CHAPTER V

BIOCHEMICAL PROPERTIES OF PARAQUAT RESISTANT MUTANTS OF *CHLAMYDOMONAS REINHARDTII*

5.1 Preparation of Protoplast and Chloroplast

5.1.1 Protoplast Formation Autolysin which is a wall hydrolytic enzyme specific for *Chlamydomonas* was prepared in the laboratory from mating reaction of different mating types of *C. reinhardtii* ,11-32/b and 11-32/c. It was found that under laboratory conditions 70-80% of mating was consistently obtained resulting in the release of autolysin into the medium. Crude autolysin preparation was determined to be 8-10 µg protein/ml medium. In order to maximize the amount of protoplast formed, the enzyme was concentrated to yield 100 µg protein/ml by ultrafiltration.

The number of forming protoplasts could be enumerated by treating the hydrolysis mixture with 0.025% Triton X-100 (final concentration). At this Triton X-100 concentration, the protoplast was totally lysed while only a small amount of cell (5%) was broken (Fig. 5.1).

The period of time for maximum protoplast formation of the wild type and paraquat resistant *C. reinhardtii* by autolysin treatment was established (Fig. 5.2). The rate of cell wall digestion by autolysin was clearly different among three strains of *C. reinhardtii*. The slowest rate was seen in PPQ-10/3 in comparison with

Figure 5.1 Effect of Triton X-100 on cell hydrolysis of *Chlamydomonas reinhardtii* 137c.

Cells ($6x10^6$ cells/ml) in 0.6 ml N-free medium were mixed with 1 ml of 0.04% Triton X-100 (5 mM EDTA, pH 8.0). After 5 min of incubation at 34°C, the remaining intact cells were counted.

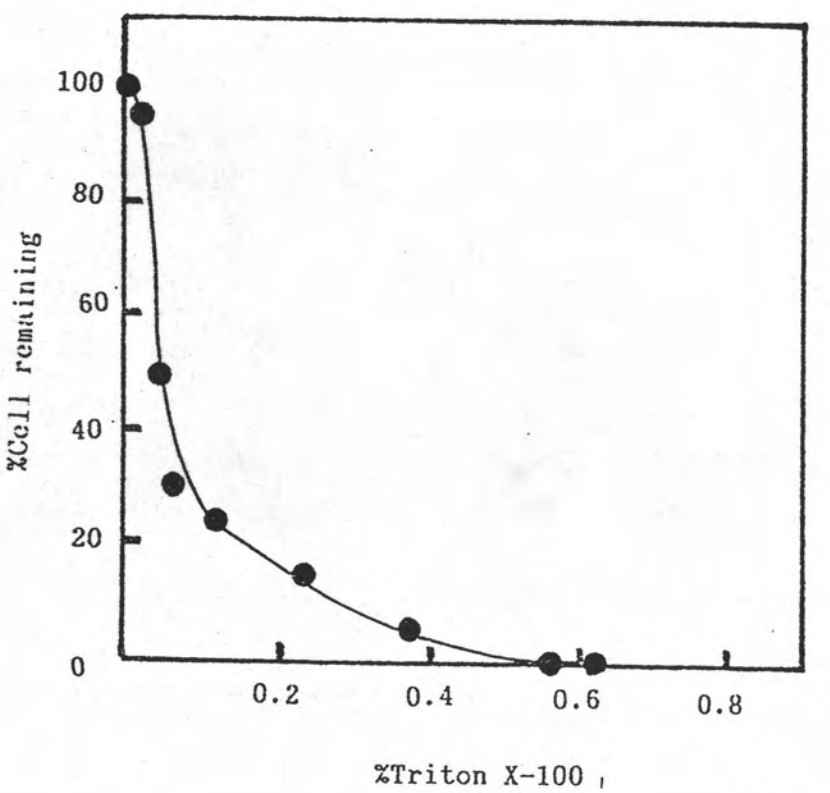
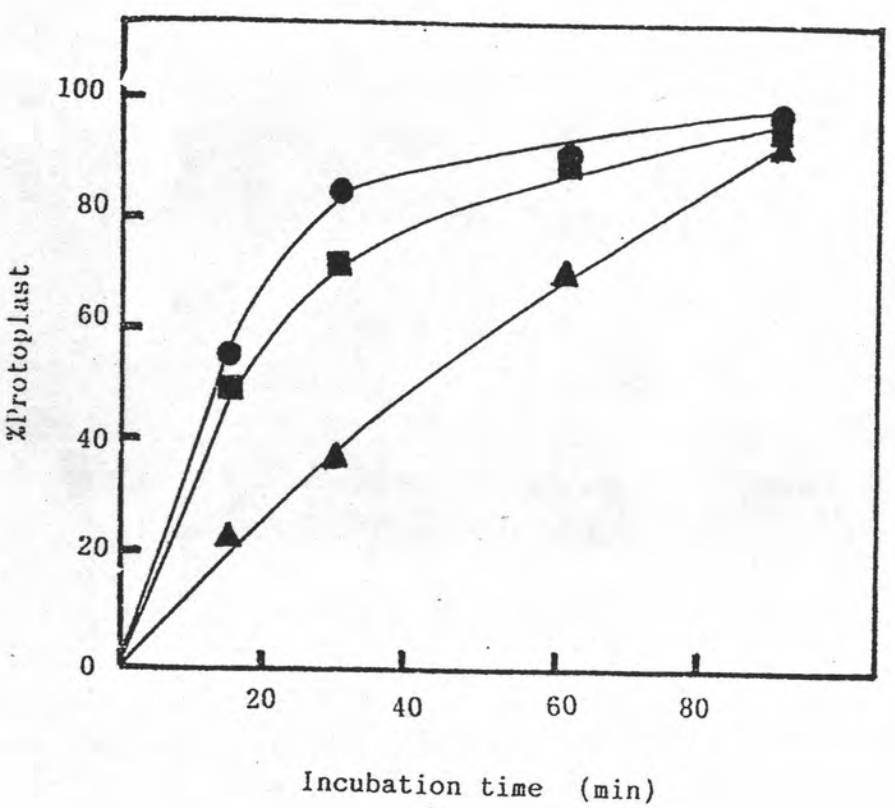


Figure 5.2 Time course of protoplast formation in Chlamydomonas reinhardtii wild type and paraquat resistant strains. Cells (logarithm phase) were incubated in autolysin solution (100 μg/ml) at 34°C. At time intervals a 0.6 ml aliquot of cell suspension was mixed with 1 ml of 0.04% Triton X-100 (5 mM EDTA , pH 8.0). Number of protoplast formed was calculated by subtracting number of the remaining cells from the total control cells without autolysin treatment.
(●) wild type (▲) PPQ-10/3 (■) UPQ-S1



that of UPQ-S1 strain and wild type respectively. However the period of time for maximum protoplast formation in all strains was approximately the same at 90 min with about 94-98% protoplast yields.

Differences in size of protoplasts were obviously observed in each strain. Protoplasts of the wild type were smaller than that of UPQ-S1 and PPQ-10/3 respectively (Fig. 5.3).

The isolated protoplasts were fairly stable. When transferred into TMP medium and kept at 37°C for 10 hours without adding cycloheximide , no cell wall regeneration could be detected.

5.1.2 Isolation of Chloroplast Protoplasts of wild type and paraquat resistant mutants of C. reinhardtii were subjected to breaking by using digitonin lysis of which the action was not specific to only protoplast membrane but also chloroplast membrane. The protoplasts were shortly exposed to digitonin (0.004%) at 30-32°C for 2 min, and the resulting material was forced through a plastic micropipette tip (1 mm in diameter as described in section 2.9). The free chloroplasts appeared as concave structures (Fig. 5.4) among the unbroken protoplasts which were indicated to be smooth spherical shape with the apparent protoplast boundaries. Approximately 60% yield of free chloroplasts could be recovered from the protoplasts under the experimental conditions.

Figure 5.3 Protoplasts of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Vegetative cells at hour 6 in light period were exposed to autolysin (100 µg/ml) for 90 min at 34°C. Pictures of protoplasts were taken under a light microscope (magnification x200).

a. wild type

b. PPQ-10/3

c. UPQ-S1

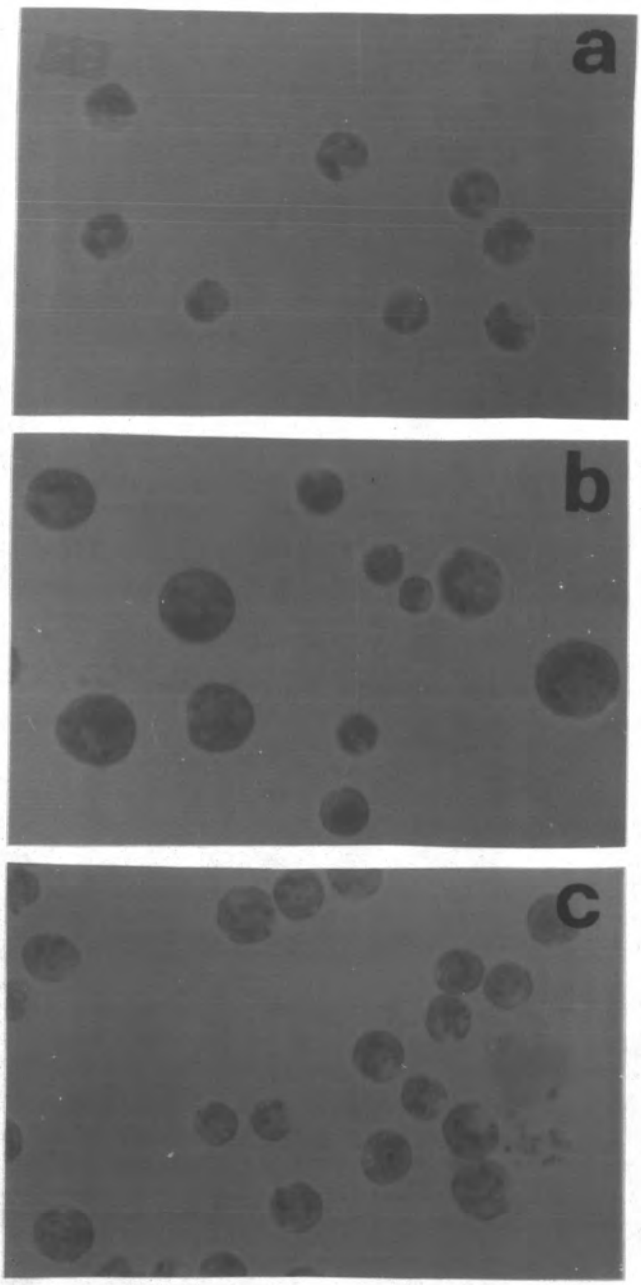
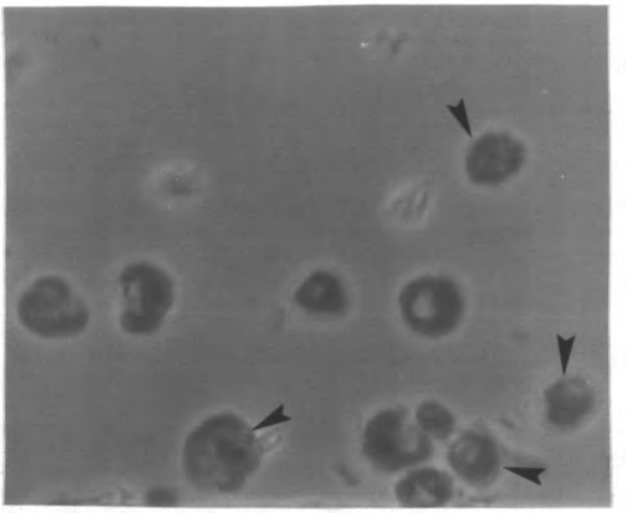

a
b
c

Figure 3.4 Illustration of Chlamydomonas reinhardtii 137c chloroplasts.

Protoplasts were shortly treated under the control action of digitonin at 30-32°C. Chloroplast appeared among the unbroken protoplasts (arrows). The pictures were taken under a phase contrast microscope (x200).



5.2 Uptake of [^{14}C]Paraquat

5.2.1 Reliability of the Method for Measurement of [^{14}C]Paraquat

To assure the reliability of the method for extraction of [^{14}C]paraquat from cells, a standard assay was first set up by using C. reinhardtii 137c cell as a model. After directly adding known amount of [^{14}C]paraquat to 10^7cells, 10% TCA solution was used to lyse cells and precipitate all cellular proteins to give a colorless clear supernatant. It was found that supplementation of non radioactive paraquat (8 mM) in 10% TCA was necessary for reproducibility of the radioactivity measurement of [^{14}C]paraquat.

Reliability of the method was judged by determing the recovery and precision value of the extraction. From Table 5.1, it was revealed that at any amount of [^{14}C]paraquat giving to cells, the radioactive paraquat was completely chased into the supernatant portion resulting in the recovery of extraction considerably 100% .

In case of the precision of the extraction method, it could be seen as well in Table 5.1 that the coefficient of variation (%c.v.) varied from 0.98-12.96 depending on the amount of [^{14}C]paraquat input to cells. However the %c.v. was in the range of acceptable precision.

5.2.2 Optimization of the Methods for [^{14}C]Paraquat uptake

Two parameters possibly affect paraquat uptake of the algal cell were determined. Effect of cell density was first evaluated by using 137c cells. In the mixture of 2.5 ml, various number of

Table 5.1 Recovery and precision of the 10% TCA extraction method for [^{14}C]paraquat.

Certain amount of [^{14}C]paraquat was added to packed cells of C. reinhardtii (10^7 cells) and incubated at 30°C for 30 min. After extraction with 10% TCA (containing 8 mM paraquat), the radioactivity was counted and calculated for %recovery and precision (within assay) of the method whereby the latter was shown as %c.v. (n = 10).

Amount of [^{14}C]paraquat added (pmol/10^7 cells)	%Recovery	%c.v.
6	105 ± 0.81	12.96
15	109 ± 1.20	7.08
33	105 ± 0.80	0.98

cells were accounted to yield $2x10^6$-$30x10^6$ cells/ml. It is indicated in Fig. 5.5a that higher cell density in assay mixture diminished paraquat uptake. Cell density at $2x10^6$-$4x10^6$ cells/ml was shown to result in maximum uptake value.

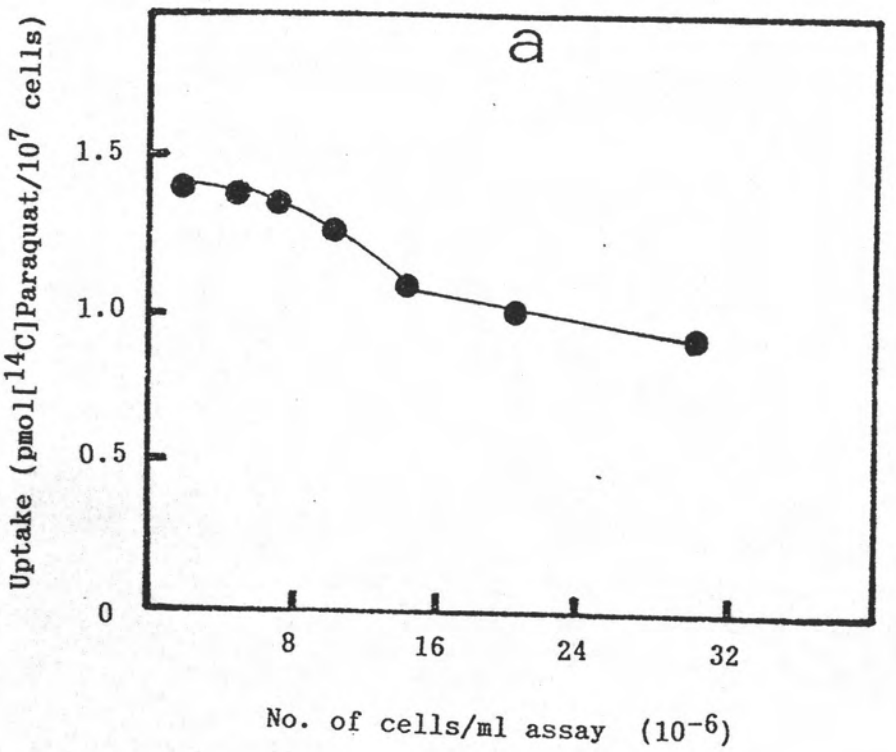
Another factor to be considered was the effect of washing. Once uptake reaction was terminated, cells were repeatedly washed with 20 folds of chilled medium, by centrifugation, to eliminate non-specific binding of [^{14}C]paraquat molecules. Results clearly demonstrate that level of [^{14}C]paraquat retained was sharply decreased to a constant value at the third washing (Fig. 5.5b). Hence throughout the uptake studies in this research project, amount of [^{14}C]paraquat in cells was traced when washings were repeated for 3 times.

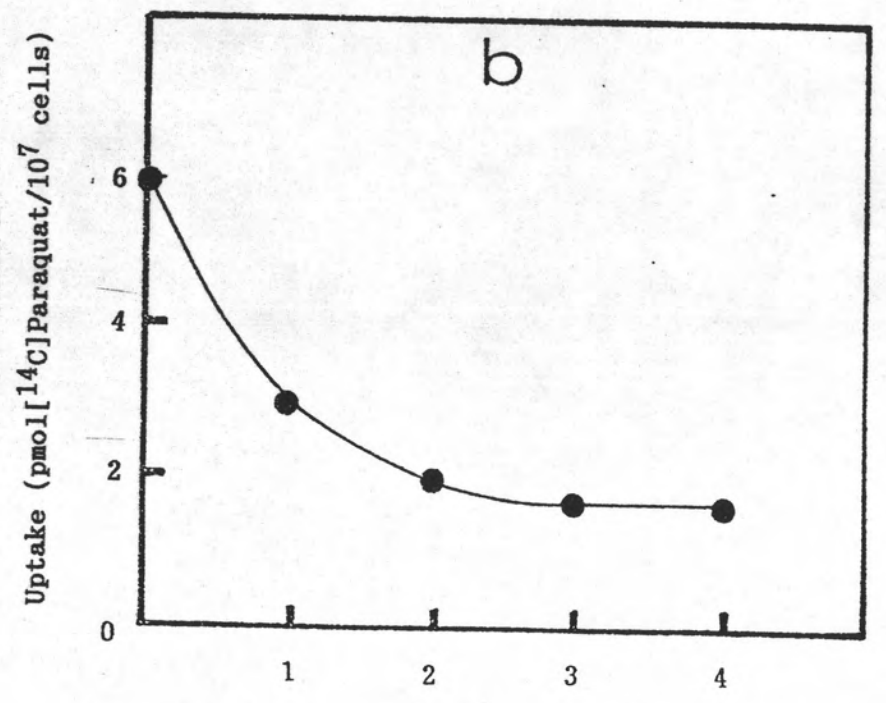
5.2.3 Time Course of [^{14}C]Paraquat Uptake The time course of uptake of 0.1 μM [^{14}C]paraquat into C. reinhardtii cells was examined at 30°C. Fig. 5.6 depicted that in all strains, the zero-time intercept did not always pass through the point of origin. This was attributed to a rapid irreversible adsorption of paraquat presumably on cell wall surface of the alga. For 137c cells, following the first phase, uptake appeared linear for approximately 15 min and reached a steady state no later than 30 min (Fig. 5.6a). On another hand, although the appearance of [^{14}C]paraquat within the UPQ-S1 cell (Fig. 5.6c) was a linear function on time during the first 15 min, the rate approaches zero at about 20-45 min and begins

Figure 5.5 Optimization of factors affecting [^{14}C]paraquat uptake in Chlamydomonas reinhardtii 137c.

a. Effect of cell density: Assay was achieved in a 2.5 ml medium containing 0.1 μM [^{14}C]paraquat with various cell concentrations. After incubation for 15 min, cells were washed 3 times and measured for cellular radioactivity.

b. Effect of washing: Cells were incubated in a 2.5 ml medium containing 0.1 μM [^{14}C]paraquat ($4x10^6$ cells/ml) for 15 min. After each washing, cellular radioactivity was measured.


a
Uptake (pmol[^{14}C]Paraquat/10^7 cells)
1.5
1.0
0.5
0
8
16
24
32
No. of cells/ml assay (10^{-6})


b
Uptake (pmol[^{14}C]Paraquat/10^7 cells)
6
4
2
0
1
2
3
4
No. of washing

to decline thereafter. The paraquat resistant PPQ-10/3 took up the herbicide in a different pattern from wild type and the paraquat resistant UPQ-S1. The profile of time course of uptake exhibited a maximum peak around 20-30 min and a decline phase after 30 min (Fig. 5.6b).

The time course profile demonstrated in this experiment that the process of paraquat uptake into the algal cells specifically in the initial phase was a linear function of time. It is the first evidence to support that the paraquat herbicide is transported into C. reinhardtii via a carrier-mediated process requiring the function of membrane protein.

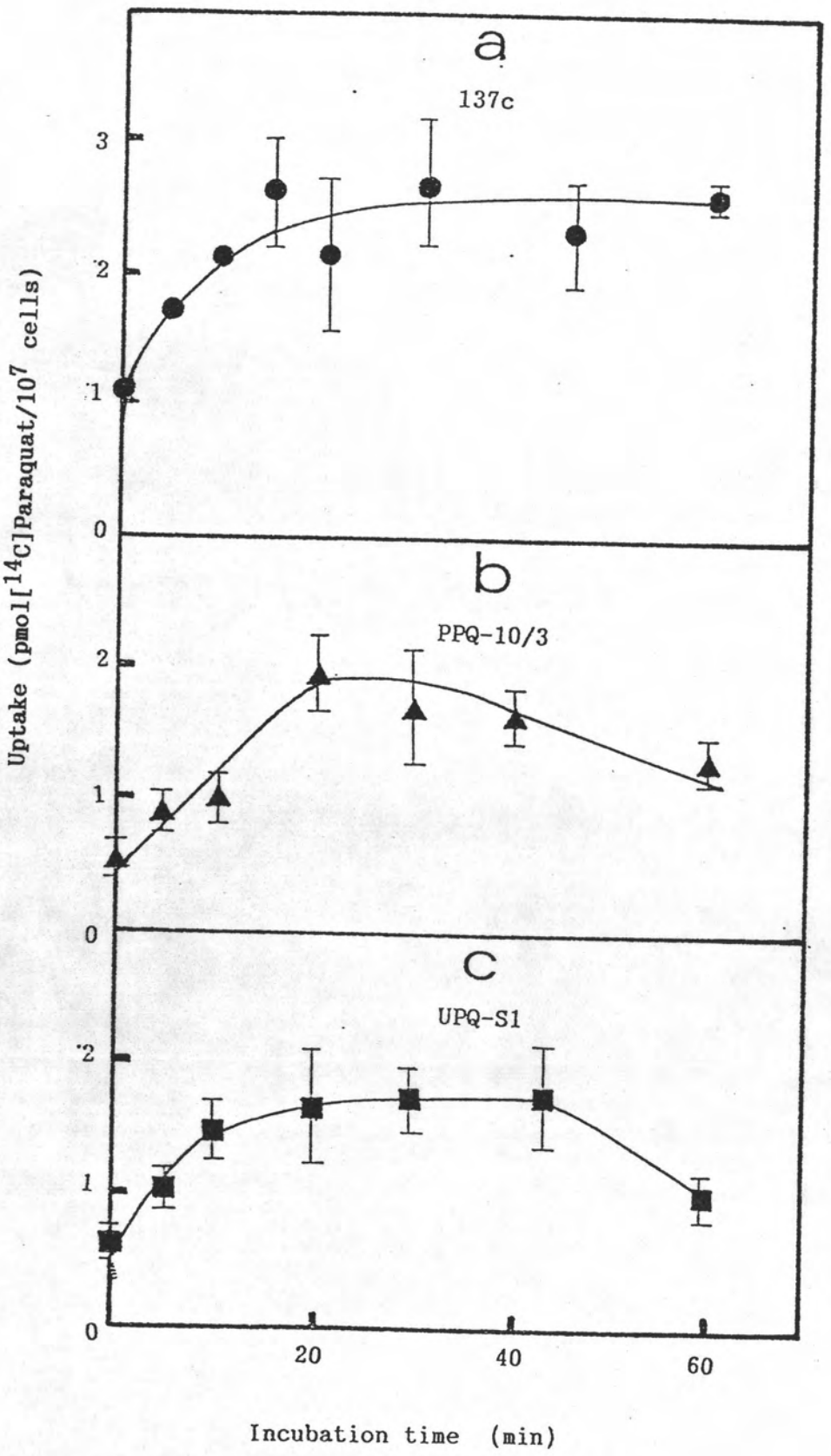
Moreover from this result, the [^{14}C]paraquat uptake reaction would be further achieved with different incubation periods in different cell types that was 30 min for 137c and UPQ-S1, whereby 20 min incubation was designed for PPQ-10/3.

5.2.4 [^{14}C]Paraquat Uptake of Whole Cell Kinetics of paraquat uptake was studied under standard conditions , in a range of low [^{14}C]paraquat concentration (0.03-0.35 µM) without light exposure. The results indicated that the maximum value of [^{14}C]paraquat penetrated into C. reinhardtii PPQ-10/3 and UPQ-S1 were approximately 6 pmol/10^7 cells, which was not significantly different from that in wild type. Nevertheless different pattern of uptake kinetics was obviously noticed. Uptake of [^{14}C]paraquat as a function of initial concentration in paraquat resistant strains, PPQ-10/3 and

Figure 5.6 Time course of [^{14}C]paraquat uptake in Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells were incubated in a 2.5 ml medium containing 0.1 μM [^{14}C]paraquat ($4x10^6$ cells/ml). At time intervals of uptake cells were collected by centrifugation, washed 3 times and measured for cellular radioactivity. Control zero time of uptake was performed by adding chilled [^{14}C]paraquat into an ice-cold reaction mixture and immediately subjected to centrifugation.

a. 137c b. PPQ-10/3 c. UPQ-S1


a
137c
b
PPQ-10/3
c
UPQ-S1
Uptake (pmol[14C]Paraquat/10^7 cells)
Incubation time (min)
0
1
2
3
20
40
60

UPQ-S1, was a typical saturable process whereby a steady state was observed at around 0.2 μM [^{14}C]paraquat (Fig. 5.7b and c). This result confirmed the finding that paraquat penetration through cell membrane is facilitated by a carrier-mediated system as interpreted in the previous section.

Whereas in wild type (137c), it remarked a strange correlation between the cellular [^{14}C]paraquat and the flux of the radioactive herbicide outside (Fig 5.7a). Results indicated that paraquat uptake in wild type cells did not possess a clear saturation curve which is an important characteristic of carrier-facilitated transport. In spite of the fact that the uptake profile consistently exhibited an unexpected declining phase, a linear correlation of the amount of intracellular [^{14}C]paraquat versus the initial concentration of [^{14}C]paraquat could be noticed at low concentration of the herbicide (0.03-0.2 μM).

5.2.5 Effect of An Energy Uncoupling Agent on [^{14}C]Paraquat Uptake Cells were subjected to starvation and energy depletion by treatment with dinitrophenol (DNP) which is a potent inhibitor for the coupling reaction of ATP synthesis.

As shown in Fig. 5.8 , DNP at 1 mM had no effect on the uptake profile of all of the 137c , PPQ-10/3 , and UPQ-S1 strains but remarkably reduced the uptake value. The results led to a conclusion that the process of [^{14}C]paraquat transport across the algal membrane was an energy dependent mechanism.

Figure 5.7 [^{14}C]Paraquat uptake in whole cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells grown in paraquat free medium were incubated in a 2.5 ml medium containing various amount of [^{14}C]paraquat ($4x10^6$ cells/ml). After a certain period of time at 30°C, cells were washed 3 times and measured for cellular radioactivity.

a. 137c b. PPQ-10/3 c. UPQ-S1

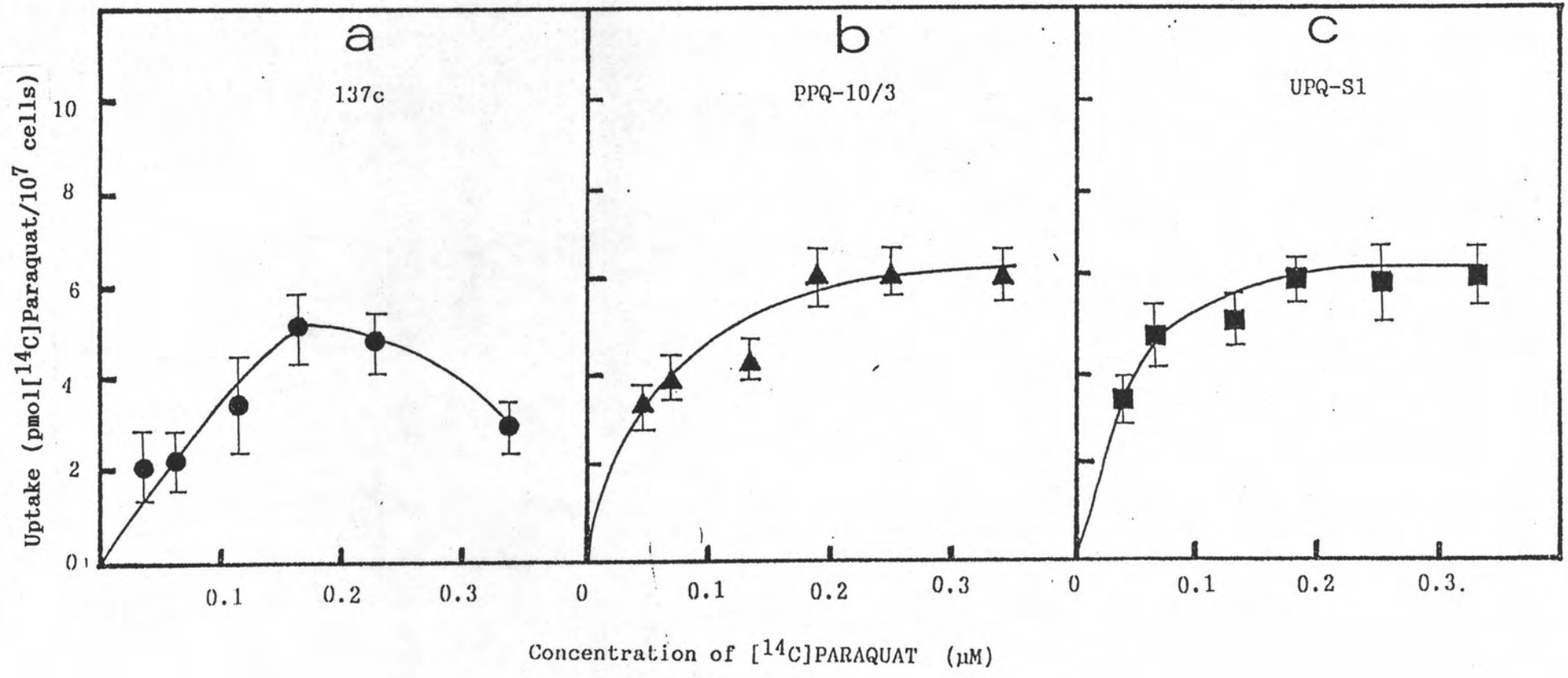

a
137c
b
PPQ-10/3
c
UPQ-S1
Uptake (pmol[14C]Paraquat/10^7 cells)
0
2
4
6
8
10
0.1
0.2
0.3
Concentration of [14C]PARAQUAT (μM)

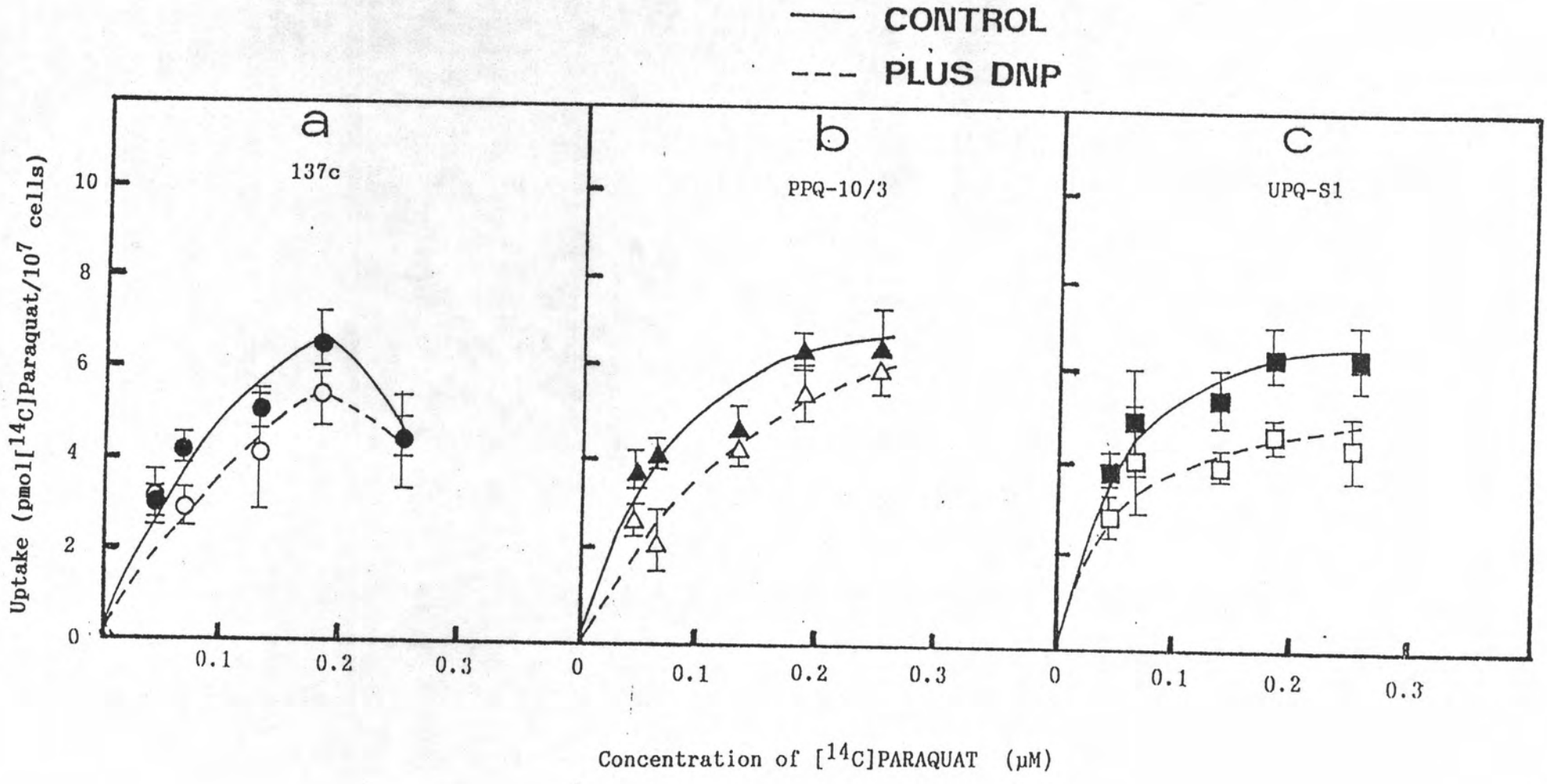
Figure 5.8 Effect of dinitrophenol (DNP) on [^{14}C]paraquat uptake in Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Active growing cells in paraquat free medium (hour 6 of light period) were undertaken energy depletion by incubation, without illumination provided, in a medium containing 1 mM DNP ($4x10^6$ cells/ml) for 3 hours at 30°C. [^{14}C]Paraquat uptake was achieved in the presence of 1 mM DNP. Control experiments were performed without DNP treatment.

a. 137c b. PPQ-10/3 c. UPQ-S1

_____ without DNP

_ _ _ _ _ _ plus DNP


CONTROL
PLUS DNP
a
137c
b
PPQ-10/3
c
UPQ-S1
Uptake (pmol[14C]Paraquat/10^7 cells)
0
2
4
6
8
10
0.1
0.2
0.3
Concentration of [14C]PARAQUAT (μM)

5.2.6 [^{14}C]Paraquat Uptake of Paraquat Pretreated Cells

Cells were cultured in a medium containing paraquat at a sublethal dose that was 0.1 µM for C. reinhardtii 137c and 1.0 µM for paraquat resistant mutants, PPQ-10/3 and UPQ-S1. At these levels of the herbicide , cells normally grew and demonstrated growth pattern similar to that without paraquat pressure as previously described in chapter 3.

As shown in Fig. 5.9 , there was an alteration in in situ [^{14}C]paraquat uptake of cells that were pre-exposed to paraquat during growth. Level of [^{14}C]paraquat detected in PPQ-10/3 (10.9 pmol/10^7 cells) and UPQ-S1 (13.6 pmol/10^7 cells) were almost 2 times higher comparing to those in non-treated cells (Fig. 5.7b and c). On the contrary, uptake value in pretreated 137c cells did not change but the uptake pattern was found markedly different. The [^{14}C]paraquat rapidly fluxed into cells and remained constant at 4.5 pmol/10^7 cells throughout the range of the herbicide tested.

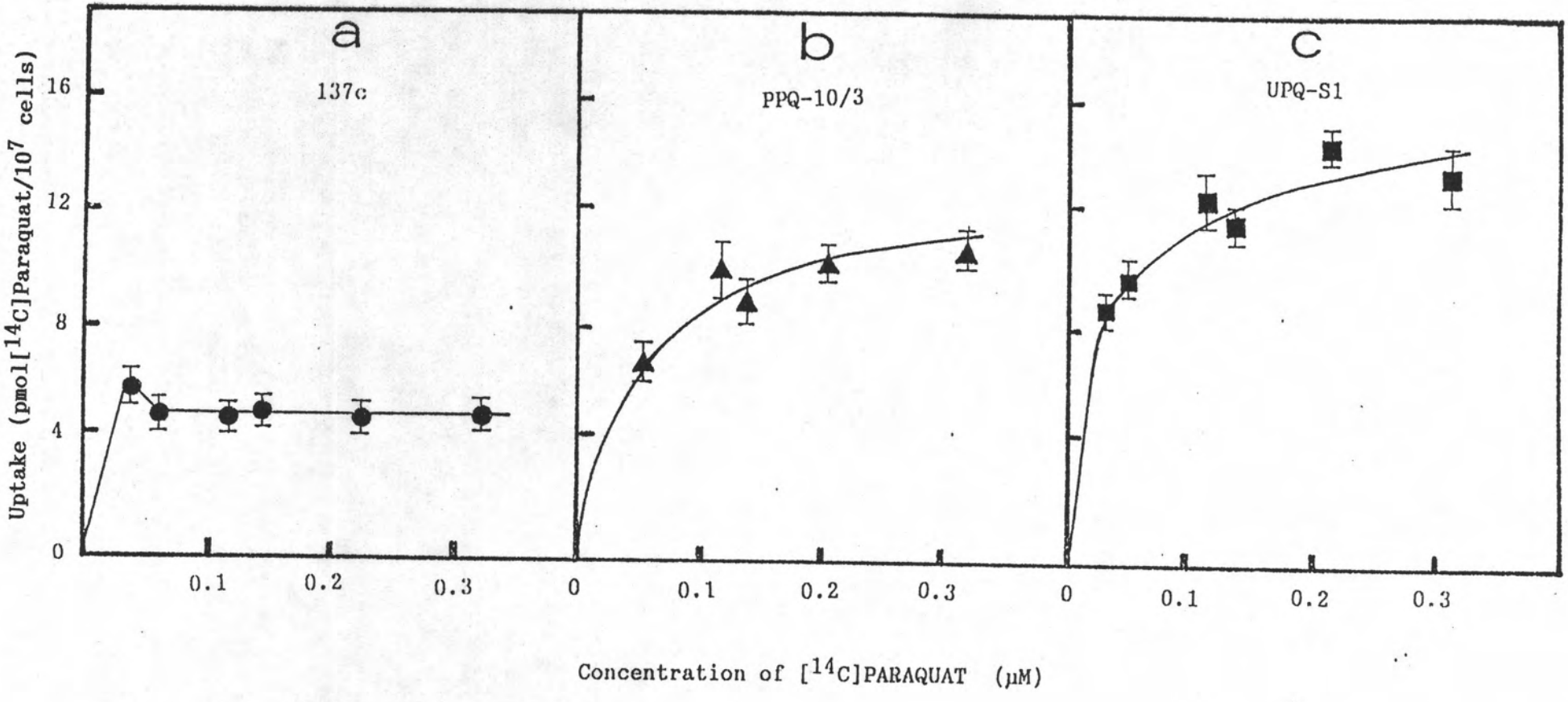
5.2.7 [^{14}C]Paraquat Uptake of C. reinhardtii protoplast

In order to investigate the mechanism of paraquat transport directly across cell membrane , the protoplasts or wall-less cells of C. reinhardtii both wild type (137c) ,PPQ-10/3 , and UPQ-S1 were used for [^{14}C]paraquat uptake studies. Protoplasts of each strain were produced by the activity of autolysin as described in section 5.1.1. The stock protoplast preparations were washed several times to dilute the effect of cycloheximide that possibly interfered

Figure 5.9 [^{14}C]Paraquat uptake in paraquat pretreated cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells grown in a medium with paraquat at sublethal doses were incubated in a 2.5 ml medium containing various amount of [^{14}C]paraquat ($4x10^6$ cells/ml). After a period of uptake at 30°C, cells were washed 3 times and measured for cellular radioactivity.

a. 137c b. PPQ-10/3 c. UPQ-S1


a
137c
b
PPQ-10/3
c
UPQ-S1
Uptake (pmol[^{14}C]Paraquat/10^7 cells)
16
12
8
4
0
0.1
0.2
0.3
Concentration of [^{14}C]PARAQUAT (μM)

with the uptake process.

Evidences from the experimental results (Fig. 5.10) illustrated interestingly differences in the pattern of the uptake of [^{14}C]paraquat. As for the wild type 137c cells, more saturable profile was established at very low concentration of [^{14}C]paraquat and the declining phase still existed and the maximum uptake value was held at the same level as found in the uptake of whole cells (6 pmol/10^7 cells) (Fig. 5.10a).

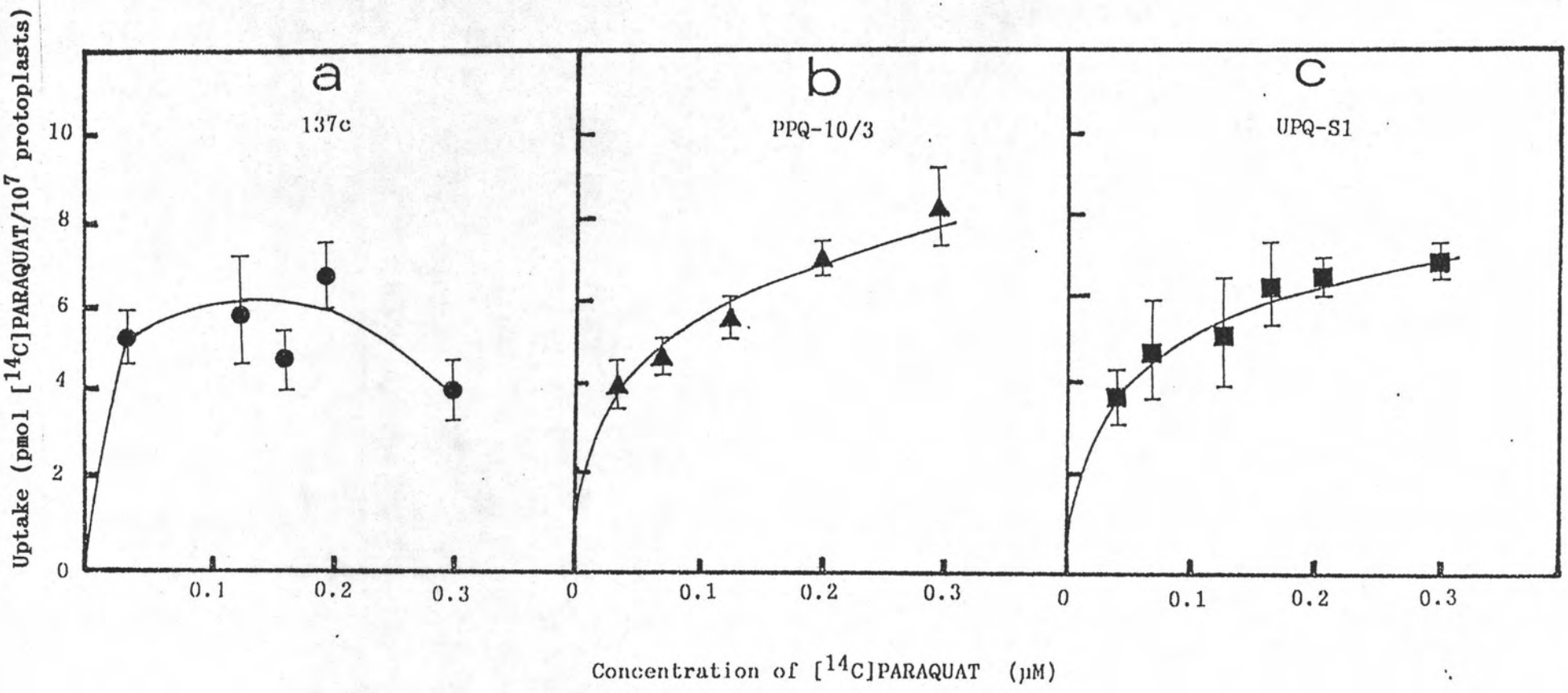
The patterns of [^{14}C]paraquat uptake into the paraquat resistant strains both PPQ-10/3 and UPQ-S1 were obviously different from those in the wild type. However the profile and quantity of radiolabelled paraquat taken up by both mutants were very much equivalent to the whole cell value (Fig. 5.10b and c).

5.2.7 Kinetic Constants for [^{14}C]Paraquat Uptake Owing to the evidences that carrier-mediated process involved in transport across membrane of paraquat, the kinetic constants for the paraquat uptake were investigated by following the Michaelis-Menten relationship of a coupled enzyme-substrate reaction. The Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) of [^{14}C]paraquat uptake were estimated in C. reinhardtii 137c, PPQ-10/3, and UPQ-S1. It was found (Fig. 5.11 and Fig. 5.12) that kinetic constants of [^{14}C]paraquat uptake in C. reinhardtii 137c could not be evaluated by a double reciprocal plot when using whole cell both in normal cells and the paraquat-pretreated cells.

Figure 5.10 [^{14}C]Paraquat uptake in protoplasts of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Protoplasts produced from cells growing in paraquat free medium were incubated in a 2.5 ml medium containing various amount of [^{14}C]paraquat ($4x10^6$ cells/ml). After a period of uptake at 30°C, protoplasts were washed 3 times and measured for the radioactivity.

a. 137c b. PPQ-10/3 c. UPQ-S1


a
137c
b
PPQ-10/3
c
UPQ-S1
Uptake (pmol [14C]PARAQUAT/10^7 protoplasts)
0
2
4
6
8
10
0.1
0.2
0.3
Concentration of [14C]PARAQUAT (μM)

However such a correlation was obtained when protoplasts of 137c were used for examination (Fig. 5.13). On the contrary, the Michaelis-Menten constant of the uptake could be measured for the saturable entry curve of [^{14}C]paraquat uptake in whole cells and protoplasts of both paraquat resistant strains (PPQ-10/3 and UPQ-S1) (Fig. 5.11, 5.12, 5.13).

Kinetic constants for [^{14}C]paraquat direct transport across membrane were compared among the wild type and resistant strains for the whole cell uptake, both normal cells and the herbicide pretreated cells, and also the protoplast uptake (Table 5.2 , 5.3, and 5.4).

There was almost no change in the K_m value of the whole cell uptake of [^{14}C]paraquat for both paraquat resistant strains which were previously grown in the absence or presence of paraquat at the sublethal dose concentration. The kinetic data provided a significant difference between the K_m value of PPQ-10/3 and UPQ-S1. As expected, the K_m of paraquat uptake for PPQ-10/3 was about 2-3 folds higher than the value for UPQ-S1. While the value of V_{max} in PPQ-10/3 was almost 2 times higher than the UPQ-S1 strain. Results from comparative values of the kinetic constants for protoplasts [^{14}C]paraqaut uptake illustrated somewhat interesting data. The K_m value of PPQ-10/3 protoplast paraquat uptake was highest (7 folds and 4 folds higher than 137c wild type cell and UPQ-S1 respectively) with 3 folds higher in V_{max} value in comparing to 137c and UPQ-S1.

Figure 5.11 A double reciprocal plot for [^{14}C]paraquat uptake in whole cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

The kinetic constants K_m and V_{max} were investigated from x-intercept and y-intercept respectively.

(●) wild type

(▲) PPQ-10/3

(■) UPQ-S1

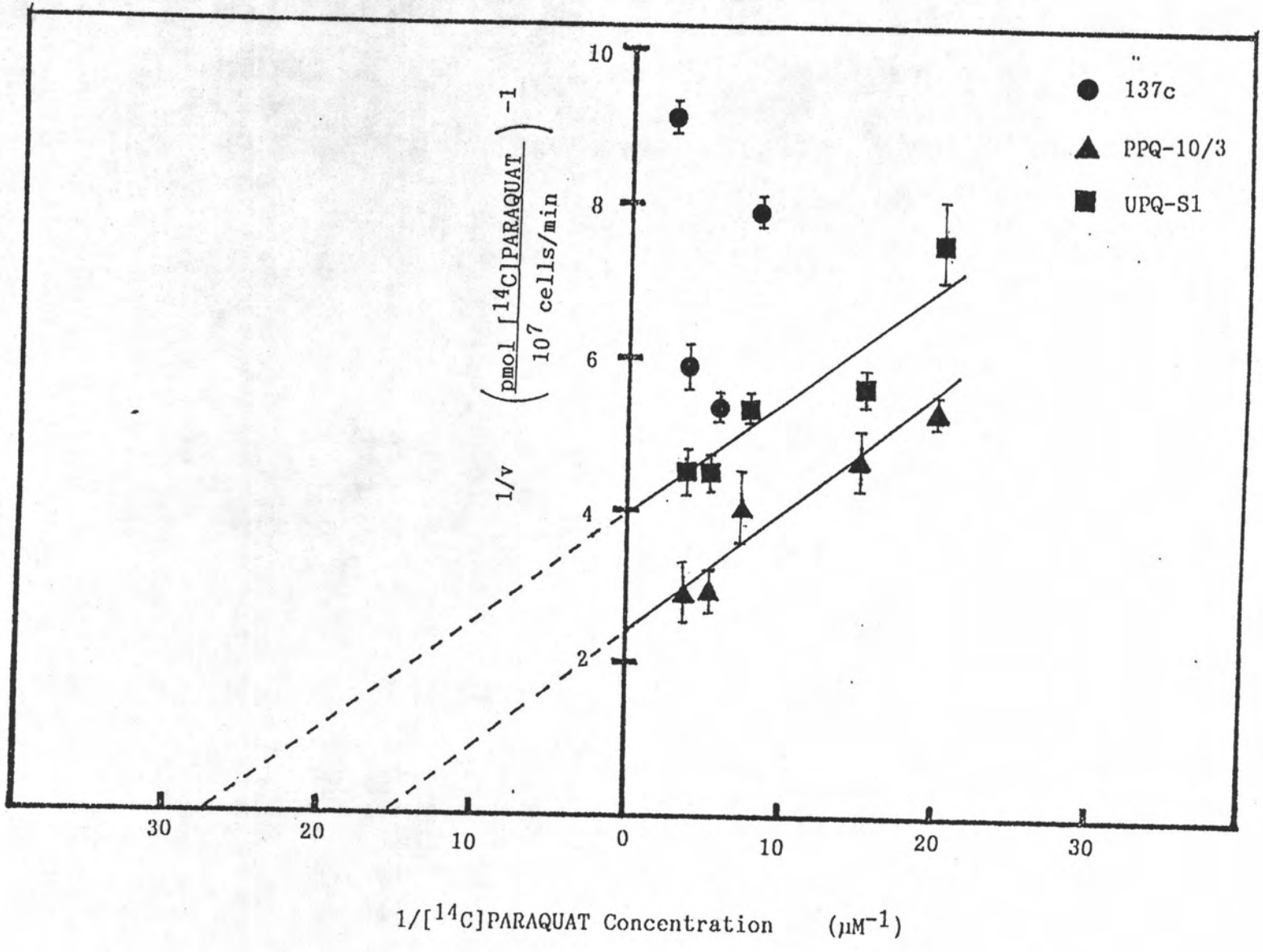

137c
PPQ-10/3
UPQ-S1
1/v (pmol [14C]PARAQUAT / 10^7 cells/min)^-1
10
8
6
4
2
30
20
10
0
10
20
30
1/[14C]PARAQUAT Concentration (μM^-1)

Figure 5.12 A double reciprocal plot for [^{14}C]paraquat uptake in cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains previously grown in the medium supplemented with sublethal dose of paraquat (0.1 μM for 137c and 1.0 μM for the resistant strains).

The kinetic constants K_m and V_{max} were investigated from x-intercept and y-intercept respectively.

(●) wild type

(▲) PPQ-10/3

(■) UPQ-S1

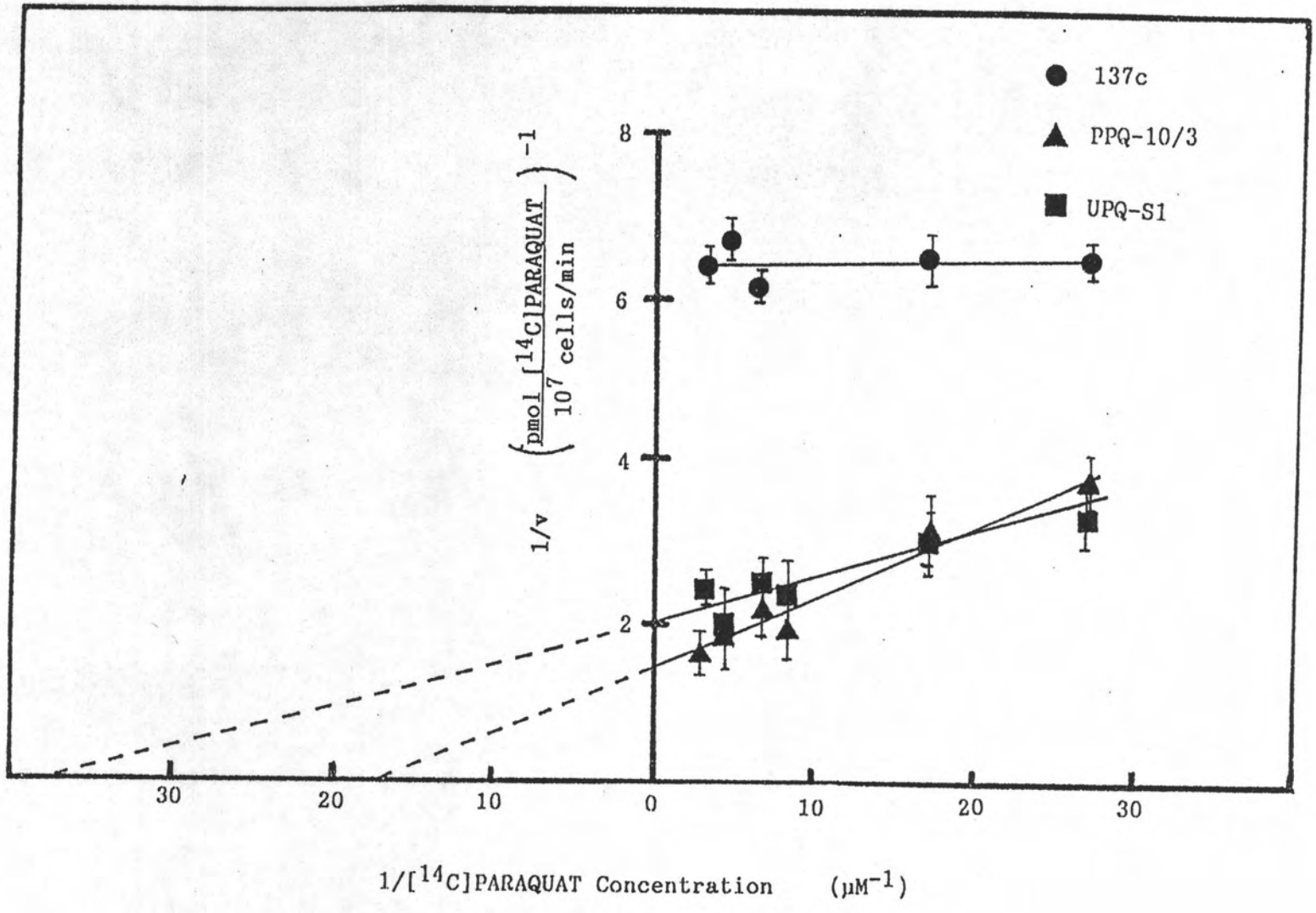

137c
PPQ-10/3
UPQ-S1
1/v $\left(\frac{\text{pmol [}^{14}\text{C]PARAQUAT}}{10^7 \text{ cells/min}}\right)^{-1}$
8
6
4
2
30
20
10
0
10
20
30
1/[^{14}C]PARAQUAT Concentration (μM^{-1})

Figure 5.13 A double reciprocal plot for [^{14}C]paraquat uptake in protoplasts of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

The kinetic constants K_m and V_{max} were investigated from x-intercept and y-intercept respectively.

(●) wild type

(▲) PPQ-10/3

(■) UPQ-S1

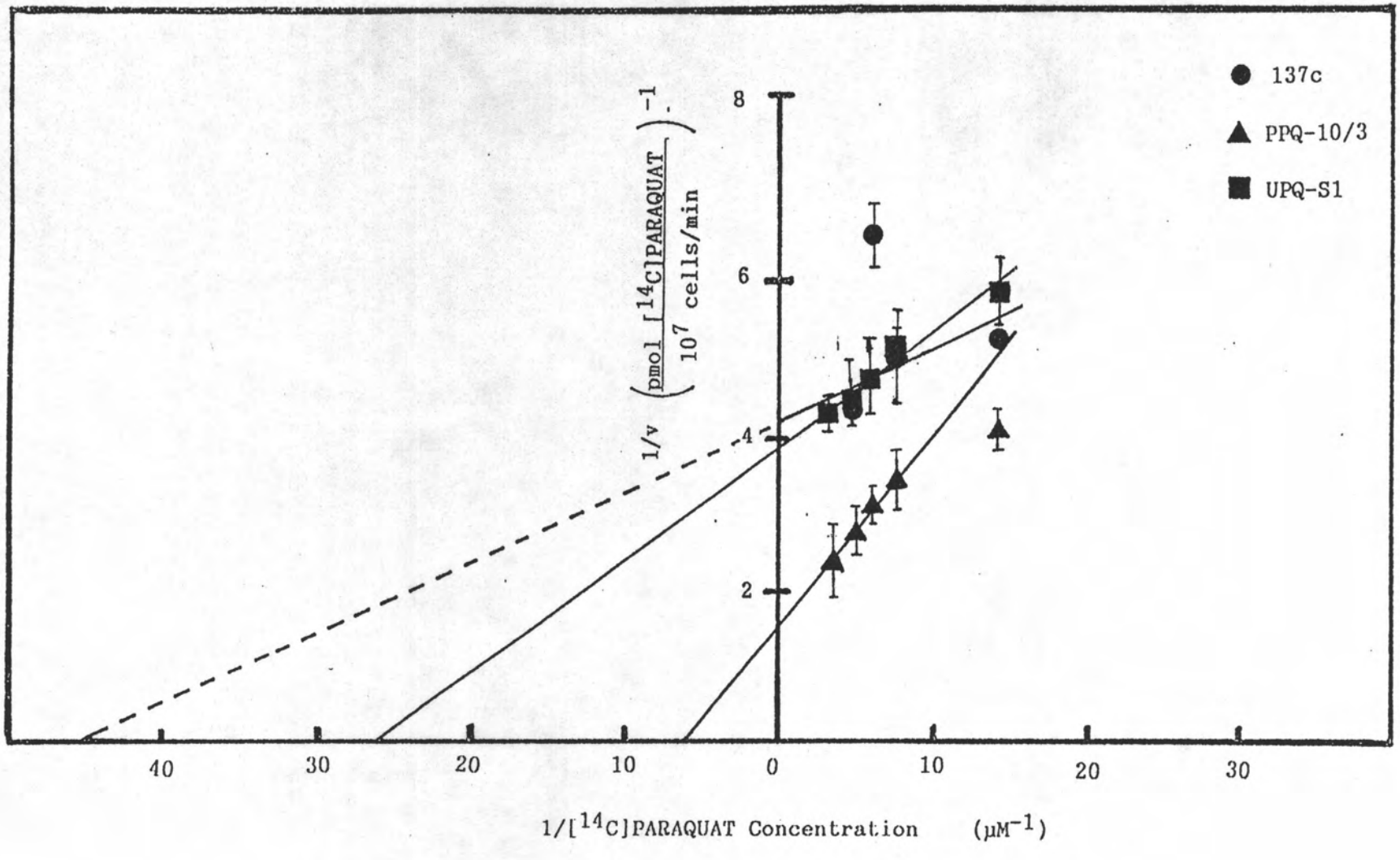

137c
PPQ-10/3
UPQ-S1
1/v $\left(\frac{\text{pmol [}^{14}\text{C]PARAQUAT}}{10^7 \text{ cells/min}}\right)^{-1}$
8
6
4
2
40
30
20
10
0
10
20
30
1/[^{14}C]PARAQUAT Concentration (μM^{-1})

Table 5.2 Comparative values of kinetic constants for [^{14}C]paraquat uptake in whole cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

The kinetic constants K_m and V_{max} were estimated as described in Fig. 5.11.

Strain	K_m (μM of [^{14}C]paraquat)	V_{max} (pmol/10^7 cells.min^{-1})
137c	***	***
PPQ-10/3	0.064	0.41
UPQ-S1	0.037	0.25

**** could not be determined

Table 5.3 Comparative values of kinetic constants for [^{14}C]paraquat uptake in cells of Chlamydomonas reinhardtii wild type and paraquat resistant strains previously grown in the medium supplemented with sublethal dose of paraquat (0.1 μM for 137c and 1.0 μM for the resistant strains).

The kinetic constants K_m and V_{max} were estimated as described in Fig. 5.12.

Strain	K_m (μM of [^{14}C]paraquat)	V_{max} (pmol/10^7 cells.min^{-1})
137c	***	***
PPQ-10/3	0.061	0.69
UPQ-S1	0.027	0.48

**** could not be determined

Table 5.4 Comparative values of kinetic constants for [^{14}C]paraquat uptake in protoplasts of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

The kinetic constants K_m and V_{max} were estimated as described in Fig. 5.13.

Strain	K_m (μM of [^{14}C]paraquat)	V_{max} (pmol/10^7 cells.min^{-1})
137c	0.022	0.24
PPQ-10/3	0.156	0.63
UPQ-S1	0.038	0.26

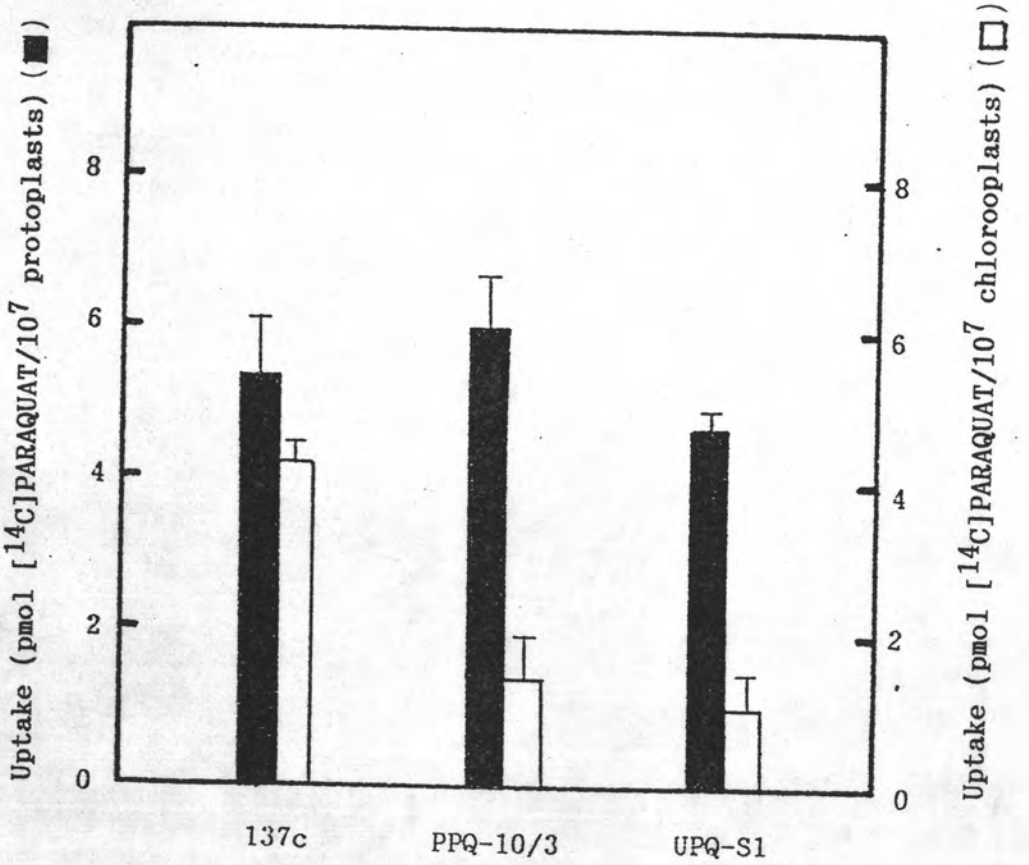
5.2.9 Distribution of [^{14}C]Paraquat into Chloroplast

C. reinhardtii has only one chloroplast per cell. It was of interest to investigate the distribution of [^{14}C]paraquat into the chloroplast after the radioactive herbicide had been taken up into the protoplasts of C. reinhardtii 137c, PPQ-10/3, and UPQ-S1. The radioactive counts of [^{14}C]paraquat detected in chloroplast isolated by digitonin lysis of the protoplasts were calculated by correction for the yield of chloroplasts (60%) with respect to the net uptake in protoplasts.

It was strikingly illustrated (Fig. 5.14) that when the protoplasts of C. reinhardtii 137c were exposed to 0.1 μM [^{14}C]paraquat, the uptake value was 5.39 ± 0.73 pmol/10^7 protoplasts of which almost 80% of the radioactivity passed through the chloroplasts (4.35 ± 0.41 pmol/10^7 chloroplasts). In comparison with the paraquat resistant C. reinhardtii strains, it was clearly demonstrated (Fig. 5.14) that only 22-23% of [^{14}C]paraquat which was taken up into the protoplasts could be transported further to the chloroplasts of both resistant strains (PPQ-10/3 and UPQ-S1).

Figure 5.14 Comparative distribution of [^{14}C]paraquat into protoplast and chloroplast of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Protoplasts were incubated in 2.5 ml reaction mixture containing 0.1 µM [^{14}C]paraquat ($4x10^6$ protoplasts/ml) and after completing the uptake procedures they were subjected to isolation of chloroplasts.



5.3 Comparative Studies of the Enzyme Content in Wild Type and Paraquat Resistant Strains

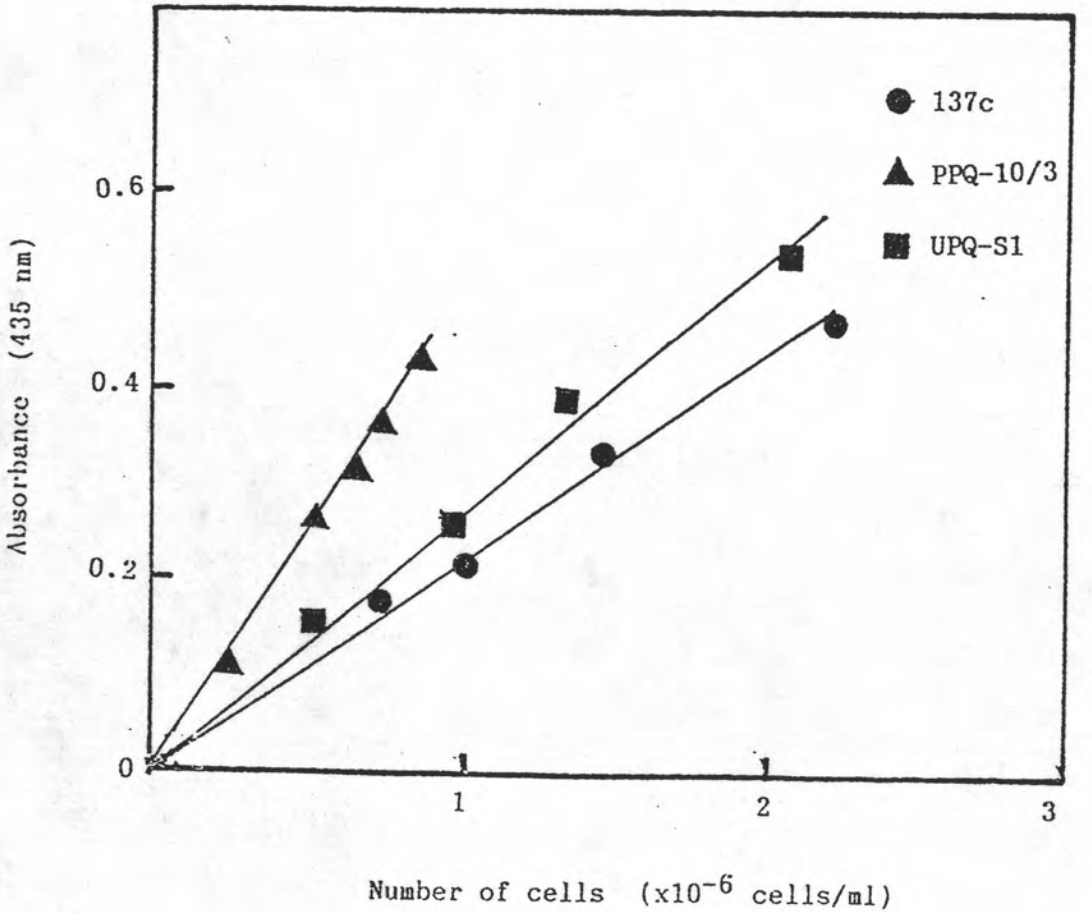
5.3.1 Determination of Cell Number Crude enzymes for the measurement of the enzyme content were prepared by either continuous disruption through a French Pressure Cell or sonication under the complete cell breaking conditions. The broken cells released chloroplastic pigments into the lysis medium, and on the basis that one cell holds a single chloroplast, the relationship between the absorbance at 435 nm of the green pigment and the number of cell was plotted. When wild type C. reinhardtii 137c and paraquat resistant PPQ-10/3 and UPQ-S1 were competely broken by sonication, a linear correlationship between cell number which was counted by haemacytometer and the absorbance at 435 nm was illustrated (Fig. 5.15) in different patterns depending on the chlorophyll content in cells.

It was the standard relationship between these two parameters that the crude enzyme lysate preparations in this research project were easily determined for its absorbance of the green pigment so that the number of cell could be estimated directly for the source of the crude extract.

5.3.2 Enzymes Content in C. reinhardtii Level of three enzymes activities namely, superoxide dismutase, catalase, and ascorbate peroxidase, were compared at three stages of growth. Starting with an inoculum of 10^5 cells/ml on day 0 (inoculation day), it had been already illustrated (Fig. 3.2) that, when based on the increase in

Figure 5.15 Correlation between the number of cells and the absorption of green pigment of Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cells ($1.5x10^6$-$12x10^6$ cells) in 6 ml of 50 mM phosphate buffer, pH 7.8) were disrupted by sonication for 3 min, centrifuged and the supernatants were measured for the absorbance at 435 nm.



cell density, culture of 137c was in mid log phase on day 1, late log phase on day 2, and in stationary phase on day 3. Cultures at the indicated times were sampled and assayed for the content of enzyme activities in terms of total units per 10^7 cells and specific activities (units per mg protein).

In Table 5.5, total units of superoxide dismutase in C. reinhardtii 137c was rather constant at approximately 30-40 units /10^7 cells through the growth period, as well as the specific activity (28-32 units/mg protein). Content of catalase (32.13 $\pm$ 0.98 units/10^7 cells at mid log phase) and ascorbate peroxidase (26.06 $\pm$ 0.87 units/10^7 cells at mid log phase) regularly decreased to a half amount when cultures propagated into stationary phase. Calculating specific activity for both enzymes gave a different pattern from the enzyme production, since the specific activity of catalase and ascorbate peroxidase were found to be maximum in the late growth phase whereas lower specific activity were detected at the mid log and stationary phase.

5.3.3 Enzyme Content in PPQ-10/3 During growth period of PPQ-10/3 under liquid culture conditions, the amount of superoxide dismutase, catalase, and ascorbate peroxidase were followed in comparison to that of the 137c wild type strain. When starting with the same inoculum size (10^5 cells/ml), the growth of PPQ-10/3 culture was measured. The growth curve was found according to the previous section (Fig. 3.9). At certain time intervals, those were

Table 5.5 Content of superoxide dismutase (SOD), catalase and ascorbate peroxidase in Chlamydomonas reinhardtii 137c, as a function of growth stages.

Cells were photosynthetically grown in paraquat free medium with an inoculum size of 10^5 cells/ml. Stage of growth was identified by counting cell density at time intervals during the culture period. (n = 3)

Growth phase	Superoxide dismutase		Catalase		Ascorbate peroxidase	
	content of activity (units/10^7 cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)
mid log	32.31 ± 0.24	28.67 ± 0.77	32.13 ± 0.98	18.56 ± 1.13	26.06 ± 0.87	39.60 ± 0.37
late log	32.33 ± 1.33	32.18 ± 0.29	26.47 ± 1.24	22.74 ± 1.74	19.64 ± 1.07	45.14 ± 2.47
stationary	40.04 ± 1.47	32.64 ± 0.45	18.67 ± 1.67	15.24 ± 1.34	13.74 ± 1.88	29.76 ± 0.51

day 2 (mid log phase), day 3 (late log phase), and day 4 (stationary phase), cells were pooled and assayed for the enzyme activities. The results were shown in Table 5.6.

Level of superoxide dismutase in PPQ-10/3 was maximum (278.73 ± 0.18 units/10^7 cells) at mid log phase , which was about 10 folds higher than that in 137c (wild type). At this growth phase the value of specific activity of superoxide dismutase in PPQ-10/3 was almost twice higher than that of the 137c strain. The superoxide dismutase content found at the stationary phase was about 4 folds lower than this maximum enzyme production (68.08 ± 0.71 units /10^7 cells). The maximum content of catalase was also found at the mid log growth phase (131.73 ± 2.0 units/10^7 cells). Corresponding to the superoxide dismutase activity, the catalase enzyme activity content measured at the mid log stage was almost about 4 folds higher than the original wild type strain. Remarkable decrease in catalase content along growth was also demonstrated in this paraquat resistant strain.

Interestingly, it was found that the content of ascorbate peroxidase of PPQ-10/3 was remarkably increased to 130 folds over the wild type enzyme. Especially at late log cultivated stage, total units of ascorbate peroxidase in PPQ-10/3 was as high as 2566.96 ± 33.16 units/10^7 cells comparing to only 19.64 ± 1.07 units /10^7 cells in wild type. In contrast to the previously mentioned enzymes, the lowest amount of PPQ-10/3 ascorbate peroxidase was determined as 633.11 ± 48.71 units/10^7 cells at mid log phase

Table 5.6 Content of superoxide dismutase (SOD), catalase and ascorbate peroxidase in Chlamydomonas reinhardtii PPQ-10/3 as a function of growth stages.

Cells were photosynthetically grown in paraquat free medium with an inoculum size of 10^5 cells/ml. Stage of growth was identified by counting cell density at time intervals during the culture period. (n = 3)

Growth phase	Superoxide dismutase		Catalase		Ascorbate peroxidase	
	content of activity (units/10^7 cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)
mid log	278.73 ± 0.18	59.09 ± 1.32	131.73 ± 2.00	27.93 ± 0.42	633.11 ± 48.71	341.39 ± 26.26
late log	165.91 ± 0.52	41.28 ± 0.71	117.65 ± 1.93	29.27 ± 0.21	2566.96 ± 33.16	892.86 ± 28.63
stationary	68.08 ± 0.71	31.12 ± 0.48	30.24 ± 0.38	13.82 ± 0.17	1838.75 ± 37.33	942.71 ± 31.07

instead of the statioanry phase.

Comparison to the remarkable increase in the amount of PPQ-10/3 ascorbate peroxidase which was detected together with the increase in specific activity of the enzyme (10-40 folds of the wild type enzyme), there was only slightly altered in the specific activity of PPQ-10/3 superoxide dismutase and almost no significant difference in catalase specific activity with respect to those of the wild type (137c).

5.3.4 Enzymes Content in UPQ-S1 Activity of superoxide dismutase, catalase, and ascorbate peroxidase were measured, according to the previous sections, in the paraquat resistant C. reinhardtii UPQ-S1 strain. Data from the growth curve of UPQ-S1 (Fig.3.14) indicated one day later growth in comparison to 137c and PPQ-10/3. However activity of enzyme detected on day 3 and day 4 cultures of UPQ-S1 could be corresponded to the results at late log phase and stationary phase of 137c and PPQ-10/3. The data were collected in Table 5.7.

The total amount of superoxide dismutase in UPQ-S1 was determined to be maximally at 123.33 $\pm$ 2.76 units/10^7 cells at late log phase and minimally at 67.54 $\pm$ 1.87 units/10^7 cells at stationary phase. The enzyme level was only in the range of 1.5-4 folds of that found in wild type cell, whereas the specific activity of superoxide dismutase from the two strains were not different.

As for catalase, there was no significant change in the

Table 5.7 Content of superoxide dismutase (SOD), catalase and ascorbate peroxidase in Chlamydomonas reinhardtii UPQ-S1, as a function of growth stages.

Cells were photosynthetically grown in paraquat free medium with an inoculum size of 10^5 cells/ml. Stage of growth was identified by counting cell density at time intervals during the culture period. (n = 3)

Growth phase	Superoxide dismutase		Catalase		Ascorbate peroxidase	
	content of activity (units/10^7 cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)
early log	98.86 ± 2.53	29.91 ± 9.5	120.62 ± 0.55	41.92 ± 0.22	33.38 ± 1.41	33.77 ± 2.11
late log	123.33 ± 2.76	30.64 ± 1.84	123.42 ± 3.16	37.12 ± 0.94	579.33 ± 2.33	286.86 ± 1.16
stationary	67.54 ± 1.87	32.26 ± 3.95	151.20 ± 0.79	52.13 ± 0.87	131.40 ± 10.11	107.14 ± 8.24

content of catalase along growth period of UPQ-S1. It was rather constant at approximately 120-150 units/10^7 cells which was 4-8 folds of the enzyme level in the wild type. This enhanced amount of UPQ-S1 catalase enzyme was parallel with slight increase in the specific activity of the enzyme (1.7-3.0 folds over the wild type catalase).

The level of UPQ-S1 ascorbate peroxidase in day 2 culture appeared not different from the wild type enzyme (33.38 $\pm$ 1.41 units /10^7cells), but it was followed by rapid increase to almost 20 times during the late log phase and then decreased to a relatively low level at stationary phase (131.40 $\pm$ 10.11 units/10^7 cells). This rythmic changes of the total units of UPQ-S1 ascorbate peroxidase was demonstrated together with the alteration in the specific activity of the enzyme.

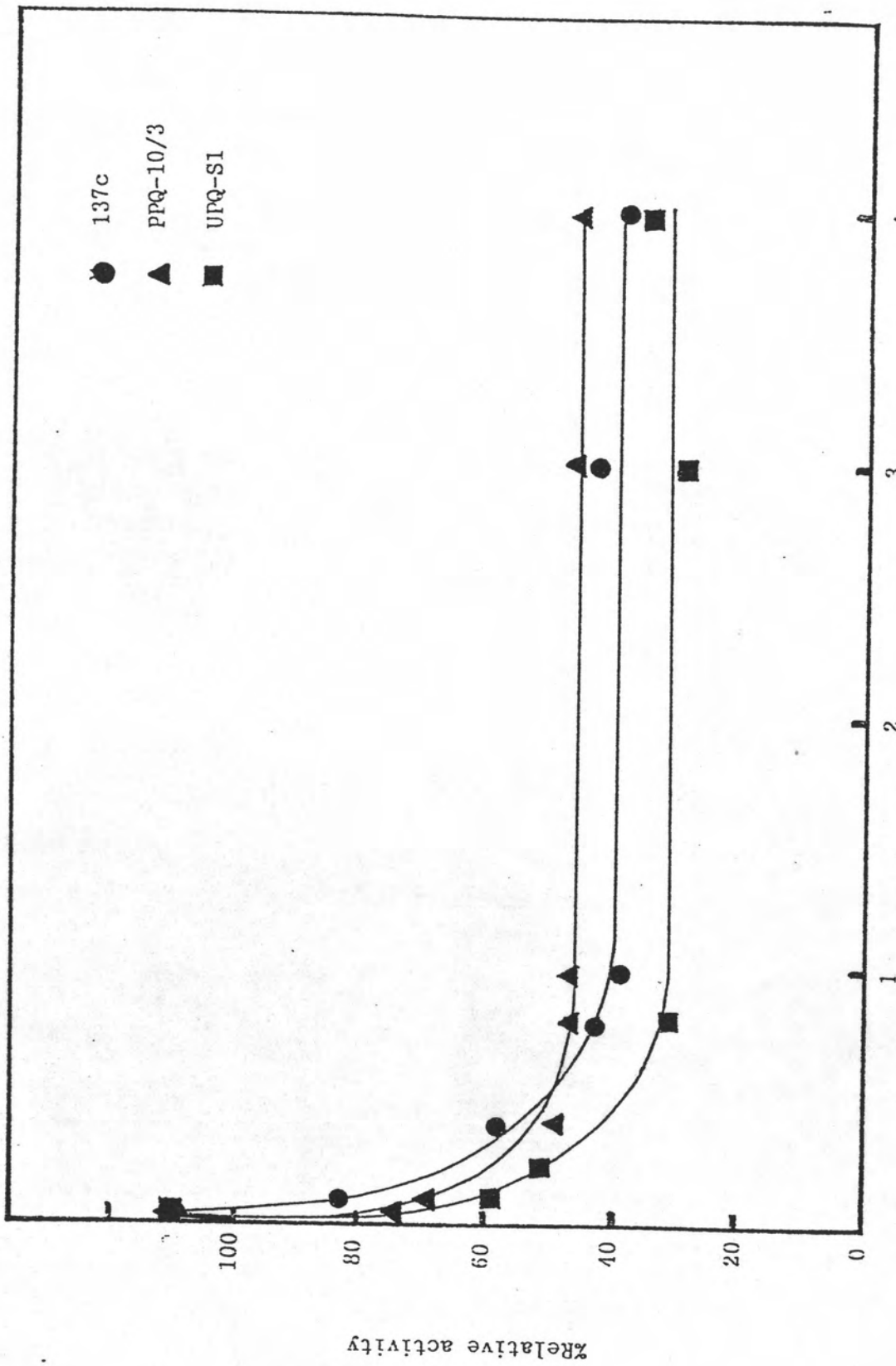
5.4 Effect of Cyanide on Superoxide Dismutase

Effect of cyanide was examined with equal units of superoxide dismutase (3 units) in crude enzymes from *C. reinhardtii* 137c, PPQ-10/3, and UPQ-S1. Similar results were obtained that addition of cyanide in the assay had two effects on the enzyme (Fig. 5.16).

At very low concentration of cyanide (0.02-0.04 mM) this compound could slightly stimulate the activity of superoxide dismutase (up to 10%) in all strains test. Continuously increase the cyanide concentrations caused a sharp drop in the superoxide dismutase activity in all sources of the crude extract. Cyanide concen-

Figure 5.16 Effect of cyanide on crude superoxide dismutase from Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Superoxide dismutase in crude extracts of C. reinhardtii was assayed in the presence of potassium cyanide at various concentrations. The activity was calculated as a percentage of activity in relation to the activity in the absence of potassium cyanide.


137c
PPQ-10/3
UPQ-S1
%Relative activity
100
80
60
40
20
0
1
2
3
4
Cyanide concentration (mM)

tration at 0.8- 1.0 mM diminished superoxide dismutase activity down to only 30-45% of the original activity. The inhibition effect of cyanide on superoxide dismutase remained constant at this level although the concentration of cyanide was increased to as high as 4 mM.

The inhibitory effects of cyanide on crude superoxide dismutases of C. reinhardtii which was illustrated here in Fig. 5.16 in this experiment seemed to be the first evidence leading to the possible existence of a superoxide dismutase isozyme which contains copper and zinc as prosthetic groups (Cu/Zn-SOD) as in the higher plants.

5.5 Effect of Paraquat Treatment on Enzymes Content in C. reinhardtii

The possibility of paraquat to alter the biosynthesis of superoxide dismutase, catalase, and ascorbate peroxidase in C. rienhardtii wild type and paraquat resistant strains was investigated by exposure of the algal cells to sublethal dose of paraquat. At these conditions there was no change in growth pattern of the alga observed. The content of superoxide dismutase was assayed as total units in cells and as units of copper/zinc-enzyme (Cu/Zn-SOD). The latter was measured in the presence of 4 mM cyanide solution.

5.5.1 Effect of Paraquat Treatment on the Target Enzymes Production of C. reinhardtii 137c

It was shown in Table 5.8 that

Table 5.8 Effect of paraquat treatment on superoxide dismutase, catalase, and ascorbate peroxidase of *Chlamydomonas reinhardtii* 137c.

Cells at log phase were harvested and transfered to fresh medium containing 0.1 μM paraquat (10^6 cells/ml). After illuminated under growth conditions for 48 hours, cells were collected to assess the activity of superoxide dismutase, catalase and ascorbate peroxidase. Control experiment was carried on simultaneously as above except that paraquat was not included. (n = 3)

Treatment	Superoxide dismutase		Catalase		Ascorbate peroxidase	
	content of activity (units/10^7 cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)
control	23.35 ± 1.35 *(7.93 + 1.26)	16.00 ± 0.71	23.16 ± 3.31	16.84 ± 2.40	12.11 ± 1.67	24.22 ± 1.68
0.1 uM paraquat	11.07 ± 1.07 *(9.20 ± 0.69)	12.25 ± 0.72	15.04 ± 0.44	15.89 ± 0.47	20.21 ± 1.48	49.29 ± 2.23

* Total units of Cu/Zn-SOD

once C. reinhardtii as the wild type cell was treated with 0.1 μM paraquat for two doubling times, the specific activity and the content of ascorbate peroxidase slightly increased whereas those of catalase did not significantly change.

The unexpected result was found in case of superoxide dismutase. Treatment of paraquat caused almost half reduction in the amount of superoxide dismutase (23.35 ± 1.35 units/10^7 cells to 11.07 ± 1.07 units/10^7 cells). The depression of superoxide dismutase content of activity in C. reinhardtii 137c cells did not seem to result from the inhibition of the copper/zinc-enzyme since the calculated level of Cu/Zn-SOD was rather constant (7.93 ± 1.26 to 9.20 ±0.69 units/10^7 cells). In addition, there was only a small decrease in the specific activity of all three types of the enzyme.

5.5.2 Effect of Paraquat Treatment on the Target Enzymes Production of C. reinhardtii PPQ-10/3 Treatment of paraquat at the sublethal concentration (1 μM) to PPQ-10/3 cells did not bring about induction of the superoxide dismutase, but on the contrary, it resulted in half reduction of total units of superoxide dismutase from the normal level (212.94 ± 4.05 units/10^7 cells to 131.58 ± 1.08 units/10^7 cells) (Table 5.9).

The occurrence of Cu/Zn-SOD in PPQ-10/3 was slightly elevated by paraquat treatment (59.42 ± 0.79 units/10^7 cells) comparing to the control level (52.42 ± 1.81 units/10^7 cells). This level of PPQ-10/3 copper/zinc-enzyme was about 7 folds of the wild

Table 5.9 Effect of paraquat treatment on superoxide dismutase, catalase, and ascorbate peroxidase of Chlamydomonas reinhardtii PPQ-10/3.

Cells at log phase were harvested and transfered to fresh medium containing 1 μM paraquat (10^6 cells/ml). After illuminated under growth conditions for 48 hours, cells were collected to assess the activity of superoxide dismutase, catalase and ascorbate peroxidase.

Control experiment was carried on simultaneously as above except that paraquat was not included. (n = 3)

Treatment	Superoxide dismutase		Catalase		Ascorbate peroxidase	
	content of activity (units/10^7 cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)
control	212.94 ± 4.05 *(52.42 ± 1.81)	65.11 ± 0.93	131.34 ± 0.67	22.60 ± 1.13	2071.43 ± 8.93	414.29 ± 5.47
1 uM paraquat	131.58 ± 1.08 *(59.42 ± 0.79)	52.35 ± 0.57	112.10 ± 1.70	20.16 ± 2.49	2857.14 ± 7.14	549.45 ± 3.37

* Total units of Cu/Zn-SOD

type enzyme. The lower in the superoxide dismutase content was also observed with a slight decrease in the specific activity of the enzyme.

Paraquat did not have any significant effect on PPQ-10/3 catalase both the enzyme content (units/10^7cells) and specific activity (units/mg proteins) of the enzyme. The only enzyme that was clearly induced by paraquat was ascorbate peroxidase. It was illustrated that total content of ascorbate peroxidase and its specific activity were enhanced by almost 2 folds.

5.5.3 Effect of Paraquat Treatment on the Target Enzymes Production of C. reinhardtii UPQ-S1 UPQ-S1 culture which had been exposed to paraquat at the sublethal dose (1 μM) for two doubling times (48 hours) contained less amount of superoxide dismutase comparing to the control value. In cells without paraquat treatment, total units of the enzyme was at the level of 107.76 ± 3.35 units/10^7 cells (Table 5.10), of which the copper/zinc-enzyme was calculated to be 40.08 ± 3.98 units/10^7 cells. No alteration in copper/zinc-superoxide dismutase could be observed after UPQ-S1 was treated with the herbicide, although the total content (units/10^7 cells) of superoxide dismutase was reduced to almost one-fourth of the original activity of the enzyme. Furthermore treatment of the herbicide had no effect on the specific activity (units/mg protein) of superoxide dismutase.

Neither content of catalase nor the catalase specific acti-

Table 5.10 Effect of paraquat treatment on superoxide dismutase, catalase, and ascorbate peroxidase of *Chlamydomonas reinhardtii* UPQ-S1.

Cells at log phase were harvested and transfered to fresh medium containing 1 μM paraquat (10^6 cells/ml). After illuminated under growth conditions for 48 hours, cells were collected to assess the activity of superoxide dismutase, catalase and ascorbate peroxidase.

Control experiment was carried on simultaneously as above except that paraquat was not included. (n = 3)

Treatment	Superoxide dismutase		Catalase		Ascorbate peroxidase	
	content of activity (units/10^7 cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)	content of activity (units/10^7cells)	specific activity (units/mg protein)
control	107.76 ± 3.35 *(40.08 ± 3.98)	27.31 ± 0.94	108.76 ± 2.36	45.59 ± 0.51	357.14 ± 10.11	253.29 ± 6.10
1 µM paraquat	83.22 ± 10.94 *(40.10 ± 7.64)	23.77 ± 4.70	96.63 ± 1.15	56.96 ± 1.90	538.88 ± 31.70	367.28 ± 21.42

*Total units of Cu/Zn-SOD

vity was affected when UPQ-S1 was exposed to the sublethal dose of paraquat. This was not the case for ascorbate peroxidase because there was a significant enhancement of both the enzyme content and its specific activity to almost 1.5 folds higher than those without paraquat treatment (357.14 ± 10.11 units/10^7 cells and 253.29 ±6.10 units/mg protein respectively).

5.6 Effect of paraquat Treatment on Isozymes of C. reinhardtii Superoxide Dismutase

5.6.1 Effect of Paraquat on Isozymes of C. reinhardtii 137c superoxide dismutase

When the whole extracts of superoxide dismutase from C. reinhardtii 137c were electrophoresed by using various amount of crude enzyme extracts (0.5-1.5 units of superoxide dismutse), the position of the enzymes were visualized by nitro blue tetrazolium staining. From control activity staining in the absence of any enzyme inhibitors, nine isozymes of wild type superoxide dismutase was seen in Fig. 5.17a (lane A-D). They were numerically coded according to their relative mobility (R_f) on gel as SOD-1, SOD-2, SOD-3, SOD-4, SOD-5, SOD-6, SOD-7, SOD-8, and SOD-9. Band of the SOD-7 isozyme was highest in the intensity of staining, indicating it to be the major isozyme of superoxide dismutase in C. reinhardtii 137c. Whereas very low in the intensity of the activity staining of SOD-1, SOD-2, SOD-3, SOD-4, and SOD-6 illustrated these isozymes to be minor isozymes of 137c superoxide dismutase, which could be visualized only when larger amount of the enzyme was used.

Figure 5.17 Zymograms of superoxide dismutase (SOD) from extracts of Chlamydomonas reinhardtii 137c.

Various units of superoxide dismutase from cells growing in paraquat free medium and from cells growing in the medium containing 0.1 μM paraquat were electrophoresed in a 10% polyacrylamide gel. Bands of the enzyme were activity stained by nitro blue tetrazolium staining.

lane A-D : SOD of cells in paraquat free medium

(A) 0.5 unit (B) 0.75 unit

(C) 1.0 unit (D) 1.5 unit

lane E-H : SOD of cells in paraquat medium

(E) 0.5 unit (F) 0.75 unit

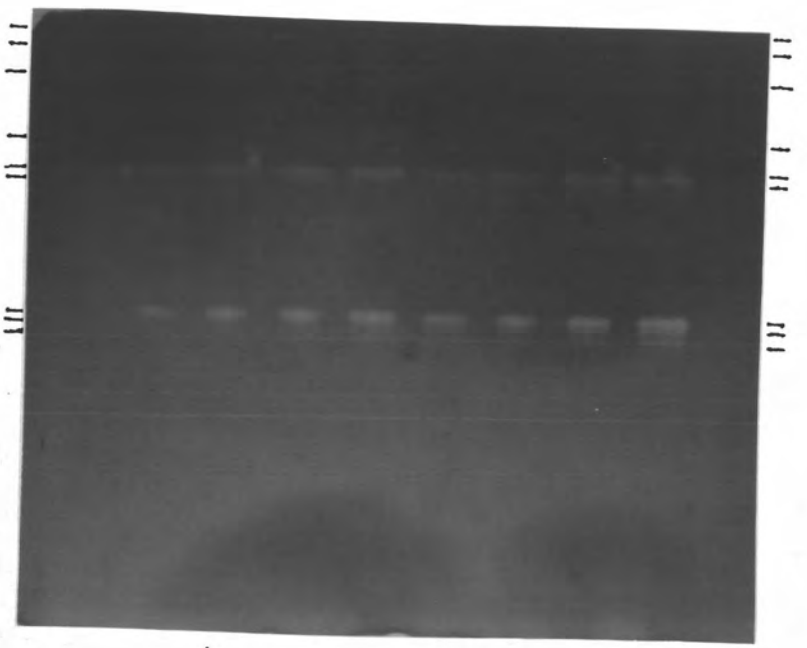
(G) 1.0 unit (H) 1.5 unit

Fig. 5.17a control activity staining

Fig. 5.17b activity staining in the presence of hydrogen peroxide

Fig. 5.17c activity stainibg in the presence of hydrogen peroxide + cyanide

Fig. 5.17a



Explanatory Drawings of SOD Isozymes

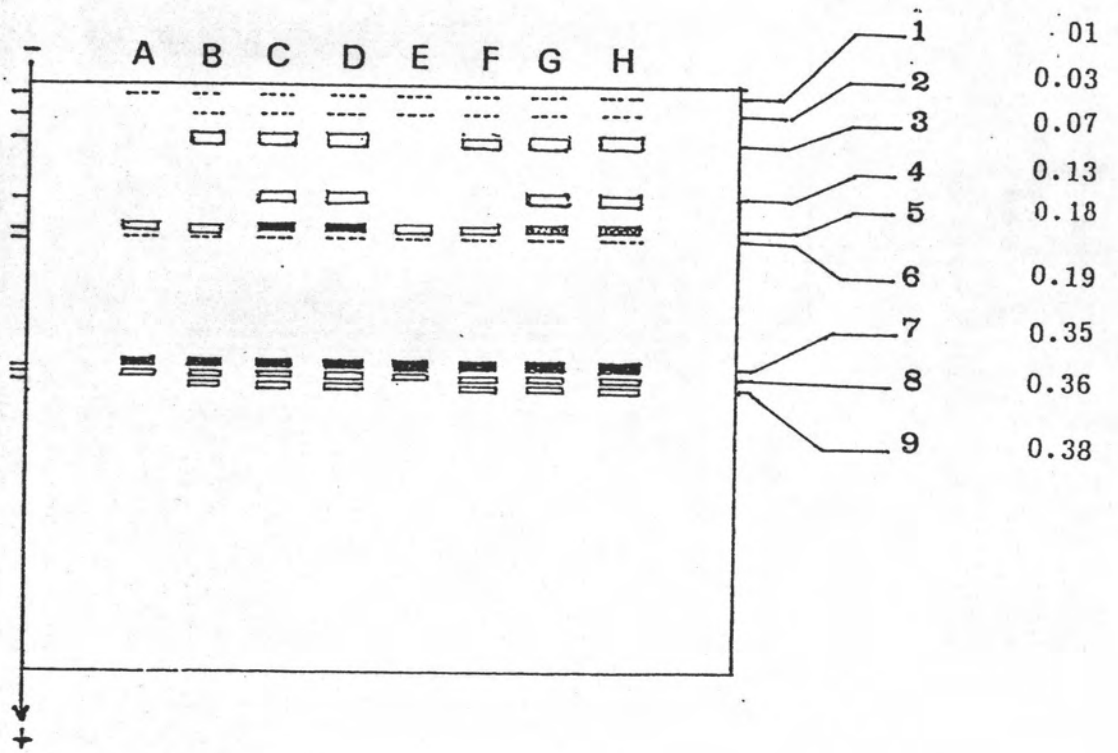
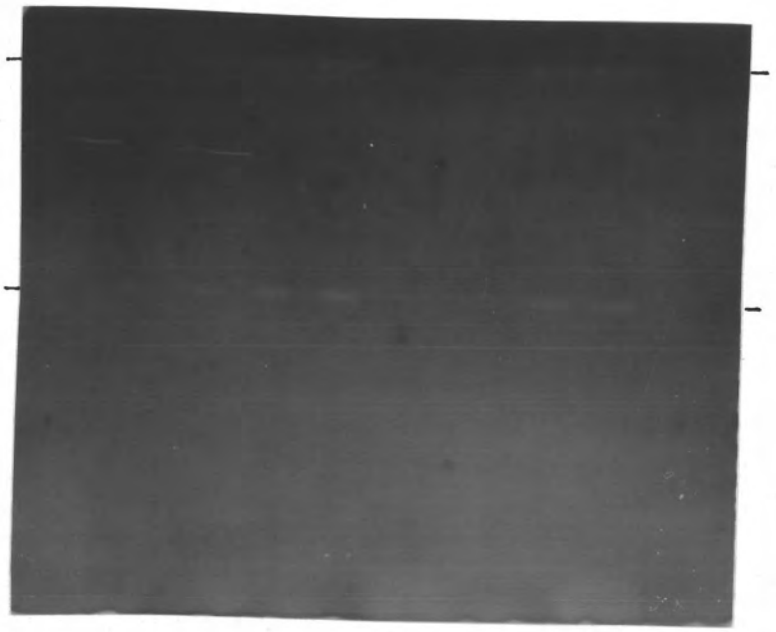


Fig. 5.17b



Explanatory Drawings of SOD Isozymes

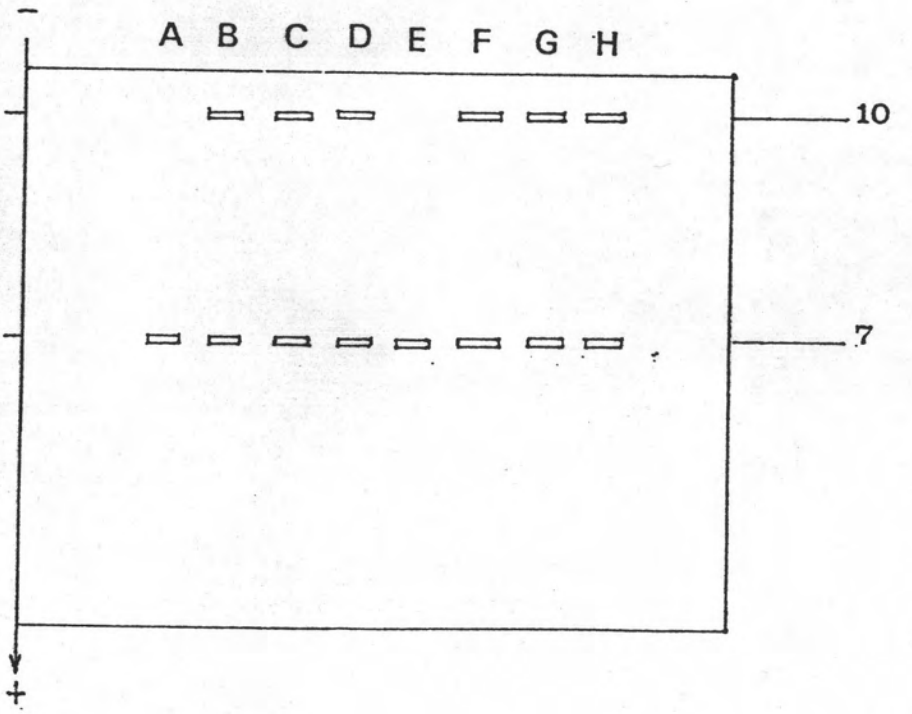
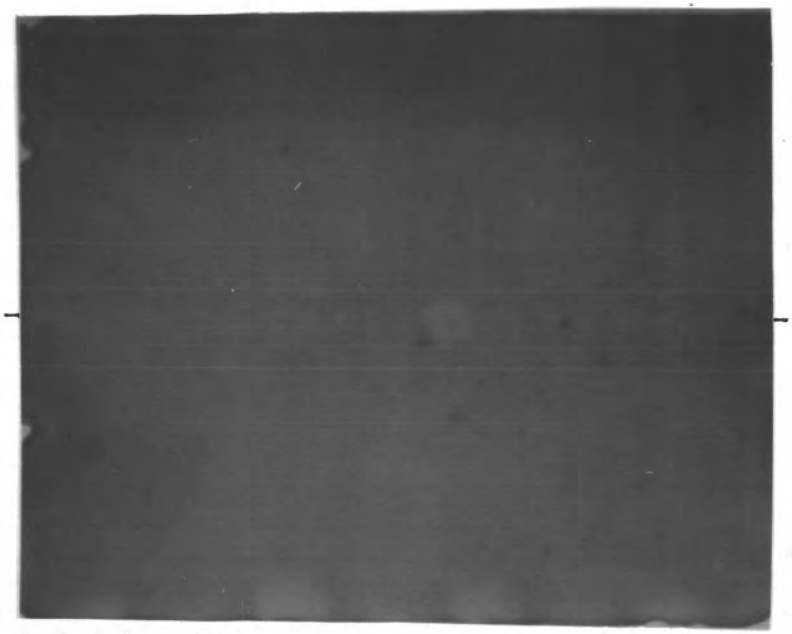
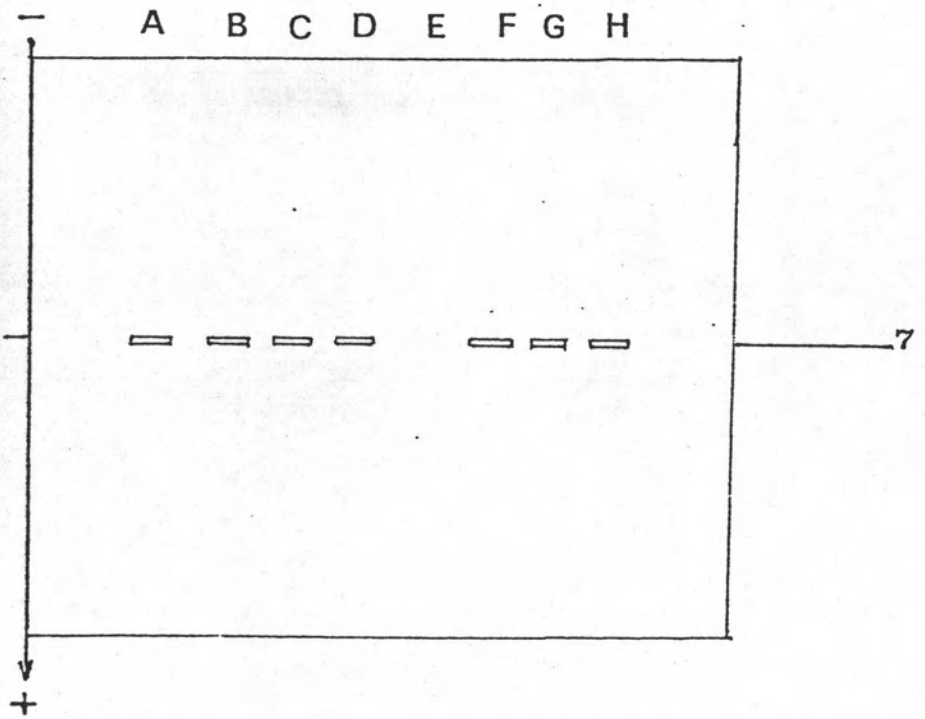


Fig. 5.17c



Explanatory Drawings of SOD Isozymes



The superoxide dismutase activity was stained in the presence of hydrogen peroxide (10 mM) to identify the superoxide dismutase containing iron as a prosthetic group (Fe-SOD). It was found in Fig 5.17b (lane A-D) that all isozymes of SOD-1 to SOD-9 were inhibited by hydrogen peroxide although band of SOD-7 still partially remained. It revealed that all of SOD-1 to SOD-9 isozymes of C. reinhardtii 137c superoxide dismutase are the iron enzymes. However under the hydrogen peroxide treatment of the activity staining, an unexpectedly new band of superoxide dismutase was detected. at R_f = 0.10. This occurred when crude enzyme of 0.75-1.5 units was studied (lane B-D, Fig. 5.17b) and the band was designated as SOD-10 lying at the distance between SOD-3 and SOD-4. This band was classified as a Cu/Zn-SOD because it disappeared when cyanide (4 mM) was included in the hydrogen peroxide treatment (lane A-D, Fig. 5.17c).

Effect of paraquat (0.1 μM) on the isozymes of wild type superoxide dismutase was investigated. Isozyme patterms were found not distinct from those in the wild type cells growing in the medium without the herbicide (lane E-H, Fig. 5.17). Totally ten isozymes were observed. However there was some significant differences in the intensity of the activity bands. Those were SOD-5 (Fe-SOD) and SOD-10 (Cu/Zn-SOD). When comparing between the equal units of superoxide dismutase of the enzyme from cells with and without paraquat treatment, it was demonstrated that SOD-5 production appeared to be less in paraquat treated cells

(lane E-H comparing to lane A-D, Fig. 5.17a). For SOD-10, its band was detected in paraquat treated cells only when 1.0-1.5 units of superoxide dismutase were examined whereas the same enzyme in paraquat non-treated cells could be visualized at only half concentration of the enzyme used (0.75 units).

5.6.2 Effect of Paraquat on Isozymes of C. reinhardtii PPQ-10/3 Superoxide Dismutase Culture of PPQ-10/3 in paraquat free medium was introduced to analysis of the isozyme patterns of superoxide dismutase. The results were shown in Fig. 5.18.

As a whole, C. reinhardtii PPQ-10/3 contained ten isozymes as commonly found in 137c strain. The identification of these isozymes by treatment with hydrogen peroxide (lane A-D, Fig. 5.18b) and treatment with hydrogen peroxide plus cyanide (lane A-D, Fig 5.18c) gave the same results as of the wild type enzyme that, among the ten isozymes, only SOD-10 was Cu/Zn-SOD whereas the others were Fe-SODs.

Intensity of the activity staining of Cu/Zn-SOD in PPQ-10/3 (lane A-D, Fig. 5.18b) were compared to those in the wild type enzyme (lane A -D, Fig. 5.17b). The higher intensity was noticed in the copper/zinc-enzyme of PPQ-10/3 (SOD-10). This was suggested an increase in the amount of the Cu/Zn-isozyme in the paraquat resistant strain, PPQ-10/3, with respect to the wild type 137c strain.

The effect of paraquat at sublethal dose (1 μM) on isozymes of PPQ-10/3 superoxide dismutase was also examined. Isozymes of the

crude extracts from those cells grown under the pressure of paraqaut (lane E-H, Fig. 5.18) were elucidated in parallel with the isozymes of cells grown without the herbicide pressure (lane A-D, Fig. 5.18). Two effects of paraquat were illustrated.

One effect was observed at bands of SOD-5 (Fe-SOD) of the enzyme from paraquat treated cells with a reduction in the intensity of band at any unit of superoxide dismutase (0.5-1.5 units) in comparison to those of the SOD-5 isozyme from extract of the herbicide non-treated cells.

Another effect was shown by the induction of SOD-10 (Cu/Zn-SOD) that its band began to be clearly observed in less amount of the total superoxide dismutase from paraquat treated PPQ-10/3 (0.5 units, lane E in Fig. 5.18b). While at the same amount of superoxide dismutase from the paraquat non-treated cells, this band of SOD-10 was relatively faded (lane A, Fig 5.18b).

5.6.3 Effect of Paraquat on Isozymes of C. reinhardtii UPQ-S1 Superoxide Dismutase Isozyme patterns of UPQ-S1 superoxide dismutase were interestingly different from those of the 137c and PPQ-10/3 strains. UPQ-S1 cell cultured in paraquat free medium contained totally nine isozymes of which five isozymes were SOD-5, SOD-7, SOD-8, SOD-9, and SOD-10 (lane A-D, Fig. 5.19a and 5.19b), in common with those of 137c and PPQ-10/3.

Another four new isozymes were emerged in the electrophoretic pattern of UPQ-S1 which were coded as SOD-11, SOD-12, SOD-13,

Figure 5.18 Zymograms of superoxide dismutase (SOD) from extracts of Chlamydomonas reinhardtii PPQ-10/3.

Various units of superoxide dismutase from cells growing in paraquat free medium and from cells growing in the medium containing 1 μM paraquat were electrophoresed in a 10% polyacrylamide gel. Bands of the enzyme were activity stained by nitro blue tetrazolium staining.

lane A-D : SOD of cells in paraquat free medium

(A) 0.5 unit (B) 0.75 unit

(C) 1.0 unit (D) 1.5 unit

lane E-H : SOD of cells in paraquat medium

(E) 0.5 unit (F) 0.75 unit

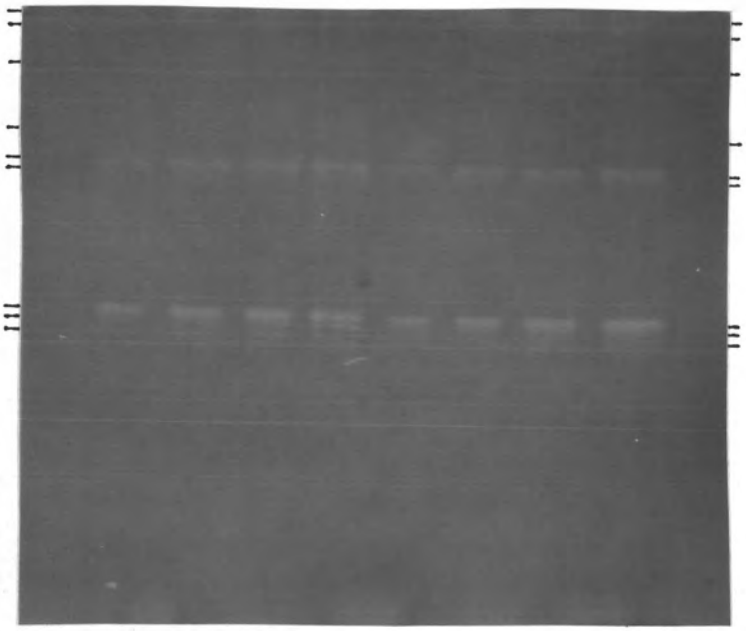
(G) 1.0 unit (H) 1.5 unit

Fig. 5.18a control activity staining

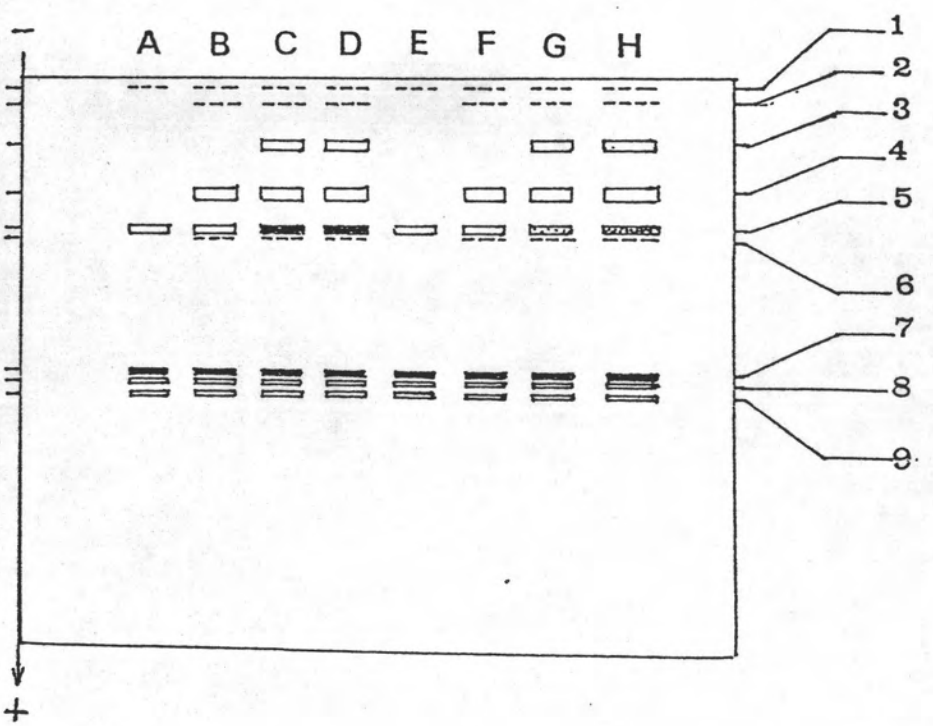
Fig. 5.18b activity staining in the presence of hydrogen peroxide

Fig. 5.18c activity staining in the presence of hydrogen peroxide + cyanide

Fig. 5.18a

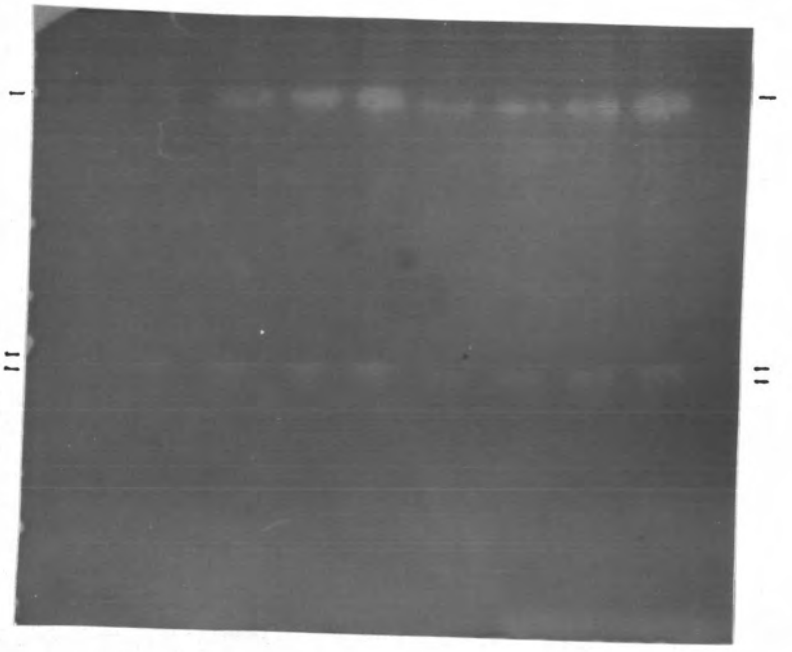


Explanatory Drawings of SOD Isozymes



	R_f
1	0.01
2	0.03
3	0.07
4	0.13
5	0.18
6	0.19
7	0.35
8	0.36
9	0.38

Fig. 5.18b



Explanatory Drawings of SOD Isozymes

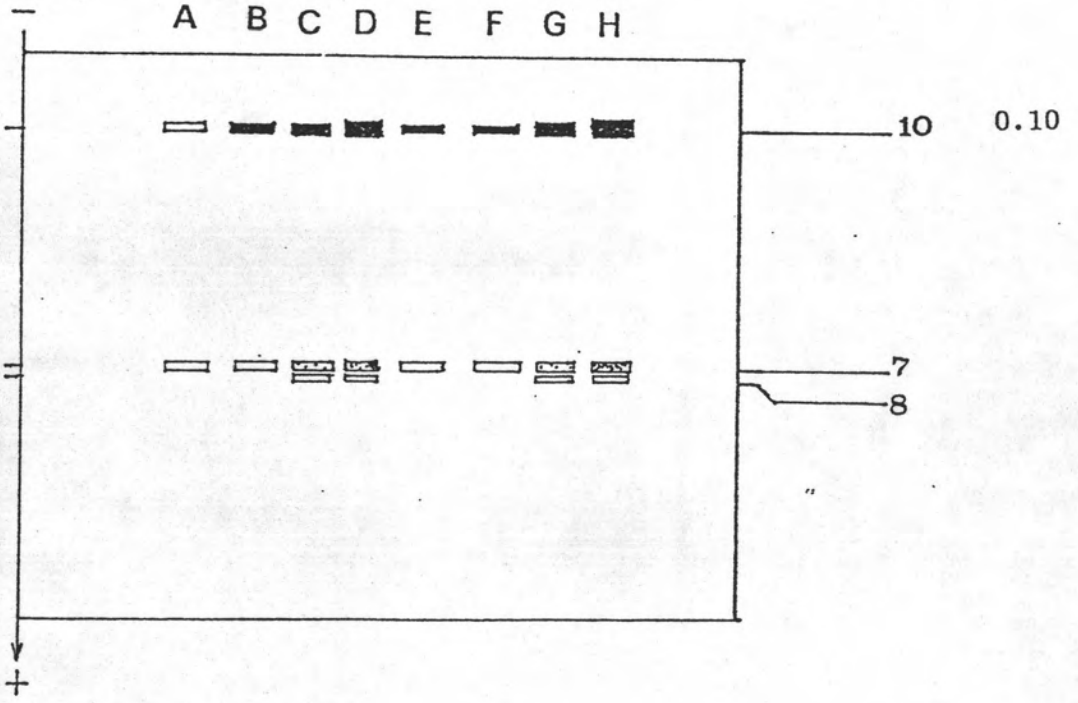
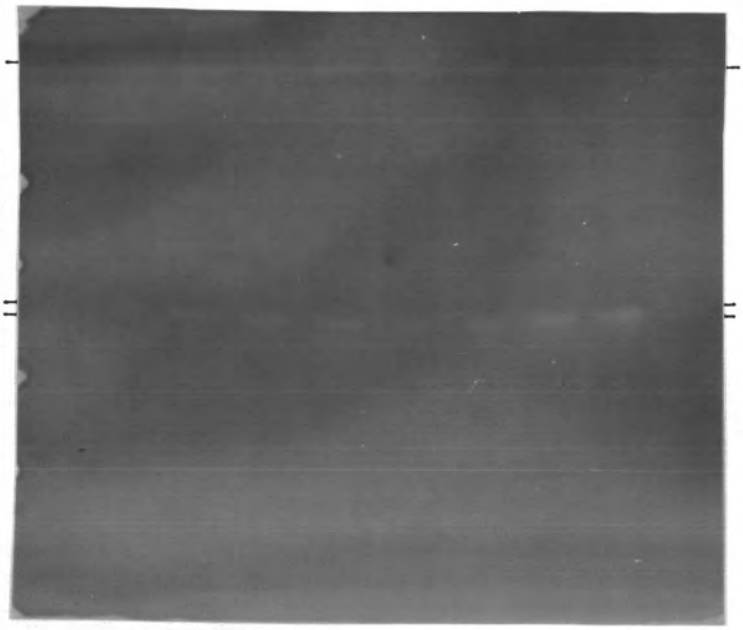
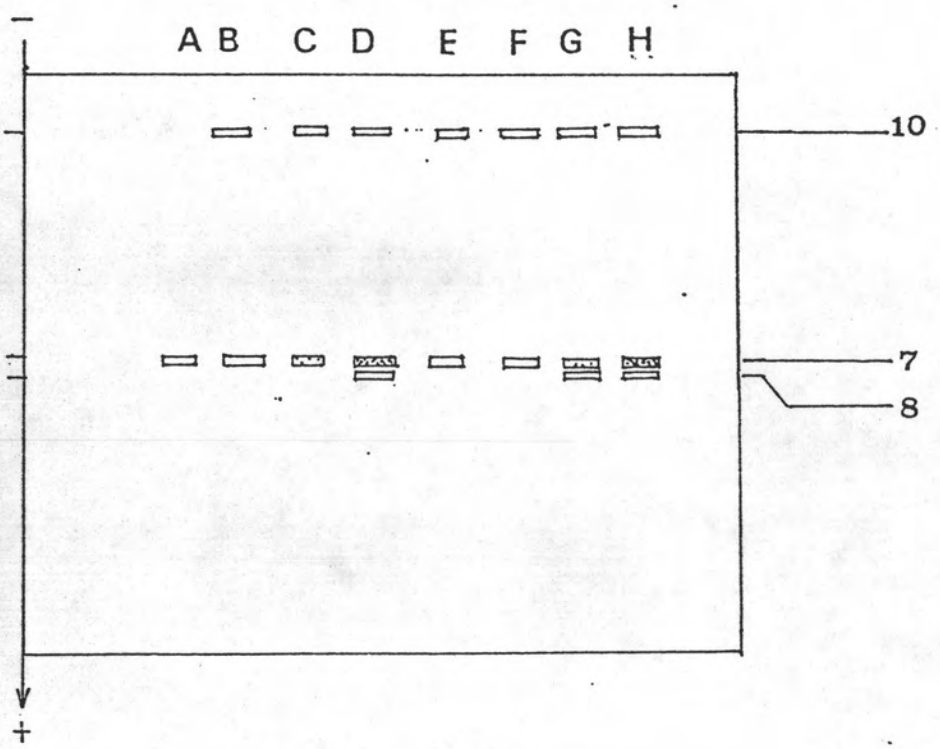


Fig. 5.18c



Explanatory Drawings of SOD Isozymes



and SOD-14. These isozymes were detected as minor isozymes simultaneously apparent with SOD-10 (Cu/Zn-SOD) when activity staining was undertaken in the presence of hydrogen peroxide (lane A-D, Fig. 5.19b). However only SOD-11 and SOD-12 were susceptible to inhibition by cyanide, indicating them to be Cu/Zn-SODs, as shown in Fig. 5.19c (lane A-D) that bands of SOD-11 and SOD-12 disappeared when cyanide was added in the hydrogen peroxide treatment. The SOD-13 and SOD-14 isozymes of superoxide dismutase found specially in UPQ-S1 paraquat resistant strain were not inhibited by cyanide and at the same time thier activities were enhanced by hydrogen peroxide. It therefore concluded that SOD-12 and SOD-13 were not Cu/Zn-SOD nor Fe-SOD.

When UPQ-S1 was cultured in the medium containing the herbicide at sublethal dose (1 μM), five isozymes of superoxide dismutase were diminished in activity staining. Those were SOD-10, SOD-11, SOD-12, SOD-13 and SOD-14. Figure 5.19b presented activity staining of superoxide dismutase in the presence of hydrogen peroxide, in which lane E-H showed isozyme patterns of the enzyme from culture with paraquat pressure. Residual activity of SOD-7 was also detected. When focusing on the SOD-10, its activity staining illustrated less in amount of the enzyme than the same isozyme of UPQ-S1 grown without paraquat pressure (lane A-D). Similar results were interpreted in case of SOD-13 and SOD-14, although the signals were not clear but the results were consistently occured for several repeated experiments, whereas bands of SOD-11 and SOD-12 could not

Figure 5.19 Zymograms of superoxide dismutase (SOD) from extracts of Chlamydomonas reinhardtii UPQ-S1.

Various units of superoxide dismutase from cells growing in paraquat free medium and from cells growing in the medium containing 1 μM paraquat were electrophoresed in a 10% polyacrylamide gel. Bands of the enzyme were activity stained by nitro blue tetrazolium staining.

lane A-D : SOD of cells in paraquat free medium

(A) 0.5 unit (B) 0.75 unit

(C) 1.0 unit (D) 1.5 unit

lane E-H : SOD of cells in paraquat medium

(E) 0.5 unit (F) 0.75 unit

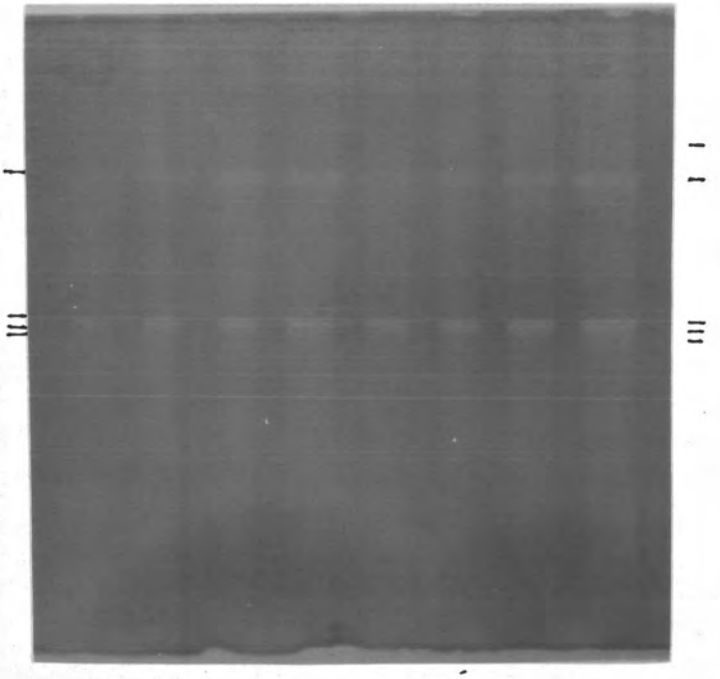
(G) 1.0 unit (H) 1.5 unit

Fig. 5.19a control activity staining

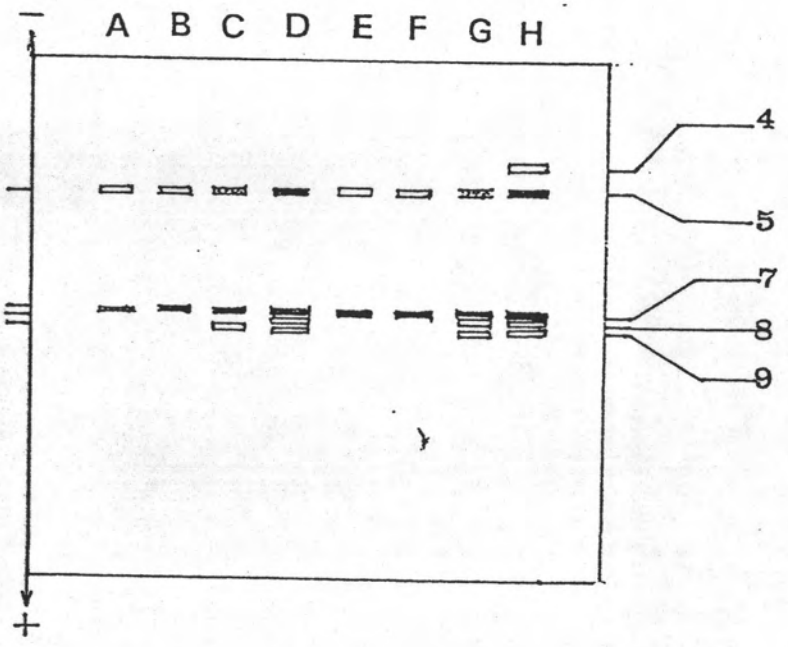
Fig. 5.19b activity staining in the presence of hydrogen peroxide

Fig. 5.19c activity staining in the presence of hydrogen peroxide + cyanide

Fig. 5.19a



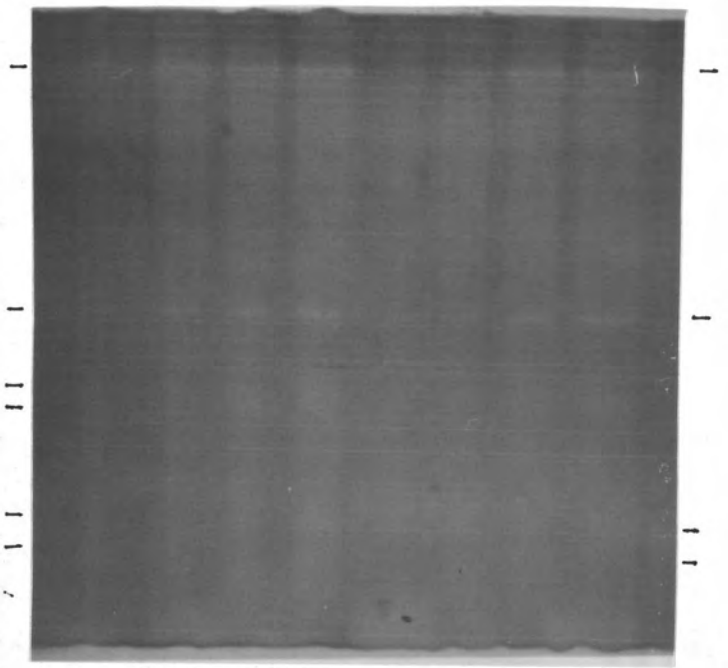
Explanatory Drawings of SOD Isozymes



R_f

0.13
0.18
0.35
0.36
0.38

Fig. 5.19b



Explanatory Drawings of SOD Isozymes

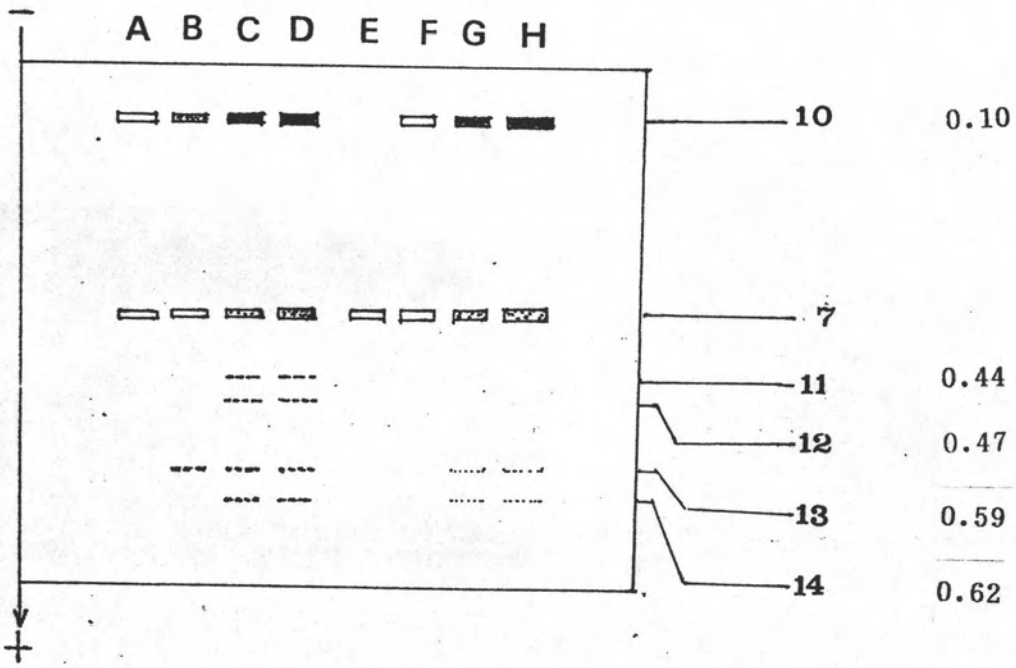
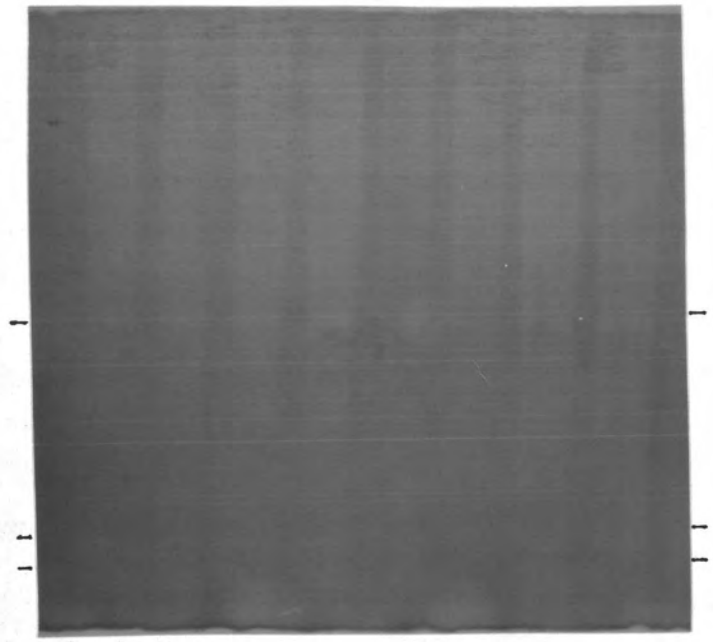
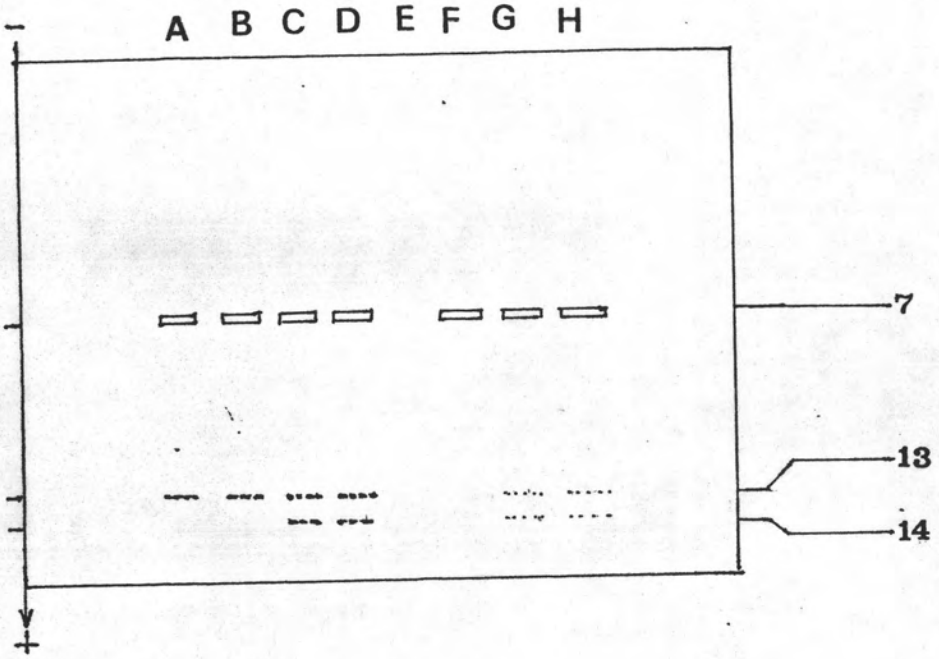


Fig. 5.19c



Explanatory Drawings of SOD Isozymes



be detected. Moreover, it was illustrated (lane H, Fig. 5.19a) that paraquat treatment caused induction of SOD-4 (Fe-SOD) in the paraquat resistant UPQ-S1.

5.7 Patterns of Cellular Proteins in Wild Type and Paraquat Resistant C. reinhardtii

In order to evaluate protein species that could be correlated with paraquat resistance in C. reinhardtii, pattern of cellular proteins in the two paraquat resistant strains were compared to that of the wild type. Polyacrylamide gel electrophoresis was performed with non-denatured proteins of the whole cell lysate and the results were depicted in Fig. 5.20.

Without paraquat pressure during growth, a certain pattern of cellular proteins was obtained in PPQ-10/3 strain (lane C) which was almost similar to the pattern in wild type (137c)(lane A). Neither extra band nor deficient band was observed. By the effect of paraquat treatment (1 μM), five protein bands were relatively disappeared in PPQ-10/3 (lane D). Those were bands at position 1, 2, 3, 4, and 5. In contrast, in case of wild type only one protein at band no. 5 (lane B) clearly lost under the paraquat pressure (0.1 μM).

For UPQ-S1, different protein patterns were obtained. Cells grown in paraquat free medium contained an enhanced amount of protein at band no. 1 and band no. 2 but a reduced amount of protein at band no. 3 and band no. 4 (lane E) with respect to the

wild type proteins. Besides inclusion of the herbicide (1 μM) in the medium led to a further decrease in protein at band no.3 and band no. 4(lane F).

Figure 5.20 Electrophoregrams of native proteins from Chlamydomonas reinhardtii wild type and paraquat resistant strains.

Cultures of C. reinhardtii, both in the presence and in the absence of paraquat at sublethal dose, were lysed by sodium dodecyl sulfate. Supernatants of the whole lysate (200 µg protein) were electrophoresed anodally under the non-denaturation system for a distance of 13 cm. Proteins were stained with coomassie brilliant blue-R.

lane A: proteins of 137c grown in paraquat free medium

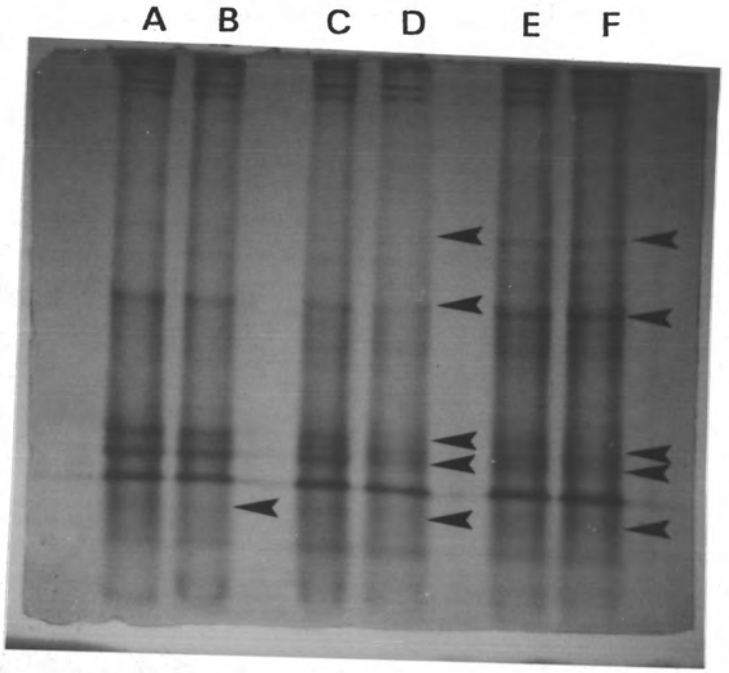
lane B: proteins of 137c grown in 0.1 µM paraquat medium

lane C: proteins of PPQ-10/3 grown in paraquat free medium

lane D: proteins of PPQ-10/3 grown in 1 µM paraquat medium

lane E: proteins of UPQ-S1 grown in paraquat free medium

lane F: proteins of UPQ-S1 grown in 1 µM paraquat medium


A
B
C
D
E
F
R_f
1
0.32
2
0.46
3
0.70
4
0.74
5
0.85

5.8 Analysis of Chloroplast DNA Restriction Patterns

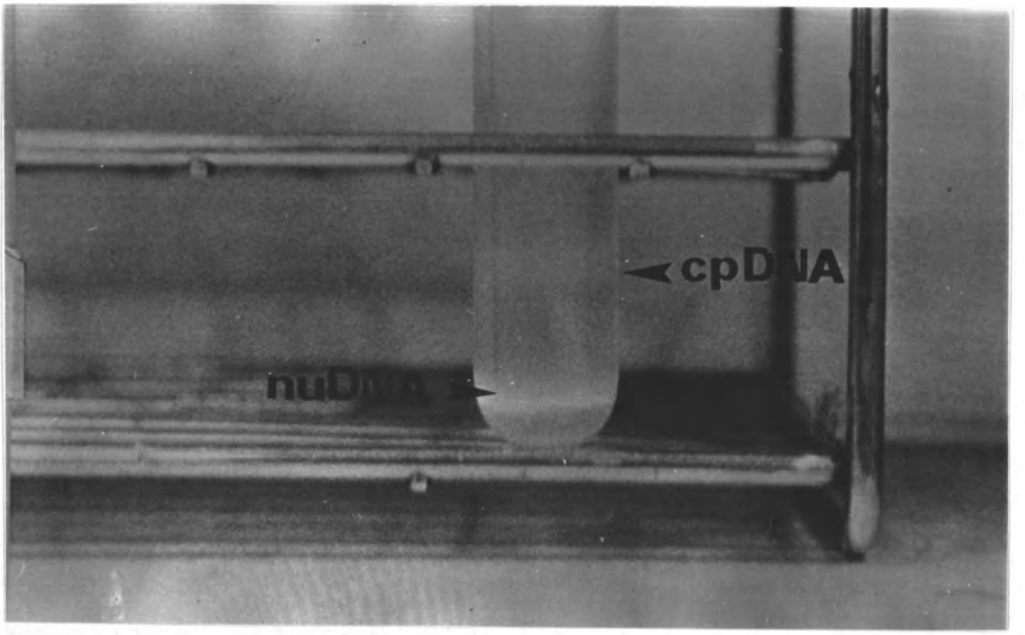
5.8.1 Purification of Chloroplast DNA For restriction endonuclease analysis, the chloroplast DNA of C. reinhardtii 137c and the paraquat resistant strain UPQ-S1 which was constructed via chloroplast DNA directed mutagenesis, were purified by ultracentrifugation in cesium chloride gradient. Two dense fluorescing bands of ethidium bromide were clearly separated, upper band and lower band (Fig. 5.21), refering to two DNA species. The buoyant densities of C. reinhardtii chloroplast DNA and nuclear DNA in cesium chlorice gradient have been studied to be 1.696 $g.cm^{-3}$ and 1.724 $g.cm^{-3}$ respectively. Therefore the upper band was considered as a chloroplast DNA rich fraction, whereas the lower band should be the nuclear DNA fraction.

5.8.2 Identification of Alterations in Chloroplast DNA In Fig. 5.22, lane 2 and lane 9 showed the purity of chloroplast DNA preparations from wild type and UPQ-S1 respectively. There was very little amount of the same DNA impurity in both preparations at 14.1 kb. The majority of the chloroplast DNA, more than 99% as judged by the fluorescence intensity, migrated to the position in the limited resolution region of gel (above 23.8 kb of DNA).

Chloroplast DNA of UPQ-S1 was digested with five restriction endonucleases, BamH I, Bgl II, EcoR I, Pst I, and Xho I. Pattern of the restriction fragments was analysed in comparing to the restriction pattern of 137c (wild type) chloroplast DNA which was digested

Figure 5.21 UV fluorescence of DNAs-ethidium bromide in cesium chloride gradient.

DNA preparation from C. reinhardtii 137c was separated by ultracentrifugation in cesium chloride gradient containing ethidium bromide at 140,000g for 40 hrs.



with the same restriction enzyme. Digestion of the DNA by all enzymes used resulted in dicrete bands.

Analysis of the BamH I and EcoR I digests (E1 and E2 pairs, Fig. 5.22) could not bring about clear conclusions because the enzymes cut chloroplast DNA into a large number of fragments comigrating together. However comparison of chloroplast DNA from UPQ-S1 mutant and wild type digested with Pst I (E3 pair, Fig. 6.18) revealed five DNA fragments which illustrated 50% or more decreasing in intensity of fluorescent signal in UPQ-S1 digest. Those fragments were at 15.5 kb, 14.8 kb, 13.5 kb, 12.4 kb, and 12.0 kb. The reference bands with no comparative difference in the fluorescence intensity between chloroplast DNA of wild type and UPQ-S1 were remarkable at 4.8 kb and 4.2 kb.

Alternative attempt was carried out by using the two extra restriction enzymes, Bgl II and Xho I (Fig. 5.23). A few discrete bands were observed. However they could not be distinguishable between the two chloroplast DNAs .

Figure 5.22 Electrophoregrams of BamH I, EcoR I, and Pst I digests of purified chloroplast DNAs of C. reinhardtii 137c and UPQ-S1.

Chloroplast DNA (0.5 µg) and digested chloroplast DNA (2 µg) were loaded on 0.7% agarose gel (16 cm in length) and electrophoresed in E buffer, pH 8.0 at a constant voltage of 3 v/cm. After staining with ethidium bromide, a photograph was taken under UV light.

In each pair of one enzyme, wild type 137c DNA is at the left, and UPQ-S1 DNA is at the right. Arrows mark the fragments with altered intensity of fluorescing in the UPQ-S1 chloroplast DNA digested with Pst I.
(E1 = BamH I, E2 = EcoR I, E3 = Pst I)

lane 1 : size markers of λ / Hind III fragments

lane 2 : 137c chloroplast DNA

lane 3 : BamH I digested 137c chloroplast DNA

lane 4 : BamH I digested UPQ-S1 chloroplast DNA

lane 5 : EcoR I digested 137c chloroplast DNA

lane 6 : EcoR I digested UPQ-S1 chloroplast DNA

lane 7 : Pst I digested wild type chloroplast DNA

lane 8 : Pst I digested UPQ-S1 chloroplast DNA

lane 9 : UPQ-S1 chloroplast DNA

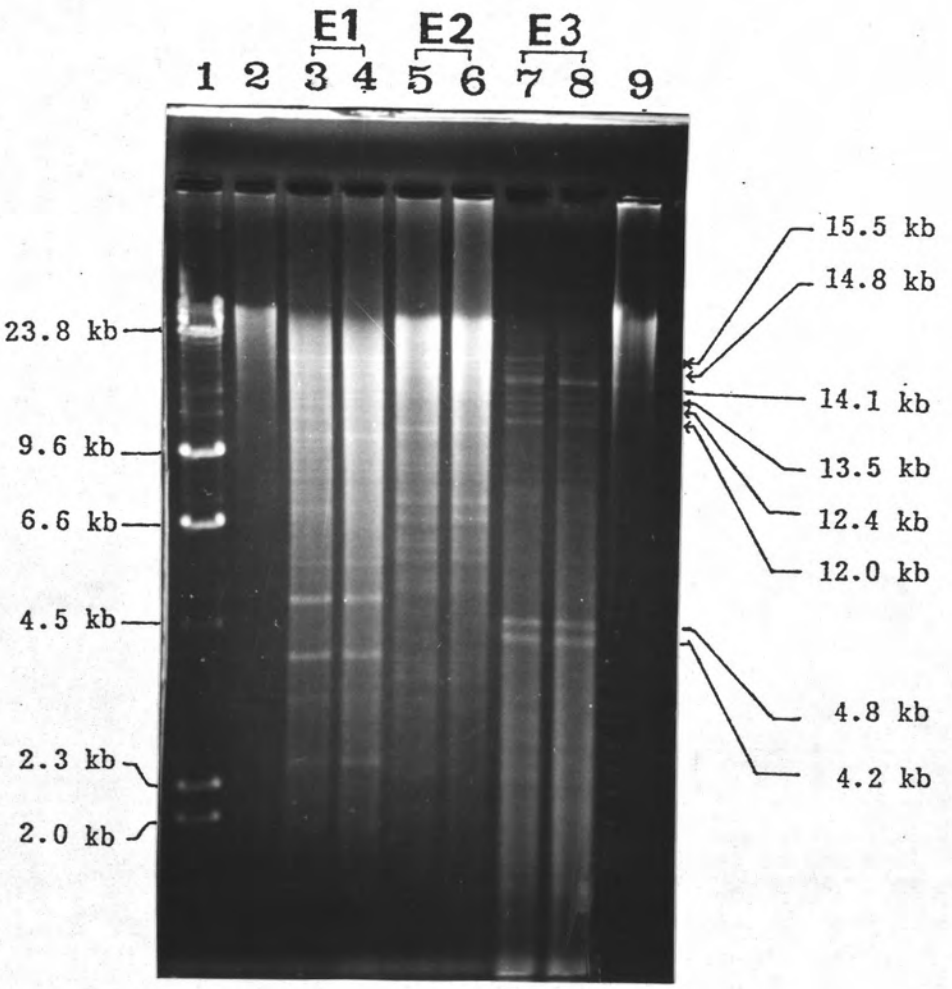

E1
E2
E3
1 2 3 4 5 6 7 8 9
23.8 kb
9.6 kb
6.6 kb
4.5 kb
2.3 kb
2.0 kb
15.5 kb
14.8 kb
14.1 kb
13.5 kb
12.4 kb
12.0 kb
4.8 kb
4.2 kb

Figure 5.23 Electrophoregrams of Bgl II and Xho I digests of purified chloroplast DNAs of C. reinhardtii 137c and UPQ-S1.

Chloroplast DNA (0.5 µg) and digested chloroplast DNA (2 µg) were loaded on 0.7% agarose gel (16 cm in length) and electrophoresed in E buffer, pH 8.0 at a constant voltage of 3 v/cm. After staining with ethidium bromide, a photograph was taken under UV light.

In each pair of one enzyme, wild type 137c DNA is at the left, and UPQ-S1 DNA is at the right.
(E4 = Bgl II, E5 = XhoI)

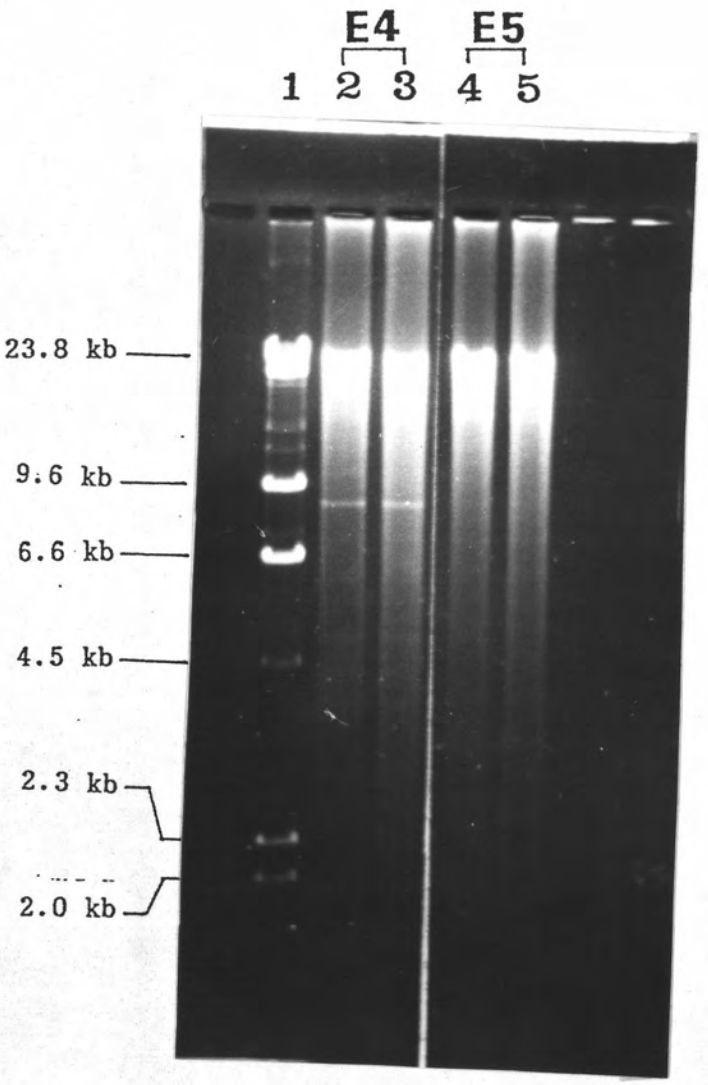
lane 1 : size markers of λ / Hind III fragments

lane 2 : Bgl II digested 137c chloroplast DNA

lane 3 : Bgl II digested UPQ-S1 chloroplast DNA

lane 4 : Xho I digested 137c chloroplast DNA

lane 5 : Xho I digested UPQ-S1 chloroplast DNA


E4
E5
1 2 3 4 5
23.8 kb
9.6 kb
6.6 kb
4.5 kb
2.3 kb
2.0 kb