CHANGES IN PHOTOSYNTHETIC APPARATUS AND ACTIVITY IN PROTOPLAST CULTURE

by

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Dedication:

I dedicate this thesis to my parents in gratitude for their moral support during my education.

CONTENT

INTRODUCTION

The possibility of obtaining protoplasts from plant tissues and of regenerating plants from them is very important for the genetics as well for the physiology of higher plants /Cocking 1972/. Since the initial report by Cocking /1960/ on the isolation of protoplasts from tomato root tip cells, many investigations have been conducted on various types of plant cells. Viable protoplasts have been successfully isolated from many different parts of plants: roots /Bawa and Torrey 1971/; Kameya and Uchimiya /1972/; root nodules /Davey et al. 1973/; flower petals /Potrykus 1971/; fruit tissues /Gregory and Cocking 1965/ endosperm /Motoyoshi 1972/; pericarp /Skene 1974/ and leaves /Takebe et al. 1968; Otsuki and Takebe 1969; Power and Cocking 1970; Davey et al. 1974a,b; Evans et al. 1972 and others/. Among the others leaf /mesophyll/ protoplasts have been more extensively studied.

By definition, the isolated protoplast is a single cell bounded by plasmalemma, and when first formed, containing all the normal cell components, although being completely freed from its cell wall. From a physiological viewpoint, however, the protoplast cannot be regarded simply as a cell lacking a cell wall /Mills and Krebs 1968/. This is because the mechanics of isolation, in conjunction with factors in their environment, undoubtedly in-

fluence its metabolism and elicit subtle ultrastructural changes. /Power and Cocking 1970/. The absence of a cell wall may affect the permeability of the cell membrane and lead to a partial leakage of solutes from the protoplast /Power and Cocking 1970/. The protoplast is also in a transient state, since most protoplasts irrespective of their immediate nutritive environment will initiate the synthesis of a new cell wall a few hours after release, and eventually revert to a single-walled cell /Pojnar et al. 1967/.

It has been shown that mesophyll protoplasts can exhibit a range of biological activities, including pinocytosis /Power and Cocking 1970, Otsuki et al. 1972/, RNA and protein synthesis /Sakai and Takebe 1970/ and virus production /Aoki and Takebe 1969; Otsuki and Takebe 1969; Takebe et al. 1971; Otsuki et al. 1972/. These findings strongly suggest that enzymatically isolated protoplasts are physiologically active, and encouraged us to study their ability for in vitro development /Nagata and Takebe 1970; Takebe et al. 1971b; Nagata and Takebe 1971/.

In the last decade a number of papers have dealt with regeneration in plant protoplasts. These works focussed mainly on cell wall regeneration, induction of cell division, callus and plant regeneration /Cocking 1972/. In most of these investigations, green leaf protoplasts were used, but changes in the photosynthetic apparatus were

not considered.

Although Nishimura and Akazawa /1975/ and Colman and Mawson /1978/ showed that freshly isolated protoplasts exhibited a high rate of photosynthetic O_2 evolution and CO_2 fixation within 9 hours after protoplast isolation, the photosynthetic activities of cultured protoplasts have not yet been investigated.

In an analogous problem which concerns the photosynthetic activity of cell cultures only limited data are available on moderately developed chloroplasts /Laetch and Stetler 1965, Sunderland and Wells 1968/, photosynthetic CO_2fixation /Laetch and Stetler 1965; Venketeswaran 1965; Mc Laren and Thomas 1970; Edelman and Hanson 1971/ and oxygen evolution /Naef 1968/.

On protoplast culture there have been only certain simple observations, that during the process of colony formation differentiated mesophyll cells reverted to meristematic ones while the chloroplasts degenerated /Nagata and Yamaki 1973/. The number of chloroplasts per cell decreased during cell divisions, suggesting that the mature plastids did not multiply /Nagata and Takebe 1970/ or that their division could not keep pace with the division of the cells /Nagata and Yamaki 1973/. After a week of protoplast incubation the cells contained highly degenerated chloroplasts /Nagata and Yamaki 1973/ and the colonies had lost their green colour and become yellowish

or creamy brown /Thomas and Davey 1975; Usui and Takebe 1969/. After a few days of culture, the plastids contained large starch grains and functioned as amyloplasts in culture /Davey et al. 1974b/.

It can be seen that only a very limited characterization of protoplast photosynthesis has appeared in the literature, and the metabolic background to the degeneration of the photosynthetic apparatus which occurs during the regeneration of photosynthetically active /somatic/ cells was unknown.

It is startling that in the large number of papers on protoplasts no attention has been paid to the profound changes in the photosynthetic apparatus during the transition from autotrophy to heterotrophy or vice versa.

THE SCOPE OF THE THESIS

The present work was designed to carry out a detailed comparison of different aspects of photosynthesis occurring during the early period of the culture of protoplast isolated from tobacco mesophyll tissue.

A. Experiments were performed on chloroplast destruction by investigating the pigment and ultrastructural changes of the chloroplast during protoplast incubation.

B. Photosynthetic activities, such as O_2 evolution, P700 oxidation and 515 nm absorbance change and respiratory O_2 uptake were followed during the first 8 days of protoplast isolation.

The data obtained are the first to report that the rapid decrease in the photosynthetic pigments does not involve a loss of photosynthetic activities. The increased starch synthesis in the chloroplasts was caused by the high sucrose content of the incubation medium and led to the destruction of the thylakoids in the chloroplasts. The rate of the respiration was markedly increased, during cell multiplication showing that by cell division the protoplast metabolism was shifted from autotrophic pathways to heterotrophic ones.

MATERIALS AND METHODS

Plant material: Fully expanded leaves of 3-4-week--old tobacco plants /Nicotiana tabacum var. Xanthi/ grown under ordinary greenhouse conditions were used for the isolation of protoplasts.

Isolation of tobacco leaf protoplasts: The surface of the leaf was sterilized by successive immersion in 70% ethanol, 4% sodium hypochlorite solution /30 minutes/ and finally washed in several changes of sterile distilled water. All subsequent treatments were carried out under aseptic conditions. The lower epidermis of the leaves was peeled off /Power and Cocking, 1970/ and then the peeled leaf pieces /2 g fresh weight/ were floated/ abaxial surface downwards /on the surface of sterile 0.4 M mannitol solution. After 3 hours of preplasmolysis, the mannitol solution was replaced by 20 ml of a sterile mixture of 1% cellulase "Onozuka" R-10, 0.25% macerozyme R-10, 0.25% potassium dextran sulphate, 0.4 M mannitol, at pH 5.8. This incubation was for 2-3 hours at 36°C. During digestion, the protoplasts were released by gentle agitation. The veins and upper epidermis, which remained undigested, were removed by filtration through a sterilized Nylon cloth /63 μ diam/. The filtrate was centrifuged at 500 rpm in a bench centrifuge for 2 min. The protoplasts pellet was

resuspended in 0.4 M mannitol and the washing was repeated 2-4 times. /Nagata and Takebe 1971/.

Culture of the protoplasts: The isolated protoplasts were counted in haemocytometer /Carl Zeiss, Jena/ and were incubated at a cell density of 1.2×10^5/ml in different culture media shown in Table 1 and Table 2.

Table 1. Composition of N-T medium /Nagata and Takebe 1971/

A. Mineral salts			
Major elements	mg/l	Minor elements	mg/l
NH_4NO_3	825	H_3BO_3	6.2
KNO_3	950	$MnSO_4 \cdot 4H_2O$	22.3
$CaCl_2 \cdot 2H_2O$	220	$ZnSO_4 \cdot 4H_2O$	8.6
$MgSO_4 \cdot 7H_2O$	1233	KI	0.83
KH_2PO_4	680	$Na_2MoO_4 \cdot 2H_2O$	0.25
$Na_2 \cdot EDTA$	37.3	$CuSO_4 \cdot 5H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	27.8	$CoSO_4 \cdot 7H_2O$	0.030

B. Organic compounds	x/l
Sucrose	10 g
meso-Inositol	100 mg
Thiamine·HCl	1 mg
1-Naphthyl acetic acid	3 mg
6-Benzylaminopurine	1 mg
d-Mannitol	0.7 mole

pH 5.8

The protoplasts were cultured at 23-25°C under a continuous illumination of 1700 lux provided by white light fluorescent tubes.

Table 2. Composition of K_3 medium /Kao et al. 1971/ modified by Nagy and Maliga /1976/

A. Mineral salts					
Elements	x/l		Elements	x/l	
$NaH_2PO_4 \cdot H_2O$	150	mg	Na_2EDTA	37.3	mg
$CaHPO_4$	50	mg	$MnSO_4 \cdot 2H_2O$	10	mg
$CaCl_2 \cdot 2H_2O$	900	mg	H_3BO_3	3	mg
KNO_3	2500	mg	$ZnSO_4 \cdot 7H_2O$	2	mg
NH_4NO_3	250	mg	$Na_2MoO_4 \cdot 2H_2O$	250	ug
$/NH_4/_2SO_4$	134	mg	$CuSO_4$	25	ug
$MgSO_4 \cdot 7H_2O$	250	mg	$CoCl_2 \cdot 6H_2O$	25	ug
$FeSO_4 \cdot 7H_2O$	27.8	mg	KI	750	ug

B. Organic compounds	x/l	
Nicotinic acid	1	mg
Thiamine·HCl	10	mg
Pyridoxine·HCl	1	mg
m-Inositol	100	mg
2,4-Dichlorophenoxy acetic acid	0.1	mg
6-Benzylaminopurine	0.2	mg
1-Naphthyl acetic acid	3	mg
Xylose	250	mg
Sucrose	0.4	mole

pH 5.8

Pigment determination: Quantitative analysis of pigments of the protoplast culture was carried out at intervals of 24 hours. An aliquot of the protoplast preparation was extracted in 80% acetone and the pigments were transferred to peroxide-free ethyl ether. The chlorophyll content was estimated spectrophotometrically by the multi-wavelength-method /French 1960/. The total carotenoid content was measured as described by Horváth et al./197la/.

Electron microscopy: The protoplasts from the culture suspension were collected on a Millipore filter /22 μm/ by vacuum filtration. The protoplast pellet was immersed in the fixative of Karnovsky /1965/ and postfixed with osmic acid /Milloning 1961/. The samples were dehydrated in a sequence of increasing ethyl alcohol and embedded in Durcupan /Fluka/. Thin sections were cut on a Reichert ultramicrotome and stained with uranyl acetate and lead citrate according to Reynolds /1963/ procedure. Electron micrographs were obtained by using a JEOL 100 B electron microscope.

Measurement of O_2 evolution and O_2 uptake: The rate of photosynthetic O_2 evolution and respiratory O_2 uptake was measured by using a Clark-type oxygen electrode /Delieu and Walker 1972/ in a temperature-controlled cuvette /Rank Bros., Cambridge, U.K./. Saturating white light was provided by a 650 W iodine-tungsten lamp /Fig. 1./ 3 ml aliquots of protoplast suspension were taken for the O_2 electrode.

The measurements were carried out at 30°C.

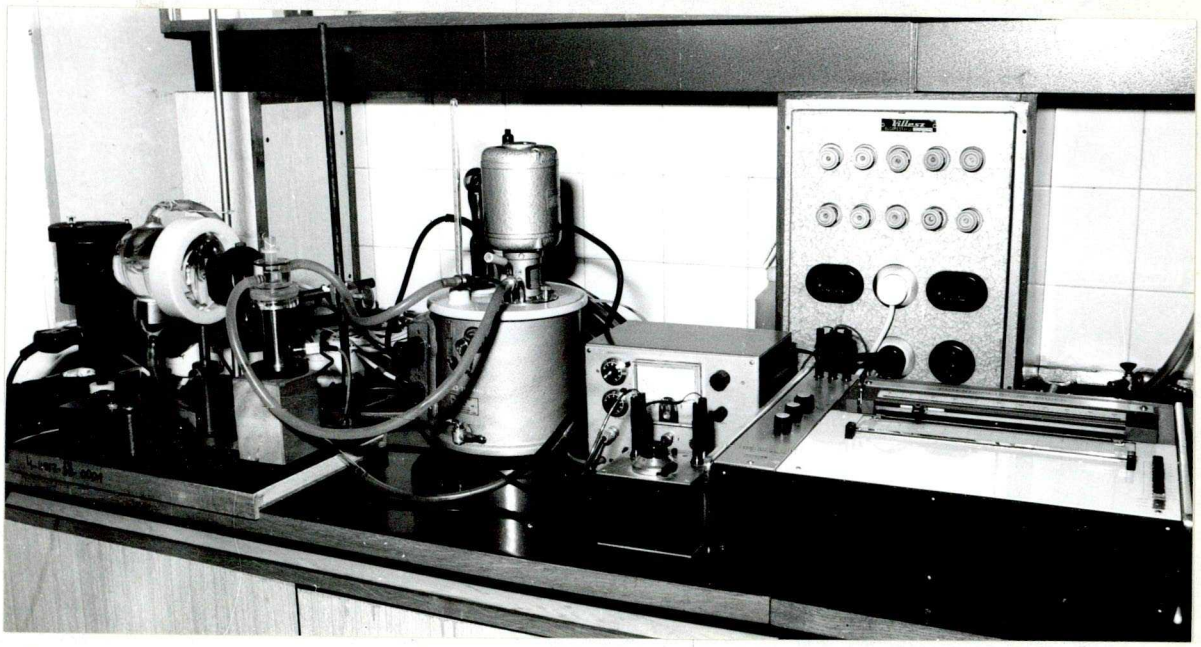

Figure 1. Oxygen electrode with the illumination attachment

Measurement of the flash-induced 515 nm absorbance change: For these measurements chloroplasts were isolated from the protoplast suspension. The protoplasts were suspended in a buffer containing 0.3 M sucrose, 0.01 M KCl, 0.05 M potassium hydrogen phosphate pH 7.2 /Anderson and Boardman, 1966/ and were forced by a gentle pressure through a needle of 0.5 mm diameter. The cell debris and chloroplasts were separated by low-speed centrifugation /300 g for 20 sec and 500 g for 2 min, respectively/. The chloroplasts were placed in a 6x10 mm cell illuminated

through a horizontal light guide by xenon flashes /General Radio, Stroboslave, 8 μs, energy 0.5 J/ perpendicular to the measuring beam. Actinic light was passed through a Calflex C /Balzers/ filter and a Schott RG 630-2 mm filter.

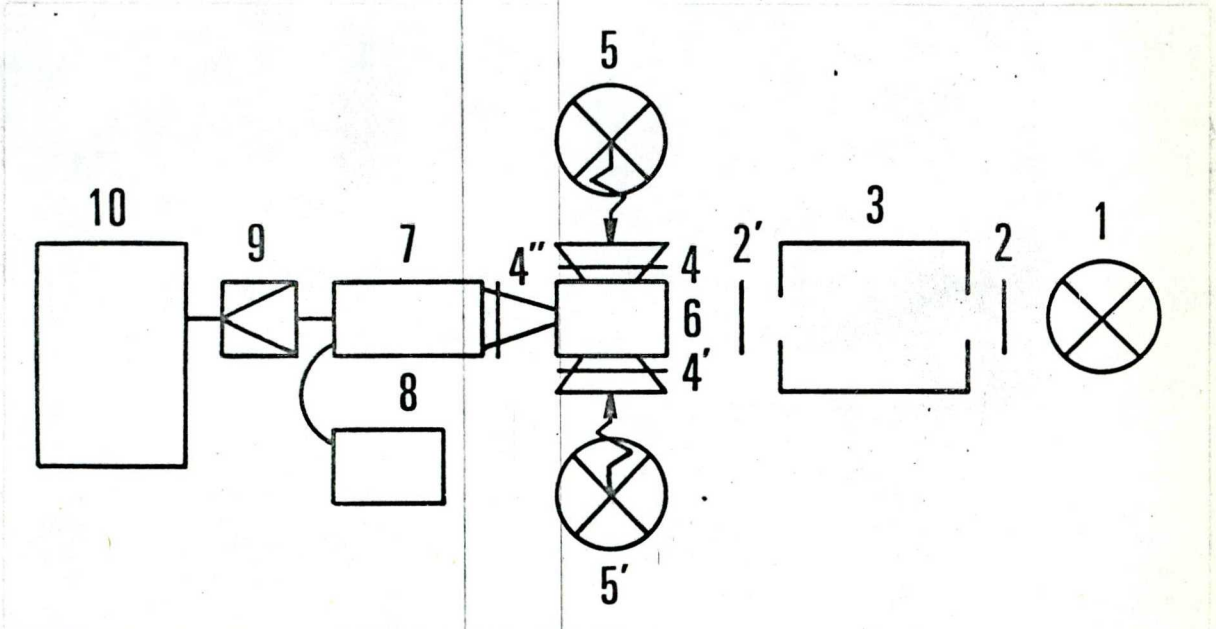

Figure 2. The block scheme of the equipment used for the measurement of 515 nm absorbance change. 1.-lamp; 2.2'-lens; 3.- Zeiss SPM 2 monochromator; 4."-Corning 4 -96 filter; 4.,4'-Schott RG 630-2 filter+light guide; 5.5'-flash light; 6.-sample; 7.-photomultiplier /EMI 9558 B/; 8.-power supply; 9.-amplifier; 10.-Intertechnique SA 40

Flashes were fired at 4 s intervals. The measuring light passed through a Zeiss SPM 2 monochromator /band width 4 nm/, and after passing the sample the light was led through a

light guide to an EMI 9558 B photomultiplier protected by a Corning 4-96 filter. The intensity of the measuring beam was about $3 \times 10^{-3} Jm^{-2} s^{-1}$ at 515 nm; 100 to 200 signals were collected in an Intertechnique SA 40 multichannel analyser. The measurements were made at 20°C.

P 700 measurement: This was carried out on sonicated protoplast material. The protoplast culture was centrifuged at 2000 rpm for 2 minutes and the pellet was resuspended in a buffer containing 0.05 M TRIS pH 7.8, 0.01 M NaCl, 0.02 M sodium ascorbate, 0.005 M cysteine HCl, and 0.4 M sucrose /Avron 1960/. The protoplasts were sonicated for 4x5 sec. The sonicated material was centrifuged at 10 000 g for 30 min. /Jacobi 1968/. The supernatant was used for the measurement of the P 700 content. An Aminco DW-2 spectrophotometer operating in the split beam mode was used. The P 700 was estimated from the decrease of absorbance around 700 nm upon illumination with blue light of saturating intensity /Marsho and Kok 1971/.

RESULTS

Morphological changes occurring during the early period of protoplast culture. The majority of the tobacco protoplasts isolated by enzymatic digestion were found to be without any visible trace of a cell wall. They were round in shape, green in colour, and glistening with chloroplasts /Fig. 3A/.

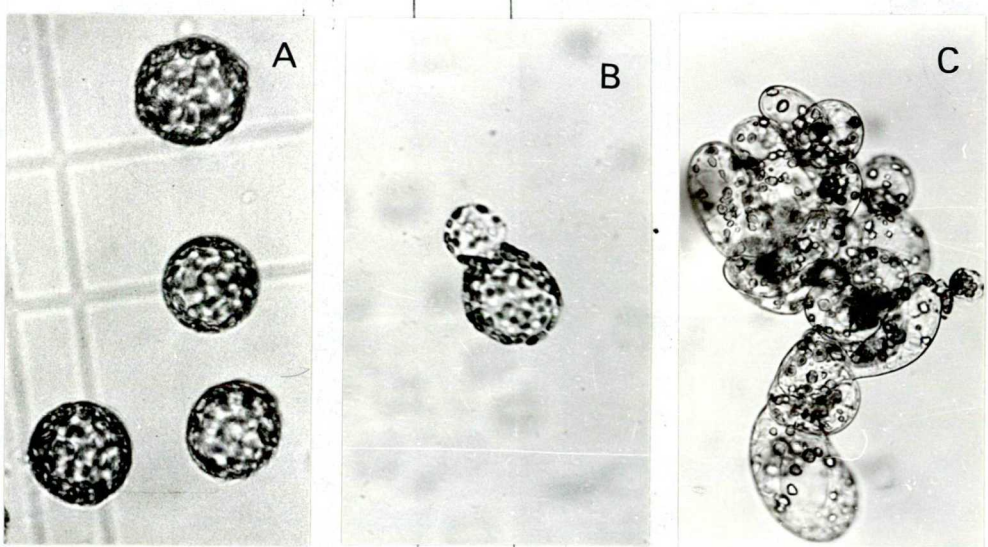

Figure 3. Cell division and colony formation by isolated mesophyll protoplasts of tobacco. A; Freshly isolated protoplasts B; The first division in a protoplast /4thday of culture/. C; A small cell colony /15thday of culture/. Magnification x 300; Protoplasts and cells were incubated in K_3 medium/

The first noticeable change in the cultured protoplast was a small increase in the size, which was evident within 24 hours of the preparation of the culture. During a lag period

of 2 to 4 days of incubation, cell wall formation took place; this was followed by cell division. Protoplasts clearly showing cell division were apparent on the 3rd day in K_3 medium and on the 6th day in N-T medium. 60--80% of the protoplasts underwent cell division at these times. The first cell division usually resulted in the formation of two equal hemispherical daughter cells. In some cases the daughter cells were different in size. /Fig. 3B/ The daughter cells which originated from the protoplasts grew in size and underwent a second division between 6th and 10th day of culture. Some of the cells had apparently gone through a third division since clusters of 6-8 cells were seen at this time. There was also an indication that daughter cells sometimes separated off and survived as single cells. After 15-20 days of incubation, subsequent divisions had produced cell colonies /Fig. 3C/ which were visible to the naked eye. The cells at this stage appeared to be rich in cytoplasm and to have thin walls, so that the boundaries between neighbouring cells were not very distinct. The cells had proliferated in three dimensions.

By this time the chloroplasts of the cultured protoplasts had lost much of their green colour and become yellowish. The chloroplasts become less and less distinct, and the number of chloroplasts per cell decreases during progressive cell division.

Changes in the pigment content during the early period of protoplast culture. Cells regenerated from green protoplasts are normally creamy-brown in colour after about two weeks of growth. It can be seen in Fig. 4. and 5. that the pigments were steadily degraded from the time of isolation until about the 10th

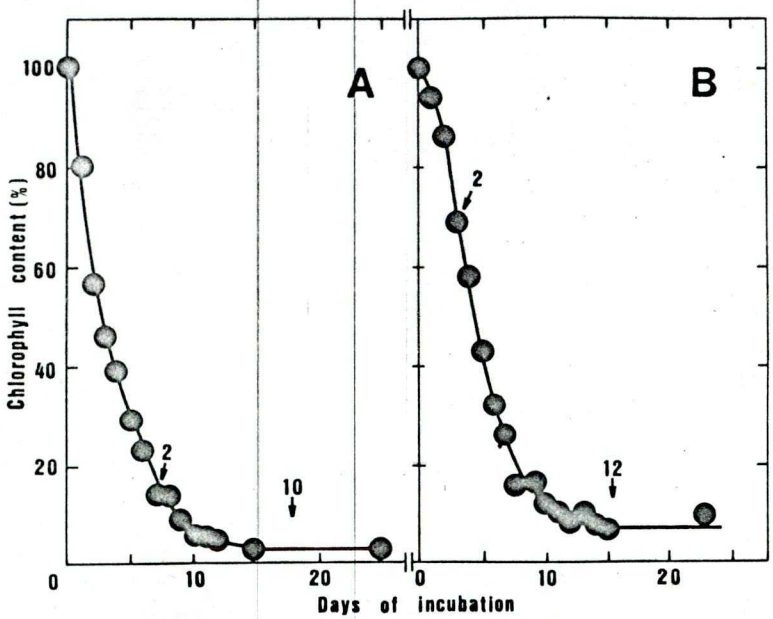

Figure 4. Change in the chlorophyll content per protoplast culture during the early period of incubation. A: protoplasts cultured in N-T medium; B: protoplasts cultured in K_3 medium. The chlorophyll content expressed in percentage of the chlorophyll content of the freshly isolated protoplast preparation. Arrows indicate the number of cells in the colonies

day of incubation. Only 30% of the chlorophylls remained by the 5-7th day of incubation. A similar pattern of degradation of the chlorophyll was exhibited in both K_3 and N-T media /Fig. 4A and B/.

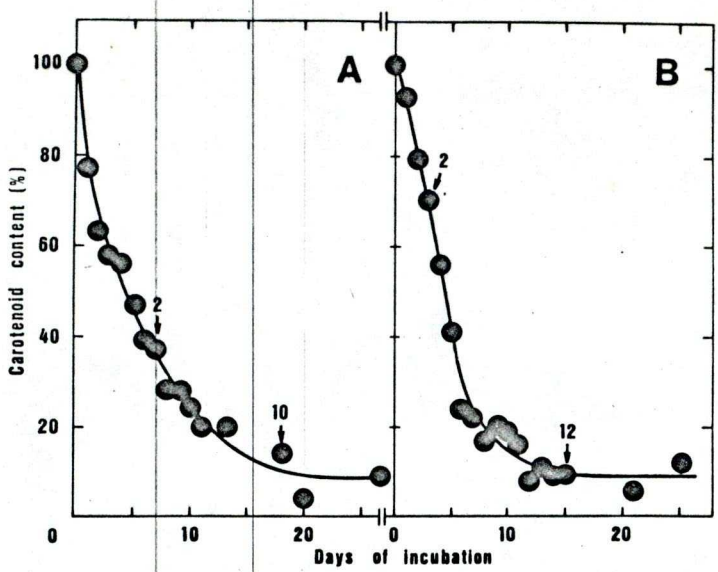

Figure 5. Change in the carotenoid content of protoplast culture during the early period of incubation. A: protoplasts cultured in N-T medium; B: protoplasts cultured in K_3 medium. The carotenoid content expressed in percentage of the content of freshly isolated protoplasts. Arrows indicate the number of cells in the colonies

The degradation of carotenoids in both media ran in the similar way to that of the chlorophyll /Fig. 5A and B/. From the graphs, pigment degradation in N-T and K_3 media, we can conclude that the differences between these media do not affect the rate of pigment decomposition occurring in the early period of protoplast culture. In contrast to the pigment degradation, the induction of protoplast division did vary according to the incubation medium used. During protoplast regeneration there was a considerable

decrease in the chlorophyll a/chlorophyll b ratio /Table 3./. Simultaneously with the chlorophyll measurements, the pH of the incubation medium was checked to

Table 3. The change of chlorophyll a/chlorophyll b ratio and the pH of the incubation media during protoplast culture.

days of incubation	Chlorophyll a/b ratio		pH of the medium
	N-T medium	K_3 medium	
0	2.91	2.79	5.6
6	2.16	2.07	5.6
11	1.75	1.46	5.6

decide whether the pigment degradation could originate from the lowering of the pH of the external medium. As it is shown on Table 3. the pH of the incubation medium was not changed within the first 11 days of the protoplast regeneration.

Ultrastructural changes of the chloroplasts occurring during the early period of protoplast culture. Parallel with the pigment degradation a gradual change could be observed in the chloroplast ultrastructure of the protoplasts during their regeneration. Following the isolation of the protoplasts an enhanced starch accumulation took place, even from the first day of incubation /Fig. 6A/.

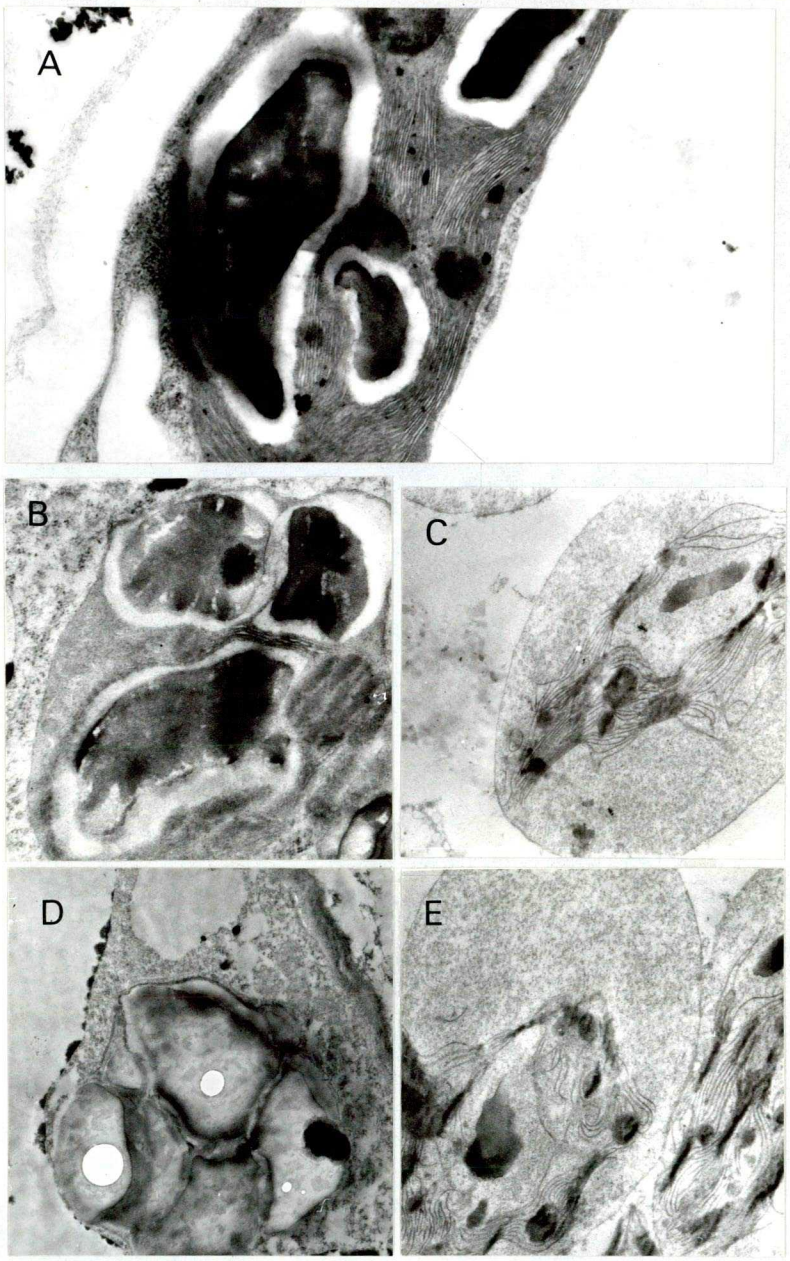

Figure 6. Ultrastructural changes of chloroplasts during leaf protoplast culture. A: Chloroplast ultrastructure after one day of protoplast incubation /x18000/; B: Chloroplast in 15-day-old culture /x15000/; C: Newly formed proplastid in the 15-day-old culture /x12000/; D: Old chloroplasts of the 30-day-old culture /x7100/; E: Proplastids formed in the 30-day-old protoplast culture /x11000/.

The enlarged starch grains appressed the chloroplast lamellae and with the further growing of the starch grains a progressive degeneration of the chloroplast lamellae occurred. After 15 days of incubation only a few, appressed, lamellae were present in the chloroplasts /Fig. 6B/. On the 10^{th}-15^{th} day of protoplast regeneration a few newly-formed proplastids appeared among the degenerated old plastids /Fig. 6C/. On the 30^{th} day of incubation only large starch grains could be found and by this time the photosynthetic lamellae had disappeared almost completely from the old chloroplasts /Fig. 6D/. The number of the newly-formed proplastids was not changed, or if there was an increase in their number it was only slight /Fig. 6E/.

Oxygen evolution and respiratory O_2 uptake during the early period of protoplast culture: An overall picture of the photosynthetic activities in protoplasts can be gained from the light-dependent oxygen evolution measured from the time of isolation until the 8^{th} day of incubation. There was an oscillation in the photosynthetic oxygen evolution, even though the colonies were maintained under continuous illumination /Fig. 7A/. The level of the respiration was not changed up to the 4^{th} day of the incubation, but after the 5^{th} day when the protoplasts started to divide, a considerable increase in the respiration was observed /Fig. 7B/.

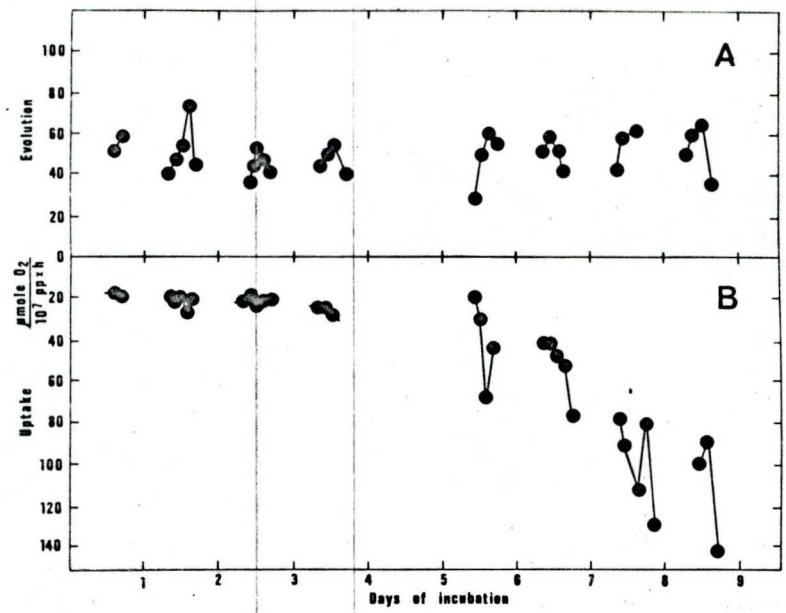

Figure 7. Photosynthetic oxygen evolution /A/ and respiration /B/ of protoplast culture during the first 8 days of their incubation

During the day the oscillation of the photosynthetic oxygen evolution exhibited a continuous increase during the morning and gradually decreased during the late afternoon hours /Fig. 8./. The maximal O_2 evolution was in the 13^{th} hour of the day. This type of periodical oscillation, known as the circadian rhythm, did not become damped but was maintained from the time of isolation up to the 8^{th} day of incubation /Fig. 7A/. Since the protoplasts exhibiting this circadian rhythm were cultured under continuous illumination, it was interesting to discover whether variation of the light and dark periods could have any influence on the oscillation of the photosynthetic oxygen evolution.

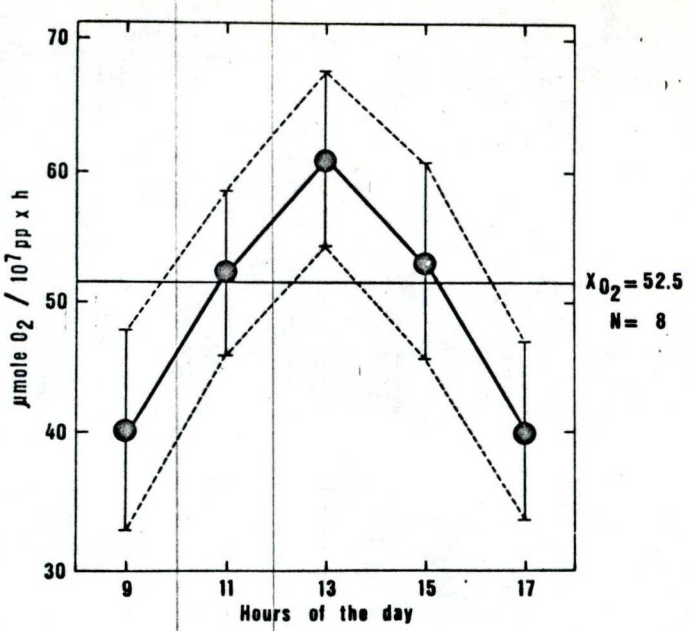

Figure 8. The circadian rhythm of O_2 evolution exhibited by the cultured protoplasts kept under continuous illumination. /Average of 8 series/

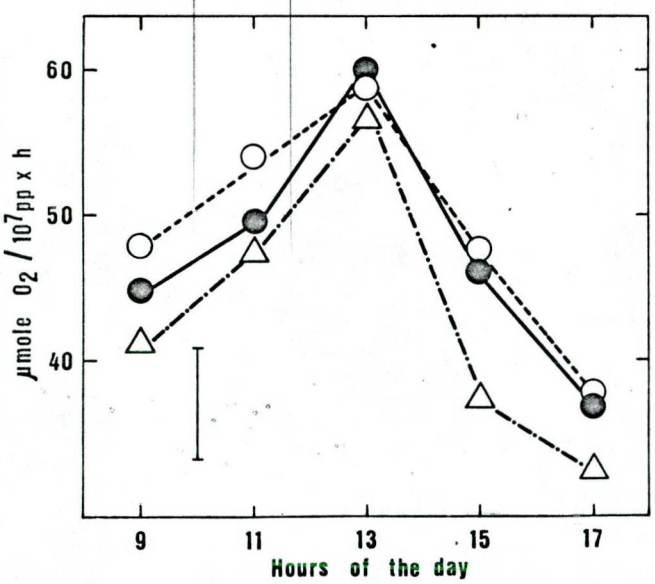

Figure 9. Effect of the variation of the light and dark periods on the photosynthetic oxygen evolution in protoplast culture. —●— control under continuous illumination. --o-- 12-hour-long light period /8^h to 20^h daily/ was followed by a 12-hour-long dark period during the night -·-Δ-·- 12-hours of darkness from 8^h to 20^h daily/ was followed a 12 hours of illumination during the night

As shown in Fig. 9. the sustained rhythm of oxygen evolution was obtained even when the cultures were incubated in an alternation from dark to light or from light to dark.

The daily average photosynthetic oxygen evolution, calculated on the basis of number of protoplasts, was at the same level on the 8th day of isolation as in the freshly isolated protoplasts.

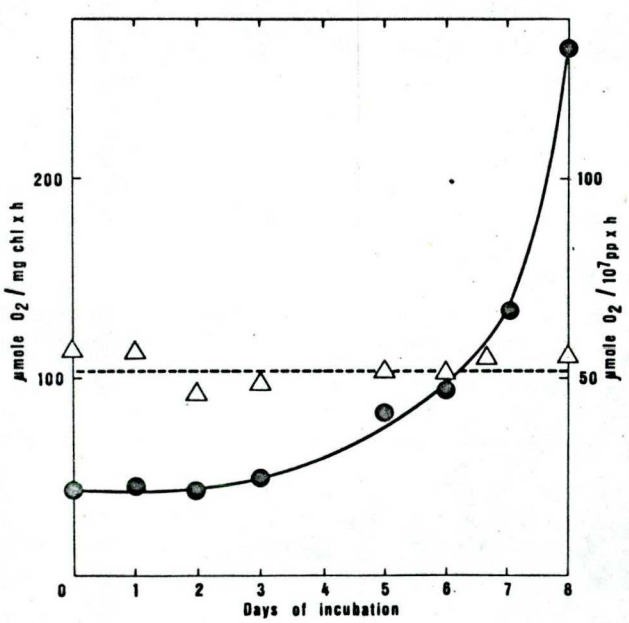

Figure 10. Changes of the daily average oxygen evolution of protoplast cultures calculated on a chlorophyll basis /—●— / or on the basis of number of the cultured protoplasts /--Δ--/

On the other hand if the oxygen evolution was calculated on a chlorophyll basis, a five-fold increase was shown in the rate of oxygen evolution /Fig. 10./. This demonstrates that the rate of oxygen evolution was not changed within the protoplasts, but the size of the photosynthetic units was much depleted during 8 days of protoplast incubation.

In contrast to the photosynthetic oxygen evolution, the respiration did not exhibit any circadian rhythm at all /Fig. 11/. The rate of respiratory O_2 uptake was unchanged during the period prior to cell divisions.

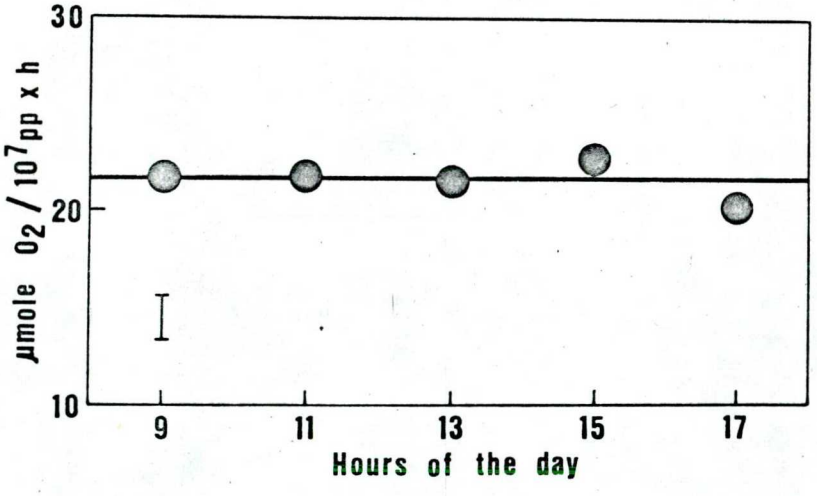

Figure 11. The rate of respirating O_2 uptake in protoplast culture measured at various hours of day /Average of 8 series/

Characterization of photosystem I during the early period of protoplast culture: The photosystem I was characterized by the chl/P 700 ratio of the chloroplasts isolated from protoplasts at different "ages" of culture. As shown in Fig. 12, the chl/P 700 ratio decreased continuously during the 8 day period of culture. This shows that degradation process occurs disproportionately in the light-

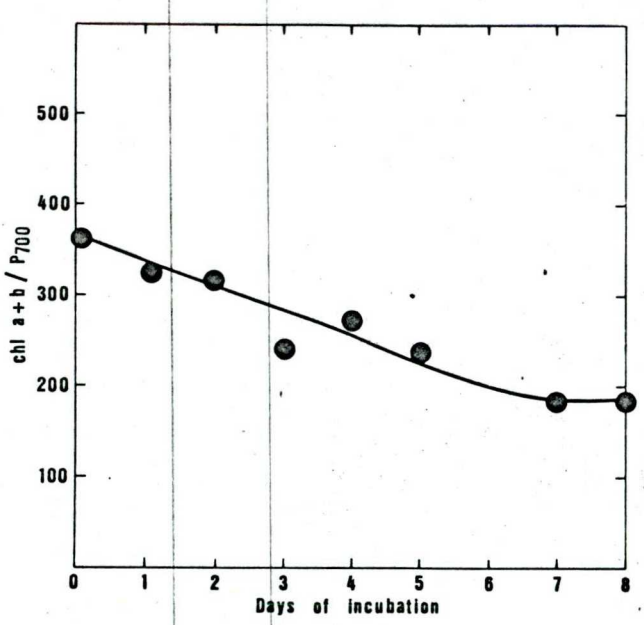

Figure 12. Change of the chl/P 700 ratio during the early period of protoplast regeneration

-harvesting pigment system. Comparing the decrease of the chl/P 700 ratio to the curve of chlorophyll degradation it can be seen that the reaction centres of photosystem I are also degraded, but to a lesser extent.

Characteristics of 515 nm absorbance changes during protoplast culture: In intact Class A chloroplasts prepared from protoplasts, the amplitude of the flash-induced 515 nm change was high and the kinetics of the signal was very complex. The 515 nm signal of Class A chloroplasts exhibited a biphasic rise: the initial very fast rise /rise time<1 msec/ was followed by a show increase of absorbance /rise time 10-20 msec/ /Fig. 13A/. The addition of 3-/3,4-dichlorophenyl/-1,1-dimethylurea inhibited the amplitude of the signal by about 50% /Fig. 13B/.

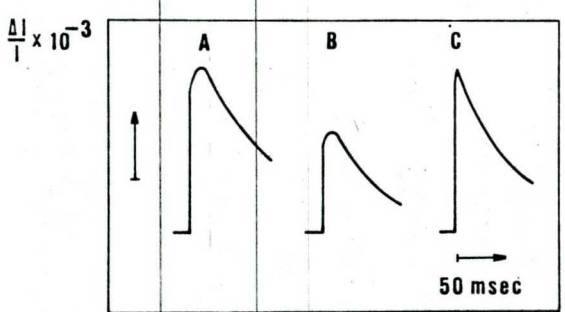

Figure 13. Oscilloscope display of flash-induced 515 nm absorbance changes of tobacco chloroplasts isolated from 2-day-old culture. A: control; B: 0.5 μM DCMU; C: 0.5 μM 3-/3,4-dichlorophenyl/-1.1-dimethylurea + 10 μM phenazine--methosulphate.

When phenazine-methosulphate was added to 3-/3,4-dichlorophenyl/-1,1-dimethylurea-treated chloroplasts the initial amplitude increased to the level of the controls and the slow rise was accelerated /Fig. 13C/. This PMS effect shows that the photosynthetic electron transport might be uncoupled to some extent like in chloroplasts aged in vitro /Roux and Faludi-Dániel 1977/.

The amplitude of the 515 nm signal calculated on a chlorophyll basis was constant during the 8 days of incubation, suggesting that the efficiency of chloroplast energization did not change during this period /Fig. 14A/.

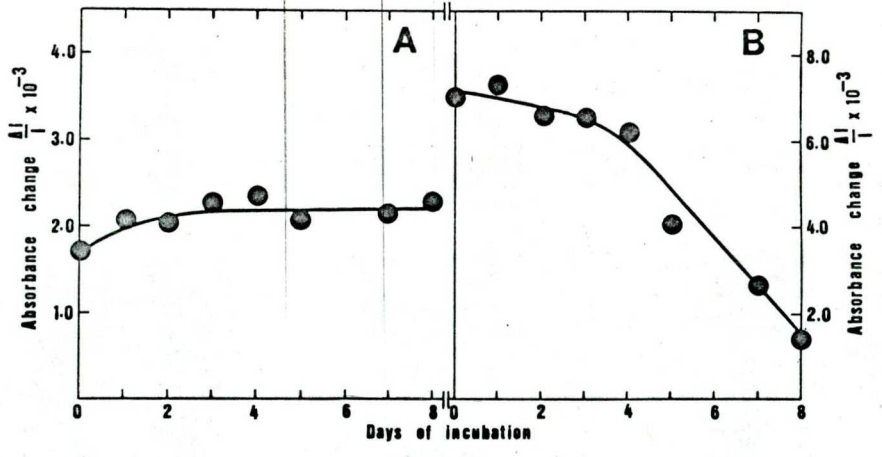

Figure 14. The effect of the age of protoplast culture on the amplitude of the 515 nm absorbance changes. An average of 100 transients were measured. A: amplitude of the signal calculated on a chlorophyll basis. B: adjustment of the optical density of the sample to 1.0 at 678 nm.

Referring the amplitude of the signal to the red maximum of the chloroplast suspension showed a decrease during the culture period /Fig. 14B/. This decrease might have been due to an increase in the nominal absorption at 678 nm caused by the enhanced starch accumulation.

DISCUSSION

Recent progress in the isolation and cultivation of plant protoplasts /Cocking 1972, Carlson et al. 1972/ warrants detailed studies on their physiological and biochemical properties. Studies on the photosynthetic activities of regenerating protoplast cultures are absent because most of the papers have dealt only with cell wall, colony and callus formation /Takebe et al. 1968, Takebe et al. 1971, Davey et al. 1974b/. These investigators found that cell division and colony formation was very much dependent on the composition of the culture medium /Nagata and Takebe 1970, Nagata and Takebe 1971/. This is in an agreement with our finding that tobacco mesophyll protoplasts start to divide earlier in the K_3 medium than in the N-T medium. After 2-3 weeks of culture the protoplasts grew into easily visible colonies of a yellow or creamy-brown color /Nagata and Takebe 1971/. The gradual decrease of the dark green colour of cultured mesophyll protoplasts is a result of the de-differentiation of the photosynthetic apparatus occurring during culture /Thomson and Davey 1975/, and/or a result of the repeated cell division, such that the mature chloroplasts can not maintain the plastid per cell ratio /Sjolund and Weier 1971/. The inbalance in the plastid per cell ratio may

result from either, the mature chloroplasts not multiplying /Usui and Takebe 1969, Nagata and Takebe 1970/ or from the division of chloroplasts being slower than the division of cells /Nagata and Yamaki 1973/. As was shown by Edelman and Hanson /1971/, the high sucrose concentration of the incubation medium reduced both chloroplast number and lamellar development. In our protoplast cultures, as shown by the micrographs, a decrease in chloroplast number per cell was also observed. The main feature, however, was structural degradation within chloroplasts. One or two days after isolation, intensive starch accumulation was observed in the chloroplasts, similar to the phenomenon decribed by Nagata and Yamaki/1973/, Nato et al./1977/, Davey et al./1974b/ and the chloroplast lamellae were strongly appressed. The intensive starch accumulation, which was forced by the high carbohydrate concentration of the culture medium, could partially destroy the lamellar system of the mature chloroplasts in tissue culture /Davey et al. 1974b/. On the 15th day of incubation only a very few appressed lamellae could be observed, and new proplastids appeared in the culture. In the 30-day-old culture the plastids contained starch only, with a few single lamellae, and the number of proplastids was increased. Davey and his co-workers /1974b/ found that the old plastids functioned as amyloplasts in the regenerating protoplasts. The

relatively rapid appearance /14 days/ of proplastids capable of rapid division may signify some restoration of the balance in the plastid per cell ratio /Sjolund and Weier 1971/.

In our protoplast cultures parallel with the ultrastructural changes an intensive destruction of pigments was observed. This pigment decomposition was influenced only slightly or not at all by the composition of the media. It was shown by measurement of the external pH that pigment destruction was not caused by the acidification of the medium. The decrease in the pigment content can be effected by the combination of cartain processes. /a/ Sucrose inhibits both chlorophyll and carotenoid synthesis /Laetch and Stetler 1965, Edelman and Hanson 1971, Pamplin and Chapman 1975/. /b/ The osmotic concentration of the medium influences pigment synthesis /Barg and Umiel 1976, Colman and Mawson 1978/./c/ As a result of their isolation as single cells, the light conditions for protoplasts become changed, and the high light intensity causes pigment destruction /Nagata and Takebe 1971, Horváth et al. 1971b/. /d/ Sugar-and starch-induced ultrastructural changes also have an effect on the pigment content /Nato et al. 1977/. These factors acting together may account for the progressive degradation of the photosynthetic pigments but the rate of pigment decomposition was so high in our case, that some additional factors may

also be involved. The characterictic change in the chlorophyll a/chlorophyll b ratio during incubation showed that the decomposition of the chlorophyll a was faster than of the chlorophyll b. Similar results were obtained by Davey et al. /1971/ during callus growth of Atropa belladonna. This may be due to the some destruction of lamellar protein which might influence the decomposition of the different chlorophyll forms. A detachment of the pigments from the lamellae might have occurred in the protoplast culture as well. This idea is supported by the changes observed in the chlorophyll/P 700 ratio.

The ultrastructural degeneration and pigment destruction may be explained by the factors discussed previously, but two other speculative explanations of chloroplast dedifferentiation are not sited out by our experiments.

According to one of these hypotheses, during dedifferentiation successive cell divisions /in mature cells/ result in the removal of inhibitors of gene action, until eventually derivative cells are taken to a "ground state". In this state the cells once again become able to express their totipotency, that is to exhibit specific morphogenetic responses. The degree of repression in vivo depends upon the position of the cells within the plant body, and therefore upon the particular functions which they are called upon to perform /Thomas and Davey 1975/. This view clearly implies that all differentiation is a process where-

-by the genetic information present in the zygote is progressively repressed but remains structurally unchanged. According to the other hypothesis, that of Sjolund and Weier /1971/ certain compounds that may be needed for the synthesis of new cytoplasmic material, for example amino acids and proteins, may be present in the chloroplasts but are in low concentration in the medium. The available pool of these compounds in the chloroplasts can be depleted and used to synthetize additional cytoplasmic material. As the rate of cytoplasmic synthesis decreases, the plastids can compete more successfully for the available compounds and re-form their membrane structures.

Interestingly enough the structural dedifferentiation occurring during the first period of protoplast regeneration was found not to be directly followed by changes in the characteristics of photosynthesis. The rate of the photosynthetic O_2 evolution calculated according to protoplast number did not change during 8 days of culture. However the amount of O_2 evolved calculated on a chlorophyll basis increased considerably which means that the size of the photosynthetic units /Schmid and Gaffron 1969/ was decreased and it was mainly the light-harvesting complex /Alberte and Thornber 1977/ which was destroyed /Chandler et al. 1972/. A similar result was

obtained by measurement of the changes of the chlorophyll/P 700 ratio during incubation. Calculating the amount of P 700 per protoplast culture showed a 30-40% degradation of the reaction centre of Photosystem I. The unchanged level of O_2 evolution per protoplast can be explained as due to the partial destruction of the two photosynthetic units being balanced by an increased electron transport rate caused by the uncoupling of electron transport and photophosphorylation /Reeves and Hall 1973/. This conclusion was confirmed by observing an increased level of respiration, which suggests that the decrease in photosynthetic ATP formation was compensated by an increase in oxidative phosphorylation. It has been shown with green cell suspension cultures that the rate of respiration increases considerably after subculture as cell division proceeds /Nato et al. 1977/ which may be a reflection of the shift from "reductive" /chloroplastic/ to "oxidative" /mitochondrial/ energetics /Chandler et al. 1972/.

During the measurement of O_2 evolution a circadian rhythmicity was observed. Circadian rhythms are biological oscillations displaying a 24 hour period under natural environmental conditions. Under constant experimental conditions /for example constant light/ the period of the rhythm usually changes slightly and stabilizes at a value more or less close to 24 hours. These rhythms are general-

ly supposed to reflect the behavior of an endogenous system, properly called a biological clock /Queiroz 1974/. However, neither the mechanism of the rhythm nor its physiological meaning have been elucidated yet /Okada et al. 1978/. Concerning higher plants it is curious that despite the extensive knowledge of the reactions of photosynthesis, only a small amount of information is published on the variations of carbon flow through those reactions with respect to the time of day /Noguchi and Tamaki 1962, Steer 1973/. In our case the cultivated protoplasts exhibited a daily oscillation in their photosynthetic O_2 production under continous illumination. The rate of O_2 evolution increased during the morning, reached a maximum at about noon and decreased steadily during the afternoon. The rhythm lasted several days in constant light and seemed to be endogenous, because it was not influenced by variations on a light-dark cycle. This type of rhythmicity seems to be a common feature of the photosynthesis of marine algae /Sweeney 1960, Driessche 1966, Karakashian and Hastings 1963, Schweiger et al. 1964, Mc Murry and Hastings 1972/. Recently Okada et al. /1978/ showed a circadian rhythm in the rate of oxygen evolution in the green marine alga Bryopsis maxima. This endogenous rhythm in photosynthesis seems to be due to changes in the chloroplast alone and to be independent

of other organelles in the cell. This is in an agreement with the finding in the present work that respiration /a mitochondrial function/ did not exhibit any circadian rhythm. Previously it was believed that the light reactions of Photosystem I and Photosystem II were not causes of the rhythmicity. /Queiroz 1974/. The circadian rhythm in the oxygen evolution of cultivated protoplasts seems to confirm the conclusion of Okada et al. /1978/ that some light reactions may participate in the time-keeping mechanism of the chloroplasts. We think that the isolated protoplast system would be an excellent one in which to study the circadian rhythmicity of higher plants.

In the chloroplasts of cultivated protoplasts the flash-induced 515 nm absorbance changes exhibited a biphasic rise similar to that of algae /Joliot and Delosme 1974/. Earlier it was thought that biphasic rise was characteristic only of algae /Junge 1974/. The biphasic rise of the 515 nm absorbance change exhibited by the chloroplasts of cultured tobacco protoplasts confirmes the recent observation that this kineticsis also a characteristic of higher plants /Horváth et al. 1978, Faludi-Dániel et al. 1978/. The amplitude of the absorbance changes calculated on a chlorophyll basis was constant during the 8 days of incubation. This suggests that the efficiency of the energization of the thylakoids was not

influenced by the cultivation of protoplasts and we can conclude that the residual thylakoids remained more or less functionally intact.

SUMMARY

In cultured protoplasts obtained from the mesophyll of tobacco leaves, structural and functional changes of chloroplasts were investigated.

Within a few days of incubation, a large proportion of the photosynthetic pigments was destroyed, and the chloroplast structure was degraded by an intense accumulation of starch in grains. These results confirm some earlyer data on single cell and/or protoplast cultures.

New results obtained in the present work:

Breakdown of the chloroplast structure continued for about 10-14 days. As callus formation proceeded, the cells containing old chloroplasts were able to form new proplastids, indicating a transition from a degenerative to a regenerative stage in the cell life.

Pigment destruction was not due to the acidification of the medium and was independent of the cell division.

As judged from the O_2 evolving capacity, P 700 oxidation and the 515 nm change, the degradation disproportionately afflicted the pigment antennae. The electron transport chain was less severely damaged.

Protoplast cultures showed a circadian rhythm in O_2 evolution which was preserved in the colonies. In contrast to the photosynthetic O_2 evolution, respiratory O_2 uptake

did not show any circadian rhythm and it was more related to the division of the cells.

Factors inducing pigment destruction and the mechanism of the biological clock contained in chloroplasts are subjects for further investigations.

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