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The mechanism of bicarbonate activation of plastoquinone reduction in photosystem II of photosynthesis

Blubaugh, Danny J., Ph.D.

University of Illinois at Urbana-Champaign, 1987

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THE MECHANISM OF BICARBONATE ACTIVATION OF PLASTOQUINONE REDUCTION IN PHOTOSYSTEM II OF PHOTOSYNTHESIS

BY

DANNY J. BLUBAUGH

B.A., Earlham College, 1980

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biology
in the Graduate College of the
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WE HEREBY RECOMMEND THAT THE THESIS BY

DANNY J. BLUBAUGH

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Govindjee

Director of Thesis Research

R. E. Reich

Head of Department

Committee on Final Examination†

Govindjee

Chairperson

Whitmarsh

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

O-517

To Keith Boehme

and

To Norma

"... a practical profession is a salvation for a man of my type; an academic career compels a young man to scientific production, and only strong characters can resist the temptation of superficial analysis."

-- Albert Einstein, quoted in:

Clark, R.W. 1971. Einstein, The Life and the Times. World Publ. Co., New York, p 51

"Processes occurring in living matter are much more complicated than the ones with which the physicist usually has to deal. For that reason the principle, familiar to physicists, that the simplest solution is probably the right one, is not applicable to biological matter, and it is not safe to assume that measurements of basic constants are correct even if they have been made by renowned scientists and have remained unchallenged for many years."

-- James Franck. 1945. Rev. Mod. Phys. 17: 112-119

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Finally, I wish to thank my beloved wife, Norma, without whose moral support and helpful assistance this thesis might never have materialized.

PREFACE

THE PURPOSE AND SCOPE OF THIS THESIS

The purpose of this thesis is to examine the requirement for bicarbonate (HCO_3^-) in photosystem II (PS II), with the objective of developing a model to explain its function. Such a model is presented in Chapter 7, after the presentation of the experiments on which it is based. This model has the virtue of being simple, yet consistent with all reported observations on the HCO_3^- effect, and has the added virtue of reconciling some observations which previously had appeared conflicting. Furthermore, it ties together some other confusing and sometimes controversial avenues of investigation which previously appeared to have no relation to the HCO_3^- requirement. The success of this model, however, will depend on further investigations to test its implications and the predictions which it raises. Many of these predictions only became apparent during the writing of this thesis, and, therefore, some crucial experiments, which I wish could be a part of this thesis, have not been performed.

Chapter 1 is a review of the HCO_3^- literature, which sets the background for the experimental chapters to follow, and provides data by which to check the validity of the model. After a description of the Materials and Methods in Chapter 2 come the experimental chapters, which are aimed at answering fundamental questions of the type that must be answered in order to develop a worthwhile model: which chemical species is involved? (Chapter 3); what does it do? (Chapter 4); how many binding sites are there? (Chapter 5); and what is the binding environment? (Chapter 6).

Until recently, not much attention has been given the HCO_3^- problem; despite its discovery nearly 30 years ago, only three laboratories come to mind where its investigation has been undertaken vigorously. These are the

laboratories of Govindjee at the University of Illinois at Urbana, where this thesis was developed; of Alan Stemler at the University of California at Davis (Dr. Stemler received his Ph.D. in Govindjee's laboratory); and of Jack J.S. Van Rensen at the Agricultural University of Wageningen in The Netherlands. The controversy between two of these laboratories (Stemler vs. Govindjee) over the existence of a major HCO_3^- effect on H_2O oxidation has probably contributed to a general aloofness toward this problem on the part of other researchers. I hope that this thesis may play some role in altering this state of affairs.

It is my impression that more attention is beginning to be paid to the HCO_3^- problem, due, in part, to the rapid advancement being made in solving other mysteries of the reaction center: these include the recent crystallization of the bacterial reaction center from two separate species; its homology with the reaction center of PS II; the new belief that Q_B, the secondary quinone electron acceptor of PS II, is an integral part of the PS II reaction center itself; the gene sequence for the Q_B apoprotein; and improved techniques for observing the Fe in PS II. This is the environment in which HCO_3^- plays its role. Perhaps because so many of the fundamental questions of photosynthesis are being answered, scientists can begin to fill in the smaller details, of which HCO_3^- is clearly one. It is my personal belief that the role of HCO_3^- will assume greater importance as we deepen our understanding of the mechanisms behind photosynthesis. If this happens, I will be proud to have been a part of the research, and to have been associated with Dr. Govindjee, who championed the problem ahead of the rest. No one who has observed the large and dramatic, reversible effect of HCO_3^- depletion can remain indifferent -- it is a phenomenon that requires an explanation.

TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER 1

INTRODUCTION

A. Historical Introduction

Photosynthesis involves the oxidation of H_2O and the reduction of CO_2. The energy required for this reaction is supplied by the light that is absorbed by the photosynthetic pigments and transferred with great efficiency to the photosystem reaction centers. In the early days of photosynthesis research, it was not known whether the O_2 that is evolved came from H_2O or from CO_2. From the overall chemical equation for photosynthesis,

$$H_2O + CO_2 + \text{light} \longrightarrow O_2 + 1/n\ (CH_2O)_n \qquad (1.1),$$

it was supposed by most early workers that CO_2 was the source of evolved O_2, with the chlorophyll somehow catalyzing the transfer of carbon from CO_2 to H_2O. An example of this was the Willstätter-Stoll hypothesis, which had hydrated CO_2 reacting with chlorophyll to yield formaldehyde and O_2 as products, with the formaldehyde then undergoing enzymatic condensation in the conversion to carbohydrates (for an excellent description of this hypothesis and its subsequent loss of favor, see Van Niel, 1949). It is now firmly established that the evolved O_2 comes from H_2O. Before introducing the role of bicarbonate in photosystem II, however, it is instructive to review the major lines of evidence for H_2O as the substrate for O_2 evolution, since some controversy over this question has continued to the present day, fueled by the requirement for bicarbonate in PS II.

The first major line of evidence for H_2O as the source of photosynthetic O_2 came from comparative studies between the green and purple sulfur-reducing bacteria and green plants (Van Niel, 1931; for a review, see Van Niel, 1941). In the bacteria, the overall reaction for photo-

synthesis is

$$2\ H_2S + CO_2 + \text{light} \longrightarrow 2\ S + 1/n\ (CH_2O)_n + H_2O \qquad (1.2).$$

Eqn. 1.1 from green plants can be rewritten as

$$2\ H_2O + CO_2 + \text{light} \longrightarrow O_2 + 1/n\ (CH_2O)_n + H_2O \qquad (1.3).$$

The similarity between Eqns. 1.2 and 1.3 is obvious; this led Van Niel to propose that photosynthesis is a light-catalyzed oxidation-reduction reaction of the form

$$2\ H_2A + CO_2 + \text{light} \longrightarrow 2\ A \text{ or } A_2 + 1/n\ (CH_2O)_n + H_2O \qquad (1.4).$$

In the green and purple bacteria A would be sulphur, whereas in higher plants and algae it would be oxygen. Thus, the implication is clear that the source of photosynthetically derived O_2 is H_2O and not CO_2.

This generalized equation for photosynthesis gained strong support with the demonstration that a variety of compounds can be photosynthetically oxidized by the purple sulfur bacteria: elemental sulfur, sulfite, and thiosulfate can all be oxidized to sulfate (Van Niel, 1931); elemental selenium is oxidized to selenate (Saposhnikov, 1937, cited by Van Niel, 1941); various organic substances can be used as hydrogen donors (c.f. Foster, 1940); and some species can use molecular hydrogen (c.f. French, 1937), all without the evolution of O_2. Only when H_2O is the hydrogen donor is O_2 evolved. Thus, green plant photosynthesis as a special case of the generalized equation became widely accepted after the pioneering work of Van Niel.

Nevertheless, it could be argued that what occurs in bacteria is not necessarily what occurs in green plants. Metzner (1975), for example, argues that since green plants, unlike the photosynthetic bacteria, require two photosystems to transfer electrons from the primary donor to the terminal acceptor, that it is not valid to compare them. Furthermore, the

bacterial photosystem, since it requires electron sources that are relatively easy to oxidize, is more similar, in this respect, to PS I of green plants than it is to the O_2-generating PS II. On the other hand, Gaffron (1940) succeeded in adapting cultures of the green algae Scenedesmus and Raphidium to the utilization of H_2, so that they photosynthesized without evolution of O_2. The H_2/CO_2 quotient was the same as in purple bacteria. Thus, at least in these species, oxygenic photosynthesis seems to fit the generalized equation of Van Niel. Nevertheless, the hypothesis that O_2 comes from H_2O needed to be tested. The test became possible with the availability of oxygen isotopes.

The isotopic composition of the evolved O_2 from photosynthesizing Chlorella cells that were supplied with H_2O or HCO_3^- enriched in ^{18}O precisely matched that of the water, not of the HCO_3^-; thus, it was concluded that the O_2 came from the H_2O (Ruben et al., 1941). Such experiments are complicated by the fact that CO_2 and H_2O are always in rapid equilibrium with H_2CO_3, according to the reaction

$$CO_2 + H_2O \text{ <----> } H_2CO_3 \qquad (1.5).$$

While CO_2 dissolved in H_2O will reach chemical equilibrium very rapidly ($t_{1/2} \approx 19$ s; Gibbons and Edsall, 1963), isotopic equilibrium between all the oxygens takes about 1000 times longer (Mills and Urey, 1940). This is because every molecule must come to equilibrium with every other molecule before isotopic equilibrium can be reached. Chemical equilibrium is dependent only on the ratio of forward to reverse rate constants; as soon as $[H_2CO_3]$ reaches its equilibrium value, the forward and reverse rates are equal. For isotopic equilibrium, on the other hand, the hydration reaction must occur many times. Because of this, the time required to reach isotopic equilibrium is very sensitive to the pH, since the greater the

ratio of $[HCO_3^-]/[CO_2]$ (i.e. the higher the pH), the fewer the number of hydration reactions in a given time. Because of the very slow oxygen exchange at the temperature (25 C) and pH ($\sim$10) of the ^{18}O experiments with Chlorella, the original investigators felt justified in ignoring this complication, as the oxygen exchange was much slower than the rate of photosynthesis.

However, Kamen and Barker (1945) pointed out that it was an unproven assumption that the isotope exchange is no more rapid inside the cells, or inside the chloroplasts, than in the outside medium. Assuming an internal pH of 6 or less, these authors calculated that the randomization of ^{18}O would be rapid enough to invalidate the conclusions.

A more careful study by Dole and Jenks (1944) confirmed the original conclusion. At isotopic equilibrium the CO_2 is slightly greater enriched in ^{18}O than is the H_2O (Webster et al., 1935). Dole and Jenks were able to sensitively measure this difference, and they showed that at isotopic equilibrium, maintained with carbonic anhydrase, the evolved O_2 had nearly the same enrichment as the H_2O, but was clearly less enriched than the CO_2. Since this experiment was done at isotopic equilibrium, it was not subject to the same criticism as the first experiments.

This line of evidence still had difficulties. It has long been presumed that the source of the earth's atmospheric O_2 is photosynthesis, yet the ^{18}O content of atmospheric O_2 is considerably greater than that of natural water (Dole, 1935). Greene and Voskuyl (1936) pointed out that the ^{18}O content of atmospheric O_2 is what would be predicted if photosynthetic O_2 were derived from the water and CO_2 together. Yosida et al. (1942) claimed to have measured an ^{18}O content of photosynthetic O_2 evolved from aqueous plants that indicated one-third of the O_2 came from CO_2. They were

able to account for this observation using a modified Willstätter-Stoll hypothesis for O_2 evolution, in which carbonic acid (i.e. hydrated CO_2) yields formaldehyde and O_2 through a peroxide intermediate. The experiment of Dole and Jenks (1944), described above, contradicts these results and shows clearly that, at least in Chlorella, the O_2 evolved is much closer in isotopic composition to the H_2O than it is to the CO_2. Still, they did show a slight increase in ^{18}O abundance compared to the H_2O; this they noted is precisely what would be predicted if there were an efficient oxygen exchange between O_2 and H_2O, although from the earlier measurements of Ruben et al. (1941) it was concluded that such an exchange did not occur. Given the complications of oxygen exchange reactions, as well as the small number of species tested, these ^{18}O labelling experiments have not escaped controversy. It is this author's opinion that the original experiments of Ruben, at al. (1941) and of Dole and Jenks (1944) were carefully done and are valid. However, he notes that even as recently as 1975 (Metzner, 1975) their validity has been challenged.

Stemler and Radmer (1975) measured the isotopic O_2 released from HCO_3^- depleted thylakoids after the addition of $HC^{18}O_3^-$ and found that only $^{16}O_2$ was evolved. This experiment was superior to the original experiments of Ruben et al. (1941) in that the appearance of $C^{18}O_2$ and $C^{16}O_2$ were monitored along with the evolution of $^{16}O_2$, and it was clearly shown that the isotopic exchange reactions were too slow for HCO_3^- or CO_2 to be the source of photosynthetic O_2. Also, since the thylakoids had been depleted of native $HC^{16}O_3^-$, the $^{16}O_2$ could only have come from $H_2{}^{16}O$. However, since the mass spectrometer used in this experiment was not sensitive enough to monitor single turnovers of the reaction centers, these authors could not rule out the remote possibility that the first few molecules of

evolved O_2 might have been derived from the reactivating HCO_3^-. This question was settled by Radmer and Ollinger (1980) who showed that even after an actinic flash, the isotopic composition of the evolved O_2 matches that of the H_2O and not of the HCO_3^-. It would seem, then, that there is little reason to doubt that H_2O is the substrate for O_2 evolution. However, the validity of even these experiments has been questioned by Stemler (1982), who holds open the possibility that PS II may be able to catalyze the hydration of CO_2 (and therefore the isotopic exchange reactions) at the O_2 evolving site.

The third major line of evidence that H_2O is the substrate for photosynthetic O_2 evolution was the classical observation by Hill (1937, 1939) that broken chloroplasts could be made to evolve O_2 in the light by the addition of ferric oxalate. This was the first successful observation of photosynthetic O_2 evolution in a system other than whole cells or an intact leaf. It was not necessary for Hill to supply the chloroplasts with CO_2; in fact, he made his observation with the chloroplasts evacuated, so any CO_2 remaining was very low in concentration. At first it was thought that some O_2 had to be present, but Hill and Scarisbrick (1940a) later found that this was simply because the small amount of ferric oxalate added was quickly reduced by organic acids present, and the O_2 was necessary to reoxidize the ferrous oxalate to ferric oxalate. If no exhaustion of the ferric salt was allowed to occur, the evolution of O_2 could continue for several hours. From the behavior of the reaction with various inhibitors, Hill and Scarisbrick (1940b) concluded that the O_2 was evolved in the "light" reaction, and that CO_2 was reduced in dark reactions that were disrupted during chloroplast isolation. The ferric salts stimulated O_2 evolution by acting as an oxidant for the light reactions. This physical

separation of the reactions leading to O_2 evolution from the reactions catalyzing CO_2 fixation was strong evidence for the non-involvement of CO_2 in O_2 evolution. Furthermore, the observed reduction of the added electron acceptor (now referred to as a "Hill oxidant"; electron transport in isolated thylakoids is called the "Hill reaction") confirmed that the light reactions of photosynthesis were oxidation-reduction reactions, with H_2O being the terminal reductant. Thus, the evolution of O_2 can be seen as a simple oxidation:

$$2\ H_2O \longrightarrow O_2 + 4\ H^+ + 4\ e^- \qquad (1.6).$$

To sum up the discussion so far, three major lines of evidence have contributed to the current picture of H_2O as the source of photosynthetic O_2 evolution: (i) by comparison with photosynthetic bacteria, which use light energy to reduce CO_2 and oxidize a hydrogen donor, green plant photosynthesis is presumed to operate by a similar mechanism, with H_2O as the hydrogen donor; (ii) ^{18}O labelling experiments have shown that the O_2 that is evolved is labelled to the same extent, or to nearly the same extent, as the H_2O, but not as the CO_2; and (iii) the light reactions of photosynthesis can be separated from the dark reactions; O_2 is evolved in the light reactions, while CO_2 is reduced in the dark reactions. The first two lines of evidence have difficulties inherent in the method that make them less than conclusive: (i) photosynthetic bacteria have major fundamental differences from green plant photosynthesis, such as in the gross architecture, in the number of photosystems, etc., and it has been argued that a direct comparison is not necessarily valid; and (ii) the complications of natural oxygen exchange between CO_2 and H_2O have cast doubt on the validity of the ^{18}O experiments in the minds of some researchers.

Given these objections to the first two lines of evidence, the third line of evidence, namely the independence of the Hill reaction on CO_2, became a cornerstone argument for H_2O as the source of photosynthetic O_2. It was therefore of great importance when Warburg and Krippahl (1960) showed conclusively that the Hill reaction was reversibly inhibited by removal of CO_2 from the membranes. Although the Hill reaction did not require that CO_2 be supplied to the membranes, Hill did not actually remove CO_2 that might have already been bound to the membranes. When this CO_2 is removed, the Hill reaction ceases. The implication is that if O_2 evolution cannot occur in the absence of CO_2, then CO_2 could remain a candidate for the source of evolved O_2. Even if H_2O is also shown to be involved, this does not preclude hydrated CO_2 (i.e. H_2CO_3 or HCO_3^-) as the source, as originally proposed by Willstäter and Stoll. This hypothesis, and variations of it, have been proposed throughout the nearly three decades since Warburg and Krippahl's discovery (e.g. see Warburg, 1964; Stemler, 1982).

Despite the above objections, the scientific community has not seriously questioned H_2O as the source of evolved O_2 since the time of Hill and Bendall's very successful "Z-scheme" (Hill and Bendall, 1960). The accumulated evidence for the light reactions of photosynthesis being a series of oxidation-reductions, with H_2O as the ultimate source of electrons, is overwhelming, and the picture is not likely to be overturned easily. It is possible, of course, to incorporate CO_2 into the mechanism of O_2 evolution without a major overhaul of our current model -- the mechanism of O_2 evolution is still one of the least understood aspects of photosynthesis -- but models such as that championed by Warburg as recently as 1964 (Warburg, 1964), in which CO_2 has more than a regulatory or

catalytic role, are probably out of vogue forever. One effect of the controversy over the past 25 years seems to have been not so much to cast doubt on the role of H_2O, as to have raised suspicion or caused indifference to the role of HCO_3^-. This is unfortunate, because although a major effect of HCO_3^- on O_2 evolution can be ruled out, it very clearly does play a major role in the reduction of plastoquinone (see Section B.2) and is, therefore, deserving of more attention than it has received. This thesis is one attempt to redress this imbalance. The remainder of this introduction will examine what is known about the role of HCO_3^- in PS II.

B. The Requirement for HCO_3^- in PS II: A Review

1. The Discovery

Credit for the discovery of the HCO_3^- requirement in the light reactions of photosynthesis is generally given to Warburg and Krippahl (1958, 1960). However, such a requirement was suggested several years earlier by Boyle (1948), who quite by accident noticed that O_2 production was halted in the H_2O to p-benzoquinone Hill reaction in ground spinach leaves when KOH was placed in the center well of a manometer to take up CO_2. This observation was shown later to be an artifact (Abeles et al., 1961), due to distillation of quinone from the main compartment of the reaction vessel into the KOH solution. Under such conditions, O_2 uptake occurs in the center well at a rate sufficient to reabsorb all of the O_2 produced by the Hill reaction. Thus, no net O_2 evolution would have been observed by Boyle, regardless of whether CO_2 were required or not. The conclusion that CO_2 was required was, therefore, unwarranted. Under the same conditions as Boyle, Abeles and coworkers (1961) found no effect on the Hill reaction by having CO_2 present or absent in the atmosphere. On the other hand, the same authors were able to confirm the observation by

Warburg and Krippahl, noting, under their conditions, a consistent difference in O_2 evolution when atmospheric CO_2 was present or absent. In these experiments the quinone concentration was lower, and there was no KOH in the center well. This, along with differences in pH, probably accounts for the failure to observe a CO_2 dependence under Boyle's conditions.

Other researchers may have noticed the requirement for HCO_3^- earlier, without fully recognizing it as such. For instance, Franck (1945) reported that broken chloroplasts evolved more O_2 when they were supplied with CO_2 than when they were flushed with N_2. Admission of CO_2 to CO_2-free suspensions always caused a sudden increase in the rate of O_2 production. Franck took this observation to justify that he was looking at real photosynthesis, with CO_2 as oxidant. However, from his protocol it is clear that he was using broken chloroplasts, which would have been missing the stromal enzymes necessary for CO_2 reduction. He did not add a Hill oxidant to his preparations; only small amounts of O_2 were evolved, but the rate was significantly higher when CO_2 was present. Later, under the same experimental conditions, Brown and Franck (1948) found that when $^{14}CO_2$ was used, there was no accumulation of the radiolabel in the chloroplasts. Therefore, they concluded that the stimulation by CO_2 was not due to CO_2 fixation in the chloroplasts, but to some other, possibly catalytic, role for CO_2.

This observation did not attract much attention until Warburg and Krippahl (1958, 1960) rediscovered the requirement for CO_2. They, too, showed that the Hill reaction was inhibited by CO_2 removal and strongly stimulated by addition of CO_2 at low partial pressure, and that there was no net reduction of CO_2 occurring simultaneously. These authors suggested that the CO_2 requirement reflects a catalytic function for CO_2 in the

mechanism of O_2 evolution. Earlier, Burk and Warburg (1950) had postulated an elaborate scheme for photosynthesis which contradicted much of the collective wisdom of the photosynthesis community, and this new finding of a CO_2 requirement was quickly pounced upon as evidence for the scheme (Warburg et al., 1959). Warburg's scheme did not separate the photochemical process from CO_2 metabolism: a photochemical reaction consumes one molecule of CO_2 and yields one molecule of O_2 per quantum; then a thermochemical back reaction consumes two-thirds of the released O_2 and releases two-thirds of the consumed CO_2. To explain the Hill reaction, in which no net reduction of CO_2 occurs (Brown and Franck, 1948; Warburg and Krippahl, 1958), Warburg and Krippahl postulated that unlike whole cells, isolated chloroplasts cannot retain the reduced CO_2, which is reoxidized by the Hill reagent. In Warburg and Krippahl's scheme the precursor of O_2 is a phosphorylated peroxide of carbonic acid, produced by the action of illuminated chlorophyll on the Hill oxidant, CO_2, H_2O, and phosphate.

Perhaps because of this insistence that H_2O is not the source of photosynthetic O_2, this observation of a CO_2 requirement, unlike the earlier observation of Franck (1945), attracted much notice, and was rapidly confirmed by several researchers (Abeles et al., 1961; Stern and Vennesland, 1962; Izawa, 1962; Good, 1963). The CO_2 effect was shown to be a general phenomenon, observable with a wide variety of Hill reagents and with a wide variety of species (Stern and Vennesland, 1962). Several observations at this time argued against the scheme of Warburg and Krippahl: (i) the stimulatory effect of CO_2 on the Hill reaction was much reduced in weak light, compared to strong light, suggesting that CO_2 was not involved in a photochemical reaction, but in a non-photochemical step (Izawa, 1962; Good, 1963); (ii) the correlation of the CO_2 dependence with

the presence of small anions suggested that HCO_3^-, not CO_2, was the important substance (Good, 1963); (iii) whereas Warburg's scheme has photophosphorylation intimately connected with CO_2 metabolism, uncouplers of phosphorylation do not relieve the impairment caused by CO_2 depletion, indicating a site of action remote from phosphorylation (Good, 1963); and (iv) whereas one would expect a greater CO_2 dependence with weaker Hill oxidants if the oxidant is involved in CO_2 metabolism, no such trend was observed (Good, 1963). Nevertheless, Warburg continued to present his scheme as though it were established fact (c.f. Warburg, 1964).

2. The Site of Action

Because of the non-independence of the HCO_3^- effect on light intensity, it was concluded that HCO_3^- acts at a non-photochemical step of the Hill reaction (Izawa, 1962; Good, 1963). The first attempt to locate this site of action was by Punnett and Iyer (1964), who looked at the effect of CO_2 on photophosphorylation. They observed that by adding relatively high concentrations of HCO_3^- to non-HCO_3^--depleted chloroplasts, they could accelerate the Hill reaction, as well as enhance the rate of phosphorylation. The ATP:$2e^-$ ratio was also increased, particularly when the pH was above 7. Thus, one of the effects of added CO_2 appeared to be to improve the coupling between electron transport and phosphorylation. However, as pointed out by Batra and Jagendorf (1965), the stimulation of the Hill reaction by HCO_3^- in the absence of either uncouplers or ADP and phosphate seems to argue, if anything, for a looser coupling. The apparent contradiction of these two observations they found difficult to rationalize (another explanation for these observations is offered in Chapter 7). Punnett and Iyer suggested that CO_2 may increase the efficiency of formation of a high energy intermediate resulting from electron transport,

but Batra and Jagendorf found that added CO_2 actually decreases the yield of the high energy state of the chloroplasts, which suggested to them that the high energy state may be in competition with the formation of ATP. Batra and Jagendorf extended the observations of Punnett and Iyer and showed that the effect observed is actually a different effect than the HCO_3^- dependence observed by Warburg and Krippahl (1958, 1960): (i) the Punnett and Iyer effect requires a relatively high concentration of HCO_3^- added to non-HCO_3^--depleted chloroplasts, whereas the Warburg and Krippahl effect requires much lower concentrations of HCO_3^- added to HCO_3^- depleted chloroplasts; (ii) uncouplers eliminate the stimulation of the Hill reaction by HCO_3^- in non-depleted chloroplasts (Batra and Jagendorf, 1965), whereas uncouplers have no effect on the HCO_3^- dependence of depleted chloroplasts (Stern and Vennesland, 1962; Good, 1963; see also Khanna, et al., 1977); (iii) added HCO_3^- stimulates phosphorylation under conditions of cyclic electron flow around PS I, supported by pyocyanine, with or without CMU (p-chlorophenyl-1,1-dimethyl urea) to block electron donation by PS II, whereas the removal of CO_2 by depletion has no effect on pyocyanine supported phosphorylation (Batra and Jagendorf, 1965); and (iv) the Warburg and Krippahl effect appears to represent a requirement for HCO_3^-, in that the rate of electron transport is depressed by removal of CO_2 and is restored by adding back the HCO_3^-, whereas the Punnett and Iyer effect is a true stimulation, in that removal of CO_2 does not inhibit phosphorylation and cyclic electron transport (Batra and Jagendorf, 1965). To this list can be added the observation that the pH optimum for the Warburg and Krippahl effect is around pH 6.5 (Khanna et al., 1977; Vermaas and Van Rensen, 1981), whereas Punnett and Iyer observed the maximal effect between pH 7.0 and 7.8.

A large number of these observations are similar to the report made later by Barr and Crane (1976), which became the topic of investigation in Chapter 5, Section I (see also Blubaugh and Govindjee, 1984). The striking similarities between these sets of observations suggest a common underlying mechanism, which is explored in more detail in Chapter 7; although the differences between the Warburg and Krippahl effect and the Punnett and Iyer effect suggest separate sites of action, they may yet be intimately related.

The first attempt to locate the site of impairment in HCO_3^- depleted chloroplasts was the study by Stemler and Govindjee (1973), which showed that HCO_3^- depletion had no effect on the rate of electron transport from the artificial PS II electron donor diphenylcarbazide (DPC) to the electron acceptor 2,6-dichlorophenolindophenol (DCPIP). DPC is believed to donate electrons to the primary donor to PS II, Z. Therefore, it was concluded that the effect of HCO_3^- depletion on the H_2O to DCPIP reaction was due to a HCO_3^- site prior to Z; that is, at the O_2 evolving locus itself. However, this result was later reinterpreted to be due to the rate-limiting donation of electrons by DPC, which obscures the HCO_3^- effect (Wydrzynski and Govindjee, 1975). This scenario was repeated a decade later when Fischer and Metzner (1981) concluded that HCO_3^- was required at the O_2 evolving site, in part because they could not observe a HCO_3^- effect in thylakoids using artificial electron donors to PS II (hydroxylamine, Mn^{2+}, tetramethylbenzidine and tetraphenylboron). Eaton-Rye and Govindjee (1984) showed that for at least two of these (hydroxylamine and benzidine), the electron transport rates supported by these donors is no greater in non-depleted controls than the rates typically obtained by HCO_3^- depletion; thus, this approach cannot be used to assign a location for the HCO_3^-

impairment. The other two donors used by Fischer and Metzner were not tested by Eaton-Rye and Govindjee, but the implication is that they, too, are probably rate-limiting in their donation.

Another problem with DPC as an electron donor is that it has an apparent effect on the membrane structure: in thylakoids that have begun to break down, DPC appears to stimulate energy trapping by PS II, perhaps by linking physically separated components (Harnischfeger, 1974). This increase in the quantum yield can mask an impairment of electron transport. However, the HCO_3^- effect can be seen with DPC and other artificial electron donors to PS II, if one looks at fluorescence instead of electron transport. Wydrzynski and Govindjee (1975) showed that HCO_3^- depletion accelerates the rise of the fluorescence transient in a manner similar to the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which is known to block electron transport after the first stable quinone acceptor, Q_A. In contrast, treatments which are known to impair the O_2 evolving mechanism, such as mild heat treatment, Tris treatment, etc., were shown to eliminate the variable Chl a fluorescence. These effects are predictable from the understanding that fluorescence is a monitor of $[Q_A^-]$ (Duysens and Sweers, 1963; Murata et al., 1966). Since HCO_3^- depletion produces a transient similar to treatment with DCMU, Wydrzynski and Govindjee concluded that HCO_3^- depletion causes a block on the acceptor side of PS II, after Q_A. In support of this argument, they showed that DPC, as well as other artificial PS II donors, restore the variable fluorescence to heat-treated and Tris-treated chloroplasts, but the effects of HCO_3^- depletion and restoration remain, even with these donor systems. Similarly, Eaton-Rye and Govindjee (1984) showed that when hydroxylamine is used to simultaneously inhibit O_2 evolution and to donate electrons to

PS II, the decay of Chl a fluorescence after a flash, which monitors the reoxidation of Q_A^-, is reversibly inhibited by HCO_3^- depletion. Thus, they reaffirmed the location of the HCO_3^- requirement to be on the acceptor side of PS II, after Q_A.

Initially, Stemler and Govindjee (1974) interpreted the effect of HCO_3^- depletion on the fluorescence transient as supportive of an impairment on the O_2 evolving side of PS II. This is because, although they observed an acceleration of the rise from the initial level F_o to the intermediate hump I (F_I), they then observed a slower rise from I to the maximum fluorescence level F_{max} (F_P). They argued that a block after Q_A would have caused a higher fluorescence at all times. However, this transient can be better understood now as a partial block after Q_A, due to a partial HCO_3^- depletion. The accelerated rise from F_o to I is due to the faster accumulation of Q_A^-, while the slower rise from I to F_{max} represents the filling of the plastoquinone (PQ) pool, which is slowed by the impairment; only when the PQ pool is reduced can $[Q_A^-]$ accumulate to its maximum level. A thorough HCO_3^- depletion causes a complete, or nearly complete, block between Q_B and the PQ pool, causing a fluorescence transient which is indeed higher at all times, up to F_{max} (Vermaas and Govindjee, 1982a).

It is now well established that the site of impairment caused by HCO_3^- depletion is on the acceptor side of PS II. Khanna and coworkers (1977) established the site as between Q_A and PQ by the use of artificial electron donors and acceptors. Neither PS I electron transport, as measured by O_2 uptake during electron transport from reduced diaminodurene (DAD_{red}) to methylviologen (MV) (DAD_{red} donates electrons after the PQ pool; MV accepts electrons from the terminal side of PS I and passes them to O_2), nor PS II

electron transport prior to Q_A, as measured by O_2 evolution during electron transport from H_2O to silicomolybdate (SiMo), is inhibited by HCO_3^- depletion. However, the PS II reduction of oxidized DAD, which accepts electrons from the PQ pool, does show the HCO_3^- dependence. These results clearly place the site of inhibition after Q_A, but before PQ.

Recently, Graan (1986) has challenged the generally accepted premise that SiMo accepts electrons from Q_A (e.g. Giaquinta and Dilley, 1975; Zilinskas and Govindjee, 1975). He argues that all available evidence concerning SiMo involvement with PS II is also consistent with SiMo simply replacing DCMU from the Q_B binding site (see also Böger, 1982). The apparent ability to replace DCMU is dependent on the redox state of SiMo; the reduced form apparently binds not at all or much less tightly than the oxidized form (Graan, 1986). Thus, SiMo may be functioning like benzoquinone and other electron acceptors which replace PQ, except that the binding affinity of the oxidized form is high enough to outcompete DCMU. Therefore, many reported observations throughout the literature, including the absence of a HCO_3^- effect in the H_2O-to-SiMo reaction, may have to be re-evaluated if Graan is correct. However, if the reduction of SiMo is rate limiting, then an impairment of electron transport after Q_A by HCO_3^- depletion would not be expected to be seen. Another possible explanation for the apparent ability of SiMo to replace DCMU is offered in Chapter 7.

Regardless of what the final outcome concerning SiMo will be, there remains ample evidence for the involvement of HCO_3^- in electron transport between Q_A and the PQ pool. Jursinic and coworkers (1976) found that HCO_3^- depletion slowed the oxidation of Q_A^-, as monitored by the decay of the Chl a fluorescence yield after an actinic flash, from a half-time of about 0.5 ms to approximately 2.6 ms. When the decay was determined as a

function of flash number (Govindjee et al., 1976), it was discovered that the oxidation of Q_A^- was even slower after the third and subsequent flashes, with a half-time of about 150 ms. This suggests that two electrons can still flow through Q_A to reduce Q_B to Q_B^{2-}, and that the reoxidation of Q_B^{2-} then becomes rate limiting. Thus, HCO_3^- depletion appears to slow down electron flow from Q_A to Q_B and to block the exchange of Q_B^{2-} with the PQ pool.

There also appeared to be a 30-50% inhibition of charge separation in these and other repetitive flash experiments (Stemler et al., 1974; Jursinic et al., 1976; Siggel et al., 1977), which prompted the suggestion that HCO_3^- depletion also inactivates a portion of the PS II reaction centers (Jursinic et al., 1976). However, an alternative explanation was offered by Jursinic and Stemler (1982), who found that a very slow component of the fluorescence decay, with a half-time of 1-2 s, was increased two- to three-fold in HCO_3^- depleted samples. They suggested that in a significant portion of the reaction centers of HCO_3^- depleted chloroplasts Q_A^- was not reoxidized in the dark time between flashes, thus keeping the reaction centers in a photosynthetically closed state. Since the increase of this very slow component occurred even after the first flash, they concluded that it was a component of the Q_A^- to Q_B electron transfer, and they suggested that HCO_3^- depletion may alter the redox potential of Q_A with respect to Q_B, or otherwise destabilize Q_B^-. It has been suggested that HCO_3^- depletion does destabilize Q_B^- by preventing the protonation of a nearby protein group (Eaton-Rye, 1987). It is also possible that this slow component represents some inactive PS II centers (e.g Graan, 1986; Garab et al., 1987), and that HCO_3^- depletion somehow raises the number of such centers, perhaps by inhibiting the binding of PQ

(e.g. Eaton-Rye, 1987; see also Chapter 4). However, as pointed out by Crofts et al. (1984), in normal active centers PQ binding and release must occur with a half-time less than 1 ms, in order to account for the observed reduction time of the PQ pool (Stiehl and Witt, 1969).

Robinson et al. (1984) confirmed the slower fluorescence decay of HCO_3^- depleted thylakoids, but obtained much faster rates, overall, than were reported previously. Presumably, this was due to a slower flash frequency (1 Hz, instead of 33 Hz) that permitted most of the very slow component to decay between flashes. After one or two flashes, Q_A^- decays with a half-time of 1.2 ms in HCO_3^- depleted thylakoids, compared to 0.23 ms in the control samples. After 3 flashes the half-time is increased to 10 ms. Eaton-Rye (1987) has extended these observations to show that at pH 7.5, the half-time of Q_A^- decay in HCO_3^- depleted thylakoids continues to increase after each flash up to 5-7 flashes, as opposed to only 3 flashes at pH 6.5. Also, after one or two flashes, the half-time is greater at the acidic pH, whereas after 4 or more flashes the half-time is greater at the alkaline pH. This behavior was explained by two separate pH-dependent processes. From a kinetic analysis of the pH dependence of the decay rate after 1-2 flashes, it was suggested that the binding of PQ is inhibited by HCO_3^- depletion, with the greatest effect occurring at acidic pH. A simultaneous inhibition of the protonation of Q_B^- was presumed to occur, which becomes more severe at alkaline pH because of the further reduced availability of H^+.

The inhibition of Q_A^- reoxidation by HCO_3^- depletion has also been shown by following the decay of the absorbance change at 320 nm, which is due to absorption by the semiquinones Q_A^- and Q_B^-, with comparable results to those obtained by the fluorescence decay experiments described above

(Siggel et al., 1977; Farineau and Mathis, 1983).

The site of HCO_3^- action has also been located at the quinone reactions by the interaction between HCO_3^- binding and herbicide binding. Khanna et al. (1981) showed that HCO_3^- depletion decreased the binding affinity of atrazine. Similarly, a variety of atrazine-type herbicides have been shown to inhibit HCO_3^- binding (Van Rensen and Vermaas, 1981; Vermaas et al., 1982; Snel and Van Rensen, 1983). Most of these herbicides appear not to be competitive with HCO_3^-, but bind closely enough to "feel" its influence. Since these herbicides are believed to inhibit PS II by replacing PQ from the Q_B site (e.g. Oettmeier and Soll, 1983), the binding of HCO_3^- at or near Q_B is presumed.

While the effect of HCO_3^- depletion on the acceptor side of PS II has been firmly established, an effect on the donor side has been a source of controversy. Numerous observations have been reported to support the idea of a major effect on the O_2 evolving complex (for a review, see Stemler, 1982). However, most of these observations have been explained without the need to invoke a site on the donor side of PS II (e.g. Vermaas and Govindjee, 1981a, 1981b, 1982b). Thus, some effects on the S-state transitions of the O_2 evolving complex (Stemler et al., 1974; Stemler, 1980) may actually be due to interactions of the S-states with the Q_B site (see e.g. Diner, 1977). Some of the observations have been shown to be artifactual, such as the apparent insensitivity of electron transport supported by artificial PS II donors (discussed above). One observation, that the rate of $H^{14}CO_3^-$ binding appears to be dependent on the pH of the lumen, rather than the external pH (Stemler, 1980), is given an alternative interpretation in Chapter 7. No firm evidence has, to date, been shown for an involvement of HCO_3^- on the donor side of PS II; in fact, numerous

studies suggest a non-involvement of HCO_3^- on the donor side (Stemler and Radmer, 1975; Khanna et al., 1977; Van Rensen and Vermaas, 1981; Khanna et al., 1981).

3. Physiological Significance

Reproducibly large effects of HCO_3^- depletion were not observed until low pH and high salt concentrations were used during the depletion procedure (Stemler and Govindjee, 1973). Since then, high concentrations of formate (HCO_2^-) have been routinely used. With the measurement by several laboratories of a dissociation constant for the HCO_3^- * PS II complex of 80 μM (Stemler and Murphy, 1983; Snel and Van Rensen, 1984; Jursinic and Stemler, 1986), it has been suggested that the stimulatory effect of HCO_3^- is no more than a simple reversal of an inhibitory effect of HCO_2^- (Stemler and Murphy, 1983). A HCO_3^- dependence in the absence of HCO_2^- has been observed (Robinson et al., 1984; Eaton-Rye et al., 1986; see also Chapter 2), but the effect is never as dramatic as when inhibitory anions are present. The observation of a HCO_3^- effect on PS II in vivo is difficult to distinguish, due to the obvious requirement for CO_2 in the Calvin cycle. Nevertheless, Ireland and coworkers (1987) have attempted to do this in leaves by examining the effect on fluorescence of a small decrease in the intercellular CO_2 concentration from an already low level. They observed a significant increase in $[Q_A^-]$ under conditions of only a small decrease in CO_2 fixation, despite a large quenching of fluorescence due to the transmembrane pH difference. They concluded that HCO_3^- is involved in the quinone reactions in vivo.

In this thesis, evidence is presented for an essential role for HCO_3^- (Chapter 5). Some errors in the determination of Kd are pointed out (Chapter 3, Chapter 5), and a model is presented for a mechanism that keeps

HCO_3^- tightly bound, even at low HCO_3^- concentrations (Chapter 7). One of the major conclusions of this thesis is that HCO_3^- is an essential requirement for electron transport through the quinones of PS II.

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CHAPTER 2

MATERIALS AND METHODS

A. Plant Material

Spinach (Spinacia oleracea) was purchased wholesale from a local produce distributor, or grown hydroponically in the laboratory of Archie Portis at the University of Illinois. The hydroponically grown spinach was, typically, darker in appearance and gave the best chlorophyll a (Chl a) fluorescence transients, but also gave lower rates of electron transport expressed on a per chlorophyll basis. Possibly, this was due to a larger antenna size in this spinach, due to lower light intensities during growth than is available to the market spinach. However, J. Whitmarsh (personal communication) has measured the antenna size in both types of spinach and found no significant difference.

The market spinach was highly variable in quality, and, judging from the size and texture of the leaves, at least two different varieties were obtained on various occasions. No studies were performed to test for differences between these varieties; thylakoids obtained from either variety were used interchangeably. It is possible that for different experiments, or under different techniques of thylakoid isolation, one or the other variety is favorable. For this thesis, however, reliance was placed on reproducibility of observations without regard for the variety or growth history of the starting plant material. Wherever feasible, an entire set of measurements was done with a single sample, in order to minimize variables. Whatever trend was noticed would then be confirmed with other samples. Particularly in the case of HCO_3^- depleted membranes, which introduces another degree of variability, there generally appeared to be no advantage in averaging measurements taken from different samples.

The data presented throughout this thesis are of representative experiments.

Another source of variability in starting plant material appeared to be seasonal in nature. Coleman (1987) showed, without much discussion, a sinosoidal variation in electron transport rates in PS II preparations. The activity ranged from about 225 to 425 μmoles of evolved O_2 per mg Chl per hour, with the lowest activity occurring in the summer months. Eaton-Rye and Govindjee (1987) showed a significant pH effect in the half-time of Q_A^- decay in HCO_3^- depleted thylakoids; this pH effect was considerably more pronounced in the winter months, being almost unobservable during the summer (Eaton-Rye, 1987). Similarly, Schreiber and Rienits (1987) have observed a seasonal variation in the degree of fluorescence quenching by ATP, with the largest effect being observed in the summer. The stimulatory effect of HCO_3^- or CO_2 on photophosphorylation was also shown to be much larger in winter-grown than in summer-grown oats (Punnett and Iyer, 1964; Batra and Jagendorf, 1965). These examples are surely not exhaustive; the awareness of seasonal variations seems to be widespread, and it is clear that the variations can be sizable and extend to a large number of photosynthetic phenomena. To this author's knowledge, however, no detailed study of such variations has ever been undertaken, nor has an attempt been made in this thesis to do so. However, it is important to be aware of such variations, as this might account for some conflicting observations between laboratories.

B. Thylakoid Isolation

All buffers were prepared to near final volume, adjusted to the appropriate pH at room temperature, and then diluted to the final volume. All isolation procedures were carried out at 7 C, to minimize enzymatic degradation of the thylakoids.

Thylakoids were typically obtained by grinding 3 to 4 of the larger fresh leaves, with midribs removed, in a grinding medium for 10 s in a Sorvall omnimixer. The grinding medium was prepared immediately before thylakoid isolation by diluting 4 ml of 10% (w/v) bovine serum albumin (BSA) and 0.8 ml of 100 mM EDTA (pH 7.5) to 80 ml with cold (7 C) isolation buffer (pH 7.5) containing 50 mM sodium phosphate and 50 mM NaCl (the final concentration of BSA was 0.5% (w/v) and of EDTA, 1 mM). The rationale for using BSA in the grinding medium is to bind any free fatty acids, which can inhibit O_2 evolution. The reason for using EDTA is to bind metal ions that may be required for some degrading enzymes that may be present after cell rupture. The concentration of EDTA chosen is low enough to not cause any noticeable inhibition of PS II through chelation of Ca or Mn. Neither BSA nor EDTA was used in the subsequent washing or resuspension of the thylakoids.

After grinding, the homogenate was filtered through 4 layers of Miracloth (a non-woven, industrial cloth designed for polishing silver) and pelleted at 3,500 x g for 7 min. The thylakoids in the pellet were washed once in cold isolation buffer, repelleted, and then resuspended in a minimum volume of the isolation buffer. Resuspension of the pellet was done by gently sucking the medium up in a pipette and blowing it onto the pellet. The chlorophyll (Chl) concentration after the final resuspension was typically 2-3 mg/ml, as determined by the spectrophotometric method of MacKinney (1941). If the thylakoids were to be frozen, then the final resuspension buffer contained 0.4 M sucrose or sorbitol as an osmoticum to prevent damage to the membranes by water crystals during freezing and to minimize protein release during storage (c.f. Volger et al., 1978).

Freezing was done with small aliquots (typically 150 µl) in glass

vials submerged in liquid N_2 in dim light. Thylakoids could be stored this way for many months without appreciable loss of activity. Thawing was done rapidly in dim light under a stream of room-temperature water and the thylakoids then kept on ice throughout the experiment. Usually, the thawed sample would last 5-6 hours, with only a small gradual loss of activity. After this time, however, loss of activity usually became noticeably accelerated. When performing long experiments, frozen thylakoids were generally preferred to non-frozen, as an identical sample could be thawed after a few hours.

C. HCO_3^- Depletion

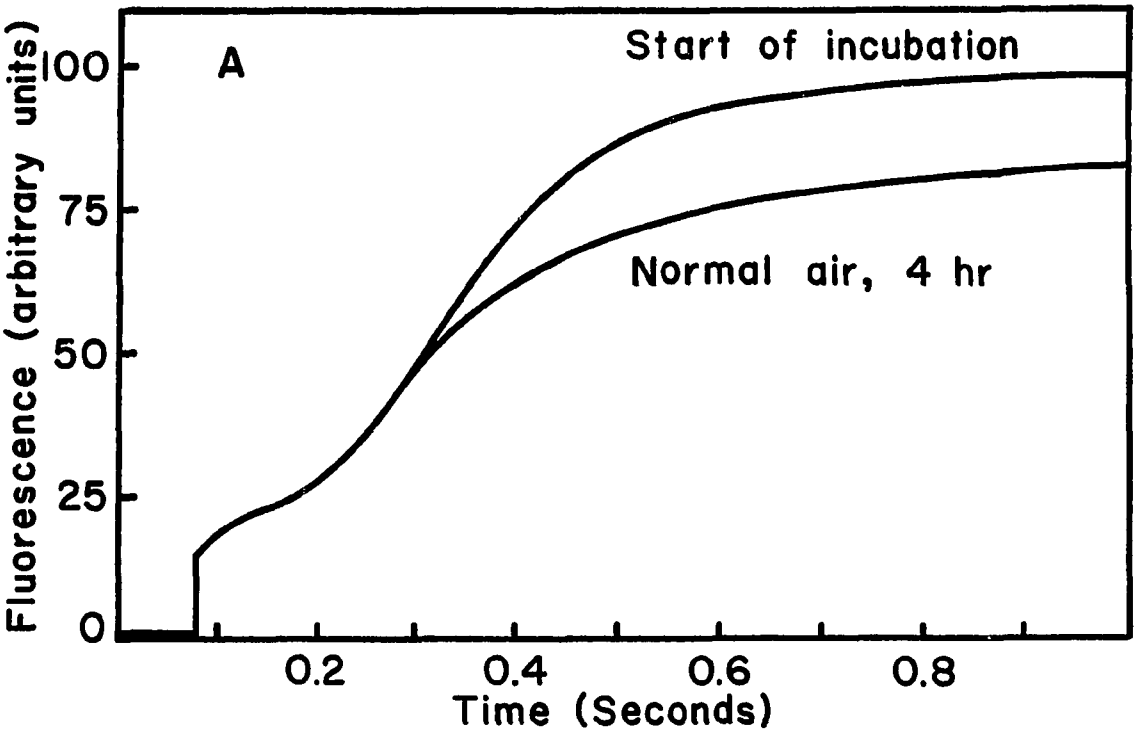
N_2 gas or a mixture of 80% N_2 and 20% O_2 (CO_2-free air) was used to purge containers and solutions of CO_2. The gas was first passed through a drying column of $CaCl_2$ and ascarite (asbestos-coated NaOH) to remove any residual CO_2. The $CaCl_2$ removes moisture which can harbor HCO_3^-, and NaOH adsorbs CO_2. The gas was then bubbled through distilled H_2O to hydrate it. Several minutes of bubbling was done before using the gas, to assure that the H_2O reservoir was itself depleted of CO_2. Hydration of the gas was necessary to prevent concentration changes by evaporation of the solutions.

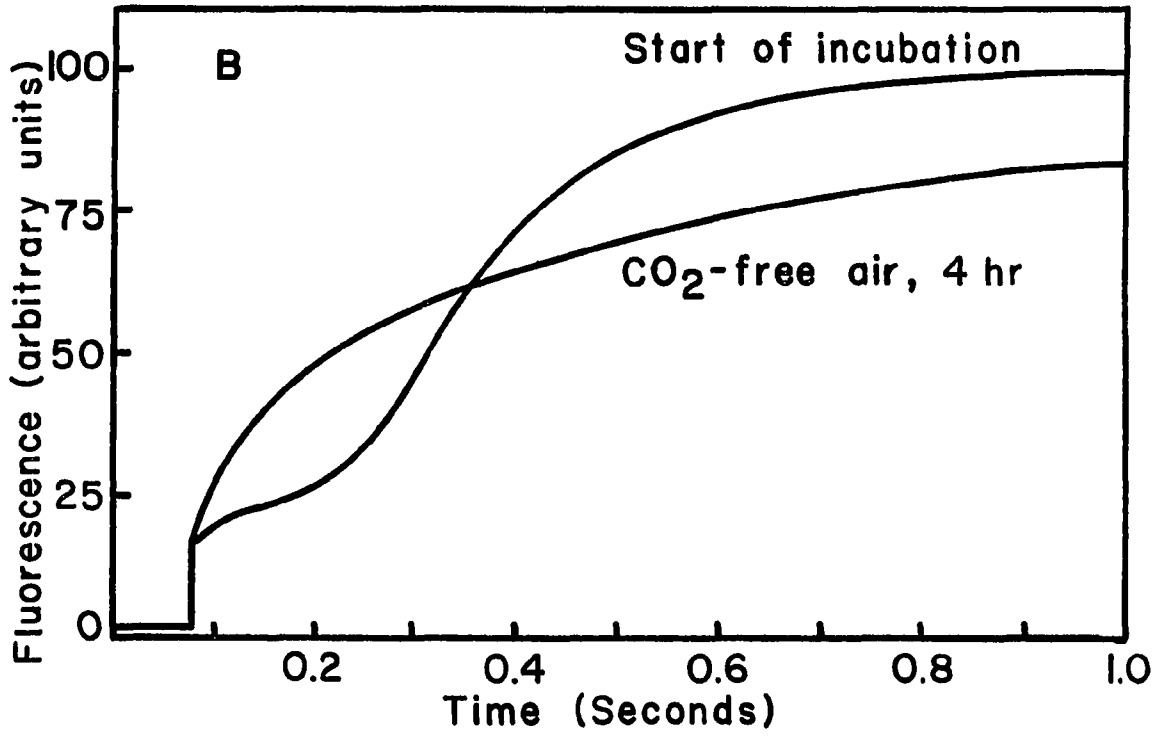
The strategy for depleting thylakoids of HCO_3^- is to suspend them in a CO_2-free medium of low pH and high salt at room temperature. A brief rationale for this strategy will be given here. The low pH causes most of the HCO_3^- to become CO_2, which can be easily flushed away in a gas stream. Too low a pH, however, also causes an irreversible loss of activity (Vermaas and Govindjee, 1982), which probably accounts for the very low control activity common in much of the earlier literature on the HCO_3^- effect (c.f. Stemler and Govindjee, 1973). Much attention has been paid to the new technique of Vermaas (1984) and of Snel and Van Rensen (1984),

because a much higher control activity is retained. The essential feature of this method is to incubate the thylakoids at a more neutral pH for a longer period of time, thus avoiding the irreversible inactivation due to low pH. Comparable success was obtained by this author using the older method by simply raising the pH to 5.5 or higher. A certain trade-off is involved; a higher pH is more favorable for overall activity, but requires a longer incubation time to achieve a good HCO_3^- depletion.

The older literature sometimes suggests a pH as low as 5.0 — this is too low, and with phosphate as the buffer there would be no buffering capacity, so the pH could drift even lower. In theory, any non-damaging pH can be used, since some of the HCO_3^- is always present as CO_2, and flushing away CO_2 will, by Lavoisier's principle of mass action, drive all of the HCO_3^- to CO_2. Of course, at higher pH it will take longer to remove all of the HCO_3^-. An illustration of this principle is the effect on the fluorescence transient of a gentle stream of CO_2-free air on a thylakoid sample at pH 6.4 (Fig. 2.1). Even without formate (HCO_2^-) or high salt, and at a pH considerably higher than normally used for HCO_3^- depletion, it is obvious that HCO_3^- is being removed from the membranes. The faster fluorescence rise under CO_2-free air is typical of HCO_3^- depleted thylakoids, due to inhibition of electron flow out of Q_A^- (Wydrzynski and Govindjee, 1975). The time required, however, to deplete the membranes by this method is quite long; even after 4 hours the effect is not maximal. The aging effect on the thylakoids is apparent as a quenching of the fluorescence in both the control and the depleted samples. The depletion time may also have been lengthened by the fact that the sample was not stirred; however, the sample did have a large surface area, as the 2 ml volume was less than 1 mm thick.

Figure 2.1. The effect of HCO_3^- depletion, in the absence of formate and at low [NaCl] (15 mM), on the variable Chl a fluorescence transient. (A) The fluorescence transient at the start of incubation and after 4 hrs of incubation under a stream of normal air. (B) The fluorescence transient at the start of incubation and after 4 hrs of incubation under a stream of CO_2-free air. The area above the curve for the HCO_3^- depleted sample is approximately 60% of that for the non-HCO_3^--depleted sample. Spinach thylakoids were suspended in 50 mM Na phosphate, pH 6.4, 15 mM NaCl, and 5 mM $MgCl_2$.


A
Start of incubation
Normal air, 4 hr
Fluorescence (arbitrary units)
100
75
50
25
0
0.2
0.4
0.6
0.8
Time (Seconds)


B
Start of incubation
CO_2-free air, 4 hr
Fluorescence (arbitrary units)
100
75
50
25
0
0.2
0.4
0.6
0.8
1.0
Time (Seconds)

Although in theory one ought to get a depletion at any pH, in our hands a good depletion at pH 7.5 could not be obtained. Similarly, thylakoids that have been depleted of HCO_3^- and then raised to a pH of 7.5 or higher, do not have much restored activity upon addition of HCO_3^- (Khanna et al., 1977). Addition of HCO_3^- to HCO_3^- depleted thylakoids has even been reported to further inhibit the Hill activity at pH 8.0 (Stemler, 1980; Stemler and Jursinic, 1983). These observations might suggest a barrier to HCO_3^-/CO_2 which is alleviated at lower pH. This idea is discussed in Chapter 7, in connection with a postulated sequestered pool of HCO_3^-.

A high salt concentration favors the removal of HCO_3^-, as illustrated in Fig. 2.2, which shows the time course of the effect illustrated in Fig. 2.1 for thylakoids suspended at two different salt concentrations. The area over the Chl a fluorescence induction curve is proportional to the size of the electron acceptor pool (for a review, see Lavorel et al., 1986; Van Gorkom, 1986), and gets smaller when there is an impairment of electron flow out of Q_A^-, as in the case of HCO_3^- depletion. Under identical conditions, the area over the curve diminishes faster (i.e. the fluorescence rise is faster) when the thylakoids are suspended in 100 mM NaCl, as opposed to only 15 mM NaCl. Thus, HCO_3^- is apparently removed from the membranes more readily at higher [NaCl].

The use of high salt concentrations by Stemler and Govindjee (1973), particularly the use of HCO_2^-, in combination with low pH, made possible the first observations of a large and reproducible HCO_3^- effect, although even the control rates in those early experiments were quite low, probably due to the low pH as described above. It has been suggested that the HCO_3^- binding site is a general anion binding site, and that even Cl^- and the

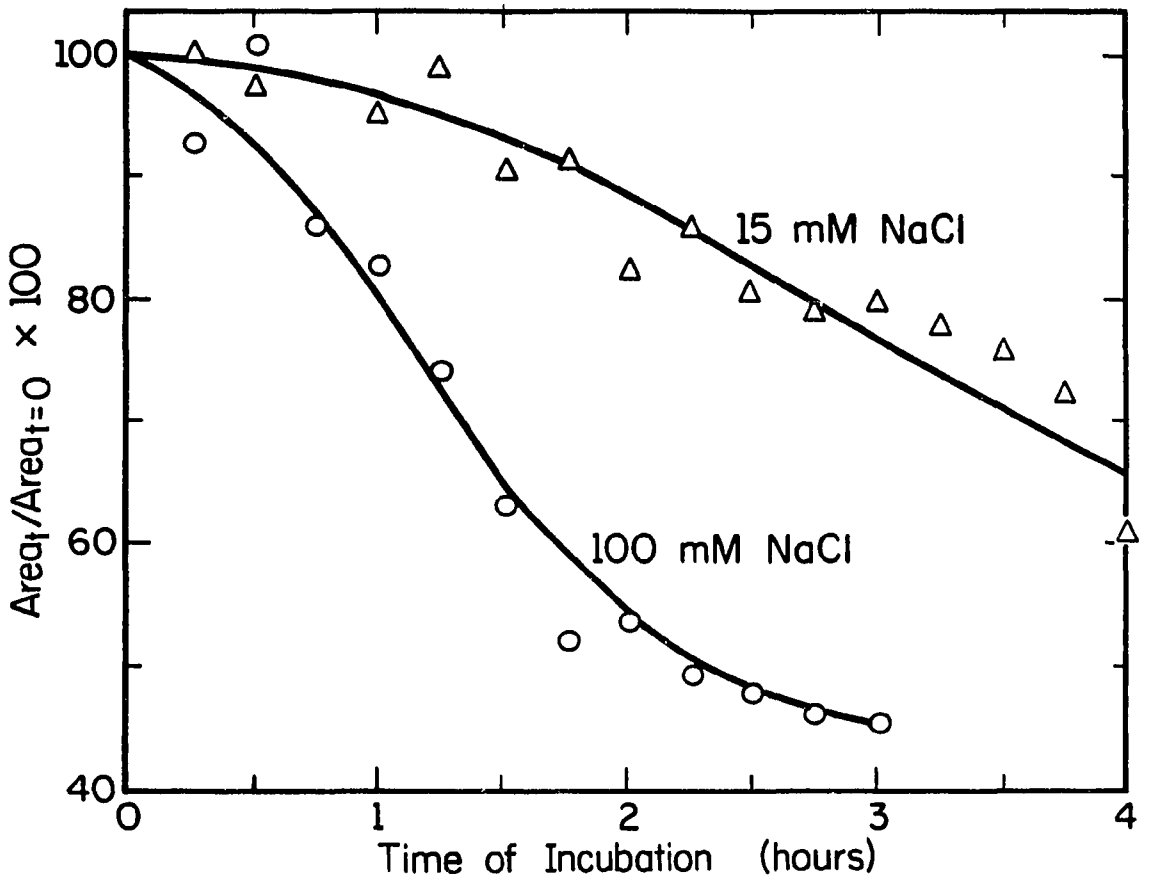


Figure 2.2. The time course of HCO_3^- depletion in the absence of formate, by incubation under CO_2-free air, under low and high salt concentrations. The area above the fluorescence transient is plotted as a function of time under CO_2-free air. $Area_{t=0}$ is the area above the transient at the start of the incubation, and $Area_t$ is the area at the time indicated. Symbols: circles, 100 mM NaCl; triangles, 15 mM NaCl.

other halides compete with the HCO_3^- (Stemler and Murphy, 1985; see also Jursinic and Stemler, 1987). While it is fairly certain that HCO_2^-, NO_2^-, and other anions that resemble HCO_3^- bind to the same site (Snel and Van Rensen, 1984), the relatively inefficient effect of Cl^- could be explained by other means than a direct competition with HCO_3^-, such as ionic strength effects, localized changes in pH at the charged surface of the membrane, etc. (see also Chapter 7). Good (1963) showed that the effects of Cl^- and acetate (CH_3COO^-) were not additive, but synergistic. This implies separate actions of the two; therefore, they are probably not competitive with each other for the same site. Nevertheless, high salt concentration does favor HCO_3^- depletion, particularly when the anion is also an analog of HCO_3^- and can compete for the binding site.

Higher temperatures also favor HCO_3^- depletion by lowering the solubility of CO_2, by accelerating the conversion of HCO_3^- to CO_2, and perhaps by accelerating other reactions by which HCO_3^- may be released from the membranes (see Chapter 7). Stemler (1977) showed that the exchange of exogenous $H^{14}CO_3^-$ with previously bound HCO_3^- is significantly greater at higher temperatures. It follows, then, that the removal of bound HCO_3^- should be easier at higher temperatures. While too high a temperature can obviously damage the membranes, a room temperature incubation can be used with advantage, particularly if the incubation time is relatively short. The method of Vermaas (1984) and of Snel and Van Rensen (1984), which uses a milder pH, involves an incubation at room temperature for 1-2 hours, without appreciable loss of activity. The exclusion of O_2 during this incubation may be a key factor in maintaining the activity at this temperature.

Following is the procedure used throughout this thesis for depleting

thylakoids of HCO_3^-. Throughout the depletion procedure and subsequent experiments, all steps were done under a stream of hydrated CO_2-free air or N_2, up until HCO_3^- was added to the sample. The depletion and resuspension media were bubbled with N_2 or CO_2-free air for at least 10 min before use. The thylakoids were resuspended in the depletion medium (CO_2-depleted 50 mM Na phosphate, 100 mM NaCl, 100 mM $NaHCO_2$, 5 mM $MgCl_2$, pH 5.5) to a [Chl] of 40-80 μg/ml, then incubated with gentle shaking for 5 min at room temperature in the dark. The depleted thylakoids were then placed on ice and pelleted by centrifugation at 3,500 x g for 7 min at 7 C. The pellet was then resuspended in reaction medium (same as the depletion medium, except pH 6.5) to the desired [Chl]. The measured [Chl] was usually 10-20% less than calculated, apparently due to a tendency of the depleted membranes to adhere to the glass. This probably reflects some altered charge properties of the depleted membranes. Some resuspension of the pellet was accomplished while squirting the medium in through a syringe, with the flow directed onto the pellet. Invariably, however, large chunks of pellet would remain suspended, and would require vortexing of the closed tube to get a homogeneous suspension. The lower the pH of the depletion procedure, the more resistant to break-up the chunks seemed to be; again, this probably reflects altered charge properties of the membranes. The time of vortexing was always kept as brief as possible, though, surprisingly, the vortexing did not appear to adversely affect activity. After restoration of the Hill activity with a saturating [HCO_3^-] the electron transport rates were, typically, around 400-600 uequivalents per mg Chl per hr (using 2,6-dichlorophenolindophenol (DCPIP) or ferricyanide as electron acceptor), and were about 80-90% of the original activity.

D. Electron Transport Rate Measurements

1. O_2 Evolution

Rates of O_2 evolution were determined polarographically using a Hansatech Pt/Ag-AgCl electrode, described by Delieu and Walker (1972), or a Yellow Springs Instruments Clark-type electrode. O_2 diffusing across a thin teflon membrane that separates the electrode from the sample chamber, is reduced at the Pt surface and induces a current flow. The rate of diffusion across the membrane, and therefore the strength of the current, is proportional to the $[O_2]$ in the solution. Thylakoids in an appropriate buffer ([Chl] = 10-15 µg/ml) were placed into the chamber and stirred magnetically. Illumination was provided by a Kodak Carousel 4200 slide projector equipped with a Corning CS3-68 yellow filter. The light was further filtered through 2 inches of a 1% solution of $CuSO_4$ to absorb infrared radiation, in order to prevent temperature changes of the electrode. The light intensity reaching the sample chamber was 2.25×10^3 W m^{-2}, as measured by a Lambda Instruments LI-185 radiometer. The temperature of the sample was maintained with a circulating, temperature-controlled water bath. 2 mM ferricyanide or 0.1 mM methyl viologen (MV) were typically used as Hill oxidants, and 10 mM methylamine along with 100 nM Gramicidin D was used as an uncoupler. If MV was used as Hill oxidant, then 1 mM NaN_3 was also added to block catalase activity, and 225 units/ml superoxide dismutase was added to scavenge O_2^- radicals. Without the latter two additions, O_2 uptake is not stoichiometric with MV reduction (Izawa, 1980).

The output current from the electrode was converted to voltage and displayed on an Esterline Angus model E1101S strip-chart recorder. Calibration of the signal was done according to the method of Delieu and

Walker (1972); prior to each set of experiments the difference was measured in the voltage induced by air-saturated water (water left in a squeeze bottle overnight) versus water to which sodium dithionite was added, which consumes all O_2. The $[O_2]$ in the air-saturated water was determined from the water temperature, using the formula of Truesdale and Downing (1954). Because $[O_2]$ in air-saturated water is dependent on temperature, and because equilibration with the air takes some time after a temperature change, the temperature of the circulating water bath was always set to the ambient temperature of the room, as determined from the temperature of the water. Thus, the water that was used for calibration was neither super-saturated nor under-saturated in O_2 at the temperature of the experiment. Rates of electron transport were determined as μmoles O_2 evolved per hour per mg Chl.

2. DCPIP Reduction

Rates of electron transport from H_2O to the dye 2,6-dichlorophenol-indophenol (DCPIP) were measured spectrophotometrically by following the disappearance of light absorbance at 600 nm as the DCPIP was reduced. Measurements were made with a Cary-14 spectrophotometer equipped with a side-illuminating actinic lamp consisting of one or two Sylvania DVY 650 W, 120 V halogen bulbs with a 5 cm water filter, a glass heat filter, and a Corning CS2-59 red filter. The intensity of this actinic light was 1.5×10^3 W m^{-2}, as measured by a YSI-Kettering Model 65 Radiometer. A 602 nm interference filter was placed between the sample and the photomultiplier to avoid noise from scattered actinic light. Another 595 nm interference filter was placed between the reference beam and the photomultiplier, as it was discovered that actinic light could reflect into the spectrophotometer and into the reference compartment.

In experiments involving HCO_3^- depleted thylakoids, the sample cuvette was filled to 4 ml and stoppered, leaving a gas space of only about 150 µl. The purpose of such a small gas space was to minimize escape of CO_2 after the addition of HCO_3^-. However, in order to illuminate the entire 4 ml of sample, some modification was necessary to the existing equipment. Specifically, two halogen bulbs were provided, instead of one, oriented so that one illuminated the bottom half of the cuvette while the other illuminated the top half. Because of the nature of the lens arrangements in the lamp, it was necessary to be able to lower the cuvette about 7 mm in order to illuminate the entire cuvette. Therefore, a new cuvette holder was built with a more compact base. All of the measuring beam still passed through the sample. Fig. 2.3 shows a typical spectrophotometric recording of DCPIP reduction in HCO_3^- depleted and HCO_3^- restored thylakoids.

A volume of 10 mM DCPIP was added to the cuvette sufficient to yield a 60 µM concentration of the anion at the pH and volume of the experiment, based upon a pKa of 5.90 (Armstrong, 1964). Since it is the anion, rather than the acid, that absorbs maximally at 600 nm, it was determined to start each experiment based on the concentration of the anion, rather than the total concentration. The concentration chosen was low enough to still be in the linear range of Beer-Lambert's law, while being high enough to permit considerable electron transport before depleting the dye. The cuvette was flushed with N_2 gas, if necessary, to remove CO_2, and a thylakoid sample containing 10-12 µg Chl/ml was added. Ordinarily, no uncoupler was needed, as DCPIP is itself an uncoupler. It was observed, however, that 10 mM methylamine and 100 nM Gramicidin D, while not improving the initial rate of electron transport, did sometimes provide a measure of stability to the trace, in that the trace remained linear for a

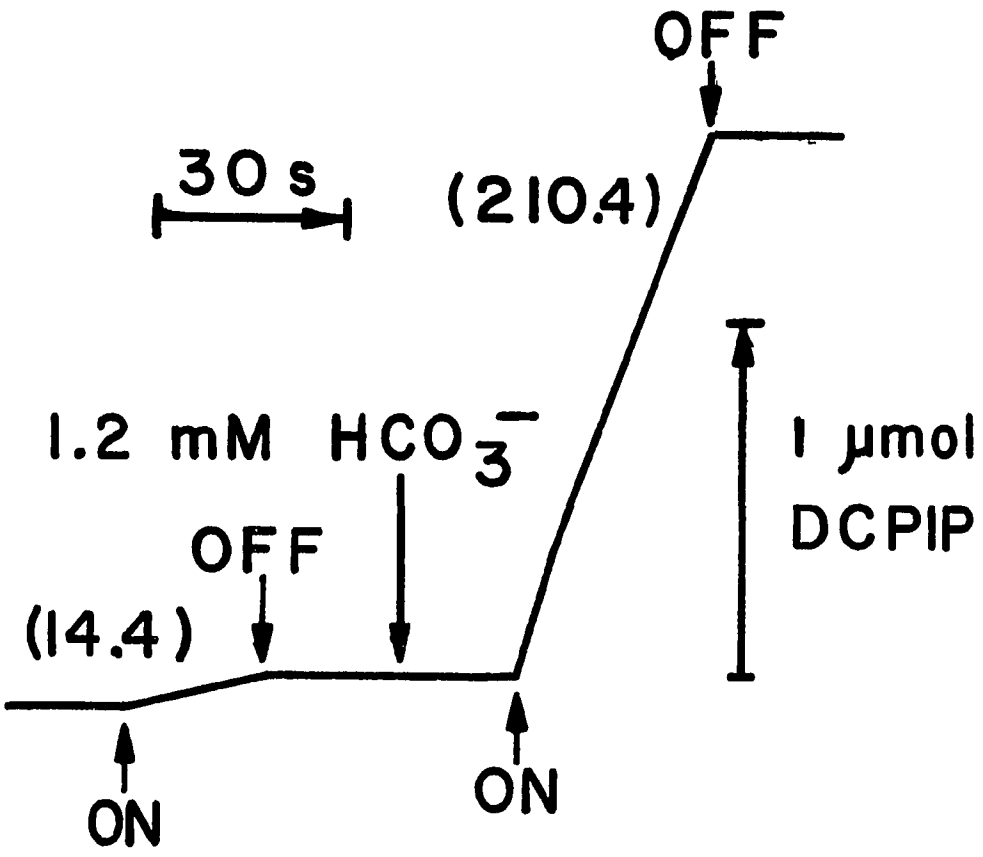


Figure 2.3. Demonstration of the HCO_3^- requirement in spinach thylakoids: electron flow from H_2O to DCPIP, as measured by the disappearance of absorbance at 600 nm. Thylakoids were depleted of HCO_3^- in a medium containing 50 mM Na phosphate, pH 5.3, 100 mM NaCl, 100 mM $NaHCO_2$, and 5 mM $MgCl_2$. The [Chl] was 12 µg/ml. The restored rate was 210 µmoles DCPIP reduced per mg Chl per hr.

longer period of time. When used, this is mentioned in the text. The sample was stirred, using a water-driven magnetic stirrer, and the absorbance was monitored at 600 nm. The absorptivity of DCPIP was calculated for the pH of the experiment according to the equation of Armstrong (1964).

E. Fluorescence Induction Curves

The 685 nm fluorescence emitted by Chl *a* in PS II undergoes a complex kinetic induction upon illumination (for a review, see Briantais *et al.*, 1986). The fluorescence rises from an initial level O (also referred to as F_o) to an intermediate level I, followed by a plateau or dip D, then another rise to a maximum level P (also known as F_{max}). In isolated thylakoids, this is the extent of the induction changes; in intact tissue, however, there are further changes, resulting in a decrease in fluorescence to a terminal level T, which is not much higher than the inital fluorescence F_o, with intervening valleys S_1, S_2 and mountains M_1, M_2. These further changes after P are not fully understood, but probably reflect a combination of (i) a redistribution of energy transfer to the two photosystems, (ii) a fluorescence quenching due to energization of the membrane, and (iii) a quenching by reduced pheophytin. Since all experiments in this thesis were done with isolated thylakoids, these changes after P will not be dealt with further here. Fig. 2.4 shows the effect of HCO_3^- depletion on the fluorescence induction in thylakoids.

The fluorescence rise from F_o to F_{max} is believed to reflect the kinetics of Q_A reduction (Duysens and Sweers, 1963; Murata *et al.*, 1966). Oxidized Q_A is a fluorescence quencher predominantly by virtue of its role as a stable electron acceptor. A lesser amount of quenching occurs due to the ability of oxidized quinones to quench non-photochemically

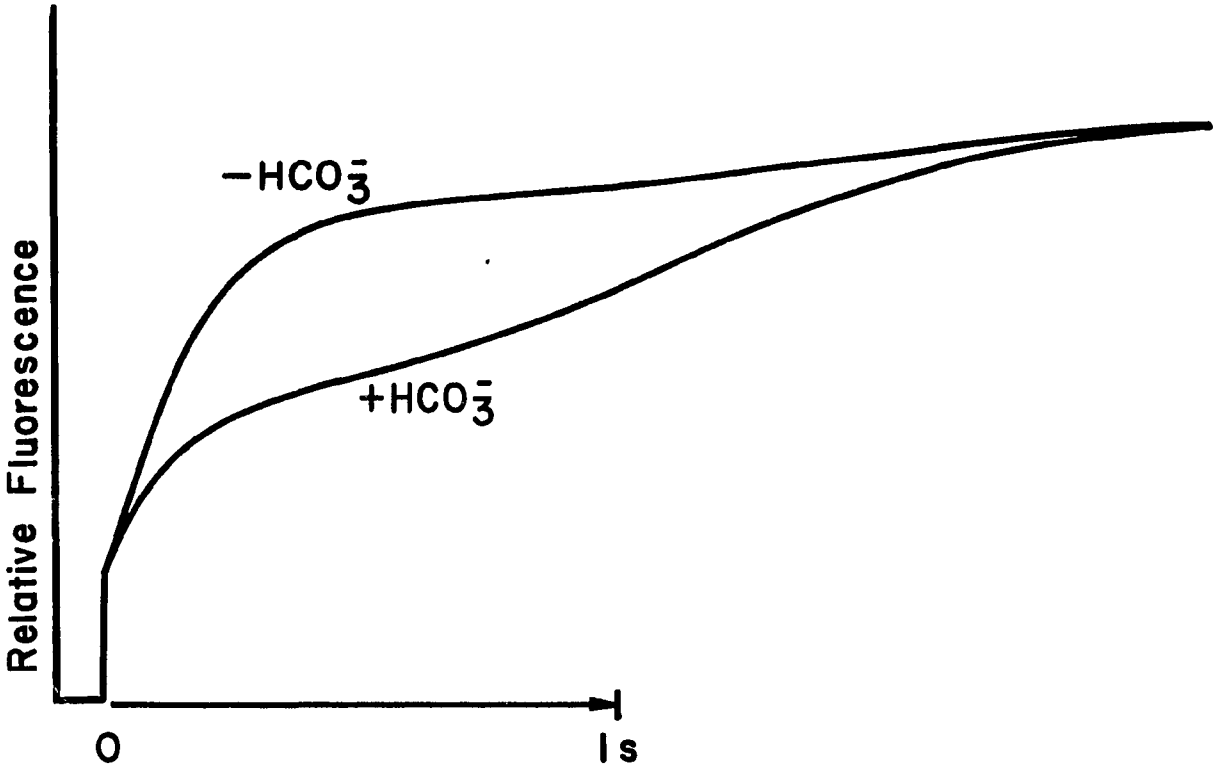


Figure 2.4. Demonstration of the HCO_3^- requirement in spinach thylakoids: the variable Chl a fluorescence intensity, measured at 685 nm, as a function of time after the onset of illumination. The [Chl] was 24 μg/ml. Upper curve: thylakoids depleted of HCO_3^- as in Fig. 2.3. Lower curve: 10 mM HCO_3^- readded.

(Vernotte et al., 1979). The rise from O to I probably reflects the initial reduction of Q_A to the point where the flow of electrons into Q_A is equal to the flow from Q_A to Q_B. The I to D plateau would thus represent a steady-state level of Q_A^-, in which the rates of oxidation and reduction are balanced. As the plastoquinone pool becomes reduced, however, electron flow out of Q_A^- is slowed, and fluorescence then rises to the F_{max} level, at which all of the Q_A is reduced (assuming the light is saturating). Since HCO_3^- depletion impairs electron flow from Q_A to Q_B and blocks electron flow from Q_B to PQ, it is obvious that the fluorescence induction should be very sensitive to HCO_3^- depletion. The accelerated rise due to HCO_3^- depletion is apparent in Fig. 2.4 (see also Wydrzynski and Govindjee, 1975).

An alternative explanation for the kinetics of the fluorescence induction curve in terms of a heterogeneity of PS II is also possible, in which the O to I phase is related to inactive reaction centers, and the I to P phase is related to active reaction centers (Govindjee, personal communication; for a review on PS II heterogeneity, see Black et al., 1986). In this regard, the shape of the fluorescence induction curve is similar to the velocity curve of a dual enzyme system in which each enzyme catalyzes formation of the same product (e.g. Segel, 1975, pp 64-71). In this case, the product would be Q_A^-. Again, since HCO_3^- depletion blocks the oxidation of Q_A^-, the observed acceleration of the fluorescence rise would be expected.

Fluorescence transients were measured with the fluorimeter described by Munday and Govindjee (1969), with significant modification. The output current from the photomultiplier was converted to voltage, then digitized with 8 bit precision by a Biomation Model 805 waveform recorder and stored

on an LSI 11 minicomputer (Digital Equipment Corporation). A program was developed in the course of this study (see section H) that permitted display of the transient on an oscilloscope, or output of the trace on a digital plotter. Computer-aided manipulations of the data were also possible, as described in Appendix I. Initially, the strategy used to capture the transient was to mechanically open the shutter and to let the rise of fluorescence from the base line to F_o provide the trigger to the Biomation recorder. A special pretrigger mode permitted capture of a user-determined number of points before the trigger, so that the baseline could be established. Later, an electronic shutter was used, with a home-built controller opening the shutter and, after a predetermined delay, triggerring the Biomation recorder to begin recording. This made possible the use of another mode on the Biomation recorder that permits a change in the time base after a predetermined number of points after the trigger. In other words, both the fast kinetic rise, and the slower rise to F_{max} could be observed on a single trace. Because of the slow component to the rise to F_{max}, the true F_{max} level could not be determined reliably by the initial strategy, but with the later strategy, this problem was overcome.

Two ml of a thylakoid sample containing 20-25 µg Chl/ml were placed into the bottom of the glass-bottomed Dewar flask which formed the sample chamber. No uncouplers or electron acceptors were used (addition of an electron acceptor eliminates the variable fluorescence by keeping the PQ pool oxidized). The Dewar flask was stoppered to prevent evaporation. If HCO_3^- depleted thylakoids were used, the stopper was fitted with an inlet and outlet for a gentle stream of H_2O-saturated, CO_2-free air or N_2 to bathe the sample. The outflow was bubbled through water for visual detection of the strength of the flow, as too great a flow would spread the

thylakoid sample to the sides of the Dewar, and alter the transient.

Illumination was provided with a General Electric DDY 750 W, 120 V lamp, filtered with a 5 cm water filter to absorb infrared radiation, or a Kodak Carousel 4200 slide projector equipped with a 9% neutral density filter. The light was further filtered with Corning CS5-56 and CS4-76 blue filters. The fluorescence was filtered with a Corning CS2-61 red filter before entering the Bausch and Lomb monochromator. The slit widths on the monochromator were set to 4 mm; this provided a band pass of 13.2 nm. Figs. 2.5 and 2.6 show the effect of light intensity on the fluorescence yield. The light intensity chosen (marked by an arrow in Fig. 2.5) is clearly saturating. At still higher light intensities, the fluorescence yield appears to decline (Fig. 2.5). This is probably an artifact due to the shutter opening: at high light intensities, the O to I rise is very fast in relation to the shutter, leading to error in the determination of F_o. The light intensity chosen is below the point at which this error becomes significant. Other problems of very high light intensity are apparent in Fig. 2.7, which shows the fluorescence induction at the maximum light intensity attainable with our equipment, and at the light intensity normally used (9% of maximum). The large 120 Hz noise, which is due to the heating and cooling of the lamp filament, is more apparent at high intensity and is more obscuring of the signal. Furthermore, because the overall transient is faster (i.e. there are higher frequency components to the signal), less filtering of this noise can be done without altering the transient signal. Also, the O to I and I to D transitions are obscured. The 120 Hz noise could be filtered out during amplification of the signal. However, an RC time constant above 0.5 ms distorts the signal appreciably in the early stages of the induction (higher frequency components of the

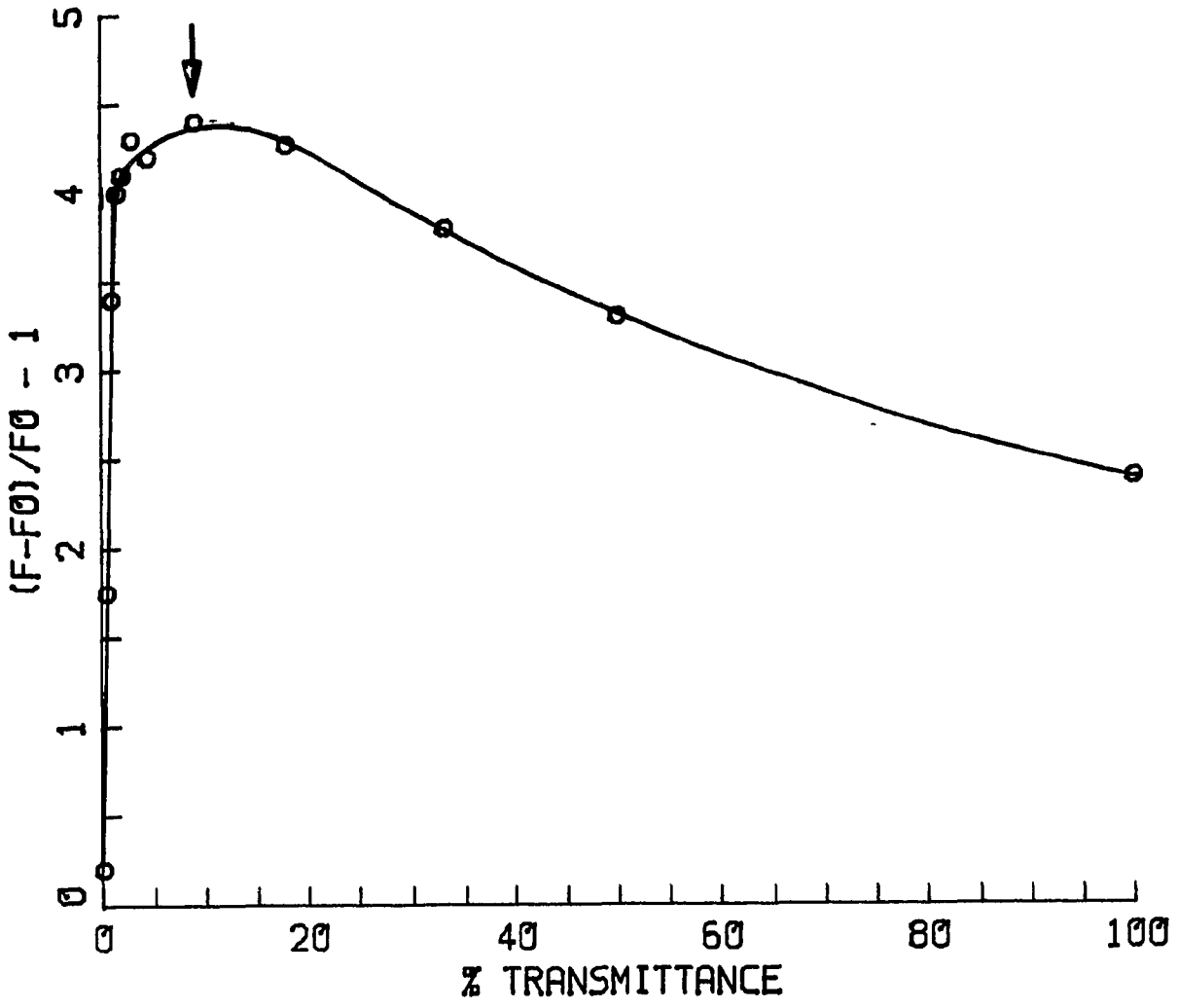


Figure 2.5. The variable fluorescence of spinach thylakoids as a function of light intensity. The light intensity was varied by placing neutral density filters in front of the projection beam. The percent transmittance of the filter is plotted on the X-axis. 100% transmittance corresponds to a light intensity of 2.9×10^3 W m^{-2}. The arrow indicates the light intensity routinely used for measurements of Chl _a_ fluorescence transients. The [Chl] was 24 µg/ml.

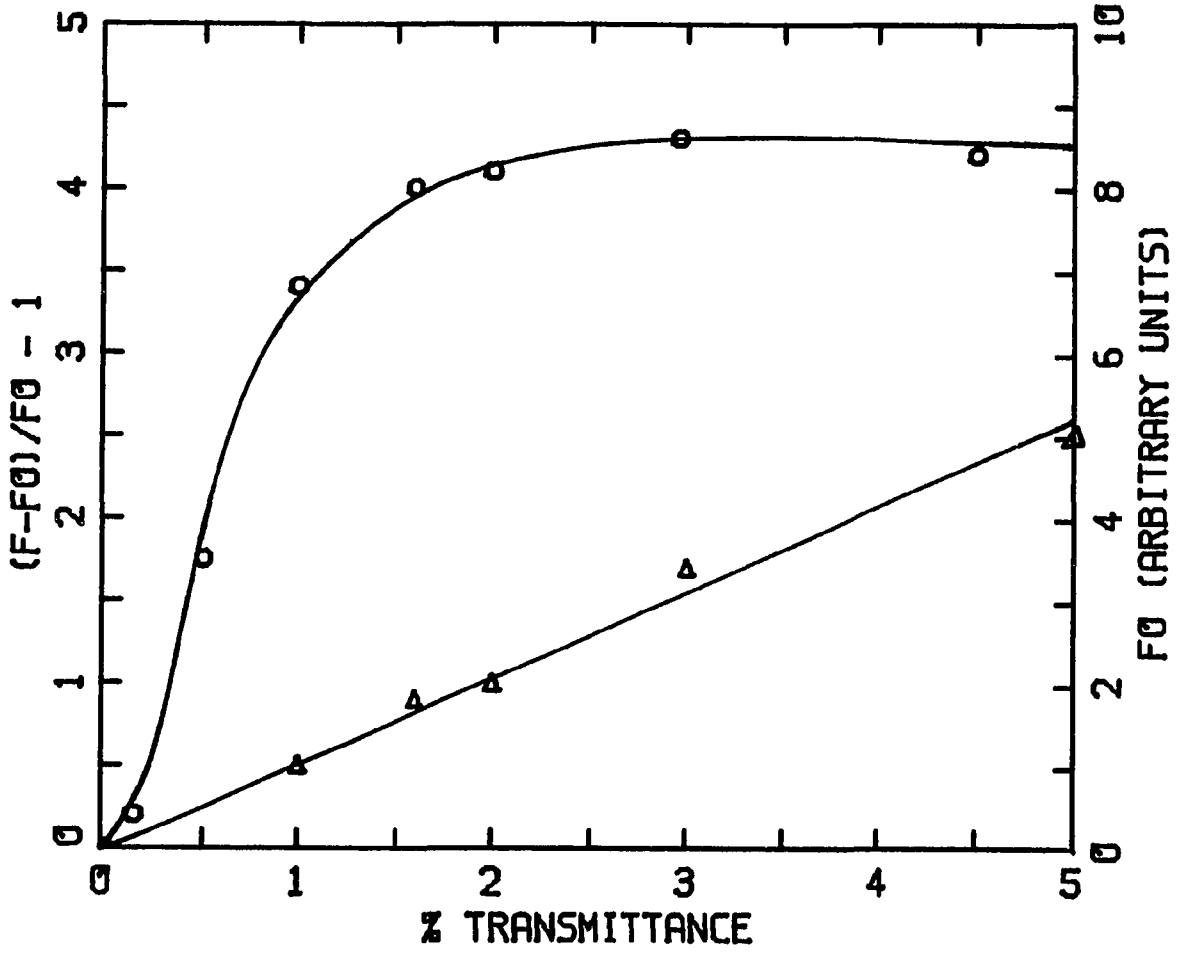


Figure 2.6. The yield of variable fluorescence (circles) and of initial fluorescence (triangles) in spinach thylakoids, as a function of light intensity. Details are the same as in Fig. 2.5.

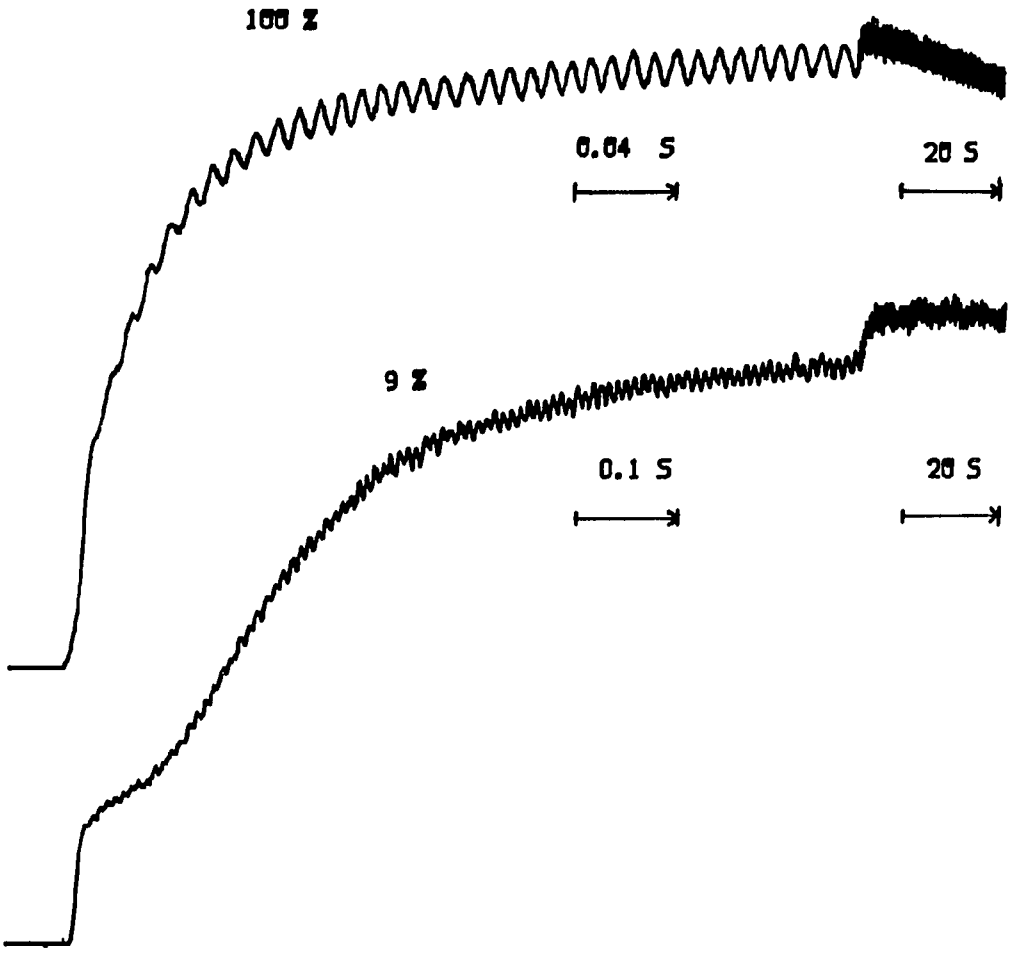


Figure 2.7. The Chl a fluorescence transient, measured at 685 nm, of a single sample of spinach thylakoids at two different saturating light intensities. The [Chl] was 24 µg/ml. Upper curve: the light intensity was 2.9 x 10^3 W m^{-2}, the intensity of the exciting light with no neutral density filter. Lower curve: the intensity of the exciting light was reduced with a 9% neutral density filter.

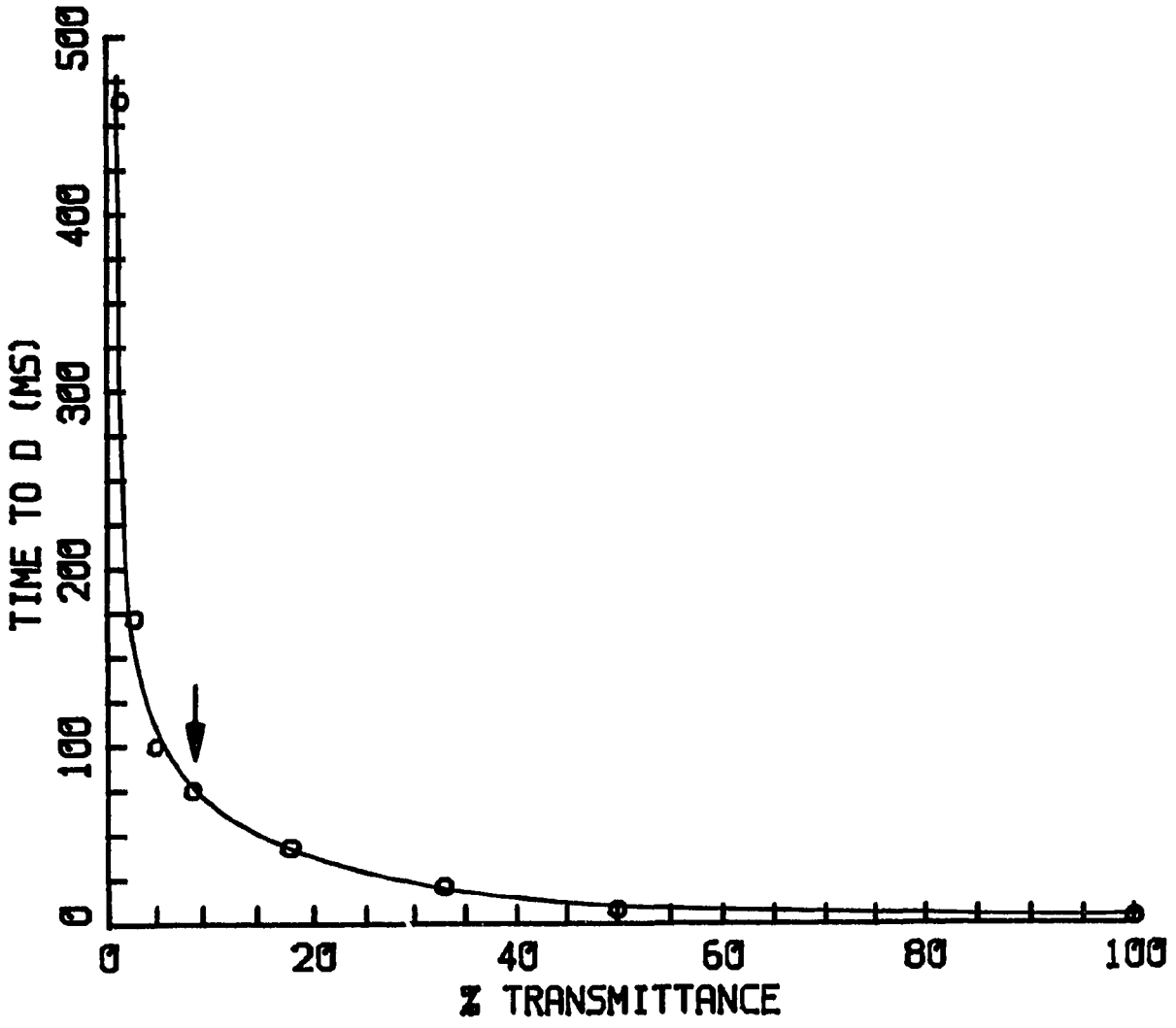


Figure 2.8. The time required to reach the D fluorescence level after the onset of illumination of spinach thylakoids, as a function of the exciting light intensity. The [Chl] was 24 μg/ml. Plotted on the X-axis is the percent transmittance of the neutral density filter placed before the exciting beam. 100% corresponds to a light intensity of 2.9×10^3 W m^{-2}. The arrow indicates the light intensity routinely used for measurements of Chl a fluorescence transients.

signal exist where changes are more rapid), and particularly obscures F_o. Therefore, only partial filtering of the signal was performed. Fig. 2.8 shows the time required to reach D, as a function of the light intensity, with the arrow indicating the light intensity ordinarily used.

F. Emission Spectra

Emission spectra were measured on the same apparatus as the fluorescence induction curves, described above. A motor moved the grating of the monochromator at a predetermined rate, and a real-time clock, programmed to tick in synchrony with this motor, was used to determine the sampling rate of the Biomation waveform recorder. Program control was essentially the same as for fluorescence induction curves, except for the use of the real-time clock, and some other minor changes described in Appendix I. The slit width of the monochromator was set to 1 mm (the bandpass was 3.3 nm), for maximum resolution, which is particularly important for separating the F_{685} and F_{695} peaks in the low-temperature spectra. Because of the more gradual changes in the spectrum, as compared to the fast kinetic rise of the fluorescence induction curve, a greater degree of noise filtering could be used; an RC filter with time constant up to 10 ms could be used without noticeable distortion of the signal. Also, since the shutter opening time is not important in determing the spectra, the shutter aperture was full open, which helped to compensate for the decreased signal size due to the more narrow slit widths.

For low-temperature spectra, 2 layers of cheesecloth, held down by a Teflon ring, were placed in the bottom of the Dewar flask before introduction of the thylakoids. Liquid N_2 could then be poured on top of the sample without the thylakoids getting pushed too far to the edges of the Dewar. There would always be some movement of the thylakoids, however,

with the result that two different experiments would have an added variable of an undetermined sample thickness. When it was desirable to be able to directly compare spectra from two different experiments, an internal standard of 5 uM fluorescein was included with the sample. The fluorescein has an emission peak at 540 nm. None of the fluorescein emission overlaps with the thylakoid emission spectrum, which begins with a sharp peak at 685 nm. Thus, normalization of the curves with respect to the fluorescein peak essentially corrects for any variation due to differences in sample thickness. Under these conditions, the lamp was filtered with Corning CS7-59 and CS4-76 blue filters, and the emission was filtered with a Corning CS3-69 yellow filter.

G. Fluorescence Decay

The decay of Chl fluorescence after an actinic flash gives information about the rate of electron flow out of Q_A^-. Measurements were made using the protocol and equipment developed by Eaton-Rye (Eaton-Rye, 1987).

H. Computer Programs and Analysis

Several programs were developed by the author to operate with the fluorimeter used for fluorescence induction measurements and for fluorescence spectra, and for kinetic analysis and plotting of other types of data. These programs are described in detail in Appendix I. The programs are designed to run on a Digital Equipment Corporation LSI 11 minicomputer, running under the RT11 operating system. Required hardware is a Data Translation digital-to-analog point plotter (model DT2771), a Data Translation real-time clock (model DT2769), a Biomation waveform recorder (model 805), a Houston Hi-plot series digital plotter, and a Hazeltine video terminal (model 1420; certain cursor functions are utilized

by the programs; other terminals could be used with only slight program modification). Two oscilloscopes are also required, of any design. The programs are written in FORTRAN, except for the subroutines which transfer data from the Biomation recorder to the computer, or which display data on the oscilloscope. These routines are written in machine language. Great flexibility is provided by the programs for storage, display, manipulation and plotting of data. The programs were used extensively for the work presented in this thesis.

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CHAPTER 3

THE ACTIVE SPECIES

A. Summary

Evidence is presented that the bicarbonate ion (HCO_3^-), not CO_2, H_2CO_3, or CO_3^{2-}, is the species that stimulates electron transport in photosystem II from spinach (Spinacia oleracea). Advantage was taken of the pH dependence of the ratio of HCO_3^- to CO_2 at equilibrium in order to vary effectively the concentration of one species while holding the other constant. The Hill reaction was stimulated in direct proportion with the equilibrium HCO_3^- concentration, but it was independent of the equilibrium CO_2 concentration. The other two carbonic species, H_2CO_3 and CO_3^{2-}, are also shown to have no direct involvement. It is suggested that HCO_3^- is the species which binds to the effector site.

B. Introduction

Originally, the requirement for CO_2 in the Hill reaction was thought to indicate an involvement of CO_2 in the O_2 evolving mechanism (Warburg et al., 1959; see also Stemler and Govindjee, 1973). Warburg and coworkers (1959) developed an elaborate scheme to show how a phosphorylated peroxide of carbonic acid (hydrated CO_2) could be the precursor to O_2 evolution. This scheme assumed that CO_2 or H_2CO_3 was the species required.

Good (1963) was the first to examine the effect of various anions on the bicarbonate (HCO_3^-) dependence, and found that small monovalent anions increased the dependence of the chloroplasts on HCO_3^-. Particularly effective were formate (HCO_2^-) and acetate ($CH_3CO_2^-$), which suggested to Good that the HCO_3^- ion is the important substance, not CO_2. Stemler and Govindjee (1973) took advantage of this suggestion to obtain the first

method for reproducibly obtaining what was then considered to be a large (4-5 fold) HCO_3^- dependence of ferricyanide (FeCy) or 2,6-dichlorophenol-indophenol (DCPIP) reduction by isolated chloroplasts. Their treatment consisted of low pH to favor the conversion of HCO_3^- to CO_2, and high salt (250 mM NaCl, 40 mM Na acetate) to compete with HCO_3^- for its binding site. Their maximum restored rates, however, were still largely inhibited with respect to non-treated chloroplasts, an effect which today can be understood as a consequence of irreversible damage by the low pH (see e.g. Vermaas and Govindjee, 1982).

A subsaturating [HCO_3^-] showed a larger stimulation of the Hill reaction at pH 6.8 than it did at pH 5.8, supporting the suggestion that HCO_3^- is the active species (Stemler and Govindjee, 1973). This experiment was not conclusive, however, since it did not rule out the possibility of a pH dependence on the binding affinity of the active site. The authors favored the conclusion that HCO_3^- is the active species, however, by another argument: They showed that HCO_3^- stimulation of the Hill reaction only occurred when the chloroplasts were incubated with HCO_3^- in the dark. No stimulation occurred while the chloroplasts were illuminated, though a subsequent dark period would restore the activity. It was suggested that HCO_3^- is released in the light at a rate that corresponds with its binding. Since CO_2 is uncharged and non-polar, it would not be expected to bind other than by covalent attachment, while the suggested exchange is more consistent with an ionic binding. Thus, HCO_3^- as the active species was thought to be more likely. This is a weak argument, however, since CO_2 can react with an amino group on a protein to form a carbamate complex which, because of its relative instability, could decompose readily to permit such an exchange. Such a carbamate formation has been demonstrated for the

regulation of ribulose-1,5-bisphosphate carboxylase by CO_2 (Lorimer et al., 1976).

The pH profile of the HCO_3^- dependence shows an optimum around pH 6.5 (Khanna et al., 1977; Vermaas and Van Rensen, 1981). While the measurements of Stemler and Govindjee (1973) are confirmed by the pH profile, the drop-off of the dependence as the pH is increased above 6.5 argues against HCO_3^- as the sole active species, since $[HCO_3^-]$ would be expected to increase with increasing pH up to around pH 8.4. Again, this argument ignores any possible pH effects on the binding environment. However, because of the close proximity of the pH optimum to the pKa of HCO_3^-/CO_2 (pKa = 6.4), it has been suggested that both CO_2 and HCO_3^- are required (Vermaas and Van Rensen, 1981).

Evidence that CO_2 is involved was obtained by Sarojini and Govindjee (1981a, 1981b) by measuring the lag time between the addition of CO_2 or HCO_3^- and the onset of O_2 evolution. At a low assay temperature (5 C) to slow the interconversion of CO_2 and HCO_3^-, and an alkaline pH of 7.3 to give a large ratio of $[HCO_3^-]$ to $[CO_2]$, the lag time was considerably shorter when CO_2 was added, compared to HCO_3^-. When carbonic anhydrase was present to accelerate the interconversion of CO_2 and HCO_3^-, the lag times became nearly identical for either species added. These results were interpreted to mean that either CO_2 was the species that was bound, or that CO_2 was required for diffusion to the active site.

Stemler (1980) concluded that CO_2, not HCO_3^-, is the binding species. In non-depleted chloroplasts inhibited by 100 mM HCO_2^-, the addition of 50 mM HCO_3^- caused a further inhibition of O_2 evolution at pH 8.0, whereas HCO_3^- partially restored the activity at pH 7.3. Because of the much larger $[CO_2]$ at the lower pH, it was concluded that CO_2 is the binding

species and that HCO_3^-, like HCO_2^-, is inhibitory. However, these measurements, like the earlier ones at pH 5.8 and 6.8 (Stemler and Govindjee, 1973), are not sufficient to determine the active species, since they ignore possible pH effects on the binding environment. While the earlier study based its conclusion on two points on the ascending side of the pH profile, this latter conclusion is based on two points on the descending portion of the pH profile, and suffers from the same limitations. The inhibition by HCO_3^- at pH 8.0 is interesting, but does not rule out HCO_3^- as the species which stimulates electron transport at lower pH, since other examples are known of activators that act as inhibitors under certain conditions (see e.g. Segel, 1975, pp 383-385). Furthermore, it is possible that the observation is artifactual: the pH profile of O_2 evolution drops off rapidly in the region of pH 8. Unless carefully controlled, addition of HCO_3^- is likely to shift the pH somewhat toward more alkaline values, and even a small shift will have a large effect on electron transport. Thus, the apparent inhibition by HCO_3^- could be a simple pH effect. Nevertheless, it is interesting to note that the stimulation of phosphorylation by HCO_3^- or CO_2 (Punnett and Iyer, 1964) becomes an inhibition at pH above 8.

Stemler (1980) observed that the rate of ^{14}C labelling of HCO_3^- depleted chloroplasts by $H^{14}CO_3^-$ decreases with increasing pH over the pH range of 6.0 to 7.8, provided that at least a 5 min incubation period is given at the pH before the $H^{14}CO_3^-$ is added. When the incubation was omitted, the rate of ^{14}C labelling was pH independent for at least two minutes. These observations led Stemler to conclude that not only is CO_2 the binding species, but that the binding occurs on the inside surface of the thylakoid membrane (i.e. at the O_2 evolving locus), as an incubation is

necessary to allow the internal pH to equilibrate after a pH jump. However, the rate of ^{14}C labelling under these experimental conditions is greater at pH 6.0 than it is at pH 6.8, which is contrary to the pH dependence of the HCO_3^- effect on the Hill reaction (Khanna et al., 1977; Vermaas and Van Rensen, 1981); therefore, another explanation should be sought that is consistent with both sets of data. An alternative explanation for these observations is presented in Chapter 7.

Because of the ambiguity concerning the species that is active in the HCO_3^- dependence, a study was undertaken to examine the stimulation of the Hill reaction by HCO_3^- at various pHs, and to take advantage of the pH dependence of $[HCO_3^-]/[CO_2]$ to effectively hold the concentration of one species constant while varying the concentration of the other. Evidence is presented that HCO_3^-, without any direct involvement by CO_2, H_2CO_3, or CO_3^{2-}, is responsible for the HCO_3^- dependence (see also Blubaugh and Govindjee, 1986).

C. Materials and Methods

Thylakoids were obtained from garden-grown spinach (Spinacia oleracea) as described in Chapter 2, except that the thylakoids were used fresh. Depletion of the membranes of HCO_3^- was performed essentially as described in Chapter 2. CO_2-free NaOH was used for the pH adjustment of solutions. A 50% NaOH solution (w/w) was sealed under N_2 and allowed to stand overnight. The insoluble Na_2CO_3 was pelleted by centrifugation, and the NaOH solution decanted under N_2.

The depletion and reaction media were prepared to final volume, acidified to a pH between 4 and 5, and bubbled with N_2 for 3-5 minutes before pH adjustment. The pH adjustment was done immediately before use, using CO_2-free NaOH while the solution was still bubbling with N_2.

After depleting thylakoids containing 2.5 mg chlorophyll (Chl) of HCO_3^-, the membranes were resuspended, under N_2, in 20 ml of reaction medium (50 mM phosphate, 100 mM NaCl, 5 mM $NaHCO_2$, 5 mM $MgCl_2$, pH 6.9). The suspended thylakoids were then distributed in 5 ml aliquots among four screw-top test tubes. While swirling the samples under N_2, a calculated volume of the same reaction medium at pH 5.2 was added to each test tube to bring the sample to the desired pH. Thus, HCO_3^- depleted thylakoids at four separate pHs were obtained, all having identical [Chl], and having gone through the same isolation and HCO_3^- depletion treatments. The measured [Chl] was 12 µg/ml in each tube, and the measured pHs were 6.31, 6.54, 6.67, and 6.87. For determination of the [Chl], 1.0 ml of the thylakoid suspension was added to 4.0 ml acetone, to yield a 1:5 dilution in 80% acetone. The [Chl] was then determined optically by the method of MacKinney (1941). The samples were kept on ice, under N_2, throughout the experiment.

A 100 mM stock solution of $NaHCO_3$ was prepared immediately before use. No effort was made to remove CO_2 from the H_2O before making the solution. The concentration of CO_2 dissolved in H_2O at room temperature and normal atmospheric pressure of CO_2, calculated from Henry's law (see Appendix II, Part A), is approximately 11 µM. At a typical pH for H_2O of 6.0 to 6.2, there is an additional 5 to 7 µM of other dissolved carbonic species in equilibrium with the CO_2 (see Appendix II, Part B). The error in the stock solution due to previously dissolved CO_2 and HCO_3^- was on the order of 0.02%.

Measurements of electron transport rates by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) were done as described in Chapter 2. A small gas space of approximately 150 µl was left in the cuvette. Escape

of CO_2 gas into this space after HCO_3^- addition introduces a small error, which was calculated to be less than 2% in the worst case (see Appendix II, Part C). The HCO_3^- was added after 1 min of dark adaptation, and the mixture was allowed to equilibrate for exactly 3 min before measuring the Hill reaction. At room temperature, the equilibration between carbonic species in aqueous solution is complete within 1 min (Cooper et al., 1968), but time must also be allowed for diffusion into the membrane and re-equilibration at the binding site. A time course of the Hill activity after the addition of a half-saturating [HCO_3^-] at pH 6.9 (Fig. 3.1) indicates that equilibrium is reached in 2 to 2.5 min. This is in close agreement with what was reported by Snel and Van Rensen (1983).

D. Results and Discussion

At equilibrium, the ratio of CO_2 to HCO_3^- in solution is dependent upon the pH, according to the reaction:

$$CO_2 + H_2O \xleftrightarrow{K_1} H_2CO_3 \xleftrightarrow{K_2} H^+ + HCO_3^- \xleftrightarrow{K_3} 2\ H^+ + CO_3^{2-} \qquad (3.1),$$

where K_1, K_2, and K_3 are equilibrium constants with the following values (Knoche, 1980): $K_1 = (1.4 \pm 0.2) \times 10^{-3}$; $K_2 = (3.2 \pm 0.4) \times 10^{-4}$ M; and $K_3 = 4.70 \times 10^{-11}$ M. CO_2 in Eqn. 3.1 refers to dissolved, not gaseous, CO_2. Thus,

$$K_1 = \frac{[H_2CO_3]}{[CO_2]}; \quad K_2 = \frac{[H^+][HCO_3^-]}{[H_2CO_3]}; \quad K_3 = \frac{[H^+][CO_3^{2-}]}{[HCO_3^-]} \qquad (3.2).$$

At alkaline pH, CO_2 is also in equilibrium with HCO_3^- through the reaction:

$$CO_2 + OH^- \longleftrightarrow HCO_3^- \qquad (3.3).$$

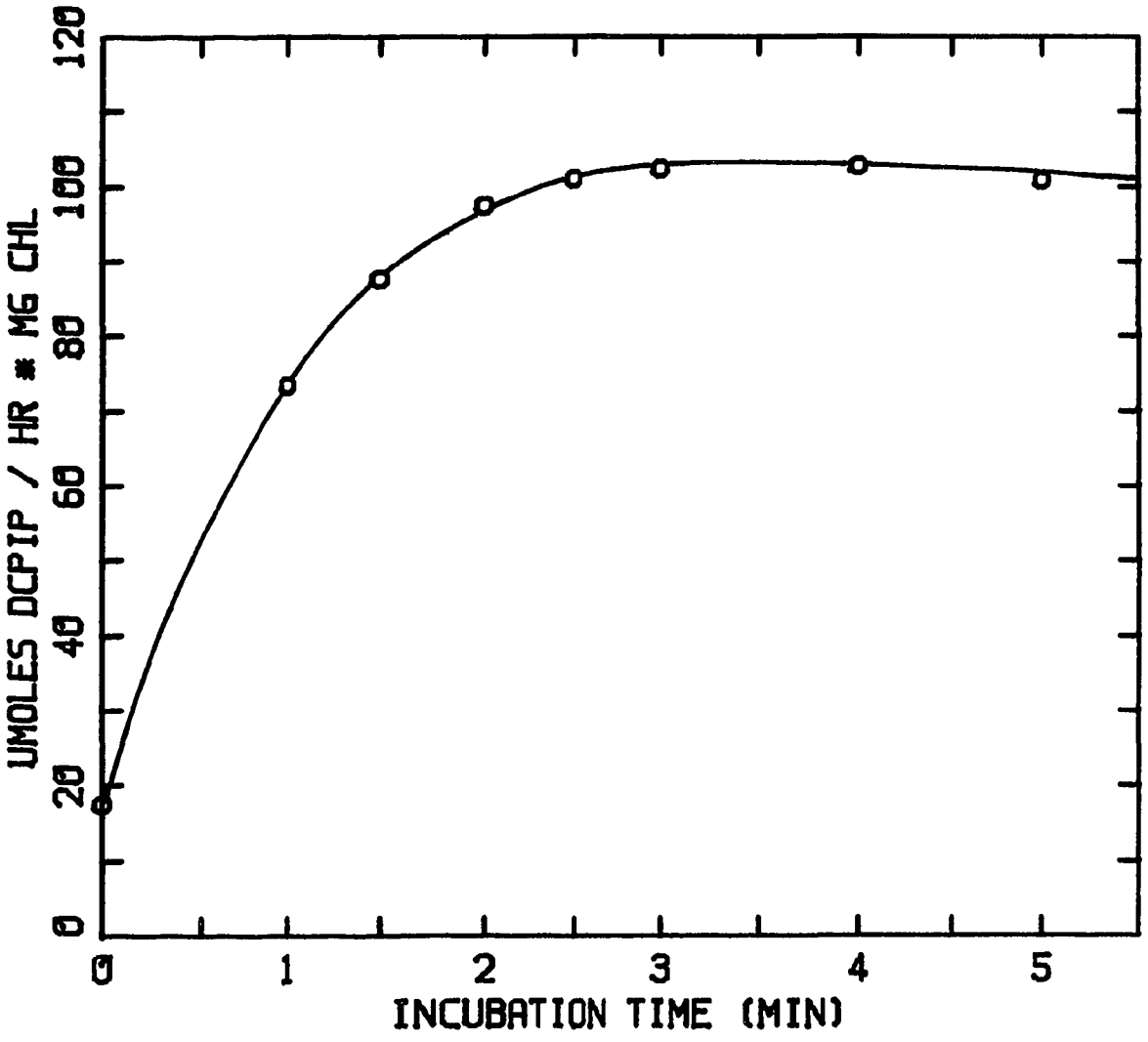

Figure 3.1. A time course for the restoration of the Hill activity after the addition of a half-saturating [HCO_3^-] to HCO_3^- depleted spinach thylakoids. The reaction medium contained 50 mM Na phosphate, pH 6.87, 100 mM NaCl, 5 mM $NaHCO_2$, and 5 mM $MgCl_2$. 0.03 mM HCO_3^- was added in the dark at time = 0, and the rate of DCPIP reduction was measured at the times indicated.

However, the contribution from Eqn. 3.3 is negligible at the pHs used in this experiment (Asada, 1982) and can be ignored. From these equations, the equilibrium (eq) concentrations of CO_2 and HCO_3^- can be calculated if the pH and the initial (i) total concentration of carbonic species are known (the derivation is given in Appendix II, Part B):

$$[HCO_3^-]_{eq} = \frac{[HCO_3^-]_i}{\frac{[H^+]}{K_1K_2} + \frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]}} \qquad (3.4)$$

$$[CO_2]_{eq} = \frac{[H^+]}{K_1K_2}[HCO_3^-] \qquad (3.5).$$

Fig. 3.2 shows the rate of DCPIP reduction, expressed as a percentage of the fully restored rate, by HCO_3^- depleted thylakoids as a function of $[CO_2]_{eq}$. It is apparent that $[CO_2]$, at which the restored rate is half maximal, is dependent on the pH. On the other hand, when the same set of data is plotted against $[HCO_3^-]_{eq}$ (Fig. 3.3), there is no apparent pH dependence; although the ratio of CO_2 to HCO_3^- at equilibrium varies nearly 4-fold over the pH range of the experiment, each curve falls on top of the others. From Eqn. 3.5 it is obvious that the ratio of CO_2 to HCO_3^- is constant at any pH, but changes proportionately with any change in $[H^+]$. The lack of pH dependence in Fig. 3.3 means that $[CO_2]$ has no apparent effect on the degree of restoration, whereas the pH dependence in Fig. 3.2 indicates that $[HCO_3^-]$ is important. The inset in Fig. 3.2 shows that the Hill activity increases with increasing $[HCO_3^-]_{eq}$, with $[CO_2]_{eq}$ constant at 0.1 mM. From the inset in Fig. 3.3, which shows the effect of $[CO_2]_{eq}$ on the Hill activity, with $[HCO_3^-]_{eq}$ constant at 0.2 mM, it is clear that the stimulatory effect of HCO_3^- is independent of the CO_2 level.

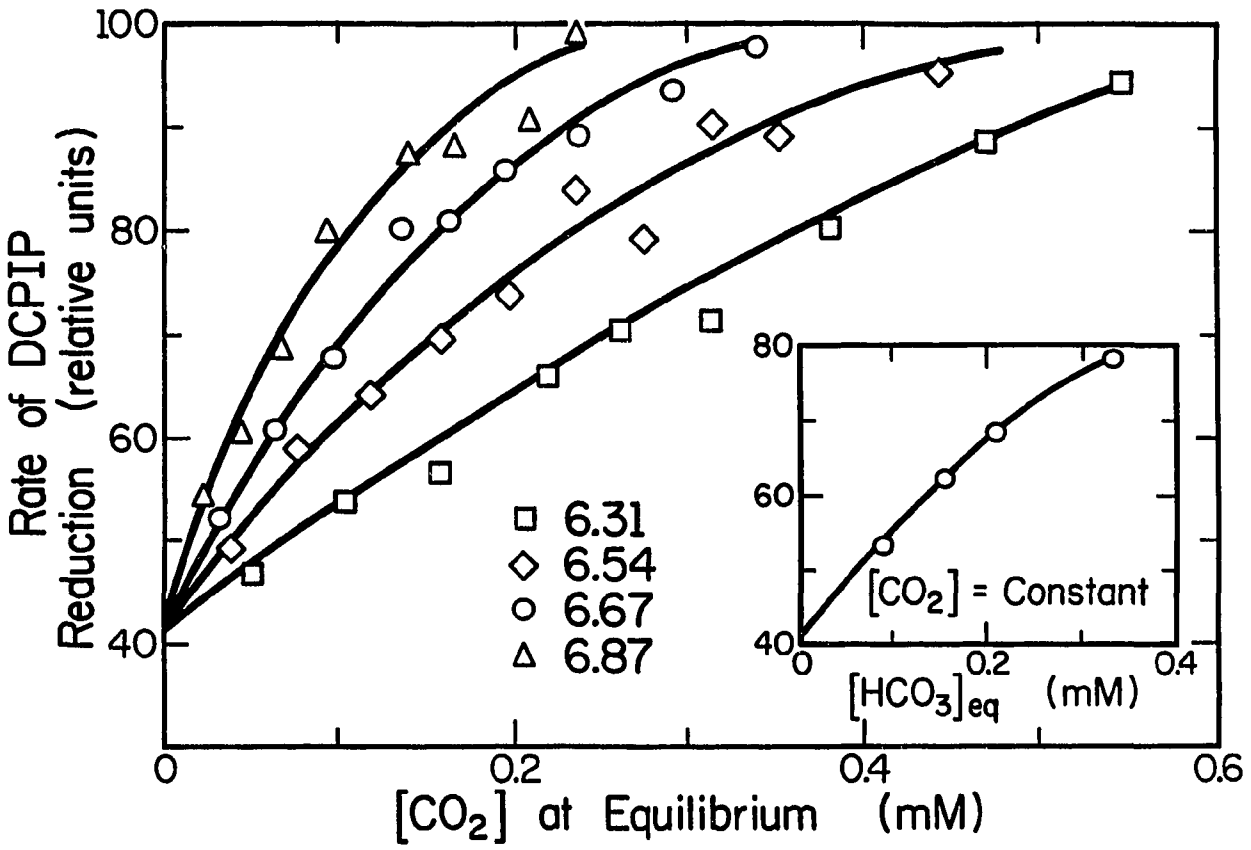

Figure 3.2. The rate of DCPIP reduction in HCO_3^- depleted thylakoids, expressed as a percentage of the control rate, as a function of the equilibrium CO_2 concentration. The reduction of DCPIP was calculated from the decrease in absorbance at 600 nm. Illumination began 3 min after the addition of $NaHCO_3$. The control rate was determined separately for each curve by adding a saturating amount of HCO_3^- (2.5 mM) to the HCO_3^- depleted samples. The control rates, in μmoles DCPIP reduced per mg Chl per hr, for each pH, were: pH 6.31, 209 (squares); pH 6.54, 212 (diamonds); pH 6.67, 191 (circles); and pH 6.87, 192 (triangles). Inset: the effect of the equilibrium HCO_3^- concentration on the Hill activity, with the CO_2 concentration held constant at 0.1 mM.

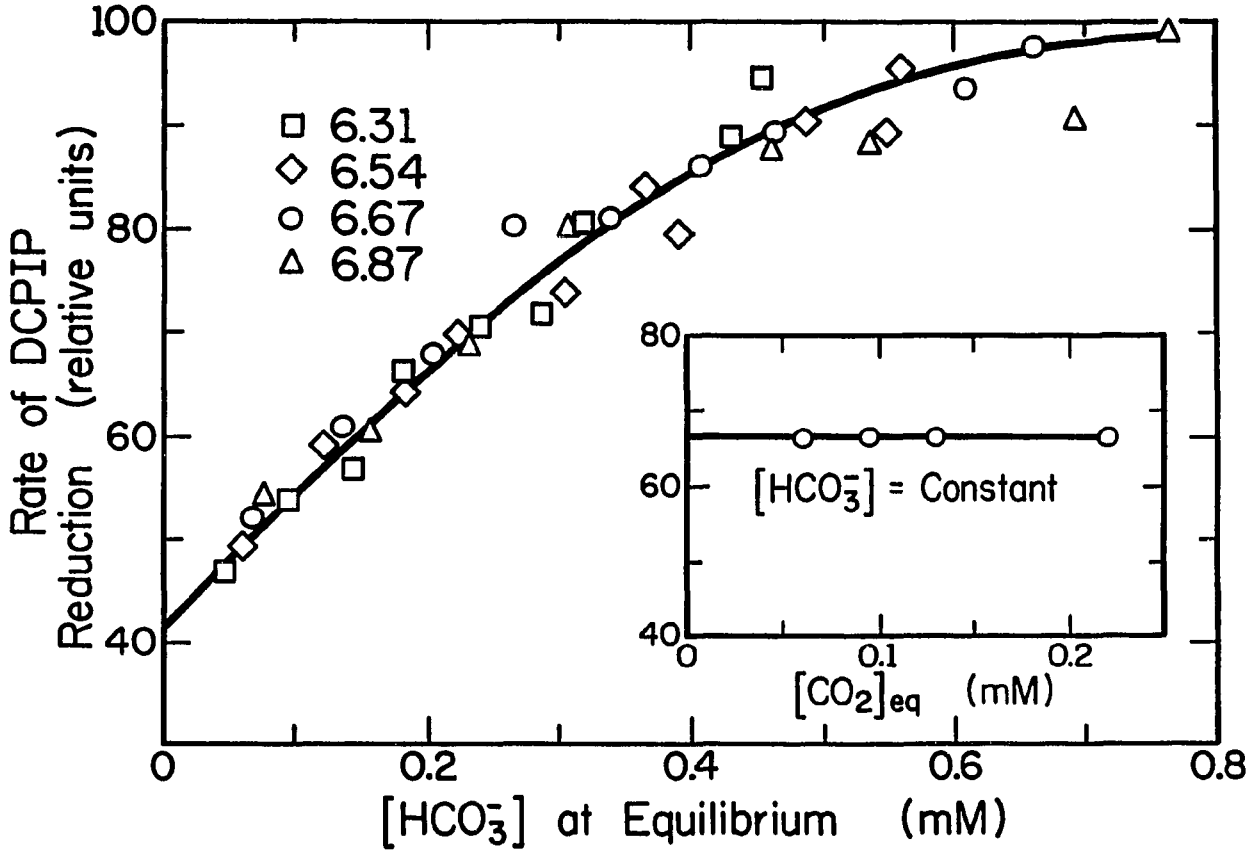

Figure 3.3. The rate of DCPIP reduction in HCO_3^- depleted thylakoids, expressed as a percentage of the control rate, as a function of the equilibrium HCO_3^- concentration. The symbols and protocol are the same as in Fig. 3.2. Inset: the effect of the equilibrium CO_2 concentration on the Hill activity, with the HCO_3^- concentration held constant at 0.2 mM.

The other two carbonic species, H_2CO_3 and CO_3^{2-}, present at extremely low concentrations, can be ruled out as having any involvement. The H_2CO_3 to CO_2 ratio at equilibrium is equal to K_1 of Eqn. 3.1 and is independent of pH. Since $[H_2CO_3]$ is directly proportional to $[CO_2]$ at all pH values, identical arguments apply for H_2CO_3 as were made for CO_2. Thus, H_2CO_3 cannot be the required species. The ratio of CO_3^{2-} to HCO_3^- at equilibrium, on the other hand, is equal to $K_3/[H^+]$. Since this ratio is inversely proportional to $[H^+]$, the data in Fig. 3.3 would be expected to show a pH dependence if CO_3^{2-} were involved in stimulating the Hill reaction. As was the case with CO_2, the lack of such pH dependence suggests that CO_3^{2-} is not involved.

The conclusion that CO_2 (or to a lesser extent, H_2CO_3) may be required for diffusion to the active site (Sarojini and Govindjee, 1981a, 1981b) is not disputed by the data presented here, since these measurements were made under equilibrium conditions and do not reflect the kinetics of HCO_3^- binding. While $[HCO_3^-]_{eq}$ is shown here to be a critical factor, $[CO_2]$ could be an important kinetic consideration if the binding site is buried beneath a hydrophobic domain.

Recently, the dissociation constant K_d for HCO_3^- has been determined to be 80 μM (Stemler and Murphy, 1983; Snel and Van Rensen, 1984; Jursinic and Stemler, 1986). Since $[CO_2]$ in photosynthesizing chloroplasts is estimated to be only 5 μM (Hesketh et al., 1982), it was suggested that under normal conditions all of the binding sites are empty, and there may be no real role for HCO_3^- in vivo (Stemler and Murphy, 1983). However, since HCO_3^-, not CO_2, is the activating species, there is no good reason to assume that the binding sites are empty. For example, if, in the vicinity of the binding site, the pH is 8 (as it is in the stroma), then $[HCO_3^-]$ in

equilibrium with 5 μM CO_2 is 220 μM (from Eqn. 3.5), well above the binding constant. Also, the determination of K_d has been made using the [HCO_3^-] that was added to the thylakoids, rather than the equilibrium [HCO_3^-]. Since the determinations were performed at pH 6.5, where nearly half of the added HCO_3^- is converted to CO_2, the K_d is too large by a factor of nearly two.

It is apparent from the data presented here, that HCO_3^-, not CO_2, H_2CO_3 or CO_3^{2-}, is the species required for PS II electron transport. This conclusion is consistent with the observed competition by anions, such as formate (HCO_2^-) and acetate ($CH_3CO_2^-$), which closely resemble HCO_3^- (Good, 1963). In fact, HCO_2^-, by itself, has been shown to inhibit electron transport in a manner similar to HCO_3^- depletion, presumably by out-competing HCO_3^- for its binding site (Robinson et al., 1984). This effect of HCO_2^- was most pronounced at lower pH values, where the [HCO_3^-] is diminished. Fig. 3.4, likewise, suggests that HCO_2^- can remove HCO_3^- from its binding site: the effect of 25 mM HCO_2^- on the Chl a fluorescence transient is identical to the effect of a partial depletion of HCO_3^- (see Stemler and Govindjee, 1974, and Chapter 1, p. 16), while the effect of 200 mM HCO_2^- is identical to the effect of a thorough HCO_3^- depletion (c.f. Fig. 2.4; see also Wydrzynski and Govindjee, 1975; Vermaas and Govindjee, 1982).

Nitrite (NO_2^-) has been shown to be as effective as formate at inhibiting PS II, apparently by competition with HCO_3^- (Stemler and Murphy, 1985; Eaton-Rye et al., 1986; see also Sinclair, 1987). The similar anion nitrate (NO_3^-), however, requires a much higher concentration to reach the same level of inhibition (Stemler and Murphy, 1985). The major difference between NO_2^- and NO_3^- is in the degree of delocalization of the negative

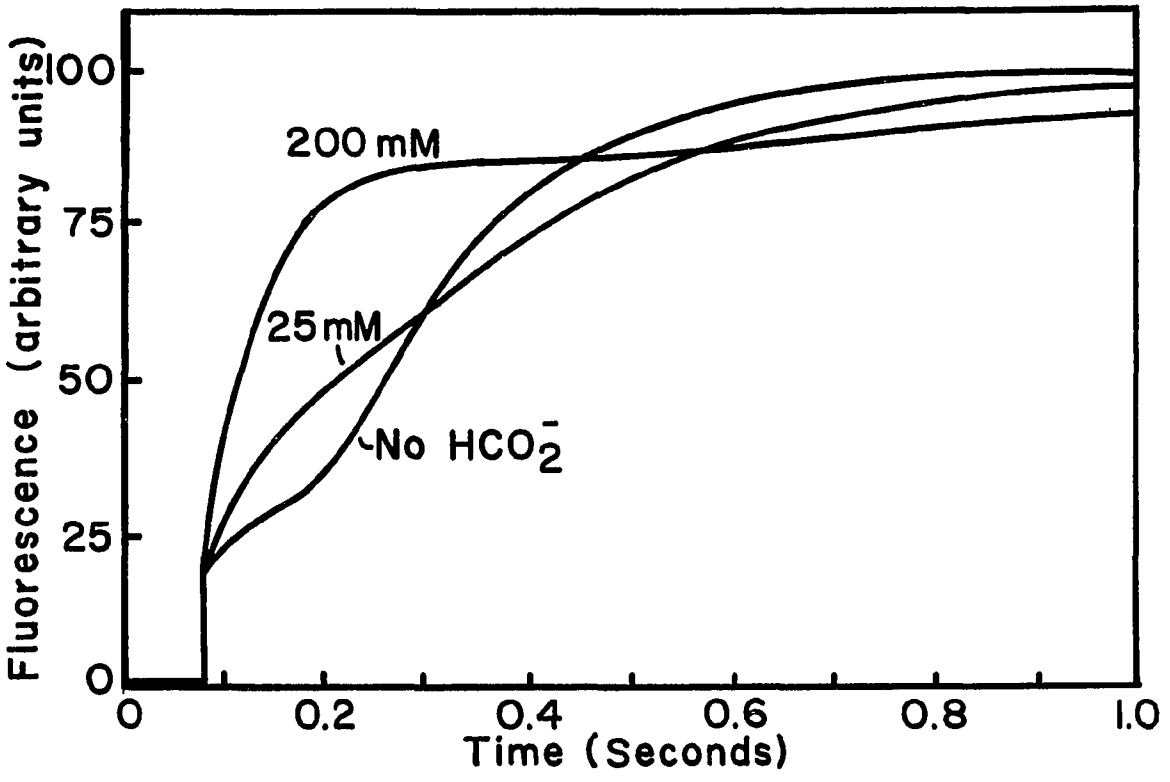

Figure 3.4. The effect of formate on the variable Chl a fluorescence transient in spinach thylakoids. Thylakoids were suspended in 50 mM Na phosphate, pH 6.4, to a final [Chl] of 25 µg/ml. The samples also contained, in order of increasing steepness of the initial fluorescence rise, no HCO_2^-, 25 mM HCO_2^- and 200 mM HCO_2^-.

charge over the molecule, as illustrated in Fig. 3.5. In this regard, NO_3^- more closely resembles CO_3^{2-}, which is shown here to be ineffective in stimulating electron transport, than it does HCO_3^-. The effective NO_2^-, on the other hand, has the same degree of charge delocalization as does HCO_3^- and HCO_2^-. Therefore, the charge density may be an important parameter affecting the affinity of an anion for the HCO_3^- binding site.

Both HCO_2^- and HCO_3^- have a carboxyl group, with the same degree of charge delocalization, but only HCO_3^- stimulates the Hill reaction. The main structural difference between the two is the presence of a hydroxyl group in HCO_3^- that is absent in HCO_2^-. It appears likely that the hydroxyl group is the functional moiety, while the carboxyl group is involved in binding. The hydroxyl group could be involved in H^+ mediation during electron transfer from Q_A to Q_B and subsequent release of PQH_2, or it may be important as a source of H bonding to effect a conformational change necessary for efficient electron transfer.

It was shown by Shipman (1981) that HCO_3^-, complexed with an arginine residue, could provide a suitable binding environment for a number of herbicides that are known to interfere with electron transfer from Q_A to Q_B. Another possibility discussed by Shipman was CO_2 complexed with a lysine residue to form a carbamate. However, the Q_B apoprotein, whose primary sequence has recently been elucidated (for a review, see Kyle, 1985) and where HCO_3^- and HCO_2^- seem to act (for a review, see Chapter 1), contains no lysine. The absence of lysine in the Q_B apoprotein is thus consistent with the conclusion that CO_2 is not the activating species. Based on hydropathy plots, a secondary structure was proposed for the Q_B apoprotein (Kyle, 1985), which had arginine-257 within the hydrophobic matrix with its positive charge uncompensated by any nearby counter charge.

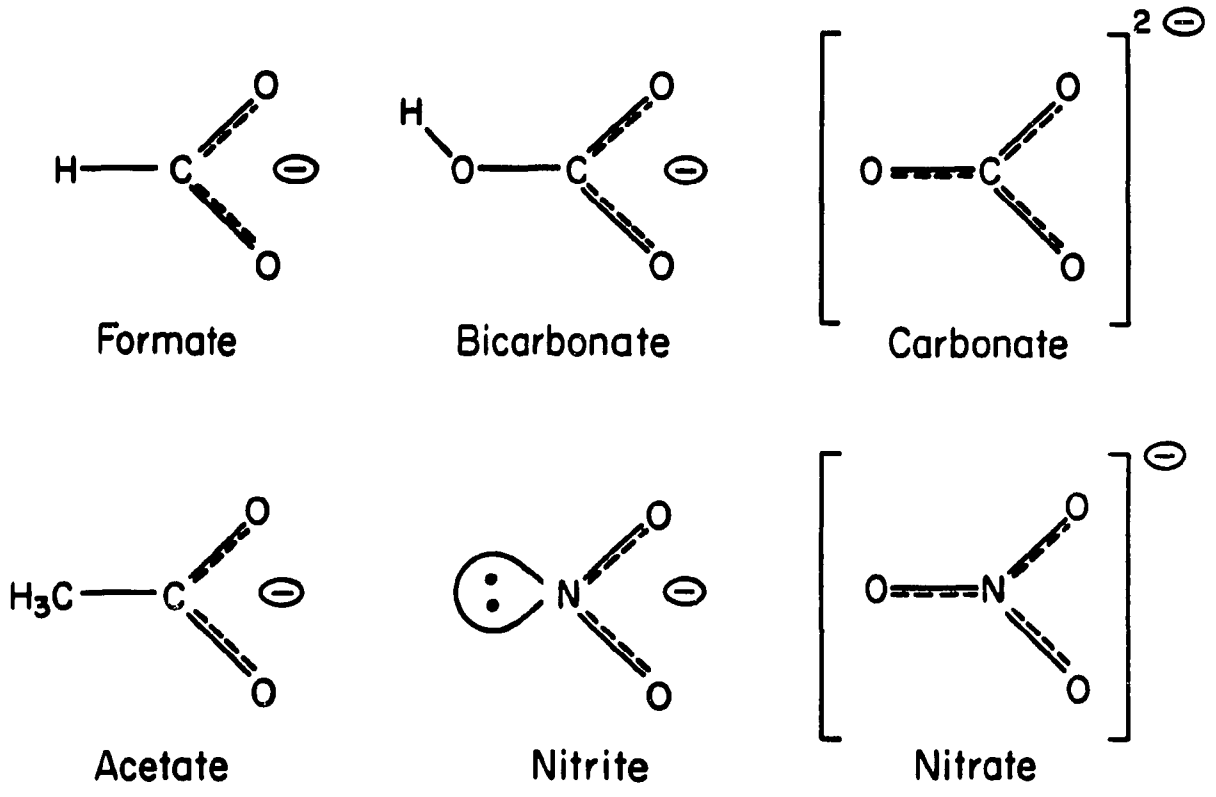

Figure 3.5. The molecular structures for several HCO_3^- analogs, showing the resonant bonds over which the charge is delocalized. Nitrate resembles carbonate, while nitrite and the others resemble bicarbonate.

This made arginine-257 appear to be a likely candidate for the binding site of HCO_3^- (e.g. Blubaugh and Govindjee, 1986). However, with the recent determination of the primary and secondary structure of the bacterial reaction center (e.g. Michel et al., 1986) and the recognition of a high degree of homology between the Q_B apoprotein of PS II and the L subunit of the bacterial reaction center (e.g. Trebst and Draber, 1986; Trebst, 1987), a more accurate estimate of the secondary structure of the Q_B apoprotein is now possible. H. Robinson (personal communication) has modeled the Q_B protein on this basis and suggests that HCO_3^- may be bound, instead, to arginine-225, as this arginine is in a more favorable location for an interaction between HCO_3^- and the bound PQ. There is also good evidence now that HCO_3^- may be bound to the non-heme Fe^{2+} in PS II (Vermaas and Rutherford, 1984; Michel and Deisenhofer, 1986). These possibilities are discussed in greater detail in Chapter 7.

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CHAPTER 4

THE FUNCTION OF HCO_3^-

A. Summary

A plastoquinone (PQ) analog that is capable of covalent attachment at the PQ binding site on the Q_B protein of photosystem II, 6-azido-5-decyl-2,3-dimethoxy-p-benzoquinone (6-azido-Q_0C_{10}), was used, in conjunction with formate incubation of spinach thylakoids, to determine whether quinone binding at the Q_B site is affected by HCO_3^- depletion. Apparently, removal of HCO_3^- from its binding site does affect the affinity of the Q_B site for the PQ analog, 6-azido-Q_0C_{10}. However, the 6-azido-Q_0C_{10} is also shown to have multiple effects that may need to be sorted out before a strict analogy with PQ can be inferred. Many of these effects are in themselves of interest in probing the workings of the quinone reactions.

B. Introduction

The photosystem II (PS II) complex of plant thylakoids is functionally connected to the cytochrome b_6/f complex (cyt b_6/f) by a mobile, intramembrane pool of plastoquinone (PQ; for reviews, see Vermaas and Govindjee, 1981; Crofts and Wraight, 1983; Whitmarsh, 1986). The secondary stable electron acceptor of PS II, Q_B, is now known to be a transiently bound PQ molecule which, after becoming doubly reduced to plastoquinol (PQH_2), exchanges with the PQ pool, as was first proposed independently by Velthuys (1981) for green plants and by Wraight (1981) for purple bacteria (ubiquinone replaces PQ in the bacteria). The PQH_2 diffuses to Cyt b_6/f, where it in turn reduces the next component of the electron transport chain, the Rieske FeS protein. The reoxidized PQ is then free to exchange again with the PQ pool. Thus, the PQ pool acts as a reservoir of "electron

shuttles" between PS II and Cyt b_6/f. At the same time, the PQ pool acts as a H^+ pump across the membrane: Since PS II reduces PQ towards the outer surface of the membrane, the H^+ ions involved in the protonation of plastoquinol must necessarily come from the stroma. Reoxidation of PQH_2 by Cyt b_6/f occurs toward the inner surface of the membrane, so the H^+ ions are deposited into the lumen. The transmembrane pH difference drives ATP formation via the coupling factor, which is an ATPase running in reverse (for an overview, see Ort, 1986). Thus, the PQ pool is at once a functional connection between two protein complexes in the electron transport chain, and a site of coupling between electron transport and photophosphorylation.

Both PQ and PQH_2 bind relatively weakly to the Q_B site, while the semiquinone, PQ^-, binds tightly. Therefore, reduction of Q_B requires two turnovers of the reaction center. The steps at which protonation of the plastoquinone occurs is not fully elucidated. Diner (1977) proposed, based on thermodynamic considerations of the equilibrium constant for dismutation of duroquinol, compared to the equilibrium constants for electron transfer through the quinones of PS II, that the protonation of Q_B should occur at the level of Q_B^{2-}. Similarly, Pulles and coworkers (1976) found from the difference absorption spectrum that Q_B^- is unprotonated, and Fowler (1977) observed a binary oscillation in H^+ uptake corresponding to the production of Q_B^{2-}. However, the oscillations observed by Fowler were very small, and were not seen at all by other workers (Hope and Moreland, 1979; Förster, et al., 1981). To account for an unprotonated Q_B^- and a lack of binary oscillation in H^+ uptake, Förster et al. (1981), proposed the protonation of a protein group to stabilize Q_B^-, as was proposed earlier for photosynthetic bacteria (Wraight, 1979). Crofts and coworkers (1984) proposed a

scheme in which a protein group near Q_A and Q_B must be protonated before Q_B^- can accept a second electron from Q_A^-. They showed that the pKa for this group appears to shift from about 6.4 to approximately 7.9 when Q_B is reduced to Q_B^-, and the oxidation of Q_A^- by Q_B^- is slowed down when this group is unprotonated. This is analogous to what occurs in photosynthetic bacteria, though the pKa's are different (Wraight, 1979).

It is tempting to assign HCO_3^- a role in this protonation, since the absence of HCO_3^- slows down the reduction of Q_B (Jursinic et al., 1976; Siggel et al., 1977), and the pKa of CO_2/HCO_3^- is about 6.4. The pKa of HCO_3^-/CO_3^{2-} is 10.2 in aqueous solution, though a bound HCO_3^-/CO_3^{2-} would be expected to have a lower pKa, due to stabilization of the negative charge upon binding to a positively charged group. Such a speculation has been made (Vermaas, 1984). However, it is not likely that HCO_3^- is the group that is undergoing the pKa shift observed by Crofts et al. (1984). For one thing, at pH's below 6.4 the putative protein group is already protonated before Q_B^- formation, and Q_A^- to Q_B^- electron transfer is not impaired (Crofts et al., 1984). If HCO_3^- is protonated it decomposes to form CO_2, which would leave the HCO_3^- site empty, and electron transfer from Q_A^- to Q_B^- would be impaired. It is possible, however, that HCO_3^- is responsible for providing a ready H^+ to this group when its pKa shifts to 7.9. If this is the case, then HCO_3^- would be serving as a H^+ buffer, in reserve for rapid protonation of this protein group, and the protein group should have a pKa higher than the HCO_3^-/CO_3^{2-}, as the H^+ donor must be the group with the lower pKa. This would imply that at a pH intermediate between the two pKa's, Q_A^- to Q_B^- electron transfer would be limited by the arrival of a H^+ from another source, perhaps the bulk phase. At still higher pH, above the pKa of the protein group, electron transfer should be

further inhibited, due to the inability of the group to become protonated. Therefore, the rate of electron transfer from Q_A^- to Q_B^- should show two titratable groups, one due to HCO_3^- and one due to the protein group, unless the two pKa's are very close in value.

In the model presented in the earlier paragraphs, in which Q_B is an exchangeable PQ, the mode of action of herbicides at this site is readily explained as a simple competition for the Q_B binding site (Velthuys, 1981; Wraight, 1981). That this is indeed the case has been elegantly demonstrated by the use of plastoquinone analogs (Vermaas and Arntzen, 1983; Vermaas et al., 1983). All of the analogs tested competed with the binding of atrazine and ioxynil. One of the analogs, 6-azido-5-decyl-2,3-dimethoxy-p-benzoquinone (6-azido-Q_0C_{10}), also supported electron transport, presumably by replacing the native PQ in its role as an electron shuttle from PS II to Cyt b_6/f (Vermaas et al., 1983). The 6-azido-Q_0C_{10} can be attached permanently by photoaffinity labeling (Yu and Yu, 1980). Ultraviolet (UV) light activates the azido group and causes the analog to become covalently linked to its binding site. When this occurs, electron transport is completely blocked from Q_A to PQ, and the herbicides are nearly totally prevented from binding. These results demonstrate that Q_B is a bound PQ that readily exchanges with other quinone analogs.

Since HCO_3^- is required for electron transport from Q_A to PQ, the question immediately arises, what role does HCO_3^- play in the binding of PQ and the release of PQH_2? The possibility that HCO_3^- may be involved in the protonation of a protein group to stabilize PQ^-, as described in earlier paragraphs, could only be part of the function of HCO_3^-. In addition to slowing the rate of electron transfer from Q_A to Q_B, HCO_3^- depletion has been shown to completely, or nearly completely, block electron transfer

from Q_B to PQ (Govindjee et al., 1976, 1984; Siggel et al., 1977; Vermaas and Govindjee, 1982). In other words, HCO_3^- depletion impairs the exchange reactions between the Q_B site and the PQ pool. There are two, non-exclusive possibilities: (i) HCO_3^- is required for PQ to bind to the Q_B apoprotein, and/or (ii) HCO_3^- is required for PQH_2 to be released from the Q_B apoprotein. This chapter describes attempts to examine the first possibility, using the photoaffinity characteristics of 6-azido-Q_0C_{10}. It is tentatively concluded that HCO_3^- depletion impairs the binding of PQ.

C. Materials and Methods

6-azido-5-decyl-2,3-dimethoxy-p-benzoquinone (6-azido-Q_0C_{10}) and 2,3-dimethoxy-5-methyl-6{10-[4-(azido-2-nitroanilinopropionoxy)]decyl}-p-benzoquinone (Q_0C_{10}-NAPA) were the generous gifts of Dr. Chang-An Yu at the Department of Biochemistry, Oklahoma State University (for the synthesis of 6-azido-Q_0C_{10}, see Yu et al., 1982; for the synthesis of Q_0C_{10}-NAPA, see Yu and Yu, 1982). The compounds were stored in ether in a refrigerator. Immediately before use, an aliquot was evaporated under a N_2 stream and redissolved in ethanol to a concentration of 100 μM.

Thylakoids were isolated from market spinach as described in Chapter 2 and frozen in liquid N_2 until use. Rates of electron transport were measured with an oxygen electrode, using ferricyanide (FeCy) as the Hill oxidant, or spectrophotometrically by following the reduction of 2,6-dichlorophenolindophenol (DCPIP), as described in Chapter 2. Fluorescence induction curves and the decay of fluorescence after an actinic flash were also measured as described in Chapter 2.

Thylakoids were suspended at a chlorophyll concentration of 20 μg/ml in pH 6.5 buffer containing 50 mM Na phosphate, 15 mM NaCl and 5 mM $MgCl_2$. NH_2OH was added to a concentration of 10 mM, and the sample was flushed

with O_2-free air (a custom tank of N_2 with 0.03% CO_2 was used for this purpose). The reason for the NH_2OH and flushing of O_2 was to prevent the oxidation of Q_B^- by the S_2 state of the O_2-evolving complex so that, when formed, Q_B^- would be long lived (see e.g. Diner, 1977). Also prevented is the oxidation of Q_B^- by O_2 (e.g. Robinson and Crofts, 1983). For formate-treated thylakoids, the pH 6.5 buffer contained 50 mM Na phosphate, 100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Na formate. Also, instead of flushing with a N_2/CO_2 mixture, the sample was flushed with N_2 that had first passed through $CaCl_2$ and ascarite to remove CO_2.

The thylakoid samples formed a thin layer (2 mm thick) on the bottom of a 600 ml beaker, which was fitted with a stopper through which the gas passed. If 6-azido-Q_0C_{10} was to be covalently attached to the Q_B site, it was added at this point and allowed to incubate in the dark for several minutes. The sample was given a single saturating flash of 3 μs half-width through the bottom of the beaker from a General Radio model 1538A strobo-slave, filtered with a Corning CS4-76 blue filter to remove UV light. The beaker was immediately placed into liquid N_2 to freeze the sample (to prevent reoxidation and release of the bound semiquinone), the stopper removed, and the sample illuminated for 1 hr with a germicidal UV lamp (General Electric G15T8), placed 15 cm above the sample. The azido group of 6-azido-Q_0C_{10} absorbs maximally around 315 nm. The excited state resulting from absorption of a UV photon is unstable and rearranges to release N_2, leaving behind a highly reactive nitrene radical, which can form a bond with any nearby functional group. This chemistry occurs at temperatures even as low as 4 K (Wasserman et al., 1964). After the UV treatment, the samples were quickly thawed under tap water and placed on ice.

To protect the thylakoids from the short-wavelength UV, the UV lamp was covered with several layers of plastic wrap, which removes wavelengths below 300 nm. Various brands of plastic wrap differ in their absorption characteristics — it was found that plastic wraps designed for microwave use were the most effective at removing the short wavelengths while still permitting passage of the 300-330 nm region in which the azido group absorbs. The brands used in this study were Reynolds and Saran wrap (for microwave ovens), used in alternating layers.

D. Results and Discussion

Fig. 4.1 shows the fluorescence induction after a variety of treatments. The traces were all from a single sample, done in the order of the numbering in the figure, with each trace representing a subsequent treatment to the sample. Trace #1 is the control thylakoids with no treatment. The characteristic O, I, D, and P levels are labeled with respect to this trace (for their meaning, see Chapter 2). Trace #2 contains 10 mM NH_2OH, which by itself causes an acceleration of the fluorescence rise. This effect has been reported previously by Izawa, et al. (1969) who interpreted it to be due to a slower back reaction of Q_A^- with the S_2 state of the O_2-evolving system. The addition of 1 μM 6-azido-Q_0C_{10} (Trace #3) causes a quenching of the chlorophyll a (Chl a) fluorescence, as expected, due to its ability to compete with the native PQ for electrons (Vermaas et al., 1983) The fluorescence rise from D to P reflects the reduction of the electron acceptor pool, with the concomitant back-up of electrons and an increased lifetime of Q_A^-. 6-Azido-Q_0C_{10}, by increasing the size of the electron acceptor pool, extends the time necessary for this to occur. An additional quenching by 6-azido-Q_0C_{10} is expected due to non-photochemical quenching by oxidized quinones in general

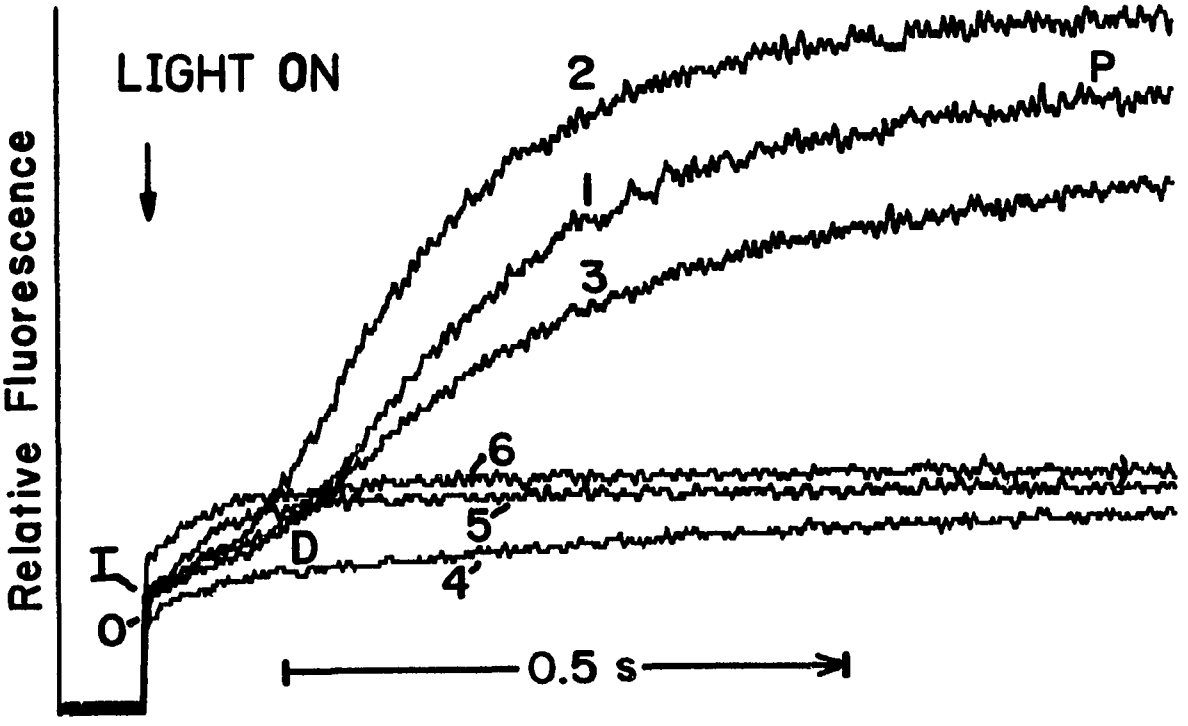


Figure 4.1. Chl a fluorescence induction curves for spinach thylakoids after various additions or treatments. Trace 1: control thylakoids in 50 mM Na phosphate, pH 6.5, 15 mM NaCl and 5 mM $MgCl_2$. The [Chl] was 20 µg/ml. Trace 2: same as 1, + 10 mM NH_2OH. Trace 3: same as 2, + 1 µM 6-azido-Q_0C_{10}. Trace 4: same as 3, but treated with UV for 15 min, as described in the text. Trace 5: same as 4, + 10 µM DCMU. Trace 6: same as 5, but with no dark adaptation. The O, I, D and P levels are marked with respect to Trace 1; their meaning is explained in Chapter 2.

(e.g. Vernotte et al., 1979). The effect of 15 min of the UV treatment (Traces #4–6) is quite dramatic. Since NH_2OH is present as an electron donor to PS II, this quenching by UV treatment is due to effects other than on the O_2 evolving mechanism. The acceptor side appears to function normally after UV treatment, as will be shown by subsequent figures, so the main damage caused by the UV treatment is probably to the reaction center itself. Trace #5 is done in the presence of 10 μM DCMU. Trace #6 is the same as Trace #5, except without a dark adaptation. The relatively large effect of dark adaptation in the presence of DCMU suggests that DCMU is not binding as tightly as usual, as some electron flow must be occurring even in its presence (since DCMU blocks electron flow after Q_A, its presence ordinarily gives a transient nearly identical to non-dark adaptation, where the electron acceptor pool is nearly completely reduced at the start of the transient). This effect on DCMU binding is expected if the 6-azido-Q_0C_{10} is covalently attached to the Q_B binding site after the UV treatment, as shown earlier for other DCMU-type herbicides (Vermaas et al., 1983). However, the percentage of centers containing covalently attached 6-azido-Q_0C_{10} must be relatively small, since some DCMU binding does occur (Trace 5 vs. Trace 4) and electron transport is occurring (Trace 4).

The electronic transition leading to the excited state of the azido group is symmetry forbidden and, therefore, occurs with very low probability (for a good description of azido chemistry, see Reiser and Wagner, 1971). Hence, a long time of UV irradiation is necessary. In subsequent experiments, the time of UV irradiation was increased from 15 min to 1 hr, and the filter to remove short-wavelength UV was increased from 8 to 20 layers of plastic wrap.

Fig. 4.2 shows the effect of 1 μM 6-azido-Q_0C_{10} on the fluorescence

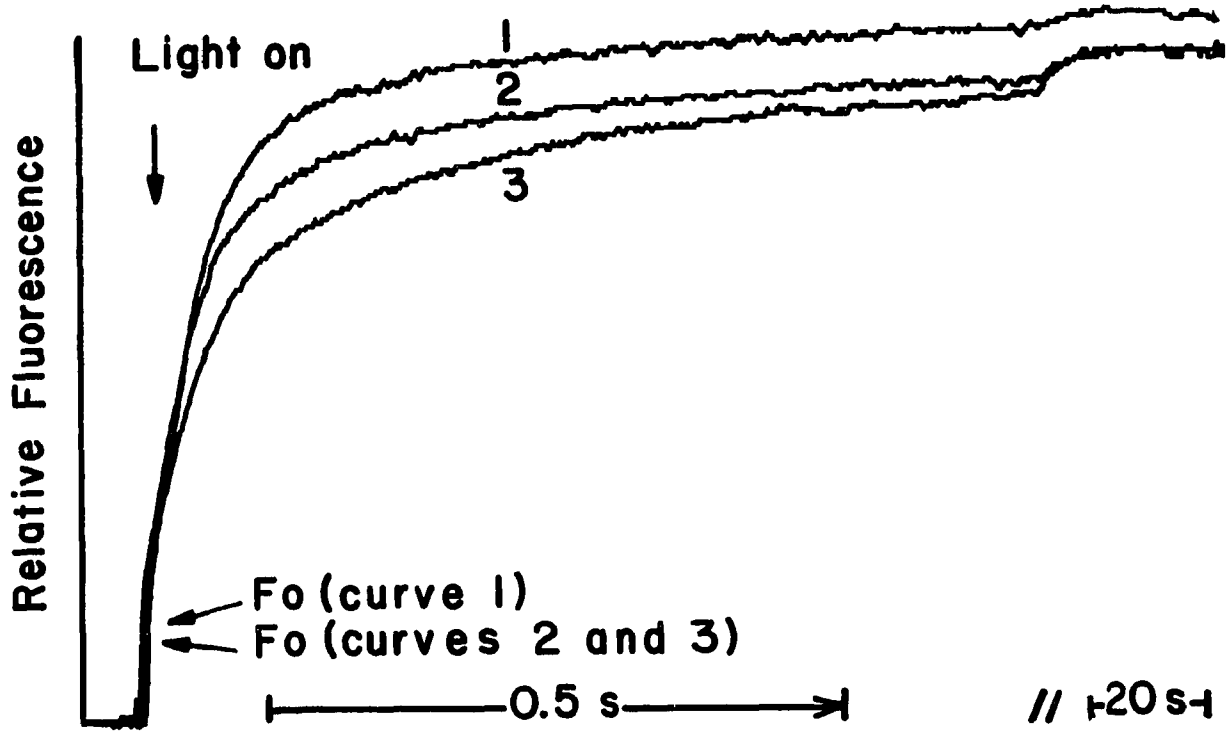


Figure 4.2. The effect of 6-azido-Q_0C_{10} on the Chl a fluorescence transient of UV-irradiated spinach thylakoids. Upper trace: UV-treated only. Middle trace: 1 μM 6-azido-Q_0C_{10} was present throughout the UV treatment. Lower trace: 1 μM 6-azido-Q_0C_{10} was added after the UV treatment. The UV treatment was done for 1 hr as described in the text.

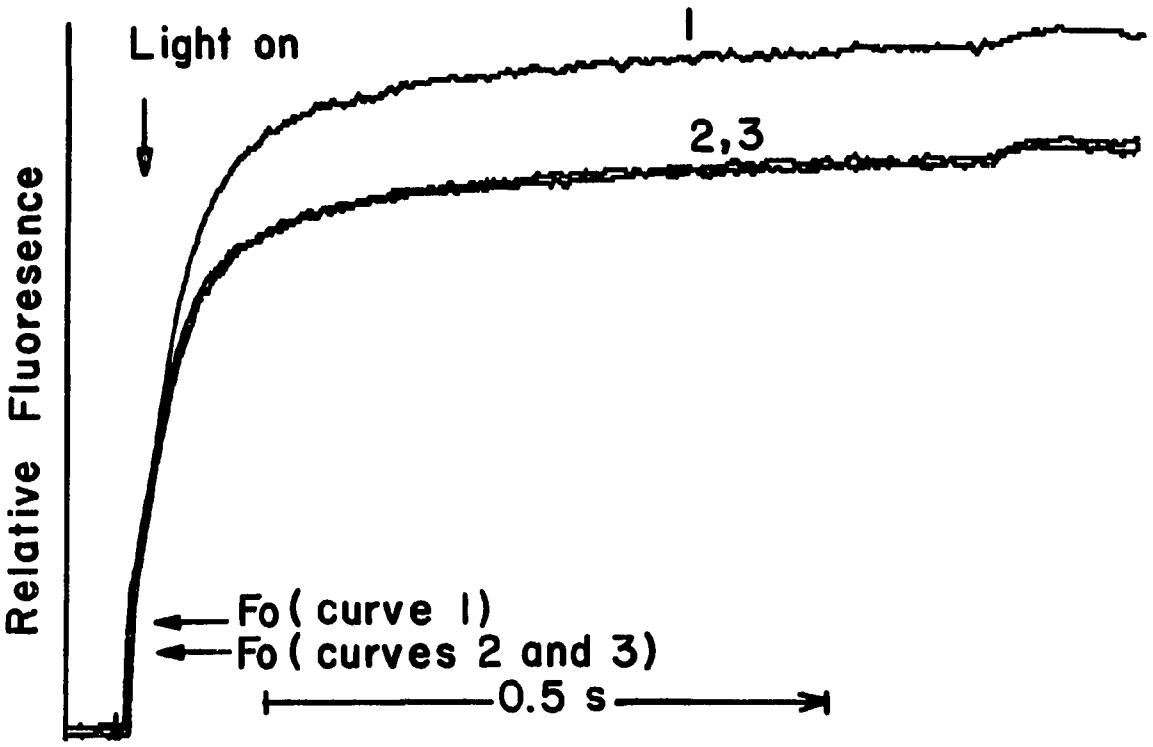


Figure 4.3. The effect of Q_0C_{10}-NAPA on the Chl *a* fluorescence transient of UV-irradiated spinach thylakoids. Conditions were identical to those of Fig. 4.2. Upper trace: UV-treated only. Lower two traces: 1 μM Q_0C_{10}-NAPA added before or after the UV treatment; there is no difference in the traces.

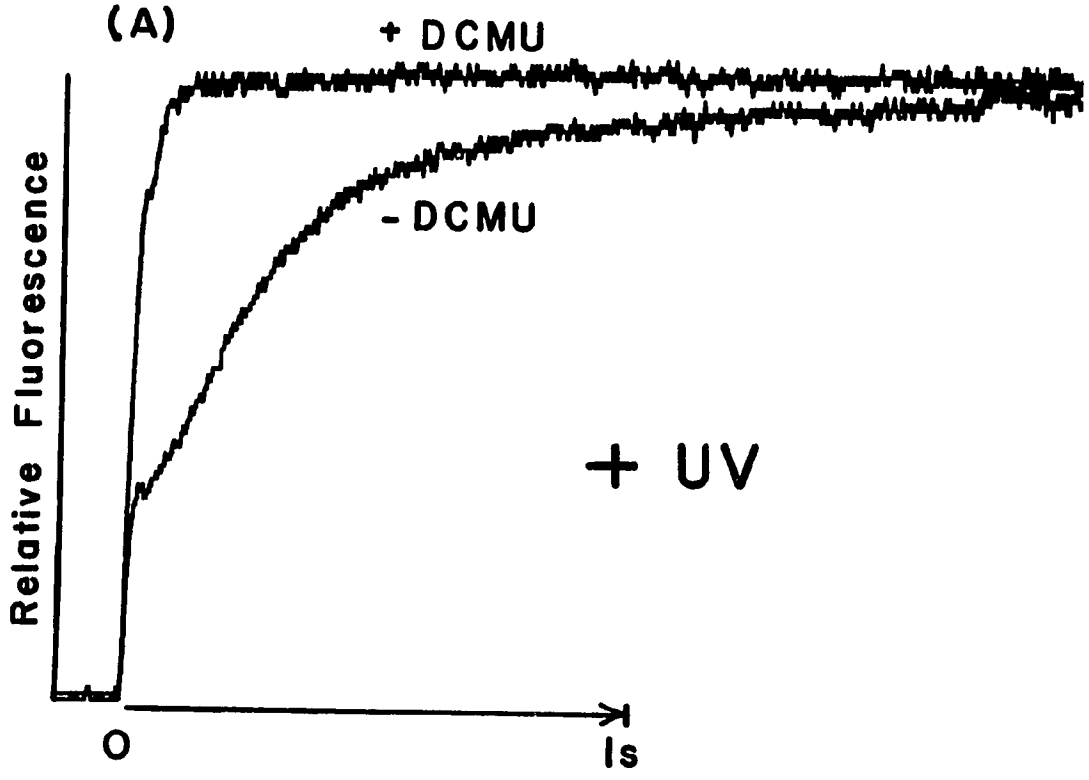
transient when added before or after the UV treatment. The 6-azido-Q_0C_{10} ought to become covalently attached when added before the UV treatment, but not when added after. When covalently attached, the fluorescence rise should be accelerated, due to the inability of the attached 6-azido-Q_0C_{10} to communicate with the pool. This is indeed what is observed, although the acceleration is smaller than would be expected (i.e. Trace 2 is not faster than Trace 1). As discussed below (see also p. 106 and pp. 113-114), 6-azido-Q_0C_{10} may be able to oxidize Q_A^- directly; this may account for the slower than expected fluorescence rise. Q_0C_{10}-NAPA, on the other hand, does not show this behavior (Fig. 4.3); whether added before or after the UV treatment, Q_0C_{10}-NAPA has no apparent effect on Q_A to Q_B electron transfer (see also p. 102). The quenching that is observable in Fig. 4.3 is due to non-photochemical quenching by the added oxidized quinone; when F_o is normalized, the traces with and without Q_0C_{10}-NAPA are identical (not shown). Q_0C_{10}-NAPA, unlike 6-azido-Q_0C_{10}, was shown by Vermaas and coworkers (1983) to be ineffective at replacing atrazine from its binding site, so it can be concluded that Q_0C_{10}-NAPA does not bind to the Q_B site. Therefore, it makes a good control for the effect shown in Fig. 4.2; the absence of any change in the kinetics of the Chl a fluorescence rise when Q_0C_{10}-NAPA is present before or after the UV treatment, compared to the obvious change when 6-azido-Q_0C_{10} is the quinone present, suggests that 6-azido-Q_0C_{10} is able to bind at the Q_B site. Furthermore, the difference in the traces when 6-azido-Q_0C_{10} is covalently attached, as opposed to non-covalently bound, suggests that 6-azido-Q_0C_{10}, like the native PQ, is able to act as an electron shuttle in and out of the Q_B site. Thus, the conclusion by Vermaas et al. (1983) that 6-azido-Q_0C_{10} is a functional analog of PQ is confirmed by these results.

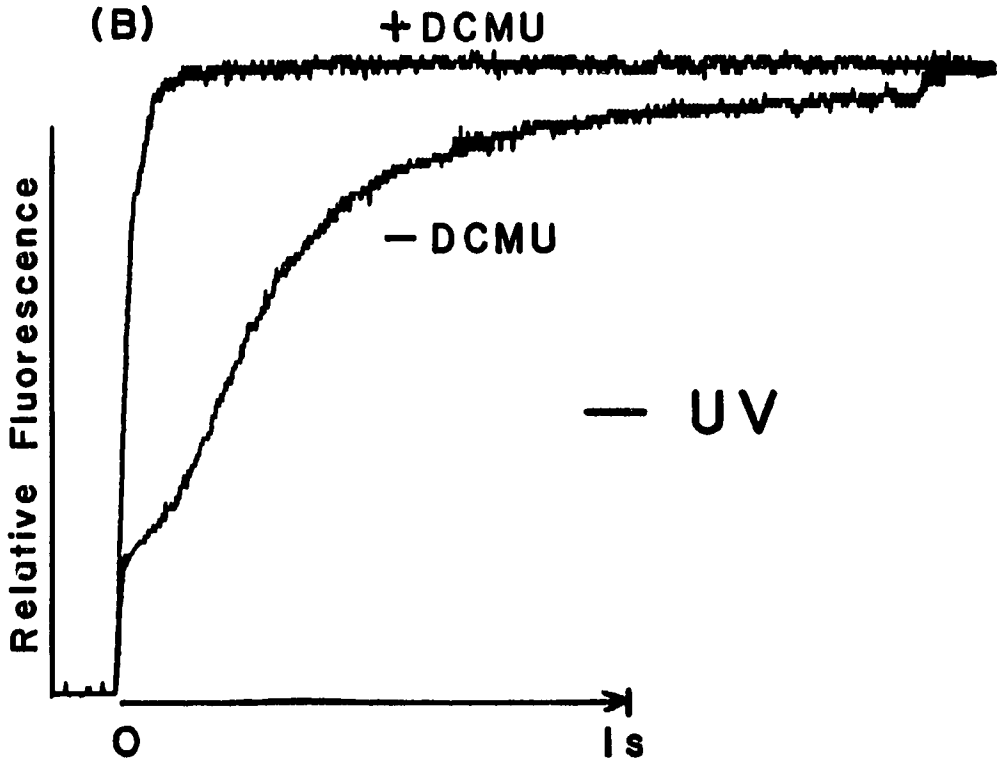
It is also worth noting that the amount of non-photochemical quenching is less with 6-azido-Q_0C_{10} than with Q_0C_{10}-NAPA. This is probably attributable to the photosynthetic reduction of 6-azido-Q_0C_{10}. Still, the percentage of centers in which the 6-azido-Q_0C_{10} is covalently attached appears to be small, as the accelerated rise is not at all like that of DCMU (Fig. 4.4). It is possible, however, that covalently attached 6-azido-Q_0C_{10} can pass electrons to the quinone pool through collisional reactions. It is also possible that non-bound 6-azido-Q_0C_{10} can accept electrons directly from Q_A^- or from the bound 6-azido-$Q_0C_{10}^-$. It has been shown, for example, that some exogenous quinones can oxidize Q_B^- directly (Zimmerman and Rutherford, 1986).

Despite the severe quenching induced by the UV treatment (Fig. 4.1), the kinetics of the variable fluorescence in UV-treated samples are normal. Fig. 4.4 shows the effect of DCMU in such samples, compared to non-UV-treated thylakoids. There is no apparent alteration in the effects of DCMU. Therefore the Q_B site is not apparently altered in the UV-treated samples (DCMU accelerates the fluorescence rise by occupying the Q_B site and preventing the reoxidation of Q_A^-). Fig. 4.5 shows the effect on the fluorescence transient when 100 mM formate was present (labelled as -HCO_3^-) during the UV treatment. 10 mM HCO_3^- reverses the effect and restores the normal transient. This is normal behavior for formate and HCO_3^- (c.f. Fig. 2.5). Therefore, the HCO_3^- binding site does not appear to be adversely affected by the UV treatment. Also shown in Fig. 4.5, for comparison, is the effect of 10 μM DCMU added after restoration of the transient by HCO_3^-.

In thylakoids in which both formate and 6-azido-Q_0C_{10} were present during the UV treatment, there is a large quenching effect by the 6-azido-Q_0C_{10} (Fig. 4.6) that is considerably larger than in identically treated

Figure 4.4. Chl *a* fluorescence transients of UV-irradiated (A) and non-UV-irradiated (B) spinach thylakoids in the presence (upper traces) and absence (lower traces) of 10 μM DCMU. The UV treatment was for 1 hr. The [Chl] was 20 μg/ml. Other details were as described in the text.


(A)
+ DCMU
- DCMU
+ UV
Relative Fluorescence
O
1s


(B)
+DCMU
— DCMU
— UV
Relative Fluorescence
O
1s

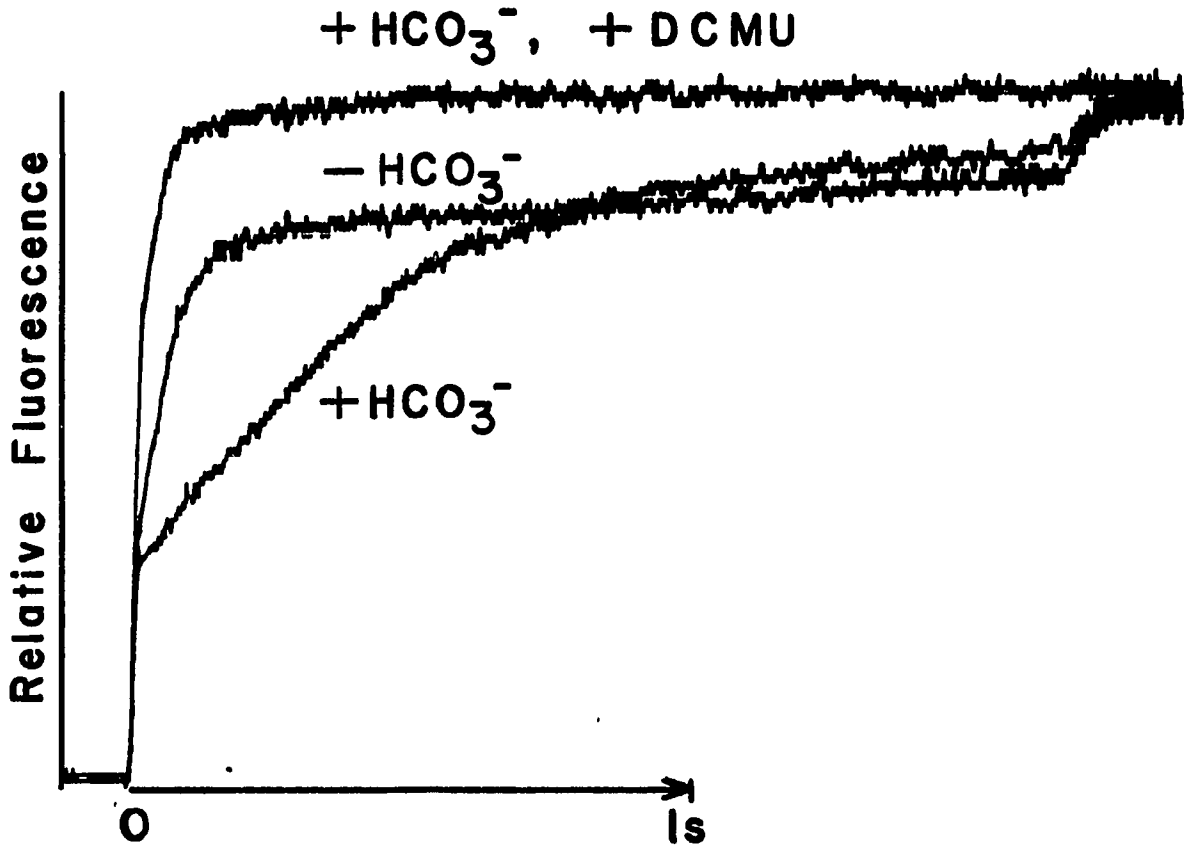


Figure 4.5. The effect of HCO_3^- on the Chl *a* fluorescence transients of formate-incubated, UV-irradiated spinach thylakoids. 100 mM $NaHCO_2$ was present throughout the 1 hr UV treatment at pH 6.5. Where indicated, 10 mM $NaHCO_3$ was added after the UV treatment. Also where indicated, 10 μM DCMU was added after the HCO_3^- addition.

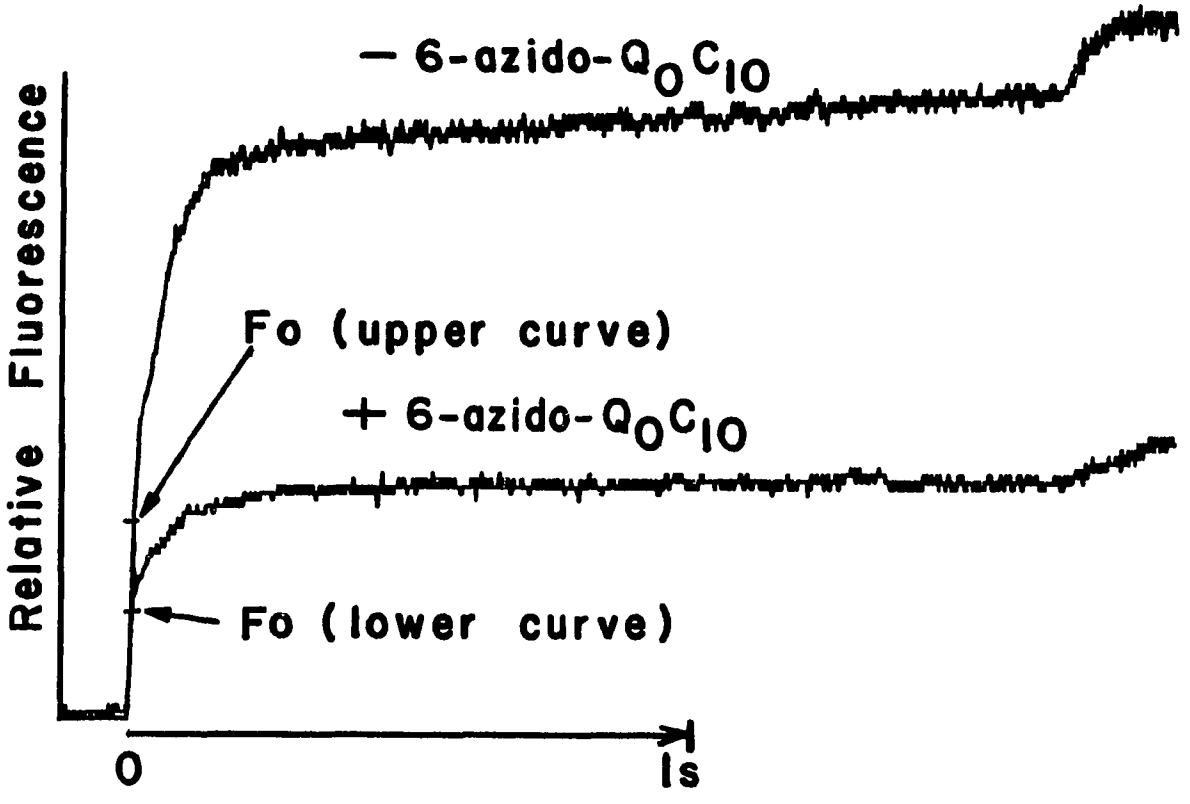


Figure 4.6. Chl a fluorescence transients of formate-incubated, UV-irradiated spinach thylakoids in the presence (lower curve) or absence (upper curve) of 2 μM 6-azido-Q_0C_{10}. Where present, the 6-azido-Q_0C_{10} was added before the UV-treatment.

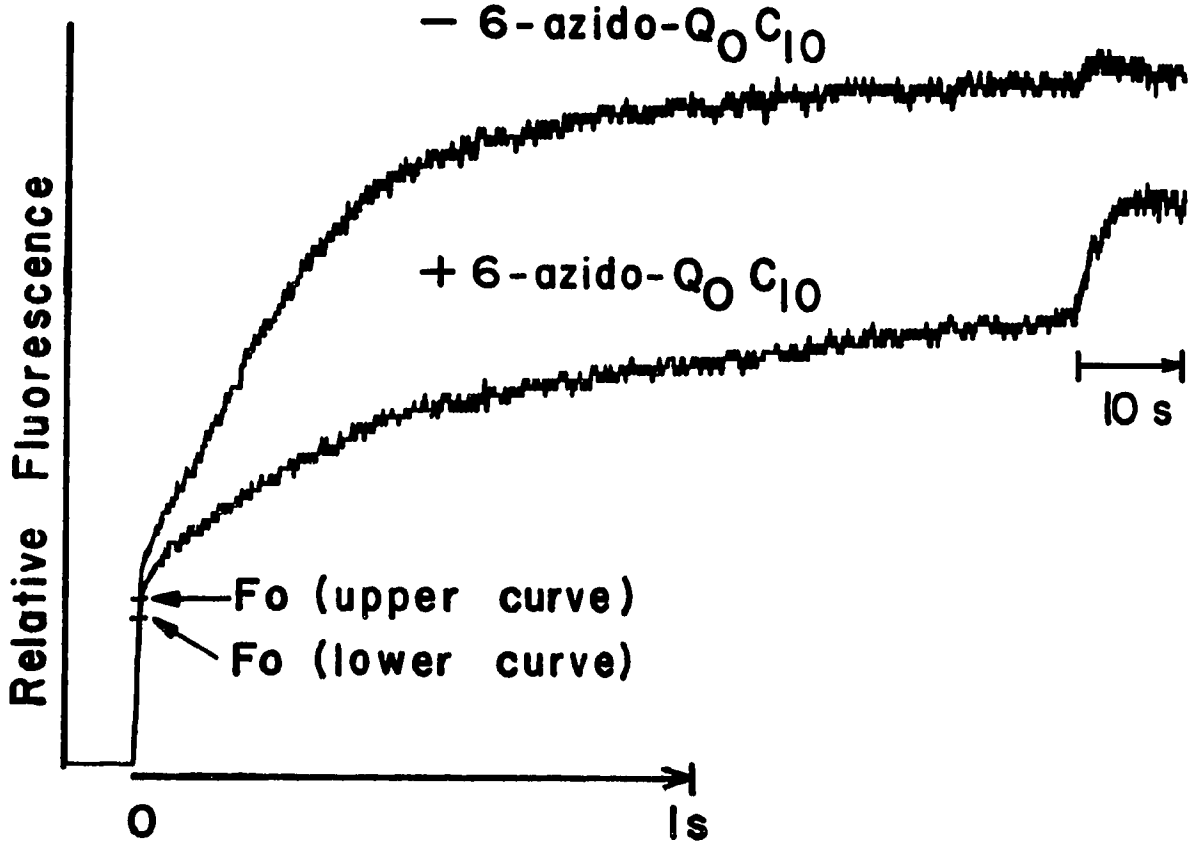


Figure 4.7. Chl a fluorescence transients of UV-irradiated spinach thylakoids in the presence (lower curve) or absence (upper curve) of 6-azido-Q_0C_{10}. Where present, the 6-azido-Q_0C_{10} was added before the UV treatment. Conditions were identical to those of Fig. 4.6, except that no formate was present.

samples without formate (Fig. 4.7). This is at least partly due to the slower reduction of the quinone pool due to HCO_3^- depletion. It may also be due to 6-azido-Q_0C_{10} accepting electrons directly from Q_A^- when formate is present. It has been shown that exogeonous quinones can oxidize the non-heme Fe^{2+} of PS II, which is then re-reduced by Q_A^-, and that formate blocks this oxidation (Zimmerman and Rutherford, 1986; see also Wydrzynski and Inoue, 1987). It is possible that formate blocks the oxidation by permitting the oxidation of Q_A^- directly by the exogenous quinones. If so, this would account for the large quenching effect of 6-azido-Q_0C_{10} in the presence of formate (Fig. 4.6).

In Fig. 4.6 there is also apparent a greater quenching of the F_o level, which may indicate a larger pool of oxidized quinones when formate was present during the UV treatment. This could be the case if formate incubation prevents 6-azido-Q_0C_{10} from binding to the Q_B site. If, indeed, formate inhibits the binding of 6-azido-Q_0C_{10}, then there should be less or no covalent attachment to the Q_B site, and the addition of HCO_3^- ought to restore the ability to reduce the quinone pool. If, on the other hand, 6-azido-Q_0C_{10} binding is not affected by formate incubation, then covalent attachment to the Q_B site will occur, and the addition of HCO_3^- would not be expected to make much difference in restoring electron flow through the Q_B site. As shown in Fig. 4.8, HCO_3^- does restore the ability to reduce the quinone pool, as evidenced by the rise in fluorescence. This suggests, then, that 6-azido-Q_0C_{10} binding may be inhibited by formate incubation. The fairly long time required to reduce the pool can be attributed to the increased size of the pool, due to the exogenous 6-azido-Q_0C_{10}. The implication of this result is that PQ binding may also be inhibited when HCO_3^- is removed from its binding site. This conclusion, however, is

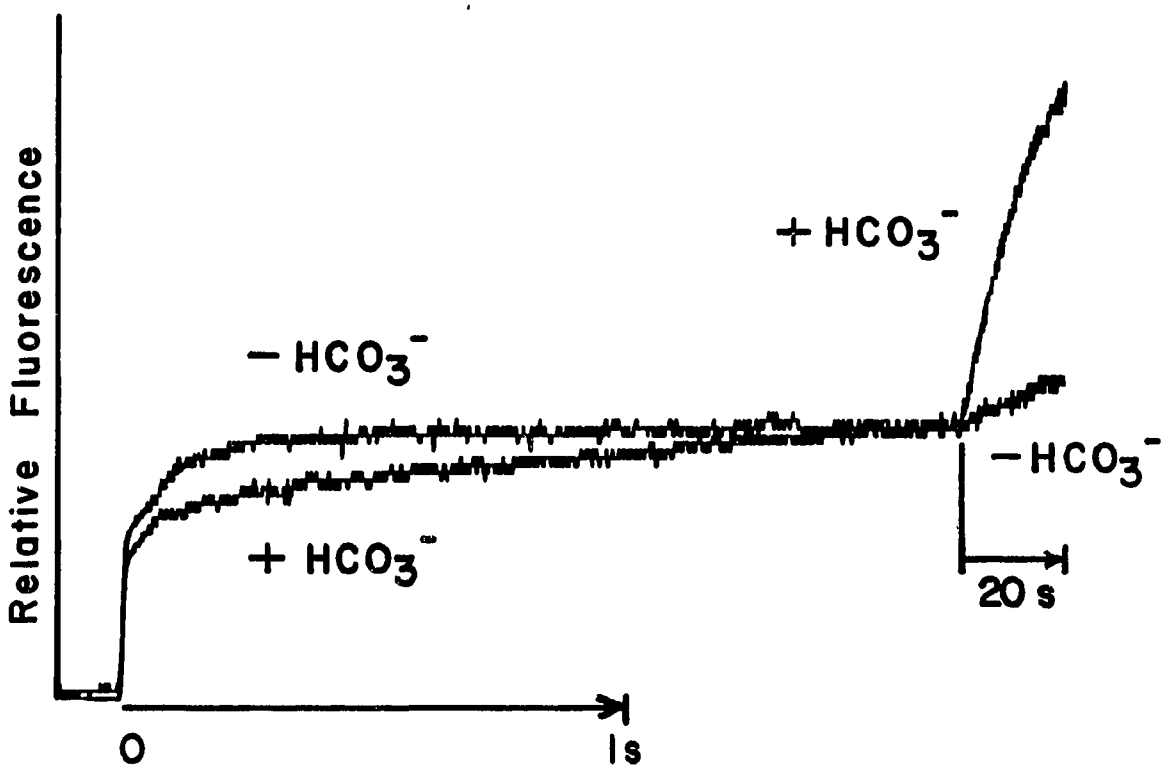
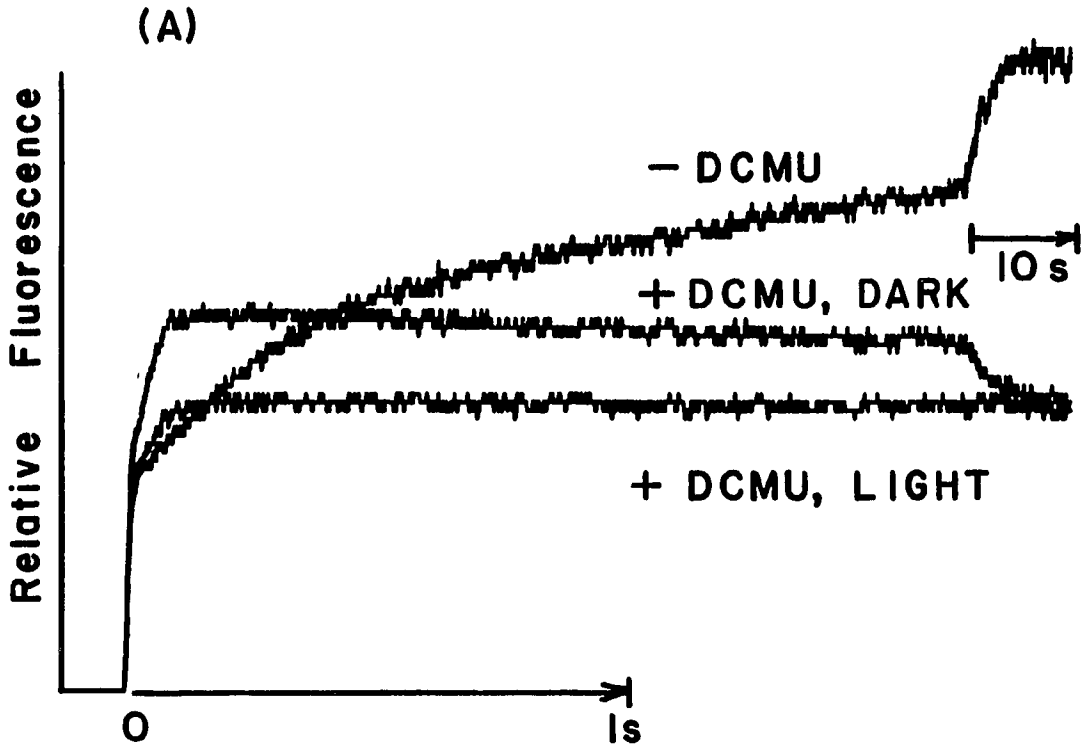


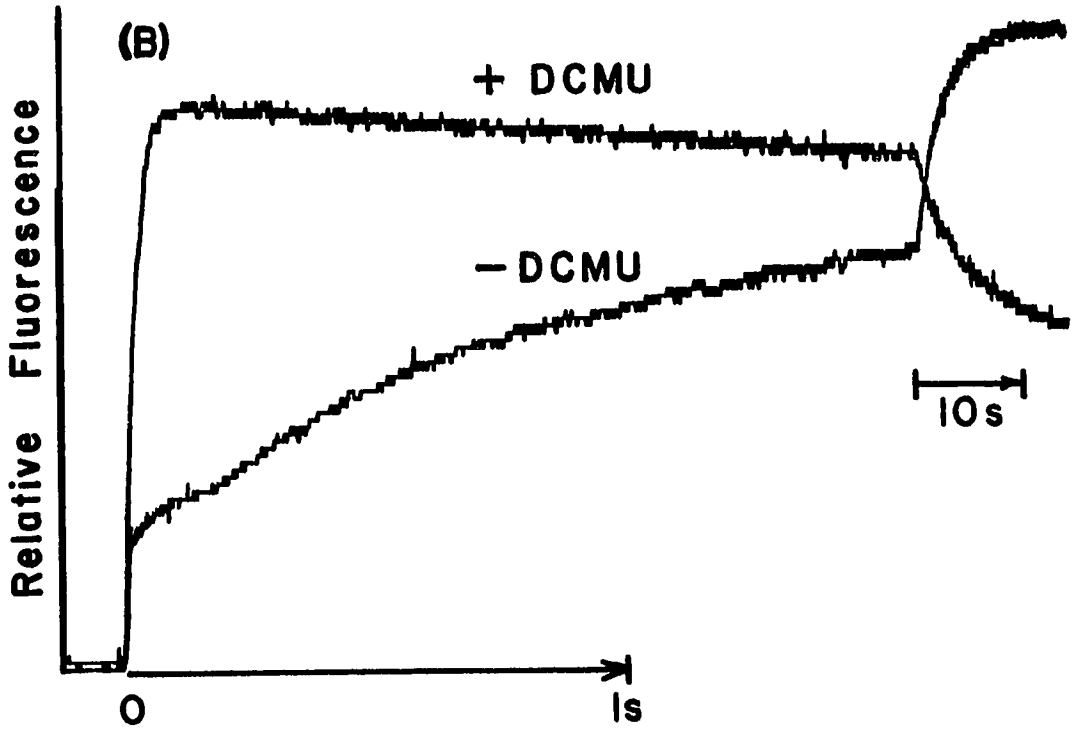
Figure 4.8. The effect of HCO_3^- on the Chl a fluorescence transients of formate-incubated, UV-irradiated spinach thylakoids, in which 2 μM 6-azido-Q_0C_{10} was present throughout the UV treatment. 100 mM formate was present throughout. Where indicated, 10 mM HCO_3^- was added after the UV treatment.

dependent on the argument that 6-azido-Q_0C_{10} is a functional analog of PQ and that the UV-treatment is effective at covalently attaching the analog to the Q_B site (Vermaas et al., 1983). As will be shown, the effects of 6-azido-Q_0C_{10} are more complicated than this.

Fig. 4.9 shows the effect of DCMU in thylakoids to which 6-azido-Q_0C_{10} is covalently bound (UV-treated) or not (non-UV-treated). In both cases, the fluorescence level induced by DCMU is lower than F_{max}. This quenching is greater in the UV-treated thylakoids. This aspect of the effect was anticipated. Competition with the 6-azido-Q_0C_{10} for the Q_B site should diminish the binding of DCMU, and DCMU binding should be diminished even more when the 6-azido-Q_0C_{10} becomes covalently attached. This was shown to be the case by Vermaas et al. (1983) for other DCMU-type herbicides, determined from binding studies of the radiolabelled herbicides. The conclusion is supported by the fluorescence data in Fig. 4.9. However, what is peculiar in the fluorescence data is that the level of fluorescence declines during illumination in the presence of DCMU. If DCMU were not completely blocking electron transport, as the above explanation would suggest, then the fluorescence should continue to rise to F_{max} (minus some non-photochemical quenching by the DCMU itself), as long as electron flow into the acceptor pool is faster than oxidation of the pool by PS I. That is, as the electron acceptor pool becomes reduced, at some point it, rather than the partial block by DCMU, would become rate limiting for the oxidation of Q_A^-. If, on the other hand, electron flow into the pool were slower than oxidation by PS I, then the fluorescence level would be expected to remain constant, as the partial block by DCMU would be the rate-limiting step to Q_A^- oxidation. In neither case would a partial block by DCMU predict a decline in fluorescence, unless (i) electron flow into Q_A

Figure 4.9. Chl a fluorescence transients of spinach thylakoids in the presence of 2 μM 6-azido-Q_0C_{10} and in the presence or absence of 10 μM DCMU. In (A) the thylakoids were UV-treated as described in the text, which is suggested to cause covalent attachment of the 6-azido-Q_0C_{10} to the Q_B site. In (B) the thylakoids were not exposed to UV radiation, and are suggested to bind 6-azido-Q_0C_{10} non-covalently.

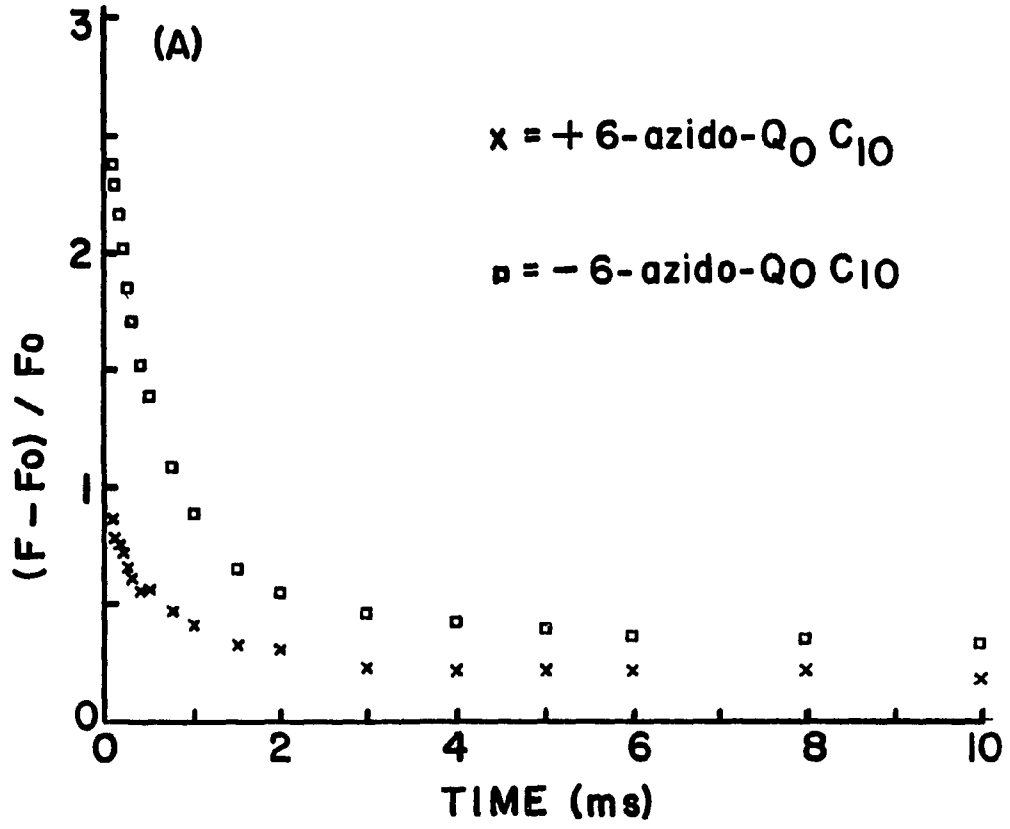

(A)
Relative Fluorescence
– DCMU
+ DCMU, DARK
+ DCMU, LIGHT
10 s
O
1s

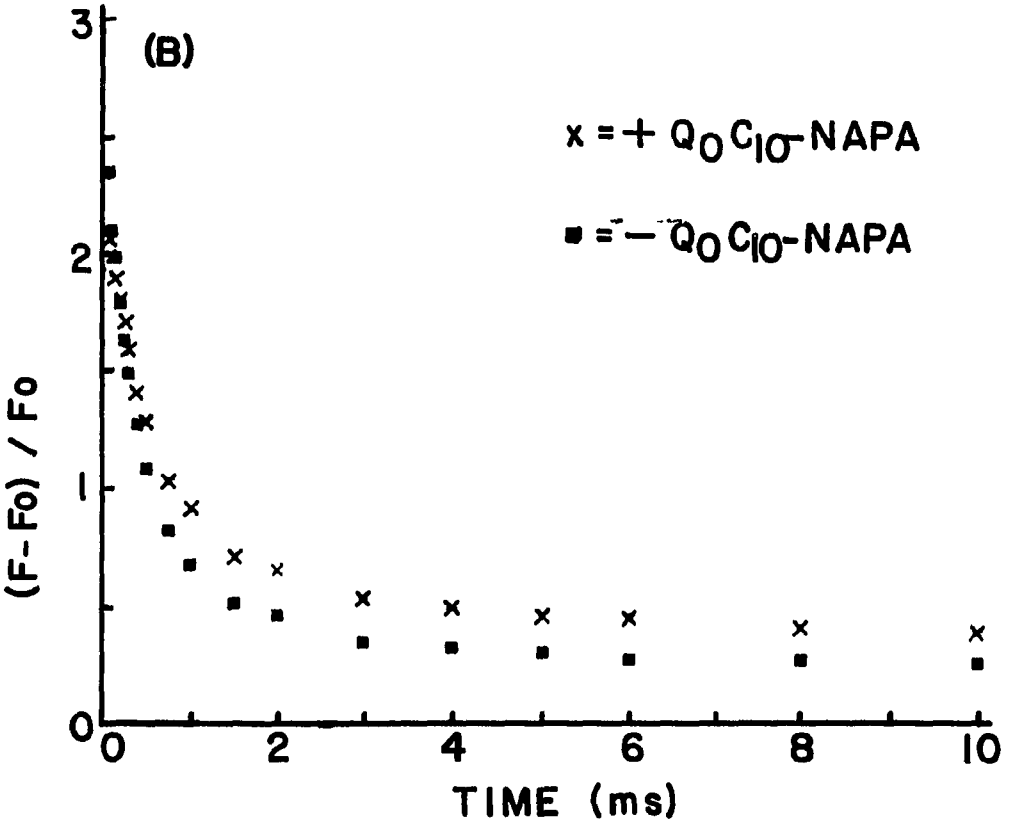

(B)
Relative Fluorescence
+ DCMU
– DCMU
10s
O
1s

is becoming diminished in the light (e.g. by an effect on the donor side of PS II or by photoinhibition of the PS II reaction center), or (ii) electron flow out of Q_A^- is accelerated in the light (e.g. by a side pathway that is activated in the light, or by diminished DCMU binding in the light). The possibility was discussed above that 6-azido-Q_0C_{10} might accept electrons directly from Q_A^- in the presence of formate. It is possible that in the absence of formate, a light-induced conformational change may have a similar effect to permit a direct oxidation of Q_A^- by 6-azido-Q_0C_{10}. This would account for the smaller than expected acceleration of the fluorescence rise when, supposedly, 6-azido-Q_0C_{10} is covalently attached to the Q_B site (Fig. 4.2).

The possibility that 6-azido-Q_0C_{10} could have an effect on the donor side of PS II was examined. Vermaas et al. (1983) had noted that there is a significantly greater miss parameter in the O_2 flash yield when 6-azido-Q_0C_{10} is present, and they conceded that there may be an effect on Z (Z, the native electron donor to P_{680}, was thought to be a bound PQ; thus, it was thought that 6-azido-Q_0C_{10} could be replacing Z in some centers to prevent the reaction center from turning over during a flash; however, more recent evidence suggests that Z is a tyrosine residue (G.T. Babcock, personal communication by Govindjee)). However, they interpreted the increased miss parameter as due to a slower Q_A^- reoxidation, since they saw no inhibitory effect of 6-azido-Q_0C_{10} on the H_2O to p-benzoquinone Hill reaction. The reoxidation of Q_A^- can be monitored directly by following the decay of the Chl a fluorescence after an actinic flash. The results of such an experiment, with and without 6-azido-Q_0C_{10}, are presented in Fig. 4.10 (A). The most immediate effect is a large quenching of the variable fluorescence (F_{var}) by 6-azido-Q_0C_{10}. This effect is not seen

Figure 4.10. Decay of the variable Chl a fluorescence after an actinic flash. In (A) the decay is shown in the presence and absence of 5 μM 6-azido-Q_0C_{10}. In (B) the decay is shown in the presence and absence of 5 μM Q_0C_{10}-NAPA.


(A)
x = + 6-azido-Q0 C10
▫ = − 6-azido-Q0 C10
(F − Fo) / Fo
TIME (ms)
0
1
2
3
0
2
4
6
8
10


(B)
x = + Q0 C10-NAPA
■ = − Q0 C10-NAPA
(F-Fo) / Fo
TIME (ms)
0
1
2
3
0
2
4
6
8
10

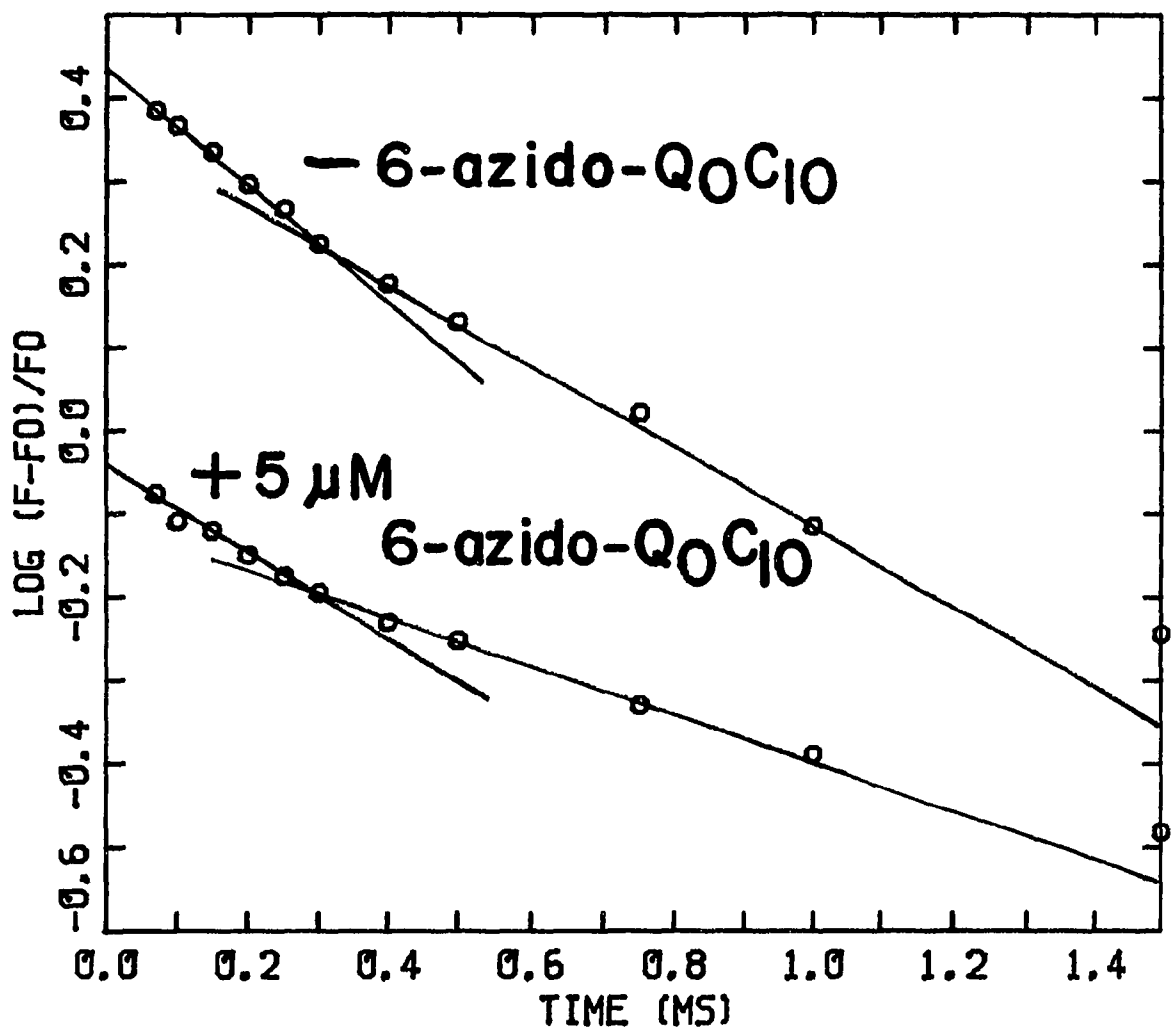


Figure 4.11. A semilog plot of the decay of the variable Chl a fluorescence after an actinic flash in the presence and absence of 5 μM 6-azido-Q_0C_{10}. Only the first 1.5 ms is shown. The data is taken from Fig. 4.10 A.

with Q_0C_{10}-NAPA (Fig. 4.10, B). The effect is not due to non-photochemical quenching by the oxidized quinone, since any non-photochemical quenching affects F_o and F equally and would, therefore, be cancelled out in the plot of F_{var}. Neither can it be due to photochemical quenching (i.e. due to 6-azido-Q_0C_{10} acting as an extremely efficient electron acceptor, to keep Q_A oxidized), for the following reasons. Since the decay is measured after a saturating flash, all of the centers are closed at t=0, and the fluorescence at t=0 is F_{max} (i.e. all Q_A is reduced). A photochemical quenching, therefore, would be observed as an accelerated decay, with F_{max} unchanged. This is not what is apparently observed in Fig. 4.10 (A). If a rapid decay process is occurring, it must be well-advanced by the first point, which was measured 70 μs after the flash. A semi-log plot of the decay (Fig. 4.11) shows the same number of decay components in both the presence and the absence of 6-azido-Q_0C_{10}, with no indication of a very fast component that could account for the quenching. On the contrary, from a comparison of the slopes in Fig. 4.11, the fluorescence decay is somewhat slower in the presence of 6-azido-Q_0C_{10}. However, it is not slow enough to account for the increased miss parameter reported by Vermaas et al. (1983). The quenching of F_{var} by 6-azido-Q_0C_{10} must be due to Q_A not going fully reduced during the flash, possibly because of a block prior to Q_A (i.e. on the donor side of PS II or between pheophytin (Pheo) and Q_A), or possibly due to an effect on the pigment system that causes excitation energy to be quenched before it is trapped. Doubling the light intensity by flashing both sides of the cuvette simulataneously did not increase F_{var} in the presence of 6-azido-Q_0C_{10}, ruling out the second possibility.

The inhibitory effect of 6-azido-Q_0C_{10} on the Hill reaction with FeCy as electron acceptor is shown in Fig. 4.12. The H_2O to DCPIP reaction,

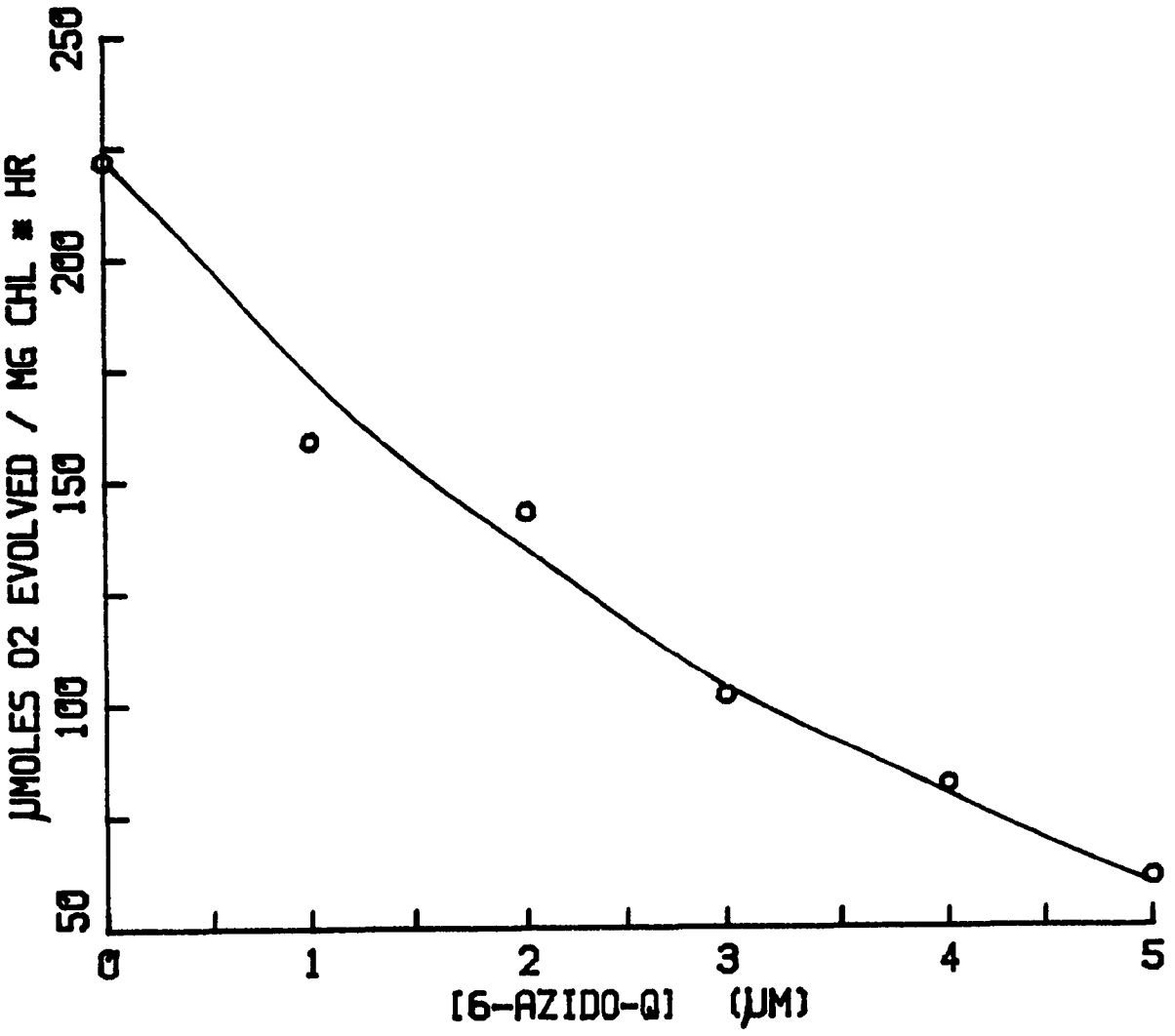


Figure 4.12. The rate of O_2 evolution as a function of 6-azido-Q_0C_{10} concentration. Spinach thylakoids were suspended in 20 mM HEPES, pH 7.5, 15 mM NaCl and 5 mM $MgCl_2$. 2 mM ferricyanide was used as an electron acceptor for the Hill reaction. Electron transport was uncoupled from photophosphorylation with 0.1 mM CH_3NH_2 and 10 nM Gramicidin D.

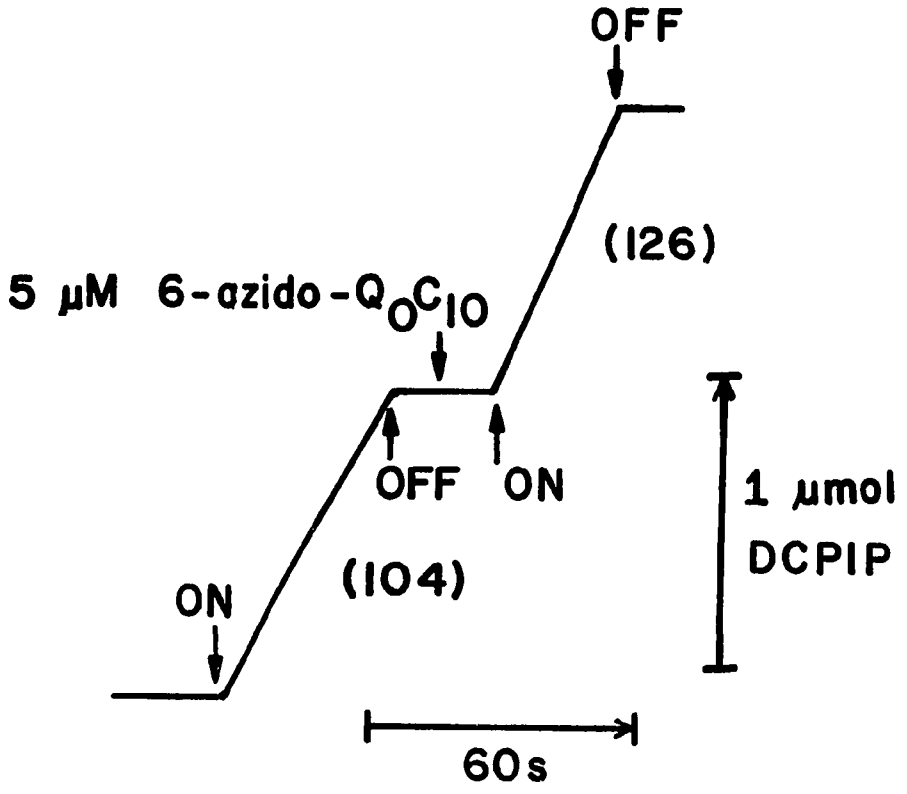


Figure 4.13. The Hill reaction, measuring electron flow from H_2O to DCPIP, showing no inhibition upon the addition of 5 μM 6-azido-Q_0C_{10}. This concentration of 6-azido-Q_0C_{10} was the largest concentration used in Fig. 4.12. The thylakoids were suspended in the same medium as in Fig. 4.12. 0.1 mM CH_3NH_2 and 10 nM Gramicidin D were present as uncouplers, and 1 μM dibromothymoquinone (DBMIB) was present to block the PS I reactions. The numbers in parentheses are the rates in μmoles DCPIP per mg Chl per hr.

however, is not inhibited; in fact, 6-azido-Q_0C_{10} appears to stimulate this reaction (Fig. 4.13). Therefore, the site of inhibition by 6-azido-Q_0C_{10} must be in PS I, not PS II. This is consistent with the observation of Vermaas et al. (1983) that the PS I partial reaction, N,N,N',N'-tetramethyl-p-phenylenediamine to methyl viologen, is inhibited by 6-azido-Q_0C_{10}. Taking all of the data into account, the most likely explanation for the quenching of F_{var} by 6-azido-Q_0C_{10} is an impairment of electron flow from Pheo to Q_A that is not rate limiting in the steady state ($Pheo^-$ to Q_A electron transfer occurs with a half-time around 200 ps; this could be increased considerably without becoming rate limiting), but which prevents all of the centers from forming Q_A^- in a 2.5 μs flash. Since the charge separation between P_{680} and Pheo is not stable, a longer lifetime for $Pheo^-$ would increase the rate of charge recombination, and this would account for the increased miss parameter in the O_2 flash yield observed by Vermaas and coworkers (1983).

Earlier, it was suggested that 6-azido-Q_0C_{10} might be accepting electrons directly from Q_A^-. Apparently, this does not occur under the conditions of Fig. 4.10, because Q_A^- oxidation is slower. However, since $[Q_A^-]$ was always low in that experiment, this could simply mean that the direct oxidation of Q_A^- by non-bound 6-azido-Q_0C_{10} occurs with a much lower probability than Q_A^- oxidation by a quinone bound in the Q_B site. On the other hand, when electron flow from Q_A^- to Q_B is impaired, as, for example, when DCMU or formate is present, or when 6-azido-Q_0C_{10} is covalently attached to the Q_B site, then the increased lifetime of Q_A^- could result in a significant degree of electron transfer from Q_A^- to non-bound 6-azido-Q_0C_{10}. This could also account for the stimulation of the the H_2O to DCPIP Hill reaction under steady-state conditions, in which the electron acceptor

pool is largely reduced and [Q_A^-] is high (Fig. 4.13). The large quenching by 6-azido-Q_0C_{10} in the presence of formate (Fig. 4.6) and the decline in the DCMU-induced fluorescence during illumination (Fig. 4.9) are not explained by the non- rate limiting inhibition of $Pheo^-$ to Q_A electron transfer, but are readily explained by a direct oxidation of Q_A^-.

It was shown in Fig. 4.9 that the DCMU-induced fluorescence level in the presence of 6-azido-Q_0C_{10} is lower when the 6-azido-Q_0C_{10} was exposed to UV than when it was not. This is consistent with a covalent attachment of the 6-azido-Q_0C_{10} during the UV treatment. Formate incubation of the thylakoids was suggested to inhibit the binding of 6-azido-Q_0C_{10}, since HCO_3^- was able to restore the variable fluorescence after UV treatment (Fig. 4.8). Since this conclusion is dependent on the assumption that 6-azido-Q_0C_{10} does indeed covalently occupy most, if not all, of the Q_B sites after UV treatment, it is worth asking whether DCMU induces a high or a low level of fluorescence in this HCO_3^- restored sample. Fig. 4.14 shows the fluorescence transient of this sample upon DCMU addition, along with the DCMU-induced transients from Fig. 4.9 for comparison. The fluorescence level in the HCO_3^- restored sample is still quenched considerably (largely due to the UV treatment), but it is higher than when 6-azido-Q_0C_{10} was exposed to UV without formate. This favors the suggestion that less 6-azido-Q_0C_{10} was bound during the UV treatment with formate. It is worth noting that there is considerably less decline of the DCMU-induced fluorescence level during illumination of the HCO_3^- restored sample than in either of the other two cases. The significance of this observation is not clear, but it may indicate a tighter binding of DCMU, or it may be that the sample was not sufficiently dark-adapted and that the level observed is already the level to which the fluorescence declines. If the latter

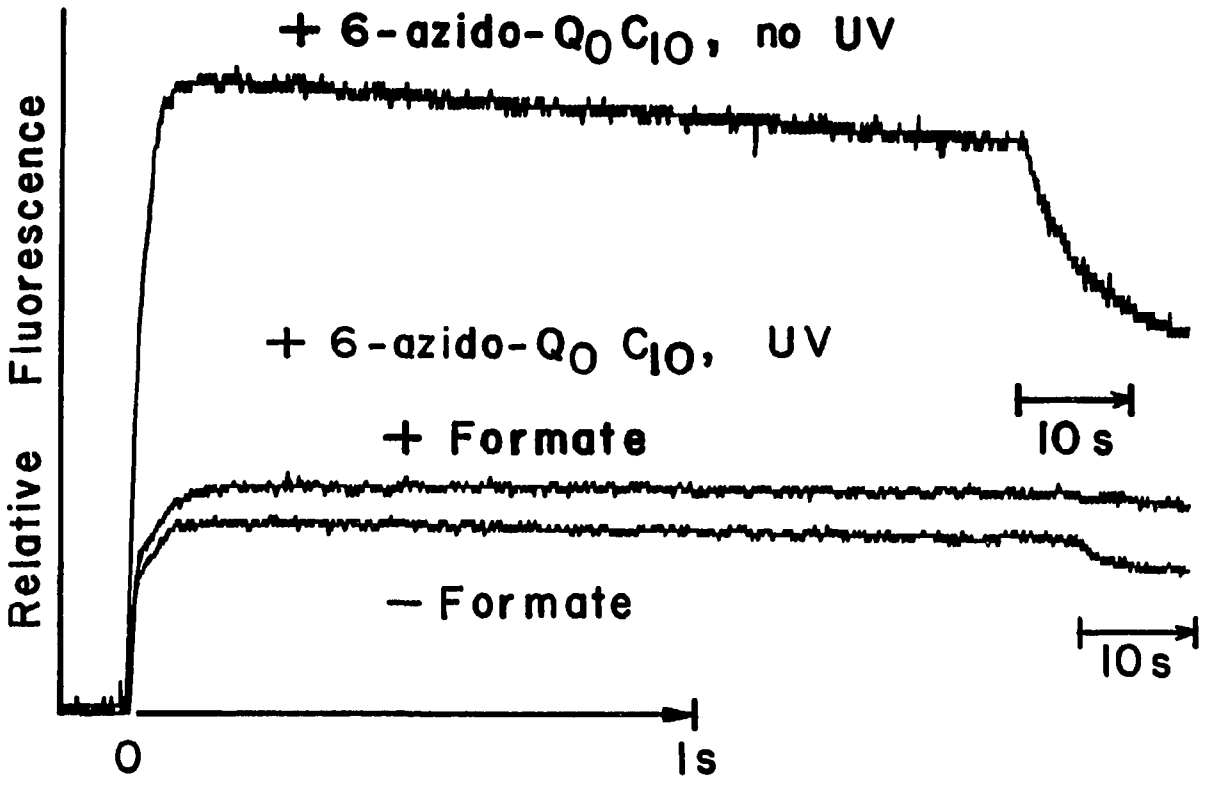


Figure 4.14. Chl a fluorescence transients in the presence of 10 μM DCMU for thylakoids from three separate treatments. Upper trace: + 2 μM 6-azido-Q_0C_{10}, without UV treatment. Middle trace: UV-treated in the presence of 2 μM 6-azido-Q_0C_{10} and 100 mM formate, then 10 mM HCO_3^- added. Lower trace: UV-treated in the presence of 2 μM 6-azido-Q_0C_{10}, without formate.

suggestion is the case, then the DCMU-induced fluorescence level is closer to that when no 6-azido-Q_0C_{10} was bound.

From the data presented in this chapter, it appears that formate incubation may inhibit the binding of 6-azido-Q_0C_{10} to the Q_B site. By implication, it is suggested that HCO_3^- depletion inhibits the binding of PQ. This argument is dependent on the conclusion that 6-azido-Q_0C_{10} is a functional analog of PQ (Vermaas et al., 1983). Much of the data presented in this chapter is supportive of that conclusion. However, 6-azido-Q_0C_{10} is shown to have multiple effects in addition to a functional competition with PQ: (i) it appears to inhibit $Pheo^-$ to Q_A electron transfer; (ii) it is probably able to oxidize Fe^{2+}, as do some other exogenous quinones (Zimmerman and Rutherford, 1986); and (iii) in the presence of formate, or after prolonged illumination, it appears to oxidize Qa- directly. Therefore, the suggestion that HCO_3^- depletion inhibits PQ binding should be viewed as tentative. It is in agreement, however, with the conclusion by Eaton-Rye (1987) that the dissociation constant for PQ binding is increased nearly 5-fold by HCO_3^-depletion.

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CHAPTER 5

THE NUMBER OF HCO_3^- BINDING SITES

A. General Introduction to Sections I and II

Until recently, it seems to have been tacitly assumed that there is a single HCO_3^- binding site involved in the regulation of electron flow through the quinones of photosystem II (PS II). Stemler (1977) measured the binding of $H^{14}CO_3^-$ to isolated thylakoids and determined that there were two pools of HCO_3^-: one was a high affinity pool existing at a concentration of approximately one HCO_3^- per 300 chlorophyll (Chl) molecules; the other was a low affinity pool at a concentration at least as large as that of the bulk Chl. Depletion of the high affinity pool was shown to be responsible for the loss of Hill activity, while the low affinity pool was assumed to be largely empty under physiological conditions and could be accounted for as non-specific binding. Assuming one PS II reaction center per 300 Chl molecules, it was concluded that one HCO_3^- is bound per PS II. However, it is now believed that a heterogeneity of PS II exists, in which 30-40% of the PS II centers are inactive (e.g. Graan and Ort, 1986; Chylla et al., 1987). The reason for this heterogeneity is unknown. The stoichiometry of active PS II to Chl is approximately 1:600 (Whitmarsh and Ort, 1984). If the inactive PS II centers are included, the stoichiometry is closer to 1:400 (J. Whitmarsh, personal communication). It is not known whether HCO_3^- binds to both types of PS II centers or only to the active centers. It is possible that the inactive centers are unable to bind HCO_3^-. If this is correct, then the data of Stemler (1977) really suggests two HCO_3^- per active PS II. In addition, the large low affinity pool may yet have a function, as will be explored in Section II.

A later study by Stemler and Murphy (1983) again concluded one HCO_3^- binding site per PS II; this time one HCO_3^- was bound per 500-600 Chl. However, there is reason to suspect that the chloroplasts were not well depleted of HCO_3^- in this later study. In the earlier study, HCO_3^- depleted thylakoids were kept in screw-capped test tubes, whereas in the later study a layer of mineral oil was used, instead, for protection against atmospheric contamination. 30% of the added $H^{14}CO_3^-$ was shown to have escaped, as $^{14}CO_2$, through this oil layer during the incubation of the thylakoids. Therefore, it is apparent that atmospheric CO_2 was also able to enter the samples through this oil layer. The conclusion that one HCO_3^- is bound per 500-600 Chl, then, is probably based on HCO_3^- binding to sites that were already partially filled, and should be considered suspect.

The best arguments for a single HCO_3^- site can be made from linear double-reciprocal plots of the Hill activity as a function of HCO_3^- concentration (Vermaas et al., 1982; Snel and Van Rensen, 1983). The linearity of these plots suggests that HCO_3^- binds to PS II according to classical Michaelis-Menten kinetics, and that probably only one binding site exists per PS II. A Hill plot of the activity as a function of HCO_3^- concentration likewise yielded a slope of 1, suggesting a single HCO_3^- site (Snel and Van Rensen, 1983). If two sites exist, they would have to be completely independent in their binding to give a linear double-reciprocal plot and a Hill coefficient of 1 (for an excellent and detailed account of the theory involved, see Segel, 1975). Similarly, Jursinic and Stemler (1986) have argued that if two sites exist, they would have to have the same binding affinity. It would seem, then, that the route of fewest assumptions is the acknowledgement of a single site.

Nevertheless, Jursinic and Stemler (1987) have recently claimed two

HCO_3^- sites per PS II, citing as evidence an earlier study (Stemler and Murphy, 1984) which concluded only one HCO_3^- site per PS II! The earlier study determined that there was one HCO_3^- bound per 500-600 Chl, and that there was an equal number of high affinity herbicide binding sites. A second, lower affinity herbicide binding site was identified in at least some of the PS II complexes (a heterogeneity of PS II was suggested), and it was suggested that the binding of herbicide to this site, rather than to the high affinity site, lowered the affinity for HCO_3^-. In the more recent paper, Jursinic and Stemler (1987) seem to consider that the low affinity herbicide binding site represents a second HCO_3^- binding site, but no further evidence is presented.

Eaton-Rye (1987) has recently proposed two HCO_3^- sites in order to explain apparent effects on both plastoquinone (PQ) binding and protonation of Q_B^-. One site is suggested to be on the non-heme Fe^{2+} in PS II and is responsible for maintaining the proper conformation for efficient PQ binding. The second site is suggested to be more difficult to deplete of HCO_3^- and is responsible for delivering a H^+ to Q_B^- or to a protein group near Q_B^-. However, Eaton-Rye (1987) has also shown that with certain assumptions, a single site of HCO_3^- binding can also be made to explain both effects.

In summary, most of the published literature suggests a single HCO_3^- binding site, though arguments can be marshalled for the possibility of two sites. Recently, it appears to have become popular to assume two sites (Jursinic and Stemler, 1987; Eaton-Rye, 1987), despite a lack of solid evidence for it. Part of the reason for this may be the desire to explain a physiological role for HCO_3^- despite a fairly large dissociation constant; the existence of a second, very tightly bound pool of HCO_3^-

would, therefore, be very satisfying (see e.g., Eaton-Rye et al., 1986). A second HCO_3^- site might also be used to marshall arguments for the involvement of HCO_3^- in photosynthetic O_2 evolution (see e.g., Stemler and Murphy, 1983).

In this chapter, the number of HCO_3^- binding sites is re-examined. The first direct evidence for multiple high affinity sites is presented in Section II. The kinetics of the restoration of the Hill activity by HCO_3^- are shown to be complex, and the linearity of the double-reciprocal plots previously published (Vermaas et al., 1982; Snel and Van Rensen, 1983) are shown to be due to an incomplete analysis. The existence of an additional low affinity site is demonstrated in Section I (see also Blubaugh and Govindjee, 1984), and in Section II is suggested to represent an intramembrane reservoir of HCO_3^-. The value of 80 μM for the dissociation constant for the binding of HCO_3^- (Stemler and Murphy, 1983; Snel and Van Rensen, 1984; Jursinic and Stemler, 1986) is shown to be based on a couple of false assumptions and, therefore, contains significant error. The picture that emerges is of two or more HCO_3^- sites which interact cooperatively to tighten the binding, and an additional intramembrane pool of HCO_3^- that raises the HCO_3^- concentration in the vicinity of the binding sites.

SECTION I: A LOW AFFINITY SITE

A. Summary

Evidence is presented for a low affinity site of bicarbonate (HCO_3^-) action in photosystem II, in addition to the high affinity site already established (for a review, see Chapter 1). Both the absence of HCO_3^- (HCO_3^- depleted thylakoids) and a high concentration of HCO_3^- (60 mM HCO_3^- added to non-depleted thylakoids) accelerate the variable chlorophyll a

fluorescence rise in the presence of 10 μM diuron (DCMU). In non-HCO_3^- depleted thylakoids the effect is independent of the order in which HCO_3^- and DCMU are added, whereas in HCO_3^- depleted thylakoids the effect is seen only when HCO_3^- is added before DCMU. It is proposed that the effect seen in HCO_3^- depleted thylakoids is due to the binding of HCO_3^- functionally near the site of DCMU binding, which is also where HCO_3^- exerts its major effect on electron transport between the primary quinone Q_A and the plastoquinone pool. It is suggested that the smaller effect seen in non-HCO_3^- depleted thylakoids is due to the binding of HCO_3^- at a second, lower affinity site. Binding at this site appears to require light, in contrast to the higher affinity site, which is inhibited by light. Bathocuproine, an inhibiter of the H_2O-to-silicomolybdate partial reaction, is synergistic with HCO_3^- in its effect on the variable chlorophyll a fluorescence of non-HCO_3^- depleted thylakoids, and may bind heterotropically with HCO_3^-. Thus, this second site of HCO_3^- binding appears to be functionally near the bathocuproine binding site.

B. Introduction

In non-HCO_3^- depleted chloroplasts, HCO_3^- stimulates whole-chain electron transport with methyl viologen (MV) as acceptor, but it inhibits the photosystem II (PS II) reduction of silicomolybdate (SiMo; Barr and Crane, 1976). It was suggested that SiMo accepts electrons, not directly from Q_A, as previously believed (e.g. Zilinskas and Govindjee, 1975; Giaquinta and Dilley, 1975), but via a side chain from Q_A, which is blocked by HCO_3^- (Barr and Crane, 1976). This observation suggests a HCO_3^- effect at a location other than the major effect at the Q_B protein.

It had been shown previously that HCO_3^- stimulates electron transport in non-HCO_3^- depleted chloroplasts, with a concomitant stimulation of

photophosphorylation (Punnett and Iyer, 1964), but it was argued that this effect was unrelated to the stimulatory effect of HCO_3^- seen in HCO_3^- depleted chloroplasts (Batra and Jagendorf, 1965). For one thing, the effect by Punnett required a significantly larger concentration of HCO_3^- than is required to stimulate non-depleted chloroplasts. Thus, the Punnett effect must be due to HCO_3^- or CO_2 binding at a second site in the membranes. The binding of HCO_3^- or CO_2 to this site has been proposed to effect a conformational change in the coupling factor CF_1 (Cohen and MacPeek, 1980). Little is known about this second site; it has generally been ignored in favor of the more dramatic HCO_3^- effect on Q_B. There is even the possibility that this second effect of HCO_3^- on CF_1 is non-specific, in that carboxylic acids in general may have similar effects on phosphorylation (Nelson et al., 1972). However, at least one effect of HCO_3^- on CF_1, the increased inhibition of phosphorylation by N-ethyl-maleimide in the presence of HCO_3^- and the decreased ability of adenylates to protect against this inhibition, is specific for HCO_3^- or CO_2 (Cohen and MacPeek, 1980).

The inhibition of the H_2O-to-SiMo reaction, as well as the stimulation of the whole-chain H_2O-to-MV reaction, was observed by Barr and Crane to require large concentrations of HCO_3^-. In this regard, the observation is similar to the effect on phosphorylation. Therefore, the hypothesis by Barr and Crane (1976) that HCO_3^- inhibits a cyclic flow of electrons around PS II, if correct, raises the interesting possibility that the Punnett effect may be more closely related to PS II than was previously supposed. In this section of the chapter, evidence is presented that supports the existence of a second, low affinity binding site for HCO_3^- or CO_2 in PS II.

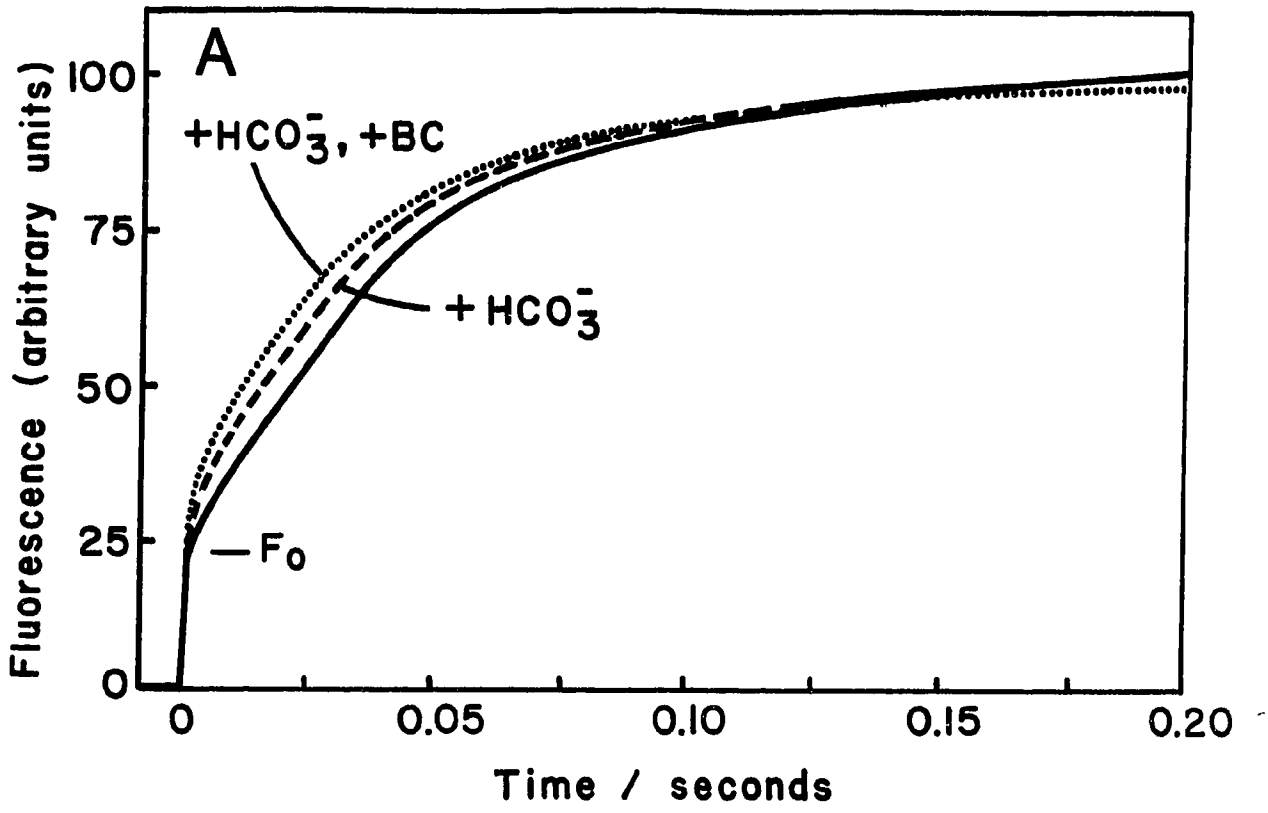
C. Materials and Methods

Thylakoids were isolated from hydroponically grown spinach as described in Chapter 2 and stored in liquid N_2. HCO_3^- depletion, measurements of rates of 2,6-dichlorophenolindophenol (DCPIP) reduction, and chlorophyll a (Chl a) fluorescence induction measurements were made as described in Chapter 2. In the experiments with non-HCO_3^- depleted thylakoids, 100 µl of saturated $NaHCO_3$ was added to the thylakoid suspension to make a final volume of 2 ml (60 mM $NaHCO_3$). This raised the pH considerably, unless a very high concentration of buffer was used, which was observed to have its own deleterious effects on the fluorescence transients. To get around this pH problem, the thylakoids were suspended in 50 mM Na phosphate, pH 7.2, for those experiments not involving the addition of HCO_3^-. For experiments where 60 mM HCO_3^- was added, the thylakoids were suspended in 50 mM Na phosphate, pH 6.6. Immediately upon addition of the HCO_3^-, which raised the pH to 7.2, a gentle gas stream at a partial pressure of CO_2 that would be in equilibrium with a 60 mM HCO_3^- solution at pH 7.2 (22% CO_2 and 78% air -- for the calculation involved, see Appendix II, Part D) was passed over the sample. With this method there was no drift of the pH; at the end of each experiment the pH was consistently 7.2.

D. Results

If, as is suggested by the hypothesis of Barr and Crane (1976), a DCMU-insensitive side chain from Q_A is blocked by HCO_3^-, then HCO_3^- should cause a faster buildup of Q_A^- and an accelerated Chl a fluorescence rise in the presence of DCMU. When 60 mM HCO_3^- was added to non-HCO_3^- depleted thylakoids in the presence of DCMU, an accelerated Chl a fluorescence rise was indeed observed (Fig. 5.1). The concentration of HCO_3^- chosen, 60 mM,

Figure 5.1. Chl *a* fluorescence transients of non-HCO_3^--depleted thylakoids in the presence of DCMU, with and without HCO_3^- and bathocuproine (BC). [A]: dashed line is with 60 mM HCO_3^-; dotted line is with 60 mM HCO_3^-, followed by 60 μM BC. [B]: dashed line is with 60 μM BC, followed by 60 mM HCO_3^-; dotted line is with 60 μM BC. The solid line in both cases is with no additions other than 10 μM DCMU. All samples were illuminated 30 s, then dark adapted for 10 min prior to measurement. Changes in pH were controlled as described in Materials and Methods. The [Chl] was 25 μg/ml.


A
+HCO3-, +BC
+HCO3-
—Fo
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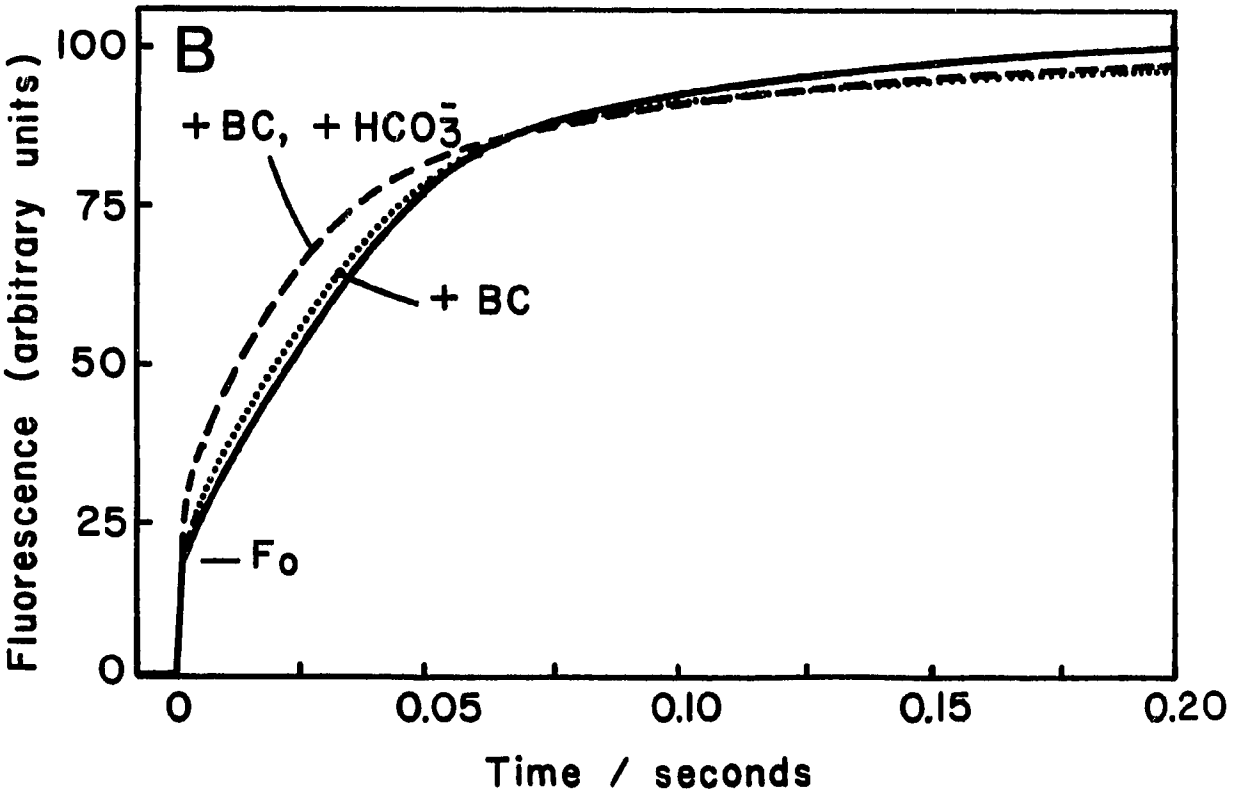
75
50
25
0
0
0.05
0.10
0.15
0.20
Time / seconds


B
+BC, +HCO3-
+BC
—Fo
Fluorescence (arbitrary units)
100
75
50
25
0
0
0.05
0.10
0.15
0.20
Time / seconds

is the concentration required to saturate the effect at pH 7.2. This effect appears to be specific for HCO_3^-, since other salts (60 mM NaCl, 60 mM $NaHCO_2$, or 60 mM Na_2SO_4) did not noticeably affect the fluorescence transient of DCMU-treated thylakoids.

Bathocuproine (4,7-dipheny1-2,9-dimethy1-1,10-phenanthroline), like HCO_3^-, was observed to inhibit the H_2O-to-SiMo partial reaction, while accelerating electron flow from H_2O to MV (Barr and Crane, 1976). Here, it is shown that bathocuproine accelerates the Chl a fluorescence rise in DCMU-treated thylakoids, as does HCO_3^- (Fig. 5.1). Interestingly, HCO_3^- and bathocuproine were observed to be synergistic in their effects on the fluorescence transient. In Fig. 5.1 (B), the effect of 60 μM bathocuproine is smaller than in Fig 5.1 (A), where the bathocuproine was added after HCO_3^- addition. Similarly, 60 mM HCO_3^-, when added after the bathocuproine, showed a larger effect than when it was added by itself. This synergism suggests a heterotropic binding of the two compounds.

This HCO_3^- effect appears to require light. When HCO_3^- was added in the dark, no effect on the fluorescence transient was observed. The fluorescence rise was accelerated only after the thylakoids had been incubated with HCO_3^- briefly in the light (Fig. 5.2). In contrast, the restoration of the Hill activity in HCO_3^- depleted chloroplasts requires a dark incubation with HCO_3^- (Stemler and Govindjee, 1973; Vermaas and Van Rensen, 1981).

In HCO_3^- depleted thylakoids, the addition of HCO_3^- causes a deceleration of the Chl a fluorescence rise in the presence of DCMU (Vermaas and Govindjee, 1982). This is opposite of the effect observed in non-HCO_3^- depleted thylakoids (Fig. 5.1), and may be due to an inhibition of the back reaction of Q_A^- with P_{680}^+ (Vermaas and Govindjee, 1982; see

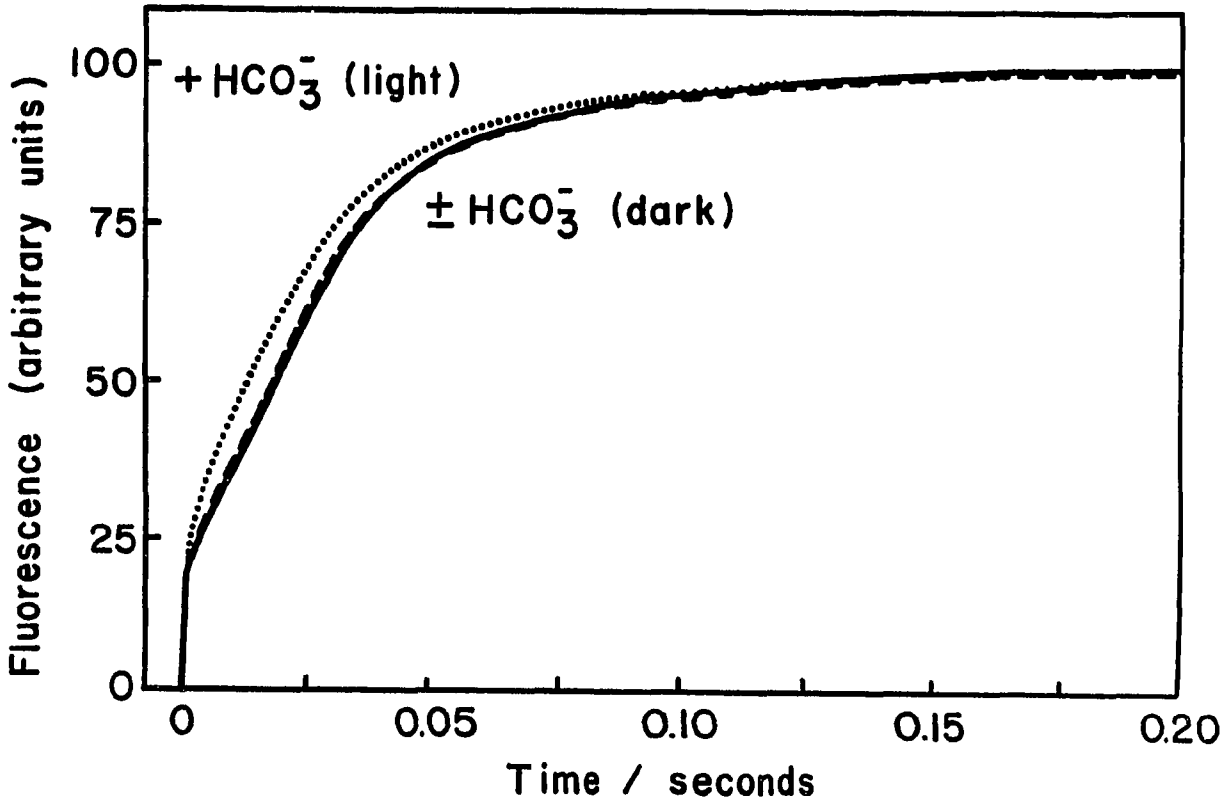
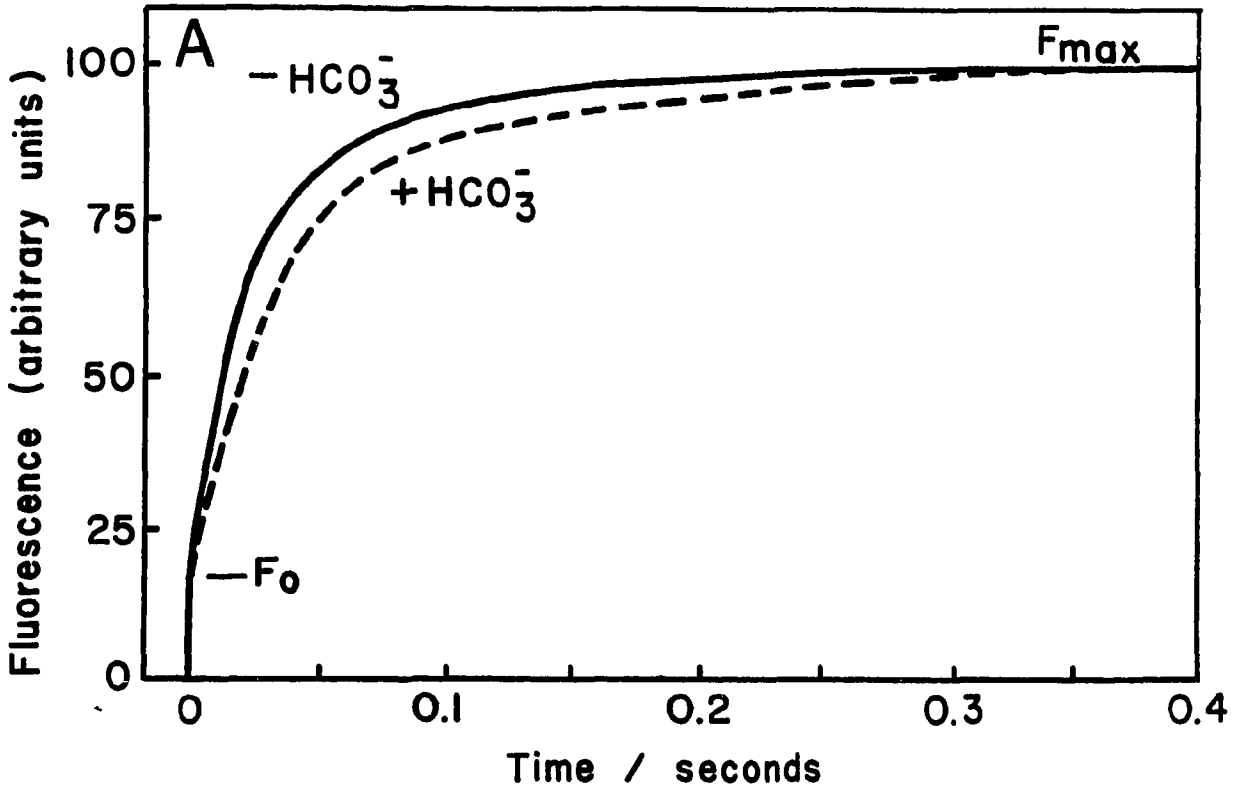
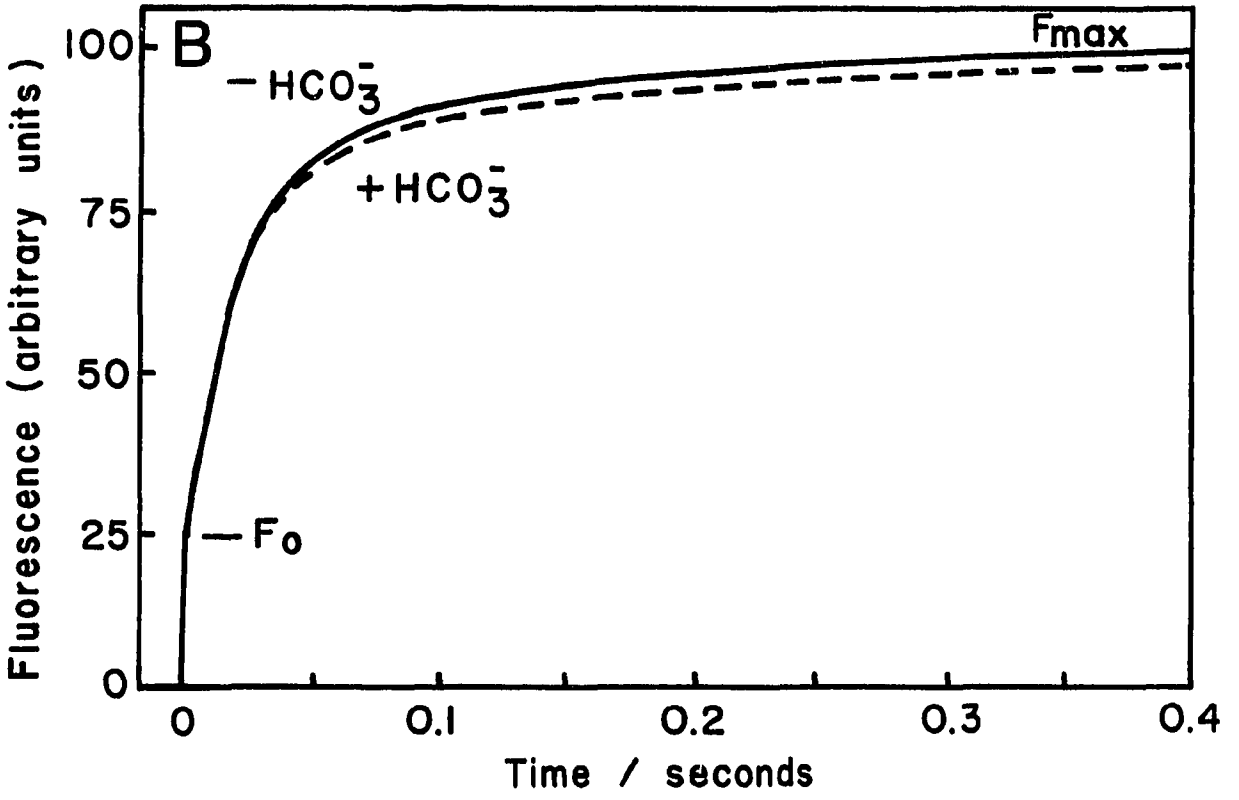


Figure 5.2. Chl a fluorescence transients of non-HCO_3^--depleted thylakoids in the presence of DCMU, with (dotted line) and without (dashed line) a brief incubation in the light. All samples were illuminated 30 s, then dark adapted for 10 min prior to measurement. 60 mM HCO_3^- was added either before the illumination (dotted line) or during the subsequent dark period (dashed line). Control thylakoids were given 10 μM DCMU, but no HCO_3^- (solid line). Changes in pH were controlled as described in Materials and Methods. The [Chl] was 25 μg/ml.

Figure 5.3. Chl *a* fluorescence transients of HCO_3^- depleted thylakoids, when HCO_3^- was added before (A) or after (B) addition of DCMU. All samples contained 10 μM DCMU, either alone (solid line) or with 12 mM HCO_3^- (dashed line). The [Chl] was 25 μg/ml.


A
Fmax
−HCO3−
+HCO3−
—Fo
100
75
50
25
0
Fluorescence (arbitrary units)
0
0.1
0.2
0.3
0.4
Time / seconds


B
Fmax
−HCO3−
+HCO3−
—Fo
100
75
50
25
0
Fluorescence (arbitrary units)
0
0.1
0.2
0.3
0.4
Time / seconds

also Eaton-Rye, 1987). This observation is confirmed in Fig. 5.3 (A), which shows the effect of 12 mM HCO_3^- on the Chl *a* fluorescence transient of HCO_3^- depleted thylakoids. Because DCMU blocks electron flow past Q_A, it was assumed that this effect was due to a site of action for HCO_3^- prior to Q_A. However, as shown here, this effect is apparently due to HCO_3^- binding near the binding site for DCMU, because the effect is only seen when HCO_3^- is added before the DCMU. When the DCMU was added first, this HCO_3^- effect was not seen (Fig. 5.3, B). DCMU is believed to bind at the Q_B binding site (*e.g.* Oettmeier and Soll, 1983), and HCO_3^- depletion has been shown previously to reduce the binding of several DCMU-type herbicides (Khanna *et al.*, 1981; Van Rensen and Vermaas, 1981; Vermaas *et al.*, 1982), indicating a close interaction between the HCO_3^- site and the herbicide binding site. Therefore, the effect of HCO_3^- on the fluorescence transient of HCO_3^- depleted thylakoids in the presence of DCMU (Fig. 5.3, A; Vermaas and Govindjee, 1982), is probably due to HCO_3^- binding on the Q_B protein, even though the effect appears to be on reactions prior to Q_A.

This observation is in contrast to the effect seen in non-HCO_3^- depleted thylakoids (Fig. 5.1), which is seen regardless of the order in which HCO_3^- and DCMU are added. Apparently, the binding of DCMU prevents HCO_3^- from reaching its site of action in HCO_3^- depleted thylakoids (see also Stemler, 1977), but not in the non-depleted samples. Therefore, it is not possible that the two effects are due simply to the HCO_3^- concentrations being on opposite sides of a concentration optimum. Rather, the effect in HCO_3^- depleted thylakoids seems to be due to HCO_3^- binding at the same site that is known to affect electron transport between Q_A and PQ (Govindjee *et al.*, 1976; Jursinic *et al.*, 1976; Siggel *et al.*, 1977; Vermaas and Govindjee, 1982), while the effect in non-depleted thylakoids

must be due to HCO_3^- or CO_2 binding at another site.

If the effect shown in Fig. 5.3 (A) is due to HCO_3^- binding at the same site that affects the quinone reactions, then it should require a dark incubation, since restoration of the Hill activity in HCO_3^- depleted thylakoids is inhibited in the light (Stemler and Govindjee, 1973; Vermaas and Van Rensen, 1981). This is indeed the case. When HCO_3^- depleted thylakoids were illuminated throughout the incubation with HCO_3^-, and DCMU was added later in the light, followed by a dark incubation, the result was the same as if the DCMU had been added first (i.e. same as Fig. 5.3, B). In other words, although HCO_3^- was added before the DCMU, it was unable to bind until the dark incubation, at which time the DCMU was already overlaying the site. This provides another contrast to the effect observed in non-HCO_3^- depleted thylakoids, since light appears to be required for HCO_3^- binding to that site (Fig. 5.2).

E. Discussion

The observations reported here are readily explained by postulating a low affinity site in addition to a high affinity site (in Section II evidence will be presented for more than one high affinity site, as well). The high affinity site at the level of Q_B is required for electron transport between Q_A and PQ (Govindjee et al., 1976; Jursinic et al., 1976; Siggel et al., 1977; Vermaas and Govindjee, 1982). Depleting HCO_3^- from this site reduces the binding of several DCMU-type herbicides (Khanna et al., 1981; Van Rensen and Vermaas, 1981; Vermaas et al., 1982), indicating a close interaction between the HCO_3^- site and the herbicide binding site. Light inhibits the binding of HCO_3^- to this site (Stemler and Govindjee, 1973; Vermaas and Van Rensen, 1981), perhaps because the ratio of Q_B^-/Q_B is higher (Stemler, 1979; see, however, Stemler et al.,

1984). The effect of HCO_3^- on the Chl a fluorescence transient of HCO_3^- depleted thylakoids in the presence of DCMU (Fig. 5.3, A; Vermaas and Govindjee, 1982) is apparently due to the binding of HCO_3^- at this high affinity site. DCMU appears to overlay this site, as HCO_3^- can bind only when it is added before the DCMU. When electron transport from Q_A^- to Q_B is blocked by DCMU, HCO_3^- apparently still allows some reoxidation of Q_A^-, either by an effect on the back reaction of Q_A^- with P_{680}^+ (Vermaas and Govindjee, 1982), or perhaps by permitting electron flow via Q_2, which requires a higher DCMU concentration for a complete block (Joliot and Joliot, 1983).

A second HCO_3^- site, of much lower affinity, is functionally near the site of bathocuproine binding. The observation that HCO_3^- accelerates the fluorescence rise in DCMU-treated thylakoids (Fig. 5.1) indicates that Q_A is reduced faster at high HCO_3^- concentrations, which is consistent with the model of Barr and Crane (1976), in which HCO_3^- inhibits a side chain from Q_A. Other models may also be possible. This second site preferentially binds HCO_3^- or CO_2 in the light, whereas the binding of HCO_3^- at the high affinity site is inhibited by light. Furthermore, the order of addition of DCMU and HCO_3^- is crucial to the high affinity site, but is of no consequence to the low affinity site.

Stemler (1977) determined, from binding studies of $H^{14}CO_3^-$ to HCO_3^- depleted thylakoids, that in addition to a fairly tightly bound pool of HCO_3^-, which is responsible for the restoration of the Hill activity, a larger low affinity pool exists of unknown function. Most of the tightly bound pool was removed when the thylakoids were washed with SiMo, but if DCMU was added first, much less of the HCO_3^- was removed by SiMo. This observation suggests that DCMU overlays the bound HCO_3^- (Stemler, 1977).

This conclusion is supported by the data presented here; the deceleration of the fluorescence rise by HCO_3^- in HCO_3^- depleted thylakoids in the presence of DCMU is seen only when HCO_3^- is added before the DCMU.

Stemler (1977) was unable to saturate the larger pool before running into solubility problems with the added HCO_3^-. He suggested that the concentration of low affinity sites was at least comparable to that of the bulk Chl. Likewise, the acceleration of the fluorescence rise by added HCO_3^- to non-depleted thylakoids in the presence of DCMU, reported here, requires large concentrations of HCO_3^-. It seems likely, then, that the low affinity site proposed here is the same site seen by Stemler (1977). In Chapter 7 it is proposed that this low affinity site creates an intramembrane reservoir of HCO_3^-.

The inhibition of the H_2O-to-SiMo reaction, as well as the stimulation of the H_2O-to-MV reaction, was also observed by Barr and Crane (1976) to saturate only at very high [HCO_3^-], though lower concentrations are reported elsewhere (Crane and Barr, 1977; Jursinic and Stemler, 1986). Similarly, the stimulation of electron transport and the enhancement of photophosporylation by HCO_3^- was observed to require 8 mM HCO_3^- for maximal effect (Punnett and Iyer, 1964). It is possible that all of these effects are due to HCO_3^- binding at the low affinity site proposed here. A possible mechanism for these diverse effects is proposed in Section II and further developed in Chapter 7.

SECTION II: THE KINETICS OF THE HCO_3^- EFFECT

A. Summary

Using HCO_3^- depleted thylakoids in which the basal activity is less than 7% of the fully restored activity after readdition of HCO_3^-, it is shown that the restored activity at a half-saturating HCO_3^- concentration

is non-linear with the chlorophyll concentration. This means that there was still some endogenous HCO_3^- remaining in these thylakoids, even though they appeared to be well depleted of HCO_3^-. A kinetic analysis of the activity curve for these thylakoids, as a function of HCO_3^- concentration, indicates that there are at least two sites of HCO_3^- in PS II, apparently with a high degree of cooperative binding. These sites are in addition to the low affinity site proposed in Section I. It is suggested that HCO_3^- is an essential activator for PS II electron transport, and that a complete removal of HCO_3^- would result in zero activity.

B. Introduction

Bicarbonate (HCO_3^-) is required for the photosynthetic reduction of plastoquinone (PQ) in photosystem II (PS II) of thylakoids. A variety of anions have been shown to compete with HCO_3^- for its binding site (Stemler and Jursinic, 1983; Stemler and Murphy, 1985), but so far only HCO_3^- has been shown to be able to sustain normal rates of electron transport through PS II. The chemical species required is HCO_3^-, not CO_2, H_2CO_3 or CO_3^{2-} (Chapter 3; Blubaugh and Govindjee, 1986). There has been some controversy over whether this HCO_3^- effect is physiologically significant, or whether it is simply an artifact resulting during the treatments to deplete thylakoids of HCO_3^-. Since formate (HCO_2^-) is routinely used to aid in depleting the membranes of HCO_3^-, it has been suggested that HCO_2^-, along with numerous other monovalent anions, inhibits PS II, and that HCO_3^-, being less inhibitory than the others, merely appears to stimulate electron transport by virtue of its competition with the more inhibitory HCO_2^- (Stemler and Jursinic, 1983; Stemler and Murphy, 1983).

Crucial to this argument has been the determination by several laboratories of an apparent dissociation constant K_d for the HCO_3^- * PS II

complex of 70–100 μM (Stemler and Murphy, 1983; Snel and Van Rensen, 1984; Jursinic and Stemler, 1986), which is rather high for physiological concentrations of HCO_3^- at pH 6.5, which is the pH optimum for the HCO_3^- effect (Khanna et al., 1977; Vermaas and Van Rensen, 1981). This could imply that the HCO_3^- sites are ordinarily empty in vivo. However, at the outer surface of the membrane, which is exposed to the stroma and towards which HCO_3^- is presumed to bind, the pH would be much higher, and so would $[HCO_3^-]$. Also, the determination for K_d has, in each case, been made on the basis of the concentration of HCO_3^- added, not on the $[HCO_3^-]$ actually present. Since about half of the added HCO_3^- is converted to CO_2 at pH 6.5, where the determinations were made, the K_d is overestimated by a factor of nearly two (Chapter 3).

Snel and Van Rensen (1984) have entertained the possibility that both HCO_3^- and HCO_2^- may be part of a regulatory mechanism to control linear electron transport, since HCO_2^- is produced in the peroxisomes. However, no solid evidence exists for the accumulation of HCO_2^- inside chloroplasts (see, however, Zelitch, 1972). Nitrite is another anion that competes for the HCO_3^- site (Stemler and Murphy, 1985; Eaton-Rye et al., 1986), and it is known to exist in chloroplasts (e.g. Anderson, 1981). However, Eaton-Rye (1987) has determined a K_d for nitrite of approximately 5 mM. Thus, the effect of nitrite under physiological conditions can be presumed to be negligible.

On the other hand, attempts to demonstrate a large and reversible HCO_3^- effect in the absence of inhibitory anions have met with only limited success (Eaton-Rye and Govindjee, 1984; Eaton-Rye et al., 1986). This does not necessarily mean that HCO_3^- has no real role other than to outcompete more inhibitory anions; it could simply reflect the difficulty

of depleting membranes of bound HCO_3^- without some help. Therefore, an important question to answer is whether there can be a tightly bound pool of HCO_3^- with a K_d much lower than what has been measured. In other words, is there more than one HCO_3^- binding site, in addition to the low affinity site discussed in Section I? There are other possibilities, as well:

(1) The K_d that has been measured could be overestimated, since it has been determined at relatively high salt concentrations, and high [NaCl] has been shown to facilitate removal of HCO_3^- (Chapter 2, Figs. 1 & 2; Eaton-Rye et al., 1986). As mentioned above, it is certain that the K_d is overestimated by at least a factor of two, due to ignoring the equilibrium conversion of HCO_3^- to CO_2 (Chapter 3).

(2) A possibility raised by Snel and Van Rensen (1984) is that HCO_3^- binding might bring the regulatory site into an active configuration which only very slowly relaxes back to an inactive state when HCO_3^- dissociates from the site. This way, a very few HCO_3^- molecules could keep all of the regulatory sites in the active state when HCO_2^- is absent.

(3) A concentrating mechanism could exist for raising the local [HCO_3^-] in the vicinity of the binding site.

This section examines the kinetics of HCO_3^- restoration of the Hill activity, and presents evidence for at least two sites of HCO_3^- binding in PS II. These sites are in addition to the low affinity site proposed in Section I. The kinetics are shown to be rather complex and are most consistent with the assumption of a high degree of cooperativity between the HCO_3^- sites and a delocalized pool of HCO_3^- with access to the binding sites. The delocalized pool may be the low affinity site discussed in Section I (see also Stemler, 1977; Blubaugh and Govindjee, 1984). The

other two sites are presumed to be actual binding sites, separate from this pool. Both the cooperativity and the delocalized pool would facilitate binding at low concentrations of HCO_3^-.

C. Materials and Methods

Thylakoids were isolated from market spinach as described in Chapter 2 and were used fresh. HCO_3^- depletion of the thylakoids and measurement of 2,6-dichlorophenolindophenol (DCPIP) reduction rates were done as described in Chapter 2. The experimental protocol was identical to that in Chapter 3, except that all measurements were made at pH 6.5. Also, 10 mM CH_3NH_2 and 10 nM Gramicidin D, both uncouplers of photophosphorylation, were added to the cuvettes before determination of DCPIP reduction rates, as these appeared to lengthen, somewhat, the initial rates of DCPIP reduction and provided better precision in the determination of the rates. The theoretical curves were generated with an LSI-11 minicomputer (Digital Equipment Corporation), using a program of personal design (see Appendix I, Part E).

D. Results and Discussion

Since HCO_3^- is the species required for the reactivation of electron transport in PS II (Blubaugh and Govindjee, 1986; Chapter 3), it is necessary, when determining K_d, to consider the actual $[HCO_3^-]$ present under the experimental conditions, rather than the $[HCO_3^-]$ added. This means that velocity curves should be plotted as a function of the equilibrium $[HCO_3^-]$, calculated from the pH and the $[HCO_3^-]$ added. This was not done in previous determinations of K_d (Stemler and Murphy, 1983; Snel and Van Rensen, 1984; Jursinic and Stemler, 1986), resulting in a value that is in error by a factor of nearly two (at pH 6.5, at which the determinations were done, nearly half of the HCO_3^- added is converted to

CO_2).

Such a velocity curve has been published in a demonstration of the active species (Blubaugh and Govindjee, 1986; Chapter 3, Fig. 3.3). In that experiment, about 40% of the fully restored activity was observed when no HCO_3^- was added. Undoubtedly, this was at least partly due to some endogenous HCO_3^- that had not been removed during the depletion procedure, since other experiments have yielded activities as low as 6-8% of the fully restored activity when no HCO_3^- is added, even when the depletion was done under identical conditions. It is common to obtain different degrees of HCO_3^- depletion from different preparations; this is one reason why results from different preparations cannot be averaged (see e.g. Van Rensen and Vermaas, 1981). The starting plant material for the experiment in Chapter 3 was extraordinarily fresh (picked from a garden on the same day as the experiment), and the formate concentration in that experiment was relatively low (5 mM); these facts may explain the reason for the less complete depletion (see discussion in Chapter 2). Whether the 6-8% of fully restored activity observable after a good depletion is also due to some tightly-bound endogenous HCO_3^-, or reflects some residual activity of PS II when all HCO_3^- has been removed, is not known. It has been suggested, however, that a thorough depletion of HCO_3^- results in a complete block of electron transport from Q_B to PQ (Vermaas and Govindjee, 1982; Govindjee et al., 1984).

If it is assumed that HCO_3^- is an essential activator (i.e. there should be zero activity when all HCO_3^- is removed), then, by extrapolation to zero activity, it can be estimated from Fig. 3.3 of Chapter 3 that an endogenous [HCO_3^-] of 0.31 mM was present. At first glance, this seems unbelievably high, considering that at the highest pH of the experiment,

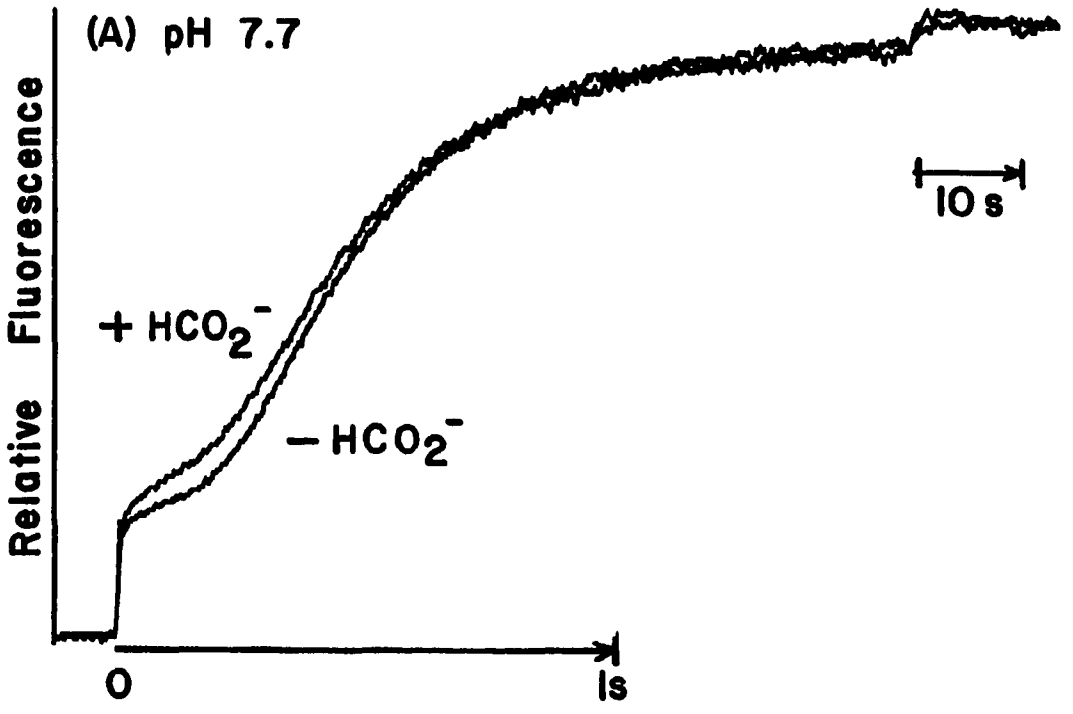
pH 6.9, the equilibrium [HCO_3^-] in an aqueous solution left open to the air would be only 0.04 mM, and would be even less at the other pHs (see Appendix II, Part A). However, it is possible that a sequestered pool of HCO_3^- exists, which keeps the [HCO_3^-] high in the vicinity of the binding site, even when the [HCO_3^-] is low in the bulk phase. This could be explained, for instance, by a large pool of low-affinity sites inside the membrane. While CO_2 would be expected to be able to cross the lipid and/or protein barrier to this pool, the exchange with the bulk phase could be limited by the availability of H^+, within this space, for the conversion of HCO_3^- to CO_2. Thus, the exchange could be rather slow. The large number of these low affinity sites would provide a buffering of the [HCO_3^-] in the vicinity of the high-affinity site(s).

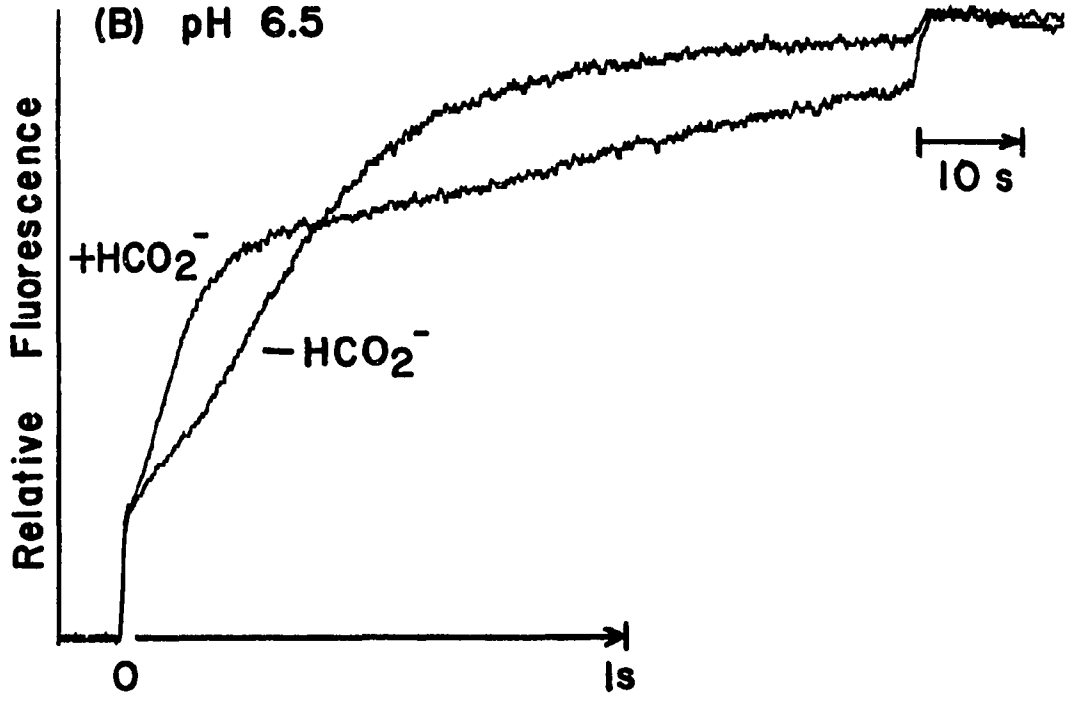
Stemler (1977) demonstrated the existence of a large low affinity pool of HCO_3^- (see also Section I of this chapter), and also showed that 30 min was required for the binding to reach equilibrium. Furthermore, the HCO_3^- bound to the high affinity site(s) does not readily exchange with the bulk phase, except at very low pH (Stemler, 1977). This may indicate the existence of some ionizable group, the protonation of which induces a conformational change that allows the release of bound HCO_3^-. Exposure of the low-affinity sites to the bulk phase could result in a significant amount of HCO_3^- exchanging with the bulk phase. Given these arguments, the 40% activity in Fig. 3.3 of Chapter 3 could be due to a fairly large amount of endogenous HCO_3^-, despite the depletion procedure. This would imply that the K_d is quite large, since only 40% of the activity would be expected to be restored to thoroughly depleted thylakoids at a [HCO_3^-] of 0.31 mM (when 5 mM formate is present), but it also implies a physiological mechanism for keeping the HCO_3^- bound, even when the bulk [HCO_3^-] is low.

As discussed in Chapter 2, were the low pH requirement simply to convert HCO_3^- to CO_2, a good depletion could theoretically be obtained at pH 7.7 by flushing away the CO_2 from solution, but all of my attempts to do so have failed. Fig. 5.4 shows, for example, the effect of 100 mM formate on the fluorescence transient at pH 7.7 and at pH 6.5. It is apparent that HCO_3^- is being removed by the formate at pH 6.5 (c.f. Fig. 2.4), but very little effect occurs at pH 7.7, even after an extended incubation. The amount of HCO_3^- present in an aqueous solution exposed to the air at pH 7.7 is only about 220 μM (see Appendix II, Part A), which is not enough HCO_3^- to outcompete 100 mM formate. The apparent lack of a formate effect is consistent with the HCO_3^- site(s) being sequestered from the bulk phase, except at low pH. As will be shown later, the bound HCO_3^- appears to be exchangeable under the conditions in which the data of Fig. 3.3 in Chapter 3 were obtained. When the equilibrium $[HCO_3^-]$ values of Fig. 3.3 are corrected to reflect an effective endogenous $[HCO_3^-]$ of 0.31 mM, then the data yields a straight line when plotted as a double reciprocal Hill plot with n=2 (Fig. 5.5). The excellence of the fit would be very coincindental, indeed, if the justification for an endogenous $[HCO_3^-]$ of 0.31 mM were invalid. This plot suggests that there are two sites of HCO_3^- binding with a high degree of cooperativity. The justification for this statement comes from the analysis of a two-substrate kinetic model (see Segel, 1975, pp 355-385). The justification will be made briefly here; a more detailed justification is contained in Appendix II, Part E.

A simple kinetic scheme for an enzyme with two substrate binding sites, each capable of producing product, is shown below:

Figure 5.4. The effect of 100 mM formate on the Chl *a* fluorescence transient of spinach thylakoids at pH 7.7 (A) versus pH 6.5 (B). The thylakoids were suspended in 50 mM Na phosphate buffer to a [Chl] of 20 μg/ml, and were dark adapted 10 min before each measurement.


(A) pH 7.7
Relative Fluorescence
+ HCO_2^-
− HCO_2^-
10 s
0
1s


(B) pH 6.5
Relative Fluorescence
+HCO_2^-
− HCO_2^-
10 s
0
1s

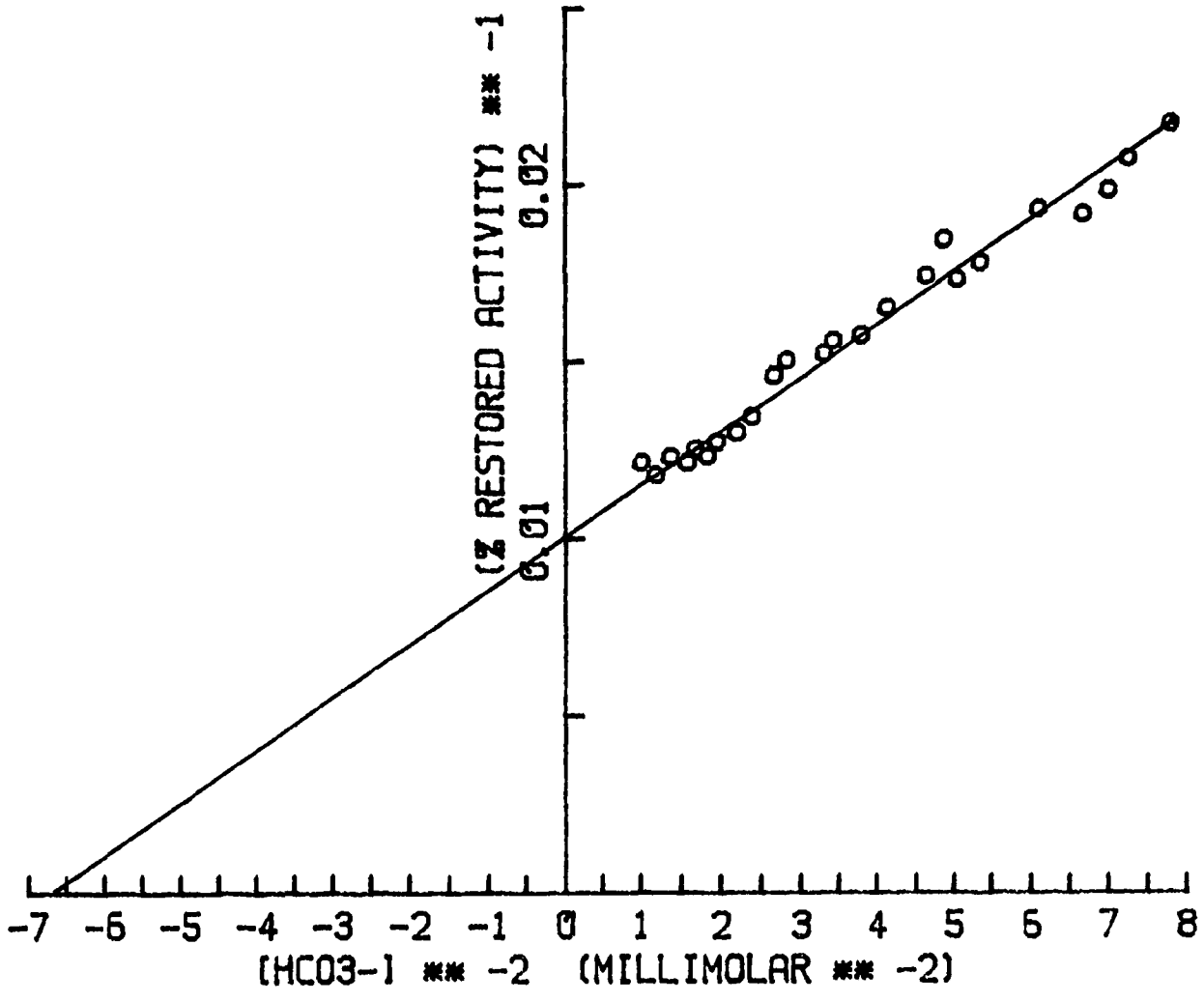
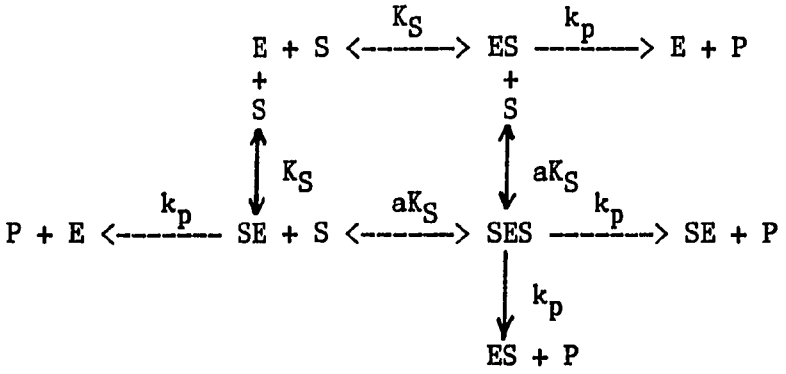


Figure 5.5. A double reciprocal Hill plot, with n=2, of the rate of DCPIP reduction, expressed as a percentage of the fully restored activity, in HCO_3^- depleted thylakoids, as a function of the equilibrium HCO_3^- concentration. The data was taken from Fig. 3.3 and corrected for an assumed endogenous [HCO_3^-] of 0.31 mM. Details of the protocol are in the legend to Fig. 3.2.



(5.1)

If there is a high degree of cooperativity between the two substrate binding sites (i.e. the cooperativity factor, a, is much less than unity), so that the second site fills immediately upon filling of the first site, then the steady state concentrations of SE and ES are close to zero, and the velocity equation is simplified to become:

$$V = \frac{V_{max}\,[S]^2}{K' + [S]^2}\,, \quad \text{where } K' = aK_S^2 \qquad (5.2).$$

This equation can be rearranged to give the linear equation:

$$\frac{1}{V} = \frac{K' + [S]^2}{V_{max}\,[S]^2} = \frac{K'}{V_{max}} \cdot \frac{1}{[S]^2} + \frac{1}{V_{max}} \qquad (5.3).$$

Thus, a plot of $1/V$ versus $1/[S]^2$ yields a straight line, with slope K'/V_{max} and intercept $1/V_{max}$.

It can be further shown that any number of cooperative binding sites, n, will give the velocity equation

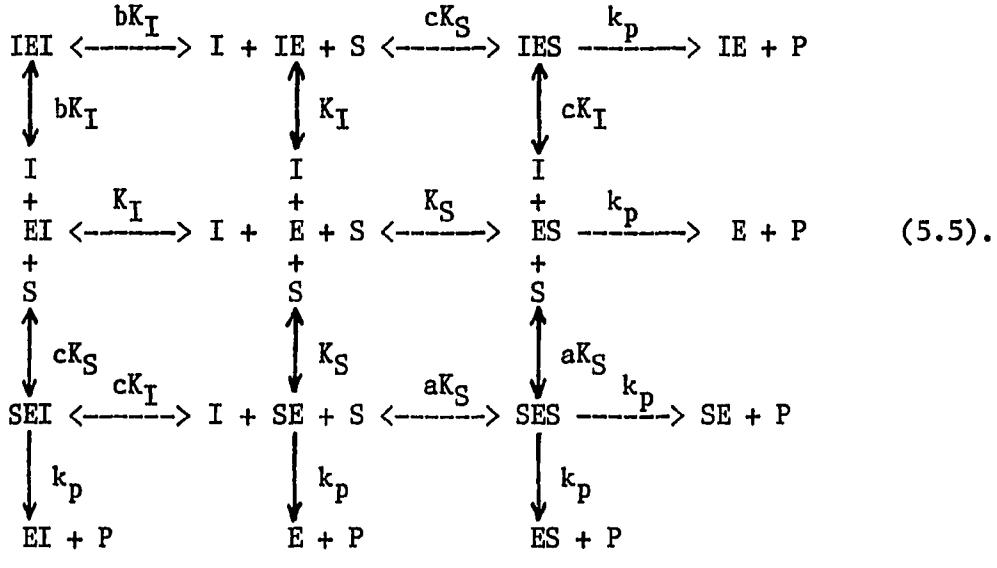
$$V = \frac{V_{max}\,[S]^n}{K' + [S]^n}\,, \quad \text{where } K' = KS^n(a^{n-1}b^{n-2}c^{n-3}\ldots z) \qquad (5.4),$$

and a,b,c, etc. are cooperativity factors,

as long as the cooperativity between the sites is sufficient to keep the

steady-state concentrations very low of those intermediates with only some of the sites filled. Thus, if a high degree of cooperativity is suspected, a plot of 1/v versus $1/[S]^n$ that gives a straight line will indicate the number of binding sites, n. It should be pointed out that n is the minimum number of sites; if the assumption of high cooperativity is not valid, linearity will be achieved with an n smaller than the number of binding sites. An extreme example of this is that any number of binding sites that are independent (i.e. a,b,c, etc. = 1) will yield a straight line with n=1, which is the well known Lineweaver-Burke plot for an enzyme that behaves Michaelis-Menten kinetics.

The data of Fig. 5.5 was obtained with 5 mM formate present. Formate is believed to be competitive with HCO_3^- (e.g. Snel and Van Rensen, 1984), so the kinetic scheme should be expanded to include an inhibitor, as shown below:



Again, if it is assumed that the cooperativity is high between substrate sites, so that not only are the concentrations of SE and ES extremely low,

but of SEI and IES, as well (i.e. the second S strongly outcompetes I), then the velocity equation is

$$V = \frac{V_{max}[S]^2}{C \cdot K' + [S]^2} \quad \text{, where } K' = aK_S^2 \text{, as before, and} \qquad (5.6).$$

$$C = 1 + \frac{2[I]}{K_I} + \frac{[I]^2}{bK_I}$$

The linear equation for the double-reciprocal plot becomes

$$\frac{1}{V} = \frac{C \cdot K'}{V_{max}} \cdot \frac{1}{[S]^2} + \frac{1}{V_{max}} \qquad (5.7).$$

The above analysis can also be shown to apply whether S is a substrate or activator, or whether S is a substrate at one site and an activator at the other, and whether or not K_S is identical for each site (see Appendix II, Part E). Since this is the case, it is not necessary to distinguish between models in which HCO_3^- is an activator or a substrate. The linearity of the plot in Fig. 5.5 suggests that there are two HCO_3^- binding sites with a high degree of cooperativity. Fig. 5.6 shows the curve obtained if the same data is plotted in a classical Lineweaver-Burke plot (n=1), and Fig. 5.7 shows the data superimposed on the velocity curve predicted from Eqn. 5.6.

This analysis, so far, has been based on the assumption that the residual activity present in HCO_3^- depleted thylakoids is due to endogenously bound HCO_3^-, and the amount of endogenously bound HCO_3^- can be estimated assuming that HCO_3^- is an essential activator. In an enzyme preparation, endogenous substrate can be detected by measuring the velocity as a function of enzyme concentration, at a constant but subsaturating

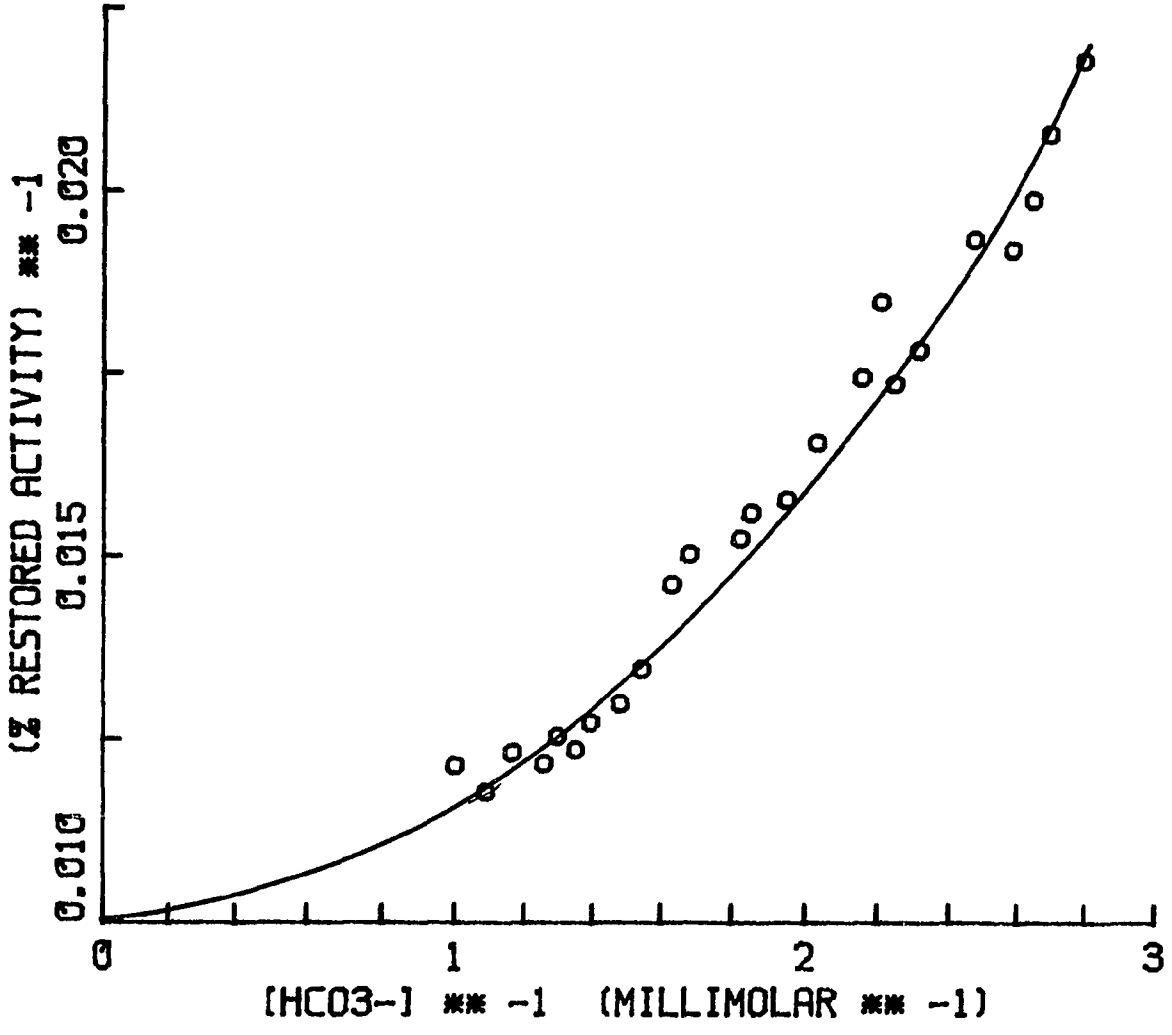


Figure 5.6. A double reciprocal Lineweaver-Burke plot of the rate of DCPIP reduction, expressed as a percentage of the fully restored rate, in HCO_3^- depleted thylakoids, as a function of the equilibrium HCO_3^- concentration. The data was taken from Fig. 3.3 and corrected for an assumed endogenous $[HCO_3^-]$ of 0.31 mM.

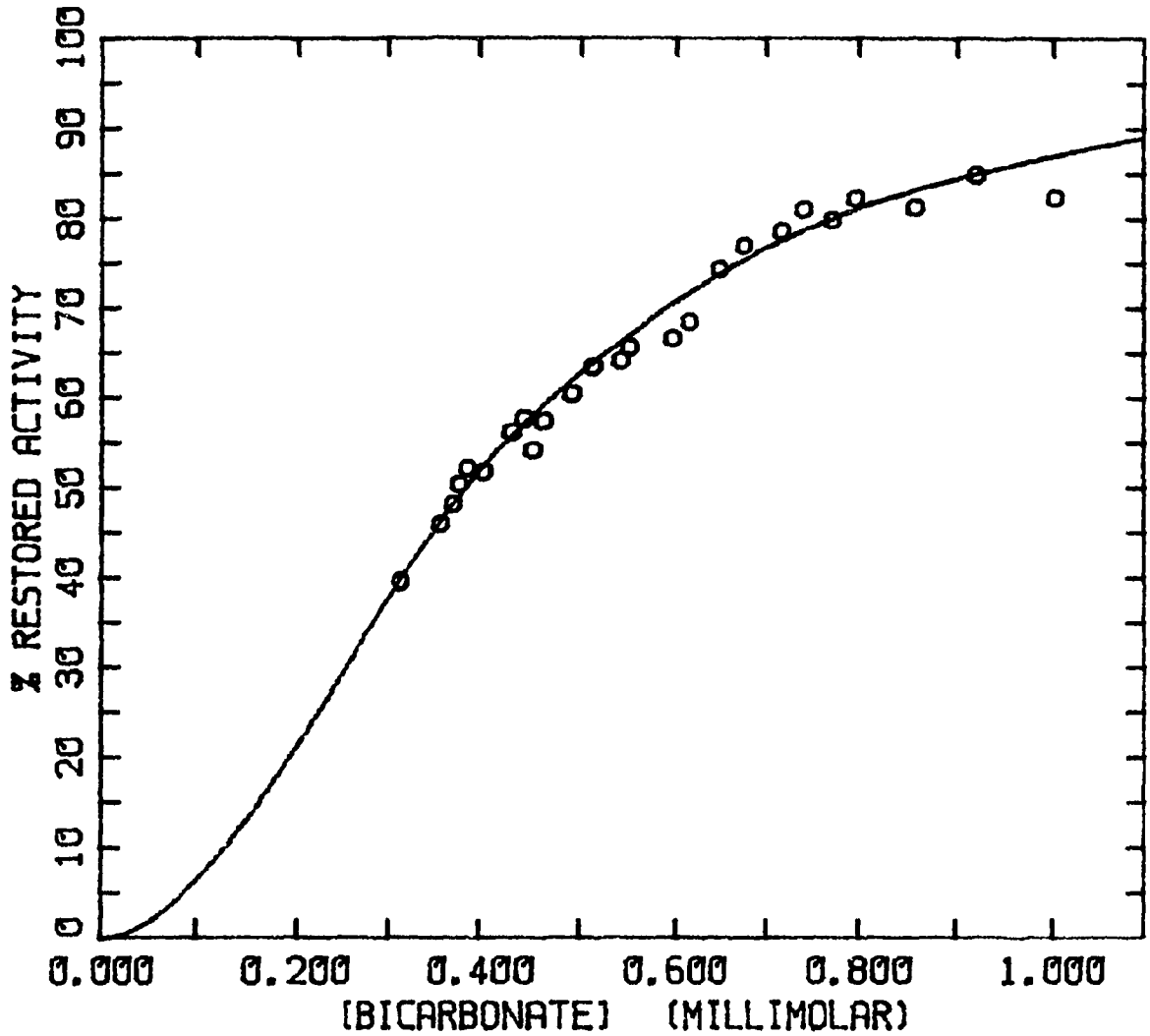


Figure 5.7. The rate of DCPIP reduction, expressed as a percentage of the fully restored rate, in HCO_3^- depleted thylakoids as a function of the equilibrium HCO_3^- concentration. The data is from Fig. 3.3, corrected for an assumed endogenous $[HCO_3^-]$ of 0.31 mM. The solid line is the predicted curve for a velocity equation of the form

$$v = \frac{V_{max}\,[S]^2}{CK' + [S]^2}$$

where $[S] = [HCO_3^-]$, V_{max} = 100% restored rate, and $CK' = 0.149\ mM^2$.

concentration of added substrate. If endogenous substrate is present, the curve will deviate from linearity, since adding more enzyme also adds more substrate. This can be demonstrated mathematically, using the velocity equation for a simple enzyme/substrate complex:

$$\frac{V}{[E]_t} = \frac{k_p [S]}{K_S + [S]} \tag{5.8}$$

for the reaction

$$E + S \underset{}{\overset{K_S}{\longleftrightarrow}} ES \xrightarrow{k_p} E + P \tag{5.9}$$

Eqn. 5.8 is linear if [S] is constant. However, $[S] = [S]_{added} + [S]_{endog}$, and $[S]_{endog} = c[E]_t$, where c is a constant. Therefore, Eqn. 5.8 becomes

$$\frac{V}{[E]_t} = \frac{k_p ([S]_{added} + c[E]_t)}{K_S + ([S]_{added} + c[E]_t)} \tag{5.10}$$

which is no longer linear for $c \neq 0$. Again, the same argument applies whether HCO_3^- is a substrate or an activator. Whatever form the velocity equation takes, $V/[E]_t$ is constant as long as both substrate and activator concentrations are constant. However, if either one increases with increasing $[E]_t$, then a plot of V vs. $[E]_t$ will not be linear.

Therefore, a plot of velocity versus [Chl] can be used to determine whether endogenous HCO_3^- exists after HCO_3^- depletion. However, the deviation from linearity will occur only if the endogenous HCO_3^- is free to exchange with the bulk phase. Otherwise, the concentration of HCO_3^- does not increase upon addition of more membranes, but rather each membrane sees the endogenous HCO_3^- present within it, regardless of the presence of other membranes (<u>i.e.</u> $[S]_{endog} \neq c[E]_t$, but rather $[S]_{endog}$ = constant).

In the following experiment, thylakoids were made fresh and depleted of HCO_3^- under identical conditions as the experiment in Chapter 3. This time, a good depletion was obtained, as the residual activity in the absence of added HCO_3^- was less than 7% of the fully restored rate. The velocity curve, plotted as a function of the equilibrium [HCO_3^-], is shown in Fig. 5.8. Fewer points were obtained than in the experiment of Chapter 3, because the sample here was also used to obtain the velocity curve as a function of [Chl] (Fig. 5.9), and it was desired to keep the measurements to under 2 hrs, so as to avoid artifacts in the velocity curves due to thylakoid aging. The first and last points determined are shown as the uppermost points in Fig. 5.9, and it can be seen that there is little change in this determination over the course of the experiment. It is also my experience that little loss of activity occurs in the first few hrs after thylakoid isolation under the conditions employed here (membranes kept on ice, and [Chl] > 2 mg/ml).

In a separate experiment, the velocity as a function of [Chl] was determined for thylakoids that were not depleted of HCO_3^- (Fig. 5.10), in order to determine at which [Chl] the actinic light starts to become limiting. It can be seen that the response is linear up to about 92 μg Chl in the 4.1 ml volume ([Chl] = 23 μg/ml). The slope of this graph gives the electron transport rate, and the scatter in the data indicates the range of experimental error in this type of measurement (rate = 323 $\pm$ 7 (avg. dev.) μmoles DCPIP reduced per mg Chl per hr). It is also worth noting that although these membranes obviously contain endogenous HCO_3^- (they are not depleted), there is no deviation from linearity because the HCO_3^- is not exchangeable with the bulk phase (Stemler, 1977).

All of the points in Fig 5.9 were made at a [Chl] at which the light

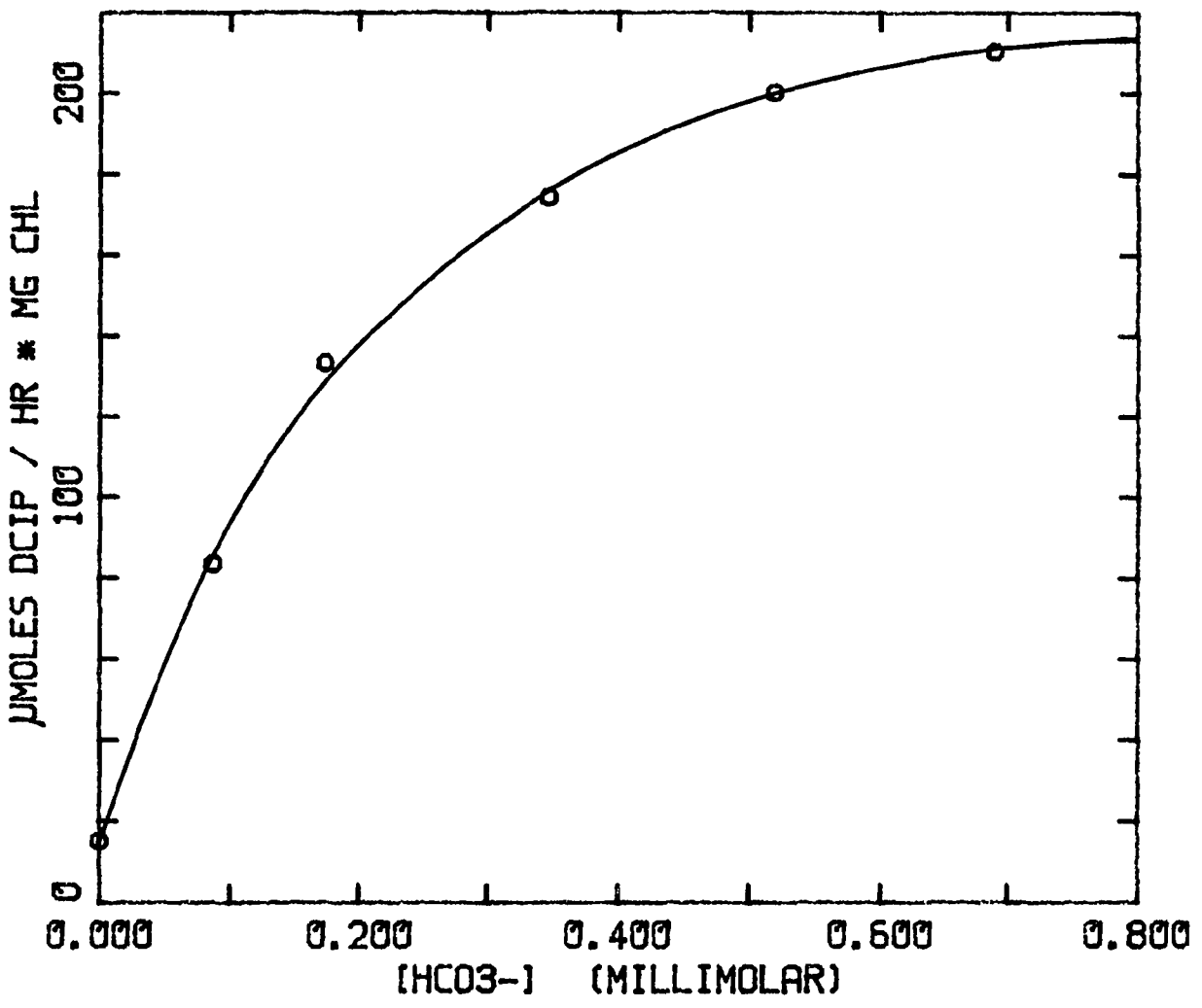


Figure 5.8. The rate of DCPIP reduction of HCO_3^- depleted thylakoids as a function of the equilibrium HCO_3^- concentration. The experimental protocol was identical to that of Fig. 3.3, except that all of the data was measured at pH 6.52. The [Chl] was 10.9 µg/ml in a 4.0 ml volume (0.0436 mg Chl).

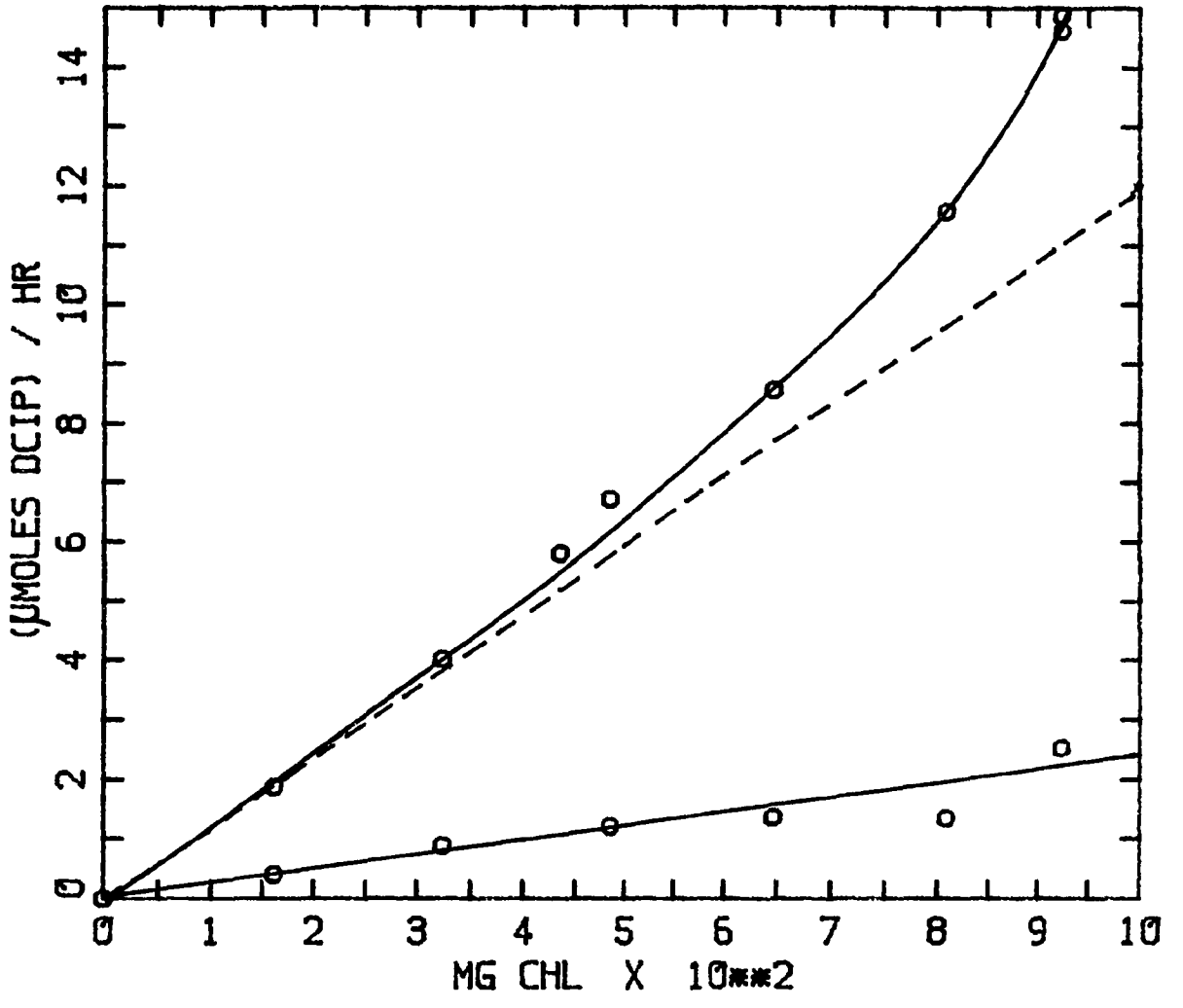


Figure 5.9. The rate of DCPIP reduction of HCO_3^- depleted thylakoids as a function of the amount of Chl in a 4.0 ml volume. The thylakoid sample used here was the same one used to obtain the data in Fig. 5.8. The slope tangent to the curve at any point is the rate of DCPIP reduction as commonly expressed (µmoles DCPIP reduced per mg Chl per hr). 0.3 mM HCO_3^- was added (0.16 mM after equilibrium) to obtain the upper curve. No HCO_3^- was added for the lower curve. The pH was 6.5.

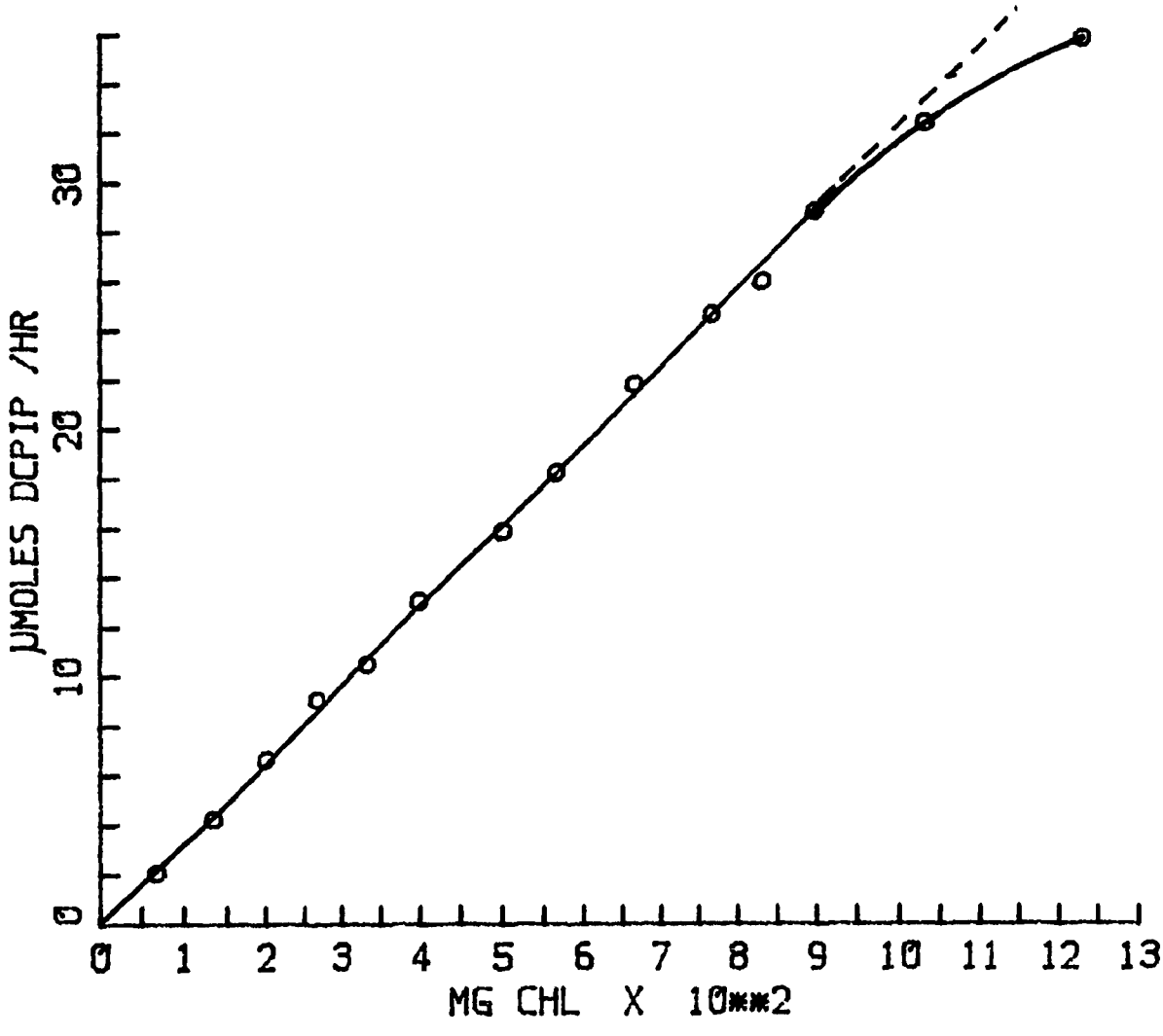


Figure 5.10. The rate of DCPIP reduction of non-HCO_3^--depleted thylakoids as a function of the amount of Chl in a 4.0 ml volume. The slope of the curve is the rate as commonly expressed on a per mg Chl basis. The curve deviates from linearity above 92 µg Chl ([Chl] = 23 µg/ml) due to attenuation of the light intensity to the point of becoming rate limiting.

is saturating. The residual activity of the HCO_3^- depleted thylakoids in the absence of added HCO_3^- appears to be linear with [Chl], whereas the addition of a constant but subsaturating concentration of HCO_3^- gives a curve that clearly deviates from linearity. This indicates that these membranes did indeed contain some endogenously bound HCO_3^-, which became exchangeable with the bulk phase after the addition of a subsaturating $[HCO_3^-]$. In the absence of HCO_3^-, the residual rate should also have deviated from linearity if the HCO_3^- were exchangeable with the bulk phase (Eqn. 5.10 applies, with $[S]_{added} = 0$). The linearity of the residual rate suggests that the endogenously bound HCO_3^- was prevented from leaving. At each [Chl], the residual rate in the absence of added HCO_3^- was first measured, then HCO_3^- was added in the dark and permitted to equilibrate for 3 min, and then the new rate was measured. Therefore, the two curves differ not only in whether or not HCO_3^- was added, but also in whether or not the thylakoids had been previously illuminated. The ability of the tightly-bound pool to exchange with the bulk phase after the addition of HCO_3^- may have been due to an increase in the ionic strength, or to a conformational change induced by the pre-illumination, or both. Raising the ionic strength does facilitate the removal of HCO_3^- during the depletion procedure (Chapter 2). Earlier, it was suggested that perhaps the reason that a low pH is required for a good HCO_3^- depletion is because a protonatable group is involved in a mechanism that holds HCO_3^- tightly, once it has been bound. Protonation of this group would presumably thwart the mechanism, and HCO_3^- could be released. A negatively charged protein group involved in a salt bridge may, therefore, be involved. Raising the ionic strength would be expected to shield this charge and could thereby facilitate the release of HCO_3^-.

Less than 7% of the fully restored activity remained in these thylakoids after HCO_3^- depletion (Fig. 5.8). It appears likely, then, that were all of the endogenously bound HCO_3^- to have been removed, the activity would have been zero. In other words, HCO_3^- may indeed be an essential activator. A double reciprocal plot of the data in Fig. 5.8, without any correction for the endogenous HCO_3^-, is apparently linear (Fig. 5.11). Without an awareness of the tightly held HCO_3^-, it would appear that the HCO_3^- site follows Michaelis-Menten kinetics. However, after a correction for the endogenous HCO_3^-, the deviation of the double-reciprocal plot from linearity should become evident. Fig. 5.12 shows such a plot, assuming that the amount of bound HCO_3^- is that amount that would be in equilibrium with 0.02 mM HCO_3^-, estimated by extrapolating the curve in Fig. 5.8 to zero activity. This is a conservative estimate, since some sigmoidicity is expected to occur at this low end of the curve (Fig. 5.7). Nevertheless, a deviation from linearity is apparent in Fig. 5.12.

Linear Lineweaver-Burke plots, such as Fig. 5.11, have been published for the HCO_3^- dependence (Vermaas et al., 1982; Snel and Van Rensen, 1983), from which the conclusion has been drawn that there is a single HCO_3^- binding site (Snel and Van Rensen, 1983). Part of the problem with these plots, aside from the lack of consideration for the possibility of endogenous HCO_3^-, is the fewness of points (usually no more than four), which makes any deviation from linearity more difficult to detect. It is possible, even, for a preparation to show a concave curve in the double reciprocal plot if the endogenous HCO_3^- is not accounted for. The data of Fig 3.2 of Chapter 3 exhibits such behavior (not shown). Such concavity is actually apparent in the data from some publications, although a straight line is invariably drawn (Snel and Van Rensen, 1983; Jursinic and Stemler,

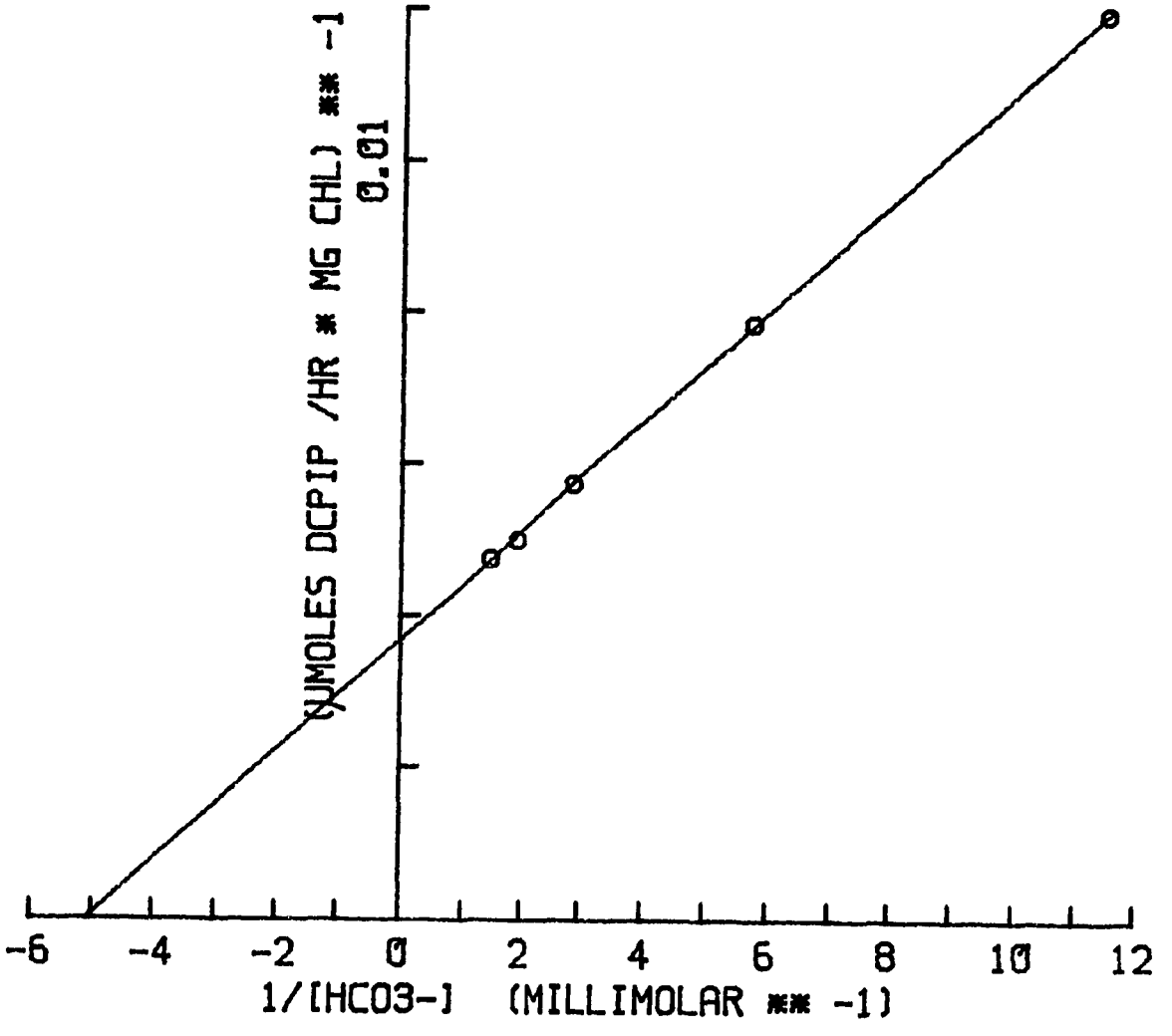


Figure 5.11. A double reciprocal Lineweaver-Burke plot of the rate of DCPIP reduction in HCO_3^- depleted thylakoids as a function of the equilibrium HCO_3^- concentration, with no correction for endogenous HCO_3^-. The data is from Fig. 5.8.

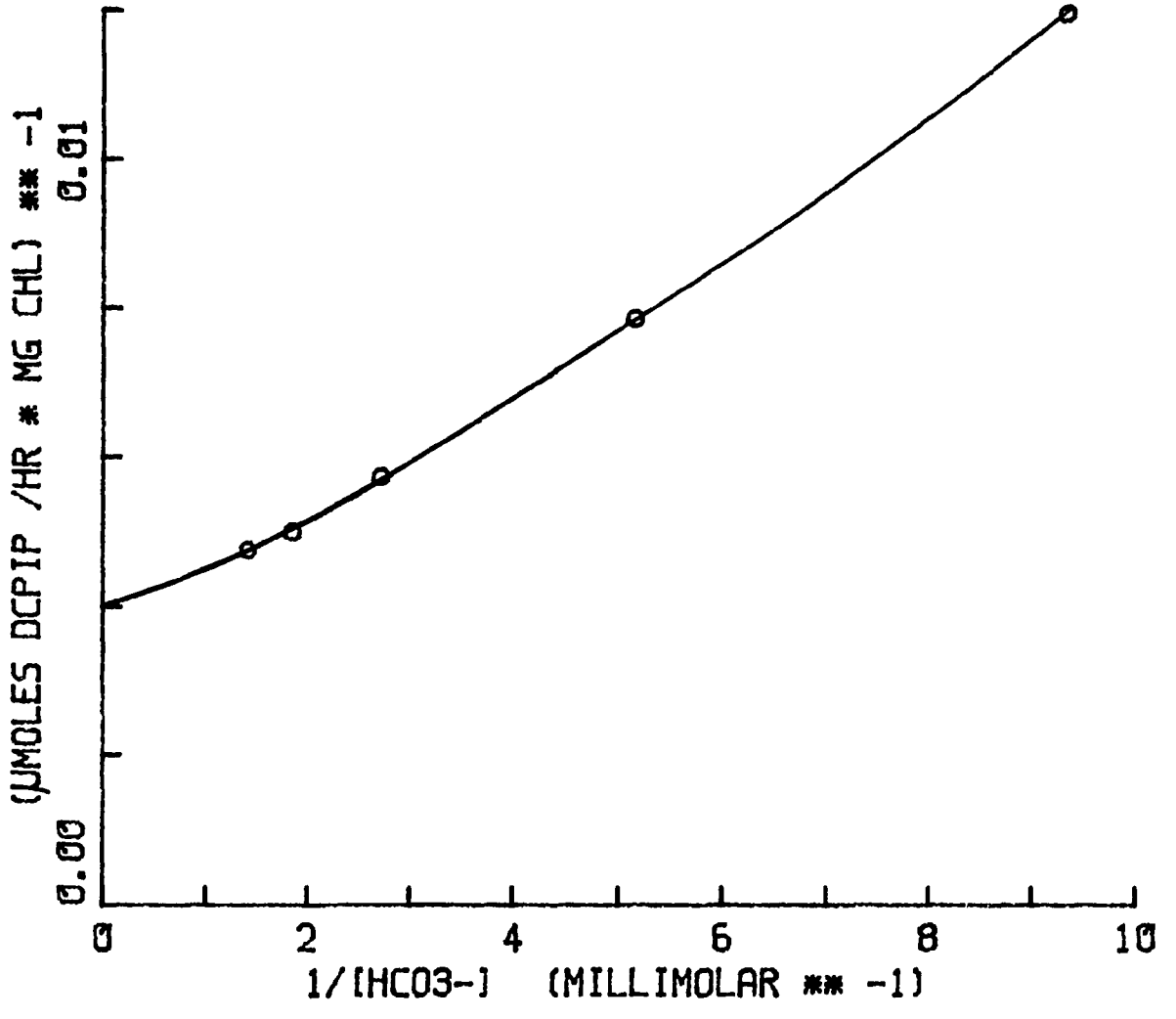


Figure 5.12. A double reciprocal Lineweaver-Burke plot of the rate of DCPIPO reduction in HCO_3^- depleted thylakoids as a function of the equilibrium HCO_3^- concentration, corrected for an assumed endogenous $[HCO_3^-]$ of 0.02 mM. The data is from Fig. 5.8.

1987).

Given the heterogeneity of PS II (for a review, see Black et al., 1986), it is conceivable that HCO_3^- binds to a single site, but with different affinities in the different types of centers. This would be analogous to a multiple-enzyme system, in which each enzyme catalyzes formation of the same product. A double reciprocal plot for such a system is not linear. However, the deviation from linearity is in the opposite direction from that shown in Fig. 5.12 (for a discussion of the kinetics of such a system, see Segel, 1975, pp. 64-71). While HCO_3^- may yet bind with different affinities to the different types of PS II centers, this model is not sufficient to explain the data presented here.

It was conservatively estimated above that an effective $[HCO_3^-]$ of at least 0.02 mM was endogenous to the membranes of Fig. 5.8. However, this concentration is insufficient to give a linear fit to a double-reciprocal plot with n=2. A linear fit is achieved instead with n=1.4. This is still sufficient to exclude the possibility of a single site, as n represents the minimum number of sites; although the actual number of sites must obviously be an integer, non-integer values for the apparent n is quite common if the cooperativity is not very high (e.g. Segel, 1975, p. 361). Of course, as explained above, the effective endogenous $[HCO_3^-]$ may be higher than 0.02 mM, due to the sigmoidicity of the curve. An excellent fit is achieved to the double-reciprocal plot with n=2, if a value of 0.1 mM is assumed for the endogenous $[HCO_3^-]$. Fig. 5.13 shows the theoretical curve for a two-site, cooperative model with an effective endogenous $[HCO_3^-]$ of 0.1 mM and the predicted curve for a Michaelis-Menten one-site model with no endogenous HCO_3^-, along with the data of Fig. 5.8. It is clear that with only a few points, clustered around the middle of the curve, it is

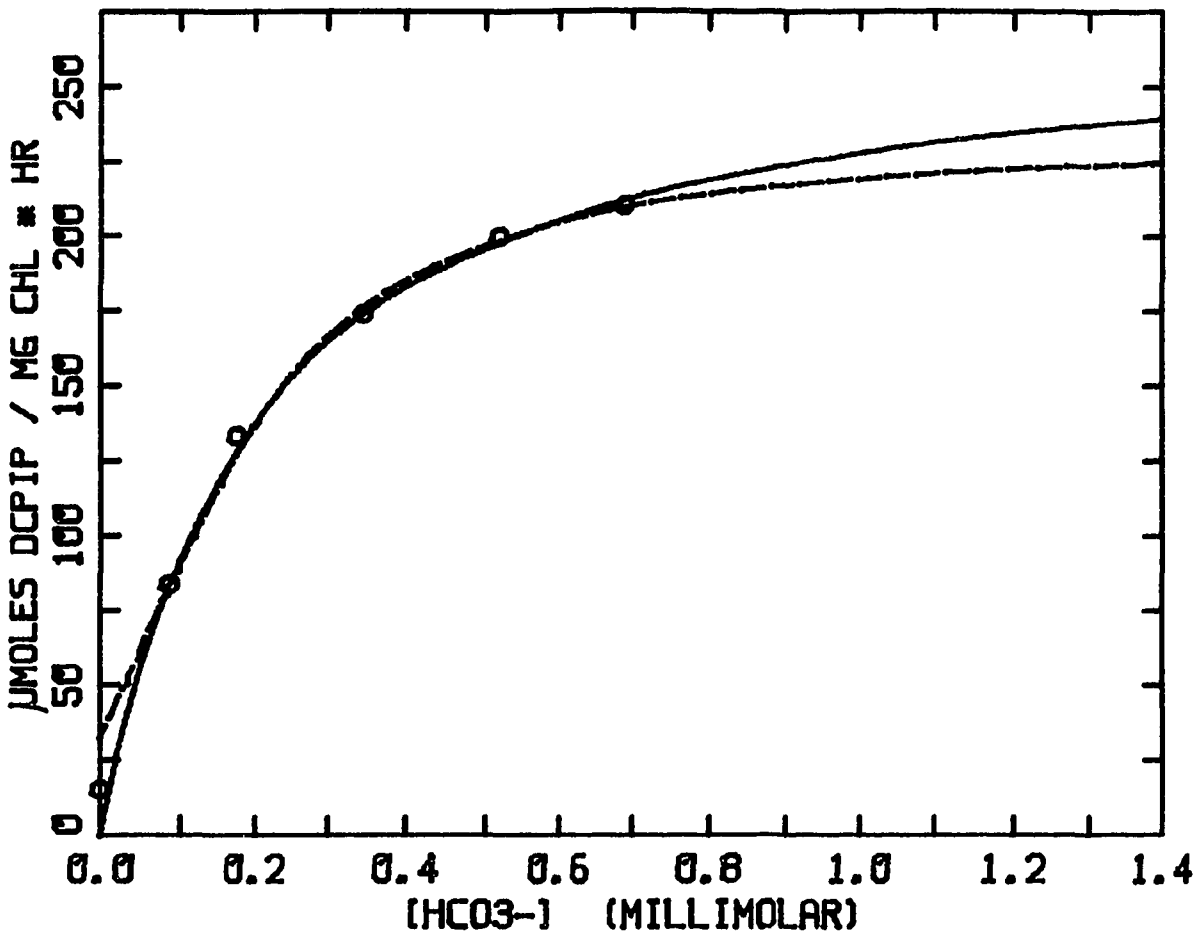


Figure 5.13. Two theoretical predictions of the data of Fig. 5.8, based on the assumption of a single HCO_3^- binding site and no endogenous HCO_3^- (solid line) or on the assumption of two HCO_3^- binding sites with cooperative binding and an endogenous [HCO_3^-] of 0.1 mM (dashed line). The velocity equations used are: Solid line:

$$v = \frac{V_{max}\,[S]}{K_S + [S]}$$

with V_{max} = 273 μmoles DCPIP reduced per mg Chl per hr and K_S = 197 μM. Dashed line:

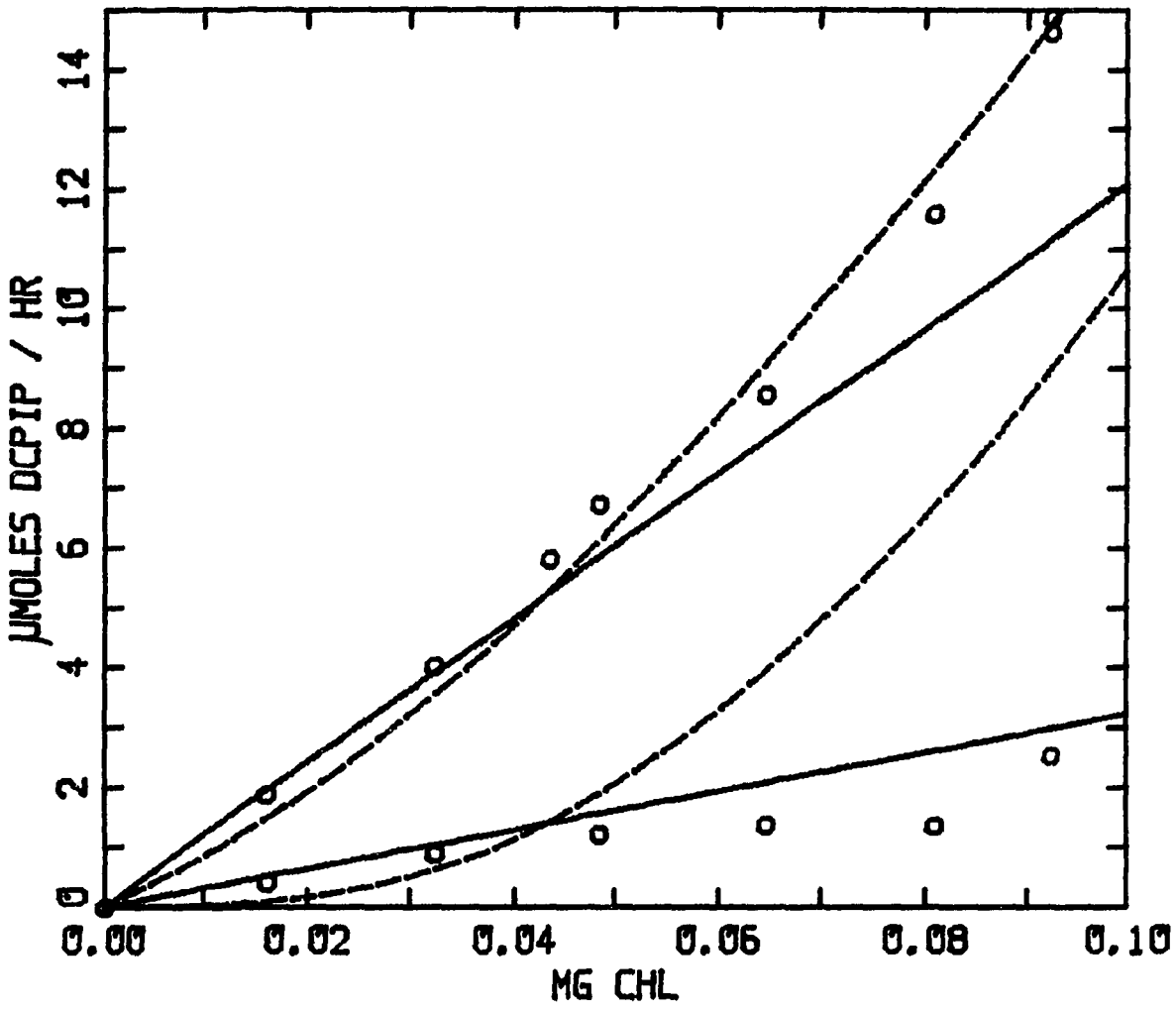
$$v = \frac{V_{max}\,[S]^2}{K' + [S]^2}$$

with V_{max} = 230 μmoles DCPIP reduced per mg Chl per hr and K' = 0.0613 mM^2.

Figure 5.14. Two theoretical predictions of the data of Fig. 5.9, based on the assumption of two HCO_3^- binding sites with cooperative binding and an endogenous $[HCO_3^-]$ of 0.1 mM. The velocity equation is

$$v = \frac{V_{max}\,([S]_{added} + [S]_{endog})^2}{K' + ([S]_{added} + [S]_{endog})^2}$$

with V_{max} = 230 μmoles DCPIP reduced per mg Chl per hr, $K' = 0.0613\ mM^2$, and $[S]_{added}$ = 0.16 mM (top pair of curves) or $[S]_{added}$ = 0 (lower pair of curves). Solid lines: the endogenous HCO_3^- is assumed to be non-exchangeable with the bulk phase, so $[S]_{endog}$ is constant at 0.1 mM. Dashed lines: the endogenous HCO_3^- is assumed to freely exchange with the bulk phase, so that $[S]_{endog} = c[Chl]$, with c = 0.1 mM / 0.0436 mg Chl. 0.0436 mg Chl was the constant amount of Chl in Fig. 5.8, from which the value of 0.1 mM for the endogenous $[HCO_3^-]$ was assumed.


μMOLES DCPIP / HR
0
2
4
6
8
10
12
14
0.00
0.02
0.04
0.06
0.08
0.10
MG CHL

impossible to discriminate between the two models, unless it is known whether or not there is endogenously bound HCO_3^-.

Since Figs. 5.8 and 5.9 were both obtained from the same preparation, it is necessary that any model chosen be able to predict both sets of data. This was attempted with the two-site model just described. As shown in Fig. 5.13, it is consistent with the velocity curve. The model's prediction of the activity vs. [Chl] curve is shown in Fig. 5.14. The dashed lines are the predictions based on the assumption that the endogenous HCO_3^- is freely exchangeable with the bulk phase. The solid lines are the predictions based on a non-exchangeable pool of HCO_3^-. The model predicts the data fairly well if it is assumed that there is no exchange until after the addition of HCO_3^-. As discussed earlier, this may be due to the increase in the ionic strength, or to the preillumination, or both.

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CHAPTER 6

OTHER ASPECTS OF THE BINDING ENVIRONMENT

A. Summary

Tetraethylthiuram disulfide (TETD; Sulfiram) inhibits the alternate oxidase of the cyanide-insensitive respiratory pathway in plant mitochondria. It also inhibits photosynthesis. Because TETD is a metal chelator, and because HCO_3^- may bind to the non-heme Fe^{2+} in photosystem II (PS II), the site of TETD inhibition in broken chloroplasts was examined. There seem to be two sites of action on the acceptor side of PS II: one is an impairment of electron transport between pheophytin (Pheo) and the first stable acceptor of PS II, Q_A. This impairment is manifested as a quenching of the variable chlorophyll a fluorescence, and a specific quenching of the F_{695} peak, relative to the F_{685} peak, in the fluorescence spectrum at 77 K. The H_2O-to-silicomolybdate partial reaction is also inhibited. The other site of inhibition is a substantial block between Q_A and the second stable electron acceptor, Q_B. This site of inhibition is shown by a several-fold increase in the half-time of Q_A^- oxidation, as monitored by the decay of the variable fluorescence after an actinic flash. There was no inhibition detected in the partial reactions associated with PS I. The inhibition of electron transport between Q_A and Q_B is similar to the effect of HCO_3^- depletion. The inhibition of $Pheo^-$ to Q_A electron transfer is similar to an effect of the plastoquinone analog 6-azido-Q_0C_{10} (Chapter 4). These effects could be due to binding of TETD to the Fe^{2+}.

B. Introduction

Tetraethylthiuram disulfide (TETD; Sulfiram) is an inhibitor of photosynthesis (Lindahl and Akerstrom, 1965), but its site of action has

never been identified. Its chemistry was well characterized, however, by Lindahl and Akerstrom (1965): TETD is an effective metal chelator and is redox active with a midpoint potential at pH 6.3 of +0.33 volts. It can also act as a sulphydryl reducing agent. Aside from the possibility of it inhibiting photosynthesis by any of these mechanisms, very little is known about its interaction with the photosynthetic system. The only other published paper dealing with this compound and photosynthesis is that by Bown et al. (1984), who showed that it stimulated the acidification of the culture medium when added to suspensions of asparagus mesophyll cells. The authors speculated that TETD may inhibit photosynthesis by lowering the intracellular pH.

TETD is also a potent inhibitor of the cyanide-insensitive respiratory pathway of plant mitochondria (Grover and Laities, 1981). Initially, the impetus for the study presented in this chapter was an hypothesis that the O_2 evolving system of photosynthesis may be mechanistically similar to an alternate oxidase working in reverse. Therefore, it was predicted that TETD would inhibit the O_2 evolving complex. However, no evidence was found for a block before the site of electron donation by artificial PS II electron donors. Instead, evidence is presented here that TETD inhibits the reoxidation of the primary quinone acceptor Q_A^-, and also has an effect on electron transfer from reduced pheophytin ($Pheo^-$) to Q_A. In this respect, it is similar to an effect of the plastoquinone analog 6-azido-Q_0C_{10} (see Chapter 4). In Chapter 4 it was suggested that the removal of HCO_3^- permits the direct oxidation of Q_A^- by the exogenous quinone. In Chapter 7, it is suggested that that this is due to a conformational change that alters the distances between the Fe^{2+} and the bound quinones. This same conformational change may be responsible for the inhibition of

electron flow from $Pheo^-$ to Q_A by 6-azido-Q_0C_{10}. A similar or identical conformational change may be responsible for the effects of TETD. Since TETD is a metal chelator, it is likely that the site of binding is the Fe^{2+}. By implication, HCO_3^- may also bind to the Fe^{2+}, and the effects of TETD may be due to removal of HCO_3^-. The evidence for TETD's site of action is presented here.

C. Materials and Methods

Tetraethylthiuram disulfide (TETD) was obtained from Sigma Chemical Co. and was used without further purification. The compound is sparingly soluble in water, and even μM amounts tend to precipitate when added to thylakoids from an ethanol-based stock solution. The most reliable method found to introduce the compound into thylakoids, without getting the artifacts associated with precipitation, was to homogenize an excess of TETD with the thylakoid suspension buffer and to use the decanted supernatant, saturated with TETD, for subsequent thylakoid suspension. The concentration of the TETD could be measured spectrophotometrically after complexing the TETD with a Cu salt, as described by Akerstrom and Lindahl (1962). It was found that the maximum concentration of TETD that could be obtained was about 130 μM, which was not quite sufficient to saturate the TETD effects. Chlorophyll a (Chl a) fluorescence induction, rates of electron transport, decay of the variable Chl a fluorescence, and the fluorescence spectrum at 77 K were all measured as described in Chapter 2.

D. Results and Discussion

Fig. 6.1 shows the effect of increasing concentrations of TETD on the Chl a fluorescence transient. The effect is saturated at slightly higher than 130 μM TETD. This concentration is approximately the limit of the

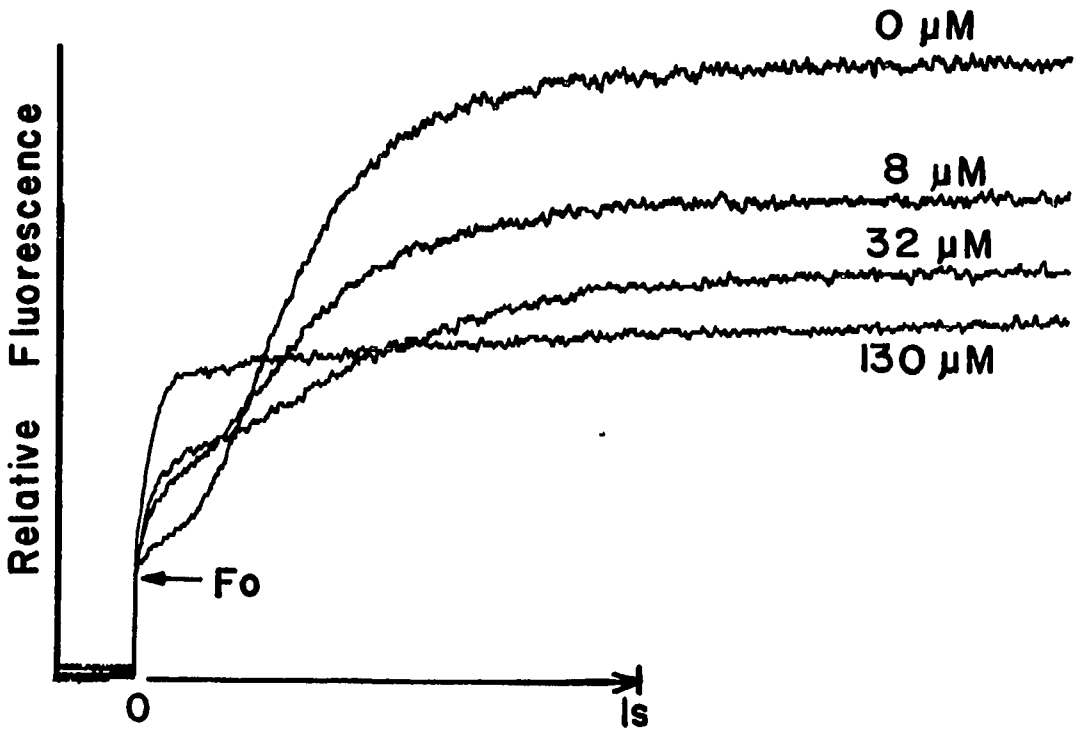


Figure 6.1. The effect of increasing concentrations of TETD on the Chl a fluorescent transient of spinach thylakoids. The thylakoids were suspended in 50 mM Na phosphate, pH 7.2, along with the indicated concentration of TETD. For details, see Materials and Methods. The thylakoids were dark adapted 5 min before measuring the transient. The [Chl] was 25 μg/ml.

solubility of TETD in H_2O; it is unknown whether higher concentrations, could they be obtained, would continue the trend. The maximum level of fluorescence, F_{max}, is quenched considerably by the TETD, but there is no effect on the initial fluorescence level, F_o. The absence of any effect on F_o suggests that the quenching is not due to non-photochemical quenching, but is due to a diminished $[Q_A^-]$ (the fluorescence yield is believed to be an indicator of $[Q_A^-]$; for a discussion, see Chapter 2). It was similarly observed that TETD has no quenching effect on the fluorescence of a Chl solution. At first glance, this effect on the fluorescence transient seems to suggest a block on the donor side of PS II.

Although F_{max} is quenched, the intermediate fluorescence level F_I is increased. The reason for this is not known, but is common in treatments that are known to inhibit the donor side of PS II. However, the F_I level in the thylakoids maximally inhibited by TETD is higher than usual for treatments that block on the donor side (c.f. Fig. 6.2), which suggests at least an additional effect on the acceptor side of PS II.

For comparison, Fig. 6.2 shows the effect on the fluorescence transient of a mild heating of the thylakoids at 45 C for 5 min, a treatment that is known to selectively inhibit the O_2 evolving complex. The addition of catechol/ascorbate, an artificial donor system to PS II, restores the variable fluorescence to these thylakoids, as is well known. The original F_{max} level is obtained, although a high F_I level remains. The reason for including these results here is to show that the catechol/ ascorbate donor system was indeed functioning. The catechol/ascorbate did not relieve the quenching effect of a subsaturating TETD concentration (Fig. 6.3), which suggests that the site of inhibition is after the site of donation by catechol/ascorbate (i.e. after the primary donor to P_{680}, Z).

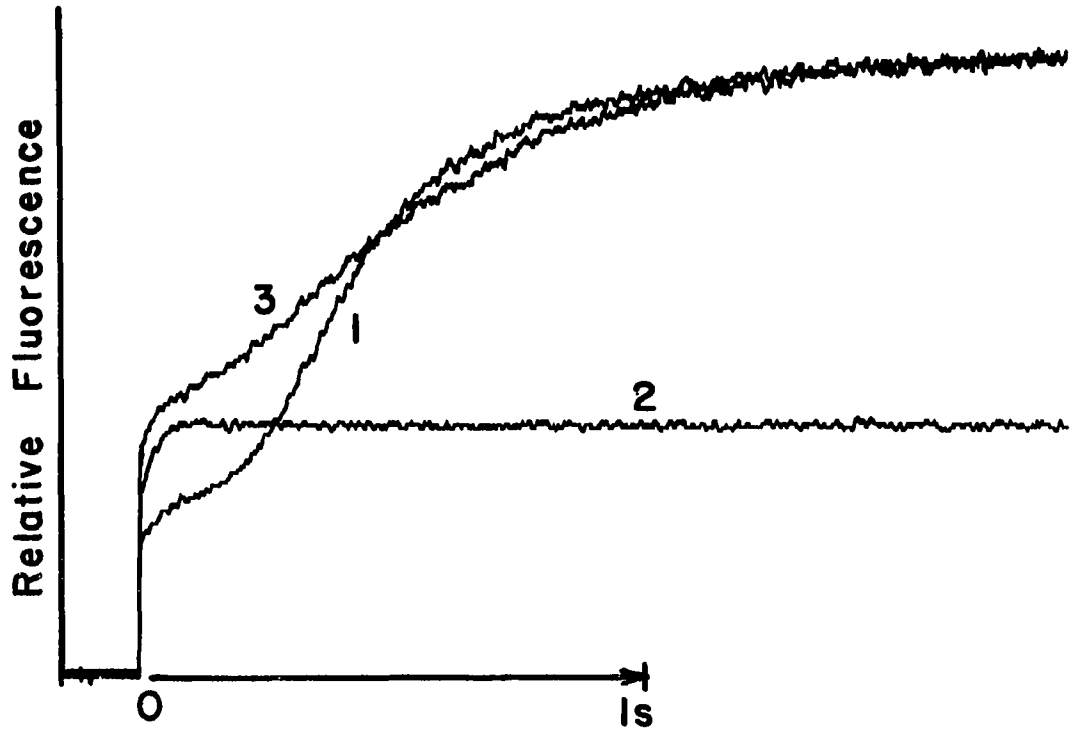


Figure 6.2. Restoration of the variable Chl a fluorescence to heat-treated thylakoids by the artificial electron donating system catechol/ascorbate. Trace 1: control thylakoids, suspended in 50 mM Na phosphate, pH 7.5. Trace 2: the thylakoids were heated in a water bath at 45 C for 5 min to impair the O_2 evolving complex. Trace 3: heat-treated thylakoids from Trace 2 with 0.5 mM catechol and 3 mM ascorbate as an electron donor system to PS II. The thylakoids werre dark adapted for 5 min prior to each measurement.

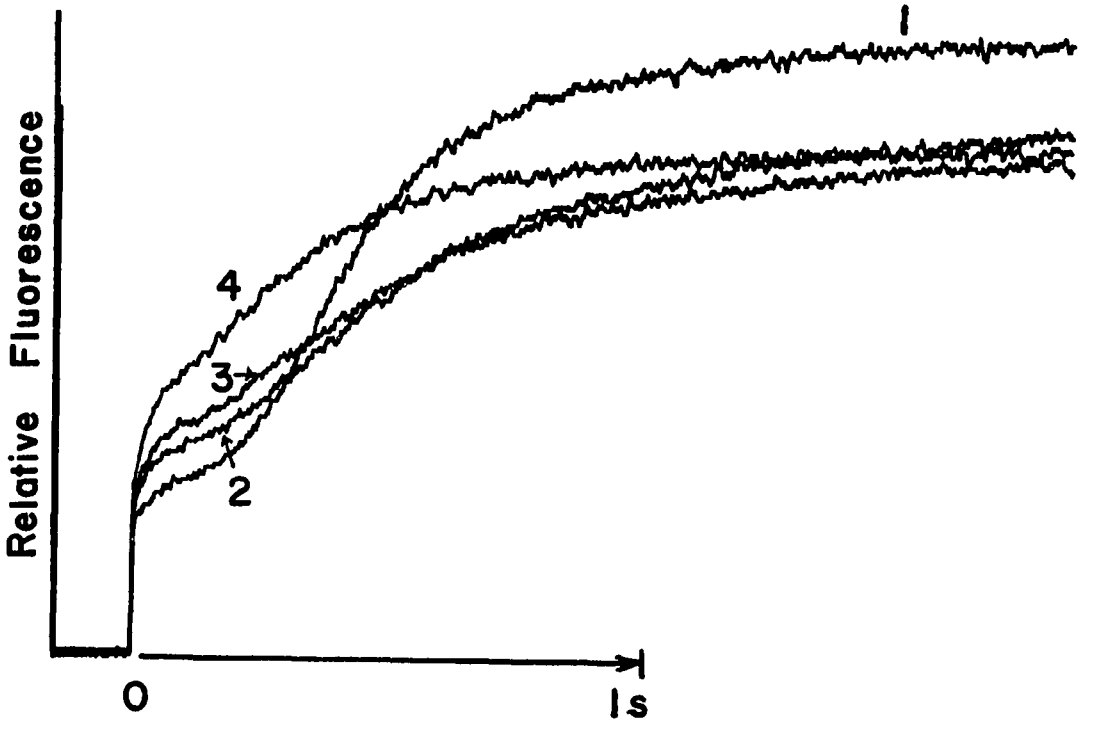


Figure 6.3. Quenching of the variable Chl a fluorescence in spinach thylakoids by 8 μM TETD and lack of restoration by catechol/ascorbate. The control thylakoids (Trace 1) were treated with TETD as in Fig. 6.1 (Trace 2). 0.5 mM catechol and 3 mM ascorbate were added during the subsequent dark adaptation (Trace 3). After a second 5 min dark adaptation, Trace 4 was measured.

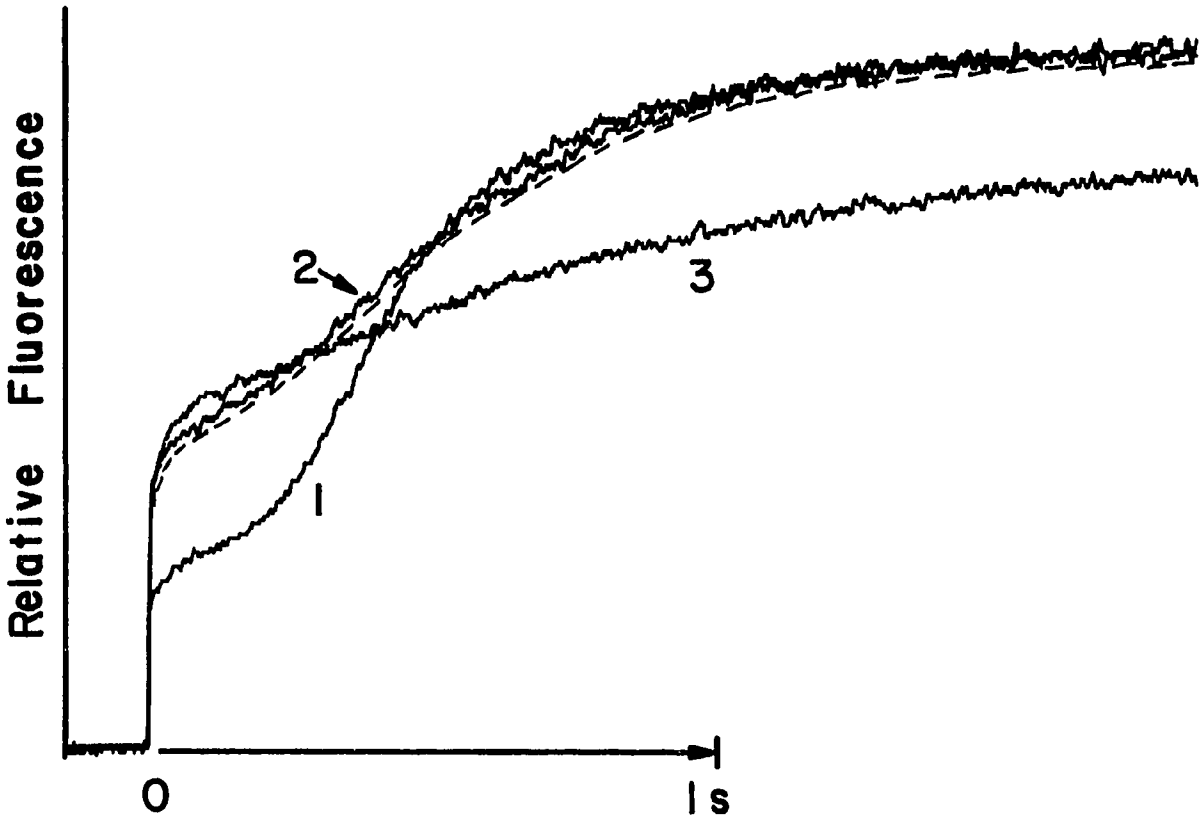


Figure 6.4. Quenching by TETD of the variable Chl a fluorescence that had been restored to heat-treated thylakoids by 0.5 mM catechol and 3 mM ascorbate. The heat-treated thylakoids were prepared as in Fig. 6.2. Trace 1: control thylakoids in 50 mM Na phospate, pH 7.2. Trace 2: heat-treated thylakoids that were devoid of any variable fluorescence (see Fig. 6.2) were given 0.5 mM catechol and 3 mM ascorbate to restore the fluorescence rise. Trace 3: 8 μM TETD added to the thylakoids of Trace 2.

Similarly, Fig. 6.4 shows that TETD still quenches the F_{max}, even after the variable fluorescence has been restored to heat-treated thylakoids by catechol/ascorbate. The original hypothesis that TETD inhibits photosynthesis by inhibiting the O_2 evolving complex is not supported by this data.

After the addition of catechol/ascorbate to the TETD-poisoned thylakoids, a longer dark time is necessary to fully oxidize the PS II acceptor pool (Fig. 6.3). This is not an effect of TETD by itself, as all of the transients in Fig. 6.3 were measured after a 5 min dark adaptation, and only the TETD + catechol/ascorbate sample was insufficiently dark adapted in that time. The significance of this is not understood. Whether there may still be an effect of TETD on O_2 evolution, in addition to an acceptor-side effect, or whether catechol/ascorbate by some other mechanism accentuates a block on the acceptor side by TETD, is not clear.

To see if TETD has an inhibitory effect on the acceptor side of PS II, the fluorescence decay after an actinic flash was measured (Fig. 6.5). This measurement is an indicator of the kinetics of the reoxidation of Q_A^-. It is clear that TETD does indeed inhibit the oxidation of Q_A^-, apparently by eliminating the fast component of the decay. This is very similar to the effect of HCO_3^- depletion (Robinson et al., 1984; Eaton-Rye, 1987).

Fig. 6.6 shows the fluorescence, as a function of flash number, at various times after the flash. In the absence of TETD, a binary oscillation is observed (i.e. at 220 μs after the flash), which is normal. An oscillation of period four, due to the turnover of the O_2-evolving system, is superimposed on the binary oscillation to create a complicated kinetics. What is of interest is how rapidly the oscillations are damped in the presence of TETD. This is consistent with an inhibited turnover of

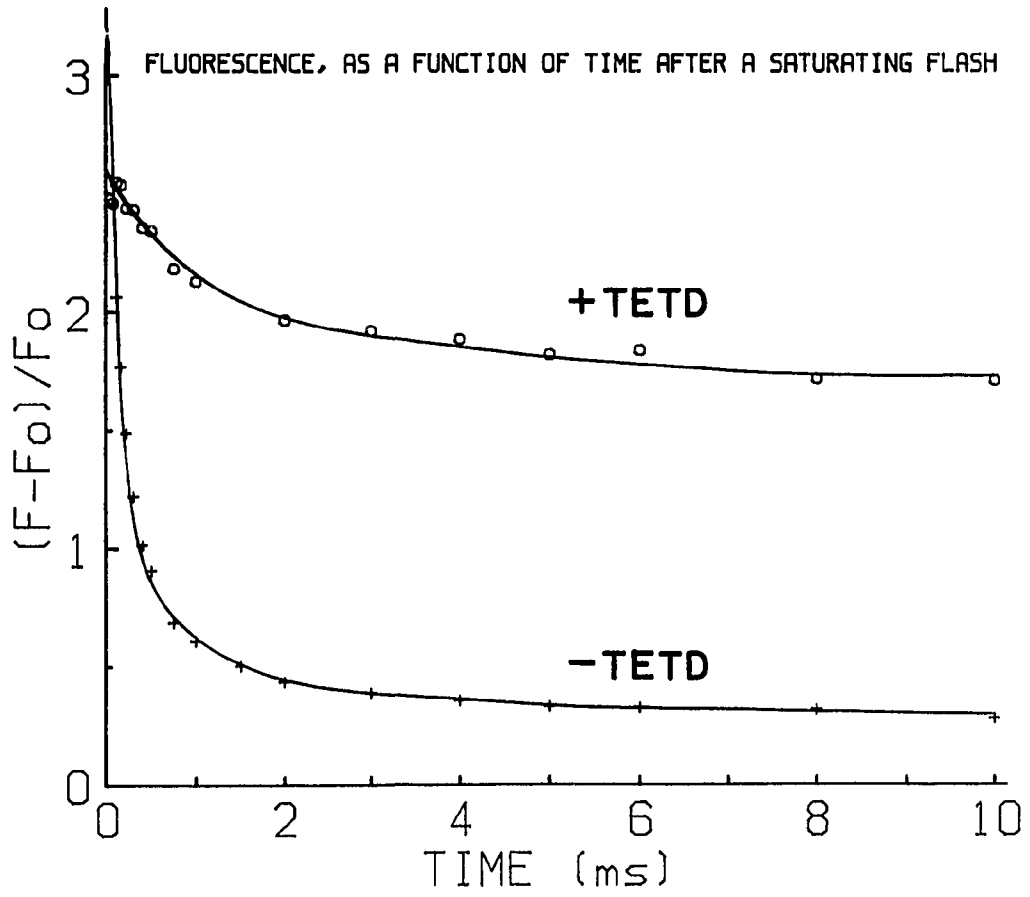
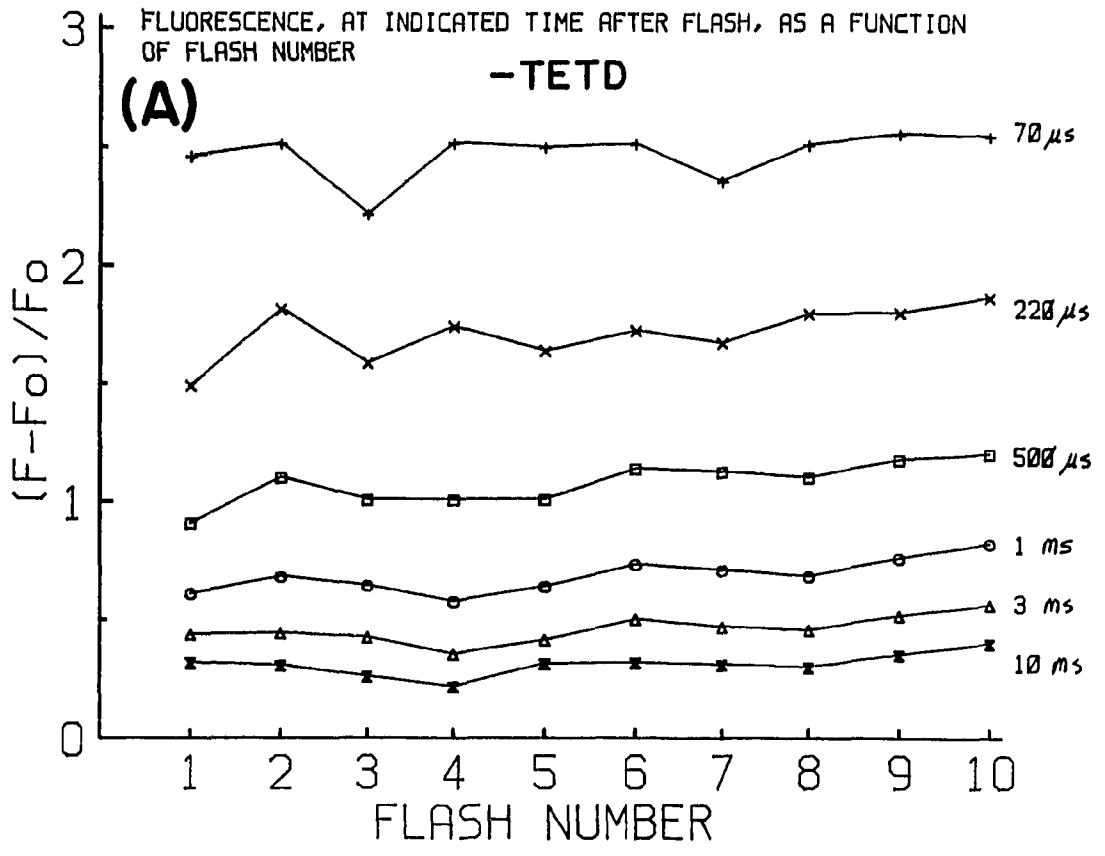
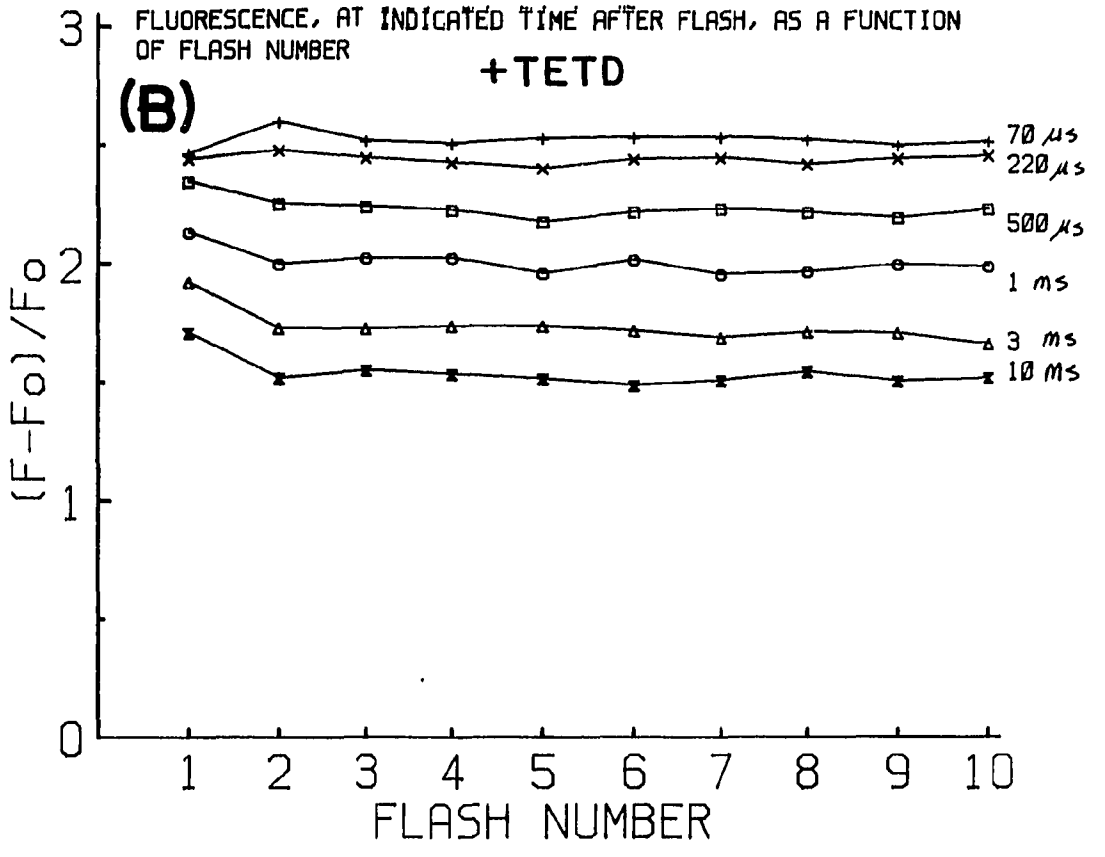


Figure 6.5. Decay of the variable Chl a fluorescence of spinach thylakoids after an actinic falsh in the presence (upper curve) and in the absence (lower curve) of 130 μM TETD. The [Chl] was 5 μg/ml.

Figure 6.6. The variable Chl *a* fluorescence yield, as a function of the actinic flash number, at the indicated times after the flash, in the absence (A) and in the presence (B) of 130 μM TETD. The [Chl] was 5 μg/ml.


FLUORESCENCE, AT INDICATED TIME AFTER FLASH, AS A FUNCTION OF FLASH NUMBER
(A)
-TETD
70 μs
220 μs
500 μs
1 ms
3 ms
10 ms
(F-Fo)/Fo
FLASH NUMBER


FLUORESCENCE, AT INDICATED TIME AFTER FLASH, AS A FUNCTION OF FLASH NUMBER
(B)
+TETD
70 μs
220 μs
500 μs
1 ms
3 ms
10 ms
(F-Fo)/Fo
FLASH NUMBER

the reaction center.

Although an inhibitory effect on Q_A^- oxidation is confirmed by Fig. 6.5, this effect does not explain the quenching of F_{max}, which is apparent in Fig. 6.5, as well as in the fluorescence transient. Since TETD has a redox potential of +0.33 volts (Lindahl and Akerstrom, 1965), there seemed the possibility that TETD might have been siphoning off electrons from Q_A^-. In this respect, the quenching of F_{max} would have been similar to the quenching by 6-azido-Q_0C_{10} (Chapter 4). However, as shown in Fig 6.7 (A), there is no O_2 evolution supported by TETD, even though some basal activity is detectable when ferricyanide (FeCy) is used as an electron acceptor. Therefore, TETD is not acting as an electron acceptor. Fig. 6.7 (B) shows the inhibitory effect of TETD on the photosynthetic reduction of silicomolybdate (SiMo). If SiMo is presumed to accept electrons from Q_A (e.g. Giaquinta and Dilley, 1975; Zilinskas and Govindjee, 1975), then this would indicate an inhibitory site prior to Q_A. Since it has already been established that there is no inhibitory site prior to Z, it can be inferred that electron flow is inhibited either between Z and P_{680}, or between $Pheo^-$ and Q_A. In the first case, the accumulation of the state $Z * P_{680}^+ * Pheo$ would account for the quenching of the F_{max} level, since P_{680}^+ is a quencher (e.g. Butler, 1972; Butler et al., 1973). In the second case the quenching would be due to accumulation of the state $Z * P_{680} * Pheo^-$, in which $Pheo^-$ is a quencher (Klimov et al., 1977).

The effect of TETD on the fluorescence spectrum at 77 K is shown in Fig. 6.8. The thylakoids contained 5 μM fluorescein as an internal standard, to which the spectra are normalized. TETD causes a specific quenching of the F_{695} and F_{735} peaks, but no quenching of the F_{685} peak (for a discussion of the fluorescence peaks, see Murata and Satoh, 1986).

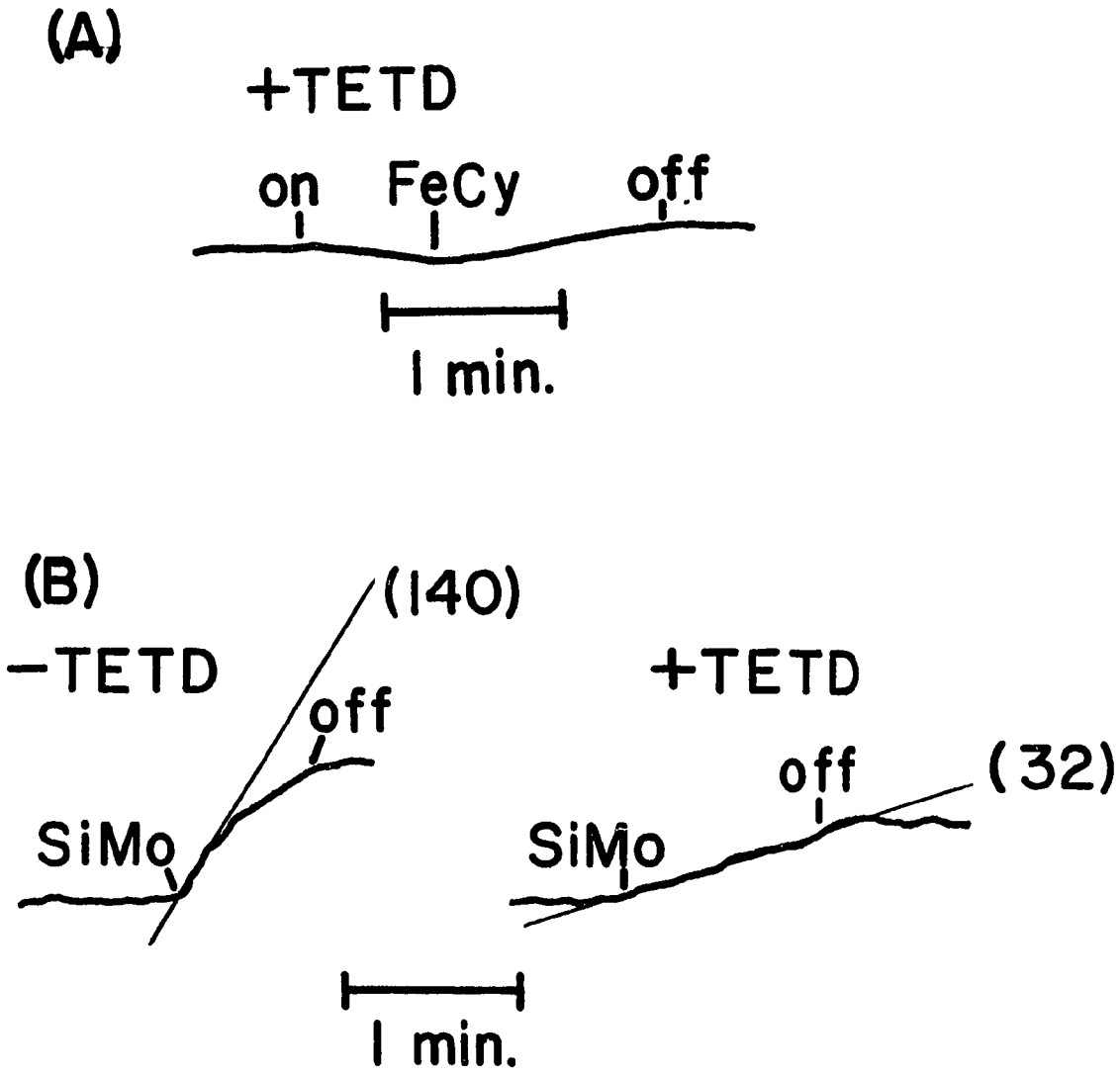


Figure 6.7. The effect of TETD on linear electron transport. In (A), O_2 evolution was measured with 130 μM TETD present, and no electron acceptor. 2 mM ferricyanide (FeCy) was added as an electron accepter after the illumination was begun. In (B) the H_2O to silicomolybdate (SiMo) partial reaction is measured in the absence (left) and in the presence (right) of TETD. The SiMo was added in the light in the presence of 10 μM DCMU. The relative rates of O_2 evolution are indicated in the parentheses beside the traces.

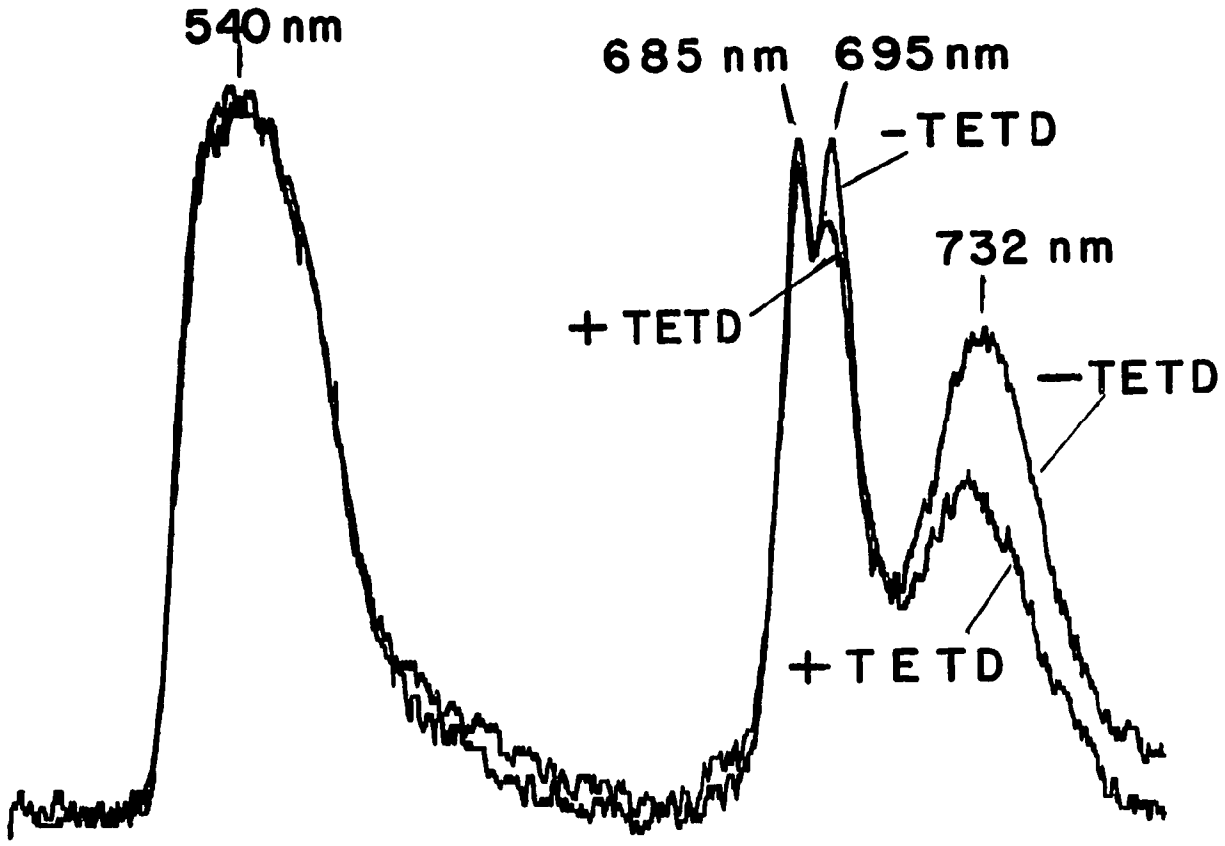


Figure 6.8. The Chl a fluorescence spectrum (uncorrected) at 77 K in the absence and in the presence of 130 μM TETD, showing the specific quenching of the F_{695} peak, relative to F_{685}. 5 μM fluorescein was present in both traces as an internal standard. The spectra are normalized with respect to the fluorescein peak at 540 nm.

A specific quenching of the F_{695} peak by $Pheo^-$ was predicted on theoretical grounds by Breton (1982) and was later demonstrated to occur under conditions in which PS II centers in the state $Z * P_{680} * Pheo^-$ had accumulated (Renger et al., 1983). Therefore, it is concluded that TETD inhibits the $Pheo^-$ to Q_A electron transfer, in addition to slowing down the oxidation of Q_A^-. The quenching of the F_{735} peak, which originates in the pigment protein complex of PS I, may indicate a decreased energy transfer from PS II to PS I.

As discussed in the introduction, the inhibition by TETD of electron transfer from $Pheo^-$ to Q_A is similar to one of the effects of the plastoquinone analog 6-azido-Q_0C_{10} (Chapter 4), which was attributed to an effect on the Fe^{2+} of PS II. HCO_3^- may also bind to the Fe^{2+}. As a metal chelator, TETD could be replacing HCO_3^- from a liganding site on the Fe^{2+}. This would account for the inhibition of Q_A^- to Q_B electron transfer.

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CHAPTER 7

THE MODEL

A. Summary

This chapter summarizes the concepts developed in the preceding chapters, along with a few others from the literature, and incorporates them into a coherent model for the role of HCO_3^- in photosystem II (PS II).

B. Two Cooperative Sites of HCO_3^- Binding

There are at least two sites of HCO_3^- binding, and probably there is a fairly high degree of cooperativity between them (Chapter 5, Section II). One site appears to be overlaid by the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) when it is present, as DCMU will prevent the effect of HCO_3^- if the DCMU is added first, but DCMU will not reverse the effect of HCO_3^- if the DCMU is added second (Chapter 5, Section I). DCMU is believed to replace PQ from the Q_B binding site (e.g. Oettmeier, 1983). One thing that PS II herbicides appear to have in common is a flat polar component with a dipole moment of 3-5 Debyes (Shipman, 1981). Thus, Shipman (1981) proposed that the herbicides bind electrostatically to a strongly polar binding site within a hydrophobic surface on the Q_B protein. HCO_3^-, bound to an arginine, could provide an appropriate electric field for such an interaction, and was considered by Shipman to be a likely part of the herbicide binding environment. The observation reported here that DCMU appears to overlay the HCO_3^- in PS II, supports this notion. Therefore, this HCO_3^- site is probably very close to the Q_B site, and it may be an arginine residue to which the HCO_3^- binds.

It has been similarly shown previously that HCO_3^- depletion lowers the binding affinity of DCMU-type herbicides (Khanna et al., 1981) and vice

versa (Van Rensen and Vermaas, 1981; Vermaas et al., 1982; Snel and Van Rensen, 1983). This interaction is only partially competitive, and was explained by Vermaas and Van Rensen (1981) as due to an overlapping of sites. Since the two HCO_3^- sites are probably cooperative, anything that affects the binding at one site will affect the binding at the second site through allosteric interactions. Thus, DCMU would be expected to alter the binding affinities of HCO_3^- at both sites. It is of interest, then, that Stemler and Murphy (1984) report that the binding of atrazine to a high affinity site (i.e. the Q_B site) seems to remove some HCO_3^- "neither competively nor noncompetitively but somehow indirectly". Similarly, the binding of HCO_3^- at the second site would be expected to affect DCMU binding, even though the sites may be spatially separated. The apparent ability of silicomolybdate (SiMo) to reduce the binding affinity of DCMU (Böger, 1982; Graan, 1986) may be due to SiMo removing HCO_3^- from this second site, rather than due to competition between SiMo and DCMU for the same site, as was suggested by Böger (1982). That SiMo does remove bound HCO_3^- was shown previously by Stemler (1977), who also observed that when the DCMU was added before the SiMo, about half as much HCO_3^- was removed.

A likely candidate for the second HCO_3^- site is the non-heme Fe^{2+} in PS II, since HCO_3^- depletion has been observed to induce a large increase in the EPR signal at g=1.82 (Vermaas and Rutherford, 1984), attributed to the Q_A^- * Fe^{2+} complex (Rutherford and Zimmerman, 1984). In bacterial reaction centers, the Fe^{2+} forms two ligands to the carboxyl group of a glutamate residue on the M subunit and one ligand to each of four histidines, two of which are in the L subunit, and two of which are in the M subunit (Michel et al., 1986). In PS II reaction centers, the Q_B protein, D_1, is highly homologous with the L subunit, while the D_2 protein

(to which Z and Q_A are believed to bind) is highly homologous with the M subunit (e.g. Trebst and Draber, 1986; Trebst, 1987). However, one major difference is that D_2 is lacking an extra loop which, in the M subunit, carries the glutamate ligand to the Fe^{2+}. Michel and Deisenhofer (1986) have suggested that in PS II, HCO_3^- takes the place of the glutamate ligand. This would explain why a HCO_3^- dependence has never been observed in the photosynthetic bacteria (Shopes and Blubaugh, unpublished). To complete the analogy, the HCO_3^- in PS II would presumably form a salt bridge between the Fe^{2+} and some residue in the D_2 protein. Thus, HCO_3^- may be required to help hold D_1 and D_2 together, via the Fe^{2+}. Removal of this HCO_3^- would be expected to induce a significant conformational change in PS II, which could disrupt electron flow through the quinones. Allostery is most often associated with polymeric enzymes. The involvement of HCO_3^- in a salt bridge to maintain the active configuration of D_1 and D_2 is, therefore, consistent with an observed cooperativity between the two HCO_3^- sites. To fully explain the cooperativity, HCO_3^- binding near the Q_B site should also induce a conformational change that brings D_1 and D_2 closer to their native structure, thereby favoring the binding of the second HCO_3^- at Fe^{2+}.

Recently, it has been shown that some exogenous quinones, when reduced by a photoact to the semiquinone, can extract their second needed electron from the Fe^{2+}, and that formate blocks this oxidation of the Fe^{2+} (Zimmerman and Rutherford, 1986). It is plausible that by removing the HCO_3^- ligand to the Fe^{2+}, formate induces a conformational change that increases the distance between the Fe^{2+} and the Q_B site, thus making electron transfer less likely. This same conformational change may then allow the exogenous quinones to accept directly from Q_A^- (see Chapter 4).

SiMo may have a similar effect; it would be able to both expose Q_A^- and act as the electron acceptor. The existence of such a conformational change upon addition of SiMo was suggested previously (Zilinskas, 1975). High concentrations of HCO_3^- inhibit the photosynthetic reduction of SiMo (Barr and Crane, 1976), perhaps by simple competition for the liganding site on the Fe^{2+}. Such a conformational change may also affect electron transfer from $Pheo^-$ to Q_A. This would account for the observed effects of tetraethylthiuramdisulfide (TETD; see Chapter 6), which, as a metal chelator, could also be binding to the Fe^{2+}. It would also explain why 6-azido-Q_0C_{10} appears to block $Pheo^-$ to Q_A electron transfer under the same conditions as it appears to accept electrons directly from Q_A^- and why formate appears to accentuate these effects (Chapter 4). It could also account for the slow rates observed for the H_2O-to-SiMo partial reaction.

Q_{400}, first identified as a high-potential component of PS II by Ikegami and Katoh (1973), and which is now known to be the Fe^{2+} (Petrouleas and Diner, 1986), is oxidized when chloroplasts are incubated with ferricyanide (FeCy) in the presence of DCMU (Ikegami and Katoh, 1973; Wraight, 1985). Like the deceleration of the chlorophyll *a* fluorescence rise in the presence of DCMU in HCO_3^- depleted thylakoids (Blubaugh and Govindjee, 1984; Chapter 5, Section I), the oxidation of Q_{400} by FeCy in the presence of DCMU is also dependent on the order of addition (Ikegami and Katoh, 1973; Bowes *et al.*, 1979). Although the oxidation of Q_{400} can be partially observed when DCMU is added first, under conditions involving the unstacking and then restacking of the grana (Wraight, 1985), observation of the full effect requires that FeCy be added before the DCMU. This suggests that FeCy, in addition to acting as a PS I electron acceptor, may be binding to the HCO_3^- site near Q_B. It may oxidize the Fe^{2+} from

this position or, alternatively, removal of HCO_3^- from this site may permit Q_B^- to oxidize the Fe^{2+}. The ability of some exogenous quinones to oxidize Fe^{2+} from the Q_B site when they become reduced to the semiquinone (Zimmerman and Rutherford, 1986) may likewise be due to an alteration in the binding affinity of HCO_3^- by these exogenous quinones. Although FeCy appears to be binding to the HCO_3^- site near Q_B (site 2, following the terminology of Eaton-Rye, 1987), it could be exerting an effect on the Fe^{2+} allosterically through the HCO_3^- site on the Fe^{2+} (site 1). FeCy is also able to oxidize Q_A^- directly in the presence of DCMU at high salt concentrations (Itoh, 1978) or at low pH (Itoh and Nishimura, 1977), two treatments which, as dicussed in section D, are likely to remove the liganding HCO_3^- from the Fe^{2+}. This supports the proposition above that a conformational change induced by the removal of HCO_3^- from site 1 permits the direct oxidation of Q_A^- by exogenous acceptors at the Q_B site. High salt concentrations actually decrease the oxidation of Q_A^- by Fecy when the pH is already low (Itoh, 1978). This is understandable with the above model: when HCO_3^- is already gone from site 1, high salt will have no additional effect, other than perhaps a competition with the Fecy for site 2.

C. The Effect on PQ Binding

The affinity of the Q_B site for PQ may be lowered by the removal of HCO_3^- (Chapter 4; see, also, Eaton-Rye, 1987). This could be due to effects of HCO_3^- at either site. Eaton-Rye (1987) has speculated that HCO_3^- binding at the Fe^{2+} (site 1) affects PQ binding, while another HCO_3^- near Q_B (site 2) is involved in the protonation of Q_B^-. If HCO_3^- binding tightens PQ binding, it follows necessarily that PQ binding also tightens HCO_3^- binding (for proof, see Appendix II, Part F). This may be sufficient

to explain the preferential binding of HCO_3^- in the dark, as opposed to the light (Stemler and Govindjee, 1973; Stemler, 1979), particularly when formate is present (Vermaas and Van Rensen, 1981). In HCO_3^- depleted thylakoids, Q_B will be largely reduced in the light (PQ^{2-}) and slow to exchange with the PQ pool. The absence of PQ in the Q_B site would make the affinity for HCO_3^- at site 1 less than it would be in the dark, when PQ would be occupying the site. While formate has a carboxyl group that could be a ligand to Fe^{2+}, it would not be able to H-bond with D_2, so it would be ineffective at providing the salt bridge that HCO_3^- can provide. Thus, formate may compete with HCO_3^- for site 1 without having the same cooperativity with PQ binding (formate would also not have the same cooperativity with the other HCO_3^- site; this might explain the large difference in binding affinity between HCO_2^- and HCO_3^-). Therefore, in the presence of formate the difference in HCO_3^- binding affinity in the light versus dark would be even more significant, since the HCO_3^- would have the added hindrance of a competitor whose binding is uninfluenced by the redox state of Q_B.

D. HCO_3^- as the Active Species at Both Sites

Neither CO_2, H_2CO_3 nor CO_3^{2-} have any apparent effect on the restoration of the Hill activity by HCO_3^- (Chapter 3). Thus, the active species is presumed to be HCO_3^- at both sites. Since the inhibitory formate (HCO_2^-) is identical to HCO_3^- in all respects except for a missing hydroxyl group, it can be presumed that the hydroxyl group is important for functioning at both sites. As discussed in section B, if site 1 is the non-heme Fe^{2+}, then the hydroxyl group here is probably involved in an H-bond with a residue on the D_2 protein, to provide the proper conformation

to the PS II reaction center. This salt bridge would be adversely affected by protonation during a low pH treatment, or by shielding of its charge by high salt concentrations. In Chapter 5, it was suggested that some negatively charged group exists that, when protonated or charge shielded, causes the release of otherwise tightly-held HCO_3^-. It is possible that HCO_3^- itself, liganded to the Fe^{2+}, is that group. Alternatively, a histidine would have a pKa in the same vicinity, so HCO_3^- could be H-bonded with an unprotonated histidine. When the conformational change occurs, the affinity for HCO_3^- at site 2 would be lowered, because of the cooperativity between sites 1 and 2.

As discussed in section B, site 2 is probably very close to the site of Q_B binding, such that DCMU binding to the Q_B site can also overlay the HCO_3^- site. An arginine residue, as suggested by Shipman (1981), is the most likely candidate for this site. Because of the very close proximity of this HCO_3^- to Q_B, and because a protein group near Q_B is protonated upon reduction of Q_B to Q_B^- (Crofts et al., 1984; see, also, the introduction to Chapter 4), it is likely that the hydroxyl group of this HCO_3^- is involved in an acid/base reaction with the protein group. Eaton-Rye (1987) has also suggested that HCO_3^- is required at this site for the protonation of a site to stabilize Q_B^-. Such a mechanism is illustrated in Fig. 7.1. A similar model has been developed by H.H. Robinson (personal communication), who suggests that HCO_3^- is bound to Arg-225 and protonates His-215. Some details differ in the two, independently obtained, models. In our model, a histidine is shown as the protein group to be protonated because of the observed pKa of 6.4 (Crofts et al., 1984) and because there is a histidine cluster around the Q_B site (Michel et al., 1986; Trebst and Draber, 1986). The pKa of this group shifts to about 7.9 upon formation of

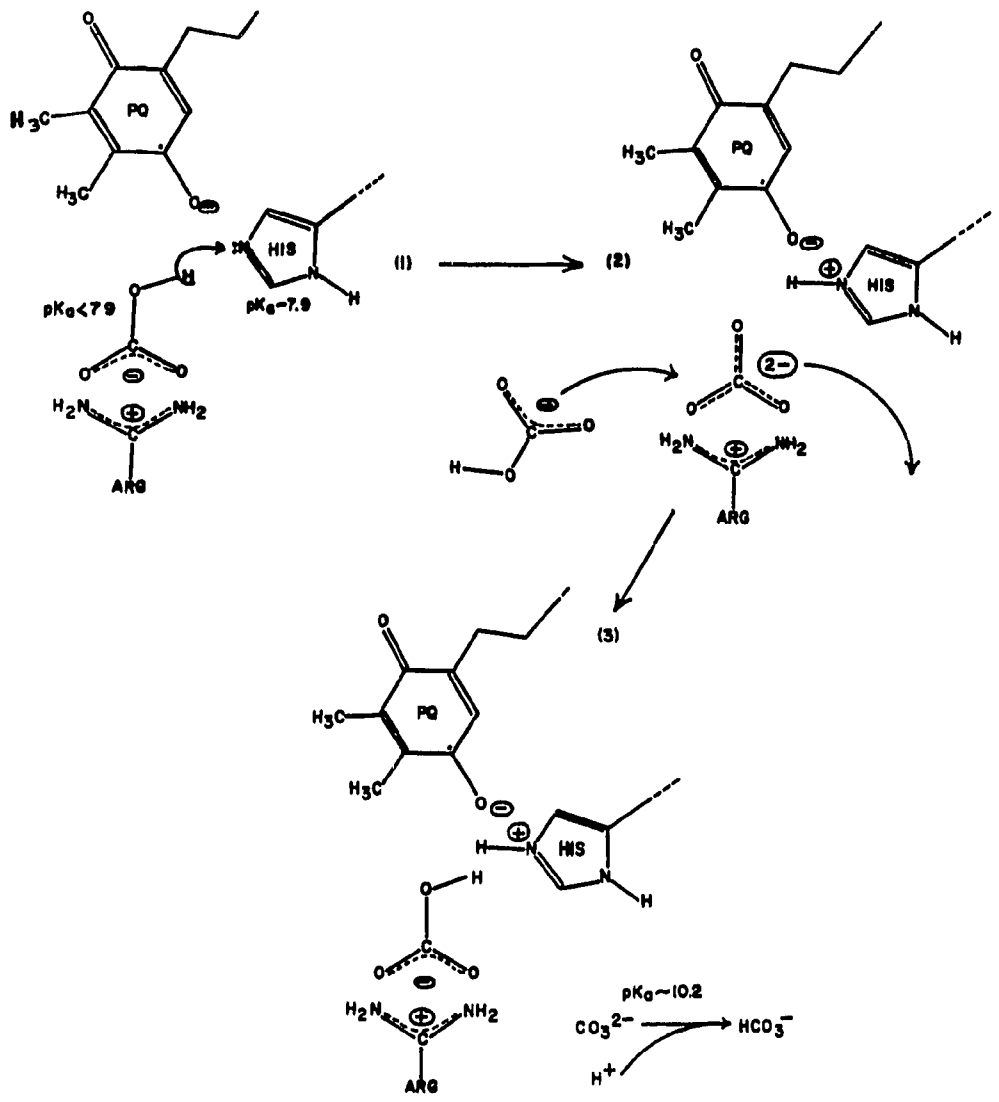

Figure 7.1. A working model for the involvement of HCO_3^- in the protonation of the secondary quinone acceptor Q_B. Protonation of a histidine near Q_B stabilizes the negative charge on Q_B^-. The binding of HCO_3^- to a positive group, most likely an arginine, lowers the pKa of HCO_3^- so that protonation of the histidine can occur. The charge on CO_3^{2-} has greater delocalization over the molecule than does HCO_3^- and binds less tightly. The CO_3^{2-} is replaced by another HCO_3^-, shifting the equilibrium sharing of the H^+ toward histidine. CO_3^{2-}, with a pKa of 10.2, picks up a H^+ readily.

Q_B^- (Crofts et al., 1984). The negative charge of Q_B^- in close proximity to the histidine would cause such a shift in the pKa by stabilizing the positive charge of the protonated histidine. The pKa of the hydroxyl group of HCO_3^- is 10.2 in aqueous solution, but because of the electron withdrawing effect of the arginine, its pKa would be shifted lower. Thus, upon reduction of Q_B to Q_B^-, a H^+ transfer would occur from HCO_3^- to the histidine.

In Chapter 3 (see, also, Blubaugh and Govindjee, 1984), it was suggested that CO_3^{2-} binds less tightly than HCO_3^-. This was inferred from the difference in binding affinity between nitrite (NO_2^-) and nitrate (NO_3^-), which closely resemble HCO_3^- and CO_3^{2-}, respectively, in their electronic structures. Thus, it is presumed in Fig. 7.1 that the CO_3^{2-} resulting after H^+ transfer is displaced by another HCO_3^-. It is possible that the CO_3^{2-} could remain in place and simply pick up a H^+ from another source. However, there would be a distinct advantage to the system if CO_3^{2-} were to be replaced as shown. Once released from its site, the pKa of CO_3^{2-} would return to 10.2, and reprotonation would be very rapid. Furthermore, the rapid replacement of CO_3^{2-} with HCO_3^- would ensure that the H^+ remains on the histidine. Thus, the equilibrium sharing of a H^+ between HCO_3^- and histidine can be shifted far in favor of the histidine by a rapid exchange of CO_3^{2-} with HCO_3^-, and H^+ uptake from the bulk phase will be more rapid if free CO_3^{2-} is the recipient.

E. An Intrathylakoid HCO_3^- Pool

It was argued above that a rapid exchange of CO_3^{2-} with HCO_3^- would be advantageous to the system. This will occur most readily if a pool of HCO_3^- is available in the vicinity of site 2. Otherwise, the instant that CO_3^{2-} is released and its pKa shifts to 10.2, it would tend to extract a H^+

from the nearest acidic group, which would be the histidine. Thus, we would be back to the situation of an equilibrium sharing of the H^+, and there would be no advantage to the release of CO_3^{2-}. With a pool, this problem would be circumvented. Furthermore, the uptake of a H^+ from the bulk phase could occur with any of the CO_3^{2-} molecules in the pool; H^+ uptake would be faster than if the CO_3^{2-} had to diffuse to the outer surface. Evidence for a low-affinity HCO_3^- site was presented in Section I of Chapter 5 (see, also, Stemler, 1977; Blubaugh and Govindjee, 1984). This low-affinity site may be such an intramembrane pool. In this respect, it is of considerable interest that HCO_3^- enhances photophosphorylation (Punnett and Iyer, 1964), since a putative intramembrane H^+ channel has been a source of debate for many years. An intramembrane pool of HCO_3^- would have H^+ buffering capacity, which might give it the characteristics of this putative H^+ channel. Both low pH and high salt concentrations have been reported to change the nature of the localized H^+ domains to a delocalized condition (Beard and Dilley, 1986). These treatments also seem to cause the release of tightly-held HCO_3^-. Thus, it is possible that a sequestered pool of HCO_3^- is involved in providing a ready source of H^+'s at the Q_B site, and at the same time is an internal H^+ buffering channel for connection of the coupling sites with the coupling factor. This is consistent with the observation that uncouplers abolish the Punnett and Iyer effect (Batra and Jagendorf, 1965).

HCO_3^- was suggested earlier to be involved in a salt bridge between the Fe^{2+} and the D_2 protein. Low pH or high ionic strength is suggested to disrupt this salt bridge, inducing a conformational change which permits the exchange of the intramembrane pool of HCO_3^- with the bulk phase. Thus, low pH or high ionic strength is likely to induce a partial HCO_3^- depletion

by permitting the HCO_3^- in the vicinity of Q_B to exchange with a larger volume. The pH optimum of the HCO_3^- effect is about 6.5 (Khanna et al., 1977; Vermaas and Van Rensen, 1981). The ascending arm is probably due to the fact that HCO_3^- is the active species (Chapter 3), and the pKa of CO_2/HCO_3^- is about 6.35. The descending arm may reflect the pKa of the group to which HCO_3^- is H-bonded in the salt bridge. This pKa would be about 6.8, suggesting a histidine residue. At pH values significantly below this, both the removal and the binding of HCO_3^- would be facilitated.

This thesis has demonstrated that HCO_3^-, not CO_2, is the binding species (Chapter 3), and all of the evidence reviewed throughout this thesis argues for the binding sites being near the outer surface of the membrane (at Fe^{2+} and Q_B). Stemler (1980), however, concluded that CO_2 is the active species and that it binds on the lumen side of the membrane, because the rate of $H^{14}CO_3^-$ binding was pH independent for the first few minutes after a pH jump, but then showed a marked pH dependence after a 5 min incubation at the new pH, with the binding being greater at lower pH values. All that is necessary to reconcile this experiment with the model presented here is to postulate that the salt bridge is disrupted by low pH on a time scale of minutes. Immediately after a jump to low pH, the HCO_3^- binding sites would still be sequestered. After an incubation at low pH, however, the sites would become exchangeable with the bulk phase, and the rate of $H^{14}CO_3^-$ binding would be accelerated.

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SUMMARY

Bicarbonate (HCO_3^-) is required for photosystem II (PS II) electron transport. Depleting thylakoids of HCO_3^- causes a reversible inhibition of more than 90% of the Hill activity. Addition of HCO_3^- restores this activity. At the start of this thesis work, it was known that the site of inhibition is at the quinone reactions on the acceptor side of PS II: electron transport is slowed between the primary and secondary quinone acceptors, Q_A and Q_B, respectively, and is blocked between Q_B and the plastoquinone (PQ) pool. Little else was known with certainty.

It was commonly speculated that HCO_3^-, rather than CO_2, was the chemical species required, based on competition by similar anions, such as formate (HCO_2^-) and acetate ($CH_3CO_2^-$). However, this was not certain, and some reports in the literature suggested that CO_2 may be the binding species. This thesis has answered this question, among others. Advantage was taken of the pH dependence of the HCO_3^-/CO_2 ratio at equilibrium in order to effectively hold the concentration of one species constant, while varying the concentration of the other. It was discovered that the Hill reaction is restored in direct proportion with the equilibrium [HCO_3^-], and is independent of the equilibrium [CO_2]. Therefore, the chemical species required for PS II electron transport must be HCO_3^-. The other carbonic species, H_2CO_3 and CO_3^{2-}, as well as CO_2, are shown in this study to play no direct role in the HCO_3^- effect. CO_2 and H_2CO_3 may be the species that actually enter the membrane, but conversion to HCO_3^- must apparently take place before binding can occur.

A velocity curve is generated of the Hill reaction as a function of the equilibrium HCO_3^-. A kinetic analysis of this curve indicates that, in

addition to a loosely bound site, there are at least two high affinity sites of HCO_3^- binding, if it is assumed that the basal activity in the absence of added HCO_3^- is due to some endogenous HCO_3^- remaining at these sites. This assumption is shown to be valid by a measurement of the activity as a function of chlorophyll (Chl) concentration, which is non-linear. This non-linearity is shown to be due to endogenously bound HCO_3^-, which is released in response to either light or high ionic strength, or both. This is an important finding, for it indicates that (i) a mechanism exists for keeping HCO_3^- tightly bound at low concentractions, (ii) HCO_3^- is apparently an essential requirement, in that zero activity is attainable if all of the HCO_3^- is removed, and (iii) the published value for the dissociation constant K_d, determined on the basis of assumed Michaelis-Menten kinetics, is in error. The importance of this finding is better appreciated in light of the current controversy over whether HCO_3^- plays any real role in vivo. This question has arisen because of the relatively high value reported for K_d. The three points made above are very relevant to this question.

Very little is known about what HCO_3^- does to stimulate electron flow. An azido analog of PQ was used in this study to probe whether PQ binding to the Q_B site is affected by HCO_3^- removal. If the azido quinone binds less when HCO_3^- is removed, then less will be covalently attached to the Q_B site after activation of the azido group by ultraviolet light. This seems to be the case, as determined by the effect of the azido quinone on the Chl a fluorescence transient. However, this conclusion is less certain than others made in this thesis, because the azido quinone is shown to have multiple effects: in addition to covalent attachment at the Q_B site, it is apparently able to accept electrons directly from Q_A^-, and it may also

impair electron flow from pheophytin (Pheo) to Q_A. These latter effects are more pronounced when HCO_3^- is removed from the membrane and may be due to conformational changes induced by the removal of HCO_3^-. A metal chelator, tetraethylthiuram disulfide (TETD) has similar effects: electron transfer from Q_A^- to Q_B is slowed, as is electron flow from $Pheo^-$ to Q_A. To explain these effects, it is suggested that one of the two bound HCO_3^-'s forms a salt bridge between the Fe^{2+} and the D_2 protein, by analogy to a glutamate residue in the bacterial reaction centers. Removal of this HCO_3^- alters the distances between Pheo, Q_A and Q_B. A second HCO_3^- is proposed to bind near enough to Q_B to be overlaid by DCMU when it binds there, as the effect of HCO_3^- on the Chl a fluorescence transient in the presence of DCMU is evident only when the HCO_3^- is added before the DCMU.

A low affinity site for HCO_3^- is also demonstrated; HCO_3^- bound to this site accelerates the Chl a fluorescence rise in non-HCO_3^--depleted thylakoids in the presence of DCMU. It is speculated that this low affinity site may also be involved in the previously observed enhancement of photophosphorylation by added HCO_3^-.

The observations reported in this thesis, along with others from the literature, are incorporated into a descriptive working model with the following features:

(1) HCO_3^- forms a salt bridge between Fe^{2+} and the D_2 protein. The carboxyl group of HCO_3^- is a bidentate ligand to Fe^{2+}, while the hydroxyl group H-bonds to a protein residue.

(2) A second HCO_3^- is involved in protonating a histidine near the Q_B site to stabilize the negative charge on Q_B^-. HCO_3^- provides a rapidly available source of H^+ for this purpose.

(3) After donation of a H^+, CO_3^{2-} is replaced by another HCO_3^-. The high

pKa of CO_3^{2-} ensures rapid reprotonation from the bulk phase.

(4) An intramembrane pool of HCO_3^- accounts for the low affintiy site. This pool is a H^+ buffering domain functionally connecting the external bulk phase with the quinones.

(5) Low pH and high ionic strength are suggested to disrupt the HCO_3^- salt bridge between Fe^{2+} and D_2. The resulting conformational change exposes the intramembrane HCO_3^- pool to the bulk phase.

APPENDIX I

DESCRIPTIONS OF COMPUTER PROGRAMS

A. FLUORE

This program is designed for capture, display, mathematical manipulation, and plotting of fluorescence induction curves. The signal current from the photomultiplier, after amplification and conversion to voltage, is digitized by the Biomation waveform recorder and displayed on an oscilloscope, under non-program control. The program permits transfer of the digitized data to the computer, and storage on floppy disk. It permits up to three traces to be displayed simultaneously on a second oscilloscope for easy comparison. The data is automatically scaled to fill the screen, and the time scale is calculated and displayed. Data taken on different sensitivity settings on the Biomation and at different recording rates can be compared, as the program corrects for the different settings during scaling of the traces. By means of two cursors which appear brightly on the oscilloscope, any portion of the traces may be expanded. An automatic y-rescale option can be put into effect, which rescales the data in the y direction after each use of the cursors. Also by use of the cursors, the user can obtain the actual X and Y values of any point. Data taken from the Biomation is automatically stored in a file on disc drive 1 (the non-system disc drive, so that the user keeps a separate data disc, which contains only data), and can be recalled at any future time. Before writing a file, however, the program checks to make sure no file of the same name already exists, and if one does, asks the user whether or not to write over the old data. Thus, the user cannot accidentally erase any previous data. A single beam is used to display the three traces; neither dual-beam nor dual-trace capabilities are required on the oscilloscope.

Thus, any available scope may be used. However, the program contains 2 scaling factors which may need to be changed when changing oscilloscopes. This is done simply by initializing the variables "xscale" and "yscale" to the proper value. The program CALIBR, described below, will determine the proper values for any scope and write them to a file that FLUORE reads upon startup. Whatever portion of the data is displayed on the scope can be plotted on the digital plotter. The program will slightly expand or diminish the trace so that tic marks correspond to whole numbers for a neater plot. For example, if 1019 points are displayed, the program will plot 1000 points. For all intents and purposes, what is seen on the scope is what is plotted. Again, the data is automatically scaled, and the user's descriptions are printed at the top of the graph. Some mathematical manipulations are also possible: using the cursors to define the boundaries, the area over the fluorescence curve can be calculated; the traces can be normalized to any point, determined by the cursors; the traces can be moved up and down or left and right, relative to each other, for comparison of any feature; and the data can be smoothed using a running average smooth.

B. SPECTR

This program is a modification of FLUORE, to allow for spectral, rather than induction, data. A new routine lets the user enter the starting and ending wavelengths and calculates the tick rate necessary for the real-time clock to synchronyze the Biomation with a motor that drives the grating of the monochromater. A Schmitt trigger is used to start the real-time clock; the user trips the trigger with any pulse-producing mechanism (the simplest device is just a battery and a switch) at the moment that the grating is at the starting wavelength. In practice, a high

degree of reproducibility is obtained. All plots and displays are expressed in terms of wavelength, rather than time, and the cursors can be used to obtain the wavelength corresponding to any point. Mathematical options include those of FLUORE, except for area over the trace, and also include creating a difference spectrum and automatically correcting a spectrum using a file of previously entered correction factors.

C. CALIBR

This program interacts with the user to determine the proper scaling factors for any oscilloscope to be used with FLUORE and SPECTR. The user adjusts the perimeter of a box, until the box encloses the space that is to hold a full trace. The program calculates the scaling factors and writes them to a file that is read by FLUORE and SPECTR during initialization.

D. GRAPH

This is a general plotting program that lets the user enter any numerical data by hand or by disc, and then will plot the data to any scale. The program is highly flexible, permitting deletion or change of data, a variety of plot symbols and/or lines, and flexible scaling and labeling of axes. Every mathematical function of which the computer is capable is included in a math package that permits the function to be performed on either the X or Y data, or both. Thus, log and semi-log plots, double reciprocals, trigonometric transformations, and many others are easily performed, without the need to re-enter data. Most of the graphs in this thesis were prepared using either this program, or the plotting routines in FLUORE and SPECTR.

E. DATFIT

This is another plotting program that permits a user-defined function to be calculated and fit to a set of data points. The data is displayed on an oscilloscope as bright points against a dimmer theoretical curve. By adjusting the values for various parameters of the function, the user can see the effect of those parameters on the theoretical curve, and compare the new curve to the data. A plot of both the data and the theoretical curve can be made on the digital plotter. The function, as well as the parameters, is set up by the user in a subroutine that is called by the main program.

APPENDIX II

FORMULA DERIVATIONS

A. Calculation of the equilibrium $[CO_2]$ and $[HCO_3^-]$ in an aqueous solution from the partial pressure of CO_2 in the gas phase above it:

Henry's Law:
$$P_B = X_B K_B \tag{A.1}$$

where P_B = partial pressure of B,

X_B = mole fraction of B in solution,

K_B = Henry's constant for B.

Substituting CO_2 for B, and rearranging:

$$X(CO_2) = \frac{P(CO_2)}{K(CO_2)} \tag{A.2}$$

Since the amount of dissolved CO_2 is small, the mole fraction can be approximated as follows:

$$X(CO_2) = \frac{\text{moles } CO_2}{(\text{moles } CO_2) + (\text{moles } H_2O)} \approx \frac{\text{moles } CO_2}{\text{moles } H_2O} \tag{A.3}$$

By definition,
$$[CO_2]_{(aq)} = \frac{\text{moles } CO_2}{\text{liter soln}} \approx \frac{\text{moles } CO_2}{\text{liter } H_2O} \tag{A.4}$$

Converting liter H_2O to moles H_2O:

$$[CO_2]_{(aq)} \cdot \frac{0.018 \text{ liters } H_2O}{\text{mole } H_2O} = \frac{\text{moles } CO_2}{\text{moles } H_2O} \approx X(CO_2) \tag{A.5}$$

Therefore,
$$X(CO_2) \approx [CO_2]_{(aq)} \cdot (0.018\ M^{-1}) \tag{A.6}$$

Substituting Eqn. A.6 into Eqn. A.2 and rearranging,

$$[CO_2]_{(aq)} \approx \frac{P(CO_2)}{K(CO_2)\cdot(0.018\ M^{-1})} \qquad (A.7)$$

At normal atmospheric pressure of CO_2, $P(CO_2) = 3.3 \times 10^{-4}$ atm (Knoche, 1980). For H_2O at 25 C, $K(CO_2) = 1.25 \times 10^6$ mm Hg (Atkins, 1978). Converting $K(CO_2)$ to units of atm,

$$K(CO_2) = 1.25 \times 10^6 \text{ mm Hg} \ \frac{1 \text{ atm}}{760 \text{ mm Hg}} = 1.65 \times 103 \text{ atm} \qquad (A.8)$$

Plugging the values for $P(CO_2)$ and $K(CO_2)$ into Eqn. A.7,

$$[CO_2]_{(aq)} \quad \frac{(3.3 \times 10^{-4} \text{ atm})}{(1.65 \times 10^3 \text{ atm})\cdot(0.018\ M^{-1})} = 11 \times 10^6 \text{ M} \qquad (A.9)$$

The $[HCO_3^-]$ in equilibrium with this $[CO_2]$ can be calculated for any pH from Eqn. B.11, below.

B. Calculation of the equilibrium (eq) $[HCO_3^-]$ and $[CO_2]$ in solution, given the pH and the initial (i) total concentration of carbonic species.

The chemical equation for the equilibration of carbonic species at moderate pH is:

$$CO_2 + H_2O \xleftrightarrow{K_1} H_2CO_3 \xleftrightarrow{K_2} H^+ + HCO_3^- \xleftrightarrow{K_3} 2\ H^+ + CO_3^{2-} \qquad (B.1)$$

By definition, the equilibrium constants are as given below, with values from Knoche (1980):

$$K_1 = \frac{[H_2CO_3]}{[CO_2]} = 1.4 \times 10^{-3} \qquad (B.2)$$

$$K_2 = \frac{[H^+]\cdot[HCO_3^-]}{[H_2CO_3]} = 3.2 \times 10^{-4} \text{ M} \qquad (B.3)$$

$$K_3 = \frac{[H^+]\cdot[CO_3^{2-}]}{[HCO_3^-]} = 4.7 \times 10^{-11}\ M \qquad (B.4)$$

From Eqn. B.3, $$[H_2CO_3] = \frac{[H^+]\cdot[HCO_3^-]}{K_2} \qquad (B.5)$$

From Eqns. B.2 and B.5, $$[CO_2] = \frac{[H_2CO_3]}{K_1} = \frac{[H^+]\cdot[HCO_3^-]}{K_1K_2} \qquad (B.6)$$

From Eqn. B4, $$[CO_3^{2-}] = \frac{K_3\cdot[HCO_3^-]}{[H^+]} \qquad (B.7)$$

If the only source of carbonic species is from the initial (i) added HCO_3^-, then $$[HCO_3^-]_i = [CO_2] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}] \qquad (B.8)$$

Substitution of Eqns. B.5, B.6 and B.4 into Eqn. B.8 gives

$$[HCO_3^-]_i = \left(\frac{[H^+]}{K_1K_2} + \frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]} \right) \cdot [HCO_3^-]_{eq} \qquad (B.9)$$

Rearrangement of Eqn. B.9 gives

$$[HCO_3^-]_{eq} = \frac{[HCO_3^-]_i}{\frac{[H^+]}{K_1K_2} + \frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]}} \qquad (B.10)$$

And from Eqn. B.6, $$[CO_2]_{eq} = \frac{[H^+]}{K_1K_2}\,[HCO_3^-]_{eq} \qquad (B.11)$$

C. Calculation of the error due to ignoring the escape of CO_2 into the 150 µl gas space in the cuvette (see Chapter 3):

From the ideal gas law, $$P(CO_2) = n(CO_2)_{(g)}\,\frac{RT}{V_{(g)}} \qquad (C.1)$$

where $P(CO_2)$ = partial pressure of CO_2,

$n(CO_2)_{(g)}$ = number of moles of CO_2 in the gas phase

R = gas constant

T = temperature (Kelvin)

$V_{(g)}$ = volume of gas phase

Substituting Henry's Law (Eqn. A.1) into Eqn. C.1 and rearranging,

$$\frac{n(CO_2)_{(g)}}{V_{(g)}} = \frac{X(CO_2)\cdot K(CO_2)}{RT} \qquad (C.2)$$

Substituting Eqn. A.6 into C.2,

$$\frac{n(CO_2)_{(g)}}{V_{(g)}} = \frac{[CO_2]_{(aq)}\cdot(0.018\ M^{-1})\cdot K(CO_2)}{RT} \qquad (C.3)$$

Substituting Eqn. B.6 into C.3 and multiplying both sides by $V_{(g)}/V_{(aq)}$,

$$\frac{n(CO_2)_{(g)}}{V_{(aq)}} = \frac{[H^+]\cdot[HCO_3^-]\cdot(0.018\ M^{-1})\cdot K(CO_2)\cdot V_{(g)}}{K_1K_2\ RT\ V_{(aq)}} \qquad (C.4)$$

Since the CO_2 in the gas space of the cuvette came from the solution, Eqn. C.4 represents the amount by which the total concentration of carbonic species in solution is reduced due to the escape of the CO_2. Therefore, the term $n(CO_2)_{(g)}/V_{(aq)}$ should be added to Eqn. B.8, which gives for Eqn. B.10,

$$[HCO_3^-]_{eq} = \frac{[H^+]}{K_1K_2} + \frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]} + \frac{[H^+]\cdot K(CO_2)\cdot(0.018\ M^{-1})\cdot V_{(g)}}{K_1K_2\cdot RT\cdot V_{(aq)}} \qquad (C.5)$$

A couple of errors still exist, both of which would make Eqn. C.5 over-corrected: (i) atmospheric CO_2 enters the gas space when the cuvette is opened to add HCO_3^-; thus, less CO_2 escapes from solution, and (ii) although the solution was sufficiently buffered to minimize any pH change, mass action toward CO_2 consumes H^+s, and any pH change which occurs will

tend toward less CO_2 formation and less loss of CO_2. Nevertheless, equation C.5 can be used to estimate the maximum error due to ignoring the escape of CO_2. In the worst case (pH 6.3, with the addition of 2.5 mM HCO_3^-), the error is less than 2%.

D. Calculation of the partial pressure of CO_2 at equilibrium with a given $[HCO_3^-]$ in solution at a given pH (e.g. see Chapter 5, Section I):

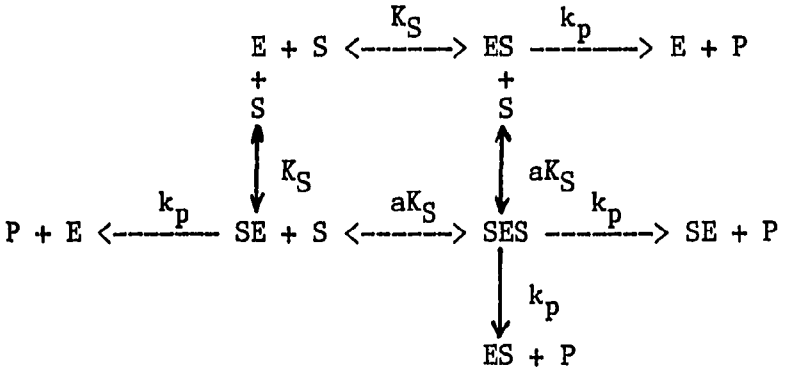
Reaarrangement of Eqn. A.7 gives

$$P(CO_2) = [CO_2]_{(aq)} \cdot K(CO_2) \cdot (0.018\ M^{-1}) \qquad (D.1)$$

The equilibrium $[CO_2]_{(aq)}$ is calculated from Eqns. B.10 and B.11 and the $P(CO_2)$ is then calculated from Eqn. D.1.

E. Kinetic analysis of various two-site schemes:

In Chapter 5, the following scheme was presented (for a thorough description of this and other kinetic schemes, see Segel (1975)):



(E.1)

A derivation of the velocity equation follows:

$$K_S = \frac{[E]\cdot[S]}{[ES]} = \frac{[E]\cdot[S]}{[SE]} \qquad (E.2)$$

$$aK_S = \frac{[ES]\cdot[S]}{[SES]} = \frac{[SE]\cdot[S]}{[SES]} \qquad (E.3)$$

From Eqn. E.2,
$$[SE] = [ES] = \frac{[S]\cdot[E]}{K_S} \qquad (E.4)$$

From Eqn. E.3, and substituting Eqn. E.4 for [ES],
$$[SES] = \frac{[S]\cdot[ES]}{aK_S} = \frac{[S]}{aK_S}\cdot\frac{[S]\cdot[E]}{K_S} = \frac{[S]^2\cdot[E]}{aK_S^2} \qquad (E.5)$$

$$[E]_t = [E] + 2[ES] + [SES] \qquad (E.6)$$

From Eqn. E.6, and substituting Eqn. E.4 for [ES],
$$\frac{[ES]}{[E]_t} = \frac{[ES]}{[E] + 2[ES] + [SES]} = \frac{\dfrac{[S]\cdot[E]}{K_S}}{[E] + \dfrac{2[S]\cdot[E]}{K_S} + \dfrac{[S]^2\cdot[E]}{aK_S}} \qquad (E.7)$$

$$= \frac{1}{[E]}\cdot\frac{\dfrac{[S]}{K_S}}{1 + \dfrac{2[S]}{K_S} + \dfrac{[S]^2}{aK_S^2}} \qquad (E.8)$$

From Eqn. E.5, and substituting Eqn. E.4 for [ES], following the same steps as above:
$$\frac{[SES]}{[E]_t} = \frac{1}{[E]}\cdot\frac{\dfrac{[S]^2}{aK_S^2}}{1 + \dfrac{2[S]}{K_S} + \dfrac{[S]^2}{aK_S}} \qquad (E.9)$$

The velocity is $$v = \frac{d[P]}{dt} = k_p[ES] + k_p[SE] + 2k_p[SES]$$

$$= 2k_p[ES] + 2k_p[SES] \qquad \text{(E.10)}$$

$$= 2k_p\,([ES] + [SES]) = 2k_p[E]\,\left(\frac{[S]}{K_S} + \frac{[S]^2}{aK_S^2}\right)$$

At saturating [S], all enzyme exists as SES, so

$$V_{max} = 2k_p[E]_t = 2k_p[E]\,\left(1 + \frac{2[S]}{K_S} + \frac{[S]^2}{aK_S^2}\right) \qquad \text{(E.11)}$$

From Eqns. E.10 and E.11,

$$\frac{V}{V_{max}} = \frac{\dfrac{[S]}{K_S} + \dfrac{[S]^2}{aK_S^2}}{1 + \dfrac{2[S]}{K_S} + \dfrac{[S]^2}{aK_S^2}} \qquad \text{(E.12)}$$

Eqn. E.12 is the velocity equation for scheme E.1. It can be simplified, if it is assumed that $a \ll 1$ (i.e. the cooperativity is high), so that [ES] = [SE] 0. Then, Eqn. E.10 becomes

$$v = 2k_p[SES] = 2k_p[E]\cdot\left(\frac{[S]^2}{aK_S^2}\right) \qquad \text{(E.13)}$$

And Eqns. E.6 and E.11 become

$$[E]_t = [E] + [SES] \qquad \text{(E.14)}$$

$$V_{max} = 2k_p[E]_t = 2k_p[E]\cdot\left(1 + \frac{[S]^2}{aK_S^2}\right) \qquad \text{(E.15)}$$

And the velocity equation reduces to

$$\frac{V}{V_{max}} = \frac{\dfrac{[S]^2}{aK_S^2}}{1 + \dfrac{[S]^2}{aK_S^2}} = \frac{\dfrac{[S]^2}{K'}}{1 + \dfrac{[S]^2}{K'}} = \frac{[S]^2}{K' + [S]^2}\ , \quad \text{where } K' = aK_S^2 \qquad \text{(E.16)}$$

This same type of analysis can be applied to an enzyme with any number of equivalent substrate binding sites. As in the example above, when the cooperativity is high, the velocity equation becomes dominated by the $[S]^n$ terms. The reader is referred to Segel (1975, pp 355-361) for a more detailed description.

A similar analysis can be applied to a two-site model in which one site is an activator site and one is a substrate site:

$$\begin{array}{ccccc} E + S & \xleftrightarrow{K_S} & ES & \xrightarrow{k_p} & E + P \\ + & & + & & \\ A & & A & & \\ \updownarrow K_A & & \updownarrow aK_A & & \\ EA + S & \xleftrightarrow{aK_S} & EAS & \xrightarrow{bk_p} & EA + P \end{array} \qquad \text{(E.17)}$$

This scheme does not assume identical dissociation constants for the two sites. However, the interaction factor, a, is necessarily the same for both routes to EAS (see Part F).

$$[E]_t = [E] + [ES] + [EA] + [EAS] \qquad \text{(E.18)}$$

$$K_A = \frac{[E]\cdot[A]}{[EA]} \; ; \quad K_S = \frac{[E]\cdot[S]}{[ES]} \qquad \text{(E.19)}$$

$$aK_S = \frac{[EA]\cdot[S]}{[EAS]} \; ; \quad aK_A = \frac{[ES]\cdot[A]}{[EAS]} \qquad \text{(E.20)}$$

From Eqn. E.19, $$[EA] = \frac{[E]\cdot[A]}{K_A} \; ; \quad [ES] = \frac{[E]\cdot[S]}{K_S} \qquad \text{(E.21)}$$

From Eqn. E.20, substituting Eqn. E.21 for [EA],

$$[EAS] = \frac{[EA]\cdot[S]}{aK_S} = \frac{[E]\cdot[A]}{K_A}\cdot\frac{[S]}{aK_S} \qquad \text{(E.22)}$$

From Eqns. E.18 and E.21, and following the same procedure as before (Eqns. E.7 - E.9),

$$\frac{[ES]}{[E]_t} = \frac{1}{[E]} \cdot \left(\frac{\frac{[S]}{K_S}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[A]\cdot[S]}{aK_AK_S}} \right) \tag{E.23}$$

$$\frac{[EA]}{[E]_t} = \frac{1}{[E]} \cdot \left(\frac{\frac{[A]}{K_A}}{\text{same denominator}} \right) \tag{E.24}$$

$$\frac{[EAS]}{[E]_t} = \frac{1}{[E]} \cdot \left(\frac{\frac{[A]\cdot[S]}{aK_AK_S}}{\text{same denominator}} \right) \tag{E.25}$$

The velocity is $v = \frac{d[P]}{dt} = k_p[ES] + bk_p[EAS]$

$$= k_p \cdot ([ES] + b[EAS]) \tag{E.26}$$

$$= k_p[E]\cdot[S] \left(\frac{1}{K_S} + \frac{b[A]}{aK_AK_S} \right)$$

Since V_{max} occurs when the enzyme is saturated with activator, as well as substrate,

$$V_{max} = bk_p[E]_t \tag{E.27}$$

From Eqns. E.26 and E.27,

$$\frac{V}{V_{max}} = \frac{\frac{[S]}{K_S} + \frac{b[S]\cdot[A]}{aK_AK_S}}{b\left(1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[A]\cdot[S]}{aK_AK_S}\right)} \tag{E.28}$$

As before, if $a \ll 1$, so that [ES] and [EA] are both ≈ 0, then Eqns. E.26 and E.28 become

$$v = bk_p[E]\frac{[S]\cdot[A]}{aK_AK_S} \tag{E.29}$$

$$\frac{V}{V_{max}} = \frac{[S]\cdot[A]}{K' + [S]\cdot[A]}, \quad \text{where} \quad K' = aK_AK_S \tag{E.30}$$

Scheme E.17 can be used to depict a one-HCO_3^--site model, in which HCO_3^- alters the affinity of the Q_B site for PQ (i.e. S = PQ, and A = HCO_3^-), or it can be used to depict a two-HCO_3^--site model, in which HCO_3^- is both a substrate and an activator (this would be analogous to the situation in carbonic anhydrase). In the first case, [S] = constant, and Eqn. E.30 reduces to

$$\frac{V}{V_{max}} = \frac{[A]}{K'' + [A]}, \quad \text{where} \quad K'' = \frac{aK_AK_S}{[S]} \tag{E.31}$$

This is functionally the same as the Michelis-Menten equation, and is not supported by the data of Chapter 5. In the second case, [A] = [S], so Eqn. E.30 reduces to

$$\frac{V}{V_{max}} = \frac{[A]^2}{K' + [A]^2}, \quad \text{where} \quad K' = aK_AK_S \tag{E.32}$$

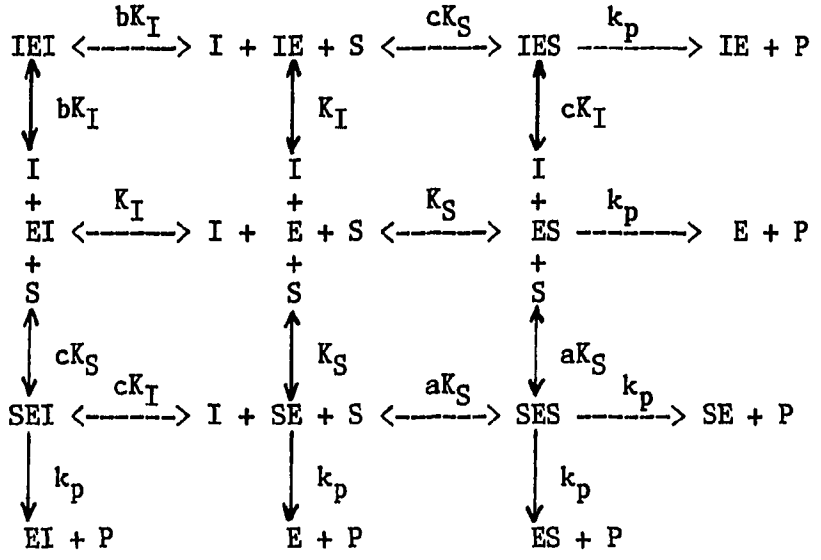
This derivation shows that the kinetic analysis of Chapter 5 is apropriate even for dissimilar dissociation constants at each site, and whether or not one site is an activator site. Segel (1975, pp. 403-404) discusses the case of multiple activation sites, and shows that

$$\frac{V}{V_{max,app}} = \frac{[A]^n}{K' + [A]^n}, \text{ where } V_{max,app} = \frac{V_{max}}{(1 + \frac{K_S}{[S]})} \tag{E.33}$$

Thus, the analysis is also appropriate if HCO_3^- is an activator at both

sites.

Finally, the analysis can be done for the case when inhibitor is present (scheme 5.5 and below):



(E.34)

The derivation follows the same steps as before:

$$K_S = \frac{[E]\cdot[S]}{[ES]} = \frac{[E]\cdot[S]}{[SE]} \; ; \quad K_I = \frac{[E]\cdot[I]}{[EI]} = \frac{[E]\cdot[I]}{[IE]} \qquad \text{(E.35)}$$

$$aK_S = \frac{[ES]\cdot[S]}{[SES]} = \frac{[SE]\cdot[S]}{[SES]} \; ; \quad bK_I = \frac{[EI]\cdot[I]}{[IEI]} = \frac{[IE]\cdot[I]}{[IEI]} \qquad \text{(E.36)}$$

$$cK_S = \frac{[EI]\cdot[S]}{[SEI]} = \frac{[IE]\cdot[S]}{[IES]} = \frac{[ES]\cdot[I]}{[IES]} = \frac{[SE]\cdot[I]}{[SEI]} \qquad \text{(E.37)}$$

From equations E.35 - E.37,

$$[ES] = [SE] \; ; \quad [EI] = [IE] \; ; \quad [IES] = [SEI] \qquad \text{(E.38)}$$

$$[E]_t = [E] + 2[ES] + 2[EI] + 2[SEI] + [IEI] + [SES] \qquad \text{(E.39)}$$

$$\frac{[ES]}{[E]_t} = \frac{1}{[E]} \cdot \frac{\frac{[S]}{K_S}}{1 + \frac{2[S]}{K_S} + \frac{2[I]}{K_I} + \frac{2[I][S]}{cK_SK_I} + \frac{[I]^2}{bK_I^2} + \frac{[S]^2}{aK_S^2}} \tag{E.40}$$

$$\frac{[EI]}{[E]_t} = \frac{1}{[E]} \cdot \frac{\frac{[I]}{K_I}}{\text{same denominator}} \tag{E.41}$$

$$\frac{[SEI]}{[E]_t} = \frac{1}{[E]} \cdot \frac{\frac{[I][S]}{cK_SK_I}}{\text{same denominator}} \tag{E.42}$$

$$\frac{[IEI]}{[E]_t} = \frac{1}{[E]} \cdot \frac{\frac{[I]^2}{bK_I^2}}{\text{same denominator}} \tag{E.43}$$

$$\frac{[SES]}{[E]_t} = \frac{1}{[E]} \cdot \frac{\frac{[S]^2}{aK_S^2}}{\text{same denominator}} \tag{E.44}$$

The velocity is

$$v = 2k_p \cdot ([SES] + [ES] + [SEI]) \tag{E.45}$$

$$V_{max} = 2k_p[E]_t \tag{E.46}$$

From Eqns. E.45 and E.46,

$$\frac{V}{V_{max}} = \frac{\left(\frac{[S]^2}{aK_S^2} + \frac{[S]}{K_S} + \frac{[I][S]}{cK_SK_I}\right)}{\text{same denominator}} \tag{E.47}$$

As before, if $a \ll 1$, so $[ES] = [SE] \approx 0$, and if $a \ll b$, so that $[SEI] = [IES] \approx 0$, then Eqn. E.39 becomes

$$[E]_t = [E] + 2[EI] + [IEI] + [SES] \tag{E.48}$$

Eqn. E.45 becomes $$v = 2k_p[SES] \tag{E.49}$$

And Eqn. E. 47 becomes

$$\frac{V}{V_{max}} = \frac{\dfrac{[S]^2}{aK_S^2}}{1 + \dfrac{2[I]}{K_I} + \dfrac{[I]^2}{bK_I^2} + \dfrac{[S]^2}{aK_S^2}} \tag{E.50}$$

Since [I] is constant, Eqn. E.50 reduces to

$$\frac{V}{V_{max}} = \frac{\dfrac{[S]^2}{aK_S^2}}{C + \dfrac{[S]^2}{aK_S^2}} = \frac{\dfrac{[S]^2}{K'}}{C + \dfrac{[S]^2}{K'}} = \frac{[S]^2}{C\,K' + [S]^2} \tag{E.51},$$

$$\text{where} \quad C = 1 + \frac{2[I]}{K_I} + \frac{[I]^2}{bK_I^2}$$

F. Proof that if HCO_3^- binding increases the affinity for PQ, then PQ binding must also increase the binding affinity of HCO_3^- by the same amount (see Chapter 5, Section II):

This can be proven quite readily, using the simplified scheme below:

$$\begin{array}{ccc} E + S & \xleftrightarrow{K_S} & ES \\ + & & + \\ PQ & & PQ \\ \updownarrow K_O & & \updownarrow aK_O \\ E{\cdot}PQ + S & \xleftrightarrow{a'K_S} & ES{\cdot}PQ \end{array} \tag{F.1}$$

Separate interaction factors are assigned for the two types of binding. The question is whether a' can be not equal to a (i.e. can a' = 1, indicating no effect of PQ on HCO_3^- binding, while a ≠ 1, indicating an effect of HCO_3^- on PQ binding?). It is shown here that a and a' must be equal.

By definition,

$$K_O = \frac{[E]\cdot[PQ]}{[E\cdot PQ]} \; ; \quad K_S = \frac{[E]\cdot[S]}{[ES]} \tag{F.2}$$

$$a'K_S = \frac{[E\cdot PQ]\,[S]}{[ES\cdot PQ]} \; ; \; aK_O = \frac{[ES]\cdot[PQ]}{[ES\cdot PQ]} \tag{F.3}$$

By rearrangement of Eqn. F.2,

$$[E\cdot PQ] = \frac{[E]\cdot[PQ]}{K_O} \; ; \quad [ES] = \frac{[E]\cdot[S]}{K_S} \tag{F.4}$$

Substitution of Eqn. F.4 into Eqn. F.3 gives

$$a'K_S = \frac{[E]\cdot[PQ]\cdot[S]}{K_O\cdot[ES\cdot PQ]} \; ; \quad aK_O = \frac{[E]\cdot[PQ]\cdot[S]}{K_S\cdot[ES\cdot PQ]} \tag{F.5}$$

Therefore,

$$a' = a = \frac{[E]\cdot[PQ]\cdot[S]}{K_O\cdot K_S\cdot[ES\cdot PQ]} \tag{F.6}$$

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VITA

Danny J. Blubaugh was born November 25, 1955, in Wichita, Kansas. He grew up in Media, Pennsylvania, where he graduated from Penncrest High School in 1973 as a fine arts major. He earned a B.A. degree with a double major in Chemistry and Biology in 1980 from Earlham College in Richmond, Indiana. In 1980 he joined the laboratory of Dr. Govindjee at the University of Illinois to study the primary reactions of photosynthesis. During his tenure at the University of Illinois, he held teaching assistantships in the Departments of Chemistry and Plant Biology, and held research assistantships in the Department of Physiology and Biophysics. He received a National Research Service Award from the National Institute of Health for the years 1981 to 1985. In 1986 he was listed in the University of Illinois' "Incomplete List of Teachers Ranked as Excellent by Their Students". In 1987 he won an award in the Annual Student Paper Competition, conducted by the University of Illinois Chapter of Sigma Xi. He will be joining the laboratory of Dr. George Cheniae at the University of Kentucky to undertake research on the O_2 evolving mechanism of photosynthesis.

He is a member of the American Society of Plant Physiologists, the American Society for Photobiology, the American Association for the Advancement of Science, and of the Sigma Xi Scientific Research Society. He has co-authored the following publications:

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