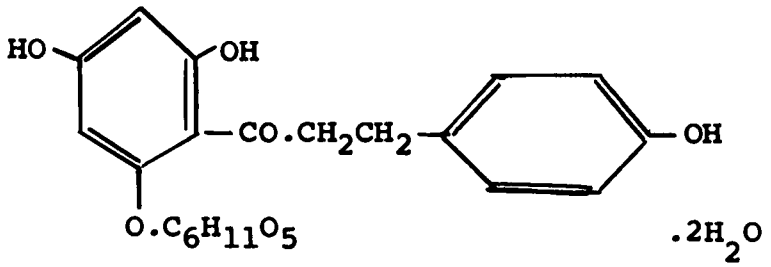
Material	μmoles CO_2/mg Chl/hr	Investigators
whole chloroplasts, 0.35M NaCl	0.25	Allen *et al* (3) 1955
whole chloroplasts, 0.5M sucrose	1.7	Holm-Hansen *et al* (104) 1959
broken chloroplasts (sugar beet)	3.5	Whatley *et al*, 1960 (105)
chloroplasts isolated by a non-aqueous technique	24.	Heber & Tyszkiewicz, 1962 (106)
whole chloroplasts (0.33M sorbitol)	36.9	Walker, 1965 (107)
whole chloroplasts (0.33M sorbitol) + HEPES buffer + PPi	155.	Jensen & Bassham, 1966 (108)

CO_2 fixation was enhanced by the addition of PPi was not explained and this poses an intriguing problem.

The effects of these buffers on various parameters of both mitochondrial and chloroplast activities have recently been studied by others (110,111,112,113), and in general, HEPES or TES were found to be superior to either Tricine or Tris buffers. HEPES may in some unknown manner reduce the leakage of essential components known to cause poor in vitro efficiencies. This is suggested by some data of Stinson and Spencer (111) who reported that the amount of cytochrome c lost from plant mitochondria was least in those isolated in HEPES buffer. The studies of Kalberer, Buchanan and Arnon (113) also demonstrated the superiority of sugars and related compounds over sodium chloride in maintaining the activity of isolated chloroplasts for CO_2 assimilation. Chloroplasts isolated by the technique of Jensen and Bassham (108) are capable of fixing CO_2 at rates comparable to those achieved in vivo. The characteristics of these preparations are also likely to be similar to those in vivo. It was felt important to study the characteristics of such preparations, and some of the results of this investigation are presented in the rest of this thesis.

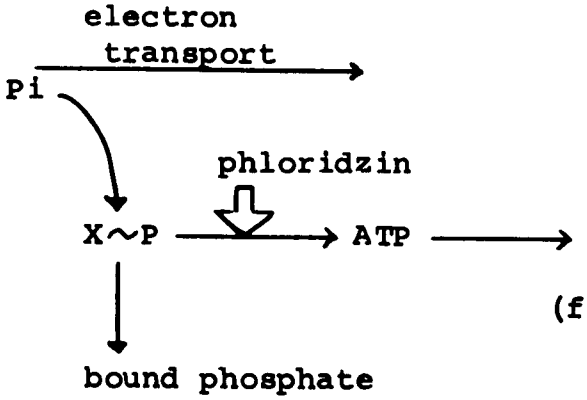
Inhibitor studies

Phloridzin (2-phloretin β-glucoside) has the following structure:



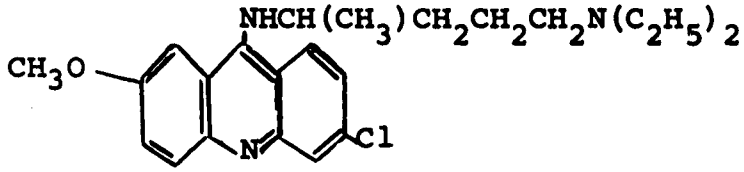
It occurs naturally as a growth-regulating agent (114) and has been used extensively for the study of cellular sugar transport. Alvarado (115) proposed that the β -D-glucopyranoside ring of phloridzin competes with actively transported sugars for a common membrane binding site. Phloridzin has also been shown to be an inhibitor of chloroplast photophosphorylation (both cyclic and non-cyclic) by Izawa et al (99). Phloretin (the aglycone) was found to be equally as inhibitory as the phloridzin. It was proposed that phloridzin inhibits at the terminal phosphorylation reaction of ATP synthesis, in a manner similar to oligomycin inhibition of mitochondrial ATP synthesis. Nobel (27) reported that 0.5mM phloridzin inhibited PSP by 99%.

The action site of phloridzin has been schematically represented as follows:



(from reference 116)

The other inhibitor used in this study was quinacrine (atebrin). It has the following structure:



.2HCl
.2H$_2$O

Quinacrine is a commonly used uncoupler in studies of PSP (32,39,81). Gromet-Elhanon and Avron (81) demonstrated that it inhibited PSP in a dark reaction in Swiss Chard chloroplasts. It did not appreciably affect the formation of high energy intermediates. An interesting report by Dilley and Vernon (33) noted that levels of quinacrine inhibitory to ATP formation, had a slightly increased effect on the degree of shrinkage in volume of spinach chloroplasts. They proposed that the light scattering increase observed was a result of the formation of high energy intermediates that may or may not lead to ATP

synthesis depending upon the presence of the phosphate-acceptor system. A quinacrine-stimulated shrinkage of chloroplasts, has also been noted by Izawa (117).

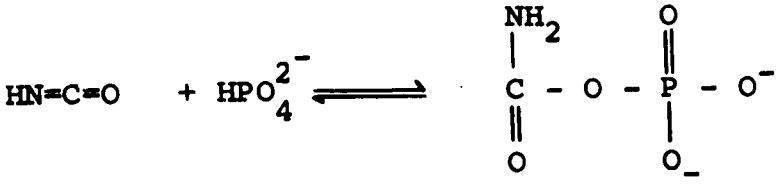
Role of Pyrophosphate

The enhancement of CO_2 fixation in isolated chloroplasts by the addition of inorganic pyrophosphate reported by Jensen and Bassham (108) is of considerable interest. In past years, the presence of PPi has been noted in several biological systems (118,119,120), and a number of roles have been proposed for it. Some of these views will be discussed in this section as an introduction to work on the mechanism by which the PPi may enhance CO_2 fixation in chloroplasts (109). Phosphorus is a unique element. It has mild and equally balanced electron accepting and donating tendencies (121), yet the accepting tendency is adequate to increase the electrophilia of the oxygen atom of the P-OH group, and therefore these ionize as acids, P-O H^+. The donating tendency is enough to make the phosphorus slightly positively charged. Orthophosphate is a highly stable molecule owing to its resonant nature. In PPi or ATP, this resonance is suppressed, and the phosphate linkages have a high group transfer potential (8-16 kcal/g.equivalent in vivo), compared to other organic phosphate compounds e.g. 3-phosphoglyceric acid that have a low group transfer potential (c. 3 kcal/g.equivalent). According to Needham (121) the organic pyrophosphates have a

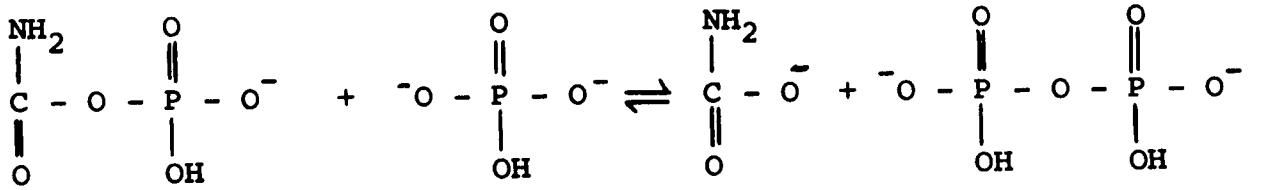
slightly lower transfer potential (12.0 kcal/g.equivalent) than the inorganic pyrophosphate (14.5 kcal/g.equivalent). This is thought to be a result of restraint on the organic pyrophosphate by the nucleotide moiety. Thus ATP pays for its high kinetic stability by a small loss in its energy of hydrolysis.

Podolsky and Morales (122) demonstrated that the hydrolysis of ATP is energetically similar to that of inorganic pyrophosphate and tri-metaphosphates. A similar conclusion was noted by Watson (123).

It has been postulated that PPi was utilized as an energy donor by early forms of life on Earth (121,124,125). Miller and Parris (124) showed that PPi could be synthesized under pre-biotic conditions by a reaction of cyanate with hydroxy apatite (at 35°C for 20 days).



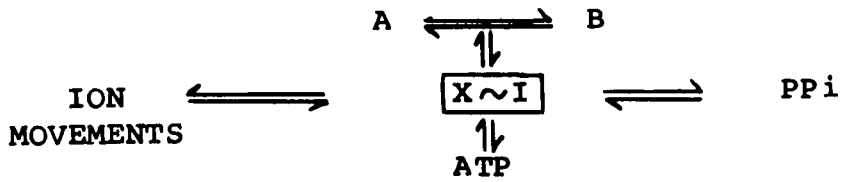
carbamyl phosphate



inorganic pyrophosphate

These reactions probably occurred on the surface of the phosphorus minerals.

In living organisms, the phosphorylation of inorganic phosphate to PPi is thought to occur at the pre-ADP level (119, 120).



A and B are electron carriers

(from reference 125)

A sequence of reactions for the synthesis of PPi in Rhodospirillum rubrum have been proposed (126) to occur as follows:

(1) $X\sim I + Pi \rightleftharpoons I + X\sim P$

(2) $X\sim P + Pi \rightleftharpoons X\begin{matrix}\sim P\\ \diagdown P\end{matrix}$

(3) $X\begin{matrix}\sim P\\ \diagdown P\end{matrix} \rightleftharpoons X - P\sim P$

(4) $X - P\sim P \rightleftharpoons P\sim P + X$

This has not yet been verified experimentally.

The presence of PPi has been shown in higher plants, algae, bacteria and animals. In plants, it is synthesized during the formation of starch, for example,

Glucose-1-phosphate + ATP $\longrightarrow$ ADPG + PPi

Kornberg (127) pointed out that enzymic reactions releasing PPi are considered irreversible as a result of the hydrolysis of PPi, so that the overall reaction favours synthesis. The formation of PPi has been reported in spinach chloroplasts by Bassham (128). The level of PPi was shown to increase in the light, and fall

rapidly in the dark. These transient changes in the levels of PPi have also been noted in Chlorella (129). The amount of PPi formed was variable, and the amount of label in PPi was between 10% and 50% of that in the ATP. The cause of this variation is not yet understood although it could be explained by the observations of Mishra and Mohantz (130). They reported that there were diurnal variations in the activity of the acid phosphatases in leaves of cowpea, for example, pyrophosphatase activity in the period from 6 a.m. to noon increased by 150%.

It is possible that the energy of the PPi molecule could be utilized in various synthetic reactions. Siu and Wood (131) reported the enzymic synthesis of phosphoenol-pyruvate from oxaloacetate and PPi. Another role was reported by M. Baltscheffsky (132) who demonstrated that ATP and PPi could induce energy-dependent spectral changes in both chromatophores and mitochondria.

Cole and Hughes (133) reported that polyphosphates could give rise to ATP in their bacterial system (also see reference 134). Of special interest is a paper by Szymona (135). He showed the presence of a polyphosphate hexokinase in cell-free extracts of Mycobacterium phlei capable of utilizing inorganic polyphosphates for the phosphorylation of D-glucose and D-glucosamine without the mediation of the adenylate system.

A role for PPi has also been reported in the transhydrogenation reaction (136) in photosynthetic bacteria.

$$NADH + NADP^{+} + PPi \longrightarrow NAD^{+} + NADPH + 2Pi$$

Oligomycin inhibited the transhydrogenation reaction induced by ATP, but had no effect when the reaction was catalyzed by either light or PPi. Recently Yamada and Kurahashi (137) demonstrated the presence of an ATP or PPi - dependent phenyl-alanine racemase in Bacillus brevis. There was an absolute requirement for either ATP or PPi, and Mg^{2+} or Mn^{2+}. The optimum PPi concentration was 0.2 - 2 mM, and the optimum for Mg^{2+} was below 0.5 mM. Above this level, inhibition occurred.

Evidence has been produced supporting the view that PPi may act as an alternative form of stored energy to ATP in photosynthetic CO_2 fixation (80), and this will be expanded in this thesis.

The Problem

The review in the preceding pages has covered selected work on several different aspects of chloroplast physiology. All these aspects have one factor in common, and that is the conservation or utilization of energy.

There are reports of inorganic pyrophosphate being synthesized in chloroplasts, and other reports of enhancement of CO_2 fixation by addition of PPi. The formation of pyrophosphate

in chloroplasts is investigated in this thesis, and some conclusions pertaining to the possible utilization of inorganic pyrophosphate as an energy donor are sought.

The mechanism by which energy is conserved in photosynthesis is shown in the literature review to be a subject of controversy. It is thought that energy is conserved as ATP, yet there are a number of reports suggesting roles, other than ATP synthesis, for high energy intermediates. Conformational changes, ion transport and light-induced pH changes all appear to be linked in some way with electron flow and energy conservation, and the role of ATP in these reactions is not clear. There is a lack of knowledge regarding both the mechanism of ATP synthesis, and the nature of its utilization in chloroplasts. These particular problems were studied, and some results are presented in the following sections.

MATERIALS AND METHODS

Chemicals and Supplies

Except where specified otherwise, chemicals used in this investigation were obtained from the Fisher Scientific Company, Edmonton, and were mainly "Fisher Certified" grade.

The ADP ("A grade" sodium salt) and ATP ("A grade" disodium salt), TES, HEPES and MES (all "A grade"), ribose 5-phosphate ("A grade" barium salt), reduced glutathione and dithiothreitol, and phloridzin were purchased from Calbiochem., Los Angeles, California.

The quinacrine HCl, iodoacetamide, oligomycin, phenazine methosulphate, ribulose 1,5-diphosphate (dibarium salt), and the β -nicotinamide adenine dinucleotide phosphate (reduced form, tetrasodium salt, type III) were obtained from the Sigma Chemical Company, St. Louis, Missouri. The magnesium chloride hexahydrate was from the Mallinckrodt Chemical Works, Montreal, P.Q., and the 3-phosphoglyceric acid (barium salt) was from the Nutritional Biochemical Corporation, Cleveland, Ohio.

Silica Gel G ('acc. to Stahl', E. Merck, Darmstadt) and the Mackery and Nagel MN-300 cellulose powder were purchased from Canadian Laboratory Supplies, Edmonton. The polyethylene glycol (as Carbowax 1500) was obtained from Analytical Engineering Laboratories, Connecticut, U.S.A., and polyvinyl

pyrrolidone (as PVP K30) was a kind gift from the Irwin Dyestuff Division of Vancouver, B.C.

The radio-isotopes ^{14}C-$NaHCO_3$ (specific activity 20.0 mc./millimole) and ^{45}Ca-$CaCl_2$ ($CaCl_2$ in 0.5 N HCl, specific activity 8.35 mc./mg.), and the fluors, 2,5-diphenyloxazole (PPO) and p-bis 2-(5-phenyloxazaloyl)-benzene (POPOP), were from the New England Nuclear Corporation. The ^{32}P-H_3PO_4 (H_3PO_4 in 0.02 N HCl, carrier-free) was purchased from both the New England Nuclear Corp., and the Atomic Energy Commission of Canada. The naphthalene for scintillation counting was supplied by Fisher Scientific, and the Cab-O-Sil (#M-5) was purchased from the Cabot Corporation, Boston, Massachusettes. Counting was done in Nuclear Chicago "Spectravial II" vials.

The water used in this investigation was either demineralized, double distilled water, or distilled water that had been passed through demineralizing and organic removal resins before redistillation from an all-glass still, and generally contained less than 2 parts per million of ionizable impurities.

Choice of Tissue, and Growth Conditions

Most studies on the photosynthetic mechanism of higher plants have utilized spinach leaves as a source of chloroplasts, and this species was chosen for this investigation. These leaves have the advantage of being soft, and are therefore

readily ruptured without recourse to harsh grinding techniques. The cell-sap is near to neutrality, and the leaves are largely or entirely free from starch granules. These are important factors when the object is to isolate intact and relatively pure preparations of chloroplasts.

The spinach (Spinacia oleracea - variety King of Denmark) was purchased from Steele-Briggs Seed Co. Ltd., Edmonton. It was found to germinate readily and evenly when sown in a mixture of black loam soil, peat and sand (3:3:1) fertilized with superphosphate. The plants were grown at 10°C in a controlled environment chamber, and were watered daily with tap-water. A bank of fluorescent cool white lamps (intensity of about 20,000 lux) were on for 13 hours and off for 11 every 24 hours.

Leaves collected from four week old plants were found to yield the most active chloroplast preparations with respect to their ability to fix carbon dioxide and synthesize endogenous ATP. Unless stated to the contrary, chloroplasts for this investigation were isolated from leaves 4 to 5 weeks old, during the light period. Leaves from plants less than 4 weeks old were too small for use.

Choice of Buffer

One of the main features of the method of Jensen and Bassham (108) is the use of recently introduced buffers (110). Most of the studies of photophosphorylation in this thesis involved the isolation and reaction of chloroplasts in Tris

or Tricine. An investigation of the effects of recent buffers (110) demonstrated the superiority of HEPES buffer over the others in studies of chloroplast CO_2 fixation. This buffer was then used for most of the remaining studies.

In a few experiments, recrystallized HEPES was used. Recrystallization involved dissolving the commercial HEPES (melting point 217°C) in boiling ethanol and water, and bringing it to its pKa at pH 5.0 with glacial acetic acid (110). This was done twice, and the product had a melting point of 233°C. (A melting point of 234°C is reported by Good et al; ref. 110). The results obtained with recrystallized HEPES did not differ from those obtained using the unrecrystallized commercial HEPES.

Chloroplast Isolation Procedure

The method introduced by Walker (107) and modified by Jensen and Bassham (108) was further adapted for this study. The essence of Jensen and Basshams procedure is the use of a rapid isolation technique and use of HEPES buffer for the reaction.

Immediately after harvest, the leaves (10-15 g.) were washed three times in distilled water at 2-5°C. They were then quickly blotted dry, and cut with a pair of scissors into small fragments, into an ice-cold mortar containing 100 ml. of grind buffer (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, and 1.0 mM disodium EDTA at pH 7.5 at 0°C). The fragmented tissue

was filtered through cheesecloth (8 layers), and the extract centrifuged at 2000 x g (5000 r.p.m.) for 50 seconds in an International Company model B-20 refrigerated centrifuge equipped with a number 870 head. The total spinning time was only one minute and thirty seconds, with manual braking applied to the head. Speed in the preparation of the chloroplasts was found essential for high activity in both CO_2 fixation and endogenous ATP synthesis studies. The pellet was suspended immediately by addition of reaction buffer, and swirling on a Vortex Mixer for 1-2 seconds. The composition of the reaction buffer was the same as the grind buffer, but with no EDTA, pH 7.5 at 20°C.

The object behind the choice of the isolation components was to retain the activity of the preparations as close to that of the in vivo state as possible. While Jensen and Bassham (108) isolated chloroplasts in MES buffer, followed by suspension and reaction in HEPES, it was found that the above procedure, where HEPES was used in both steps gave chloroplasts just as active in CO_2 fixation. Nor were isoascorbate, inorganic pyrophosphate and inorganic phosphate (108) routinely added.

Components of the reaction mixtures are shown in the legends of the appropriate figures and tables. When additions were made to the basic reaction buffer, the pH was readjusted with concentrated HCl or NaOH with a Radiometer pH meter.

Chlorophyll Determination

The method devised by Arnon (138) was followed. After isolation of the chloroplasts, 0.2 ml. was added to 24.8 ml. of acetone:water (4:1 v/v). The mixture was centrifuged at 1000 x g for 2 minutes in an International HN centrifuge run at full speed, and the density of the supernatant was measured in 1 cm. matched quartz cuvettes in a Beckman DU2 spectrophotometer (Beckman Instruments Company) at 652 mμ. The concentration of the chlorophyll (a+b) was calculated by the method of Arnon (138).

Photophosphorylation Assay

For the study of photophosphorylation, 0.2 ml. of chloroplasts containing about 0.2 mg. chlorophyll were added to 1.8 ml. of reaction buffer solution (0.01M HEPES, 0.33M sorbitol, 1.0mM $MgCl_2$, 1.0mM $MnCl_2$) with 1.0mM ^{32}P-KH_2PO_4 (containing 2-8 x 10^5 counts per minute). Other components are as indicated in the appropriate tables and figures. The stock ^{32}P-KH_2PO_4 was purified (139) by dilution with 1N HCl and heating to 100°C for one hour in order to convert any meta- or pyro-phosphoric acids to ortho-phosphoric acid. The hot solution was passed through a column (1 cm. x 10 cm.) of activated charcoal, and upon cooling, the radio-active solution was adjusted to neutrality by addition of NaOH.

The experiments were conducted in Warburg reaction flasks with one or two sidearms, used for the addition of

reactants to the flasks. Prior to use, the flasks were cleaned thoroughly by immersion in chromic acid solution for at least 24 hours. They were then drained, rinsed and allowed to soak for a further hour in hot detergent solution. They were again rinsed with hot tap water, and finally with double distilled water. They were oven-dried, and then stored until use. The reactions were performed in a lucite water-bath held at $20^{o} \pm 0.5^{o}C$. They were illuminated from below by a bank of three hundred watt incandescent spot lamps, providing an intensity of approximately 30,000 lux. The vessels were argon-flushed during the experiments. The reactions were initiated by turning the light on, and terminated by turning the light off and simultaneously adding 0.2 ml. of aqueous 20% (w/v) trichloroacetic acid from the flask sidearms to the reaction vessel. The samples were then centrifuged at 1000 x g for one minute (International HN centrifuge at full speed, situated in a refrigerated container). The adenosine tri-phosphate was then assayed by the method of Nielsen and Lehninger (140).

i. Isobutanol-benzene Extraction Procedure

This method for measuring the incorporation of radio-activity from inorganic ^{32}Pi into the organic phosphate fraction is based on the extraction of the unesterified ^{32}Pi complexed with molybdate (in a solvent phase), while the organic phosphate remains in the aqueous phase (140).

The supernatant obtained after completion of the experiment was added to 1.2 ml. of cold acetone, and allowed to stand in the dark for 10 minutes in a refrigerator. Then 2.5 ml. of a chilled solution of water saturated with a 1:1 mixture of isobutanol-benzene (IBB) was added, followed by 7.0 ml. of a chilled 1:1 mixture of isobutanol and benzene saturated with water. The tubes were inverted twice to mix the contents.

Rubber stoppers and Parafilm were subject to attack by the IBB reagent. It was found that polyethylene "capalls" (Canadian Laboratory Supplies) were resistant, and these were used to cap the test-tubes used in this assay.

From a graduated buret, 0.8 ml. of an acid-molybdate reagent (141), prepared by dissolving 5 g. of ammonium molybdate in 40 ml. of 10N H_2SO_4, and made up to a final volume of 100 ml. with distilled water, was slowly added to each tube and again mixed by inversion. The tubes were placed in a refrigerator for a further 5 minutes, and they were then shaken for 30 seconds in a reciprocating shaker (Eberbach Corporation, Ann Arbor, Michigan) at full speed. The tubes were then allowed to stand until the phases had separated, and then the upper solvent phase containing the non-esterified ^{32}P-KH_2PO_4 was removed under vacuum through a pasteur pipet into a trap. A 0.02 ml. aliquot of 0.02M KH_2PO_4 was added to the remaining aqueous layer, followed by a further 7.0 ml. of the IBB solvent system, saturated with water. The

tubes were again vigorously shaken for 30 seconds, and after phase separation the upper layer was again removed. An aliquot of the aqueous phase was pipetted with a 0.25 ml. volumetric micropipet into a scintillation vial for counting.

The radio-isotopes were counted in a Nuclear Chicago Corporation Unilux II Liquid Scintillation Counter. On occasions a Nuclear Chicago Model I, or a Beckman 200B Liquid Scintillation Counter were used. A polyether solvent system (142) containing 1,4-dioxane, anisole and 1,2-dimethoxyethane (6:1:1 v/v) with 12g/litre PPO and 0.5g/litre POPOP was found to give good efficiencies, but in the bulk of the work a fluor solution containing 100g. napthalene, 7g. PPO and 0.3g. POPOP in 1 litre of 1,4-dioxane (143) was used, as this was found to be preferable for dissolving aqueous solutions. Normally 10 ml. of fluor solution were added to each vial from a Packard constant volume dispenser. The scintillation vials were emptied immediately after use, and rinsed with water. They were then thoroughly washed by several rinses with hot water, followed by a 24 hour soak in detergent solution. They were then individually scrubbed in hot detergent, rinsed in tap water, and finally washed with distilled water. Sample vials were occasionally checked for background activity, and were not found to vary significantly from a mean of about 30 counts per minute.

ATP was taken to be that fraction of the esterified phosphate synthesized in the light resulting from the presence of the cofactors of ATP synthesis, ADP, inorganic phosphate and magnesium. Photophosphorylation was defined as the ATP formed in the light minus that formed in the dark.

ii. Adsorption of Nucleotides onto "Norite"

Photophosphorylation was also assayed by adsorption of the radio-active nucleotides onto activated charcoal ("norite", obtained from the Sigma Chemical Company), and then counted directly in a scintillation counter (144). The "norite" was activated by the method used by Barker and Hollinshead (145). "Norite" was added to about 20 ml. of 6N HCl and allowed to stand for 3 hours, and then it was washed with water by decantation until the washings were at pH 5.0. The charcoal was suspended in a volume of 0.1N HCl to give a solution containing 10 mg. norite per ml. One ml. of this suspension was added to the reaction mixture following IBB extraction and allowed to stand at room temperature for 10 minutes. The charcoal was then recovered either by centrifugation at 1000 x g or by pouring onto a layer of celite (1 cm. thick) previously washed with 6N HCl and water, and supported on a sintered glass disc. The charcoal was washed three times with 3 ml. water, and was then suspended in 10 ml. fluor solution containing about 0.5 g. Cab-O-Sil, and counted for activity.

iii. Luciferase Assay

In a few experiments, the luciferase assay for ATP (146) was used. Luciferase (from Firefly tails) was supplied by the Worthington Biochemical Corporation, Freehold, New Jersey, as a partially purified dry powder. These preparations were dissolved in 5 ml. distilled water to give a suspension containing 0.02M $MgSO_4$ and 0.05M arsenate buffer, pH 7.0. These salts, are necessary for optimal activity of the luciferase, and were present in the commercial enzyme preparation. One ml. of cold distilled water was added to 1 cm. quartz cuvettes, followed by 0.5 ml. of the ATP-containing solution. This was placed in the path of the photomultiplier tube of a DU2 Spectrophotometer, and 0.5 ml. of the luciferase solution was added and mixed. The spectrophotometer was set at zero transmittance with the light source off. After 15 seconds following addition of the enzyme, the intensity of the light response was measured as the percentage transmittance. The response of the enzyme to ATP was found to vary considerably from one enzyme preparation to another, and consequently the response of each preparation of luciferase to a known concentration of ATP was tested, and the results used to calculate the level of ATP in the unknown samples.

The presence of ADP was also found to initiate a light response in the presence of luciferase, and it was

concluded that there may have been an ATP-generating system present in the Worthington Luciferase. The presence of such a system has also been noted by others e.g. references 147 and 148. Therefore a control containing ADP at the concentration used in the PSP experiments was also made, and subtracted from the total ATP-induced light emission to give a measure of photophosphorylation.

Thin Layer Chromatography

The glass plates 10 x 10 cm., were found to require thorough cleansing in order to obtain uniform coating. A coating apparatus (Desaga, Heidelburg, Germany, supplied by Canadian Laboratory Supplies Limited) was used, adjusted to give a 0.25mm. thick layer. When more than 25 lambda were added per spot, 0.50 mm. thick layers were made. Prior to use the washed glass plates were wiped with acetone, and then coated with the cellulose slurry (15g. per 60 ml. distilled water, homogenized for 30 seconds in a Sorvall Omnimixer operated at full speed) and allowed to dry overnight. Silica gel layers were made in the same way (for 5 plates, 25g. silica gel were shaken with 50 ml. distilled water).

Aliquots from the reaction mixtures were evaporated under reduced pressure to about 0.1 ml. Then 10 lambda of these concentrated extracts were spotted on cellulose plates

with a capillary micropipet, and dried with a warm air flow from a hair dryer. Authentic samples were also spotted on the plates.

ATP and ADP were chromatographed using an isobutyric acid:ammonia:water (57:4:39 v/v) solvent for 2 hours. The plates were dried and sprayed with Hanes and Isherwood Reagent (149) consisting of 1g. ammonium molybdate in 8 ml. distilled water, plus 3 ml. concentrated HCl and 3 ml. concentrated perchloric acid (70%), and diluted to 100 ml. with acetone. After spraying, the plates were either exposed to ultraviolet radiation for 10 minutes, or sprayed with aqueous 1% stannous chloride in 10% HCl. The phosphorylated compounds showed up as dark blue areas.

For separation of ATP and PPi, two-way chromatography with (a) ethyl acetate:acetic acid:water (3:3:1 v/v) and (b) methanol:formic acid:water (16:3:1 v/v) on cellulose-coated plates was used. The chromatograms were run for two hours in one direction and then for two hours in the other direction. The plates were then dried and sprayed. Phosphate anions were separated by a solvent system consisting of methanol:concentrated ammonia:10% TCA:water (50:15:5:30), on silica gel plates (150).

Spots corresponding to ATP and PPi were scraped off the plates and added to a fluor gel (with Cab-O-Sil) and the activity counted.

3-phosphoglyceric acid was chromatographed in a similar manner with equal volumes of propionic acid:water (18:22) and butanol:water (370:25) as the solvent system. The plates were sprayed as above, and heated for 10 minutes at 150°C for development of the blue colour. Aniline hydrogen phthalate (151) was also found to be a useful reagent for identification of authentic samples of 3-PGA (0.93 g. aniline and 1.6 g. phthalic acid to 100 ml. with water-saturated n-butanol). After spraying the plates were heated to 105°C for 5 minutes.

CO_2 Fixation Assay

The reactions were conducted in the presence of radioactive bicarbonate, and were terminated by addition of 0.2 ml. 20% trichloroacetic acid (w/v). Aliquots of up to 0.25 ml. were added to empty scintillation vials, and the unreacted bicarbonate removed by warming the vials to about 50°C for 5 minutes in a fume cabinet. Then 10 ml. of fluor were added to each vial, and the samples counted in the scintillation counter.

Calcium and Phosphate Uptake by Chloroplasts

After incubation with the particular isotope to be studied, the chloroplasts were removed either by a "Millipore" Filter (white, plain filter 25 mm., diameter, with 0.8 micron pores $\pm$ 0.05 microns, obtained from Millipore Limited,

Montreal, P.Q.) held by a micro-syringe filter holder (also from Millipore Limited), or by centrifugation of the chloroplasts at 1000 x g followed by two washings with buffer solution, and finally counting in the scintillation counter. With the "Millipore" filter, a blank without chloroplasts was made and the counts of this were subtracted from the experimental values, to give an indication of the total ion uptake. The ion uptake by chloroplasts in the dark was subtracted from the total ion uptake, to give the light-induced ion uptake.

Scintillation Counting

In liquid scintillation systems, energy may be lost by quenching before it reaches the photomultiplier tube. Two of the most ubiquitous quenchers are dissolved oxygen and water. Also many unknown quenchers are found in biological tissues.

In this investigation standard quenching curves were prepared (by the channels ratio method) by using the same quenching agents as present in the samples to be examined. To each sample, a known amount of isotope (either ^{14}C-toluene, ^{32}P-KH_2PO_4, or ^{45}Ca-$CaCl_2$) was added with a 50 µl. fixed-needle Hamilton Syringe. Various amounts of quenching agent (prepared from plant tissue by carrying out a "dummy-run" in the absence of isotope) were added to these flasks, fluor was added and the samples counted. Two channels were used in the Nuclear-Chicago

Instruments, one was "optimized" for ^{14}C or ^{45}Ca counting, and the other for ^{32}P counting. Both operated in the integral (L-∞) mode with the upper discriminator set at its highest voltage and its lower gates set at the lowest voltage. The data obtained were used to ascertain any variations in the counting efficiency within the samples from a particular experiment. The channels ratio from a series of samples was routinely calculated, and if necessary, adjustments were made to reach a common degree of counting efficiency. In the case of the high energy beta emitter, ^{32}P (1.71 millielectron volts) no significant variation was noted within samples from a particular experiment, while with ^{14}C (0.156 mev) and ^{45}Ca (0.256 mev) slight variations in the degree of quenching were noted, and adjustments were made for these.

RESULTS AND DISCUSSION

CHLOROPLAST ISOLATION TECHNIQUES

i. Choice of Tissue

Attempts were made to obtain chloroplast preparations from spruce needles, in order to compare results with other photosynthesizing tissues. The chlorophyll-containing fragments were completely inactive, and this was found to be a result of the high level of tannins and other phenolic compounds in the tissue, that are inhibitory to photophosphorylation.

Polyethylene glycol (152) and polyvinyl pyrrolidone (153) have been reported to inactivate phenolic compounds. By addition of these compounds to the grind and reaction buffers, low levels of ATP synthesis could be observed, but reproducibility was difficult to achieve.

In young spinach leaves, the level of phenolic compounds is low, and for this and the other reasons given in Materials and Methods (pages 46-47), spinach tissue was used throughout this investigation. Lower levels of ATP synthesis were found with old spinach tissue. It was noted that when chloroplasts were isolated from old leaves (8 weeks), the addition of 0.1% polyvinyl pyrrolidone in the grind buffer increased the level of PMS-catalyzed ATP synthesis by 15-20%. It is possible therefore, that one of the factors involved in inducing low activities in chloroplasts isolated from old leaves, is the increase in the

endogenous level of phenolic compounds. Utilization of the phenolic-complexing agents may enable future investigations of a wider variety of plant tissues than is possible at present.

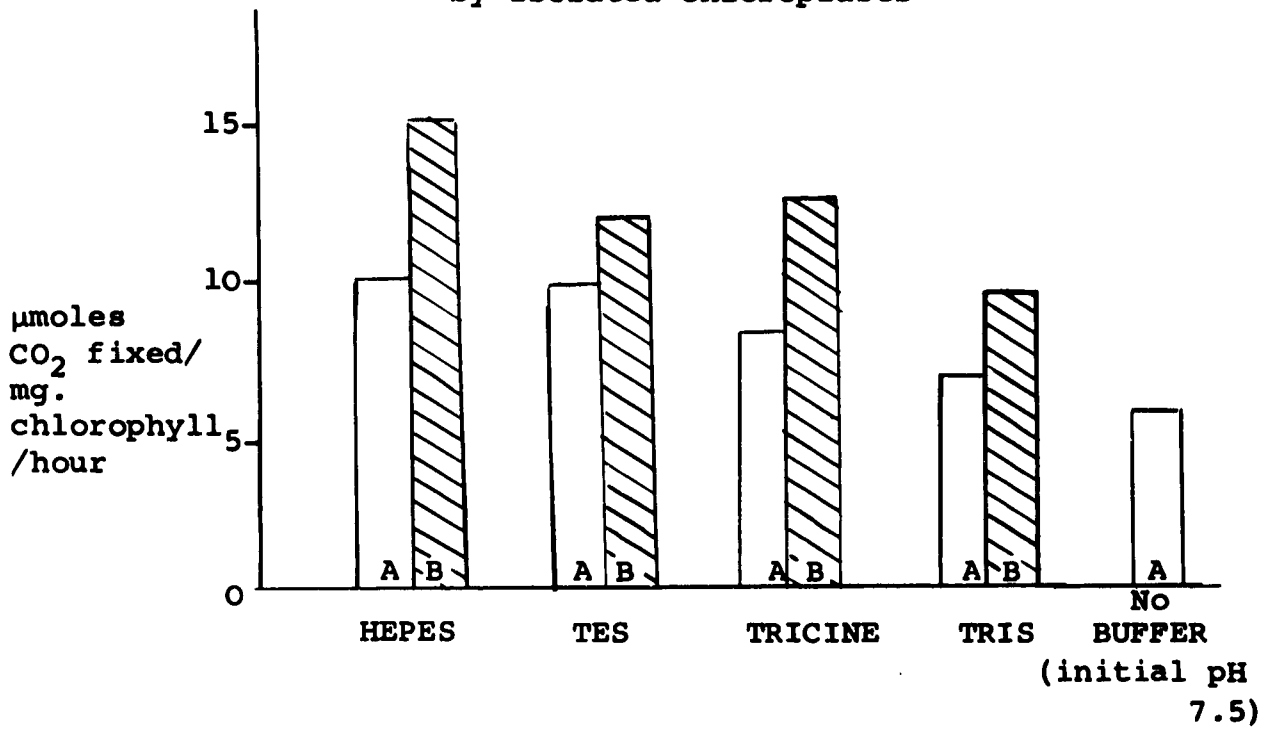
The spinach chloroplasts isolated for this study were examined under the phase-contrast optics of a light microscope, and were found to be between 50 and 70% intact. Chloroplasts were judged intact if they exhibited a refractive appearance, whilst those with well-defined grana were presumed to have lost their outer membranes (154). Retention of the outer membrane appears to be vital in order to prevent leakage of intermediates essential for either endogenous ATP synthesis or CO_2 fixation.

ii. Choice of Buffer

In the Introduction, recent methods for the isolation of chloroplasts capable of high rates of CO_2 fixation were discussed (page 35). Activities approaching the *in vivo* rate have been reported by Jensen and Bassham (108). Their main innovations over the method of Walker (107) were the use of HEPES (110) and the addition of inorganic pyrophosphate. Using these techniques, the chloroplasts isolated in the course of this thesis were found capable of rates of CO_2 fixation comparable to those of Jensen and Bassham (108).

The effects of a number of buffers on the CO_2 fixation ability of spinach chloroplasts were tested in this investigation. The results in Figure VII indicate the low efficiency of CO_2

Figure VII. Effects of buffer on the rate of CO_2 fixation by isolated chloroplasts



In Experiment A chloroplasts were isolated from 4 week old leaves in a medium 0.33 M in sorbitol, 1.0 mM in $MgCl_2$, 1.0 mM in $MnCl_2$, 1.0 mM in EDTA, pH was adjusted to 7.5 with NaOH. They were added to a reaction mixture of the same composition (no EDTA), including one of the buffers shown above, at pH 7.5, and a concentration of 0.01 M, and 25 mM ^{14}C-$NaHCO_3$ (1.2×10^6 cpm). The reaction was at 20°C for 5 minutes at a light intensity of 30,000 lux in an argon atmosphere. It was terminated by addition of TCA (see Methods).

The procedure in Experiment B was the same, except the chloroplasts were isolated in 0.01 M HEPES buffer, pH 7.5 (with other components as before), and then reacted in the different buffers as indicated above. Chlorophyll concentration was 0.2 mg./flask in both A and B.

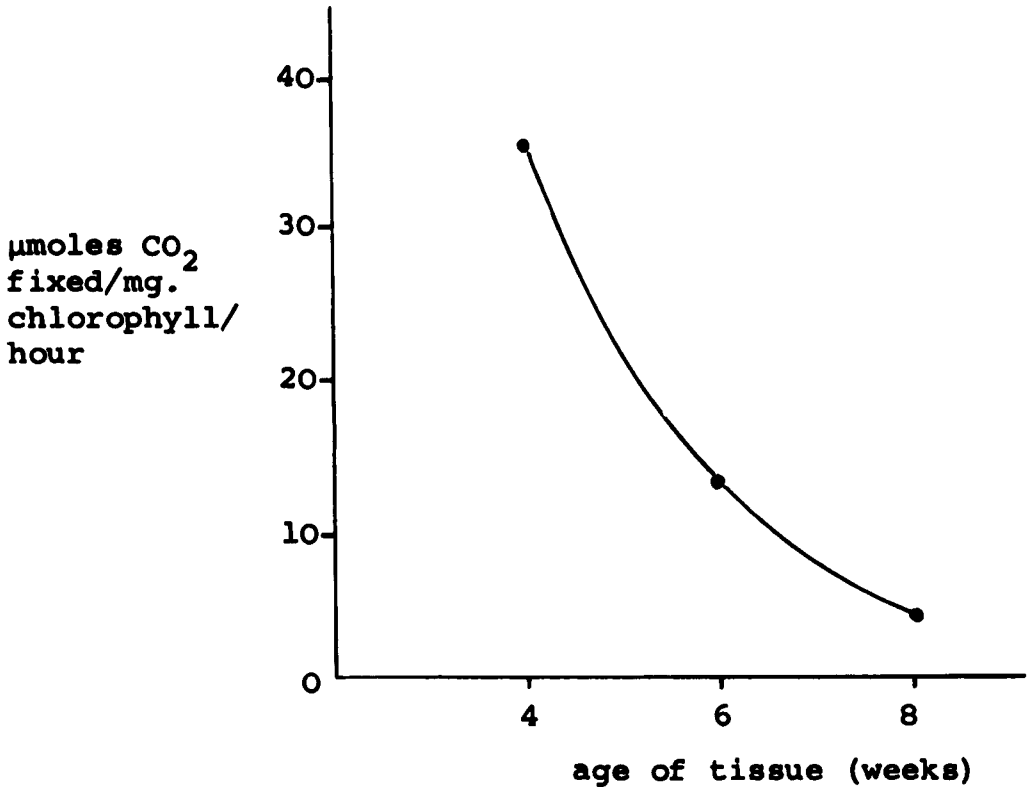
assimilation in the Tris; HEPES in both the grind and reaction buffer was found to give the best results. Tris buffer has been shown to act as an uncoupler of ATP synthesis in spinach chloroplasts (64) and this uncoupling may explain its poor efficiency in these experiments. The effects of these buffers have been tested on a variety of biochemical systems (110-113) and the same general results were reported. The choice of buffer for investigations of chloroplast physiology is of considerable importance, and it is apparent that much more knowledge is necessary regarding the variety of effects of buffers on biological systems.

iii. Effects of Ageing

(a). in vivo

The results in Figure VIII show the effects of age of tissue on the ability of the chloroplasts to fix CO_2. The leaves in all cases appeared healthy and showed no apparent signs of senescence apart from the presence of a darker green colour. This decrease in CO_2 fixation with age has also been reported by Kalberer *et al* (113), who also noted a similar decrease with discs from older leaves. The decrease in ability to fix CO_2 could be a result of a change in ability of the chloroplasts to withstand the isolation procedure, or an increased permeability of the chloroplast membranes with age. In this regard, data with ageing whole leaves would be of interest.

Figure VIII. The effects of age of plant on the ability of the isolated chloroplasts to fix CO_2



Chloroplasts were isolated from tissue of various ages and added to reaction buffer (0.01 M HEPES; 0.33 M sorbitol; 1.0 mM $MgCl_2$; 1.0 mM $MnCl_2$; 25 mM ^{14}C-$NaHCO_3$ (1.2 x 10^6 cpm pH 7.5). The reaction was for 5 minutes at 30,000 lux at 20°C, in an argon atmosphere. The total volume of the reaction mixture was 2.0 ml. The reaction was terminated by addition of 0.2 ml. 20% TCA. The chlorophyll concentration was adjusted to give 0.22 mg./flask. Leaves from plants less than 4 weeks old were too few and undeveloped for isolation.

(b). *in vitro*

When chloroplasts were isolated from 4 week old leaves and aged in the dark at room temperature before the light reaction, there was a marked decrease in ability to fix CO_2. This is shown in Table VI, and is probably a result of leakage of intermediates of the Carbon Cycle through the chloroplast membrane. The addition of either reduced glutathione or Na isoascorbate to the reaction buffer increased the levels of CO_2 fixed, but did not prevent this decline in ability noted with *in vitro* ageing (Table II). It was concluded from these studies that young tissue should be used, chloroplast isolation should be done speedily, and subsequent studies be made immediately following the isolation procedure.

INORGANIC PYROPHOSPHATE AS A POSSIBLE ENERGY SOURCE FOR CO_2 FIXATION

i. Effects of ATP and PPi on CO_2 Fixation

In Table III the effects of ATP and PPi on CO_2 fixation can be seen. Both compounds greatly stimulated CO_2 fixation, and the effects were generally equivalent. Maximum increases were at 5 mM in both cases, and above this concentration, no additional stimulation was observed.

It was postulated that both ATP and PPi may be acting as sources of energy for CO_2 fixation, and the effects of these

Table II. The effects of reduced glutathione or Na isoascorbate on the decline in CO_2 fixation ability of ageing chloroplasts

Chloroplast Preparation	Ageing time (min) in dark	µmoles CO_2 fixed /mg. chlorophyll/hour			
		0	5	10	15
No. 1	No GSH	17	8.5	4.2	0.5
	5 mM GSH	32	19	6	1.2
No. 2	No isoascorbate	2.3	1.8	0.4	0.2
	2 mM isoascorbate	6.2	4.7	1.3	0.6

Chloroplasts were isolated from 5 week old leaves. After centrifugation, the chloroplast pellet was immediately suspended in 3 ml. reaction mixture (0.01 M HEPES; 0.33 M sorbitol; 1.0 mM $MgCl_2$; 1.0 mM $MnCl_2$ pH 7.5), aliquots (0.2 ml) were reacted with or without GSH or isoascorbate as shown for 5 minutes at 30,000 lux and 20°C. Chloroplasts were aged in the dark at 20°C, and reacted at 5 minute intervals in an argon atmosphere.

Chloroplast concentration (mg. chlorophyll/flask):
No. 1 = 0.22
No. 2 = 0.25

Table III. The effect of added ATP or PPi on CO_2 fixation in a light dependent system

	μmoles CO_2 fixed/mg. chlorophyll/hour				
chloroplast preparation	No. 1	No. 2	No. 3	No. 4	No. 5
control	11.5	12.9	11.1	17.5	16.3
ATP	19.4	17.5	25.4	57.6	36.9
PPi	17.8	19.6	25.8	66.0	35.7

The reaction buffer was 0.01 M in HEPES, 0.33 M in sorbitol, 1.0 mM in $MgCl_2$, 1.0 mM in $MnCl_2$, pH 7.5. Reaction time 5 min. at 30,000 lux and 20°C, in an argon atmosphere. In experiments No.'s 1 and 2, solutions were 1 mM in ATP or PPi, in No. 3, 3 mM, and in No.'s 4 and 5, 5 mM. The chloroplasts and 50 μmoles ^{14}C-$NaHCO_3$ (1.2×10^6 cpm) were added at the initiation of each reaction. Total volume of reaction mixture was 2.0 ml.

Chloroplast concentration (mg. chlorophyll/flask):
No. 1 = 0.18; No. 2 = 0.10; No. 3 = 0.18; No. 4 = 0.21; No. 5 = 0.15

two compounds on CO_2 fixation in this system were therefore studied in detail.

M. Baltscheffsky (155) reported a synergistic relationship between ATP and PPi as sources of energy in Rhodospirillum rubrum chromatophores. However, in spinach chloroplasts the effects of ATP and PPi were found to be additive, but not synergistic (Table IV).

ii. Effects of Ageing on ATP and PPi-enhanced CO_2 Fixation

The decline in ability of isolated chloroplasts to fix CO_2 with age has already been noted. The data in Table V show that this decline is not altered by the presence of ATP and PPi, although these compounds are still able to enhance the fixation of CO_2 in chloroplasts isolated from tissue of all ages studied.

In Table VI the effects of ageing chloroplasts in vitro at room temperature on their ability to fix CO_2, and to respond to added ATP and PPi are given. A similar trend to that shown in Table V is apparent. It is of interest that the degree of enhancement found upon addition of either ATP or PPi was highest in chloroplasts aged in vitro for the longest time. These data point out the importance of standardization of technique in experiments of this nature, if valid comparisons are to be drawn.

Table IV. The effect of ATP with or without PPi on CO_2 fixation

Treatment	μmoles CO_2 fixed/mg. chlorophyll/hour
Control	28.3
PPi (1 mM)	53.3
ATP (1 mM)	53.3
PPi (1 mM) + ATP (1 mM)	74.6

Chloroplasts were isolated from 4 week old plants, and were suspended in buffer (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 25 mM ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm, pH 7.5). The total volume of the reaction mixture was 2 ml. Reaction time was for 5 mins. at 30,000 lux at 20°C. PPi and ATP were present in the reaction buffer as shown in the Table. Chlorophyll concentration was 0.15 mg./flask. The flasks were flushed with argon during the experiment.

Table V. The effect of age of plant on the ability of chloroplasts isolated from spinach leaves to fix CO_2 and respond to ATP and PPi

Age (weeks)	ATP (mM)	PPi (mM)	µmoles CO_2 fixed /mg. chlorophyll/hour	
			prep'n No. 1	No. 2
	0	0	7.6	61.2
4	5	0	31.8	74.2
	0	5	24.3	74.7
	0	0	3.1	20.8
6	5	0	7.6	36.4
	0	5	6.2	38.4
	0	0	2.4	4.2
8	5	0	8.4	5.7
	0	5	6.7	5.6

Chloroplasts were isolated from leaves of various ages, and the reaction was made in 2 ml. buffer pH 7.5 (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 25 mM ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm, and ATP or PPi as shown). The reaction was at 20°C, 30,000 lux for 5 minutes, in an argon atmosphere.

Chloroplast concentration (mg. chlorophyll/flask)

Age	Prep'n No. 1	No. 2
4	0.34	0.25
6	0.30	0.32
8	0.38	0.30

Table VI. The effect of *in vitro* ageing of isolated chloroplasts on their ability to fix CO_2 and respond to added ATP and PPi

Age (min)	ATP (mM)	PPi (mM)	µmoles CO_2 fixed /mg. chlorophyll/hour	stimulation factor
	0	0	41	
0	5	0	92	x2.26
	0	5	89	x2.18
	0	0	16.5	
5	5	0	45	x2.75
	0	5	47.5	x2.29
	0	0	4	
10	5	0	18	x4.5
	0	5	12	x3.0
	0	0	0.1	
15	5	0	2.0	x16.6
	0	5	1.2	x10.0

Chloroplasts were isolated from 20 g. leaves of 4 week old plants. After centrifugation, the chloroplast pellet was immediately suspended in 3 ml. reaction mixture (without ATP or PPi) as in Table II. Aliquots (0.2 ml.) were added to the first series ("age 0") and reacted for 5 min. at 30,000 lux. The remaining chloroplasts were aged at room temperature in the dark. At 5 min. intervals the aged chloroplasts were added to the flasks, and reacted as shown above. Each aliquot contained 0.21 mg. chlorophyll. The reaction buffer was 0.01 M in HEPES, 0.33 M in sorbitol, 1.0 mM in $MgCl_2$, 1.0 mM in $MnCl_2$, 25 mM in ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm, and ATP or PPi as shown. The flasks were flushed with argon during the course of the experiments.

iii. Effects of Pi and PPi on CO_2 Fixation

The mechanism by which pyrophosphate exerts its effect on CO_2 uptake by photosynthesizing chloroplasts is not known. There have been reports that low levels of inorganic phosphate enhanced CO_2 fixation (2,113), and it is possible that a chloroplast pyrophosphatase may release Pi from the pyrophosphate and this may serve to stimulate CO_2 fixation. However, in our system (Table VII) we were unable to demonstrate any stimulation of CO_2 fixation by Pi, but rather an inhibition was observed.

This phosphate-induced inhibition of CO_2 fixation is probably related to the carboxylation step. Indeed Pi has been reported to act as a competitive inhibitor of ribulose 1,5 diphosphate carboxylase (156). Inhibition of CO_2 fixation by inorganic phosphate above 0.6 mM has been reported by others (113), and inhibition of oxygen evolution by phosphate (above 50 μM) in chloroplasts has recently been reported by Cockburn, Baldry and Walker (157). The level of endogenous orthophosphate in spinach chloroplasts has been shown to be 4-25 mM (158), and it is likely that in the intact chloroplast there is compartmentation of the phosphate, so that it may not be at these levels at the sites of CO_2 fixation.

The effect of phosphate alone, pyrophosphate alone, and a combination of them both, on CO_2 fixation is shown in

Table VII. Effect of inclusion of inorganic phosphate in the assay medium on CO_2 fixation

	μmoles CO_2 fixed /mg. chlorophyll/hour	
	chloroplast preparation	
μM KH_2PO_4	No. 1	No. 2
0	410	342
1	350	308
5	294	294
50	266	268
500	123	256
2000	74	244
5000	66	164

Buffer composition was 0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 25 mM ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm, pH 7.5. KH_2PO_4 was added to the assay medium to give the concentrations shown. There was a 5 min. light reaction at 30,000 lux and 20°C. The chlorophyll concentration was 0.20 mg./flask in No. 1 and 0.25 mg. in No. 2. The flasks were flushed with argon during the reactions.

Table VIII. It is apparent from these data that inhibition of CO_2 fixation occurred with Pi at the concentrations used, however there was a large enhancement of CO_2 fixation with PPi. Surprisingly, when PPi was added to a solution containing inhibitory concentrations of Pi, the degree of inhibition was not increased, as would be expected if PPi was simply being hydrolysed to give free Pi. The possibility that PPi stimulation may not be a result of hydrolysis to phosphate has also been noted recently by Jensen and Bassham (159). This suggests that PPi may function in some other manner, possibly as an energy source. It is also possible that PPi may be hydrolyzed in a chloroplast compartment separate from the locale of added Pi. The concept of compartmentation presents a fascinating problem, and presents a large field for future study.

Recently, a similar effect of PPi on the evolution of O_2 by intact spinach chloroplasts has been reported (157). Inorganic pyrophosphate failed to produce the inhibitory effects noted for Pi, and in the presence of PPi, the Pi inhibition was less severe.

iv. Effects of pH on ATP and PPi-enhanced CO_2 Fixation

As both ATP and PPi appeared to have similar effects on CO_2 fixation, the effects of a number of factors on the induced CO_2 assimilation were tested. First, the effects of pH were studied, and the results are given in Table IX.

The data in Table IX indicated that the optimal pH for CO_2 fixation was 7.75 - 8.00. Of interest is the fact that

Table VIII. Effects of inorganic phosphate and inorganic pyrophosphate on CO_2 fixation

Inorganic phosphate	Inorganic pyrophosphate	μmoles CO_2 fixed /mg. chlorophyll/hour chloroplast preparation No. 1	No. 2	No. 3
-	-	26	23	74
+	-	6	18	68
-	+	49	64	172
+	+	14	47	84

Chloroplasts from 4 week old leaves were incubated for 5 min. at 30,000 lux at 20°C in 2 ml. buffer (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 25 mM ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm, pH 7.5). Pi was 2.5 mM in experiment 1, and 5 mM in 2, 3; PPi was 5 mM when present.
The chlorophyll concentration was 0.17 mg./flask in experiment 1, 0.19 mg. in experiment 2, and 0.21 mg. in experiment 3. The flasks were flushed with argon during the course of the experiments.

Table IX. The effects of pH on the ability of isolated spinach chloroplasts to fix CO_2, and respond to the presence of 1 mM ATP or 1 mM PPi

Chloroplast preparation	Buffer	pH	μmoles CO_2 fixed /mg. chlorophyll/hour		
			Control	ATP (1 mM)	PPi (1 mM)
No. 1	MES	6.00	3.2	7.2 (+125)	2.7 (-16)
	MES	6.25	4.5	8.2 (+ 82)	3.0 (-33)
	MES	6.50	4.9	9.2 (+ 88)	4.1 (-16)
No. 2	HEPES	6.75	12.0	24.0 (+100)	13.4 (+12)
	HEPES	7.00	23.4	41.1 (+ 76)	22.5 (- 4)
	HEPES	7.25	27.6	58.0 (+110)	33.4 (+21)
	HEPES	7.50	39.0	72.0 (+ 85)	82.5 (+112)
No. 3	HEPES	7.50	17.7	41.7 (+136)	29.7 (+68)
	HEPES	7.75	21.3	48.4 (+127)	34.3 (+62)
	HEPES	8.00	26.4	56.0 (+112)	37.2 (+41)
	HEPES	8.25	18.4	33.3 (+ 81)	18.9 (+ 3)

Chloroplasts from 4 week old plants were suspended in buffers of the appropriate pH's, either 0.01 M in MES or 0.01 M in HEPES, 0.33 M sorbitol, 1.0 mM in $MgCl_2$, 25 mM in ^{14}C-$NaHCO_3$ (1.2×10^6 cpm) and made 1 mM in ATP or PPi as indicated. Reaction for 5 mins. at 30,000 lux, 20°C. The chlorophyll concentration was 0.18 mg./flask in No. 1, 0.18 mg. in No. 2 and 0.13 mg. in No. 3. The flasks were flushed with argon during the reaction. The figures in parentheses indicate the % change induced by the presence of ATP or PPi.

stimulations by both ATP and PPi varied with pH, and in both cases the optimal pH for maximum enhancement of CO_2 fixation was at 7.50. However, at the extremes of the pH's studied, the PPi effect appeared to be far more limited than that resulting from ATP. Below pH 7.00 and above pH 8.00 there was no stimulation by inorganic pyrophosphate, while the stimulation by ATP was noted at all the pH values studied. This dependency on pH may signify the presence of a pH-sensitive PPi - utilizing enzyme system in the chloroplasts, or perhaps an effect of pH on the permeability of the chloroplast membrane to ATP or PPi.

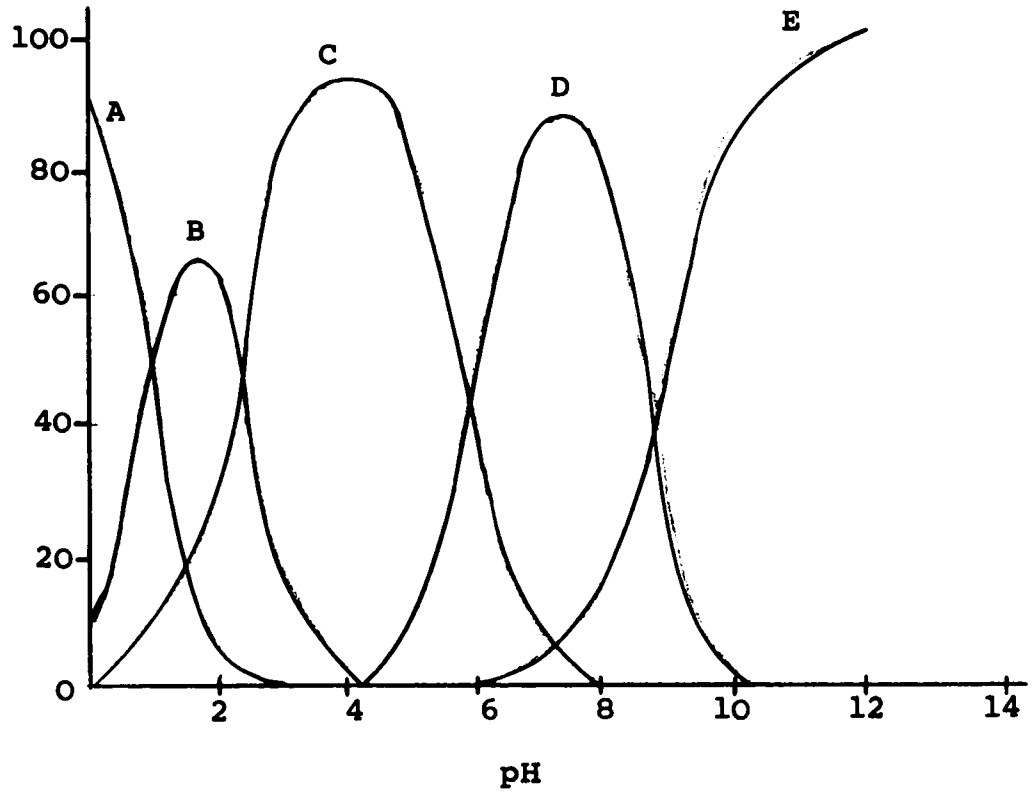
The degree of hydrolysis of Na pyrophosphate in solution has been shown to be dependent on the hydrogen ion concentration (160), and this is shown diagrammatically in Figure IX.

This pH effect would presumably also apply to the added ATP, and it is possible that the effects observed in Table VIII were merely a result of pH directly affecting the hydrolysis of the PPi or ATP. This is unlikely in these experiments because of the large changes in effect noted with very small pH changes, and the reported stability of PPi at pH 7 to 8 (182).

v. Effects of pH on ADP and PPi-enhanced CO_2 Fixation

The effect of ADP on the pyrophosphate-induced stimulation of CO_2 fixation was tested at different pH values, and the results are shown in Table X. At pH 7.25 and pH 7.50,

Figure IX. Effect of pH on distribution of anionic species of pyrophosphate



A. $H_4P_2O_7$ (least stable)

B. $H_3P_2O_7^{1-}$

C. $H_2P_2O_7^{2-}$

D. $HP_2O_7^{3-}$

E. $P_2O_7^{4-}$ (Most stable)

(from reference 160)

Table X. Effects of pH on the action of PPi and ADP on CO_2 fixation

ADP (mM)		μmoles CO_2 fixed/mg. chlorophyll/hour			
		pH 7.25	pH 7.50	pH 7.75	pH 8.00
0	Control	60.8	71.6	98.0	94.0
0	PPi (1.25 mM)	73.2	102.0	114.4	104.0
2.5	PPi (1.25 mM)	71.2	95.2	121.6	110.4
5.0	PPi (1.25 mM)	70.4	88.8	122.0	132.4

The chloroplasts were isolated from 4 week old plants, and were suspended in buffer of the appropriate pH in 0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 25 mM ^{14}C-$NaHCO_3$ (1.2×10^6 cpm) and made 1.25 mM in PPi as shown. Reaction for 5 min. at 30,000 lux and 20°C. The chlorophyll concentration was 0.17 mg./flask. The reaction flasks were flushed with argon during the experiments.

ADP slightly inhibited the stimulation resulting from the presence of 1.25 mM PPi, while at pH 7.75 and 8.00, ADP enhanced the effects of PPi on CO_2 fixation. It has been noted (126) that ADP stimulates the activity of pyrophosphatase in chromatophores at pH 7.5. PPi hydrolysis might then result in levels of Pi inhibitory to CO_2 fixation. However, caution should be employed in the interpretation of experiments with microorganisms, especially when relating them to chloroplasts of higher plants. At higher pHs enough ATP may be formed from the added ADP to increase the PPi effect on CO_2 fixation. In separate experiments, ADP (2 mM) was found to increase CO_2 fixation in the absence of added ATP or PPi, while 5 mM ADP had no effect. If these stimulations of CO_2 fixation result from ATP synthesis, the reactions should be blocked by an energy transfer inhibitor. Phloridzin was chosen, for it is believed to act as an inhibitor of a phosphorylation reaction close to the site of ATP synthesis (99). Phloridzin also inhibits the enzymic hydrolysis of ATP (183). It was found (Table XI) that the stimulation with ADP could be completely inhibited by phloridzin (0.25 mM).

vi. Effects of ADP on ATP and PPi-induced CO_2 fixation

The results in Table Xa demonstrate that ADP was capable of increasing the levels of CO_2 fixed both in the

Table Xa. Effect of ADP on ATP and PPi-induced CO_2 fixation

	ADP (mM)	µmoles CO_2 fixed/mg. chlorophyll/hour	
		Experiment No. 1	2
Control	0	70.4	23.4
Control	0.5	81.6	34.3
ATP (3 mM)	0	85.7	50.4
ATP (3 mM)	0.5	102.0	67.2
PPi (3 mM)	0	105.8	66.7
PPi (3 mM)	0.5	112.9	82.9

Chloroplasts (No. 1 = 0.22 mg. chlorophyll; No. 2 = 0.26 mg. chlorophyll) were added to 2.0 ml. reaction buffer (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$ pH 7.75) in ADP, ATP, and PPi were included as shown in the Table. The reaction was for 5 mins. at 30,000 lux and 20°C.
The atmosphere was air.

Table XI. Effects of phloridzin on the fixation of CO_2 by isolated spinach chloroplasts in the presence of ADP

	μmoles CO_2 fixed/mg. chlorophyll/hour		
	ADP (mM)		
phloridzin (mM)	0	0.5	1.0
0	24.1	33.4	40.6
0.25	19.5	20.7	19.0
0.50	15.8	15.6	14.9

Chloroplasts were preincubated for 5 mins. with or without phloridzin, in 0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, pH 7.5, and then the reaction was started by adding 25 mM ^{14}C-$NaHCO_3$ (1.2×10^6 cpm), and ADP and turning on the lights for 5 mins. The flasks were flushed with argon during the experiment. The chlorophyll concentration was 0.19 mg./flask.

control flasks and in the presence of ATP and PPi. The ADP may be utilized either by a chloroplast adenylate kinase or in photophosphorylation to yield ATP that can then be used for CO_2 fixation. Interpretation of this experiment is complicated because of the paucity of information relating to the penetration through the chloroplast membrane of ADP, ATP, and PPi, and the various sites at which these compounds may be utilized.

Information on the endogenous levels of Pi and nucleotides in chloroplasts (isolated by a non-aqueous technique) is available (158). These levels were all very variable from one preparation to another, e.g. the adenylate concentration varied from 0.15 to 0.5 mM. Bassham (128) noted that the amount of labelled PPi in spinach chloroplasts was between 1/10 and 1/2 that of the ATP. Although the determination of these levels in the chloroplasts isolated in this thesis would provide interesting information, it was felt that the levels would vary with each isolation procedure.

vii. Effect of Mg^{2+} on ATP and PPi-enhanced CO_2 Fixation

In order to test the hypothesis that Mg^{2+} may be necessary for the enhancement of CO_2 fixation by ATP or PPi, the experiments shown in Table XII were performed. The results indicate that the optimal Mg^{2+} concentration for CO_2 fixation in the absence of added ATP or PPi was 0.25 mM.

Table XII. Effects of Mg^{2+} concentration on CO_2 fixation in the presence of ATP or PPi

	μmoles CO_2 fixed/mg. chlorophyll/hour					
	Control		ATP (2.5 mM)		PPi (2.5 mM	
$MgCl_2$ (mM)	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
0	15.3	25.3	23.8	28.8	23.5	42.8
0.25	18.1	34.4	25.1	35.9	24.5	40.9
0.50	14.4	29.2	24.0	33.5	20.6	37.5
1.00	10.4	10.7	21.4	13.4	18.2	15.0
5.00	6.7	9.2	12.8	9.4	9.2	9.4
10.00	5.2	-	10.9	-	6.6	-

Chloroplasts were isolated from 4 week old plants in Mg^{2+} free buffer, 0.01 M HEPES, 0.33 M sorbitol, 1.0 mM EDTA, pH 7.5 and assayed in 0.01 M HEPES and 0.33 M sorbitol, pH 7.5 plus Mg^{2+}, ATP (2.5 mM) and PPi (2.5 mM) as shown and 25 mM ^{14}C-$NaHCO_3$, 1.2×10^6 cpm. The flasks were flushed with argon during the experiment. The chlorophyll concentration was 0.18 mg./flask in No. 1, and 0.22 mg./flask in No. 2.

This level of Mg^{2+} also gave the highest fixation of CO_2 in the presence of either ATP or PPi. Higher levels of Mg^{2+} were noted to be inhibitory to CO_2 fixation.

The hydrolysis of polyphosphates is catalyzed by magnesium ions. A co-ordinated complex is formed that increases the positive charge on the phosphorus atom, thereby facilitating attack by water or hydroxyl ions (160). A non-enzymic transphosphorylation reaction between ATP and Pi, catalyzed by Mn^{2+}, has been demonstrated by Lowenstein (161).

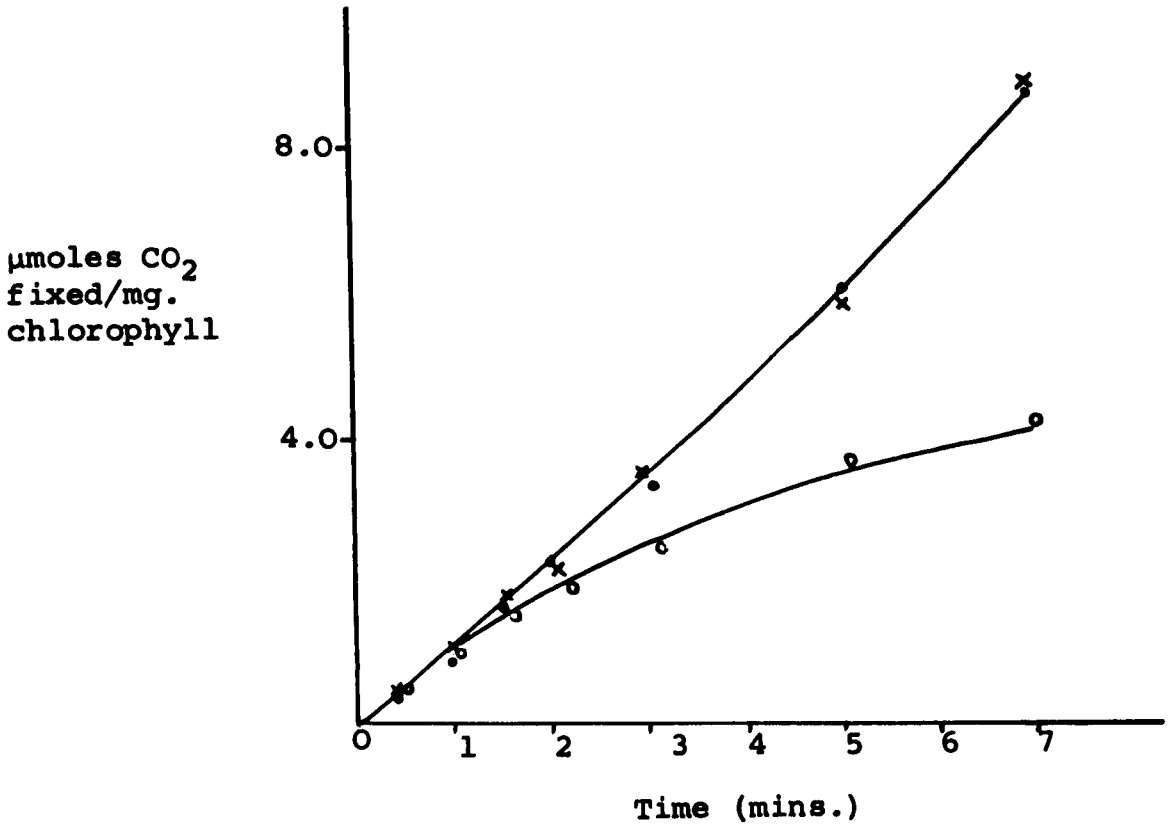
viii. A temporal study of the effects of ATP and PPi on CO_2 fixation

The data in Figure X show that the initial kinetics (60 μmoles CO_2 fixed/mg. chlorophyll/hour) were not influenced by the presence of either exogenous ATP or PPi. With time, the effects of these high energy compounds became apparent. This could be a result of a utilization of added ATP or PPi to drive CO_2 fixation, or an effect of these two compounds on the chloroplast membrane permeability to endogenous substrates required in CO_2 fixation.

ix. Effects of Phloridzin on ATP and PPi-enhanced CO_2 Fixation

The effect of an energy transfer inhibitor, phloridzin, was next tested on the ATP and PPi-induced increases in CO_2 fixation, and the results are shown in Table XIII. Other

Figure X. A temporal study of the effects of ATP and PPi on CO_2 fixation



Chloroplasts were isolated from 6 week old leaves (0.36 mg. chlorophyll/flask), and reacted in 0.01 M HEPES buffer pH 7.75 (0.33 M sorbitol, 1.0 mM $MgCl_2$, 12 mM ^{14}C-$NaHCO_3$, 6.0 x 10^5 cpm). ATP (3 mM) and PPi (3 mM) were included as shown in the Figure. The reaction was at 20°C, and a light intensity of 30,000 lux. Total volume was 2.0 ml. Aliquots were taken at the time intervals shown, and the amount of fixed ^{14}C measured. The atmosphere was air.

Key: o Control
● ATP (3 mM)
x PPi (3 mM)

Table XIII. The effects of phloridzin on the ATP and PPi induced increases in CO_2 fixation

	μmoles CO_2 fixed/mg. chlorophyll/hour							
	chloroplast preparation No.							
	1		2		3		4	
Phloridzin	ATP	PPi	ATP	PPi	ATP	PPi	ATP	PPi
-	25.4	25.7	45.2	33.2	12.1	11.1	31.1	30.9
+	18.7	20.5	25.6	23.2	9.5	9.0	14.7	15.1
Control 1	11.1		14.8		7.2		11.5	
Control 2	8.9		11.8		5.76		7.7	

The reaction mixtures (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 25 mM ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm) were 5 mM in ATP or PPi (pH 7.5). In experiments 1, 2, and 3, solutions were 0.25 mM in phloridzin where indicated, and 0.50 mM in No. 4. There was a 5 min. reaction in the light (30,000 lux) at 20°C. Control 1 was made in the absence of phloridzin, ATP and PPi. The values for Control 2 were based on an assumed 20% inhibition of the basal rate by phloridzin (0.25 mM) and 33% by 0.50 mM phloridzin. The flasks were flushed with argon during the experiments. The chloroplast concentration was 0.18 mg. chlorophyll/flask in No. 1, 0.17 mg. in No. 2, 0.12 mg. in No. 3 and 0.15 mg. in No. 4.

studies (Table XXXII) demonstrated that phloridzin (0.25 mM) inhibited CO_2 fixation by 0-20%. From the data in Table XIII it was calculated that both the ATP and PPi induced increases in CO_2 fixation were inhibited by 60 ± 7% by 0.25 mM phloridzin, and by 80 ± 4% by 0.50 mM phloridzin. These figures are based on the percentage increases observed over the control value (no phloridzin). A more significant calculation based on the % changes induced by phloridzin on the ATP and PPi-enhancements of CO_2 fixation was also made, using two control values. One control value was obtained in the absence of phloridzin, and the other was based on a 20% inhibition of the basal CO_2 fixation by 0.25 mM phloridzin, or 33% by 0.50 mM phloridzin. In this case 0.25 mM phloridzin was found to inhibit the ATP-induced increase in CO_2 fixation by 37 ± 15% and the PPi-induced increase by 25 ± 10%. 0.5 mM phloridzin inhibited both reactions equally by 62 ± 2%. These inhibitions are greater than those noted for phloridzin on the basal CO_2 fixation (Table XXXII), and may signify a role for ATP and PPi as energy sources. However, the effect of phloridzin on the basal rate of CO_2 fixation in the presence of added ATP is not known, and therefore these data cannot be considered conclusive.

It is of interest that PPi (6.7 mM) has been observed to inhibit photophosphorylation and the ATP $\rightleftharpoons$ ^{32}Pi exchange

in the light, and to stimulate ATPase activity in chromatophores of Rhodospirillum rubrum (162). The relationship of this work to chloroplasts of higher plants is not yet known, but presents an exciting possible mechanism for the action of exogenous PPi, if it is in fact utilized as an energy source.

x. Studies on Dark CO_2 Fixation

All the reactions discussed have involved a 5 minute light period. Attempts were made to obtain dark fixation of CO_2 in this same chloroplast system to see if ATP or PPi could serve as an energy source in the absence of light.

The results in Table XIV indicate that ATP was able to stimulate the dark fixation of CO_2 in the presence of ribose 5-phosphate (cf. reference 163), whereas under the same conditions PPi had no effect. Different chloroplast preparations showed considerable variation in their ability to respond to either ATP or PPi, and often no stimulation could be observed with either one. The cause of this variability may be related to variations in the permeability of the chloroplast membrane to the different substrates (164). No appreciable assimilation of ^{14}C was found in the isolated chloroplasts unless ribose 5-phosphate or ribulose diphosphate was added. D-ribose had no effect.

The influence of magnesium ions on the effects of ATP or PPi in the dark was also tested, and the results are shown in Table XV. The ATP effect was shown to have an absolute requirement for Mg^{2+}, while there was still no effect with the PPi.

Table XIV. Dark CO_2 fixation in isolated spinach chloroplasts

Preparation No.	Ribose 5-P (1 mM)	NADPH (0.1 MM)	ATP (2 mM)	PPi (2 mM)	µmoles CO_2 fixed/mg.^{2} chlorophyll/ hour
1	+	-	-	-	0.44
	+	+	-	-	0.50
	+	+	+	-	1.30
	+	+	-	+	0.50
2	+	-	-	-	1.84
	+	-	+	-	6.6
	+	-	-	+	1.2

Chloroplasts isolated from 4 week old plants were assayed in 0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 25 mM ^{14}C-$NaHCO_3$ (1.2×10^6 cpm), 0.1 mM phenazine methosulphate; other reactants are shown in the table. Reactions were completely in the dark at 20°C for 10 min., in the air. The chlorophyll concentration was 0.15 mg./flask in No. 1 and 0.31 mg. in No. 2.

Table XV. Effect of $MgCl_2$ on the dark fixation of CO_2 by isolated chloroplasts

Ribose 5-P (1 mM)	$MgCl_2$ (2 mM)	ATP (2.5 mM)	PPi (2.5 mM)	µmoles fixed/mg. chlorophyll/hour
+	-	-	-	1.2
+	-	+	-	1.05
+	+	+	-	8.5
+	-	-	+	0.95
+	+	-	+	1.05

Chloroplasts were isolated in 0.01 M HEPES, 0.33 M sorbitol, pH 7.5, and assayed in the same buffer, 25 mM in ^{14}C-$NaHCO_3$ (1.2×10^6 cpm); with R5P, $MgCl_2$, ATP, and PPi (at the levels shown). Reactions were completely in the dark at 20°C for 10 mins. in the air. The chlorophyll concentration was 0.27 mg./flask.

When the dark fixation of CO_2 was driven by the presence of ribulose 1,5-diphosphate, ATP and PPi were both shown to stimulate the fixation of CO_2 in 2 experiments, but had no effect at all in 4 other experiments.

xi. Effects of ADP and DTT on the incorporation of ^{32}Pi into ATP and PPi

Some results are presented in Table XVI that indicate the formation of ^{32}P-pyrophosphate in spinach chloroplasts, when no ADP was added. In the presence of exogenous ADP, the activity located in PPi was decreased, and was found in large amounts in ATP. Dithiothreitol (10 mM) appeared to induce the synthesis of PPi. The synthesis of PPi may involve a reversal of the pyrophosphatase known to be present in chloroplasts (134). In micro-organisms the activity of this enzyme was stimulated by reductants such as GSH or cysteine (165). It is possible that pyrophosphatase reversal may be involved in PPi synthesis.

ION UPTAKE BY CHLOROPLASTS

i. Calcium Ion Uptake - the effects of ATP and PPi

The light-induced uptake of calcium ions by spinach chloroplasts has been described (28,90,94,95). This ion transport occurs under conditions favouring the light-triggered, magnesium-dependent ATPase that is enhanced by the addition of sulphydryl groups (40). In the previous section, PPi appeared to operate as an energy source in a manner similar to ATP, and

Table XVI. The effects of ADP and DTT on the incorporation of ^{32}Pi into ATP and PPi

Additions	^{32}P-PPi (cpm)			^{32}P-ATP (cpm)		
	Chloroplast Preparation No.					
	1	2	3	1	2	3
No ADP	63	140	118	66	61	86
ADP (10 mM)	55	120	92	490	400	560
No ADP + 10 mM DTT	150	157	-	56	43	-
Control (dark) no ADP	47	68	-	50	40	-

Chloroplasts were added to buffer pH 7.5 (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, and 5 mM ^{32}P-KH_2PO_4, 7.5×10^5 cpm). The ADP and DTT were added as shown in the Table. The reaction was at 20°C, and 30,000 lux for 5 mins. and was stopped by addition of TCA after a 5 minute light reaction. The samples were centrifuged at 1000 x g for 1 min. and then 20 lambda aliquots were spotted onto cellulose thin layer plates. Separation was by 2 way chromatography with I Methanol; formic acid; water (16:3:1 v/v) for 1 1/2 hours and II acetic acid; ethyl acetate; water (3:3:1 v/v) for 1 hour (see Methods). The flasks were flushed with argon during the experiments. The chlorophyll concentration was 0.16 mg./flask in No. 1, 0.21 mg. in No. 2, and 0.25 mg. in No. 3.

thus it was of interest to determine its effect on the energy-dependent uptake of calcium ions in spinach chloroplasts. Both ATP and PPi were found to stimulate the light-induced uptake of calcium ions. This is illustrated by the data in Table XVII.

The addition of Triton X-100 (to preparation 3) appeared to enhance the uptake of calcium ions, especially in the presence of ATP. Neumann and Jagendorf (166) demonstrated a stimulation of electron transport by low concentrations of Triton X-100, and this was considered to be a result of an uncoupling of a rate-limiting step concerned with PSP.

The effect of PPi on calcium uptake was found difficult to assay by the Millipore technique at high chloroplast concentrations (more than 0.25 mg. chlorophyll). Under these conditions, very high background counts were recorded, presumably a result of the chloroplasts blocking passage through the millipore filter of a ^{45}Ca-calcium pyrophosphate complex. This problem was overcome by reducing the amount of chloroplasts added by a factor of 10. The reproducibility of the Millipore technique was then found to be quite good ($\pm$ 10%).

It has been reported that the uptake of calcium ions was only slightly affected by the inhibitor phloridzin at concentrations severely inhibitory to PSP (27). This work was repeated, and it was found that calcium ion uptake was increased by incubating chloroplasts in the presence of 0.25 mM or 0.5 mM

Table XVII. Spinach chloroplast calcium ion uptake induced by light in the presence of ATP or PPi

	μmoles $CaCl_2$/mg. chlorophyll		
	Experiment No.		
	1	2	3
Control	83.3	261.2	303.5
ATP (5 mM)	106.4	303.5	402.4
PPi (5 mM)	122.3	482.4	390.6
Dark control (ATP, 5 mM)	-	136.0	136
Dark control (PPi, 5 mM)	-	147.3	147.3

Chloroplasts (0.03 mg. chlorophyll) were added to reaction buffer pH 7.5 (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 5 mM GSH, 1.0 mM ^{45}Ca-$CaCl_2$, 8.5 x 10^5 cpm) and reacted for 5 mins. at 30,000 lux at 20°C. Total volume 2.0 ml. The atmosphere was air.
Experiment 3 used the same chloroplasts as 2, except 40 μg. Triton X-100 were present.
In Experiment 1, the ^{45}Ca uptake was assayed by the millipore apparatus, and in 2 and 3 chloroplasts were centrifuged and washed before counting (see Methods).

phloridzin by 17% and 41% respectively. These concentrations were later noted to be extremely inhibitory to ATP synthesis in both PMS-catalyzed cyclic PSP (light-dependent and light-triggered mechanisms) and in endogenous PSP. This work adds weight to the supposition that the energy for ion uptake may be derived from a high energy state produced prior to ATP synthesis. Added ATP and PPi may increase calcium ion uptake by inducing the formation of this high energy state. It is interesting to note that a similar effect of phloridzin has been reported by Crofts (97). He noted that at a concentration that completely inhibited PSP, phloridzin slightly increased the extent and rate of both proton and ammonium ion uptake in spinach chloroplasts.

ii. Phosphate Ion Uptake - the effect of ATP and PPi

It appeared from the data in Table XVIII that the presence of ATP decreased the uptake of phosphate anions into the chloroplasts, both in the dark and light. The effect of PPi was tested in Experiment 3 and was found to have a similar effect to the ATP in the light reaction. When $CaCl_2$ (1 mM) was present, the amount of phosphate taken up into the chloroplasts was further decreased.

iii. Conclusion

It was deemed of interest to investigate the mechanism by which the addition of inorganic pyrophosphate

Table XVIII. The effects of ATP, PPi, and light on the uptake of ^{32}P-H_3PO_4 by isolated spinach chloroplasts

	Treatment	^{32}P-uptake by isolated chloroplasts (μmoles/mg. chlorophyll)		
		Experiment No.		
		1	2	3
Light reaction	control	0.64	2.4	16.6
	ATP (0.5 mM)	0.24	1.3	6.91
	PPi (0.5 mM)	-	-	5.2
Dark reaction	Control	0.96	1.6	-
	ATP (0.5 mM)	0.45	1.3	-

Chloroplasts (0.08 mg. chlorophyll) were added to reaction buffer pH 7.5 (0.01 M HEPES, 0.33 M sorbitol, 1.0 mM $MgCl_2$, ^{32}P-H_3PO_4, 5.0 x 10^5 cpm, 1.0 mM PMS, and ATP and PPi as shown in the Table). The reaction was for 5 minutes at 30,000 lux or in the dark, at 20°C, in the air.

could increase the level of CO_2 fixed by isolated chloroplasts. A number of parameters were varied, and it was concluded that both ATP and PPi were affected similarly by a variety of factors. Of special interest was the severe inhibition of the ATP and PPi induced increases in CO_2 fixation by an energy transfer inhibitor, phloridzin. The initial kinetics of CO_2 fixation were not affected by either added ATP or PPi. When the stoichiometry of these ATP and PPi-induced increased in CO_2 fixation were studied, 3 μmoles of ATP or PPi were found to increase the amount of CO_2 fixed by between 0.1 to 1.2 μmoles.

The additions of ATP and PPi were also found to increase the uptake of calcium ions by isolated chloroplasts. The ATP and PPi could be acting as energy sources, or may simply complex with the internal Ca^{2+} and hence lower the energy needed for the entry process of calcium. Both ATP and PPi were shown to decrease the uptake of added H_3PO_4 by chloroplasts. In view of other observations on the inhibition of CO_2 fixation by Pi, it is possible that ATP and PPi may stimulate CO_2 fixation, at least in part, by decreasing the uptake of Pi into the chloroplasts.

Many of these conclusions are tentative, but do provide some insight into the mechanism by which added PPi may enhance CO_2 fixation. In addition to the proposed functions for added ATP and PPi there are a number of other possibilities, including a non specific function resulting from their polyvalent anionic nature, or some indirect function in which the high energy phosphate bonds help to maintain some essential chloroplast structure or enzyme.

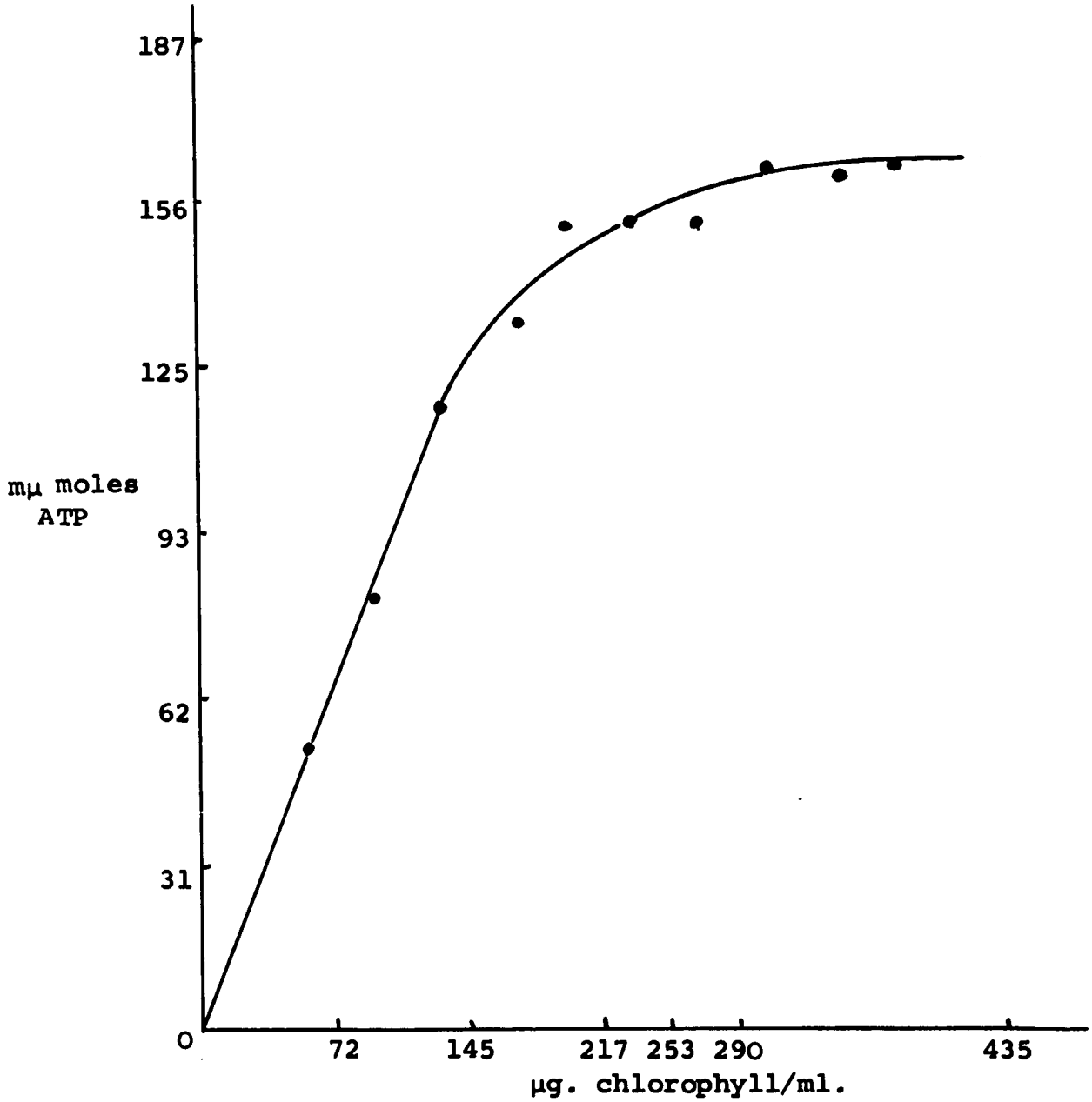
ATP SYNTHESIS IN ISOLATED CHLOROPLASTS

ATP was assayed by a variety of methods, but predominantly by the IBB extraction procedure. This method was found to give reproducible results, for example, when chloroplasts were incubated with ^{32}P-KH_2PO_4 in the dark, or in the light for 5 minutes, and the esterified phosphate assayed by IBB extraction, the standard error was only $\pm$ 6% for 9 identical reaction flasks. Results obtained following "norite" adsorption of the radioactive nucleotides were the same as those from the IBB extraction. The luciferase assay for ATP was found useful for confirmation of the results from IBB extraction.

i. Effect of Chloroplast Concentration on ATP Synthesis

The data presented in Figure XI demonstrate the effects of chloroplast concentration on the synthesis of ATP. Initially, there was a linear response to increased levels of chloroplasts, and above 250 μg. chlorophyll per ml. no further increase in ATP synthesis occurred. This phenomenon may have been a result of limiting light intensity or PMS concentration at the higher chloroplast concentrations. In most of the studies reported in this thesis, the amount of chloroplasts added was less than 125 μg. chlorophyll per ml., so that chloroplast concentrations would be limiting. The levels of ATP synthesis observed in these experiments are low. This could be a result of using intact chloroplasts (cf reference 184).

Figure XI. The effect of chloroplast concentration on photophosphorylation



Chloroplasts were isolated as described in Materials and Methods in buffer at pH 7.8 at 0°C (Tricine, 0.05 M; sucrose, 0.4 M; $MgCl_2$, 1.0 mM; EDTA, 1.0 mM). Various concentrations of chloroplasts were added to the reaction flasks. These contained buffer (as above, but with no EDTA) pH 7.8 at 20°C that was 5 mM in ^{32}P-KH_2PO_4 (1.5×10^5 cpm), 0.1 mM in phenazine methosulphate (PMS) and 10 mM in ADP. The final volume was 2 ml. The reaction proceeded for 10 minutes in the light (30,000 lux) at 20°C under an argon atmosphere, and was terminated by the addition of 0.2 ml. 20% trichloroacetic acid (w/v). The ^{32}P-ATP was assayed by the method of Nielsen and Lehninger (140).

ii. Effect of an Argon Atmosphere on ATP Synthesis

The data in Table XIX indicate that the formation of ATP is reduced in an argon flushed atmosphere. This effect has been demonstrated by others, and it was postulated that oxygen may act catalytically in PSP by reoxidizing the PMS, when this is present in limiting concentrations (167,168). That PMS-catalyzed cyclic PSP is dependent on the oxidation-reduction state of the electron carrier has been demonstrated by Zweig and Avron (169).

iii. Effect of PMS Concentration and Temperature on ATP Synthesis

The amount of ATP synthesized in these experiments was also found to be related to the concentration of PMS, the catalyst for cyclic PSP. This is illustrated by the data in Table XX. These data also indicate that under these conditions, ATP can be synthesized, at low temperatures, although at a lower rate. This ability of chloroplasts to generate ATP at low temperatures has also been noted by Hall and Arnon (170). They pointed out that at low temperatures inhibitory to oxidative phosphorylation, ATP could still be synthesized by PSP. It is possible that PSP may serve to provide ATP for metabolic process in the phytoplankton or snow algae of polar regions, under conditions unfavourable to oxidative phosphorylation.

Table XIX. Effect of an argon atmosphere on the light-dependent synthesis of ATP

Atmosphere	μmoles ADP added	mμmoles ATP formed
Air	0	0
Air	0.5	23
Air	1.0	49
Argon	0	0
Argon	0.5	14
Argon	1.0	28

Chloroplasts were added to 0.01 M HEPES buffer at pH 7.5 that was 0.33 M in sorbitol, 1.0 mM in $MgCl_2$, 1.0 m in M EDTA 0.1 mM in phenazine methosulphate, 5 mM in KH_2PO_4, and ADP as shown, to give a final volume of 2 ml. The reaction was for 5 mins. at 30,000 lux at 20°C. The ATP was assayed by the luciferase procedure. The chlorophyll concentration was 0.12 mg./flask.

Table XX. Effect of phenazine methosulphate on ATP formation by the light-dependent mechanism of PSP at two temperatures in spinach chloroplasts

PMS (μmoles)	mμmoles ATP formed			
	reaction temperature			
	2°C		20°C	
	chloroplast preparation No.			
	1	2	1	2
0.1	50	12	88	80
0.2	72	63	112	130
0.4	115	78	147	170

Chloroplasts were isolated from freshly picked 6 week old spinach leaves as shown in Methods (in 0.4 M sucrose; 0.05 M Tricine; 1.0 mM $MgCl_2$; 1.0 mM EDTA, pH 7.8). The reaction medium was the same, except there was no EDTA, and included 10 μmoles ^{32}P-KH_2PO_4 (1.5×10^5 cpm) and 20 μmoles ADP (pH 7.5). The final volume was 2.0 ml. Phenazine methosulphate was added as shown in the Table; the reaction time was for 10 minutes (30,000 lux) at the two temperatures. The esterified phosphate was then assayed by the IBB extraction procedure. The chloroplast concentration was 0.16 mg. chlorophyll/flask. The atmosphere was argon.

iv. The Mechanism of ATP Synthesis

(a). a phosphorylated intermediate ?

The enzymic hydrolysis of ATP could represent a partial reaction in the mechanism of ATP formation. Two types of light-activated ATPase have been noted, and it is therefore not too surprising that there should be two light-activated mechanisms of ATP synthesis (171). These may reflect two or more different locations in the electron transport chain for ADP phosphorylation (12,172,173) or perhaps two mechanisms are operative at the same site.

It is not clear whether the precursor of ATP synthesis in PSP is a carrier $\sim$ ADP or a carrier $\sim$ Pi. As ATP can be generated in a dark reaction following a short light period, this 2 phase reaction can be used to study formation of intermediate in PSP. The results in Table XXI are from experiments in which ADP or ^{32}Pi was added in the light, followed by ^{32}Pi or ADP in the dark. When the light reaction was conducted with ADP and the ^{32}Pi added in the dark, the amount of esterified phosphate was lower, than when the light reaction was conducted in the presence of ^{32}Pi. This may be indicative of a phosphorylated high energy intermediate being synthesized in the light, that is then able to phosphorylate ADP in the dark, or may be a result of an adenylate kinase phosphorylating some endogenous ADP in the light reaction.

Table XXI. Effects on spinach chloroplast PSP of addition of ADP in the light or in the dark following a short light reaction

Light (30 seconds)	Dark (60 seconds)	mμmoles ATP
ADP (μmoles)	^{32}Pi (μmoles)	
0	10	16
10	10	24
20	10	34
30	10	25
40	10	17
^{32}Pi (μmoles)	ADP (μmoles)	
10	0	16
10	10	50
10	20	63
10	30	53
10	40	42

Chloroplasts were added to Tricine buffer (0.05 M) pH 7.5 (0.4 M in sucrose, 1.0 mM in $MgCl_2$, and 0.1 mM in PMS). There was a pre-illumination period prior to addition of ADP or ^{32}Pi as shown above. (The activity of the ^{32}Pi was 1.1 x 10^5 cpm). Then a 30 second light reaction at 30,000 lux was given, followed by a 60 second dark reaction during which the ADP or ^{32}Pi were added to the respective flasks. The reaction was terminated by addition of 0.2 ml. 20% trichloroacetic acid, and ^{32}P-ATP was assayed by the IBB extraction procedure. The chloroplast concentration was 0.17 mg. chlorophyll/flask. The flasks were flushed with argon.

In these experiments, the length of the light period was found to be important. A 30 second light period at 30,000 lux was used; if this was increased to 60 seconds the yield of ATP formed in the following dark period was reduced by about 50%.

Similar evidence for the formation of carrier$\sim$ Pi was given by Kahn and Jagendorf (22). More recently, these conclusions were queried by Jagendorf and Hind (174). They pointed out that there could be some endogenous ADP present in the chloroplasts during the light reaction with ^{32}Pi that could drive ATP synthesis. They concluded that the major high energy state was a non-phosphorylated compound designated X_e. There is also likely to be a considerable amount of endogenous inorganic phosphate present in the chloroplasts (158), probably at higher levels than the endogenous ADP (especially following a 2-5 minute pre-illumination period).

It is evident that more critical experiments are required to confirm the nature of the high energy intermediate of PSP, especially in view of data presented by Avron and Jagendorf (79) that indicated the formation of a carrier$\sim$ ADP. This conclusion was based on the somewhat indirect evidence that in order to show arsenate uncoupling in chloroplasts, ADP was required. The formation of a carrier$\sim$ ADP has also been demonstrated in chromatophores of Rhodospirillum rubrum (175).

The data in Table XXI also indicate that high levels of ADP (greater than 20 μmoles) inhibited the synthesis of ATP. This effect of ADP has been shown by others (118). In this regard, it is interesting to note that Wessels and Baltscheffsky (134) reported an inhibition of a spinach chloroplast ATPase by high levels of both AMP and ADP.

Hinkson and Boyer (23) have presented evidence for the presence of phosphorylated compounds synthesized in chloroplasts more rapidly than is ATP. They reported that the incorporation of ^{32}Pi into this material was considerably decreased by increased levels of ADP. Higher levels of ADP also decreased the total yield of ^{32}P-ATP in their experiments.

(b). Effect of pH on ATP synthesis

In Table XXII, the effect of varying the pH of the reaction mixture on the dark phosphorylation of ADP is demonstrated. The optimum was found close to pH 7.75. However, it is apparent from the work of Nobel (101) on the effect of pH on the endogenous synthesis of ATP in pea chloroplasts, that the results obtained will depend on the combined effects of the pH, the type of buffer used, and the effects of Cl^- or Na^+ ions (depending on whether HCl or NaOH was used to adjust the buffer pH). With a Tris-HCl buffer, the optimum pH for endogenous ATP synthesis was near to pH 7.9, while with TES-NaOH buffer, the optimum pH was from 7.9 - 8.2 (101).

Table XXII. Effect of pH on spinach chloroplast dark phosphorylation of ADP following a 20 second light period

pH					
7.0	7.5	7.75	8.0	8.5	9
mμmoles ATP					
34	70	77	64	32	5

Chloroplasts were added to 0.01 M HEPES buffer (0.33 M sorbitol, 1.0 mM $MgCl_2$, 0.1 mM PMS, 2.0 mM GSH, and 5 mM ^{32}P-KH_2PO_4, 1.0×10^5 cpm) adjusted to different pH value, and incubated at 30,000 lux for 20 seconds. Then, in the dark, 20 μmoles ADP was added to the flasks, and after a further 60 seconds dark period, 0.2 ml. 20% TCA was added to terminate the reaction. ^{32}P-ATP was assayed by the IBB procedure. The chlorophyll concentration was 0.18 mg./flask. The flasks were flushed with argon during the experiments.

(c). Effect of thiol protectants

The data in Table XXIII indicate that the presence of reduced glutathione (GSH) in both the grind and reaction buffers stimulated the light-triggered reaction. A similar effect with dithiothreitol (176) is shown in Table XXIV. These stimuli by GSH and DTT are especially interesting in view of the sulphydryl-dependent nature of the light-triggered ATPase system of chloroplasts (40). This ATPase activity is thought to be more related to a non-cyclic electron flow phosphorylation site than to a cyclic electron flow site (172). These data indicate that -SH group protectants enhance ATP formation, but do not differentiate between the light-dependent or light-triggered reactions of ATP generation. The results given in Table XXV show that it is the light-triggered reaction that is protected. In these experiments, GSH was only added in the dark period following the light reaction.

(d). Effects of iodoacetamide

It was then postulated that if this system was sensitive to sulphydryl group protectants, it might be inhibited by a sulphydryl group inhibitor, for example iodoacetamide (177). This compound severely inhibits CO_2 fixation (178), probably by inhibiting glyceraldehyde 3-phosphate dehydrogenase (179), but it appears to have very little effect on the light-dependent synthesis of ATP (177).

Table XXIII. Effect of addition of reduced glutathione on the light-triggered synthesis of ATP

ADP (μmoles) added	mμmoles ATP formed	
	+ GSH	No GSH
10	20	12
20	21	13
30	23	11

Chloroplasts were isolated in 0.05 M Tris-HCl buffer at pH 7.8, (0.4 M sucrose, 1.0 mM $MgCl_2$, and 1.0 mM EDTA). The reaction buffer was the same except there was no EDTA, and there was 5 mM GSH present where indicated, 0.1 mM PMS and 5 mM ^{32}P-KH_2PO_4 (2.1×10^5 cpm). The total volume was 2.0 ml. ADP was added in the dark after a 30 second light reaction at 20°C under an argon atmosphere. After 60 seconds in the dark the reaction was stopped with TCA, and assayed by the IBB procedure. The chlorophyll concentration was 0.14 mg./flask. The flasks were flushed with argon.

Table XXIV. Effect of dithiothreitol on the light-triggered synthesis of ATP

DTT (mmoles)	mμmoles ATP formed
0	246
15	280
40	340

The method was as for Table XXIII, except DTT was substituted for GSH, and 20 μmoles ADP was added to all the flasks in the dark period. The chlorophyll concentration was 0.2 mg./flask. The atmosphere was argon.

Table XXV. The effect of GSH on the dark phosphorylation of ADP

GSH (mM) added in the dark	mμmoles ATP formed in the dark after a 30 sec. light reaction	
	Chloroplast preparation No. 1	2
0	2.1	5.1
0.5	2.6	5.1
2.5	2.8	6.0
5.0	5.3	11.1

Chloroplasts were isolated in 0.05 M Tris-HCl buffer at pH 7.8 (0.4 M sucrose, 1.0 mM $MgCl_2$, 1.0 mM EDTA). The reaction buffer was the same, except there was no EDTA. After a 30 second light reaction in the presence of 0.1 mM PMS, and 5 mM ^{32}P-KH_2PO_4 (4.3×10^5 cpm), the chloroplasts were reacted in the dark for 60 seconds with 20 μmoles ADP and GSH. The esterified phosphate was assayed by the IBB method. The chlorophyll concentration was 0.15 mg. in No. 1 and 0.18 mg. in No. 2. The flasks were flushed with argon.

The results in Table XXVIa indicate that the iodoacetamide had no inhibitory effect on ATP synthesis by a light-dependent reaction. However, in Table XXVIb it is apparent that iodoacetamide severely inhibited the light-triggered reaction. These experiments were repeated, and analyzed by thin layer chromatography.

The ^{32}P-ATP zones were then counted for activity in a scintillation counter, and the iodoacetamide inhibition of the light-triggered reaction of ATP synthesis was again demonstrated. This inhibition was not alleviated by incubating the chloroplasts with 5 mM GSH (Table XXVII).

The interpretation of these studies is complicated by the inhibition of PSP (including the light-dependent reaction) by p-chloromercuribenzoate (PCMB), reported by Jagendorf and Avron (168). However, they did notice the presence of a certain amount of phosphorylation that was resistant to PCMB concentrations that completely inhibited flavin mononucleotide catalyzed phosphorylation. They suggested from these studies that there were two kinds of phosphate esterification when PMS was the cofactor. The results presented in this section of the thesis support this hypothesis. This could explain the high phosphorylation rates associated with PMS-catalyzed systems.

Table XXVI. The effects of iodoacetamide on the light-triggered and light-dependent synthesis of ATP by a PMS-catalyzed system

(a). Light-dependent reaction:

ADP (μmoles)	mμmoles ATP			
	no IAM		5 mM IAM	
	chloroplast preparation No.			
	1	2	1	2
0	0	(22)	0	(25)
1.0	44	(57)	44	(85)
2.0	44	(106)	46	(124)

The chloroplasts were isolated as given in the Methods section. They were pre-incubated with the IAM in the dark for 10 minutes, in HEPES (0.01 M), sorbitol (0.33 M), $MgCl_2$ (1.0 mM), $MnCl_2$ (1.0 mM, PMS (0.1 mM) buffer (pH 7.75). The reaction was initiated by adding the ADP and 10 μmole, ^{32}P-KH_2PO_4 (9.2×10^5 cpm) and turning the light on. The reaction lasted for 5 minutes in the light. ATP was assayed by the luciferase method (Figures in parentheses are from a separate experiment assayed by IBB extraction, and norite adsorption). Chlorophyll concentration was 0.18 mg./flask in No. 1 and 0.20 mg. in No. 2. The atmosphere was argon.

(b). Light-triggered reaction:

chloroplast prep'n No.	IAM (mM)					
	0	0.5	1.0	1.5	2.5	5.0
	mμmoles ATP					
1	23.0	20.2	18.4	16.1	13.9	11.7
2	18.3	15.8	13.2	7.5	-	4.2

The reaction conditions were the same as for the light-dependent reaction, except the ADP (30 μmoles) was added in the dark after the 30 second light reaction, and reacted for 60 seconds. TCA was added and the esterified ^{32}P-Pi extracted by the IBB method. The chlorophyll concentration was 0.2 mg./flask in both experiments. The atmosphere was argon.

Table XXVII. The effect of a 10 minute preincubation of chloroplasts with iodoacetamide on the light-triggered synthesis of ATP, in the presence of reduced glutathione

Iodoacetamide (mM)	mμmoles ATP	
	No GSH	+ GSH
0	26.0	38.0
1.0	19.8	34.5
10.0	18.0	25.0

Chloroplasts were isolated in Tris (0.05 M) buffer pH 7.8 (0.4 M sucrose, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, and 1.0 mM EDTA). The reaction buffer was the same (except there was no EDTA, and GSH was present as shown), and was 5 mM in ^{32}P-KH_2PO_4 (2.1×10^5 cpm), and 0.1 mM in PMS. The light reaction was for 30 seconds, at 30,000 lux, and was followed by addition of 30 μmoles ADP (in 100 lambda buffer, pH 7.8) in the dark. The reaction was terminated after 60 seconds in the dark, and assayed by the IBB method. The chlorophyll concentration was 0.10 mg./flask (no GSH), and 0.13 mg. (+GSH). The flasks were flushed with argon during the experiments.

INHIBITOR STUDIES

i. Effects of Phloridzin and Quinacrine on PSP

The effects of phloridzin and quinacrine were tested on both the light-dependent and light-triggered mechanisms of PMS-catalyzed ATP synthesis, and on the light-dependent "endogenous" synthesis of ATP in isolated spinach chloroplasts. The data presented in Table XXIX demonstrate that these compounds severely inhibited ATP synthesis in the PMS-catalyzed system. This effect of phloridzin was also shown by Izawa et al (99) who indicated that similar concentrations of phloridzin would inhibit both non-cyclic and PMS-catalyzed cyclic PSP to the same extent. The degree of inhibition induced by phloridzin is similar to that demonstrated by Nobel (27), and the results with quinacrine are in agreement with those of Bennun and Avron (171) who studied the two ATPase systems of chloroplasts.

The results in Figure XII demonstrate the inhibition by phloridzin of the "endogenous" synthesis of ATP (in the absence of any exogenous catalyst of either non-cyclic or cyclic PSP). It is interesting to note that

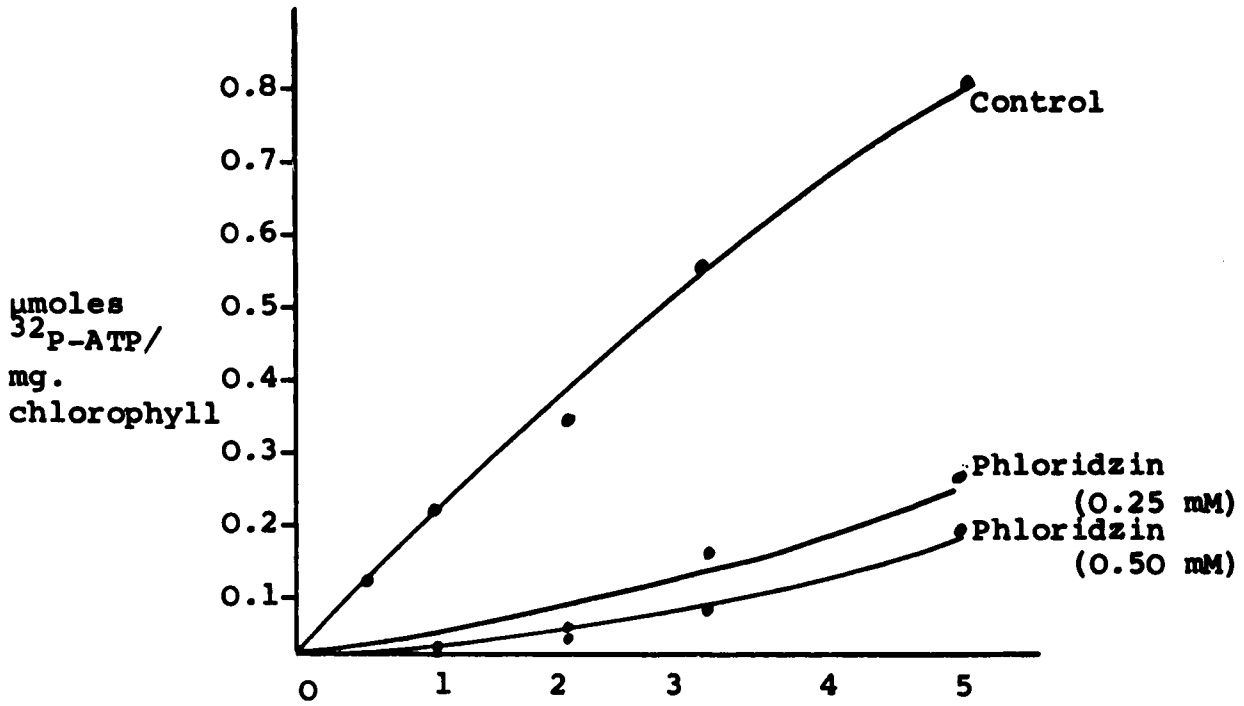
Table XXIX. Effects of phloridzin and quinacrine on ATP formation in spinach chloroplasts

Inhibitor (μM)	% inhibition	
	light-dependent reaction	light-triggered reaction
Phloridzin 50	52	42
Phloridzin 125	59	62
Phloridzin 250	66	70
Phloridzin 500	77	95
Quinacrine 5.0	33	30
Quinacrine 10.0	68	55
Quinacrine 25.0	95	74
Quinacrine 50.0	99	90

In the light-dependent reaction, 0.2 ml. of the chloroplast suspension (from 4 - 6 weeks old leaves) were added to reaction buffer (0.01 M HEPES pH 7.5, 0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$) and incubated in the dark with or without inhibitor, for 5 minutes. The reaction buffer was also 1.0 mM in ADP, 1.0 mM in KH_2PO_4 and 0.1 mM in phenazine methosulphate (PMS). The total volume was 2 ml. The light reaction (30,000 lux) lasted for 5 minutes. ATP was assayed by the luciferase method.

For the light-triggered reaction the same buffer was used. It was 0.1 mM in PMS, 2.0 mM in glutathione and 1.0 mM in KH_2PO_4. After the 5 minutes preincubation with or without inhibitor, 2.0×10^5 cpm ^{32}Pi were added, the light was turned on for 20 seconds, and then ADP was added in the dark to give a concentration of 10 mM, and the reactions were allowed to proceed in the dark for a further 1 minute. ^{32}P-ATP was then assayed by the IBB extraction procedure. Each level of inhibitor was studied in two or more separate experiments. The flasks were flushed with argon during these experiments. The chlorophyll concentration was 0.2 mg./flask.

Figure XII. The effect of phloridzin on "endogenous" ATP synthesis of isolated spinach chloroplasts



Chloroplasts (0.5 mg. chlorophyll) were isolated from 5 week old leaves; the reaction was followed in twice-recrystallized HEPES buffer (0.01 M) pH 7.75 (0.33 M sorbitol, 1.0 mM $MgCl_2$, 0.4 mM ADP, 0.4 mM ^{32}P-KH_2PO_4 (7.5×10^4 cpm); 25 mM $NaHCO_3$). The phloridzin was added at zero time, there was no preincubation with the inhibitor. The reaction was at a light intensity of 30,000 lux, and 20°C; 0.25 ml. aliquots were taken at the times shown and added to 0.5 ml. acetone and 0.1 ml. 20% TCA. The ATP was assayed by the IBB extraction procedure. The atmosphere was air.

in this study, the phloridzin completely inhibited ATP synthesis for the first minute. Although there was then a slow alleviation of inhibition, even after 5 minutes 0.5 mM phloridzin inhibited "endogenous" ATP synthesis by 75%. The very low levels of ATP synthesis could be a result of using intact chloroplasts (184).

In other experiments, the effects of phloridzin were tested on the esterification of phosphate in the absence of added ADP. The esterified phosphate was measured by the IBB extraction procedure. This method will assay both nucleotides and sugar phosphates (140) from the reaction mixture. Surprisingly, the data in Table XXX indicate that phloridzin, at concentrations capable of severely inhibiting ATP synthesis, had no effect on the esterification of phosphate in experiments in which no ADP was added. There would presumably be a small amount of endogenous ATP present, but the data in Figure XII demonstrated that endogenous ATP synthesis was severely inhibited by phloridzin. It was concluded that

Table XXX. Effects of 0.25 mM phloridzin and 40 mM dithiothreitol on the esterification of orthophosphate in a light triggered reaction

Experiment Number	Phloridzin	DTT	Secs. in dark after light reaction			
			5	30	90	150
			μmoles esterified Pi/mg. chlorophyll			
1	+	-	90.4	112	82.2	101
	-	-	95.9	115	65.6	110
2	+	-	34.3	41.1	20.4	21.7
	-	-	27.4	22.0	2.2	19.1
3	+	+	182	268	208	187
	+	-	145	154	87	116

Chloroplasts (0.2 mg. chlorophyll) were added to Tricine buffer (0.05 M) pH 7.5 (0.4 M sucrose, 1.0 mM $MgCl_2$, 0.1 mM PMS). There was a 5 minute preincubation in the dark; then ^{32}Pi (3.8×10^5 cpm) was added, mixed, and light was turned on for 20 seconds and then the reaction proceeded in the dark for the times shown in the Table. The esterified phosphate was assayed by IBB extraction. The flasks were flushed with argon during the experiments.

The degree of inhibition induced by phloridzin is similar to that demonstrated by Nobel (27), and the results with quinacrine are in agreement with those of Bennun and Avron (171) who studied the two ATPase systems of chloroplasts.

The results in Figure XII demonstrate the inhibition by phloridzin of the "endogenous" synthesis of ATP (in the absence of any exogenous catalyst of either non-cyclic or cyclic PSP). It is interesting to note that in this study, the phloridzin completely inhibited ATP synthesis for the first minute. Although there was then a slow alleviation of inhibition, even after 5 minutes 0.5 mM phloridzin inhibited "endogenous" ATP synthesis by 75%.

In other experiments, the effects of phloridzin were tested on the esterification of phosphate in the absence of added ADP. The esterified phosphate was measured by the IBB extraction procedure. This method will assay both nucleotides and sugar phosphates (140) from the reaction mixture. Surprisingly, the data in Table XXX indicate that phloridzin, at concentrations capable of severely inhibiting ATP synthesis, had no effect on the esterification of phosphate in experiments in which no ADP was added. There would presumably be a small amount of endogenous ATP present, but the data in Figure XII demonstrated that endogenous ATP synthesis was severely inhibited by phloridzin. It was concluded that

ii. Effects of Inhibitors on CO_2 Fixation

(a). characteristics of the system

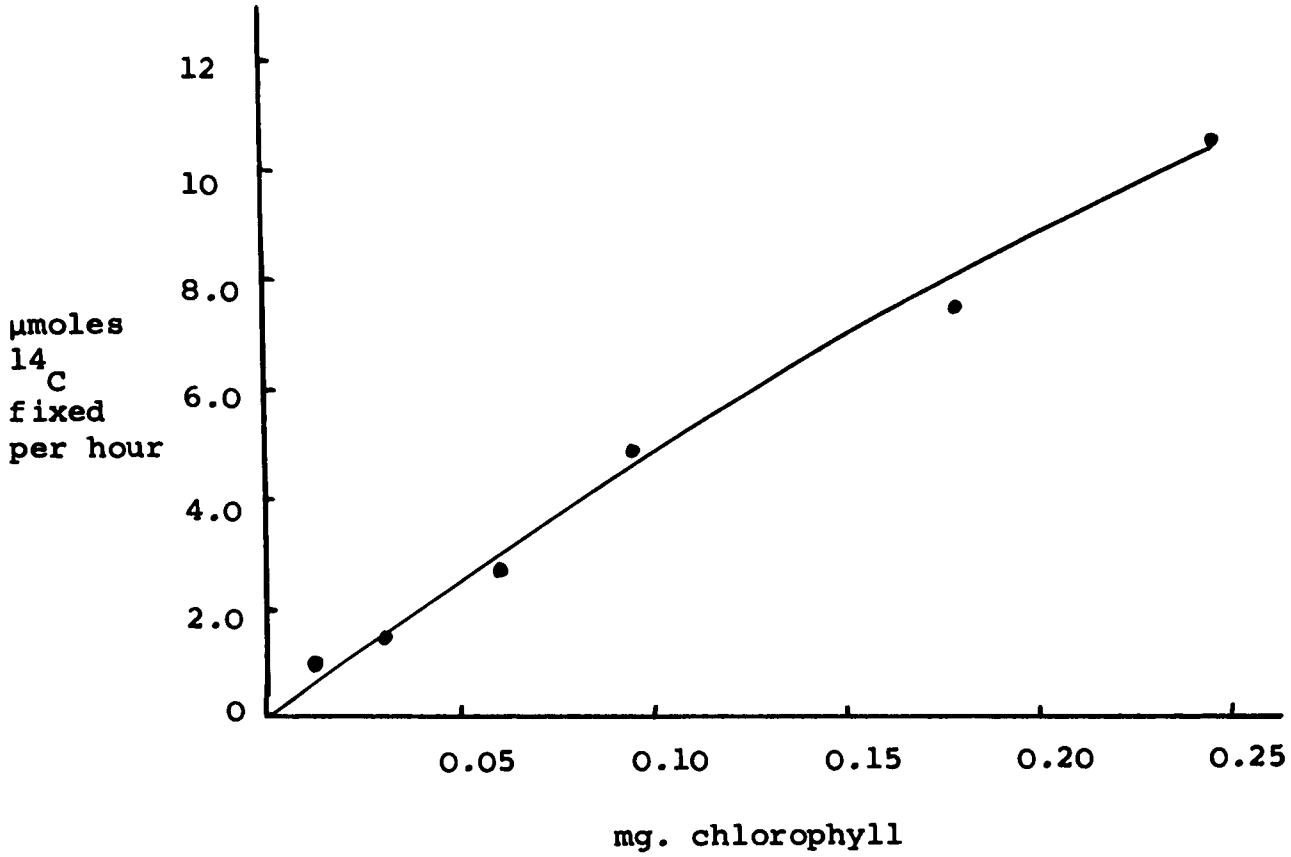
The data in Figure XIII demonstrate the effects of chloroplast concentration on CO_2 fixation. The reaction was noted to be linear with increasing chlorophyll concentration. The reaction was also found to be linear for the first 5 minutes, in a temporal study of CO_2 fixation in this system (Figure XIV).

There was no initial lag in CO_2 fixation in any of these reactions, however a lag in the initial kinetics of CO_2 fixation has been observed by others e.g. references 179 and 180. Bamberger and Gibbs (179) noted that this "induction period" could be eliminated by addition of carbon cycle intermediates. Thus, this lag period is probably a result of leakage through the chloroplast membranes of certain carbon cycle intermediates. The absence of such a lag may indicate the integrity of the chloroplasts and the prevention of excessive leakage through the chloroplastmembrane. Jensen and Bassham (159) using similar chloroplast isolation techniques to ours, also showed no lag period in CO_2 fixation following onset of the light reaction.

(b). effects of addition of carbon-cycle intermediates

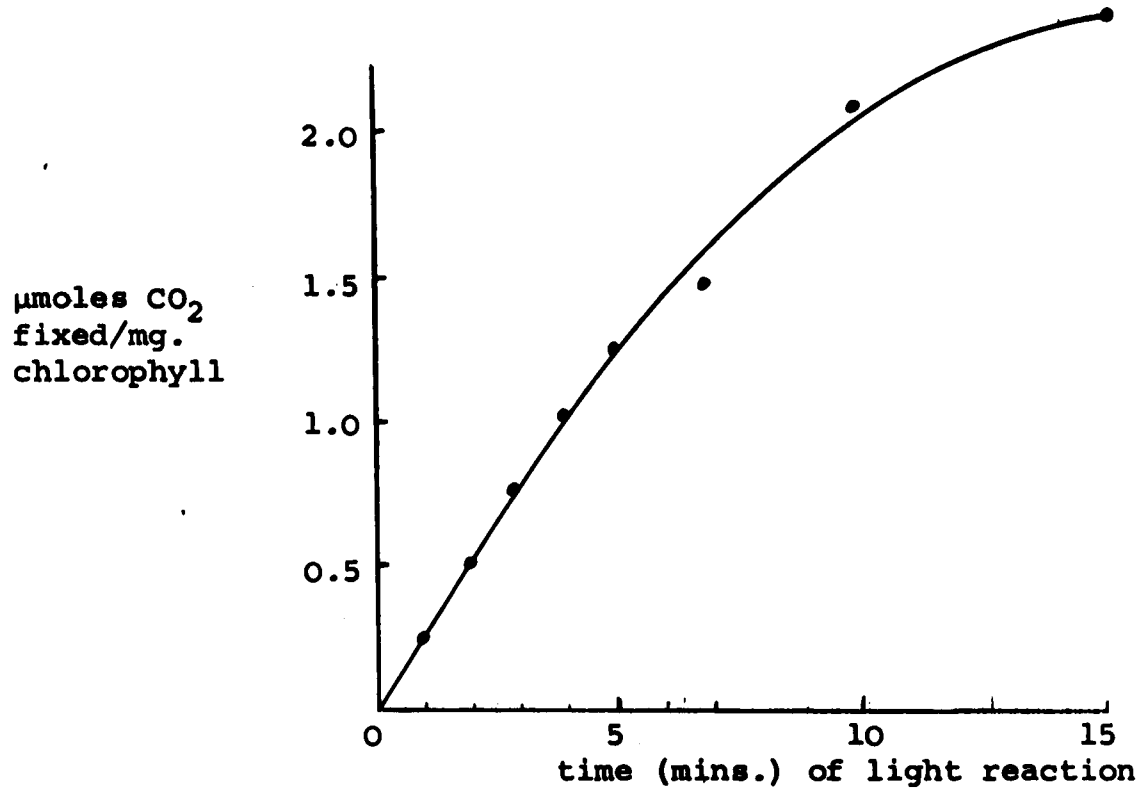
The addition of ribose 5-phosphate (R5P) was found to stimulate CO_2 fixation. However the addition of 3-phosphoglyceric

Figure XIII. The effect of chloroplast concentration on CO_2 fixation



Chloroplasts were isolated from 5 week old leaves, and reacted at different concentrations in HEPES (0.01 M) buffer pH 7.5 (0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 12 mM ^{14}C-$NaHCO_3$ (0.6×10^6 cpm)); the total volume was 2 ml. The reaction was for 5 minutes at 20°C (light intensity of 30,000 lux). The flasks were flushed with argon during the experiment.

Figure XIV. Effect of length of light reaction on CO_2 fixation



Chloroplasts (0.22 mg. chlorophyll) isolated from 4 week old leaves were added to 0.01 M HEPES buffer (0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 25 mM ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm) pH 7.5. They were reacted at a light intensity of 30,000 lux and at 20°C. Aliquots were taken at various time intervals and assayed for fixed ^{14}C. The atmosphere was air.

acid (3PGA) was observed to inhibit CO_2 fixation (Table XXXI and Figure XV). R5P presumably stimulates CO_2 fixation by increasing the substrate supply for the carboxylase enzyme.

The inhibition of CO_2 fixation by 3PGA has been noted by others (180,181). Gibbs, Latzko, Everson and Cockburn (180) reported that chloroplasts with low rates of CO_2 fixation were stimulated in this activity by additions of 3PGA, while inhibition occurred with chloroplasts photosynthesizing at high rates. The data in Figure XV also show that 3PGA completely inhibited CO_2 fixation for one minute, followed by a slow release from the inhibition. This release could be a result of the added 3PGA being metabolized to other intermediates of the reductive pentose phosphate cycle of photosynthesis, thereby lowering the concentration of the 3PGA to more physiological levels. The 3PGA may act in this system by inhibiting an enzyme of the carbon cycle.

(c). effects of inhibitors

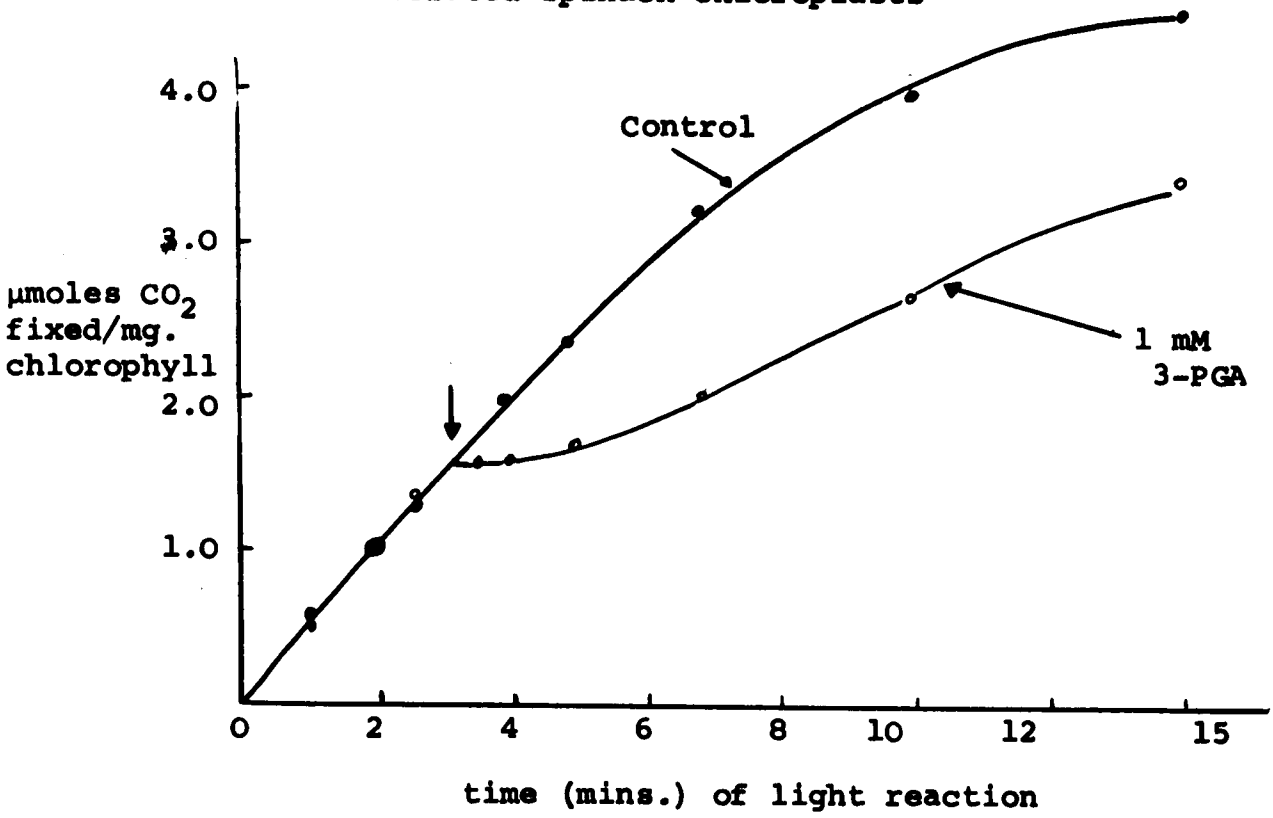
The effects of a number of inhibitors on CO_2 fixation by isolated spinach chloroplasts were tested. Parachloro-mercuribenzoic acid (0.15 mM) and iodoacetamide (5.0 mM) severely inhibited CO_2 fixation, when reacted with chloroplasts for 5 minutes, with no pre-incubation period. Oligomycin (2.5 μg/ml) was shown to have no effect on CO_2 fixation under these conditions. Phenazine methosulphate (0.1 mM) also had no effect on CO_2 fixation.

Table XXXI. Effects of 3-PGA and R5P on CO_2 fixation

Addition	Time (minutes)							
	1	2.5	3.5	4.0	5.0	7.0	10.0	15.0
	percentage of control value							
3-PGA (1 mM)	100	100	88	81	72	62	66	66
R5P (1 mM)	100	100	100	103	103	106	107	110
R5P (2 mM)	100	100	101	104	107	106	122	122

Chloroplasts isolated from 4 week old leaves were reacted in 0.01 M HEPES buffer pH 7.5 (0.33 M sorbitol, 1.0 mM $MgCl_2$, 20 mM ^{14}C-$NaHCO_3$ (1.1×10^6 cpm)). The light was turned upon addition of the chloroplasts, and at 3 minutes, 3-PGA or R5P was added. Aliquots were taken at the intervals shown and assayed for fixed ^{14}C. The atmosphere was air. The chlorophyll concentration was 0.18 mg./flask.

Figure XV. The effect of added 3-PGA on CO_2 fixation in isolated spinach chloroplasts



Chloroplasts (0.24 mg. chlorophyll) were added to 0.01 M HEPES buffer pH 7.5 (0.33 M sorbitol, 1.0 mM $MgCl_2$, 20 mM ^{14}C-$NaHCO_3$ (1.1×10^6 cpm)). The reaction was at 20°C and a light intensity of 30,000 lux. At 3 minutes, 3-PGA was added, at the times shown, aliquots were taken and assayed for fixed ^{14}C. The atmosphere was air.

(d). effects of phloridzin and quinacrine on CO_2 fixation

The effects of the inhibitors phloridzin and quinacrine on CO_2 fixation were next studied in detail. The concentrations of these inhibitors used in these experiments, were sufficient to severely inhibit both PSP and ATPase activity. The results shown in Table XXXII demonstrate that these two substances did not severely inhibit CO_2 fixation.

The degree of inhibition varied from one chloroplast preparation to another, quite markedly in the case of quinacrine. It appeared that the age of the tissue was of importance in determining the degree of inhibition. In the case of phloridzin, an average of 6% inhibition for 4 experiments (Table XXXII - preparations 1-4) was noted for chloroplasts isolated from 6 week old leaves, while tissue 2 weeks older yielded chloroplasts that were slightly more susceptible to the phloridzin (Table XXXII preparations 5-7). This variability of effect with age may reflect, among other things, changes in membrane permeability or a changing ability of the chloroplasts to withstand the isolation procedures without rupturing. It is of interest that there was a trend with both the inhibitors, for preparations that showed the least inhibitory effect, to be those that gave the highest levels of CO_2 fixed, presumably reflecting the intact nature of the chloroplasts.

Table XXXII. Effects of phloridzin and quinacrine on CO_2 fixation by isolated spinach chloroplasts

A. Phloridzin (mM)		0	0.25
Age of tissue	chloroplast preparation #	μmoles CO_2 fixed/mg. chlorophyll/hour	
6 weeks	1	79.2	79.2
	2	64.2	59.5
	3	48.3	47.6
	4	44.6	39.1
8 weeks	5	23.6	20.9
	6	55.5	44.1
	7	41.6	33.9
B. Quinacrine (μM)		0	10
6 weeks	8	89.3	88.7
	9	77.4	57.2
	10	56.4	50.2
	11	36.5	33.1
8 weeks	12	69.6	75.0
	13	36.5	33.8
	14	51.4	36.8

0.2 ml. chloroplasts (0.2 - 0.25 mg. chlorophyll) were added to 1.8 ml. buffer solution (0.33 M sorbitol; 0.01 M HEPES; 1.0 mM $MgCl_2$; 1.0 mM $MnCl_2$; pH 7.5). The samples were preincubated in the dark for 5 minutes. After addition of 50 μmoles ^{14}C-$NaHCO_3$ (1.2×10^6 cpm) to the reaction mixture, the samples were exposed to light (30,000 lux) for 5 mins. at 20°C, under an atmosphere of argon. The experiment was terminated by addition of 0.2 ml. 20% TCA, and the unreacted bicarbonate removed by heating to 50°C. Aliquots were then counted in a scintillation counter.

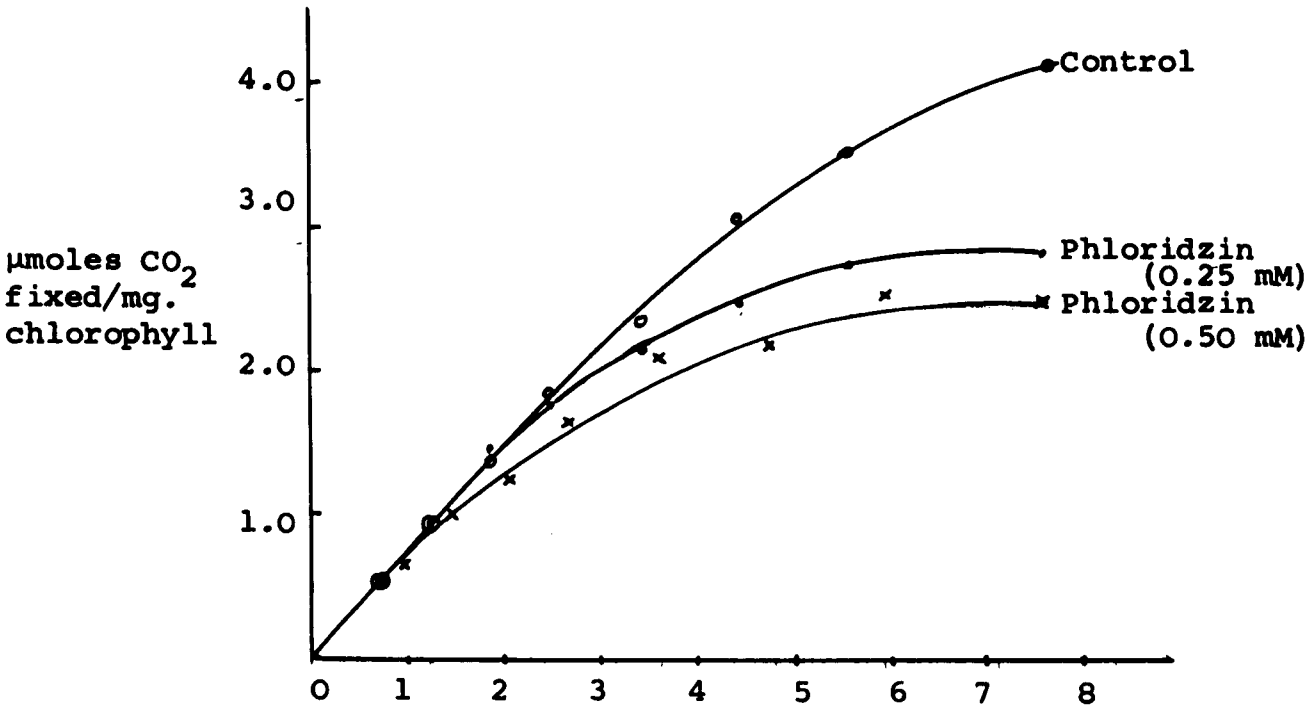
The effects of phloridzin on the kinetics of CO_2 fixation were investigated and the results are given in Figure XVI. It was concluded from these experiments that the initial kinetics of CO_2 fixation were unaffected by the presence of concentrations of phloridzin sufficient to severely inhibit both the PMS-catalyzed and endogenous synthesis of ATP, even after a 5 minute pre-incubation in the $\begin{matrix}\text{light or}\\ \text{dark}\end{matrix}$ with the inhibitor. The enzymic hydrolysis of any endogenous ATP present in the chloroplasts would have been largely inhibited by the phloridzin (99). It is unlikely therefore, that the initial rates of CO_2 fixation (circa 2 μmoles CO_2 fixed/mg. chlorophyll in 3 minutes) could be driven by ATP present in the chloroplasts prior to isolation.

(e). conclusion

The inhibition of CO_2 fixation noted after 2 to 3 minutes (Figure XVI) may be a result of a failure in the supply of the necessary substrates for the carboxylation mechanisms, or an accumulation of some compound inhibitory to this mechanism.

A primary product of the CO_2 uptake process is thought to be 3PGA, and its metabolism is dependent upon a supply of ATP. In our system, both the synthesis and hydrolysis of ATP are largely inhibited by the action of the phloridzin, and thus 3PGA may accumulate. The addition of 3PGA has already been shown to inhibit CO_2 fixation (see Figure XV), and thus the

Figure XVI. A temporal study of the effect of phloridzin on CO_2 fixation



Chloroplasts were added to 0.01 M HEPES (0.33 M sorbitol, 1.0 mM $MgCl_2$, 15 mM ^{14}C-$NaHCO_3$ 7.5 x 10^5 cpm, and phloridzin at the concentrations shown) pH 7.5. Immediately following chloroplast addition, the flasks were exposed to light (30,000 lux) and aliquots were taken and added to 0.1 ml. 20% TCA. Fluor was added, and the samples were counted. Similar results were obtained when the chloroplasts had been preincubated in the dark for 5 minutes with the phloridzin prior to the light reaction. The chlorophyll concentration was 0.16 mg./flask. The atmosphere was air.

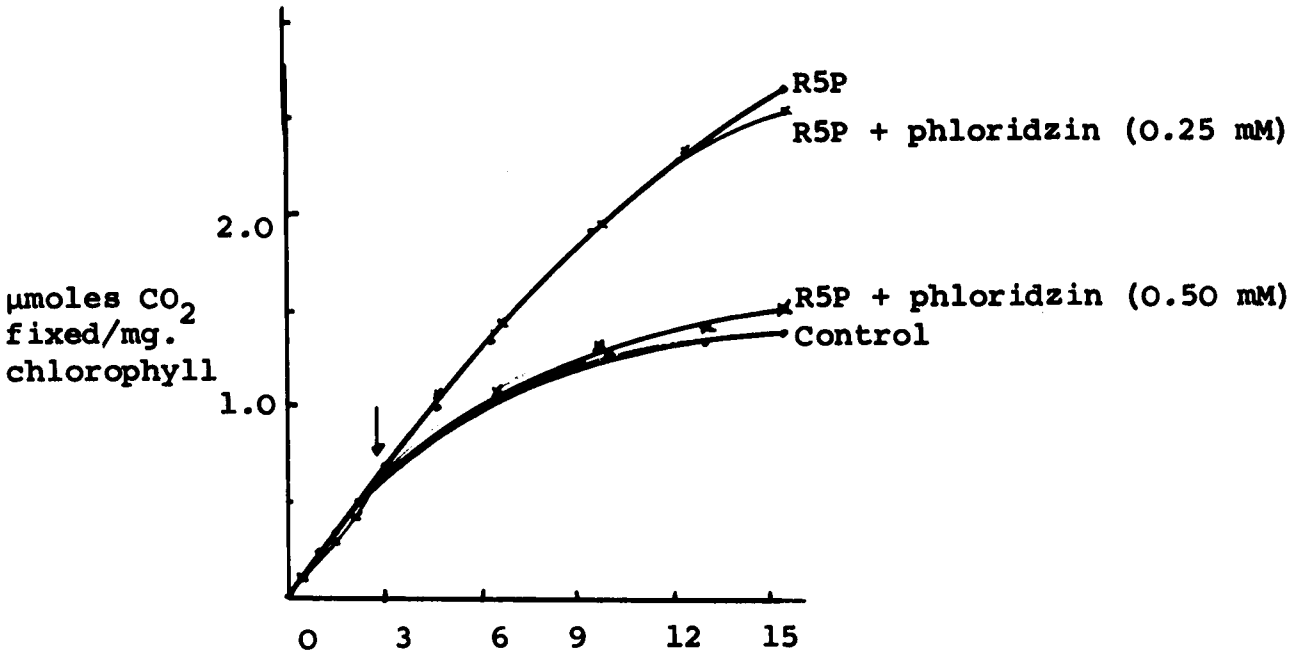
slow-developing phloridzin-induced inhibition of fixed CO_2 noted in Figure XVI, may be a result of 3PGA accumulation.

It is postulated, therefore, that the energy for the phosphorylation of ribulose 5-phosphate (Ru5P) may be derived from a high-energy intermediate formed prior to the synthesis of ATP. This conclusion is consistent with the often-observed 1:1 stoichiometry of ATP:NADPH in studies of non-cyclic PSP (60,61). It is also possible that added ADP could compete with Ru5P, and under certain conditions, ATP could be generated for use in other reactions in the chloroplast e.g. protein synthesis.

R5P has been shown to stimulate CO_2 fixation (Table XXXI) and if ATP were necessary for this stimulation, the reaction would be inhibited by phloridzin. However, as can be seen in Figure XVII, 0.25 mM phloridzin had no effect on the R5P-induced stimulation of CO_2 fixation. Higher concentrations of phloridzin (0.5 mM) decreased the R5P-induced stimulation of CO_2 fixation, but not below the control values obtained in the absence of both phloridzin and R5P. This work is consistent with the view that the phosphorylation of Ru5P could occur when ATP synthesis was largely inhibited.

In Table XXXIII both $^{32}P\text{-}H_3PO_4$ and $^{14}C\text{-}NaHCO_3$ were added to chloroplasts, and after the reaction the two isotopes were counted. Phloridzin slightly inhibited the amount of

Figure XVII. The effect of R5P and phloridzin on CO_2 fixation



Chloroplasts were added to 0.01 M HEPES (0.33 M sorbitol, 1.0 mM $MgCl_2$, 25 mM ^{14}C-$NaHCO_3$, 1.2 x 10^6 cpm, with or without phloridzin) pH 7.5, and reacted at 20°C and a light intensity of 30,000 lux. After 3 minutes in the light, R5P (4.0 mM) was added, and the reaction continued. Aliquots were taken at intervals and assayed for fixed CO_2. The chlorophyll concentration was 0.18 mg./flask. The flasks were flushed with argon.

Table XXXIII. The effects of phloridzin on the assimilation of CO_2 and Pi in isolated chloroplasts

Treatment	Minutes 5	10
	fixed ^{14}C cpm/0.25 ml. reaction mixture	
no phloridzin	640	1370
phloridzin (0.25 mM)	580	1070
	esterified phosphate cpm/0.25 ml. reaction mixture	
no phloridzin	1130	2530
phloridzin (0.25 mM)	3040	3070

Chloroplasts isolated from 5 week old leaves were added at zero time to 0.01 M HEPES buffer (0.33 M sorbitol, 1.0 mM $MgCl_2$, 10 mM ^{14}C-$NaHCO_3$, 7.5 x 10^5 cpm, ^{32}P-H_3PO_4, 7.5 x 10^5 cpm, with or without phloridzin) pH 7.5. Samples were taken after 5 and 10 minutes in the light, and added to 0.2 ml. 20% TCA. The esterified phosphate was determined by IBB extraction (140).

^{14}C-bicarbonate fixed, but considerably increased the amount of esterified phosphate.

These solutions were then assayed for ^{32}P-3-PGA by thin layer chromatography. The results are shown in Table XXXIV. It was found that at 5 minutes the 3-PGA represented about 25% of the total ^{32}P-activity both with and without phloridzin. However, at 10 minutes in the absence of phloridzin the amount of labelled 3-PGA was 28% of the total ^{32}P-activity, while with phloridzin, 3-PGA constituted about 70% of the total activity. It does appear therefore, that phloridzin induces a slow increase in the level of 3-PGA.

These studies suggest that the phosphorylation of R5P may occur when the synthesis of ATP is inhibited by an energy transfer inhibitor. It would be of interest to investigate the effects of phloridzin on the R5P kinase system, and test whether or not the utilization of ATP in this reaction could be inhibited. This is of importance because of the uncertain nature of the term "ATPase" in chloroplasts.

The phosphorylation of 3-PGA presumably requires ATP and in the absence of ATP, 3-PGA will accumulate and may then prevent further CO_2 fixation. Bassham (181) suggested several years ago that the photosynthetic carbon reduction cycle may be mediated by an organized system of multi-functional enzymes, and proposed a direct link between the electron transport system and the carbon reduction cycle. The results presented in this thesis support this view.

Table XXXIV. The effects of phloridzin on the accumulation of 3-PGA during photosynthesis

	^{32}P-3 PGA cpm/0.25 ml.	
	minutes	
phloridzin (0.25 mM)	5	10
-	300	700
+	700	2200

Chloroplasts were isolated from 5 week old leaves, and added at zero time to 0.01 M HEPES buffer (0.33 M sorbitol, 1.0 mM $MgCl_2$, ^{32}P-H_3PO_4, 7.5 x 10^5 cpm, with or without phloridzin) pH 7.5. The reaction was at 20°C and at a light intensity of 30,000 lux. Samples were taken after 5 and 10 minutes in the light, and assayed for 3-PGA by thin layer chromatography. The chlorophyll concentration was 0.25 mg./flask.

SUMMARY AND CONCLUSIONS

Considerable interest has arisen in recent years concerning the mechanisms of energy conservation and utilization in isolated chloroplasts. Energy conservation has been explained in terms of two major hypotheses; the chemi-osmotic theory and the chemical intermediate theory. The main difference between these two lies in the mechanism by which a high energy intermediate is formed. Once formed, this intermediate may have a role in chloroplast endergonic reactions in addition to ATP synthesis.

Roles for high energy intermediates were studied in this thesis. It was deemed necessary in this work to use chloroplasts exhibiting maximal activities of CO_2 fixation and the recent advances in the methodology of chloroplast isolation were first investigated.

The common practice of using chloroplasts isolated from tissue of uncertain origins, for example, spinach purchased from a supermarket, was found to be injudicious. In the HEPES/sorbitol isolation method, it was found essential to use young leaves (4 to 5 weeks old) for maximal activity. It was also important to isolate the chloroplasts very rapidly, and to use them immediately after centrifugation. Chloroplasts isolated by this technique were found capable of fixing 100 to 170 μmoles CO_2 fixed/mg. chlorophyll/hour.

The practice of including inorganic pyrophosphate in the reaction buffer to secure high CO_2 fixation rates was investigated. PPi was found to induce very similar responses to those induced by added ATP in these chloroplast preparations. The level of the stimulation attained with either ATP or PPi was noted to be dependent upon both the age of the plant tissue, and the age of the isolated chloroplasts. It has been thought by others that PPi increased the CO_2 fixation rate merely by supplying inorganic phosphate. In our study, the addition of Pi was found to inhibit CO_2 fixation, and yet this inhibition was reduced when PPi was added. This suggested an alternative role for PPi other than as a donor of Pi.

The pH optima for both the ATP- and PPi-induced increases in CO_2 fixation were identical. The increase in CO_2 fixation resulting from the addition of PPi was found to be more sensitive to small changes in pH than the stimulation of CO_2 fixation by ATP. Both ATP and PPi were utilized more readily in the presence of magnesium ions, but high concentrations of Mg^{2+} were found to inhibit both the ATP and PPi-induced increases in CO_2 fixation.

The dark fixation of CO_2 was also studied, and it was found to be stimulated by addition of ribulose 1,5 diphosphate or ribose 5-phosphate. ATP, but not PPi, was able to increase this dark fixation of CO_2 in a magnesium-requiring reaction.

Isolated chloroplasts were noted to vary in their ability to respond to exogenous ATP and PPi in this dark reaction.

It is thought that PPi is formed during photosynthesis, e.g. in starch synthesis. Our studies indicated that when chloroplasts were reacted in the light with radioactive Pi, in the absence of ADP, there was accumulation of label in PPi. This decreased when ADP was added.

It appeared from these studies that PPi could be formed during photosynthesis, and might be utilized (as a substitute for ATP) to drive CO_2 fixation. The enhancement of CO_2 fixation by both ATP and PPi was partially inhibited by phloridzin, an energy transfer inhibitor. The precise mechanism by which phloridzin inhibits ATP synthesis and hydrolysis is, however, not understood. The possibility of compartmentation of the Pi, PPi and ATP within the chloroplasts was considered.

The light-induced uptake of calcium ions was studied. This uptake was enhanced by addition of phloridzin, and also by addition of either ATP or PPi. It was concluded that exogenous ATP and PPi may operate by reversing electron transport to synthesize a high energy intermediate, or by combining with calcium ions within the chloroplasts and thereby lowering the energy requirement for calcium uptake.

The process of photophosphorylation has been suggested to involve at least two different reactions. The synthesis of ATP was studied in isolated chloroplasts by addition of ADP, Pi and Mg^{2+}. A catalyst such as PMS was found to induce higher rates of ATP synthesis presumably by the "cyclic" mechanism. The levels of ATP formed were very low, possibly resulting from the use of intact chloroplasts. The dark phosphorylation of ADP following a short light period could be inactivated by the sulphydryl group inhibitor, iodoacetamide. The light phosphorylation was not affected by this compound. The former reaction was activated by sulphydryl group protectants e.g. GSH or DTT.

In light-dark transition experiments, the presence of ^{32}Pi in the light followed by a dark addition of ADP gave higher levels of ^{32}-P-ATP than when ^{32}P was added in the dark after a short light reaction with ADP. This could either be a result of the light-induced formation of a high energy phosphorylated intermediate, or the presence of an active adenylate kinase phosphorylating any endogenous ADP that may be present following the light preincubation.

Phloridzin and quinacrine were shown to severely inhibit ATP synthesis in both a PMS-catalyzed cyclic system and in endogenous PSP (in which no catalyst was added). At a concentration inhibitory to ATP synthesis, phloridzin

slightly stimulated the esterification of inorganic phosphate in the absence of exogenous ADP.

A temporal study of CO_2 fixation showed that phloridzin had no initial effect on the reaction, however after 2 minutes a slow inhibition developed. It was proposed that the phosphorylation of Ru5P could be driven by a high energy intermediate when ATP synthesis and hydrolysis were inhibited. It also appeared that the addition of R5P stimulated CO_2 fixation, even when 0.25 mM phloridzin was present in the system. On the other hand, when exogenous 3-PGA was added to the chloroplasts, an immediate cessation of CO_2 fixation was noted. It was postulated that phloridzin may inhibit CO_2 fixation by causing an accumulation of 3-PGA. Chromatographic studies of the ^{32}P-labelled products of photosynthesis in chloroplasts incubated with phloridzin suggested that 3-PGA did indeed accumulate in these systems.

These results were interpreted as suggesting a direct link between the electron transport chain and the carbon reduction cycle. Thus high energy intermediates may have a number of roles in addition to the synthesis of ATP. These could include the initial steps in CO_2 fixation, ion uptake, and volume changes in chloroplasts.

REFERENCES

1. R. Lumry, J.D. Spikes and H. Eyring. "Photosynthesis". Ann. Rev. Plant Physiol. 5, 271-340 (1954).

2. D.I. Arnon, M.B. Allen and F.R. Whatley. "Photosynthesis by isolated chloroplasts". Nature 174, 394-396 (1954).

3. M.B. Allen, D.I. Arnon, J.B. Capindale, F.R. Whatley and L.J. Durham. "Photosynthesis by isolated chloroplasts III. Evidence for complete photosynthesis". J. Amer. Chem. Soc. 77, 4149-4155 (1955).

4. J.A. Bassham and M. Calvin. "The Path of Carbon in Photosynthesis". Englewood Cliffs, New Jersey, Prentice Hall (1957).

5. A. Frenkel. "Light induced phosphorylation by cell-free preparations of photosynthetic bacteria". J. Amer. Chem. Soc. 76, 5568-69 (1954).

6. M.B. Allen, F.R. Whatley and D.I. Arnon. "Photosynthesis by isolated chloroplasts. VI. Rates of conversion of light into chemical energy in photosynthetic phosphorylation". Biochim. Biophys. Acta 27, 16-23 (1958).

7. Biochemistry of Chloroplasts, Vol. I and II. Ed. T.W. Goodwin, Academic Press, New York (1967).

8. Harvesting the Sun. Photosynthesis in Plant Life. I.M.C. Symposium. Academic Press, New York (1967).

9. "Energy Conversion by the Photosynthetic Apparatus". Brookhaven Symp. in Biology 19, Upton, New York. Brookhaven Nat'l. Lab. (1967).

10. A. San Pietro and C.C. Black. "Enzymology of Energy Conversion in Photosynthesis". Ann. Rev. Plant Physiol. 16, 155-174 (1965).

11. D.I. Arnon. "Photosynthetic Activity of Isolated Chloroplasts". Physiol. Rev. 47 (3), 317-354 (1967).

12. B. Kok. "Photosynthesis, the Path of Energy" in Plant Biochemistry, Chapter 35, Ed. J. Bonner and J.E. Varner. Academic Press, New York (1965).

13. J.A. Bassham. "Photosynthesis: The Path of Carbon" in Plant Biochemistry, Chapter 34, ed. J. Bonner and J.E. Varner. Academic Press, New York (1965).

14. L.P. Vernon and M. Avron. "Photosynthesis". Ann. Rev. Biochem. 34, 269-297 (1965).

15. "Photosynthetic Mechanisms of Green Plants". Natl. Acad. Sci. N.R.C. Publ. 1145. Washington, D.C. (1963).

16. E.C. Slater. "An evaluation of the Mitchell hypothesis of chemi-osmotic coupling in oxidative and photophosphorylation". European J. Biochem. 1, 317-334 (1967).

17. L. Ernster and C.P. Lee. "Biological oxidoreductions". Ann. Rev. Biochem. 33, 729-790 (1964).

18. P. Mitchell. "Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism". Nature 191, 144-148 (1961).

19. P. Mitchell. "Chemi-osmotic coupling in oxidative and photosynthetic phosphorylation". Biol. Rev. 41, 445-502 (1966).

20. B. Chance, C.P. Lee and L. Mela. "Control and Conservation of Energy in the cytochrome chain". Feder. Proc. 26 (5), 1341-54 (1967).

21. P.D. Boyer. "Phosphohistidine". Science 141, 1147-52 (1963).

22. J.S. Kahn and A.T. Jagendorf. "The pathway of ATP formation in photophosphorylation". Biochem. Biophys. Res. Commun. 2, 259-263 (1960).

23. J.W. Hinkson and P.D. Boyer. "The light-induced formation of rapidly phosphorylated compounds in chloroplasts". Arch. Biochem. Biophys. 110, 16-22 (1965).

24. Y.K. Shen and G.M. Shen. "Studies on photophosphorylation. II. The "light intensity effect" and intermediate steps of photophosphorylation". Biol. Abstr. 42, 7459 (1963). (Scientia Sinica (Peking) 11, 1097-1106 (1962).

25. G. Hind and A.T. Jagendorf. "Separation of light and dark stages in photophosphorylation". Proc. Natl. Acad. Sci. U.S. 49, 715-722 (1963).

26. A.T. Jagendorf and E. Uribe. "Photophosphorylation and the chemi-osmotic hypothesis". Brookhaven Symp. 19, 215-245 (1966).

27. P.S. Nobel. "Calcium uptake, ATPase and photophosphorylation by chloroplasts in vitro". Nature 214, 875-877 (1967).

28. B.C. Mayne and R.K. Clayton. "Luminescence of chlorophyll in spinach induced by acid-base transition". Proc. Natl. Acad. Sci. 55, 494-497 (1966).

29. R.A. Dilley and L.P. Vernon. "Changes in light absorbance and light-scattering properties of spinach chloroplasts upon illumination". Biochemistry 3, 817-824 (1964).

30. A.T. Jagendorf. "The chemi-osmotic hypothesis of PSP" in Harvesting the Sun. Ed. A. San Pietro and C.C. Black. p. 69-78, 1967. Academic Press, New York.

31. J. Neumann and A.T. Jagendorf. "Light-induced pH changes related to phosphorylation by chloroplasts". Arch. Biochem. Biophys. 107, 109-119 (1964).

32. A.T. Jagendorf and J. Neumann. "Effect of uncouplers on the light-induced pH rise with spinach chloroplasts". J. Biol. Chem. 240, 3210-3214 (1965).

33. R.A. Dilley and L.P. Vernon. "Ion and water transport processes related to a light-dependent shrinkage of spinach chloroplasts". Arch. Biochem. Biophys. 111, 365-375 (1965).

34. A.T. Jagendorf and E. Uribe. "ATP formation by acid-base transition of spinach chloroplasts". Proc. Natl. Acad. Sci. U.S. 55, 170-177 (1966).

35. R.E. McCarty and E. Racker. "Effects of an antiserum to the chloroplast coupling factor on photophosphorylation and related processes". Feder. Proc. 25, 226 (1966).

36. E. Uribe and A.T. Jagendorf. "On the localization of organic acids in acid induced ATP synthesis". Plant Physiol. 42, 697-705 (1967).

37. E. Uribe and A.T. Jagendorf. "Organic acid specificity for acid induced ATP synthesis in chloroplasts". Plant Physiol. 42, 706-711 (1967).

38. J.H. Kaplan, E. Uribe and A.T. Jagendorf. "ATP hydrolysis caused by acid-base transition of spinach chloroplasts". Arch. Biochem. Biophys. 120, 365-375 (1967).

39. A. Bennun and M. Avron. "Light-dependent and light-triggered ATPases in chloroplasts". Biochim. Biophys. Acta 79, 646-648 (1964).

40. B. Petrack, A. Craston, F. Sheppy and F. Farron. "Studies on the hydrolysis of ATP by spinach chloroplasts". J. Biol. Chem. 240, 906-914 (1965).

41. B.C. Mayne and R.K. Clayton. "Luminescence of chlorophyll in spinach chloroplasts induced by acid-base transitions". Proc. Natl. Acad. Sci. U.S. 55, 494-497 (1966).

42. R.A. Reid, J. Moyle and P. Mitchell. "Synthesis of ATP by a proton motive force in rat liver mitochondria". Nature 212, 257-258 (1960).

43. R.S. Cockrell, E.J. Harris and B.C. Pressman. "Synthesis of ATP driven by a potassium gradient in mitochondria". Nature 215, 1487-1488 (1967).

44. B. Chance and L. Mela. "Proton movements in mitochondrial membranes". Nature 212, 372-376 (1966).

45. P. Mitchell and J. Moyle. "Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria". Nature 208, 147-151 (1965).

46. C.S. Rossi, E. Carafoli, J. Bielawski and A.L. Lehninger. "Proton movements across the mitochondrial membrane supported by hydrolysis of ATP". European J. Biochem. 2, 332-340 (1967).

47. W.S. Lynn and R.H. Brown. "P:O and ADP:O ratios and quantum yields in chloroplasts with use of chloranil as electron acceptor". J. Biol. Chem. 242, 418-426 (1967).

48. W.S. Lynn and R.H. Brown. "Competition between phosphate and protons on phosphorylation and cation exchange in chloroplasts". J. Biol. Chem. 242, 426-432 (1967).

49. B. Chance, M. Nishimura, M. Avron and M. Baltscheffsky. "Light-induced intravesicular pH changes in Rhodospirillum rubrum chromatophores". Arch. Biochem. Biophys. 117, 158-166 (1966).

50. B. Chance and L. Mela. "Hydrogen ion concentration changes in mitochondrial membranes". J. Biol. Chem. 241, 4588-4599 (1966).

51. H. Baum. "Energetics of coupled events involving small compartments". Nature 214, 1326-1327 (1967).

52. P. Mitchell. "Proton current flow in mitochondrial systems". Nature 214, 1327-1328 (1967).

53. B. Chance and L. Mela. "A hydrogen ion concentration gradient in a mitochondrial membrane". Nature 212, 369-372 (1966).

54. S.J.D. Karlish and M. Avron. "Relevance of proton uptake induced by light to the mechanism of energy coupling in photophosphorylation". Nature 216, 1107-1109 (1967).

55. O. Warburg and G. Krippahl. "Die chemischen gleichungen der photosynthese". Z. Physiol. Chem. 332, 225-243 (1963).

56. M. Stiller. "The path of carbon in photosynthesis". Ann. Rev. Plant Physiol. 13, 151-170 (1962).

57. M.D. Hatch and C.R. Slack. "Photosynthesis by sugar-cane leaves. A new carboxylation reaction, and the pathway of sugar formation". Biochem. J. 101, 103-112 (1966).

58. D.I. Arnon, F.R. Whatley and M.B. Allen. "Assimilatory power in photosynthesis". Science 127, 1026-34 (1958).

59. M. Avron, D.W. Krogmann and A.T. Jagendorf. "The relation of photosynthetic phosphorylation to the Hill reaction". Biochim. Biophys. Acta 30, 144-153 (1958).

60. J.F. Turner, C.C. Black and M. Gibbs. "Studies on photosynthetic processes. I. The effect of light intensity on TPN reduction, ATP formation and CO_2 assimilation in spinach chloroplasts". J. Biol. Chem. 237, 577-579 (1962).

61. M. Stiller and B. Vennesland. "Photophosphorylation and the Hill reaction". Nature 191, 677-678 (1961).

62. N.E. Good. "Reassessment of the stoichiometry of photophosphorylation". Nature 188, 661-662 (1960).

63. T. Punnett. "The stoichemistry of PSP". Natl. Acad. Sci. N.R.C. Publ. 1145, 619-624 (1963).

64. C.A. Fewson, C.C. Black and M. Gibbs. "Further studies on the photo-chemical production of reduced TPN and ATP by fragmented spinach chloroplasts". Plant Physiol. 38, 680-685 (1963).

65. W.S. Lynn and R.H. Brown. "P/2e$^-$ ratios approaching 4 in isolated chloroplasts". J. Biol. Chem. 242, 412-418 (1967).

66. F.R. Whatley. "Some recent results in photophosphorylation". Can. J. Bot. 43, 105-117 (1965).

67. A.T. Jagendorf and M. Avron. "Cofactors and roles of photophosphorylation by spinach chloroplasts". J. Biol. Chem. 231, 277-290 (1958).

68. L.P. Vernon and W.A. Zaugg. "Photoreductions by fresh and aged chloroplasts: requirement for ascorbate and 2,6 dichlorophenolindophenol with aged chloroplasts". J. Biol. Chem. 235, 2728-2733 (1960).

69. J.H.C. Smith and C.S. French. "The major and accessory pigments of photosynthesis". Ann. Rev. Plant Physiol. 14, 181-224 (1963).

70. G. Hind. "The site of action of plastocyanin in chloroplasts treated with detergent". Biochim. Biophys. Acta 153, 235-240 (1968).

71. J.A. Gross, A.M. Shefner and M.J. Becker. "Distribution of chlorophylls in chloroplast fragments". Nature 209, 615 (1966).

72. J.M. Anderson and N.K. Boardman. "Fractionation of the photochemical system of photosynthesis". Biochim. Biophys. Acta 112, 403-421 (1966).

73. J.S. Kahn and A.T. Jagendorf. "The ATP-ADP exchange enzyme from chloroplasts. Further purification and antigenic properties". Biochim. Biophys. Acta 58, 149-154 (1962).

74. J.S. Kahn. "The relation of the ATP-ADP exchange activity to photophosphorylation and to 3-PGA kinase in spinach chloroplasts". Biochim. Biophys. Acta 79, 421-423 (1964).

75. C. Carmeli and M. Avron. "A light-triggered ATP-Pi exchange reaction in chloroplasts". European J. Biochem. 2, 318-326 (1967).

76. G.E. Skye, N. Shavit and P.D. Boyer. "The catalysis by modified chloroplasts of the Pi⇌ATP, Pi⇌HOH and ATP⇌HOH exchange reactions in the absence of light". Biochem. Biophys. Res. Commun. 28, 724-730 (1967).

77. K.G. Rienits. "A light and cysteine-activated ATP-Pi exchange reaction in chloroplasts and its' relationship to ATPase and PSP". Biochim. Biophys. Acta 143, 595-606 (1967).

78. W.S. Zaugg and L.P. Vernon. "Light-induced dark ^{32}P - ATP formation by Rhodospirillum rubrum chromatophores. ATP⇌Pi exchange activity". Biochemistry 5, 34-40 (1966).

79. M. Avron and A.T. Jagendorf. "Evidence concerning the mechanism of ATP formation by spinach chloroplasts". J. Biol. Chem. 234, 967-972 (1959).

80. J.R. Vose and M. Spencer. "Energy sources for photosynthetic carbon dioxide fixation. Biochem. Biophys. Res. Commun. 29, 532-537 (1967).

81. Z. Gromet-Elhanon and M. Avron. "Effect of inhibitors and uncouplers on the separate light and dark reactions in photophosphorylation". Plant Physiol. 40, 1053-1059 (1965).

82. R.E. McCarty and E. Racker. "Partial resolution of the enzymes catalyzing PSP. III. Activation of ATPase and ^{32}P-labelled Pi-ATP exchange in chloroplasts". J. Biol. Chem. 243, 129-137 (1968).

83. A.R. Schultz and P.D. Boyer. "The fate of oxygens of phosphate in photophosphorylation". Arch. Biochem. Biophys. 93, 335-337 (1961).

84. N. Shavit, G.E. Skye and P.D. Boyer. "Occurrence and possible mechanism of ^{32}P and ^{18}O exchange reactions in photophosphorylation". J. Biol. Chem. 242, 5125-5130 (1967).

85. H. Kushida, M. Itoh, S. Izawa and K. Shibata. "Deformation of chloroplasts on illumination in intact spinach chloroplasts". Biochim. Biophys. Acta 79, 201-203 (1964).

86. L. Packer. "Volume changes and contractility of mitochondrial and chloroplast membranes". Annals N.Y. Acad. Sci. 137, 624-640 (1966).

87. R.A. Dilley and A. Rothstein. "Chloroplast membrane characteristics". Biochim. Biophys. Acta 135, 427-443 (1967).

88. L. Packer, A.C. Barnard and D.W. Deamer. "Ultrastructure and photometric evidence for light-induced changes in chloroplast structure in vitro". Plant Physiol. 42, 283-293 (1967).

89. G. Hind and A.T. Jagendorf. "Light scattering changes associated with the production of a possible intermediate in photophosphorylation". J. Biol. Chem. 240, 3195-3201 (1965).

90. E.L. Gross and L. Packer. "Ion transport and conformational changes in spinach chloroplast grana". Arch. Biochem. Biophys. 121, 779-789 (1967).

91. T. Ohnishi. "De changement de volume du chloroplaste, accompagné de photophosphorylation et les proteines ressemblantes à l'actine et à myosine extraites du chloroplaste". J. Biochem. 55, 494-503 (1964).

92. J.A. Young and L. Packer. "On the occurrence of a contractile protein in spinach chloroplasts". Biochim. Biophys. Acta 126, 400 (1966).

93. A.P. Brown. "Volume contraction of isolated pea chloroplasts promoted by bivalent cations". Biochem. J. 102, 791-801 (1967).

94. A.R. Crofts, D.W. Deamer and L. Packer. "Mechanisms of light-induced structural change in chloroplasts. II. The role of ion movements in volume changes". Biochim. Biophys. Acta 131, 97-118 (1967).

95. P.S. Nobel and L. Packer. "Light-dependent ion translocation in spinach chloroplasts". Plant Physiol. 40, 633-640 (1965).

96. P.S. Nobel. "Calcium uptake, ATPase and photophosphorylation by chloroplasts in vitro". Nature 214, 875-877 (1967).

97. A.R. Crofts. "Amine uncoupling of energy transfer in chloroplasts. I. Relation to ammonium ion uptake". J. Biol. Chem. 242, 3352-3359 (1967).

98. P.S. Nobel, S. Muraki and A. Takamiya. "Localization of light-induced strontium uptake in spinach chloroplasts". Plant and Cell Physiol. 7, 263-275 (1966).

99. S. Izawa, G.D. Winget and N.E. Good. "Phloridzin, a specific inhibitor of photophosphorylation and phosphorylated-coupled electron transport in chloroplasts". Biochem. Biophys. Res. Commun. 22, 223-226 (1966).

100. L. Packer. "Effect of nigericin upon light-dependent monovalent cation transport in chloroplasts". Biochem. Biophys. Res. Commun. 28, 1022-28 (1967).

101. P.S. Nobel. "A rapid technique for isolating chloroplasts with high rates of endogenous photophosphorylation". Plant Physiol. 42, 1389-1394 (1967).

102. L.-V von Stedingk. "Light-induced reversible pH changes in chromatophores from Rhodospirillum rubrum". Arch. Biochem. Biophys. 120, 537-541 (1967).

103. S. Izawa and G. Hind. "The kinetics of the pH rise in illuminated chloroplasts". Biochim. Biophys. Acta 143, 377-390 (1967).

104. O. Holm-Hansen, N.G. Pon, K. Nishida, V. Moses and M. Calvin, "Uptake and distribution of radioactive carbon from labelled substrates by various cellular components of spinach leaves". Physiol. Plantarum. 12, 475-501 (1959).

105. F.R. Whatley, M.B. Allen, A.V. Trebst and D.I. Arnon. "Photosynthesis by isolated chloroplasts. IX. Photosynthetic phosphorylation and CO_2 assimilation in different species". Plant Physiol. 35, 188-193 (1960).

106. U. Heber and E. Tyszkiewicz. "The rate of photosynthesis in isolated chloroplasts". J. Exp. Bot. 13, 185-200 (1962).

107. D.A. Walker. "Correlation between photosynthetic activity and membrane integrity in isolated pea chloroplasts". Plant Physiol. 40, 1157-1161 (1965).

108. R.G. Jensen and J.A. Bassham. "Photosynthesis by isolated chloroplasts". Proc. Natl. Acad. Sci. (U.S.) 56, 1095-1101 (1966).

109. J.R. Vose and Mary Spencer. "Effects of ATP and PPi on carbon dioxide fixation by isolated spinach chloroplasts". Submitted for publication, March, 1968.

110. N.E. Good, G.D. Winget, W. Winter, T.N. Connelly, S. Izawa and R.M.M. Singh. "Hydrogen ion buffers for biological research". Biochemistry 5, 467-477 (1966).

111. R.A. Stinson and Mary Spencer. "An evaluation of the effects of five buffers on respiratory parameters of isolated mitochondria". Can. J. Biochem. 46, 43-50 (1968).

112. C. Bucke, C.W. Baldry and D.A. Walker. "Photosynthetic CO_2 fixation by isolated chloroplasts in Good's buffers". Phytochem. 6, 495-499 (1967).

113. P.P. Kalberer, B.B. Buchanan and D.I. Arnon. "Rates of photosynthesis by isolated chloroplasts". Proc. Natl. Acad. Sci. (U.S.) 57, 1542-1549 (1967).

114. L. Sarapuu. "Seasonal changes of a growth regulating agent in apple trees". Chem. Abstr. 66, 75143v (1967). (Fiziol. Aktiv. Veshchestra Ikh Primen. Rastenievod., Dokl. Nauch. Konf. 1963, 183-190 (1965)).

115. F. Alvarado. "Hypothesis for the interaction of phloridzin and phloretin with membrane carriers for sugars". Biochim. Biophys. Acta 135, 483-495 (1967).

116. A. Kylin and J-E. Tillberg. "Action sites of the inhibitor-complex from potato and of phloridzin in light-induced energy transfer in Scenedesmus". Z. Pflanzenphysiol. 57, 72-78 (1967).

117. S. Izawa. "The swelling and shrinking of chloroplasts during electron transport in the presence of phosphorylation uncouplers". Biochim. Biophys. Acta 102, 373-378 (1965).

118. J. Sykes and J.A. Gibbon. "ATP production in the light and the dark by vesicle preparations isolated from Chlorobium thiosulphatophilum L660". Biochim. Biophys. Acta 143, 173-186 (1967).

119. H. Baltscheffsky and L-V von Stedingk. "Bacterial photophosphorylation in the absence of added nucleotide. A second intermediate stage of energy transfer in the light-induced formation of ATP". Biochem. Biophys. Res. Commun. 22, 722-728 (1966).

120. H. Baltscheffsky, L-V. von Stedingk, H-W. Heldt and M. Klingenberg. "Inorganic pyrophosphate formation in bacterial photophosphorylation". Science 153, 1120-1122 (1966).

121. A.E. Needham. "Phosphorus compounds" in The Uniqueness of Biological Materials, p. 401-416. Pergamon Press, London, 1965.

122. R.J. Podolsky and M.F. Morales. "The enthalpy change of ATP hydrolysis". J. Biol. Chem. 218, 945-959 (1956).

123. J.D. Watson. Molecular Biology of the gene, Chapt. 5 "Coupled reactions and group transfers". (W.A. Benjamin, Inc., New York, 1965).

124. S.L. Miller and M. Parris. "Synthesis of inorganic pyrophosphate under primitive earth conditions". Nature 204, 1248-1250 (1964).

125. H. Baltscheffsky. "Inorganic pyrophosphate and the evolution of biological energy transformation". Acta Chem. Scand. 21, 1973-1974 (1967).

126. M. Baltscheffsky, H. Baltscheffsky and L-V. von Stedingk. "Light-induced energy conversion and the pyrophosphatase reaction in chromatophores from Rhodospirillum rubrum". Energy Conversion by the Photosynthetic Apparatus in Brookhaven Symposium in Biology No. 19 (1966), pp. 246-253.

127. A. Kornberg. In "Horizons in Biochemistry" Eds. M. Kasha and B. Pullman. pp. 251-264. Academic Press, New York, 1962.

128. J.A. Bassham. "Energy Conversion by the photosynthetic apparatus" Discussion p. 254 in Brookhaven Symposium in Biology No. 19 (1966).

129. T.A. Pedersen, M. Kirk and J.A. Bassham. "Light-dark transients in levels of intermediate compounds during photosynthesis in air-adapted chlorella". Physiol. Plantarum 19, 219-231 (1966).

130. D. Mishra and B. Mohantz. "Diurnal variation of the acid phosphatase activity in the leaves of cowpea". Planta 75, 239-243 (1967).

131. P.M. Siu and H.G. Wood. "Phosphoenol pyruvic carboxy-transphosphorylase, a CO_2 fixation enzyme from propionic acid bacteria". J. Biol. Chem. 237, 3044-3051 (1962).

132. M. Baltscheffsky. "Inorganic pyrophosphate as an energy donor in photosynthetic and respiratory electron transport phosphorylation systems". Biochem. Biophys. Res. Commun. 28, 270-277 (1967).

133. J.A. Cole and D.E. Hughes. "Metabolism of polyphosphates in Chlorobium thiosulphatophilum". J. Gen. Microbiol. 38, 65-72 (1965).

134. J.S.C. Wessels and H. Baltscheffsky. "Adenosine triphosphatase activity in chloroplasts". Acta Chem. Scand. 14, 233-246 (1960).

135. M. Szymona. "Purification properties of a new hexokinase utilizing inorganic polyphosphate". Chem. Abstr. 58, 1674e (1963). (Acta biochim. polonica 9, 165-181 (1962)).

136. D.L. Keister and N.J. Yike. "Studies on an energy-linked pyridine nucleotide transhydrogenase in photosynthetic bacteria. I. Demonstration of the reaction in Rhodospirillum rubrum". Biochem. Biophys. Res. Commun. 24, 519-525 (1966).

137. M. Yamada and K. Kurahashi. "ATP and PPi-dependent phenyl alanine racemase of Bacillus brevis Nagano". J. Biochem. 63, 59-69 (1968).

138. D.I. Arnon. "Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaxis". Plant Physiol. 24, 1-15 (1949).

139. M. Avron. "Photophosphorylation by Swiss-chard chloroplasts". Biochim. Biophys. Acta 40, 257-272 (1960).

140. S.O. Nielsen and A.L. Lehninger. "Phosphorylation coupled to the oxidation of ferrocytochrome c". J. Biol. Chem. 215, 555-571 (1955).

141. J.B. Martin and D.M. Doty. "Determination of inorganic phosphate. Modification of Isobutyl alcohol procedure". Anal. Chem. 21, 965-967 (1949).

142. Liquid Scintillation Counting. Nuclear Chicago Corporation Publication No. 711580 (1966), p. 17.

143. D.W. Schindler. "A liquid scintillation method for measuring carbon-14 uptake in photosynthesis". Nature 211, 844-855 (1966).

144. R.K. Crane and F. Lipmann. "The effect of arsenate on aerobic phosphorylation". J. Biol. Chem. 201, 235-243 (1952).

145. G.R. Barker and J.A. Hollinshead. "The degradation of RNA in the cotyledons of Pisum arrense". Biochem. J. 103, 230-237 (1967).

146. B.L. Strehler in Methods of Enzymatic Analysis. Ed. H. Bergmeyer, p. 559. Academic Press, New York (1963).

147. H. Holmsen, I. Holmsen and A. Bernhardsen. "Microdetermination of ADP and ATP in plasma with the firefly luciferase system". Anal. Biochem. 17, 456-474 (1966).

148. W.M. Balfour and F.E. Samson, Jr. "Transphosphorylases in the firefly lantern". Arch. Biochem. Biophys. 84, 140-142 (1959).

149. C.S. Hanes and F.A. Ishenwood. "Separation of the phosphoric esters on the filter paper chromatogram". Nature 164, 1107-1112 (1949).

150. H. Seiler. "Dünnschicht-chromatographie von Anionen: phosphate". Helv. Chim. Acta 44, 1753-1755 (1961).

151. D.C. Mortimer. "Paper chromatographic separation of some biologically important phosphate esters". Can. J. Chem. 30, 653-660 (1952).

152. B.J. Miflin and R.H. Hageman. "Photosynthetic phosphorylation by maize chloroplasts". Plant Physiol. 38, 66-70 (1963).

153. W.D. Loomis and J. Battaile. "Plant phenolic compounds and the isolation of plant enzymes". Phytochem. 5, 423-438 (1966).

154. D. Spencer and H. Unt. "Biochemical and structural correlations in isolated spinach chloroplasts under isotonic and hypotonic conditions". Australian J. Biol. Sci. 18, 197-210 (1965).

155. M. Baltscheffsky. "Inorganic pyrophosphate and ATP as energy donors in chromatophores from Rhodospirillum rubrum". Nature 216, 241-244 (1967).

156. J.M. Paulsen and M.D. Lane. "Spinach ribulose diphosphate carboxylase I. Purification and Properties of the enzyme". Biochemistry 5, 2350-2357 (1966).

157. W. Cockburn, C.W. Baldry and D.A. Walker. "Some effects of inorganic phosphate on O_2 evolution by isolated chloroplasts". Biochim. Biophys. Acta 143, 614-624 (1967).

158. K.A. Santarius and U. Heber. "Changes in the intracellular levels of ATP, ADP, AMP and Pi, and the regulatory function of the adenylate system in leaf cells during photosynthesis". Biochim. Biophys. Acta 102, 39-54 (1965).

159. R.G. Jensen and J.A. Bassham. "Photosynthesis by isolated chloroplasts II. Effects of addition of cofactors and intermediate compounds". Biochim. Biophys. Acta 153, 219-226 (1968).

160. J.D. McGilvery and J.P. Crowther. "The hydrolysis of the condensed phosphates". Can. J. Chem. 32, 174-185 (1954).

161. J.M. Lowenstein. "Transphosphorylations catalyzed by bivalent metal ions". Biochem. J. 70, 222-230 (1958).

162. T. Horio, K. Nishikawa, M. Katsumata and J. Yamashita. "Possible partial reactions of the photophosphorylation process in chromatophores from Rhodospirillum rubrum". Biochim. Biophys. Acta 94, 371-382 (1965).

163. C.P. Whittingham, R.G. Hiller and M. Bermingham. "The production of glycollate during photosynthesis" in Photosynthetic Mechanisms of Green Plants. Natl. Acad. Sci. N.R.C. publication 1145, 675-683 (1963).

164. E.J. Johnson and B.S. Bruff. "Chloroplast integrity and ATP-dependent CO_2 fixation in Spinacia oleracea". Plant Physiol. 42, 1321-1328 (1967).

165. A.J. D'Eustachio, E. Knight Jr., and R.W. Hardy. "Stimulation of inorganic pyrophosphatase activity in Clostridium pasteurianum by reductants". J. Bacteriol. 90, 288-289 (1965).

166. J. Neumann and A.T. Jagendorf. "Uncoupling photophosphorylation by detergents". Biochim. Biophys. Acta 109, 382-389 (1965).

167. D.I. Arnon. "Cell-free photosynthesis and the energy conversion process" in Light and Life. Ed. W.D. McElroy and B. Glass. p. 502. The Johns Hopkins Press, Baltimore, U.S.A. (1961).

168. A.T. Jagendorf and M. Avron. "Inhibitors of photosynthetic phosphorylation in relation to electron and oxygen transport pathways of chloroplasts". Arch. Biochem. Biophys. 80, 246-257 (1959).

169. G. Zweig and M. Avron. "Dependence of photophosphorylation by isolated chloroplasts on the oxidation-reduction state of N-methyl phenazinium methyl sulphate (phenazine methosulphate)". Nature 208, 190-191 (1965).

170. D.O. Hall and D.I. Arnon. "Photosynthetic phosphorylation above and below 0°C". Proc. Natl. Acad. Sci. U.S. 48, 833-839 (1962).

171. A. Bennun and M. Avron. "The relation of the light-dependent and light-triggered ATPases to photophosphorylation". Biochim. Biophys. Acta 109, 117-127 (1965).

172. C.C. Black. "Evidence supporting the theory of two sites of photophosphorylation in green plants". Biochem. Biophys. Res. Commun. 28, 985-990 (1967).

173. H. Baltscheffsky and D.Y. de Kiewiet. "Existence and localization of 2 phosphorylation sites in PSP of isolated spinach chloroplasts". Acta Chem. Scand. 18, 2406-2408 (1964).

174. A.T. Jagendorf and G. Hind. "Studies on the mechanism of photophosphorylation" in Photosynthetic Mechanisms in Green Plants. Natl. Acad. Sci. N.R.C. Publ. 1145, 599-610 (1963).

175. T. Horio, K. Nishikawa and J. Yamashita. "Synthesis and possible character of a high-energy intermediate in bacterial photophosphorylation". Biochem. J. 98, 321-329 (1966).

176. W.W. Cleland. "Dithiothreitol, a new protective agent for SH groups". Biochemistry 3, 480-482 (1964).

177. J. Webb. Enzyme and Metabolic Inhibition, Vol. III, Chapt. I. Academic Press, New York (1963).

178. D.I. Arnon, M.B. Allen and F.R. Whatley. "Photosynthesis by chloroplasts IV. General concept and comparison of 3 photochemical reactions". Biochim. Biophys. Acta 20, 449-468 (1956).

179. E.S. Bamburger and M. Gibbs. "Effects of phosphorylated compounds and inhibitors on CO_2 fixation by intact spinach chloroplasts". Plant Physiol. 40, 919-927 (1965).

180. M. Gibbs, E. Latzko, R.G. Everson and W. Cockburn. "Carbon mobilization by the green plant" in Harvesting the Sun. ed. A. San Pietro, F.A. Greer and T.J. Army. p. 111-130. Academic Press, New York (1967).

181. J.A. Bassham. "Kinetic studies of the photosynthetic carbon reduction cycle". Ann. Rev. Plant Physiol. 15, p. 116 (1964).

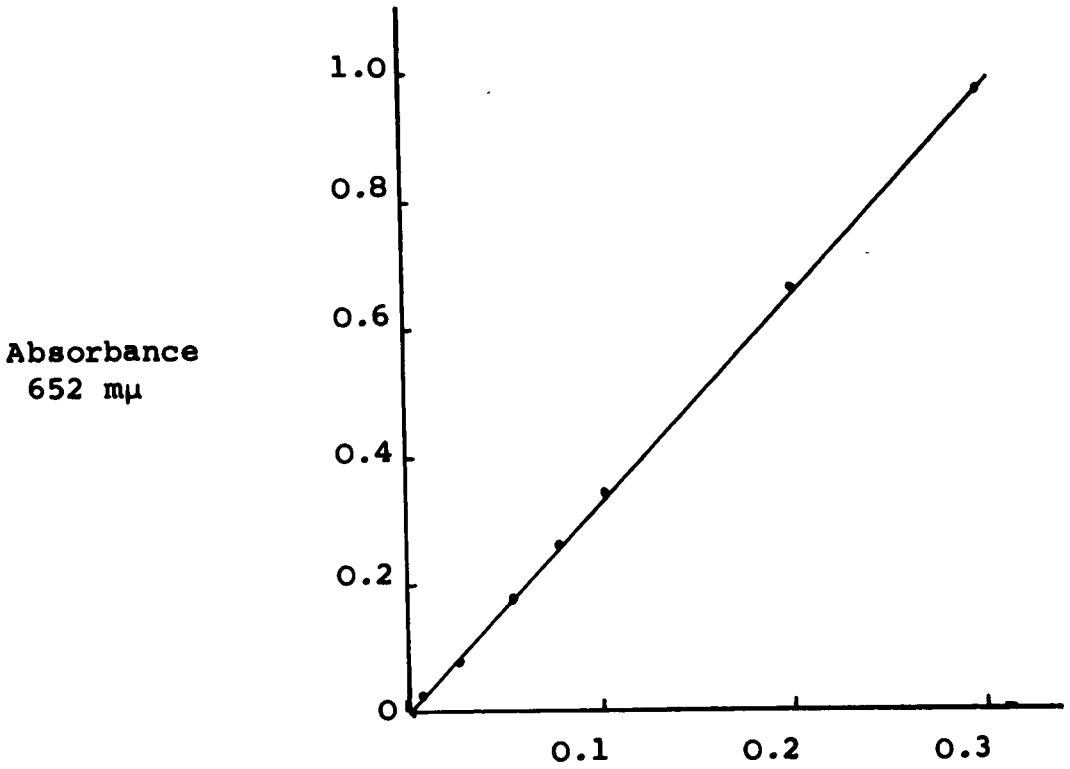
182. A.F. Rega, R.A. Caro and R. Radicella. "Stability of ^{32}P-labelled pyrophosphate". Radiochim. Acta 2, 218-219 (1964).

183. S. Izawa, T.N. Connolly, G.D. Winget and N.E. Good. "Inhibition and uncoupling of photophosphorylation in chloroplasts" in Brookhaven Symposium in Biology No. 19 (1966), pp. 169-187.

184. D.A. Walker. "Correlation between photosynthetic activity and membrane integrity in isolated pea chloroplasts". Plant Physiology 40, 1157-1161 (1965).

APPENDIX

i. Chlorophyll Assay



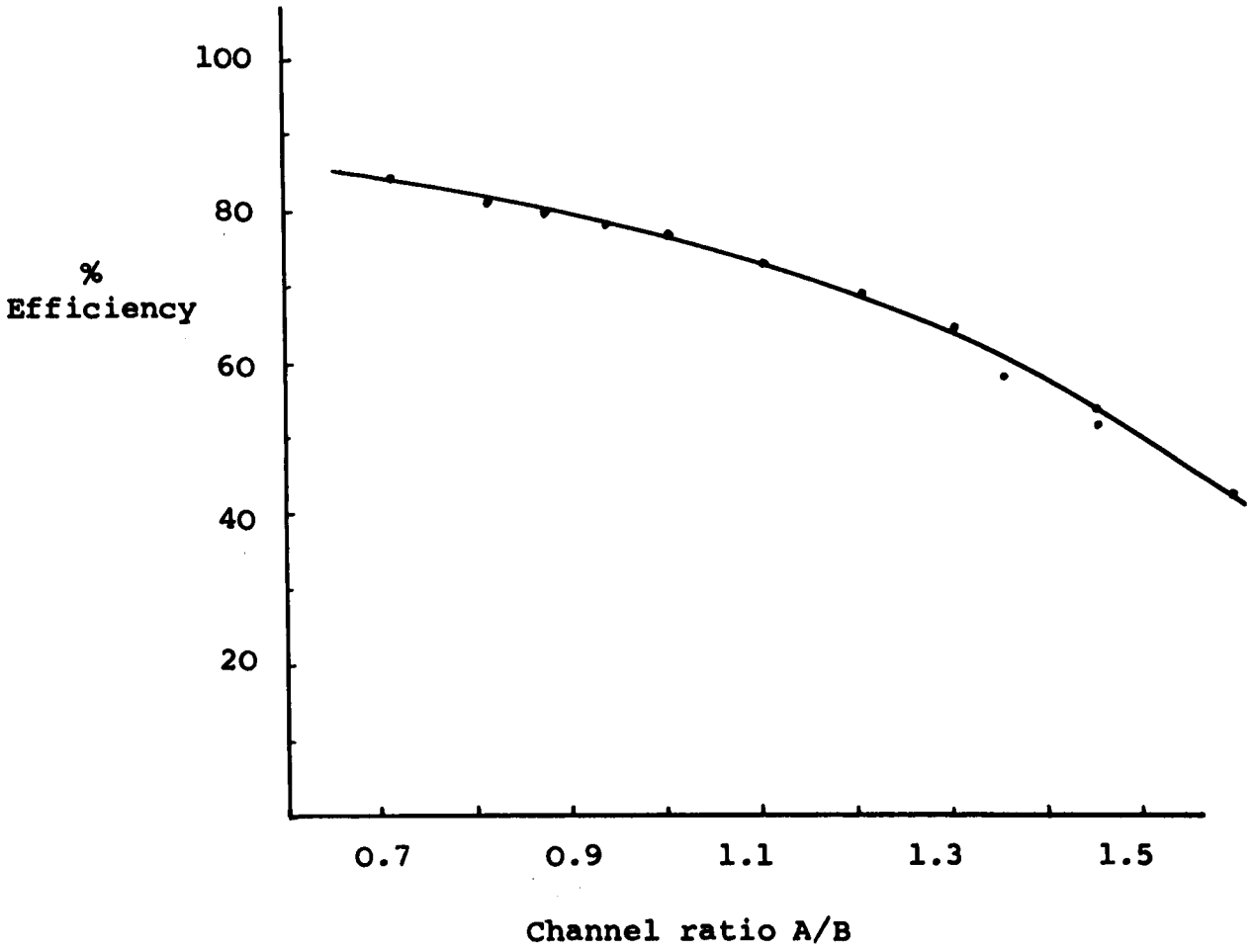
Chloroplasts were isolated from 10 g. spinach leaves as shown in Methods. They were suspended in 1 ml. of reaction buffer and aliquots were assayed for chlorophyll (a+b) by the method of Arnon (138).

$$\text{mg. chlorophyll/ml.} = \frac{\text{chlorophyll absorbance } (652\ m\mu)}{34.5} \times 1000 \times \frac{25}{0.2 \times 1000}$$

(Arnon, reference 138, plotted the light absorption data for chlorophyll a and b, and found the curves intersected at 652 mμ, with a specific absorption coefficient of 34.5).

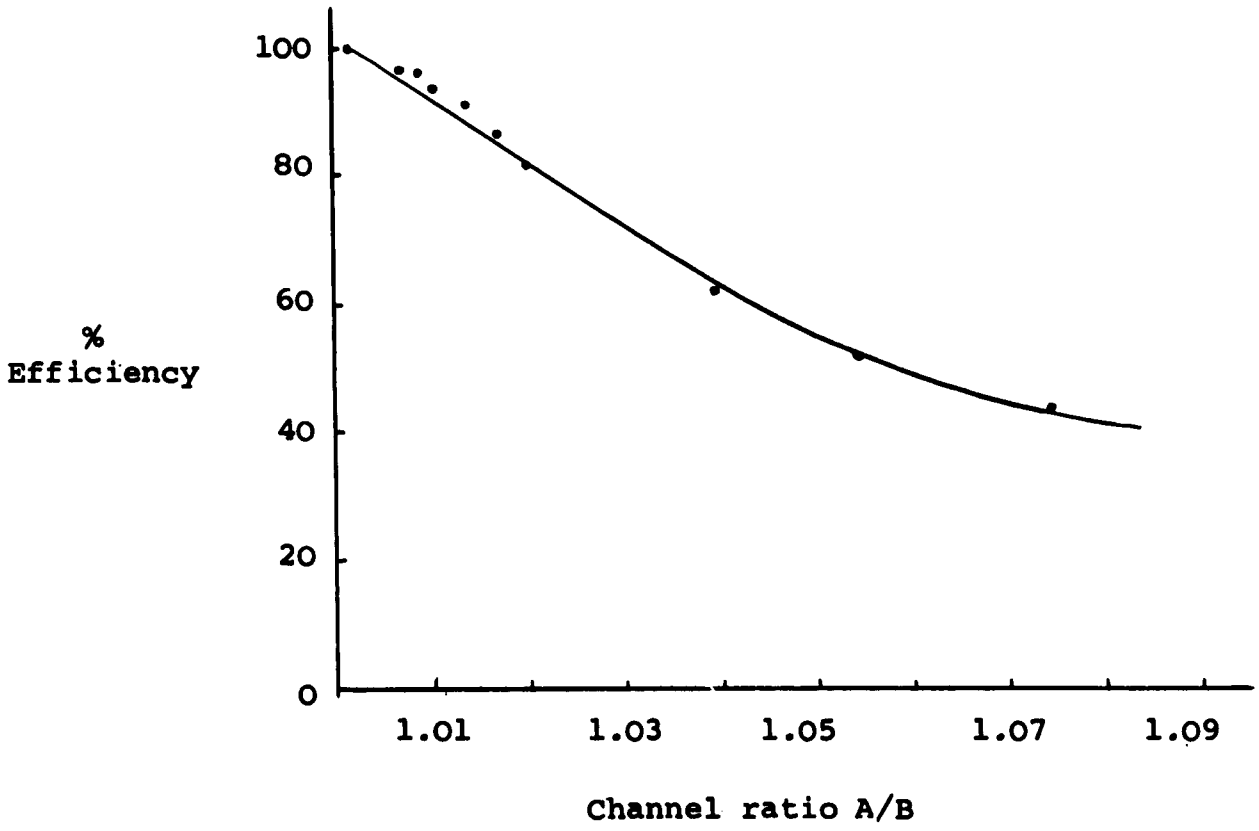
ii. Quenching curves

a. ^{14}C-$NaHCO_3$



Samples were quenched with quenching mixture described in the Methods section.
Nuclear Chicago Unilux II. L - U, L = 0.0, U = 9.9
Attenuation A = B250
B = D750. Counting time 10 minutes.

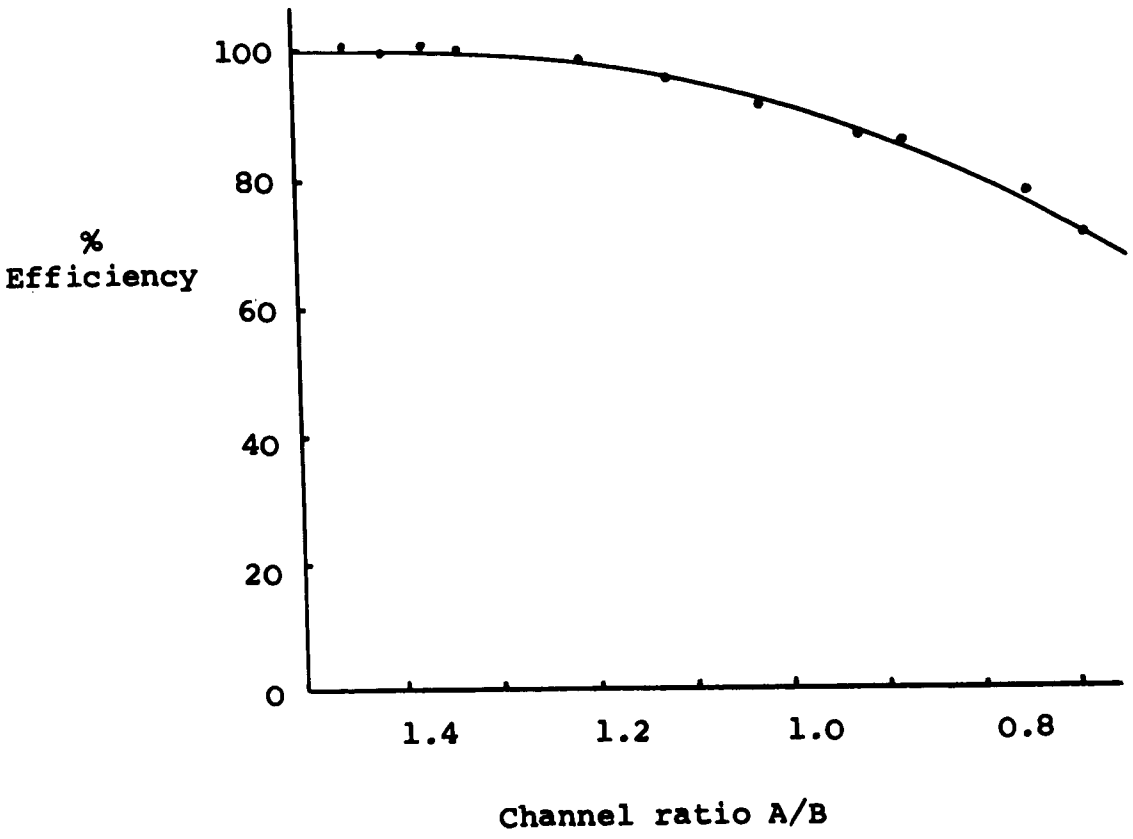
b. ^{45}Ca-$CaCl_2$



Samples were quenched with increasing concentrations of chloroplasts isolated as in Methods. The flask with no quenching agent was taken as 100% efficient in this experiment.

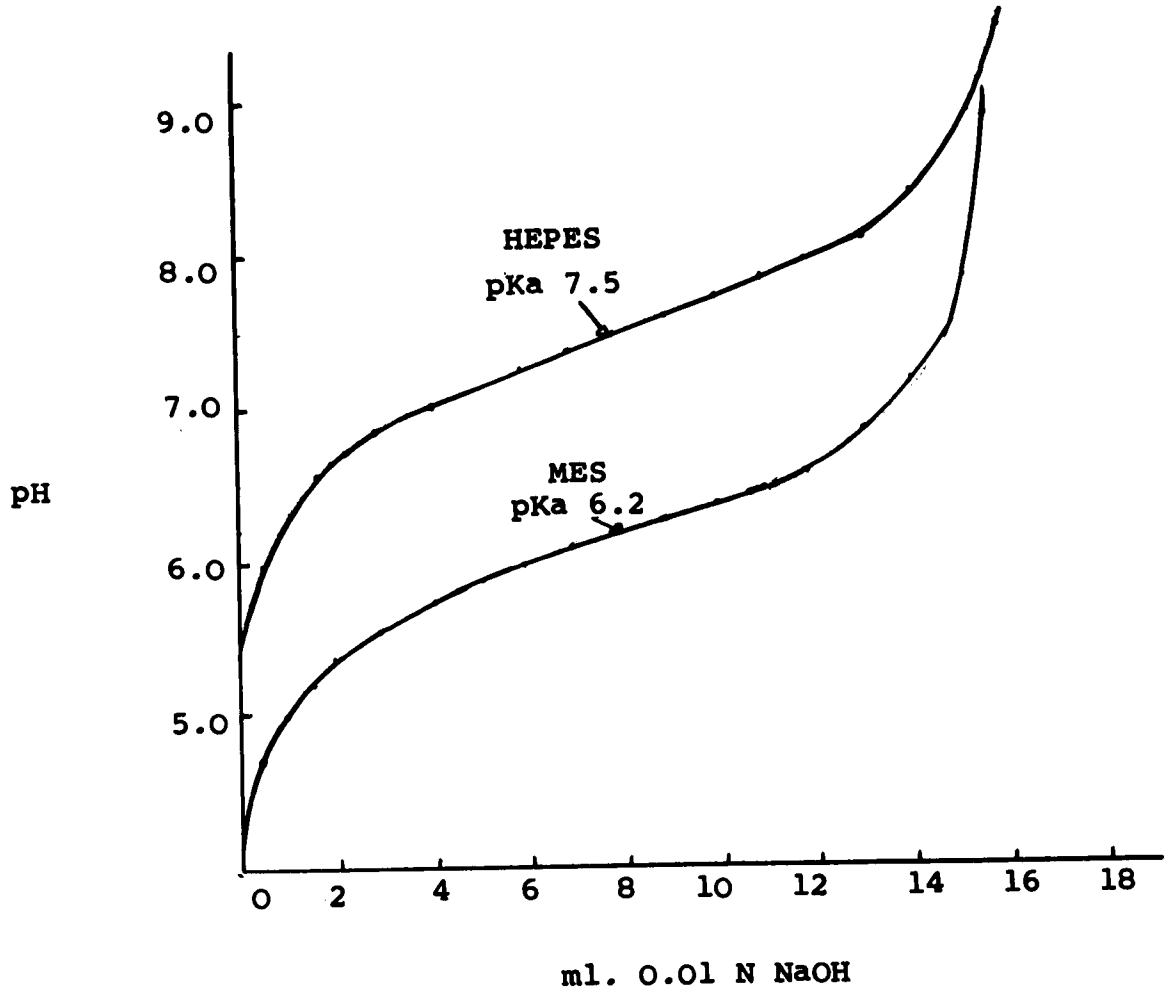
Nuclear Chicago Unilux II. L - U, L = 0.0, U = 9.9
Attenuation A = B250
B = D700. Counting time 10 minutes.

c. ^{32}P-H_3PO_4



Samples were quenched with varying amounts of the aqueous phase obtained from the IBB extraction procedure. A known amount of ^{32}P-H_3PO_4 was added to each flask, and the flask to which no quenching agent was added was taken to be 100% efficient.
Nuclear Chicago Unilux II. L - U, L = 0.0, U = 9.9
Attenuation A = D750
B = H050. Counting time 10 minutes.

iii. Buffering ranges of HEPES and MES



0.01 M HEPES and 0.01 M MES were titrated with 0.01 N NaOH to ascertain the effective buffering range of these buffers. The pKa values obtained were very close to those reported in the literature.

iv. Reproducibility of Extraction procedures

a. IBB extraction

	cpm in aqueous phase				
Experiment 1	290	290	320	295	
Experiment 2	7100	6400	6800	6900	
Experiment 3	7000	6500	6800	6200	6900

0.21 mg. chloroplasts were added to 0.01 M HEPES buffer (0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MgCl_2$, 0.1 mM PMS, 5 mM ADP, 2 mM ^{32}P-KH_2PO_4, 3.1 x 10^5 cpm) pH 7.5, to give a total volume of 2.0 ml. In Experiment 1 the reaction was in the dark for 1 minute, and then the ^{32}P-KH_2PO_4 was extracted by the IBB procedure. In Experiments 2 and 3 the reaction was conducted in the light (30,000 lux) for 4 minutes prior to IBB extraction. 4 or 5 flasks were used in each of these experiments.

b. Extraction of ^{32}P-H_3PO_4 by the Millipore Filter

Flask No.	cpm/millipore filter
1	3140
2	3440
3	3270

In this experiment chloroplasts (0.18 mg. chlorophyll) were reacted in 0.01 M HEPES buffer (0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$), with ^{32}P-H_3PO_4 (2.0×10^5 cpm) in the light for 5 minutes at 20°C. Then the chloroplasts were removed by the Millipore filter and washed with 10 ml. of buffer (as for reaction buffer) at 0°C. The millipore filters were then placed into scintillation vials containing 10 ml. of fluor, and counted.

c. Extraction of ^{45}Ca-$CaCl_2$ by the Millipore Filter

Flask No.	cpm ^{45}Ca-$CaCl_2$/millipore
1	2900
2	2200
3	2600

In this experiment, the chloroplasts (0.02 mg. chlorophyll) were reacted in 0.01 M HEPES buffer (0.33 M sorbitol, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$) with 1.0 mM ^{45}Ca-$CaCl_2$ (8.5×10^5 cpm) for 15 minutes in the dark. Then the chloroplasts were removed by Millipore filter, and washed with 10 ml. of buffer (as for reaction buffer) at 0°C. The millipore filters were then placed in scintillation vials containing 10 ml. fluor solution and counted.

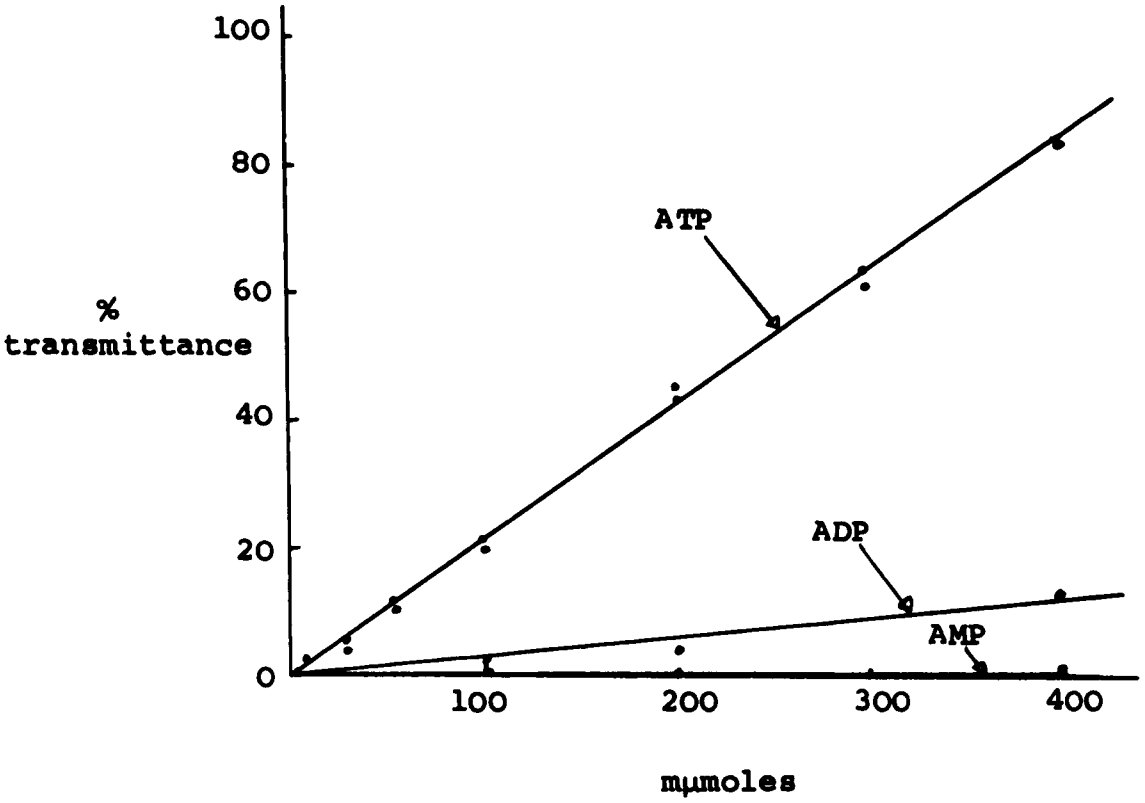
v. Thin Layer Chromatography

	R_f	Solvent
ATP	0.35	1
	0.02	2
	0.16	3
PPi	0.59	3
	0.27	2
3-PGA	0.45	4

Cellulose coated plates were prepared as shown in Methods. Authentic samples were added, and the plates were placed into tanks containing 100 ml. of solvent.

Solvent 1. isobutyric acid/ammonia/water 57/4/39 v/v
2. ethyl acetate/acetic acid/water 3/3/1 v/v
3. methanol/formic acid/water 16/3/1 v/v
4. propionic acid/water (18/22 v/v); butanol/water (370/25 v/v) 50/50 v/v

vi. Luciferase Assay of ATP



Varying amounts of ATP, ADP or AMP were added to a Worthington Luciferase solution. The intensity of the light emission was measured in a DU2 spectrophotometer as described in the Methods. Arbitary units could be used in place of % transmittance.

vii. Efficiency of ATP solvent extraction

^{14}C-ATP (cpm x 10^{-3}) added	recovered after IBB extraction Experiment No. 1	2	3
1.50	1.50	1.45	1.30
15.0	11.0	14.6	9.00
150.0	125.0	104.0	130.0

Known amounts of ^{14}C-ATP were added to 2.0 ml. 0.01 M HEPES buffer pH 7.5 (0.33 M sorbitol, 1.0 mM $MgCl_2$). The ATP was extracted by the IBB procedure, and the aqueous phase was counted for activity.

viii. Quenching of Luciferase Reaction by Chloroplasts

Chloroplasts (0.05 mg. chlorophyll in 0.5 ml. HEPES (0.01 M) buffer) were added to a cuvette containing 1.0 ml. distilled water and 0.5 ml. Worthington Luciferase. They were mixed, and 100 mμmoles ATP added. The intensity of the light reaction was measured in a DU2-spectrophotometer. The presence of the chloroplasts were found to quench the light reaction by about $5 \pm 2\%$.