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STUDIES OF ENERGY CONSERVATION IN CHLAMYDOMONAS REINHARDII

by

John Mottley B.Sc., M.Sc.

in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF WARWICK

School of Molecular Sciences

September 1976

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ACKNOWLEDGEMENTS

The author would like to thank his supervisor Dr. D.E. Griffiths for the use of laboratory facilities and an additional six months financial assistance to allow him to complete this work.

Appreciation is also extended to Professor D.R. Davies and Mrs. V. Brewster of the University of East Anglia, for training in tetrad analysis techniques and gifts of the C.reinhardii strains.

The author would also like to thank his colleagues in the School of Molecular Sciences, particularly Dr. W.E. Lancashire, Dr. R. Houghton, Mr. B.P.M. Menco, Mr. K. Cain and Dr. E. Griffiths for useful discussions. The 'loan' of certain equipment and chemicals would have been very difficult without the help of Mrs. M. West.

Financial assistance for two years from the S.R.C. council is appreciated. In this regard, gratitude is, of course, also extended to the British taxpayer.

The author is deeply grateful for the encouragement given to him by his parents.

SUMMARY

An attempt was made to gain an insight into the energy conservation process in Chlamydomonas reinhardii. This organism possesses both mitochondria and a chloroplast and so respiration and photosynthesis can be studied in the same cell. The growth of C.reinhardii under different trophic conditions was characterised. The sensitivity of the wild-type (Wt^+) strain to a wide range of inhibitors was determined in order to select potentially useful inhibitors for the isolation of drug-resistant mutants. The effect of these inhibitors on energy conservation was determined using chloroplast preparations from pea (Pisum sativum) and the $CW15^+$ (cell wall-less) strain of C.reinhardii. The reported modes of action of the triorganotins and alkylguanidines on photosynthetic energy conservation were confirmed and, in some cases, extended.

Mutant strains of C.reinhardii, which are resistant to inhibitors of oxidative and photosynthetic phosphorylation, may provide an insight into the energy conservation process. Drug-resistant strains of C.reinhardii were isolated and divided into five major classes on the basis of their cross-resistance characteristics. The $TP\text{-}8^+$ (triorganotin-resistant) mutant was specifically resistant to trimethyltin and triethyltin but more sensitive than the Wt^+ strain to other inhibitors. The alkylguanidine-and ethidium bromide-resistant mutants exhibited pleiotropic resistance patterns to other membrane-active inhibitors. The resistance phenotypes of all the mutant strains were inherited in a Mendelian fashion and were generally present under different trophic conditions. The resistance in these mutants may be due to change(s) in one or more of the cell membranes but the exact locus of resistance was not determined. However, the resistance of the $EBr\text{-}6^+$

(ethidium bromide-resistant) strain may be due to reduced uptake of ethidium bromide.

Attempts to localise the mode of resistance of the TP-8^{+} strain at the sub-cellular level were unsuccessful.

GENERAL ABBREVIATIONS

ADP/O	adenosine 5'-diphosphate:oxygen ratio.
AES	automatic external standard.
ATP	adenosine 5'-triphosphate.
ATP-ase	adenosine 5'-triphosphatase.
c (prefix)	centi (10^{-2}x).
CPM	counts per minute.
cv.	variety (of an organism).
DNA	deoxyribonucleic acid.
DPM	disintegrations per minute.
DW	dry weight.
equiv.	equivalent (weight).
g	gram(s).
$g_{av.}$	average acceleration due to gravity (9.81m.sec^{-2}).
K_m	Michaelis constant.
l	litre.
lb	pound (weight).
log	logarithm.
m	metre.
m (prefix)	milli (10^{-3}x).
M	molar (concentration).
min	minute (time).
n (prefix)	nano (10^{-9}x).
OD	optical density.
Pi	inorganic (ortho) phosphate.
PS1	photosystem 1.
PS2	photosystem 2.
RNA	ribonucleic acid.
RuDP-carboxylase	ribulose-1,5-diphosphate carboxylase.

sec	second (time).
sp., spp.	species (singular and plural).
μ (prefix)	micro (10^{-6}x).
UV	ultraviolet.
Wt	wild-type.
λ	wave-length.

Other abbreviations are explained in the text.

CHEMICAL ABBREVIATIONS AND SOURCES.

Except for those in the following list, all chemicals mentioned in the text were obtained from Sigma Chemical Co.

Compound	Source
Acridine orange	Calbiochem
ADP	Boehringer-Mannheim
agar	Oxoid
Antimycin A	Calbiochem
Atractyloside	Calbiochem
Aurovertin	Shell Research Ltd.
p-Benzoquinone	May and Baker
Cl-methyl dibutyltin	Tin Research Inst.
Dibutyltin diacetate	Tin Research Inst.
DCCD (N,N'-dicyclohexylcarbodiimide)	BDH
Diethyltin dichloride	Tin Research Inst.
DNP (2,4-dinitrophenol)	BDH
Dio-9	Netherlands Fermentation
Dioctyltin dichloride	Tin Research Inst.
EDTA (ethylenediaminetetraacetic acid)	Hopkins and Williams.
Ethidium bromide (EB) (2,7-diamino-9-phenyl-phenanthidium-10-ethyl-bromide)	Calbiochem
Galegine sulphate (3-methyl-2-butenylguanidine sulphate)	Aldrich Chemical Co.
'1799' (1,1,5,5-trifluoromethyl-1,5-hydroxy-pentan-3-one)	Du Pont
Malonate	Fisons
Methyl viologen (MV) (N,N'-dimethyl-γ,γ'-dipyridylium dichloride)	Koch-Light Laboratories Ltd.
MNN (N-methyl-N-nitroso-N'-nitroguanidine)	Koch-Light Laboratories Ltd.
NADH (reduced nicotinamide adenine dinucleotide)	Boehringer-Mannheim

Compound	Source
Nigericin	Eli Lilly
Peptone	Oxoid
Phlorizin (4,4',6'-trihydroxy-2'-glucosid-odihydrochalcone)	Pfaltz and Bauer Inc.
PMS (methyl phenazine methosulphate)	BDH
Potassium cyanide	Hopkins and Williams
Potassium ferricyanide	May and Baker
Proflavine (3,6-diaminoacridinium chloride hydrochloride)	Calbiochem
Rhodamine B	BDH
Rhodamine 6G	BDH
SDS (sodium dodecyl sulphate)	BDH
Sodium acetate	Hopkins and Williams
Sodium arsenate	BDH
Sodium azide	Fisons
Spectinomycin (decahydro-4a,7,9-trihydroxy-2-methyl-6,8-bis(methylamino)-4H-pyrano [2,3-b] -benzodioxin-4-one)	Upjohn Co.
TBT (tributyltin chloride)	BDH
TcHT (tricyclohexyltin hydroxide)	Dow Chemical Co.
TES (N-tris(hydroxymethyl)methyl-2-amino-methane sulphonic acid)	General Biochemicals
TET (triethyltin sulphate)	Tin Research Inst.
Tetrabutyltin	Tin Research Inst.
Tetraphenylboron	BDH
TMT (trimethyltin chloride)	BDH
TPT (tripropyltin chloride)	BDH
TPhT (triphenyltin chloride)	BDH
Tributyltin oxide	Tin Research Inst.
TTFB (4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole)	Shell Research Ltd.
Valinomycin	Calbiochem
Venturicidin	Glaxo
Yeast extract	Difco

CHAPTER I

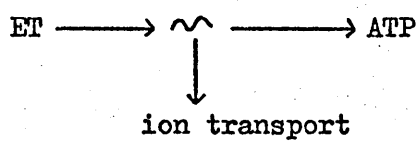
Introduction

(a) General Introduction

Energy conservation in living organisms is the process whereby cells harvest the energy required for cellular function and the central rôle of ATP as the principal chemical energy store in cells is well established. In organisms which possess both mitochondria and chloroplasts, such as Chlamydomonas reinhardii, ATP is synthesised during respiration and photosynthesis. However, oxidative and photosynthetic phosphorylation have proved to be among the most difficult biochemical processes to resolve.

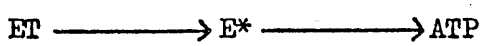
There are three main hypotheses regarding the coupling of electron transport to phosphorylation : the chemical, the conformational and the chemiosmotic mechanisms.

The chemical hypothesis (Slater, 1953) proposes that electron transport (ET) generates the formation of a high energy intermediate (~) which is used for the synthesis of ATP :



According to this hypothesis, ion transport phenomena, including those resulting in the formation of a proton gradient across the mitochondrial or thylakoid membrane, are secondary processes driven by the high energy intermediate.

According to the conformational hypothesis (Boyer, 1965), energy derived from electron transport is primarily conserved as a conformational change in a respiratory protein (E) and this conserved energy is then used in ATP synthesis :-



The chemiosmotic hypothesis (Mitchell, 1961, 1966[a]) proposes that proton translocation coupled directly to electron transport produces a proton gradient across the thylakoid or inner mitochondrial membrane and that ATP synthesis results from the dissipation of this gradient through a transmembrane ATP-ase:

$$ET \longrightarrow H^{+} \longrightarrow ATP$$

However, none of these proposals has yet been fully established as accounting for energy conservation in mitochondria and chloroplasts but their relative merits are discussed by Greville (1969), Schwartz (1971) and Slater (1971).

There have been four main approaches to the analysis of electron transport and associated phosphorylation in mitochondria and chloroplasts. Firstly, there have been attempts at isolation of the various components followed by their reassociation. Secondly, the kinetics of oxidation and reduction of the various electron carriers and the transport of ions across the organelle membrane have been examined. Thirdly, analysis of the effects of a wide variety of inhibitors on electron transport and phosphorylation have been used in conjunction with the above studies. Fourthly, there has been a combined biochemical and genetic approach to the problem of energy conservation, which has yielded valuable information regarding the biogenesis of the individual components of the system.

For genetic studies of energy metabolism both haploid and diploid organisms have specific advantages. With haploid organisms, genetic changes are more easily detected because a homologous gene cannot mask their effect. This is a major reason for the great popularity of bacterial systems for genetic studies. On the other hand, mutations involving vital functions are more difficult to introduce into haploid

cells since they usually die from the loss of the single copy of an essential gene function. Yeasts and certain algae and fungi are thus very attractive organisms for genetic study since they can be cultivated both in the diploid and haploid stage. Mutants of eucaryotic micro-organisms have been used in some investigations of the coupling of electron transport to phosphorylation, (e.g. :- Beck et al, 1968; Kovac & Hrusovska, 1968), and there is extensive literature on Saccharomyces, Neurospora and Chlamydomonas reinhardii mutants in which electron transport is affected. However the genetic approach in eucaryotic cells is complicated by the interacting DNA systems of the nucleus, mitochondria and, in the case of unicellular algae, of the chloroplast.

One important class of mutants consists of those with increased resistance to inhibitors which have a known and specific action on some aspect of mitochondrial or chloroplast function. To date, studies with mutants resistant to inhibitors of energy conservation have concentrated upon specific inhibitors such as oligomycin (Parker et al, 1968; Stuart, 1970; Avner and Griffiths, 1970, 1973 [ab]; Wakabayashi and Gunge, 1970; Mitchell et al, 1971, 1973; Griffiths et al, 1972; Shannon et al, 1973; Rowlands and Turner, 1974), antimycin (Grimmelikhuijzen et al, 1975), azide and DNP (Goto & Anraku, 1974), bongkrekic acid (Perkins et al, 1973; Lauquin et al, 1973), triethyltin (Griffiths et al, 1972; Lancashire and Griffiths, 1971, 1975[a]), venturicidin (Lancashire and Griffiths 1975[b]) and alkylguanidines (Brunner et al, 1973). The majority of these studies have used the yeast S.cerevisiae which, although a useful test organism, is atypical of eucaryotes in being able to survive without respiring mitochondria. Consequently it would be desirable to extend these studies to obligately aerobic and photosynthetic organisms.

The single-celled alga C.reinhardii has proven to be a very

useful experimental organism in this respect. The life-cycle is extremely simple and yet includes the characteristic features of higher organisms, viz: vegetative growth, differentiation of gametes, fertilisation and meiosis. Several classes of mutants, which have lesions in photosynthetic electron transport, have already been isolated and characterised both biochemically and genetically and have yielded invaluable information in respect of this process (Levine, 1969; Levine and Goodenough, 1970). Only very limited work, however, has been carried out on either mitochondrial mutations of this alga (Alexander et al., 1974) or on mutations directly affecting the terminal reactions of photosynthetic phosphorylation (Sato et al., 1971). Consequently there is a general lack of information relating to C.reinhardii mutants with lesions in either oxidative or photosynthetic phosphorylation. In the case of the latter process, this gap has not even been narrowed by comparable work on other suitable eucaryotic photosynthetic organisms, where there is almost a total lack of research on the isolation and characterisation of mutants resistant to inhibitors of oxidative and photosynthetic phosphorylation.

This thesis describes preliminary attempts at a biochemical genetic approach to the study of oxidative and particularly photosynthetic phosphorylation in C.reinhardii. In particular, this thesis records the results of the phenotypic and genotypic characterisation of mutants isolated as resistant to inhibitors of oxidative and photosynthetic phosphorylation.

(b) Mode of action of inhibitors used in these studies

(1) Organotin compounds

With the possible exception of mercurials, organotin compounds have received more attention for their biological effects and mode of action than the organic derivatives of any other metal. Several reviews have appeared (Barnes and Stoner, 1959; Ingram et al., 1960;

Sijpesteyn et al, 1969; Poller, 1970; Luijten, 1972; Thayer, 1974), which detail their general biocidal properties. Organotin compounds are now widely used as pesticides, e.g. :- trialkytin and triphenyltin salts are used as fungicides, algicides, molluscicides, wood preservatives and marine anti-fouling paints. A striking feature of tin compounds is that, unlike for example lead, mercury or arsenic, toxicity is only manifest in organotin compounds, inorganic tin being virtually non-toxic (Barnes and Stoner, 1959; Ingram et al, 1960)

Whilst all organotin compounds are toxic, their effect varies according to the number and type of organic groups present. Alkyls tend to be more toxic than aryls and triorganotins are more toxic than di- or tetra- organotins (Thayer, 1974). Dialkyltin compounds have a somewhat different mode of action from trialkyltins in that they react with sulphydryl groups and inhibit enzymes such as α-keto acid oxidases (Aldridge and Cremer, 1955).

In contrast, triorganotin compounds are known to interfere specifically with oxidative and photosynthetic phosphorylation (Aldridge and Street, 1964; Kahn, 1968; Stockdale et al, 1970), their effect depending on the pH and ionic composition of the assay medium. In an alkaline, chloride-free sucrose medium, triorganotin compounds have a predominantly oligomycin-like action on a step in the energy transferring chain of oxidative phosphorylation (Aldridge and Rose, 1969; Stockdale et al, 1970; Coleman and Palmer, 1971). However in an acidic, chloride-containing medium, a chloride-hydroxide exchange reaction is mediated by triorganotin compounds (Selwyn et al, 1970), which results in a depletion of substrate anions in the mitochondria (Manger, 1969; Stockdale et al, 1970; Harris et al, 1973; Skilleter, 1975) and an increase in the internal acidification (Dawson and

Selwyn, 1974). The latter effect is probably responsible for the considerable inhibition of DNP-stimulated oxidation of succinate- or NAD^+-linked substrates (Coleman and Palmer, 1971; Dawson and Selwyn 1974). It appears that in order to be effective in catalysing the chloride-hydroxide exchange reaction, organotins must have three carbon-tin bonds, an n-octanol : water partition coefficient greater than unity and one atom or group of atoms bonded to the tin atom which is readily exchangeable with the chloride ion (Wulf and Byington, 1975). The demonstration that oligomycin is also capable of mediating Cl^- transport in mitochondria (Ariel and Avi-dor, 1973) underlines the similarity of the mode of action of triorganotin compounds and oligomycin as chloride-dependent uncouplers as well as energy transfer inhibitors.

Compared to the work on respiration, much less research has been reported concerning the effect of organotin compounds on photosynthesis. Tributyltin (TBT) was found to be a specific inhibitor of photophosphorylation in isolated Euglena and spinach chloroplasts (Kahn, 1968, 1970). Very low concentrations were required for complete inhibition and there appeared to be a stoichiometric binding relationship of one mole of TBT to 60-120 moles of chlorophyll. Kahn (1968) postulated that TBT binds to chloroplasts close to or at the site of binding of the coupling factor (CF_1) and thus prevents the loss, by hydrolysis, of a high energy intermediate in CF_1-deficient chloroplasts. This was indicated by a marked stimulation by low concentrations of TBT of both photophosphorylation and the light-dependent 'pH rise' in chloroplasts deficient in CF_1. This effect is similar to that of DCCD in spinach chloroplasts except that this compound also directly affects NH_4Cl-uncoupled photosynthetic electron transport (Mc Carty

and Racker, 1967), whilst TBT only indirectly affects coupled electron transport and maximal inhibition is independent of ADP and Pi and is reversed by uncouplers (Kahn, 1968). Certain other photosynthetic energy transfer inhibitors, such as Dio-9 (Mc Carty et al, 1965) and Phlorizin (Izawa et al, 1966), require ADP and Pi for maximal inhibition and so it appears that the binding site of TBT lies between that of DCCD and Dio-9 (Kahn, 1968).

A hypothesis to account for the inhibition of photophosphorylation by triorganotin compounds has recently been proposed by Watling - Payne and Selwyn (1975) and extended by Gould (1976). This hypothesis is based on the following observations : (1) At very low concentrations, TPhT inhibits ATP synthesis and coupled electron transport but not basal (-Pi) or uncoupled electron transport. (2) Membrane-bound ATP $\rightleftharpoons$ Pi exchange and Mg^{2+} -dependent ATP-ase activities of chloroplasts are sensitive to TPhT but the Ca^{2+} - and Mg^{2+}- dependent ATP-ase activities of isolated CF_1 are not. (3) The light-driven proton pump in chloroplasts is stimulated by low levels of TPhT at the same stoichiometry (2-2.5 TPhT molecules/ 100 chlorophyll molecules) as inhibition of ATP synthesis. (4) Low levels of TPhT restore the proton-pump activity of illuminated CF_1-depleted chloroplasts.

These observations led Gould (1976) to propose that TPhT molecules block the membrane proton channels located at or near the CF_1 attachment sites (Fig.1.1B). This results in the inhibition of ATP formation and membrane-bound ATP-ase and ATP $\rightleftharpoons$ Pi exchange activities. Stimulation of light-driven proton uptake in chloroplasts with CF_1 attached would then be due to the blocking of proton leakage through the CF_1. Depletion of CF_1 from chloroplasts results in opening of proton channels, producing a proton-leaky membrane (Fig. 1.1C), which are blocked on

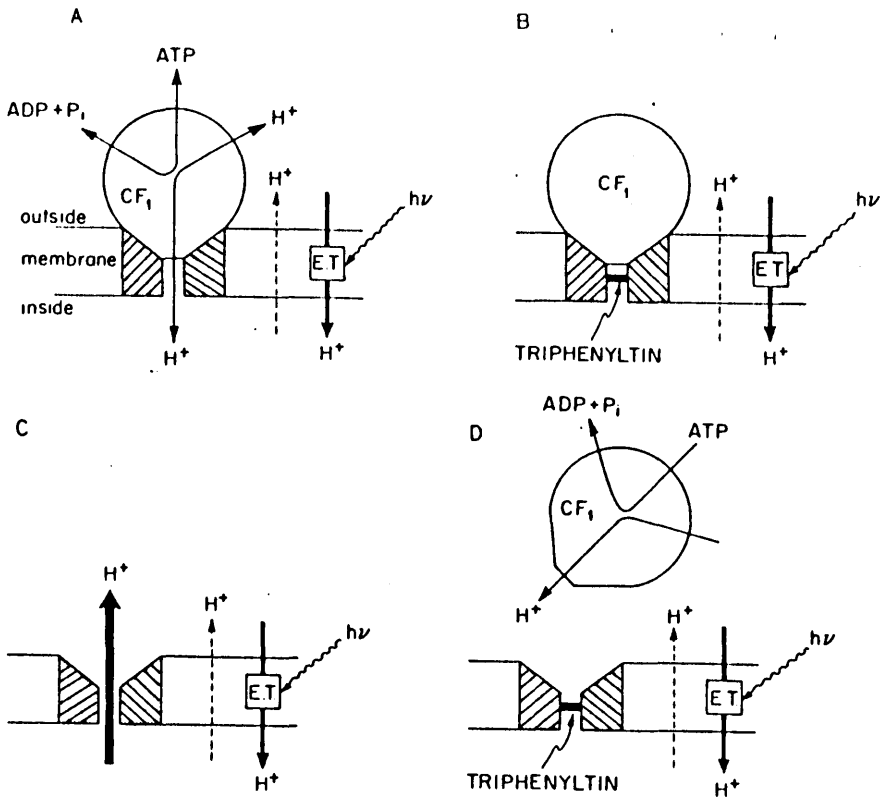


Figure 1.1. Simplified scheme for energy-linked reactions in chloroplasts and their inhibition by triphenyltin.

CF_1, chloroplast coupling factor; E.T., the chloroplast electron-transport chain.

The shaded area represents a tightly bound component of the thylakoid membrane containing a proton carrier or channel. (A) Vectoral pathways of protons coupled to electron transport, ATP formation and ATP hydrolysis. The dashed line indicates passive (non-coupled) diffusion through the membrane. (B) Blocking of the membrane carrier or channel by triphenyltin. Note that the net internal accumulation of protons should be higher since leakage outward through the coupling factor is effectively stopped. (C) Removal of coupling factor by EDTA wash. Note that net proton uptake is abolished due to the high rate of proton leakage through the now un-gated membrane channel or carrier. (D) Blocking the proton channel or carrier with triphenyltin in EDTA-washed chloroplasts. Note that net proton uptake is restored, and also that the ATP-ase activity of solubilised coupling factor is unaffected by triphenyltin.
(Diagram and legend taken from Gould, 1976).

addition of TPhT (Fig.1.1D). This produces a reconstitution of the light-induced proton uptake. The ATP-ase activities of the CF_1 would then be insensitive to TPhT due to lack of association of the CF_1 with the TPhT binding sites on the chloroplast membrane.

The applicability of this hypothesis to the mechanism of action of other triorganotin compounds is not clear. TBT inhibits isolated CF_1 Ca^{2+} - dependent ATP-ase only at high concentrations and elicits reconstitution of the 'pH rise' in illuminated CF_1 -deficient chloroplasts (Kahn, 1968). TMT does not reconstitute this 'pH rise' which suggests that the 'pseudo-recoupling' process requires a somewhat hydrophobic region of the thylakoid membrane for binding of the triorganotin compounds (Watling-Payne and Selwyn, 1975). The relationship between energy-transfer inhibition by other triorganotin compounds and their ability to block proton-conducting channels requires further investigation.

Although the major effects of triorganotin compounds are as energy transfer inhibitors, it has recently been found that trialkyltin compounds can catalyze a chloride-hydroxide exchange reaction across the thylakoid membrane under suitable conditions (Watling and Selwyn, 1970, 1974). This is similar to the effect produced in mitochondria and explains the uncoupling activity of triorganotin compounds in Cl^--containing media, especially at acid pH values (Watling-Payne and Selwyn, 1974). This uncoupling involves an exchange of internal Cl^- ions for external OH^- ions in response to the pH component of the electrochemical potential gradient set up on illumination of chloroplasts (Fig. 1.2). The result is a dissipation of the pH gradient, while the electro-chemical gradient is released by the electrogenic natural uniport of Cl^- ions to the inside of the thylakoids (Watling

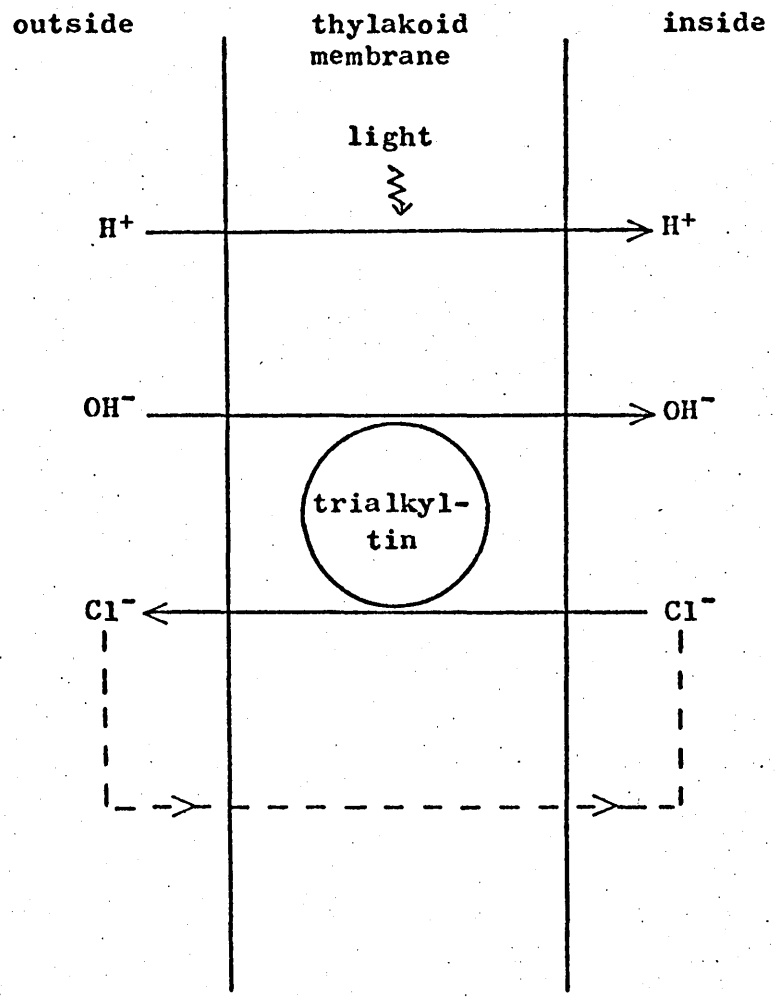


Figure 1.2. Postulated mechanism for triorganotin-mediated uncoupling via Cl^--OH^- exchange. (After Watling-Payne and Selwyn, 1974).

- Payne and Selwyn, 1974).

Watling-Payne and Selwyn (1974) tested the effect of a range of organotins on various reactions associated with photophosphorylation in pea chloroplasts. They found that low concentrations of a series of organotins inhibited ADP-stimulated oxygen evolution in a pH- and concentration-dependent manner. At pH 7.6, all the test compounds produced inhibition of state 3 (in the presence of ADP and Pi) oxygen evolution at low concentrations and of CCCP-uncoupled electron transport at higher concentrations. However, at intermediate concentrations, TBT and particularly TET produced uncoupling and stimulation of basal electron transport via the catalysis of the chloride-hydroxide exchange reaction. Using a selected concentration of triorganotin compounds, it was generally found that, at pH values of 7.6 and 8.0, photophosphorylation was inhibited, at pH values of 7.0 and 6.5, electron transport was stimulated and, at pH 6.0, electron transport was inhibited.

In almost all the studies on the effect of triorganotin compounds on energy transfer, the general order of decreasing effect appears to be TBT > TPhT > TPT > TET > TMT (Lynn, 1968; Watling-Payne and Selwyn, 1974; Skilleter, 1975; Wulf and Byington, 1975), which suggests a highly hydrophobic site of action of these compounds (Watling-Payne and Selwyn, 1974).

(2)Guanidine derivatives

Guanidine and its derivatives were the first inhibitors to be introduced which inhibited respiration coupled to phosphorylation without having any effect on the non-phosphorylating respiration of mitochondria (Hollunger, 1955). Subsequent research has revealed some differences in the effects of various guanidine derivatives, which suggests a somewhat different mechanism or site of action of these compounds.

Pressman (1963) showed that the effectiveness of alkylguanidines increases with increasing size of the alkyl substituent up to $C_{12}H_{23}$. They were found to differ in their effects from oligomycin in the following ways: (1) they are much more effective in inhibiting the phosphorylation at site I than at the other sites, (2) inhibition sets in more slowly, (3) although DNP reverses the inhibition by guanidines, it does not occur instantaneously, (4) octylguanidine (OG) inhibits the dicoumarol-stimulated respiration in the presence of oligomycin, and (5) the guanidines do not inhibit DNP-induced ATP-ase activity.

Whereas alkylguandines are specific for phosphorylation site I in mitochondria, other substituted guanidines possess specificity for different sites; phenylethylbiguanide is specific for site II (Pressman, 1962;) and synthalin (decamethylenediguanide) is specific for site III (Guillory and Slater, 1965).

Chappell (1963) showed that inhibition by galegine (methyl butenylguanidine) and hexylguanidine is greater on respiration limited by ADP or Pi, suggesting that these guanidines combine with a non-phosphorylated high-energy intermediate of oxidative phosphorylation. In contrast, oligomycin inhibits respiration equally well whether added in the active or the 'resting' (due to lack of ADP) phases of respiration.

From the foregoing, earlier workers concluded that the alkylguanidines act at a point closer to the respiratory chain than oligomycin. In support of this view was the observation that oligomycin almost completely inhibits the ATP-ADP exchange activity associated with site III phosphorylation (Wadkins and Lehninger, 1963), whilst alkylguanidines have little effect (Guillory and Slater, 1965).

More recent work has indicated that certain guanidine derivatives may have an indirect effect on oxidative phosphorylation. For example,

Schäfer (1974) suggested that the inhibition of oxidative phosphorylation by biguanidine derivatives is due to induced physical changes within the membrane phopholipids rather than a result of direct interaction with functional intermediates of energy conservation. Likewise, Davidoff (1974) has suggested that the cellular effects of biguanidine derivatives may be due to interference with mitochondrial Ca^{2+} transport. However, since it has been shown that the various classes of guanidine derivatives produce somewhat different effects in mitochondria, these proposed modes of action may not apply to all the alkylguanidines. Similarly, it has been shown that OG and synthalin inhibit K^+ transport in whole yeast cells at similar concentrations to those that affect respiration (Pẽna, 1973) but the rôle, if any, of this phenomenon in the inhibition of mitochondrial respiration has not yet been resolved.

Recently Gómez-Puyou et al. (1976) have shown that the more lipophilic alkylguanidines act directly on the F_1 ATP-ase complex in mitochondria. Similar inhibitory activities were obtained against ATP-ase activity in submitochondrial particles and soluble extracted F_1 particles. This suggests that unspecific interactions of alkylguanidines with the mitochondrial membrane are not responsible for their action on oxidative phosphorylation, particularly since inhibition of the ATP-ase activity could account for the inhibition of oxidative phosphorylation (Papa et al, 1975). This could also account for the differences in modes of action of alkylguanidines and oligomycin which acts at the level of the hydrophobic components of the mitochondrial membrane (Lardy et al, 1958). The fact that K^+ prevents the action of OG on F_1 (Gómez-Puyou et al, 1976) may be related to the antagonistic effects of this compound on K^+ transport and respiration in yeast cells (Pẽna, 1973). Likewise, the effects of histones on mitochondrial

respiration and swelling is considered to be related to their effect on mitochondrial K^+ transport (Johnson et al, 1966). Histone-induced swelling of mitchondria in the absence of ATP was inhibited by OG at concentrations which inhibit oxidative phosphorylation but not by uncouplers, such as CCCP and DNP, or electron transport inhibitors, such as amytal, azide or antimycin A (Schwartz et al, 1966). In addition, histone stimulated oligomycin - and aurovertin - inhibited respiration but had no effect on respiration inhibited by OG (Johnson et al, 1966). Consequently, the modes of action of histone and OG on respiration may be related through a common effect of these substances on K^+ transport in mitochondrial membranes.

The mode of action of guanidine derivatives on photosynthesis is much less well documented than their effect on mitochondria. Avron and Shavit (1965) found that OG was an uncoupler of photosynthetic electron transport but that non-cyclic photophosphorylation was more sensitive than cyclic photophosphorylation. The stimulation of non-cyclic electron transport was also much greater in the absence of Mg^{2+}, ADP and Pi than in their presence (Avron and Shavit, 1965).

Gross et al. (1968) found that synthalin was a photosynthetic energy transfer inhibitor but, unlike OG, was much more effective in the presence than in the absence of phosphate. The inhibition by synthalin was reversed by various uncouplers and the light-induced proton uptake was not affected at concentrations which inhibited phosphorylation, which suggested that synthalin acts on a later intermediate (Gross et al, 1968). However, the effect of synthalin resembled that of OG in having a greater effect on non-cyclic photophosphorylation than on cyclic photophosphorylation and also in the respect of a decreased inhibition of cyclic photophosphorylation with decreasing light intensities (Avron and Shavit, 1965; Gross et al.

1968). The latter indicates that the differential effects of synthalin and OG on non-cyclic and cyclic photophosphorylation were not due simply to a difference in the control rates of ATP synthesis in the two pathways.

Consequently it appears that the various classes of guanidine derivatives may differ in their mechanisms or site of action in the energy transfer chains of both mitochondria and chloroplasts. The marked site specificity of guanidines indicates that there may be a difference in the terminal steps of energy transfer between the cyclic and non-cyclic systems in chloroplasts and between the three ATP-conserving sites in mitochondria. This acts in favour of a theory involving purely chemical coupling as opposed to chemiosmotic coupling, particularly in view of further evidence of similar differential effects of uncouplers such as FCCP (Avron and Shavit, 1965).

(3) Ethidium bromide

The trypanocidal drug ethidium bromide (EB) inhibits DNA template function in bacteria (Waring, 1964; Richardson, 1973; Richardson and Parker, 1973) and inhibits the functioning of the DNA polymerase enzyme (Aktipis and Kindelis, 1974). The dye intercalates *in vitro* between base pairs of native double-stranded DNA (Le Pecq and Paoletti, 1967; Crawford and Waring, 1967) and RNA (Kreishmann *et al*, 1971; Aktipis and Martz, 1974; Jones and Kearns, 1975) and prevents the initiation of RNA chains catalyzed by DNA-dependent-RNA polymerase (Richardson, 1973; Richardson and Parker, 1973). In general, transcription of covalently closed circular DNA is more readily inhibited by EB than is the transcription of linear DNA (Richardson and Parker, 1973).

The phenotypic result of EB treatment varies to some extent depending on the cell type but generally includes a decrease in respiratory activity due to cessation of cytochrome oxidase activity

and cytochrome a-a_3 and b formation (Naum and Pious, 1971; Soslau and Nass, 1971; King et al, 1972; Mahler and Perlman, 1972; Sato et al, 1973; Klietman et al, 1973), lesions that are associated with the inner mitochondrial membrane. Ethidium bromide is also known to selectively degrade mitochondrial DNA (mt-DNA) (Goldring et al, 1970) and to induce respiration-deficient cytoplasmic mutations in facultative aerobes such as the yeast Saccharomyces cerevisiae (Slonimiski et al, 1968) by inhibiting de novo synthesis of respiratory enzymes (Mahler et al, 1971).

The effects of EB on the structure and function of mammalian cells (Soslau and Nass, 1971), protozoans (Meyer et al, 1972), algae (Flechtner and Sager, 1973; Nass and Ben-Shaul, 1973), and petite-negative yeasts (Crandall, 1973; Luha et al, 1974), are generally completely reversible under suitable conditions, whereas EB-induced petite formation in the petite-positive yeast S.cerevisiae may be genetically stable or reversible under certain conditions. For example, elevated temperatures reverse EB petite induction, which suggests that a heat-sensitive membrane - mt DNA- EB complex is involved (Perlman and Mahler, 1971). Consequently, the primary action of EB in petite induction may occur at the level of the mitochondrial membrane and this is further supported by the prevention of EB petite induction by anaerobiosis or treatment with oligomycin (Bech-Hansen and Rank, 1972). EB is known to bind to mitochondria to produce an energy-linked transition in the membrane (Azzi and Santato, 1971) and may alter the attachment sites of mt-DNA, which are known to occur on the mitochondrial membrane (Nass, 1969[ab]).

Bastos and Mahler (1974) have postulated that the mitochondrial energy coupling device provides a close and indispensable link between energy transduction by the mitochondrial membrane and the genetic

capabilities of mt-DNA. They proposed a hypothesis for the mechanism of action of EB in the petite-positive yeast S.cerevisiae, as follows: (a) When mt-DNA of a sensitive strain of the yeast is exposed in vivo to EB, it is converted to large (mass = 12.5 x 10^6 daltons) fragments containing the covalently linked dye, (b) This product is recognised and degraded by specific nuclease(s) with an absolute requirement for intramitochondrial ATP and which requires some components of the mitochondrial ATP synthetase (ATP-ase) system for its function. (c) Coincident with or as a consequence of (b), the mitochondrial ATP-ase becomes activated in a manner similar to the response of this system toward lipophilic uncouplers.

The latter point is interesting in view of reports concerning the effect of EB on the respiration of isolated mitochondria. Miko and Chance(1975) showed that EB stimulated state 4 respiration of pigeon heart mitochondria and also lowered the respiratory control ratio, stimulated the ATP-ase activity and partially released the inhibition of state 3 respiration by oligomycin. Earlier Razin and Mager (1964) had also shown that EB inhibited ATP-dependent purine incorporation into reticulocytes and red-blood corpuscles in a similar way to the uncouplers DNP and CCCP and that the reduction of internal ATP levels correlated with the reduction in purine incorporation. EB was also found to inhibit the ATP-P^{32} exchange reaction, stimulatethe mitochondrial ATP-ase activity and decrease the P/O ratio of rat-liver mitochondria (Razin and Mager, 1964). However, it is difficult at present to relate the effect of EB on respiration with its specific effect on mt-DNA since the response towards EB of mt-DNA from petite-positive and petite-negative cells is as yet unresolved. Bastos and Mahler (1974) are of the opinion that the specific mt-DNA degrading properties of EB are not elicited in petite-negative cells, whereas other workers

have provided contrary evidence (Whittaker et al, 1972; Flechtner and Sager, 1973). Likewise, it is difficult to compare the effects of EB on whole cell respiration in mammalian cells (Miko and Chance 1975), bacteria (Tomchick and Mandel, 1964) and yeasts (Pẽna and Ramirez, 1975) in view of the recent findings which implicate EB in a competitive inhibition of K^+ transport in S.cerevisiae cells and hence it mimics to some extent the effects of K^+ on whole-cell respiration (Pẽna and Ramirez, 1975).

Little work has so far been carried out on the effect of EB on chloroplast DNA (c-DNA). The DNA and ultrastructure of Acetabularia chloroplasts appear to be largely unaffected by high concentrations of the drug (Heilporn and Limbosch, 1971). Similarly, Nass and Ben-Shaul (1973) found that EB inhibited cell division in Euglena but only the ultrastructure of the mitochondria and not of the chloroplasts was affected. Although some chloroplast functions were inhibited, e.g. :- chlorophyll synthesis, the effect on mitochondrial function was much greater and could conceivably have been a direct cause of any effect on chloroplast function via a reduction in essential exports from the EB-sensitive mitochondria to the chloroplasts (Nass and Ben-Shaul, 1973). However, Flechtner and Sager (1973) reported an EB-induced reversible loss of c-DNA from C.reinhardii; growth of the alga for 1-2 doublings in the presence of sub-lethal concentrations of EB reduced the c-DNA to 15% of the control value followed by reappearance of the c-DNA during post-treatment growth in the absence of the drug. The decrease in c-DNA was apparently due to reduction in both synthesis of new c-DNA and degradation of pre-existing c-DNA (Flechtner and Sager, 1973). This correlates with the EB-sensitivity of in vitro DNA synthesis in chloroplasts of C.reinhardii (Ho et al., 1974). No loss of c-DNA genetic markers was observed, however, even with EB concentrations that

produced a 99.9% death rate in the cell population. The reversibility of the EB effect on c-DNA was suggested to be due to genetic redundancy and that EB probably preferentially degrades the reiterated sequences, leaving one or more intact copies (Flechtner and Sager, 1973). The presence of sequestered 'master-copies' has also been the interpretation for the reversible effect of EB on mt-DNA of the obligate aerobe Kluyveromyces lactis (Luha et al, 1971; Whittaker et al, 1972). However this is questionable in view of the results of Bastos and Mahler (1974), which indicates that the reversible nature of the EB-induced effects on petite-negative yeasts is due to the inability of the mt-DNA to form an intermediate in the series of reactions leading to DNA degradation.

(c) The Biology of C.reinhardii

(1) Cytology.

C.reinhardii is a unicellular, haploid, heterothallic green alga whose principal virtues as an experimental cell include ease of cultivation in the laboratory, a simple and rapid life cycle and well-defined Mendelian and cytoplasmic genetic systems. The algae exhibits many of the cytological structures of higher plant and animal cells and has been well characterised by several investigators (Sager and Palade, 1954, 1957; Schotz et al, 1972). The cells of C.reinhardii are typically ovoid in shape and approximately 8 x 15µm in diameter (Fig.1.3) with a plasma membrane, cellulose cell-wall and a polysaccharide-containing capsule. Two thirds of the cell is occupied by a posteriorly-located single green cup-shaped chloroplast bounded by a double membrane and containing a pyrenoid surrounded by a number of starch plates. The anterior third of the cell contains the nucleus, two large contractile vacuoles and two flagella, each of about 10µm in length. In addition, electron microscopical observations reveal the presence of mitochondria, endoplasmic reticula and Golgi bodies. The

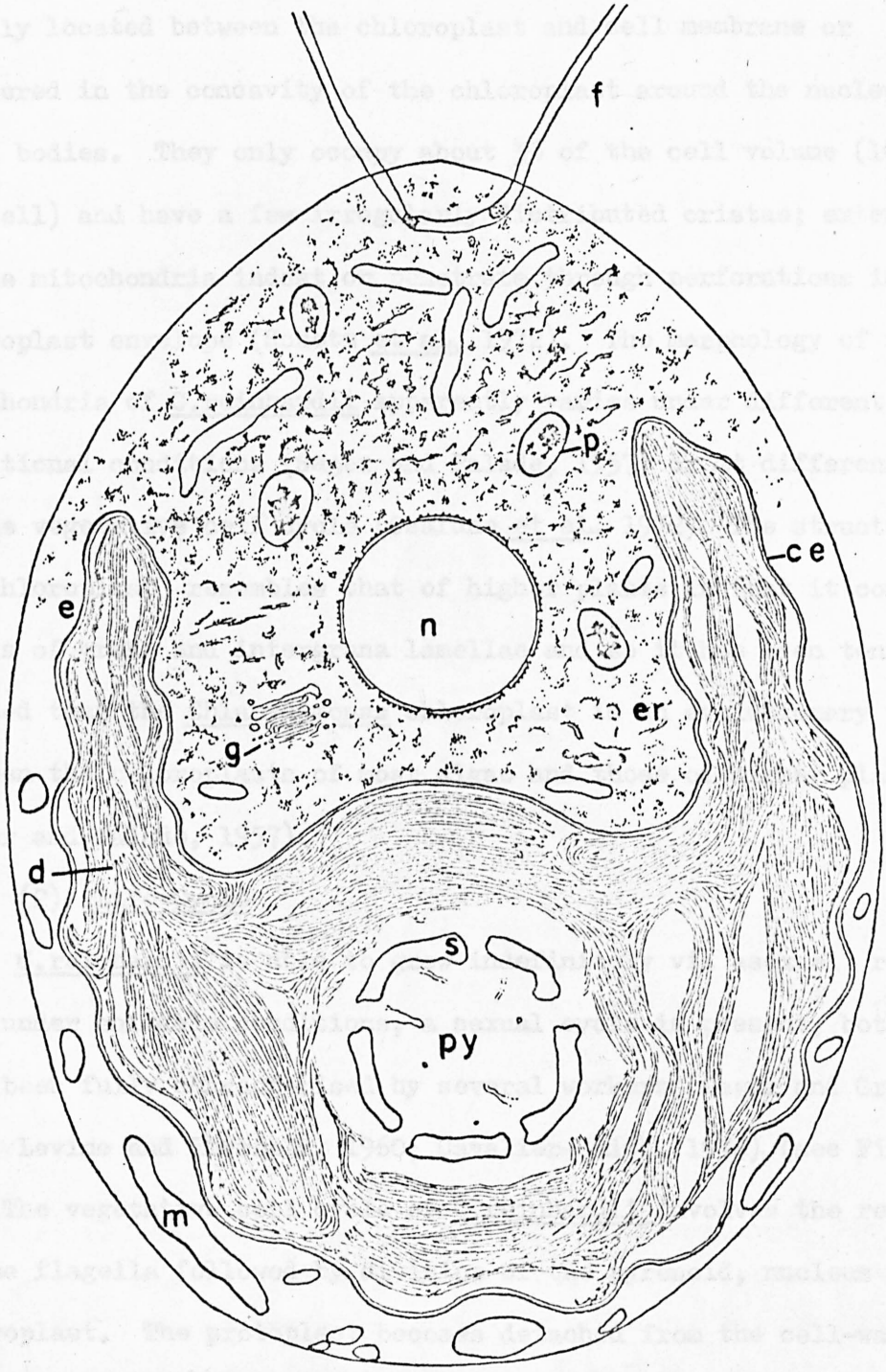


Figure 1.3. Diagram of the normal green cell of C. reinhardii as seen at low magnification in the electron microscope.

The chloroplast is shown surrounded by the double envelope(ce) within which the eye-spot (e), pyrenoid (py) and starch plates (s) are located. The cytoplasm also contains other organelle systems, including mitochondria (m), Golgi bodies (g), endoplasmic reticula (er) and vacuoles containing metaphosphate (p). The nucleus (n) is surrounded by a double membrane with pores and a dense coating of ribosomes on its outer surface. (From Sager, 1960).

mitochondria are generally rod-shaped or filamentous and are usually located between the chloroplast and cell membrane or scattered in the concavity of the chloroplast around the nucleus and basal bodies. They only occupy about 3% of the cell volume (10 - 15 per cell) and have a few irregularly distributed cristae; extensions of the mitochondria indent or penetrate through perforations in the chloroplast envelope (Sohotz et al, 1972). The morphology of the mitochondria of C.reinhardii apparently varies under different nutritional conditions (Sager and Palade, 1957) or at different stages in the vegetative cell cycle (Osafune et al, 1972). The structure of the chloroplast resembles that of higher plants in that it contains stacks of grana and intergrana lamellae and so it has been tentatively assumed that the Chlamydomonas chloroplast is an evolutionary transition between the chloroplasts of most algae and those of higher plants (Sager and Palade, 1957).

(2) Life-Cycle

C.reinhardii is able to grow indefinitely via asexual reproduction and, under suitable conditions, a sexual cycle is present; both cycles have been fully characterised by several workers (Sager and Granick, 1954; Levine and Ehersold, 1960; Cavalier-Smith, 1974) (see Fig.1.4).

The vegetative cell cycle of C.reinhardii involves the regression of the flagella followed by division of the pyrenoid, nucleus and chloroplast. The protoplast becomes detached from the cell-wall and eventually divides into 2,4 or 8 daughter cells, which grow two motile flagella and remain enclosed by the mother cell-wall until liberation occurs.

C.reinhardii is a heterothallic, haploid, isogametic alga and the two mating types are arbitrarily designated plus (mt^+) and minus (mt^-). Clones may be propogated indefinitely in the vegetative state

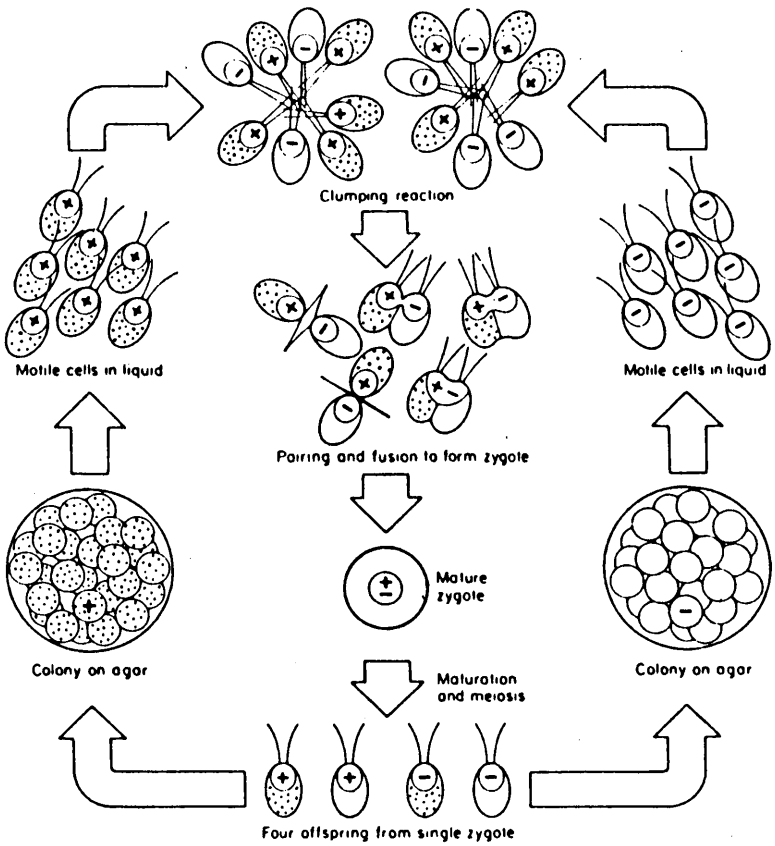


Figure 1.4. The life-cycle of C. reinhardii.

The diagram shows the segregation of the mating type, denoted by + and -, and a nuclear gene pair denoted by the presence and absence of shading. Pairs of cells of opposite mating type fuse to form zygotes and, after a period of maturation, the zygotes germinate with the release of 4 or 8 zoospores (meiotic products). (After Sager, 1960).

but when the two mating types are mixed under suitable conditions of nitrogen depletion and light intensity, fusions occur between single cells of opposite mating type, leading to binucleate zygote formation (Fig.1.4). If the zygote is placed in the dark for several days it does not divide vegetatively but developes a thick wall and undergoes meiosis to produce 4 or 8 haploid zoospore products capable of vegetative growth. Alternatively, if the zygote is immediately placed in continuous light, it may divide mitotically to give rise to stable diploid vegetative strains which behave phenotypically as mt^- (Gillham, 1963; Ebersold, 1967).

(3) Genetic Systems

Two clearly defined genetic systems have been characterised in C.reinhardii. Genes belonging to one system exhibit typical Mendelian inheritance and fall into 16 linkage groups (Levine and Goodenough, 1970), but genes belonging to the other system exhibit uniparental (UP) inheritance (Sager, 1954, 1972; Gillham, 1969).

Mendelian System

Over 100 mutations have been obtained in C.reinhardii which are transmitted in a Mendelian fashion, i.e. :- show 1:1 segregation of parental allele pairs in crosses. These genes are responsible for functions related to photosynthesis (Levine, 1969; Levine and Goodenough 1970), the flagellar apparatus (Starling, 1969; McVittie, 1972), amino-acid metabolism (Eversole, 1956; Loppes, 1969[a]), cell-wall biogenesis (Davies and Plaskitt, 1971; Hyams and Davies, 1972), the vegetative cell-cycle (Howell and Naliboff, 1973) and drug-resistance (Sager, 1954).

There is some doubt as to the exact number of haploid chromosomes in the nucleus of C.reinhardii since various estimates of 8 (Buffaloe, 1958; Levine and Folsome, 1959), 16 (Wetherell and Kraus, 1956; Mc Vittie and Davies, 1971) and 18 ± 2 (Schaechter and De Lamater, 1955) have

been reported. The number of nuclear linkage groups in C.reinhardii appears to be 16 (Levine and Goodenough, 1970), which agrees with a haploid chromosome number of 16.

It has been suggested by Levine and Goodenough (1970) that up to 8 of the 16 Mendelian linkage groups may be located in the chloroplast rather than the nucleus. This hypothesis was based on the fact that only 8 chromosomes were detected in the nucleus of C.reinhardii (Levine and Folsome , 1959) and that 8 of the 16 known linkage groups contain genes responsible for photosynthetic function, which contrasts with the even distribution of mutations to auxotrophy and non-motility over all the linkage groups. Additional evidence was based on the assumption that c-DNA must be inherited biparentally if the experimental findings of Chiang(1968) on the meiotic transmission of both parental c-DNA's were valid (Levine and Goodenough, 1970). However, Mc Vittie and Davies (1971) have pointed out that there is ample evidence for the presence of 16 nuclear chromosomes in C.reinhardii and the work of Sager and Ramanis (1967) shows that non-Mendelian genes can indeed be inherited biparentally. In addition, if half the genome of C.reinhardii was located in the chloroplast, the amount of c-DNA should approximate that of the nucleus when in fact it only constitutes 6-14% of the total (Sager and Ishida, 1963; Chiang and Sueoka, 1967). Another piece of evidence against the hypothesis of Levine and Goodenough is that if the 8 Mendelian linkage groups involved in photosynthesis resided in the chloroplast then 1:1 segregation would have to occur in the C-DNA during meiosis. This could only occur if there is only one copy of each gene in the plastid (Kirk, 1972) and biochemical evidence indicates that there are many copies (Bastia et al, 1970, 1971; Wells and Sager, 1971).

Consequently it can be concluded that the available evidence

indicates that the 16 known Mendelian linkage groups of C.reinhardii probably reside in the nucleus and that they are responsible for a variety of cellular functions in this alga.

Uniparental System

Non-Mendelian mutations exhibiting UP inheritance in C.reinhardii were first recognised by Sager (1954), who found that mutants resistant to 500 µg/ml streptomycin (sr-2 mutation) were inherited uniparentally, whereas mutants resistant to 100 µg/ml of the antibiotic (sr-1 mutation) were transmitted in a Mendelian fashion in crosses. Later work produced a series of antibiotic-resistant mutants that show UP inheritance and these have been arranged together in a linkage group or chromosome, which is probably circular (Sager and Ramanis, 1971). In crosses, the mt^+ or maternal parent transmits its UP markers to all four meiotic products in 90% or more of the zygotes (maternal zygotes). UP genes from the mt^- or paternal parent are transmitted in 10% or less of the zygotes (exceptional zygotes). Two kinds of exceptional zygotes occur viz., paternal exceptional zygotes, which only transmit UP genes from the mt^- parent, and biparental exceptional zygotes, which transmit UP genes from both parents to the meiotic products (Sager, 1972). Unlike Mendelian genes, which only segregate during meosis, UP genes segregate during both the meiotic and postmeiotic mitotic divisions (Gillham, 1969; Sager, 1972). Studies on the segregation and recombination of UP genes in biparental zygotes and their progeny (Sager and Ramanis, 1965), together with experiments on the inheritance of c-DNA in maternal zygotes (Sager and Lane, 1972), have led to explanations of UP inheritance in C.reinhardii (Sager, 1972; Gillham et al., 1974).

There is some evidence that UP genes are localized on c-DNA but this has not been rigorously proven and a thorough understanding

of the UP system has been hampered by lack of success in defining the precise cellular location(s) of the UP genome(s). C.reinhardii chloroplasts were originally shown to contain DNA by Ris and Plaut (1962) and Sager and Lane (1972) showed that, during mating, the β-band (chloroplast) DNA from the mt^+ parent is preserved and replicated, while that from the mt^- parent is degraded soon after zygote formation. The latter results, however, could not be confirmed by Chiang (1968, 1971) whose work indicated that the c-DNA from both parents is passed on through the zygotes to the zoospores. In addition, mitochondria have not been eliminated as a possible site for UP genes (Schimmer and Arnold, 1970; Behn and Arnold, 1973), particularly since they are known to contain DNA (Ryan et al., 1974^{ab}). If UP genes do occur on c-DNA then there are probably many copies of the same genes. Studies on the renaturation kinetics of C.reinhardii c-DNA indicate that its information content is about $1\text{-}2 \times 10^8$ daltons (Wells and Sager, 1971; Bastia et al., 1971), which suggests the presence of 20-30 copies of the genome per chloroplast and information sufficient to code for the structures of 165-330 proteins of average molecular weight 40,000 (Kirk, 1972).

It can be concluded that C.reinhardii has at least two genetic systems, a Mendelian and a UP system. The major difference between the two systems appears to lie in the rules governing their inheritance, segregation and recombination. The Mendelian system probably resides in the nucleur DNA and the UP system may reside in c-DNA but this has not yet been fully determined. If the UP system proves not to reside in c-DNA, it may be localised in mt-DNA, in a (minor) species of c-DNA other than β-band DNA, or in some other satellite DNA.

CHAPTER 2

Growth Experiments on C.reinhardii

Introduction

C.reinhardii is an obligate aerobe that can grow in the light with CO_2 as the sole carbon source (phototrophic growth), in the dark with sodium acetate as the carbon and energy source (heterotrophic growth) or in the light in the presence of both acetate and CO_2 (mixotrophic growth). The alga is an 'acetate organism' i.e., it grows best in acetate solutions but not on many other organic acids or sugars (Sager and Granick, 1953; Stross, 1960), which is probably due to impermeability of the cells to organic molecules larger than acetate (Hutner and Provasoli, 1951).

Numerous Mendelian mutations are known in C.reinhardii which cannot grow phototrophically because they are unable to carry out one or more of the reactions associated with photosynthesis (Levine and Goodenough, 1970). On the other hand, heterotrophic and mixotrophic growth can be obtained without these functions. Since C.reinhardii is an obligate aerobe, heterotrophic growth is not possible without respiration but it is not altogether clear whether this is also true of mixotrophic and phototrophic conditions. It may be that photophosphorylation provides sufficient energy to make growth possible without mitochondrial respiratory functions. Previously defined growth conditions may, therefore, be used as approximate guides to the differential functioning of mitochondria and chloroplasts in cells of C.reinhardii.

It was considered important in two respects to characterise the growth of C.reinhardii under different conditions. Firstly, it may provide more insight into the functioning of mitochondria and chloroplasts under different growth conditions, and secondly it was

necessary to fully characterise the batch growth of C.reinhardii under the same standardised conditions as employed for subsequent growth and biochemical experiments.

The second part of Chapter 2 deals with the effect of several inhibitors on the growth of the alga, which gave an indication as to which drugs would be useful for the isolation of drug-resistant mutants.

Materials and Methods

Strains, Media and Growth of C.reinhardii

(a) General growth techniques

The mt^+ and mt^- strains of C.reinhardii (stock 137c) were originally obtained from Professor R.P. Levine via Professor D.R. Davies. Except where indicated in the text, the alga was grown on a modification (Davies, personal communication) of the minimal medium of Sueoka (1960) (Appendix Table 1), either alone (M medium) or supplemented with 24.5mM sodium acetate (A medium) and/or yeast extract and peptone (YAP medium). For growth on solid medium, the liquid medium was supplemented with 1.5% Difco bacto-agar prior to autoclaving for 15 min at 15 lbs/sq.inch. All working and stock cultures of Wt and mutant strains were grown on YAP or A media and stored in the dark at +4°C. These cultures were routinely sub-cultured every 3 weeks.

Growth in the light was achieved by placing the plates or flasks in a fume cupboard fitted with a thermostatically-controlled fan heater (Xpelair) which maintained the ambient temperature at 25°C. Illumination was from two sides and from above by Philips white fluorescent lamps which gave a light intensity of approximately 4000 lux at the vessel or plate surface; agar plates were arranged on a series of transparent perspex shelves.

For growth in liquid medium, a large loopful of inoculum from a healthy plate culture was aseptically transferred to a 100 ml volume of liquid A medium in a 250 ml Erlenmeyer flask and shaken for 72 h to early stationary phase on a mark V orbital shaker (L.H. engineering). This 'starter' culture was then either used directly or, for larger-scale growth, transferred in 75 ml aliquots (5% inoculum) to 2 l Erlenmeyer flasks containing 1.5 l A medium and grown for a further 72 h to early stationary phase.

(b) Determination of growth curves

Growth in liquid media was assessed by aseptically transferring a 2.5 ml inoculum of 'starter' culture to 50 ml of medium in a 500 ml Erlenmeyer sidearm flask and shaken at 250 revs./min. Growth rates were routinely measured in terms of increased optical density using an Eel Colorimeter (Evans Electroselenium Ltd.). Readings were taken at 520 nm, thus using the instrument as a modified turbity meter.

For routine liquid phototrophic growth, the cultures were aerated by passing a mixture of 95% air and 5% CO_2 through a Dreschel bottle containing sterile distilled water and then through a length of sterilised glass tubing containing a small wad of non-absorbent cotton wool and fitted into a rubber bung. Liquid heterotrophic growth was carried out in a light-proof Gallenkamp orbital shaker maintained at a constant temperature of 25°C, whilst heterotrophic growth on agar plates was carried out in a Gallenkamp constant temperature incubator at 25°C.

Calculation of growth rates and extents in liquid culture.

Growth rates in liquid culture were expressed as the reciprocal doubling time ($\frac{1}{t_d}$). The population doubling time was determined, according to Monod (1949), either directly from a $\log_2$ plot of OD or from the following formula :-

$$t_d = \frac{t_2 - t_1}{\log_2 b_2 - \log_2 b_1}$$

where b_2 is the final and b_1 the initial OD reading during the time interval t_2-t_1 .

Optical density was approximately proportional to DW of the cultures (Fig.2.11). Growth extents were calculated by subtraction of the initial OD at time t_1 of a liquid culture from the final OD at time t_2 .

Dry weight, chlorophyll and protein estimates.

Dry weights of whole cells were measured by vacuum filtration onto pre-weighed Whatman GF/C glass fibre filters and dried to constant weight in an oven at 110 - 120°C for 2-3 days.

Chlorophyll was estimated according to the method of Arnon (1949).

Protein determinations on chloroplast or whole-cell preparations were carried out by the method of Lowry et al. (1951) after extraction of the chlorophyll. 0.1 ml of chloroplast suspension was diluted with 20 ml 80% acetone and centrifuged at 1.5×10^3 $g_{av.}$ for 5 min. The green supernatant was decanted off and the protein residue resuspended in 1.0 ml distilled water and re-centrifuged at 1.5×10^3 $g_{av.}$ for 5 min. The final bleached residue was then used for protein determinations. It was found that, by this method, 80% of the protein was recovered after washing in water and so each final protein estimate was corrected for by a factor of 1.25.

pH Measurements

pH changes during batch culture of C.reinhardii were measured with a Pye pH meter (model 79) fitted with a Pye Ingold combined glass and reference pH electrode (type E07). The electrode was calibrated against standard phosphate buffer (pH 7.0) and the culture was stirred continuously throughout the readings. Uninoculated sterile control flasks showed no change in pH during the period of the experiment.

Total cell numbers

Total cell numbers were obtained by serial dilution of the cell suspension to approximately 1×10^6 cells per ml. One drop of a 36% formaldehyde solution per 10 ml of suspension was then added to immobilise the cells and the suspension shaken. Quadruplicate counts were taken with a Neubauer counting chamber.

Viable cell numbers

Viable cell numbers were estimated by aseptic serial dilutions

of the growing culture in fresh sterile A medium. Replicate plates were spread with 0.1 ml of a suitable dilution to give approximately 500 cells per plate. The agar plates used were of the same type (A or M medium) as the growing culture and they were incubated under phototrophic, mixotrophic or heterotrophic conditions depending on the source of the test culture. Colony counts were assessed after 1 week under phototrophic and mixotrophic conditions or 2 weeks under heterotrophic conditions.

Packed cell volume

Packed cell volumes were measured by accurately pipetting a known quantity of cell culture into graduated haematocrit tubes. Sufficient volume of culture was added to give readings of at least $2mm^3$ and replicate tubes were centrifuged at 1.5×10^3 $g_{av.}$ for 5 min in a BTL bench centrifuge.

(c) Effect of inhibitors on growth

The effect of drugs on growth in liquid media was carried out as for the determination of growth curves in liquid culture except with the addition of the drug after autoclaving and cooling the media to room temperature. The Eel colorimeter was adjusted to zero with blank tubes containing sterile medium and drug only.

Inhibitor-containing agar plates were prepared by adding the drug to autoclaved media after cooling to $50^{\circ}C$. Equal quantities of the stock drug solvent were added to the control and drug media. All plates were used within 24 h of pouring. Early stationary-phase cells from mixotrophically-grown 'starter' cultures were applied to the drug plates using a modification of the drop-out technique (Wilkie and Lee, 1968). The apparatus used consisted of a tray into which 27 aluminium test-tube caps had been cemented so as to conform to the size of a 9 cm petri dish. These trays were sterilised for 15 min

at 15 lbs /sq.inch and, after cooling, 1 ml of the culture was placed in each cap. The replicator consisted of a perspex block holding 27 metal rods fixed in the same pattern as the test-tube caps in the tray. The cell suspensions were inoculated onto the surface of the solid media using the flame-sterilised replicator. A wide range of drug concentrations were tested and the sensitivity of C.reinhardii to each drug was then defined as the minimum concentration of drug which inhibited growth after 1 weeks incubation in the light or 2 weeks incubation in the dark. The extent of colony growth was assessed on a 0 to 4 point scale where 0 represented no growth and 4 represented excellent growth. The minimum inhibitory concentration (MIC) was taken as the concentration at which point 1 growth occurred or the range between the concentrations at which 0 point growth and 2, 3 or 4 point growth occurred. At least three determinations of the MIC were made in order to obtain a reasonably accurate estimate.

Results

(a) Growth characteristics of C.reinhardii during batch culture

The characteristics of batch cultures of C.reinhardii grown under various trophic conditions are shown in Figs. 2.1 to 2.11. The results show a general sigmoidal relationship between time and many of the parameters used to measure growth. Figures 2.1 and 2.2 show that aerating batch cultures of C.reinhardii with a mixture of air and CO_2 greatly enhanced phototrophic growth but had little effect on mixotrophic or heterotrophic growth. Subsequent experiments (Figs.2.3 to 2.11) were carried out with aeration of phototrophic cultures only and they show that, in general, the growth rates and extents generally decreased in the order mixotrophic > phototrophic > heterotrophic.

Optical density measurements of phototrophic and mixotrophic growth revealed a lag phase of approximately 20 h before the beginning of the exponential phase of growth (Figs.2.1 and 2.2), which appeared to be slightly longer than when growth was assessed using some of the other parameters, such as cell number. These cultures began to enter stationary phase at around 60 - 70 h when measured by OD but somewhat earlier, particularly with mixotrophic growth, when assessed by other parameters, such as chlorophyll, protein and cell numbers.

Heterotrophically-grown cultures had a much longer lag phase than the other growth conditions. In most cases this amounted to 40 - 50 h in length, although it was longer when assessment was by chlorophyll and packed cell volume and shorter when measured by cell numbers. Stationary phase in these cultures was attained after about 70 h (OD), although at a later time when chlorophyll, protein, packed cell volume and DW were estimated and at slightly earlier times when measurement was by cell numbers.

Optical density was related to increase in DW throughout the

Legend to Figures 2.1. to 2.10.

Growth characteristics of C. reinhardii during batch culture.

—— Phototrophic growth with no aeration.

•—• Phototrophic growth with 95% air : 5% CO_2.

▲—▲ Mixotrophic growth with no aeration.

×—× Mixotrophic growth with 95% air : 5% CO_2.

△—△ Heterotrophic growth with no aeration.

o—o Heterotrophic growth with 95% air : 5% CO_2.

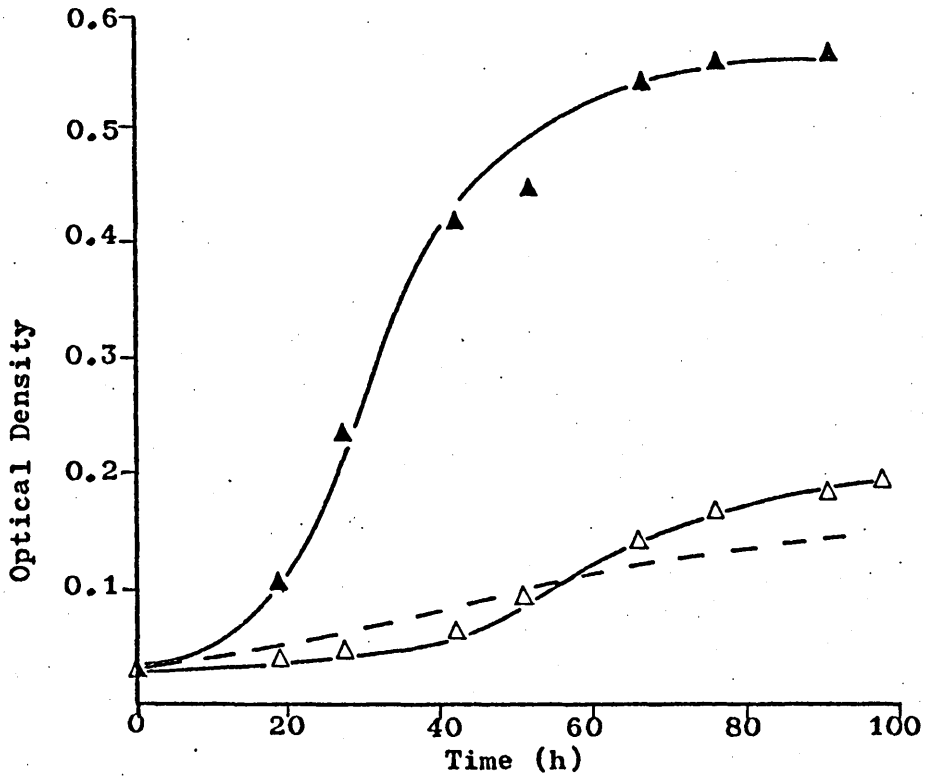


Figure 2.1. Increase in optical density during the batch culture of C. reinhardii.

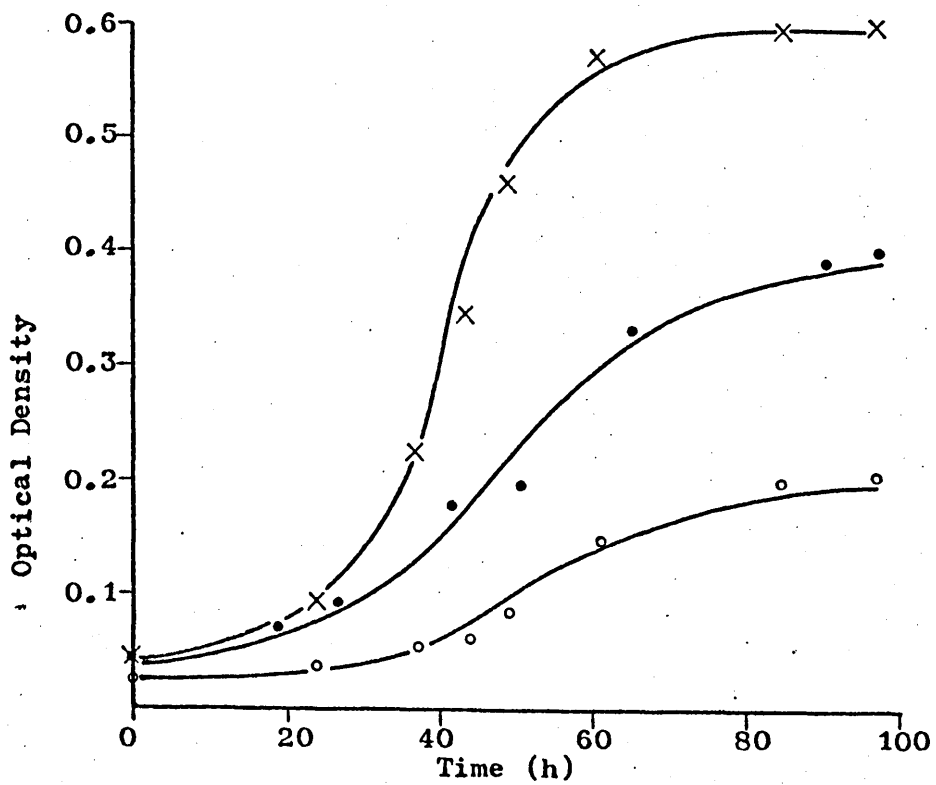


Figure 2.2. Increase in optical density of aerated batch cultures of C. reinhardii.

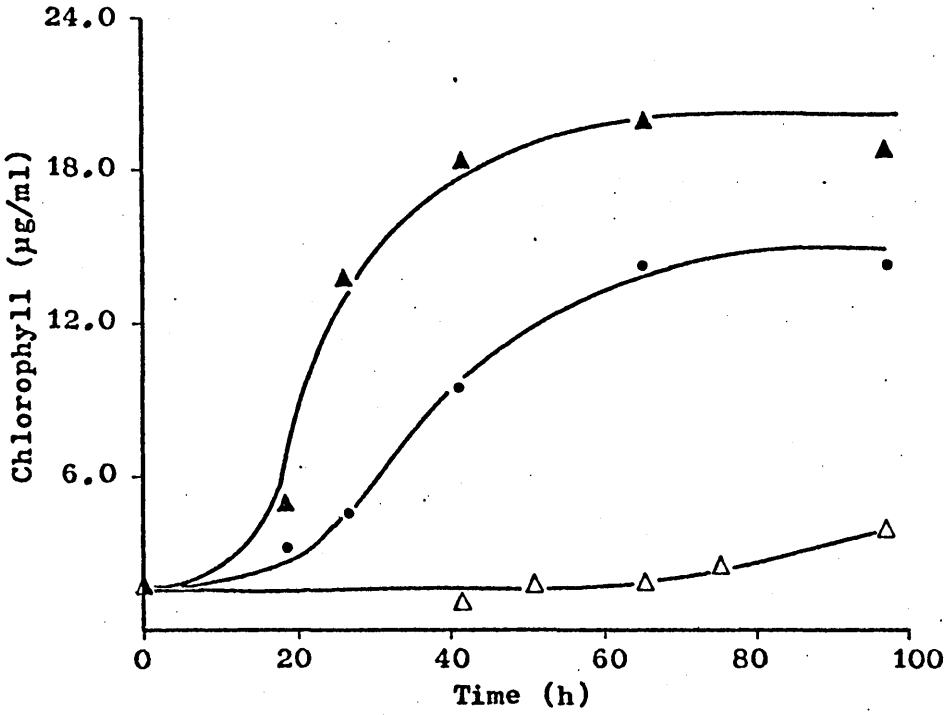


Figure 2.3. Increase in chlorophyll during the batch culture of C. reinhardii.

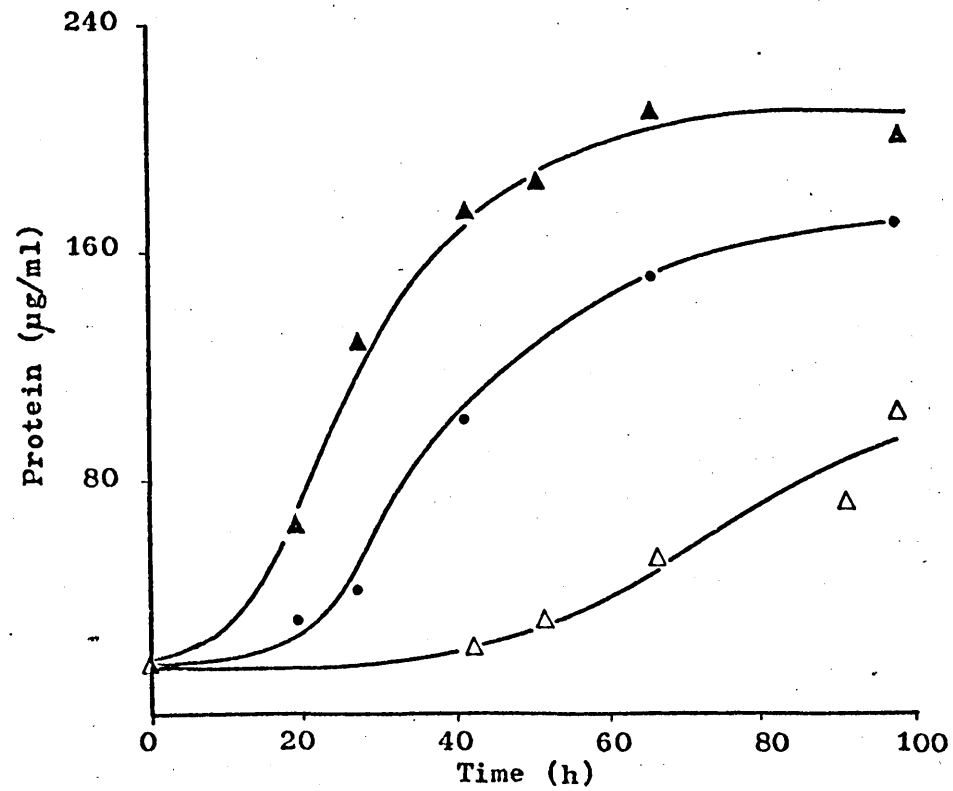


Figure 2.4. Increase in protein during the batch culture of C. reinhardii.

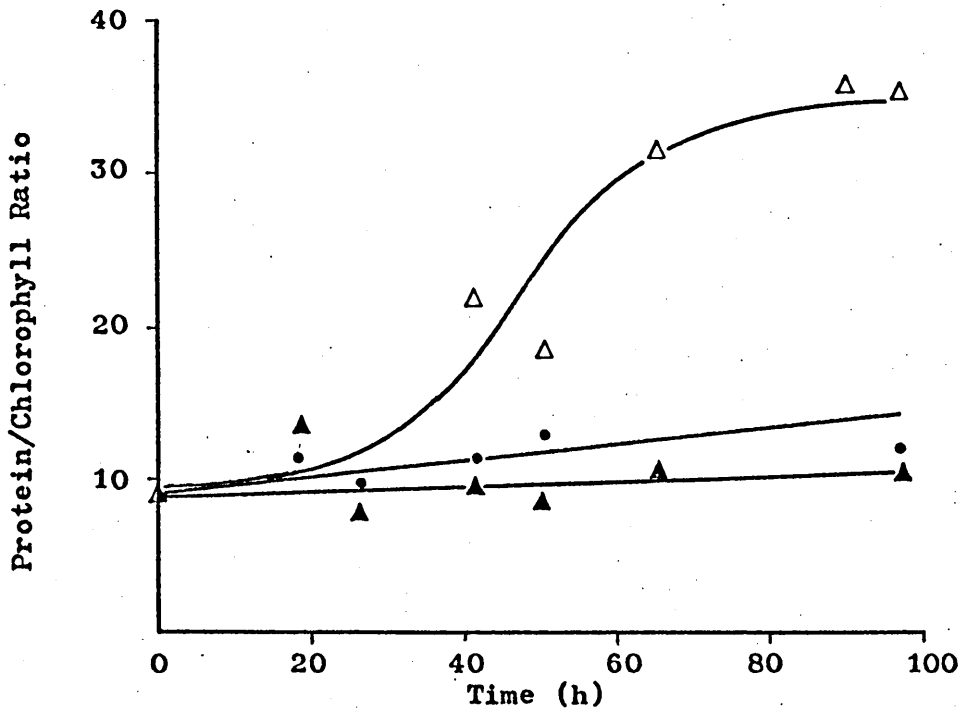


Figure 2.5. Changes in the protein/chlorophyll ratio during the batch culture of C. reinhardii.

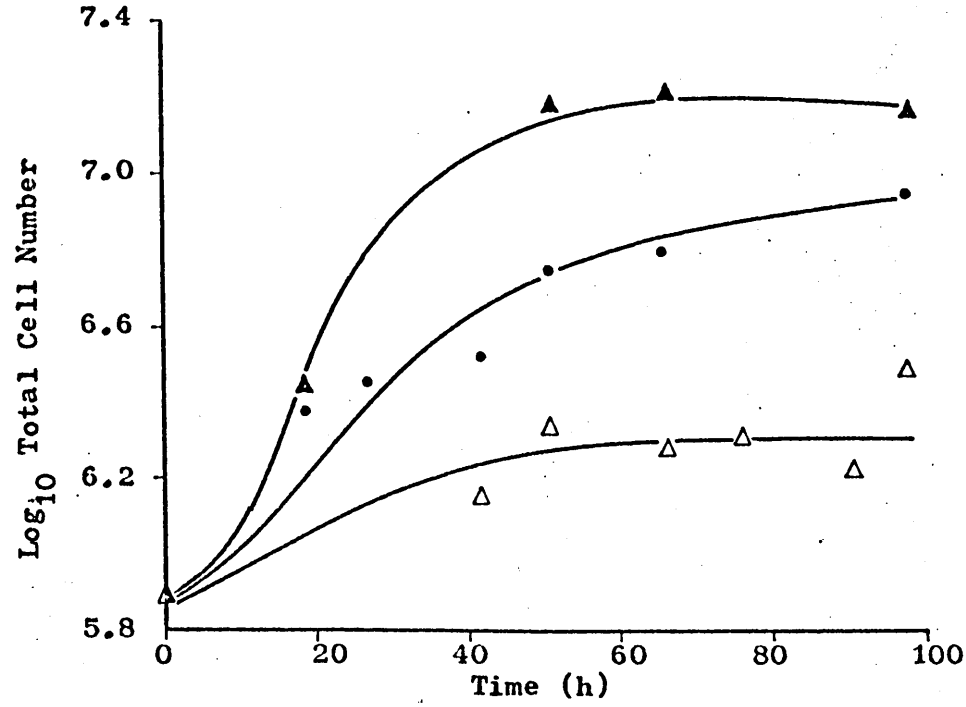


Figure 2.6. Increase in total cell numbers during the batch culture of C. reinhardii.

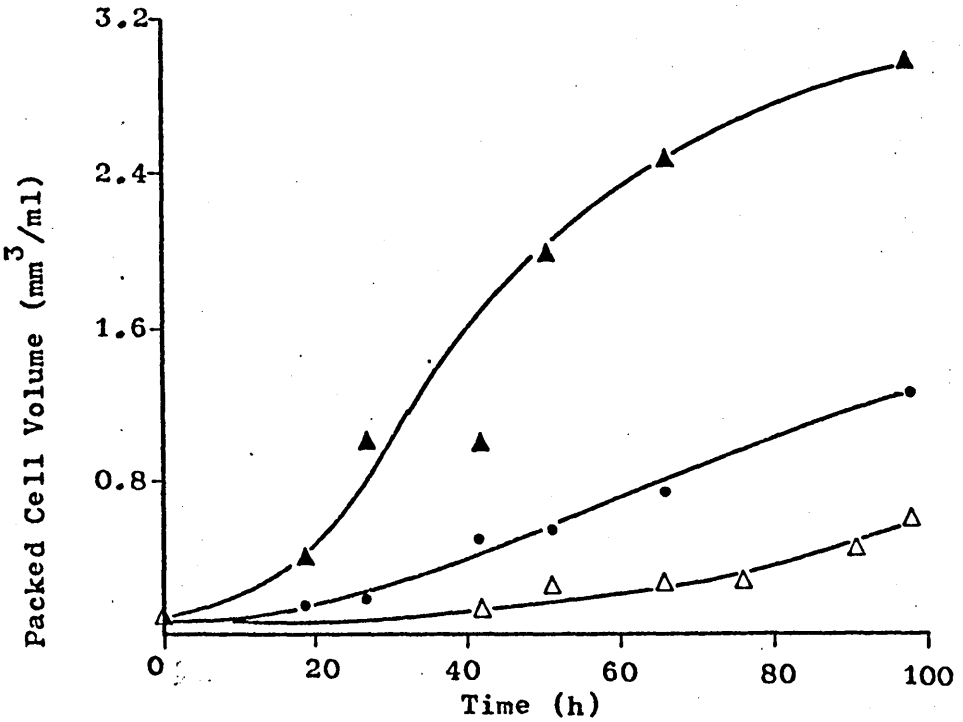


Figure 2.7. Increase in packed cell volume during the batch culture of C. reinhardii.

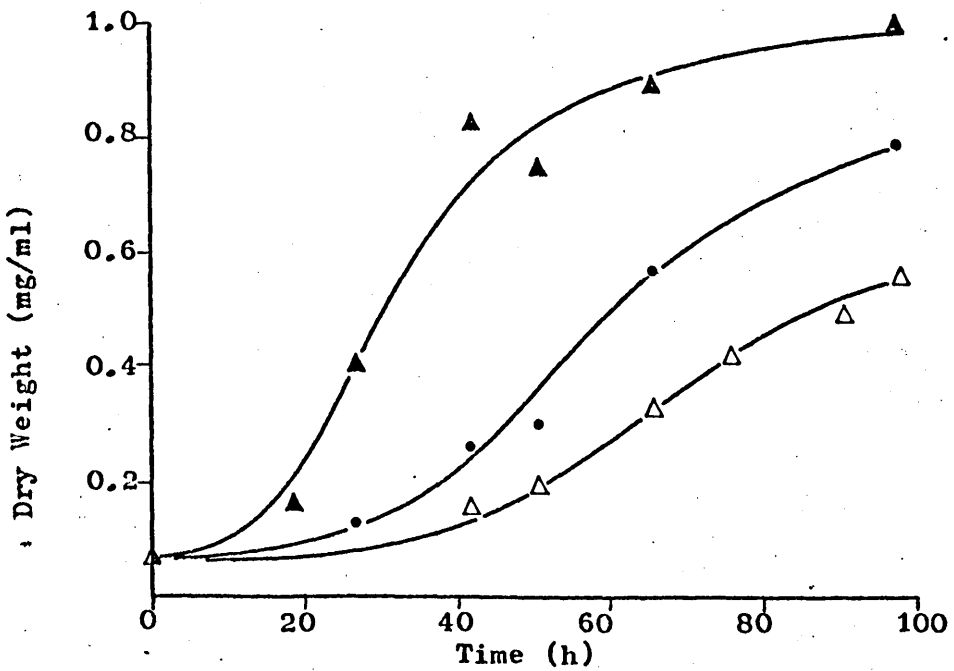


Figure 2.8. Increase in dry weight during the batch culture of C. reinhardii.

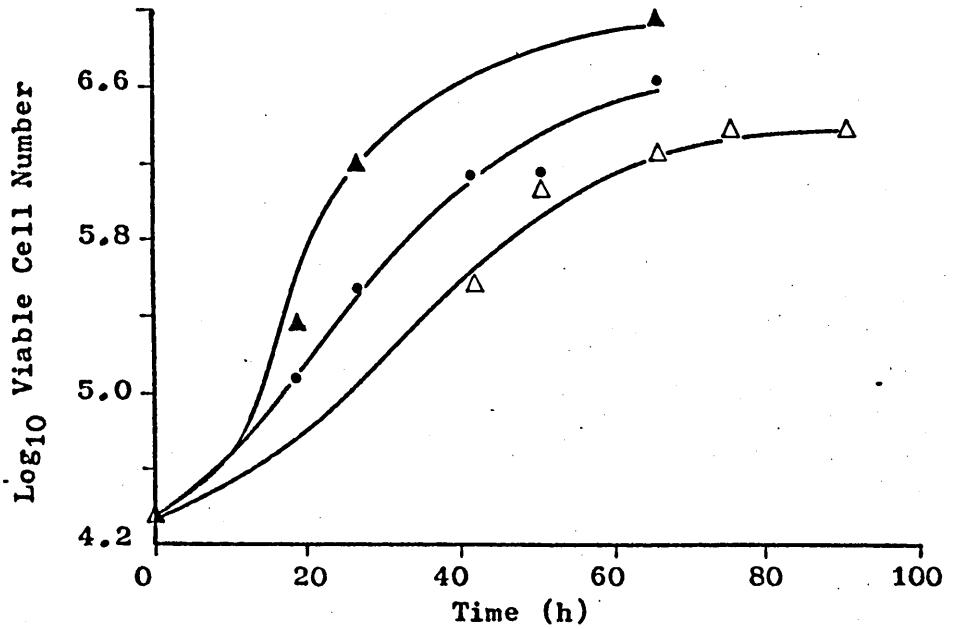


Figure 2.9. Increase in viable cell numbers during the batch culture of C. reinhardii.

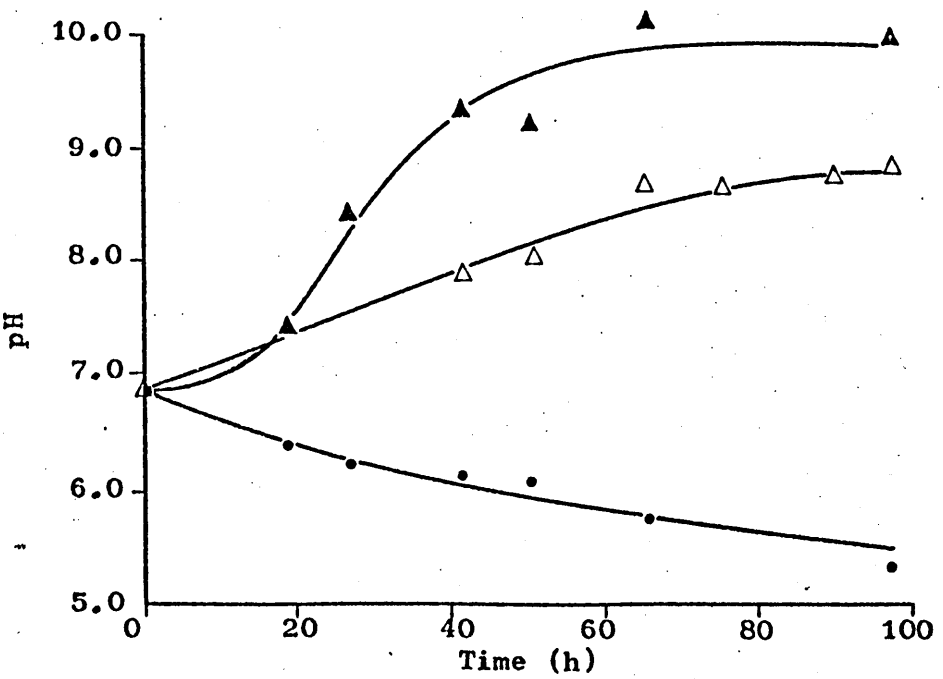


Figure 2.10. Changes in medium pH during the batch culture of C. reinhardii.

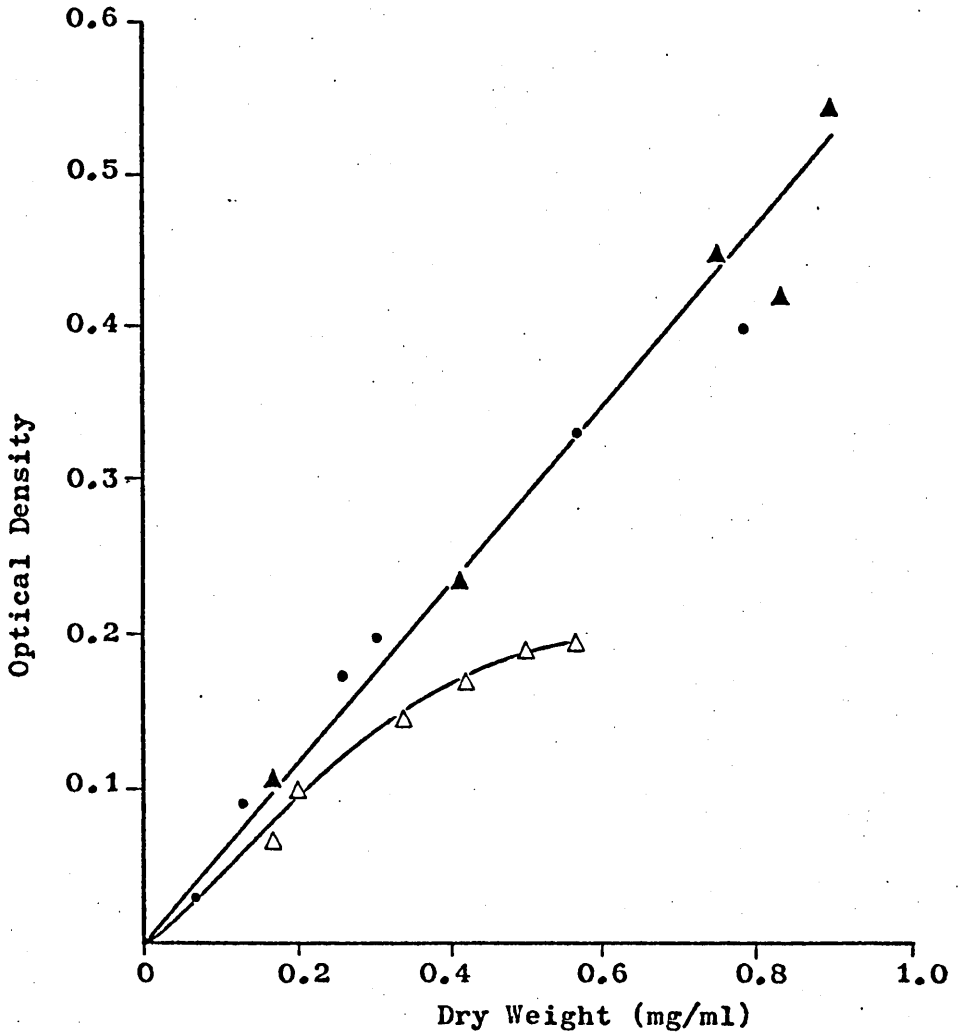


Figure 2.11. Relationship between the optical density and dry weight of batch cultures of C. reinhardii.

batch growth of the alga with a slight deviation in the case of heterotrophic growth (Fig. 2.11).

The protein/chlorophyll ratio of phototrophic and mixotrophic cells remained about constant around a value of 10 except for a slight increase during phototrophic growth (Fig. 2.5). Heterotrophic growth resulted in a marked sigmoidal increase in this ratio, reaching a maximum at stationary phase.

Marked pH changes in the media were found to occur during batch culture (Fig. 2.10). All three types of media had an initial pH of 6.9 and this changed to approximately 10.0, 8.8 and 5.3 for stationary phase mixotrophic, heterotrophic and phototrophic cultures respectively.

(b) Effect of sodium acetate on batch growth.

Figures 2.12 to 2.13 show the effect of different acetate concentrations on the growth characteristics of C.reinhardii in batch culture. A saturating acetate concentration of about 49 mM was found for the growth rates and extents during heterotrophic growth. Inhibition of either growth parameter was not evident at higher concentrations up to a concentration of 122 mM. Maximum growth rates and extents for mixotrophic cultures were produced by 24 mM and 49 mM respectively but higher concentrations produced growth inhibition. The final pH of the culture media after growth on different acetate concentrations is shown in Fig. 2.14. Heterotrophic growth produced an increase in pH with increased acetate concentrations up to a maximum pH of 9.25. A different trend occurred during mixotrophic growth with a maximum pH of 10.3 occurring at low acetate concentrations and then the final pH decreased at higher concentrations to give a final pH of 9.3 at 122 mM acetate. Phototrophic growth (no acetate) produced a decrease in pH.

Legend to Figures 2.12. to 2.14.

Effect of sodium acetate on the batch growth of C. reinhardii.

▲—▲ Mixotrophic growth .

Δ—Δ Heterotrophic growth .

Final (stationary phase) pH, growth rates and growth extents were measured simultaneously on the same batch cultures. The initial pH of each medium was 6.9.

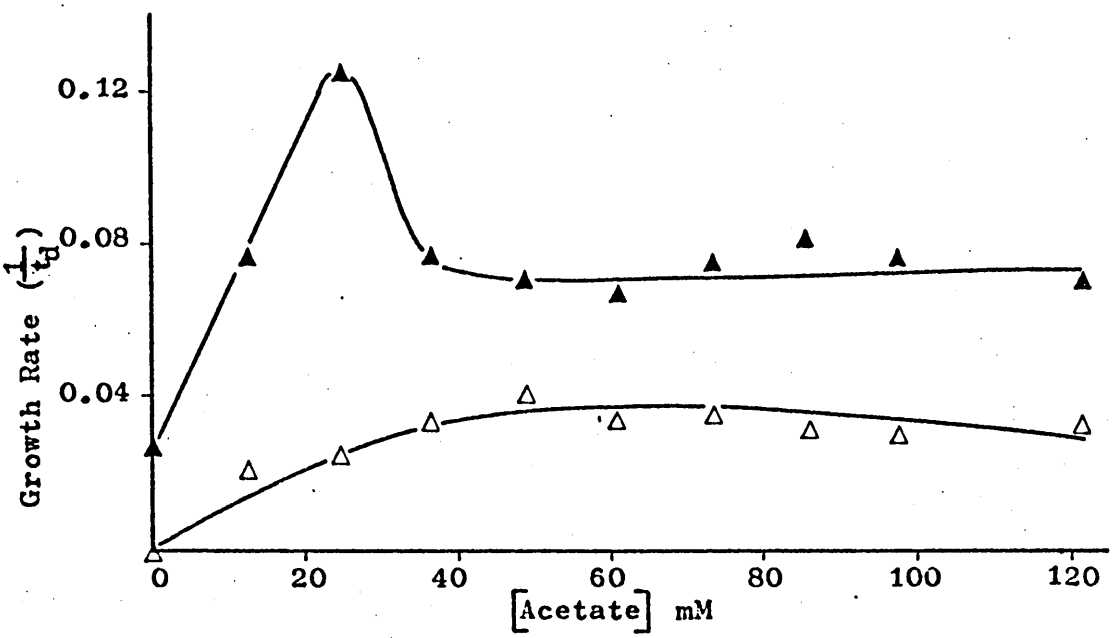


Figure 2.12. Effect of acetate concentration on the growth rate of C. reinhardii in batch culture.

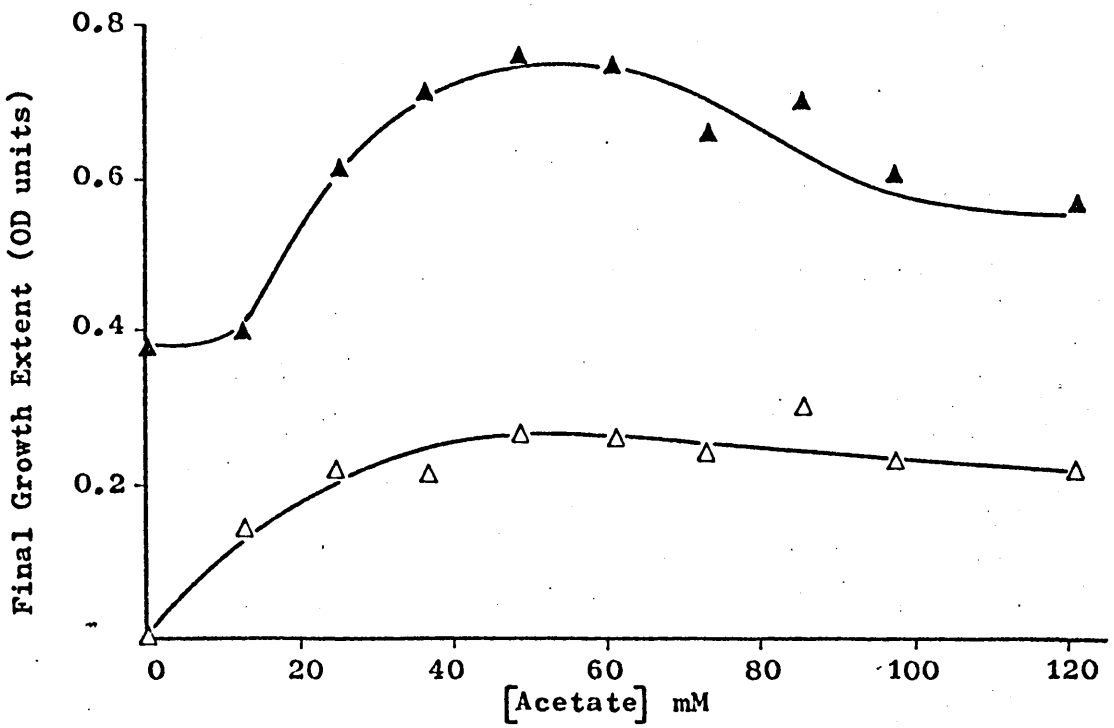


Figure 2.13. Effect of acetate concentration on the extent of growth of C. reinhardii in batch culture.

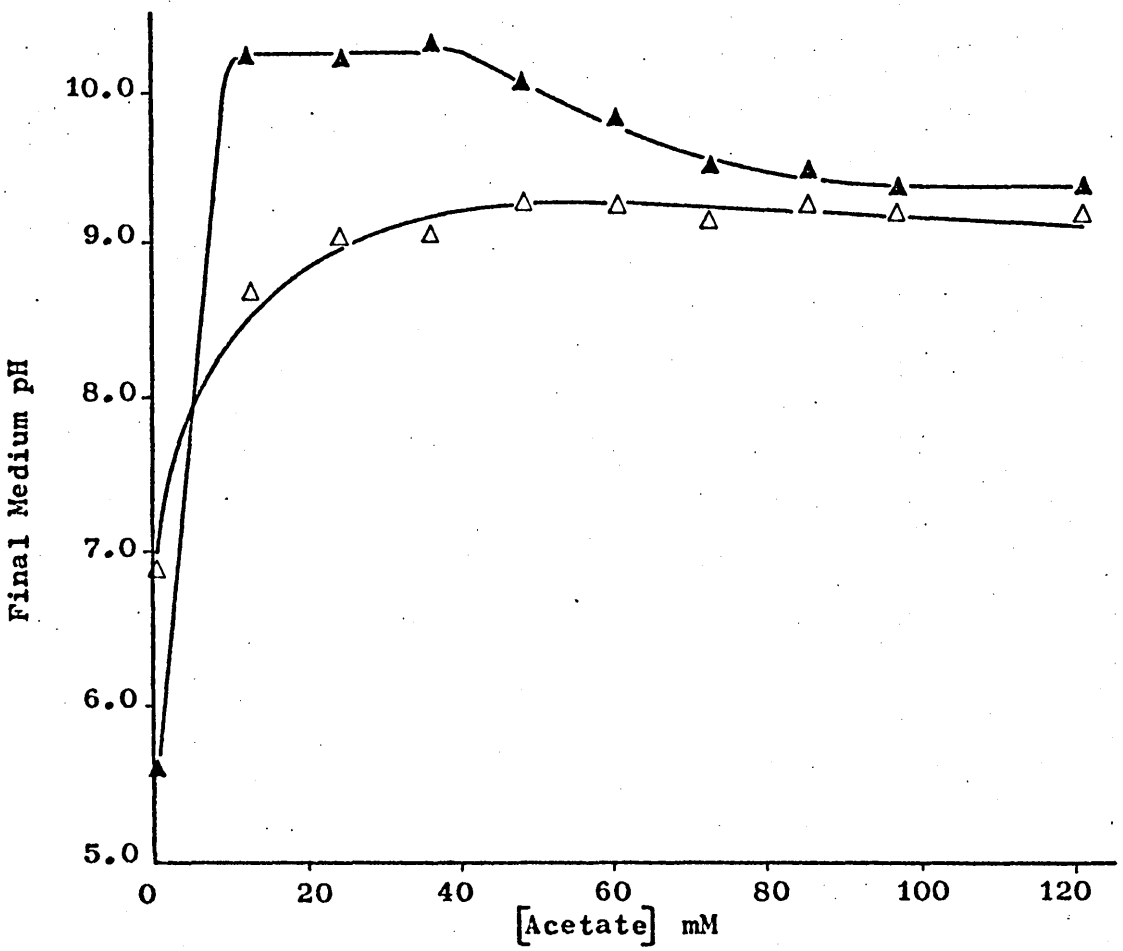


Figure 2.14. Effect of acetate concentration on the final pH of the medium during the batch culture of C. reinhardii.

(c) Effect of various inhibitors on growth on solid media.

The effect of various inhibitors on the growth of C.reinhardii on solid media is shown in Table 2.1. The compounds are arranged into loose groupings depending on their reported biochemical mode of action and their MIC values are only approximate within the range of the concentrations tested.

The results indicate that C.reinhardii was insensitive to low concentrations of many of the inhibitors although some, e.g. :- the triorganotins, alkylguanidines, EB and CTAB, inhibited growth at relatively low concentrations. Phototrophic growth appeared to be differentially inhibited by spectinomycin whilst heterotrophic growth was relatively sensitive to a wide range of the inhibitors, particularly valinomycin, TBT, TPhT, oligomycin, antimycin A, acriflavine, Dio-9, dicyclohexyl-18-crown-6 and robenzidine. The response of mixotrophic growth did not differ greatly from that of phototrophic growth except in the cases of spectinomycin and 1799, where it was more resistant, and with TMT, where it was more sensitive.

Legend to Table 2.1.

Effect of various inhibitors on the growth of C. reinhardii on solid media.

* The minimum inhibitory concentrations of the drugs are expressed in μM except where indicated.

+ P = Phototrophic growth.
M = Mixotrophic growth.
H = Heterotrophic growth.

Table 2.1

Compound \ Growth+	Minimum Inhibitory Concentration*		
	P	M	H
Energy transfer inhibitors			
TMT chloride	20.5	10.2	10.2
TET sulphate	3.9	3.5	3.0
TPT chloride	1.8	1.8	1.1
TBT chloride	1.2	1.2	0.3
TPhT chloride	1.0	0.8	0.1
TBT oxide	0.2 - 1.7	-	< 0.2
TcHT hydroxide	2.6	2.6 - 26.0	0.3 - 2.6
Octylguanidine	19.3	16.9	14.5
Dodecylguanidine	7.6	7.6	7.6
Galegine sulphate	> 113	113 - 284	425
Oligomycin	> 25 µg/ml	> 25 µg/ml	10 µg/ml
Phlorizin	> 229	> 229	> 229
Venturicidin	> 126	> 126	32 - 126
DCCD	121 - 242	> 242	> 242
Dio-9	25 - 50 µg/ml	25 - 50 µg/ml	1 - 5 µg/ml
Robenzidine	> 75	> 75	3 - 30
Aurovertin	> 10 µg/ml	> 10 µg/ml	1 - 5 µg/ml
Uncouplers			
DNP	> 1,358	> 1,358	> 1,358
Atebrin	> 106	> 106	> 106
CCCP	14.6	14.6	8.5
'1799'	90	> 128	51
Na Arsenate	> 1,603	> 1,603	> 1,603
Tetraphenylboron	> 292	> 292	> 292
TTFB	32 - 79	32 - 79	32 - 79
Adenine nucleotide translocase inhibitors			
Atractyloside	> 119	> 119	> 119
Rhodamine 6G	111 - 222	22 - 111	< 22

Table 2.1 (Continued)

Compound \ Growth+	Minimum Inhibitory Concentration*		
	P	M	H
Ionophores			
Valinomycin	>45	>27	9
Nigericin	5 μg/ml	7.5 μg/ml	5 μg/ml
Gramicidin	>50 μg/ml	>50 μg/ml	>50 μg/ml
Dicyclohexyl-18-crown-6	>269	>269	54
Electron transport inhibitors			
KCN	>3,840	>3,840	>3,840
Antimycin A	>18	>18	0.09 - 0.9
Amytal	>1,105	>1,105	>1,105
Rotenone	>127	>127	>127
Inhibitors of DNA function			
Proflavine	>314	>314	>314
Acriflavine	>100 μg/ml	>100 μg/ml	10 μg/ml
Acridine orange	>419	>419	140
Spectinomycin	45	60	60
Rifampicin	>358	>358	287
Ethidium bromide	19.0	19.0	12.7
Detergents			
SDS	173 - 347	347 - 694	173 - 347
Sodium deoxycholate	1,449	1,449	1,208
CTAB	7 - 14	14 - 27	<7
Miscellaneous			
Rhodamine B	21 - 104	21 - 104	21 - 104
Diethyltin dichloride	>403	>403	>403
Tetrabutyltin	29 - 144	3 - 29	3
Dioctyltin dichloride	>240	120 - 240	24 - 120
Dibutyltin diacetate	29 - 143	29 - 143	3 - 29
Cl-methyl-dibutyltin	79	79	79

Discussion

Growth of C.reinhardii in batch culture under various trophic conditions produced sigmoidal growth curves irrespective of the parameter used to measure growth. Growth was greatest in the presence of both sodium acetate and light (mixotrophy) but aeration with 5% CO_2 only significantly enhanced growth when acetate was absent in the light (phototrophy). Growth on acetate in the dark (heterotrophy) was much reduced compared to mixotrophic growth.

Since C.reinhardii is an obligate aerobe and possesses mitochondria, it would be expected that these organelles function during all growth conditions whilst the chloroplasts would only be able to function in photosynthesis during growth in the light. This could account for the reduced growth under heterotrophic conditions but the relationship between mitochondrial and chloroplastic functions during growth under different conditions does not appear to be a simple additive one in C.reinhardii. The effect of various combinations of light, acetate and CO_2 on the growth of the alga may be, at least partially, due to repression/derepression systems operating under various growth conditions in response to changes throughout growth of various environmental factors such as pH, light, nutrients, O_2 and CO_2.

Heterotrophic growth would appear to be wholly dependent on acetate as a substrate since starch reserves are not mobilized to a great extent in dark-grown Chlamydomonas species (Ricketts, 1972) and starch breakdown in the y - 1 mutant of C.reinhardii requires continuous illumination (Ohad et al., 1967). Consequently the stationary phase of heterotrophic growth may be caused largely by the exhaustion of acetate, whereas the lag phase may represent an adaptation to acetate as the sole carbon and energy source by increased synthesis or activation of the necessary enzymes for its utilization. This is

indicated by an increased activity of succinate dehydrogenase when the y - 1 photosynthetic mutant of C.reinhardii is transferred from mixotrophic to heterotrophic conditions (Ohad et al, 1967). In addition, the glyoxylate enzymes isocitrate lyase and malate synthase, which are considered to be required for growth on acetate (Haigh and Beevers, 1964) and have reduced activity during phototrophic growth, are preferentially synthesised prior to growth on acetate and increase 7 - 10 fold in activity during heterotrophic growth (Harrop and Kornberg, 1966). Likewise, high isocitrate lyase activity was detected in heterotrophically-grown cells of C.dysosmos, which can grow on acetate as the sole carbon and energy source, but was absent in mutant strains which were capable of respiring acetate but not capable of using it for heterotrophic growth (Haigh and Beevers, 1964).

The pH increase during heterotrophic growth was probably caused largely by uptake of acetate since the other factors which commonly cause pH increases in culture media, namely photosynthetic CO_2 and nitrate uptake (Pirt, 1975), do not occur in the dark in C.reinhardii (Thacker and Syrett, 1972[b]). This is also indicated by the fact that the growth extents of heterotrophic cultures on different acetate concentrations approximately parallel the final pH of the medium. The increase in the pH of the heterotrophic medium probably contributed in turn to the stationary phase in these cultures through restricted uptake, at high pH values, of charged acetate and mineral ions (Hutner and Provasoli, 1951).

The metabolism of phototrophically- and mixotrophically-grown cells probably differed a great deal from that of heterotrophically grown cells due largely to the influence of light. Light is known to inhibit respiration to some extent in C.reinhardii, either through competition of cyclic photophosphorylation for available ADP (Teichler-

Zallen et al, 1971) or through PSI oxidants acting as alternative sinks for respiratory electrons (Healey and Myers, 1971). This inhibitory effect on respiration probably occurs under both phototrophic and mixotrophic conditions since it is not modified by the presence of acetate (Healey and Myers, 1971). The longer lag phase of phototrophic compared to mixotrophic growth may have been partly due to the change from the mixotrophic conditions found in the 'starter' cultures to the phototrophic conditions in the assay flasks. The enhancement of phototrophic growth by CO_2 is likely to have been brought about partly through photosynthetic carbon fixation since RuDP carboxylase activity is consistently somewhat higher in phototrophic cells compared to mixotrophic cells (Togasaki and Levine, 1970; Harris et al, 1974;), perhaps due to a slight inhibitory effect of acetate on its activity. The increased growth of mixotrophic cultures compared to heterotrophic cultures probably arose via photosynthesis, utilizing atmospheric or perhaps respiratory CO_2.

The decrease in pH of phototrophic media during growth was probably due to uptake of ammonium ions from the medium which would be replaced by hydrogen ions (Pirt, 1975). The low pH values would also favour passive CO_2 uptake by the cells since dissolved CO_2 at acid values is primarily in the form of CO_2 and H_2CO_3, both of which are readily absorbed and utilized by algal cells for photosynthesis (Round, 1970). The stationary phase of phototrophic cultures was probably brought about by the limiting pH eventually attained in these cultures.

The development of alkaline conditions during mixotrophic growth means that the available carbon for photosynthesis would be in the form of bicarbonate ions (Round, 1970), which can be absorbed and utilized by C.reinhardii through the presence of carbonic anhydrase

induced by low CO_2 concentrations (Nelson et al, 1969). However, at pH 10 and above, carbonate anions predominate and these are not utilisable by algae (Round, 1970) and may have contributed largely to the stationary phase in mixotrophic cultures at this restrictive pH. The high pH values would also limit photosynthetic CO_2 fixation (Orth et al, 1966) and acetate uptake for respiration (Hutner and Provasoli, 1951). However, the effect of pH values greater than 9 would appear to be primarily on photosynthesis, as opposed to respiration, since the stationary phase of heterotrophic cultures occurs at about pH 9.

The metabolism of phototrophic and mixotrophic cells may also differ due to induction of photorespiration. High pH values (8 - 9), along with low CO_2 tension, high O_2 tension and high light intensity, are known to enhance glycolate formation in C.reinhardii, which may be excreted or metabolised further depending on the CO_2 available to the cells (Orth et al, 1966; Nelson and Tolbert, 1969, 1970; Cooksey, 1971). If only air is available, glycolate is formed and further metabolised, whereas aeration with 1% CO_2 results in excretion of the glycolate into the culture medium (Nelson and Tolbert, 1969; Cooksey, 1971). Some of the mixotrophic culture conditions, such as high O_2 tension, low CO_2 tension and high pH, may have been conducive for photorespiration but it would have been absent in dark-grown cells (Tolbert, 1974). Photorespiration may also have occurred in phototrophic cultures since it has been found that a medium pH of 4 - 6 may induce glycolate production and excretion by C.reinhardii during batch culture (Allen, 1956; Orth et al, 1966). If this is so then glycolate excretion may have contributed to the decline in the pH of the phototrophic culture medium and could have perhaps helped to neutralise the alkaline conditions found in mixotrophic cultures. It is unlikely that excreted

glycolate or sugars (Allen, 1956) would be reabsorbed and utilised by the cells during growth since 'acetate' organisms, such as C.reinhardii, are generally specific in their utilisation of acetate as a substrate (Droop, 1974).

The increase in medium pH during mixotrophic growth was probably mainly due to uptake of acetate by the cells but, since the final pH of the medium did not parallel the growth extents at low acetate concentrations (Figs. 2.13 and 2.14), there appears to be another factor involved. Selective uptake of nitrate ions relative to ammonium ions would not appear to be involved since, when ammonium nitrate is used as the nitrogen source for algae, ammonium ions are first absorbed followed by nitrate ions (Morris, 1974). It may be that uptake of bicarbonate from the medium, under conditions of limited CO_2 supply, enhanced the alkalinity. That light-induced uptake of bicarbonate by C.reinhardii cells is accompanied by a pH increase in the medium has been shown by Neumann and Levine (1971). This bicarbonate uptake for photosynthesis may be greater at the lower acetate concentrations and result in a high final pH even though the growth extent is low.

The lack of any inhibitory effect of acetate on heterotrophic growth (Figs. 2.12 to 2.14) suggests that high acetate concentrations affect photosynthesis as opposed to respiration. This inhibitory effect may not apply to photosynthetic oxygen evolution which is unaffected by the presence of acetate (Russel and Gibbs, 1966; Healey and Myers, 1971; Levine and Duram, 1973; Morris et al, 1974). That acetate may have a concentration-dependent effect on photosynthesis is indicated by the fact that the mixotrophic growth rate was inhibited at lower acetate concentrations (>24 mM) than was growth extent (>61 mM). Perhaps the lower acetate concentrations competitively inhibit the

rate of photosynthetic carbon uptake whilst higher concentrations directly inhibit the enzymes involved in CO_2 fixation, such as RuDP carboxylase. This is also suggested by the possible inhibitory effect of acetate on the activity of this enzyme (Togasaki and Levine, 1970; Morris et al, 1974), which if reduced or completely lost would also mean reduction or the complete loss of photosynthetic bicarbonate uptake and the associated pH rise in the medium (Neumann and Levine, 1971).

The mechanism of the possible inhibition of RuDP carboxylase activity is unknown but may be through control by enzymes of the glyoxylate cycle. These enzymes are normally absent or reduced in activity in phototrophic and mixotrophic cultures of Chlamydomonas spp. (Haigh and Beevers, 1964; Harrop and Kornberg, 1966) but high acetate concentrations may override the inhibition of these enzymes, which may in turn control photosynthetic carbon metabolism.

Chlorophyll synthesis by C.reinhardii proceeds very slowly in the dark but is very rapid in the light, which shows the unsuitability of chlorophyll as a method of estimating growth under different conditions. The fact that at least some chlorophyll is synthesised in the absence of light indicates that C.reinhardii differs from higher plants in its ability to convert chlorophyll precursors to chlorophyll. Although shortage of nitrogen usually results in greatly diminished cell chlorophyll contents (Droop, 1974) and nitrate and ammonium assimilation by cells of C.reinhardii is greater in the light than in the dark (Thacker and Syrett, 1972[a]), the heterotrophic cells do not appear to be nitrogen depleted. This is shown by the large increase with growth of the protein/chlorophyll ratio in heterotrophic cultures (Fig.2.5). However, there is a possibility that under conditions of no nitrate assimilation by heterotrophic cells of C.reinhardii

(Thacker and Syrett, 1972[b]), the somewhat restricted nitrogen supply may be channelled by the cell into protein for growth at the expense of chlorophyll synthesis, which is not required for growth in the dark. That the acetate/nitrate ratio of the medium is of prime importance in the control of chlorophyll synthesis in algae has been shown previously (Ellis et al, 1975). This effect may be due to the induction of the glyoxylate cycle by acetate in the dark and then metabolite repression or inhibition of the enzyme(s) involved in the synthesis of chlorophyll precursors (Ellis et al, 1975). Absence or reduction in activity of the glyoxylate cycle enzymes under phototrophic and mixotrophic conditions (Haigh and Beevers, 1964; Harrop and Kornberg, 1966) would then allow chlorophyll synthesis to proceed normally.

An alternative hypothesis would be the presence of both a 'dark'- and a 'light' - dependent pathway of chlorophyll synthesis. The former pathway would then operate under heterotrophic conditions and would be absent in the y-1 mutant of C.reinhardii, which is incapable of chlorophyll synthesis in the dark. This hypothesis is supported by the findings that sub-lethal concentrations of streptomycin inhibit chlorophyll synthesis in dark-grown but not light-grown cells of C.reinhardii (Sager and Tsubo, 1962). Protochlorophyll is formed in both light-and dark-grown cells of the y-1 mutant of C.reinhardii and the light-induced conversion of protochlorophyll to chlorophyll is not inhibited by streptomycin (Sager, 1961). Consequently the light and dark pathways of chlorophyll synthesis may diverge after protochlorophyll formation.

It would appear from the foregoing that the growth of C.reinhardii under various trophic conditions may produce interacting changes in metabolism. The shifting of metabolism gives rise to changes in the characteristics of the media, such as pH, which may in turn affect

the overall metabolism of the cells. These changes in metabolic pattern and environmental factors should be taken into account when comparing various aspects of algal growth, e.g. :- response to inhibitors, under different conditions.

The effect of various inhibitors on the growth of C.reinhardii on solid media shows that many of the compounds were insufficiently toxic at low concentrations to be of any use in the selection of resistant mutants. This low toxicity of many of the inhibitors may be related to the characteristic low permeability of many 'acetate' flagellates to molecules larger than sodium acetate (Stross, 1960; Round, 1970). However, this is an oversimplification since toxicity was not always related to size of the molecules, for example DNP was non-toxic whilst TPhT exhibited a high toxicity. Likewise toxicity of the trialkyltins and alkylguanidines increased up the homologous series which was probably related to increased lipid solubility.

The mode of action of these compounds undoubtedly played a part in their toxicity to whole cells. For example, the insensitivity of the cells to KCN under mixotrophic and heterotrophic conditions may reflect the virtual absence of cytochrome a_3 in the vegetative cells of C.reinhardii (Hiyama et al, 1969). However, the KCN insensitivity of phototrophic growth is not consistent with the known inhibitory effects of low KCN concentrations on light-induced oxygen evolution in whole cells of the alga (Neumann and Levine, 1971). In addition, the high sensitivity of heterotrophic growth towards antimycin A may indicate that the cyanide-insensitive respiration in this alga, which develops after germination of the zygospores (Hommersand and Thimann, 1965), is due to a change in the electron transport chain at a point beyond the antimycin-sensitive b/c_1 segment. The inhibitory effect of Dio-9 is consistent with the known inhibitory effect of

similar concentrations on the whole cell respiration and photosynthesis of C.reinhardii (Neumann and Levine, 1971).

The differential inhibition by spectinomycin of phototrophic, compared to mixotrophic or heterotrophic growth, may reflect the specific inhibition of chloroplast ribosomes by this inhibitor (Surzycki et al., 1970). The lack of any differential effect of rifampicin on chloroplast function is surprising in view of the reported specificity of this inhibitor for cDNA transcription (Surzycki, 1969) but may have been due to light inactivation of this light-sensitive compound.

The generally greater sensitivity of heterotrophic growth, compared to phototrophic and mixotrophic growth, may reflect a specific inhibition of mitochondrial function by some of the compounds. This is likely with antimycin A and oligomycin which are known to be more effective inhibitors of oxidative than of photosynthetic phosphorylation (Baltscheffsky, 1960; Izawa and Good, 1972). Likewise, the greater sensitivity of heterotrophic growth towards acriflavine, and perhaps EB, may reflect their differential inhibition of mitochrondrial protein synthesis in C.reinhardii (Stegeman and Hoober, 1973; Alexander et al., 1974). This specificity for heterotrophic growth is unlikely to be due simply to pH changes occurring in solid media since pH increases during growth of cells on solid mixotrophic media were more marked than those under heterotrophic conditions. The generally slower growth of cells on solid media in the dark, compared to those in the light, may perhaps have contributed to increased sensitivity of heterotrophic growth. It should be pointed out that growth on solid media does not detect any differential effect of inhibitors on growth rates but only on the presence or absence of growth. Consequently any differential inhibition of growth rates would have been masked by the nature of the assay system.

The more toxic inhibitors, such as the triorganotins and alkylguanidines, were selected as potential candidates for the selection of inhibitor-resistant mutants (Chapter 4) and their modes of action on some aspects of energy conservation in chloroplasts were examined (Chapter 3).

CHAPTER 3

Effect of Selected Energy Transfer Inhibitors on Isolated Chloroplasts

Introduction

Triorganotin and alkylguanidine compounds are known to affect energy transfer in mitochondria. The mode of action of the various triorganotin salts differs according to the pH and ionic composition of the assay medium. Energy transfer inhibition occurs in alkaline sucrose media devoid of chloride ions (Stockdale et al., 1970; Coleman and Palmer, 1971), whilst an uncoupling effect predominates in a medium containing chloride ions, particularly at acid pH values (Selwyn et al., 1970; Coleman and Palmer, 1971; Dawson and Selwyn, 1974). The effect of the various guanidine derivatives on mitochondrial energy transfer is governed mainly by the type of substituent but most possess phosphorylation site specificity and their mode of action appears to differ from that of oligomycin (Pressman, 1962, 1963; Chappell, 1963; Guillory and Slater, 1965).

Much less work has been carried out on the mode of action of these compounds on photosynthetic energy transfer. Only two reports relating to the effects of guanidine derivatives on photosynthetic energy transfer (Avron and Shavit, 1965; Gross et al., 1968) and only five that investigate the effects of triorganotins on this process (Lynn, 1968; Kahn, 1968, 1970; Watling and Selwyn, 1970, 1974) were known to the author at the beginning of this study.

Triorganotin compounds and the guanidine derivatives octyl-(OG) and dodecyl-(DG) guanidine were chosen as candidates for the selection of resistant mutants of C.reinhardii because of their known mode of action on photosynthetic energy transfer and because of their high toxicity to whole cells of the alga (Table 2.1). However, before progressing onto the isolation of resistant mutants against these

compounds, it was considered necessary to first confirm their inhibitory action on isolated chloroplasts. Preliminary experiments with chloroplasts and chloroplast fragments isolated from C.reinhardii indicated that they could only be isolated in an uncoupled state and exhibited some ATP-ase activity and no photosynthetic control. Consequently isolated pea chloroplasts were used as the standard test material in most assays since they could be isolated with reasonably tight photosynthetic control. In addition, the effect of the inhibitors on the Ca^{2+}-dependent ATP-ase activity could be conveniently assayed using the high yields of purified chloroplasts from the $CW15^{+}$ strain of C.reinhardii.

Materials and Methods

(a) Preparation of pea chloroplasts.

Except where indicated in the text, chloroplasts were isolated from pea leaves by a modification of the method of West and Wiskich (1973). 14 day-old pea seedlings (Pisum sativum L. cv. 'Meteor') were grown in Bower's compost. 15g of leaves were ground in a Waring blendor at full speed for 5 sec in 75 ml of an ice-cold medium containing 0.25M sucrose, 1 mM $MgCl_2$, 0.5% BSA and 30 mM TES buffer (adjusted to pH 7.3 with NaOH). The brei was filtered through 6 layers of cheesecloth and the filtrate centrifuged for 1 min at $2.5 \times 10^3 g_{av.}$ in a MSE Mistral 6L centrifuge fitted with a 12 x 50 ml swing-out head. The centrifuge was accelerated from rest to $2.5 \times 10^3 g_{av.}$ and back to rest in a total elapsed time of 1 min 40 sec. For 'Class 1' (unbroken) chloroplasts, the sedimented chloroplasts were gently re-suspended to a concentration of approximately 1.5 mg chlorophyll/ml in 0.25M sucrose, 0.5% BSA and 25 mM Hepes buffer adjusted to pH 7.6 with NaOH. For 'Class 2' (broken) chloroplasts, the pellet was gently resuspended in 20 ml of medium containing 25 mM sucrose, 0.5% BSA and 25 mM Hepes/NaOH buffer (pH 7.6) and centrifuged at $2.5 \times 10^3 g_{av.}$ for 1 min. Finally the chloroplasts were gently resuspended in 25 mM KCl, 5mM $MgCl_2$ and 1 mM Hepes buffer (pH 7.6).

All chloroplast preparations were checked for their degree of intactness under the phase-contrast microscope. The 'Class 1' preparations consisted of approximately 50 - 75% as bright and highly-reflecting chloroplasts with smooth outlines and non-resolved grana. Most of the 'Class 2' chloroplasts were larger, more regular in shape and more granular in appearance than the 'Class 1' chloroplasts.

(b) Electron transport activities of pea chloroplasts and the effect of inhibitors.

Oxygen evolution and uptake were measured with a Clark-type

oxygen electrode (Rank Bros., Bottisham, Cambs., UK.) incorporated into a glass-lined perspex reaction vessel maintained at a temperature of 25° C. The assay medium for oxygen evolution with $K_3Fe(CN)_6$ as the electron acceptor contained 0.25 M sucrose, 5 mM $MgCl_2$, 12 mM KH_2PO_4, 2.64 mM $K_3Fe(CN)_6$ and 25 mM Hepes/NaOH buffer (pH 7.6). Oxygen partial pressure was reduced to a minimum by flushing with water-saturated N_2 and the perspex lid lowered. The assay medium for oxygen uptake with methyl viologen (MV) as the electron acceptor was the same as with $K_3Fe(CN)_6$ except that 0.8 mM MV and 0.8 mM NaN_3 replaced the $K_3Fe(CN)_6$ and the medium was not flushed with N_2.

'Class 1' chloroplasts were added at a concentration of 60 µg chlorophyll/ml and sufficient ADP (adjusted to pH 7.6 with NaOH) was added to maintain state 3 for the duration of each assay. Where inhibitors were added as ethanolic solutions, the control rates were determined with the addition of the same quantity of drug-free solvent. The final volume of the assay medium was 2.5 ml. Red light ($\lambda_{max} \geq 650$ nm) was provided at a saturating intensity of 3×10^4 lux (see Appendix Fig.1) at the vessel surface by a Rank Aldis Tutor 1000 projector fitted with a red glass filter and the chloroplast suspension was stirred throughout the assays with a magnetic follower. The electrode current was recorded on a Servoscribe 2 (RE 520.20) potentiometric recorder.

The mean control value ($\pm$ standard deviation) for the state 3 rate of oxygen evolution (in the presence of ADP, Pi and $K_3Fe(CN)_6$) over 55 separate observations was 27.2 $\pm$ 8.4 µ moles O_2/mg chl./h .

(c) Light-induced pH changes in pea chloroplasts and the effect of inhibitors.

Light-induced pH changes were measured with a Model 33 B-2 Vibron electrometer (Electronic Instruments Limited) with the pH electrode (Pye Unicam Ingold) inserted into the vessel of a Clark-type oxygen

electrode maintained at a constant temperature of 25° C. The assay medium contained 25 mM KCl, 2.14 mM p-benzoquinone, 5 mM $MgCl_2$ and 1 mM Hepes/NaOH buffer (pH 7.6). 'Class 2' chloroplasts were added at a concentration of 200 µg/ml to give a final assay volume of 2.8 ml. Red light ($\lambda_{max} \geq 650$nm) was provided at a saturating intensity of 3×10^4 lux at the vessel surface by a Rank Aldis Tutor 1000 projector fitted with a red glass filter and the chloroplast suspension was stirred throughout the assays with a magnetic follower. The electrode current was recorded on a Servoscribe 2 potentiometric recorder. Recorder deflections were related to hydrogen ion concentration using a constructed standard curve produced by the addition of small aliquots of a standard 0.01N HCl solution to the complete assay system in the dark. Stock solutions of inhibitors were adjusted to pH 7.6 prior to addition.

The mean control values ($\pm$ standard deviation) over a series of 22 independent observations for the light-induced proton changes were as follows; extent of proton uptake (ΔH^+) = 0.52$\pm$ 0.09 µ equivs. H^+/mgchl. ; rate of light-induced proton uptake = 10.35$\pm$ 2.74 µ equivs. H^+/mgchl./min ; rate of dark proton loss = 3.6$\pm$ 1.5 µ equivs. H^+/mgchl./min.

(d) Light-induced transmission changes in pea chloroplasts and the effect of inhibitors.

'Class 2' chloroplasts were prepared as described previously except that the final chloroplast pellet was resuspended in a medium containing 25 mM KCl, 5 mM $MgCl_2$ and 1 mM Hepes buffer (adjusted to pH 7.6 with NaOH).

The reaction medium consisted of the resuspension medium plus 2.14 mM p-benzoquinone and chloroplasts to a concentration of 10 µg chlorophyll/ml. Any deviations from this medium composition are indicated in the text. The final volume in each case was 2.8 ml.

The reaction medium was not stirred throughout the assays but, after making additions, the medium was rapidly mixed with a glass micro-spatula before illumination. Inhibitors were added as either aqueous or ethanolic solutions and the control rates determined with the same quantity of drug-free solvent. Transmission changes were measured with an Eppendorf spectrophotometer (Netheler and Hinz, Hamburg) modified to allow illumination at 90° to the actinic light source (Fig.3.1) The chloroplast suspension was placed in a 1.0 cm light-path cuvette with four optical faces and illuminated with light at 546 nm which had passed through a 1 mm x 4 mm slit. The unabsorbed light at 546 nm was selected by placing a Kodak 'Wratten' 58 gelatine filter between the cuvette and photomultiplier. Illumination at 90° was achieved by using a Rank Aldis Tutor 1000 projector with the light passing through a red glass filter ($\lambda_{max} \geq 650$ nm) fixed into the side of the spectrophotometer lid. The light was directed onto the cuvette by means of a black metal tube and stray light was reduced by a rubber seal between the tube and the spectrophotometer lid. The temperature of the reaction medium was maintained constant by passing water at 25° C through the water-jacketted cuvette holder.

Light-induced changes in transmission were measured at least twice for each treatment and the mean value taken. The mean control values ($\pm$ standard deviation) for the light-induced transmission changes over a series of 20 separate observations were as follows (in Δ% transmission/mgchl.) : with KCl = 17 ± 3; with sodium acetate = 632 ± 164.

(e) Isolation of chloroplasts from C.reinhardii strain $CW15^+$.

The $CW15^+$ (cell-wall-less) strain of C.reinhardii was obtained from Professor D.R. Davis and chloroplasts were prepared by a modification of the method of Keller (personal communication, 1975). 4.5 l of early

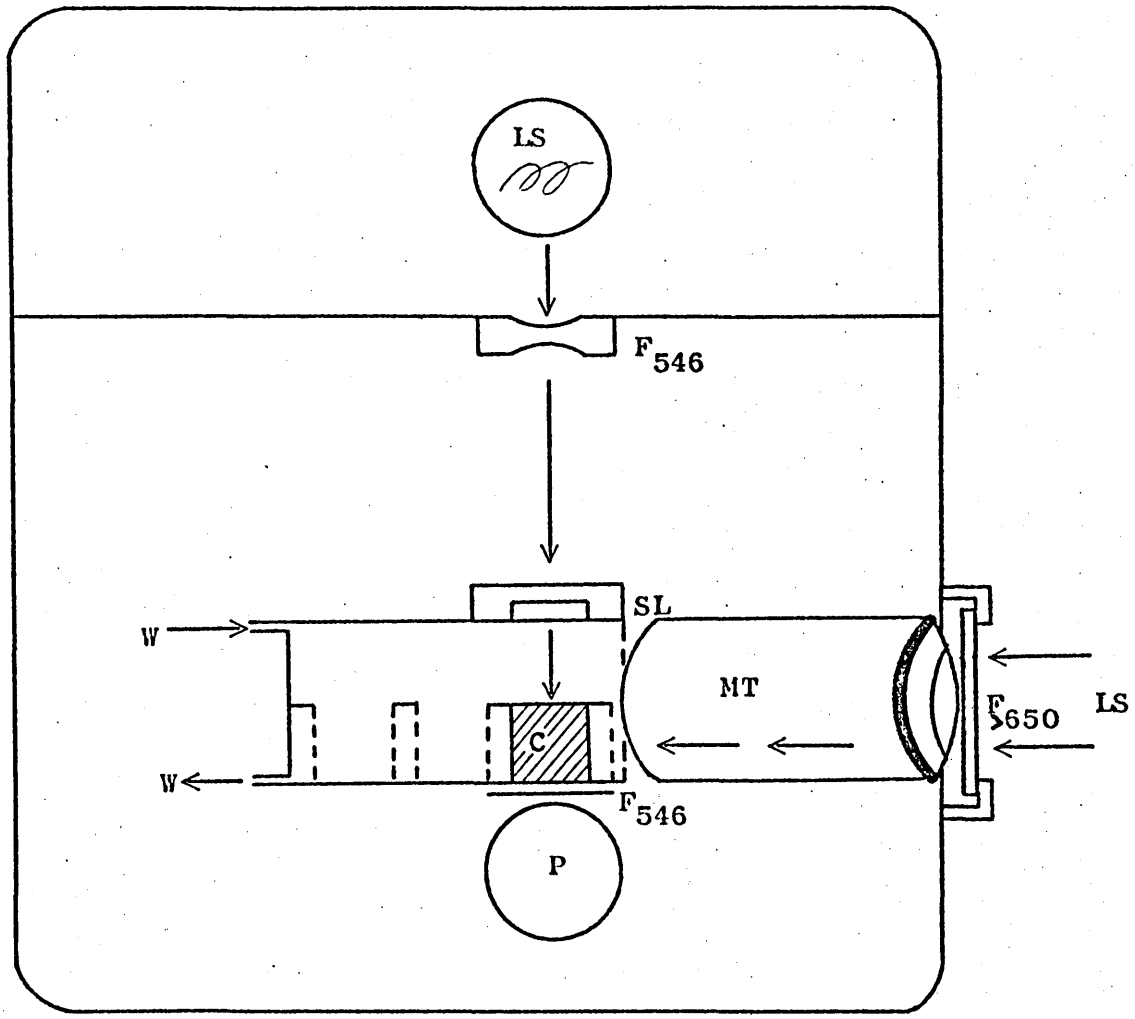


Figure 3.1. System used to measure light-induced transmission changes in chloroplasts. (Not to scale).

LS Light source.
W Constant temperature water.
C Cuvette.
P Photomultiplier.
MT Metal tube.
SL Slit.
F_{546} Filter for monochromatic light at 546nm.
$F_{>650}$ Filter for monochromatic light at >650nm.

stationary-phase cells (72 h old) were harvested at 2.5 x $10^3 g_{av.}$ for 5 min at 0° C using a Sorvall RC 2-B centrifuge. The cells were washed once and resuspended in T_1 buffer (2mM KCl, 10 mM tris/H_2SO_4 buffer, pH 8.0) at a concentration of 0.5 mg chlorophyll/ml. The suspension was left to plasmolyse on ice in the dark for 12 - 15 h and the suspension reharvested at 2.5 x $10^3 g_{av.}$ for 5 min at 0° C. The pellet was gently resuspended in an equal volume of T_2 buffer, consisting of 10 mM tris/H_2SO_4 pH 8.0 and 20 mM KCl, and layered over a discontinuous sucrose gradient, consisting of 25 ml each of 2.0 M and 1.5 M sucrose dissolved in T_2 buffer. The gradients were centrifuged in a SW 25.2 swing-out head of a Beckman L - 2.50 centrifuge at 8 x $10^3 g_{av.}$ for 90 min. The chloroplasts at the 2.0 M - 1.5 M interphase (see Fig.3.2) were collected from the top with a Pasteur pipette, diluted 4 fold with T_2 buffer and centrifuged at 2.5 x $10^3 g_{av.}$ for 5 min at 0° C. The chloroplast pellet was washed twice, resuspended in 2 volumes of T_2 buffer and sonicated for 10 - 30 sec on an MSE ultrasonicator at low power and no.3 amplitude. Chloroplast breakage was checked at 10 sec intervals until approximately 95% breakage was achieved. The sonicated chloroplast suspension was diluted 4 fold with T_2 buffer and centrifuged twice at 480 $g_{av.}$ for 5 min at 0° C to remove unbroken chloroplasts. The residue was discarded and the supernatant re-centrifuged at 2 x 10^4 $g_{av.}$ for 20 min at 0° C. Finally the resultant pellet was resuspended in T_2 buffer to a concentration of 0.5 mg chlorophyll/ml with a Ten-Broeck glass homogenizer.

Appearance of the sucrose gradients.

The sucrose gradients used for purifying the chloroplasts derived from $CW15^+$ consisted of 2 green layers and a pellet (see Fig.3.2). The band between the sample layer and 1.5 M sucrose (B_1) consisted of chloroplast fragments, while the one between the 1.5 M and 2.0 M

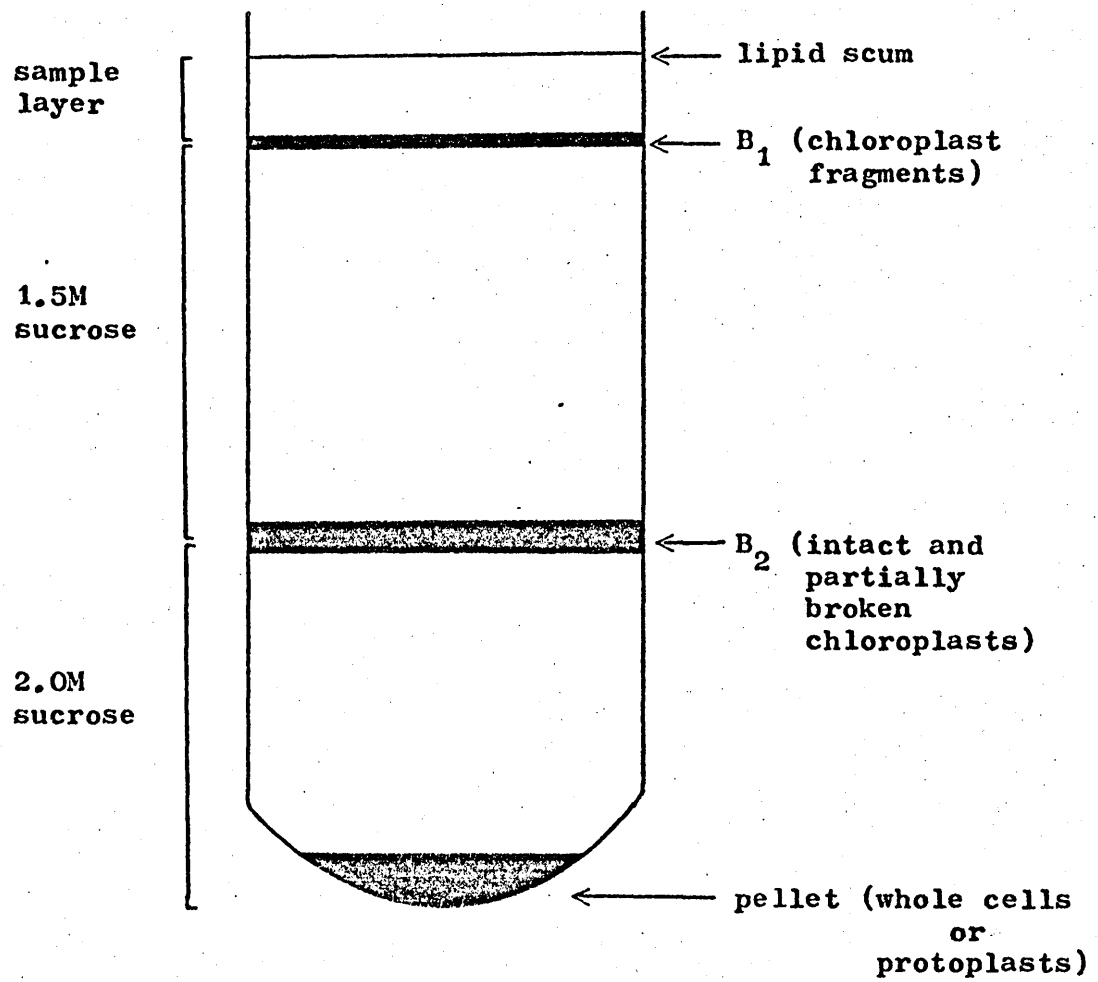


Figure 3.2. Appearance of the discontinuous sucrose density gradients used for the separation of whole chloroplasts from the $CW15^+$ strain of C. reinhardii.

layers (B_2) consisted of a mixture of intact chloroplasts and chloroplasts with broken membranes. The pellet consisted mainly of whole cells (protoplasts). The purity of the chloroplast band was determined by phase-contrast microscopy and by treatment with 0.01% Triton X - 100 which caused immediate lysis of chloroplasts but not of protoplasts. A further criterion was the low respiratory activity of the B_2 chloroplast band. Electron microscopy has previously shown the absence of contaminating organelles and membranes in chloroplasts prepared by this method (Ho et al., 1974).

(f) The Ca^{2+}-dependent chloroplast ATP-ase activity of C.reinhardii ($CW15^+$) and the effect of inhibitors.

The heat-activated, Ca^{2+}-dependent ATP-ase activity of chloroplast fragments was measured by estimating colorimetrically the inorganic phosphate liberated from ATP. The assay procedure used was a modification of the methods of Vambutas and Racker (1965) and Sato et al. (1971).

The reaction mixture for the activation stage contained chloroplast fragments equivalent to 50 µg chlorophyll and the following in µ moles : tris/SO_4 buffer (pH 8.0) 100; disodium EDTA (pH 8.0) 5; ATP (pH 8.0) 1 and distilled water to a final volume of 0.7 ml. After incubation in the dark at 60° C for 1.5 min, the tubes were rapidly placed on ice and the following components in µ moles quickly added for the reaction stage : ATP (pH 8.0) 5; $CaCl_2$ 10. The final volume was 1.9 ml. $CaCl_2$ was omitted from the control tubes. The concentrations of EDTA and $CaCl_2$ for the activation and reaction stages respectively and the activation time at 60° C were found to be optimal or near-optimal for the reaction (Appendix Figs. 2 and 3). The reaction tubes were incubated at 36° C for 10 min in the dark and terminated by the addition of 0.1 ml of 50% TCA. The precipitate was removed by centrifugation and 1 ml of the clear supernatant assayed for the presence

of inorganic phosphate by the method of Fiske and Subbarow (1925). Inhibitors were added as an aqueous or ethanolic solution at the beginning of the reaction stage and the control tubes contained an equal volume of the drug-free solvent.

At least two determinations of the effect of each inhibitor were made on each of two separate chloroplast preparations and the mean value taken. The mean control rate for the ATP-ase activity over a series of 26 estimations was 78.6 ± 29.9 μ moles Pi liberated per mg chl. per h.

Results

(a) Electron transport activities of pea chloroplasts and the effect of inhibitors.

The efficacy of various methods used to isolate and determine the photosynthetic activity of pea chloroplasts is shown in Table 3.1. The rate of oxygen evolution on illumination in the absence of ADP was slow and has been termed state 2 (basal) (Watling-Payne and Selwyn, 1974), whilst the ADP-stimulated oxygen evolution was rapid and termed state 3 (West and Wiskich, 1973). The rate after ADP had been phosphorylated was slow due to the accumulation of non-phosphorylated intermediates or the high energy state and is termed the state 4 rate (West and Wiskich, 1973). This control of oxygen evolution by the level of phosphate acceptor(ADP) in the medium has been defined as photosynthetic control (PC) (West and Wiskich, 1968). The methods in Table 3.1 are arranged in order of increasing PC ratios which gives an idea of the increasing 'tightness' of coupling between photophosphorylation and electron transport in these preparations. Photosynthetic control values with potassium ferricyanide as the electron acceptor were higher than those with methyl viologen (MV) and the use of 0.25 M sucrose (West and Wiskich, 1973), as opposed to 0.4 M (West and Wiskich, 1968), also increased state 3 rates and the PC and ADP/O ratios. When 0.4 M sucrose was used during the preparation, purifying the chloroplasts by centrifugation through a layer of 1.0 M sucrose (West and Wiskich, 1968) slightly improved the PC ratio with MV as the electron acceptor but decreased the ADP/O ratio. Chloroplasts prepared and assayed in 0.3 M sorbitol (Watling-Payne and Selwyn, 1974), using MV as the electron acceptor, were insufficiently active.

A polarograph tracing of oxygen evolution by chloroplasts prepared and assayed essentially by the method of West and Wiskich (1973) is shown in Fig.3.3. Maximum PC ratios were usually around

Table 3.1. Efficacy of methods used to isolate and determine the photosynthetic activity of pea chloroplasts.

Method Reference	Modification of Methods	State 3 Rate (μ moles O_2/mgchl/h)	PC Ratio	ADP/O Ratio
West and Wiskich(1968)	Chloroplasts not purified through 1.0M sucrose.Electron acceptor = MV	+ 13.00	1.53	0.81
Watling-Payne and Selwyn (1974)	Electron acceptor = MV	+ 12.38	1.71	0.68
West and Wiskich(1968)	Electron acceptor = MV	+ 10.83	1.83	0.66
West and Wiskich(1973)	0.25M sucrose used in prep.medium.Electron acceptor = MV	+ 17.33	1.87	1.12
West and Wiskich(1968)	Electron acceptor = $K_3Fe(CN)_6$	- 12.38	2.93	1.04
West and Wiskich(1973)	0.25M sucroeused in prep.medium.Electron acceptor=$K_3Fe(CN)_6$	- 24.64	3.13	1.12

* Rate in presence of ADP and Pi.

+ = O_2 uptake by chloroplasts.

- = O_2 evolution by chloroplasts.

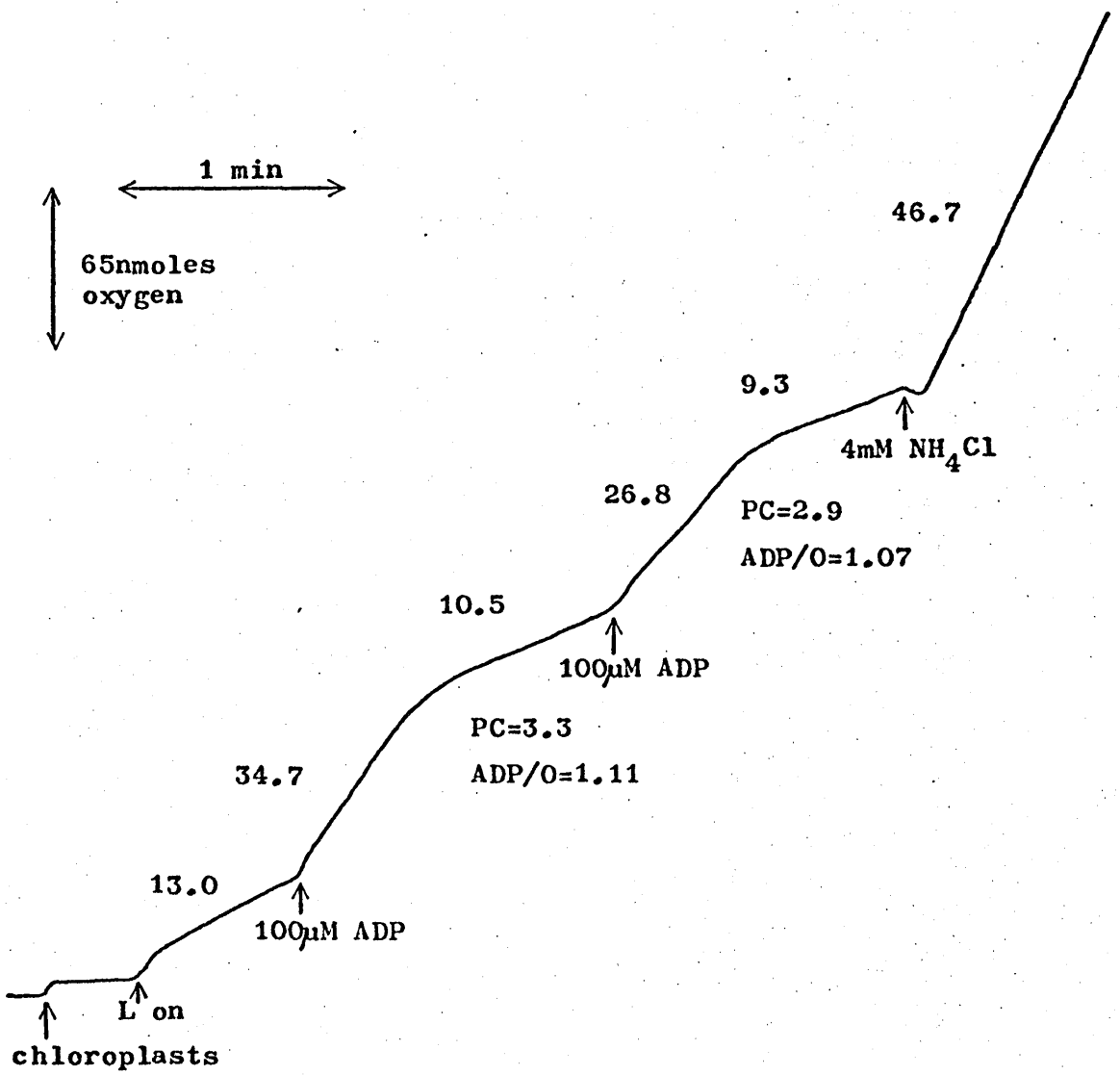


Figure 3.3. Polarographic tracing of oxygen evolution by pea chloroplasts.

Numbers along trace represent the rates in µmoles oxygen per mg chlorophyll per hour.
PC=photosynthetic control ratio.

a value of 3.3 but these decreased during subsequent additions of ADP to the same suspension, due to decreased values for both state 3 and state 4 rates. ADP/O ratios usually had maximum values of around 1.1, although values as high as 1.43 were observed in some preparations. Ammonium chloride uncoupled both state 4 (Fig.3.3) and state 3 (Fig.3.4) rates with maximal effect at 8.0 mM (Appendix Fig.1). Various other compounds also affected state 3 as shown in Table 3.2. Ethidium bromide inhibited state 3 at 406 μM and Dio-9 and phlorizin, which are energy transfer inhibitors of photophosphorylation (McCarty et al., 1965; Izawa et al., 1966), also inhibited state 3. The inhibition by all three compounds was maximally relieved by NH_4Cl. Sodium arsenate, in the absence of added phosphate, slightly stimulated state 3 and this was further enhanced by NH_4Cl but not maximally. Ethanol, at the maximum concentration used in the assays involving ethanol-soluble inhibitors (0.8% v/v), only slightly inhibited state 3.

The effect of a series of triorganotin compounds on state 3 is shown in Figs. 3.4 and 3.5. In a low chloride, sucrose medium, the major effect was inhibition of state 3, which was maximally relieved by NH_4Cl (Fig. 3.4 a), but there was no inhibition in NH_4Cl-uncoupled chloroplasts (Fig.3.4 b). In a medium lacking sucrose and containing high chloride concentrations, as used for the assay of light-induced pH changes, the tin compounds produced an initial stimulation of state 3 followed by a time-dependent inhibition (Fig.3.4 c). Similar effects were found with all the triorganotins but the concentration required to inhibit state 3 depended on the organic group in the following order of decreasing proportional activities at the I_{50} values : TBT (1.0) > TcHT (1.6) > TPhT (6.3) > TPT (10.0) > TET (421) > TMT (1,057) (Fig. 3.5 and Table 3.4).

The effect of the alkylguanidines on state 3 oxygen evolution

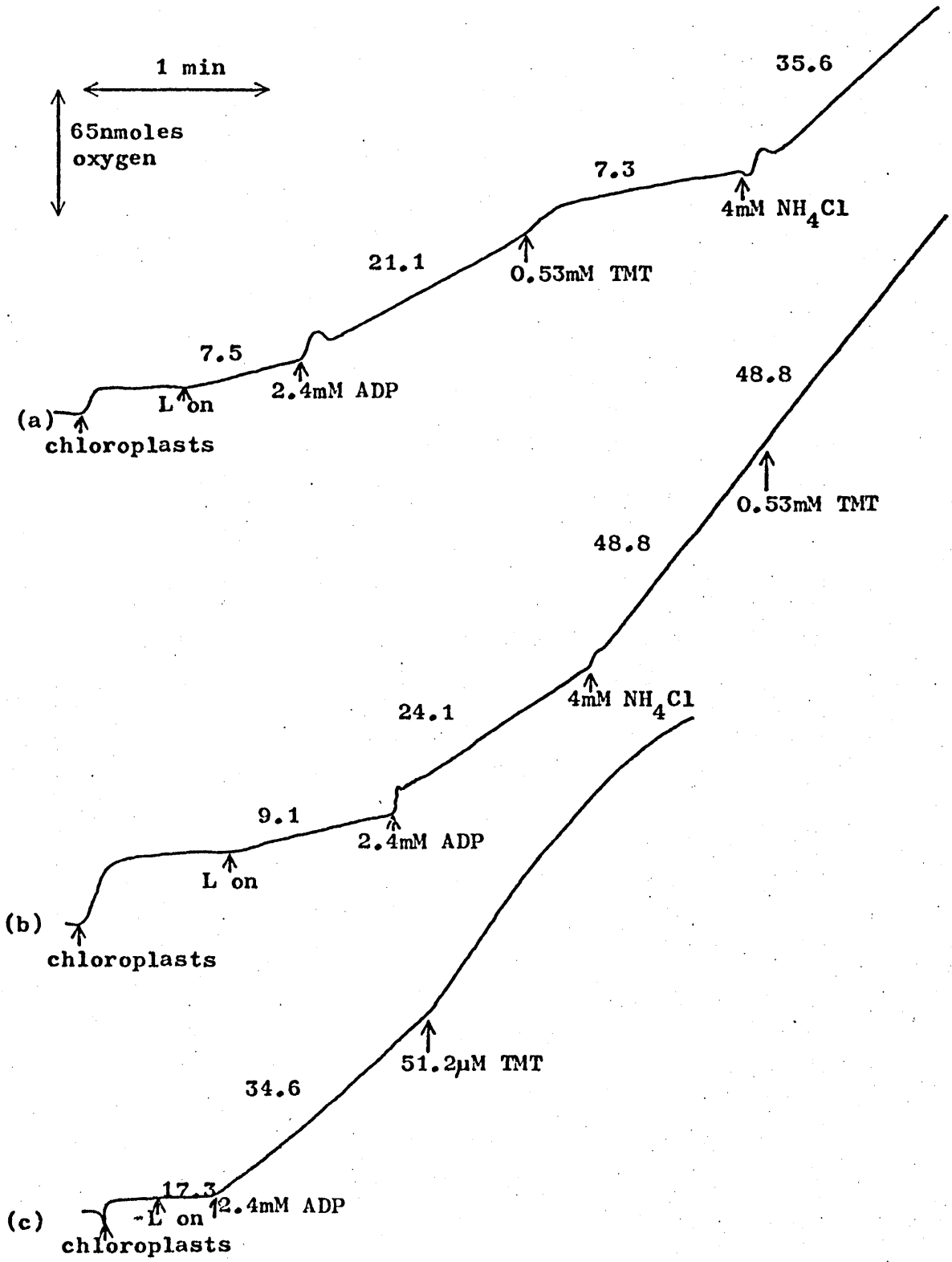


Figure 3.4. Effect of TMT on the state 3 oxygen evolution of pea chloroplasts.

(a)&(b) In a medium containing 0.25M sucrose, 2.64mM $K_3Fe(CN)_6$, 5mM $MgCl_2$, 12mM KH_2PO_4 and 25mM HEPES/NaOH buffer (pH 7.6).

(c) Same medium except 25mM KCl replaced the sucrose and 1mM HEPES/NaOH buffer (pH 7.6) was used.

Table 3.2 Effect of various compounds on the state 3 oxygen evolution of pea chloroplasts.

Compound and Concentration	Control State 3 O_2 Evolution Rate•	State 3 Rate• + Compound	State 3 Rate• + Compound + 4mM NH_4Cl
EB(406 μM)	23.4	14.7	52.4
Arsenate(41.8mM)	14.4▲	20.3	27.1
Ethanol*(0.8%v/v)	37.2	33.1	-
Dio-9(10 μg/ml)	29.4	13.5	47.9
Phlorizin(45.8 μM)	26.2	18.1	40.3

• State 3 rate in μ moles O_2/mg chl./h.

▲ State 3 O_2 evolution rate determined in the absence of Pi.

* Maximum concentration used for the addition of drugs.

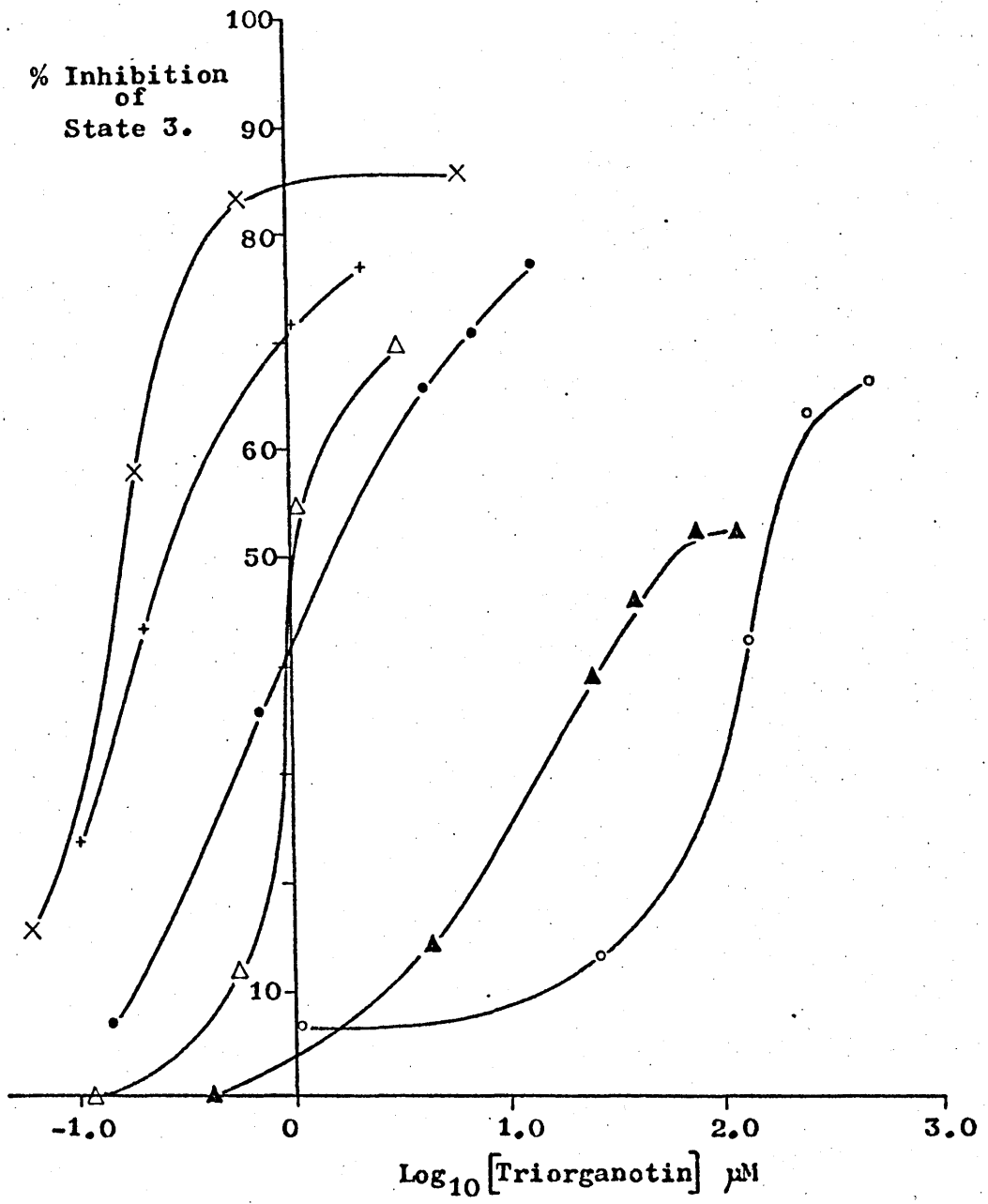


Figure 3.5. Inhibition by triorganotins of the state 3 oxygen evolution of pea chloroplasts.

o—o TMT.
▲—▲ TET.
•—• TPT.
Δ—Δ TPhT.
+—+ TcHT.
×—× TBT.

is shown in Figs. 3.6 to 3.8. Figure 3.6 shows that the control and TPT -inhibited state 3 rates were stimulated by octylguanidine (OG) and dodecylguanidine (DG). At lower concentrations than those required to uncouple, the alkylguanidines inhibited state 3 and this inhibition was maximally relieved by NH_4Cl (Fig. 3.7 a and b). At higher concentrations than those required to uncouple, state 3 was initially stimulated but this was followed by a gradual inhibition of state 3 and the inhibition was not relieved by NH_4Cl (Fig. 3.7 c). The overall concentration-dependent effect of the alkylguanidines on state 3 is shown in Fig. 3.8. Low concentrations of these compounds inhibited state 3 and the inhibition was relieved by NH_4Cl. Intermediate concentrations stimulated state 3 whilst higher concentrations produced inhibition. DG required lower concentrations than OG to produce comparable effects.

(b) Light-induced pH changes in pea chloroplasts and the effect of inhibitors.

The effect of TMT on the light-induced pH changes in pea chloroplast suspensions is shown in Figs. 3.9 to 3.11. When TMT was added to the assay medium before illumination, both the rate of rise in pH of the medium and the final extent of the steady state were inhibited. However, the rate of pH decline in the dark was markedly stimulated (Fig. 3.9). The effect of a range of TMT concentrations on the pH changes is shown in Fig. 3.10. TMT maximally inhibited the rate of rise about 50%, the extent about 30% and the dark decline rate was maximally stimulated by approximately 240%.

Addition of low concentrations of TMT in the light, after the pH rise had reached a steady state, resulted in an immediate sharp decline in medium pH, which eventually reached a steady state at a lower value than the original extent (Fig. 3.11 a). Higher TMT concentrations

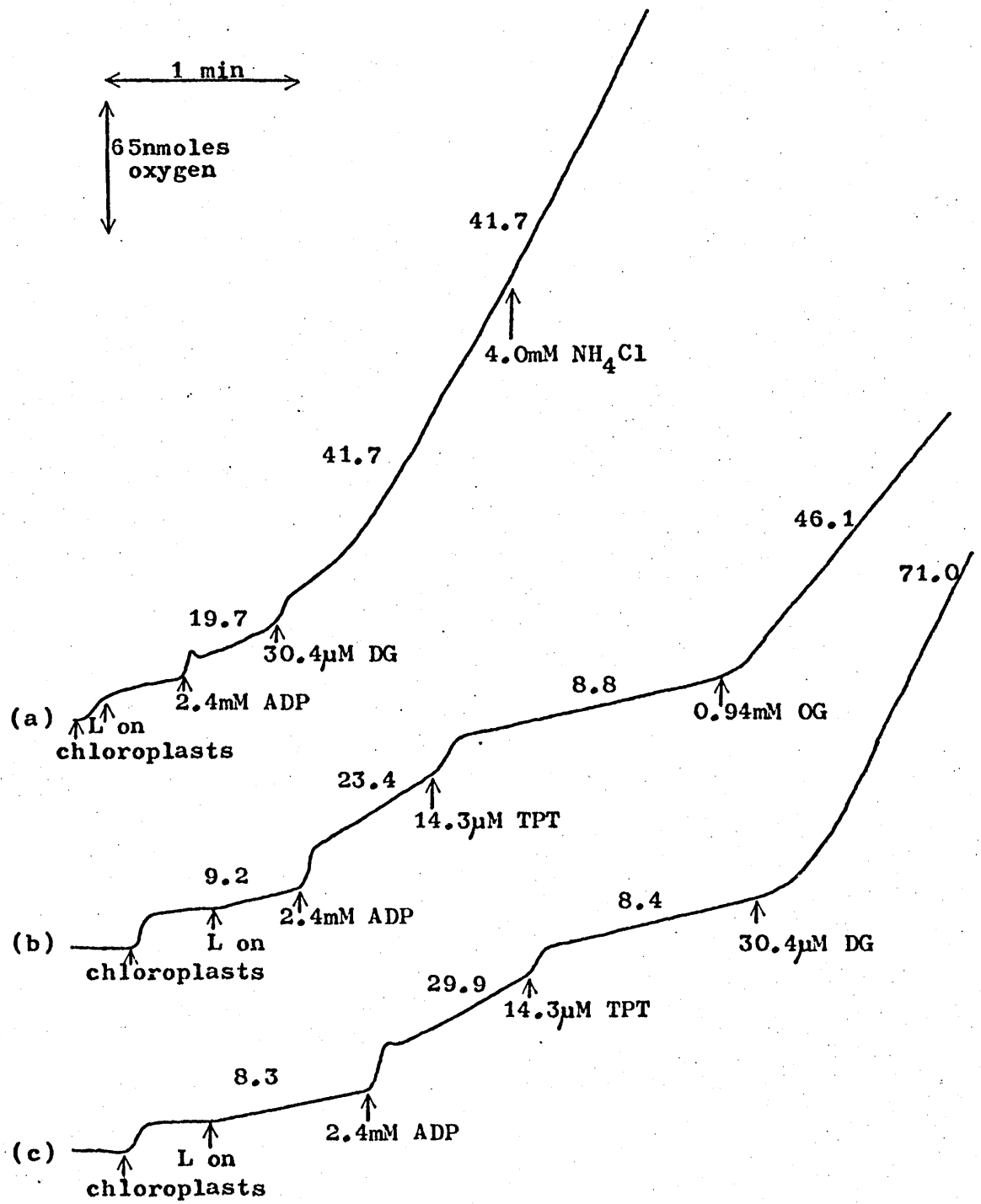


Figure 3.6. Uncoupling effect of alkylguanidines on control and tripropyltin-inhibited state 3 oxygen evolution of pea chloroplasts.

The numbers along the traces represent the rates in μmoles oxygen per mg chlorophyll per hour.

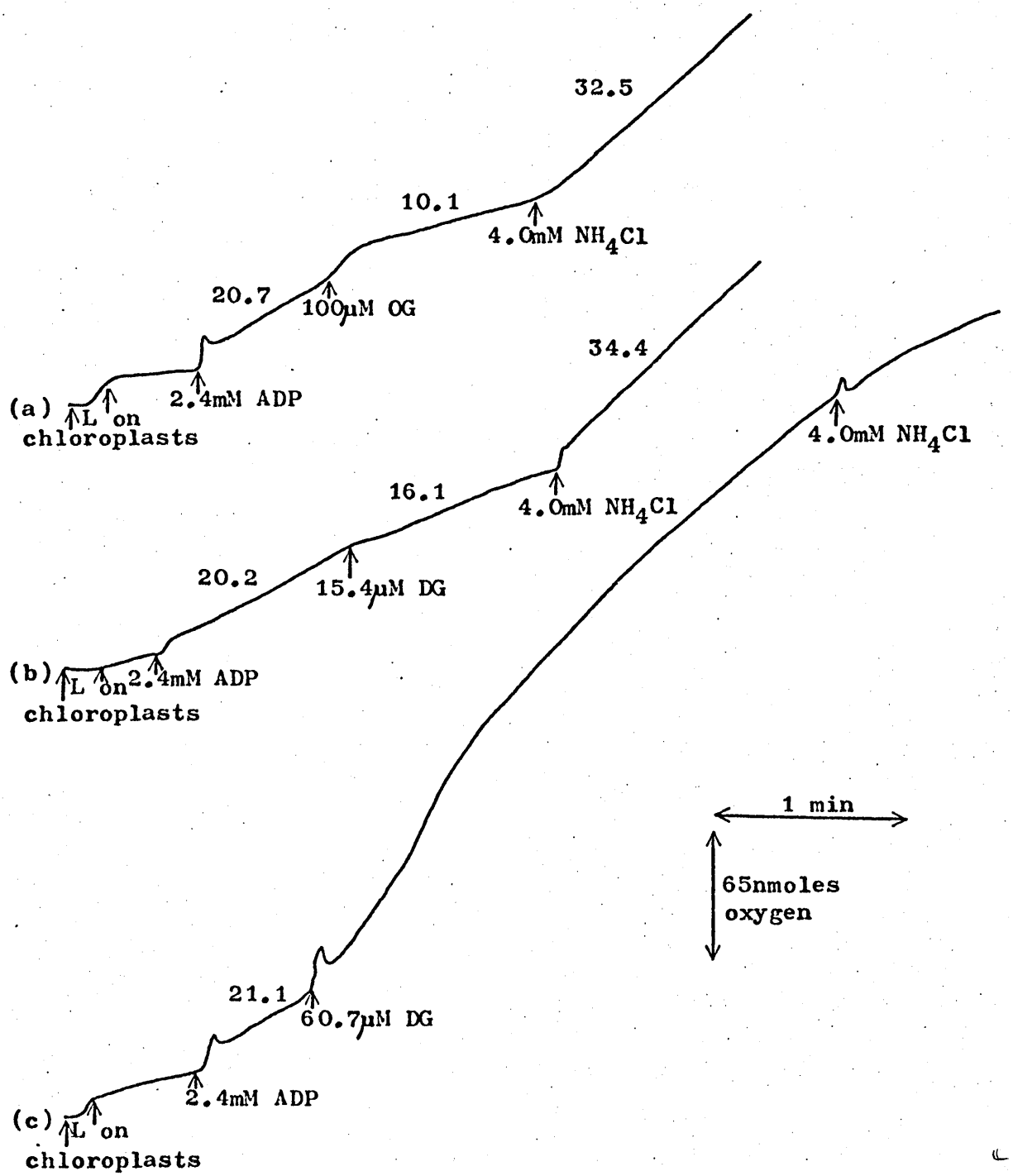


Figure 3.7. Inhibitory effects of alkylguanidines on the state 3 oxygen evolution of pea chloroplasts.

The numbers along the traces represent the rates in μmoles oxygen per mg chlorophyll per hour.

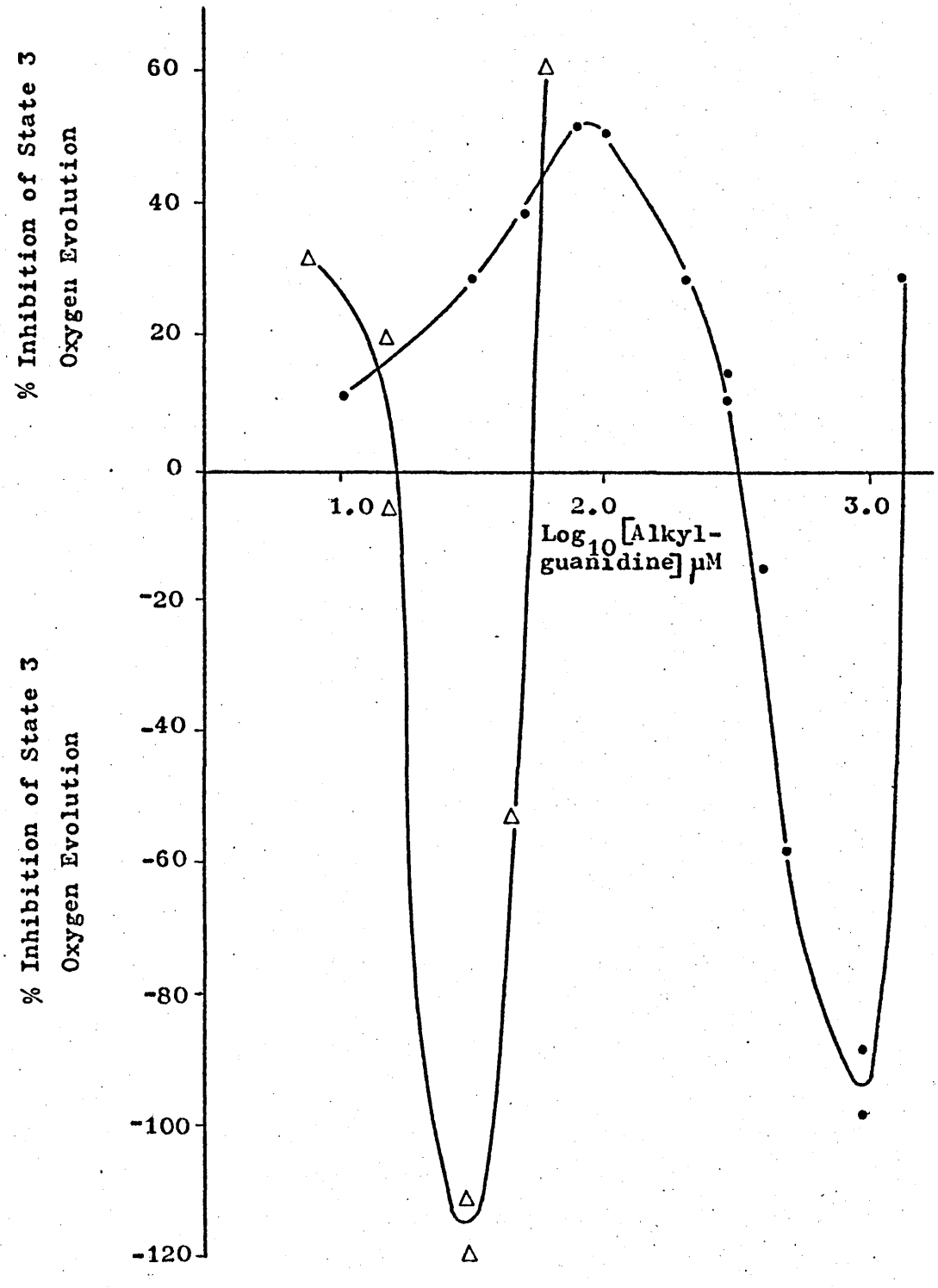


Figure 3.8. Effect of alkylguanidines on the state 3 oxygen evolution of pea chloroplasts.

•—• octylguanidine.

△—△ dodecylguanidine.

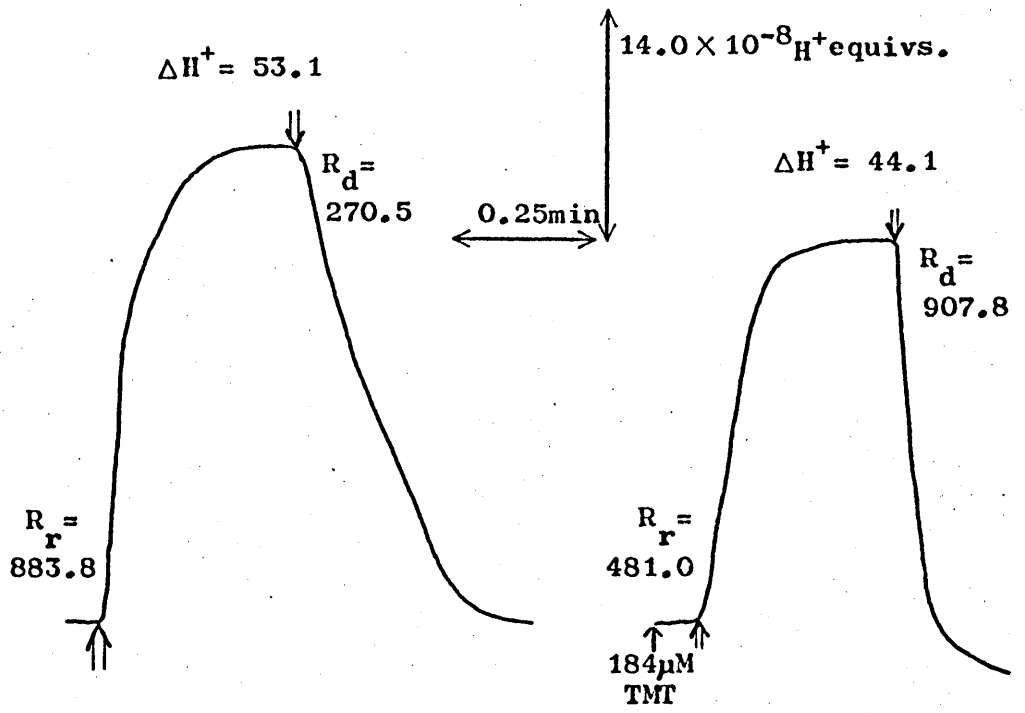


Figure 3.9. Effect of TMT on the light-induced pH changes of a pea chloroplast suspension.

The traces show the light-induced pH changes that occur on illumination of a pea chloroplast suspension in the absence (a) and presence (b) of TMT added prior to illumination.

R_r = initial rate of proton uptake (H^+equivs. $\times 10^{-8}$/ mg chl./min) on illumination.

R_d = initial rate of proton loss (H^+equivs. $\times 10^{-8}$/ mg chl./min) on switching off the light.

ΔH^+ = maximum proton uptake (H^+equivs. $\times 10^{-8}$/mg chl.) on illumination.

⇑ = illumination on.

⇓ = illumination off.

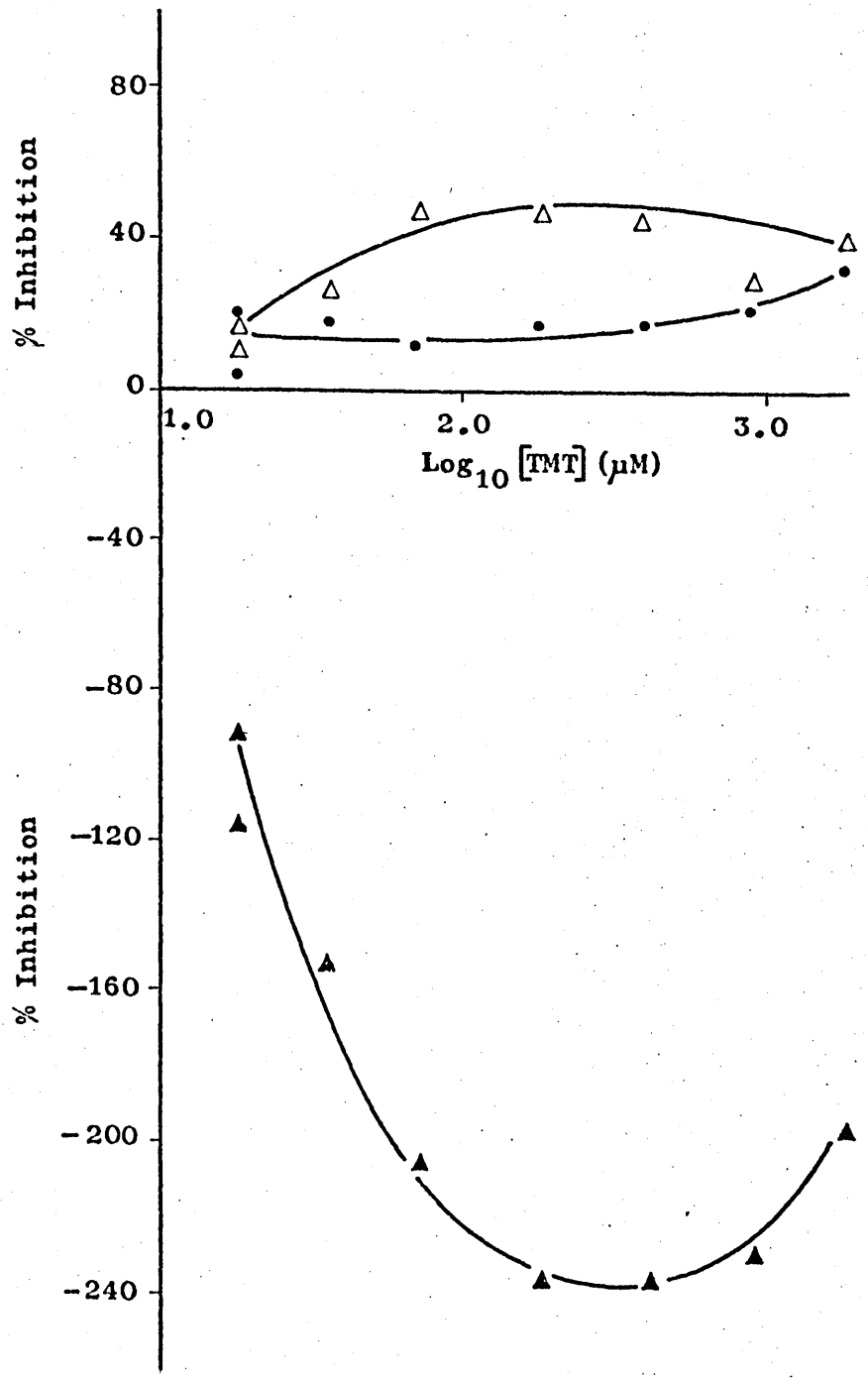


Figure 3.10. Effect of TMT concentration on the light-induced pH changes of pea chloroplast suspensions.

△—△ Rate of light-induced H^+ uptake.

•—• Extent (ΔH^+) of light-induced H^+ uptake.

▲—▲ Rate of H^+ loss in the dark.

Legend to Figure 3.11.

TMT-induced decay of the light-induced pH changes of pea chloroplast suspensions.

In traces a to e, aliquots of an aqueous solution of TMT were added where indicated to the following final concentrations: (a) 9.2μM, (b) 18.3μM, (c) 45.7μM and (d) 457.3μM.
In traces e and f, aliquots of distilled H_2O and an aqueous solution of TMT were added to give the final indicated concentrations.

⇑= illumination on.
⇓= illumination off.
↓= additions.

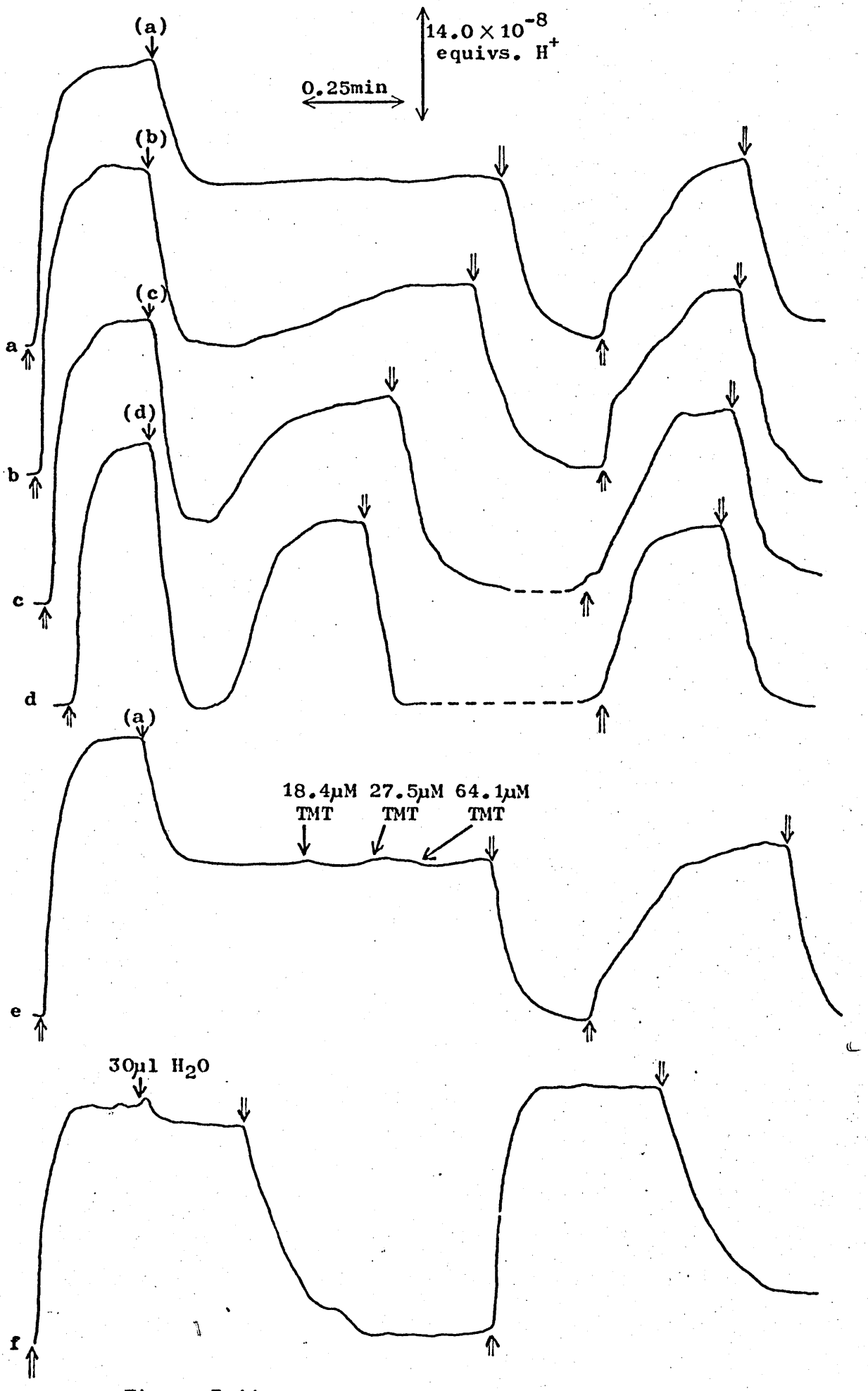

(a)
14.0 × 10⁻⁸
equivs. H⁺
0.25min
(b)
(c)
(d)
a
b
c
d
(a)
18.4μM TMT
27.5μM TMT
64.1μM TMT
e
30μl H₂O
f

Figure 3.11.

produced similar effects except for an 'overshooting' of the rapid decline. This 'overshooting' was followed by a slower spontaneous increase in medium pH to eventually reach a new steady state at a lower pH value than the original uninhibited level (Fig. 3.11 b to d). The degree of 'overshooting' was proportional to the concentration of TMT added but the final steady state level was approximately the same irrespective of the TMT concentration. Turning off the light resulted in a decline to the baseline medium pH and re-illumination resulted in a pH rise to the inhibited steady state level. This final inhibited steady state level was not decreased by further additions of TMT even when low TMT concentrations were initially used to produce the inhibited level (Fig. 3.11 e). Addition of water (the TMT solvent) to the uninhibited steady state did not cause significant alteration of the medium pH (Fig. 3.11 f).

(c) Light-induced transmission changes in pea chloroplasts and the effect of inhibitors.

The effect of triorganotin compounds on the light-induced transmission changes of pea chloroplast suspensions is shown in Figs. 3.12 and 3.13. Light-induced transmission changes were absent when the assay medium contained KCl (Fig. 3.12 a) but were stimulated in the presence of sodium acetate (Fig. 3.12 c). Triorganotins stimulated transmission changes in a medium containing KCl (Fig. 3.12 b), depending on their concentration (Fig. 3.13). The triorganotins did not stimulate transmission changes in the same medium that was used for the assay of state 3 oxygen evolution rates (Fig. 3.12 e). All the triorganotin compounds were active in the KCl medium but did not show exactly the same order of activity, nor such a difference in activity between the different triorganotins, as in the inhibition of state 3 oxygen evolution (Fig. 3.5). TMT and TET were the least active in

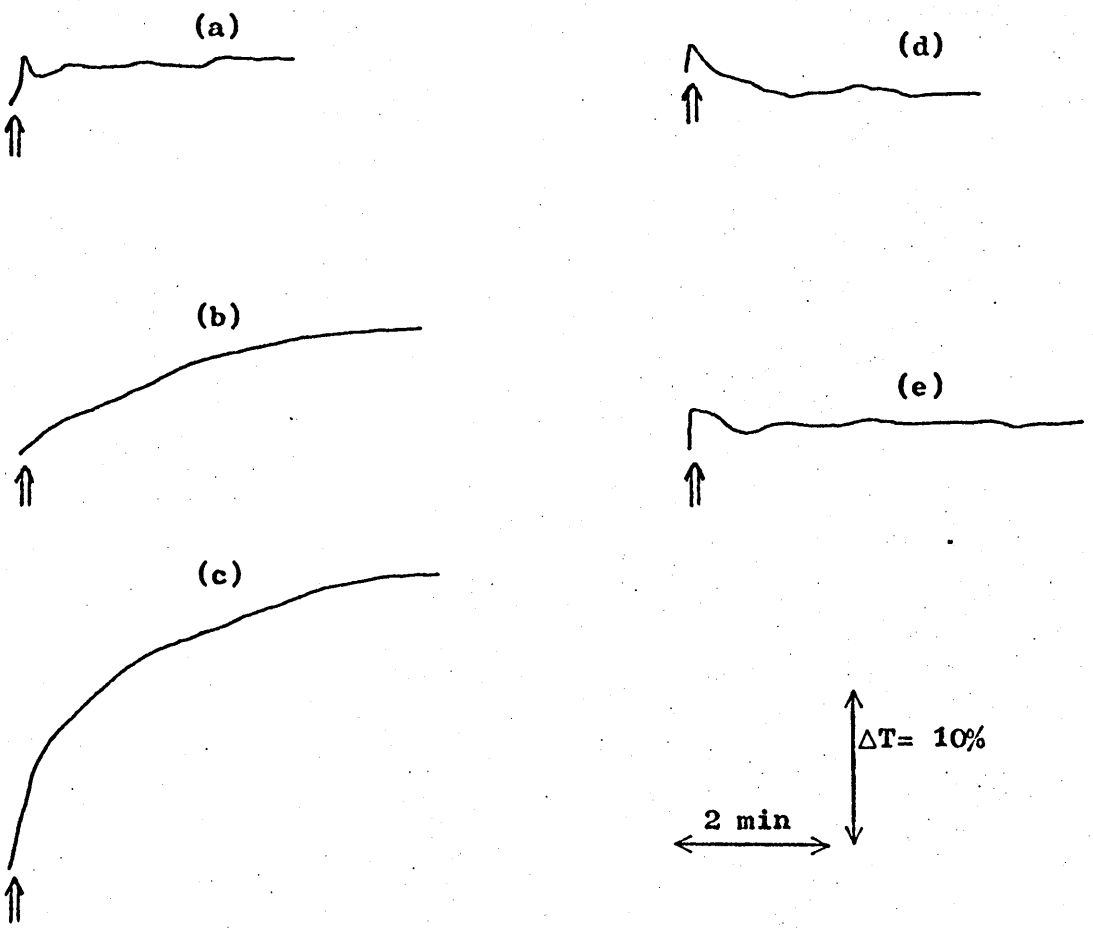


Figure 3.12. Light-induced transmission changes of pea chloroplast suspensions.

All traces were with chloroplasts (10µg chl./ml) suspended in the following media:-

(a) 25mM KCl, 5mM $MgCl_2$, 2.14mM p-benzoquinone and 1mM Hepes/NaOH buffer pH 7.6. The final volume was 2.8ml.

(b) Same as (a) plus 0.24µM TPT.

(c) Same as (a) plus 100mM sodium acetate.

(d) 0.25M sucrose, 5mM $MgCl_2$, 12mM KH_2PO_4, 2.4mM ADP, 2.64mM $K_3Fe(CN)_6$, 25mM Hepes/NaOH buffer pH 7.6. The final volume was 2.5ml.

(e) Same as (d) plus 0.24µM TPT.

⇑ Actinic illumination on.

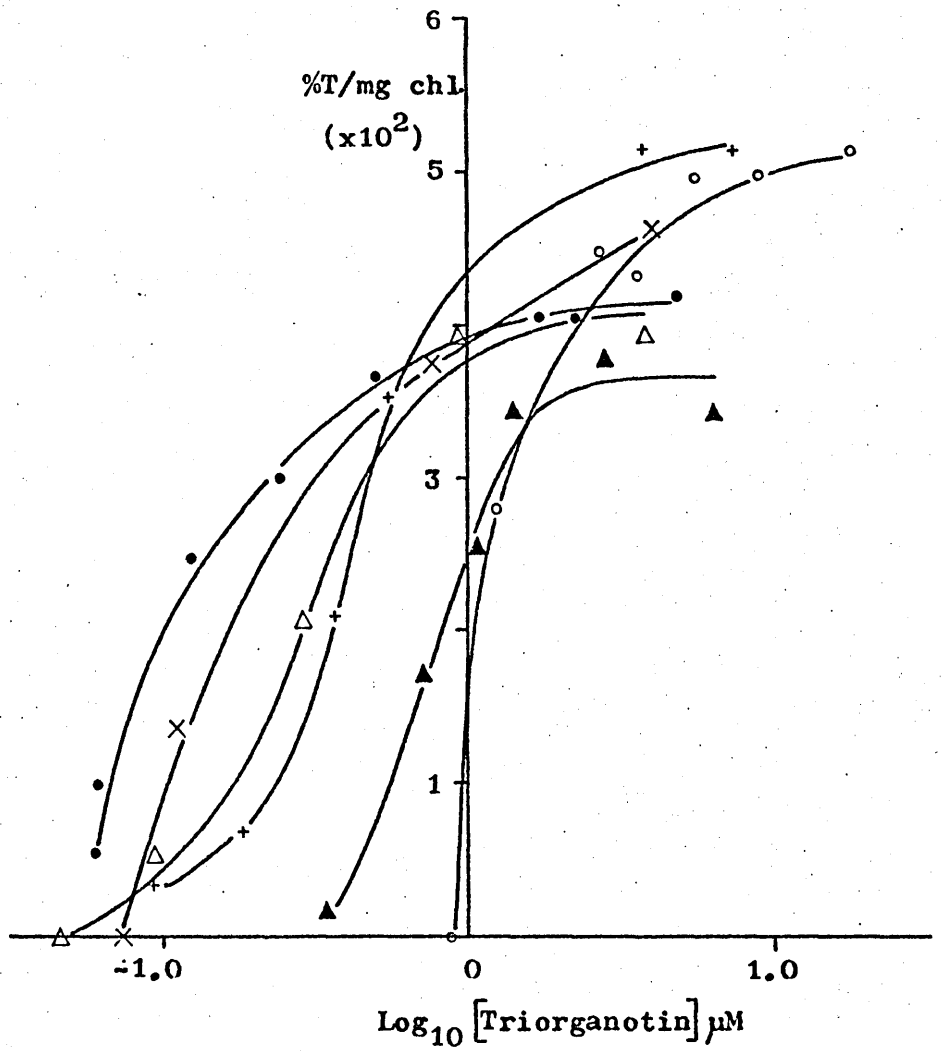


Figure 3.13. Effect of triorganotin compounds on the light-induced transmission changes of pea chloroplast suspensions.

o—o TMT.
▲—▲ TET.
•—• TPT.
△—△ TPhT.
+—+ TcHT.
×—× TBT.

inducing transmission changes whilst TcHT and TPhT were intermediate and TBT and TPT the most active. Ethidium bromide and the alkylguanidines were inactive in this assay over a wide concentration range (2.8 to 726.7 μM) and ethanol did not affect the activity up to a concentration of 2.5% v/v, which was the maximum concentration used when testing ethanol-soluble inhibitors.

(d) The Ca^{2+}-dependent chloroplast ATP-ase activity of C.reinhardii ($CW15^{+}$) and the effect of inhibitors.

The inhibition of the Ca^{2+}-dependent ATP-ase activity of chloroplast fragments from C.reinhardii (strain $CW15^{+}$) by the triorganotin and alkylguanidine compounds is shown in Figs. 3.14 and 3.15. The results show a low inhibitory activity for TMT and particularly TcHT, whilst the other compounds were more inhibitory than these and had similar activities to each other. The triorganotins were far less active in this assay than in any of the others and OG was somewhat less active than was DG.

The effect of various other inhibitors on the Ca^{2+}-dependent ATP-ase activity is shown in Table 3.3. Ethanol at 2.6% produced some inhibition of the ATP-ase activity so the effect of ethanol-soluble inhibitors was taken as the effect over and above this background inhibition level. At the concentrations tested, all the compounds produced a small degree of inhibition, although ephrepeptin gave complete inhibition at quite low concentrations. At concentrations lower than those required to inhibit, some of the inhibitors, viz., Dio-9, phlorizin, NH_4Cl and ephrepeptin, stimulated the ATP-ase activity.

The relative effects of the various triorganotin and alkylguanidine compounds on chloroplast activities are summarised in Table 3.4. For more meaningful comparisons, the values are expressed as the specific activity of each compound on a μ moles inhibitor per mg chlorophyll basis. Although this may not allow accurate cross-comparison of the

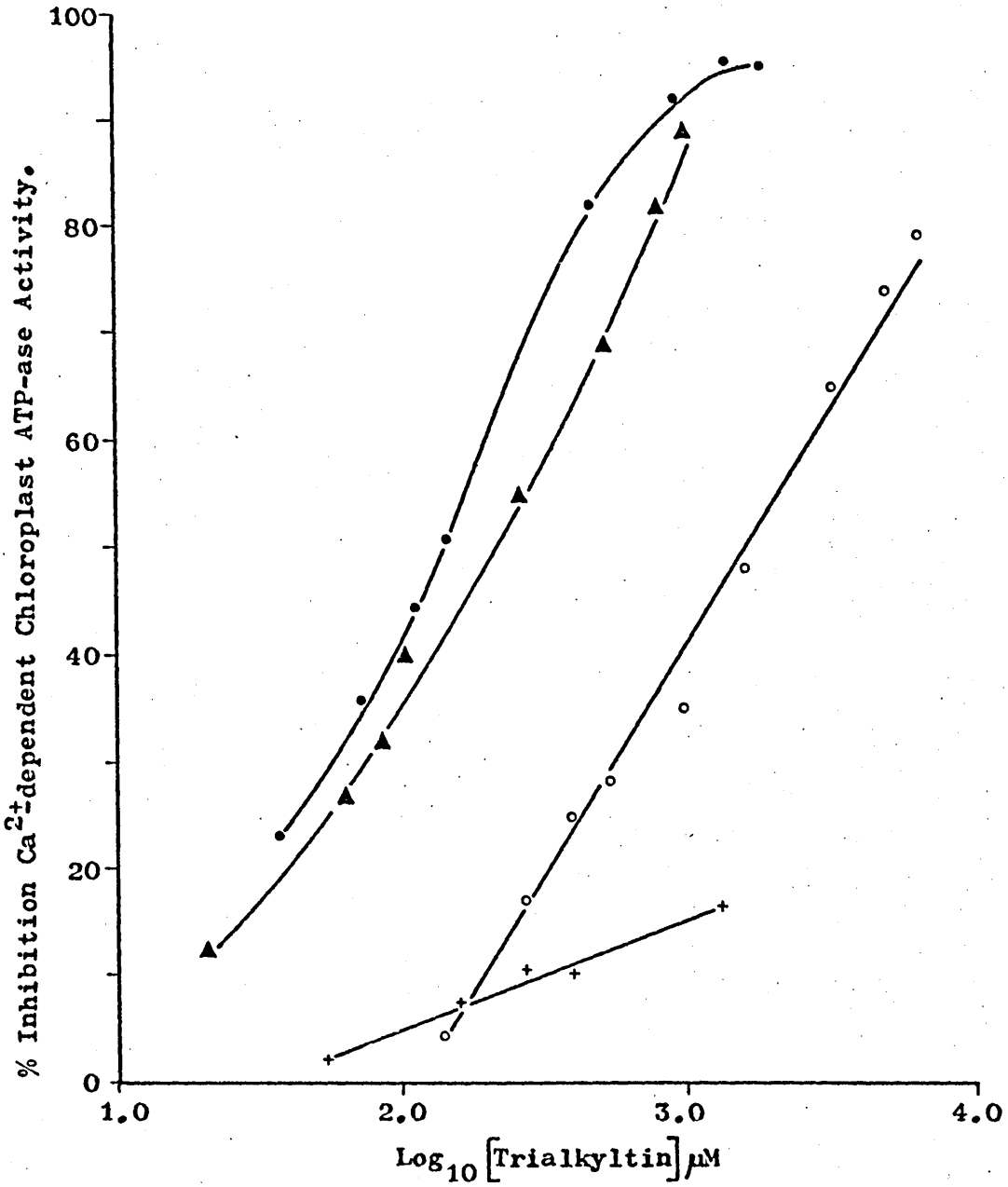


Figure 3.14. Inhibition by trialkyltin compounds of the Ca^{2+}-dependent ATP-ase activity of C. reinhardii (strain $CW15^{+}$) chloroplast fragments.

o—o TMT.
▲—▲ TET.
•—• TPT.
+—+ TcHT.

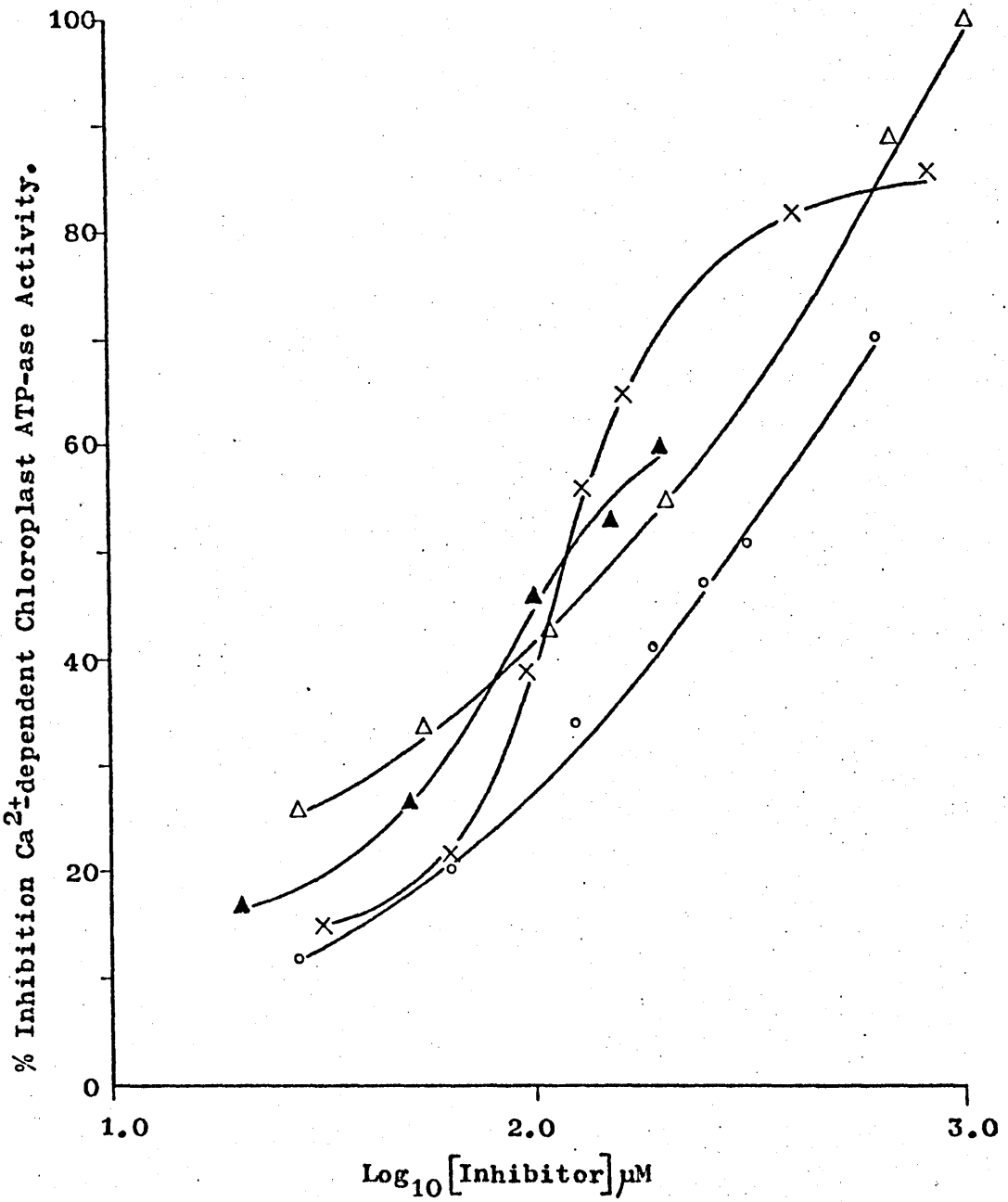


Figure 3.15. Inhibition by triorganotins and alkylguanidines of the Ca^{2+}-dependent ATP-ase activity of C. reinhardii (strain $CW15^{+}$) chloroplast fragments.

×—× TBT.
△—△ TPhT.
○—○ OG.
▲—▲ DG.

Table 3.3. Effect of various inhibitors on the Ca^{2+}-dependent ATP-ase activity of chloroplast fragments from C.reinhardii (strain $CW15^{+}$).

Inhibitor	ATP-ase Activity (μ moles Pi liberated/mg chl./h)
None	83.8
Ethanol (2.6%* v/v)	64.2
CCCP (6.6 μM)	61.2
CCCP (32.9 μM)	59.4
Dio-9 (13.2 μg/ml)	70.1
Dio-9 (65.8 μg/ml)	57.0
Phlorizin (30.2 μM)	70.7
Phlorizin (301.6 μM)	79.0
NH_4Cl (0.5 mM)	74.8
NH_4Cl (5.3 mM)	57.6
Ephrepeptin(2.1 ng/ml)	77.0
Ephrepeptin(21.1 ng/ml)	62.3
Ephrepeptin(210.5 ng/ml)	41.7
Ephrepeptin(5.3 μg/ml)	0

* Maximum concentration used for the addition of inhibitors.

Table 3.4. Summary of the effect of inhibitors on various chloroplast activities.

Inhibitor	n-Octanol : H_2O Partition Coefficient▲	Ca^{2+}-ATPase (I^{*}_{50})	State 3 O_2 Evolution (I^{*}_{50} or $^{\bullet}S_{100}$)	pH Decay Rate($S^{\bullet}_{100}$)	Transmission ($S^{+}_{2.5\%}$)
TMT	0.51	71.6	2.6^{*}	89.0×10^{-3}	116.0×10^{-3}
TET	3.7	8.0	1.1^{*}	40.0×10^{-3}	100.0×10^{-3}
TPT	52.0	5.7	$25.0 \times 10^{*-3}$	7.5×10^{-3}	14.0×10^{-3}
TPhT	1.2×10^{4}	5.7	$15.7 \times 10^{*-3}$	6.5×10^{-3}	38.0×10^{-3}
TcHT	2.0×10^{4}	7.6×10^{4}■	$4.0 \times 10^{*-3}$	3.5×10^{-3}	45.0×10^{-3}
TBT	1.3×10^{3}	4.5	$2.5 \times 10^{*-3}$	1.75×10^{-3}	21.0×10^{-3}
OG	-	11.3	$24.9^{\bullet}$	N.T.	N.A.
DG	-	4.8	$0.40^{\bullet}$ and $0.63^{\bullet}$	N.T.	N.A.

Values are in μ moles inhibitor per mg chlorophyll.

▲According to Wulf and Byington (1975). The value for TET refers to the bromide derivative.

$^{*}I_{50}$ = Dose required to inhibit activity by 50%.

$^{\bullet}S_{100}$ = Dose required to stimulate activity to twice the control value.

$^{+}S_{2.5\%}$ = Dose required to give 2.5% ΔT/mg chlorophyll.

■Insufficiently soluble in assay medium to give 50% inhibition so value is extrapolated from Fig. 3.14.

N.T., not tested

N.A., not active.

activities of any one inhibitor in the different assay systems, it does indicate that the Ca^{2+}-ATP-ase was far less sensitive to the triorganotin compounds than the other chloroplast activities. This table also shows that in all the assays, except for the transmission changes where TPT was the most active, the order of decreasing inhibitory activity of the trialkyltins was TBT > TPT > TET > TMT. Consequently, the activity may be related to the n-octanol : H_2O partition coefficient which increases through this series. TPhT and TcHT usually had specific inhibitory activities between TET and TBT except for the surprising inactivity of TcHT towards the Ca^{2+}-ATP-ase activity. OG and DG appeared to show more correspondence in their activities in the ATP-ase and state 3 assays than did the triorganotins, although an accurate comparison is not possible due to the triphasic nature of the effect of these compounds on state 3 oxygen evolution.

Discussion

Pea chloroplasts were isolated with reasonably high values for the PC and ADP/O ratios and the oxygen uptake of these chloroplasts was markedly stimulated by uncouplers. This indicates that the non-cyclic electron transport in these chloroplasts was coupled to ATP synthesis and hence provides a simple and rapid method of determining the effect of inhibitors on the coupling mechanism. This was confirmed by the uncoupler-reversible inhibition of state 3 oxygen evolution by known energy-transfer inhibitors, such as Dio-9 (Mc Carty *et al.*, 1965) and phlorizin (Izawa *et al.*, 1966), and the stimulation by arsenate in the absence of added phosphate (Avron and Jagendorf, 1959). Ethidium bromide at a concentration of 406 μM produced an NH_4Cl-reversible inhibition of state 3 oxygen evolution, which indicates that EB may be an energy transfer inhibitor at this concentration. This is interesting in view of the reported *in vitro* uncoupling properties of EB on mitochondrial respiration (Miko and Chance, 1975), which may be linked to the action of EB on mt-DNA degradation (Bastos and Mahler, 1974). However, the effects of EB on photosynthetic energy transfer have not been previously reported and require further investigation.

The triorganotin compounds inhibited state 3 oxygen evolution and the inhibition was relieved by uncouplers. Maximum inhibition occurred to about the level of the basal (state 2) rate and NH_4Cl-stimulated electron transport was not inhibited which indicates that, under the conditions of this assay, the triorganotin compounds behaved as electron transfer inhibitors. The activity of the triorganotin compounds depended on the organic group in a way which may be related to their n-octanol : H_2O partition coefficients i.e., except for TcHT and TPhT, inhibitory activity and partition coefficients generally increased up the homologous series from TMT to TBT (Table 3.4). This

may indicate a highly hydrophobic site of action of the trialkyltins, with the more polar compounds being less active due to their having a lower affinity for the inhibitory site of action.

Very similar results were found by Watling-Payne and Selwyn (1974), using a similar assay system, except that TET was found to be less inhibitory than TMT. In addition, these authors found a slight inhibition of state 2 oxygen evolution by TMT, TET, TPT and TBT within the same concentration range that state 3 was inhibited, which is uncharacteristic of many photosynthetic energy transfer inhibitors (Good et al., 1966). However, this inhibition was not due to a direct inhibitory effect on electron transport since uncoupled electron transport was not inhibited at concentrations that inhibited state 3 (Watling-Payne and Selwyn, 1974; Gould, 1976). This was also shown with NH_4Cl-uncoupled electron transport (e.g., Fig. 3.4). However, the affect, if any, of the state 2 inhibition on the state 3 inhibition is not clear, particularly since a general lack of inhibition of state 2 by TMT and TPhT has been reported (Gould, 1976).

When chloroplasts were suspended in a medium containing high Cl^- concentrations, addition of the triorganotin compounds before illumination resulted in inhibition of the rate of rise and extent of the pH change but a stimulation of the dark decay rate. Similar qualitative effects were produced by all the triorganotins but at different relative concentrations (e.g., effect on dark decay in Table 3.4). These effects are consistent with the operation of a $Cl^- - OH^-$ exchange reaction mediated by the triorganotins, which results in a dissipation of the pH component of the electrochemical potential gradient produced on illumination (Watling-Payne and Selwyn, 1970, 1974). Except with TMT and TET, this uncoupling effect occurred at around similar

concentrations to those that inhibited state 3 (Table 3.4) and so may have affected this state 3 inhibition. However, the uncoupling effect was probably minimal since the light-induced transmission changes were not stimulated by triorganotins in the medium used for measuring the inhibition of state 3 oxygen evolution (Fig. 3.12 e).

Addition of low concentrations of triorganotin compounds to illuminated chloroplast suspensions, after the steady state pH gradient had been reached, resulted in an immediate sharp decline in the pH level followed by equilibration at a new steady state at a lower pH level to the original one. Higher triorganotin concentrations produced progressively greater 'overshoot' during the initial decline but the final steady state level in the light was approximately the same irrespective of the triorganotin concentration.

The reason for the transient 'overshooting' is not clear. The uncoupling Cl^- - OH^- exchange reaction is presumably involved in the effect but whether it is involved directly in the 'overshooting' phenomenon remains to be investigated. The 'all or nothing' nature of the initial sharp decline, whereby further triorganotin additions have no further effect, may indicate that the Cl^- - OH^- exchange reaction is saturated at relatively low concentrations. The fact that initial additions of high triorganotin concentrations eventually results in recovery to a similar steady state level, as when low triorganotin concentrations are initially added, supports this view. However, the extent of the 'overshooting' increases with increasing concentrations of triorganotins and is absent at low concentrations, which may indicate a secondary action of higher triorganotin concentrations.

The reversible nature of the 'overshoot' is interesting in view of an analogous effect produced in chloroplasts by tetraphenylboron (TPB). This effect is completely reversible and varies with the

concentration of TPB added, although at higher concentrations TPB inhibits the extent of H^+ uptake (Horton and Packer, 1968). The exact mechanism of the TPB effect was not found although it may involve a light-dependent, irreversible uptake of TPB^- by chloroplasts and a change in the permeability characteristics of the chloroplast membrane towards H^+ and K^+ due to a reaction of TPB^- with protonated amino groups in the membrane (Horton and Packer, 1968; Fiolet and van Dam, 1973). The slow 'inactivation' would then be a side-reaction in which the protons in the membrane react with TPB (Fiolet and van Dam, 1973). Whether a similar mechanism is also involved in the action of triorganotin compounds, or whether the 'overshooting' simply results from a combination of the Cl^- - OH^- exchange reaction and the non-specific detergent-like properties of higher triorganotin concentrations (Stockdale et al., 1970), requires further clarification.

The fact that the triorganotin compounds enhanced the light-induced transmission changes in a KCl-medium provides further evidence that the Cl^- - OH^- exchange mechanism occurs under similar conditions to those used for assaying the light-induced pH changes. A mechanism to account for the enhancement of the light-induced shrinkage by weak (e.g., acetic) acid anions has been proposed by Crofts et al. (1967). Illumination results in an increase in the internal H^+ concentration which shifts the equilibrium in the direction of the undissociated acid and increases its concentration within the thylakoid compartment. The undissociated acid then diffuses out into the medium, resulting in osmotic shrinkage of the thylakoids. However, when chloroplasts are suspended in a medium composed of salts of strong inorganic acids in the range pH 7.5 - 8.0, the completely dissociated ions do not move in response to the light-induced proton gradient and produce little change in light-scattering (Watling-Payne and Selwyn, 1970).

Further addition of triorganotin compounds, which are active in the Cl^- - OH^- exchange reaction, results in a large change in light scattering which is similar to that observed in a sodium acetate medium. This is due to a movement of OH^- ions, in exchange for Cl^- ions, into the intra-thylakoid space in response to the light- induced pH gradient (Watling-Payne and Selwyn, 1970). There is a general correlation between light scattering and transmission changes when chloroplasts undergo volume changes in vitro (Packer and Murakami, 1972). Consequently, the observed effects of triorganotin compounds on the light-induced transmission changes of chloroplasts suspended in a KCl medium reflects the volume changes of the thylakoids due to the operation of a Cl^- - OH^- exchange mechanism.

The relative efficacy of the different triorganotin compounds at the $S_{2.5\%}$ level reveals some differences in their activities compared to their effect on the light-induced pH rises. Tripropyltin was the most active compound in the transmission studies but TBT was most active in stimulating the dark decay of the light-induced pH rises. The reason for this discrepancy is unknown but, if for optimal activity in these assays a compound should have some property mid-way between that for TPT and TBT (e.g., partition coefficient), then the difference in activity between the two compounds in the two assays would in fact be smaller than would at first appear.

The triorganotin compounds, except for TcHT, had a similar general order of activity against the Ca^{2+}-dependent ATP-ase activity of C.reinhardii chloroplasts as in the other assays, i.e., TMT was least active and TBT was most active. However, the triorganotins were much less inhibitory in this assay than in any of the others. This may be due to the use of different chloroplast systems in the various assays which may not allow direct comparison. In addition,

the presence of EDTA in the ATP-ase assay medium may lead to uncoupling of chloroplasts by removal of the chloroplast coupling factor (Jagendorf and Smith, 1962; Avron, 1963) and render them relatively insensitive to the effects of inhibitors, such as triorganotins, which require an intact CF_1/membrane system (Gould, 1976). This is supported by findings that both TBT and TPhT are much less active in inhibiting the Ca^{2+}-dependent ATP-ase of isolated coupling factor than in inhibiting photophosphorylation (Kahn, 1968; Gould, 1976). However, in spite of the high triorganotin concentrations required to inhibit the Ca^{2+}-dependent ATP-ase activity of the chloroplast fragments, the inhibition could well be a specific reaction with the enzyme since the same concentration of TBT has been found to be required to obtain complete inhibition in crude as compared to purified, enzyme preparations (Kahn, 1968).

The reason for the striking absence of inhibition of the Ca^{2+}-dependent ATP-ase by TcHT is unknown. The fact that this compound was active in the other assays indicates that the site of action of this compound is located in the chloroplast membrane and that it has no direct effect on the CF_1 even at very high concentrations. The cyclic nature of the molecule may indicate a more specific ionophoretic property of this compound.

The mode of action of the alkylguanidines appears to be concentration-dependent. At relatively low concentrations (up to 3.4×10^{-4}M for OG and up to 1.6×10^{-5}M for DG), they behaved as energy transfer inhibitors and the inhibition of state 3 oxygen evolution was relieved by uncouplers such as NH_4Cl. At intermediate concentrations (1.6×10^{-5}M to 5.3×10^{-5}M for OG and 3.4×10^{-4}M to 9.4×10^{-4}M for OG), they uncoupled and maximally stimulated state 3 and relieved the inhibition produced by the triorganotins. This uncoupling effect was not due

to catalysis of a Cl^- - OH^- exchange, as with the triorganotins, since the alkylguanidines did not stimulate the light-induced transmission changes in a KCl medium. At relatively high concentrations, a time-dependent inhibition of state 3 electron transport occurred and this inhibition was not relieved by uncouplers. OG has been previously found to behave as a photosynthetic energy transfer inhibitor at low concentrations (5×10^{-5}M) and an uncoupler at higher concentrations (2×10^{-4}M) (Gross et al., 1968).

The effect of the alkylguanidines on state 3 oxygen evolution was paralleled by their effects on the Ca^{2+}-dependent ATP-ase activity of C.reinhardii chloroplast fragments. Inhibition of the ATP-ase by OG occurred at least between 2.5×10^{-5} M and 6.3×10^{-4}M and for DG over the range 2.0×10^{-5}M to 2.0×10^{-4}M. Consequently, inhibition of the ATP-ase may have occurred over the whole alkylguanidine concentration range irrespective of whether they behaved as energy transfer inhibitors, uncouplers or electron transport inhibitors. However, comparisons between the effects of inhibitors in different assay systems, using chloroplasts from different sources, may not be justifiable. In addition, the chloroplast fragments used in the ATP-ase assay would have been uncoupled by the EDTA in the assay medium so any stimulation of activity by the alkylguanidines may have been masked. However, a parallel between the degree of uncoupling and the extent of inhibition of different chloroplast ATP-ase activities has been previously observed with OG (Bennun and Avron, 1965).

The inhibition of electron transport by high concentrations of alkylguanidines may be due to a non-specific detergent-like action on the chloroplast membranes as proposed for the action of OG on mitochondrial respiration (Mitchell, 1966[b]). The inhibition of the Ca^{2+}-dependent ATP-ase may be due to a more specific action on the

chloroplast coupling factor in a similar way to their effect on the F_1 of mitochondria (Goméz-Puyou et al., 1976). The greater activity of DG, compared to OG, on the Ca^{2+}-dependent ATP-ase activity suggests a hydrophobic site of action of these compounds, which has also been inferred from the relative inhibitory properties of different guanidine derivatives on the respiration and ATP-ase activities of mitochondria (Pressman, 1963; Goméz-Puyou et al., 1976).

In conclusion, it appears that the triorganotin and alkylguanidine compounds are active against the energy-conserving mechanism of chloroplasts. Previous reports that the triorganotin compounds may behave primarily as uncouplers, via a Cl^- - OH^- exchange mechanism, or principally as energy transfer inhibitors, depending on the composition of the assay medium, have been confirmed. The alkylguanidines also have a multiple effect on chloroplast energy conservation but this is concentration-dependent. The energy transfer inhibition at relatively low concentrations may be due to a direct effect on CF_1 activity. Intermediate concentrations uncouple electron transport from photophosphorylation and relieve the inhibition of coupled electron transport produced by triorganotin compounds. In view of the reported mode of action of TBT and TPhT in blocking a H^+-conducting channel through the chloroplast membrane at the level of the CF_1 attachment site (Kahn, 1968; Gould, 1976), it is possible that this mechanism may also apply to some extent to the other triorganotins. It may be that the release of the triorganotin inhibition of coupled electron transport by alkylguanidines is due to a non-specific release of the H^+ gradient by a more direct effect of these compounds on the chloroplast membrane. The detergent-like property of the alkylguanidines (Mitchell, 1966[b]) is consistent with this hypothesis in view of the known uncoupling effects of other detergents (Neumann and Jagendorf, 1965;

Izawa and Good, 1965). Higher inhibitory concentrations of alkylguanidines may then have a more direct effect on electron transport, either through direct interaction with the electron transport intermediates or a greater non- specific detergent-like effect on the thylakoid membrane.

CHAPTER 4

Isolation and Whole-cell and Genetic Characterisation of Inhibitor-resistant Mutants of C.reinhardii.

Introduction

The isolation and study of mutants that have affected function of the mitochondria and chloroplasts has been a most useful tool in the understanding of these organelles. For example, the use of photosynthetic mutants of algae has proven very effective in elucidating nuclear and chloroplast control of chloroplast structure and function (Levine and Goodenough, 1970; Surzycki et al., 1970; Gillham, 1974) and the nature and organization of the different components involved in photosynthesis (Levine, 1968, 1969). One important class of such mutants consists of those with increased resistance to inhibitors which have a known and specific action on some aspect of respiration or photosynthesis.

Both Mendelian and non-Mendelian mutations are known in C.reinhardii that make the alga resistant to, or dependent upon, antibiotics that inhibit protein synthesis by 70S (bacterial) ribosomes (Sager, 1954, 1972; Sager and Tsubo, 1961; Gillham, 1965; Sager and Ramanis, 1965, 1967, 1971; Gillham and Fifer, 1968; Gillham, 1969). A significant proportion of these mutants have been found to have altered chloroplast ribosomes (Mets and Bogorad, 1972; Davidson et al., 1974; Schlanger and Sager, 1974; Brugger and Boschetti, 1975) and so could be of immense use in the determination of the rôle of chloroplast ribosomes in chloroplast function and biogenesis.

The isolation and characterisation of mutants of C.reinhardii resistant to drugs which directly affect other processes in the cell besides chloroplast protein synthesis has been very limited. This chapter describes work which attempted to isolate mutants of

C.reinhardii that were resistant to inhibitors of energy conservation (e.g., organotins, alkylguanidines and ethidium bromide) and DNA replication (e.g., ethidium bromide). These mutants were then partially characterised at the whole-cell level in order to obtain indications as to their mode of resistance and hence to assess their potential use in the elucidation of the nature of energy conservation in this alga.

Materials and Methods

(a) General methods.

Growth of C.reinhardii.

Drug-resistant mutants were derived from the mt^+ stock of C.reinhardii (strain 137c). Growth in liquid or solid media in the presence or absence of drugs was carried out as described in the Materials and Methods of Chapter 2.

Dry weight, total cell numbers and chlorophyll estimations.

The estimation of DW, total cell numbers and chlorophyll in whole-cell suspensions of C.reinhardii were carried out as described in the Materials and Methods of Chapter 2.

(b) Survival curves of C.reinhardii (Wt^+) following mutagenesis.

UV treatment.

UV mutagenesis was carried out on mid log-phase cells (49 h old) of the mt^+ strain from 1.5 l batch cultures of A medium. The cells were washed and resuspended in sterile M medium to a concentration of 2.35×10^6 cells per ml and 10 ml aliquots irradiated in a 7.5 cm crystallizing dish using a Sylvania G8T5 germicidal lamp with maximum output at 254 nm. The distance from the lamp to the culture surface was 10 cms and the suspension was stirred continuously during irradiation. At regular time intervals up to 120 secs, 0.1 ml aliquots of the suspension were withdrawn, diluted with sterile M medium and spread onto replicate plates of solid M, A and YAP media to give a concentration of 4×10^3 cells per plate. To prevent photoreactivation, the entire operation from irradiation to plating was performed in a dark-room illuminated only by a lamp covered with a Kodak 'Wratten' yellow safelight filter and the plates were stored for 12 h in the dark at room temperature before subsequent growth under light or dark conditions. Colony growth was assessed after one week in the

light or two weeks in the dark.

MNN treatment.

Early stationary-phase cells of C.reinhardii mt^+ (72 h old) were washed once in sterile 0.1 M citrate buffer, pH 5.0, and finally resuspended in this buffer to a concentration of approximately 1 x 10^8 cells per ml. 5.0 mls of a filter-sterilised stock solution of MNN dissolved in citrate buffer, pH 5.0, were added to give a final MNN concentration of 50 µg per ml and the suspension shaken in a 250 ml conical flask at 25° C in the dark. At regular time intervals up to 20 min, aliquots of the suspension were removed, quickly diluted 100 fold with sterile A medium to stop the reaction and spread onto replicate plates of solid M, A and YAP media to give a concentration of 700 cells per plate. Colony growth was assessed after one week in the light or two weeks in the dark.

(c) Isolation of drug-resistant mutants.

Drug-resistant mutants of the mt^+ strain of C.reinhardii were isolated either by spontaneous selection or by induction with UV light or MNN. Attempts were made to isolate mutants under phototrophic, mixotrophic and heterotrophic growth conditions using M and A media containing the following drug concentrations:-
TMT(102 µM), TET(15 and 30 µM), EB(25,51,127,254,380 and 507 µM), OG(48,96 and 241 µM), DG(19,38 and 76 µM), venturicidin(315 µM), Dio-9(100 µg/ml), TPhT(3 µM), atebrin(529 µM), TPT(18 µM), CCCP(49 and 97 µM) and TBT(15 µM).

Spontaneous selection.

Early stationary-phase cells from 1.5 l mixotrophic cultures of C.reinhardii (mt^+) were washed, resuspended in fresh sterile A medium and evenly spread onto drug-containing agar plates to give 1 x 10^8 cells per plate.

U.V. induction.

UV induction of mutants was carried out as previously described for the determination of survival rates. The cell culture was exposed to the UV light for 60 secs, which gave a survival rate of approximately 1 - 10%. Cells were then spread onto drug-containing agar plates at a concentration of 1 x 10^7 cells per plate and left in the dark at room temperature for 24 h before subsequent growth in the light or dark.

MNN induction.

MNN induction of mutants was carried out as previously described for the determination of survival rates. The cells were treated with MNN for 10 min to give a survival rate of less than 20%. The treated cells were then immediately diluted 10 fold with sterile A medium to stop the reaction, centrifuged aseptically and resuspended in a few mls of sterile A medium. A portion of the suspension was immediately spread onto drug-containing agar plates at a concentration of 3.6 x 10^7 cells per plate. A second portion of the suspension was diluted with sterile A medium containing 2 x 10^4 units of penicillin G and grown for a further 72 h to stationary phase. These regrown cells were then centrifuged and spread onto drug-containing agar plates at a concentration of 3 - 4 x 10^7 cells per plate.

In all cases of selection and induction procedures, the prepared plates were incubated in the light or dark at 25°C until sufficient mutant colony growth had occurred to allow the individual colonies to be transplanted onto fresh solid drug-free media. The colonies were then sub-cloned and a single colony used as the stock culture for each mutant strain.

(d) Growth response of Wt$^+$ and mutants to inhibitors.

Cross-resistance patterns.

Wild-type and mutant cultures were grown mixotrophically in 10 ml aliquots of A medium in 25 ml conical flasks. This allowed simultaneous growth of large numbers of cultures. Growth on drug plates was assessed by a modification of the drop-out technique (Wilkie and Lee, 1968), as described in the Materials and Methods of Chapter 2.

Effect of inhibitors on growth rates and extents.

The effect of TMT, TET and EB on growth rates and extents in liquid culture was determined as described in the Materials and Methods of Chapter 2.

(e) Effect of various conditions on the response of Wt^+ and mutants to inhibitors.

Temperature.

The effect of different temperatures on the response of Wt and mutant strains to inhibitors was carried out on solid media as described in the Materials and Methods of Chapter 2. The minimum and maximum restrictive growth temperatures for C.reinhardii, under all growth conditions, were found to be 10°C and 35°C respectively so temperatures of 15°C, 25°C and 30°C were used where possible. Experiments under phototrophic and mixotrophic conditions were carried out in an illuminated fume cupboard and experiments under heterotrophic conditions were carried out in a Gallenkamp constant temperature incubator.

Spectinomycin.

The effect of spectinomycin on the response of the Wt and mutant strains to inhibitors was carried out on solid media as described in the Materials and Methods of Chapter 2. A concentration of 45 μM spectinomycin was used in the presence of the drugs as this concentration was found to specifically inhibit phototrophic, but not

mixotrophic and heterotrophic, growth (Tables 2.1 and 4.5).

Deoxycholate.

The effect of deoxycholate on the response of the Wt and mutant strains to inhibitors was carried out on solid media as described in the Materials and Methods of Chapter 2. A concentration of 725 μM deoxycholate was used in the presence of the drugs since this concentration was found to be the maximum concentration that had no visible effect on growth of the Wt or mutant strains on solid media. This concentration was also found to produce only slight cell-disruption of the $CW15^+$ (cell wall-less) strain of C.reinhardii when suspended in liquid A medium at a concentration of 5 mg DW per ml (Fig. 4.10).

(f) Effect of inhibitors on whole-cell respiration and oxygen evolution.

Early stationary-phase cells (72 h old) from mixotrophic cultures of Wt^+, $TP\text{-}8^+$ and $POG\text{-}10^+$ strains of C.reinhardii were harvested by centrifugation at room temperature for 5 min at $4 \times 10^3 g_{av.}$ The cells were washed once in A medium and finally resuspended in A medium at a concentration of 1 mg chlorophyll per ml. Oxygen evolution and uptake were measured with a Clark-type oxygen electrode incorporated into a glass-lined perspex reaction vessel and maintained at a temperature of 25°C. The assays were carried out in A medium to give a final cell concentration of 40 μg chlorophyll per ml in a final volume of 2.5 ml and the cell suspension was stirred continuously throughout the assays. Oxygen uptake was determined with the reaction vessel covered with a light-proof cloth. Light for the measurement of net oxygen evolution was supplied by a Rank Aldis Tutor 1000 projector giving a light intensity of 3.3×10^4 lux at the vessel surface.

(g) Uptake of inhibitors.

Ethidium bromide.

The uptake of EB by whole cells of Wt and mutant strains was

assayed by the time-course disappearance of EB from the supernatant of a cell suspension. Early stationary-phase cells (72 h old) from mixotrophically-grown cultures were aseptically washed and resuspended in fresh sterile A medium to a concentration of 50 mg DW per ml. EB was added to give a final concentration of 125 μM and the suspension was shaken on a Gallenkamp shaker at 25°C in the light. 2.0 ml aliquots of the suspension were withdrawn at fixed intervals, rapidly placed in tubes of a BTL microangle centrifuge and centrifuged for 5 min. 1.0 ml of the supernatant was then added to 1.0 ml of a 48.6 mM sodium dodecyl sulphate (SDS) solution in distilled water to give a final SDS concentration of 24.3 mM. This concentration of SDS was found to give maximum fluorescence enhancement of a wide range of EB concentrations (Appendix Fig.4). The solutions were rapidly mixed and the concentration of EB determined by measuring the fluorescence intensity using an Eppendorf photometer with a fluorimeter attachment (Netheler and Hinz, Hamburg). The excitation and emission wavelengths were 313 + 366 nm and 590 - 3000 nm respectively.

Standard curves relating the concentration of EB with the fluorescence intensity of different solutions were constructed (Appendix Fig. 5). The photometer was adjusted to maximum sensitivity in the presence of a standard solution of EB, equivalent to half the EB concentration used in the uptake experiments, dissolved in 24.3 mM SDS. Any reduction in the fluorescence intensity of the EB in the supernatants could therefore be related to a reduction in concentration by multiplying the uptake data by a factor of 2.

$[^{113}Sn]$ TET Chloride

Radio-labelled TMT was unavailable so the uptake of $[^{113}Sn]$TET chloride by whole cells of the Wt^+ and $TP\text{-}8^+$ strains was investigated. Mid log-phase cells (48 h old) were sedimented at 3×10^3 $g_{av.}$ for

5 min at room temperature, washed once and finally resuspended in fresh A medium to a concentration of 10 mg DW per ml. $[^{113}Sn]$TET was added to 5.0 mls of the suspension to give a concentration of 9.8 µM $[^{113}Sn]$TET and the suspension stirred and maintained at a constant temperature of 25°C throughout the assays.

Replicate 250 µl aliquots of the suspension were removed at intervals and rapidly filtered through Whatman GF/C glass fibre filters using a vacuum pump and glass manifold filtering system. The filters and cells were washed once with 2.0 ml of distilled water and the wet filters and cells placed in an empty liquid scintillation vial.

Several procedures for bleaching of the cells were tried to prevent AES values becoming too low (Appendix Table 2). Hydrogen peroxide and dil. H_2SO_4 added either directly to the filter and cells alone or to the filter and cells immersed in scintillation fluid (1.0 l toluene : 0.5 l Triton X - 100 : 7.0 g 2-(4'-t-butylphenyl)-5-(4"-biphenyl) —1,3,4-oxadiazole) were found to be poor bleaching procedures. Sodium hypochlorite was an excellent bleaching agent but produced high erroneous counts. A satisfactory bleaching procedure was found to be the addition of 0.5 ml chlorine water to the wet filter and cells placed in an empty scintillation vial. After 1 h the scintillation fluid was added to the bleached filter and cells. This procedure did not cause significant loss of volatile radioactive compounds from the vial (Appendix Table 2).

Gamma emission of $[^{113}Sn]$TET solutions was estimated with a Packard tri-carb liquid scintillation spectrometer (model 2425). A standard curve relating AES values to the final calculated DPM was constructed (Appendix Fig.6), since a true value of the DPM of standard $[^{113}Sn]$TET solutions in quenched samples was not obtained

directly (Appendix Fig.7). A percentage correction factor was thus obtained for each AES value of quenched samples and this held true for both bleached and unbleached samples (Appendix Figs. 6 and 8). A satisfactory method for measuring $[^{113}Sn]$TET in the presence of bleached cells was thus developed using the AES correction factor (Appendix Fig. 8).

(h) Genetic analysis.

Tetrad analysis.

Crosses, maturation of zygotes and tetrad analysis were carried out essentially according to the methods of Ebersold and Levine (1959).

For mating, haploid cells from vegetative palmelloid cultures of opposite mating type were suspended separately in 10 ml of sterile nitrogen-free A medium (Appendix Table 1). The cultures were shaken in the light for 24 h to induce motility and sexual competence. Each mating-type suspension was mixed with the opposite one in equal proportions and left in the light for 3 h to allow mating and fusion. The zygote suspension was then poured onto A medium, illuminated for 24 h and placed in darkness for 5 days. Following this maturation period, the vegetative cells were scraped from the agar surface with a flame-sterilized scalpel blade and the remaining vegetative cells killed by exposing the plate to chloroform vapour for 40 secs. The more 'sticky' zygospores remained on the cleared area of the plate and were streaked into a single row on fresh A medium using a flame-sterilized wire loop. Single zygospores were separated from the main body of vegetative cells and zygospores under low-power magnification using a flame-sterilized length of fine wire. The plate was then illuminated for 24 h to allow zygospore germination. Each zygospore produced 4 or 8 meiotic products which were separated with the aid of a microscope and micromanipulator (Singer Instrument Co. Limited).

Each meiotic product usually produced a single colony on illumination for 5 days (Appendix Fig.9), which was then streaked onto fresh agar medium prior to phenotypic characterisation.

The determination of the mating type of a colony was determined by suspending the colony in nitrogen-free A medium and shaking the culture in the light for 24 h. Two separate drops of the culture were then placed on A agar medium and each one mixed with a drop of either Wt^+ or Wt^- from nitrogen-free A medium. The plates were placed in the light for a further 24 h, followed by incubation in the dark for 5 days. The plates were then assessed for the presence and absence of zygospores.

Sub-clonings of zoospore progeny

In some cases, after separation of the single zygospores from the vegetative cells, the meiotic products were not separated out but allowed to grow together as a single colony. These single colonies were then sub-cloned onto fresh medium and approximately 16 of the resultant individual colonies were chosen at random and tested for drug resistance.

Results

(a) Survival curves of C.reinhardii (Wt^+) following mutagenesis.

The survival curves for C.reinhardii (Wt^+) cells following treatment with two different mutagenic agents are shown in Figs. 4.1 and 4.2. Increasing the time of exposure to the mutagens resulted in a decrease of survival rates. There was a distinct lag phase in the lethal effects of UV light but the time-response curves for MNN were more linear. In general, the lethal effects of the mutagens decreased in the following order of growth conditions : YAP(L) < M(L) < A(L) < A(D) ≤ YAP (D) and were particularly marked under dark conditions.

(b) Isolation of drug-resistant mutants.

Drug-resistant mutants were isolated either spontaneously or by induction with the two mutagens. Regrowth of the cells after mutagenesis considerably increased the number of drug-resistant colonies obtained, particularly after MNN treatment. The total number of mutant colonies examined for their cross-resistant characteristics after induction or spontaneous selection were as follows : triorganotin-resistant (75), EB-resistant (41) and alkylguanidine-resistant (56). Five major classes of resistant mutants were obtained from all the mutant colonies that proved to have stable, resistant phenotypes and one representative of each class was selected for further study (Table 4.1). Attempts to isolate mutants on other drugs proved unsuccessful or the isolated colonies were insufficiently resistant and cross-resistant to other drugs to warrant further investigation.

(c) Growth response of Wt^+ and mutants to inhibitors.

Cross-resistance patterns.

The cross-resistance patterns of the five major classes of drug-resistant mutants are shown in Table 4.2. These values represent the

Legend to Figures 4.1 and 4.2.

Survival of C. reinhardii cells after treatment with UV light and MNN.

The cells were treated as described in the Materials and Methods and subsequently grown under the following conditions:-

+—+ YAP medium in the light.

•—• A medium in the light.

△—△ M medium in the light.

▲—▲ YAP medium in the dark.

o—o A medium in the dark.

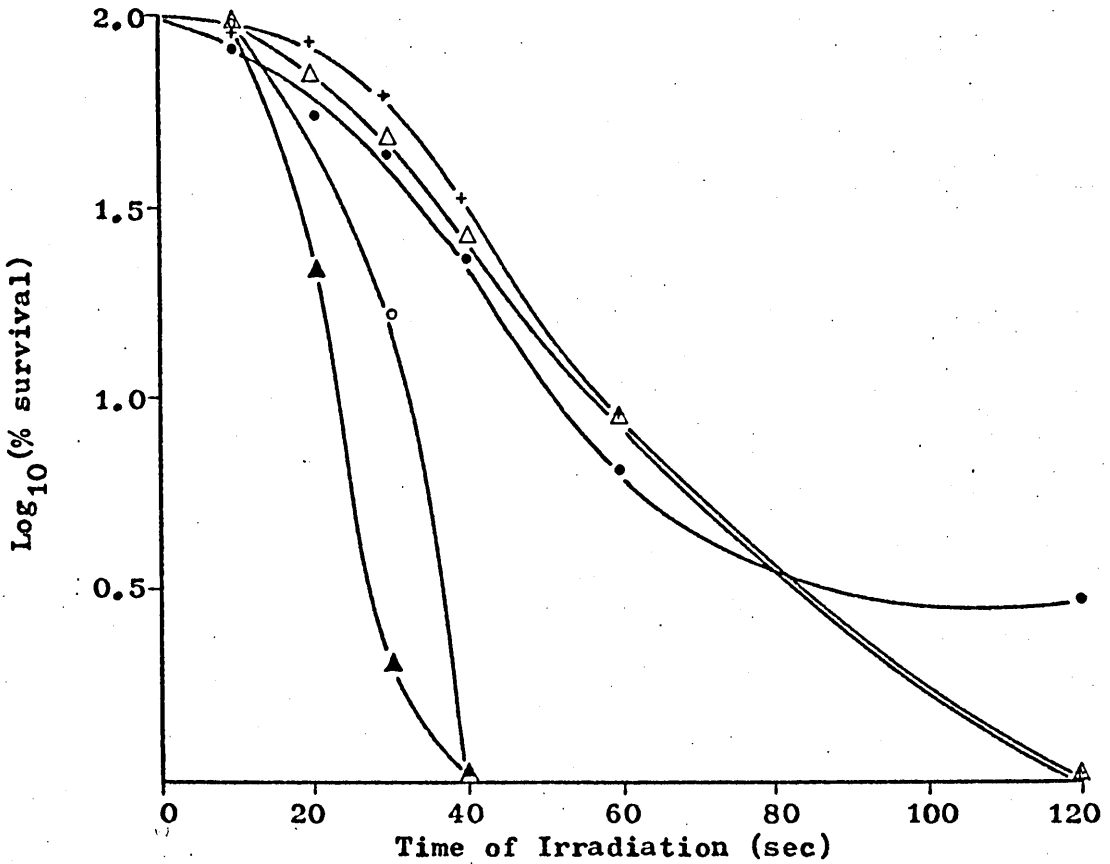


Figure 4.1. Survival of C. reinhardii cells after treatment with UV light.

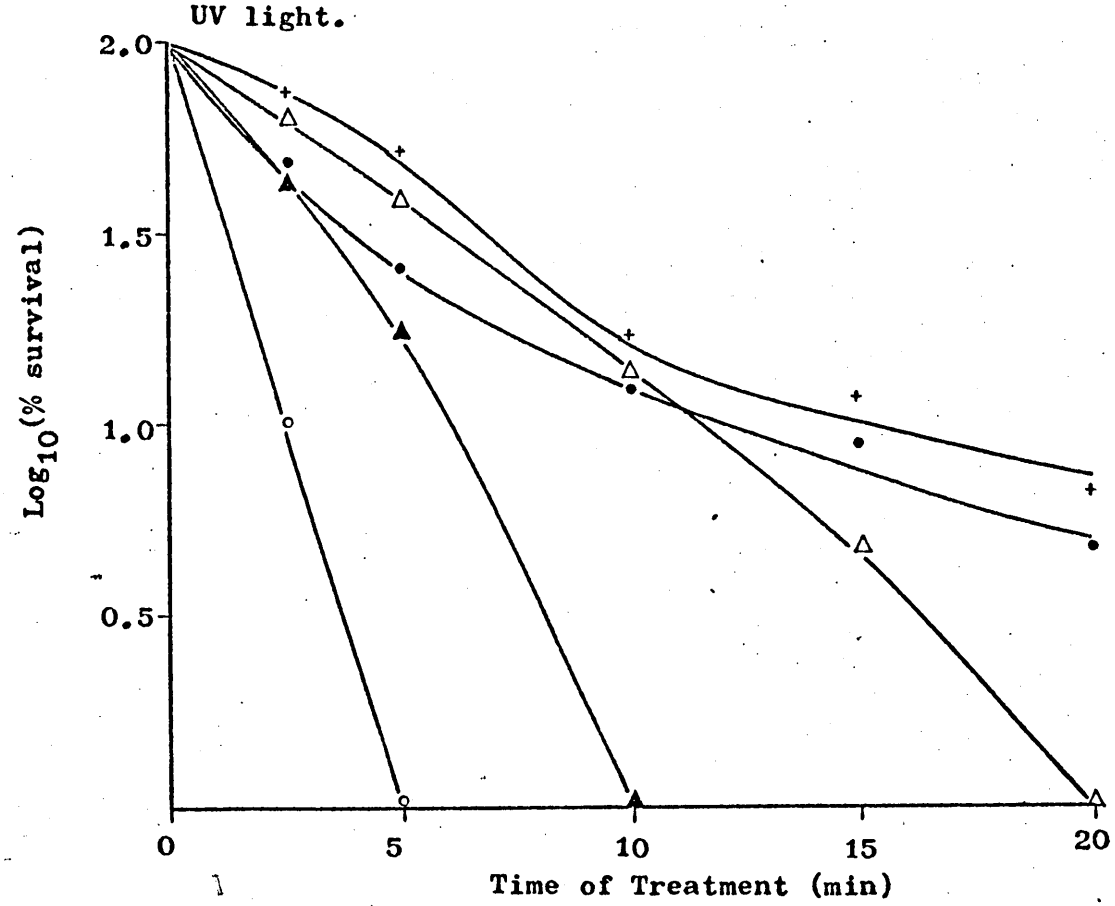


Figure 4.2. Survival of C. reinhardii cells after treatment with MNN.

Table 4.1. Origin of the drug-resistant mutants selected as representative of their class.

Mutant	Origin
TP-8$^+$	Spontaneous selection on 14.8 µM TET under phototrophic growth conditions.
EBr-6$^+$	MNN induction on 50.7 µM EB under phototrophic growth conditions.
POG-10$^+$	MNN induction on 96.4 µM OG under phototrophic growth conditions.
POG-13$^+$	Spontaneous selection on 48.2 µM OG under phototrophic growth conditions.
MD-8$^+$	Spontaneous selection on 75.9 µM DG under phototrophic growth conditions.

Legend to Table 4.2.

Cross-resistance patterns of the drug-resistant mutants of C.reinhardii.

The results represent the mean values for at least three determinations on each drug for each strain.

P = phototrophic growth.
M = mixotrophic growth.
H = heterotrophic growth.
N.D.= not determined.
U.K.= unknown. The inhibitors were not tested in sufficiently high or low concentrations to reach the MIC of either the Wt^+ or mutant strains.

The cross-resistances of all the strains towards diethyltin dichloride, tetraphenylboron, phlorizin, sodium arsenate, KCN and atractyloside were also tested but all their fold resistance values are classified as U.K.

Table 4.2.

Strain / Compound	Fold Cross-resistance to Inhibitors (Wt^+ = 1.0)														
	TP-8$^+$			EBr-6$^+$			POG-10$^+$			POG-13$^+$			MD-8$^+$		
	P	M	H	P	M	H	P	M	H	P	M	H	P	M	H
TMT chloride	10	20	10	2.3	3.8	3.5	0.9	1.5	1.0	1.1	1.3	1.0	0.5	1.0	1.0
TET sulphate	3.5	3.2	2.0	2.0	2.5	3.3	1.0	1.5	0.7	1.0	0.8	0.3	1.5	0.8	1.0
Tetrabutyltin	1.0	1.0	0.5	1.0	13.6	10.0	1.0	1.0	0.5	1.0	5.5	1.0	0.2	1.0	5.5
Dibutyltin diacetate	0.3	0.3	1.0	1.7	1.0	5.5	1.0	0.2	1.0	1.0	1.0	1.0	0.3	0.2	1.0
TBT chloride	0.5	0.3	0.5	2.5	2.5	2.5	1.3	0.5	0.5	0.8	0.3	0.4	0.5	0.1	2.0
TcHT hydroxide	0.5	0.1	0.3	3.3	1.0	1.0	0.5	0.8	1.0	0.5	0.8	1.0	0.5	0.8	1.0
TPhT chloride	0.1	0.1	0.1	N.D.	N.D.	N.D.	0.3	0.3	<0.5	0.3	0.1	1.0	0.3	0.1	2.0
TBT oxide	0.2	N.D.	>0.5	2.0	N.D.	>2.5	1.2	N.D.	U.K.	1.0	N.D.	U.K.	0.2	N.D.	>1.0
Dioctyltin dichloride	<0.1	0.4	1.0	U.K.	>1.3	2.5	<0.8	0.4	1.0	<0.8	1.0	1.0	<0.5	0.4	1.0
Cl-methyl-dibutyltin	<0.7	<0.7	<0.7	<0.1	U.K.	<0.2	<0.7	U.K.	<0.7	<0.7	<0.7	U.K.	<0.3	<0.7	U.K.
Octylguanidine	1.0	1.1	1.1	1.0	0.9	1.3	4.0	9.1	10.3	3.3	4.9	4.5	3.0	3.0	5.7
Dodecylguanidine	0.8	1.8	1.3	0.8	1.0	1.0	4.5	4.5	4.5	1.0	1.0	1.0	1.5	2.0	2.0

Table 4.2. (continued)

Compound \ Strain	Fold Cross-resistance to Inhibitors (Wt^+ = 1.0)														
	TP-8$^+$			EBr-6$^+$			POG-10$^+$			POG-13$^+$			MD-8$^+$		
	P	M	H	P	M	H	P	M	H	P	M	H	P	M	H
Galegine sulphate	U.K.	0.6	0.5	U.K.	1.0	0.7	U.K.	>2.1	>1.0	U.K.	>2.1	>1.0	U.K.	>2.1	1.0
Ethidium bromide	0.6	0.8	1.1	12.5	8.3	7.6	7.9	4.0	2.8	1.1	0.6	0.8	6.3	2.9	1.3
Aurovertin	U.K.	U.K.	1.7	U.K.	U.K.	1.7	U.K.	U.K.	1.7	U.K.	U.K.	1.7	U.K.	U.K.	1.7
TTFB	1.0	0.6	0.3	1.0	1.0	0.6	0.4	1.0	0.4	1.0	1.0	1.0	0.4	1.0	0.6
CCCP	0.8	0.8	1.0	0.8	0.8	1.0	0.8	0.8	1.0	0.8	0.8	1.0	0.8	0.8	1.0
Atebrin	0.1	0.1	0.1	N.D.	N.D.	N.D.	1.0	1.2	1.0	1.0	1.0	<0.5	0.8	<0.4	0.6
Proflavine	0.1	<0.1	0.2	U.K.	U.K.	1.0	U.K.	U.K.	1.0	U.K.	U.K.	0.5	U.K	U.K.	0.3
Acriflavine	0.1	<0.1	<0.1	U.K.	U.K.	1.0	U.K	U.K.	2.5	U.K.	U.K.	2.5	U.K.	U.K.	2.0
Acridine orange	0.2	0.2	1.0	U.K.	U.K.	1.0	U.K.	U.K.	2.0	U.K.	U.K.	2.0	U.K.	U.K.	2.0
SDS	1.0	0.3	0.4	1.0	1.0	1.0	0.4	0.2	0.4	1.0	0.7	1.0	<0.1	0.2	0.4
CTAB	N.D.	N.D.	N.D.	4.3	1.7	U.K.	1.0	<0.3	>3.0	1.0	<0.3	U.K.	>5.7	>2.7	>1.0
Deoxycholate	1.0	1.0	1.0	1.5	1.2	1.5	1.0	1.0	0.8	1.0	1.0	1.0	0.8	1.0	1.2

Table 4.2. (continued)

Compound \ Growth Condition	Fold Resistance of TP-8$^+$ Strain to Inhibitors (Wt$^+$ = 1.0)		
	P	M	H
Oligomycin	< 0.4	< 0.7	1.0
Rhodamine B	1.0	1.0	1.0
Rhodamine 6G	0.5	< 0.3	U.K.
Amytal	U.K.	U.K.	< 0.2
DNP	< 0.6	< 1.0	< 0.4
Dicyclohexyl-18-crown-6	U.K.	U.K.	1.2
Nigericin	0.6	1.0	0.6
Venturicidin	< 0.1	< 0.6	0.8
Rotenone	< 0.8	< 0.4	< 0.2
Antimycin A	U.K.	U.K.	< 0.03
Dio-9	0.6	1.0	1.0
Robenzidine	< 0.2	< 0.8	N.D.
DCCD	0.7	< 0.5	< 0.5
'1799'	1.0	< 1.0	< 1.0
Rifampicin	< 1.0	< 1.0	< 1.0

ratio of the MIC of the mutant strain to the MIC of the Wt^+ strain for each inhibitor. However, due to some variation in the response of different cultures of the same strain to different inhibitors, mutant strains with values of between 0.5 and 2.0 were arbitrarily considered to be insignificantly different from the Wt^+ strain in their response to a particular drug.

The results show that the TP-8^+ mutant was resistant under all growth conditions to TMT and TET only but generally more sensitive than the Wt^+ strain to many of the other inhibitors, including most of the other organotin compounds. The EBr-6^+ strain proved to be mainly resistant to EB under all growth conditions, although with some resistance to the organotin compounds, particularly tetrabutyltin. This strain was also resistant to CTAB, at least under phototrophic conditions. The POG-10^+ mutant was resistant to EB and the three guanidine derivatives as well as being slightly resistant to CTAB, acriflavine and acridine orange. The resistance was again generally present under all growth conditions. This strain was also slightly hypersensitive under certain growth conditions, to some of the other inhibitors, such as some of the organotin compounds, TTFB, SDS and CTAB. The POG-13^+ mutant was slightly more sensitive than the Wt^+ strain to atebrin, CTAB and some of the organotins, except tetrabutyltin to which it was resistant under mixotrophic conditions. This mutant was also resistant to OG under all growth conditions and slightly resistant to galegine sulphate and acriflavine. The MD-8^+ strain exhibited greatest resistance to OG, EB, CTAB and tetrabutyltin but was relatively more sensitive to some of the organotins and perhaps TTFB, atebrin, proflavine and SDS.

The relationship between the toxicity of some of the triorganotins towards the Wt^+ and TP-8^+ strains and their n-octanol : H_2O partition

coefficients is shown in Figs. 4.3 and 4.4. Data for TcHT was not included in this figure due to an insufficiently accurate estimation of the MIC for this compound. The results for the other triorganotins show that their toxicity towards both strains increased with increasing partition coefficient and that the nature of the relationship depended on the growth conditions of the alga. The relationship between the logarithm of the inverse of the MIC and the logarithm of the partition coefficient was approximately linear for heterotrophic growth in both strains but non-linear for the other growth conditions.

Effect of inhibitors on growth rates and extents.

The effect of trialkyltins and EB on the growth of the Wt^+ and two of the drug-resistant strains of C.reinhardii is shown in Figs. 4.5 to 4.8 and Table 4.3. The results show a sigmoidal relationship between the inhibition of growth rates and extents for all the strains and the inhibitor concentration. The growth response of the mutant strains in liquid culture reflected their response on agar plates in both their tolerance levels relative to the Wt^+ strain (fold resistance) (Table 4.3) and in their resistance under all growth conditions. In addition, the I_{50} values for the different inhibitors were very similar for both the growth rates and extents with all the strains tested (Table 4.3). There was a difference in the I_{50} values with EB for the growth rates and extents of the mixotrophically-grown EBr-6^+ mutant (Table 4.3), which also exhibited a more flattened inhibition curve with respect to both rates and extents (Figures 4.7 and 4.8). The mixotrophic growth of this strain was not completely inhibited by EB even at very high concentrations.

(d) Effect of various conditions on the response of Wt^+ and mutants to inhibitors.

The effect of various factors on the inhibitor tolerance levels of the Wt^+ and various mutant strains is shown in Tables 4.4 to 4.6.

Legend to Figures 4.3 and 4.4.

Figure 4.3. Relationship between the toxicity of triorganotin compounds to C. reinhardii Wt^+ strain and their n-octanol:water partition coefficients.

●—● Phototrophic growth.
△—△ Mixotrophic growth.
▲—▲ Heterotrophic growth.

Figure 4.4. Relationship between the toxicity of triorganotin compounds to C. reinhardii $TP\text{-}8^+$ strain and their n-octanol:water partition coefficients.

●—● Phototrophic growth.
△—△ Mixotrophic growth.
▲—▲ Heterotrophic growth.

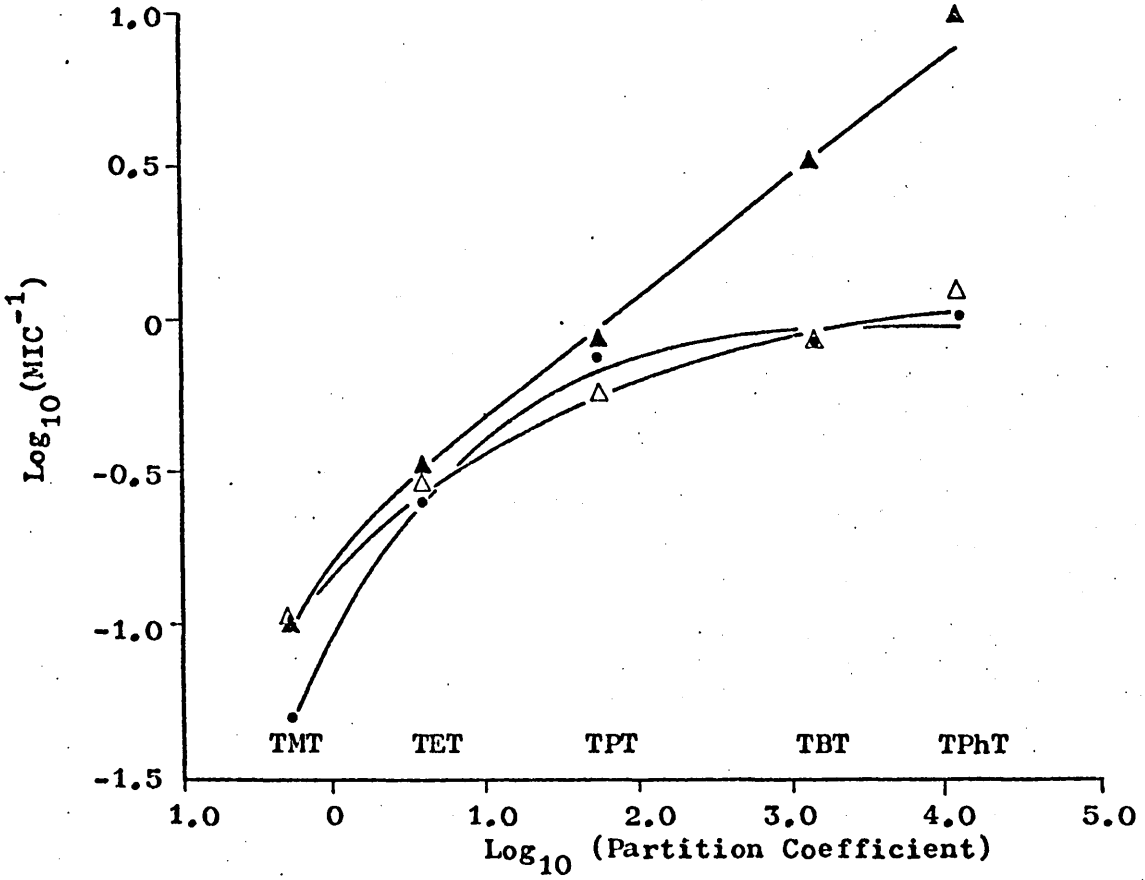


Figure 4.3.

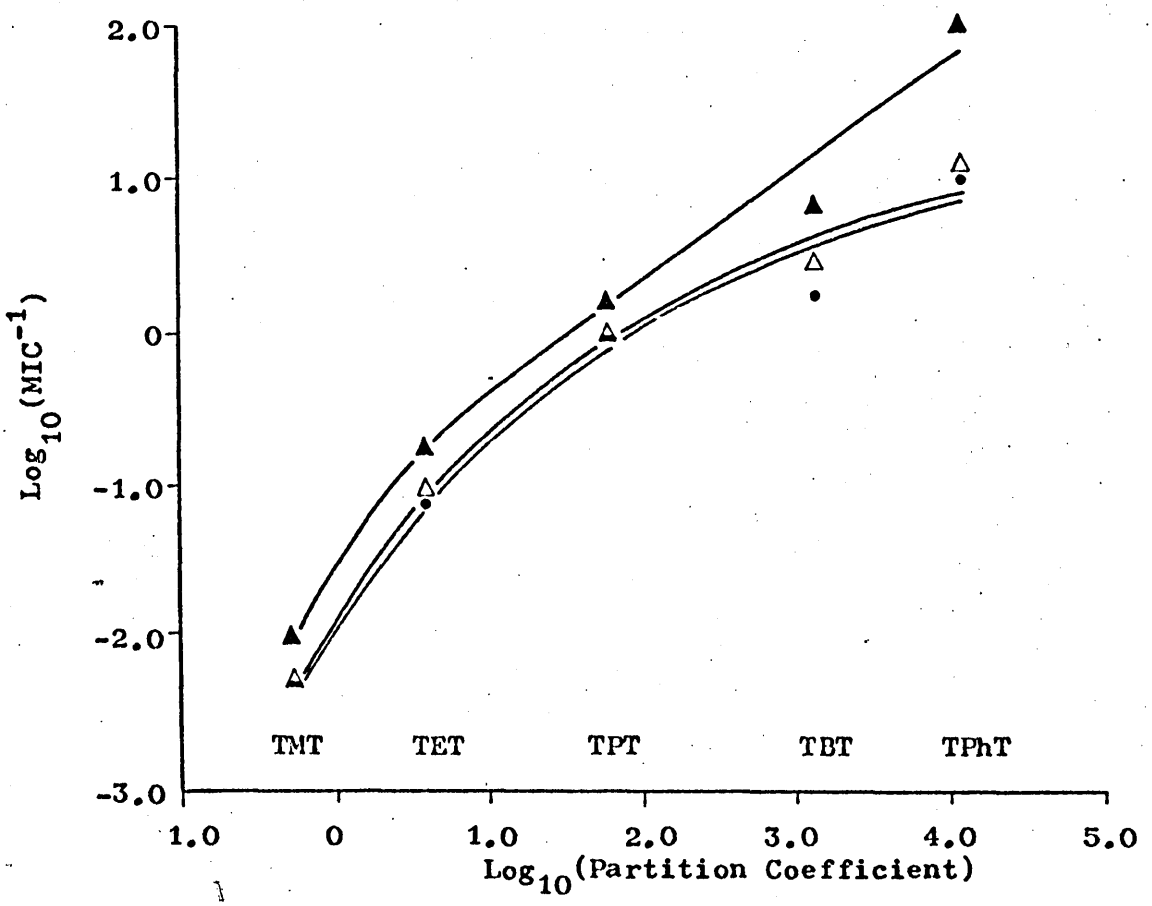


Figure 4.4.

Legend to Figures 4.5 to 4.8.

Figure 4.5. Trimethyltin inhibition of the growth rates of the Wt^+ and $TP\text{-}8^+$ strains of C. reinhardii.

Figure 4.6. Trimethyltin inhibition of the growth extents of the Wt^+ and $TP\text{-}8^+$ strains of C. reinhardii.

Figure 4.7. Ethidium bromide inhibition of the growth rates of the Wt^+ and $EBr\text{-}6^+$ strains of C. reinhardii.

Figure 4.8. Ethidium bromide inhibition of the growth extents of the Wt^+ and $EBr\text{-}6^+$ strains of C. reinhardii.

△—△ Phototrophic growth of the Wt^+ strain.
▲—▲ Mixotrophic growth of the Wt^+ strain.
•—• Heterotrophic growth of the Wt^+ strain.

+—+ Phototrophic growth of the mutant strain.
o—o Mixotrophic growth of the mutant strain.
×—× Heterotrophic growth of the mutant strain.

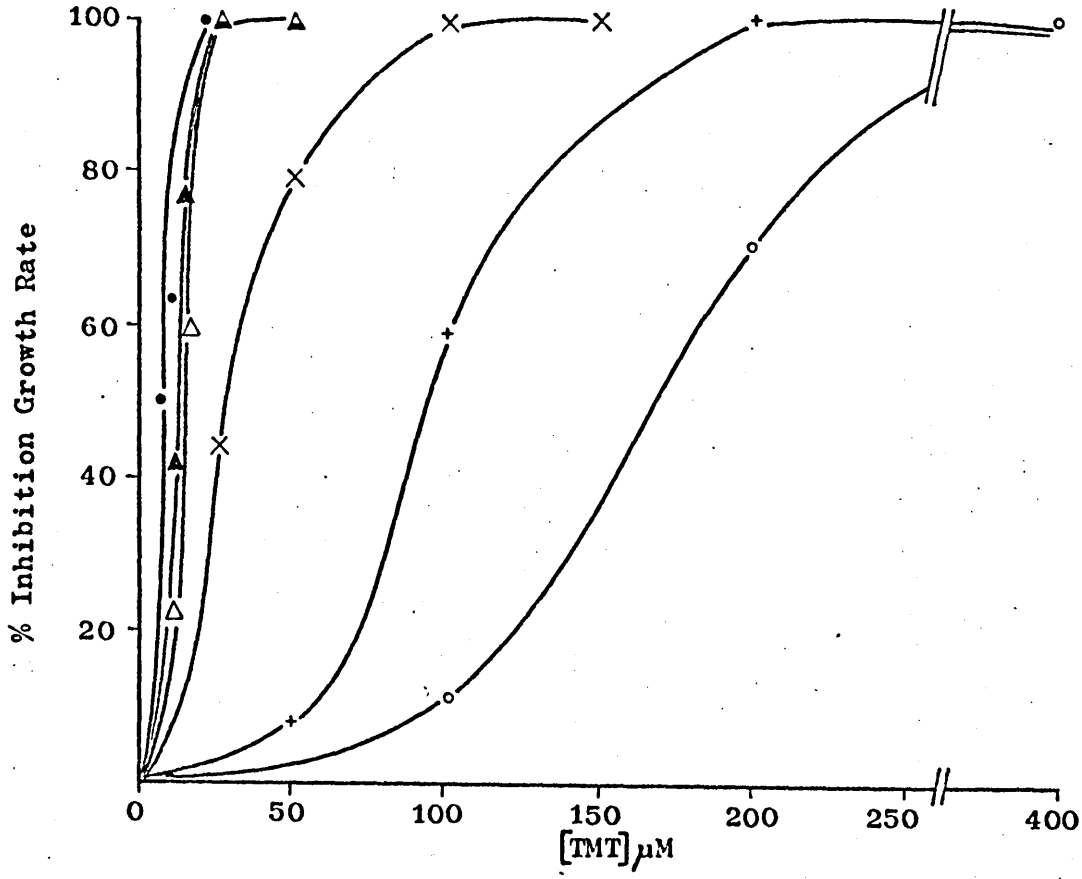
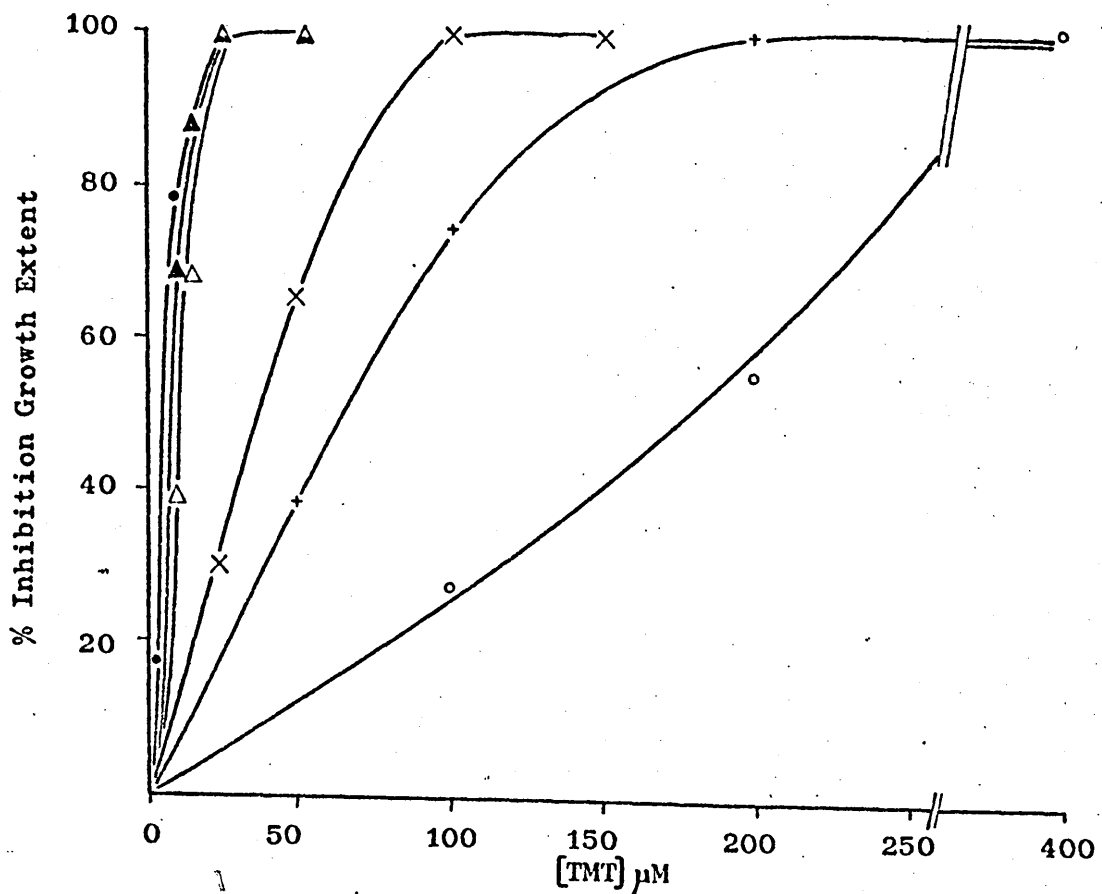


Figure 4.5.



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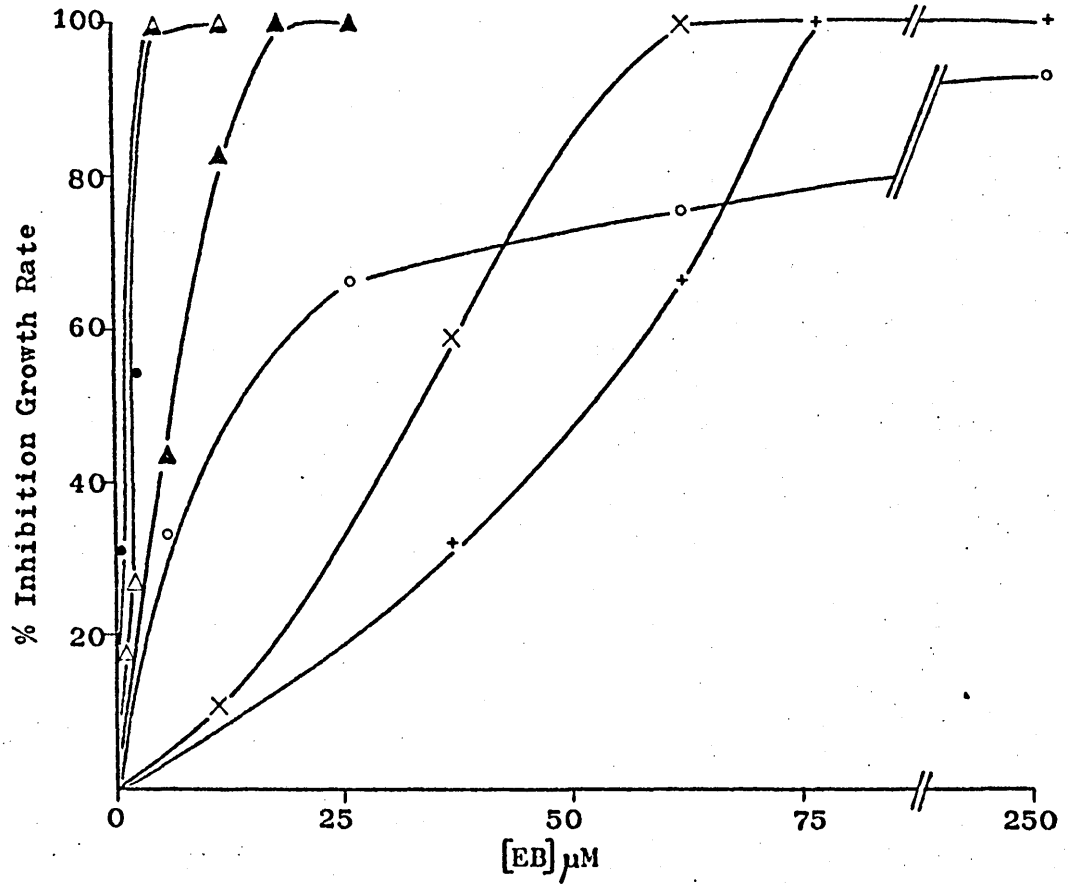


Figure 4.7.

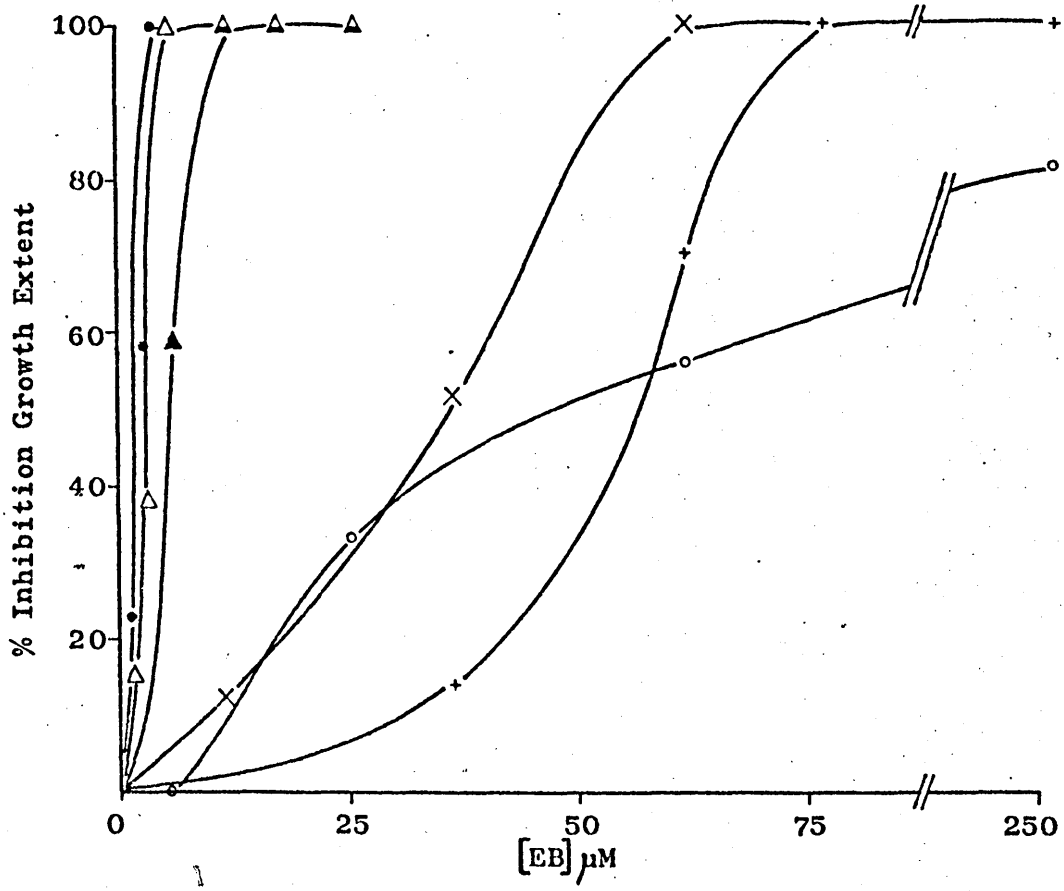


Figure 4.8.

Table 4.3. Summary of the inhibitory effects of trialkyltins and ethidium bromide on the growth of the Wt^+ and drug-resistant strains of C.reinhardii in liquid batch culture.

Strain and Growth Parameter"		I_{50} Values (μ moles/litre) for Inhibitors under Different Growth Conditions*							
		TMT			TET^+		EB		
		P	M	H	M	H	P	M	H
Wt^+	R	15.0	12.5	7.3	1.9	1.2	2.9	6.3	2.1
	E	10.0	7.5	5.0	2.9	1.3	3.8	5.4	2.5
$TP\text{-}8^+$	R	96.3	168.8	30.0	3.6	3.1	-	-	-
	E	63.8	185.0	37.5	6.0	2.1	-	-	-
$EBr\text{-}6^+$	R	-	-	-	-	-	52.5	12.5	32.9
	E	-	-	-	-	-	53.3	50.0	36.3

* P = phototrophic growth, M = mixotrophic growth, H = heterotrophic growth

+ The effect of TET on phototrophic growth was not tested.

" R = exponential growth rate.

E = final growth extent in terms of OD units.

Table 4.4. Response of the Wt^+ and drug-resistant strains of C.reinhardii to inhibitors at different temperatures.

Strain		Minimum Inhibitory Concentration"(μM)					
		Wt^+	$TP\text{-}8^+$	$EBr\text{-}6^+$	$POG\text{-}10^+$	$POG\text{-}13^+$	$MD\text{-}8^+$
TMT	25P*	13 - 26	103	26 - 51	13	13 - 26	5 - 13
	30P	13 - 26	103	26 - 51	5 - 13	13 - 26	5 - 13
	25M	5 - 13	103	26 - 51	13	13	5 - 13
	30M	5 - 13	103	13 - 26	5 - 13	5 - 13	5 - 13
	15H	13	103	13 - 26	5 - 13	5 - 13	5 - 13
	25H	13	103	26	5 - 13	5 - 13	5 - 13
	30H	5 - 13	103	13 - 26	5 - 13	5 - 13	5 - 13
OG	25P	12 - 24	12 - 24	12 - 24	48 -120	48 -120	48 -120
	30P	12 - 24	12 - 24	12 - 24	>120	48 -120	48 -120
	25M	12 - 24	12 - 24	12 - 24	>120	48 -120	48
	30M	12 - 24	12 - 24	12 - 24	>120	48 -120	48 -120
	15H	12	12	5 - 12	>120	48 -120	24 - 48
	25H	12	12	12	>120	48 -120	48 -120
	30H	12	12	12	>120	48 -120	48 -120
EB	25P	19	19	>101	>101	19 - 26	>101
	30P	19 - 26	26 - 64	>101	>101	19 - 26	101
	25M	19	26	>101	>101	13 - 19	64
	30M	26 - 64	26 - 64	>101	>101	26	101
	15H	26	26 - 64	101	26 - 64	13	26
	25H	19	19 - 26	101	26 - 64	13	19
	30H	13	19	101	26	13	19

*Numbers represent growth temperature in degrees Celsius.
P = phototrophic, M = mixotrophic, H = heterotrophic.

"Inhibitor concentrations used were as follows (in μM) :- TMT : 3,5,13, 26, 51, 103 and 128; OG : 5, 12, 24, 48 and 120 ; EB : 7, 13, 19, 26, 64 and 101.

Table 4.5. Response of the Wt^+ and drug-resistant strains of C.reinhardii to inhibitors in the presence and absence of spectinomycin.

Strain		Minimum Inhibitory Concentration" (μM)					
		Wt^+	$TP\text{-}8^+$	$EBr\text{-}6^+$	$POG\text{-}10^+$	$POG\text{-}13^+$	$MD\text{-}8^+$
Spectinomycin•	P*	45	45	45	45	45	45
	M	60	60	60	60	60	60
	H	60	60	60	60	60	60
TMT	M	13 - 51	>128	13 - 51	13 - 51	13 - 51	5 - 13
	H	13	51 -128	51	13	13	13
TMT + Spectinomycin	M	5 - 13	>128	13 - 51	13	5 - 13	<5
	H	5 - 13	51 -128	13 - 51	5 - 13	5 - 13	5 - 13
OG	M	12 - 24	12 - 24	12 - 24	120 -240	48 - 64	48 - 64
	H	12	12 - 24	12 - 24	120 -240	48 - 64	48 - 64
OG + Spectinomycin	M	5 - 12	5 - 12	5 - 12	120 -240	48 - 64	48 - 64
	H	12	12 - 24	12 - 24	120 -240	48 - 64	48 - 64
EB	M	26	7	>190	127 -190	13	64
	H	7 - 13	7	>190	26 - 64	7 - 13	13
EB + Spectinomycin	M	13 - 26	7	>190	127 -190	7	26 - 64
	H	<7	7	>190	26 - 64	7	7

*P = phototrophic, M = mixotrophic, H = heterotrophic.

"Inhibitor concentrations used were as follows (in μM) :- TMT : 5, 13, 51 and 128; OG : 5, 12, 24, 48, 64,120 and 240; EB : 7, 13, 26, 64, 127 and 190.

•The concentration of spectinomycin used together with the other inhibitors was 45 μM.

Table 4.6. Response of the Wt^+ and drug-resistant strains of C.reinhardii to inhibitors in the presence and absence of deoxycholate•.

Strain		Minimum Inhibitory Concentration" (μM)					
		Wt^+	$TP\text{-}8^+$	$EBr\text{-}6^+$	$POG\text{-}10^+$	$POG\text{-}13^+$	$MD\text{-}8^+$
TMT	P*	13 - 51	>128	51	13	13 - 51	<5
	M	5 - 13	>128	51	5 - 13	5 - 13	<5
	H	5 - 13	>128	13 - 51	5 - 13	5 - 13	5 - 13
TMT + Deoxycholate•	P	<5	51 -128	5	<5	<5	<5
	M	<5	>128	13	<5	<5	<5
	H	<5	51 -128	5 - 13	<5	<5	<5
OG	P	24 - 48	24 - 48	24 - 48	>241	48 -120	48 -120
	M	24 - 48	24 - 48	24	>241	241	48
	H	12 - 24	12 - 24	24 - 48	120-241	48 -120	120-241
OG + Deoxycholate•	P	5 - 12	5 - 12	5 - 12	24 - 48	5 - 12	5 - 12
	M	24 - 48	24 - 48	24 - 48	24 - 48	24 - 48	24 - 48
	H	12 - 24	24	24	12 - 24	12 - 24	12 - 24
EB	P	13 - 26	7	>127	>127	13 - 26	>127
	M	13 - 26	7	>127	64 -127	13 - 26	64 -127
	H	13	7	>127	26 - 64	7	13 - 26
EB + Deoxycholate•	P	13 - 26	<7	>127	64 -127	13 - 26	>127
	M	26 - 64	7	>127	64 -127	13 - 26	64 -127
	H	<7	<7	<7	<7	<7	<7

* P = phototrophic, M = mixotrophic, H = heterotrophic.

" Inhibitor concentrations used were as follows (in μM) :- TMT: 5, 13, 51 and 128; OG : 5, 12, 24, 48, 120 and 241; EB : 7, 13, 26, 64 and 127.

• The concentration of deoxycholate used together with the inhibitors was 725 μM.

Different temperatures between 15°C and 30°C had little effect on the response of the various strains to the various inhibitors under different growth conditions (Table 4.4). Spectinomycin, at a concentration which preferentially inhibited phototrophic growth of all the strains, likewise appeared to have little effect on the resistance of the mutant strains relative to the Wt^+ strain (Table 4.5). The effect of a sub-lethal concentration of deoxycholate on the tolerance levels of the various strains was irregular (Table 4.6). Although this surface-active agent substantially increased the inhibitory levels of TMT for all the strains, except $TP\text{-}8^+$, its effect on reducing the MIC of the other inhibitors appeared to depend on both the strain and growth condition. For example, only the phototrophic growth of the Wt^+, $TP\text{-}8^+$ and $EBr\text{-}6^+$ strains was rendered more sensitive to OG in the presence of deoxycholate, whereas this also occurred under other growth conditions with the other strains. A similar anomaly was the considerable reduction in the MIC of EB for most of the strains under heterotrophic conditions only. Although the effect of deoxycholate on the MIC of the inhibitors was not apparently uniform, the results do show a possible modification of the tolerance levels of the various strains to the different inhibitors.

(e) Effect of inhibitors on whole-cell respiration and oxygen evolution.

The effect of inhibitors on the whole-cell respiration and light-induced oxygen evolution of the Wt^+ and two of the drug-resistant strains of C.reinhardii is shown in Table 4.7. The results do not indicate any major differences in the response of the drug-resistant strains relative to the Wt^+ strain. This applies to both dark respiration and light-induced oxygen evolution. In addition, TMT and TET exhibited little differential inhibition of either dark

Table 4.7. Effect of inhibitors on the whole-cell respiration and light-induced oxygen evolution of the Wt^+ and drug-resistant strains of C.reinhardii.

Strain	Inhibitor and Concentration (μM)		Percentage Inhibition*	
			Dark Respiration•	Net Light-induced O_2 Evolution▲
Wt^+	TMT	512	28.5 ± 16.3	24.0 ± 11.5
$TP\text{-}8^+$	TMT	512	23.0 ± 0.7	19.0 ± 7.0
Wt^+	TMT	1,280	44.1 ± 12.4	36.8 ± 14.4
$TP\text{-}8^+$	TMT	1,280	28.0 ± 4.5	21.5 ± 10.3
Wt^+	TMT	2,560	54.5 ± 0.7	38.0 ± 19.1
$TP\text{-}8^+$	TMT	2,560	41.0 ± 0.7	45.5 ± 6.3
Wt^+	TET	8	29.3 ± 5.1	17.5 ± 8.4
$TP\text{-}8^+$	TET	8	10.5 ± 8.5	15.0 ± 9.9
Wt^+	TET	197	31.0 ± 14.0	19.8 ± 4.5
$TP\text{-}8^+$	TET	197	42.0 ± 2.0	30.0 ± 18.2
Wt^+	OG	241	46.4 ± 23.5	76.0 ± 15.5
$POG\text{-}10^+$	OG	241	42.8 ± 28.5	94.2 ± 4.0

* Second series of figures in each column represent the standard deviation of the mean percentage value.

• Oxygen uptake in the dark. Control rates in μ moles O_2 uptake/mg chl./ were 40.1 ± 15.5, 38.3 ± 12.8 and 47.5 ± 14.1 for the Wt^+, $TP\text{-}8^+$ and $POG\text{-}10^+$ strains respectively.

▲ Calculated from formula : $\% \text{ inhibition} = \frac{L_{r''} - R_{r''}}{L_{r'} - R_{r'}} \times 100$

where $L_{r''}$ = net O_2 evolution in light in presence of inhibitor.

$L_{r'}$ = net O_2 evolution in light in absence of inhibitor.

$R_{r''}$ = dark respiration rate when illumination terminated in presence of inhibitor.

$R_{r'}$ = dark respiration rate when illumination terminated in absence of inhibitor.

Control rates in μ moles O_2 evolution/mg chl./h were 105.3 ± 48.1, 95.6 ± 32.4 and 110.4 ± 13.9 for the Wt^+, $TP\text{-}8^+$ and $POG\text{-}10^+$ strains respectively.

respiration or light-induced oxygen evolution but CG at 241 μm appeared to inhibit light-induced oxygen evolution to a greater extent than dark respiration. However, in most experiments, respiration and light-induced oxygen evolution were not stimulated by uncouplers, such as CCCP, '1799' and DNP, which may indicate that ATP synthesis was not tightly coupled to electron transport in whole cells of this alga. Preliminary trials showed that populations of different ages and grown under different nutritional conditions were equally unresponsive to uncouplers in the oxygen electrode. Consequently little can be concluded from these experiments concerning the effect of the inhibitors on energy transfer in the different strains.

(f) Uptake of inhibitors.

Ethidium bromide.

The time-course uptake of EB by the Wt^+ and $EBr\text{-}6^+$ strains of C.reinhardii is shown in Fig. 4.9 and Table 4.8. EB uptake by both strains was initially rapid within the first 30 min, followed by a slow gradual uptake up to at least 8 h. The results show that the Wt^+ strain absorbed nearly 2.5 times the quantity of EB from the medium relative to the $EBr\text{-}6^+$ strain during the first hour of incubation (Fig.4.9). However, the rate of uptake of EB from the medium was approximately equal in both strains between 1 and 8 h of incubation. Uptake of EB by both strains increased when the EB concentration in the medium was raised from 125 μM to 1.25 mM, although the $EBr\text{-}6^+$ strain still absorbed less EB than the Wt^+ strain (Table 4.8). Deoxycholate, at concentrations that caused low percentage lysis of liquid suspension of the $CW15^+$ strain (0.72 and 0.97 μM) (Fig.4.10), increased the uptake of EB by both strains (Table 4.8).

$[^{113}Sn]$TET Chloride.

The uptake of $[^{113}Sn]$TET by the Wt^+ and $TP\text{-}8^+$ strains of C.reinhardii

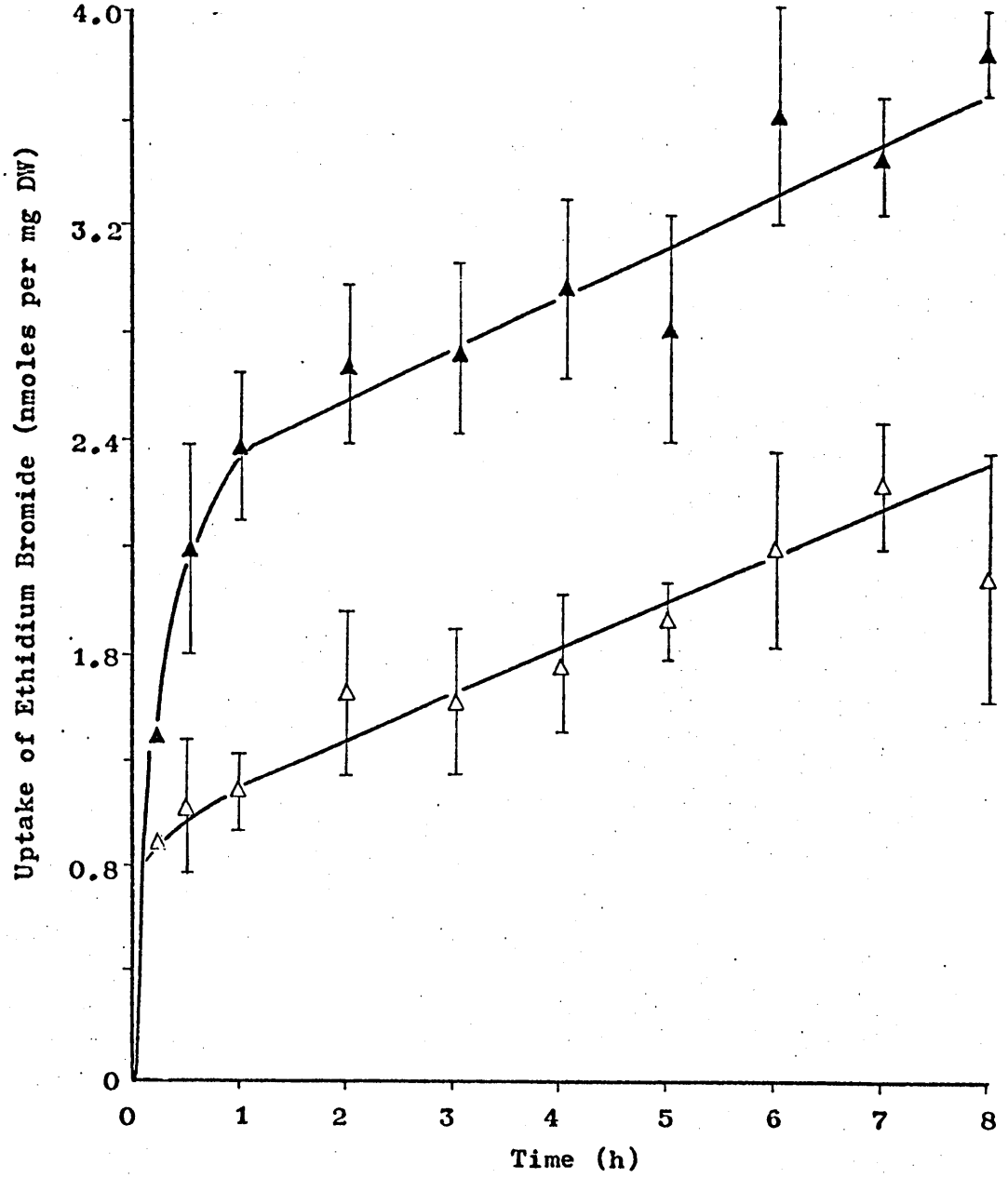


Figure 4.9. Uptake of ethidium bromide by the Wt^+ and $EBr\text{-}6^+$ strains of C. reinhardii.

The vertical lines represent the limits of the standard deviation of the mean values for each time.

▲—▲ Wt^+.

△—△ $EBr\text{-}6^+$.

Table 4.8. Uptake of ethidium bromide by the Wt^+ and $EBr\text{-}6^+$ strains of C. reinhardii.

Time (h)	Uptake of EB* (nmoles/mg DW)							
	Wt^+ 1.25mM EB	$EBr\text{-}6^+$ 1.25mM EB	Wt^+ 125µM EB	Wt^+ 125µM EB + 0.72µM D"	Wt^+ 125µM EB + 0.97µM D"	$EBr\text{-}6^+$ 125µM EB	$EBr\text{-}6^+$ 125µM EB + 0.72µM D"	$EBr\text{-}6^+$ 125µM EB + 0.97µM D"
0	0	0	0	0	0	0	0	0
0.5	—	—	1.78	2.30	3.06	1.02	1.58	1.98
1	55.5	24.8	1.94	2.92	3.24	1.04	1.98	2.26
2	59.1	40.9	2.38	3.04	3.30	1.30	2.06	2.36
4	56.9	42.8	2.96	3.14	3.30	1.56	2.12	2.40
6	55.2	43.2	3.32	3.62	3.66	2.42	3.26	3.18
8	56.8	45.1	—	—	—	—	—	—

* The results are the means of two replicates for a single experiment.

" D = deoxycholate.

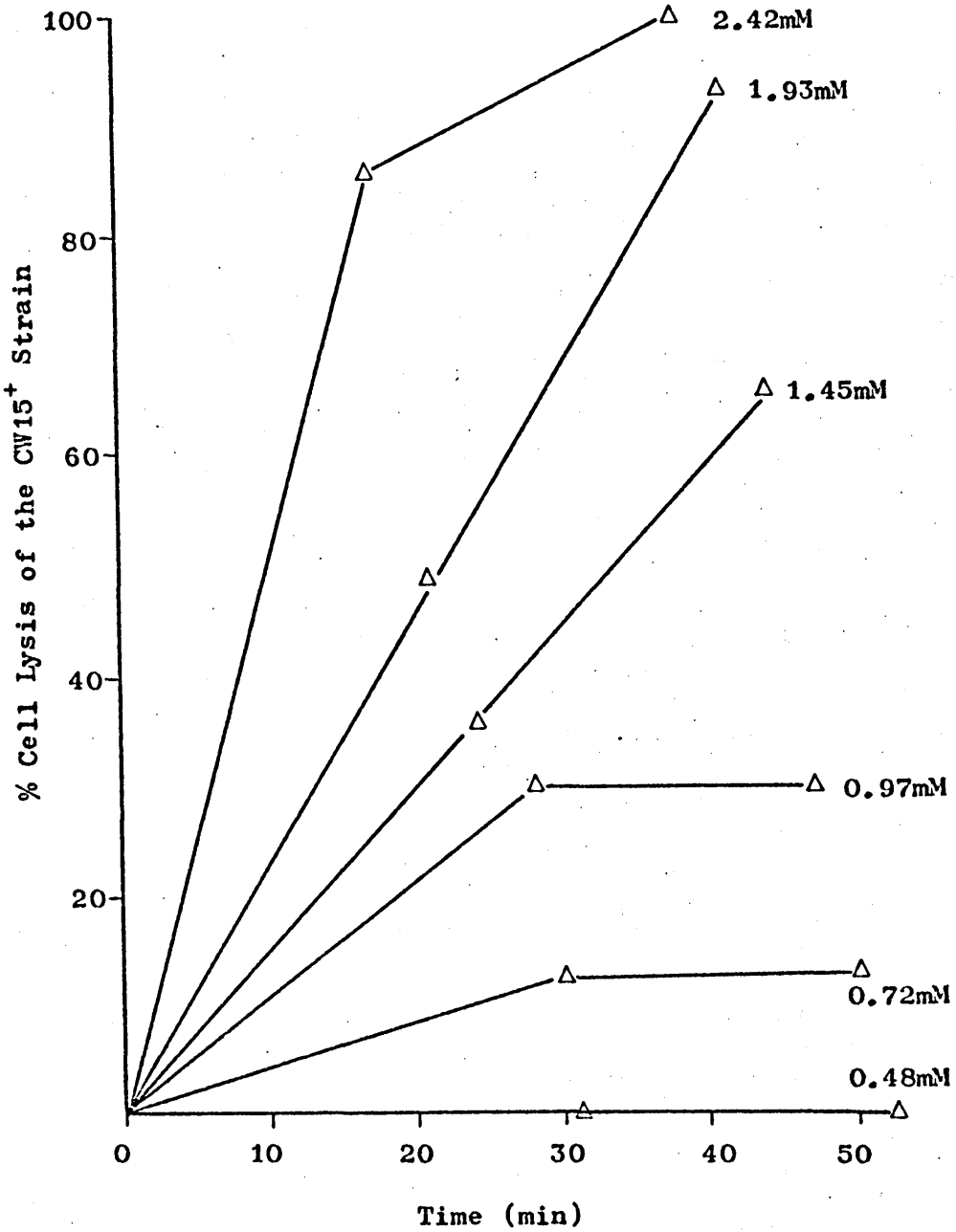


Figure 4.10. Effect of sodium deoxycholate on cell lysis of the $CW15^+$ (cell wall-less) strain of C. reinhardii in liquid suspension.

The figures along each trace represent the various concentrations of deoxycholate. There was no visible cell lysis of the Wt^+ strain of C. reinhardii at any of these concentrations.

is shown in Fig. 4.11. Both strains absorbed [^{113}Sn]TET in similar amounts during the first 25 min. After this time, the quantity of [^{113}Sn]TET in the cells of both strains gradually decreased.

(g) Genetic analysis

The results of the genetic analysis of some inhibitor-resistant mutants of C.reinhardii are shown in Tables 4.9 and 4.10. Table 4.9 shows the tetrad analysis of the progeny from crosses involving the (+) type mutant strains and the Wt^- strain. All the trialkyltin-resistant mutants in this table exhibited similar cross-resistance patterns to the TP-8^+ strain. Although many of the tetrads were incompletely viable, the results from the surviving progeny generally showed a 1 : 1 ratio in the resistance/sensitivity of the progeny for all the mutant strains. The mt phenotype was also inherited in a 1 : 1 fashion when examined in some of these tetrads.

These tetrad analysis results are further supported by parallel analysis of zoospore colony sub-clonings (Table 4.10), which shows that of the individual cells of colonies formed from the progeny of single zygotes, approximately half were drug resistant and half drug sensitive.

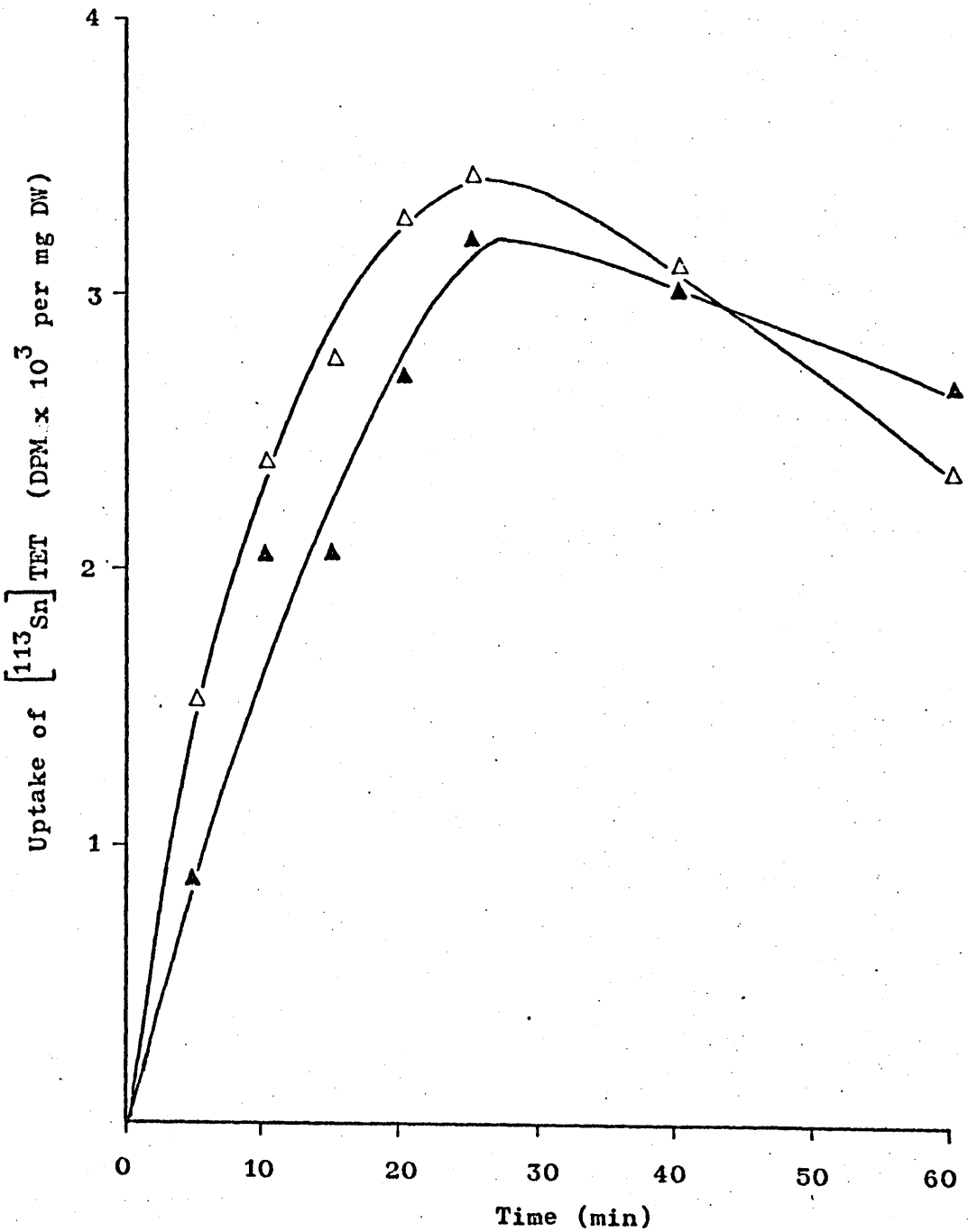


Figure 4.11. Time-course uptake of $[^{113}Sn]$TET by whole cells of the Wt^+ and TP-8^+ strains of C. reinhardii.

▲—▲ Wt^+.

△—△ TP-8^+.

Legend to Table 4.9.

Tetrad analysis of the drug-resistant mutants of C. reinhardii.

• Numbers in parenthesis refer to the number of tetrads with the particular resistant/sensitive progeny ratio indicated.

* S/D refers to the number of progeny that survived out of the total number of progeny that were dissected from a single zygospore.

Table 4.9.

Cross \ *S/D	Number of Drug-resistant and Drug-sensitive Progeny*									
	2/4	3/4	4/4	2/8	3/8	4/8	5/8	6/8	7/8	8/8
Trialkyltin-resistant										
TP-8^{+}x Wt^{-}			2:2(1)							
TP-9^{+}x Wt^{-}					2:1(1)	2:2(1)		4:2(1)		
TP-12^{+}x Wt^{-}			2:2(1)	1:1(1)						
TP-17^{+}x Wt^{-}				1:1(1)						4:4(1)
TP-30^{+}x Wt^{-}			2:2(1)							
TM-2^{+}x Wt^{-}	1:1(1)	2:1(1)		1:1(1)						
EB-resistant										
EBr-6^{+}x Wt^{-}	1:1(2)						4:1(1)	4:2(1)	4:3(1)	4:4(2)
								2:4(1)	3:4(1)	
								3:3(1)		
OG-resistant										
POG-10^{+}x Wt^{-}	1:1(2)	2:1(1)			1:2(1)	4:0(4)		2:4(1)	4:3(1)	4:4(2)
		1:2(1)				2:2(8)		5:1(1)		
POG-13^{+}x Wt^{-}	1:1(2)		2:2(1)			1:3(1)		4:2(1)		4:4(2)
						2:2(7)		3:3(1)		
						4:0(4)				
						0:4(3)				
MD-8^{+}x Wt^{-}	0:2(1)					2:2(1)		2:4(1)		
						1:3(1)				
Wt^{+}x Wt^{-}			0:4(1)				0:5(1)			0:8(1)

Table 4.10. Zoospoore colony sub-clonings of selected inhibitor-resistant mutants of C.reinhardii.

Cross	No Separate Zygote Colony Sub-clonings	Total No Colonies Examined	Mean % Resistance
Trialkyltin-resistant			
TP-8$^+$ x Wt$^-$	8	149	56
TP-12$^+$ x Wt$^-$	3	46	53
TP-17$^+$ x Wt$^-$	3	48	34
TP-30$^+$ x Wt$^-$	26	416	47
TP-36$^+$ x Wt$^-$	10	155	46
TM-1$^+$ x Wt$^-$	2	32	25
TM-2$^+$ x Wt$^-$	8	166	41
EB-resistant			
EBr-6$^+$ x Wt$^-$	24	190	61
OG-resistant			
POG-10$^+$ x Wt$^-$	11	87	39
POG-13$^+$ x Wt$^-$	15	120	52
Wt$^+$ x Wt$^-$	2	32	0

Discussion

Sigmoidal survival curves with a distinct lag phase were obtained after treatment of the cells of C.reinhardii Wt^+ with UV light, in contrast to the more linear curves obtained with MNN. This lag phase may be indicative of the presence of repair systems that specifically correct some of the UV-induced lesions but not those produced by MNN treatment (Hyams and Davies, 1972). The percentage survival of the cells after treatment with the two mutagens depended on the growth conditions of the alga. Both mutagens were most lethal when the treated cells were subsequently grown in the dark and least effective when the cells were subsequently grown on YAP medium in the light. This difference in lethality is unlikely to be due to a photoreactivation of the repair processes in the light-grown cells since the cells were held for 12 hours in the dark prior to growth in the light. A small contribution to the generally greater survival rates in the light may have been made by the compensatory power of photosynthesis. Cells with mutations involving reduced respiratory function may be able to depend on photosynthesis for growth, whereas this function would not be present under dark conditions. That cells of C.reinhardii, with mutations of mitochondrial function, are able to undergo a limited number of cell divisions to form small colonies under phototrophic and mixotrophic conditions has been suggested by the work of Alexander et al. (1974). In addition, the generally greater survival rates of cells plated on YAP medium in the light may have been due, in part, to the survival of any auxotrophic mutants that may have been induced. However, in the absence of data on the frequencies of respiratory and auxotrophic mutations in this experiment, it is impossible to assess the full contribution of these mutations to the survival rates on different media. The slower growth rate of

cells in the dark may have played a part in the lethal effects of the mutagens. Mutation frequencies of C.reinhardii, induced by certain mutagens, are known to be influenced by post-mutagenic multiplication of the cells (Loppes, 1969[b]), but the effect of the rate of cell multiplication on either mutation frequencies or survival rates has not so far been investigated in this alga.

Inhibitor resistant mutants of C.reinhardii were isolated either through spontaneous selection or by induction with UV light or MNN. The 172 resistant colonies examined were further sub-divided into five major classes on the basis of their different cross-resistance patterns.

When the mt^+ type of each inhibitor-resistant mutant was crossed to the Wt^- strain, a 1 : 1 segregation of the resistant and sensitive progeny was apparent. This Mendelian inheritance indicates that the mutations are located on nuclear chromosomes (Sager, 1954). Consequently it would appear that the five major classes of inhibitor-resistant mutations isolated in this study were probably of nuclear origin. This predominance of nuclear mutations is unlikely to be a consequence of the isolation methods since cytoplasmic gene mutations have also been previously isolated either spontaneously or by induction with UV light or MNN (Sager, 1954; Gillham, 1965).

The drug-resistance phenotype in all these mutants could arise through several possible modifications in the cell (Lancashire and Griffiths, 1975[a]):-

(a) Exclusion of the inhibitors from the cell due to modification of the cell-wall or plasma membrane permeability.

(b) Detoxifying mechanisms (constitutive or induced) in the cytoplasm of the cell or the matrix of the cell organelles.

(c) Exclusion of the inhibitor from the mitochondria and/or

the chloroplast due to changes in their membrane permeability towards the inhibitors.

(d) Modification at the site of action of the inhibitors.

The TP-8$^+$ strain was only resistant to TMT and TET and had similar or greater sensitivity than the Wt$^+$ strain for a wide spectrum of other chemically and functionally unrelated inhibitors. The toxicity of some of the triorganotin compounds towards whole cells of both the Wt$^+$ and TP-8$^+$ strains increased in a way that was related to the n-octanol : H_2O partition coefficient of these inhibitors. A similar relationship existed for the alkylguanidine-resistant mutants POG-10$^+$, POG-13$^+$ and MD-8$^+$, which were more resistant to OG than to DG. This relationship is analogous to that found for the effect of these compounds on energy transfer reactions in chloroplasts (Chapter 3) and thus may reflect differences in the intrinsic activity of these compounds on energy conservation in whole cells. However, other factors that are also influenced by the partition coefficient of the compounds, such as permeability of the plasma and organelle membranes, are equal possibilities for the observed differences in activity of the compounds. Triorganotin-resistant mutants of S.cerevisiae have also been shown to possess high levels of resistance to TMT and TET but low levels of resistance to TPT, TBT, TcHT and TPhT (Lancashire and Griffiths, 1971). Except for TET, to which resistance was highest, the fold resistance of these mutants decreased with increasing partition coefficient of the compound. This basic similarity in the resistance patterns of the triorganotin-resistant mutants of S.cerevisiae and C.reinhardii is interesting in view of the reported low level resistance of growth, respiration and the ATP-ase activity of sub-mitochondrial particles of the triorganotin-resistant mutants of S.cerevisiae (Lancashire and Griffiths, 1971, 1975[a]). However, the

apparent lack of resistance of the dark respiration and light-induced oxygen evolution in the TP-8^+ mutant of C.reinhardii, coupled to the hypersensitivity of the mutant to triorganotins, other than TMT and TET, perhaps indicates a somewhat different mode of resistance in this mutant.

Inhibitor resistance in the mutants, both on solid and in liquid media, was generally present under all growth conditions, which indicates that mechanisms common to, or outside, both the mitochondria and chloroplasts are responsible for the resistance phenotype. However, there were growth condition-dependent differences in the fold resistances of some of the mutant strains; for example, the TP-8^+ mutant was from 2 to 4 times more resistant to TMT under mixotrophic conditions, both on solid and in liquid culture, than under phototrophic or heterotrophic conditions. Consequently the cellular environment may influence the resistance phenotype of this mutant, perhaps via an effect on the physiological state of the cell or organelles. The reason for this is not known and is open to several interpretations. One possibility is that if TMT impairs both oxidative and photosynthetic functions, then the overall ATP economy of the cell may be less affected under growth conditions that allow both mitochondria and chloroplasts to function most efficiently (mixotrophic growth). Under conditions where growth is limited by a reduction in the functioning of any one of the energy conserving organelles (phototrophic and heterotrophic conditions), there may be less compensatory power in the cells to support growth. Another possibility is an effect of growth conditions on the permeability of the plasma membrane via an effect on the peripherally-situated outer membranes of the mitochondria and chloroplast.

It is possible that the differential resistance under different growth conditions is in some way a function of the difference in medium

pH which is known to develop during growth (Chapter 2). However, there were similar inhibitions of growth rates and extents by TMT and these would be measured at different medium pH values (at mid-log phase and stationary phase respectively). In addition, the fact that phototrophic and heterotrophic culture conditions develop wide variations in medium pH but still allow comparable inhibition by TMT would discount a simple relationship. However, an effect of medium pH on the drug resistance of the strains cannot be discounted in view of the known effects of pH on microbial metabolism and permeability (Pirt, 1975). The reported effects of pH on the biocidal activity of the triorganotins is contradictory. Antifungal activity is pH independent (Kaars Sijpesteyn et al., 1962), whilst pH influences the inhibition of growth and whole-cell respiration in S.cerevisiae by TET (Lancashire and Griffiths, 1975[a]).

In order to obtain more information on the resistance phenotypes of the various drug-resistant mutants, the effect of various factors on the tolerance levels of these strains was examined. The results show that the resistant phenotypes were not temperature-sensitive. In addition, spectinomycin, which may produce a concentration-dependent specific inhibition of chloroplast ribosome function (Surzycki et al., 1970), did not greatly affect the resistance phenotypes at concentrations which inhibited phototrophic, but not mixotrophic or heterotrophic, growth. Consequently the resistance of the mutant strains does not apparently depend upon chloroplast ribosome function.

Deoxycholate generally increased the sensitivity of the mutant strains to the inhibitors which may be due to enhanced uptake of the drugs by way of an increased permeability of the plasma membrane. The same concentration of deoxycholate increased the uptake of EB by both the Wt^+ and $EBr\text{-}6^+$ strains. The mode of action of deoxycholate,

in disrupting various components of the plasma membrane (Ehrhart and Chauveau, 1975), is consistent with an increased permeability of the membrane and a potentiation of the toxicity of inhibitors by this (Flavin and Slaughter, 1974) and other (St.John et al., 1974) surfactants. However, the potentiation of the toxicity of drugs to both the Wt^+ and mutant strains is not conclusive unless deoxycholate lowers the resistance of the mutant strain to the same level as the Wt^+ strain. This was not always the case in experiments on solid media and the uptake of EB by $EBr\text{-}6^+$, in the presence of deoxycholate, was consistently somewhat lower than for the Wt^+ strain. However, the fact that EB uptake by the $EBr\text{-}6^+$ strain was generally lower than for Wt^+, in the absence of deoxycholate, lends support to decreased permeability being responsible for resistance in this mutant strain. Deoxycholate did not decrease the resistance of $TP\text{-}8^+$ to TMT, which may indicate that this mutant may not have altered plasma membrane permeability. This is supported by the similar uptake of $[^{113}Sn]$TET by the $TP\text{-}8^+$ and Wt^+ strains, although the low resistance of $TP\text{-}8^+$ to TET would be expected to produce at most a small difference in $[^{113}Sn]$TET uptake.

The biphasic nature of the uptake kinetics of EB (Fig. 4.9) has several possible interpretations. The initial rapid uptake may be due to binding of the dye to the plasma membrane or cell-wall and the slower progressive uptake may be due to permeation of the plasma membrane and entry of EB into the cytaplasm. However, preliminary experiments showed that the enhancement of EB fluorescence, normally found when EB binds to membranes (Gitler et al, 1969), did not occur when the cells of either Wt^+ or $CW15^+$ were added to solutions of EB. An alternative explanation is that EB may initially rapidly diffuse through the plasma membrane and then more slowly permeate the peripherally-

situated membranes of the mitochondria and chloroplast. Whatever was the cause of the biphasic uptake kinetics, it would appear that the reduced removal of EB from the medium by the EBr-6^+ strain was manifested during the initial rapid phase since the rate of removal during the later slower phase was the same as with the Wt^+ strain. The fact that uptake of EB by C.reinhardii increased at higher exogenous EB concentrations (Table 4.8), possibly suggests that a diffusion-like process is involved. However, the uptake of low concentrations of EB by whole cells of different species of yeast is also concentration dependent and is increased by the presence of fermentable substrate (Pẽna and Ramirez, 1975; Celis et al., 1975). In addition EB appears to be taken up via the energy-requiring cation transport system of the cell (Pẽna and Ramirez, 1975). The uptake of EB in these experiments on yeast was measured up to 20 min, which may correspond to the initial rapid phase of uptake or adsorption by the cells of C.reinhardii. Although EBr-6^+ took up less EB than the Wt^+ strain, the 2.5 fold difference in uptake does not fully account for the 8 - 12 fold resistances found in liquid and on solid media. However, the high cell concentrations used in the uptake assays do not correspond to those during growth of the alga and may influence the uptake characteristics.

The cross-resistance patterns for the EB-and alkylguanidine-resistant mutants are interesting in view of similar mutants of other microorganisms. EB-resistant mutants of S.cerevisiae, which were cross-resistant to CTAB and/or acriflavine, have been previously isolated (Bech-Hansen and Rank, 1972). Glucose repression decreased the resistance, suggesting that resistance in these mutants was due to decreased permeability of the mitochondrial membrane. Similarly, one cytoplasmic and two different classes of nuclear mutants resistant

to EB and cross-resistant to OG and/or synthalin have been isolated from the yeast K.lactis (Brunner et al., 1973), some of which were probably permeability mutants (Celis et al., 1975).

The EB-and alkylguanidine-resistant mutants exhibited a broad pleiotropic response to four main classes of inhibitors, namely organotins, alkylguanidines, surfactants such as SDS and CTAB, and membrane-active dyes such as EB and acriflavine. This pleiotropic response towards the various inhibitors may be explained by a common mode of action of these compounds on cell membranes. The reported membrane activity of the various inhibitors may be related as follows:-

(1) All these classes of inhibitors contain both polar and non-polar regions in their molecules.

(2) Triorganotins and alkylguanidines have a detergent-like effect on membranes, particularly at higher concentrations (Mitchell, 1966[b]; Stockdale et al., 1970).

(3) It has been suggested that certain guanidine derivatives may act as analogous of polyamines, such as spermine (Brunner et al., 1973). The membrane ATP-ase activity of Bacillus subtilis is affected by both CTAB and spermine (Rosenthal and Buchanan, 1974) and OG inhibits the histone-induced swelling and stimulation of respiration in mitochondria (Schwartz et al., 1966; Johnson et al., 1966).

(4) Both EB and alkylguanidines competitively inhibit the uptake of monovalent cations, such as K^+, by yeast cells in a substrate-dependent manner (Pẽna, 1973; Pẽna and Ramirez, 1975) and triorganotins affect anion transport across mitochondrial (Selwyn et al., 1970) and chloroplast (Watling and Selwyn, 1970) membranes.

(5) EB and acriflavine preferentially inhibit mitochondrial or chloroplast DNA replication and transcription and cause degradation of the DNA, depending on the concentration and growth conditions

(Stegeman and Hoober, 1973; Flechtner and Sager, 1973; Ho et al., 1974; Alexander et al., 1974). The site of action of EB in degrading mt-DNA is considered to be at the level of the membrane attachment site of the mt-DNA and the specific mt-DNA degrading properties result in an activation of the mitochondrial ATP-ase (Bastos and Mahler, 1974). Both EB and acridine orange affect respiration in isolated mitochondria (Grimwood and Wagner, 1974; Miko and Chance, 1975). Analogous to this relationship is the finding that nalidixic acid inhibits the in vitro c-DNA synthesis of C.reinhardii (Ho et al., 1974) and that a nalidixic acid-resistant mutant of this alga is hypersensitive to the surface-active agent phenethyl alcohol (Robreau et al., 1973).

(6) All five classes of inhibitors are known to affect energy conservation in chloroplasts and mitochondria.

Consequently, the pleiotropic resistance patterns of all the inhibitor-resistant mutants indicates change(s) in one or more of the cell membranes since the inhibitors to which they are resistant are known to be membrane-active in one way or another. However, the exact locus of the lesion(s) is unknown, although it may be at the plasma membrane in the EBr-6^+ strain. The mutations may involve a change in a single membrane-bound enzyme, such as an ATP-ase, or a general membrane component. A resistant mitochondrial or chloroplast ATP-ase may account for the change in response to those inhibitors that affect both the DNA functions of these organelles and the energy conservation and ion transport functions of their membranes. Alternatively, resistance due to the lesion of a basic structural unit of a membrane, such as a phospholipid or structural protein, may be the result of a non-specific change of the whole membrane and hence a change in the

interaction of the membrane with diverse types of inhibitors.

It is interesting that most of the compounds to which the EB and alkylguanidine-resistant mutants showed a broad pleiotropic response are cationic. Consequently, their membrane activity may, at least initially, involve a binding to negatively-charged sites with a resultant effect on the membrane potential and the binding and transport of substrate and inorganic cations. This may perhaps account for the reported effects of these compounds on respiration and cation transport in whole cells and subcellular organelles. Genetic modification of the negatively-charged binding sites may then confer resistance to whole cells or organelles to the inhibitory action of these compounds, but would also be expected to change their respiratory and substrate-and ion-transporting functions. The findings that guanidine derivatives, CTAB and EB bind to negative electrostatic sites, which are probably p-lipids, and inhibit cation transport in various types of membranes (Gitler et al., 1969; Pẽna, 1973; Davidoff, 1974; Schäfer, 1974; Pẽna and Ramirez, 1975), may be relevant in this respect.

The difference in the nature of the pleiotropic resistance patterns of the various classes of EB- and alkylguanidine-resistant mutants may be indicative of subtle differences in the nature of the binding sites of the various inhibitors. The single nuclear gene mutations present in the various classes may, in some cases, be allelic and hence a change in the gene may result in a change in the binding site(s) of several functionally related inhibitors. Alternatively, if some of the genes are not allelic with the others, they may result in binding changes that confer resistance to a more limited group of inhibitors.

CHAPTER 5

Sub-cellular Characterisation of the Wild-type and TP-8^{+} Strains of C.reinhardii.

Introduction.

The selection of drug-resistant strains is a commonly used technique in microbial genetics to obtain mutants which are defective in particular processes. The underlying rationale is that if an inhibitor is specific for a particular process, resistant strains may be insensitive due to a change in that process. Other mechanisms are of course possible, the most common of which is reduced permeability to the drug. The triorganotin compounds are highly effective inhibitors of oxidative and photosynthetic phosphorylation amd mutants of C.reinhardii that are resistant to these inhibitors may provide material for the study of energy conservation simultaneously in both mitochondria and chloroplasts in the same cell.

Although drug-resistant mutants have been used extensively in the study of the structure and biogenesis of the mitochondrial ATP synthetase (ATP-ase) complex, very little work has so far been attempted of an analogous study in chloroplasts. This has probably resulted from the lack of three primary requisites in a study of this kind : (a) A suitable photosynthetic organism amenable to both genetic and biochemical manipulation. (b) Specific inhibitors of photosynthetic phosphorylation that are sufficiently toxic to whole cells of the organism. (c) Isolation of mutants of the organism that are resistant to the inhibitory effects of the drugs. The drug resistant mutants of C.reinhardii that have been isolated in this study are the only mutants known to the author that satisfy all these criteria. However, the problems associated with the use of C.reinhardii in studies of this kind are two fold : (1) obligate aerobic properties of the cells

that do not allow rapid screening of mutants that have lesions in oxidative or photosynthetic phosphorylation and (2) isolation of mitochondria and chloroplasts exhibiting tight coupling between electron transport and phosphorylation.

In order to determine whether the trialkyltin-resistant mutant TP-8$^+$ of C.reinhardii would be of use in the study of energy transfer in this alga, the response to trialkyltins of sub-cellular preparations of the Wt$^+$ and TP-8$^+$ strains were compared. This was of interest in view of the findings that certain mutants of S.cerevisiae and Aspergillus nidulans, that are resistant to triorganotins, oligomycin or venturicidin, exhibit resistance to these inhibitors at the level of the mitochondria, sub-mitochondrial particles and purified mitochondrial ATP-ase (Rowlands and Turner, 1974; Griffiths and Houghton, 1974; Lancashire and Griffiths, 1975[a]; Griffiths et al., 1975). Although the drug-resistance phenotypes of S.cerevisiae were cytoplasmically inherited, nuclear mutations associated with oligomycin resistance in A.nidulans also results in a resistant mitochondrial ATP-ase activity (Rowlands and Turner, 1974). In addition, the TET - and oligomycin - resistant mutants of S.cerevisiae that were resistant at the mitochondrial ATP-ase level were those that showed greatest specificity in their resistance to triorganotins or oligomycin respectively (class 2 mutants) (Lancashire and Griffiths, 1975[a]; Griffiths and Houghton, 1975). Consequently, the similar specificity of the TP-8$^+$ strain of C.reinhardii for resistance to TMT and TET may indicate a resistance phenotype at the sub-cellular or even at the ATP-ase level.

Materials and Methods

(a) Electron transport activities.

Early stationary-phase cells (72 h old) of the Wt^+ and $TP\text{-}8^+$ strains were harvested by centrifugation at 4×10^3 $g_{av.}$ for 5 min at 0°C. The following procedure was carried out at 0°C. The cells were washed three times in a modified medium of Gorman and Levine (1965), which contained 20 mM KCl, 2.5 mM $MgCl_2$, 1 mM Mg Na_2 EDTA, 1 mM reduced glutathione, 0.25 M sucrose, 0.1% BSA and 10 mM Hepes/NaOH buffer (pH 7.5), and resuspended in this medium at a ratio of 1 vol. medium to 2 vol. pelletted cells. The cells were broken in a French press (American Instrument Co.Inc.) at a pressure of 5×10^3 lbs /sq.inch and immediately diluted with 3 vols. of the resuspension medium. The resultant suspension was centrifuged three times at 1.8×10^3 $g_{av.}$ for 8 min to remove unbroken cells and the supernatant was sedimented at 2×10^4 $g_{av.}$ for 15 min. The final pellet was resuspended in the above medium at a concentration of approximately 1.0 mg chlorophyll/ml.

Oxygen uptake was measured with a Clark-type oxygen electrode incorporated into a glass-lined perspex reaction vessel maintained at a temperature of 25°C. Except where indicated in the text, the assay medium for photosynthetic oxygen uptake contained 0.1 mM methyl viologen (MV), 1.0 mM KCN, 4.1 μM rotenone and 0.4 mM malonate. The assay medium for measuring NADH oxidation contained 0.9 mM NADH and 0.4 mM malonate. Succinate oxidation was assayed in a medium containing 10 mM sodium succinate and 4.1 μM rotenone. All three assay media contained chloroplasts to a concentration of 40 μg chl/ml and resuspension medium (pH 7.0) to a final volume of 2.5 mls.

The mean control rates ($\pm$ standard deviations), over a series of 6 determinations, for the Wt^+ and $TP\text{-}8^+$ strains respectively were

as follows (in μ moles O_2 uptake/mg chl./ h):- MV oxidation : 62.4 $\pm$ 22.5 and 55.4 $\pm$ 11.0 ; succinate oxidation : 10.5 $\pm$ 4.8 and 5.8 $\pm$ 0.74; NADH oxidation : 25.5 $\pm$ 8.3 and 30.3 $\pm$ 5.9.

(b) Light-induced transmission changes of chloroplast fragments.

Chloroplast fragments were prepared according to the method of Gorman and Levine (1965), using early stationary-phase cells (72 h old) of the Wt^+ and $TP\text{-}8^+$ strains. The cells were harvested at 4 x $10^3 g_{av.}$ for 5 min at 0°C and washed twice in a resuspension medium containing 20 mM KCl, 2.5 mM $MgCl_2$, 1 mM Mg Na_2EDTA, 1 mM reduced glutathione, 0.1% BSA and 0.01 M phosphate buffer pH 7.5. The paste of cells was ground in a chilled mortar and pestle with acid-washed sand to approximately 90% cell breakage. The resulting paste was diluted with a little resuspension medium and the supernatant further diluted three fold with resuspension medium. The supernatant was then centrifuged three times at 1.8 x 10^3 $g_{av.}$ for 8 min at 0°C and the pellet discarded each time. Finally, the supernatant was diluted five fold and centrifuged at 2 x 10^4 $g_{av.}$ for 20 min at 0°C. The final pellet was resuspended in the resuspension medium to a concentration of 1.0 mg chlorophyll/ml.

Except where indicated in the text, the assay medium contained 20 μm methyl phenazine methosulphate (PMS), 100 mM KCl or 100 mM sodium acetate, chloroplast fragments at a concentration of 10 μg chlorophyll/ml and 10 mM Hepes/NaOH buffer (pH 8.0) to a final volume of 3.0 ml. The light intensity used was 3 x 10^4 lux. The light intensity and concentrations of acetate and chloroplasts used were found to be optimal for the assay (Appendix Figs. 10 and 11).

Transmission changes were measured with a modified Eppendorf spectrophotometer as described in the Materials and Methods of Chapter 3.

The mean control values ($\pm$ standard deviation), over a series

of 19 determinations, for the light-induced transmission changes of the Wt^+ and TP-8^+ strains respectively were as follows (in % Δ transmission/mg chl.):- with KCl : 497 ± 60 and 618 ± 83 ; with sodium acetate : 677 ± 39 and 776 ± 73.

(c) Light-induced pH changes in chloroplast fragments.

Chloroplast fragments were prepared as for the light-induced transmission assays except that the final pellet was washed twice and resuspended in 10 mM KCl. pH changes were measured with a Pye pH meter (Model 290) with the pH electrode (Pye Unicam Ingold) inserted into a water-jacketted glass vessel maintained at a constant temperature of 25°C.

Except where indicated in the text, the assay medium contained the following in a final volume of 3.0 ml : 17.0 mM KCl, 0.67 mM P-benzoquinone and chloroplasts to a concentration of 133.0 µg chlorophyll per ml. Inhibitor solutions were adjusted to pH 7.0 and the assay medium was initially adjusted to pH 7.0 after addition of the inhibitors in the dark. The suspension was illuminated with white light from a Daray 1200 bench light at an intensity of 52×10^3 lux. The initial pH of the medium and the concentrations of KCl and chloroplasts used were found to be near optimal for the activity of the chloroplasts in this assay (Appendix Figs. 12, 14 and 15). The light intensity used was supra-optimal for the extent (ΔH^+) and rate of light-induced H^+ uptake (Appendix Fig. 13). Recorder deflections were related to hydrogen ion concentration using a constructed curve produced by the addition of small aliquots of a standard 0.01N HCl solution to the complete assay system in the dark. The assay medium was initially stirred after addition of all the components but, to reduce excessive scatter in the recordings, the pH changes were assayed without stirring.

The mean control values (± standard deviation), over a series

of 12 determinations, for the light-induced proton changes were as follows:- Wt^+ : extent of proton uptake (ΔH^+) = 0.066 ± 0.015 μ equivs. H^+/mg chl. ; rate of light-induced proton uptake = 0.696 ± 0.130 μ equivs. H^+/mg chl./min; rate of dark proton loss = 0.661 ± 0.153 μ equivs. H^+/mg chl./min. $TP\text{-}8^+$: extent of proton uptake (ΔH^+) = 0.079 ± 0.017 μ equivs. H^+/mg chl. ; rate of light-induced proton uptake = 0.849 ± 0.138 μ equivs. H^+/mg chl./min; rate of dark proton loss = 0.838 ± 0.089 μ equivs. H^+/mg chl./min.

(d) Ca^{2+}-dependent ATP-ase activity of chloroplast fragments.

Chloroplast fragments were prepared from both the Wt^+ and $TP\text{-}8^+$ strains essentially by the method of Levine and Gorman (1966). Early stationary-phase cells (72 h old) were harvested at 4 x $10^3 g_{av.}$ for 5 min at 0°C. The following procedure was carried out at 0°C. The cells were washed three times in a resuspension medium containing 20 mM KCl in 10 mM tris/H_2SO_4 buffer, pH 8.0, and resuspended in this medium in a ratio of 1 vol. buffer to 1 vol. cells. The suspension was sonicated with an MSE ultrasonicator to approximately 90% cell breakage and then centrifuged three times at 480 $g_{av.}$ for 6 min to remove unbroken cells. The resultant supernatant was further centrifuged for 2 x 10^4 $g_{av.}$ for 20 min and the residue resuspended in the resuspension medium, using a glass homogeniser, at a concentration of 1.0 mg chlorophyll/ml.

The Ca^{2+}-dependent ATP-ase activity and its inhibition by drugs were assayed as described for the purified chloroplasts of the $CW15^+$ strain in the Materials and Methods of Chapter 3. The conditions of the assay were optimal or near optimal (Appendix Figs. 16 to 21), namely final concentrations of 3.2 mM ATP, 5.3 mM $CaCl_2$, 10 min incubation at 36°C, an assay pH value of 8.0, activation for 1.5 min at 60°C and 7.1 mM disodium EDTA (activation stage concentration). The kinetic

experiments shown in Appendix Figs. 16 to 21 were carried out with the other conditions at optimum levels.

The mean control rates for the ATP-ase activity over a series of 15 determinations were 62.1 $\pm$ 9.4 and 52.3 $\pm$ 9.8 μ moles Pi liberated/mg chl./h for the Wt^+ and $TP\text{-}8^+$ strains respectively.

Results

(a) Electron transport activities.

The electron transport activities of sub-cellular preparations of C.reinhardii Wt^+ are shown in Tables 5.1 and 5.2. Respiratory and non-cyclic photosynthetic electron transport rates were low or absent in the absence of the appropriate electron donors or substrates (Table 5.1). Addition of MV and KCN in the light stimulated MV oxidation approximately 20 fold and addition of NADH or succinate in the dark likewise stimulated the respiratory electron transport activities. Addition of ADP, Pi and uncouplers had no effect on the oxygen uptake rates, indicating that electron transport activities were not tightly coupled to phosphorylation in these preparations. The inhibitors used to selectively inhibit NADH oxidation (rotenone) or succinate oxidation (malonate) were only partially selective in their activity (Table 5.2). However, the use of these inhibitors, along with omission of the appropriate substrates, probably resulted in the selective inhibition of either NADH or succinate oxidation. Likewise, the use of KCN, malonate and rotenone in the assay of MV oxidation was probably sufficient to completely inhibit respiratory electron transport in the absence of NADH and succinate.

The effect of TMT on the oxygen uptake coupled to MV, succinate and NADH oxidation in the Wt^+ and $TP\text{-}8^+$ strains of C.reinhardii is shown in Figs. 5.1, 5.2 and 5.3 respectively. Very high concentrations of TMT were required to inhibit these activities and relatively low concentrations stimulated MV oxidation in both strains (Fig.5.1). There was no apparent difference between the two strains in the response to TMT of non-cyclic photosynthetic electron transport (Fig.5.1), respiratory succinate oxidation (Fig. 5.2) or respiratory NADH oxidation (Fig. 5.3). The control (uninhibited) electron transport

Table 5.1. Electron transport activities of sub-cellular preparations from C.reinhardii (strain Wt^+).

Additions \ Electron Donor	Oxygen Uptake (μmoles/mg chl./h)		
	MV	NADH	Succinate
Control*	2.9	0	0
+ Light	25.2	-	-
+11.7mM Succinate	-	-	14.6
+ 0.32mM NADH	-	26.6	-
+ 0.12mM MV	51.8	-	-
+ 1.1mM KCN	66.5	-	-
+ 8.9mM KH_2PO_4	67.0	25.9	14.1
+ 4.3mM ADP	66.8	23.1	14.8
+ 0.22μM DNP	-	22.0	14.0
+10.7μM CCCP	58.5	-	14.1

* Control contained only chloroplasts (40 μg chl./ml) and resuspension medium to a final volume of 2.5 ml.

Table 5.2. Effect of inhibitors on the electron transport activities of sub-cellular preparations from C.reinhardii (strain Wt^+).

Additions \ Electron Donor	% Inhibition of Electron Transport		
	MV	NADH	Succinate
Rotenone (2.1 μM)	-	24	-
Rotenone (4.1 μM)	0	58	26
Rotenone (15.2μM)	-	75	-
Malonate (0.4 mM)	0	14	42
KCN (1.1 mM)	-	80	65

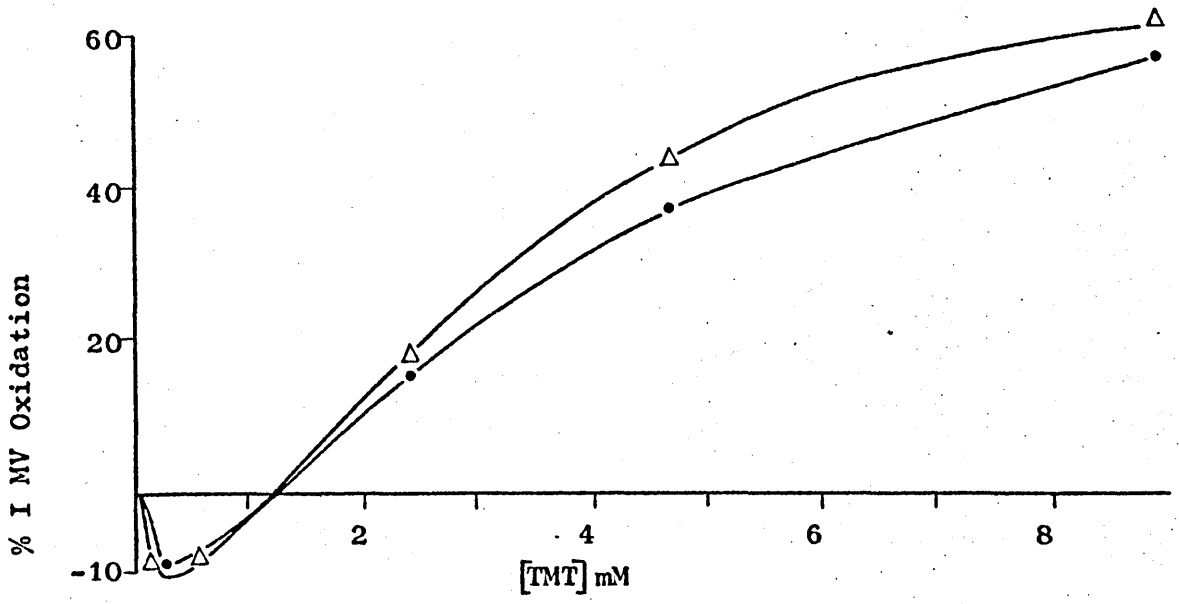


Figure 5.1. Effect of TMT on the non-cyclic photosynthetic electron-transport activities of extracts from the Wt^+ and TP-8^+ strains of C. reinhardii.

•—• Wt^+, Δ—Δ TP-8^+.

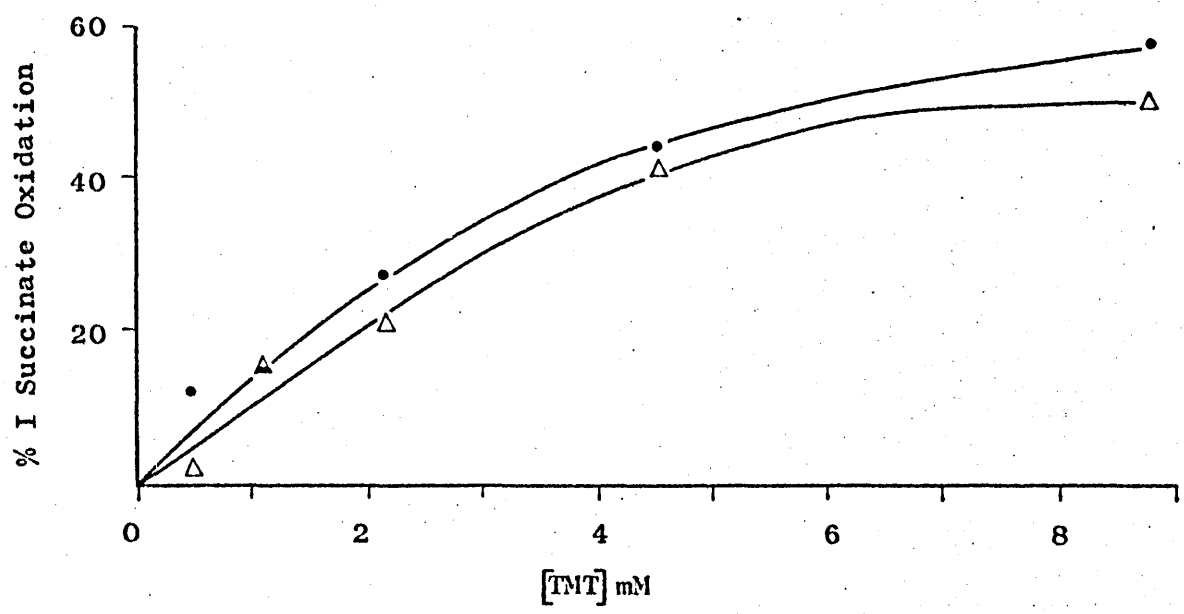


Figure 5.2. Effect of TMT on the succinate oxidation of extracts from the Wt^+ and TP-8^+ strains of C. reinhardii.

•—• Wt^+, Δ—Δ TP-8^+.

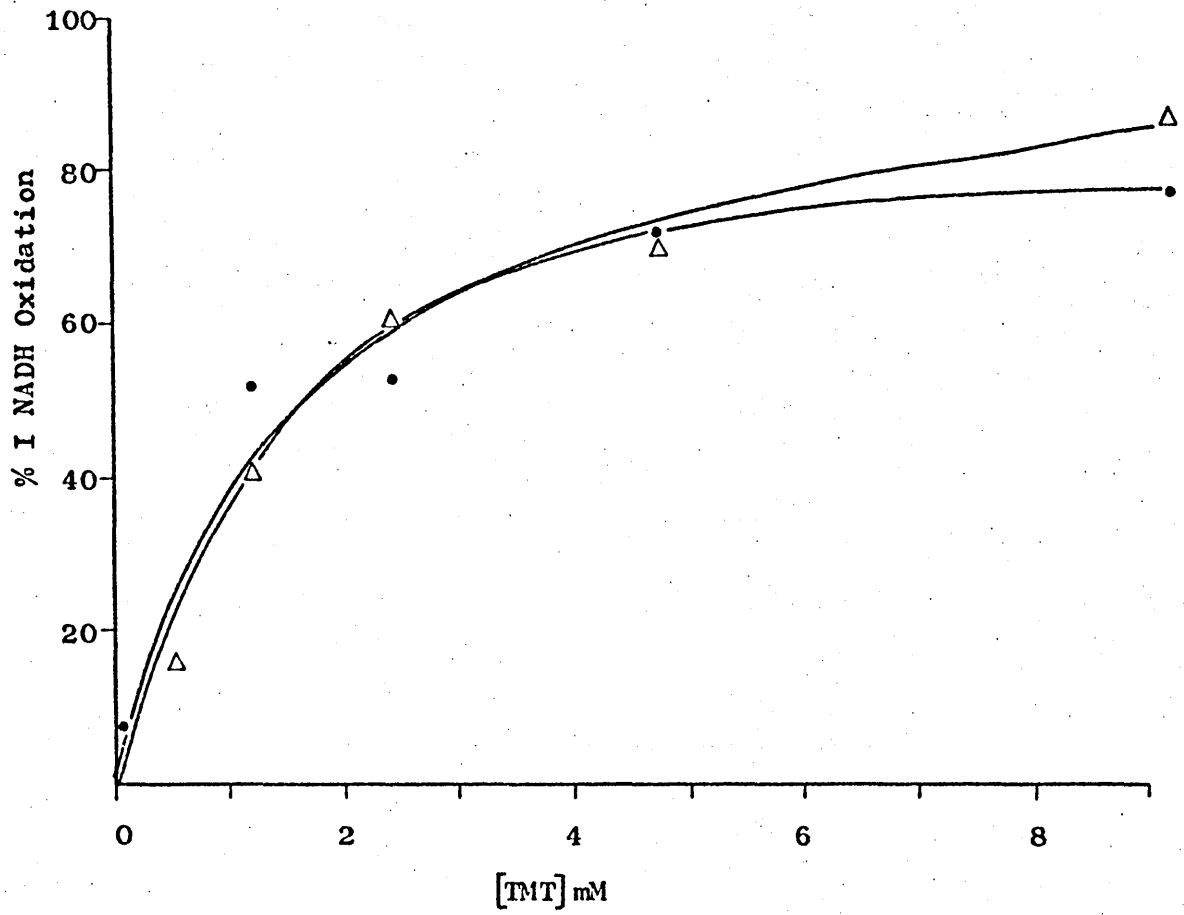


Figure 5.3. Effect of TMT on the NADH oxidation of extracts from the Wt^+ and $TP\text{-}8^+$ strains of C. reinhardii.

•—• Wt^+.
Δ—Δ $TP\text{-}8^+$.

rates of both strains were also similar.

(b) Light-induced transmission changes of chloroplast fragments.

The light-induced transmission changes of chloroplast fragments from the Wt^+ and TP-8^+ strains are shown in Fig. 5.4 and Table 5.3. A small light-induced transmission change occurred with chloroplasts suspended in only buffer and PMS (Fig. 5.4 b). These transmission changes were stimulated by sodium acetate (Fig. 5.4c) but not by KCl (Fig. 5.4d). Triorganotins, at low concentrations, slightly stimulated the transmission changes in the presence of KCl, although they inhibited the changes at higher concentrations (Fig. 5.4 e and f, and Table 5.3). There was no apparent difference between the two strains in either the specific activities of the chloroplast fragments or in their response to triorganotins.

(c) Light-induced pH changes in chloroplast fragments.

The effect of TMT on the light-induced pH changes of the Wt^+ and TP-8^+ strains is shown in Fig. 5.5. In both strains, the rate of light-induced proton uptake was most sensitive, the rate of dark proton loss intermediate, and the extent of proton uptake least sensitive to inhibition by TMT. There was little difference in the inhibitory activity of TMT towards both strains in this assay. The control (uninhibited) proton changes of both strains were also similar.

(d) Ca^{2+}-dependent ATP-ase activity of chloroplast fragments.

The Ca^{2+}-dependent ATP-ase activity of both the Wt^+ and TP-8^+ strains were equally inhibited by high concentrations of TMT (Fig.5.6). The control (uninhibited) ATP-ase activities were also similar in both strains.

Table 5.4 summarises the inhibitory activity of TMT on the chloroplast fragments of the two strains. On a mg of chlorophyll

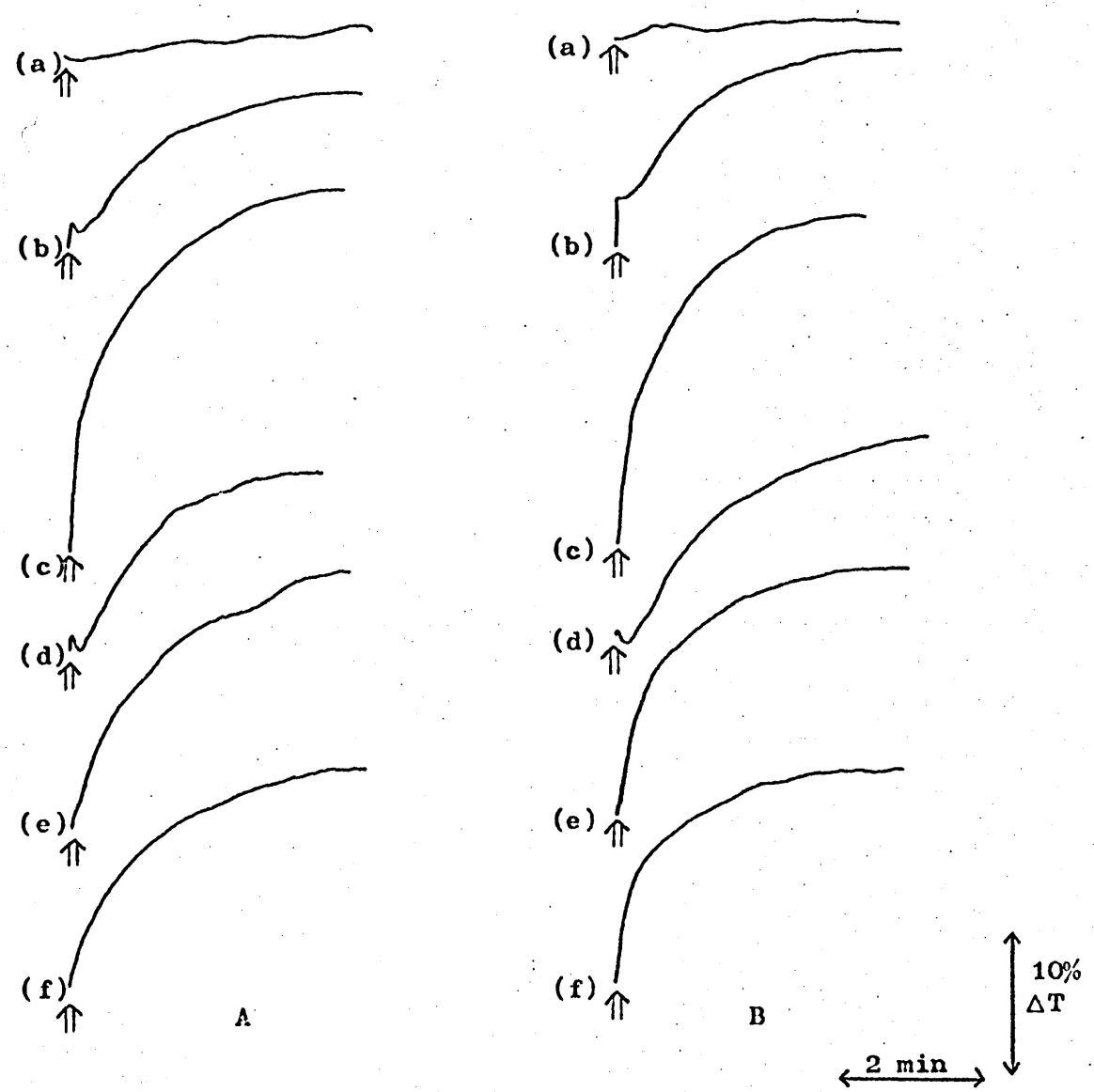


Figure 5.4. Light-induced transmission changes of chloroplast fragments from C. reinhardii.

Series 1 and 2 are with the Wt^+ and $TP\text{-}8^+$ strains respectively, with the following assay components:

(a) 20μM PMS in Hepes/NaOH buffer, pH 8.0. (b) 20μM PMS and chloroplasts (10μg/ml). (c) 20μM PMS, chloroplasts (10μg/ml) and 100mM sodium acetate. (d) 20μM PMS, chloroplasts (10μg/ml) and 100mM KCl. (e) Same as (d) plus 0.24μM TPT. (f) Same as (d) plus 0.34μM TMT.

⇑ Actinic illumination on.

Table 5.3. Effect of triorganotins on the light-induced transmission changes of chloroplast fragments from the Wt^+ and $TP\text{-}8^+$ strains of C.reinhardii.

Strain / Triorganotin	Transmission Changes* (% of Control)	
	Wt^+	$TP\text{-}8^+$
TMT (0.3 μM)	115	113
TMT (2.6 μM)	108	113
TMT (4.3 μM)	86	92
TMT(15.3 μM)	69	69
TET (0.1 μM)	116	108
TET (1.3 μM)	84	85
TPT (0.2 μM)	126	137
TBT (0.3 μM)	160	148
TPhT(0.9 μM)	110	106
TPhT(2.6 μM)	93	88

*Carried out in a medium containing 100 mM KCl.

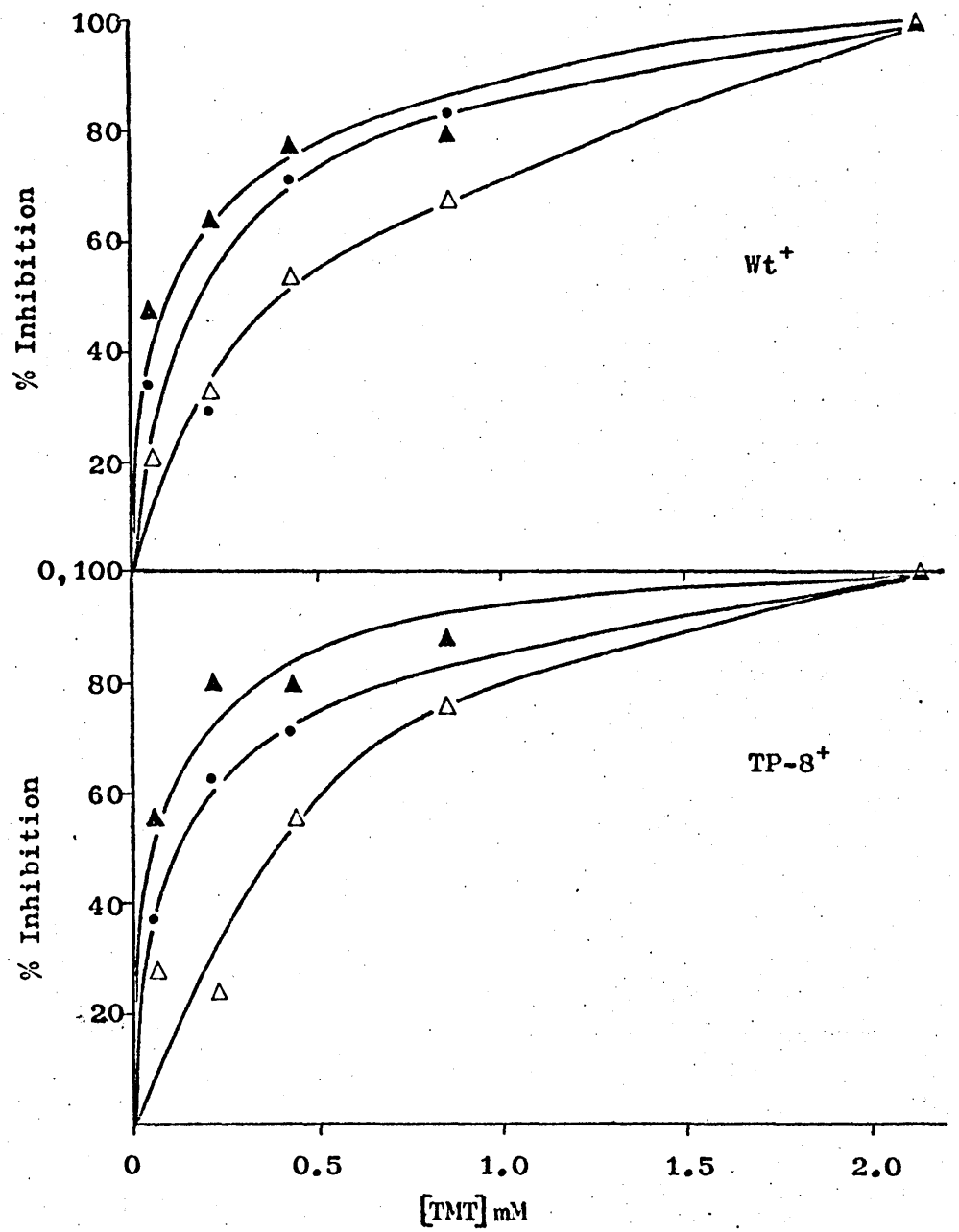


Figure 5.5. Effect of TMT on the light-induced pH changes of suspensions of chloroplast fragments from the Wt^+ and TP-8^+ strains of C. reinhardii.

▲—▲ Rate of light-induced H^+ uptake.

△—△ Extent (ΔH^+) of light-induced H^+ uptake.

•—• Rate of H^+ loss in the dark.

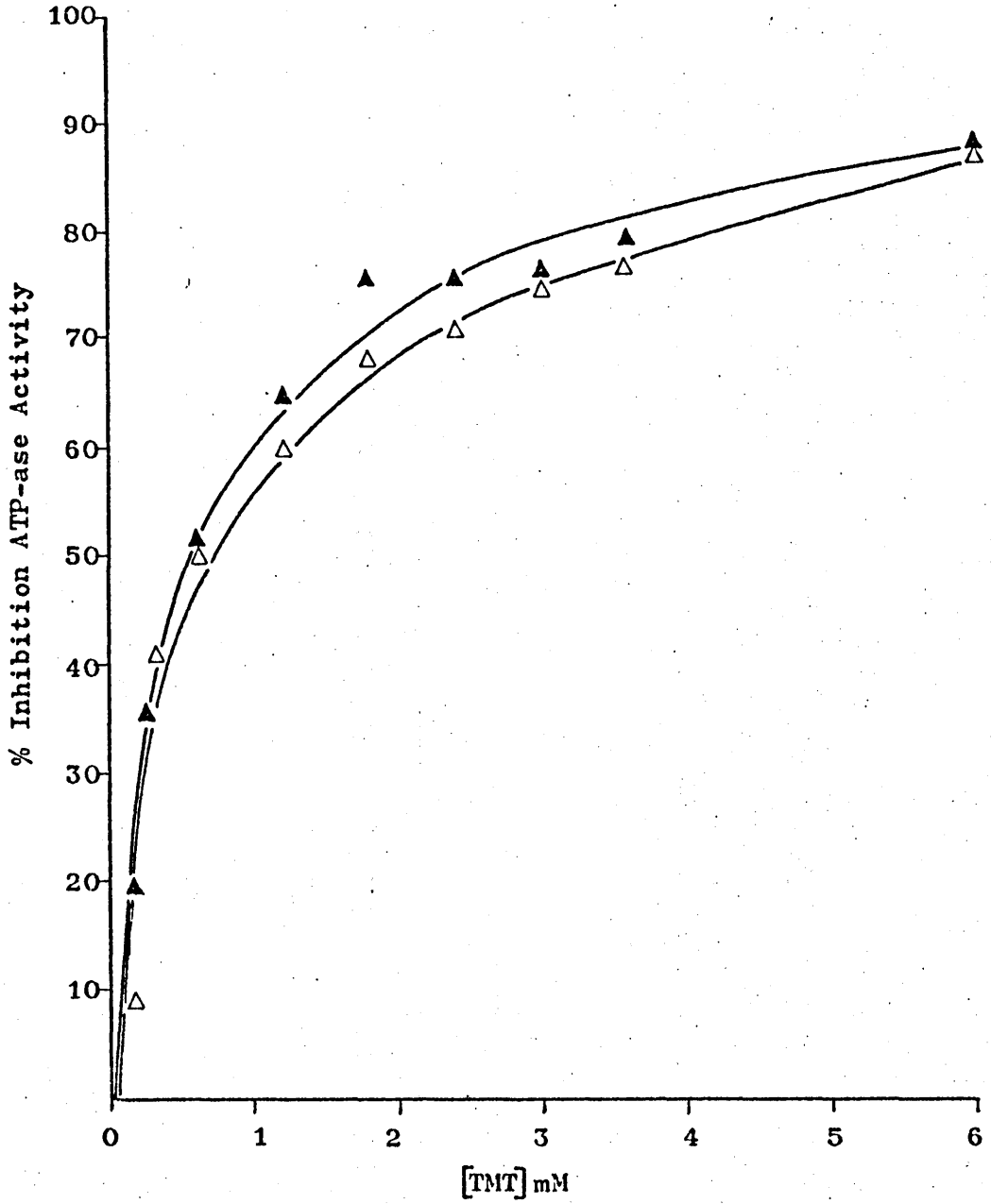


Figure 5.6. Effect of TMT on the Ca^{2+}- dependent ATP-ase activity of chloroplast fragments from the Wt^{+} and TP-8^{+} strains of C. reinhardii.

▲—▲ Wt^{+}.

△—△ TP-8^{+}.

Table 5.4. Summary of the effect of TMT on the chloroplast fragments of the Wt^+ and $TP\text{-}8^+$ strains of C. reinhardii.

Strain	[TMT] (μmoles/mg chl.) Required to Produce 50% Inhibition of Activity						
	Electron Transport			Light-induced pH Changes			Ca^{2+}-dependent ATP-ase
	MV Oxidation	Succinate Oxidation	NADH Oxidation	Rate of H^+ Uptake	Extent of H^+ Uptake (ΔH^+)	Rate of H^+ Dark Loss	
Wt^+	430	370	120	1.35	9.02	3.83	19
$TP\text{-}8^+$	340	470	100	0.68	8.35	2.26	23

basis, the light-induced proton changes were most sensitive to inhibition by TMT and the Ca^{2+}-dependent ATP-ase activity less sensitive. Much higher TMT/chlorophyll ratios were required to inhibit the respiratory and non-cyclic photosynthetic electron transport activities. In the electron transport assay, MV and succinate oxidation were inhibited by similar concentrations of TMT but both were less sensitive to TMT than was NADH oxidation.

Discussion

The various activities of the sub-cellular preparations from C.reinhardii responded similarly to the inhibitory effects of TMT in both the Wt^+ and TP-8^+ strains. Consequently, the TMT resistance in the TP-8^+ strain does not appear to be localised at the level of the Ca^{2+}-dependent chloroplast ATP-ase, respiratory and non-cyclic photosynthetic electron transport, or the high-energy intermediate of photosynthetic energy transfer.

The high concentrations of TMT required to inhibit the test reactions compared to those required to inhibit state 3 oxygen evolution in pea chloroplasts (Chapter 3) may indicate a non-specific mode of inhibition by TMT in these preparations. That high (>1mM) concentrations of TMT can produce non-specific denaturation of chloroplast structure, with concomitant inhibition of uncoupled electron transport, has been observed by Watling-Payne and Selwyn (1974). This may be related to the detergent-like effect on mitochondria of high concentrations of triorganotins (Stockdale et al., 1970).

The Cl^- —› OH^- exchange reaction mediated by the triorganotins in pea chloroplasts (Chapter 3) was not stimulated to the same extent in the chloroplast preparations from C.reinhardii. The light-induced transmission changes in a KCl-containing medium were only slightly stimulated by triorganotins at concentrations that induced large changes in pea chloroplasts. In addition, the dark decay rate of the light-induced proton gradient in a KCl medium were inhibited by TMT at concentrations which stimulated this rate in pea chloroplasts (Chapter 3). Consequently it would appear that the Cl^- —› OH^- exchange reaction mediated by the triorganotins was operative to only a small extent in the chloroplast preparations from C.reinhardii. The small stimulation of MV oxidation at low TMT concentrations may

have been due to the slight Cl^- — OH^- exchange reaction present, since a similar stimulation of basal non-cyclic electron transport by TMT has been attributed to this uncoupling mechanism (Watling-Payne and Selwyn, 1974).

The reason for the low rates of the Cl^- — OH^- exchange reaction in these preparations is not clear but, since the reaction proceeds in response to the pH gradient generated on illumination (Watling-Payne and Selwyn, 1974), it may be related to the very low extents of proton uptake in these chloroplast fragments. The extent of proton uptake at pH 8.0 in similar preparations was approximately 0.035 μ equivalents H^+ per mg chl. for the Wt^+ strain of C.reinhardii (Appendix Fig.15), as compared to an average of 0.5 μ equivs. H^+ per mg chl. for the pea chloroplasts at pH 7.6 (Chapter 3). Alternatively, the low exchange reaction rates may be partly due to the decrease in osmotic response of chloroplast preparations with decreasing size of the particles (Gross and Packer, 1967).

Comparison of the inhibitory effect of TMT on the various activities of the sub-cellular preparations of C.reinhardii (Table 5.4) reveals a more specific effect of this compound on the light-induced pH changes than on either the Ca^{2+}-dependent ATP-ase activity or electron transport. The significance of this is not clear, particularly since the ATP-ase assays were carried out at a different pH (8.0) than either the pH changes (7.0) or electron transport (7.0) assays. However, it may indicate a more specific effect of TMT on the thylakoid membrane as opposed to a direct effect on either the CF_1 or intermediates of the electron transport chain. The fact that the rate of light-induced proton uptake and dark proton loss were more sensitive to TMT than the extent of proton uptake may indicate a blockage of the proton-conducting channels in the thylakoid membrane, analogous to that

reported for TPhT (Gould, 1976). However, since the extent of proton uptake was not stimulated by TMT, as would be expected of a TPhT-like mode of action, TMT may have a somewhat different inhibitory mechanism than that of TPhT. It is possible that the molecules of TMT, which are smaller than those of TPhT, only partially block the proton-conducting channels and hence affect the rate, but not the extent, of proton movement across the thylakoid membrane.

In conclusion, it appears that the potential use of the TP-8^+ mutant of C.reinhardii in the study of energy conservation is uncertain. Although this mutant is resistant to the growth-inhibitory effects of compounds that affect both respiratory and photosynthetic energy transfer, the exact cellular site of resistance has not been localised. Further work is required to determine the relative response to TMT of ATP synthesis associated with both respiration and photosynthesis in the Wt^+ and TP-8^+ strains. In this regard, conditions for producing high, reproducible rates of photophosphorylation in chloroplast preparations from C.reinhardii have recently been developed (Brand et al., 1975). In addition, a method for isolating mitochondria from the $CW15^+$ strain has also been found (Ryan et al., 1973, 1974). Consequently, both respiratory and photosynthetic energy conservation in C.reinhardii has become much more accessible to both genetic and biochemical analysis in vivo and in vitro. This is illustrated by the fact that a Mendelian mutant (F-54) of C.reinhardii, which is incapable of both cyclic and non-cyclic photophosphorylation, has a lesion in the terminal steps of photosynthetic energy transfer at the level of the Ca^{2+}-dependent ATP-ase (Sato et al., 1971). Since the coupling enzymes involved at all sites of ATP synthesis are affected in F-54, it appears that the enzymes are either identical or similar enough to be equally affected by the same genetic lesion. It would be

interesting to investigate the effect, if any, of this single Mendelian gene mutation on other ATP-ase activities of both chloroplasts and mitochondria to determine whether the same gene is involved in their biogenesis.

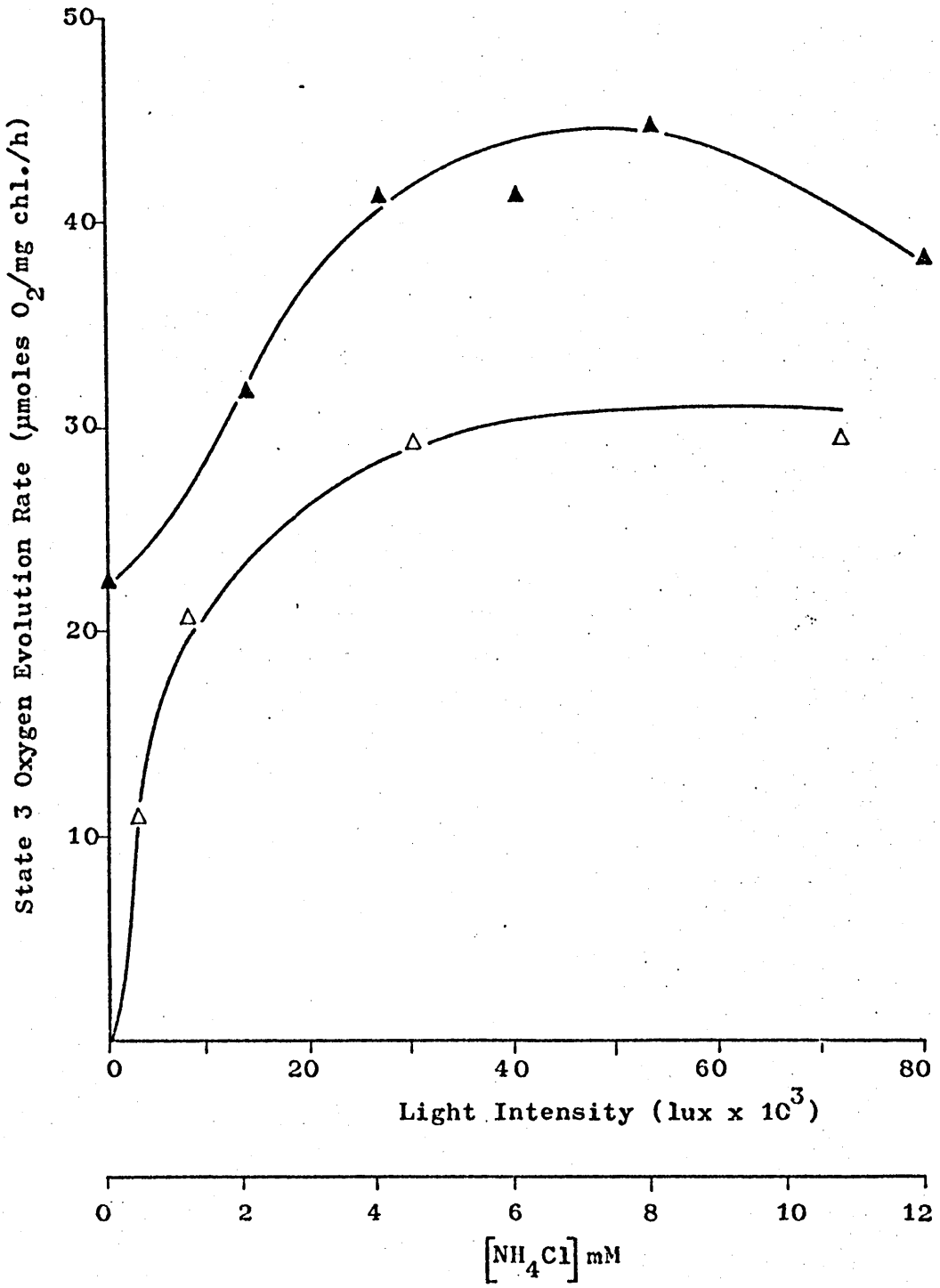
The use of mutants of C.reinhardii, with lesions in energy conservation, are becoming more promising both from the point of view of unravelling the biogenetic relationship between respiratory and photosynthetic energy conservation and in solving the perennial problem of the mechanism of energy transfer.

APPENDIX

Appendix Table 1. Composition of media used for the growth of C.reinhardii.

Stock Solution	Component	Quantity per Litre of Medium*			
		M	A	YAP	Nitrogen-free A
Beijerink's Nitrogen-free	$MgSO_4 \cdot 7H_2O$	-	-	-	0.10
	$CaCl_2 \cdot 2H_2O$	-	-	-	0.05
Beijerink's High Salt	$MgSO_4 \cdot 7H_2O$	0.10	0.10	0.10	-
	$CaCl_2 \cdot 2H_2O$	0.05	0.05	0.05	-
	NH_4NO_3	0.40	0.40	0.40	-
Phosphate buffer	K_2HPO_4	0.72	0.72	0.72	0.72
	KH_2PO_4	0.36	0.36	0.36	0.36
Trace elements	Na_2EDTA	5.00	5.00	5.00	5.00
	$ZnSO_4 \cdot 7H_2O$	2.20	2.20	2.20	2.20
	H_3BO_3	1.14	1.14	1.14	1.14
	$MnCl_2 \cdot 4H_2O$	0.51	0.51	0.51	0.51
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.11	0.11	0.11	0.11
	$CuSO_4 \cdot 5H_2O$	0.16	0.16	0.16	0.16
	$FeSO_4 \cdot 7H_2O$	0.49	0.49	0.49	0.49
	$CoCl_2 \cdot 6H_2O$	0.16	0.16	0.16	0.16
Supplements	Sodium acetate	-	2.00	2.00	2.00
	Yeast extract	-	-	4.00	-
	Peptone	-	-	5.00	-

* The trace element components are in mg/litre. All other values refer to quantities in g/litre.

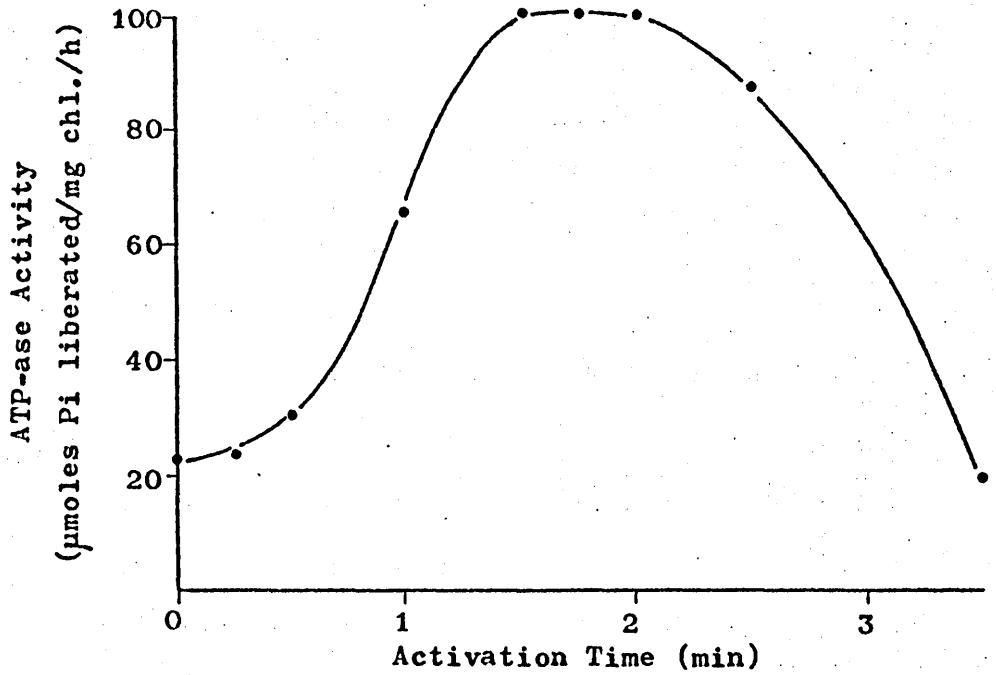


Appendix Figure 1. Effect of light ($\lambda_{max} \geq 650nm$) intensity and ammonium chloride concentration on the state 3 oxygen evolution of pea chloroplasts.

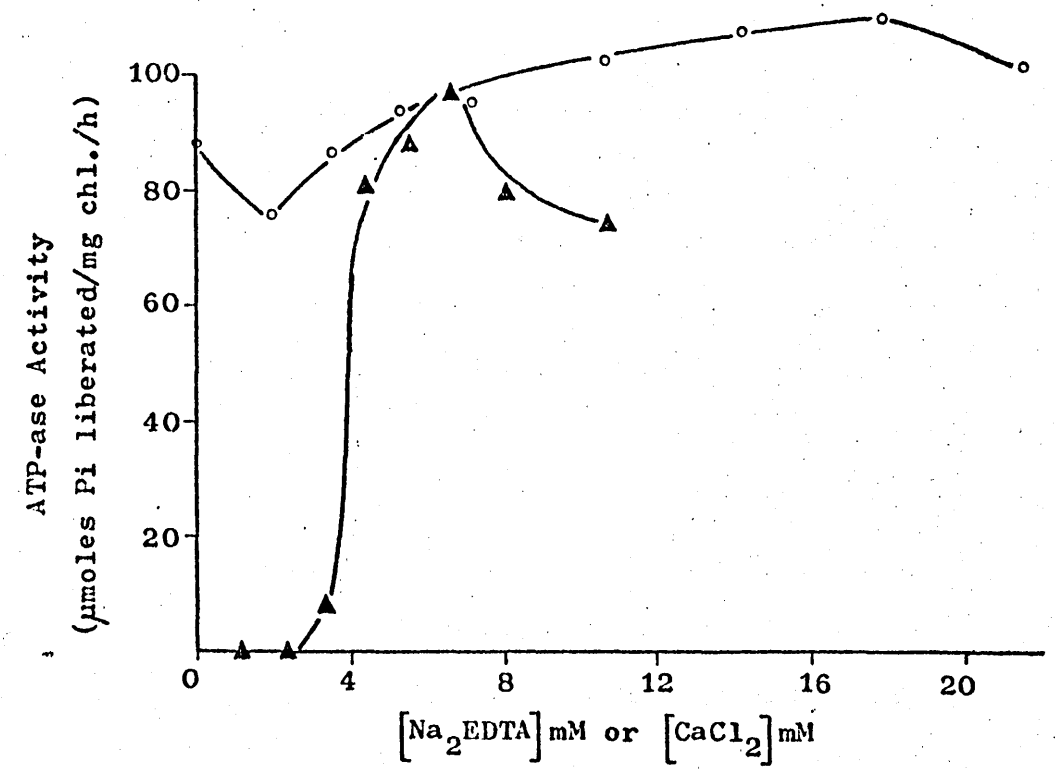
Chloroplast preparation and assay methods are described in the Materials and Methods of Chapter 3, using $K_3Fe(CN)_6$ as the electron acceptor.

△—△ Light intensity.

▲—▲ NH_4Cl concentration.



Appendix Figure 2. Time-dependent activation at 60°C of the Ca^{2+}-dependent ATP-ase activity of C. reinhardii ($CW15^{+}$) chloroplast fragments.



Appendix Figure 3. Effect of EDTA and calcium chloride on the heat-activated Ca^{2+}-dependent ATP-ase activity of C. reinhardii ($CW15^{+}$) chloroplast fragments.

o—o Na_2EDTA.

▲—▲ Calcium chloride.

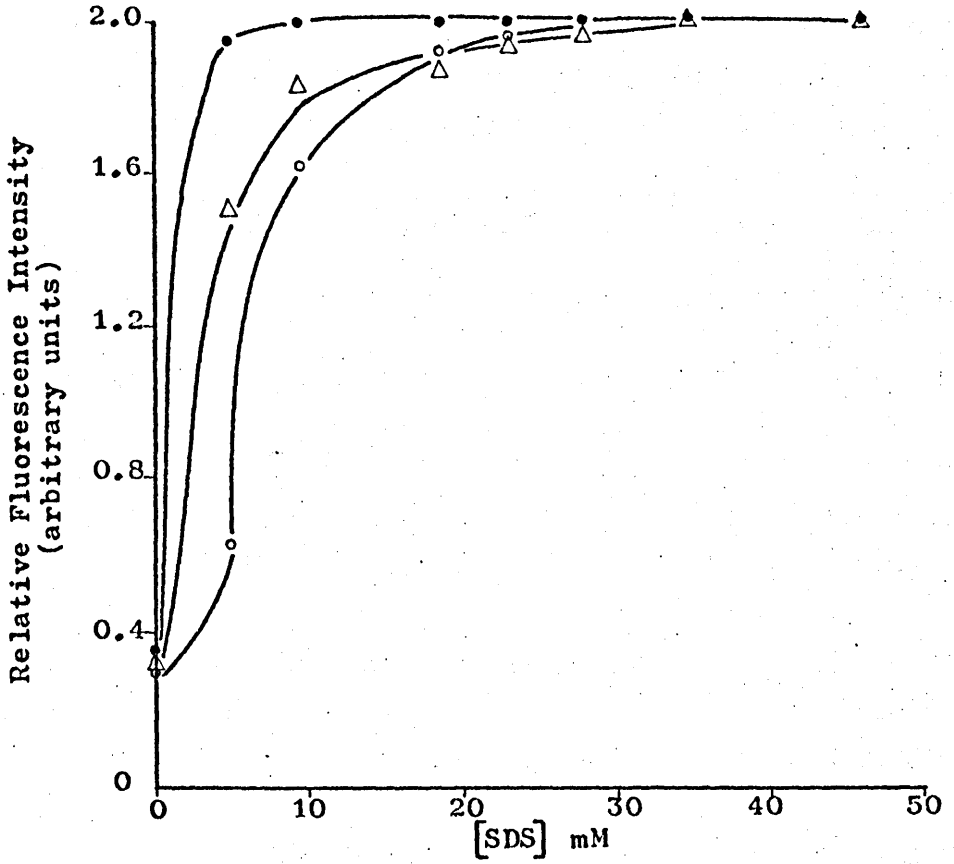
Legend to Appendix Figures 4 and 5.

Appendix Figure 4. Effect of different SDS concentrations on the fluorescence intensity of various ethidium bromide solutions.

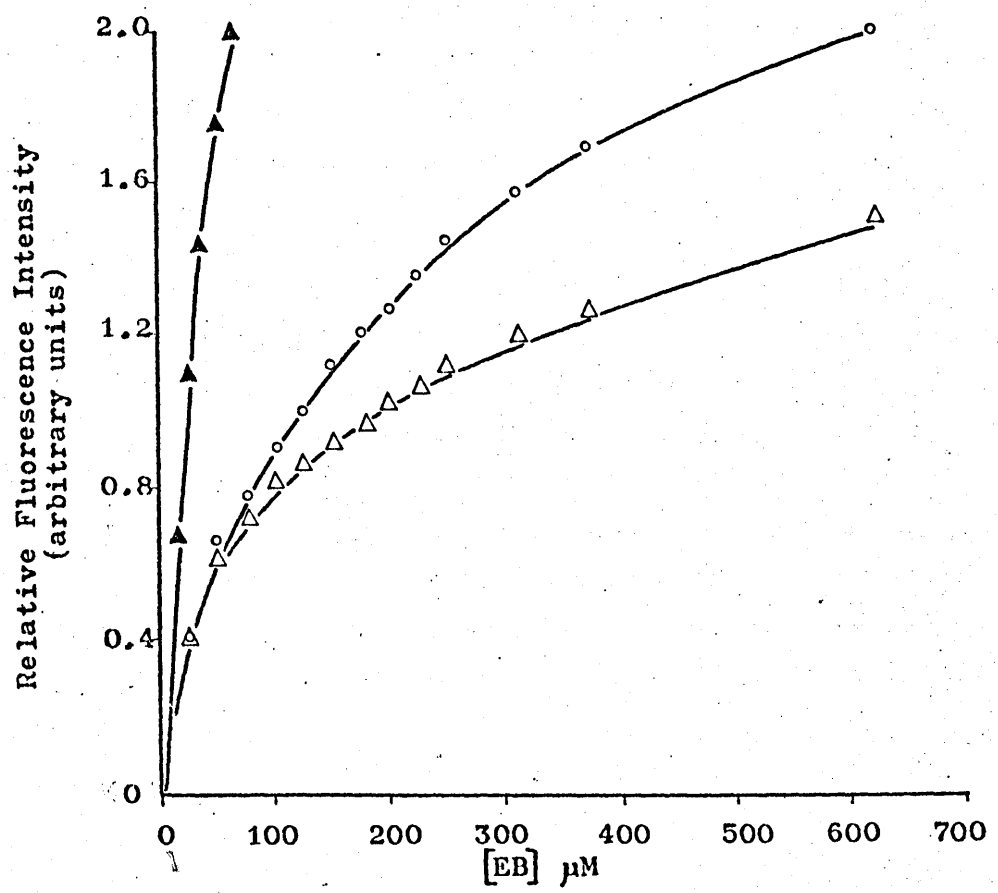
The fluorescence intensity of different standard EB solutions containing various SDS concentrations was determined by adjusting the fluorimeter to maximum sensitivity (to give an intensity of 2.0 arbitrary units) with the indicated EB solutions containing 46.1mM SDS. The fluorescence intensity, relative to the maximum of 2.0, of the standard EB solutions, in the presence of lower SDS concentrations, was then determined. The standard EB solutions used were:- •—• 50μM, Δ—Δ 200μM, ○—○ 625μM.

Appendix Figure 5. Relationship between the relative fluorescence intensity and ethidium bromide concentration in the presence or absence of SDS.

The fluorescence intensity of different standard EB solutions in the presence or absence of SDS was determined by adjusting the fluorimeter to maximum sensitivity with solutions of 625μM or 62.5μM EB in the presence or absence of 24.3mM SDS. In the absence of SDS, the fluorimeter could only be adjusted to give a maximum reading of 1.5 arbitrary units with a solution of 625μM EB. In the presence of SDS, both 625μM and 62.5μM EB solutions could be adjusted to give 2.0 arbitrary units. The fluorescence intensity of lower EB concentrations, relative to maximum values of 2.0 or 1.5, where appropriate, was then determined. The standard EB solutions used were:- ▲—▲ 62.5μM (plus 24.3mM SDS), ○—○ 625μM (plus 24.3mM SDS), Δ—Δ 625μM (no SDS).



Appendix Figure 4.



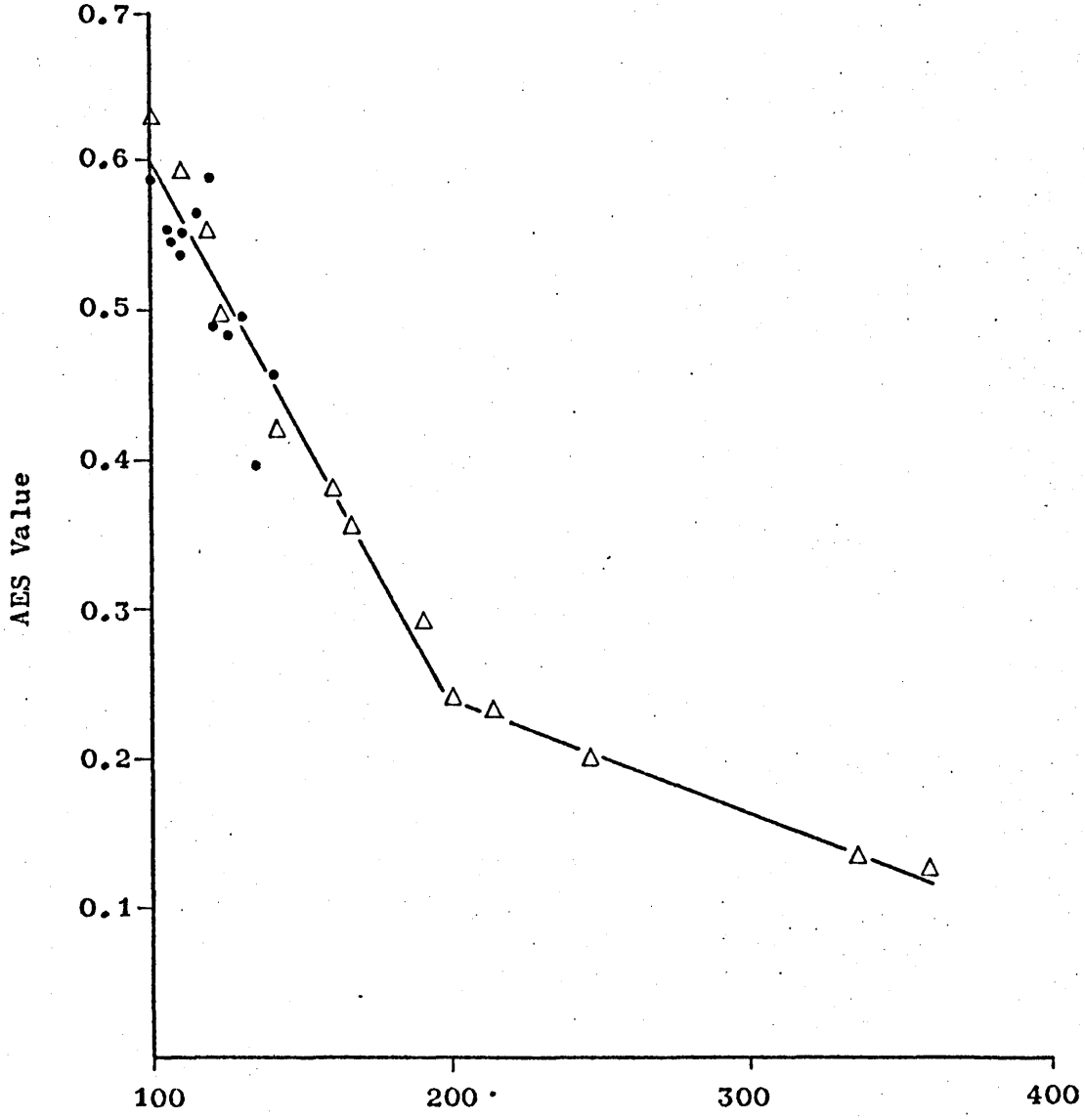
Appendix Figure 5.

Appendix Table 2. Efficiency of various preparative procedures for the liquid scintillation counting of whole cells of C.reinhardii.

Preparative Procedure*	AES• Value	CPM•
Blank vial + scintillant	0.6266	165
F+C+ scintillant	0.1340	602
F+C+ 0.5ml NaOCl + scintillant	0.4286	24162
F+C+ scintillant + 0.5ml NaOCl	0.4735	5732
Blank vial + scintillant + 0.5ml NaOCl	0.4704	9399
F+C+ 1.0ml H_2O_2 + scintillant	0.0227	689
F+C+ scintillant + 1.0ml H_2O_2	0.1584	9641
Blank vial + scintillant + 1.0ml H_2O_2	0.3850	214
F+C+ 0.5ml 10% H_2SO_4+ scintillant	0.2147	125
F+C+ scintillant + 0.5ml 10% H_2SO_4	0.0783	165
Blank vial + scintillant + 0.5ml 10% H_2SO_4	0.4949	162
F+C+ 0.5ml Cl_2/H_2O + scintillant	0.5078	190
F+C+ scintillant + 0.5ml Cl_2/H_2O	0.1204	128
Blank vial + scintillant + 0.5ml Cl_2/H_2O	0.5698	198
F+ 10µl 414 µM $[^{113}Sn]$ TET + scintillant	0.6362	38301
F+ 5µl 414 µM $[^{113}Sn]$ TET + scintillant	0.6355	19505
F+ 10µl 414 µM $[^{113}Sn]$ TET +0.5ml Cl_2/H_2O + scintillant	0.5827	36822
F+ 5 µl 414 µM $[^{113}Sn]$ TET +0.5ml Cl_2/H_2O + scintillant	0.5924	18143

*Refers to order in which the various components were added to the vials F = glass fibre filter, C = cell suspension.

•Results were the mean of three replicates for each treatment.



Appendix Figure 6. Relationship between the AES value and the calculated DPM of quenched samples of $[^{113}Sn]$ TET.

△—△ Using unbleached cells of C. reinhardii as quenching agent.

•—• Using chlorine-water bleached cells of C. reinhardii as quenching agent.

Legend to Appendix Figures 7 and 8.

Appendix Figure 7.

△—△ AES value.

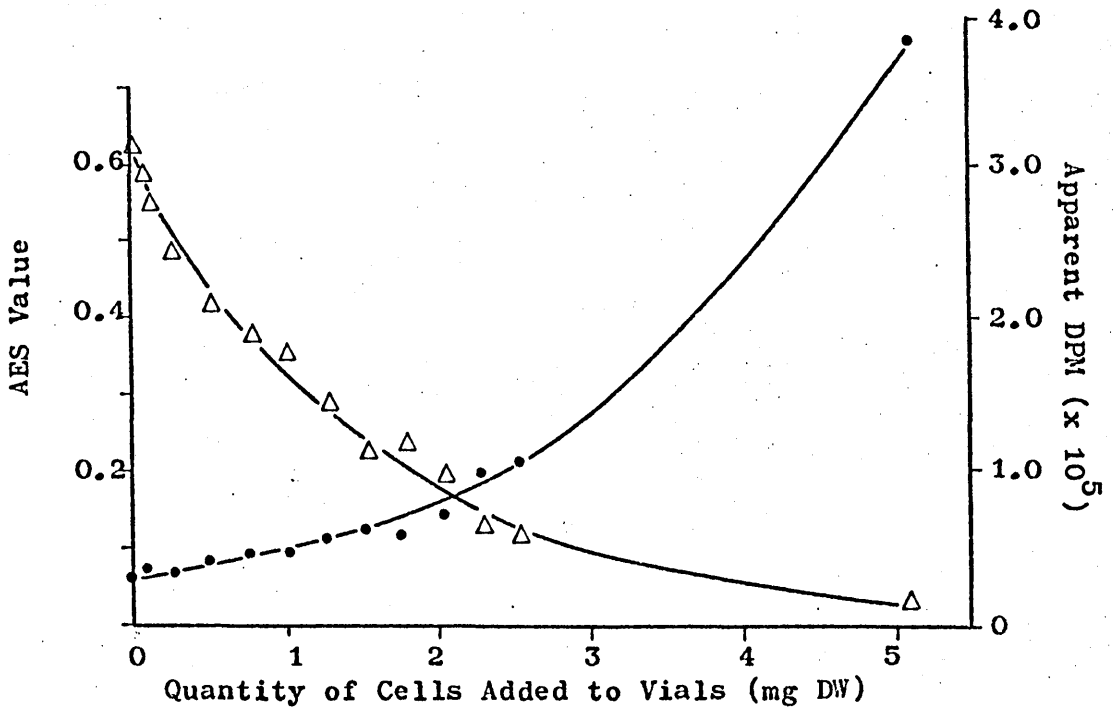
•—• DPM calculated directly from CPM and AES value.

Appendix Figure 8.

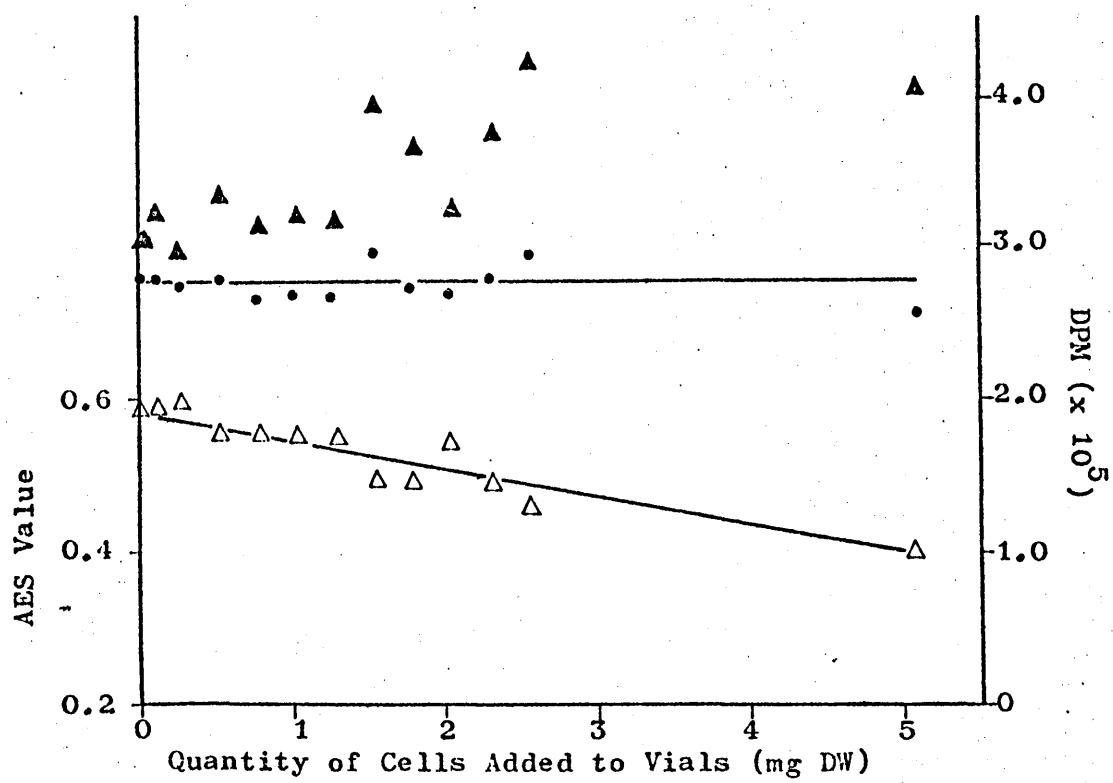
△—△ AES value.

▲—▲ DPM calculated directly from CPM and AES value.

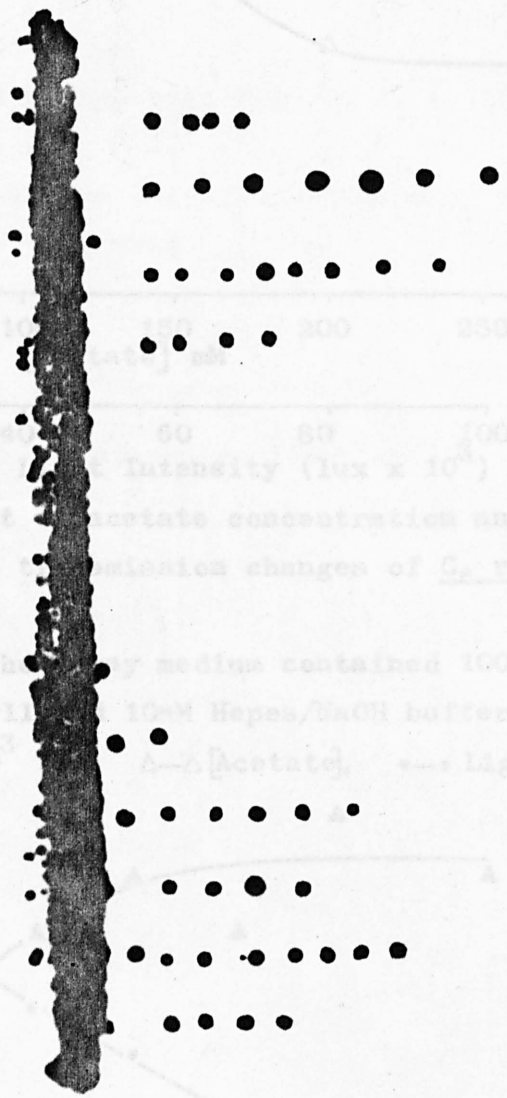
•—• DPM calculated from the CPM, AES value and correction factor using Appendix Fig. 6.



Appendix Figure 7. Effect of the quantity of unbleached cells added to standard $[^{113}Sn]$ TET solutions on the AES values and apparent DPMs.

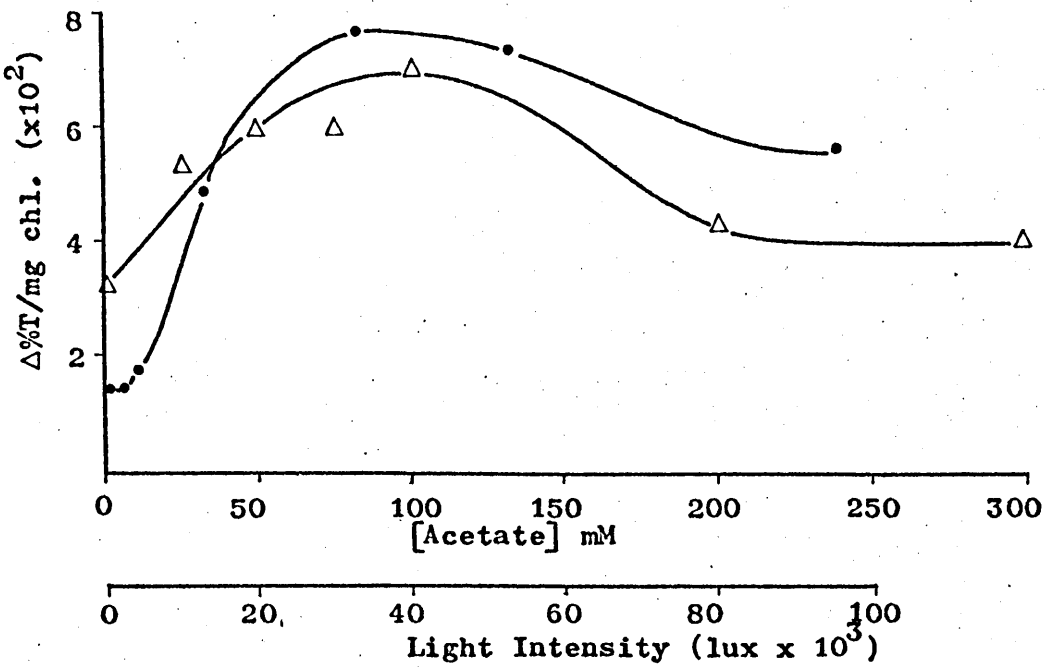


Appendix Figure 8. Effect of the quantity of chlorine-water-bleached cells added to standard $[^{113}Sn]$ TET solutions on the AES values and apparent and true DPMs.

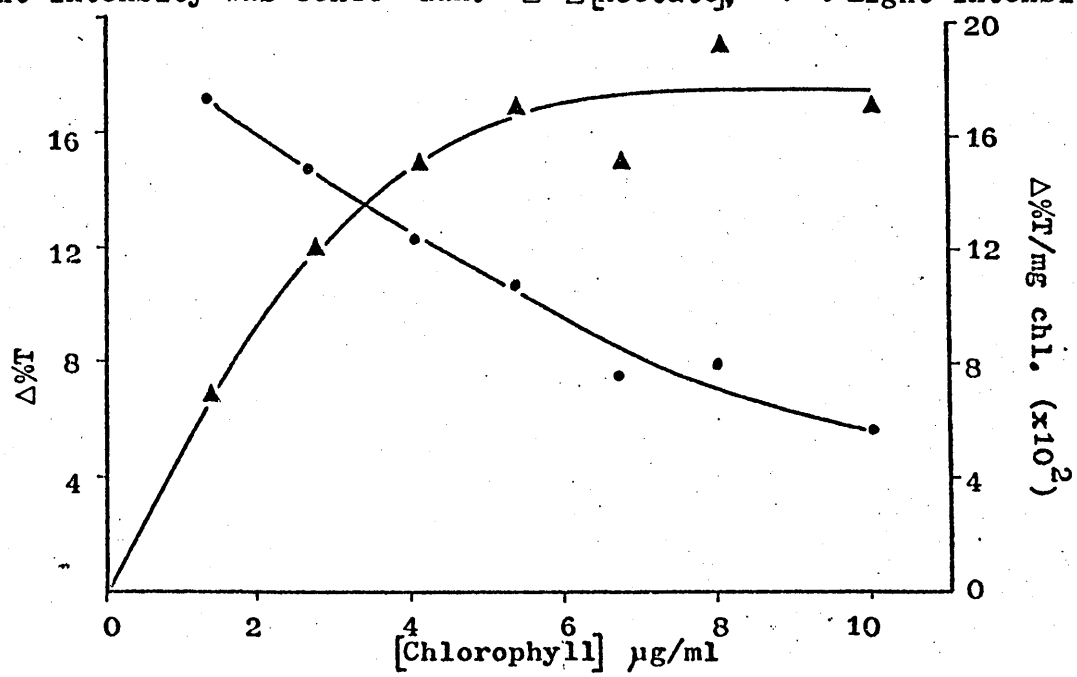


Appendix Figure 9. Colony formation on agar plates used for tetrad analysis.

Zygospores were streaked along the agar surface and single zygospores separated to the right of the line. After maturation of the zygospores, the daughter cells were separated along a line at right angle to the streak and subsequently developed into separate colonies.



Appendix Figure 10. Effect of acetate concentration and light intensity on the light-induced transmission changes of C. reinhardii (Wt^+) chloroplast fragments.
Except where indicated, the assay medium contained 100mM acetate, 20 μM PMS, 10μg/ml chlorophyll and 10mM Hepes/NaOH buffer, pH 8.0. The light intensity was $53x10^3$ lux. △—△ [Acetate], •—• Light intensity.



Appendix Figure 11. Effect of chloroplast concentration on the absolute (Δ%T) and specific (Δ%T/mg chl.) activity of the light-induced transmission changes of C. reinhardii (Wt^+) chloroplast fragments. The assay medium contained 100mM acetate, 20μM PMS, 10mM Hepes/NaOH buffer, pH8.0. The light intensity was $53x10^3$ lux.
▲—▲ Δ%T, •—• Δ%T/mg chl.

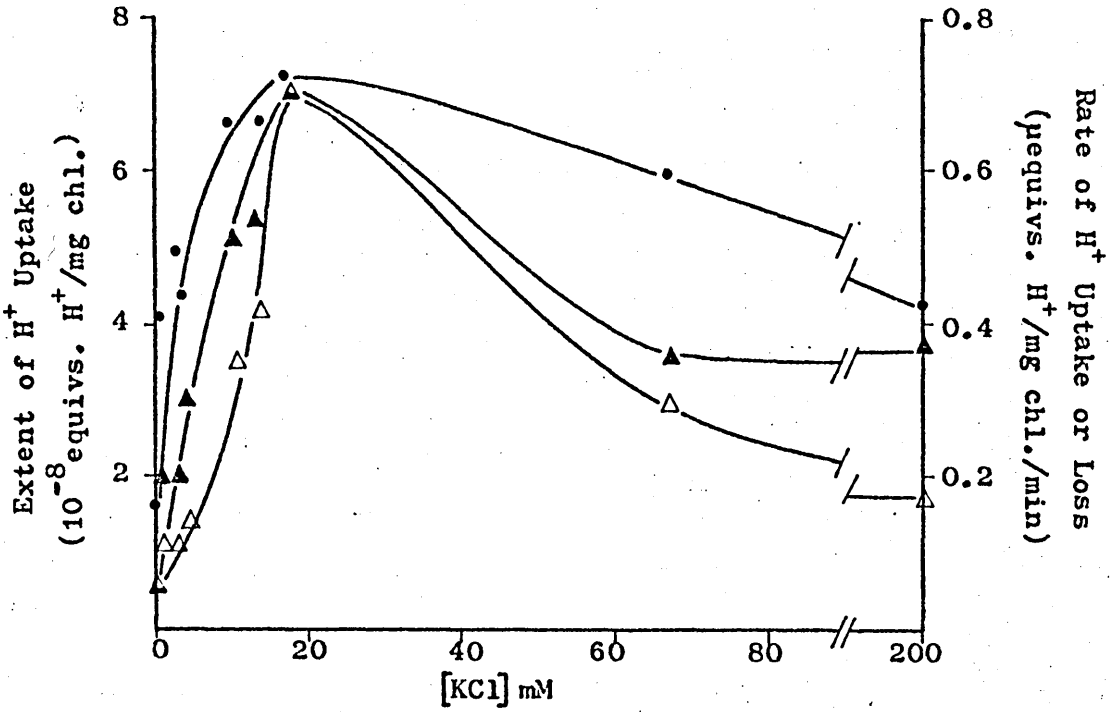
Legend to Appendix Figures 12 to 15.

Effect of various conditions on the light-induced H^+ uptake of *C. reinhardii* chloroplast fragments.

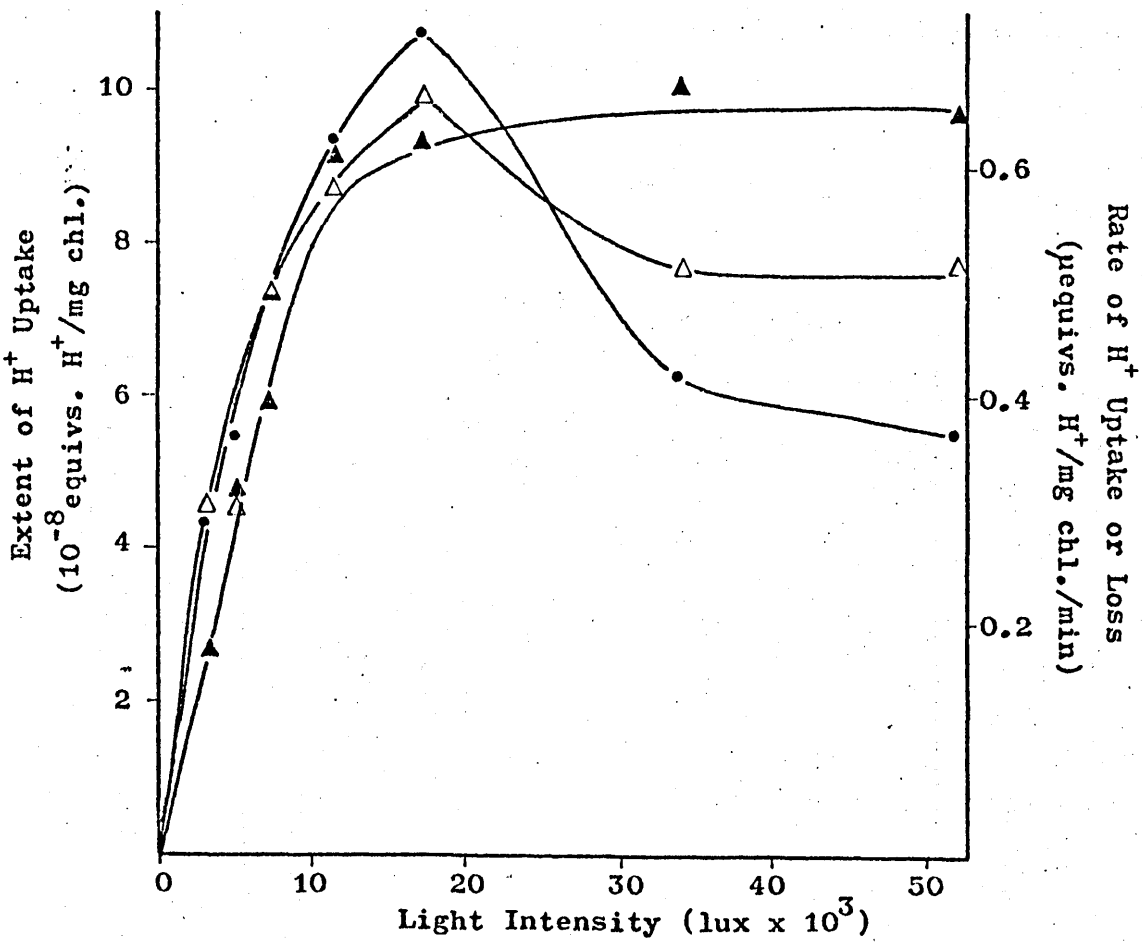
•—• Extent of H^+ uptake (ΔH^+).

△—△ Rate of H^+ uptake on illumination.

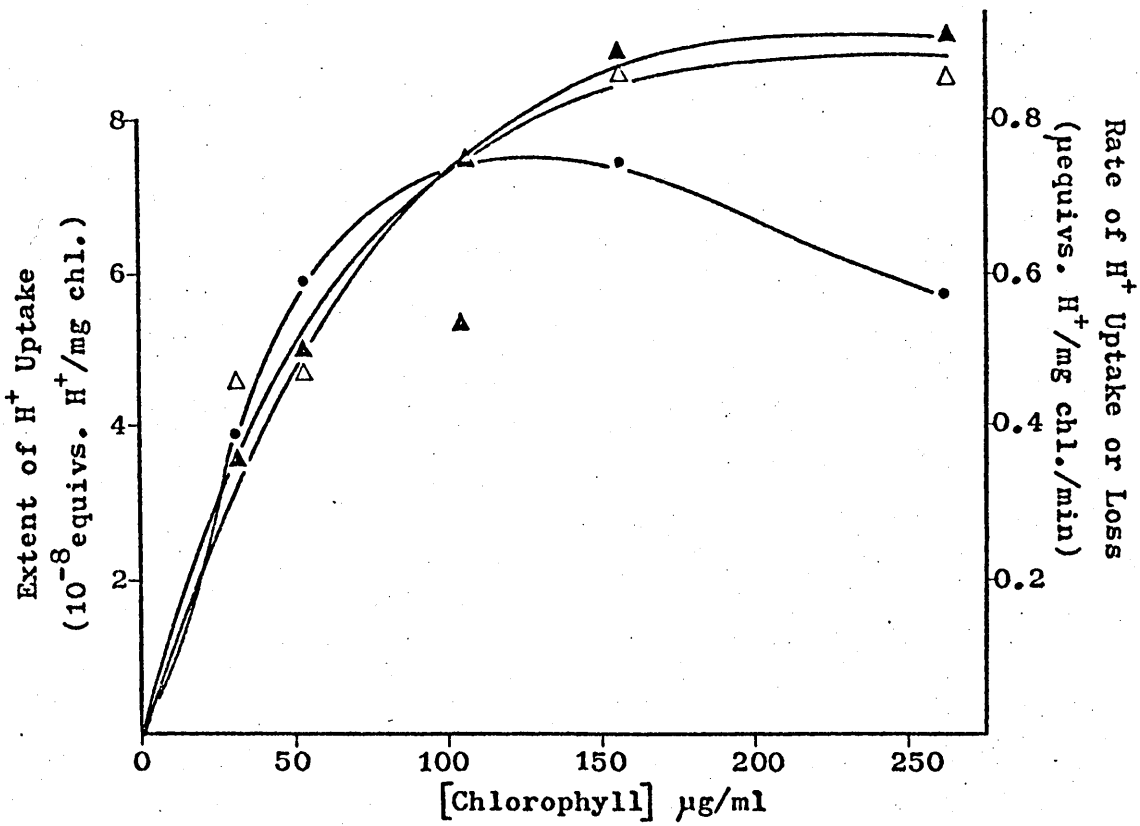
▲—▲ Rate of H^+ loss on switching off the illumination.



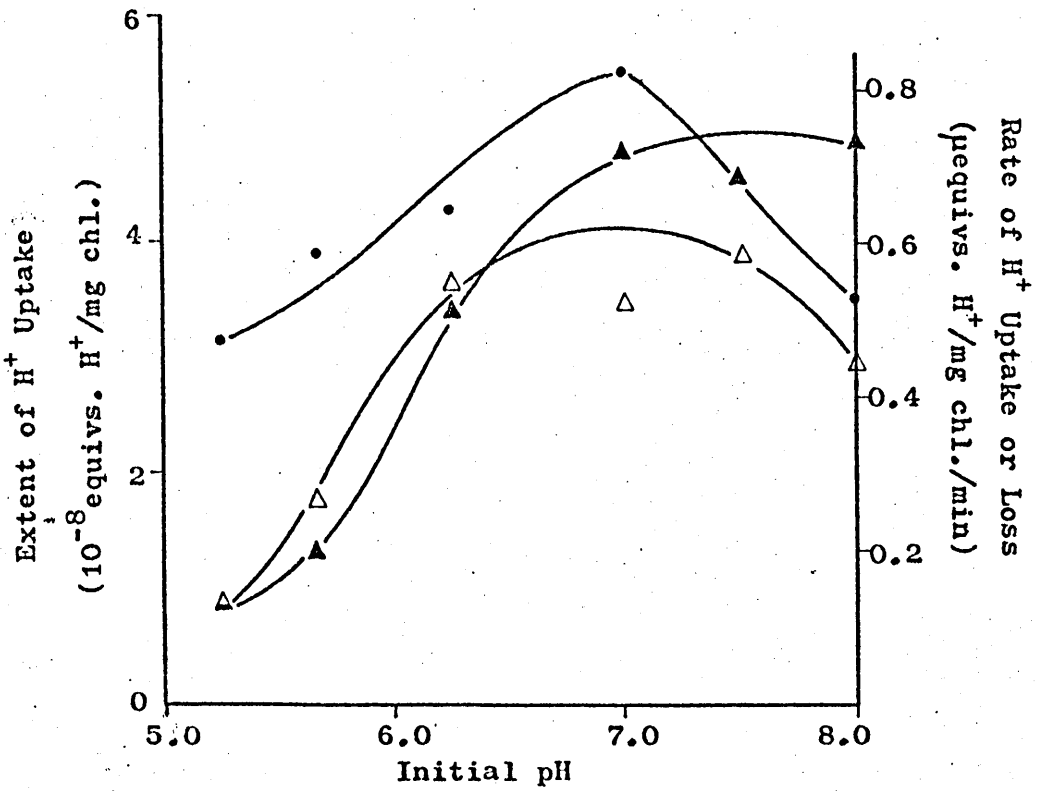
Appendix Figure 12. Effect of potassium chloride concentration on the light-induced H^+ uptake of C. reinhardii chloroplast fragments.



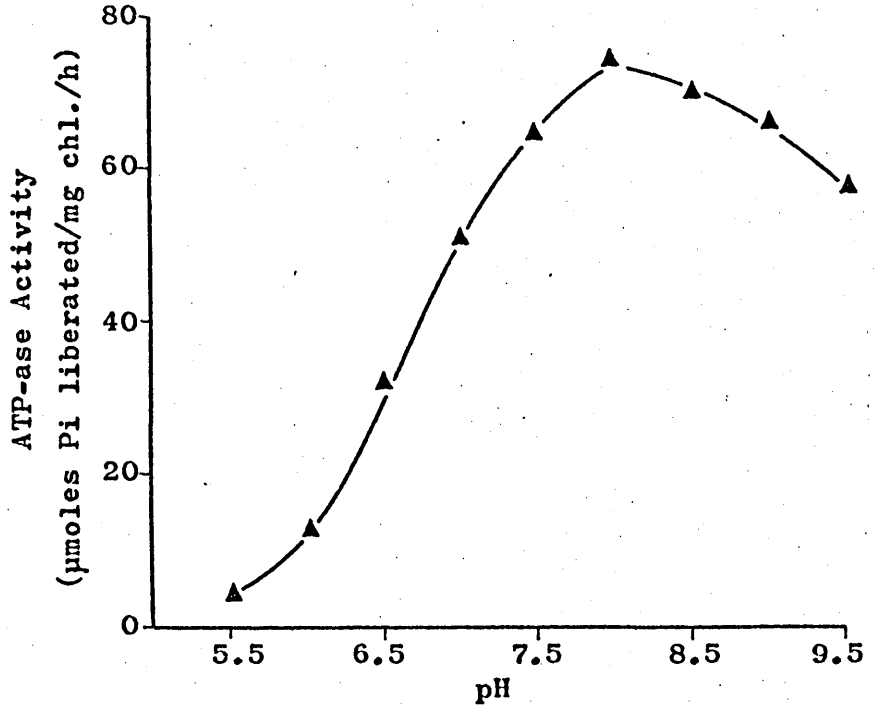
Appendix Figure 13. Effect of light intensity on the light-induced H^+ uptake of C. reinhardii chloroplast fragments.



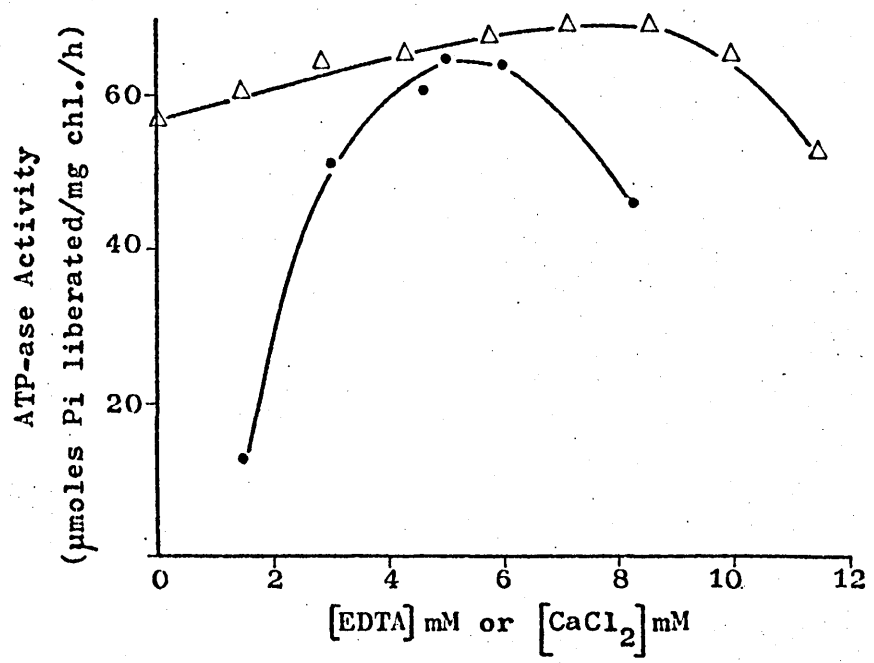
Appendix Figure 14. Effect of chloroplast concentration on the light-induced H^+ uptake of C. reinhardii chloroplast fragments.



Appendix Figure 15. Effect of initial pH on the light-induced H^+ uptake of C. reinhardii chloroplast fragments.

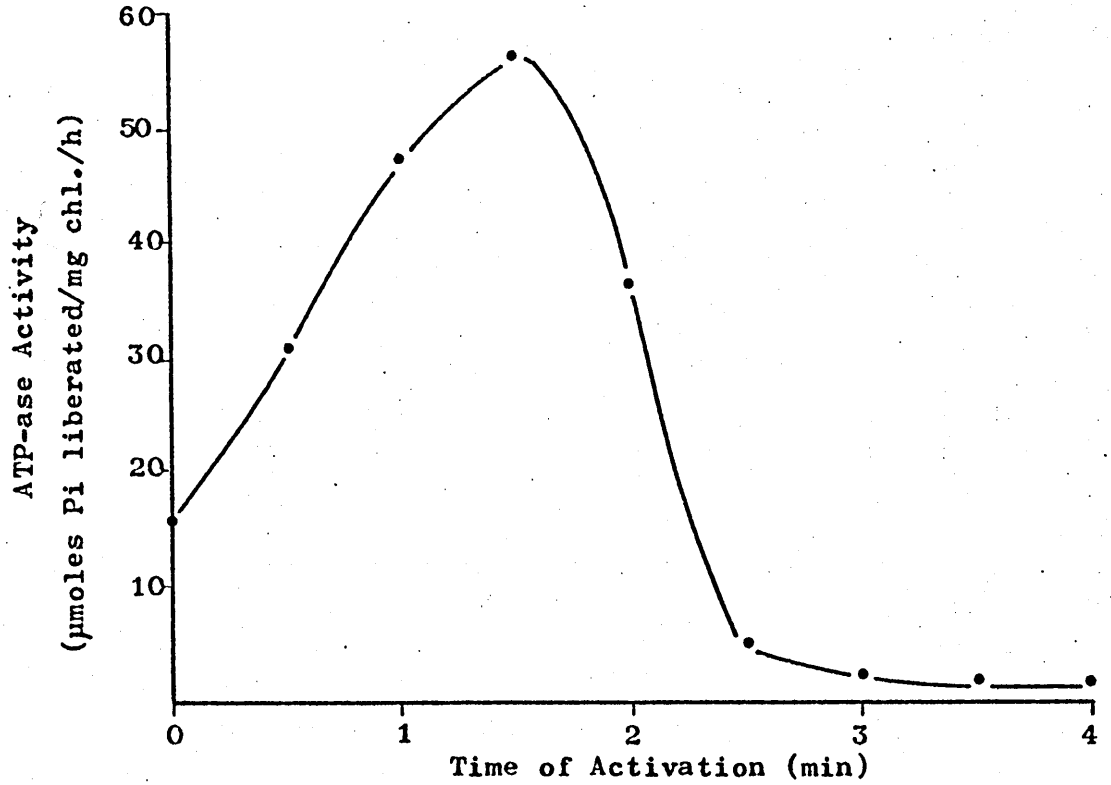


Appendix Figure 16. Effect of pH on the heat-activated, Ca^{2+}-dependent ATP-ase activity of C. reinhardii chloroplast fragments.

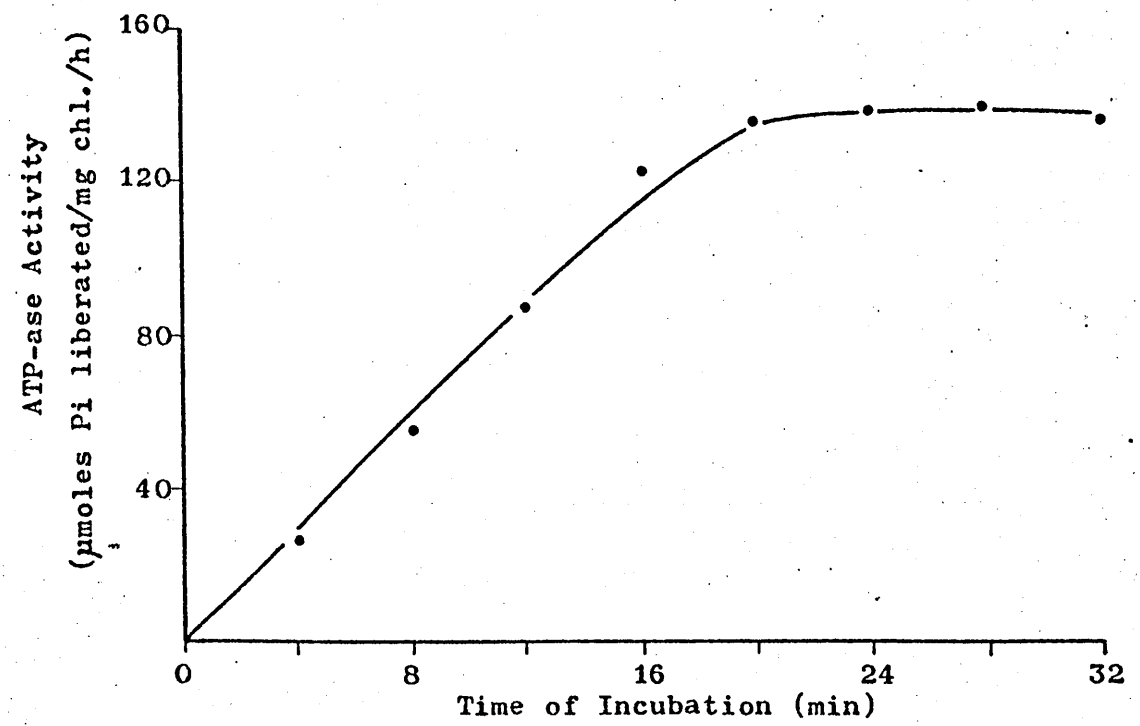


Appendix Figure 17. Effect of EDTA and calcium chloride on the heat-activated, Ca^{2+}-dependent ATP-ase activity of C. reinhardii chloroplast fragments.

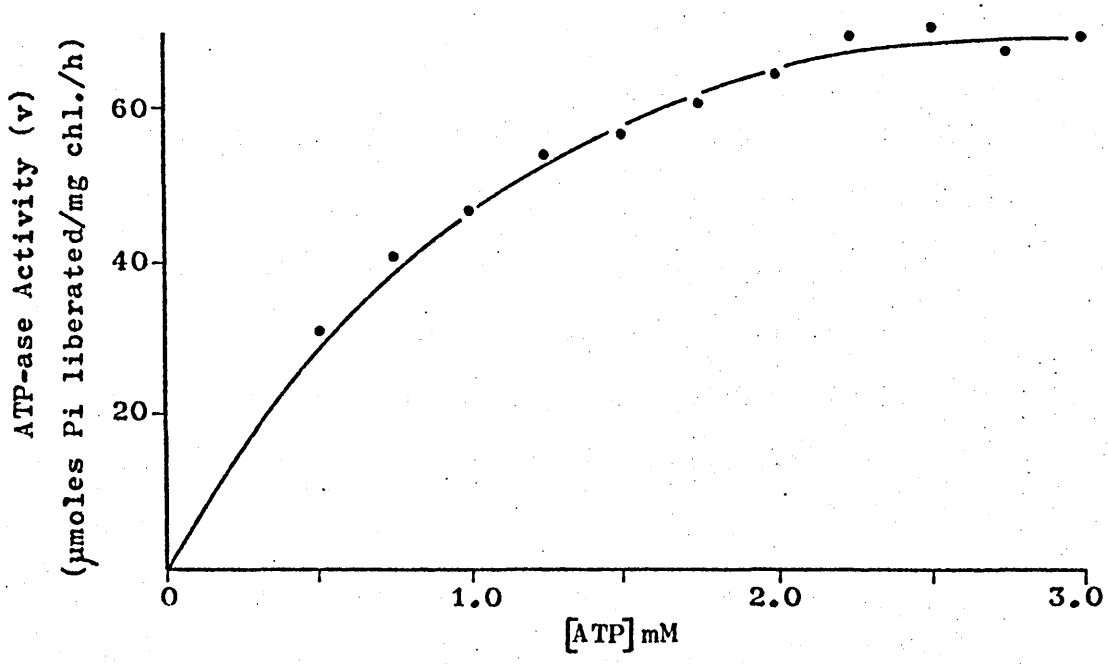
△—△ EDTA. •—• Calcium chloride.



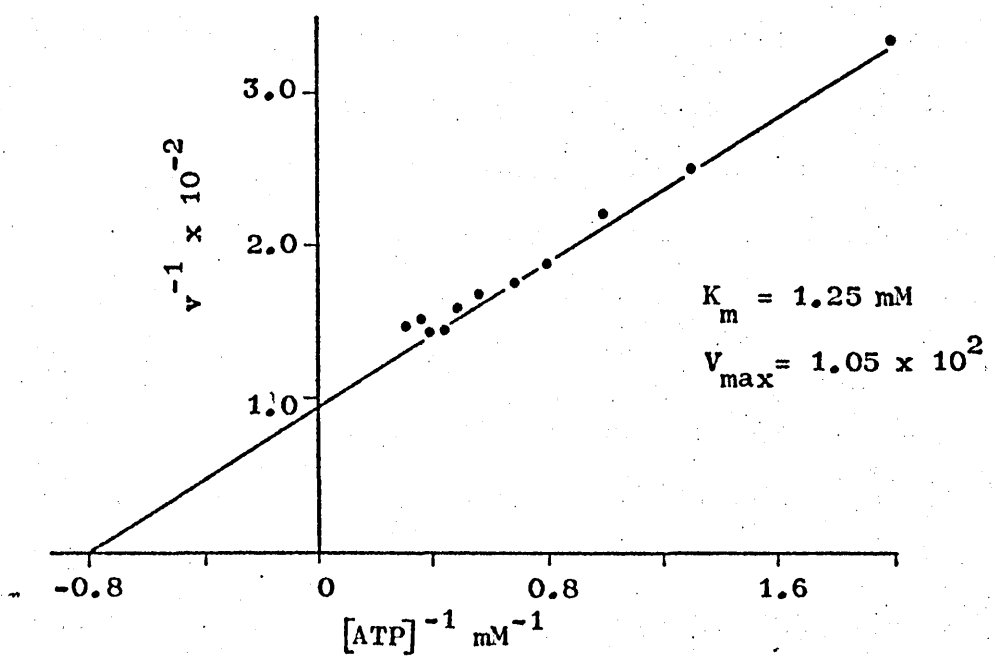
Appendix Figure 18. Effect of incubation time at 60°C on the activation of the Ca^{2+}-dependent ATP-ase activity of C. reinhardii chloroplast fragments.



Appendix Figure 19. Effect of incubation time at 36°C on the heat-activated, Ca^{2+}-dependent ATP-ase activity of C. reinhardii chloroplast fragments.



Appendix Figure 20. Effect of ATP concentration on the activity of the Ca^{2+}-dependent ATP-ase of C. reinhardii chloroplast fragments.



Appendix Figure 21. Lineweaver-Burk plot of the dependence of chloroplast ATP-ase activity on ATP concentration.

The values were obtained from Appendix Fig. 20.

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