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Accumulation of Chlorophyll, Chloroplastic Proteins, and Thylakoid Membranes during Reversion of Chromoplasts to Chloroplasts in *Citrus sinensis* Epicarp[1]

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ABSTRACT

***In vitro* culture of pericarp segments from fruit of *Citrus sinensis* (L.) Osbeck cv Valencia was used to determine the temporal sequence in development of chloroplasts from chromoplasts during regreening of the epicarp. Regreening of chromoplasts closely resembled greening of etioplasts, except that regreening proceeded much more slowly. Chlorophyll, the light-harvesting chlorophyll *a/b* binding protein of photosystem II, the chlorophyll *a* binding protein of reaction center P-700 of photosystem I, thylakoid membranes, and adenosine triphosphate synthetase were all detected at very low levels in degreened epicarp. All of these increased in parallel during regreening of the epicarp. Ribulose 1,5-bisphosphate carboxylase (RuBPCase) levels were high in degreened epicarp and declined for the first 10 days of culture before reaccumulating in the regreening segments. Light was necessary for the accumulation of all of the chloroplastic components. A lack of exogenous nitrogen did not prevent the accumulation of any chloroplastic component except RuBPCase, although accumulation of the other components was reduced. Sucrose at 150 millimolar in media lacking nitrogen markedly inhibited the accumulation of chlorophyll and light-harvesting chlorophyll *a/b*-protein.**

Redifferentiation of chromoplasts to chloroplasts has been observed in regreening of several plant organs (6, 9, 19) including the epicarp of *Citrus sinensis* (L.) Osbeck fruit (23). During the reversion of chromoplasts to chloroplasts, Chl accumulates and new thylakoid membranes are formed within the chromoplast. Plastids develop that resemble chloroplasts of immature green fruit, except that many of the plastoglobuli formed during differentiation of chloroplasts to chromoplasts are still present in the redifferentiated chloroplasts (23).

Development of chloroplasts in higher plants has most commonly been studied using etioplasts generated by growing seedlings in darkness. Etioplasts lack Chl, thylakoid membranes, the Chl *a/b*-binding protein of PSII (LHCP[3]; 7), and the Chl *a*-binding protein of reaction center P-700 of PSI (CPI; 26). Dark grown seedlings contain the chloroplastic proteins RuBPCase (18, 22, 27) and CF_1 (7). Upon exposure to light, etioplasts rapidly become photosynthetically competent chloroplasts, accumulating Chl, thylakoids, and new polypeptides including LHCP and CPI (1, 18, 26, 27).

Reversible interconversions between chloroplasts and chromoplasts can be conveniently studied by culturing pericarp segments from citrus fruit on agar media (11). Media with high sugar concentrations and lacking N promote differentiation of chloroplasts into chromoplasts, while media with low sugar concentrations or any of several sources of N promote redifferentiation of chromoplasts into chloroplasts.

This paper presents results of experiments using cultured segments of pericarp from *C. sinensis* (L.) Osbeck cv Valencia to determine the temporal sequence of appearance of Chl, thylakoids, and certain chloroplastic proteins during redifferentiation of chloroplasts from chromoplasts. Chl, LHCP, CPI, and thylakoid membranes were present in very low abundance in chromoplasts of *C. sinensis*. The reaccumulation of these components during development of chloroplasts from chromoplasts resembled chloroplast development from etioplasts, except for the time required. RuBPCase and CF_1 were present in chromoplasts of *C. sinensis* prior to regreening on agar media. RuBPCase levels decreased markedly for the first 10 d of culture, but reaccumulated in segments that regreened. Light was required for regreening and for the reaccumulation of RuBPCase after the initial loss. The absence of N from low sugar media reduced the accumulation of Chl, thylakoids, and Chl-binding proteins and completely prevented the reaccumulation of RuBPCase. High concentrations of sucrose (150 mM) in the absence of N inhibited the accumulation of LHCP and regreening, but this inhibition was largely overcome by the inclusion of KNO_3 in the culture medium.

MATERIALS AND METHODS

Pericarp Culture. Segments of pericarp 1 cm in diameter were prepared from fruit of *Citrus sinensis* (L.) Osbeck cv Valencia as previously described (11). The segments were kept on agar media described by Murashigi and Tucker (17), except that 60 mM KNO_3 was the sole source of N, the sucrose concentration was reduced to 15 mM, growth regulators were deleted, the fungicide benomyl (methyl 1-[butylcarbamoyl]-2-benzimidazole-carbamate) was added (11, 25), and the media were buffered at pH 5.5 with 150 mM Mes (adjusted with KOH). Segments were also cultured on similar media with N deleted, or with 150 mM sucrose.

Chlorophyll Determination. Chl in the epicarp of each segment was measured *in situ* by the difference in *A* at 675 and 735 nm

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[3] Abbreviations: LHCP, light-harvesting Chl *a/b*-protein; CPI, Chl *a* binding protein of reaction center P-700 of PSI; RuBPCase, ribulose 1,5-bisphosphate carboxylase; CF_1, α-subunit of adenosine triphosphate synthetase.

($\Delta A_{675-735}$) using an integrating sphere reflectometer attachment on a Bausch and Lomb model 340 spectrophotometer as previously described (11). Absorbance values were converted to nmol Chl using a standard curve made by comparing Chl extracted with dimethylformamide (16) from several segments with their mean $\Delta A_{675-735}$. By this method, we were able to efficiently measure Chl in individual regreening segments long before Chl could be detected by extraction from individual segments. Chl *a* to Chl *b* ratios were determined in regreened segments by extracting Chl in dimethylformamide and using the formulas developed by Moran (16) to calculate the abundance of each pigment.

Electron Microscopy. Sections of epicarp excised from the center of pericarp segments were fixed 1 h in 1% (w/v) glutaraldehyde in agarless culture medium and then transferred to agarless culture medium containing 3% (w/v) glutaraldehyde for 1 to 2 h. Samples were postfixed in 5% (w/v) $KMnO_4$ for 30 min, dehydrated with an acetone series, and embedded in Spurr's resin (20). Thin sections were mounted on carbon coated 200 mesh grids and stained 4 min with 10% (w/v) uranyl acetate followed by 2 min in 0.2% (w/v) lead citrate. Sections were viewed at 75 kV with a Hitachi H-500 electron microscope.

Thylakoid Membrane Estimations. Relative thylakoid membrane content of the plastids was estimated by measuring the total length of membranes in micrographs of 10 plastids at each sampling.

Water-Soluble and SDS-Soluble Protein Determinations. Subsets of four pericarp segments were randomly selected at each sampling and the epicarp removed from the mesocarp with a razor blade. Samples of epicarp were weighted (0.5–1.0 g) and ground in 25 ml sodium phosphate buffer (pH 7.8) with mortar and pestle, and the suspension centrifuged at 5000*g* and 0°C for 10 min. Supernatants were decanted, and the pellets rehomogenized three times in buffer, recentrifuged, and the supernatants combined with the original supernatant. The last supernatant contained no detectable protein. The remaining pellets were then ground three times in 10 ml of 0.5% (w/v) SDS followed by centrifugation at 5000*g* for 10 min at 0°C. Protein in the extracts was determined by the method of Lowry *et al.* (14) using BSA as a standard.

Protein Isolation and Polyacrylamide Gel Electrophoresis. The epicarp were removed from pericarp segments with a razor blade, weighed, and frozen in liquid N_2. The segments were lyophilized and stored at −20°C until used. Segments were homogenized with a Brinkman polytron in 3:1 (v:w) homogenization buffer (0.4 M sucrose, 10 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 100 mM Tris-HCl [pH 8.0]), filtered through two layers of cheesecloth, and centrifuged at 10,000*g* for 10 min. The supernatant was made 2% (w/v) SDS and frozen at −20°C until used. The pellet (containing the membrane fraction) was resuspended in homogenization buffer, brought to 2% (w/v) SDS, and frozen at −20°C until used. All samples were heated to 100°C for 1 min prior to gel electrophoresis.

Equal amounts of protein (40–50 μg per sample lane) were separated by electrophoresis through 7.5 to 15% gradient polyacrylamide gels in the presence of 0.1% SDS and then either stained with Coomassie brilliant blue R or transferred to cyanogen bromide paper for antibody hybridization as described by Mayfield and Taylor (15). The antisera to LHCP, CPI, CF_1, and RuBPCase were prepared from maize proteins as described by Harpster *et al.* (10). Amounts of individual proteins on blots were estimated by comparing densitometrically the autoradiograms of samples with a serial dilution of protein from the most regreened tissue. Exposures were chosen that remained within the linear range of the film, and the amount of protein expressed in relative units.

RESULTS

Chlorophyll Accumulates in Pericarp Segments Cultured in the Light. When *Citrus sinensis* pericarp segments, nearly devoid of Chl, were cultured 20 d under continuous fluorescent light they accumulated Chl in the epicarp to about 9 nmol/segment or 11 nmol/cm² (Fig. 1), whereas segments kept in the dark failed to accumulate Chl. For comparison, the Chl content of fruit left on the tree ranged from 3 to 12 nmol/cm². The ratio of Chl *a* to Chl *b* averaged 2.7 ± 0.1 in regreening segments. Segments kept under continuous light on media lacking N accumulated Chl at a slower rate than similar segments on media with nitrogen.

Thylakoid Membrane Accumulation Progressed with Chl Accumulation. Initially, chromoplasts in the epicarp of *C. sinensis* fruit were very similar to those in electron micrographs presented by Thomson *et al.* (23). The chromoplasts contained numerous large plastoglobuli and a few swollen, thylakoid fragments. Electron micrographs of plastids from epicarp of segments cultured in the light for 10, 20, and 26 d are shown in Figure 2. Between 5 and 15 d, plastids appeared similar to those at 10 d. Thylakoid membranes at 5 d were fragmentary and not well organized, but became progressively more orientated and longer by 10 and 15 d, although they remained swollen with no grana apparent. The greatest degree of organization occurred between 15 and 20 d. During this time, membrane appression occurred, the thylakoids orientated along one axis, and an extensive array of grana developed. By 20 d, the plastids resembled normal chloroplasts, except that small plastoglobuli still remained. These plastids were very similar to those observed by Thomson *et al.* (23) in the epicarp of fruit that had regreened *in situ*.

The relative abundance of thylakoids in reforming chloroplasts increased approximately 3-fold (Fig. 3) over 20 d of culture, after a slight decrease in thylakoid content during the initial 5 d. The thylakoid content of plastids in epicarp of segments kept on media lacking N failed to increase until after 10 d of culture.

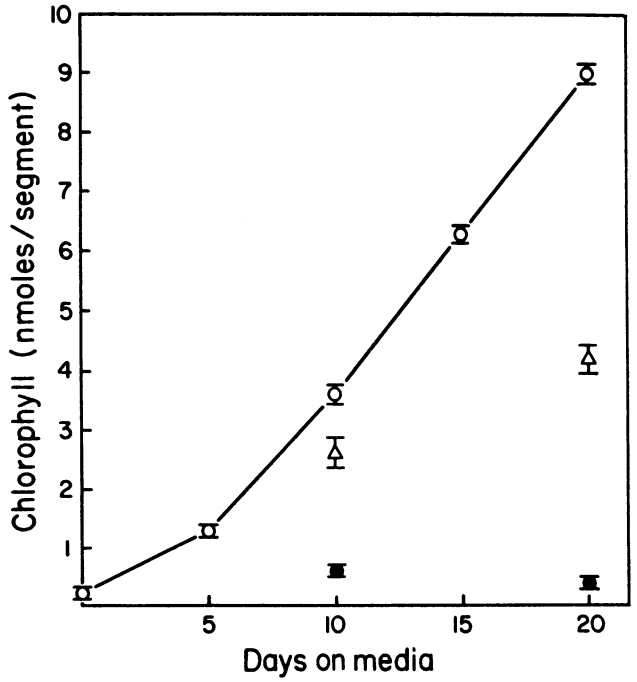

FIG. 1. Accumulation of Chl in epicarp of *C. sinensis* pericarp segments regreening *in vitro*. Pericarp segments were kept 20 d in the dark (●), under continuous fluorescent light between 4 and 12 W m^{-2} (○) and under continuous fluorescent light on media without nitrogen (△). Each value represents the mean ± SE of 10 individual segments.

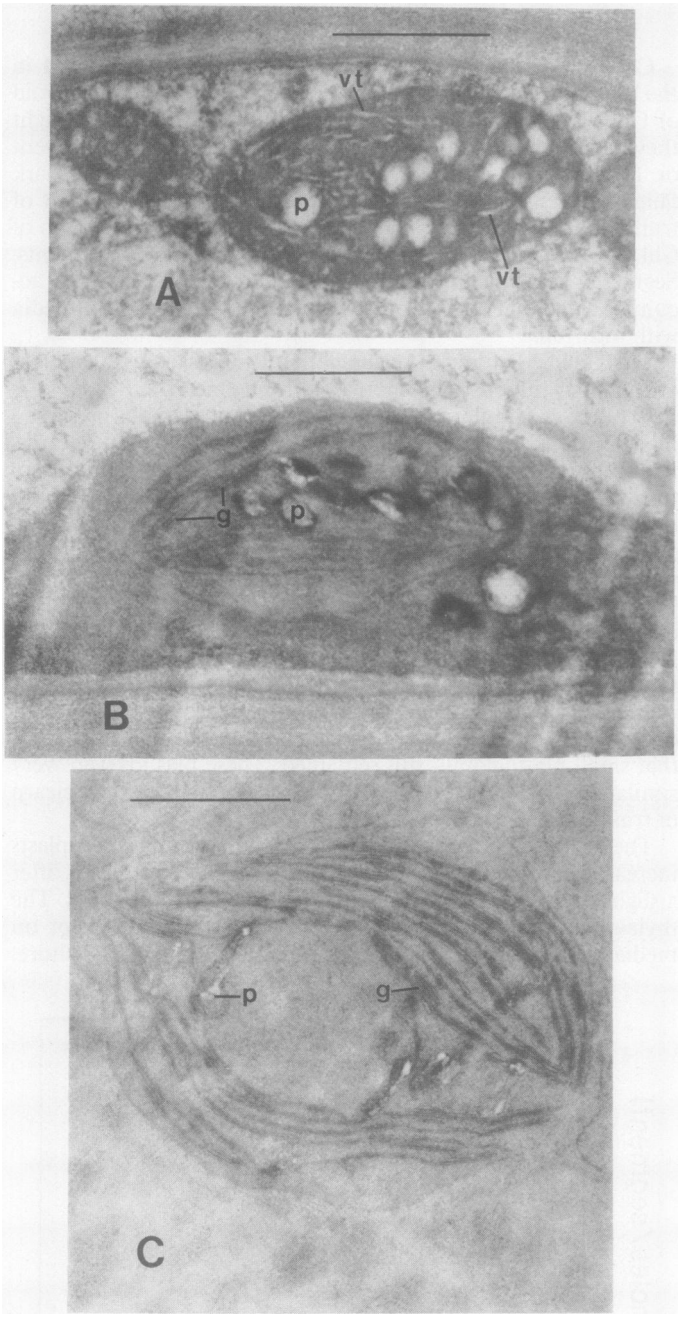

FIG. 2. Electron micrographs of plastids at different stages of reversion from chromoplast to chloroplast in epicarp of *C. sinensis* (L.) Osbeck peel segments. A, Plastid after 10 d. Thylakoids are vesiculated (vt) and interspersed among plastoglobuli (p), although somewhat orientated along one axis (× 24,000). B, Plastid after 20 d. Thylakoids are appressed, normal appearing, and grana (g) are present. Plastoglobuli are still present, but less well defined (× 24,000). C, Plastid after 26 d, with well defined thylakoids and grana. Plastoglobuli are very much reduced in size and number (× 24,000; bars = 1 μm).

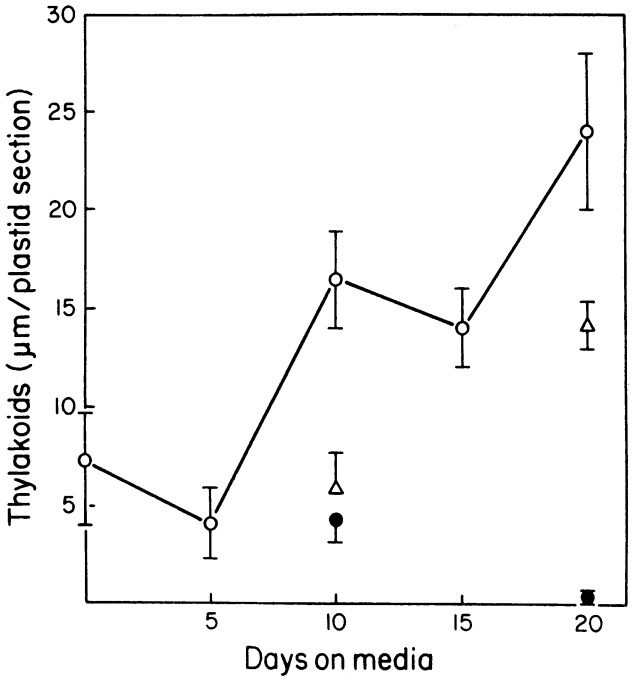

FIG. 3. Accumulation of thylakoid membranes in epicarp of *C. sinensis* pericarp segments regreening *in vitro*. Each value represents the mean ± SE of 10 plastids from 10 epicarp. Symbols as in Figure 1.

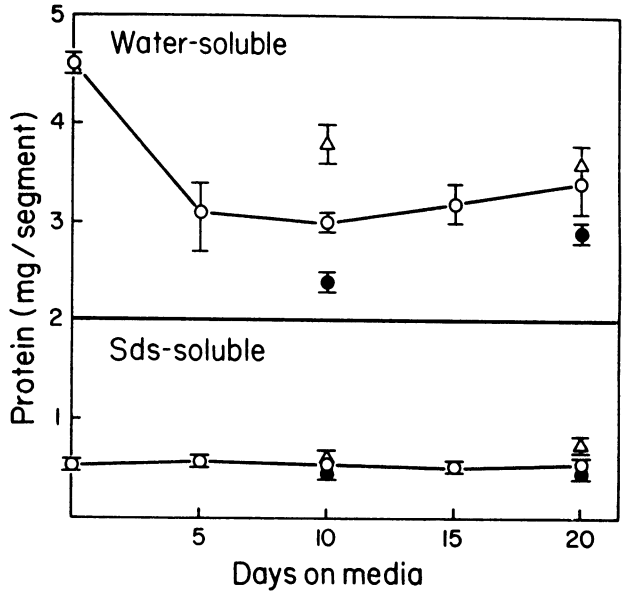

FIG. 4. Total water-soluble and SDS-solubilized protein in epicarp of *C. sinensis* pericarp segments regreening *in vitro*. Each value represents the mean ± SE of 3 subsets of epicarp. Symbols as in Figure 1.

After 10 d, the thylakoid content increased to about half the amount of segments kept on media with N. Epicarp plastids of segments kept in the dark on media with N lost thylakoid membranes throughout the study period.

Total Water-Soluble and SDS-Solubilized Protein Content. Water-soluble protein decreased for the first 5 d of regreening of pericarp segments kept in the light on media containing N (Fig. 4). After this initial loss, there was a slight increase in water-soluble protein. Somewhat more water-soluble protein was lost from the epicarp of segments cultured in the dark while a lack of N in the media tended to slow the initial loss of protein. The content of SDS-solubilized protein did not change significantly during the 20 d of culture, except for a slight increase in the epicarp of segments kept on media lacking nitrogen (Fig. 4).

PAGE Profiles of Membrane and Soluble Proteins. The PAGE profile of proteins isolated from epicarp segments prior to culture on agar media shows numerous proteins present in both the water-soluble (Fig. 5-I) and membrane (Fig. 5-II) fractions. Several proteins are present in both the membrane and water-soluble fractions of the uncultured segments that are not present in the

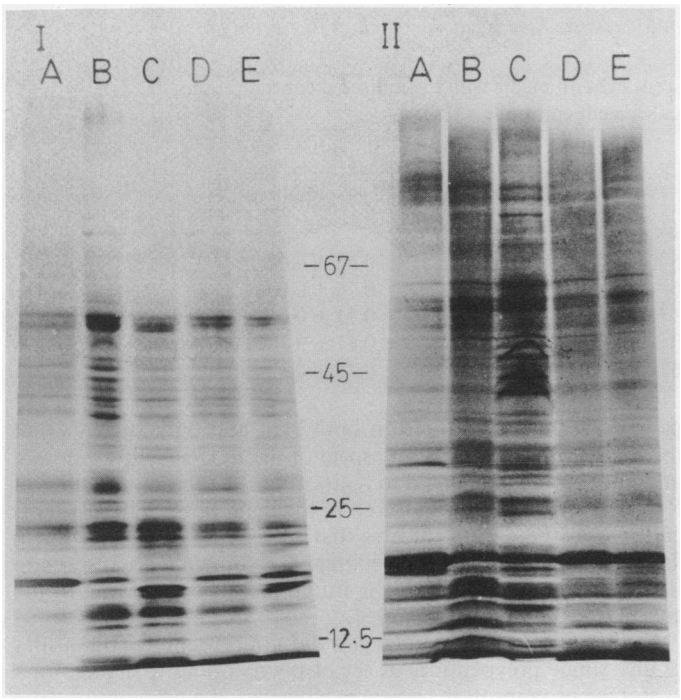

FIG. 5. PAGE of water-soluble (I) and membrane (II) proteins from *C. sinensis* epicarp during *in vitro* regreening on medium containing N. A, Initial; B, light, 10 d; C, light, 20 d; D, dark, 10 d; E, dark, 20 d. Equal amounts of protein were added to each lane.

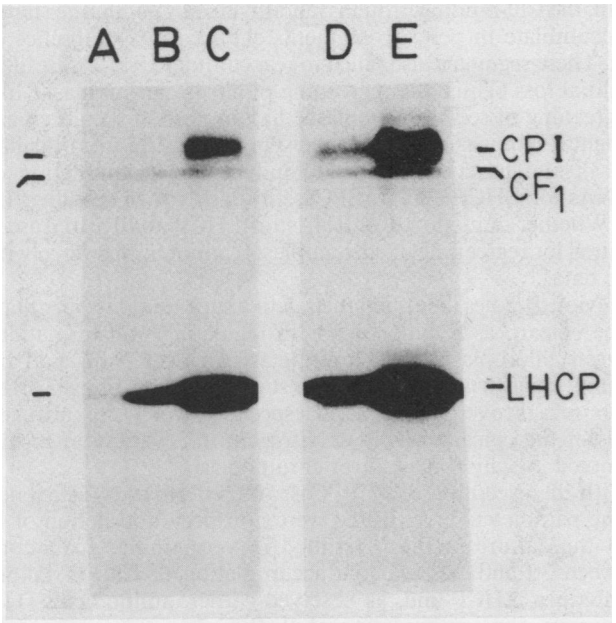

FIG. 6. Protein blot of membrane proteins separated by PAGE. The blot was probed with antisera raised against LHCP, CPI, and CF_1. Protein isolated from epicarp of *C. sinensis* pericarp segments before (A) and after regreening 10 d (B) and 20 d (C) on medium lacking N, and 10 d (D) and 20 d (E) on medium containing 60 mM KNO_3. Equal amounts of protein were added to each lane.

segments after regreening, and also several protein bands that are present in the regreened tissue, such as the LHCP protein (Fig. 6), are not apparent in the uncultured segments. The majority of the protein bands in both the membrane and water-soluble protein fractions appear to be similar in quantity in both uncultured and cultured epicarp segments.

Chl-Binding Proteins Accumulate during Regreening of Cultured Pericarp. Low levels of LHCP were detected in epicarp of segments prior to culture, but CPI was not detected. The amount of both LHCP and CPI increased several-fold in the epicarp of segments cultured in the light (Fig. 6). LHCP increased about 60-fold and in close parallel to the increase in Chl over the study period, while segments cultured in the dark failed to accumulate LHCP (Fig. 7). Segments cultured in the light for 20 d in the absence of nitrogen had significantly less Chl-binding protein than segments cultured on media with N (Figs. 6, 7). Accumulation of LHCP and Chl was strongly inhibited by 150 mM sucrose in media lacking N (Table I). This inhibition was overcome by inclusion of 60 mM KNO_3.

RuBPCase and CF_1 Content. The epicarp of *C. sinensis* contained the chloroplastic enzymes RuBPCase and ATPase prior to culture on agar media (Figs. 6 and 8). The level of RuPBCase decreased for the first 10 d in segments cultured in the light or dark and in the presence or absence of N (Fig. 8). Segments cultured in the light on media containing N reaccumulated levels of RuBPCase equal to those in chromoplast prior to culture, while segments cultured in the dark or in light on media lacking N failed to reaccumulate RuBPCase. The levels of CF_1 remained fairly constant in segments cultured in the light (Fig. 6), but decreased during 20 d culture in the dark (data not shown).

DISCUSSION

Examination of the electron micrographs confirms that regreening of *Citrus sinensis* (L.) Osbeck cv Valencia epicarp *in vitro* closely resembles natural, *in situ* regreening as described by Thomson *et al.* (23). During regreening, chloroplasts appeared to be formed by reversion of chromoplasts. New thylakoids, which eventually organized into well developed grana membranes, appeared as invaginations of the plastid membrane. Plastoglobuli, formed in chromoplasts during degreening, were reduced in number and size after extensive regreening, but were not entirely absent from reformed chloroplasts.

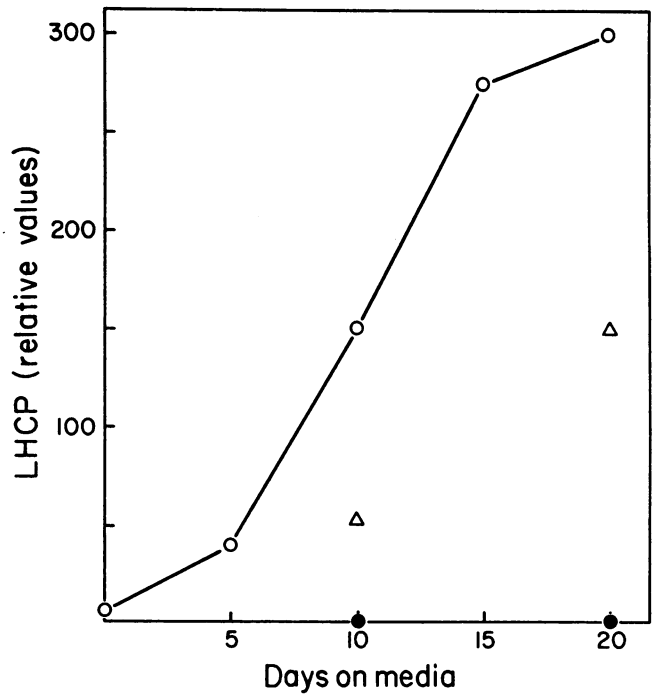

FIG. 7. Relative abundance of LHCP per equivalent amount of SDS-solubilized protein from the epicarp of *C. sinensis* pericarp regreening *in vitro*. The amount of protein was estimated as described in "Materials and Methods." Symbols as in Figure 1.

Table I. *Sucrose Effects on Chl, Proteins and LHCP in C. sinensis Epicarp during 20 days Regreening in Vitro*

Segments were cultured 20 d under continuous fluorescent light on media with the indicated concentrations of KNO_3 and sucrose. Each value is the amount (±SE) per segment epicarp.

Addition to Medium		Chl	LHCP	Protein Fractions	
KNO_3	Sucrose			Water-soluble	SDS-solubilized
mM		*nmol*	*relative*	*mg*	
initial		0.2 ± 0.1	5	4.8 ± 0.1	0.54 ± 0.04
0	15	4.2 ± 0.2	206	3.6 ± 0.1	0.74 ± 0.09
0	150	1.1 ± 0.2	51	4.1 ± 0.2	0.71 ± 0.06
60	150	8.2 ± 0.2	310	5.0 ± 0.2	0.67 ± 0.10
60	15	9.0 ± 0.2	306	3.8 ± 0.4	0.55 ± 0.12

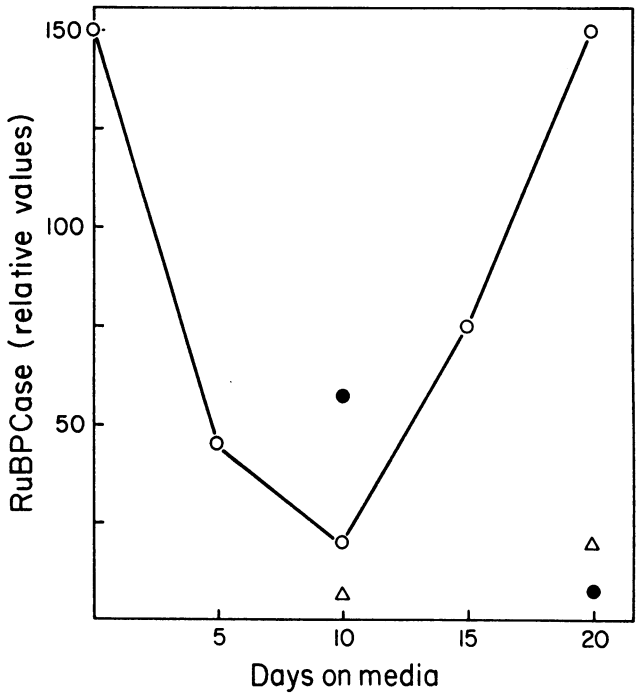

FIG. 8. Relative abundance of RuBPCase per equivalent amount of water-soluble protein from epicarp of *C. sinensis* pericarp regreening *in vitro*. The amount of protein was estimated as described in "Materials and Methods." Symbols as in Figure 1.

Reversion of chromoplasts to chloroplasts also resembles greening of seedling etioplasts, except that regreening of citrus epicarp proceeds at a much slower rate. Citrus regreening required 20 d *in vitro* and several weeks *in situ* (12) to reach Chl levels attained after only a few hours of etioplast greening (4). At the time of culture, Chl, thylakoid membranes, and Chl-binding proteins were all detected in epicarp chromoplasts, although at low levels compared with regreened tissue. In segments cultured in continuous light, Chl, thylakoids, LHCP, and CPI all increased several-fold over the study period. Chl and LHCP increased in very close parallel; however, the thylakoid membrane content may not have increased until after a 5-d delay, and LHCP accumulated for 15 d before appression and stacking took place. During greening of etiolated seedlings, thylakoid stacking was closely associated with the appearance of LHCP (2, 5, 21).

The chloroplastic proteins RuBPCase and ATPase were present in epicarp chromoplasts prior to culture, as in etioplasts of maize and cucumber (18), again suggesting that chromoplasts and etioplasts have some similarities. However, during the first 10 d of regreening of citrus epicarp, the level of RuBPCase decreased while RuBPCase rapidly accumulated during greening of etiolated seedlings (18). The initial loss of RUBPCase in pericarp segments cultured in both light and dark suggests that the loss of this enzyme is an aspect of the *in vitro* culture system and not specifically related to regreening. The reaccumulation of RuBPCase in segments cultured in the light, but not in segments cultured in the dark, indicates that regreening of citrus epicarp and greening of etiolated seedlings are similar with respect to RuBPCase accumulation if the initial loss of the enzyme in the cultured segments is discounted.

Examination of the stained gels did not reveal large quantities of any single polypeptide, or set of polypeptides that might act as storage proteins in the epicarp. However, the presence of large amounts of RuBPCase in the chromoplasts, which initially decreased before reaccumulating, suggests that this protein might act as a storage protein in citrus chromoplasts. The initial decline in total water-soluble protein content during regreening *in vitro* could, in part, be accounted for by this loss of RuBPCase.

Chl, the Chl-binding proteins, and thylakoid membranes failed to accumulate in pericarp segments cultured in the absence of light. These segments also failed to reaccumulate RuBPCase after its initial loss. Thus, the regreening of citrus chromoplasts, like the greening of seedling etioplasts, has an absolute requirement for light. Light is necessary for the synthesis of Chl from Pchlide (8, 13), and has been shown to induce the accumulation of mRNAs for LHCP and RuBPCase in dark grown seedlings (3, 24). Whether induction of mRNAs for LHCP and RuBPCase is required for regreening of chromoplasts cannot be answered with these data.

Previous studies identified the relative sugar and nitrogen state of the epicarp as a major factor in regulating transformations between chloroplasts and chromoplasts in degreening and regreening citrus fruit (11, 12). High sugar concentrations favored chromoplasts over chloroplasts especially when the nitrogen status of the epicarp was low. Nitrogen, in a variety of forms, promoted the chloroplasts over chromoplasts.

With the exception of RuBPCase, the effects of N deficiency on the parameters investigated were nonspecific. Deletion of N from the culture medium resulted in very similar reductions (between 30 and 50%) in the accumulation of Chl, thylakoid membranes, LHCP and, as observed earlier, amino acids (11). In contrast, the accumulation of RuBPCase was very slight in the absence of exogenous N. This differential effect of N deficiency on the appearance of RuBPCase and LHCP may reflect differences in N availability in the plastid and cytoplasm, and the need for plastid polypeptide synthesis for RuBPCase and not for LHCP synthesis.

From our limited data on Chl and LHCP, high sucrose concentrations appear to enhance the effects of N deficiencies. Reductions in the rate of accumulation of Chl and LHCP by deletion of N from the media were greatly magnified by increased sucrose concentrations. This effect was completely overcome by inclusion of 60 mM KNO_3.

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