PHYSIOLOGICAL CHARACTERISATION OF A MUTANT OF THE HALOTOLERANT ALGA *DUNALIELLA PARVA*

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SUMMARY

A mutant of the halotolerant green alga Dunaliella parva CCAP 19/9 was isolated, which leaks large amounts of glycerol into the medium. The success of Dunaliella in high salinity environments is largely due to the special osmoregulatory mechanism which involves the synthesis and degradation of the compatible solute glycerol. This allows the cell to adapt to changes in the salt concentration in the surrounding medium. Despite the high loss of glycerol under isotonic conditions the mutant grows at the same rate as the wild type and has the same level of intracellular glycerol during the period of leakage. There is however, a difference in the rates of oxygen evolution and uptake, which indicates that the mutant requires more energy to maintain a constant glycerol level. When levels of deposition of the storage product starch were compared between mutant and wild type, less starch was present in mutant cells. This suggests that the wild type converts more fixed CO_2 from photosynthesis into starch.

The basis of the leakage may be in the lipid composition. The whole cell lipids of the mutant lack two lipids found in the wild type. It is quite likely that the lack of one of these lipids in the membrane affects glycerol retention.

In continuous (chemostat) culture the effect on glycerol leakage of temperature, salinity, pH, light intensity and high levels of CO_2 was examined. It was found that high levels of CO_2 stop leakage of glycerol completely and lower levels of CO_2 can induce leakage again. Both the mutant and wild type take up organic compounds, ^{14}C-glycerol, ^{14}C-glucose and ^{14}C-acetate are transported into the cell by active transport systems. However, no evidence was found for metabolism of these compounds and the cells could not be induced to grow heterotrophically.

CONTENTS

ABBREVIATIONS

CCAP	Culture Collection of Algae and Protozoa
CCCP	carbonylcyanide-m-chlorophenyl hydrazone
DGDG	digalactosyldiacylglycerol
DGTS	diacylglyceryltrimethylhomoserine
dpm	disintegrations per minute
Downshock	a decrease in the osmotic potential of the medium
ECV	extracellular volume
fl	femtolitre (x 10^{-15})
ICV	intracellular volume
MGDG	monogalactosyldiacylglycerol
mut	mutant
M.W.	molecular weight
OD	optical density
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	polyethyleneglycol
PEP	phosphoenolpyruvate
PG	phosphatidylglycerol
PI	phosphotidylinositol
Rf	ratio to solvent front
SQDG	sulphoquinovosyldiacylglycerol
TCA	trichloroacetic acid
TLC	thin layer chromatography
Tris	tris[hydroxymethyl]aminomethane
Upshock	an increase in the osmotic potential of the medium
v/v	volume per volume

wt	wild type
w/v	weight per volume
µCi	microCurie
µE m^{-2} s^{-1}	microEinstein per metre squared per second

CHAPTER 1 INTRODUCTION

1.1. Extreme environments

There are many ecosystems with extreme environmental conditions, such as high salinity, low or high temperature, acidic or alkaline pH, high atmospheric pressure etc., in which the organisms that live there have to adopt special strategies in order to survive. Since extreme environments require special adaptational abilities of the organisms, the number of species found in any extreme environment is low and competition for resources is not as fierce as in "low stress" environments, in which more species are able to live and compete against each other (Grant and Long 1981, Edwards 1990).

1.2. High salinity environments

High salinity environments, are often inland lakes, such as the Dead Sea in Israel or the Great Salt Lake in Utah, in which the rate of evaporation exceeds the rate of input of fresh water (Larsen 1980). Thus they have a salinity above that of sea water (> 0.5 M NaCl) up to saturated salt concentration (> 5 M NaCl). As these salt lakes are found in very hot climates, some of the smaller ones may dry out frequently, so making the environmental

conditions even more severe. The chemical composition of the water in the Dead Sea differs from that of the Great Salt Lake in that it has an extremely high level of magnesium and a lower level of sodium than the Great Salt Lake. Both lakes have a high concentration of chloride (Grant and Ross 1986).

1.3. Microorganisms found in high salinity environments

The organisms of high salt environments are, with the exception of a few invertebrates, exclusively microorganisms, i.e. bacteria, algae and fungi. There are two separate groups of salt tolerant bacteria, the halotolerant eubacteria, such as Halomonas elongata, Micrococcus halobius and Ectothiorhodospira halophila and in the Archaebacteria the Halobacteriaceae such as Halobacterium salinarium (Kushner 1988). The latter group require about 2 M NaCl to be present to allow growth and they differ from the eubacteria in fundamental aspects of their cell organisation (Juez 1988). In the fungi, the yeasts Debaryomyces hansenii, Saccharomyces rouxii and Candida famata have the ability to grow in high salinity habitats (Brown 1978 and Larsson et al. 1990). Amongst algae the microalga Dunaliella has been found to be most tolerant to high salt conditions (Gilmour 1990).

1.4 Distribution of Dunaliella

Dunaliella is a genus of halotolerant unicellular, wall-less green algae, which have the ability to grow in salinities ranging from 0.1 M to saturated salt (> 5 M NaCl)(Ben-Amotz and Avron 1981). However, no single species is known which will grow across the entire salinity range and Dunaliella species have been classified into two groups: halotolerant species usually found in marine environments, they have an upper salinity limit of 2.0 M NaCl and halophilic species which are found in hypersaline lakes and grow in salinities greater than 2.0 M NaCl (Ginzburg and Ginzburg 1985). Later work from the same laboratory suggests that this distinction is not clear cut, since both halotolerant and halophilic species can be trained to grow at salinities from 0.5 to 3.5 M NaCl (Ginzburg et al. 1990). Dunaliella spp. found in hypersaline lakes, in particular D. salina, D. viridis and D. parva are probably the most halotolerant eukaryotic microorganisms (Brock 1975). In 1936 D. salina and Halobacterium were the first microorganisms to be isolated from the Dead Sea, previously believed to be lifeless (Volcani 1944).

Dunaliella is the major and possibly the only primary producer in the Great Salt Lake (Post 1977) and is the main food source of the brine shrimp Artemia salina. Most of the photosynthetic production of the Great Salt Lake

can be attributed to Dunaliella according to Stephens and Gillespie (1976), who found that growth is only limited through selfshading and lack of nitrogen.

The ability of the genus Dunaliella to grow in a wide range of salinities has made this alga very successful in these high salt ecosystems, where only few other microorganisms can exist. But Dunaliella has not only the ability to grow in high salinities, it is also able to adapt quickly to a change in salt concentration in the surrounding water. This ability is particularly important in the estuarine or rock pool environments, where the salinity can change rapidly (McLuskey 1989).

1.5. Adaptation of microorganisms to high salt environments

In order to grow in a high salinity environment, microorganisms must be able to elevate their internal osmotic potential to equal that of the medium. Otherwise water will be drawn out of the cells leading to dehydration and death. There are two basic strategies adopted by microorganisms to grow in high salinities: the first is to adapt the cellular metabolism to high salt concentrations inside the cells and tolerate the salt in the cytoplasm, the second is to exclude the salt from the cell and produce organic osmolytes known as compatible solutes (Gilmour 1990). The first strategy is found in

the Halobacteriaceae (Archaebacteria), where they accumulate KCl in response to the high external salinity. The intracellular enzymes are salt tolerant and can function under high salt conditions, indeed they cannot function in the absence of molar concentrations of salt and this is the reason they require 2 M NaCl for growth (Brown 1983). The eubacteria, fungi and algae have adopted the second strategy for life in high salinities, they have "normal", i.e. salt sensitive enzymes (Brown 1976) and exclude salt from the inside of the cell. However, in order to maintain the osmotic balance they must synthesise or take up compatible solutes. These are organic compounds, which have a number of functions (Borowitzka 1981).

1. Many have a role in osmoregulation, as they are accumulated to high levels inside the cell. They regulate the water activity of the cell and in this way minimize the flux of water in and out of the cell, thus the balance of external osmotic potential is maintained, excluding salt from the cell.

2. Due to their role in intracellular water regulation compatible solutes have no effect on the metabolic processes inside the cell, even when present in high concentrations.

3. In vitro, they protect enzymes from inhibition due to high salt concentrations, thus during a transient increase in intracellular salt concentration after an upshock, compatible solutes may actually protect the cell.

Because compatible solutes are accumulated in high concentrations, they have to be highly soluble. Therefore they are mainly sugars, polyols, amino acids and betaines (Yancey et al. 1982).

1.6. Compatible solutes in algae

The compatible solutes found in algae fall into three major groups (Table 1.1). Group one includes the polyhydric alcohols (polyols), such as glycerol, mannitol and sorbitol. In group two are glycosides (sugar molecules with glycerol side chains), such as galactosylglycerides, floridoside and isofloridoside. The third group includes the amino acids glutamate and proline (Ben-Amotz and Avron 1983). The pattern of distribution of compatible solutes in the different groups of algae is directly related to the evolution of the various algal groups (Figure 1.1). The evolutionary tree of algae splits into 2 major branches, the Chlorophytes and Chromophytes. The compatible solutes found in the Chlorophytes are glycerol, sucrose, proline and mannitol. In the group of the chromophytes the major

TABLE 1.1. Salt tolerance and accumulation of compatible solutes in algae (Ben-Amotz and Avron 1983)

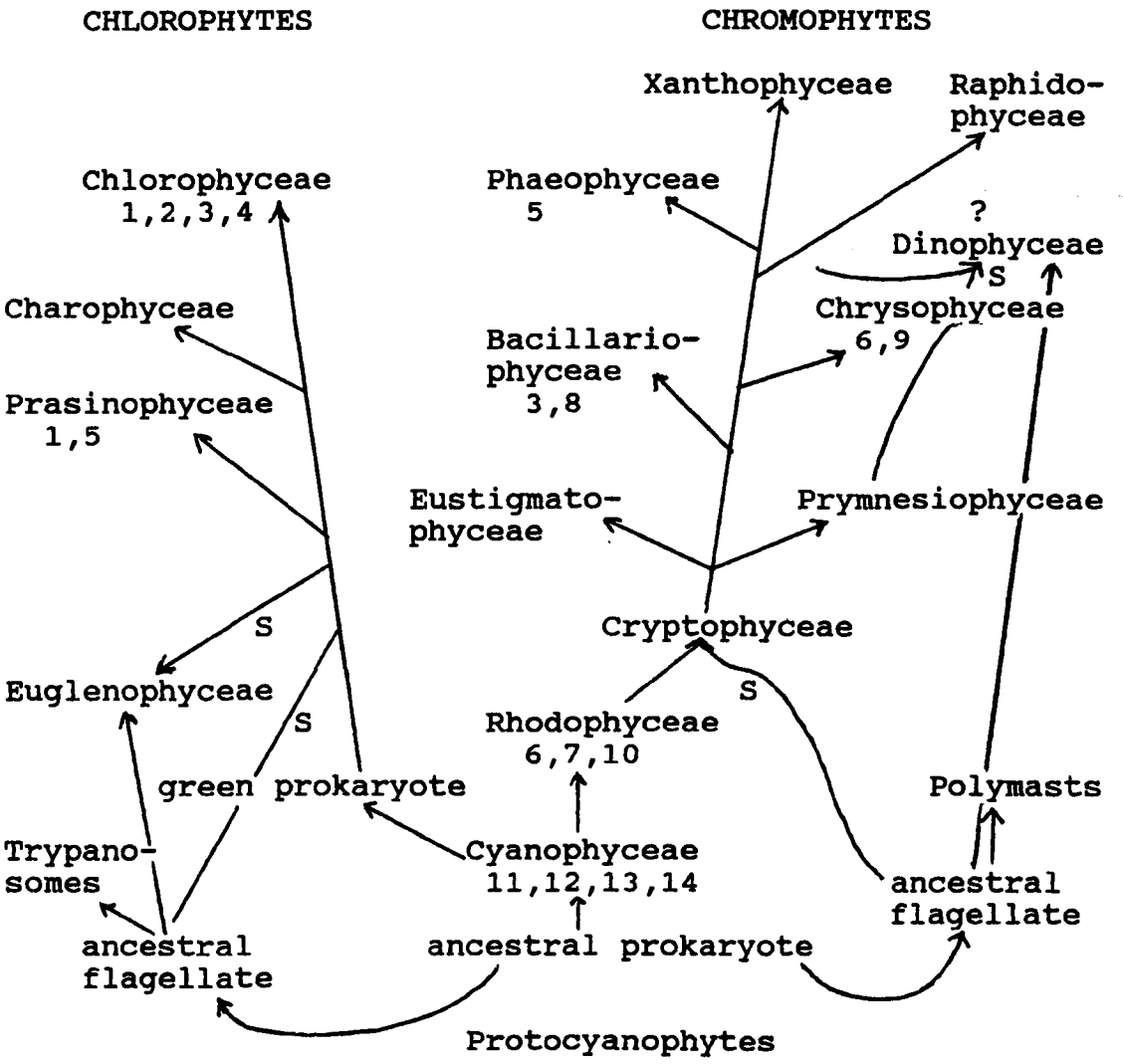
Organism	Range M NaCl	Compatible solute
CYANOPHYCEAE		
Aphanotheca halophytica	1.2 -5.1	amino acids, polyols, carbohydrates
Synechococcus sp. glucosylglycerol	0.03-1.2	
RHODOPHYCEAE		
Centroceras clavulatum	0.39-0.78	digenaside
Corallina officinalis	0.39-0.78	floridoside
Grateloupia filicina	0.39-0.78	floridoside, isofloridoside
Griffithsia monilis	0.23-0.93	digenaside
Hypenea valentiae	0.39-0.78	floridoside, isofloridoside
Iridophycus flaccidum	0.3 -0.6	floridoside, isofloridoside
Lomentaria umbellata	0.39-0.78	floridoside, isofloridoside
Porphyra perforata	0.3 -0	floridoside, isofloridoside
P purpurea	0.04-1.8	floridoside, isofloridoside
P. umbilicalis	0.3 -3.6	floridoside
Rhodymenia foliifera	0.39-0.6	floridoside, isofloridoside
CHRYSOPHYCEAE		
Monocrysis luteri	0.15-1.0	Cyclohexanetetrol
Ochromonas malhamensis (*Poteriochromonas stipitata*)	0.01-0.15	isofloridoside
Paraphysomonas vestita	0.01-1.6	ND
EUSTIGMATOPHYCEAE		
Monallantus salina	0.04-0.6	mannitol, proline
WHOI Car-A	0.04-0.6	mannitol, proline
PHAEOPHYCEAE		
Ascophyllum nodosum	0.05-0.55	mannitol
Colpomenia sinuosa	0.39-0.78	mannitol
Dictyota dichotoma	0.39-0.78	mannitol
Ecklonia radiata	0.39-0.78	mannitol
Fucus (various species)	0.04-0.55	mannitol
Pilayella littoralis	0.04-1.8	mannitol
Scytosiphon lomentaria	0.39-0.78	mannitol
PRASINOPHYCEAE		
Asteromonas gracilis	0.5 -4.5	glycerol
Platymonas subcordiformis	0.2 -1.5	mannitol
P. suecica	0.05-1.5	mannitol

TABLE 1.1 (cont)

BACILLARIOPHYCEAE		
Cyclotella cryptica	0.13-0.53	proline
C. meneghinana	0.1 -0.45	proline
Cylindrotheca fusiformis	0.57-0.7	mannose
Navicula sp.	0.25-2.75	proline
Phaeodactylum tricornutum	0.14-0.77	proline
CHLOROPHYCEAE		
Botryococcus sp.	0.4 -2.83	ND
Chlamydomonas sp.	0.34-1.71	glycerol
Chlorella emersonii	0.01-0.33	proline, sucrose
Dunaliella (various species)	0.1 -5.5	glycerol
Klebsormidium marinum	0.04-0.27	sorbitol, proline
Stichococcus bacillaris	0.39-0.6	sorbitol, proline
S. chloranthus	0.04-0.6	sorbitol, proline

ND= not determined

Figure 1.1. Algal evolution and compatible solutes



Compatible solutes

1. glycerol
2. sucrose
3. proline
4. sorbitol
5. mannitol
6. isofloridoside
7. floridoside
8. mannose
9. cyclohexanetetrol
10. digenaside
11. glucosylglycerol
12. polyols
13. amino acids
14. carbohydrates

S= symbiotic event

adapted from South and Whittik (1987)

compatible solutes are isofloridoside, floridoside, cyclohexanetetrol, digenaside and mannose. The only overlap with the Chlorophytes is the presence of mannitol in the Phaeophyceae and proline in the Bacillariophyceae, otherwise the compatible solutes of the groups are quite distinct from each other.

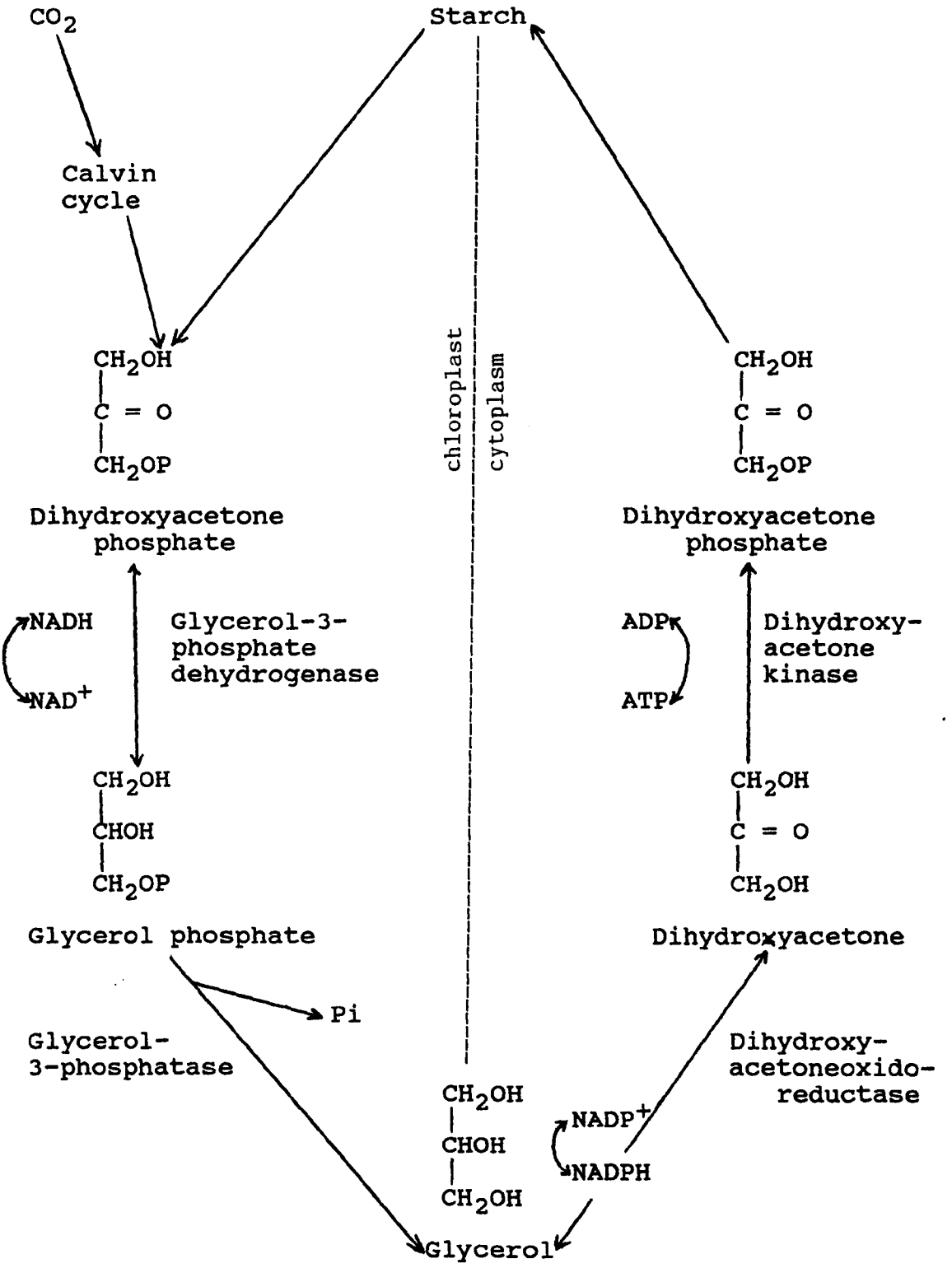
1.7. Osmoregulation in Dunaliella

The compatible solute utilized by Dunaliella is glycerol, which is accumulated to high concentrations from photosynthetically fixed CO_2 or by break down of the storage product starch (Craigie and MacLachlan 1964, Wegmann 1971, Ben-Amotz and Avron 1973, Borowitzka and Brown 1974). Glycerol protects the cells from dehydration in high salinity environments and enables the salt sensitive chloroplastic and cytoplasmic enzymes to function normally (Ben-Amotz and Avron 1972, Borowitzka and Brown 1974). There is a linear relationship between the salt concentration in the surrounding medium and the level of intracellular glycerol (Ben-Amotz and Avron 1973, 1978,1980, Borowitzka et al. 1977). Not only are Dunaliella cells able to grow in high salt concentrations, they are also able to adapt to a change in salinity. This is aided by their ability to rapidly adjust the intracellular osmotic pressure by glycerol synthesis or degradation (Ben-Amotz and Avron 1981). After a hypertonic shock to a higher salinity, the cells

produce and accumulate glycerol above the original level. After a hypotonic shock to a lower salt concentration, the glycerol level inside the cell is lowered by converting the glycerol to osmotically inactive starch or in more extreme cases by excreting it into the medium, in a process which requires energy (Zidan et al. 1987). The process of osmotic adjustment to the new salinity begins minutes after the salt shock but takes about 90 min to return cell metabolism to normal (Ben-Amotz and Avron 1973, Brown and Borowitzka 1979) and is independent of light and protein synthesis (Ben-Amotz and Avron 1981, 1990)

During the 1970's and 1980's, work was carried out on the pathway of glycerol synthesis and degradation culminating in the proposal of the glycerol cycle shown in Figure 1.2 (Ben-Amotz and Avron 1981). The input to the cycle is dihydroxyacetone phosphate (DHAP) which can be generated from photosynthetically fixed CO_2 via the Calvin cycle or from starch. DHAP is converted to glycerol phosphate and then to glycerol. When glycerol is no longer required, it is removed by a different pathway via dihydroxyacetone back to DHAP (Figure 1.2). Three of the enzymes of the cycle are unique to this pathway, dihydroxyacetone reductase, dihydroxyacetone kinase and glycerol-1-phosphatase. More recent work has concentrated on the localisation of the enzymes in the chloroplast or cytoplasm (Gimmler and Lotter 1982, Haus and Wegmann 1984, Marengo et al. 1985). The evidence suggests that

FIGURE 1.2. The glycerol cycle in Dunaliella



adapted from Gilmour (1990) and Ben-Amotz and Avron (1981, 1990)

the synthesis of glycerol takes place in the chloroplast, whereas the removal reactions are in the cytoplasm (Fig. 1.2).

The way in which changes in salinity are sensed and osmoregulation is initiated is not clear, but a number of possibilities have been suggested. It may well be that the physical change of the cellular volume and thus the folding (Einspahr et al. 1988) or enlarging (Curtain et al. 1983) of the plasma membrane is the trigger. Another possible explanation is that the cell might sense changes in external salt concentration via the activity of the plasma membrane H^+-ATPase (Oren-Shamir et al. 1989). The activation of the ATPase and the consequent proton flux from the cell results in an increase in internal pH (Goyal et al. 1987, Goyal and Gimmler 1989, Kuchitsu et al. 1989). This might be significant in osmoregulation as a number of enzymes in the glycerol cycle have an alkaline pH optimum (Goyal et al. 1987). The ATPase consumes ATP and this leads to an increase in inorganic phosphate inside the cell (Bental et al. 1990), which could also play a role in osmoregulation. It is also possible that the decrease in ATP concentration or the increase in ADP concentration stimulates the enzymes involved in recovery.

1.8. Algal and Cyanobacterial Biotechnology

Algal and cyanobacterial biotechnology has been of interest for a number of years, starting in the 1940's and gaining in importance ever since. Commercial utilization of algae includes a wide range of species, microalgae, macroalgae and blue-green algae (cyanobacteria). The products and uses of algae in industry are very varied (Table 1.2). Algae are utilized in the production of health foods, vitamins, fine chemicals, oils, polysaccharides, single cell protein and have also found application in aquaculture and as soil conditioners (see Table 1.2 for references).

Much of algal biotechnology is still in the developmental stages and a lot of research has to be done before the products are commercially available. However, "new" algal products and applications are being found all the time and the field of algal biotechnology is advancing fast (Cresswell et al. 1989). Not all of the algal products found to date are commercially viable, quite often the product can be produced cheaper by other means. There is however likely to be a future for many algal biotechnological processes, particularly the ones which replace the use of oil, as oil prices are likely to increase in the near future and oil reserves will eventually run out altogether. So it becomes more r

TABLE 1.2. Algal and cyanobacterial commercial products

Organism	product/ commercial use	reference
Chlamydomonas (Chlorophyceae)	soil inocula, fertilizer	1
Chlorella (Chlorophyceae)	health food, amino acids	2,3
Dunaliella (Chlorophyceae)	beta-carotene, glycerol, solvents, protein feed food supplement	4-12
Gracilaria (Rhodophyceae)	agar	13
Haematococcus (Chlorophyceae)	astaxanthin, food colouring	14
Laminaria (Phaeophyceae)	alginates, food	15
Macrocystis (Phaeophyceae)	alginates	16
Porphyra (Rhodophyceae)	food	15
Porphyridium (Rhodophyceae)	fine chemicals, polysaccharides	3,17
Scenedesmus (Chlorophyceae)	single cell protein, hydrocarbon compounds	3,18
Spirulina (Cyanophyceae)	health food, food colouring, animal feed	3,19
Tetraselmis (Chlorophyceae)	aquaculture	20,21

TABLE 1.2 (cont) References

1. Bennemann (1989)
2. Oh-Hama and Miyachi (1988)
3. Richmond (1986)
4. Borowitzka and Borowitzka (1988)
5. Chen and Chi (1981)
6. Nakas et al. (1983)
7. Ben-Amotz and Avron (1980)
8. Ben-Amotz and Avron (1981)
9. Ben-Amotz and Avron (1989)
10. Ben-Amotz and Avron (1990)
11. Ben-Amotz et al. (1988)
12. Mokady et al. (1989)
13. Hansen (1984)
14. Kobajashi et al. (1991)
15. Van der Meer (1988)
16. Borowitzka (1986)
17. Vonshak (1988)
18. Soeder and Hegewald (1988)
19. Richmond (1988)
20. Laing and Helm (1981)
21. De Pauw and Persoone (1988)

more important to extract and produce substances by oil-independent means.

In recent years people have become more and more health conscious and are in increasing numbers aware of the need for a healthy diet.The world health food market is worth billions of pounds and more research is undertaken all the time to bring more "natural" products to the attention of the customer. Health foods derived from algae are beginning to appear in the shops and especially beta-carotene containing powders and vitamin tablets (see section 1.8) are big sellers, particularly since it was claimed that beta-carotene has anti-cancer properties (Harvey 1988).

1.9. Biotechnology of Dunaliella

The biotechnology of Dunaliella is quite far advanced,since it is the only microalga licensed by the United States as a food source (R. Righelato, personal communication). A number of companies are growing Dunaliella commercially all over the world in outdoor open ponds.

Beta-carotene, which is the most valuable product produced by Dunaliella, is accumulated in large amounts inside the cell and gives the alga a red colour, which predominates in the salt lakes. The highest levels of

accumulation of beta-carotene are found in D. salina where it can make up to 14% of the dry weight (Ben-Amotz et al 1982) and to a lesser extent in D. bardawil. The production of glycerol from Dunaliella is at present not commercially viable, because the costs are too high compared to other means of glycerol production from petroleum industry products (Gilmour 1990).

An "all microbial" production of ethanol or solvents such as acetone or butanol by bacteria, using Dunaliella biomass as substrate is still in the experimental phase (Williams et al. 1978). It is technically feasible but at the moment hopelessly uneconomic. Ben-Amotz and Avron (1981) suggest that Dunaliella is a perfect theoretical model system for biosolar energy conversion, whereby Dunaliella cells are completely utilized for the extraction of beta-carotene and glycerol and the remaining biomass is used as a high protein animal feed. The commercial cultivation of Dunaliella however poses a number of problems and various factors have to be taken into account to make the project viable. Growth rate and yield have to be finely balanced as, eg. the maximum beta-carotene and glycerol accumulation is under stress conditions and so takes place at suboptimum growth conditions, which consequently results in a lower growth rate (Chen and Chi 1981).

Open air pond systems are not without drawbacks, as the cultures are exposed to the weather and rainfall brings a

change in salinity (Oswald 1988) and consequently a decrease in the carotenoid accumulation. The design of an open culture system has to take into consideration the requirement of the cells for light, but must also ensure that the algae are not exposed to excessive levels of irradiation, which despite the presence of the carotenoid pigments might lead to damage and photoinhibition (Ben-Amotz and Avron 1989). Mixing of the cultures in the ponds has to be ensured for the even distribution of nutrients and removal of excess O_2, as well as the prevention of stratification in the pond and sinking of the algal cells and often a single paddle or pump is used (Borowitzka and Borowitzka 1989). For the commercial cultivation of Dunaliella the ponds or raceways have a surface area of 1-4 km^2 and a depth of 10-20 cm (Ben-Amotz and Avron 1989).

Theoretically, Dunaliella could be grown indoors using large illuminated fermentors, but the cost of the lighting makes this uneconomical. Other factors, such as corrosion of the fermentor by the high salt present, are also important. Nevertheless there is interest in growing Dunaliella indoors, but only if this could be achieved by heterotrophic growth on cheap carbohydrate substrates.

1.10. Aims of the project

A mutant of *Dunaliella parva* which leaks large amounts of glycerol into the medium was isolated. The mutant was characterised and grown in continuous culture. The feasibility of heterotrophic growth was examined and an investigation of the molecular basis of the glycerol impermeability of the plasma membrane was carried out.

2.1. Algal cultures

Dunaliella parva CCAP 19/9 wild type was obtained from the Culture Collection of Algae and Protozoa, the marine part of which is now based at the Scottish Marine Biological Association, Oban, UK.

The Dunaliella parva 19/9 mutant, which leaks large amounts of glycerol into the medium, arose spontaneously from a wild type culture grown in continuous culture in a CO_2 limited chemostat.

2.2. Growth conditions of Dunaliella

2.2.1. Batch culture

The cultures were grown in 250 ml flasks containing 150 ml of growth medium (see Appendix), containing either 0.4 M or 1.5 M NaCl as described by Hajibagheri et al. (1986) and Blackwell and Gilmour (1989). Mutant and wild type cells were kept as separate lines in these two salinities at all times and had been adapted to them for over two years.

The temperature in the growth room was maintained at 25^0C and the light intensity was 20-25 µE m^{-2} s^{-1}. The cultures were aerated by occasional shaking, usually every two days. Cells were used for experiments after 3 to 14 days.

2.2.2. Continuous culture in the Chemostat

In continuous culture the cells were grown in a 1 or 2 litre volume LDH chemostat with mechanical stirrer and overflow system, in which the working volume was 800 ml and 1.5 l respectively.

All growth parameters were controlled, the temperature was maintained around 30^0C ($\pm$ 2 %), in hot weather it was necessary to connect the cold finger to the chemostat (tap water is passed through a tube within the culture pot) to prevent overheating. The pH was maintained between 7.6 and 7.8 by monitoring the pH with a pH electrode connected via a control box to peristaltic pumps which pumped either 1 M NaOH or 0.2 M HCl into the culture pot. Air was bubbled through the chemostat at a rate of 2.5 l min^{-1}.

The culture was illuminated with 2 light rings (Philips TLE 22 W/29, 190 µE m^{-2} s^{-1}) around the chemostat. The culture was mixed with the mechanical stirrer at 500 rpm, whereby shearing of the Dunaliella cells was not a

problem. The medium flow from a 20 l medium vessel was controlled by a medium pump, with a flow rate of 0.24 to 0.32 ml min^{-1}.

The medium in the chemostat was the 1.5 M NaCl medium used in batch culture (see Appendix), but because of the interference of Tris buffer with the acetylacetone glycerol test, no buffer was added. The pH of the medium was controlled entirely by the pH electrode and control box. The culture pot, filled with the appropriate medium, the 20 l medium vessel and the overflow medium collection vessel were autoclaved for 20 minutes at 15 psi.

For the inoculation of the chemostat a 10 to 14 day old mutant batch culture grown in 1.5 M NaCl medium was used, the size of the inoculum was between 40 and 60 ml, depending on the age of the culture. Medium flow was not started until the third day after inoculation to allow the culture to become established and to be actively growing. Samples were collected in sterile 15 ml Bijou bottles via the sampling port on the chemostat and chlorophyll content and glycerol leakage into the medium were determined, using the methods described in sections 2.3 and 2.6.

2.3. Chlorophyll and cell number determination

The chlorophyll content of *Dunaliella* cells was determined after extraction of the chlorophyll by 80 % (v/v) acetone. The method of McKinney (1941) and Arnon (1949) was used, whereby a 5 ml aliquot of cells is centrifuged for 10 minutes at high speed in a bench centrifuge (3000 g). The supernatant is discarded and the pellet is resuspended in 1 ml of distilled water, to break open the cells. After the addition of 4 ml of acetone the cells are whirlimixed and centrifuged again for 5 minutes at 3000 g. The absorption of the supernatant is measured at 645 and 663 nm using an LKB Ultraspec spectrophotometer with 80 % acetone as the blank.

Chlorophyll content of the cells is calculated using the following equation:

$$OD_{645} \times 202 = Y$$

$$OD_{663} \times 80.2 = X$$

$$Y + X \div 2 = \mu g \text{ chlorophyll per 5 ml sample}$$

In addition, the equations of Lichtenthaler and Wellburn (1983) were also used to calculate chlorophyll contents (see section 2.4). In this method OD_{646} is used instead of OD_{645}, but this is an insignificant difference. Despite the different equation used in the two methods, the chlorophyll values calculated were found to be similar.

Cell counts were generated using a standard haemocytometer ($1mm^2$ lined grid with a depth of 0.02 mm). The cells were killed by adding 10 µl of Grams' Iodine to a 1 ml sample and at least five replicates were made for each cell number determination.

2.4. Carotenoid determination

Carotenoids were calculated from spectrophotometer scans with the LKB Ultraspec using samples from chemostat cultures extracted in 80 % acetone (v/v). To enable accurate determination a grid was constructed for reading the ODs from the scan.

The following calculation was used according to Lichtenthaler and Wellburn (1983):

chlorophyll a = $12.21\ OD_{663} - 2.81\ OD_{646}$

chlorophyll b = $20.13\ OD_{646} - 5.03\ OD_{663}$

carotenoids µg per 5 ml sample $= \frac{1000\ OD_{470} - 3.27\ C_a - 104\ C_b}{229}$

2.5. Starch assay

Starch was assayed using the method described by Takeda and Hirokawa (1978).

Reagents:

1. KI reagent (0.03 M Potassium Iodide and 0.01 % (w/v) Iodine)

2. 17.5 % (v/v) perchloric acid

Procedure:

10 ml samples of algae were centrifuged at 3000 g for 10 minutes, the pellets were resuspended in 1.5 ml of 17.5 % (v/v) perchloric acid and were incubated at 45 ^{0}C for 50 min. After incubation 1.5 ml of the KI reagent was added and the cells were spun down for 5 minutes in the bench centrifuge at maximum speed (3000 g).

Absorption was read at 560 nm using 17.5 % (v/v) perchloric acid as blank. A standard curve was prepared

with 0 to 250 µg soluble starch in 17.5 % (v/v) perchloric acid.

2.6. Glycerol determination

Two methods for the determination of glycerol were used:

a) The specific enzymatic assay for glycerol, either as commercial test kit from Boehringer-Mannheim Lewes, UK, or as a slightly modified method, based on the test kit.

b) The non-specific acetylacetone assay as described by Ben-Amotz and Avron (1978).

2.6.1. Glycerol determination using the enzyme test:

a. Principle of the method:

The test is for the determination of glycerol in foodstuffs and other materials and is based on the following reactions:

Glycerol is phosphorylated by ATP to glycerol-3-phosphate, catalyzed by glycerokinase (GK)

$$\text{glycerol} + \text{ATP} \xrightarrow{GK} \text{glycerol-3-phosphate} + \text{ADP}$$

The ADP formed is reconverted by phosphoenolpyruvate and pyruvate kinase (PK) into ATP with the formation of pyruvate.

$$ADP + PEP \xrightarrow{PK} ATP + pyruvate$$

Pyruvate is reduced to L-lactate by NADH with the oxidation of NADH to NAD in the presence of lactate dehydrogenase (LDH).

$$pyruvate + NADH + H^+ \xrightarrow{LDH} L\text{-}lactate + NAD^+$$

The amount of NADH oxidized as a result of this reaction is stoichiometric to the amount of glycerol present. NADH is determined by reading the OD at 340 nm.

b. Procedure:

The following solutions have to be prepared, based on the commercial enzymatic test kit:

1. A buffer solution (100 mM glycylglycine buffer pH 7.4, 0.2 M $MgCl_2.6\ H_2O$, NADH, ATP, PEP) was prepared by adding 7 mg NADH, 22 mg ATP and 11 mg PEP to 11 ml of glycylglycine and $MgCl_2.6H_2O$ solution, this forms solution 1 in the assay, which is only stable for 2 to 3 days, so it was freshly made up on the day of the experiment.

2. Enzyme suspension, consisting of pyruvate kinase and lactate dehydrogenase (700 and 1000 U ml^{-1} respectively) from Sigma Chemical Company.

3. Glycerokinase suspension (37 U ml^{-1}) from Sigma.

4. Glycerol standard solution containing 0.39 mg ml^{-1}

All four solutions are ready to use in the Boehringer Mannheim commercial enzyme test kit, whereby solution 1 contains in addition an unknown quantity of stabilizers.

The determination of glycerol was carried out, using 4 ml glass cuvettes as shown in Table 2.1. After mixing the absorption in each cuvette was read at 340 nm against air at intervals until a steady value is reached. At this point 0.01 ml of suspension 3 was added to all cuvettes, after mixing the absorption was monitored until the readings had stabilized.

To calculate the glycerol content of the sample the following equation is used:

$$\frac{\text{final volume (3.03 ml) x molecular weight (92.1)}}{\text{e x d x v x 1000}} \times \text{OD}$$

where,

e = extinction coefficient for NADH (6.3 l $mmol^{-1}$ cm^{-1})

d = pathway of cuvette (1 cm)

Table 2.1: Determination of glycerol using the enzyme test

	Blank	Standard	Sample
solution 1	1.0 ml	1.0 ml	1.0 ml
distilled H_2O	1.9 ml	1.8 ml	1.9 ml
1 M NaOH*	0.02 ml	0.02 ml	0.02 ml
growth medium	0.09 ml	0.09 ml	--
30 % TCA	0.01 ml	0.01 ml	--
solution 4	--	0.1 ml	--
sample	--	--	0.1 ml
suspension 2	0.01 ml	0.01 ml	0.01 ml

* Addition of NaOH is necessary to neutralize the acidified samples, if the commercial test kit solutions are not used

v = volume of sample (ml)

eg. for the modified enzyme method where NaOH is added:

$$\frac{2.79}{e} \times \text{OD - blank} = \text{concentration of glycerol } (g\ l^{-1})$$

The dilution of the sample during preparation by the addition of TCA has to be taken into consideration.

2.6.2. Glycerol determination using the acetylacetone test

The following reagents are used:

a. Periodate reagent - 15.4 g ammonium acetate is dissolved in 180 ml of 2 % (v/v) acetic acid. After dissolving 130 mg of sodium periodate in this solution, 20 ml of glacial acetic acid is added.

b. Acetylacetone reagent - 1 ml of acetylacetone + 99 ml isopropanol. This reagent has to be stored in the dark.

0.1 ml of samples or standards (0-40 µg glycerol ml^{-1}) and 0.1 ml of distilled water (90 µl of Tris-free medium and 10 µl of 30 % (v/v) TCA for the standards and blank) were added to test tubes, 0.1 ml of distilled H_2O was used as blank. 1 ml of periodate reagent was added to all tubes

and they were incubated at room temperature for 5 minutes. 2.5 ml of acetylacetone reagent was then added and the samples, standards and blank were incubated at 40-50 ^{0}C for 20 minutes. After cooling on ice the absorption was read at 410 nm against the reagent blank.

2.6.3. Preparation of samples for glycerol assays

Samples were prepared in the same way for both enzymatic and acetylacetone assays. For the assay of glycerol in the cells and the medium 4.5 ml samples were placed in 5 inch test tubes, 0.5 ml of 30% TCA was added with mixing and samples were stood at room temperature for 15 minutes. At the same time 5 ml samples were added to 5 inch test tubes for the determination of intracellular glycerol. All test tubes were then centrifuged in a bench centrifuge at maximum speed (3000 g) for 10 minutes. After centrifugation 1 ml of supernatant from the TCA killed cells and 0.9 ml of supernatant of the intact cells was added to 1.5 ml Eppendorf tubes. 0.1 ml of 30 % TCA was then added to the supernatant of the unkilled sample and all the tubes were whirlimixed. Samples were then taken for the glycerol assays.

2.7. Measurement of oxygen evolution and oxygen uptake

The measurements of oxygen evolution and uptake were made using a modified Clark oxygen electrode as described by Gilmour (1982). A temperature of $30^{0}C$ was maintained in the chamber of the electrode by circulating water from a constant temperature water bath. The chamber was illuminated by a Philips 12 V, 100 W projector lamp with a photon flux density of 50 $\mu E\ m^{-2}\ s^{-1}$.

Calibration of the oxygen electrode was carried out by the addition of sodium dithionite to 1 ml of growth medium of appropriate salinity in the chamber, thus removing the molecular oxygen. The solubility values of oxygen in different salinities determined by Schlieper (1972) and Gilmour (1982) were used and are given in the Appendix. Rates of oxygen evolution and uptake were calculated using the following equation:

$$\frac{\text{Oxygen content in 1 ml medium at 30 }^{o}C}{\text{No. chart units between 0 and 100 \%}} \times \frac{\text{No. chart units oxygen change}}{\text{Time (min)}} \times \frac{60}{\text{mg chl. ml}^{-1}}$$

Measurements of oxygen evolution and uptake were routinely determined using three to four day old cultures, which after chlorophyll determination (section 2.3) were harvested by centrifugation at 3000 g for 10 minutes and concentrated to give a chlorophyll content of 30 $\mu g\ ml^{-1}$. 1 ml samples were dispensed into the chamber of the oxygen electrode and kept in the dark for 5

minutes, before oxygen evolution and uptake were measured. For the salt shock experiments the cells were resuspended in the appropriate medium straight after centrifugation and the measurements were made immediately and then monitored over a period of four hours.

2.8. Growth on organic compounds in the dark

Mutant and wild type batch cultures were set up with 150 ml medium in 250 ml flasks containing 1.5 M NaCl and

1. basic growth medium (see Appendix)

2. basic growth medium plus 0.1 % (w/v) sodium acetate or
   0.1 % (w/v) glucose or
   0.1 % (w/v) glycerol

3. basic growth medium plus Euglena gracilis medium which contains:-
   0.1 % (w/v) sodium acetate,
   10 ml $CaCl_2$ solution, containing 1g/l,
   0.1 % (w/v) lab lemco (Oxoid)
   0.2 % (w/v) tryptone (Oxoid) and
   0.2 % (w/v) yeast extract (Oxoid).

The flasks were kept in the dark at 25 ^{0}C with occasional shaking by hand.

2.9. Uptake of organic compounds

The uptake of ^{14}C-glycerol, ^{14}C-glucose and ^{14}C-acetate by Dunaliella cells was measured by an adaptation of the method of Gimmler and Schirling (1978). The sample is centrifuged through high density silicone oil and forms a pellet at the bottom of an Eppendorf tube, leaving the supernatant suspended on top of the oil layer.

Three day old cells from three replica batch culture flasks were harvested and concentrated by centrifugation at 3000 g for 10 minutes in a bench centrifuge to give 30 µg chlorophyll ml^{-1}. Three 5 ml samples were placed in sterile 50 ml flasks stoppered with cotton wool bungs. The cultures were inoculated with 5 µl of one of the labelled compounds (see section 2.10) and were manually shaken throughout the experiment to ensure even distribution of the labelled substances in the flasks. The flasks were then incubated in a waterbath at either 20 ^{o}C or 30 ^{0}C, and depending on the experiment were either illuminated with a Philips TLE 22 W/29 light ring to provide a light intensity of 20-25 µE $m^{-2}s^{-1}$, wrapped in aluminium foil for total darkness or left in daylight for ambient light conditions (5-10 µE $m^{-2}s^{-1}$).

The sampling was started after 5 minutes and an aliquot of 0.3 ml from each sample was placed on top of 0.3 ml of oil in an Eppendorf tube. For cells grown in 0.4 M NaCl Dow Corning 550 silicone oil was used (BDH Chemicals Ltd)

and for 1.5 M NaCl grown cells the Dow Corning 710 silicone oil (BDH) was used. The different oils are necessary due to the different densities of 0.4 and 1.5 M NaCl medium. The Eppendorf tubes were then spun in the microfuge at 6000 g for 1 minute. 100 µl of the supernatant was taken and added to a plastic scintillation vial containing 5 ml of scintillation cocktail (Safe Fluor S from Lumac LSC, Belgium). The tip of the Eppendorf tube, containing the pellet, was cut off and transferred to an Eppendorf tube containing 0.3 ml distilled H_2O. The tip was placed open end down and the Eppendorf tubes were centrifuged to transfer pellet from the tip into distilled water. The Eppendorf tip was then discarded and the pellet was resuspended by whirlimixing and 100 µl of the sample was then added to a scintillation vial containing 5 ml of cocktail. The vials were then counted for 1 minute each in a Beckman LS 1801 scintillation counter, which was set up to give dpm readings due to a preset quench curve.

For all uptake experiments, each point is the mean of three independent replicates, the standard errors were always less than 5 % of the mean unless otherwise stated in the figure legend.

2.10. Calculation of Extracellular Volume (ECV) and Intracellular Volume (ICV) of Dunaliella cell pellets

1. Two 14 day old cultures grown in 0.4 M NaCl were combined and concentrated to give 1 ml samples with a chlorophyll content of 30 µg/ml. 22 µl of $^{3}H_2O$ (25 µCi ml^{-1}, see section 2.11) were added to one 1 ml sample and 40 µl of ^{14}C-dextran (6.75 µCi ml^{-1}, see section 2.11) to another.

2. After 5 minutes 0.3 ml from each sample were taken and spun through 0.3 ml of silicone oil (Dow Corning 550) in a plastic Eppendorf tube for 1 minute at high speed (6000 g) in the microfuge.

3. 50 µl of the supernatant was taken and placed in a scintillation vial containing 5 ml of scintillation cocktail and counted. The tip of the Eppendorf tube containing the pellet was cut off and resuspended in 0.3 ml of distilled water by spinning at 6000 g for 15 seconds in the microfuge. 0.3 ml was taken and placed in a scintillation vial containing 5 ml of scintillation cocktail and counted.

4. Calculation of the cell volume (Rottenberg 1979):

$$\text{Pellet volume} = \frac{\text{DPM } {}^{3}H_2O \text{ pellet}}{\text{DPM } {}^{3}H_2O \text{ supernatant} \times 6} \times 300 = y \text{ µl}$$

$$ECV = \frac{DPM\ ^{14}C\text{-dextran pellet}}{DPM\ ^{14}C\text{-dextran supern} \times 6} \times 300 = z\ \mu l$$

ICV = pellet volume minus ECV (= y - z µl)

2.11. Concentrations of labelled compounds used

a. ^{14}C-glycerol stock solution:

165 µCi µmol^{-1}, 50 µCi ml^{-1}

0.25 µCi = 1.5 nmol

Routinely 5 µl of ^{14}C-glycerol stock was used for each 5 ml algal sample. Therefore, it contained 1.5 nmol of ^{14}C-glycerol ≡ 300 nM = 0.3 µM = 0.3 µmoles l^{-1}. The total dpm present in 5 µl of ^{14}C-glycerol stock was 555 000.

b. ^{14}C-glucose stock solution:

246 µCi µmol^{-1}, 200 µCi ml^{-1}

1 µCi ≡ 4.1 nmol

5 µl of ^{14}C-glucose stock was used for each 5 ml algal sample. Therefore, it contained 4.11 nmol of ^{14}C-glucose ≡ 822 nM = 0.822 µM = 0.822 µmoles l^{-1}. The total dpm present in 5 µl of ^{14}C-glucose stock was 2.22×10^6.

c. ^{14}C-acetate stock solution:

53 µCi µmol^{-1}, 200 µCi ml^{-1}

1 µCi ≡ 18.9 nmol

5 µl of ^{14}C-acetate stock was used for each 5 ml algal sample. Therefore, it contained 18.9 nmol of ^{14}C-acetate ≡ 3780 nM = 3.78 µM = 3.78 µmoles l^{-1}. The total dpm present in 5 µl of ^{14}C-acetate stock was 2.22×10^6.

d. ^3H_2O stock solution

5 mCi ml^{-1} diluted to 25 µCi ml^{-1}

e. ^{14}C-dextran stock solution

50 µCi ml^{-1} diluted to 6.75 µCi ml^{-1}

All of the above were obtained from Amersham except for ^{14}C-dextran which came from New England Nuclear.

2.12. Calculation of the amount of labelled compounds taken up

An example of this calculation is shown below for uptake of ^{14}C-glycerol by the mutant grown in 0.4 M NaCl. The incubation period was 3 hours at 30 ^{0}C (Fig. 5.1).

10899 dpm in a 300 µl sample containing 2.03 x 10^6 cells.
ECV = 0.11 µl, amount of ^{14}C-glycerol in supernatant = 110 dpm μl^{-1}, therefore correction for ECV = 12 dpm.
10877 dpm in 2.03 x 10^6 cells
2.22 x 10^6 = µCi, therefore
4.90 x 10^{-3} µCi in 2.03 x 10^6 cells
0.25 µCi = 1.5 nmol (section 2.11), therefore
0.0294 nmol / 2.03 x 10^6 cells
= 0.0294 nmol / 2.03 x 10^6 x 66 x 10^{-15} l*
= 0.0294 nmol / 1.34 x 10^{-7} l
= 2.194 x 10^5 nmol/l
= 0.22 mmol/l
= 0.22 mM

* Volume of one cell adapted to 0.4 M NaCl, see section 2.10)

2.13. Hexokinase assay

The assay used to determine the presence of hexokinase in Dunaliella was a modification of the method described by

Parry and Walker (1966), in which the formation of ADP is measured by coupling it to the oxidation of NADH, in the presence of phosphoenolpyruvate (PEP) and an excess of both pyruvate kinase and lactate dehydrogenase.

The enzyme activity was measured using a Pye-Unicam SP1800 double-beam spectrophotometer, which maintains a constant temperature of 30 ^{0}C in the cuvette housing during the experiments. This spectrophotometer was connected to a Pye-Unicam AR.25 linear recorder. The cuvettes used in this experiment were 4 ml quartz cuvettes with a 1 cm light path. The assay mixture used is shown in Table 2.2. All assay components, except for the ATP and glucose are added and incubated at 30 ^{0}C for 2 minutes. ATP is then added and a linear rate of ATPase activity is obtained over 3 minutes. Finally glucose is added and the decrease in absorbance at 340 nm is read against a water blank for a further 3 minutes. The final activity is corrected for ATPase activity. Samples of cells containing 30 μg ml^{-1} chlorophyll were sonicated before assaying. A positive control was run using hexokinase from Boehringer Mannheim, Lewes, UK.

Table 2.2: Assay mixture used to determine hexokinase activity

Assay mixture (in 3 ml) contained:-

200mM triethanolamine hydrochloride buffer (pH 7.5)	1.5 ml
150 mM $MgSO_4$	0.15 ml
100 mM ATP	0.15 ml
1 M KCl	0.3 ml
50 mM PEP	0.045 ml
15 mM NADH	0.05 ml
1 M glucose	0.3 ml
pyruvate kinase.(2 mg ml^{-1})	0.005 ml
lactate dehydrogenase.(5 mg ml^{-1})	0.005 ml
extract and water to final volume	3.0 ml

2.14. Lipid extraction using Thin Layer Chromatography

a. Method 1 - phospholipid fraction

For the extraction of the lipids from the cells (Bligh and Dyer 1959), 1 x 150 ml batch cultures of the mutant and the wild type of 19/9 grown at 1.5 M NaCl were centrifuged in four glass 50 ml centrifuge tubes in a bench centrifuge at 3000 g for 10 minutes. The resulting pellets were each resuspended in 10 ml of chloroform: methanol: water (1: 2: 0.8 v/v) solution, whereby it was assumed and taken into account that 1 ml of cell water was already associated with the pellets. The cell suspension was incubated for 15 minutes at room temperature in 5 inch test tubes. After incubation the tubes were centrifuged at 3000 g for 10 minutes and the supernatant was collected in 2 x 50 ml glass tubes.

2.67 ml of chloroform and 3-5 ml (depending on cell density of the culture) of distilled water were added to the glass tubes and a 2 phase system, with the lipids and pigments in the lower phase, was established. The samples were left on ice for 10 minutes to settle. The lower phase was then dried under reduced pressure in the rotary evaporator and re-dissolved in 1 ml of chloroform.

The lipids were fractionated on a silicic acid column (3 g silicic acid, 100-200 mesh) by passing the following solvents through the column:

1. neutral lipids : 40 ml chloroform

2. glycolipids : 25 ml acetone : chloroform (4:1 v/v)

3. phospholipids : 25 ml methanol : chloroform (1:1 v/v)

The phospholipids, which are the membrane lipids separated into fraction 3, were dried under reduced pressure and resuspended in 1 ml of chloroform.

TLC plates (Merck silica gel 60, 0-25 mm thick) were activated in an oven at 105 ^{0}C for 60 minutes. The TLC plates were run with three standards, phosphatidylcholine, (Sigma product no. P5388), phosphatidylethanolamine (Sigma product no. P8798) and phosphatidylglycerol (Sigma product no. P5531). The solvent mixture in the tank consisted of chloroform : methanol : acetic acid : water (100 : 20 : 12 : 2.5 v/v/v/v). At the end of development the plates were air dried and exposed to iodine vapour to locate the lipids on the plates.

b. Method 2 - for total lipids

In this method the TLC plates were run with the total lipids, without separating them in the silicic acid column. The previously mentioned standards PC, PE and PG were also used in this method. The development solvents used in these experiments were, as in method 1 chloroform : methanol : acetic acid : water, but in the proportions of 170 : 30 : 20 : 7 (v/v/v/v/).

2.15. Isolation of the plasma membrane

The plasma membrane fraction was isolated according to the method of Peeler et al. (1989) using a dextran-PEG-2 phase system.

1. Two 150 ml batch cultures grown in 1.5 M NaCl were combined and centrifuged in the bench centrifuge at 3000 g for 10 minutes. The following isolation procedure was carried out at 4 ^{0}C.

2. The pellet was resuspended in 40 ml of disruption buffer (400 mM mannitol, 2 mM EDTA, 1 mM $MgCl_2$, 100 mM Na_2HPO_4 pH 8.0).

3. The cells were disrupted in the French Press, by running them through twice.

4. The disrupted cell suspension was then centrifuged in the bench centrifuge at 3000 g for 3.5 min. The resulting pellet, which contains the chloroplast membranes was kept in the freezer for future use.

5. 10 ml of the supernatant was added to each of two glass centrifuge tubes, which contained 15 ml of the dextran-PEG-2 phase system. This comprises of 10 % w/v dextran, MW 500 000, 10 % w/v PEG, 178 mM mannitol, 0.89 mM EDTA, 0.44 mM $MgCl_2$, 44.6 mM Na_2HPO_4 pH 8.0, and 320 mM NaCl

6. The tubes were mixed by shaking and centrifuged at 3000 g for 10 min.

7. The upper PEG rich phase was removed and centrifuged at 150 000 g for 1 h. The resulting pellet contained the plasma membrane fraction.

8. The lower, dextran rich green phase contained all cellular membranes, except for the plasma membrane and chloroplasts.

CHAPTER 3 COMPARISON OF THE PHYSIOLOGY OF DUNALIELLA PARVA 19/9 MUTANT AND WILD TYPE

3.1. Introduction

The success of Dunaliella in high salinity environments depends on the ability to balance the high salt content of the surrounding medium by synthesizing glycerol (section 1.6). The following study was initiated when a spontaneous mutant of D. parva 19/9 arose in a continuous culture. This mutant was characterised by its inability to retain glycerol within the cell and it was subsequently isolated and purified by conventional microbiological techniques of picking single colonies from solid media. The two strains have been maintained separately in 0.4 and 1.5 M NaCl medium for the entire duration of this project. As the electron micrographs (Figs. 3.1 and 3.2) show, there is no obvious detectable difference in the physical appearance between mutant and wild type. Mutant and wild type cells are of similar size and volume (see Table 5.4).

In order to determine whether the large loss of glycerol puts the mutant at a disadvantage when compared to the wild type, leakage data, growth rates, intracellular glycerol concentrations and starch contents, as well as their response to an osmotic shock, were investigated and compared between the two strains.

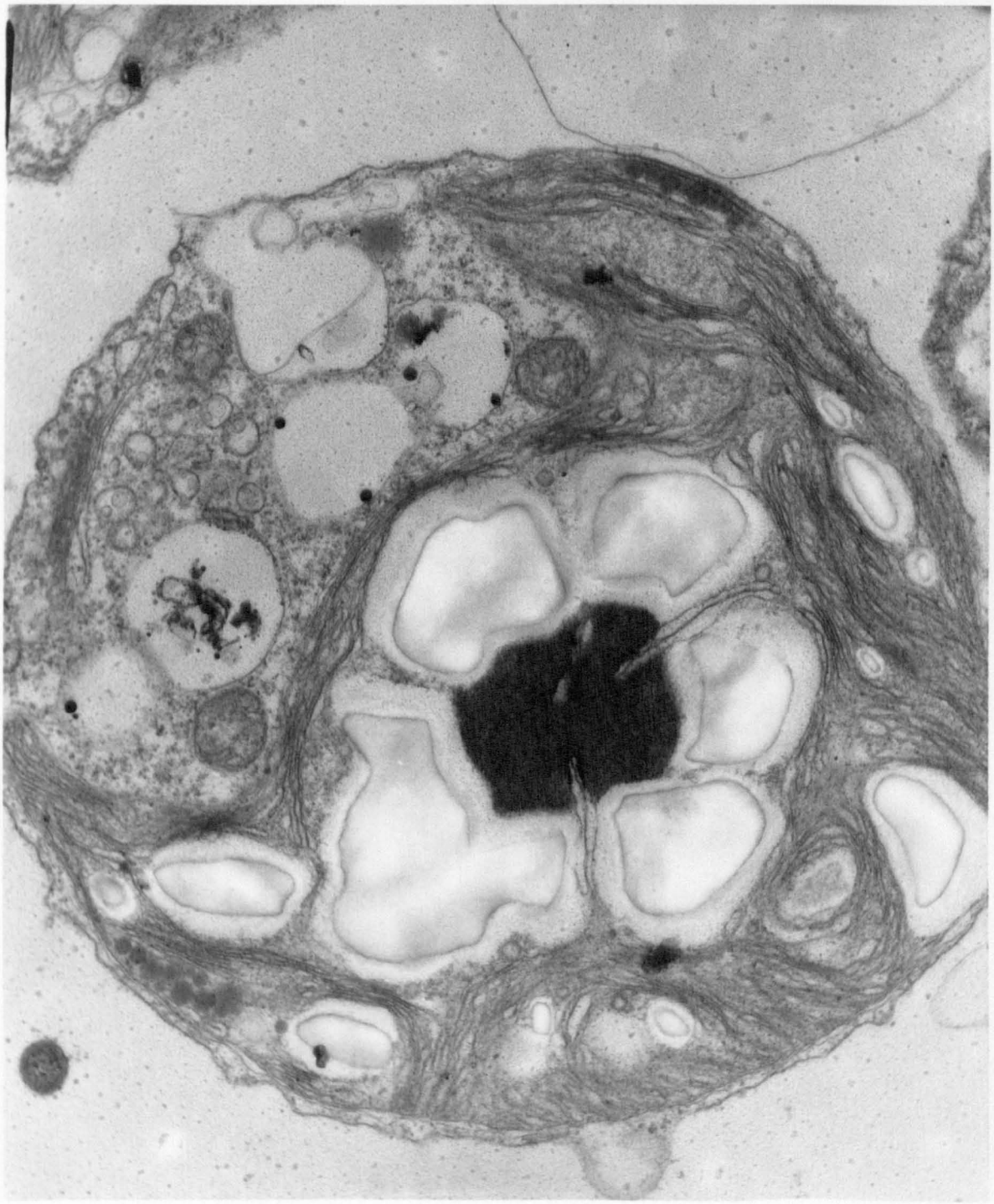


FIGURE 3.1 Electron micrograph of the mutant of Dunaliella parva in 0.4 M NaCl. The magnification is x 30000.

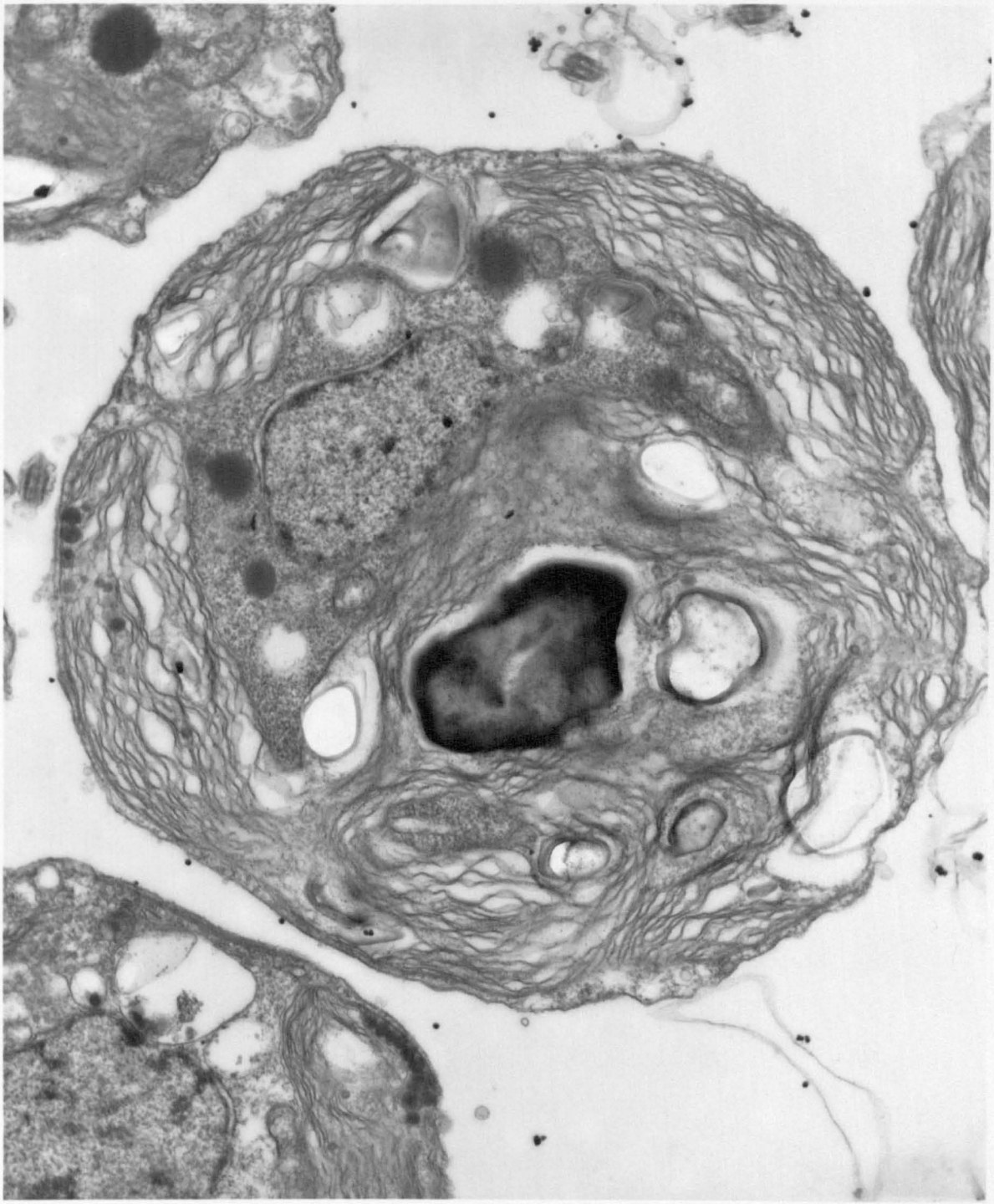


FIGURE 3.2 Electron micrograph of the wild type of Dunaliella parva in 0.4 M NaCl. The magnification is x 30000.

3.2. Results

3.2.1. Glycerol leakage

A constant level of glycerol must be maintained within the cell to balance the external osmotic potential due to NaCl. However, the mutant of D. parva leaks up to 75 % of the glycerol synthesized into the medium when grown in a salinity of 1.5 M NaCl (Table 3.1). In wild type cultures only 17 % of the glycerol produced was found in the medium, this is probably due to lysed cells. In 0.4 M NaCl medium wild type cells show no leakage at all, whereas mutant cells leak up to 73 % of their glycerol into the medium at this salinity (Table 3.1). Percentage leakage is calculated by determining the amount of glycerol in the cells plus medium and in the medium only, after the cells have been removed by centrifugation (section 2.6.3).

3.2.2. Growth rates

When growth rates of mutant and wild type were compared over a period of 14 days, no significant difference could be found at 0.4 M NaCl and at 1.5 M NaCl the mutant grew slightly faster than the wild type (Figure 3.3). Both mutant and wild type show a faster growth rate in medium

TABLE 3.1. Percentage glycerol leakage of Dunaliella parva mutant and wild type cells grown in 0.4 and 1.5 M NaCl batch culture. Glycerol was measured in cells plus medium and in the medium only and the percentage leakage calculated from the data.

STRAIN	SALINITY	TIME (days)		
		3	8	15
wild type	0.4 M NaCl	0 %	0 %	0 %
mutant	0.4 M NaCl	0 %	50 %	73 %
wild type	1.5 M NaCl	0 %	15 %	17 %
mutant	1.5 M NaCl	0 %	62 %	75 %

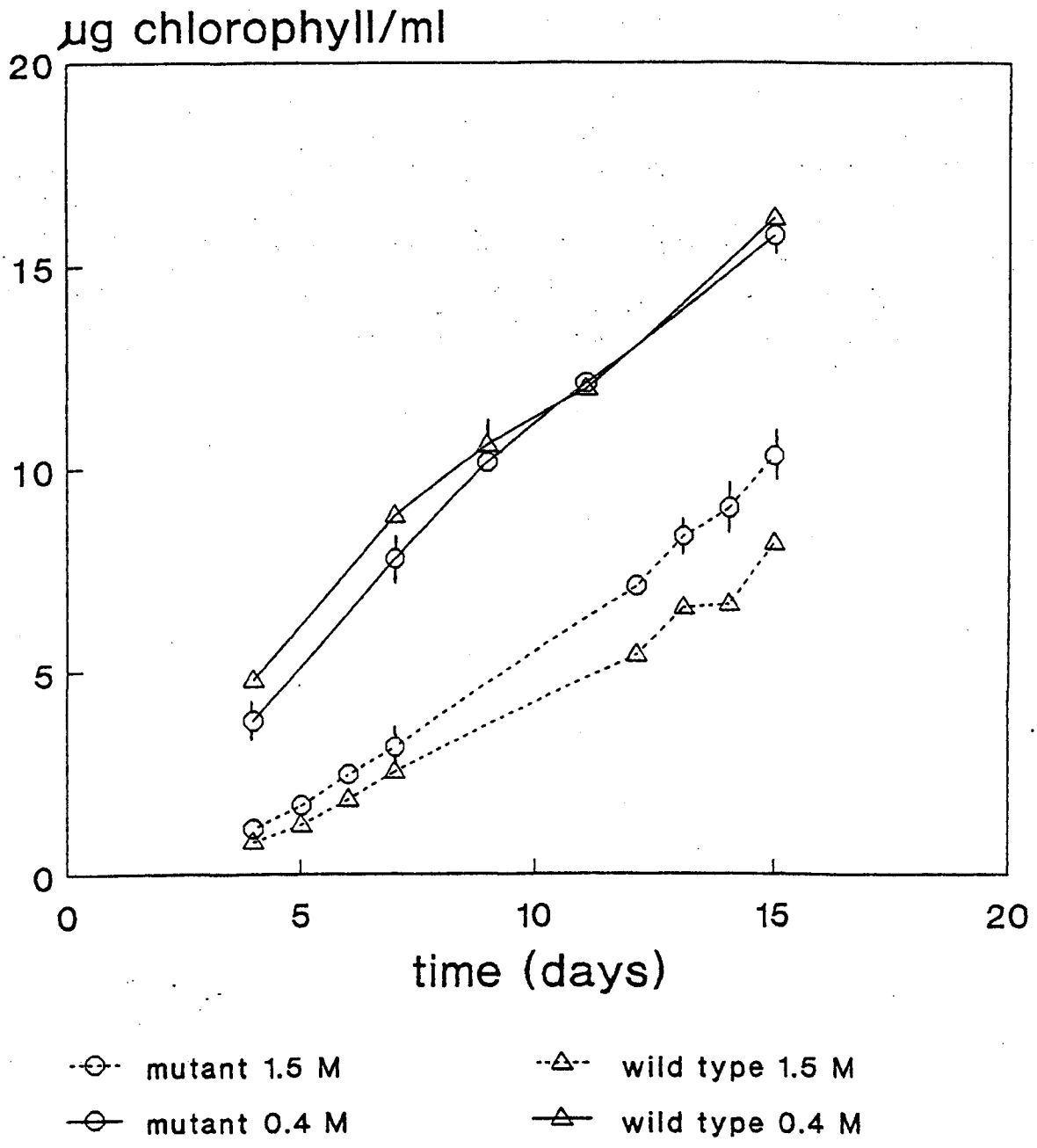


FIGURE 3.3 Growth curve of mutant and wild type cells in batch culture at 0.4 M and 1.5 M NaCl. Each point is the mean of nine replicates and the error bars represent plus or minus one standard error. If no error bars are shown, they are smaller than the symbols.

containing 0.4 M NaCl, compared to 1.5 M NaCl, indicating that the higher salinity imposes some stress on the cells.

3.2.3. Oxygen evolution and uptake

The leakage of intracellular glycerol by the mutant means that glycerol must be synthesized continuously at a high level to compensate for the loss. This implies that the mutant will require more energy than the wild type to grow, particularly at high salinity. In order to test this hypothesis rates of oxygen evolution and uptake were measured in mutant and wild type cells adapted to growth at 0.4 and 1.5 M NaCl (Table 3.2). In a salinity of 1.5 M NaCl the mutant shows higher oxygen evolution and uptake rates than the wild type. However, mutant and wild type cells grown in 0.4 M NaCl show the same rate of oxygen evolution and uptake, which probably reflects the lesser energetic stress imposed on the mutant at 0.4 M NaCl.

3.2.4. Intracellular glycerol and starch

When the level of intracellular glycerol of mutant and wild type cells was compared over a range of salinities from 0.1 to 3.5 M NaCl (Fig. 3.4), both were found to have very similar glycerol concentrations inside the cells. The amount of intracellular starch found in both

TABLE 3.2. O_2 evolution in the light and O_2 uptake in the dark of Dunaliella parva mutant and wild type cells grown in 0.4 and 1.5 M NaCl (rates in µmoles O_2 mg^{-1} chlorophyll h^{-1}). O_2 evolution was not corrected for photorespiration. The data are expressed as the mean of six replicates plus or minus standard error.

STRAIN	SALINITY	OXYGEN EVOLUTION	OXYGEN UPTAKE
wild type	0.4 M NaCl	29.77 ± 1.3	12.95 ± 0.5
mutant	0.4 M NaCl	29.45 ± 2.1	12.51 ± 0.8
wild type	1.5 M NaCl	23.03 ± 4.2	8.23 ± 1.9
mutant	1.5 M NaCl	34.87 ± 2.2	11.71 ± 1.9

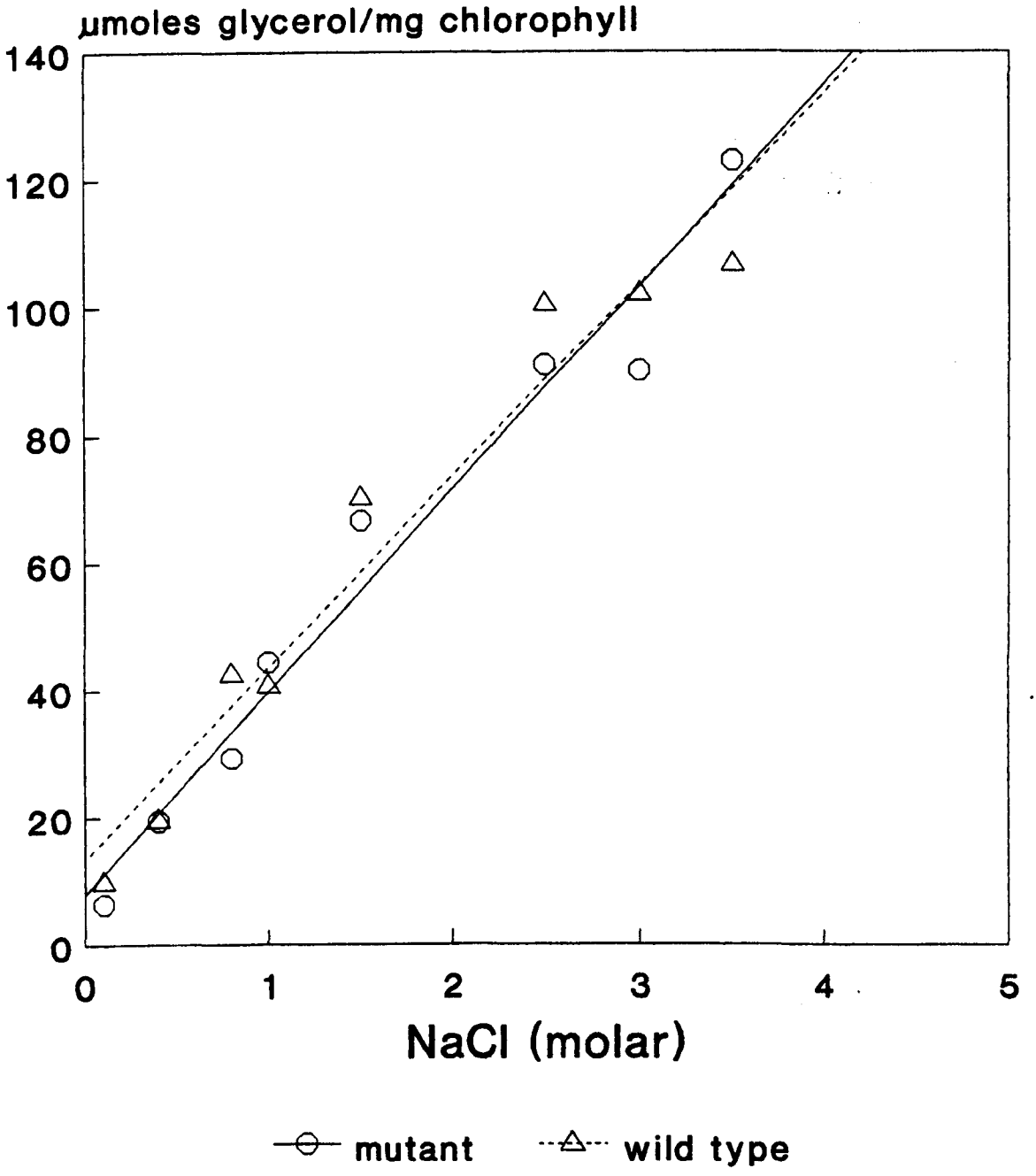


FIGURE 3.4 Comparison of the amount of intracellular glycerol found in mutant and wild type cells grown at a range of salinities. The correlation coefficients are 0.983 and 0.974 for mutant and wild type respectively.

strains after 8 days growth in a range of salinities is shown in Figure 3.5. The wild type showed higher levels of starch inside the cells throughout the range of salinities (0.1 to 3.5 M NaCl). Figure 3.6 shows the time course of starch deposition in mutant and wild type cells grown at 0.4 M NaCl. It is clear that the wild type deposits more starch at all times over the 14 day growth period.

3.2.5. Growth rates of mutant and wild type after osmotic shocks

The growth rate of mutant and wild type cells after an osmotic shock from 0.4 M to 1.5 M NaCl in comparison with unshocked controls growing in 0.4 M NaCl is shown in Figure 3.7. Both mutant and wild type show a similar rate of growth after the upshock, although they grow more slowly than the control cells grown at 0.4 M NaCl. When cells of both strains are shocked from 1.5 M NaCl to 2.5 M NaCl, both mutant and wild type grow at a slightly slower rate than control cells in 1.5 M NaCl (Fig. 3.8). Mutant and wild type cells, which have received a hyperosmotic shock by changing the salinity from 1.5 M to 3.5 M NaCl exhibit an even more reduced rate of growth when compared to the 1.5 M NaCl controls, but again there is no evidence that there is any difference between the shocked mutant and wild type cells (Fig. 3.9). The growth rate of mutant and wild type cells following a

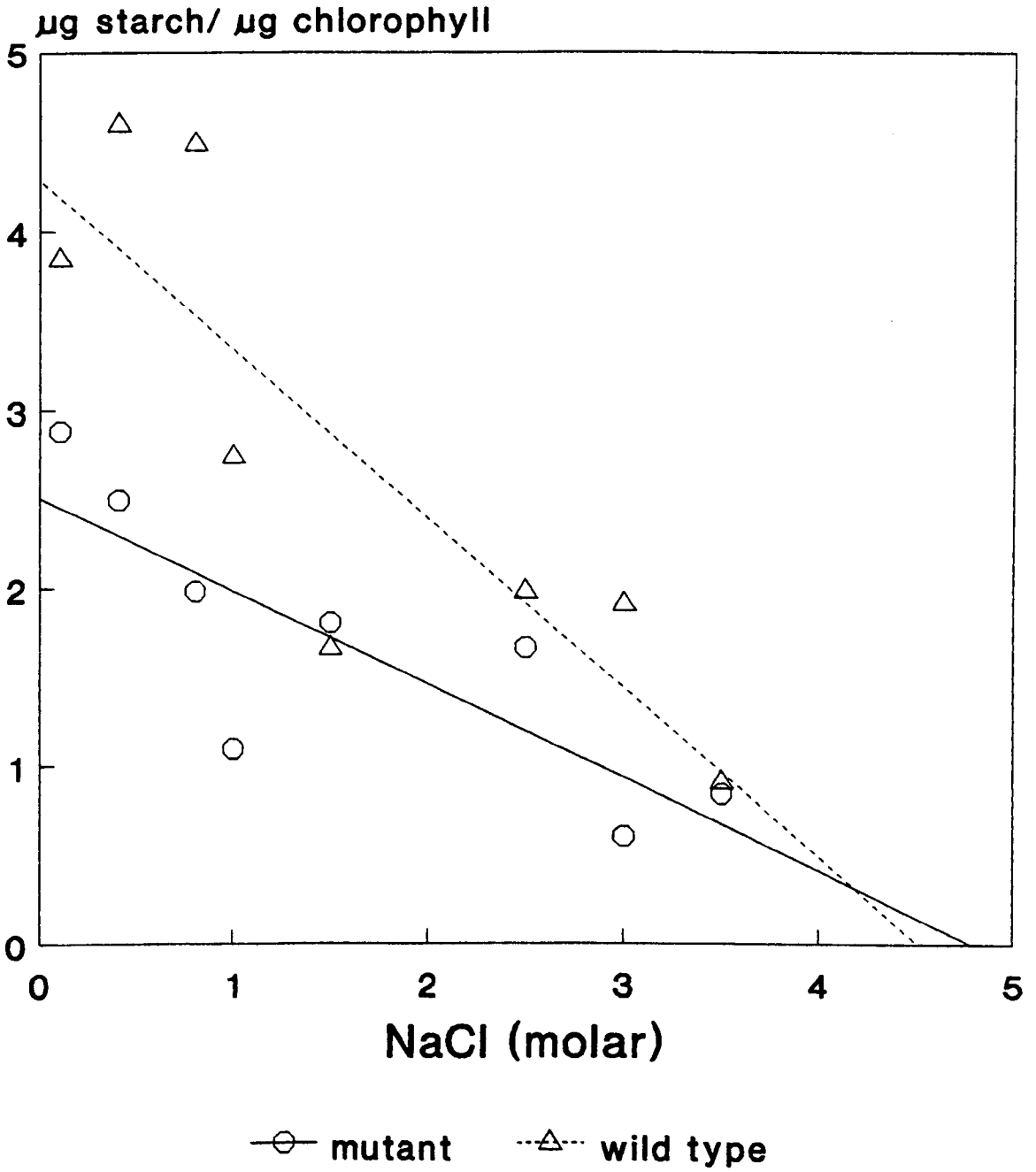


FIGURE 3.5 The amount of intracellular starch in mutant and wild type cells grown at various salinities. The correlation coefficients are 0.828 and 0.858 for mutant and wild type respectively.

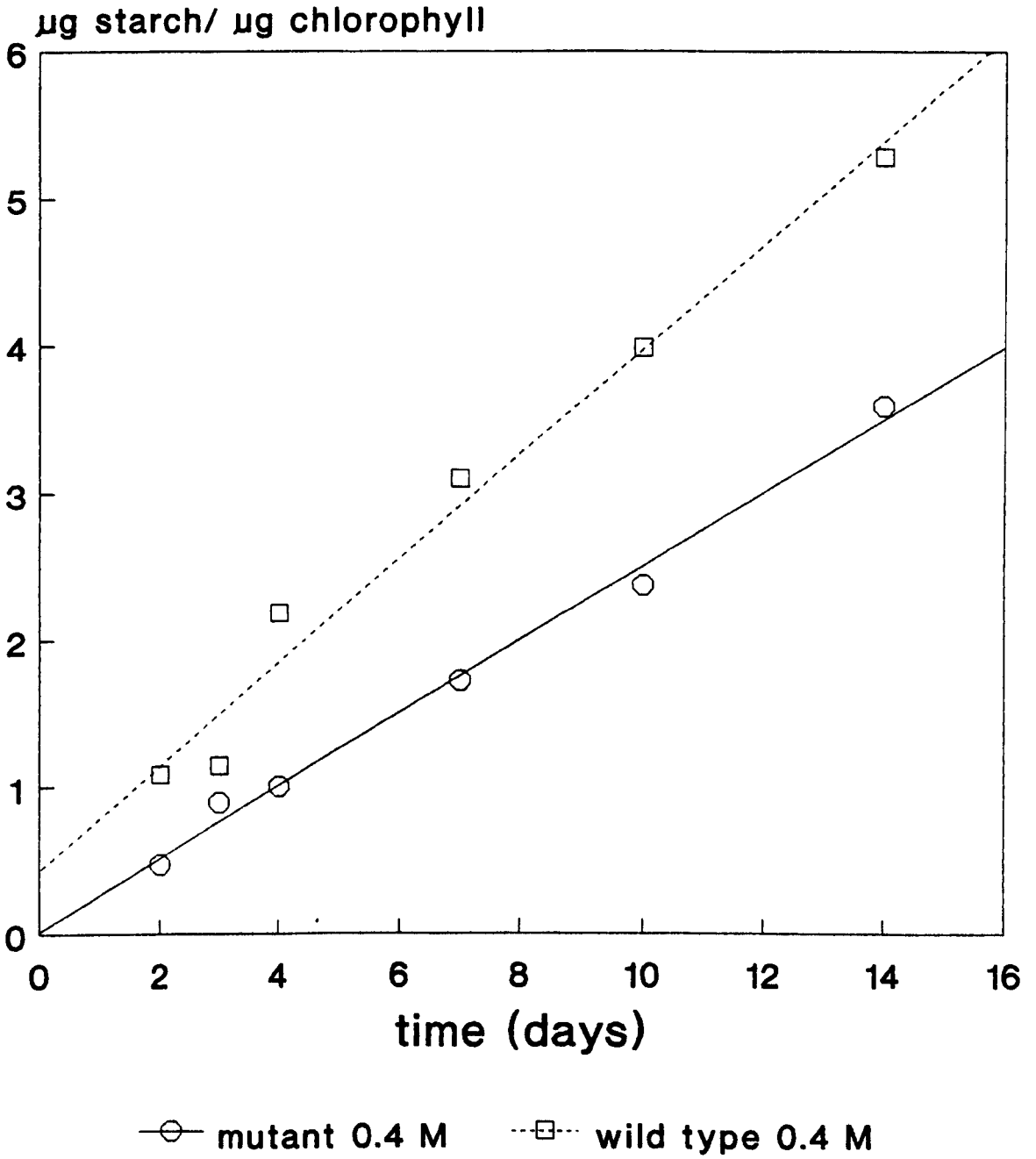


FIGURE 3.6 Starch deposition of mutant and wild type grown in 0.4 M NaCl.

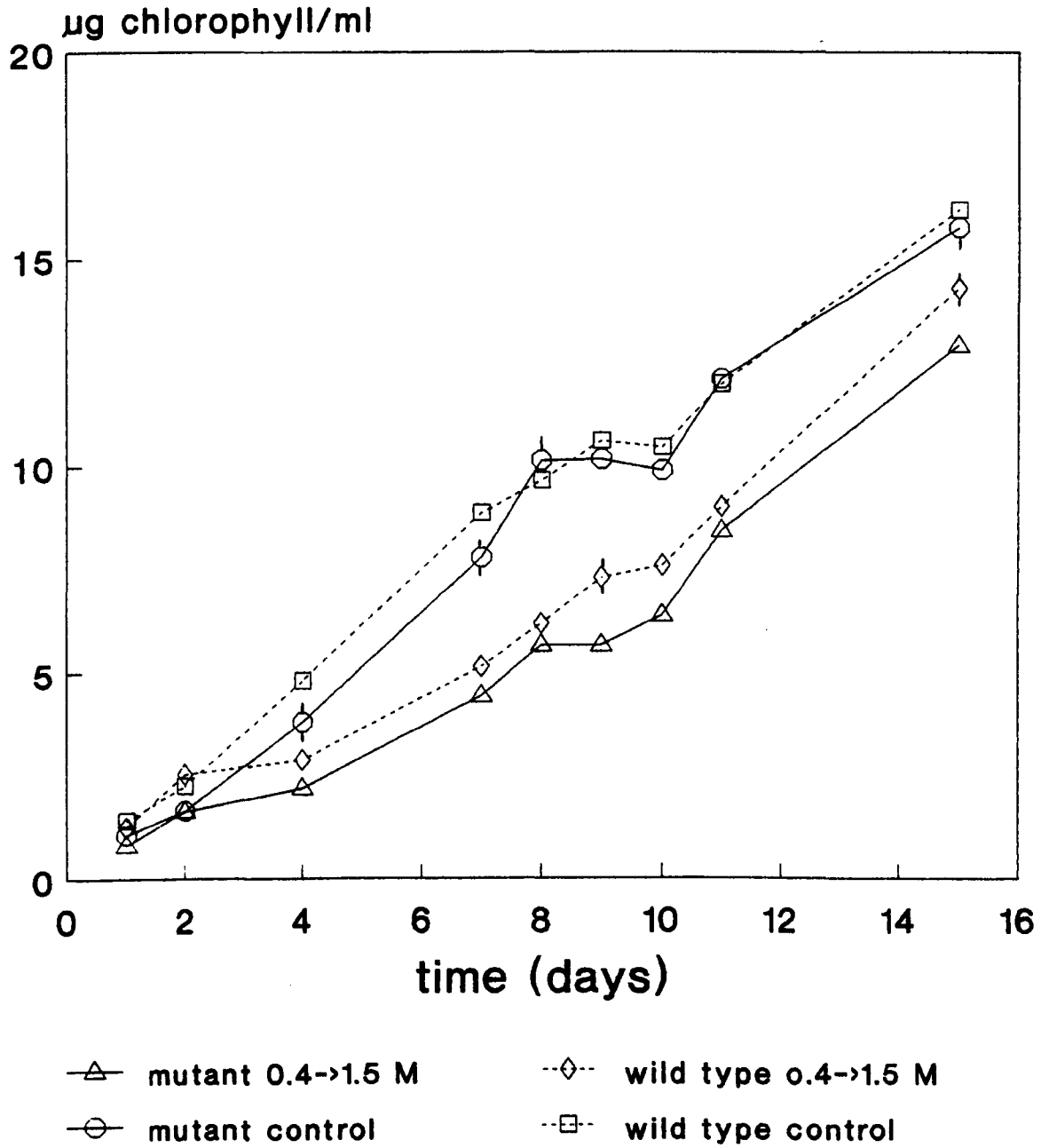


FIGURE 3.7 Growth rates of mutant and wild type after a hyper-osmotic shock from 0.4 M NaCl to 1.5 M NaCl. Controls remained in 0.4 M NaCl.

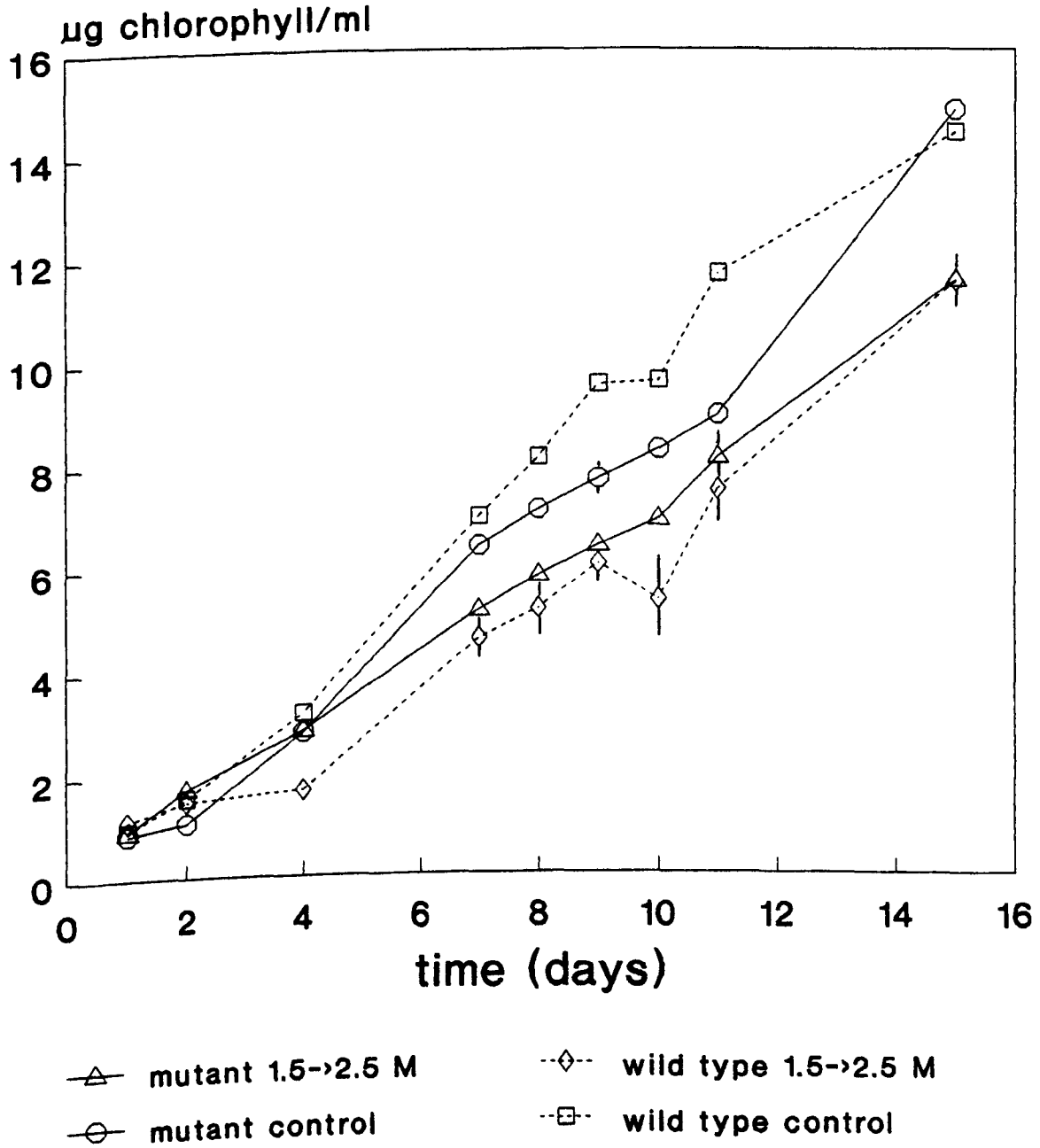


FIGURE 3.8 Growth rates of mutant and wild type after transfer from 1.5 M NaCl to 2.5 M NaCl. Controls remained in 1.5 M NaCl.

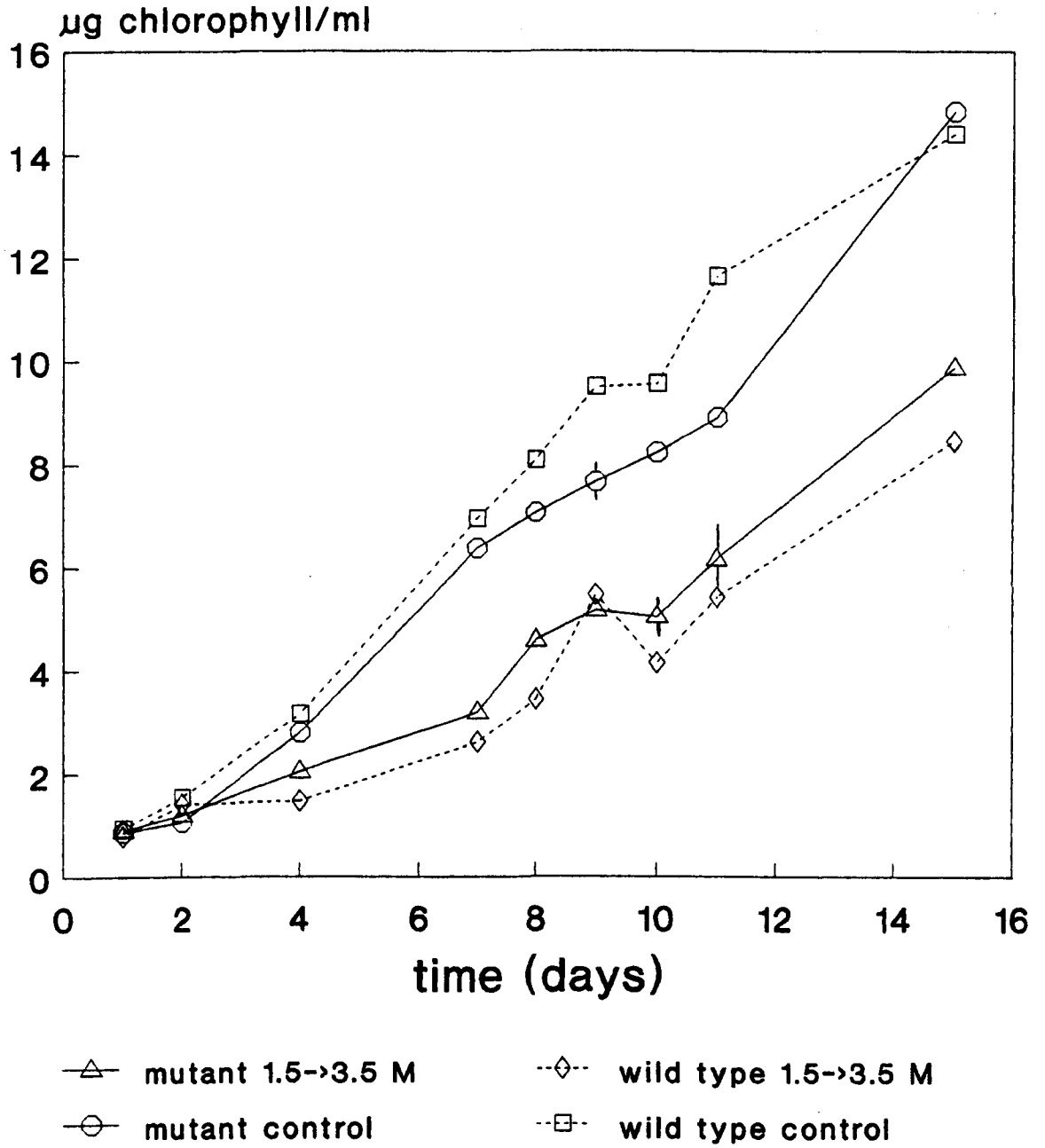


FIGURE 3.9 Growth rates of mutant and wild type after transfer from 1.5 M NaCl to 3.5 M NaCl. Controls remained in 1.5 M NaCl.

hypoosmotic shock from 1.5 M to 0.4 M NaCl is shown in Figure 3.10. Shocked mutant cells grow at the same rate as mutant and wild type control cells in 1.5 M NaCl medium, however shocked wild type cells grow at a slightly slower rate.

3.2.6. Oxygen evolution and uptake after osmotic shock

In the following experiment mutant and wild type cells grown in 0.4 M NaCl were transferred to 1.5 M NaCl medium and 1.5 M NaCl grown cells to 0.4 M NaCl. Oxygen electrode measurements were carried out at 5 min, 60 min and 240 min after the shock (Table 3.3). After the upshock, mutant and wild type cells immediately show much reduced rates of oxygen uptake and evolution. The O_2 evolution rate of mutant cells is decreased to 45 % of the control rate and the uptake rate is decreased to 66 % of the control rate. Rates of wild type cells are down to 64 % and 52 % of the control rates respectively. After 60 min both mutant and wild type cells have recovered and O_2 evolution and O_2 uptake are close to control rates (Table 3.3).

On exposure to the downshock from 1.5 M to 0.4 M NaCl, both mutant and wild type show reduced rates of oxygen evolution immediately after the shock (Table 3.3). Mutant rate decreases to 71 % and wild type rate to 41 % of the control rates. However, the rate of oxygen uptake by the

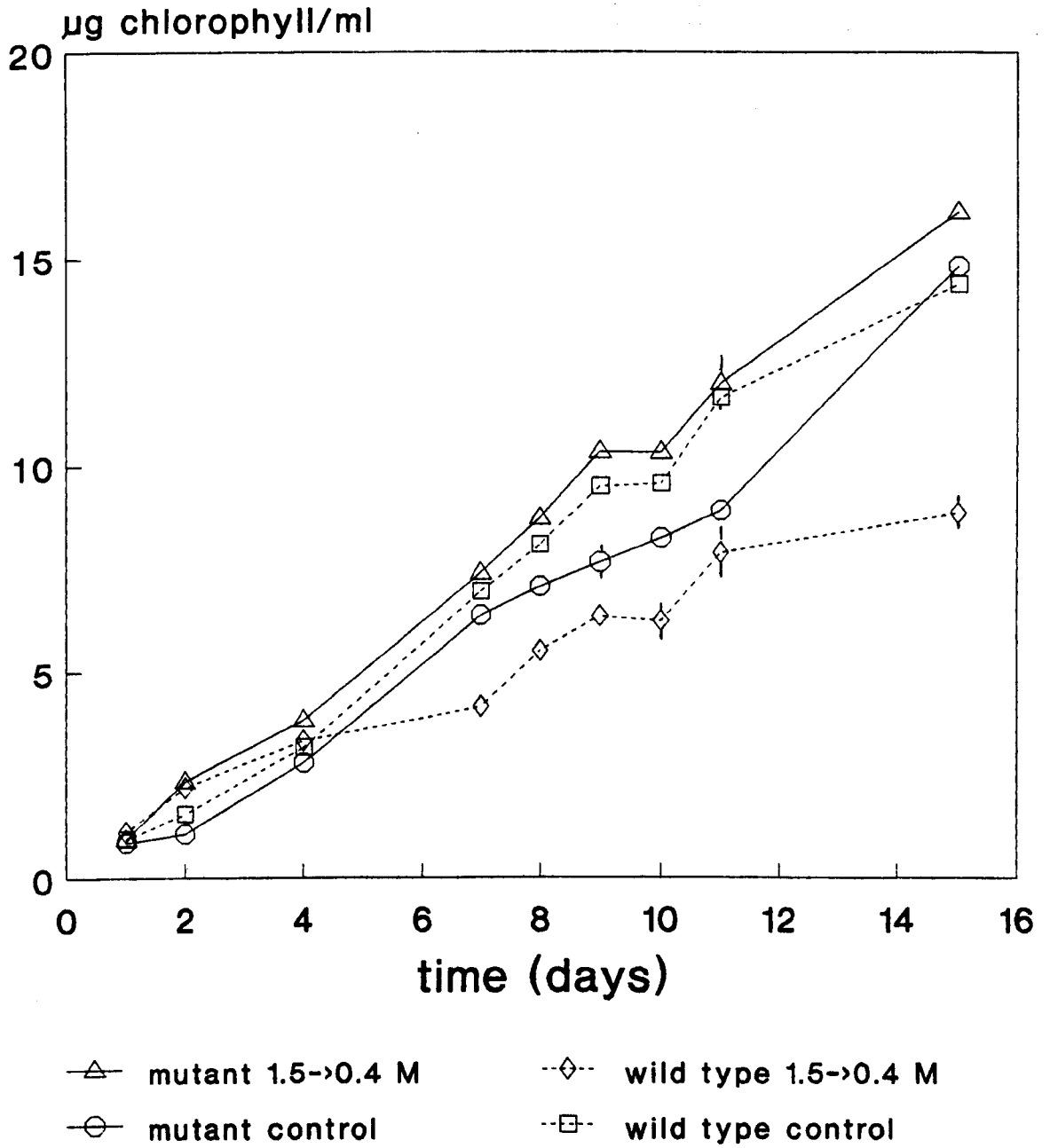


FIGURE 3.10 Growth rates of mutant and wild type after transfer from 1.5 M NaCl to 0.4 M NaCl. Controls remained in 1.5 M NaCl.

mutant is the same as the control and the wild type rate is much higher than the control. The rates of O_2 evolution and O_2 uptake shown by the mutant decrease over the 4 hour duration of the downshock experiment. This contrasts with complete recovery of the O_2 evolution rate by the wild type over the same period and the elevated rate of O_2 uptake shown by the wild type throughout the downshock experiment (Table 3.3).

3.3. Discussion

Dunaliella cells normally retain the compatible solute glycerol within the cell and it is assumed that this is essential for the proper functioning of their osmoregulatory system (section 1.6). However, the mutant of Dunaliella parva 19/9, which we have isolated leaks large amounts of glycerol into the medium under isotonic conditions (Table 3.1). The leakage is not due to cell lysis as the growth rate of the mutant is the same as that of the wild type during the period of glycerol leakage (Table 3.1, Figure 3.3). Rates of oxygen evolution and oxygen uptake in mutant cells grown in 1.5 M NaCl are higher than those of the wild type in the same salinity. However, mutant and wild type rates are the same at 0.4 M NaCl. This indicates clearly that the high salinity imposes stress on mutant cells, which require more energy to produce glycerol at a higher rate in order to compensate for the leakiness of the membrane. Despite

TABLE 3.3. Oxygen evolution in the light and oxygen uptake in the dark by the mutant and wild type after hyper- and hypo-osmotic shocks (rates in µmoles O_2 mg^{-1} chlorophyll h^{-1}). O_2 evolution was not corrected for respiration. The data refer to the mean of six replicates plus or minus standard error.

Time	Salinity	Oxygen evolution	Oxygen uptake
<u>Mutant</u>			
control	1.5 M NaCl	56.6 ± 3.23	18.28 ± 2.1
5 min	1.5 -> 0.4 M	40.0 ± 2.83	18.26 ± 3.2
60 min		43.67 ± 1.87	13.04 ± 0.13
240 min		29.94 ± 3.18	7.58 ± 0.44
<u>Wild type</u>			
control	1.5 M NaCl	38.94 ± 2.69	15.95 ± 3.23
5 min	1.5 -> 0.4 M	15.89 ± 2.13	22.28 ± 1.82
60 min		30.86 ± 0.58	22.49 ± 0.49
240 min		37.02 ± 1.46	23.1 ± 0
<u>Mutant</u>			
control	0.4 M NaCl	38.45 ± 1.04	9.45 ± 0.94
5 min	0.4 -> 1.5 M	17.45 ± 3.21	6.28 ± 0.8
60 min		35.23 ± 0.84	10.5 ± 1.14
240 min		45.36 ± 5.3	8.41 ± 0.97
<u>Wild type</u>			
control	0.4 M NaCl	42.4 ± 2.69	12.91 ± 3.41
5 min	0.4 -> 1.5 M	27.02 ± 1.19	6.78 ± 0.18
60 min		40.62 ± 0.43	10.03 ± 0.18
240 min		36.28 ± 1.53	12.94 ± 0.49

the large loss of glycerol by mutant cells, the amount of glycerol found inside the cells is the same as in wild type cells up to a salinity of 3.5 M NaCl (Figure 3.4). Without the ability of the mutant to produce sufficient amounts of glycerol to balance the external salinity and make up for the glycerol lost into the medium, the mutant cells could not grow in high salinity environments. The wild type accumulates the storage product starch in higher amounts than the mutant (Figs. 3.5 and 3.6). This indicates, that a larger proportion of fixed CO_2 must be converted to glycerol in the mutant cells to make up for the loss of glycerol to the medium. In the wild type excess CO_2 fixed is converted to the storage product starch.

Growth rates after osmotic shocks reflect the severity of the shock and the salinity of the new medium, in which the cells were resuspended. Both mutant and wild type cells adapt quickly to a higher or lower salt concentration, but the rate of growth following the shock decreases particularly after an increase in salinity (Figs. 3.7 - 3.10). The immediate response to osmotic shocks is reflected in O_2 evolution and uptake data shown in Table 3.3, whereby the rates are much reduced initially and recover to normal levels within the first hour after the shock. However, mutant cells, which have higher rates in 1.5 M NaCl and which were transferred from 1.5 M to 0.4 M NaCl show a steady decrease to only 50 % of the control rates after 4 hours (Table 3.3). This

indicates again, that at 0.4 M NaCl the cells are less stressed and the mutant adapts to the lower salinity with reduced rates of photosynthesis and respiration. Wild type cells however, actually show increased rates of O_2 uptake, following the downshock. The reason for this is not clear, but it might be connected to the bioenergetics of the conversion of glycerol into starch following the osmotic downshock (Kaplan et al. 1980).

The leakage data agree with earlier data of Enhuber and Gimmler (1980) who also found substantial leakage from another strain of D. parva. Enhuber and Gimmler commented on the higher energetic costs that this growth strategy would entail. This seems to be the case in our study of the mutant of 19/9 and is reflected in the higher rates of oxygen evolution and uptake at 1.5 M NaCl when compared to the wild type (Table 3.2). In contrast to the earlier data, Gimmler and Hartung (1988) reported on the low permeability of the plasma membrane of D. parva for glycerol. It is suggested that this impermeability is due to the unusual lipid composition of the plasma membrane of Dunaliella. (Evans et al. 1982, Sheffer et al. 1986, Peeler et al. 1989). The lipid composition of mutant and wild type cells of D. parva 19/9 is shown in Chapter 6.

The results from these experiments show that the leaky mutant is not at a disadvantage when compared to the wild type. This suggests that wild type cells do not utilize all the energy they produce and are running their

cellular metabolism below full capacity. This may be an adaptation which enables these microorganisms to cope with any sudden stress imposed on the cells by changes in the extreme environments they live in (Hard and Gilmour 1991).

CHAPTER 4 THE MUTANT GROWN IN CONTINUOUS CULTURE IN THE CHEMOSTAT

4.1. Introduction

Cultivation of microorganisms in continuous culture is very useful for the study of the physiology and genetics of these organisms. The most common type of continuous culture system used in the laboratory, the chemostat, is based on the continuous addition of fresh medium (in which one of the nutrients is set to limit growth) to a culture vessel. The size of the culture vessel can range from test tube size to thousands of litres, and it has an overflow device to control culture volume (Tempest 1970). Various physical parameters including pH, temperature, O_2 and CO_2 are continuously controlled. Once equilibrium is reached the culture is in a steady state in which cell number and nutrient availability are constant, controlled by the flow rate of the fresh medium into the chemostat and the concentration of the limiting nutrient in the influent medium. The chemostat allows growth rate (which depends on rate of medium addition) and population density (which depends on concentration of limiting nutrient in the influent medium) to be adjusted independently of each other. The limiting nutrient can be any essential element, but is commonly the carbon source.

In contrast to the nutrient limited culture of bacteria in the chemostat, the cultivation of phototrophic microorganisms, such as microalgae, is often limited by the light intensity or the concentration of CO_2. Light energy supplies the energy required for photosynthesis and CO_2 or HCO_3^- is used as the sole source of carbon (see section 4.2.5). All other nutrients needed for growth are present in excess of the cultures' requirements (Lee at al. 1985). The data in this chapter describe the growth of the mutant of D. parva 19/9 in the chemostat under CO_2 limited condition (section 2.2.2). Various parameters (e.g. temperature, pH and salinity) were changed one at a time to see their effect on glycerol leakage.

4.2. Results and Discussion

4.2.1. Effect of temperature on glycerol leakage

These experiments were carried out to determine, whether increased temperature has a direct effect on the glycerol loss by the mutant. A rise in temperature increases the speed at which chemical and enzymatic reactions in the cell take place. However, at high temperatures proteins, nucleic acids and other cellular components become inactivated and eventually irreversibly damaged.

Figure 4.1 shows that in the first few days after inoculation and on reaching steady state growth in

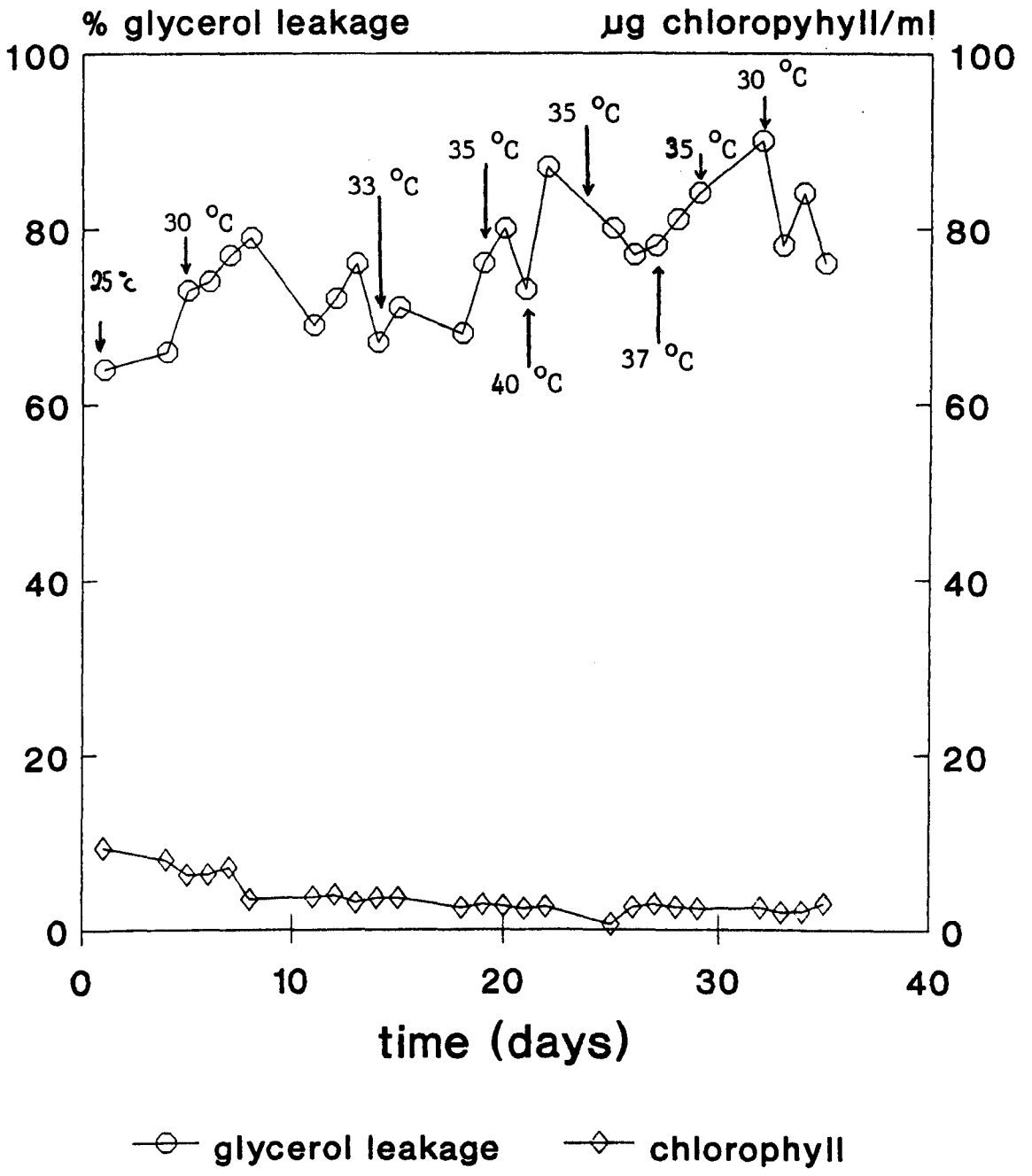


FIGURE 4.1 The effect of increased temperature on the glycerol leakage and chlorophyll content of a mutant culture grown in the chemostat (dilution rate 0.0096 h^{-1})

continuous culture in 1.5 M NaCl medium the glycerol leakage of the mutant fluctuates between 64 and 79 %. The percentage leakage figure is derived from the amount of glycerol found in the medium as a percentage of total glycerol present in the medium and inside the cells. A slow increase in temperature from 30 to 40 ^{0}C over a period of 8 days indicated a trend of increased glycerol leakage into the medium up to a level of 87 %. One day after increasing the temperature to 40 ^{0}C the cells were dying with only a small number of cells in the culture motile and viable. After decreasing the temperature to 35 ^{0}C the culture recovered and the glycerol leakage fell over the following two days down to 77 %. An increase in temperature by 2 ^{0}C again showed an increased leakage of up to 84 % and 2 days after the temperature was switched back to 35 ^{0}C the loss of glycerol increased up to 90 %. The culture recovered after changing the temperature back to 30 ^{0}C and the leakage fell to 76 %.

The results indicate that at around 37 ^{0}C the cells are at their upper temperature limit for metabolic processes to proceed normally and that at 40 ^{0}C they experience damage to their membranes and consequent disruption of metabolic functions as suggested by Gimmler et al. (1978). The high amount of glycerol found in the medium following a rise in temperature to 40 ^{0}C is due to increased leakage through the plasma membrane because of a re-organisation of the membrane lipid components (Wegmann et al. 1980) and also lysis of dying cells which

releases intracellular glycerol into the medium. Very few cells survive this temperature stress and the culture is on the verge of collapse as the chlorophyll data show (Fig. 4.1). A decrease to a lower temperature allows the culture to recover slowly over the following 2 to 3 days.

Over the time span of the experiment the leakage data are rather variable and the link between increased temperature and glycerol loss is probably no more than a trend. However, further experimentation may show a direct connection between the temperature and the loss of glycerol into the medium.

4.2.2. Effect of salinity on glycerol leakage

The effect of increased salt concentration on the glycerol loss by the mutant was investigated. As salinity of the medium increases, more glycerol is produced by the cells to adapt to the change in osmotic pressure (see section 1.7). This could lead to increased glycerol loss by the mutant as the cells will have a higher turnover of glycerol.

The culture growing in steady state at 1.5 M NaCl leaked 76 to 77 % of the glycerol produced into the medium over a period of 5 days (Fig. 4.2). On the fifth day medium containing 3 M NaCl was connected at a flow rate of 0.27 ml min^{-1} to slowly raise the salinity in the chemostat.

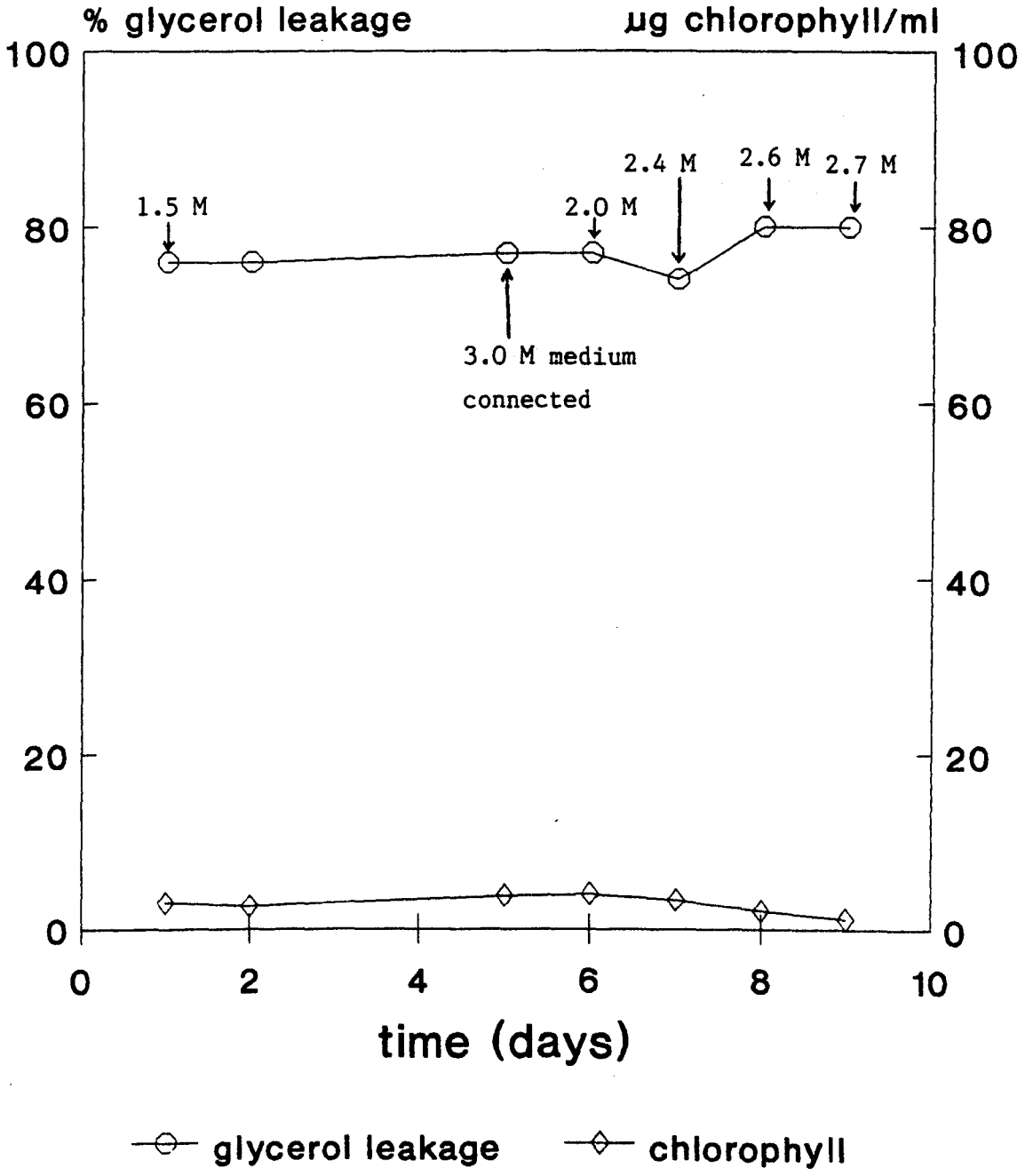


FIGURE 4.2 Effect of increased salinity on the glycerol leakage and chlorophyll content of a mutant culture grown in the chemostat (dilution rate = 0.0108 h^{-1})

Over the following days the salt concentration increased up to 2.7 M which resulted only in small changes in glycerol loss up to 79 %. However, above 2.7 M NaCl the cells had reached their upper salt tolerance limit in continuous culture, the culture died and the experiment was terminated.

Over the time span of the experiment despite the increase in salinity, the leakiness of the mutant membrane remained virtually unchanged until the upper salinity limit of the cells was reached. This indicates that the percentage of glycerol leaked into the medium is stable, irrespective of the actual amount of glycerol present in the cells. As the salinity increases in the medium so does the concentration of intracellular glycerol, which means that at the same percentage leakage the amount of glycerol lost into the medium increases. Over the period of the experiment the glycerol found in the medium increased from 1.31 umoles glycerol ml^{-1} to 1.74 umoles glycerol ml^{-1} with the dilution rate remaining unchanged.

4.2.3. Effect of high pH on glycerol leakage

On reaching steady state when grown at pH 7.8 to 8.0 (normal setting in other experiments) the mutant cells leaked around 86 % of glycerol into the medium (Fig. 4.3). As the pH was further increased step by step to 8.4 over the following days the percentage leakage remained

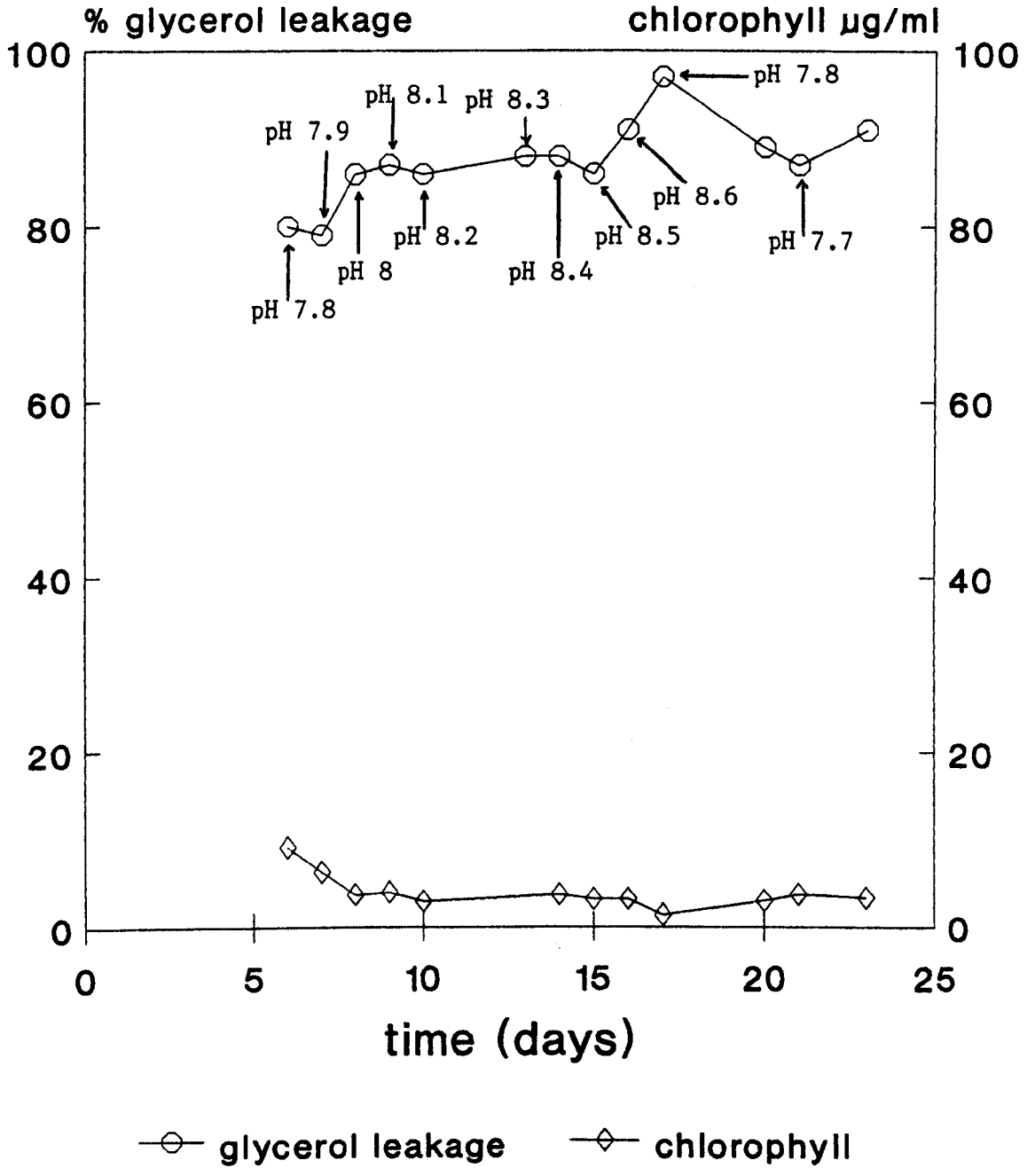


FIGURE 4.3 The effect of increased pH on the glycerol leakage and chlorophyll content of a mutant culture grown in the chemostat (dilution rate 0.0096 h^{-1})

unchanged. When the pH was increased to 8.5 the mutant leaked 91 % and after changing the pH to 8.6, 97 % of the glycerol was lost into the medium. At this stage the culture was dying and only recovered after adjusting the pH to 7.8, which resulted in a drop in leakage to 87 % over a period of 4 days.

It is evident that the upper pH limit for Dunaliella cells is below pH 8.5/8.6. This agrees with batch culture data, where after approximately 14 days the pH was found to be around pH 8.5 and this high pH, together with decreased nutrient levels and shading due to high cell density, stopped growth.

4.2.4. Effect of light intensity on glycerol leakage and carotenoids

Carotenoids are orange-yellow pigments associated with chlorophylls in the thylakoid membrane. Carotenoids have a role in light harvesting, whereby particularly the carotenoids, beta-carotene and the xanthophylls, transfer absorbed light energy to chlorophyll a with high efficiency (Young and Britton 1990a). In the algae this efficiency varies from class to class, in the green algae it is nearly as high as chlorophyll absorbed light (Goodwin 1980). The second and probably the most important function of carotenoids is that of protection of the photosynthetic apparatus against photosensitized

oxidation. The pigment-protein complexes scatter excess energy in high light conditions, which results in singlet excited chlorophyll giving rise to the triplet-state chlorophyll (^{3}CHL), which passes energy to oxygen, so forming the highly reactive and destructive oxygen species $^1O_2$ (singlet-oxygen). Carotenoids protect by either quenching the energy from ^{3}CHL, thus preventing the formation of singlet-oxygen in the first instance or by reacting directly with the $^1O_2$ once it is formed (Young and Britton 1990a and b). The third function of carotenoids, particularly beta-carotene, is suggested to be that of a "carbon sink" (Borowitzka and Borowitzka 1988) coming into use when metabolic pathways are inhibited because of lack of substrate compounds and photosynthetic products. The excess carbon is diverted to beta-carotene since it is a neutral compound. The accumulation of carotenoids depends on growth conditions which impose a stress on the culture, such as high salinity, high temperature, high light intensity or nitrate limitation, so that the growth rate is low (Ben-Amotz and Avron 1983, Ben-Amotz 1987, Borowitkza and Borowitzka 1988). High levels of lead and copper have also been reported to induce high accumulation of beta-carotene (Pace 1977). Amongst the Dunaliella species, D. salina has the highest content of beta-carotene, up to 14 % of dry weight (Ben-Amotz et al. 1982, Borowitzka et al. 1984). This is of great commercial interest in the field of algal biotechnology (see section 1.9, Ben-Amotz and Avron 1990).

The mutant was grown in CO_2 limited continuous culture with a light intensity of 190 µE m^{-2} s^{-1}. On reaching steady state the leakage of glycerol increased from 59 to 75 % (Fig. 4.4). The carotenoids also increased from 0.364 to 0.443 µg carotenoids μg^{-1} chlorophyll. The amount of carotenoids was calculated from absorption spectra such as these shown in Figure 4.5. The addition of a further light ring, which raises the illumination of the culture to 230 µE m^{-2} s^{-1} resulted in higher synthesis of carotenoids to 0.468 µg μg^{-1} chlorophyll, whereby the glycerol loss decreased slightly to 71 % (Figure 4.4). Upon illumination of the culture with 260 µE m^{-2} s^{-1} the carotenoids increased to 0.574 µg ug^{-1} chlorophyll over the following 5 days. The glycerol leakage during this time span increased in the first instance to 89 %, then fell to 71 % to go up to 84 % on the next day. When the light intensity was switched back to 230 µE m^{-2} s^{-1}, carotenoids decreased over the next 6 days to just below the original level at the beginning of the experiment of 0.356 ug carotenoids/ µg chlorophyll. The glycerol loss increased initially to 93 %, then fell to 90 %.

The data shown in Figures 4.4 and 4.5 clearly demonstrate an increase in carotenoid content of the cells with increasing light intensity, as has been previously demonstrated (Loeblich 1982, Ben-Amotz and Avron 1983). With the increase in carotenoids, a trend of increased

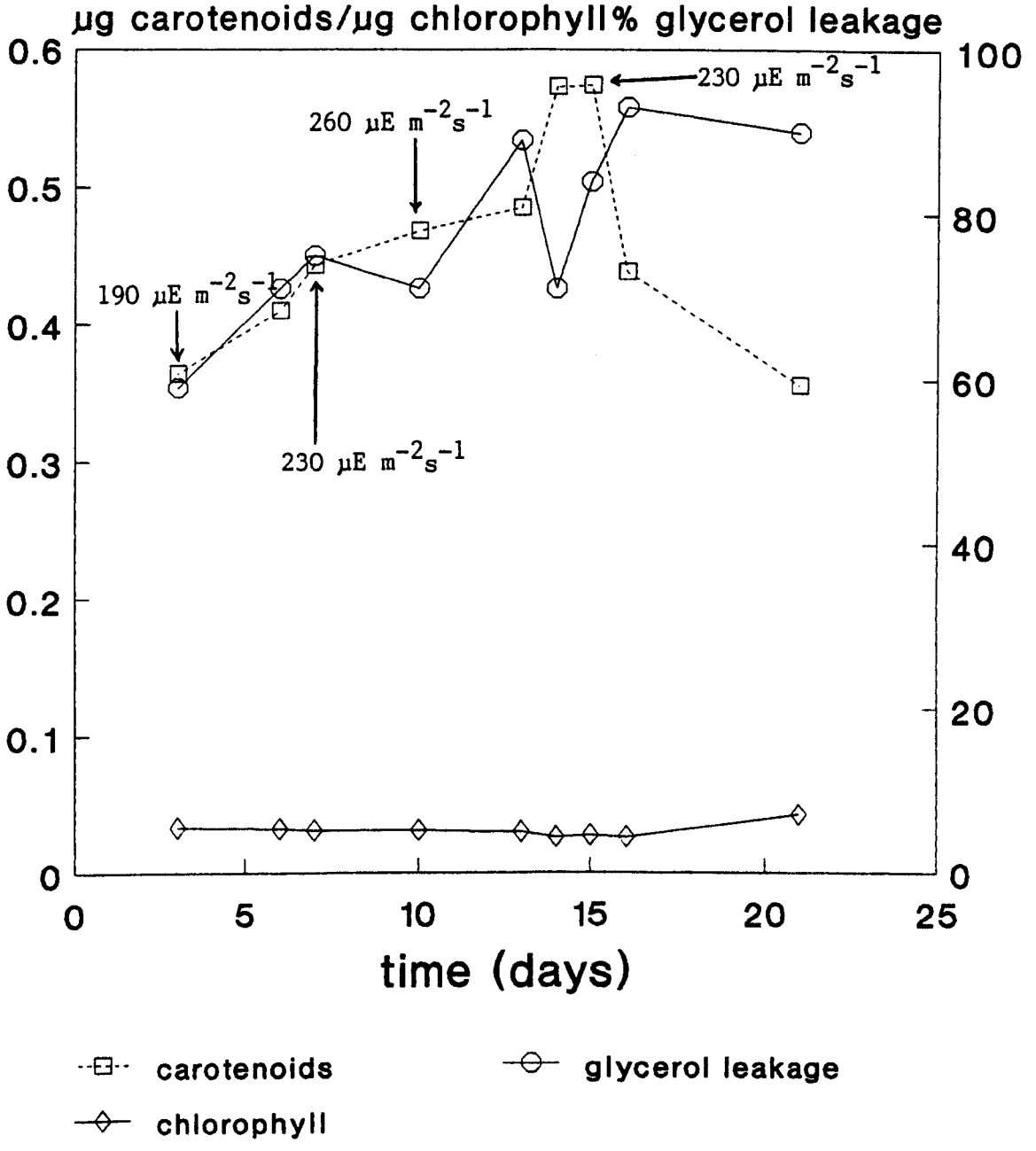


FIGURE 4.4 The effect of increased illumination on glycerol leakage, carotenoid content and chlorophyll content of a mutant culture grown in the chemostat (dilution rate 0.0128 h^{-1})

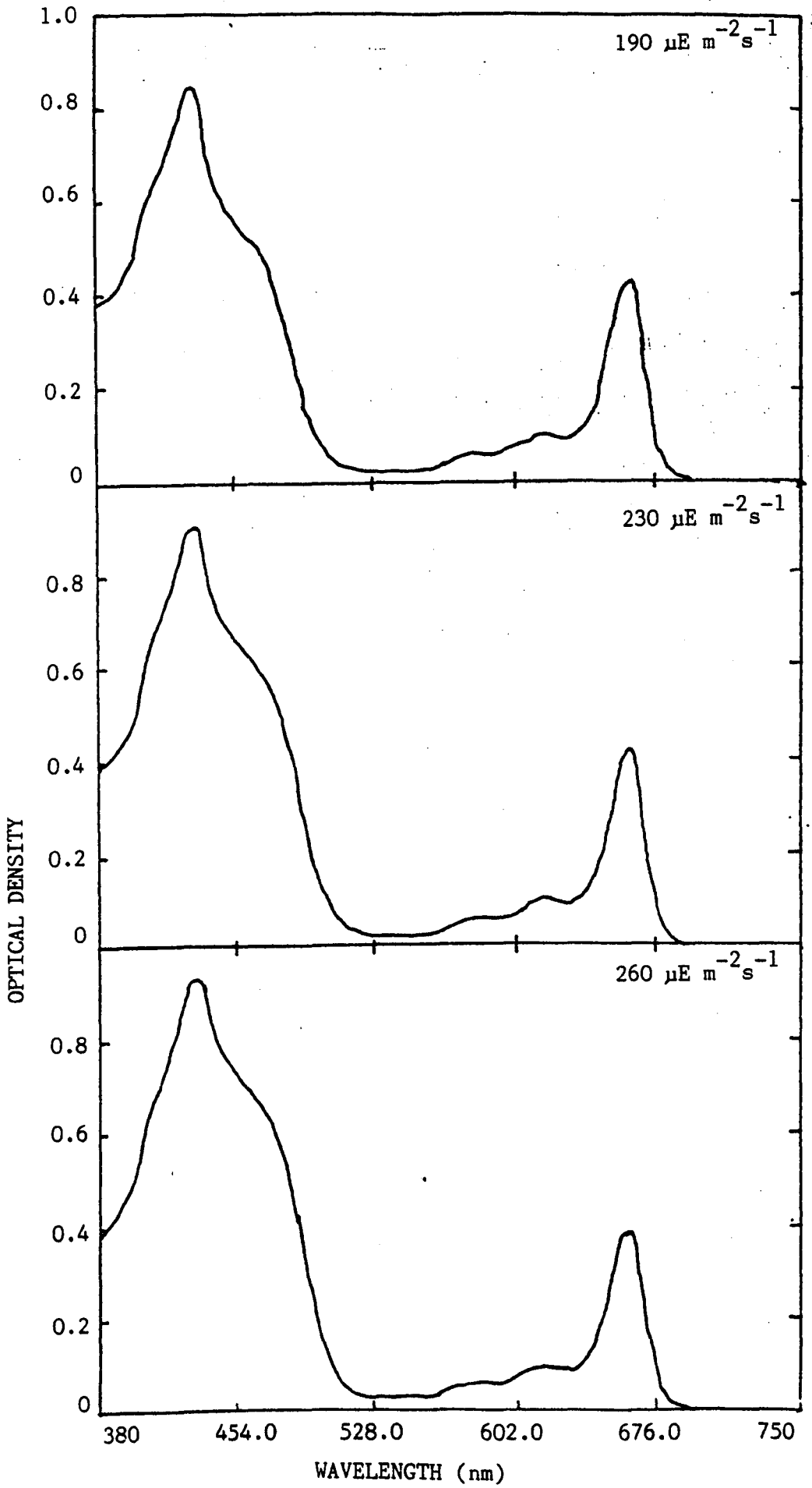


FIGURE 4.5 The effect of increased irradiance on the spectral composition of a mutant culture grown in the chemostat.

glycerol leakage can be observed (Figure 4.4). It appears that at the highest light intensity used (260 $\mu E\ m^{-2}\ s^{-1}$) that the cells were damaged by the high irradiance. However the data in Table 4.1 show that this is not the case, since the chlorophyll content of the culture remained high throughout the experiment. Therefore the glycerol lost into the medium is not due to dying cells. However, the variability of the leakage data from this experiment does not allow a definite conclusion on whether there is a direct connection between the carotenoid accumulation and the glycerol loss.

4.2.5. Effect of high CO_2 on glycerol leakage

Strict photoautotrophs require the presence of inorganic carbon in the medium and Dunaliella cells utilize CO_2 and HCO_3^- as carbon sources. These are made available in the chemostat by bubbling air through the culture, which contains 0.03 % (v/v) CO_2 and by the addition of $NaHCO_3$ at the concentration of 1 g l^{-1} to the medium. In contrast, the natural high salt habitats of Dunaliella, such as the Great Salt Lake, have CO_3^{2-} as the main inorganic carbon source, some HCO_3^- and relatively little CO_2 are present (Post 1977). Growth is greatly stimulated by the addition of extra inorganic carbon, either as CO_2 or $NaHCO_3$ (Borowitzka and Borowitzka 1988), whereby CO_2 assimilation and the rate of photosynthesis are limited by light intensity (Street and Cockburn 1972). The flux

of CO_2 into the cell is thought to be by passive flux across the plasma membrane indicating a constant membrane CO_2 conductivity during photosynthesis and respiration. There is also an active transport system for CO_2 found in cyanobacteria and algae (Espie et al 1988, Sultemeyer et al. 1989, Miller et al. 1988). This carrier mediated uptake depends on demand by photosynthesis (Gimmler et al. 1990). The permeability coefficient of algal membranes is much lower than that of higher plants (Raven 1988) and this is due either to the difference in the membrane lipid composition, the thickness of the plasma membrane (which depends on the chain length of the fatty acids in the membrane lipids) or the rate of active CO_2 transport across the membrane (Gimmler et al. 1990). The conductivity of the plasma membrane is increased by illumination, which means that the resistance of CO_2 diffusion is lowered in higher plants and in algae in the light (Gimmler et al. 1990).

Under conditions of low CO_2 (0.03 % as in air) Dunaliella cells utilize the carbon more efficiently than cells grown in 5 % CO_2. The low CO_2 grown cells have a CO_2 concentrating mechanism and accumulate inorganic carbon inside the cell, D. salina was found to accumulate CO_2 to a concentration of 20 times that of the inorganic carbon present in the medium (Zenvirth and Kaplan 1981). The proportion of internally concentrated CO_2 decreases with increasing CO_2 concentration in the medium.

The following experiments were designed to find out the effect of high levels of CO_2 on the glycerol leakage. After growing the cells to steady state on 0.03 % CO_2 (in air) and 1 g l^{-1} $NaHCO_3$, the percentage of CO_2 was increased to 2 % (Fig. 4.6, Exp. 1). After addition of the increased level of CO_2 the growth rate increased and the percentage leakage fell initially from 62 to 42 % and over the next 4 days down to around 2 % and after a slight increase to 6 % decreased to zero leakage. The cells could not be induced to leak again, despite switching back to low CO_2 in normal air or by increasing the temperature to 35 ^{0}C. After termination of this experiment the chemostat was re-inoculated with cells from the previous run and the cells were grown on 0.03 % CO_2. No loss of glycerol into the medium occurred at any time, even an increase in temperature up to 45 ^{0}C over 10 days resulted in no leakage (data not shown) and the experiment was terminated. After setting the chemostat up again with the leaky mutant the cells were grown to steady state, where the leakage of glycerol was found to be 91 to 94 % (Fig. 4.6, Exp. 2). Two days after the addition of 5 % CO_2 the leakage dropped sharply to 42 % and then down to zero the day after. At this point the experiment was terminated, the chemostat was set up again and was freshly inoculated with leaky mutant cells (Fig. 4.6, Exp. 3). After the culture had reached steady state with a leakage of 67 %, 5 % CO_2 was bubbled through the vessel. After an initial increase of the glycerol loss up to 96 % the leakage dropped immediately down to 0 %

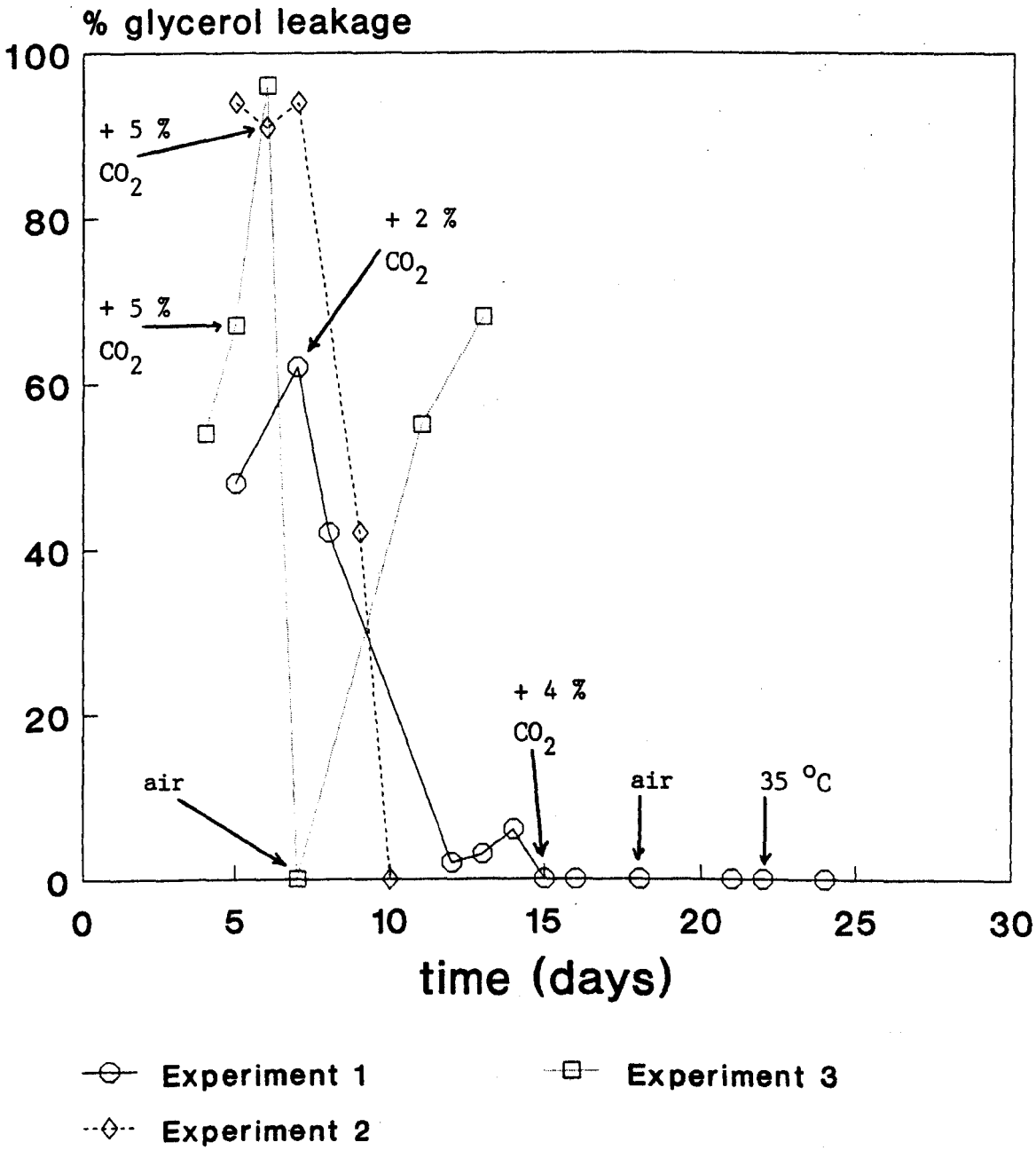


FIGURE 4.6 The effect of increased CO_2 concentration on glycerol leakage of a mutant culture grown in the chemostat (dilution rate between 0.0096 h^{-1} and 0.0256 h^{-1}).

within two days of adding the high carbon dioxide. At this stage the air supply was switched back on replacing the high CO_2. This resulted after 4 days in a return to glycerol loss through the plasma membrane of 55 % and two days later 68 % (Fig. 4.6, Exp. 3).

From the results of these experiments it can be seen that the leakage of glycerol varies considerably from experiment to experiment. However, it is clear that the concentration of CO_2 has a profound effect on the leakiness of the mutant membrane. A concentration of 2 % CO_2 has a slower effect on glycerol loss in that reaching the point of no leakage takes longer than when 5 % CO_2 is used. Once the culture stops leaking glycerol, leakage can only be induced again if the CO_2 level is reduced before a threshold point is reached. Thus, if the CO_2 level is decreased as soon as zero leakage is established, the process is reversible and leakage returns. However, if three days pass at zero leakage, then leakage cannot be re-established and even high temperature treatment, which would normally induce Dunaliella cells to leak (Wegmann et al. 1980), has no effect. In fact, the cells seem to be less sensitive to these high temperatures (compare Figs 4.1 and 4.6). Once the threshold point is reached the changes in the plasma membrane are irreversible and leakage cannot be induced again. An analysis of the lipid content of wild type and mutant cells is presented in Chapter 6.

CHAPTER 5 HETEROTROPHIC GROWTH OF DUNALIELLA PARVA AND THE UPTAKE OF ORGANIC COMPOUNDS

5.1. Introduction

Heterotrophic growth of microalgae has been of interest for a number of years, particularly by the algal biotechnology industry. Algal cultures which grow without the need of illumination make the production process much cheaper as large stainless steel fermentors can be used. In addition the likelihood of contamination is much lower in these closed systems when compared with open ponds (see section 1.9). In order to grow heterotrophically, the cells have to be able to grow independently from illumination and they have to be able to take up and utilise the organic carbon source provided in the growth medium. There are a large number of carbon compounds which are utilised by cells, the most commonly used are probably acetate and glucose. Attempts to grow Dunaliella cells heterotrophically have been unsuccessful (Ginzburg 1969, Coughlan 1977, Ukeles and Rose 1976, Borowitzka and Borowitzka 1988). There are however a number of heterotrophic strains of the closely related green alga Chlamydomonas (Harris 1989). The following experiments were designed to investigate heterotrophic growth of Dunaliella parva 19/9 mutant and wild type and uptake of organic compounds into the cells. The possibility that the leaky mutant may take up organic compounds more

readily than the wild type is considered in these experiments.

5.2 Results and Discussion

5.2.1. Heterotrophic growth

Simple growth experiments in batch culture were carried out. A number of different carbon and nitrogen sources and media were used in the attempt to grow mutant and wild type cells heterotrophically (Table 5.1). However, over a period of 14 days no growth took place. The cells, which were inoculated at the beginning of the experiment died after 8 to 14 days without dividing. It appears that the cells were able to live on their storage products for a number of days.

5.2.2. Uptake of organic compounds

There are many species of microalgae which take up organic compounds into the cells, glycerol, glucose and acetate for example are taken up by a number of diatoms (Cooksey 1978) and chlorophytes (Ukeles and Rose 1976). Uptake of organic compounds by cells of Dunaliella have been reported by Ginzburg (1969) who found that ^{14}C-sucrose and ^{14}C-inulin were taken up by D. parva and by Zidan (1988) who observed accumulation of 3-O-

TABLE 5.1. Heterotrophic growth of *Dunaliella parva* in two media with different nitrogen sources (5mM) and carbon souces (0.1 % w/v)

growth medium*	nitrate	urea	ammonium	glycyl-glycine
	-	-	-	-
+acetate	-	-	-	-
+glucose	-	-	-	-
+glycerol	-	-	-	-
+Egm +a	-	-	-	-
+Egm +glu	-	-	-	-
+Egm +gly	-	-	-	-
+Egm +a +v	-	-	-	-
+Egm +glu +v	-	-	-	-
+Egm +gly +v	-	-	-	-

* = Basic growth medium (see Appendix)
Egm = *Euglena gracilis* medium (see section 2.8)
a = acetate
glu = glucose
gly = glycerol
v = vitamin solution (100 µg l^{-1} thiamine, 0.5 µg l^{-1} biotin and 0.5 µg l^{-1} cyanocobalamin)

methylglucose in D. tertiolecta. An organic compound can enter the cell by diffusion or active transport. Hellebust (1978) suggested Na^+-driven glucose uptake took place in the diatom Cyclotella cryptica, whereas Ginzburg and Richman (1985) and Kwon and Grant (1971), suggested glucose uptake by Dunaliella was a passive process.

The transport studies presented below are concerned with the uptake of ^{14}C-glycerol, ^{14}C-glucose and ^{14}C-acetate by both mutant and wild type.

5.2.3. Uptake of ^{14}C glycerol

5.2.3.1. Effect of temperature and light intensity

Glycerol, since it is the compatible solute, is probably one of the most likely organic carbon compounds to be taken up by Dunaliella cells and ^{14}C labelled glycerol was used in the following transport studies. The experiments were carried out using the silicone oil centrifugation method as described in section 2.9. Figure 5.1 shows the uptake of glycerol by the mutant and the wild type cells grown in 0.4 M NaCl and incubated at 30 ^0C in ambient light for the duration of the experiment. The wild type takes up glycerol more quickly within the first 5 minutes of the experiment and uptake levels off after about 30 minutes. However over the time span of 6 hours the glycerol uptake by the mutant is very similar

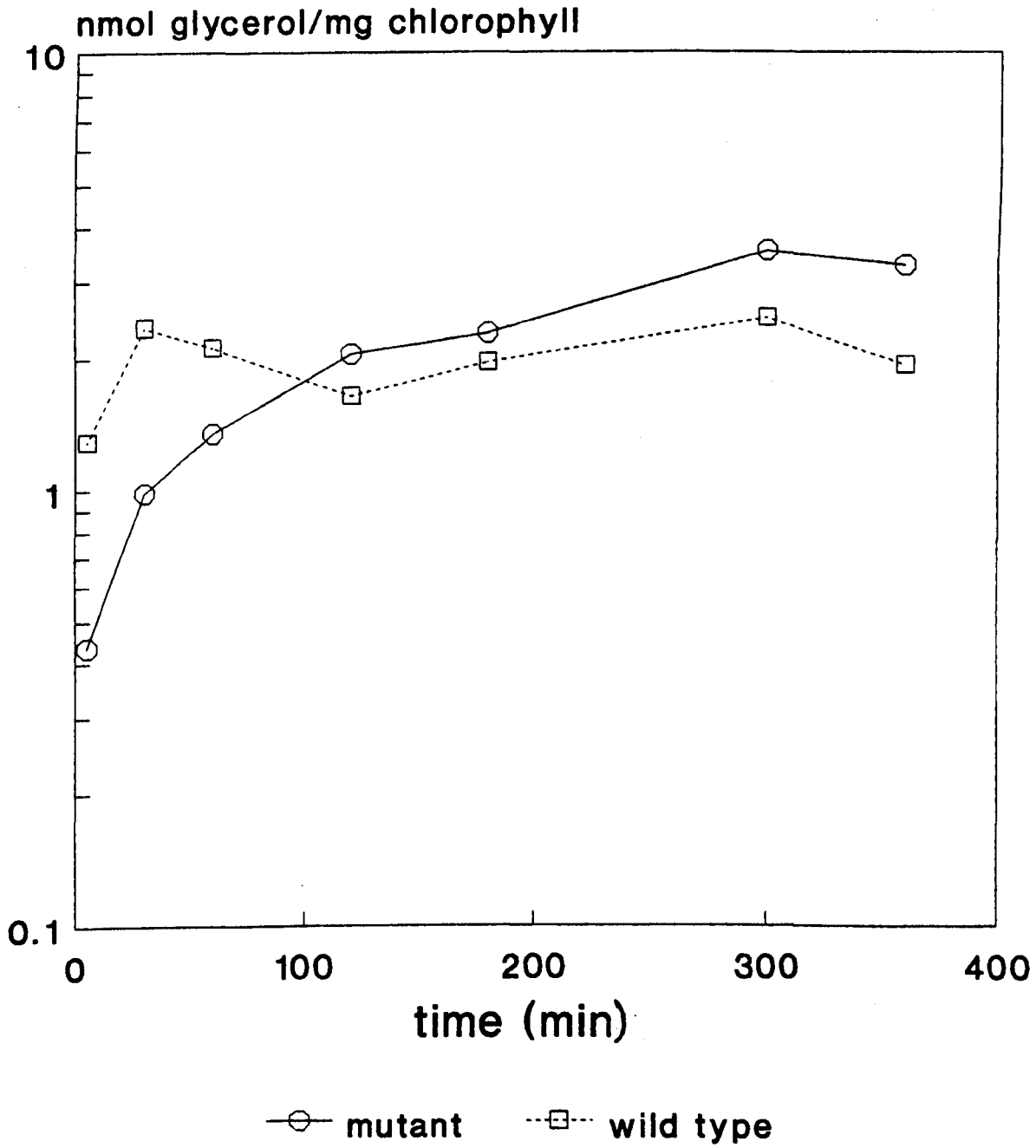


FIGURE 5.1 ^{14}C-glycerol uptake by mutant and wild type in 0.4 M NaCl at 30 ^{o}C in ambient light.

to that of the wild type. Mutant and wild type cells grown in 1.5 M NaCl show a lower level of uptake when compared to 0.4 M NaCl grown cells (Fig. 5.2) and the larger errors indicate that the cells are stressed by the high salinity. In order to determine the dependence of the glycerol transport into the cells on light and temperature, the uptake experiments were carried out in three different light regimes, dark, ambient and light, at two different temperatures, 20 and 30 ^{0}C using mutant cells (Figs 5.3 and 5.4). As expected the cells show increased uptake at the higher temperature, but there is no difference in uptake in light, dark or ambient light at 20 ^{0}C and only a slight difference at 30 ^{0}C, whereby the cells in ambient illumination show the highest uptake over 6 h and the cells in the dark show the lowest. Wild type cells also exhibit higher uptake rates at 30 ^{0}C when compared to 20 ^{0}C (Figs. 5.5 and 5.6) and at both temperatures the cells in the dark are slightly below the level of uptake in ambient and light conditions over the period of the experiment. At 20 ^{0}C ambient illuminated cells show higher uptake than cells in the light, however this is not the case at 30 ^{0}C, where there is no apparent difference.

Unless otherwise stated, ambient light at 30 ^{0}C were used for all other transport assays.

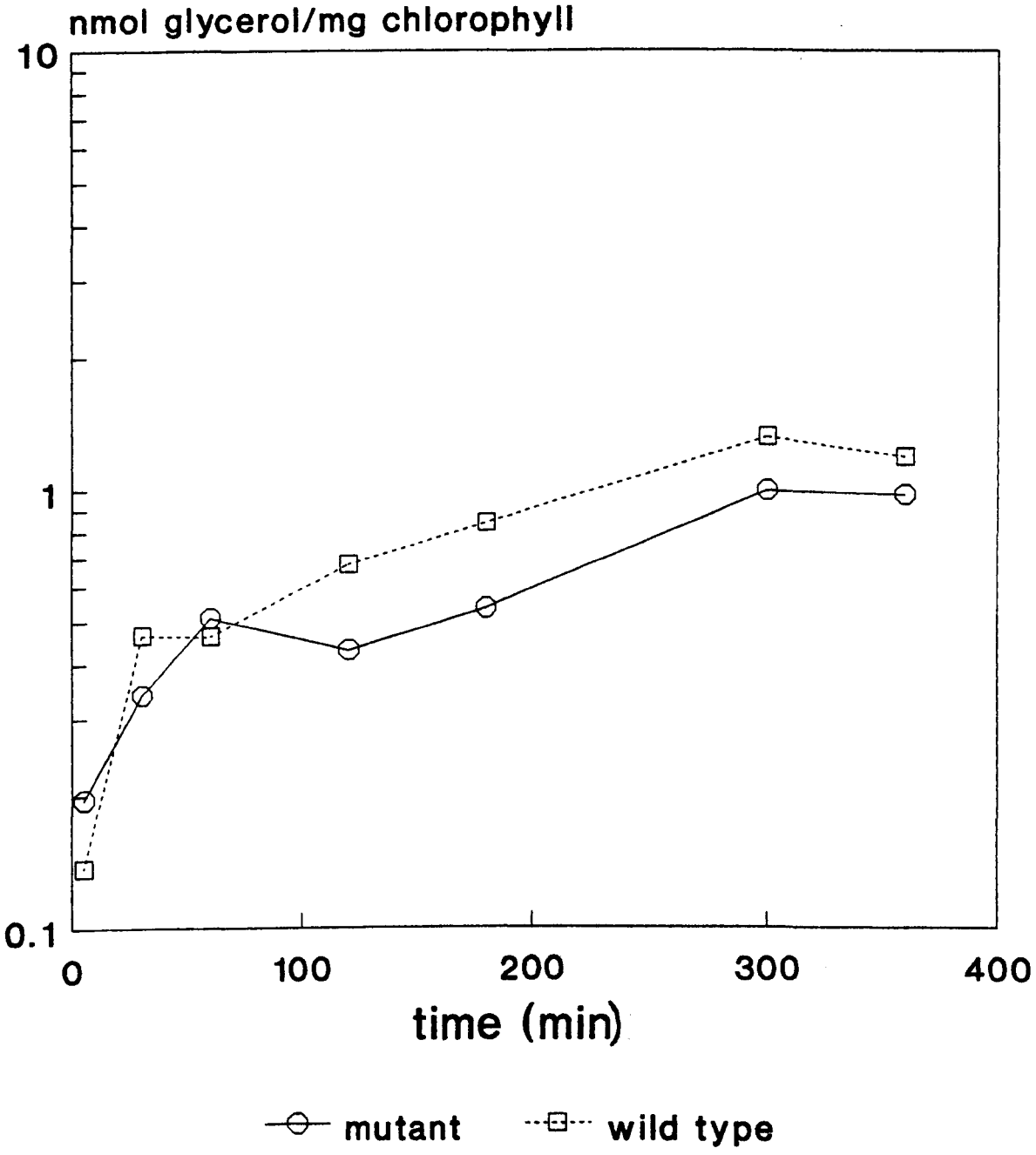


FIGURE 5.2 ^{14}C-glycerol uptake by mutant and wild type in 1.5 M NaCl at 30 ^{o}C in ambient light.
Due to the stress imposed on the cells by the high salinity the errors were around 15 %.

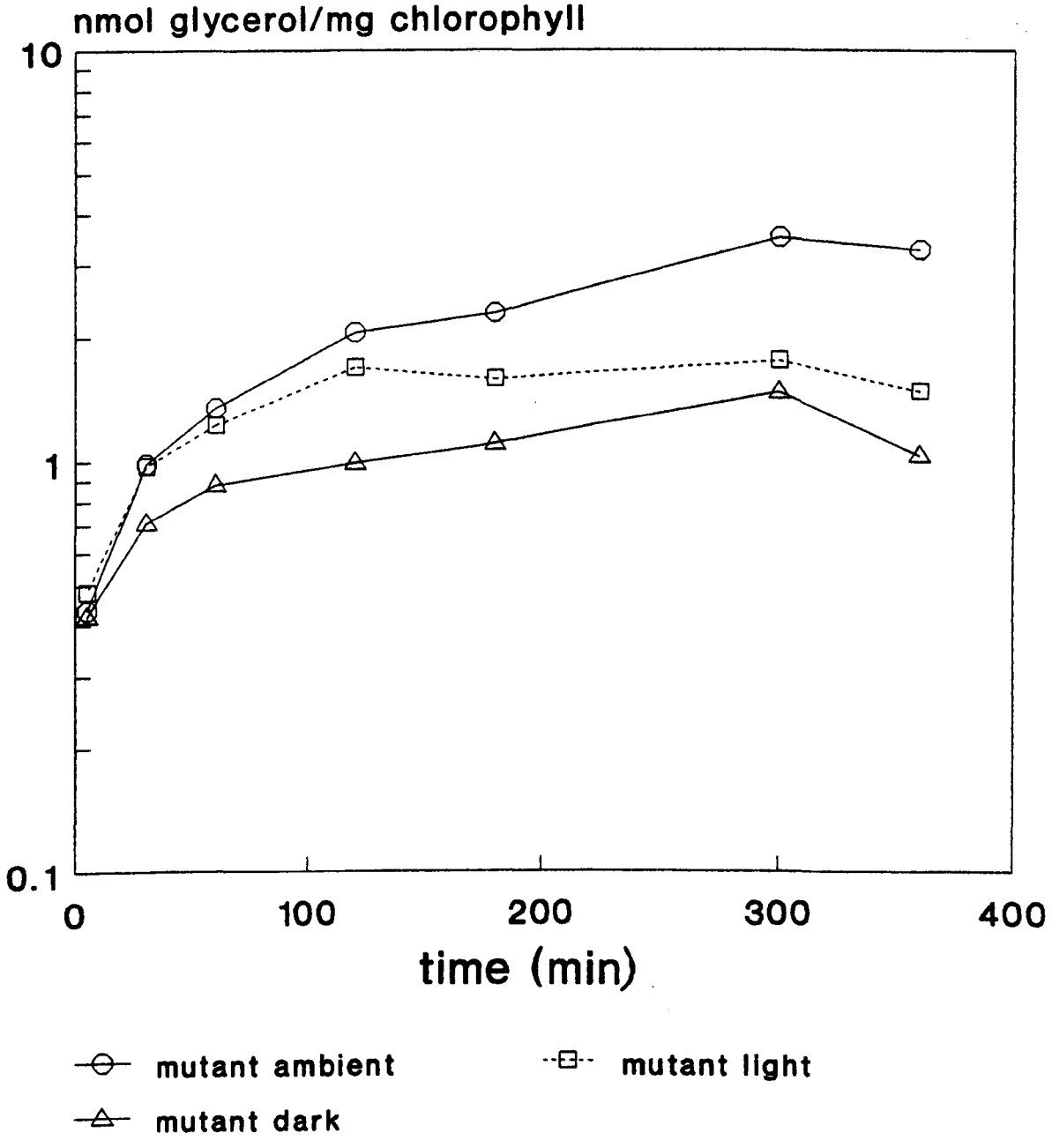


FIGURE 5.3 The effect of light intensity on ^{14}C-glycerol uptake by the mutant in 0.4 M NaCl at 30 ^{o}C.

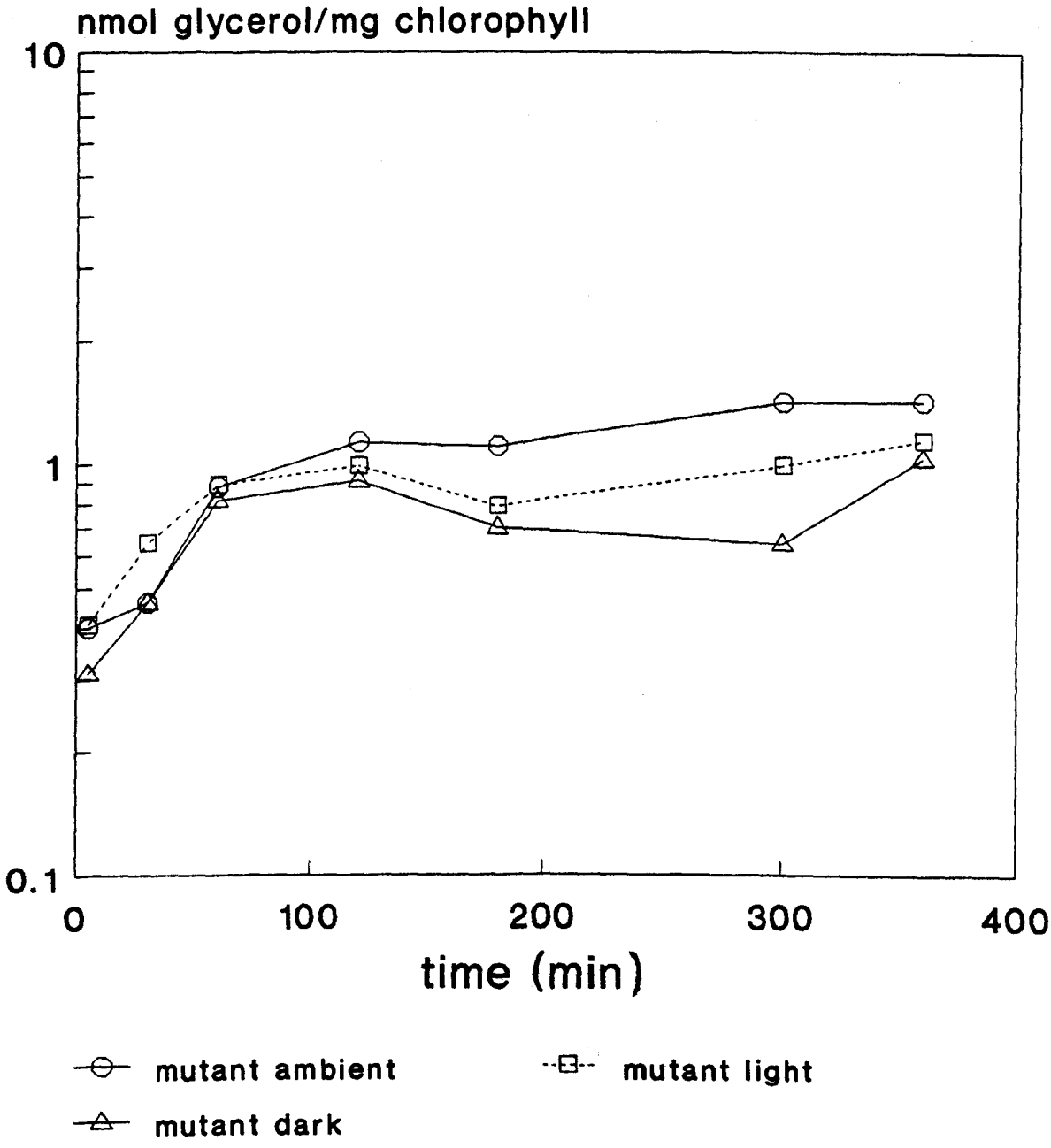


FIGURE 5.4 The effect of light intensity on ^{14}C-glycerol uptake by the wild type in 0.4 M NaCl at 20 ^{o}C.

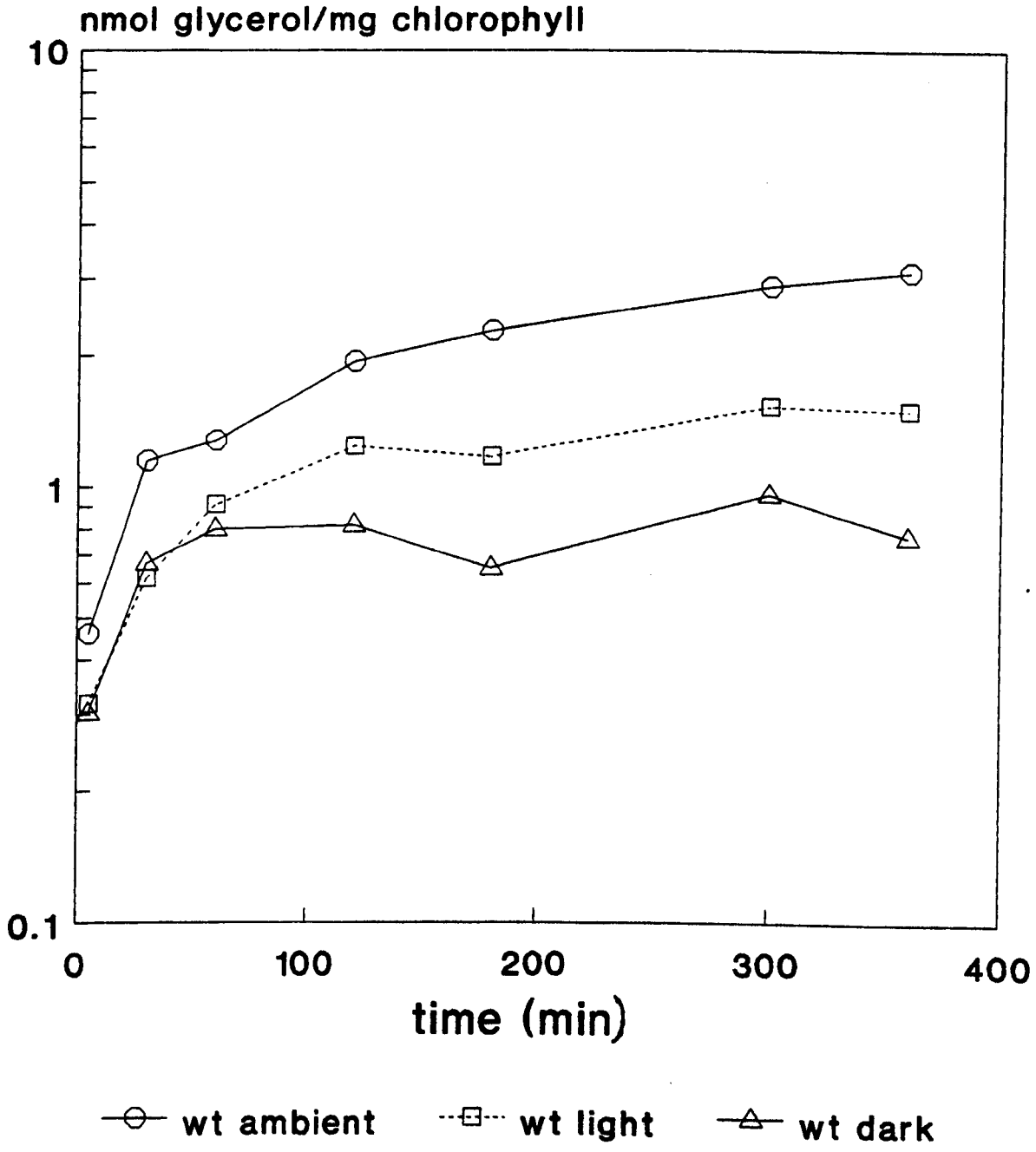


FIGURE 5.5 The effect of light intensity on ^{14}C-glycerol uptake by the wild type in 0.4 M NaCl at 20 ^{o}C.

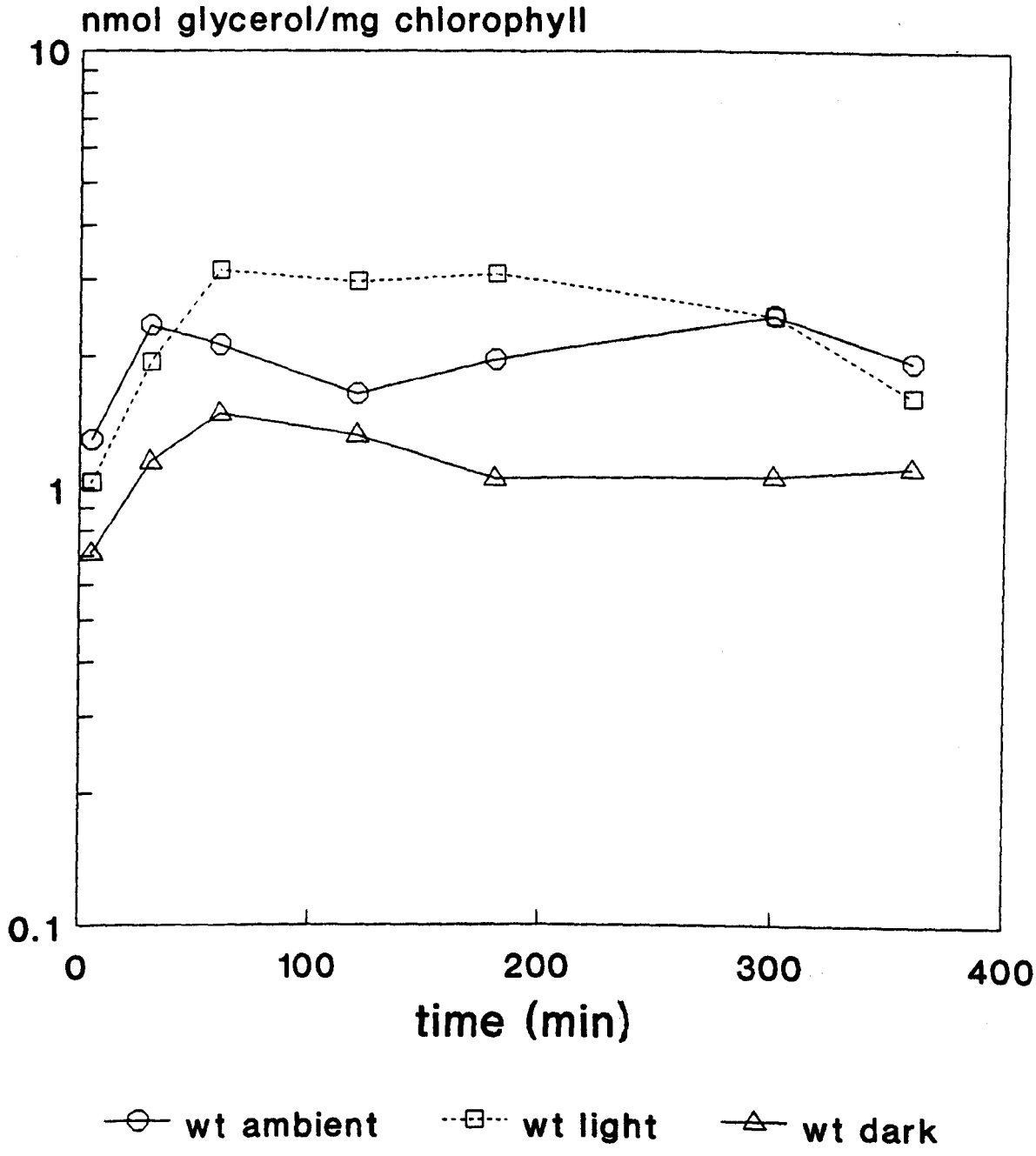


FIGURE 5.6 The effect of light intensity on ^{14}C-glycerol uptake by the wild type in 0.4 M NaCl at 30 ^{o}C.

5.2.3.2. Inhibition of uptake

It is important to establish whether the uptake is by active transport and to check for the possibility of the labelled glycerol binding to the cell surface and so falsifying the pellet counts and uptake rates. To check for active transport or binding the uncoupler CCCP and iodine were used. CCCP uncouples the proton motive forces from ATP synthesis by allowing protons to cross the membrane without traversing the ATPase. Iodine rapidly kills the cells. Iodine and CCCP were added to 0.4 M NaCl grown mutant and wild type cells before the ^{14}C-glycerol was added. In both cases, no transport of labelled glycerol took place as indicated by very low counts (which are probably due to background counts) in the pellets compared to the controls (Figs. 5.7, 5.8). The effect of iodine and CCCP on the transport of glycerol across the membrane agrees with oxygen electrode data. Both iodine and CCCP inhibit photosynthesis and respiration of the cells completely, at the concentrations used in the transport assays.

5.2.3.3. Saturation of the transport system

As part of the characterisation of the transport system, different concentrations of hot and cold glycerol at a constant ratio of 1:11 were supplied to 0.4 M NaCl grown

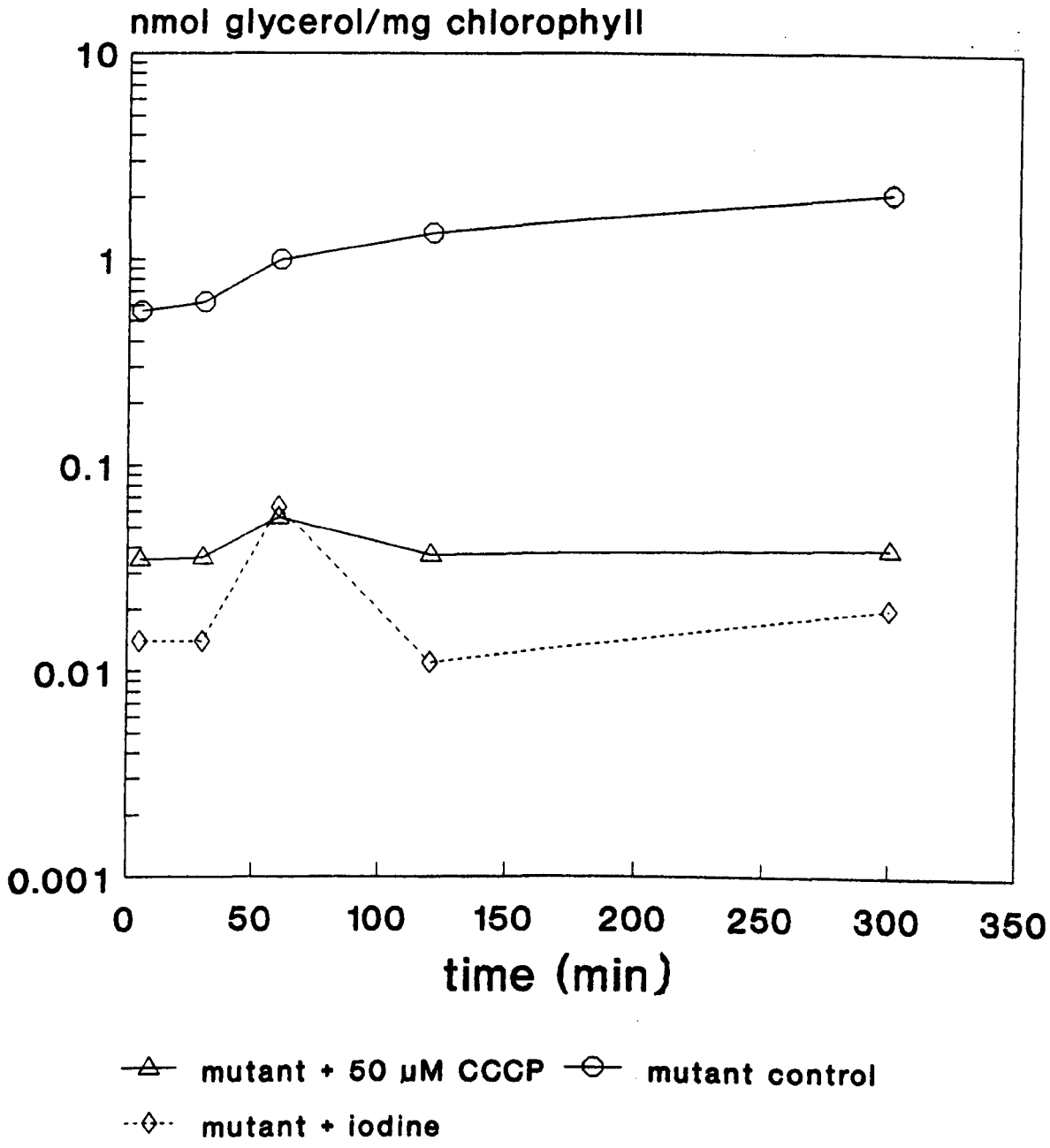


FIGURE 5.7 The effect of CCCP and iodine on ^{14}C-glycerol uptake by the mutant grown in 0.4 M NaCl.

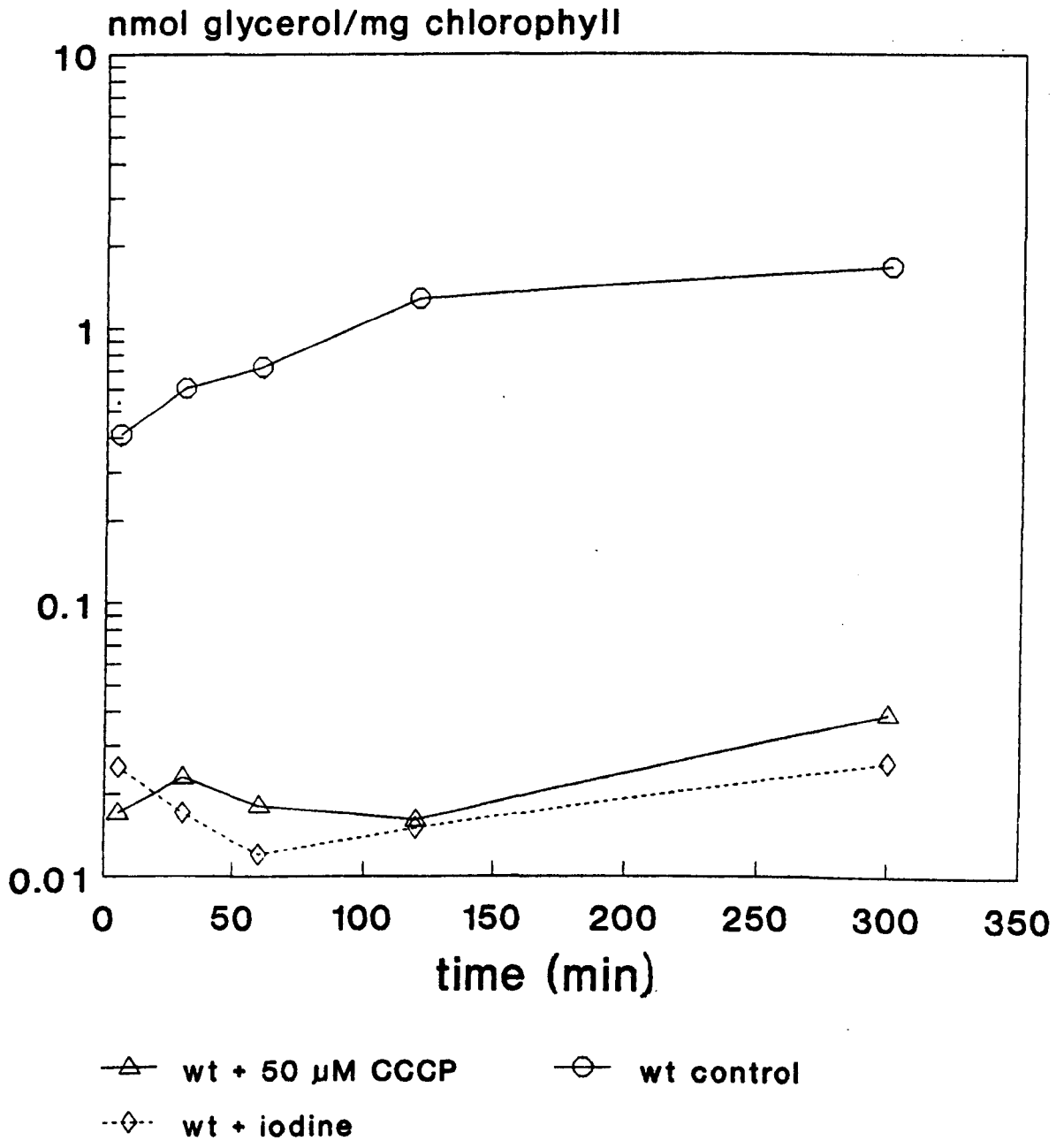


FIGURE 5.8 The effect of CCCP and iodine on ^{14}C-glycerol uptake by the wild type grown in 0.4 M NaCl.

mutant cultures (Fig. 5.9). The rate of uptake is much higher in cultures with a concentration of 1.65 uM than with 0.3 µM glycerol. It seems however, that the transport system has reached saturation point at about 1.65 µM since, the uptake rate in the 3.3 and 6.6 uM samples was not significantly higher.

5.2.3.4. Uptake of glycerol following osmotic shocks

The following experiments were carried out to establish the effect of hyper- and hypo-osmotic shocks on the transport of glycerol into the cell. In the upshock experiments 0.4 M NaCl grown mutant and wild type cells were resuspended in 0.8 M, 1.0 M, 1.5 M and 2.0 M NaCl media and the labelled glycerol was added immediately after the resuspension of the cells in the fresh medium. Uptake was then monitored over a period of 5 hours (Figures 5.10 and 5.11). The rate of glycerol uptake following a hyperosmotic shock depends on the degree of the shock, the more severe the shock the lower the rate of uptake and the cells take longer to recover. Cells transferred from 0.4 M to 2 M NaCl are severely shocked and show only a very slight rate of uptake over the 5 hour time span of the experiment. Mutant and wild type cells after a shock from 0.4 M to 0.8 M and from 0.4 M to 1.0 M NaCl recover quickly within minutes and glycerol uptake is just below that of the control (Figs. 5.10 and 5.11). The effect of hyperosmotic shocks on the glycerol

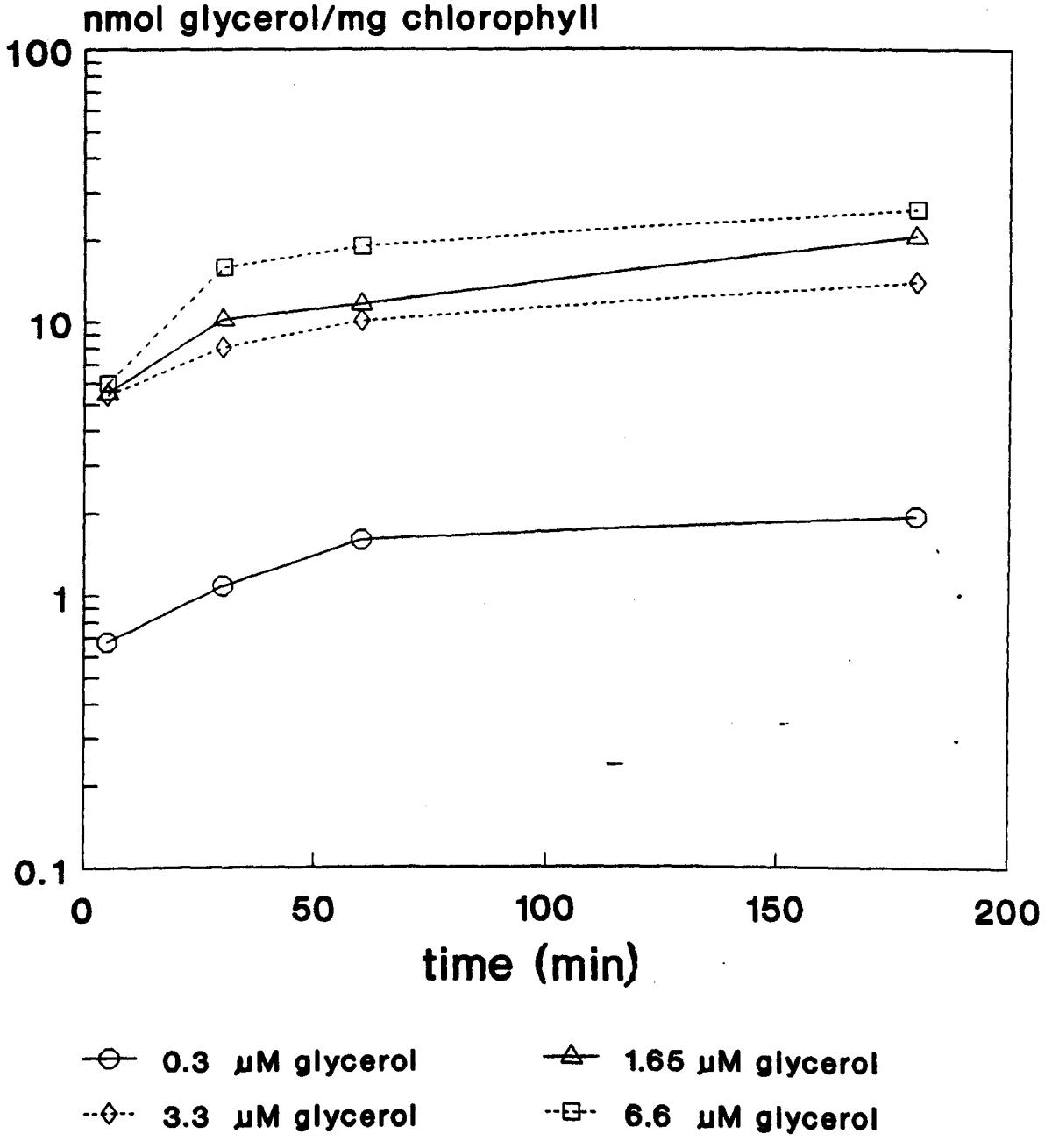


FIGURE 5.9 The effect of different concentrations of glycerol on uptake of ^{14}C-glycerol by the mutant. A ratio of hot : cold glycerol of 1 : 11 was maintained at each concentration.

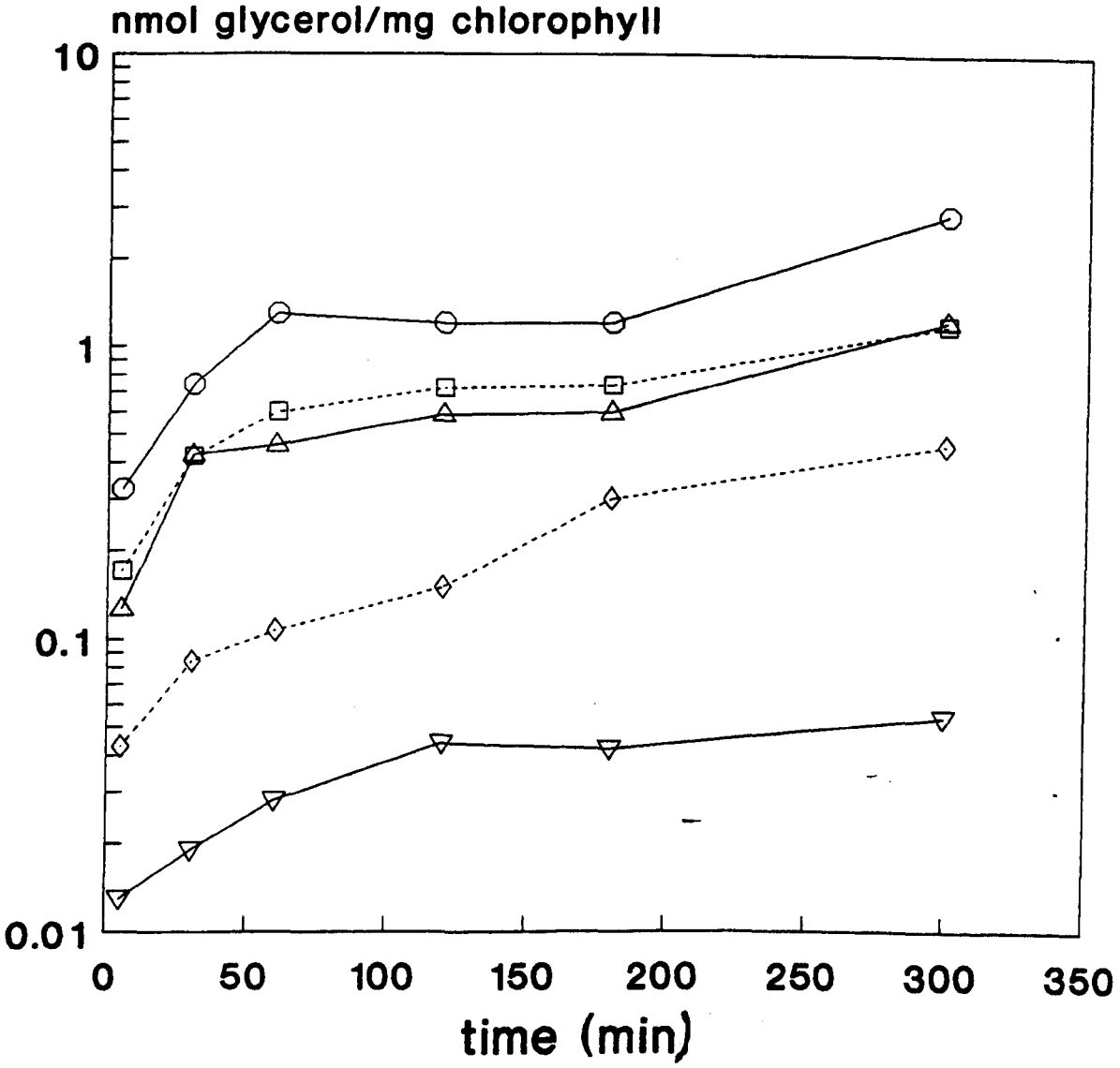


FIGURE 5.10 ^{14}C-glycerol uptake by the mutant following hyper-osmotic shocks.

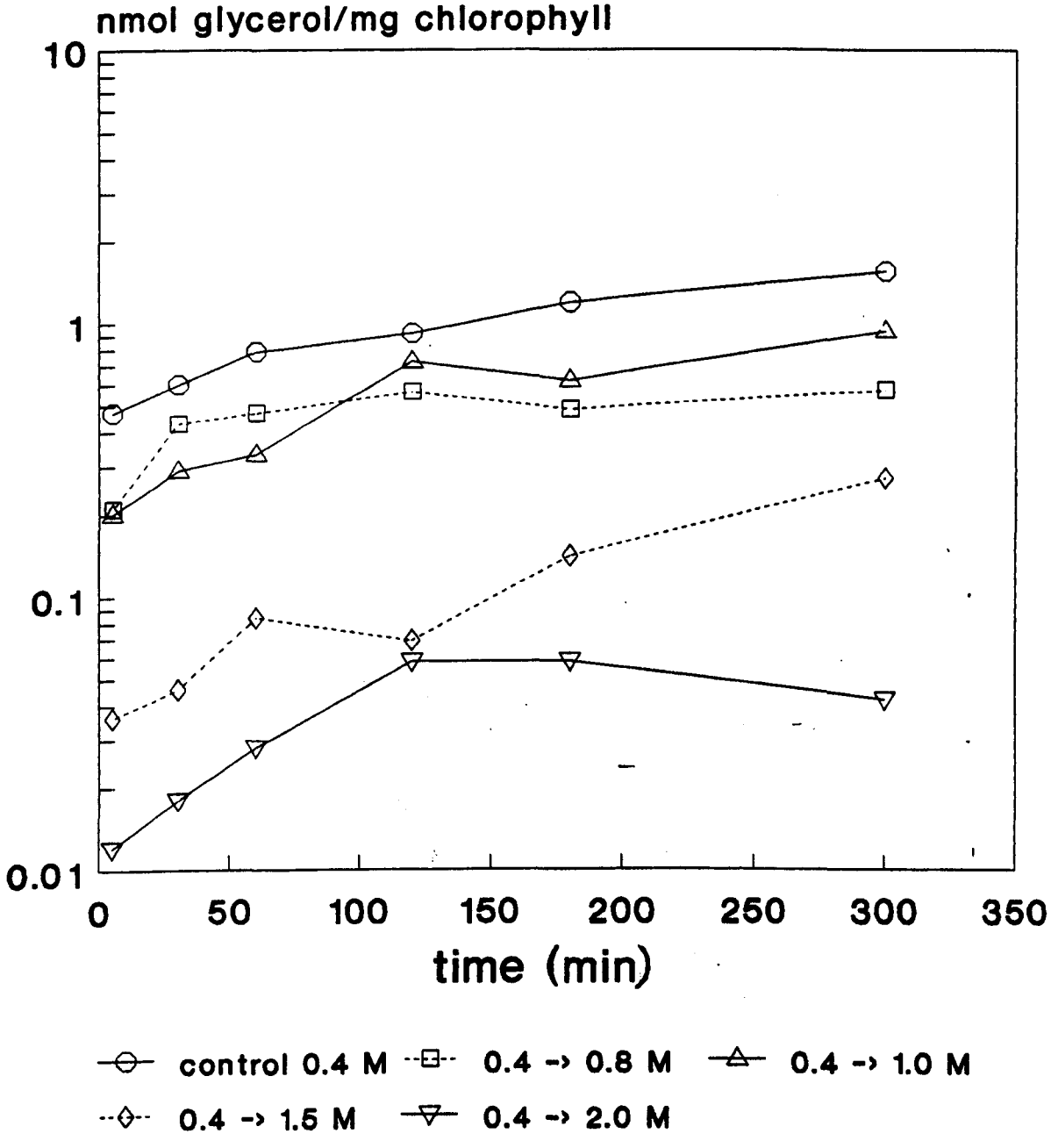


FIGURE 5.11 ^{14}C- glycerol uptake by the wild type following hyperosmotic shocks.

uptake by the cells can also be seen by following the disappearance of ^{14}C-glycerol from the supernatant after silicone oil centrifugation (Section 2.9). These data are shown in Figures 5.12 and 5.13 and the extent of disappearance of the labelled glycerol from the supernatant agrees with the glycerol found in the pellet of the sample. In fact the low level of glycerol uptake by shocked mutant cells is well illustrated in Figure 5.12 since the supernatant levels of ^{14}C-glycerol remain virtually unchanged after salinity increases to 1.5 and 2.0 M NaCl.

In section 3.2.6, O_2 evolution and uptake measurements were used as a measure of stress imposed on the cells by salinity shocks. Parallel experiments were carried out using the range of salinities used in these uptake experiments. Measurements were made after 5 min and Table 5.2 shows that 0.4 M NaCl grown cells resuspended in salinities up to 1.0 M NaCl show little or no inhibition of O_2 evolution or uptake. Increasing the salinity from 0.4 to 1.5 M NaCl inhibits these processes by about 50 % and a salinity increase from 0.4 to 2.0 M NaCl inhibits O_2 evolution and uptake completely immediately after the shock. The data agree with the uptake data shown in Figures 5.10 and 5.11.

The hypoosmotic shock experiments were carried out by resuspending 1.5 M NaCl grown cells in 0.4 M, 0.8 M and 1.0 M NaCl before adding the labelled glycerol to the

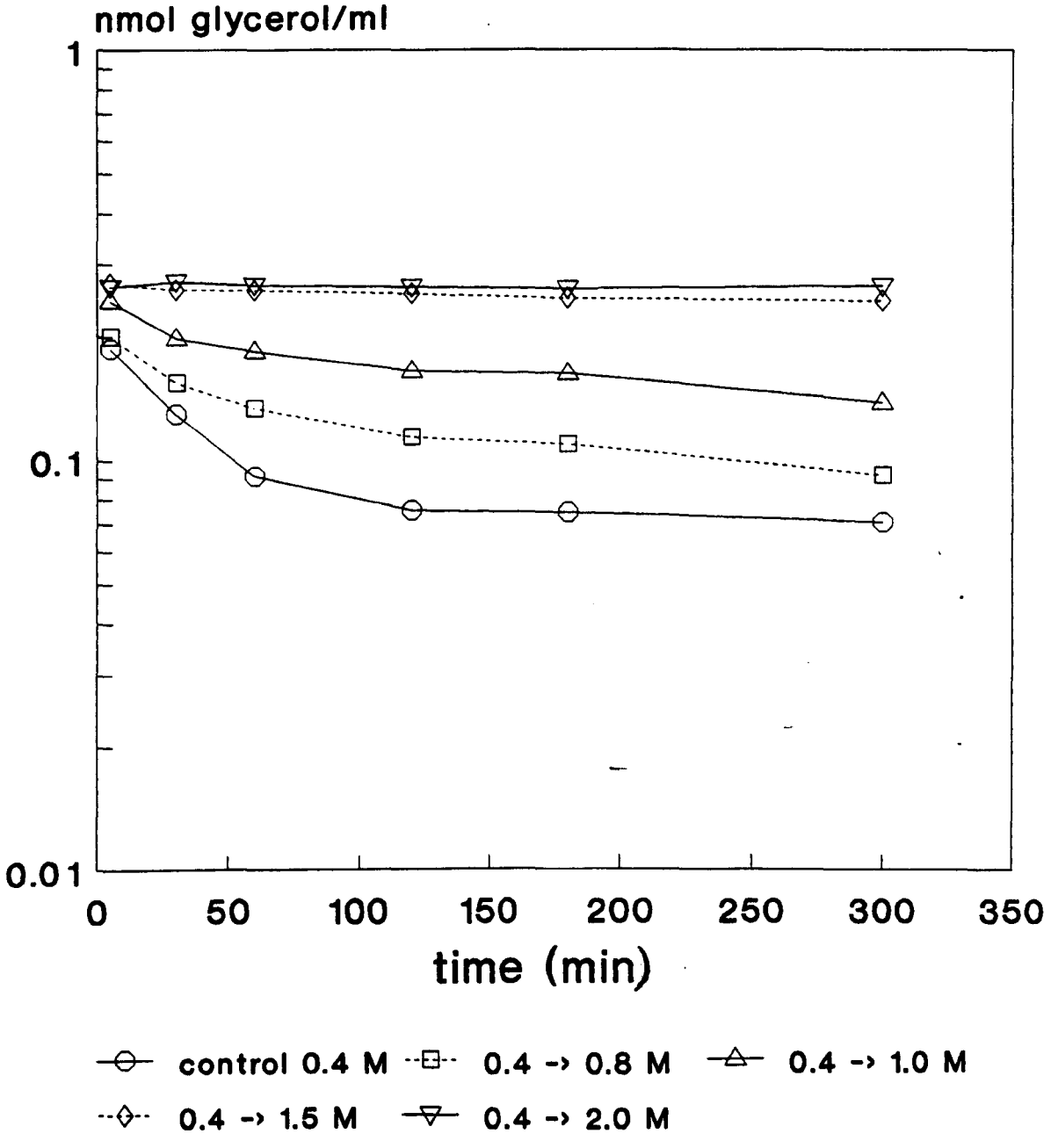


FIGURE 5.12 Amount of ^{14}C-glycerol present in the medium of a mutant culture following hyperosmotic shocks (see Fig. 5.10).

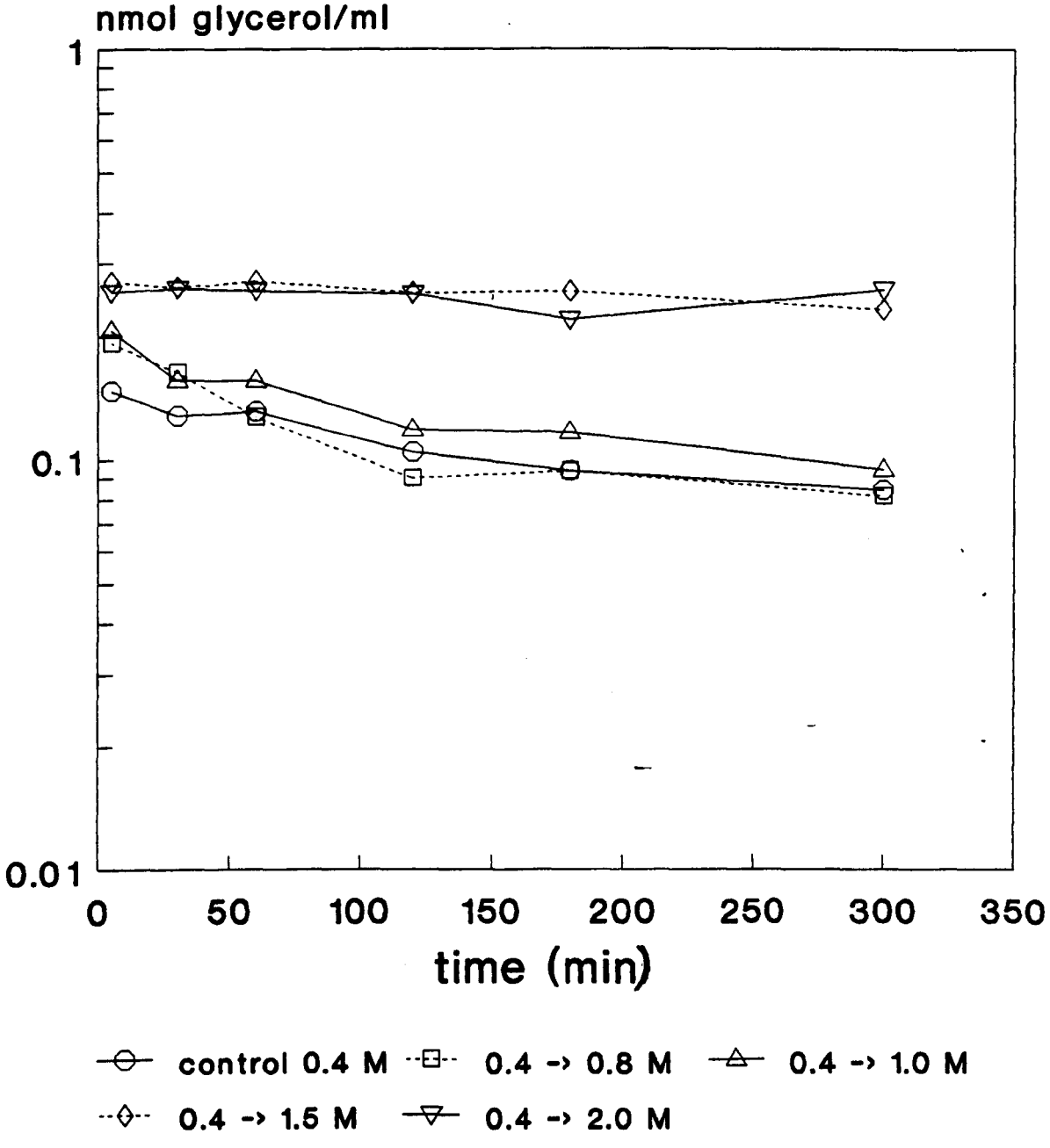


FIGURE 5.13 Amount of ^{14}C-glycerol present in the medium of a wild type culture following hyperosmotic shocks (see Fig. 5.11).

samples. Uptake of the ^{14}C-glycerol was monitored over 5 hours. After a downshock the mutant cells only take up very small amounts of glycerol (Figure 5.14) and the uptake does not increase with recovery from the osmotic shock. This is in contrast to wild type cells (Figure 5.15), where the rate of glycerol transport is initially the same as the control, but levels off 2 hours after the shock, whereas the uptake in the control increases further. Rates of oxygen evolution and oxygen uptake of mutant and wild type cells after a downshock are shown in Table 5.3. In both mutant and wild type the rate of oxygen uptake is little affected by downshocks except after a downshock from 1.5 to 0.4 M, where there is a significant increase in O_2 uptake by wild type cells (see section 3.2.6. There is a significant effect of downshocks on photosynthesis with the wild type showing slightly greater inhibition. This effect is similar to that seen with an equivalent upshock (Tables 5.2 and 5.3). There is no explanation from the O_2 data of the different uptake rates shown by mutant and wild type after downshocks (Figs 5.14 and 5.15).

5.2.3.5. Competition for the transport system

The following experiment was designed to find out if the transport system is specific for glycerol or whether it is a general transport system, which takes up other organic compounds as well. 0.3 µM of labelled glycerol

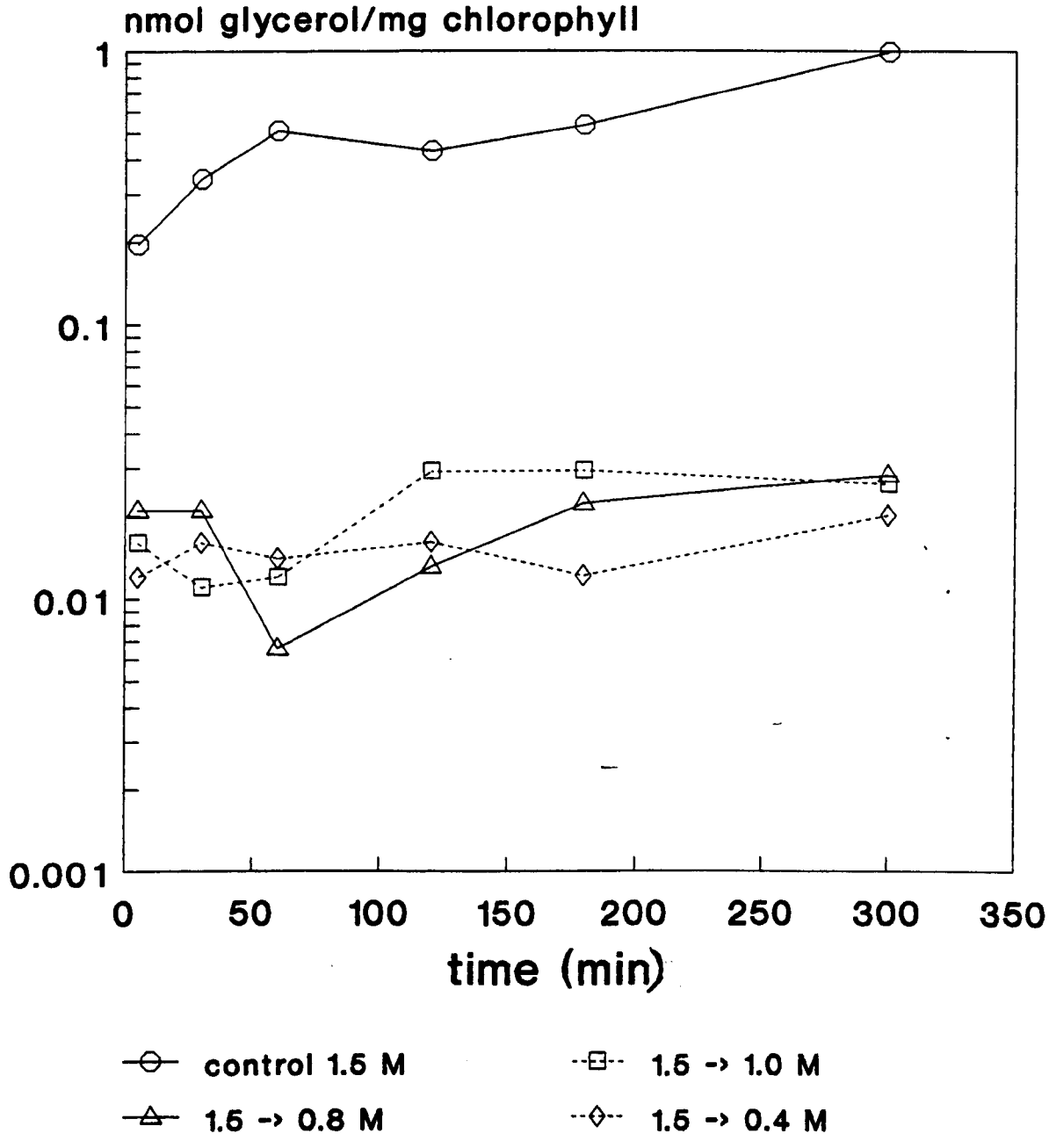


FIGURE 5.14 ^{14}C-glycerol uptake by the mutant following hypoosmotic shocks.

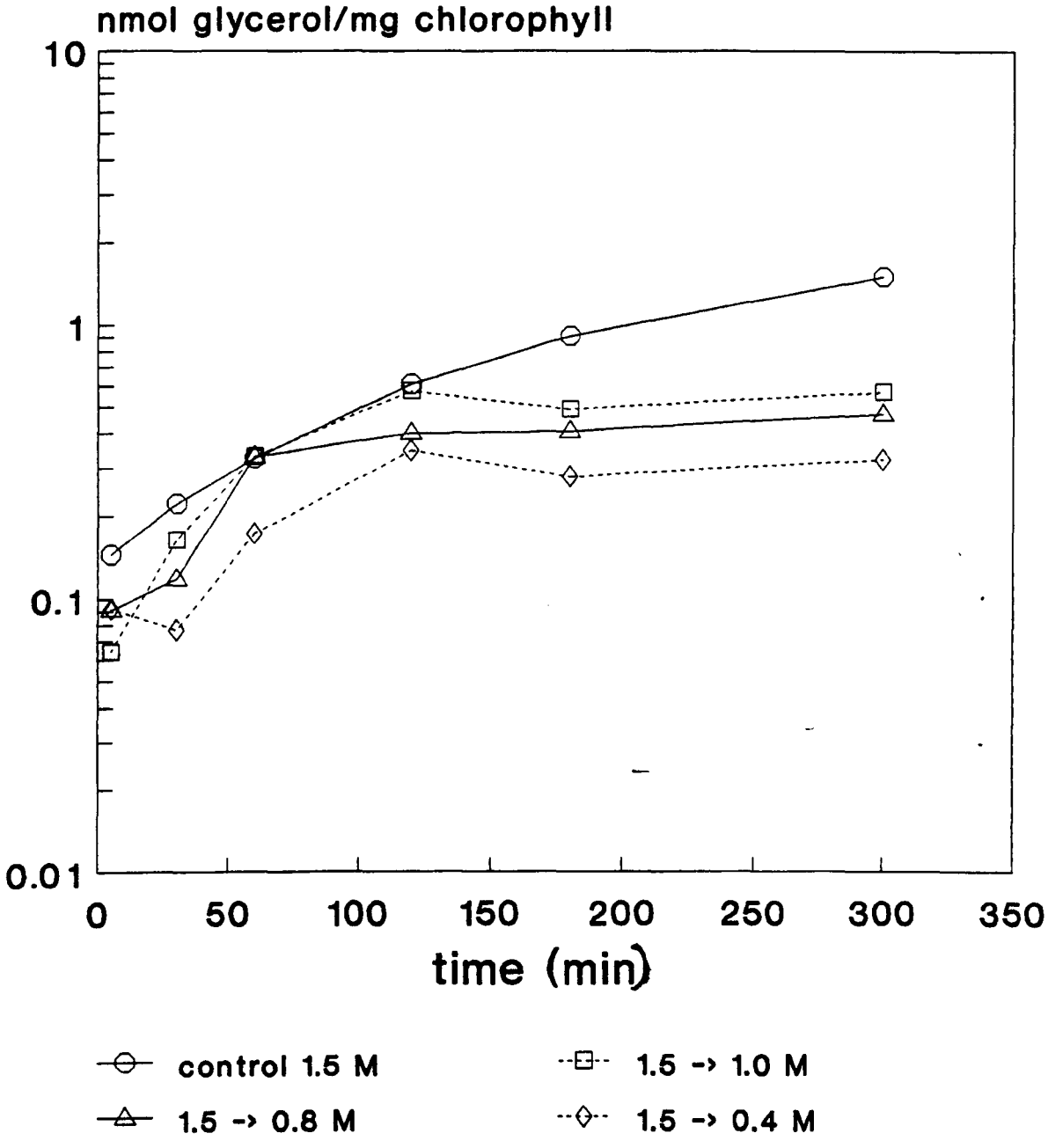


FIGURE 5.15 ^{14}C-glycerol uptake by the wild type following hypoosmotic shocks.

TABLE 5.2. The effect of a hypertonic salt shock on oxygen evolution and uptake in mutant and wild type cells. The measurements were made 5 min after the shock and the results are presented as percentage of control rates. Oxygen evolution was measured in a light intensity of 50 $\mu E\ m^{-2}\ s^{-1}$, the control rates (n = 3) were 46.21 ± 2.4 and 42.4 ± 2.7 µmoles O_2 evolved mg^{-1} chl h^{-1} for mutant and wild type respectively. O_2 uptake was measured in the dark, the control rates (n = 3)were 9.8 ± 1.2 and 12.9 ± 3.4 µmoles O_2 taken up mg^{-1} chl h^{-1} for mutant and wild type respectively.

Mutant

	oxygen evolution	oxygen uptake
0.4 M NaCl control	100	100
0.4 -> 0.8 M NaCl	116.4 ± 5.5	83.4 ± 7.2
0.4 -> 1.0 M NaCl	107.0 ± 6.5	83.2 ± 8.1
0.4 -> 1.5 M NaCl	55.5 ± 17.3	53.2 ± 18.7
0.4 -> 2.0 M NaCl	0	0

Wild type

	oxygen evolution	oxygen uptake
0.4 M NaCl control	100	100
0.4 -> 0.8 M NaCl	96.5 ± 6.3	92.8 ± 6.8
0.4 -> 1.0 M NaCl	81.5 ± 4.8	90.6 ± 6.2
0.4 -> 1.5 M NaCl	63.7 ± 2.8	52.5 ± 1.4
0.4 -> 2.0 M NaCl	0	0

TABLE 5.3. The effect of hypotonic salt shocks on oxygen evolution and uptake in mutant and wild type cells. Measurements were made 5 min after the shock. All conditions were as in Table 5.2. Control rates of oxygen evolution were 51.9 ± 3.2 and 38.9 ± 2.7 μmoles mg^{-1} chl h^{-1} for mutant and wild type respectively. Control rates for oxygen uptake were 18.3 ± 2.1 and 16.0 ± 3.2 μmoles mg^{-1} chl h^{-1} for mutant and wild type respectively.

Mutant

	oxygen evolution	oxygen uptake
1.5 M NaCl control	100	100
1.5 -> 1.0 M NaCl	76.2 ± 10.3	98.3 ± 5.4
1.5 -> 0.8 M NaCl	79.6 ± 15.4	90.2 ± 3.5
1.5 -> 0.4 M NaCl	67.9 ± 3.5	100.0 ± 17.5

Wild type

	oxygen evolution	oxygen uptake
1.5 M NaCl control	100	100
1.5 -> 1.0 M NaCl	58.0 ± 8.5	88.8 ± 5.9
1.5 -> 0.8 M NaCl	57.5 ± 13.4	91.2 ± 7.8
1.5 -> 0.4 M NaCl	40.8 ± 5.5	139.7 ± 11.4

was used for the control cells of mutant and wild type. Hot glycerol of the same concentration as that of the controls and cold glucose at a concentration of 3μM was added to 0.4 M NaCl grown cells. The uptake is shown in Figure 5.16, mutant and wild type cells with and without additional cold glucose show the same rates of uptake, which levels off after 1 hour.

5.2.3.6. Incorporation of the labelled glycerol

The question of the fate of the glycerol once it is taken up into the cell is a very interesting one. Glycerol may be utilized by the cells as an additional source of carbon and some may be released from the cells as ^{14}C-CO_2. Alternatively, it might be converted to the storage product starch. Another possibility, which has been investigated in the following experiments, is that the labelled glycerol is incorporated into the lipid fraction of the cell, where it could act as a carbon sink. A control uptake experiment was carried out, whereby after 3 hours a sample was taken and the total amount of glycerol present in the cells in terms of mg chlorophyll was determined as in previous experiments. Pigments were then extracted from the culture in 80 % (v|v) acetone as described in section 2.3. The total glycerol present in the cells was found to be 5.37 nmoles glycerol/ mg chlorophyll. The extraction showed that 3.4 nmoles glycerol/ mg chlorophyll were present in the

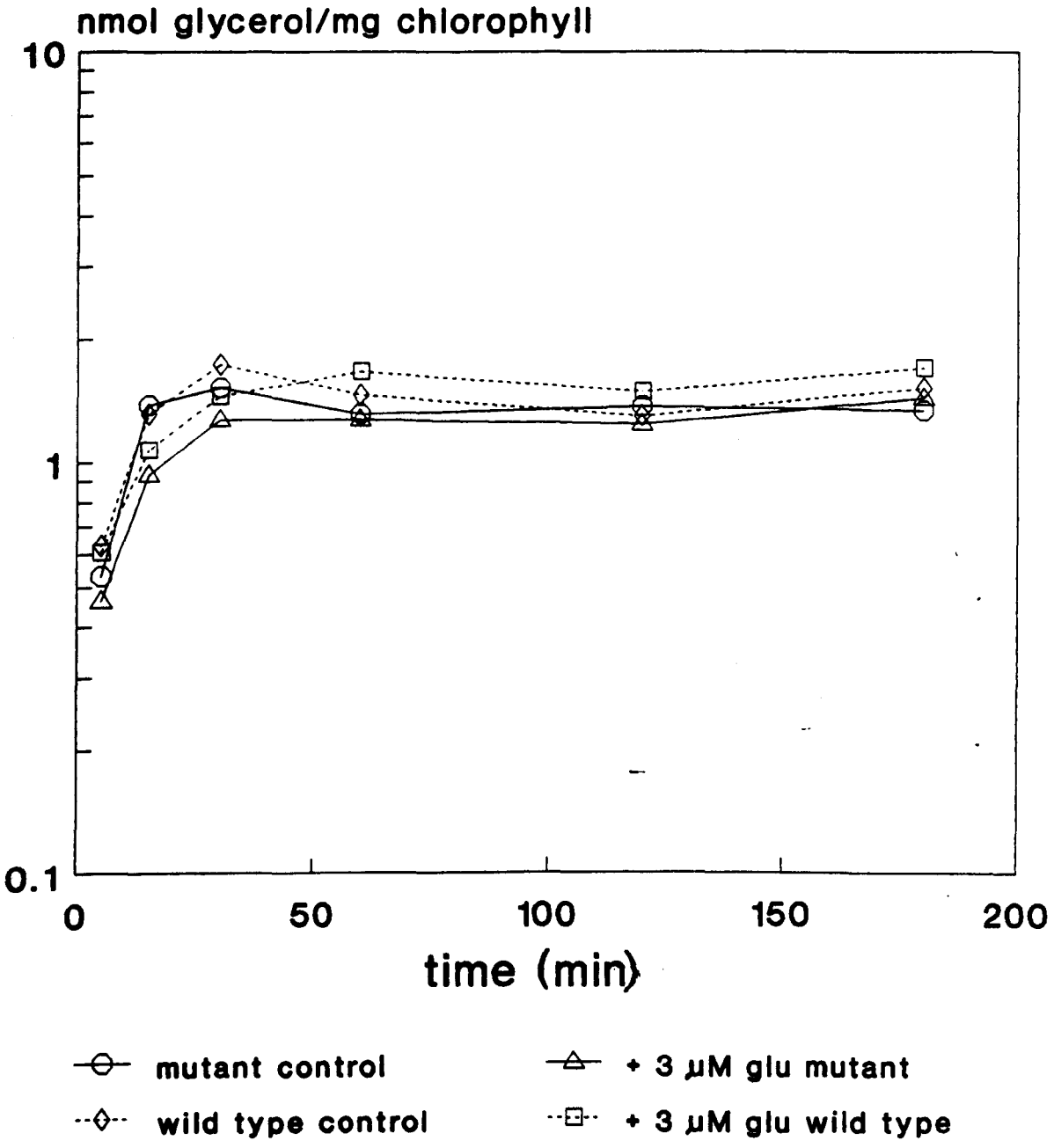


FIGURE 5.16 The effect of high concentrations of unlabelled glucose on the ^{14}C-glycerol uptake by mutant and wild type cells in 0.4 M NaCl. The ratio of ^{14}C-glycerol : unlabelled glucose is 1:11.

organic solvent fraction. This means that 63 % of the labelled glycerol is apparently incorporated into the lipid fraction of the cells.

5.2.3.7. Amount of glycerol taken up per cell

In order to determine whether it is feasible for Dunaliella cells to grow on glycerol heterotrophically, it is of interest to calculate the concentration of ^{14}C-glycerol inside an individual cell. To do this it is necessary to accurately determine the uptake of glycerol into the cells. In all the previous experiments, no account has been taken of the ^{14}C-glycerol which is present in the pellet after centrifuging through silicone oil, but which is trapped between the cells. This glycerol has not been taken up by the cells and therefore our previous experiments have over-estimated the uptake. The data shown for cells poisoned with CCCP or iodine suggest that this is not a large discrepancy (Figs 5.7 and 5.8), but nevertheless it should be accounted for before determining the internal concentration of glycerol. Therefore, the values for the ECV and ICV of pellets of both mutant and wild type cells grown at 0.4 M and 1.5 M NaCl were calculated as described in section 2.10 and are shown in Table 5.4. Cell counts were carried out with samples containing 30 $\mu g\ ml^{-1}$ chlorophyll, the concentration which was used throughout all the transport studies (Table 5.4). From these cell counts and the dpm

TABLE 5.4a. Determination of cell volumes of mutant and wild type cells grown at 0.4 and 1.5 M NaCl using the method described in section 2.10.

	Pellet Volume (µl)	ECV (µl)	ICV (µl)	Cell No. per 0.3 ml	Cell Volume (fl)
0.4 M mut	0.243	0.110	0.133	2.03×10^6	66
0.4 M wt	0.235	0.101	0.134	1.67×10^6	80
1.5 M mut	0.383	0.159	0.224	2.50×10^6	90
1.5 M wt	0.359	0.144	0.215	2.69×10^6	80

TABLE 5.4b. Representative concentrations of intracellular glycerol calculated from uptake of ^{14}C-glycerol.

0.4 M NaCl mutant 3 hours at 30 °C (Fig 5.1)	0.22 mM
0.4 M NaCl wild type 300 min at 30 °C (Fig 5.1)	0.17 mM
1.5 M NaCl mutant 300 min at 30 °C (Fig 5.2)	0.05 mM
1.5 M NaCl wild type 300 min at 30 °C (Fig 5.2)	0.06 mM

values of the pellets corrected for the ^{14}C-glycerol in the ECV, the concentration of labelled glycerol taken up into the cell was calculated. An example of this calculation is shown in section 2.12, which confirmed that the counts in the ECV were negligible. The intracellular concentration of ^{14}C-glycerol was found to be around 0.2 mM for both mutant and wild type at 0.4 M NaCl, but considerably lower, around 0.05 mM, for both strains at 1.5 M NaCl (Table 5.4).

5.2.3.8. Discussion

In order for organisms to grow heterotrophically in the dark, they have to be able to take up organic carbon compounds and utilize them in cell metabolism. Both the mutant and wild type of Dunaliella parva 19/9 take up glycerol into the cells at similar rates when it is present in the medium (eg. Figs. 5.1, 5.2). The glycerol transport system appears to be an active transport system, which is energy driven, since it is inhibited by the uncoupler CCCP as well as by iodine (Figs. 5.7, 5.8). Furthermore, the increase in the rate of uptake, when the temperature is increased by 10 ^{0}C to 30 ^{0}C also indicates active transport, diffusion rates would quite likely be the same at both temperatures (Figs. 5.3 to 5.6). Glycerol uptake is largely independent of light energy, it takes place in high light (20-25 $\mu E\ m^{-2}\ s^{-1}$), dark and ambient light (5-10 $\mu E\ m^{-2}\ s^{-1}$) over the period of the

experiment (Figs. 5.3 to 5.6). Uptake in the dark over a long time span is one of the necessary prerequisites of heterotrophic growth.

Dunaliella cells start to adapt to changes in salinity within minutes and the duration of the recovery phase depends on the severity of the shock (Gimmler and Moeller 1981, Ginzburg et al. 1990, 1991). Uptake of glycerol following osmotic shocks (Figs. 5.10, 5.11, 5.15) follows the same pattern of return to the original rates (Kessly and Brown 1981). The notable exception is the rate of uptake in mutant cells following a downshock, despite recovery from the shocks as indicated by the rates of oxygen uptake and evolution (Table 3.3), no significant uptake of glycerol takes place (Fig. 5.14). This makes sense, as the cells dispose of glycerol following a hypo-osmotic shock and there is no requirement for additional glycerol at this point and thus no glycerol is taken up. This would suggest that the transport system can be switched on and off dependent on the circumstances and particular requirements of the cells.

When organic carbon compounds are taken up, the cells either utilize them and they enter one of the metabolic pathways, or they are stored in original or a converted form. The data in section 5.2.3.6 suggest that more than half of the glycerol taken up by the mutant and wild type cells is incorporated into the lipids of the cell. The

rest may be utilized in cell metabolism, but we have no evidence for or against this hypothesis. The data shown in section 5.2.3.7 show that the uptake of ^{14}C-glycerol into the cells is not large. In any case it is probably not feasible for the cells to grow on glycerol heterotrophically, since it is unlikely that the cells could utilise glycerol as a compatible solute and as a sole source of carbon for growth. Possibly glucose is a more viable candidate.

5.2.4. Uptake of ^{14}C-glucose

5.2.4.1. Effect of light intensity

Mutant and wild type cells of D. parva 19/9 grown in 0.4 M NaCl take up labelled glucose into the cell (Fig. 5.17). Mutant and wild type show very similar uptake rates over the duration of the experiment (3 hours). Figure 5.18 shows the uptake by cells grown in 1.5 M NaCl, mutant cells appear to have a slightly higher rate, however as in Figure 5.2 the larger errors indicate that the cells are stressed in this high salt concentration. Glucose transport by the wild type takes place at the same rate in the light and in the dark (Fig. 5.19), the rate is however, slightly lower than in ambient light conditions (Fig. 5.18).

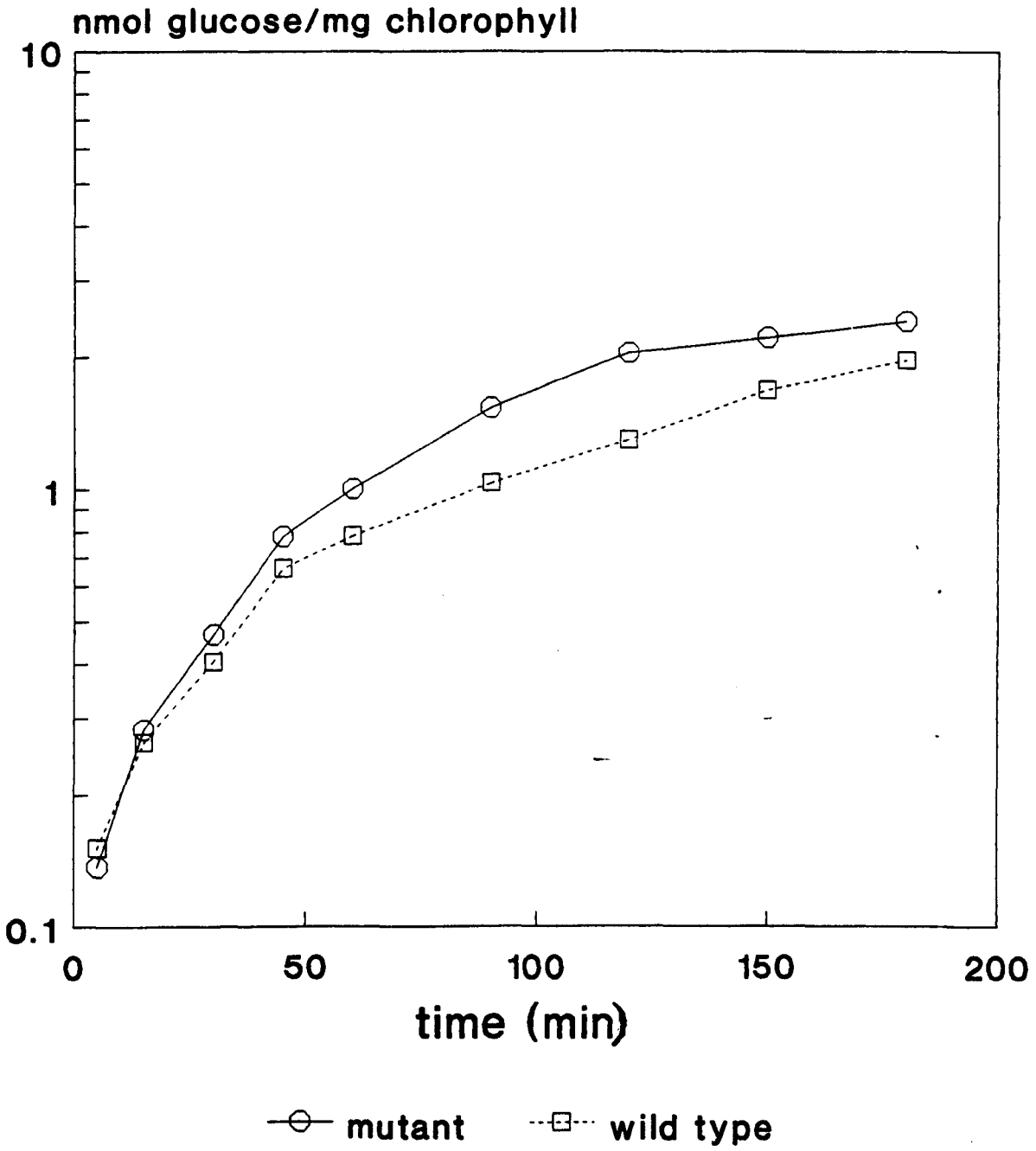


FIGURE 5.17 ^{14}C-glucose uptake by mutant and wild type in 0.4 M NaCl.

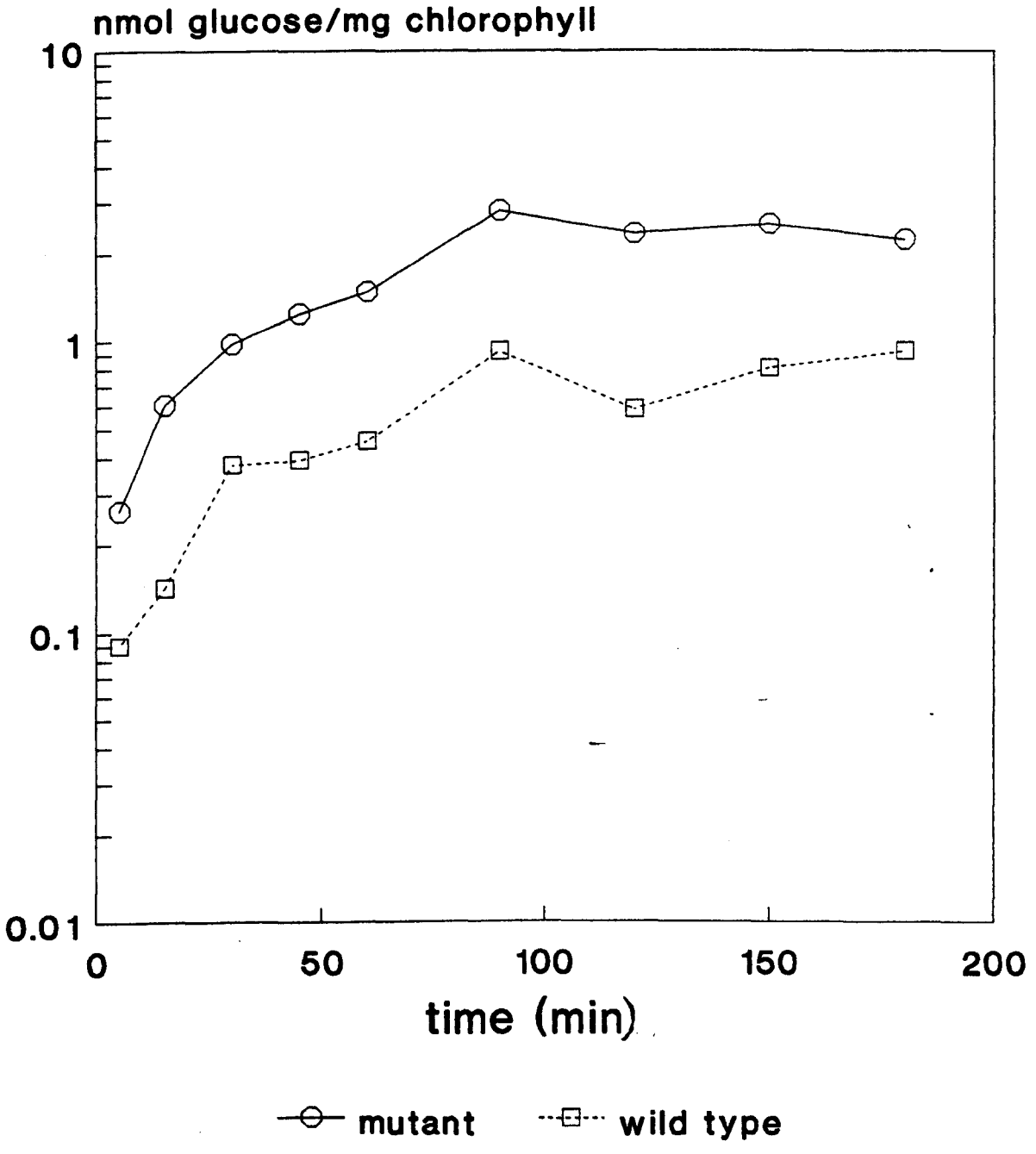


FIGURE 5.18 ^{14}C-glucose uptake by mutant and wild type in 1.5 M NaCl.

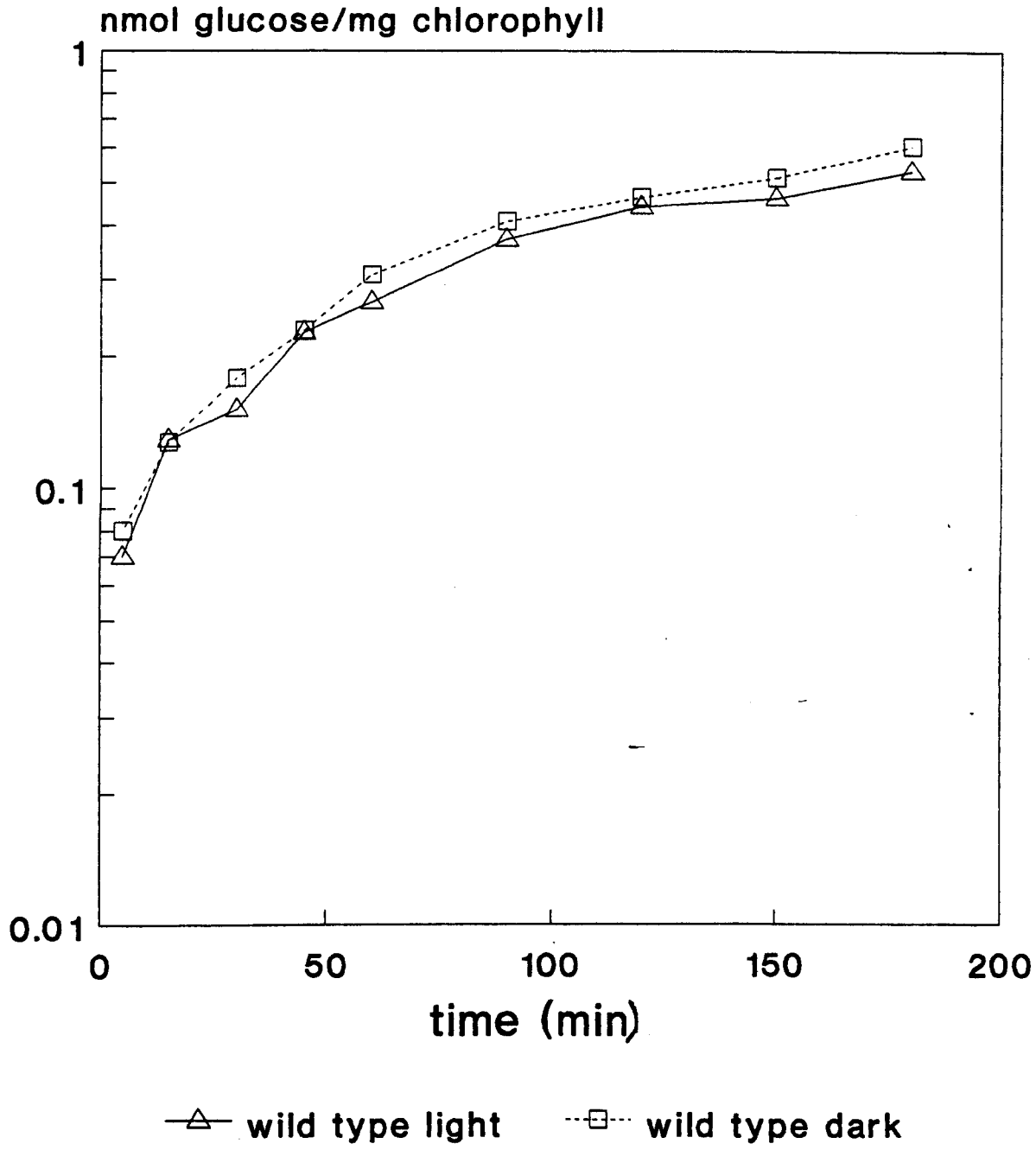


FIGURE 5.19 ^{14}C-glucose uptake by the wild type in light and dark in 1.5 M NaCl.
Due to the stress imposed on the cells by the high salinity the errors were around 15 %.

5.2.4.2. Inhibition of uptake by CCCP

The inhibition of glucose uptake by CCCP is shown in Figure 5.20. Transport does not take place in the presence of 50 µmol CCCP over the 3 hour time span of the experiment in either mutant or wild type cells. Both of the controls have uptake rates similar to the experiment shown in Figure 5.17.

5.2.4.3. Saturation of the transport system

Four different concentrations of glucose were used to establish the point of saturation of the uptake system, the ratio of hot to cold glucose was kept constant (Fig. 5.21). The glucose uptake by the mutant is saturated around a concentration of 4.47 µM, since higher concentrations of glucose (8.93 and 17.86 µM) do not show higher amounts of glucose being taken up over a period of 3 hours.

5.2.4.4. Competition for the uptake system

The transport study, which is shown in Figure 5.22 was designed to establish whether the presence of cold glycerol (up to 20 times glucose concentration) has any effect on the ^{14}C-glucose uptake. The results indicate the same uptake rates in all 4 samples in the experiment,

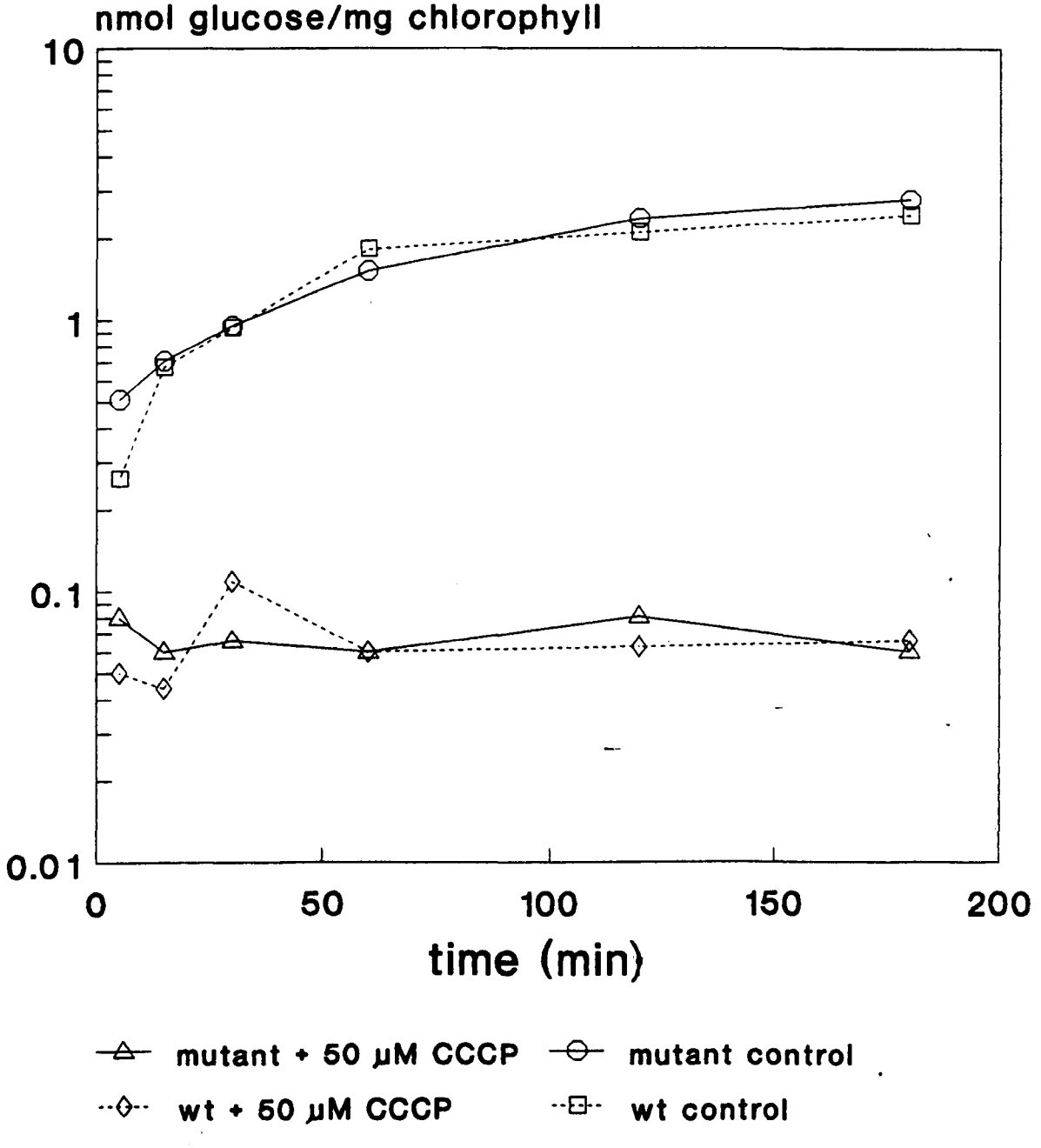


FIGURE 5.20 The effect of CCCP on ^{14}C-glucose uptake by mutant and wild type in 0.4 M NaCl.

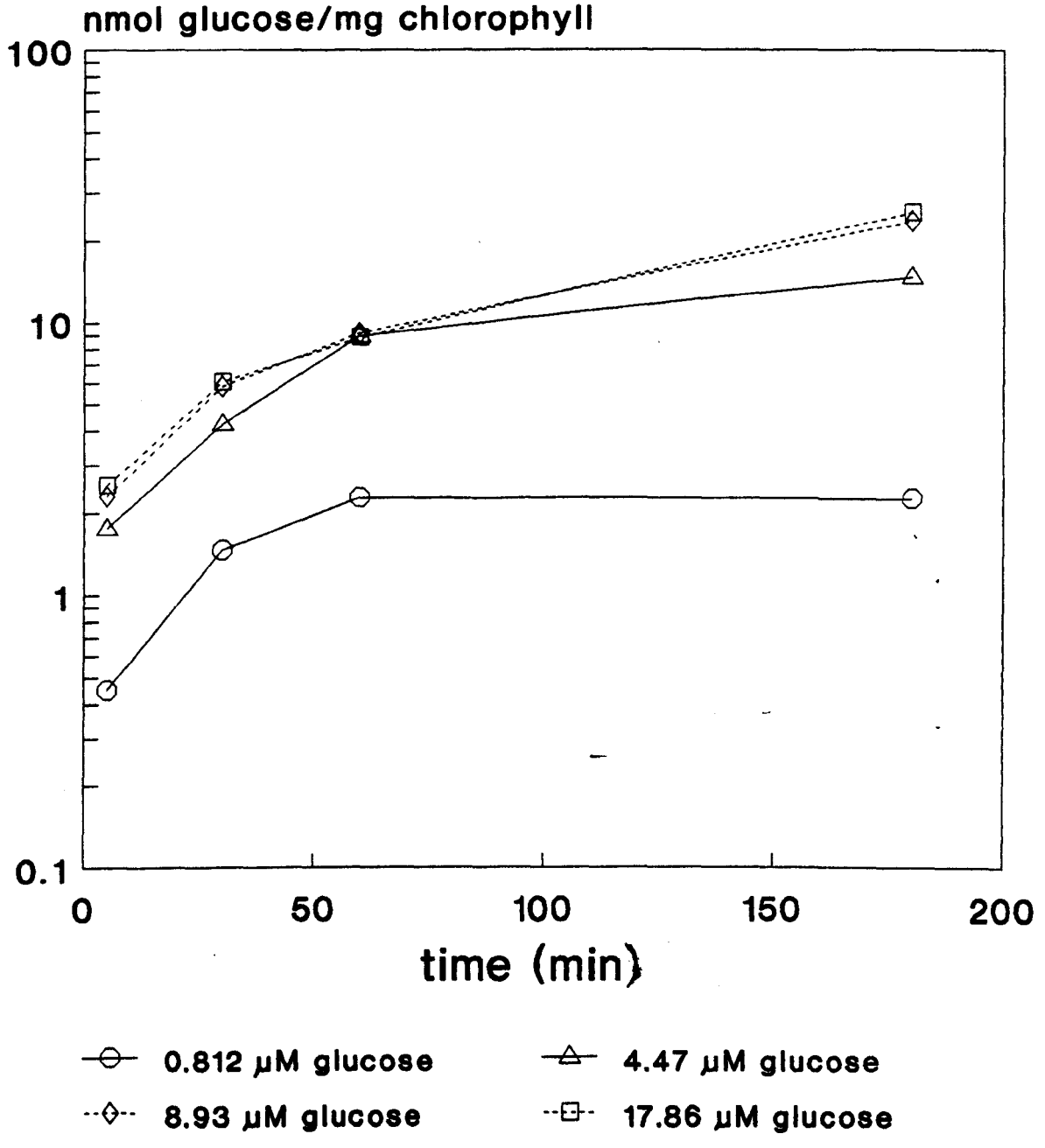


FIGURE 5.21 The effect of different concentrations of glucose on uptake of ^{14}C-glucose by the mutant. A ratio of hot : cold glycerol of 1 : 11 was maintained at each concentration.

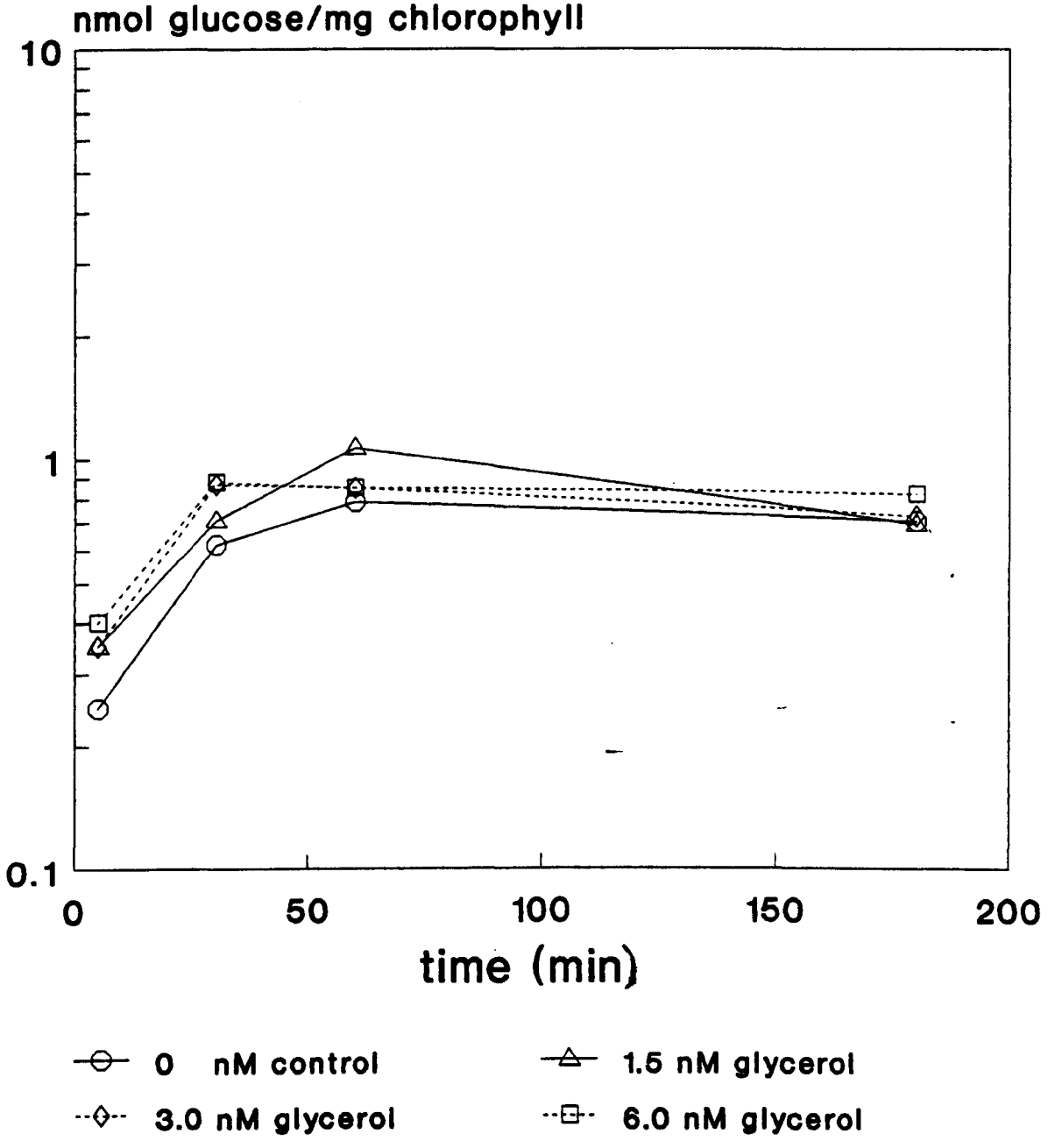


FIGURE 5.22 The effect of high concentrations of unlabelled glycerol on ^{14}C-glucose uptake by the mutant. The ratio between ^{14}C-glucose and unlabelled glycerol is 1 : 11.

thus there is no evidence that glucose and glycerol are taken up by the same transport system.

5.2.4.5. Incorporation of the labelled glucose

This experiment was carried out following the same procedure as with the labelled glycerol, see section 5.2.3.6. Of the glucose taken up after 3 hours 88.32 % was found to be incorporated into the organic solvent soluble fraction of the cell.

5.2.4.6. Amount of glucose taken up per cell

The actual concentration of labelled glucose taken up by a single cell in a representative experiment (Figure 5.17) after 3 hours has been calculated as 0.16 mM of glucose in mutant and 0.13 mM in wild type cells (see section 5.2.3.7).

5.2.4.7. Hexokinase assay

Mutant and wild type cells were assayed for the presence of hexokinase as described in section 2.13. The enzyme hexokinase has to be present in the cells, if glucose is to be utilised via glycolysis, since it catalyses the first step in which glucose is converted into glucose 6-

phosphate. No hexokinase activity could be detected in either mutant or wild type cells.

5.2.4.8 Discussion

The uptake system for glucose in Dunaliella parva 19/9 appears to be quite similar to that for glycerol (Figs. 5.17 and 5.18). The uptake is independent of light intensity (Fig. 5.19). The system is, as is the glycerol transport system, an active one, since it is inhibited by CCCP (Fig. 5.20). This is in contrast to the findings of Kwon and Grant (1971) and Ginzburg and Richman (1985) who did not find an active transport system for glucose in D. tertiolecta or D. parva, but agrees with a report by Hellebust (1978) who found a CCCP inhibited active transport system for glucose in the diatom Cyclotella cryptica. Rates of uptake are saturated at a concentration of around 4.47 µM (Fig. 5.21). The glucose transport system is separate to that of glycerol, because additional glycerol does not compete for uptake into the cell (Fig. 5.22). This agrees with the effect of glucose on ^{14}C-glycerol uptake (Fig. 5.16). A high proportion of the glucose transported into the cell is incorporated into the lipid fraction. This might be a response to the lack of hexokinase which suggests that glucose cannot be metabolised as an energy source. The absence of hexokinase in Dunaliella parva is in contrast to the findings of Kwon and Grant (1971) who found hexokinase

activity in D. tertiolecta cells. The actual amount of glucose transported into a single cell by the glucose uptake system is similar to the amount of glycerol taken up.

5.2.5. Uptake of ^{14}C-acetate

5.2.5.1. Transport of acetate in two different salinities

The uptake of acetate by cells in 0.4 M NaCl is shown in Figure 5.23. Wild type cells seem to take up a higher amount of acetate at the beginning of the experiment, however the mutant reaches the same level of the wild type cells after a period of 60 minutes. Both mutant and wild type uptake levels off after the first hour. Figure 5.24 shows uptake of cells grown in 1.5 M NaCl. Wild type cells take up the labelled acetate at a faster rate than mutant cells and uptake levels off after 60 minutes. The mutant uptake takes place at a slower rate, but it reaches the same level as the wild type after 3 hours. Both mutant and wild type take up higher amounts of acetate in a salinity of 1.5 M NaCl when compared to the uptake in 0.4 M NaCl (Figs. 5.23 and 5.24)

5.2.5.2. Incorporation of labelled acetate

After extraction of the cells in 80 % acetone, 49.5 % of the acetate taken up by 0.4 M NaCl grown mutant cells was

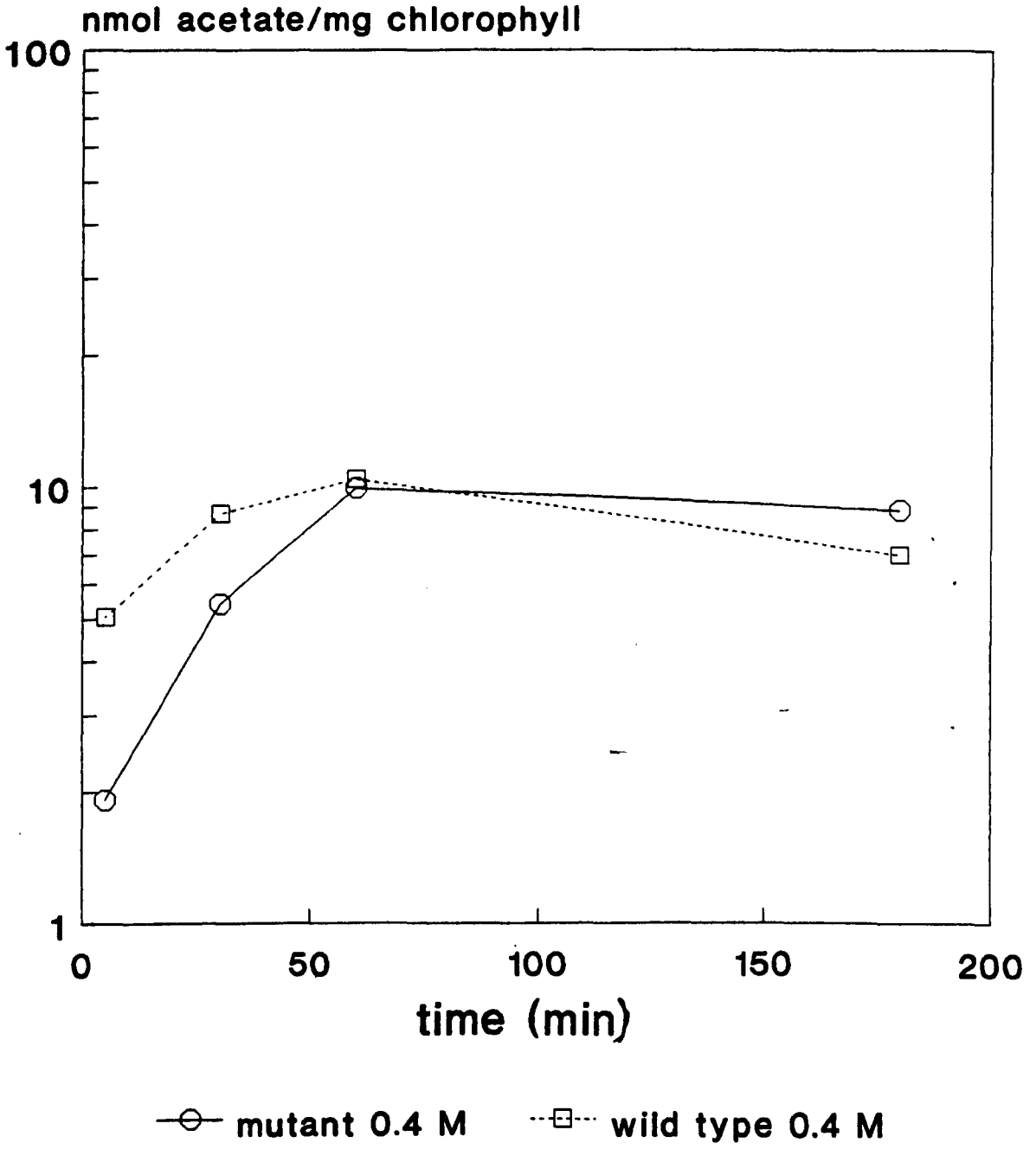


FIGURE 5.23 ^{14}C-acetate uptake by mutant and wild type in 0.4 M NaCl.

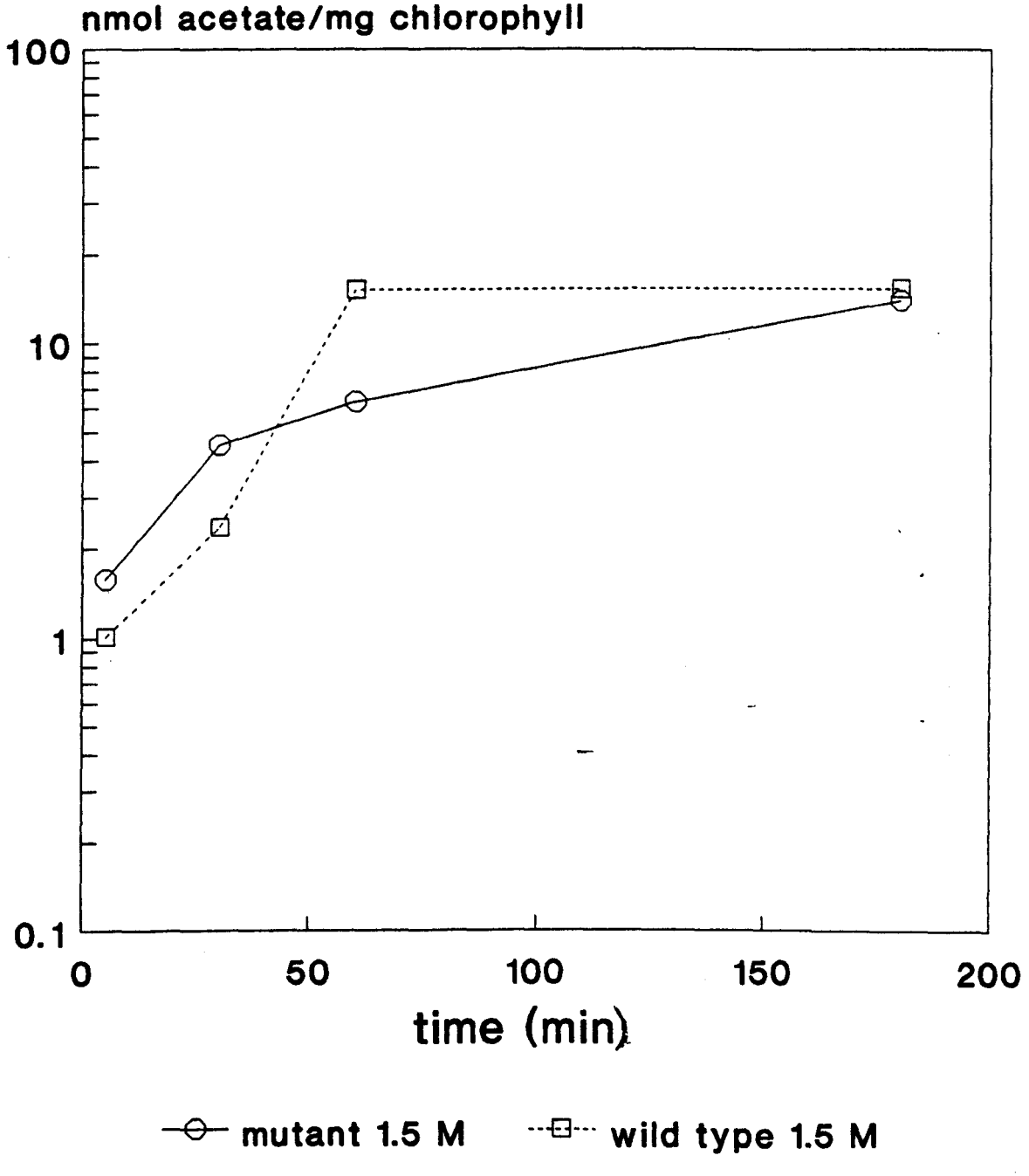


FIGURE 5.24 ^{14}C-acetate uptake by mutant and wild type in 1.5 M NaCl.

found to be incorporated in the organic solvent fraction after 3 hours of the experiment.

5.2.5.3. Amounts of acetate taken up per cell

The amount of acetate taken up by a single cell was calculated from the transport data in Figure 5.23. After 1 hour, mutant and wild type cells were found to have taken up acetate to a concentration of 0.66 and 0.7 mM acetate respectively (see section 5.2.3.7.).

5.2.5.4 Discussion

A large number of microalgae take up acetate from the medium (Ukeles and Rose 1976) and D. parva 19/9 cells were found to take up acetate readily. Overall mutant and wild type cells show very similar rates of labelled acetate uptake (Figs. 5.23 and 5.24), whereby the uptake is higher in cells grown in 1.5 M NaCl medium (Fig. 5.24). The relatively large error bars again indicate the stress the cells are under in such a high salt concentration. Nearly 50 % of the acetate taken up is found in the organic solvent fraction. The actual acetate concentration in a single cell after 1 hour is about 3 times as high as the concentration of glycerol and glucose taken up after 3 hours. This also means that more acetate is incorporated in the lipid fraction in

comparison to glycerol and glucose. More work on the acetate uptake system is required in order to characterise it further and to determine, if it is similar to the glycerol and glucose transport systems.

5.3. Conclusion and Future Work

Despite the presence of uptake systems which transport carbon compounds into the cell that are potential organic carbon sources for heterotrophic growth, it appears that D. parva 19/9 cannot grow in the dark. There are two possible explanations for this. Firstly the organic compound is taken up in insufficient amounts to sustain growth without photosynthesis over a long period of time or secondly the cells may not be able to utilize the organic compound in its central metabolic pathways once it is taken up. Future work on these uptake systems should include a more detailed account of the fate of the labelled substances. In particular, measurement of $^{14}CO_2$ being evolved would be evidence for some metabolism of the compounds. Further information on the acetate and possibly other organic compound transport systems would also be beneficial.

These results are quite significant for the commercial exploitation of Dunaliella, since they confirm that the commercial cultivation of Dunaliella is not feasible in fermentors without illumination.

CHAPTER 6 COMPARISON OF THE LIPID COMPOSITION OF MUTANT AND WILD TYPE DUNALIELLA PARVA CELLS

6.1. Introduction

6.1.1 Surface layers of Dunaliella

The plasma membrane surrounds the cytoplasm of the cell and consists of a lipid bilayer and proteins, which are arranged within the lipids, according to the Singer and Nicolson "Fluid Mosaic Model" (Hopkins 1978). The membrane is a selective barrier between the cell and the environment, controlling inward transport of metabolites and outward disposal of waste products. It is also a receiver and transducer of information, whereby signals are generated, which influence events and processes within the cell. There is no cellulose cell wall in these algae, but to the outside of the plasma membrane there is a glycocalyx layer, formed by the sugar residues of the oligosaccharide parts of the membrane glycoproteins (Oliveira et al. 1980). This cell coat is thought to have a protective function against adhering bacterial contaminants and plays a major role in recognition and reception of extracellular signals. The sugar residue filaments of the glycocalyx react with the substrate and so influence transport across the membrane and possibly bind cations in an exchangeable form.

6.1.2. The plasma membrane of Dunaliella

The plasma membrane of Dunaliella is a normal lipid bilayer (Curtain et al. 1983) with unusual permeability properties (Ginzburg 1969, Ginzburg and Richman 1985, Gimmler et al. 1989). It must withstand high concentration gradients of salt on the outside of the cell and glycerol inside. Glycerol, which is of low molecular weight, usually passes freely across biological membranes. However, the plasma membrane of Dunaliella is impermeable to glycerol, thus retaining the compatible solute within the cell, preventing extensive leakage into the medium (Gimmler and Hartung 1988).

The ability of Dunaliella cells to withstand changes in external solute concentration without lysis of the cells is due to a physical change in the ordering of lipids in response to higher or lower levels of charged phospholipid head groups in the membrane. As the membrane shrinks or expands lipids move in and out of a membrane material reservoir, which is either associated with the plasma membrane or originates from the fusion of small vesicles from the cytoplasm of the cell (Curtain et al. 1983, Maeda and Thompson 1986). Einspahr et al.(1988) reported initial membrane folding after a hyperosmotic shock. These physical changes transduce information about exterior osmotic changes via membrane enzymes, which trigger glycerol synthesis or degradation. The

plasma membrane ATPase is thought to play a major part in the initiation of this recovery mechanism (Oren-Shamir et al. 1989) coupled with a change in the concentration of inorganic phosphate within the cell due to a change in volume (Bental et al. 1990) and an increase in the internal pH (Goyal and Gimmler 1989, Kuchitsu et al. 1989). Despite the change in the number of lipid molecules after a salt shock, the number of proteins in the membrane remains constant (Curtain et al. 1983).

6.1.3. Lipid classes in eukaryotic algae

The lipid and fatty acid composition of algae is species specific and is dependent on environmental conditions, such as temperature, light intensity, salinity, nutrient availability and nitrogen concentration (Ben-Amotz et al. 1985).

The lipid classes found in eukaryotic algae (Wood 1988) are:

1. neutral lipids, such as triacyglycerols, steroids (including sterols and sterol esters) and hydrocarbons,

2. polar lipids, which include phospholipids, mono- and di-galactosylacylglycerols and plant sulpholipids,

3. photosynthetic pigments, the chlorophylls and carotenoids and

4. fatty acids as components of lipids, these occur only in bound form, mostly as glycerol esters.

The total lipid content of algae can be a high proportion of the cell, Ben-Amotz et al. (1985) reported that the lipid content of the green alga Botryococcus braunii makes up 45 % of the organic weight, which increases to 55 % under nitrogen deficient conditions.

6.1.4 Lipid composition of the plasma membrane of Dunaliella

The lipid composition of the plasma membrane differs from that of whole cells (Sheffer et al. 1986). The major lipids of Dunaliella plasma membrane and chloroplasts are the phospholipids phosphatidyl-ethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylinositol (PI) as well as diacylglyceryltrimethylhomoserine (DGTS), a polar betaine lipid and the glycolipids monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulphoquinovosyldiacylglycerol, and a number of neutral lipids. Free sterols are also present, accounting for 55 % of the total and being the biggest membrane component (Peeler et al. 1989). Whereas Peeler et al. reported the

presence of PI, Sheffer et al. did not. However, the biggest controversy is the presence or absence of sterol peroxides, which account for 22 % of the D. salina plasma membrane fraction, isolated by Sheffer et al. (1986), but which Peeler et al. (1989) did not detect. Sheffer et al. claim that the high proportion of sterol peroxides is responsible for the unusual permeability properties and determines the membrane structure.

6.2. Results and Discussion

6.2.1. Plasma membrane isolation

The isolation of the plasma membrane of Dunaliella cells has proved to be very difficult in the past for a number of reasons. The identification of the isolated fraction requires either markers, such as the vanadate sensitive, K^+ stimulated membrane specific ATPase (Gimmler et al. 1989, Gilmour et al. 1985) or a probe, such as sulforhodamine B (Sheffer and Avron 1986), in order to check for contamination of the fraction with other cellular membranes, such as chloroplasts and mitochondria. Two different isolation methods have been developed, differential centrifugation using sucrose gradient (Sheffer and Avron 1986, Gimmler et al. 1989), or using a dextran-polyethylene glycol two phase system (Peeler et al. 1989).

The plasma membrane of the mutant and wild type was isolated according to the method of Peeler et al. (1989) as described in section 2.15. However, the plasmalemma fractions obtained were not large enough to carry out extensive characterisation of the composition. It was nevertheless possible to carry out a crude check of the isolated fraction for contamination through pigments by extraction with 80 % acetone, no impurities were found.

The lipid composition of the membrane is responsible for the retention of the intracellular glycerol (Peeler et al. 1989) and the leaky mutant may be deficient in one or more lipids or have a different composition to the wild type. Total lipids were extracted from whole cells according to Bligh and Dyer (1959) and phospholipids were separated from the bulk lipids by column chromatography (section 2.14). TLC plates were run of the separated phospholipids and also total lipids and it was found that the mutant lacked two of the major phospholipids, PG and PE, whereas the wild type had no PE in the membrane (Fig. 6.1). This is quite surprising, as PE is thought to be one of the major plasma membrane lipids. However, it is in agreement with the findings of Evans et al. (1982), who found PE to be present in Dunaliella tertiolecta, but not D. parva 19/9. The difference between mutant and wild type lipid composition lies in the lack of PG and the unidentified lipid with the Rf of 0.44 in the mutant, all other lipids are the same as in the wild type and comprise of MGDG, DGTS, DGDG, SQDG, PI, PC, one other

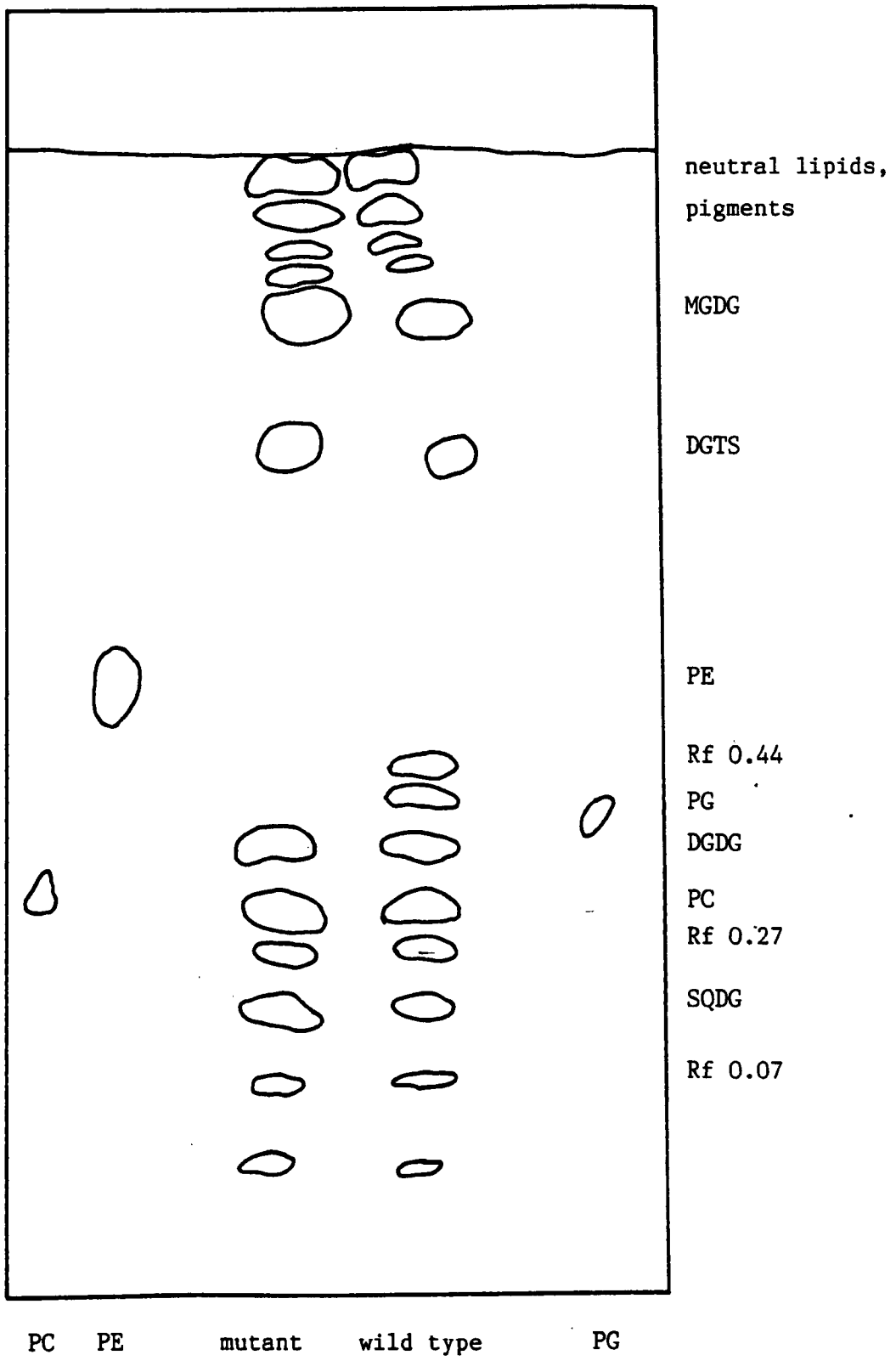


FIGURE 6.1 Lipid composition of the leaky mutant and wild type after separation by TLC. Authentic standards of PC, PE and PG were run on the same plate.

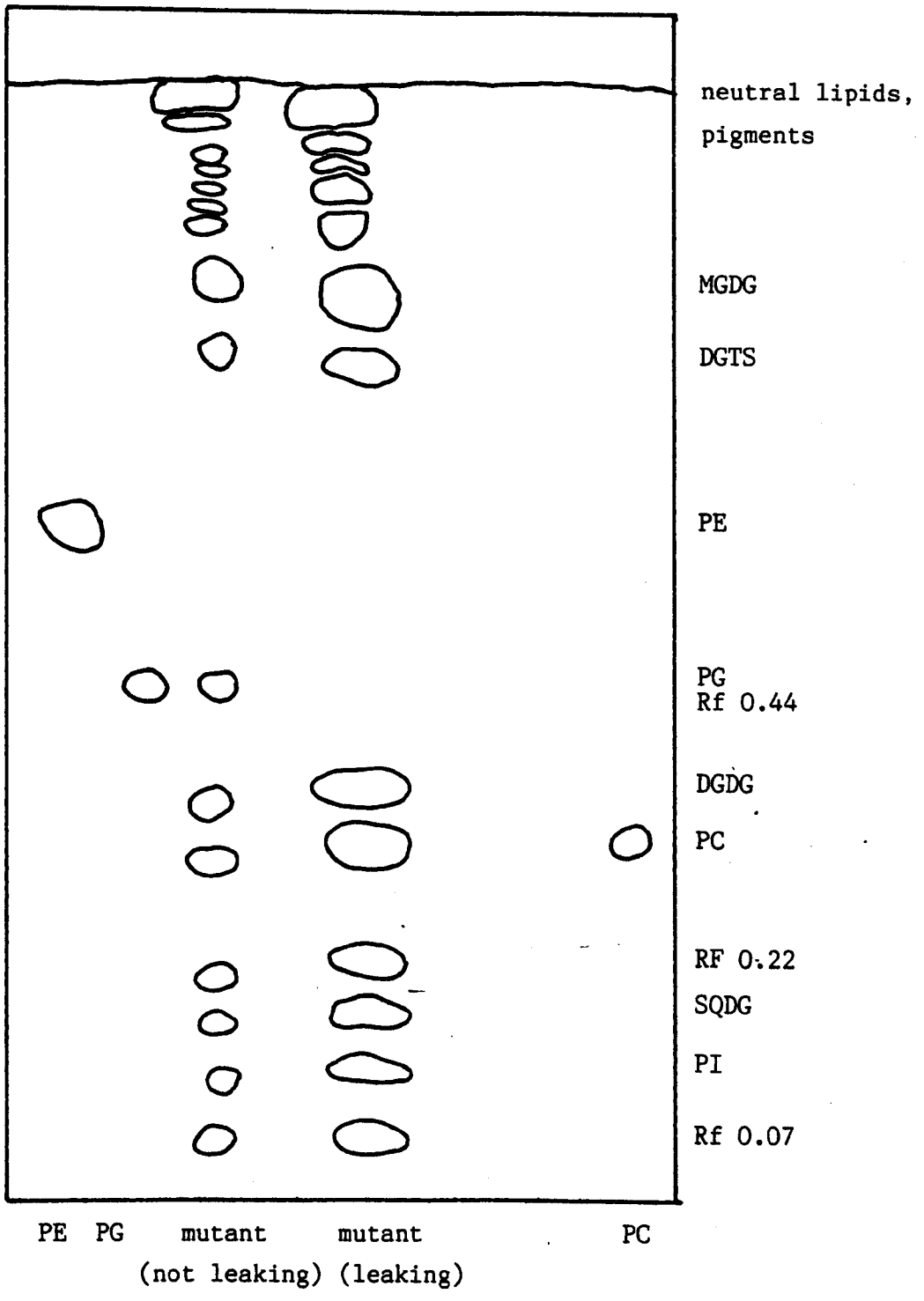


FIGURE 6.2 Lipid composition of the non-leaking mutant grown in high CO_2 in the chemostat and the leaky mutant grown in batch culture. The lipids were separated by TLC.

unidentified lipid with Rf of 0.27, neutral lipids and pigments (Fig. 6.1).

6.2.2. The lipid composition of the mutant grown in the chemostat in high CO_2 conditions

The mutant was grown in continuous culture in the chemostat in medium containing 1.5 M NaCl with the addition of 5 % CO_2 as described in Chapter 4 (section 4.2.5). After 3 days of bubbling CO_2 through the culture the glycerol leakage stopped and the whole cell lipids of the mutant cells were extracted by the Bligh and Dyer (1959) method, and separated by TLC (section 2.14). The lipids extracted from a mutant batch culture grown at 1.5 M NaCl were used as a control. Comparison of the lipid extracts from the chemostat (not leaking glycerol) and batch culture (leaking glycerol) showed the presence of PG only in the mutant grown in the chemostat, all other lipids were identical (Figure 6.2) When compared to the wild type lipid composition (Figure 6.1) the unidentified lipid at Rf 0.44 is not present in mutant cells grown in chemostat culture or batch culture. It appears that the availability of high CO_2 changes the lipid composition of the mutant in such a way that PG is now present. However there is still a difference in the presence of the unidentified lipid (Rf= 0.44) in the wild type and this

suggests that the unidentified lipid (Rf= 0.44) plays no major role in glycerol retention.

These results suggest that the lack of PG in the mutant may be responsible for the leakage of glycerol. However, further experiments are necessary to prove this hypothesis. Since our data relate to whole cells, it will be necessary to demonstrate that PG is present in the plasma membrane of the wild type and not only in the chloroplast membranes. If so, reconstitution experiments could be attempted to prove the importance of PG in glycerol retention.

CHAPTER 7 CONCLUSIONS

A leaky mutant of Dunaliella parva 19/9 was characterised in this study. It was found that the mutant leaks up to 75 % of the intracellular glycerol into the medium (Table 3.1). Despite the high loss of glycerol, the mutant shows the same growth rate as the wild type over the period of leakage (Fig. 3.2). Intracellular glycerol levels are the same in mutant and wild type (Fig.3.4) which makes sense since the internal osmotic pressure must balance the external osmotic pressure of the high salt concentrations. The wild type was found to have higher deposition rate and levels of starch inside the cell than the mutant (Figs. 3.5, 3.6). At 1.5 M NaCl the mutant has higher rates of oxygen evolution and oxygen uptake than the wild type (Table 3.2) indicating the stress imposed on the mutant cells by the salinity of the medium.

The phase of recovery following osmotic shocks reflects the severity of the shock and is completed after about 60 minutes (Table 3.3). Growth rates of mutant and wild type are very similar and depend on the salinity of the new medium in which the cells were resuspended and they decrease with increasing salinity (Figs. 3.7. to 3.10).

When the mutant was grown in continuous culture in the chemostat, the effect of high temperature (Fig. 4.1), salinity (Fig. 4.2), pH (Fig. 4.3), light intensity (Fig.

4.4) and high CO_2 (Fig. 4.6) on glycerol leakage was examined. Only high concentrations of CO_2 showed a marked effect on glycerol leakage, the mutant stopped leaking after bubbling 5 % CO_2 through the culture and leakage could be induced again after decreasing the concentration of CO_2, if this was not done immediately the cells showed zero leakage and leakage could not be re-established (Fig. 4.6).

Both mutant and wild type did not grow heterotrophically on organic carbon sources (Table 5.1), however organic compounds, such as glycerol, glucose and acetate are taken up into the cell by active transport systems (Figs. 5.1, 5.2). These transport systems are independent of light intensity (Figs. 5.3, 5.4, 5.19) and are inhibited by CCCP and iodine (Figs. 5.7, 5.8, 5.20). A large percentage of the compounds taken up are incorporated into the cell biomass (section 5.2.3.6., 5.2.5.5 and 5.2.7.2). However evidence of metabolism of the labelled substances could not be found and thus requires further investigation.

When the total lipid composition of mutant and wild type were compared, the mutant was found to lack two lipids, PG and one unidentified lipid with the Rf of 0.44 (Fig. 6.1). The lipid composition of the high CO_2 non-leaking mutant from the chemostat show a change in lipid composition in that PG is now present, however the unidentified lipid at RF 0.44 is not ((Fig. 6.2). These

data implied that PG was the key to glycerol retention by the plasma membrane.

The mutant is clearly not at a disadvantage when compared to the wild type even under stressed conditions, such as high salinity or following osmotic shocks. The only differences found are in lower starch deposition and the higher rates of oxygen uptake and evolution, as the mutant has a higher requirement for energy to maintain a constant intracellular glycerol level. This indicates that cells normally run below full capacity and do not utilize all the energy available. This may allow them to survive any sudden stress imposed on the cells

Future work would include further characterisation of the transport systems, particularly that of acetate. An investigation of the fate of the labelled compounds once inside the cells should be carried out in order to establish if they are metabolised and $^{14}CO_2$ trapping experiments would be the most useful in this respect. Furthermore the isolation and characterisation of the plasma membrane of mutant and wild type, as well as that of a high CO_2 (non-leaking) mutant should be carried out in order to examine and compare the lipid composition. These experiments are essential since the whole lipid analysis carried out in Chapter 6 do not unequivocally prove that PG is the key component in glycerol retention by the plasma membrane.

The work in this thesis suggests that the leaky mutant of D. parva 19/9 may have a role to play in the commercial production of glycerol by Dunaliella. At the moment the low selling price of glycerol (less than $ 2 kg^{-1} from petroleum by-products) means that the savings on harvesting costs due to use of the leaky mutant would still not make the process commercially viable. However, it may be that in future when the price of oil increases, the mutant may allow comercial production of glycerol by Dunaliella to become a reality.

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APPENDIX

Dunaliella Growth Media

All solutions were prepared using distilled water and stored at room temperature. The reagents were all "Analar" grade.

Stock Solutions:

4.0 M KCl

2.0 M $MgCl_2.6H_2O$

1.0 M $CaCl_2.2H_2O$

2.4 M $MgSO_4.7H_2O$

4.0 M $NaNO_3$

0.5 M Na_2SO_4

0.1 M NaH_2PO_4

1.5 mM FeEDTA*

1.0 M Tris*

* Both solutions were adjusted to pH 7.6 before addition to the medium

Micronutrient Supplements:

185.0 mM H_3BO_3

7.0 mM $MnCl_2.4H_2O$

0.8 mM $ZnCl_2$

0.02 mM $CoCl_2$

0.0002 mM $CuCl_2$

0.02 mM $CoCl_2$ was prepared by adding 1 ml of a 2 mM stock solution to H_2O to a final volume of 100 ml.

0.0002 mM $CuCl_2$ was prepared by adding 0.1 ml of a 0.2 mM stock solution to H_2O to a final volume of 100 ml.

Volumes of Stock Solutions added to the Medium (ml l^{-1}):

Stock Solution	Volume	Final Concentration (mM)
KCl	2.5	10
$MgCl_2$	10.0	20
$CaCl_2$	10.0	10
$MgSO_4$	10.0	24
$NaNO_3$	1.25	5
Na_2SO_4	48.0	24
NaH_2PO_4	1.0	0.1
FeEDTA	1.0	0.0015
Micronutrients	1.0	-
Tris.HCl	20.0	20

23.38 g l^{-1} or 87.66 g l^{-1} NaCl were added to give 0.4 M or 1.5 M NaCl medium respectively.

After adjusting the pH to 7.8 with either HCl or NaOH, and making the solution up to its final volume, 1 g l^{-1} of solid $NaHCO_3$ was added.

The medium was then autoclaved for 20 minutes at 15 psi.

Solid Media

Nutrient Plates:

Nutrient Broth No.2 (Oxoid) with added NaCl to give 0.4 M or 1.5 M NaCl concentration.

To solidify the solution, 1.6% (w/v) of bacteriological agar (Oxoid) was added and the medium was then autoclaved

for 20 minutes at 15 psi. After cooling to 50 ^{o}C the plates were prepared by pouring the nutrient solution into 9 cm^3 plastic Petri dishes.

Media Plates and Slopes:

Media plates and slopes were prepared by adding 1.6% (w/v) of agar (Oxoid) to 0.4 M or 1.5 M medium. After autoclaving for 20 minutes at 15 psi and following cooling to 50 ^{o}C, the medium was dispensed into Petri dishes for medium plates. For slopes the solution was poured into sterile 25 ml universal bottles or 5 or 6 inch test tubes with cotton wool bungs. The bottles and test tubes were rested at an angle so that the setting agar formed a slope.

The Solubility of Oxygen in Water

The solubility of oxygen in water changes with salinity and temperature. For calibration of the Clark type oxygen electrode, the following oxygen solubility values were used. Salts other than NaCl were not taken into consideration:

Salinity (M NaCl)	O_2 concentration at 30 ^{o}C (μmoles ml^{-1})
0	0.2202
0.4	0.2084
0.8	0.1835
1.0	0.1776
1.4	0.1633
1.5	0.1598
1.8	0.1468
2.0	0.1397
2.5	0.1231