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CONVERSION OF TIGHTLY BOUND ADP TO BOUND ATP ON CHLOROPLAST MEMBRANES AND PARTIAL CHARACTERIZATION OF SPINACH CHLOROPLAST BINDING SITES

BY

JERRY SZYJEWICZ

A dissertation submitted to the Graduate
Faculty in Biochemistry in partial fulfillment of the
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August 15, 1983
Date

Aaron Lukton
Chairman of Examining Committee

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Supervisory Committee

ABSTRACT

[^{3}H]-Adenine nucleotides were exchanged into the binding sites of spinach chloroplasts. A maximum of 3.7 nmoles of [^{3}H]-nucleotides/mg chlorophyll remained bound to the membranes following repeated washings (centrifugation and resuspension in fresh medium) of the labeled chloroplasts. Passage of similarly labeled chloroplasts through a Sephadex G-50 column removed additional bound [^{3}H]-nucleotides; 1.98 nmoles of [^{3}H]-nucleotides/mg chlorophyll remained bound to the membranes. Two types of binding sites were identified in these chloroplasts: 1) the exchangeable site(s) - these sites slowly shed their nucleotides in the dark (0.87 nmoles of [^{3}H]-nucleotides/mg chlorophyll dissociated from the chloroplasts in the dark 30 min following elution from the Sephadex G-50 column). An additional 0.14 nmoles of [^{3}H]-nucleotides were lost from the membranes in the dark following the addition of free ADP or ATP or upon excitation of the chloroplasts in the absence of added nucleotides; 2) the non-exchangeable site(s) - 0.89 numoles of bound [^{3}H]-nucleotides/mg chlorophyll remain uneffected by the conditions that brought about the release of nucleotides from the exchangeable site(s). These nucleotides exchanged with medium nucleotides, ATP or ADP, only following excitation of the chloroplasts ($t_{\frac{1}{2}}$ = 0.2-0.5 s). This exchange was inhibited by 20 mM NH_4Cl or by 1 mM NBD.Cl and showed a requirement for Mg^{++}.

[^{3}H]-ADP at the non-exchangeable site was converted to [^{3}H]-ATP which remained firmly attached to the chloroplasts when the chloroplasts were illuminated in the presence of inorganic phosphate but in the absence of added nucleotides. Formation of bound [^{3}H]-ATP from bound [^{3}H]-ADP was also observed when labeled chloroplasts that were passed through Sephadex G-50 were incubated in the dark with ATP. ATP mediated phosphorylation of bound

[^{3}H]-ADP was inhibited by free ADP, the ATP analogue AMPPNP, and by the uncouplers NH_4Cl (20 mM) and NBD.Cl (1 mM). Conversion of bound ADP to ATP was catalyzed by a chloroplast membrane componant, possibly CF_1, and not by enzymes such as adenylate kinase (AK). The rate of phosphorylation of bound [^{3}H]-ADP on the non-exchangeable site ($t_{\frac{1}{2}} \sim 5.0$ s) and the rate of exchange of labeled nucleotides from this site with free nucleotides were too slow to serve as intermediates in photophosphorylation.

Pyruvate kinase and phosphoenolpyruvate inhibited ATP $\rightleftharpoons$ [^{32}Pi] exchange by more than 80% in the presence of 1 mM ATP and 5 mM [^{32}Pi] under conditions of photophosphorylation. These results are explained by release of ADP (ATP hydrolysis product) from the hydrolytic site followed by photophosphorylation of the free ADP + [^{32}Pi] to [^{32}P]-ATP on a second catalytic site. Similar rates of ATP hydrolysis and [^{32}Pi] incorporation into ATP provide additional support that ATP hydrolysis and [^{32}Pi] incorporation into ATP provide additional support that ATP synthesis and ATP hydrolysis occur on two separate catalytic sites.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor Vida Vambutas for her help in the many discussions and criticisms of this work as it progressed; to Dr. C.S.P. Jenkins for his helpful criticism of the writing of this thesis and for his unrelenting support; to Dr. A. Lukton for his support; to Dr. H. Penefsky and Dr. B. Beattie for their helpful criticism of my work.

I acknowledge also my deepest gratitude to Freda and Danielle Schevitz for their patience and support.

ABBREVIATIONS

CF_1	chloroplast coupling factor
BF_1	bacterial coupling factor
MF_1, F_1	mitochondrial coupling factor
DPN^+ (NAD)	diphosphopyridine nucleotide (nicotinamide-adenine dinucleotide)
TPN^+ (NADP)	triphosphopyridine nucleotide (nicotinamide-adenine dinucleotide phosphate)
DNP	2,4-dinitrophenol
FMN	flavin mononucleotide
DPIP	2,6-dichlorophenol indophenol
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
PMS	phenazinemethosulfate
SDS	sodium dodecyl sulfate
F_2DNP	1,5-difluoro-2,4-dinitrobenzene
DTP	dimethyl-3,3'-dithiobispropionimidate dihydrochloride
DCCD	N,N'-dicyclohexylcarbodiimides
Nbf-Cl (NBD.Cl)	4-chloro-2-nitrobenzofurazon (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole)
Arylazido- -alanyl ATP	3-0- 3-[(N-4-azido-2-nitrophenyl)amino]propionyl ATP
NbS^6ITP	6-[(3-carboxy-4-nitrophenyl)thio]-9- -D-ribofuranozylpurin 5'-triphosphate
AMPPNP	adenylyl imidodiphosphate
FSBA	p-fluorosulfonylbenzoyl-5'-adenosine
TNP-ATP	2',3'-O-(2,4,6-trinitrophenyl)-ATP
ITP	inosine triphosphate
NEM	N-ethylmaleimide

PK	pyruvate kinase
PEP	phosphoenol pyruvate
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetate
DTT	dithiothreitol
AK	adenylate kinase

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INFORMATION

Historical background

The coupling between respiration and formation of phosphate bonds was first observed by Engelhardt (1) in 1930, in experiments with blood hemolysates. In 1937, Lipman (2) reported that phosphorylation of adenylic acid was associated with the dehydrogenation of pyruvic acid by *Bacillus delbrukii*. At that time, Warburg (3) demonstrated that the formation of ATP was coupled to the enzymatic oxidation of 1,3 diphosphoglycerate; Kerbs and Johnson (4) demonstrated one of the principle steps in carbohydrate metabolism, the citric acid cycle; and Hill (5) discovered that isolated chloroplasts rapidly reduced ferricyanide in light and evolve oxygen in the process.

In 1939, Banga and Ochoa (6) showed that pyruvate was rapidly oxidized by ground pigeon brain dispersions. In the same publication, they also recognized that "adenine nucleotide", inorganic phosphate and Mg^{++} (or Mn^{++}) were important factors in the oxidation of pyruvate. They "not only increased the oxidative removal of pyruvate considerably, but made its oxidation more complete." In subsequent publication, Ochoa (7,8) and others (9-13) showed in more detail that phosphorylation and oxidation of pyruvate were coupled reactions. Based on the number of phosphates incorporated per oxygen consumed (P:O ratio) that were obtained during the oxidation of pyruvate, Ochoa (8) correctly concluded that each of the primary dehydrogenation steps involved in the oxidation of one molecule of pyruvate to carbon dioxide and water generates three phosphate bonds. A total of fifteen phosphate bonds were formed per molecule of pyruvate oxidized. Thermodynamic measurements showed that the free energy (ΔF) of complete oxidation of glucose to carbon dioxide and water, liberated 688 Kcal, more than enough needed to generate the predicted 36 phosphate bonds, 430 Kcal.

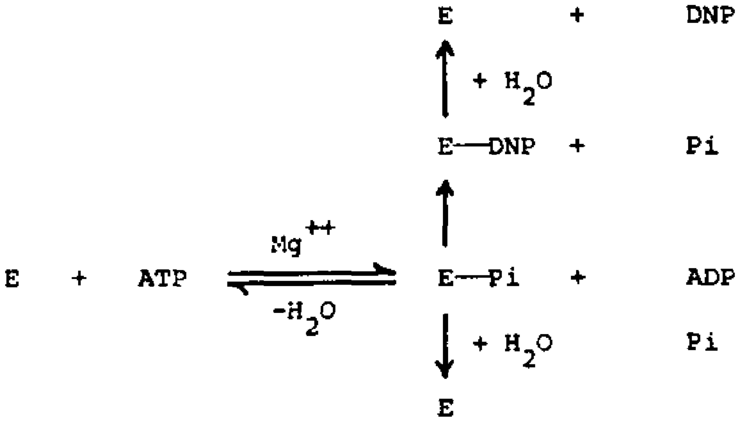
1. Respiration and phosphorylation

The importance of oxygen in cellular respiration and the role of iron containing compounds in biological oxidation was realized by Warburg (14) in the late 1920s. This was, primarily through observations of cyanide and carbon monoxide intervention with respiration and utilization of oxygen. However, the basis for the existence of a sequence of respiratory carriers between substrate and oxygen was supplied by Keilin with discovery of the cytochromes; initially identifying the spectra of cytochromes b, c, and a (15) and later by his discovery of cytochrome oxidase (16). It was quickly suspected by Keilin and Warburg that the cytochromes acted in an ordered sequence (17). This was demonstrated experimentally by Ball's (18) oxidation reduction studies in which he showed the reduction potential of cytochromes b, c and a to be -0.04, +0.27, and +0.29 mv respectively. Cytochrome a_3 was placed at the end of the chain because it was shown to be the one that reacts with oxygen (rev. 19). A link between the coenzymes (DPN and TPN) and the cytochromes was indicated from the inhibition of coenzyme dependent substrate oxidation by cyanide (20). Warburg and Christian (21) were able to observe that oxidation of the coenzymes could be linked to oxygen only through the "old yellow enzyme" (flavoproteins). Theorell (22) succeeded in linking the coenzymes to cytochrome c through the "old yellow enzyme". Nearly two decades later, with the aid of pure succinate dehyrogenase (23), the involvement of flavoproteins in the succinate pathway was demonstrated; and the flavoproteins were shown to be indispensable in the passage of hydrogen from $DPNH_2$ to cytochrome c (24). Lockhart and Potter (25) demonstrated that cytochrome c was essential for the passage of hydrogen from the coenzymes to oxygen; and the role of cytochrome b, although more equivocal, was also shown to be required for the reduction of cytochrome c. The picture that was emerging at the time was summarized in 1946 by Ochoa (27):

1) Pyruvic acid → ? → cytochrome system → O_2
2) Acetyl
3) Isocitric acid → TPN → Flavoprotein II → cyt. c → cyt. ox. → O_2
4) Oxalosuccinic acid
5) α-ketoglutaric acid → ? → cyt. c → cyt. ox. → O_2
6) Succinic acid → ? → cyt. c → cyt. ox. → O_2
7) Fumaric acid
8) Malic acid → TPN → Flavoprotein I → ? → cyt. c → cyt. ox. → O_2
9) Oxaloacetic acid

Although Ochoa suspected that the energy librerated during the passage of e^- from $TPNH_2$ or $DPHN_2$ to oxygen was utilized for the generation of high energy phosphate bonds (26), it was Friedkin and Lehninger (27), 1948, who demonstrated that aerobic incubation of $DPNH_2$ with "particulate matter" of rat liver in the presence of ATP, Mg^{++}, cytochrome c and [^{32}P] resulted in esterification of the label into organic phosphate. They thus removed the site or aerobic phosphorylation from the Kreb's cycle and raised questions concerning the enzymes involved in electron transport and phosphorylation. Pardee and Potter (28) in a study on factors which effect the maintenance of oxidative phosphorylation in tissue fractions from rat kidney concluded from their studies that the loss of phosphorylation capacity was due to loss of labile components in mitochondria. The role of intact mitochondria in oxidation of pyruvate, other carbohydrates, fatty acids and some amino acids to carbon dioxide and water, and its role in phosphorylation was soon realized (29,30).

It was commonly observed that aged mitochondria or tissue preparations caused more rapid ATP hydrolysis than fresh preparations. Kielley and Kielley (31) observed that mitochondrial fractions from rat liver caused the liberation of phosphate from ATP. The ATPase activity was associated with particles obtained from dissociated mitochondria. These preparations required Mg^{++} for ATPase activity and were inhibited by ADP. Lardy and Wellman (32) observed that 2,4 dinitrophenol greatly enhanced the liberation of phosphate from ATP in intact mitochondria. The observation that DNP also was a potent inhibitor of oxidative phosphorylation (10,33), led Lardy and Wellman to believe that the ATPase activity and phosphorylation were opposite sides of the same reaction. They proposed that DNP caused the breakdown of the high energy intermediate according to the following scheme (32):



It is not an accident that a phosphate-enzyme intermediate was proposed since the only known mechanisms of ATP formation involved a phosphate intermediate, i.e. phosphoenolpyruvate and diphosphoglycerate linked phosphorylation of ADP. Enzyme-Pi, acetyl-Pi thioesters as well as others have been proposed at one time or another to be intermediates in phosphorylation (34). This chemical hypothesis of phosphorylation was summarized by

Slater (35) in 1953) as follows:

$$AH_2 \xrightarrow{\qquad} A{\sim}X \xrightarrow{+\ Y} X{\sim}Y \xrightarrow{+\ Pi} X{\sim}P \xrightarrow{ADP} ATP$$

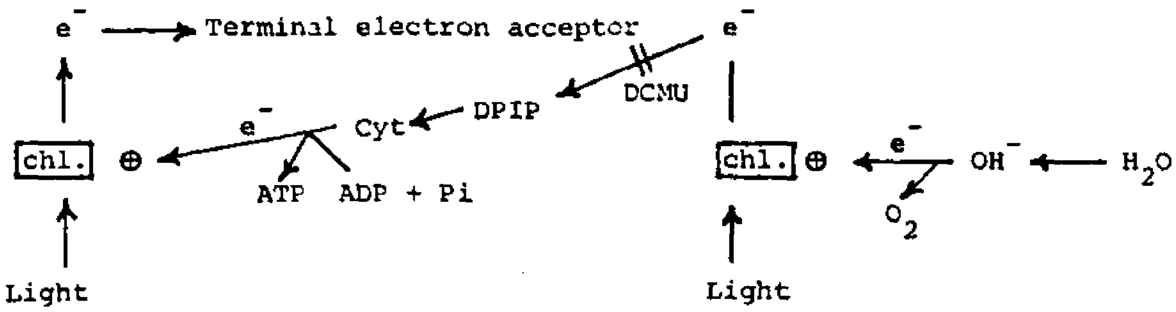
A~X is a high energy intermediate in the respiratory chain, while X~Y is an intermediate removed from the respiratory chain and free to form the high energy phosphate intermediate. To date, however, no high energy intermediates have been identified which can account for the observed patterns of ATP synthesis.

2. Photophosphorylation and the chloroplast electron transport system

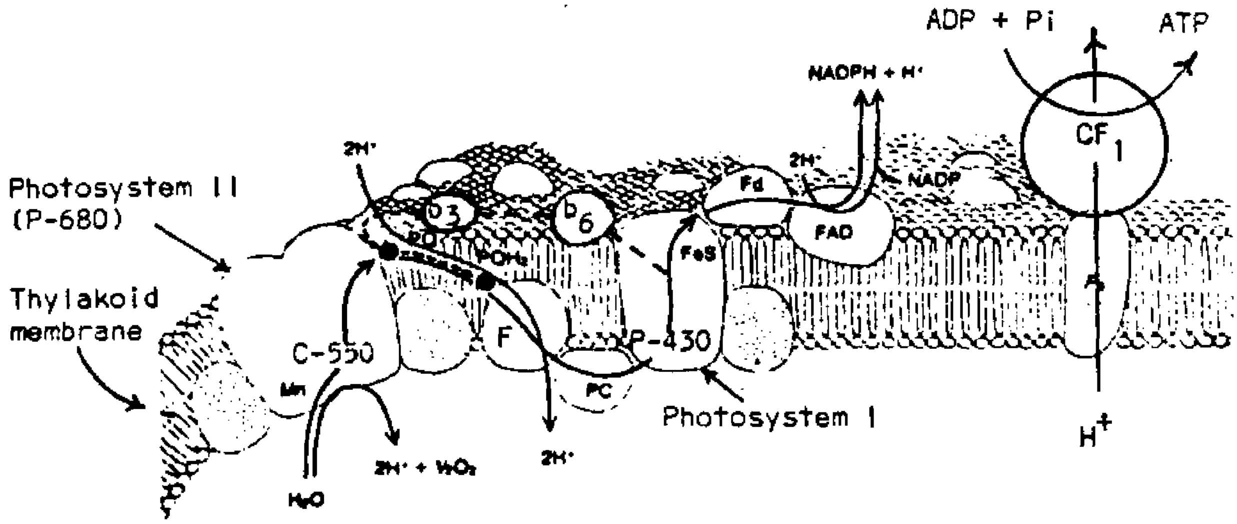
In 1954 light dependent phosphorylation of ADP was reported by Arnon et al (36) in spinach chloroplasts. For photophosphorylation to proceed the chloroplasts required AMP or ADP, Pi, Mg^{++}, ascorbate and light. Since no e^- acceptor was used in these experiments the rate of photophosphorylation was slow, 3 umoles per hour per mg chlorophyll. The rate of photophosphorylation increased by more than ten fold when small amounts of flavin mononucleotides (FMN) and vitamin K were added to the reactions (36). Photophosphorylation of ADP was also demonstrated in the same year in chromatophore fragments of Rhodospirillum rubrum, Frankel (37).

Data indicating that phosphorylation in chloroplasts was coupled to electron transport, as in mitochondria, began to emerge in 1954 with observations that the cofactors FMN and vitamin K stimulated photophosphorylation (36) and that illumination of chloroplasts resulted in photophosphorylation and simultanious oxidation of cytochrome F (38). Arnon (36) noted that photophosphorylation in the presence of these cofactors occured without oxygen evolution or an electron acceptor, which led him to propose that electron flow during photophosphorylation takes place in a closed loop: light absorbed by chlorophyll ejects a high energy electron. The resulting positive "hole" is filled by movement of an electron from cytochrome F, while the high energy electron flows from a high to a low potential through a cofactor to cytochrome F. This cyclic electron flow, according to Arnon, was coupled to phosphorylation of ADP to ATP (39).

According to Hill and Bendall (40), however, electron flow in chloroplasts was linear similar to the mitochondrial system, where cytochromes F and b_6 served the same function cytochromes c and b in mitochondria and cytochrome oxydase had its counterpart in a chlorophyll containing complex in chloroplasts. In this model light absorbed by chlorophyll was used for the photo-oxidation of water to produce oxygen and transport hydrogen against its thermochemical gradient. The reverse stepwise transfer of hydrogen through cytochromes F and b_6 was coupled to phosphorylation of ADP to ATP. Hill and Bendall (40) proposed that two or three light driven reactions may be required for transport of hydrogen from water to an acceptor (NADP). Experimental evidence for two separate light driven reactions in green plants was later demonstrated by Losada et al (41).They showed that electron flow from water to NADP could be separated into two distinct photochemical reactions: addition of the electron donor 2,6 dichlorophenol indophenol (DPIP) to chloroplasts, where participation of water as an electron donor was blocked with the inhibitor DCMU, resulted in photophosphorylation and NADP reduction rates which were similar to controls (which did not include DCMU or DPIP). However, in the absence of DCMU, DPIP acted as an electron acceptor in the light; in a reaction that resulted in rapid oxygen evolution and with inhibition of photophosphorylation. Substituting DPIP with a terminal electron acceptor such as ferricyanide or NADP restored oxygen evolution and photophosphorylation. DPIP was envisioned to act as an electron carrier between two photosystems as follows (41):



A more detailed description of the electron transport system in plant chloroplasts is summarized in the next scheme (42):



Photons of light are absorbed by electrons in photosystems I and II, promoting the electrons to a high energy singlet which rapidly decays to a more stable singlet capable of interacting with the primary electron acceptors in photosystems I and II, denoted respectively by P430 and cytochrome C_{550} (C_{550}). In photosystem II absorption of photons of light are coupled to photolysis of water to oxygen and the formation of H^+ within the thylakoids. From C_{550}, electrons flow from a high (negative) to a low (positive) redox potential sequentially through cytochrome b_{559} (b_3), plastoquinone (PQ), cytochrome f (F), plastocyanine (PC) to photosystem I. From photosystem I electron flow proceeds to P430, ferredoxin (Fd), ferredoxin-NADP reductase (FAD) to reduce NADP or some other linear electron acceptor, i.e. methyviolygen or potassium ferricyanide. The described path of electron flow is called linear electron flow, and it is accompanied by rapid oxygen evolution. In the presence of a cyclic electron, i.e. phenazinemethosulfate (PMS) or pyocyanin, electrons from P430 flow through cytochrome b_6 back to cytochrome b_3, then back again to photosystem I. Passage of a pair of electrons through plastoquinone to cytochrome f is coupled to the translocation of a pair of protons into the thylakoids to generate a protomotive force (42). During cyclic electron flow, photosystem II is bypassed and oxygen evolution is supressed.

3. The chemiosmotic coupling between electron transport and ATP synthesis

Formation of ATP is a complex process which consists of a number of different parts (refer to the previous figure). In chloroplasts, this involves photo-excitation of the photosystems, which induces the flow of electrons through the electron transport system in the thylakoid membranes. This is accompanied by the vectorial transport of protons into the thylakoid vesicles to generate a protomotive gradient across the vesicle membrane. The back flow of these protons through the ATP synthase, CF_o-CF_1, complex provides the energy for ATP synthesis. CF_o is a thylakoid membrane hydrogen ion channel and CF_1 (chloroplast coupling factor) contains the ATPase activity. There is little doubt that the chemiosmotic hypothesis proposed by Mitchel (43) also provides the unifying picture for the overall mechanism of ATP formation in mitochondria, bacteria and chloroplasts. That the chemiosmotic gradient is the driving force for ATP synthesis has been amply demonstrated. For example vesicles containing CF_o-CF_1 but no e^- transport capacity were shown to synthesize ATP with an artificially induced proton gradient. Artificial ΔpH was obtained by successive incubations of vesicles at high and low pH (44). Conversely compounds which prevent formation of the transmembrane hydrogen gradient such as uncouplers, are potent inhibitors of ATP synthesis. In the same line, energy transfer inhibitors such as DCCD (oligomycin in mitochondria) which react with CF_o subunits to block proton flux through CF_o are also potent inhibitors of ATP synthesis (45). The degree of linking between electron flow, Δ pH and ATP synthesis is still a matter of debate. The idea that there may be a close link between electron flow and ATP synthesis, in chloroplasts, is indicated from the observation that very short pulses of light of insufficiant duration to generate a significant proton motive force (PMF) still result in ATP synthesis. Boyer suggests that e^- flow induces a conformational change in membrane proteins which is associated with a pK change in the side chains. Different ionization of the side chains on the two sides of the membrane leads to translocation of protons and ATP synthesis. The flow of protons through the CF_o-CF_1 complex is reversible. Hydrolysis of ATP reverses the hydrogen flux, regenerating a partial ΔpH and under some conditions results in limited back electron flow.

4. Isolation of CF_1

As indicated in the historical portion of this introduction, significant evidence was accumulated which showed that ATPase and phosphorylation activities were closely related reactions. Bronk and Kielley (47) demonstrated that the ATPase activity was still present in mitochondria disrupted by sonication. Racker showed later that ATPase activity in submitochondrial particles could be stimulated by tryptic digestion, suggesting that mitochondrial ATPase existed in latent form (48). Other experiments showed that mitochondria or bacterial extracts could be fragmented into a particulate fraction and a soluble fraction. The particulate fraction was capable of e^- transport but no phosphorylation. The addition of the soluble component to the particulate fraction restored oxidative phosphorylation (49). Pullman et al (50) in 1960, isolated a Mg^{++} dependent ATPase from mitochondria in soluble form. In a subsequent paper, they demonstrated that ATPase and the coupling factor activities resided on the same protein (51). Chloroplast coupling factor ATPase was subsequently isolated and characterized by Vambutas and Racker (52).

The remainder of this report summarizes the structural studies that have been carried out on the coupling factor, including subunit composition, function of subunits and catalytic properties. Chloroplast coupling factor will be emphasized but there will be significant references to other coupling factors particularly in areas where CF_1 has not been well characterized.

Structure

1. Morphology

Electron micrograph of thylakoids obtained with negative staining (53,56), positive staining (55), and freeze etching (54) techniques show CF_1 to have a spherical structure of about 100 Å in diameter which is protruding from the outer surface of the thylakoid membranes. With negative staining micrographs, CF_1 appears to be connected to the membrane by a stalk (57). However, the detection of membrane components that correspond to CF_1 by freeze etching indicates that CF_1 may also be partially embedded in the membrane (54).

2. Molecular weight

There is no general agreement on the exact molecular weight of coupling factors from plants, animals or bacteria. Molecular weights for beef heart F_1, based on sedimentation equilibrium centrifugation, range from 280,000 to 360,000 (58,61). The variation has been attributed to dissociation of enzyme subunits during purification and centrifugation procedures (59), or to the presence of non-protein material on the enzyme (62). Reports of bacterial BF_1 molecular weights have also been variable 345,000 for *E. coli* and 385,000 for *Streptococcus faccalis* (65). Spinach chloroplast CF_1 was reported to have a molecular weight of 325,000 using sedimentation equilibrium centrifugation (63). In a more recent study, comparable values of 320,000-330,000 have been obtained for CF_1 from light scattering and small angle X-ray scattering measurements (64). Recent findings that may have an important effect on molecular weight determination, show that binding of nucleotides to the subunits of *E. coli* BF_1 and that interaction of coupling factor subunits can result in "packing" of the subunits into a tighter conformation (67). Dunn (66) has determined that this results in significant increase in the sedimentation coefficient. It also increases electrophoretic mobility and reduces the frictional ratio of the bound as compared to the unbound subunit. Nearly a 15% difference in molecular weight of the α subunit of *E. coli* was observed depending whether the subunit was ATP bound or free (66). The molecular weight of the coupling factor is a critical measurement, since small differences in molecular weight have an important bearing on the determination of enzyme subunit stoichiometry.

3. Subunit molecular weights

SDS-Polyacrylamide gel electrophoresis reveals that CF_1 is composed of five different subunits. The subunits are designated α, β, γ, δ, and ϵ in order of decreasing molecular weights. Coupling factors from plant, animal or bacteria appear to share the same five subunits. Molecular weights measurements of the subunits from CF_1 indicate approximate values of 60,000, 56,000, 37,000, 17,500, and 13,000 in order from the α to ϵ subunits (68,72). Bacterial BF_1 subunit molecular weights are nearly identical to those of CF_1 (70). Most of these molecular weight determinations were obtained from the subunit mobilities in SDS-polyacrylamide gel electrophoresis. However, Knowles and Penefsky (60) have found significant molecular weight differences when other methods were used. They found, for example, a close agreement in molecular weights obtained by gel filtration, amino acid analysis and equilibrium sedimentation. Average molecular weights by these methods were 54,000, 50,000, 33,000, 17,000, and 5,700 (monomers). These values were somewhat different from those obtained by SDS-polyacrylamide gel electrophoresis: 59,000, 54,000, 33,000, and 13,000. No value was given for the ϵ subunit. Whichever of these methods reflects the more accurate subunit molecular weight remains to be determined.

4. Subunit stoichiometry

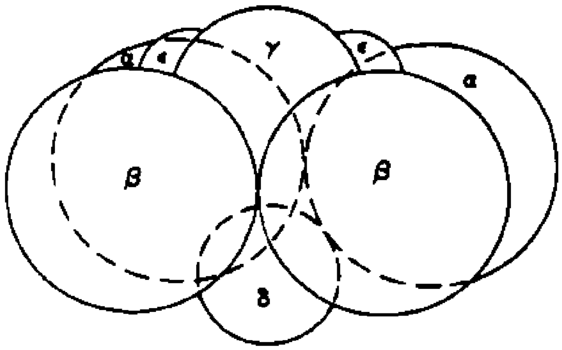
The stoichiometry of the subunits in CF_1, F_1 or BF_1 have not been conclusively demonstrated. Several different methods indicate that CF_1 has the composition $\alpha_2\beta_2\gamma\delta\epsilon_2$. Jagendorf et al (69) estimated the subunit composition of 2:2:1:1:2 from the relative absorbtion of dye by subunit polypeptides following gel electrophoresis, compared to the dye absorbed by known amounts of purified subunits. They came to the same conclusion from amino acid analysis of whole CF_1 compared to its subunits. Similar subunit composition was obtained from radioactivity incorporated into each subunit following analysis by SDS-polyacrylamide gel electrophoresis of CF_1 that had been isolated from pea plants grown in $[^{14}C]O_2$ (71) and from analysis of aggregates formed after treatment of

CF_1 with cross-linking agents (72). Based on CF_1 subunit stoichiometry of $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$, the sum of the subunit molecular weights is 311,000 which compares well with the molecular weight for CF_1 obtained by sedimentation equilibrium, 325,000. The picture of the subunit composition of F_1 is less clear. Senior and Brook (73) determined the molecular weight of the subunits by SDS-polyacrylamide gel electrophoresis to be 53,000, 50,000, 25,000, 12,700, and 7,500 (59). They concluded that F_1 has subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. Muller _et al_ (61) found two binding sites for aurovertin on F_1. They also concluded that a significant portion of F_1 was non-protein which led them to propose a molecular weight of 326,000 for F_1 rather than 360,000. Based on these two determinations and subunit molecular weights, they arrived at F_1 subunit composition of $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$. Similar stoichiometry for the three largest subunits was determined by Baird and Hammes (74).

Subunit composition of bacterial BF_1 is equally confusing although reports tend more towards an $\alpha_3\beta_3$ with greater variability for the smaller subunits. For example, growing _E. coli_ or _Solmonella typhemurium_ on $[^{14}C]$-protein hydrolysate, Bragg and Haw (70) determined, by a combined use of cross-linking agents and analysis of subunit aggregates by SDS-polyacrylamide gel electrophoresis for $[^{14}C]$ incorporation and staining intensities, that the subunit composition for these bacteria is $\alpha_3\beta_3\gamma\delta\epsilon$. A number of other reports are in agreement with this arrangement (82,84). However, from analysis of cold denatured fragments of _E. coli_ ATPase, Vogel and Steinhart (81) suggest an $\alpha_2\beta_2$ stoichiometry for the large subunits. Electron micrograph or negatively stained BF_1 from _Streptococcus faecalis_ indicates that the enzyme consists of six nearly identical subunits in a hexagon, suggesting a $\alpha_3\beta_3$ composition (75). Similar hexagonal arrangemnt was found in thermophylic bacterium PS3 (80). Additional indications of subunit compositions are shown in the following section.

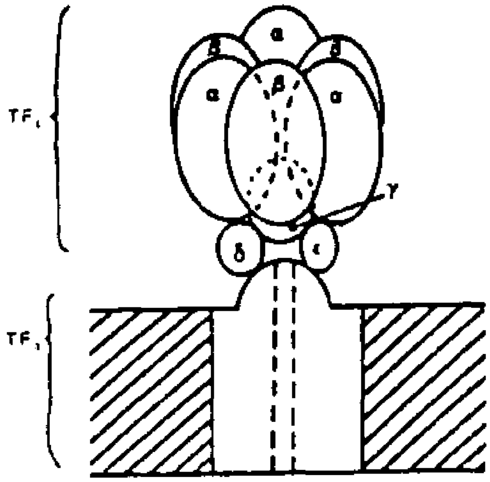
5. Spatial relationship of subunits

This information comes primarily through two types of studies, cross-linking of enzyme subunit to determine subunit proximity and from reconstitution studies. Examples of cross-linking agents used are F_2DNP, which is a bifunctional crosslinking agent capable of forming a 3 Å bridge between an amino group and a tyrosine phenolic group (76); DTP is an 11 Å long bifunctional imidoester which contains in its carbon chain a disulfite bond that is easily cleaved (77); (O-phenonthroline)$_2$CuII (78), and H_2O_2 (79) also act as reversible cross-linking agents by oxidizing adjacent sulfhydryl groups. Such agents were used by Baird and Hammes (72) on latent CF_1. The products of the reactions were subjected to SDS-polyacrylamide gel electrophoresis. The subunit composition of the cross-linked aggregates were then determined by taking advantage of the reversible nature of the crosslinking agents followed by a second dimensional SDS-polyacrylamide gel electrophoresis of the unlinked products. The resulting fragments showed no ϵ_2, $\delta\epsilon$, δ_2, $\gamma\delta$ or $\delta\epsilon_2$. Combinations of $\alpha\epsilon$, $\beta\epsilon$, $\alpha\delta$, α_2, $\alpha\beta$, $\beta\delta$, and β_2 dimers were found; $\alpha\beta\gamma$, $\alpha\beta_2$, and $\gamma\epsilon_2$ trimers; $\alpha_2\beta\gamma$ and $\alpha_2\beta_2$ tetramers were also found. From these studies they concluded a subunit stoichiometry for CF_1 $\alpha\beta\gamma$, $\alpha\beta_2$, of $\alpha_2\beta_2\gamma\delta\epsilon_2$ with the following arrangement of subunits (72):



Formation of α_2 and β_2 dimers argues against a $\alpha\beta\alpha\beta$ alternating arrangement. Similar methodology was applied to beef heart F_1. Resulting fragments suggest $\alpha_2\beta_2\gamma_2\delta\epsilon$ stoichiometry with the same arrangement of the large subunits as in CF_1 (74). Interestingly, using similar cross-linking procedure for E. coli and S. typhimurium no α_2 or β_2 were detected, suggesting that in these bacteria the α and β subunits have an alternating arrangement. The placement of the smaller subunits were as in CF_1 (70).

The first successful reconstitution of a coupling factor from its subunits was achieved by Yoshida et al (82) using the exceptionally stable subunits from the thermophilic bacterium PS3. They added the subunits in various combinations to ^{+}H permeable F_o-vesicles. F_o is the membrane counterpart of F_1, a multisubunit complex which is postulated to constitute a transmembrane DCCD sensitive hydrogen ion channel. Both ϵ and δ subunits bound to the F_o-vesicles but with no effect on the H^+ permeability of the vesicles. When the γ subunit was added to the $\epsilon\delta$-F_o-vesicles the permeability of the vesicles was greatly reduced. Addition of β subunits to this complex restored partial DCCD sensitive ATPase activity. This activity was greatly enhanced when the α subunits were also included. The proposed subunit structure for PS3 ATPase (82) is:



Later similar results were obtained with E. coli subunit reconstitution experiments. Minimum subunit requirements in this studies for ATPase activity was $\alpha\beta\gamma$, but complete restoration of phosphorylation capacity also required the δ, ϵ and F_o vesicles (84).

In summary, it can be stated with some degree of certainty that the hydrophobic δ subunit, in the presence of Mg^{++}, is responsible for the attachment of BF_1, CF_1 or MF_1 to their respective membrane F_o counterparts (82-85). Small angle X-ray scattering indicates that the δ subunit is an ellipsoid with half axis 25 Å x 90 Å (88). Antibody studies indicate that CF_1 ATPase activity appears to require both the α and β subunits. Tryptic digestion which removes the three smaller subunits does not impair CF_1 ATPase (68) suggesting that this activity resides in the large subunits. More specifically, the ATPase activity has been associated with the β subunits (86): however, restoration of phosphorylation appears to require both the α and β subunits (87). The ϵ subunit in CF_1 is considered to be on inhibitor protein since its removal by either heat, sulfhydryl reagents or trypsin activates CF_1-ATPase (68,89).

Binding sites

1. Nucleotide binding to the coupling factor

The possibility that beef heart ATPase contains very tight binding sites emerged in 1965 when 0.8 moles of bound nucleotides per mole F_1 was found to be stable to repeated ammonium sulfate precipitations (100). The binding of nucleotides to chloroplast coupling factor was later demonstrated in 1971 by Roy and Moudrianakis (90). Incubation of [^{14}C]-ADP with CF_1 followed by gel filtration showed the label to be associated with CF_1 at a ratio of 2ADP/CF_1. These bound nucleotides were placed in the role of catalytic intermediates. The finding of ATP and AMP following chromatography of CF_1-[^{14}C]-ADP complex led Roy and Moudrianakis to propose that the mechanism for ATP synthesis was through a terminal transphosphorylation, similar to that of adenylate kinase. In subsequent publications the same authors reported that energized chloroplasts incorporate [^{3}H]-AMP plus inorganic phosphate into bound [^{3}H]-ADP. This observation was explained in terms of AMP in the role of a primary phosphate acceptor in ATP synthesis according to the scheme:

$\mathrm{AMP} + \mathrm{Pi} + \mathrm{E} \xrightarrow{1} \mathrm{E}^{\mathrm{ADP}} + \mathrm{ADP}_2 \xrightarrow{2} \mathrm{E}^{\mathrm{ADP}_1}_{\mathrm{ADP}_2} \xrightarrow{3} \mathrm{E}^{\mathrm{AMP}}_{\mathrm{ATP}}$. Step 1 being the energy dependent step (91).

Tight binding sites for ADP were also established for rat liver F_1 (92), beef heart F_1(103), in chromatophores from Rhodospirillum rubrum (97) as well as in coupling factors from a variety of other sources (98,99). The number of nucleotide binding sites in beef heart F_1 was determined by Harris et al (94) in 1973, to be five, three moles of ATP and two moles of ADP per mole of F_1. In later experiments, Harris and Slater (96), using the energy transducing coupling factor from chloroplasts, bound approximately 3 nmoles of bound nucleotides per mg chlorophyll, 2.5 nmoles of ATP and 1.3 nmoles of ADP also per mg chlorophyll. This is equivalent to 1.9 and 1.0 moles of ATP and ADP respectively per mole of CF_1. The number of bound nucleotides in energy transducing mitochondrial particles were later revised to three (95). The first characterization of the high affinity nucleotide binding sites in isolated beef heart F_1 was carried out by Hilborn and Hammes (103); studying the equilibrium binding of ADP and its analogue ^{6}ethano-ADP, they found two binding sites for ADP and its analoque with the dissociation constant for ADP of 0.28 uM and 47 uM. Extending their studies to chloroplast coupling factor, Hammes et al (101) found similarly, two binding sites for ADP and the ATP analoque AMPPNP. The binding constants for ADP were 1.82 uM and 100 uM. A third binding site for AMPPNP was also discovered in the heat activated CF_1 (101). At about the same time, 1975, Garret and Penefsky (102) discovered that passing beef heart F_1 through a Sephadex column equilibrated with 50% glycerol removes practically all bound nucleotides from the enzyme. F_1 treated in this fashion was capable of binding more than 4 moles of ADP and almost 5 moles of AMPPNP per mole of F_1 as compared to 2 moles of ADP and one mole of ATP per mole F_1 in untreated enzyme. It was their conclusion that of the 5 binding sites in F_1, three sites exhibited very high affinity for nucleotides and did not exchange readily while two other sites with less affinity engaged in reversible binding. In a recent publication by Cross and Nalin (104), the number of nucleotide binding sites per F_1 were revised to 6 sites. They incubated the enzyme which has been depleted of all endogenous nucleotides with $[^{14}C]$-ADP. The mixture was centrifuged through Sephadex columns to remove unbound nucleotides than chased with cold ATP. Following this treatment 3 moles/mole F_1 of $[^{14}C]$-labeled nucleotides remained bound to the enzyme. Subsequent incubation with $[^3H]$-AMPPNP resulted in the binding of 3 additional moles of the analogue/mole F_1 without displacement of any of the $[^{14}C]$-labeled nucleotides. Based on these results, binding sites for $[^{14}C]$-nucleotides were characterized as non-exchangeable sites and [H]-nucleotides incorporated into the exchangeable sites. Similar studies have not been performed with chloroplast CF_1. As a result, the number of characteristics of the binding sites in CF_1 remains more obscure.

2. Bound phosphate

Beef heart F_1 was shown to contain a binding site for inorganic phosphate with a dissociation constant of 80 uM at pH 7.5 (105). Kasahara and Penefsky (106) have also found that ADP, ATP or AMPPNP inhibited Pi binding suggesting that the Pi site was near a nucleotide binding site. In the presence of Mn^{++}, SO_4^{--}, and aurovertin, compounds which enhance the binding of phosphate, an additional phosphate binding site was detected. pH Optimum for phosphate binding is near 6.0. The binding constant at that pH is 27 uM as compared to 285 uM at pH 8.1 (105, 106). Similar pH optimum was found for phosphate binding to CF_1 (107). Hammes *et al* (108) found that incubation of CF_1 and labeled [^{32}P]-ATP in the presence of Mg^{++} results in rapid incorporation of 1 mole of ATP/mole CF_1. This is followed by the rapid hydrolysis of ATP to bound ADP and Pi. The dissociation constant for this Pi was very slow, $t_{\frac{1}{2}}$ = 24 h. Activation of the Ca^{++} ATPase resulted in more rapid release of Pi. The low optimum pH for Pi binding suggests that monovalent form of Pi is the binding species (106). It may also be the ionic form which is required for phosphorylation.

3. Binding of nucleotides to coupling factor subunits

To date, only the α and β subunits of coupling factors have been shown to contain nucleotide binding sites. The initial attempts to localize the ATP binding site in mitochondrial ATPase followed the discovery that 4-chloro-2-nitrobenzofurazon (Nbf-cl) inactivated mitochondrial ATPase (109). Using [^{3}H]-Nbf-cl, Racker (110) showed that the inhibitor was associated with the β subunit and the ATPase site. Hammes and Cantley (101) found that heat activation of CF_1 ATPase resulted in the exposure of an additional binding site for AMPPNP. They also found that reaction of heat activated enzyme with Nbf-cl also prevented the binding of this analoque, thus indicating that this third site is on the β subunit. The binding sites were later explored in more detail using various alkylating and photo-affinity analoques of the adenine nucleotides. For example, the photo-affinity labeled analoque of ATP, 8-azido-ATP, is slowly hydrolyzed by F_1. Exposure of the F_1-8-azido-ATP complex to light results in covalent attachment of the analoque to the enzyme. When the analoque was reacted with F_1 in the absence of Mg^{++}, SDS-polyacrylamide gel electrophoresis showed that the label was found exclusively on the β subunits, but when Mg^{++} was included, both α and β subunits were labeled (111,116). Using 8-azido-ADP in a similar experiment, the label was associated with the α subunit when Mg^{++} was included; but in the absence of Mg^{++}, both the α and β subunits were labeled (112). These labeling patterns appear to suggest that ADP may be more selective for the α subunits and ATP for the β subunits. Although either nucleotide can bind to the α or β subunits (119). The ATPase activity was completely inhibited when 2 moles of either the ATP or ADP analoque was incorporated per mole of F_1. After repeated labeling of F_1 with 8-azido-ADP, 4 moles of label were recovered per mole of F_1, two on the α and two on the β subunits. Two additional unlabeled nucleotides were also discovered in the same preparation using the Luciferin-luciferase method for the detection of ATP, suggesting 6 moles of bound nucleotides per mole of F_1 (111). Other analoques of ATP, NbS^6ITP (113) and the mixed anhydride of $[^3H]$-ATP and mesitylenecarboxylic acid (115) have potent inhibitory effects on F_1-ATPase and are found to be exclusively associated with the β subunits. Stripping F_1 of its endogenous nucleotides or using energy transducing F_1 on submitochondrial particles with the capacity to exchange all their nucleotides results in the incorporation of the ATP analogues, FSBA or arylazido-β-alanyl-ATP, into both α and β subunits with concomitant inhibition of ATP$\rightleftharpoons$Pi exchange (114,117).

In chloroplast coupling factor, the latent enzyme reacts with the photoaffinity label, arylazido-ATP, stoichiometrically mole for mole. This label is found on the β subunit. However when arylazido-ADP was used, the label was incorporated equally into the α and β subunits (118) suggesting ADP binding sites on both the α and β subunits. Wagenvoord et al (116) have reported the incorporation of 4 moles of 8-azido-ADP per mole of CF$_1$ after repeated labelings in the presence of Ca^{++}. Such results, although not a direct proof, is consistent with CF$_1$ subunit stoichiometry of $\alpha_2\beta_2$; and the finding of 6 binding sites on F$_1$ suggests a $\alpha_3\beta_3$ stoichiometry for the mitochondrial enzyme. A single binding site for ADP or ATP per α or β subunit was shown in the BF$_1$ subunits of E. coli (84) and the thermophilic bacterium PS3 (67).

4. Cooperative interaction between catalytic subunits

The fact that coupling factors contain two or three pairs of α and β subunits indicates that the enzyme also contains a certain amount of symmetry. X-Ray diffraction studies of BF$_1$ crystals from the thermophilic bacterium PS3 show that such symmetry does in fact exist (122). Since catalytic activity is commonly associated with the larger subunits there should be as many catalytic sites as α and/or β subunits. Experimental evidence however have shown this is not the case. For instance, interaction of NBD.Cl with a single β subunit, one mole NBD.Cl/mole F$_1$ or CF$_1$, results in complete inhibition of ATPase activity (109). Similar results are obtained when one mole of DCCD interacts with one mole of E. coli BF_1 (123) and one mole of efrapepin per mole of mitochondrial F_1 inhibits both ATP hydrolysis and ATP synthesis (124). These observations in which a reaction between an inhibitor and a single site on the enzyme induces inactivation of 2 or 3 potentially identical sites are taken as indications of interaction between sites (whatever happens on one site appears to effect all sites).

Further indications of asymmetry between catalytic sites comes from the kinetics of nucleotide interaction with the binding sites. Almost as a rule, the multiple binding sites on the coupling factor have greatly varied affinities for a given nucleotide (125,126). In CF_1 two unequal sites for ADP have been found in addition to a third site with its own unique properties (101,103). Such heterogeneity in binding sites has been attributed to cooperative subunit interaction. Catalytic site cooperativity is also indicated from oxygen exchange experiments; for example, the rate of loss of labeled oxygen from $[^{18}O]$-γ-phosphate of ATP to water during ATP hydrolysis is more rapid at low substrate (ATP) concentrations than at high ATP concentrations. This is explained by ATP binding on one site promoting product release from a second site, and thereby reducing the amount of exchange between $[^{18}O]$ from γ-Pi of ATP with water (145). Other evidence for site cooperativity comes from experiments which show that ADP + Pi binding on one site promotes the release of ATP from a second site or that binding of ATP promotes the release of ADP + Pi during hydrolysis (140,143). The addition of adenine nucleotides also increases the rate of release of bound Pi (125) and the rate of AMPPNP binding and release from the high affinity sites is influenced by binding of nucleotides on the other sites (62). Using the ATP analogue TNP-ATP, Grubmeyer and Penefsky (126) demonstrated that the rate of hydrolysis of [^{32}P)-TNP-ATP on one site was increased 15-20 fold by binding of TNP-ATP on a second site (127). In the same line, incorporation of [γ^{32}P]-ATP into one hydrolytic site of F_1 at excess enzyme concentrations resulted in slow [γ^{32}P]-ATP hydrolysis. This rate increased by 10^6 when excess cold ATP was permitted to bind to a second unoccupied site (128,129).

Kagawa et al (67) took a different approach to the study of subunit cooperativity. Using hydrogen exchange kinetics between water and peptide groups of purified α and β subunits of the thermophilic bacterium PS3, they found that when the α and β subunits were brought into contact, "tightening" of the α but not of the β subunit occurred. ATP binding to the β subunit results in tightening of that subunit. This tightening was enhanced by interaction of this β subunit with either an α subunit or another β subunit. Tightening of subunit conformation was determined from decreased proton exchange activity. The role of catalytic site cooperativity in ATP synthesis will be discussed further on.

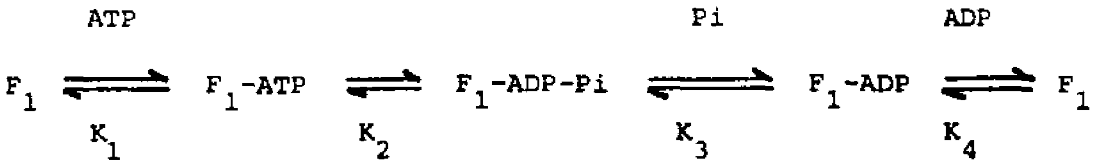
Catalytic activities

1. ATPase

The ATPase activity is one of the more useful markers of the coupling factor. This capacity to hydrolyze ATP is expressed in the membrane bound energy transducing enzyme as well as in the isolated enzyme. In the later, the ATPase is cold labile. ATP hydrolysis appears to be regulated/inhibited by a small peptide, and for complete expression of the ATPase activity, it is essential that this protein be removed. An inhibitor protein was initially identified by Pullman and Monroy (120) in mitochondria where it appears to be distinct from the other five subunits of F_1. F_1 is the only enzyme which loses the inhibitory protein upon warming of the enzyme at alkaline pH (120). Activation of chloroplast and microbial ATPases require somewhat more drastic treatment such as high concentrations of sulfhydryl reagents (130), incubation at elevated temperatures (131) or proteolytic digestion with trypsin (52). None of these procedures, however, result in the ATPase activity comparable to the mitochondrial enzyme. Trypsin, the most effective activator of Ca^{++}-CF_1-ATPase, yields approximately 35 umoles ATP hydrolyzed per mg per min (133) as compared to nearly 100 umoles/mg/min for F_1 (132). In CF_1, the ATPase inhibitor protein is most likely the ϵ subunit. This is indicated by observations which show that heat activation of CF_1-ATPase follows the loss of the δ and ϵ subunits (89). However, of the two subunits only the ϵ subunit contains sulfhydryl groups, hence limiting sulfhydryl activation of ATPase to this subunit. Chloroplasts usually retain a very low level of Mg^{++} dependent ATPase activity in the dark ($\sim$1 umole ATP hydrolyzed/mg chlorophyll/h). Exposure of chloroplasts to light or other forms of excitation energy, results in increased ATPase activity, approximately 10 umoles/mg/h. The pH optimum of CF_1 Ca^{++} and Mg^{++} ATPases and F_1 ATPases are 8.0-8.5 (52).

ATPases from most sources have the capacity to hydrolyze other purine nucleotides, UTP as well as a large number of analoques of ATP. The capacity to hydrolyze GTP, ITP or N^6-ethanoadenosine 5-triphosphate suggests that the amino group on the 6 position of the purine moiety is not critical for catalytic activity. However modification of N-1 results in enzyme inhibition. The ribose ring of ATP can also withstand considerable modification and still remain a substrate for F_1 ATPase (62,134).

The kinetics of ATP hydrolysis was recently demonstrated in some detail by Grubmeyer, Cross and Penefsky (128). This is briefly summarized in the following scheme:



$$F_1 \underset{K_1}{\overset{\text{ATP}}{\rightleftharpoons}} F_1\text{-ATP} \underset{K_2}{\rightleftharpoons} F_1\text{-ADP-Pi} \underset{K_3}{\overset{\text{Pi}}{\rightleftharpoons}} F_1\text{-ADP} \underset{K_4}{\overset{\text{ADP}}{\rightleftharpoons}} F_1$$

The equilibrium constant for each of the steps are : $K_1 = 10^{12}\ M^{-1}$, $K_2 = 0.5$, $K_3 = 6 \times 10^{-4}$ M, and $K_4 = 3 \times 10^{-7}$ M. The above describes the steps of ATP hydrolysis on a single site of F_1 since the forward and reverse rate constants are based on measurements of excess enzyme to substrate concentration, which permits no more than one site occupancy per F_1. Two important conclusions were drawn from these results: $k_2 = 0.5$ suggests the synthesis of ATP from ADP + Pi at the catalytic site can occur with no expenditure of free energy; the high affinity of ATP for the catalytic site suggest that expenditure of energy is required to dislodge ATP from the catalytic site, such energy can be supplied from the chemiosmotic gradient and/or from substrate binding on other sites (96,138). These observations as well as oxygen exchange results constitute important evidence that the ATPase site may also be the site of ATP synthesis.

2. Exchange reactions

Nucleotide exchange properties with coupling factors were previously discussed. The following are other forms of exchange reactions that are catalyzed by the coupling factor.

Cohn (135) in 1953, first reported evidence for a mitochondrial catalyzed reaction in which $[^{18}O]$ labeled oxygen from inorganic phosphate rapidly exchanged with water oxygen. The exchange was uncoupler sensitive.

Subsequent studies by others showed that mitochondria also catalyze ATP $\rightleftharpoons$ Pi (136), ATP $\rightleftharpoons$ H_2O and ATP $\rightleftharpoons$ ADP exchange. These reactions appear to be associated with partial reactions of reversible formation of ATP by way of the reaction ADP + Pi $\rightleftharpoons$ ATP + H_2O (137). Two distinct exchange reactions have been identified in the energy transducing coupling factor: (1) medium exchange, involves the exchange of reactants at the catalytic site with the same reactants in the medium; for instance, ATP $\rightleftharpoons$ Pi exchange requires the hydrolysis of γ-phosphate of ATP by the ATPase, exchange of phosphate at the catalytic site with labeled phosphate in the medium followed by reincorporation of the labeled phosphate into bound ADP. Medium exchange is uncoupler sensitive and is inhibited by energy transfer inhibitors such as oligomycin (or DCCD in chloroplasts); and (2) intermediate exchange, which involves rapid interconversion of ADP, Pi and ATP at the catalytic site; for example, hydrolysis of [^{18}O]-γ-phosphate from ATP results in rapid displacement of phosphate [^{18}O] with water oxygens, Pi $\rightleftharpoons$ H_2O exchange. The high rate of this exchange in comparison to ATP $\rightleftharpoons$ Pi exchange and its insensitivity to uncouplers indicate that it is a separate component from medium exchange (138). This residual Pi $\rightleftharpoons$ H_2O exchange in submitochondrial heart vesicles was resistant to high concentrations of As, a competitive inhibitor of Pi, suggesting that medium Pi was not involved in the exchange and that the exchange occurred with enzyme bound intermediates (139,140). The observed responses of medium and intermediate exchange to uncoupler has led to the formulation of the conformational coupling hypothesis of ATP synthesis (discussed under Mechanisms of ATP synthesis).

Intermediate Pi $\rightleftharpoons$ H_2O exchange was also demonstrated in chloroplasts by Wimmer and Rose (141). This was observed under conditions of ATP synthesis and light dependent ATP hydrolysis. In addition, they have also shown that $\beta\gamma$ [^{18}O]-oxygen bridge to β non-bridge [^{18}O] scrambling during ATP hydrolysis occurs at rates similar to intermediate Pi$\rightleftharpoons$$H_2O$ exchange, supporting reversible hydrolysis mechanism of ADP, Pi and ATP at the catalytic site.

Using submitochondrial particles, Rosing et al (140) demonstrated that under conditions of net ATP hydrolysis in the presence of an ADP trapping system, pyruvate kinase plus PEP, the medium ATP$\rightleftharpoons$$H_2O$ exchange, ATP$\rightleftharpoons$Pi exchange, and medium Pi$\rightleftharpoons$$H_2O$ exchange were inhibited. Intermediate Pi$\rightleftharpoons$$H_2O$ exchange remained largely unaffected by this treatment. These results were interpreted to indicate that ATP release on one site was coupled to the binding of ADP + Pi on a second site. In somewhat similar experiments, it was demonstrated that medium exchange, catalyzed by submitochondrial particles under phosphorylation conditions, was nearly completely inhibited when hexokinase and glucose were added to the reaction solution. Two conclusions that came from these experiments were: that energy derived from ATP hydrolysis can drive medium exchange and that for the release of ADP and Pi on one site, ATP has to bind to a second site (142,143).

Substrate concentrations have an important effect on intermediate exchange. During ATP synthesis, for example, at saturating ADP and Pi concentrations, mitochondrial particles exhibit low intermediate exchange, but when the concentration of either ADP or Pi were limited to the concentrations in the Km level an increase in intermediate exchange was observed (143). Russo et al (144) have demonstrated, under conditions of hydrolysis with an ATP regenerating system, that as the concentration of ATP is reduced, the rate of intermediate exchange may increase 40-200%. Choate et al (145) extended these observations to purified soluble F_1; they found that at high ATP concentrations, F_1 catalyzed little or no intermediate $Pi \rightleftharpoons H_2O$ exchange; but at low ATP concentrations, $<$10 uM, there was a marked increase in exchange. These results are again interpreted in terms of site cooperativity. At low ATP concentrations, ADP and Pi formed at the catalytic site continue $Pi \rightleftharpoons H_2O$ exchange. At higher concentrations, ATP binding to a second site accelerates the release of ADP + Pi, hence lowering the rate of intermediate $Pi \rightleftharpoons H_2O$ exchange.

3. Conformational change

Incubation of chloroplasts in [^{3}H]-water results in energy dependent incorporation of [^{3}H] into CF_1. This is observed when chloroplasts were illuminated or when a pH gradient was artificially imposed across the thylakoid membranes. The reaction was uncoupler sensitive and the amount of [^{3}H] incorporated was inhibited by the presence of nucleotides. These experiments by Ryrie and Jagendorf (146) were taken to indicate that upon energization of the thylakoid membranes, CF_1 undergoes a conformational change exposing hidden amino acid residues to hydrogen exchange.

Illumination of thylakoids in the presence of N-ethylmaleimide (NEM) leads to incorporation of the alkylating reagent into the γ subunit of CF_1 with concomittant inhibition of photophosphorylation (147). The dependence of this incorporation upon energy again indicates a conformational change in the γ subunit and exposure of the group susceptible to alkylation. McCarthy and Fagan (147) further observed that nucleotides inhibited the incorporation of NEM into the γ subunit, suggesting that the energy induced conformational changes in that subunit exposing the nucleotide binding site(s).

Membrane bound coupling factors labeled with fluorescent probes such as aurovertin show a fluorescence shift when the membranes are energized. Fluorescent shifts are interpreted in terms of energy induced conformational changes in the coupling factor. An important observation in these experiments is the rapid rate of the fluorescent shift in response to an energized state. This rate was in the range which suggested the possible involvement of the conformational change in catalytic activity (62). The intensity of fluorescence and changes in polorization fluorescence were also observed in aurovertin-F_1 complex in response to ADP, ATP, Mg^{++} or Pi additions (148,149).

Nucleotides bound to soluble CF_1 or membrane associated CF_1 exchange slowly in the dark with soluble nucleotides (90,150). In the light, however, exchange of nucleotides on thylakoid bound CF_1 is rapid and uncoupler sensitive (96). This energy dependent exchange is explained by an enzyme conformational change, which either exposes bound nucleotide or changes their binding kinetics, so that they are more easily exchanged with medium nucleotides. Other catalytic activities of thylakoid bound CF_1 have also been shown to be dependent on energy induced structural changes; for example, membrane bound CF_1 or soluble CF_1 ATPase activity may be activated and greatly accelerated either by illumination of thylakoids (151) or by removal of the ϵ subunit from the soluble enzyme (89), suggesting that activation of ATPase activity is coupled to an energy dependent change in the ϵ subunit in the native state. Other changes in subunit conformation are also observed in response to nucleotide binding. For example, binding of ATP to the α subunit of E. coli BF_1 results in a significant increase in the electrophoretic mobility and a decrease in the frictional coefficient of the bound subunit (66). Similarly, binding of ATP to the β subunit of the thermophilic bacterium PS3 BF_1 or addition of β to α subunits results in decreased hydrogen exchange between water and the amino acid residue on the β subunit (67).

4. ATP formation (mechanisms)

Although a considerable amount of information has been accumulating over the years on the structure and activities of the coupling factor, a clear mechanism of the steps that lead to ATP formation remains unknown. Because of the complexity of the subject and the many contradictory interpretation of experimental results, the following part of the introduction is limited to a few commonly described alternative models of ATP synthesis (rev. 57,62,87,152).

ATP synthesis mediated by a high energy covalent intermediate of ADP or phosphate is generally ruled out, primarily because no such high energy intermediate has ever been identified. In addition, a significant amount of experimental evidence is not consistent with a high energy intermediate. For example, observations by Boyer (153) that ADP and not Pi provides the bridge oxygen between β and γ phosphates of ATP; the absolute dependence of Pi $\rightleftharpoons$ H_2O exchange on ADP (154); the observations by Webb et al (155) that hydrolysis of γ-($^{16}O^{17}O^{18}O$)-thio-phosphate-ATP by F_1 goes through inversion of configuaration; initial appearance of (^{32}P) in the γ position of ATP during photophosphorylation (156,157), in addition to recent evidence that ADP bound to soluble CF_1 can be phosphorylated by medium Pi (107), plus the deeper understanding of the events of ATP hydrolysis and ATP synthesis are all inconsistent with a high energy intermediate in ATP synthesis.

The chemiosmotic hypothesis of Mitchel proposes that ATP synthesis is driven by the translocation of protons through the F_0-F_1 complex (43), 159). A close link between the proton gradient and phosphorylation is indicated by the observations that ATP synthesis results in a decrease in the transthylakoid ΔpH (160) and ATP hydrolysis regenerates a partial ΔpH and can induce reverse electron flow (161). According to Mitchel, two protons are pumped to the catalytic center of CF_1 through the transmembrane proton channel, CF_o, combine with phosphate oxygen at the active site, converting it to the cation form. ADP, also at the active site and protected from the protons, is in the anion form. ADP reacts with the phosphate by nucleophilic substitution reaction with displacement of water from the phosphate (162). This mechanism is supported by the observed low pH optimum for Pi binding to the coupling factor F_1 (106) and the low pH optimum for the phosphorylation of CF_1 bound ADP by medium Pi (107).

The possibility that the proton gradient is not involved in the condensation of ADP + Pi to ATP was initially raised by Boyer (163) in 1965. He proposed instead that energy in the form of the electrochemical gradient is conserved in a high energy protein conformational changes, with possible coupling of this conformational change to high energy intermediate, such as an acyl-S-compound. This model proposed that energy stored in the form of a conformational change occurred prior to ATP synthesis. Synthesis of ATP was at the expense of the high energy conformational state (164). In subsequent years the conformational coupling hypothesis took on a different shape; this followed observations of rapid, uncoupler insensitive $Pi \rightleftharpoons H_2O$ exchange during ATP hydrolysis and ATP synthesis by submitochondrial particles. According to Boyer, this could be explained if the reaction steps involved in ATP synthesis are readily reversible requiring no energy input. Further observations that $ATP \rightleftharpoons Pi$ exchange and medium $ATP \rightleftharpoons H_2O$

exchange were sensitive to the uncoupler 2,4 DNP led Boyer to propose that energy was required for substrate binding or product release (138, 165). This model for phosphorylation gained important support from the findings of Harris and Slater (96) who showed that mitochondrial and chloroplast coupling factor contained tightly bound ADP and ATP. From the ratio of bound ATP to bound ADP, they estimated that the free energy change associated with ATP synthesis is approximately zero; and the proposed requirement for energy in the substrate binding and product release steps only was also supported by the observation that exchange of these bound nucleotides with medium nucleotides is an energy dependent process and is uncoupler sensitive (96). Recently, the finding by Grubmeyer et al (128) that ATP at the hydrolytic site of F_1 dissociates to ADP + Pi with an equilibrium constant of 0.5 toward ATP formation was taken as further evidence that no energy is expended during ATP synthesis. Observations that the dissociation or association constants for nucleotide binding to some tight binding sites are very low, in the order of 0.1 uM (84,128) have also been incorporated into the conformational coupling hypothesis of ATP synthesis by proposing that binding energy may contribute towards ATP formation (104) and that energy input in the form of the chemiosmotic gradient is associated with conversion of high affinity site(s) to lower affinity site (s); so as to permit exchange of product ATP with new substrate ADP and Pi.

Another important feature of the conformational model is that it explains ATP synthesis in terms of cooperative interaction between catalytic centers. In this model, known as the alternating site model, ATP release from one catalytic site is coupled to binding of ADP + Pi on a second site or release of ADP + Pi during ATP hydrolysis is coupled to binding of ATP on a second site (140,143,145).

In the transphosphorylation model of ATP synthesis, AMP acts as the primary phosphate acceptor. AMP plus phosphate are condensed by the coupling factor in an energy dependent step to form bound ADP. The β phosphate of this ADP is then transfered to substrate ADP bound to a second site on the coupling factor, in an energy independent step, to generate ATP and regenerate AMP. (Experiments which led to the formulation of this model are discussed under Bound nucleotides.) As predicted by this model, ATP synthesis in the presence of ADP and [^{32}P] results in the initial formation of β labeled ADP. This part of the mechanism is the most controversial since findings which show such initial β labeling can also be attributed to the adenylate kinase (AK) reaction 2ADP $\rightleftharpoons$ ATP + AMP. Such involvement by AK seems to be supported by observations of initial γ labeling in experiments where AK was suppressed (166) or where acid-base transition was used to drive ATP synthesis (167). However, observations of rapid conversion by excited thylakoids of [^{3}H]-AMP plus Pi into bound [^{3}H]-ADP in the presence of ADP (168) and rapid exchange of AMP plus Pi with CF$_1$ bound ADP (169) suggest the possibility that AMP participates in CF$_1$ catalyzed reactions.

In summary, a substantial amount of evidence suggests that ATP formation is coupled to an energy dependent conformational change, which according to Boyer, is associated predominantly with binding and release of reactants. Enzyme conformational change may also have an important regulatory function (170). Whether energy is required for the actual esterification of inorganic phosphate and ADP to ATP is more controversial. According to the conformational hypothesis, no energy is required for this step. However, the low optimum pH for phosphate binding (106) and the low optimum pH for the slow rate of bound ATP synthesis by soluble CF_1 (107) suggests that localized proton pools at the catalytic site may infact be required for synthesis of ATP in line with Mitchel's predictions. Further, observations that little or no bound ATP is synthesized by soluble coupling factor in the presence of ADP + Pi at pH which is optimum for ATPase activity (pH 8.0-8.5) (50,52,107) contradicts proposals that ATP synthesis and hydrolysis are freely reversible reactions which are independent of PMF. Even the uncoupler insensitive intermediate $Pi \rightleftharpoons H_2O$ exchange in mitochondrial particles, usually taken as strong evidence against energy requirement for ATP formation, may also be explained in terms of Mitchel's hypothesis as follows: ATP hydrolysis at the catalytic site generates a proton that is confined in the catalytic site. Such a proton may then be used reversibly to regenerate ATP. This localized reversible reaction may be protected from uncouplers. If one assumes, however, that the translocation of protons is coupled to an enzyme conformational change within the model of catalytic site cooperativity, ATP hydrolysis on one site would induce a conformational change on a second site which is coupled to the release of hydrolysis products and causes the translocation of a proton from that second site into vesicles to regenerate an uncoupler sensitive Δ H. According to this model, at high ATP concentrations, hydrolysis is very fast which results in rapid conformational changes and proton translocation. The protons at the catalytic site are therefore short lived which explains the low rate of intermediate $Pi \rightleftharpoons H_2O$ exchange (145) and the large ΔH formation in chloroplasts which is observed at high ATP concentrations. At low ATP concentrations, slow ATP hydrolysis results in longer lives protons at the catalytic site. This permits more reversals of the reaction H_2O + ATP $\rightleftharpoons$ ADP + Pi hence the larger Pi $\rightleftharpoons$ H_2O exchange. Based on observations such as have been described above, it may be concluded that the actual mechanism of ATP synthesis may incorporate different features from different models.

OBJECTIVES

The objective of this study is to characterize CF_1 bound nucleotides in more detail in an energy transducing system.

SUMMARY

Labeled adenine nucleotides were incorporated into the binding sites of spinach chloroplasts. The chloroplasts were subjected to gel filtration, a process which removed free and "loosely" bound nucleotides. The remaining nucleotides appeared to reside on two distinct sites. On the first site (exchangeable site), the nucleotides slowly exchange with free nucleotides. They were also released in the light in the absence of added nucleotides. On the second site (non-exchangeable site), both light and free nucleotides were required to induce exchange on nucleotides from this site. Bound [^{3}H]-ADP on the non-exchangeable site was slowly phosphorylated to bound [^{3}H]-ATP in the light in the absence of added free nucleotides. Uncoupler sensitive phosphorylation of bound [^{3}H]-ADP to bound [^{3}H]-ATP was also observed in the dark in the presence of added ATP.

CONVERSION OF TIGHTLY BOUND ADP TO BOUND ATP
ON CHLOROPLAST MEMBRANES AND
PARTIAL CHARACTERIZATION OF SPINACH CHLOROPLASTS
BINDING SITES

INTRODUCTION

The role of the bound nucleotides on coupling factors from various sources has been studied in great detail (rev. 62,87,152). In general, their function has been catagorized in either a regulatory role (153,177, 188) or as catalytic intermediates (107,173,175). The number of nucleotide binding sites that has been found on CF_1 is either three (101,186) or four (116). Only one nucleotide remains CF_1 bound after extensive Sephadex chromatography. To date only the α and β subunits have been shown to have nucleotide binding sites (87); one binding site per subunit (84,119). A number of studies indicate that the active site, as determined by ATPase activities and exchange kinetics, are found on the β subunits (101,110, 174). The role of bound nucleotides on the α subunits have been more obscure. Antibodies either to the α or β subunits inhibit phosphorylation (68). Alkylating ATP analogues such FSBA (114) or alylazido alanyl-ATP (117) which are specific for the α and β subunits are potent inhibitors of ATP $\rightleftharpoons$ Pi exchange. Reconstitution of bacterial coupling factor from its subunits shows that both α and β subunits were essential for restoration of phosphorylation capacity (82). As a result, the role of bound nucleotides on the α subunits in phosphorylation cannot be excluded.

The demonstration of direct involvement of bound nucleotides in phosphorylation has been slowly evolving. Magnuson and McCarthy (178) showed that tightly bound ADP on chloroplasts was partially phosphorylated to ATP in a light dependent reaction. In other reports (178), ADP was found tightly bound to chloroplast coupling factor after light dependent hydrolysis of ATP. From this it was inferred that ADP was at the catalytic site. In rapid kinetic experiments by Rosen and Boyer (121), a short lived chloroplast bound ATP was observed during active phosphorylation.

The most direct demonstration of bound ADP participation in ATP synthesis was recently shown in spinach chloroplasts CF_1 by Feldman and Sigman (107). ADP exchanged into one of CF_1 binding sites was phosphorylated to form bound ATP when the CF_1-ADP complex was incubated with high concentrations of inorganic phosphate at pH 6.0.

Also, critical to the studies of the function of the bound nucleotides have been the exchange properties of medium nucleotides with bound nucleotides. Medium ADP, ATP and their analogues readily equilibrate with exchangeable nucleotides on the enzyme. A number of inhibitors which prevent exchange also block catalytic activity. For example, phenylglyoxal inactivates beef heart mitochondrial F_1 ATPase; and it also causes the loss of one exchangeable nucleotide binding site (104). The antibiotic efrapepin competes with ADP and Pi binding and it also inhibits ATPase activity and oxidative phosphorylation (124,176). In intact chloroplast thylakoids a number of inhibitors and uncouplers have been identified which have a potent inhibitory effect on exchange of nucleotides with CF_1 binding sites and photophosphorylation (177-179).

We have further investigated this apparent linkage between exchange and phosphorylation using spinach chloroplasts. A system was used in which specific binding sites on chloroplasts were vacated of their nucleotides: such a system with fewer bound nucleotides has permitted the greater characterization of the remaining bound nucleotides. Their exchange properties are discussed and conditions are shown whereby bound ADP is phosphorylated to generate bound ATP.

METHODS

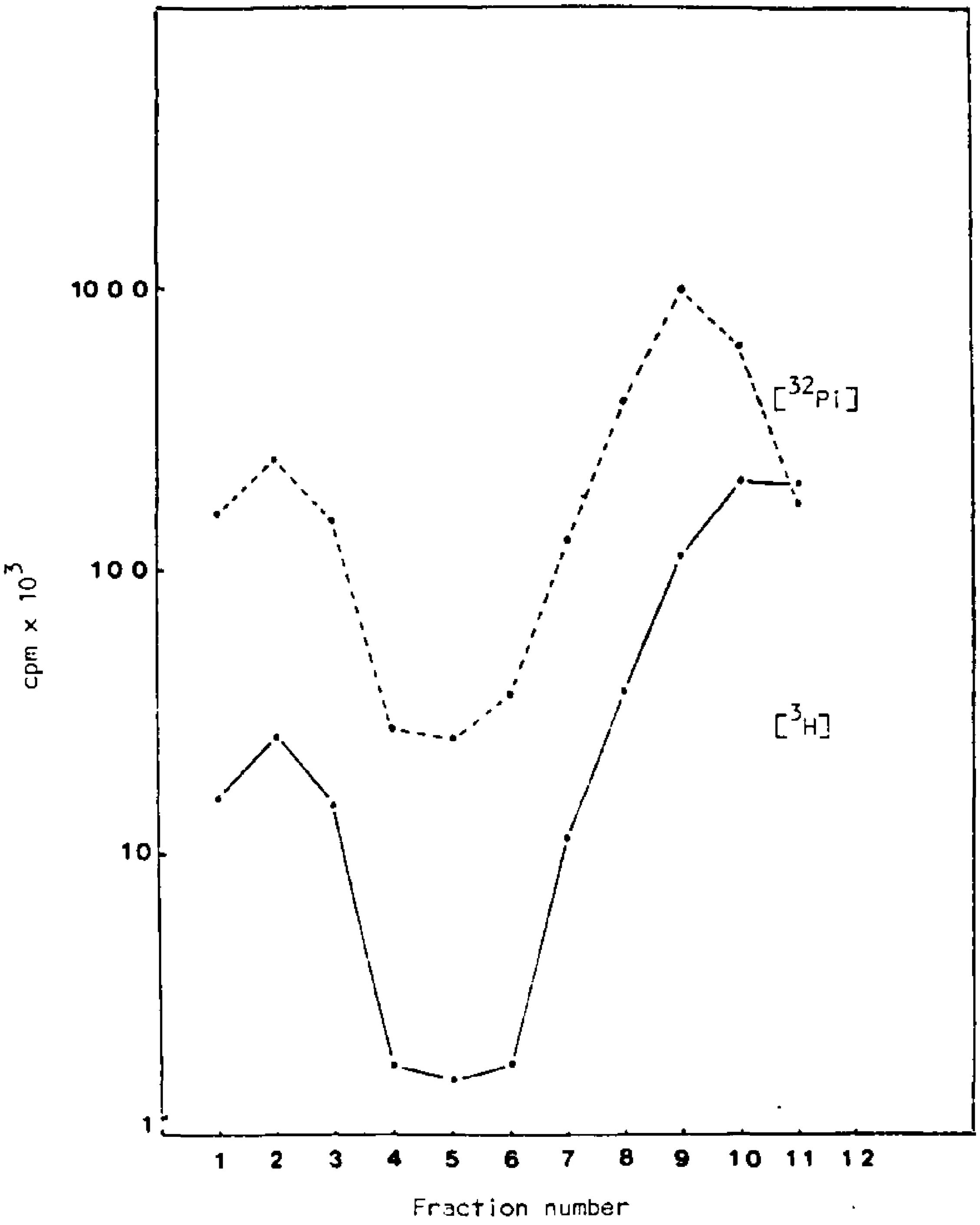
Labeling of chloroplast bound nucleotides: Chloroplasts were isolated from 60 g deveined spinach leaves as described by Avron (180) and chlorophyl content was estimated according to Arnon (181). After the chloroplast suspension was pelleted at 10,000 g for 5 min, the resulting pellet was suspended in one to two milliliters of resuspension medium which contained 5 mM Hepes buffer, pH 7.6, 0.2 M sucrose, 10 mM NaCl, 2 mM $MgCl_2$, and 0.4% defatted bovine serum albumin. The resulting suspension, 7-10 mg chlorophyll/ml, was supplemented with 1 u mole of $[^3H]$-AMP (50 uCi), 0.25 u moles phenazine methasulfate (PMS) and 2 u moles inorganic phosphate. The final volume was approximately 1.5 ml. The mixture was gently homogenized in a tissue homogenizer, warmed to 15° C in a water bath and exposed to light for 2 min. During the illumination period the chloroplast suspension was gently homogenized to obtain uniform exposure of all chloroplasts to light. A minimum of 10 cm of water between the sample and the light source was used as an infrared filter. At the end of the light exposure, the chloroplasts were immediately cooled to 0° C in an ice slurry.

Separation of thylakoid bound nucleotides from free nucleotides: Exposure of chloroplasts to light in the presence of $[^3H]$-AMP, Pi, and an electron acceptor results in a rapid incorporation of the label into the tight nucleotide binding site of CF_1 (91). The thylakoid bound nucleotides were separated from free nucleotides by passage of the chloroplast suspension through Sephadex G-50 packed in a column (40-120u particle size; column 1.5 cm x 50 cm). Prior to use, the Sephadex G-50 was equilibrated with resuspension medium containing 5 mM Hepes buffer, pH 7.6, 0.2 M sucrose, 10 mM NaCl, 2 mM $MgCl_2$, and 0.3% defatted bovine serum albumin. Approximately five void volumes of this medium were passed through the column prior to chloroplast application The flow rate was adjusted to 1 ml/min with a peristaltic pump. The chloroplasts were eluted with resuspension medium at 0-5° C and in the dark. The only light source was a 15 watt fluorescent lamp with a green filter. Chloroplasts eluted in the void volume. A typical separation profile of bound from free nucleotides is shown in Fig 1. The first two fractions were pooled. The combined volume was approximately 5 ml which was then used in the described experiments.

Separation of labeled chloroplasts from labeling medium was also performed by repeated washings of chloroplasts. Chloroplasts were labeled as described above. The labeled mixture was initially diluted to 50 ml with 50 mM Tris buffer, pH 7.6, containing 0.4 M sucrose, 10 mM NaCl, 0.03% bovine serum albumin, and 4 mM $MgCl_2$, and then centrifuged for 5 min at 10,000 g at 0-5° C. After removal of the supernatant, the pellet was rinsed once with 2.5 ml of the dilution buffer and resuspended in 3 ml of the same buffer with a tissue homogenizer. The mixture was then diluted to 30 ml and centrifuged as before. The washing procedure was repeated 3-4 times until similar counts were obtained in the supernatants of two consecutive washings. Bound nucleotides versus free nucleotides in the last wash medium was usually 4:1 respectively. Further washings of the chloroplasts were avoided to minimize possible damage to membranes and the loss of chloroplast bound nucleotides. The pellet was finally resuspended in 4 ml resuspension medium containing 5 mM Hepes buffer, pH 7.8, 10 mM NaCl, 2 mM $MgCl_2$, 0.2 M sucrose, and 0.3% bovine serum albumin.

Fig 1. Passage of labeled chloroplasts through Sephadex G-50. Chloroplast suspension (1.5 ml/11 mg chlorophyll) isolated as described in the Methods was supplemented with [^{3}H]-AMP 25 uCi, AMP 1 umole, Pi 2.0 umoles, [^{32}Pi] (1 x 10^{7} cpm), and PMS 0.25 umoles. The mixture (~2 ml) was exposed to light for 2 min with continuous mixing in a homogenizer. A sample (~1.5 ml) of the reaction mixture was applied to a Sephadex G-50 column (1 cm x 50 cm). The flow rate through the column was adjusted to 1 ml/min. Two milliliter fractions were collected. The first fraction was collected when the green band began to elute. Concentrations of chlorophyll in these fractions were about 0.6 mg/ml. Aliquots of these fractions were counted for [^{3}H] and [^{32}P].

Fig 1.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cpm x 10^3
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5
6
7
8
9
10
11
12
Fraction number
[^{32}Pi]
[^{3}H]

Exchange measurements: Labeled chloroplasts following elution through Sephadex and containing approximately 0.05 mg chlorophyll in 0.1 ml were injected into 0.9 ml reaction medium which contained 15 mM tricine buffer, pH 8.0, 10 mM NaCl, 4 mM $MgCl_2$, 50 uM PMS, and other additions as indicated in the legends of the figures and tables. Reaction times were controlledwith an electronic timer connected in series with the light source. At the end of the illumination period, the reaction tubes (1.5 ml polypropylene microcentrifuge tubes) were immediately centrifuged in an Eppendorf microcentrifuge for 1 min. The supernatant was removed and the resulting pellet was resuspended in 1 ml 9M urea. Both supernatant and pellet were saved for scintilation counting and for labeled nucleotide composition analysis. The supernatant was counted directly. The pellet was counted in either one of two ways: in the first procedure, 0.4 ml of pellet suspension was supplemented with 0.1 ml 10% SDS, and after vigorous mixing, 0.01 ml was counted; a small amount of quenching was encountered using this procedure; in the second method, 0.4 ml of urea denatured chloroplast suspension was supplemented with 0.1 ml 35% perchloric acid and centrifuged. The clear supernatant was counted. All procedures, with the exception of the light reaction, were performed under a green safe light. The reaction time (light or dark exposure) did not include sample handling time. Approximately 15% of the bound label was lost during this period in the presence of medium nucleotides when compared to reactions in the absence of nucleotides. The significance of this will be discussed in the results section. Excluding this small initial loss, there was no additional loss of bound nucleotides even after prolonged incubations.

Dark phosphorylation of CF_1 bound $[^3H]$-ADP: Labeled chloroplasts, prepared by gel filtration as described earlier, were incubated with media of indicated compositions, Table III. The reaction conditions were identical to those used in the exchange studies. At the end of the reaction times, however, the samples ($\sim$1 ml) were poured into 15 ml of medium which has been precooled to approximately -0.5° C in a salt ice slurry. The dilution medium consisted of 30 mM tricine buffer, pH 8.0, 10 mM NaCl, 2 mM $MgCl_2$, and 0.1 M sucrose. The chloroplasts were centrifuged at 0-2° C for 2 min at 10,000 g. The supernatant (0.5 ml) was counted and the pellet was denatured with 1 ml 9M urea. At this concentration, urea is an effective quenching agent. This is indicated from tests which show that preincubation of chloroplasts with 3 M urea resulted in the complete inhibition of photophosphorylation and 75% inhibition of ADP$\rightleftharpoons$ATP exchange. Preincubation with 6M urea completely inhibited photophosphorylation and ADP$\rightleftharpoons$ATP exchange activities in the chloroplasts. Both the supernatant and the pellet were stored at -20° C. Each was later analyzed for their labeled nucleotide composition. Prior to chromatography, carrier AMP, ADP, and ATP, 0.5 umoles of each, were added to the denatured chloroplasts, and 0.5 ml of the mixture was chromatographed on polyethyleneimine cellulose (PEI) columns as follows.

Chromatographic separation of mixed nucleotides: Nucleotide analysis is based on a modified method McCarthy et al (158), using PEI cellulose columns (1 cm x 2 cm). The PEI resin was thoroughly de-fined and degased before use. An initial layer of sand or glass beads, 50-100 u in diameter, was added to the columns to a height of 0.5 cm. The PEI resin suspended in 0.1 M HCl was layered on top of that. The resin was then packed down with a porous polyethylene disc fitted through the top of the column. The column was connected to a peristaltic pump which maintained the flow rate through the column at 0.2 ml/min. Prior to the addition of the sample, 1.0 ml of distilled water was passed through each column. The added sample (approximately 0.5 ml) was permitted to run into the column. This was followed with an additional 0.5 ml of water. Samples which had been denatured in 9 M urea were applied directly to the columns. Urea, even at this high concentration, did not effect the adsorption or elution of the nucleotides. Chlorophyll or other constituents in the denatured chloroplast suspension were not removed since these also did not interfer with the separation of the nucleotides. AMP was eluted with 0.3 M LiCl, ADP with 1 M LiCl, and ATP with 1.5 M LiCl. Two milliliter fractions were collected. The recovery of total nucleotides was nearly 95%. The individual nucleotides were recovered in less than 3 milliliters of effluent.

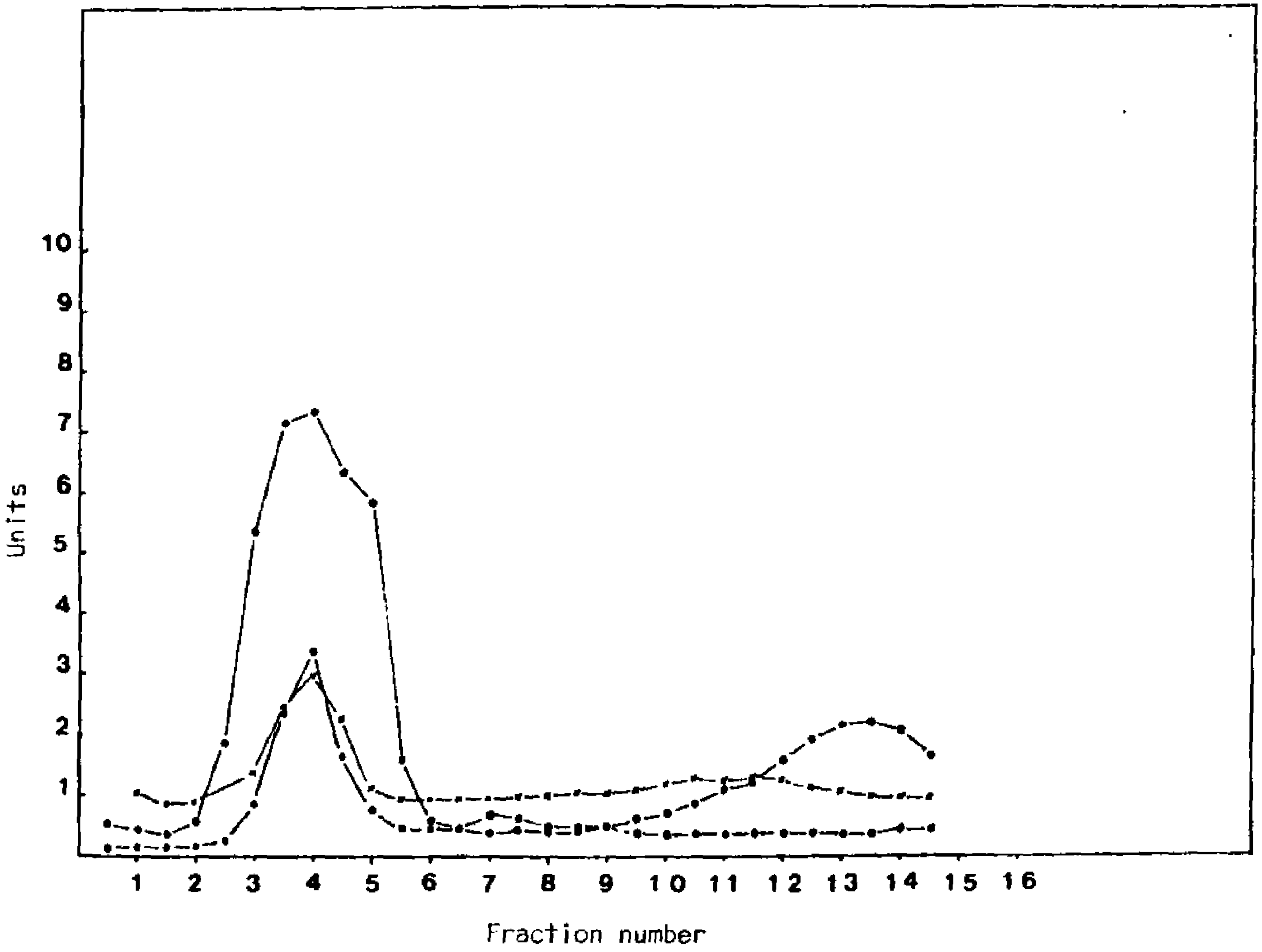
<u>Photophosphorylation</u> includes all assays for [^{32}P] incorporation. This was determined in either of two ways, as indicated. [^{32}P] incorporation into total organic fraction was determined by the triethylamine-ammonium molybdate precipitation method of Shugino and Miyoski (182). This reagent was added either directly to the reaction, using it as a reaction terminating agent or to urea-terminated reactions. The [^{32}Pi] quantitatively precipitated by the reagent was removed by centrifugation. The supernatant containing nucleotides incorporated [^{32}Pi] was supplemented with cold inorganic phosphate, approximately 0.1 umole/ml of Shugino reagent, to remove traces of unprecipitated [^{32}Pi]. The sample was centrifuged again and the precipitate discarded. To the supernatant, excess cold phosphate was then added, 10 umoles, to remove

unreacted molybdate reagent. (It was found that unreacted Shugino reagent may cause increased quenching during scintilation counting, presumably by a reaction with free phosphate formed from the breakdown of phosphorylated products) Following centrifugation, a sample of the clear supernatant was added to a scintilation vial.

[^{32}P] incorporation into ATP was determined by chromatography of reaction products on PEI columns. For these measurements, reactions were terminated with twice the volume of 10 M urea followed by vigorous mixing and rapid cooling in an ice slurry in the dark. With this treatment, it was found that urea is an effective quenching agent. Carrier AMP, ADP, and ATP, 0.1 umoles each, were added to 0.5 ml of sample, which was chromatographed as previously described. To 2 ml of ATP fractions, 1 ml Shugino reagent was added to remove traces of free inorganic phosphate. Following centrifugation, the excess ammonium molybdate reagent was removed as before by adding excess Pi. The precipitate was discarded and the label in the supernatant was measured by scintilation counting.

[^{3}H]-Nucleotide incorporation into CF$_1$. Chloroplasts (15.0 mg chlorophyll/ml) derived from 200 g of deveined spinach leaves were labeled as previously described with [^{3}H]-AMP 0.1 ml (250 uCi/ml), AMP 0.1 ml (10 mM), Pi 0.2 ml (10 mM), PMS 0.05 ml (5 mM), and light in a final volume of 3 millilters. The binding site of [^{3}H]-nucleotides on chloroplasts was determined as follows: labeled chloroplast suspension (3.0 ml) was applied to a Sephadex G-50 column (3 cm x 40 cm). The resin was pre-equilibrated with resuspension medium; the chloroplasts were eluted with the same resuspension medium and elution of chloroplasts was as previously described. The eluted chloroplasts (15 ml) were divided into three parts. To one part, 0.1 ml resuspension medium was added; to the second part, 0.1 ml ATP (0.1 M) was added; and to the third part, 0.1 ml ADP (0.1 M) was added. Each of these was kept in the dark for 15 sec and then centrifuged at 10,000 g for 2 min at 0-5° C. The supernatants were discarded and the pellets were resuspended at 0-5° C in 10 mM NaCl to a concentration of 0.1 mg chlorophyll/ml. The extraction of CF_1 from the thylakoid membranes was according to Lien and Racker (133). The extracted CF_1 was concentrated by ultrafiltration at room temperature from 100 ml to 7 ml. Three milliliters aliquots were then subjected to a sucrose gradient centrifugation (5-25%) in a Beckman SW 27 rotor at 26,000 rpm for 24 h at room temperature. This procedure was also according to Lien and Racker (133). One milliliter fractions were collected from the bottom of the nitrocellulose centrifuge tubes. Each fraction was then analyzed for radioactivity, protein concentration and ATPase activity. $[^3H]$-Nucleotides in each fraction were determined by scintillation counting; protein concentration by the method of Lowry et al (183) and ATPase activity was calculated from the rate of ATP hydrolysis (133), release of Pi (190). The results are shown in Fig 2. The fractions containing the greatest ATPase activity were pooled. Two milliliters of this were supplemented with 0.1 ml of AMP, ADP, and ATP (5 mM each), then analyzed for $[^3H]$-nucleotides on PEI columns as previously described. Regardless whether ATP, ADP or neither was added to the chloroplasts prior to CF_1 extraction, the results showed that CF_1 associated label in every case was $[^3H]$-ADP with insignificant amounts of $[^3H]$-AMP or $[^3H]$-ATP (data not shown).

Fig 2. Binding of labeled nucleotides to CF_1. Thylakoids were labeled with $[^3H]$-AMP (specific activity 65,601 cpm/nmole) and Pi as described in Methods. Bound nucleotides were separated from free by gel filtration through Sephadex G-50 (bead size 40-120 um; column size 3 cm x 40 cm). CF_1 was then extracted from the eluted chloroplasts according to Racker *et al* (133). The samplex were concentrated by ultrafiltration. Approximately three milliliters of the concentrate was layered on a sucrose gradient (5-25%) and centrifuged for 24 h on a SW-27 rotor at 26,000 rpm. One milliliter fractions were collected. Aliquots of 0.2 ml were analyzed for ATPase activity, trypsin activated Ca^{++} ATPase (o———o). Aliquots of 0.1 ml were used to determine protein concentration by the method of Lowry *et al* (×———×), and 0.1 ml from each fraction was counted in a scintillation counter (●———●). The units in the figure are: ATPase, umoles ATP hydrolyzed/10 min/sample; Protein concentration, ug x 10^2/ml; and Labeled nucleotides, nmole x 10^{-2}/ml.


10
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Fraction number

Fig 2.

RESULTS

Composition of bound nucleotides in Sephadexed chloroplasts.

Labeled chloroplasts were subjected to gel filtration in order to separate chloroplast-bound nucleotides from free nucleotides. The chloroplast-bound nucleotides, 1.98±0.45 (n = 6) nmoles per mg chlorophyll consisted of 0.48 nmoles AMP, 1,22 nmoles of ADP and 0.28 nmoles of ATP per mg chlorophyll. The amount of AMP varied from one preparation to the next. AMP was also easily removed by an additional centrifugation of the Sephadexed chloroplasts suspension. The origin of this AMP is not known: possibly, it is a loosely bound nucleotide which elutes with the chloroplasts. Alternatively, it may arise from ADP or ATP that was discharged from the high affinity site(s) into the medium followed by dephosphorylation to AMP. As will be shown, a fraction of chloroplast-bound nucleotides is gradually lost into the suspension medium where the free nucleotides are susceptible to modification by endogenous enzymes.

When labeled chloroplasts were subjected to the successive wash technique to separate the bound nucleotides from free, the amount of [^{3}H]-nucleotides that was bound to the chloroplasts was 3.7 nmoles per mg chlorophyll, nearly twice the amount found for Sephadexed chloroplasts. This value is more or less in agreement with similar determinations in other reports (96,184). Exposure of these labeled chloroplasts to light for 30 s in medium containing 1 mM ADP results in the exchange of nearly 90% of the chloroplast-bound label into the reaction medium. The observations suggest that Sephadex technique can be used to remove a fraction of bound nucleotides from the membranes.

Dark- and light-dependent exchange of bound nucleotides.

The length of the Sephadex column (1 cm x 50 cm) was used for the separation of chloroplast-bound nucleotides from free was amply long to achieve complete separation. Yet 30 min after gel filtration nearly one half of the label that eluted with the chloroplasts was typically found free in the suspension medium. From results summarized in Fig 1, it was determined that immediately after collection of chloroplasts, labeled with [^{3}H]-AMP and [^{32}Pi] as described in Methods, nearly 85% of the [^{3}H] and 90% of the [^{32}P] label in the chloroplast was bound. This value was obtained by subtracting the amount of label in the trail following the chloroplast peak from the nucleotides in the chloroplast peak. It was assumed that the trail represents free nucleotides and that a similar amount was also present in the chloroplast peak. The label in the trail was assumed to represent the gradual discharge of bound nucleotides from the chloroplasts during their passage through the column. The large amount of free label found in the chloroplast suspension 30 min after gel filtration may have resulted from continuous discharge of chloroplasts-bound label following elution of chloroplasts. The process of shedding bound-nucleotides appears to be complete within 30 min since no significant additional loss of bound nucleotides was detected in the time interval between 30 and 150 min after the chloroplast elution from the column. 1.03 (n = 6) nmoles of nucleotides per mg chlorophyll remained firmly attached on the chloroplasts CF$_1$. These observations suggest the presence of two different binding sites in Sephadexed chloroplasts, the exchangeable site(s) which slowly lose bound nucleotides in the dark and non-exchangeable site(s) which retain bound nucleotides under similar conditions. Chloroplasts, aged for at least 30 min were used in the subsequent experiments.

In addition to the bound nucleotides that were lost into the suspension medium following the aging of chloroplasts, about 14% (n = 6) of the remaining bound label consistently exchanged in the dark with added ADP or ATP, as compared to a medium without added nucleotides (Tables I and II). These results indicate that a small amount of bound label was not lost to the medium from an exchangeable site in aged chloroplasts. The exchange of this residual label was apparently induced by medium nucleotides. Such dark exchange is not surprising and appears to be consistent with observed exchange of medium nucleotides into soluble CF_1 binding sites (101,184). One millimolar NBD.Cl or 20 mM NH_4Cl did not inhibit the residual dark exchange. The remaining chloroplast-bound nucleotides, 0.89 (n = 6) nmoles per mg chlorophyll, appear to be unaffected by the addition of either ADP or ATP so long as the reaction is kept in the dark suggesting that these nucleotides reside at a non-exchangeable site.

Rapid exchange of nucleotides at the 'non-exchangeable site' occurs upon illumination of chloroplasts by a high intensity white light (Tables I and II). In a 500 millisec light flash, nearly 50% of these bound nucleotides were exchanged with medium ADP or ATP. In controls with no medium nucleotides, the discharge of bound nucleotides was only 7% of the dark controls. As expected this energy dependent exchange was inhibited by 20 mM NH_4Cl or by 1 mM NBD.Cl. This exchange was not inhibited by 1 mM phloridzin and it showed a requirement for Mg^{++} (EDTA inhibited).

Fig 3 shows energy dependent exchange of nucleotides on the non-exchangeable site in greater detail. Labeled aged chloroplasts were pulsed into medium that contained either ADP, ATP or both. In a time-dependent exposure to light ranging from 100 millisec to 10 s, there was rapid exchange of the bound nucleotides with medium nucleotides with an esti-

Table I. Exchange of chloroplast bound nucleotides. Labeled aged chloroplasts, 0.67 mg chlorophyll/ml, were injected into reaction mixture (0.9 ml) which contained 4 mM $MgCl_2$, 10 NaCl, 15 mM tricine buffer, pH 8.0, 50 uM PMS, and the indicated additions. The reactions were carried out at room temperature for 0.5 s. Following the dark or light incubation, the contents of the reaction were poured into 15 ml reaction mixture minus the indicated additions which had been precooled to 0° C. The diluted mixture was centrifuged and the amount of label in the pellet and supernatant was measured as described in the experimental procedure.

TABLE I

Additions	Bound nucleotides (n moles/mg chlorophyll)	Released nucleotides (n moles/mg chlorophyll)
Dark reaction		
1. Reaction mixture	1.04	-
2. 5 mM Pi	1.04	-
3. ADP 1 mM + Pi 5 mM	0.90	1.20
4. ADP 1 mM + ATP 10 mM + Pi 5 mM	0.90	1.35
5. ATP 1 mM + Pi 5 mM	0.83	1.34
6. ATP 1 mM + Pi 5 mM + NBD.Cl 1 mM	0.83	-
7. ATP 1 mM + Pi 5 mM + NH_4Cl 20 mM	0.84	-
Light reaction		
1. Reaction mixture	0.95	-
2. 5 mM Pi	0.92	-
3. ADP 1 mM + Pi 5 mM	0.60	1.64
4. ADP 1 mM + ATP 10 mM + Pi 5 mM	0.44	1.73
5. ATP 1 mM + Pi 5 mM	0.54	1.63
6. ATP 1 mM + Pi 5 mM + NBD.Cl 1 mM	0.79	-
7. ATP + Pi 5 mM + NH_4Cl	0.78	-

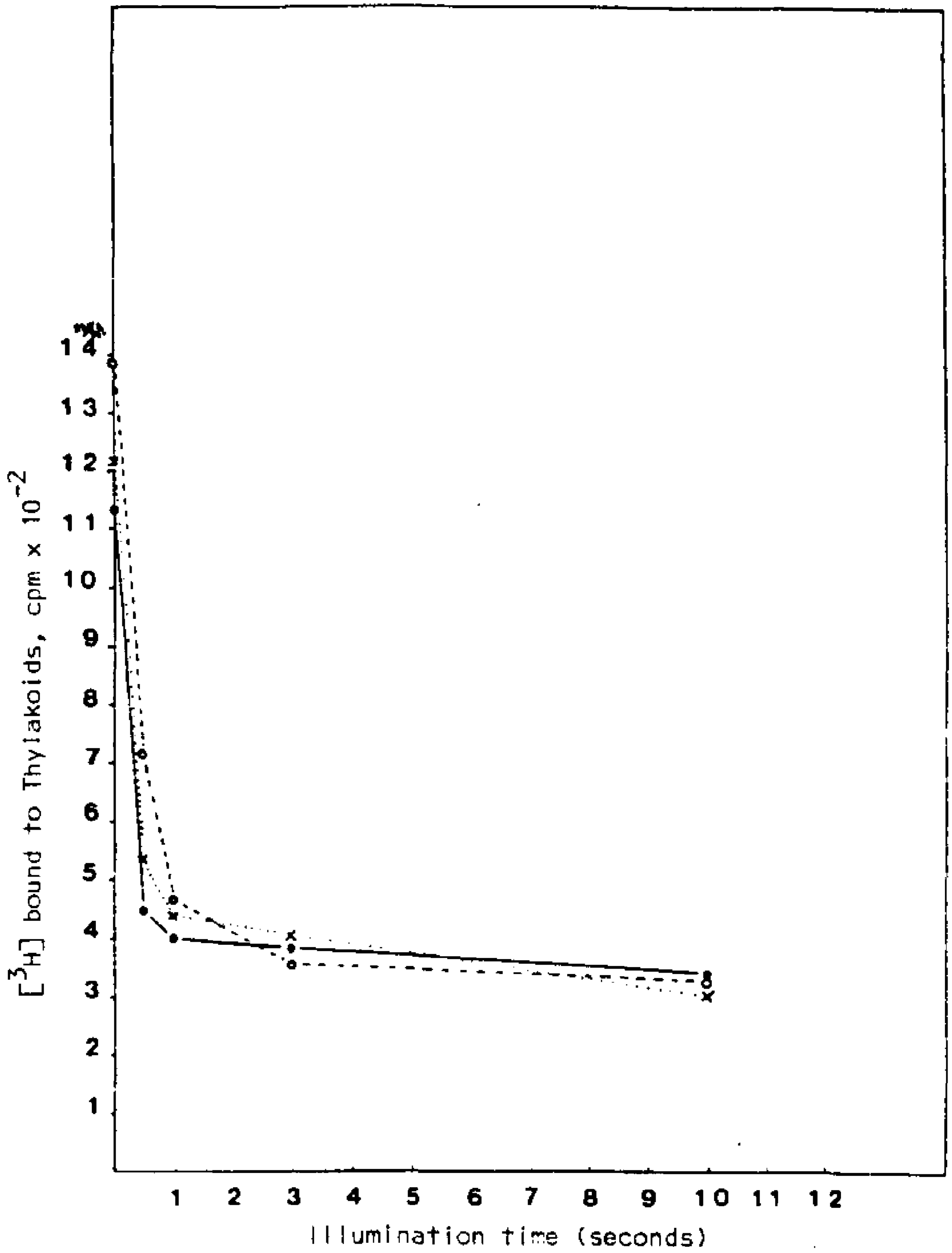
Table II. Exchange of chloroplast bound nucleotides. Labeled aged chloroplasts (0.1 ml, 43 ug of chlorophyll) were pulsed into reaction medium (0.9 ml) containing: 4 mM $MgCl_2$, 10 mM NaCl, 15 mM tricine buffer, pH 8.0, 50 uM PMS, and other additions as shown in the table. The dark and light reactions were incubated at room temperature for 0.5 s. Chloroplasts were immediately pelleted in a microcentrifuge for 1 min. The pellets and supernatants were separated and the amount of label in each was determined as described in Methods.

TABLE II

	Additions	Bound nucleotides (n moles/mg chlorophyll)	Released nucleotides (n moles/mg chlorophyll)
	<u>Dark reaction</u>		
1.	Reaction mixture	0.70	-
2.	5 mM Pi	0.71	-
3.	ADP 1 mM + Pi 5mM	0.64	0.88
4.	ADP 1 mM + ATP 10 mM Pi 5 mM	0.60	1.10
5.	ATP 1 mM + Pi 5 mM	0.57	0.96
6.	ATP 1 mM + Pi 5 mM + phloridzin	0.60	-
7.	ATP 1 mM + Pi 5 mM + EDTA 5 mM	0.60	-
	<u>Light reaction</u>		
1.	Reaction mixture	0.67	-
2.	5 mM Pi	0.65	-
3.	ADP 1 mM + Pi 5 mM	0.41	1.13
4.	ADP 1 mM + ATP 10 mM + Pi 5 mM	0.43	1.42
5.	ATP 1 mM + Pi 5 mM	0.39	1.34
6.	ATP 1 mM + Pi 5 mM + phloridzin	0.33	-
7.	ATP 1 mM + Pi 5 mM + EDTA 5 mM	0.63	-

Fig 3. Time- and energy-dependent exchange of thylakoid bound nucleotides. Labeled aged chloroplasts, 54 mg chlorophyll (0.1 ml) were pulsed into 0.9 ml reaction medium of the same electrolyte composition as described in the legend of Table II. The reaction medium also included either 1 mM ADP (○—○), 1 mM ADP plus 10 mM ATP (×—×) or 1 mM ATP (●—●). Upon addition of nucleotides, the mixture was immediately exposed to light for the indicated time intervals. Zero times and controls were kept in the dark for a time equal to the longest light exposure (10 sec). Following illumination, the reaction mixtures were immediately centrifuged for 1 min at 11,000 rpm in an Eppendorf Microcentrifuge. The supernatant and pellet were separated and the radioactivity in the pelleted chloroplasts was measured following denaturation with perchloric acid as described in Methods.

Fig 3.



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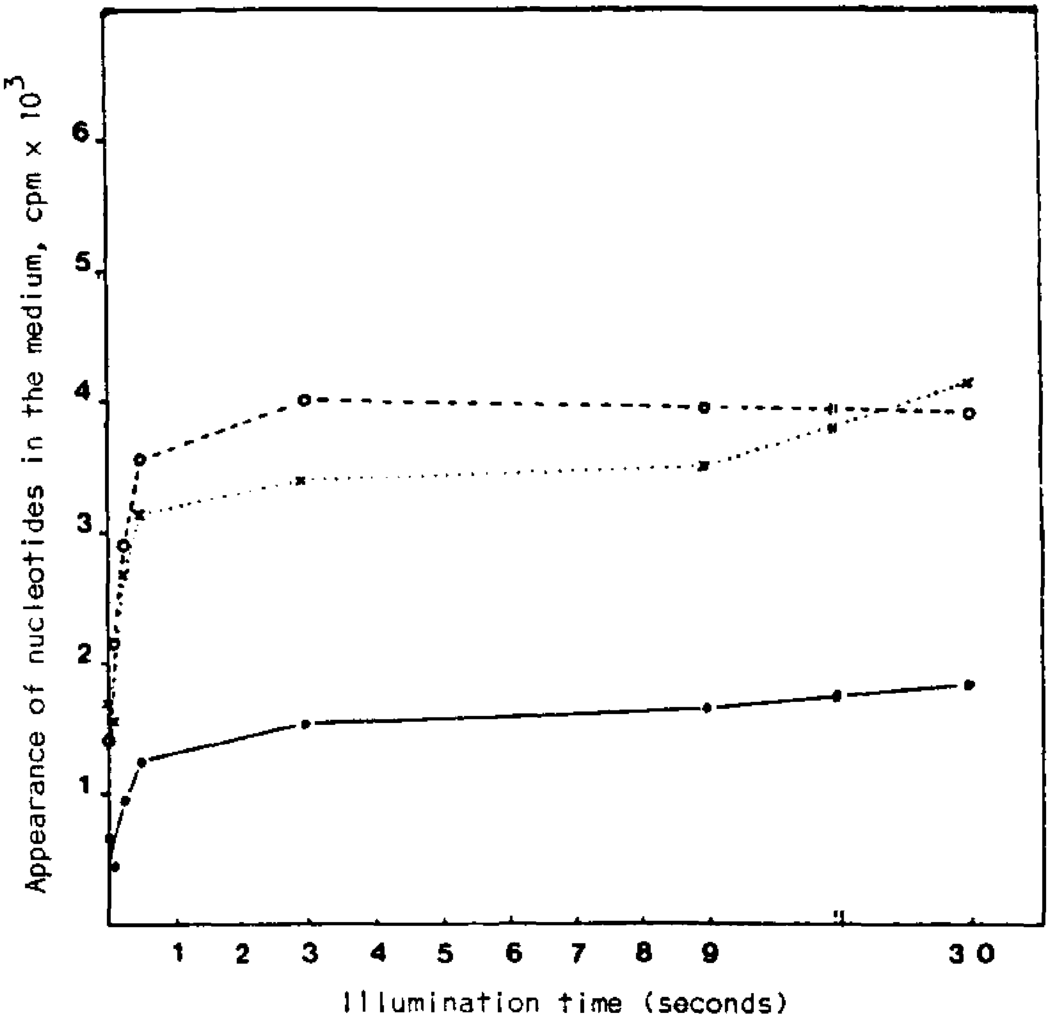
6
5
4
3
2
1
[^{3}H] bound to Thylakoids, cpm x 10^{-2}
1
2
3
4
5
6
7
8
9
10
11
12
Illumination time (seconds)

mated exchange rate, $t_{\frac{1}{2}}$, of 250 ms. This is similar to the rate of ($t_{\frac{1}{2}} < 1s$) obtained by Shoshan and Selman (191) for the exchange of adenine nucleotides from light and DTT activated thylakoids and for activation of ATPase activity. The calculated rate of exchange from the data in Fig 3 was 13.3 umoles/mg chlorophyll/h.

Exchange of bound nucleotides with medium ADP + Pi, AMP + Pi or inorganic phosphate is shown in Fig 4. Again it is observed that in the dark phase of the reaction (t = 0) more bound label is discharged into reaction medium which contains nucleotides than into medium with only inorganic phosphate. In the light phase of the reaction, in time intervals ranging from 100 msec to 30 s, there was rapid exchange of bound nucleotides with medium ADP + Pi (exchange rate constant was $2\text{-}4.5^{-1}$). With AMP + Pi in the reaction medium, the bound nucleotides exchanged as rapidly as with ADP + Pi but the extent was slightly less than with ADP. The similar exchange rates with these two nucleotides suggests a direct effect of AMP + Pi on the exchange of bound nucleotides. It is unlikely that AMP + Pi were modified so rapidly to form medium ADP by endogenous enzymes as to elicite the observed exchange rates, particularly since both ATP and adenylate kinase were found only in trace amounts in these chloroplast preparations: 0.28 nmoles/mg chlorophyll ATP, most of which was in the bound form, and 0.02 units of adenylate kinase (unit = umoles ATP formed/mg chlorophyll/min). A possible mechanism for this exchange may involve AMP + Pi condensation to bound ADP on one site with simultaneous ejection of bound $[^3H]$-ADP from the same site or a neighboring site. However in view of the recent report of Moudrianakis (197) of rapid AK activity in washed chloroplasts, one cannot exclude that at least part of this exchange was caused by AK dependent formation of ADP from AMP and ATP.

Fig 4. The exchange of bound nucleotides (appearance of bound nucleotides in the medium). Aged chloroplasts, 30 ug chlorophyll (0.1 ml) bearing [^{3}H]-bound nucleotides were supplemented with 5 mM $MgCl_2$, 10 mM NaCl, 15 mM tricine buffer, pH 8.0, 50 uM PMS, and where indicated: 5 mM Pi (●—●), 1 mM AMP plus 5 mM Pi (x--x), and 1 mM ADP plus 5 mM Pi (o--o). The mixtures, final volume 0.8 ml, were exposed to light for the indicated time periods. Controls, t = 0, were protected from light for 30 sec. Following light or dark reactions, the samples were immediately centrifuged as described in the legend of Fig 3; 0.05 ml of the supernatant were counted directly.

Fig 4.



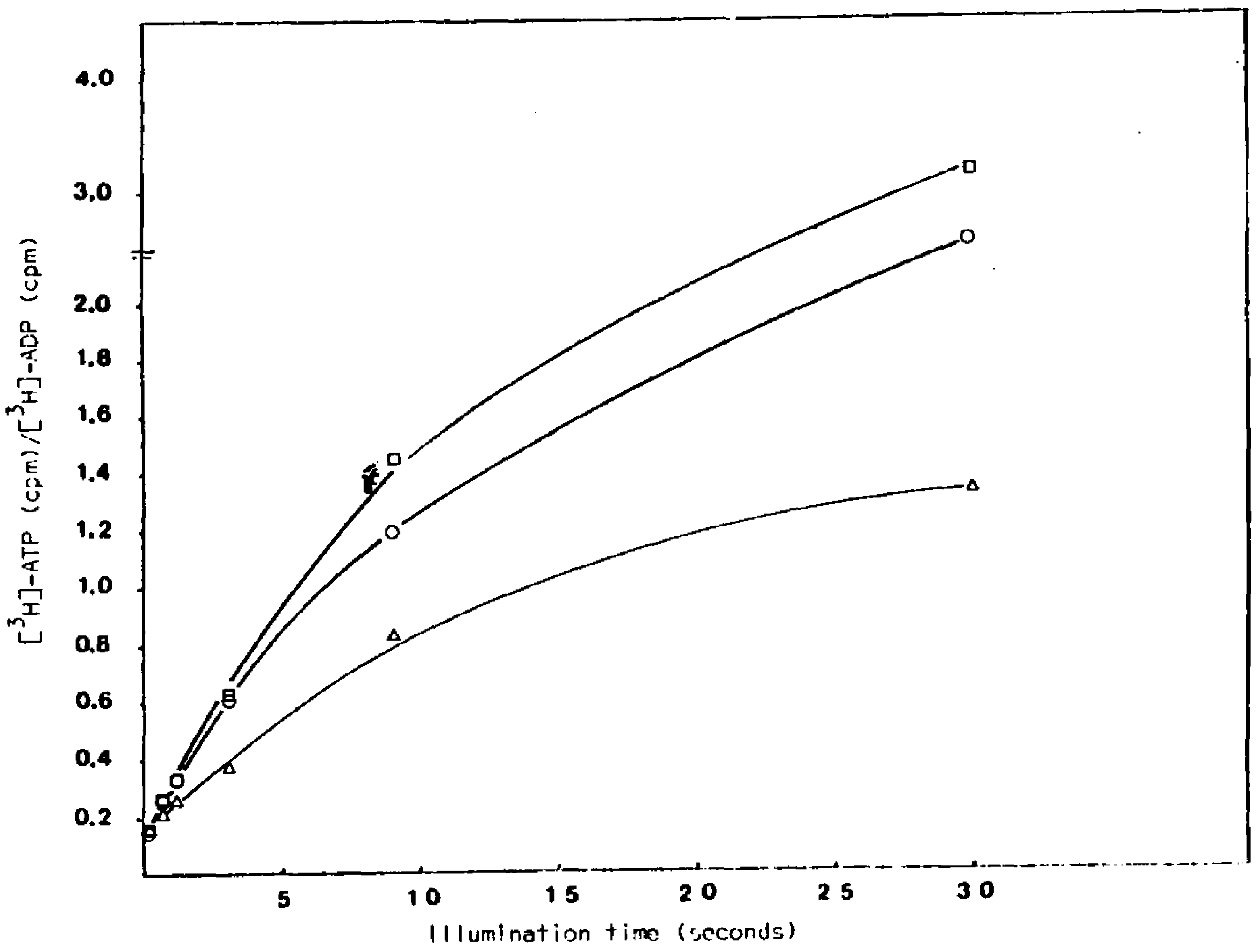
Appearance of nucleotides in the medium, cpm x 10^3
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
30
Illumination time (seconds)

With inorganic phosphate alone in the reaction medium (Fig 4), exposure of the chloroplast to light caused a significant portion of the bound nucleotides to be released into the medium (32%). The magnitude of this release, however, was substantially less than when ADP or AMP were also present. A comparison of the light-dependent release of bound nucleotides in medium containing Pi, to the dark exchange (t = 0) of bound nucleotides with added medium nucleotides, shows bound label losses of similar magnitude (32% vs 24%). This similarity suggests that the residual bound nucleotide of the exchangeable site may have been involved in each case. Although the percentage or residual bound nucleotides varied somewhat from one experiment to the next (seldom above 25%), the amount of dark residual bound nucleotide exchange in the presence of free nucleotides and the amount of bound nucleotides lost in the light in the absence of free nucleotides were always comparable. Results in Tables I, II, and III support these observations.

Energy dependent phosphorylation of bound [^{3}H]-ADP.

In the experiments summarized in Figs 5 and 6, the exchange rates of bound [^{3}H]-nucleotides and the rates of their phosphorylation were analyzed in parallel. Since the amount of labeled nucleotides per mg chlorophyll was constant in each sample, the rate of exchange may be expressed as the ratio of [^{3}H]-free/[^{3}H]-bound; and the rate of [^{3}H]-ADP phosphorylation to [^{3}H]-ATP may also be conveniently expressed as the ratio of [^{3}H]-ATP/[^{3}H]-ADP. The total label, bound and free, in the control samples (no additions) was 10,611 cpm/mg chlorophyll (1.76 nmoles/mg chlorophyll). The free nucleotides amounted to 4277 cpm/mg chlorophyll (0.71 nmoles/mg chlorophyll and the bound fraction amounted to 6382 cpm/mg chlorophyll (1.06 nmoles/mg chlorophyll). The nucleotide composition

Fig 5. Light-dependent conversion of bound [^{3}H]-ADP to [^{3}H]-ATP. Chloroplasts (0.3 ml chlorophyll) eluted from Sephadex G-50 and pre-labeled with [^{3}H]-adenine nucleotides were added to reaction solutions consisting of 15 mM tricine-NaOH buffer, pH 8.0, 4 mM $MgCl_2$, 10 mM NaCl and 50 uM PMS. Where indicated, the reaction medium also included 5 mM Pi (○–○), 1 mM AMP plus 5 mM Pi (□–□) or no further additions (△–△). The reaction mixtures, final volum one milliliter, were exposed to light or kept in the dark for the specified times. The reactions were terminated by cessation of illumination and addition of two milliliters of 9 M urea followed by immediate chilling to 0° C. One milliliter sampes were analyzed for nucleotide composition as described in Methods. The results are expressed as the ratio of cpm of [^{3}H]-ATP/cpm in [^{3}H]-ADP.



[^{3}H]-ATP (cpm)/[^{3}H]-ADP (cpm)
4.0
3.0
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5
10
15
20
25
30
Illumination time (seconds)

Fig 5.

Fig 6. Light-dependent exchange of bound nucleotides (appearance of nucleotides in the medium). The reaction conditions were the same as in Fig 5. Labeled chloroplasts (0.1 ml) were added to 0.5 ml of medium and illuminated. Following the reactions in the light, the samples were immediately centrifuged. The label content in the pellet and supernatant were determined. The amounts of [^{3}H]-AMP were substracted and only the quantity of labeled [^{3}H]-ADP + [^{3}H]-ATP in the supernatant and pellet are shown. The results are expressed as the ratio of [^{3}H]-free (supernatant)/[^{3}H]-bound (pellet). (■), 1 mM AMP plue 5 mM Pi; (●), 5 mM Pi.

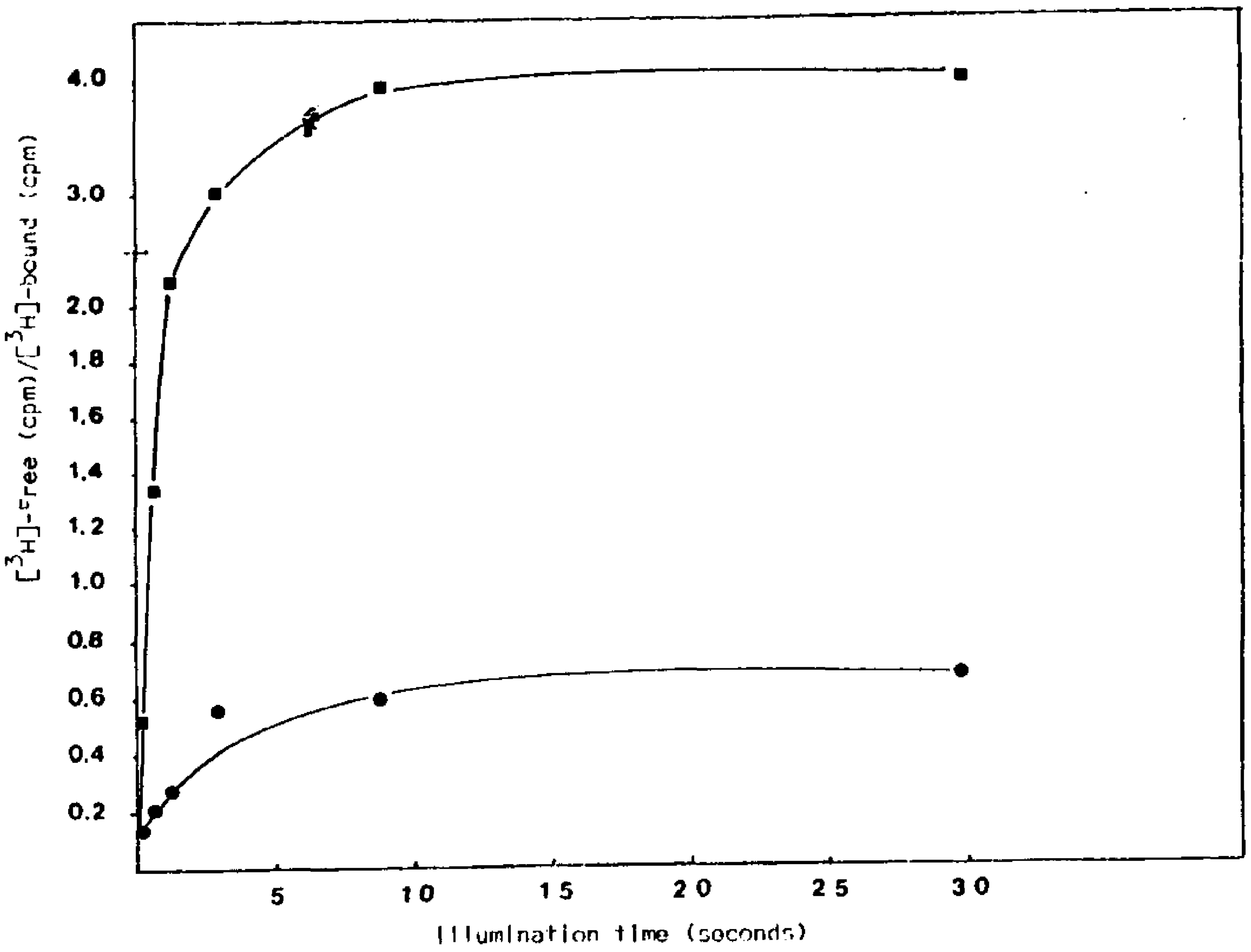

4.0
3.0
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
[^{3}H]-free (cpm)/[^{3}H]-bound (cpm)
5
10
15
20
25
30
Illumination time (seconds)

Fig 5.

of the free fraction was 3127 cpm/mg chlorophyll (0.52 nmoles/mg chlorophyll) of [^{3}H]-AMP and 1150 cpm/mg chlorophyll (0.19 nmoles/mg chlorophyll) of [^{3}H]-ADP and [^{3}H]-ATP combined. The bound fraction consisted of [^{3}H]-ADP and [^{3}H]-ATP. No significant amount of [^{3}H]-AMP was present (usually $<$ 0.05 nmoles/mg chlorophyll). In the results of Fig 6, the values of [^{3}H]-AMP were substracted and only the quantity of label in [^{3}H]-ADP and [^{3}H]-ATP is indicated in the ratio [^{3}H]-free/[^{3}H]-bound. The results of exchange in Fig 6 and the rate of phosphorylation in Fig 5 may therefore be compared based only on the changes in the [^{3}H]-ADP and [^{3}H]-ATP levels. [^{3}H]-AMP does not appear to interfer with exchange or phosphorylation of [^{3}H]-ADP As shown in Table IV (pages 85 & 86), the levels of [^{3}H]-ADP + [^{3}H]-ATP remained constant through the duration of the experiment (30 s). Superimposing Fig 5 and Fig 6 shows that when labeled aged chloroplasts are injected into medium containing AMP + Pi and exposed to light, the ratio of [^{3}H]-free/[^{3}H]-bound increases more rapidly, $t_{1/2}$ = 0.5 s[‡], than the [^{3}H]-ATP/[^{3}H]-ADP ratio, $t_{1/2}$ = 5.4 s[‡‡]. This indicates that the rate of release of bound [^{3}H]-ADP in the presence of AMP + Pi is much more rapid than the rate of phosphorylation of [^{3}H]-ADP. This observation further suggests that the synthesis of [^{3}H]-ATP from precursor [^{3}H]-ADP occurs after [^{3}H]-ADP has been released from the binding site. On the other hand, when ADP and AMP were omitted and inorganic phosphate alone was present in the reaction medium, the ratio of [^{3}H]-ATP/[^{3}H]-ADP increased initially at the same rate ($t_{1/2}$ = 5.4 s) as the ratio of [^{3}H]-free/[^{3}H]-bound ($t_{1/2}$ = 5.4 s). Then, with

---

[‡] $t_{1/2}$ is the initial exchange velocity of [^{3}H]-free/[^{3}H]-bound extrapolated to a ratio of one.

[‡‡] $t_{1/2}$ is the initial phosphorylation velocity of [^{3}H]-ATP/[^{3}H]-ADP extrapolated to a ratio of one.

longer illumination periods (greater than 3 s), the ratio of [^{3}H]-ATP/[^{3}H]-ADP continued to increase while the ratio of [^{3}H]-free/[^{3}H]-bound leveled off. Phosphorylation of [^{3}H]-ADP to [^{3}H]-ATP continued at a slow rate without further release of bound nucleotides. Whereas initial (t = 0) the fraction of [^{3}H]-ATP and the fraction of free label were nearly identical at 0.18, following a 30 s light exposure the fraction of [^{3}H]-ATP increased to over 2.5 while the fraction of free label increased only to 0.65, indicating that in the absence of medium nucleotides (with inorganic phosphate as the only addition) bound [^{3}H]-ADP was converted to bound [^{3}H]-ATP. It is not known whether phosphorylation of bound [^{3}H]-ADP occured on the binding site prior to release or from exchange of medium [^{3}H]-ATP with bound [^{3}H]-ADP. In the absence of either AMP or Pi, the rate of [^{3}H]-ATP was greatly reduced (Fig 5). The small amount of [^{3}H]-ATP formation that was found may have resulted from esterification of trace amounts of inorganic phosphate which was not completely removed by the passage of chloroplasts through the Sephadex column.

ATP mediated phosphorylation of bound ADP.

Phosphorylation of bound [^{3}H]-ADP to [^{3}H]-ATP was indicated when labeled aged chloroplasts were incubated in medium which contained ATP. The degree of such phosphorylation appeared to be directed releated to the ATP/ADP ratio in the medium. The results in Table III show the composition of chloroplast-bound nucleotides. The dark reactions show that incubation of labeled chloroplasts with ADP and ATP, each 1 mM, results in no significant increase in the amount of bound [^{3}H]-ATP when compared to an identical reaction with only 1 mM ADP. The fractions of [^{3}H]-ATP, expressed as the [^{3}H]-ATP/[^{3}H]-ADP ratio, for the two reactions were 0.47 and 0.43 respectively. When the medium concentration of ATP was increased to 10 mM while maintaining the ADP concentration at 1 mM, the ratio increased to 1.47. The ratio increased further to 2.45 with 1 mM ATP and no ADP in the medium. Total counts that remained chloroplast bound in the dark were nearly identical for all experiments and independent of the nucleotide composition in the reaction mixture.

ATP dependent bound [^{3}H]-ADP phosphorylation appears to be uncoupler sensitive. In the presence of 20 mM NH_4Cl, 1 mM ATP failed to induce the phosphorylation of bound ADP (dark sample 8). Similarly, 1 mM NBD.Cl also inhibited bound ADP phosphorylation (dark sample 7). Instead of the ratio of 2.46 for [^{3}H]-ATP/[^{3}H]-ADP with 1 mM ATP, the ratio remained at 0.58 and 0.56 with inhibitors. In unpublished experiments, we found that NBD.Cl can inhibit the activities of CF_1 by two different paths. The first is the known reaction of NBD.Cl with CF_1 ATPase (NBD.Cl reacts with the β subunit with concomitant inhibition of ATPase activity (110), and a second reaction involves NBD.Cl interaction with an as yet unidentified thylakoid membrane component. This

Table III. Dark phosphorylation of thylakoid bound [^{3}H]-ADP. Thylakoids with bound [^{3}H]-nucleotides were prepared as described in Methods. 0.2 ml Aliquiots of the labeled thylakoids (0.58 mg chlorophyll/ml) were added to reaction mixtures containing 15 mM tricine buffer, pH 8.0, 4 mM $MgCl_2$, 10 mM NaCl, and 50 mM PMS plus other components as indicated in the Table. After light or dark exposure, each sample was diluted with 15 ml of reaction mixture, precooled to 0° C, and centrifuged to remove the free nucleotides. The pelleted membranes containing the bound nucleotides were denatured by the addition of 1 ml of 10 M urea. The denatured pellets were analyzed for [^{3}H]-AMP, [^{3}H]-ADP, and [^{3}H]-ATP.

TABLE III

	Additions							
	ADP mM	ATP nM	Pi mM		$[^3H]$-ADP nmoles/mg	$[^3H]$-ATP nmoles/mg	ADP + ATP Total nmoles/mg	$\frac{[^3H]\text{-ATP}}{[^3H]\text{-ADP}}$
<u>DARK Incubation of labeled chloroplasts 0.5 s</u>								
1.	1.0	0	5		0.49	0.21	0.70	0.41
2.	1.0	1.0	5		0.42	0.19	0.61	0.47
3.	1.0	10.0	5		0.29	0.40	0.69	1.40
4.	0	1.0	5		0.17	0.44	0.61	2.56
5.	0	0	5		0.44	0.36	0.81	0.81
6.	0	0	0	Reaction mixture	0.43	0.35	0.78	0.81
7.	0	1.0	5	NBD.Cl 1 mM	0.44	0.25	0.69	0.56
8.	0	1.0	5	NH_4Cl 20 mM	0.46	0.27	0.74	0.58
<u>LIGHT Incubation of labeled chloroplasts 0.5 s</u>								
1.	1.0	0	5		0.25	0.17	0.42	0.68
2.	1.0	1.0	5		0.26	0.23	0.49	0.87
3.	1.0	10.0	5		0.21	0.18	0.39	0.86
4.	0	1.0	5		0.23	0.19	0.42	0.82
5.	0	0	5		0.40	0.25	0.65	0.62
6.	-	-	-		-	-	-	-
7.	0	1.0	5	NBD.Cl 1 mM	0.38	0.22	0.60	0.60
8.	0	1.0	5	NH_4Cl 20 mM	0.35	0.23	0.58	0.66

<u>Dark phosphorylation of thylakoid bound $[^3H]$-ADP.</u>

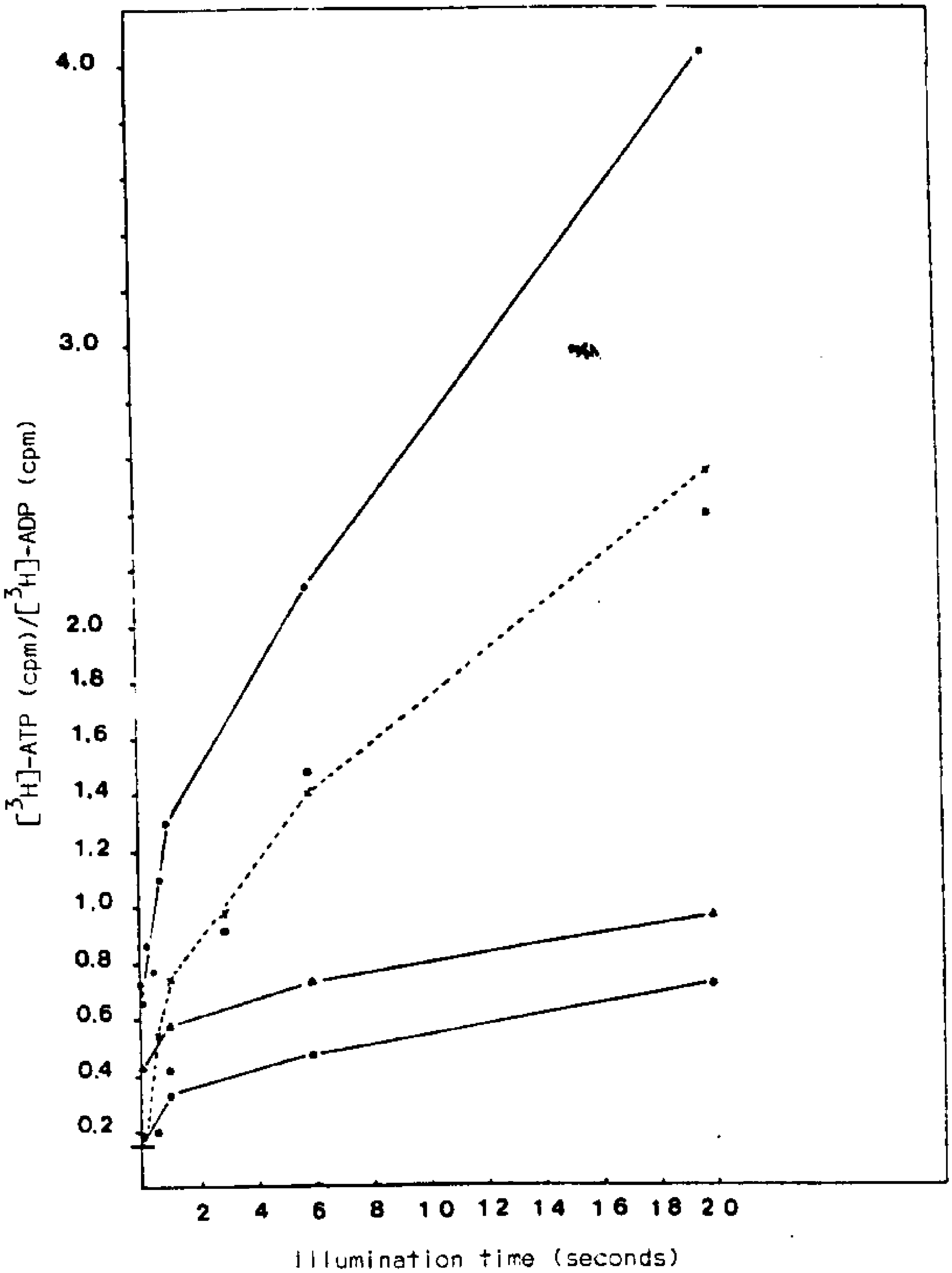
interaction causes the collapse of the intrathylakoid Δ pH). In either case, however, NBD.Cl may inhibit the formation of a high energy state, e.g. in reaction one, NBD.Cl inhibits ATP hydrolysis and reverses protein flow; in reaction two, NBD.Cl acts as an uncoupler by discipating ΔpH. From the dark reactions of Table III, it can be again observed that control chloroplasts (dark samples 5 and 6) retain more bound label, 18%, than chloroplasts incubated with nucleotides (dark samples 1-4) supporting previous observations that the 'excess' bound label is on the exchangeable site. Analysis of the chloroplast bound nucleotides reveals that the [^{3}H]-ATP/[^{3}H]-ADP ratio in controls (dark samples 5 and 6) is 0.81 while in samples with either 1 mM ADP or 1 mM ADP plus 1 mM ATP this ratio was 0.41 and 0.47, suggesting a higher proportion of bound [^{3}H]-ATP in the controls. Following a 15 s light exposure, 19% of the bound label was lost in control samples (dark and light samples 5). This value is almost identical to the 18% dark exchange of bound label in the presence of medium nucleotides suggesting as before that the same nucleotides on the exchangeable sites were involved in the two reactions. The light exposure also resulted in a decrease in the [^{3}H]-ATP/[^{3}H]-ADP ratios of the controls from 0.81 to 0.62 indicating that the lose of bound nucleotide from the exchangeable site was mostly [^{3}H]-ATP.

Time-dependent and light phosphorylation of bound [^{3}H]-ADP

Labeled chloroplasts, eluted from Sephadex G-50, aged for approximately 45 min at 0° C in the dark were reacted in the presence of various nucleotides as shown in Fig 7. Photophosphorylation patterns of chloroplast associated [^{3}H]-ADP were measured in a time dependant reaction ranging from 0.1 to 20 s. The control sample shows the labeled nucleo-

Fig 7. Photophosphorylation of 'Bound' $[^3H]$-ADP in medium containing nucleotides. Labeled thylakoids, 0.7 mg chlorophyll/ml, were prepared as described in Methods. Thylakoids, 70 ug chlorophyll, were added to medium which contained 20 mM tricine buffer, pH 8.0, 4 mM $MgCl_2$, 10 mM NaCl, 50 uM PMS, 10^{-5} M A_2P_5, 2.5 mM Pi, and other components as indicated: 1 mM ADP (□——□), 1 mM ADP plus 10 mM ATP (△——△), 1 mM ATP (●——●), 1 mM AMPPNP (×----×), and 1 mM AMP (■   ■). The reaction mixture (final volume 1 mL) were exposed to light for the indicated durations. Dark samples (t = 0) were kept in the dark for 20 s. The reactions were terminated by the addition of two milliliters of 10 M urea followed by storage of the mixtures at 0° C in the dark. The samples were analyzed on PEI columns for labeled nucleotide composition. Control (▬) reflects nucleotides composition of thylakoids prior to reaction. The results are expressed as the $[^3H]$-ATP/$[^3H$-ADP] ratio.

Fig 7.


4.0
3.0
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
$[^3H]$-ATP (cpm)/$[^3H]$-ADP (cpm)
2
4
6
8
10
12
14
16
18
20
illumination time (seconds)

tide composition, expressed as the $[^{3}H]$-ATP/$[^{3}H]$-ADP ratio, prior to illumination. This control ratio was 0.15. This is not significantly different from the $[^{3}H]$-ATP/$[^{3}H]$-ADP ratio of 0.17 found when the labeled chloroplasts were incubated in the dark with 1 mM ADP. As the fraction of ATP in the dark reaction medium was increased, the ratio of $[^{3}H]$-ATP/$[^{3}H]$-ADP also increased. With 1 mM ADP plus 10 mM ATP in the medium, the $[^{3}H]$-ATP/$[^{3}H]$-ADP fraction increased to 0.41 and with 1 mM ATP, the ratio increased further to 0.71. The lower $[^{3}H]$-ATP/$[^{3}H]$-ADP ratios of Fig 7 compared to the ratios shown in Table III may have resulted from differences in experimental procedures. In Fig 7 for instance, the $[^{3}H]$-ATP/$[^{3}H]$-ADP ratios reflect the composition of label nucleotides in the complete reaction medium; in contrast, the $[^{3}H]$-ATP/$[^{3}H]$-ADP ratio in Table III shows only chloroplast bound nucleotide compositions.

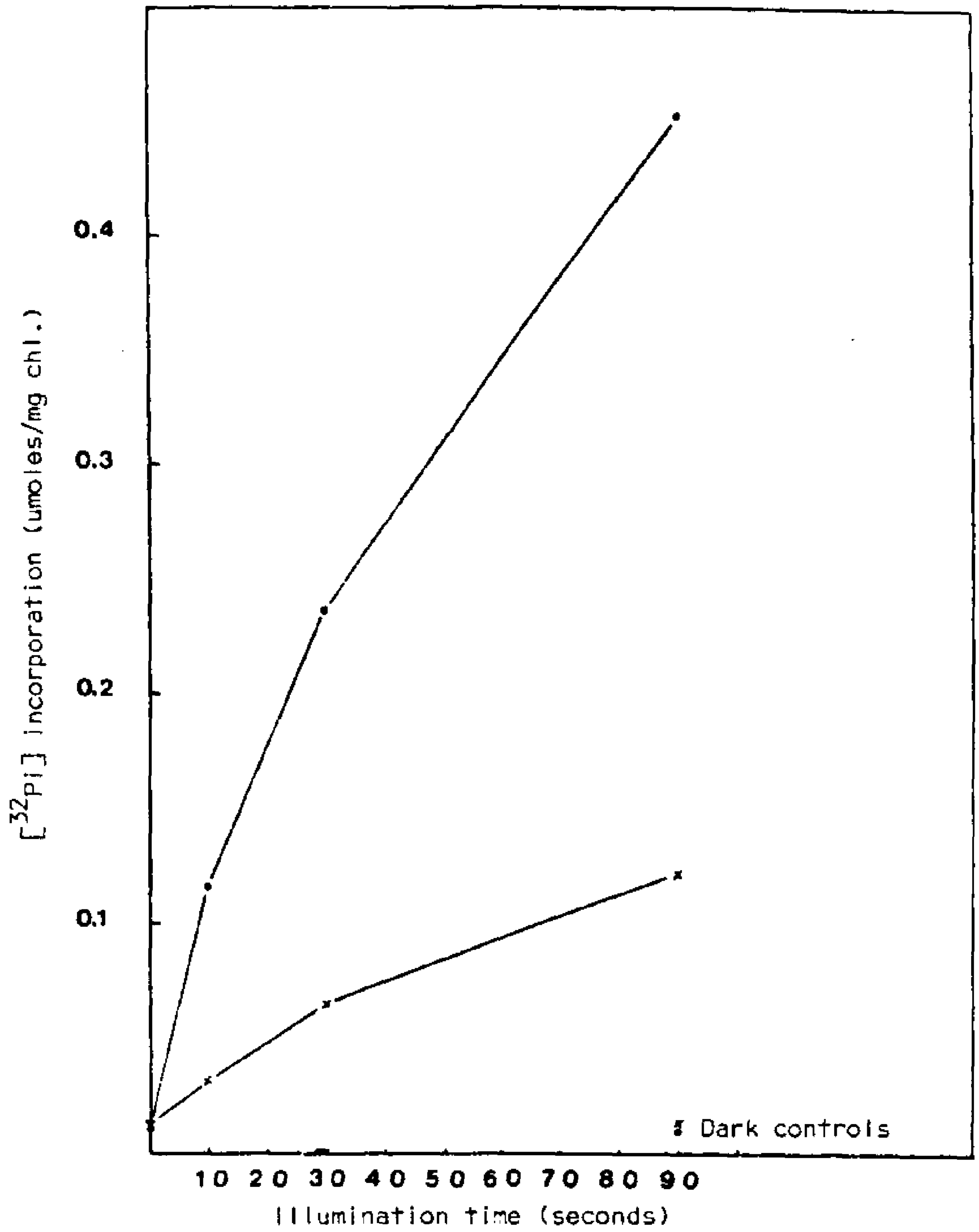
Exposure of chloroplasts to light in the presence of 1 mM ADP plus 2.5 mM Pi resulted in a two phase pattern of $[^{3}H]$-ADP phosphorylation. The first phase consists of a rapid pulse of $[^{3}H]$-ADP phosphorylation. This phase appears to be over in one second and was followed by a second phase of slower more gradual phosphorylation of $[^{3}H]$-ADP. The second phase of this reaction most likely represents the phosphorylation of exchanged $[^{3}H]$-ADP which had equilibrated with free unlabeled ADP. From the slope of the increase in the $[^{3}H]$-ATP/$[^{3}H]$-ADP ratio between 6 and 20 s, the rate of photophosphorylation was calculated to be 640 umoles/mg chlorophyll/h which is in the normal range of photophosphorylation. However, using the slope of $[^{3}H]$-ATP/$[^{3}H]$-ADP between zero and one second (in the presence of ADP and Pi), the photophosphorylation rate was 4370 umoles/mg/h. This rate is over five times above the upper normal range of photophosphorylation. Such an initial surge in

[^{3}H]-ADP phosphorylation can be explained only if a fraction of bound [^{3}H]-ADP was phosphorylated prior to its release from the binding site and diluted in the pool of medium ADP. This is consistent with the previous observations in Figs 5 and 6 where bound [^{3}H]-ADP can be phosphorylated on the non-exchangeable site under energized conditions. The short duration of this phosphorylation in the presence of ADP and Pi may be the result of rapid exchange of the bound [^{3}H]-ADP with medium ADP before significant phosphorylation of bound [^{3}H]-ADP had occurred. Similar observations were made when 1 mM ADP plus 10 mM ATP were in the reaction mixture.

Substituting 1 mM ATP with 1 mM AMPPNP eliminated the dark increase in the [^{3}H]-ATP/[^{3}H]-ADP ratio (at t = 0). In the light reaction, the [^{3}H]-ATP/[^{3}H]-ADP slope generated in the presence of AMPPNP had an intercept of 0.15, identical to the control. This suggests that the non-hydrolysable analogue cannot serve as a substitute for the ATP induced dark phosphorylation of bound [^{3}H]-ADP (Table III) suggesting further that medium ATP hydrolysis is an essential factor in the dark phosphorylation of bound [^{3}H]-ADP. Phosphorylation patterns of [^{3}H]-ADP by illuminated chloroplasts in the presence of 1 mM AMPPNP and 1 mM ATP appear to be similar, particularly in the early phases of the reactions (zero to one second) where there was an initial rapid and almost identical rise in the [^{3}H]-ATP production. In the absence of medium ADP, however, it is difficult to ascertain whether bound [^{3}H]-ADP or medium [^{3}H]-ADP was phosphorylated. Between 1 and 20 s, the rates of [^{3}H]-ADP phosphorylation were slower, more so in the presence of AMPPNP. Substrate limitation is expected to be more severe with the non-hydrolyzable analogue, hence the lower rates of [^{3}H]-ADP phosphorylation at the long reaction intervals.

Fig 8. Time course of $[^{32}P] \rightleftharpoons$ ATP exchange inhibition by pyruvate kinase in excited chloroplasts. Chloroplasts (0.021 mg chlorophyll) were incubated with 1 ml reaction medium containing 50 mM tricine-NaOH, pH 8.0, 10 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, 5 mM $[^{32}Pi]$ (23,363 cpm/nmole), and 50 uM PMS. In the indicated sample (×——×), the medium was also supplemented with 5 mM phosphoenolpyruvate and pyruvate kinase. The reaction was exposed to excitation light energy or kept in the dark for the shown times. The reactions were terminated by the addition of 0.1 ml 35% perchloric acid. An aliquot (0.2 ml) was analyzed for $[^{32}P]$ incorporation into organic phosphate (190) as described in Methods.

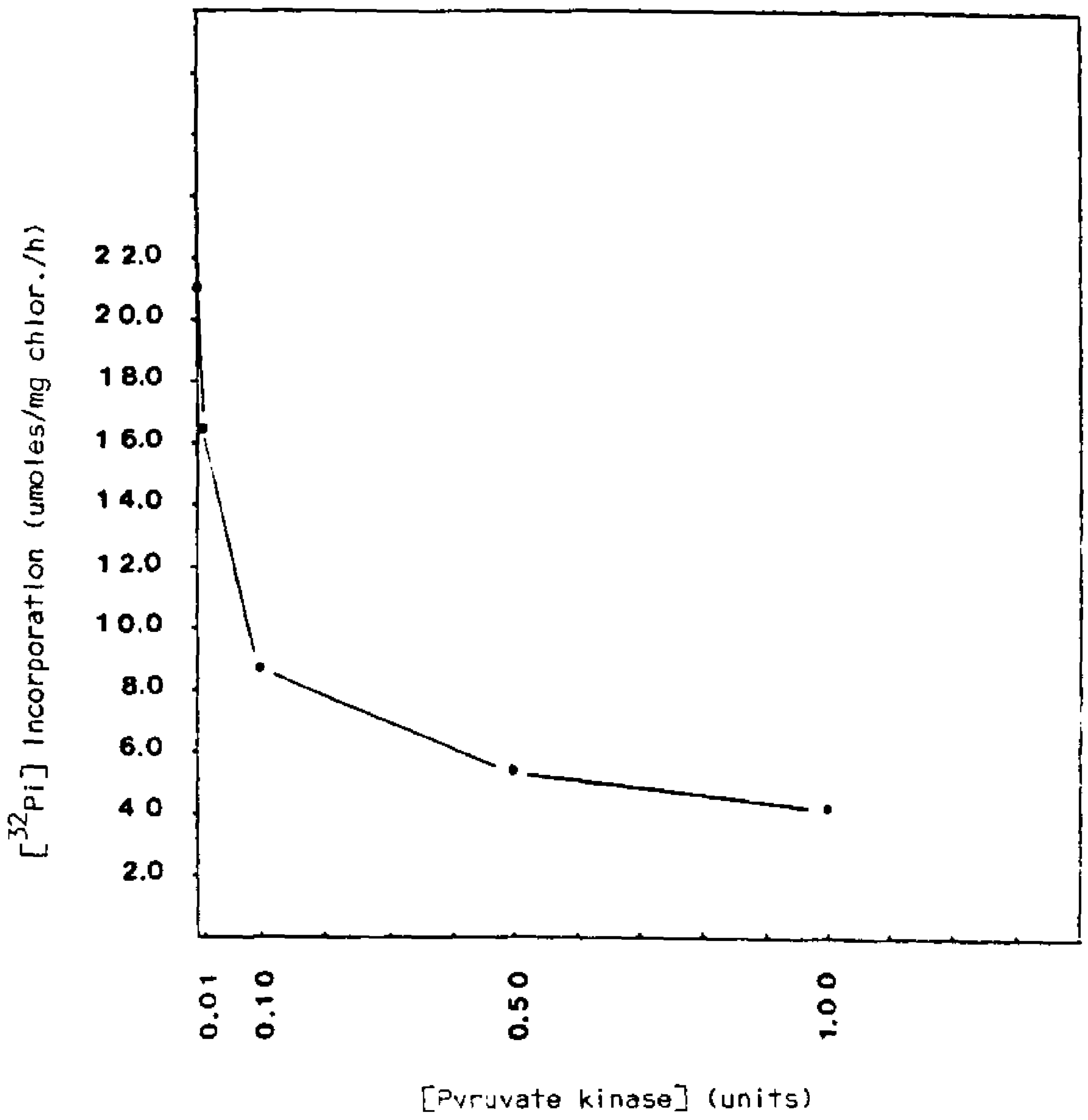
Fig 8.



[^{32}Pi] incorporation (umoles/mg chl.)
0.4
0.3
0.2
0.1
x Dark controls
10
20
30
40
50
60
70
80
90
Illumination time (seconds)

Fig 9. Inhibition of energy dependent [^{32}Pi] $\rightleftharpoons$ ATP exchange by pyruvate kinase in spinach chloroplasts. Chloroplasts, 0.19 mg chlorophyll, were incubated in one milliliter of reaction medium containing 5 mM [^{32}Pi] (2267 cpm/nmole) and other additions as shown in Fig 10. The highest pyruvate kinase concentration was arbitrarily set at unity. The pyruvate kinase additions are dilutions thereof. Controls contain phosphoenolpyruvate but not pyruvate kinase. Samples, exposed to light for 30 s, were also accompanied by a dark control. [^{32}Pi] Exchange into organic phosphate (light minus the dark rates) were determined as described in Fig 10.

Fig 9.



2 2.0
2 0.0
1 8.0
1 6.0
1 4.0
1 2.0
1 0.0
8.0
6.0
4 0
2.0
[^{32}Pi] Incorporation (umoles/mg chlor./h)
0.01
0.10
0.50
1.00
[Pyruvate kinase] (units)

ATP $\rightleftharpoons$ [^{32}P] exchange

Spinach chloroplasts catalyze a rapid light-dependent ATP$\rightleftharpoons$[^{32}P] exchange. When spinach chloroplasts were exposed to light in the presence of 1 mM ATP, 5 mM [^{32}Pi] and the electron carrier PMS, the rate of [^{32}Pi] incorporation into organic phosphate was 20.4 umoles/mg chlorophyll/h (Fig 9). Including pyruvate kinase and phosphoenolpyruvate in the reaction medium had a powerful inhibitory effect on the rate of ATP$\rightleftharpoons$ [^{32}P] exchange reaction (Fig 8). Fig 9 shows that inhibition of ATP$\rightleftharpoons$[^{32}P] exchange was related to the concentration of pyruvate kinase (PK) in the reaction medium. At elevated PK, [^{32}Pi] incorporation was inhibited by more than 80%, The possibility that ATP$\rightleftharpoons$[^{32}P] exchange may be completely eliminated at even higher PK concentrations was not tested, but according to experiments by Kayalar et al (140) in mitochondrial particles, ATP$\rightleftharpoons$ [^{32}P] exchange was nearly eliminated at very high PK levels. These results suggest that under conditions of photophosporylation, ATP hydrolysis results in rapid release of ADP into the medium and ATP$\rightleftharpoons$Pi exchange occurs primarily as a result of phosphorylation of medium ADP by [^{32}P]. Reverse phosphorylation of hydrolysis product, ADP on the hydrolytic site, following Pi (bound)$\rightleftharpoons$ [^{32}Pi] (medium) exchange, does not appear to be the major path for ATP$\rightleftharpoons$ [^{32}Pi] exchange; since in such a mechanism ADP would be protected at the catalytic site and ATP$\rightleftharpoons$ [^{32}Pi] would not be very susceptible to PK and PEP inhibition. Two alternate mechanisms which are more consistent with these results are: 1) the catalytic site cooperativity model, where binding of substrate ATP on one site accelerates the release of product ADP and Pi from a second catalytic site (127,185) followed by photophosphorylation of this ADP by [^{32}P]; and 2) these results may also be used as an argument in favor of separate catalytic sites for ATP synthesis and ATP hydrolysis. The high degree of ATP $\rightleftharpoons$ [^{32}P] exchange inhibition by PK and PEP suggests that ATP hydrolysis is largely irreversible under the described conditions. The rapid rate of [^{32}P] incorporation into ADP following ATP hydrolysis (20.4 umoles/mg chlorophyll/h) (Fig 9) as compared to reported rates of γ-[^{32}P]-ATP hydrolysis by excited chloroplasts, 10 umoles/mg chlorophyll/h (191)) suggests that ADP is phosphorylated almost as fast as it is being released from that ATPase site. It is very unlikely that ATP hydrolysis and ATP synthesis can be catalyzed by the ATPase site simultaneously at the same velocity in the two opposite directions.

Adenylate kinase activity in chloroplasts that were passed through a Sephadex G-50 column

The use of chloroplasts instead of pure CF$_1$ in the reported experiments raises the possibility that the observed phosphorylation of bound [^{3}H]-ADP to bound [^{3}H]-ATP was catalyzed by chloroplast enzymes other than CF$_1$. Contribution by adenylate kinase (AK)-like activity may, for example, result in the scrambling of labeled nucleotide between AMP, ADP and ATP by the reversible reaction (194): $2ADP \rightleftharpoons AMP + ATP$. Conversion of free [^{3}H]-ADP to [^{3}H]-ATP (or [^{3}H]-AMP) may therefore have taken place if AK were present in the labeled chloroplasts purified by column chromatography. Label scrambling may occur without a net charge in the concentrations of the labeled nucleotides. Such label scrambling may be detected with unlabeled nucleotide 'traps'. For instance, the addition of exogenous unlabeled ATP, as in the experiments shown in Table III and Fig 7, results in rapid exchange of AK-bound [^{3}H]-ATP with free ATP, trapping released [^{3}H]-ATP in a pool of unlabeled ATP. This is a sensitive method for measuring [^{3}H]-ATP formation. Similarly in the presence of added unlabeled

AMP (with no other nucleotide additions), the formation of [^{3}H]-AMP by AK may also be detected by such a label trapping technique.

AK activity in spinach chloroplasts (from direct assay) was found to be 16.3 umoles ADP formed/mg chlorophyll/h. AK activity was determined from the rate of the reaction: $2ADP \rightleftharpoons ATP + AMP$. The amount of ATP was assayed by the incubation of chloroplasts (0.1 mg chlorophyll) with 1 mM ADP in the presence of 5 mM glucose, saturating amounts of hexokinase and glucose-6-phosphate dehydrogenase, 0.5 units of each (1 unit = one umole of ATP formed/min), 2 mM NAD, 10 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$ and 20 mM Tris buffer, pH 8.0. The rate of NADH formation was monitored by the change in optical density at 340 nm. The passage of chloroplasts through a Sephadex G-50 column, as described in the methods, reduced AK activity tenfold to 1.2 umoles/mg chlorophyll/h. It appeared therefore that AK (MW 22,000 (195)) was removed as a result of chromatography on Sephadex G-50 which has an exclusion MW of 30,000 (196). Although AK activity appeared to be greatly reduced following the passage of chloroplasts through the Sephadex G-50, this activity was not completely eliminated.

In a second approach, the activity of AK was determined by label trapping techniques. Advantage was taken of the observation that nearly 25% (0.48 nmoles/mg chlorophyll) of the label in aged chloroplasts was free [^{3}H]-AMP which can be susceptible to conversion by AK into free [^{3}H]-ADP or [^{3}H]-ATP. A label trap for [^{3}H]-AMP (by the addition of unlabeled AMP to the chloroplasts) should therefore result in a time dependent accummulation of [^{3}H]-AMP which should reflect the endogenous AK activity. As shown in Table IV, exposure of chloroplasts to light in the presence of either AMP + Pi (1 mM each), Pi (1 mM) or controls, did not change the levels of [^{3}H]-AMP over the duration of the reaction (0.5-30 s). There

Table IV. Nucleotide composition of results in Fig 5. The amounts of [^{3}H]-AMP were determined only in control samples (i.e. reaction mixtures lacking AMP or Pi) and not in samples containing added AMP + Pi or Pi only. Values marked with * were calculated by substracting the amount of [^{3}H]-ATP from the mean of the total amount of label in the control sample. The amount of labeled chloroplasts in all samples was assumed to be identical.

TABLE IV

	Control		Pi		AMP + Pi	
	nmoles/mg chlorophyll		nmoles/mg chlorophyll		nmoles/mg chlorophyll	
Exposure time (s)	$[^3H]$-AMP	$[^3H]$-ADP + $[^3H]$-ATP	*$[^3H]$-AMP	$[^3H]$-ADP + $[^3H]$-ATP	*$[^3H]$-AMP	$[^3H]$-ADP + $[^3H]$-ATP
30 (dark)	0.44	1.13	0.57	1.19	0.75	1.01
0.5	0.46	1.14	0.43	1.33	0.62	1.14
1.0	0.74	1.34	0.46	1.30	0.70	1.06
3.0	0.51	1.29	0.59	1.17	0.62	1.14
9.0	0.48	1.34	0.49	1.27	0.65	1.11
30.0	0.48	1.22	0.53	1.23	0.56	1.20
Mean	0.52	1.24	*0.51	1.25	*0.65	1.11

was however, a 20% increase in the [^{3}H]-AMP label in the reaction performed in the presence of AMP + Pi as compared to controls, suggesting the presence of AK.

However, while the levels of [^{3}H]-AMP remained largely unchanged during the indicated reaction interval, the amount of [^{3}H]-ATP that was formed from [^{3}H]-ADP in the same reaction period (Fig 5) increased by more than four-fold, from 0.2 to 8.5 nmoles/mg chlorophyll. This suggests that the formation of [^{3}H]-ATP from [^{3}H]-ADP in the presence of AMP + Pi or Pi alone was an energy dependent reaction which was too rapid to be accounted for by the observed low levels of AK.

Using similar label trapping techniques, the results in Table V reveal that adding labeled chloroplasts to the reaction solutions in which the ATP concentrations were varied from 0-20 mM while maintaining the ADP concentration at 1 mM caused no significant decrease in the [^{3}H]-AMP levels. Since the reaction mixture was exposed to light for 5 s, it can be assumed that most of the bound nucleotides were exchanged into the medium and the released [^{3}H]-ATP and [^{3}H]-ADP trapped in their respective pools of unlabeled nucleotides. Only [^{3}H]-AMP remained in trace amounts (0.24 nmoles/mg chlorophyll) and undiluted. Disappearance of [^{3}H]-AMP, as a result of AK catalyzed phosphoryl transfer from ATP to [^{3}H]-AMP should have been easily detected. The absence of a change in [^{3}H]-AMP levels at high ATP concentrations, Table V, suggests that AK activity during the reaction period was negligible. In comparison, under the same reaction conditions, the amount of [^{3}H]-ATP that formed from [^{3}H]-ADP increased by nearly 65% from 0.66 nmoles/mg chlorophyll to 1.03 nmoles/mg chlorophyll. In view of the small changes in the [^{3}H]-AMP levels under the conditions which favored its removal by AK, it is unlikely that under the same conditions AK catalyzed the phosphorylation of [^{3}H]-ADP.

Table V. The effects of free nucleotides on chloroplast-associated labeled nucleotides. Labeled, Sephadex-treated chloroplasts, 76 ug chlorophyll, were added to the reaction medium which contained 20 mM tricine-NaOH buffer, pH 8.0, 5 mM $MgCl_2$, 50 uM PMS and the indicated concentrations of ADP, ATP and Pi. The reaction mixtures (final volume, 1 ml) were exposed to light for 5 s, then terminated by the addition of 1.5 ml of 10 M urea, followed by immediate storage at 0° C in the dark. The samples were analyzed for labeled nucleotide composition. The control reflects the composition of the labeled nucleotides of chloroplasts prior to reaction.

TABLE V

Additions				nmoles/mg chlorophyll			
ADP mM	ATP mM	Pi mM		$[^3H]$-AMP	$[^3H]$-ADP	$[^3H]$-ATP	$[^3H]$-ATP/$[^3H]$-ADP
0	0	0	(control)	0.21	1.52	0.34	0.22
1	0	5.0		0.29	1.50	0.66	0.44
1	1.0	5.0		0.24	1.35	0.75	0.56
1	2.5	5.0		0.21	1.29	0.91	0.71
1	5.0	5.0		0.25	1.28	0.93	0.73
1	10.0	5.0		0.23	1.21	0.94	0.78
1	20.0	5.0		0.23	1.14	1.03	0.90

Table VI. <u>The effect of diadenosine pentaphosphate (A_2P_5) on ATP dependent dark phosphorylation</u>. Labeled thylakoids, 70 ug chlorophyll, were added to the reaction medium containing 20 mM tricine-NaOH buffer, pH 8.0, 4 mM $MgCl_2$, 10 mM NaCl, 50 uM PMS, 2.5 mM Pi and the indicated additions of nucleotides and diadenosine pentaphosphate (A_2P_5). The mixture (final volume, 1.0 ml) was illuminated with white light for 5 s. The reaction mixtures were terminated with twice the volume of 10 M urea followed by storage at 0° C in the dark. Samples were analyzed for labeled nucleotide composition on PEI columns as described in the methods.

TABLE VI

Additions					
ADP mM	ATP mM	Pi mM	A_2P_5 mM	$[^3H]$-ADP + $[^3H]$-ADP cpm/mg chlorophyll x 10^3	$[^3H]$-ATP/$[^3H]$-ADP
1	0	2.5	0	18.3	0.47
1	0	2.5	0.01	17.1	0.48
1	1	2.5	0	17.7	0.60
1	1	2.5	0.01	17.6	0.66
1	10	2.5	0	18.1	0.72
1	10	2.5	0.01	18.9	0.72
0	1	2.5	0	-	-
0	1	2.5	0.01	17.1	2.23

More likely explanations for the observed [^{3}H]-ATP formation are either light dependent phosphorylation of [^{3}H]-ADP or ATP dependent dark phosphorylation of bound [^{3}H]-ADP or a combination of the two reactions.

Further, Table VI shows the effects of diadenosine pentaphosphate (A_2P_5) on ATP dependent phosphorylation of bound [^{3}H]-ADP. A_2P_5 inhibition of spinach chloroplast AK has been reported by McCarthy (166). The inhibition patterns of spinach AK by A_2P_5 were not determined. At the concentration used in the experiments given in Table VI (10^{-5}M), A_2P_5 inhibited the formation of ATP by more than 90% when rabbit muscle AK, approximately 0.1 units (1 unit = 1 umole ATP formed/min), was incubated with 1 mM ADP. Similar inhibition of chloroplast AK was assumed in the reaction samples of Table VI. However, the direct confirmation of this assumption was not made and positive control samples were not obtained. While the amount of [^{3}H]-ADP conversion to [^{3}H]-ATP increased with increased medium ATP concentration, there was no detectable difference between reactions in which A_2P_5 was included and reactions where the inhibitor was omitted. At least partial inhibition of this reaction would have been expected in the presence of 0.01 mM A_2P_5 if this ATP dependent phosphorylation of bound [^{3}H]-ADP had been catalyzed by AK.

In summary, a number of different approaches to measuring the AK activity appears to indicate that the AK activity in spinach chloroplasts following gel filtration is greatly reduced. More importantly are the results from the label trapping experiments (Tables IV and V) which show that the amount of label scrambling that can be attributed to AK is too small to account for the large amount of [^{3}H]-ADP phosphorylation to [^{3}H]-ATP in the presence of ATP (Table III) or in a light reaction in the presence of Pi (Fig 5). Although an effort was made to demonstrate that contamination by AK is not a major factor in bound [^{3}H]-ADP phosphorylation, it does not exclude contamination by other systems in spinach chloroplasts that use nucleotides as reaction substrates. Any such reaction which is reversible may result in label redistribution between different nucleotides. Such contamination is a problem if free nucleotides were being studied. The observed ATP-dependent dark phosphorylation of bound [^{3}H]-ADP to form bound [^{3}H]-ATP occured on the thylakoids without release into the medium. It was therefore not accessible to modification by other enzymes.

DISCUSSION

Conversion of tightly CF_1-bound ADP to ATP on thylakoid membranes.

In this report, we show that [^{3}H]-ADP bound on a high affinity site of thylakoid bound CF_1 can be converted to [^{3}H]-ATP which remains firmly bound. We observed the synthesis of bound ATP from bound ADP in two unrelated experiments. Synthesis of bound ATP was indicated when chloroplasts bearing bound [^{3}H]-ADP were illuminated in a reaction solution supplemented with inorganic phosphate, 1 mM, in the absence of added nucleotides. Under these conditions bound [^{3}H]-ADP was phosphorylated with retention of the product [^{3}H]-ATP on the thylakoid membranes (Figs 5 and 6). Phosphorylation of bound [^{3}H]-ADP to bound [^{3}H]-ATP was also observed when chloroplasts containing bound labeled ADP were incubated in the dark with ATP (Table III). It is of interest that both reactions have in common the conditions where exchange of bound nucleotides is highly restricted; i.e. the reaction in the presence of light energy but in the absence of medium nucleotides or the reaction in the dark in the presence of ATP. A further similarity between the two phosphorylation reactions was their dependence on energy. In the first case, the protomotive force was generated by the exposure of chloroplasts to light; in the second case by ATP hydrolysis (189). Using conditions which drives phosphorylation but restricts exchange may have permitted the isolation of the bound ATP.

The analysis of the results in Figs 5 and 6 show that although the exchange (loss) of bound nucleotides was greatly restricted in the absence of medium nucleotides (in the presence of Pi only), it was not completely eliminated. Nearly 30% of the bound nucleotides were discharged from the chloroplasts into the medium during a 5 s light exposure, Fig 4. Following this initial loss of bound nucleotides, however, the amound of remaining nucleotides bound to the thylakoid membranes (between 5 and 30 s of light exposure) remained essentially the same at 0.89 nmoles/mg chlorophyll. Based on these observations, the bound nucleotides appear to reside on two different sites on CF_1. Those that released their nucleotides upon excitation of chloroplasts, the exchangeable sites, and those that retained their nucleotides, the non-exchangeable site. We further observed that while the amound of bound nucleotides on the non-exchangeable site remained constant with continued illumination of chloroplasts. The amount of ATP on that site continued to increase in the same period, suggesting either that phosphorylation of bound [^{3}H]-ADP has occured on its original non-exchangeable site site or that exchange has occured between midium [^{3}H]-ATP and bound [^{3}H]-ADP, where medium [^{3}H]-ATP was formed from medium [^{3}H]-ADP during the light reaction.

Unlike the above observations where the origin of [^{3}H]-ATP on the non-exchangeable site may be questioned, phosphorylation of bound [^{3}H]-ADP in the absence of light, in medium that contained ATP (Table III), can occur only at the original binding site. Only the chloroplast bound nucleotides are shown in Table III. Unbound nucleotides were removed following the reactions. Exchange of bound [^{3}H]-ADP with free nucleotides in the dark followed by its phosphorylation to [^{3}H]-ATP on a second site is therefore highly unlikely. Had such an exchange occurred at any point during the reaction, the [^{3}H]-ADP released would have been immediately diluted and lost in the pool of free ADP (Sample 3, 1 mM ADP + 10 mM ATP, Table III). Further, the uniform concentrations of the chloroplast bound nucleotides following the dark reactions (Dark Samples 1,2,3,4,7, and 8 of Table III) indicate that the bound nucleotides remained firmly attached to the chloroplast in the dark reactions. This was independent of nucleotide composition in the reaction medium. While the amount of these bound nucleotides remained essentially constant, the ratio of [^{3}H]-ATP bound/[^{3}H]-ADP bound increased more than 5-fold from 0.41 to 2.56 as the percentage of ATP in the reaction medium increased from 0 to 100. On the basis of the above observations, the involvement of soluble enzymes such as adenylate kinase in the conversion of bound [^{3}H]-ADP to bound [^{3}H]-ATP can be largely ruled out. In addition, as shown in Tables IV and V, the rate of label scrambling between soluble adenine nucleotides that can be attributed to adenylate kinase activity was too low to be measured. Therefore, adenylate kinase activity in such chloroplast preparations as used in the experiments shown in Table III (i.e. performed under similar conditions as those of the experiments shown in Tables IV and V) could not account for the large amount of chloroplast-bound [^{3}H]-ATP which was formed from bound [^{3}H]-ADP in the dark. A possible mechanism for the formation of bound [^{3}H]-ATP from bound [^{3}H]-ADP may involve the binding of free ATP (or ADP) to a site other than the non-exchangeable site, most likely the exchangeable site(s); and as shown recently by Girault et al (184), ATP binding to the exchangeable sites of CF_1 can lead to its hydrolysis. It is therefore possible that the energy derived from such ATP cleavage is conserved and used for phosphorylation of bound ADP on the non-exchangeable site. As expected from such a model, interference with ATP binding, cleavage or formation of a high energy state, would result in the inhibition of bound [^{3}H]-ADP phosphorylation. In agreement with these prediction, displacement of ATP with ADP resulted in the inhibition of bound [^{3}H]-ADP phosphorylation. ADP competes with ATP binding to CF_1 (93). As the fraction of ADP was increased relative to ATP, the amount of [^{3}H]-ADP phosphorylation decreased proportionately (Table III, dark samples 1-4), similarly substituting the non-hydrolyzable analogue of ATP, AMPPNP, failed to induce bound ADP phosphorylation (Fig 7). As shown in Table III, synthesis of bound [^{3}H]-ATP was uncoupler sensitive (20 mM NH_4Cl inhibited ATP dependent phosphorylation of bound [^{3}H]-ADP). NBD.Cl (1 mM) was equally effective in preventing this phosphorylation. Rienitz et al (192) and Davenport and McCarthy (189) have shown that thylakoids hydrolyze ATP in the dark after illumination which can lead to regeneration of transthylakoid proton gradient. The latter have further shown that this gradient promotes ATP ⇌ Pi exchange and can be used for ATP synthesis. In line with these observations, it appears that the uncoupler, NH_4Cl, dissipated the hydrogen ion gradient formed by hydrolysis of ATP, thereby inhibiting phosphorylation of bound [^{3}H]-ADP. This sensitivity to uncouplers indicates that phosphorylation of bound [^{3}H]-ADP requires a hydrogen ion gradient, as would be predicted by the Mitchel hypothesis (43).

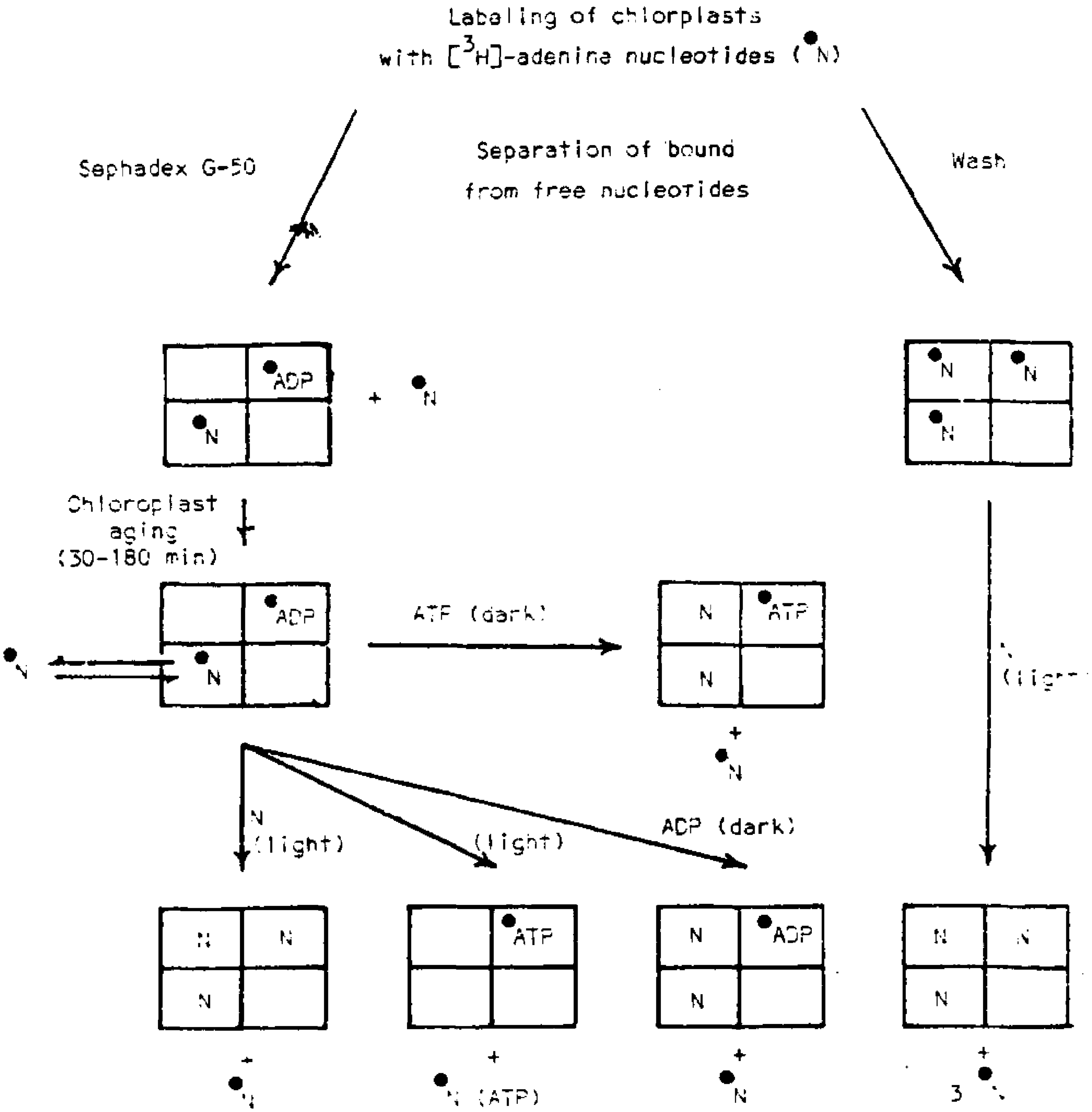
The apparent energy requirement (in the form of a proton motive force) for the phosphorylation of bound ADP to bound ATP differs from other models of ATP synthesis which propose energy input requirement for substrate binding and product release steps and not for condensation of ADP + Pi (96,138). This difference maybe reconciled if energy supplied by ATP hydrolysis generates a local pH change at the catalytic site of the enzyme. The pH change may either induce a conformational change in the enzyme or enhance Pi binding which subsequently may in turn stimulate the phosphorylation reaction. The low pH optimum (pH 6.0) for Pi binding, observed by Kasahara and Penefsky (106) and the low pH optimum (pH 6.0) for phosphorylation of bound ADP by Pi observed by Sigman and Feldman (107) in soluble CF_1, support the above arguments. Our results are in agreement with the requirement of energy for the exchange of the phosphorylated intermediate.

The function, if any, of bound [^{3}H]-ATP formed on the non-exchangeable site in photophosphylation is not clear. Based on the following evidence however, we propose that the non-exchangeable site can not serve as the major path of photophosphorylation: 1) Lack of rapid and quantitative generation of [^{3}H]-ATP on the non-exchangeable site following energization of thylakoids by exposure to light. As shown in Fig 5, phosphorylation of bound [^{3}H]-ADP, $t_{\frac{1}{2}}$ of approximately 5 s, is much too slow to be consistent with photophosphorylation; 2) Slow formation of bound [^{3}H]-ATP during photophosphorylation conditions in the presence of ADP and Pi (Fig 7). The small initial surge of [^{3}H]-ATP formation that was observed during the illumination period of 0-1 s suggests that nucleotides on the non-exchangeable site exchanged with free nucleotides before significant phosphorylation of bound [^{3}H]-ADP could have taken place. It appears therefore that bound [^{3}H]-ADP phosphorylation is slow regardless of whether Pi only or ADP + Pi are in the reaction medium (exchange of bound with free nucleotides, $t_{\frac{1}{2}}$ = 0.25 s, Fig 3, is more rapid than the rate of [^{3}H]-ADP phosphorylation, t = 5.0 s, Fig 5); 3) Slow exchange of nucleotides from the non-exchangeable site. The measured exchange rates of nucleotides on the non-exchangeable site with free nucleotides was at best 13.3 umoles/mg chlorophyll.h (Figs 3 and 4). This rate is substantially lower than the rates of photophosphorylation, under similar conditions, typically in the range of 500 umoles/mg chlorophyll/ h. It appears therefore that slow exchange of nucleotides on the non-exchangeable site can not accommodate the rapid exchange of nucleotides that are required during photophosphorylation. These results are basically in aggreement with Bickle-Sandkotter and Strotman (177) and Dunham <u>et al</u> (188) who have similarly concluded that the exchange of nucleotides on chloroplast high affinity sites are too slow to be consistent with photophosphorylation. What other function is served by the non-exchangeable site and by the observed conversion of bound [^{3}H]-ADP to bound [^{3}H]-ATP on that site is currently unclear. It is possible that it may have a regulatory role or some catalytic function. Further discussion of the non-exchangeable site is given in the following sections.

Further characterization of CF_1 binding sites.

In this study, the nucleotide binding sites on spinach chloroplasts were defined in more detail. Fig 10 is a schematic summary of nucleotide binding properties to thylakoid membranes. From the amount of energy dependent labeled nucleotides incorporation into chloroplast binding sites, a maximum of 3.7 nmoles of nucleotides were found to be bound to the chloroplasts/mg chlorophyll. Since the total amount of chloroplast bound nucleotides were not determined, it is possible that additional binding sites were also present which did not exchange their nucleotides under the described conditions and that such sites were not revealed by the labeled nucleotide exchange reactions. Those labeled nucleotides which were incorporated into the chloroplast binding sites exchanged when chloroplasts were exposed to light in the presence of medium nucleotides. Only the CF_1-bound nucleotides are known to exchange under these conditions. It is also generally agreed that thylakoid bound nucleotides are associated with CF_1. Additionally, based on measurements by Strotman (186) showing 1.3 nmoles CF_1/mg chlorophyll, 3.7 nmoles/mg translates to 3 moles of bound nucleotides/mole CF_1 which is in agreement with other similar determinations in chloroplasts and isolated CF_1 (96,116,184, 191). However, as of yet, it has not been firmly established that all chloroplast bound nucleotides are associated with CF_1. Passing labeled chloroplasts through a Sephadex column results in the retention of approxi-

Fig 10. Schematic summary of adenine nucleotide binding properties to chloroplast membranes. The shaded areas represent the hypothetical non-exchangeable binding site(s) and the light areas show the exchangeable sites. Labeled $[^3H]$-nucleotides (N) incorporation into CF_1 binding sites, the wash and gel filtration procedures for separation of bound from free labeled nucleotides are described in Methods. The other steps are given in the text. Washed chloroplasts were used shortly after preparation without the aging of chloroplasts. N denotes unlabeled nucleotides.


Labeling of chlorplasts
with [3H]-adenine nucleotides (●N)
Sephadex G-50
Separation of bound
from free nucleotides
Wash
●ADP
●N
+ ●N
●N
●N
●N
Chloroplast
aging
(30-180 min)
●ADP
●N
●N
ATP (dark)
N
●ATP
N
+ ●N
(lig
N
(light)
(light)
ADP (dark)
N
N
N
+ ●N
●ATP
+ ●N (ATP)
N
●ADP
N
+ ●N
N
N
N
+ 3 ●N

mately 2 nmoles of labeled bound nucleotides/mg chlorophyll. Extraction of CF_1 from these labeled chloroplasts shows that part of this label was associated with CF_1 (approximately 0.05 nmoles of labeled nucleotides/nmole of CF_1 (M.W. 325,000)).

The two nmoles of nucleotides/mg chlorophyll were observed to be chloroplast bound immediately after gel filtration; 30 min thereafter, however, almost one half of the bound label dissociated from the chloroplasts suggesting that the nucleotides on these Sephadex-treated chloroplasts were bound to two distinct binding sites. One binding site slowly loses its nucleotides to medium free of nucleotides, in the dark (the exchangeable site) (Fig 1, Table I). The other binding site appears to require both medium nucleotides and an excited state for their exchange (non-exchangeable site). Two distinct nucleotide binding sites on chloroplasts are also suggested from the properties of the small amount of residual bound nucleotides (approximately 0.2 nmoles/mg chlorophyll, Fig 4 and Table III) which remain associated with the aged chloroplasts. These nucleotides differ from nucleotides on the non-exchangeable site in that they exchange in the dark in the presence of medium nucleotides and a similar amount of residual nucleotides also appear to be lost from the chloroplasts following exposure to light in the absence of free nucleotides. These residual nucleotides may represent the remnant of nucleotides which were discharged from the exchangeable binding site in the dark following the elution of chloroplasts from the Sephadex column; and the amount of these nucleotides that remained bound may reflect their equilibrium state with the released nucleotides. Although only two types of binding sites appear to be indicated in chloroplasts which were subjected to gel filtration, other binding sites may also be present. However any such site(s) with reduced affinity for adenine nucleotides may have been vacated of their nucleotides by gel filtration and no residual nucleotides at such a site(s) would be detected.

Proposed role of chloroplast binding sites in photophosphorylation.

A significant amount of evidence suggest that the high affinity binding site(s) on chloroplast membranes may be the same as the ATPase site(s) on chloroplast coupling factor, CF_1. For example, CF_1 ATPase, which appears to reside on the β subunit of CF_1 (110), is latent. Its activation by light, DTT (130), heat (131) or tryptic digestion (52) is corrolated with the removal of the ϵ subunit (89). This treatment also exposes an additional nucleotide binding site and an NBD.Cl reaction site (101). In thylakoids, the resistance of bound nucleotides on the non-exchangeable site (shaded areas, Fig 10) to removal by gel filtration, prolonged aging, energized state in the absence of free nucleotides or dark exchange with added nucleotides bears resemblance to ATPase latency and the protected third binding site in soluble CF_1; this similarity suggests that in both systems, the same site may be involved. Similar interpertations are also offered by Selman and co-workers (188,191) based on results in which they observed that light and DTT activates the latent thylakoid bound CF_1 ATPase and also exposes the high affinity non-exchangeable site for exchange with medium nucleotides. Without such activation, in the dark, this site appears to be protected against exchange and the ATPase activity is latent.

Although it remains to be proven, based on circumstantial evidence such as that given above, it is conceivable that the non-exchangeable site is the ATPase site on CF_1. The slow phosphorylation of bound nucleotides on the non-exchangeable site in addition to the slow exchange of nucleotides from such a site as was observed in this report and by others (177,188), do not counter the above model and may infact lend support to it. For instance, the exchange rate of nucleotides from the non-exchangeable site was found to be 13.3 umoles/mg chlorophyll/h (Fig 3). This rate is comparable to the rate of ATP hydrolysis, 10 umoles/mg chloroplast/h (191) and to the rate of ATP $\rightleftharpoons$ [^{32}Pi] exchange, 20.4 umoles/mg chlorophyll/h (Fig 9), but not to the rate of photophosphorylation which was more than an order of magnitude faster. These results appear to be best explained from the standpoint of separate catalytic sites for ATP hydrolysis and ATP synthesis.

Other evidence to support the concept that ATP hydrolysis and synthesis occur on separate sites: 1) Simultaneous and nearly equivalent ATP hydrolysis and ATP synthesis by excited chloroplasts in the presence of ATP and Pi (see ATP $\rightleftharpoons$ [^{32}Pi] exchange in the Results Section) indicate that it is unlikely that a single site (the ATPase or the non-exchangeable site) catalyzes both reactions. Since the exchange reaction was performed in the presence of 1 mM ATP + Pi with no added ADP, it appears that the similar rates for ATP synthesis and hydrolysis found were achieved if two separate sites were involved; 2) The inhibition of energy dependent ATP $\rightleftharpoons$ [^{32}Pi] exchange by pyruvate kinase and phosphoenol pyruvate; and 3) Similar rates of [^{3}H]-ADP photophosphorylation by excited chloroplasts in the presence of ATP or AMPPNP (Fig 7, reaction intervals between 0 and 1 s) also argue against the ATPase (non-exchangeable) site being the site of photophosphorylation. Others have cited similar results with AMPPNP as evidence against same site catalysis for ATP synthesis and ATP hydrolysis (171,193).

An additional finding in these experiments is that the residual bound nucleotide on the exchangeable site is in a large part in the form of ATP (Table III, dark samples 5 and 6). This ATP was stable in the dark (during the time course of the experiment, approximately 3 min). From the decrease in the [^{3}H]-ATP/[^{3}H]-ADP ratio following exposure of the chloroplasts to light (Table III, light samples 5 and 6), it appears that bound [^{3}H]-ATP was preferentially lost from the exchangeable site. The energy dependent release of residual nucleotides from the exchangeable site, although slow in the absence of medium nucleotides, $t_{\frac{1}{2}} > 0.5$ s (Fig 4), may be greatly accelerated by the addition of free nucleotides. More rapid movements of nucleotides in and out of the exchangeable sites may infact indicate that they are the sites of photophosphorylation.

The model used in these experiments can supplement studies with isolated coupling factor. Subjecting labeled chloroplasts to gel filtration effectively separates bound from free nucleotides and also results in fewer occupied binding sites ; two binding sites were identified. The fact that an intact ATP synthetase complex was used has permitted the study of the binding sites in detail not possible with the isolated enzyme. This is particularly true in the area of energy conversion during ATP synthesis. The use of Sephadexed labeled chloroplasts can therefore serve as an important model for continued studies of the steps in ATP synthesis.

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