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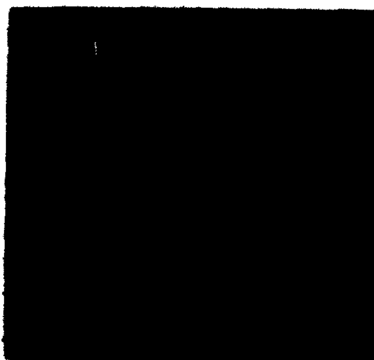

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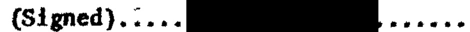
(Signed).....  .....

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ASPECTS OF PHOTO-METABOLISM IN
THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS*

by

Errol Radcliffe Jansz, B.Sc. (Hon.)

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT DALHOUSIE UNIVERSITY.

MARCH 1972

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Aspects of Photo-Metabolism in the Blue-Green Alga *Anacystis nidulans*" submitted by Errol Radcliffe Jansz in partial fulfilment of requirement for the degree of Doctor of Philosophy.

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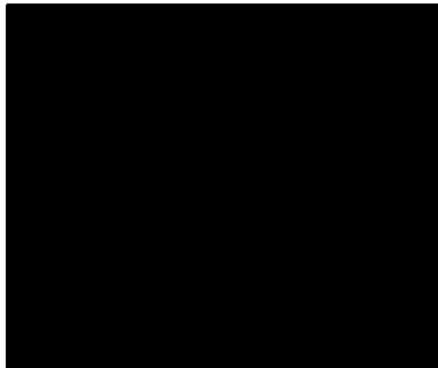

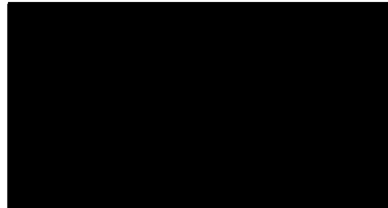

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ABSTRACT

PART I. Cold shock (0-3°) had a marked effect on photosynthesis (O_2 evolution and CO_2 fixation) of Anacystis nidulans. The labelling pattern (after short-term $^{14}CO_2$ fixation) of cold-shocked cells was similar to normal cells. The effect of cold shock on photosynthesis was not "all or none" when individual cells were considered. Cold shock caused a marked decline in viability and also a change in selective permeability resulting in the loss of 25% of the total dialysable material. Cold shock of cell-free extracts caused a decline in photophosphorylation. No decline in photosynthetic electron flow was observed. The effect of cold shock on whole cells is explained by either or both the leakage of cell material and the decline in photophosphorylation.

PART II. Studies of short-term $^{14}CO_2$ fixation by Anacystis nidulans showed that more than 90% of the C fixed passed through the Calvin cycle. A second, minor pathway of $^{14}CO_2$ fixation, involved PEP carboxylase. Evidence for this pathway was obtained from short-term $^{14}CO_2$ fixation studies, from the position of label in aspartate, and from enzyme studies in extracts. No evidence could be obtained for a transcarboxylation between the product of PEP carboxylation and RDP. Fixed C could also appear rapidly as glutamate-1-^{14}C. The reductive carboxylation of succinic acid could not be observed in extracts; it appeared that glutamate was synthesized from OAA-4-^{14}C via the Krebs cycle.

ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
F6P	D-fructose-6-phosphate
FDPase	D-fructose-1,6-diphosphate phosphatase
NAD(H)	oxidized (reduced) nicotine amide adenine dinucleotide
NADP(H)	oxidized (reduced) nicotine amide adenine dinucleotide phosphate
OAA	oxaloacetate
PEP	phosphoenolpyruvate
PGA	phosphoglyceric acid
SDP	D-sedoheptulose-1,7-diphosphate
RDP	Ribulose-1-5-diphosphate
CCCP	carbonyl cyanide m-chlorophenylhydrazone
chl.	chlorophyll
chl.*	chlorophyll (corrected)
cyt.	cytochrome
CMU	N-(p-chlorophenyl)-N'(dimethyl)-urea
DCMU	N-(3,4,dichlorophenyl)-N'(dimethyl)-urea
DCPIP	2,6-dichlorophenol-indophenol
Fd	ferridoxin
FMN	flavin mononucleotide
MV	methyl viologen
PMS	phenazine methosulphate

PPNR	photosynthetic pyridine nucleotide reductase
phyco.	phycocyanin
P.S.I.	photosystem I
P.S.II	photosystem II
PSP	photophosphorylation
V	initial velocity (of enzyme reaction)
BAW	butanol:acetic acid:water::4:1:5
EDTA	ethylenediaminetetraacetic acid
P	phosphate
Pi	inorganic phosphate
POPOP	1,4-bis[2-(5-phenyloxolyl)]-benzene
PPO	2,5-diphenyloxazolone
TCA	trichloroacetic acid
tris	tris(hydroxy-methyl)aminomethane
μM	micromoles
μ<u>M</u>	micromolar
mV	millivolts
μCi	microcurie
mCi	millicurie
nm	nanometer

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INTRODUCTION

The introduction is divided into five parts, entitled: Part A, blue-green algae and *Anacystis nidulans*; Part B, cold sensitivity; Part C, electron flow and photophosphorylation; Part D, photosynthetic CO_2 fixation; Part E, the scope of the thesis. In each case well known and generally accepted views are presented only in outline and discussion is mainly confined to areas that have direct bearing on this thesis and to more recent developments in each field of study.

PART A

Blue-green algae and *Anacystis nidulans*

The blue-green algae or *Cyanophyta* thrive in a wide variety of environments, including frigid lakes and hot springs. They can be marine, freshwater or terrestrial; free living or found growing in association with higher plants or fungi. They are of some importance ecologically, for example, as nitrogen fixers and in recent times they have been a subject of interest in the area of water pollution control.

(1) Characteristics. The salient characteristics of these organisms have been documented by Smith (1955), and Fritsch (1935) among others. The blue-green algae: (i) are prokaryotic, (ii) lack chloroplasts, (iii) have no chlorophyll b, but have an accessory pigment, c-Phycocyanin, (iv) move (if at all) only by gliding, (v) do not possess any mode of sexual reproduction, (vi) are reported to have gas vacuoles, (vii) can be unicellular or

filamentous, (viii) generally have a mucilaginous sheath, (ix) have a cell wall of the bacterial type, which as a result can be digested by lysozyme (Crespi et al., 1962), (x) are generally considered to be gram negative (Drew and Meyer, 1964), but there is some disagreement (Echlin and Morris, 1965), (xi) have a high level of unconjugated pterins (Hatfield et al., 1961).

(2) Photosynthetic apparatus of blue-green algae. The photosynthetic apparatus of blue-green algae consists of sheet-like lamellae composed of double membranes (Lang, 1968). The membranes are closed at both ends to form sac-like structures which could be called thylakoids. The lamellae (thylakoids) are either peripheral or permeate the cell, but are not aggregated to form grana as in chloroplasts. The lamellae content of blue-green algae depends directly on the conditions of growth of the organism (Allen, 1968b) and it is these lamellae which contain chlorophyll.

(3) Anacystis nidulans - Taxonomy and Morphology. Anacystis nidulans is a unicellular, rod shaped, blue-green alga of 0.5 μ in diameter. It is generally accepted as a member of the family Chroöcoccaceae. However it is believed by some (Pringsheim, 1968; Drouet, Silva, 1962) to be able to form short filaments and hence Drouet (Silva 1962) has classified it as belonging to the Oscillatoriaceae and renamed the organism as Phormidium mucicola. Pringsheim (1968) has renamed it Lauterbournia nidulans. However Allen and Stanier (1968) have defended its classification as a member of the Chroöcoccaceae. This view was also supported by Kunisawa and Cohen-Bazire (1970) who found that filaments of mutant Anacystis nidulans were multi-

nucleate coenocytes. The organism was found by Van Baalen (1957) and Forrest et al. (1957) to be very cold sensitive.

(4) Occurrence of pteridines in blue-green algae. Blue-green algae and light-grown photosynthetic bacteria have a high level of pteridines (Maclean et al., 1966 b), about 1/10% of dry weight in *Anacystis nidulans* (Maclean et al., 1966 b). The function of this high level of pteridines is not known. The only well documented role of unconjugated pterins is their role as cofactors for hydroxylation of phenylalanine, tyrosine and tryptophan. However, Nugent and Fuller (1967) showed that a known inhibitor of pteridine utilization prevented the synthesis of both coloured carotenoids and chlorphyll in *Rhodospirillum rubrum* and that the addition of 10^{-4} *M* biopterin allowed the recovery of chlorophyll content and the "complete recovery of photosynthetic activity". Further, Wu and Myers (1967) have shown that biopterin can function in the photoreduction of NADP, and Fuller and Nugent (1969) showed that biopterin could be photoreduced by bacterial chromophores. Maclean et al. (1965) showed that photophosphorylation in spinach chloroplasts was stimulated by pteridines. All this indicated that pteridines could be involved in photosynthesis. For recent speculations on their role in this connection see Fuller et al. (1971).

PART B

Cold sensitivity

Most of the work in the literature on the effect of cold on biological material, relevant to this discussion, can be divided into two parts: (i) the effect of near freezing temperatures on bacteria, (ii) the effect of freezing on tissues.

The former is closer to the main point of this study, but is almost entirely concerned with viability. The second line of study has very little in common with this investigation, since freezing could produce physical damage of quite a different type. It is considered here only because of the observed effect on photophosphorylation.

(1) Effect of cold shock on viability. It has been known for some time that bacteria (principally gram negative), when grown exponentially are sensitive to cold shock. The degree of sensitivity varies from organism to organism but generally 1/2 - 4 hr. cold shock at 0° can be sufficient to produce a significant decrease in viability. The effect was shown in E. coli (Sherman and Albus, 1923), Pseudomonas aeruginosa (Farrell and Rose, 1968) Pseudomonas pyocyanea and Salmonella typhimurium (Gorrill and McNeil, 1960), Aerobacter aerogenes (Strange and Dark, 1962), Serratia marcescens (Strange and Ness, 1963) and also in the gram positive bacterium Streptomyces hydrogenans (Ring, 1965).

Several factors affect the viability of cold-shocked bacteria such as: (i) nature of diluent, (ii) rate of cooling, a rapid rate of cooling being necessary to get an effect on viability. Protection from loss of viability can be brought

about in several ways, the effectiveness of these methods seem to vary from organism to organism.

Maynell (1958) showed that _E. coli_ was protected from loss of viability by the use of 0.3M sucrose as diluent, _Pseudomonas pyocyanea_ (Gorrill and McNeil, 1960) was protected by sucrose or full strength Ringers solution. _Pseudomonas aeruginosa_ (Farrell and Rose, 1968) was protected by 5mM $MgCl_2$, while _Aerobacter aerogenes_ was more susceptible to cold in saline than distilled water (Strange and Dark, 1962). The viability of this organism after cold shock in saline, was increased by the addition of several compounds including: (i) sucrose, (ii) small quantities of divalent metal ions, (iii) amino acids, (iv) concentrated cold shock filtrates.

Other factors that influenced viability were: (i) cell density, (ii) nature of growth medium (Strange and Ness, 1963).

Studies have also shown that cold shock caused the release of small molecules. These included U.V. absorbing compounds, amino acids and ATP (Strange and Dark, 1962). However Strange and Ness (1963) provided data to show that it was difficult to make correlations between excretion of material and viability. Further, Farrell and Rose (1968), using psychrophilic and mesophilic pseudomonads, showed that both excreted approximately the same quantity of U.V. absorbing material, although only the mesophile showed appreciable loss in viability. No data to show the effect of protective substances on the excretion of cell material is available. However, data from the osmotic cold shock of yeasts (Patchings and Rose, 1971) showed that although Mg^{2+} protected yeasts from loss of viability it in

fact increased the release of cell material.

Some correlations have been made between viability after cold shock and fatty acid composition of the cell membranes. This was done by Farrell and Rose (1968), who showed that when pseudomonads were grown at low temperatures, the percentage of unsaturated fatty acids increased and the organism became more resistant to the cold. Farrell and Rose (1968) hypothesized that cooling caused freezing of the fatty acids of the membrane which either led to the formation of channels in the membrane or alternatively, distorted the protein molecules of the membrane; either of which would alter permease activity and lead to a loss of low molecular weight compounds. With the increase of the level of unsaturated fatty acids, the melting points of the lipids in the membrane will decrease, hence the membrane will be less susceptible to freezing.

The above refers to microorganisms not cooled to below the freezing point of water. However, another theory has been put forward by Levitt and Dear (1970) to explain the effect of freezing on cell viability. Briefly this theory proposes that freezing causes a "hole" to be formed in the semi-permeable membrane and this results in the efflux of cell material. The holes are said to originate from denatured or damaged proteins and are irreversible as they are fixed by intramolecular S-S bonds from SH oxidation or disulphide exchange.

(2) Effect of cold shock on photosynthesis. The only case of cold shock on photosynthesis is that shown by *Anacystis nidulans*. This was studied by Van Baalen (1957) and Forrest et

al. (1957), who showed that on subjecting the organism to 4° for less than an hour, its photosynthetic capacity declined markedly. It was also shown that there was a loss of pteridines, glutamic acid and small quantities of other amino acids from the cell. It was believed that the loss in photosynthetic capacity might be related to the loss of pteridines from the cell. It was also pointed out by Van Baalen that cell-free extracts prepared from cold-shocked cells showed lower Hill reaction activity as measured by DCPIP reduction.

It has also been shown by Heber (1970) that thylakoid membranes obtained from osmotic shock of spinach chloroplasts (which were capable of high rates of ATP synthesis), on freezing to -25° lost their capacity to synthesize ATP. They also lost their proton pump activity. This loss was protected by sucrose. Heber (1970), has isolated from a frost-hardy plant (hardy spinach), three heat stable protein fractions, which in small quantities protected thylakoid membranes from loss in capacity to synthesize ATP.

Summary. It appears that one of the fundamental effects of cold shock is to damage membranes, and in bacteria this has been connected with fatty acid composition of membranes. There is no agreement on whether the loss in cell constituents is responsible for decline in viability.

PART C

Electron flow and photophosphorylation

The primary event in photosynthesis can be described as a light activated transfer of electrons against a chemical potential gradient, which results in the splitting of water and the generation of reducing power and ATP.

At present there are two schemes of electron flow in photosynthesis: (i) two light reactions in series (the Hill and Bendall scheme), (ii) the three light reaction formulation of Arnon.

This section of the introduction is devoted to an outline of these two schemes, as well as a summary of more recent studies which may have some bearing on either or both these formulations. The section also includes a brief outline of the sites of action of artificial electron acceptors and donors used in this study, and finally a discussion of the results obtained in the field using blue-green algae. Other important topics such as: (i) quantum requirements and associated theories, (ii) the mechanism of ATP synthesis, (iii) the structure of chloroplasts, and (iv) the physical fractionation of chloroplasts are not discussed as they have no direct bearing on the work presented in this thesis.

(1) <u>Historical</u>. The concept that photosynthesis in plants and algae requires the co-operation of two light reactions arose from the studies of enhancement by Emerson et al. (1957), i.e. that the low efficiency of light, between 680 nm and 700 nm

could be increased by providing light of a shorter wavelength. Blinks (1959), found that a sudden substitution of one beam of light for another of a different wavelength caused a disturbance in the time course of oxygen evolution (chromatic transients). Myers and French (1960), showed that the "Emerson enhancement effect" can also be produced by rapidly alternating light sources and concluded that the photochemical products of one light reaction persisted for several seconds; sufficiently long to interact with the products of a second light reaction.

(2) The two-light reactions in series formulation (Hill and Bendall Scheme). The representation that photosynthesis is driven by two light reactions was put forward by Hill and Bendall (1960) and is shown in Figure 1 (Bishop, 1971).

The scheme has been reviewed by Vernon and Avron (1965), Gibbs (1967), Boardman (1968), and Bishop (1971). The scheme involves: (i) the photolysis of water by a short wavelength photoreaction, which results in the increase of the negative potential of electrons to about 0 volts, (ii) the electrons are then passed down a series of electron acceptors until they reach the donor for the long wavelength photoreaction, (iii) this latter photoreaction raises the negative potential of the electrons to about -0.5 volts and this ultimately leads to the reduction of ferridoxin and NADP. The whole process is called noncyclic electron flow and results in the formation of 1 NADPH per pair of electrons transferred. It also results in the formation of ATP; noncyclic photophosphorylation.

PHOTOSYSTEM II PHOTOSYSTEM I

+0.8
0.4
0
E_o' (volts)
+0.4
+0.8
H_2O
O_2
chl.
Q
PQ
Cyt.b-559
Cyt.f
PC
chl.
$Cyt.b_6$
FRS
Fd
f_p
NADP+

Figure 1. The Hill and Bendall scheme (modified from Bishop, 1971). PQ, plastoquinone; PC, plastocyanin ; Q, quencher; FRS, ferridoxin reducing substance; f_p, ferridoxin NADP reductase.

The number of molecules of ATP synthesized per pair of electrons transferred is commonly believed to be 1, but P/2e ratios of more than unity have been reported by Winget et al. (1965), Isawa and Good (1968) and others. The specific site(s) of noncyclic photophosphorylation have not been conclusively shown. This is especially uncertain due to recent doubts of the participation of cytochromes between the photoreactions.

In addition to noncyclic electron flow, cyclic electron flow can also take place. This happens at a high ratio of reduced to oxidized ferridoxin and results in the donation of electrons from photosystem I to some point in the electron transfer chain (E.T.C.). Cyclic photophosphorylation is of physiological significance (Arnon, 1967) since a ratio of ATP: NADPH of more than one is necessary for CO_2 fixation, and additional ATP is also needed for other physiological processes.

The site(s) of cyclic photophosphorylation have not been conclusively shown either. From studies of Lee et al. (1969) and several others, the existence of a site specific for cyclic photophosphorylation was suggested. Studies with specific inhibitors have led to the same conclusion.

The participation of plastoquinones in the electron transfer chain was shown by Bishop (1959), Krogmann (1961), Henniger and Crane (1963, a,b), Amesz (1964) and others; while the participation of plastocyanin was shown by Katoh (1963), Katoh and Takamiya (1963), de Kouchkovsky and Fork (1964) and many others. In both cases the evidence presented included redox. changes in red and far-red light (= 700 nm) and extraction and reconstitution studies.

Studies of absorption changes at 420 nm, by Duysens (1962), indicated the participation of cytochromes between the photosystems. This was confirmed by more detailed studies of the redox. changes of cytochromes by several groups of workers. Levine and Gorman (1966) demonstrated this very clearly using mutants of Chlamydomonas reinhardii. Cramer and Butler (1967) provided evidence consistent with Cyt. b-559 and Cyt. f being between the photoreactions. They also showed that Cyt. b_6 (Cyt. b-563) was reduced by photosystem I.

The involvement of ferridoxin as a mediator in the transfer of electrons to NADP was shown by Whatley et al. (1963), while the existence of an acceptor receiving electrons from photosystem I before ferridoxin was shown by Yocum and San Pietro (1969), and named the Ferridoxin reducing substance (FRS). This is believed to be similar to the cytochrome reducing substance (CRS) shown by Fugita and Myers (1965, 1966).

Recent studies have indicated that a pigment, C550, is either equivalent to the quencher (Q) of photoreaction I or an excellent index for it (Erixson and Butler, 1971; Butler and Okayama, 1971).

The Hill and Bendall scheme is also supported by evidence from several other sources.

(3) The three photoreaction formulation of Arnon. A large part of the above evidence is also consistent with the scheme of Arnon. The notable exception is the evidence relating to the antagonistic effects of red and far-red light on the redox. state of components of the electron transfer chain.

The three light reaction formulation of Knaff and Arnon (1969) can be summarized as follows (Figure 2): (i) photosystem I and II are not connected, (ii) photosystem II consists of two photoreactions connected by an electron transfer chain. The chain consists of Q (pigment C550), plastoquinone, Cyt. b-559, and plastocyanin. The system is involved in noncyclic photophosphorylation, (iii) photosystem I contains Cyt. b_6 and Cyt. f and is only involved in cyclic photophosphorylation.

Evidence presented by Arnon's group to support the concept of two unconnected photosystems falls in two categories: (i) evidence presented to support the original two separate light reaction hypothesis of Arnon. This included (a) the effect of an inhibitor of photophosphorylation, desaspidin, (b) the lack of enhancement of NADP reduction in broken chloroplasts; (ii) recent evidence from the redox changes of cytochromes.

Arnon (1965), Gromet-Elhanon and Arnon (1965) and Arnon (1967) presented evidence that a specific inhibitor, desaspidin, inhibited cyclic photophosphorylation and also photophosphorylation catalyzed by DCPIP/ascorbate but did not affect photophosphorylation with water as electron donor. This line of evidence was criticized by Avron and Gromet-Elhanon (1966) who showed that inhibition by desaspidin was related to the redox state of the environment (the more reducing the environment, the more effective the inhibitor). Although the criticism has been rejected by Tsujimoto et al. (1966), this line of evidence cannot be given much weight.

The second line of evidence was the lack of enhancement of NADP reduction in broken chloroplasts (McSwain and Arnon, 1968).

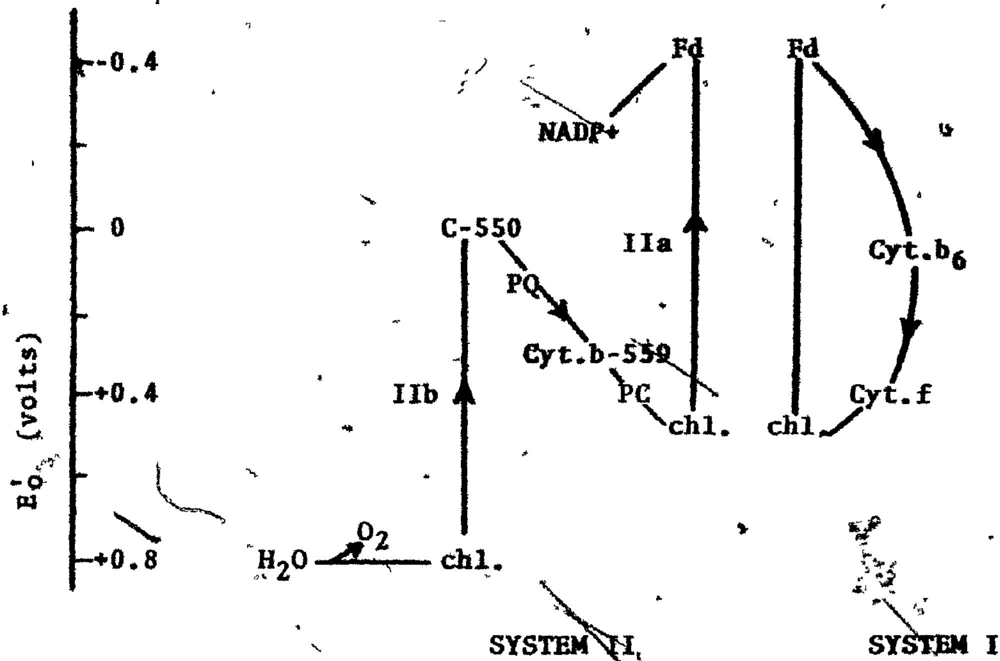


Figure 2. The three light reaction scheme (modified from Knaff and Arnon, 1969). IIa and IIb, two short wavelength photoreactions; I, long wavelength photoreaction; C-550, primary acceptor for IIb; PQ, plastoquinone; PC, plastocyanin; f_p, ferridoxin NADP reductase.

Enhancement would be expected on the Hill and Bendall scheme but not on the Arnon scheme. However, recently Sane and Park (1971) have reported that enhancement of NADP reduction could be obtained and have presented evidence to show that it was dependent on the presence of factors in the PPNR fraction used in these studies.

Knaff and Arnon (1969, a,b,c) provided evidence for the oxidation of Cyt. b-559, (red light was more effective than far-red light), when the reduction was blocked either by using low temperature or by washing with tris buffer. They also showed that a new pigment, C550 was reduced by PS 11 at low temperatures.

Knaff and Arnon (1969 c) showed that DCMU inhibits photooxidation of Cyt. b-559 and that plastocyanin was necessary for its photooxidation by red light. Similar studies showed that plastocyanin was not required for either the photoreduction of C-550 by red light, (Knaff and Arnon, 1971) or the photooxidation of Cyt. f by far-red light (Knaff and Arnon, 1970). This indicated that Cyt. b-559 and C-550 did not form a part of the same photoreaction.

Knaff and Arnon (1971) showed that chloroplasts lacking plastocyanin reduced Cyt. b-559 and that photoreduction took place preferentially in red light. They also showed that DCMU stimulated C-550 reduction but inhibited C-550 oxidation and Cyt. b-559 reduction. Thus Knaff and Arnon concluded that Cyt. b-559 was further away from photoreaction 11b than C-550 and that DCMU acts at a site between them. This conclusion, however, was apparently contradictory to the inhibition of Cyt. b-559 oxidation by DCMU (Knaff and Arnon, 1969).

Recent studies of Knaff and McSwain (1971) have shown that there was a red rise for Cyt. f oxidation and a red drop for Cyt. b-559 oxidation and C550 reduction. All this evidence lends support to the three light reaction scheme and was contrary to what is expected from the conventional Hill and Bendall formulation.

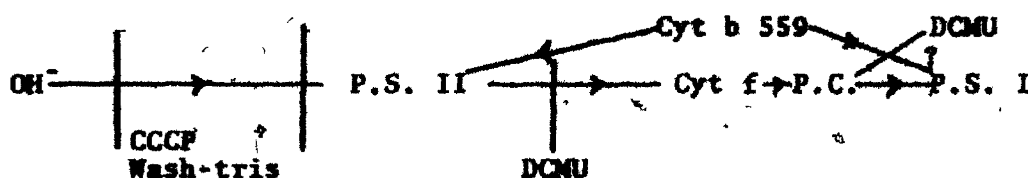
(4) <u>Recent developments in electron transport (from laboratories other than Arnon's)</u>. Confirmation of evidence of Arnon for the pigment C550 was reported by Erixson and Butler (1971). They concluded that C550 was either the primary acceptor for photoreaction II or an excellent index for it. Bendall and Sofrova (1971) reached the same conclusion but they named this pigment P546. These two groups also confirmed the site of action of DCMU; between the primary and secondary acceptor of photoreaction II.

The more significant advance however was the confirmation of Arnon's data on the photooxidation of Cyt. b-559 preferentially by red light. This confirmation was reported by Erixson and Butler (1971) and Bendall and Sofrova (1971). Both studies were carried out at -196°. Hiller et al. (1971) working at room temperature, (in the presence of carbonyl cyanide m-chlorophenyl-hydrozone-CCCP), obtained nearly the same result but in this case oxidation of Cyt. b-559 took place nearly as well in far-red light. Hiller and co-workers also found that if aged chloroplasts of a mutant ~~[illegible] they could obtain Cyt.~~ b-559 reduction in red light.

Interpretations of the results varied. Arnon and co-workers

concluded that the data lent support to the three light reaction concept, while the other groups sought to explain the data by modification of the Hill and Bendall scheme or attributed the data to deviations from the normal process brought about by abnormal reaction conditions (low temperature, disruption of membrane systems by washing with tris and the use of CCCP).

Hiller et al. (1971) suggested that Cyt. b-559 was not a normal component of the electron transport chain and was probably on a bypass. They stated that under conditions of the primary oxidant being inhibited (either with CCCP or by washing with tris), Cyt. b-559, (which is generally in the reduced state) directs electrons to either excited state and becomes oxidized. The scheme was represented as follows:



On the other hand, Bendall and Sofrova (1971) placed Cyt. b-559 between the photolysis of water and photoreaction II. This was consistent with the data of Butler and Okayama (1971) and Brisson and Butler (1971).

However, Brisson and Butler (1971) theorized that under normal conditions Cyt. b-559 lay between photosystems II and I but, at low temperatures or after washing with tris, electrons cannot be removed from water and under these conditions Cyt. b-559

functioned as an electron donor. They also argued that the concomitant oxidation of Cyt. b-559 and reduction of C550 indicated that under the conditions both pigments functioned as a part of the same photoreaction, (which was in effect, the reverse of the conclusion reached by Arnon).

Summary. Arnon's conclusions based on evidence from desaspidin and from lack of enhancement for NADP reduction are no longer acceptable. However, recent studies on the redox. changes of cytochromes with red and far-red light have thrown the generally accepted views on electron flow into a state of confusion. The net result is that some of the advantages held by the Hill and Bendall scheme over the three light reaction scheme appear to be nullified. Due to this it is not possible to reach a firm conclusion in deciding which of the two concepts is more accurate.

(5) Sites of action of artificial electron donors and acceptors.

(a) Ferricyanide accepts electrons after the site of ATP synthesis and assays for photosystem II.

(b) Reduced Cyt. c donates electrons to photosystem I (Kok et al., 1963) and assays for photosystem I.

(c) There is some disagreement in the literature about the site of action of DCPIP. According to Sun and Sauer (1971), who studied quantum efficiencies, DCPIP accepts electrons after photosystem II. However, Lien and Bannister (1971) reported that the site of DCPIP reduction was dependent on the presence of plastocyanin. If plastocyanin was present, then a fast DCPIP reduction took place after photosystem I, but in the absence of

plastocyanin a slow DCPIP reduction took place after photosystem II.

(d) Methyl viologen accepts electrons after photosystem I, (Vernon and Avron, 1965), and is involved in noncyclic electron flow.

(e) PMS accepts electrons after photosystem I (Kok and Hoch, 1961; Hauska et al., 1970) and passes the electrons down to a point in the electron transfer chain, i.e. it is involved in cyclic electron flow. It catalyses cyclic photophosphorylation.

(6) Studies on photophosphorylation and electron flow using blue-green algae. Studies with blue-green algae indicated that electron transport and photophosphorylation were the same as in higher plants. However, reports have indicated that the photosynthetic apparatus of blue-green algae was very susceptible to physical disruption; in fact most of the early studies were carried out in the presence of high molecular weight polymers, Carbowax (Van Baalen, 1957; Fredricks and Jagendorf, 1964) and Ficoll (Susor and Krogmann, 1964).

One feature which stands out with respect to the photosynthetic apparatus of blue-green algae is the ease with which components of electron transport and photosynthetic phosphorylation are lost. Fredricks and Jagendorf (1964) with *Anacystis nidulans* and Susor and Krogmann (1964) with *Anabaena variabilis* showed that when photosynthetic particles were extracted with water, a loss of Hill activity resulted. Hill activity was restored on addition of a factor from the extract.

High rates and coupling ratios for noncyclic photophos-

phorylation (and also in the case of Anacystis nidulans, reasonable rates of NADP reduction) were not observed in early studies. However, with the development of the lysozyme technique of preparing cell-free extracts of blue-green algae, Biggins (1967) with Phormidium luridum, and Lee et al. (1969) with Anabaena variabilis, were able to obtain rates of ATP synthesis and NADP reduction comparable to those obtained with chloroplasts. However, results with Anacystis nidulans were not as good, especially with respect to noncyclic photophosphorylation. Some of the results from photochemical experiments with blue-green algae are summarized on Table 1 and 2. It was interesting that addition of ferridoxin and ferridoxin:NADP-oxidoreductase was not necessary for maximal rates in Phormidium luridum (Biggins, 1967).

Biggins (1967), Lee et al. (1969) and others have observed that the apparatus for cyclic photophosphorylation was much more resistant to physical disruption than was the apparatus for noncyclic photophosphorylation, and preparation of cell-free extracts by grinding, homogenization or sonication caused a loss in capacity for noncyclic photophosphorylation. This implied a loss or dislocation of a coupling factor in the phosphorylating apparatus which was uniquely required for noncyclic photophosphorylation.

Biggins (1967), using Phormidium luridum found that if the photosynthetic lamellae were washed with dilute buffer, a specific protein factor was lost. There was also a loss in cyclic photophosphorylation. A similar situation was reported by

Table 1. Studies on electron flow using blue-green algae.

Organism/method of prepn. of cell-free extract	Subtrate/ cofactor	Rates (µEq./mg. chl./hr.)	Reference
Anabaena variabilis			
Homogenization	$K_3Fe(CN)_6$	465	Susor & Krogmann (1964)
	NADP	464	
	DCPIP	172	
lysozyme	$K_3Fe(CN)_6$	257	Lee et al. (1969)
	NADP	275*	
Anabaena cylindrica			
homogenization	Cyt.c (Red)	49	Fugita & Myers (1965)
Phormidium luridum			
lysozyme	NADP	298	Biggins (1967)
	$K_3Fe(CN)_6$	386	
Anacystis nidulans			
grinding	$K_3Fe(CN)_6$	150	Fredricks & Jagendorf (1964)
	DCPIP,Cyt.c	+	
	NADP	-	
homogenization	DCPIP	170*	Van Baalen (1957)
sonication	Cyt.c (Red)	17	Fugita & Myers (1966)
	"	25*	Brown (1969)
homogenization	NADP	45	Black et al. (1963)
lyophilization & lysozyme	$K_3Fe(CN)_6$	150*	Gerhardt & Santo (1966)
	NADP	100*	Bothe (1969)
	NADP	160*	Bothe & Berzborn (1970)

* Approximate values calculated from data.

Table 2. Studies on photophosphorylation using blue-green algae

Organism/method of prepn. of cell-free extract	Substrate/ cofactor	Rates (μM ATP mg.chl./hr.)	Reference
Anabaena variabilis			
grinding	PMS	200	Petrack (1959)
homogenization	PMS	130-800	
	FMN	240	Duane et al. (1965)
	DCPIP/ ascorbate	93	
	NADP, $K_3Fe(CN)_6$	0	Lee et al. (1969)
sonication	NADP, $K_3Fe(CN)_6$	0	
lysozyme	$K_3Fe(CN)_6$	171	
	NADP	107	"
	PMS	480	
Phormidium luridum			
grinding	PMS	250-350	Biggins (1967)
	NADP, $K_3Fe(CN)_6$	0	
lysozyme	PMS	238	
	PMS/ascorbate	357	
	$K_3Fe(CN)_6$	181	"
	NADP	132	
	DCPIP/ ascorbate	187	
Anacystis nidulans			
homogenization	NADP, FMN	-	Black et al. (1963)
	PMS	+(low)	
lyophilization & lysozyme	PMS	150	Gerhardt & Santo (1966)
	Menadione	70	
	$K_3Fe(CN)_6$	30*	
	NADP	32	Bothe (1969)
	Pd(DCMU)	80	

* Approximate values calculated from data.

Lee et al. (1969) with Anabaena variabilis which underwent a similar loss after sonication of particles in dilute buffer.

Another peculiarity of blue-green algae was that noncyclic photophosphorylation and electron transport were so loosely coupled that, uncouplers of photophosphorylation, or the presence or absence of ADP and inorganic phosphate, had no effect on the rate of electron flow (Lee et al. 1969).

Another point of interest was that Holton and Myers (1963, 1967, a,b) found a low potential c type cytochrome in Anacystis nidulans. The physiological function of this cytochrome is not known.

PART D

Photosynthetic carbon dioxide fixation

Studies with *Anacystis nidulans* have shown that sugars were synthesized by the Calvin Cycle (Kindel and Gibbs, 1963, Kandler, 1961). However, these studies provided no insight into the primary CO_2 accepting reaction. The nature of this reaction was interesting especially in view of (i) the note by Richter (1961) that aspartate was one of the first labelled products of CO_2 fixation by *Anacystis nidulans* and (ii) the current interest in the Hatch-Slack-Kortschak Pathway.

This section of the introduction outlines the pathways of CO_2 fixation in higher plants and bacteria, with special emphasis on CO_2 incorporating reactions, i.e RDP carboxylase and the Hatch-Slack-Kortschak pathway.

(1) The reductive phospho-pentose cycle.

(a) An outline of the cycle.

The main pathway of CO_2 fixation in most higher plants, algae and photosynthetic bacteria is a cyclic series of reactions called the "Calvin Cycle", or the reductive phospho-pentose cycle. The cycle was deduced by Calvin and his co-workers during 1946-1955 and has been discussed by Calvin and Bassham (1962) and Bassham (1964). In its simplest form the Calvin cycle consists of four stages: (i) The carboxylation of three molecules of RDP to give six molecules of 3-phospho-glyceric acid (PGA). (ii) PGA is then reduced to triose phosphate. (iii) A series of reactions then converts five of the six triose phosphate molecules to 3 pentose phosphate molecules. (iv) The pentose phosphates

are phosphorylated to regenerate RDP. The net result is that for every turn of the cycle three molecules of CO_2 are fixed and this carbon is drained off either as PGA or hexose phosphate to form sugar or amino acid.

(b) Evidence for the cycle from kinetic studies and isolation of enzymes.

The primary line of evidence was from kinetic studies of the labelling of compounds in the living cell following the introduction of $^{14}CO_2$. These studies were carried out under steady state conditions and proved to be an invaluable tool in the elucidation of the nature of the cycle. The main principle was that the order of appearance of labelled compounds reflected the order of their synthesis. Although the technique has many limitations it was generally successful.

The second and supporting line of evidence came from the study of enzymic activity in cell-free extracts. This was done by Racker, Hurwitz, Weissbach, Horecker and others, (e.g. Racker, 1955, 1957; Hurwitz et al., 1956; Horecker et al., 1956). Most photosynthetic organisms were found to have adequate activity of the relevant enzymes.

(c) Some apparent anomalies.

Early criticism of the Calvin cycle came mainly from Stiller (1962) but one anomaly pointed out by Kandler and Gibbs (1956) warranted more attention. They pointed out that the C-4 of glucose was more highly labelled than C-3. This was later explained by Bassham (1964) as being due to a small highly radioactive pool of glyceraldehyde 3-phosphate reacting with a larger (and consequently lower specific active) pool of dihydroxy-

acetone phosphate. A related anomaly was that the label in C-1 and C-2 of glucose was more than that in C-5 and C-6. This was explained by Bassham as being due to the reversibility of the transketolase reaction. The above is an illustration of some of the problems of this type of kinetic study. Problems could also arise as a result of the presence of: (1) unstable intermediates, (2) more than one pool of a particular intermediate, (3) multi-enzyme complexes.

Bassham (1964) has listed several reasons for believing that some enzymes of the cycle exist as an organized system. As a result of this some intermediates could not be isolated in measurable quantities, e.g. 3-phosphoglyceraldehyde and 4-phosphoerythrose. This also allowed considerable quantities of radioactive material to pass through the cycle without saturating all the free intermediates.

The other type of early criticism was the lack of adequate activity in some of the Calvin cycle enzymes in certain organisms. Among these enzymes were fructose diphosphate phosphatase (FDPase), sedoheptulose diphosphate phosphatase (SDPase)(Peterkofsky and Racker, 1961). But it has been shown since, that at least in the case of FDPase, the enzyme was ferridoxin-activated (Buchanan, 1967). Reports have also been made by Richter (1961) and Fewson et al. (1962) that FDP aldolase was absent from Anacystis nidulans, however this enzyme has been demonstrated by Van Baalen (1965) and later by Willard and Gibbs (1968). In any case, failure to demonstrate enzymic activity is not an overwhelming argument against the occurence of a reaction in vivo.

However the most prominent argument against the Calvin cycle has been the low activity and high K_m of RDP carboxylase. This is discussed below.

(d) Ribulose diphosphate carboxylase and CO_2 fixation.

The enzyme was isolated and purified from many sources. Akazawa et al. (1969) and Anderson et al. (1968) compared the molecular weights of the enzymes from various sources. Its molecular weight from most plants, algae and some photosynthetic bacteria was in the range of $5\text{-}5.5 \times 10^5$, but enzymes from *Rhodopseudomonas palutris* and *R. spheroides*, and *Hydrogenomonas facilis* and *Rhodospirillum rubrum* were much smaller.

Wishnick et al. (1970) showed that the enzyme contained copper, but that the apo-enzyme was fully active. The same authors reported the presence of 8 sub-units in the spinach enzyme, each divisible into one large and one small peptide chain.

The enzyme was activated by Mg^{2+} (generally a requirement) and was inhibited by inorganic phosphate (Wishnick and Lane, 1971). However, Anderson et al. (1968) reported that the *Rhodospirillum* enzyme did not require magnesium for its activity. The specificity of the divalent ion required was generally dependent on the source of the enzyme. It was suggested by Wishnick et al. (1970) that the divalent ion played a role in the binding of the six carbon intermediate.

Although there is little doubt that this enzyme is the carboxylating enzyme of the Calvin cycle, an outstanding feature is its rather high Km for HCO_3^-. Substrate concentrations for half maximal activity of the order of 11 mM - 20 mM have been

reported for the purified enzyme (Paulsen and Lane, 1966; Racker, 1957).

Cooper et al. (1969) showed that the substrate for RDP carboxylase was CO_2 and that the Km was 0.45 mM. However this value appears rather high, considering that the concentration of CO_2 in a solution in equilibrium with air is of the order of 8 μM (Hatch and Slack, 1970).

In vivo the problem of CO_2 concentration can be resolved by a CO_2 concentrating mechanism at the site of carboxylation. Alternatively, the kinetic properties of the enzyme (or even the mechanism of the reaction) may differ in vivo due to (i) allosteric activation or (ii) its environment in the chloroplast. However there is no evidence for this type of activation of RDP-carboxylase by metabolites. Although Mg^{2+} reduced the Km for HCO_3^- (Sugiyama et al. 1968), the change in Km appeared to be at the expense of Vmax. which decreased. There was also an accompanying downward shift in pH optimum.

Studies with isolated chloroplasts showed that the Km for HCO_3^- was very much lower than that of the free enzyme (Hatch and Slack, 1970). There was also an observation that the CO_2 fixing capacity of chloroplasts varied considerably when prepared in different ways even though there was little difference in the content of photosynthetic enzymes. In addition, the rate of CO_2 fixation in chloroplasts was five-fold that of RDP carboxylase isolated from them. This might represent a difference in the kinetic properties between the free enzyme and the chloroplast-bound species. The difference however can

be equally well explained by the operation of a CO_2 activating mechanism (Latzko and Gibbs, 1968), or an increase in supply of CO_2.

Recently, Champigny and Miginiac-Maslow (1971) and Miginiac-Maslow and Champigny (1971) showed that antimycin A was antagonistic toward the inhibition of carbon dioxide fixation by inorganic phosphate. They suggested on the basis of this and other evidence that a high energy intermediate took part in the transport of HCO_3^- into the chloroplast at the expense of photophosphorylation.

Another way of increasing the supply of CO_2 has been suggested by the studies of Reed and Graham (1968), who connected the CO_2 fixing capacity of *Chlorella* with its carbonic anhydrase content. They found that *Chlorella* grown at high CO_2 levels had low carbonic anhydrase levels and when the organism was transferred to a low CO_2 atmosphere it could not fix CO_2 into the Calvin cycle until the carbonic anhydrase level increased. It was possible that carbonic anhydrase played some role in the supply of CO_2 to RDP carboxylase.

Some of the anomalies for the low activity of RDP carboxylase can be explained by the reported light activation of this enzyme. Two theories of light activation have been put forward. Wildner and Criddle (1969) showed the presence of a light activation factor. The kinetic effect of this factor was to increase maximal velocity of the enzyme. Jensen and Bassham (1968) suggested that Mg^{2+} might be involved in the light activation of RDP carboxylase *in vivo*. They suggested that light caused a movement of H^+ from stroma to thylakoid and a movement of Mg^{2+}

in the other direction. This was based on the data of Dilley and Vernon (1965) who showed that there was a light-dependent movement of H^+ into the chloroplast and movement of Mg^{2+} out of the chloroplast. However the possibility suggested by Bassham and Jensen might not be of significance because as was earlier pointed out, Mg^{2+} lowers Km by lowering Vmax. Recently Jensen (1971) showed that Mg^{2+} can take the place of light in CO_2 fixation by broken chloroplasts and that its main effect was on Ribulose-5-phosphate kinase which it activated 25-100 fold. He also provided evidence to show that RDP carboxylase was activated 2-3 fold and that the presence of Mg^{2+} lowered the Km for HCO_3^- to 0.4 mM - 0.6 mM. The author suggested that some factor or orientation in the local environment of the carboxylase in the chloroplast gave the enzyme more affinity for CO_2 and hypothesized that Mg^{2+} could do the same thing in broken chloroplasts.

The idea that Mg^{2+} was involved in light activation in chloroplasts was consistent with the studies of Len and Nobel (1971) who found that chloroplasts isolated from plants kept in the dark ("dark plants") were less active in CO_2 fixation than those in the light ("light plants"). On addition of 5 mM Mg^{2+} however, the photosynthetic phosphorylation of "dark plants" increased 3 fold and the rate of CO_2 fixation 2-7 fold to bring it to the same level as the "light" plants". The authors also suggested that in addition to efflux of Mg^{2+} from thylakoid to stroma, the Mg^{2+} concentration would be increased by efflux of water from the chloroplasts.

(e) Other methods of control.

In the previous section some methods of control of the Calvin cycle were discussed. These mainly dealt with RDP carboxylase. Bassham (1971) has discussed other possible methods of control of CO_2 fixation. These included: (i) The flux of ions, in and out of chloroplasts as well as within the chloroplast itself. Since this involves H^+ flux too, pH is affected. (ii) Transport of intermediates across chloroplast membrane. (iii) Activation and inhibition of enzymes; (a) light activation of FDP^{ase} and SDP^{ase} (by reduced ferridoxin), (b) light activation of ribulose-5-phosphate kinase (Mg^{2+} and production of substrate-ATP), (c) light activation of glyceraldehyde 3-phosphate dehydrogenase (Ziegler et al., 1968), (d) activation of the production of 6-phosphogluconate in the dark. (iv) Regulation by NH_4^+ of the quantity of carbohydrate and protein synthesized. This last point was shown by Kanazawa et al (1970) in Chlorella. NH_4^+ inhibited F6P-UDPG-transglycosylase and thereby prevented sucrose synthesis. At the same time NH_4^+ also stimulated pyruvate kinase. This had the net effect of increasing amino acid synthesis.

(2) The C-4 dicarboxylic acid pathway. The C-4 dicarboxylic acid pathway or the Hatch-Slack-Kortschak pathway has been reviewed recently by Walker and Crofts (1970) and Hatch and Slack (1970). The pathway was first suggested by the work of Kortschak (1965), who showed that after very brief exposure to $^{14}CO_2$, maize and sugar cane leaves gave rise to malate and aspartate as the first major labelled products.

On the basis of the studies with sugar cane, Hatch and Slack (1966) proposed the following mechanism for the pathway. (1) PEP is carboxylated to produce oxaloacetate. (2) The oxaloacetate formed is rapidly equilibrated with pools of aspartate and malate which are in effect the first stable radioactive products of the pathway. (3) The C-4 COOH of a dicarboxylic acid (probably oxaloacetate) is transferred to an acceptor in the Calvin cycle (probably RDP or a compound derived from it) to form PGA and pyruvate. (4) The pyruvate regenerates PEP. In essence, what the pathway is doing, is providing CO_2 to the Calvin cycle in another form, perhaps more efficiently.

The presence of the proposed pathway as judged by the appearance of label initially in malate and aspartate has been found in many species of tropical plants and these include species of Gramineae, Cyperaceae, Amaranthaceae, Portulaceae, Chenapodiaceae - Hatch et al. (1967), Johnson and Hatch (1968), Osmund (1967). All plants sharing this type of labelling have several common morphological features. (a) Two types of chloroplasts (mesophyll and bundle sheath). (b) Bundle sheath chloroplasts have no grana or have poorly developed grana. (c) The bundle sheath cells are tightly packed, (Berry et al. 1970). (d) The bundle sheath chloroplasts accumulate starch.

(a) Evidence for pathway.

The main line of evidence was the rapid labelling of aspartate and malate in the C-4 and the transfer of this label during pulse-chase experiments into the C-1 of PGA and subsequently to sucrose and glucose (Hatch and Slack, 1966; Johnson and Hatch, 1969). Evidence was also produced suggesting that

RDP was implicated as the acceptor of CO_2 from the C-4 dicarboxylic acids (Johnson and Hatch, 1969).

A study of enzymic activities revealed that these plants had PEP carboxylase activity sufficient to support photosynthesis (Johnson and Hatch, 1968; Slack and Hatch, 1967). Further, Hatch and Slack (1970) showed that the sugar cane enzyme had a low Km for HCO_3^- (0.4 mM) and it was likely that the enzyme, like that of peanut cotyledons (Maruyama and Lane, 1962), used HCO_3^- as substrate. However, Waygood et al. (1969) showed that maize PEP carboxylase used CO_2 as substrate and had a Km HCO_3^- of 0.25 mM.

The C-4 plants also contained 2-10 fold higher levels of aspartate aminotransferase and NADP-specific malate dehydrogenase than did C-3 plants (Slack and Hatch, 1967). Hatch and Slack (1969, 1968) also showed that the C-4 plants also had a pyruvate-Pi dikinase and a pyrophosphatase for the conversion of pyruvate to PEP. However, the transcarboxylase required to complete the connection with the Calvin cycle was not demonstrated by this group of workers. The only evidence for a transcarboxylase enzyme came from Pan and Waygood (1971).

(b) Localization of enzymes involved in CO_2 fixation in C-4 plants.

The localization of photosynthetic enzymes in C-4 plants has been extensively studied by Slack et al. (1969) and others. The study was made possible by methods developed to separate bundle sheath and mesophyll chloroplasts (Baldry, 1968; Slack et al., 1969). These studies showed that the enzymes for oxaloacetate, malate and aspartate synthesis as well as pyruvate-Pi dikinase and pyrophosphatase were localized mainly in the

mesophyll chloroplasts. According to Hatch and Slack (1970), RBP carboxylase was attached to the chloroplast membrane, and therefore stategically located to encounter HCO_3^-. Thus, the mesophyll chloroplasts were clearly equipped to synthesize C-4 dicarboxylic acids.

On the other hand Calvin cycle enzymes were low in the mesophyll chloroplasts. The reverse was true in the bundle sheath chloroplasts, that is, the dicarboxylic acid synthesizing enzymes were low whereas enzymes involved in the Calvin cycle and the malic enzyme were present in large quantities. The location of RDP carboxylase in C-4 plants was confirmed by Bjorkman and Gahl (1969).

(c) The C-4 dicarboxylic acid pathway - a mechanism for translocation of CO_2.

The lack of evidence for a transcarboxylation reaction as well as suggestive evidence from the localization of enzymes made it likely that the formation of C-4 dicarboxylic acids was a mechanism to trap CO_2 in the mesophyll and transfer it in the form of C-4 dicarboxylic acids to the CO_2-deprived bundle sheath chloroplasts by way of plasmodesmata. The theory was proposed by Berry et al. (1970) who postulated that once the dicarboxylic acids passed into the vascular bundle chloroplasts they were decarboxylated and the CO_2 utilized by the Calvin cycle. In other words, the dicarboxylic acid pathway was a mechanism to circumvent the morphological disadvantage of having the enzymes of the main CO_2 fixing process localized in an area of poor gas exchange.

The theory was supported by several lines of evidence.

Bjorkman et al. (1969) demonstrated that two species of Atripex fixed CO_2 in different ways. One showed the presence of a C-4 dicarboxylic acid pathway while the other fixed CO_2 directly into the Calvin cycle (C-3 plant). The plants showed the expected morphological characteristics (the C-3 plant having only mesophyll chloroplasts which were freely accessible to gas exchange). Hybrids of these two plants showed intermediate enzyme activities, labelling patterns and morphology.

Further evidence came from the work of Downtown et al. (1970) and Downtown and Pyliotis (1971). They showed that the mesophyll cells of C-4 plants, like all the chloroplast-containing cells of C-3 plants, could reduce tetranitro blue tetrazolium chloride (TNBT), while the vascular bundle sheath cells could not. This implied that the latter could not perform a Hill reaction. Downtown et al. also showed that the bundle sheath chloroplasts lost their grana as they lost the power to reduce TNBT during ontogeny.

Anderson et al. (1971) showed that the vascular bundles of C-4 plants had poor grana development, low photosystem II activity and low photosystem II pigments.

Arntzen et al. (1971) showed that only grana-containing particles were capable of the Hill reaction and proton pump activity and that in the grana-containing particles, ATP formation was inhibited by proton pump inhibitors. This was also shown with respect to bundle sheath and mesophyll chloroplasts. The bundle sheath chloroplasts however, were able to carry out PMS-stimulated photophosphorylation. Put together, this evidence showed that bundle sheath chloroplasts made ATP but had no

source of reducing power.

It has been shown by Downtown (1970) that C-4 plants could be divided into "aspartate formers" and "malate formers", depending on which dicarboxylic acid most of the label enters. Downtown also showed that aspartate formers had some grana in their vascular bundle chloroplasts and were capable of some photosystem II activity. He proposed that in the "malate formers" the fixed CO_2 is transferred to the bundle sheath as malate and that once there, the malate is acted upon by the malic enzyme (localized in the bundle sheath) which results in the formation of the needed reducing power and CO_2. While in the case of the "aspartate formers", the reducing power is formed in the bundle sheath chloroplasts, and the only function served by the transfer of aspartate is the transport of CO_2. The enzyme that decarboxylates aspartate was not specified but Edwards et al. (1971) showed that some C-4 plants with low malic enzyme (generally "aspartate formers", Downtown, 1970) had high PEP carboxykinase levels in their bundle sheath chloroplasts.

Evidence for two different modes of carbonyl transfer in C-4 plants was presented by Downtown (1970) who showed that "aspartate formers" showed a post-illumination burst of CO_2 while malate formers did not. Recently, Downtown (1971) showed by pulse-chase experiments that both aspartate and malate were converted into Calvin cycle intermediates.

Although overall evidence seems to indicate that the C-4 dicarboxylic acid pathway is primarily a mode of transfer of CO_2 (and reducing power too in some cases), from the data of Hatch and Slack (1966), the decarboxylation step must result in

the CO_2 formed being part of a pool that is preferentially and quantitatively converted to the Calvin cycle intermediates in a light-dependent reaction. In spite of all the evidence for translocation of CO_2 it is difficult to distinguish this from a transcarboxylation, as initially suggested by Hatch and Slack (1966, 1970). The critical evidence seems to be the presence of specific decarboxylating enzymes and the lack of Hill activity in the bundle sheath chloroplasts. However Pan and Waygood (1971) concluded that a transcarboxylating enzyme did exist.

(3) CO_2 fixation by photosynthetic bacteria. There is ample evidence for the operation of the Calvin cycle in several autotrophically grown photosynthetic bacteria (Gibbs, 1967).

Fuller et al. (1961) and Fuller and Anderson (1957) showed that *Chromatium Strain D* fixed CO_2 by the carboxylation of PEP in addition to the Calvin cycle. Bachofen et al. (1964), Buchanan and Evans (1965), Buchanan et al. (1967) and Evans et al. (1966) showed that the reductive tricarboxylic acid cycle may contribute to CO_2 fixation in *Chlorobium thiosulfatophilum* and *Rhodospirillum rubrum*. They proposed that this type of CO_2 fixation occurred in parallel with the Calvin cycle, and served as a source of amino acids and precursors of lipids and porphyrins. Evidence for the reductive tricarboxylic acid cycle was based on the kinetics of labelling of aspartate and glutamate and the detection of ferridoxin dependent pyruvate synthetase and α-ketoglutarate synthetase in cell-free extracts of these organisms. Citrate lyase activity was also detected. However, the relative

contribution of this mode of CO_2 fixation appeared to depend on how the organism was grown. Anderson and Fuller (1967a,b,c) showed that pathways other than the Calvin cycle were dominant in Rhodospirillum rubrum when the organism was grown in the presence of acetate or malate. However the Calvin cycle predominated when the organism was grown autotrophically.

(4) CO_2 fixation in blue-green algae. Anacystis nidulans was used in the rather short study with blue-green algae. Kandler (1961) provided data to show that most of the carbon dioxide fixed by A. nidulans passed through the Calvin cycle. Kindel and Gibbs (1963) showed that the glucose-polysaccharide in the organism was synthesized by the Calvin cycle. However Richter (1961) showed that the first labelled products formed when A. nidulans was exposed to radioactive CO_2 were aspartate, glutamate and PGA.

Although it was apparent that the bulk of the CO_2 fixed and the sugars synthesized arose from the Calvin cycle, the data of Richter suggested that the C-4 dicarboxylic acid pathway could be operative. This was of particular interest since all the data accumulated using this organism was as consistent with a C-4 dicarboxylic acid pathway as a conventional Calvin cycle.

The early label in glutamate too was of interest, because of the data on photosynthetic bacteria and also the presence of a large pool of free glutamate in Anacystis nidulans observed in the course of this study.

PART E

The scope of the thesis

This thesis is presented in two parts: Part I, The effect of cold shock on _Anacystis nidulans_; Part II, The CO_2 incorporating reactions of _Anacystis nidulans_.

The effect of cold shock on _Anacystis nidulans_ was studied mainly from the standpoint of photosynthesis. The main question asked was, why does photosynthesis decline after cold shock? This section of the work was divided into two parts: A, Whole cell studies; B, Studies with cell-free extracts.

The whole cell studies included: (i) the overall effects of cold shock on photosynthesis, (ii) attempts to restore photosynthesis in cold shocked suspensions, (iii) a study of the effects of cold shock on viability and the leakage of cell constituents in order to determine if they paralleled the decline in photosynthetic capacity.

Cell-free extract studies were confined to experiments designed to determine if the decline in photosynthetic capacity could be explained on the basis of the effect of cold shock on electron flow and photophosphorylation.

These studies showed that an answer to the main question could be provided.

In the second part of the thesis, the CO_2 incorporating reactions of _Anacystis nidulans_ were studied. This line of study was undertaken because it appeared possible that CO_2 incorporation in _Anacystis nidulans_ was different from that shown by other algae. This section includes a study of CO_2 incorporation by

whole cells and cell-free extracts of the organism. It was hoped that this study would show if pathways of CO_2 incorporation other than the Calvin cycle. (such as the carboxylation of PEP, a 'Hatch-Slack type' transcarboxylation or the reductive carboxylation of succinic acid) are present in the organism.

The study included: (i) the kinetics of CO_2 incorporation into whole cells, (ii) determination of the label in oxaloacetate, (iii) determination of the position of label in glutamic acid and aspartic acid, (iv) attempts to show the above mentioned enzymic activities in cell-free extracts.

Results showed that there appeared to be a minor CO_2 incorporating pathway in addition to the Calvin cycle.

ORGANISMS, METHODS AND MATERIALS

PART A

Organisms and general methods

(1) Organisms, *Anacystis nidulans* and *Anabaena variabilis* were obtained from the laboratory of Professor J. Myers of the University of Texas. Stock cultures were maintained at room temperature under constant illumination on the agar medium of Allen (1968, a) and sub-cultured every month.

(2) Growth of cultures. The medium of Hughes et al. (1958), as modified by Allen (1968, a), was used. The medium contained, (in g./liter), $NaNO_3$, 1.5; K_2HPO_4, 0.039; $MgSO_4.7H_2O$, 0.075; Na_2CO_3, 0.020; $CaCl_2$, 0.027; $Na_2SiO_3.9H_2O$, 0.058; EDTA, 0.001; citric acid, 0.006; ferric citrate, 0.006; microelements, 1 ml. The microelements consisted of, (in g./liter), H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.391; $CuSO_4.5H_2O$, 0.79; $Co(NO_3)_2.6H_2O$, 0.0494; pH of medium 7.8.

The cultures were grown by the method of Maclean et al. (1972) at 37° in a constant temperature room. The culture vessel was a 3 liter Fernbach flask closed tightly with a rubber stopper. The culture (300 ml.) was stirred rapidly with a magnetic bar to ensure maximum spray, thus facilitating illumination and gas exchange. Illumination was provided by two, 20 watt "cool white" fluorescent lamps placed horizontally about 3 cm. from the liquid level.

For generation of CO_2, once a day, a candle was burned in the flask until it extinguished itself. Burning time was about 1 min. and resulted in 3-5% CO_2 in the atmosphere.

The cultures were innoculated with cells from growing cultures such that the initial culture density was about 100 Klett cell units per ml. (to be defined later). During the first day of growth the lights were placed about 8 cm. from the culture and moved to 1 cm. away on the second day. The cultures grew linearly with an increase of about 100 Klett absorbtion units per day till it reached about 1000 absorbtion Klett units. A typical growth curve is shown on Figure 3. Since the effects under study were affected by the age of the cells and possibly also by the conditions of growth, a strict regimen for the growth of the organism was followed. Cells to be used were harvested after a fixed time, generally 3 days, and unless otherwise stated, experiments were carried out using cells of this age. Although cultures at this stage of growth had a cell density of only 300-400 Klett absorption units, they were more photosynthetically active.

Anabaena variabilis was grown in a similar way, however, the culture was not stirred but swirled around a few times a day.

Occasionally during routine tests for contamination, organisms giving rise to white colonies on agar were detected. These colonies were indistinguishable from Anacystis nidulans by microscopic examination. In some instances, the white colonies eventually became blue-green after longer periods

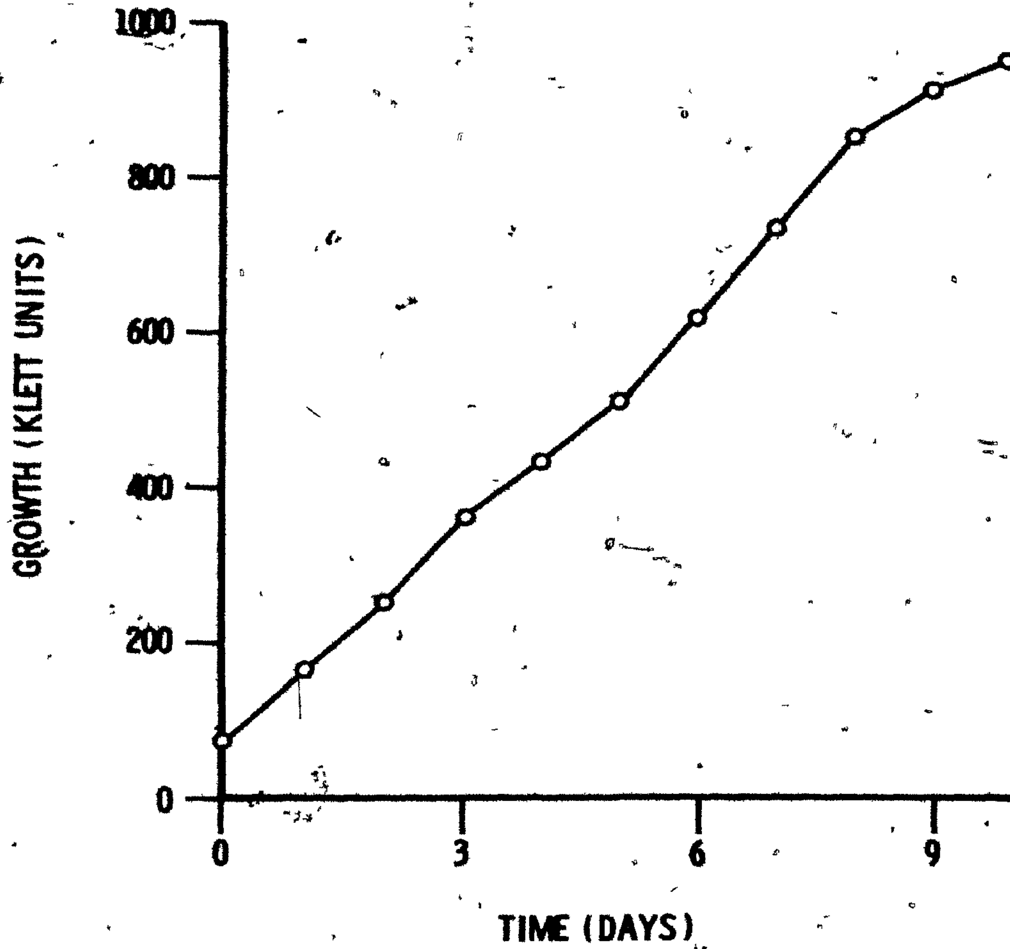


Figure 3. Typical growth curve of *Anacystis nidulans*. For details, see text.

(5 days) of incubation, and in one case a white colony purified by a second streaking on agar showed on the third streaking exclusively blue-green colonies. Cultures used had either no white colonies or a low white colony count (less than .1%).

(3) <u>Measurement of cell mass</u>. Cell mass was measured routinely on a Klett colorimeter equipped with a No. 66 red filter. There was a linear relationship between cell mass and Klett reading to 250 absorption units (Maclean et al., 1972). One Klett unit of cells was the cell mass in 1 ml. of a suspension of one Klett absorption. Five hundred fifty Klett cell units was equivalent to 1 mg. dry wt. (Maclean et al., 1972). The packed cell volume of 100,000 Klett units was 0.5 ml. The latter was determined by centrifuging a concentrated cell suspension in a narrow-stemmed centrifuge tube until there was no further contraction in volume of the cell pellet, (about 40 min. at 6000 *g*). Thirty-thousand Klett units contained approximately 1 mg. chlorophyll. Chlorophyll was determined spectroscopically at 663 nm after extraction with 80% acetone (Myers and Kratz, 1955).

PART B

Cold shock experiments I - whole cells

(1) Preparation of cold-shocked cells. Cells were harvested, washed twice with, and suspended in, growth medium at cell densities varying from 10^8 cells per ml. to 5×10^9 cells per ml. (5000 Klett units per ml.). Samples of this suspension (less than 5 ml.) in test tubes 1-1 1/2 [illegible] diameter were immersed in an ice-water bath (in the dark) till the temperature fell to 0° and then kept in a cold room at 3°. For other temperatures, centrifuge compartments were used as constant temperature baths (after the cells were brought down to the required temperature by immersing in ice). Control samples were kept in the dark at 37°. A similar procedure was followed for cell-free extracts.

(2) Measurement of viability. Two methods were used.

(i) The method of Allen (1968, a) i.e. the direct determination of viable counts by growth of single colonies. Colony counts were made after incubation for three days at 37°. A few drops of water were added daily to the cover plate to compensate for evaporation. Illumination was provided by two, 20 watt "cool white" fluorescent lamps placed 10 cm. away from the plates. The method was found to be satisfactory by comparing viable counts with total counts (in a hemocytometer), Table 3.

Viability of cold-shocked cells was tested by cold treat-

Table 3. Determination of viable counts by the method of Allen. Observed standard deviations calculated from multiple sets of plates.

	Number of cells	
	Calculated (Hemocytometer)	Experimental viable cells
1.	59[illegible]	606 ± 95
2.	474	400 ± 49

ment at 10^8 cells per ml. followed by serial dilutions to give 100-400 colonies per plate.

(ii) The optical density method of Maclean et al. (1972). Cold-shocked cells were grown at 10^8 cells per cup and their optical density compared (after two days) with control cups containing 10^8, 10^7, 10^6 and 10^5 cells per cup. Percent viability was calculated by plotting a standard curve of log. (cell conc.) against optical density and expressed as a percentage of that of the 10^8 sample, after two days growth. This standard curve is shown on Figure 4. The plot was made in this way in order to correct for the variation in rates of growth of cell suspensions over a series of experiments. The viability in cold-shocked suspensions was then estimated from the standard curve by using the optical density of its cups and that of the corresponding 10^8 control.

(3) Measurement of Photosynthesis. Photosynthesis was measured by (i) oxygen evolution, (a) manometric method, (b) polarographic method; (ii) carbon dioxide fixation.

Manometric method. The general procedure of Kratz and Myers (1955) was followed using a Gilson Differential Respirometer equipped for photosynthesis (two banks of 8, 30 watt tungsten lamps located 7 cm. from the flasks). The temperature was 37°. Cells were suspended in 5 ml. of 0.1 M $NaHCO_3$-K_2CO_3 buffer (Warburg buffer #9). Equilibration which generally took 30-45 min. was done with the cells in the main chamber until the thermobarometer of bicarbonate buffer showed

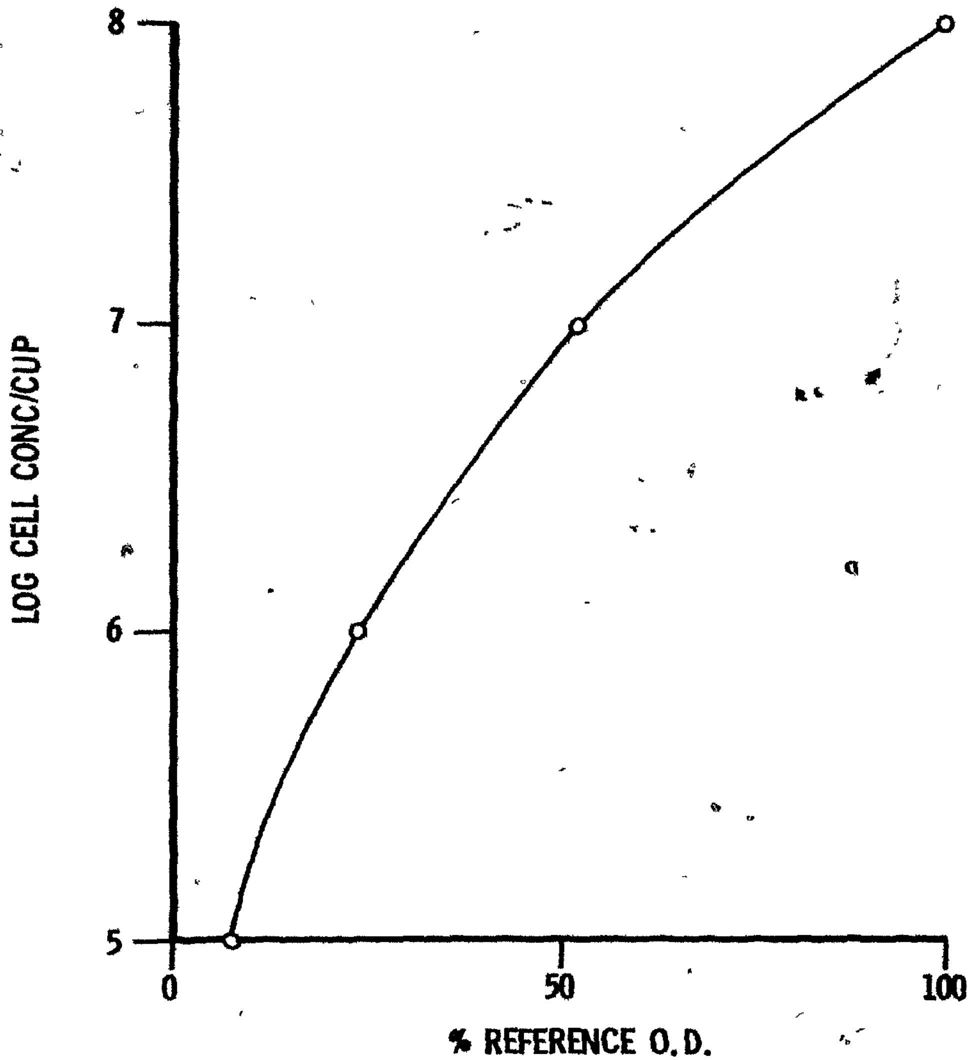


Figure 4. Standard curve for optical density method of estimating viability; optical density taken after two days growth at room temperature. Reference optical density was that of 10^8 cells/innoculum. For details, see text.

no gas exchange. Values shown are corrected for dark respiration, which was of the order of 5% photosynthesis. The rate of photosynthesis was a linear function of cell mass. This was established by measuring photosynthesis with mixtures of active and inactive cells. The latter was obtained by exposing cells to a temperature of 55° for 30 min., which treatment destroyed photosynthetic activity but did not alter pigmentation. This is shown in Table 4. Generally about 3000 Klett units of 3 day old cells per flask were used and the rate of oxygen evolution was about 600 µl/hr. This was less than the rates obtained by Kratz and Myers (1955), but was considered satisfactory as the physiological rate of photosynthesis of the cells growing under these culture conditions was approximately 150 µl/hr. (assuming 36 hr. doubling time). The activity of cells from cultures older than 3 days was found to be somewhat less. Estimations of the rate of photosynthesis of duplicate samples was found to correspond to within 5%. Photosynthesis was measured over a period of 10 min., and time course studies over this period showed that the rate of oxygen evolution was linear.

In earlier experiments due to a miscalculation of the percent CO_2 in equilibrium with the buffer, the reaction vessels were pre-gassed with 5% CO_2 before equilibration. This resulted in a lowering of pH. However, using this method, a linear standard curve was obtained up to about 7000 Klett units per flask (Figure 5) with similar rates of photosynthesis to the other method. Some of these results are retained in the

Table 4. Standard curve for manometric oxygen evolution. For details see text.

Klett units per flask (viable cells)	Oxygen evolved (µl/hr.)
1200	144
2400	336
3600	504
4800	708
6000	888

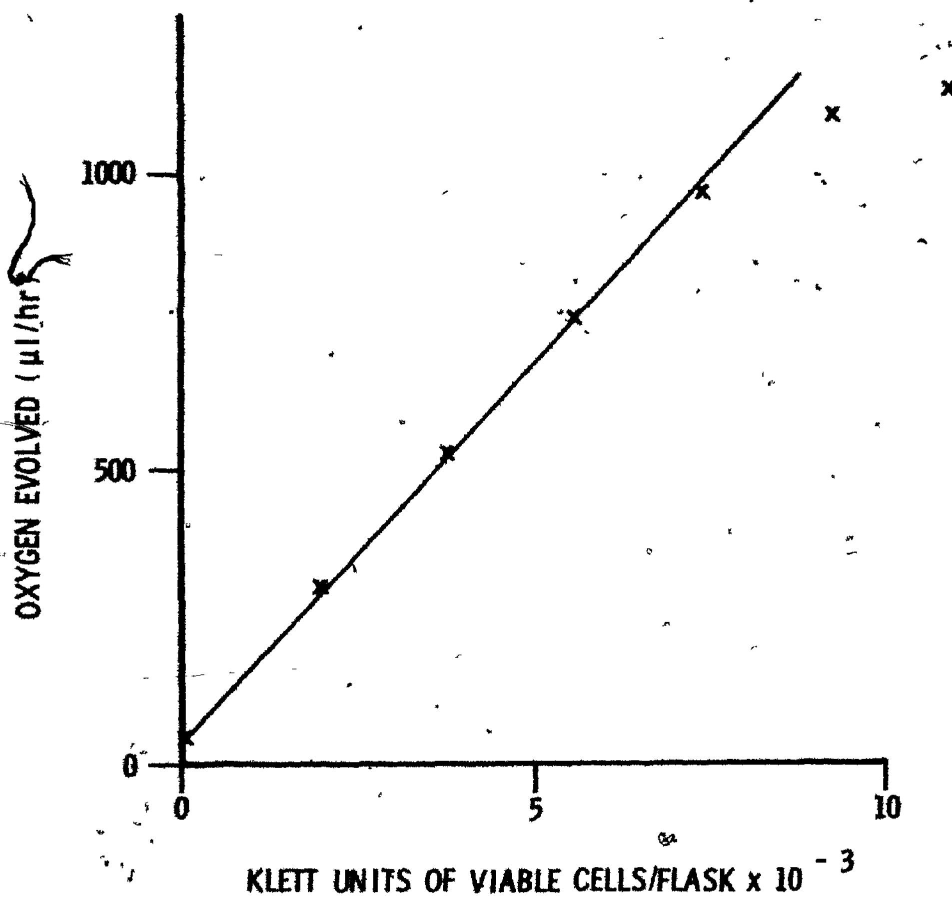


Figure 5. Standard curve for Manometric estimation of photosynthesis (Method II). For details, see text.

text and are marked as being done by method II. Percent photosynthesis of cold-shocked cells calculated using each method was similar.

Polarographic method. The apparatus was an Interscience Corp. oxygen polarograph, thermostated at room temperature and equipped with a Yellow Springs Co. teflon-protected platinum electrode connected to a 10 mV recorder. The rate of diffusion of oxygen through the teflon membrane was proportional to concentration of dissolved oxygen and only a small fraction of the oxygen was used in the production of current. To prevent oxygen loss to the atmosphere during photosynthesis measurements, the reaction chamber had to be well-sealed with vaseline applied to the smooth surfaces. Also the set screw (which otherwise allowed considerable gas leakage) was permanently sealed to the transparent lucite chamber. After each photosynthesis measurement the "addition chamber" was removed thereby exposing the reaction mixture to the atmosphere and, while stirring continued, excess oxygen was allowed to diffuse from the suspension. The value recorded by the apparatus depended on the state of the teflon membrane, the rate of stirring and the position of the sensitivity control, all of which made it desirable to restrict detailed comparison to results obtained in one day. The instrument was calibrated with water containing known concentrations of oxygen. These different concentrations of oxygen in water were obtained by equilibrating air with water at 3°, 25° and 37° and oxygen with water at 25°. A value for zero oxygen content was determined

with dithionite. Linearity of voltage with oxygen concentration was observed. As a result absolute rates of photosynthesis could be calculated. Illumination was provided by a 100 watt tungsten lamp placed about 7 cm. away from the reaction chamber. Cells were suspended in a mixture of 1.3 ml.,of 0.1 M $NaHCO_3/K_2CO_3$ buffer (pH 9) and 1 ml. of growth medium. Linearity between photosynthesis and number of viable cells was observed. This is shown in Figure 6. Again, as in manometry each point represents a mixture of live and dead cells. Routinely 3000-3500 Klett units of cells were used. Rates of photosynthesis were in the range of 200 µl/3000 Klett units/hr.

Carbon dioxide fixation. Cells were suspended in growth medium (5 ml.) in 50 ml. Erlenmeyer flasks and shaken in a wrist action shaker for 2-4 hr. at 37° under illumination similar to growth. The gas phase was 8% CO_2 (containing 6 µCi of $^{14}CO_2$) in air. The cells were harvested and washed in distilled water until there were negligible counts in supernatant. The cells were then bleached for 2 hr. at 45° with 10% H_2O_2 to yield a colourless suspension which had no quenching characteristics when counted in a scintillation counter (Unilux 1, model 6850). As in other methods of photosynthesis discussed, a nearly linear standard curve was obtained on plotting Klett units of live cells against photosynthetic activity. This is shown in Figure 7. For cold shock studies, 3600 Klett units of cells were used.

(4) Cell autoradiography. Cells (approximately 10,000 Klett

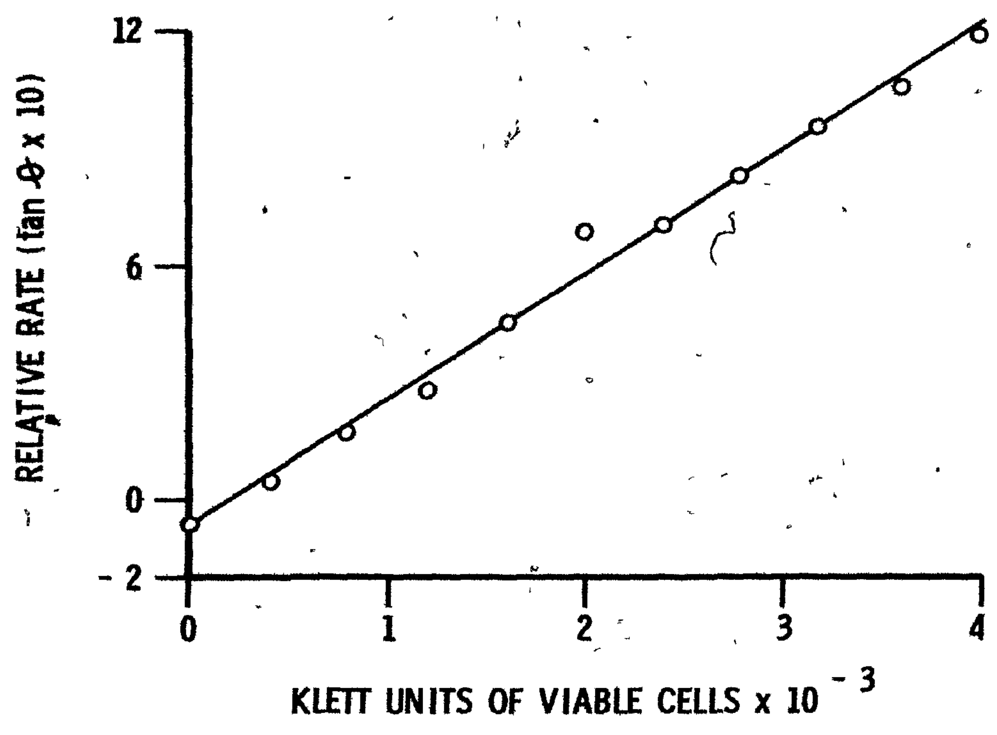


Figure 6. Standard curve for Polarographic estimation of photosynthesis. For details, see text.

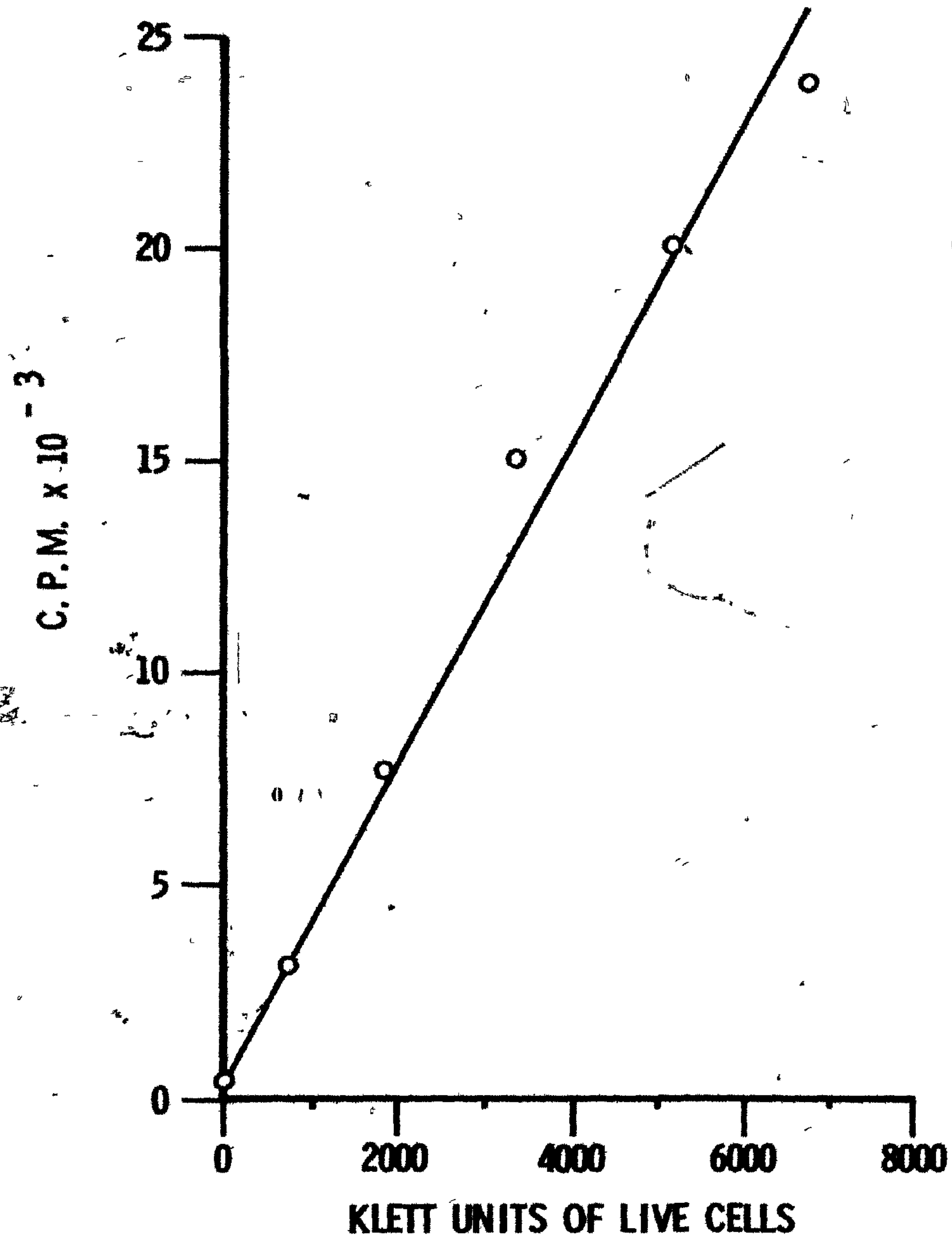


Figure 7. Standard curve for $^{14}CO_2$ fixation. For details, see text.

units) were exposed to 30-80 µCi $^{14}CO_2$ for 5 hr. under conditions similar to $^{14}CO_2$ fixation. The cells were washed twice in water and resuspended in water to a final concentration of 10^7 cells per ml. A drop of this suspension was smeared on a microscope slide, air-dried and fixed in 95% ethanol. The slide was dipped in a mixture of Illford Liquid Nuclear Emulsion and water (1:3) at 40-44°, dried in air and sealed in a dark box for 1, 2 or 3 days. The slides were developed by dipping in a 25% solution of Kodak Ectaflow Reagent for 75 sec., washed twice in water, fixed for 2 min. using 15% Kodak Fixer and washed again twice with water (the second wash containing one drop of Kodak Photoflow per 10 ml.).

The slides were then examined under the microscope for radioactivity, which appeared as spots (grains) over the cells. The number of inactive cells per 1000 cells was determined. Random fields of the slides were photographed and the number of spots for each cell counted. There was usually no difficulty in observing cell outlines but occasionally in doubtful instances clarification was accomplished by taking two photographs, one in the plane of focus of the cells and another in that of the grains.

(5) Preparation of uniformly labelled cells. For ^{14}C, cultures were grown in 5% CO_2 (containing 0.5 mCi $^{14}CO_2$) in air. For ^{33}P the cultures were grown for one day and on the second day, 1 mCi $H_3{}^{33}PO_4$ (carrier-free) was added. Cultures were grown under the usual conditions (page 41). Isotope incorporation

into the washed cells was determined by the procedure described under $^{14}CO_2$ fixation. Total dialysable material was determined after killing the cells by heating for 30 min. at 65°. Radioactivity in the supernatants of cold-shocked suspensions was determined after passing the supernatant through a 0.5 μ filter to remove any remaining whole cells and cell debris.

(6) Estimation of pteridines. Pteridines were estimated by the method of Maclean et al. (1965 a, 1966 b) and Patterson et al. (1956). This involved adding acetic acid to pH 4, warming for a few minutes, followed by oxidation of the pteridine side chain to pterin-6-COOH acid with alkaline permanganate. The product was estimated (after the precipitation of $KMnO_4$ as MnO_2) in 1 N borate buffer, pH 9, by fluorescence in an Aminco-Bowmann spectrophotofluorometer (excitation 360 nm, emission 450 nm). Frequent checks for quenching were negative. Generally, samples of 4000-20,000 Klett units were used for pteridine estimation. Fluorescence was linear with pteridine content in the range of pteridine concentrations estimated - 0.02-0.05 μmoles/5 ml. (Maclean and Morton, 1970). In the present study it was confirmed that the error at these concentrations was about 10%. Pteridines were estimated in the cells or in the supernatants, as indicated in the results.

(7) The methyl viologen - Mehler reaction. The reaction was studied manometrically in a Gilson Respirometer by following gas uptake at 30°. The reaction mixture (modified from Chua, 1971) contained KCN, 1 mM; Methyl viologen, 2 mM; Phosphate

buffer, 12 mM; cells (*Anacystis nidulans*) 2000 Klett cell units in 5 ml. of growth medium, final pH 7.2. Gas exchange was followed after 30 min. equilibration. The reaction time was 10 min. with illumination followed by a further 10 min. in the dark.

PART C

Cold shock experiments II - cell-free extracts

(1) Preparation of cell-free extracts. The method was based on procedures of Vance and Ward (1969), Biggins (1967) and Lee et al. (1969) for the preparation of cell-free extracts by lysozyme digestion. Freshly harvested cells (100,000 Klett cell units) were washed twice in water and suspended in 5 ml. of 0.4 M sucrose - 0.03 M phosphate buffer, pH 6.8, containing 10 mg. lysozyme and 3-4 mg. EDTA (Mahler and Fraser, 1956). The cells were shaken in a Gilson Respirometer at 37° for 3 hr. This gave a protoplast preparation, which was centrifuged and washed 4 times with sucrose-phosphate buffer or sucrose-Tris buffer (depending on the experiment) to eliminate inhibitors of photosynthesis in commercial lysozyme (Lee et al., 1969), and also EDTA. Some lysis took place during the washing. The protoplasts were lysed by adding small quantities of water (4 ml.) followed by vigorous mixing for 1 min., solid sucrose was added such that the final concentration was 0.4 M and the appropriate buffer then added (the exact strength and pH of the buffer depended on the system to be assayed). The extract was then centrifuged at 6000 g. for 5 min. and the supernatant decanted (the scum over the residue was usually retained). The treatment resulted in the liberation of about 40-60% of the total chlorophyll and phycocyanin. The ratio of optical densities of phycocyanin:chlorophyll (620 nm:678 nm) in the extract was approximately constant (1.20-1.50), the phycocyanin

being slightly higher than that for whole cells. This preparation will be referred to as the "protoplast lysate". A second extract of the residue gave almost 90% lysis, but was not used in experiments. Optical densities of extracts were measured at 678 nm, 620 nm and 740 nm and the volume of extract to be used in the experiments calculated by the method of Van Baalen (1957); more accurate chlorophyll content in reaction mixtures were determined by the formula of Myers and Kratz (1955), (assuming that the extinction coefficient of chlorophyll in water at 678 nm is approximately equal to that in acetone at 663 nm).

The photosynthetic lamellae could be separated from the extract by centrifugation at 100,000 g. for 30-60 min. This gave a pellet referred to as the "lamellae fraction".

(2) Assays for electron flow and photophosphorylation.
Protoplasts were washed in sucrose-phosphate buffer for electron flow experiments and sucrose-0.1 M tris for photophosphorylation experiments. The change in buffer for the latter had to be made in order to calculate accurate specific activities of inorganic phosphate.

Assays for electron flow were done either manometrically (ferricyanide reduction), or spectrophotometrically (ferricyanide and DCPIP reduction and Cyt. c oxidation).

For manometric experiments, illumination conditions were as for measurement of photosynthesis, while for spectrophotometric determination of electron flow and photophosphorylation, illumination was provided by a 650 watt tungsten lamp placed

25-30 cm. distant. Heating was prevented by filtering the light through a Roux bottle filled with water. Some photophosphorylation experiments were also carried out with illumination provided by three, 20 watt "cool white" fluorescent lamps placed 4 cm. away.

Reaction vessels were either 3 ml. cuvettes (DCPIP reduction and Cyt. c oxidation), or 5 ml. glass syringes (photophosphorylation). Both types of vessels were used for ferricyanide reduction. All spectrophotometric measurements were made with a Cary-14 recording spectrophotometer. The assays were done at room temperature (except manometric determination of oxygen evolution, which was done at 30°).

When CMU was used, it was introduced into the extract by mixing 0.5-1 mg. of the pure powder with the extract (20 ml.) for about 15 min. using a Vortex mixer. Only a part of the CMU dissolved, but the treatment resulted in almost complete inhibition of ferricyanide reduction and no inhibition of PMS mediated photophosphorylation. The procedure was followed to avoid the use of ethyl alcohol, which was not desirable. All assays were done with crude extracts in 0.3-0.4 M sucrose. Specific details for each method are given below.

(a) <u>Assays for electron flow.</u>

(i) <u>Ferricyanide reduction</u>. <u>Manometric</u>. (Adapted from Susor and Krogmann, 1964). Reaction mixture (5 ml.) contained (in µmoles), phosphate buffer (pH 7.8), 80; $MgCl_2$, 80; NaCl, 40; chlorophyll, 0.06-0.25 mg.; $K_3Fe(CN)_6$, 10-20; 0.05 ml. Hughes and Gornham microelements. Oxygen evolution was

followed over a period of about 20 min. in a Gilson Respirometer.

Spectrophotometric estimation. (Adapted from the method of Süsor and Krogmann, 1964). Reaction mixtures (3-4 ml.) contained Tris-HCl (pH 7.8), 40 mM; $MgCl_2$, 20 mM; $K_3Fe(CN)_6$, 0.5-1 mM; Phosphate buffer, (pH 7.8), 5 mM; NaCl, 5 mM; ADP, 5 mM; chlorophyll, 15-40 µg. Ferricyanide reduction was estimated at 420 nm either directly when reaction mixture was contained in cuvettes or from the TCA-precipitated supernatants (see noncyclic photophosphorylation). Reaction time was 3-6 min., and all readings were corrected for dark reduction of $K_3Fe(CN)_6$.

(ii) DCPIP reduction. (Adapted from the method of Van Baalen, 1957). Reaction mixtures (3.0 ml.) contained (in µmoles) DCPIP, 0.3; NaCl, 40; phosphate buffer, (pH 7.5), 150; chlorophyll, 10-30 µg. The extracts were equilibrated for about 3 minutes until the rate of dark reduction of DCPIP decreased to a very low level. All readings were corrected for dark reduction. Reaction time was 4-5 min. Measurements were made at 550 nm and/or 600 nm. There were signs of a low level of autooxidation of DCPIP.

(iii) Cytochrome c. oxidation. (Adapted from Fugita and Myers, (1965) and Brown, 1969). The reaction mixtures (3 ml.) contained (in µmoles) phosphate buffer, (pH 7.3), 150; methyl viologen, 0.5; 50% reduced Cyt. c, 0.14-0.2; $MgCl_2$, 3; $CaCl_2$, 3; chlorophyll, 10-30 µg; and CMU. The extract was equilibrated in the dark until endogenous reduction of Cyt. c,

decreased to an insignificant level. Generally samples to be compared were given exactly the same period of dark preincubation before the light was turned on. Cyt. c reduction was followed at 550 nm. The reaction time was 9 min.

(b) Assays for photophosphorylation.

(i) PMS-Ascorbic dependent (cyclic) photophosphorylation. The methods of Avron (1960), Biggins (1967), and Krogmann (1965 and 1969) were used as guides in the choice of reagent and cofactor concentrations. The reaction mixtures (3 ml.) consisted of (in μmoles) Tris-maleate buffer, (generally pH 7.6), 200; NaCl, 15; $MgCl_2$, 35; ADP, 15; phosphate buffer, 20; PMS, 0.1; ascorbate, 100; $^{32}P_i$, 0.5-1 μCi; chlorophyll, 40-55 μg; and CMU. All reagents were adjusted to the appropriate pH. The reaction was initiated by injecting the $^{32}P_i$ and phosphate buffer into the syringe (from the needle end) and terminated by ejecting 0.5-0.7 ml. aliquots into 0.1 ml. of ice-cold 30% TCA at 2-3 min. intervals. The total reaction time was 8-10 min. Each sample was centrifuged for 10 min. and 0.6 ml. of the supernatant was assayed for ATP. A typical time course is shown in Figure 8.

(ii) Noncyclic photophosphorylation. Reaction mixtures were as for the spectrophotometric estimation for $K_3Fe\ (CN)_6$ reduction but the reaction was initiated by injecting the $K_3Fe\ (CN)_6$ (final conc. approximately 1 mM) phosphate buffer (containing 0.5 μCi ^{32}P) and ADP into the syringes. Samples were taken at 2, 4 and 6 min. As in cyclic photophosphorylation, ATP (and $K_3Fe\ (CN)_6$) were determined in TCA supernatants.

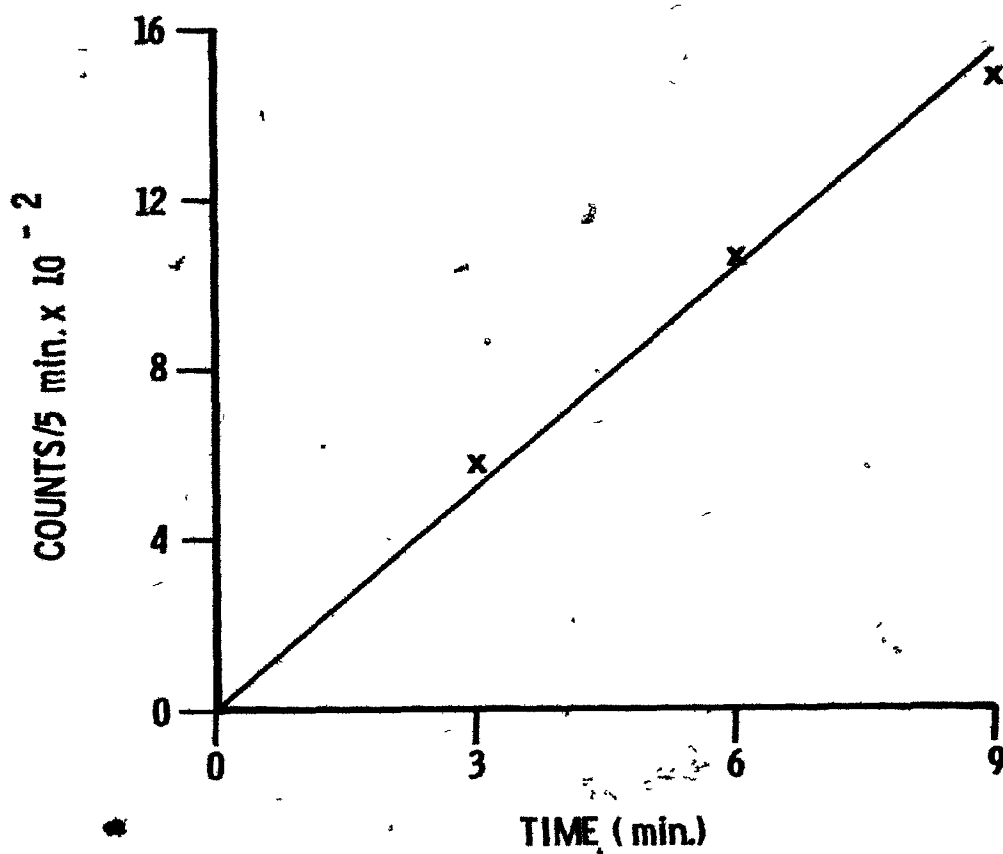


Figure 8. A typical time course for incorporation of ^{32}P in PMS-Ascorbate catalyzed photophosphorylation. For details, see text.

(iii) Assay for ATP. ATP (incorporation of $^{32}P_i$ into organic phosphate) was assayed by the method of Avron (1960). To each sample, acetone (1.2 ml.) was added and the mixture allowed to stand for 10 min. The following were then added in order: water saturated with a mixture of isobutanol:benzene 1:1 (to increase the volume of supernatant to 2.5 ml.); isobutanol:benzene, 1:1 saturated with water (6 ml.) and ammonium molybdate, (0.8 ml. of 5% solution in 4 N H_2SO_4). The mixture was then shaken gently and allowed to stand for 5 min. at room temperature. The rest of the procedure was carried out at 0°. This involved extracting the phosphomolybdous acid into the organic layer by 30 sec. of vigorous mixing in a Vortex mixer. After a second extraction with solvent, 0.6 ml. of the aqueous phase was transferred to a stainless steel planchet 2.0 cm. in diameter and counted with a Geiger-Muller counter (probe, 2.5 cm. end window diameter). One unextracted sample was routinely made up to the same final volume as the extracted aqueous phase (3.3 ml.) and 0.6 ml. counted in order to determine total counts per sample. Since the μmoles in inorganic phosphate (Pi) and μg chlorphyll (chl) per sample were known, the rate of ATP formation in μmoles/mg. chl./hr. could be determined by the following formula (Avron, 1960).

$$\mu\text{moles ATP/mg. chl./hr.} = \frac{\text{c.p.m./min illumination} \times \mu\text{m Pi} \times 1000 \times 60}{\text{c.p.m. total} \times \mu\text{g chl.}}$$

PART D

Carbon dioxide fixation I - whole cells

(1) Short term labelling with $^{14}CO_2$. This was done adopting the general procedure of Calvin and co-workers. The washed cells were suspended in a special medium consisting of 1/10 strength medium of Allen (but less Na_2CO_3, Na_2SiO_3 and 1/100 strength with respect to $NaNO_3$) together with 300 µM potassium phosphate buffer (pH 7.8) per 100 ml. The salt content in the medium was lowered to facilitate subsequent chromatography. Two general procedures were adopted. Method A. The cells were placed in a "lollipop" of dimensions 200 sq. cm. surface area and 1 cm. thick. Illumination was provided by two tungsten lamps (650 watts) placed on either side 20 cm. away. Two Roux bottles filled with water were placed between the lights and the "lollipop" to act as a heat filter. The suspension (50,000 Klett cell units) was illuminated for 10 min., the light switched off, and then 850 µCi (12.8 µmoles) of $Na_2{}^{14}CO_3$ was added and a dark control sample taken immediately. (The entire dark period lasted about 1.5 min.). The light was then switched on and samples removed at fixed intervals. The samples were drained into boiling ethanol such that the ethanol in the extract was 80%. The residue was washed successively in ethanol, ethanol:water::1:1 and water and the combined extract was flash evaporated at 35-40° to 1 ml. final volume. Method B. The cell suspensions (15,000 Klett cell units in 20 ml.) were placed in a series of Erlenmeyer flasks equilibrated

in the light for 5 min. and then 150 μCi (2.3 μmoles) of $Na_2{}^{14}CO_3$ added. The light used was a 650 watt tungsten lamp placed 20 cm. away (with a Roux bottle placed in between as heat shield). After the required time, boiling ethanol was poured into the cells to give an 80% alcoholic extract. The rest of the procedure was as in Method A. The advantages of Method B were that (a) shorter periods of labelling time were achieved and (b) the dark period was eliminated.

(2) Estimation of oxaloacetate after short-term labelling. When oxaloacetate was to be estimated, the cells were killed by pouring the contents of each Erlenmeyer flask (20 ml.) into 90% ethyl alcohol containing 0.25 N HCl and 0.04% 2,4-dinitrophenylhydrazine cooled to -78°. Approximately 1 mg. of carrier oxaloacetate was added and the extract allowed to warm to room temperature. After 1 hr. at 25°, the cell debris was removed by centrifuging, washed as before, the pooled extracts concentrated to 10 ml., then extracted and purified by the method of Friedmann and Haugen (1943), i.e. first by extracting with 3 portions ethyl acetate (15 ml.), then 1 N Na_2CO_3 (20 ml.) was added to the combined ethyl acetate fraction to extract the keto acids into the aqueous phase. The water extract was washed with ethyl acetate, acidified and re-extracted into ethyl acetate (3, 10 ml. portions). The ethyl acetate was evaporated at room temperature and the keto acids separated by paper chromatography using butanol:ethanol:0.5 N NH_3:: 7:1:2. The recovery of carrier oxaloacetic acid was complete.

(3) Separation of radioactive compounds.

(a) Soluble fraction.

Aliquots (usually 50 µl) containing 20,000 to 900,000 c.p.m. were spotted on chromatograms (Whatman no. 1) and run in two dimensions with (i) phenol saturated with water and (ii) butanol:acetic acid:water (BAW)::4:1:5. In most cases a short run (1 inch) of BAW was performed before the first dimension, to clear the spotting area of chlorophyll. This was found to help considerably in the separation of phosphate esters.

(b) Insoluble fraction.

The water and ethanol unextractable residue was hydrolysed for 1-2 hr. with 1 N HCl by the method of Kindel and Gibbs (1963). The supernatant and water washings of the hydrolysate were combined and the amount of radioactivity in it and the residue determined. The supernatant was flash evaporated to 1 ml. and chromatographed using the same solvents as in (a). The supernatant was also run through an amino acid exchange column (Beckman Amino acid Analyser, model 120c), and distribution of radioactivity in eluate determined by collecting samples every 2 min. with a fraction collector.

(4) Quantitation of radioactivity. The radioactive areas on the chromatogram were detected by autoradiography (Benson et al., 1950) with "Kodak" "No Screen" X-ray film. Spots with about 4000 c.p.m. per sq. inch could be observed after 4 days of exposure. Radioactivity on the spots was determined quantitatively by two methods. (1) Counting radioactivity directly off the paper with a Geiger-Muller counter (Nuclear

Chicago, model 183) equipped with a probe of end window diameter, 2.5 cm. and (2) by cutting the paper and counting the radioactivity with a liquid scintillation counter using 10 ml. of scintillation fluid (0.3 g POPOP and 5 g PPO per liter of toluene). Several precautions were taken when radioactivity was estimated by these methods. Counting efficiency, when the liquid scintillation system was used, was 85-90% as determined with standard samples of ^{14}C toluene. When radioactive standards were spotted on Whatman no. 1 chromatography paper of 7 sq. cm. or less, activity detected was found to be proportional to absolute activity and counting efficiency was about 65%. This allowed the chromatography paper to be cut up and counted directly. When the Geiger-Muller counter was used areas of 2 cm. square were counted at a time. Trials with standards (after chromatography) showed that counts were proportional to radioactivity. The validity of these two methods of counting was further tested by comparing results of the two methods for the same chromatographed samples. One such comparison is shown in Figure 9. The linear relationship with low percent error except at very low radioactivity levels enabled results to be interconverted without significant error. The efficiency of the Geiger-Muller counter was approximately 3% of that of the liquid scintillation counter. However, above characteristics of the Geiger-Muller counter held true only after chromatography; direct application and counting gave a spuriously high activity, closer to 5% and was not as reproducible. This was probably

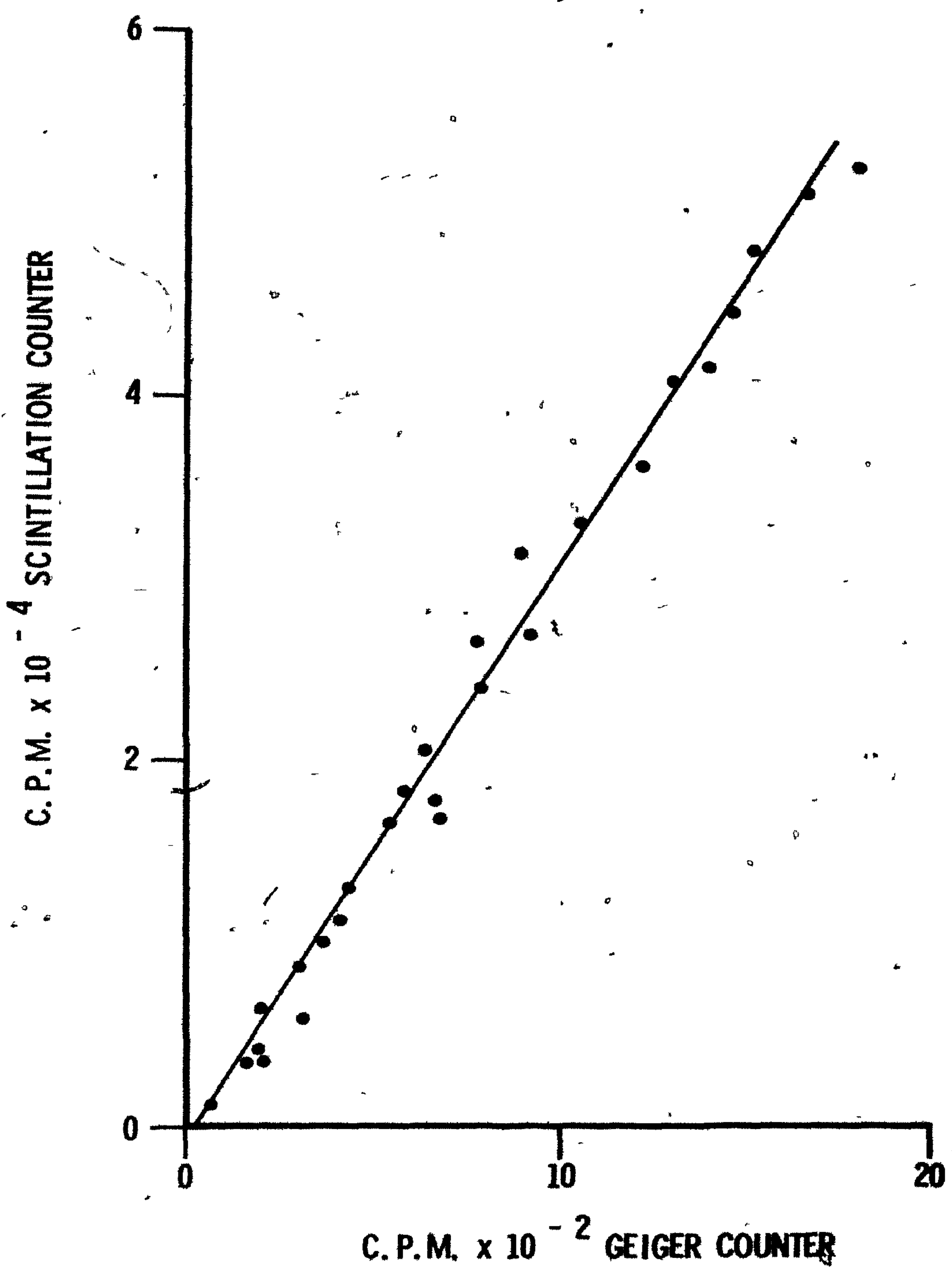


Figure 9. Relationship between radioactivity estimated by a Geiger-Muller counter and by scintillation counter. For details, see text.

due to uneven distribution of radioactivity throughout the thickness of the paper. It was also noticed that the origin of the chromatogram gave a spuriously high activity both on X-ray film and on the Geiger counter. This was found to be due to material sitting on top of the paper and was easily recognized by counting the reverse side of the paper.

Radioactivity of solutions were estimated by drying aliquots of material with a heat lamp on Whatman no. 1 chromatography paper of standard size (about 7 sq. cm.). The samples were then counted with a scintillation counter.

Estimation of radioactivity from the ion exchange column was done after evaporating 0.4 ml. of each fraction to dryness in a stream of hot air. The radioactivity in the samples was determined using a scintillation counter.

(5) Identification of labelled compounds. Labelled compounds were identified by their R_F values in the two solvents described above. On some occasions re-chromatography in a third solvent (propanol:1% NH_3::2:1) was done. The identity of the more important compounds was confirmed by co-chromatography with radioactive or chemical standards. Radioactive standards were used for (a) phosphoglyceric acid, (b) malate, (c) citrate, (d) glucose, (e) fumarate, (f) succinate, (g) α-ketoglutarate. The ninhydrin test was used for amino acids, mainly (a) aspartate, (b) glutamate, (c) alanine. The phosphomolybdic acid test (Block et al., 1958d) was performed for the glyceraldehyde-3-phosphate standard.

(6) Tests for recovery. Authentic ^{14}C containing citrate, malate, fumarate, succinate, α-ketoglutarate and phosphoglyceric acid were put through the entire isolation procedure of the short-time labelling method in order to evaluate their recovery. A similar recovery was done for non-radioactive glutamate and aspartate. Table 5 gives the results of these recovery experiments. In these recovery experiments about 1-3% of the total activity was found to remain at the origin. In all cases except citrate and α-ketoglutarate nearly all the radioactivity recovered was localized in one spot. In the case of citrate the radioactivity was concentrated in 3 closely placed spots containing 60, 33 and 5 per cent respectively of the total radioactivity recovered. Due to this, in all experiments, the sum of the radioactivity in these areas was counted as citrate. The radioactivity from α-ketoglutarate spread out into a number of spots whose position was not reproducible.

(7) Estimation of total glutamate. The chemical estimation was adapted from the general procedure of Block et al. (1958a) i.e. by spraying with 1% ninhydrin in acetone, followed by heating in an oven for 20 min. at 100°. The spot was then dissolved in 70% acetone and the glutamate determined spectrophotometrically at 570 nm. Absolute values were determined by comparison of the optical densities with a standard curve (shown in Figure 10).

(8) Estimation of radioactivity in C-1 of glutamate. The glutamic acid was chromatographed in parallel with 1-^{14}C

Table 5. Percent recovery of radioactive compounds. For details see text.

Compound	% Recovery
PGA	90-100
Citrate	62
Malate	60
Fumarate	72
Succinate	70
α-ketoglutarate	-
Aspartate	90-100
Glutamate	90-100

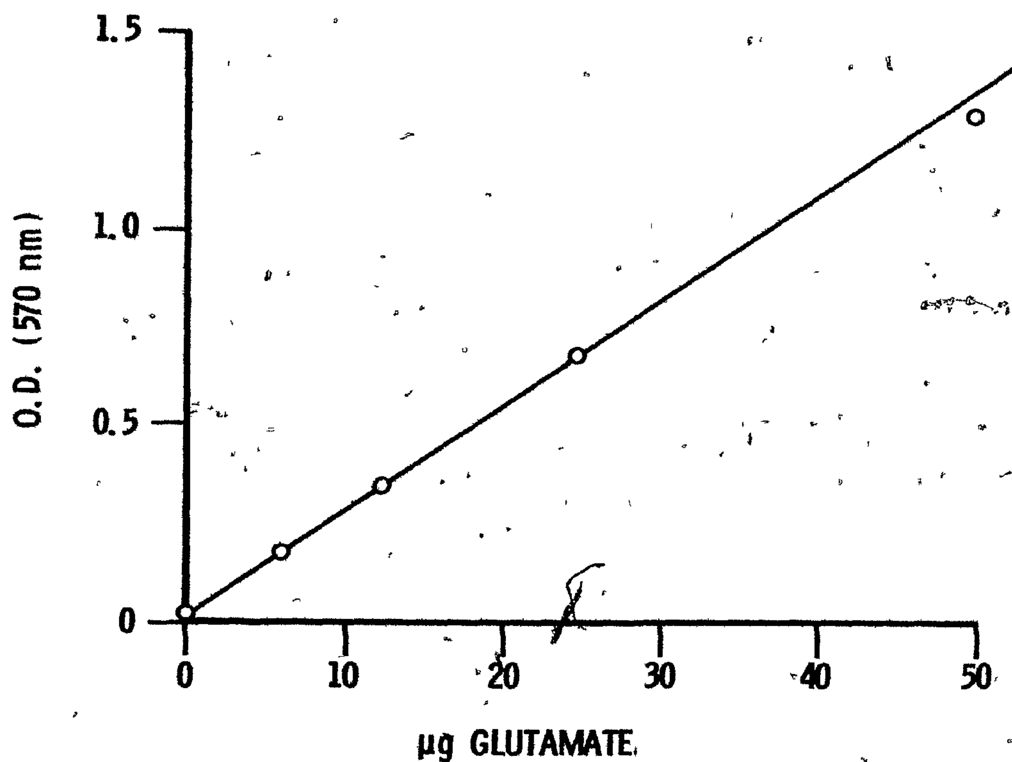


Figure 10. Standard curve for Glutamate determination. Estimation done on ninhydrin reaction product in 70% acetone. For details, see text.

and 5-^{14}C labelled glutamate (all samples contained the same chemical quantity of glutamate - 50 µg). The papers were then sprayed with 1% ninhydrin in acetone, dried in a fume hood for about 36 hr. and then held in a tank saturated with acetic acid vapour, for 6-18 hr. The radioactive areas were counted, before and after the treatment, with a Geiger-Muller counter. Under these conditions standard glutamate-1-^{14}C retained only 2-3% of its radioactivity, while glutamate-5-^{14}C retained 80-90% of its radioactivity. Using this data the isotope in C-1 of glutamic acid was determined.

(9) Determination of radioactivity in C-4 of aspartic acid. The labelled aspartic acid was purified by paper-chromatography, the paper cut up and mixed with cold aspartic acid (1 mg.) to give a sample of known specific activity. This was dissolved in 0.1 M phosphate buffer, pH 7.5 (7.5 ml.), α-ketoglutaric acid, (2 molar excess) and 50-100 units of glutamic-oxaloacetic transaminase was added. The mixture was allowed to stand for 8 hr. at 37° and excess 2,4 dinitrophenylhydrazine (in 3 N HCl) was added. The mixture was then heated for 25 min. at 85° to convert the remaining oxaloacetate-phenylhydrazone to the corresponding pyruvate derivative (Hatch et al., 1967). The pyruvate-phenylhydrazone was purified by the method of Block et al. (1958c) i.e. by solvent extraction successively into $CHCl_3$, Na_2CO_3 and $CHCl_3$, the chloroform contained 20% ethanol. The final $CHCl_3$ extract was evaporated at room temperature, an aliquot chromatographed with butanol:ethanol: 0.5 N NH_3::7:1:2 and the hydrazone identified by comparison

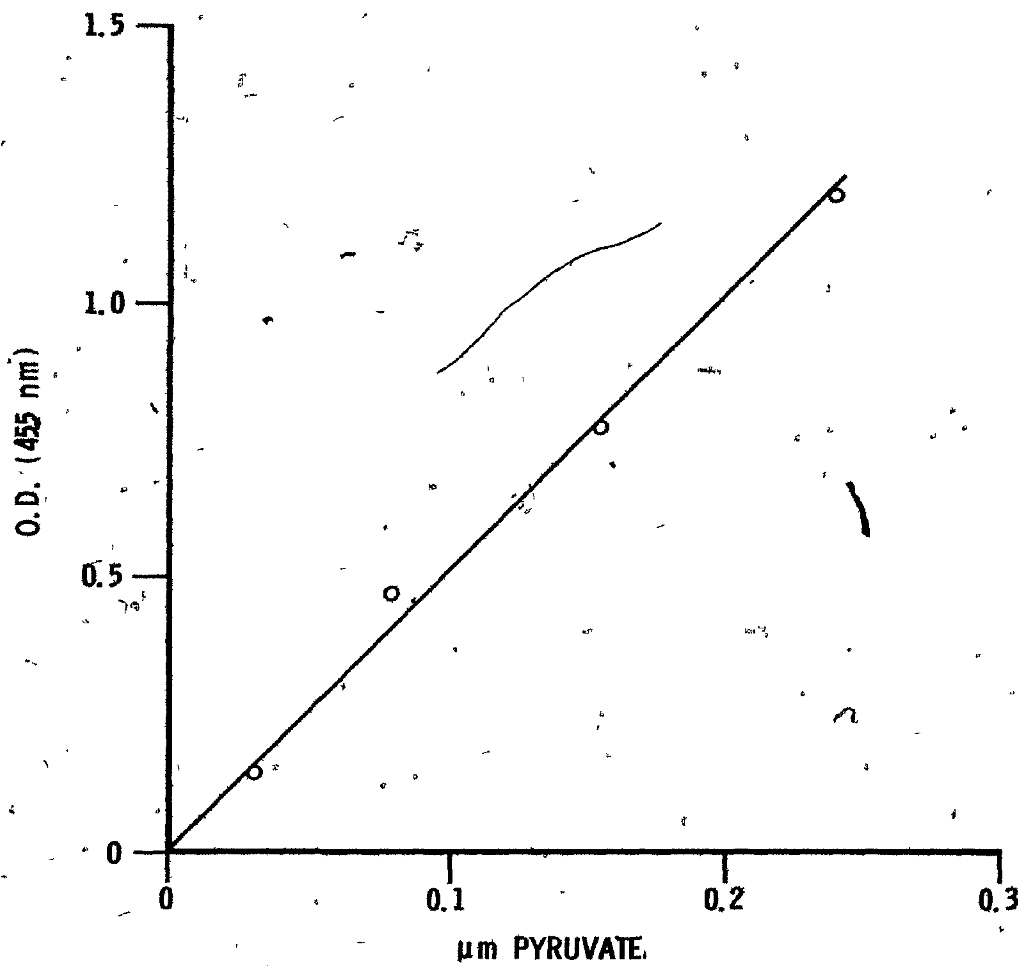


Figure 11. Standard curve for pyruvate-phenylhydrazones. Phenylhydrazone standards in 3 ml. of 1 N NaOH. For details, see text.

with a standard.

The major pyruvate spot (0.5-1 μmole) was marked off and counted with a Geiger-Muller counter (samples of low radioactivity were counted on parallel samples with a scintillation counter). The chemical pyruvate was determined by the method of Block et al. (1958c) i.e. a spot was cut up into small pieces and mixed thoroughly with 1 N NaOH (10-20 ml.) and estimated spectrophotometrically at 455 nm with the use of a standard curve for pyruvate-phenylhydrazone (Figure 11). The overall yield was 30-50%.

PART E

Carbon dioxide fixation II - cell-free extracts

(1) Assay for RDP carboxylase (carboxydismutase). This was done by the method of Slack and Hatch (1967), on the unfractionated protoplast lysate. In the preparation of the protoplasts the use of phosphate buffer was avoided and tris buffer was used since inorganic phosphate was a known inhibitor of the enzyme. A degree of purification could be achieved by centrifuging out the chlorophyll containing particles at 6,000 g for 30 min. and using the supernatant. CO_2 was introduced as Na_2CO_3 and the concentration and pH of the buffer were chosen such that the final pH of the reaction mixture was 8.00-8.20. Unless otherwise stated, reaction mixtures contained (mM), Tris-HCl, 100; Na_2CO_3, 25 (containing 5-7 μCi ^{14}C); $MgCl_2$, 7.5-10; mercaptoethanol, 4.6; RDP, 0.5-0.8; in 1.25-1.6 ml. extract containing 40-65 μg chlorophyll. When the concentration of Na_2CO_3 was varied, the concentration of the buffer was raised to 200 mM. The light source when used, was three, 20 watt fluorescent lamps placed 5 cm. distant. The reaction was initiated by injecting sodium carbonate into the reaction vessel (a 2 ml. glass syringe). The reaction system was mixed for 30 sec. and the timing started. This procedure was adopted in order to allow for a short lag which has been observed (Wishnick and Lane, 1971). The reaction was terminated by ejecting aliquots (0.25-0.5 ml.) into 0.05 ml. of 40% TCA. A part of the aliquot was placed on aluminium

planchet, (2.5 cm. in diameter), covered with a disc of tissue paper ("Kleenex") of weight 1.4 mg./cm.^{2}, dried with a heat lamp for 10 min. and counted with a Geiger-Muller probe of the same diameter. Counting efficiency was 3% and the duplicate aliquots agreed to within 10%. Uptake of ^{14}C was nearly linear and a typical time course is shown in Figure 12. Rates were calculated from samples taken at 3,6 and 9 min. Very occasionally a lag was still evident, and in these instances calculations were based on incorporation from 3-9 min. Products were identified by two dimensional chromatography using the same solvents as in Part C (3). Since the ratio of chlorophyll to phycocyanin (phyco) in the extract was about 10% less than in the cells, rates were calculated in terms of corrected chlorophyll in extract where,

$$\text{Chl* O.D.} = \text{Corrected chl* O.D.}$$

$$= \text{O.D. phyco in extract} \times \frac{\text{O.D. chl-cells}}{\text{O.D. phyco cells}}$$

The second term was generally, $\frac{1}{1.2 - 1.3}$

The weight of chl* can then be calculated from the extinction coefficient of chlorophyll.

Rates were then estimated by the following formula:

$$\mu\text{moles of } CO_2 \text{ fixed/mg chl* min.}$$

$$= \text{c.p.m./min.} \; \frac{\text{Vol. reaction mixture}}{\text{Vol. "sample"}} \times \frac{100}{3} \times \frac{\mu\text{moles}}{\text{c.p.m.}} \frac{CO_3^{2-}}{CO_3^{2-}} \times \frac{1000}{\mu\text{g chl*}}$$

The purity of RDP was checked by chromatography in BAW (4:1:5)

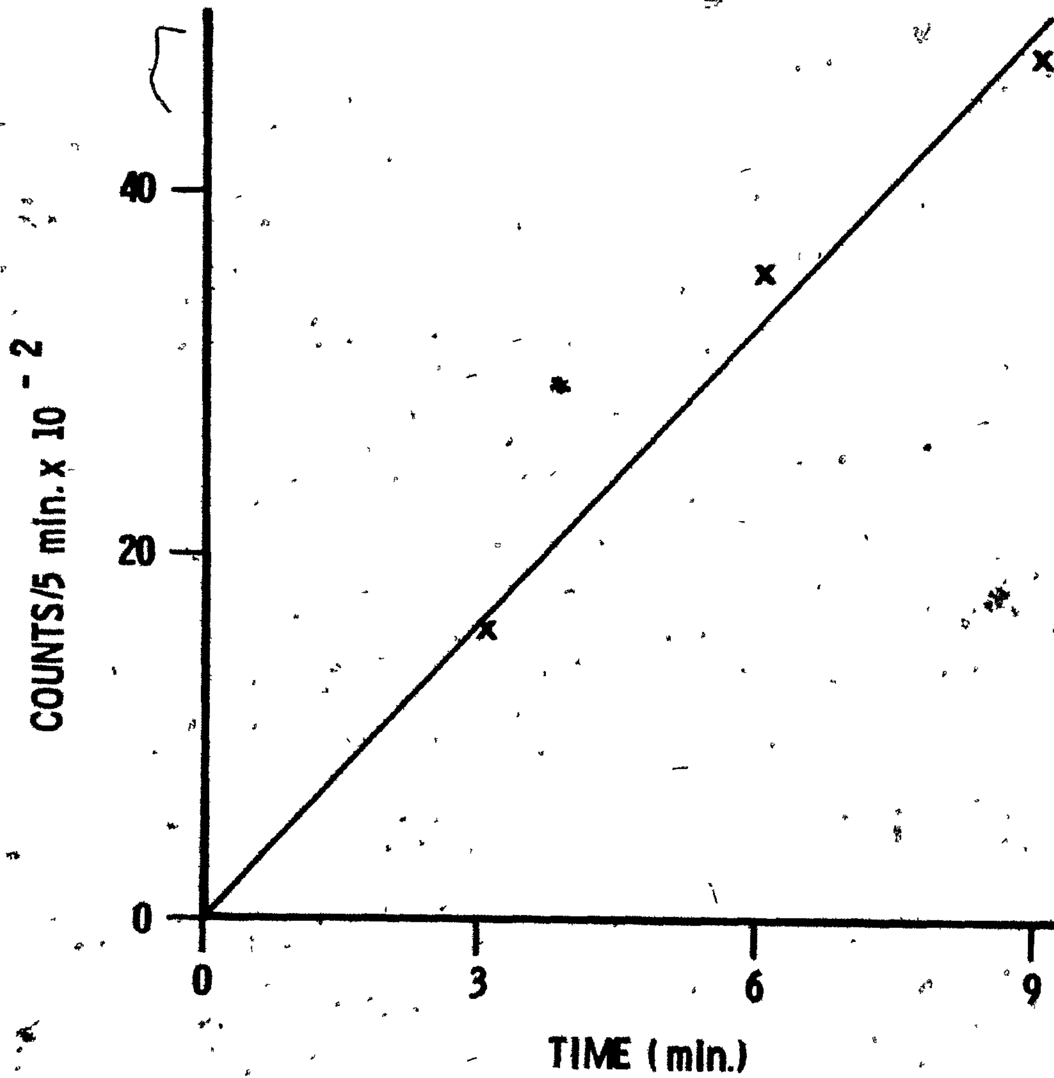


Figure 12. A typical time course for incorporation of ^{14}C in the RDP carboxylase assay. For details, see text.

and the phosphomolybdic acid test. This showed only one spot which corresponded to a sugar diphosphate.

(2) Assay for PEP carboxylase. The general experimental procedure was as in the RDP carboxylase assay. Unless otherwise stated reaction mixtures consisted of (in mM) Tris HCl, 100; $MgCl_2$, 7.5; Mercaptoethanol, 4; Na_2CO_3, 12.5-15; PEP, 2-5; Glutamate, 3.2; Glutamic-oxaloacetic transaminase, 17.5 units; contained in a volume of 1.45-1.65 ml. (40-65 μg chlorophyll). The final pH was 8.0-8.1. When the Na_2CO_3 or PEP concentration was varied the concentration of the buffer was raised to 200 mM. Reaction rates were generally nearly linear over a reaction time of 9 min. (Figure 13), although occasionally the rate decreased between 6 to 9 min. The reaction was initiated by adding sodium carbonate (2.5-5.0 μCi ^{14}C). The rest of the procedure was as in the RDP carboxylase assay. The purity of PEP was assayed by the method recommended by "Sigma" Chemical Company by converting the PEP to pyruvate by pyruvate kinase and determining NADH oxidation at 340 nm using lactic dehydrogenase.

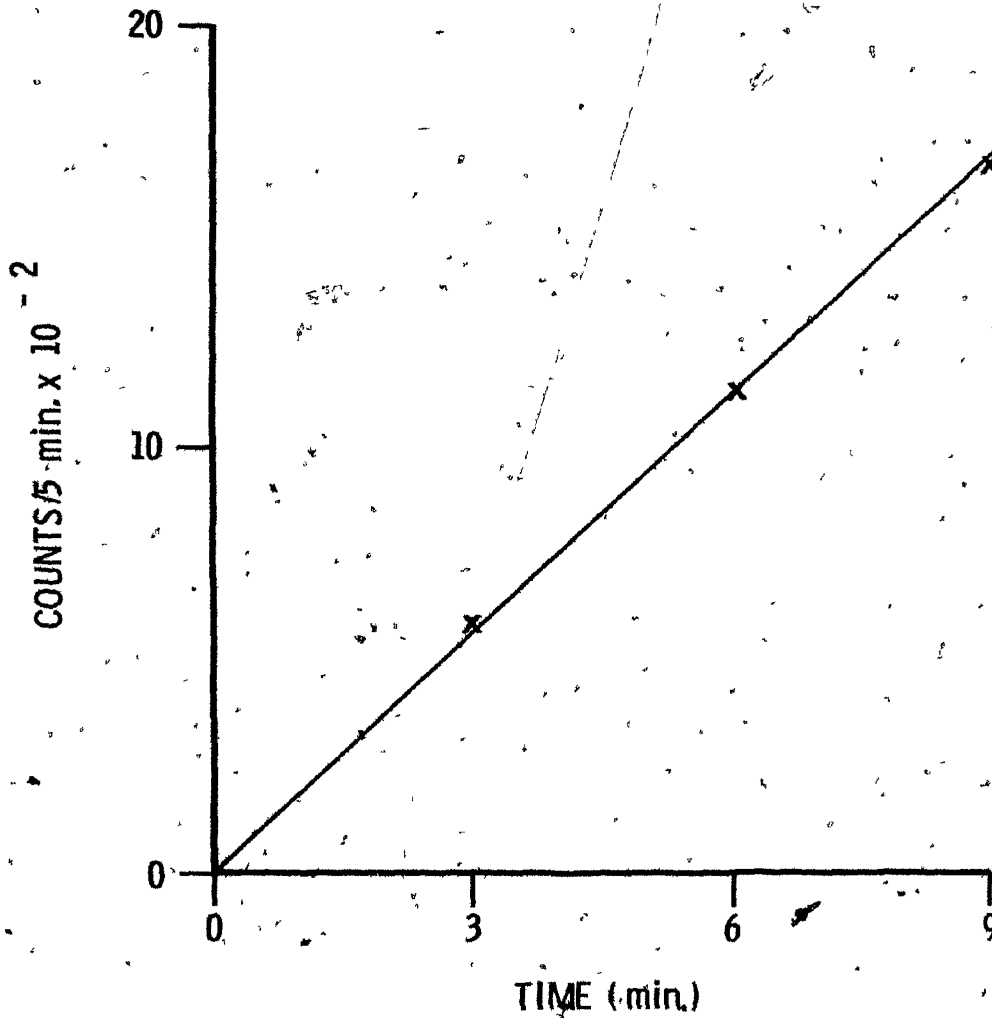


Figure 13. Typical time course for ^{14}C incorporation in the PEP carboxylase assay. For details, see text.

PART E

Materials

Whenever possible reagent grade chemicals were used. Distilled water was used in all experiments and all glassware was cleaned in chromic-sulphuric acid.

$H_3{}^{33}PO_4$, glutamate-1-^{14}C, glutamate-5-^{14}C were purchased from New England Nuclear Corp.; $Na_2{}^{14}CO_3$ and $H_3{}^{32}PO_4$ were purchased from Atomic Energy of Canada; aspartate-4-^{14}C, succinate-1-^{14}C, fumarate-2,3-^{14}C and PGA-U-^{14}C were all purchased from Calbiochem.; malate-4-^{14}C and α-ketoglutarate-1-^{14}C were purchased from Calatomic; citrate-1,5-^{14}C and glucose-U-^{14}C were purchased from Amersham-Searle Corp. All radiochemicals except Na_2CO_3 and $H_3{}^{32}PO_4$ were chromatographed two dimensionally (using 50,000 c.p.m.) and their purity tested by detection of the radioactive areas with Kodak "No Screen" X-ray film. The radiochemicals except α-ketoglutarate (which spread out into a number of spots) gave rise to only one main spot, impurities (if any) contained less than a few percent of the total radioactivity.

ADP (di Na salt, equine muscle, Catalog. No. A 0127), dl glceraldehyde-3-phosphate (G 5251), PEP (tri Na salt, p 7002), NADP (mono Na·T 9102), NADPH (di Na, T 1003), RDP (tetra Na, R 8250), ATP (di Na, A 3127), cis oxaloacetic acid (hydroxy-fumarate, 340-58), DCPIP (D 1878), MV (M 2254), and PMS (P 9625) were all purchased from Sigma Chemical Co. CMU was obtained by the courtesy of E.L. du Pont de Nemours and Co.

Pyruvate kinase (rabbit skeletal muscle, type II, p 1506), lactic dehydrogenase (beef heart, type III, L 2635), glutamic-oxaloacetic transaminase (G 2751), lysozyme (egg-white grade III, L 7001), Cytochrome-c (horse heart type IV, C 7752), and Ferridoxin (spinach, type III, F 5875) were all purchased from Sigma Chemical Co.

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RESULTS

PART I

Effect of cold shock on *Anacystis nidulans*

(A) Whole cell studies.

(1) Effect of cold shock on photosynthesis. The first set of experiments was designed to determine the effect of cold shock on photosynthesis. Photosynthesis was measured by (i) oxygen evolution, (a) manometrically, (b) polarographically; and by (ii) CO_2 fixation.

Manometric estimation of oxygen evolution. This was essentially a repetition of the studies of Van Baalen (1957) and Forrest et al. (1957).

Results of this experiment (Figure 14), showed that photosynthesis declined rapidly to about 35% the control value after 15 min. at 0°-3°. Further cold treatment caused a further decline in photosynthesis and after 2 hr. the photosynthetic capacity was only 15% of the control. Cell suspensions held at 37° showed no decline in photosynthesis.

Several experiments of this type with three day old cultures gave similar results, with the photosynthetic capacity after 15 min. cold shock being in the range of 35-50% control. However, it was observed that older cultures were more resistant to cold shock, for example one old culture yielded 85% and 27% control rates of photosynthesis after 15 and 60 min. of cold shock respectively.

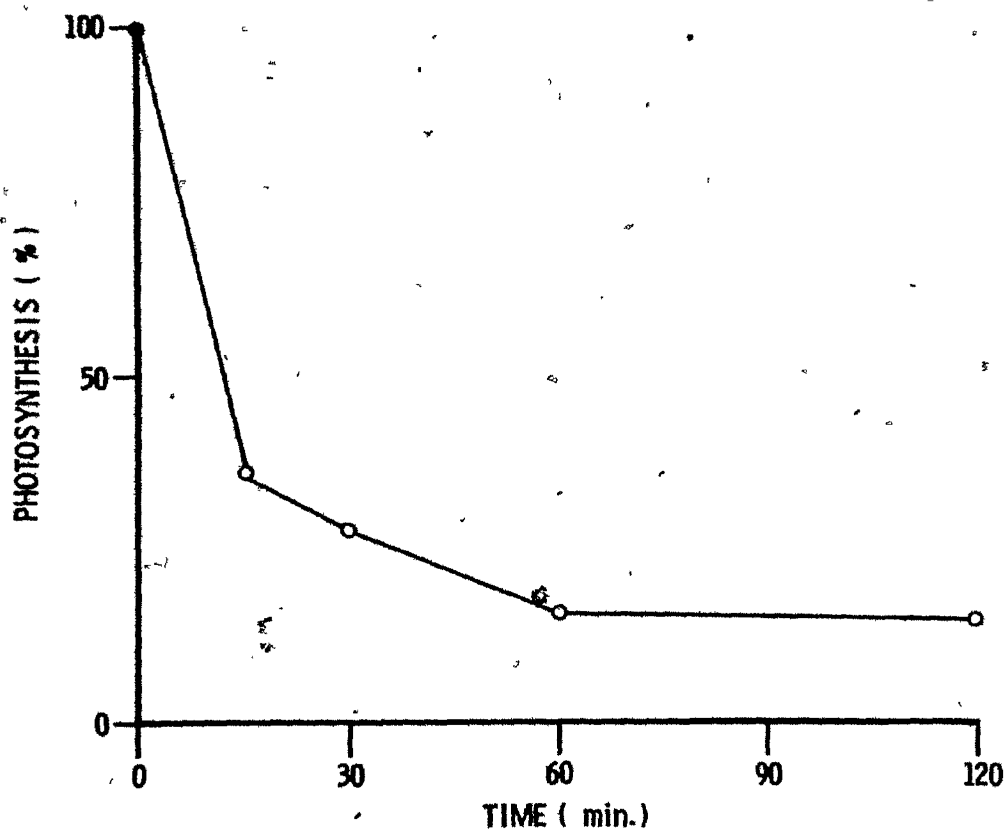


Figure 14. Effect of cold shock on photosynthesis. Estimation, manometric at 37°. Time refers to time in the cold (0-3°). Rate of photosynthesis of control, 660 µl/3000 Klett cell units/hr. For experimental details see Methods.

Photosynthesis was usually measured 45 min. after the cells were cold-shocked. In the case of cells cold-shocked for 15 min. or less, the rate of photosynthesis showed no significant decline during the following 1-2 hr. at 37°. However, cells cold-shocked for longer periods of time (1-2 hr.) showed a further slight decline in photosynthetic rate after the cells were removed from the cold.

Carbon dioxide fixation. Carbon dioxide fixation was studied next in order to determine whether gas exchange was a true reflection of the photosynthetic capacity of cold-shocked cells. This was done by comparing $^{14}CO_2$ incorporation in control and cold-shocked cells. Results (Table 6) showed that there was reasonable agreement between photosynthesis measured by CO_2 uptake and oxygen evolution. The experiment was conducted with an old culture.

Measurement of photosynthesis by polarography. This type of measurement allowed the determination of photosynthesis soon after cold shock. Results followed the same general pattern as for the manometric method. Figure 15, shows that the effect of cold shock (15 min.) on photosynthesis was fully expressed within 5 min. after removal of cells from the cold.

(2) Short term CO_2 fixation by cold-shocked cells. The experiment was designed to determine if cold shock affected the extent or the path of CO_2 fixation after short periods of photosynthesis. The experiment was done using the same technique as CO_2 fixation studies (Methods, pg. 66).

Table 6. Decline of oxygen evolution and CO_2 uptake after cold shock, expressed as a percentage of a control kept at 37°. Manometric oxygen evolution measured by Method II (pg. 49). For experimental details see Methods.

Cold shock time (min.)	Oxygen evolution (% control)	CO_2 uptake (% control)
2	97	96
15	85	91
30	64	55
60	27	12

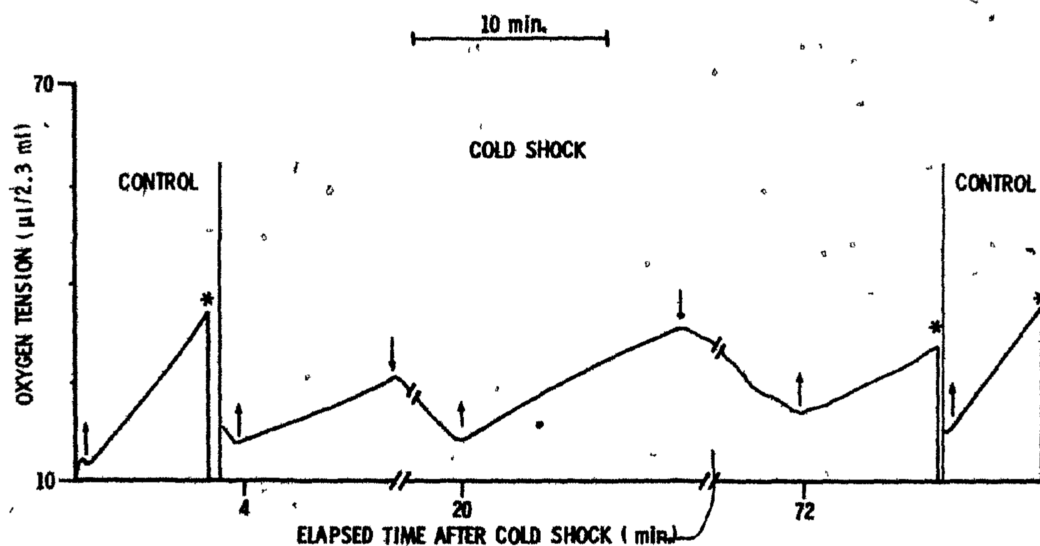


Figure 15. Polarographic estimation of photosynthesis to determine the effect of holding cold-shocked cells at room temperature. Adapted from the original tracing. ↑ Reaction chamber sealed, light on. ↓ Chamber opened, light off and solution agitated to remove oxygen. * cell suspension changed. Control suspensions were measured before and after cold-shocked suspensions. For experimental details see Methods.

<u>Table 7</u>. Short term labelling of cold-shocked cells. Duration of cold shock, 15 min. Control cells kept at 37°. * identified by R_F only. † identified by co-chromatography. For experimental details see pg. 66.

	C.P.M. x 10^{-2}			
	Cold shock		Control	
	17 sec.	60 sec.	17 sec.	60 sec.
Hexose* and Pentose P and PGA	401	3408	1529	8027
Triose P†	8	119	33	284
PEP*	7	30	45	289
Aspartate†	27	217	76	1026
Glutamate†	3	96	8	203
Citrate†	14	209	13	296
Malate†	<1	55	<1	50
Alanine†	<1	14	<1	117
Insoluble material	54	1354	237	4468

Results of the experiment (Table 7) showed that cold-shocked cells, as expected, incorporated less isotope than control cells. However, the general pattern of labelling in control and cold shocked cells was similar. The only compounds that did not show a proportionate decline in ^{14}C incorporation were citrate and malate but the label in these two compounds was low.

(3) Cell-autoradiography. Observations made up to this stage raised the possibility that the culture was composed of two kinds of cells; those sensitive to cold shock and those resistant to it. Such a situation would have important bearing on the interpretation of results and information was needed to determine whether, for an individual cell, the effect was all or none.

The answer to this question was provided by cell autoradiography where photosynthesis, as measured by incorporation of $^{14}CO_2$, could be assessed for individual cells.

Control and cold-shocked cells were allowed a period of photosynthesis, following which cell-autoradiography was performed. A typical autoradiogram is shown in Figure 16. It shows the grains over the cells and in some cases the cells too. The grains over the individual cells were then counted (Figure 17). When the number of grains per cell was more than 12, accurate counting was impossible and because of this, high counts were grouped together as in Table 8.

The fraction of inactive or poorly active cells (those with no grains over them) was higher in cold-shocked cells

Figure 16. A typical cell autoradiogram. For experimental details see Methods.

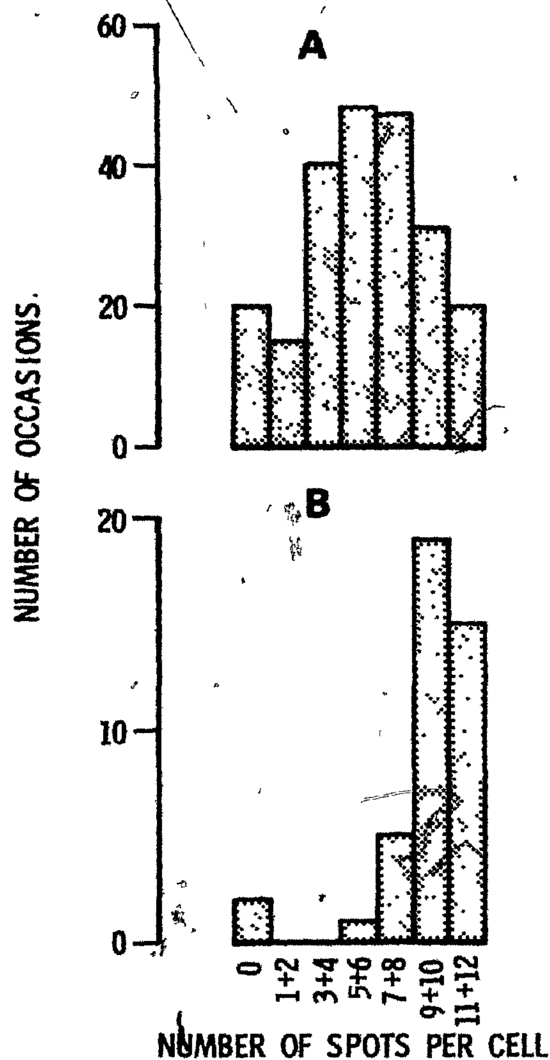


Figure 17. Distribution of grains over cells after 3 days autoradiography. Grains of cold-shocked cells (A) and control cells (B). The remainder of the distribution pattern is shown in Table 8.

Table 8. Number of grains per cell when grain count was high. The table represents the upper part of the distribution curve shown in Figure 17. Number of occasions are expressed as a percentage of the total number of cells counted in each case. Duration of cold shock, 30 min.

Grains per cell	No. of occasions	
	Control	Cold shock
13-15	14	6
16-20	17	2
>20	9	4
>>20	6	1

than in control cells.

It was clear, however, that the major effect of cold shock was to decrease the average value for grains over each cell and this showed that the effect was not 'all or none' with respect to each cell. The average number of grains per cell was 6.8 and 14 for cold shock and control cells respectively, i.e. just less than 50%. Calculations based on total incorporation of radioactive carbon dioxide into the cells gave a value of 41%. The correlation was considered satisfactory especially in view of the approximations made in counting grains.

(4) Effect of cold shock on viability. The effect of cold shock on viability was studied in order to determine if the effect of cold shock on photosynthesis was accompanied by a loss in viability.

The method of Allen (1968 a) for growing colonies from single cells proved satisfactory (Methods, pg. 45), cells grew rapidly with a doubling time of 3-5 hr. Results (Figure 18) showed that there was a dramatic loss in viability after cold shock at 0°-3°. The Figure shows that after 15 min. exposure to the cold, viability declined to 2%. Zero time on the figure represents the time taken for the temperature to fall to 3° (about 1.5 min.). This loss in viability was reproducible, generally 3 day old cultures showed less than 10% viability after 15 min. cold shock. Control samples held at 37° showed no loss in viability over the period of the

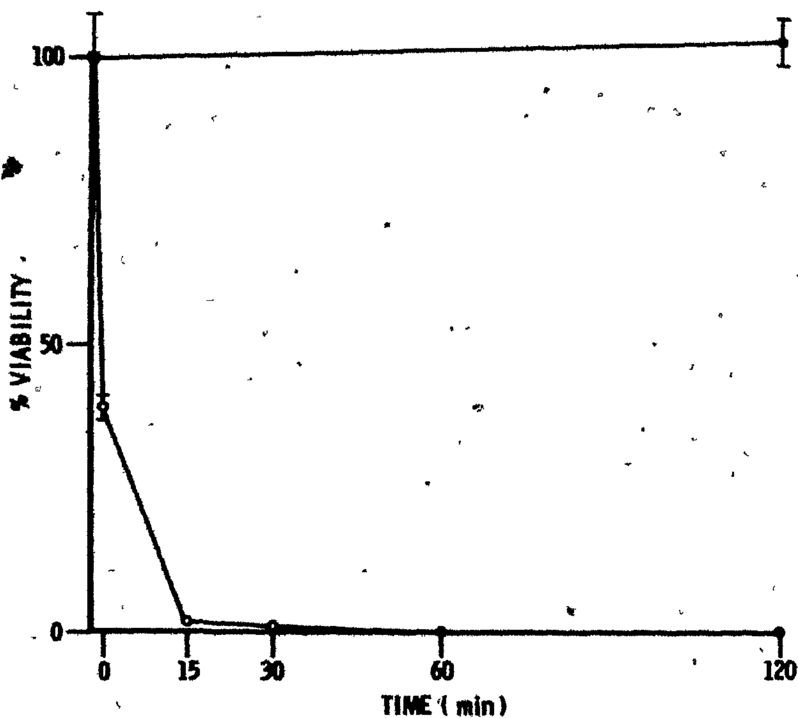


Figure 18. Effect of cold shock on viability. Time refers to time in cold. O——O, cold shock (0-3°); ●——●, control (37°). Vertical bars represent standard deviations calculated from total number of cells counted. Experimental procedure as in Methods.

experiment, but it was noticed that, if the cells were held in phosphate buffer (instead of growth medium) at 37°, there was some loss of viability.

As with photosynthesis, older cultures were more resistant to the cold than younger ones and with an older culture, viability of 50% was observed after 15 min. cold shock.

The age of culture effect on viability was confirmed using the optical density method (Methods, pg. 47). Results (Figure 19) showed that acquisition of resistance with age was a gradual process. The conclusion was based on the assumption that cold-shocked cells grew at the same rate as control cells.

Cold shock of cells at a cell density of 10^{10} cells/ml. instead of the customary 10^8 cells/ml. resulted in approximately the same percentage survival.

Since cold sensitivity varied somewhat from culture to culture, a strict comparison of the decline in photosynthetic capacity and viability could be made only if cold shock was performed using the same cell suspension. Photosynthesis was measured manometrically by method II (pg. 49). The results (Table 9) showed that for a given cell suspension, the effect of cold on viability was much more pronounced than on photosynthesis. Repeated experiments gave the same conclusion. This supported the conclusion drawn in the cell autoradiography experiment, which showed that all the cells were affected by cold shock.

The effect of temperature on viability was next studied.

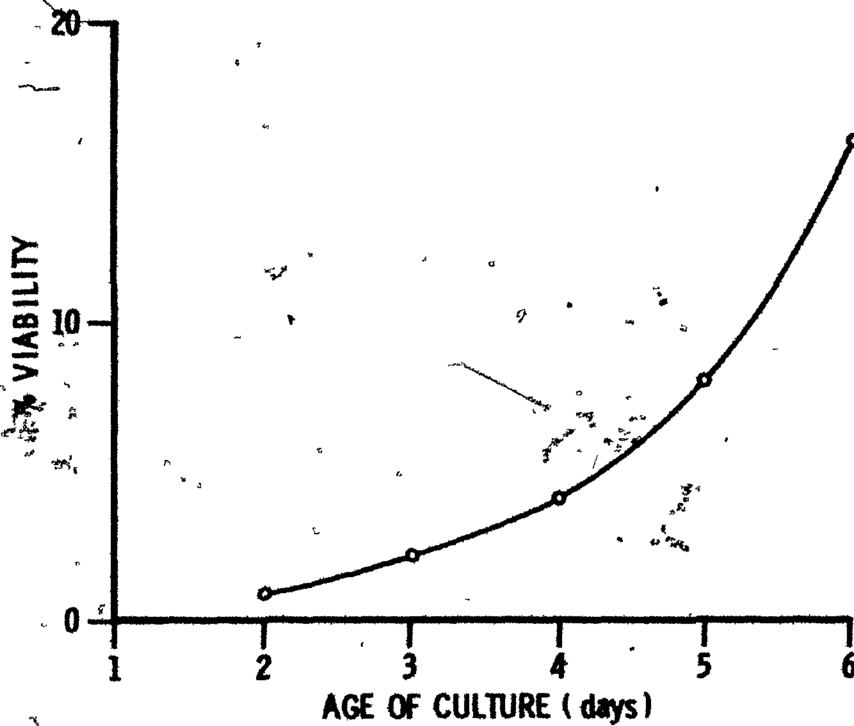


Figure 19. Effect of age of culture on viability after cold shock. Duration of cold shock, 15 min. Viability calculated by optical density method. For experimental details see Methods.

<u>Table 9</u>. Effect of cold shock on photosynthesis and viability using the same cell suspension. For experimental details see Methods, pg. 47, 49.

Time of cold shock (min.)	Viability (% control)	Photosynthesis (% control)
2	38	72
15	2	38
30	1	36
60	<0.2	28
120	<0.2	22

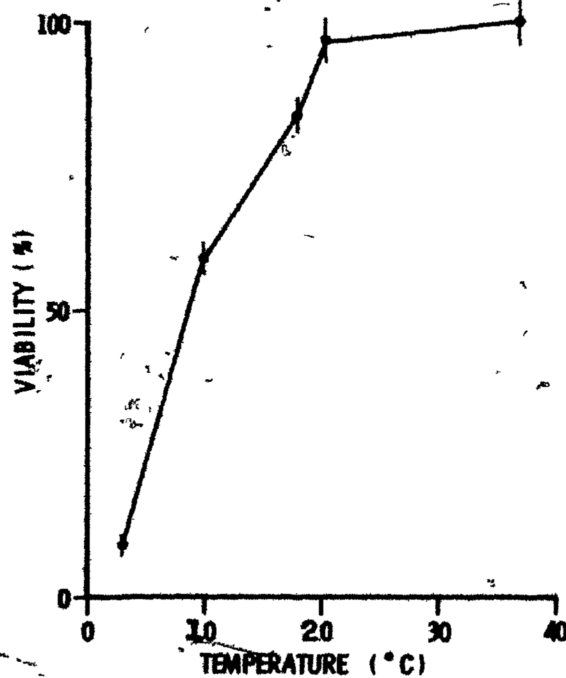


Figure 20. Effect of temperature on viability. Duration of cold shock, 60 min. Vertical bars represent standard deviations. For experimental details, see Methods.

Results indicated that there was significant loss in viability at 10° and at lower temperatures there was a further loss in viability. Time of cold shock in each case was 60 min. Results are shown in Figure 20.

(5) Release of cell components after cold shock. It was known that pteridines and glutamic acid were released from cold-shocked cells (Van Baalen, 1957). The main objective of this set of experiments was to classify the types of compounds released after cold shock and to determine what fraction of the total cell material they comprised.

(a) General aspects.

The release of ^{14}C and ^{33}P from labelled cells during cold shock is shown in Figure 21. The figure shows that about 25% of the dialysable material is quickly lost from the cell. Further time in the cold did not lead to increased excretion.

What was referred to as "total dialysable material" was the dialysable material lost from the cells when they were held at 65° for 30 min. This was generally about 2% of the total incorporated ^{14}C. It was assumed that the "total dialysable material" consisted of nearly all the available small molecules. However, it was possible that the value for "total dialysable material" was high, since the fraction could have contained some small molecules that arose as a result of degradation.

About 0.4-0.6% of the total cell carbon was lost during cold shock. This value was in close agreement with the value

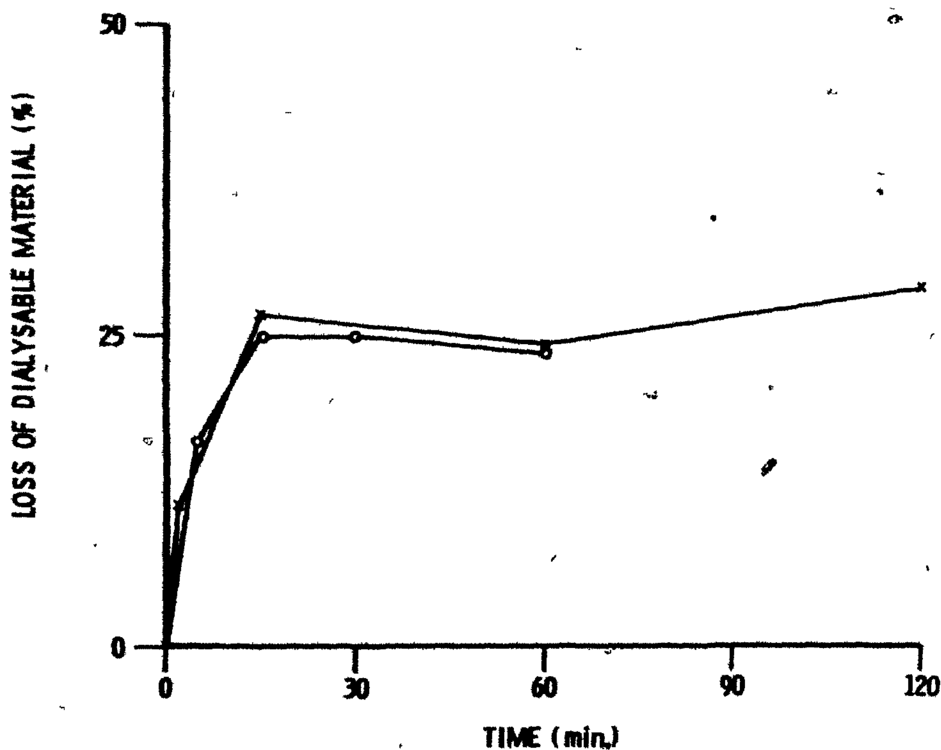


Figure 21. Excretion of material following cold shock, expressed as a percentage of "total dialysable" material; O——O, excretion of ^{14}C from uniformly labelled cells. X——X, excretion of ^{33}P from uniformly labelled cells. 100% is 2.04×10^4 c.p.m., for ^{14}C; and 2.86×10^5 c.p.m., for ^{33}P; per unit aliquot. For experimental details see Methods.

of 0.6% which was arrived at by the chemical estimation of glutamate lost from the cell (glutamate accounts for approximately half the carbon excreted - see below).

At least 99% of the cold shock supernatant was dialysable, which indicated that all the excreted material consisted of small molecules.

The leakage of material was also followed as a function of time after cold shock using ^{33}P labelled cells. This is shown in Table 10.

(b) Classification of labelled products and extent of leakage of radioactive fractions.

The supernatant from the 15 min. cold-shocked sample of the ^{14}C labelled cells was separated by two dimensional chromatography and identified by autoradiography (Methods, pg. 68). Table 11 shows the composition of the material lost after cold shock.

The main excretion product was glutamic acid which constituted almost half of the radioactive carbon lost. Other ninhydrin positive spots made up a further 20.6% of the ^{14}C material. The only notable non-amino acids were PEP, citrate, malate and pteridines. There was notably a lack of label in areas where phosphate esters (other than PEP) normally ran, although some of the phosphate esters may have remained at the origin.

The remaining radioactivity was not identified and of this only 4% was marked on the autoradiogram as definite spots.

Amino acids were identified using chemical standards and

Table 10. Excretion of ^{33}P from labelled cells after 15 min. cold shock, when cold shock cells were held at 37°. Time refers to that at 37°. Centrifugation time of 10 min. was not included. 100% dialysable was 2.86×10^{-5} c.p.m./aliquot. For experimental details see Methods.

Time after cold shock (min.)	Excretion of ^{33}P (% total dialysable)
0	26.5
40	35
80	33

Table 11. Radioactivity in excretion products expressed as a percentage of ^{14}C lost from labelled cells. Time of cold shock, 15 min. * identified by R_F, pteridines identified by fluorescence in U.V. light. † identified by co-chromatography. For experimental details see Methods.

Compound	^{14}C lost (% Total)
Glutamate†	46
Aspartate†	3.8
Cystine*	2.7
Alanine‡	1.0
Ser. gly. $aspNH_2$*	1.0
Other ninhydrin positive spots	12.1
Pteridines	2.5
PEP*	2.7
Citrate and malate*	2.5
Other phosphate esters*	<1
Origin	2.0

were chemically quantitated by the ninhydrin reaction. The ratio of optical density at 570 nm of glutamic acid to other ninhydrin positive areas was 1:0.52, which was in the same order of magnitude as determined by ^{14}C leakage.

Chemical tests for leakage of ninhydrin positive material also showed that about 65% of the total ninhydrin positive material leaked out of the cell within 60 min. of cold shock. Controls kept at room temperature released only 3-5% of ninhydrin positive material into the supernatant, in the same period of time. Of the free glutamic acid, 50-80% was lost during cold shock, but only a small fraction of the available alanine was lost from the cells. Glutamic acid was not excreted in detectable quantities (5%) from control samples kept at room temperature.

On separation of the ^{33}P-labelled supernatants by one dimensional chromatography (BAW::4:1:5), the ^{33}P compounds separated into 4 radioactive areas which are called fractions F1, F2, F3, F4 in order of increasing R_f. The fractions (Table 12) contained - F1, sugar diphosphates; F2, sugar monophosphates; F3, triose-P and PGA; F4, inorganic phosphate and PEP. (Fractions F2 and F3 merged into one another and were best considered together.)

It was interesting to note that the composition of the fractions did not change much with cold shock time. A similar distribution of radioactivity was observed when the sample cold-shocked for 15 min. was allowed to stand at 37° for 80 min.

<u>Table 12</u>. Distribution of radioactivity in ^{33}P fractions for different cold shock times. 100% represents (in counts x 10^{-5}/4 min./aliquot): 2 min., 1.55; 15 min., 3.14; 60 min., 2.81; 120 min., 3.41; For experimental details see Methods.

Fraction	Radioactivity (%)			
	2 min.	15 min.	60 min.	120 min.
F1	6	8.5	5.5	7.5
F2	34	27.5	30	30
F3	5	6	5	4
F4	55	58	59.5	58.5

<u>Table 13</u>. ^{33}P material released as a function of total available material in each fraction. 100% represents (in counts x 10^{-5}/4 min./aliquot): F1, 2.36; F2, 0.94; F3, 0.30; F4, 5.89. For experimental details see Methods.

Fraction	Radioactivity (% total available)
F1	11
F2	64
F3	23
F4	27.5
F1, 2, 3 and 4	27

If the fractions of the 15 min. cold shock supernatant were expressed as a percentage of the total available material in each fraction then it was evident that there was selective loss of some fractions (Table 13). This table showed that F2 was lost selectively and F1 retained selectively. This could reflect some compartmentation. The same conclusion was reached when the loss of glutamate was considered. Glutamate was lost to the extent of 50-80%, while overall ^{14}C was lost to the extent of only 25%.

Two dimensional chromatography of ^{33}P material resulted only in the resolution of a triose-phosphate spot.

It was concluded from these experiments that cold caused a lesion which resulted in the loss of about 0.5% of the total carbon which probably represented about 25% of the total dialysable material. The material lost was probably qualitatively representative of the small molecules of the cell. However, it appeared that quantitatively there was some selectivity in the loss.

(6) Attempts to restore photosynthesis in cold-shocked cells. The observation that a large fraction of low molecular weight compounds was lost from the cell following cold shock suggested experiments to determine if concentrated supernatants from cold-shocked cells could restore photosynthesis. In order to do this supernatants from many cold shock cell suspensions were pooled and concentrated by freeze-drying. The final concentration of the extract was approximately equal to the

intracellular concentration of intermediates (as calculated from packed cell volume). This extract was tested for its effect on photosynthesis. The concentrated cold shock supernatants completely inhibited photosynthesis in cold-shocked cells. Control cells were inhibited to the extent of 18%.

Other attempts to restore photosynthesis included the study of the effects of specific additives. Compounds added included, (1) electron acceptors, NADP and $K_3Fe(CN)_6$, (2) CO_2 acceptors, PEP and RDP. The rationale for using electron acceptors was that, if electron flow was not affected directly by cold and if the lowering of photosynthetic capacity was due to the loss of intermediates, then the addition of an electron acceptor might increase oxygen evolution. The rationale for using CO_2 acceptors was similar but in this case any recovery in photosynthetic capacity was dependent not only on electron flow but also on the generation of ATP. Other attempts to restore photosynthesis included the addition of ATP and also the major excretion product, glutamic acid.

All these additions failed to restore photosynthesis. The results are shown in Table 14.

The concentration of reagents used was sufficient to support measurable photosynthesis over the reaction time. Other studies using manometric method II (pg. 49) gave similar results. No change was observed at varying concentration of reagents but in some cases high concentrations (e.g. 9 m*M* glutamate) inhibited.

One exception was $K_3Fe(CN)_6$ which seemed to increase gas

Table 14. The effect of additives on the photosynthetic capacity of cold-shocked cells. Photosynthesis measured manometrically (Methods, pg. 47). Reaction volume, 5 ml.; Klett cell units per flask, 2650; time of cold shock, 20 min.

Additive	Conc. (mM)	Oxygen evolved (µl./10 min.)	
		Control	Cold shock
(a)			
-	-	94	40
PEP	1.5	97	41
Glutamate	2.5	-	41
(b)			
-	-	88	36
NADP	3	89	36
$K_3Fe(CN)_6$	20	90	34
RDP	1.5	92	38
ATP	3	91	36

evolution when tipped into the reaction vessel immediately before beginning the measurement of photosynthesis. However it was not possible to repeat this and the effect obtained was believed to be an artifact.

Results of experiments in this section indicated that these compounds could not restore photosynthesis in cold-shocked cells.

(7) Variation of cold shock medium and its effect on photosynthetic capacity. Studies with bacterial systems have shown that the cold shock medium has a profound effect on viability (see Introduction, pg. 4) and as a result it was interesting to determine if the use of media that protect bacteria from loss of viability, could also protect the photosynthetic capacity of *Anacystis nidulans* from the cold.

In these studies 5 mM $MgCl_2$, full strength Ringers solution (g/l : NaCl, 9.0; $CaCl_2$,0.25; KCl,0.42), 0.3 and 0.4 M sucrose were used as cold shock media with growth medium as control. Results of these experiments showed that cold shock in any of these media did not result in protection of photosynthetic capacity. In fact slightly lower rates of photosynthesis (when compared to cold shock in growth medium) were observed in each case.

The conclusion is that these media have no protective effect on photosynthesis.

(8) Excretion of pteridines. One of the reasons for undertaking the study of excretion of pteridines was to find out

whether, from the concomitant decline in photosynthesis and loss of pteridines from the cell (Forrest et al., 1957; Van Baalen, 1957) evidence could be uncovered which would link these compounds to photosynthesis. The experiments were modelled on those of Forrest and coworkers and pteridine loss was measured in the supernatant after various periods of cold shock. However, it was soon noticed that more pteridines escaped from the cell during the period of manometry. Results showed (Figure 22) that there was a progressive increase of pteridine in the supernatant with time of cold shock. However, if the pteridines were measured in the cell about 1 hr. after cold shock (coinciding with the time photosynthesis was measured), less pteridines were found to be retained in the cell. Controls kept at 37° did not excrete pteridines.

The progressive loss of pteridines from the cell after cold shock was interesting and this was studied further. Results showed that there was in fact a loss of pteridines during manometry, especially just after tipping the cell suspension into the main vessel. Several experiments gave similar results. On the other hand photosynthesis of cold-shocked cells remained nearly constant. A plot of the minimum percent pteridines in the cell and photosynthesis (percent control) showed that there was some relationship between photosynthetic decline and pteridines lost from the cell (Figure 23).

However, work on this line was discontinued for several reasons. (1) It was found that all the pteridines lost from

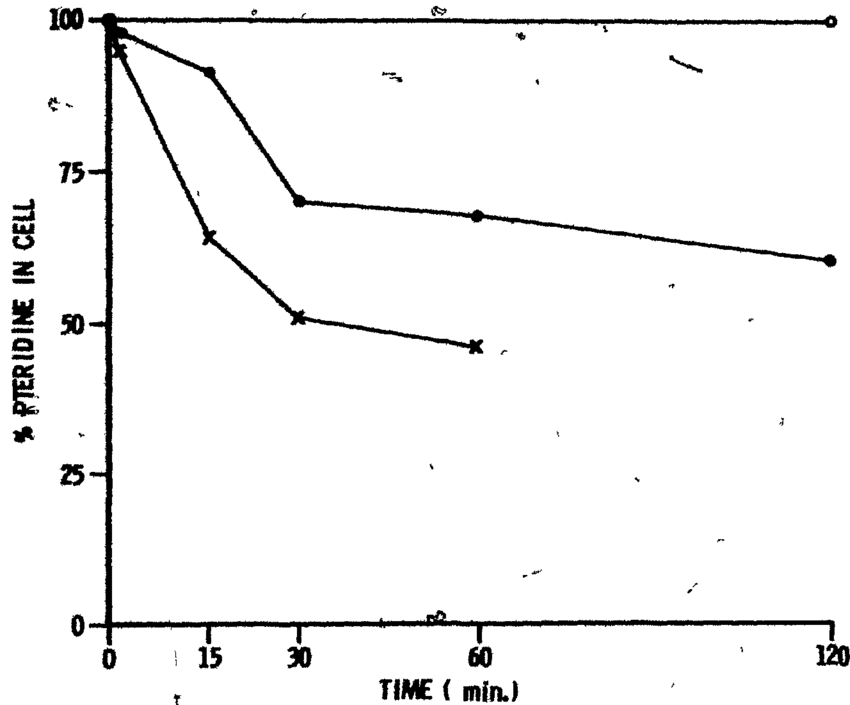


Figure 22. Effect of cold shock on pteridine excretion. O---O, control cells held at 37°; ●—●, cold-shocked cells. Percent pteridine in cell determined from pteridine in supernatant, immediately after cold shock; X—X, pteridines determined in cell 1 hr. after cold shock, coinciding with the time photosynthesis was generally measured. For experimental details see Methods.

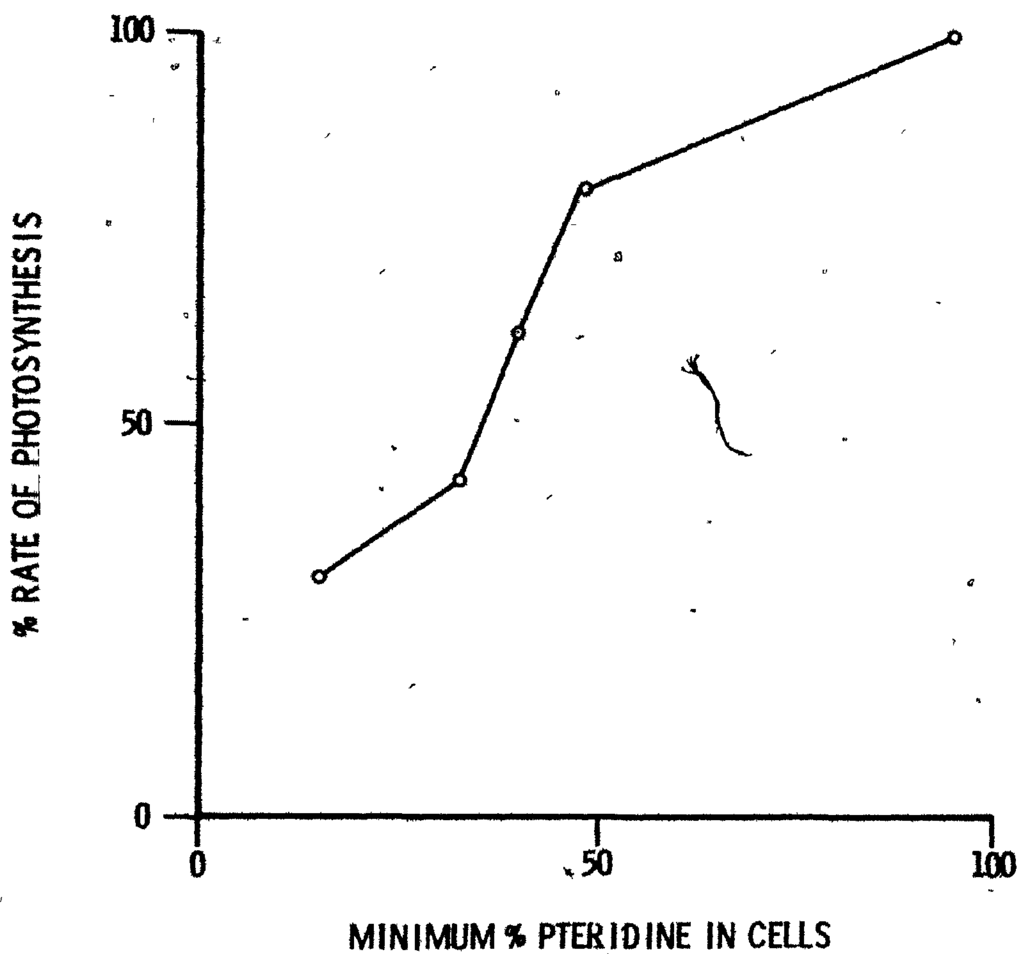


Figure 23. Relationship between percent rate of photosynthesis and percent minimum pteridine remaining in the cell. Data from different experiments. For experimental details see Methods.

the cell could not be recovered in the supernatant (possibly due to photolysis during manometry). (2) Pteridines in contact with cold-shocked cells tended to produce a yellow fluorescent derivative and it was uncertain whether this pteridine could be assayed quantitatively by the methods of analysis used. Other theoretical reasons for the discontinuation of this approach are given in the Discussion.

(9) Effect of cold shock on Anabaena variabilis. Similar cold treatment of *Anabaena variabilis* showed that the photosynthesis of this organism was not susceptible to cold shock. No pteridines were detected in the supernatant.

(10) Summary of observations made using whole cells. (a) It was confirmed that cold shock had a marked effect on photosynthesis (O_2 evolution and CO_2 fixation) in *Anacystis nidulans*. (b) *Anabaena variabilis* did not show this effect. (c) It was found that the effect of cold on photosynthesis was not "all or none" with respect to individual cells of the population. (d) The effect of cold was rapidly manifested and also shown over very short periods of photosynthesis. (e) Cold shock caused a loss of viability and a breakdown of selective permeability. (f) There was no special reason to believe that pteridine loss was the cause of photosynthesis decline. (g) Electron acceptors and CO_2 acceptors could not restore photosynthesis.

(B) Cell-free extract studies.

Since cold had some effect on the limiting membrane, it was possible that it might also have some effect on the photosynthetic membranes. Studies of Van Baalen (1957), who found that cell-free extracts prepared from cold-shocked cells had lower Hill activity (as measured by DCPIP reduction) and those of Gerhardt and Santo (1966) who advised that cell-free extracts of *Anacystis nidulans* be kept at room temperature in order to observe photophosphorylation, supported this possibility. As a result it was decided to study the effect of cold shock on electron flow and photophosphorylation in cell-free extracts of *Anacystis nidulans*.

Cell-free extracts were prepared by lysozyme digestion in the presence of EDTA. Three hr. of incubation at 37° resulted in protoplasts, most of which retained the characteristic shape of the cell. When the osmotic pressure of the medium was lowered, most of the cells lysed. It was of interest that when these protoplasts were subjected to cold shock even in the presence of 0.4 M sucrose, there was some lysis, causing the release of phycocyanin.

Cold shock was performed after the preparation of cell-free extracts, in 0.4 M sucrose, thus the effect observed could be interpreted as a direct effect of cold.

(1) Effect of cold shock on electron flow.

(a) Electron flow using ferricyanide as acceptor. - Photosystem II.

Preliminary studies were performed by measuring oxygen evolution. Rates of oxygen evolution were in the range of 120-190 µEq./mg.chl./hr. The gas evolution was light catalyzed and inhibited by CMU (Figure 24).

A study of the effect of cold shock on the system showed (Figure 25) that cold had very little effect on oxygen evolution and the apparent lower activity of the cold-shocked samples was due to the slight lag at the start of the reaction. Repetition of this experiment confirmed the conclusion that cold shock had no effect on the system.

Further experiments using ferricyanide as electron acceptor were done by following ferricyanide reduction spectrophotometrically at 420 nm. Rates of reduction observed in these experiments were high (generally in the range of 450-550 µM/mg.chl./hr. and on one occasion a rate as high as 700 was observed). These rates were similar to those obtained with Phormidium luridum (Biggins, 1967) and Anabaena variabilis (Susor and Krogmann, 1964) but higher than those previously obtained with Anacystis nidulans (Introduction, pg. 21).

Checks were made to determine if there were any inherent errors in the procedure. It was observed that there was some dark reduction at 420 nm but this was small (Figure 28), and results were corrected for it. It was also observed that there was some loss of phycocyanin absorption (620 nm) during the experiment, however, this was of little significance since phycocyanin had very low absorption at 420 nm. Further, determination of ferricyanide reduction in trichloroacetic acid

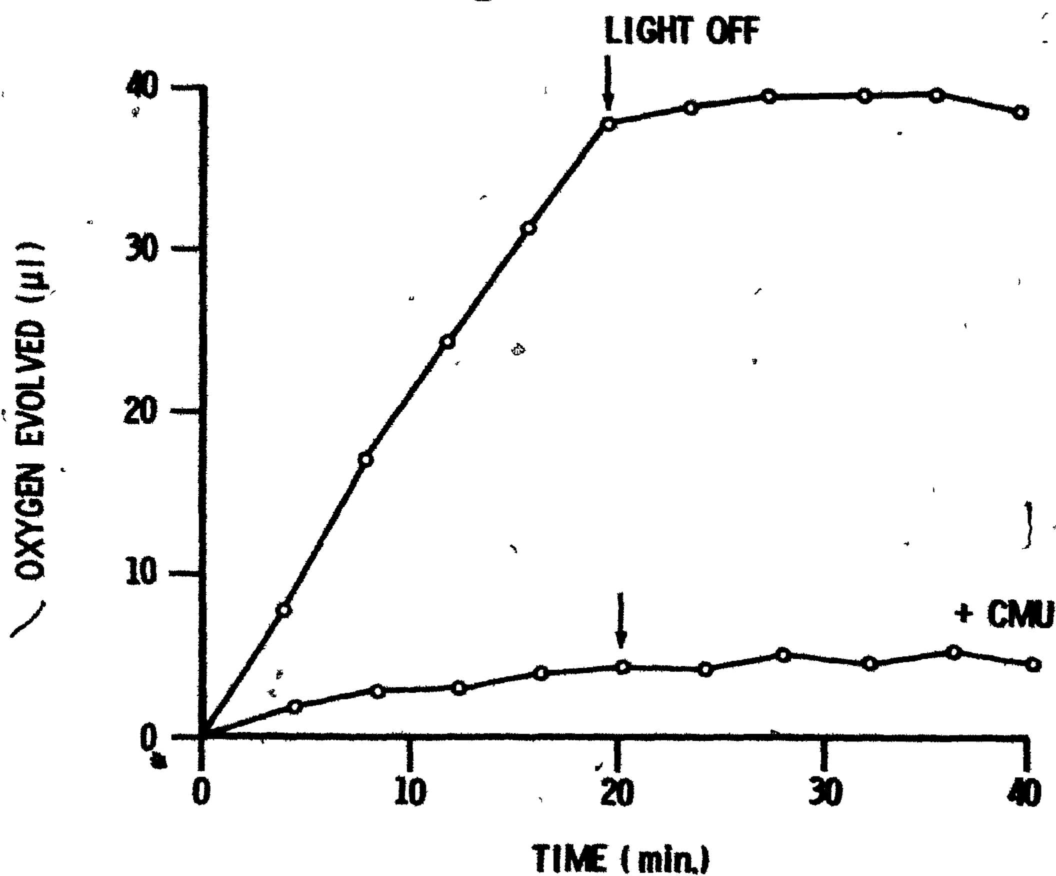


Figure 24. Oxygen evolution in a cell-free extract using $K_3Fe(CN)_6$ as electron acceptor. $K_3Fe(CN)_6$, 20 µmoles; chlorophyll, 120 µg; in 5 ml. ↓, Light off. For further experimental details see Methods.

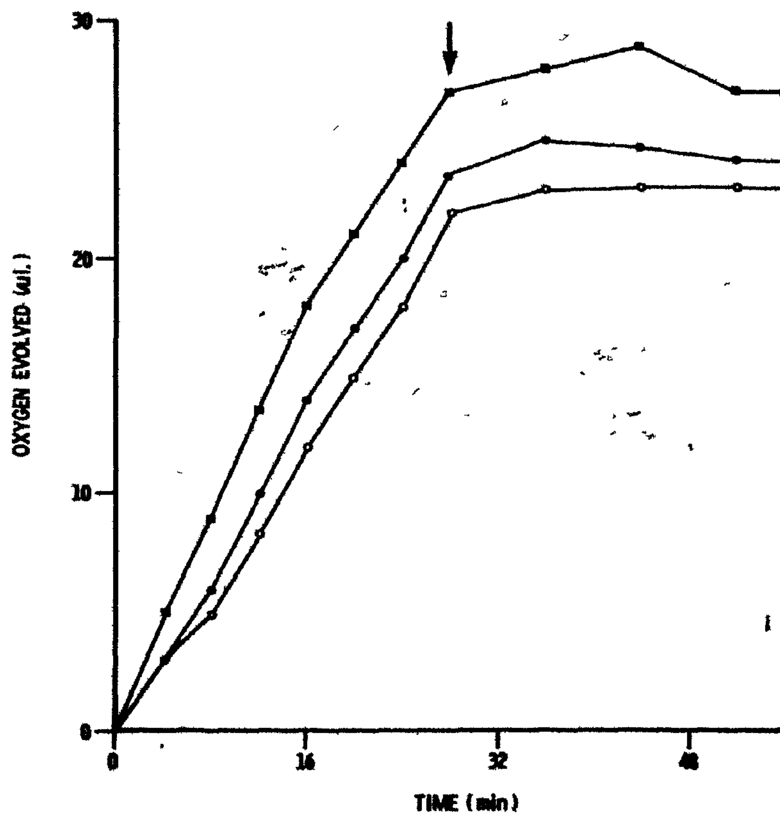


Figure 25. Effect of cold shock on photosystem II. $K_3Fe(CN)_6$, 10 µmoles; chlorophyll, 60 µg; ↓, Light off; ■——■, control (room temperature); ●——●, 15 min. cold shock; O---O, 60 min. cold shock. For further experimental details see Methods.

supernatants (which has a blank optical density of less than 0.04) gave results similar to direct determination of optical density changes in the extract. Further evidence that absorbence at 420 nm measured true physiological photoreduction of ferricyanide was its 80-90% inhibition with CMU and the high P/2e ratios obtained (see section (2) (b)).

Preliminary experiments showed that the rate of ferricyanide reduction was proportional to concentration of extract (Table 15).

Other preliminary experiments showed that:

(1) pH could be varied between 7.2 and 8 without significantly changing the rate of ferricyanide reduction.

(2) The presence of ADP did not increase the rate of ferricyanide reduction (Figure 26).

(3) Sucrose concentrations from .04 M - 0.4 M had no effect on the reduction of ferricyanide.

(4) Mg^{2+} stimulated the rate of reaction almost two fold (Figure 27).

Similar overall results have been obtained by Susor and Krogmann (1964) using Anabaena variabilis.

The effect of cold shock on ferricyanide reduction was studied next. Results (Figure 28) showed that 30 min. cold shock had no effect on ferricyanide reduction. Similar results were obtained in the absence of ADP and inorganic phosphate. In addition, extracts and protoplasts could be stored in a refrigerator (approximately 6°) for more than 24 hr. with retention of more than 75% of their activity.

Table 15. Effect of concentration of extract on the rate of ferricyanide reduction. $K_3Fe(CN)_6$, 2 μmoles; reaction time, 3 min. For experimental details see Methods.

Chlorophyll (μg./3 ml.)	Ferricyanide reduced (μmoles/min.)
5	0.042
10	0.090
16.7	0.166
25	0.254
36	0.354

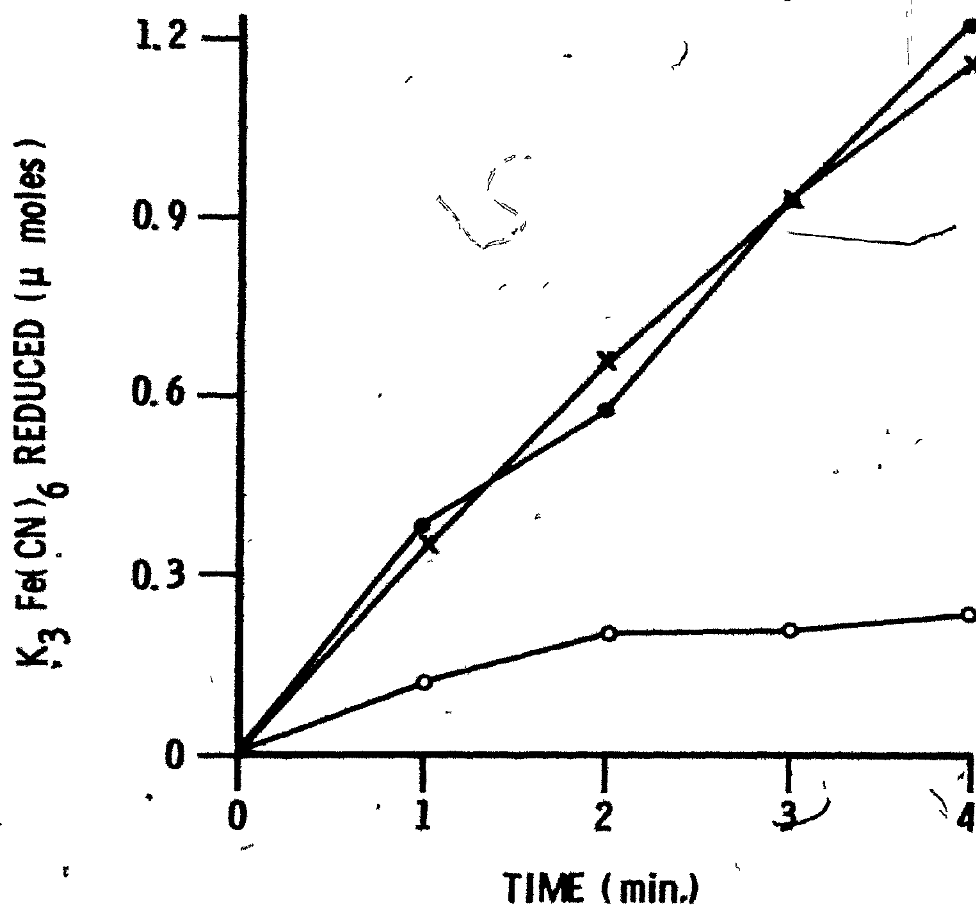


Figure 26. Effect of ADP on $K_3Fe(CN)_6$ reduction. $K_3Fe(CN)_6$, 2 μmoles; chlorophyll, 40 μg; in 5 ml. ●—●, +ADP; X—X, -ADP; O—O, ADP+CMU. Data not corrected for dark reduction. Ferricyanide was measured in the TCA supernatant. For further experimental details see Methods.

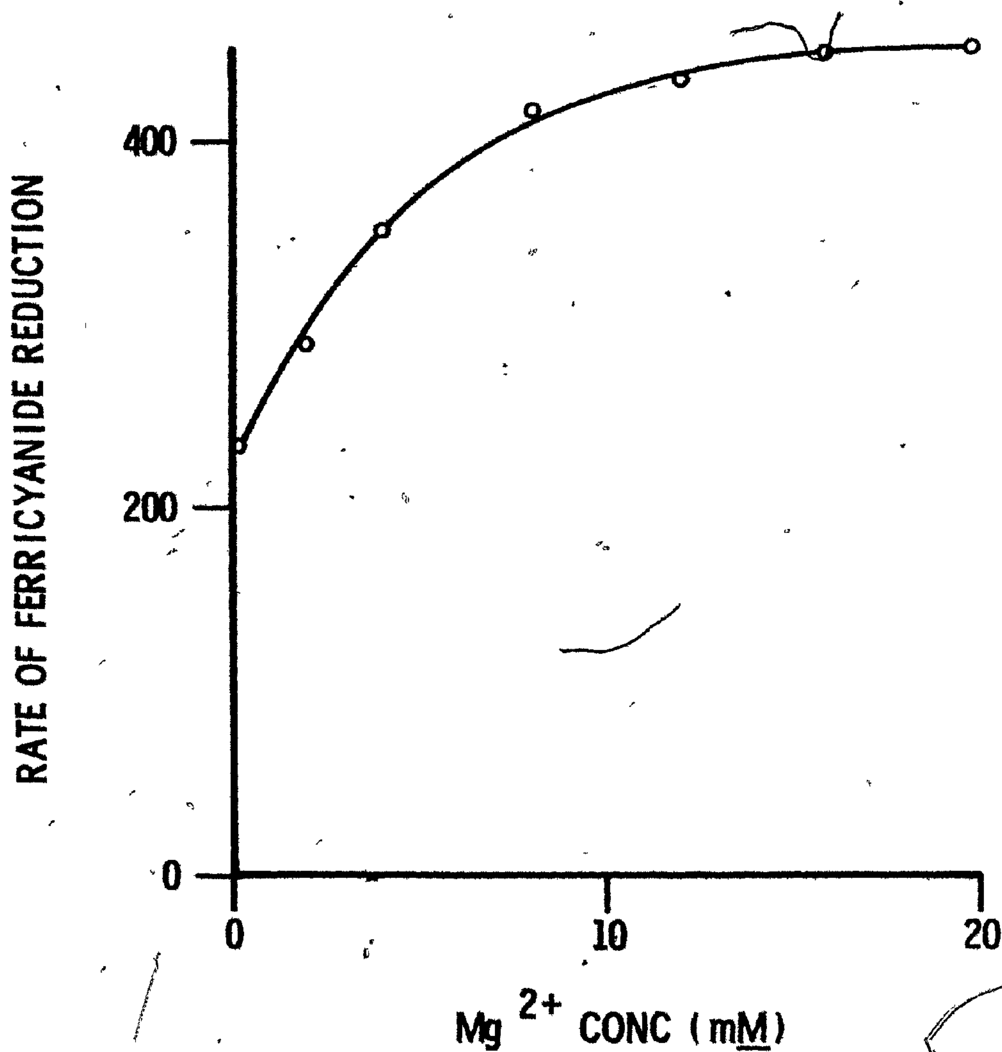


Figure 27. Effect of Mg^{2+} on $K_3Fe(CN)_6$ reduction. Rates expressed in µmoles/mg.chl./hr. $K_3Fe(CN)_6$, 1.5 µmoles; chlorophyll, 20 µg; in 3 ml. Rates calculated over a period of 2 min. For other experimental details see Methods.

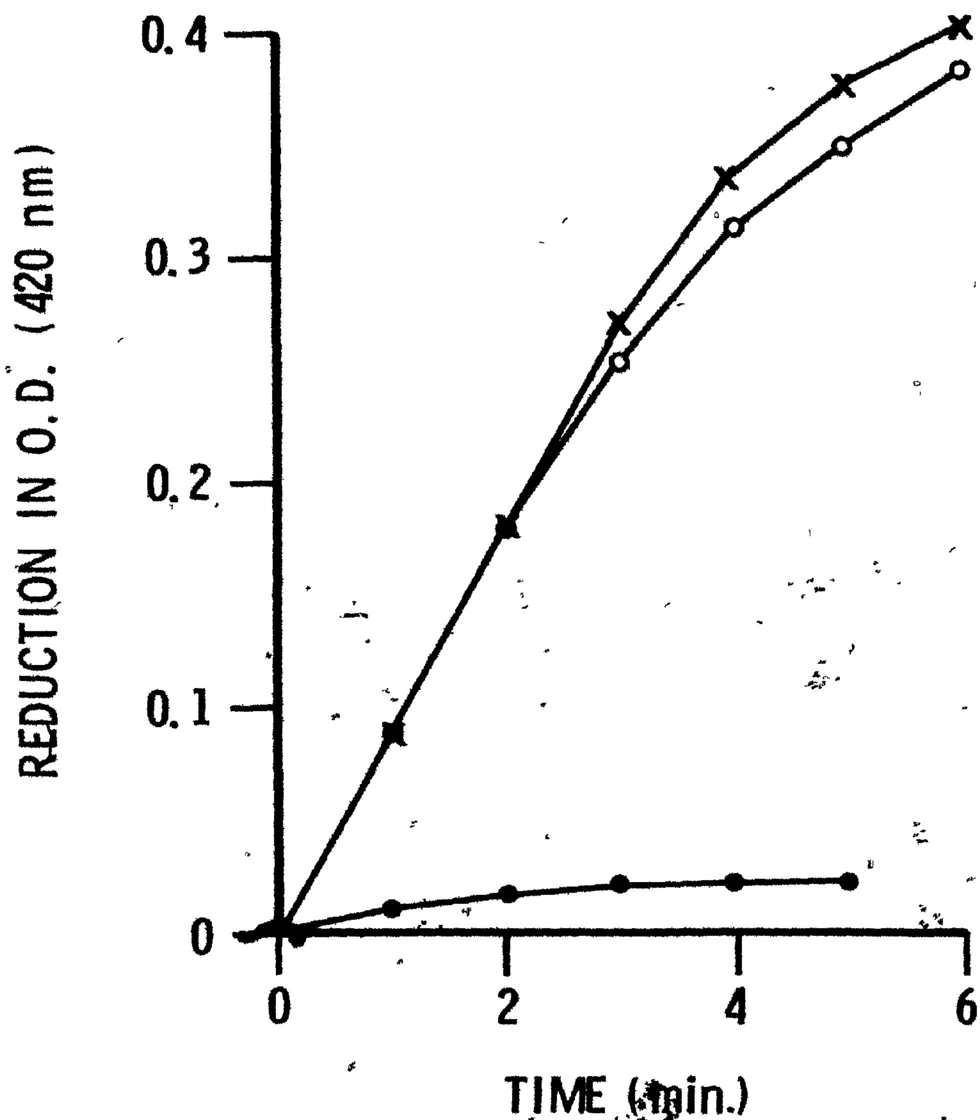


Figure 28. Effect of cold shock on $K_3Fe(CN)_6$ reduction. Ferricyanide, 1.5 µmoles; chlorophyll, 21 µg; in 3 ml. O——O, control (room temperature); X——X, 30 min. cold shock; ●——●, dark reduction. For other experimental details see Methods.

Cold shock was found to have no effect on ferricyanide reduction even when it was carried out in water instead of 0.4 M sucrose.

All this shows that cold shock has no effect on photosystem II.

It was not possible to prepare suitable cell-free extracts from cold-shocked cells using the lysozyme digestion procedure, since the protoplasts were found to lyse during digestion as well as during washing, hence losing soluble proteins. Such extracts have low Hill reaction capacity, most likely due to loss of water soluble material.

(b) Electron flow using reduced cytochrome-c as electron donor (photosystem I).

After preliminary experiments, it was decided to use concentrations of 0.07 mM Cyt. c (50% reduced) and 0.16 mM methyl viologen as electron donor and acceptor respectively. Trials showed that these concentrations gave nearly linear rates of reduction over the full length of the experiment, with a maximum rate of 15-20 µM reductant/mg.chl./hr. (using E-max. Cyt. c, red.-ox. = 19,000).

Rates were very low compared to ferricyanide reduction but were comparable to those previously reported in the literature for Anacystis nidulans by Fugita and Myers (1966) and Brown (1969). Interference from the dark reduction of Cyt. c was minimized as described in Methods (pg. 62).

The effect of cold shock on the system was next studied (Figure 29). The results showed that cold had no effect on the

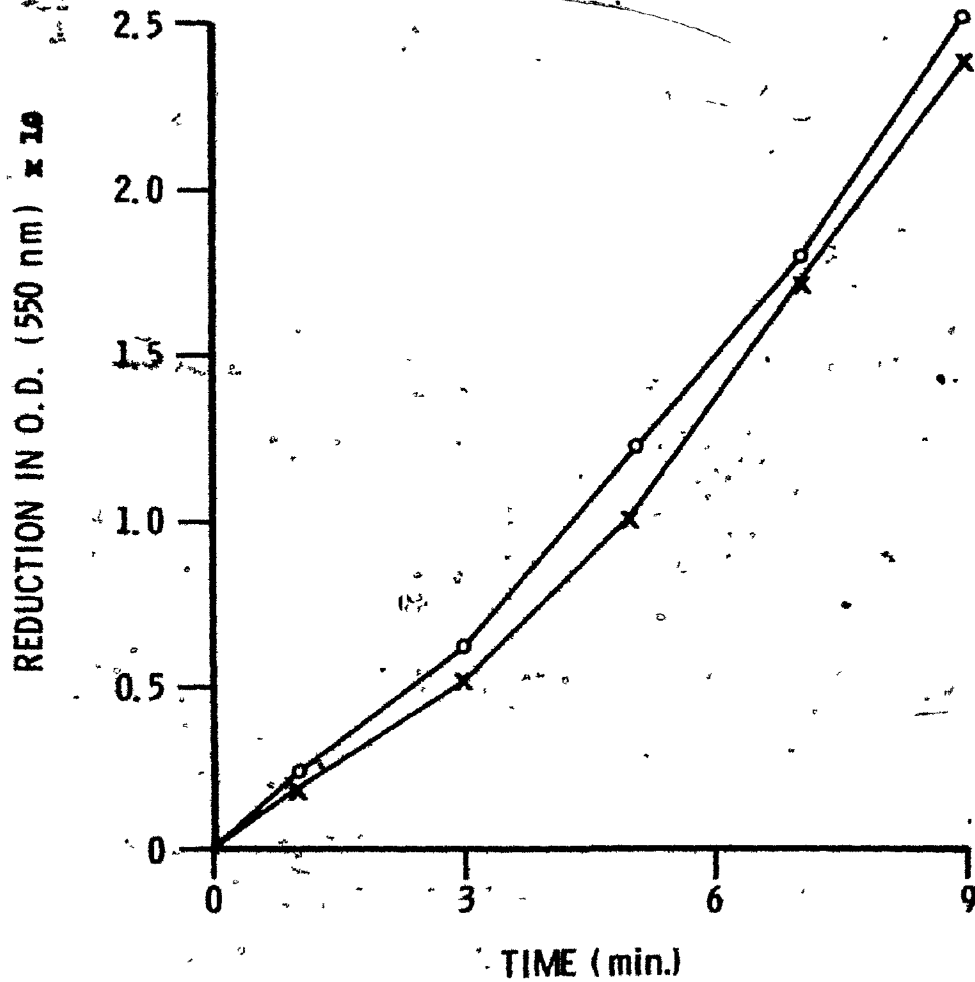


Figure 29. Effect of cold shock on photosystem I. Cyt. c, 0.20 μmoles (50% reduced); Methyl viologen, 0.5 μmoles; chlorophyll, 20 μg; in 3 ml. O——O, 30 min. cold shock; X——X, control. For further experimental details see Methods.

oxidation of Cyt. c. This showed that photosystem I was not affected by cold shock.

(c) Electron flow using DCPIP as electron acceptor (photosystem I + II, see Introduction pg. 18).

Preliminary experiments showed that the rates of DCPIP reduction, over the concentrations used (0.08 mM - 0.13 mM), showed little variation. In each case, the reaction was characterized by a slight decrease in rate as the reaction proceeded, irrespective of the concentration of DCPIP used. Interference from dark reduction of DCPIP was minimized by adopting the procedures described in Methods (pg. 62). DCPIP reduction was inhibited 80-90% by CMU and about 90% of the reduction in optical density at 550 nm could be regenerated with ferricyanide. This showed that most of the reduction in optical density observed was due to true photoreduction of DCPIP.

Rates of reduction were 120-150 µEq. reductant/mg.chl./hr. during reaction time of 4 min. but initial rates of 200 have been observed. These rates were similar to those obtained by Van Baalen (1957).

The effect of cold shock on DCPIP reduction was studied next. Results (Figure 30) showed that cold shock had no effect on DCPIP reduction. The same result was obtained at limiting DCPIP concentrations, Table 16. This latter experiment was designed to determine if cold shock changes the affinity of DCPIP for reduction. Results clearly showed that this was not the case and that the rate of DCPIP reduction by control

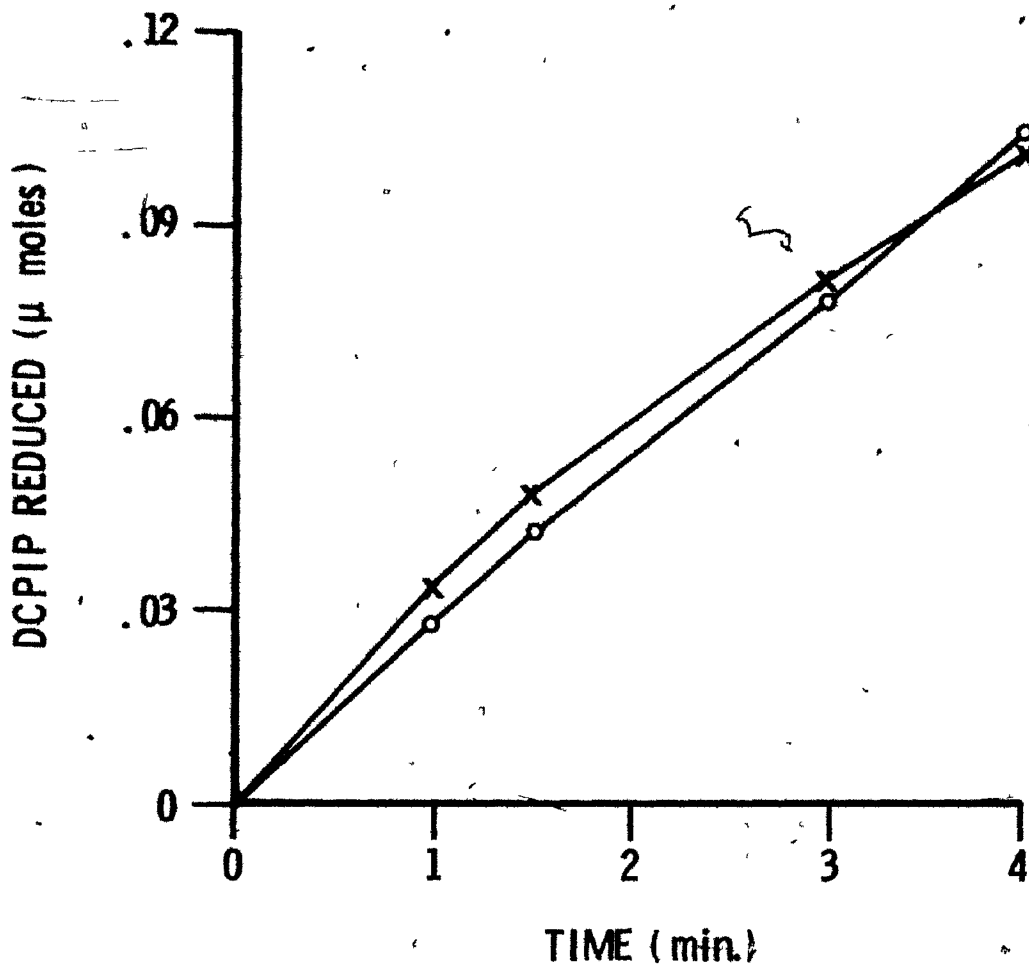


Figure 30. Effect of cold shock on DCPIP reduction. DCPIP, 0.3 μmoles; chlorophyll, 22 μg; in 3 ml. X—X, control; O—O, 30 min. cold shock. For further experimental details see Methods.

Table 16. The effect of cold shock on DCPIP reduction at limiting DCPIP conc., DCPIP, .15-.17 µmoles; chlorophyll, 30 µg.; oxidized DCPIP at end of reaction, .015-.020 µmoles. For further experimental details see Methods.

Time (min.)	Reduction in O.D. (550 nm)	
	Cold shock (30 min.)	Control
1	0.135	0.145
2	0.225	0.240
3	0.285	0.300
4	0.325	0.340

and cold shock samples were almost identical throughout the time course even after nearly all the DCPIP (approx. 90%) was reduced.

(d) Attempts to estimate methyl viologen and NADP reduction (combined photoreactions).

Attempts to follow methyl viologen reduction spectrophotometrically by the method of Kok were not successful due to technical difficulties in obtaining an anaerobic atmosphere.

Attempts to follow NADP reduction by the method of Biggins (1967 b) were not successful either because no reduction could be observed even after the addition of ferridoxin. It was probable that this was due to a deficiency of ferridoxin:NADP transhydrogenase. However, the reason why work on this line was discontinued was due to a light-catalyzed oxidation of NADPH observed in these extracts. This effect could not be abolished by: (a) washing the particles, (b) dialysis (overnight), (c) rotenone.

(2) Effect of cold shock on photophosphorylation.

(a) Cyclic photophosphorylation. - (PMS-ascorbate mediated).

Preliminary studies showed that the pH optimum of the system was 7.5-7.6. It was also found that the formation of organic phosphate was dependent on the presence of light, ADP and PMS. The presence of CMU did not inhibit (Table 17). The low rates of photophosphorylation in Table 17, A and B were due to the low light intensity used. Ascorbate was also required; in its absence, only very low levels of photophosphorylation were observed (Table 17 C). The results also

<u>Table 17</u>. Characteristics of PMS-ascorbate mediated (cyclic) photophosphorylation. Chlorophyll: A, 41 µg; B, 46 µg; C, 55 µg; pH: A and C, 7.6; B; 7.2; illumination: A and B, three, 20 watt "cool white" fluorescent lights; C, 650 watt tungsten lamp (Methods, pg. 63). T1 and T2 refer to experiments done with extracts held at room temp. for 20 and 40 min. respectively. For further experimental details see Methods.

Expt.	Conditions	Rate (µM ATP/mg. chl./hr.)
A	complete	98
	-ADP	0.6
	dark	0.6
B	complete	68
	-CMU	54
	-PMS	10
C	complete	128
	" T1	134
	" T2	124
	-ascorbate	11

showed that the system retained activity when held at room temperature.

Cold shock had a marked effect on PMS-ascorbate photophosphorylation. After 45 min. cold shock only about a third of the total phosphorylating activity remained. In order to find out if this effect was unique to Anacystis nidulans, a cell-free extract of Anabaena variabilis was treated in the same way. Results (Figure 31) showed that PSP in Anacystis nidulans was much more sensitive to cold shock than Anabaena variabilis.

The rate of photophosphorylation obtained in these experiments compared favourably with the best in the literature for Anacystis nidulans (see Introduction, pg. 22).

(b) Noncyclic photophosphorylation (with $K_3Fe(CN)_6$ as electron acceptor).

Assay conditions were the same as those for ferricyanide reduction. Preliminary experiments showed that the system was inhibited by CMU (Table 18). They also showed the stability of the system over a period of 45 min. Other trials also showed that light was a requirement.

In these experiments, radioactivity in zero time samples was subtracted.

Next, the effect of cold shock was studied. Results are shown in Table 19 and Figure 32. As in cyclic photophosphorylation cold had a marked effect on ATP synthesis. ATP synthesis declined to about 40% after 15 min. of cold shock. Samples held at room temperature showed no such loss (Figure 32). The rate of photophosphorylation obtained was better than any

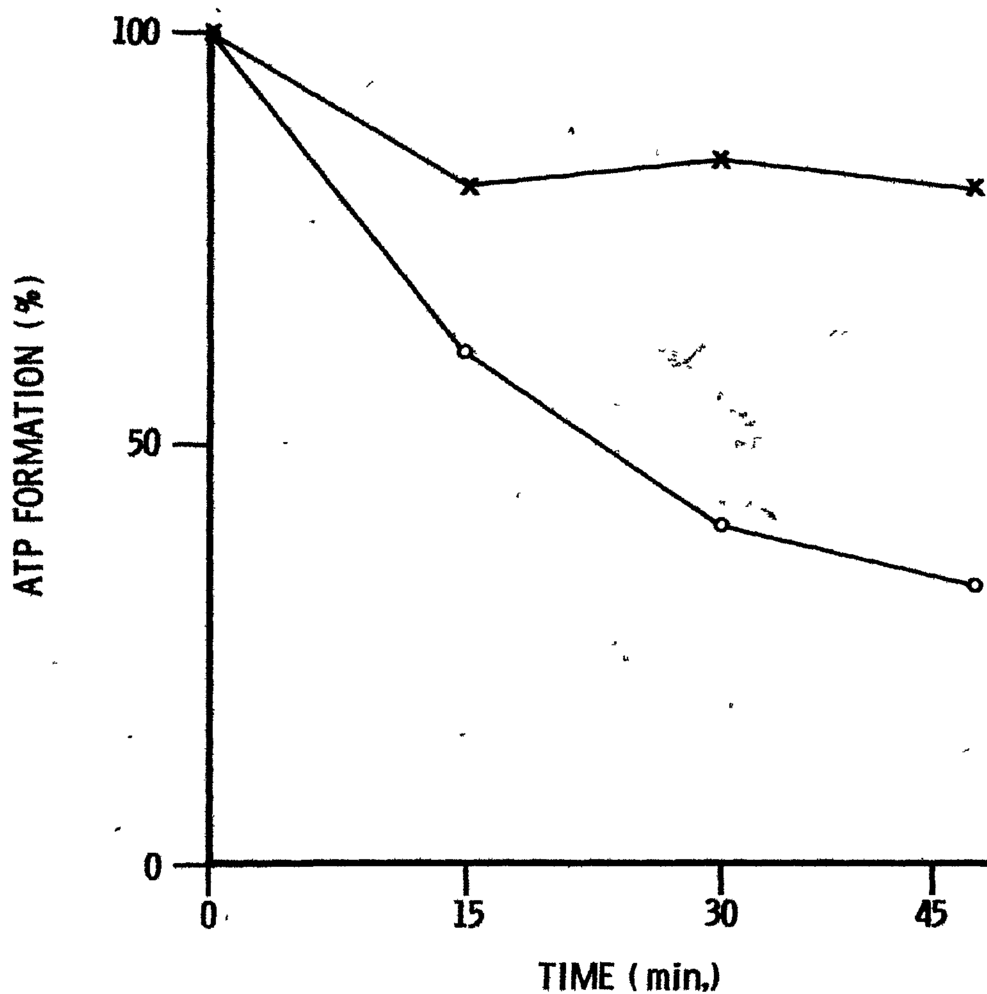


Figure 31. Effect of cold shock on cyclic PSP. Ascorbate, 100 µmoles; PMS, 0.1 µmoles. CMU was present. Chlorophyll, for Anabaena, 33 µg; for Anacystis, 49 µg; in 3 ml. X——X, Anabaena; O——O, Anacystis. Time refers to time in cold. Control rates, 190 and 165 µM ATP/mg.chl./hr. for Anabaena and Anacystis respectively. For further experimental details see Methods.

Table 18. Characteristics of noncyclic photophosphorylation. Chlorophyll, 18.3 μg; $K_3Fe(CN)_6$, 1.4 μmoles; T1 refers to time the extract was held at room temperature before experiment was performed (45 min.). For further experimental details see Methods.

Conditions	Rate of PSP (μM/mg. chl./hr.)
Complete	155
T1	164
CMU	7.8

Table 19. Effect of cold on P/2e ratio, Conditions as in Figure 32. Rates were calculated after 6 min. illumination.

Rate	Control	Cold shock (30 min.)
μM ATP/mg.chl./hr.	190	66
μM $K_3Fe(CN)_6$/mg.chl./hr.	459	464
P/2e	0.83	0.28

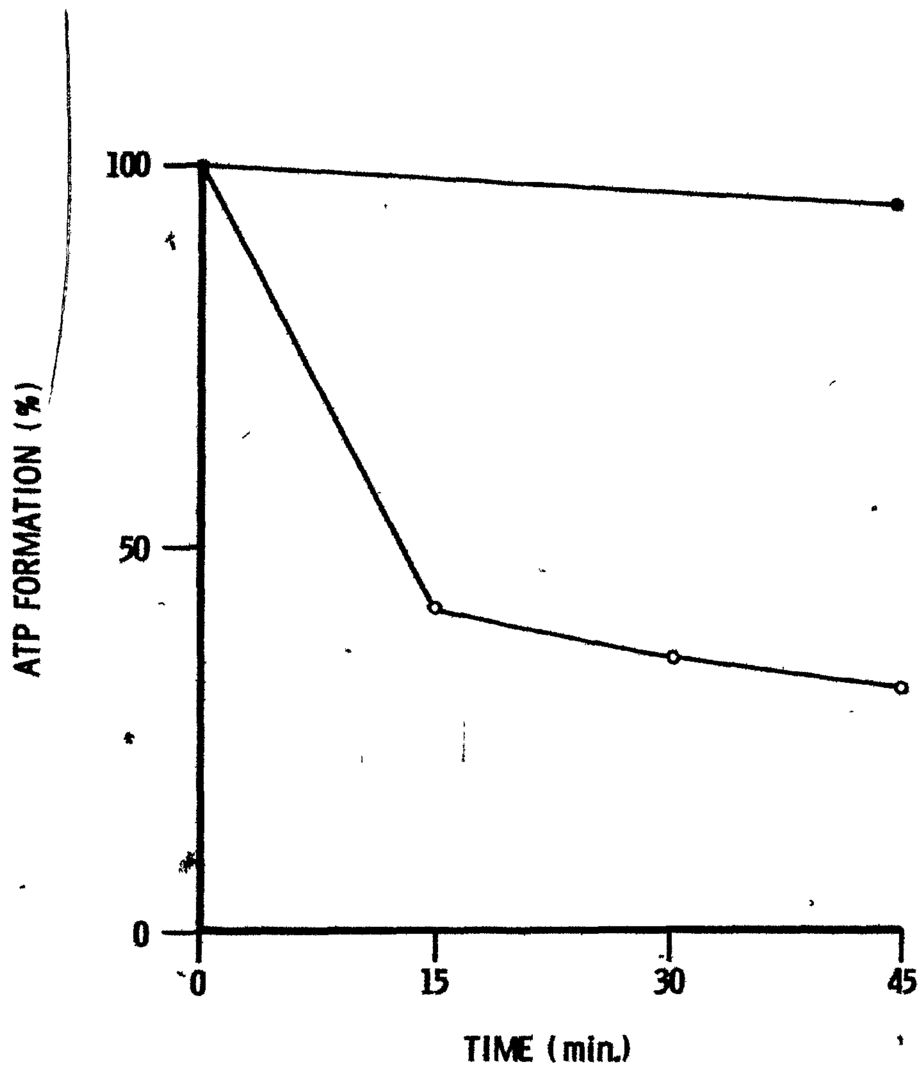


Figure 32. Effect of cold shock on noncyclic PSP. Ferricyanide, 0.7 mM; chlorophyll, 34.5 μg in 3.7 ml. O—O, cold shock (0°); ●—●, control, room temperature. Control rate, 190 μM ATP/mg.chl./hr. Time refers to time in cold. For further experimental details see Methods.

data in the literature for the organism, and coupling ratios (Table 19) were high. The effect of cold was to lower the P/2e ratio, (Table 19); 30 min. cold shock resulted in a P/2e ratio of about 0.3 (which was similar to the previous best P/2e ratios reported by Gerhardt and Santo, 1966 using cell-free extracts prepared by lyophilization and lysozyme digestion).

The results suggested that low P/2e ratios reported in the literature may be due to exposing cells, protoplasts or extracts to the cold during some stage of preparation.

(3) Effect of cold shock on PEP-carboxylase activity. In Part II B the characteristics of the carboxylating enzyme are reported; their sensitivity to cold shock was also investigated. On subjecting the system to cold shock, it was found that it was sensitive, losing up to 50% of its activity after 15 min. at 0° (Figure 33). A similar system from Anabaena variabilis showed no cold sensitivity. Since the effect could not explain the loss in photosynthetic capacity of Anacystis nidulans, the system was not studied further. It was interesting to note that the other CO_2-fixing enzyme (RDP carboxylase) was not cold sensitive.

(4) Summary of cell-free extract studies. Cell-free extract studies showed that cold shock had a marked effect on photophosphorylation. The general pattern of the decline in photophosphorylation was very similar to those obtained for photosynthesis with whole cells. Photophosphorylation of Anabaena variabilis (whose photosynthesis was not sensitive

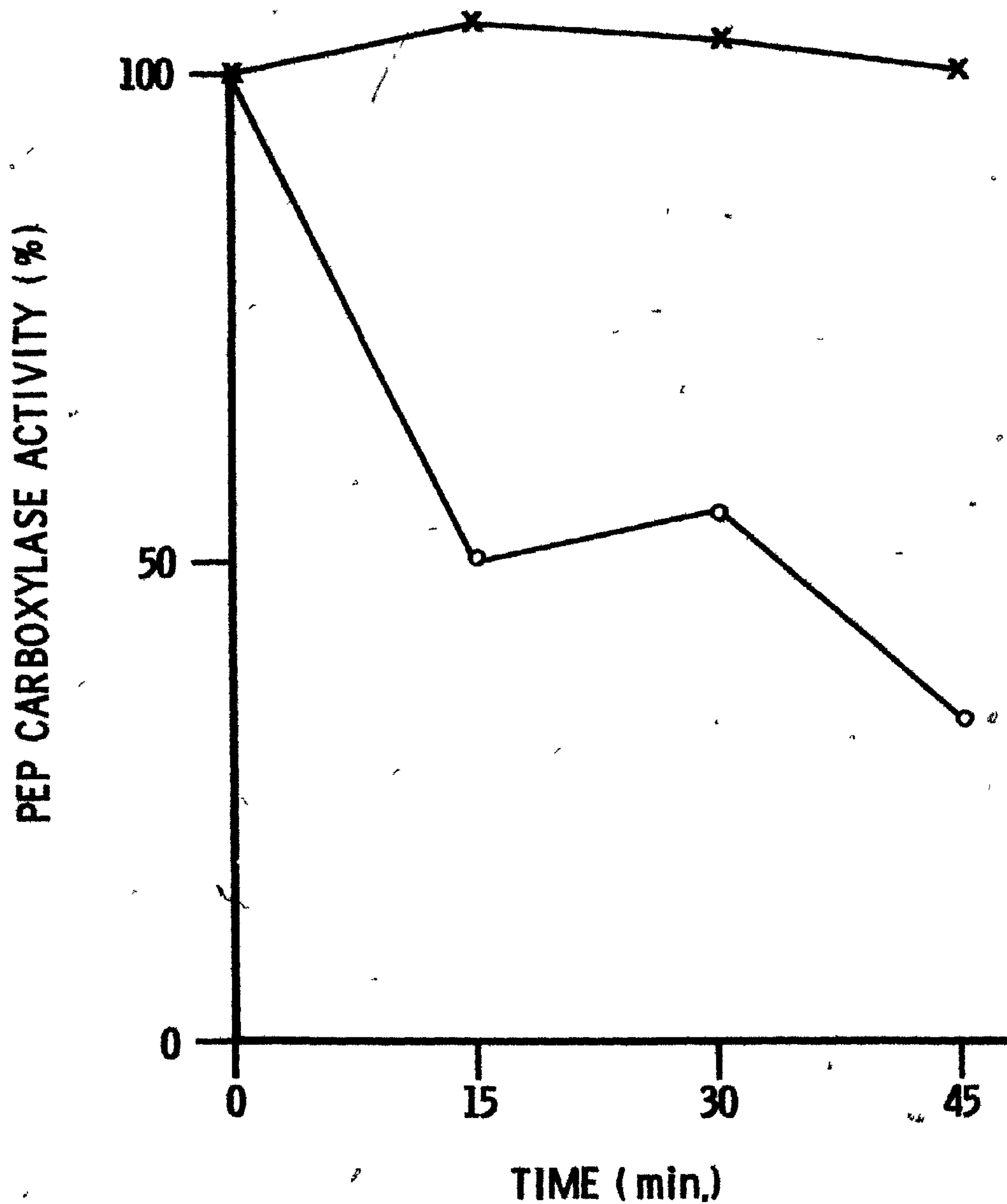
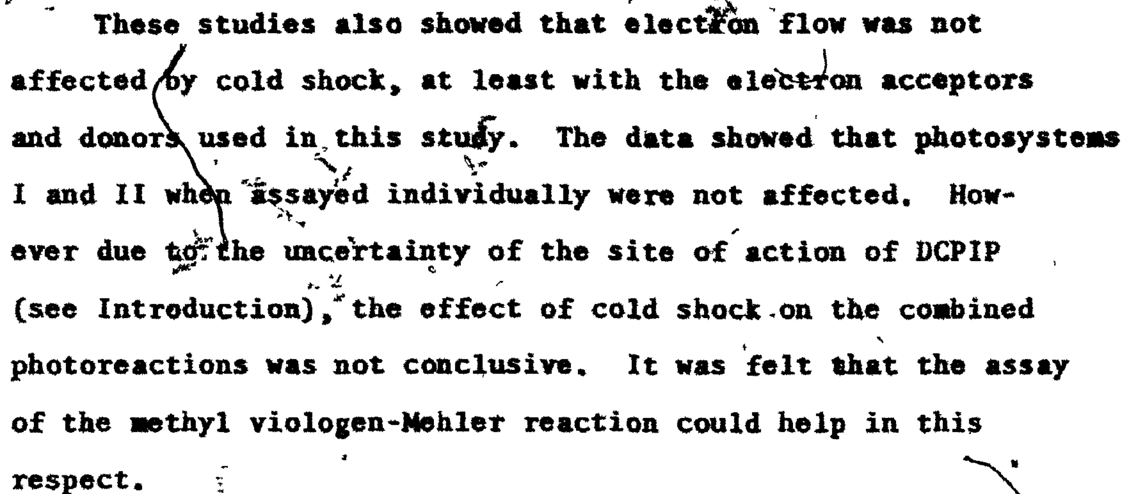


Figure 33. Effect of cold shock on PEP carboxylase activity. chl.*: Anabaena, 22 µg; Anacystis, 41 µg; final pH, 8.1. Control rates: Anacystis, 0.17 µM.C/mg.chl.*/min.; Anabaena, 0.49 µM.C/mg.chl.*/min.; X—X, Anabaena; o—o, Anacystis; time refers to time in cold. For further experimental details see Methods.

to cold shock) was relatively resistant to cold shock.



These studies also showed that electron flow was not affected by cold shock, at least with the electron acceptors and donors used in this study. The data showed that photosystems I and II when assayed individually were not affected. However due to the uncertainty of the site of action of DCPIP (see Introduction), the effect of cold shock on the combined photoreactions was not conclusive. It was felt that the assay of the methyl viologen-Mehler reaction could help in this respect.

(C) <u>Effect of cold shock on the methyl viologen-Mehler reaction.</u>

The reaction involved the reduction of methyl viologen by photosystem I with the concomitant evolution of half a molecule of oxygen per pair of electrons transferred. The reduced methyl viologen is then oxidized giving H_2O_2. The net result is the uptake of a molecule of oxygen per pair of electrons transferred. As a result the overall process involved the uptake of oxygen at half the rate of oxygen evolution in photosynthesis, provided catalase activity was absent. In this experiment, KCN was used to inhibit catalase (Chua, 1971) and oxygen uptake was followed manometrically (Methods, pg. 57).

This method was used to study the effect of cold shock on electron flow in whole cells. It was useful as it allowed the study of the combined photosystems, a study that was not possible in cell-free extracts.

The overall characteristics of the system are shown in Table 20. The results showed that methyl viologen, KCN and light were required for gas uptake. The results also showed that cold shock did not inhibit the Mehler reaction.

The high level of gas evolution in the dark for the 'complete' cold shock sample (Table 20 A) was not observed in other experiments. The rate of oxygen uptake was in the range of 120 µmoles/mg.chl./hr. which was comparable to rates calculated from whole cell photosynthesis.

Table 20. Characteristics of the methyl viologen-Mehler reaction. Time of cold shock, 15 min.; Klett cell units/vessel, 2000; control cells were held at room temperature. (For further experimental details, see Methods).

Expt.	Cold shock	Reaction Mixture	Oxygen uptake (µl./10 min.) Light	Dark
A	-	complete	26	0
	-	KCN omitted	3	0
	+	complete	30	-8
	+	KCN omitted	5	0.5
B	-	complete	32	0.5
	-	M.V. omitted	4.5	0
	-	dark	5	-1
	+	complete	32	-0.5

Table 21. The effect of cold shock on the methyl viologen Mehler reaction at low concentrations of methyl viologen. Conditions same as Table 20, but time of cold shock, 30 min. In all samples, dark gas uptake, 2-3 μl/10 min.

Conc. of M.V. (mM)	Oxygen uptake (μl./10 min.)	
	Control	Cold shock
0.2	27.4	35.0
0.4	30.8	36.5
0.6	27.8	32.2

The next table shows the effect of cold shock on the Mehler reaction at lower concentrations of methyl viologen. The results (Table 21) showed again that cold shock did not cause a loss in gas uptake, in fact, the cold shock samples had higher rates of gas uptake.

The results of these experiments supported previous conclusions that cold shock had no effect on electron flow. The method had theoretical complications and therefore firm conclusions were difficult to make, but the experiment had value since the expected rate of gas uptake was observed; further the observations were in harmony with previous experiments.

PART II

Carbon dioxide incorporating reactions of Anacystis nidulans

(A) Whole cell studies.

The experiments were designed to show the various paths by which $^{14}CO_2$ was incorporated. The experimental procedure essentially consisted of the addition of high specific activity $^{14}CO_2$ (as Na_2CO_3) to cell suspensions and the separation and identification of the labelled products formed. Incorporation of $^{14}CO_2$ in glucose and phosphate esters were assumed to be equivalent to fixation by the Calvin cycle and the labelling in aspartate and glutamate were assumed to be due to possible alternate routes, subject to the position of label in the two amino acids.

It should be noted, however, that the experiments were not done under the steady state and therefore the relative physiological significance of the different routes of CO_2 incorporation cannot be evaluated here.

The average rates of photosynthesis were equivalent to about 1000 µl. CO_2 fixed/mg. chl./hr. (about equal to that observed with standard growth conditions) and at the end of the experiment approximately 15% of the added CO_2 was utilized.

Data in this section is presented in tables rather than figures, because only the labelling in the initial sample was of critical interest.

Identities assigned to compounds were based only on chromatography. Occasions where authentic standards were not

used are indicated in the tables.

(1) Distribution of isotope among compounds in the ethanol-soluble fraction. A preliminary experiment designed to evaluate pool-size of intermediates was first done. This involved the incorporation of $^{14}CO_2$ into cell suspensions over a period of 15 min. (Table 22). The experiment did not serve its purpose because even after that period of time, some of the intermediates (e.g. glutamic acid) had not attained complete labelling. This was deduced from knowledge gained previously in the study of the leakage of intermediates from the cell (Part I, (A), (5)). This indicated that glutamic acid had a very low turnover rate.

In the next experiment (which will be referred to hereafter as E-1) a cell suspension was mixed with $^{14}CO_2$ (as $Na_2{}^{14}CO_3$) in the dark (Methods, pg. 66, method A) following which the light was switched on. Results of the experiment showed (Table 23) that incorporation in the dark was mainly into glutamic and aspartic acids (Figure 34) while the phosphate esters contained very little label. After the light was switched on, the two amino acids were labelled faster and the labelling continued through all the samples taken. The phosphate esters were not labelled to a great extent in the first few seconds after the light was switched on (Figure 35) but became prominent after 21 sec. (Figure 36) and soon accounted for the bulk of the CO_2 fixed. At the same time, the label in the ethanol-insoluble fraction increased considerably. (This was identified as mainly polymer glucose, see next section).

Table 22. $^{14}CO_2$ fixation for 15 min. Na_2CO_3, 170 µmoles (0.5 µCi). Other conditions as in Methods (method A, pg. 66). Radioactivity expressed per 50 µl aliquot. (Total volume 1 ml.). *identified from R_F only. † identified by co-chromatography with authentic standards.

Compound	Radioactivity c.p.m. x 10^{-2}
PGA†	26
Sugar phosphates*	171
PEP*	07
Triose P†	11
Aspartate†	40
Glutamate†	39
Alanine†	06
Citrate + Glycolate(?)	10
Fumarate†	16
Succinate†	05

Table 23. Distribution of radioactivity in B-1. Counts expressed per 50 µl aliquot. * identification by R_F only. † identified by co-chromatography with authentic standards. For details see Methods.

	Counts/10 min. 10^{-3}				
Compound	dark	8 sec.	21 sec.	36 sec.	54 sec.
PGA†	21	11.2	260	720	1420
Hexose* mono P.		16.0	180	938	2000
PEP*	6(?)	4.8	12	62	90
Triose P.†	-	3.2	16	34	62
Sugar di P.*	-	8.0	36	80	300
Pentose* mono P.	-	6.5	38	132	266
UDPG area*	-	3.2	20	72	260
Aspartate†	53	163	321	530	912
Glutamate†	14	41.5	51	111	128
Citrate†	-	3.0	16	25	84
Ethanol insoluble	-	13	57	280	1390

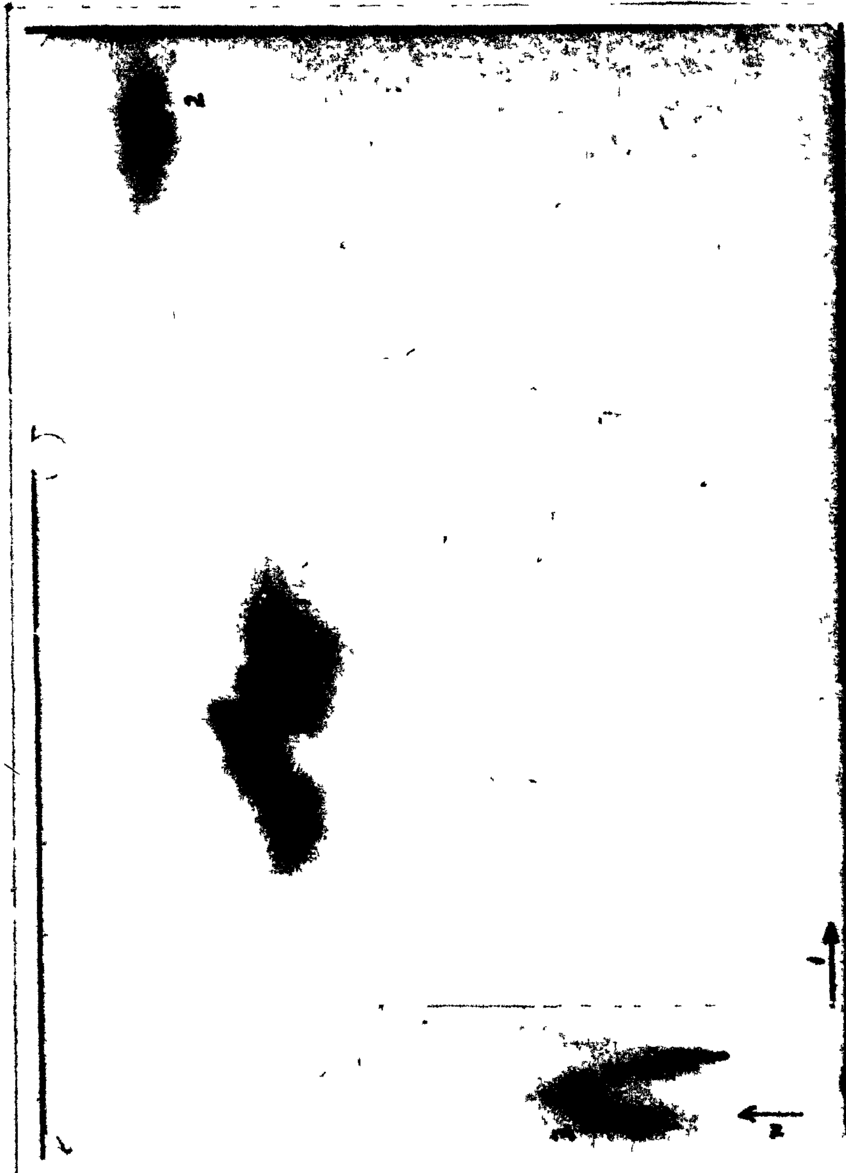


Figure 34. Autoradiogram of chromatogram, 80 sec. pre-illumination sample of E-1. 1, aspartate; 2, glutamate; 3, phosphate esters; 1⟶, phenol-water; 2⟶, BAW. For experimental details see Methods.

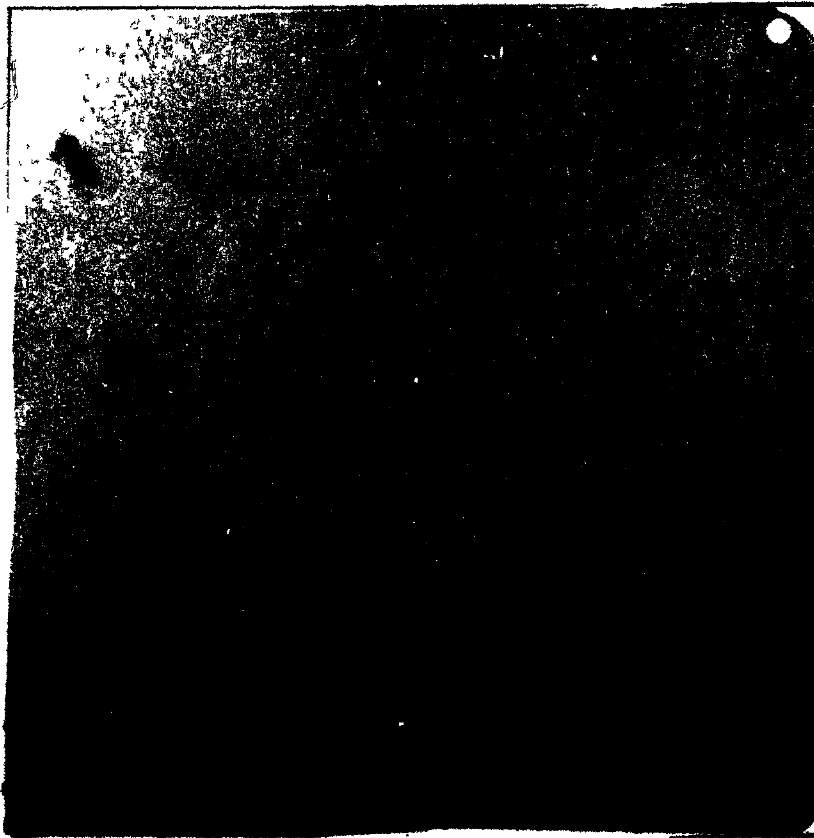


Figure 35. Autoradiogram of chromatogram, 8 sec. photosynthesis of E-1. 1, aspartate; 2, glutamate; 3, phosphate esters; 1⟶, phenol-water; 2⟶, BAW. For experimental details see Methods.

Figure 36. Autoradiogram of chromatogram, 21 sec. photosynthesis of E-1. 1, aspartate; 2, glutamate; 3, PGA; 4, hexose monophosphates; 5, sugar diphosphates; 6, PEP; 7, citrate; 8, triose P; 1⟶, phenol-water; 2⟶, BAW. For experimental details see Methods.

The experiment showed that labelled aspartic acid and glutamic acid could be formed without significant operation of the Calvin cycle. This type of initial labelling into aspartate and glutamate is called type II labelling.

In another experiment (which will be called E-2) a dark preincubation was avoided (Methods, pg. 66, method B) and radioactive carbonate was added to a preilluminated sample. The results of this experiment (Table 24) showed that although there was some lag in the labelling of the phosphate esters, the labelling in these compounds exceeded by far the labelling in aspartate, even in the earliest sample (Figure 37). This indicated that the Calvin cycle could operate without the labelling of aspartate. After 17 sec. the labelling of the total Calvin cycle intermediates was linear. The labelling in aspartate in the experiment was initially slow but ultimately reached the same level as E-1. In later samples a whole spectrum of intermediates was labelled (Figure 38). This type of labelling (initial Calvin cycle intermediate labelling) is called type I labelling.

One problem encountered in these experiments was the large quantity of radioactivity that remained at the origin. This was greater in the type I pattern experiments in which radioactivity at the origin was as much as 15% of the total isotope in the ethanol soluble fraction. On hydrolysis of this spot with 1 N HCl at 100° for 1 hr., followed by chromatography, a 'smear' of radioactivity was obtained. It was believed that this represented a mixture of cell wall poly-

Table 24. Distribution of radioactivity in E-2. Counts expressed in terms of a 50 µl aliquot. * identification by R_F only. † identified by co-chromatography with authentic standards. For details see Methods.

	Counts/10 min.x10^{-3}			
Compound	4 sec.	17 sec.	52 sec.	60 sec.
Hexose mono P.*+PGA†	270	1379	6074	6151
Pentose P.*	12	92	796	871
UDPG area*	2	14	316	270
PEP*	1	45	159	289
Aspartate†	14.5	76	960	1026
Glutamate†	2	8	197	203
Citrate†	-	13	221	296
Malate†	-	<1	40	50
Glycolate(?)*	-	13	148	248
Alanine†	-	<1	66	117
Ethanol insoluble	35	237	3605	4468

Figure 37. Autoradiogram of chromatogram, 4 sec. photosynthesis of E-2. 1, aspartate; 2, phosphate esters; 1⟶, phenol-water; 2⟶, BAW. For experimental details see Methods.

Figure 38. Autoradiogram of chromatogram, 60 sec. photosynthesis of E-2. 1, aspartate; 2, glutamate; 3, PGA+sugar phosphates; 4, PEP; 5, pentose monophosphates; 6, alanine; 7, UDPG area; 8, a,b, citrate; 9, malate; 10, fumarate; 11, unknown (glycolate?); 1⟶, phenol-water; 2⟶, BAW. For experimental details see Methods.

Table 25. Distribution of radioactivity in experiment done at two light intensities. Low light - 3,20 watt 'cool white' fluorescent lights. High light - 650 watt tungsten lamp. * identification by R_F only. † identification by co-chromatography. Counts expressed per 150 µl aliquot. For experimental details see Methods.

	Counts/10 min. 10^{-3}			
	low light		high light	
Compound	6 sec.	20 sec.	7 sec.	20 sec.
PGA†	10.5	61	18.2	291
Sugar phos.* (mono + di)	24.1	232	30.8	714
Triose P.*	1.0	5.7	1.5	49
PEP*	-	1.1	-	37
Glutamate†	-	0.6	-	7.5
Citrate†	-	-	-	7.5
Aspartate†	4.2	44	123.0	366
Malate†	-	5.7	-	13.5
Ethanol insoluble	2	33	1	190

Figure 39. Autoradiogram of chromatogram, 7 sec. of high light intensity. 1, aspartate; 2, PGA; 3, sugar phosphates; 1⟶, phenol-water; 2⟶, BAW. For experimental details see Methods.

saccarides, sugar diphosphates and degradation products. Due to possible degradation of key intermediates during isolation, the percent recovery of all these intermediates was determined. In general, the recovery of key intermediates was high (see Methods) and the data shown was corrected for this loss.

On repeating method B at two light intensities, a mixed pattern of labelling was observed for low light intensity, while a type II pattern was observed for high light intensity. The high light intensity seemed to affect the labelling in the Calvin cycle intermediates only after 20 sec. (Table 25, Figure 39).

Next it was decided to study the composition of the ethanol-insoluble fraction and determine the label in C_4 of aspartate and C_1 of glutamate.

(2) Components of the ethanol-insoluble fraction. On hydrolysis of the ethanol-insoluble fraction with HCl and chromatography (Methods, pg. 68), it was found (Table 26) that the bulk of the label in the later samples (60 sec.) was in glucose. Glucose has been shown by Kindel and Gibbs (1963) to be formed by the Calvin cycle.

However at shorter times an unknown spot (X) with low R_f in both solvents was the major radioactive compound. Its R_g (R_F with reference to glucose) was, in phenol-water, 0.57; and in BAW, 0.30. The nature of this compound(s) was not studied further. There was considerable activity in another unknown spot (Y) with R_g, 2.4 in phenol water, 3.5 in BAW (Figure 40). This spot was inadvertantly run off the paper in

Figure 40. Autoradiogram of chromatogram of hydrolyzed ethanol-insoluble fraction. 60 sec. photosynthesis of E-2. 1, glucose; 2, aspartate; 3, unknown X; 4, unknown Y; 1⟶, phenol-water; 2⟶, BAW. For experimental details see Methods.

Table 26. HCl hydrolysate of ethanol insoluble fraction from E-1 and E-2. For experimental details see Methods.

Experiment	Compound	C.P.M. x 10^{-2}			
		8 sec.	21 sec.	36 sec.	54 sec.
E-1	Glucose	1	3	132	834
	Aspartate	2	2	10	17
	Unknown X	2	9	34	64
		4 sec.	17 sec.	52 sec.	60 sec.
E-2	Glucose	-	48	2110	3620
	Aspartate	-	3	50	75
	X	21	55	260	300
	Y	-	13	320	480

Table 27. Effect of ninhydrin on authentic 1-^{14}C and 5-^{14}C labelled glutamate and a test sample (mixture of 36 and 54 sec. samples of E-1). For experimental details see Methods.

	Counts/5 min.				
	glutamate-1-^{14}C		glutamate-5-^{14}C		Unknown
	a	b	a	b	
Before spray	3362	1546	3584	2070	2135
After spray	88	45	3024	1707	113
Label remaining (%)	2.7	2.9	84	83	5.3

the hydrolysate of E-1. There was some label in aspartic acid. Several other low radioactive spots were present. The 60 sec. sample from E-2 is shown in Figure 40. In E-1 the hydrolysate of the 54 sec. sample was passed through an amino acid separation column. This resulted in a major peak in the 15 min. fraction which contained 60-70% of the total activity. This peak was probably glucose and the only radioactive amino acid was aspartic acid (41 min.). A small peak at 23 min. was probably one of the unknown spots.

(3) Estimation of labelling in glutamate-1-C. The radioactive isotope in C-1 of glutamate was determined by decarboxylation with ninhydrin (Methods, pg. 72). Controls used were glutamate-1-^{14}C and glutamate-5-^{14}C. Results showed (Table 27) that glutamate-5-^{14}C retained over 80% of its radioactivity with this treatment while glutamate-1-^{14}C retained less than 3% of its radioactivity. The unknown sample, (mixture of 36 and 54 sec. sample of E-1) lost most of its radioactivity on treatment with ninhydrin. It was estimated that the percent of ^{14}C in C-1 of glutamate was at least 95. (This was consistent with glutamic acid arising from either the reductive carboxylation of succinyl CoA or via C-4 labelled oxaloacetic acid and the citric acid cycle).

(4) Estimation of labelling in aspartate-4-C. The procedure involved the conversion of aspartic acid of known specific activity into pyruvate (resulting in the loss of C-4 of aspartate). The specific activity of pyruvate (c.p.m./μM)

Table 28. Percent radioactivity in C-4 of aspartate. 95% confidence limits with respect to counting of radioactivity are shown.

Experiment	Time (sec.)	Radioactivity in C-4 of aspartate (%)
E-1	21	>99
	36	96 ± 0.8
	54	91 ± 0.9
E-2	17	90 ± 2.0
	52	82 ± 1.2
	60	80 ± 1.2

was then determined. Results showed that the pyruvate isolated (C-1, 2, 3 of aspartate) had very little label, i.e. most of the label was lost (as CO_2) and therefore, was in the C-4 of aspartate. This was consitent with aspartate arising via the carboxylation of PEP.

(5) Determination of label in oxaloacetate. The reason why this experiment was done was that if a "Hatch-Slack" transcarboxylation" occurred (see Introduction) in *Anacystis nidulans*, it was possible that oxaloacetate would be more highly labelled than aspartate. Hence radioactivity in oxaloacetate (isolated as a phenylhydrazone) was determined after short periods of exposure to radioactive $^{14}CO_2$.

Results (Table 29) showed that very little labelled oxaloacetate was formed. Most of the radioactivity was in the phosphate esters. Low label in oxaloacetate was also observed by Hatch et al. (1967) using several plants having a C-4 dicarboxylic acid pathway. It was noticed that although the counts in oxaloacetate was low they appeared to increase slightly with time.

A repeat experiment gave similar results.

(6) Summary of CO_2 fixation in whole cells. These experiments suggested the existence of independent pathways of CO_2 fixation into sugars and at least aspartate (and possibly glutamate). This was supported by kinetic studies as well as the position of label in aspartate and glutamate.

Table 29. Label in oxaloacetate compared to total incorporation and label in aspartate. All counts expressed in terms of a 50 µl aliquot. OAA was determined in a 250 µl aliquot counted for 10 min. For experimental details see Methods.

	C.P.M. x 10^{-2}		
Time (sec.)	Total	Asp.	OAA
7	70	2	0.12
16	178	7	0.15
30	603	12	0.35

(B) Cell-free extract studies.

It was decided to examine cell-free extracts to determine if the carboxylating enzymes PEP carboxylase and RDP carboxylase could be detected, and if so, whether the enzymes are present in sufficient quantities to support CO_2 fixation in whole cells. The study of PEP carboxylase was of special interest because (1) the low label in oxaloacetate and (2) because OAA could be involved in a hypothetical transcarboxylation reaction giving CO_2 to the Calvin cycle (Hatch and Slack, 1970; see Introduction). The low label in oxaloacetate did not rule out such a possibility, because the reaction might occur through a derivative of oxaloacetate (Pan and Waygood, 1971) or involve enzyme-bound oxaloacetate. Hence it was also decided to determine if CO_2 incorporation by RDP could be stimulated by PEP, because if such was the case it would be strong evidence for a transcarboxylation reaction.

(1) Ribulose-1-5 diphosphate (RDP) carboxylase. The enzyme was assayed by following the incorporation of $^{14}CO_2$ into fixed C (Wishnick and Lane, 1971; for details, see Methods, pg. 78). A preliminary study (two dimensional chromatography and autoradiography of reaction products with standard radioactive PGA) established that most of the radioactivity fixed was in PGA. It was next decided to study the kinetic parameters of the enzyme. All Michaelis constants determined are termed "Apparent" since all studies were conducted with cell-free extracts which were not purified. Results showed that the

"apparent" activity of the enzyme was in the range of 0.8-1.1 µM ^{14}C fixed/mg.chl./min. (1000-1500 µl CO_2/mg.chl./hr.) which was sufficient for the rates of growth observed in our cultures, but was only 15-25% of the maximum rate of photosynthesis observed in manometry.

At an early stage it was noticed that protoplasts washed with phosphate buffer showed lower enzymic activity. This was probably due to the known inhibitory action of inorganic phosphate (Wishnick and Lane, 1971). All subsequent experiments were conducted using tris buffer as the washing reagent. Preliminary studies also showed that the system did not lose enzymic activity over a period of one hour at room temperature.

Studies with this system showed (Table 30, A, B) that the enzyme was subject to substrate inhibition.

Preliminary studies also showed that the activity of the enzyme varied very little (less than 10%) from pH 7.9-8.15.

The "apparent" Km for HCO_3^- was determined next. Results (Figure 41) on a Lineweaver-Burk plot showed a concentration for half maximal activity, of 11 mM. These kinetic characteristics were very similar to those obtained by others, for plant enzymes (see Introduction). No co-operative effects were observed at these levels of CO_2 concentration.

The "apparent" Km for RDP was also determined from a Lineweaver-Burk plot (Figure 42). The data when fitted into a hyperbola (by computer) gave an "apparent" Km of 0.063 mM which is somewhat lower than what has been previously reported (Wishnick and Lane, 1971) for the spinach enzyme.

Table 30. Substrate inhibition of RDP carboxylase. Reaction mixtures contained, A: Na_2CO_3, 16 mM; $MgCl_2$, 7.5 mM; chl.*, 36 μg; final pH, 8.1 in 1.6 ml. B: same as A except Na_2CO_3, 25 mM; chl.*, 40 μg. Inorganic phosphate in washing medium in A only. For further experimental details see Methods.

Expt.	Conc. of RDP (mM)	Initial velocity (μM.C/mg. chl.*/min.)
A	0	<0.01
	0.24	0.38
	0.48	0.39
	1.50	0.20
B	0.28	0.76
	0.42	0.80
	0.56	0.73
	0.84	0.68

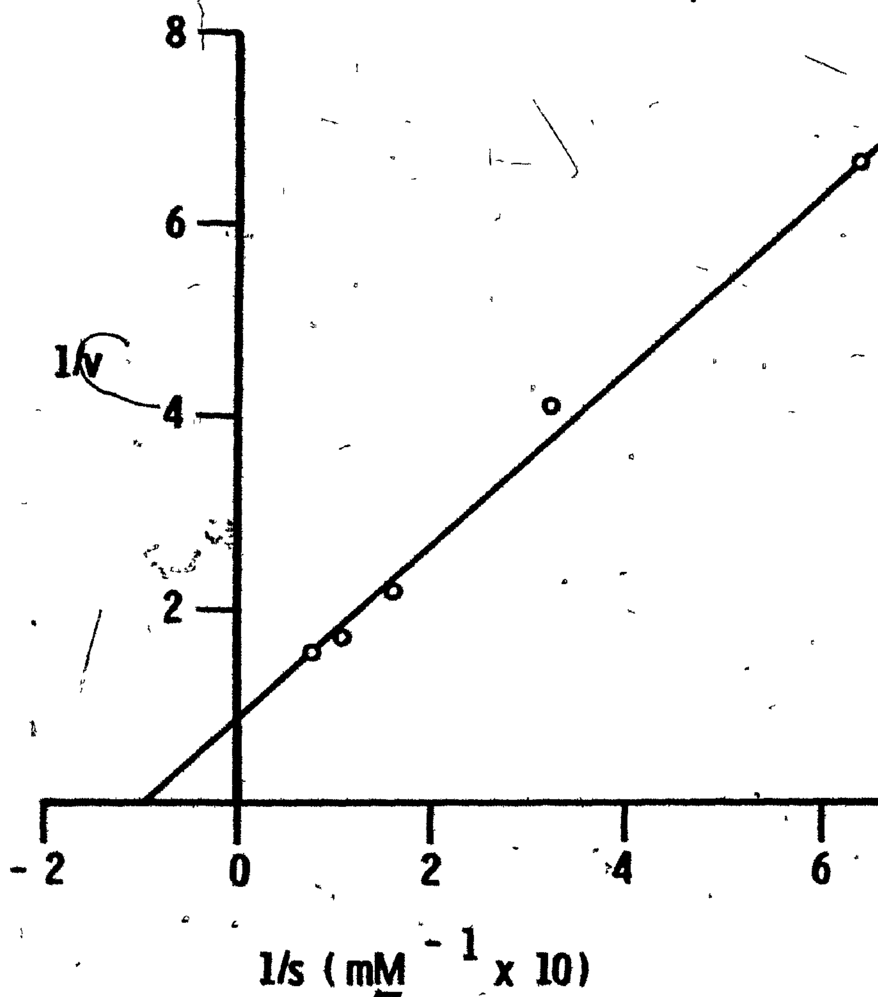


Figure 41. Lineweaver-Burk plot for HCO_3^-. Enzyme, RDP carboxylase. RDP, 0.5 mM; $MgCl_2$, 10 mM; extract, 64 µg. chl.*; Tris-HCl, 0.2 M; final pH, 8.1; in 1.6 ml. V (initial velocity) expressed as µM. C fixed/mg.chl.*/min. For further experimental details see Methods.

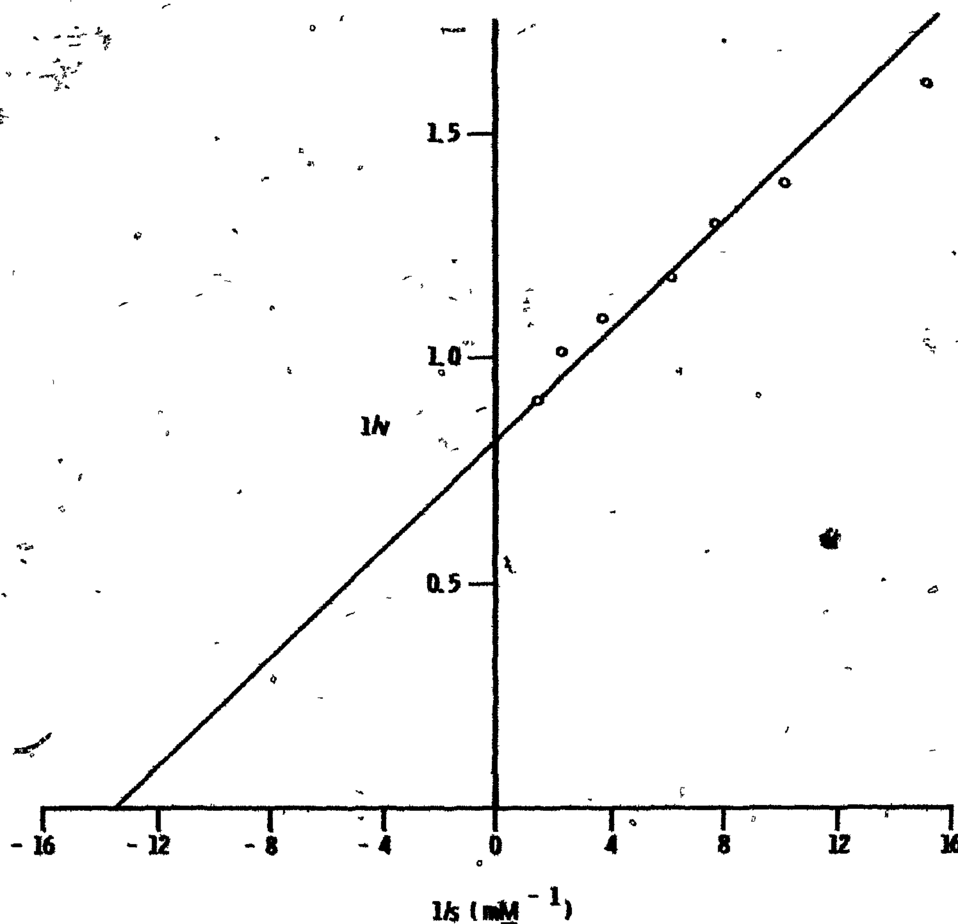


Figure 42. Lineweaver-Burk plot for RDP Enzyme, RDP carboxylase; HCO_3^-, 44 mM; $MgCl_2$, 7.5 mM; extract, 44 µg.chl.; Tris-HCl; 0.2 M; pH, 8.05; in 1.6 ml. V (initial velocity) is expressed as µM C fixed/mg.chl.*/min. For further experimental details see Methods.

Table 31. Location of RDP carboxylase activity. Conditions as in Table 30B except RDP, 0.66 mM; chl., 50 μg. For experimental details see Methods.

Fraction	Enzyme Activity (μM.C fixed/mg.chl.*/min.)
Complete	0.86
Supernatant	0.75
Chlorophyll particles	0.14

After one wash only 16% of the activity remained with the chlorophyll-containing fragments (Table 31). This showed that the enzyme was soluble.

An attempt to determine if the enzyme was light-stimulated, showed that this was not the case. There were, however, possible reasons why the experiment was unsuccessful. (1) The light used (see pg. 78) may not have been intense enough because the extract was highly coloured. (2) Mechanisms of light activation are believed to occur which may not be possible in cell-free extracts (Introduction pg. 29).

(2) Phosphoenolpyruvate (PEP) carboxylase. The enzyme was studied by following the incorporation of $^{14}CO_2$ into fixed carbon by carboxylation of PEP and conversion of the oxaloacetate formed into aspartate by transamination (Methods, pg. 81). A preliminary study (two dimensional chromatography and autoradiography with an authentic standard) showed that nearly all the radioactivity isolated (>95%) was in aspartate.

Characteristics of the enzyme assay were studied next and this showed (Figure 43) that the assay required PEP, glutamate, and glutamic-oxaloacetic transaminase for maximum activity.

Preliminary studies also showed that 2 mM PEP was sufficient to give linear reaction rates over the reaction time with 60 μg chl. These studies also showed that 5 units of transaminase and 3.2 mM glutamate were saturating under these reaction conditions.

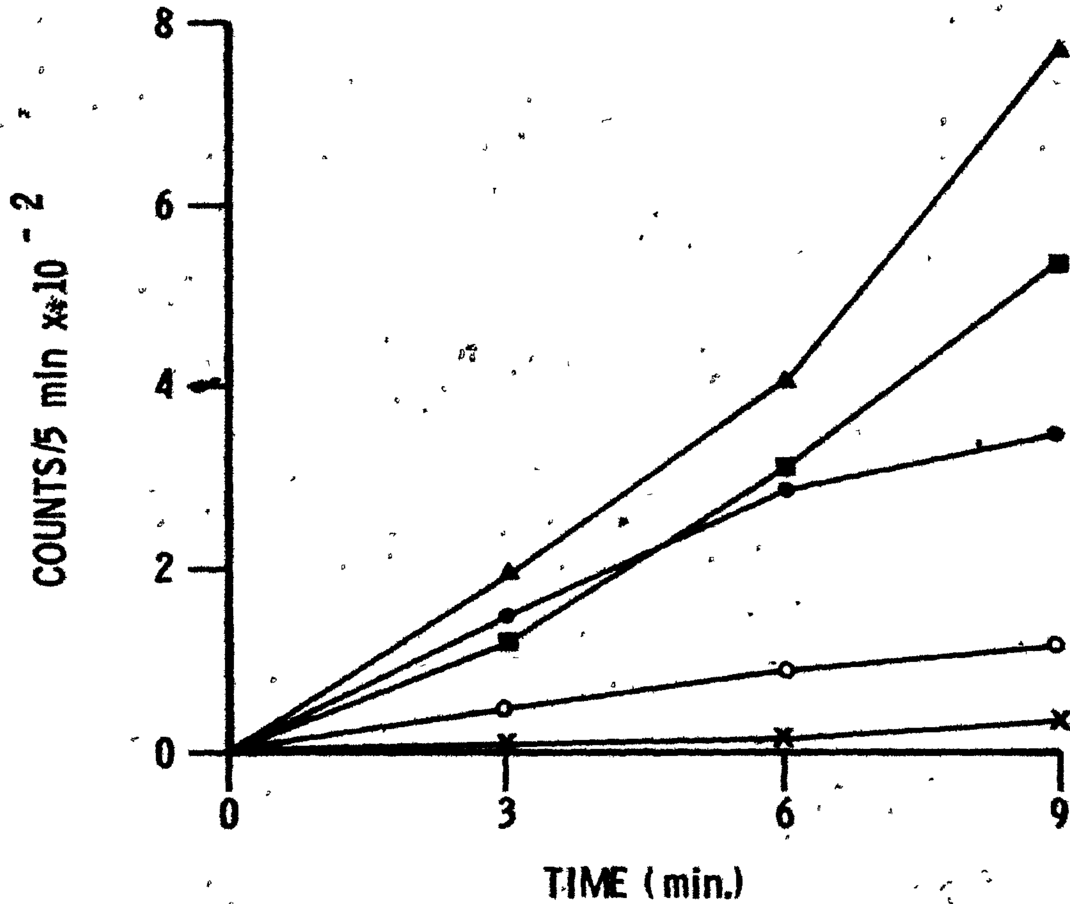


Figure 43. Characteristics of the PEP carboxylase assay system. Na_2CO_3, 6 mM; PEP, 1.2 mM; Tris-HCl, 0.1 M; glutamic acid, 3.2 mM; glutamic-oxaloacetic transaminase, 17.5 units; extract, 42 μg chl.*; pH, 8.1; X——X, PEP, glutamate and transaminase omitted; O——O, glutamate and transaminase omitted; ●——●, glutamate omitted; ■——■, transaminase omitted; ▲——▲, complete reaction mixture. For further experimental details see Methods.

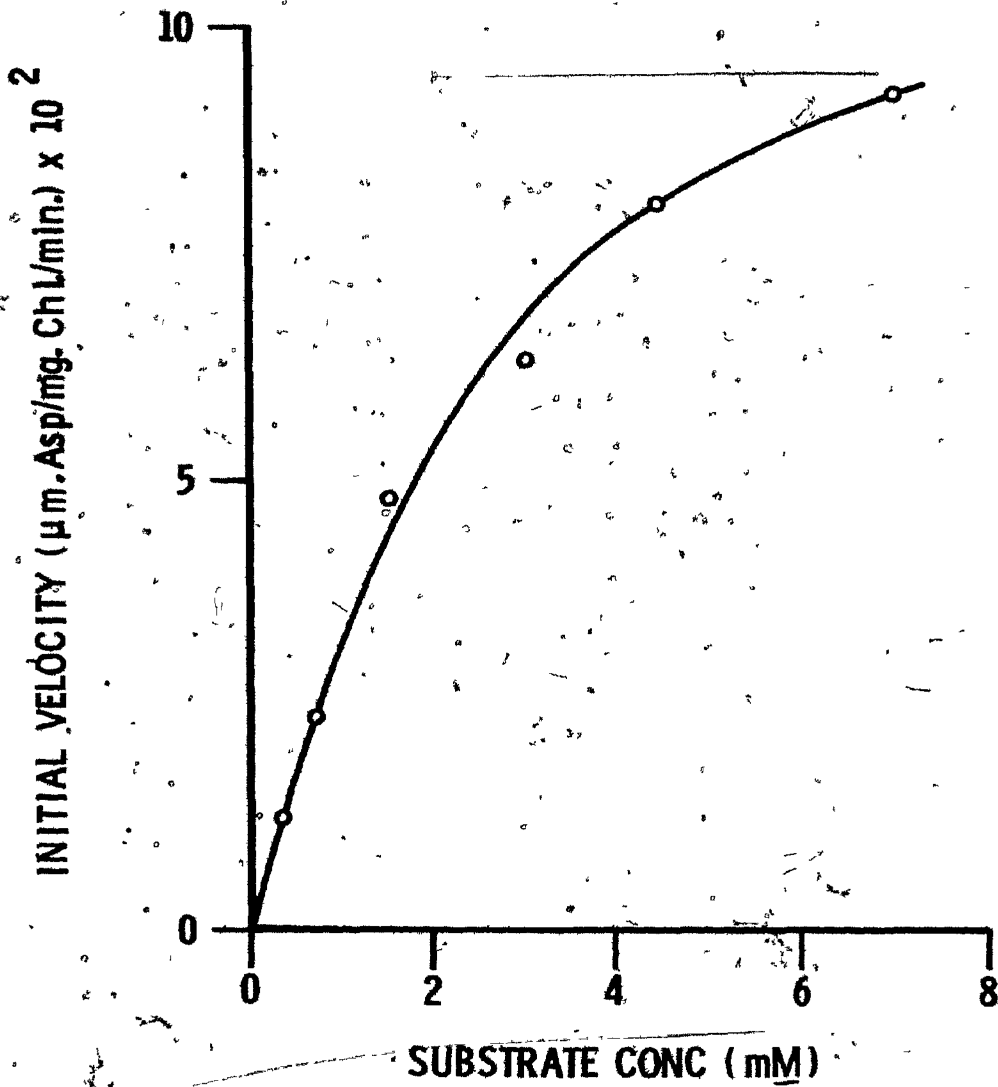


Figure 44. Substrate curve for HCO_3^-. Enzyme, PEP carboxylase; conditions as in Figure 45.

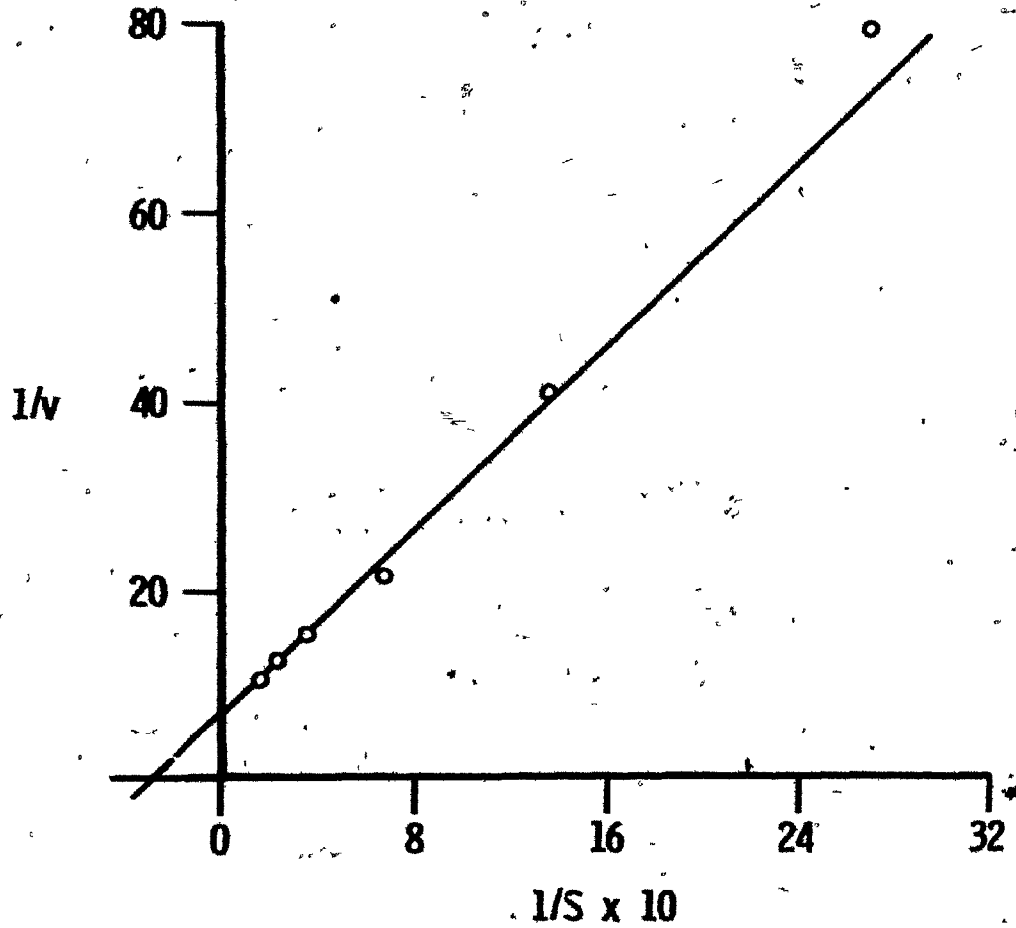


Figure 45. Lineweaver-Burk plot for HCO_3^-. Enzyme, PEP carboxylase; PEP, 3.4 mM; extract, 39 μg chl.*; pH, 8.0 in 1.6 ml. V (initial velocity) expressed as μM. C fixed (aspartate)/mg.chl.*/min. S expressed as mM. For further experimental details see Methods.

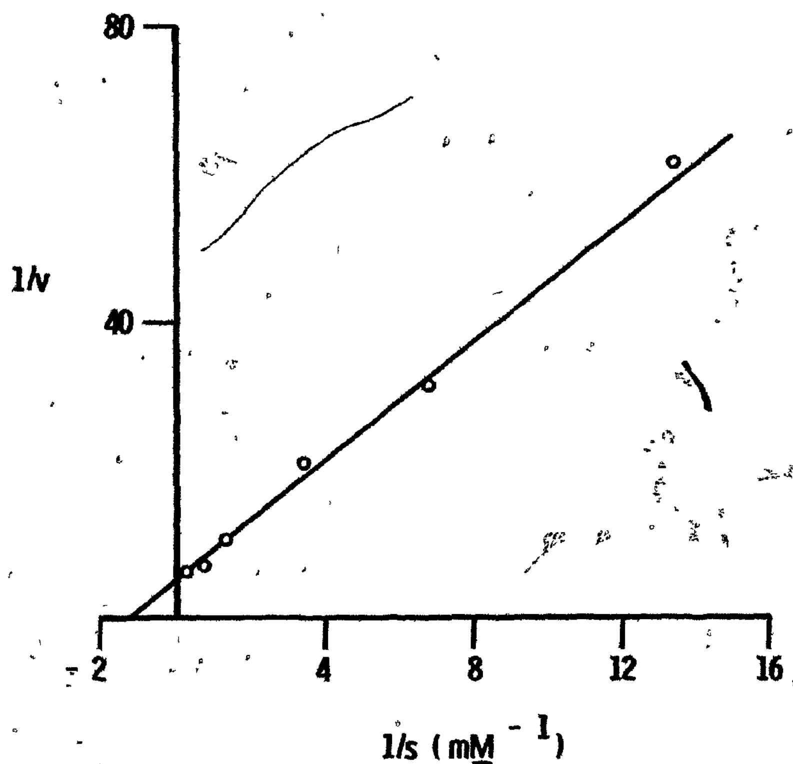


Figure 46. Lineweaver-Burk plot for PEP. Enzyme, PEP carboxylase; Na_2CO_3, 12.5 mM; extract, 55 µg. chl.*; pH, 8.1, in 1.6 ml. V(initial velocity) expressed as µM. C fixed (aspartate)/mg.chl.*/min. For further experimental details see Methods.

Table 32. Location of PEP carboxylase activity. The reaction mixture (1.6ml.) contained Na_2CO_3, 6.4 mM; glutamic acid, 6.4 mM; glutamic-oxaloacetic transaminase, 10 units; PEP, 1.2 mM; chl.*, 58 µg. Other conditions as in Methods (pg. 81).

Fraction	Enzyme Activity (µM C fixed/mg.chl.*/min.)
Complete	0.050
Supernatant	0.050
Chlorophyll particles	0.004

The activity of this enzyme varied from extract to extract and was in the range of 0.1-0.5 μM C fixed/mg. chl.*/min. These rates were sufficient to account for the asparate formation shown in whole cell studies.

A substrate curve for HCO_3^- concentration showed hyperbolic kinetics (Figure 44) and a Lineweaver-Burk plot showed (Figure 45) an "apparent" Km of 3.6 mM. This was higher than that of plant enzymes e.g. in maize, Waygood et al. (1969) observed a Km of 0.25 mM.

A Lineweaver-Burk plot for PEP concentration (Figure 46) gave an "apparent" Km of 0.7 mM which was similar to that of plant enzymes but much lower than for bacterial enzymes, (Maeba and Sanwal, 1969).

The enzyme, like RDP carboxylase, was soluble and in this case very little enzyme activity remained attached to the particles after one wash (Table 32). In fact, the activity (.004 μM/mg.chl.*/min.) was very close to the blank value obtained by mixing $Na_2{}^{14}CO_3$ with the extract.

The low activity in the experiment was probably due to the sub-optimal concentration of PEP and bicarbonate used. On comparison with the data on RDP carboxylase, it seemed that PEP carboxylase was less firmly bound to the lamellae (if bound at all).

(3) <u>Assay for a PEP-stimulated transcarboxylation reaction.</u> The assay medium was that of RDP carboxylase but a lower concentration of HCO_3^- (3 mM) was used in order to minimize direct carboxylation of RDP to form PGA. The rationale behind

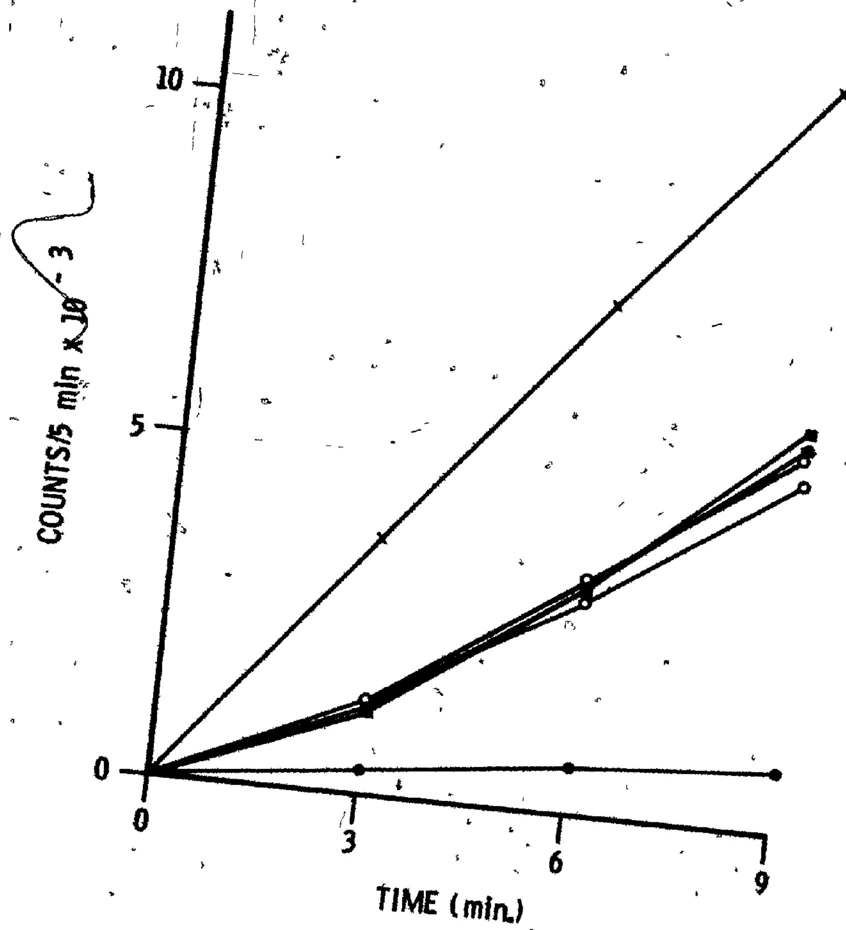


Figure 47. Test for oxaloacetate-RDP transcarboxylase activity. General procedure that of RDP carboxylase assay but HCO_3^-, 3 mM; PEP, 2.8 mM; RDP, 0.4 mM; extract, 59 µg. chl.*; pH, 8.05, in 1.6 ml. ●—●, PEP; ■—■, RDP, X—X, PEP+transaminase system; O—O, RDP and PEP.

Table 33. Analysis of products of carboxylation reaction mixtures. Conditions as for Figure 47.

Reaction mixture	Products Counts/10 min. x 10^{-3}		
	Aspartate	PGA	R_F - 0.45
PEP and transaminating system	118.0	0.5	<0.1
PEP	1.4	0.8	1.0
RDP	0.7	56.1	0.4
RDP + PEP	3.7	46.6	1.5

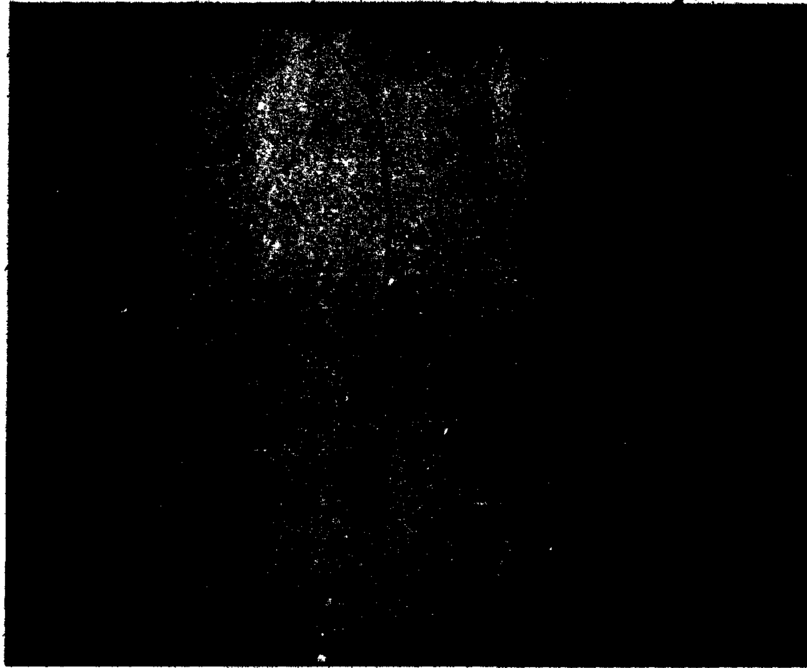


Figure 48. Autoradiogram of chromatogram of Table 33. 1, PEP and RDP; 2, PGA standard; 3, PEP and transaminating system; 4, RDP; 5, aspartate standard; 6, PEP. For experimental details see Figure 47.

the experiment was to determine if, at low concentrations, PEP carboxylase could stimulate incorporation of $^{14}CO_2$ into PGA., Such a situation would be expected if oxaloacetate (or an intermediate of the PEP carboxylation reaction) acted as a CO_2 donor to RDP carboxylase. However, results showed (Figure 47) that even though the extract contained PEP carboxylase activity there was no stimulation of CO_2 incorporation, showing that such a transcarboxylation activity was absent in these extracts. The products of the reaction were chromatographed, autoradiographed, and counted on a scintillation counter. (Table 33, Figure 48). This further confirmed that PEP did not stimulate the formation of PGA.

(4) Assay for the reductive carboxylation of succinic acid. The assay was modelled on that of Buchanan et al. (1967). The reason for the experiment was to find out if the enzyme for converting succinyl-CoA into α-ketoglutarate (α-ketoglutarate synthetase) was present in *Anacystis nidulans*.

The assay system was provided with a source of reducing power, substrate and co-factors and a method of trapping any α-ketoglutarate formed.

Reducing power was generated in the form of reduced ferridoxin (Fd) by illuminating lamellae fragments of *Anacystis nidulans*. DCPIP-ascorbate was used as the electron donor system and Fd was used as the acceptor. CMU was present and a helium atmosphere was maintained to prevent reoxidation of reduced Fd. NADPH was also provided as an alternate source of reducing power.

The reaction mixture was also provided with substrates for the synthesis of succinyl-CoA, i.e. succinic acid, coenzyme A and ATP. It was reasoned that if the enzyme was present succinyl-CoA would be reductively carboxylated with $^{14}CO_2$ in the presence of reduced ferridoxin and thiamine pyrophosphate to form radioactive α-ketoglutaric acid. The latter could then be trapped as glutamic acid using glutamic-oxaloacetic transaminase and aspartic acid. Reaction times were 30 and 60 min.

Results, however, showed that although ^{14}C was incorporated, the bulk of the radioactivity was in the phosphate esters. The total activity was the same in the presence and absence of succinic acid. After one dimensional chromatography, the radioactive glutamate band was cut and the radioactivity in it was estimated. The activity was the same in the presence and absence of succinic acid.

(5) Summary of cell-free extract studies on CO_2 fixation. These studies showed that (a) sufficient PEP carboxylase was present to account for the formation of aspartate, at rates observed in whole cells, (b) extracts had enough RDP carboxylase activity to account for 15-25% whole cell rates of photosynthesis. No evidence could be obtained for either (c) the reductive carboxylation of succinic acid or [d] the presence of a PEP-stimulated oxaloacetate-RDP transcarboxylation.

DISCUSSION

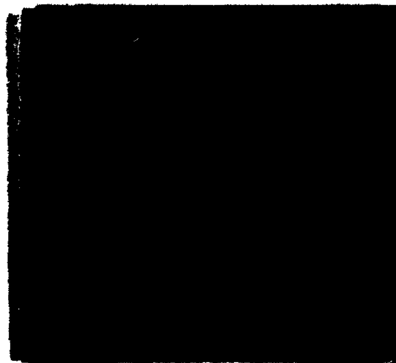
PART I

Effect of cold shock on *Anacystis nidulans*

The main question asked in this study was, Why does photosynthesis decline after cold shock?

A preliminary study confirmed that cold shock had a marked effect on O_2 evolution, 15 min. cold shock caused a decline to about 35% control rates. Longer periods of cold shock (1-2 hr.) caused a further slow decline to 15-20%. The next question asked was, Is oxygen evolution by cold-shocked cells a true reflection of their photosynthetic capacity? This was important since gas evolution may not have been a true reflection of CO_2 fixation because: (1) the observed rate of oxygen evolution might have been lower than CO_2 fixation due to a contribution of a Mehler type reaction (autooxidation of a reduced intermediate to form H_2O_2) in cold-shocked cells, (2) the observed rate may have been high due to O_2 evolution being coupled to the reduction of some unknown compound(s). However, the results showed that CO_2 fixation paralleled oxygen evolution (except at low percent photosynthesis values). This showed that oxygen evolution of cold-shocked cells represented true CO_2 fixation. The deviations at low percent photosynthesis are discussed later.

The next question asked was, Is the effect of cold shock fully expressed when the cells are removed from the cold? The answer was yes. This was shown by polarography with cells cold-shocked for 15-30 min. Results showed that cold-



shocked cells showed a loss in photosynthetic capacity immediately after being removed from the cold and that this level of photosynthesis continued constant during the next 1-2 hr. after cold shock. The same conclusion was reached from the manometric experiments. An exception was the case of cells cold-shocked for long periods of time (1-2 hr.) where photosynthesis tended to decline slightly (5 percentage units) during 1-2 hr. after cold shock. This could explain some of the deviations between CO_2 fixation and O_2 evolution observed in the later samples of CO_2 fixation, as O_2 evolution was measured over a period of 10-20 min. while CO_2 fixation was measured over a period of 2 hr. What the above experiment indicated was, that the lesion caused by cold was rapid and not dependent on subsequent incubation at room temperature.

The next question asked was, Is there any difference in the extent of labelling or in the labelling pattern of cold-shocked and control cells after short periods of photosynthesis? The experiment showed that after only 17 sec. of photosynthetic incorporation of $^{14}CO_2$, the radioactivity of cold-shocked cells was only 25-30% that of control (similar to that observed for longer periods of photosynthesis). This narrowed down the effect on photosynthesis to a direct one on a photosynthetic process (i.e. electron flow, photophosphorylation or CO_2 fixation) as opposed to, for example, the gradual build up of intermediates which were not further metabolized due to a defect (caused by cold) in a pathway not directly involved in photosynthesis (i.e. lipid, protein or nucleic acid synthesis).

This conclusion is consistent with results from manometric and polarographic experiments.

The pattern of labelling in control and cold-shocked cells was similar; the cold-shocked cells showing lower label in both the phosphate esters and aspartate, which represent different carboxylation pathways (see Part II of thesis). The label in citrate and malate, however, was higher than expected. The possible significance of this is discussed later.

All the above observations could be explained if only a part of the population of cells was completely inactivated. Hence the next question asked was, Are all the cells of the population affected or is the population comprised of cold-sensitive and cold-resistant fractions? The question was answered by cell autoradiography. Results showed that the distribution of grains over cold-shocked cells could be explained to a large extent by the random decay of isotope. This suggested that nearly all the cells of the population were affected. What was shown with certainty was that the effect was not "all or none". This was easily visualized when the data was considered from the point of view of inactive or poorly active cells (those with no grains). These cells comprised of only about 10% of the total population in the cold shock suspension, while the overall loss in photosynthetic capacity in the suspension was over 50%. The conclusion reached was that cold shock was not, on a cell basis an "all or none" effect, at least with respect to photosynthesis.

The next questions asked were: Does (a) cold shock have

any effect on viability? and (b) If so, how does the decline in viability compare with decline in photosynthesis? The answer to (a) was yes, viability was affected, and to (b) the answer was that, the effect of cold shock on viability was much more marked than its effect on photosynthesis. This confirmed the conclusion of autoradiography, viz. nearly all the cells of the population were affected by cold shock.

The increased resistance to cold shock with age of culture was interesting because superficially similar results have been observed with bacteria, where the cells are most sensitive during exponential growth (Introduction, pg. 4). However, in Anacystis nidulans, exponential growth was not a requirement for cold sensitivity (growth under these culture conditions was nearly linear).

Van Baalen (1957) and Forrest et al. (1957) showed that pteridines and amino acids were excreted with cold shock. The next questions asked were: (i) What is the composition of the cold shock supernatant? (ii) What fraction of the cell material is excreted? (iii) Does excretion of cell material parallel photosynthetic decline? The answers to these questions were obtained by studying the excretion of isotope from ^{14}C- and ^{33}P-labelled cells. These studies showed that about 0.5% of the total cell material (25% "total dialysable" material) was lost from the cell following cold shock; more than 99% of this material was dialysable. The loss was rapid and there was little further increase of isotope in the supernatant when the cells were cold-shocked for more than 15 min. In

this respect, the pattern of leakage of cell constituents differed from that of photosynthesis decline.

When 15 min. cold-shocked cells were held at room temperature, there was very little increase in isotope (^{33}P) in the supernatant. This was in harmony with the observations on photosynthesis (which did not decrease further when 15 min. cold-shocked cells were held at room temperature). These excretion studies also showed that the major excretion products were amino acids, glutamate comprising of nearly 50% of the excreted material. Other substances lost from the cell included phosphate esters, citric acid cycle intermediates and pteridines.

The glutamate lost from the cell was 50-80% of that available and in fraction 2 of the phosphate esters, the loss was approximately 60%; while the average loss of ^{14}C and ^{33}P was only about 25%. On the other hand, fraction 1 of the phosphate esters was lost to the extent of about 10% and very little of the available alanine was lost from the cell.

All this indicated that there might be intracellular compartmentation which prevented the loss of some components, or alternatively, the change in the membrane could be complex leading to selective release of different compounds from the same pool. The former alternative is suggested by examination of electron micrographs of Anacystis nidulans (Allen, 1968 b; Hansell, 1970, using cells from these cultures) where the photosynthetic lamellae could be expected to function as a permeability barrier.

In spite of the loss of a large quantity of small molecules, sufficient intermediates were held in the cell to support some measure of photosynthesis, which shows that at least some of these intermediates (e.g. Calvin cycle compounds) must have been retained within the cell after cold shock. It appeared likely that the reactions involved in photosynthesis were located such that they were not affected by permeability changes. Even in the absence of compartmentation by the photosynthetic lamellae, the intermediates involved in CO_2 fixation could still be held in the cell by being enzyme-bound. Further, the products of the reaction might be quickly converted to polymerized material. This is supported by the concept that at least some of the enzymes of the Calvin cycle are present in complexes (Bassham, 1964; Karm and Moudrianakis, 1969) and also by the data in the second part of this thesis, which shows that polymer glucose was formed very rapidly from CO_2.

The loss of cell material could explain (at least in part) the loss of photosynthetic capacity in cold-shocked cells. In fact, it is difficult to visualize a situation where photosynthesis would not decline despite the loss of this quantity of cell constituents. It was possible, however, that the effect on the limiting membrane was not the only effect of cold shock on photosynthesis.

It seems possible that the decline in viability might be due to the loss of cell material, but conclusions are more difficult to draw with respect to viability (Introduction pg. 4) because organisms with vastly different survival

following cold shock excrete approximately the same quantity of cell material. It is quite feasible that the viability of cold-shocked cells depends mainly, not on the loss of material but on the ability of the cell to repair the damage caused by cold shock. Since the effect on viability was not of primary interest, it was not studied further.

It was suggested by Van Baalen (1957) and Forrest et al. (1957) that loss in photosynthetic capacity after cold shock could be connected to the loss of pteridines. The next question asked was, Can any evidence be provided to support this suggestion? Experiments yielded ambiguous results. Although there was some definite correlation between pteridine loss and photosynthetic decline after cold shock, it was found that pteridines continued to be lost after cells were removed from the cold. It was felt that this might be used to show that pteridine loss and photosynthetic decline did not parallel each other, but it soon became apparent that several theoretical complications could arise in interpreting this type of result. (1) It was possible that there were at least two different pools of pteridines, one active and one inactive and it was possible that only the loss in the active pool might parallel photosynthetic decline. (2) It was possible that some of the pteridines were converted to an inactive form before they were excreted (e.g. by oxidation) hence decline in photosynthetic capacity could parallel this inactivation rather than simple excretion. Because of this uncertainty there was little reason to pursue the study, especially since pteridines comprised only about 2.5% of the carbon lost from the cell.

The increase in permeability with cold shock was not studied any further but the change in selective permeability might possibly be connected with the fatty acid composition of the membranes of _Anacystis nidulans_. Nichols et al. (1965) showed that _Anacystis nidulans_ has no poly-unsaturated fatty acids in its membranes, while the membranes of _Anabaena variabilis_ (which is not cold sensitive) contained these fatty acids. This concept is consistent with the theory of bacterial cold sensitivity (Farrell and Rose, 1968).

Studies with varying cold shock media (those known to protect bacteria from loss in viability) did not protect photosynthesis. This was not of any great significance, but it did suggest that it was unlikely that the effect of cold was due to change in activity of water in the cell due to increased permeability.

Up to this point the study was concentrated on the effect of cold shock on the overall process of photosynthesis. Conclusions thus far were that cold affected nearly all the cells and that the loss of photosynthetic capacity was due to a decrease in a photosynthetic process as opposed to a defect in a metabolic process other than that involved in CO_2 fixation. One reason why this decline could take place was the loss of selective permeability of the membrane, which could affect photosynthesis through either the loss of cell constituents or the general loss of cellular integrity. Work then proceeded to determine if cold shock affected any reaction sequence in photosynthesis.

The next question asked was, Can electron acceptors increase the rate of oxygen evolution in whole cells after cold shock? If this was so, it would mean that electron flow was not the main cause of photosynthetic decline. However, this was not the case as no effect could be obtained either with electron acceptors (ferricyanide or NADP) or CO_2 acceptors (RDP or PEP). The conclusion reached was that either electron flow was affected or that the acceptors were inaccessible to the reaction site.

The studies of Gerhardt and Santo (1966) and Van Baalen (1957) suggested that cold shock might have an independent effect on the photosynthetic membranes. Hence the next question was, What is the effect of cold shock on electron flow and photophosphorylation? Cold shock had no effect on any system of electron flow studied. The results in this section have a different theoretical meaning depending on what scheme of electron flow is followed. Ferricyanide assays for P.S.II on the Hill and Bendall scheme and probably for P.S.IIb on the Arnon scheme (see Introduction for schemes). Cyt. c assays for P.S.I on either scheme, while DCPIP assays for P.S.II alone or P.S.II and I on the Hill and Bendall scheme. The observed rates of reduction of DCPIP in this study were such that if the conclusions of Lien and Bannister (see Introduction, pg. 18) are correct, the system must have at least some contribution from P.S.I. One might assume that DCPIP can similarly assay for P.S.IIb or P.S.IIb and IIa on the Arnon scheme but this is not known.

In the light of the above considerations, it was deduced that P.S.I and P.S.II (IIa, Arnon), when assayed individually, appeared not affected by cold shock. What could not be said from these experiments was whether the combination of P.S.I and II (IIa and IIb, Arnon) was affected by cold shock. It was hoped that the methyl viologen-Mehler reaction could provide a solution for this, as the system should assay for the overall NADP-reducing activity. The experiment showed that cold shock did not reduce the capacity of this system and suggested that cold had no effect on electron flow.

This was contrary to the statements of Van Baalen (1957), who observed that cold-shocked cells had a lower capacity for the reduction of quinone and also that cell-free extracts prepared from cold-shocked cells showed a lower Hill reaction as measured by DCPIP reduction. In the former case such an assay could be complex because quinone could have different permeability to cold-shocked and control cells and hence different substrate concentration curves. Secondly, the rate of quinone reduction in intact Anacystis cells is much greater than the whole cell photosynthetic rate (Van Baalen, 1957) and hence it was possible that if there is an effect of cold shock on quinone reduction, that this may not have a significant effect on the overall rate of photosynthesis.

The second observation of Van Baalen was more puzzling since in the studies presented here, cold shock had no effect on the reduction of the same acceptor (DCPIP). It was unlikely that this was due to a different site of action of DCPIP in the

two procedures, since approximately the same rates of reduction were observed in both cases. A possible explanation however, is that the effect observed by Van Baalen was a cumulative effect of both cold shock and homogenization of the cold-shocked cells. This was not investigated further because marked effects were obtained on photophosphorylation in cold-shocked cell-free extracts.

With the data of Van Baalen in mind, it may be said that although cold might have some effect on electron flow, it is unlikely that this is a major effect of cold shock.

Cold shock had a marked effect on photophosphorylation (both cyclic and noncyclic). The general pattern of the effect was very similar to the decline in photosynthesis of whole cells. Photophosphorylation in Anabaena variabilis (which is not cold-sensitive) was relatively unaffected (in addition to this study, the insensitivy of Anabaena photophosphorylation to cold shock can be deduced by the experimental procedures of Duane et al., 1965; and Lee et al., 1969). Another point that is of significance in this connection is the data of Gerhardt and Santo (1966) in which cell-free extracts of lyophilized cells of Anacystis nidulans (exposed to the cold only as intact cells) showed low P/2e ratios. It appears more than a coincidence that cold-shocked cell-free extracts gave similar low P/2e ratios.

Hence it was highly suggestive that the loss in photosynthetic capacity of whole cells was indeed caused by the decline in photophosphorylation. This was theoretically

feasible as a loss of ATP synthesis would necessarily cause a decline in CO_2 fixation by the Calvin cycle and hence decrease the rate of photosynthesis.

The mechanism by which cold affects photophosphorylation was not studied further, but it must represent a direct effect of cold shock on the photosynthetic membranes, perhaps causing a change in their conformation or the loss or dislocation of a coupling factor. Such an effect on the photosynthetic membranes was also suggested by the studies of Van Baalen (1957). The effect of cold shock on PEP carboxylase was also of interest. It could have been considerably more important had a Hatch-Slack transcarboxylation occurred (Part II of thesis). The nature of the effect was not studied further, as it was clear that the pathway to aspartate accounted for less than 10% of the total carbon fixed (and therefore could not explain the loss of photosynthetic capacity in whole cells). It could provide an explanation, however, for the lower labelling of aspartate observed during short term photosynthesis in $^{14}CO_2$. On the other hand, the lower label in aspartate could also be explained by the loss of glutamate from the cell. The second explanation was more satisfactory as it was in harmony with the proportionately higher label in citrate and malate.

Further questions originating from the work presented here are: (1) How does cold shock affect photophosphorylation? (2) How does cold shock affect PEP carboxylase activity? (3) How does cold shock affect viability? Can viability be protected in any way? If so, what is the mechanism of protection?

(4) Are these the only cold-sensitive processes in the organism?

(5) Does protein synthesis and nucleic acid synthesis take place in cold-shocked cells?

Conclusions. The question asked was, Why does photosynthesis decline after cold shock? The answer provided here is that either or both (1) the loss of selective permeability (loss of cell material) and (2) the loss of photophosphorylation can cause the decline in photosynthetic capacity.

PART II

CO_2 incorporating reactions of Anacystis nidulans

The main question asked in this section was, How is CO_2 incorporated in Anacystis nidulans?

The first question asked was, What is the general pattern of CO_2 incorporation in Anacystis nidulans? Studies presented here with whole cells suggested that the bulk of the CO_2 fixed in Anacystis nidulans passed through the Calvin cycle. This was consistent with the data of Kandler (1961). However, it was also observed in these studies that under some conditions, there was an initial high incorporation of $^{14}CO_2$ into aspartate and sometimes also into glutamate, which has also been recorded by Richter (1961). These studies showed that aspartate and glutamate could be formed without the operation of the Calvin cycle (in type II labelling) and also that there could be extensive labelling of the Calvin cycle intermediates without significant isotope in the two amino acids (in type I labelling). This suggested that these two types of labelling were the result of different carboxylating reactions.

The initial labelling in aspartate is not unique to Anacystis nidulans as it was observed by Fuller et al. (1961) with Chromatium, Hatch and Slack (1966) with C-4 plants, and by Graham and Wittingham (1968) with Chlorella. The initial labelling of aspartate in C-4 plants is probably due to a CO_2 translocation mechanism (see Introduction, pg. 34), while

that in Chlorella occurs at very low CO_2 levels and the switch to the Calvin cycle is observed only after 1-2 hr. photosynthesis, (unlike Anacystis nidulans where the change occurs within a few seconds). The switch in pattern in Chlorella was believed to occur in parallel with an induction of the synthesis of carbonic anhydrase (Read and Graham, 1968; Graham and Wittingham, 1968). It is clear that the last two observations are probably not related to the situation in Anacystis nidulans.

The nature of the factors that decide which type of labelling occurred was unknown but could possibly involve one or more of a number of variables such as (i) enzyme levels, (ii) availability of substrate, (iii) a control mechanism involving for example, NH_4^+ (Kanazawa et al., 1970) or some other metabolite.

Since the study was conducted under non-steady state conditions, the general physiological significance of the initial label in aspartate and glutamate cannot be assessed.

Although it was likely that CO_2 incorporation into aspartate represented true CO_2 fixation, it was possible that the label in aspartate reflected only an equilibration with CO_2 involving no net fixation. It was equally possible that aspartate was just an index of the flow of ^{14}C through oxaloacetate which could participate in a transcarboxylation reaction (Introduction pg. 32).

Although the lag in the synthesis of sugars and Calvin cycle intermediates was probably an artifact due to non-steady state conditions, it can be also explained on the basis of a

transcarboxylation reaction.

The early label in glutamate was reminiscent of the situation in Rhodospirillum rubrum and Chlorobium thiosulfatophilum where a reductive citric acid cycle is operative (Introduction pg. 37).

Another interesting feature in these experiments was that an unknown compound(s) X in the ethanol insoluble fraction was labelled extremely fast. It was possible that this compound could be the result of yet another independent carboxylation reaction. If this was not the case it probably arose from an intermediate closely connected with CO_2 fixation.

Studies up to this point indicated that the bulk of the CO_2 fixed passed through the Calvin cycle but that there probably was independent CO_2 fixation into aspartate and possibly also into glutamate.

The next question asked was, What is the distribution of isotope in aspartate? Results suggested that nearly all the isotope was in C-4, indicating that aspartate was formed by the carboxylation of a 3-C compound (most likely PEP).

The next question asked was, What is the distribution of isotope in glutamate? The results showed that nearly all the isotope was in C-1. This was consistent with two pathways, either (1) the reductive carboxylation of succinic acid which is known to occur in some photosynthetic bacteria (Buchanan et al., 1967) and also suspected to occur to a small extent in some green algae (Gibbs, 1967) or alternatively (2) the label could also come from oxaloacetate-4-^{14}C which can be stereo-

specifically converted to glutamate-1-^{14}C (Lehninger, 1970). The latter alternative was feasible since aspartate was labelled in C-4 and hence one would expect oxaloacetate to be labelled in the same position. Secondly, Richter (1961, b) found that under conditions of Mn^{2+} starvation the label in aspartate increased and the label in glutamate decreased and since isocitric dehydrogenase (in yeast) is stimulated by Mn^{2+} (Kornberg, 1955) the observations were consistent with glutamate being formed by the citric acid cycle. The only observation that was not consistent with this pathway was the kinetic data which showed that the label in citrate was too low to account for the label in glutamate at early sample times (see Results, E-1). However, this type of argument is not entirely free of complications because there could be preferential convertion of labelled citrate to glutamate without prior equilibration with the entire pool of citrate (which would not be surprising considering the stereospecificity of the reactions). Moreover, a similar situation (low label in citrate, high label in glutamate), has been observed by Bassham and Kirk (1960) in Chlorella. It should be noted however, that if glutamate was formed in this way, then the incorporation of ^{14}C into glutamate actually represents no net fixation of CO_2 but rather a net loss of cell material because for every C atom incorporated two must be lost.

The next question asked was, Is the isotope in oxaloacetate higher than in aspartate? The answer was no, in fact the label in oxaloacetate was extremely low (a similar situation

was also observed by Hatch et al. (1967) using several C-4 plants). This seemed to indicate that (1) oxaloacetate was not an intermediate in the synthesis of aspartate, or (2) an enzyme-bound oxaloacetate existed which resulted in the preferential conversion of the newly synthesized oxaloacetate into aspartate, or (3) oxaloacetate has a small pool-size and is completely saturated with label. The latter was not consistent with the progressively increasing label in oxaloacetate, but could not be ruled out since non-steady state conditions prevailed and the increase in radioactivity in oxaloacetate could have represented an increase in pool-size.

At this point it was decided to terminate whole cell studies and to look for, in cell-free extracts, the enzymic activities suggested by the whole cell studies.

Whole cell studies had shown that the bulk of the CO_2 was fixed via the Calvin cycle and as a result the next question asked was, Has the organism sufficient RDP carboxylase activity to account for whole cell rates of photosynthesis? These studies showed that cell-free extracts contained sufficient RDP carboxylase activity to account for only 15-25% of the manometric photosynthetic rate. A more striking feature was the high Km of CO_2 for the enzyme. However, these observations are common to most photosynthetic organisms and theories have been advanced to reconcile them with the central role of RDP carboxylase in photosynthesis.

The next question asked was, Is PEP carboxylase present in sufficient quantities to account for the observed whole cell

rates of synthesis of aspartate? The answer was yes. This showed that aspartate could be formed by the carboxylation of PEP.

What is the significance of this pathway? Is it an indep[illegible]ent pathway (as it appears to be, at least to a small exte[illegible] in most photosynthetic organisms e.g. Chlorella, Smith et al., 1961) or is it an index of a transcarboxylation involving an intermediate in PEP carboxylation. The former alternative could be of significance since Anacystis nidulans is believed to have an incomplete citric acid cycle (Smith et al., 1967), the break being between α-ketoglutaric acid and succinic acid. If this is the case, the carboxylation of PEP could be of considerable significance, because it could account for the synthesis of C-4 dicarboxylic acids as well as precoursors of porphyrins. An alternate route for the synthesis of these compounds is the glyoxalate cycle for which enzymes are known to be present (Pearce and Carr, 1967).

The next question asked was, Is there a PEP stimulated transcarboxylation reaction in Anasystis nidulans? Results showed that no stimulation of RDP carboxylase can be obtained even at low [CO_2] by the addition of PEP, which tends to suggest that a product of PEP carboxylase does not donate CO_2 to RDP carboxylase. Since the presence of a transcarboxylation reaction was thus unlikely, what is the significance of the preferential initial labelling in aspartate? It was possible that at the low concentration of CO_2 used, its supply will be low, at least initially and if the Km of PEP carboxylase is

less than that of RDP carboxylase <u>in vivo</u> (as it is <u>in vitro</u>) then it could be visualized that PEP carboxylase might use the bulk of the CO_2 present initially. Alternatively, this effect could be due to the localization of the enzyme. If PEP carboxylase is located closer to the periphery of the cell than the photosynthetic lamellae, the enzyme would be more strategically located than RDP carboxylase to encounter CO_2.

Then why the variation in labelling pattern from experiment to experiment? To this, there is no answer except to speculate that slightly differing reaction conditions might allow CO_2 to enter the cell at different rates or alternatively, the enzymes, the substrates or effectors may vary from one batch of cells to another to such an extent that such changes in labelling pattern can occur.

The final question asked was, Can any evidence be obtained for the reductive carboxylation of succinic acid? The answer appeared to be no, but complexity of the assay did not allow firm conclusions to be made.

<u>Conclusions</u>. The Calvin cycle appears to be responsible for the bulk of the CO_2 fixed by <u>Anacystis nidulans</u>. Although the quantitative significance of the pathway could not be assessed, CO_2 could also be fixed by the carboxylation of PEP. The major product of the reaction was aspartate. No evidence could be obtained for a "Hatch-Slack transcarboxylation". Although no evidence could be obtained for α-ketoglutarate synthetase activity, glutamate-1-^{14}C can be one of the first

products of photosynthesis. It is possible that glutamate arose from oxaloacetate-4-^{14}C via the citric acid cycle.

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