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University of Illinois at Urbana-Champaign
Ph.D., 1977
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PARTIAL CHARACTERIZATION OF ADENINE NUCLEOTIDE
BINDING TO ISOLATED CHLOROPLAST COUPLING FACTOR

BY

DAVID LEE VANDERMEULEN

B.A., Central College, 1970

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biophysics
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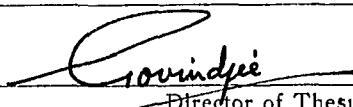
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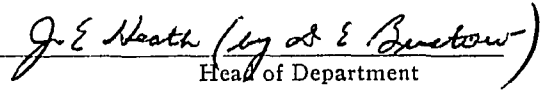
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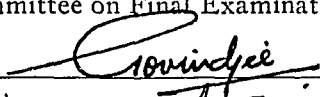
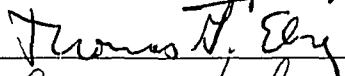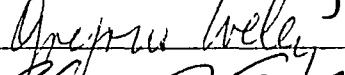BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

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† Required for doctor's degree but not for master's

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DEDICATION

To my parents, without whom neither I
nor this thesis would have been possible.

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CF_1	chloroplast coupling factor
Chl	chlorophyll
εAMP	$1,N^6$-etheno adenosine monophosphate
εADP	$1,N^6$-etheno adenosine diphosphate
εATP	$1,N^6$-etheno adenosine triphosphate
FeCy	ferricyanide
K_d	dissociation constant
K_1	smallest apparent K_d in a two binding site system
K_2	largest apparent K_d in a two binding site system
p	polarization of fluorescence
PMS	N-methylphenazonium methylsulfate
SDS	sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

The major function of the photosynthetic process in plants and photosynthetic bacteria is the conversion of solar energy into chemical energy. Much of this process as a whole has already been elucidated (1-3), but many of the mechanistic details are not known at the molecular level. As in most biological processes, structural and functional changes in photosynthesis appear to be closely related. The chloroplast of green plants is composed of flattened membraneous sacs called thylakoids; these sacs are often arranged in stacks, referred to as grana (4). The thylakoid membranes contain the pigments, enzymes and other molecules necessary to support "uphill" electron transfer from water, through two different photosystems, to nicotinamide adenine dinucleotide phosphate ($NADP^+$). The reduced product (NADPH) is used as reductant in the subsequent conversion of phosphoglyceric acid to phosphoglyceraldehyde during the Calvin cycle for CO_2 fixation.

Linked to electron transport from water to $NADP^+$ is the production of ATP (adenosine triphosphate, also required for the Calvin carbon cycle) from adenosine diphosphate (ADP) and inorganic phosphate (P_i) utilizing the coupling factor (CF_1) protein or ATP-synthetase enzyme found on the exterior

of the thylakoid membranes (5-7). Membrane-bound or isolated CF_1 protein is seen as a 90-100 Å particle in electron micrographs (8,9). The purified CF_1 complex has a molecular weight of 325,000 (10), and can be dissociated by SDS-electrophoresis into five different types of subunit peptide chains, whose approximate molecular weights are: alpha (59,000), beta (56,000), gamma (37,000), delta (17,500) and epsilon (13,000), (11-13). Chemical cross-linking studies of CF_1 point to a minimal subunit stoichiometry of $(alpha)_2$, $(beta)_2$, $(gamma)_1$, $(delta)_1$, $(epsilon)_2$, and a possible structural model derived from the data was presented (14). (Further details concerning the characteristics of isolated and membrane-bound CF_1 are summarized by Jagendorf (15). Photosynthetic energy conservation in general and photophosphorylation have also been reviewed (15-18).)

It was the intent of this study to investigate and partially characterize some aspects of the mechanism of photophosphorylation, especially as regards the involvement of CF_1, using fluorescence analysis in conjunction with other standard techniques. Considering first the process of energetic coupling between electron transfer and phosphorylation, it is known that electron transport induces ionic pumping leading to ion gradients and an electrical potential across the thylakoid membrane. If inhibitors are added that dissipate the ion gradients and/or membrane potential, ATP production

is no longer measurable; thus, these components have in the past been assumed to be important for the formation of the so-called "high energy intermediate," although there is doubt as to what role, if any, the membrane potential component plays in steady-state photophosphorylation. The exact form of the "energized intermediate state" is not known, but there are several theories postulated that are under consideration by investigators in photosynthesis. These include the well-known "chemical," "chemiosmotic" and "conformational" coupling hypotheses, as well as various modifications.

A. Summary of proposed models of phosphorylation

We will not consider the chemical hypothesis as such in detail here. For literature on this model, see refs. 19-26. This theory, as far as chloroplasts and mitochondrial energetics is concerned, has been seriously questioned by many, since a thorough search has failed to produce convincing evidence for covalent intermediates; see ref. 27 for literature on this point. In addition, Chaney and Boyer (28) measured the relative rates of transfer of the two parts of P_i, i.e., the exchange between phosphate and water oxygen atoms, and the exchange of the phosphate atom with ATP; since no discrepancy in rates was detected, it was concluded that either there is no pool of a phosphorylated, covalent intermediate, or its size (within their quantitative limits) is

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less than 3 per Cyt f (i.e., less than the estimated number

of CF_1 molecules). Thus, with the limitations of the present

evidence, it is suggested that in chloroplast phosphorylation

the only phosphorylated intermediate may be enzyme-bound P_i

(phosphorylated CF_1 protein).

Green and Ji (29,30) proposed that the "downhill"

ATPase reaction could be reversed by directly utilizing the

energy of a coupled exergonic reaction. In this view, the

complex of ADP plus phosphate bound to CF_1 might be "forced"

to react, yielding ATP and water. According to Weber (31),

such a one-step scheme is not viable, since in order to bring

the two reactions together in space and time to produce ATP

from ADP (against the thermodynamic gradient), the so-called

"catalytic enzyme complex" would have to have a lifetime

long enough to allow bound ATP to follow the thermodynami-

cally more favorable route of hydrolysis.

Various aspects of data and theories relating to the

involvement of conformational changes of CF_1 in energy trans-

duction have been summarized by Boyer et al. (32) and

Jagendorf (33). According to a recent form of the conforma-

tional coupling hypothesis by Boyer and coworkers (34-36)

and Harris and Slater (37), ATP is formed at coupling sites

with limited or even no energization from electron transfer.

If it is assumed that the dissociation equilibria of ADP-CF_1

and P_i-CF_1 complexes are much higher than the ATP-CF_1 complex

in the inactive (no energy yet) state, Harris and Slater

(37) predicted that the equilibrium of the ATPase reaction

would be shifted to the formation of firmly bound ATP. Under

usual experimental conditions, the ATP/ADP ratio is normally

only about 10^{-8} at equilibrium. However, (a) an effective

increase in the local concentration of ADP and P_i that may

result from their binding close to each other, (b) a decrease

in water activity at the catalytic site, and/or (c) the pre-

ferential tight binding of ATP at the catalytic site, could,

in Boyer's view (35), increase the ATP/ADP ratio by several

orders of magnitude to about one or greater, thus allowing

formation of ATP with little or no energy input. Subse-

quently, bound ATP is thought to be liberated to the medium

by means of an energy-requiring protein conformational change

which transforms a "tight" enzyme-ATP complex into a "loose"

complex. Experimental data obtained with submitochondrial

particles was interpreted by Boyer as supporting his proposal

(36); other experiments in which the release of tightly bound

radioactive-labelled adenine nucleotides from CF_1 by the

action of either light-induced photosynthetic electron trans-

port or an acid-base transition demonstrated energy dependency

of this adenine nucleotide exchange on CF_1, and were taken as

support for the conformational hypothesis (38,39).

As Rosing et al. (40) point out, though, it was never

demonstrated that reactions or release of the tightly bound

nucleotides occurred with sufficient rapidity to qualify as
intermediates in the main path of ATP synthesis, or were in-
volved in initial ATP formation. Some preliminary data of
Bachofen et al. (41) was inconsistent with the model pre-
sented above; bound ATP in chloroplasts could not, under
their experimental conditions, be released by light, a pH
jump or by the addition of ATP. The recent data of Rosing
et al. (40) and Smith et al. (42) have also demonstrated that
at least a portion of this model may not be tenable for
chloroplasts. Using rapid mixing and quenching techniques,
it was demonstrated that the reactions converting (a) bound
ATP plus bound AMP to bound ADP, or (b) bound ADP plus medium
P_i to ATP are both only slow side reactions (42). Similar
millisecond mixing and quenching experiments (40) showed that
most or all of the initial ATP arises from medium ADP and
medium P_i. With no or low amounts of added medium ADP, the
tightly bound ADP present in thylakoid membranes is released
to the medium on energization and then subsequently forms ATP
(by an as yet undetermined mechanism). Rosing et al. (40)
state that these results eliminate the possibility that con-
version of the ADP tightly bound to CF_1 is a step in the main
pathway of chloroplast phosphorylation as previously suggested
(37,38,43-47).

Recently, Boyer (48) extended the conformational
hypothesis presenting a possible mechanism whereby a proton

gradient (an integral part of Mitchell's chemiosmotic hypothesis, discussed below) could be coupled to ATP synthesis. Inspired in part by experimental findings of his coworkers (34,36), he suggested that energy input may drive ATP synthesis by a change in the binding of reactants at the catalytic site. Thus, in photophosphorylation, conformational changes may be coupled to exposure of charged groups on CF_1 (unspecified proton accepting groups, perhaps a carboxyl on CF_1) to different sides of the membrane. A cycle of charged group exposure or movement may be coupled to proton translocation across the membrane, according to Boyer (Fig. 1A).

In the framework of Mitchell's chemiosmotic hypothesis (49), a membrane is considered essential as the energy of oxido-reduction is stored in the form of a proton gradient and the attendant transmembrane electric potential (together, the protonmotive force or pmf). This proton gradient is formed as a natural consequence of electron and hydrogen acceptors arranged on the different sides of the membrane (Fig. 1B). The gradient is supposed to account for the entire free energy of hydrolysis of ATP. The central functional agent in this coupling proposal is an anisotropic membrane-bound ATPase. A stoichiometric relation of two protons translocated for each ATP formed, as predicted by Mitchell, has been observed in some (but not all) cases (52), as well as a functional ATPase (CF_1) on the lamellar outside surface

Fig. 1A.   Diagram of Boyer's scheme of conformational coupling of phosphorylation to a proton gradient. $B^{\ominus}$ represents a proton accepting group, of which a number, n, change exposure from one side to the other of a membrane coupled to conformation and catalytic changes at the phosphorylation site that drive ATP synthesis.   The positive charge, depicted at a membrane pore, is supposed to prevent hydronium ion diffusion, but allow changes in exposure of $B^{\ominus}$ and free diffusion of water.   Changes in binding and catalytic properties of the phosphorylation site are represented by the transitions in stages A,B,C, and D; Boyer suggests that this conversion, through steps 1 to 4, respectively, serves to drive ATP synthesis coupled to translocation of n protons across the membrane. (Fig. 1A is taken from Fig. 1 of ref. 48.)

Fig. 1B.   Diagram of one possible representation of "vectorial electron flow" across the thylakoid membrane with two proton liberation sites inside (in the loculus).   This scheme is supported by studies on the topography of the chloroplast membrane (50).   Each native coupling site (energy-conserving site) is comprised of an electrogenic part in the photosystem (charge transfer across the membrane in the primary photoact), and an electron neutral proton translocating part, together completing a so-called "Mitchellian loop."   (Fig. 1B is taken from Fig. 2 of ref. 51.)

Fig. 1C.   Diagram of a scheme for ATP synthesis compatible with Mitchell's "chemiosmotic molecular mechanism" for a proton translocating ATPase.   The perspective here is magnified compared to Fig. 1B.   The active center is proposed to be in the interface region between the lipophilic HF_o and the more hydrophilic CF_1 components of the ATPase complex.   See text for further details.   (Fig. 1C is adapted from Fig. 4 of ref. 27.)

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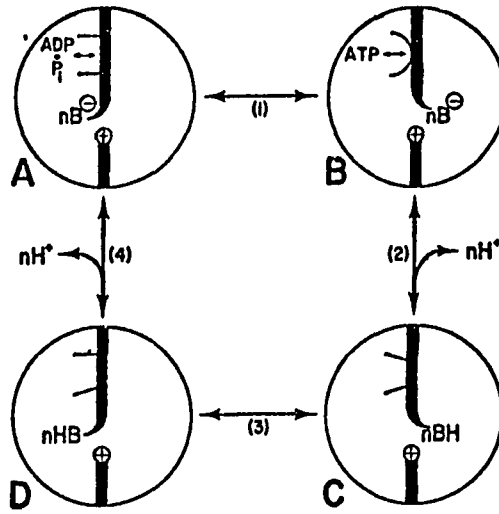
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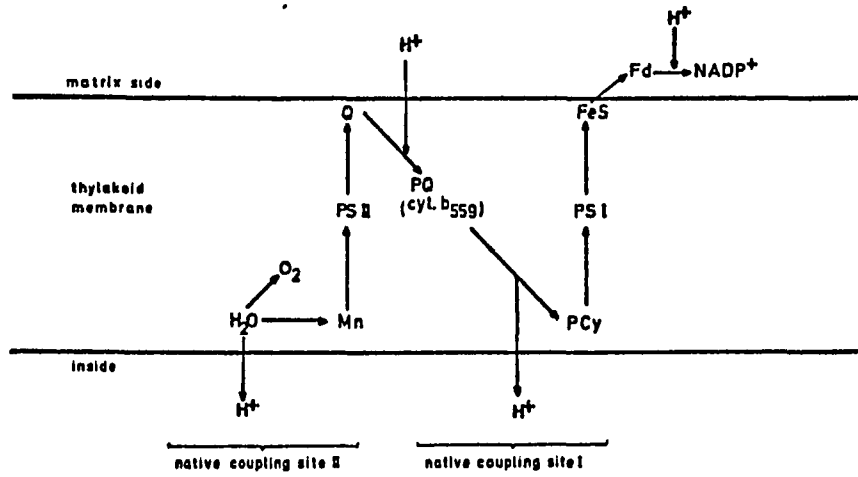
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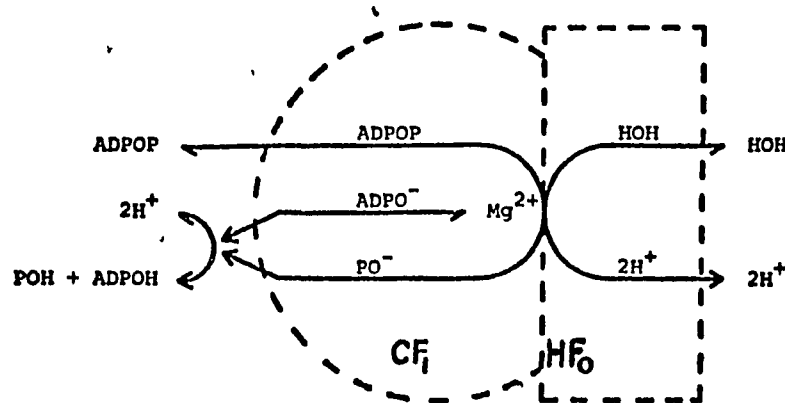
(53), which can bind adenine nucleotides and perhaps act in transphosphorylation reactions (46,47). Whether the proton pumping is obligatory to the energy coupling or a by-product has not yet been definitely established. But in experiments consistent with some kind of chemiosmotic mechanism, Uribe and Jagendorf (54,55) found that the collapse of an artificially imposed pH gradient in the dark could result in net ATP synthesis if ADP and P_i were provided. (The relation between hydrogen ion gradients and possibly relevant membrane configurational changes has been reviewed (17).)

Support for this model was obtained in another system, the "purple membrane" of Halobacterium halobium, containing the rhodopsin-like protein bacteriorhodopsin which catalyzes light-induced proton transport during photophosphorylation (56). When the purple membrane was incorporated with lipid vesicles, addition of mitochondrial ATPase and hydrophobic proteins to these vesicles results in a model system exhibiting light-driven ATP synthesis (57). When a diffusion potential was established across the chloroplast membrane by providing a high external concentration of salt in which the cation was made more permeant than the anion (using KCl plus valinomycin) at suboptimal values of proton gradient, a further net post-illumination synthesis of ATP was obtained (58); Schuldiner et al. (58) proposed that the effect of the diffusion potential was via an increase of the electrochemical

gradient of protons which can be coupled to the synthesis of

ATP. Further evidence taken as support for the chemiosmotic

mechanism in chloroplasts was obtained by Witt and coworkers,

especially for flash-induced, non-steady-state conditions

(for a review, see ref. 59); quantitative relationships be-

tween electrical and proton gradients and either the kinetics

(60) or energetics (60-62) were presented. Recent data of

Witt et al. (63) also shows that a large artificial, pulsed

electrical field could induce ATP synthesis in chloroplasts

(under conditions where there should be no pH gradient across

the membrane) with yields on the same order as those induced

by light.

Mitchell has recently proposed a molecular mechanism

for the chemiosmotic hypothesis (27). He assumes that the

fundamental chemical process catalyzed by the reversible

ATPase is the interchange of nucleophilic OH^- and $ADPO^-$ groups

on the electrophilic phosphate phosphorous center. The equi-

librium for the reaction ATP plus HOH on one side and ADP plus

POH on the other normally lies far toward the direction of

hydrolysis. According to Mitchell, the direction could be

"poised" in favor of ATP synthesis by a mechanism in which the

reaction takes place between functionally separated phases in

the membrane; for example, the process may employ both the

lipophilic and more exterior segments of the coupling factor

protein complex (Fig. 1C). In this view, the active center

for phosphorylation lies on the interface region between
these "microphases," so that the gamma-phosphorous center of
ATP becomes specifically accessible on one side (but not on
the other) to the hydrogen ions that are at a relatively high
potential (on the right in Fig. 1C) in the aqueous phase when
an ATP-poising pmf is applied across the coupling membrane.
Briefly, then, Mitchell's revised theory involves the follow-
ing three steps:

(1) PO^- and $ADPO^-$ enter the CF_1 complex

(2) PO^- is protonated to POH and then POH_2^+ (thus promoting
the withdrawal of OH^- in ATP synthesis), as a "trigonal,
bipyrimidal pentavalent transitional intermediate" is
formed

(3) ADPOP leaves together with HOH.

The crucial aspect concerns step (2) in which protonation
occurs at a site where there is both PO^- and $ADPO^-$ but, while
$ADPO^-$ is left as it is, PO^- becomes POH_2^+. In this way POH_2^+
produced by successive protonations can somehow attack $ADPO^-$
without being inhibited by attack of protons on $ADPO^-$. Ac-
cording to Mitchell, this is accomplished by a strict spatial
restriction at the CF_1 complex, with PO^- being accessible to
protons from one side of the active center, while $ADPO^-$ is
not accessible from the other side (Fig. 1C).

Boyer (64) has criticized certain aspects of Mitchell's
detailed molecular mechanism. He states that (a) addition of

an OH^- ion to the terminal phosphoryl group of ATP for

hydrolysis, (b) the formation of a trinegative $O = PO_3^{3-}$

species, (c) the addition of a second proton to an OH group

on phosphorous to give an oxonium ion (OH_2^+), and (d) the

pentavalent transitional intermediate--all of which Mitchell

proposes--are very unlikely and not compatible with available

chemical and thermodynamic information concerning the cleav-

age and synthesis of phosphate esters. Continuing the debate,

Mitchell (65) presented a rebuttal of the above objections of

Boyer (64), followed by further clarification and maintenance

by Boyer of his ground (48), to which the interested reader

is referred for details.

Two recent papers by Mitchell present a specific (66)

and a more general (67) model for incorporating the proton-

motive force of the chemiosmotic mechanism with the redox

transfers in the respiratory chain in mitochondria, as well

as in electron transfer chains in photosynthetic bacteria

and in chloroplasts. A detailed discussion of these formula-

tions, however, is beyond the scope of this Introduction and

not directly related to the data of this thesis. Moreover,

there is serious question as to the viability of such trans-

membrane gradient models on experimental grounds (see discus-

sion in succeeding two paragraphs). And, on a theoretical

basis, Weber (31) has stated that the chemiosmotic hypothesis

as such cannot serve as a valid mechanism since it is not

consistent with the "principle of detailed balance"--the independence of each enzyme catalyzed reaction from all the others.

On the basis of present evidence it appears probable that protons or a proton activity gradient of some nature is involved in photophosphorylation. The theory of Mitchell, and other modifications, generally rely on a transmembrane gradient. Data were cited above which have been taken as support for a chemiosmotic mechanism functioning in energy transduction for some ATPase systems, and perhaps for chloroplasts under certain experimental conditions. On the other hand, it should be noted that recent data (68) shows that in mitochondria phosphorylation proceeds without a protonmotive force, i.e., the measured pmf (transmembrane proton plus electrical gradient) was only a fraction of that required for phosphorylation. In another system, using the Ca^{2+}-ATPase from sarcoplasmic reticulum, Knowles and Racker (69) observed ATP synthesis even though there was no ion gradient across the membrane; they proposed that the energy derived from ion-protein interaction drives the formation of ATP.

Moreover, a recent careful and thorough study by Ort and coworkers (70,71) appears to rule out the involvement, in steady-state photophosphorylation in chloroplasts, of proton gradients or electrical potential differences across the membrane which separates the medium from the greater part

of the internal aqueous space (loculus). Ort et al. (70,71)
found that photophosphorylation begins abruptly at full
steady-state efficiency and rate as soon as the time of
illumination exceeds about 4-5 msec in control chloroplasts,
or as soon as ca. 50 msec are exceeded if valinomycin plus
KCl are present. With or without valinomycin, permeant buf-
fers have little or no effect on the time of illumination
required to initiate phosphorylation, and yet the buffer
systems used delay acidification of the inside of the
thylakoid (bulk inner aqueous phase) by at least one or two
orders of magnitude! (Previously, Lynn (72) observed that
even though imidazole at the concentrations used did not
inhibit phosphorylation, internal acidification, as measured
with neutral red, was inhibited.) Ort et al. (71) suggested
that the protons produced by electron transport can be used
"directly" for phosphorylation, i.e., without even entering
the lumen (inner aqueous phase) of the lamellar system;
several previously unsolved problems apparently resolved by
these findings are discussed by the authors (71).

This concept involving intramembrane events (proton
activity gradient within the membrane itself) is is general
agreement with (but not necessarily specifically endorsing)
the previous suggestions by Williams ((73), see discussion
of his ideas below). Izawa and coworkers (74,75) also sug-
gested this idea on the basis of the difference in inhibition

of photophosphorylation sites I and II (e.g., see Fig. 1B) by low pH as compared to the lack of inhibition of accumulation of protons in the thylakoid (74), and on the basis of the apparent total "uselessness" (not available for phosphorylation) of protons ultimately released by reduction of the artifical system II donor I^- (75). Also, Lynn (72) found that maintenance of both a high reducing potential (low concentration of oxidized terminal acceptors) and a high internal concentration of protons within the chloroplast is required for phosphorylation. Both electron flow (72) and phosphorylation (76,77) proceed during the post-illumination period (and in an acid-base experiment, according to Lynn). On this basis Lynn (72) suggested that the oxidation and deprotonation of reduced, proton sensitive carriers within chloroplasts (presumably inside the membrane), either during or following illumination cause phosphorylation. The exact nature of an intramembrane mechanism proposed by these various authors is nevertheless still obscure.

Williams (73) suggests that membranes are not just "inert divisions," but energy transducing membranes are "packed with protein catalyst blocks which do not necessarily equilibrate rapidly with the surrounding phases." A major difference between the theories of Mitchell and Williams is found in their description of phases. With Mitchell, the energy of non-equilibrium is stored in the bulk aqueous

phases, not in the membrane--thus, it is a "chemiosmotic" hypothesis. With Williams, there is no equilibrium in the membrane, and energy is stored in phases that constitute the "separate blocks" of the membrane; the connection to osmotic energy is then a secondary matter, as for phosphorylation. Both theories use the generation of high proton activities in space, such that pH equilibrium between different internal and/or external regions of the cell are not established. Part of the difference in the two conceptions, then, rests in their views of the nature of internal and external phases in a biological system and the way membranes actually divide spaces. Oxidation-reduction in one phase is coupled to proton and hydroxyl production in their respective phases. Proton production is in turn coupled (in some unknown manner) to ATP production in its phase. The question of interest is--where are these phases located and how do the coupling events proceed?

Since $\Delta G \sim RT \ln [H^+]$, production of a proton gradient (between bulk phases) is accounted for energetically by the free energy decline during electron transport. But, as Williams states (73), the amount of protons generated depends on the relative bulk of the phases in Mitchell's view, i.e., the number of mitochondria (or chloroplasts) per unit volume. If the efficiency of ATP production in its phase is fixed, then the amount of H^+ and OH^- generated in the open phases

depends on the volume of the oxido-reduction phase, implying
that the P:O ratio should depend considerably on the organelle
concentration, which according to Williams has not been found.
Viewed from a different angle, charges should not, in Williams'
view, be discharged into the medium, as in osmotic theories,
for then there is the problem of equilibration of the energy
of a single cell (or its outer side) with the whole of the
volume in which it is suspended. Williams solves the "pro-
blem" by strictly limiting the volume into which the energy
carrying entity (e.g., an ion) can diffuse. One can imagine
this restricted volume involving the ATP-synthetase complex
(cf., for example, Mitchell's interface region discussed above
which separates a basic and electrically negative phase from
an acidic and electrically positive phase).

Williams (73) and Mitchell (49), as well as Boyer (35),
have proposed that since phosphorylation of ADP is a conden-
sation reaction, it could be driven by stabilizing water
(i.e., decreasing the water activity in the phase containing
the ATPase, such as a non-aqueous region of the membrane).
However, Weber (31) states that reduction of water activity
alone will not lead to the expected synthesis of ATP because
the equilibrium constant in the phase with low water activity
becomes proportionally reduced.

Williams (73) observes that proton concentration
changes (influx for energized chloroplasts) may reflect a

"leak" of charge from the site of phosphorylation. Internal charge gradients are, in this view, established prior to ATP formation; ATP formation and osmotic gradients both are considered possible as alternate or simultaneous, but nevertheless different, paths. Thus, ATP formation can be induced by "loading the osmotic system," just as ATP can provide energy for a gradient. (We note here that Weber shows that while ATP production may depend on maintaining an "effector" or osmotic gradient, it is possible that the energy source for this may be found in the hydrolysis of a certain fraction of ATP itself; ref. 78.) The problem with the basic chemiosmotic idea, according to Williams, is that greater energy is needed to set up the osmotic system than can be recovered as ATP, so that the proposed mechanism is both inefficient and not very cyclic. As far as Uribe and Jagendorf's acid-base experiment (54,55) is concerned, the question is how much energy is expended initially in setting up the large chemical gradient compared to the amount of ATP formed? Williams believes that "such large external energy stores cannot be maintained by chloroplasts or mitochondria and that organelles must for this reason avoid this very method of generating ATP."

The fact that protons accumulated in the inner aqueous phase can be used for phosphorylation with fairly high efficiency (79-81), or that chloroplasts can be made to synthesize ATP by applying a large (pulsed) external electrical

field (63), does not imply that a transmembrane proton gradient or electrical potential difference is the actual in vivo "high energy intermediate." This is underscored by the recent data of Ort et al. (71), discussed above, which demonstrate that steady-state photophosphorylation can proceed without a transmembrane potential (see also refs. 82-84) or transmembrane proton gradient. In addition, the data of Lynn (72) suggested to him that electron flow is the cause of phosphorylation in postillumination or acid-base experiments, and that an imposed pH gradient, in the presence of large anions which prolong the pH-induced flow of electrons, is also capable of causing cyclic phosphorylation (e.g., via endogenous protonated mobile electron carriers, which could flux during transport of H^+, OH^- and anions across chloroplast membranes, leading to reduction and oxidation of various portions of the electron chain, depending upon the local internal pH). It was observed (72) that this concept is consistent with the fact that the half-life of the high-energy intermediate state ("Xe") is longer than the half-life for ATP formation (77). Thus, some of the strongest supportive data for the chemiosmotic view has been interpreted in quite another way.

The problem of the size of the energy store required, as well as the efficiency and reversibility, for the energy transduction process is approached from a different angle

in the mechanism proposed by Weber (31,78). In his model,
ATP could be formed in a series of one or more phosphate
group-transfer reactions in which formation of a compound
with high phosphate transfer potential is coupled to oxido-
reduction in a two-step process. First, by "direct uptake"
of phosphate from a donor of zero transfer potential, an
intermediate of less than maximum transfer potential is gen-
erated (termed low energy phosphate donor). Second, this
low energy donor is promoted to the level of high energy
intermediate (high energy phosphate donor) by coupling to
the decline of free energy in an osmotic or concentration
gradient. The addition of the osmotic energy to the chem-
ical free energy occurs by means of a protein, through
negative interactions of ligands that become bound to the
protein. These ligands or "protein-bound effectors" could
be, for example, the phosphate group plus another ion of the
same charge or an ion that interacts negatively with some
charged group involved in phosphate binding. This positive
free energy of interaction results in an increase in the
transfer potential of phosphate bound to the protein as the
system is "pushed" to an unfavorable conformation. Though
the energy of interaction is not large (2-4 kcal), it is
enough to cause destabilization of the effector binding and
"tip the energetic scales" in favor of formation of the high
energy phosphorylated intermediate that leads to ADP

phosphorylation. As in the case of the other hypotheses dis-
cussed, the components of the reaction sequence are restricted
to certain compartments; the latter could presumably be
located within the energy transducing membrane, as long as an
effector gradient can be maintained by an independent energy
source across the boundary between compartments.

Since Weber's theory ascribes an important and funda-
mental role to a "chemical" intermediate, the previously
mentioned problem with the "pure chemical" hypothesis noted
above could be raised here also (at least as far as chloro-
plast data is concerned). On the other hand, as Racker (85)
has pointed out, perhaps phosphorylated "chemical" inter-
mediates do not accumulate in mitochondria or chloroplasts
because they are either hydrolyzed or rapidly dephosphorylated
by ADP. (The "lag time" for the formation of ATP after
initiation of either photophosphorylation (70) or acid-base
induced phosphorylation (42) was measured to be 3-7 msec,
which indicates the limiting time for the existence of any
intermediate.) Thus, instead of phosphoenzyme, one finds,
for example, bound ATP as suggested by Boyer and Slater (35,
86). Moreover, in the case of the Na^+-K^+ and Ca^{2+} pumps in-
volving phosphorylation of ADP, phosphorylated intermediates
have been documented (87-90).

Thus, it is apparent that there is no general consen-
sus as to what the molecular mechanism for ATP formation in
chloroplasts is or should be. The picture is far from clear

yet; one can probably say more about what is not involved in photophosphorylation than what is. It is possible that, as Racker has said, the true mechanism may comprise a "merger of the chemiosmotic, conformational and chemical hypotheses in a compromise formulation" (Weber's model, as well as Boyer's in a different and less complete way, being specific examples) in which a proton activity gradient formed during electron transport is responsible for a conformational change in CF_1; according to Racker this would perhaps allow the uptake of P_i and the formation of phosphorylated protein, which, however fleetingly, serves as chemical intermediate prior to ATP formation (85).

B. The problem

Some of the evidence presented thus far indicates that the substrates of photophosphorylation (ADP and P_i) may interact rather directly with one another, as well as with the coupling enzyme, in the process of ATP formation. Since there is currently little information available on the binding of P_i, ADP or ATP to the ATP-synthetase enzyme ("latent CF_1"), how the substrates and products of phosphorylation interact upon binding, and how the binding characteristics depend upon the medium environment (e.g., proton and salt concentration), we considered it important to obtain as much of this information as possible with a relatively well-defined system (isolated CF_1).

Fluorescence methods were chosen in this study. Specifically, we monitored the binding of fluorescent-modified nucleotides by measuring the polarization of fluorescence. This technique provided a convenient and fairly direct method of examining the kinetics of binding and unbinding (release) of the nucleotides to CF_1, the latter by observing the competition between the fluorescent and non-fluorescent forms (demonstrated to be essentially equivalent functionally in chloroplasts).

Also, since an energy-linked conformational change in membrane-bound CF_1 was inferred on the basis of light-induced or acid-base induced incorporation of tritium into CF_1 (91), and since this type of change is important in a number of the mechanistic concepts summarized above, an attempt was made to monitor rather directly the conformational state of isolated CF_1 as detected by the intensity and polarization of fluorescence of the tyrosine residues intrinsic to CF_1.

C. Use of fluorescence methods

Compounds whose fluorescence is sensitive to their environment (fluorescent probes) have come under fairly wide use in the investigation of proteins and their conformational changes as well as membrane structures involved in a variety of biological phenomena. Their use both in model systems and in more physiological situations, including lipid/detergent micelles, mitochondria and sub-mitochondrial particles,

erythrocyte membranes, etc. has been reviewed (92-96). These are substances which are not generally found in the natural system (i.e., "extrinsic" fluorescent probes), but added to extract new information, as opposed to an "intrinsic" probe such as endogenous chlorophyll or aromatic residues of membrane proteins.

We have attempted in this study to use both types of probes, and to some extent as they interrelate, to aid in elucidating the molecular mechanism involved in photosynthetic energy conservation. Fluorescence of the tyrosine residues of coupling factor can be viewed as an intrinsic probe of the protein state, whereas the fluorescent-modified nucleotides provide information from the extrinsic approach. However, because we have shown that fluorescent ADP and ATP substitute well for the naturally occurring nucleotides involved in the in vivo processes (see Results, chapter III, section A), they can in a very real sense also be considered as "intrinsic" probes.

Two broad classifications of fluorescence experimentation exist: those in which the polarization of exciting and/ or emitting light is monitored, and those in which this is not a significant factor. In the first catgory, one may discover information concerning the microviscosity of a particular site, the flexibility of rotation in protein conjugates and complexes, the degree of orientation of fluorescent

elements in model or physiological systems, and the binding
of small molecules to large proteins; see Weber (95) for fur-
ther information. In this study, we have concentrated on
this category and taken advantage of the power of the
fluorescence polarization technique to investigate the pro-
perties of the binding of relatively small molecules (fluo-
rescent-modified nucleotides, MW = ca. 600 or less) to the
high molecular weight (ca. 325,000), slowly rotating coupling
factor macromolecule (CF_1). Compounds which rotate freely in
solution, upon becoming bound to a large protein, are ex-
pected to demonstrate a significant increase in the polariza-
tion of fluorescence as they become relatively more
"immobilized." Thus, positive values of polarization of
fluorescence of added nucleotides are taken as a measure of
binding to CF_1. There is ample precedent for use of this
approach in the study of binding (97-99).

D. Limitation of scope

No significant light-induced change in fluorescence of
the modified adenine nucleotides was observed in chloroplasts,
capable of supporting either photophosphorylation or light-
dependent ATPase activity. This is expected for several
reasons: (1) the nucleotides active in on-going phosphoryla-
tion are transiently and non-covalently bound; (2) nucleo-
tides involved in CF_1 interactions would, under typical
experimental conditions, be a small fraction of the total

fluorescent population; (3) chlorophyll absorbs in the spectral regions used for both excitation and emission of the fluorescent nucleotides, thereby making measurements of any potential changes in fluorescence of fluorescent ADP or ATP in chlorophyll suspensions technically virtually impossible; and, (4) the fluorescence properties of all the adenine nucleotides are identical (100), so that, unfortunately, interconversion between the various forms during phosphorylation cannot be studied by spectral means.

Due to the complexity of the thylakoid membrane system, it is in this case more difficult to gain useful information about photophosphorylation than with isolated CF_1. Thus, the scope of the present investigation was limited to a study of the properties of isolated CF_1. In particular, coupling factor in its "unactivated" (non-ATPase) or "latent" ATP-synthetase form was utilized for the following two reasons.

(1) Fluorescent-modified ATP is not a good substitute for ATP in the ATPase or ATP-P_i exchange reactions (101) and therefore comparisons between binding properties obtained by the fluorescence polarization technique and enzymatic activity (the obvious advantage of using this non-physiological "conformation" of CF_1) would be quite questionable.

(2) There are fundamental differences (listed below) between the latent (ATP-synthetase) and activated or

treated (ATPase) forms of chloroplast CF_1 that indicate that the relationship between conclusions based on experiments with modified CF_1 and the mechanism of photophosphorylation (which requires unmodified or untreated CF_1) would be less than direct:

(a) heat, trypsin or other destructive perturbation is required to transform soluble CF_1 into an ATPase enzyme (15); there is a structural-chemical alteration as indicated by the appearance of two additional -SH groups per mole with these treatments (102);

(b) the ATPase activity of soluble CF_1 is calcium dependent and inhibited by magnesium, whereas photophosphorylation is magnesium dependent (8,102);

(c) CF_1 activated by heat cannot rebind to CF_1-depleted chloroplast membranes to reconstitute photophosphorylation as in the case of "unactivated" soluble CF_1; and,

(d) inhibition studies with antisera to the subunits of CF_1 have shown that anti-alpha or anti-gamma inhibit photophosphorylation, but anti-alpha plus anti-gamma are required to inhibit the calcium ATPase (103).

Since soluble CF_1 detached from the thylakoid is already one step away from the fully intact in vivo situation, we did not

want to introduce further complications by altering the protein preparation structurally and functionally in the present work. Experiments done with ATPase-CF_1 may prove, in the future, to be beneficial to the study of _in vivo_ energy transduction. In the present thesis, we have concentrated on a partial characterization of isolated, latent CF_1.

The disadvantage of the approach undertaken in this thesis is that it is rather static and necessarily cannot take into account any mechanistic details that are dependent on the complete, energized membrane system. On the other hand, the advantage is that the sample studied is much simpler and more well-defined, and the derived interpretation with such systems is often more clear-cut. It is anticipated that only through an integration of data obtained using both approaches (complex, complete system, as well as simpler, fragmented system) will the mechanism of biological energy transduction be uncovered.

E.   Interaction of nucleotides with coupling factor

The coupling factor protein (CF_1) plays a central role in the process of energy transduction (5,102,104). In experiments of this thesis, the interaction of this enzyme with its proposed substrates (_i.e._, adenine nucleotides) was studied by using the fluorescent analogs of AMP, ADP, and ATP (105). Some spectroscopic properties of these fluorophores and their biological activities (in other systems) have

already been described (100,106-108). The chemical structure
of the fluorescent analog of ATP, abbreviated as εATP (3-β-
$\underline{D}$ -ribofuranosylimidazo (2,1-$\underline{i}$) purine 5'-triphosphate or
1,N^6-ethenoadenosine triphosphate), is shown below. The
analogs of ADP and AMP differ from the structure shown only
in having one or two of the terminal phosphate groups removed
from εATP for εADP and εAMP, respectively.
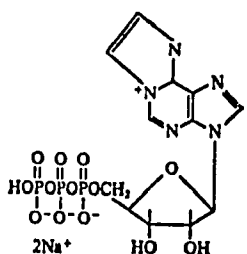
εATP

Recently, the fluorescent compounds εADP and εAMP-PNP
were used in conjunction with the non-fluorescent nucleotides
to partially characterize binding sites on soluble chloro-
plast CF_1 (109); in this study, the ATPase function of CF_1
was investigated, and a model was proposed in which nucleo-
tide binding sites on heat-activated CF_1 act as allosteric
conformational "switches" for the ATP hydrolysis activity of
solubilized CF_1. In the present work, however, attention has
been focused on the unmodified CF_1 protein as it may relate
to the forward process of phosphorylation, and, therefore
there is only limited comparison between the two studies.
(The work of Cantley and Hammes (109) had come to our

attention after this study had been undertaken and much of the data collected.) A comparative study of CF_1-ligand complexes has also been made using circular dichroism and chemical isolation (110).

It has been demonstrated that εADP acts nearly as well as ADP as substrate for photophosphorylation, although εATP substiutues poorly for ATP in the ATPase reaction using heat-activated CF_1, but does function like ATP in stabilizing CF_1 against heat inactivation (101). In this thesis, the above result for εADP phosphorylation was confirmed. In addition, for the first time, εADP and εATP were shown to substitute well for ADP and ATP in several other CF_1-mediated chloroplast reactions (ADP inhibition of electron transport, ATP inhibition of electron transport, ATP stimulation of light-induced proton uptake), thus demonstrating that the fluorescent-modified nucleotides are good functional analogs, and data obtained using these compounds can be interpreted with confidence.

It was further shown that: (a) fluorescence polarization, using a photon counting instrument, can indeed be used to study binding of adenine nucleotides to isolated coupling factor in its ATP-synthetase form, as these nucleotides (εADP and εATP) bind well to CF_1 at two sites; (b) there is no binding of εAMP to CF_1 under a variety of conditions; (c) the cofactors of photophosphorylation (ADP, ATP, P_i, Mg^{2+})

interact directly at the level of binding to CF_1; (d) the affinity of CF_1 for the adenine nucleotides is dependent on the ionic (salt) content of the medium; (e) binding of εADP and εATP to CF_1 is sensitive to pH, with maximal binding observed at pH 8, and it appears that isolated CF_1 undergoes essentially irreversible changes when the pH is shifted from this optimum; and (f) the kinetics of binding of εADP and εATP to isolated CF_1 are similar, but the release of εATP from CF_1 when an excess of non-fluorescent ATP is present, is very much slower than the rate of release of εADP in the presence of excess ADP.

Finally, for the first time, the fluorescence of the coupling factor protein itself (utilizing the intrinsic tyrosine residues) was measured under different conditions in an attempt to monitor directly the conformational state of CF_1; a reversible cation-induced change, as well as an adenine nucleotide dependent change, in the polarization of CF_1 fluorescence was demonstrated.

We consider it premature to specify a particular mechanism of phosphorylation (e.g., chemical, chemiosmotic, conformational) on the basis of the above results, although they are nevertheless pertinent in their consideration. Further work in this area, employing both chloroplasts and simpler systems such as isolated CF_1 may permit a more definitive correlation in the future.

CHAPTER II

MATERIALS AND METHODS

A. Chloroplast preparation

Class II chloroplasts were isolated from lettuce
(Lactuca sativa, var. Romaine), unless otherwise indicated,
by homogenizing in a buffer containing 50 mM phosphate or
Tricine-NaOH (pH 7.8), 10 mM NaCl plus 10 mM KCl, and 400 mM
sucrose, and filtering through several layers of cheesecloth
and a 10 µm mesh cloth; the filtrate was centrifuged at
1500 x g for 10 min. The chloroplast pellet was washed in
hypotonic medium (homogenizing buffer, minus sucrose) to
rupture the outer chloroplast membrane envelope, and then
resuspended in a small volume of homogenizing medium. Chloro-
phyll concentration was determined by the method of Mackinney
(111) with a Cary 14 spectrophotometer.

B. Chloroplast activity

Ferricyanide reduction (Hill activity) was measured by
following the decrease in absorbance at 420 nm on a Cary 14
spectrophotometer when the chloroplast suspension was simul-
taneously illuminated with saturating (7×10^2 watts/m^2) red
actinic light (passed through Corning C.S. 2-64 and heat
filters); the extinction coefficient used for calculation of
the amount of ferricyanide reduced was 1000 $cm^{-1}M^{-1}$ as given
by Trebst (112).

Photophosphorylation and light-induced proton flux were measured essentially as described by Dilley (113), by monitoring the rate of alkalinization of the medium using a recording pH meter; illumination was with saturating $(6 \times 10^2$ watts/m^2) white light passed through a heat filter. Details of reaction mixture are given in the appropriate Table legends.

C. Preparation of coupling factor protein

Coupling factor protein (CF$_1$) was isolated from lettuce (or in a few experiments, spinach) leaves by a procedure based on the method of Strotmann et al. (114). Our specific preparation procedure (also see flow chart on p. 36) typically began with approximately 8-12 (depending on size and condition) large heads of market lettuce (Lactuca sativa, var. Romaine); leaves were deveined and washed in a large bath of chilled deionized water. The washed leaves were homogenized briefly (several seconds) in a large Waring blendor containing 0.8-1.0 liter of homogenizing medium (10 mM Na$_4$P$_2$O$_7$, 350 mM sucrose, pH 7.5). The homogenate (about 1.5 liter) was strained through six layers of cheesecloth and centrifuged in six 250 ml plastic bottles at ca. 1200 x g for 10 minutes using a refrigerated (0 - 4°C) International Centrifuge Model PR-2. This provided the chloroplast fragments.

The chloroplast pellets were washed four times in 1.5 liters of 10 mM Na$_4$P$_2$O$_7$, pH 7.5 (a small artist's brush was

used for resuspension) to wash thylakoid membranes free of
ribulose-1,5-diphosphate carboxylase and minor proteins.
The washed pellets were then resuspended in a medium contain-
ing 2 mM Tricine-NaOH and 300 mM sucrose at pH 8 (to give a
final volume of 80 ml) to release CF_1 from the thylakoids.
After gently stirring this suspension during stripping of
CF_1 at 12-15°C for 10 minutes, the sample was centrifuged at
38,000 rpm (ca. average centrifugal force = 100,000 x g) in
a Beckman L3-50 ultracentrifuge (type 42 rotor) for 10 min-
utes at ca. 12°C. The pellet containing chloroplasts was
discarded, and 2.5 ml of 1.5 M Tricine-NaOH (pH 8), 0.8 ml
of 0.2 M EDTA (pH 8) and 0.22 g of ATP were added to the
supernatant to help stabilize CF_1. The supernatant was then
centrifuged at 100,000 x g for 40 min. and the chloroplast
pellet discarded. Solid $(NH_4)_2SO_4$ was slowly added, with
stirring, to the supernatant to give a final concentration
of 2 M (roughly 50% saturation at 4°C) in order to concen-
trate CF_1. After storage at 4°C overnight, the precipitate
was separated from sucrose and collected by centrifugation
(10,000 x g for 10 min.). The pellet was dissolved in a
small volume (several ml) of 40 mM Tricine-NaOH (pH 8), 4 mM
ATP, 2 mM EDTA and 2 M $(NH_4)_2SO_4$. This medium is known to
maintain the stability of CF_1 for up to several months when
storage is at 4°C (115). (Since the pH of Tricine buffer is
temperature dependent (negative $\Delta pK_a/°C$), this was allowed

FLOW CHART FOR CF_1 PREPARATION

Deveined, washed lettuce leaves
|
homogenized in blendor with PP_i-sucrose buffer
|
homogenate strained through cheesecloth and centrifuged
at 1200 x g for 10 minutes

resuspend chloroplast fragments in
10 mM $Na_4P_2O_7$ (pH 7.5) and centrifuge   discard
at 1200 x g for 10 minutes to wash   supernatant
thylakoids free of ribulose-1,5-
diphosphate and minor proteins
|
repeat washing procedure 3 more times
|
resuspend final washed pellet in 2 mM Tricine-NaOH (pH 8) plus
0.3 M sucrose to release CF_1 from the thylakoid membranes
|
centrifuge at 100,000 x g for ca. 10 minutes

discard   add to supernatant small volumes of
pellet   concentrated stabilizing agents to give
final concentrations of 40 mM Tricine-NaOH
(pH 8), 4 mM ATP and 2 mM EDTA
|
centrifuge at 100,000 x g for ca. 40 minutes

discard   add slowly, with stirring, solid $(NH_4)_2SO_4$
pellet   to supernatant (containing CF_1 to give
final concentration of 2 M
|
store overnight at 4°C
|
centrifuge at 10,000 x g for 10 minutes

dissolve pellet in few ml of 40 mM   discard
Tricine-NaOH (pH 8), 4 mM ATP, 2 mM EDTA,   supernatant
2 M $(NH_4)_2SO_4$ and store at 4°C

(see text for further details)

for by adjusting the pH to ca. 7.9 when adding $(NH_4)_2SO_4$ at 12-15°C before cooling to 3-4°C.)

For use in fluorescence experiments, aliquots of stored CF_1 (collected from 2-4 repetitions of the above procedure, p. 36) were centrifuged for 5 min. at ca. 10,000 x g, dissolved in a small volume (ca. one ml) of 30-40 mM Tricine-NaOH (pH 8), centrifuged for 2 min. at ca. 10,000 to remove any undissolved material, and then desalted and stripped of bound nucleotides on a Sephadex G-50 column (1 x 50 cm) at ca. 12-15°C, since unprotected CF_1 in solution is cold (0-10°C) labile (115,116). Protein concentration was determined by the Lowry method (117). A molecular weight of 325,000 for CF_1, as found by Farron (10), was used in determining the molarity.

The data of Fig. 2 shows that the yield (mg CF_1 released per mg chlorophyll) of CF_1 obtained by the above method is 0.12 mg/ml when the chlorophyll concentration is 0.5 mg/ml during the 0.3 M sucrose sucrose incubation step; this agrees well with the value obtained by Strotmann (personal communication; see also (114)). However, we found that this yield can be increased if the chlorophyll concentration is increased; at a chlorophyll concentration of 1.5 mg/ml, 0.3 mg/ml of CF_1 was obtained.

Our preparation was checked by comparing the fluorescence spectra of soluble CF_1 with a solution of tyrosine in

Fig. 2. Concentration of coupling factor protein (CF_1)
released as a function of chlorophyll concentration during
incubation in 0.3 M sucrose. Spinach chloroplast suspensions
were first washed four times in sodium-pyrophosphate buffer
(pH 7.4), resuspended in buffer containing 0.3 M sucrose and
3 mM Tricine-NaOH (pH 8), and then subjected to high-speed
centrifugation (20,000 x g, 15 min.) to remove all chloroplast
material before measuring protein concentration in the super-
natant. Data points with error bars represent averages of at
least three separate values, other points represent a single
experiment.
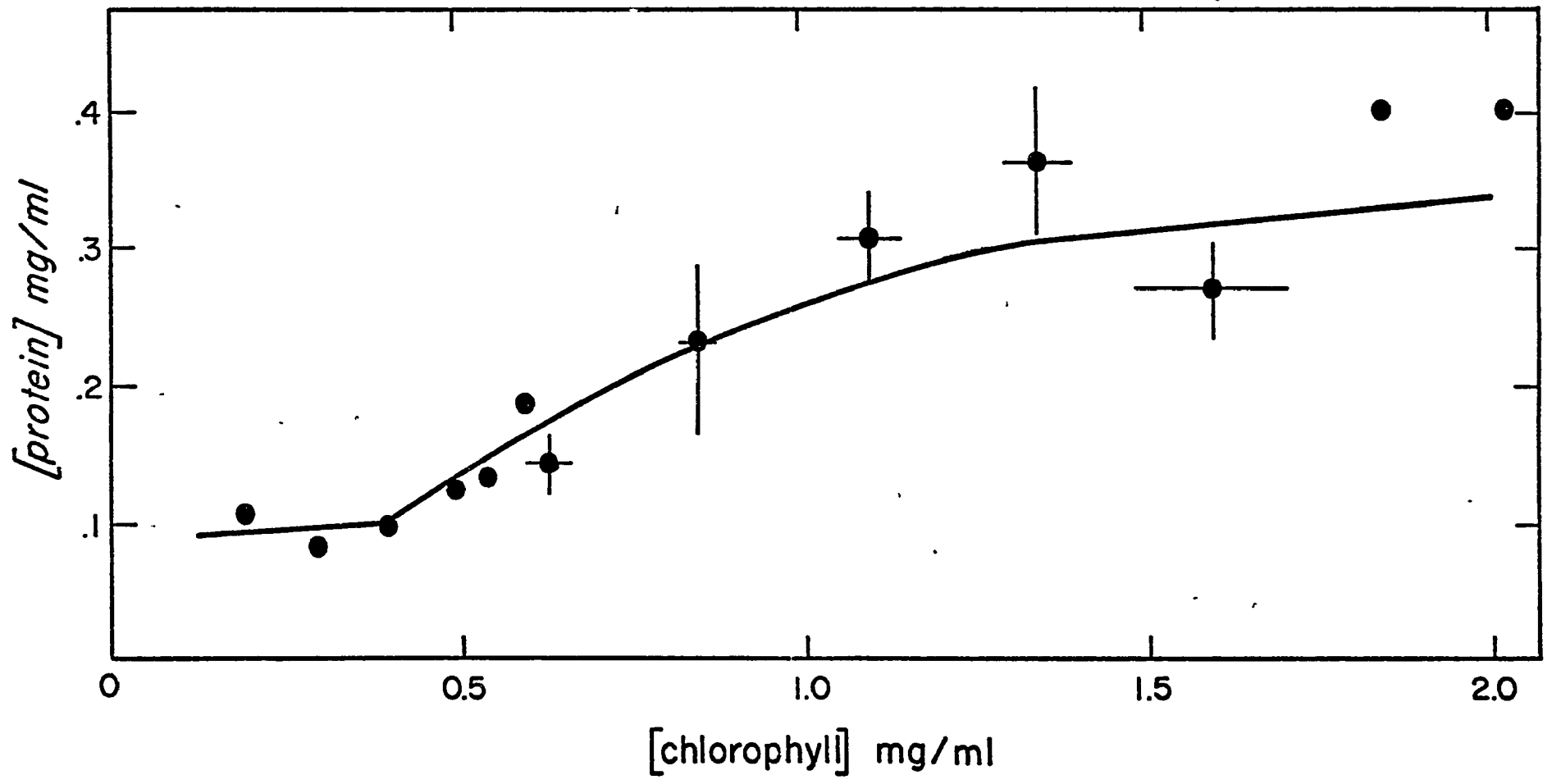
the same buffer (25 mM Tricine-NaOH, pH 8.1), since CF_1 is known to contain tyrosine, but not tryptophan, residues (10). Both samples, when excited at 270 nm, had identical emission maxima at 305 nm (Fig. 3). In addition, Lien and Racker (115) found that the ratio of fluorescence at 300 and 350 nm was correlated directly with the purity of CF_1 preparations; all fractions with a 300:350 nm intensity ratio exceeding 1.85 were consistently found to be homogenous by polyacrylamide gel electrophoresis—with the procedure used in this study, ratios of ca. 2 were typical.

The activity of CF_1 isolated by the method used here was checked by its ability to rebind to thylakoid membranes depleted of CF_1 and restore photophosphorylation; see Shoshan and Shavit (119) for a study on factors affecting the reconstitution of photophosphorylation in chloroplast membranes. Data in Table 1 show that these CF_1 preparations can restore phosphorylation with an efficiency as high as 90% (sample reconstituted at 0.11 mg Chl/ml) and at least 65% (at 0.18 mg Chl/ml) compared to control.

D. Chemicals

The fluorescent nucleotides $1,N^6$-ethenoadenosine mono-, di- and triphosphate (εAMP, εADP, εATP) were purchased from P & L Biochemicals, Inc., Milwaukee, Wis.; Tricine, AMP, ADP, ATP, KH_2AsO_4, PMS (N-methylphenazonium methylsulfate), and dithioerythritol were from Sigma, St. Louis, Mo. Purity of

Fig. 3. Fluorescence emission spectrum of isolated chloroplast CF_1 (1.35 mg/ml, part A) and tyrosine (0.4 mg/ml, part B) in 25 mM Tricine-NaOH (pH 8.1). Excitation was at 270 nm, half-bandwidth = 7 nm and emission half-bandwidth = 8 nm (part A); excitation and emission half-bandwidths = 6 nm (part B). Ordinate scales for A and B are not directly comparable; spectra are not corrected for variation of instrument response with wavelength. Spectra were measured with a Hitachi Perkin-Elmer MPF-2A spectrofluorometer. The slightly broader spectrum of the CF_1 sample is likely to be due to a combination of concentration differences, different interaction of tyrosine with environment in the protein and in solution, and wider bandwidths necessary for measurement of weaker signal, and contribution of a slight amount of tryptophan impurity (very slight amounts will be weighted relatively strongly due to the much higher quantum yield of tryptophan compared to tyrosine in proteins containing both, see Konev (118)).
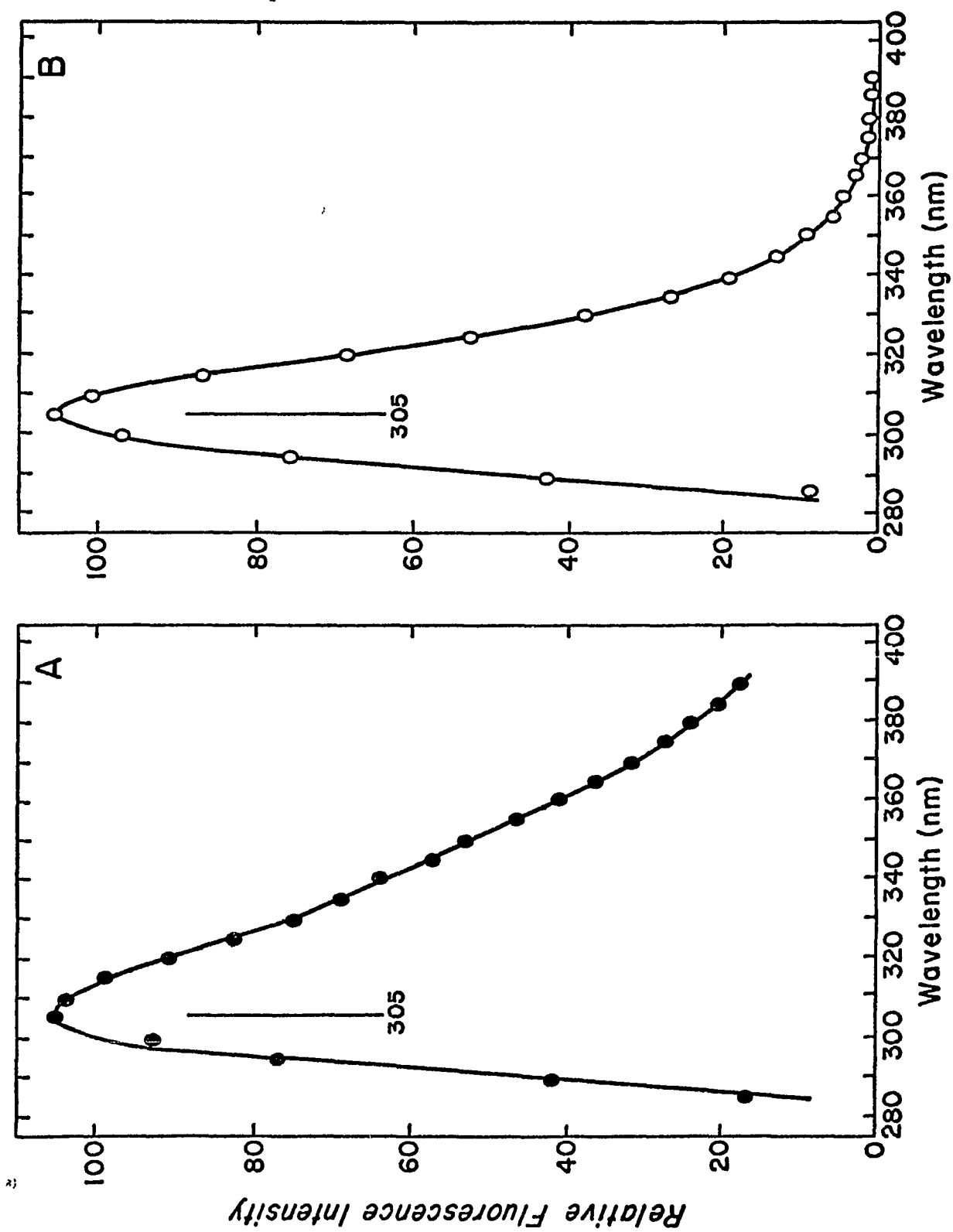
TABLE 1

RECONSTITUTION OF PHOTOPHOSPHORYLATION

Sample	$\dfrac{\mu\text{moles ATP}}{\text{mg Chl} \cdot \text{hr}}$
Control at 4 mg Chl/ml	490
Control at 0.18 mg Chl/ml	370
Sample reconstituted at 0.18 mg Chl/ml	220
Control at 0.11 mg Chl/ml	260
Sample reconstituted at 0.11 mg Chl/ml	240
Chloroplasts in sucrose-tricine buffer (before centrifugation)	70
Sucrose-washed chloroplasts ($-CF_1$)	0

Reconstitution protocol: Chloroplasts were washed four times with 10 mM $Na_4P_2O_7$ (pH 7.4) and resuspended in 2 mM Tricine, 0.3 M sucrose buffer (pH 7.8): [Chl] = 0.7 mg/ml. After centrifugation for 15 minutes at 20,000 x g, the chloroplast pellet ($-CF_1$) was resuspended in 2 mM Tricine, 0.3 M sucrose (pH 7.8): [Chl] = 1.3 mg/ml. The supernatant contained CF_1 at 0.12 mg/ml protein. Samples were reconstituted by incubating (with stirring) 5 ml of the CF_1 supernatant solution with sucrose-washed chloroplasts ($-CF_1$) at the concentration indicated in this Table, plus 10 mM $MgCl_2$. At the same time, aliquots of control chloroplasts were diluted to the chlorophyll concentration of the reconstituted samples. Photophosphorylation activity of reconstituted and control samples were assayed at ca. 50 μg Chl/ml in a reaction mixture containing 3.3 mM NaH_2PO_4, 3.3 mM $MgCl_2$, 17 mM KCl, 1.7 mM ADP, 0.67 mM dithioerythritol and 25 μM PMS.

fluorescent nucleotides was assured by thin-layer chromato-graphy.

E. Polarization of fluorescence

Fluorescence polarization measurements on fluorescent nucleotides were made on a T-format polarization instrument in Professor G. Weber's laboratory, basically similar to that described by Weber (120), but with photon counting (121). This apparatus allowed the convenient subtraction of back-ground scattering interference at low signal-to-noise values. A 450 Watt xenon arc, cooled by air draft, was the light source. The 310 nm exciting light was selected by a Bausch and Lomb grating monochromator (1 mm slitwidth; 1.67 nm/mm dispersion) and passed through a Corning glass C.S. 7-54 filter. The fluorescence emission was filtered through a Corning glass C.S. 0-52 filter, which also blocks out the exciting light from detection by the photomultiplier tube.

For measurements of polarization of protein fluores-cence, the same photon counting instrument was used, except the exciting wavelength was 280-285 nm and the fluorescence emission was filtered through a Corning C.S. 0-54. Initial base levels of polarization of protein fluorescence varied somewhat with the particular preparation and condition, and so the data from different experiments were normalized to the polarization with no addition for easier comparison. All ex-periments were made at 20°C.

Values of $I_{\parallel}$ and $I_{\perp}$, where $I_{\parallel}$ is the intensity of the emission light polarized parallel to the exciting beam (which is polarized perpendicular to the plane of observation), and $I_{\perp}$ is the intensity of the emission light polarized perpendicular to the exciting beam, were obtained by repeated counting over 20-30 second intervals. Unless otherwise indicated, the polarization values reported here are the average of 2-3 such determinations. The polarization of fluorescence (p) is defined as $(I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$.

F. Fluorescence intensity

Relative intensity of fluorescence of the modified adenine nucleotides was measured on the photon counting instrument described above, under the same conditions and settings. (See section II.G for use of fluorescence intensity in calculations of binding parameters.) Fluorescence emission of proteins was measured on a Hitachi Perkin-Elmer MPF-2A spectrofluorometer; further details are given in figure legends.

G. Calculation of binding parameters

Data for the binding of the fluorescent-modified nucleotides to CF_1 was calculated and plotted according to the equation derived by Klotz (122), namely, $S/Dx = 1/n + K/nD(1-x)$, where n is the number of adsorbing sites, $K(=K_d)$ is the equilibrium constant of dissociation of the complex;

S and D are the total protein and ligand concentrations, respectively, and x is the fraction of ligand bound. The reciprocal of the intercept of a plot of S/Dx vs. 1/D(1-x), i.e., the [protein]/[bound ligand] vs. 1/[free ligand], gives n, and K/n is the slope. The polarization data was analyzed for binding according to this method in the range of nucleotide concentration from ca. 2 to 35 μM with CF_1 at concentrations of 4 to 8 μM (further details given in figure legends of chapter III). The essential features of the binding plot are illustrated in Fig. 4. The ordinate is unitless since the protein and ligand concentrations cancel out and x is a fraction; the abscissa is expressed in μM^{-1}. Since the slope (from which the K_d is directly calculated) is the change in ordinate value divided by the change in abscissa value, the K_d will then be appropriately expressed in units of μM. If the ordinate intercept is 0.5, the number of binding sites is the reciprocal, or 2. The K_d's for these sites are found by determining the respective slopes and dividing by n. In this thesis, K_1 will refer to the region of smaller slope (smaller K_d or stronger binding) and K_2 will refer to the region of steeper slope (greater K_d or weaker binding). If the two sites are of intrinsically the same affinity, non-linearity observed in the binding plot is probably due to electrostatic interactions between the two negatively charged ligands as first one, and then the second nucleotide

Fig. 4. Typical Klotz binding plot for ligand-protein system with two binding sites (n = 2) of relatively stronger (site 1) or weaker (site 2) affinity. The total protein concentration (S) is kept constant while the total ligand concentration (D), and therefore the fraction of ligand bound (x) and unbound (1-x), is varied. S/Dx = [protein]/[bound ligand] and $1/D (1 - x)$ = 1/[free ligand].
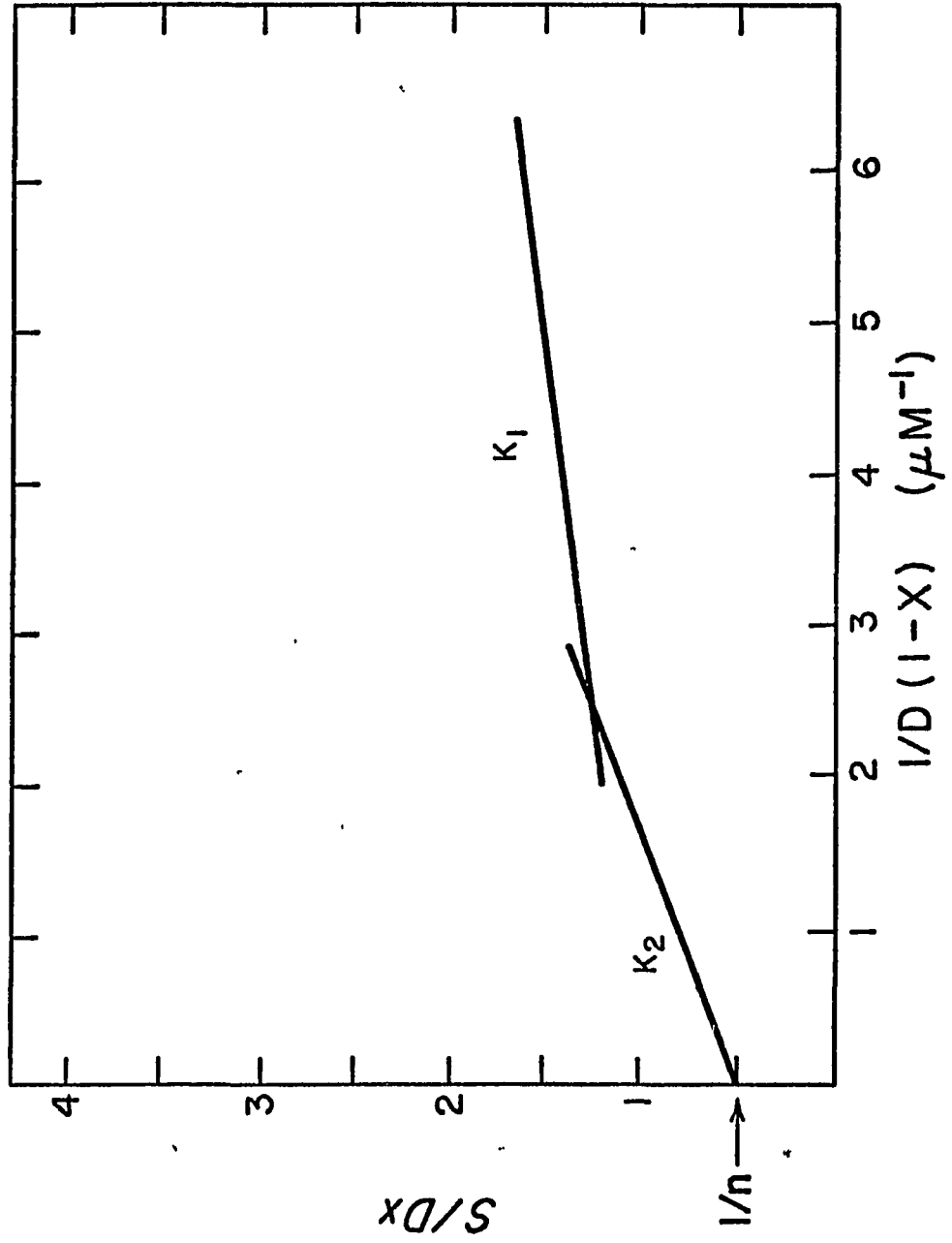
binds (122). In this case the "true" K_d will in the first
approximation be some average of K_1 and K_2; this will gener-
ally not have much effect on the results reported here since
for both ϵADP and ϵATP the values of K_1 and K_2 fall within
a very narrow range (1-3 μM).

The fraction of ligand bound, x, was calculated accord-
ing to the relation $x = R (p/p_b) - R + 1$. R is the fluores-
cence intensity of the ligand-protein system relative to that
of the ligand alone at the same concentration and pH, computed
using the sum of the parallel and perpendicular components of
fluorescence intensity when the sample was excited with light
polarized in a plane perpendicular to the axis of observation
(99); p is the observed polarization at a given ligand con-
centration; p_b is the value for complete binding of ligand,
determined by extrapolation of the raw binding data (polariza-
tion of fluorophor vs. added concentration of ligand, at low
concentrations of ϵADP or ϵATP, p_b = 0.20 for ϵADP and 0.25
for ϵATP). Reasonable confidence can be placed in the values,
especially since a portion of the binding data for ϵADP was
confirmed independently using a forced dialysis technique
(109) with good agreement. (For literature on the use of the
polarization method in binding studies, see refs. 97-99.)

We note further that the method of monitoring binding
used in this study has the simplifying advantage of rapidity
(minimizing possible complications of any components becoming

destroyed or converted), since a complete polarization measurement, including corrections for scattering, can be completed within a few minutes after combining the reactants. Moreover, the kinetics of unbinding or release of adenine nucleotides from CF_1 could be monitored in a unique and convenient way by following the decrease in polarization of the fluorescent-nucleotide bound to CF_1 when an excess of non-fluorescent nucleotide was added.

CHAPTER III

RESULTS

A. Chloroplast reactions

In order to determine the validity of using fluorescent-modified nucleotides in binding studies with isolated, unmodified, coupling factor (CF_1), several chloroplast reactions were measured to see to what degree the analogs can substitute for the unmodified nucleotides. In this way, the interactions of ADP and ATP with (membrane-bound) CF_1 in its native, untreated, ATP synthetase state could be examined; isolated CF_1 per se cannot perform phosphorylation.

In Table 2, data are presented for the initial rates of photophosphorylation of εADP and ADP for cyclic (N-methyl phenazonium methyl sulfate, PMS) and non-cyclic (H_2O to methyl viologen) reactions in spinach chloroplasts. The rates of εADP phosphorylation (at concentrations of nucleotide ranging from 0.2 mM to 1.7 mM) compared to ADP phosphorylation are between about 60-85%. Using 50 μM PMS, which causes cyclic electron flow around photosystem I, and with 1.5 mM εADP, 270 μmoles of ATP/mg Chl/hr were produced compared to 410 μmoles of ATP/mg Chl/hr with ADP (Table 2A, top two rows) giving a 65% efficiency for the fluorescent nucleotide. For other values, see lines 3-6 in Table 2A (60%), and lines 1-2 in Table 2B (85%). Using 0.8 mM methyl viologen,

TABLE 2

COMPARISON OF RATES OF PHOTOPHOSPHORYLATION
USING ADP OR εADP AS SUBSTRATE

	Nucleotide	$\dfrac{\text{moles ATP or ATP}^*}{\text{mg Chl} \cdot \text{hr}}$	Percent of Control
A	1.5 mM ADP	410	100
	1.5 mM εADP	270	65
	0.75 mM ADP	380	100
	0.75 mM εADP	230	60
	0.3 mM ADP	365	100
	0.3 mM εADP	220	60
B	0.2 mM ADP[†]	290	100
	0.2 mM εADP	245	85
	0.2 mM ADP	190	100
	0.2 mM εADP	170	89
	1.7 mM ADP	150	100
	1.7 mM εADP	90	60

<u>Reaction</u> <u>conditions</u>:   (A) Cyclic photophosphorylation was measured in a medium containing 3 mM NaH_2PO_4, 3 mM $MgCl_2$, 17 mM KCl and 50 µM PMS at pH 7.8 using spinach chloroplasts at ca. 50 µg Chl/3 ml.   (B) [†]Lines 1-2 are with cyclic electron transport, measured in the medium of part A.   Lines 3-6 are with non-cyclic electron transport, with 0.8 mM methyl viologen replacing PMS.   Oat chloroplasts used in part B.

[*]Values for rates of phosphorylation are ca. ± 10 moles ATP/ mg Chl/hr; shown here is representative data of 3 experiments.

which accepts electrons only from the primary acceptor X of

pigment system I (123), and, thus, involves non-cyclic elec-

tron flow from H_2O to methyl viologen using both pigment

systems, 1.7 mM εADP led to a net production of 90 εmoles of

εATP/mg Chl/hr against 150 μmoles of ATP/mg Chl/hr with ADP,

giving a 60% efficiency for εADP (Table 2B, lines 5-6);

0.2 mM nucleotide gave a still higher relative efficiency

(ca. 89%, Table 2B, lines 3-4). This data (Table 2), then

agrees well with the data of Shahak et al. (101), and indi-

cates that εADP is a suitable analog to use in the study of

phosphorylation-related properties of coupling factor.

Adenine nucleotides are known to exert a regulatory

effect on the rate of electron transport and proton trans-

port, both mediated by the interaction of ADP or ATP with

CF_1 (124-127). In coupled chloroplasts, electron transport

is inhibited under non-phosphorylating conditions (e.g., by

addition of ADP or ATP, without P_i, the effect saturating at

concentrations as low as 50-100 μM in the presence of Mg^{2+})

and the inhibition is partially reversed under phosphorylat-

ing conditions (ADP, P_i, Mg^{2+} added). The data of Table 3A

(lines 1-2) show that 100 μM ADP inhibits the rate of fer-

ricyanide reduction by 22% in lettuce chloroplasts under

non-phosphorylating conditions. (Without the addition of

nucleotide, ferricyanide reduction in our chloroplasts was

300 ± 8 μmoles FeCy reduced per mg Chl per hr.) If the ADP

TABLE 3

EFFECTS OF ADENINE NUCLEOTIDES ON FERRICYANIDE REDUCTION

Addition		μmoles FeCy reduced per mg Chl per hr	Percent Inhibition
A	no ADP	300 ± 8	----
	100 μM ADP	233	22.4
	500 μM ADP	235	21.7
	500 μM ADP + 1 mM KH_2PO_4	257	14.3
	100 μM ATP	224	25.5
B	no εADP	300 ± 8	----
	100 μM εADP	261	13.0
	500 μM εADP	263	12.4
	500 μM εADP + 1 mM KH_2PO_4	275	8.7
	100 μM εATP	242	19.2

Reaction conditions: Lettuce chloroplasts equivalent to
45 μg Chl/2 ml were suspended in a medium containing 15 mM
Tricine-NaOH (pH 8.3), 100 mM sucrose, 6 mM $MgCl_2$ and 0.6
mM $K_3Fe(CN)_6$. At this concentration of FeCy, it accepts
electrons mostly from photosystem I. The decrease in
absorbance at 420 nm was measured during exposure of chloro-
plasts to saturating (7 x 10^2 watts/m^2) red light using a
Corning C.S. 2-64 glass·filter; values for FeCy reduction
correspond to initial rates. Shown here in representative
data of 3 experiments. The range of error indicated in the
data represents the approximate minimum resolution of the
measurement system.

concentration is increased to 500 μM, no further inhibition is produced (line 3), which confirms previously reported data on the saturation of this effect (125). This inhibition can also be obtained by εADP, although to a smaller extent (ca. 13%, lines 1-3, Table 3B); this shows a 60% effect as compared to that of ADP.

If 1 mM KH_2PO_4 (P_i) is present in the medium to allow photophosphorylation to proceed, a partial recovery (ca. 40%) of the rate of electron transport was observed with 500 μM ADP (line 4, Table 3A); εADP substituted for ADP in this reaction, and gave a recovery rate of 70% (line 4, Table 3B) compared to the unmodified nucleotide.

ATP also lowers the rate of ferricyanide reduction; addition of 100 μM ATP results in an inhibition of about 25% (line 5, Table 3A). This decrease is also produced by εATP, with activity of ca. 75% (line 5, Table 3B) compared to unmodified ATP.

In a related phenomenon, it was shown previously (124, 126) that ATP at a low concentration (10 μM) stimulates the extent of the proton gradient by as much as 2-3 fold in spinach chloroplasts. This is due to a reduction in the rate of proton efflux at the level of CF_1. In Table 4, data for the effect of 10 μM ATP or εATP on the extent of net proton uptake in lettuce chloroplasts is shown; typically, the extent of stimulation was in the range of 1.5-2.0 fold. An

average of three experiments showed that ca. 0.11 μmoles H^+/ mg Chl increased to ca. 0.18 μmoles H^+/mg Chl when 10 μM ATP was added, giving a 64% stimulation (line 4, Table 4); an increase from ca. 0.11 to 0.17 μmoles H^+/mg Chl was observed when 10 μM ATP was added, giving a stimulation of 56%. Thus, the fluorescent-modified ATP substituted well for ATP in these experiments, with about 85% of the activity of the unmodified nucleotide.

Finally, an attempt was made to estimate the functional efficiency of εAMP compared to AMP by investigating an unusual reaction reported recently for spinach chloroplasts. First, Mukohata and Yagi (128) showed that arsenate (As) plus ADP (catalyzing arsenylation) could substitute for phosphate (P_i) plus ADP (catalyzing phosphorylation) in restoring the ADP-inhibited rate of ferricyanide reduction. However, they also demonstrated that AMP + ATP + As partially restored the inhibition of ferricyanide reduction by ATP, ATP + As, or ATP + AMP, whereas AMP + As produced little or no inhibition of electron transport with nucleotide concentrations up to 100 μM. This reaction facilitated by the combined presence of ATP + AMP + As was termed "quasi-arsenylation"; it is interesting that phosphate could not substitute for arsenate in this reaction. The "quasi-arsenylation" reaction was nevertheless useful for our purposes since AMP is somewhat functional here, although the relationship to in vivo photophosphorylation, if

TABLE 4

EFFECTS OF ADENOSINE TRIPHOSPHATE AND ITS
FLUORESCENT ANALOG ON PROTON UPTAKE

μmoles H^+/mg Chl*		Percent Stimulation
-ATP	+ATP	
0.087	0.151	73
0.120	0.191	59
0.129	0.206	60
Average 0.112	0.183	64
-εATP	+εATP	
0.085	0.130	53
0.096	0.153	59
0.140	0.220	57
Average 0.107	0.168	56

Reaction conditions: Lettuce chloroplasts equivalent to
130 μg Chl/3 ml were suspended in a medium containing 1.5 mM
Tricine-NaOH (pH 8), 5 mM $MgCl_2$, 25 mM NaCl, 25 mM KCl and
25 μM PMS, then illuminated with white light passed through
a heat filter and a circulating water bath surrounding the
reaction chamber. ATP or εATP concentration was 10 μM.

*Values for proton uptake are ±0.006 μmole, indicating the
approximate minimum resolution of the measurement system.

any, is unclear. Using lettuce chloroplasts from two separate preparations, we were able to observe only little or no quasi-arsenylation, even when our preparation showed good inhibition by ATP and ADP, which was restored by the "usual" arsenylation reaction (data not shown). However, using pea chloroplasts, we did observe substantial restoration of the ATP-inhibited electron transport rate by AMP; εAMP substituted for AMP. As shown in Table 5, in the presence of 1 mM KH_2AsO_4 (As), the rates of ferricyanide reduction were 288 and 210 μmoles/mg Chl/hr when 50 μM AMP and 75 μM ATP were added, respectively. However, addition of 75 μM ATP and 50 μM AMP together yielded a value of 261 μmoles FeCy/mg Chl/hr. This restoration by AMP was calculated as follows:

$$\% \text{ restoration} = \frac{\%\text{inhibition}(+ATP) - \%\text{inhibition}(+ATP+AMP)}{\%\text{inhibition}(+ATP)};$$

in calculating %inhibition, control rates were the values with AMP(εAMP) + As. Such a calculation demonstrated a 65% restoration by AMP, whereas εAMP was 30% as efficient as AMP.

Thus, the results of this section show that the fluorescent-modified nucleotides εADP and εATP can substitute well (65-85%) for the unmodified nucleotides in various chloroplast reactions: ADP phosphorylation (Table 2), ADP inhibition of electron transport and restoration of this inhibition by P_i (Table 3), ATP inhibition of electron transport (Table 3), and ATP stimulation of the extent of proton

TABLE 5

RESTORATION OF ATP INHIBITED ELECTRON TRANSPORT
BY QUASI-ARSENYLATION

	Addition	μmoles FeCy reduced per mg Chl per hr
A	50 μM AMP	288 $\pm$ 7*
	50 μM εAMP	276
	75 μM ATP	210
	75 μM ATP + 50 μM AMP	261
	75 μM ATP + 50 μM εAMP	221
	Addition	Percent Restoration[†] FeCy Reduction
B	ATP + AMP	65
	ATP + εAMP	20

Reaction conditions: Pea (dwarf, progress #9) choroplasts equivalent to 46 μg Chl/2 ml were suspended in a medium containing 15 mM Tricine-NaOH (pH 8.3), 100 mM sucrose, 6 mM $MgCl_2$, 0.6 mM $K_3Fe(CN)_6$ and 1 mM KH_2AsO_4 (As). Ferricyanide reduction was measured as in Table 3.

*Electron transport rates are the average of three measurements. [†]See text for details.

transport (Table 4). The εAMP was also found to substitute (Table 5) for AMP in restoration of ATP inhibition of electron transport by quasi-arsenylation (requiring ATP + AMP + As), but with less efficiency (ca. 30%) than for the other nucleotides.

B. Binding of nucleotides to coupling factor protein

In attempting to understand the mechanism of photophosphorylation it is important to have information on the binding to CF_1 of the primary substrates and cofactors involved in the reaction and some idea of how they interact. Up to this point, this has not been systematically investigated. For this reason, using polarization of fluorescence as a convenient way to monitor nucleotide binding (see chapter II for further details), the interaction of εAMP, εADP, εATP, P_i and Mg^{2+} with isolated CF_1 was studied.

Polarization values of nucleotide fluorescence (excitation at 310 nm) significantly above the zero level were observed when εADP was added to a solution of CF_1 isolated from lettuce chloroplasts as described in chapter II. Fig. 5 shows the kinetics of binding of εADP (filled circles) and εATP (open circles) to CF_1. In both cases, essentially maximal binding is achieved in ca. 5 minutes. Since this was the approximate time interval between successive additions of εADP and εATP in titrations made to ascertain binding constants (this chapter, presented below), it is the equilibrium

Fig. 5.   Time course of the binding of εADP (4.9 µM,
filled circles) and εATP (4.2 µM, open circles) to 7 µM CF_1.
The lower curve (squares) is the time course of binding of
4.9 µM εADP to CF_1 (from the same stock preparation as the
upper curves) which had first been titrated in steps from pH
8 to 7 with 1 N HCl and then from pH 7 to 8 with 1 N NaOH be-
fore adding εADP.   Medium contained 40 mM Tricine-NaOH (pH 8)
and 5 mM $MgCl_2$.   Shown is representative data from three ex-
periments.   In the early part of the time scale here, and in
some subsequent kinetic experiments (Figs. 17 and 24), there
is more scatter in the data because of insufficient time (due
to limiting time of data acquisition) to make 3 repetitions
for individual points as was normally done.

εATP (control CF$_1$)

εADP (control CF$_1$)

εADP (CF$_1$ first titrated from pH 8→7→8)

Polarization of εADP or εATP Fluorescence

Time After εADP or εATP Addition (min)
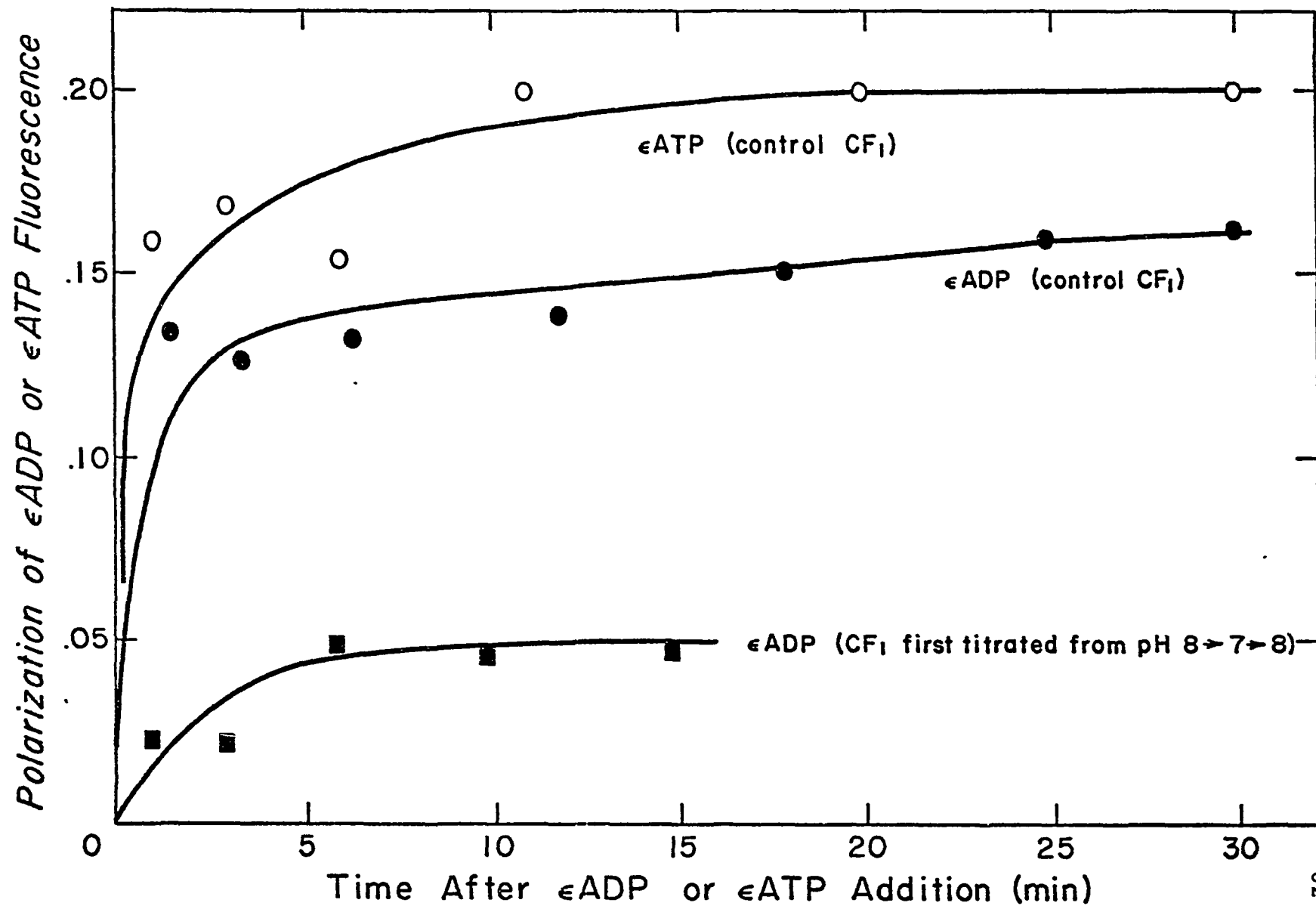
values that are being determined.

When a solution of CF_1 from the same preparation as Fig. 5 was subjected to pH titration from pH 8 to 7 and back to pH 8, before εADP binding was measured (Fig. 5, filled squares), the kinetics were similar (within error of measurement), but the maximal level of polarization of εADP-CF_1 attained was considerably less (one third of control) indicating some irreversible effects. Fig. 6 shows the pH dependence of binding of εADP and εATP to CF_1 in the range pH 7-8.5; maximal binding under these conditions was at pH 8. The changes in polarization during pH titration were essentially not reversible (not shown, but see Fig. 5, square symbols).

Further support for an irreversible effect on CF_1 was found with measurements of the effect of pH changes on the intensity of fluorescence of the tyrosine residues intrinsic to CF_1 protein. During titration from pH 8 to 7, a change (20% increase, see Fig. 7) in intensity of protein fluorescence at 310 nm was observed, indicating perhaps an (as yet unspecified) alteration in the structural state of CF_1; titration from pH 7 back to 8 demonstrated that this effect also was not reversible. A small irreversible change in the clarity of the CF_1 solution was also noted upon visual inspection, perhaps representing nonspecific aggregation, denaturation or separation of CF_1 complex into subunits. (Lien et al. (13) also observed such changes with a cold

Fig. 6. pH dependence of the binding of εADP (4.9 μM, filled circles) and εATP (4.2 μM, open circles) to CF_1 (two experiments with 4.5 and 6.5 μM CF_1 were normalized together). Maximum polarization values were 0.16 and 0.20 for εADP-CF_1 and εATP-CF_1, respectively. Changes in pH were made by titrating with 1 N HCl and 1 N NaOH. Medium contained 40 mM Tricine-NaOH (pH 8) and 5 mM $MgCl_2$.

% Maximum Polarization of Fluorescence

pH of Medium

● εADP – CF$_1$
○ εATP – CF$_1$

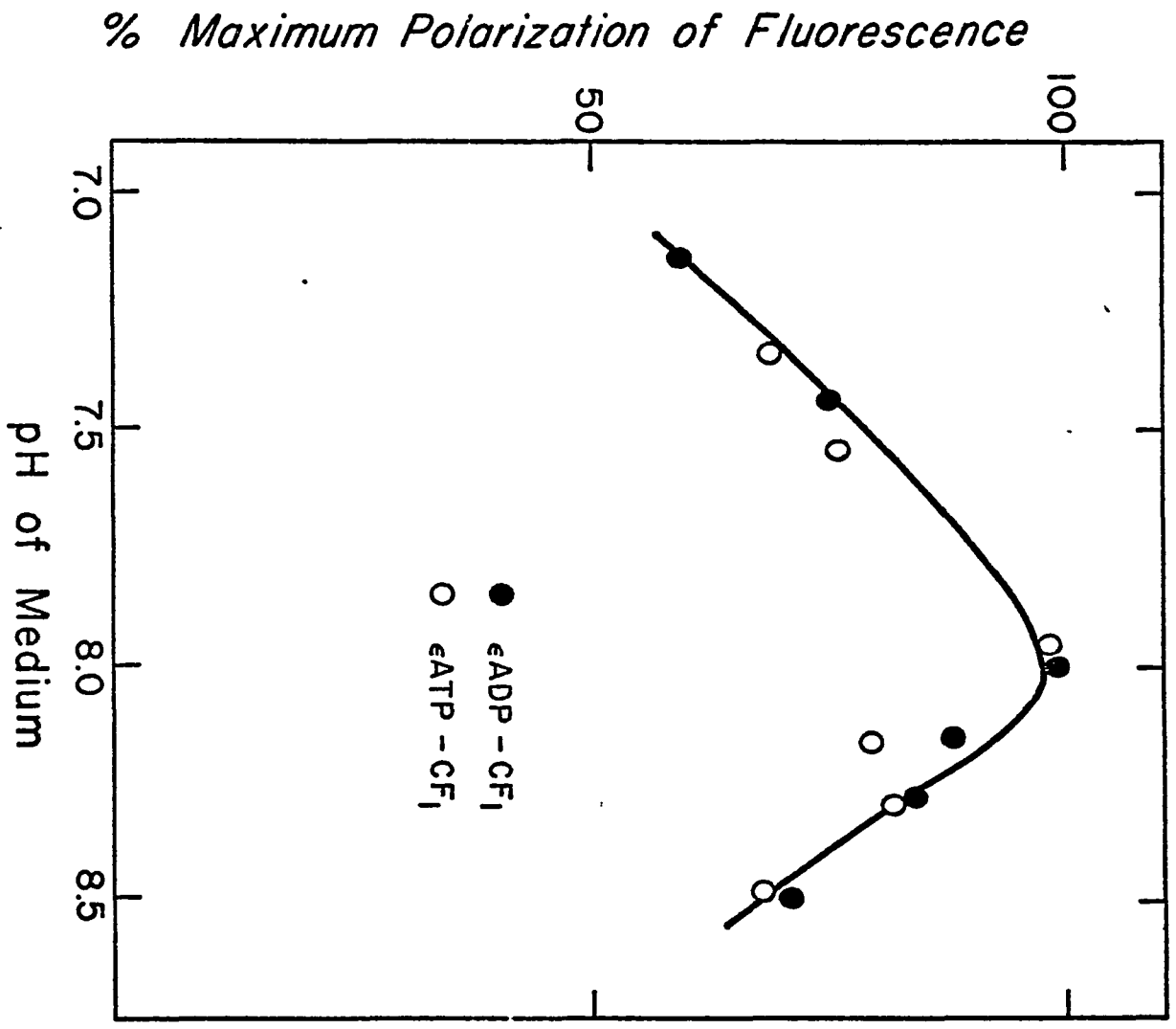Fig. 7. pH dependence of the intensity of CF_1 protein
fluorescence. Excitation was at 275 nm and fluorescence
emission was monitored at 310 nm (excitation and emission
half-bandwidths = 12 nm with a Hitachi Perkin-Elmer MPF-2A
spectrofluorometer. Changes in pH were made by titrating
with 1 N HCl and 1 N NaOH. The CF_1 solution contained 40 mM
Tricine-NaOH (pH 8), 5 mM $MgCl_2$ and 6.5 μM CF_1.
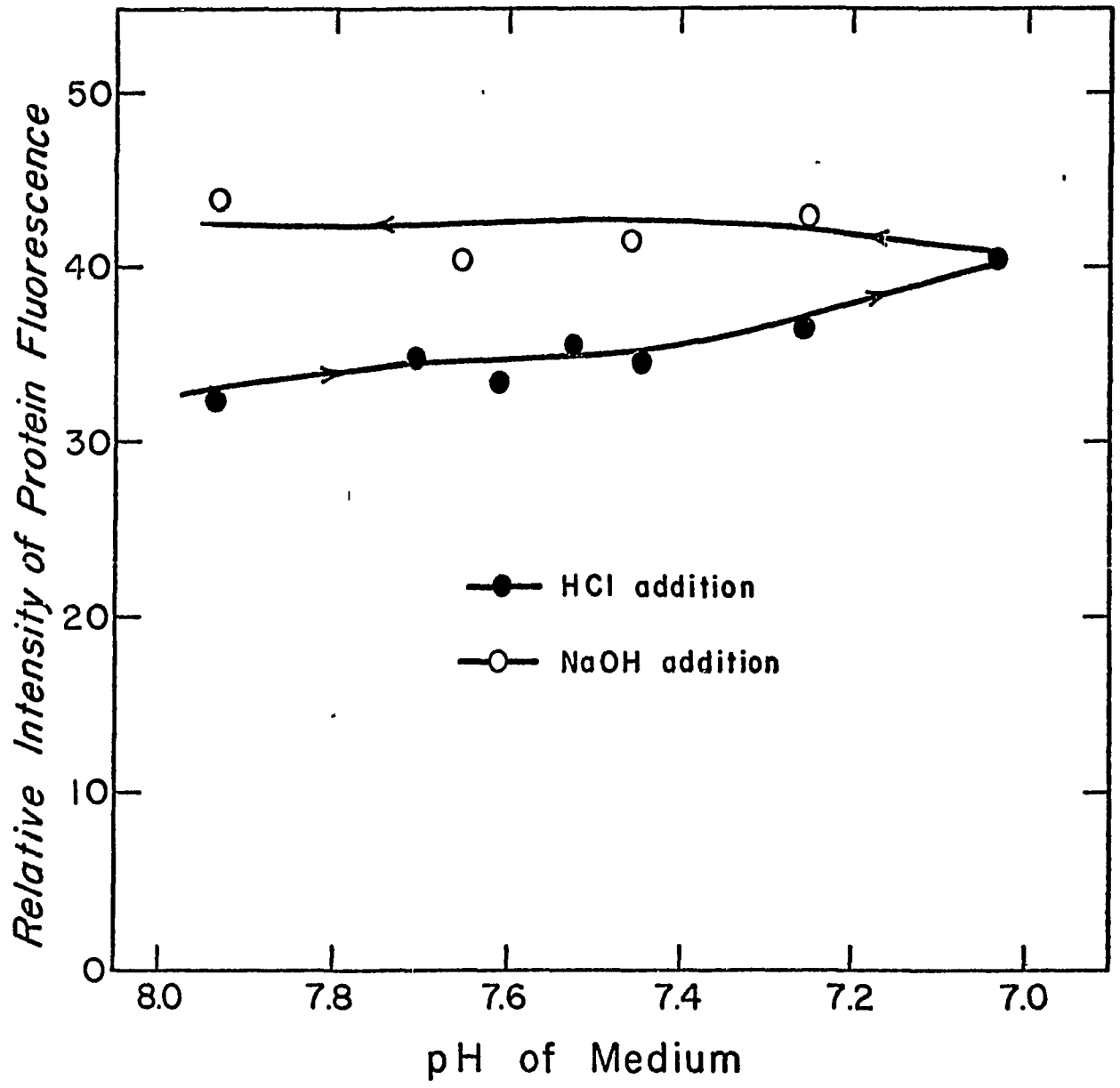
inactivation treatment at pH 6.5; irreversible inactivation of isolated CF_1 could be prevented by including glycerol plus ATP in the medium, but we could not test the effects of these factors since ATP at the high concentrations necessary completely prevents observation of binding of the fluorescent nucleotides to CF_1.) Isolated, unprotected CF_1 under our conditions is apparently quite sensitive to changes in the pH of the environment, as indicated both by binding of fluorescent nucleotides to CF_1 and by protein fluorescence (Figs. 5-7).

1. εADP

As the concentration of ligand (εADP) added to a solution of CF_1 is increased from 2-30 μM, the polarization decreases from the maximum (for a given ionic condition) toward p = 0 as a greater percentage of the total fluorescing population remains unbound (Fig. 8). Comparing the different curves of Fig. 8, it can be seen that the ionic status of the medium substantially affects the polarization curves. The best binding of εADP was obtained with 5 mM $MgCl_2$ (open circles), whereas 10 times lower $MgCl_2$ concentration (open squares), high ionic strength [100 mM NaCl added with 5 mM $MgCl_2$ (filled circles) or with 0.5 mM $MgCl_2$ (filled squares)], and no added salts (other than buffer, triangles) resulted in lower polarization. The degree of εADP binding varied in the order 5 mM $MgCl_2$ > 0.5 mM $MgCl_2$ > 5 mM $MgCl_2$ + 100 mM NaCl >

0.5 mM $MgCl_2$ + 100 mM NaCl > buffer only. Going from buffer only to 5 mM $MgCl_2$ + buffer, polarization at 2 μM εADP increased by about a factor of 2.

If the fluorescence intensity data for the same conditions as Fig. 8 are plotted, a smaller, but corresponding trend was observed, suggesting that there is a small quenching of εADP fluorescence correlated with increasing binding strength (Fig. 9); this trend is also observed in data for other conditions to be presented below. Although the differences in intensity of nucleotide (εADP and εATP) fluorescence observed with different additions were relatively small in Fig. 9 (as well as Figs. 13, 20, 26, presented below), the possible effect of such differences on the K_d's was accounted for by including the appropriate ratio of intensities (e.g., between bound and unbound fractions of fluorescent ligands) in the binding curve calculations (see section II.G); thus, the reported values are the corrected ones.

When the data of Fig. 8 was analyzed for binding constants (see chapter II and figure legend for details), the binding curves of Fig. 10 were obtained, which show [protein]/ [bound εADP] vs. 1/[free εADP]. Coupling factor shows two binding sites for εADP of fairly high affinity with 5 mM $MgCl_2$, one (K_1 = 0.5 μM) being apparently somewhat tighter than the other (K_2 = 2 μM) (open circles). However, it may well be that the observed heterogenity is a reflection of the negative

Fig. 8. Polarization of εADP fluorescence as a function
of εADP concentration in the presence of isolated CF_1 in dif-
ferent ionic media. Medium contained 4.1 μM CF_1 and 30 mM
Tricine-NaOH, pH 8 (Δ), or buffer plus the following additions:
5 mM $MgCl_2$ (0), 0.5 mM $MgCl_2$ (□), 5 mM $MgCl_2$ + 100 mM NaCl (●),
or 0.5 mM $MgCl_2$ + 100 mM NaCl (■). In this and the following
figures 9-26, the wavelength of excitation was 310 nm, and
fluorescence emission was filtered through a Corning C.S. 0-52
glass filter placed before the photomultiplier. See chapter
II for further experimental details.
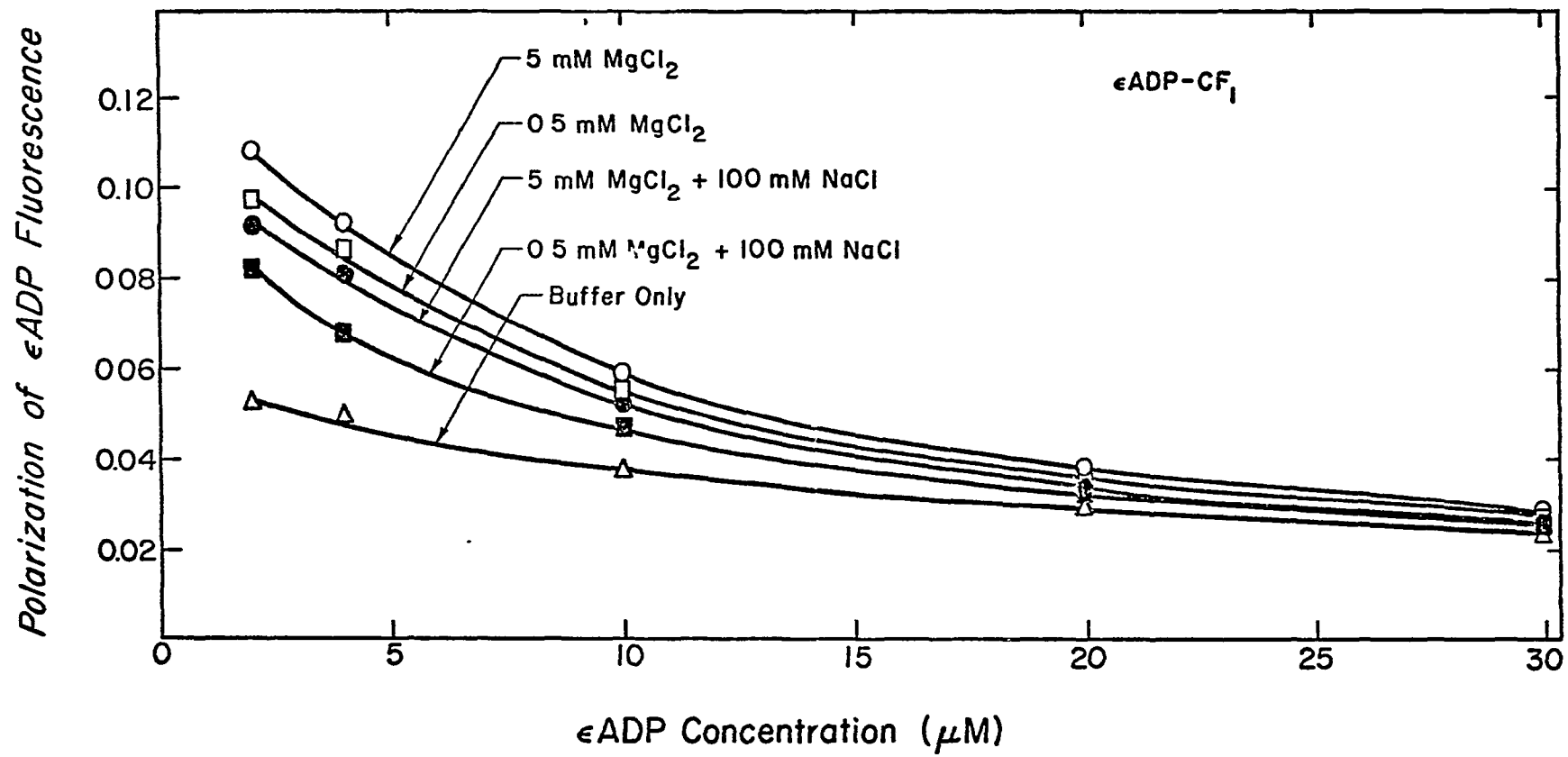
Fig. 9. Relative intensity of εADP fluorescence as a function of εADP concentration in a solution of CF_1 in different ionic media. Medium contained 4.1 μM CF_1 and 30 mM Tricine-NaOH, pH 8 (Δ), or buffer plus the following additions: 5 mM $MgCl_2$ (o) or 0.5 mM $MgCl_2$ ($\square$).
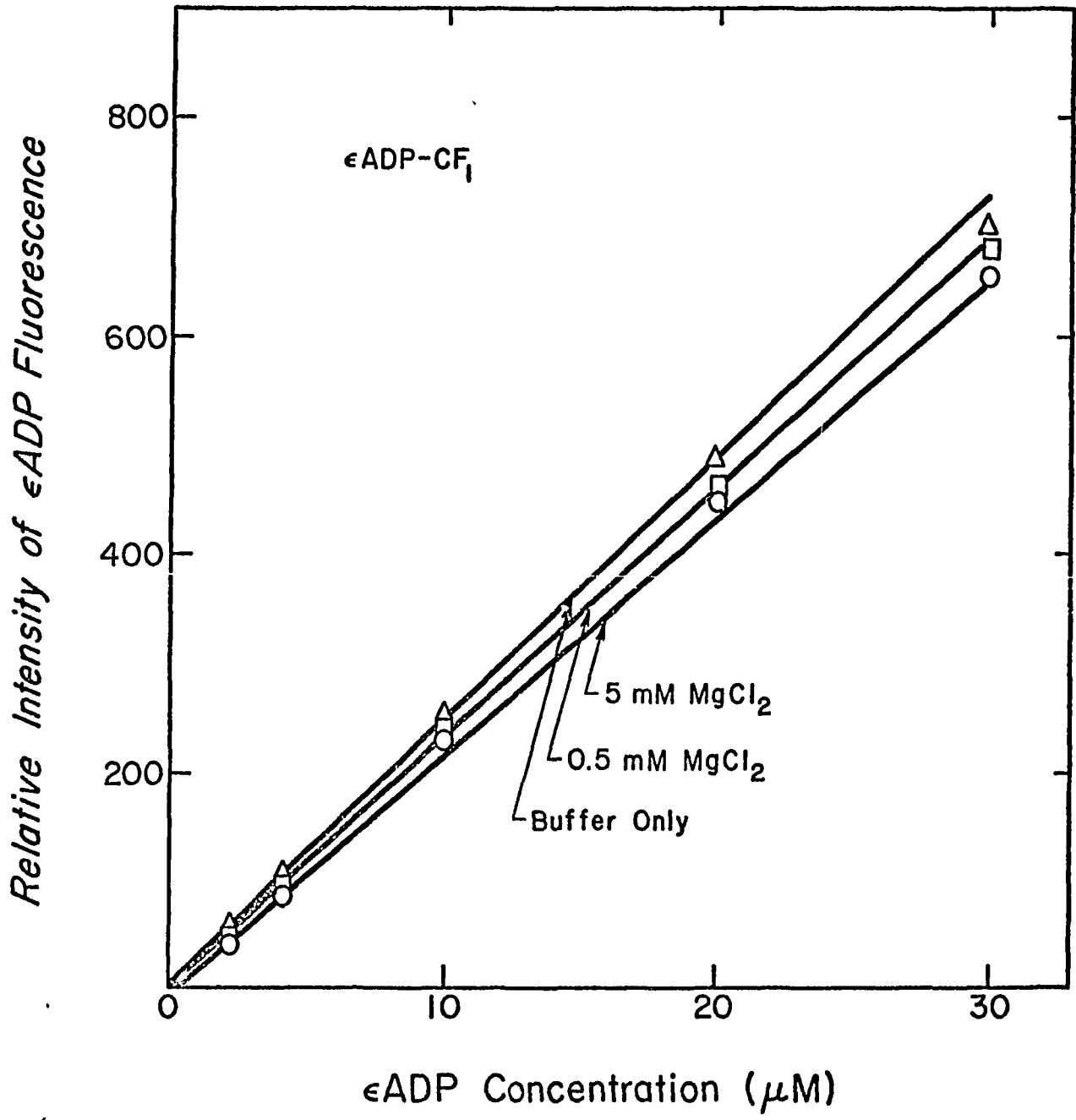
Fig. 10. Klotz binding plot for ADP-CF$_1$ as a function of ionic content. See legend of Fig. 8 for experimental details. See chapter II for explanation of binding curves. Calculated values for dissociation constants (K$_d$) are: K$_1$ = 0.5 μM, K$_2$ = 2 μM (5 mM MgCl$_2$, open circles); K$_1$ = 2.8 μM, K$_2$ = 6 μM (5 mM MgCl$_2$ + 100 mM NaCl, filled circles); K$_1$ = 6 μM, K$_2$ = 8 μM (30 mM Tricine-NaOH only, triangles). K$_1$ and K$_2$ refer to regions to the right and left of the break in binding curves, respectively.

εADP–CF$_1$

Buffer Only

5 mM MgCl$_2$ + 100mM NaCl

5 mM MgCl$_2$

$\dfrac{\text{[protein]}}{\text{[bound εADP]}}$

1/ $\Big[$ free ADP$\Big]$ (μM^{-1})

75
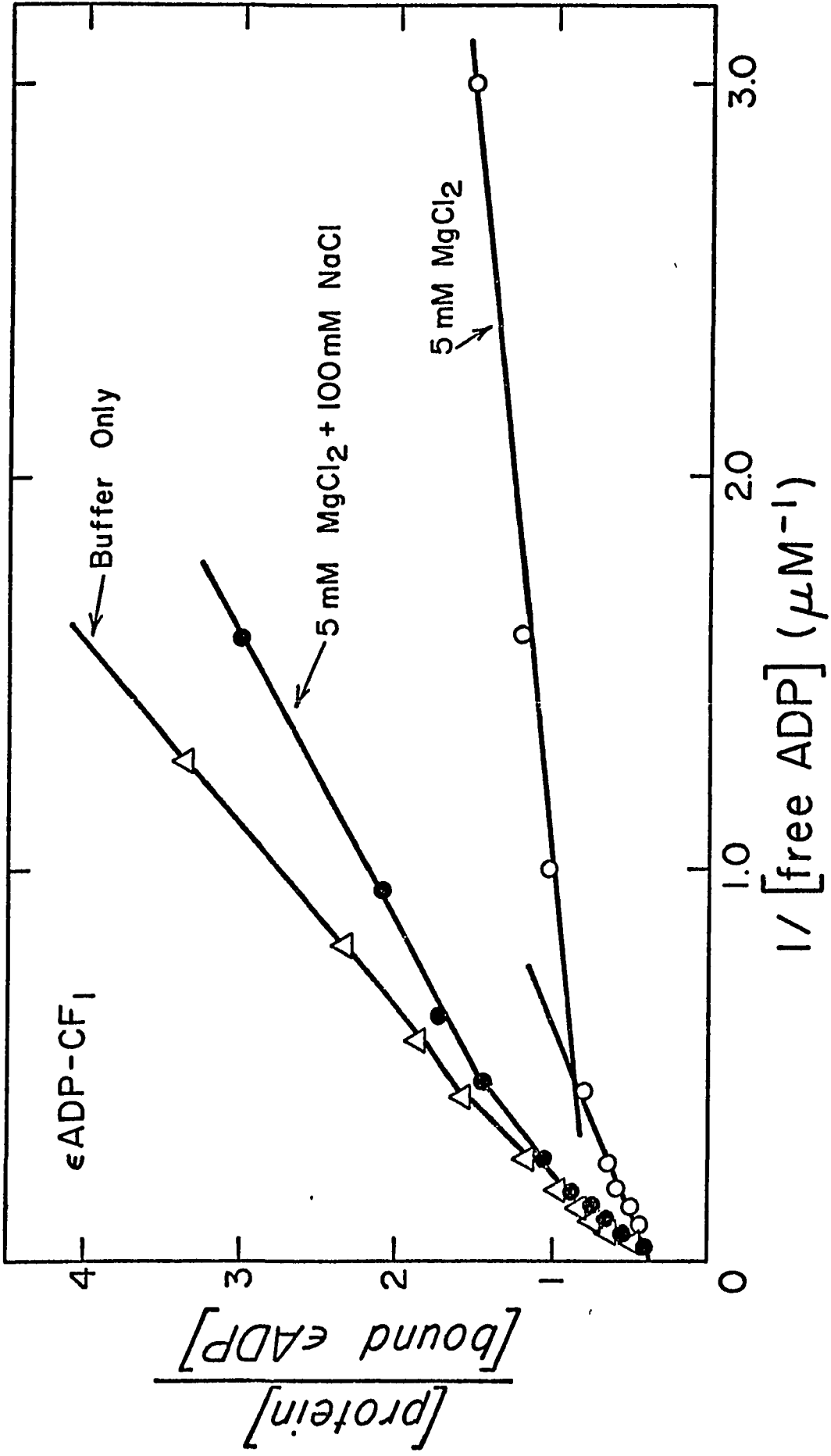
interaction upon binding to two sites of essentially the same affinity, rather than an indication of sites of intrinsically different K_d's.

As the $MgCl_2$ concentration is reduced from 5 mM to 0.5 mM and/or the ionic strength is increased by about an order of magnitude (100 mM NaCl added, filled circles) the affinities are correspondingly lowered (K_1 = 2.8 µM; K_2 = 6 µM. With buffer only (triangles), the dissociation constants are further increased (K_1 = 4 µM; K_2 = 6 µM) and the "degree of separation" between the sites is decreased (K_2/K_1 is ca. 4 for 5 mM $MgCl_2$, but reduced to 1.5 for no $MgCl_2$). Thus, it appears that Mg^{2+} acts not only in facilitating stronger εADP binding, but the heterogenity or negative interaction between the two sites as well. High ionic strength (0.1 M - 0.5 M) media are known to enhance the lability of CF_1 (as monitored by ATPase activity) in the cold, and to a lesser extent at room temperature (7). Possibly, a related, but not necessarily identical, change in CF_1 producing a conformation less favorable for εADP binding would explain the effect of 0.1 M NaCl observed in Figs. 8 and 10. The solubility of many proteins is augmented by concentrations of salt in this range, but it seems unlikely that this would cause a decrease in binding; on the other hand, added salt causes precipitation of proteins, but only at concentrations an order of magnitude higher. Thus, these latter non-specific

effects, at least are probably not operative here.

The binding of εADP to CF_1 in the presence of $MgCl_2$, $CaCl_2$ and $MgCl_2 + P_i$ is compared in Fig. 11. The binding affinity with 5 mM $MgCl_2$ ($K_1 = 1.5$ µM; $K_2 = 2.5$ µM, open circles), appears to be slightly greater than with 5 mM $CaCl_2$ ($K_1 = 2.5$ µM; $K_2 = 5$ µM, open squares). Qualitatively, the same difference was observed in different experiments, although there exists a small variance in quantitative values of K_1 and K_2, corresponding to some variability in preparations and the limitations and experimental errors inherent in the fluorescence measurements. For example, under conditions of the experiment of Fig. 10, K_1 and K_2 and 5 mM $MgCl_2$ were 0.5 µM and 2 µM. No large significance should be attached to these small differences.

If phosphate (0.5 mM KH_2PO_4) is present with 5 mM $MgCl_2$ (Fig. 11, filled circles), the affinity of both the magnesium mediated binding sites is substantially reduced to an apparently low value ($K_1 = K_2 = 6.5$ µM). (Reminder: K's given are dissociation, not association, constants.) The curves for magnesium dependent binding ($\pm P_i$) of εADP to CF_1 isolated from spinach were also calculated (using the data of Fig. 2 from a preliminary publication of the author, see ref. 129); these values exhibit a trend similar to that shown above: $K_1 = 1$ µM, $K_2 = 2$ µM for 5 mM $MgCl_2$ and $K_1 = K_2 = 6$ µM for 5 mM $MgCl_2 + 0.5$ mM KH_2PO_4. Thus, the divalent

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Fig. 11. Klotz binding plot for ϵADP-CF_1. See chapter II for explanation of binding curves. Medium containing 5.7 µM CF_1 and 25 mM Tricine-NaOH (pH 8) plus the following additions affected the K_d's as indicated: 5 mM $MgCl_2$ (open circles, $K_1 = 1.5$ µM, $K_2 = 2.5$ µM), 5 mM $MgCl_2$ + 0.5 mM KH_2PO_4 (filled circles, $K_1 = K_2 = 6.5$ µM), and 5 mM $CaCl_2$ (open squares, $K_1 = 2.5$ µM, $K_2 = 5$ µM).

79
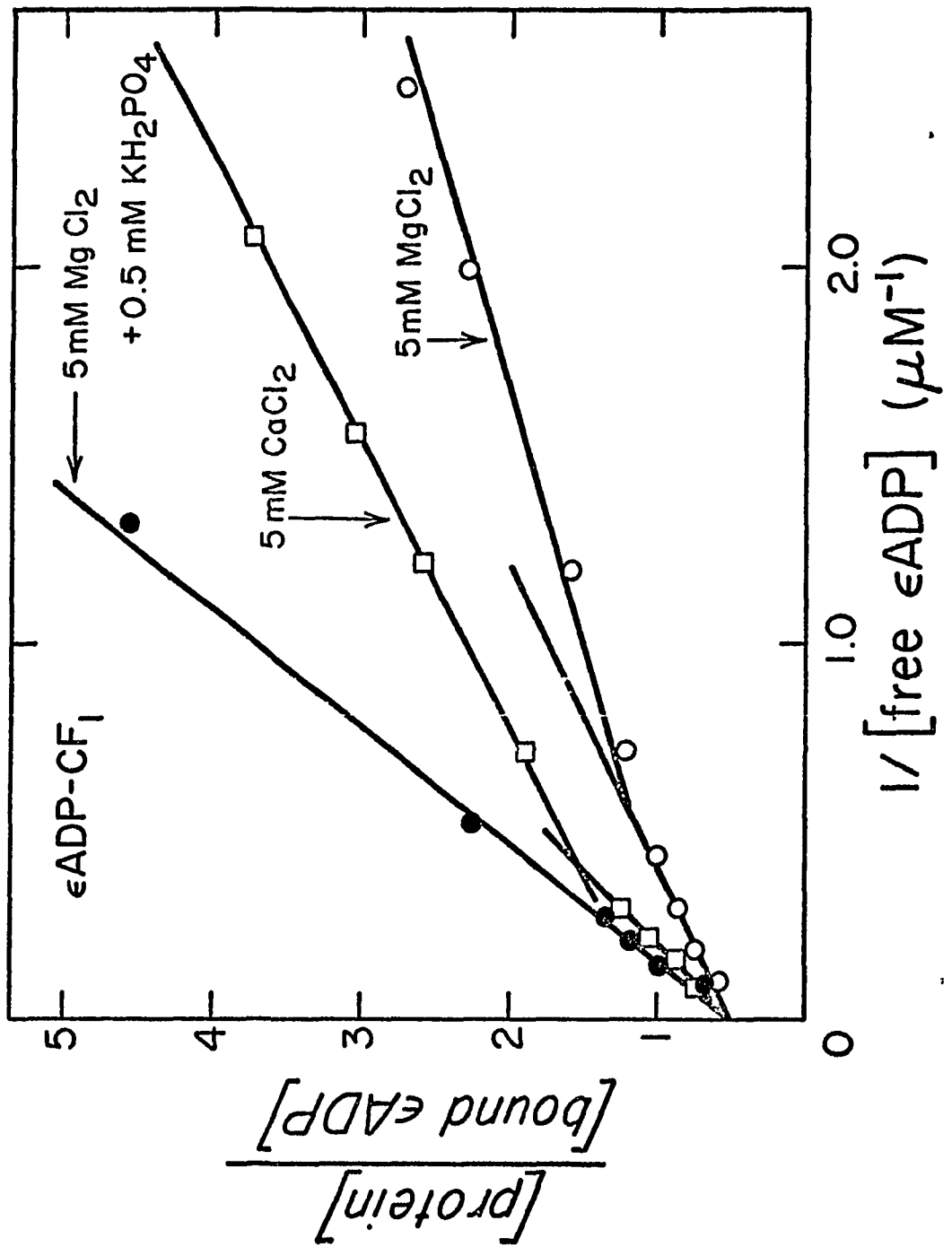
cations Mg^{2+} and Ca^{2+} both facilitate εADP binding to CF_1 at 2 sites with only small difference, whereas P_i exhibits a substantial negative interaction with both εADP sites.

The effect of the end product of photophosphorylation (ATP) on the binding of εADP to CF_1 was investigated. At relatively low concentration (10 μM), ATP has sizable effect on εADP binding as indicated by a decrease (ca. 60% at 10 μM εADP) in polarization of fluorescence (Fig. 12; open circles, without, and filled circles with 10 μM ATP). A small effect of 10 μM ATP on the intensity of εADP fluorescence was also observed (Fig. 13).

We note here that the actual polarization value observed here with 5 mM $MgCl_2$ at εADP concentration of ca. 2.5 μM approaches 0.20, whereas in Fig. 8 it appears to be lower (ca. 0.12). These differences between different preparations were due to several factors: concentration of CF used (4.1 μM in Fig. 8 and 8.5 μM in Fig. 12) and on the intrinsic differences in CF_1 preparations, of necessity from different available sources in which the variability could not be controlled. However, the conclusions obtained in this work are not affected by these differences in absolute values because the same trends were observed with different treatments in all cases.

Binding curves for the data of Fig. 12 and a similar experiment using 20 μM ATP are presented in Figs. 14 and 15.

Fig. 12. Effect of 10 μM ATP on the polarization of
εADP fluorescence in the presence of isolated CF_1. Medium
contained 8.5 μM CF_1, 30 mM Tricine-NaOH (pH 8) and 5 mM
$MgCl_2$; open circles: no further additions, filled circles;
10 μM ATP was added first and 10 minutes allowed before εADP
additions.

εADP–CF$_I$

Control

+10 μM ATP

εADP Concentration (μM)

Polarization of εADP Fluorescence

Fig. 13.   Effect of 10 μM ATP on the intensity of εADP fluorescence in the presence of isolated CF_1.   Same conditions as Fig. 12.

ϵADP-CF$_1$

+10 μM ATP

Control
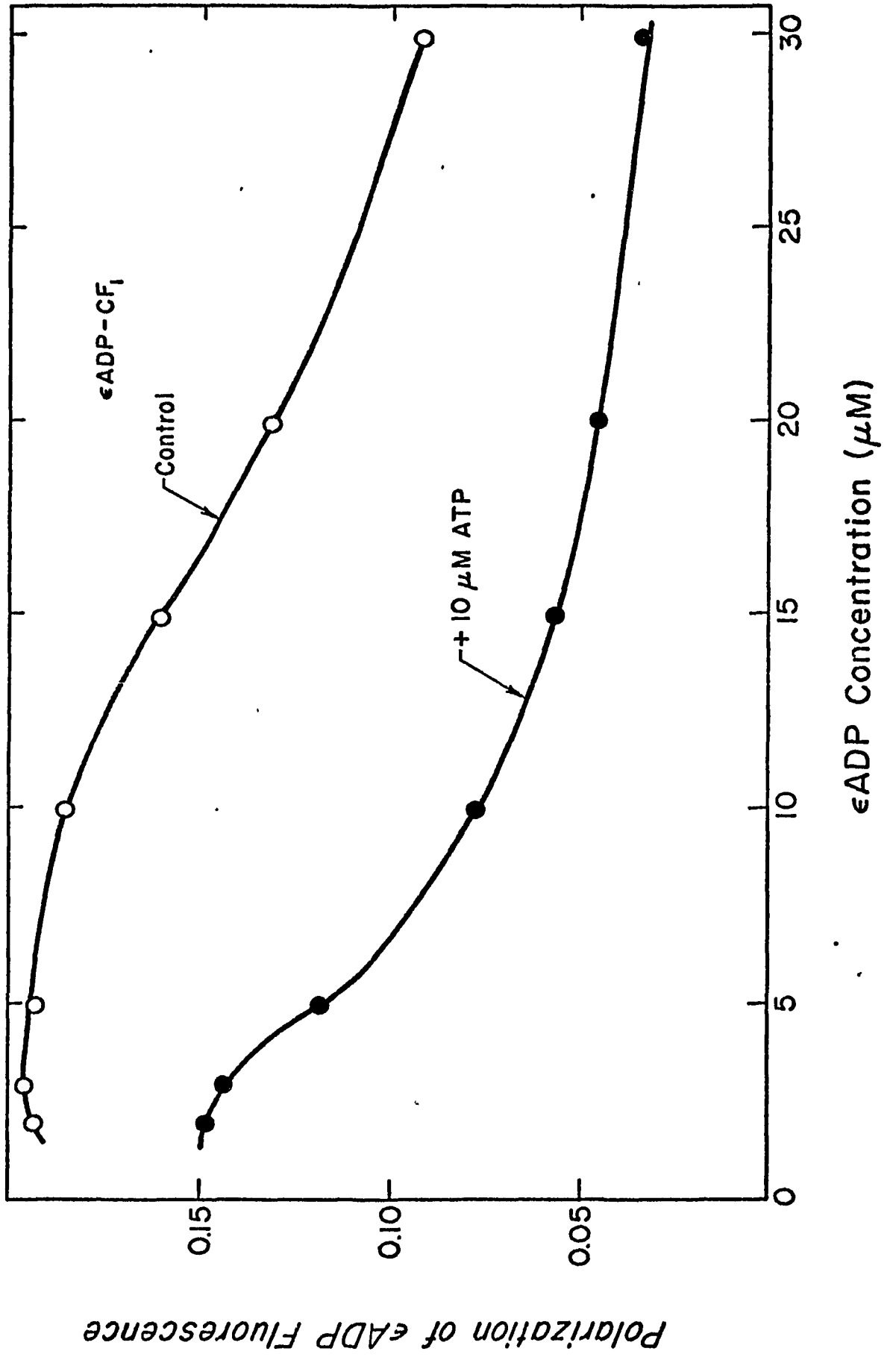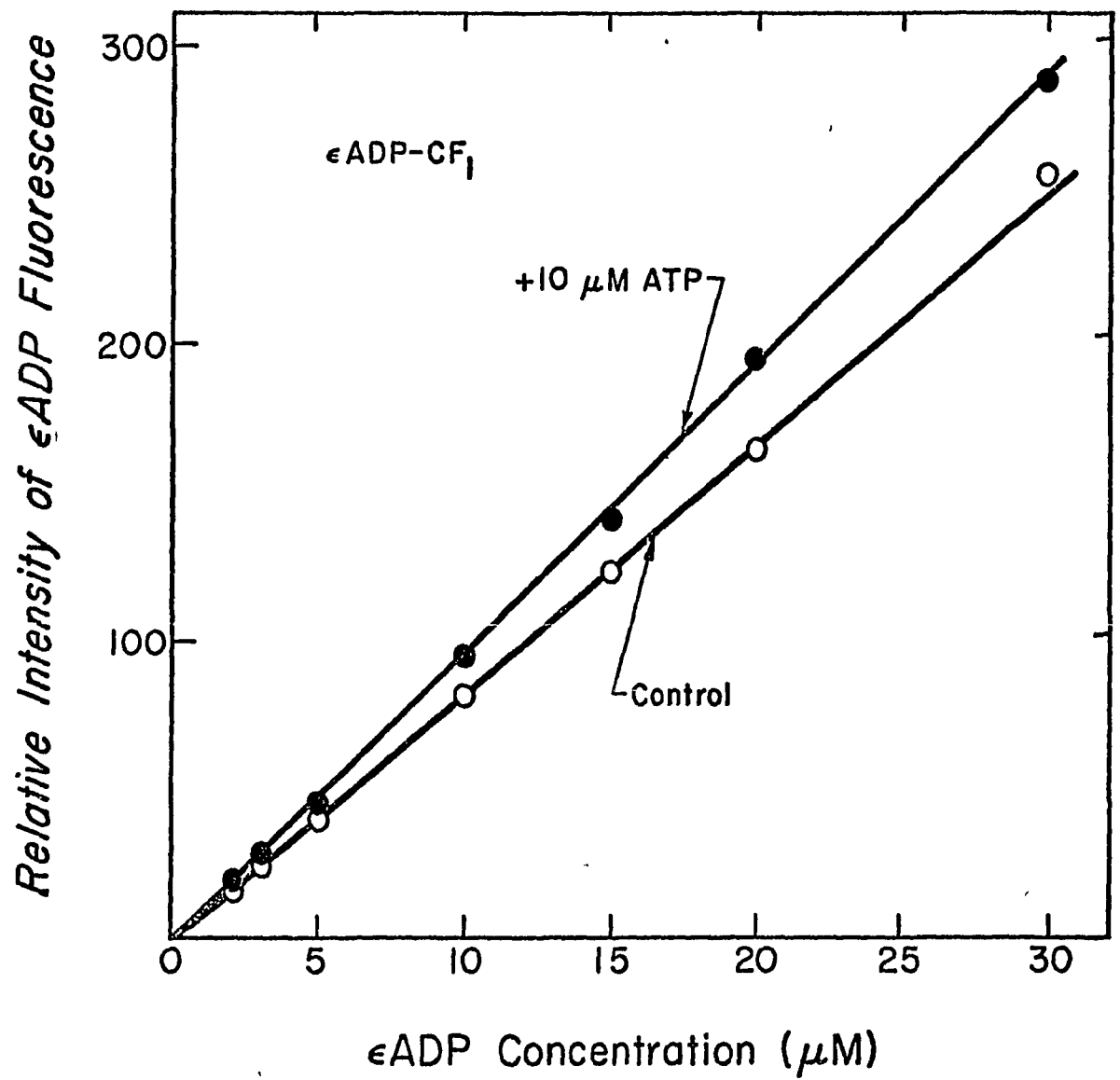
Fig. 14. Effect of 10 μM ATP on Klotz binding plot for εADP-CF$_1$. (See legend of Fig. 12 for experimental details.) See chapter II for explanation of binding curves. Calculated values for K$_d$'s are: K$_1$ = 0.5 μM, K$_2$ = 1.5 μM (-ATP, open circles) and K$_1$ = 2.6 μM, K$_2$ = 11 μM (+10 μM ATP, filled circles).
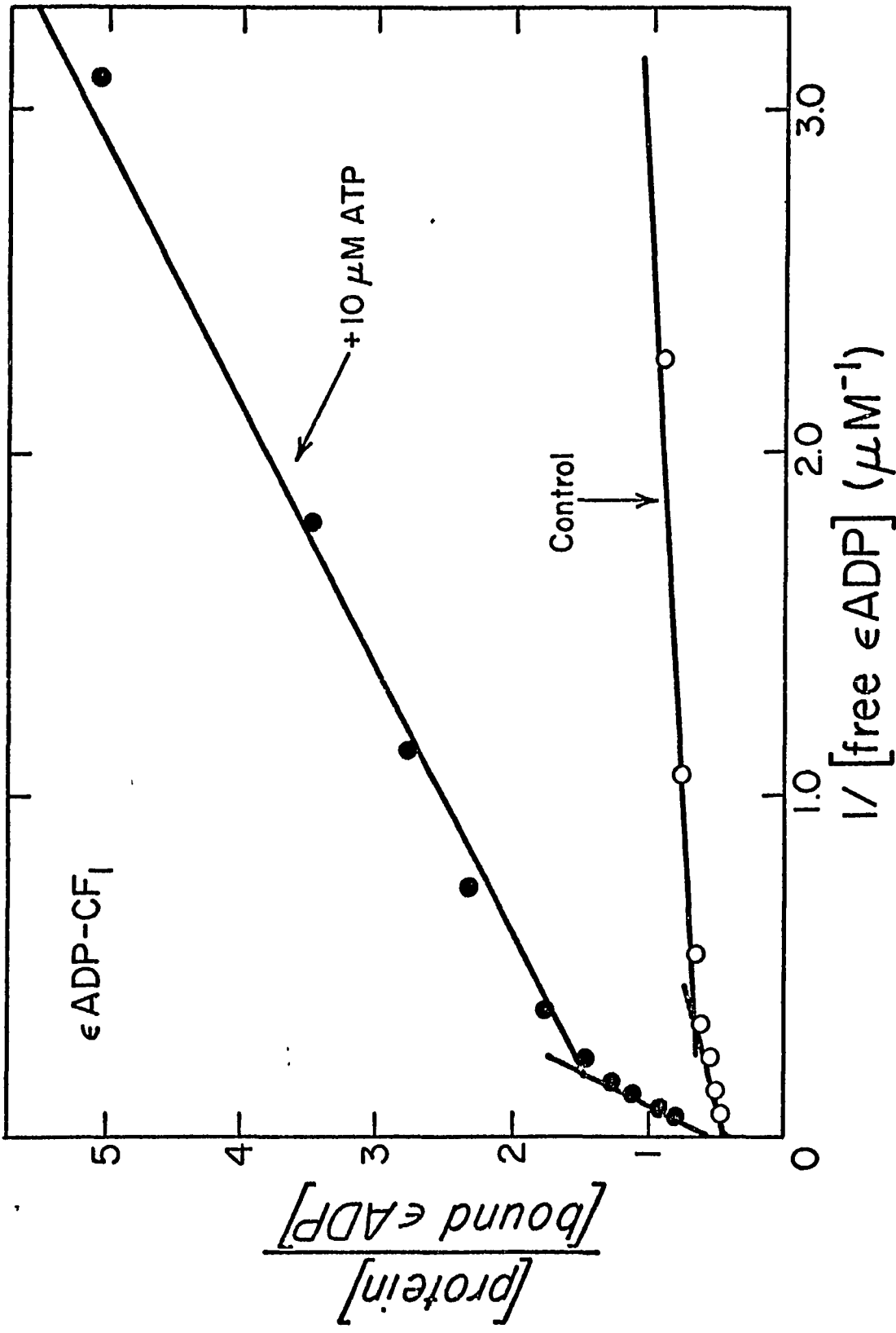
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Fig. 15. Effect of 20 µM ATP on Klotz binding plot
for εADP-CF$_1$. See chapter II for explanation of binding
curves. Medium containing 30 mM Tricine-NaOH (pH 8), 5 mM
MgCl$_2$ and 6 µM CF$_1$. Calculated values for K$_d$'s are: K$_1$ =
0.7 µM, K$_2$ = 2 µM (-ATP, open circles) and K$_1$ = 25 µM, K$_2$ =
30 µM (+20 µM ATP, filled circles).

εADP–CF$_I$

Control

+20μM ATP

$\dfrac{[\text{protein}]}{[\text{bound } \epsilon\text{ADP}]}$

1/ [free εADP] (μM^{-1})
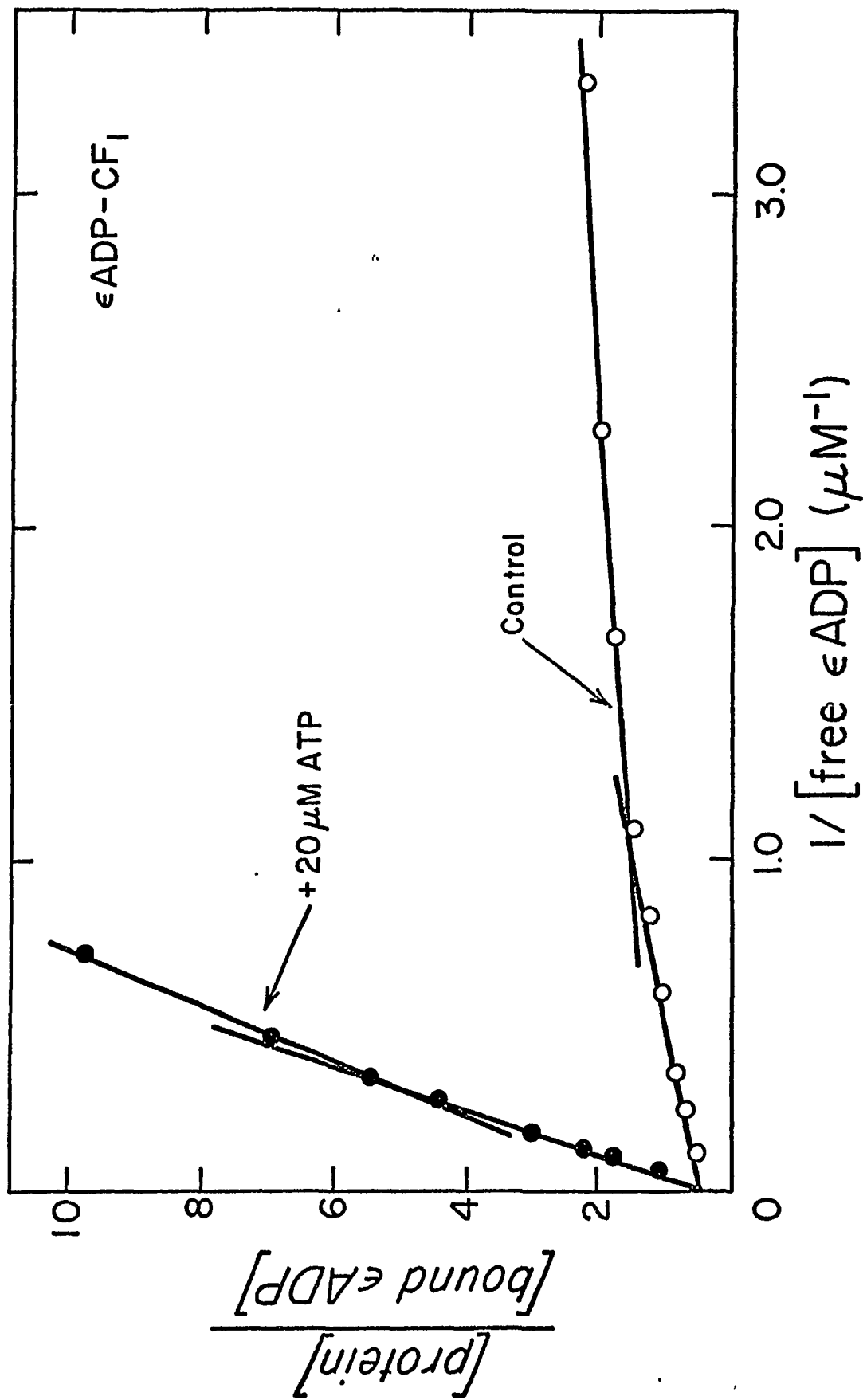
From Fig. 14, it is clear that the presence of 10 μM ATP reduces the binding affinity of εADP to CF_1 by about an order of magnitude for both sites: K_1 = 0.5 μM, K_2 = 1.5 μM (-ATP, open circles) and K_1 = 2.6 μM, K_2 = 11 μM (+ATP, filled circles). Increasing the ATP concentration to 20 μM (Fig. 15) affects εADP binding further (K_1 = 25 μM, K_2 = 30 μM, filled circles). Thus, it is evident that ATP exhibits a strong negative interaction with both εADP sites on CF_1.

Since it is known that both ADP and ATP form complexes with Mg^{2+}, one might suggest that the effect of either or both of these nucleotides on the binding curves for εADP (or εATP, see section B.2. below) is due simply to a reduction in the amount of available Mg^{2+} necessary for maximal binding (Fig. 8). However, using association constants for formation of Mg-ATP (K_a = 10^4) and Mg-ADP (K_a = $10^{3.1}$) and the concentrations of ADP or ATP (10^{-5} M) and $MgCl_2$ (5 x 10^{-3} M) used, one can calculate that ATP and ADP would reduce the concentration of free Mg^{2+} by only ca. 5 x 10^{-4} M and 5 x 10^{-5} M, respectively. A comparison with Fig. 8 readily shows that even decreasing $MgCl_2$ concentration by nearly ten times the calculated value of Mg-ATP cannot account for the dramatic changes induced in εADP binding by 10 μM nucleotide (Mg-ADP would have even less effect).

In the experiment converse to that of Fig. 12, the effect of 10 μM ADP on the polarization of εADP-CF_1 was

investigated. With a solution of CF_1 that produces a typical polarization (binding) curve (Fig. 16, open circles) in the range of εADP concentration from 2 to 35 μM, addition of 10 μM ADP (filled circles) decreases the level of εADP polarization to a value close to zero (less than 10% of control). Thus, the negative interaction of unmodified ADP with εADP binding (Fig. 16) is apparently stronger than the interaction of ATP (at the same concentration) with εADP (Fig. 12).

In an experiment in which the order of addition of nucleotide was reversed an excess of ADP or ATP was added to a solution of 2 μM εADP after it had achieved maximal binding to CF, and the time course of the effect on εADP fluorescence polarization was followed. Under these conditions (Fig. 17), even up to 30 minutes after the addition of ATP, there was no change in polarization of εADP fluorescence. On the other hand, addition of 10 μM or 20 μM ADP caused a substantial decrease in polarization, proportional to the ADP concentration. After ca. 30 minutes, with a tenfold excess of ADP (20 μM), the polarization was reduced to nearly zero (ca. 0.025). When 20 μM ADP was added first and allowed to equilibrate with CF_1 before adding εADP, within 30 minutes the polarization of fluorescence of εADP-CF_1 equilibrated to the same value (ca. 0.025) as when ADP was added after εADP; this trend was also observed with 10 μM ADP, as expected (not shown). Thus, in comparing the effects of unmodified ADP and ATP, these results

Fig. 16. Effect of 10 μM ADP on the polarization of εADP fluorescence in the presence of isolated CF_1. Medium contained 30 mM Tricine-NaOH (pH 8), 5 mM $MgCl_2$ and 3.6 μM CF_1, plus 10 μM ADP (filled circles) or no further addition (open circles).

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Polarization of εADP Fluorescence vs. εADP Concentration (μM)

εADP–CF₁

Control

+ 10 μM ADP
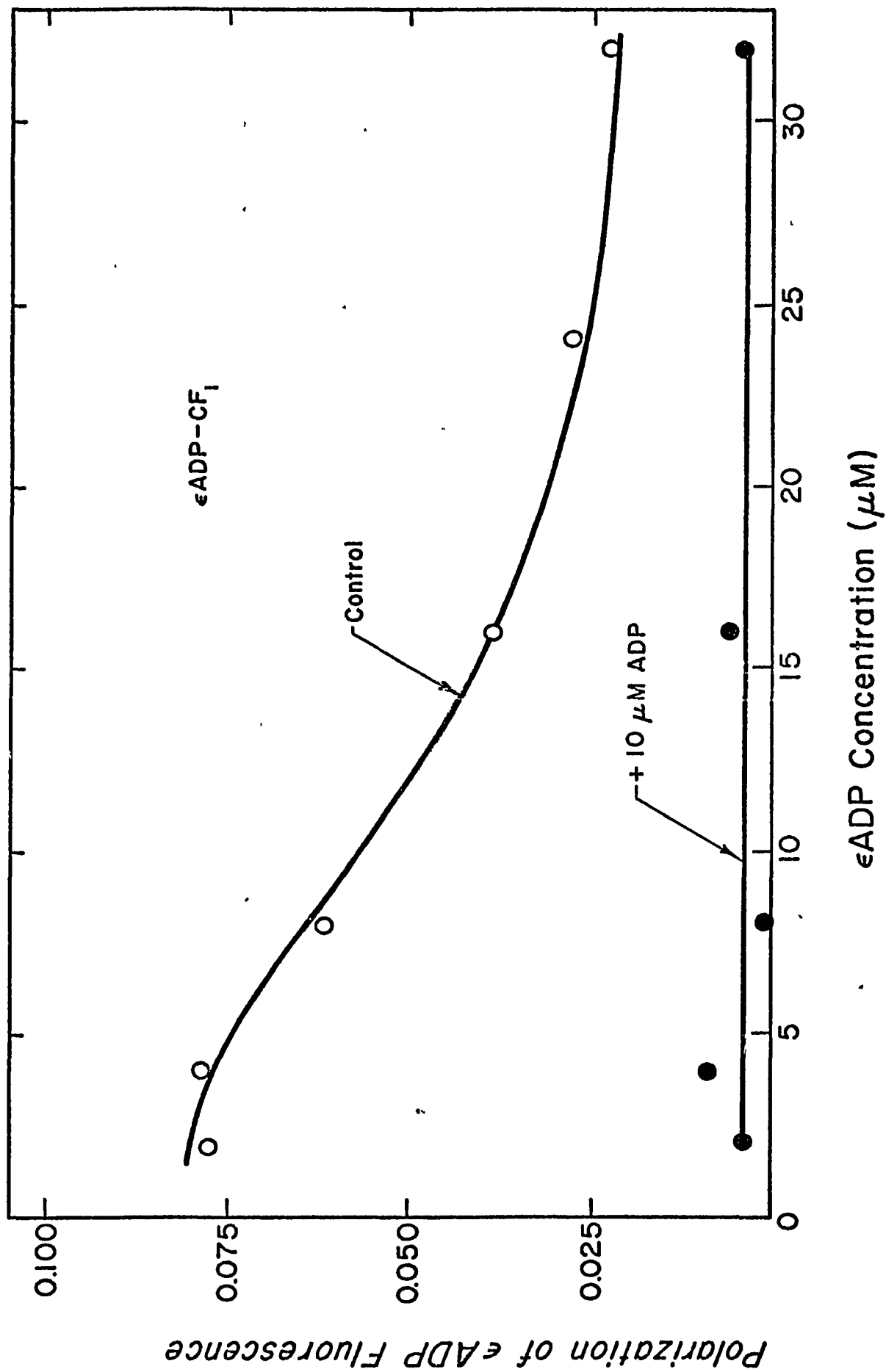
Fig. 17. Time course of the effect on the polarization of εADP fluorescence of addition of an excess of ADP or ATP to a solution containing 2 μM εADP bound to CF_1. Experiments with square symbols (7.3 μM CF_1 used) were normalized, at level of maximum polarization, to data obtained in another set of experiments indicated by circular symbols (6.9 μM CF_1 used). ADP or ATP was added at the point indicated, at the concentrations shown in the figure. (Vertical error bars indicate the approximate uncertainty in polarization introduced by the effect ($\leq$2%) of added ADP or ATP on the background scattering intensity subtracted in the calculation of fluorescence polarization.) In the experiment in which 20 μM ADP was added first (filled circles), a 15 minute interval was allowed for equilibration before adding 2 μM εADP. Medium contained 5 mM $MgCl_2$ and 30 mM Tricine-NaOH (pH 8).
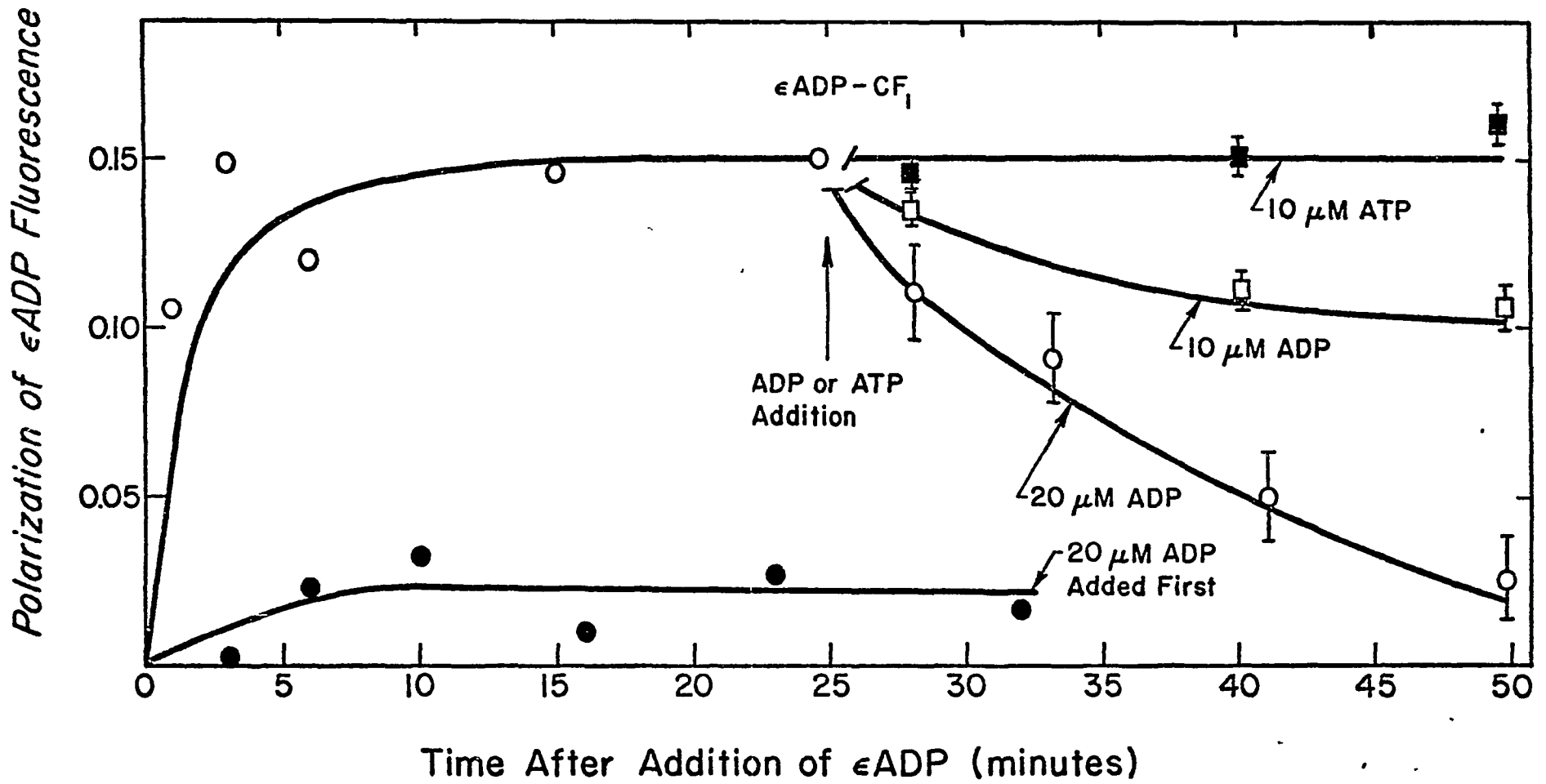
are consistent with the degree of effect these nucleotides have on the ϵADP binding curves discussed above (Figs. 12 and 16), i.e., ADP has a stronger apparent interaction with ϵADP than ATP does. It was observed that ADP equilibrates directly with the ϵADP binding sites, but ATP interacts poorly with ϵADP sites (Fig. 17).

Since the polarization of ϵADP fluorescence is reduced in the presence of 10 μM ATP in Fig. 12, there is an apparent contradiction with the lack of effect of 10 μM ATP on ϵADP fluorescence in Fig. 17, even when present in the CF_1 solution for 25 minutes, which is longer than the incubation period of Fig. 12. However, there is a difference in the order of adding ATP and ϵADP in the two cases. When ATP is added first, it can bind to CF_1, presumably at a non-ϵADP regulatory or allosteric site, such that the K_d for ϵADP binding is increased (Figs. 14 and 15); this proposed effect may be mediated by an ATP-induced change in CF_1 conformation. On the other hand, if added first, ϵADP can bind to CF_1 so as to prevent the ATP-dependent decrease in polarization of ϵADP fluorescence, possibly mediated indirectly by an ADP-induced change in the ATP regulatory site via a change in CF_1 conformation. Support for an ADP-induced change in CF_1 conformation is indicated by polarization of protein fluorescence (presented below, section III.G, Table 7). Moreover, the suggestion of an ADP-induced effect on the ATP binding is consistent with the decrease in the K_d for binding of ϵATP to

to CF_1 which we observed under the same conditions (i.e.,
when ADP was added first; presented below, section III.B.2).
In conclusion, although these suggestions may not be a unique
explanation of these ADP-ATP binding interactions, they are
internally consistent and provide a simple interpretation of
the data.

2. εATP

Analogous to the experiments with εADP, the binding of
εATP to CF_1 was also investigated. Fig. 18 presents a com-
parison of the effects of 5 mM $MgCl_2$ (open circles), 5 mM
$CaCl_2$ (open squares), and 5 mM $MgCl_2$ plus P_i (0.5 mM KH_2PO_4,
filled circles). If $MgCl_2$ or $CaCl_2$ is present in the medium
there are two binding sites on CF_1 for εATP ($K_1 \sim 2$ µM, $K_2 \sim$
4 µM). As in the case of ADP, phosphate substantially in-
creases the dissociation constant for both Mg-εATP binding
sites ($K_1 = K_2 = 7$ µM). Both the divalent cations Mg^{2+} and
Ca^{2+} facilitate εATP binding to CF_1 at sites with $K_d \sim 2$ µM
and P_i exhibits a substantial negative interaction with both
sites.

The effects of ADP, the nucleotide substrate for photo-
phosphorylation, on the binding of εATP to CF_1 were also
examined in analogy to the results of Figs. 12-15. Again, at
relatively low concentration (10 µM), ADP significantly
affects εATP binding as indicated by a decrease (ca. 60% at
10 µM ATP) in polarization (Fig. 19; open circles, minus ADP;

Fig. 18. Klotz binding plot for εATP-CF$_1$. See Materials and Methods for explanation of binding curves. Medium containing 5.7 μM CF$_1$ and 25 mM Tricine-NaOH (pH 8), plus the following additions affected the K_d's as indicated: 5 mM MgCl$_2$ (open circles, K_1 = 2.2 μM, K_2 = 4 μM), 5 mM MgCl$_2$ + 0.5 mM KH$_2$PO$_4$ (filled circles, K_1 = K_2 = 7 μM, and 5 mM CaCl$_2$ (open squares, K_1 = 1.8 μM, K_2 = 4 μM).

Figure shows a plot of $\frac{[protein]}{[bound\ \epsilon ATP]}$ versus $1/[free\ \epsilon ATP]\ (\mu M^{-1})$ for $\epsilon ATP-CF_1$ under three conditions: $5\,mM\ MgCl_2 + 0.5\,mM\ KH_2PO_4$, $5\,mM\ MgCl_2$, and $5\,mM\ CaCl_2$.

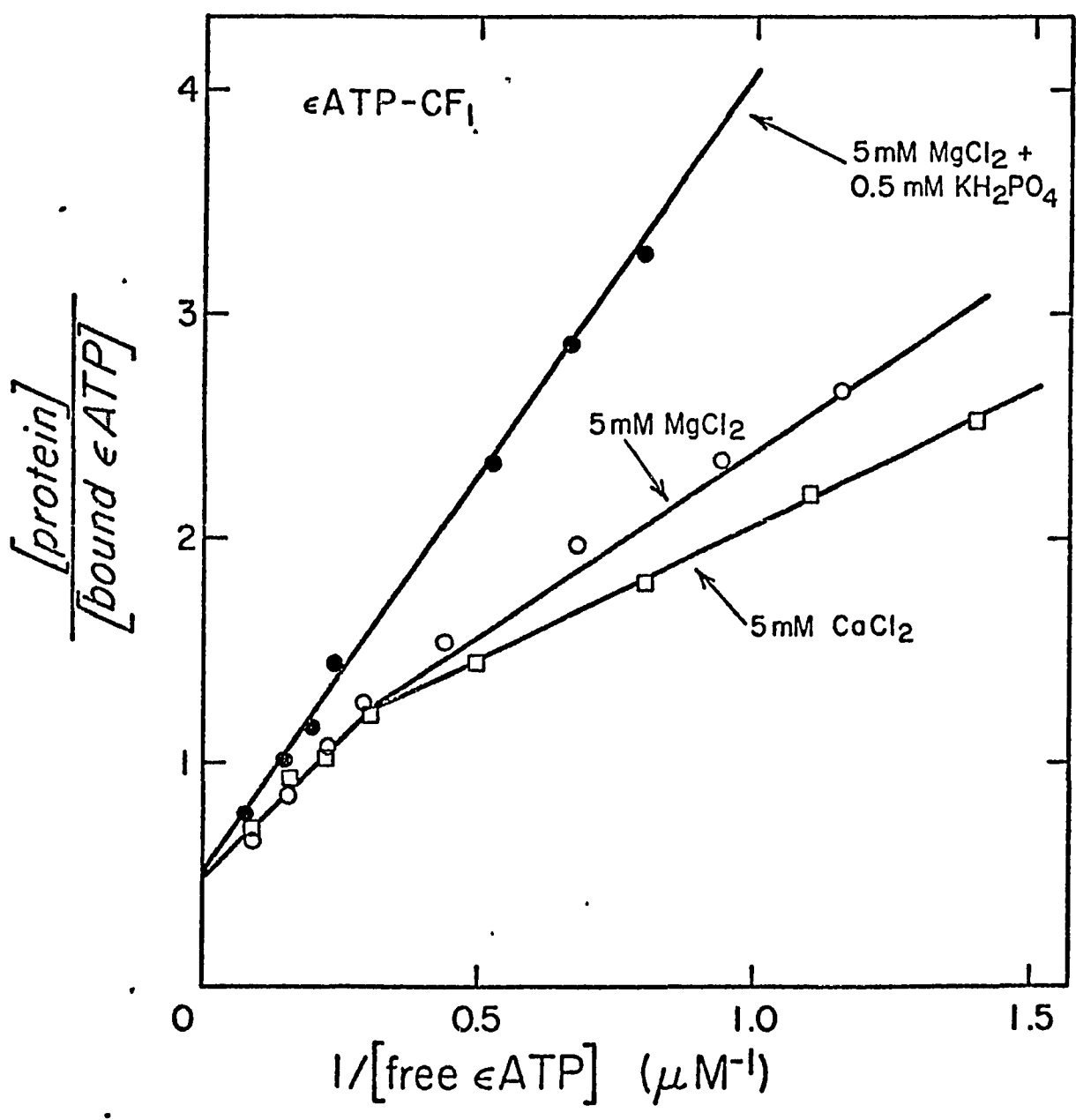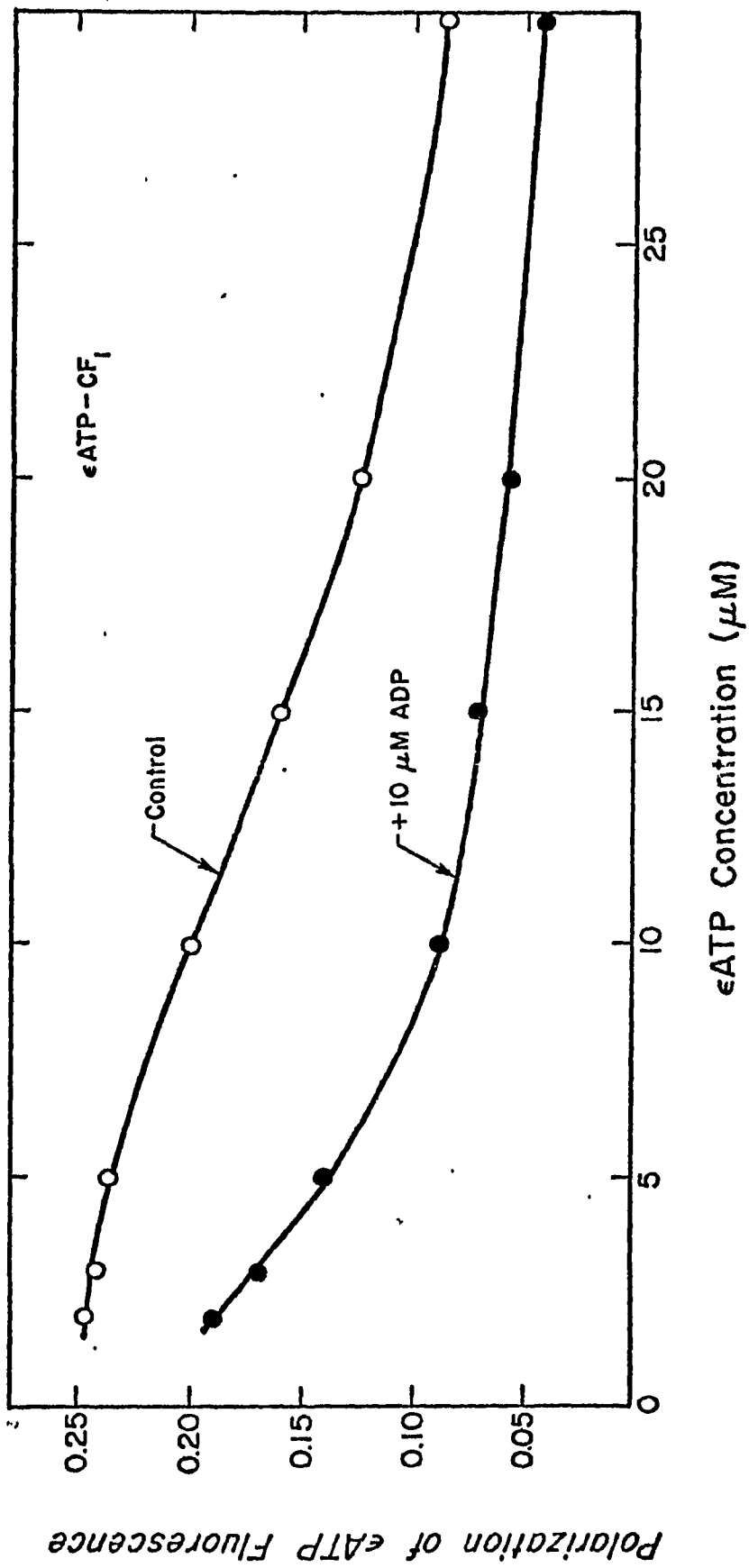Fig. 19. Effect of 10 μM ADP on the polarization of
εATP fluorescence in the presence of isolated CF_1. Medium
contained 8.5 μM CF_1, 30 mM Tricine-NaOH (pH 8) and 5 mM
$MgCl_2$; open circles, no further additions; filled circles,
10 μM ADP was added first and 10 minutes allowed before
εATP additions.

εATP–CF$_1$

Control

+10 μM ADP

εATP Concentration (μM)

Polarization of εATP Fluorescence

filled circles, plus 10 µM ADP). A small effect of ADP on intensity of εATP fluorescence was also observed (Fig. 20). Binding curves for the results of Fig. 19 and a similar experiment using 20 µM ADP are presented in Figs. 21 and 22. It is evident from Fig. 21 that the presence of 10 µM ADP reduces the binding affinity of εATP to CF_1 by about an order of magnitude: K_1 = 0.6 µM, K_2 = 4 µM (-ADP, open circles) and K_1 = 2.5 µM, K_2 = 16 µM (+10 µM ADP, filled circles). By increasing the ADP concentration to 20 µM (Fig. 22), εATP binding at the tighter site is further reduced, with an apparent tendency for the dissociation constants for the two sites to become closer to each other (in terms of binding affinity), i.e., K_1 = K_2 = 18 µM. Thus, it is evident that ADP exhibits a strong negative interaction with both εATP sites on CF_1.

In the experiment converse to that of Fig. 19, the effect of 10 µM ATP on the polarization of εATP-CF_1 was investigated. In a solution of CF_1 that yields a typical polarization (binding) curve (Fig. 23, open circles) in the range of [εATP] from 2 to 35 µM, addition of 10 µM ATP decreases the level of εATP polarization to a value close to zero or less than 5% of control (Fig. 23, filled circles). Thus, the negative interaction of unmodified ATP with εATP binding (Fig. 23) is apparently stronger than the interaction of ADP (at the same concentration) with εATP (Fig. 19).

Fig. 20. Effect of 10 μM ADP on the relative intensity of εATP fluorescence in the presence of isolated CF_1. Same conditions as Fig. 19.
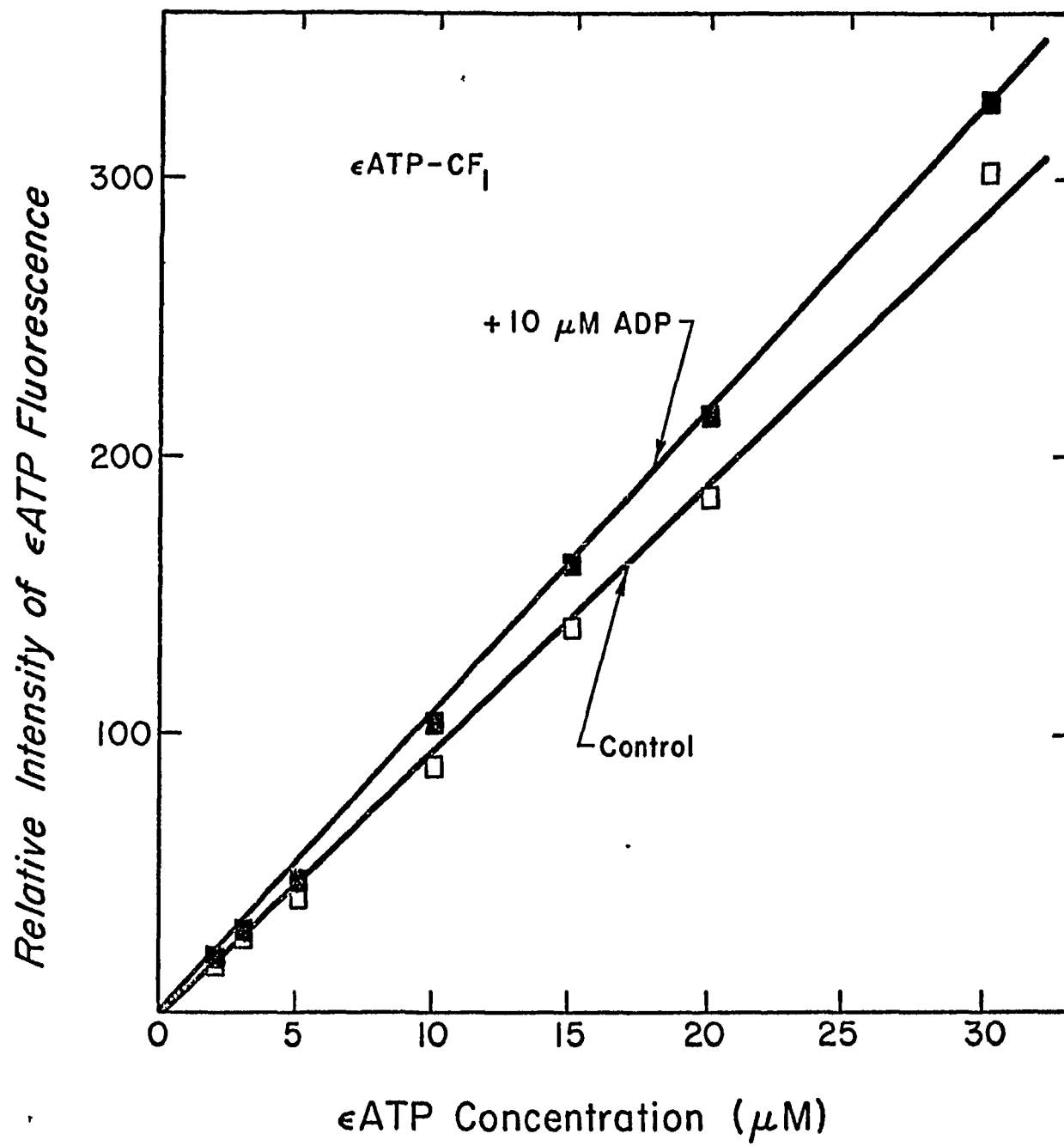
Fig. 21. Effect of 10 µM ADP on the Klotz binding plot for εATP-CF$_1$. (Fig. 19 legend has details.) See chapter II for explanation of binding curves. Calculated values for K_d's are: K_1 = 0.6 µM, K_2 = 4 µM (-ADP, open circles) and K_1 = 2.5 µM, K_2 = 16 µM (+10 µM ADP, filled circles).
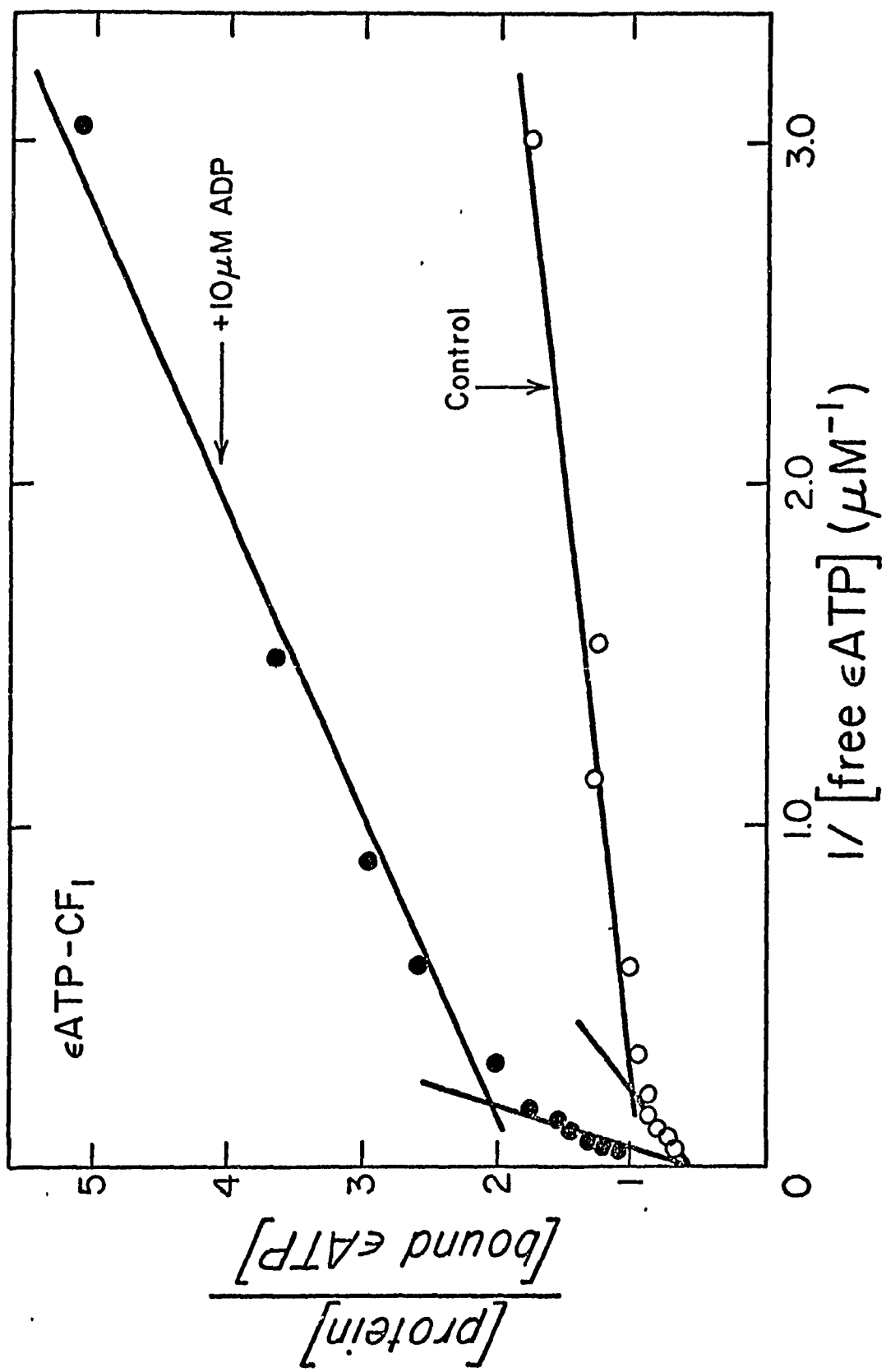
Fig. 22. Effect of 20 μM ADP on the Klotz binding plot for εATP-CF$_1$. See Materials and Methods for explanation of binding curves. Medium contained 30 mM Tricine-NaOH (pH 8), 5 mM MgCl$_2$ and 6 μM CF$_1$. Calculated values for K$_d$'s are: K$_1$ = 0.75 μM, K$_2$ = 2 μM (-ADP, open circles) and K$_1$ = K$_2$ = 18 μM (+20 μM ADP, filled circles).

Fig. 23.   Effect of 10 µM ATP on the polarization of εATP fluorescence in the presence of CF_1.   Medium contained 30 mM Tricine-NaOH (pH 8), 5 mM $MgCl_2$ and 3.6 µM CF_1, plus 10 µM ATP (filled circles) or no further addition (open circles).

Polarization of εATP Fluorescence vs εATP Concentration (μM). Curves labeled εATP-CF₁, Control, and +10 μM ATP.

In an experiment in which the order of addition of nucleotide was reversed, an excess of unmodified ADP or ATP was added to a solution of 2 µM εATP after it had achieved maximal binding to CF_1, and the time course of the effect on εATP fluorescence polarization was followed. Under these conditions (Fig. 24), there was a slow, small decrease in polarization of ATP fluorescence when a 10 µM or 20 µM ATP was added. In this case 10 µM ADP also caused a small decline in polarization, which within experimental error, was not very different from the effect of 10 µM ATP. In all cases, ca. 30 minutes after addition of unmodified nucleotide, there was only about a 16% decrease from the initial, maximal level. When 20 µM ATP was added first and allowed to equilibrate with CF_1 before adding εATP, even after 30 minutes the polarization of fluorescence of εATP-CF_1 did not reach the value attained when ATP was added after εATP; this trend was also observed with 10 µM ATP (not shown). The slow attainment of equilibrium under these conditions (Fig. 24) is discussed below at the end of this section.

Dowex-2 is a cross-linked polystyrene ion exchange resin which has a very high affinity for negative ions (130), including nucleotide anions. If εATP bound to CF_1 is readily accessible to the medium, Dowex-2 would be expected to complex with the anion and prevent binding, thus causing, without delay, a decrease in polarization of εATP fluorescence.

Fig. 24. Time course of the effect on the polarization of εATP fluorescence of addition of an excess of ADP or ATP to a solution containing 2 μM εATP bound to CF_1. Experiments with square symbols (6.8 μM CF_1 used) were normalized, at the level of maximum polarization, to data obtained in another set of experiments indicated by circular symbols (6.9 μM CF_1 used). ADP or ATP was added at the point indicated, at the concentrations shown in the figure. (See the legend of Fig. 17 for explanation of error bars.) In the experiment in which 20 μM ATP was added first (filled circles), a 15 minute interval was allowed for equilibration before adding 2 μM εATP. Medium contained 5 mM $MgCl_2$ and 30 mM Tricine-NaOH (pH 8). Approximately one mg Dowex-2X was added to ATP-CF_1 solution as indicated in the figure; after mixing, measurement of fluorescence polarization was not made until after a 2 minute interval to allow Dowex particles to settle. Before use, the Dowex-2X was washed according to the procedure outlined in Brewer et al. (130).
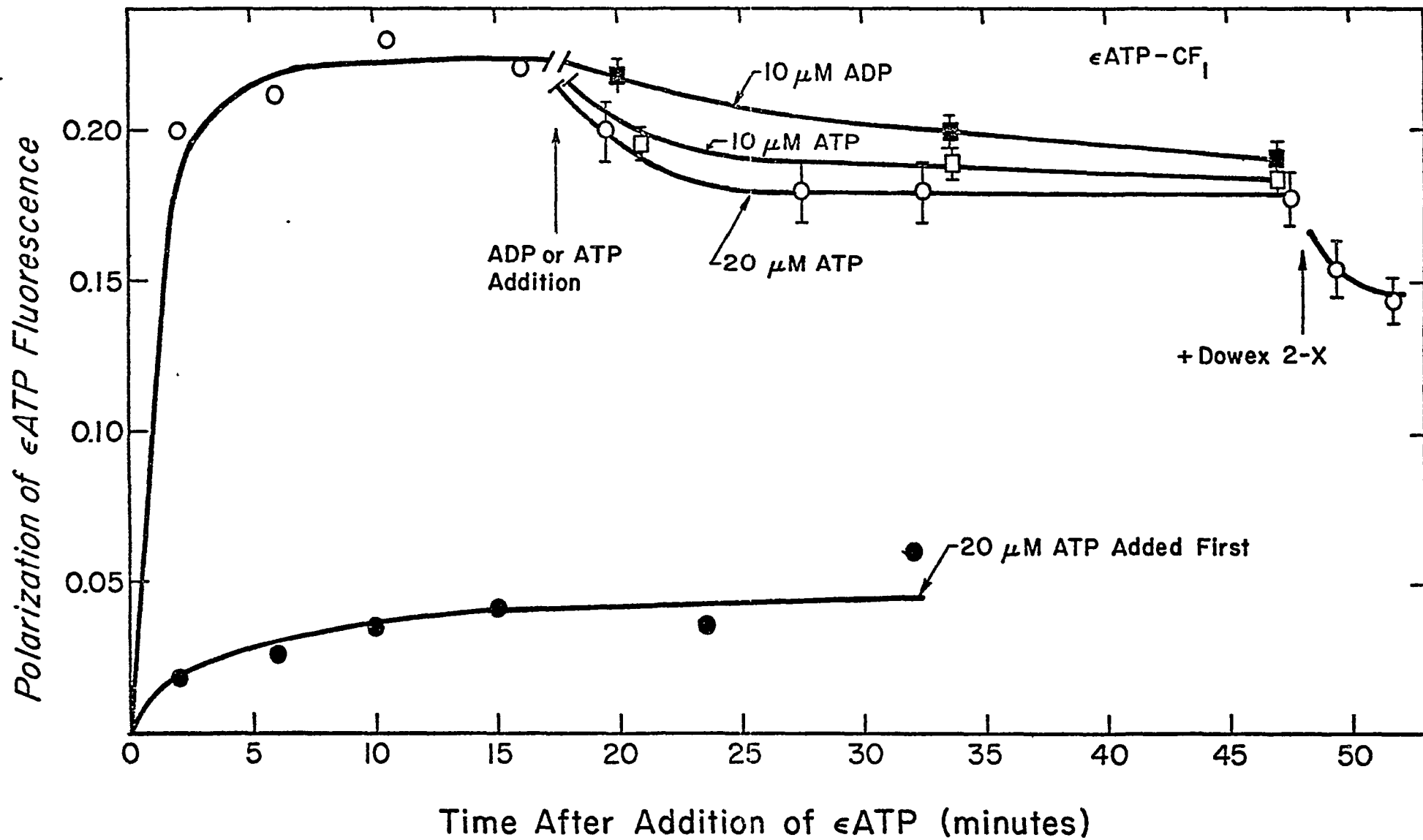
When ca. one mg of Dowex-2 was added to the εATP-CF$_1$ sample
just described (Fig. 24), after a few minutes the polarization
had decreased, but only to about half the initial, maximal
level. Based on the exchange capacity of this resin (ca. 3
milliequivalents per g), the binding capacity of one mg of
Dowex-2 is about 1000-fold in excess of the amount of εATP in
a 2 μM solution of 2 ml volume. One would expect this to
assure a rapid removal of εATP bound at relatively exterior
regions of the CF$_1$ complex. Since a rather slow, incomplete
removal was observed (Fig. 24), it appears that a significant
portion of the εATP becomes bound to CF$_1$ rather tightly and/or
at a relatively unexposed site.

The relatively slow time course for "unbinding" or
release of εATP in Fig. 24 suggests that a small part of the
effect ADP has on the calculated K_d's for εATP binding sites
(Figs. 21 and 22), and to a lesser extent the effect of ATP
on the K_d's for εADP binding sites (Figs. 14, 15 and 17), may
be due to kinetic factors (e.g., equilibrium not entirely
achieved during binding experiment). However, it is still
evident that the binding of ADP and ATP to sites on CF$_1$
show a sizable negative interaction, even though the "true"
K_d's may be slightly different, the latter being difficult to
ascertain (e.g., due to problems with CF$_1$ stability or
enzymatic conversion of nucleotides with experiments involving
hours rather than minutes).

3. εAMP

The polarization of fluorescence of εAMP in the presence of CF_1 was investigated under a variety of conditions to check for possible binding. Fig. 25 shows that coupling factor preparations which demonstrate good "control" binding of εADP to CF_1 do not show binding to AMP; for εAMP, the polarization of fluorescence was essentially zero with 5 mM $MgCl_2$, 5 mM $MgCl_2$ + 0.5 mM KH_2PO_4, 5 mM $MgCl_2$ + 10 μM ADP or 5 mM $MgCl_2$ + 10 μM ATP present (see figure legend for details). A corresponding trend was observed with measurements of intensity of εAMP and εADP fluorescence under the same conditions (Fig. 26); data for εAMP, evidently not bound to CF_1 all lie on the same line, representing relatively "unquenched" fluoresecence, whereas the fluorescence intensity of εADP, bound to CF_1 (εADP-CF_1), is quenched somewhat relative to εAMP. (The fluorescence quantum yields of εAMP and εADP are known to be identical; ref. 100.)

It is evident that there is essentially no binding of εAMP to isolated CF_1, regardless of the presence of cofactors of photophosphorylation. Since the relative "functional" efficiency of εAMP relative to the unmodified nucleotide (at least in one particular reaction, see section III.A., Table 5) was not as high as for εADP or εATP (30% vs. 65-85%), one may have less confidence in extrapolating the results of this section for εAMP to AMP. However, no binding of AMP to isolated CF_1 was found independently (110, 131).

Fig. 25. Polarization of εAMP and εADP fluorescence in the presence of isolated CF_1. Medium contained 30 mM Tricine-NaOH (pH 8), 5 mM $MgCl_2$ and 3.3 µM CF_1 (top, A) or 4.5 µM CF_1 (bottum, B), plus εADP (◼) or εAMP (●). Polarization of εAMP fluorescence was also measured in the above medium plus 0.5 mM KH_2PO_4 (◓), 10 µM ADP (0), and 10 µM ATP (◒).
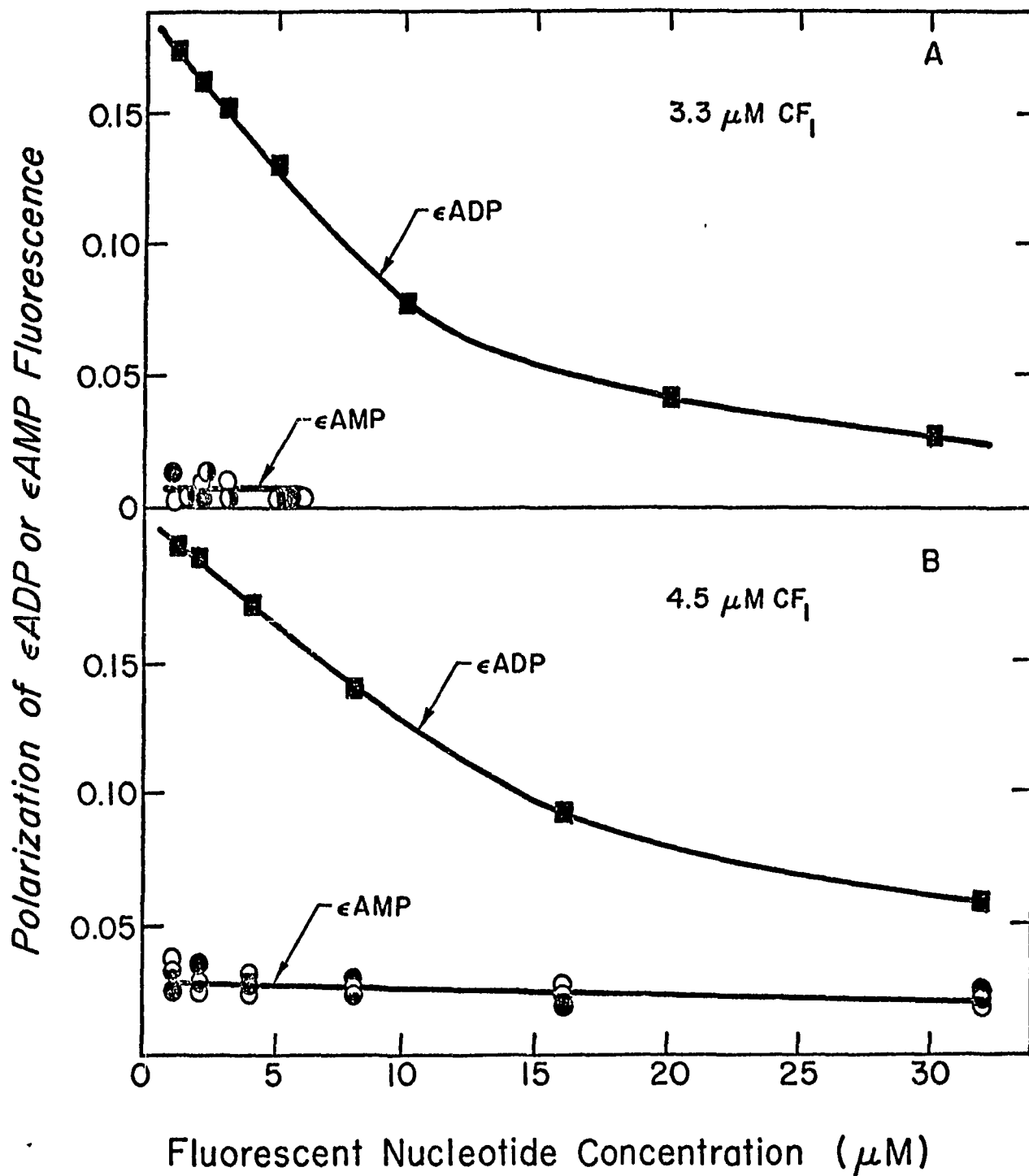
Fig. 26. Relative intensity of εAMP and εADP fluorescence in the presence of isolated CF_1. Same conditions as in Fig. 25. The numbers on the right ordinate are for B, and on the left for A.

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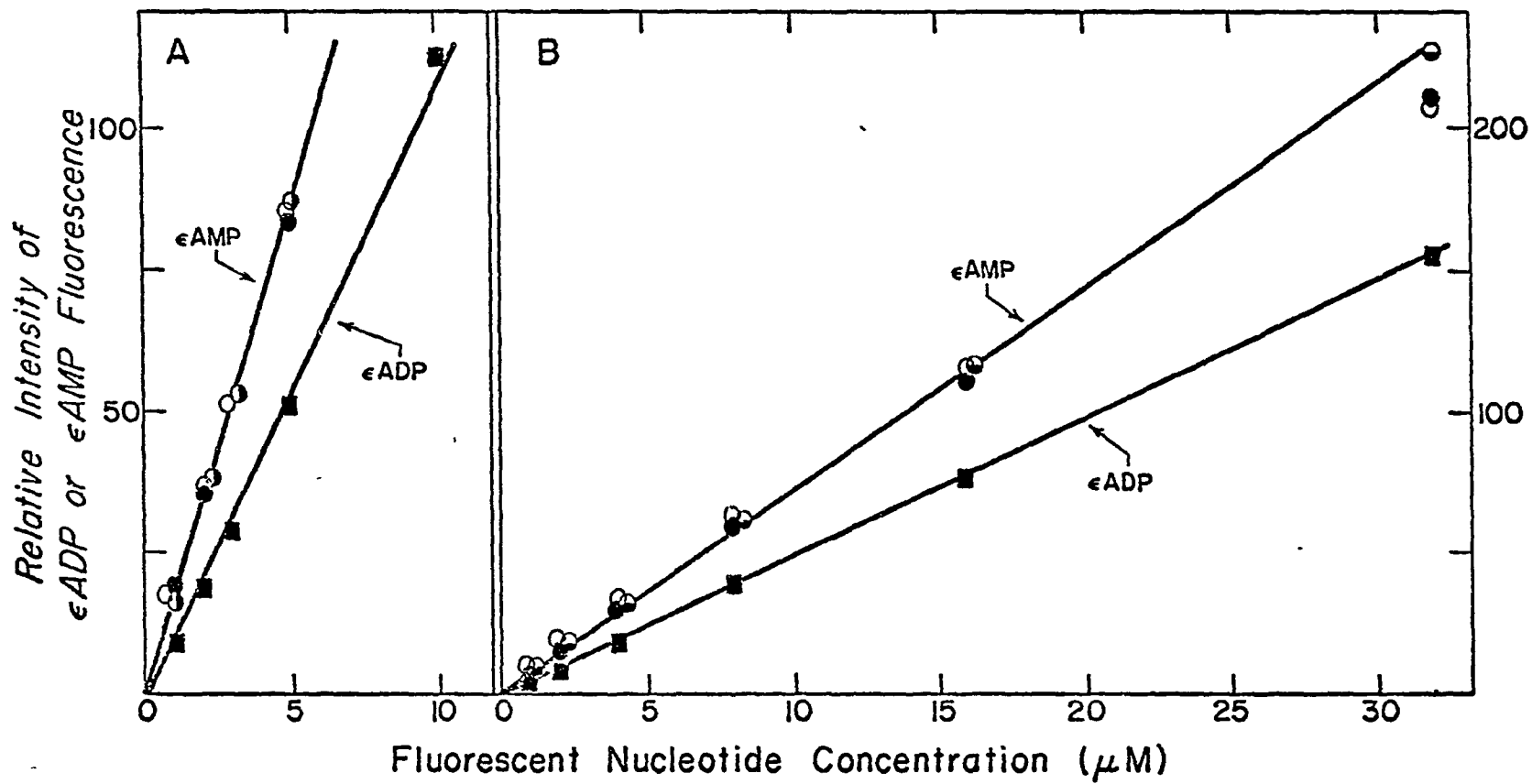
C. Polarization of fluorescence of tyrosine of coupling
factor protein

Since it is probable that the substrates or cofactors
of phosphorylation might induce functional changes in the con-
formation of CF_1 (32,33,91), the polarization of fluorescence
of the tyrosine residues in CF_1 was measured as an approach
to monitor possible alterations in the physical state of the
protein. In contrast to tryptophan, the emission spectrum of
tyrosine is relatively insensitive (118) to changes in its
environment (e.g., polarity), and therefore spectral changes
could not be utilized.

The addition of divalent cations to a solution of CF_1
produced a significant (ca. 20%) increase in polarization of
tyrosine fluorescence (Fig. 27). $MgCl_2$ is somewhat more
efficient than $CaCl_2$ in producing this effect in the con-
centration range 2.5 to 20 mM, although 30 mM $CaCl_2$ response
is the same as that for 30 mM $MgCl_2$. The concentration for
half-maximal effect is ca. 5 mM $MgCl_2$ under these conditions.
With KCl, the resulting changes were much smaller (<4%) in
the same concentration range (Fig. 27); results with KH_2PO_4
were similar to the KCl data (not shown).

Addition of $MgCl_2$, in the concentration range used in
Fig. 27, had little or no effect on both ultraviolet absorp-
tion (280 nm) by CF_1 and the intensity of protein fluores-
cence (not shown). This indicates that the observed salt

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Fig. 27. The effect of various salts on the polarization of coupling factor (tyrosine) fluorescence. Medium contained 25 mM Tricine-NaOH (pH 8.2) and 3.6 μM CF_1. See chapter II (section E) for details of measurement.
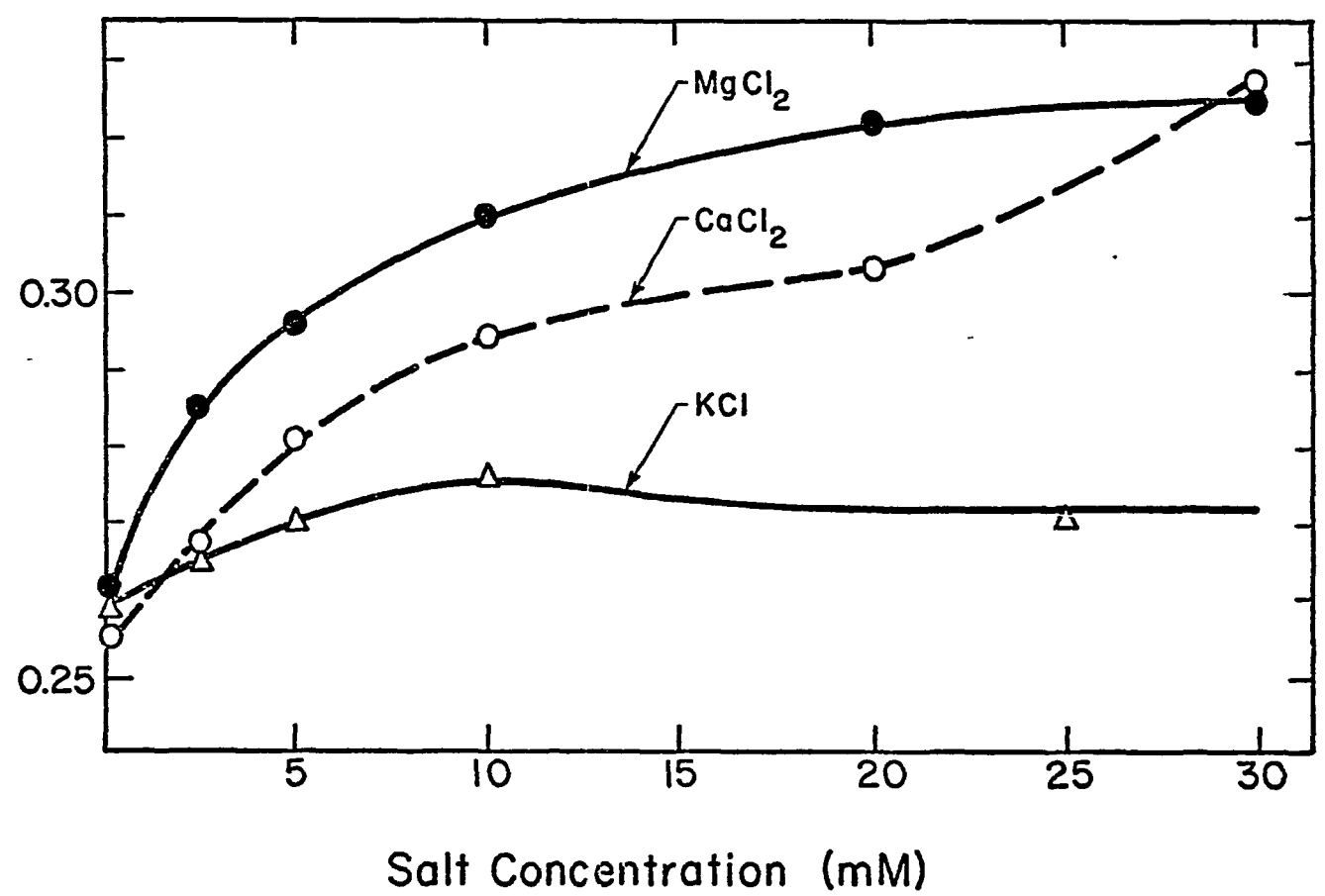
induced changes in polarization of CF_1 fluorescence are not due merely to a decrease in the lifetime of tyrosine fluorescence, which could also account for the observed changes. For example, a shorter lifetime would result in fluorescence being emitted before molecular motions causing decrease in polarization can occur. Rather, it is probable that these changes are due to an alteration in protein structure in which, for example, the mobility of one or more tyrosine residues of CF_1 is reduced.

The magnesium induced increase in polarization of fluorescence is reversible, as shown by the addition of 5.0 to 7.5 mM EDTA (Table 6). This indicates that the effect of the salt here is not an artifact produced by some irreversible destructive effect (cf. effect of pH shift, section III. B., Fig. 7).

The data of Table 7 demonstrate that 20 μM ADP causes an increase in polarization of CF_1 (tyrosine) fluorescence above the saturated $MgCl_2$ (Table 7A) and $CaCl_2$ (Table 7B) enhanced level (ca. 31%) to ca. 34% and 33%, respectively.

If 15 mM KH_2PO_4 is present (Table 7C), the polarization increase produced by either 15 mM $MgCl_2$ or 15 mM $MgCl_2$ + 20 μM ADP addition is smaller (29% and 30%, respectively). Actual enhancement in polarization of fluorescence is 0.03 (line 3, Table 7C) instead of 0.05 (line 3, Table 7A) with 15 mM $MgCl_2$, and is 0.04 (line 4, Table 7C) instead of 0.08

TABLE 6

EFFECTS OF EDTA AND $MgCl_2$ ON POLARIZATION
(p) OF CF_1 FLUORESCENCE

Additions	p*	Change From Control
none	0.260	----
2.5 mM $MgCl_2$	0.274	0.014
5.0 mM EDTA	0.263	0.003
5.0 mM $MgCl_2$	0.276	0.016
7.5 mM EDTA	0.262	0.002

Reaction conditions: Medium contained 25 mM Tricine-NaOH (pH 8) and 0.2 µM CF_1. Additions were made sequentially in the same sample, in the order listed.

*Instrumental fluctuations introduce error in p values of ca. 0.003. Results are average of two experiments. See chapter II, section E for further details of measurements.

TABLE 7

EFFECT OF ADP ON THE POLARIZATION (p) OF CF_1 FLUORESCENCE

	Additions	p^*	Change From Control
A	none	0.260	----
	$MgCl_2$ (5 mM)	0.290	0.030
	$MgCl_2$ (15 mM)	0.312	0.052
	ADP (20 µM)	0.341	0.081
B	none	0.260	----
	$CaCl_2$ (5 mM)	0.283	0.023
	$CaCl_2$ (15 mM)	0.313	0.053
	ADP (20 µM)	0.327	0.067
C	none	0.260	----
	KH_2PO_4 (15 mM)	0.274	0.014
	$MgCl_2$ (15 mM)	0.290	0.030
	ADP (20 µM)	0.302	0.042

Reaction conditions: Medium contained 25 mM Tricine-NaOH (pH 8.2) and 5.4 µM CF_1. In each experiment (A,B,C) the indicated additions were made sequentially in the same sample; note that the number in parentheses refer to the total concentration in the sample at that point in the addition sequence.

*Instrumental fluctuations introduce error in p values of ca. 0.004.

(line 4, Table 7A) with 15 mM $MgCl_2$ + 20 µM ADP; these differences are significant.

Thus ADP as well as divalent cations exert some control on the conformation of CF_1, and it appears that the negative interaction of P_i and ADP presented above in binding curves (Fig. 11) is also evident in the effects on tyrosine fluorescence polarization--indicating conformation changes of CF_1.

CHAPTER IV

DISCUSSION AND SUMMARY

Since investigation of the molecular mechanism of phosphorylation in chloroplasts is at a rather primitive stage yet, the aim of the present work was to find out basic information concerning one particular key component in this reaction, namely, the coupling factor enzyme (CF_1) that facilitates ATP synthesis. Since the gap between what is known of nucleotide, ionic, and conformational effects observed in functioning chloroplasts and what is specifically known concerning the binding and interaction of these factors with CF_1 has been rather large, we have in the present work investigated the latter in a systematic detailed study. The intact chloroplast, although fully functional, is so complex that it is difficult to ascertain what specifically is occurring at the molecular level. Therefore, we have undertaken the present study using a relatively simpler, more well-defined system, i.e., the CF_1 component as isolated from the chloroplast, in which the number of species involved is considerably reduced, and the data obtained can be interpreted with more confidence regarding which components are interacting and how.

The activity of the CF_1 enzyme that is isolated by the preparation procedure used in this investigation was checked

and assured by its ability to rebind to membranes stripped of CF_1 and restore photophosphorylation with high efficiency (Table 1). Substantial purity of our preparation was also assured by means of an established fluorescence test; see Fig. 3 and section II.C.)

Unfortunately, the enzyme activity cannot be measured when not membrane-bound, except when functioning as an ATPase, and then only when further treated (the ATPase activity of membrane-bound CF_1 is also not expressed without further modification). Since the interaction of nucleotides, ions and ATPase-CF_1 has already been studied rather more fully (e.g., 7, 13, 109, 132, 133), and due to further apparent problems in relating this to the phosphorylation mechanism, as discussed in section I.D., we have concentrated our attention on the "latent," ATP-synthetase form of CF_1. Of course, using this approach the data will of necessity not relate directly to the step in the phosphorylation of ADP in vivo which requires "membrane energization." At the same time, soluble, latent CF_1 is apparently quite close to the in vivo situation, since isolated coupling factor, without further modification can be rebound to thylakoid membranes and be shown to function in the formation of ATP. In addition, any differences that might be observed between bound and unbound CF_1 may prove to be useful in determining what characteristics are functionally significant in photophosphorylation; the

viability of this suggestion should be tested more fully as more data on both complete and fragmented chloroplast systems is acquired in the future, but some of our data is discussed in this light, below.

Pursuing this course of investigation, then, the interaction of adenine nucleotides with CF_1 was studied utilizing primarily the fluorescent-modified forms of these compounds (ϵAMP, ϵADP, ϵATP). Measurement of binding by the fluorescence polarization method (section I.C., II.G.) was chosen since (a) it provides a convenient and fairly rapid means of determining binding, and (b) ϵADP and ϵATP, while essentially the same as ADP and ATP functionally, are labelled (by fluorescence) as compared to the unmodified nucleotides, and therefore, the binding to or release from CF_1 of adenine nucleotides can easily be monitored, even in the presence of excess nucleotide. The ability of ϵAMP, ϵADP and ϵATP to substitute functionally for AMP, ADP, and ATP, respectively, was first determined by comparative measurements of several reactions dependent on (membrane-bound) CF_1 (Tables 2-5 and section III.A.).

Although ϵAMP substituted less well (ca. 30%, Table 5) for AMP than ϵADP and ϵATP for ADP and ATP, respectively (ca. 65-85%), it was found that ϵAMP, under a variety of conditions, shows little or no binding to isolated CF_1 (Figs. 25, 26 and section III.B.3.); moreover, since a previously

discovered chloroplast transphosphorylation reaction involving
AMP (46,47) has recently been shown to be a slow side reaction
(42), εAMP was not studied further in the present work.

On the other hand, εADP and εATP both bind well to
CF_1 at two sites (e.g., see Figs. 5,11,18). The binding was
complete within about 5 minutes after nucleotide addition in
both cases using a medium containing 5 mM $MgCl_2$ at pH 8. This
is in contrast to the very slow (1 to 2 hr time course) bind-
ing of ^{14}C-labelled ADP to CF_1 (46); however, in this case,
only very tightly bound enzyme-ADP complexes (able to pass
through Sephadex column intact) which were insensitive to the
presence of divalent cations were studied, and as discussed
above (section I.A.) reactions involving such tightly bound
species are evidently not in the main path of chloroplast
phosphorylation (42). In another study with isolated CF_1
(109), 1/2 to 2 hr incubation was also allowed; however, in
this case 0.1 M NaCl was included in their reaction medium,
which we have shown (Figs. 8 and 10) suppresses binding of
εADP to CF_1.

Binding of the substrate for phosphorylation, εADP,
was found to be sensitive not only to the ionic strength of
the medium (effect of 0.1 M NaCl mentioned above), but to
some degree also the concentration of activating cation (Mg^{2+},
Fig. 8). Komatsu and Murakami (134) showed that Mg·ADP com-
plex, rather than free ADP, is the active form of the

substrate in photophosphorylation. The effect of Mg^{2+} observed here in the binding of εADP to CF_1 is apparently primarily not an effect directly involving concentration of Mg·ADP, since (a) some binding of εADP to CF_1 is observed even in the absence of added $MgCl_2$, and (b) 0.5 mM $MgCl_2$ is already about 100 times the concentration needed for a 1:1 complex of Mg·ADP, and yet increasing $[MgCl_2]$ to 5 mM has a further effect in binding. Since we have shown that $MgCl_2$ in the millimolar range does reversibly change the physical state of CF_1, as detected by polarization of protein (tyrosine) fluorescence (Table 6, Fig. 27), it seems likely that the observed $MgCl_2$ effect on binding of εADP is at least partially due to a change in conformation of CF_1.

With both Mg^{2+} and Ca^{2+}, the binding of εADP and εATP to sites of CF_1 is similar with K_d's on the order of 1-3 µM (Figs. 11 and 18). It seems unlikely, then, that the known specificity of the photophosphorylation reaction for Mg^{2+} (15,135) is due strictly to an effect on adenine nucleotide binding per se; rather, it may be that cation-dependent differences in CF_1 conformation are involved, evidence for which was obtained using polarization of protein (tyrosine) fluorescence (Fig. 27, Table 7) as an indicator of the structural state of CF_1.

The interaction of CF_1 with proton flux in thylakoid membranes is suggested on the basis of experiments in which

CF_1 is removed from the membrane and a subsequent more rapid rate of proton efflux after illumination and lower net proton uptake is observed (136); also, an interaction of ATP (1-10 μM) with CF_1 (as demonstrated using an antibody specific for coupling factor), causes a retardation of proton efflux in chloroplasts (125, 127). Schmid and coworkers (137) showed that removal of CF_1 from the thylakoid results in an increase in the conductance for both (a) protons and (b) other ions; the first of these effects was found to be largely reversible by reincorporating CF_1 into the membrane, whereas the second "side effect" could only be weakly reversed. In addition, Schmid et al. (137) found that the ADP accelerated decay of the electric potential, as measured by the 520 nm absorption change, was inhibited by antibody to CF_1, and interpreted this data as evidence that the synthesis of ATP under flash excitation is coupled to a translocation of protons across a special pathway through CF_1 (as postulated by Mitchell (41)). However, Girault and Galmiche (138) discovered that the faster dark decay of the 520 nm change under phosphorylating conditions can be reproduced equally well by adding ATP alone; moreover, even though εADP is efficiently phosphorylated into εATP, neither of these fluorescent nucleotides was effective at all on the 520 nm absorbance change dark decay, which thus has no direct link with phosphorylation (138).

Certain of the models (e.g., Mitchell and Boyer; Fig. 1) for phosphorylation summarized previously (section

I.A.), consistent with some of the data of the previous para-
graph, specifically include the interaction of protons or a
proton activity gradient with the coupling enzyme as a crit-
ical step in the energy transduction mechanism. Boyer (48),
for example, suggested the possible change in binding of
reactants at the catalytic site. To test the viability of
such proposals, i.e., by seeking to determine if CF_1 in a
well-defined system exhibits any interesting effect by pro-
tons, the effect of pH changes on the binding of εADP and
εATP was investigated. It was found that the binding of
these adenine nucleotides to CF_1 was maximal at pH 8 (Fig. 6)
at which CF_1 was isolated, where the protein is generally
considered to be most stable and where photophosphorylation
is maximal (i.e., pH 8-8.5, see ref. 15); shifting from pH 8
resulted in reduced binding. However, since this pH effect
on binding was not reversible (allowing time intervals of
several minutes between additions of acid or base; see also
Fig. 5), and since an irreversible pH effect on the structural
state of CF_1 (e.g., as detected by protein fluorescence) was
documented (Fig. 7), it appears that the observed changes are
not directly related to enzyme mechanism. These essentially
negative results are evidently another example of how isolated,
soluble CF_1 is more sensitive than the membrane-bound enzyme,
and it is concluded that any H^+-CF_1 interactions affecting
adenine nucleotide binding and/or CF_1 conformation which may

be important for phosphorylation require the thylakoid membrane for expression.

During the course of the complete reaction sequence (as yet unknown) in which P_i is esterified to ADP to form ATP, these compounds must interact with, and presumably bind to, coupling factor, the ATP-synthesizing enzyme. Since the information was as yet undetermined, the nature of the effects of ADP, ATP and P_i on the binding characteristis of $\epsilon ADP-CF_1$ and $\epsilon ATP-CF_1$ was investigated (Figs. 11-24). It was found that both 500 μM P_i (Fig. 11) and 10 μM ATP (Fig. 14) increase the K_d's for the $\epsilon ADP-CF_1$ binding sites by approximately four-fold and six-fold, respectively. Similarly, both 500 μM P_i (Fig. 18) and 10 μM ADP (Fig. 21) increase the K_d's for the $\epsilon ATP-CF_1$ binding sites by about three-fold and four-fold, respectively. It is apparent that there is strong negative interaction among these anion-cofactors at the level of binding to CF_1, indicating they are close functionally, and most likely, in physical proximity as well.

Data further indicating interaction of ADP and P_i with CF_1 in affecting the physical state of CF_1 was obtained by monitoring the polarization of tyrosine fluorescence of the protein (Table 7). ADP was shown to cause an increase in the polarization of protein fluorescence above the saturated cation (e.g., 10-15 mM $MgCl_2$) enhanced level; this change was reduced by half when P_i (Table 7A,7C) was present. Thus, the

negative interaction of P_i and ADP is also evident in the effects on CF_1-tyrosine fluorescence polarization and the associated conformation changes, which are probably a physical or conformational expression of the ADP + P_i binding interaction.

In this light, we note several chloroplast reactions that Jagendorf (15) has summarized which indicate that the P_i binding site on CF_1 may have rather broad specificity and CF_1 apparently exhibits one or more pronounced conformational and functional changes only when both ADP and P_i (or certain other analog anions) are present: (a) uncoupling of phosphorylation by arsenate, competitive with P_i; (b) light-dependent inhibition of phosphorylation by sulfate, also competitive with P_i; (c) light-dependent $KMnO_4$ inhibition of phosphorylation; (d) a slowing and inhibition of the light-dependent tritium-incorporation indicated conformational change; and (e) suppression of light-dependent N-ethylmaleimide (NEM) inhibition of phosphorylation, in which a conformational change exposes an -SH group in the gamma subunit of CF_1. Thus, it appears that our data on ADP and P_i interaction in binding to CF_1 provides evidence directly at the level of CF_1 for a possible molecular basis for the membrane-dependent conformational effects listed above. In addition, while quite speculative, the effects of ADP + P_i on the NEM effect (reaction (e) above) suggest that ADP and/or P_i interact with

CF_1 in at least one instance at the gamma subunit; while of course requiring further testing, this suggestion seems plausible since it is known that antiserum to the gamma (or alpha) subunit alone is sufficient to inhibit phosphorylation (103). (In this connection, we also note that Grebanier and Jagendorf (139) have utilized two of these light-dependent CF_1 reactions to show the lack of site-specificity of coupling factor; similarly, using electron microscopy, Miller and Staehlin (140) found tnat CF_1 was located only on unstacked grana regions and they also concluded that the high energy intermediate was in common to all coupling factor molecules.)

This type of negative interaction in general, perhaps involving phosphate, was proposed in a model for phospnorylation by Weber (31, 78). More specifically, interaction between ADP and P_i was proposed by Boyer (35), which, he suggests, might be due to close binding and thereby result in an effective increase in the local concentration of ADP and P_i. This is then supposed to contribute to an increase in the ATP/ADP ratio from 10^{-8} to perhaps nearly one, catalyzing ATP formation with essentially no energy input; release of ATP, in his view, then requires energy. (It is noted here that recent experiments using an uncoupler in oxidative phosphorylation of submitochondrial particles were consistent with an energy-linked conformational change promoting P_i and ADP binding in a mode competent for ATP synthesis (141).)

Although our data presented above is on the surface consistent with such a concept, the existence of such a mutual interaction does not prove that ATP can be formed as Boyer suggests. There appear to be two problems. (1) It is not clear that an increase by P_i of the K_d for ADP binding by less than an order of magnitude would result in such a dramatic increase in the ATP/ADP ratio [when the concentration of P_i is already clearly in excess of ADP, which is in the same approximate range, 2-35 µM, as the K_m for ADP in phosphorylation (20-50 µM in spinach chloroplasts)]; and, (2) we also found that P_i also increases the K_d for ATP.

Another suggestion included by Boyer (35) as an alternate way of explaining how ATP might be formed (before release) with little or no energy input states that there is a preferential tight binding of ATP at the catalytic site. Our data doesn't support this concept, however, since the K_d's for both εADP-CF$_1$ and εATP-CF$_1$ binding sites are similar. Other data, for and against this conformational coupling proposal, and summarized in section I.A., should also be considered in this context. (It may be that in chloroplasts, as in the recent findings for submitochondrial particles (139), energy input linked to a conformational change, in the complete system, is required to promote P_i and ADP binding to a mode competent for phosphorylation, a possibility that should be tested.)

Do ADP and ATP bind to CF_1 at the same sites? Since εADP and εATP bind to CF_1 with similar kinetics (Fig. 5) and K_d's (Figs. 11 and 18), if ADP and ATP are interchangeable at the same sites, then they should exhibit similar competition for both εADP and εATP sites. However, it was found that (a) at equivalent concentrations, ADP reduces the binding of εADP to CF_1 more strongly than ATP (Figs. 12 and 16) and similarly, ATP reduces the binding of εATP to CF_1 more strongly than ADP (Figs. 19 and 23) and (b) ADP binds well at the εADP site in causing the release of bound εADP from CF_1, whereas ATP does not (Fig. 17). Thus, the evidence indicates that the binding sites for ADP and ATP on CF_1 are probably not the same, and in any case, these nucleotides do not exchange with CF_1 interchangably. Any model for phosphorylation in chloroplasts which is contingent upon equivalent sites for ADP and ATP on CF_1 when not energized would, therefore, be questionable.

The data for exchange of ATP or ADP with εATP bound to CF_1, while more complex, is not antithetic to the above conclusion (Fig. 24). An excess of ATP or ADP caused an inefficient, slow release of εATP from CF_1 (Fig. 24) as compared to the release of εADP from CF_1 (Fig. 17). Even when the strong anion binding resin Dowex-2 was added such that there was a large excess of exchange capacity, only a moderate increase in the extent of removal of εATP was observed (Fig. 24). Thus, while ATP substitutes well for εATP and when added first

suppresses the binding of εATP, some rearrangement seems to prevent the efficient release from CF_1 of εATP (already bound to a relatively interior region), by the competition of ATP added <u>second</u>. If the εATP site(s) on CF_1 is involved in the process of phosphorylation, this data is not inconsistent with an energy-requiring step needed to effectively release ATP from the coupling enzyme during net phosphorylation; though Boyer's proposal (35) includes such a step, the present data does not of course prove that it, or any other part of his model, is valid.

The kinetics of binding of εADP and εATP to CF_1 with 5 mM $MgCl_2$ were not unusually slow (Fig. 5), but with the limitations of measurements we could only say that maximal binding is attained within a few minutes. On the other hand, the kinetics of release of εADP and εATP from CF_1 when excess ADP or ATP were added in competition were relatively slow (at least minutes, Figs. 17 and 24) compared to the rate at which chloroplast phosphorylation proceeds (several milliseconds after initiation by light or acid-base treatment (42,70)). This suggests that CF_1 may have to be bound to the hydrophobic HF_0 protein and/or thylakoid membrane for fully functional adenine nucleotide binding to be expressed.

In conclusion, we would like to emphasize the following points. Table 8 summarizes the results so that the reader can get, at one glance, most of the pertinent information discussed here.

(1) Fluorescent nucleotides (ϵADP and ϵATP) replace the natural nucleotides rather efficiently in several chloroplast reactions (Table 8.A);

(2) ϵAMP does not bind to CF_1 (Table 8.C.1);

(3) the latent isolated coupling factor protein, which re-constitutes phosphorylation well when added to CF_1-depleted chloroplasts (Table 8.B), binds (within $\sim$ 5 minutes) both ϵADP and ϵATP at two cation-sensitive sites each of similar binding affinity, though the ϵADP and ϵATP sites are apparently not the same (Table 8.C.2, 8.C.3);

(4) polarization of fluorescence of nucleotides and coupling factor suggest that cation-induced changes of conformation exist (Table 8.C, 8.D);

(5) binding of CF_1 to nucleotides is maximal at pH 8 where phosphorylation in chloroplasts is also maximal, but changes, with isolated CF_1, at other pHs are not directly related to enzyme action (Table 8.C, 8.D.1);

(6) interaction of P_i with CF_1 in the presence of nucleotide occurs as monitored by dramatic increase in K_d's for ϵADP-CF_1 and ϵATP-CF_1 binding sites (Table 8.C), and by decrease in polarization of fluorescence of CF_1 (Table 8.D.2), suggesting negative interaction of adenine nucleotides and P_i on CF_1; and,

(7) interaction of CF_1 with ϵADP and ATP on the one hand,

TABLE 8

SUMMARY OF RESULTS

EXPERIMENT	RESULTS	REFERENCES
A. Chloroplast Reactions	Activity of Fluorescent Nucleotides Compared to Unmodified Compounds	section III.A.
1. photophosphorylation	ADP/ADP 70%	Table 2
2. effects of nucleotides on electron transport	AMP/AMP 30% ADP/ADP 65% ATP/ATP 75%	Table 5 Table 3 Table 3
3. effects of nucleotides on proton transport	ATP/ATP 8₅%	Table 4
B. Isolated Coupling Factor Purity and activity tests	 highly pure based on protein fluorescence ratio test; CF_1 preparation can facilitate reconstitution of phosphorylation, 90%	section II.C., Figure 3 Table 1

TABLE 8 (continued)

EXPERIMENT	RESULTS	REFERENCES
C. Polarization of Nucleotide Fluorescence		
1. εAMP	no binding to CF_1 under variety of conditions	section III.B.3; Figs. 25,26
2. εADP		section III.B.1
a. kinetics	binds at two sites within ~5 minutes	Figs. 5,10,11,17
b. cations	for both Mg^{2+} & Ca^{2+}, K_d's for εADP-CF_1 are ~0.8-2 µM; K_d's increase ~5-10 fold as [$MgCl_2$] decreases from 5 mM to 0 mM; 0.1 M NaCl increases K ~4-fold	Figs. 10,11
c. P_i	500 µM P_i increases K_d's for εADP-CF_1 ~5-fold	Fig. 11
d. ADP	causes release of εADP from CF_1	Fig. 17
e. ATP	20 µM ATP increases K_d for εADP-CF_1 20-fold	Fig. 15
f. pH	maximum binding at pH 8; irreversible effect with pH changes	Figs. 5,6

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TABLE 8 (continued)

EXPERIMENT	RESULTS	REFERENCES
C. Polarization of Nucleotide Fluorescence		
3. ϵATP		section III.B.2
a. kinetics	binds at two sites within ~5 minutes	Figs. 5,18,24
b. cations	for both Mg^{2+} and Ca^{2+}, K_d's for ϵATP-CF_1 are ~1-3 μM; two sites with Mg^{2+} and Ca^{2+}	Fig. 18
c. P_i	500 μM P_i increases K_d's for ϵATP-CF_1 ~4-fold	Fig. 18
d. ADP	20 μM ADP increases K_d for ϵATP-CF_1 ~20-fold	Fig. 22
e. ATP	release of ϵATP from CF_1 is much slower than in the case of ϵADP (see Table 8. C.2.d)	Fig. 24
f. pH	maximum binding at pH 8; irreversible effect with pH changes	Fig. 6
D. CF_1 Protein Fluorescence		section III.C
1. intensity	irreversible effect by changes in pH	Fig. 7
2. polarization		
a. cations	reversible increase observed with 0-15 mM added salt, in the order $MgCl_2 > CaCl_2 > KCl$	Fig. 27 Tables 6,7
b. ADP + P_i	ADP causes increase above cation-saturated level; P_i reduces ADP increase	Table 7A,C

and with εATP and ADP on the other, as indicated by sizable increases in the K_d's for εADP-CF_1 and εATP-CF_1, respectively, suggests a negative interaction of ADP and ATP on CF_1 (Table 8.C).

(8) the kinetics of release of εADP and εATP from CF_1 when excess ADP or ATP were added in competition were relatively slow compared to the rate at which in vivo photophosphorylation proceeds; this suggests that CF_1 may have to be bound to the hydrophobic HF_o protein and/or the thylakoid membrane for fully functional adenine nucleotide binding to be expressed.

On the basis of the characteristics of the binding of ADP and ATP with CF_1 found in this investigation, and summarized in this chapter, it would be premature to delineate a detailed model for the mechanism of energy transduction in chloroplasts. We feel the data of this thesis does, however, lay a substantial foundation in the study of the interaction of adenine nucleotides with the "latent" coupling enzyme; moreover, profitable and instructive correlation with existing data and proposed models was made at several points, and the information obtained will be pertinent in further consideration of detailed mechanism and in directing the course of future investigation. It is anticipated that a synthesis of work employing intact chloroplasts and extensions of the present study using a simpler system will afford a more complete understanding of photophosphorylation.

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(2) Is there a triplet state in photosynthesis? (1973) J. Sci. Indus. Res. 32, 62-69 (review).

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