The Effect of Osmotic Stress on Photosynthesis in the Unicellular Green Alga *Dunaliella tertiolecta*

by

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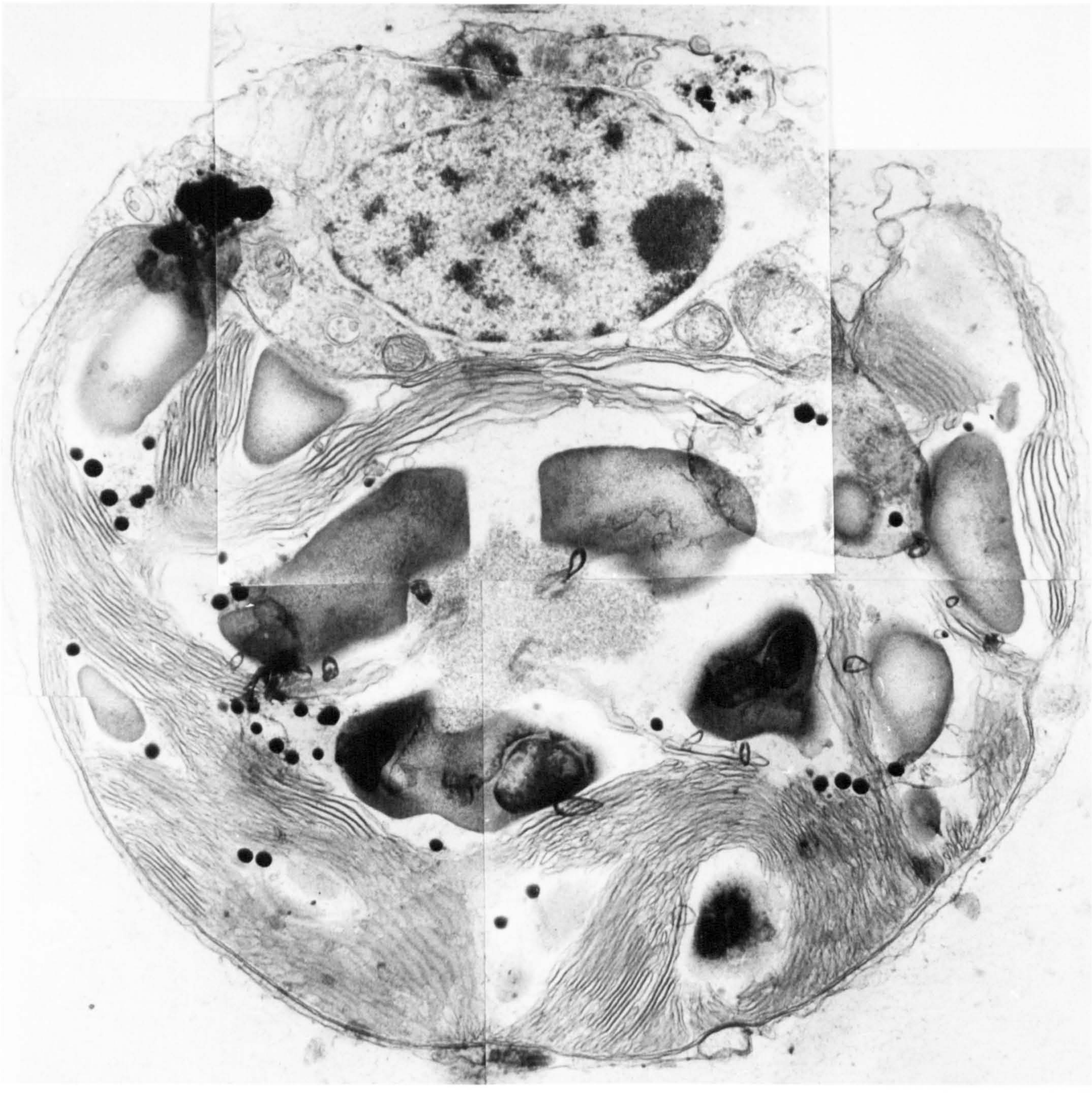
CONTAINS

PULLOUTS



FRONTISPIECE. An electron micrograph of Dunaliella tertiolecta, magnification x 36,000.

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SUMMARY

The unicellular green alga Dunaliella tertiolecta can withstand large variations in the external salinity of its growth medium from 0.09 to 1.71 M NaCl. The osmoregulatory mechanism which allows this wide adaptation to fluctuating external salinity is the production or removal of intracellular glycerol. By investigating the evolution and uptake of oxygen by the alga using an oxygen electrode, it was demonstrated that the rate of photosynthesis was greatly reduced during the first 100 min after an increase in external salinity. This was shown to be significant, since a large amount of glycerol was synthesised while the rate of photosynthesis was low. It was therefore concluded that at least in the initial stages after an increase in external NaCl, the production of glycerol must be via the degradation of starch, the storage product of D. tertiolecta.

The mechanism of the NaCl-induced inhibition of photosynthesis was studied using measurements of chlorophyll fluorescence emissions and determinations of the kinetics of the absorption change at 519 nm. These measurements indicated that at least four processes were involved with the NaCl-induced decrease in the photosynthetic rate: (a) there is an immediate increase in excitation energy going to PSI, the mechanism of the spillover of light energy from PSII to PSI can be initiated in the dark; (b) the rate of photosynthetic electron transport is slowed down; (c) the rate of the primary photochemical reactions at PSII are decreased and (d) there is an increased rate of ion flux through the thylakoid membrane which it is suggested is mediated by an influx of external Na^+ ions into the cell.

The effect of decreasing the external salinity was also investigated and it was found that the rate of photosynthesis was decreased by a similar

amount to that induced by an external salinity increase. However, it was clearly demonstrated that the mechanism of the inhibition of photosynthesis induced by decreasing the external NaCl concentration was not based on the four processes listed above, but depends on a different mechanism which is not yet understood.

Other osmotica besides NaCl were used to increase the osmolality of the growth medium. In the short term (100 min), increasing the KCl concentration induced glycerol synthesis and inhibited photosynthesis in a similar way to that caused by increasing the NaCl concentration. However, over a period of a few days high external concentrations of KCl proved fatal for the algal cells. It appears that KCl toxicity may be due to the KCl-induced loss of intracellular glycerol to the medium. Increasing the external sucrose concentration also induces glycerol synthesis by D. tertiolecta. Sucrose which exerts only an osmotic stress has a similar, but smaller inhibitory effect on the rate of photosynthesis than that caused by increasing the NaCl concentration which exerts an ionic and osmotic effect. The increase in ion flux across the thylakoid membrane can only be accelerated by increasing the concentration of external sucrose if a minimum amount of NaCl is also present. Increasing the external ethylene glycol concentration does not induce glycerol synthesis, and it does not appear to exert an osmotic stress on the cells. However, it does inhibit the overall process of photosynthesis to the same extent as sucrose, although it does not affect any of the specific photosynthetic processes studied. Increasing the external glycerol concentration appears to exert an osmotic stress since it behave in a similar fashion to sucrose as far as the inhibition of specific processes of photosynthesis is concerned. For obvious reasons this cannot be confirmed

by measuring the intracellular glycerol content in the presence of a large excess of extracellular glycerol. Nevertheless, the results present indirect evidence for the relatively low permeability of the plasmalemma of D. tertiolecta to glycerol.

Abbreviations

Wherever possible the symbols and units in this thesis conform to the recommendations of the IUPAC (SI units). Some of the other abbreviations used are:-

chl	chlorophyll
DCMU	3 - (3', 4' - dichlorophenyl) - 1, 1 - dimethylurea
FCCP	carbonyl cyanide - p - trifluoromethoxy phenyl hydrazone
Fm	maximum fluorescence
Fv	variable fluorescence
LH a/b complex	light-harvesting complex containing equimolar amounts of chlorophylls a and b
Os kg^{-1}	osmolality in osmoles per kilogram
PSI	photosystem I
PSII	photosystem II
PSU	photosynthetic unit
Q	primary electron acceptor of PSII
$\Delta \psi$	electrical gradient or membrane potential
Δ pH	proton concentration gradient
$^{o}/_{oo}$	parts of sodium chloride per thousand parts of seawater

CHAPTER 1

INTRODUCTION

1.1 General Introduction

In order to survive, plant or animal cells must be able to maintain a suitable internal environment which enables their metabolic functions to operate satisfactorily. Metabolic functions are regulated by enzymes which need a stable supply of water within the cell. If organisms are subjected to conditions whereby water is lost from their cells, their enzymes are inhibited and metabolic functions breakdown.

The methods used by plants or animals to overcome this problem can be termed osmoregulation i.e. the control of osmolality (Appendix C) within an organism. In this thesis, this term will be used to indicate a process whereby an organism regulates the amount of intracellular water in response to a change in environmental water content e.g. the addition of salt to the organisms environment would induce a water stress on the organism (by increasing the osmolality of the medium) and a consequent loss of water from its cells. Osmoregulation is the process which enables the organism to recover this water loss; it equally well applies to the cell having to excrete water in the case where the environmental salt content has become less.

The subject of this thesis is the relationship between photosynthesis and osmoregulation in the unicellular green alga Dunaliella. However, it would be useful to compare the methods of osmoregulation in higher plants (section 1.2), single-celled microorganisms (section 1.3) and algae (section 1.4) before considering osmoregulation in Dunaliella in some detail (section 1.5). A brief description of photosynthesis in the alga is given in section 1.6 and finally, the commercial

importance of Dunaliella is indicated in section 1.7.

The unusual properties of cells of Dunaliella which are involved with their ability to withstand a wide range of salinities in their growth medium have been reviewed recently by Brown and Borowitzka (1979).

1.2 Osmoregulation in Higher Plants

Osmoregulation in higher plants is a highly complex process because it involves the interaction of regulatory mechanisms at the level of the cell with those of tissues and organs (Hellebust, 1976; Wyn-Jones et al., 1977; Flowers, Troke and Yeo, 1977; Greenway and Munns, 1980). Two main sub-divisions of higher plants can be delimited with respect to osmoregulation i.e. halophytes and glycophytes.

1.2.1 Halophytes

Halophytes can be defined as plants which survive to complete their life cycle at salinities in excess of 300 mM, although they can grow (sometimes less successfully) at lower salinities. They are distinguished from glycophytes by their ability to accumulate ions to high concentrations (Flowers et al., 1977). This mechanism of ion accumulation (normally NaCl) allows the plants to increase their cellular solute concentration to match that found in their environment. Most halophytes possess special mechanisms which allow them to regulate their internal NaCl concentration; these include salt glands (which excrete concentrated salt solutions), continuous enlargement of succulent tissues and salt-accumulating epidermal bladder cells (Hellebust, 1976).

Another more general mechanism of NaCl regulation in halophytes is based on the observation that most of the ions accumulated by the plants are found in the cell vacuoles. This has been concluded from

direct measurements of the ion content in the different cell compartments, and from indirect measurements based on the inhibition of enzymes isolated from halophytes which were subjected to NaCl concentrations that would be present in the cytoplasm, if no compartmentation of ions took place (Flowers et al., 1977).

If cytoplasmic ion concentrations are low relative to those in the vacuole, some other solutes must be accumulated in the cytoplasm if it is not to lose water to the vacuole. The experimental evidence cited by Hellebust (1976) and Flowers et al. (1977) suggests that the accumulation of organic metabolites in the cytoplasm allow it to retain water. These organic compounds appear to act as compatible solutes (Aitken and Brown, 1972; Brown and Simpson, 1972). A compatible solute has two functions: firstly, it is osmotically active and therefore allows the cell (or part of the cell) to remain in osmotic equilibrium with its surroundings i.e. it prevents water loss, and secondly it protects enzyme activity against the effects of high salt concentration and low water availability (Brown, 1976 and 1978a; Brown and Borowitzka, 1979). Pollard and Wyn-Jones, (1979) have shown that whilst organic compounds such as glycinebetaine do not inhibit enzyme activity up to a concentration of 500 mM, similar concentrations of NaCl greatly reduced enzyme activity.

The types of organic compounds found in halophytes are proline, sorbitol, reducing sugars, quaternary ammonium compounds, α-amino nitrogen compounds and glycinebetaine (Ahmad, Larher and Stewart, 1979; Cavalieri and Huang, 1979; Coughlan and Wyn-Jones, 1980; Jefferies, Rudmik and Dillon, 1979; Treichel, 1979).

1.2.2 Glycophytes

Glycophytes have no specialised mechanisms for dealing with high

external salt concentrations and they can regulate their salt content only to a moderate extent by limiting ion transfer to the shoot, either by possible re-export of ions to the soil via the roots or by re-distribution of ions in different organs. The major osmotic components of glycophytes are potassium salts of organic acids and sugars with NaCl having a lesser role (Hellebust, 1976; Greenway and Munns, 1980).

1.3 Water Stress in Single-Celled Microorganisms

A brief summary of the responses of single-celled microorganisms to water stress is given here. For more detailed information, the recent reviews by Brown (1976), Gould and Measures (1977), Brown (1978a) and Kushner (1978) should be consulted.

In comparison to higher plants, the mechanisms of osmoregulation in single-celled microorganisms are simpler and therefore better understood. Most microorganisms grow optimally when water is freely available provided that nutrients are present in acceptable amounts. However, the amount of stress (due to water loss) that microorganisms can withstand varies greatly (Brown, 1976; Gould and Measures, 1977). Table 1 shows the range of salt tolerance and salt optima for representative microorganisms. This table illustrates the important distinction between halophilic and halotolerant organisms. The halobacteria and Dunaliella viridis can tolerate similar ranges of NaCl concentration, but the optimum growth of the halobacteria takes place at much higher salt concentrations than that of D. viridis. Therefore, the halobacteria are halophilic organisms whereas D. viridis is halotolerant.

1.3.1 Yeasts/Cont...

TABLE 1

Salt (NaCl) Relations of some Representative Microorganisms

(adapted from Brown, 1976)

	Salt Tolerance (M)	Salt Optimum (M)
Non-halophilic bacteria		
Enterobacteriaceae	0 - 0.7	0.2 - 0.3
Pseudomonas	0 - 0.7	0.2 - 0.3
Lactobacillus plantarum	0 - 1.6	low
Staphylococcus aureus	0 - > 3	0.2
Moderately halophilic bacteria		
Micrococcus halodenitrificans	0.2 - 4.0	1.0
Halophilic and extremely halophilic bacteria		
Ectothiorhodospira halophila	1.5 - 5.1	1.9 - 3.8
Halobacteria	2.6 - saturated	3.4 - 5.0
Marine and halophilic algae		
Dunaliella tertiolecta	0.2 - 1.5	0.2
Dunaliella viridis	1.7 - saturated	1.7
Halophilic actinomycetes		
Actinospora halophila	2 - saturated	/
Yeasts		
Debaryomyces hansenii	0 - 2.7	/

/ not determined

1.3.1 Yeasts

Another type of organism shown in Table 1 which can withstand water stress are the xerotolerant yeasts e.g. Debaryomyces hansenii (Gustafsson and Norkrans, 1976) and Saccharomyces rouxii (Gould and Measures, 1977). These yeasts accumulate glycerol as a compatible solute (section 1.2.1) in response to high salt concentrations in the medium (Edgely and Brown, 1978; Gustafsson, 1979; Adler and Gustafsson, 1980).

The osmolality (Appendix C) of the medium can be increased using NaCl or sugars, all of these compounds exert a water stress on the microorganism. However, the tolerance of an organism is not necessarily the same when stressed with either NaCl or sugars. S. rouxii can withstand relatively larger water stresses than the halobacteria if the osmolality of the medium is raised using sugars (Table 1). However, if NaCl is used to exert the water stress, S. rouxii has only the same tolerance as the non-halophilic bacterium Staphylococcus aureus shown in Table 1 (Brown, 1976).

1.3.2 Halophilic Bacteria (Table 1)

The mechanism of osmoregulation in the halophilic bacteria is based on the observation that as the level of salt is raised in the medium, the level of salt increases inside the cells. The intracellular composition of salts is unlike that in the medium, however. While the growth medium contains Na^+ in several hundredfold excess over K^+, the cells concentrate K^+ to over 2M concentration, and the intracellular K^+ concentration is several times the Na^+ concentration (Lanyi, 1974). In contrast to the situation in halophilic higher plants (section 1.2.1), there is no possibility of keeping the ions in a separate cell compartment

away from the site of metabolic activity. Thus, the enzymes of halophilic bacteria must be able to function in the presence of a high concentration of salt. In fact, many of the enzymes isolated from the halobacteria require a high concentration of salt ($\sim$ 2M) for optimum activity (Larsen, 1962; Lanyi, 1974).

1.3.3 Non-halophilic Bacteria

Table 1 shows that the non-halophilic bacteria differ greatly in their tolerance to NaCl in the growth medium. However, an increase in the salt concentration of the growth medium induces a characteristic increase in the levels of particular pool amino acids e.g. glutamic acid, γ-aminobutyric acid and proline. It has been shown that during osmotic stress the fundamental reaction is an increase in pool glutamate (Measures, 1975). In halophilic bacteria, K^+ is accumulated in response to an increase in the salt concentration of the growth medium (section 1.3.2), whilst in non-halophilic bacteria K^+ accumulation depends on the amino acid involved. In the cases where glutamic acid is accumulated, K^+ must also be accumulated to maintain electrical neutrality, because glutamic acid is charged at physiological pH's. However, in the case of proline accumulation there is no concomitant rise in K^+, because proline is uncharged at physiological pH's (Measures, 1975).

1.4 Osmoregulation in Algae

This subject has been reviewed recently by Cram (1975), Hellebust (1976), Zimmermann (1978), Kauss (1979) and Raven, Smith and Smith (1980).

Algae are found in habitats of widely different osmolalities (Appendix C), and have accordingly evolved osmoregulatory mechanisms to deal with environments which are either very saline, very dry, subject

to rapidly fluctuating salinities, or of very low salt content (Hellebust, 1976). Algae can broadly by split into three groups depending on how they osmoregulate: (a) coenocytic algae with large vacuoles e.g. Valonia, use inorganic ions as their osmotic solutes, whereas (b) smaller microbial algae e.g. Dunaliella, employ organic molecules (compatible solutes - section 1.2.1) as their osmotic solutes and (c) multicellular algae with both vacuolate and relatively less vacuolate cells, which use both inorganic and organic solutes e.g. Griffithsia (Bisson and Kirst, 1979). It is now believed, however, that both types of regulation are present in every osmoregulating plant system, but that their respective (macroscopically dominating) contribution to the osmotic relations of the whole cell depends on the relative dimensions of the vacuole with respect to the cytoplasmic compartment (Zimmermann, 1978).

The algal cell parameter controlled by the accumulation of inorganic or organic solutes is not always the same. In algae with rigid cell walls the parameter controlled is the turgor pressure, whereas in wall-less cells it is the volume (Bisson, 1982).

1.4.1 Coenocytic Algae

This rather homogeneous group includes the coenocytic algae e.g. Valonia, plus the species with giant cells e.g. Hydrodictyon. Table 2 shows that algae in this group have different concentrations of Na^+, K^+ and Cl^- in their vacuolar sap, depending on whether they grow in freshwater or seawater. That osmoregulation takes place by changing the inorganic ion content of the vacuole has been demonstrated in Codium decorticatum (Bisson and Gutknecht, 1975), Lamprothamnium (Bisson and Kirst, 1980a and b) and Valonia macrophysa (Hastings and Gutknecht, 1974 and 1976). In Valonia utricularis, Kirst (1980a) found that in addition to the regulation of the ion content, sucrose was accumulated as the external osmolality was increased.

TABLE 2

Concentrations of the Major Ions in the Vacuolar Sap of Some Coenocytic and Giant-Celled Algae and in the Water Surrounding Them (from Kauss, 1979a)

Species	Ion Concentration (mM) Na^+	K^+	Cl^-	Turgor (bar)	Reference
FRESHWATER					
Freshwater*	1.2	0.07	1.2	/	
Chara corallina	47	74	106	6	(a)
Nitella flexilis	27	80	135	6	(b)
Hydrodictyon africanum	17	40	38	?	(c)
MARINE					
Seawater	485	11	590	/	(d)
Valonia ventricosa	44	625	643	2	(d)
Codium decorticatum+	257	295	454	2.3	(e)
Halicystis parvula	415	9	579	0.5	(f)

*Mean value from references (a), (b) and (c).

+Vacuolar sap plus cytoplasmic solutes.

References

(a) Hope and Walker, 1975

(b) Kishimoto and Tazawa, 1965

(c) Raven, 1967

(d) Gutknecht and Dainty, 1968 and Gutknecht, 1968

(e) Bisson and Gutknecht, 1975 and 1977

(f) Greaves and Gutknecht, 1976

The author suggested that the sucrose was most likely located in and limited to the cytoplasm, which would be similar to the situation proposed for halophilic higher plants (section 1.2.1).

1.4.2 Multicellular Algae

In this category of algae, many of the species are known to use both organic compounds and inorganic ions as a means of osmoregulation (Table 3). Wiencke and Lauchli (1981) found that K^+, Cl^-, floridoside (O - α - D - galactopyranosyl - (1 → 2) - glycerol) and isofloridoside (O - α - D - galactopyranosyl - (1 → 1) - glycerol) are the main osmotically active solutes in Porphyra umbilicalis. They concluded that floridoside and isofloridoside are probably loacted in the cytoplasm, whereas Cl^- may be mainly localised in the vacuoles. Localisation of K^+ varied with the osmolality of the external medium. The function of the organic compounds in P. umbilicalis is likely to be that of compatible solutes (section 1.2.1). Table 3 shows that the galactosylglycerols, floridoside and isofloridoside play an important role in osmoregulation in red algae. However, Kremer (1979) found no evidence of their involvement in the osmoregulation of the red algae Porphyra, Chondrus, Iridea, Dumontia or Rhodymenia.

1.4.3 Microbial Algae

Table 4 lists the microbial algae which are known to regulate their internal osmolality using organic solutes. Some of them also regulate their ion content. The typical sequence of events is that after an increase in the external NaCl concentration, inorganic ions (often K^+) enter the cell to restore the osmotic balance; this usually takes less than 15 - 20 min. Then over a longer time period (perhaps a few hours)

TABLE 3

Solutes Involved in Osmoregulation in Multicellular Algae

Rhodophyta	
Polysiphonia lanosa	K^+ and dimethylsulphoniopropionate (Reed, 1982)
Griffithsia monilis	K^+, Na^+, Cl^- and digeneaside (Bisson and Kirst, 1979)
Iridophycus flaccidum	floridoside (Kauss, 1968)
Porphyra perforata	K^+ (Eppley, 1958), floridoside and isofloridoside (Kauss, 1968)
Porphyra purpurea	floridoside (Reed, Collins and Russell, 1980) and KCl (Reed, Collins and Russell, 1981)
Porphyra umbilicalis	K^+, Cl^-, floridoside and isofloridoside (Wiencke and Lauchli, 1980 and 1981)
Chlorophyta	
Ulva lactuca	K^+, Cl^- and β-dimethylsulphoniopropionate (Dickson, Wyn-Jones and Davenport, 1980)
Ulothrix fimbriata	sucrose and glutamic acid (Brown and Hellebust, 1980)
Enteromorpha intestinalis	sucrose (Brown and Hellebust, 1980)
Phaeophyta	
Pilayella littoralis	mannitol (Reed, 1980)
Ascophyllum nodosum	mannitol + salts (Munda, 1967)
Fucus spp.	mannitol (Munda, 1967).

digeneaside is 2 - D - glyceric acid - α - D - mannopyranoside

floridoside is O - α - D - galactopyranosyl - (1 → 2) - glycerol

isofloridoside is O - α - D - galactopyranosyl - (1 → 1) - glycerol.

TABLE 4

Microbial Algae which Respond to Changes in the Osmolality of the Medium by Altering their Internal Concentration of Organic Solutes

ORGANISM	MAJOR ORGANIC SUBSTANCE(S) WHOSE CONCENTRATION ALTERS	SOLUTES WHOSE CONCENTRATION CHANGES IN ADDITION	REFERENCES
Chlorophyceae:			
Asteromonas gracilis	glycerol	/	Ben-Amotz and Grunwald (1981)
Brachiomonas submarina	glycerol	amino acids	Hellebust (1982)
Chlamydomonas pulsatilla	glycerol	amino acids	Hellebust (1982)
Chlorella emersonii	proline	sucrose and Na^+	Setter and Greenway (1979) Greenway and Setter (1979a and b
Chlorella pyrenoidosa	sucrose	/	Hiller and Greenway (1968)
Chlorella salina	proline	K^+	Kirst (1977c)
Dunaliella parva	glycerol	/	Ben-Amotz and Avron (1973a)
Dunaliella tertiolecta	glycerol	sucrose	Frank and Wegmann (1974)
Dunaliella viridis	glycerol	K^+	Borowitzka and Brown (1974)
Hyalococcus dermatocarponis	sucrose and proline	sorbitol	Brown and Hellebust (1980a)
Klebsormidium flaccidum	sucrose	glutamic acid and aspartic acid	Brown and Hellebust (1980a)
Prasinophyceae:			
Platymonas subcordiformis	mannitol	K^+, Na^+ and Cl^-	Kirst (1975, 1977a, 1977c and 1979)
Platymonas suecica	mannitol	/	Hellebust (1976)
Chrysophyceae:			
Poterioochromonas malhamensis	isofloridoside	amino acids + K^+	Kauss (1979a)
Bacillariophyceae:			
Cyclotella cryptica	proline	K^+ and other amino acids	Liu and Hellebust (1976b and c)
Cylindrotheca fusiformis	mannose	/	Paul (1979)
Phaeodactylum tricornutum	proline	other amino acids	Besnier et al. (1969) Schobert (1977 and 1980)
Prymnesiophyceae:			
Pavlova lutheri	cyclohexanetetrol	/	Craigie (1969)
Cyanophyceae:			
Aphanothece halophytica	carbohydrates, polyols and amino acids	K^+	Miller et al. (1976) Yopp, Miller and Tindall (1978)
Synechococcus sp.	glucopyranosylglycerol	/	Borowitzka et al. (1980)

organic molecules are synthesised and the internal ion content decreases back to normal. This basic type of osmoregulation has been demonstrated in Platymonas subcordiformis (Kirst, 1979) and Aphanothece halophytica (Yopp, Miller and Tindall, 1978). It also occurs in Chlorella emersonii, although in this case the initial osmotic balance is restored by sucrose instead of an inorganic ion (Greenway and Setter, 1979b). It seems likely that a similar mechanism takes place in the other algae in Table 4, but has not yet been demonstrated.

Table 4 also illustrates three other important points: (a) taxonomically speaking, a large variety of algae use organic solutes for osmoregulation, (b) the range of organic solutes is relatively small i.e. mainly amino acids, sugar alcohols and polyols, and (c) only a small proportion of algae have been studied in such a way as to reveal their method of osmoregulation.

The two algae which have been most extensively studied are Dunaliella and Poterioochromonas: Dunaliella will be discussed in some detail in section 1.5, but to conclude this section a brief account will be given of osmoregulation in Poterioochromonas.

1.4.4 Osmoregulation in Poterioochromonas

Poterioochromonas is a genus of freshwater flagellates whose cells are unwalled. One species of the genus, P. malhamensis has been extensively studied by Kauss and co-workers (Kauss, 1979a). An increase in the osmolality of the external medium causes the cells to shrink, however, P. malhamensis can recover its original cell volume by increasing the internal concentration of isofloridoside (O - α - D - galactopyranosyl - (1 → 1) - glycerol) (Kauss, 1967a and b). Isofloridoside production accounted for about 70 - 80% of the observed volume regained by the cells

after stress. The remaining 20 - 30% is due to an increase in the level of free amino acids (mainly alanine) and K^+ plus its unknown counterion in about equal proportions (Kauss, Luttge and Krichbaum, 1975). The relative contribution of the three different substances used for osmotic regulation in Poterioochromonas does not appear to be constant, but subject to some variation, although isofloridoside was always found to be the major constituent changing under stress conditions. After the addition of an osmotically active substance to the medium, there is no indication that an increase in the internal K^+ concentration takes place during the initial recovery period, as it does in some other algae (section 1.4.3). This is probably due to the fact that there is only a 1 - 3 min. delay in initiating isofloridoside production after the onset of the osmotic stress (Kauss, 1979a).

Biochemical studies on osmotic regulation were therefore mainly performed on the mechanisms involved in isofloridoside metabolism. The speed of the onset of isofloridoside production and the fact that inhibitors of protein synthesis do not influence the rate of isofloridoside formation indicate that regulation of the isofloridoside-pathway does not involve the de novo synthesis of enzymes, but occurs instead by activation of pre-existing enzymes (Kauss, 1967a; Schobert, Untner and Kauss, 1972). Figure 1 shows that the various studies using both physiological and biochemical methods have allowed the formulation of a hypothetical pathway of isofloridoside formation (Kauss 1979a; Kauss and Thomson, 1982). The key enzyme of the special isofloridoside pathway is a galactosyltransferase able to transfer a galactosyl unit from UDP-galactose to sn-glycerol-3-phosphate. The most recent research on Poterioochromonas has concentrated on identifying the signal which triggers the production or degradation of isofloridoside. Among the hypothetical possibilities were pH-shifts or such amplifying substances as cyclic AMP or Ca^{++}

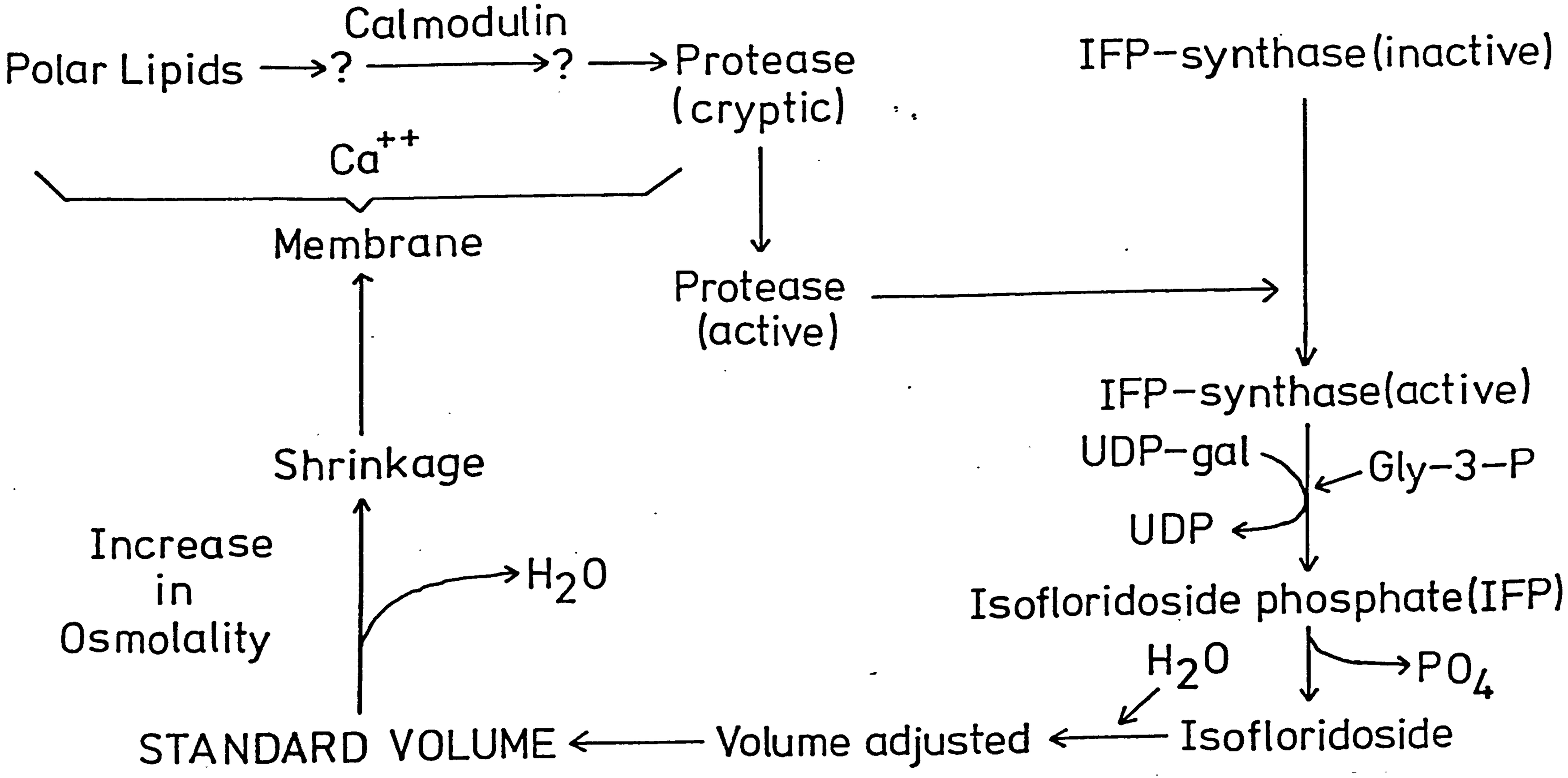


FIGURE 1. The proposed pathway of isofloridoside formation in Poterioochromonas (adapted from Kauss and Thomson, 1982).

(Kauss, 1979b). As Figure 1 shows, it now seems that the sensing of volume changes by the alga involves a Ca^{2+}-regulated system which controls the activation of isofloridoside-phosphate synthase via a protease (Kauss, 1981; Spang, Claude and Kauss, 1981; Kauss and Thomson, 1982).

1.5 Osmoregulation in Dunaliella

It was known as long ago as 1936 that the genus of green algae Dunaliella was very unusual: in this year their presence was noted in the Dead Sea in Israel, which previous to this had been thought to be lifeless (Wilkansky, 1936). The alga is described as closely resembling Chlamydomonas, except in the nature of the cell envelope: like Poterioochromonas (section 1.4.4), Dunaliella has no cell wall. Species of Dunaliella are found worldwide and have been recorded in salt marshes, inland salt springs, lagoons and saline pools (Butcher, 1959). A full morphological description of D. tertiolecta is given in section 2.1.

Dunaliella spp. are most often found either in conditions of high salt e.g. the Dead Sea or in conditions of fluctuating salt content e.g. the intertidal zone of the shore line. Thus, for sometime now, Dunaliella has been known to be unusually euryhaline, that is to say the cells are able to tolerate a wide variation in the osmolality of their environment. Dunaliella can thrive in media containing from 0.1M NaCl to saturated salt concentrations (Ben-Amotz and Avron, 1981).

1.5.1 Mechanism of Osmoregulation

The first indication as to how this euryhalinity is achieved by Dunaliella was provided by Craigie and McLachlan (1964). They described the photosynthetic production of glycerol as the major soluble carbohydrate in D. tertiolecta under stressed and non-stressed conditions. Their data

also showed that as the salinity increased, glycerol production and the photosynthetic rate increased as well.

Prior to this observation and for some years after, the osmotic balance of Dunaliella was thought to be mediated by high salt concentration inside the cell in a similar method to that employed by halophilic bacteria (section 1.3.2) (Marre and Servattaz, 1959; Trezzi, Galli and Bellini, 1965; Ginzburg, 1969). Various attempts to determine the intracellular concentration of NaCl did not yield a conclusive result. However, this idea was largely discredited when Johnson et al. (1968) showed that enzymes isolated from Dunaliella would not catalyse their reactions in the presence of high concentrations of salt, but would do so in the presence of high concentrations of glycerol. Thus, this indirect experiment indicated the advantages of a high level of internal glycerol in response to an increase in salinity as had been found by Craigie and McLachlan (1964).

During the late 1960's and throughout the 1970's, attempts were made to elucidate the process by which Dunaliella produces glycerol in response to a salinity increase, and also how it removes glycerol from the cell in response to a salinity decrease. Wegmann (1969 and 1971) showed the influence of osmotically active substances on photosynthetic glycerol production and on gas exchange in D. tertiolecta. He looked at the percentage of ^{14}C incorporated into glycerol over the range of 0.17 - 4M NaCl and found that it increased to a distinct maximum at 2.8M NaCl and then declined. Increasing the concentration of osmotic substances produced oxygen consumption, accompanied by CO_2 evolution. These processes also showed distinct maxima at 2.8M NaCl. The respiratory quotient showed that a fermentation process occurred at higher salinities. Wegmann suggested that glycerol formation was a protective mechanism for the survival of Dunaliella in its natural habitat, i.e. glycerol could

prevent structural damage when cells were dried. He did not believe, however, that glycerol formation was an osmoregulatory mechanism as it was partly excreted or lost into the medium.

Ben-Amotz and Avron (1972) showed that enzymic and photosynthetic reactions of cell-free preparations from D. parva were inhibited rather than stimulated by the salt concentration optimal for growth. This observation agreed with the findings of Johnson et al. (1968) using D. viridis. Therefore, the cells must maintain a considerably lower salt concentration internally than that present in the surrounding medium.

Ben-Amotz and Avron (1973a) demonstrated very clearly that when the extracellular salt concentration of the algal suspension was increased or decreased, the intracellular glycerol level varied accordingly. As no leakage of intracellular glycerol was observed above 0.6M NaCl, these alterations in glycerol content were interpreted as due to the metabolic formation and degradation of intracellular glycerol. Ben-Amotz and Avron claimed that these results indicated the existence of a new-type of algal osmoregulation, in which the osmotic balance depends on the synthesis and degradation of intracellular glycerol in response to the external salt concentration. Table 4 lists other algae which use a similar method of osmoregulation.

The conclusions of Ben-Amotz and Avron (1973a) were largely supported by the work of Borowitzka and Brown (1974), Borowitzka, Kessly and Brown (1977) and Gimmler and Schirling (1978). The work of Heimer (1973) and Borowitzka and Brown (1974) confirmed that enzymes isolated from D. parva, D. tertiolecta and D. viridis were inhibited by NaCl solutions normally encountered in vivo. They were not inhibited by glycerol concentrations until very high molarities of glycerol were reached i.e. approximately 4 - 5M. Borowitzka and Brown (1974) used the term compatible solute (section 1.2.1) to describe the role of glycerol in Dunaliella, since

they believed that although intracellular glycerol must necessarily contribute to the osmotic status of the algae, its primary function in influencing their salt relations is considered to be in maintaining enzyme activity under conditions of high extracellular salt concentration and hence low (thermodynamic) water activity. In fact, they found that the presence of glycerol partially protected the activity of enzymes isolated from Dunaliella, from inhibition by high salt concentrations. Schobert (1977a) suggested that various polyols (e.g. glycerol) and the imino acid proline can allow complete hydration of biopolymers (which include enzymes) to be maintained even with a reduced number of available water molecules. The polyols can replace water molecules by means of their water like hydroxyl-groups and proline is postulated to associate via its hydrophobic part with hydrophobic side chains of biopolymers, thereby converting them into hydrophilic groups which bind water molecules using hydrogen bonding.

Borowitzka et al. (1977) showed that glycerol could be accumulated in the dark in response to a salinity increase and also that glycerol was produced in response to an increase in the extracellular sucrose concentration as well as the extracellular NaCl concentration.

Gimmler and Schirling (1978) showed that, in contrast to earlier assumptions, both glycerol and Na^+ contribute to the compensation of the high external osmolality induced by concentrated salt media. Latorella and Vadas (1973) found evidence for a light driven H^+/Na^+ exchange which allowed the cells to grow in high salinities. Muller and Wegmann (1978) suggested that sucrose may play an important role in osmoregulation in D. tertiolecta above 298K. These authors are not suggesting that glycerol is not important in Dunaliella, but that it is perhaps not as important as first thought. Jones and Galloway (1979) found that D. tertiolecta expends energy producing an excess amount of glycerol over that

required for osmoregulation, leading to a reduction in the growth rate for the organism. On the basis of these results, it appears that the osmoregulatory mechanism may not be the simple scheme of balancing external osmolality with internal accumulation of glycerol, as originally envisioned (Ben-Amotz and Avron, 1973a).

1.5.2 Hypothetical Mechanisms for Glycerol Production in Dunaliella

There is little doubt that the main substance involved in osmoregulation in Dunaliella is glycerol. Gimmler, Schirling and Tobler (1977) have suggested that the Dunaliella cell undergoes a three stage process when the salinity is changed (section 1.4.3) e.g. for a salinity increase the following is seen:-

(a) the cell immediately shrinks, due to the rapid loss of water.

(b) an equilibrium of external and internal osmolality is reached after 2 minutes, during this stage the cell behaves as a perfect osmometer.

(c) during the next 15 - 180 minutes, the cell returns to approximately its original volume by the synthesis of glycerol.

As already stated, it is generally believed that glycerol production in response to increasing salinity can take place in the light or the dark (Ben-Amotz and Avron, 1973a; Ben-Amotz, 1974; Ben-Amotz, 1975; Borowitzka et al., 1977). However, Frank and Wegmann (1974) found no glycerol formation in D. tertiolecta in the dark after a large increase in salinity, but this observation has not been confirmed.

Therefore, it has been hypothesised that two different metabolic pathways may be responsible for glycerol formation: one using photosynthetic products and the other via the metabolic degradation of starch, the storage product in Dunaliella (Ben-Amotz, 1975). Figure 2

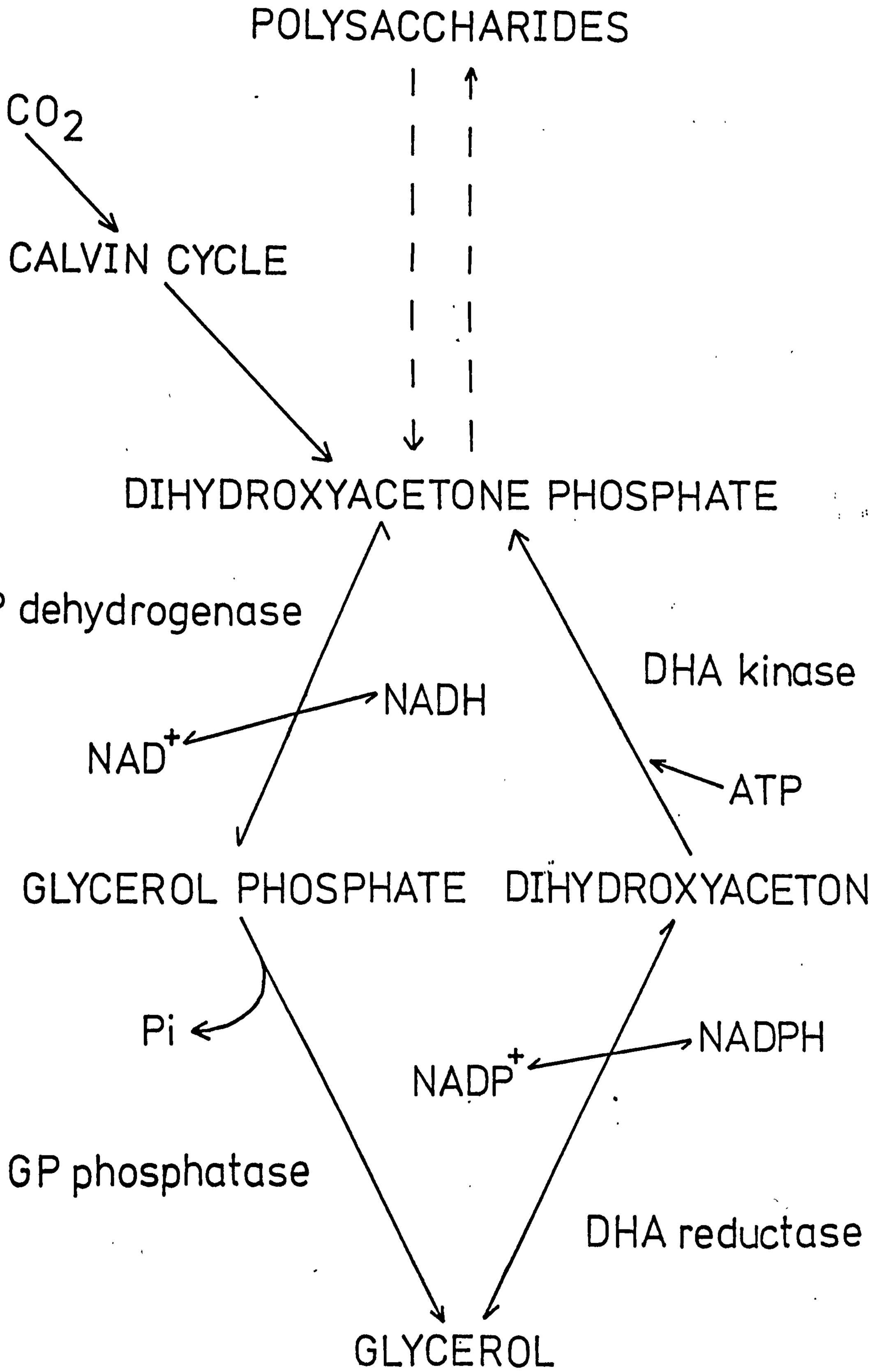


FIGURE 2. A hypothetical pathway of glycerol synthesis and removal in Dunaliella (adapted from Ben-Amotz et. al., 1982).

illustrates a proposed scheme of glycerol synthesis in Dunaliella (Ben-Amotz and Avron, 1981; Ben-Amotz, Sussman and Avron, 1982). Dihydroxyacetone phosphate (DHAP) derived from the Calvin cycle during photosynthesis or from the degradation of starch is hydrolysed to glycerol phosphate which is then converted to glycerol. At very high concentrations of intracellular glycerol and when the cells are exposed to hypotonic conditions, glycerol can be converted back to DHAP via an intermediate compound dihydroxyacetone (DHA) (Figure 2). Three enzymes have been isolated from Dunaliella which could take part in this hypothetical scheme of events: DHA reductase (Ben-Amotz and Avron, 1973b; Ben-Amotz and Avron, 1974; Borowitzka and Brown, 1974; Borowitzka et al., 1977), DHA kinase (Lerner and Avron, 1977; Lerner, Sussman and Avron, 1980) and glycerol-1-phosphatase (Sussman and Avron, 1981). The same enzymes were found in another halotolerant alga which osmoregulates with glycerol, Aster omonas gracilis (Table 4) (Ben-Amotz and Avron, 1980; Ben-Amotz et al., 1982).

The method by which these enzymes are regulated by external salinity changes is not known. However, there are some theories as to how it might take place. Wegmann (1978) suggested that as osmotic stress removes water from the chloroplast, the chloroplast shrinks, leading to an increase in the concentration of the chloroplast contents. The activation of an inactive enzyme precursor could be induced by the concentration increase of the protein itself, an ion or an organic intermediate, thus starting glycerol synthesis which increases the osmolality within the chloroplast. The chloroplast then regains its original size, and the activation of glycerol synthesis is turned off. Experiments concerning osmotically induced membrane alterations suggest an activation mechanism connected to the chloroplast envelope, which is not yet understood (Wegmann, 1978).

Recently, Gimmler, Wiedemann and Moller (1981) have observed a NaCl-induced decrease in starch synthesis in the light. They suggested that this was due to the increase of the endogenous inorganic phosphate level (resulting from enhanced glycerol synthesis (Figure 2) and ATP consumption) together with the decrease of the phosphoglyceric acid pool. The decrease of the ATP-level and the phosphorylation potential (which certainly have to be elaborated more quantitatively as they vary with the experimental conditions) may be a primary signal in the osmoregulation of D. parva and one primary sensing site may be the transport ATPase at the plasmalemma. This hypothesis is being investigated at the present time (Gimmler et al., 1981).

1.6 The Mechanism of Photosynthesis in Dunaliella

In section 1.5.1, the method of osmoregulation in Dunaliella was described; the production or degradation of intracellular glycerol in response to a salinity increase or decrease respectively. The effect of osmoregulation on the metabolism of Dunaliella is the subject which this thesis attempts to discuss. One convenient way to investigate the metabolism of the alga is to study it's photosynthetic reactions. The process of photosynthesis is clearly implicated in the various hypotheses put forward to explain the mechanism of glycerol production (section 1.5.2). Therefore, the study of the photosynthetic reactions of Dunaliella can allow us to investigate the role of photosynthesis in osmoregulation and can also tell us something about the effect of osmoregulation on the overall metabolism of the alga.

In this section, the basic process of photosynthesis will be summarised briefly (section 1.6.1), with reference to experiments carried out on Dunaliella (section 1.6.2). The photosynthetic parameters measured in Dunaliella in this project will be briefly described in section 1.6.3.

1.6.1 Brief Summary of Photosynthesis

The general equation of photosynthesis can be written as:-

$$CO_2 + 2H_2A \xrightarrow[\text{CHLOROPHYLL}]{\text{LIGHT}} (CH_2O) + H_2O + 2A.$$

(CH_2O) represents stored organic matter and H_2A represents an oxidisable substrate or electron donor (Clayton, 1980). In the different classes of photosynthetic bacteria, different substances can be used as electron donors e.g. H_2, H_2S and a variety of simple organic compounds (Clayton and Sistrom, 1978). However, in higher plant photosynthesis, H_2O alone serves as the electron donor and the following equation can be written:-

$$CO_2 + 2H_2O \xrightarrow[\text{CHLOROPHYLL}]{\text{LIGHT}} (CH_2O) + H_2O + O_2,\ \Delta G = 469\ kJ\ mole^{-1}.$$

Thus, only in this special case where H_2O is the electron donor, is O_2 evolved as a by-product of photosynthesis, consequently no O_2 is evolved in bacterial photosynthesis because H_2O is not the electron donor. In addition to higher plants, O_2 evolution is found in all classes of algae, including the cyanobacteria (blue-greenalgae), and it can be used as a measure of the overall process of photosynthesis. This will be discussed further in Chapter 4.

Photosynthesis can be divided into two sections; light reactions or primary processes which are light driven and the 'dark' reactions or secondary processes which take place in the light or the dark.

The primary processes of photosynthesis appear to be very similar, if not identical in all the O_2-evolving organisms and they are involved with the initial photosynthetic reaction i.e. the absorption of light. Light is absorbed by various classes of pigments of which the carotenoids and the chlorophylls are the most important. These light-harvesting or antenna pigments transfer their energy to specialised chlorophyll a

molecules which are found in reaction centres (Duysens, 1951 and 1952; Govindjee and Govindjee, 1974).

Emerson and Arnold (1932a and b) first proposed the idea of a photosynthetic unit (PSU) which they suggested consisted of a reaction centre and associated light-harvesting or antenna chlorophyll molecules plus the 'dark' chemical machinery. They studied the O_2 evolution of Chlorella in response to brief flashes of light and found that with weak flashes the yield corresponded to 1 O_2 per about eight quanta absorbed. Strong flashes which were sufficient to excite every molecule of chlorophyll at least once, gave a maximal yield of about 1 O_2 per 2,400 chlorophyll molecules. Emerson and Arnold hypothesised that 2,400 molecules of chlorophyll cooperate in serving a single reaction centre which produces O_2. It is now known that the primary photochemical reaction involves single quanta driving single electron transfers (see below). Thus the results of Emerson and Arnold (1932a and b) can be re-interpreted so that one quantum absorbed by any chlorophyll molecule in a set of 300 (2,400 ÷ 8) can produce one photochemical event and eight such events are needed for the evolution of one O_2 molecule. Thus, according to this interpretation one PSU consists of 300 chlorophyll molecules in association with one reaction centre. The PSU need not necessarily be thought of as a distinct package of antenna pigment plus reaction centre, isolated from neighbouring PSU's. Possibly the antenna pigments form a matrix studded with reaction centres in the ratio of 300 chlorophyll molecules to one reaction centre. These contrasting views have been called the 'puddle' and 'lake' models respectively (Govindjee and Govindjee, 1974).

In O_2 - evolving organisms, the definition of a PSU is complicated by the fact that there are two kinds of photochemical system, photosystem I

and photosystem II (see later). The modern idea of the photochemical apparatus is that it consists of three main parts: a major antenna component (light-harvesting complex containing equimolar amounts of chlorophylls a and b i.e. LH a/b complex) and a component for each photosystem consisting of a reaction centre and a subantenna of chlorophyll a. For an entire chloroplast it seems that there is one reaction centre of each kind (photosystem I and photosystem II) per about 300 molecules of chlorophyll a and 100 of chlorophyll b (Clayton, 1980).

Thus having briefly summarised the structure of the photochemical apparatus, the function of the various components will now be examined.

The primary photochemical reaction of charge separation takes place in the reaction centres. The specialised molecules of chlorophyll a act as the primary electron donor (P), an electron acceptor (A) and a secondary electron donor (D) are also present, and the following reaction takes place (Mathis and Paillotin, 1981):-

$$D\ P\ A \xrightarrow{h\nu} D\ P^{*}\ A \longrightarrow D\ P^{+}\ A^{-} \longrightarrow D^{+}\ P\ A^{-}$$

When a quantum of light energy (hv) is captured by the reaction centre chlorophyll, an electron is promoted to a higher energy level i.e. an excited state denoted by P^{*}. De-excitation of this excited state can occur by four competing processes <u>in vivo</u> (Chapter 5), one of which is trapping by a reaction centre ($D\ P^{+}\ A^{-}$) and another is that the energy can be disipated via fluorescence. At room temperature, fluorescence is largely associated with photosystem II (see below) and it can be measured and used to estimate the efficency at which photosystem II is operating. This experimental technique will be fully described in Chapter 5.

The primary photochemical reaction results in the separation of one strong reducing equivalent and one strong oxidising equivalent ($D^{+}\ P\ A^{-}$).

The evolution of one molecule of O_2 requires that four electrons be removed from H_2O and for this to take place four quanta of light must be utilised to drive four photochemical charge separations as shown in the equation:-

$$2\ H_2O \xrightarrow{4\ h\nu} 4e^- + 4\ H^+ + O_2$$

Emerson and Arnold (1932a and b) obtained a figure of eight quanta of light absorbed for the production of one O_2 molecule, and by the 1950's, it was generally accepted that eight or more quanta are needed for the evolution of one O_2 molecule and concomitant reduction of one molecule of CO_2. This implied that two quanta cooperate in the separation of one strong reducing equivalent and one strong oxidising equivalent (Clayton, 1980).

Experimental evidence was accumulated throughout the 1940's and 1950's which suggested the operation in series of two light reactions to provide the necessary energy for the production of a sufficiently reduced electron acceptor that can serve as a reductant of CO_2 (Clayton, 1980). This idea was most clearly suggested by the work of Emerson (1958) who demonstrated that far-red light (> 680 nm) was inefficient in promoting photosynthesis (the 'red drop'), but was extremely efficient in enhancing the photosynthetic efficiency of red light (< 680 nm). Hill and Bendall (1960) proposed a scheme to account for these experimental results which is known today as the 'Z' scheme. However, most of the credit goes to Duysens and Amesz (1962) who provided the first convincing evidence for the 'Z' scheme and formulated the outline of it shown in Figure 3, which has stood the test of time (Avron, 1981). Duysens and Amesz showed that far-red light which excites predominantly what they termed photosystem I (PSI) reduces CO_2 via $NADP^+$ and oxidises cytochrome f, whereas red light which excites predominantly photosystem II

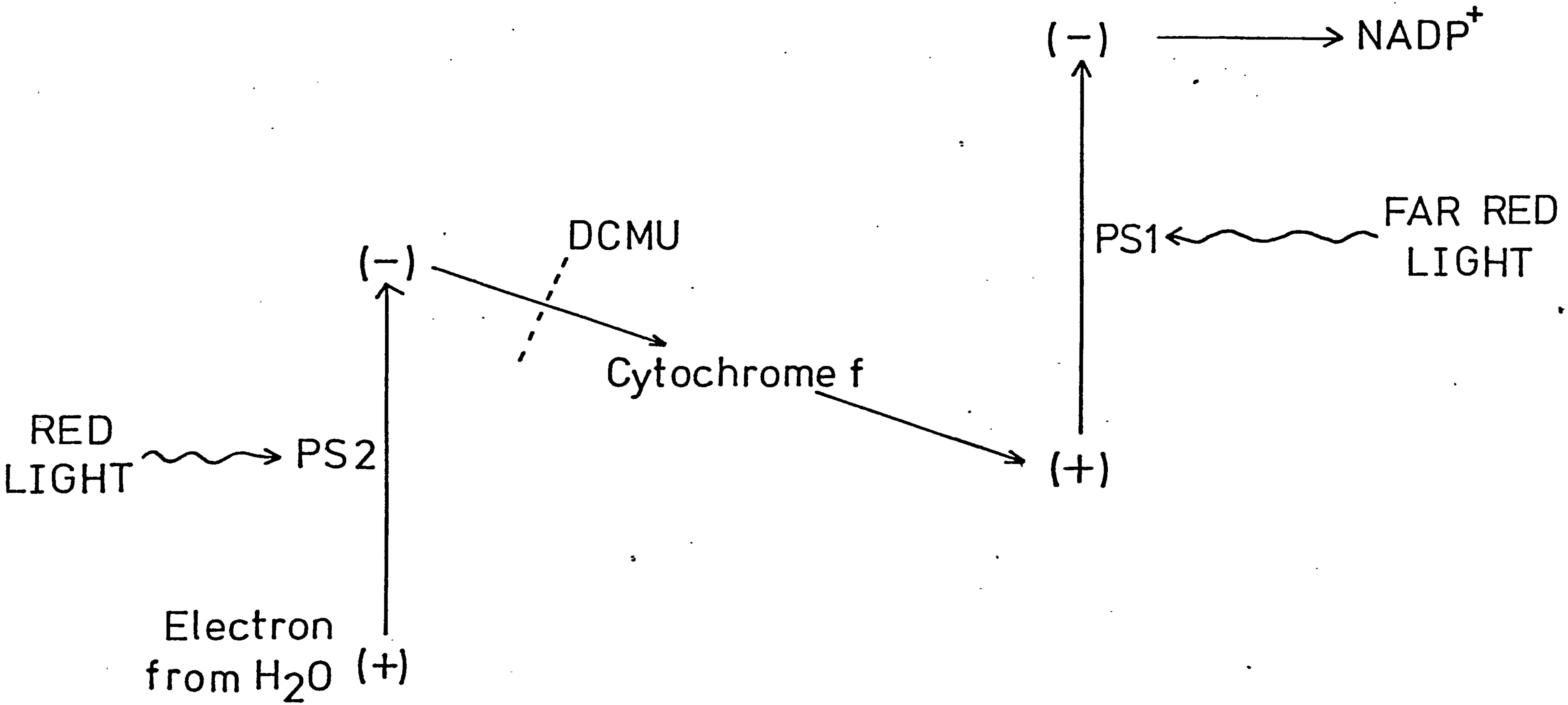


FIGURE 3. The basic outline of the 'Z' scheme of photosynthetic electron transport (adapted from Clayton, 1980).

produces O_2 from H_2O and reduces cytochrome f. The reduction of cytochrome f by PSII was blocked by the herbicide 3 - (3',4', dichlorophenyl), -1, 1 - dimethylurea (DCMU) (Figure 3).

There are thus two types of reaction centres in O_2 - evolving organisms: the PSI reaction centre and the PSII reaction centre. They are present in a nearly 1 : 1 ratio and function in series to drive electrons from H_2O to $NADP^+$ (Mathis and Paillotin, 1981). Both types of reaction centres contain specialised molecules of chlorophyll a, possibly organised as a dimer. There are two types of specialised chlorophyll a molecules; in PSII reaction centres there is P680 (the approximate maximum wavelength of its light-induced absorption decrease is 680 nm) and in PSI reaction centres there is P700 (Clayton 1980; Mathis and Paillotin, 1981).

Quanta absorbed by the light-harvesting a/b complex (i.e. LH a/b complex, the major antenna component) may go to one photosystem or the other, in varying proportions, but always mainly to PSII. It appears that the amount of quanta entering each photosystem can be regulated to achieve the optimal rates of operation for the two photosystems. Two regulatory devices seem to operate, (a) divalent cations or far-red light (which excites PSI) increase the coupling between the LH a/b complex and PSII and diminish the flow of quanta to PSI and (b) when PSII receives an excess of quanta, the trapping by its reaction centres is attenuated and quanta are shunted to PSI i.e. spillover (Clayton, 1980) (Chapter 5).

It is now well established that the mechanism of photosynthetic electron transport is based on the action of two photosystems operating in series i.e. 'Z' scheme. During the 1960's and 1970's a flurry of activity in the field resulted in the discovery of many intermediate

electron carriers and of their sequential order. Figure 4 shows the modern complex picture of the 'Z' scheme and as can be seen not all of the components have been chemically identified.

The region from H_2O to P680 is not well characterised because it is easily inactivated by experimental manipulation (Avron, 1981). However, chloride and particularly manganese have been implicated in the O_2-evolving process (Radmer and Cheniae, 1977). The first stable electron acceptor associated with PSII is Q which is thought to be a plastoquinone molecule with special properties. Horton and Croze (1979) found evidence to suggest that there are two components of Q with different midpoint redox potentials i.e.-247 mV (Q_L) and -45 mV (Q_H). They suggested that the two components could act in series or in parallel. There is also some indirect evidence that an electron acceptor is present prior to Q, denoted by I in Figure 4, which may be a molecule of pheophytin a (Clayton, 1980; Mathis and Paillotin, 1981). However, Horton and Croze (1979) speculated that Q_L is equivalent to I.

The plastoquinone pool contains about 10 molecules per electron transport chain. They accept electrons from Q via another molecule R, which is also probably a plastoquinone. The role of this intermediate plastoquinone molecule appears to be to allow the build up of 2 electrons to take place (R^{2-}), before they are passed together to a molecule of the plastoquinone pool (PQ) where the following reaction occurs (Cox and Olsen, 1982).

$$PQ + 2e^- + 2H^+ \longrightarrow PQH_2$$

The plastoquinone pool is followed by a recently discovered iron-sulphur protein (Malkin and Posner, 1978; White, Chain and Malkin, 1978). The electron transport components between the iron-sulphur protein and P700 are cytochrome f, which was the first component described (Figure 3),

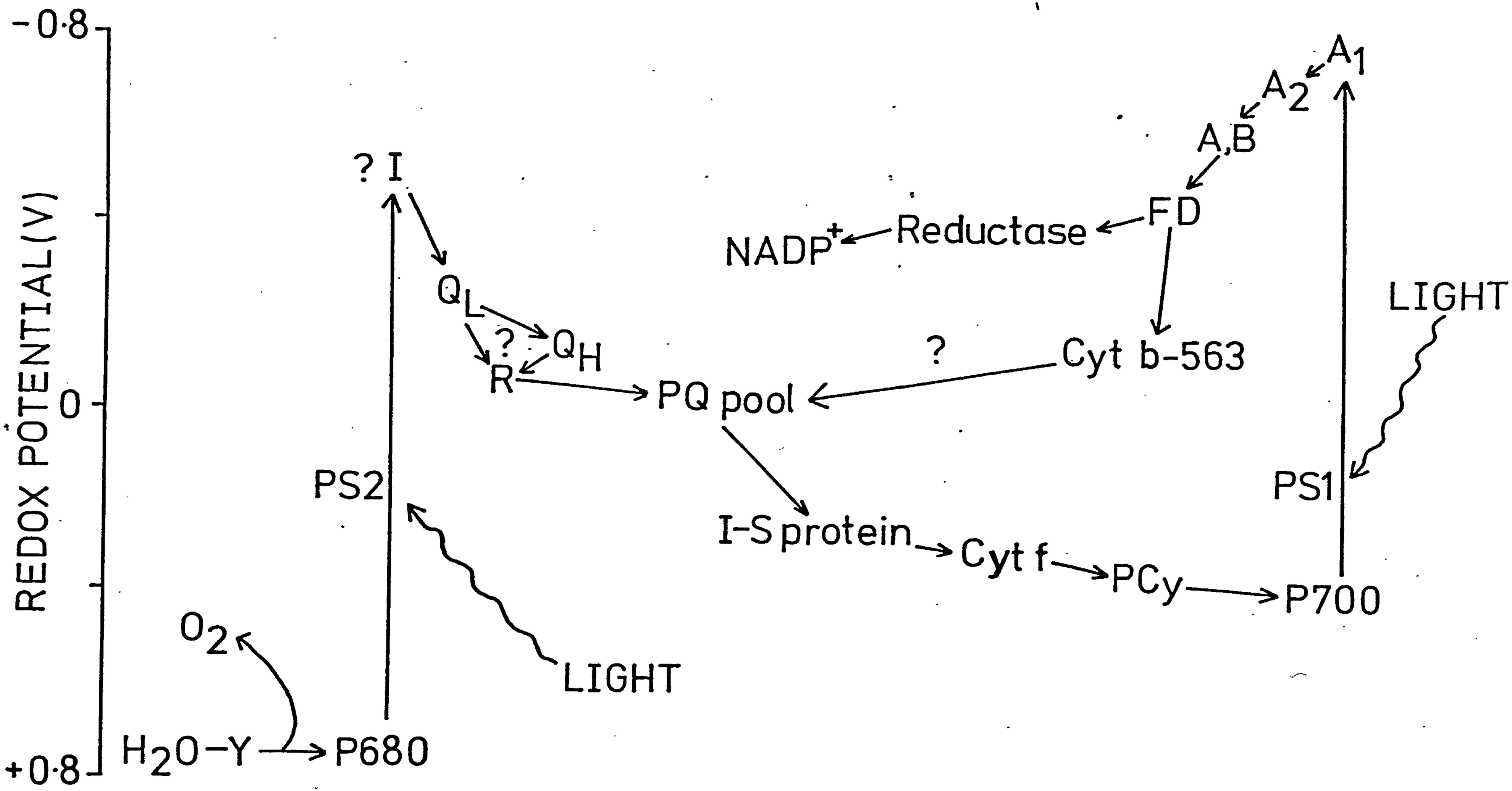


FIGURE 4. The photosynthetic electron transport scheme ('Z' scheme). PQ is plastoquinone; I-S is iron-sulphur; Cyt is cytochrome; PCy is plastocyanin; and FD is ferredoxin (adapted from Clayton, 1980).

and plastocyanin (Avron, 1981).

The primary electron acceptor of PSI called A_1 in Figure 4, is not well characterised, although it has properties similar to a chlorophyll a dimer (Shuvalov, Dolan and Ke, 1979). The secondary electron acceptors are iron-sulphur proteins, A_2(X) and A,B(P430) which quickly pass on electrons to ferredoxin (Sauer et al., 1978). These early electron acceptors associated with PSI have been resolved with the aid of electron paramagnetic resonance (epr) studies. This technique involves the use of microwave spectroscopy to look at the electron spins of atoms and molecules and permits the detection of an unpaired electron or electrons (Evans, 1977).

For the 'dark' reactions of photosynthesis to proceed, ATP and NADPH must be fed into them. NADPH can clearly be generated directly from the photosynthetic electron transport chain when electrons pass from ferredoxin to ferredoxin NADP reductase and on to $NADP^+$ (Figure 4). This does not explain where the ATP comes from, however energy is lost in the 'energetically downhill' parts of photosynthetic electron transport and in some way this must be utilised to form the high energy bond of ATP.

Experiments have shown that the rate of photosynthetic electron transport is coupled to ATP production, e.g. if there is little ADP present in a chloroplast preparation to take part in the $ADP + P_i \rightarrow ATP$ reaction, there is a low rate of electron transport. However, if ADP is added to the preparation the rate of electron transport is increased, this phenomenon is called 'photosynthetic control'. This observation can help to identify the sites in the electron transport chain which are coupled to ATP production, because in the absence of phosphorylation (due to a lack of ADP say), the rate limiting step

for electron transport must be the energy-coupling site. Thus, the electron carriers preceeding the coupling site will be overreduced, whereas the electron carriers following the site should be overoxidised (Avron, 1981). This type of experiment led to the identification of a coupling site between the plastoquinone pool and cytochrome f (Figure 4), and it is also generally accepted that a second coupling site is found in the region between H_2O and Q or R (see later).

Two types of photosynthetic electron transport are possible as is shown in Figure 4. Non-cyclic electron transport ($H_2O \rightarrow$ P680 $\rightarrow$ P700 $\rightarrow$ $NADP^+$) evolves O_2 and produces ATP and NADPH, whereas cyclic electron flow (cyclic around P700) produces only ATP, and neither reduces $NADP^+$ nor evolves O_2. Figure 4 indicates that cyclic electron flow is made possible by cytochrome b-563 accepting electrons from ferredoxin and donating them to the plastoquinone pool. A midpoint redox potential of -110 mV for cytochrome b-563 agrees well with its hypothesised role in cyclic electron transport, but other experimental evidence to support this conclusion is not wholly convincing (Slovacek, Crowther and Hind, 1980; Cox and Olsen, 1982).

The most widely accepted scheme to explain how ATP is produced by electron transport is the chemiosmotic hypothesis (Mitchell 1961 and 1966). This hypothesis is based on the fundamental observation that only particles or vesicles surrounded by a membrane can synthesise ATP. The postulates of the hypothesis are as follows:-

(a) reactions occur in or on a proton impermeable membrane that encloses an inner space.

(b) electron transfer involves the alternation of electron carriers (e.g. cytochromes) and hydrogen carriers (e.g. quinones); reactions occur on opposite sides of membranes. This implies that electron transfer is coupled to proton translocation.

(c) the influx of protons makes the concentration of H^+ inside greater than the concentration of H^+ outside and it induces a transmembrane electrical potential (positive inside).

(d) protons efflux through a membrane bound proton permeable unidirectional ATPase.

In the case of O_2-evolving photosynthetic organisms, the proton impermeable membranes are the thylakoid membranes of the chloroplast which enclose inner spaces. The electron transfer involving the alternation of electron and hydrogen carriers is the photosynthetic electron transport scheme shown in Figure 4 which takes place in the thylakoid membrane. The chemiosmotic hypothesis suggests that as electrons pass along the electron transport chain, protons are pumped into the inner thylakoid space. Therefore a coupling site is a part of the electron transport chain where protons are taken up on the outside of the thylakoid, carried across the membrane and released into the thylakoid interior, as electron transfer occurs. This proposal requires that electron transport takes place vectorially across the thylakoid membrane (Avron, 1981).

The evidence for vectorial transport of electrons in the thylakoid membrane has been reviewed by Trebst (1974), Trebst (1980) and Cox and Olsen (1982). It is based on investigations of the components of the membrane by chemical probes and antibodies to determine their localisation in the membrane and also on experiments using inhibitors of electron transport. This has allowed a scheme to be postulated which would result in protons being transported across the membrane as predicted by the chemiosmotic theory (Figure 5).

A possible mechanism to explain how the plastoquinone pool/cytochrome f coupling site operates is incorporated into Figure 5. A

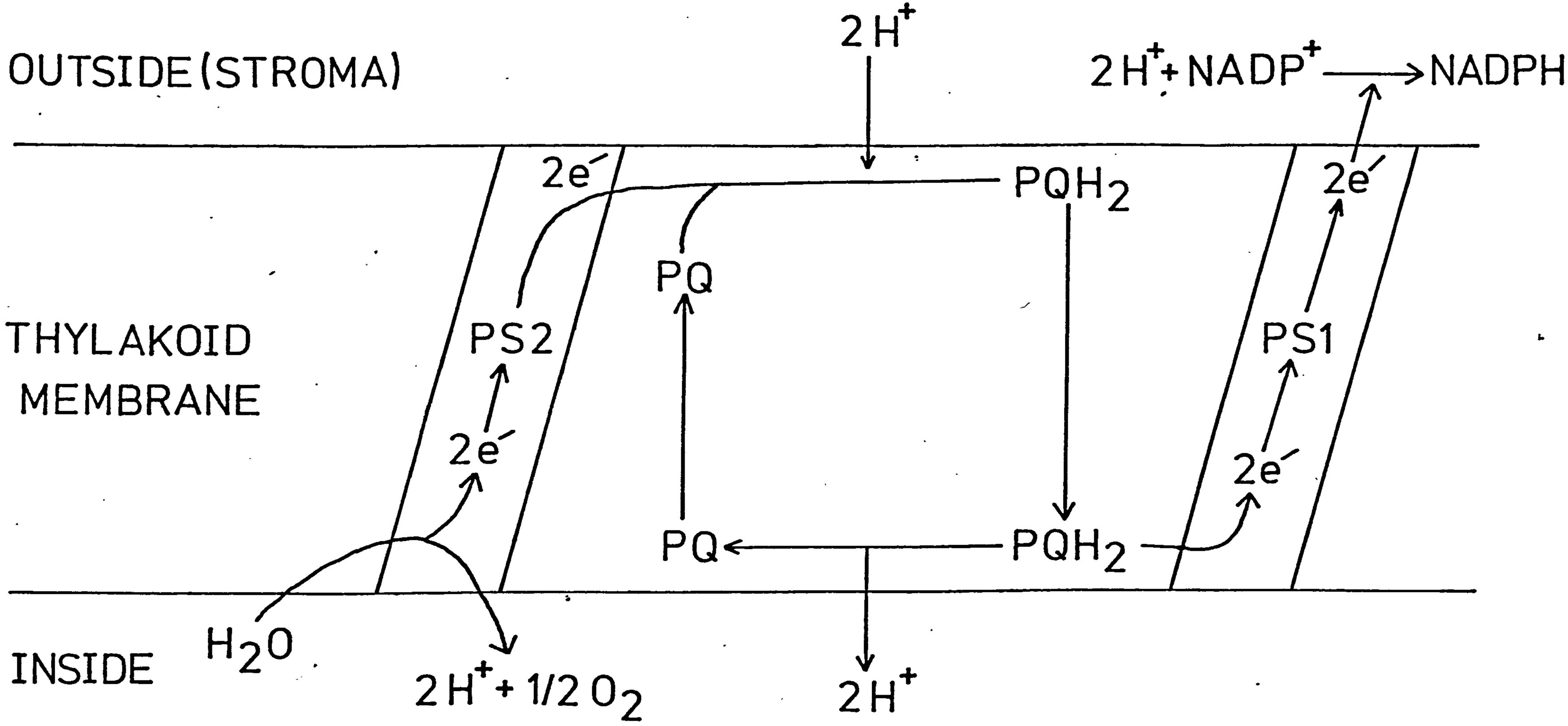


FIGURE 5. A possible pathway for vectorial electron transport in the thylakoid membrane, PQ is a molecule of the plastoquinone pool (adapted from Cox and Olsen, 1982).

molecule of the plastoquinone pool receives electrons at the outside of the thylakoid membrane, and therefore the accompanying protons are taken up from the chloroplast stroma. The hydrogen atoms then cross the membrane in association with plastoquinone molecules. Cox and Olsen (1982) present evidence in favour of a more complex plastoquinone pathway for crossing the membrane than is shown in Figure 5. At the inside of the thylakoid membrane the electrons are passed on to cytochrome f via the iron-sulphur protein and the protons are released into the inner thylakoid space. Figure 5 also explains the reason for a coupling site of electron transport and ATP production between H_2O and Q. As a result of the photochemical reaction of PSII, H_2O is oxidised. The electrons are released into the electron transport chain and the protons are released into the inner thylakoid space. The terminal electron acceptor $NADP^+$ is found at the outside of the membrane, and when it receives electrons from ferredoxin NADP reductase it takes up the accompanying protons from the stroma. Thus the net result is a transport of protons across the membrane.

One of the most controversial aspects of the scheme shown in Figure 5 is the position in the membrane of plastocyanin. The model of vectorial electron transport would place it towards the inside of the membrane, but the experimental evidence does not entirely support this assumption (Trebst, 1980).

The transport of protons across the thylakoid membrane results in the establishment of an electrochemical gradient. The electrochemical gradient is made up of two components: a proton concentration gradient with the inner thylakoid compartment more acidic (ΔpH) and an electrical gradient or membrane potential ($\Delta\psi$) which is positive inside (Avron, 1981). Figure 6 illustrates in a simplified form how the chemiosmotic theory

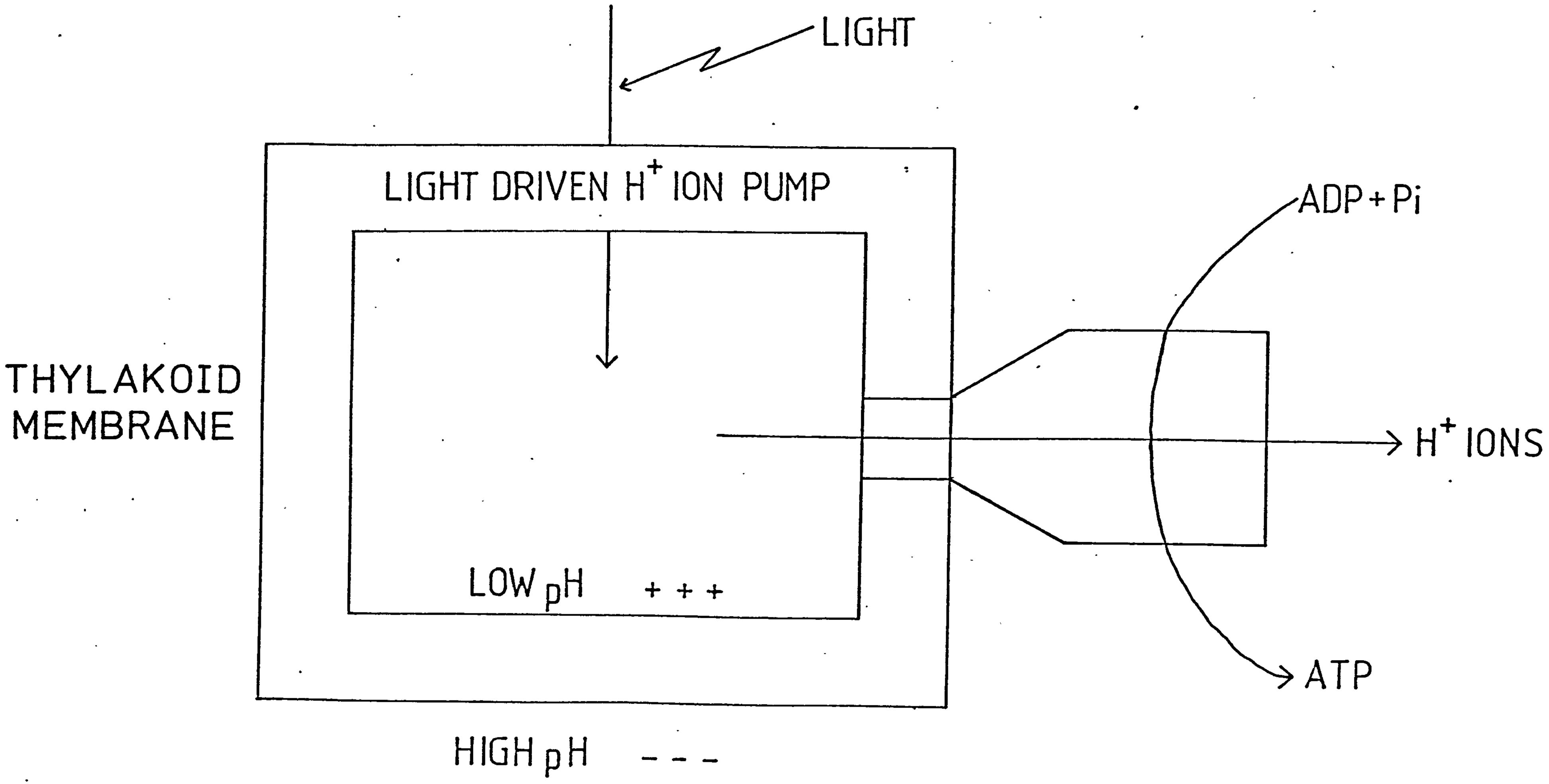


FIGURE 6. A simplified representation of the chemiosmotic theory as it applies to thylakoids. The light driven H^+ ion pump is the 'Z' scheme.

predicts that a build up of H^+ inside the thylakoid could be channeled through a proton permeable membrane bound ATPase to produce ATP. The formation of ATP when protons are passed through the ATPase is a process that is not well understood (Clayton, 1980).

The amount of energy which could be made available for ATP production by light-induced H^+ uptake is known as the proton motive force (pmf). This could be in the form of all $\Delta\psi$ or all ΔpH or a combination of both. Theoretical calculations can be made to approximate the amount of the pH difference or the size of the membrane potential that would be needed to drive ATP synthesis. Assuming standard conditions (pH = 7, temperature = 298K) and deciding on set concentrations for ATP, ADP and inorganic phosphate, approximately 240 mV of $\Delta\psi$ or 4 units of ΔpH would allow ATP synthesis to take place. If dark-adapted chloroplasts are given brief flashes of light it appears that initially the pmf is in the form of a large $\Delta\psi$ of around 200 mV. However, Hinkle and McCarty (1978) found in isolated choroplasts under continuous light (steady-state conditions) that the $\Delta\psi$ was negligible, but a ΔpH of 3.5 units was formed which would produce ATP. Thus photophosphorylation is driven by the tansmembrane electrical potential only for the first few seconds after a chloroplast preparation is exposed to light and then this role is taken over by the transmembrane pH gradient (Avron, 1981).

The experimental evidence to support the theory that the pmf drives ATP synthesis has been reveiwed by Jagendorf (1975). Jagendorf and Uribe (1966) demonstrated that ATP could be produced in the dark if chloroplasts were quickly transferred from acid conditions (pH4) to basic conditions (pH8). Gräber, Schlodder and Witt (1977) showed that 200 mV of $\Delta\psi$ can yield quantitatively significant ATP formation when imposed artificially.

Compounds are known which can uncouple photophosphorylation from electron transport i.e. in their presence electron transport proceeds but ATP production is abolished. The 'classic' uncouplers e.g. dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone (CCCP) act as electrogenic proton carriers. They transfer protons down the electrochemical gradient from the inner thylakoid space into the stroma of the chloroplast, and in doing so abolish the $\Delta\psi$ and ΔpH. However, at concentrations only a little higher than those that cause uncoupling, they inhibit photosynthetic electron transport (Hinkle and McCarty, 1978; McCarty, 1980). Gramicidin also acts as an uncoupler, this molecule forms a pore through the thylakoid membrane and allows ions with one positive charge to pass through. It does not inhibit electron transport even at high concentrations (Walker and Crofts 1970; McCarty, 1980). In the presence of uncouplers, electron transport takes place at its maximum rate, since it cannot be slowed down by 'back' pressure from a build up of H^+ in the thylakoid interior i.e. there is no 'photosynthetic control'.

Another class of molecules called ionophores can affect the electrochemical gradient set up by light-induced proton pumping. Ionophores allow certain types of ions to cross membranes in response to the electrochemical gradient (Walker and Crofts, 1970). There are two types of ionophore commonly employed in photosynthetic research. The first group are typified by valinomycin which carries K^+ or NH_4^+ across membranes but not Na^+ or H^+ (Clayton, 1980). This results in the discharging of the $\Delta\psi$, but has no effect on ΔpH (Walker and Crofts, 1970). Telfer and Barber (1974) found that high concentrations of valinomycin can inhibit photosynthetic electron transport and affect the H^+ permeability of the thylakoid membrane (i.e. uncouple). The other type of ionophore can be represented by nigericin which can transport K^+ across membranes, but

only in exchange for H^+ i.e. it accelerates the approach to equilibrium of H^+/K^+ across the membrane. Therefore in the presence of K^+, nigericin abolishes the ΔpH, but does not affect Δψ (Walker and Crofts, 1970). Clayton (1980) suggested that it should be possible to measure the relative proportions of the pmf due to the ΔpH or the Δψ by using valinomycin and nigericin. If both of these ionophores are added together, they act as an uncoupler because both the ΔpH and the Δψ are abolished and there is no ATP production.

It is possible to investigate the membrane potential (Δψ) set up across the thylakoid membranes by looking at the change in the absorption of light of 519 nm. The mechanism of the absorption change is thought to be due to the electric field generated by the initial charge separations at the two photosystems causing a change in the absorption properties of chlorophyll a, chlorophyll b and carotenoid molecules found in the thylakoid membranes. This phenomenon will be discussed in detail in Chapter 6.

The 'dark' reactions or secondary processes of photosynthesis are involved in 'fixing' CO_2 into carbohydrates using the ATP and NADPH generated by the light reactions. The process of fixation can occur in two main ways with either a three carbon (C_3) or a four carbon (C_4) first product (Hatch, Osmond and Slatyer, 1971). The C_4 plants can be divided into the tropical grasses which produce the C_4 acids in the light and the crassulacean acid metabolism plants (e.g. Crassulaceae and Bromeliaceae) which accumulate malic acid (C_4) in the dark (Edwards and Huber, 1981; Osmond and Holtum, 1981). The type of carbon-fixation normally found in other plants and algae is the C_3 carbon reductive cycle (Robinson and Walker, 1981). However, it has recently been suggested that a C_4-type of photosynthesis is found in some algae

(Beardall et al., 1976).

1.6.2 Photosynthesis in Dunaliella

A lot of investigations into photosynthesis in Dunaliella have been connected with osmoregulation and have already been mentioned (section 1.5). However, there are many published papers which discuss photosynthesis in Dunaliella without referring to osmoregulation. Some of the most relevant results from these papers are summarised in this section.

Cox and Olsen (1982) indicated that the copper-containing electron transport component plastocyanin may be replaced by a soluble c-type cytochrome in algal chloroplasts (Figure 4). This was shown in the green alga Scenedesmus and it was noted that the concentration of plastocyanin and the c-type cytochrome vary inversely in response to the cupric ion concentration of the growth medium (Bohner and Boger, 1978; Bohner, Bohme and Boger, 1980; Bohner et al., 1980). However, Bohner et al. (1980) found that in D. parva, variations in the copper concentration change the plastocyanin content only a little, and no soluble c-type cytochrome could be detected, in contrast to the situation with Scenedesmus. As cytochrome f is also present, Dunaliella, with respect to these three redox proteins resembles a higher plant (Figure 4).

The effect of changing the light intensity on the size of the photosynthetic unit (PSU) was measured in Dunaliella (section 1.6.1). Perry, Talbot and Alberte (1981) measured the ratio of light-harvesting pigments to P700 (reaction centre of PSI) in D. euchlora. They found that there was no change in the PSU size when the light intensity was decreased from 300 to 4 μmoles $m^{-2}s^{-1}$. Falkowski et al. (1981)

measured the ratio of light-harvesting pigments to reaction centres of both PSI and PSII. They found that cells of D. tertiolecta contained more chlorophyll a (associated with reaction centres) and had a lower ratio of chlorophyll a to chlorophylls b and c when the light intensity was decreased from 500 to 30 μmoles $m^{-2}s^{-1}$. The cellular content of PSI and PSII reaction centres increased in parallel as chlorophyll a increased, so that PSU sizes changed only slightly and the ratio of PSI:PSII reaction centres remained constant at approximately 1:1.

The effect of growing Dunaliella under a 12:12 hour light:dark regime instead of continuous light was investigated and it was found that cells had a higher photosynthetic rate and a higher photosynthesis: respiration ratio under the 12:12 hour light:dark regime (Humphrey, 1979). Wallen and Geen (1971a and b) showed that in D. tertiolecta the photosynthetic rate and the total pigment concentration were higher in blue light and lower in green light compared with white light of the same intensity.

Several investigations have been made into specific components of the photosynthetic electron transport chain. The electron paramagnetic resonance (epr) properties of PSI iron-sulphur centres were determined in D. parva (Hootkins, Malkin and Bearden, 1981). It was possible to photoreduce two iron-sulphur centres at cryogenic temperatures whose epr signal was unusually temperature dependent, indicating that they differed from higher plant (spinach) iron-sulphur centres (section 1.6.1). Von Kameke and Wegmann (1977 and 1978) isolated two manganese containing subchloroplast particles from Dunaliella and partially characterised them and Noro (1978) looked at the effect of various concentrations of manganese on the growth and chlorophyll content of D. tertiolecta. Two ferredoxins have been isolated and purified from D. salina, their amino acid sequences were compared with two different types of

ferrodoxins, (a) those representative of other plants, and (b) one isolated from the halophilic organism Halobacterium. Although D. salina has halophilic properties, the ferredoxin isolated from the alga did not show any special homology to Halobacterium ferredoxin (Hase et al., 1980).

The photosynthetic properties of Dunaliella are also studied in the USSR, but the papers are usually only available in abstract form. Myronyuk, Masyuk and Akopyants (1980a and b) have studied the catalase activity of D. salina and Balnokin and Medvedev (1980) have isolated chloroplasts from D. salina and D. maritima and looked at the effect of ions on the rate of electron transport.

An attempt to isolate intact chloroplasts from D. marina was made by Kombrink and Wober (1980a and b). They chemically-induced lysis of the cells with DEAE-dextran and then centrifuged them in a linear sucrose density gradient. The chloroplasts isolated by this method were intact by morphological and biochemical criteria, however they did not 'fix' CO_2 nor photoreduce 3-phosphoglycerate nor demonstrate CO_2-dependent O_2 evolution. These defects are primarily due to an inhibition of electron transport at PSI (Kombrink, Wober and Walker, 1980).

It appears that the type of carbon-fixation normally found in Dunaliella is the C_3 carbon-reductive cycle (Robinson and Walker, 1981). However, Mukerji, Glover and Morris (1978) showed that the type of carbon-fixation method could vary in D. tertiolecta, depending on the physiological state of the alga.

1.6.3 Measurements of Photosynthesis

Most of the experiments described in this thesis are concerned with the effect of ionic and osmotic stress on the photosynthetic processes of

Dunaliella. The organism was chosen for two reasons: firstly, it is euryhaline and is thus able to respond to ionic and osmotic stress, and secondly because a number of photosynthetic parameters may be studied in the alga without the need to disrupt the cell. Three of these parameters are:-

(a) O_2 evolution - allows us to look at the photosynthetic process as a whole (Chapter 4).

(b) chlorophyll fluorescence - can be used specifically to look at PSII or to determine the relative activity of the two photosystems. It can also give us information about individual components of the electron transport chain e.g. the redox state of Q, the primary electron acceptor of PSII shown in Figure 4 (Chapter 5).

(c) light-induced pigment absorption change at 519 nm - this allows us to measure the permeability of the thylakoid membrane to ions, this is important in the context of the chemiosmotic theory of ATP production (Chapter 6).

The background theory to each of these experimental systems is described in full in the relevant chapter.

1.7 Commercial Aspects of Glycerol Production by Dunaliella

Even before Craigie and McLachlan (1964) demonstrated the salinity-induced production of glycerol by Dunaliella, it had been suggested that mass cultivation of algae like Dunaliella could be used as an alternative food source (Eddy 1956; Gibor, 1956). Recently, it was shown that under conditions of optimum light intensity and temperature and in high salinity, 85% of the dry weight of Dunaliella is glycerol (Ben-Amotz, 1977), and in a medium of 1.5 M NaCl, the intracellular concentration of glycerol is 2.1 M (Ben-Amotz and Avron, 1973a). This appeared to make Dunaliella

a very promising candidate for solar energy production and it was suggested that the alga could be used as animal feed because it is a good concentrated protein source (Gibbs and Duffus, 1976; Ben-Amotz, 1977).

Various groups also observed that D. salina and D. bardawil were capable of producing β-carotene in large amounts if grown under conditions of high light intensity and high salt concentration (Drokova, 1961; Loeblich, 1970; Semenko and Abdullaev, 1980; Ben-Amotz and Avron, 1981; Loeblich, 1982). In Israel at this moment, Dunaliella is being grown under semi-industrial conditions to produce glycerol, β-carotene and protein (Ben-Amotz and Avron, 1981). The alga is also being grown on a commercial scale in Western Australia to produce β-carotene (M.A. Borowitzka, personal communication). A recent industrial study suggests that using Dunaliella may be the best way to produce glycerol commercially (Chen and Chi, 1981). However, Gudin and Chaumont (1980) claimed that at present it would not be economically viable to produce glycerol from Dunaliella, but it may be economical to produce certain high value products from the alga, of which β-carotene may be one.

CHAPTER 2

MATERIALS AND METHODS

2.1 Classification and Description of Dunaliella

Dunaliella is a genus of unicellular green algae which belong to the order Volvocales (some authorities reserve this order for the colonial genera and place unicells like Dunaliella in a separate order, Chlamydomonadales), in the class Chlorophyceae of the division Chlorophyta (Round, 1973).

Dunaliella is very similar to Chlamydomonas morphologically except that Dunaliella has no cell wall, and is surrounded only by a plasmalemma with a glycocalyx covering (Oliveira, Bisalputra and Antia, 1980; Hoshaw and Maluf, 1981). Dunaliella is an oval biflagellate, flagella are simple and are 1.5 - 2 times as long as the cell. Figure 7 shows an election micrograph of the alga, the flagella are not shown and the cell is round not oval, both of these changes are probably connected with the fixation procedure. A detailed description of Dunaliella tertiolecta, the species used in this project, is given below. It is based on my own observations and the work of Butcher (1959).

The cells of D. tertiolecta are 9-11 × 5.5-7μ, generally uncompressed, ovoid or elliptical; anterior end tapering either gradually or suddenly into an acute apex, posterior is rounded. The chloroplast is campanulate, rather yellowish-green, always rugose, pyrenoid is central to sub-basal surrounded completely by a starch sheath. The stigma is central, rather large and diffuse. The nucleus is small and central. Cells also contain numerous small refractive globules distributed evenly throughout the cell.

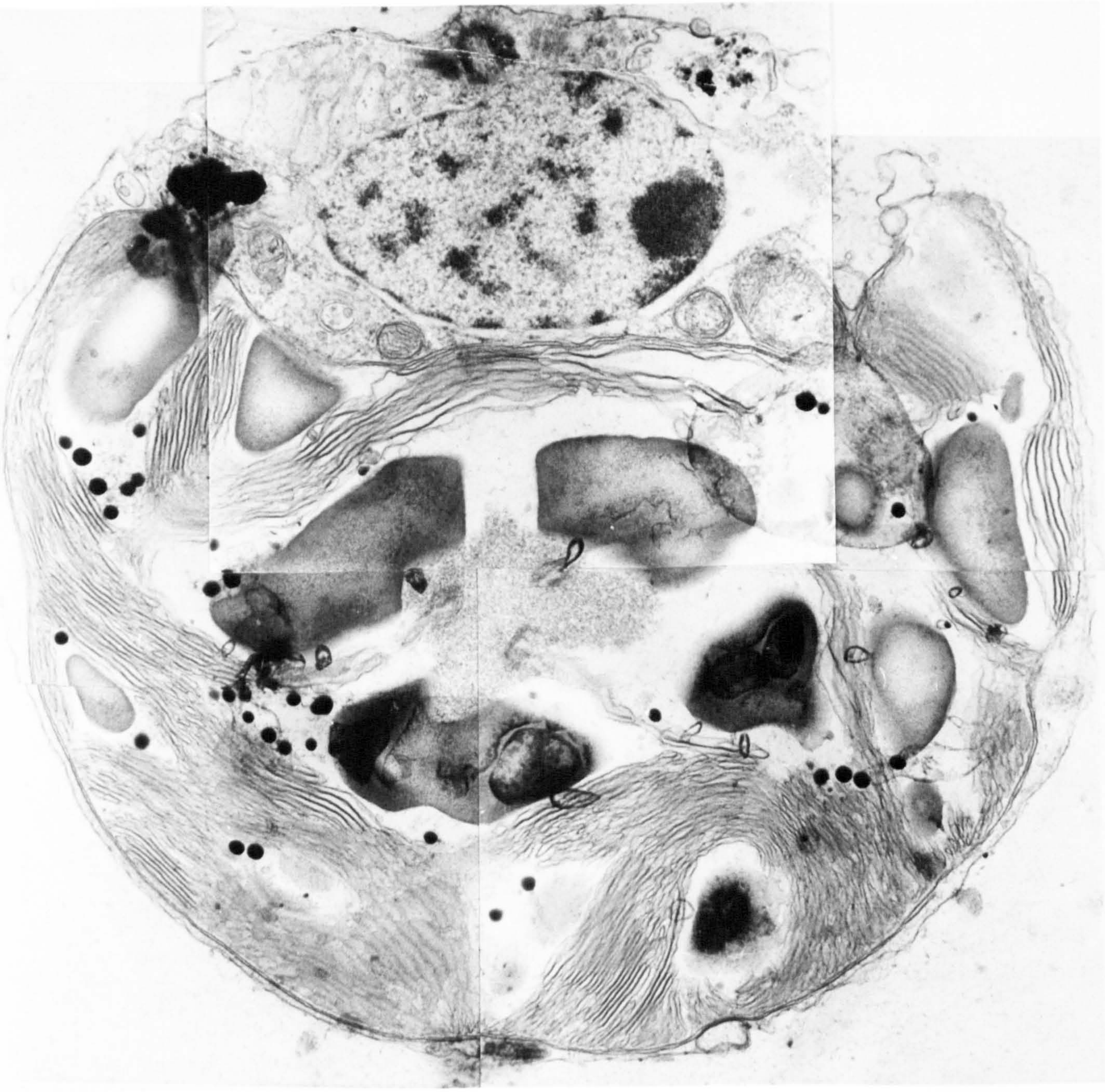


FIGURE 7. An electron micrograph of Dunaliella tertiolecta, magnification x 36,000.

The taxonomy of the genus Dunaliella is a difficult and confused subject e.g. D. tertiolecta is similar in many ways to D. parva and D. primolecta. The salient taxonomic feature appears to be the presence of the very small colourless refractive globules throughout the cytoplasm of D. tertiolecta which are not found in D. parva or D. primolecta (Butcher, 1959).

2.2 Culture Conditions

Dunaliella tertiolecta, strain number 19/6A was obtained from the Culture Centre of Algae and Protozoa, Cambridge, England. This alga was identified from the description by Butcher (1959) as described in section 2.1.

The alga, supplied as an agar slope, was transferred to Boney's liquid culture medium, which consists of a seawater base enriched with nutrients (Appendix A). The cells were transferred to the liquid medium using an innoculating loop under sterile conditions. The best growth was obtained if the alga was first transferred to 5ml of Boney's medium in a test-tube plugged with non-absorbent cotton wool; the alga was then allowed to thicken up before being transferred to 150ml of Boney's medium in a 250ml flask plugged with cotton wool. The thickening up process took 2-3 days.

Initial 150ml cultures were set up in 250ml flasks in a constant temperature growth room at 301K. The alga was grown under continuous light of intensity 3.5-5Wm^{-2} supplied by four 20 watt fluorescent lamps (Thorn white 3500). The spectral intensity distribution emitted by these lamps is shown in Figure 8. No air was bubbled through the cultures and they were not agitated. New cultures were started by adding 10, 15 or 20ml of algal cells which had been growing for 4-6 weeks to 150ml of fresh Boney's medium (Figure 9).

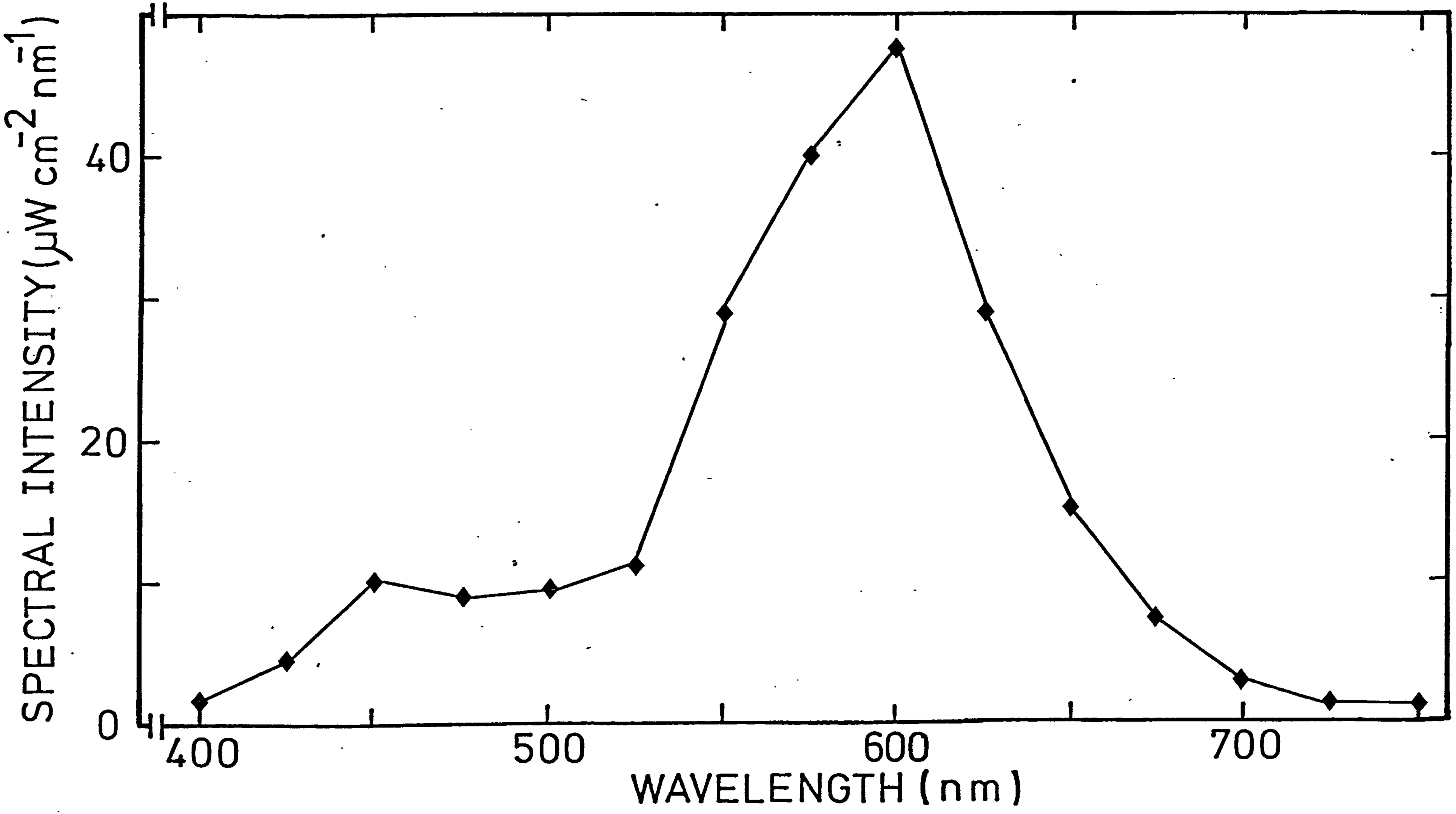


FIGURE 8. The spectral intensity distribution of light falling on the algal cultures.

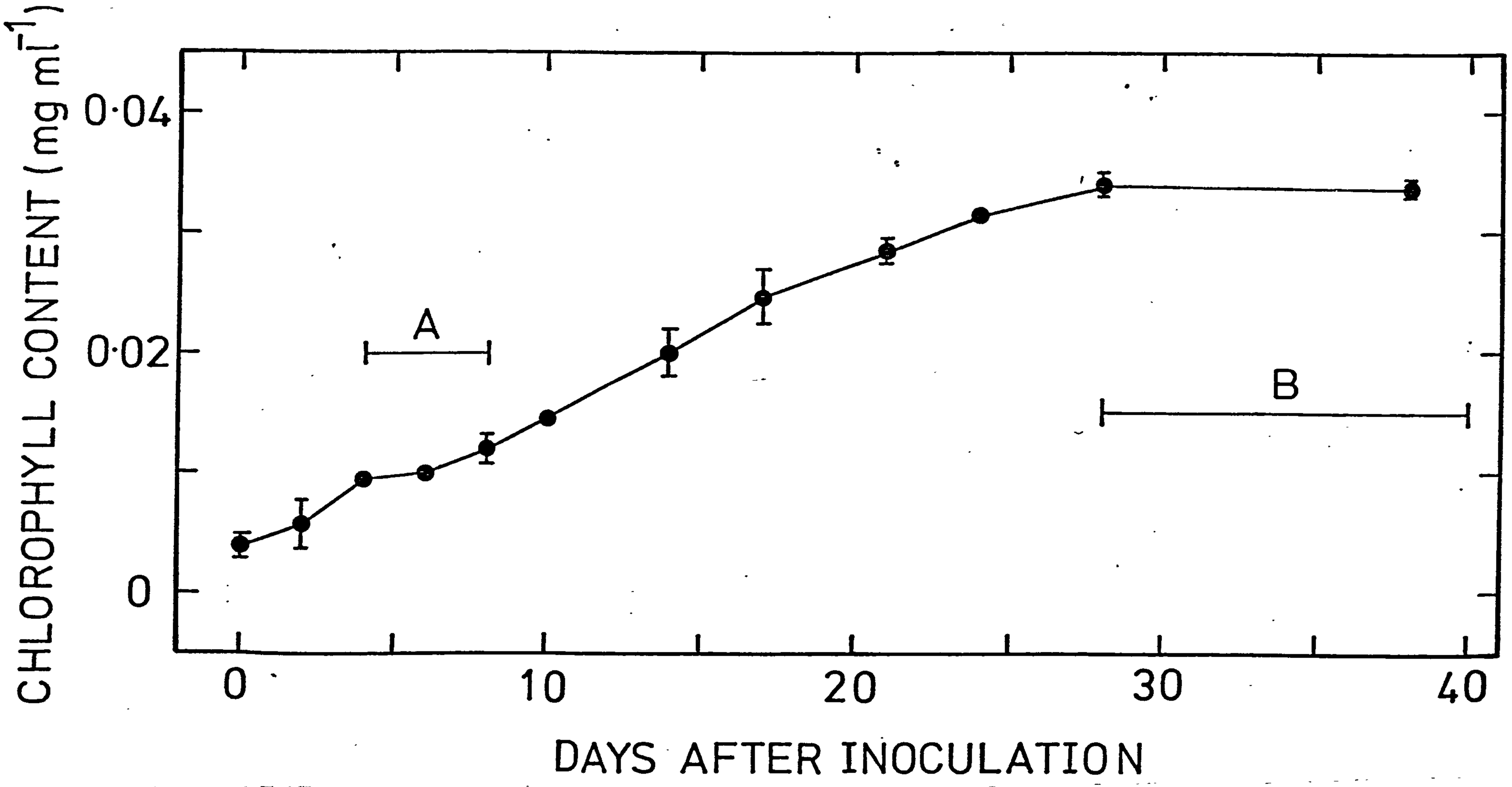


FIGURE 9. The growth curve of D. tertiolecta after an initial inoculation of 15 ml of 4 to 6 week old cells. Inoculations of 10 or 20 ml produced identical curves. A: 4 to 8 day period when cells were used for experimental purposes; and B: 4 to 6 week period when stationary phase cells were taken to inoculate fresh cultures.

2.3 Determination of Chlorophyll Content

The chlorophyll content was measured by extracting the pigments in hot methanol (Holden, 1965). 10ml of the algal culture was centrifuged at 3,500 × g for 5 min; the supernatant was discarded and the pellet was resuspended in redistilled methanol. The tube of chlorophyll solution was covered with aluminium foil and placed in a warm water bath (318K) for 10 min. The solution was then passed through Whatman No.1 filter paper and the filtrate collected in an aluminium foil-covered test-tube. The optical density of the resulting chlorophyll solution was measured in a spectrophotometer against a redistilled methanol blank at 650 and 665nm. The equation used to determine the chlorophyll content was:-

$$C(\text{mg ml}^{-1}) = 25.5 \times OD_{650} + 4 \times OD_{665}$$

corrected for the dilution factor.

2.4 Growth Curve of Dunaliella

To investigate the kinetics of the growth of D. tertiolecta, the chlorophyll content of algal cultures was measured at various times after inoculation. Figure 9 shows the growth curve for initial inoculations of 10, 15 or 20ml of 4-6 week old algal cells (section 2.2). The alga was used for experimental purposes when the chlorophyll content had reached 10µg ml^{-1}, thus cultures were used for experiments 4-8 days after being innoculated (Figure 9).

2.5 Determination of Salinity

The salinity of the seawater medium was determined by the method of Harvey (1963). 0.16M silver nitrate was titrated against a 10ml sample of seawater medium of unknown salinity which contained three

drops of 0.41M potassium chromate as an indicator. The halogens in the solution were precipitated as white solids and the colour turned pale yellow. The end-point was reached when the solution turned a persistent peach or dirty orange colour. The salinity of the sample in ‰ NaCl was numerically equal to the volume in ml of silver nitrate solution required to titrate the 10ml sample (Barnes, 1959; Harvey, 1963). The values obtained in ‰ NaCl could easily be converted to molarity of NaCl (Appendix B).

2.6 Salinity Adjustments

Using the method of Harvey (1963) the salinity of Boney's medium was found to vary from 20 to 30 ‰ NaCl due to differences in the salinity of the seawater base. Therefore the salinity of each batch of Boney's medium was determined and it was adjusted to 25 ‰ NaCl (0.43M) by the addition of solid NaCl or distilled water. Thus the alga was normally grown at 0.43M NaCl. However, for experimental purposes, it was necessary to increase or decrease the salinity of Boney's medium. This was achieved by addition of NaCl or of distilled water respectively (Appendix B).

2.7 Osmotic Adjustments

Instead of NaCl being added to the growth medium, some of the experiments required that other osmotica were added. To achieve comparable results some measure of osmotic stress was necessary and the parameter chosen was osmolality which has the units osmoles kg^{-1} (Appendix C). The other osmotica used were potassium chloride (KCl), sucrose, glycerol, and ethylene glycol. Wolf, Brown and Prentiss (1977) give tables whereby the osmolality of the above solutions can be related to the osmolality of NaCl. The amount of KCl, sucrose, glycerol

and ethylene glycol added to the growth medium is shown in Appendix C. However, in all cases, KCl, sucrose, ethylene glycol or glycerol was added to the basic Boney's medium and thus 0.43M NaCl was always present.

2.8 Determination of Intracellular Glycerol Content

2.8.1 Chemical Method

A rapid chemical method for estimating glycerol concentration in fermentation solutions was proposed by Lambert and Neish (1950). Glycerol is quantitatively oxidised to formaldehyde by periodic acid, using a 5 minute oxidation period to minimise oxidation of glucose. The iodate and periodate are then reduced to iodide by a large excess of sodium arsenite and the formaldehyde is then determined directly in the oxidation mixture by the colour reaction with chromotropic acid. This procedure was employed in the presence of increased concentrations of NaCl and KCl, but not in the presence of large sucrose or ethylene glycol concentrations because the latter compounds interfered with the colour change of the test. The exact method followed in the present work is described below.

Three 150ml cultures of D. tertiolecta were combined and the chlorophyll content of the algae was determined as already described (section 2.3). The algae were centrifuged at 3,500 × g for 5 min so that 150µg of chlorophyll were present in each sample, then the algae were resuspended in 10ml of 5mM sodium dihydrogen phosphate buffer pH7.5 containing 0.43M NaCl and centrifuged at 3,500 × g for 5 min. The centrifugation was repeated and the algae were resuspended in 10ml of 16mM phosphate buffer, 2mM magnesium chloride pH7.5 which contained NaCl or KCl to change the osmolality of the external medium (Ben-Amotz and Avron, 1973a).

The samples were incubated at 298K in a water bath either in the dark or under continuous light ($9.2Wm^{-2}$). 1ml of 30% trichloroacetic acid was used to terminate the incubation. The deproteinised samples were then centrifuged as before and the amount of glycerol present in the clear supernatant was determined (Lambert and Neish, 1950). 1ml of clear supernatant from each sample was taken and pipetted into separate 100ml volumetric flasks. Distilled water was added to each flask to make them up to 20ml. In addition a set of glycerol standards from 2.17 to 8.69 µmoles ml^{-1} was set up and made up to 20ml with distilled water. A blank flask was also set up which contained only 20ml of distilled water. 1ml of 5M sulphuric acid was added to each flask and then 5ml of 0.1M sodium periodate. Exactly 5 min. later, 5ml of 1M sodium arsenite was added to each flask with mixing. 5-10 min. later each flask was made up to 100ml with distilled water and thoroughly mixed. 1ml from each flask was pipetted into fresh test-tubes which were covered with aluminium foil and 10ml of chromotropic acid reagent (Appendix D) was added to each test-tube. The test-tubes were then placed in a 363K water bath for 30 min. The tubes were removed and cooled on ice. The contents of each tube were made up to 15ml with distilled water to prevent evaporative changes in volume between samples. The optical density of the samples was measured at 570nm in a spectrophotometer against the blank which had no glycerol or algae present. A typical calibration curve is shown in Figure 10.

2.8.2 Enzymatic Method

In the presence of large amounts of sucrose or ethylene glycol, an enzymic method of glycerol determination was used. The principle of the test is:-

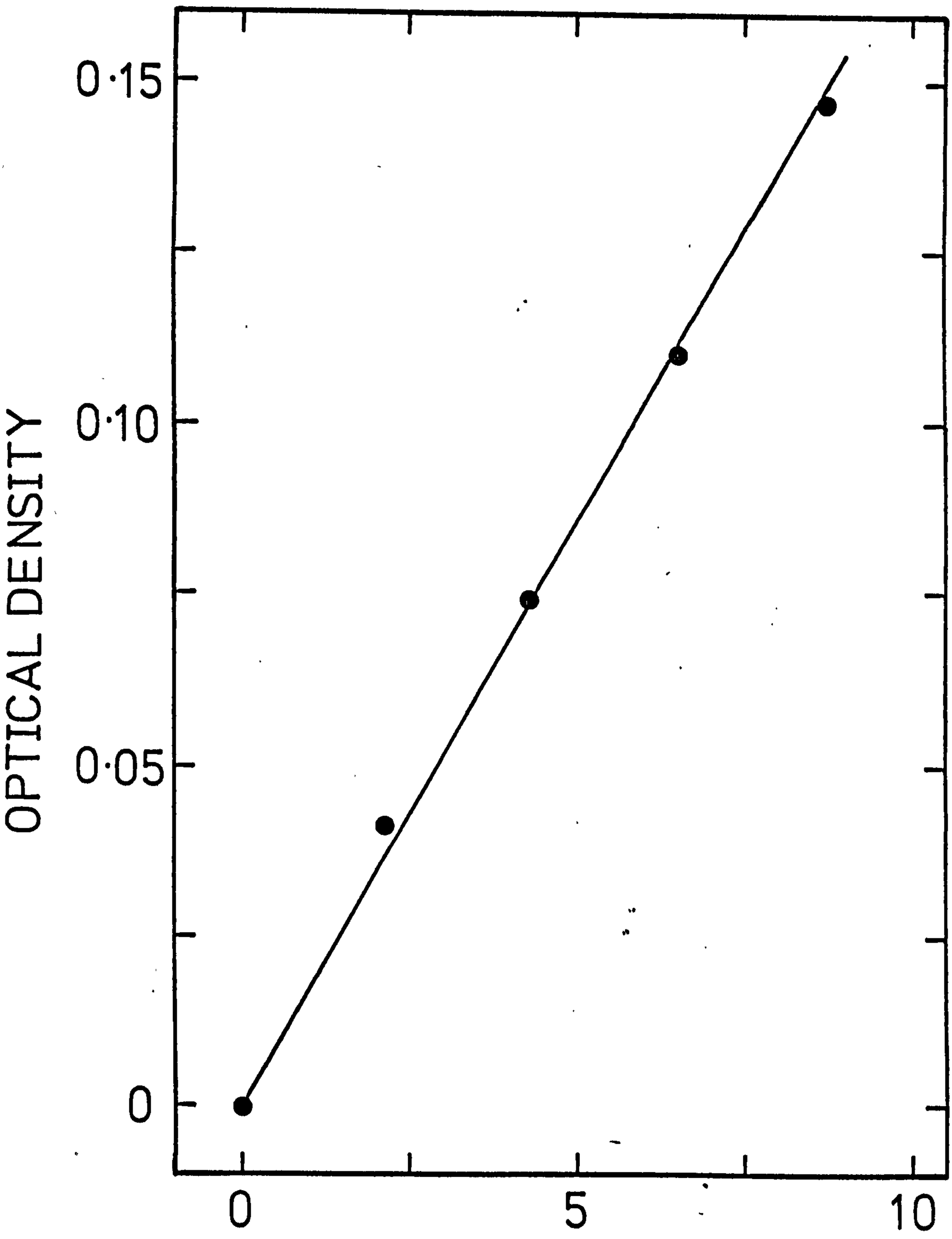


FIGURE 10. A typical calibration curve for the chemical method of glycerol determination.

$$\text{(a)}\quad \text{Glycerol} + \text{ATP} \xrightarrow{\text{GLYCEROKINASE (EC 2.7.1.30)}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{(b)}\quad \text{ADP} + \text{Phosphoenolpyruvate} \xrightarrow{\text{PYRUVATE KINASE (EC 2.7.1.40)}} \text{ATP} + \text{Pyruvate}$$

$$\text{(c)}\quad \text{Pyruvate} + \text{NADH}^{+} + \text{H}^{+} \xrightarrow{\text{LACTATE DEHYDROGENASE (EC 1.1.1.27)}} \text{Lactate} + \text{NAD}^{+}$$

The amount of NADH consumed in the above reaction is stoichiometric with the amount of glycerol present. NADH is determined by means of its absorption at 340nm (Eggstein and Kuhlmann, 1974).

The method of extracting the algae for glycerol analysis was as described in section 2.8.1 except that (a) only 100µg of chlorophyll was present in each sample, (b) for incubation the algae were re-suspended in 1ml of 16mM phosphate buffer, 2mM magnesium chloride pH7.5, which contained NaCl, sucrose or ethylene glycol to raise the osmolality, and (c) 0.1ml of 30% trichloroacetic acid was used to terminate the incubation.

The amount of glycerol present in the clear supernatant extracted from the algae was determined using the enzymatic test kit for glycerol determination in foodstuffs (Boehringer-Mannheim). Appendix D shows the amounts of reagents used in the enzymatic determination of glycerol. Glycerol standards were set up from 1.09 to 4.34 µmoles ml^{-1}. All of the reagents shown in Appendix D were added to 10mm glass cuvettes except the glycerokinase suspension. After 5-7 min. the optical density of each cuvette was measured against a distilled water blank at 340nm. This was repeated 4 or 5 times till a steady reading was obtained. The glycerokinase suspension was now added and measurements of the optical density were taken after 5, 10, 12 and 15 min. A typical calibration curve is shown in Figure 11.

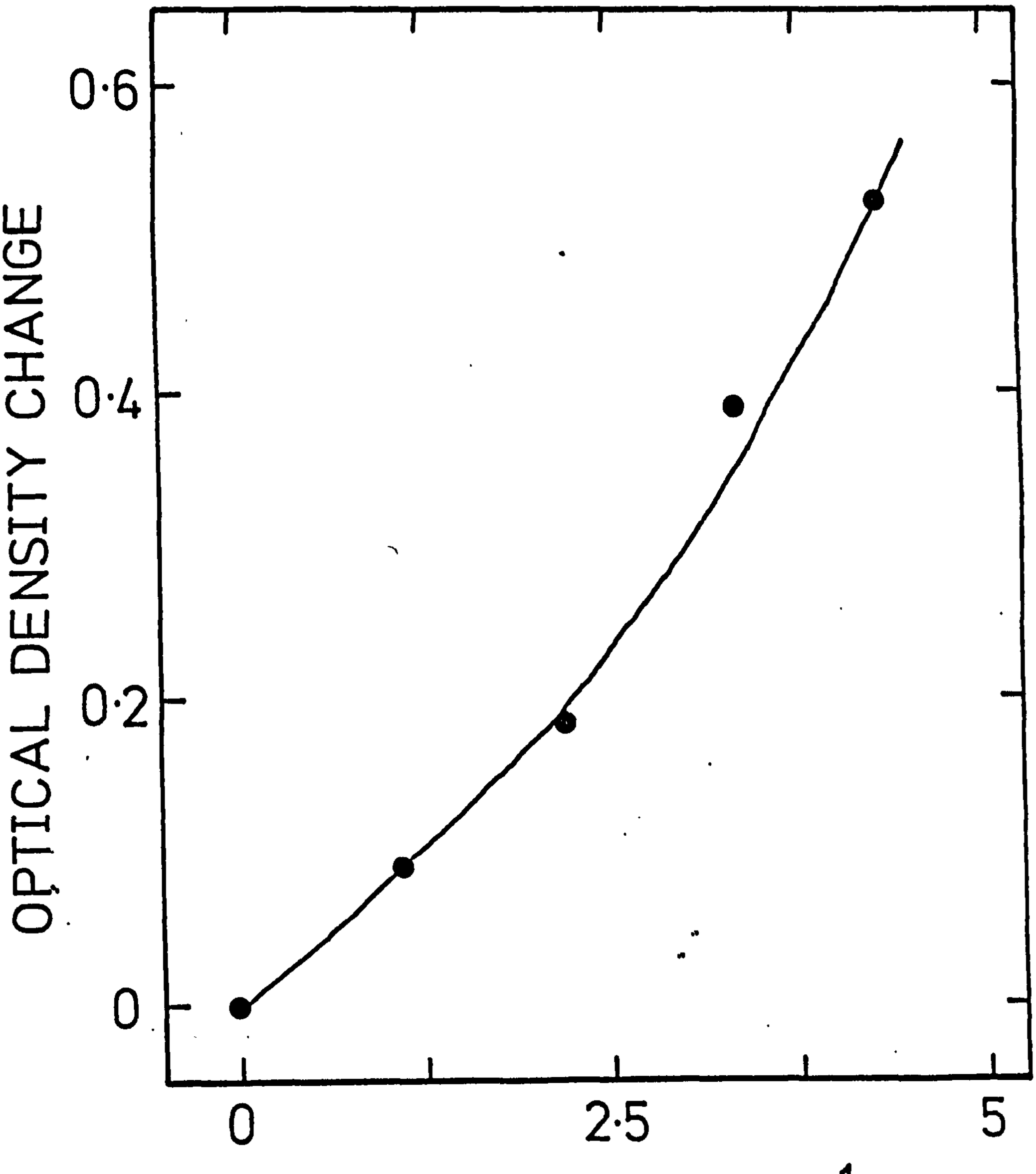


FIGURE 11. A typical calibration curve for the enzymatic method of glycerol determination.

2.9 Measurement of Oxygen Evolution or Uptake

Oxygen evolution or uptake was measured in a modified Clark oxygen electrode (Rank Bros., Bottisham, Cambridge, England) of the type described by Delieu and Walker (1972).

The apparatus consisted of platinum wire sealed in plastic as the cathode, and an anode of circular silver wire bathed in a saturated KCl solution. The electrodes were separated from the reaction mixture by an oxygen-permeable teflon membrane. The reaction mixture in the plastic container was stirred constantly with a small magnetic stirring rod. When a voltage was applied across the two electrodes using the polarising meter, the platinum electrode became negative with respect to the reference electrode and the oxygen in the solution is thought to undergo electrolytic reduction at the cathode:-

$$4H^{+} + O_2 + 4e^{-} = 2H_2O.$$

The flow of current in the circuit when the polarising volts were set between 0.5 and 0.8V varied in a linear relationship to the partial pressure of the oxygen in solution. The instrument was usually operated at a polarising voltage of about 0.65V. The current flowing was measured by connecting the electrode to a sensitive potentiometric chart recorder.

The reaction chamber was kept at a constant temperature by circulating water from a temperature-controlled water bath. Where applicable a light source was used to illuminate the reaction chamber. The intensity of this light was 140Wm^{-2} as measured by a U.D.T. Model 40X Opto-Meter. A diagram of the layout of the apparatus is shown in Figure 12 and a diagram of the electrode is shown in Figure 13.

Calibration of the oxygen electrode consisted of determining the zero

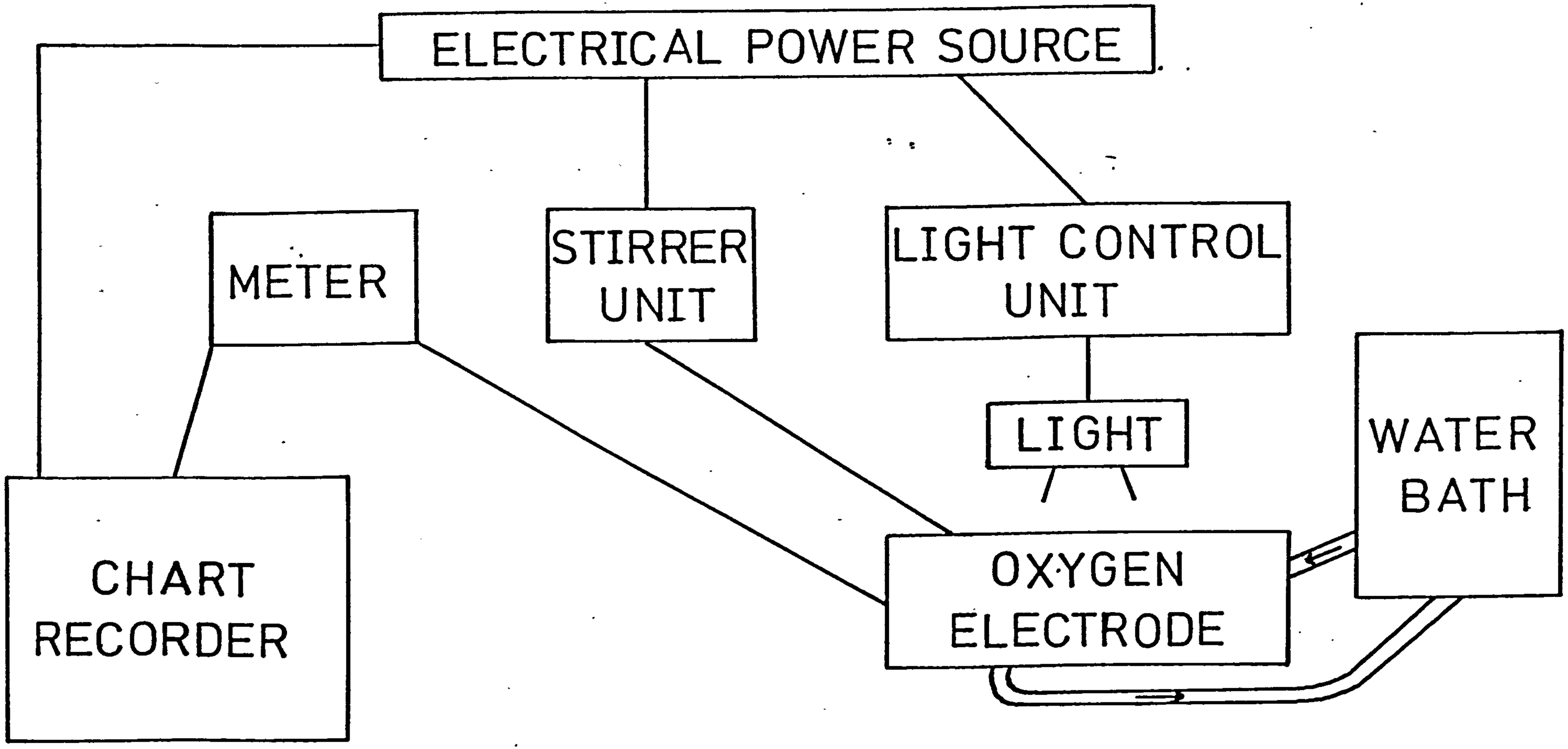


FIGURE 12. The layout of the apparatus used in the oxygen electrode experiments.

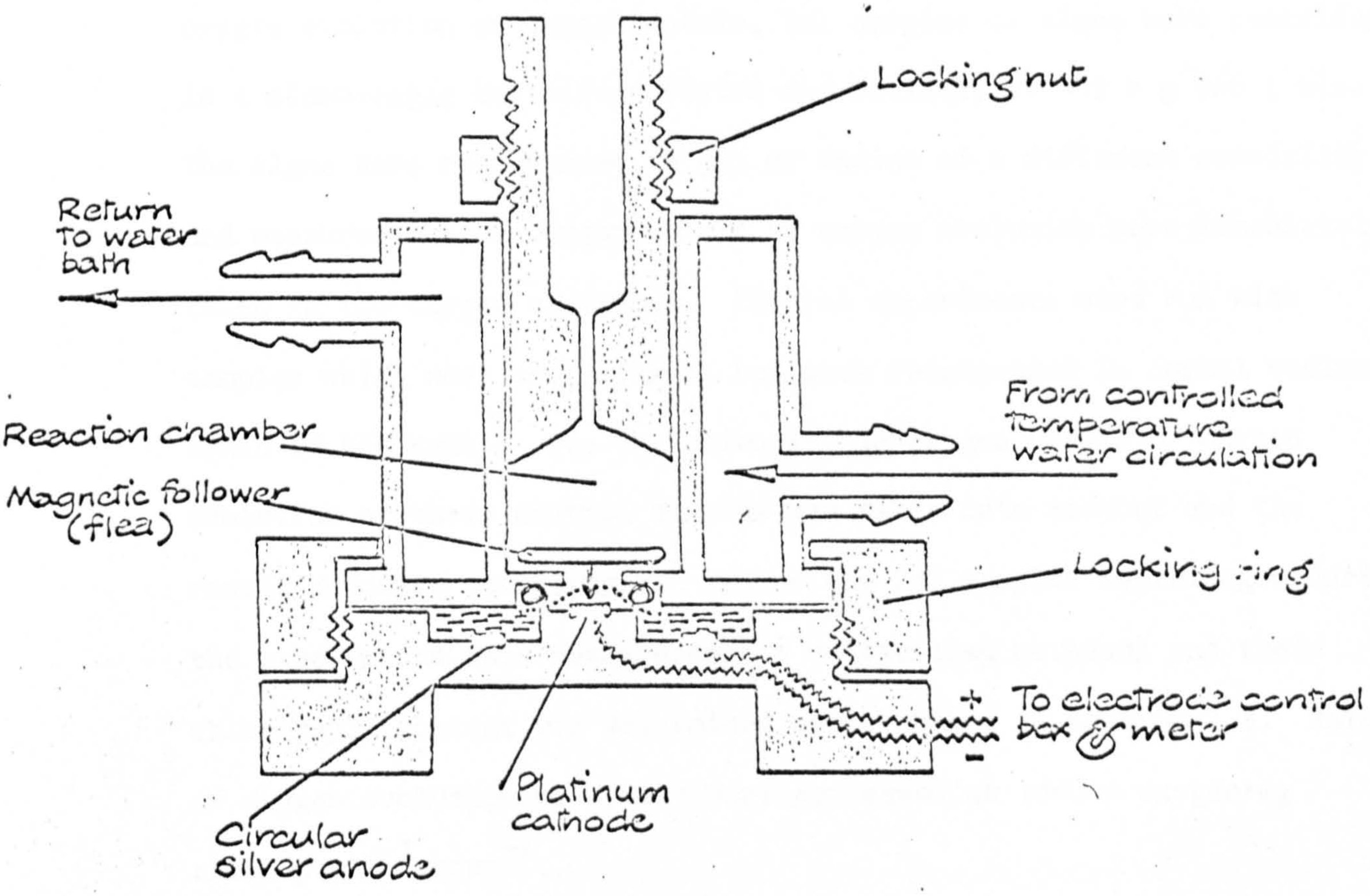


FIGURE 13. A diagram of the oxygen electrode.

oxygen level (by addition of sodium dithionite) and the concentration of oxygen in air-saturated Boney's medium.

One to three days before an experiment took place, 100ml of algae were centrifuged at 536 × g for 15 min. The algae were resuspended in fresh Boney's medium and placed in an illuminated ($9.2Wm^{-2}$) constant temperature water bath at between 294 and 297K. The water bath was connected to the water jacket surrounding the reaction chamber of the oxygen electrode (Figures 12 and 13).

To determine the effect of changes in the external osmolality on oxygen evolution or oxygen uptake, 2ml samples of algae were centrifuged in a micro-angle centrifuge (Baird and Tatlock) at 492 × g for 1 min. The algae were resuspended in 2ml of medium of a different osmolality and measurements of oxygen uptake or oxygen evolution were immediately taken in the oxygen electrode. Control experiments were run with samples which were centrifuged, but were resuspended in normal medium again (0.43M NaCl). The variation in the oxygen uptake or oxygen evolution of these control samples was taken into account and the result adjusted accordingly. Two of the 2ml samples centrifuged during the experiments were resuspended in redistilled methanol and their chlorophyll content was determined as described in section 2.3. Rates of oxygen evolution or uptake were expressed as μmoles oxygen•mg $chlorophyll^{-1}$•$hour^{-1}$.

To express the results in this way the concentration of oxygen present in the solutions must be known. The oxygen concentration values for different salinities of seawater were tabulated in the literature (Carpenter, 1966; Strickland and Parsons, 1968). However, these oxygen concentration values did not go above a salinity of 0.85M NaCl. As oxygen electrode experiments were performed up to 1.71M NaCl, it was necessary to determine the oxygen concentrations of the higher salinities.

Moreover, experiments were performed using KCl, sucrose, ethylene glycol and glycerol additions to seawater, thus the oxygen concentration values for these solutions had to be determined, as they were not available in the literature. Therefore the chemical method of Winkler (1888) was used to determine the oxygen content of the solutions.

The principle of the Winkler test is that when concentrated solutions of divalent manganese and alkaline potassium iodide are added to the water sample, white manganous hydroxide is first formed and then oxidised to manganic hydroxide by the molecularly dissolved oxygen. The brown manganic hydroxide settles to the bottom of the bottle. Sulphuric acid is then added and this dissolves the manganic hydroxide and iodine is liberated. The liberated iodine takes up the excess iodide and forms I_3^-. This solution is then titrated against thiosulphate and the end-point of the titration is indicated by a starch solution (Schlieper, 1972).

The experimental procedure of the Winkler test was as described by Martin (1972). The thiosulphate solution was calibrated as described by Martin (1972) except that a standard solution of potassium dichromate was used instead of potassium bi-iodate solution.

2.10 Measurement of Chlorophyll Fluorescence

The induction of chlorophyll fluorescence intensity of dark-adapted algae was measured using a laboratory built fluorimeter (Figure 14). Light from a 150 watt quartz-iodine lamp (driven by a Coutant stabilised DC supply) was filtered through Corning 4.76, Corning 4.96 and Calflex C filters to give broad-band blue actinic light. Fluorescence of the algal sample was detected at right angles from the exciting beam with an EMI 9659B (extended S20) phototube protected with a Balzers 695nm interference filter and a Schott RG 695

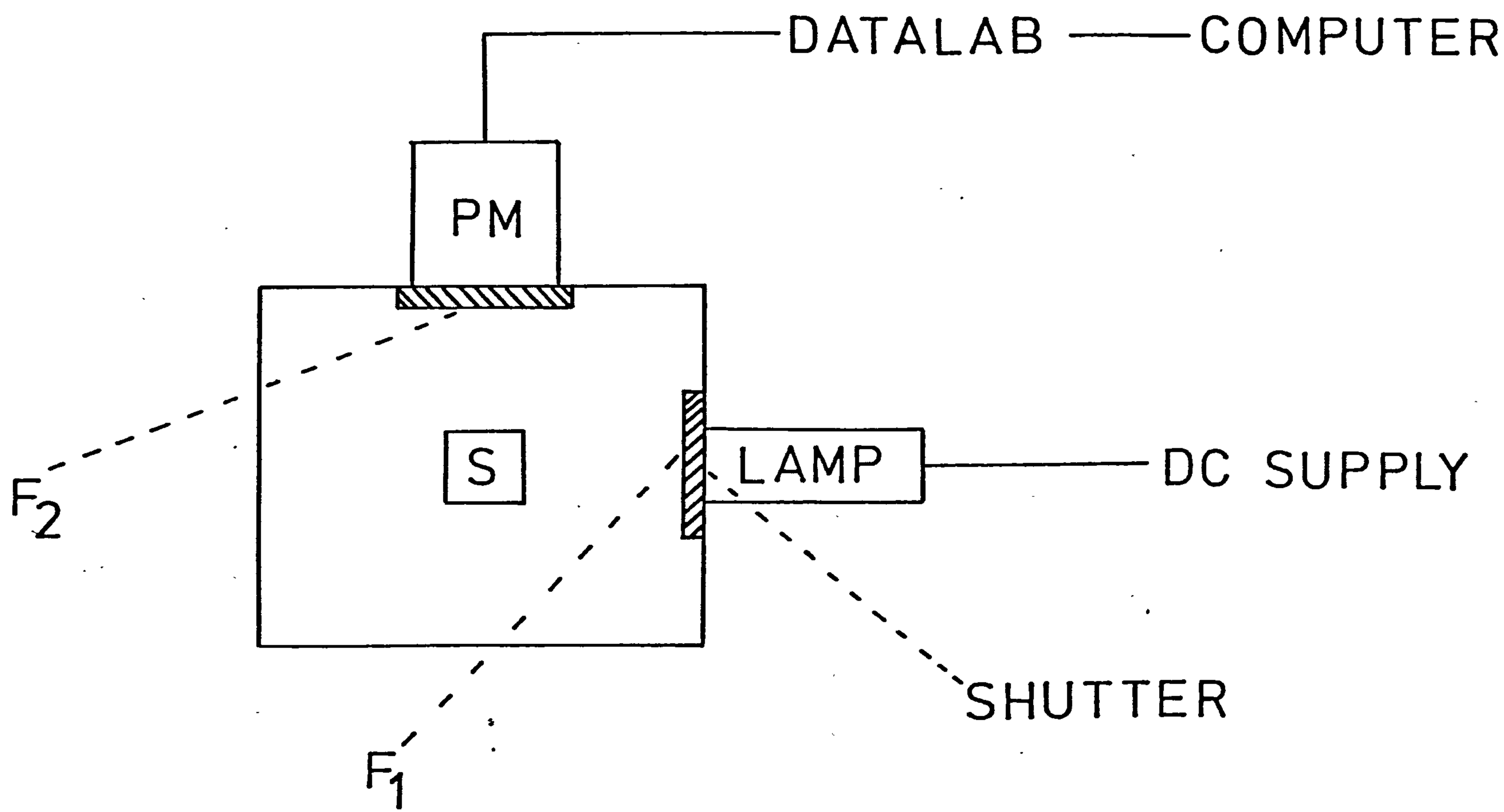


FIGURE 14. The basic apparatus used to measure chlorophyll fluorescence. S is the algal sample in a cuvette, F_1 and F_2 are the actinic and analysis filters respectively and PM is the photomultiplier.

cut-off filter. The photocurrent was passed through a current-to-voltage converter, amplified and fed into a Datalab DL 922 transient recorder (Data Laboratories Ltd, Mitcham, Surrey, England). The data was either plotted on to a potentiometric chart recorder to give a permanent record or it was transferred to the memory of a Digital Equipment Corporation PDP11-34 mini-computer and then plotted out on a Tektronix 4662 plotter to give a graphical record. A solenoid-operated shutter with an opening speed of approximately 2ms was used to switch on the light into the reaction chamber. The actinic light intensity in the reaction chamber was 1.2 Wm^{-2}. The transient was recorded for the first 100ms after the shutter opened. A solution of milk of approximately the same turbidity as the algal suspension was used to determine the amount of actinic light which was detected in the absence of algae.

The experimental procedure for the measurement of chlorophyll fluorescence was that firstly the chlorophyll content of the algal culture was assayed by the hot methanol method (section 2.3). Then, enough algae were centrifuged in a bench-top centrifuge (M.S.E.) at 3,500 × g for 5 min, to give a pellet containing 30µg of chlorophyll. The algal pellet was immediately resuspended in 3ml of the test solution, and placed in a 10mm pathlength cuvette. The cuvette was then placed in the reaction chamber and the algae were dark-adapted for 5 min. 3-(3', 4',-dichlorophenyl)-1, 1-dimethylurea (DCMU) was then added to give a final concentration of 12µM. This operation was carried out in as low a light intensity as possible. The cuvette was replaced in the reaction chamber, and the induction of chlorophyll fluorescence intensity was taken immediately.

In some experiments, chlorophyll fluorescence was measured in the absence of DCMU for much longer time periods than 100ms. On

these occasions, the data was directly plotted on to a potentiometric chart recorder and was not fed into the Datalab transient recorder.

A further series of fluorescence measurements were made in collaboration with Dr. Neil Baker and Andy Weber at the Biology Department, University of Essex. The apparatus allowed us simultaneously to measure the fluorescence kinetics at 695 and 720 nm at room temperature (293K). In addition, fluorescence spectra measured from 650 to 800 nm were recorded at 77K. In all the experiments an exciting beam of 440 nm with an intensity of 100 μmol. photons $m^{-2}s^{-1}$(27 Wm^{-2}) was used. The entrance and exit slits were 1.25 mm and 5 nm respectively. More details of this experimental system can be found in the papers by Bradbury and Baker (1981a and b).

For the 293K kinetic measurements at 695 and 720 nm, cells were centrifuged and resuspended in the test solution, then spotted on to filter paper to form a 'leaf-like' sample. They were then dark-adapted for 5 min before the fluorescence measurements were taken. The filter paper always remained damp with the suspending medium during the periods of dark-adaptation and fluorescence measurement. The simultaneous measurement of fluorescence emission at 695 and 720 nm was made possible by using bifurcated fibre optic light pipes attached to a perspex light-mixing rod.

For the 77K fluorescence emission spectra, the algae were centrifuged and resuspended in the test solution. They were dark-adapted for 5 min at 293K, then immersed in liquid nitrogen for 5 min prior to measurement. 0.5 ml of cell suspension was frozen to produce a 0.5 cm thick pellet for spectral measurement.

2.11 Measurement of the Field-Indicating Absorption Change at 519 nm

The light-induced absorption change at 519 nm was measured in a rapidly-responding single-beam spectrophotometer (Figure 15). Light from a 150 watt tungsten-halogen projector bulb, was passed through an Applied Photophysics M380 monochromator to give a weak measuring beam of light at 519 nm with an intensity of 0.1 Wm^{-2}. The beam was passed through the algal sample in a 10 mm pathlength cuvette and was detected by an EMI 9659B (extended S20) photomultiplier supplied with a high voltage of 700-800V from a Brandenburg 475R H.T. source. The actinic illumination was provided by flashes of 3 μs duration and 0.5J energy (electrical) generated by a General Radio model 1539 'Stroboslave' perpendicular to the axis of the measuring beam. A Wratten 92 red filter was placed over the actinic source to give a suitable cut-off wavelength and intensity. A set of blue-green filters were placed in front of the photomultiplier tube to eliminate the scattered actinic light. Normally these were a Corning 4.96 filter plus a Barr and Stroud 523 nm interference filter, but occasionally a second 4.96 filter was also used. The photocurrent was passed through a current-to-voltage converter, amplified and fed into a Datalab DL922 transient recorder.

Due to the small size of the signal, it was necessary to sum a number of measurements and this was achieved by transferring the signal from the Datalab transient recorder to the memory of the PDP11-34 mini-computer. In this way further signals were added and plotted on a Tektronix 4662 plotter to give a graphical record. The sequence of events during the accumulation of the signal is shown in Figure 16. This type of signal averaging allows an improvement in the signal to noise ratio as is illustrated in Figure 17. For this type of system to be effective the noise associated with the signal must be randomly

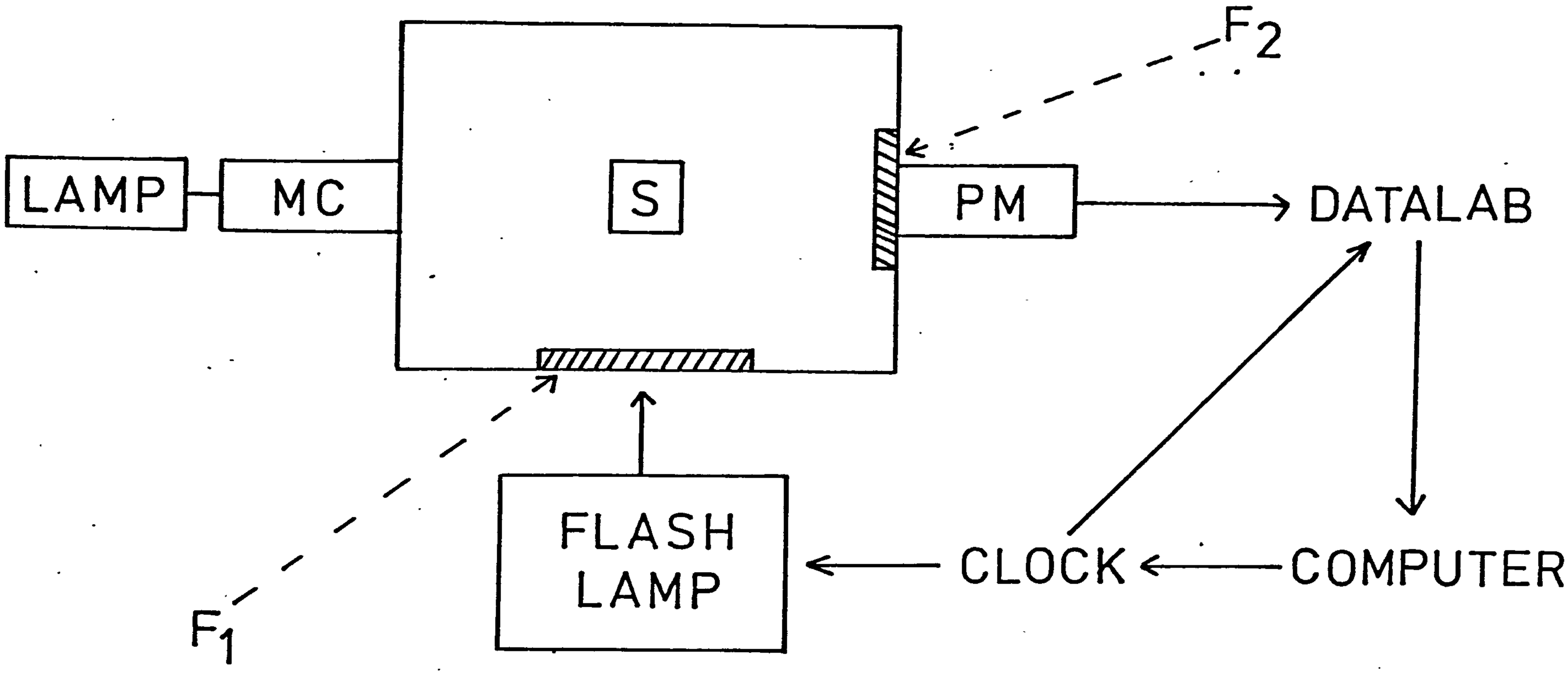


FIGURE 15. The apparatus used to measure the light-induced absorption change at 519 nm. MC_1 is the monochromator; S is the algal sample; PM is the photomultiplier; F_1 is the actinic filter; and F_2 are the blue-green analysis filters.

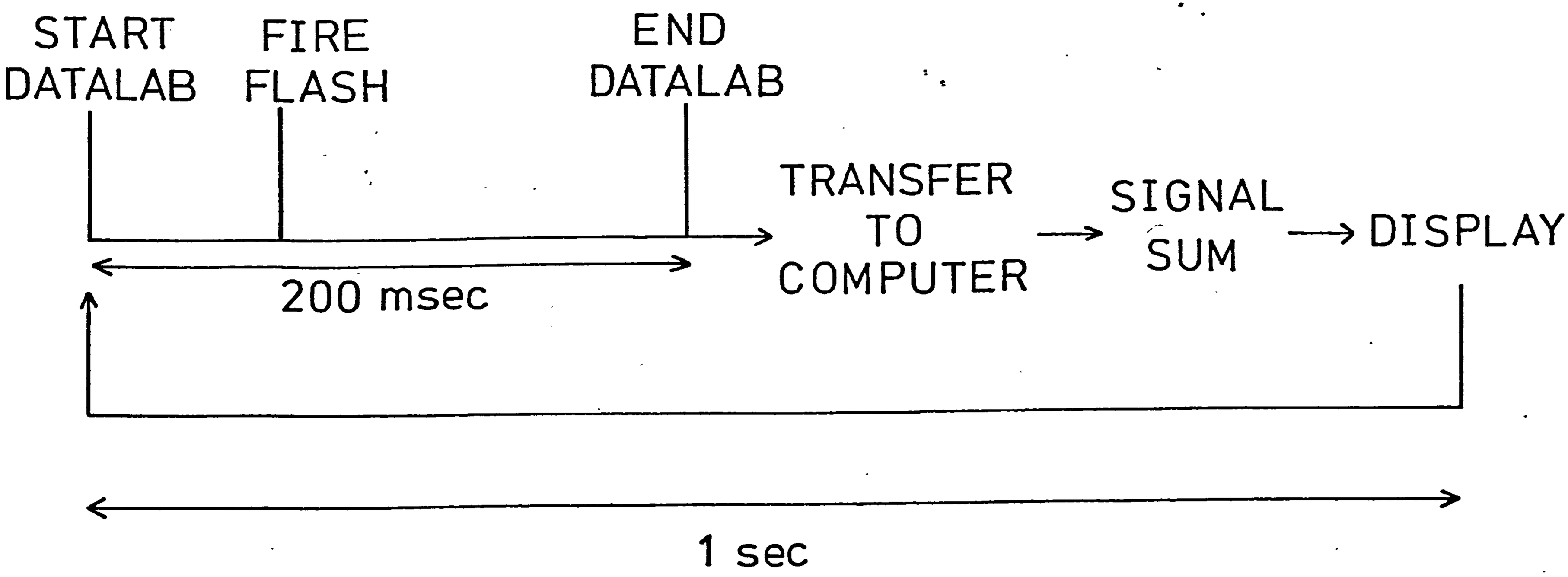


FIGURE 16. The sequence of events during the accumulation of the signal in the absorption change experiments.

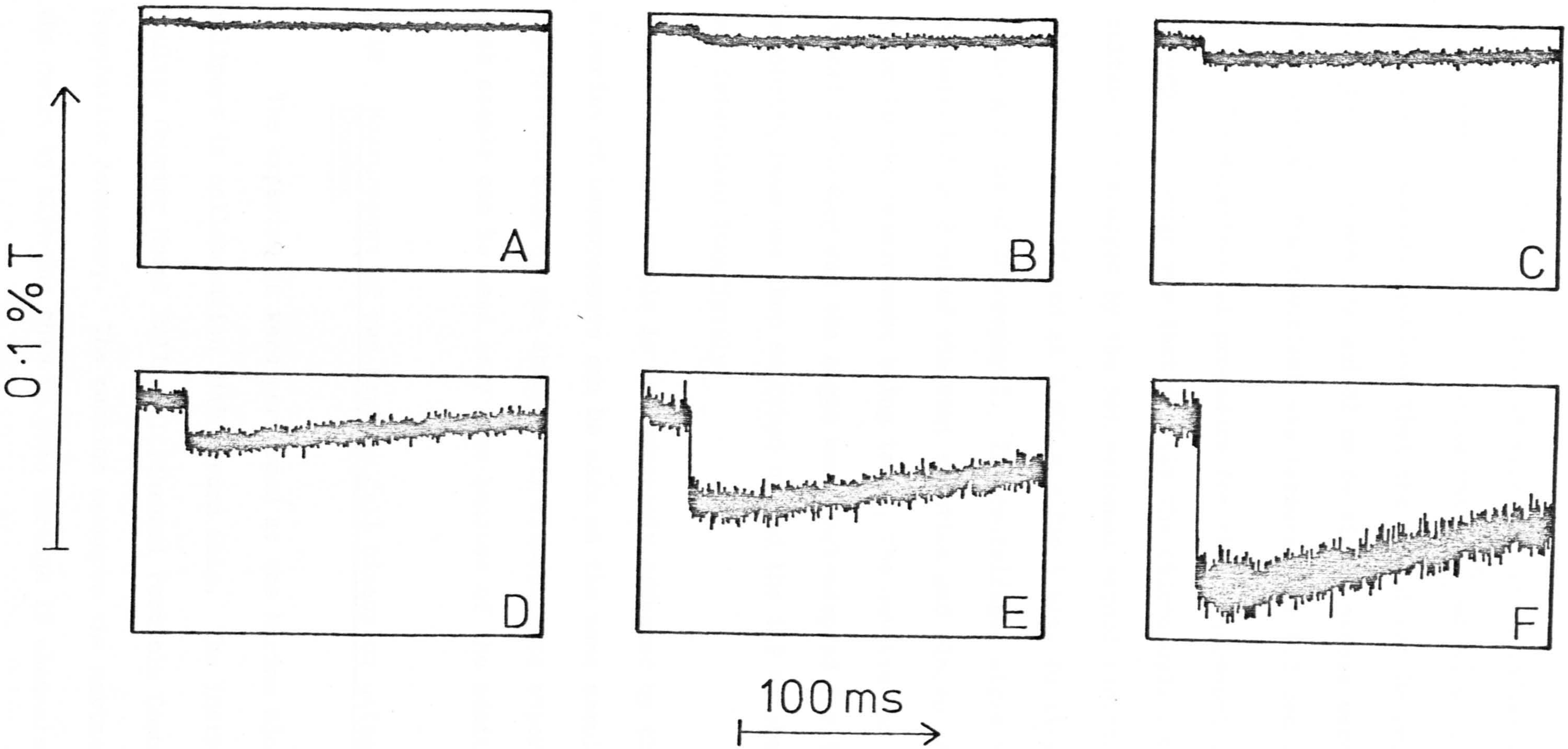


FIGURE 17. The effect of increasing the number of signal sweeps summed on the signal to noise ratio. A: 1 sweep; B: 4 sweeps; C: 8 sweeps; D: 16 sweeps; E: 32 sweeps; F: 64 sweeps. % T is the percentage change in transmission. The algae were resuspended in 0.43 M NaCl (normal growth medium) for 5 min in the dark prior to the measurements.

distributed in time so that it cancels out as individual signals are summed. The signal to noise ratio $\left(\frac{S}{N}\right) \propto \sqrt{n}$, where n is the number of signals summed, provided that the signal can be repeated at will. The flash rate was 1Hz and 32 or 64 signal sweeps were summed for each sample. The transient was measured for 0.2 sec (Figures 16 and 17).

The experimental procedure for the measurement of the 519 nm absorption change was that firstly the chlorophyll content of the culture was assayed by the hot methanol method (section 2.3). Enough algae were centrifuged at 3,500 × g for 5 min, to give a pellet containing 90 µg of chlorophyll. The centrifuged algae were then resuspended in 3 ml of the test solution and placed in a 10 mm cuvette. Prior to the measurement being taken, the cuvette was placed in the reaction chamber and the algae were dark-adapted for 5 min. The measuring beam was then switched on and the 519 nm absorption change was determined immediately.

The algal sample is not adversely affected by this process and a series of measurements can be made on the same sample. This is not the case in some of the chlorophyll fluorescence experiments where each sample can be used only once because of the addition of DCMU.

2.12 Measurement of the Maximum Cell Dimension using a Coulter Counter

The experiments were performed at the Marine Biological Station, Millport in collaboration with Gordon Gale. The instrument used was a Coulter Counter Model TAII, Multichannel Particle Counter with Population Accessory. The machine measures the maximum dimension of the cells by allowing them to pass through 16 channels of different sizes. The instrument was calibrated using PDVB latex particles with a maximum dimension of 13.5µ and from this the size range of the

channels can be calculated.

The experimental procedure was that a known volume of cells was centrifuged and resuspended in 3 ml of the test solution. After 5 min, 1.5 ml of the sample was added to a large volume of filtered seawater (of normal salinity i.e. approximately 30 ‰ or 0.5 M NaCl) already present in the machine. The number of cells which passed through each channel was immediately determined. Before the addition of the algae, a count of the particles present in the filtered seawater was made, and this background count was subtracted from the count with algae present. After approximately 2 hours, the remaining 1.5 ml of the algal sample was treated in a similar fashion. During this 2 hour period, the samples were kept in ambient room light.

2.13 Determination of Standard Errors

Most of the experiments in this study were repeated more than once and the standard errors of the results were calculated as follows:-

$$\text{Standard error} = \sqrt{\frac{s^2}{n}}$$

where,

$$s^2 = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}$$

x is the individual value of each observation.

n is the number of observations.

Results were recorded as $\bar{x} \pm$ the standard error where $\bar{x}$ is the mean of the x's. Thus when the results were graphed, the means were the points on the graph and the error bars were two times the standard error i.e. one standard error above and below the point.

CHAPTER 3

GLYCEROL SYNTHESIS AND CELL SIZE AND SHAPE CHANGES

3.1 Introduction

Glycerol synthesis by Dunaliella in response to an increase in the external NaCl concentration was first observed by Craigie and McLachlan (1964). This observation was confirmed in various species of Dunaliella: D. tertiolecta (Wegmann, 1971; Borowitzka and Brown, 1974; Jones and Galloway, 1979), D. parva (Ben-Amotz and Avron, 1973a; Gimmler and Schirling, 1978), D. salina (Ben-Amotz and Avron, 1978), D. viridis (Borowitzka and Brown, 1974) and D. bardawil (Ben-Amotz and Avron, 1980). A decrease in the NaCl concentration of the external medium was reported to cause a decrease in the intracellular glycerol content of the alga (Ben-Amotz and Avron, 1973a; Brown and Borowitzka, (1979). The results presented in section 3.2 demonstrate that the strain of D. tertiolecta used in this project behaved in a similar fashion to other species of Dunaliella with respect to its intracellular glycerol content.

Section 3.3 shows the results of Coulter counter experiments to determine if rapid volume decreases occur in cells of D. tertiolecta in response to increases in the external osmolality. The change in shape of D. tertiolecta cells in response to an increase in the external salinity is also shown in section 3.3.

For all the experiments described in this chapter, the algae were grown in a basic medium (Appendix A) of 0.43 M NaCl (0.8 Os kg^{-1}) and transferred to test media which contained NaCl, KCl, sucrose, ethylene glycol or glycerol. The test media were made up by adding these substances to the basic medium to raise the osmolality to

3.2 Os kg^{-1}. Thus each substance exerted an osmotically equivalent stress (Appendix C). In some experiments the salinity of the test medium was decreased to 0.1 M NaCl (0.2 Os kg^{-1}) or to zero (distilled water) (Appendix B).

The implications of the results are briefly discussed in section 3.4.

3.2 Glycerol Production by D. tertiolecta

The effect on glycerol production by D. tertiolecta of increasing or decreasing the osmolality of the growth medium was determined. In Figures 18, 19 and 22-24 the value given for the glycerol content includes the glycerol found in the cells and the glycerol found in the external medium. However, the salinity decrease data in Figure 18 show the amount of intracellular glycerol only. Figures 20 and 21 differentiate between intracellular and extracellular glycerol.

Figure 18 shows that an increase in the external NaCl concentration from 0.4 to 1.7 M causes an increase in the glycerol content from 20 to 50 µmoles mg chl^{-1} during the 160 min after the salinity was increased. Cells which were treated identically, but were not subjected to a salinity increase (transferred from 0.4 to 0.4 M NaCl) showed no increase in glycerol content. However, algae which were transferred from 0.4 to 0.1 M NaCl lost glycerol from inside the cell. The glycerol loss took place within 20 min of the salinity decrease and there was little change in the intracellular glycerol level over the next 80 min (Figure 18). After a salinity decrease to 0.1 M, the amount of glycerol in the cells plus the amount in the external medium was between 14.8 and 19.4 µmoles mg chl^{-1} (data not shown). This suggests that the majority of the glycerol lost was excreted into the medium and was not

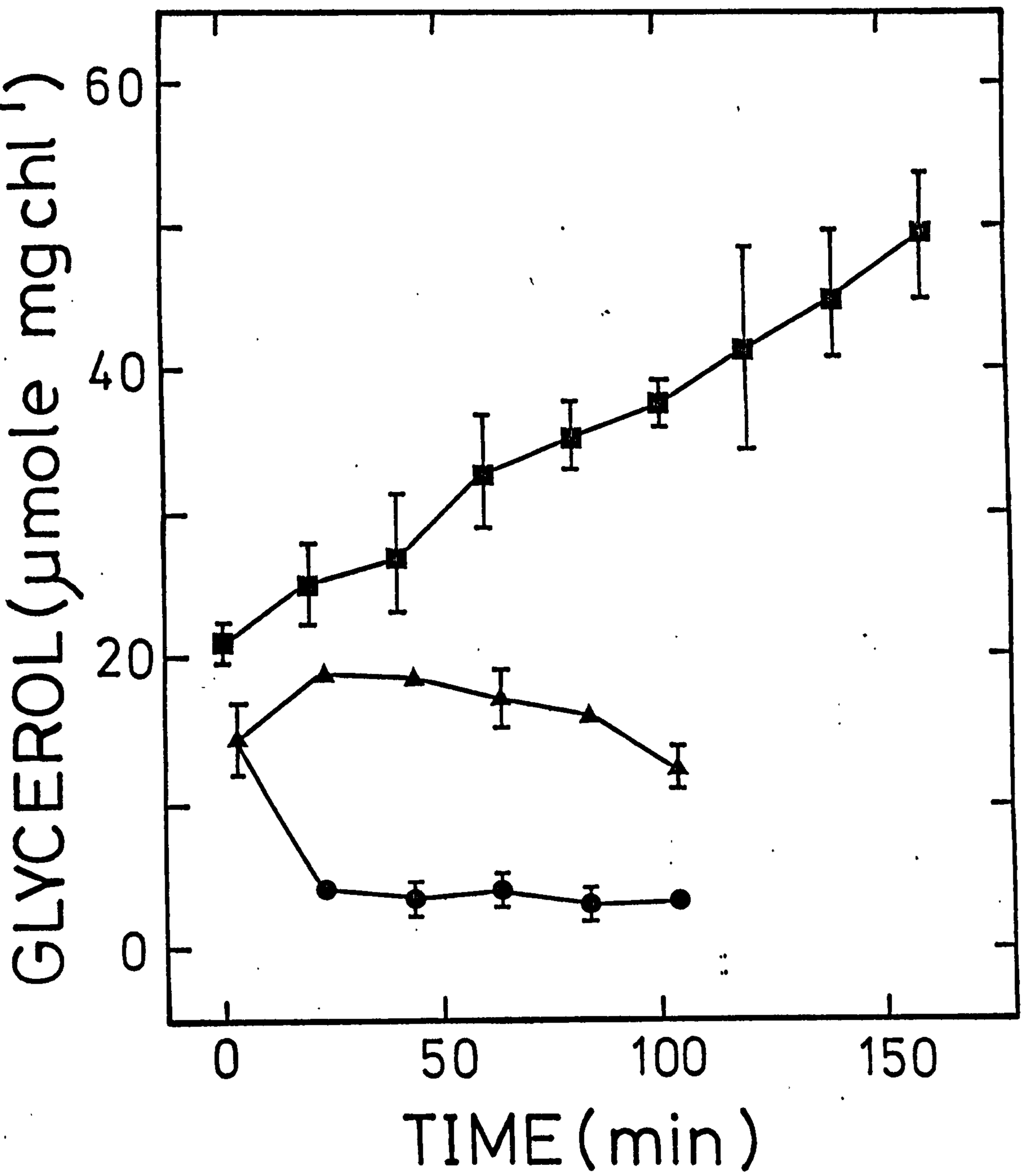


FIGURE 18. The effect of increasing or decreasing the external NaCl concentration on the glycerol content of the algal cells plus the growth medium.

▲——▲ 0.4 M NaCl (0.8 Os kg^{-1}), control

■——■ 1.7 M NaCl (3.2 Os kg^{-1})

●——● 0.1 M NaCl (0.2 Os kg^{-1}), cells only

Glycerol was determined by the chemical method. Each point represents the mean of at least three determinations.

metabolised internally (Figure 18).

Figure 19 shows that KCl can also induce glycerol synthesis in D. tertiolecta when it is added to the growth medium. In this case, an osmotically equivalent solution of KCl induces less glycerol production by the algae than NaCl (Figures 18 and 19). However, the KCl-induced increase in glycerol synthesis is of the same order as that induced by NaCl (Figures 21A and B).

Figures 20 and 21 differentiate between total glycerol (i.e. cells + medium) and glycerol found only in the medium. Figure 20 shows that after an increase in salinity from 0.4 to 1.7 M NaCl, the glycerol produced is retained within the cell and is not lost to the medium during the 360 min period after the salinity increase. Figure 21A confirms this result over the shorter time period of 160 min, however Figure 21B shows that it is not the case in a KCl medium of the same osmolality. The amount of glycerol found in the medium is very much higher in the case of KCl and it increases as the overall glycerol content increases. The amount of glycerol found in the medium expressed as μmoles ml^{-1} is shown in Table 5 and indicates clearly that the concentration of extracellular glycerol is significantly higher in the presence of KCl. In addition Table 5 shows that the % of total glycerol in the medium falls after an increase in external NaCl concentration, this appears to be due to an increase in intracellular glycerol with only a small amount lost to the medium. With an increase in KCl concentration the % of total glycerol in the medium stays much the same indicating that a significant proportion of the glycerol synthesised intracellularly is being lost to the medium.

Figure 22 shows that the addition of sucrose to the growth medium induces a larger glycerol content after 160 min than a NaCl

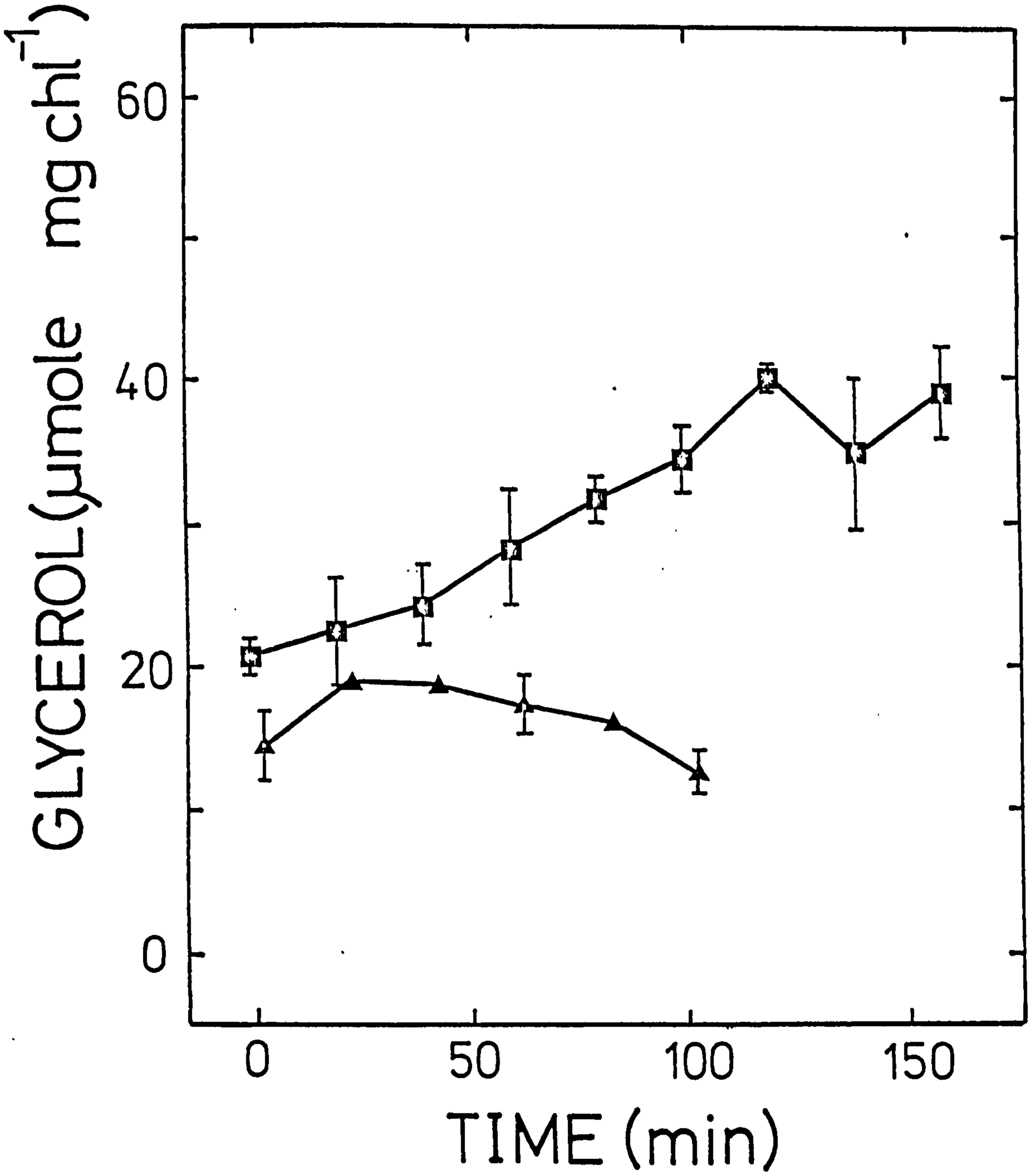


FIGURE 19. The effect of increasing the external KCl concentration on the glycerol content of the algal cells plus the growth medium.

▲——▲ 0.4 M NaCl (0.8 Os kg^{-1}), control

■——■ control plus 1.3 M KCl (3.2 Os kg^{-1})

Glycerol was determined by the chemical method. Each point represents the mean of at least three determinations.

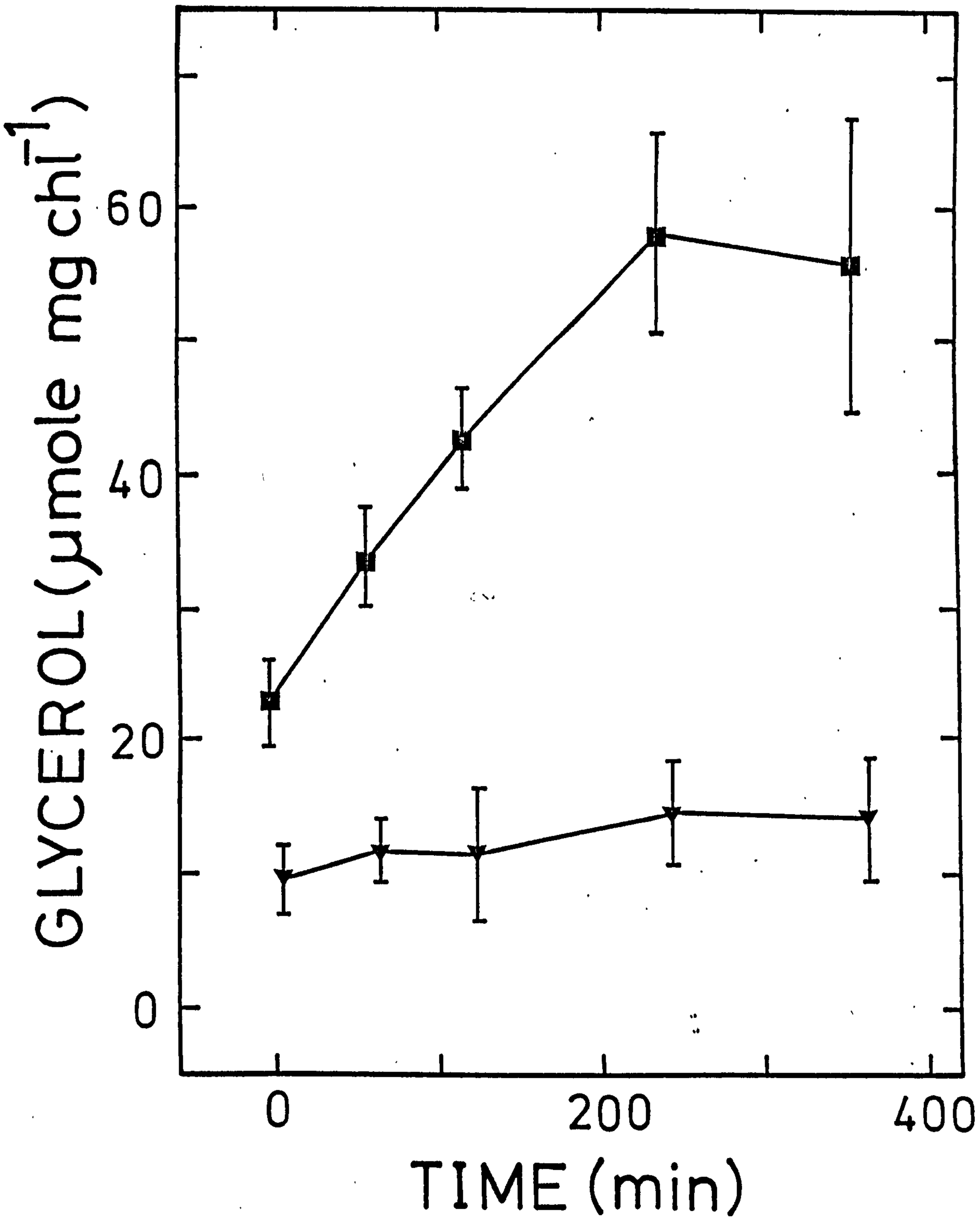


FIGURE 20. The amount of glycerol found in the growth medium after an increase in the external NaCl concentration.

■——■ 1.7 M NaCl (3.2 Os kg^{-1}), cells + medium

▼——▼ 1.7 M NaCl (3.2 Os kg^{-1}), medium only

Glycerol was determined by the chemical method. Each point represents the mean of three determinations.

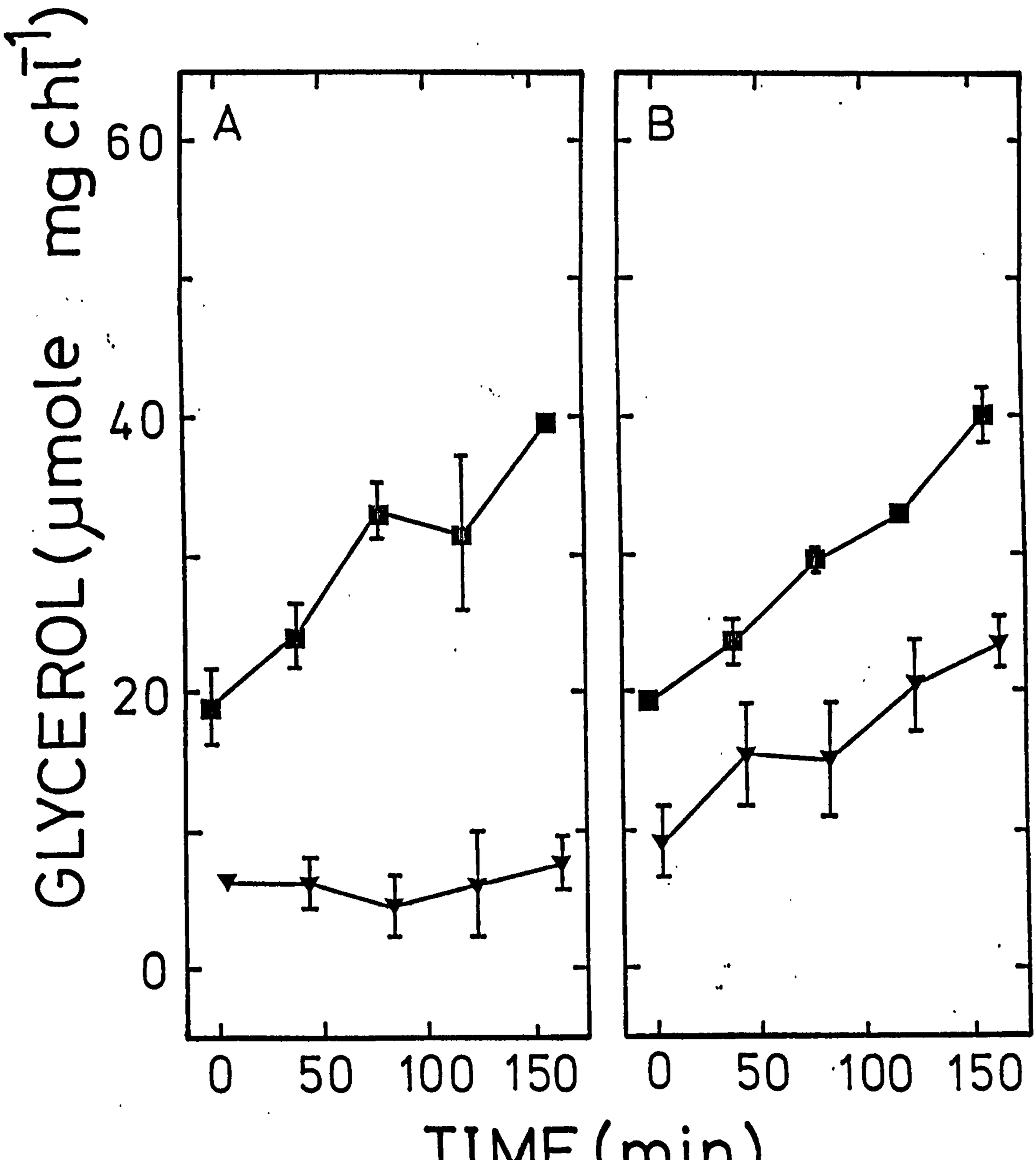


FIGURE 21. The amount of glycerol found in the growth medium after an increase in the external osmolality to A: 1.71 M NaCl (3.2 Os kg^{-1}); and B: 0.4 M NaCl + 1.3 M KCl (3.2 Os kg^{-1}). The glycerol content was determined in the cells + medium (■———■) and in the medium only (▼———▼). Glycerol was determined by the chemical method. Each point represents the mean of three determinations.

TABLE 5

The Amount of Glycerol Found in the External Medium After an Increase in the External NaCl Concentration From 0.4 M (0.8 Os kg^{-1}) to 1.7 M (3.2 Os kg^{-1})

TIME (min)	Glycerol in Medium (μmoles ml^{-1})	% of Total Glycerol in Medium
0	1.5 ± 0.4	40.6 ± 6.2
60	1.8 ± 0.3	36.5 ± 9.3
120	1.7 ± 0.7	29.0 ± 13.0
240	2.2 ± 0.6	27.5 ± 9.2
360	2.1 ± 0.7	30.2 ± 12.5

(Data from Figure 20)

The Amount of Glycerol Found in the External Medium After an Increase in the External Osmolality From 0.8 to 3.2 Os kg^{-1} Using Either NaCl or KCl

	NaCl		KCl	
TIME (min)	Glycerol in Medium (μmoles ml^{-1})	% of Total Glycerol in Medium	Glycerol in Medium (μmoles ml^{-1})	% of Total Glycerol in Medium
0	1.0 ± 0.1	35.8 ± 7.3	1.4 ± 0.4	48.5 ± 13.3
40	0.9 ± 0.3	27.3 ± 9.6	2.3 ± 0.6	65.6 ± 14.5
80	0.7 ± 0.3	13.1 ± 6.3	2.3 + 0.6	50.7 ± 11.5
120	0.9 ± 0.6	21.0 ± 11.8	3.1 ± 0.5	62.0 ± 10.7
160	1.2 ± 0.3	19.5 + 5.1	3.5 ± 0.3	59.3 ± 6.7

(Data from Figure 21)

Each value is the average of three determinations plus or minus the standard error.

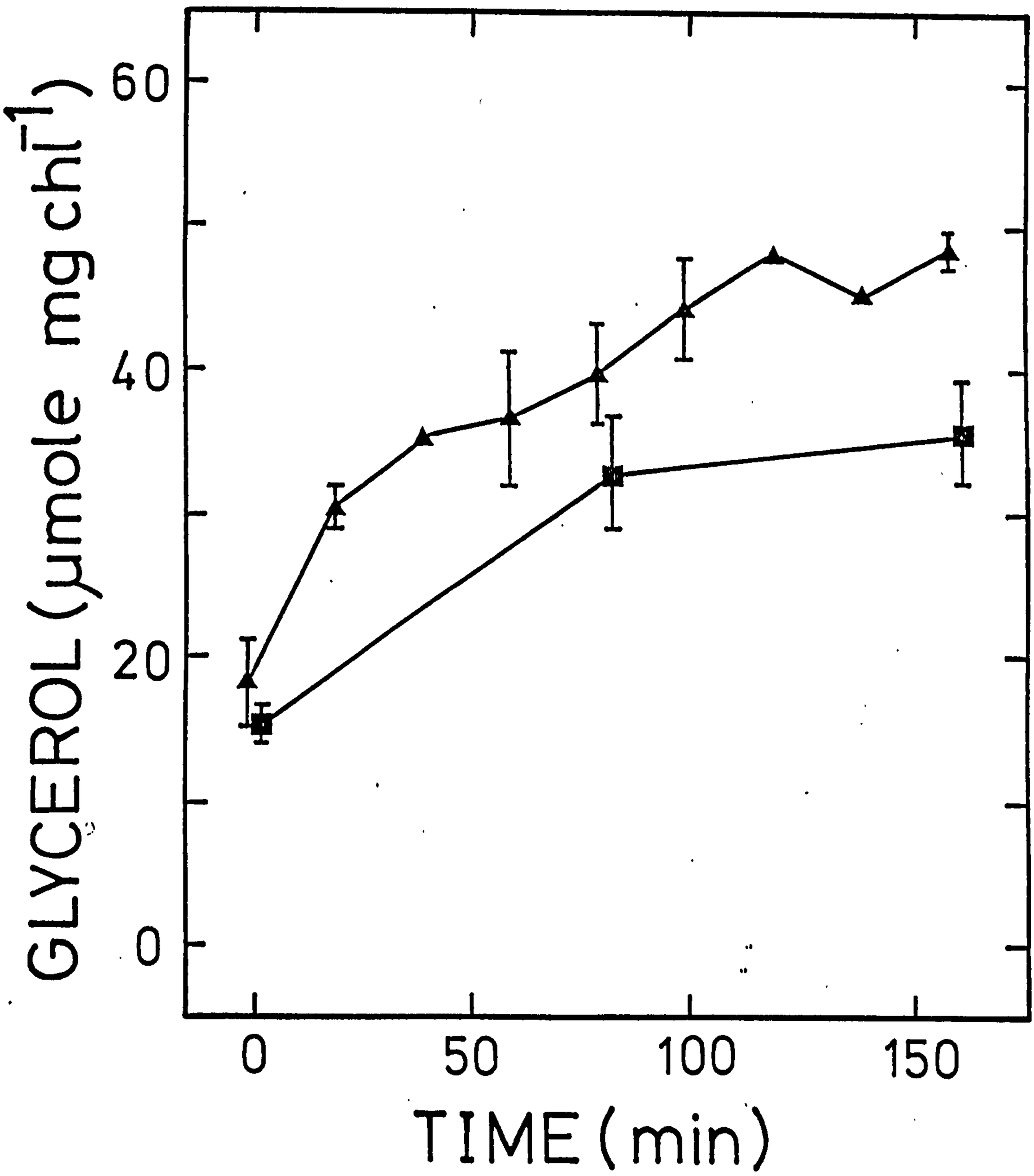


FIGURE 22. The effect of increasing the external sucrose concentration on the glycerol content of the algal cells plus the growth medium.

■——————■ 1.7 M NaCl (3.2 Os kg^{-1})

▲——————▲ 0.4 M NaCl + 1.4 M sucrose (3.2 Os kg^{-1})

Glycerol was determined by the enzymatic method. Each point represents the mean of three determinations.

medium of the same osmolality. However, an ethylene glycol medium of the same osmolality did not induce any glycerol synthesis by D. tertiolecta (Figure 23).

Figure 24 shows that glycerol can be produced in the dark in response to a salinity increase from 0.4 to 1.7 M NaCl, although the amount synthesised was decreased. A similar amount of glycerol production was also possible in the light in the presence of the inhibitor of photosynthetic electron transport, DCMU. After 100 min in the dark, the % glycerol produced compared to that in the light was 57.2 $\pm$ 10.5, in the presence of DCMU, the % was 60.1 $\pm$ 11.7. Therefore just over half the glycerol production usually found in response to a NaCl increase took place in the presence of DCMU or in the dark.

3.3 Cell Size and Shape Changes

To determine if volume changes occur in D. tertiolecta in response to an increase in the osmolality of the medium, the cells were measured in a Coulter counter (particle size analyser). The Coulter counter electrically senses the displacement of electrolyte as the cells pass through the orifice. The maximum dimension of the cells were determined and presented as a histogram of the % of cells found within the previously calibrated size ranges (section 2.12). Figure 25 shows that after 5 min there is almost no change in cell size after an increase in osmolality from 0.8 to 3.2 Os kg^{-1} exerted by NaCl or glycerol and only a very small decrease in cell size when the same osmolality is applied using KCl or sucrose. After 2 hours the cell size has returned to normal in the presence of these osmotica. The case of ethylene glycol is interesting since there is a small cell size increase after 5 min at 3.2 Os kg^{-1} and after 2 hours there is still

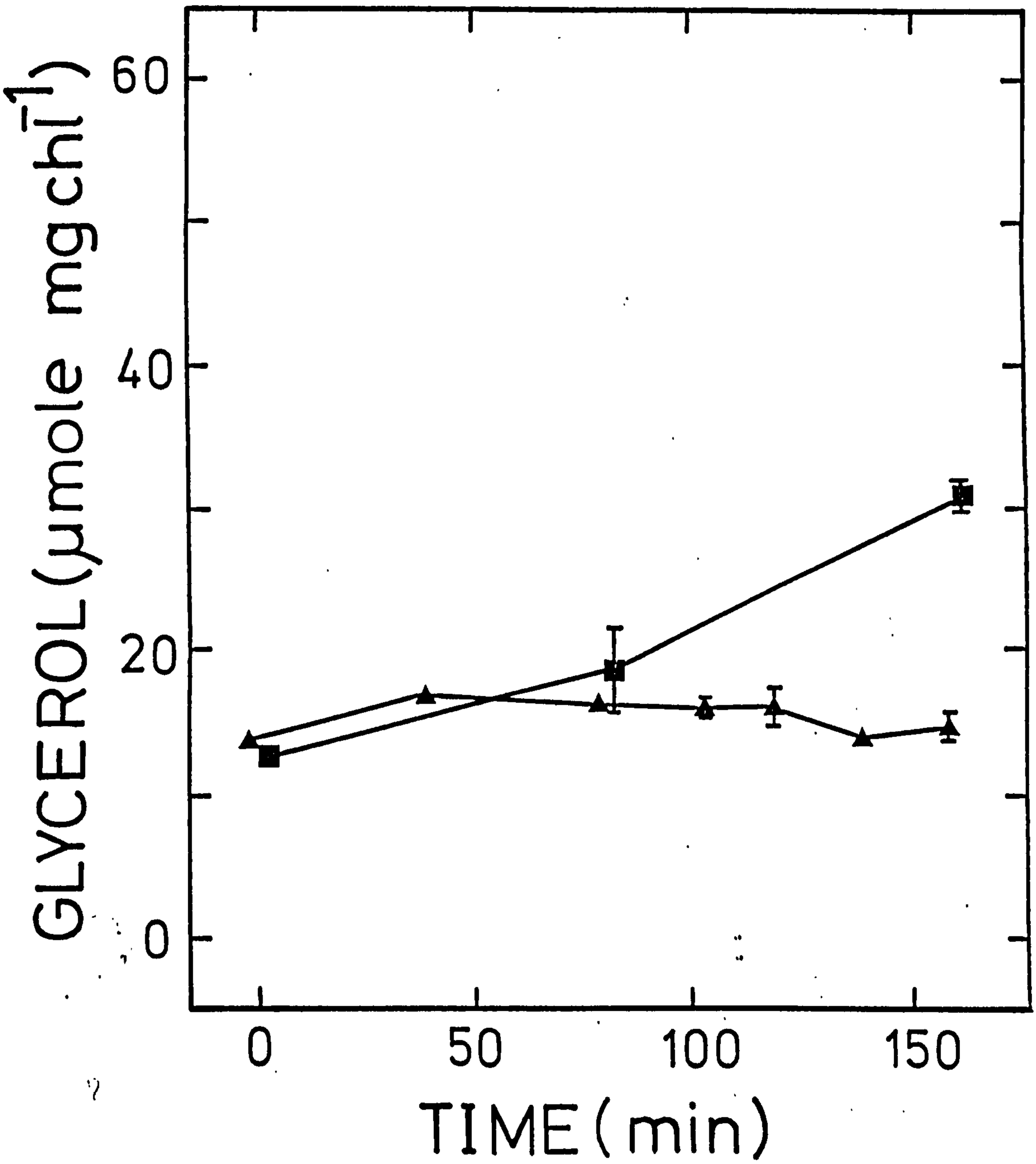


FIGURE 23. The effect of increasing the external ethylene glycol concentration on the glycerol content of the algal cells plus the growth medium.

■——■ 1.7 M NaCl (3.2 Os kg^{-1})

▲——▲ 0.4 M NaCl + 2.1 M ethylene glycol (3.2 Os kg^{-1})

Glycerol was determined by the enzymatic method. Each point represents the mean of three determinations.

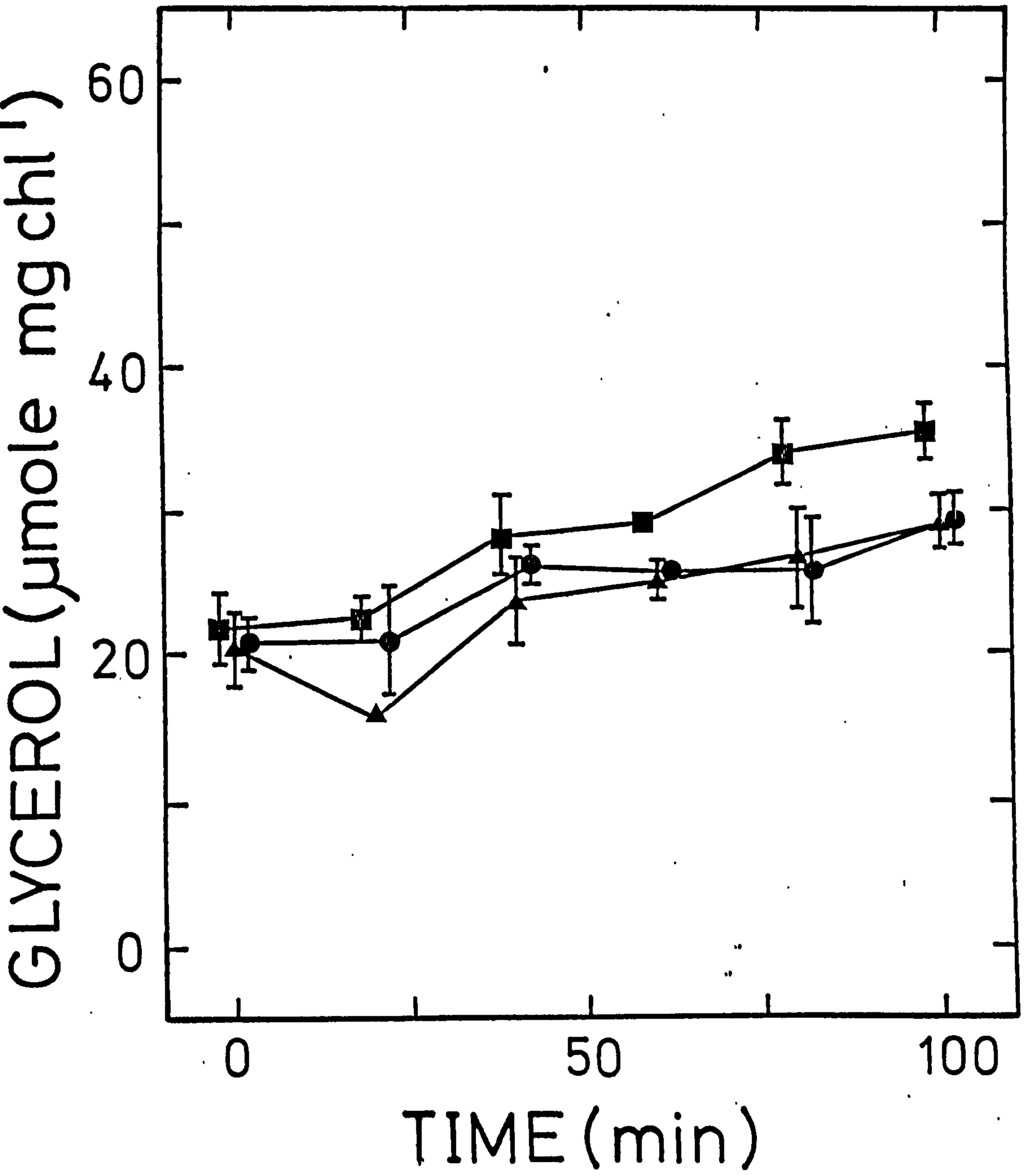


FIGURE 24. The effect of increasing the external NaCl concentration to 1.7 M (3.2 Os kg^{-1}) on the glycerol content of the algal cells plus the growth medium under the following conditions:-

■——■ in the light

▲——▲ in the dark

●——● in the light, in the presence of 17 μM DCMU

Glycerol was determined by the chemical method. Each point represents the mean of at least three determinations.

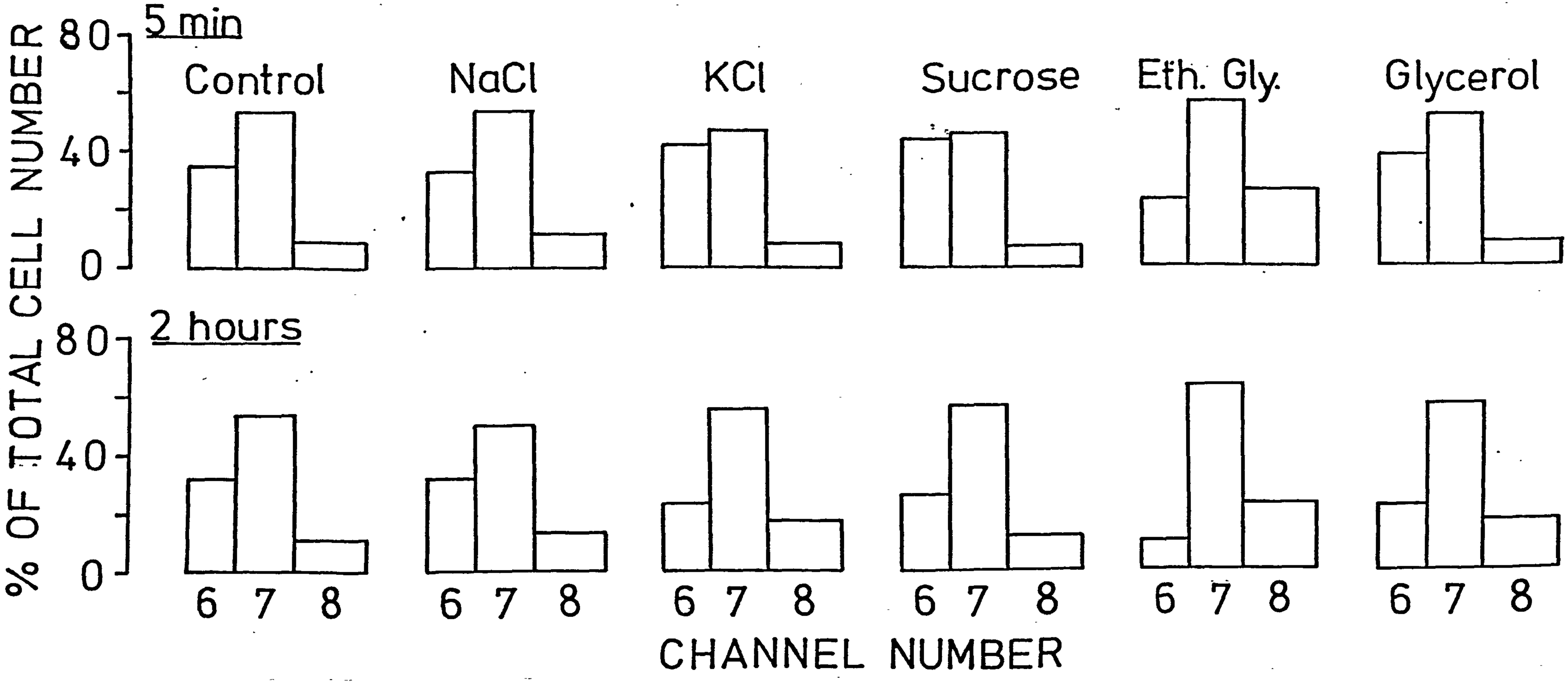


FIGURE 25. The effect of increasing the external osmolality on the maximum cell dimension. The control cells were resuspended in 0.4 M NaCl (0.8 Os kg^{-1}), the others were resuspended in 3.2 Os kg^{-1} exerted by the osmotica indicated. The total number of cells counted in each sample was from 25,000 to 30,000. The size range measured was from 5.4 to 6.8 μ (channel 6), 6.8 to 8.5 μ (channel 7) and 8.5 to 10.7 μ (channel 8). The data shown are from one experiment only, but a repeat experiment produced similar results.

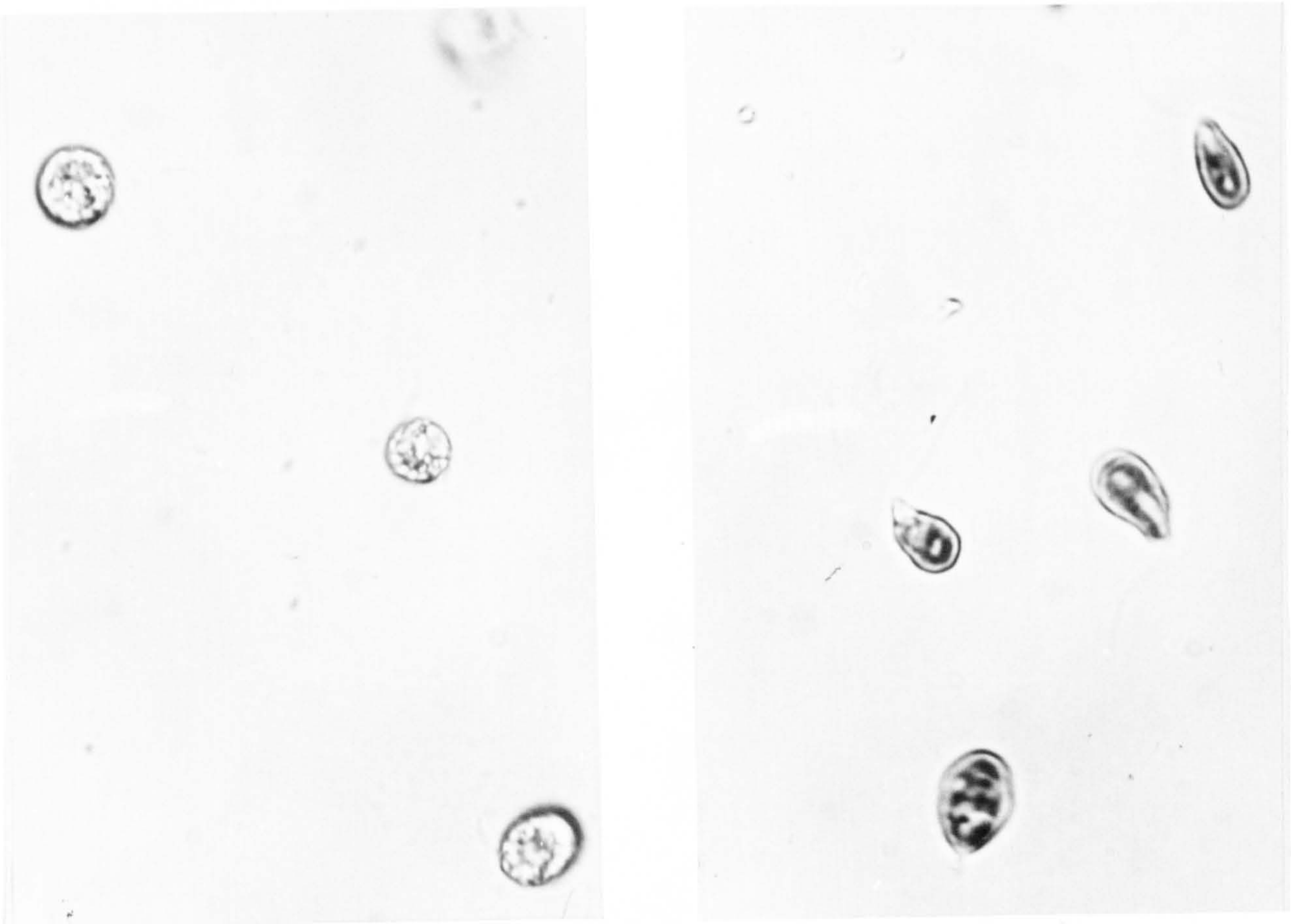
evidence that the cell size is larger than in the control sample.

An explanation of the surprisingly small changes shown by the Coulter counter is suggested by the photographs shown in Figures 26A and B. The cells in Figure 26A have been resuspended in 0.4 M NaCl for 5 min and those in Figure 26B have been resuspended in 1.7 M NaCl for 5 min. The change in shape of the stressed cells is quite clear and is shown diagrammatically in Figure 26C. This evidence suggests that the volume decrease due to the salt stress exhibits itself in the form of a decrease in the width of the cell. This is supported by the results shown in Table 6 which indicate clearly that the length of the cell decreases only slightly in response to an increase in the osmolality of the medium, simultaneously the cell width decreases significantly. Since the cells are in general longer than they are broad, it is the length of the cells which was being measured in the Coulter counter experiments, since the instrument determines the maximum dimension of cells.

Figure 27 shows that a salinity decrease to 0.1 M NaCl (0.2 Os kg^{-1}) does not disrupt the cells, but resuspending them in distilled water does. Within 10 min of being placed in distilled water all the cells have burst due to a large uptake of water from the medium.

3.4 Discussion

D. tertiolecta was shown to produce glycerol in response to an increase in the salinity of the growth medium (Figure 18). The glycerol was shown to accumulate intracellularly and little was lost to the growth medium in the first few hours after the salinity increase (Figures 20 and 21A, Table 5). The results described for D. teriolecta in section 3.2 agree very well with the published work (section 3.1).



A B

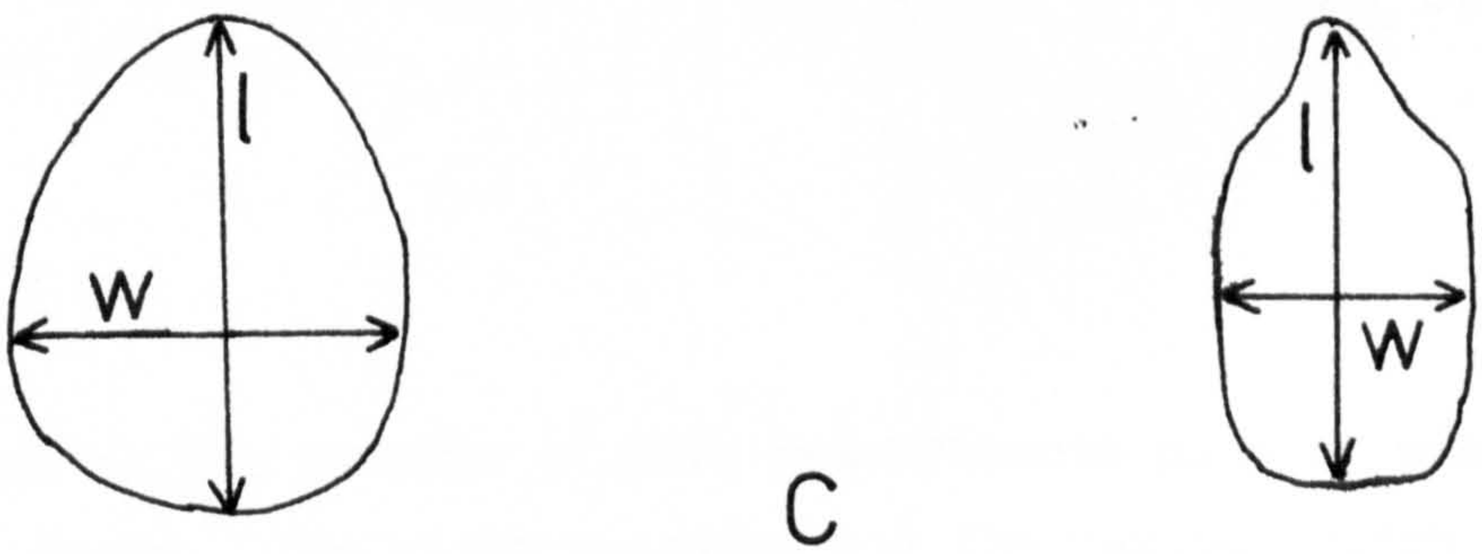


FIGURE 26. Light microscope photographs of D. tertiolecta, magnification x 100 (oil immersion). The algae were resuspended for 5 min in A: 0.4 M NaCl (0.8 Os kg^{-1}), control; and B: 1.7 M NaCl (3.2 Os kg^{-1}); at which time the photographs were taken. C: the line drawings represent the change in shape and indicate where the measurement were made that are shown in Table 6.

TABLE 6

Relative Dimensions of D. tertiolecta Cells

Determined in the Light Microscope

Suspending Medium	Length	Width
0.4 M NaCl (0.8 Os kg^{-1})	5.3 ± 0.3	4.2 ± 0.2
1.7 M NaCl (3.2 Os kg^{-1})	4.8 ± 0.2	2.9 ± 0.2
0.4 M NaCl + 1.3 M KCl (3.2 Os kg^{-1})	4.7 ± 0.2	2.9 ± 0.1
0.4 M NaCl + 1.4 M Sucrose (3.2 Os kg^{-1})	4.5 ± 0.2	2.5 ± 0.2

Each value is the average of five measurements plus or minus the standard error. The width measured was the maximum width indicated in Figure 26C. The values are expressed in eyepiece graticule units.

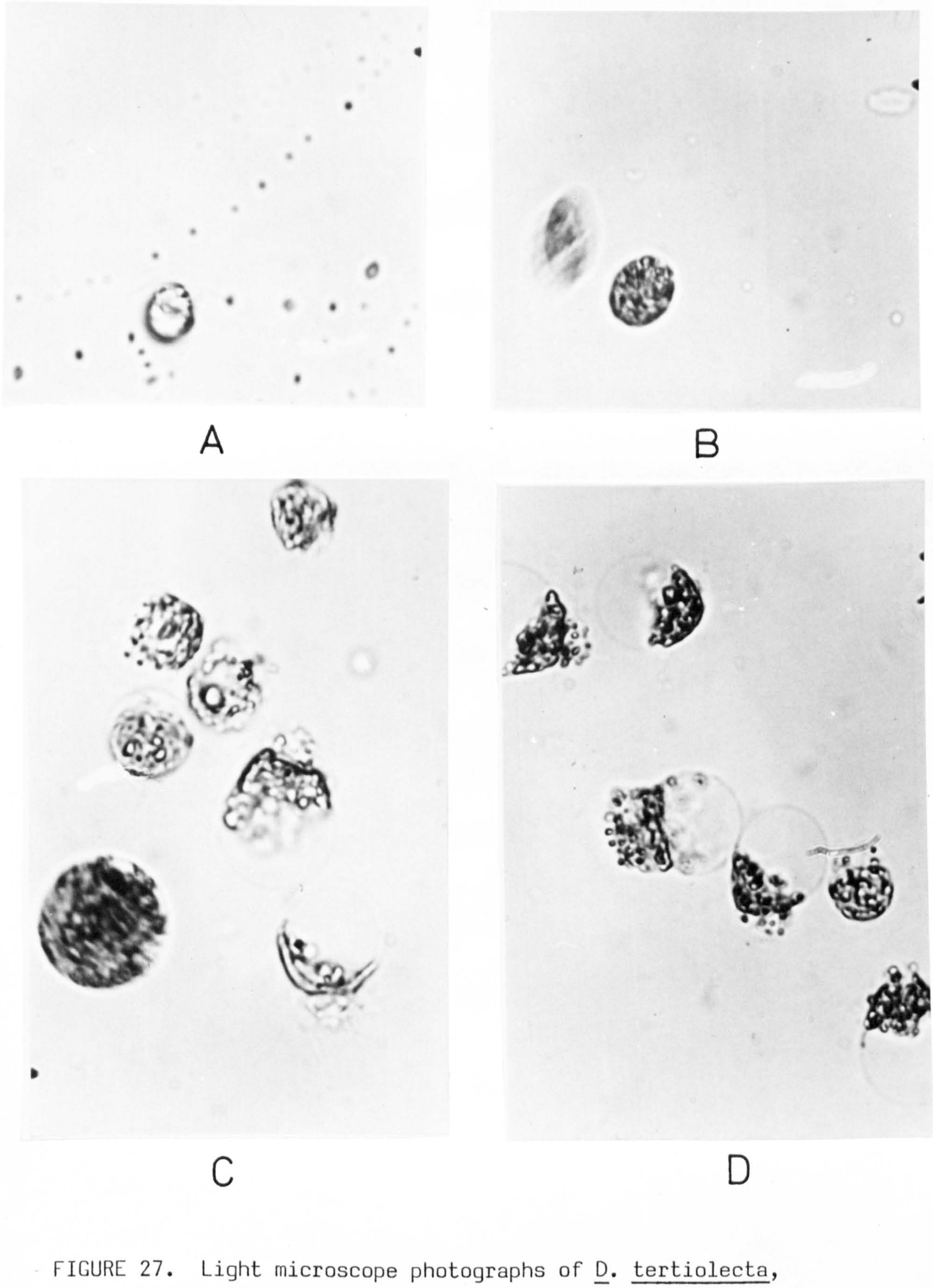


FIGURE 27. Light microscope photographs of D. tertiolecta, magnification x 100 (oil immersion). The algae were resuspended in A and B: 0.1 M NaCl (0.2 Os kg^{-1}) for 5 min; C: distilled water (0 Os kg^{-1}) for 5 min; D: distilled water for 10 min; at which times the photographs were taken.

However, Ben-Amotz and Avron (1973a) and Brown and Borowitzka (1979) indicated that glycerol synthesis in Dunaliella was completed within 90 min of the increase in salinity. Figure 20 shows that D. tertiolecta produces glycerol for about 4 hours after a salinity increase.

Glycerol production in response to an increase in salinity also took place in the presence of DCMU or in the dark (Figure 24). Although in both cases just over half the amount of glycerol normally produced was synthesised by the end of the 100 min period. Glycerol synthesis by Dunaliella in the dark was also observed by Ben-Amotz (1975) and Borowitzka et al. (1977). However, Frank and Wegmann (1974) reported that D. tertiolecta did not synthesise glycerol in the dark after an increase in the external salinity from 1.0 to 2.6 M. DCMU blocks the photosynthetic electron transport chain between Q and R and therefore stops noncyclic electron flow, but does not inhibit cyclic electron flow (Chapter 1, Figure 4). In the dark, there is clearly no photosynthetic electron transport. Therefore the close correlation between the amount of inhibition due to DCMU (40%) and that due to incubating the algae in the dark (43%) seems to indicate no role for cyclic electron transport in glycerol production.

Figures 18 and 19 suggest that the onset of glycerol synthesis in response to an osmolality increase is very rapid, since at the zero time readings there is already evidence of glycerol production. Given that the maximum delay before 30% trichloroacetic acid was added to the cells was 1 - 2 min (section 2.8.1), this suggests that glycerol synthesis begins within 2 min of the osmotic stress being applied. This immediate induction of glycerol production by osmotic stress was also found by Borowitzka et al. (1977) and Brown and Borowitzka (1979).

KCl also stimulated glycerol production in D. tertiolecta: this

had not been demonstrated previously in Dunaliella (Figure 19). It was also observed that if the cells were grown in the high concentration of KCl used in Figure 19 (1.3 M KCl + 0.4 M NaCl), they died within 2 - 3 days. KCl toxicity had previously been demonstrated in Dunaliella by McLachlan (1960) and Johnson et al. (1968). A possible reason for KCl toxicity is illustrated in Figure 21B and Table 5, where it is shown that a large proportion of the glycerol synthesised intracellularly by the algae is lost to the external medium. Thus it seems that a cyclic process occurs in the presence of a large external concentration of KCl i.e. glycerol synthesised is lost to the medium whereby it adds to the stimulation for more production of intracellular glycerol which is then lost to the medium. This process is energetically extremely wasteful.

Wallen and Geen (1971a and b) reported that exposure to blue light enhanced protein production and reduced carbohydrate production in D. tertiolecta. Jones and Galloway (1979) reduced the intracellular glycerol content of D. tertiolecta by growing the alga in blue light and this produced a significant increase in the growth rate over that in white light at a non-saturating light intensity of 33 μmoles $m^{-2}s^{-1}$. They concluded that a reduction in the glycerol content did not affect the alga's ability to osmoregulate, therefore more glycerol was being synthesised than was necessary for osmoregulation (Jones and Galloway, 1979). This suggested that even in the case of increased NaCl concentration glycerol production can under certain circumstances be energetically wasteful.

Sucrose was also capable of inducing glycerol production in D. tertiolecta (Figure 22). It was of the same magnitude as NaCl-induced glycerol production and indicated that the cells could respond to an osmotic stress, the only ions present being those of the basic

medium. Sucrose-induced glycerol production in Dunaliella was also demonstrated by Wegmann (1971) and Borowitzka et al. (1977). The amount of glycerol in the medium was not measured in the case of sucrose. However, it can be implied from the fact that there is no indication of rapid cell death that sucrose does not induce glycerol leakage to the same extent as KCl (Chapter 5, Figure 48).

Ethylene glycol induced no glycerol production in D. tertiolecta (Figure 23). This osmoticum also had a surprising effect on cell size as measured by the Coulter counter. The maximum dimension of the cells appeared to increase when ethylene glycol was added to the growth medium (Figure 25). This suggests that ethylene glycol may be permeable to the plasmalemma of the algae and therefore it would not exert an osmotic stress. This could account for the lack of glycerol production in the presence of ethylene glycol (Figure 23).

Sucrose - and ethylene glycol - induced glycerol production was measured using an enzymatic determination method because these osmotica interfered with the chemical determination method used in all the other experiments. The enzymatic method appears to be specific for glycerol (section 2.8.2). However, a careful comparison of the values obtained for NaCl-induced glycerol production by both methods (Figures 18, 22 and 23) suggests that the chemical method measures not only glycerol but also other related compounds. This would not be surprising because of the relatively non-specific nature of the chemical method (section 2.8.1).

Many factors can influence the measurement of maximum dimensions made using a Coulter counter. The orientation and shape of the cell as it passes through the orifice and the effects of the electrical field intensity on the cell may all have an effect on the measurements obtained (Rabinowitch, Grover and Ginzburg, 1975; Gimmler, Schirling

and Tobler, 1977). The changes in size and shape of the cells during their life cycle must be taken into account in longer term experiments (Gimmler et al., 1977; Bruggemann, Weiger and Gimmler, 1978). These limitations must be borne in mind when discussing results obtained with a particle size analyser like a Coulter counter.

Gimmler et al. (1977) used a Coulter counter to measure the changes in volume of D. parva cells when the external NaCl concentration was varied. They found that rapid volume changes took place which were due to water movement alone i.e. the plasmalemma of the alga acted as a differentially-permeable membrane. The authors concluded that after 2 min an equilibrium of the external and internal osmolality was reached and at this stage the cells behave as perfect osmometers as described by the Boyle Van't Hoff relation. Over the next two hours the cell volume returned to normal due mainly to glycerol synthesis or removal. Figure 25 shows that evidence for cell volume decreases in response to an increase in the external osmolality was found only to a very small extent in this study. This is explained as being due to the Coulter counter used in this study only measuring the length of the cell (maximum dimension) when it appears that it is the width of the cell which decreases in response to the volume decrease (Figure 26 and Table 6). Thus it has not been possible to repeat the results of Gimmler et al (1977) because the maximum dimension of the cell was not a suitable indicator of the volume of the cell. However, the Coulter counter experiments confirm that ethylene glycol does not act in the same way as the other osmotica used (Figure 25).

Ginzburg (1969) provided evidence for two distinct compartments in the Dunaliella cell, by examining the permeability of the cell towards ^{14}C-dextran and ^{14}C-inulin. Her results can be interpreted as

suggesting an outer compartment bounded by a fairly permeable membrane (plasmalemma) and an inner compartment enclosed by a largely impermeable membrane (chloroplast envelope). Rabinowitch et al. (1975) also measured the change of cell volume of Dunaliella using a particle size analyser. They found volume changes similar to Gimmler et al. (1977) which were consistent with the Boyle Van't Hoff relation. They determined the nonosmotic volume to be 60-80% of the total cell volume. The 20-40% of the cell volume which responds to the external osmolality can be described using two kinetic coefficients of volume change which were tentatively identified with the plasmalemma (accounts for 60-70% of the volume change) and the chloroplast envelope (accounts for 30-40% of the volume change). These data taken in conjunction with Figure 7 (which shows that a large part of the cell volume is taken up by the cup-shaped chloroplast) and Figure 26B (which shows a constriction of the cell around the chloroplast) leads to the suggestion that the osmotically available water is in the cytoplasm and the nonosmotic water is found inside the chloroplast (Ginzburg, 1978). This however is only a hypothesis because Gimmler et al. (1977) found a value of only 20-40% for the nonosmotic volume.

Figure 18 indicated that a salinity decrease to 0.1 M NaCl caused a depletion of intracellular glycerol. The loss of glycerol took place rapidly and it was suggested that it may be excreted into the medium. Figure 27 shows that at 0.1 M NaCl the cells are still structurally intact, thus the glycerol loss was not caused by disruption of the cell. In addition it was found that the algae could survive in 0.1 M NaCl for 3-4 weeks. However, if the cells were resuspended in distilled water then they burst within 10 min (Figure 27).

The subject of glycerol leakage by Dunaliella is a controversial

one. Several authors have found appreciable loss of ^{14}C-labelled organic material by Dunaliella without changing the external salt content; a significant proportion of this material appears to be glycerol (Hellebust, 1965; Craigie et al., 1966; Huntsman, 1972). There seems little doubt that at low salinities glycerol leakage can occur (Ben-Amotz and Avron, 1973a). The disagreement arises on whether glycerol is lost from the cells when they are fully adapted to a medium of fairly high salinity e.g. 1.5 M NaCl. Frank and Wegmann (1974) suggested that D. tertiolecta lost glycerol to the medium in appreciable amounts, but Ben-Amotz and Avron (1973a) did not find glycerol leakage in D. parva above 0.6 M NaCl. Jones and Galloway (1979) found significant loss of glycerol to the medium in exponentially growing cells suspended in 1.0 M NaCl. Wegmann, Ben-Amotz and Avron (1980) showed that glycerol leakage could be induced by high temperature, they found essentially none is released below 313 K, 50% release occurs at 323 K and complete loss of intracellular glycerol was found at 333 K. The authors concluded that a temperature dependent component of the cell membrane must be necessary for glycerol retention. Enhuber and Gimmler (1980) made a detailed study of the ability of Dunaliella to retain glycerol. They compared the ability of Dunaliella cells to retain glycerol against that of other plant and animal cells and surprisingly found that the plasmalemma of Dunaliella was not especially impermeable to glycerol. They proposed a theoretical model for glycerol efflux into the medium and concluded that Dunaliella follows a strategy of tolerating a large efflux of glycerol into the medium instead of avoiding this efflux by having a plasmalemma that is impermeable to glycerol (Enhuber and Gimmler, 1980). However, it has been suggested recently that the calculations of glycerol efflux by Enhuber and Gimmler are in error and that the glycerol loss is small (Ben-Amotz, 1982 and

personal communication).

The results shown in Figure 21 indicate that KCl but not NaCl can induce substantial leakage of glycerol into the external medium. This suggests that the mechanism of glycerol retention by Dunaliella is adversely affected by a high external concentration of K^+.

If glycerol loss to the medium is of the order suggested by Jones and Galloway (1979) and Enhuber and Gimmler (1980) then it will have an inhibitory effect on the growth of the algae in laboratory cultures. It would also have to be taken into account when the feasibility of commercial production of glycerol by Dunaliella is examined (section 1.7). The ecological effect of glycerol leakage in the natural habitats of the alga is likely to be variable. In rock pool and estuarine habitats where the salinity changes are short-lived it is unlikely to be significant. However, in habitats of extreme salinity e.g. Dead Sea in Israel, glycerol excretion may be ecologically important.

CHAPTER 4

OXYGEN EVOLUTION AND UPTAKE

4.1 Introduction

In Chapter 3 it was demonstrated that D. tertiolecta synthesised glycerol intracellularly when the external NaCl concentration was increased (Figures 18, 20 and 21A). Glycerol production in Dunaliella can take place in the light or the dark (Figure 24; Ben-Amotz and Avron, 1973a; Borowitzka, Kessly and Brown, 1977). Therefore, Ben-Amotz (1975) suggested that two different metabolic pathways could be involved with the synthesis of glycerol in Dunaliella: one using photosynthetic products and the other by way of the metabolic degradation of starch, the storage product of the alga. An indication of the relative contribution of these processes to glycerol production can be deduced by looking at the effect of an increase in external NaCl concentration on the rate of photosynthesis and respiration. If the algae are illuminated and subjected to increased concentrations of extracellular NaCl, the photosynthetic rate might be expected to increase if this was the major pathway of glycerol synthesis in the light. This type of salt-induced stimulation of photosynthesis was found in D. parva by Bruggemann, Weiger and Gimmler (1978). Therefore, measurements of the rate of photosynthesis and respiration in D. tertiolecta were made 5 and 100 min after extracellular salinity increases up to 1.71 M (3.2 Os kg^{-1}). The algae were kept in an illuminated (9.2 W m^{-2}) controlled temperature water bath at 294-297 K between measurements (section 2.9). The same measurements were made when the external osmolality was increased up to approximately 3.2 Os kg^{-1} using KCl,

sucrose, ethylene glycol or glycerol, and also when the external salinity was decreased down to zero (distilled water).

The rates of photosynthesis and respiration were determined using an oxygen electrode by measuring the oxygen evolution in the light and the oxygen uptake in the dark (section 2.9). Photosynthesis was taken as being equal to the oxygen evolution in the light corrected for the oxygen uptake in the dark; respiration was assumed to equal the rate of dark oxygen uptake. The accuracy of rates of photosynthesis determined by this method depends on the rate of photorespiration being equal to the rate of respiration in the dark. However, Jackson and Volk (1970) and Samish et al. (1972) have suggested that photorespiration frequently occurs at rates considerably greater than dark respiration. If this is correct, the rates of photosynthesis quoted in this study will be underestimates.

The results obtained using the oxygen electrode are expressed in μmoles oxygen evolved or taken up mg chlorophyll^{-1} hour^{-1}. To calculate these results, the oxygen content of the test solutions had to be known and these were determined using the Winkler test (section 2.9). The oxygen concentration values for the test media used in section 4.2 are shown in Appendix E.

4.2 Results

Figure 28 shows that increasing the external NaCl concentration from 0.43 to 0.67 M causes a small decrease in the photosynthetic rate after 5 min. Larger increases in the external salinity lead to greater inhibition until at 1.71 M NaCl (3.2 Os kg^{-1}) the photosynthetic rate is only 10% of the control. After 100 min, there is a recovery of

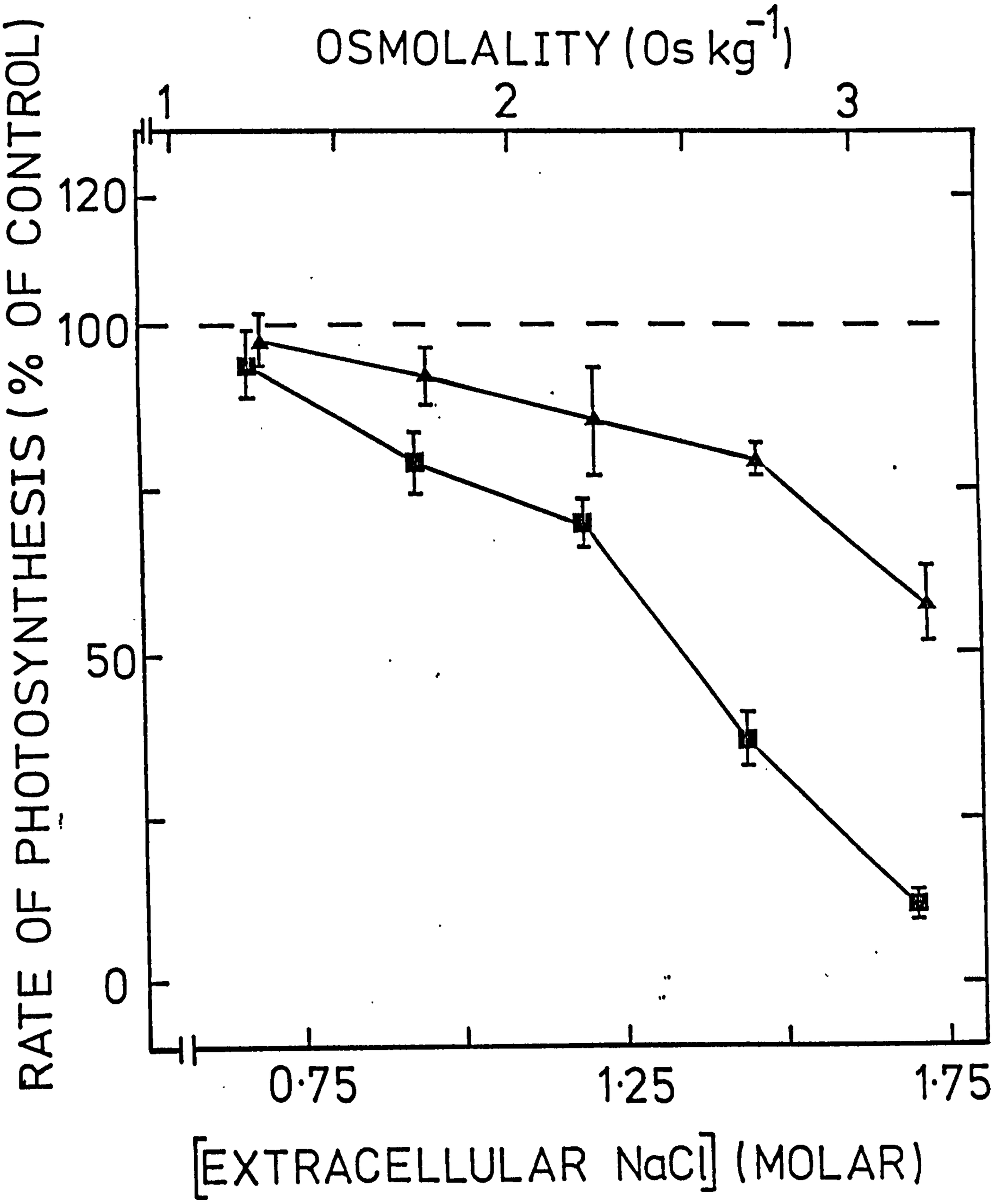


FIGURE 28. The effect of increasing the external NaCl concentration on the rate of photosynthesis. The algae were resuspended in the salinities indicated for 5 min (■——■) or 100 min (▲——▲). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 19.2 to 75.6 μmoles of O_2 evolved mg chl^{-1} h^{-1}.

photosynthetic rate, but at all the increased salinities the rate is still inhibited. Figure 29 indicates that during the same period increasing the salinity inhibits the respiration rate, but not to the same extent as photosynthesis is inhibited.

Decreasing the external NaCl concentration has a similar effect on the photosynthetic rate as increasing the salinity (Figure 30). However, there is less recovery at low salinities within the 100 min time period. Figure 31 shows that decreasing the salinity down to 0.09 M NaCl (0.16 Os kg^{-1}) can stimulate the respiratory rate after 5 min, but at the end of 100 min the stimulation has disappeared and is replaced by a small inhibition of respiration. In distilled water, there is clear inhibition of respiration after both 5 and 100 min.

Increasing the external KCl concentration causes a decrease in the photosynthetic rate after 5 min (Figure 32). There is a recovery after 100 min, but there is still inhibition at all levels of increased KCl concentration. The inhibition of the rate of photosynthesis caused by increasing the external osmolality using KCl is similar to the inhibition induced by NaCl (Figure 32). Increasing the external KCl concentration causes small amounts of inhibition and stimulation of the respiration rate; only at 1.28 M KCl (3.1 Os kg^{-1}) after 100 min is there substantial inhibition (Figure 33).

Figure 34 indicates that increasing the external sucrose concentration can cause a reduction in the photosynthetic rate after 5 min, which does not recover to any great extent within 100 min of the osmolality being increased. However, the maximum decrease in the photosynthetic rate caused by sucrose was 50% compared to 90% for NaCl (Figures 28 and 34). This could partly be explained by the fact that

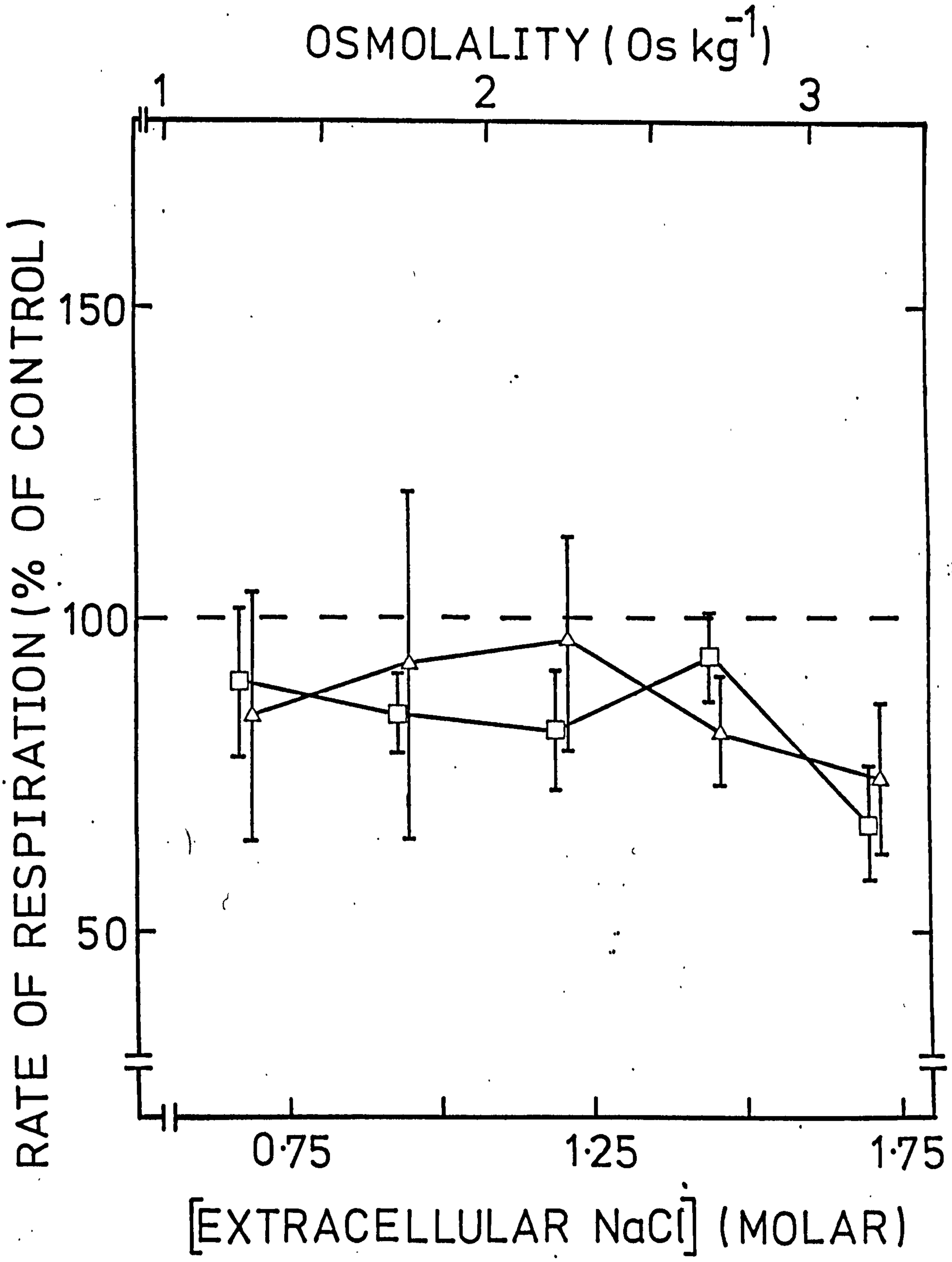


FIGURE 29. The effect of increasing the external NaCl concentration on the rate of respiration. The algae were resuspended in the salinities indicated for 5 min (□——□) or 100 min (△——△). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 2.1 to 9.3 μmoles O_2 taken up mg chl^{-1} h^{-1}.

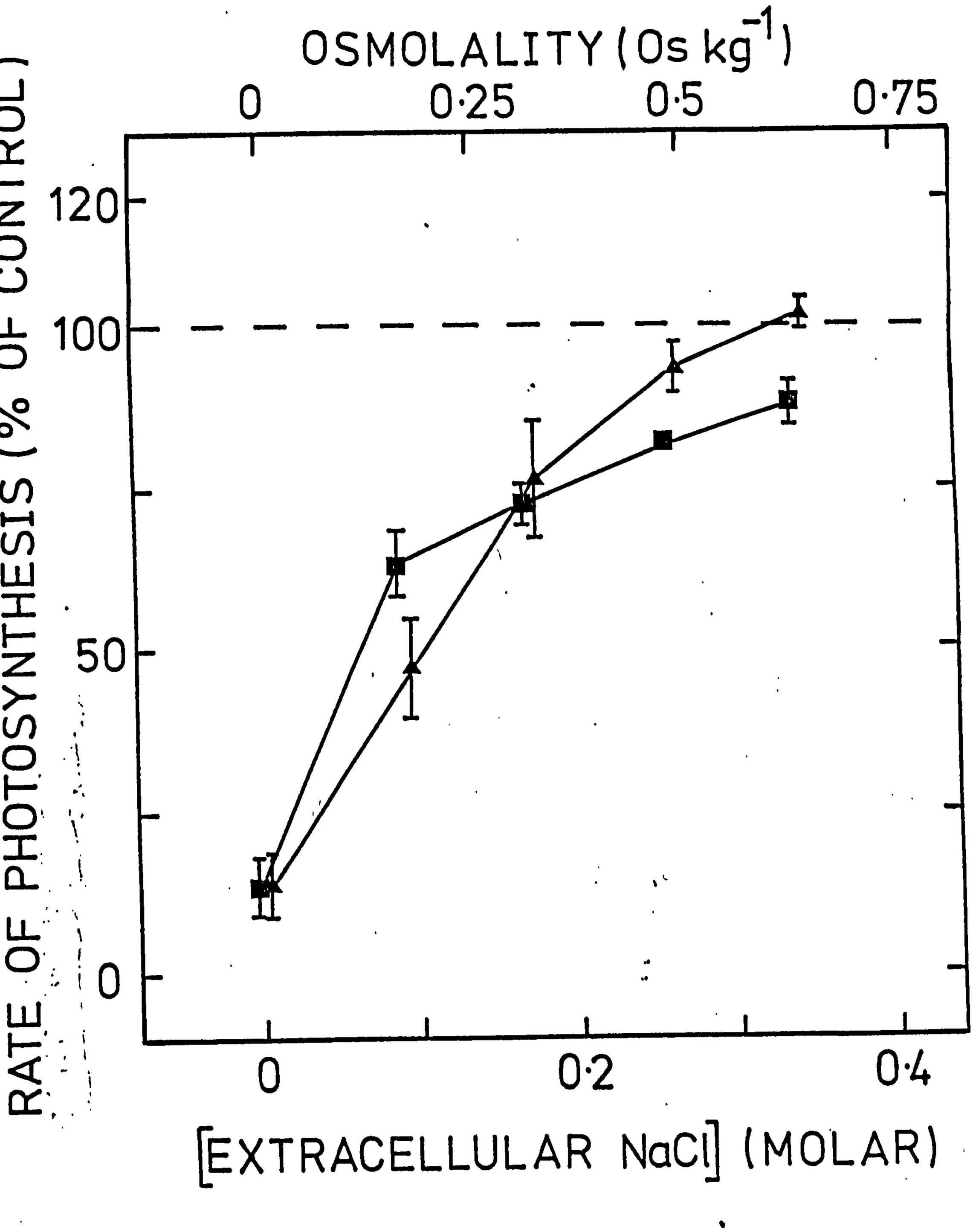


FIGURE 30. The effect of decreasing the external NaCl concentration on the rate of photosynthesis. The algae were resuspended in the salinities indicated for 5 min (■——■) or 100 min (▲——▲). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 27.7 to 67.7 μmoles O_2 evolved mg chl^{-1} h^{-1}.

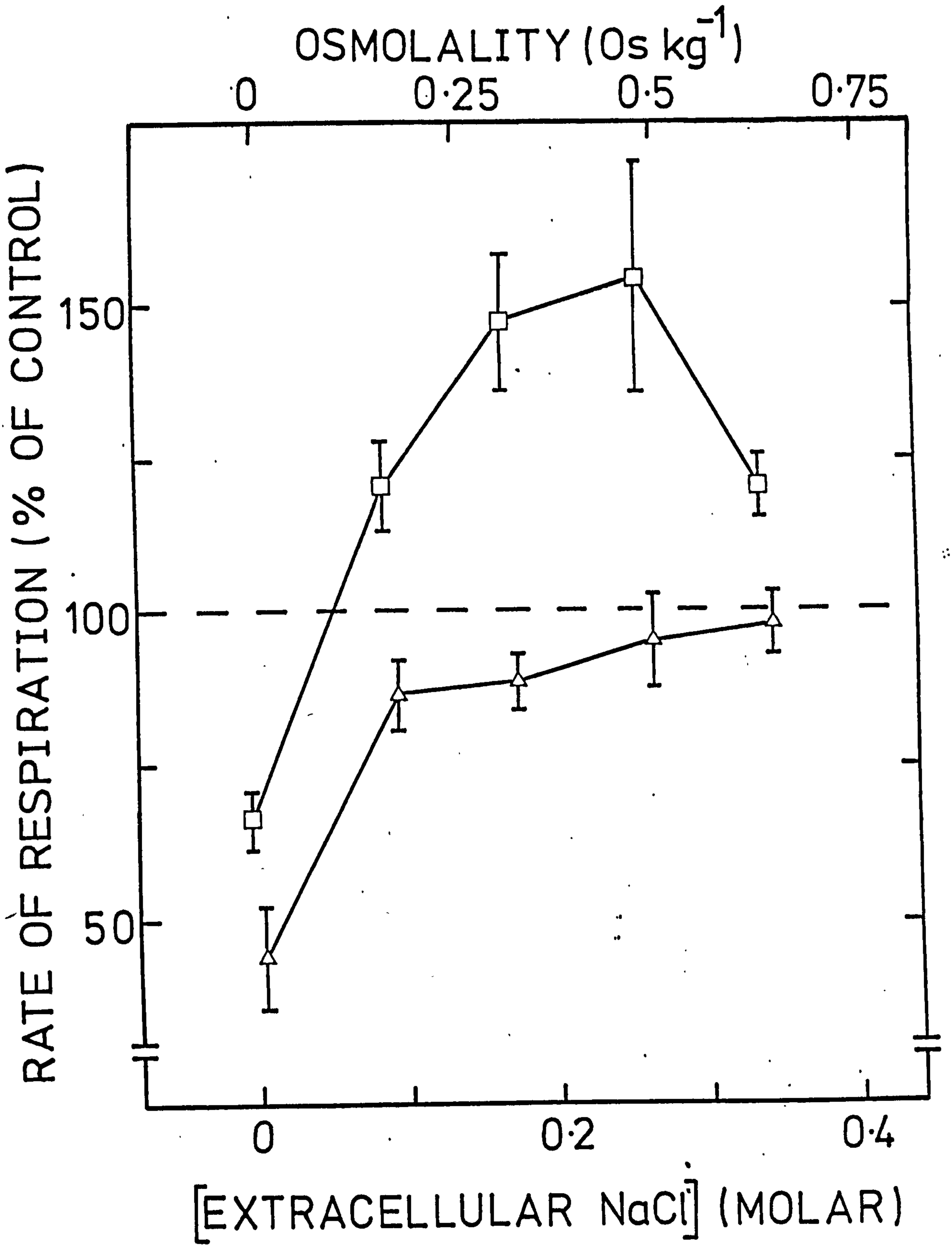


FIGURE 31. The effect of decreasing the external NaCl concentration on the rate of respiration. The algae were resuspended in the salinities indicated for 5 min (□——□) or 100 min (△——△). Each point represents the mean of four determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 2.7 to 8.7 μmoles O_2 taken up mg chl^{-1} h^{-1}.

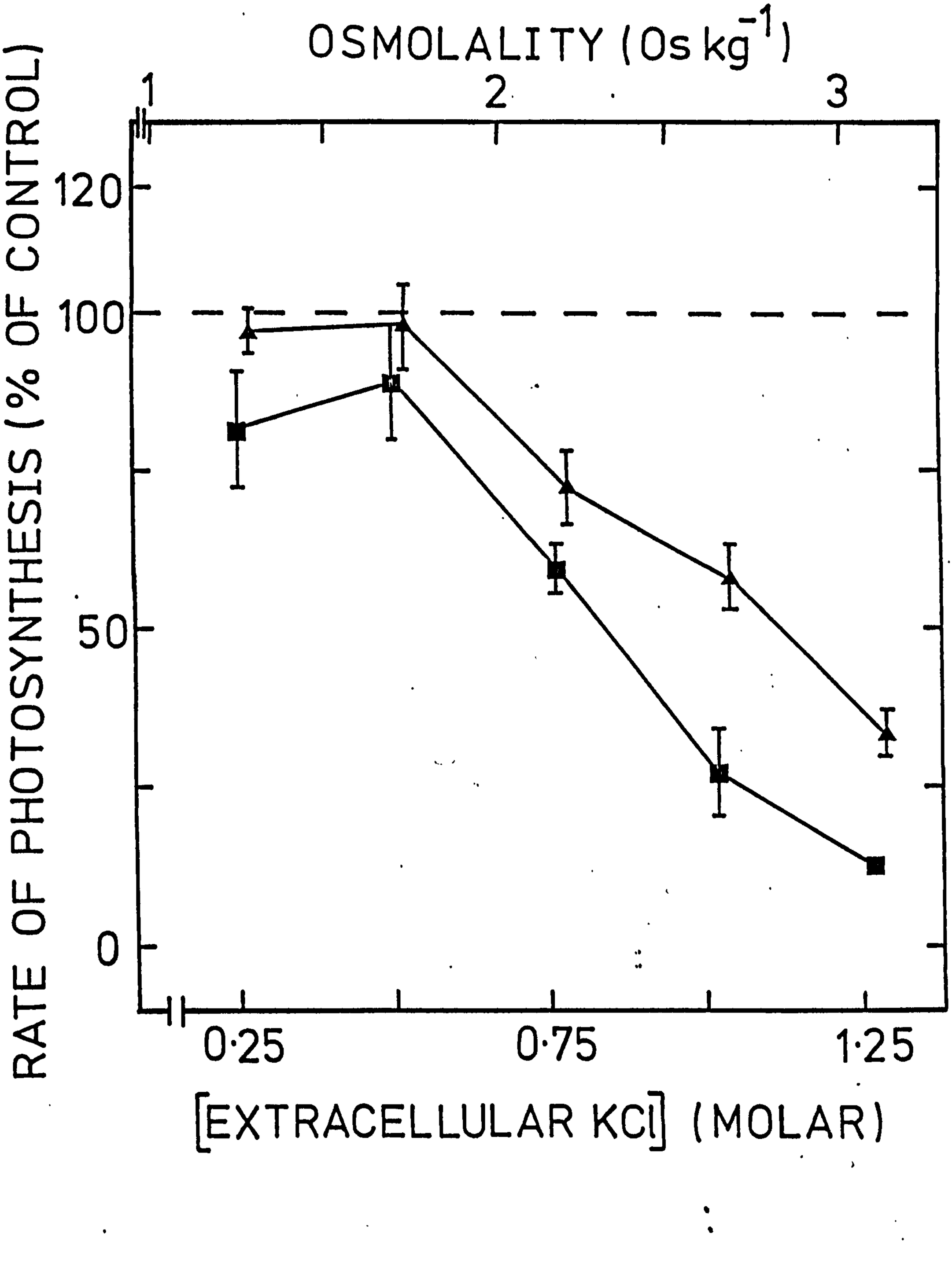


FIGURE 32. The effect of increasing the external KCl concentration on the rate of photosynthesis. The algae were resuspended in 0.43 M NaCl plus the concentration of KCl indicated for 5 min (■——■) or 100 min (▲——▲). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 30.9 to 79.1 μmoles O_2 evolved mg chl^{-1} h^{-1}.

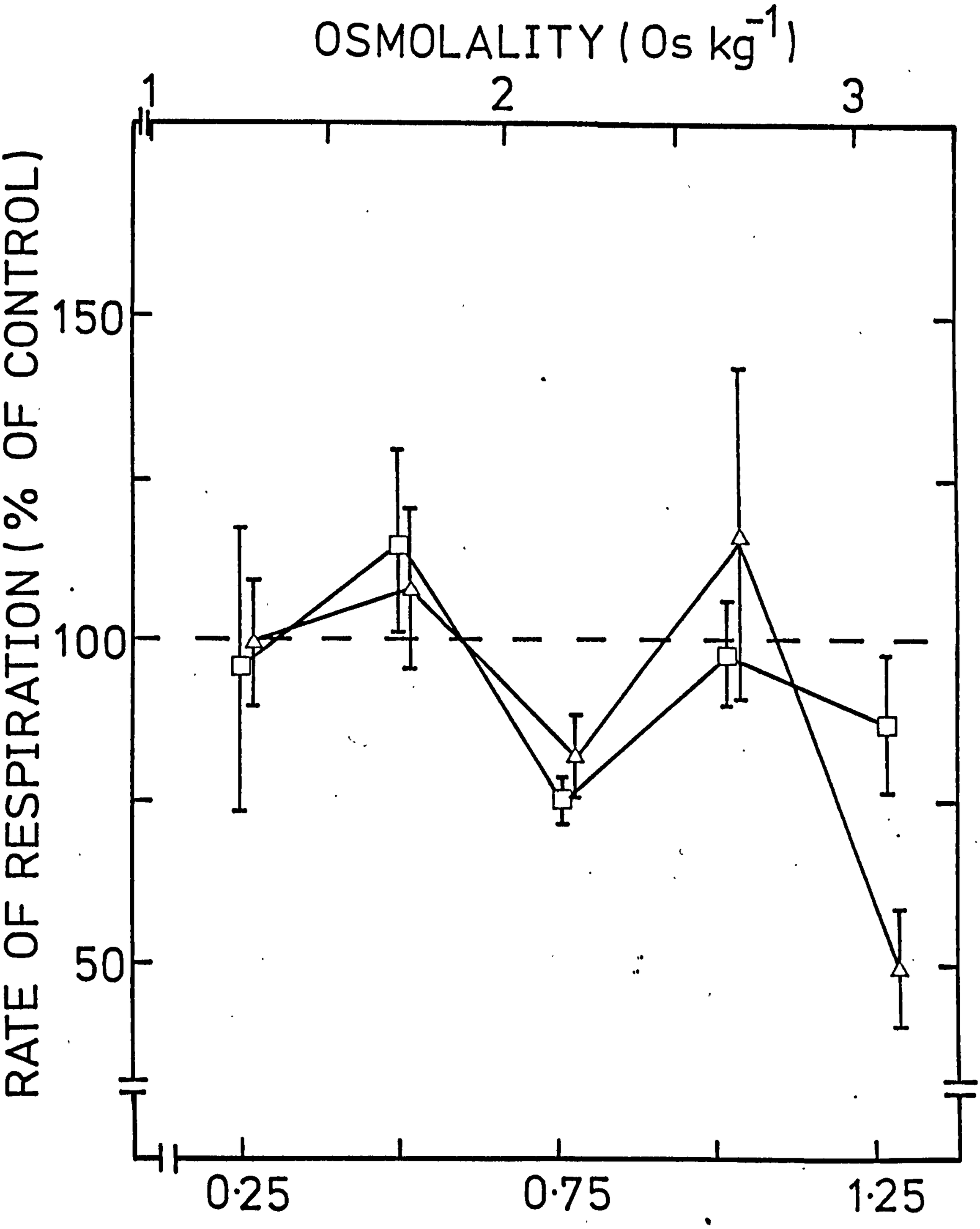


FIGURE 33. The effect of increasing the external KCl concentration on the rate of respiration. The algae were resuspended in 0.43 M NaCl plus the concentration of KCl indicated for 5 min (□——□) or 100 min (△——△). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 0.7 to 21.2 μmoles O_2 taken up mg chl^{-1} h^{-1}.

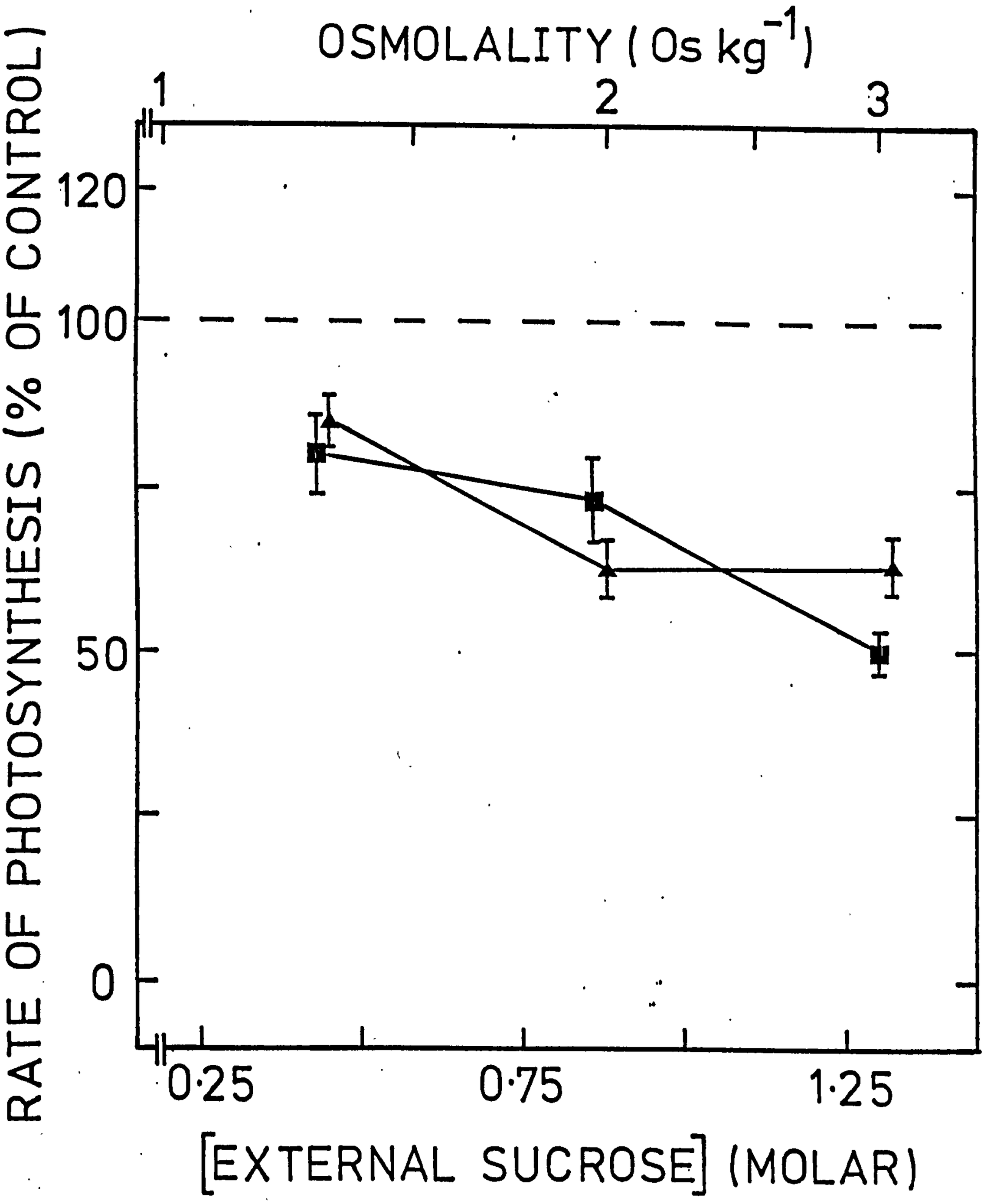


FIGURE 34. The effect of increasing the external sucrose concentration on the rate of photosynthesis. The algae were resuspended in 0.43 M NaCl plus the concentration of sucrose indicated for 5 min (■——■) or 100 min (▲——▲). Each point represents the mean of four determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 28.5 to 69.0 μmoles O_2 evolved mg chl^{-1} h^{-1}.

the maximum osmolality exerted by sucrose was 3.0 Os kg^{-1} compared with 3.2 Os kg^{-1} for NaCl. However, it is unlikely that this small discrepancy in the external osmolality would account for the large difference in the inhibition of photosynthesis induced by NaCl and sucrose (Figures 28 and 34). Figure 35 shows that increasing the external osmolality using sucrose inhibits respiration, but that the amount of inhibition is largely independent of the osmolality exerted by the sucrose solution.

Increasing the external ethylene glycol concentration decreases the photosynthetic rate after 5 min for concentrations above 0.88 M (1.7 Os kg^{-1}) (Figure 36). There is a recovery at the higher osmolalities after 100 min, but at all the increased osmolalities except the lowest there is still some degree of inhibition. The maximum inhibition of the rate of photosynthesis due to ethylene glycol is similar to that induced by sucrose (Figure 34), but less than that caused by NaCl or KCl (Figures 28 and 32). Figure 37 shows that after 5 min ethylene glycol decreases the respiratory rate, this takes place to about the same extent for all the concentrations of ethylene glycol used. After 100 min the inhibition of respiration has turned to a stimulation at all the concentrations except at 1.30 M ethylene glycol (2.2 Os kg^{-1}).

Figure 38 shows that increasing the extracellular glycerol content decreases the rate of photosynthesis after 5 min. There is some recovery of the rate after 100 min, but it is still inhibited at all levels of increased external glycerol. The lowest rate of photosynthesis found after 5 min was at 1.92 M glycerol (3.2 Os kg^{-1}) and this was 25% of the control value. Thus, glycerol inhibits photosynthesis more than sucrose and ethylene glycol (Figures 34 and 36), but less than NaCl and

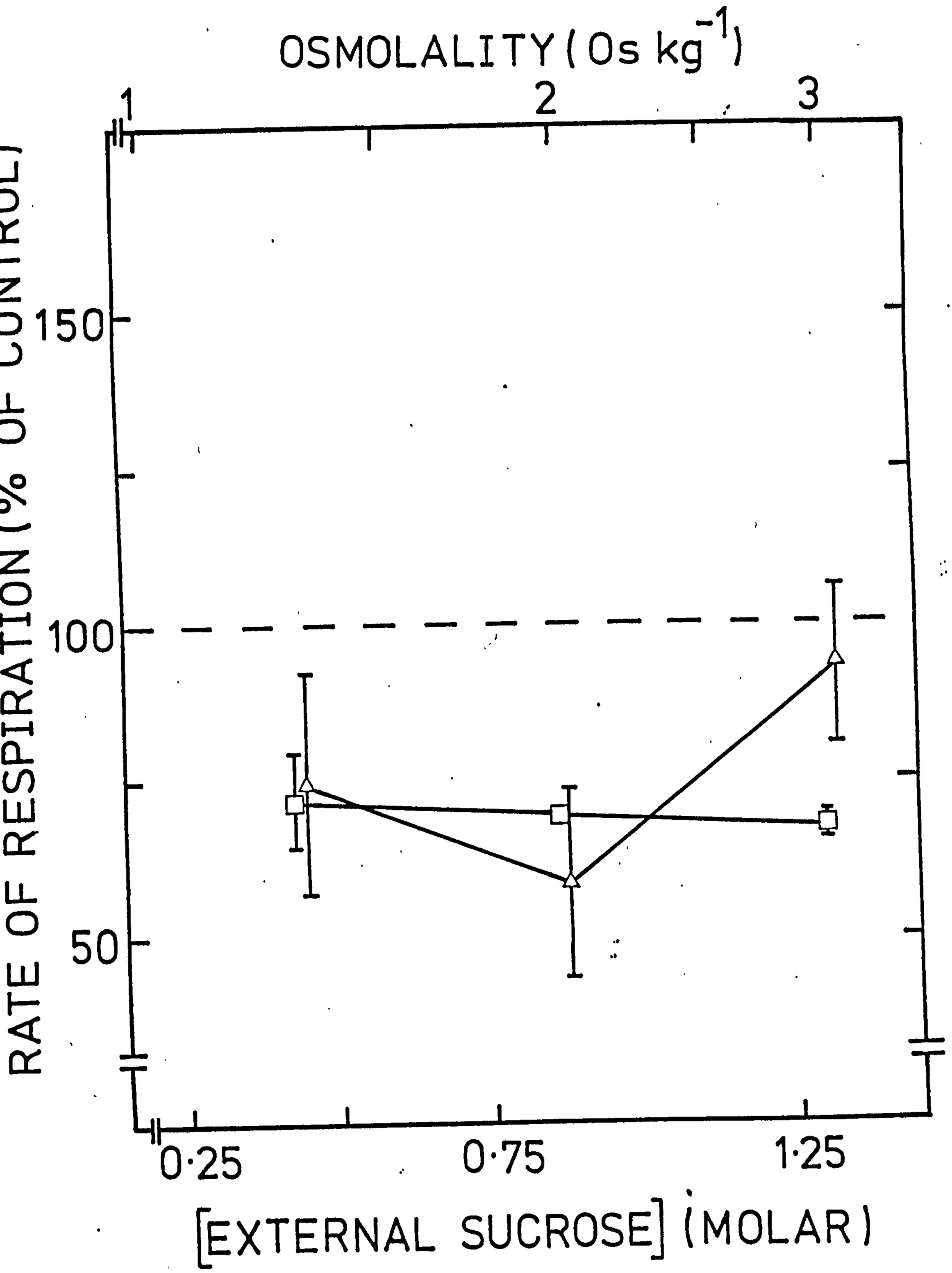


FIGURE 35. The effect of increasing the external sucrose concentration on the rate of respiration. The algae were resuspended in 0.43 M NaCl plus the concentration of sucrose indicated for 5 min (□——□) or 100 min (△——△). Each point represents the mean of three determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 2.8 to 19.2 μmoles O_2 taken up mg chl^{-1} h^{-1}.

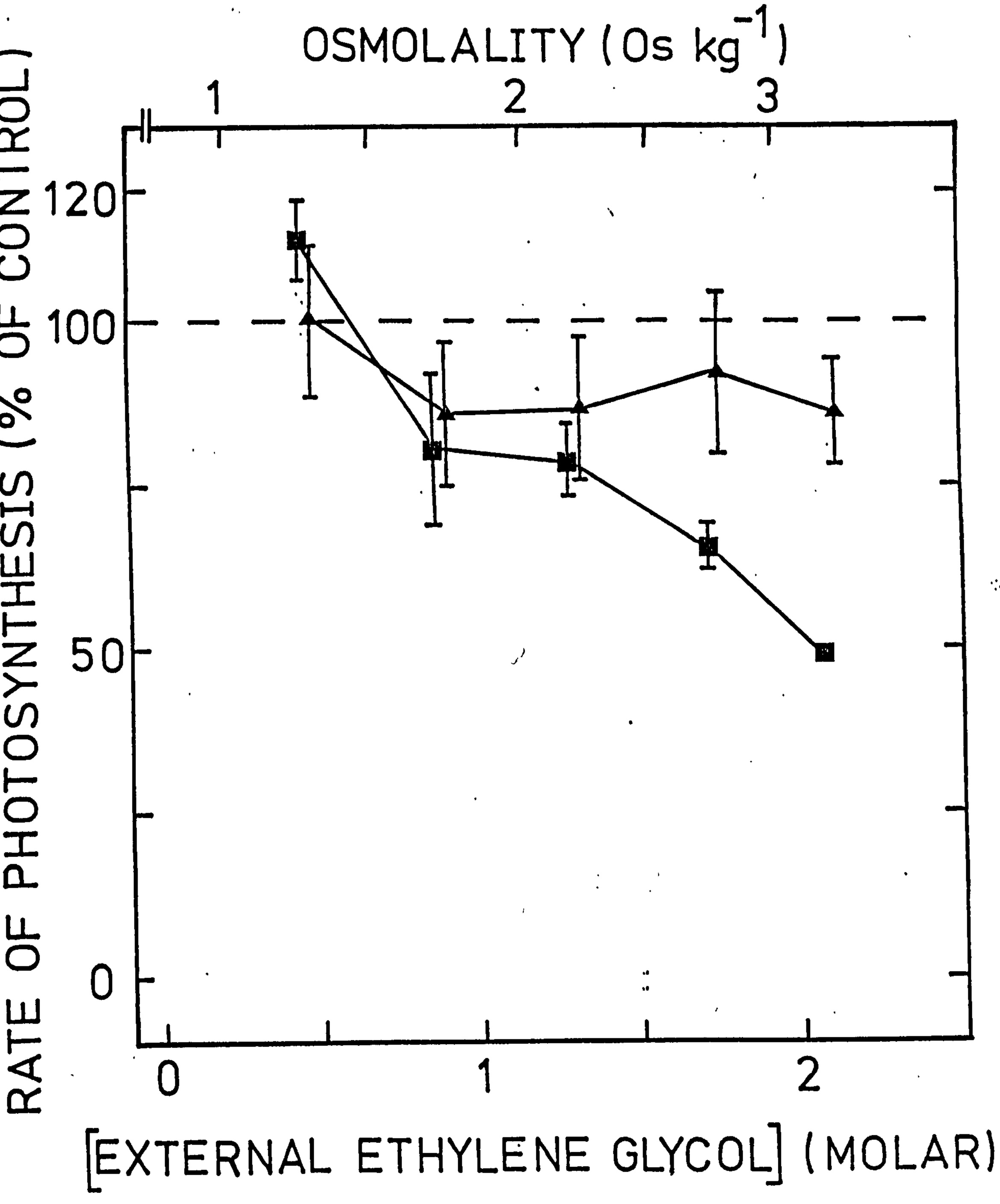


FIGURE 36. The effect of increasing the external ethylene glycol concentration on the rate of photosynthesis. The algae were resuspended in 0.43 M NaCl plus the concentration of ethylene glycol indicated for 5 min (■——■) or 100 min (▲——▲). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 12.2 to 57.2 μmoles O_2 evolved mg chl^{-1} h^{-1}.

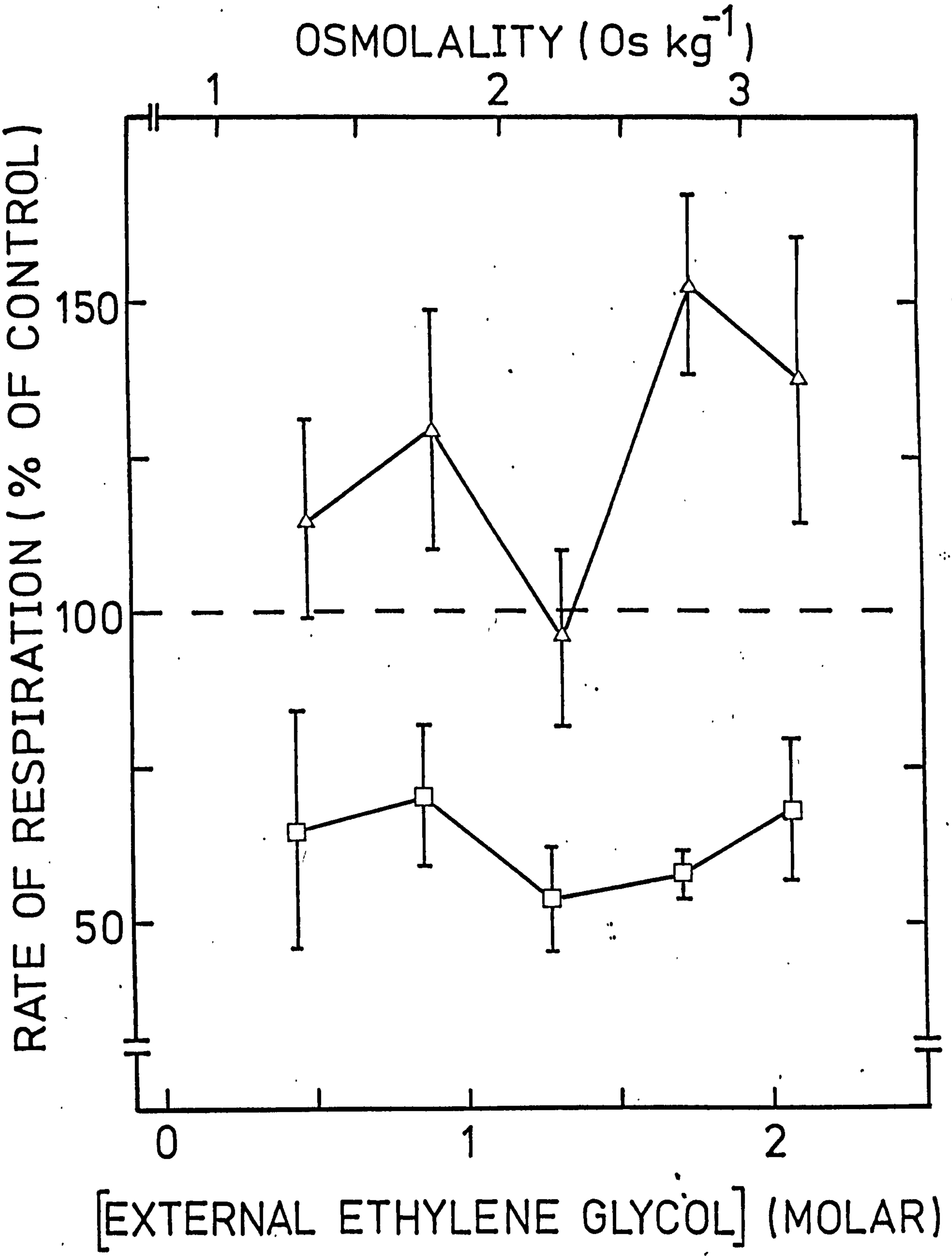


FIGURE 37. The effect of increasing the external ethylene glycol concentration on the rate of respiration. The algae were resuspended in 0.43 M NaCl plus the concentration of ethylene glycol indicated for 5 min (□——□) or 100 min (△——△). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 0.8 to 14.9 μmoles O_2 taken up mg chl^{-1} h^{-1}.

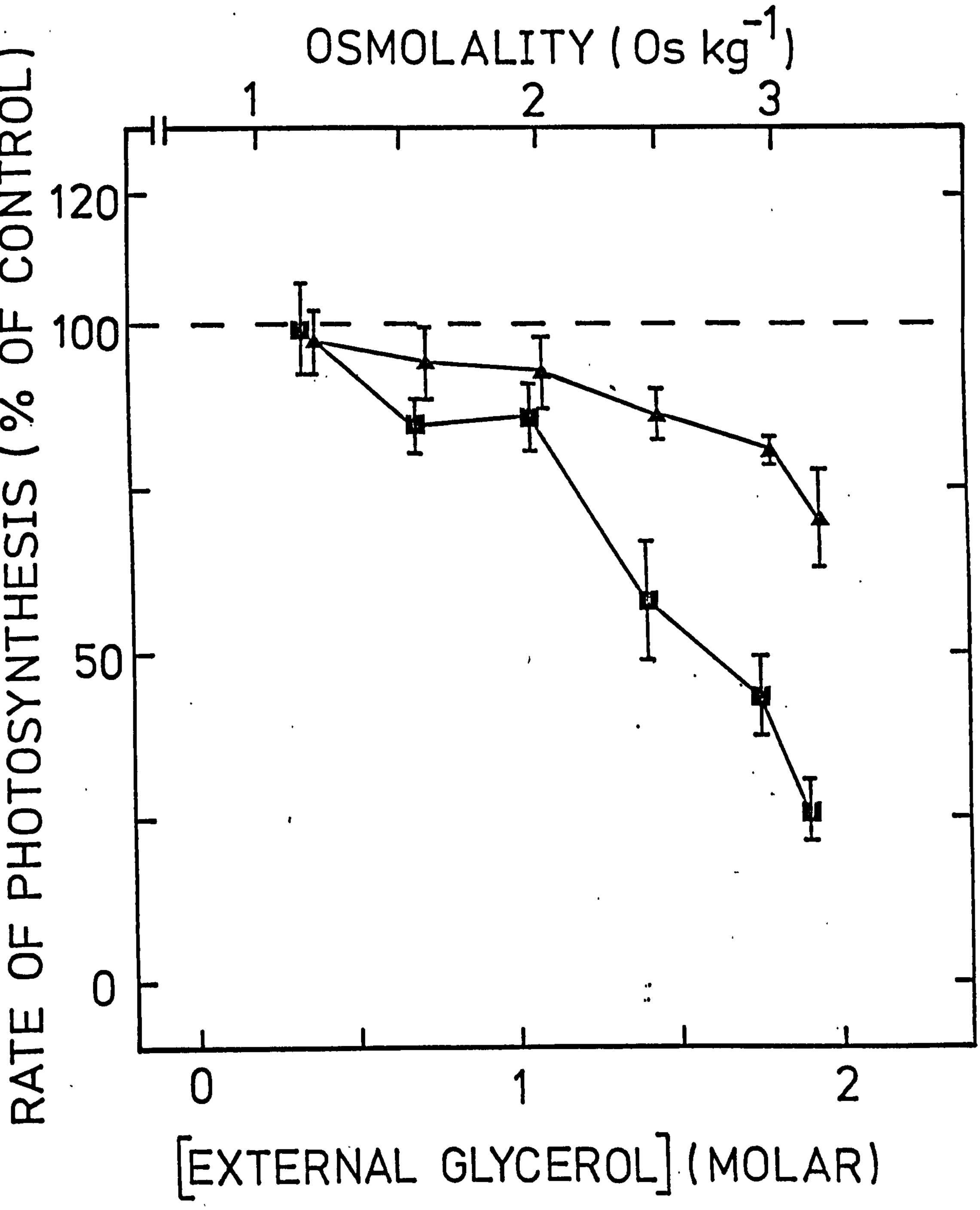


FIGURE 38. The effect of increasing the external glycerol concentration on the rate of photosynthesis. The algae were resuspended in 0.43 M NaCl plus the concentration of glycerol indicated for 5 min (■——■) or 100 min (▲——▲). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 29.2 to 63.4 μmoles O_2 evolved mg chl^{-1} h^{-1}.

KCl (Figures 28 and 32). Figure 39 shows that increasing the extracellular glycerol concentration above 0.71 M (1.6 Os kg^{-1}) inhibits the respiratory rate after 5 min; especially at higher osmolalities where the inhibition persists over the 100 min period. At lower osmolalities there is some stimulation of respiration after 100 min.

A. Ben-Amotz (personal communication) suggested that the photosynthetic rate decreases when the external salinity is increased because the oxygen content of the solutions of high NaCl concentration reach saturation level. Thus, if oxygen was evolved by the algae it would not be detected since the oxygen reading was already at the maximum level. Dr. Ben-Amotz suggested that pretreating the algal sample by bubbling nitrogen through it would lower the initial oxygen concentration and allow the detection of oxygen evolution by the algae that might otherwise be masked. This experiment was carried out and the results are presented in Table 7. They show that a small increase in the rate of light-induced oxygen evolution is found at 1.71 M NaCl (3.2 Os kg^{-1}) when the suspending medium is bubbled with nitrogen (with the algae present) to reduce the oxygen concentration to a low level. However, the difference is only of the order of 5% of the control and not significant. Table 7 also shows that bubbling the medium (and algae) with nitrogen did increase the rate of photosythesis found in the presence of 1.32 M KCl, but the higher rate was still only 15% of the control value. A small increase in the rate of photosynthesis was also found with a pretreatment of nitrogen bubbling at 3.2 Os kg^{-1} exerted by ethylene glycol or glycerol, but in neither case was the increase large. When the suspending medium (and algae) is bubbled with nitrogen in the presence of 1.38 M sucrose, there is

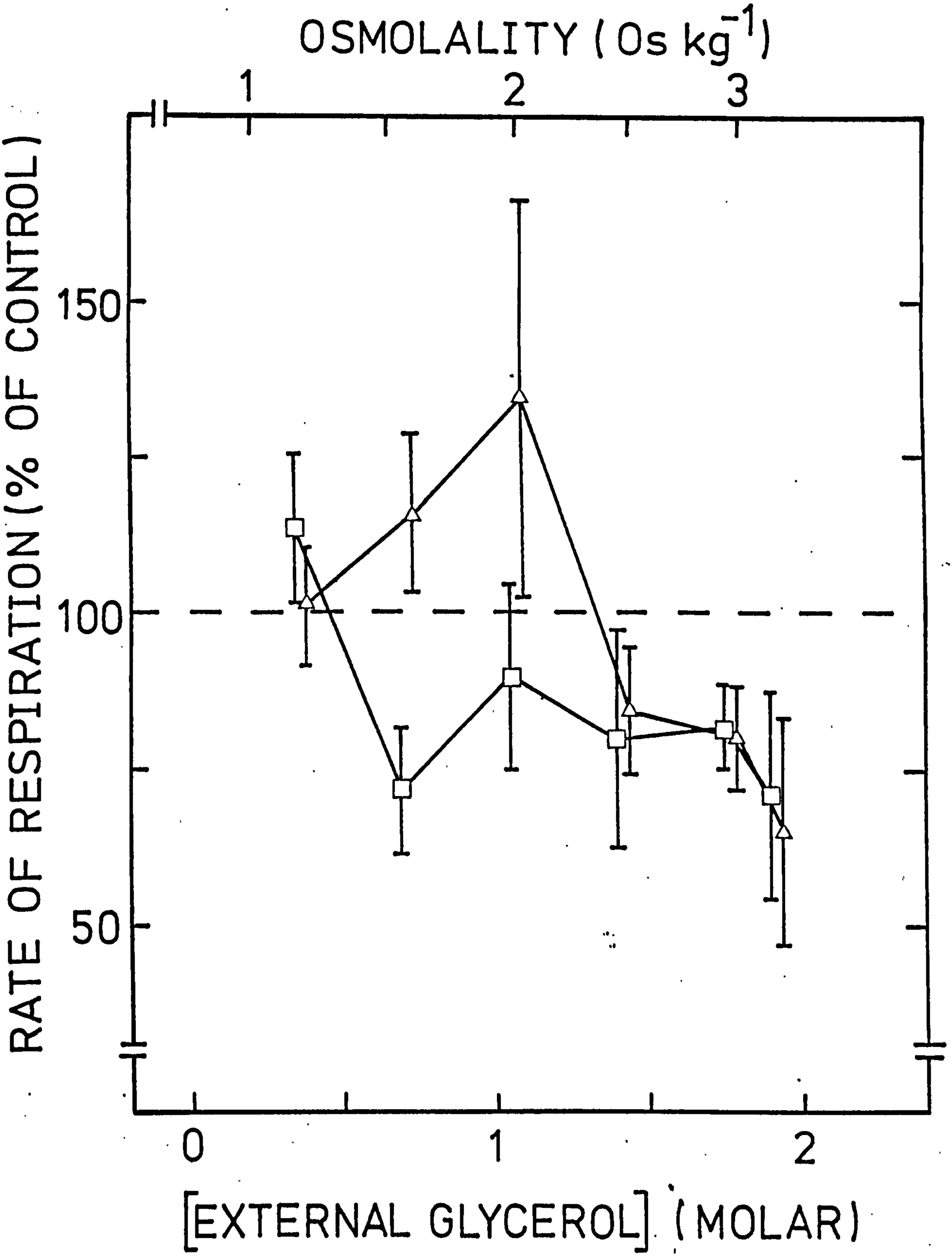


FIGURE 39. The effect of increasing the external glycerol concentration on the rate of respiration. The algae were resuspended in 0.43 M NaCl plus the concentration of glycerol indicated for 5 min (□——□) or 100 min (△——△). Each point represents the mean of five determinations. The control rate in 0.43 M NaCl (0.8 Os kg^{-1}) ranged from 2.7 to 26.8 μmoles of O_2 taken up mg chl^{-1} h^{-1}.

TABLE 7

The Effect of Bubbling Nitrogen Through the Algae on the Inhibition of Light-Induced Oxygen Evolution Caused by Increasing the External Osmolality to 3.2 Os kg^{-1}

	Rate of Light-Induced Oxygen Evolution (% Control)	
	No Pretreatment	Bubbled with Nitrogen
1.71 M NaCl	18 ± 5.6	24 ± 8.6
1.32 M KCl	7	15
1.38 M Sucrose	79	63
2.09 M Ethylene glycol	75	78
1.92 M Glycerol	57	64

The oxygen evolution from control samples resuspended in 0.43 M NaCl (0.79 Os kg^{-1}) were measured in the presence or absence of the pretreatment. The percentage values shown in the Table were calculated using the relevant control value. In the case of NaCl, the results of four experiments are expressed as the mean plus or minus the standard error. For KCl and sucrose the data are the mean of two experiments and for ethylene glycol and glycerol, the results of a single experiment are shown.

The experimental technique was to dark-adapt the algae for 3 min following their resuspension in the test solution. Then the light was turned on and oxygen evolution was monitored for 5 min. When the algae were pretreated with nitrogen the bubbling was done in the reaction chamber of the oxygen electrode before the algal sample was dark-adapted. After bubbling with nitrogen, the oxygen concentration of the media had been reduced to a low level only slightly above the 0% value which was determined using sodium dithionite.

in fact greater inhibition of photosynthesis than in the absence of the pretreatment (Table 7). Therefore, there is no evidence for a large increase in the rate of photosynthesis in algae suspended in 3.2 Os kg^{-1} when the oxygen concentration of the suspending medium is reduced by bubbling it with nitrogen. Thus, the inhibition of photosynthesis observed at 3.2 Os kg^{-1} does not appear to be due to the test media being saturated with oxygen.

In addition, experiments were carried out in the oxygen electrode in the absence of algae. A seawater-solution of 1.71 M NaCl was placed in the reaction chamber and the amount of oxygen present was adjusted using sodium dithionite (which removes oxygen) and hydrogen peroxide (which adds oxygen). It was found that the 1.71 M NaCl solution was not saturated with oxygen because when hydrogen peroxide was added the oxygen content increased. When the oxygen saturation point was reached, the chart recorder trace began to oscillate and bubbles of oxygen could be seen in the solution in the reaction chamber. Neither oscillations of the recorder trace nor bubbles of oxygen in the reaction chamber were ever observed during the experiments described earlier in the section. This confirms that oxygen saturation was not a problem at the osmolalities used in this project.

4.3 Discussion

In section 4.1, it was stated that the rate of photosynthesis was equal to the amount of light-induced oxygen evolution corrected for the dark oxygen uptake and that respiration was equal to the amount of dark oxygen uptake. This can only be assumed if oxygen evolution and uptake occurs in processes directly connected with photosynthesis and

respiration respectively. This however does not appear to be strictly true. Kaplan, Schreiber and Avron (1980) indicated that when starch is converted to glycerol, oxygen should be evolved. This oxygen evolution although not coming from the light-induced splitting of water may nevertheless be dependent on the photosynthetic electron transport chain for the production of ATP and NADPH required for the starch to glycerol conversion. Also when glycerol is converted back to starch oxygen uptake would be expected if the NADPH produced in this reaction was oxidised by molecular oxygen (Kaplan et al., 1980). Thus, it must be borne in mind that photosynthetic oxygen evolution may include oxygen evolved when starch is converted to glycerol and that respiration may include oxygen uptake due to the glycerol to starch conversion. The relevance of these secondary processes will be discussed during this section. Photosynthesis will still be used to describe the light-induced evolution of oxygen corrected for dark oxygen uptake, but instead of respiration the general term dark oxygen uptake will be used.

The results presented in section 4.2 have shown clearly that during the 100 min period after a large increase in the external osmolality exerted by NaCl, KCl or sucrose, photosynthesis is inhibited (Figures 28, 32 and 34). The extent of the decrease in the photosynthetic rate was always highest at the maximum osmolality exerted i.e. approximately 3.2 Os kg^{-1}. It was 90% for NaCl and KCl, and 50% for sucrose. The amount of glycerol production induced by an osmolality of 3.2 Os kg^{-1} exerted by NaCl, KCl or sucrose is shown in Figures 20, 19 and 22 respectively (section 3.2). In each case a steady-state value for the glycerol content (cells plus medium) had been approached. If it is assumed that glycerol synthesis was complete and the new

intracellular glycerol level had been reached, then the percentage glycerol produced within 100 min compared to the amount of glycerol synthesised to reach the new steady-state level can be calculated. The values obtained were 50% (NaCl), 70% (KCl) and 86% (sucrose). However, since the time allowed to reach the assumed steady-state glycerol level was longer for NaCl (360 min) than for KCl or sucrose (160 min), it seems reasonable to conclude that at least 50% of the glycerol synthesis necessary to produce the new stable intracellular glycerol level has taken place within 100 min of an osmolality increase to 3.2 Os kg^{-1} induced by these osmotica (Figures 19, 20 and 22). In any case, there is a substantial amount of glycerol synthesis during this 100 min period; it is 17, 14 and 26 µmoles of glycerol produced mg $chlorophyll^{-1}$ for NaCl, KCl and sucrose respectively (Figures 20, 19 and 22). Thus this amount of glycerol synthesis takes place at an osmolality of 3.2 Os kg^{-1} while the rate of photosynthesis is greatly decreased (Figures 28, 32 and 34).

The effect on dark oxygen uptake of an increase in the external osmolality exerted by NaCl, KCl or sucrose was also determined in section 4.2 (Figures 29, 33 and 35). The results were rather variable probably because the rate of oxygen uptake in the control samples could be very low which led to large percentage changes. Figures 29 and 35 showed that the rate of dark oxygen uptake was inhibited during the 100 min after the osmolality was increased using NaCl or sucrose. There was a small amount of stimulation in the case of KCl, but this could not be correlated with increasing the osmolality (Figure 33). Thus, with respect to NaCl, KCl and sucrose, it appears that dark oxygen uptake is moderately inhibited during the period of glycerol production.

Increasing the external glycerol concentration inhibits the rate

of photosynthesis (Figure 38), and glycerol appeared to have the same effect on the cell size changes measured in the Coulter counter as NaCl, KCl or sucrose (Figure 25). This suggests that glycerol can exert an osmotic stress on the cells. If this is so it would be indirect evidence to support the hypothesis that the plasmalemma of the alga was relatively impermeable to glycerol (section 3.4). The rate of dark oxygen uptake is also inhibited by high external glycerol concentrations after 5 and 100 min, which would support the hypothesis. However at the end of 100 min, there is stimulation of dark oxygen uptake at the low external glycerol concentrations (Figure 39).

Increasing the external concentration of ethylene glycol also inhibits the rate of photosynthesis (Figure 36). However, in the Coulter counter experiments, ethylene glycol appeared to induce a small increase in the cell size as opposed to the small decreases observed with the other osmotica (Figure 25). Moreover, ethylene glycol did not induce any glycerol synthesis (Figure 23), and this suggests that it does not exert an osmotic stress on the cells. The inhibition of photosynthesis observed could be due to the direct effect of ethylene glycol on the intracellular environment. This theory is supported by the inhibition of dark oxygen uptake by ethylene glycol after 5 min. However, at the end of 100 min the rate of oxygen uptake is stimulated by all bar one of the ethylene glycol concentrations (Figure 37). This makes it unlikely that the direct interference of ethylene glycol with the intracellular environment is responsible for the inhibition of photosynthesis.

Decreasing the external salinity was shown to result in a loss of intracellular glycerol content (Figure 18). This reduction in the intracellular glycerol content can occur in two ways; either by excretion or leakage of glycerol into the medium, or by glycerol being metabolically

converted to starch where it is osmotically inactive. Figure 30 shows that decreasing the external salinity had a similar inhibitory effect on the rate of photosynthesis as an increase in the external salinity (Figure 28). However, decreasing the external salinity had a stimulatory effect on the rate of dark oxygen uptake after 5 min at all the decreased salinities except distilled water (Figure 31). This may indicate a role for respiration in the metabolic conversion of glycerol to starch. However, Kaplan et al. (1980) also found an increase in the rate of dark oxygen uptake in D. salina in response to a salinity decrease, which they attributed directly to the conversion of glycerol to starch. Starch synthesis from glycerol requires ATP but it produces NADPH, as already mentioned this NADPH can be oxidised by molecular oxygen resulting in oxygen uptake (Kaplan et al., 1980). Thus, the increase in dark oxygen uptake as the salinity is decreased may be caused by a greater conversion of glycerol to starch which decreases the glycerol content of the cells. Further decreases in the salinity may cause less stimulation of dark oxygen uptake because a greater proportion of the glycerol being lost is excreted or leaked into the medium rather than being converted to starch (Figure 31). The data quoted in section 3.2 for glycerol excretion at 0.09 M NaCl (0.16 Os kg^{-1}) would tend to support this suggestion, but more data on the relative leakage or excretion of glycerol at various levels of decreased salinity in conjunction with measurements of the dark oxygen uptake of the alga are needed to confirm it. The process which stimulates the rate of oxygen uptake in the dark must be complete 100 min after the salinity is decreased, since at this time the stimulation of the dark oxygen uptake has been replaced by a small amount of inhibition. In the absence of salt (distilled water) the cells are disrupted within 10 min (Figure 27), but they are still capable of a reduced rate of dark oxygen uptake 100 min

after being placed in distilled water (Figure 31).

Brown and coworkers made a similar study of the oxygen evolution and uptake of Dunaliella when exposed to increased or decreased external salinity (Brown and Borowitzka, 1979; Kessly and Brown, 1981). They made their measurements during the period of intracellular glycerol adjustment using the halotolerant species D. viridis and the marine species D. tertiolecta; both species responded in essentially similar ways. They found a clear inhibition in the amount of light-induced oxygen evolution which in their study resulted in oxygen uptake in the light. This agrees with the results shown in Figure 28, although oxygen uptake in the light was only rarely observed in this project. However, it may well be that if the algae in this study had been subjected to a slightly larger external NaCl increase, oxygen uptake in the light would have been observed regularly. Brown and Borowitzka (1979) found that for D. viridis it took about 48 hours for the oxygen evolution completely to return to normal after a salinity increase from 1.5 to 4.0 M. This agrees with some preliminary long term experiments carried out on D. tertiolecta during this project (data not shown).

However, Kessly and Brown (1981) using D. tertiolecta found that a decrease in the external NaCl concentration from 1.6 to 0.53 M did not inhibit oxygen evolution very much. This contrasts markedly with Figure 30 where substantial inhibition of the photosynthetic rate was found by decreasing the salinity from 0.43 to 0.09 M. The difference in results may be due to the fact that Kessly and Brown normally grew D. tertiolecta in a medium of 0.17 M NaCl. Thus, at the end of their salinity decrease experiments, the salinity was still three times that of the normal growth medium. Whereas in this project the salinity decreases represented actual decreases when compared to the salinity of

the normal growth medium (0.43 M NaCl).

Brown and coworkers reported that dark oxygen uptake persisted after either an increase or decrease in salinity. Their results for the effect of increasing the external salinity on the rate of oxygen uptake are in general agreement with those shown in Figure 29 i.e. that dark oxygen uptake appears to be partially reduced after an increase in the external salinity. However, Brown and coworkers found that decreasing the external salinity from 1.6 to 0.53 M caused a reduction in oxygen uptake immediately after the salinity decrease. This contrasts with the results shown in Figure 31 which showed a stimulation of the dark oxygen uptake induced by decreasing the salinity. This could well be due to the different methods of decreasing the salinity employed in the two studies which were outlined above.

Wegmann (1971) used a Warburg apparatus to estimate the effect of increasing the external osmolality on the oxygen evolution or uptake of D. tertiolecta. In this study, cells were incubated in the new osmolality for 15 min before the measurements were made. Wegmann also found that with the algae illuminated increasing the external NaCl concentration caused a decline in oxygen evolution, then oxygen consumption accompanied by CO_2 evolution. These processes showed distinct maxima at 2.8 M NaCl (cells were normally grown at 1 M NaCl). The author suggested that the respiratory quotient indicated that a fermentation process took place at higher salinities. However, Wegmann (1981) found that although the photosynthetic rate was diminished after a salinity increase, the percentage of ^{14}C photosynthetically incorporated into glycerol was greatly increased. The carbon flow into glycerol could exceed 80% after a salinity increase from 1.0 to 1.7 M.

The results of the present study and the work by Brown and

Borowitzka (1979), Kessly and Brown (1981), and Wegmann (1971) suggests that Dunaliella produces intracellular glycerol in response to an increase in the external osmolality whilst the rate of photosynthesis is very low. There is evidence to suggest that dark oxygen uptake is also inhibited during this period, but to a lesser extent. Thus, the initial part of the glycerol production appears to come mainly via the breakdown of starch, the storage product of the alga. Although, there is no evidence for the oxygen evolution which Kaplan et al. (1980) suggested occurs when starch is converted to glycerol. Photosynthesis certainly seems to contribute to the initial glycerol production as suggested by the ^{14}C-incorporation results of Wegmann (1981). However, the significance of its role will depend on the severity of the inhibition of photosynthesis. It is certainly true, however, that the rate of photosynthesis does not need to return to normal before it can contribute to glycerol synthesis. This is illustrated by Figure 24 which shows that during the 100 min after a salinity increase to 1.71 M, glycerol production in the light is faster than in the dark or when noncyclic photosynthetic electron transport is inhibited by DCMU. During this period the rate of photosynthesis has only recovered to about 60% of the control value (Figure 28). Unfortunately, the information available does not allow the calculation of the proportion of initial glycerol synthesis that depends on photosynthetic products and the proportion that depends on the degradation of starch.

In contrast to this hypothesis, there is evidence which suggests that photosynthesis is in fact stimulated by an increase in the external NaCl concentration. Craigie and McLachlan (1964) using a ^{14}C-incorporation method found that the photosynthetic rate increased as the salinity of the medium increased. However, the cells had been growing in the higher

salinities for 5 weeks before the measurements were made and were thus fully adapted to the media. This long term stimulation of photosynthesis by higher external salinities was also found by Gimmler, Weidemann and Moller (1981). This phenomenon will be discussed further in Chapter 7.

A stimulation of photosynthesis by increasing the osmolality of the medium was found by Ben-Amotz and Avron (1972) and Frank and Wegmann (1974) using oxygen electrode measurements. Unfortunately, neither publication states whether the algae have been newly resuspended in the media of high osmolality or whether they have been allowed to adapt to them before the measurements were taken. Thus, these data lose a lot of their usefulness.

However, Gimmler's group have found evidence for an increase in photosynthetic oxygen evolution 5 min after an increase in the salinity of the medium from 1.5 to 2.5 M (Bruggemann et al., 1978). This was shown to persist for two hours after the salinity increase. Dark oxygen uptake rates measured at the same time behaved in a similar fashion to those described in this thesis. This initial stimulation in photosynthetic rate after a salinity increase was confirmed by Gimmler et al. (1981) who found a higher amount of CO_2-fixation and a larger reduction of p-benzoquinone (measure of noncyclic electron transport) 15 min after a salinity increase from 1.5 to 2.5 M. In the same experiment decreasing the salinity inhibited both of these processes. Therefore, the results concerning an immediate salinity-induced stimulation of photosynthesis disagree with the results found in this study. One possible explanation for this is that different species of Dunaliella were used, Gimmler's group used the halotolerant D. parva (normally grown at 1.5 M NaCl), whereas the marine D. tertiolecta (normally grown at 0.43 M NaCl) was used in this study.

Gimmler et al. (1981) pointed out that the immediate salt-induced increase in photosynthesis found with D. parva is not present if the salinity is increased to higher levels than approximately 3 M, although the cells can withstand transfers to these higher levels of external salinity. Thus, the initial increase in the photosynthetic rate is not essential for osmoregulation in D. parva. Kaplan et al. (1980) also found that the rate of oxygen evolution in D. salina increased immediately after an increase in the salinity from 1.5 to 2.1 M. Both D. parva and D. salina are halotolerant species, therefore, it is tempting to suggest that this immediate stimulation of photosynthesis in response to a moderate increase in salinity is a characteristic of halotolerant species of Dunaliella, due to their adaptation to high external salt concentrations. It was not found by Brown and Borowitzka (1979) using the halotolerant species D. viridis, however the only increase in salinity used in this study was from 1.5 to 4.0 M which is too large to allow the stimulation of photosynthesis according to Gimmler et al. (1981). It could therefore be characteristic of halotolerant Dunaliella species, but more information is needed to confirm this.

The apparent salt-induced stimulation of photosynthesis in some Dunaliella species may in fact be due to starch being converted to glycerol, because Kaplan et al. (1980) suggested that this process would evolve oxygen. However, Gimmler et al. (1981) also measured the increased photosynthetic rate as an increase in the amount of CO_2-fixation which would not be expected if the majority of the glycerol was coming from starch.

The results described in this chapter have shown clearly that the rate of photosynthesis of the marine D. tertiolecta is inhibited by increasing the external salt concentration from 0.43 to 1.71 M NaCl.

There is no evidence for an immediate salt-induced stimulation of photosynthesis at any of the intermediate salinity increases (Figure 28). The inhibition of photosynthesis persists during at least the first 100 min after an increase in the external NaCl concentration from 0.43 to 1.71 M; by the end of 100 min a significant amount of glycerol synthesis has taken place.

CHAPTER 5

CHLOROPHYLL FLUORESCENCE

5.1 Background Theory

Each time a photon of light energy is captured by an antenna chlorophyll molecule, an electron is promoted to a higher energy level i.e. an excited state (Govindjee and Govindjee, 1974). De-excitation of these excited states can occur by four competing processes in chlorophyll in vivo:-

1. internal conversion to heat (nonradiative decay) - rate constant k_H
2. fluorescence - rate constant k_F
3. energy transfer from PSII to PSI (under normal circumstances PSI does not fluoresce at room temperature) - rate constant k_T
4. trapping by a reaction centre i.e. photochemistry - rate constant k_P.

Each process is given a rate constant and the quantum yield of fluorescence (ϕ_F) can be expressed as:-

$$\phi_F = \frac{k_F}{k_H + k_F + k_T + k_P}$$

By definition the sum of the quantum yields, $\phi_H + \phi_F + \phi_T + \phi_P = 1$.

At physiological temperatures, most of the fluoresance is emitted by the chlorophyll a of PSII with its maximum around 685 nm (Papageorgiou, 1975). In contrast, the yield of PSI fluorescence at room temperature is low (Boardman, Thorne and Anderson, 1966; Vredenberg and Slooten, 1967). PSI fluorescence can be measured at cryogenic temperatures (section 5.4), but the following discussion relates to room temperature fluorescence.

The amount of fluorescence emanating from algae or chloroplasts can be measured. The simplest way to interpret these measurements is to think of

fluorescence as an inverse measure of photochemistry i.e. if fluorescence increases, photochemistry decreases and vice versa. Duysens and Sweers (1963) suggested a mechanism that would result in an inverse relationship between fluorescence and photochemistry. They proposed that the amount of fluorescence depended on the redox state of Q, the primary electron acceptor of PSII. If it is assumed that k_p (rate of photochemistry) was much larger than the other processes, then if Q is oxidised a quantum of light would be trapped by the reaction centre and used for photochemistry; in this state the reaction centre is said to be open. Therefore in its oxidised state Q quenches the fluorescence yield (originally called Q to denote its quenching of fluorescence). However, if Q is reduced, then a quantum trapped by the reaction centre cannot be used for photochemistry and it is returned to the antenna chlorophyll; in this state the reaction centre is said to be closed. If A is the number of reaction centres which are open, the value of k_p can be multiplied by the proportion of open reaction centres. This can be expressed in terms of the quantum yield of fluorescence (ϕ_F):-

$$\phi_F = \frac{k_F}{k_H + k_F + k_T + k_P A}$$

There are two extreme cases of this equation:-

all reaction centres open, $\phi_F = \frac{k_F}{k_H + k_F + k_T + k_P.1}$ = Fo (minimum level of fluorescence)

all reaction centres closed, $\phi_F = \frac{k_F}{k_H + k_F + k_T}$ = Fm (maximum level of fluorescence)

When all the reaction centres are open, A = 1, Q is oxidised, and the fluorescence level is low (Fo). However, when all the reaction centres are closed, A (and thus k_p) = 0, Q is reduced, and the fluorescence level is high.

These theoretical predictions can be related to experimental observations. Dark-adapted algae have all their reaction centres open; when they are exposed to light the initial fluorescence measured is Fo. In the light, the reaction centres begin to close and the fluorescence yield begins to increase towards Fm. However, electron transport to PSI will reoxidise Q and therefore quench fluorescence (Figure 4, Chapter 1). Thus to get an accurate measure of Fm, DCMU is added to the preparation. DCMU blocks electron transport between Q and R, therefore, in its presence Q cannot be reoxidised and the maximum fluorescence level is reached. The Fm level is thus largely determined by the concentration of Q in the algal sample. A typical fluorescence induction curve after dark-adaptation and the addition of DCMU is shown in Figure 40. The amount of fluorescence measured as the reaction centres close is equal to Fm minus Fo, this is called the variable fluorescence Fv.

In the experimental system, Fo and Fm can be measured and therefore Fv is also known. An estimate of the photochemical efficiency of PSII can be deduced from the measured fluorescence parameters as follows:-

$$\frac{Fm - Fo}{Fm} = \frac{Fv}{Fm} = \frac{\dfrac{k_F}{k_F + k_H + k_T} - \dfrac{k_F}{k_F + k_H + k_T + k_P}}{\dfrac{k_F}{k_F + k_H + k_T}}$$

Whence,

$$\frac{Fv}{Fm} = \frac{k_P}{k_F + k_H + k_T + k_P} = \phi_P max$$

$\phi_P max$ is the maximum photochemical efficiency of PSII and has a value between 0 and 1, and the lower its value the less efficiently PSII is operating.

However, there are two main objections to this simple relationship

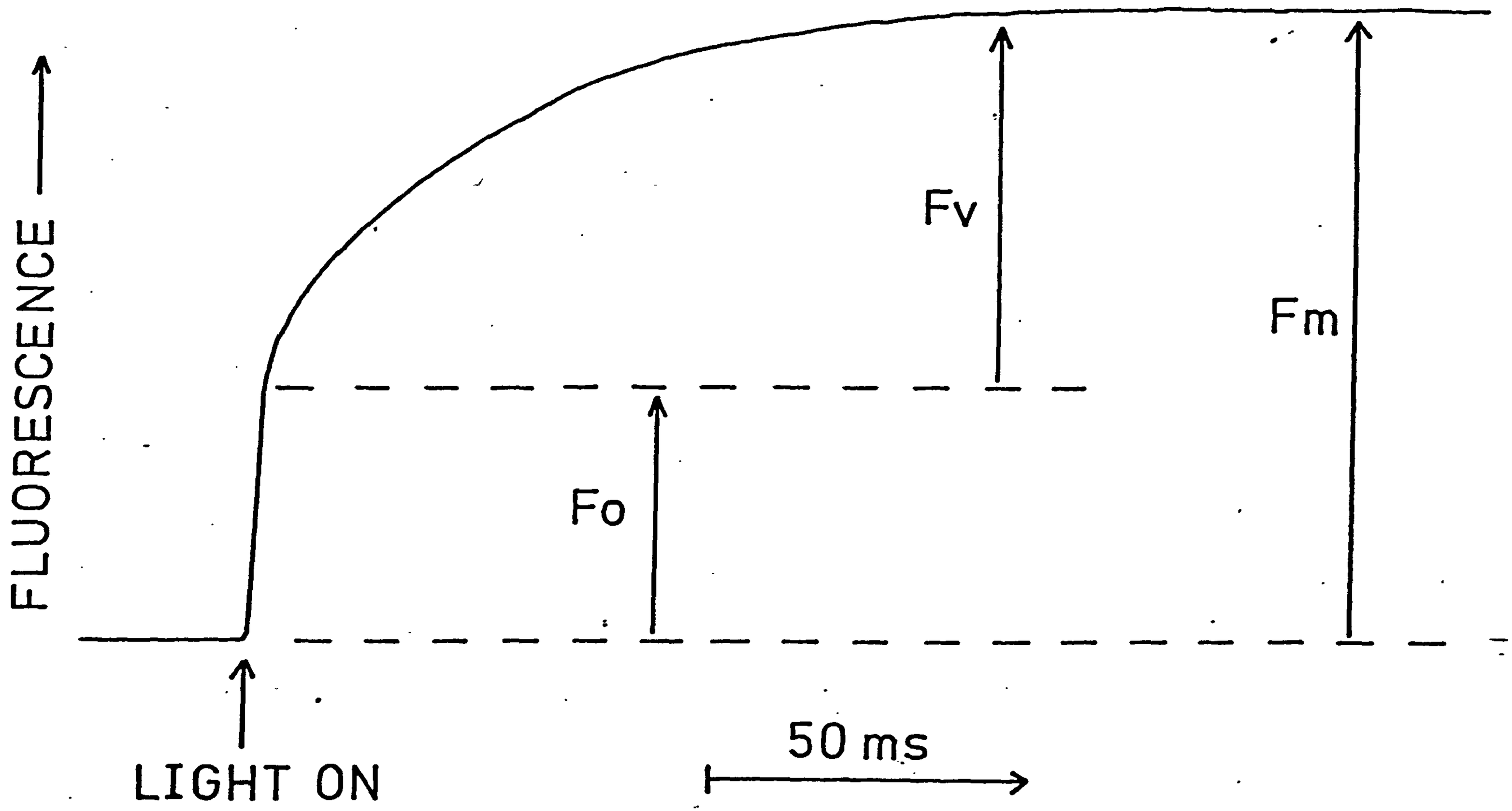


FIGURE 40. A typical fluorescence induction curve for dark-adapted algae in the presence of DCMU.

connecting fluorescence and photochemistry:-

(a) the usual experimentally measured increase in fluorescence from Fo to Fm is in the order of 3 to 5 fold. However, Haehnel et al. (1982) state that theoretical expectations based on a quantum yield of photochemistry of approximately 0.95 when Q is oxidised (i.e. reaction centre open) suggest that when photochemistry is blocked (by e.g. DCMU) the Fm level should be 20-fold higher than Fo (Kok and Hoch, 1961; Sun and Sauer, 1971). Clayton (1969) and Lavorel and Joliot (1972) suggested that this could be due to a constant fluorescence emanating from either PSI chlorophyll or from a photochemically inactive form of chlorophyll, which would be present at the Fo level and therefore make the Fo level appear larger. However, Kitajima and Butler (1975) did not find any evidence for this 'constant fluorescence' in chloroplasts at 77K.

(b) the simple inverse relationship outlined above predicts that Fv/Fm is equal to the maximum yield of photochemistry ($\phi_p$max). Therefore artificial quenchers of fluorescence and photochemistry would be expected to quench Fo and Fm in such a way as to decrease the ratio Fv/Fm in proportion to the decrease in $\phi_p$max. This is found when dibromothymoquinone is used as the artificial quencher, since its mode of action is simply to compete with reaction centres for the quanta of light passing around the antenna chlorophyll molecules (Kitajima and Butler, 1975). However, it is not true of ferricyanide which quenches Fm while Fo is unaffected, and although Fm is quenched, the primary photochemistry is largely unaffected (Butler and Kitajima, 1975). Thus, in the case of ferricyanide Fv/Fm does not equal $\phi_p$max.

To try to explain these two discrepancies, Butler and Kitajima (1975) and Butler (1978) proposed a new bipartite model for the photochemical apparatus (Figure 41). In addition to the processes assigned rate constants

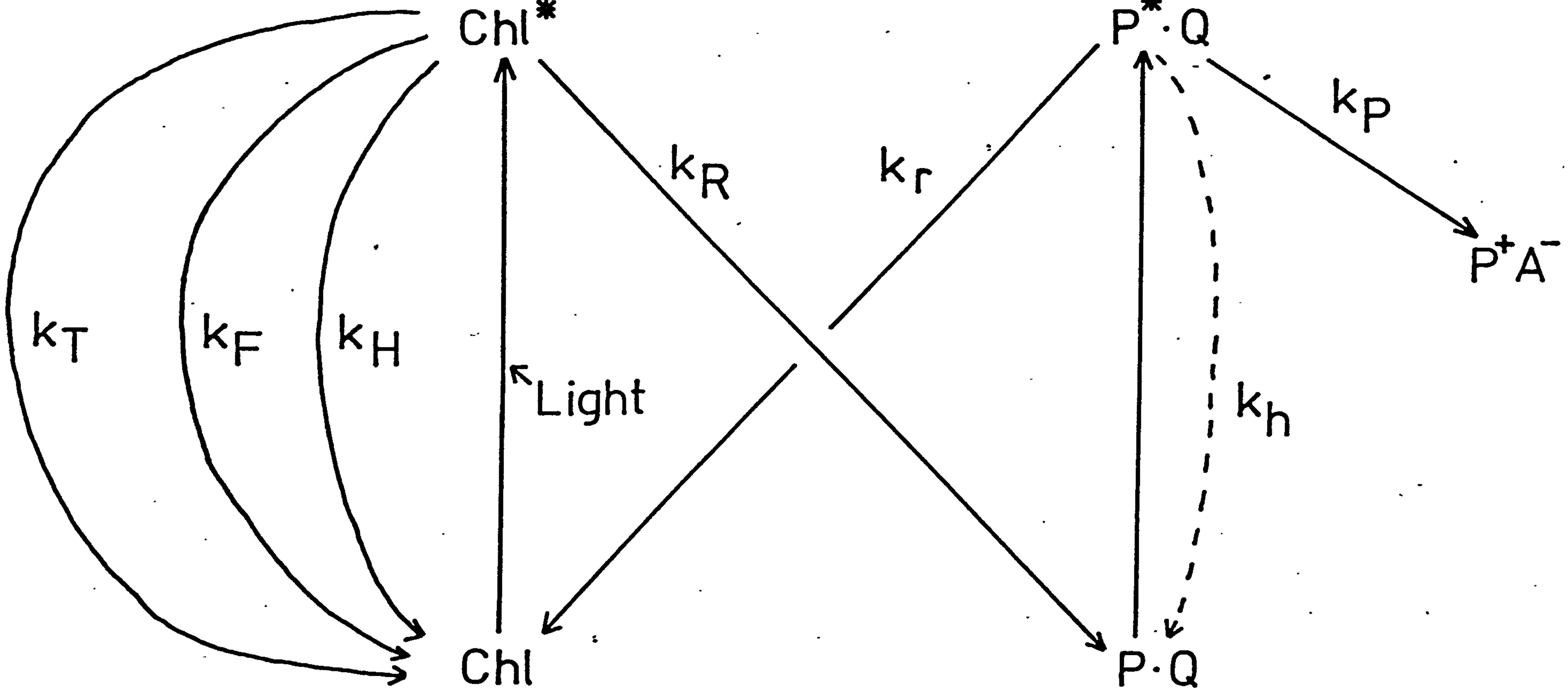


FIGURE 41. The bipartite model for the fate of excitation energy in the photochemical apparatus (adapted from Butler, 1978). See text for the definitions of the rate constants. Other symbols represent:- *: the excited state; Chl: antenna chlorophyll molecule; P·Q: primary electron donor (P) and primary electron acceptor (Q) of the PSII reaction centre.

at the beginning of the chapter, the following processes are added to the bipartite model:-

1. transfer of energy from antenna chlorophyll to the reaction centre - rate constant k_R

2. the back transfer of energy from the reaction centre to antenna chlorophyll - rate constant k_r

3. internal conversion to heat (nonradiative decay) in the reaction centre - rate constant k_h.

The discrepancies in the level of F_m and the specific quenching of Fv can be rationalised by considering the process with rate constant k_h i.e. internal conversion to heat within the reaction centre (Figure 41). If ferricyanide increased the rate of k_h, then this would quench Fv without affecting Fo; it would not however, affect the rate of photochemistry providing that $k_p \gg k_r$ or k_h in the open reaction centres. Internal conversion to heat within the reaction centre could also account for the ratio Fm/Fo being 3 to 5 instead of the theoretically expected 20, in conjunction with energy transfer to PSI. Butler and Kitajima (1975) suggested that two types of energy transfer from PSII to PSI are possible, one type from the antenna chlorophyll (k_T) and the other type from the reaction centre (equivalent to k_h) (Figure 41). Thus energy transferred from PSII to PSI from the antenna chlorophyll decreases both Fm and Fo, however PSII to PSI energy transfer from the reaction centre decreases Fm but not Fo.

Experimental evidence to support the bipartite model has been found by Haehnel et al. (1982) who studied the fluorescence lifetime on a picosecond time scale of chlorophyll a in chloroplasts and green algae. With the reaction centres open (Fo), they found that fluorescence is dominated by two fast components which probably represent the transfer of light energy from PSII antenna chlorophyll molecules and from the LH a/b complex (page 22) to

the PSII reaction centres. When the reaction centres are closed (Fm) the fast fluorescence components remain, because transfer to the reaction centres continues due to the primary electron donor (P) of PSII (page 22) being rapidly rereduced by water. However, at Fm a slow fluorescence component is now dominant, and it may reflect the kinetics of back transfer from the reaction centres to the antenna chlorophyll (Haehnel et al., 1982). The data concerning the two fast components of fluorescence emission support the tripartite model of the photochemical apparatus, which is similar to the bipartite model except that it separates the LH a/b complex from PSII (Butler and Strasser,1977; Butler, 1978). However, the distinction between the bipartite and tripartite models need not concern us here, suffice to say that the results of Haehnel et al. (1982) support the concept of the bipartite model which embraces the idea of internal conversion at the reaction centre (k_h) as another alternative pathway of de-excitation of the excited state (Figure 41).

The fluorescence measurements which are presented in section 5.2 are expressed as Fv/Fm. If the simple model of Duysens and Sweers (1963) is used Fv/Fm equals $\phi_P$max as already described. However, the preceding discussion shows that this simple relationship is no longer tenable. The factor of internal conversion to heat at the reaction centre must be taken into account when the Fv/Fm results are interpreted. This can be done by introducing the rate constant for trapping at a closed reaction centre (k_h) into the equation (Figure 41). As already mentioned, due to the rapid rereduction of the primary electron donor of PSII by water, the rate of excitation of reaction centres can be assumed to stay constant irrespective of whether the reaction centre is open or closed. Therefore, the processes that occur in the excited reaction centre (P^*Q) can be examined in isolation (Figure 41). If the reaction centres are open (Q is oxidised) the majority

of the quanta go to photochemistry i.e. $k_p > 0.9$. However, if the reaction centres are closed there are only two possible pathways for the excitation energy i.e. transfer back to the antenna chlorophyll (k_r) or internal conversion to heat within the reaction centre (k_h). An estimate of the amount of internal conversion within the reaction centres has been made by van Grondelle and Duysens (1980). They assumed that when all the reaction centres are open $k_p > 0.9$. If the fluorescence yield was to increase by a factor of 4 upon illumination, $\phi_p max = Fv/Fm = 4 - 1/4 = 0.75$. If $k_p > 0.9$, then $k_p/k_p - k_h > 0.9/0.75$ or $k_h > 0.17\ k_p$ (Figure 41). Thus, this predicts that almost 20% of the excitation energy can be lost to internal conversion in the closed reaction centre, and this specifically quenches Fm (van Grondelle and Duysens, 1980). Therefore, the following relation can be established:-

$$\frac{Fv}{Fm} = \frac{k_P}{k_P + k_H + k_T + k_F} \div \frac{k_P}{k_P - k_h}$$

$$= \phi_P max \div \frac{k_P}{k_P - k_h}$$

Thus, a decrease in Fv/Fm may mean that the efficiency of photochemistry ($\phi_p max$) is being decreased, but it may also mean that the level of photochemistry remains the same while the amount of internal conversion to heat within the reaction centre (k_h) is being increased. Alternatively both processes may be responsible for a part of the decrease in Fv/Fm. This must be kept in mind when the Fv/Fm results are interpreted.

In section 5.3.1, results are presented from experiments in which the fluorescence kinetics were measured in the absence of DCMU over a long time scale i.e. 2 min (compared to 100-200 ms for Fv/Fm determinations). The characteristic waves of fluorescence found during the first 2 min after dark-

adapted algae are illuminated are known as the Kautsky effect (Papageorgiou, 1975; Lavorel and Etienne, 1977). As already mentioned, the initial rise in fluorescence is due to the closing of PSII reaction centres, it appears that initially PSII is overdriven due to its close coupling with the LH a/b complex (page 24). However, a fall in the fluorescence yield then takes place which can be correlated with Q becoming oxidised and it may well represent a spillover of energy to PSI to approach the optimal rates of operation of the two photosystems (page 24). The effect of increasing or decreasing the external osmolality on the Kautsky effect in D. tertiolecta is shown in section 5.3.1.

The results of similar long term fluorescence measurement experiments are shown in section 5.3.2, but in this case the algae are exposed sequentially to light which excites either PSII only or both PSII and PSI. This allows the investigation of the effect of external osmolality changes on the regulatory mechanisms that should be present in D. tertiolecta to keep the photosystems operating efficiently (page 24).

In the long term fluorescence experiments, the level of fluorescence is decreased after a few minutes illumination. This quenching can be relaxed by the addition of DCMU and the effect of external osmolality changes on the kinetics of DCMU-induced relaxation of fluorescence quenching is shown in section 5.3.3.

In section 5.3.4, the effect of increasing the external osmolality on the distribution of light energy between the photosystems was determined by simultaneous measurements of fluorescence emission at 695 nm (correlated with PSII) and 720 nm (correlated with PSI).

Finally, to complement the room temperature fluorescence results, excitation spectra were determined from 650 to 800 nm at 77 K (section 5.4).

5.2 Determination of Fv/Fm

Previously, the method of measuring the fluorescence parameters Fo (minimum fluorescence) and Fm (maximum fluorescence) was outlined (sections 2.10 and 5.1). The algal sample was dark-adapted for 5 min and DCMU was added immediately before the light was switched on. Under these conditions, a fluorescence induction curve was obtained which was similar to that shown in Figure 40. Thus, from the induction curve, Fo and Fm can be measured and the variable fluorescence Fv (= Fm - Fo) can be calculated. The results presented in this section concern measurements of the fluorescence parameters after algal samples had been subjected to external osmolality changes. Measurements of Fm and Fo were made 5 and 100 min after the osmolality was changed. Between measurements the algae were kept in ambient room light.

Figure 42 shows that increasing the external concentration of NaCl to 1.2 M (2.2 Os kg^{-1}) or above decreases Fv/Fm after 5 min, however below 1.2 M NaCl there is little inhibition. The maximum decrease in Fv/Fm after 5 min is found at 1.71 M NaCl (3.2 Os kg^{-1}) where the ratio is 65% of the control value. After 100 min there is a recovery of Fv/Fm at all the increased salinities and only at 1.71 M NaCl was there substantial inhibition. At external NaCl concentrations of 1.2 M or less, there is a slight stimulation of Fv/Fm after 100 min (Figure 42).

Figure 43 shows that decreasing the external salinity (except to distilled water) does not decrease Fv/Fm after 5 or 100 min, in fact there is a small amount of stimulation. There is also a stimulation of Fv/Fm when exposed to distilled water for 5 min. Figure 27 showed that after 5 min in distilled water many of the cells were disrupted, thus disruption of the cells does not immediately decrease fluorescence. However after

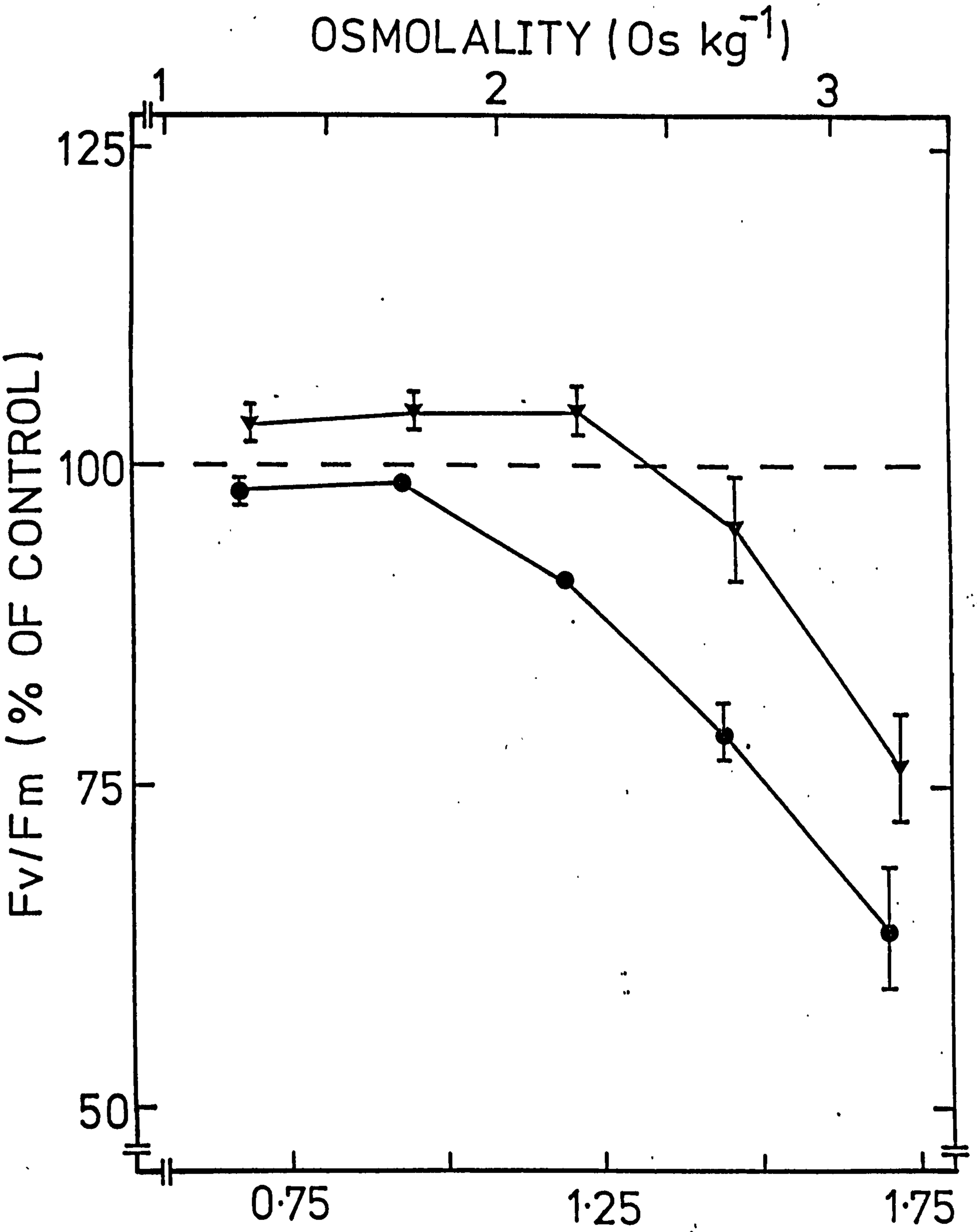


FIGURE 42. The effect of increasing the external NaCl concentration on Fv/Fm. The algae were resuspended in the salinities indicated for 5 min (●——●) or 100 min (▼——▼). Each point represents the mean of three determinations. The Fv/Fm level at 0.43 M NaCl (control) was 0.69 ± 0.009 after 5 min and 0.71 ± 0.003 after 100 min.

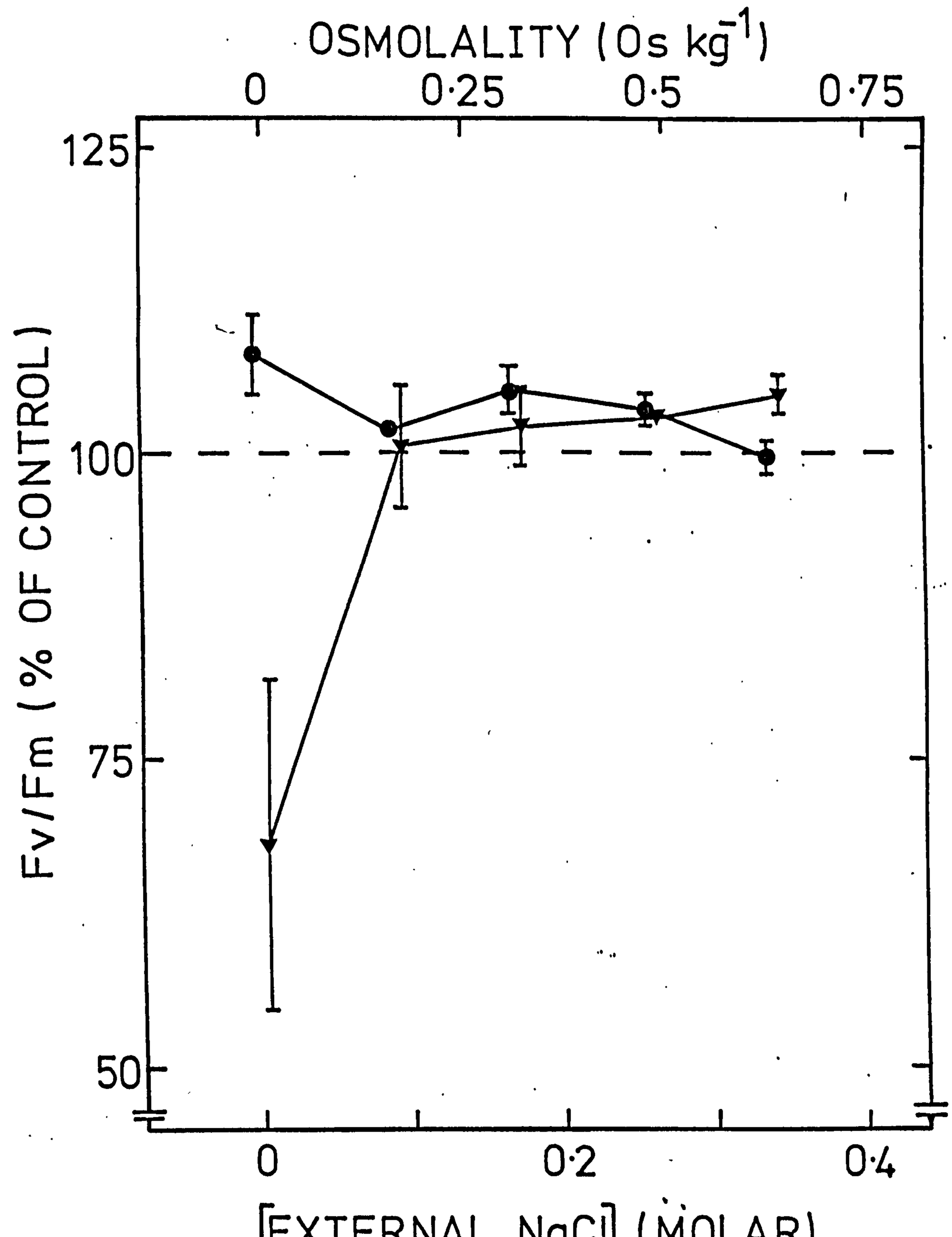


FIGURE 43. The effect of decreasing the external NaCl concentration on Fv/Fm. The algae were resuspended in the salinities indicated for 5 min (●——●) or 100 min (▼——▼). Each point represents the mean of three determinations. The Fv/Fm level at 0.43 M NaCl (control) was 0.60 ± 0.014 after 5 min and 0.71 ± 0.003 after 100 min.

100 min in distilled water when disruption of the cells would be complete (Figure 27) there is a decrease in Fv/Fm, but it is still almost 70% of the control value (Figure 43).

Figure 44 shows that increasing the external KCl concentration has a similar effect on the Fv/Fm ratio as NaCl additions (Figure 42). Two slight differences are apparent, one is the small stimulation at low external concentrations of KCl after 5 min and the other is the lower value of Fv/Fm at KCl concentrations below 0.77 M KCl (2.2 Os kg^{-1}) after 100 min (Figures 42 and 44).

Figure 45 indicates that increasing the external sucrose concentration decreases the Fv/Fm ratio after 5 min, but not to the same extent as NaCl does (Figure 42). However, after 100 min at the increased sucrose solutions there has been little or no recovery of Fv/Fm. The levels of Fv/Fm in sucrose solutions are now similar to those in NaCl solutions after 100 min except for a lack of stimulation of Fv/Fm at the low osmolalities (Figures 42 and 45).

Figure 46 shows that increasing the external ethylene glycol concentration decreases the Fv/Fm ratio only slightly after 5 min. The maximum decrease was only 8.5% compared to 36% for NaCl (Figure 42). After 100 min, there was a slight stimulation of Fv/Fm at all concentrations of external ethylene glycol used.

Figure 47 shows that increasing the extracellular glycerol concentration above 1.41 M (2.4 Os kg^{-1}) decreases Fv/Fm. The maximum decrease is only 17% against 36% for NaCl (Figure 42). After 100 min there has been a recovery at the higher external glycerol solutions, although no stimulation is displayed at the lower osmolalities. The Fv/Fm values after 100 min in all the glycerol solutions used are

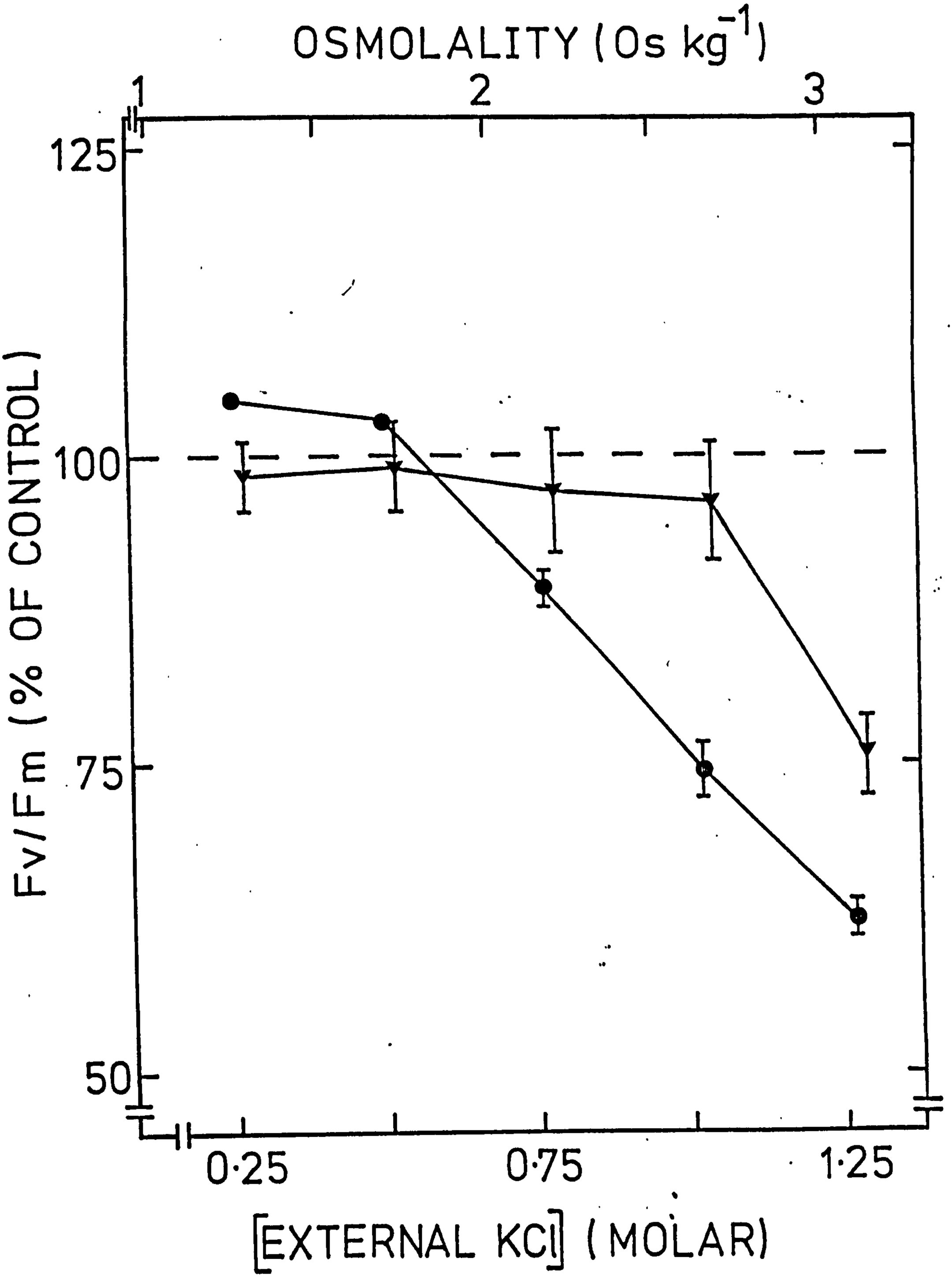


FIGURE 44. The effect of increasing the external KCl concentration on Fv/Fm. The algae were resuspended in 0.43 M NaCl plus the concentration of KCl indicated for 5 min (●——●) or 100 min (▼——▼). Each point represents the mean of three determinations. The Fv/Fm level at 0.43 M NaCl (control) was 0.58 ± 0.006 after 5 min and 0.73 ± 0.005 after 100 min.

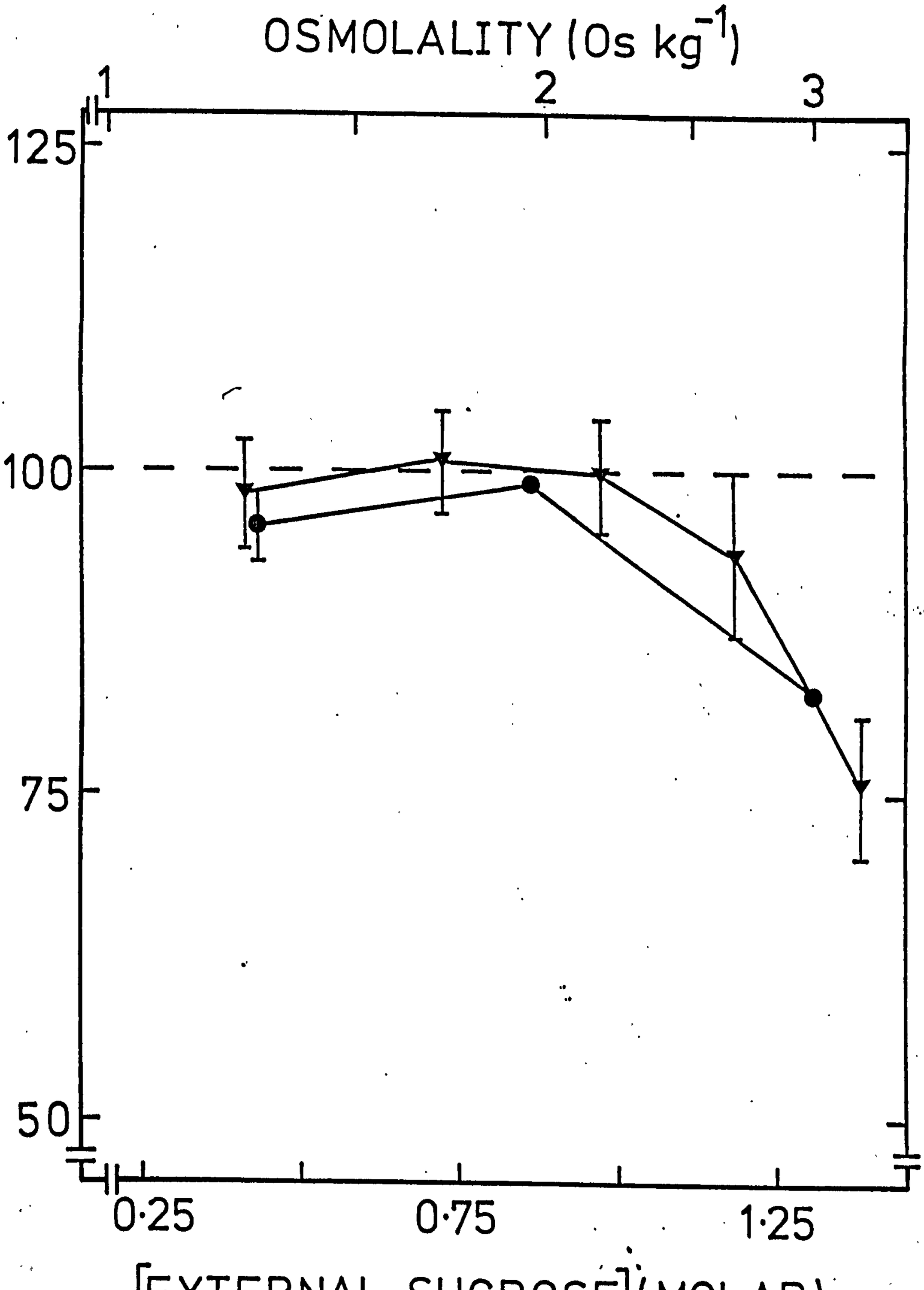


FIGURE 45. The effect of increasing the external sucrose concentration on Fv/Fm. The algae were resuspended in 0.43 M NaCl plus the concentration of sucrose indicated for 5 min (●——●) or 100 min (▼——▼). Each point represents the mean of three determinations. The Fv/Fm level at 0.43 M NaCl (control) was 0.58 ± 0.010 after 5 min and 0.73 ± 0.005 after 100 min.

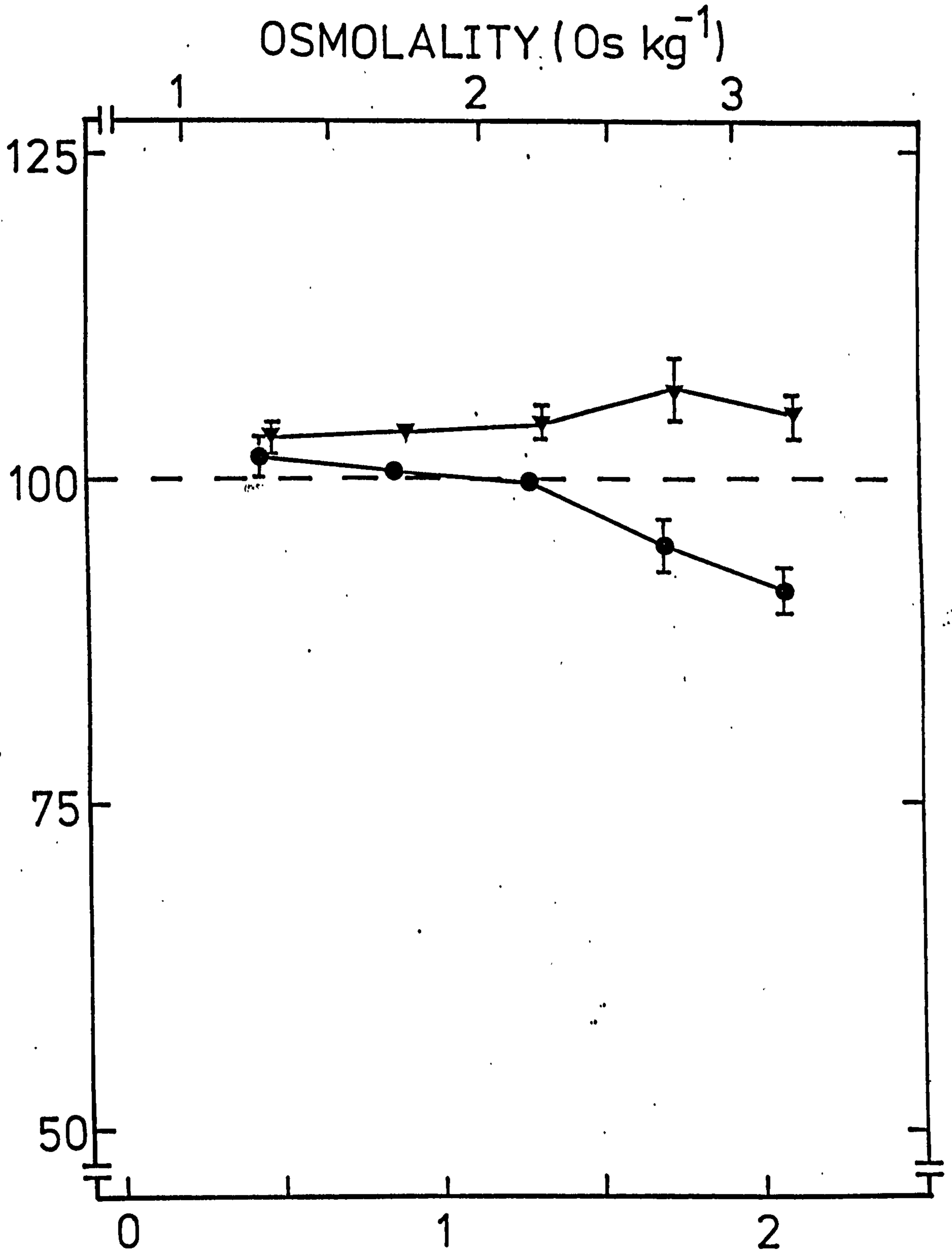


FIGURE 46. The effect of increasing the external ethylene glycol concentration on Fv/Fm. The algae were resuspended in 0.43 M NaCl plus the concentration of ethylene glycol indicated for 5 min (●——●) or 100 min (▼——▼). Each point represents the mean of three determinations. The Fv/Fm level at 0.43 M NaCl (control) was 0.58 ± 0.004 after 5 min and 0.58 ± 0.002 after 100 min.

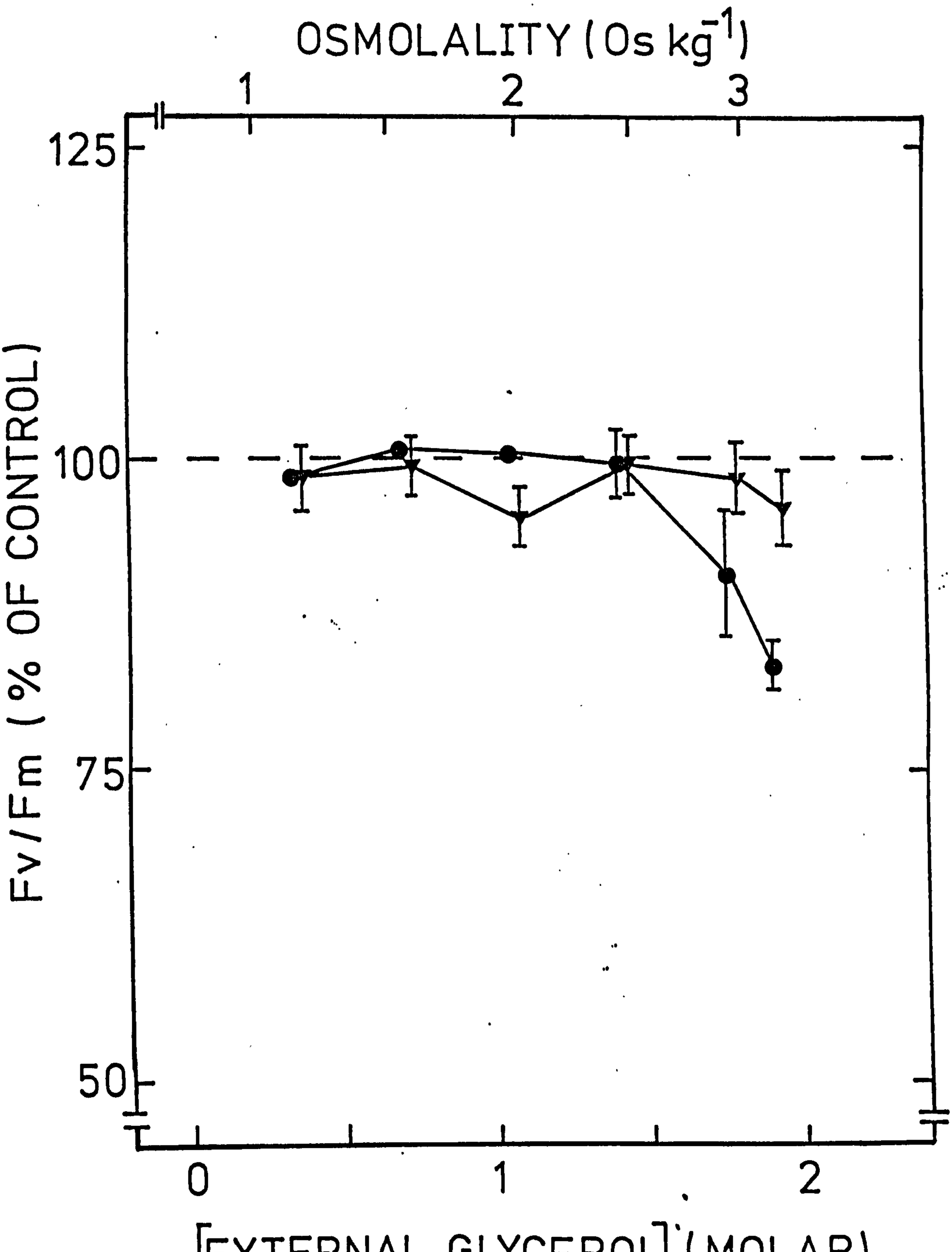


FIGURE 47. The effect of increasing the external glycerol concentration on Fv/Fm. The algae were resuspended in 0.43 M NaCl plus the concentration of glycerol indicated for 5 min (●——●) or 100 min (▼——▼). Each point represents the mean of three determinations. The Fv/Fm level at 0.43 M NaCl (control) was 0.62 ± 0.009 after 5 min and 0.62 ± 0.010 after 100 min.

at least 95% of the control value (Figure 47).

The effect of the uncoupler FCCP on Fv/Fm was determined. It was found that the addition of FCCP (to a final concentration of 2.2 μM) at the beginning of the 5 min dark adaptation period to a 3 ml algal sample (containing 30 μg of chlorophyll) reduced the Fv/Fm ratio to 15% of the control value (data not shown).

Experiments were performed to investigate the effect of long term exposure to high external osmolality on the Fv/Fm ratio in D. tertiolecta. The effect on Fv/Fm of increasing the external osmolality to 3.2 Os kg^{-1} using either NaCl, KCl or sucrose was monitored over a 21 hour period (Figure 48). The results show that in the presence of sucrose the alga recovers quicker than in NaCl or KCl. Complete recovery takes place with both the increased NaCl and sucrose media but not with KCl. There is an initial recovery after increasing the KCl concentration which is similar to the recovery in NaCl, but after 6 hours Fv/Fm begins to decrease again in the KCl solution and after 21 hours it has fallen to a low value (Figure 48).

As already mentioned (section 3.4), it was observed that the cells die after 2-3 days exposure to a high KCl concentration. However, the Fv/Fm determinations indicated that this was true only if the external KCl concentration was above 0.51 M. Below this level the Fv/Fm ratio returns to normal and the cells can continue to grow (data not shown).

5.3 Long Term Fluorescence Changes

5.3.1 Kautsky Phenomena

When algae, leaves or intact chloroplasts are exposed to light after a period of dark adaptation, characteristic waves of fluorescence

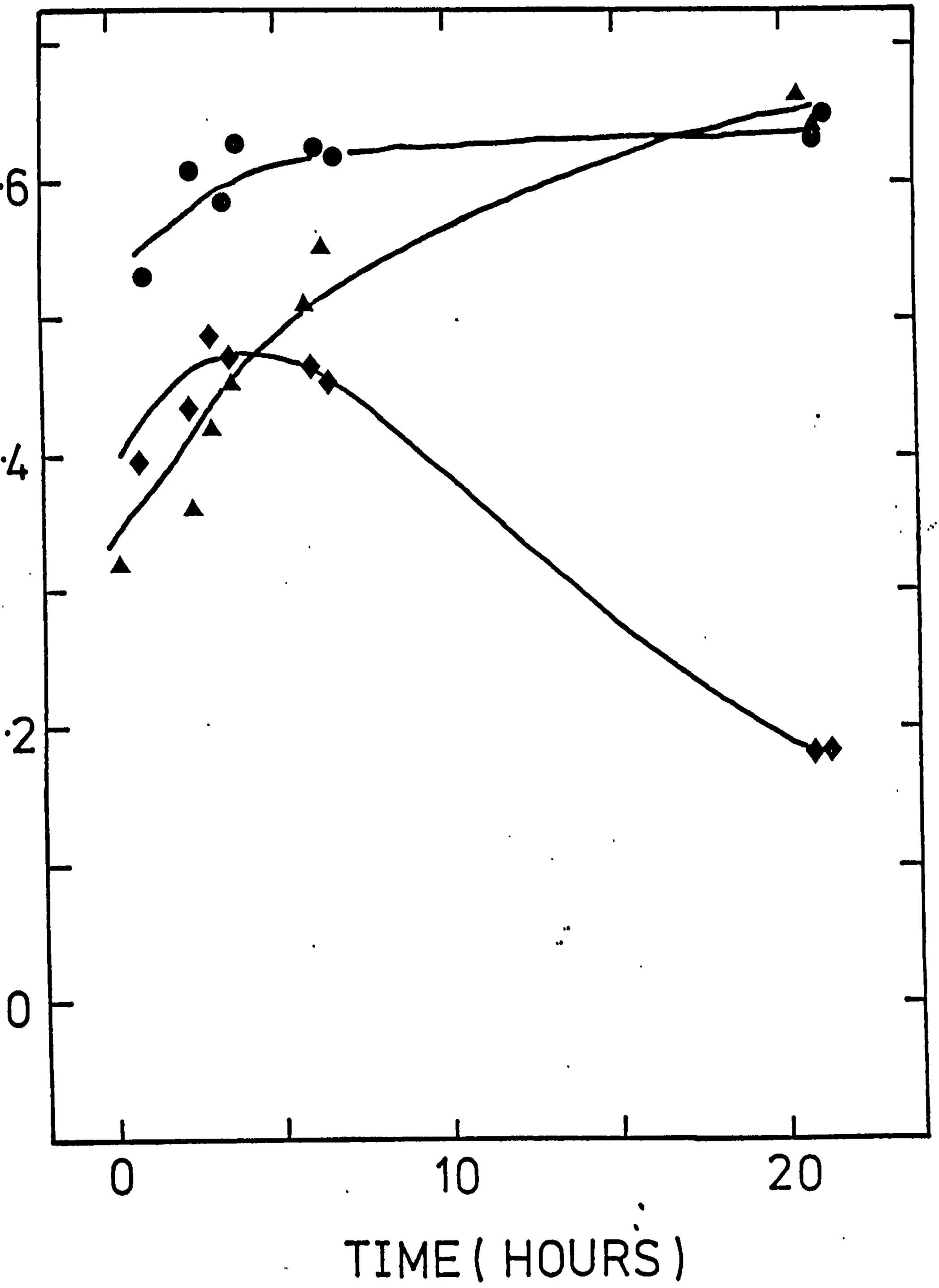


FIGURE 48. The long term effect of increasing the external osmolality to 3.2 Os kg^{-1} on Fv/Fm. The algae were resuspended in 0.43 M NaCl plus 1.28 M NaCl (▲——▲), 1.28 M KCl (◆——◆) or 1.38 M sucrose (●——●). The experiment was repeated five times and the data shown are from one representative experiment.

emission can be detected over a time scale of a few minutes which are known as the Kautsky effect (Kautsky, 1931). A typical curve of Kautsky fluorescence from D. tertiolecta is shown in Figure 49. The various peaks and troughs are assigned letters to simplify comparison of the experimental data following the convention of Lavorel (1959), and Munday and Govindjee (1969). Figure 49 shows the fluorescence induction curve of dark-adapted algae resuspended in normal growth medium (0.43 M NaCl). The initial feature of the curve is the rapid rise to I when the light is switched on. This includes the Fo level which cannot be seen because the time scale is too long (Figure 40). There is then a dip from I to D, followed by a rise in fluorescence to P. From P the fluorescence falls to S (the so-called quasi steady-state level), this is the final level of fluorescence from chloroplasts (Krause et al., 1982). However, in algae and leaves, S is followed by a slow rise to a secondary peak M and finally a slow decline to the terminal fluorescence level T (Papageorgiou, 1975). The terminal fluorescence level T has not been reached in Figure 49 after 2 min.

It is a very difficult task to assign specific photosynthetic processes to explain the Kautsky fluorescence changes (Lavorel and Etienne, 1977). However, a simplified account will be attempted here to allow easier interpretation of the results. Other possibilities will be discussed in section 5.5. As before (section 5.1) the initial fluorescence rise to I can be correlated with the reduction of Q at PSII. The decrease in fluorescence exhibited between I and S (via D and P) is probably due to a reoxidation of Q by PSI (Figure 49). At the moment of highest fluorescence (I), PSII may be overdriven, therefore, it can be suggested that the fall in fluorescence from I to S is due to an increase in the spillover of excitation energy from PSII to

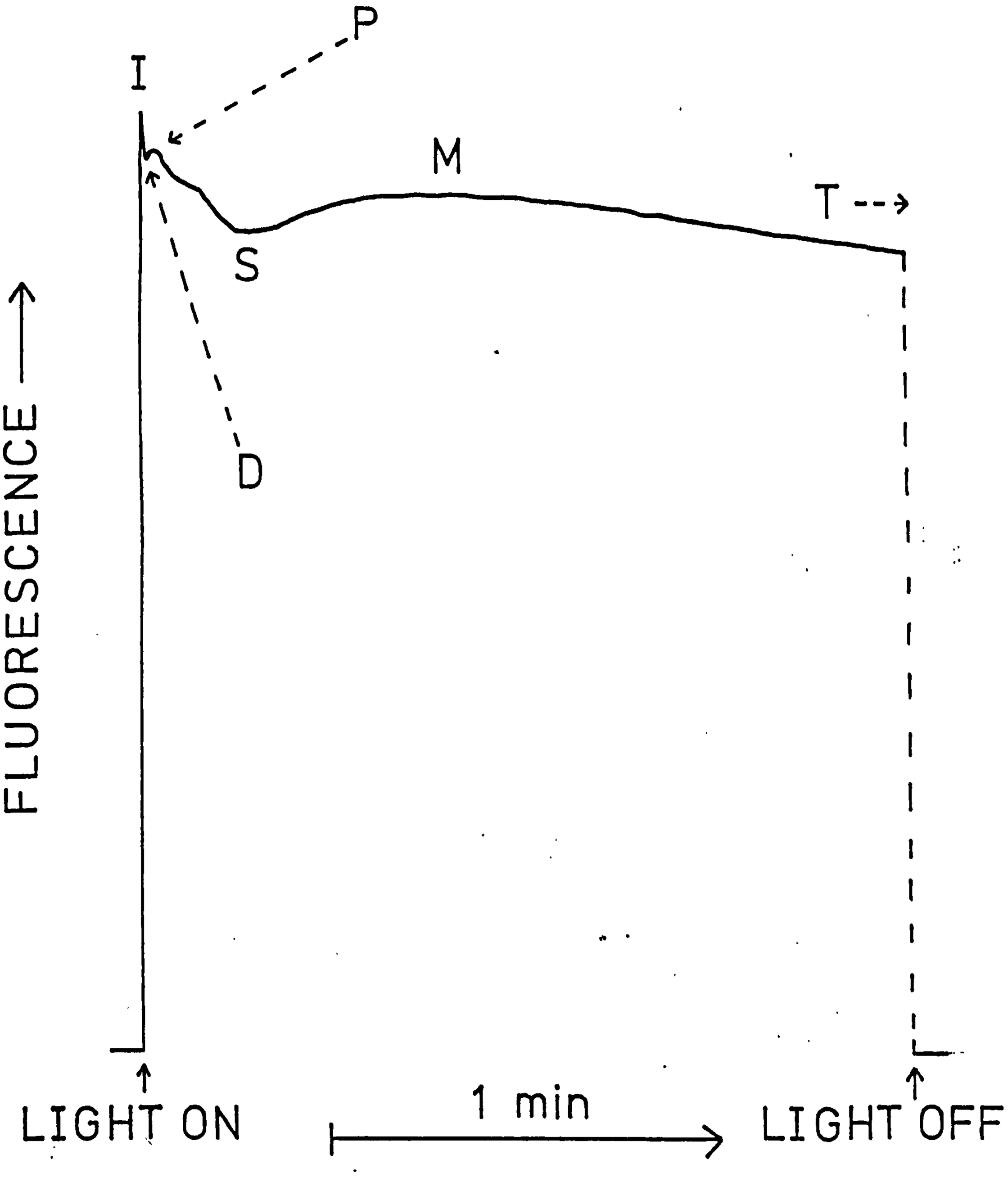


FIGURE 49. A typical fluorescence induction curve showing the Kautsky phenomena. The quality of light used predominantly excited PSII i.e. Light 2 (Page 47).

PSI so that both photosystems can approach their optimal rate of operation. The slow SMT transition can be thought of as fine tuning of the system to reach the steady-state situation with photosynthesis operating efficiently (Figure 49).

Figure 50 shows the effect of increasing the salinity on the Kautsky phenomena after 5 min. The first change as the NaCl concentration increases is that the small fluorescence increase from D to P progressively declines until at 1.20 M (2.2 Os kg^{-1}, Trace D) it has almost completely disappeared. During the increase from 0.43 M NaCl (Trace A) to 1.20 M NaCl (Trace D) the secondary SMT phase has also disappeared. Thus at 1.20 M NaCl, there is a large decrease from peak fluorescence at I to a low steady-state level. Increasing the salinity further to 1.45 and 1.71 M NaCl (Traces E and F respectively) show a similar fluorescence induction curve to 1.20 M NaCl, but with a greater degree of fluorescence quenching being displayed. In addition, the initial fluorescence peak at I does not decrease as quickly at the higher salinities (Figure 50, Traces E and F). Figure 51 shows that after 100 min the magnitude of the fluorescence induction has decreased at all salinities including the control (Trace A), but especially so above 0.94 M NaCl (1.7 Os kg^{-1}, Trace C). The shape of the curve has returned completely to normal at 0.68 M NaCl (Trace B) and it is showing signs of doing so at the higher salinities. Trace F shows that even at 1.71 M NaCl (3.2 Os kg^{-1}) the SMT transition is beginning to return and a shoulder at the D to S phase is recognisable (Figure 51).

Figure 52 shows that decreasing the salinity (except to distilled water) does not have as great an effect on Kautsky fluorescence parameters after 5 min as increasing the NaCl concentration has (Figure 50). The peak at the D to P region disappeared but at all decreased salinities

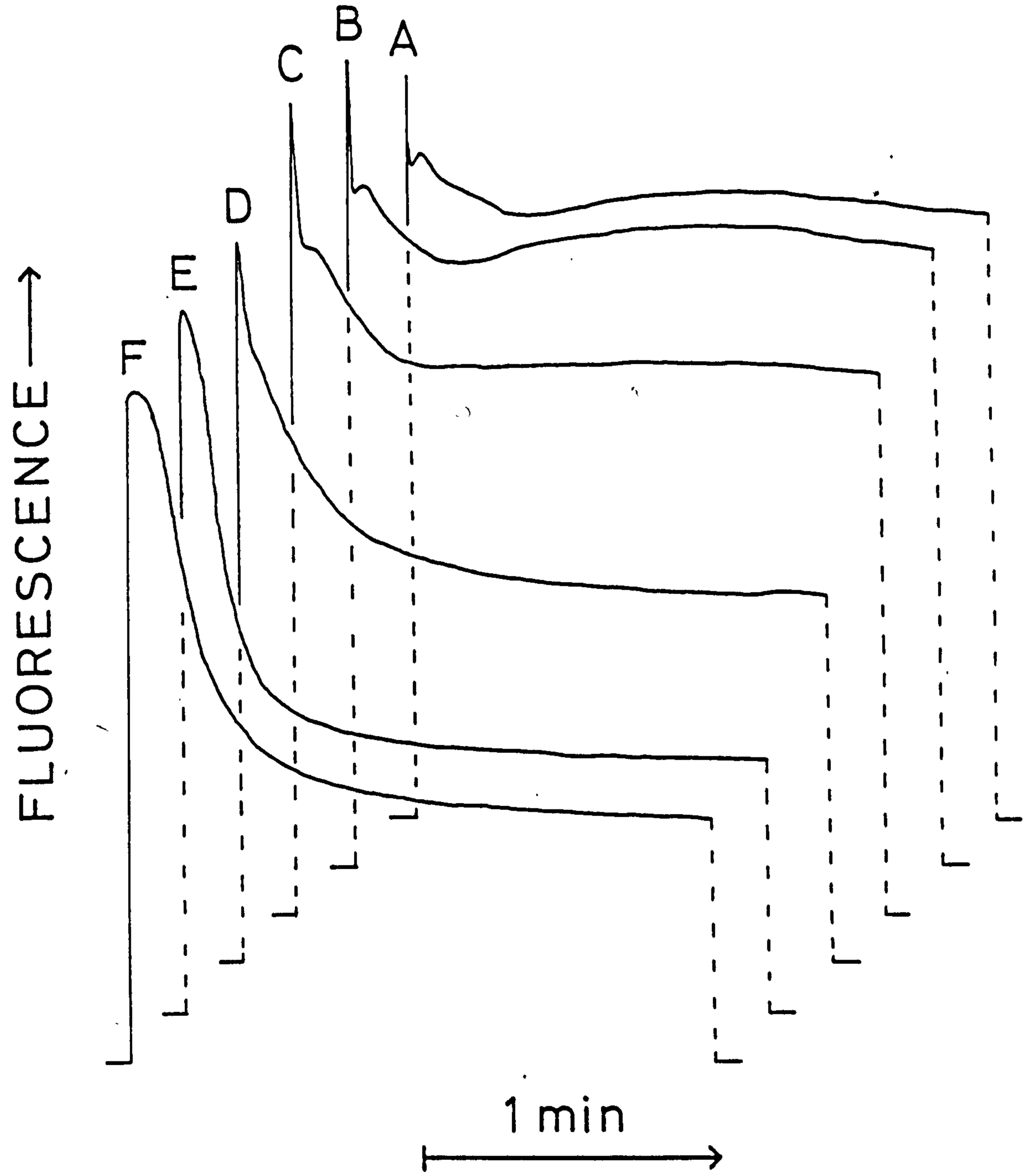


FIGURE 50. The effect of increasing the external NaCl concentration on the Kautsky phenomena. The fluorescence emission was measured after the algae were resuspended for 5 min (in the dark) in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.68 M NaCl (1.3 Os kg^{-1}); C: 0.94 M NaCl (1.7 Os kg^{-1}); D: 1.20 M NaCl (2.2 Os kg^{-1}); E: 1.45 M NaCl (2.7 Os kg^{-1}); F: 1.71 M NaCl (3.2 Os kg^{-1}).

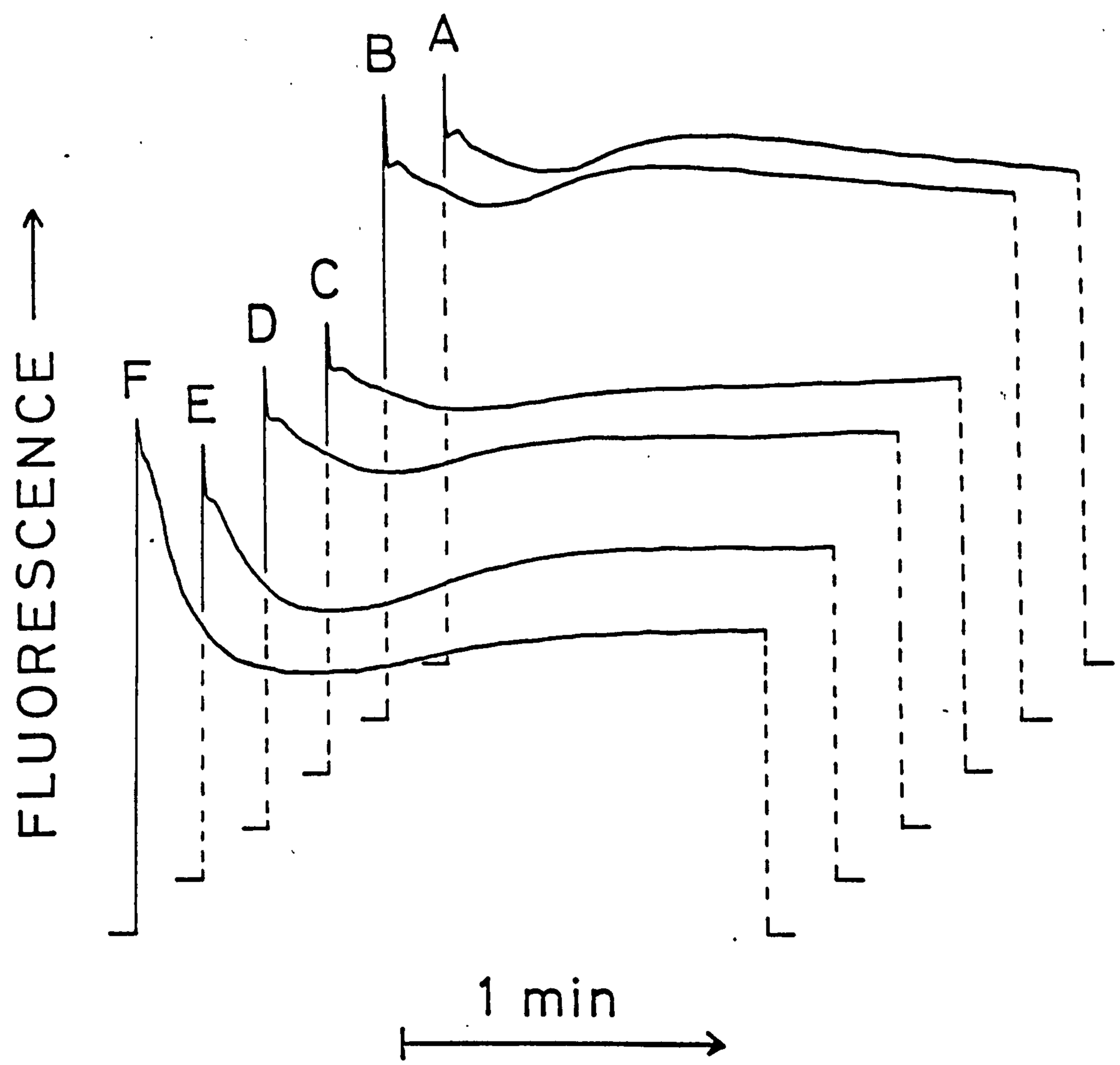


FIGURE 51. The effect of increasing the external NaCl concentration on the Kautsky phenomena. The fluorescence emission was measured after 100 min at A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.68 M NaCl (1.3 Os kg^{-1}); C: 0.94 M NaCl (1.7 Os kg^{-1}); D: 1.20 M NaCl (2.2 Os kg^{-1}); E: 1.45 M NaCl (2.7 Os kg^{-1}); F: 1.71 M NaCl (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement.

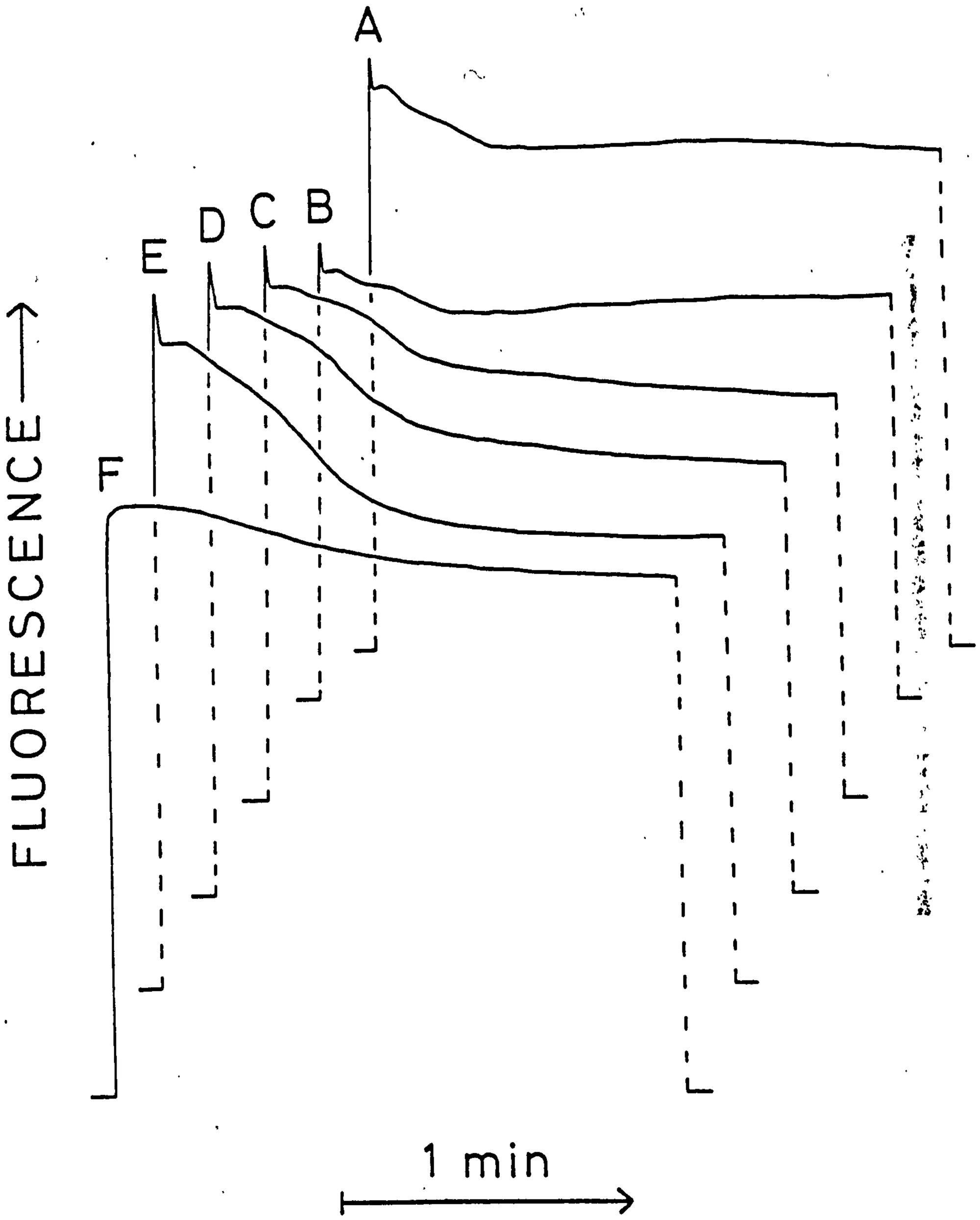


FIGURE 52. The effect of decreasing the external NaCl concentration on the Kautsky phenomena. The fluorescence emission was measured after the algae were resuspended for 5 min (in the dark) in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.34 M NaCl (0.6 Os kg^{-1}); C: 0.26 M NaCl (0.5 Os kg^{-1}); D: 0.17 M NaCl (0.3 Os kg^{-1}); E: 0.09 M NaCl (0.2 Os kg^{-1}); F: distilled water (0 Os kg^{-1}). Trace F is scaled down by a factor of 2.

(except distilled water) it was still present as a plateau. The SMT transition was affected by all decreases in salinity and increased fluorescence quenching took place especially at 0.09 M NaCl (0.16 Os kg^{-1}, Trace E). However this was less than that induced by high external NaCl concentrations. Also, the peak at I declined rapidly in all decreased salinities (except distilled water) in contrast to the high salinity situation (Figure 50). Distilled water (Trace F) showed a large increase in the initial peak I (Trace F is scaled down by a factor of 2 in Figure 52). There is little fluorescence quenching at distilled water and there is no evidence of the usual Kautsky phenomena. After 100 min the Kautsky fluorescence parameters have returned to normal at all decreased salinities except distilled water (Figure 53). In distilled water there is a constant level of fluorescence which does not decay during the 2 min measurement period (Trace F); at this time all the cells are known to be disrupted (Figure 27).

Figure 54 shows that after 5 min the effect of increasing the KCl concentration on the Kautsky phenomena was virtually identical to the effect of increasing the NaCl concentration (Figure 50). After 100 min, at all increased KCl concentrations (and in the control) considerable reduction in the magnitude of fluorescence is evident (Figure 55). It is also clear that compared to NaCl after 100 min (Figure 51), there is much less recovery of the Kautsky characteristics in the case of KCl, even though the NaCl and KCl solutions exert the same external osmolalities (Figure 55).

After 5 min in the presence of increased external sucrose concentrations, the Kautsky parameters are similar to those induced by increased NaCl concentrations except that the D to P transition persists to a higher osmolality and most strikingly the peak at I

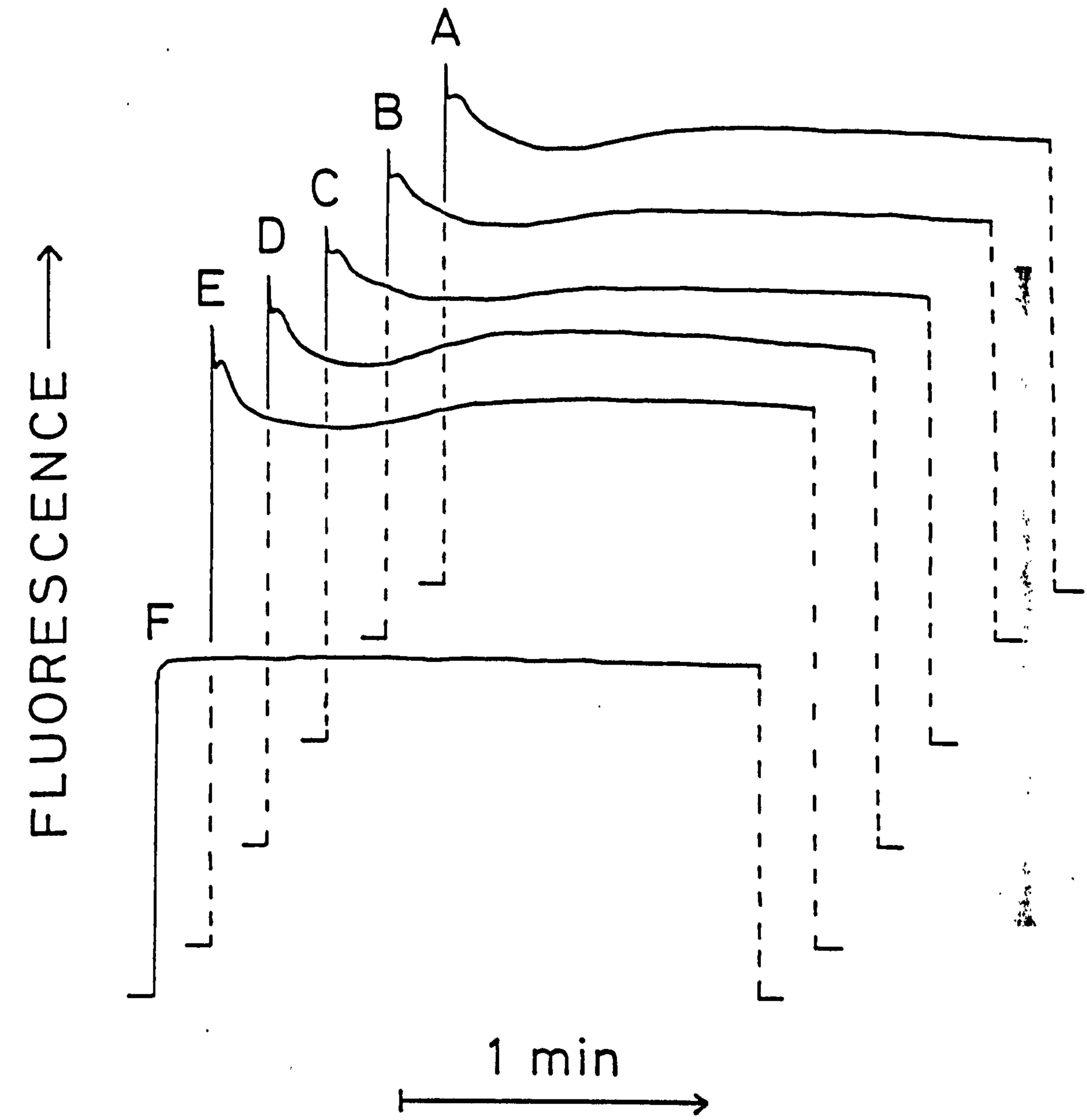


FIGURE 53. The effect of decreasing the external NaCl concentration on the Kautsky phenomena. The fluorescence emission was measured after 100 min at A: 0.43 M NaCl (0.8 Os kg^{-1}), control; 0.34 M NaCl (0.6 Os kg^{-1}); C: 0.26 M NaCl (0.5 Os kg^{-1}); D: 0.17 M NaCl (0.3 Os kg^{-1}); E: 0.09 M NaCl (0.2 Os kg^{-1}); F: distilled water (0 Os kg^{-1}). Trace F is scaled down by a factor of 2. The algae were dark adapted for 5 min prior to measurement.

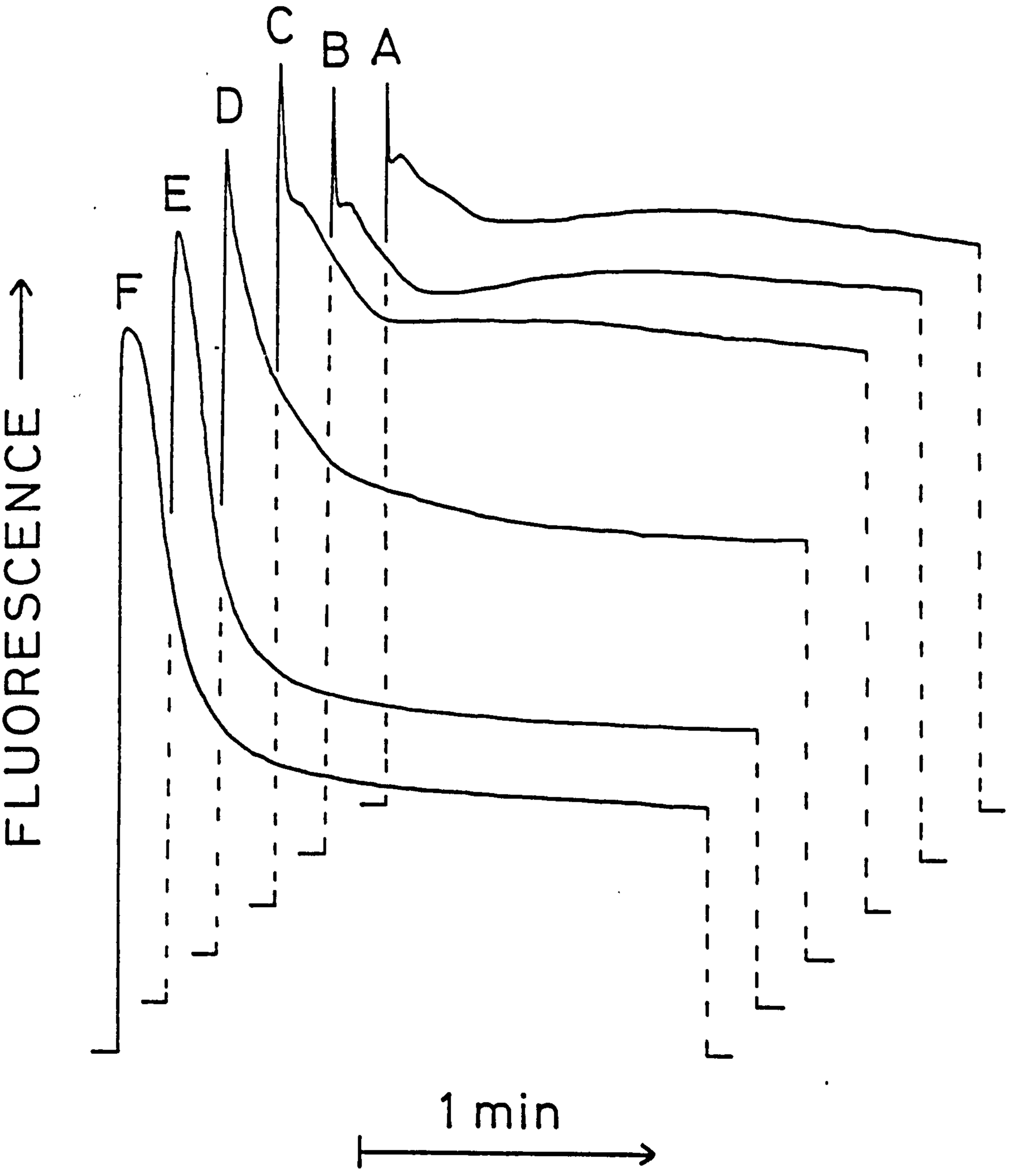


FIGURE 54. The effect of increasing the external KCl concentration on the Kautsky phenomena. The fluorescence emission was measured after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.26 M KCl (1.3 Os kg^{-1}); C: 0.51 M KCl (1.7 Os kg^{-1}); D: 0.79 M KCl (2.2 Os kg^{-1}); E: 1.03 M KCl (2.7 Os kg^{-1}); F: 1.32 M KCl (3.2 Os kg^{-1}).

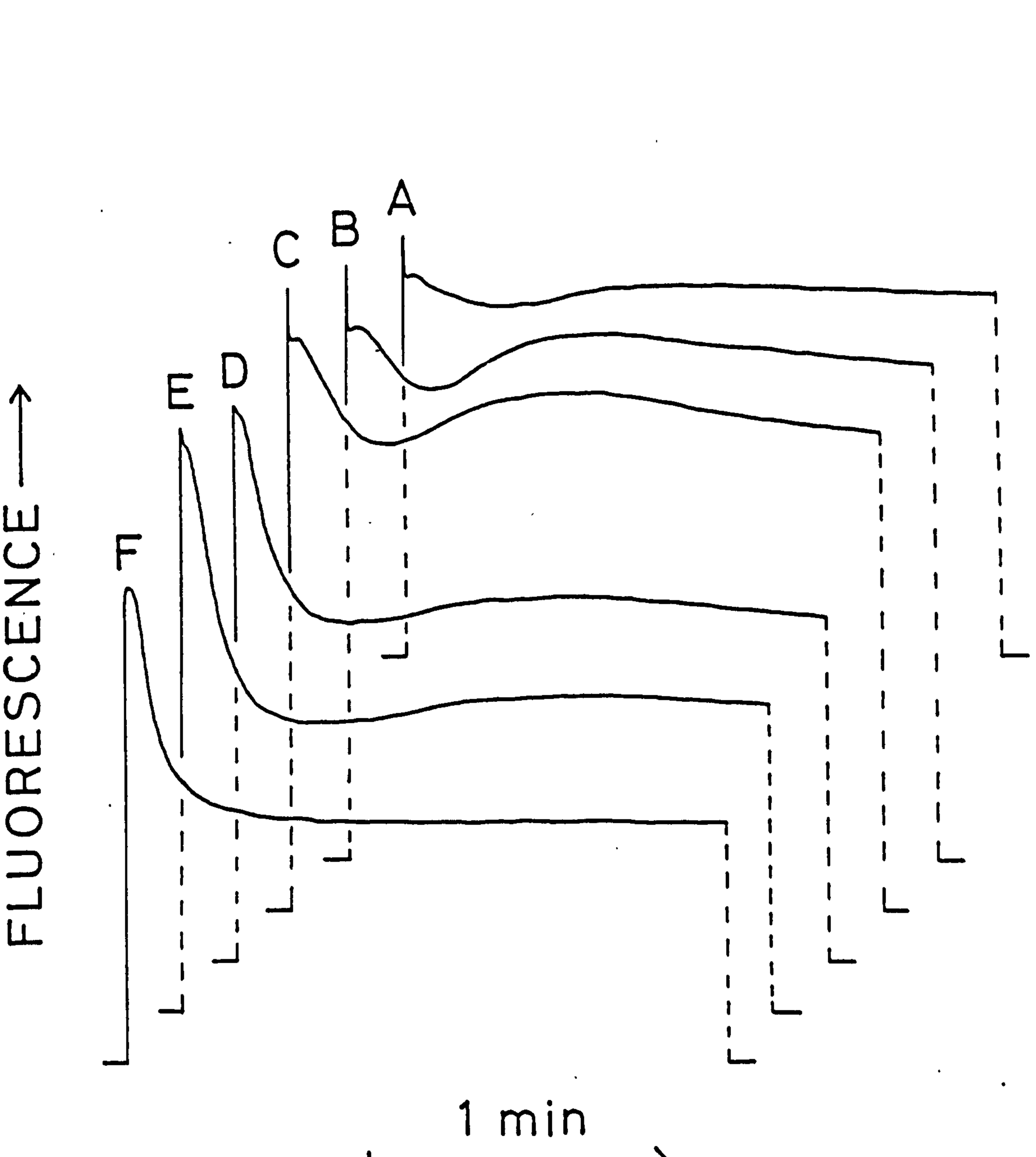


FIGURE 55. The effect of increasing the external KCl concentration on the Kautsky phenomena. The fluorescence emission was measured after 100 min at 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.26 M KCl (1.3 Os kg^{-1}); C: 0.51 M KCl (1.7 Os kg^{-1}); D: 0.79 M KCl (2.2 Os kg^{-1}); E: 1.03 M KCl (2.7 Os kg^{-1}); F: 1.32 M KCl (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement.

decays rapidly at all levels of increased sucrose concentrations (Figure 56). After 100 min the fluorescence emission is quenched at all sucrose solutions and in the control (Figure 57). However, the Kautsky phenomena have returned to normal at external sucrose concentrations of less than 0.72 M (1.7 Os kg^{-1}, Traces B and C). At the higher osmolalities the fluorescence curve has almost returned to the shape of the control (Figure 57).

Figure 58 shows that 5 min after increasing the external ethylene glycol concentration up to 1.30 M (2.2 Os kg^{-1}, Trace D), there is very little effect on the Kautsky phenomena. Even at the highest external osmolality of 3.2 Os kg^{-1} (Trace F), the D to P peak is still present as a plateau and the amount of quenching is moderate. The peak at I decreases rapidly in all ethylene glycol concentrations after 5 min (Figure 58). After 100 min in increased ethylene glycol concentrations (and in the control), there is no overall quenching and the Kautsky fluorescence changes have returned to normal except perhaps at the highest external osmolality (Trace F) where the D to P fluorescence increase is still only a plateau (Figure 59). Thus the effect of ethylene glycol on Kautsky fluorescence is much less than that of NaCl, KCl or sucrose.

Figure 60 indicates that increasing the external glycerol concentration has a similar effect on the Kautsky phenomena after 5 min as NaCl increases have (Figure 50), except that the second highest osmolality exerted (Trace E) has less effect than the equivalent trace in Figure 50. This is probably due to the different osmolalities exerted by the respective Trace E's i.e. 2.4 Os kg^{-1} for glycerol and 2.7 Os kg^{-1} for NaCl. Thus when this difference is taken into account

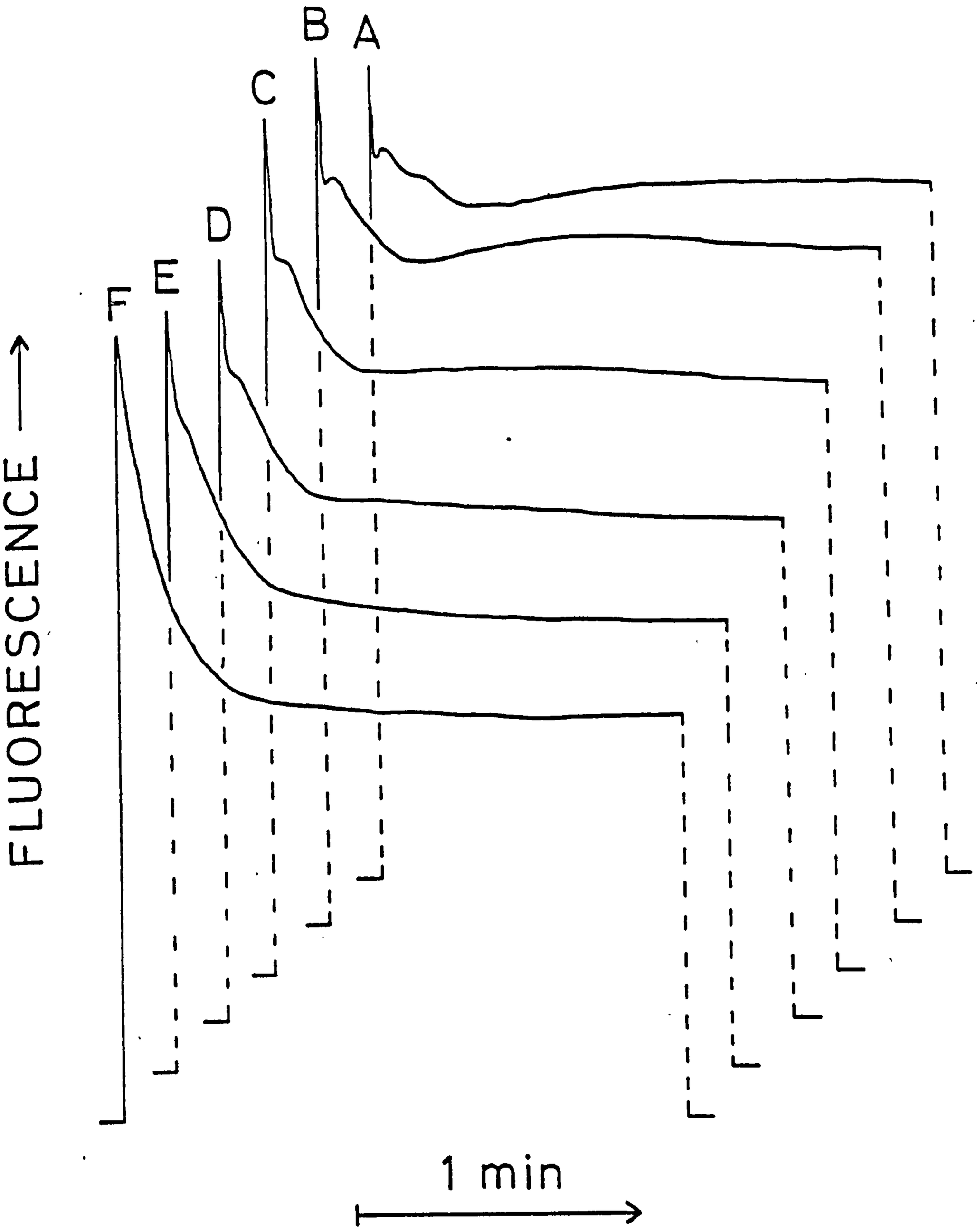


FIGURE 56. The effect of increasing the external sucrose concentration on the Kautsky phenomena. The fluorescence emission was measured after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.42 M sucrose (1.3 Os kg^{-1}); C: 0.72 M sucrose (1.7 Os kg^{-1}); D: 0.97 M sucrose (2.2 Os kg^{-1}); E: 1.18 M sucrose (2.6 Os kg^{-1}); F: 1.38 M sucrose (3.2 Os kg^{-1}).

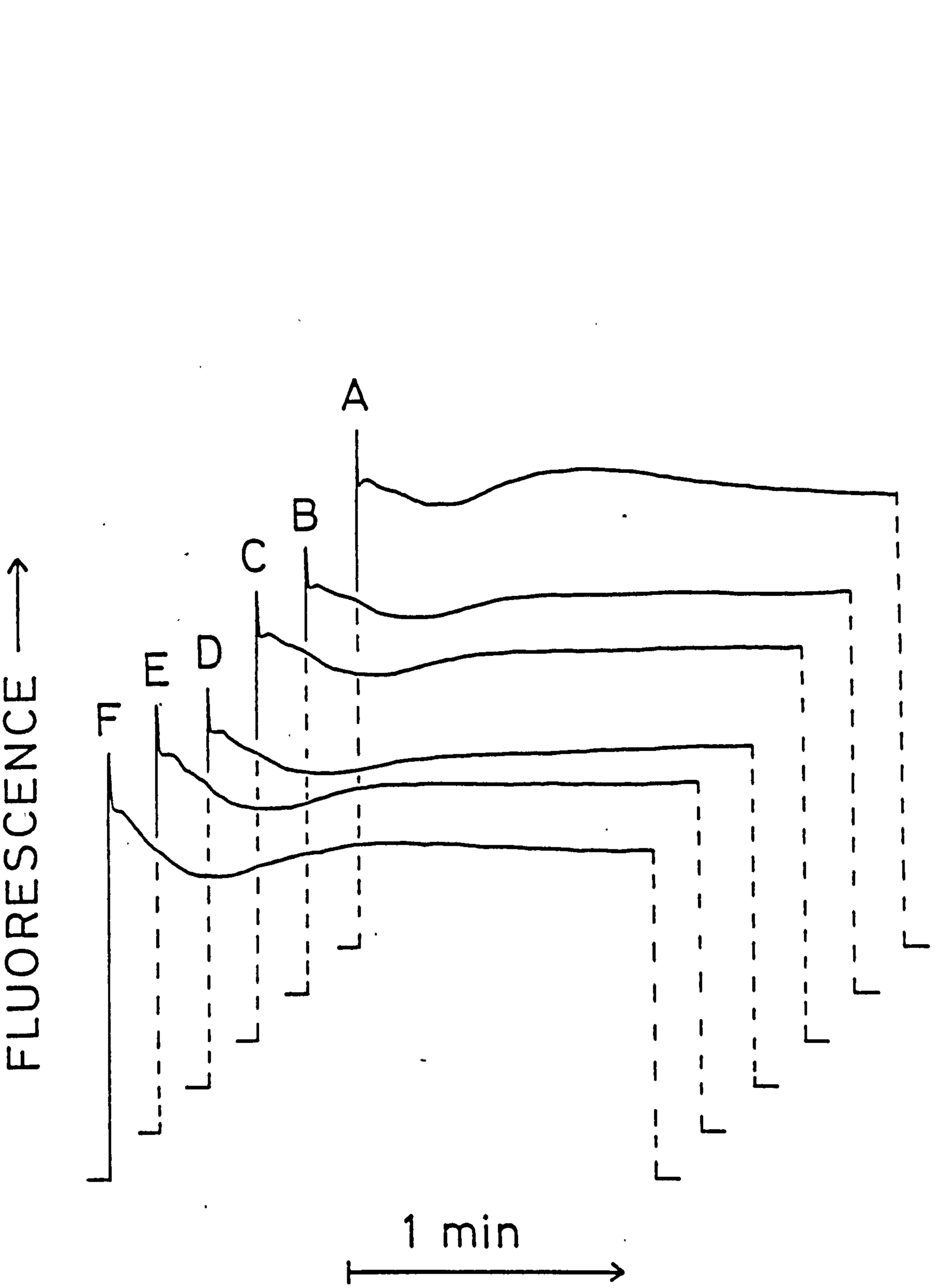


FIGURE 57. The effect of increasing the external sucrose concentration on the Kautsky phenomena. The fluorescence emission was measured after 100 min at 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.42 M sucrose (1.3 Os kg^{-1}); C: 0.72 M sucrose (1.7 Os kg^{-1}); D: 0.97 M sucrose (2.2 Os kg^{-1}); E: 1.18 M sucrose (2.6 Os kg^{-1}); F: 1.38 M sucrose (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement.

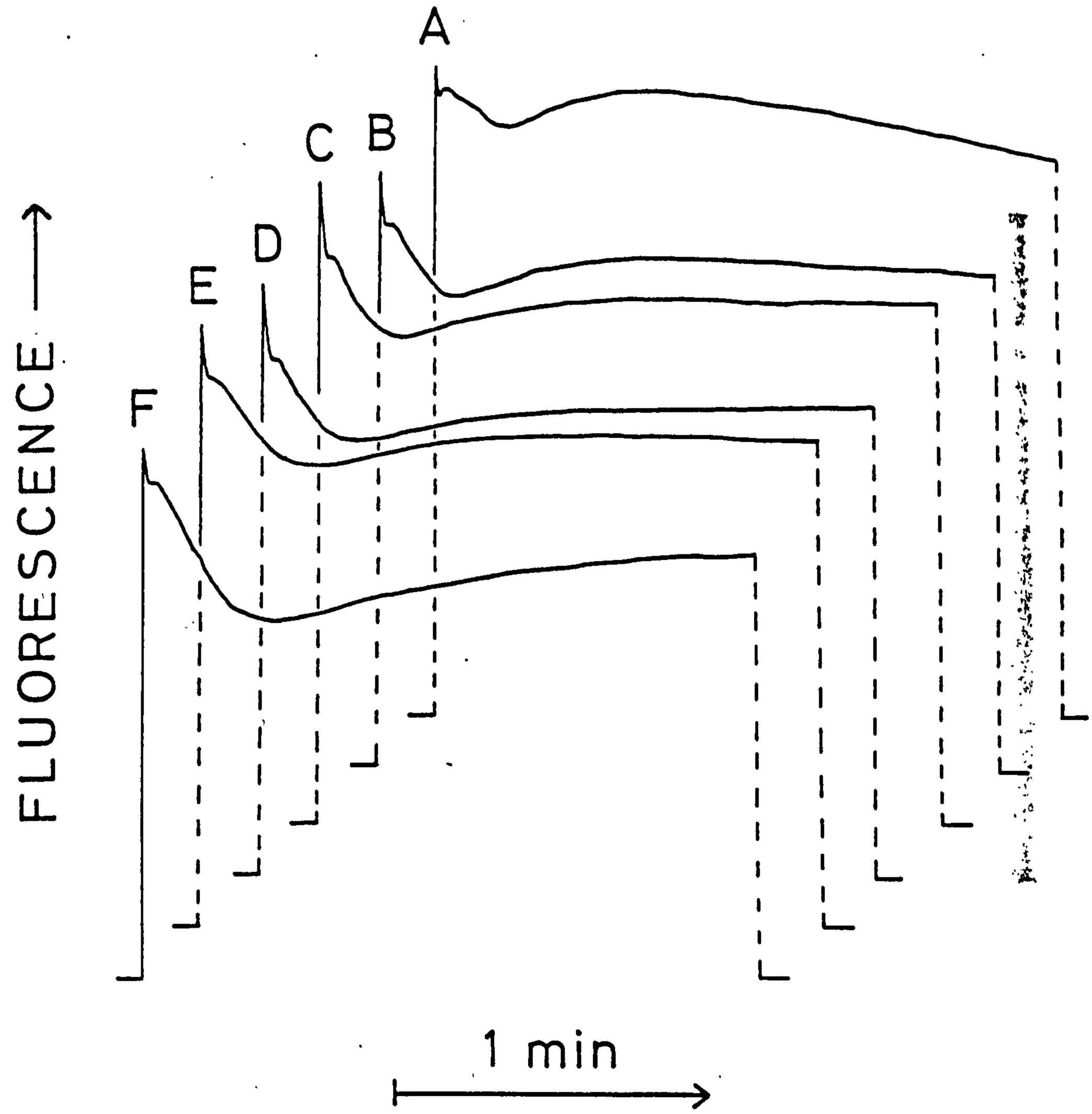


FIGURE 58. The effect of increasing the external ethylene glycol concentration on the Kautsky phenomena. The fluorescence emission was measured after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.46 M ethylene glycol (1.3 Os kg^{-1}); C: 0.88 M ethylene glycol (1.8 Os kg^{-1}); D: 1.30 M ethylene glycol (2.2 Os kg^{-1}); E: 1.73 M ethylene glycol (2.7 Os kg^{-1}); F: 2.09 M ethylene glycol (3.2 Os kg^{-1}).

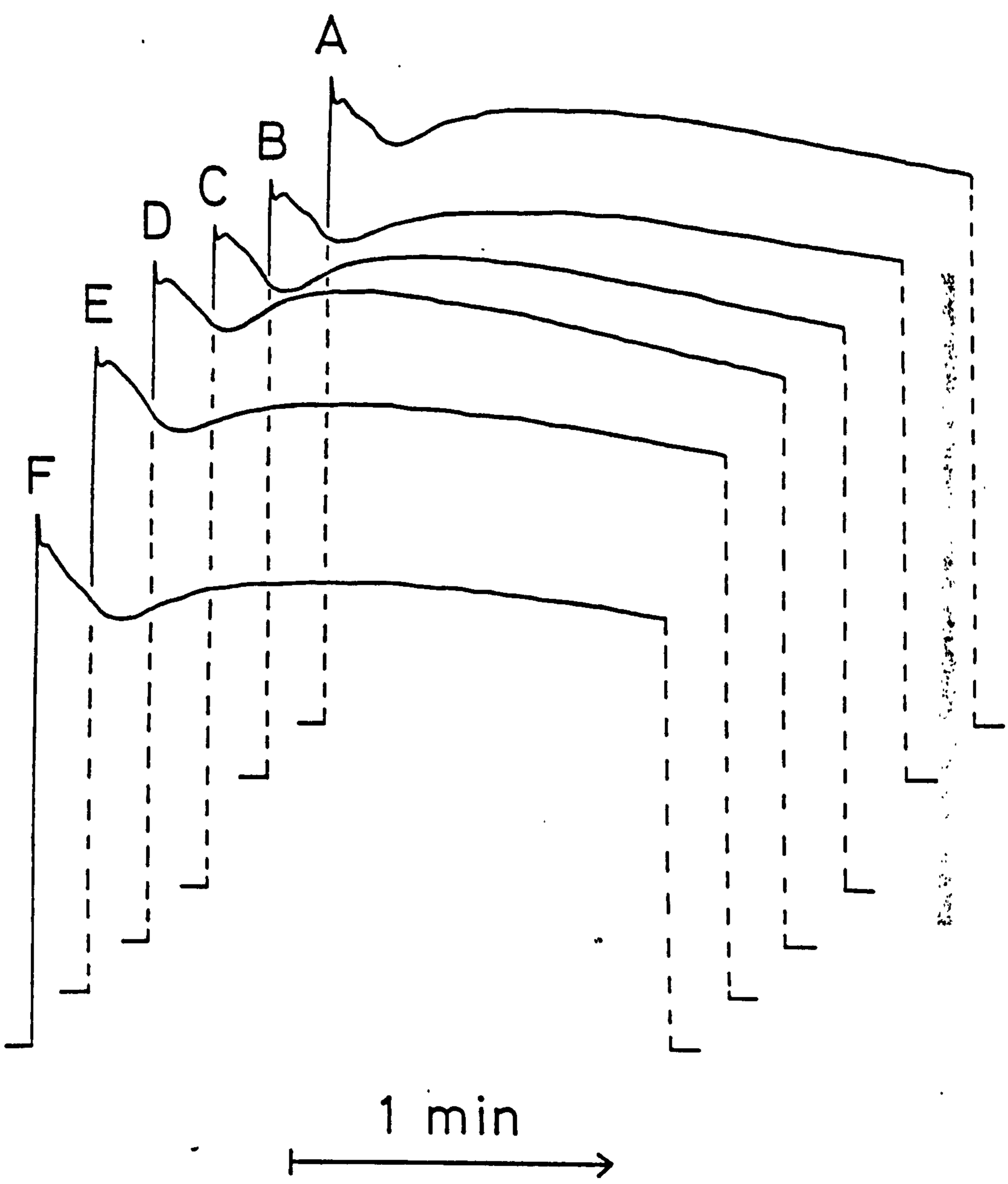


FIGURE 59. The effect of increasing the external ethylene glycol concentration on the Kautsky phenomena. The fluorescence emission was measured after 100 min at 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.46 M ethylene glycol (1.3 Os kg^{-1}); C: 0.88 M ethylene glycol (1.8 Os kg^{-1}); D: 1.30 M ethylene glycol (2.2 Os kg^{-1}); E: 1.73 M ethylene glycol (2.7 Os kg^{-1}); F: 2.09 M ethylene glycol (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement.

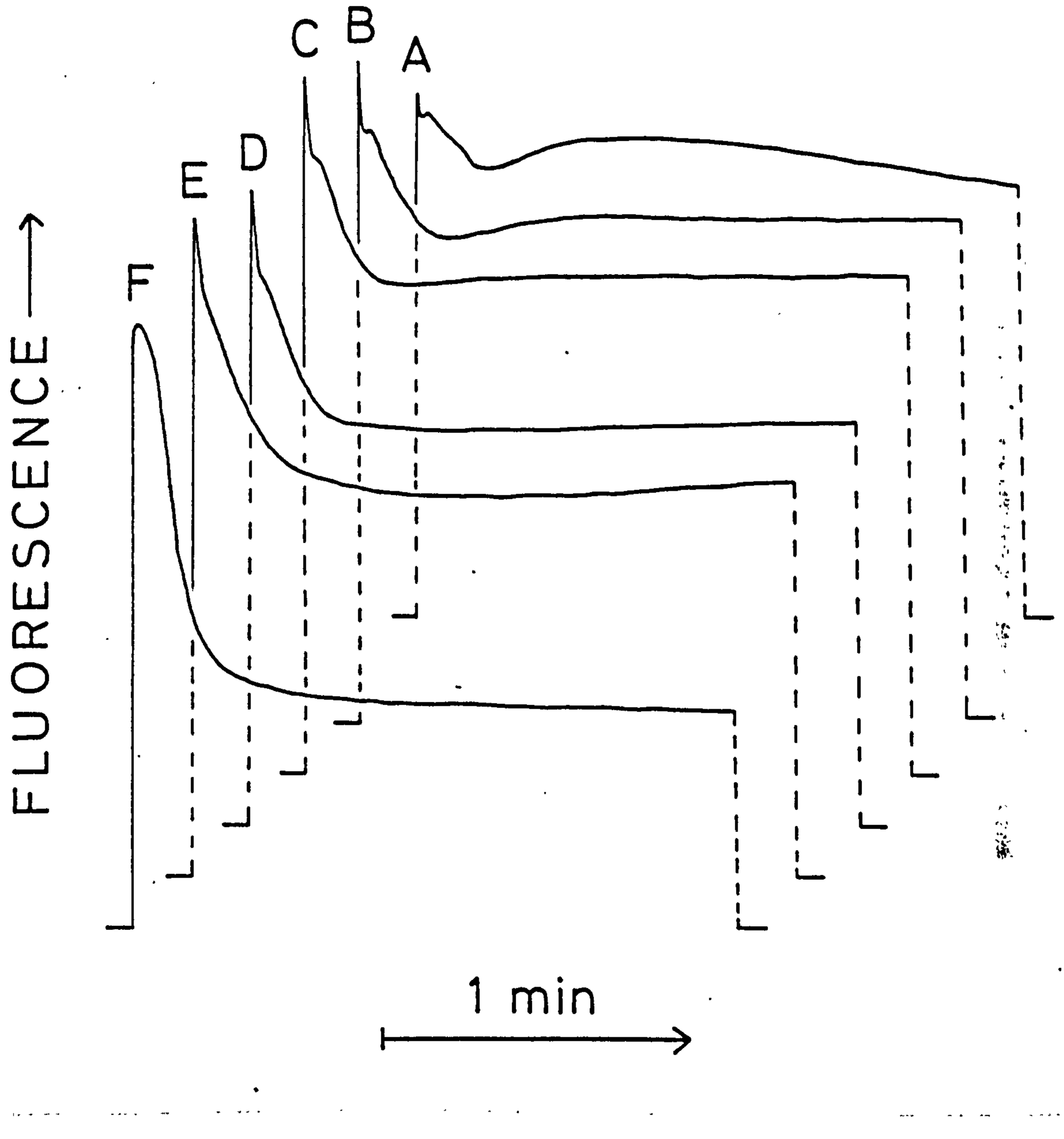


FIGURE 60. The effect of increasing the external glycerol concentration on the Kautsky phenomena. The fluorescence emission was measured after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.35 M glycerol (1.2 Os kg^{-1}); C: 0.71 M glycerol (1.5 Os kg^{-1}); D: 1.06 M glycerol (2.0 Os kg^{-1}); E: 1.42 M glycerol (2.4 Os kg^{-1}); F: 1.92 M glycerol (3.2 Os kg^{-1}).

the effect of glycerol and NaCl on Kautsky fluorescence after 5 min is very similar. Figure 61 shows that after 100 min at the increased glycerol concentrations there has been substantial recovery of the Kautsky fluorescence characteristics; the degree of recovery is similar to that found in the presence of increased external NaCl concentrations (Figure 51).

The uncoupler FCCP was added to algal samples at the beginning of the 5 min dark adaptation period to see if it could reproduce the changes in Kautsky fluorescence induced by increasing the external salinity. Figure 62 shows that increasing the concentration of FCCP added to the algae at 0.43 M NaCl (normal growth medium) induces the D to P transition to disappear and causes a slow decline from the peak at I, which are both characteristic of the NaCl effect (Figure 50). However the SMT transition is always visible in the presence of FCCP and the degree of quenching in this region is much less than that in the presence of high external salt concentrations (Figures 50 and 62). Another uncoupler CCCP has a similar effect to FCCP (data not shown).

D, L-glyceraldehyde (a relatively specific inhibitor of photosynthetic carbon assimilation according to Walker, 1981) does not have any effect on the Kautsky fluorescence induction curve of D. tertiolecta. An increase in the intensity of the exciting beam (4.8 W m^{-2} instead of the usual 1.3 W m^{-2}) has no effect on the shape of the Kautsky fluorescence curves except that the fluorescence decrease from P to S is larger in the higher light intensity (data not shown).

Figure 63 shows fluorescence induction curves measured at a much faster time scale. The fluorescence level was measured for approximately 3.5 s and a split time base was used to enable the O fluorescence level

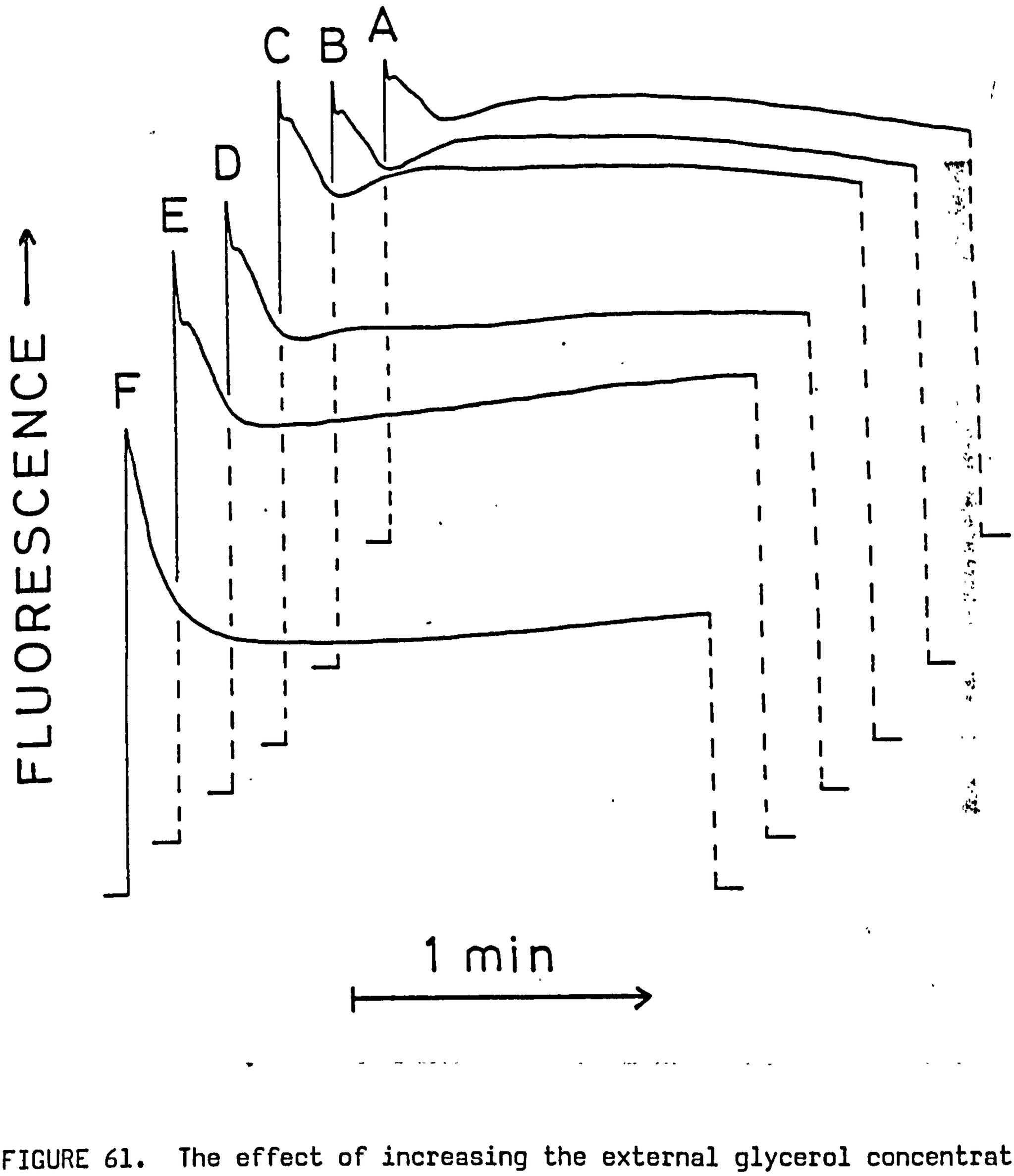


FIGURE 61. The effect of increasing the external glycerol concentration on the Kautsky phenomena. The fluorescence emission was measured after 100 min at 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.35 M glycerol (1.2 Os kg^{-1}); C: 0.71 M glycerol (1.5 Os kg^{-1}); D: 1.06 M glycerol (2.0 Os kg^{-1}); E: 1.42 M glycerol (2.4 Os kg^{-1}); F: 1.92 M glycerol (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement.

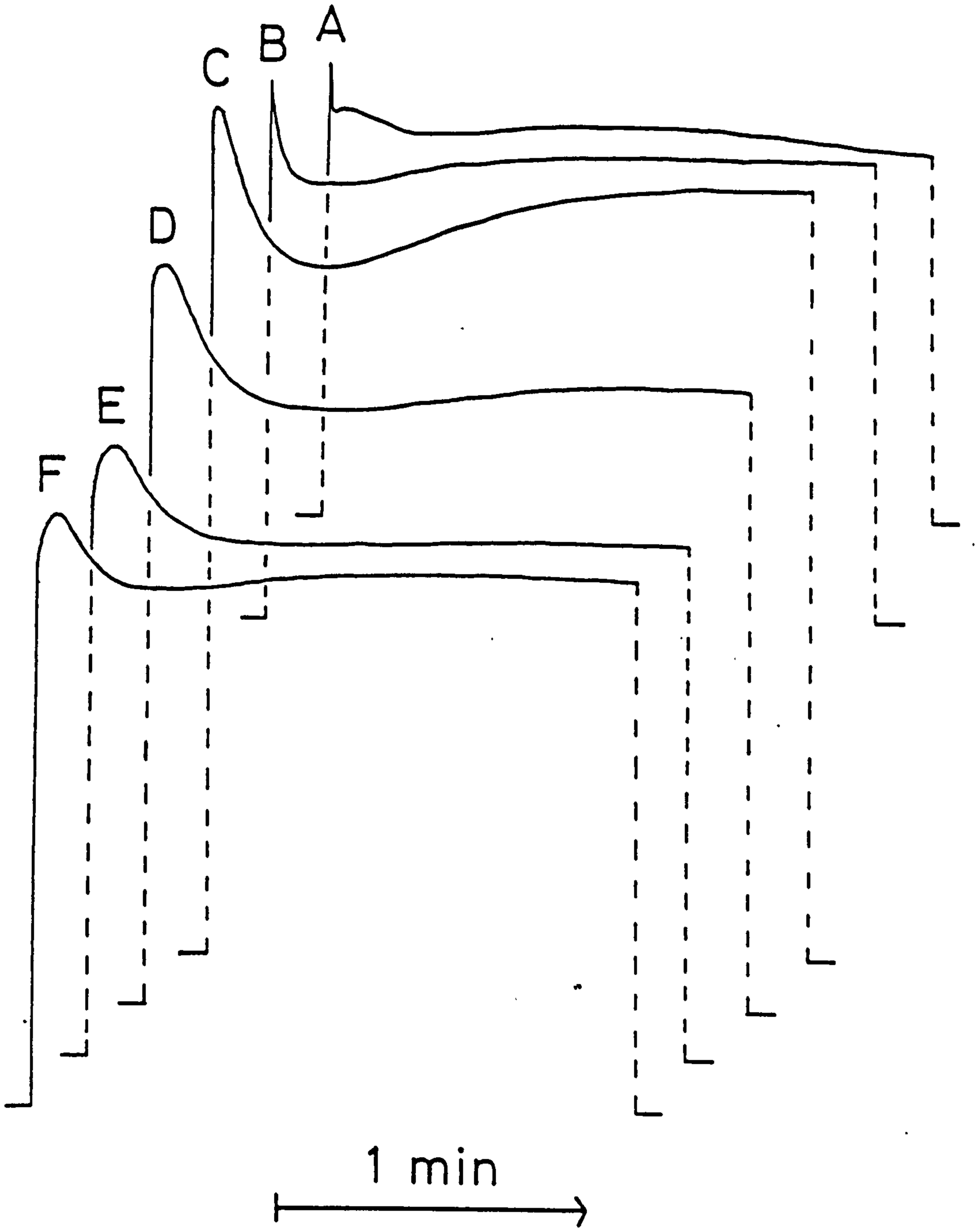


FIGURE 62. The effect of the uncoupler FCCP on the Kautsky phenomena. The fluorescence emission was measured after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl (normal growth medium) plus A: 6 µl of absolute alcohol, control; B: 0.5 µM FCCP; C: 2 µM FCCP; D: 9 µM FCCP; E: 17.5 µM FCCP; F: 26 µM FCCP. The FCCP was dissolved in absolute alcohol.

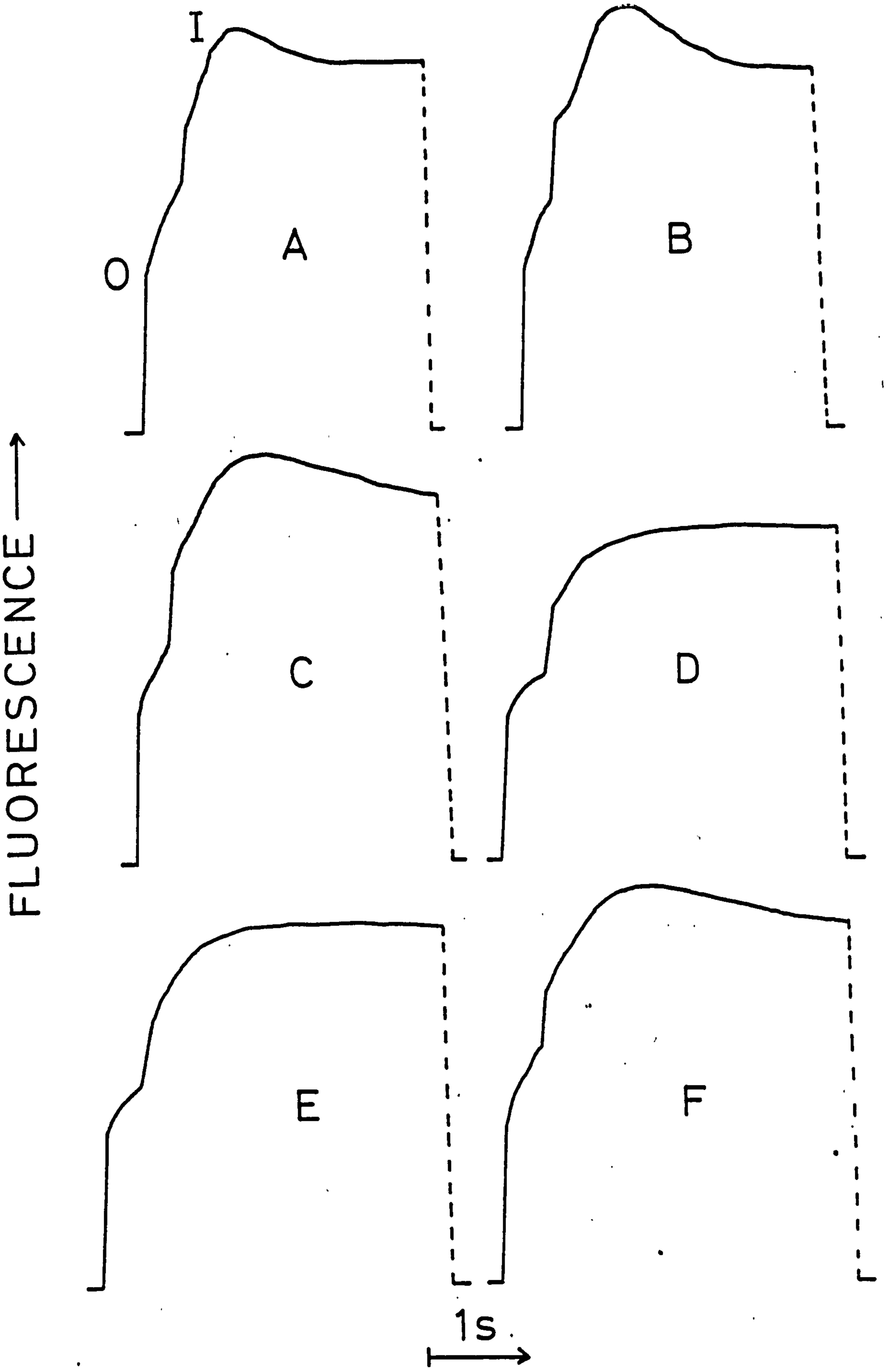


FIGURE 63. The effect of increasing the external osmolality on the initial Kautsky phenomena. The fluorescence emission was measured for approx. 3.5s after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.25 M NaCl (1.3 Os kg^{-1}); C: 0.77 M NaCl (2.2 Os kg^{-1}); D: 1.28 M NaCl (3.2 Os kg^{-1}); E: 1.32 M KCl (3.2 Os kg^{-1}); F: 1.38 M sucrose (3.2 Os kg^{-1}). A split time base was used, the first 12.5% of the trace represents 0.05s and the rest represents 3.5s.

(equivalent to Fo) of the Kautsky curve to be recognisable. Trace A is a control showing the O level of fluorescence before reaching the peak fluorescence at I which then decays towards D. The O (or Fo) level does not change very much in response to increases in the external osmolality, only at the higher NaCl concentrations (Figure 63, Traces C and D) is it decreased slightly. The faster time scale also allows us to determine more accurately the effect of osmotic stress on the rate of the decline of fluorescence from the peak at I. It appears that a small increase in the NaCl concentration actually causes an increase in the rate of decline i.e. the spike I in earlier Figures in this section would be sharper (Figure 63, Trace B). However, high NaCl and KCl concentrations (Traces D and E) cause the decline from peak I to be slower as was illustrated already in Figures 50 and 54. Trace F shows that a sucrose solution exerting a similar high external osmolality does not reduce the rate of the decline in I as much as NaCl or KCl (Traces D and E). Figure 63 shows that 1.38 M sucrose (3.2 Os kg^{-1}) has an effect equivalent to 1.20 M NaCl (2.2 Os kg^{-1}, Trace C).

5.3.2 State 1 - State 2 Transitions

Noncyclic photosynthetic electron transport can only operate at maximum efficency if both PSI and PSII are receiving an approximately equal amount of light energy (Figure 4, Chapter 1). As already suggested in section 5.3.1, PSII appears initially to be overdriven, this is shown by the rapid rise in fluorescence as Q (primary electron acceptor of PSII) becomes reduced. This imbalance in the excitation energy reaching the two photosystems can be accentuated by initially exposing the dark-adapted algae to blue-green light which is preferentially absorbed by PSII. (Blue-green light has been used to induce

fluorescence in all of the experiments already described in this chapter). This PSII light (Light 2) is modulated and the modulated fluorescence is detected by a photomultiplier and lock in amplifier. This allows the introduction of light of approximately 718 nm which preferentially excites PSI (Light 1), but is not modulated and is thus not seen by the detector system. This is necessary since the wavelength of fluorescence emitted is around 685 nm. This experimental method is described in detail by Chow et al. (1981).

Using this experimental system, the algae can be induced to alter the rate of spillover from PSII to PSI to maintain a balance in excitation energy entering each photosystem. If Light 2 is switched on, the algae slowly adjust to the imbalance by allowing light energy to spillover to PSI. This state of high spillover is called State 2. If instead Light 1 is switched on, spillover is kept to a minimum and the algae are said to be in State 1 (Chow et al., 1981; Allen et al., 1981).

A typical fluorescence induction curve involving State 1 - State 2 transitions in D. tertiolecta is shown in Figure 64. When Light 2 is switched on the initial fluorescence characteristics are different from those seen in section 5.3.1 (Figure 49). This is not due to the light quality since the same filters were used initially in the experiments illustrated in Figures 49 and 64. However, the light intensity employed in the State 1 - State 2 experiments (Figure 64) was 2 to 4 W m^{-2}, whereas in the previous Kautsky fluorescence experiments (Figure 49) it was 1.3 to 1.5 W m^{-2}. Thus, the higher light intensity coupled with the longer time scale lead to the different initial fluorescence characteristics in the two sets of experiments.

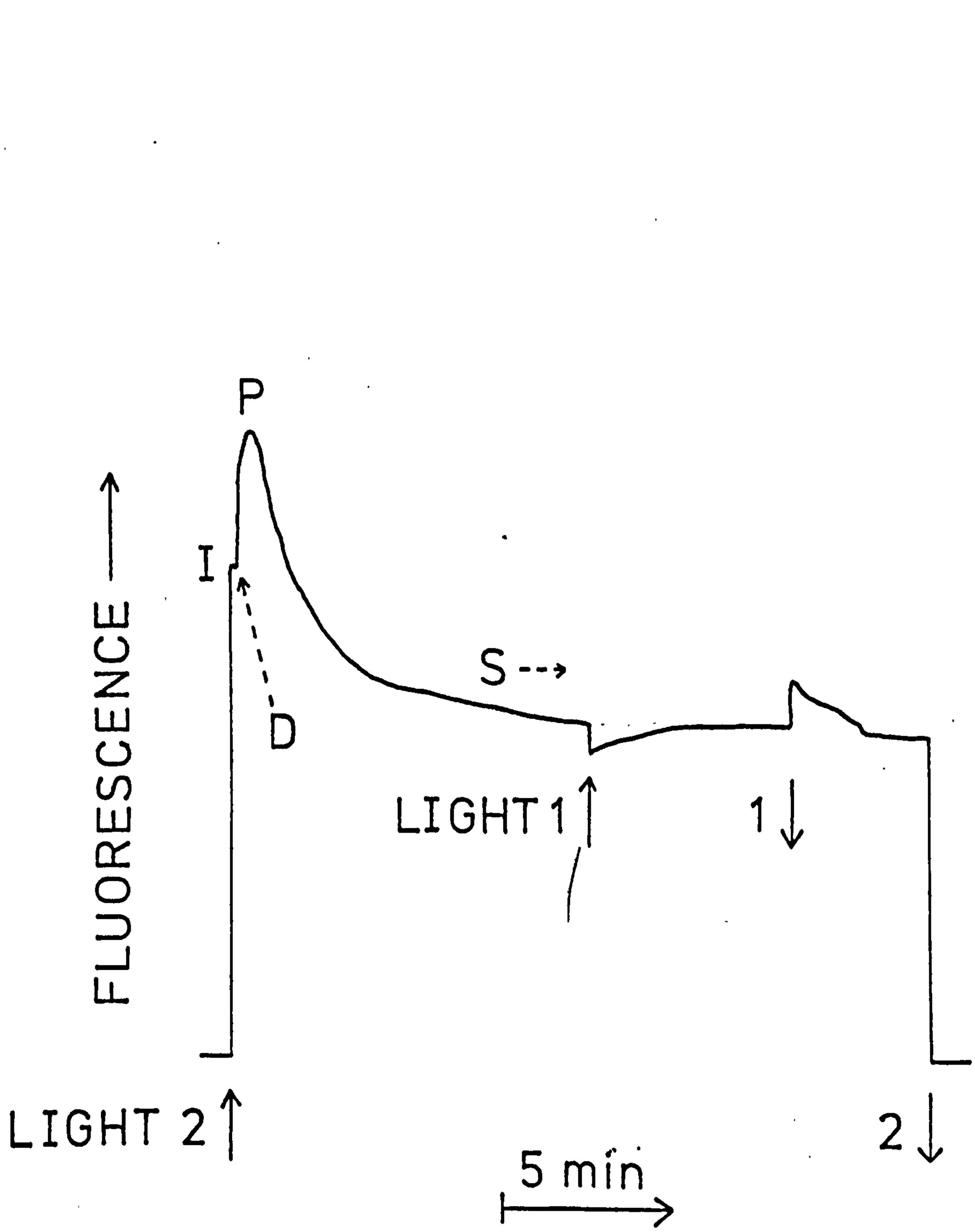


FIGURE 64. A typical State 1 - State 2 fluorescence induction curve. The filters for Light 2 were as described on page 47 and for Light 1 a 718 nm interference filter was used.

In the State 1 - State 2 experiments I and D occur at a much lower level of fluorescence than the peak P (Figure 64), and this is considered to be the classic form for a Kautsky curve (Papageorgiou, 1975; Lavorel and Etienne, 1977). In the presence of Light 2 there is a large fluorescence decrease from P towards S which can be connected with an increase in spillover from PSII to PSI. Due to the higher light intensity this is larger in State 1 - State 2 experiments than in the Kautsky experiments (Figures 49 and 64). After 10 min exposed to Light 2, the level of fluorescence is still decreasing which suggests that the algae are not quite fully adapted to the State 2 condition. At this point, Light 1 is switched on and there is an immediate fall in fluorescence (due probably to a rapid reoxidation of Q), which confirms that PSI was still slightly underdriven in Light 2 i.e. the State 2 condition was not fully established. After the initial decrease in fluorescence induced by Light 1, the fluorescence level increased slowly towards the level found in Light 2. This slow increase in fluorescence in the presence of both Light 1 and Light 2 can be correlated with a reduction in Q brought about by a decrease in spillover from PSII to PSI.

When Light 1 is switched off there is an immediate rise in the fluorescence level which can be correlated with Q rapidly becoming reduced in the absence of Light 1. However, there is then a rapid fall in fluorescence which is probably due to an increase in spillover from PSII to PSI; which enhances PSI activity and this causes reoxidation of Q. Thus the control of spillover of excitation energy between PSII and PSI can allow both photosystems to drive noncyclic electron

transport efficiently (Figure 64; Chow et al., 1981).

Five min after being resuspended in 0.43 M NaCl (Figure 65, Trace A), the algae show the typical State 1 - State 2 transitions as described for Figure 64. The only difference is that in Figure 65 (and all subsequent Figures) the light regime was 6 min Light 2, followed by 6 min of both Light 1 and Light 2, and finally 4 min of Light 2 only again. An increased external salinity of 1.20 M NaCl (2.2 Os kg^{-1}) induced the transitions illustrated in Trace B of Figure 65 after 5 min. It appeared that after Light 1 was switched on there was little decrease in spillover since the small fluorescence rise in the presence of Light 1 and Light 2 did not take place. Also when Light 1 was switched off, after the initial increase in fluorescence, there was only a small subsequent decrease indicating that an increase in spillover from PSII to PSI does not take place to any great extent. At 1.71 M NaCl (3.2 Os kg^{-1}) after 5 min (Figure 65, Trace C), when Light 1 is switched on the fluorescence level continues to decrease suggesting that the degree of spillover in Light 2 is less in the presence of high external NaCl concentration i.e. the algae are less adapted to the State 2 condition in high salt than in the control (Figure 65, Traces A and C). There is no spillover change in the presence of both Light 1 and Light 2 i.e. there is no small increase in fluorescence. In addition, when Light 1 is switched off again in the presence of 1.71 M NaCl, the fluorescence exhibits a slow rise which indicates a slow increase in the reduction of Q; there does not appear to be any spillover from PSII to PSI which would be expected when Light 1 is turned off. After 100 min the State 1 - State 2 transitions have returned to normal at 1.20 M NaCl. However, even after 100 min exposure to 1.71 M NaCl, the shape shown in Figure 65, Trace C persists although after Light 1 is switched off there

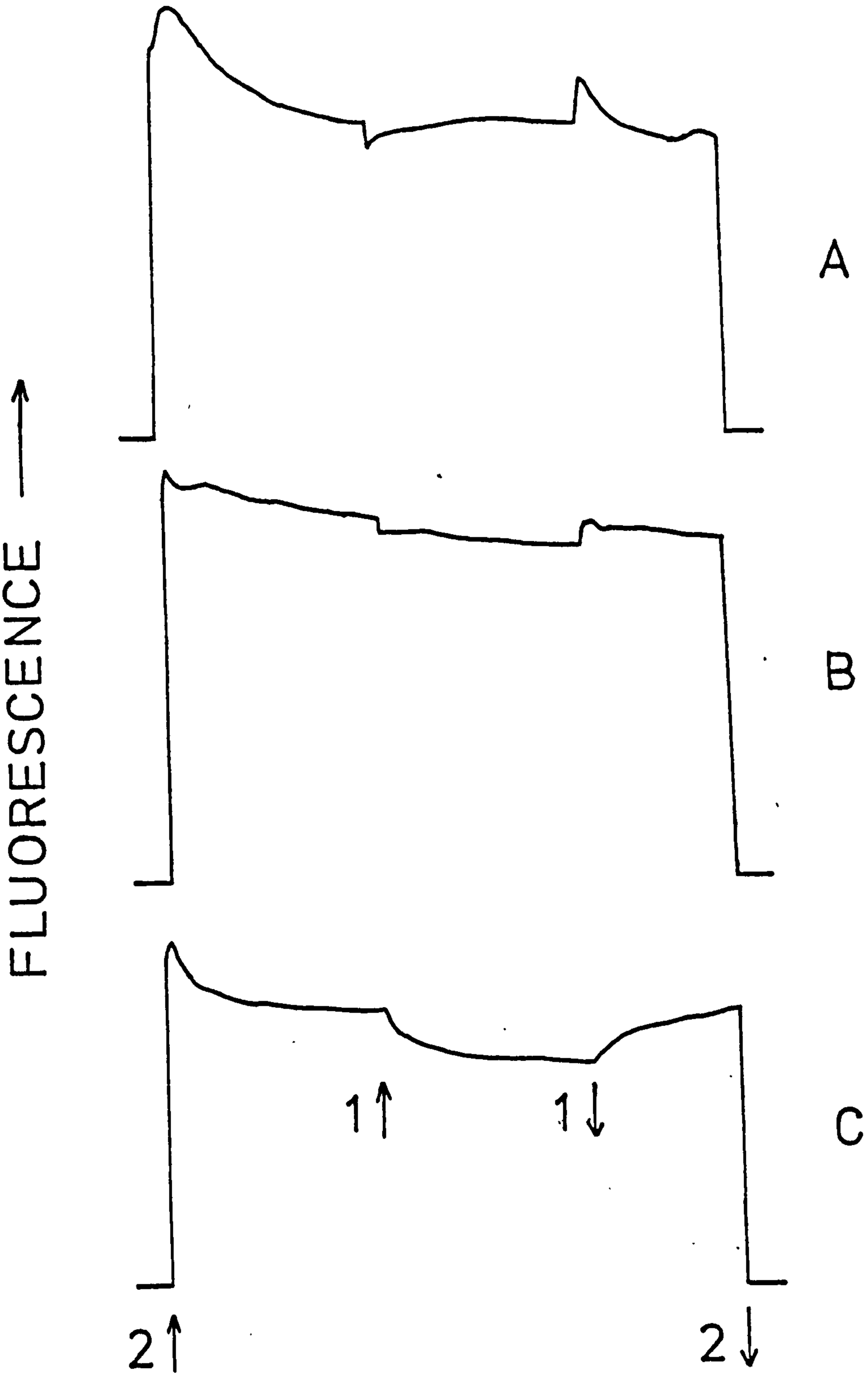


FIGURE 65. The effect of increasing the external NaCl concentration on State 1 - State 2 fluorescence. The algae were resuspended for 5 min (in the dark) in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 1.20 M NaCl (2.2 Os kg^{-1}); C: 1.71 M NaCl (3.2 Os kg^{-1}).

is only a very small slow increase in fluorescence; there is no evidence of spillover changes. It took about 4 to 6 hours for the trace to return to normal in 1.71 M NaCl (data not shown).

The control Trace A in Figure 66 shows one or two unusual features compared to other control curves (Figure 64 and Figure 65, Trace A), but it shows similar spillover changes. 5 min after decreasing the salinity to 0.17 or 0.09 M NaCl (Traces B and C respectively), there are no alterations in the spillover changes like those induced by increasing the NaCl concentration (Figure 65). However, although the fluorescence level varies in a similar manner to the control, the spillover changes seem to overshoot in lower salinities e.g. when Light 1 is switched on the fluorescence increases to a level above that found at the end of 6 min in Light 2 (Figure 66, Traces B and C). After 100 min at the decreased salinities the transitions have returned to normal except in the case of 0.34 M NaCl where there is still a slightly larger increase in fluorescence when Light 1 and Light 2 are both switched on (data not shown).

Figure 67 shows that after 5 min, increased concentrations of KCl have a similar effect to that induced by increased NaCl concentrations (Figure 65). After 100 min at 0.79 M KCl (2.2 Os kg^{-1}), the trace has returned to normal, but this is not true of 1.32 M KCl (3.2 Os kg^{-1}). After 9 hours exposure to 1.32 M KCl, the normal reaction to the switching on of Light 1 has returned, but when Light 1 is switched off, there is still no spillover change just a small slow increase in the fluorescence level (data not shown).

Figure 68 shows that the addition of sucrose does not affect the State 1 - State 2 transitions as much as increasing the NaCl or KCl

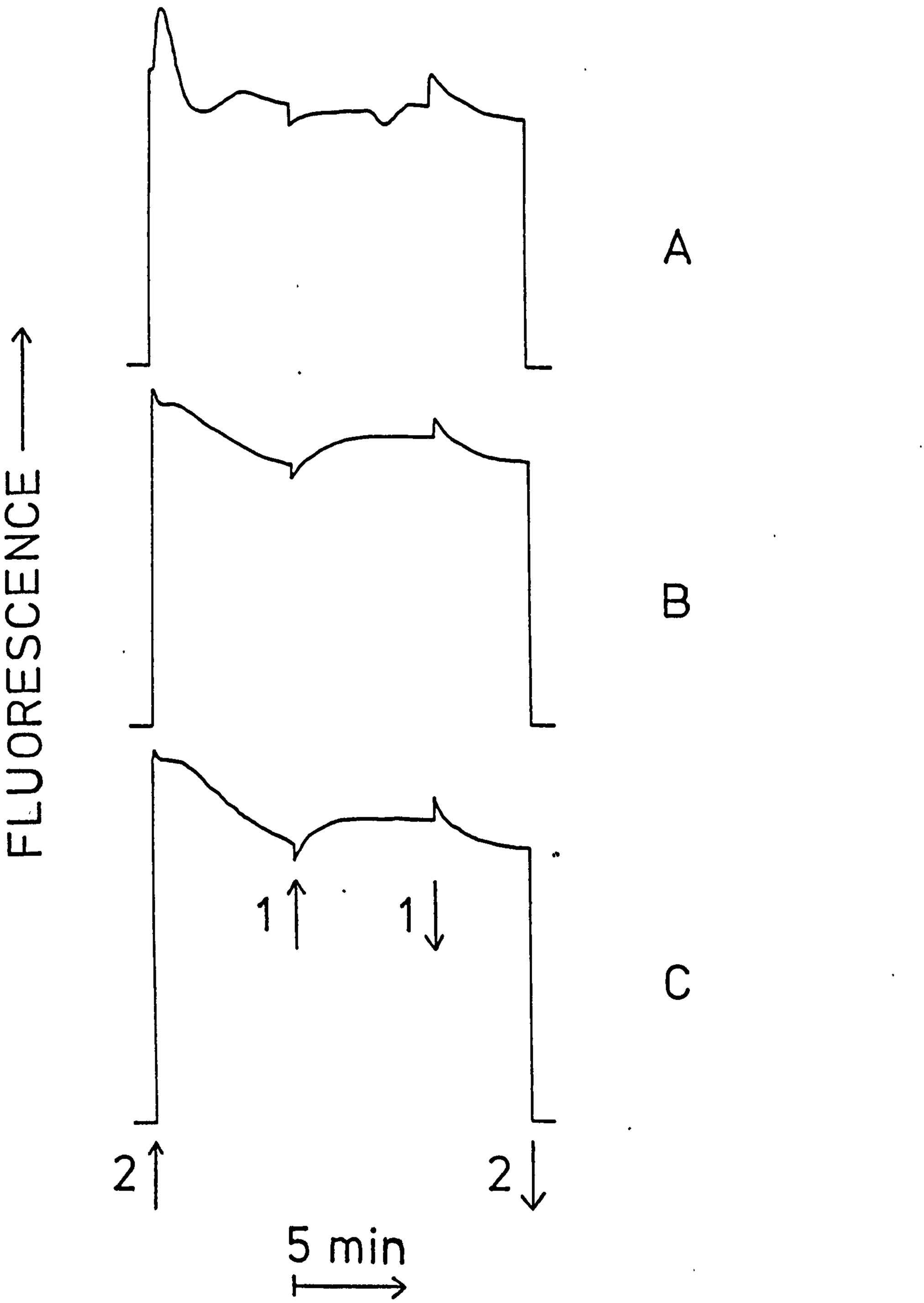


FIGURE 66. The effect of decreasing the external NaCl concentration on State 1 - State 2 fluorescence. The algae were resuspended for 5 min (in the dark) in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.26 M NaCl (0.5 Os kg^{-1}); C: 0.09 M NaCl (0.2 Os kg^{-1}).

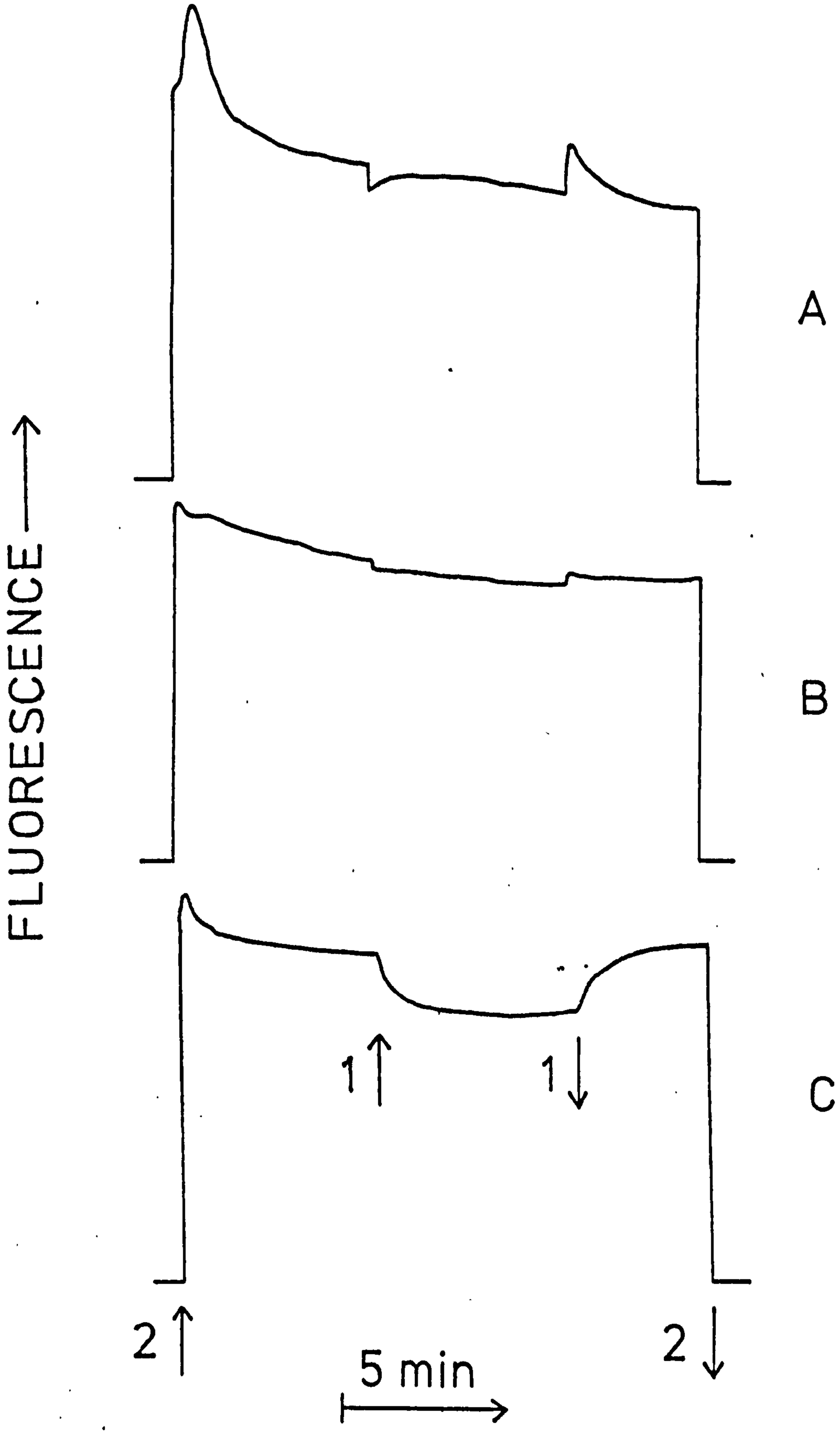


FIGURE 67. The effect of increasing the external KCl concentration on State 1 - State 2 fluorescence. The algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.79 M KCl (2.2 Os kg^{-1}); C: 1.32 M KCl (3.2 Os kg^{-1}).

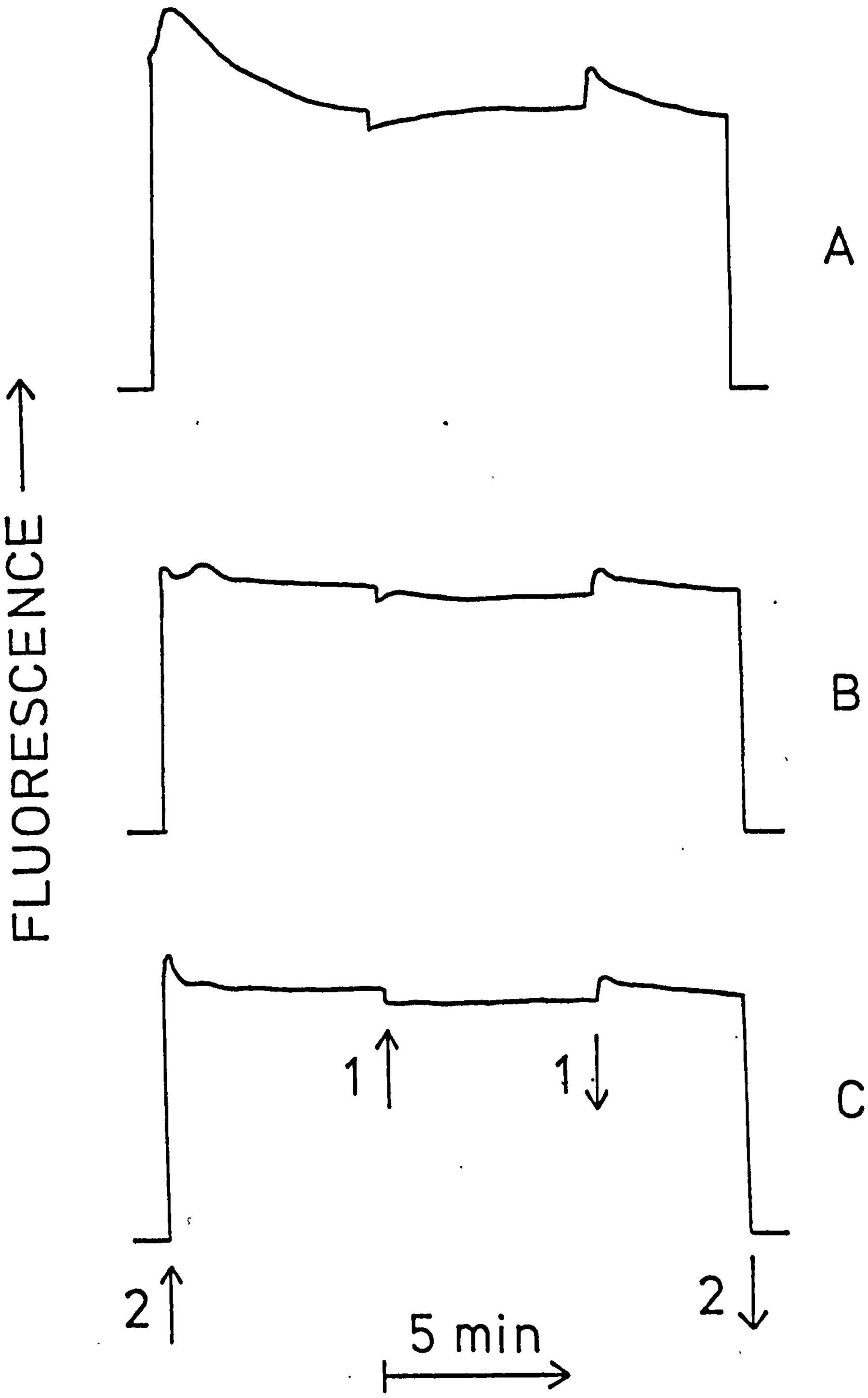


FIGURE 68. The effect of increasing the external sucrose concentration on State 1 - State 2 fluorescence. The algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.97 M sucrose (2.2 Os kg^{-1}); C: 1.38 M sucrose (3.2 Os kg^{-1}).

concentration does (Figures 65 and 67). Even at the highest osmolality used i.e. 1.38 M sucrose (3.2 Os kg^{-1}), there is no sign of a complete breakdown of the spillover process as has been suggested by the NaCl and KCl data. After 100 min exposure to sucrose solutions the State 1 - State 2 transitions have returned completely to normal (data not shown).

5.3.3 DCMU-Induced Relaxation of Fluorescence Quenching

It has been demonstrated in the preceding sections that when chlorophyll fluorescence is measured there is an initial peak (at I or P) which falls to a lower level within a few minutes of the start of the illumination of the algae (Figures 49 and 64). Krause, Vernotte and Briantais (1982) demonstrated that if DCMU is added to an algal or chloroplast sample at the point of high fluorescence quenching (i.e. the point of low fluorescence emission), then the quenching is relaxed and a biphasic increase in fluorescence occurs. This is illustrated using D. tertiolecta in Figure 69, where it can be seen that the fluorescence level after the addition of DCMU is higher than the initial fluorescence peak I. Krause et. al. (1982) also found this using Chlorella and suggested that it is due to the rapid onset of noncyclic electron transport after the light is switched on preventing a strong transitory reduction of Q. Krause et. al. performed experiments which allowed them to suggest that the rapid phase of DCMU-induced relaxation of fluorescence quenching is due to the reduction of that part of Q which is in the oxidised state before the addition of DCMU. They attributed the slow phase of relaxation to a decrease in 'energy-dependent' quenching which is related to the intrathylakoid H^+ concentration i.e. the larger the Δ pH gradient across the thylakoid membrane the greater the slow phase of the relaxation of

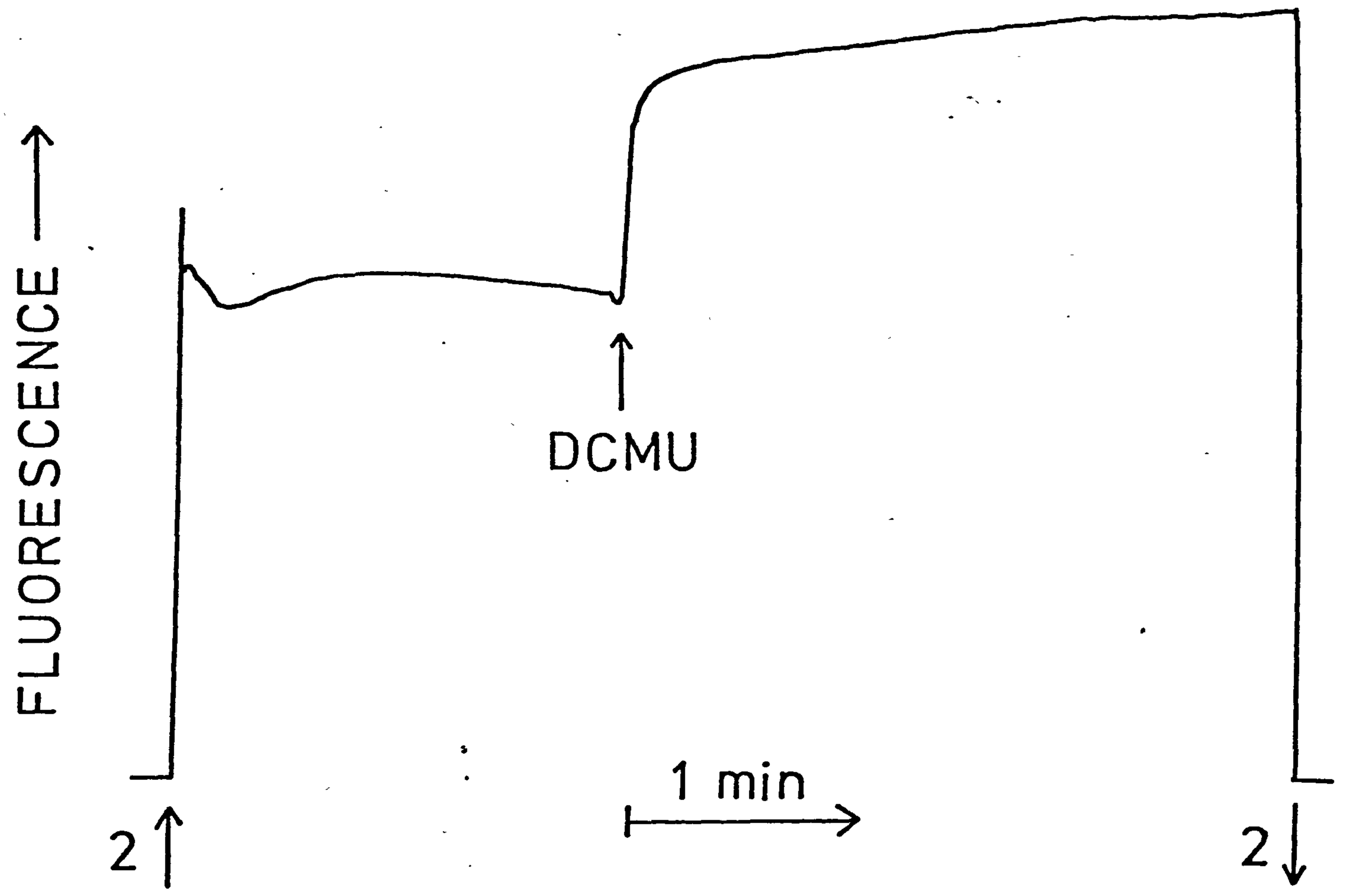


FIGURE 69. The effect of DCMU on the fluorescence emission of the alga in the presence of Light 2, showing the biphasic relaxation of quenching.

quenching (Briantais et. al., 1979; section 1.6.1). Therefore in this section DCMU is used to relax fluorescence quenching which has been induced by changing the external osmolality to find out whether oxidised Q or a large Δ pH gradient is mainly responsible for the osmotically-induced quenching.

The results shown in Table 8 were calculated from fluorescence curves like the one shown in Figure 69. The algae were exposed to light which mainly excited PSII (Light 2); thus they were becoming adapted to State 2 when the DCMU was added (to a final concentration of 10 μM) after 2 min illumination. The extent of the fast phase of DCMU-induced relaxation of fluorescence quenching is expressed as a percentage of the fluorescence level before the DCMU addition. The extent of the slow phase of relaxation is expressed as a percentage of the fluorescence level before the DCMU addition plus the amount of fluorescence attributed to the fast phase of relaxation. This is necessary to remove the possibility that non-photochemically induced changes are occurring (Bradbury and Baker, 1981b; Krause et. al., 1982). Table 8 shows that increasing the external osmolality using NaCl, KCl or sucrose decreases the fast phase of DCMU-induced relaxation of quenching (which depends on the redox state of Q) and increases the slow phase (which depends on the extent of the Δ pH gradient). These effects are seen most clearly with NaCl and KCl; sucrose also causes them to a lesser extent. Decreasing the external osmolality also causes the slow phase to increase, but does not affect the fast phase relaxation of quenching induced by DCMU. In distilled water there was no DCMU-induced relaxation of fluorescence quenching (Table 8).

A similar series of experiments were carried out using DCMU to

Table 8

DCMU-Induced Relaxation of Chlorophyll Fluorescence Quenching in the Presence of Light 2

Test Medium	Fluorescence Level Before DCMU addition (arbitrary units)	Extent of Fast Phase		Extent of Slow Phase	
		Units	Percentage	Units	Percentage
0.43 M NaCl (control)(0.8 Os kg^{-1})	26	10.5	40	6	16
0.68 M NaCl (1.3 Os kg^{-1})	23	10	43	6.5	20
1.20 M NaCl (2.2 Os kg^{-1})	18.5	7	38	6.5	25
1.45 M NaCl (2.7 Os kg^{-1})	12	2.5	21	14.5	100
1.71 M NaCl (3.2 Os kg^{-1})	10.5	1.5	14	14.5	121
0.26 M KCl (1.3 Os kg^{-1})	20	13.5	68	2	6
0.79 M KCl (2.2 Os kg^{-1})	16.5	7.5	45	4.5	19
1.06 M KCl (2.7 Os kg^{-1})	9.5	2.5	26	12	100
1.32 M KCl (3.2 Os kg^{-1})	9.5	1.5	16	13	118
0.42 M Sucrose (1.3 Os kg^{-1})	23	12	52	4	11
0.97 M Sucrose (2.2 Os kg^{-1})	21	7.5	36	4.5	16
1.18 M Sucrose (2.6 Os kg^{-1})	19	7	37	3.5	13
1.38 M Sucrose (3.2 Os kg^{-1})	14	3.5	25	8.5	49
0.34 M NaCl (0.6 Os kg^{-1})	20	7	35	4	15
0.17 M NaCl (0.3 Os kg^{-1})	19.5	5.5	28	18	72
0.09 M NaCl (0.17 Os kg^{-1})	17.5	6	34	15.5	66
Distilled water(0 Os kg^{-1})	51	/	/	/	/

relax fluorescence quenching when the algae were becoming adapted to State 1 (i.e. the degree of spillover from PSII to PSI was decreasing). The DCMU is added (to a final concentration of 10 μM) after the algae have been exposed to 6 min of Light 2 and then 6 min of Light 1 and Light 2 (Figure 70). In these experiments, Light 1 was not switched off until the end of the experiment, when both light sources were turned off together. Table 9 shows that algae resuspended in 0.43 M NaCl (normal growth medium) in the presence of both Light 1 and Light 2 have a different balance of the two quenching components Q and Δ pH from the algae exposed to Light 2 only (Table 8). As would be predicted, in the presence of Light 1 and Light 2, Q is mainly oxidised and thus the Q-dependent fast relaxation of quenching phase is much larger in these algae, whereas the Δ pH dependent slow phase is similar to that found in the presence of Light 2 only. Table 9 indicates that the fast phase is decreased by increasing the external osmolality using NaCl, KCl or sucrose; it occurs to a lesser extent in the case of sucrose. Increasing the external osmolality has very little effect on the slow relaxation phase. Decreasing the external osmolality also decreases the extent of the fast phase of the DCMU-induced fluorescence increase in a similar fashion to increased sucrose concentrations, but it does not affect the slow relaxation phase which is dependent on the Δ pH. Distilled water abolishes the DCMU-induced relaxation of fluorescence quenching (Table 9).

5.3.4 Simultaneous Measurement of Fluorescence Emission at 695 and 720 nm

The fluorescence emission of the algae can be measured simultaneously at 695 and 720 nm using an exciting beam of 440 nm wavelength

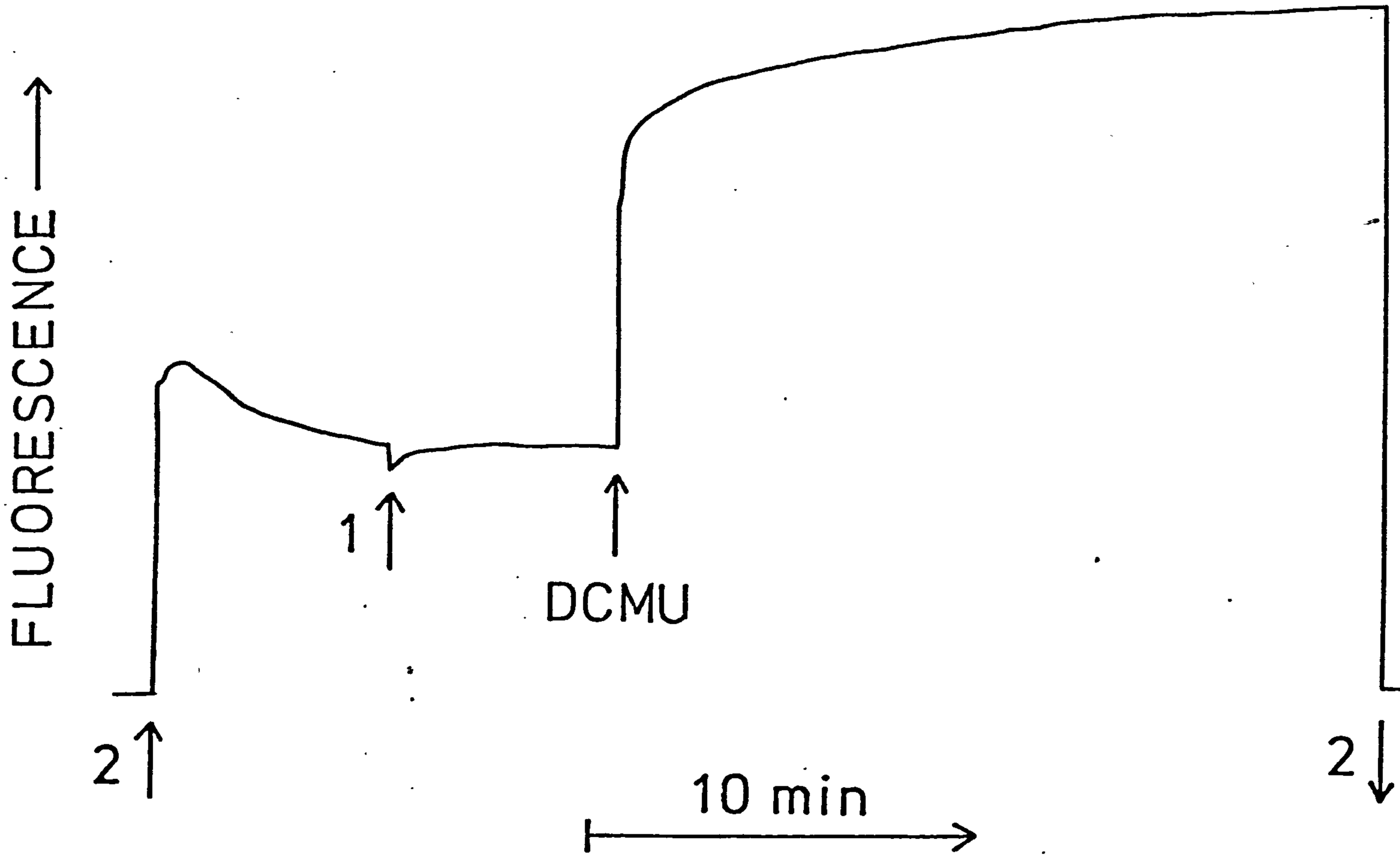


FIGURE 70. The effect of DCMU on the fluorescence emission of the alga in the presence of Light 1 and Light 2, showing the biphasic relaxation of quenching.

Table 9

DCMU-Induced Relaxation of Chlorophyll Fluorescence Quenching in the Presence of Light 1 and Light 2

Test Medium	Fluorescence Level Before DCMU Addition (arbitrary units)	Extent of Fast Phase		Extent of Slow Phase	
		Units	Percentage	Units	Percentage
0.43 M NaCl (control)(0.8 Os kg^{-1})	14	18	129	6.5	20
0.68 M NaCl (1.3 Os kg^{-1})	14	16.5	118	6	20
1.20 M NaCl (2.2 Os kg^{-1})	12	13	108	3.5	14
1.45 M NaCl (2.7 Os kg^{-1})	11.5	7.5	65	4	21
1.71 M NaCl (3.2 Os kg^{-1})	13.5	6.5	48	3	15
0.26 M KCl (1.3 Os kg^{-1})	9	14	156	2	9
0.79 M KCl (2.2 Os kg^{-1})	10	9.5	95	3	15
1.06 M KCl (2.7 Os kg^{-1})	11.5	10.5	91	4.5	20
1.32 M KCl (3.2 Os kg^{-1})	12.5	6.5	52	3	16
0.42 M Sucrose (1.3 Os kg^{-1})	11.5	14.5	126	4	15
0.97 M Sucrose (2.2 Os kg^{-1})	12	13	108	3	12
1.18 M Sucrose (2.6 Os kg^{-1})	10.5	10	95	3	15
1.38 M Sucrose (3.2 Os kg^{-1})	11	9.5	86	3.5	17
0.34 M NaCl (0.6 Os kg^{-1})	12	13	108	7	28
0.17 M NaCl (0.3 Os kg^{-1})	14	12.5	89	6.5	25
0.09 M NaCl (0.17 Os kg^{-1})	15	11	73	6.5	25
Distilled water (0 Os kg^{-1})	35	/	/	/	/

light (section 2.10). The emission at 695 nm can be attributed mainly to PSII and the 720 nm emission mainly to PSI (Lavorel and Etienne, 1977). A similar chlorophyll fluorescence induction curve is found at both wavelengths, they exhibit the initial peak P and a fall in fluorescence to the terminal value T (Bradbury and Baker 1981b). The curves are normalised at the fluorescence peak P so that the ratio of fluorescence at 720 nm (F720) over the fluorescence at 695 nm (F695) is 1.0. Then the ratio of F720/F695 is determined at the steady-state level T. Table 10 shows the effect of increasing the external osmolality on F720/F695 at T. It is clear that 5 min after increasing the external osmolality using NaCl, KCl or sucrose, the F720/F695 ratio has increased significantly; sucrose had a smaller effect than NaCl or KCl (Table 10). This indicates that PSI is apparently absorbing a larger share of the excitation energy at high osmolalities. After 4 hours at the increased osmolalities, the F720/F695 ratio has returned to below the control value in the case of sucrose and to just above the control value in the case of NaCl or KCl (Table 10).

5.4 Fluorescence Emission Spectra at 77K

The fluorescence emission spectra of D. tertiolecta were determined from 650 to 800 nm at 77K using an exciting beam of 440 nm wavelength light (section 2.10). At low temperature there is a much higher level of fluorescence associated with PSI chlorophyll. The fluorescence emission at 683 and 694 nm is mainly associated with PSII, emission at longer wavelengths is mostly dependent on PSI (Papageorgiou, 1975; Lavorel and Etienne, 1977). Thus, this technique can be used to compare the amount of excitation energy associated with each photosystem. Figure 71 shows a typical low temperature spectrum for algae resuspended

Table 10

Simultaneous Measurement of Chlorophyll Fluorescence Emission at 695 and 720 nm

Test Medium	Ratio of F720/F695	
	At P	At T (steady-state)
0.43 M NaCl (0.8 Os kg^{-1})(control), 5 min	1.0	1.007 ± 0.019 (3)
1.71 M NaCl (3.2 Os kg^{-1}), 5 min	1.0	1.282 (2)
1.71 M NaCl (3.2 Os kg^{-1}), 4 hours	1.0	1.019 (5)
1.32 M KCl (3.2 Os kg^{-1}), 5 min	1.0	1.252 (1)
1.32 M KCl (3.2 Os kg^{-1}), 30 min	1.0	1.258 (1)
1.32 M KCl (3.2 Os kg^{-1}), 4 hours	1.0	1.022 (1)
1.38 M Sucrose (3.2 Os kg^{-1}), 5 min	1.0	1.171 (1)
1.38 M Sucrose (3.2 Os kg^{-1}), 4 hours	1.0	0.976 (1)

Number of replicates indicated in brackets.

The algae were dark-adapted for 5 min prior to measurement.

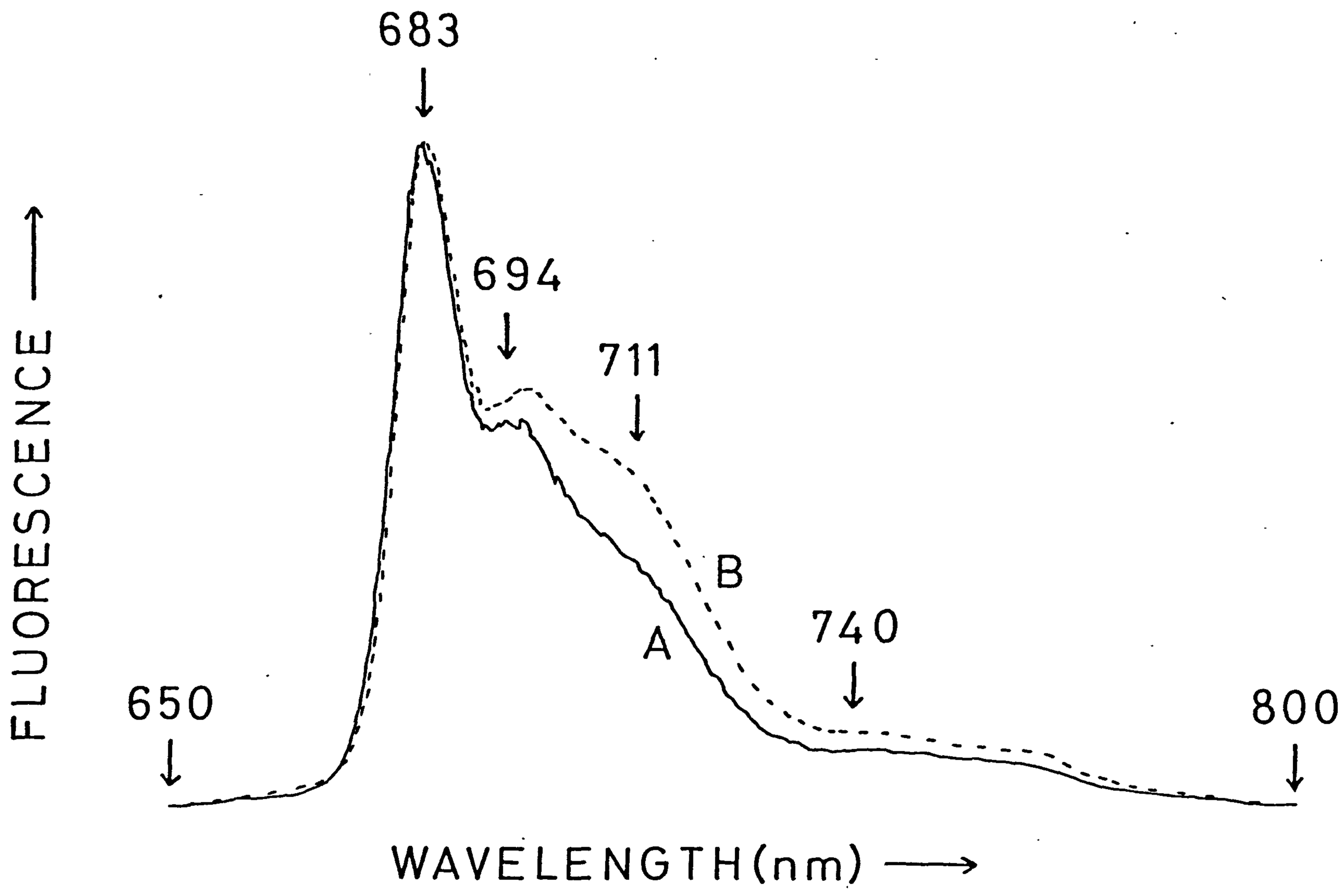


FIGURE 71. Fluorescence emission spectra determined from 650 to 800 nm at 77K. The algae were resuspended in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; and B: 1.71 M NaCl (3.2 Os kg^{-1}). The experimental procedure was as described in Table 11.

in normal growth medium (Trace A). Superimposed on this control trace is the spectrum found 5 min after an increase in the external salinity to 1.71 M (Trace B). The initial part of the spectra including the 683 nm fluorescence peak are virtually identical, but the algae exposed to a high external salt concentration show increased fluorescence at 694 nm (mainly connected with PSII) and 711 and 740 nm (mostly associated with PSI). This type of experiment was also carried out with KCl and sucrose and the results are expressed in Table 11 as ratios of the longer wavelength fluorescence emissions over the emission at 683 nm. It can be clearly seen that 5 min after increasing the external osmolality using NaCl or KCl, there is an increase in the excitation energy in chlorophyll species which emit fluorescence at 694, 711 and 740 nm (Table 11). After 5 min exposure to a high concentration of sucrose, there is no increase in the emission at 694 nm, and compared to NaCl or KCl relatively smaller increases in emission at 711 and 740 nm. After almost 5 hours, the algae suspended in the sucrose solution have returned completely to the control emissions. In the case of NaCl, the algae have almost returned to the control values of fluorescence emission after 5 hours. However, the algae resuspended in KCl have not recovered to any extent after 4 hours (Table 11).

5.5 Discussion

The first part of the discussion will be used to attempt an interpretation of the chlorophyll fluorescence changes found when the external NaCl concentration is increased (section 5.5.1). Section 5.5.2 will compare the conclusions made on the basis of the NaCl data with the effects of increasing the external osmolality using KCl, sucrose, ethylene glycol or glycerol. Finally, the effect of decreasing

Table 11

Characteristics of Fluorescence Emission at 77K

Test Medium	Fluorescence Emission Ratios		
	F694/F683	F711/F683	F740/F683
0.43 M NaCl (0.8 Os kg^{-1})(control), 5 min[a]	0.585	0.386	0.097 (2)
1.71 M NaCl (3.2 Os kg^{-1}), 5 min[a]	0.628	0.496	0.124 (1)
1.71 M NaCl (3.2 Os kg^{-1}), 2.25 hours	0.625	0.424	0.118 (1)
1.71 M NaCl (3.2 Os kg^{-1}), 4.75 hours	0.556	0.400	0.111 (2)
1.32 M KCl (3.2 Os kg^{-1}), 5 min[a]	0.612	0.533	0.138 (1)
1.32 M KCl (3.2 Os kg^{-1}), 2 hours	0.614	0.520	0.114 (1)
1.32 M KCl (3.2 Os kg^{-1}), 4 hours	0.607	0.566	0.131 (1)
1.38 M Sucrose (3.2 Os kg^{-1}), 5 min[a]	0.569	0.425	0.118 (1)
1.38 M Sucrose (3.2 Os kg^{-1}) 2.5 hours	0.600	0.436	0.114 (1)
1.38 M Sucrose (3.2 Os kg^{-1}), 4.75 hours	0.580	0.372	0.095 (1)

Number of replicates indicated in brackets.

a After resuspension in the test medium, the algae were dark-adapted for 5 min at 293 K. They were then immersed in liquid nitrogen and illuminated for 5 min at 77K to close the reaction centres so that the emission spectra are taken at the F max level of fluorescence.

the external salinity will be briefly discussed in section 5.5.3.

5.5.1 NaCl

In Chapters 3 and 4, it was demonstrated that increasing the external NaCl concentration inhibited photosynthesis during the period of intracellular glycerol synthesis. Thus, in this chapter the effect of increasing the external NaCl concentration has been determined on various fluorescence parameters to investigate possible mechanisms for the salt-induced inhibition of photosynthesis.

Increasing the NaCl concentration decreased the Fv/Fm ratio after 5 min (section 5.2), which may indicate that the photochemical efficency of PSII was decreased by high external salt concentrations. However, it may also indicate that high external salt concentrations increase the internal conversion to heat at the reaction centre (k_h) and therefore photochemistry would be unaffected (section 5.1). However, even if it is assumed that k_h does not increase, the maximum fall in the photochemical efficency of PSII induced by high salt after 5 min is only 36% (Figure 42). Thus, this inhibition would not account for the 88% inhibition of the photosynthetic rate found in the presence of high salt after 5 min (Figure 28). Therefore, it is clear from these results that NaCl-induced inhibition of photosynthesis cannot completely be explained on the basis of a specific inhibition of PSII photochemistry. In fact owing to the way in which the Fv/Fm data must be interpreted, it is possible that the photochemical efficency of PSII is not decreased at all by salt stress (section 5.1).

Thus to enable further investigation of the NaCl-induced inhibition of photosynthesis, other fluorescence parameters were studied. The

simultaneous measurement of the fluorescence emission at 695 and 720 nm at room temperature and the fluorescence emission spectra determined at 77K, provided evidence of an increased proportion of excitation energy going to PSI after 5 min at high external NaCl concentrations (Tables 10 and 11). These observations can be taken as an indication of increased spillover from PSII to PSI. They also indicate that the spillover change can take place in the dark at room temperature (Tables 10 and 11). Thus the spillover change could not have been induced in the normal way (Allen et al., 1981; Horton and Black, 1981; Chow et. al., 1981). Therefore, it appears that algae stressed with NaCl for 5 min in the dark are adapted to the condition of increased spillover (State 2).

In section 5.3.1, the effect of high external NaCl concentrations on the long term fluorescence parameters of the Kautsky effect was determined. Figure 50 shows that 5 min after increasing the external NaCl concentration, the Kautsky fluorescence curve had been modified in three ways:-

(a) the initial fluorescence peak I which decays rapidly in the control samples, declines progressively more slowly as the external salt concentration is increased,

(b) the D to P transient fluorescence rise progressively diminishes, then disappears as the salt concentration increases,

(c) the slow fluorescence changes (SMT transition) disappear with increasing NaCl concentration and are replaced with a much higher level of fluorescence quenching especially above 1.20 M NaCl.

It is difficult to assign specific photosynthetic processes to the Kautsky fluorescence changes, but this has been attempted by Papageorgiou

(1975). Using his conclusions as a guideline, the Kautsky fluorescence changes found in high NaCl concentrations can be analysed as follows.

The initial fluorescence rise to I is not affected to any great extent by high salt suggesting that the initial reduction of Q takes place normally (Figure 50). However, in high salt the fluorescence decrease from I to D takes place more slowly. The I to D decrease in fluorescence is thought to be due to the reoxidation of Q by PSI, therefore, the results suggest that the rate of electron transport from PSII to PSI is decreased by high salt.

The D to P fluorescence increase disappears as the NaCl concentration is increased: the D to P part of the fluorescence curve has been connected to the occurrence of rapid spillover changes (Schreiber and Vidaver, 1976a and b). The disappearance of these rapid light-induced spillover changes could be due to spillover changes having already taken place during the 5 min dark adaptation period as already suggested (Tables 10 and 11).

The fluorescence decline from P to S has been extensively studied recently (Briantais et. al., 1979; Bradbury and Baker, 1981b; Walker, 1981). From the work of these authors, three general processes can be connected with the P to S decay in fluorescence emission:-

(a) the reoxidation of Q possibly mediated by increased spillover of excitation energy from PSII to PSI.

(b) the acidification of the thylakoid space i.e. an increase in the Δ pH across the thylakoid membrane.

(c) the thermal deactification (internal conversion to heat) of excited PSII chlorophyll molecules.

Therefore, the increase in fluorescence quenching in the P to S region

induced by increasing the NaCl concentration could be caused by an increase in one or more of these processes. However, the previous data (Tables 10 and 11) indicating increased spillover to PSI in the dark suggest that the reoxidation of Q is involved, although this may be affected by the apparent slower rate of electron transport in the presence of high salt.

Concentrations of the uncoupler FCCP which induce a rapid decay of the 519 nm absorption change (section 1.6.1 and Chapter 6) cause similar changes in the fast Kautsky fluorescence parameters to those induced by high salt concentrations (Figure 62, Traces E and F). FCCP causes a slow decline in fluorescence from the peak at I and the D to P transient fluorescence rise disappears. Thus, in common with high salt, uncoupling appears to slow down the rate of electron transport and abolish the rapid light-induced spillover changes. However, in contrast to high concentrations of external NaCl, FCCP does not induce a large amount of fluorescence quenching from I to S, and the SMT transition is still present. Walker (1981) has attributed the slow SMT fluorescence change to the effects of carbon assimilation on both the adenylate status of the stroma and the Δ pH gradient across the thylakoid membrane. Therefore it is perhaps surprising that the uncoupler FCCP does not affect the SMT change to a greater extent (Figure 62).

To try to distinguish between the possible mechanisms of NaCl-induced fluorescence quenching, DCMU was added to the algae after 2 min exposure to Light 2, when the NaCl-induced quenching was high (Figure 50). Krause et. al. (1982) demonstrated that the biphasic rise in fluorescence after DCMU addition can be attributed to two processes. The fast DCMU-induced fluorescence rise is due to the reduction

of that part of Q which was in an oxidised state before DCMU addition and the slow DCMU-induced fluorescence rise is due to a release of 'energy dependent' fluorescence quenching which depends on the Δ pH gradient. The results of the experiments in which DCMU is used to relax fluorescence quenching are shown in Table 8 and indicate that at high NaCl concentrations (above 1.45 M), the quenching is dominated by the portion thought to be connected with the Δ pH gradient (Krause et. al., 1982). High external salt concentrations decrease the amount of quenching due to Q being oxidised, suggesting that in high salt Q is more reduced than in the control. This is probably due to the slower rate of electron transport in high salt, which was inferred from the Kautsky fluorescence curves. A careful examination of Table 8 shows that the final fluorescence level in the presence of high salt after the addition of DCMU is only 62% of the final DCMU-induced fluorescence level in the control. Therefore, there is evidence for internal conversion to heat of excited PSII chlorophyll molecules playing a role in NaCl-induced fluorescence quenching. Thus it appears from these results that the NaCl-induced quenching is due to an internal conversion to heat of excited PSII chlorophyll molecules and to the presence of a large Δ pH gradient across the thylakoid membrane. However, it should be borne in mind that a build up of secondary metal cation gradients could also contribute to the Δ pH part of the fluorescence quenching (Barber, 1976).

In section 5.3.2, it was shown that the normal response of fluorescence to the transitions between State 1 and State 2 were lost in algae stressed by high salt concentrations. It was found that there was apparently not enough spillover from PSII to PSI to oxidise Q (and therefore reduce fluorescence) to the normal extent (Figure 65).

This can however be interpreted in a different way when the fact that the changes needed for spillover can take place in the dark in response to salt stress is taken into account (Tables 10 and 11). Therefore, when Light 2 is switched on to salt-stressed algae, there is a smaller fluorescence peak because some of the light is being immediately spilled over to PSI. Thus, under these circumstances a large fluorescence decrease would not be expected and in fact it is not found (Figure 65). When Light 1 is switched on there is normally a rapid decrease in fluorescence due to the oxidation of Q by electron transport driven by PSI. However, in high salt the rapid reoxidation of Q is replaced by a slow reoxidation based presumably on a slower rate of electron transport. In the control, when Light 1 is switched off there is an immediate increase in fluorescence due to the rapid reduction of Q by PSII. However, in high salt there is only a slow reduction of Q by PSII when Light 1 is turned off. This cannot be explained by a salt-induced decrease in the rate of electron transport, but it can be explained by Q being more reduced in high salt or by the increased salt-induced spillover to PSI initiated in the dark still operating and preventing a rapid reduction of Q in Light 2 (Figure 65).

Therefore, it appears that although a spillover change is found in the dark adaptation period after a salt stress, there does not seem to be any light-induced spillover changes characteristic of State 1 - State 2 transitions taking place in high NaCl concentrations. It is thought that the State 1 - State 2 transitions depend on the phosphorylation of the LH a/b complex (page 22) catalysed by a protein kinase. If the LH a/b complex is phosphorylated spillover is increased (State 1 → State 2) and if it is dephosphorylated, spillover is decreased (State 2 → State 1). Furthermore, the redox state

of plastoquinone appears to control the activity of the protein kinase in an unknown way (Allen et.al., 1981; Chow et. al., 1981; Horton and Black, 1981). Therefore the lack of light-induced spillover changes in high salt could be due to two possible reasons: (a) the phosphorylation of the LH a/b complex may be inhibited by e.g. a lack of ATP or b) the message from plastoquinone to the protein kinase to switch on the phosphorylation of the LH a/b complex may not be sent because plastoquinone may be insufficiently reduced. The NaCl-induced decrease in electron transport could be implicated in both of these hypothetical processes.

Earlier in the discussion the effect of DCMU-induced relaxation of NaCl-induced fluorescence quenching was described (Table 8). In this experiment the algae were illuminated by Light 2 and were at least partially adapted to State 2. Thus to complement these results the same experiment was carried out during the State 1 - State 2 transition experiments, the DCMU being added when the algae had been exposed to Light 1 and Light 2 together for 6 min; i.e. when they were mainly adapted to State 1 (Figure 70). Table 9 shows as expected that the quenching due to oxidised Q is much larger in algae in State 1 than in State 2 (Table 8). The quenching due to Δ pH was similar in control algae in State 1 and State 2. In State 1 algae subjected to high external NaCl concentrations, the amount of quenching due to oxidised Q was decreased, thus as predicted from the State 1 - State 2 transition data (Figure 65), Q remains more reduced in high salt. In State 1 algae high NaCl concentrations slightly decrease the quenching due to the Δ pH, in contrast to the increase in the Δ pH quenching found with high salt in State 2 algae (Tables 8 and 9). In State 1 and State 2 algae, the final DCMU-induced fluorescence level is much smaller

in the presence of high salt (60% of the control value). This may indicate a role for the internal conversion to heat of excited PSII chlorophyll molecules in fluorescence quenching induced by NaCl, but it may also suggest a role for an increased transfer of excitation energy to PSI which would result in the lower fluorescence yield.

In summary, it appears that when the algae are subjected to increased NaCl concentrations an unknown change in the properties of the photochemical apparatus occurs which can take place in the dark. This leads to an increase in the excitation energy going to PSI when the light is switched on. Therefore, spillover occurs very rapidly which leads to either a large degree of fluorescence quenching or a decrease in the fluorescence peak, depending on the light intensity and time scale used (Figures 50 and 65). The increase in fluorescence quenching induced by NaCl is probably due to two processes: (a) an internal conversion to heat of excited PSII chlorophyll molecules and (b) an increased amount of excitation energy being absorbed by PSI due to increased spillover. An increase in the Δ pH gradient is also implicated in algae adapted towards the State 2 condition, but it is not found at all in algae mostly adapted to the State 1 condition. The usual light-induced spillover changes are not seen in high salt probably because of the reduction in the rate of electron transport induced by NaCl. The observation that Q seems to remain more reduced in high NaCl concentrations is also likely to be due to the decrease in electron transport.

In conclusion, in high NaCl concentrations there appears to be a loss of control over the excitation energy entering the two photosystems and a decrease in the rate of electron transport. These processes may well account for the inhibition of the rate of light-

induced O_2 evolution found in high salt concentrations (Figure 28).

5.5.2 KCl, Sucrose, Ethylene Glycol and Glycerol

In this section a brief comparison will be made between the effects on the fluorescence parameters of D. tertiolecta of increasing the external osmolality using NaCl and of increasing the osmolality using KCl, sucrose, ethylene glycol or glycerol.

Increasing the external KCl concentration has an identical effect on the fluorescence measurements after 5 min to that seen with increasing the external NaCl concentration. However, over the longer time period of 100 min, smaller rates of recovery could be detected in high concentrations of KCl e.g. Figures 51 and 55. The most striking example of the longer term inhibition by KCl is shown in Figure 48. This was a comparison of the Fv/Fm ratio made over 20 hours in the presence of NaCl, KCl or sucrose. Both NaCl and KCl (and to a much lesser extent sucrose) decrease the Fv/Fm ratio immediately after the osmolality increase. However, in the presence of high KCl, after an initial recovery the Fv/Fm ratio falls to a low level and the cells die. This lack of recovery was noticed for KCl in almost all the fluorescence studies. It suggests that after an initial period of trying to adapt to the increased KCl concentration, the algae fail to do so and the cells die.

In contrast to KCl, and as already mentioned above, increasing the external osmolality using sucrose has less effect on the fluorescence parameters than does NaCl. Even after only 5 min the sucrose effect is less (e.g. Figures 50 and 56 or 65 and 68). Figure 56 showed that sucrose in common with NaCl abolishes the D to P fluorescence rise and

produces a similar amount of quenching to NaCl which obscures the SMT transition. However, there is no slow decay of fluorescence from the peak at I induced by sucrose, which indicates that there is no slowing down of electron transport. A close examination of the kinetics of the decline of the initial fluorescence peak revealed that 1.38 M sucrose (3.2 Os kg^{-1}) has a similar effect on the rate of decline of the fluorescence peak as 1.20 M NaCl (2.2 Os kg^{-1}) (Figure 63). After 100 min, a more or less complete recovery is found at all the sucrose concentrations used (e.g. Figure 57). Thus, although some of the NaCl-induced effects can be reproduced by the osmotic stress exerted by sucrose, the magnitude of the sucrose effect is certainly less.

Increasing the external ethylene glycol concentration has virtually no effect on the fluorescence parameters (e.g. Figures 42 and 46, or 50 and 58).

Increasing the external glycerol concentration has an effect that approached the magnitude of the NaCl effect but did not quite equal it (e.g. Figures 50 and 60). However, it seemed that glycerol had the most effect on the fluorescence parameters of the non-ionic solutions used.

5.5.3 Salinity Decreases

Decreasing the external salinity, unless it was to distilled water had little effect on the fluorescence parameters (e.g. Figures 42 and 43 or 50 and 52). Decreasing the salinity had an interesting effect on the State 1 - State 2 transitions (Figure 66). It did not abolish spillover like increasing the salinity, instead it made the normal fluorescence changes more pronounced. This may indicate that

the influx of water into the cells that accompanies a decrease in salinity causes a decrease in the efficency of the feedback mechanism that controls the magnitude of spillover changes.

CHAPTER 6

FIELD-INDICATING 519 nm ABSORPTION CHANGE

6.1 Introduction

The light-dependent reactions of photosynthesis produce the ATP and NADPH which are necessary for the reduction of CO_2 to sugars and other useful organic molecules. In section 1.6.1 the current theories on how NADPH and ATP are produced via photosynthesis were outlined. $NADP^+$ is reduced directly from the photosynthetic electron transport chain (Figure 4). The phosphorylation of ADP was also shown to depend on electron transport, and Mitchell's chemiosmotic theory was described in section 1.6.1 to show how vectorial electron transport in the thylakoid membrane could be coupled to H^+ ion transfer into the inner thylakoid space (Figure 5). The uptake of H^+ from the stroma occurs at two separate sites, one of which is connected with PSII (reduction of plastoquinone) and the other is dependent on PSI (reduction of $NADP^+$). Two sites of H^+ release into the inner thylakoid space can be characterised, one at PSII (splitting of water) and the other connected with PSI (oxidation of plastoquinone). Therefore, two interconnected pathways of light-induced H^+ transport across the thylakoid membrane can be observed (Figure 5). In addition, it has been suggested that there is a third pathway of H^+ transport across the membrane. This was proposed by Mitchell (1976) in the form of a protonmotive quinone cycle (Q-cycle). The basis of this mechanism is that electrons lost by plastosemiquinone (PQH) at the inside of the membrane are transported back across the membrane by two b-type cytochromes resulting in H^+ uptake from the stroma (Cox and Olsen, 1982).

A modification of this scheme was proposed by Velthuys (1980) who suggested that cytochrome b_6 (equivalent to cytochrome b_{563}, section 1.6.1) is reduced by plastoquinone (PQH_2) at the inside of the membrane and at the same time another one electron acceptor (possibly the recently discovered iron-sulphur protein, section 1.6.1) is reduced as well. When two cytochrome b_6 molecules have been reduced, they move across the membrane and reduce a plastoquinone molecule resulting in H^+ uptake from the stroma (Velthuys, 1980). Other modifications of the Q-cycle have been suggested (Crofts and Wood, 1978; Crowther and Hind, 1980; Bouges-Bocquet, 1981), but most of these mechanisms would result in two H^+ ions being taken up for every electron passing from PSII to PSI. However, the current experimental evidence for this is far from convincing (Cox and Olsen, 1982).

The influx of H^+ sets up an electrochemical potential gradient across the thylakoid membrane which can be used to phosphorylate ADP by the passage of H^+ ions through a membrane-bound ATPase (Figure 6). The mechanism of phosphorylation is not fully understood, but it appears to depend either on the direct effect of protons on the ATPase or on the conformational changes of the ATPase induced by H^+ efflux (McCarty, 1979).

The electrochemical gradient across the thylakoid membrane can be made up of two components, a proton concentration gradient with the inner thylakoid compartment more acidic (Δ pH) and an electrical gradient or membrane potential ($\Delta\psi$) which is positive inside (Avron, 1981). The amount of energy that could be made available by light-induced H^+ pumping into the thylakoid inner space is known as the proton motive force (pmf). In section 1.6.1, by making certain assumptions about the concentration of ATP, ADP and inorganic phosphate, it was shown that approximately 4 units of Δ pH or 240 mV of $\Delta\psi$ would be necessary for

phosphorylation to take place. There is now good experimental evidence that the imposition of either an artificial Δ pH gradient or an artificial membrane potential ($\Delta\psi$) can lead to ATP production in the dark (Jagendorf, 1975; Gräber, Schlodder and Witt, 1977).

Although the postulated electrochemical potential gradient is found across the thylakoid membrane, attempts have been made to measure the portion due to $\Delta\psi$ using microelectrodes (Vredenberg and Tonk, 1975). Other methods used to determine the Δ pH across the thylakoid membrane include the use of radioactively labelled probes or the use of fluorescent amines (Rottenberg, Grunwald and Avron, 1972; Schuldiner, Rottenberg and Avron, 1972). However, one of the most convenient methods for determining the transmembrane electric field ($\Delta\psi$) is to examine the absorption changes which take place when chloroplasts or algae are illuminated. Some of the light-induced changes in absorption reflect processes such as the oxidation/reduction of cytochrome f and plastocyanin or the formation of carotenoid triplets (Bouges-Bocquet, 1977; Joliot, Delosme and Joliot, 1977). However, other absorption changes are belie ved to be due to the light-induced electric field which is set up across the thylakoid membrane and which induces a change in the absorption properties of chlorophyll b and carotenoid molecules imbedded in the thylakoid membrane (Junge, 1977). Field-dependent absorption changes can be explained in terms of electrochromism which was first observed in the absorption spectra of dyes exposed to electric fields (Platt, 1961). The electric field needed to cause such electrochromic changes is strong, of the order of 10^7 V m^{-1}. However, biological membranes are very thin and an electric field of the magnitude of 10^7 V m^{-1} can be produced by a voltage of 100 mV across a membrane 10 nm thick. Thus, electrochromic absorption changes are relevant to

the thylakoid membranes which are in the order of 5 nm thick (Junge, 1977).

The effect of a strong electric field is to cause a shift in an absorption band by a few tenths of a nanometer; this is illustrated in Figure 72A. A difference spectrum can be calculated from the absorption in the presence or absence of the electric field and Figure 72B shows a typical difference spectrum obtained with a dye. A similar difference spectrum can be obtained in chloroplasts or algae by measuring the electrochromic bandshift at different wavelengths of low-intensity light, in the presence or absence of actinic light i.e. a light-minus-dark difference spectrum. Figure 73 shows that a light-minus-dark difference spectrum for chloroplasts is more complex than that of a dye due to more than one type of pigment molecule contributing to the electrochromic absorption change and each pigment has more than one absorption band. It can be seen that the major absorption increase believed to be of electrochromic origin takes place at approximately 520 nm, and for this reason many researchers have concentrated on measuring light-induced absorption changes at approximately 520 nm.

Junge (1977) has reviewed the evidence which links the electrochromic absorption change at 520 nm to the light-induced separation of charge across the thylakoid membrane. The evidence can be split up into three main categories:-

1. spectroscopic evidence - this is based on the similarity between the electrochromic difference spectrum for chlorophylls and carotenoids <u>in vitro</u> measured in the presence or absence of an electric field (Schmidt, Reich and Witt, 1971), and that found for the light-induced electric field-indicating difference spectrum in chloroplasts (Figure 73; Emrich, Junge and Witt, 1969)

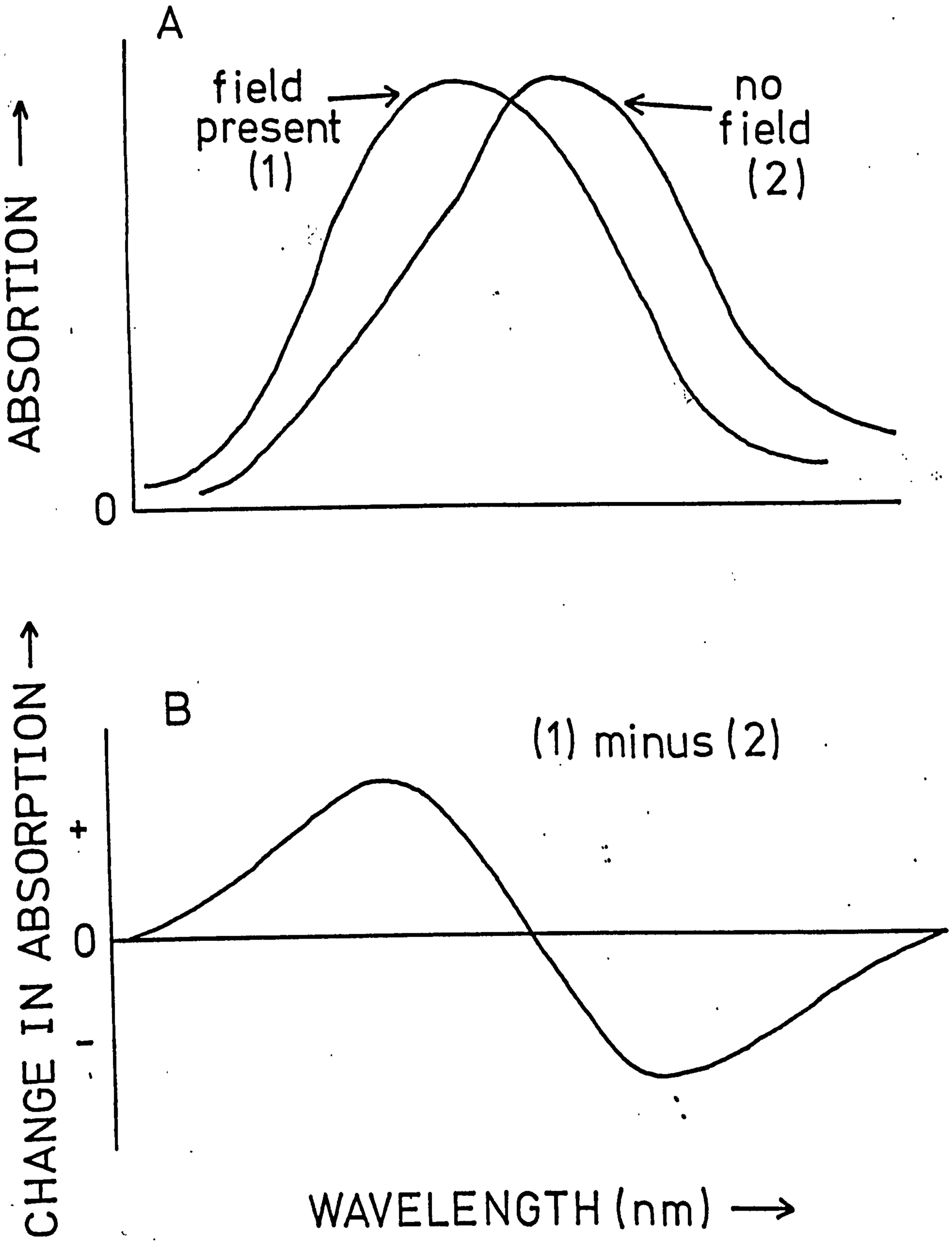


FIGURE 72. The effect of a high electric field on the absorption spectrum of a dye. A: the electrochromic band shift caused by the field and B: the difference spectrum (from Junge, 1977).

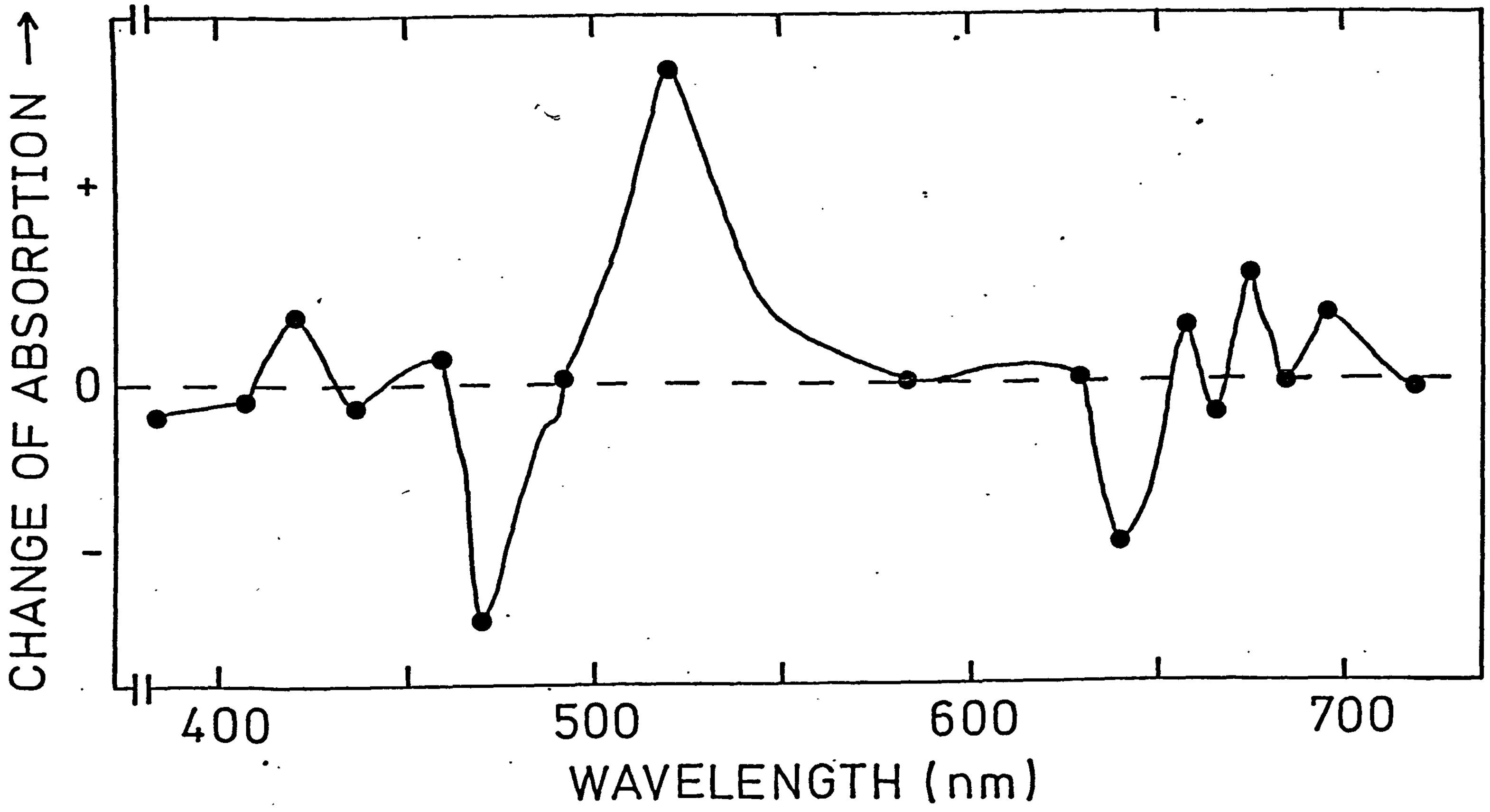


FIGURE 73. A light minus dark difference spectrum of the electrochromic absorption changes observed in chloroplasts (approximate drawing from Junge, 1977).

2. kinetic evidence - this is based on the observation that the decay rate of the absorption increase can be accelerated by uncouplers (section 1.6.1) which allow the H^+ ions to pass down the electrochemical gradient. An example of this is illustrated in Figure 74, which shows the effect of the uncoupler FCCP on the absorption change in D. tertiolecta. Junge and Witt (1968) observed that the decay of the absorption change can be accelerated by any treatment which increases the permeability of the thylakoid membrane to ions e.g. osmotic shock or ion transporting ionophores (section 1.6.1). Rumberg and Siggel (1968) reported an acceleration of the decay of the absorption change under phosphorylating conditions which indicates the influence of 'photosynthetic control' on electron transport. A lack of substrate for photophosphorylation (e.g. ADP) slows down electron transport due to the build up of a 'back pressure' from H^+ ions in the thylakoid inner space (section 1.6.1).

3. evidence from artificially induced diffusion potentials - this has been found most convincingly in bacterial chromatophores by Jackson and Crofts (1969). They used the K^+ - specific ionophore valinomycin (section 1.6.1) and by adding KCl to the chromatophore suspension they could impose a diffusion potential. The spectrum was almost identical to a light-induced one and the absorption change varied linearly with the diffusion potential (Jackson and Crofts, 1969). The linear relationship was surprising since the theory (Junge, 1977) predicts that the change in absorption should be proportional to the field squared. This discrepancy could be accounted for by the presence of a permanent field across the membrane, but to account for the pseudo-linear relationship it would have to be of the order of 2V (Clayton, 1980). This type of experiment has also been carried out with

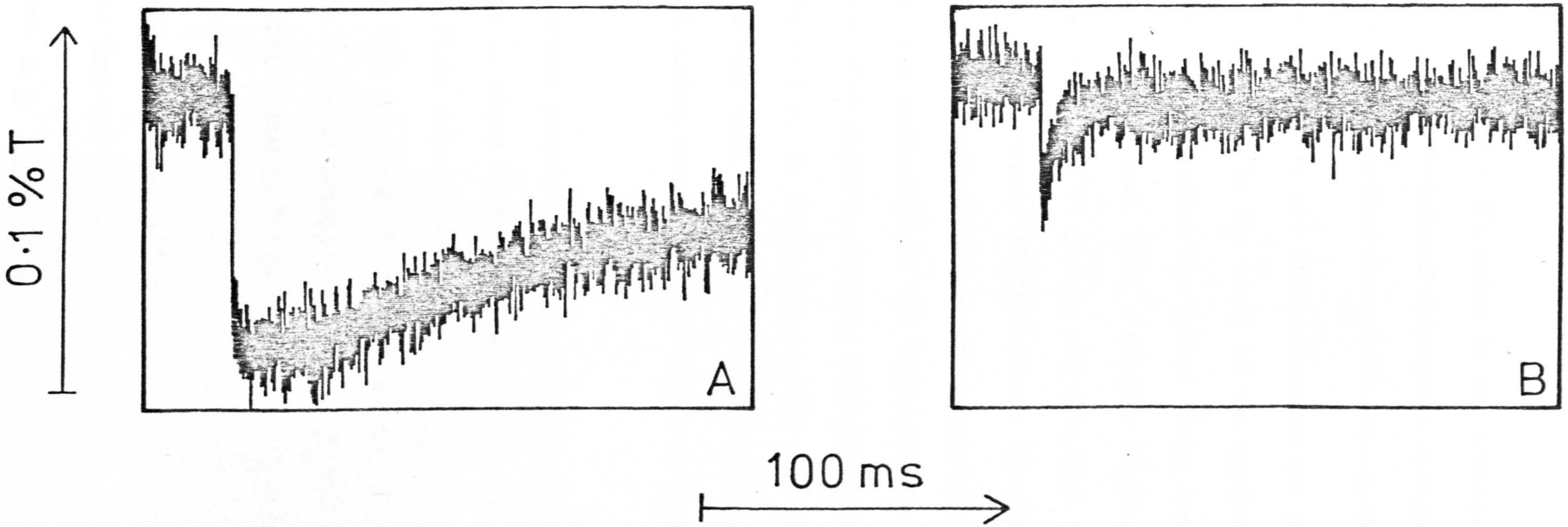


FIGURE 74. The effect of the uncoupler FCCP on the field-indicating 519 nm absorption change. The algae were resuspended in 0.43 M NaCl (normal growth medium) in the dark for 5 min, with no additions (Trace A) or in the presence of 65 μM FCCP (Trace B), before the kinetics of the absorption change were determined. % T is the percentage change in transmission.

chloroplasts using a KCl pulse in the presence of Mg^{2+}; the difference spectrum of the KCl-induced absorption changes was identical to the light-induced absorption changes (Schapendonk and Vredenberg, 1977).

However, there are limitations to the use of electrochromic absorption changes in the study of the transmembrane electric field. Light-scattering changes may appear to be extremely similar to electrochromic changes, with a similar light-minus-dark difference spectrum. Junge (1977) points out that this makes them difficult to distinguish from truely electrochromic changes, but it can be argued that if the scattering changes are responding to the electric field, they are also indicators of the light-induced $\Delta\psi$. However, the changes associated with light-scattering normally take place over time intervals in excess of 1 s. In this project, all the 519 nm absorption change determinations were made using short flashes of actinic light and the field-indicating change was meaured for a maximum of 200 ms (section 2.11).

A second problem which is relevant is that absorption changes occurring in algae between 470 and 530 nm may not be linked with the electric field set up across the thylakoid membrane. Junge (1977) suggests that the lack of response of the decay rate of the absorption change in algae to ionophores like valinomycin casts doubt on whether the absorption change in algae actually measures the transmembrane electric field. However, this lack of effect of valinomycin is not surprising, it can be explained by the inability of the ionophore to penetrate into the algae.

Even in D. tertiolecta which lacks a cell wall, there is no effect of the uncoupler gramicidin (data not shown) (section 1.6.1),

but as illustrated in Figure 74, the uncoupler FCCP does accelerate the rate of decay of the absorption change in D. tertiolecta as would be expected. FCCP also appears to decrease the extent of the absorption change, due presumably to the induction of a very rapid decay phase (Figure 74). Therefore, not all of the treatments which affect the absorption change in chloroplasts do so in algae because of the difficulty involved in penetrating the outer membranes of algae. However, the characteristic effect of FCCP or CCCP, together with the similarity of the light-minus-dark difference spectrum suggest that the 519 nm absorption change can be used to study the transmembrane electric field in algae (Musto and Hipkins, 1981).

The 519nm absorption change can be measured under two different conditions i.e. flashing or continuous actinic light. The distinction is important since photophosphorylation in chloroplasts appears to be driven by the membrane potential ($\Delta\psi$) only for a few seconds after being exposed to light, and then this role is taken over by the Δ pH gradient (Avron, 1981; section 1.6.1). As already stated, all the experiments described in section 6.2 were conducted using short flashes of light to excite the absorption change, which was then followed for 200 ms (section 2.11). Therefore, it is likely that the membrane potential ($\Delta\psi$) was the dominating part of the electrochemical potential gradient in D. tertiolecta over this time scale.

Figure 74, Trace A shows that the absorption change measured at 519 nm in D. tertiolecta is made up of three distinct phases similar to those found by Joliot and Delosme (1974) using Chlorella and Chlamydomonas. These phases can be described as follows (Figure 74, Trace A):-

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1. a large absorption increase (transmission decrease) which occurs in less than 1 μs; the size of the absorption increase reflects the separation of charge at the reaction centres of PSI and PSII (Junge and Witt, 1968).

2. a slow absorption increase which takes place within 40 ms. This slow increase has been found in both algae and chloroplasts and is associated with PSI (Slovacek and Hind, 1978; Horvath et. al., 1979; Schapendonk, Vredenberg and Tonk, 1979; Musto and Hipkins, 1981). Joliot and Delosme (1974) were the first to suggest that the slow absorption increase could be connected with a third electron transfer across the membrane in addition to the charge separations at the reaction centres. Thus, the slow phase of absorption increase has been taken as evidence for the Q-cycle or a similar scheme operating in algae or chloroplasts. However recently, Olsen and Barber (1981) have suggested that the slow phase is due to the delocalisation of charge brought about by the release of H^+ and OH^- into the aqueous phases.

3. a relatively slow absorption decrease with a half-time in the order of 250 to 300 ms (Gilmour, Hipkins and Boney, 1982). The rate of decay of the absorption change reflects how permeable the membrane is to protons and other ions as illustrated in Figure 74 (Junge and Witt, 1968).

One possible reason for the different phases of the absorption change could be the inhomogeneity of the algal sample. This cannot be completely disregarded, but in the case of D. tertiolecta the cells are motile and will tend to keep the sample well mixed during the short period of preparation and measurement. There is no evidence for rapid phototactic movements by the alga which could interfere with the measurements.

Therefore, in section 6.2 results are presented on the effect of changing the external osmolality on the field-indicating absorption change measured at 519 nm in D. tertiolecta. In section 6.3 the 519 nm absorption change is used to indirectly measure the effect of osmotic stress on PSI while PSII is chemically blocked. The results are discussed in section 6.4.

6.2 Effect of Changing the External Osmolality on the 519 nm Absorption Change

In this section the effect of changing the osmolality on the electrochromic 519 nm absorption change will be determined. Figure 75, Trace A shows the normal absorption change in algae resuspended in 0.43 M NaCl (growth medium) for 5 min illustrating the three main features of the absorption change described in section 6.1. Increasing the external NaCl concentration to 0.68 or 0.94 M has little effect on the absorption change except that at 0.68 M the initial fast absorption increase is larger than in the control (Figure 75, Traces B and C). Trace D shows that increasing the salinity to 1.20 M abolishes the slow absorption increase (slow phase). Further salinity increases to 1.45 or 1.71 M cause a significant decrease in the initial absorption change; they also abolish the slow phase and accelerate the rate of decay of the absorption change (Figure 75, Traces E and F). These effects are more pronounced at the highest external salinity used (Trace F) and they suggest that high NaCl concentrations decrease the amount of charge separation at the reaction centres and increase the ion flux through the membrane (Figure 75). Figure 76 shows that the absorption change has returned to normal after 100 min at increased salinities up to 1.45 M NaCl; in fact at 1.45 M NaCl after 100 min there appears to be an increased amount of the slow phase absorption

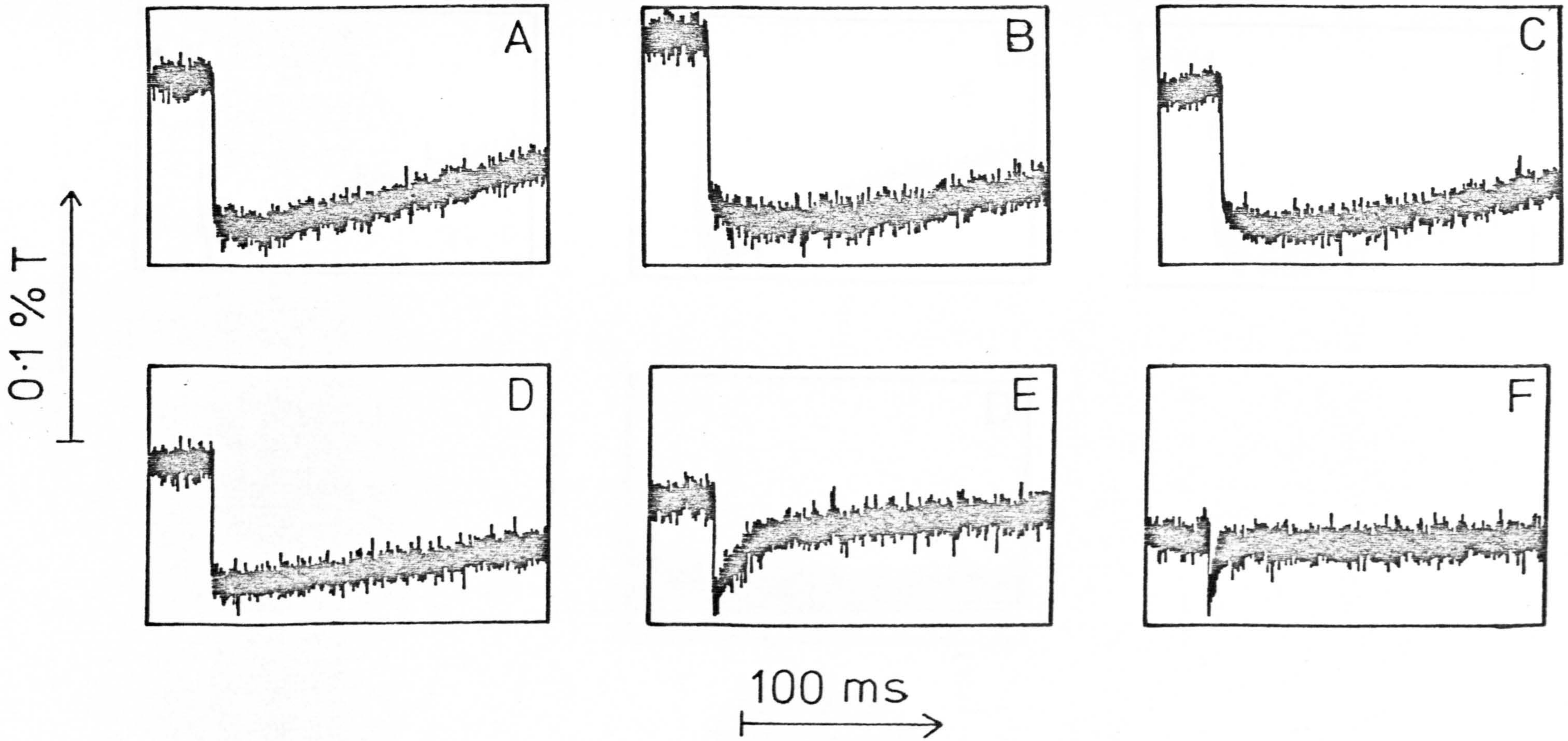

FIGURE 75. The effect of increasing the external NaCl concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.68 M NaCl (1.3 Os kg^{-1}); C: 0.94 M NaCl (1.7 Os kg^{-1}); D: 1.20 M NaCl (2.2 Os kg^{-1}); E: 1.45 M NaCl (2.7 Os kg^{-1}); F: 1.71 M NaCl (3.2 Os kg^{-1}). % T is the percentage change in transmission.

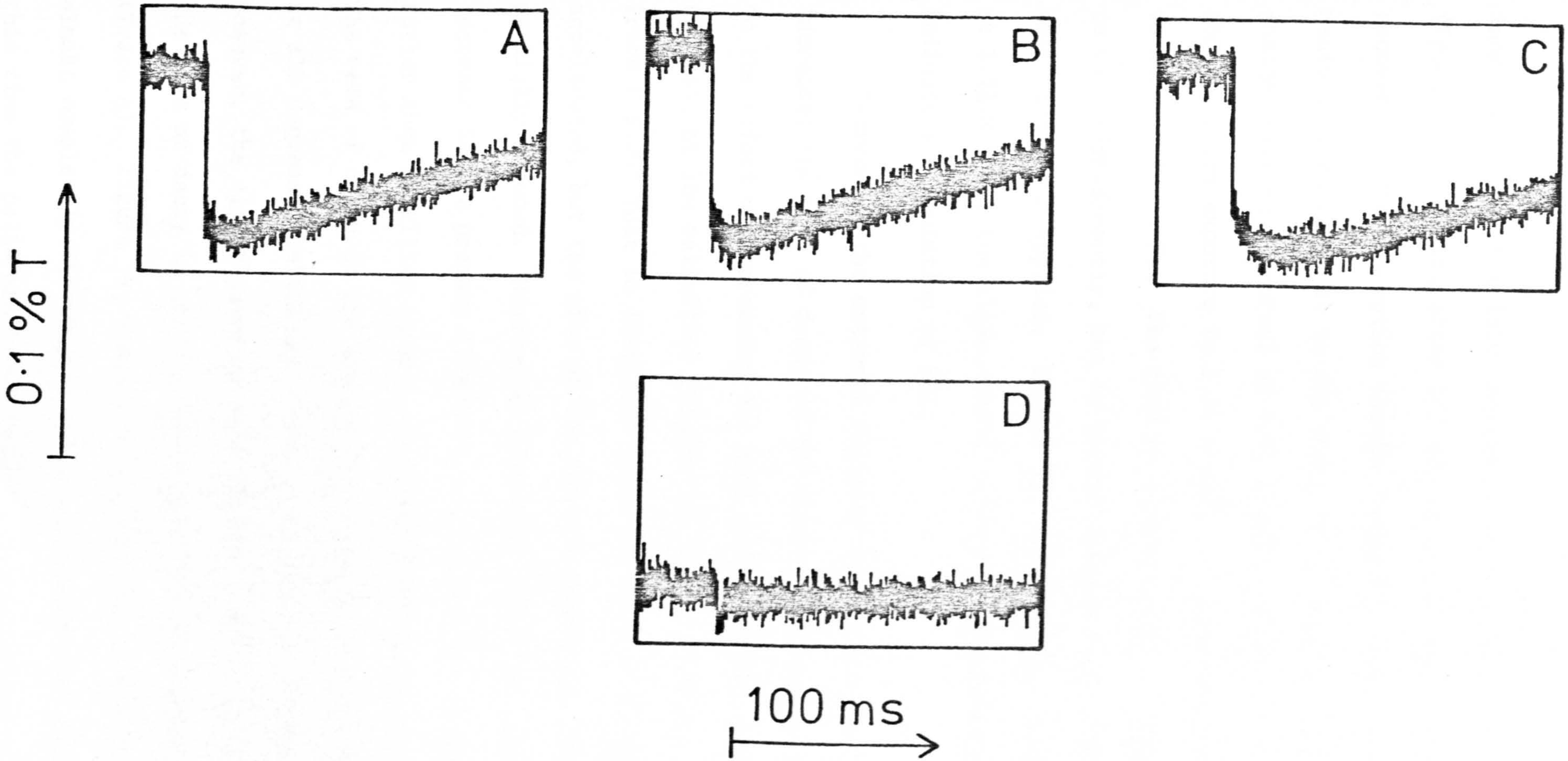

FIGURE 76. The effect of increasing the external NaCl concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after 100 min at A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.68 M NaCl (1.3 Os kg^{-1}); C: 1.45 M NaCl (2.7 Os kg^{-1}); D: 1.71 M NaCl (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to the measurement. % T is the percentage change in transmission.

increase present possibly indicating an increase in PSI activity (Trace C). However, after 100 min at 1.71 M NaCl there has been no recovery of the absorption change (Trace D). Thus, longer term experiments were carried out to see when, if at all, the 519 nm absorption change returned to normal at 1.71 M NaCl. Figure 77 shows that after about 4 hours exposure to 1.71 M NaCl the absorption change has returned to normal (Trace E). The time to full recovery at high salt was found to vary significantly, but is always between 4 and 8 hours after the salt stress was applied. Figure 77, Trace F shows that after 320 min at 1.71 M NaCl, the algae exhibit a very large slow phase which may indicate a stimulation of PSI.

Decreasing the external salinity (except to distilled water) increases the rate of decay of the absorption change, but in contrast to the effect of increasing the NaCl concentration, the slow phase is not lost at low salinities (Figure 78, Traces B to E). Figure 78, Trace F shows that in distilled water the rate of decay is greatly accelerated, but the size of the initial absorption increase is only partially reduced. However at distilled water the slow absorption increase is not present (Trace F). After 100 min at decreased salinities (other than distilled water), there has been little change (Figure 79). The rate of decay of the absorption increase is still relatively fast at all decreased salinities except distilled water (Traces B to E). However, the significance of this observation is diminished because the rate of decay in the control sample is also faster than normal (Trace A). Figure 79, Trace F shows that the absorption change has almost completely disappeared after 100 min at distilled water. At this time the cells are known to be disrupted (Figure 27).

Figure 80 shows that the effect of increasing the KCl concentration

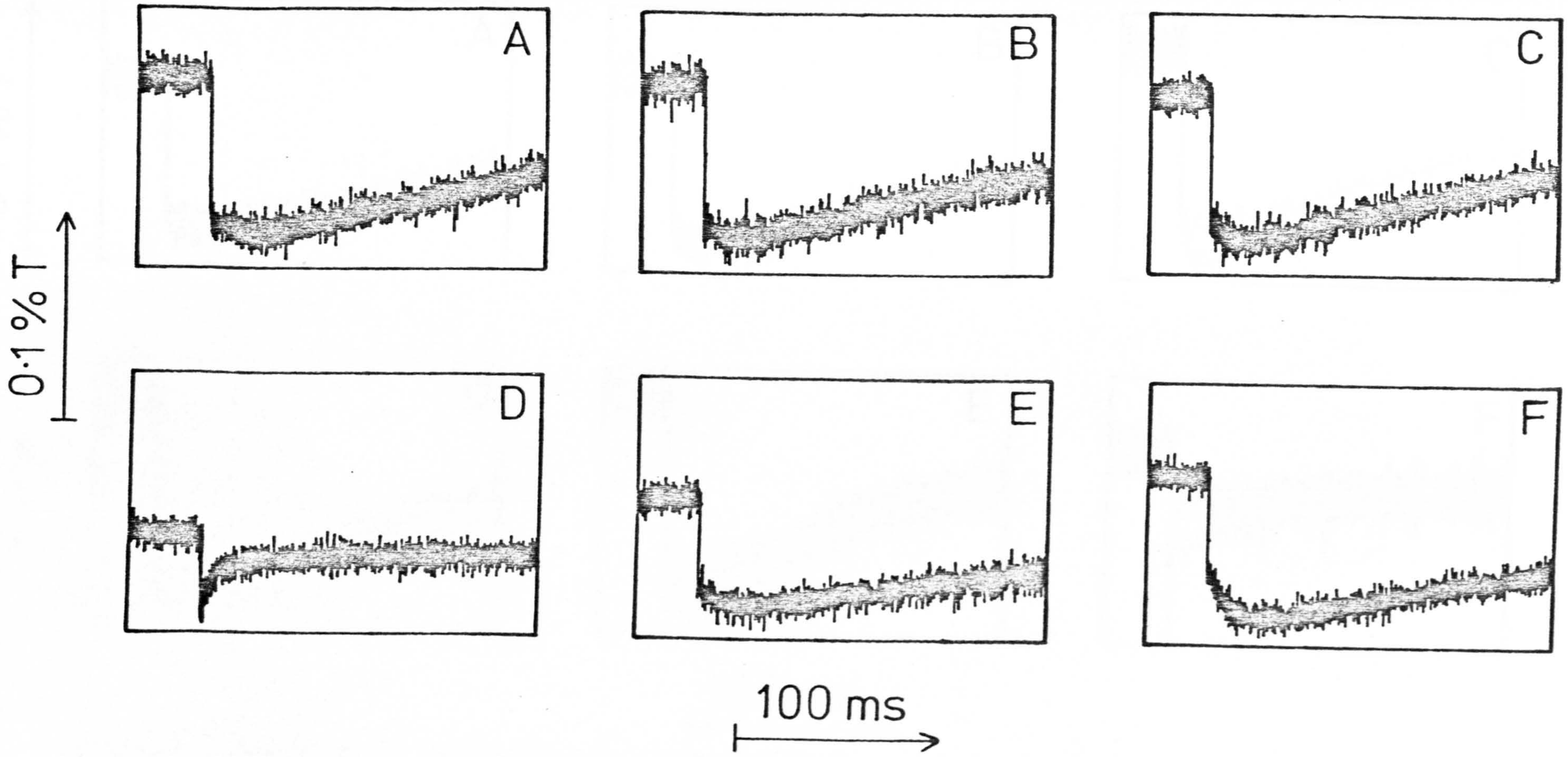

FIGURE 77. The long term effect of increasing the external NaCl concentration on the field-indicating 519 nm absorption change. The algae were resuspended in A: 0.43 M NaCl (0.8 Os kg^{-1}), control for 90 min; B: 0.43 M NaCl for 230 min; C: 0.43 M NaCl for 280 min; D: 1.71 M NaCl (3.2 Os kg^{-1}) for 110 min; E: 1.71 M NaCl for 250 min; F: 1.71 M NaCl for 320 min; at which times the kinetics of the absorption change were determined. The algae were dark-adapted for 5 min prior to the measurement. % T is the percentage change in transmission.

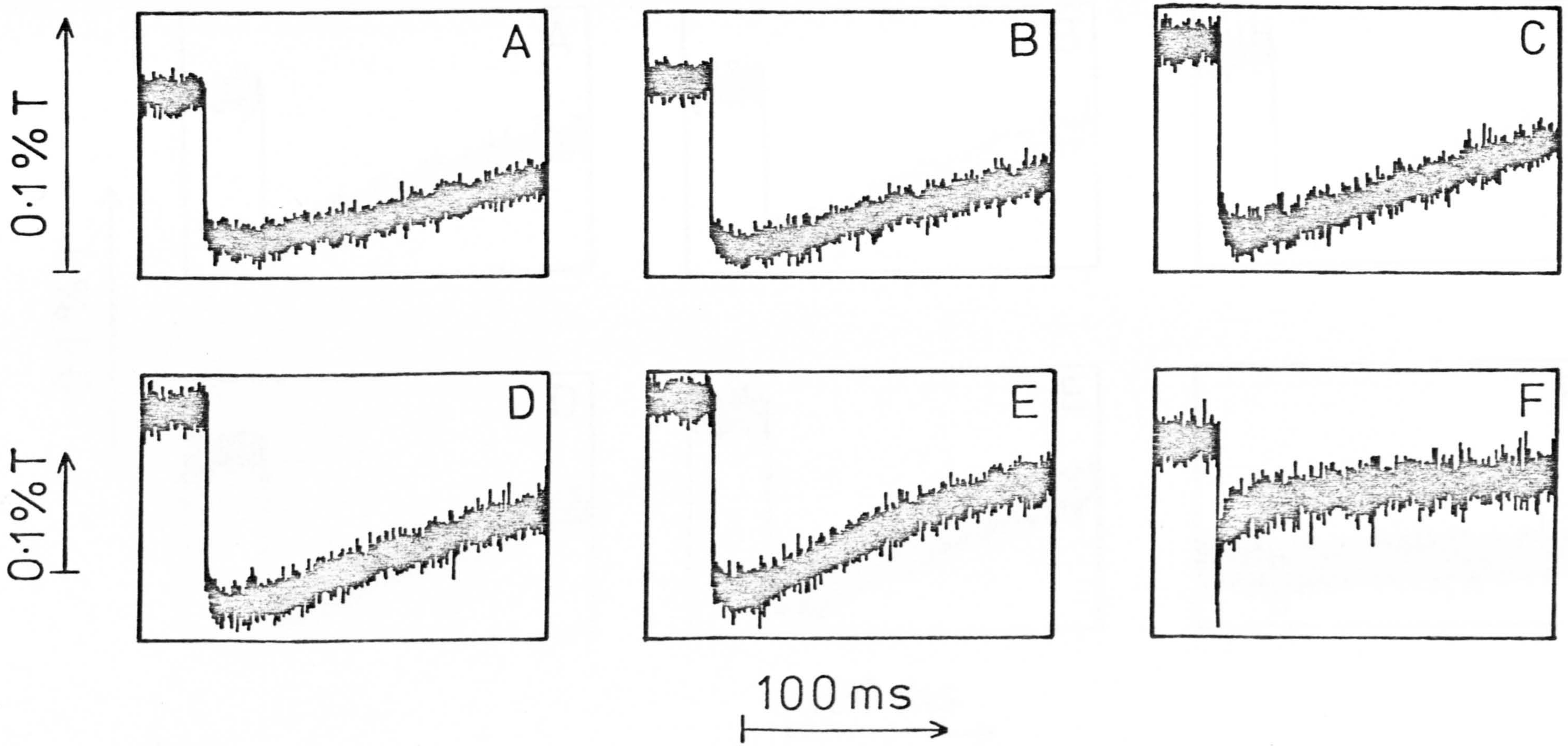

FIGURE 78. The effect of decreasing the external NaCl concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.34 M NaCl (0.6 Os kg^{-1}); C: 0.26 M NaCl (0.5 Os kg^{-1}); D: 0.17 M NaCl (0.3 Os kg^{-1}); E: 0.09 M NaCl (0.2 Os kg^{-1}); F: distilled water (0 Os kg^{-1}). % T is the percentage change in transmission; the upper value refers to Traces A to D, the lower value refers to Traces E and F which were determined on a different day.

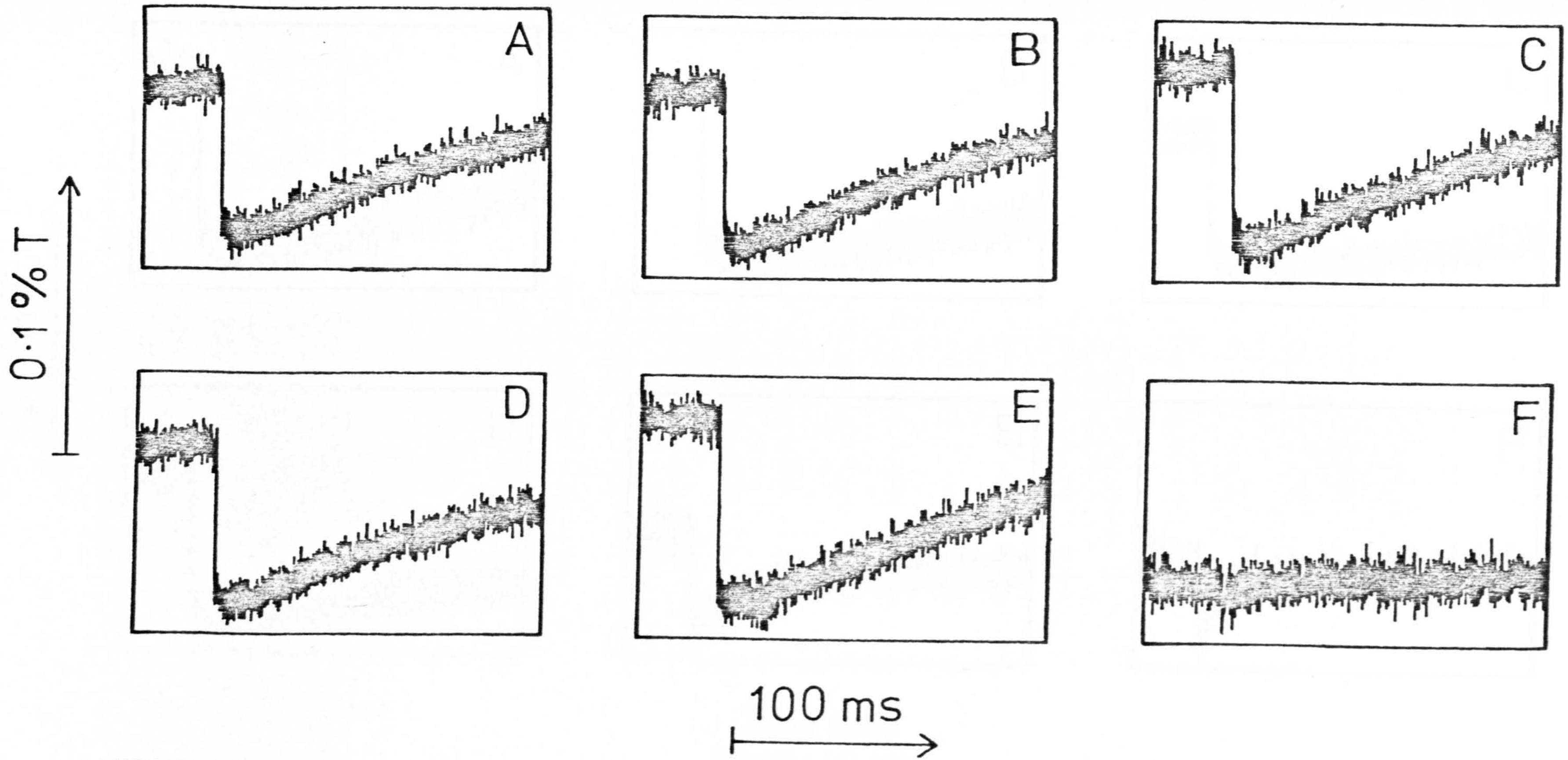

FIGURE 79. The effect of decreasing the external NaCl concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after 100 min at A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.34 M NaCl (0.6 Os kg^{-1}); C: 0.26 M NaCl (0.5 Os kg^{-1}); D: 0.17 M NaCl (0.3 Os kg^{-1}); E: 0.09 M NaCl (0.2 Os kg^{-1}); F: distilled water (0 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement. % T is the percentage change in transmission.

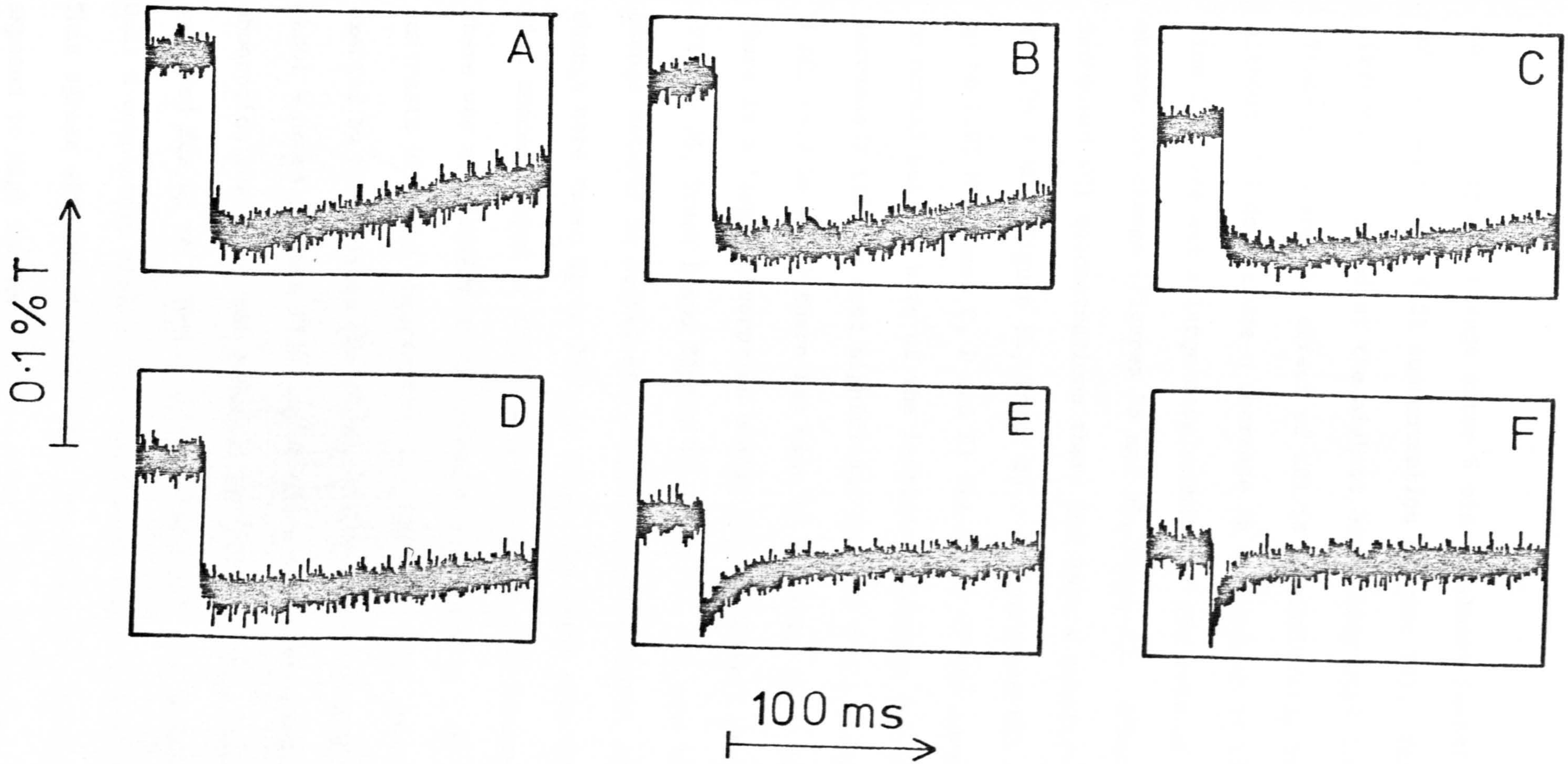

FIGURE 80. The effect of increasing the external KCl concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.26 M KCl (1.3 Os kg^{-1}); C: 0.51 M KCl (1.7 Os kg^{-1}); D: 0.79 M KCl (2.2 Os kg^{-1}); E: 1.03 M KCl (2.7 Os kg^{-1}); F: 1.32 M KCl (3.2 Os kg^{-1}). % T is the percentage change in transmission.

on the absorption change after 5 min is almost identical to the effect of increasing the NaCl concentration (Figure 75). The only major difference is found at the highest osmolality used i.e. 3.2 Os kg^{-1} (Trace F), where the effect of KCl is significantly less than NaCl, although KCl does cause a decrease in the extent of the initial absorption increase and a large acceleration of the rate of decay of the absorption change (Figures 75 and 80, Trace F). After 100 min at increased KCl concentrations there has been a complete recovery only at 0.26 M KCl (Figure 81, Trace B). At increased KCl concentrations up to 1.03 M (Traces C, D and E) the shape of the curve has returned to normal but the size of the initial absorption change is still decreased; this is most significant at 1.03 M KCl (Trace E). At 1.32 M KCl (3.2 Os kg^{-1}) there has been no recovery after 100 min, although there is a larger absorption change than with NaCl at the same stage (Figure 76, Trace D and Figure 81, Trace F). To see if the absorption change returns to normal at 1.32 M KCl, measurements of the absorption change were taken up to 330 min after the algae were exposed to this high concentration of KCl. Figure 82 shows that during this period there was no recovery of the absorption change at 1.32 M KCl. This contrasts with the observation that the same high external osmolality exerted by NaCl allows the absorption change to return to normal after about 4 hours (Figure 77). Experiments were also performed where the absorption change in the presence of increased KCl concentrations was measured for up to 3 days. It was found that KCl concentrations above 0.51 M completely abolish the absorption change within the 3 day period. This agrees with observations that the cells die within this period if exposed to high concentrations of KCl (data not shown).

The effect on the absorption change of exerting an osmotic stress

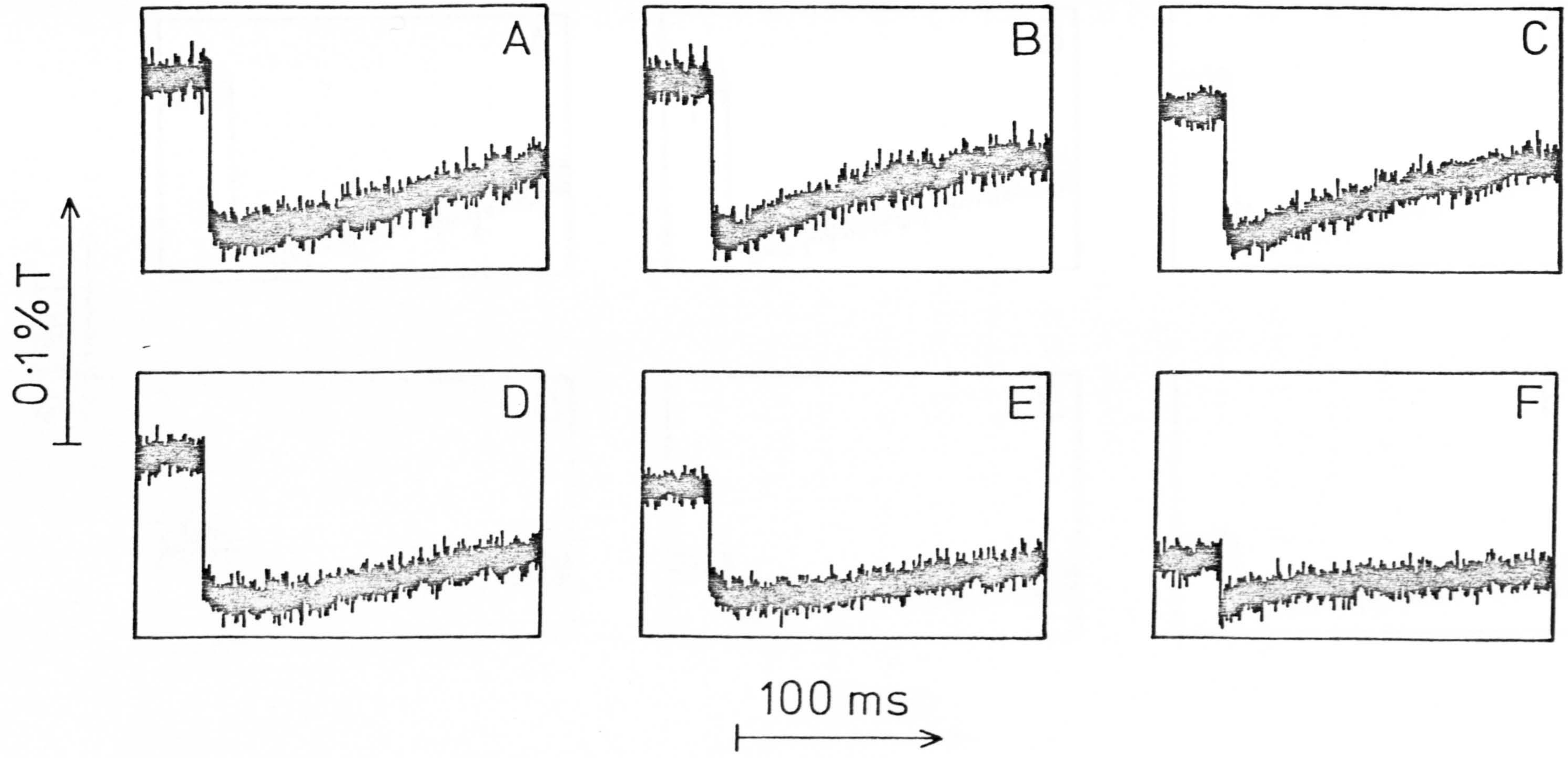

FIGURE 81. The effect of increasing the external KCl concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after 100 min at 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.26 M KCl (1.3 Os kg^{-1}); C: 0.51 M KCl (1.7 Os kg^{-1}); D: 0.79 M KCl (2.2 Os kg^{-1}); E: 1.03 M KCl (2.7 Os kg^{-1}); F: 1.32 M KCl (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement. % T is the percentage change in transmission.

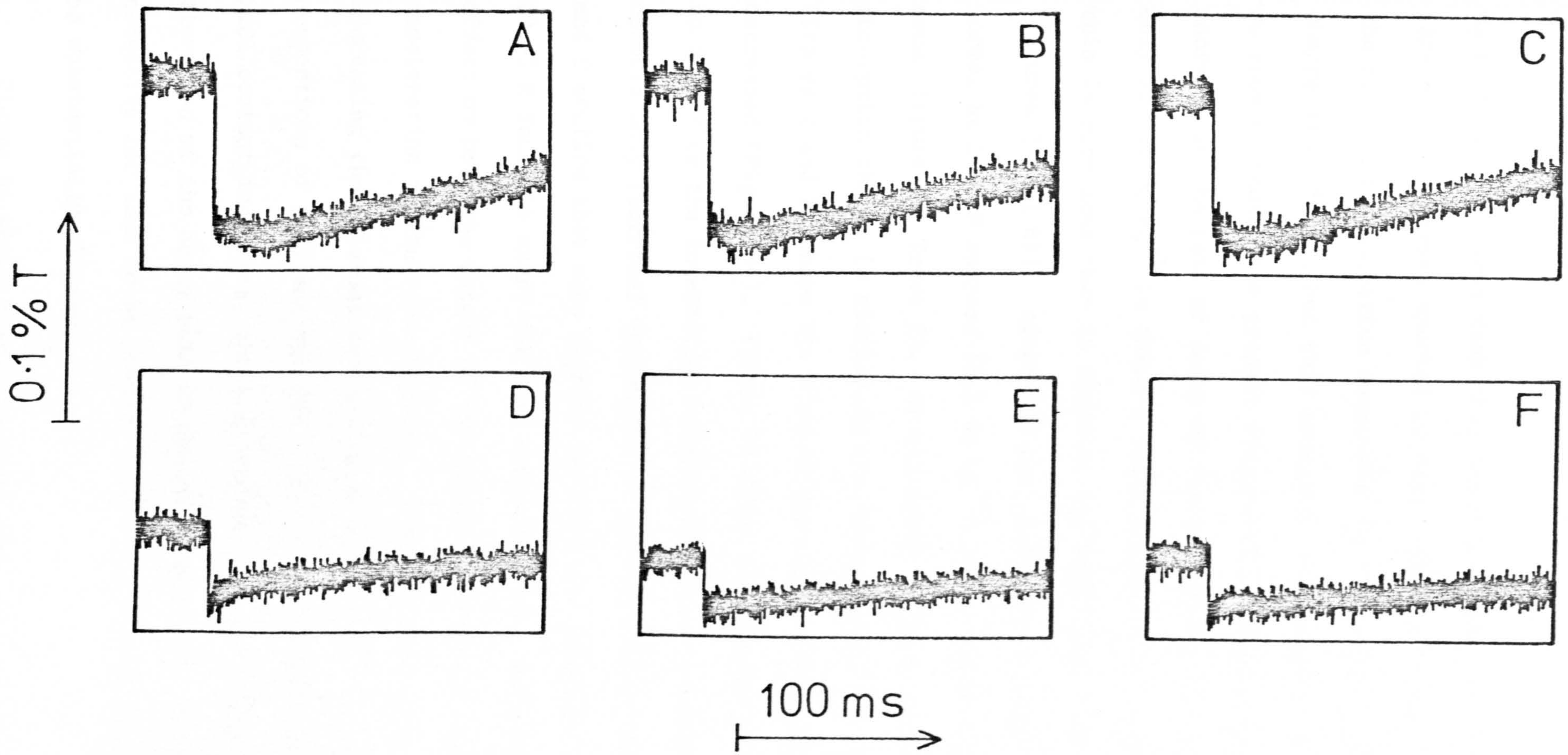

FIGURE 82. The long term effect of increasing the external KCl concentration on the field-indicating 519 nm absorption change. The algae were resuspended in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control for 90 min; B: no addition for 230 min; C: no addition for 280 min; D: 1.32 M KCl (3.2 Os kg^{-1}) for 120 min; E: 1.32 M KCl for 260 min; F: 1.32 M KCl for 330 min; at which times the kinetics of the absorption change were determined. The algae were dark-adapted for 5 min prior to measurement. % T is the percentage change in transmission.

using sucrose is less than that induced by the same osmolality of ionic (and osmotic) stress exerted by NaCl (Figures 75 and 83). Increasing the sucrose concentration decreases the size of the initial absorption change after 5 min, but this effect is not proportional to the external sucrose concentration present (Figure 83). Significantly there is little increase in the rate of decay of the absorption change induced by sucrose; only at 3.2 Os kg^{-1} is there a small acceleration of the decay rate, but this is much less than is found at the same osmolality exerted by NaCl (Figures 75 and 83). After 100 min, the rate of decay has returned to normal at 1.38 M sucrose (3.2 Os kg^{-1}) and a larger slow phase can be seen (Figure 84, Trace F). At all other sucrose concentrations the absorption change is similar to the control except at 0.42 and 0.97 M (Traces B and D) where the size of the rapid absorption change is decreased (Figure 84). Figure 85 shows the results of an experiment to determine the interaction between the ionic and osmotic (NaCl) and osmotic only (sucrose) components of the osmolality increase. Traces B and C confirm that when sucrose and NaCl are added to the basic medium (0.43 M NaCl) to exert the same osmolality then NaCl has a much larger effect on both the extent of the initial absorption increase and on the acceleration of the rate of decay. Traces D to F show the effect of increasing the external osmolality to 3.2 Os kg^{-1} using different proportions of NaCl and sucrose. It can be clearly seen that as the NaCl content increases, the size of the initial change decreases and the acceleration of the decay rate increases (Figure 85). This illustrates the necessity for ions to be present before the 519 nm absorption change can be substantially altered.

Figure 86 shows that increasing the external concentration of ethylene glycol has almost no effect on the absorption change after

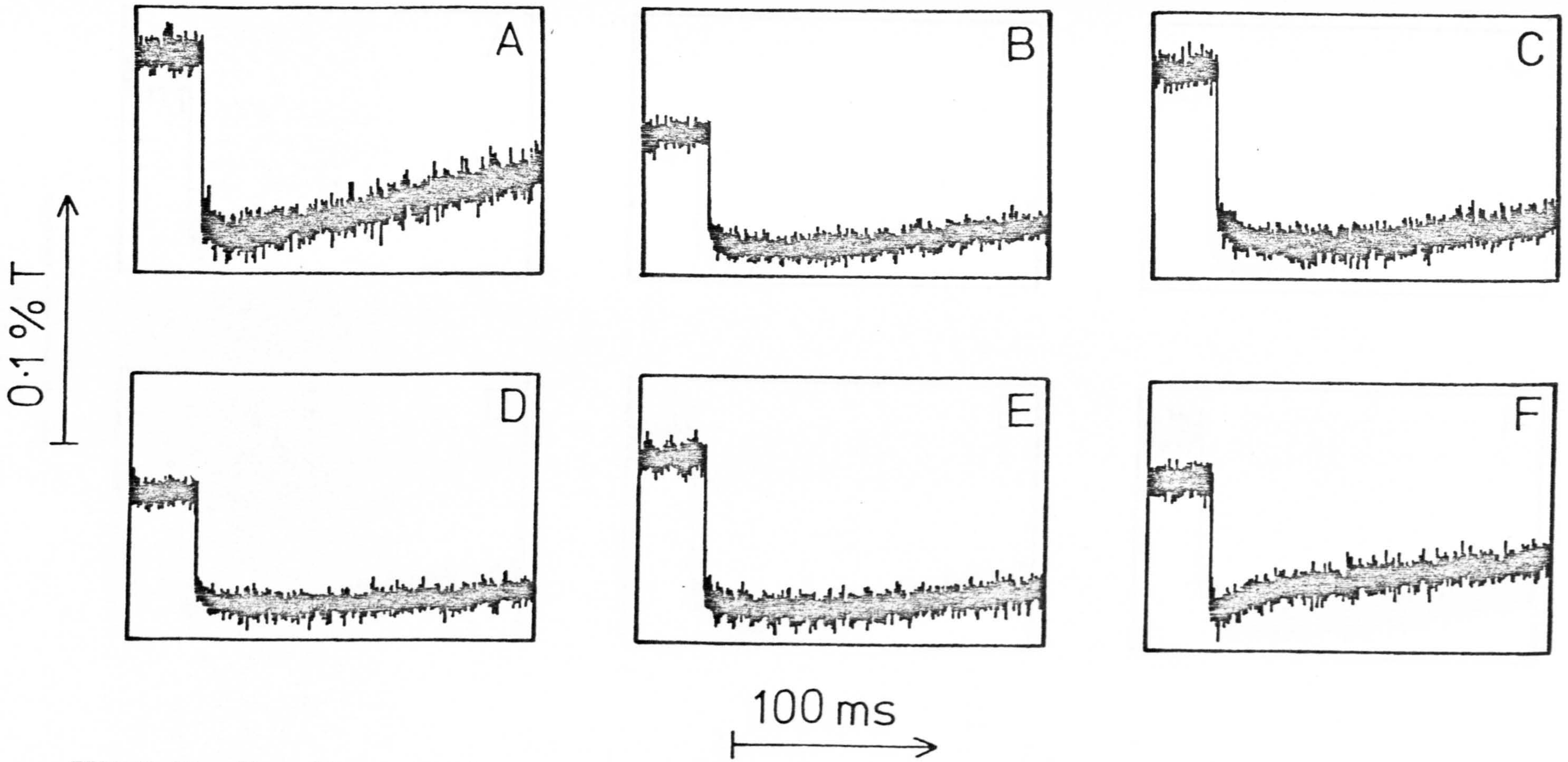

FIGURE 83. The effect of increasing the external sucrose concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.42 M sucrose (1.3 Os kg^{-1}); C: 0.72 M sucrose (1.7 Os kg^{-1}); D: 0.97 M sucrose (2.2 Os kg^{-1}); E: 1.18 M sucrose (2.6 Os kg^{-1}); F: 1.38 M sucrose (3.2 Os kg^{-1}). % T is the percentage change in transmission.

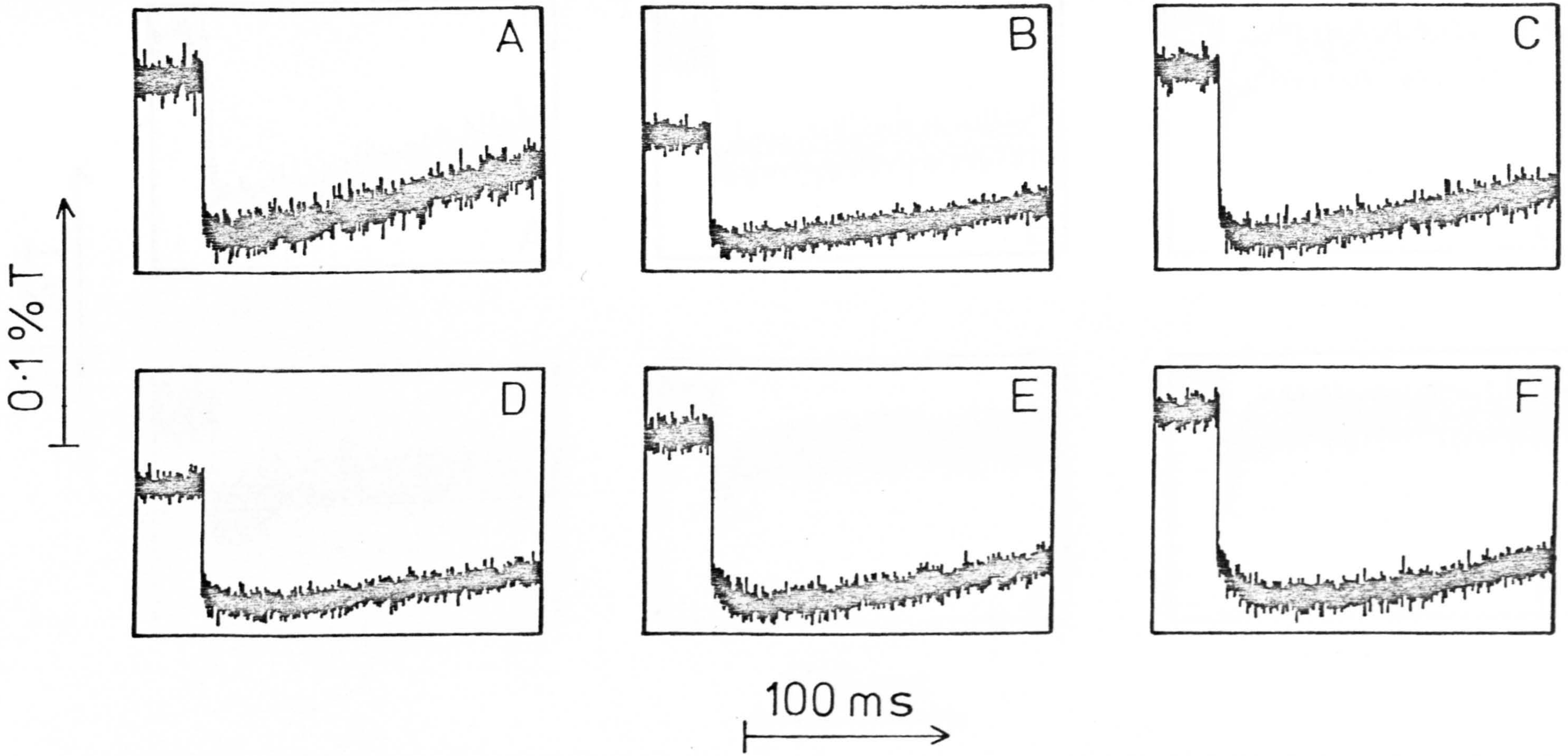

FIGURE 84. The effect of increasing the external sucrose concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after 100 min at 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.42 M sucrose (1.3 Os kg^{-1}); C: 0.72 M sucrose (1.7 Os kg^{-1}); D: 0.97 M sucrose (2.2 Os kg^{-1}); E: 1.18 M sucrose (2.6 Os kg^{-1}); F: 1.38 M sucrose (3.2 Os kg^{-1}). The algae were dark-adapted for 5 min prior to measurement. % T is the percentage change in transmission.

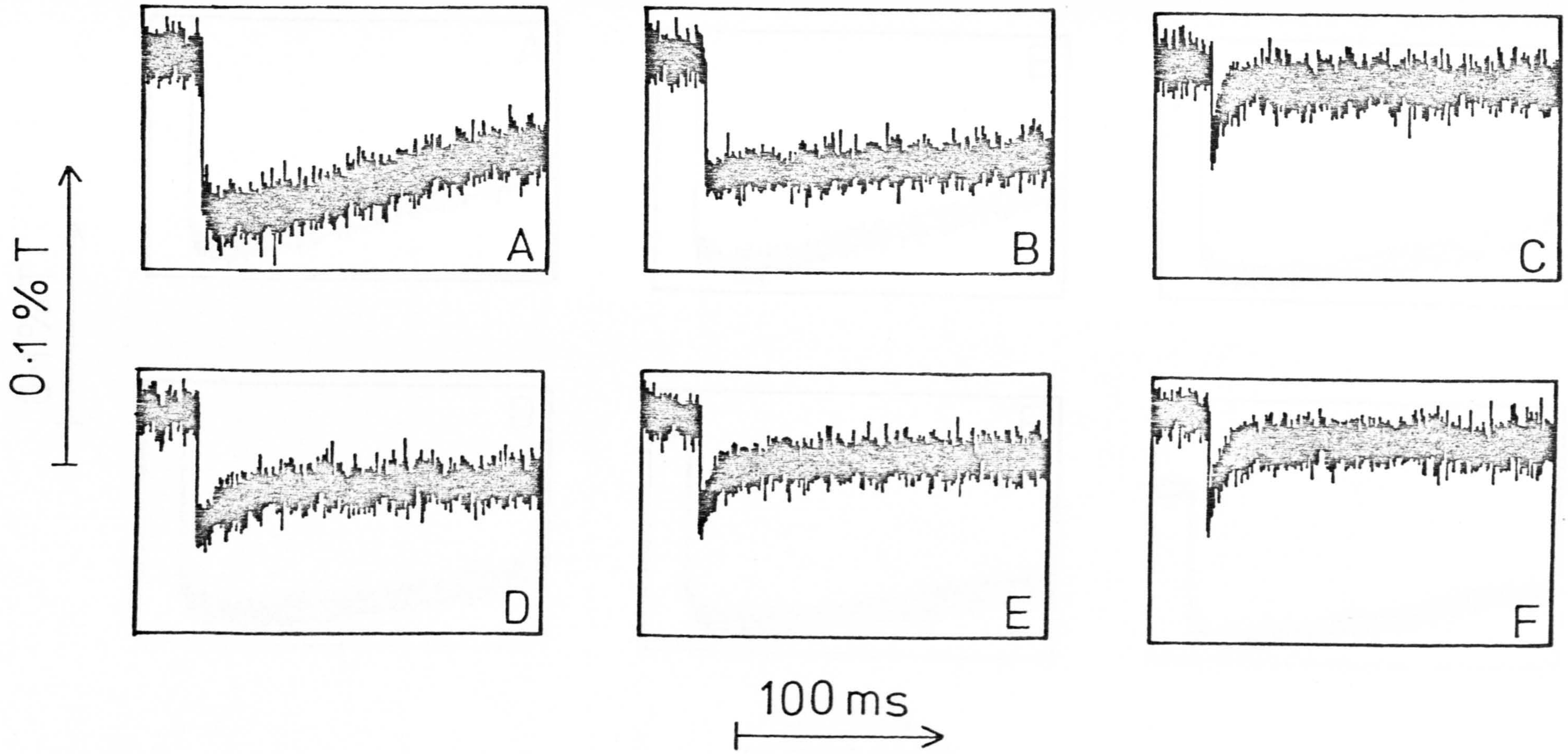

FIGURE 85. The effect of different mixtures of NaCl and sucrose on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 1.38 M sucrose (3.2 Os kg^{-1}); C: 1.28 M NaCl (3.2 Os kg^{-1}); D: 0.25 M NaCl and 1.18 M sucrose (3.2 Os kg^{-1}); E: 0.51 M NaCl and 0.97 M sucrose (3.2 Os kg^{-1}); F: 0.77 M NaCl and 0.72 M sucrose (3.2 Os kg^{-1}). % T is the percentage change in transmission.

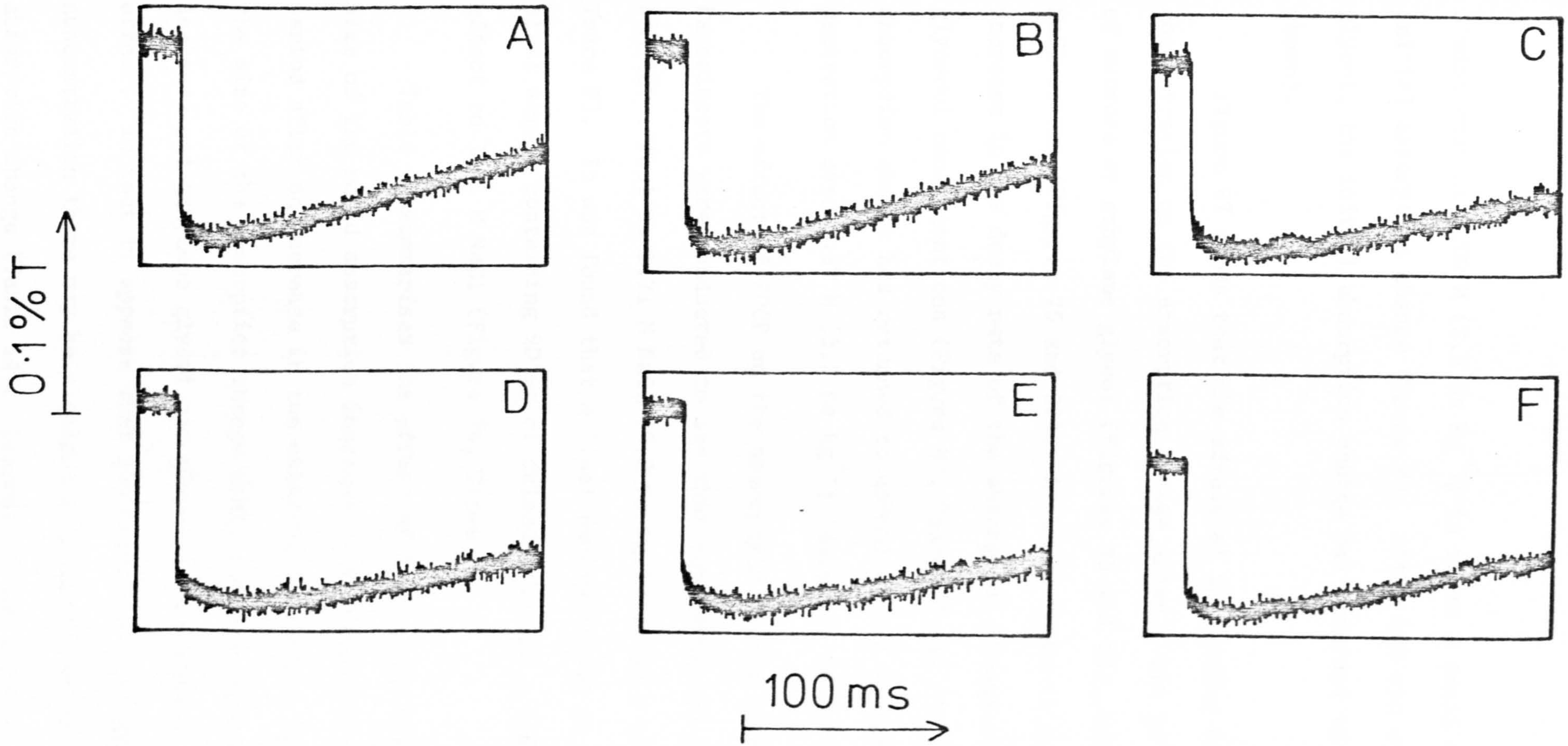

FIGURE 86. The effect of increasing the external ethylene glycol concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.46 M ethylene glycol (1.3 Os kg^{-1}); C: 0.88 M ethylene glycol (1.8 Os kg^{-1}); D: 1.30 M ethylene glycol (2.2 Os kg^{-1}); E: 1.73 M ethylene glycol (2.7 Os kg^{-1}); F: 2.09 M ethylene glycol (3.2 Os kg^{-1}). % T is the percentage change in transmission.

5 min; only at 2.09 M (3.2 Os kg^{-1}) is there a small decrease in the initial absorption change (Trace F). After 100 min at 2.09 M ethylene glycol, the initial absorption change has returned to normal (data not shown).

Figure 87 shows that the effect of increasing the external glycerol concentration on the absorption change after 5 min is greater than that of sucrose or ethylene glycol (Figures 83 and 86), but less than that of NaCl or KCl (Figures 75 and 80). Although there is a substantial increase in the decay rate of the absorption change at high external glycerol concentrations (Figure 87, Traces E and F), after 100 min the absorption change has returned to normal at the highest glycerol concentration used 1.92 M (3.2 Os kg^{-1}) (data not shown).

The effect of FCCP on the absorption change is shown in Figure 74. Experiments were conducted to see what concentration of FCCP had a similar effect to 1.71 M NaCl (3.2 Os kg^{-1}) after 5 min (Figure 75, Trace F). It was found that a final concentration of 65 µM FCCP in an algal sample containing 90 µg of chlorophyll had a nearly identical effect to 1.71 M NaCl (Figure 74, Trace B).

Table 12 summarises the effect of increasing the osmolality on the size of the rapid absorption increase. It shows that within the 100 min period after an increase in the external osmolality NaCl and KCl decrease the size of the absorption change most, sucrose induces a moderate decrease and ethylene glycol and glycerol have almost no inhibitory effect. In fact it appears that 100 min after increasing the glycerol concentration there may be a slight stimulation of the size of the absorption change (Table 12). Decreasing the salinity (except to distilled water) causes a stimulation of the initial absorption change

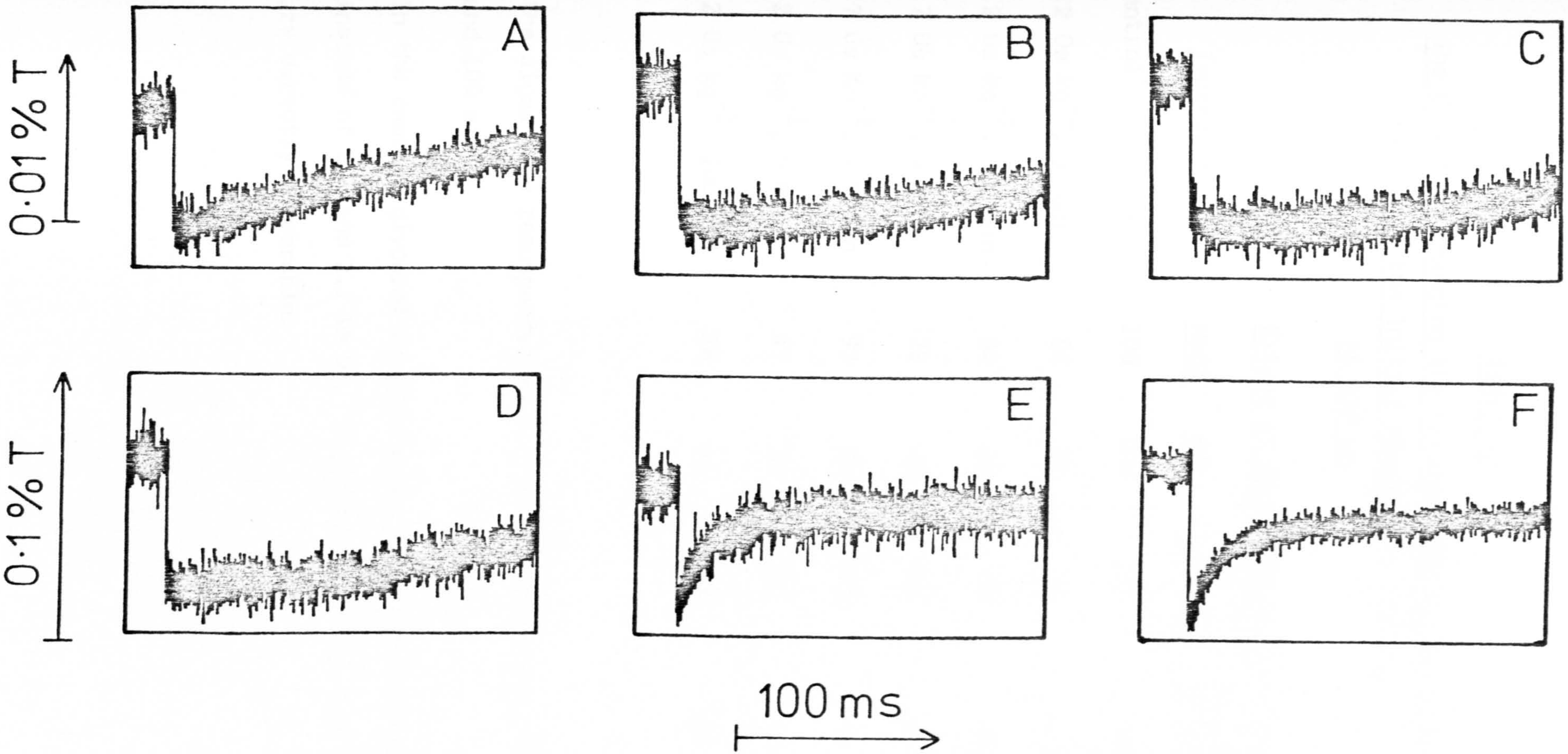

FIGURE 87. The effect of increasing the external glycerol concentration on the field-indicating 519 nm absorption change. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in 0.43 M NaCl plus A: no addition (0.8 Os kg^{-1}), control; B: 0.71 M glycerol (1.5 Os kg^{-1}); C: 1.06 M glycerol (2.0 Os kg^{-1}); D: 1.42 M glycerol (2.4 Os kg^{-1}); E: 1.77 M glycerol (3.0 Os kg^{-1}); F: 1.92 M glycerol (3.2 Os kg^{-1}). % T is the percentage change in transmission; the upper value refers to Traces A to E, the lower value refers to Trace F which was determined on a different day.

TABLE 12

The Effect of Changing the External Osmolality on the Extent of the Initial Absorption Increase at 519 nm

Treatment	Extent of Rapid Absorption Increase (% Control)				
	NaCl	KCl	Sucrose	Ethylene glycol	Glycerol*
Control+	100	100	100	100	100
2.2 Os kg^{-1}, 5 min	86	72	56	100	90
2.2 Os kg^{-1}, 100 min	94	88	74	99	/
2.7 Os kg^{-1}, 5 min	78	64	80	93	93
2.7 Os kg^{-1}, 100 min	99	68	100	97	134
3.2 Os kg^{-1}, 5 min	47	54	76	76	93
3.2 Os kg^{-1}, 100 min	28	41	83	105	104

+ The algae were resuspended in 0.43 M NaCl (0.8 Os kg^{-1}) for 5 and 100 min.

* In the case of glycerol an osmolality of 2.4 and 3.0 Os kg^{-1} was exerted instead of 2.2 and 2.7 Os kg^{-1} respectively. The 3.2 Os kg^{-1} results are directly comparable.

after 5 and 100 min. At distilled water the absorption change is decreased especially after 100 min (Figures 78 and 79; data not shown).

6.3 Effect of Changing the External Osmolality on the Absorption Change While PSII is Chemically Blocked

The 519 nm absorption change can be used as an indirect measure of the rate of photochemistry at PSI. To do this PSII must be blocked, this can be achieved by adding DCMU and hydroxylamine to the algae and illuminating them for 5 min (Bennoun and Joliot, 1969; Bennoun, 1970; Joliot and Delosme, 1974). The experimental procedure was to add DCMU and hydroxylamine to a 3 ml algal sample (containing 90 µg of chlorophyll) to final concentrations of 10 µM and 100 µM respectively. The algae were then illuminated for 5 min at a light intensity of 8.6 W m^{-2} and finally dark-adapted for 5 min before the measurement of the absorption change (section 2.11).

The efficiency of the blocking of PSII was determined by measuring the chlorophyll fluorescence of the algae in the presence of 10 µM DCMU and in the presence of 10 µM DCMU and 100 µM hydroxylamine. The samples were pre-illuminated for 5 min at 8.6 W m^{-2} in the latter case (section 2.10). The area above the fluorescence curve was compared in the two treatments (Figure 40) and the inhibition of PSII was estimated to be 36% $\pm$ 3.8 (8 determinations). The decrease in the extent of the rapid absorption change induced by DCMU and hydroxylamine was found to be 37% $\pm$ 4.6 (6 determinations). Figure 88 illustrates the decrease in the absorption change caused by the inhibitors at 0.43 M NaCl (Traces A and B). Due to the small size of the signal, 128 signal sweeps were summed for each sample (section 2.11). Figure 88 also shows that the effect of increasing the external NaCl concentration on the rate of decay of

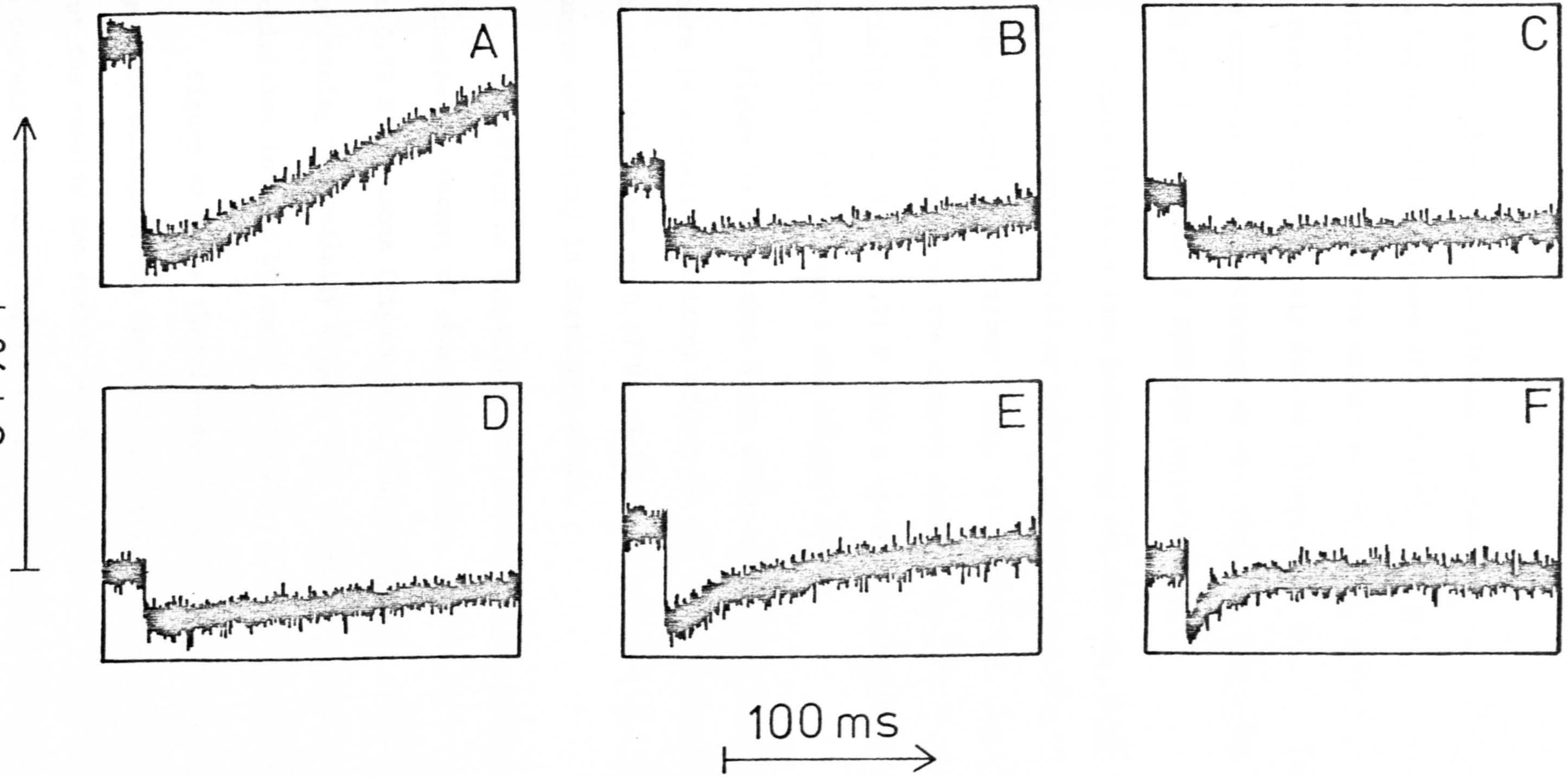

FIGURE 88. The effect of increasing the external NaCl concentration on the field-indicating 519 nm absorption change in the presence of DCMU and hydroxylamine. The kinetics of the absorption change were determined after the algae were resuspended for 5 min (in the dark) in A: 0.43 M NaCl (0.8 Os kg^{-1}), control; B: 0.43 M NaCl (0.8 Os kg^{-1}) plus 10 μM DCMU and 100 μM hydroxylamine; C: 0.68 M NaCl (1.7 Os kg^{-1}) plus DCMU and hydroxylamine; E: 1.45 M NaCl (2.7 Os kg^{-1}) plus DCMU and hydroxylamine; F: 1.71 M NaCl (3.2 Os kg^{-1}) plus DCMU and hydroxylamine. % T is the percentage change in transmission.

the absorption change is similar to that found in the absence of DCMU and hydroxylamine (Figure 75). For this reason , the results in this section refer only to the extent of the rapid absorption change which is taken as being largely due to charge separation at PSI. The results are expressed as a percentage of the control value found at 0.43 M NaCl in the presence of DCMU and hydroxylamine.

Figure 89 shows that increasing the external NaCl concentration by a small amount to 0.68 or 0.94 M causes a decrease in the absorption change induced by PSI after 5 min. At 1.20 M NaCl the absorption change has almost returned to the control value. Further increases in the salinity to 1.45 and 1.71 M have a small stimulatory effect on charge separation at PSI after 5 min (Figure 89).

Figure 90 shows that 5 min after decreasing the external salinity, there is a small inhibitory effect on the PSI-induced absorption change at salinities down to 0.17 M. Below this salinity the inhibition is larger especially in distilled water.

The effect of increasing the external KCl concentration is to increase the amount of charge separation at PSI after 5 min, especially at 0.79 M and above (Figure 91). In contrast to the effect of increasing the salinity (Figure 89), there is only a small amount of inhibition induced by small increases in KCl concentration (Figure 91).

Figure 92 shows that increasing the external sucrose concentration tends to stimulate the absorption change induced by PSI after 5 min, but the results are rather variable. There is however no evidence of a decrease in charge separation at PSI induced by increased concentrations of sucrose.

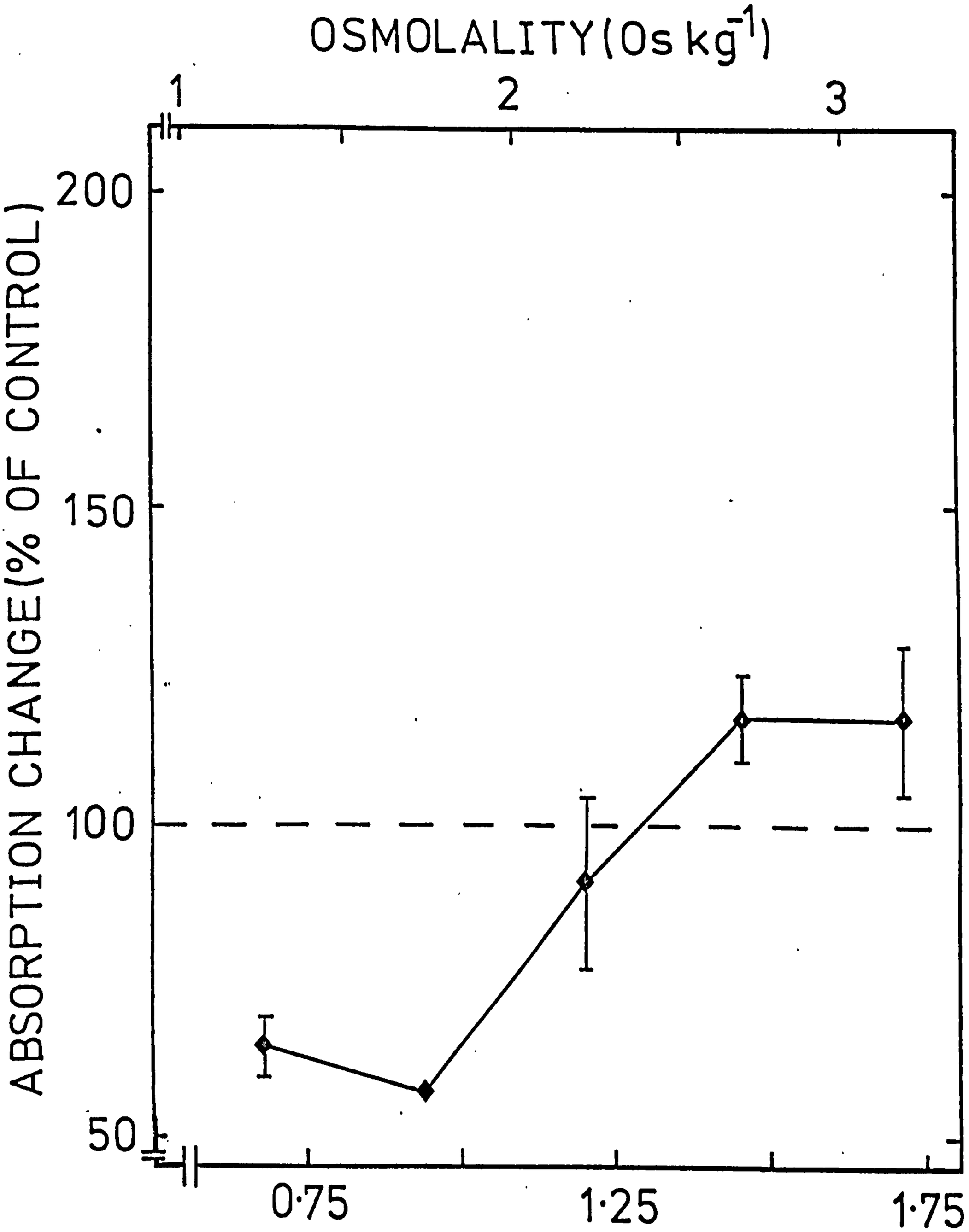

FIGURE 89. The effect of increasing the external NaCl concentration on the extent of the initial 519 nm absorption change while PSII was chemically blocked. The algae were resuspended in the salinities indicated for 10 min prior to measurement. The change in absorption in the control (0.43 M NaCl, 0.8 Os kg^{-1}) Δ I/I = 6.9 x 10^{-5} $\pm$ 1.4 x 10^{-6}. Each point represents the mean of three determinations.

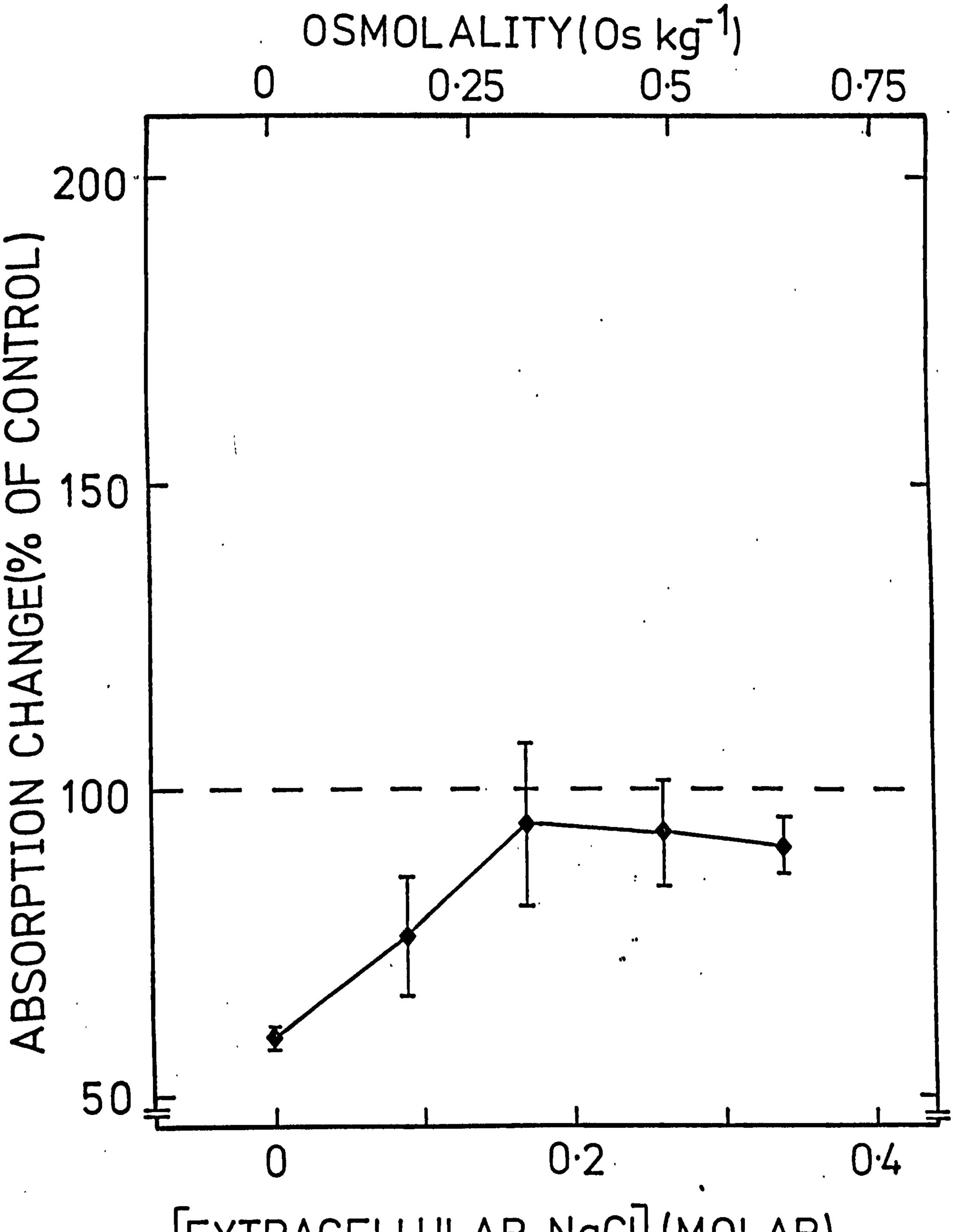

FIGURE 90. The effect of decreasing the external NaCl concentration on the extent of the initial 519 nm absorption change while PSII was chemically blocked. The algae were resuspended in the salinities indicated for 10 min prior to measurement. The change in absorption in the control (0.43 M NaCl, 0.8 Os kg^{-1}) $\Delta I/I = 6.9 \times 10^{-5} \pm 1.4 \times 10^{-6}$. Each point represents the mean of three determinations.

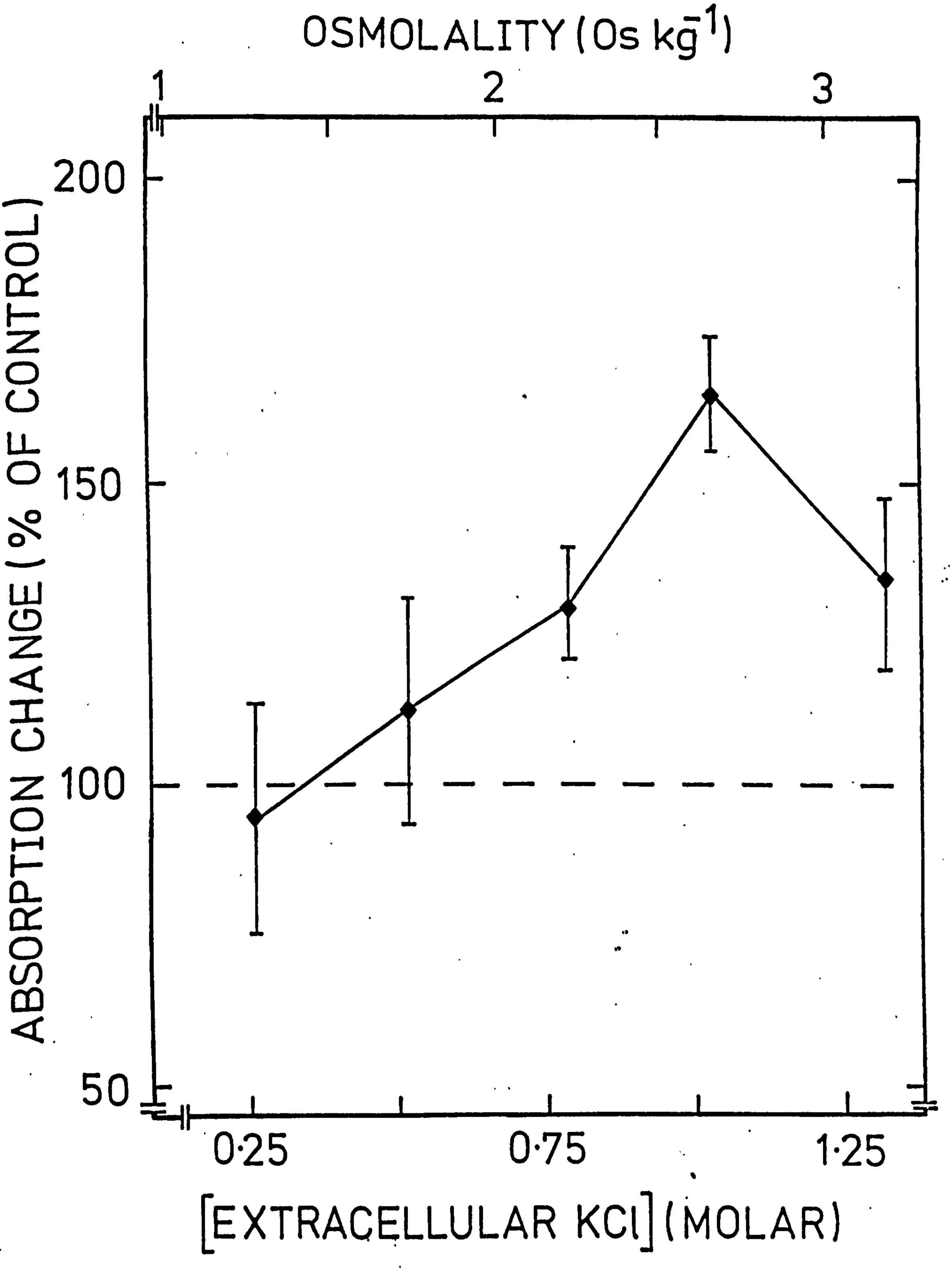

FIGURE 91. The effect of increasing the external KCl concentration on the extent of the initial 519 nm absorption change while PSII was chemically blocked. The algae were resuspended in 0.43 M NaCl plus the concentration of KCl indicated for 10 min prior to measurement. The change in absorption in the control (0.43 M NaCl, 0.8 Os kg^{-1}) $\Delta I/I = 6.1 \times 10^{-5} \pm 6.7 \times 10^{-6}$. Each point represents the mean of three determinations.

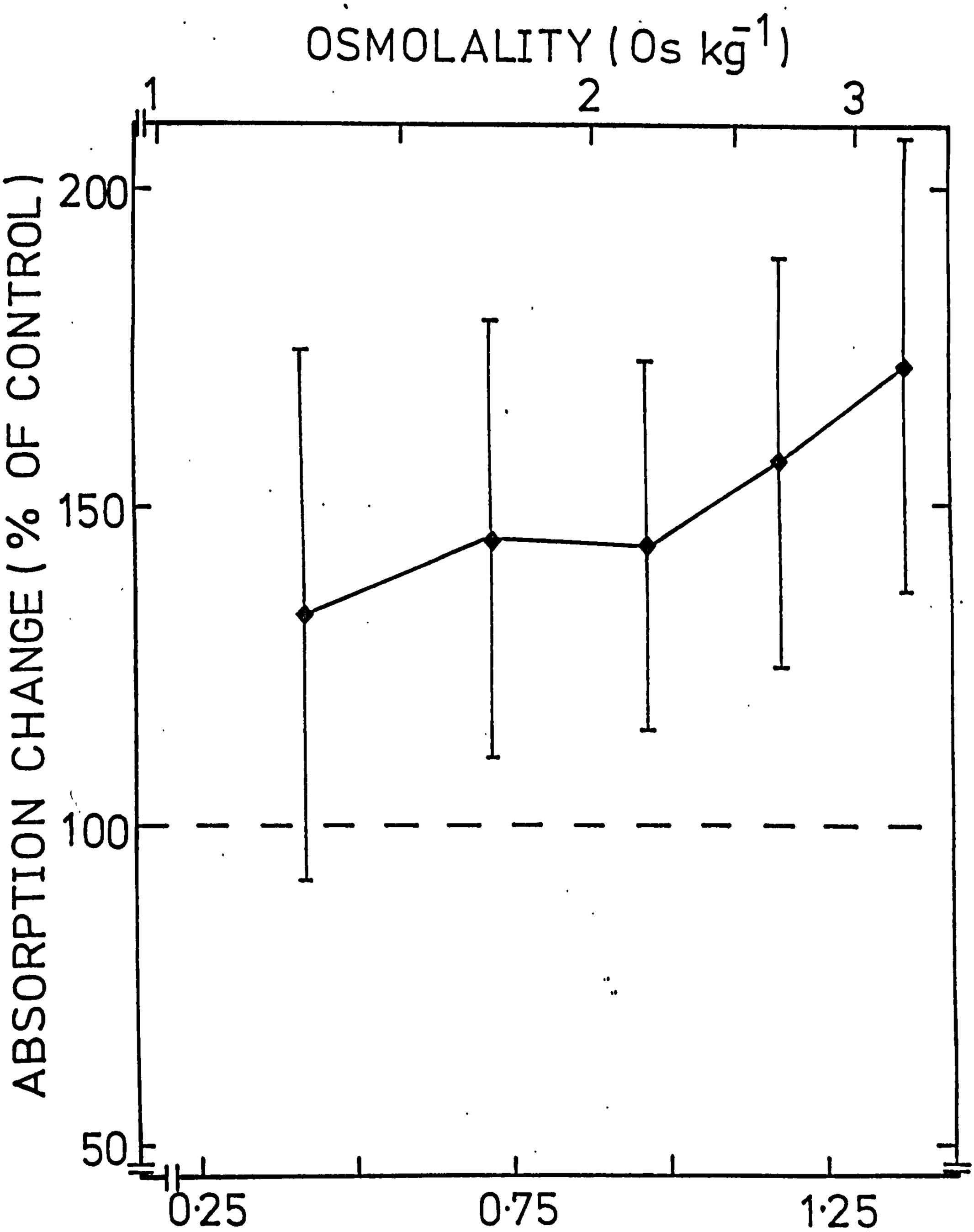

FIGURE 92. The effect of increasing the external sucrose concentration on the extent of the initial 519 nm absorption change while PSII was chemically blocked. The algae were resuspended in 0.43 M NaCl plus the concentration of sucrose indicated for 10 min prior to measurement. The change in absorption in the control (0.43 M NaCl, 0.8 Os kg^{-1}) $\Delta I/I = 4.5 \times 10^{-5} \pm 8.0 \times 10^{-6}$. Each point represents the mean of three determinations.

6.4 *Discussion*

The 519 nm absorption change has been used to investigate the effect of increasing the external osmolality on *D. tertiolecta*. In section 6.1, the three main features of the absorption change were related to photosynthetic processes as follows: (a) the large absorption increase which takes place rapidly reflects the separation of charge at the reaction centres of PSI and PSII, (b) the slow phase absorption increase associated with PSI may reflect the presence of a third electron transfer step across the thylakoid membrane, and (c) a relatively slow absorption decrease, the rate of which indicates the ion flux through the thylakoid membrane (Joliot and Delosme, 1974; Junge, 1977).

The results shown in section 6.2 indicate that osmotica like NaCl or KCl which exert an ionic and osmotic stress decrease the amount of charge separation at the reaction centres and increase the net ion flux through the thylakoid membrane (Figures 75 and 80). However, osmotica such as sucrose, ethylene glycol and glycerol which exert only an osmotic stress do not affect these processes to the same extent (Figures 83, 86 and 87). Ethylene glycol can be considered separately, since it alone of the osmotica used does not induce glycerol synthesis (Figure 23). KCl and glycerol will also be considered later in the discussion to allow a direct comparison of the effects of NaCl and sucrose.

Figure 75 shows that increasing the NaCl content up to 1.20 M NaCl (2.2 Os kg^{-1}) does not increase the net ion flux through the thylakoid membrane (Traces B, C and D). However, when sucrose is added to these NaCl solutions to make the osmolality up to 3.2 Os kg^{-1}, then algae resuspended in these NaCl-sucrose solutions show an increased rate of ion flux (Figure 85, Traces D, E and F). However, sucrose added to the basic growth medium (0.43 M NaCl) cannot increase the ion flux through

the thylakoid membrane to any extent (Figure 85, Trace B and Figure 83). Therefore, it appears that the presence of ionic stress is necessary to increase the ion flux, but osmotic stress exerted by sucrose can also accelerate the ion flux providing that a minimum amount of NaCl (approximately 0.68 M) is present. However it is possible to argue that the small amount of increased ion flux found when sucrose is added to the growth medium (Figure 83, Trace F) is due to the 0.43 M NaCl already present. Thus, there may be no minimum cut-off point for the NaCl concentration, but above 0.68 M NaCl (in the presence of an overall 3.2 Os kg^{-1} stress) the ion flux is significant (Figure 85, Trace D). Therefore, it seems that the two components of increased osmolality i.e. osmotic and ionic stress, can interact to produce the increased ion flux.

The mechanism whereby the ion flux is increased could possibly be due to the effect of increased osmolality on the membrane-bound ATPase. It is possible to detach certain peripheral proteins from the thylakoid membrane which are involved with the coupling of phosphorylation to electron transport (Figure 6; McCarty, 1979). Removal of the coupling factor could account for the increased ion flux through the membrane, if this was induced by Na^+ ions then they need not enter the thylakoid to increase the ion flux. However, the interaction of ionic and osmotic stress suggests that it is the osmotic stress that affects the thylakoid membrane making it leaky to ions; this could be induced by either NaCl or sucrose. The reason for the lack of acceleration of the rate of decay induced by sucrose is that suitable ions to pass down their electrochemical gradient (and decrease the field) are not present. These ions are however present when the NaCl concentration is increased which implicates the Na^+ or Cl^- ions.

The necessity for the presence of ions suggests that the externally increased concentration of NaCl may be responsible for increased influx of Na^+ and Cl^- ions into the cell. Ginzburg (1981b) using D. parva investigated the fluxes of Na^+, K^+ and Cl^- between the cells and the medium when the external salinity was increased from 0.5 to 1.0 M NaCl. In response to the salinity increase the cell volume decreases due to the rapid efflux of water. After 2 min the concentration of Na^+ and Cl^- ions has increased and after allowances were made for the volume decrease it was found that there had been a large influx of $Na^+$and Cl^- into the cell. This influx of ions was not dependent on the cell metabolism since it occurred equally well at 277 and 298K (Ginzburg, 1981b). Similar results indicating a Na^+ influx into cells of D. parva after an increase in the external salinity were also found by Gimmler and Schirling (1978). Therefore, a similar Na^+ and Cl^- influx into D. tertiolecta could be responsible for the effects on the 519 nm absorption change. If this is to be true, the ions must cross both the plasmalemma of the alga and the chloroplast envelope. Also Figures 76 and 77 showed that the increase in ionic flux was only temporary and it recovers in a time that approximately equals that needed for glycerol synthesis to be complete i.e. 4 to 6 hours at 3.2 Os kg^{-1} (Figure 20). Thus it may be that as the glycerol is synthesised and the alga begins to regain its original volume, that the Na^+ and Cl^- ions are expelled from the cell. Indeed Ginzburg (1981b) found that after the increase to 1.0 M NaCl, the Na^+ and Cl^- content of the cell had returned to normal after 2-3 hours. At least in the case of Na^+ the removal appears to be an active process dependent on metabolic energy (Ginzburg, 1981a). Thus it seems possible that the synthesis of intracellular glycerol allows the alga to restore the ionic balance in the chloroplast.

The decrease in the charge separation induced by increased NaCl concentrations is larger than that induced by increased sucrose concentrations except for a rather anomalous result at 2.2 Os kg^{-1} (Table 12). These results confirm that increasing the external osmolality can decrease the efficiency of the primary photochemical reactions as tentatively suggested for PSII by the Fv/Fm chlorophyll fluorescence results (section 5.2). This suggests that part of the salt-induced inhibition of light-induced oxygen evolution occurs at the level of the primary processes.

The other osmotica used which can induce glycerol synthesis i.e. KCl and glycerol gave essentially similar results to NaCl and sucrose respectively. The main difference between the effect of NaCl and KCl was the lack of recovery of the absorption change when exposed to high KCl concentrations (Figure 82). This can also be connected to the findings of Ginzburg (1981b), since she observed that K^+ did not take part in the ionic fluxes after an increase in the external NaCl concentration. Thus it may be that when the external KCl concentration is increased, K^+ influx into the cell occurs which cannot be reversed by the metabolic processes used to remove Na^+. Gimmler and Schirling (1978) showed that increased external K^+ concentration does lead to a large influx of K^+ into the cells.

There were two main differences between the effect of sucrose and glycerol on the absorption change. Firstly, glycerol accelerated the rate of ion flux to a greater extent than sucrose (Figure 87), unfortunately the effect of combined solutions of NaCl and glycerol was not determined. Secondly, glycerol did not decrease the amount of charge separation to the same extent as sucrose (Table 12).

Increasing the external ethylene glycol concentration has almost

no effect on the absorption change (Figure 86). This is in agreement with its inability to induce glycerol synthesis (Figure 23). Figure 86 supports the hypothesis that ethylene glycol does not exert an osmotic stress on the algal cells.

Decreasing the external salinity stimulates the amount of charge separation at the reaction centres (Figure 78). It also induces a faster rate of decay, but this has different kinetics to the decay rate acceleration induced by increasing the external salinity (Figures 75 and 78). In the presence of decreased external salinities, the slow phase absorption increase is not lost except when the algae are exposed to distilled water.

The slow phase of the absorption increase is associated with PSI (Musto and Hipkins, 1981; section 6.1). The chlorophyll fluorescence results in Tables 10 and 11 (Chapter 5) suggested that the amount of light energy entering PSI may be increased immediately after the external NaCl content is increased to 1.71 M. However, there is no enhancement of the slow phase immediately after the NaCl concentration is increased to 1.71 M NaCl, instead there is a rapid decay of the absorption change (Figure 75, Trace F). This may be the result of the increased ion flux obscuring any increase in the slow phase which might be expected to be present reflecting the increased efficiency of PSI. Evidence to support this theory comes from the kinetics of the absorption change after the algae have become adapted to high salt (Figure 76, Trace C and Figure 77, Trace F). These traces show an increased amount of slow phase possibly indicating that the enhancement of PSI efficiency continues throughout the period of adaptation to salt stress. However, the fluorescence results shown in Tables10 and 11 indicate that the amount of light energy entering PSI has returned to normal by the end of about 4 to 5 hours.

In section 6.3 the effect of increasing the external osmolality on charge separation was measured while only PSI was photochemically active (Joliot and Delosme, 1974). The results confirmed that high external osmolalities exerted by NaCl, KCl and sucrose, increase the amount of photochemical activity associated with PSI (Figures 89, 91 and 92). This qualitatively agrees with the findings shown in Tables 10 and 11 (Chapter 5) obtained by measuring fluorescence emission. It also suggests that the decrease in charge separation induced by NaCl, KCl and sucrose when both photosystems are active is due to inhibition at PSII (Table 12). Figure 90 shows that no increase in the photochemical activity of PSI is induced by decreasing the external salinity. This is in agreement with earlier fluorescence experiments which indicated that the distribution of light energy between the photosystems was not affected by decreasing the salinity (Figure 66).

CHAPTER 7

CONCLUSIONS

7.1 Introduction

In section 1.1 it was stated that the subject of this thesis was the relationship between photosynthesis and osmoregulation in Dunaliella. It will have become apparent to the reader that the subject matter covered can be split into two parts. The first part concerned the basic osmoregulatory mechanism of the alga i.e. glycerol metabolism and its relationship to the major cellular processes of photosynthesis and respiration (Chapters 3 and 4). The major finding of this section of the investigation was that an increase or decrease in the external osmolality inhibited the rate of photosynthesis in Dunaliella during the period of glycerol adjustment. The second part of the project was instigated to try to determine the reasons for the decrease in photosynthesis, and it consisted of a detailed investigation of some of the photosynthetic parameters that can be measured in intact cells (Chapters 5 and 6).

In sections 7.2 and 7.3 of this brief concluding chapter, the important effects of increasing the salinity will be summarised for glycerol metabolism (section 7.2) and for photosynthesis (section 7.3). In section 7.4, a summary of the results of all the osmotica used will be presented along with a description of the major trends. Finally, some broad conclusions will be made, which reflect on the current theories of osmoregulation in Dunaliella (section 7.5).

7.2 Glycerol Metabolism

Since Craigie and McLachlan (1964) first demonstrated glycerol production by Dunaliella in response to an increase in the external salinity, a good deal of work has been carried out on the glycerol metabolism of Dunaliella. There are however some areas where contrasting results have been found. Ben-Amotz and Avron (1973a) using D. parva and Brown and Borowitzka (1979) using D. viridis and D. tertiolecta both state that the new intracellular glycerol level is reached within 90 min of the increase in salinity. However, the results shown in Chapter 3 (Figure 20) for D. tertiolecta indicate that glycerol synthesis occurs for about 4 hours after the salinity is increased. It is important to know the length of time needed for glycerol synthesis since the rate of cellular processes during this period of internal adjustment of glycerol will be of interest to the study of osmoregulation.

The results described in Chapter 4 (Figure 28) show very clearly that the photosynthetic rate is decreased by increased external salinities; the lower rate of photosynthesis continued for at least 100 min after the salinity was increased. Therefore, a major part of the glycerol production took place at a time when the photosynthetic rate was low. It is generally agreed that glycerol can be produced by Dunaliella in the dark (Ben-Amotz, 1975; Borowitzka, Kessly and Brown, 1977; Chapter 3, Figure 24), although Frank and Wegmann (1974) failed to observe glycerol synthesis in the dark. Thus, it was natural to suggest that the initial glycerol synthesis could come via the breakdown of starch, the storage product of the alga (Brown and Borowitzka, 1979). However, Wegmann (1981) has shown that a higher percentage of photosynthetically fixed ^{14}C enters the internal pool of glycerol 10 min after a salinity increase. The unknown factor is what proportion

of the initial glycerol synthesis is due to photosynthetic products and what proportion is due to the degradation of starch (section 4.3).

There are two additional factors in the relationship between photosynthesis and glycerol production. Firstly, photosynthesis can apparently be stimulated by moderate increases in salinity in halotolerant members of the genus Dunaliella i.e. D. parva and D. salina (Kaplan, Schreiber and Avron, 1980; Gimmler, Wiedemann and Moller, 1981). There is however no evidence for this process in the marine D. tertiolecta (Chapter 4, Figure 28).

The second point concerns the long term stimulation of photosynthesis in high salt concentrations which was first observed by Craigie and McLachlan (1964). More recently, Gimmler et. al., (1981) looked at this process in detail and suggested that it is due to Dunaliella switching over to a more efficient type of metabolism in high salt concentrations. Cells of D. parva adapted to increased external salinity have higher amounts of chlorophyll per cell and they have a low amount of starch compared to cells grown in lower salinities (Gimmler et. al.,1981). These observations elegantly illustrate how a halotolerant alga like D. parva can withstand prolonged periods at high salinity. Long term growth measurements were not carried out to any extent in the present work, so it is not known whether the marine D. tertiolecta may also show this higher photosynthetic efficiency at high salinity. In general, some of the interesting questions still to be answered about Dunaliella concern the possible differences between halotolerant and marine species (Brown and Borowitzka, 1979).

The controversial question of whether glycerol is lost to the medium or not after the algae are adapted to media of fairly high salinity (> 1.5 M NaCl) was discussed in section 3.4. Results presented

in Chapter 3 showed only a very small loss of glycerol to the medium during the period of glycerol production in response to an increase in salinity (Figure 20). Nevertheless, when extrapolated over much longer time scales, the small leakage may become significant. Thus, more information is needed on this subject to reconcile the opposing views (Enhuber and Gimmler, 1980; Ben-Amotz, 1982).

7.3 Photosynthesis

When the NaCl-induced inhibition of photosynthesis was observed (Chapter 4, Figure 28), it was decided to examine some photosynthetic parameters to pinpoint which part of the process was inhibited by increasing the external NaCl concentration (Chapters 5 and 6). However, the situation turned out to be more complex than first envisaged. It appears that the immediate effect of an increase in the salinity on photosynthesis is to increase the amount of excitation energy going to PSI; the unknown mechanism which increases the spillover of light energy to PSI can occur in the dark (Chapter 5, Tables 10 and 11; Chapter 6, Figure 89). The increase in external NaCl concentration also immediately slows down the rate of electron transport (sections 5.3.1 and 5.3.2) and increases the rate of ion flux through the thylakoid membrane (Chapter 6, Figure 75). The rate of the primary photochemical reactions at PSII are decreased (Chapter 5, Figure 42; Chapter 6, Figure 75) and there is a large increase in fluorescence quenching (Chapter 5, Figure 50).

It is clear therefore that the inhibition of one single process cannot account for the effect of increasing the external salinity on photosynthesis in D. tertiolecta. However, the results summarised above implicated a number of processes which are involved in the

NaCl-induced inhibition of photosynthesis. First of all, the increased spillover of light energy to PSI could upset the balance of energy entering the photosystems and decrease the efficiency of photosynthetic electron transport from H_2O to $NADP^+$ (Chow et. al., 1981). There is also evidence that the primary electron acceptor of PSII, Q, remains more reduced when the algae are exposed to high NaCl concentrations (Chapter 5, Tables 8 and 9), which would not be expected if PSI was overexcited at the expense of PSII. The explanation appears to be that the rate of electron transport between the two photosystems is slowed down (independently of any possible decrease due to the unequal excitation of the photosystems), and this results in Q being mainly reduced. This could also explain why the internal conversion to heat at PSII increases, since although less light energy is entering PSII, Q remains mostly reduced, and as a consequence the majority of PSII reaction centres are closed and the excess light energy is lost by internal conversion (section 5.1). The excess light energy is not re-emitted as fluorescence, since the fluorescence level is quenched by high NaCl concentrations (Chapter 5, Figure 50).

The increase in internal conversion could explain the quenching of fluorescence, but another factor was found to be important in State 2 - adapted algae i.e. an increased level of the Δ pH gradient across the thylakoid membrane in response to salt stress (Chapter 5, Table 8; Krause et. al.,1982). However, in Chapter 6 it was found that there was an increase in the flux of ions through the thylakoid membrane induced by increased salinity (Figure 75). These observations involving the Δ pH gradient and the increased ion flux taken together suggest that ions other than H^+ are involved in the increased flux. This is in agreement with the interaction of NaCl and sucrose in

inducing the increased ion flux described in section 6.4. The suggestion is feasible since Gimmler and Schirling (1978) and Ginzburg (1981b) demonstrated Na^+ and Cl^- influx into D. parva when the external salinity was increased.

7.4 A Comparison of the Effects of the Other Osmotica Used

A brief summary of the results described in this thesis is given in Table 13. It shows that the results of increasing the KCl concentration are similar in the short-term (up to 100 min) to those found when the NaCl concentration is increased. However, in the long term (2 to 3 days), the cells die when exposed to high concentrations of KCl (Chapter 5, Figure 48). The toxicity of KCl seems to be due to at least two processes. The first is that although intracellular glycerol is synthesised in response to increased KCl concentration, the majority of it is lost to the external medium (Chapter 3, Figure 21B). The second process is based on the observation that increasing the KCl concentration causes increased ion flux through the thylakoid membrane (Chapter 6, Figure 80). By analogy with NaCl, it can be suggested that an influx into the cells of K^+ ions occurs; this has been demonstrated by Gimmler and Schirling (1978) for D. parva. Therefore, although excess intracellular Na^+ ions can be removed (Ginzburg, 1981b), perhaps K^+ ions cannot because the glycerol synthesised is lost to the medium and thus does not restore the intracellular ionic balance.

Increasing the external osmolality using sucrose which exerts only an osmotic stress has a similar, but smaller effect than that induced by increasing the concentration of NaCl or KCl, which exert both an ionic and osmotic stress (Table 13). The only salt-induced effects sucrose does not induce to any extent are the slowing of electron

TABLE 13

SUMMARY OF RESULTS

	Increasing NaCl	Increasing KCl	Increasing Sucrose	Increasing Ethylene glycol.	Increasing Glycerol	Decreasing NaCl*
Glycerol synthesis/ removal (Ch.3)	synthesis	synthesis, but lost to medium	synthesis	no synthesis	N.D.	removal
Volume changes (Coulter counter) (Ch.3)	no change	small decrease	small decrease	small increase	small decrease	N.D.
Photosynthesis (Ch.4)	inhibited (90%)	inhibited (90%)	inhibited (50%)	inhibited (50%)	inhibited (75%)	inhibited (55%)
Dark oxygen uptake (Ch.4)	inhibited (35%)	inhibited (50%) but variable	inhibited (40%)	inhibited, 5min(45%) stimulated, 100min(55%)	inhibited (35%) but variable	stimulated, 5min(55%) inhibited, 100min(15%)
Fv/Fm fluorescence (Ch.5)	inhibited (35%)	inhibited (35%)	inhibited (25%)	little effect	inhibited (15%)	no effect
Kautsky fluorescence (Ch.5)	slower electron transport + quenching	slower electron transport + quenching	quenching only	little effect	slower electron transport + quenching	quenching only
State 1 - State 2 fluorescence (Ch.5)	loss of light-induced spillover changes	loss of light-induced spillover changes	moderate loss of spillover changes	N.D.	N.D.	light-induced spillover changes present
F720/F695 and 77K fluorescence (Ch.5)	increased amount of light energy going to PSI	increased amount of light energy going to PSI	increased amount of light energy going to PSI	N.D.	N.D.	N.D.
519 nm absorption change (Ch.6)	decreased charge separation +increased ion flux	decreased charge separation +increased ion flux	decreased charge separation (variable)	little effect	increased ion flux	small increase in ion flux
Charge separation at PSI (Ch.6)	stimulation (15%)	stimulation (65%)	stimulation (70%) but variable	N.D.	N.D.	inhibition (25%)

N.D. - not determined

* Decreasing NaCl does not include results from resuspension in distilled water, which can be taken as inhibitory to all processes.

Figures in brackets represent the maximum percentage inhibition/stimulation during the 100 min period.

transport (Chapter 5, Figure 56) and the acceleration of ion flux (Chapter 6, Figure 83). Therefore, this may imply that these two effects are closely connected. The relationship between osmotic and ionic stress deserves further experimentation.

Increasing the ethylene glycol concentration does not induce glycerol synthesis and it has very little effect on any of the specific photosynthetic parameters measured (Table 13). Paradoxically, it does inhibit the overall process of photosynthesis, in a way which is not presently understood. Increasing the ethylene glycol concentration also slightly increased the cell volume of D. tertiolecta, underlining the fact that it has a fundamentally different effect from the other osmotica used (Chapter 3, Figure 25).

For obvious reasons, the effect of increasing the external glycerol concentration on intracellular glycerol production cannot be measured. However, the results summarised in Table 13 suggest that high external concentrations of glycerol would initiate intracellular glycerol production i.e. glycerol appears to be capable of exerting an osmotic stress. This is indirect evidence to support the suggestion that the cells of D. tertiolecta are relatively impermeable to glycerol.

Decreasing the external salinity inhibits photosynthesis in a similar fashion to the inhibition induced by increasing the salinity. However, in almost every other respect decreasing the salinity has a different effect to that of increasing the salinity (Table 13). One of the few effects that they both induce is quenching of fluorescence (Chapter 5, Figure 52). Therefore, the mechanism of the inhibition of photosynthesis at low salinities is different from that at high salinities.

7.5 Conclusions

The results suggest that D. tertiolecta synthesises intracellular glycerol in response to an increase in the external NaCl concentration. During the first 100 min after a salinity increase, photosynthesis is inhibited, which suggests that the production of glycerol by D. tertiolecta depends initially on the degradation of starch. The inhibition of photosynthesis appears to be due to a combination of an imbalance of light energy entering the photosystems, a decrease in electron transport, a loss of light energy at PSII due to internal conversion to heat and increased ion flux across the thylakoid membrane. Despite these problems the alga maintains its production of glycerol which is dependent on the availability of ATP (Kaplan et. al., 1980; Gimmler et. al., 1981). Therefore, future work should investigate exactly how Dunaliella retains metabolic energy to drive glycerol production while the cell metabolism is disrupted to the extent described in this thesis.

APPENDIX A

Composition of Enriched Seawater Medium (Boney's medium)

The following nutrient solutions were made up:-

Solution A

50 ml of 0.4% Na_2NO_3

2 ml of 1.47 g l^{-1} $MnSO_4:4H_2O$

2 ml of 0.0023 g l^{-1} $CuSO_4:5H_2O$

2 ml of 0.064 g l^{-1} $CoCl_2:6H_2O$

2 ml of 0.23 g l^{-1} $NaMoO_4:2H_2O$

Solution B

2 ml of 4.98 g l^{-1} $ZnSO_4:7H_2O$

Solution C

15 ml of 2.6 g l^{-1} tetrasodium salt of EDTA (ethylene diamine tetraacetic acid) + 0.12 g l^{-1} $FeSO_4:7H_2O$

Solution D

1.5 ml of 15 g l^{-1} $Na_2HPO_4:12H_2O$

Solutions A, B, C and D were autoclaved in separate flasks at 15 lbs in^{-1} for 15 min. Approximately 1 litre of seawater was filtered through Whatman No. 1 chromatography paper and then autoclaved at 15 lbs in^{-1} for 15 min. After the seawater and the nutrient solutions had been allowed to cool, 923.5 ml of filtered autoclaved seawater was measured out and nutrient solutions A, B, C and D were added to the seawater. The medium was normally made up in 5 litre batches and stored in a cold room. The 'raw' seawater was also stored in a cold room prior to being filtered and autoclaved.

APPENDIX B

Salinity Adjustments

The salinity of each batch of Boney's medium (B.M.) was adjusted to 25‰. (0.43M), therefore the following table can be drawn up.

Composition of the Medium	Salinity (‰).	Molarity of NaCl	Osmolality (Os kg^{-1})
25 ml of distilled water (D.W.)	0	0	0
20 ml of D.W. + 5 ml of B.M.	5	0.09	0.16
15 ml of D.W. + 10 ml of B.M.	10	0.17	0.31
10 ml of D.W. + 15 ml of B.M.	15	0.26	0.48
5 ml of D.W. + 20 ml of B.M.	20	0.34	0.63
30 ml of Boney's medium (B.M.)	25	0.43	0.79
30 ml of B.M. + 0.45g NaCl	40	0.68	1.26
30 ml of B.M. + 0.90g NaCl	55	0.94	1.72
30 ml of B.M. + 1.35g NaCl	70	1.20	2.20
30 ml of B.M. + 1.80g NaCl	85	1.45	2.70
30 ml of B.M. + 2.25g NaCl	100	1.71	3.24

Salinity (‰) = Molarity of NaCl x 58.45

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APPENDIX C

The Weight in Grams of NaCl, KCl, Sucrose, Ethylene Glycol and Glycerol Added to 30 ml of Boney's Medium, the Values Expressed in Terms of Molarity and Osmolality

NaCl			KCl			Sucrose			Ethylene Glycol			Glycerol		
g $30ml^{-1}$	Molarity	Osmol-ality	g $30ml^{-1}$	Molarity	Osmol-ality	g $30ml^{-1}$	Molarity	Osmol-ality	g $30ml^{-1}$	Molarity	Osmol-ality	g $30ml^{-1}$	Molarity	Osmol-ality
-	0.43	0.79	-	0.43	0.79	-	0.43	0.79	-	0.43	0.79	-	0.43	0.79
0.45	0.68	1.26	0.58	0.69	1.25	4.3	0.85	1.27	0.86	0.89	1.25	0.98	0.78	1.15
0.90	0.94	1.72	1.16	0.95	1.72	7.4	1.15	1.71	1.64	1.31	1.77	1.96	1.14	1.54
1.35	1.20	2.20	1.77	1.22	2.21	10.0	1.40	2.16	2.42	1.73	2.17	2.95	1.49	1.95
1.80	1.45	2.70	2.37	1.49	2.71	12.1	1.61	2.64	3.22	2.16	2.69	3.92	1.85	2.41
2.25	1.71	3.24	2.95	1.75	3.19	14.2	1.81	3.19	3.89	2.52	3.18	4.90	2.20	2.96
												5.30	2.35	3.15

$$\text{Osmolality (Osmoles kg}^{-1}) = \frac{\text{freezing point depression, }^{\circ}C}{1.86}$$

All solutions contain 0.43 M NaCl, thus to calculate the molarity of KCl, sucrose, ethylene glycol or glycerol in a given solution, subtract 0.43 from the values given above.

APPENDIX D

Additional Information on the Glycerol Determination Assays

Chromotropic acid reagent for chemical determination

1 g of 4,5-dihydroxy-2,7-napthalenedisulphonic acid (chromotropic acid) was dissolved in 100 ml of distilled water and the solution was filtered. 300 ml of concentrated sulphuric acid was carefully added to 150 ml of distilled water, cooled and added to the sulphonic acid solution to make 500 ml. This solution was stored in a stoppered flask covered with aluminium foil, and it was prepared fresh every two weeks.

Reagents for enzymatic determination

Solution 1 - glycylglycine buffer (pH 7.4), NADH, ATP, phosphoenolpyruvate, magnesium sulphate and stabilisers.

Suspension 2 - pyruvate kinase (600 U ml^{-1})

Suspension 3 - glycerokinase (85 U ml^{-1})

	Blank	Glycerol Standard	Unknown Sample
Buffer solution 1	1.00 ml	1.00 ml	1.00 ml
Distilled water	1.89 ml	1.79 ml	1.90 ml
*1.71 M NaCl or 1.38 M sucrose in 16 mM sodium phosphate buffer pH 7.5	0.10 ml	0.10 ml	-
Sample or standard solution	-	0.10 ml	0.10 ml
*30% trichloroacetic acid	0.01 ml	0.01 ml	-
Suspension 2	0.01 ml	0.01 ml	0.01 ml
Suspension 3	0.01 ml	0.01 ml	0.01 ml
Total	3.02 ml	3.02 ml	3.02 ml

*These solutions are added to the blank and the glycerol standards because they are present in the unknown samples.

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APPENDIX E

The Effect of Increasing the Concentration of NaCl, KCl, Sucrose, Ethylene Glycol or Glycerol on the Oxygen Content of Boney's Medium

Osmolality (Os kg^{-1})	Oxygen Concentration (μmoles ml^{-1})				
	NaCl	KCl	Sucrose	Ethylene Glycol	Glycerol*
0.8 (growth medium)	0.23	0.23	0.23	0.23	0.23
1.3	0.21	0.21	0.19	0.22	0.22
1.7	0.20	0.20	0.17	0.21	0.21
2.2	0.19	0.19	0.15	0.21	0.21
2.7	0.18	0.18	0.13	0.21	0.20
3.2	0.16	0.16	0.10	0.21	0.20
					0.19

*The osmolalities of the glycerol solutions were 1.2, 1.5, 2.0, 2.4, 3.0 and 3.2 Os kg^{-1} as shown in Appendix C.

The oxygen concentration values of solutions with salinities less than that of the normal growth medium (0.43 M NaCl) were calculated from the data of Strickland and Parsons (1968).

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+reference unavailable.

*only an abstract or summary of the reference was consulted.

ERRATA

Table of Contents: for Eratta read Errata.

Page III, line 25: for behave read behaves.

Page 30, line 19 : for tansmembrane read transmembrane.

Page 81, line 19 : for fluoresance read fluorescence.

Page 88, line 13 : insert initially between which and excites.

Page 107, line 23: for efficency read efficiency.

Figure 72A : for Absortion read Absorption.

Page 123, line 15: for meaured read measured.

Figure 88, legend: delete C: 0.68 M NaCl (1.7 Os kg^{-1}) plus DCMU and hydroxylamine, and insert C: 0.68 M NaCl (1.3 Os kg^{-1}) plus DCMU and hydroxylamine; D: 0.94 M NaCl (1.7 Os kg^{-1}) plus DCMU and hydroxylamine.

Page 133 : for 5 min read 10 min on this page.

Page 148, lines 18 and 20: for lbs in^{-1} read lbs in^{-2}