Plant Physiol. (1983) 73, 243–249
0032-0889/83/73/0243/07/$00.50/0

# Nutritional Control of Regreening and Degreening in Citrus Peel Segments[1]

Received for publication December 27, 1982 and in revised form April 27, 1983

ALBERT HUFF
*Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721*

## ABSTRACT

**A method for reversibly regreening and degreening citrus epicarp *in vitro* using peel segments was developed.**

**Peel segments from mature degreened fruit promptly regreened when kept in light upon agar medium containing low (15 millimolar) concentrations of sucrose. Higher concentrations of sucrose inhibited this regreening, but $NO_3^-$ and certain amino acids included in the media overcame the inhibition by sucrose. However, L-serine strongly inhibited regreening. In the presence of nitrogen, sucrose promoted regreening.**

**Peel segments from green fruit remained green on media with low concentrations of sucrose and on media with high concentrations of sucrose and 60 millimolar $KNO_3$, but degreened in response to high concentrations of sucrose in the absence of nitrogen. Nitrate overcame the degreening effects of high sucrose concentrations in both light and dark. Peel segments were reversibly degreened and regreened by transferring the segments between appropriate media.**

**Nitrate in the media markedly reduced the levels of endogenous sugars in the epicarp and increased endogenous amino acid levels. Sucrose in the media increased endogenous sugar levels and, in the presence of nitrate, increased endogenous amino acid levels. In the absence of nitrogen, high sucrose concentrations reduced endogenous amino acid concentrations.**

Plastids in higher plants exist in a wide variety of forms that can, to some degree, interconvert (20, 30). Major questions of plastid biology are what factors determine the form of a plastid in a plant part at any particular time and what factors bring about transformations from one form to another. In this regard, the development of chloroplasts from proplastids and etioplasts is well studied and several reviews are available (20, 24, 30), whereas transformations between other plastid forms have been less well studied. In some plants, apparently reversible interconversions between chloroplasts and chromoplasts have been noted (9, 13, 14, 31) that might be well suited for studying those factors that determine between these two plastid forms.

Citrus fruit degreen during winters in response to cool air and soil temperatures below 13°C (35). Some late season fruit, most notably Valencia oranges (*Citrus sinensis* [L.] Osbeck), left unharvested into late spring and summer will regreen commencing at the stem end, and in response to the warmer temperatures (5). This regreening of the epicarp seems to be a partial reversion of chromoplasts to chloroplasts rather than formation of new chloroplasts from proplastids; a conclusion based upon the appearance of plastid forms intermediate between chromoplasts and chloroplasts, and the absence of any proplastid-like forms (31). Similar observations have indicated chromoplasts to chloroplast transformations in the root cortex of *Daucus carota* L. (13), the sepals of *Nuphar luteum* Sibth et Sm. (14), *Chrysosoplenium alternifolium* and *Chr. oppositifolium* (29), the spathe of *Zantedeschia elliottiana* Eng. (14), and subepidermal tissues of *Cucurbita pepo* fruit (9).

In addition to temperature, the degreening and regreening of citrus fruit are affected by nitrogen fertilization (18, 19), exogeneously applied gibberellins (7), and certain internal factors such as variety, rootstocks, and number of seeds per fruit (11).

An *in vitro* method of studying this interconversion of chromoplasts and chloroplasts in citrus peels has been developed and a preliminary report presented (15). Further *in vitro* studies have shown that the degreening and regreening of citrus peels can be regulated nutritionally. An abundance of nitrogen promotes the chloroplast form, whereas an abundance of sugars promotes the chromoplast form. A nutritional explanation for the degreening and regreening of certain citrus fruit is proposed.

## MATERIALS AND METHODS

Fruit of *Citrus sinensis* L. Osbeck (cv Valencia) and *C. paradisi* Macf. (cv Marsh) were surface-sterilized by a 5-min soak in 1% (w/v) NaOCl and rinsed in sterile water. A wide section of peel was cut from the equatorial region (usually) with a razor blade and held submerged in a shallow tray of sterile water while discs were cut out with a 10-mm-diameter cork borer.

Peel segments thus prepared were placed epicarp side up on 8 ml of agar media in covered 18- × 150-mm culture tubes and placed under continuous fluorescent lighting (Sylvania Cool White) of between 4 and 12 w $m^{-2}$ the following morning. Temperatures were kept between 29 and 30°C. Basal medium (B) was that of Murashigi and Tucker (23), except that growth regulators, $NH_4NO_3$, $KNO_3$, and sucrose were deleted, agar was increased to 1.5% (w/v), and the fungicide benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate] was added as described by Vakis *et al.* (32) to reduce the incidence of molds that are difficult to eliminate by surface sterilization. Recently, 150 mM Mes buffer (pH 5.5) has been added to the media. Also, many segments will not survive if the total solute concentration is below about 250 mM.

To evaluate the nutritional requirements for regreening of peel segments, elements and vitamins were selectively deleted from medium B with 20.6 mM $NH_4NO_3$, 18.8 mM $KNO_3$, and 146 mM sucrose added. In nitrogen-deficient media, potassium was restored with $KHCO_3$ and in potassium-deficient media, phosphorous and nitrogen were restored with 1.25 mM $NH_4H_2PO_4$ and 8.77 mM $NH_4NO_3$. Chloride and sulfate ions were restored with KCl and $K_2SO_4$ in Ca- and Mg-deficient media, respectively, and Mg, Zn, and Mn were restored as chloride salts in media with sulfate ions deleted. In phosphorous-deficient media, $KH_2PO_4$ was replaced with KCl. Other elements were deleted

[1] Paper No. 3701, Arizona Agricultural Experiment Station, University of Arizona.

without restoration of counter ions.

Relative Chl content of the peel segments was determined *in situ* on subsets at the time they were placed on media and again after 7 and 14 d by measuring the difference in $A$ at 675 and 735 nm ($\Delta A_{675-735}$) using an integrating sphere reflectometer attachment to a Bausch and Lomb model 340 spectrophotometer (16). This allowed estimations of Chl content in single 10-mm-diameter segments at a level well below that detectable by extraction, and enabled routine Chl estimations to be made quickly and efficiently for hundreds of discs. This was especially important for regreening peel segments. However, at high Chl concentrations, the sensitivity of the *in situ* measurements decreased making them less useful for degreening studies. Generally, each reported value represents the mean $\Delta A_{675-735}$ of a 10-segment subset.

To determine the sugar and amino acid contents, epicarps (0.4–1.2 g) were separated from mesocarps of three or four peel segments, and homogenized in 80% ethanol with mortar, pestle, and sand. These homogenates were transferred to 50-ml centrifuge tubes, centrifuged 20 min at 12,100$g$ and 0°C, and the supernatant collected. The residue was rinsed three times with 80% (v/v) ethanol, centrifuging between rinses, and each rinse added to the original supernatant.

Most ethanol was removed from the combined supernatants by evaporating to less than 20 ml and diluting to 100 ml with deionized $H_2O$. One-ml aliquots were then assayed for reducing sugars by Cu (II) reduction (22) and assayed for sucrose by the increase in reducing sugars after treating a 1-ml aliquot with 0.86 IU of invertase for 2 h at room temperature. Amino acids were assayed in 1-ml aliquots by the method of Yemm and Cocking (34).

## RESULTS

**Regreening of Citrus Epicarp *In Vitro*.** Segments of peel from *C. sinensis* fruit regreened somewhat linearly for 10 d when kept under continuous fluorescent light on B media with $NH_4NO_3$, $KNO_3$, and sucrose added (Fig. 1). In common with other greening phenomena, the regreening of citrus epicarp required light and none occurred in the dark. Maximum regreening during 13 d under continuous fluorescent light required between 2 and 4 w $m^{-2}$ and higher light intensities up to about 12 w $m^{-2}$ had no further effects (Table I).

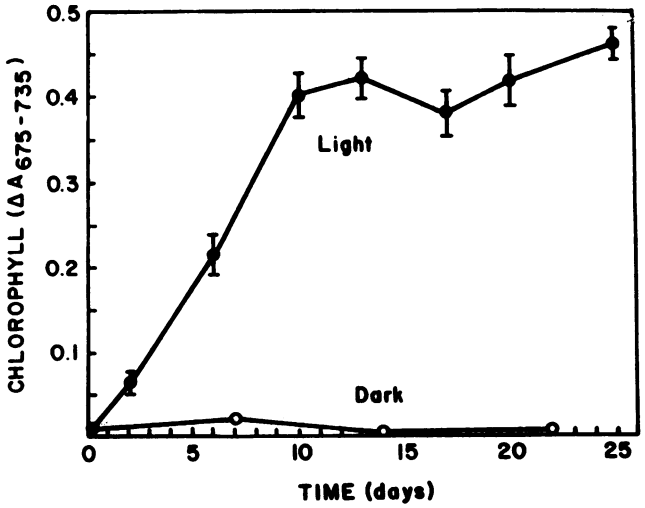


FIG. 1. Regreening of *C. sinensis* peel segments *in vitro*. Peel segments were placed on B media with 146 mM sucrose, 20.6 mM $NH_4NO_3$, and 18.8 mM $KNO_3$. Segments were kept in darkness or under continuous fluorescent light. Each value represents the mean ± SE of 10 segments.

Table I. *Light Intensities Required for Maximum Regreening*

Segments of *C. sinensis* peel were kept 13 d under continuous fluorescent light and on B media with 30 mM $NH_4NO_3$ and 37 mM sucrose. Each Chl value represents the mean of 10 ± SE.

| Light Intensity | Chl |
|---|---|
| w $m^{-2}$ | $\Delta A_{675-735}$ |
| 0 | 0.12 ± 0.01 |
| 2.0 | 0.38 ± 0.02 |
| 4.0 | 0.45 ± 0.01 |
| 8.4 | 0.44 ± 0.02 |
| 11.7 | 0.41 ± 0.03 |

Table II. *Nutritional Requirements for Regreening of Citrus Peel Segments*

*C. sinensis* peel segments were kept 13 d under continuous fluorescent light on B media with 20.6 mM $NH_4NO_3$, 18.8 mM $KNO_3$, and 146 mM sucrose, and similar media with the indicated deletions. Each value represents the mean of 10 ± SE.

| Deleted Components | Chl |
|---|---|
| | $\Delta A_{675-735}$ |
| None | 0.42 ± 0.01 |
| Nitrogen | 0.09 ± 0.02 |
| Iron | 0.41 ± 0.01 |
| Potassium | 0.52 ± 0.02 |
| Magnesium | 0.50 ± 0.04 |
| Calcium | 0.46 ± 0.03 |
| Phosphorous | 0.41 ± 0.01 |
| Sulfur | 0.42 ± 0.01 |
| Glycine | 0.48 ± 0.01 |
| Inositol | 0.46 ± 0.03 |
| Nicotinic acid, pyridoxine, and thiamine | 0.49 ± 0.01 |
| Minor elements | 0.45 ± 0.05 |

**Nutritional Requirements for Regreening.** When segments of *C. sinensis* peel were kept 13 d on complete media with 146 mM sucrose, and media with various components deleted (singly or in combination), only nitrogen was observed necessary for regreening (Table II). Deletion of any other component from the media did not significantly reduce the degree of regreening compared with segments on complete media. In addition, segments of *C. sinensis* and *C. paradisi* peel have regreened on media containing only sucrose and $KNO_3$, with all other components deleted (*e.g.* Fig. 5).

Inasmuch as nitrogen was apparently both necessary and sufficient for regreening of citrus epicarp, experiments were conducted to determine the efficacy of selected inorganic and organic sources of nitrogen. *C. paradisi* peel segments readily regreened on media with 60 mM N supplied as nitrate salts (Table III), although salts of the divalent cations $Ca^{2+}$ and $Mg^{2+}$ elicited somewhat more regreening than salts of monovalent cations. In contrast, 60 mM $NH_4Cl$ was a poor source of nitrogen and did not promote regreening. In addition, $NH_4^+$ appeared at 7 d to have inhibited regreening of segments on media with 30 mM $NH_4NO_3$; however, after 14 d, similar segments contained only slightly less Chl than segments on media containing other monovalent salts.

L-Glutamine was the most effective source of nitrogen used to promote regreening, while L-glutamate and L-alanine were only slightly less effective (Table III). Glycine and L-arginine behaved much as $NH_4NO_3$. By 7 d, regreening of peel segments on media supplemented with either of these amino acids lagged considerably behind other peel segments, but by 14 d had nearly caught up with those on media with $KNO_3$. L-Asparagine promoted regreening of segments to only about one-third that of L-gluta-

Table III. *Regreening of C. paradisi Peel Segments with Various Sources of Nitrogen*

Peel segments were kept 7 and 14 d under continuous fluorescent light on B media including 150 mM sucrose and 100 mM Mes (pH 5.5) plus the indicated nitrogen source and mannitol to maintain constant solute concentrations for each experiment. Amino acid solutions were filter-sterilized and added to autoclaved media. Each value represents the mean of 10 ± SE.

| Nitrogen Source | Chl | |
|---|---|---|
| | 7 d | 14 d |
| Experiment a | $\Delta A_{675-735}$ | $\Delta A_{675-735}$ |
| Initial | 0.005 ± 0.002 | |
| None | 0.08 ± 0.01 | 0.13 ± 0.03 |
| $KNO_3$ (60 mM) | 0.19 ± 0.01 | 0.35 ± 0.02 |
| $NaNO_3$ (60 mM) | 0.20 ± 0.01 | 0.33 ± 0.02 |
| $NH_4NO_3$ (30 mM) | 0.09 ± 0.01 | 0.30 ± 0.02 |
| $NH_4Cl$ (60 mM) | 0.05 ± 0.01 | 0.12 ± 0.02 |
| $Ca(NO_3)_2$ (30 mM) | 0.23 ± 0.01 | 0.41 ± 0.02 |
| $Mg(NO_3)_2$ (30 mM) | 0.19 ± 0.02 | 0.45 ± 0.03 |
| Experiment b | | |
| Initial | 0.009 ± 0.002 | |
| None | 0.04 ± 0.01 | 0.08 ± 0.02 |
| $KNO_3$ (60 mM) | 0.18 ± 0.01 | 0.34 ± 0.01 |
| L-Glutamate (60 mM) | 0.13 ± 0.01 | 0.43 ± 0.03 |
| L-Alanine (60 mM) | 0.19 ± 0.02 | 0.41 ± 0.03 |
| L-Glutamine (60 mM) | 0.15 ± 0.01 | 0.54 ± 0.03 |
| L-Asparagine (60 mM) | 0.08 ± 0.01 | 0.18 ± 0.03 |
| Glycine (60 mM) | 0.06 ± 0.01 | 0.35 ± 0.02 |
| L-Arginine (60 mM) | 0.07 ± 0.01 | 0.30 ± 0.03 |
| L-Serine (60 mM) | 0.015 ± 0.004 | 0.027 ± 0.003 |

mine.

L-Serine was not only the poorest source of nitrogen used, but appeared to be inhibitory to regreening. To determine if serine was indeed inhibitory, *C. paradisi* peel segments were cultured on B media with 15 mM sucrose, 60 mM $KNO_3$ as a source of nitrogen, and L-Serine. L-Arginine was also included because arginine was reported to antagonize the promotion of oat leaf senescence by serine (26). In these experiments (Table IV), serine strongly inhibited regreening, while arginine enhanced, rather than diminished, the effects of serine.

**Sugar Inhibition of Regreening.** The preceding experiments, which defined a requirement for nitrogen, were done with media containing about 150 mM sucrose. When segments were later kept on media with only 15 mM sucrose, it was found that there was less need for exogenous nitrogen and that considerable regreening took place in absence of nitrogen in the media. In fact, regreening of peel segments from both *C. sinensis* and *C. paradisi* fruit on media without nitrogen was progressively more inhibited by sucrose concentrations up to about 150 mM (Fig. 2). Beyond that concentration, there was little further inhibition.

To be certain that the observed inhibition was due to the specific action of sugars and not an osmotic effect, *C. paradisi* peel segments were cultured on media with increasing concentrations of mannitol (Table V). Solute concentrations of up to 500 mM (348 mM mannitol) had little effect on regreening; however, between 500 and 600 mM there was a slight decrease in the amount of regreening. Therefore, the results shown in Figure 2 are not due to an osmotic effect.

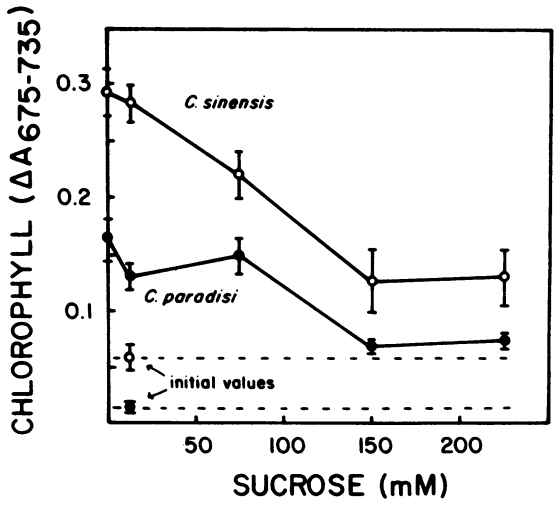


FIG. 2. Inhibition of regreening by sucrose in the absence of nitrogen. Chl was determined in peel segments kept 14 d under continuous fluorescent light, on B media with 150 mM Mes (pH 5.5) and the indicated concentrations of sucrose. Broken lines represent Chl in peel segments at the time they were placed on media. Each value represents the mean ± SE of 10 segments.

Table IV. *Serine Inhibition of Regreening*

*C. paradisi* peel segments were cultured for 7 and 14 d on B media with 60 mM $KNO_3$, 15 mM sucrose, plus indicated additions and mannitol to adjust to constant solute concentrations. Each value represents the mean of 10 ± SE.

| Addition to Media | Chl | |
|---|---|---|
| | 7 d | 14 d |
| | $\Delta A_{675-735}$ | $\Delta A_{675-735}$ |
| Initial | 0.000 ± 0.003 | |
| None | 0.27 ± 0.02 | 0.59 ± 0.02 |
| L-Serine (60 mM) | 0.09 ± 0.02 | 0.11 ± 0.02 |
| L-Serine (60 mM) + L-arginine (60 mM) | 0.04 ± 0.01 | 0.04 ± 0.01 |

In Figure 2, it is apparent that in the absence of nitrogen and at reduced sugar levels, *C. sinensis* peel segments regreen to a greater extent than *C. paradisi* peel segments, whereas at 150 mM sucrose, both regreen to about the same extent. This is confirmed by data presented in Table VI, which are averages of

Table V. *Osmotic Effects on Regreening of C. paradisi Peel Segments*

Peel segments were cultured for 14 d under continuous fluorescent light on B media containing 15 mM sucrose, 60 mM $KNO_3$, and the indicated concentrations of mannitol. Each value represents the mean of 10 ± SE.

| Mannitol | Chl |
|---|---|
| *mM* | $\Delta A_{675-735}$ |
| Initial | 0.019 ± 0.003 |
| 48 | 0.49 ± 0.02 |
| 148 | 0.54 ± 0.01 |
| 248 | 0.53 ± 0.02 |
| 348 | 0.52 ± 0.01 |
| 448 | 0.46 ± 0.02 |

several experiments. On medium B (no nitrogen) and low concentrations of sucrose, *C. sinensis* peel segments regreened at about twice the rate as did *C. paradisi* peel segments. On high sucrose media, regreening of both species was inhibited to about the same low level. Furthermore, *C. sinensis* peel segments on low concentrations of sugars appeared to regreen at a nearly maximal rate, as inclusion of nitrate did not significantly promote further regreening. In contrast, inclusion of nitrate in low sucrose media increased *C. paradisi* regreening about 2-fold and to approximately that same maximal rate. *C. sinensis* peel segments required exogenous nitrogen only when kept on media containing high sugar concentrations.

The promotion of *C. paradisi* regreening by nitrogen at low sucrose concentrations required that at least some sugar be included in the media. On media lacking sugars, nitrate had no effect on regreening (Table VII), but increased regreening over 2-fold in the presence of low concentrations of sucrose, glucose, and fructose. At high sucrose concentrations, where regreening was much reduced, inclusion of nitrogen overcame most of the inhibition. Indeed, the slight inhibitions caused by 150 mM sucrose in nitrate containing media (BN) seen in Table VI are averages, and 150 mM sucrose in the presence of nitrogen often promoted regreening in comparison with 15 mM sucrose.

The relationship between nitrogen and sugars is further amplified by determinations of endogenous concentrations of sugars and amino acids in epicarps of peels that have regreened on media with and without nitrate and with 15 and 150 mM sucrose (Fig. 3). When peel segments were kept 14 d on media lacking nitrogen and with low sucrose concentrations, endogenous sugar and amino acid contents of the epicarp declined considerably from their initial values and the Chl content increased markedly. Peel segments kept 14 d on similar media with 150 mM sucrose had either the same or higher endogenous sugar contents and somewhat lower amino acid contents than they had initially, and much lower levels of Chl than segments kept on media with 15 mM sucrose.

When nitrate was included in the media, the endogenous sugar contents were markedly reduced whether on media with 15 or 150 mM sucrose. At 15 mM sucrose, inclusion of nitrate in the media reduced the content of sugars in epicarp to their lowest values, and Chl levels usually increased to their highest values. With 150 mM sucrose in the nitrate-containing media, endogenous sugar levels were higher, but were still considerably less than in epicarp of peel segments on similar media lacking nitrogen. High sucrose concentrations in nitrate-containing media also strongly promoted increases in endogenous amino acid content. This is in contrast to the effect of sugars in media lacking nitrogen in which endogenous amino acid levels were further suppressed. Thus, after 14 d on media, there was an inverse relationship between the endogenous sugar content of the epicarp (promoted by sucrose in the media) and the degree of regreening. Also, high Chl levels were usually associated with higher endogenous amino acid contents, which were promoted by nitrate in the media and by sucrose when combined with nitrate.

**Sucrose Promotion of Degreening.** In addition to inhibiting regreening of citrus epicarp, high sugar concentrations promoted degreening of peel segments from immature fruit placed on media lacking nitrogen (Fig. 4). At 15 mM sucrose, there was only a slight loss of Chl from *C. sinensis* peel segments, whereas at 150 and 300 mM sucrose there were progressively greater losses of Chl over a 21 or 28-d period.

*C. sinensis* peel segments that were nearly degreened after 21 d on 300 mM sucrose promptly regreened upon transfer to media containing 60 mM $KNO_3$ and only 15 mM sucrose. Similar results were observed with *C. paradisi* peel segments (Fig. 5), although in the absence of nitrogen, 150 mM sucrose was sufficient to cause a nearly 90% loss of Chl in 21 d, whereas 300 mM sucrose was required to elicit a similar response in *C. sinensis* peel segments.

**Nitrate Inhibition of Degreening.** In the presence of exogenous nitrate, 150 mM sucrose did not induce degreening of *C. paradisi* peel segments (Fig. 5). In addition, 60 mM $KNO_3$ greatly reduced loss of Chl from peel segments allowed to degreen in the dark, where Chl synthesis is absent (Fig. 6). When *C. paradisi* peel segments that had been degreened on high sucrose media were transferred to media that contained, in addition to benomyl, only 60 mM $KNO_3$ and 29 mM sucrose (Fig. 5), they regained their initial Chl levels in 12 d and in 20 d had nearly as much Chl as those peel segments that were kept on media containing nitrogen for the entire time. Those peel segments transferred to

Table VI. *Increase in Chl during Regreening of C. sinensis and C. paradisi Peel Segments Responding to Combinations of Sucrose and Potassium Nitrate*

Segments were cultured 14 d under continuous fluorescent light on B media with 15 or 30 mM sucrose (low) and 146 or 150 mM sucrose (high) and similar media with 60 mM $KNO_3$ (BN). Values are averages of experiments done during a 2-year period. Inasmuch as initial Chl contents varied, values reported are average increases ($\Delta\Delta A_{675-735}$ ± SE)[a] in Chl. The number of experiments are given in parenthesis.

| Species | Chl Increase | | | |
|---|---|---|---|---|
| | Low sucrose | | High sucrose | |
| | B | BN | B | BN |
| | $\Delta\Delta A_{675-735}$ | $\Delta\Delta A_{675-735}$ | $\Delta\Delta A_{675-735}$ | $\Delta\Delta A_{675-735}$ |
| *C. paradisi* | 0.18 ± 0.02 | 0.37 ± 0.01 | 0.09 ± 0.02 | 0.30 ± 0.01 |
| (*n*) | (18) | (57) | (9) | (23) |
| *C. sinensis* | 0.37 ± 0.04 | 0.41 ± 0.03 | 0.11 ± 0.02 | 0.37 ± 0.03 |
| (*n*) | (8) | (17) | (6) | (14) |

[a] $\Delta A_{675-735}$ after 14 d − $\Delta A_{675-735}$ initially.

Table VII. *Sugar Requirement for Nitrate Enhancement of C. paradisi Regreening*

Peel segments were cultured 14 d under continuous fluorescent light on B media with 150 mM Mes (pH 5.5) and the indicated amounts of sugars, and similar media with 60 mM $KNO_3$ (BN). Each value represents the mean of 10 ± SE.

| Sugar, Concn. | Chl | |
|---|---|---|
| | B | BN |
| | $\Delta A_{675-735}$ | $\Delta A_{675-735}$ |
| Initial | 0.013 ± 0.001 | |
| 0 | 0.16 ± 0.02 | 0.18 ± 0.03 |
| Sucrose, 15 mM | 0.13 ± 0.01 | 0.33 ± 0.02 |
| Glucose, 30 mM | 0.16 ± 0.02 | 0.32 ± 0.03 |
| Fructose, 30 mM | 0.20 ± 0.02 | 0.35 ± 0.02 |

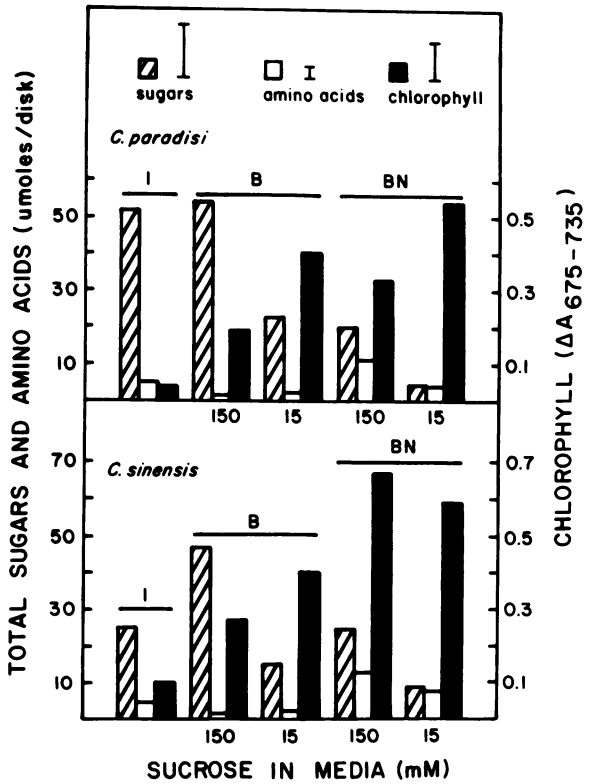


FIG. 3. Endogenous sugar and amino acid content after regreening *in vitro*. Peel segments from *C. paradisi* and *C. sinensis* were sampled initially (I) and after 14 d under continuous fluorescent light on the following four media: B with 15 mM sucrose, B with 150 mM sucrose, B with 60 mM $KNO_3$ (BN) and 15 mM sucrose, and BN with 150 mM sucrose. Chl values represent the means of 10 segments. Sugar and amino acid values represent means of three sets of three or four segments. Values differ significantly (Duncan's multiple range test) if by an amount equivalent to the vertical line at the top of the figure and alongside that block defining the parameter represented.

fresh media lacking nitrogen continued to lose Chl until 97% was lost after 45 d.

## DISCUSSION

Initial attempts at culturing peel segments infallibly resulted in death of the segments within 48 h. Presumably, failure was due to release of highly toxic oils from glands in the epicarp, and preparation of peel segments under water was attempted with the idea that these oils would float away from the segments before cells were damaged. This method proved successful, but

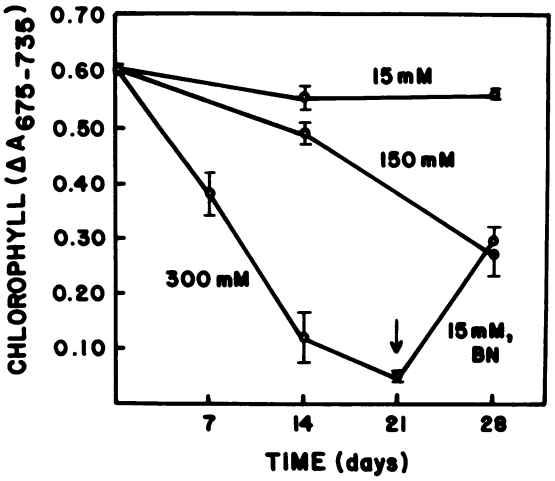


FIG. 4. Degreening of *C. sinensis* peel segments from immature fruit and its promotion by sucrose. Peel segments were kept under continuous fluorescent light and on B media with 15, 150, and 300 mM sucrose, and mannitol to maintain the combined sucrose and mannitol concentrations at 300 mM. After 21 d, peel segments degreened on 300 mM sucrose were transferred to BN (B with 60 mM $KNO_3$) media containing 15 mM sucrose (arrow). Each value represents the mean ± SE of 10 segments.

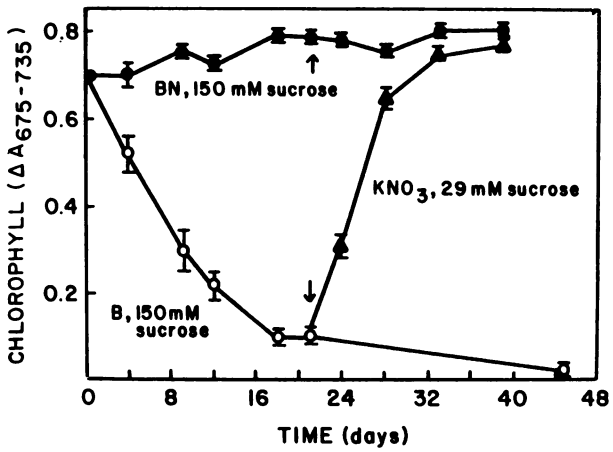


FIG. 5. Reversible degreening and regreening of *C. paradisi* peel segments in relation to exogenous nitrogen and sucrose. Peel segments from immature fruit were kept on B and BN (B with 60 mM $KNO_3$) media under continuous fluorescent light. At 21 d (arrows), peel segments were transferred to fresh media or media of new composition; (▲), media contained only 60 mM $KNO_3$, benomyl, 29 mM sucrose, and agar. Each value represents the mean ± SE of 10 segments.

whether or not for the presumed reason is not known.

In several experiments, mannitol was used to reduce osmotic potentials, later Mes was included in all media to eliminate the need for mannitol while increasing the buffering capacity of the media. There was no significant change in results ($P > 0.5$; *df* 118) after inclusion of Mes.

An important difficulty with this system has been the need to include a fungicide in the media. In experiments with the fungicide left out, loss of segments to mold was very high; however, there was no significant difference ($P > 0.4$; *df* 18) in the amount of Chl accumulated in segments that survived 10 d on media without benomyl compared with segments on media with benomyl.

The interconversion of chloroplasts and chromoplasts in the epicarp of citrus fruit seems determined by metabolic conditions as defined by the cellular status of carbon and nitrogen. Degreening and regreening of citrus epicarp *in vitro* is a fully reversible

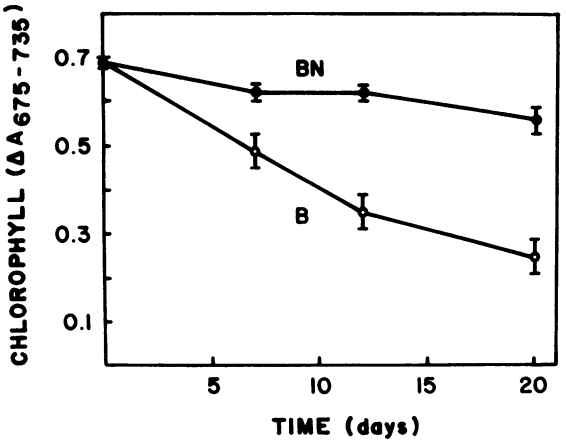


FIG. 6. Nitrate inhibition of Chl loss in the dark. *C. paradisi* peel segments from immature fruit were kept in the dark on B media with 146 mM sucrose and similar media with 60 mM $KNO_3$(BN). Each value represents the mean ± SE of 10 segments.

process largely dependent upon the availability of nitrogen and abundance of sugars. High concentrations of sugars in epicarp not supplied with nitrogen promote transformations to chromoplasts, whereas low concentrations of sugars promote the chloroplast form and are associated with higher internal amino acid concentrations. High sugar levels cause green peel segments to degreen and, when internal sugar concentrations are kept high by inclusion of sucrose in the media, reversion of chromoplasts to chloroplasts is markedly suppressed.

Although nitrogen in the media promotes regreening of peel segments, it is not always necessary and seems mainly to counter the effects of high sugar concentrations. When segments are placed on low sugar media, regreening is often as great without added nitrogen as with nitrogen. Since *C. paradisi* peels contain as much as 400 mmol N/kg, and 46 mmol N/kg as nitrate (28), endogenous nitrogen supplies are apparently adequate for regreening when endogenous sugar concentrations are reduced. Thus, *in vitro* regreening of peels is less a provision of materials such as nitrogen than removal of the peel segments from factors in the fruit that promote degreening. Evidence reported here indicates that high sugar concentrations in degreened epicarp are responsible for chromoplasts rather than chloroplasts.

It is important to note that these relations are most apparent after the peel segments have been cultured on media. Values obtained for peel segments at the time they are placed on media do not always agree with those obtained after a 14-d period. For example, peel segments used to obtain the results presented in Figure 3 contained much less Chl initially (I) than would be expected on the basis of their initial sugar and amino acid content. These results suggest other influential factors in the epicarp, such as hormones, in addition to sugars and amino acids.

Similar behavior has been well documented in certain algae (1, 2, 27), where the presence or absence of a chloroplast could be predetermined by the C/N ratio of the growth media, and the greening of some callus in tissue culture (8, 10, 17). The degreening and regreening of citrus peel segments apparently represents the first example of the control by C/N ratios of plastid form in differentiated higher plant tissues, and control of interconversions between chromoplasts and chloroplast; although both greening and degreening processes require adequate supplies of carbohydrate as a source of energy (20).

While the more effective sources of nitrogen are likely precursors of Chl (4, 12), promotion of regreening obtained by inclusion of nitrogen cannot be ascribed to the provision of substrate for Chl synthesis, inasmuch as exogenous nitrate reduced the rate of degreening in the dark when Chl synthesis had ceased. Nitrogen must hinder the degradation of Chl and disruption of the chloroplast. Aoki, Matsuka, and Hase (1) originally proposed that the carbon-nitrogen control of chloroplast development in *Chlorella protothecoides* might be explained by a carbonaceous bleaching agent that would react with nitrogen to form a product that does not promote bleaching. Kirk and Tilney-Bassett (20) have proposed a similar explanation for control of chloroplast development in which Chl or an intermediate in Chl synthesis is an inhibitor of Chl synthesis and consequently chloroplast development. The inhibiting effect of Chl is neutralized by nitrogen in the form of Chl-binding proteins. The effect of nitrogen noted here could be in part an indirect consequence of the reduction in endogenous sugar levels.

To explain the effect of nitrate on Chl loss in the dark, nitrogen would have to not only inactivate an inhibitor of Chl synthesis, but also inactivate a promoter of Chl and chloroplast degradation. These could be the same substance.

Shibaoka and Thimann (26) suggested that the conversion of chloroplasts to chromoplasts in senescent leaf tissue could result from the appearance of specific serine containing proteases. L-Serine, which promotes senescence in oat leaves (26), does inhibit regreening of epicarp. However, the two systems are not completely analogous because L-alanine, which also promoted senescence of oat leaves, promotes regreening of citrus epicarp, and L-arginine does not antagonize L-serine in regreening citrus epicarp as it does in senescence of oat leaves.

Whatever the mechanism, degreening and regreening of citrus fruit in response to temperatures and other environmental factors could be explained by the effects those factors have on peel sugars and nitrogen. During winter, soil and air temperatures decline and nitrogen uptake by citrus is markedly reduced (6, 33). Also, translocation of nitrogen from the roots of citrus trees appear to be reduced (33) and peels of *C. paradisi* cease to accumulate nitrogen (19). The epicarp of navel oranges (*C. sinensis*, cv Washington Navel) actually lose nitrogen from November through February while degreening and then regain the lost nitrogen (mostly as soluble nitrogen and presumably as free amino acids) from April through June (21). Warmer temperatures in the spring would increase nitrogen uptake, renewing the flux of nitrogen to the fruit. Increasing levels of nitrogen fertilization increases the level of nitrogen in the peels (3).

Whereas the nitrogen flux to the fruit is reduced by low temperatures, sugar concentrations in citrus peels continue to increase (21) and, in the epicarp of *C. paradisi*, reducing sugars in particular accumulate in response to mean low weekly temperatures below about 10°C (25). By mid-February, in Florida, total sugars no longer accumulate in the epicarp of *C. paradisi*, and with warming temperatures there is a marked drop in reducing sugars with a concomitant increase in sucrose. In the experiments described here, total sugars were reported although they were predominantly reducing sugars. Sucrose supplied in the media was readily inverted to glucose and fructose in the peels. Thus, a distinction between sugars could not be made and the sugar effect could be specific to reducing sugars without being apparent under the experimental conditions employed. Degreening and regreening of citrus fruit in response to cool winter and warm spring temperatures could therefore reflect a reduction in nitrogen flux accompanied by an accumulation of reducing sugars in the winter followed by a renewed nitrogen flux and diminished suppression of chloroplast development by reducing sugars in the spring.

The influence that other factors have on regreening could also be mediated through their effects on the nitrogen and sugar status of the epicarp. For example, $GA_3$, which delays degreening and enhances regreening of citrus, tends to reduce sugar levels in the fruit and peel (7, 21), although not to a large degree.

*Acknowledgments*—I thank Nancy Simons, Rick Houghton, Adam Kartman, Lynda Delph, and Jon Pierre Michaud for their skilled technical assistance.

## LITERATURE CITED


1. AOKI SM, M MATSUKA, E HASE 1965 De- and re-generation of chloroplasts in the cells of *Chlorella protothecoides*. V. Degeneration of chloroplasts induced by different carbon sources, and effects of some antimetabolites upon the process induced by glucose. Plant Cell Physiology 6: 487–497
2. APP AH, AT JAGENDORF 1963 Repression of chloroplast development in *Euglena gracilis* by substrates. Protozoology 10: 340–343
3. BOUMA D 1959 The development of the fruit of the Washington Navel orange. Aust J Agric Res 10: 804–817
4. BEALE SI 1978 δ-Aminolevulinic acid in plants: its biosynthesis, regulation, and role in plastid development. Annu Rev Plant Physiol 29: 95–120
5. CAPRIO JM 1956 An analysis of the relation between regreening of Valencia oranges and mean monthly temperatures in southern California. Proc Am Soc Hortic Sci 67: 222–235
6. CHAPMAN HD, ER PARKER 1942 Weekly absorption of nitrate by young bearing orange trees growing out of doors in solution cultures. Plant Physiol 17: 336–376
7. COGGINS JR CW, HZ HIELD, MJ GARBER 1960 The influence of potassium gibberellate on Valencia orange trees and fruit. Proc Am Soc Hortic Sci 76: 193–198
8. DALTON CC, HE STREET 1977 The influence of applied carbohydrates on the growth and greening of cultured spinach (*Spinacia oleracea* L.) cells. Plant Sci Lett 10: 157–164
9. DEVIDÉ Z, N LJUBEŠIĆ 1974 The reversion of chromoplasts to chloroplasts in pumpkin fruits. Z Pflanzenphysiol 73 S: 296–306
10. EDELMAN J, AD HANSON 1971 Sucrose suppression of chlorophyll synthesis in carrot callus cultures. Planta 98: 150–156
11. EL-ZEFTAWI BM 1977 Factors affecting pigment levels during regreening of Valencia orange. J Hortic Sci 52: 127–134
12. GASSMAN M, J PLUSCEC, L BOGORAD 1968 δ-Aminolevulinic acid transaminase in *Chlorella vulgaris*. Plant Physiol 43: 1411–1414
13. GRÖNEGRESS P 1971 The greening of chromoplasts in *Daucus carota* L. Planta 98: 274–278
14. GRÖNEGRESS P 1974 The structure of chromoplasts and their conversion to chloroplasts. J Microscopie 19: 183–192
15. HUFF A 1980 *In vitro* regreening and degreening of citrus flavedo. Plant Physiol 65: S–67
16. JAHN OL, R YOUNG 1976 Changes in chlorophyll a, b, and the a/b ratio during color development in citrus fruit. J Am Soc Hortic Sci 101: 416–418
17. JASPERS EMJ 1965 Pigmentation of tobacco crown-gall tissue cultured *in vitro* in dependence of the composition of the medium. Physiol Plant 18: 933–940
18. JONES WW, TW EMBLETON 1959 The visual effect of nitrogen nutrition on fruit quality of Valencia oranges. Proc Am Soc Hortic Sci 73: 234–236
19. JONES WW, CW VAN HORN, AH FINCH 1945 The influence of nitrogen nutrition of the tree upon the ascorbic acid content and other chemical and physical characteristics of grapefruit. Univ Ariz Agric Exp Stn Bull 106: 456–484
20. KIRK JTO, RAE TILNEY-BASSETT 1978 The Plastids. Their Chemistry, Structure, Growth and Inheritance. Elsevier/North-Holland Biomedical Press, Amsterdam
21. LEWIS LN, CW COGGINS JR, CK LABANAUSKAS, WM DUGGER JR 1967 Biochemical changes associated with natural and gibberellin $A_3$ delayed senescence in the Navel orange rind. Plant Cell Physiol 8: 151–160
22. MARAIS JP, JL DEWIT, GV QUICKE 1966 A critical examination of the Nelson-Somogyi method for the determination of reducing sugars. Anal Biochem 15: 373–381
23. MURASHIGE T, DPH TUCKER 1969 Growth factor requirements of citrus tissue culture. Proc First Int Cit Symp 3: 1155–1161
24. POSSINGHAM JV 1980 Plastic replication and development in the life cycle of higher plants. Annu Rev Plant Physiol 31: 113–129
25. PURVIS AC, W GRIERSON 1982 Accumulation of reducing sugar and resistance of grapefruit peel to chilling injury as related to winter temperatures. J Am Soc Hortic Sci 107: 139–142
26. SHIBAOKA H, KV THIMANN 1970 Antagonisms between kinetin and amino acids: experiments on the mode of action of cytokinins. Plant Physiol 46: 212–220
27. SHIHIRA-ISHIKAWA I, E HASE 1964 Nutritional control of cell pigmentation in *Chlorella protothecoides* with special reference to the degeneration of chloroplasts induced by glucose. Plant Cell Physiol 5: 227–240
28. SINCLAIR WB 1972 The Grapefruit, Its Composition, Physiology and Products. 3. Composition of Peel, Rag and Seeds. University of California Division of Agricultural Sciences
29. SITTE P 1974 Plastiden-metamorphose und Chromoplasten bei *Chrysosplenium*. Z Pflanzenphysiol 73 S: 243–265
30. THOMSON WW, JM WHATLEY 1980 Development of nongreen plastids. Annu Rev Plant Physiol 31: 375–394
31. THOMSON WW, LN LEWIS, CW COGGINS 1967 The reversion of chromoplasts to chloroplasts in Valencia oranges. Cytologia 32: 117–124
32. VAKIS N, W GRIERSON, J SOULE, LG ALBRIGO 1970 A tissue culture technique for studying chilling injury of tropical and subtropical fruits. Hort Science 5: 472–473
33. WALLACE A 1953 Nitrogen absorption and translocation by citrus cuttings at different root temperatures. Proc Am Soc Hortic Sci 61: 89–94
34. YEMM EW, EC COCKING 1955 The determination of amino acids with ninhydrin. Analyst 80: 209–213
35. YOUNG LB, LC ERICKSON 1961 Influence of temperature on color change in Valencia oranges. Proc Am Soc Hortic Sci 78: 197–200