Ultrastructural Studies on the Stability of Chloroplasts in Attached Leaves of Spinach (Spinacia oleracea L.) Subjected to High Temperature Conditions

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by

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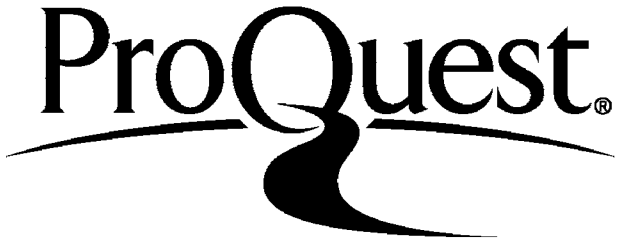

DECLARATION

The work presented in this thesis has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

signed .....
Candidature

Date ..13. Nov. 1980.....

This is to certify that the work here submitted was carried out by the candidate himself. Due acknowledgement has been made for any assistance received.

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Supervisor

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ABSTRACT

The literature relating to ultrastructural studies of chloroplast development in the leaves of angiosperm plants up to chloroplast senescence is reviewed, and attention is also given to the effects of high temperature on chloroplast ultrastructure and components. Ultrastructural changes are observed in the developing plastids of the primary leaves of spinach (Spinacia oleracea L.) under 14 h light/ 10 h dark conditions at normal growing temperature (22°C) and at high temperature (30°C) and also in complete darkness at 22°C. Under these three conditions plastid development proceeds from amyloplasts with the continuous formation of small segments of lamella which at later stages unite and develop grana. Under normal temperature conditions only an association is observed between the plastid and endoplasmic reticulum at very early stages of plastid development and amoeboid plastids are seen at an intermediate stage of plastid development. At high temperature the chloroplasts become swollen and some of them burst, liberating their contents free in the cell cytoplasm. In the dark the plastids develop into etioplasts having prolamellar bodies with typical paracrystalline structure. Swelling of the plastid lamellae is observed at both high temperature and in complete darkness. Plastid division is not seen under high temperature conditions.

Ultrastructural changes of the leaf chloroplasts during their growth and senescence is studied in detail under normal growing temperature of the plant (22°C) and also under higher temperature conditions (25°C, 30°C, and 35°C) starting with 4-weeks old plants. At normal growing temperature during the growth of the young chloroplasts to maturity, both the size and number of grana show a continuous increase along with the corresponding increase in the chlorophyll content and leaf fresh weight. When the leaf begins to show senescence the chloroplasts show a change in shape and a reduction in size. The terminal ends of the thylakoids first show club-shaped swellings

and this is followed by the breaking of the lamellae at various places resulting in the development of very variable irregularly-shaped stroma lamellae with characteristic ring or hook-shaped or looped lamellae. This extensive development of the abnormal lamellar system with such peculiar arrangements is hitherto unknown in the senescing chloroplasts of higher plants. Osmiophilic globules also show a great increase both in number and size attaining largest size in chloroplasts of the completely senesced leaf tissue. Under high temperature conditions all these changes are enhanced and brought about earlier as the temperature is increased.

The effects of high temperature on chloroplast ultrastructure are further exploited by keeping the plants at higher temperatures for successively longer time intervals. The ultrastructural changes are therefore investigated in newly expanded leaves on plants kept at the 4 temperature conditions. Two conclusions have been drawn from this investigation: (i) the higher the temperature to which the plant is subjected, the quicker the structural changes and abnormalities which the newly differentiated chloroplast shows and (ii) the lower the temperature to which the plant is subjected, the longer the time it requires for showing plastid structural changes and abnormalities. The structural changes shown by the newly formed chloroplasts includes the change in shape, reduction in size of the chloroplast, reduction in size of the interconnecting stroma lamellae, disappearance of starch grains and appearance of a few vesicles, increase in number and size of osmiophilic globules, club-shaped swellings at the terminal ends of the thylakoids and breaking of lamellae. All these structural changes of the chloroplasts are followed by the swelling of the chloroplasts which later burst at particular points thus liberating the contents free in the cell cytoplasm.

The transformation of the chloroplasts into etioplasts in complete darkness has also been investigated in the attached leaves under normal growing temperature of the plant and also under high temperature

conditions (30°C). Prolamellar body formation with typical para-crystalline structure is observed under normal temperature conditions after 128 hours of darkness. At 30°C the prolamellar body formation was not observed but instead several large vesicles are produced.

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C O N T E N T S

ABBREVIATIONS

1. Used in text

A^{o}	-	Angstrom
cm	-	centimeter
g	-	gram
h	-	hour
M	-	molar
mg	-	milligram
ml	-	millilitre
mm	-	millimeter
nm	-	nanometer
µm	-	micrometer
δ	-	delta
RNA	-	ribose nucleic acid
DNA	-	deoxy ribose nucleic acid

2. Used in experimental regime

L:D	-	14 h light/10 h dark
DD	-	complete darkness

3. Used on electron micrographs

CW	-	cell wall
ER	-	endoplasmic reticulum
G	-	globule
GB	-	Golgi body
M	-	mitochondria
P	-	plastid
PB	-	prolamellar body
S	-	starch grain
V	-	vesicles

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C H A P T E R I

GENERAL INTRODUCTION AND LITERATURE REVIEW

Although a great deal of effort has been devoted to the study of the ultrastructural and biochemical changes associated with chloroplast development, it is evident that the structure and biochemistry of mature chloroplast is still far from being understood. It is a well known fact that the general pattern of plastid development in a growing green plant progresses from a simple organelle, the proplastid, to more complex forms such as chloroplasts, etioplasts or amyloplasts (Kirk and Tilney-Bassett, 1967). The structure and development of chloroplasts has been the subject of study for many years (Schimper, 1885; Frey-Wyssling et al., 1958; Gunning and Jagoe, 1967; Whatley, 1978).

Most recent investigations of chloroplast development have been carried out on economically important species, i.e. angiosperms, and have described the changes occurring in the embryo following seed germination. In particular such studies have generally been concerned with the sequence of fine structural changes which take place as the proplastid, or the etioplast initially formed in the dark, differentiates into the mature chloroplast during leaf development. Cran and Possingham (1972) and Whatley (1978) have demonstrated the possibility that chloroplasts may dedifferentiate into proplastids under certain conditions of growth. According to them the changes in the plastid structure are not always unidirectional but often follow a cyclic pattern. Such a cyclic scheme of plastid development carries with it certain functional implications which are as listed below (Whatley, 1978).

(i) The thylakoids of mature chloroplasts exist in a state of dynamic

equilibrium. This is maintained by a continuous turnover of the thylakoids such that the amount of thylakoid renewal is in balance with that of thylakoid degradation.

(ii) Mature chloroplasts, under appropriate conditions, are capable of undergoing dedifferentiation. Dedifferentiation may involve changes in balance between the system of thylakoid renewal and degradation, in such a way that degradtion outstrips renewal.

(iii) Redifferentiation may then involve a further change in balance such that thylakoid renewal now superpasses degradation.

(iv) Senescence is probably a separate process superimposed on the general plastid cycle. Plastids may senesce not only after chloroplast maturation but also at intermediate stages.

(v) The progress of plastid developmental cycle in one organ can be influenced by factors transported from other organs and may be under hormonal control.

External growth factors like irradiation, temperature and moisture are also responsible for changing the structural and functional integrity of the plant cell and hence of the plastids. Amongst these environmental factors temperature does play an important role in governing the structural and functional changes of the developing and mature chloroplasts. Aside from the consideration that some plants show the ability to adapt to the prevailing temperature regimes during growth, many plants possess genotypic adaptation to a particular growth temperature and any change in the growth temperature will therefore affect the normal structural and functional ability of the plant. High temperature

affects the growth of the whole plant mainly through its effect on protein biosynthesis (Bernstam, 1978), enzyme denaturation (Feierabend and Mikus, 1977), destruction of lipid architecture (Molotkovsky and Zhestkova, 1965) and membrane permeability (Emmett and Walker, 1973). Therefore any change in the growth temperature condition of such plants above the normal growth temperature should reflect a change in the chloroplast structure and function. Most of the recent studies conducted in evaluating the structural and functional changes on the development and growth of chloroplasts are with respect to light (Bradbeer et al., 1970; 1974a, b, c; Cran and Possingham, 1972a, b; 1974a, b) and very little attention has been given to the fine structural changes of the plastids under high temperature conditions.

In spinach (Spinacia oleracea L.) ultrastructural work on growth and senescence of chloroplasts under different conditions of light, dark and temperature has mostly employed leaf discs (Cran and Possingham, 1972a, b; 1974a, b; Rose et al., 1974, 1975). Recently Hurkman (1979) has done some ultrastructural studies in attached and detached, ageing primary leaves of wheat.

Therefore, the aim of the present investigations has been to evaluate the effect of high temperature on the ultrastructure of chloroplasts of intact spinach (Spinacia oleracea L.) leaves during:

1. *Plastid development in the primary leaves of the seedlings.*
2. *Ageing of chloroplasts.*
3. *Senescence of chloroplasts.*
4. *Plastid development in leaves newly differentiated at various stages of plant growth.*

5. *Prolamellar body formation.*

ULTRASTRUCTURAL CHANGES DURING CHLOROPLAST DEVELOPMENT

In angiosperm plants under suitable light conditions proplastids normally differentiate into chloroplasts which produce chlorophyll, but in the absence of light these plants fail to produce chlorophyll and the proplastids of their cells develop into "etioplasts" (Kirk and Tilney-Bassett, 1967). On subsequent exposure to light however, etioplasts undergo ultrastructural changes and develop into chloroplasts. The differentiation of chloroplasts in green tissues of plants has been the subject of various studies which have been reviewed by Kirk and Tilney-Bassett (1967). Lately, this differentiation process has been studied extensively in normal whole tissues, as well as in tissue cultures (Blackwell et al., 1969) and in isolated etioplasts (Wellburn and Wellburn, 1971; Wrischer, 1973; Kohn and Klein, 1976; Wellburn et al., 1977).

The lamellae of chloroplasts originate by the invagination of the inner plastid membrane, by disaggregation of prolamellar bodies (Gunning, 1965) or from membrane-bound inclusions (Rosinski and Rosen, 1972) termed thylakoidal bodies (Hurkman and Kennedy, 1977). The suggested role of thylakoidal bodies in the formation of lamellae is based firstly on the close association of lamellae with these plastid inclusions (Jenson and Valdovinos, 1967; Mohr and Stein, 1969; Cran and Possingham, 1974a; Hoefert and Esau, 1975), and secondly on the observation that thylakoidal bodies are absent from the mature chloroplasts (Srivastava, 1966; Israel and Steward, 1967; Stetler and Laetsch, 1969; Arnott and Harris, 1973; Ames and Pivorun, 1974).

1. *Biogenesis of Chloroplast Lamellae in Etioplasts*

In 1965 Gunning found that in oat seedlings growing in the dark the cells in or near the meristem contained proplastids which like other proplastids were relatively undifferentiated, containing just a few single thylakoids. As the meristematic cells developed into mesophyll cells of the etiolated leaf, the proplastids developed into etioplasts. The most conspicuous and characteristic structure of the etioplast is the prolamellar body; the process of formation and spatial organization of this structure has been the subject of several studies recently reviewed by Gunning and Steer (1975).

The prolamellar body

In dark grown plants the leaves do not contain chloroplasts with a normal membrane system. Examining dark grown seedlings with the light microscope, Strugger (1950) found a dense body in the centre of the plastids which he termed the "primary granum". The first electron microscopic observation was made by Heitz (1954) and Leyon (1954) and later Hodge, McLean and Mercer (1956) examining the lamellar formation in higher plants found the same body in the leaves of the etiolated plants. They proposed that it had a role in the formation of chloroplast lamellae and termed it the "prolamellar body". This term has gained wide acceptance and will be used in the present investigation.

The prolamellar body appears to be a complex network of interconnected tubular membranes having the appearance of a crystal lattice. It is, therefore, referred to as crystalline-like or para-crystalline

or quassi-crystalline. Several models have been proposed for the structural arrangement of tubules and the process of formation of the prolamellar body. According to von-Wettstein (1958) this is composed of interconnected tubules and he proposed a model of tubules arranged in a cubic lattice. The beads thus would represent points where six tubules meet. Granick (1961) also drew it as a three dimensional cubic lattice of interconnected tubules. In contrast, Menke (1962, 1963) proposed that the prolamellar body of Chenopodium cosmos is a system of helically coiled but not interconnected tubules. Schnepf (1964) has described the mode by which such tubules are brought into contact with double lamellae (thylakoids) in greening Avena sativa plastids, and surmised that the prolamellar body has a helically coiled structure. Gunning (1965) and Gunning and Jagoe (1967) presented a model of the prolamellar body in Avena sativa similar to that of von Wettstein's model with three dimensional lattice structure that is composed of interconnected tubules lying in three major axes of a simple cube type of lattice (six tubules meeting at one point).

A somewhat complex model for the prolamellar body has been suggested by Wehrmeyer (1965a, b, c; 1967) after examining serial sections through prolamellar bodies of Phaseolus vulgaris. In contrast to Gunning, he interpreted the major portion of the crystalline prolamellar body structure as a six sided crystal lattice comparable to the zinc-blend crystal lattice. He found concentric and non-concentric types in etiolated leaves of Phaseolus vulgaris. In the concentric type the basic units form a system of tetrahedrally branched tubules joined to five- and six-membered rings. The centre of the lattice has the shape of a pentagonal dodecahedron. The non-concentric prolamellar bodies

follow either the crystal lattice of zinc-blend or wurzite or a combination of both. It is now agreed by Gunning and Steer (1975) that the cubic lattice is not very common and most prolamellar bodies are undoubtedly based on tetrahedrally branched tubules arranged in one or other of the lattice types described by Wehrmeyer (1965). Ikeda (1968) analyzed the fine structure of prolamellar bodies of Phaseolus vulgaris and confirmed Wehrmeyer's interpretation of the prolamellar body as an aggregate of hexagonal units. He presented a model consisting of an aggregate of hexagonal units, as seen in surface view and concluded that the lattice structure was more complex than the cubic model of Gunning (1965) but showed a close resemblance to the hexagonal model. Weier and Brown (1970) proposed a model of the prolamellar body of Phaseolus vulgaris which agrees closely with that of Wehrmeyer (1965a, b, c) and is identical to that described by Ikeda (1968). According to Weier and Brown (1970) the major portion of the crystalline prolamellar body is constructed of tubules, equal in length. The basic structural unit is a six-sided star module with four tubules uniting at each of the nodes. From these models it appears that the basic unit of prolamellar body is a tetrahedron (four tubules meeting at one point), with the tubules forming six-membered rings. The most common prolamellar body, therefore, contains hexagonal rings, but irregular patterns are also found. The appearance of the prolamellar body may be altered by the plane of the section and the pattern may be affected by the presence of more than one centre of symmetry in a prolamellar body (Wehrmeyer, 1967).

Relatively little information is available regarding the formation

of prolamellar bodies during the development of the etioplast. According to Gunning and Jagoe (1967) the proplastid contains just a few single thylakoids but as the etioplast differentiation proceeds the development of connecting arms between the thylakoids marks the formation of a crystalline centre. Gunning (1965) suggested that the crystal lattice is formed by the deposition of membranous material around ribosomes; addition of further ribosomes is followed by deposition of more membranes. However, in still earlier work (Hodge et al., 1956; Mühlethaler and Frey-Wyssling, 1959; Röbbelen, 1959; von Wettstein, 1959; Eriksson et al., 1961), it was reported that during the development of the etioplasts, there was an accumulation of vesicles which were produced by invagination of the inner plastid membrane. It was thought that the crystalline centre was formed by the fusion of these vesicles. However, no mass of vesicles has been observed during the etioplast differentiation in oat leaves (Gunning and Jagoe, 1967). According to Newcomb (1967) the prolamellar body is formed by the convergence of groups of tubules.

Englebrecht and Weier (1967) and Weier and Brown (1970) further rejected the earlier view of prolamellar body formation by the fusion of discrete vesicles derived from the inner plastid envelope. Weier and Brown (1970) concurred with the report of Engelbrecht and Weier (1967) that sheets of membranes, rather than vesicles, arise from the inner component of the plastid envelope. According to Weier and Brown (1970), in Phaseolus vulgaris in which the lamellae are porous, the prolamellar body arises through the contraction of these porous lamellae and the formation of interconnecting tubules linking each

lamella to the one above it. Thus, it may be said that the prolamellar body is the result of an accumulation of membrane products due to the blockage of light-requiring reactions that normally take place in the process of greening of seedlings or plant organs. The mechanism of its formation is not clear, but it seems to involve changes in continuous membrane system rather than a breaking off of discrete pieces (vesicles) which migrate and then re-fuse to form a continuous membrane again (Weier and Brown, 1970).

Prolamellar body transformation, conversion and thylakoid formation

When the etioplast is exposed to light, it undergoes several structural changes. Use of glutaraldehyde fixation, a technique that appears to preserve certain cellular structures lost with other fixation techniques, reveals that the structural changes, when etioplast is illuminated, are brought about by two continuous processes: the prolamellar body transformation with the loss of paracrystalline structure; and the prolamellar body conversion with the complete disappearance of prolamellar body as lamellae are produced. von Wettstein (1958) divided these processes into three steps, (a) the tubes of the prolamellar body are transformed into vesicles, (b) the vesicles are dispersed and arranged into primary layers, and (c) the vesicles are fused into discs and the discs aggregated into grana. This idea of Wettstein has not been accepted as all recent reports suggest that the prolamellar body does not, on illumination, form vesicles which disperse and ultimately fuse to form new thylakoids (Gunning and Jagoe, 1967; Lemoine, 1968). In cotyledons of Carthamus tinctorius, using permanganate fixation the prolamellar body was at

no stage observed being transformed into vesicles nor was vesicular fusion observed in the formation of new thylakoids; rather the whole process is accompanied by a direct transformation from the tubules into lamellar thylakoids (Gunning and Jagoe, 1967). Thus the break-up of the prolamellar body to form primary lamellae is identical to what is generally referred to as the second step in lamellar construction and it has a high-energy light requirement (Bogorad, 1967).

The time taken for the transformation of the prolamellar body from a paracrystalline to the non-crystalline state has been reported to vary from plant to plant and tissue to tissue. Laetsch and Price (1969) found that the prolamellar bodies in etioplasts of mesophyll cells of Saccharum officinarum require seven hours of light for this transformation even though etioplasts of the bundle sheath in the same species are transformed by very brief periods of light. Gunning and Jagoe (1967) reported that when etiolated Avena sativa leaves are exposed to light at an intensity of 750-1000 foot candles for five minutes, all the protochlorophyllide is reduced to chlorophyllide. Electron microscopic studies of the etioplasts show the order and regularity of crystalline centres have been largely lost by this exposure to light, although the continuity of the membrane surface is retained (Gunning and Jagoe, 1967).

The earlier model described by von Wettstein (1958) for the conversion of the prolamellar body and thylakoid formation involved the dispersal of vesicles from the prolamellar body throughout the plastid. These vesicles would first become arranged in layers, and

then fuse to form the several primary thylakoids of the plastids. This model envisaged conversion of the prolamellar body to thylakoids before the formation of extensive grana began. In the case of Avena sativa, Gunning and Jagoe (1967) have shown that, as greening proceeds, there is an increasing tendency for the tubular connections of the crystalline centre to pinch-off, forming a set of two-dimensional, double-membraned, sheets which extend parallel to each other out into the stroma from the prolamellar body. These sheets, or primary lamellae, have many perforations and so can give the appearance in section of a row of vesicles. The presence of perforated or reticulate sheets has also been reported by many workers (Englebrecht and Weier, 1967; Lemoine, 1968; Weier and Brown, 1970; Klein and Schiff, 1972; Bradbeer et al., 1974a, b, c). The first and obvious difference between the development of chloroplast and etioplast from proplastid is that in the etioplast the developing thylakoids are perforated (Weier and Brown, 1970; Klein and Schiff, 1972). This structural change does not appear to require chlorophyll formation, since it takes place in the lag phase of chlorophyll synthesis (Koski, 1950; Klein and Bogorad, 1964). Gunning and Jagoe (1967) suggested that as the illumination continues, the pores or perforations in the double-membraned sheets disappear. They further concluded that by the end of the lag period of greening, no large areas of membrane have been produced de-novo. This suggests that the membranous structures present in the plastids at this stage of development have been produced largely by rearrangements of the membranes originally present in the crystalline centre. It appears that the prolamellar body is the sole contributor of the building material to the primary thylakoids. However, after the lag

period, there appears to be a good correlation between chlorophyll synthesis and membrane production (Virgin et al., 1963).

The mechanism by which the prolamellar body tubules form perforated sheets and finally smooth thylakoids is not understood. According to the most authors some of the connections between tubules of the para-crystalline prolamellar body are broken, giving rise to the disordered appearance of the transformed prolamellar body. Some connections, however, remain intact and new fusions may occur as the membranes extend outward from the prolamellar body. The time taken for the completion of prolamellar body transformation varies from a few minutes to several hours and depends upon the intensity of illumination (Erriksson et al., 1961; Virgin et al., 1963). The complete conversion of prolamellar body into primary thylakoids varies from 1 - 28 hours of illumination and this depends upon the intensity of light being used, cell type, and species.

The prolamellar conversion is closely related to the shift in the absorption maximum of the chlorophyll from 684 nm to 672 nm (chloro-phyll-684 to chlorophyll-672) (von Wettstein, 1967; Henningsen, 1970). They found that if the etiolated leaves were illuminated briefly, then placed again in the dark for one hour at $0^{o}C$ or $10^{o}C$, there was no special shift and no prolamellar body conversion. Both processes were accelerated when the temperature was raised from $23^{o}C$ to $30^{o}C$.

Prolamellar body transformation and protochlorophyllide reduction

There is evidence which shows that protochlorophyllide is associated with prolamellar bodies and that it accumulates along with the formation

of paracrystalline prolamellar bodies (Kahn, 1968; Henningsen and Boyton, 1969; 1970). Although these workers found the occurrence of paracrystalline prolamellar bodies and protochlorophyllide together under certain conditions, yet, there is really no direct evidence for a causal connection. For example, in a mutant of Zea mays, Millerd et al. (1969) could find protochlorophyllide in the absence of prolamellar bodies and paracrystalline prolamellar bodies where chlorophyll rather than protochlorophyllide was accumulating. Bradbeer et al. (1970) showed that under flashing light, the prolamellar bodies were found to retain their paracrystalline structure, even though rapid synthesis of chlorophyll and thylakoid formation took place. Boardman et al. (1970) and Treffry (1970) reached the same conclusions after examining the transformation of prolamellar bodies both under red light and during low temperature greening.

Henningsen and Boynton (1969, 1970) found an accumulation of protochlorophyllide with the reappearance of prolamellar bodies when the etioplast was exposed to light for four hours. They concluded that the protochlorophyllide may be required for the formation of the paracrystalline prolamellar body. They confirmed their conclusion when they put back the etiolated leaves in the dark after a brief exposure to light and found a correlation between resynthesis of protochlorophyllide and re-formation of the paracrystalline prolamellar body. They suggested that a change from open lattice prolamellar bodies to normal paracrystalline prolamellar bodies during growth in the dark may result from the rapid accumulation of protochlorophyllide occurring at the same time.

Virgin et al. (1963) found that although protochlorophyllide reduction takes place roughly at the same time as the prolamellar body transformation, the two processes may not go hand in hand. Klein et al. (1964), using isolated leaves of Phaseolus vulgaris, found a rough parallel between the etioplasts which have undergone this structural change and the proportion of the protochlorophyllide which had been reduced to chlorophyllide. Further, Weier et al. (1970) showed that the pigment concentration and prolamellar body transformation were not necessarily associated. They found that under low light intensity (about 100 f.c. or 1070 lux) protochlorophyllide reduction took place very rapidly (20 seconds) whereas prolamellar body transformation took 1 - 2 hours. After a few more hours the prolamellar bodies reformed, even though chlorophyll synthesis continued. Thus it is still not entirely clear whether the structural change is an immediate and necessary consequence of the photoreduction of protochlorophyllide.

Grana formation

In the greening process, following a lag period, the membranous structures of the plastids are largely produced by the rearrangements of membranes originally present in the crystalline centre (Gunning and Jagoe, 1967; Kirk and Tilney-Basset, 1967). Most models of grana formation do not show any association of prolamellar body with the newly formed grana, although Virgin et al. (1963) categorized grana formation as a separate stage occurring after vesicle dispersal and fusion.

As chlorophyll synthesis accelerates, the single double-membraned sheets duplicate themselves (Gunning and Jagoe, 1967). This duplication has been shown by various workers to take place by thylakoidal invagination, overlapping growth, sliding growth, budding, or spiral growth of thylakoids or a combination of these (Wehrmeyer and Röbbelen, 1965; Paolillo and Reighard, 1967; Salema and Abreu, 1972). From here onwards chlorophyll synthesis seems to go hand-in-hand with thylakoid formation. On illumination, the primary lamellae over certain regions become double, thus producing in effect, a 'stack' of two thylakoids (Gunning and Jagoe, 1967). And over extended illumination, there are produced 6 - 8 or even more thylakoids stacked one on top of the other somewhat similar to a pile of coins in appearance.

2. *Biogenesis of Chloroplast Lamellae from Plastid Inclusions*

Investigators of chloroplast development have proposed several different schemes for the biogenesis of chloroplast lamellae (Mühlethaler and Frey-Wyssling, 1959; von Wettstein and Kahn, 1960; Gunning and Jagoe, 1965). Much of the information concerning this development has been gained following the ultrastructure changes that occur in plastids when etiolated tissue is exposed to light, as described above. There has not, however, been much recent work comparing the development of chloroplasts in light grown plants with the process observed in etiolated tissue. The prolamellar body has been discussed only in terms of lamella formation in etiolated tissue. Its appearance and role in chloroplasts in light grown tissue is not yet understood.

The presence of membrane-bound bodies within the chloroplast has been reported in recent papers (Gerola et al., 1965; Srivastava, 1966; Flemion et al., 1967; Israel and Steward, 1967; Marinos, 1967; Hurkman and Kennedy, 1977). The idea that these membrane-bound bodies are involved in lamella formation is based on the fact that the lamellae are associated closely with them (Cran and Possingham, 1974a; Hoefert and Esau, 1975; Jenson and Valdovinos, 1967; Mohr and Stein, 1969) and that these inclusions are absent from mature chloroplasts (Srivastava, 1966; Israel and Steward, 1967; Stetler and Laetsch, 1969; Arnott and Harris, 1973; Ames and Pivorun, 1974). No direct involvement of these inclusion bodies with membrane synthesis has been recorded although Newcomb (1967) has noted an association with a tubular complex.

Membrane bound plastid inclusions were observed in different plant tissues by various workers for example, in the cambium of ash (Froxinus americana) (Srivastava, 1966); peach shoot apices (Prunus persica) (Flemion et al., 1967); cultured carrot cells (Daucus carrota) (Israel and Steward, 1967); potato tuber buds (Solanum tuberosum) (Marinos, 1967); bean root tips (Phaseolus vulgaris) (Newcomb, 1967); tobacco (Nicotiana tabacum) and tomato (Lycopersicum) flower pedicels (Jenson and Valdovinos, 1967); sunflower (Helianthus tuberosus) crown gall tissue (Gee, Sun and Dwyer, 1967); tobacco leaves (Stetler and Laetsch, 1969); apical meristem of tobacco (Hurkman and Kennedy, 1977). These inclusions were first observed by Girola and Dasu (1960) as granular component of Helianthus tuberosum plastid and were termed as "opaque bodies". Later, such plastid inclusions were referred to variously as the "intralamellar inclusion" (Srivastava, 1966), the "intraplastid body" (Marinos, 1967), the "membrane-bound sac" (Newcomb, 1967), the "central granular component" (Jenson and Valdovinos, 1967), the "pre-thylakoidal body" (Israel and Steward, 1967), the "stroma centre" (Lee and Thompson, 1973) and the "thylakoidal body" (Hurkman and Kennedy, 1977).

Nature and composition of plastid inclusions

The exact nature and chemical composition of these inclusions is yet a question for further investigations. With the exception of Lee and Thompson (1973) who found these inclusions to be crystalline in nature but possessing no surrounding membrane, all other investigators are of the opinion that these inclusions are membrane-bound (Srivastava, 1966; Jenson and Valdovinos, 1967; Ames, 1972; Hurkman and Kennedy,

1977; Nessler and Mahlberg, 1979a, b). Newcomb (1967) reported that his so-called "membrane-bound sac" is granular in nature and is surrounded by a single membrane. This single membraned concept of plastid inclusion was further supported by Marinos (1967). However, Jenson and Valdovinos (1967) were of the view that these plastid inclusions are double-membraned and the membranes are a part of the thylakoid system, and also showed that these inclusions are spherical-shaped bodies of relatively large size (1.2-1.4 μm in diameter) as compared to the whole plastid (1.6-2.2 μm). Israel and Steward (1967) also observed a similar pattern of plastid inclusions.

The composition of these inclusions differs among plant species. They may consist of protein (Stetler and Laetsch, 1969; Ames and Pivorum, 1974; Hoefert and Esau, 1975), lipid and protein (Srivastava, 1966; Cran and Possingham, 1974a) or phenolic compounds (Flemion et al., 1967; Gifford and Stewart, 1968; Israel, Mapes and Steward, 1969). Since chloroplasts can synthesize protein (Boulter, Ellis and Yarwood, 1972), it is possible that the protein of these plastid inclusions is synthesized within the plastid (Hurkman and Kennedy, 1977). This suggestion is consistent with studies which show that some lamellar protein is synthesized by chloroplast (Eaglesham and Ellis, 1974). However, the protein could have originated as subunits synthesized in the cytoplasm which were then assembled within the chloroplast (Vigil and Ruddat, 1970). Several authors (Marinos, 1967; Newcomb, 1967; Burgess, 1970) have speculated that the mechanism for accumulation of cytoplasmic protein within plastids that store protein involves transport via invagination of the inner plastid membrane. Lichtenthaler (1966, 1968)

has equated the presence of osmiophilic droplets with the state of plastid development and has associated their frequency with membrane synthesis or degradation. In his view the lipid moeity of membrane may largely be derived from osmiophilic droplets. In contrast, Israel and Steward (1967), in addition to their prethylakoidal body, found another plastid inclusion which they termed "globular centre". This plastid inclusion is a spherical entity not visibly membrane-bounded, but has lipid droplets dispersed on its surface. According to Israel and Steward (1967) as the thylakoids enlarge, their association with the lipid droplets of the globular centre suggests that this body plays an integral part in chloroplast development. Nessler and Mahlberg (1979a) suggested that the electron-dense, membrane-bound inclusion in laticifer plastids of Papaver somniferum may be composed of lipoprotein.

Origin of plastid inclusions

Very little information is available regarding the origin of membrane-bound plastid inclusions and no generalized agreement has so far been made in this aspect. Marinos (1967) has reported that in dormant buds of the potato tubers plastids can accumulate phytoferritin, lipid droplets, nucleic acids and protein. He suggests that they are capable of a wide range of biochemical tasks which he considers to be a malfunctional state imposed upon them by existing physiological conditions. Gifford has suggested (Clowes and Juniper, 1968) that such plastid components as observed by Marinos might be transferred to cytoplasm as membrane-bound inclusions. Marinos (1967) findings also get support from the work of Israel and Steward (1967)

who suggest these plastid inclusions (pre-thylakoidal bodies) are the result of a complex of factors that stimulate the growth of the cells. However, Hurkman and Kennedy (1977) proposed that in tobacco stem tips the formation of a thylakoidal-body involves the following sequence of events: protein accumulation within swollen, primary lamellae which originate by invagination of the inner plastid membrane; these lamellae then coalesce to form a single membrane-bound inclusion. A similar sequence of events resulted in the formation of the membrane-bound protein inclusions in proteoplasts of developing primary leaves of mung bean (Hurkman and Kennedy, 1976). Nessler and Mahlberg (1979a) have shown that these electron-dense inclusions in laticifer plastids of Papaver sommiferum appeared to arise from the accumulation of material within an invagination of the inner plastid membrane.

Grana formation

Membrane-bound bodies may have a role in the formation of plastid lamellae (Stetler and Laetsch, 1969; Rosinski and Rosen, 1972; Hoefert and Esau, 1975; Hurkman and Kennedy, 1977), but since the nature of their contents is not yet certain, the exact mode of the formation of these lamellae is not yet fully explored. According to Israel and Steward (1967) the contents of the membrane-bound body (thylakoidal body) seem to unwind in the form of a long continuous, spiralling, membrane-bounded sack. The boundary surface of the sack consists of a layer (probably lipid) which is about 30 Å thick. Where these abut upon each other as in the formation of grana, there is a further thin surface layer (possibly protein) which separates the adjacent thylakoids. This layer, presumably double (approximately 20 Å), contains inter-

digitating proteinaceous material at the outer surface of the thylakoid (Mühlethaler, 1972). Although the plasmalemma and the thylakoid membrane have different dimensions they may still be composed of similar substances (Mühlethaler, 1972). However, the developmental sequence observed by Israel and Steward (1967) suggests that a limited amount of synthesis in the prethylakoidal body provides the substances for the later transformation of this material into chloroplast grana under the influence of light. The globular centre observed by Israel and Steward seems to make up some essential contribution to the transformation of the thylakoidal material into chloroplast grana. Since the lipid surface of the thylakoid might reasonably become much attenuated as the thylakoids extend it may be that the globular centre acts as a source of such materials, for they are very conspicuous between the stacked thylakoids which constitute the grana. However, Stetler and Laetsch (1969) found that, while the lamellae are continuous with the membrane that limits the body, there is little evidence in tobacco tissue for the unwinding observed by Israel and Steward (1967).

CHLOROPLAST REPLICATION

In 1936, Kusunoki and Kawasaki made the first and apparently only well-documented report on the fission of a higher plant chloroplast within a living cell of Utricularia vulgaris based upon the direct observation of the event. Chloroplasts in mesophyll cells of higher plants are biphasic organelles consisting of a stationary component where chlorophyll is located within a system of grana and intergrana thylakoids, the system being surrounded and interpenetrated by a translucent mobile phase. This mobile phase is that part of the chloroplast which imparts the dynamic pleomorphic behaviour to chloroplasts (Wildman et al., 1962). For example, chloroplasts may fuse to produce two stationary components enclosed by a single mobile phase. Fusion might be followed by separation of the single mobile phase to restore the original appearance of two discrete chloroplasts with individual mobile phases. Consequently, it is important to stress that, while the separation of mobile phases to yield two discrete chloroplasts might superficially resemble fission or reproduction and it is seen with considerable frequency, the division of the stationary component has never been seen (Honda et al., 1971).

Rate of chloroplast replication

Recently, several reports on the rate of increase in numbers of chloroplasts per cell have appeared which were based upon counts of plastids per cell at different times of developmental stages of leaves. In etiolated tobacco tissue culture, illumination induced chloroplast formation at the rate of 10 chloroplasts per cell per 24 hours (Boasson

and Laetsch, 1969). For growing spinach leaves, chloroplast reproduction was at the rate of about 25 chloroplasts per palisade cell per 24 hours (Possingham and Saurer, 1969).

The results of Possingham (1973) suggest that in spinach the sequence of events in greening leaf discs is that the etioplasts first increase in size and with accompanying thylakoid production from immature plastids, the cells increase in size, and then chloroplast division takes place. Support for this work also comes from studies with tobacco and a number of other higher plant species in which it seems that factors or conditions which govern cell elongation also influence chloroplast division (Honda et al., 1971; Kameya, 1972). Early as 1955 Granick suggested cell enlargement was a precondition for chloroplast replication and found that the number of chloroplasts per cell may be related to the cell surface area.

Effects of light and dark on chloroplast replication

Light is known to be necessary for growth and replication of higher plant chloroplasts (Boasson, Bonner and Laetsch, 1972; Possingham and Smith, 1972) and has a differential effect on the two processes (Possingham and Rose, 1976). The stimulative effect of light on the spinach chloroplast was observed by Possingham and Smith (1972) and Possingham (1973). A similar effect of light on tobacco chloroplasts was also observed by Boasson, Bonner and Laetsch (1972). Possingham et al. (1975) observed that in spinach relatively high light intensity (4-6 mW Cm^{-2}) is required for chloroplast replication which is inhibited in low light intensity. In addition to the number, the size of the

chloroplasts is also reduced at low light intensity and a close relationship seems to occur between both chloroplast number per cell and cell area (in section), and the daily quantity of light to which the tissue is exposed (Possingham and Smith, 1972).

The data presented by Possingham and Smith (1972) and Possingham (1973a) show that plastid division does occur in the dark but following illumination, a significant increase in plastid number occurs along with the increase in plastid area and chlorophyll content. These results suggest that some of the biochemical steps which are a prerequisite for chloroplast replication may occur both in dark and in light. It seems clear, however, that light is necessary for the final stages of chloroplast replication and this may be because plastid-RNA synthesis proceeds in the dark but is stimulated by light (Ingle, 1968; Smith, Stewart and Berry, 1970).

In addition to the quantity of light, quality of light does play an important part in chloroplast replication. Possingham and Smith (1972) and Possingham (1973b) have shown that over the intensity range 0.1-5 mW. Cm^{-2} both red (632 nm) and blue (488 nm) laser light stimulate chloroplast devision to the same extent as white light. By contrast low intensities (0.22-0.65 mW Cm^{-2}) of both white or green (525 nm) light are ineffective for chloroplast replication but permit normal chlorophyll synthesis. The large plastids of discs grown in green light divide when exposed to high intensity white light. The nature of the light receptor system for plastid replication in chloroplasts have not been established, however, the high intensity requirement for

replication and inhibition of this process by the phosphorylation inhibitor DCMU suggests it could be chlorophyll itself (Boasson, Bonner and Laetsch, 1972; Possingham, 1976).

Effects of temperature on chloroplast replication

Very little information is available on the effect of high and low temperatures on chloroplast replication. Possingham and Smith (1972) in an experiment with cultured leaf discs of spinach observed that the discs cultured at the highest temperatures (40°C day, 35°C night) died soon after transfer to these conditions. Discs grown at lowest temperature (12°C continuous) were low in fresh weight, had small cells and contained the fewest but also the largest chloroplasts. Chloroplast number per cell was at a maximum at approximately 300 degree hours above 10°C per day (25°C day, 20°C night) and was reduced as the temperature was either raised or lowered. Plastid size was at a maximum in the discs grown at the lowest temperature (12°C) and fell as temperatures were raised.

DNA-synthesis and chloroplast replication

Studies involving light and electron microscope autoradiography indicate that the DNA of spinach chloroplasts is associated with grana lamellae and with the nearby clear areas containing fibrillar material (Rose, Cran and Possingham, 1975; Possingham and Rose, 1976; Rose and Possingham, 1976). According to Rose and Possingham (1976), on average about 20 percent of the area of a chloroplast section is occupied by lamellar membranes, whereas almost 50 percent of the grains occur here. They did not find any uniform distribution of grains throughout the

chloroplast. Very little association of DNA was found with the envelope membranes of either the chloroplast or the etioplast (Herrmann and Kowallik, 1970). It is suggested (Possingham and Rose, 1976; Rose and Possingham, 1976) that the association of c-DNA molecule with the lamellar system of chloroplasts ensures an orderly segregation of c-DNA to daughter chloroplasts during binary fission by constriction.

The studies of Rose, Cran and Possingham (1975) on dark grown spinach show that there is no absolute requirement for light for DNA synthesis to occur in chloroplasts. This was also concluded from work with dark-grown Chlamydomonas (Chiang, 1971). It does appear, however, that in algae, chloroplast DNA synthesis is usually stimulated by light (Iwamura, 1962; Cook, 1966; Chiang and Sueoka, 1967) and this may include a specific type of DNA. But Rose, Cran and Possingham (1975) suggested that the light has a greater and more immediate effect on nuclear-DNA synthesis rather than on chloroplast-DNA synthesis.

ULTRASTRUCTURAL CHANGES OF CHLOROPLAST DURING AGEING AND SENESCENCE

Interest in the senescence of green tissues has centred largely upon the detectable metabolic changes. However, a little attention had been paid to the accompanying changes in the chloroplast fine structure. Therefore the most recent published work on the behaviour of chloroplasts during ageing and senescence has been orientated towards electron microscopy rather than the biochemical changes. Many workers (Dodge, 1970; Hurkman and Kennedy, 1975; Hurkman, 1979) have shown that chloroplasts are the first organelles to show some changes in the structure but were the most persistent and could still be identified when all other cell organelles had degenerated. However, the pattern of events was found to be slightly different in the artificially induced senescence of wheat leaves where the endoplasmic reticulum and the mitochondria appeared to be the first organelles to show structural changes (Shaw and Manocha, 1965), and the swelling of endoplasmic reticulum profiles was followed by a disappearance of endoplasmic reticulum and ribosomes and the mitochondria were invariably retained but with swollen and distorted cristae.

Shape and size of chloroplast

The work of various workers (Barton, 1966; Butler, 1967; Dodge, 1970; Hurkman and Kennedy, 1975; Hurkman, 1979) have shown that during the ageing and senescence of leaves from the fully expanded green state to the yellow terminal condition, the shape of chloroplast alters and the volume decreases. The fully senescent plastids of Phaseolus were shown to reduce to one-quarter of their previous size

(Barton, 1966). In Nicotiana, Ljubešić (1968) noted a considerable reduction in the chloroplast volume and in Betula, Dodge (1970) found that chloroplasts in the yellow senescent leaves have less than one-fifth of the volume of a young green chloroplast. Hurkman and Kennedy (1975) showed that in senescent tobacco leaves, plastids were much smaller (1-2 μm long) and the normal leaf plastids. Dodge presumed that this reduction in the chloroplast size could be accounted for by the loss of most of the stroma and chlorophyll from the chloroplast and some of the proteins from the membrane system. Cran and Possingham (1974b) working on cultured spinach leaf discs suggested that chloroplast degeneration in mature leaf discs may be a consequence of the tissue not containing or having access to substances produced by either dividing or expanding cells. It is therefore possible that mature leaves are critically dependent on a transport system for substances which will retard senescence, while young leaves may produce them themselves.

Chloroplast lamellae

Associated with these changes in the chloroplast shape and volume, there is an initial increase in the number of chloroplast lamellae and size of grana (Dodge, 1970) which is followed by re-orientation of the thylakoidal system (Hurkman and Kennedy, 1975; Hurkman, 1979).

The early stages of senescence appear to be a well-ordered process affecting mainly the chloroplasts. Barton (1966) found that in Phaseolus leaf chloroplasts in regions adjacent to the dense

globules a characteristic swelling occurred at the terminus of each thylakoid, which appeared as a club-shaped structure. Since the distortion was only observed adjacent to globules in senescing cells it is interpreted as being a stage in the weakening of the thylakoid membrane prior to breakdown. Dennis et al. (1967) also observed such granal swelling in Brussels sprout leaf chloroplasts and referred to it as a loosening of grana structure which eventually disorganizes and disappears. According to Dennis and co-workers (1967), Ljubĕsić (1968), Mittelheuser and Van Steveninck (1971), Mlodzianowski and Ponitka (1973) the stroma lamellae were preferentially lost relative to grana lamellae and finally, the lamellar system was reduced to membrane vesicles or, in some plastids, to membrane sheets. During these lamellar changes, the stroma degenerated and osmiophilic globules increased in both number and size (Greenwood et al., 1963; Barton, 1966; Hurkman and Kennedy, 1975; Hurkman, 1979). The chloroplast envelope remained intact until the thylakoidal system was reduced to membrane vesicles (Cran and Possingham, 1974b; Hurkman, 1979).

Mittelheuser and Van Steveninck (1971) observed similar stages of chloroplast breakdown not only in naturally senescing wheat leaves, but also in detached leaves. Butler (1967) noted a rapid breakdown of thylakoid in detached cotyledons of cucumber than in attached cotyledons. However, in other studies (Shaw and Manocha, 1965; Haber et al., 1969; Hurkman, 1979), the sequences of changes during degeneration of chloroplasts in detached, ageing leaves was less complex than in naturally senescing leaves. In detached leaves, chloroplasts become swollen, and thylakoids as well as membranes of

the plastid envelope dilated, osmiophilic globules increased in size and the stroma deteriorated. Finally, the swollen plastid envelope ruptured. All these stages occurred without significant disorganization of the lamellar system or any increase in the number of osmiophilic globules.

Although it is well established that the early stages of senescence mainly affect the chloroplasts and the breakdown of the thylakoids within the chloroplast occurs in certain areas only, the remaining unaffected grana presumably maintaining normal photosynthesis. Such features would be more consistent with the idea of a specific enzyme system (Barton, 1966) causing thylakoid breakdown at the onset of senescence, rather than an overall autolysis of cellular membranes, or wholesale liberation of hydrolytic enzymes (de Duve, 1959). Such an enzyme system may be synthesized de-novo or, on the other hand might be activated (Barton, 1966). It would presumably increase in activity in each chloroplast, then diffuse to the other parts of the cell. Such an enzyme system might be under the control of chloroplast RNA (Barton, 1966). The other possibility for the initial damage of the cell could be a failure of the cellular repair mechanism due to loss of ribosomes, export of necessary precursors, or the presence of a metabolic block in an essential synthetic pathway (Butler, 1967). All these possibilities are interrelated. A relatively small initial change would have a cumulative effect. Breakdown could start simultaneously at a number of points, and in fact the ribosomes and chloroplasts in attached tissues start to change at about the same time. In detached tissues the ribosomes persist for some time, so in this instance,

at least, ribosome loss is unlikely to be directly responsible for the other structural changes. The characteristic repeatable pattern of structural and metabolic changes suggests that senescence is genetically programmed (Butler and Simon, 1968).

Chloroplast envelope

Chloroplasts are surrounded by two separate envelope membranes which differ in function and chemical composition from the internal thylakoid membrane system (Heldt and Rapley, 1970; Douce et al., 1973; Poincelot, 1973; Cobb and Wellburn, 1974; Mackender and Leech, 1974; Mendiola-Morgenthaler and Morgenthaler, 1974; Pineau and Douce, 1974; Joy and Ellis, 1975; Sprey and Laetsch, 1975, 1976; Priestley and Woodhouse, 1980). The inner and the outer envelope membranes of chloroplasts are completely separated by an electron-transparent interspace. In isolated chloroplasts, the width of this interspace depends on the tonicity of the isolation medium. As in isolated mitochondria (Hackenbrock, 1966) conformational stages of isolated chloroplasts vary from condensed and normal to disrupted forms with decreasing molarity of sorbitol (Heldt and Sauer, 1971).

In chloroplasts of naturally senescing leaves, the first indications of ageing are the presence of osmiophilic globules (Barton, 1966), and reorientation of the thylakoidal system (Hurkman, 1979). The membranes of the grana and intergrana lamellae then become distended and later dissociate into distinct vesicles. Concurrent with these membrane changes, osmiophilic globules increase in size and number and the stroma breaks down. Finally, the chloroplast envelope ruptures

and the plastid contents disperse. However, when we consider the details of the changes and processes involved in the rupture of the chloroplast envelope, we find that several factors, independent or in association, are responsible for this occurrence. Ashton, Gifford and Bisalputra (1963) speculated that swelling of chloroplasts results from changes in the plastid membranes or in membrane associated processes. Anderson and Schaelling (1970) suggested that alteration of membrane permeability causes chloroplasts to become round and turgid. According to Hurkman (1979), in wheat leaves several consistent changes occur during cell senescence which include plasmolysis of cells, swelling of chloroplasts and dilatation of chloroplast lamellae. Harnischfeger (1970) has found that the underlying cause for structural degradation of chloroplast in vitro as seen by morphological appearance, volume changes and alterations in energy transfer seems to be largely the osmotic force which arises from the difference in solute concentration, composition and chemical potential between plastid and isolation medium. According to him during disintegration none of the events described above occur independently but are interconnected and influence each other.

Cellular degradation is more rapid in detached ageing leaves than in naturally senescing leaves. In chloroplasts of detached, ageing leaves the envelope is lost before the lamellar breakdown, but in chloroplasts of naturally senescing leaves, the envelope remains intact until lamellae are reduced to membrane vesicles (Hurkman, 1979). This difference indicates an earlier change in membrane permeability in cells of detached leaves than in naturally senescing

leaves and suggests that membrane integrity is necessary for the complex sequence of changes leading to lamellar breakdown in chloroplasts of naturally senescing leaves. Since less time is involved for chloroplasts to deteriorate in detached, ageing leaves, perhaps the enzymes necessary for lamellar breakdown are not synthesized or are destroyed, probably by vacuolar hydrolases (Matile and Winkenbach, 1971), before they can act on chloroplast membranes.

Osmiophilic globules

The most conspicuous feature of chloroplast breakdown is the accumulation of the osmiophilic globules. Small globules of this type are generally regarded as a normal feature of the stroma but a further increase in the number and size of the globules with age of mature chloroplasts has been observed (Greenwood et al., 1963; Barton, 1966; Hernandez-Gill and Schaedle, 1973; Hurkman and Kennedy, 1975; Hurkman, 1979). Lichtenthaler (1968) suggested that plants can be divided into two groups according to whether senescence of chloroplast results in an increased number of small lipid globules or an increase in the size of globules already present. However, most workers (Hurkman and Kennedy, 1975; Hurkman, 1979) have shown the corresponding increase in both the number and size in the same chloroplast. Hurkman, (1979) also found that the osmiophilic globules formed in chloroplasts of naturally senescing attached leaves were more numerous than in detached leaves where lamella degradation was not marked.

The closeness of osmiophilic globules to thylakoids (Barton, 1966; Dennis et al., 1966) and their appearance as a thylakoid break-

down product (Ikeda and Ueda, 1964; Barton, 1966; Butler, 1967) have led to the suggestion that osmiophilic globules are repositories for lipids resulting from lamella breakdown. However, it is not evident whether the globules also contain lipoidal material from other decomposing membrane systems. They also do not show any tendency to fuse together when pushed into close contact within the plastids (Barton, 1966). Lichtenthaler (1967, 1969a, b) and Barr and Arntzen (1969) have shown a correlation between lipoquinone content and the number of osmiophilic globules in ageing plastids. They found that the concentration of ε *-Tocopherylquinone (lipoquinone) was highest in mature or senescent tissues and the osmiophilic globule fraction was enriched in* ε *-Tocopherylquinone. Additionally, Lichtenthaler (1969a, b) has shown that osmiophilic globules contain lipid soluble carotenoids. The disappearance of chlorophylls from the senescing plastids due either to their decomposition, or, as seems very unlikely, translocated from the plastid. This change in distribution of the pigments in plastids would account for gradual yellowing of the tissues during senescence (Barton, 1966). The globules of mature chloroplasts of Vicia faba (Greenwood et al., 1963) and spinach (Bailey and Whyborn, 1963) have been found to contain a mixture of lipids and other substances, not all of which are to be found in chloroplast membranes. The evidence would thus seem to indicate that although the globules in senescent chloroplasts may in part be membrane breakdown products, or precursors, they probably also represent a general store of insoluble lipid material, not necessarily connected with membrane formation or breakdown (Bailey et al., 1966).*

Arnott and Harris (1973) found vesicles in tobacco chloroplasts which are derived from unique membrane discs in the stroma of developing plastids and termed them "plastosomes". In agreement with Arnott and Harris, Hurkman and Kennedy (1975) showed that the plastosomes are intermediate developmental stages preceding the appearance of osmiophilic globules. Arnott and Harris have also shown a correlation between the appearance of plastosomes and a decrease in granal volume. Similar to osmiophilic globules, plastosomes are partly lipid and may function in the isolation and/or release of metabolites during plastid development in tobacco.

In addition to lipid deposition within chloroplast sub-organelles, osmiophilic material also accumulates in the cytoplasm adjacent to chloroplasts. Mittelheuser and Van Steveninck (1971) reported that the formation of spherical lipid bodies often distended the tonoplast and these bodies were occasionally observed between the chloroplast and plasma membrane. In contrast, cytoplasmic deposits in senescing tobacco leaves are amorphous masses adjacent to chloroplasts, between the chloroplast and cell wall. Mlodzianowski and Kwinthiewicz (1973) observed osmiophilic deposits adjacent to mitochondria, as well as chloroplasts, in detached ageing leaves of Kohlrabi. They attributed deposition of osmiophilic material on the surface of these organelles to the action of lipophanerase.

Lipids and fatty acids

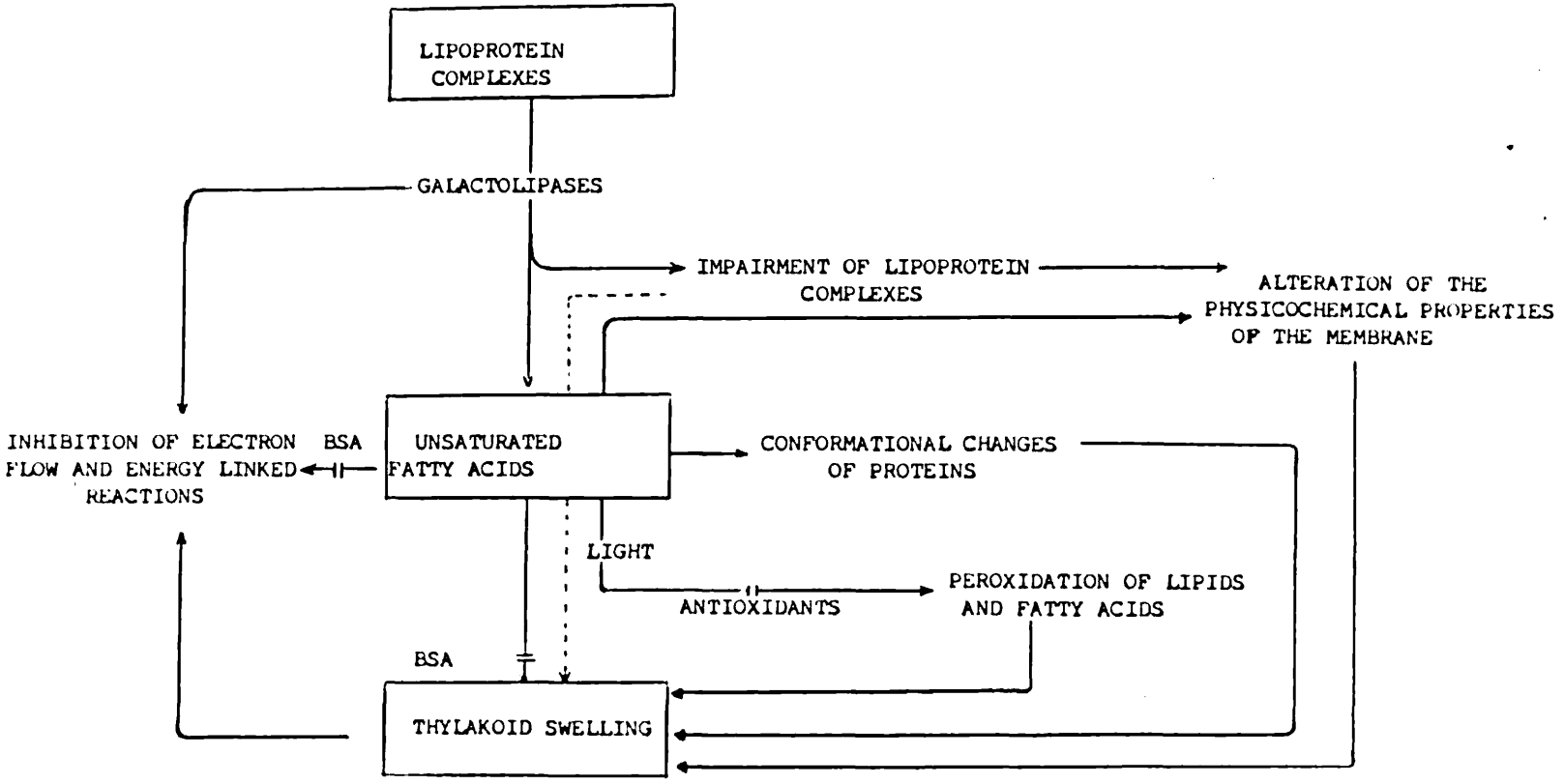
Approximately 50 percent (w/w) of the dry weight of spinach chloroplasts is lipid (Lichtenthaler and Park, 1963) and almost 40

percent (w/w) of these lipids are fatty acids (Coustantopoulos and Kenyon, 1968). Lipids may play important roles in chloroplast membrane constitution, since each membrane (grana, stroma or envelope membrane) consists of a lipid layer sandwiched by two protein layers.

During ageing, a release of endogenous unsaturated free fatty acid takes place due to hydrolysis of lipids (McCarty and Jagendorf, 1965; Siegenthaler, 1969, 1972, 1973; Siegenthaler and Rawyler, 1977). These acids impair the integrity of the thylakoid membranes and their photochemical reactions. Siegenthaler (1972) has shown that when isolated spinach chloroplast is incubated for a few hours under appropriate conditions (darkness or light, 20°C, pH 8, etc.), chloroplasts undergo irreversible swelling. The release of free unsaturated fatty acids coupled with some other factors are responsible for this swelling (as shown in the Text Figure 1). Chloroplast swelling could be due to interaction of fatty acids with the tertiary structure of protein, inducing a conformational change (Mosolov, 1964).

Since glaactolipids are the major lipids in the thylakoid membrane, the release of free fatty acids during the ageing is mostly due to galactolipase activities. McCarty and Jagendorf established that linolenic acid was released due to hydrolysis of galactolipids and that it was inhibitory to the Hill reaction, 2,6-dichlorophenol indophenol (DCIP) and ferricyanide and to cyclic photophosphorylation. Galactolipase activity in spinach chloroplasts was observed by these workers at pH 6 only. It has also been established that both the release of free fatty acids and the loss of Hill activity were pH

TEXT Figure 1. *Reaction with induced thylakoid swelling in the course of ageing. (cited from Siegenthaler, 1972).*


LIPOPROTEIN COMPLEXES
GALACTOLIPASES
IMPAIRMENT OF LIPOPROTEIN COMPLEXES
ALTERATION OF THE PHYSICOCHEMICAL PROPERTIES OF THE MEMBRANE
INHIBITION OF ELECTRON FLOW AND ENERGY LINKED REACTIONS
BSA
UNSATURATED FATTY ACIDS
CONFORMATIONAL CHANGES OF PROTEINS
LIGHT
ANTIOXIDANTS
PEROXIDATION OF LIPIDS AND FATTY ACIDS
BSA
THYLAKOID SWELLING

dependent (McCarty and Jagendorf, 1965; Coustantopoulos and Kenyon, 1968). According to the latter, the free fatty acid content of spinach chloroplasts isolated at pH 5.8-pH 8.0, has been found to vary between 3.1% and 5.5% and the losses of Hill activity were least during incubation at pH 5.8 and greatest during incubation at pH 8. The major free fatty acids released at pH 5.8 were saturated, while those released at pH 7 or 8 were mainly unsaturated acids (α-linolenic acid and hexadecatrienoic acid). The results of McCarty and Jagendorf are in absolute contradiction to those of Coustantopoulos and Kenyon and they attributed the difference to the use of different suspending media and a lower temperature. Coustantopoulos and Kenyon also showed that the increase of temperature of the incubation medium further increased the amount of free fatty acids and decreased the Hill activity which was found to be almost zero at 37°C after two hours of incubation. However, it has been found that there was an increase in the more fluid lipids (unsaturated and short chained) when blue-green algal cells were grown at lower temperature (Fork et al., 1979).

Ageing caused a shift in the pH optimum towards acidity in all the electron flow systems studied by Siegenthaler and Rawyler (1977). This finding is similar to the observation of Punnett (1957) who found that a high pH optimum was characteristic of high Hill activity preparations and that the pH optimum decreased as the chloroplasts lost their activity. Now, it is known that any uncoupling treatment (i.e. ageing) causes a similar acid shift of electron flow (Trebst, 1974). The inhibition of electron transport during ageing could also be the result of the loss of lipids themselves which are essential

for the functioning of the membrane (Siegenthaler and Rawyler, 1977). The release of lipids, but not of free fatty acids, is enhanced under alkaline conditions (Heise and Jacobi, 1973) and may account for the greater inactivation of electron flow activity at pH 8 than at pH 7. However, Shaw et al. (1976) have shown recently that when precautions are taken to prevent binding of fatty acids released by a galacto-lipid lipase to subchloroplast particles, large amounts of lipid may be removed without a marked effect on electron flow and thylakoid membrane structure.

SPECIFIC EFFECTS OF LIGHT AND DARK

Most of the ultrastructural studies of chloroplasts have been done with respect to light and dark responses. Light exerts a decisive effect on the development of the plastids since pigment synthesis, membrane synthesis, and synthesis of other plastid components are induced by light. The prolamellar body might serve as a system for membrane storage under conditions where an extensive membrane area would not be used, that is where photosynthesis is limited. Light conditions which are unfavourable for photosynthesis, e.g. low light intensity or darkness, favour formation of prolamellar bodies or paracrystalline centres.

A. High light intensity

The available data (viz. published electron micrographs of chloroplasts) from plants grown under different light intensities suggest that at higher light intensities there may be fewer lamella per chloroplast and a smaller proportion of the lamellae occurs in the grana. Ballantine and Forde (1970) have found in leaves of Glycine max grown in a controlled environmental cabinet, that under higher light intensity (22 mW Cm^{-2} irradiation) the chloroplasts had very rudimentary or small grana, while under lower light intensity (9 mW Cm^{-2} irradiation) the chloroplasts had well formed grana. They found a striking contrast between the abundance of grana in leaves which developed under low light intensity, and the paucity of grana in leaves from light intensities similar to those outdoors and they noticed good agreement between chloroplast fine structure of cabinet-grown plants and those grown out-

doors or in the glasshouse. They also noticed that chloroplasts from high temperature (27.5°C - 22.5°C) and high light had grana consisting of two or three appressed lamellae, while grana from lower temperature (20.0°C - 12.5°C) were confined to occasional lamellae overlaps. Björkman and co-workers (1972) also reported similar findings in Atriplex patula *grown under 20, 6.3, and 2 mW Cm^{-2} irradiations. Cran and Possingham (1974a) found that when young spinach leaf discs (having immature chloroplasts) were cultured in high intensity white light, the grana increased in size and frequency, and the chloroplasts increased in number and length; whereas when the mature leaf discs (having mature chloroplasts) were cultured under these same light conditions there occurs a disorganization of the original mature chloroplast and loss of chloroplast lamellae. Numerous osmiophilic droplets appeared within the stroma and, in a few chloroplasts, membrane-bound crystals may be detected. In a similar experiment with high light intensity, Cran and Possingham (1974b) observed that chloroplasts from mature leaf disc culture rapidly lost their structural integrity. After two days a considerable increase in the number of starch grains was observed. After 7 days of culture the chloroplasts were degenerate and took two forms. In the first, there was a considerable loss of thylakoidal material with no granal organization remaining. The thylakoids which persisted were simple and often swollen. Starch grains were present in lesser number than previously and numerous osmiophilic droplets were observed. The second type of plastid was one in which there had been considerable disruption of the entire plastid structure on culture. In such organelles all membranes*

including the plastid envelope had a thickened, coarse appearance, starch grains remained in situ but osmiophilic droplets were seldom seen.

B. Low light intensity

Plants grown in low light intensities show well-developed chloroplasts with large grana. The extreme development of chloroplasts in plants grown in low light intensities has been reported in the deep-shade plant Alocasia macrorrhiza (Anderson et al., 1973), which receives an average irradiation of 0.1 mW Cm^{-2} and possess massive chloroplasts, each with a large amount of lamellae and very large grana stacks having a high ratio of total length of grana to stroma. Chloroplasts of such plants contain more chlorophyll per chloroplast, an increased proportion of chlorophyll 'b' to chlorophyll 'a' and a very big increase in the amount of grana lamellae per chloroplast as compared for example, with Atriplex patula (Björkman et al., 1972) or Phaseolus vulgaris (Ballantine and Forde, 1970) which are not specially adapted to shade conditions. The extent of grana formation appears to be related to the total chlorophyll content of the chloroplast. Grana formation may simply be a means of achieving a higher density of light-harvesting assemblies and hence a more efficient collection of light quanta. The high chlorophyll content of the shade plant chloroplasts is no doubt a consequence of the need for these plants to capture all available light quanta reaching the leaves on the floor of the rain forest. Shade plants are also enriched in chlorophyll 'b' relative to chlorophyll 'a', as compared with sun species. This increases further the light-harvesting capabilities of

the shade plant by extending the wavelength range over which quanta are absorbed (Anderson et al., 1973).

C. Darkness

When Cran and Possingham (1972a, 1974a) cultured discs of young spinach leaves in darkness, there was a limited increase in plastid number and a breakdown of the granal organization. Within four days the thylakoids were noticeably swollen and after 14 days in culture most of the original thylakoids had vesiculated and prominent prolamellar bodies were produced within the stroma.

However, when mature leaf discs were cultured in darkness the thylakoid structure persisted for a longer period (Cran and Possingham, 1974a, b), although chlorophyll was lost to the same extent as in white light. After 7 days in darkness much of the granal organization of the chloroplasts remained. After a further 7 days the thylakoids disappeared, being replaced by numerous osmiophilic droplets and membrane-bound crystals.

Under dark conditions, although chlorophyll was lost to the same extent as in light, the chloroplasts retained their structural integrity for a longer period (Cran and Possingham, 1974a, b). It would appear that chlorophyll loss and membrane breakdown are not necessarily closely correlated. It is possible that light reactions of photosynthesis accelerate membrane destruction at high light intensity (Cran and Possingham, 1974b).

SPECIFIC EFFECTS OF TEMPERATURE

Temperature, light and other environmental factors can affect the chloroplast ultrastructure of developing and mature leaves of normal plants. Despite ample work on the effects of conditions of illumination on chloroplast ultrastructure during development, ageing, and senescence, very little is known concerning the influence of temperature. A general inhibition of the normal chloroplast production under high temperatures (above 32°C) has been shown by various workers (Pringsheim and Pringsheim, 1952; Feierabend and Mikus, 1977), while low temperatures (10°C - 16°C) can inhibit chloroplast development in chilling cold-sensitive plants such as sugarcane (Faris, 1927), sorghum (Slack et al., 1974), and maize (McWilliam and Naylor, 1967). Temperatures intermediate between these extremes may also affect differentiation of the photosynthetic apparatus. Erriksson et al. (1961) have described three steps of grana formation: (1) tubules of the prolamellar body and transformed into vesicles, (2) the vesicles are dispersed and arranged in primary layers by a higher energy requiring, temperature independent process, and (3) the vesicles fuse into discs and their aggregation into grana is correlated with chlorophyll synthesis, has a high energy requirement and is temperature dependent. Aside from considerations of genotypic adaptation to temperature, many plants show an ability to acclimate to the prevailing temperature regime during their growth and the rate and temperature optimum of photosynthesis in leaves and the leaf anatomy can vary according to the growth temperature and light intensity (Björkman and Holmgren, 1963; Balantine and Forde, 1970; Forde et al., 1975; Slatyer, 1977).

A. Plant Growth

The morphological development of the leaf is controlled by both genotype and environment. This allows a degree of plasticity which may be controlled to some extent by the varying internal nutrients and hormonal environment of the leaf (Treharne and Eagles, 1970; Atkin, Barton and Robinson, 1973). Temperature affects the growth of the whole plant mainly through its effect on respiration and on morphology, the primary photosynthetic reactions being temperature independent. The temperature at the shoot apex and the root exerts a large effect on leaf extension (Watts, 1972; Peacock, 1975; Woodward, 1979) which is also influenced by the temperature in the leaf region (Milthorpe and Davidson, 1966) and thus air temperature, through its influence on cell extension, will influence leaf extension. Woodward (1979) has shown that in Phleum bertolonii DC and Phleum alpinum L., an increase in temperature results in a decline in cell area and an increase in cell number, also the period of extension for a particular leaf decreases with increasing temperature, through the higher frequencies of cell division. The effects of temperature on the number and area of the leaves alter the leaf area ratio, and changes in leaf thickness alter the rate of photosynthesis per unit leaf area (Friend et al., 1962; Woodward, 1979). The net effect of an increase in temperature will therefore depend on the extent of dry matter loss through increased respiration, and dry matter increase through increases in the leaf area ratio. Feierabend and Schrader-Reichhardt (1976) working on rye leaves and Feierabend and Mikus (1977) working on wheat and barley found that although growth of leaves (elongation and

extension) was not severely affected under high temperature conditions, light grown leaves contain less protein and dry weight at 32°C than at 22°C. They expected this because of the lack of chloroplast protein synthesis which under permissive conditions at 22°C, is highest in light. Furthermore, it appears that the lack of photosynthetic carbohydrate production becomes a major limiting factor at 32°C. Parallel to these results Ballantine and Forde (1970) had shown that soybean plants under high temperature conditions grew more rapidly than those at lower temperature, had a high final dry weight, and showed a higher rate of leaf expansion on the main stem.

B. Chloroplast Ultrastructure

Very little information is available concerning the chloroplast ultrastructure at the elevated temperatures from the normal growth temperature. Recent evidence suggests that biomembranes are the most heat-susceptible sites in cells (Heber and Santarius, 1973). Santarius (1974) showed that the thylakoid membranes are much more susceptible to heat than soluble enzymes in chloroplasts. Smillie et al. (1978) have shown that when barley plants are grown at 32°C their chloroplasts contain large multilamellar grana more disorientated with respect to one another than grana from chloroplasts in plants grown at lower temperatures. Guard cell thylakoids appeared to be reduced in number and some were swollen. In the chlorotic leaf parts no grana were found and the plastids from this region contained, besides the matrix and often large accumulations of osmiophilic globules, only single thylakoids, vesicles and tubular structures.

Ballantine and Forde (1970) found that the soybean chloroplast responded differently to controlled environmental conditions like light and temperature. According to them the grana are more numerous under low light and high temperature conditions than under high light and high temperature. Also they found less starch under high temperature at all light conditions.

Since photosynthetic reactions take place in the lipo-protein matrix of thylakoid membranes, the physical phase of the membrane protein and lipids which changes by the effect of temperature must play an important role in the proper functioning of these reactions.

Protein

A decrease in protein content was observed by Feierabend (1970a) and later by Feierabend and Schrader-Reichhardt (1976) in rye leaves, at high temperature conditions, because of lack of chloroplast protein synthesis. The most convincing explanation given for this is the prevention of 70S ribosome formation at the elevated temperatures, since, in the absence of 70S ribosomes, chloroplast protein synthesis can no longer proceed. The deficiency of chloroplast ribosomes at higher temperatures was found to be independent of light conditions as it occurred also in completely dark-grown seedlings (Feierabend and Mikus, 1977).

The first electron microscopic indication that the formation of 70S ribosomes is prevented at high temperature came from the work of Feierabend et al. (1969) in rye seedlings. This was followed by Feierabend and Schrader-Reichhardt (1976), Hans-Achin and Feierabend

(1976) and Rademacher and Feierabend (1976) who found that the gradual decrease in the 70S ribosome content and, in parallel, of the chlorophyll content in the leaf tips of rye was accompanied by a corresponding decrease of the size and of the internal membrane structure of the plastids, until, finally, in the chlorotic cells grana were completely absent. The few, sometimes aggregated, vesicles and tubules of the chlorotic plastids may be, at least partially, related to the membrane material of the prolamellar bodies.

Although the biogenesis of the thylakoid and grana membrane architecture is not yet understood in detail, it is clear that several proteins of the thylakoid membranes and of the chlorophyll-protein complex must be synthesized by chloroplast protein sythesis on 70S ribosomes (Machold and Aurich, 1972; Eaglesham and Ellis, 1974; Ellis, 1975; Nielson, 1975). Most of the envelope proteins were shown to be synthesized on cytoplasmic ribosomes (Joy and Ellis, 1975; Feierabend and Schrader-Reichhardt, 1976). This is further supported by the finding that 70S ribosome-deficient plastids of rye leaves grown at 32°C were similar to the plastids of 70S ribosome-deficient mutants (Börner et al., 1972; Lerbs and Wollgiehn, 1975) both being surrounded by a normal double-membrane envelope. The swelling of the chloroplasts under high temperature conditions probably occurs as a result of conformational changes in the actomyosin-like protein of chloroplasts, which occurs through the action of free fatty acids on the tertiary structure of the protein (Mosolov, 1964).

Lipids

Lipids show a change of phase with a change of temperature. When

lipids are in lamellar or vesicular structures in water at low temperatures they are in the solid state and are in the liquid-crystalline (smectic) state at high temperatures. It has been established for model membranes that the temperature of the phase transition from the liquid-crystalline state to the solid state depends upon the lipid species as well as the fatty acid composition (Barratt et al., 1969; Ladbrook and Chapman, 1969; Träuble, 1971; Chapman, 1974). In general, the higher the degree of unsaturation of the fatty acid the lower the temperature for phase transition. A similar correlation between the phase transition temperature and the fatty acid composition has been found in biological membranes where drastic changes of physiological activities are observed at the phase transition temperatures. This has been observed as changes in the rates of growth, transport and respiration in the bacterium Escherichia coli (Overath et al., 1970; Wilson et al., 1970), as well as by changes in rates of phosphorylation and respiration in mitochondria (Lyons et al., 1964; Kemp et al., 1969; Lyons and Raison, 1970; Raison et al., 1971).

In the photosynthetic systems of chloroplasts or thylakoid membranes, however, there has not been a good demonstration of a relationship between the transition of the physical phase of membrane lipids and photosynthetic activities. In chloroplasts of higher plants and most algae there is a high content of unsaturated lipids (Benson, 1964). This suggests that the transition of the physical phase of lipids in these plants would occur far below room temperatures. In the blue-green alga Anacystis nidulans, however, there is a high degree of saturation of the major fatty acids (Hirayama, 1967),

suggesting that the transition of physical phase of lipids may occur at room temperatures. Moreover, it has been found by Holton and co-workers (1964) that the fatty acid content of Anacystis changes with room temperature. Fork et al., (1979) have shown that in blue-green alga Synechococcus lividus there is a general increase in the more fluid lipids, in all of the lipid classes when the cells were grown at lower temperature.

The effect of heat on partial destruction of chloroplast membrane lipids may presumably be owing to the activation of lipases (Molotkovsky and Zhestkova, 1965). They also recorded derangement of the chloroplast structure (swelling) and the simultaneous suppression of photochemical activity (inhibition of Hill reaction) which they found is related not so much to the destruction of the lipid ingredients of the membranes as to the direct action of the free fatty acids so formed.

Chlorophyll

The chlorophyll content of seedling leaves in which the initially present protochlorophyll has been converted to chlorophyll 'a' by the action of light, is only a small fraction of that present in the mature leaf. There is a short interval during which no further chlorophyll is formed, which may be related to morphological changes in the developing chloroplasts (Withrow et al., 1956). The rate of further accumulation of chlorophyll 'a' and the beginning of chlorophyll 'b' accumulation is then approximately autocatalytic (Blaauw-Jensen et al., 1950), reaching a steady level in the mature leaf. At this time the chlorophyll is in a state of turnover, as shown by the use of radioactive tracer

(Godev and Shlik, 1955). The maximum chlorophyll content reached in the leaf thus represents a balance between the rates of formation and destruction.

The accumulation of chlorophyll 'a' takes place through the action of light on protochlorophyll which is continuously formed in the leaf (Koski et al., 1951). In etiolated barley leaves (Virgin, 1955) the rate of protochlorophyll production is itself dependent on light intensity. Smith and Young (1956) suggested that the autocatalytic nature of chlorophyll accumulation results from the photosynthetic production of protochlorophyll precursors by the chlorophyll already present.

While studying the effect of temperature on chlorophyll accumulation in etiolated wheat seedlings, Lubimenko and Hubbenet (1932) found that the highest rate of formation and greatest chlorophyll content was found at 26°C and the lowest amount of chlorophyll was found below 4°C or above 44°C. They thought this effect of temperature may be brought about through the temperature control of protochlorophyll formation. Virgin (1955) also reported very similar temperature coefficients for this reaction. The failure of chlorophyll development in Marquis wheat at 35°C has been attributed to a block, either in the pathway of chlorophyll synthesis immediately before protochlorophyll, or in the hydrogen donor system involved in the protochlorophyll-chlorophyll transformation (Friend, 1960). A similar phenomenon also takes place in some strains of Euglena, where chlorophyll formation is prevented at a temperature of 34°C - 35°C (Brawerman and Chargaff, 1959).

Feierabend and Mikus (1977) recorded a severe inhibition of chlorophyll accumulation in the leaves of various crop plants like wheat, oat, barley at the temperature ranges between 28°C and 34°C where the leaf growth was still normal. However, in peas they observed a complete chlorosis of leaves at 33°C, with a general reduction in leaf growth. In all the above instances, they noted that the high temperature-grown chlorotic leaves were deficient of chloroplast rRNA indicating a deficiency of chloroplast ribosomes. This has been discussed above in relation to the effects upon protein synthesis (p. 50).

TEXT Figure 2. *A 6-week old spinach plant growing in a pot at 22°C in environmental controlled cabinet.*

C H A P T E R II

MATERIALS AND METHODS

GENERAL CONSIDERATIONS AND ELECTRON MICROSCOPY

1. Plant Material

The plant material selected for the present study is Spinacia oleracea L., var. Sigma leaf, commonly known as spinach, which belongs to the family Chenopodiaceae. This is a small herbaceous plant (up to about 30 cm) and is grown commercially because of its high nutritive value. The selection of this plant for the present study was based on the following considerations.

(i) This plant has a short vegetative phase (about 2.5 months) and therefore the plant material is readily available.

(ii) Since the plant is a small herb, it can be grown in controlled environment cabinets.

(iii) The plant grows well under controlled conditions.

(iv) Each cell of the green leaf contains many chloroplasts which possess well developed lamellar system (both stroma and grana).

(v) The chloroplasts of this plant show normal ultrastructure when the plant is grown at room temperature, therefore it facilitates the study of high temperature effect on chloroplast ultrastructure.

For all the investigations of the present study, only the intact attached leaves were used because of the following advantages.

(i) The whole plant can grow for a longer period at temperatures sufficiently higher above the normal growth temperature of this plant.

(ii) In the intact leaf the ultrastructural changes of the plastid can be followed during the natural senescence and can be compared

with the ultrastructural changes of the plastids caused by high temperature conditions.

(iii) The ultrastructural changes of the plastids can be followed completely during the whole vegetative phase of the plant.

(iv) The effect of various temperatures on the ultrastructure of the plastids of the newly differentiated leaves can be studied only by using the whole plant.

2. *Growing Conditions*

(a) *Selection of plant growth temperatures*

Preliminary to the main experimental studies a series of observations were made on seed germination and plant growth in the controlled environmental cabinets at temperatures ranging from 20°C to 35°C by increments of 2°C. It was noticed that both seed germination and plant growth are optimal at 22°C temperature in 14 hours light/10 hours dark as compared to other temperatures. Therefore 22°C is employed as the normal temperature for both seed germination and plant growth in spinach for the present study. At a temperature of 35°C young plants survive for only a week or two and the seeds do not germinate at all at this temperature. In between these two temperature limits (22°C and 35°C) two more temperature conditions were selected for the present study. These are 25°C, which gives a slight increase of temperature above the normal growth and 30°C, which appears to be about the highest temperature at which growth of the spinach plant can be maintained.

(b) *Raising the seedlings*

Seeds were obtained from Messrs. Suttons Seeds Limited during the years 1977, 1978 and 1979. Seeds were sown in small pots (9 cm X 15 cm) in a mixture of John Innes seed compost and placed in growth cabinets under long day conditions (14 hours light/10 hours dark). Day temperatures were 22°C and night temperatures about 20°C. Light intensity at the level of plants was about 7500 Lux. The relative humidity of the cabinet was maintained at 80% with a humidifier.

Seedlings usually emerge from the soil within 6 - 7 days. Until the fourth day the cotyledons, the primary leaves and the hypocotyl hook are all enclosed inside the seed coat after which they start emerging out of the seed coat and the soil.

For all the investigations of the present study only the tips of the leaves or the primary leaves were used as the experimental material for electron microscopy because of the following considerations.
(i) The plastids are mature at the tip of the spinach leaf and immature at the base (Cran and Possingham, 1972b).
(ii) The visual observations of the present study show that the tip of the spinach leaf is highly sensitive to high temperatures and is the first to be affected by high temperature (TEXT Figure 3).

The behaviour of the whole plants and the leaves in particular was observed carefully from all the plants under each temperature condition, during the vegetative phase and also during senescence and the photographs were taken at important stages of sampling.

TEXT Figure 3. *A 6-week old spinach plant grown at 22°C for the first four weeks followed by its transfer to 35°C for two weeks. The plant shows drying of the leaf tips under high temperature conditions.*

3. Electron Microscopy

(a) Fixation

Tissue from the tip of the leaf not more than 1 mm square was cut with a sharp blade on a sheet of dental wax in a drop of fixative. The cut tissue was then transferred directly to the fixative. All the fixing operations were done at room temperature.

The fixative used was 6% glutaraldehyde buffered by 0.1/0.15M phosphate at pH 7.2. The vials containing fixative were subjected to reduced pressure by means of a vacuum pump in order to remove air from the samples which otherwise float on the surface of the fixative. The specimens were left for 2 - 4 hours in glutaraldehyde and then they were washed very thoroughly through several rinses of 0.1/0.15M phosphate buffer at room temperature to remove excess glutaraldehyde with a minimum of four rinses in one hour and then were left overnight in the last rinse at $4^{o}C$. The specimens were next post-fixed for one hour in 1% osmium tetroxide buffered to pH 7.2 at room temperature.

1% osmium tetroxide was prepared in 6% glutaraldehyde (prepared from a 25% solution as supplied by TAAB laboratories) from 2% solution supplied by Messrs. B.D.H. in 10 ml. vials.

(b) Dehydration

After treatment with osmic acid the specimens were then rinsed thoroughly in distilled water with a minimum of two rinses, the first one rapid and the second for 15 minutes. Then the specimens were passed through grades of acetone series followed by propylene oxide

as follows:

30% acetone	*15 minutes*
50% acetone	*15 minutes*
70% acetone	*15 minutes*
90% acetone	*15 minutes*
100% acetone, two changes	*30 minutes*
50:50 propylene oxide:acetone	*10 minutes*
100% propylene oxide, two changes	*15 minutes each*

Propylene oxide was added gradually to the specimens in 100% acetone, and brought to a concentration of about 50% propylene oxide over a period of about 10 - 15 minutes.

(c) Infiltration and embedding

Resin mixture

The embedding resin introduced by TAAB laboratories (Reading, England) was selected for all experiments. This is a developed epoxy resin without any published composition. This resin is claimed to have the following valuable characteristics:

1. *relatively low viscosity*
2. *good cutting characteristics*
3. *can be used with uncoated grids*
4. *thermostability with little or no shrinkage upon polymerization*

A wide range of hardnesses could be obtained by using different proportions of the hardners, DDSA and MNA. BDMA was used as the accelerator. For most of the work in the present investigation the resin mixture used was made up according to the following schedule:

TAAB resin	*20 g.*
DDSA	*10 g.*
MNA	*10 g.*
BDMA	*0.8 g.*

Process of infiltration

After two changes of propylene oxide, the resin mixture was added to the tube containing specimens according to the following schedule:

10:1 propylene oxide:resin	*overnight (on rotator)*	
50:50 propylene oxide:resin	*4 hours*	*"*
100% resin-specimens removed to 100% resin	*overnight*	*"*
Fresh resin	*one day*	*"*

Fresh resin was filled in embedding moulds, specimens were placed in the centre of fresh resin in embedding moulds with desired orientation of specimens.

Polymerisation

The moulds filled with resin and containing specimens were placed in an oven at 60°C for 40 - 48 hours.

The specimen blocks which were released by flexing the moulds were later cut out and stuck to resin blanks with araldite adhesive for mounting in the microtome.

(d) Sectioning

Sections were cut with a glass knife on a Huxley ultramicrotome. Glass knives used were made on an LKB knife maker. Sections were

floated on distilled water. Sections which showed silver/gold to grey interference colours were picked up on copper grids (150 and 200 mesh) coated with formvar film. Some sections were also picked up on freshly-cleaned uncoated copper grids (200 and 300 mesh). Sections showing grey to silver interference colours were generally used, but difficulty in handling old, starchy, or protein-filled material occasionally necessitated the utilization of pale gold sections.

(e) Staining

The sections were stained on the grids, usually with lead citrate (Reynolds, 1963) for 2 - 10 minutes,
(i) The sections of the primary leaves were pre-stained with 1% uranyl in 70% acetone were stained with lead citrate only for 2 - 3 minutes.
(ii) Other sections were stained only with lead citrate for 5 - 10 minutes.

All staining was carried out by floating grids face downwards on a drop of stain placed on a dental wax slab, kept in a covered petri dish either with filter paper soaked in saturated solution of sodium hydroxide or with sodium hydroxide pellets. The staining with lead citrate in the presence of sodium hydroxide was carried out to achieve an atmosphere of low CO_2 tension, which might otherwise lead to contamination with lead carbonate which appears as black insoluble deposits on the sections.

After the staining, the section containing grids were rinsed thoroughly with distilled water, usually 20 - 30 gentle squirts from

a wash bottle were sufficient for proper washing of sections, after which the grids were touched on the edge of a filter paper disc to drain them.

(f) Examination in microscope

Sections were examined in an AEI (Associated Electrical Industries) Corinth 275 microscope.

Electron micrographs were taken on Ilford N4 E 50 and the films were processed in Ilford Bromophen and Hypam. All the photographs for this thesis were printed personally in the Department of Botany of Bedford College.

C H A P T E R III

THE DEVELOPMENT OF CHLOROPLASTS IN THE PRIMARY LEAVES OF SPINACH AT NORMAL AND HIGH TEMPERATURE AND IN DARKNESS

INTRODUCTION

The aim of this experiment has been to investigate the behaviour of the chloroplast ultrastructure during its development in the greening primary leaves of spinach seedlings under different temperature and light conditions. During the course of these investigations it was observed that both seed germination and plant growth of spinach is optimal at 22°C temperature as compared to other temperatures ranging from 20°C to 35°C (see Methods p. 59 above). Therefore 22°C is employed as the "normal" temperature for both seed germination and plant growth in spinach under the conditions used in this laboratory. The highest temperature at which 'normal' growth will occur is 30°C; at 35°C the seeds do not germinate at all. The following combinations of temperature, light and darkness were used for this experiment:

(i) 22°C temperature at 14h light/10h dark (L:D)

(ii) 22°C temperature at complete darkness (DD)

(iii) 30°C temperature at 14h light/10h dark (L:D)

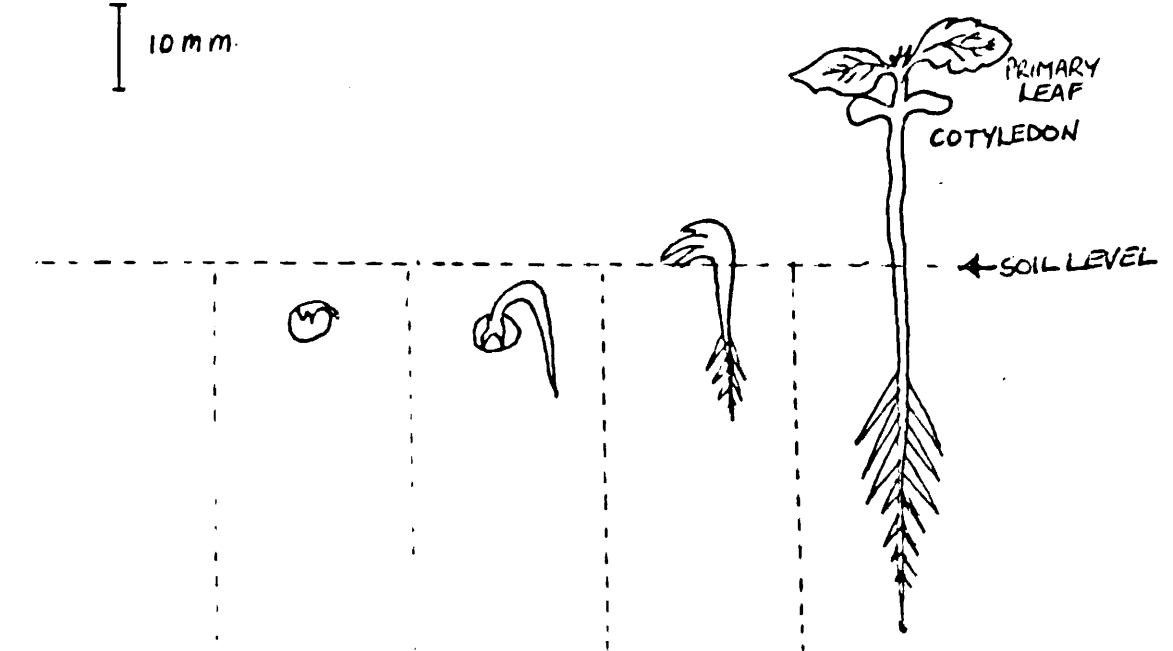
Synchronous developmental changes associated with lamellar production in the plastids of the primary leaves of spinach last for a period of one week following germination and result in the change of proplastids into mature chloroplasts. Ultrastructural changes in the developing spinach plastids were observed in detail during this period of rapid synchronous development under the conditions (i, ii, iii) noted above. All the samples for electron microscopy were taken from the tips of the primary leaves.

The cotyledons and primary leaves of spinach remain inside the

TEXT Figure 4. *Stages in the development of the seedling of Spinacia oleracea L. The stages refer to sampling stages.*

SCALE SIZE

10 mm



DAYS	4	5	6	7
STAGE	I	II	III	IV

seed coat until the fourth day from sowing, after which they start to emerge and by the seventh day the primary leaves have expanded and are green in colour. The stages taken for the study of plastid ultrastructure of the primary leaves of spinach following germination are as shown in TEXT Figure 4.

OBSERVATIONS

Stage I - Under all three growing conditions the seeds remain below the soil during the first four days of germination. Until the fourth day, the hypocotyl hook is still enclosed inside the seed coat, and the cotyledons and the enclosed primary leaves are ivory white in colour. Plastids of the primary leaves under the 14h light/10h dark regime at both 22°C and 30°C and in complete darkness are undistinguishable at this stage but after this there is a marked divergence in their ultrastructure.

The cells of the primary leaves contain many mitochondria and lipid droplets dispersed in the cytoplasm (Fig. 1). Amyloplasts appear to be grouped around the nucleus. These plastids consist of individual starch grains, or clusters of two or three enclosed by a double membrane. Some of the amyloplasts also show the presence of a few lamellae which at this stage do not follow any regular arrangement (Fig. 2). An association between the plastid and the endoplasmic reticulum was observed in some of the cells during this stage of seedling growth at 22°C temperature under light/dark conditions only (Fig. 3).

Stage II

22°C and 30°C L:D

The cell ultrastructure is still similar at these two temperatures. The plastid envelope membranes are not clearly defined (Figs. 4, 5, 6) as compared to those of mature spinach chloroplasts. Lipid droplets are no longer present in the cytoplasm. Each plastid contains one or

more starch grains, often very large (1.0 µm long) and occupying most of the plastid space. Granal stackings are not present in any plastid at this stage of development. Very rarely, in plastids developing at 22°C L:D, prolamellar bodies were observed (Fig. 5). The prolamellar bodies formed at this stage do not appear to have any crystalline structure.

22°C DD

Each plastid at this stage contains two or more large starch grains and the plastid envelope membranes are not well-defined (Fig. 7). Prolamellar bodies are common and appear to consist of an accumulation of vesicles. No evidence was found for the formation of vesicles by the invagination of the inner plastid membranes as described by Hodge et al. (1956), Muhlenthaler and Frey-Wyssling (1959), Röbbelen (1959), von Wettstein (1959), Erriksson et al. (1961). The cell cytoplasm contains many mitochondria and some cytoplasmic whorls (Fig. 7).

Stage III - At this stage, six days from sowing the seed, the seedling just emerges out of the soil and is slightly green in colour.

22°C L:D

Some plastids appear to have the form usually referred to in the literature as 'amoeboid' (Whatley, 1974) in that they are elongated or cup-shaped with irregular outlines (Figs. 8, 9). Such amoeboid plastids were not observed under complete darkness or at high temperature in the light/dark grown seedlings. Most of the plastids at this stage generally appear to be elongated or rounded (sub-spherical) in shape, although sometimes constricted but without any irregularity

of outline (Figs. 9, 10, 11). Internally the extent of stroma lamellar system gradually increases during this stage as does the frequency of occurrence of the incipient grana. The lamellae are perforated. The vesicles which appear to be produced from the plastid lamellae, rather than invaginations of inner plastid membrane are common and persist during this stage. Starch grains are present in most of the plastids other than the amoeboid type.

$30^{o}C$ L:D

At $30^{o}C$ the plastids are mostly rounded (sub-spherical) and clearly enclosed in a double-membrane envelope. The stroma lamellae show an increase with grana usually two or three thylakoids in thickness. Both stroma and grana lamellae show a characteristic swelling which is of much greater extent in the thylakoids than in the stroma lamellae (Fig. 12). Small vesicles are common, and these plastids possess a small number of osmiophilic globules.

$22^{o}C$ DD

In complete darkness at $22^{o}C$ the plastids are also spherical in shape. The plastid envelope which is double-membraned has a wavy outline (Fig. 13). The stroma lamellae are perforated and there are no grana. The lamellae show a characteristic swelling at certain places. Small vesicles are also common in the plastids. However, the most characteristic features of plastids under these conditions of development are the many large osmiophilic globules and the prolamellar bodies. The prolamellar bodies have a poorly developed, perhaps non-crystalline, structure (Fig. 13). The cell cytoplasm contains many mitochondria and Golgi bodies (Fig. 13).

Stage IV

22°C L:D

By day seven the primary leaves of light/dark grown seedlings at 22°C have emerged completely out of the soil, have expanded considerably and are green in colour. Plastids at this stage generally appear slightly elongated and bi-convex in shape. Internally the stroma lamellar system, although well developed, shows discontinuities and swelling at certain places. Many small grana with stacking up to five are connected by short fret-membranes. Starch grains are commonly present; a small number of vesicles can be seen in some of the plastids (Figs. 14, 15). Osmiophilic globules, when present, are very small in size. Plastid division by constriction is also commonly seen (Fig. 15).

30°C L:D

The leaves at 30°C are a paler green than at 22°C. Internally the plastids now show many destructive processes. The lamellae are severely broken between grana (Fig. 17). The number and size of starch grains are generally reduced while in some plastids no starch grains are found. Plastids under these conditions assume a variety of shapes appearing bi-convex, (Fig. 16) or nearly spherical and are swollen. Some of the plastids show bursting of the envelope and thus the contents are liberated into the cell cytoplasm (Fig. 17). Plastid division was not observed in these conditions.

22°C DD

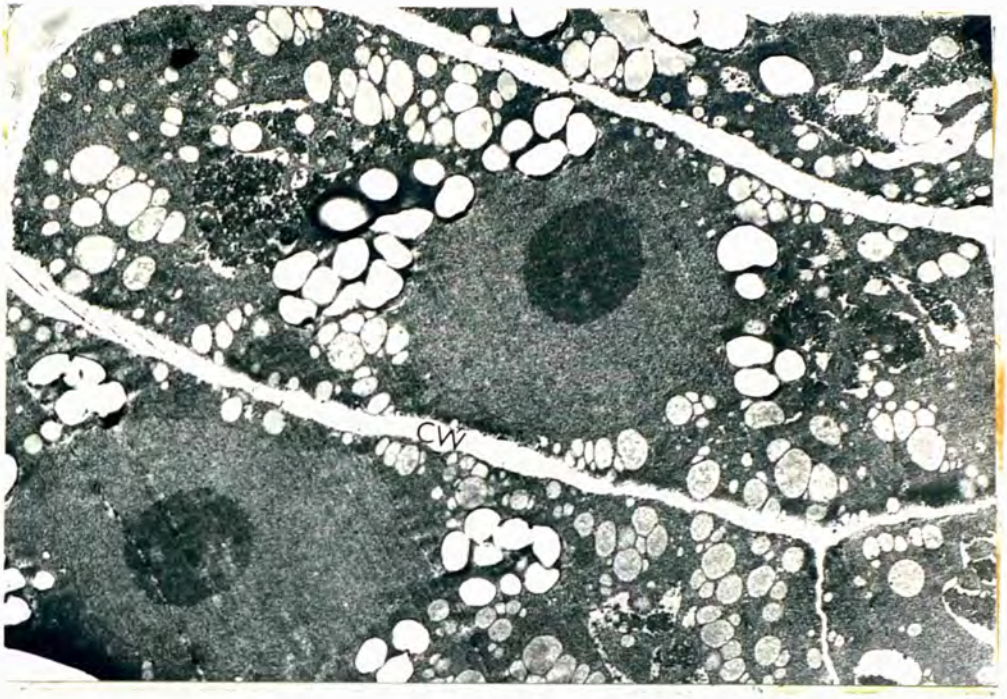
In completely dark grown seedlings (22°C) it is observed that the plastids in the primary leaves retain a typical etioplast ultrastructure.

The etioplast ultrastructure differs in many ways from the plastid ultrastructure observed in 22°C light/dark conditions. From the cross-section the etioplast appears to be spherical or bi-convex in shape. Although there is an extensive development of perforated stroma lamellae and many small incipient grana (Figs. 18, 19), the stroma also shows many clear areas. The prolamellar bodies are large, highly organized structures, typical of etioplasts. Etioplast division is also commonly observed (Fig. 20). Some of the etioplasts at this stage of development contain a few osmiophilic globules. Two or three large starch grains are present in almost all the etioplasts; and there are many vesicles (Figs. 18, 19) which are much bigger than those produced at 22°C in light/dark conditions.

PLATE 1 — *Transverse section through primary leaf of a young seedling four days after sowing at 22°C under L:D conditions.*

Fig. 1. — *Cell structure showing starch grains clustered around the nucleus.*
X 6,075.

Fig. 2. — *Amyloplast surrounded by double-membrane and showing the presence of few lamellae.* (L)
X 25,800.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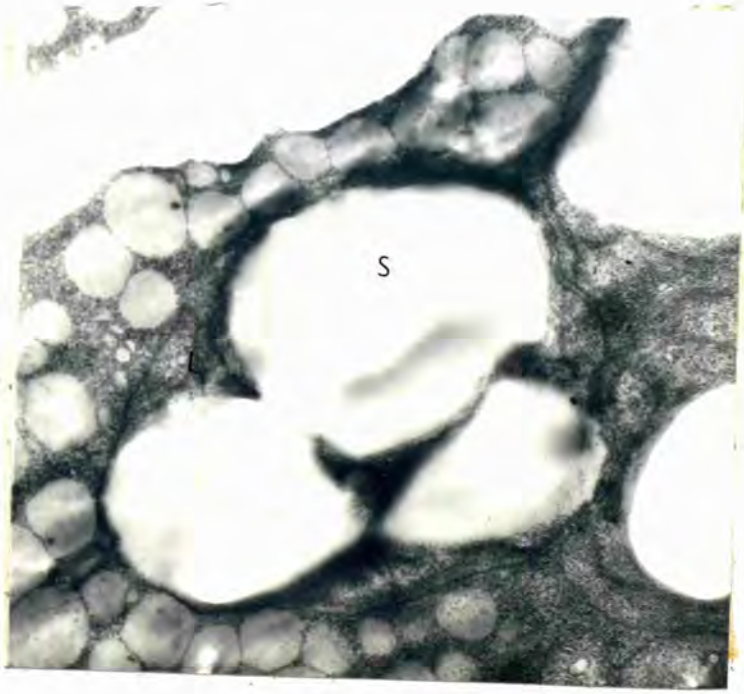

S

PLATE 2 *Section through the cell of the primary leaf of a young seedling four days after sowing at 22°C under L:D conditions.*

Fig. 3. *A plastid surrounded by Endoplasmic Reticulum.*
X 103,200.

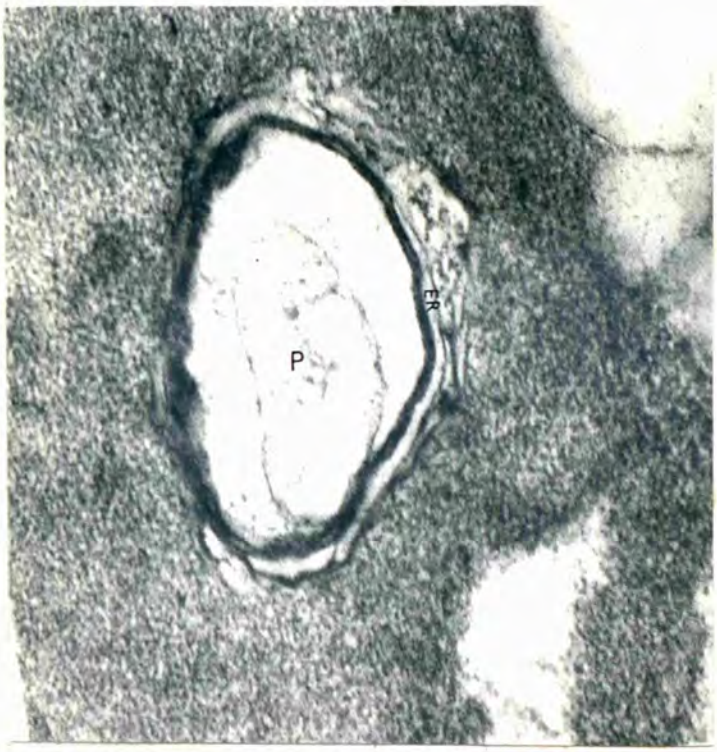
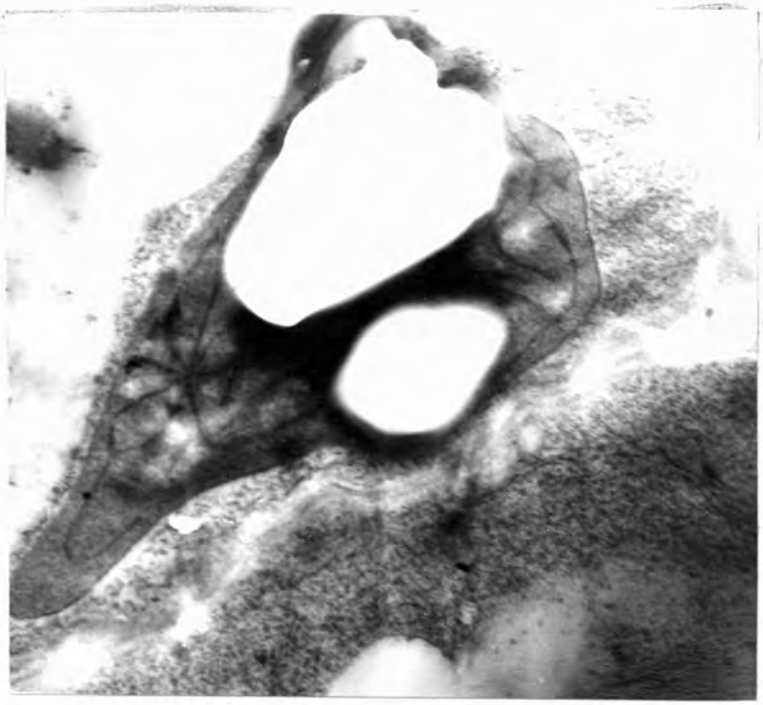

P
ER

PLATE 3 — *Plastid from the primary leaf of light/dark grown young seedling at 22°C, five days after sowing.*

Fig. 4. — *Plastid showing poorly-defined membranes and large starch grains. X 25,800.*

Fig. 5. — *Plastid showing poorly-defined membranes and large prolamellar bodies which do not possess para-crystalline structure. X 43,000.*



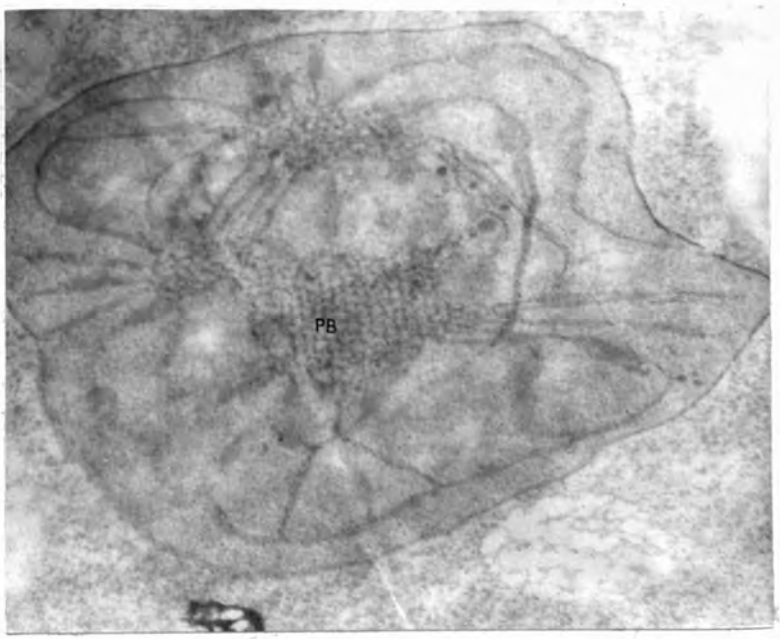

PB

PLATE 4 *Plastid from the primary leaf of light/ dark grown young seedling at 30°C, five days after sowing.*

Fig. 6. *Plastid with few lamellae and large starch grains. Plastid membranes are not well-defined.*
X 68,000.

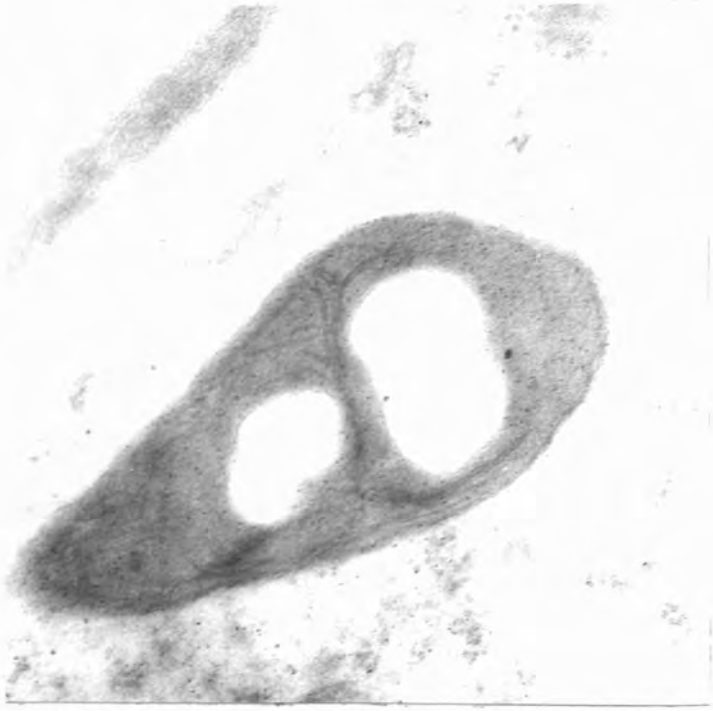


PLATE 5 *Transverse section through the cells of the primary leaf of a young seedling after five days of sowing in complete darkness at 22°C.*

Fig. 7. *Plastids, each with many big starch grains. Lamellar system is not well developed. Accumulation of vesicles at certain places give the appearance of prolamellar bodies.*
X 10,320.

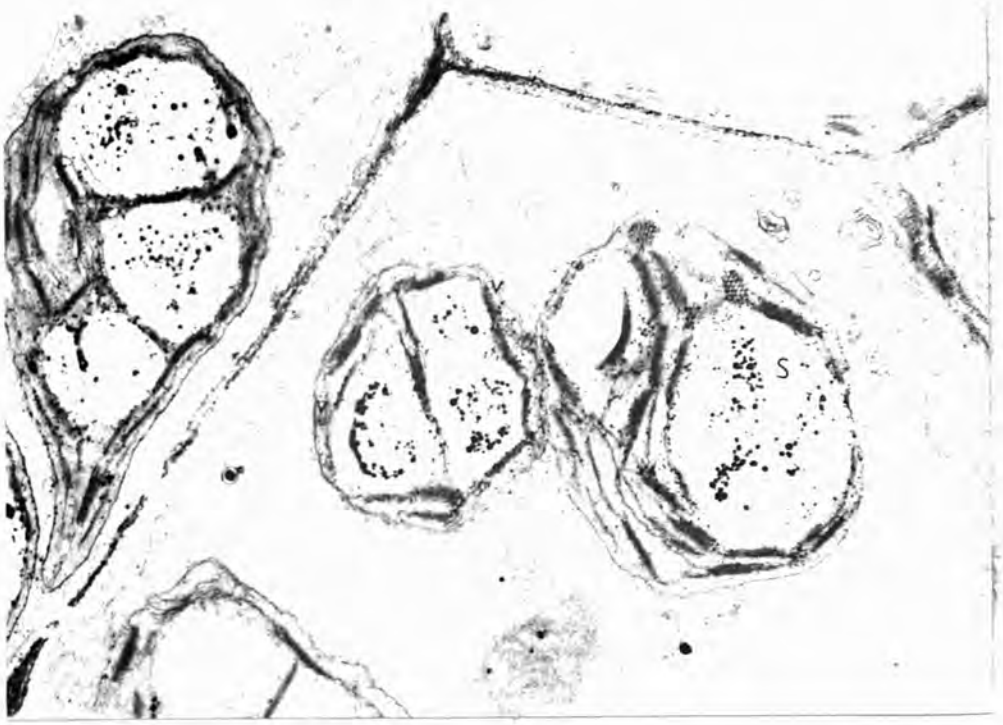
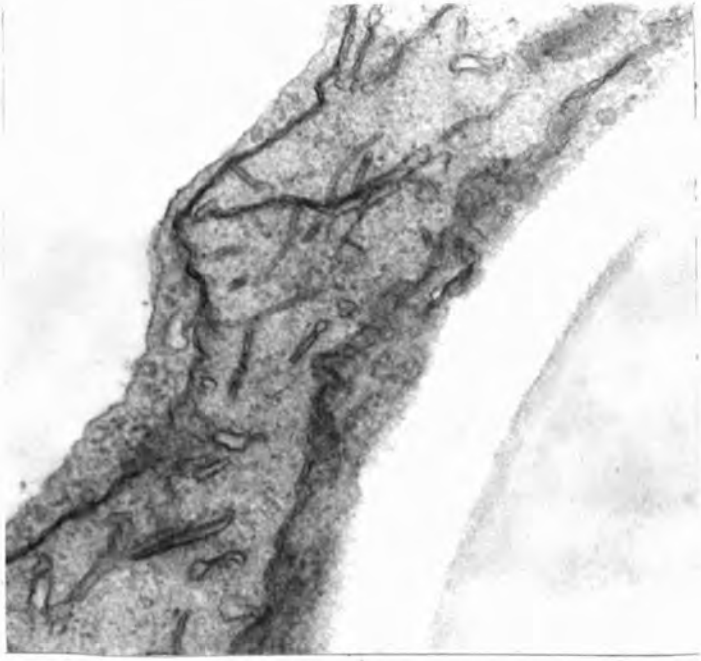

S

PLATE 6 — *Section through the primary leaf of a light/dark grown seedling after six days of sowing at 22°C.*

Figs. 8. & 9. — *Part of an amoeboid plastid with irregular outlines and showing the presence of many short lamellae which do not possess any particular arrangement.*
X 43,000.



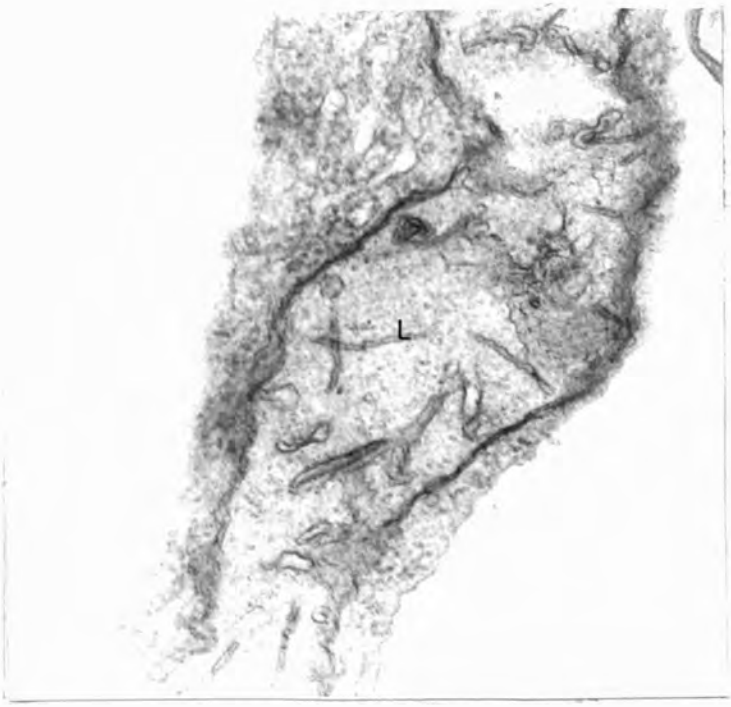
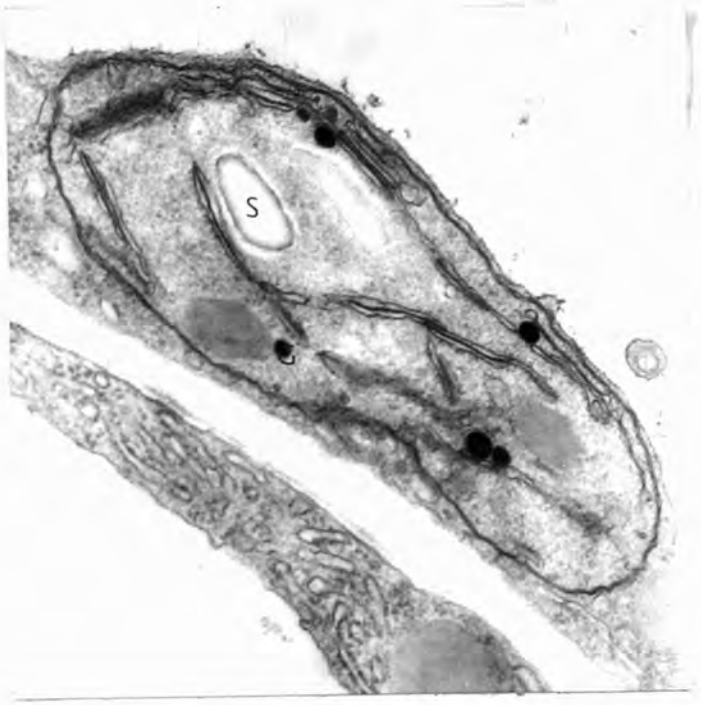

L

PLATE 7 — *Section through the primary leaf of a light/dark grown seedling after six days of sowing at 22°C.*

Fig. 10. — *Elongated plastid with distinct double-membraned envelope and showing few starch grains, few stroma lamellae with incipient grana and few vesicles at the terminal ends of the lamellae. X 25,800.*

Fig. 11. — *Rounded plastids having few starch grains, incipient grana and vesicles. A plastid shows constriction at both sides probably preliminary to division. X 10,320.*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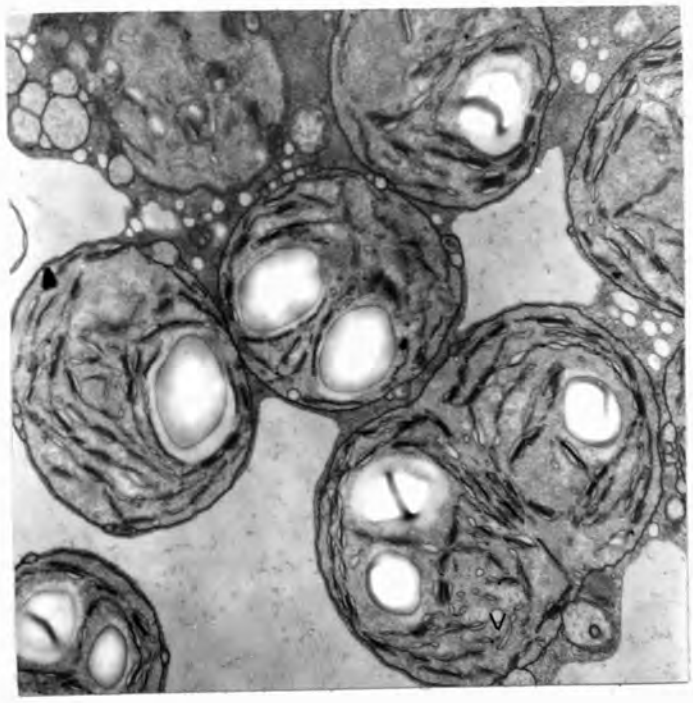
V

PLATE 8 *Section through the primary leaf of a light/dark grown seedling after six days of sowing at 30°C.*

Fig. 12. *Rounded plastid enclosed by a double-membraned envelope and showing swollen incipient grana and many small vesicles. X 43,000.*

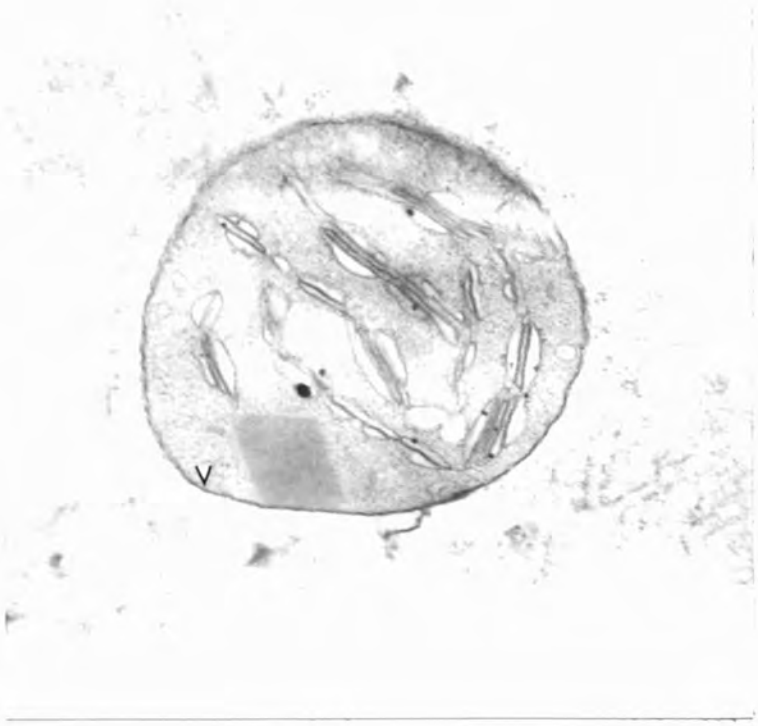
V

PLATE 9 *Section through the primary leaf of a completely dark grown seedling after six days from sowing at 22°C.*

Fig. 13. *Rounded plastid enclosed by a double-membraned envelope which shows wavy outline. Stroma lamellae do not bear any grana and the prolamellar body does not possess para-crystalline structure. Osmiophilic globules are numerous and densely stained.*
X 68,800.

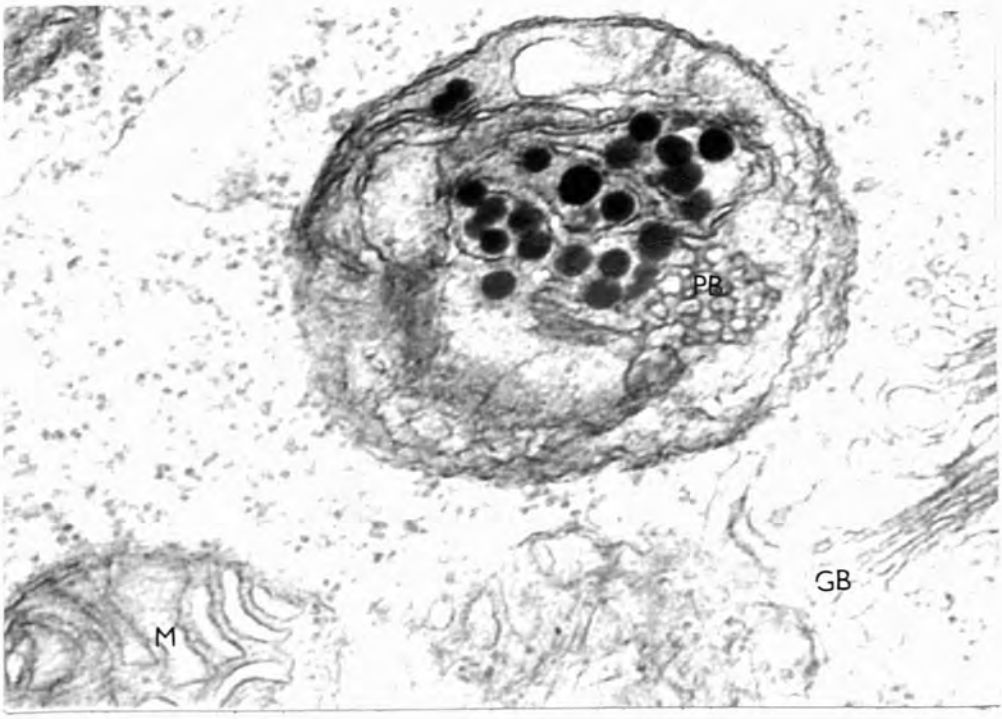
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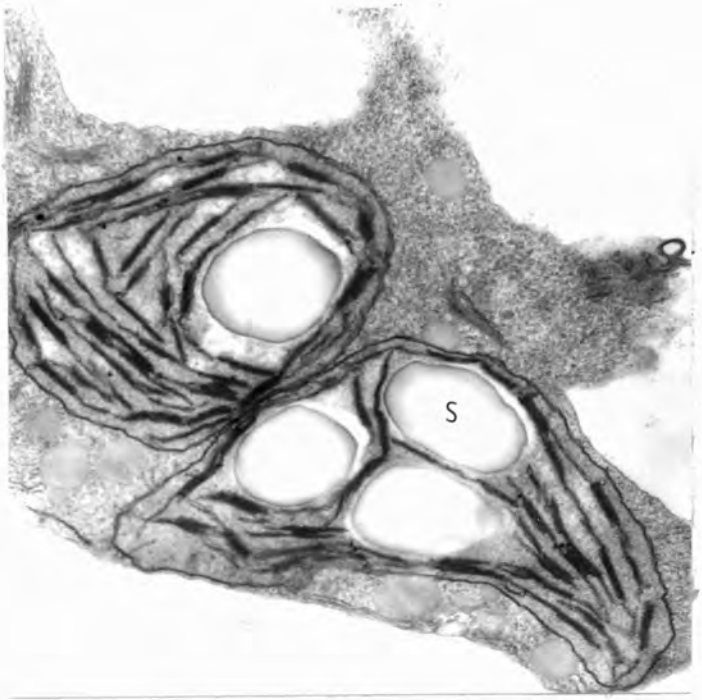
PLATE 10 *Chloroplasts from a light/dark grown primary leaf after seven days from sowing at 22°C.*

Fig. 14. *Stroma lamellae showing the continuity and having many small grana with a maximum thylakoidal stacking up to five. Starch grains are big. Chloroplasts also have a few, small vesicles at certain places.*
X 17,200.

Fig. 15. *A dividing chloroplast.*
X 17,200.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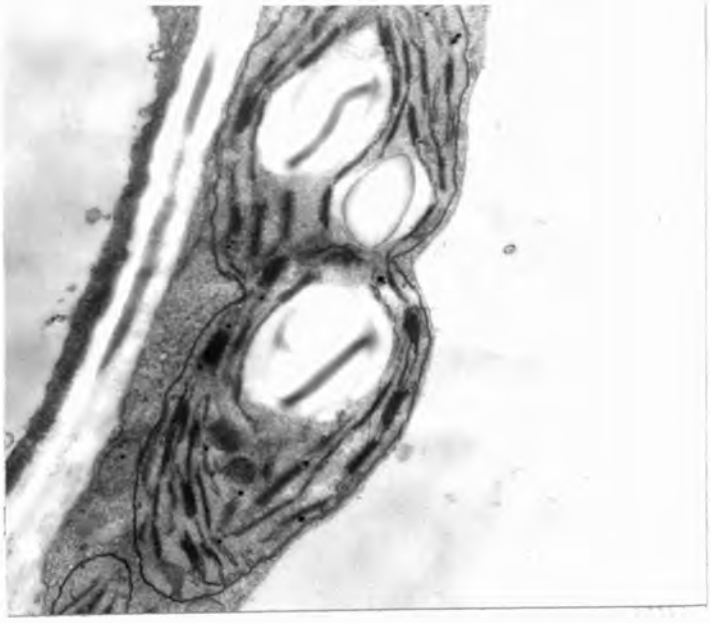
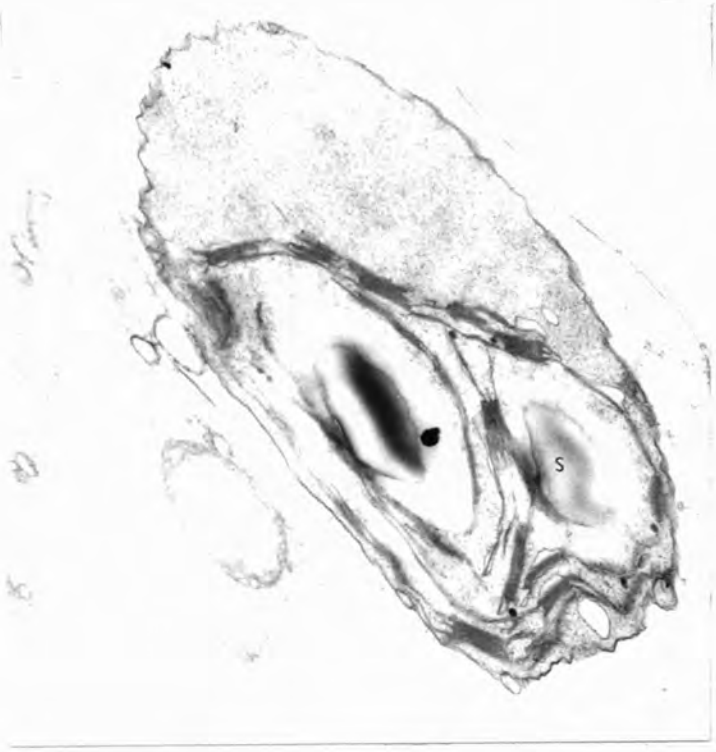


PLATE 11 *Chloroplasts from a light/dark grown primary leaf after seven days from sowing at 30°C*

Fig. 16 *Swollen chloroplast with intact stroma and grana lamellae and large starch grains. Plastid envelope shows corrugated outline.*
X 25,800

Fig. 17. *A burst chloroplast with lamellae broken between grana and many small vesicles. Starch grain is very much reduced in size.*
X 25,800.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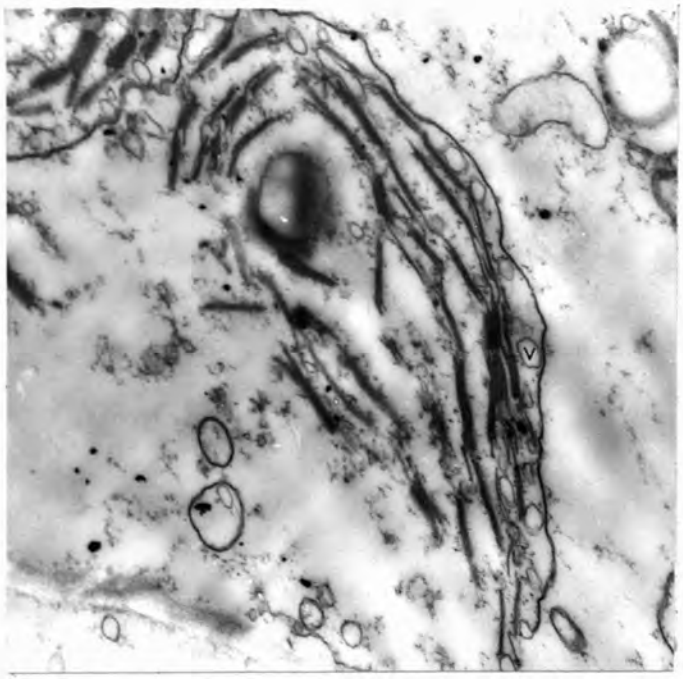
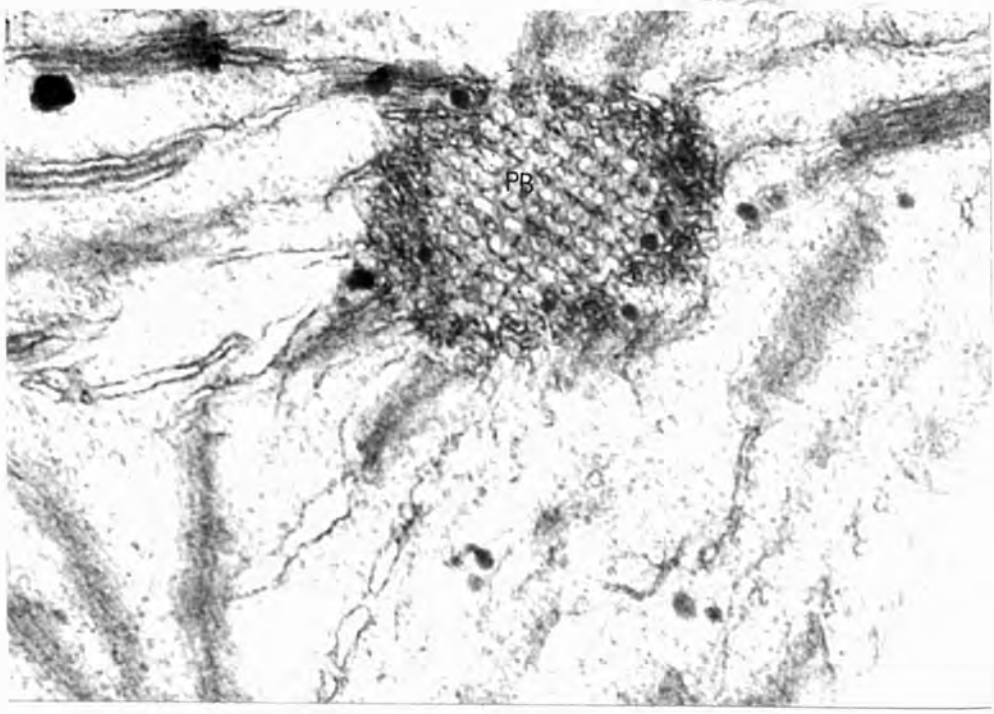

V

PLATE 12 *Section through the etioplast of completely dark grown primary leaf.*

Fig. 18. *Etioplast showing prolamellar body with characteristic paracrystalline structure. Some of the lamellae show swellings and few vesicles are also present in the etioplast.*
X 97,200.

Fig. 19. *Etioplast with well developed pro-lamellar body. Grana are large and show swellings at the terminal ends of the thylakoids. Many small vesicles are also present in the etioplast. Starch grain is big in size.*
X 60,750.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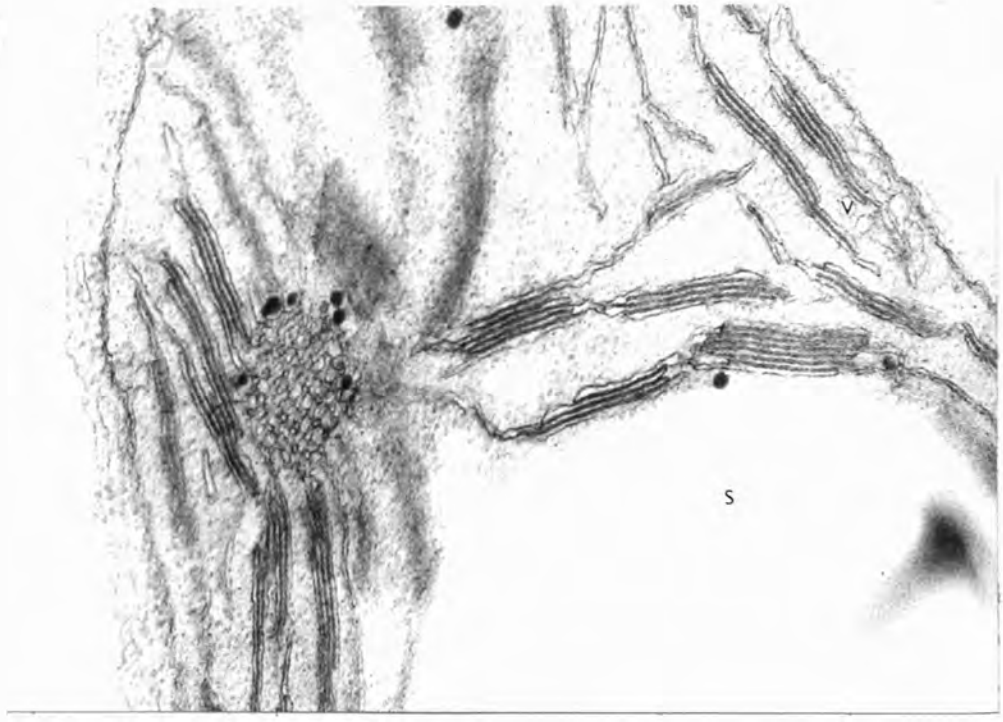
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PLATE 13 *Section through the cell of a completely dark grown primary leaf.*

Fig. 20. *A dividing etioplast.*
X 60,750.

DISCUSSION

Whatley (1978) has shown that certain aspects of development observed during seed germination are a repetition in reverse of those seen in the final stages of seed ripening. For example, changes in the plastid structure and the number of mitochondrial cristae, the behaviour of lipid droplets, starch, Golgi bodies and endoplasmic reticulum. There have been several reports on ultrastructural aspects of plastid development both during seed ripening, and during germination. Opik (1966, 1968) and Bain and Mercer (1966a, b) studied both these processes in the cotyledons of Phaseolus vulgaris and Pisum sativum respectively. Hallam (1972) and Hallam, Roberts and Osborne (1972) reported on changes in radicles of Secale cereale.

Whatley (1974) has shown that in primary leaves of Phaseolus vulgaris two phases of starch accumulation occur. The first, during seed ripening which persists for several weeks and results in the formation of a single, large, oval starch grain in each plastid. This phase is followed by loss of starch during plastid regression. The second phase of starch accumulation occurs during seed germination and results in the formation of several small spherical starch grains in each plastid. Such an accumulation during germination appears in the primary leaves of spinach as shown in the present investigations.

In the primary leaves of spinach an association between plastid and endoplasmic reticulum is observed during germination on the third day. A similar association has previously been reported in plastids

of elongating pollen tubes (Larson, 1965), in radicles of germinating Pisum sativum *(Perner, 1965; Yoo, 1970) and in the primary leaves of* Phaseolus vulgaris. *Endoplasmic reticulum has also been observed as a complete sheath around immature plastids of* Equisetum telmateia *and here the association persists throughout the development, becoming progressively less complete as the plastid matures (Whatley, 1971b). The plastid-endoplasmic reticulum association as observed by Whatley (1974) in the primary leaves of* Phaseolus vulgaris *is not observed earlier than 4 hours or later than 24 hours from the start of imbibition so she regards this as a transitory stage in chloroplast development.*

Although the exact role of endoplasmic reticulum is not yet fully understood, Bain and Mercer (1966b), while working on Pisum sativum *cotyledons postulated that it may have assisted the intracellular transport of substances, since the vascular tissue is not extensive in the cotyledons and many cells are up to 20 cells away from the vascular supply or from the axis: but since the soluble substances are not lost from the cotyledons during this time, this role seems unlikely. Whatley (1974) pointed out that the continuity of the endoplasmic reticulum around protein bodies and plastids, at the time when protein bodies are beginning to degenerate, suggests that the probable function of the endoplasmic reticulum here is protein transport.*

Opik (1966, 1968) has shown that in Phaseolus vulgaris *the plastids of cotyledons develop prolamellar bodies before becoming mature chloroplasts during seed ripening, after which the chloroplasts regress*

to proplastids. But according to Whatley (1974) this sequence is arrested in the primary leaves of Phaseolus vulgaris, for these plastids never develop prolamellar bodies or stroma lamellae but remain as proplastids throughout. Subsequent to germination, however, chloroplasts in both cotyledons and primary leaves follow a similar developmental sequence through prolamellar body and stroma lamellar development. According to some authors, the formation of prolamellar body takes place by the accumulation of vesicles which are produced by the invagination of the inner plastid membrane (Hodge et al., 1956; Mühlethaler and Frey-Wyssling, 1959; Röbbelen, 1959; von Wettstein, 1959; Erriksson et al., 1961). However, according to Gunning (1965), Gunning and Jagoe (1967), and Cran and Possingham (1972b), the prolamellar body is not formed from the vesicular outgrowths of the plastid envelope. Cran and Possingham (1972b) further suggested that they may be formed de novo as a result of continued membrane synthesis within the plastids, this being directed to tubule rather than to lamellar production. The electron micrographs of the present investigation reveal that in the spinach plastids vesicles are produced only after the development of stroma lamellae and grana, and also only to any great extent when the tissue is in the dark. Therefore in spinach the prolamellar bodies are likely to be produced from the outgrowths of thylakoids. But since under high temperature conditions the vesicle formation is abundant yet no prolamellar body is formed therefore the exact mode of formation of prolamellar bodies is however, still a question for further investigations. In the plastids of the primary leaves of spinach the prolamellar body formed at 22°C temper-

ature under light/dark conditions is of a very simple type without any clear crystalline structure.

Another type of plastid which appears in the primary leaves of the spinach seedling is an elongated, round or cup-shaped plastid with irregular outlines. This is a type known to occur frequently in meristematic tissue (Kirk and Tilney-Bassett, 1967) and suggested by Newcomb (1967) to be a feeding stage, but it has been given little consideration as a significant developmental stage. Whatley (1974) called this the 'amoeboid stage' and referred to it as the second transitory stage in chloroplast development. In the present investigation this stage can be recognized only at 22°C temperature under light/dark conditions. Whatley (1974) also suggested that it is likely that the amoeboid stage is a common intermediate in the development of different plastid types. But in the primary leaves of spinach seedlings growing in complete darkness the amoeboid formation is not observed at any stage during etioplast formation. Amoeboid plastids are elsewhere found to occur in shoot apices, root tips (Newcomb, 1967), cotyledons (Opik, 1966, 1968), cambium (Evert and Deshpande, 1970), sieve elements and companion cells (Behnke, 1973), epidermal cells (Steffen, 1964), tissue culture (Blackwell et al., 1969) and dormant tubers (Tullet et al., 1969). Steffen (1964) suggested that the amoeboid form is an intermediate stage during chromoplast development from several precursor plastid types. Sjölund and Weier (1971) present the data on plastid changes in the tissue culture of Streptanthus tortuosus which suggest that mature chloroplasts degenerate to an amoeboid form from which they can subsequently regenerate.

The present investigation shows that at the time the seedling just emerges from the soil, the lamellae of spinach plastids of the primary leaves are perforated and swollen. This increase in the area of the thylakoids of incipient grana is presumed to have resulted from the dispersal of the prolamellar bodies to form thylakoids. Such increases in the thylakoid area are also noticed by Montes and Bradbeer (1974) when the dark grown maize leaves are illuminated. Whatley (1974) has suggested that though the swelling of the lamellae may be an artefact of embedding, nevertheless, it reflects a major ultra-structural change in the lamellae at this time. In light the perforated lamellae become more continuous and this stage she regarded as the third transitory stage in chloroplast development.

Under high temperature conditions when the spinach seedling emerges in light, in addition to the formation of normal stroma and grana lamellae certain peculiar changes take place. The plastids become swollen and assume a variety of shapes presumably because of the alteration of the membrane permeability of the plastid envelope (Anderson and Schaelling, 1970). At this stage, when both the stroma and grana lamellae are intact, very few osmiophilic globules are present. The envelopes of some plastids burst even when both stroma and grana lamellae do not show much disruption. Many small vesicles are seen scattered in the cytoplasm and also in the near vicinity of the plastid (Fig. 16). These vesicles are presumed to be formed by the broken up lamellae of the plastids (Barton, 1966; Hurkman, 1979). Molotkovsky and Zhestkova (1965) have shown that the swelling of the plastids at high temperature is a result of the conformational changes

of an actomyosin-like protein. The effect of heat on chloroplast ultrastructure is discussed in more detail in Chapter IV. No plastid division is observed in any cell under high temperature conditions but division is commonly seen in both light and complete darkness at normal temperature (22°C).

The pattern of etioplast development in the primary leaves of spinach is similar to that already recorded by Berger and Feierabend (1967), Bradbeer et al. (1974), Whatley (1974). Although the amount of stroma lamellar material appears to be similar in both light and completely dark grown seedlings the etioplasts contain much larger prolamellar bodies. This suggests that the total amount of membranous material produced may be less in the light/dark grown seedlings than in the dark at this stage of seedling growth. When the stroma lamellae of the plastids of light grown seedlings are markedly increasing (day six to seven), the overall length of the plastids also increases rapidly.

Membrane-bound bodies have not been observed in the spinach plastids, at any stage of development under any of the treatments given in the present investigation. A very frequent presence of the membrane-bound bodies in almost all the cells have been reported earlier by Stetler and Laetsch (1969) in the developing plastids of Nicotiana tabacum and by Ireland (1971) in developing plastids of Phaseolus vulgaris.

C H A P T E R IV

PART-A

THE EFFECTS OF HIGH TEMPERATURE ON CHLOROPLAST ULTRASTRUCTURE DURING THE GROWTH AND SENESCENCE OF ATTACHED LEAVES OF *Spinacia oleracea*

INTRODUCTION

In this section are described the ultrastructural changes in spinach leaf chloroplasts at different stages of development of the leaf and finally during its senescence under normal growing conditions, and also when the plant is subjected to increased temperature conditions above those allowing normal growth. When the growing temperature of the plant is raised beyond the limits of normal growth it brings about destructive changes in the plant cell, particularly in the chloroplasts. Comparison is made between the destructive effect of high growing temperature on spinach chloroplasts and their senescence under normal growing temperature.

METHOD

The plants were raised at 22°C temperature and at 14h light/10h dark conditions until they were four weeks old so that they could withstand the high temperature conditions. Four groups, each of 20 plants were used in the experiment. One of these groups was kept at 22°C and the remaining three were transferred to the cabinets maintained at 25°C, 30°C and 35°C temperatures respectively; all remaining under the 10h dark regime. The plants kept at 22°C temperature conditions were treated as the control for the higher temperature conditions used.

The first fully opened leaf (TEXT Figure 5) of each of the 20 plants under each treatment was tagged to mark the age of the leaf. The samples for electron microscopy were collected from the tagged

TEXT Figure 5 *Seedling of Spinacia oleracea at 4 weeks from seed at 22°C showing the leaf (L) which was tagged for the experimental study.*

leaves weekly until the leaves started showing senescence, after which the samples were taken daily. Since in spinach leaf the plastids are mature at the tip of the leaf when still immature at the base (Cran and Possingham, 1972b and also confirmed from observation in the present investigation) the samples for electron microscopy were always collected from the tip of the leaves.

OBSERVATIONS

(i) 0 Day samples

The chloroplasts in the young photosynthetic leaf grown in 14h light/10h dark under controlled conditions at 22°C are large, and possess the normal grana-fretwork system comprising the internal membrane system with lamellae stacked into large grana intercornected with intergrana (fret) membranes which are quite long in some chloroplasts. The stroma comprising the internal matrix material has a granular appearance, rather evenly distributed, presumably due to fraction-1 protein which is the major constituent of proteinaceous stroma (Moyse, 1967). Most of the chloroplasts are very long (6.5 - 7 μm in length), appearing biconvex, plano-convex or concavo-convex in form and are surrounded by a double membraned envelope as usual. The stroma of these chloroplasts contain few starch grains and osmiophilic globules if present are very small in size (maximum size is 0.05 μm). Dividing chloroplasts by constriction are also seen in some cells.

(ii) 7 Days after transfer

22°C

The chloroplasts in the 7 days old leaf grown at 22°C are virtually identical in shape and size to those of the newly differentiated leaf and are all longer than broad. In their fine structure these plastids are also similar to those of newly differentiated leaves. There is a normal grana fretwork system with very well developed grana having a maximum stack of 25, very long fret membranes connect the two grana, especially near the envelope (Fig. 21). In some chloroplasts the grana fretwork system is slightly pushed towards the one surface leaving a space towards the other surface between the double membraned plastid envelope and the grana fretwork. This space which is filled with stroma is granular in appearance and may be called 'stroma space' (Fig. 22). There is a slight increase in both number and size of the densely stained osmiophilic globules in the 7 days old leaf and small starch grains can also be seen in some of the chloroplasts (Fig. 21).

25°C

The chloroplasts in the 7 days old leaf grown at 25°C also show a very similar fine structure to those under normal temperature conditions, except that several large starch grains (1.0 μm long) appear in the stroma resulting in the distortion of the shape of the chloroplast (Fig. 23). Many thylakoids are present within the chloroplasts and are organized into grana regions. The osmiophilic globules did not show any further increase in size or number as compared to those at 22°C conditions.

30°C

The chloroplasts in the 7 days old leaf grown at 30°C possess a normal structure and shape. However, they differ in two ways from those at 22°C and 25°C; firstly, at 30°C the granal development of plastids is very extensive (Figs. 25, 26) and the grana are mostly connected by long fret membranes; secondly, starch grains are of very limited occurrence and are very much reduced in size (0.25 μm long). Plastid division is not observed in any cell under these conditions. Osmiophilic globules do not show much increase over those at 25°C.

35°C

After 7 days at 35°C the chloroplasts assume a typical bi-convex shape and appear to be swollen. Each chloroplast contains large multilamellar grana (Fig. 27), more disorientated with respect to one another than grana from chloroplasts in plants grown at the lower temperatures (22°C and 25°C) for the same period and the lamellae show a characteristic swelling at various places. In some of the plastids there are small club-shaped swellings at the free ends of each thylakoid (Fig. 28). In a few cells, some undifferentiated or partially differentiated plastids occur. These plastids contain few, long lamellae and a few large osmiophilic globules (Fig. 29). Some of the chloroplasts also contain some small grana mostly of no more than two or three thylakoids (Fig. 28). Osmiophilic globules are more numerous and much larger (0.13 μm). No starch grain was observed in any chloroplast at this temperature. The double membraned envelope still persists enclosing all the chloroplastic material inside it.

(iii) 15 Days after transfer

22°C

Even after 15 days at 22°C the chloroplasts retain nearly the same structure as those after 7 days at this temperature. In each cell the chloroplasts lie embedded in the cytoplasm near the cell wall and are closely placed in a ring. In most of the chloroplasts the stroma space is quite large (Fig. 30). Osmiophilic globules at this stage of leaf development show some increase in size (0.08 μm) as compared to those after 7 days of leaf growth at this temperature condition.

25°C

Most of the chloroplasts after 15 days at 25°C are concavo-convex in shape with a large stroma space on the concave side and a network of grana and stroma lamellae towards the convex surface. Both the number of grana per plastid and the number of thylakoids per granum have visually increased and also the stroma lamellae are extensively developed and aligned in arrays parallel to the long axis of the chloroplast. As many as 35 thylakoids can be seen stacked in one granum (Fig. 31). Osmiophilic globules show a slight increase in both number and size (0.08 μm). Starch grains are present only in very few of the chloroplasts and are small in size (Fig. 32).

30°C

After 15 days at 30°C the chloroplasts appear to be shrunken (4 μm long) although they retain an extensive development of thylakoids. Two types of chloroplast were observed under this temperature treatment. In one type the double membraned plastid envelope has extended on one

side resulting in a characteristic plano-convex shape with a large stroma space between the grana-fretwork system and the extended plastid envelope (Fig. 33). The other types of plastid are bi-convex in section. In some of the chloroplasts of this stage there are seen some broken intergrana connections, hanging free in the stroma (Figs. 34, 35). The free ends of these lamellae appear as hooks (Fig. 35) or loops (Fig. 34). Osmiophilic globules are large (0.1 μm) and numerous. Starch grains are rare and very small (0.2 μm long) (Fig. 33).

35°C

At 35°C after 15 days the chloroplasts show great changes from their condition in the 7 days old leaf at this temperature. Almost all the chloroplasts appear rounded and most of them have lost some or all of their envelope, with the chloroplast contents lying more or less free in the cytoplasm of the cell (Fig. 37). Their fine structure shows loosened lamellae and grana, though often still interlinked with one another, and numerous. Osmiophilic globules, some of which also occur, are dispersed free in the cytoplasm of the cell.

(iii) 21 Days after transfer

22°C

The electron micrographs of plastids of leaves grown under normal temperature conditions (22°C) reveal that even after 21 days the plastids are very similar to those of newly differentiated leaves. The chloroplasts are elongated with a large stroma space, possess starch grains only in a few of the chloroplasts and the comparatively large grana are interlinked by long lamellae. Several large densely

stained globules are now present in each chloroplast. Membrane whorls are also common at this stage of leaf growth (Fig. 39). In some plastids which appear rounded the grana fretwork system assumes a deep arc or U-shape (Fig. 40).

$25^{o}C$

The fine structure of leaf plastid at this 21 day stage of leaf growth also shows many similarities to those of its earlier stages and to those at $22^{o}C$. The grana, each of which is composed of many thylakoids, are interconnected by large numbers of lamellae (Fig. 41). Some large, densely stained osmiophilic globules are present in these chloroplasts. The chloroplasts at this stage have the same size as those in the earlier stages but have the shape of a bi-convex lens without any stroma space inside it. No starch grains were detected in any of the chloroplasts at this stage.

$30^{o}C$

The plastids show reduction in size (5.5 μm long) and assume various shapes. Both stroma and grana lamellae, however, remain well developed, the grana in some plastids being composed of as many as 50 thylakoids or even more (Fig. 43) and are interconnected by many fret membranes. In a few plastids the interconnecting lamellae are broken down at certain places (Fig. 43). Starch grains are completely absent in these plastids but osmiophilic globules are numerous (Figs. 42, 43).

$35^{o}C$

The leaves of the plant under $35^{o}C$ temperature conditions at this stage of growth shrivel completely and therefore further sampling was not possible.

(iv) 28 Days after the transfer

22°C

Under the normal 22°C temperature conditions the shape of the chloroplasts do not seem to alter much from those of the 15 days and 21 days old leaves grown under these conditions. They are still elongated, concavo-convex or plano-convex in shape. Presence of large stroma space is a common feature of all the chloroplasts (Fig. 45). In some chloroplasts a single large starch grain is seen in each chloroplast (Fig. 44). However, the most striking feature of the fine structure of these chloroplasts is that the granal stacks have continued to increase in depth to occasionally as many as 55 layers (Fig. 45), though 15 - 25 layers are more common. This stage of leaf growth under normal temperature conditions also approximately coincides with the peak in chlorophyll content (TEXT Figure 8). Osmiophilic globules have further increased in both number and size (0.12 μm).

25°C

At 25°C after 28 days the chloroplasts show an increase in the thylakoidal system similar to that at 22°C but this is accompanied by an excessive increase of stroma space and irregular stroma lamellae which form vesicles, hooks and loops, either by curving inwards and/or joining the other end of the granum (Fig. 46). Starch grains are present only in few chloroplasts and are reduced in size (0.3 μm long). Osmiophilic globules show a further increase both in number and size.

30°C

After 28 days at 30°C the striking feature of the cells is the almost complete absence of the cytoplasmic contents apart from the resistant

plasmalemma, and a very few greatly altered chloroplasts. The chloroplasts are shrunken and rounded, and contain a large number of densely stained osmiophilic globules (maximum size - 0.2 μm) and patches of broken lamellae (Figs. 47, 48). Other cytoplasmic organelles for instance mitochondria with distorted crista are observed rarely among the chloroplasts.

(v) Ultrastructure of chloroplasts during senescence

The premature senescence of leaves on plants transferred to temperatures of 30°C and 35°C has determined the period of observation in these cases to be 28 days and 15 days respectively. We may compare the senescent state at normal temperatures with the effects observed after a shorter time at the higher temperatures.

The extensive increase in the thylakoid system of the chloroplast observed through 28 days of growth from the small greening leaf at 22°C is followed by the early stages of senescence in the yellow-green leaf which is characterized by an increase in irregular stroma lamellae forming vesicles, hooks and loops. This is illustrated in the 35 days old leaf in Figs. 50 and 51. The remaining extensive thylakoid system and grana, however, indicate that photosynthesis is continuing at this stage. The chloroplasts assume a variety of shapes and include several large dense globules. The starch grains observed at 28 days have now completely disappeared.

In the intermediate stages of senescence at normal growth temperatures, a typical swelling of the thylakoids occurs and in the

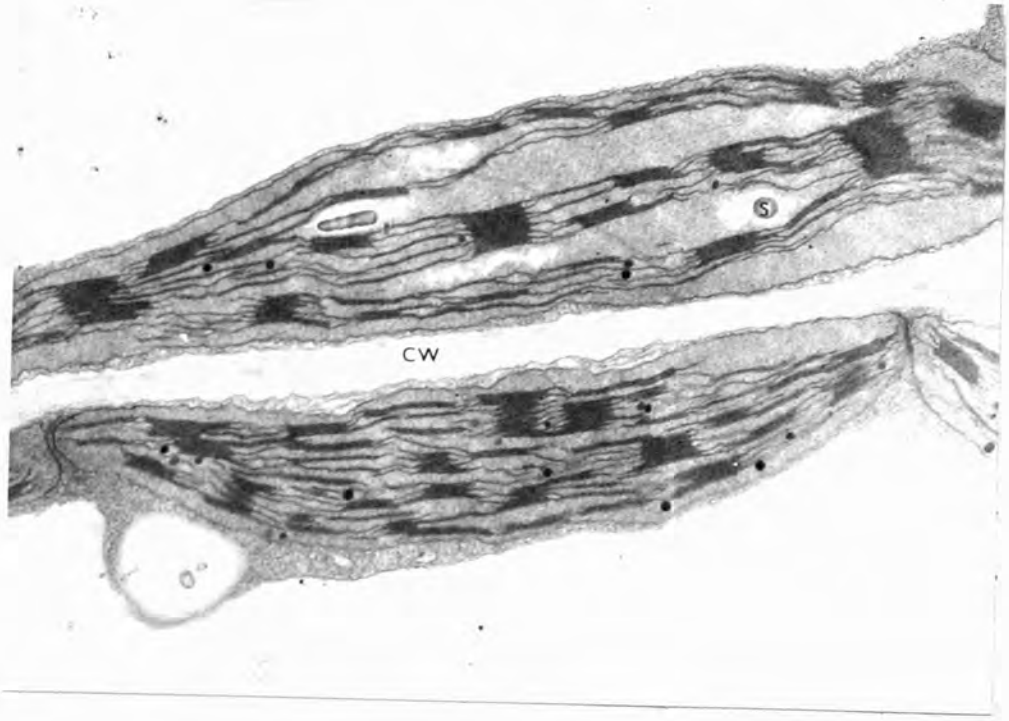
TABLE 1 - Summary of structural changes in chloroplasts of leaf

TEMPERATURE	Number of days at indicated temperature since transfer from 22°C				
	0 DAY	7 DAYS	15 DAYS	21 DAYS	28 DAYS
22°C Original Growing Temperature	Elongated chloroplasts; bi-convex or plano-convex with well developed lamellar system. Starch grains present in most of the chloroplasts. No osmiophilic globule. No stroma space.	Elongated chloroplasts, plano-convex; well developed grana. Very small starch grains and osmiophilic globules. Figs. 21 22	Mostly unchanged from 7 day. Well developed grana (to 25 stackings). Stroma space on flat side of plano-convex chloroplasts. Small starch grains and osmiophilic globules. Fig. 30	Mostly unchanged. A few rounded with arc- or "U"-shaped grana-fret system and large stroma space A few with large starch grains. Some large globules. Fig. 38 39 40	Range of shapes unaltered. Large stroma space. Increase in stacking of grana. Mostly 15-25 but up to 55 thylakoids with many fret-membranes. Large globules. Large starch grains. Figs 44 45
25°C		Elongated chloroplasts, bi-convex or plano-convex. Well-formed grana and several large starch grains; few small densely stained globules. Fig. 23	Some irregularity in shape but mostly concavo-convex with well developed grana and stroma space. Up to 35 thylakoids per granum. Osmiophilic globules increase in number but still small. Starch grains are of small size. Figs. 31 32	Similar to 22°C chloroplasts. Bi-convex shape with well developed grana. A few large globules. No starch grains. Fig. 41	Some irregularity in shape; an increase in thylakoidal system but also extensive stroma space. Irregular stroma lamellae with large loops. Osmiophilic globules large. Fig. 46
30°C		Mostly plano-convex, with well-formed grana and stroma space. Some increase in osmiophilic globules. Starch rare and grains very small. Fig. 25 26	Chloroplasts shrunken in size but mostly plano-convex with stroma space. Very small starch grains and several globules which are now large. Figs. 33 34 35	Chloroplasts shrunken in size; variously shaped. Some disorganization of lamellae (breakings, loops) between the well developed grana (up to 50 thylakoids). Numerous osmiophilic globules. Figs. 4 42	Plastids rounded with irregularly shaped masses of stacked lamellae and numerous large clustered globules. SENESCENT. (most of the other cell contents have now disappeared). Figs. 47 48 49
35°C		Chloroplasts mostly oval or plano-convex. Lamellae swollen in places with typical club-shaped swelling at the terminal ends of the thylakoids. Large osmiophilic globules. No starch grains. Some small rounded undifferentiated plastids with few lamellae and large globules. Figs. 27 28 29	Chloroplast envelope broken down; contents rounded or partially dispersed in cytoplasm; lamellae loosening in grana. Figs. 36 37	No observations: Leaf tissue dead.	No observations.

PLATE 14 *Plastids from 7 days old leaf of the plant growing at 22°C.*

Fig. 21. *Elongated plastids with multi-thylakoidal grana which are connected by long fret membranes. Small starch grains present in a plastid.*
X 24,300.

Fig. 22. *An enlargement showing a granum composed of 25 thylakoids and connected by many stroma lamellae. Clear stroma space is also seen.*
X 97,200.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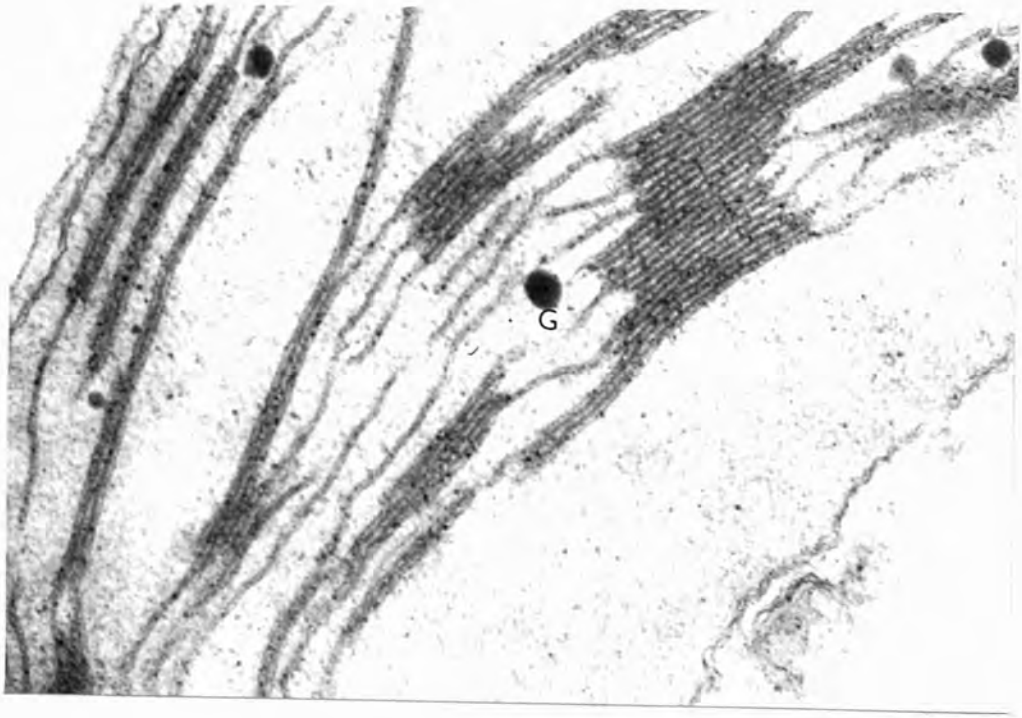
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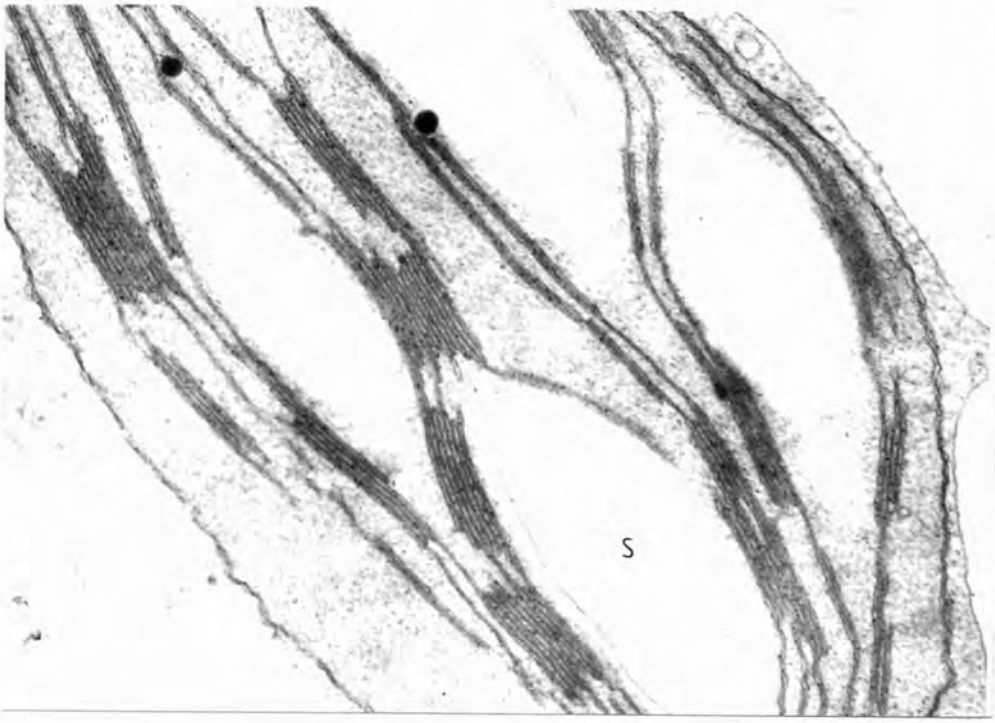
PLATE 15 *Chloroplast from 7 days after transfer to 25°C.*

Fig. 23. *Section through a chloroplast showing many big starch grains and well developed grana and stroma lamellae. X 60,750.*

Fig. 24. *An elongated plastid apparently in late stage of division by constriction. X 24,300.*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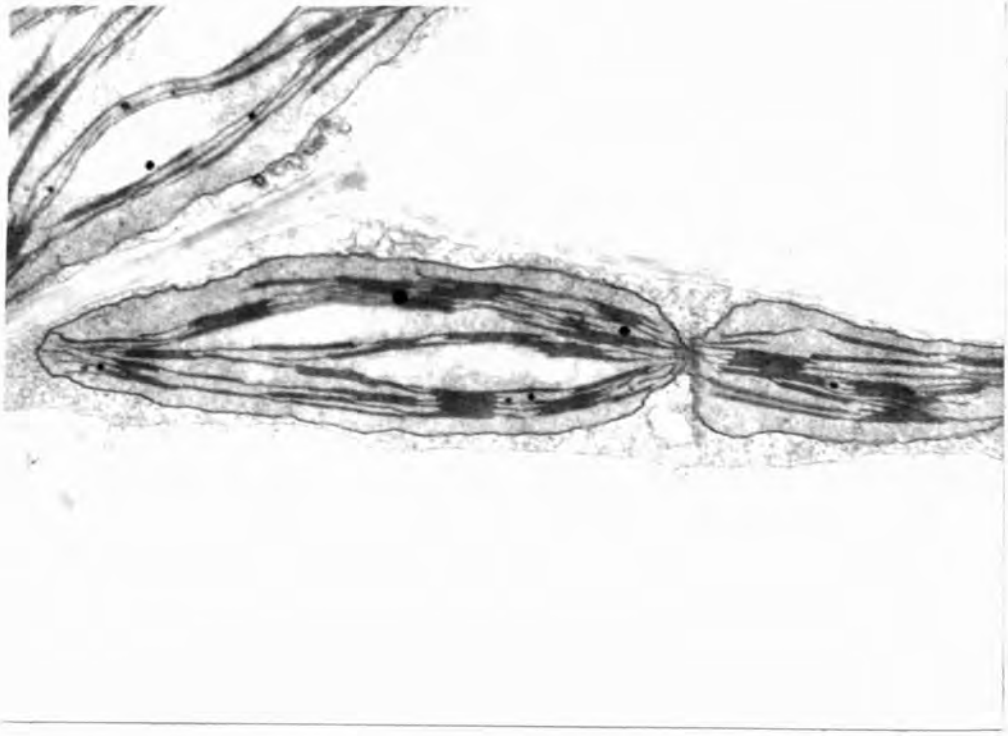
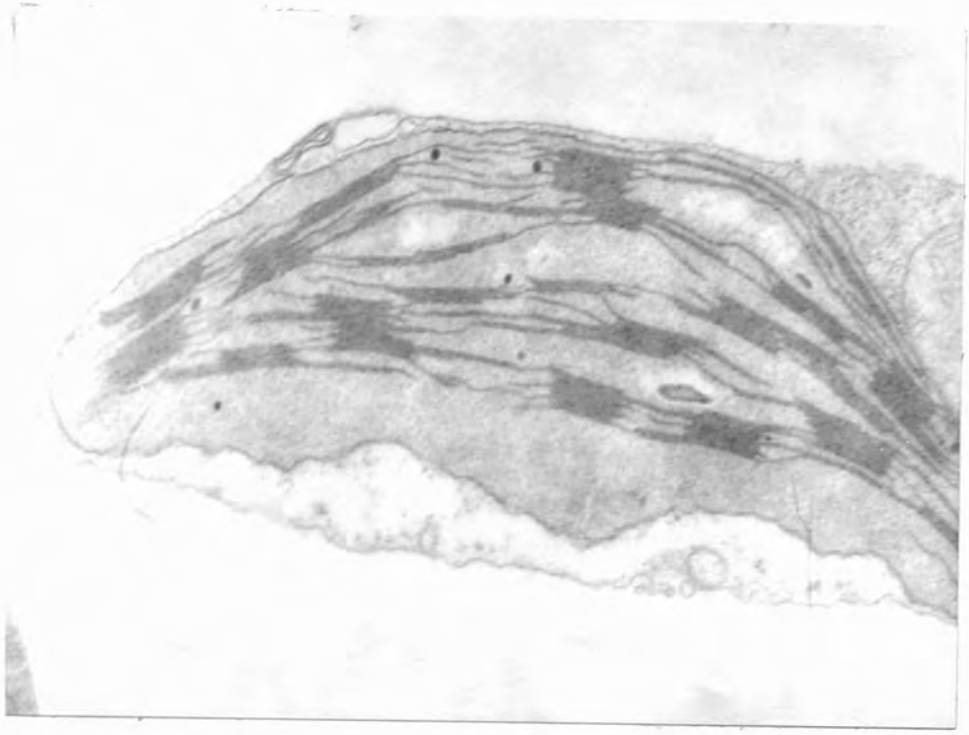


PLATE 16 *Chloroplasts from 7 days after transfer to 30°C.*

Fig. 25. *Chloroplast possesses well developed lamellar system and a small stroma space. A single starch grain appears to be very small in size.*
X 36,450.

Fig. 26. *Plano-convex chloroplast shows many multithylakoidal grana which are connected to each other by many fret membranes.*
X 60,750.



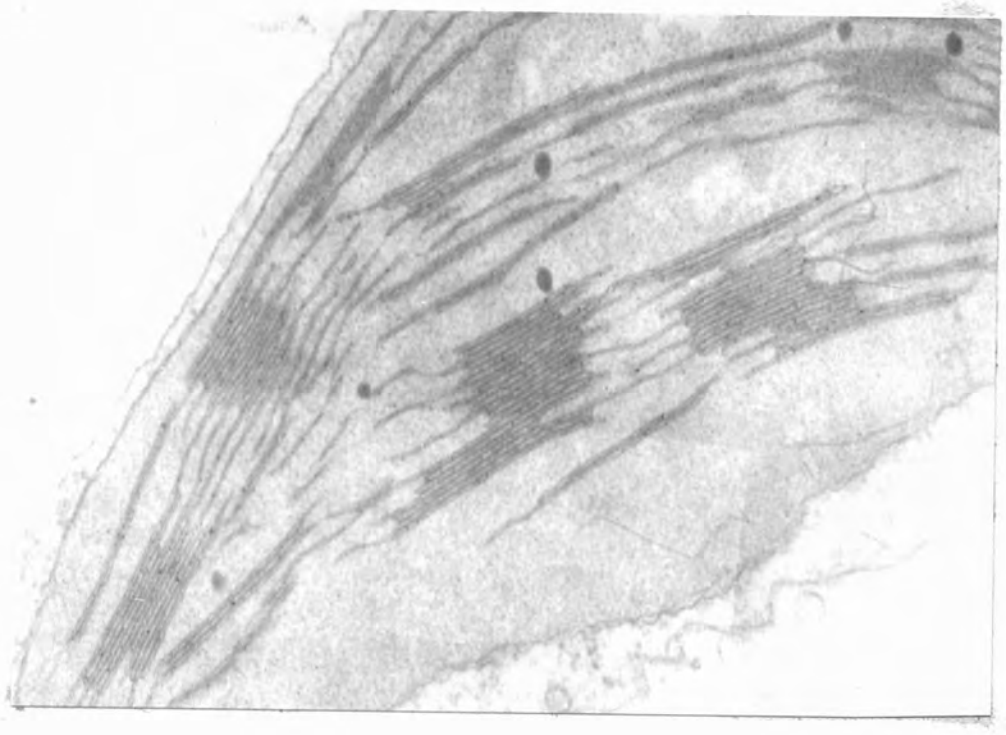
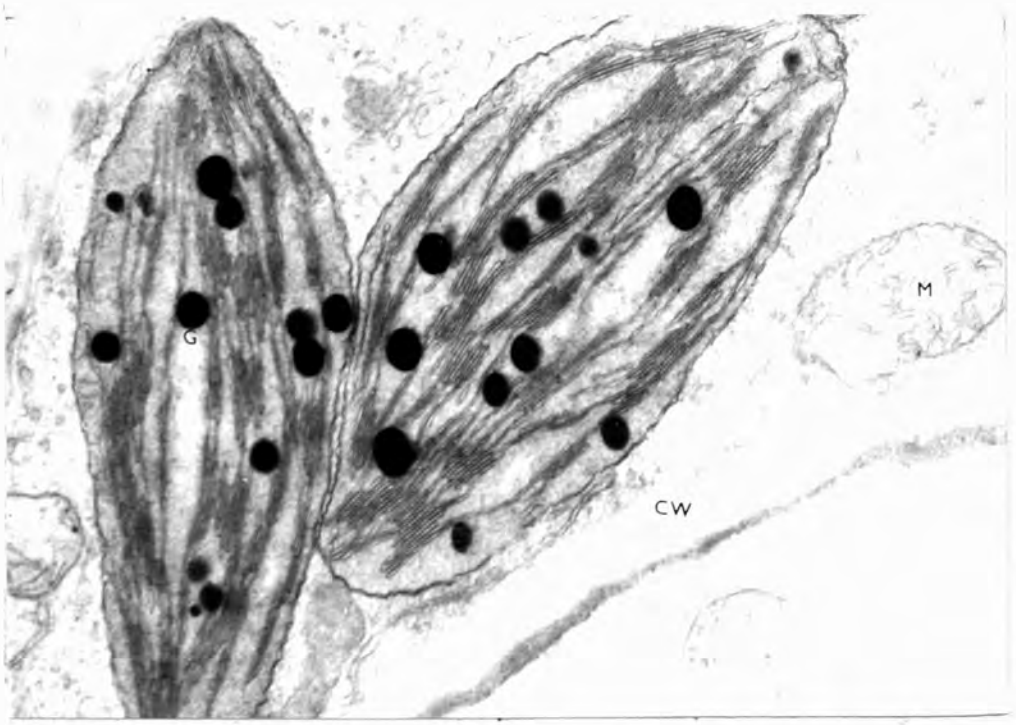


PLATE 17 *Sections through the cells of the leaf 7 days after transfer to 35°C.*

Fig. 27. *Oval-shaped chloroplasts with many, multithylakoidal grana. The lamellae show a slight swelling. Each chloroplast contains many large densely stained osmiophilic globules.*
X 36,450.

Fig. 28. *An enlargement of part of a chloroplast showing club-shaped swellings at the ends of thylakoids.*
X 36,450.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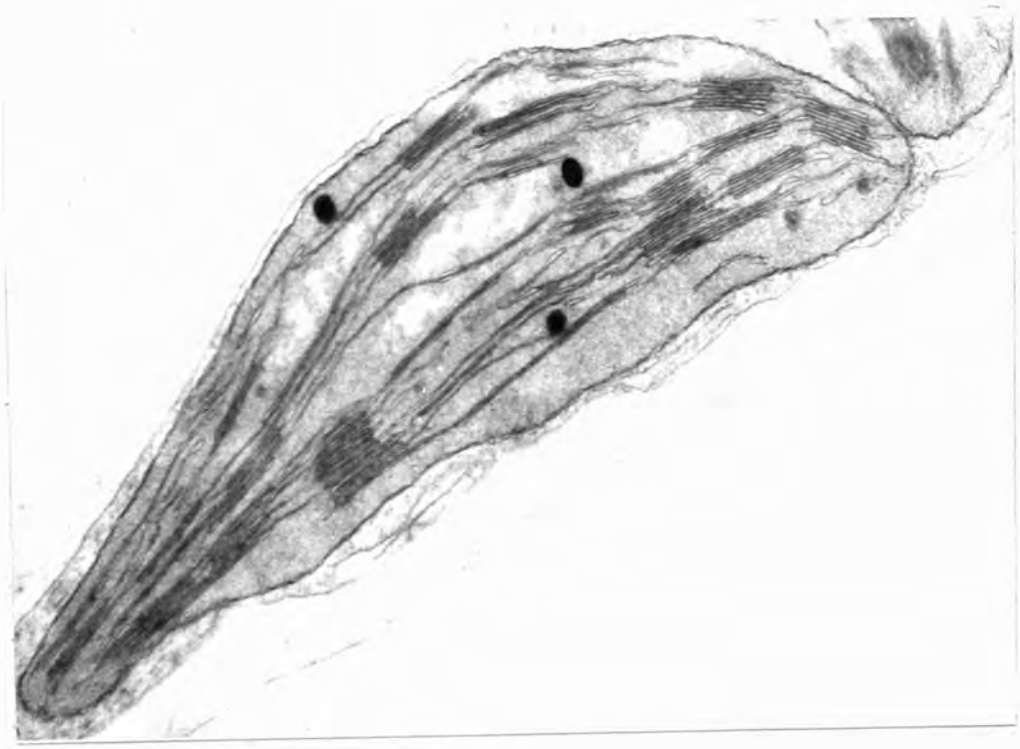

PLATE 18 *Section through the cell of the leaf 7 days after transfer to 35°C.*

Fig. 29. *An undifferentiated or partially differentiated plastid with few lamellae and large densely stained osmiophilic globules. The double membraned plastid envelope shows wavy outline.*
X 60,750.

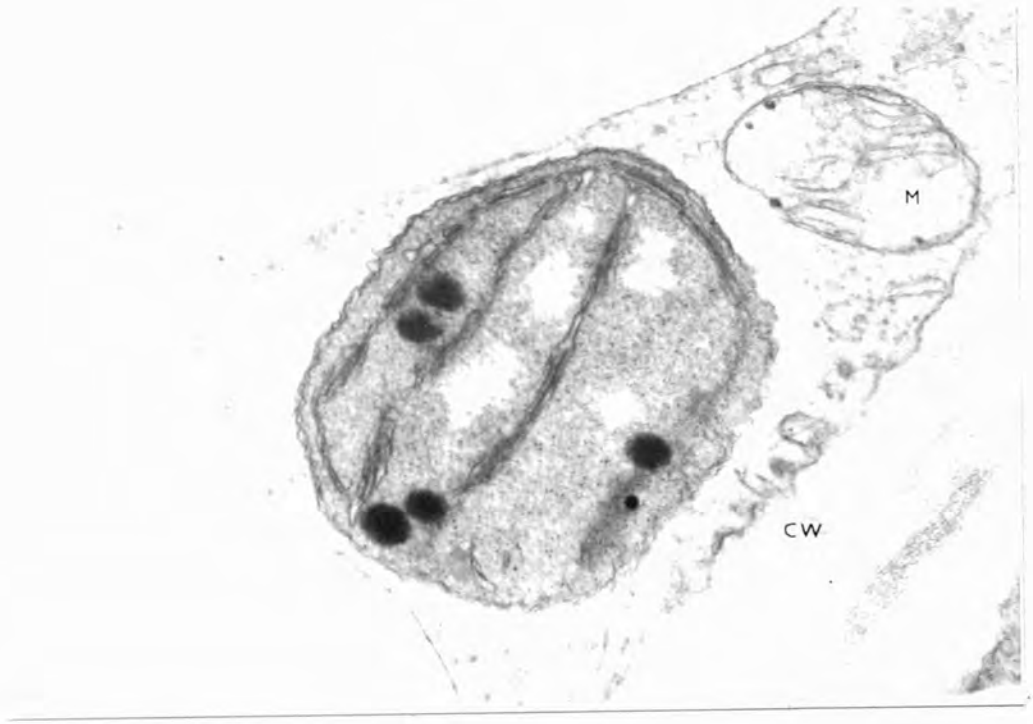
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PLATE 19 — *Section through the chloroplast of 15 days old leaf of the plant growing at 22°C.*

Fig. 30. — *Plano-convex chloroplast showing well developed grana and stroma lamellae and few starch grains. X 60,750.*

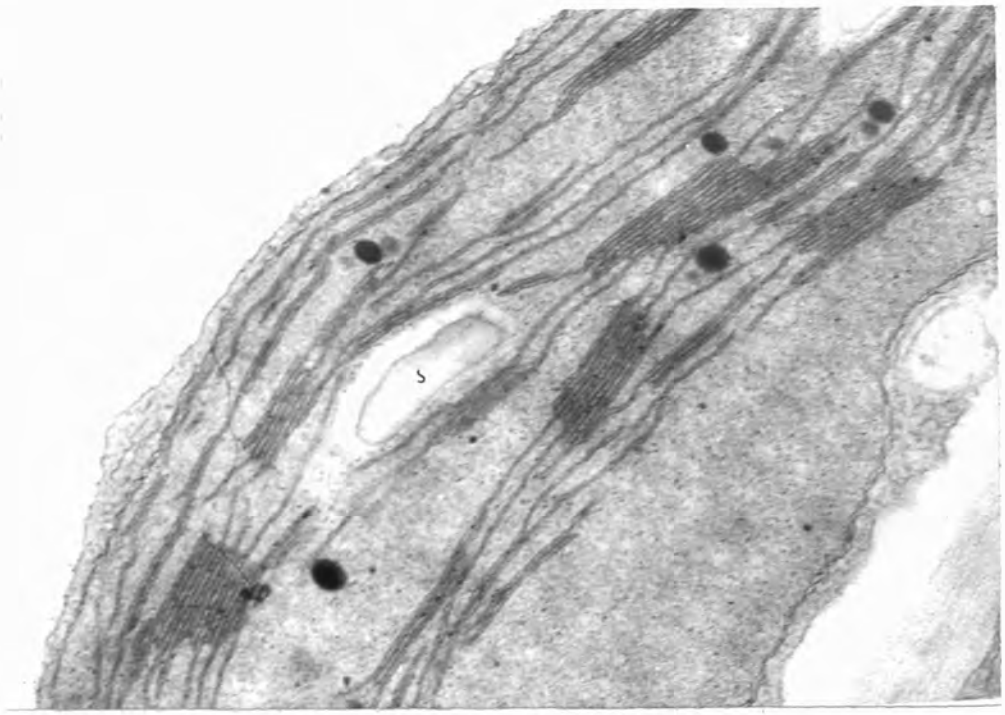

PLATE 20 *Chloroplasts from leaf 15 days after transfer to 25°C.*

Fig. 31. *Concavo-convex chloroplast with a network of well developed stroma and grana lamellae. The stroma lamellae are aligned in arrays parallel to the long axis of the chloroplast.*
X 36,450.

Fig. 32. *Chloroplast with large stroma space and a highly reduced starch grain. Both stroma and grana lamellae are well developed.*
X 60,750.


M

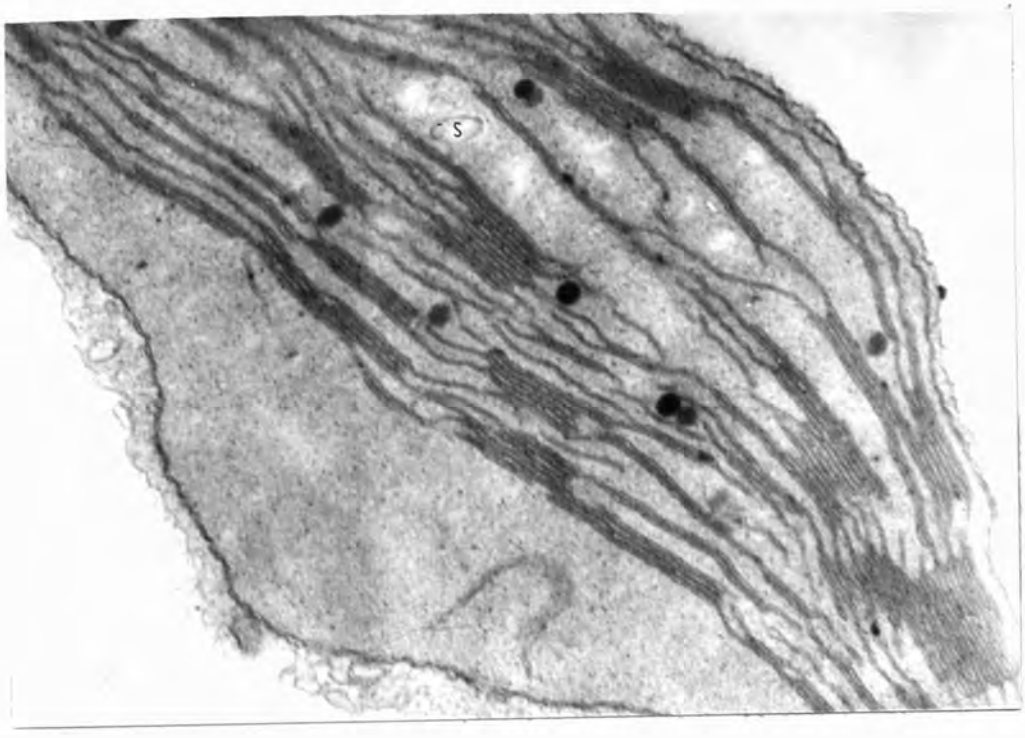
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PLATE 21 *Chloroplast from leaf 15 days after transfer to 30°C.*

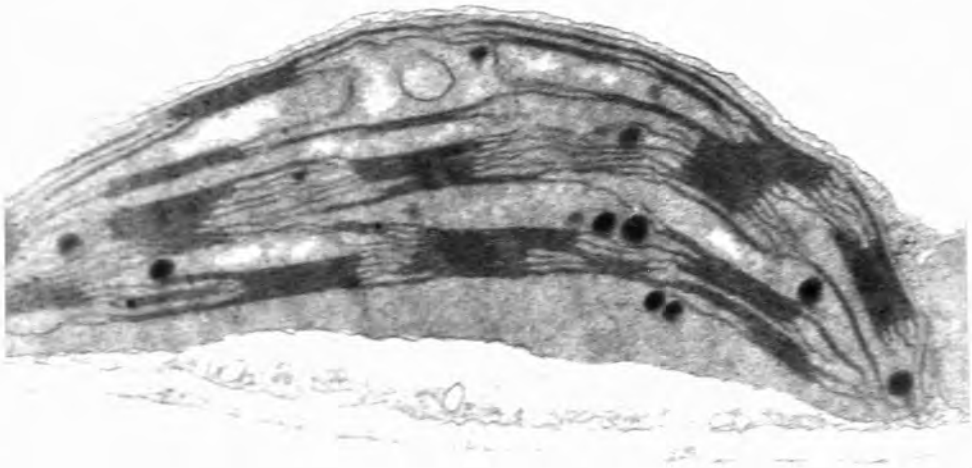
Fig. 33. *Small chloroplast with well developed stroma and grana lamellae, a small starch grain and large osmiophilic globules.*
X 24,300.


M
S

PLATE 22 *Chloroplasts with broken lamellae 15 days after transfer to 30°C.*

Fig. 34. *Chloroplast shows a reduction in size. Both stroma and grana lamellae are well developed. Some of the broken interconnecting lamellae give the appearance of loop and bridge-like connections. X 36,450.*

Fig. 35. *Section through the chloroplast showing the presence of hook-like lamellae formed from the broken interconnecting lamellae. Grana are multithylakoidal. Many large densely stained osmiophilic globules are present in the chloroplast. X 60,750.*



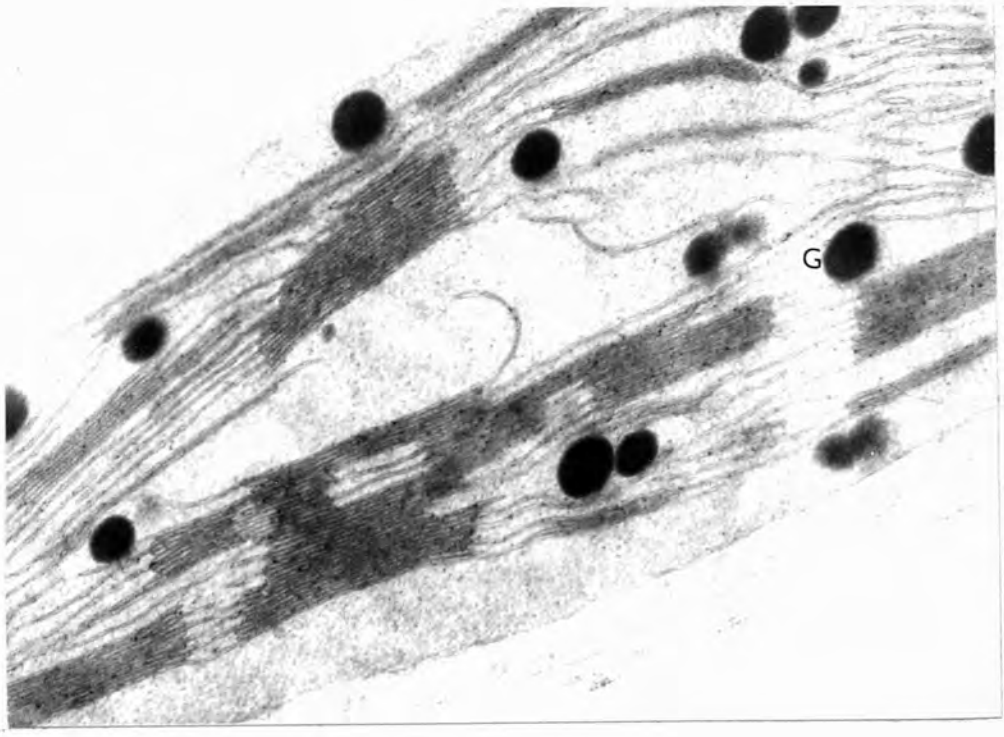

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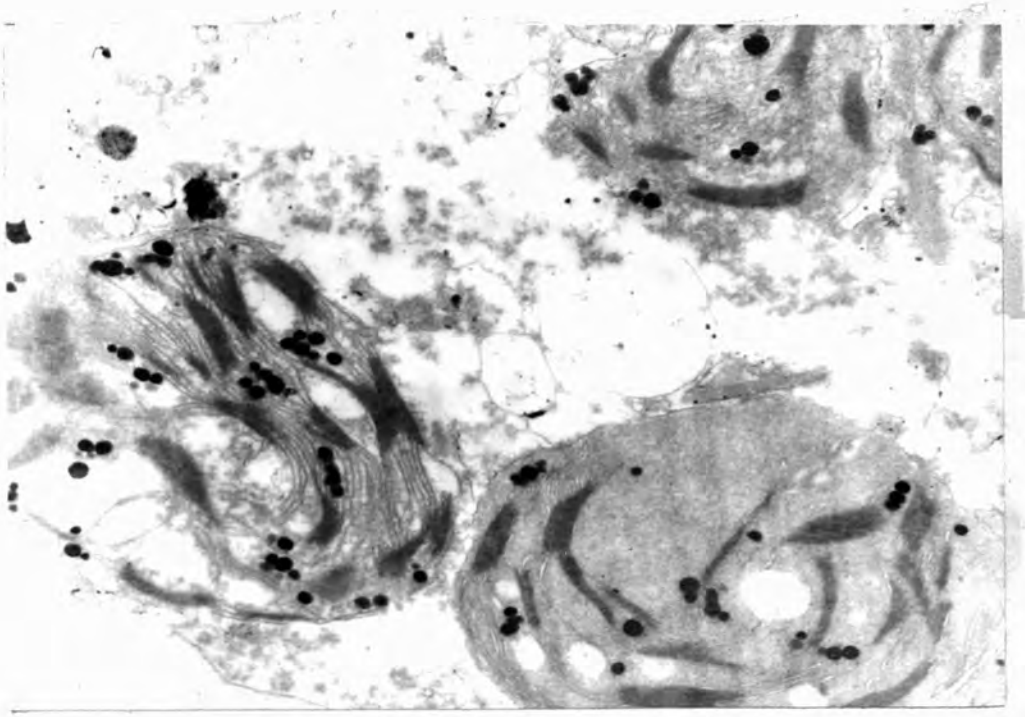
PLATE 23 *Chloroplasts with broken envelope membranes 15 days after transfer to 35°C.*

Fig. 36. *Rounded plastids partly or completely without the plastid envelope, a disrupted lamellar system, and many large osmiophilic globules.*
X 24,300.

Fig. 37. *Chloroplasts showing the complete loss of plastid envelope and the plastid contents lying free in the cell cytoplasm. The grana are completely disrupted.*
X 24,300.



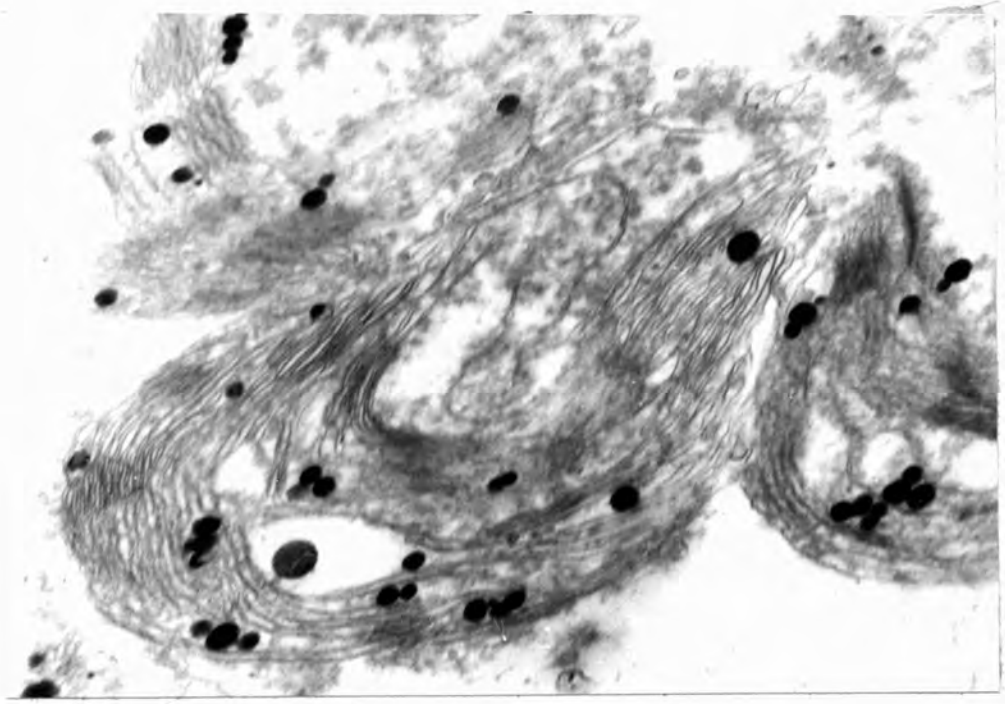
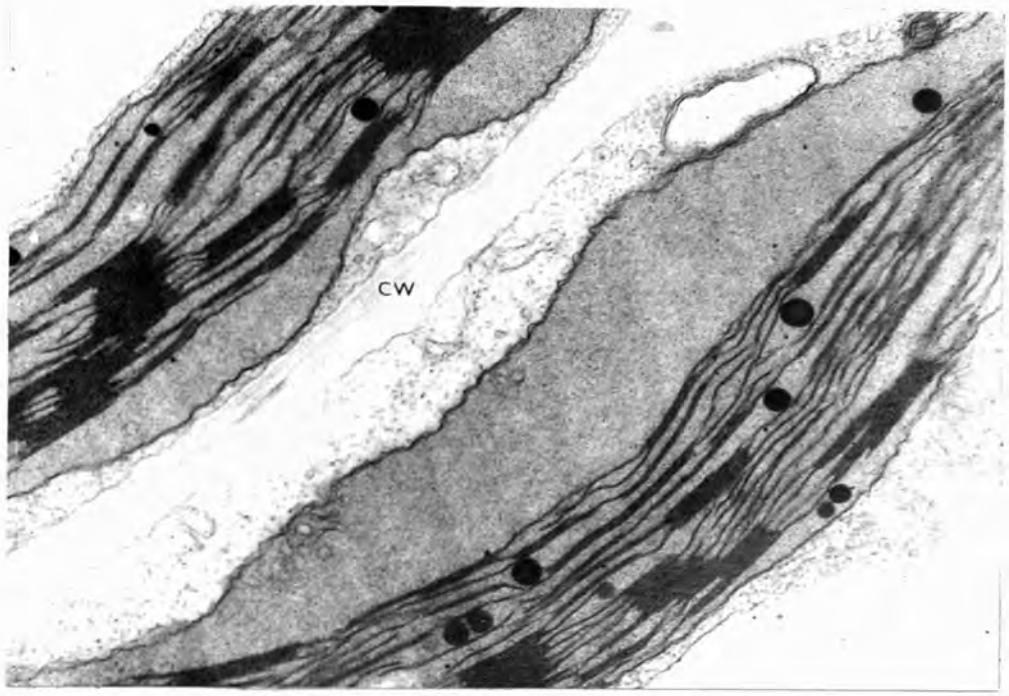


PLATE 24 *Chloroplasts from 21 days old leaf of the plant growing at 22°C temperature conditions.*

Fig. 38. *Elongated chloroplasts with well developed grana and stroma lamellae aligned in arrays parallel to the long axis of the chloroplast. Each chloroplast has many large osmiophilic globules.*
X 36,450.

Fig. 39. *Section through a plano-convex chloroplast with a large stroma space. Membrane whorls, which appear to lie in the stroma space, may lie in pockets of cytoplasm in indentations of the chloroplast envelope.*
X 60,750.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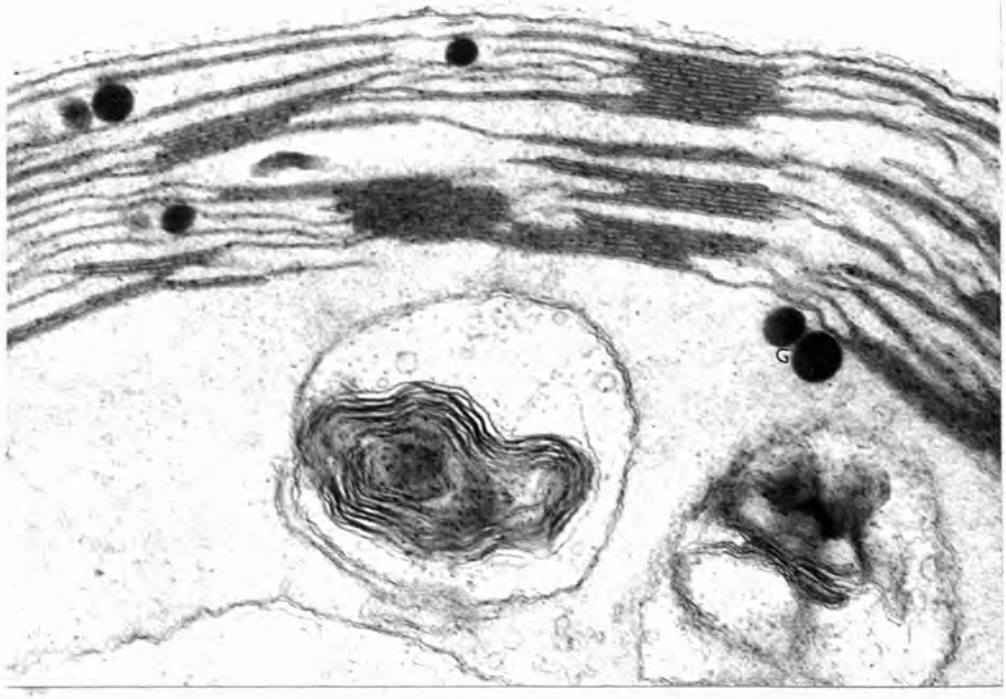
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PLATE 25 *Chloroplasts from 21 days old leaf of the plant growing at 22°C temperature conditions.*

Fig. 40. *Chloroplast showing deep arc-shaped grana fretwork system and many large osmiophilic globules. The 'hole' is probably a pocket of cytoplasm lying in an indentation of the plastid envelope.*
X 36,450.

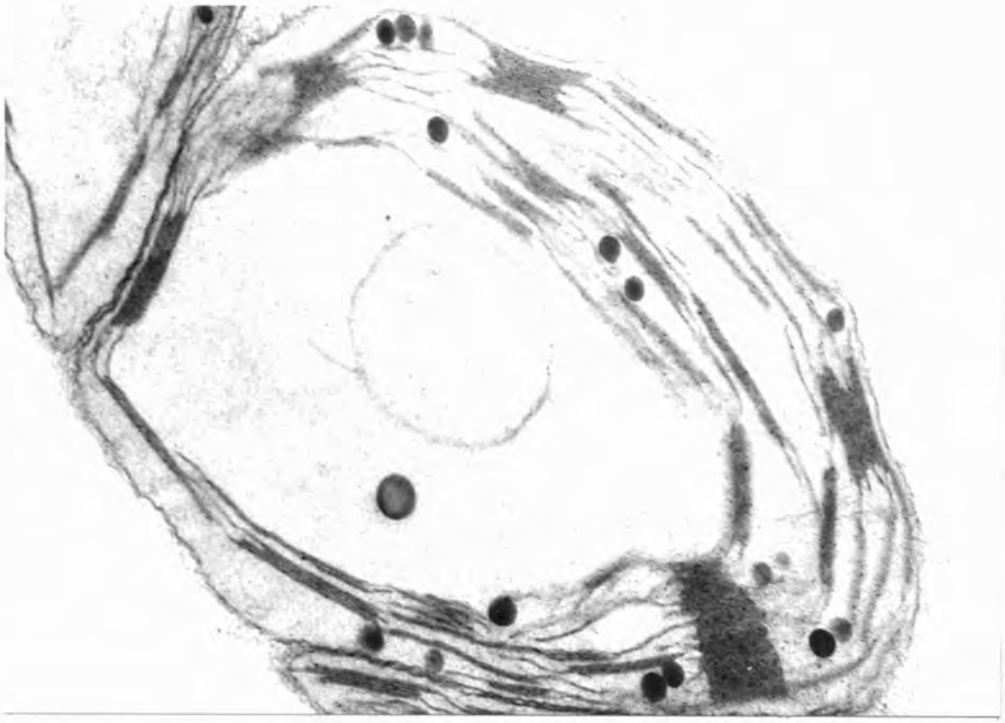


PLATE 26 *Section through the cells of leaf 21 days after transfer to 25°C.*

Fig. 41. *Chloroplast showing well developed grana which are connected by many stroma lamellae. Osmiophilic globules are large and densely stained.*
X 36,450.

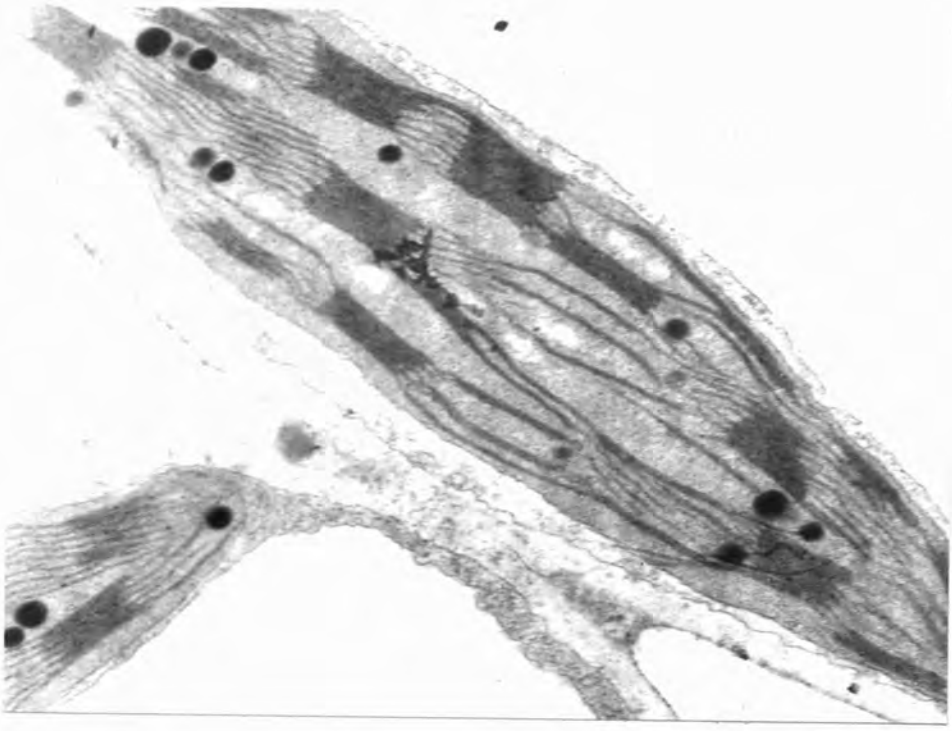
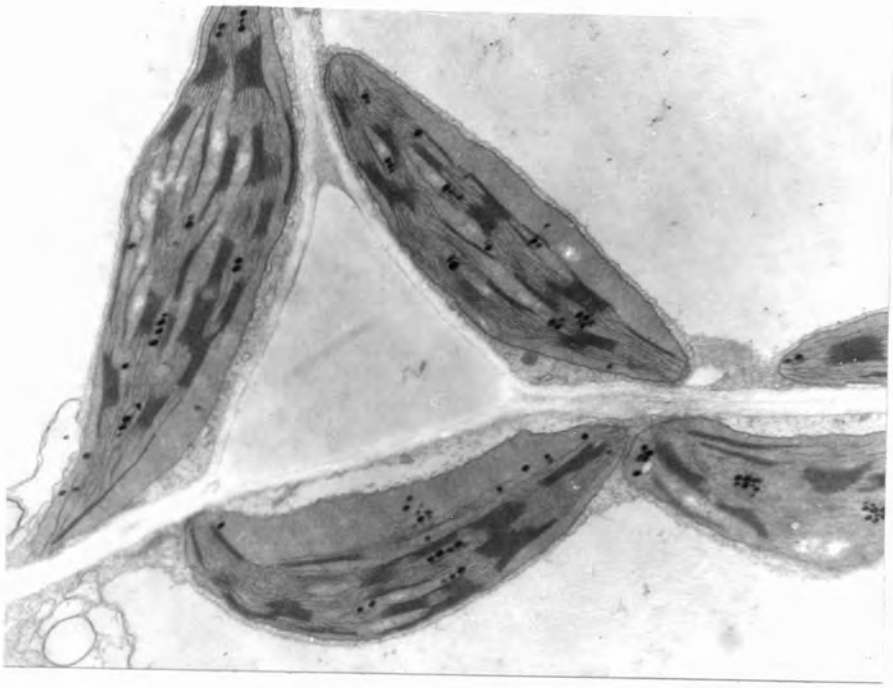


PLATE 27 *Chloroplasts showing reduction in size and possessing various shapes 21 days after transfer to 30°C.*

Fig. 42. *Each chloroplast contains many multi-thylakoidal grana connected by many long fret membranes.*
X 14,580.

Fig. 43. *Irregular-shaped chloroplast showing a very large granum. Some of the inter-connecting lamellae are broken and form hooks.*
X 24,300.



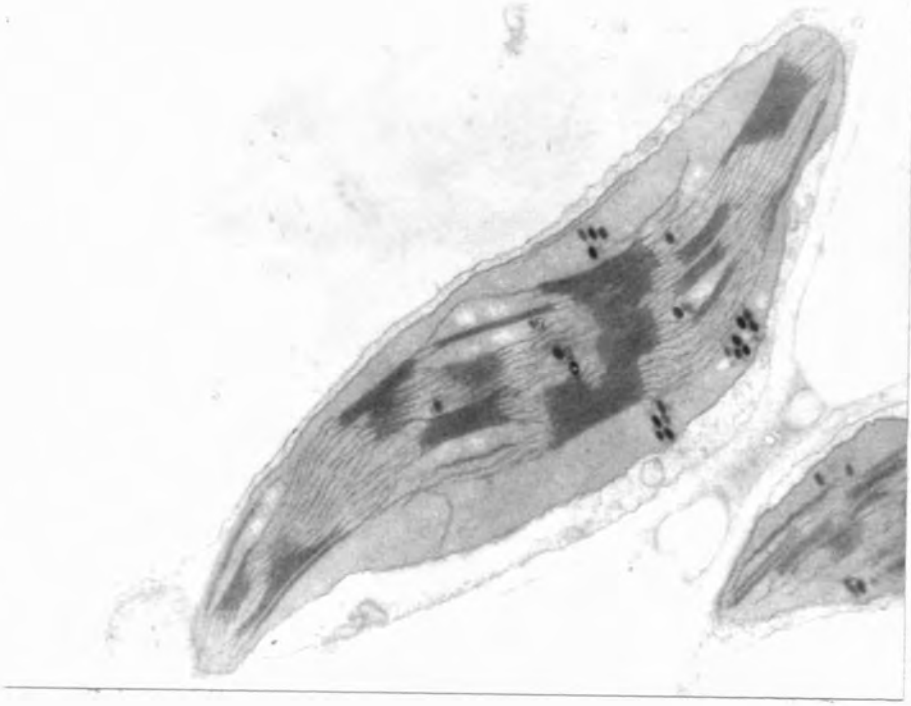
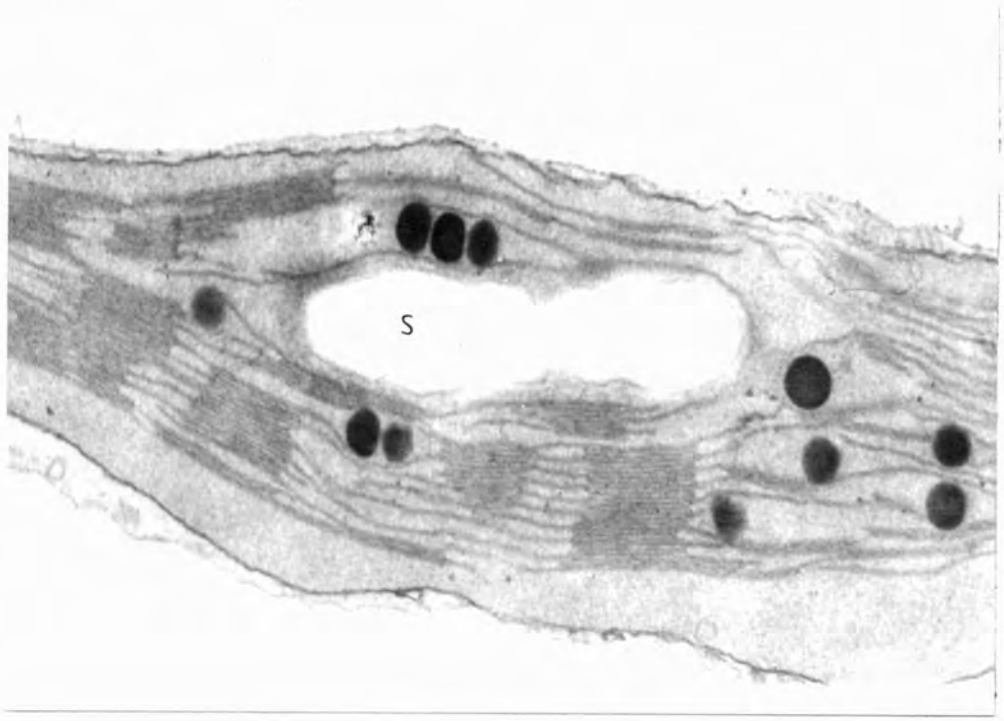


PLATE 28 *Sections through the chloroplasts of 28 days old leaf of the plant growing at 22°C temperature conditions.*

Fig. 44. *Chloroplast showing well developed lamellar system and a single large starch grain. Osmiophilic globules are densely stained and large in size. X 60,750.*

Fig. 45. *Section through a chloroplast showing a very large granum stacked with 55 thylakoids. The grana are connected by many long fret membranes. X 97,200.*


S

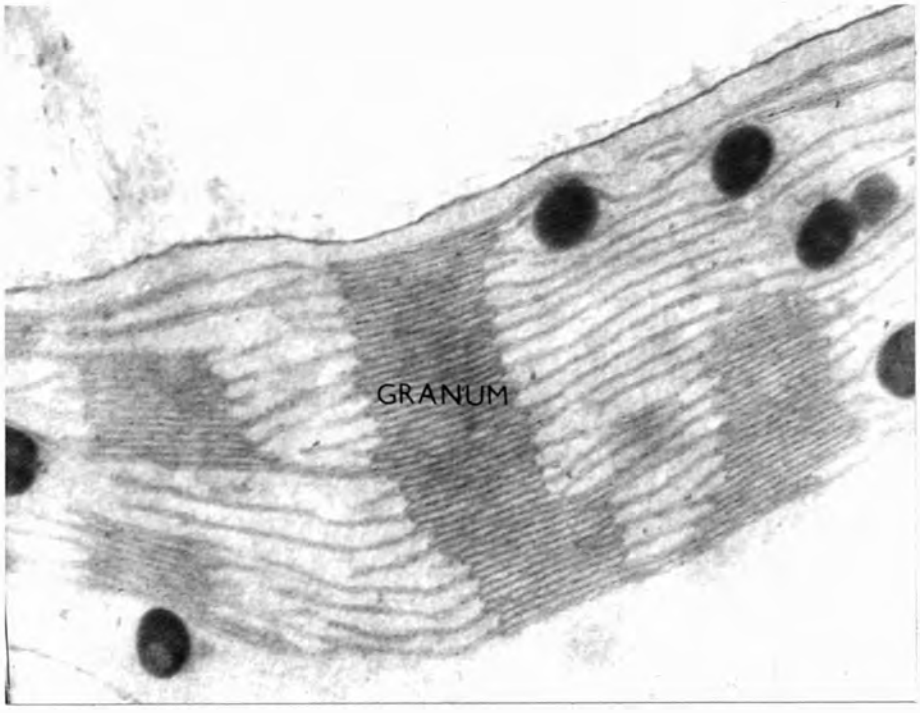

GRANUM

PLATE 29 *Irregular shaped chloroplast from a section of the leaf 28 days after transfer to 25°C.*

Fig. 46. *Chloroplast with irregular shaped stroma lamellae forming vesicles, hooks and loops, and a very small starch grain.*
X 36,450.


M
S

PLATE 30 *Chloroplast of leaf 28 days after transfer to 30°C.*

Fig. 47. *Chloroplast showing nearly spherical shape with completely distorted structure. Chloroplast contains many large osmiophilic globules and patches of broken lamellae. X 24,300.*

Fig. 48. *Enlargement of part of Fig. 47. X 97,200.*


CW

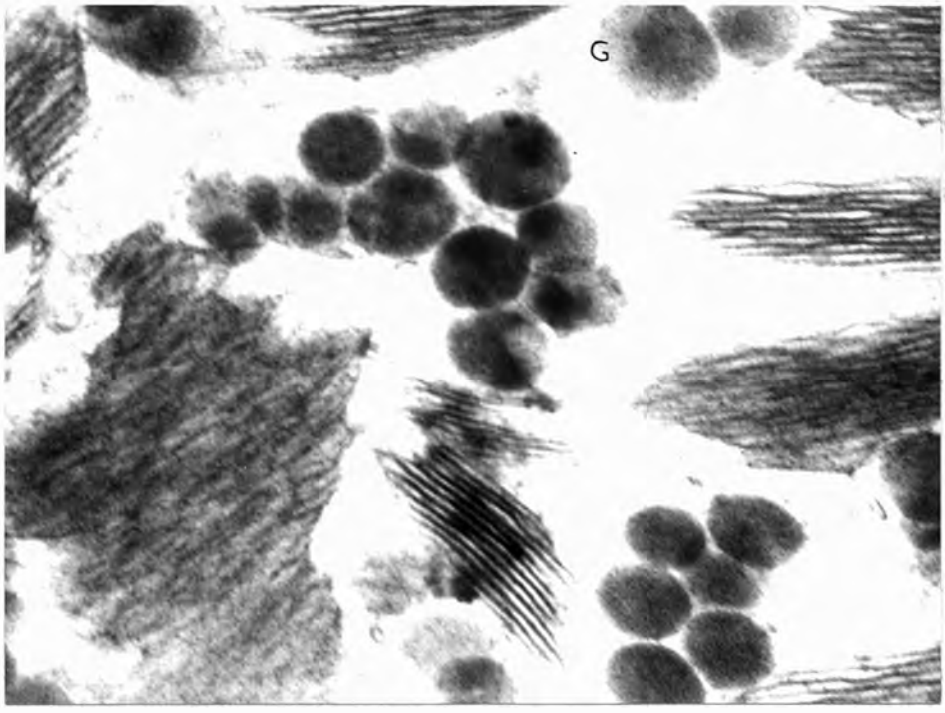
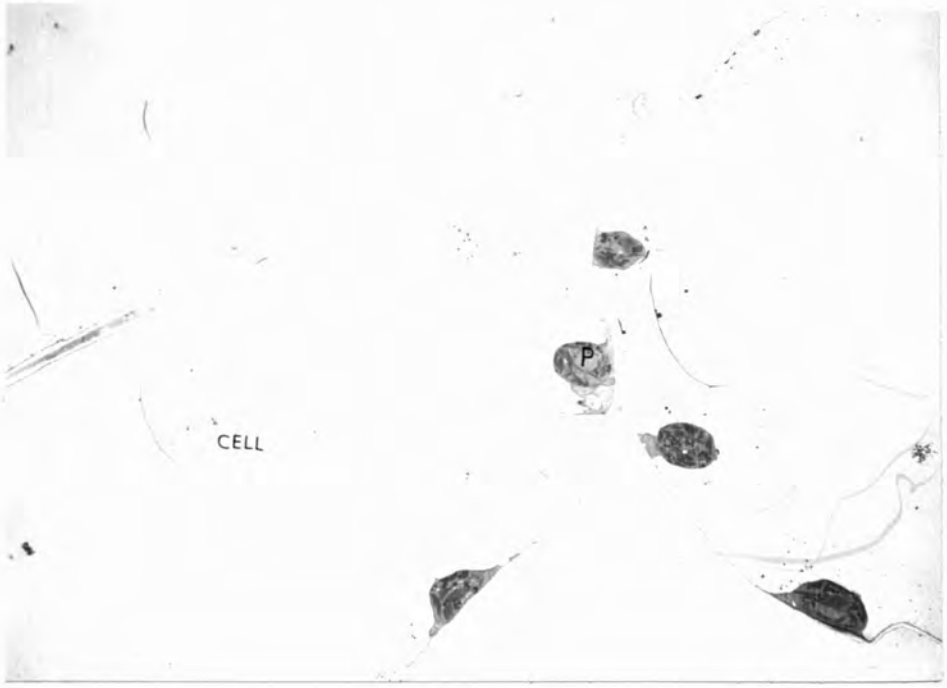

G

PLATE 31 *Section through the cells of leaf 28 days after transfer to 30°C.*

Fig. 49. *Section shows cells completely devoid of its contents other than plastids which have the distorted structure shown in Figs. 47 and 48. X 2,430.*


P
CELL

regions adjacent to the dense globules a characteristic swelling which appears as a club-shaped structure in section (Figs. 52 and 53) occurs at the terminus of each thylakoid. The number and size of densely stained globules show a much greater increase (maximum size - 0.2 μm). In the cytoplasm of the cell, mitochondria are still present and appear to be normal even at this stage of leaf senescence. Vacuoles are still there and with a layer of cytoplasm and chloroplasts pushed to the periphery of the cells. These structures show a general similarity with those of non-senescing cells.

In cells taken from completely yellowed leaf tissue (37 days after transfer) almost all the internal contents have disappeared and are consequently difficult to fix and embed. However, a consistent feature of the cells at this stage is the retention of plasmalemma. Most of the cells also contain small groups of greatly altered chloroplasts. Amongst these groups of plastids, other cell organelles can also be identified in a highly altered form. For instance, mitochondria are invariably retained but with swollen and distorted cristae. Chloroplasts (Fig. 54) have rounded, often retaining an intact bounding membrane which now encloses several groups of large electron-dense globules and irregular masses of stacked lamellae. Stroma lamellae has by this time been completely broken down, together with the ribosomal material of the stroma and the starch grains. If we compare Figure 54 with Figure 47, which shows the chloroplast after 28 days at 30°C, we see the states are identical. We infer from this that the normal process of senescence occurs at the higher temperatures but is hastened.

In the leaf of the plant after 35 days of transfer to the intermediate temperature of 25°C the onset of senescence is also clearly a little earlier. The chloroplasts at 22°C show only the first structural abnormalities characteristic of early stages of senescence, here at 25°C a more advanced senescent state is apparent.

The chloroplasts at this stage, at 25°C, become rounded with large (maximum size - 0.2 µm), very dense osmiophilic globules (Figs. 55 and 56). The grana remain well-formed but the stroma lamellae have become irregular and broken and form hooks, loops and bridges at their free ends (Fig. 55). Some of the chloroplasts under these conditions show curvatures of the grana-fretwork system where they become arc-shaped (Fig. 56). A number of distorted mitochondria are still present near the chloroplasts (Fig. 56). A number of small, densely stained globules are deposited, bead-like, along the chloroplast envelope. In places they appear to lie between the double lamellae of the membrane (Fig. 56 'b'). Subsequently the senescent chloroplasts lose the outer membranes and the disorganized contents are partially free in the disorganized cytoplasm.

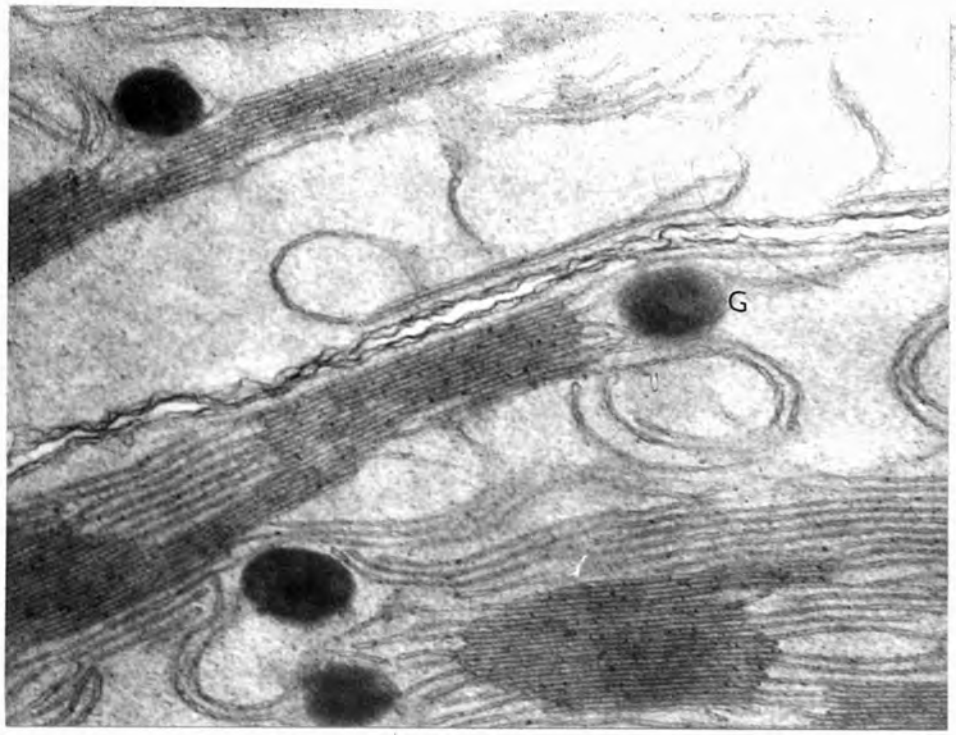
TABLE 2 - Summary of structural changes of chloroplasts of leaf during senescence

TEMPERATURE	Number of days at indicated temperature since transfer from 22°C		
	35 DAYS	36 DAYS	37 DAYS
22°C Original Growing Temperature	Abnormally shaped chloroplasts. Irregular stroma lamellae forming hooks, loops and bridges. Osmiophilic globules very large. Starch grains absent. Figs. 50, 51	Shrunken chloroplasts with broken lamellae. Free ends of lamellae show typical swellings. Very few grana. Osmiophilic globules large and numerous. Figs. 52, 53	Chloroplasts, rounded and shrunken. Lamellar system completely disrupted. Lamellae appear in the form of patches. Osmiophilic globules large and numerous. Envelope still intact. Fig. 34
25°C	Chloroplasts swollen. Stroma and grana lamellae still intact. Some broken lamellae forming loops, hooks and bridges. Lamellar system sometimes pushed to one side. Osmiophilic globules large, some appear to lie alongside chloroplast envelope. Figs. 55, 56	Chloroplast envelope ruptured even when the lamellar system is not completely distorted.	No observations.

PLATE 32 *Chloroplasts with abnormal lamellar system from 35-day old yellow-green leaf on 65-days old plant grown at 22°C.*

Fig. 50. *Section through the chloroplasts with irregular lamellar system forming loops and bridges. Some of the lamellae are broken and hang free in the stroma in the form of hooks. Few grana are still intact and are interconnected by many fret membranes. Osmiophilic globules are very large. Starch grains are absent.*
X 97,200.

Fig. 51. *An irregular-shaped chloroplast showing extensive development of curled lamellae spread throughout the stroma. Osmiophilic globules are very large.*
X 60,750.


G

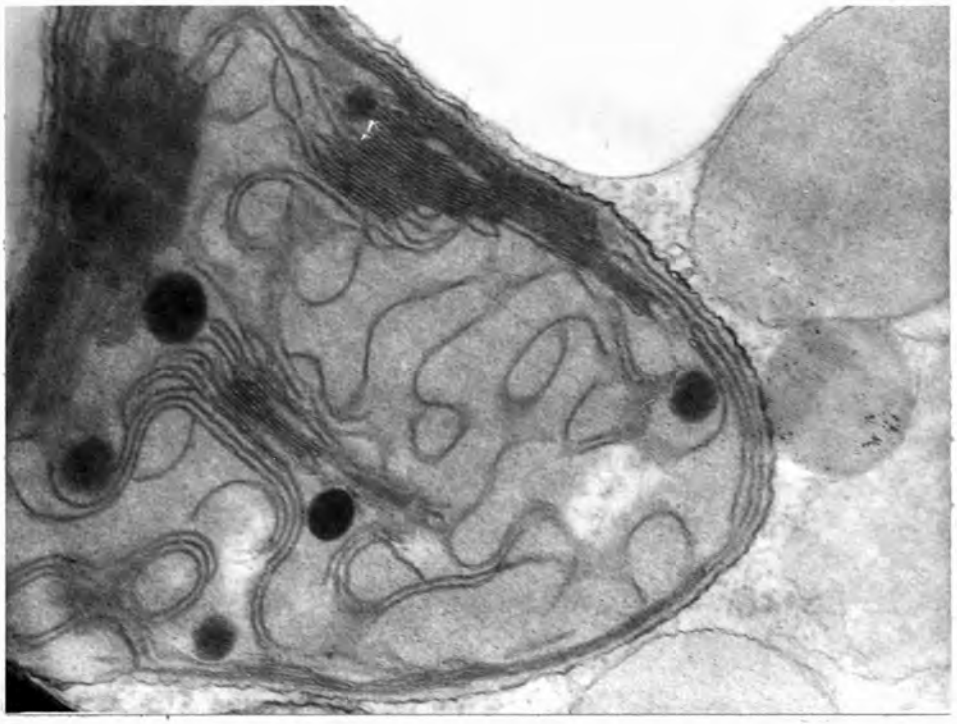


PLATE 33 — *Chloroplast of 36 day old yellow leaf on 66-day old plant grown at 22°C.*

Fig. 52. — *A chloroplast showing reduction in size and abnormal organization of lamellar system. Most of the lamellae are broken and show characteristic parting along their length and typical club-shaped swelling at the terminal ends of each lamella. Osmiophilic globules are very large.*
X 36,450.

Fig. 53. — *An enlargement of Fig. 52.*
X 97,200.


M

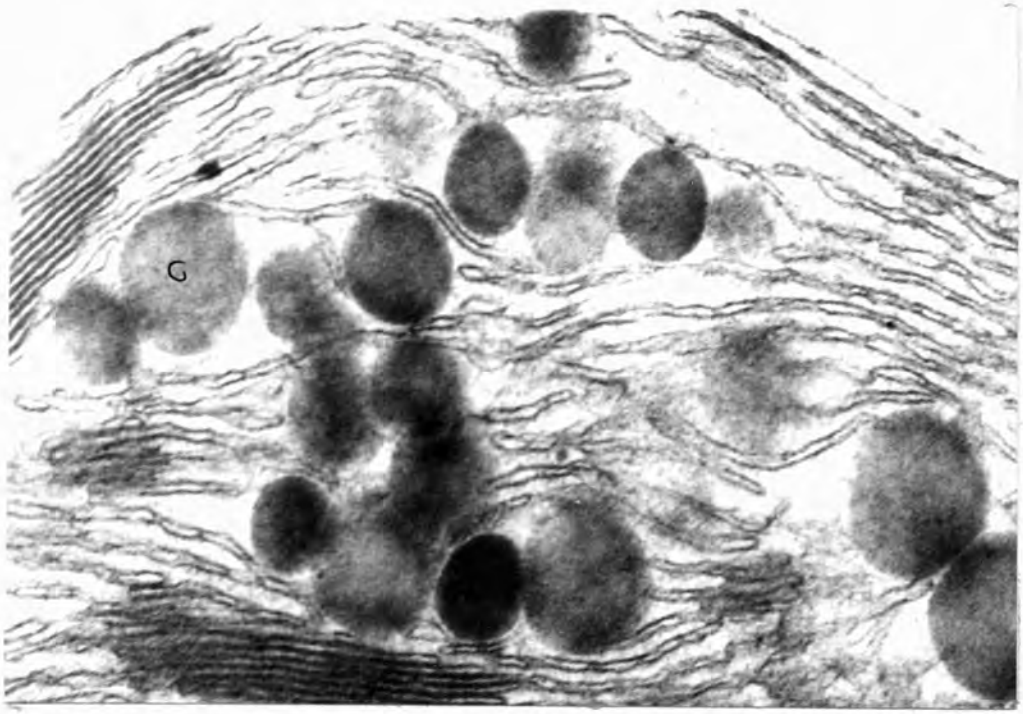

G

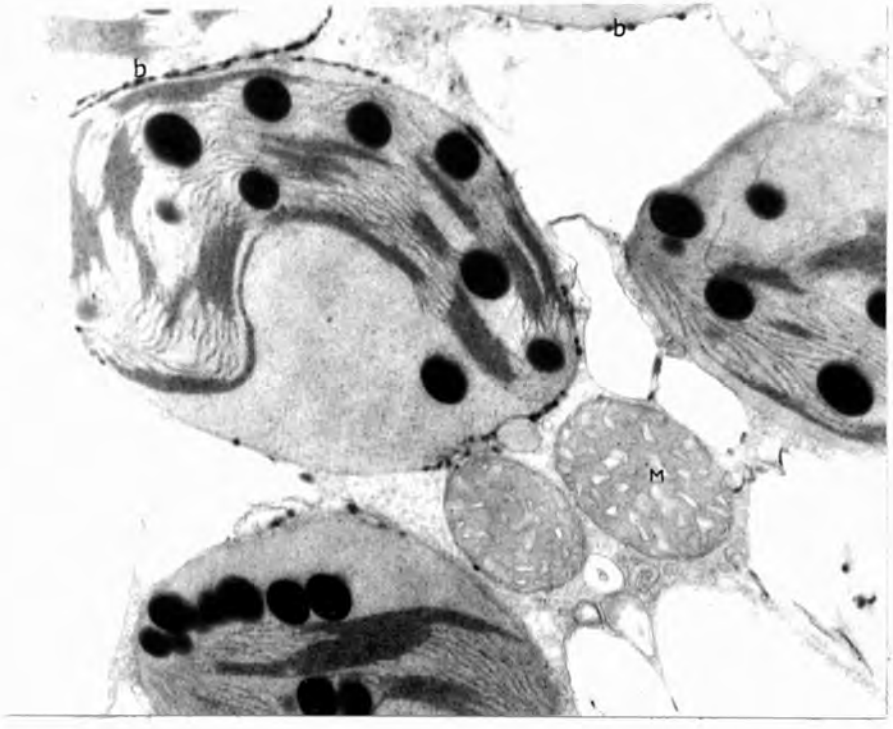
PLATE 34 *Plastid from 37-day old dry leaf from 67-day old plant growing at 22°C.*

Fig. 54. *Rounded plastid showing complete disorganization of the lamellar system. Broken down lamellae now form independent patches. Osmiophilic globules are numerous and very large.*
X 36,450.

PLATE 35 *Rounded chloroplasts of the 35-day old yellow-green leaf on 65-day old plant grown at 22°C for the first 30 days and then transferred to 25°C.*

Fig. 55. *Chloroplast showing normal grana but most of the interconnecting lamellae are broken forming loops, hooks and bridges. Osmiophilic globules are very large.*
X 60,750

Fig. 56. *Section through the cell showing that in some of the chloroplasts the lamellar system is pushed to one side. The two envelope membranes are indistinguishable from each other and a deposition of small densely stained globules appear on the surface of the envelope. Osmiophilic globules are very large.*
X 60,750


b
b
M

PART-B

OBSERVATIONS ON FRESH WEIGHT AND CHLOROPLYLL CONTENT OF LEAVES DURING GROWTH AT DIFFERENT TEMPERATURES

Parallel to the sampling for electron microscopy additional samples of the growing leaves were taken to determine the fresh weight and chlorophyll content.

THE EFFECT OF GROWING TEMPERATURE ON THE FRESH WEIGHT OF ATTACHED LEAVES

METHOD

The samples for electron microscopy, fresh weight and chlorophyll content were taken at weekly intervals until the leaves were completely yellow in colour. To mark the age of the leaves, the newly differentiated leaves were tagged just before putting the four weeks old plants in the different temperature controlled cabinets.

Five replications of the leaves were taken at each stage of sampling and under each treatment. The whole spinach leaf was cut carefully from the base with a sharp razor blade and pressed gently between the two folds of soft, absorbant tissue so as to remove any surface water from it and then gently cleaned from both the surfaces and weighed on a fractional balance.

RESULTS

At both 22°C and 25°C the fresh weight of the leaf increases up to 21 days of leaf age. Although there is a little difference up to the seventh day the increase in leaf fresh weight at 22°C between the seventh day and twenty-first day is almost twice the

TABLE 3 - Fresh weight of leaf (g) during its growth at different temperatures

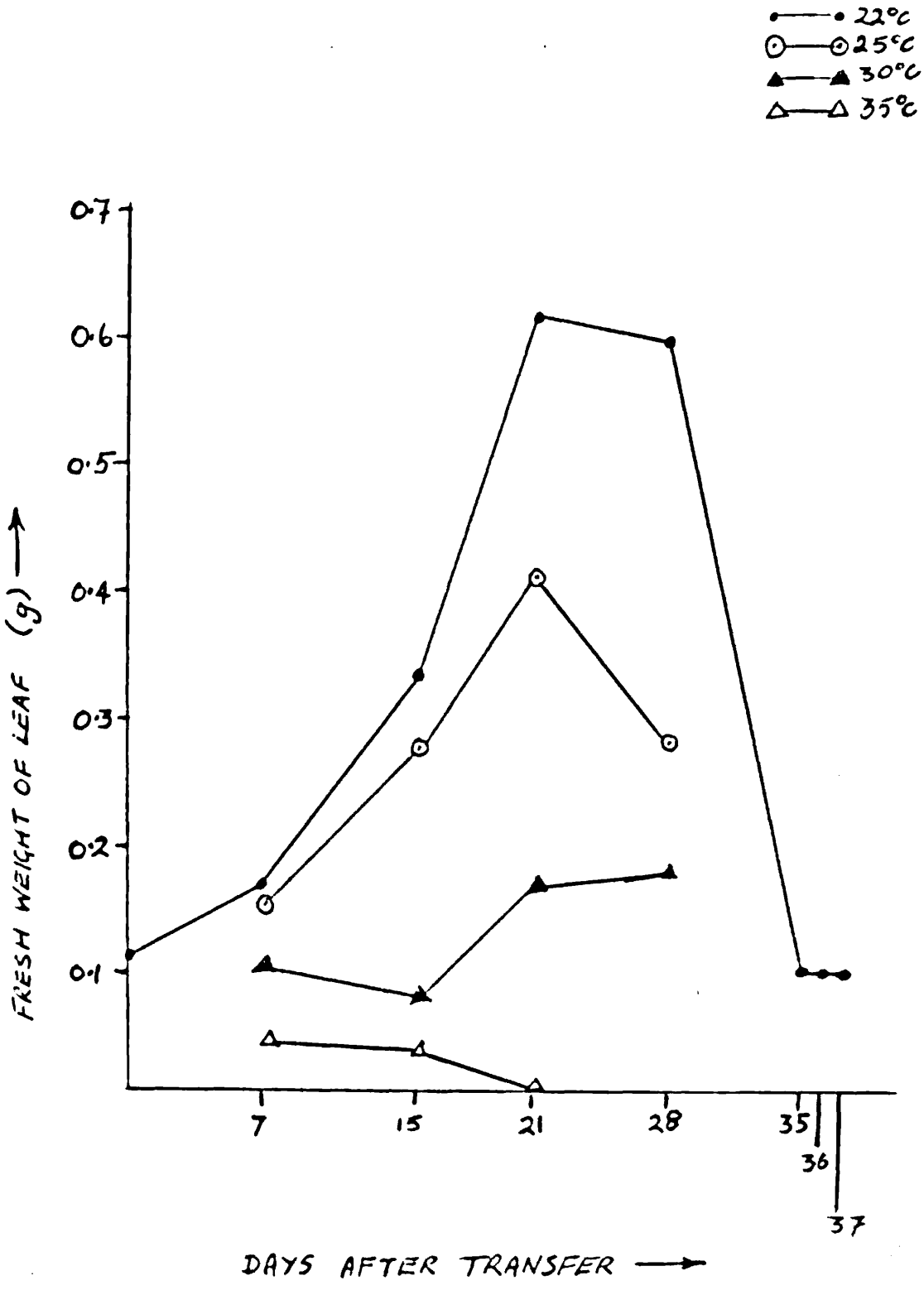
TEMPERATURE	No. of days at indicated temperature since transfer from 22°C							
	0 DAY	7 DAYS	15 DAYS	21 DAYS	28 DAYS	35 DAYS	36 DAYS	37 DAYS
* 22°C	0.11	0.167	0.333	0.610	0.591	0.100	0.100	0.100
25°C	-	0.150	0.274	0.410	0.278	-	-	-
30°C	-	0.104	0.077	0.174	0.184	-	-	-
35°C	-	0.042	0.037	0.010	-	-	-	-

* Original growing temperature

increase at 25°C. The leaf fresh weight at 30°C shows a reduction up to the fifteenth day after which there is a small increase. However, the leaf fresh weight at 35°C shows a continuous reduction throughout. The visual observations have shown that the increase in area of the leaves of the plants subjected to 35°C temperature conditions is very little in comparison to leaves growing under 22°C and 25°C temperature conditions. The increase in area of leaves at 30°C, although less than at 22°C and 25°C temperature conditions respectively (TEXT Figure 7.), is much greater than at 35°C temperature conditions.

The results also show that just before the onset of senescence at 22°C and 25°C there is an abrupt reduction of the fresh weight of leaf.

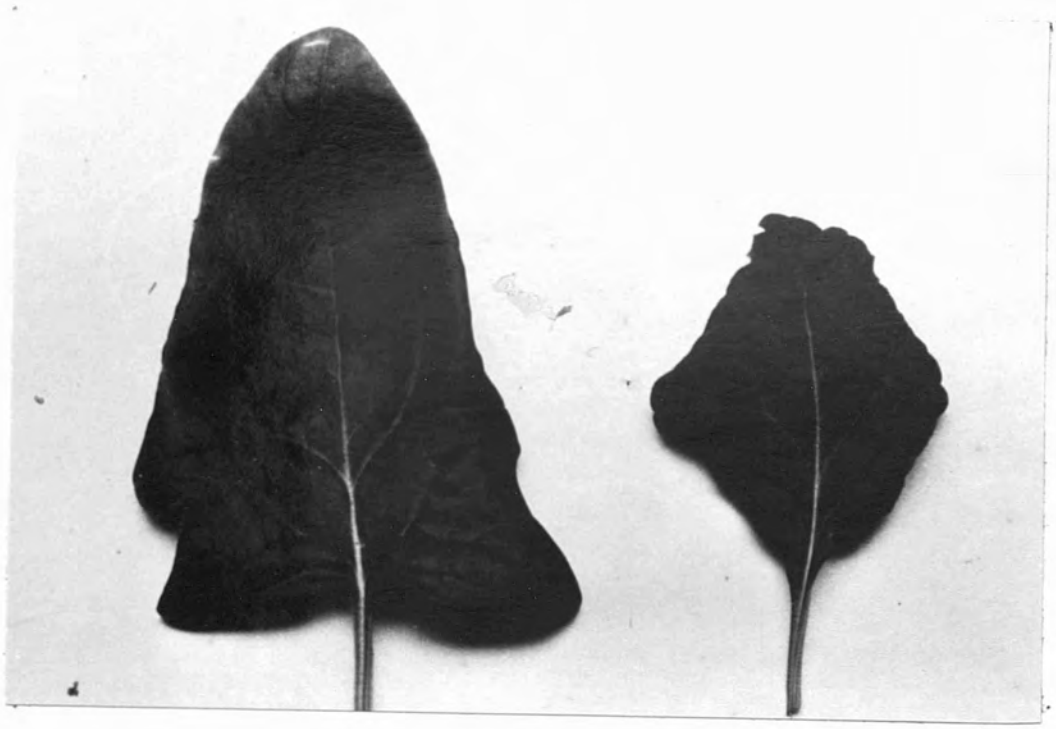
TEXT Figure 6. *Fresh weight of leaf (g) during its growth at different temperatures.*


22°C
25°C
30°C
35°C
0·7
0·6
0·5
0·4
0·3
0·2
0·1
FRESH WEIGHT OF LEAF (g)
7
15
21
28
35
36
37
DAYS AFTER TRANSFER

TEXT Figure 7 *Typical leaves on seven week old plants grown under the following conditions:*

A. *22°C temperature conditions for seven weeks*

B. *22°C for four weeks followed by three weeks at 30°C*



THE EFFECT OF GROWING TEMPERATURE ON THE CHLOROPHYLL CONTENT OF LEAVES

METHOD

Samples from the leaf tips weighing 0.1 g (or the complete leaf if the leaf weighed less than 0.1 g) were taken in replications of five, at each stage of sampling and homogenized in 5 ml of 80% acetone. The homogenate was then centrifuged at 5,000 rpm for five minutes and the supernatent was collected. Two more washings of the residue were given with 4 ml of 80% acetone each to extract the complete colour. All the supernatent was combined and the total volume made to 20 ml. Optical density was taken on a Beckman Spectrophotometer at 645 nm and 663 nm using 80% acetone as blank. The amount of total chlorophyll was calculated from the formula given below (Kirk, 1968):

$$\text{mg total chlorophyll/g tissue} - (20.2(D_{645}) + 8.02(D_{663})) \times \frac{V}{1000x\ W}$$

where,

V - Total volume made (ml)

W - Weight of sample (g)

D - Optical Density at specified wavelength

RESULTS

The date for chlorophyll content show that during different stages of leaf growth under normal temperature conditions ($22^{o}C$)

TABLE 4 - Total chlorophyll (mg/g fresh weight) during growth of leaf at different temperatures

TEMPERATURE	No. of days at indicated temperature since transfer from 22°C							
	0 DAY	7 DAYS	15 DAYS	21 DAYS	28 DAYS	35 DAYS	36 DAYS	37 DAYS
* 22°C	2.125	2.175	2.24	2.323	2.535	2.733	0.9	0.46
25°C	-	2.075	2.156	2.240	2.250	1.500	-	-
30°C	-	1.96	2.05	1.980	1.40	-	-	-
35°C	-	2.530	2.375	1.600	-	-	-	-

* Original growing temperature

there is an almost linear increase in the chlorophyll content up to 35 days of leaf age which is followed by a sudden decline during senescence of the leaf (Table 4; TEXT Figure 8).

There is an apparent increase in chlorophyll content of the leaves at 35°C after the newly differentiated leaf has been given this temperature treatment for seven days but later on the chlorophyll content clearly decreases continuously.

Both 25°C and 30°C temperature treatments show a slight decrease in the chlorophyll content in the first week of treatment which is followed by an increase up to the fourth week at 25°C temperature conditions but only up to the second week at 30°C temperature.

Total chlorophyll per leaf

When the results are plotted as total chlorophyll per leaf we see that the chlorophyll content generally follows the same trend as that of fresh weight changes of the leaves grown at the respective temperature conditions (cf. TEXT Figures 6, 9). The only difference observed is that the total chlorophyll content of the whole leaf grown at 30°C shows a little decrease between the 21st and 28th days whereas the fresh weight of the leaf has increased slightly. This result reflects the large decrease in chlorophyll per gram fresh weight. The important difference observed between the total chlorophyll and that per gram fresh weight at 35°C reflects the large decrease in fresh weight of the leaf at this high temperature during the first week. The apparent increase in chlorophyll could well

be solely due to dehydration. Unfortunately, we do not have any information on dry weight.

TEXT Figure 8. *Total chlorophyll (mg/g, fresh weight) during growth of leaf at different temperatures.*

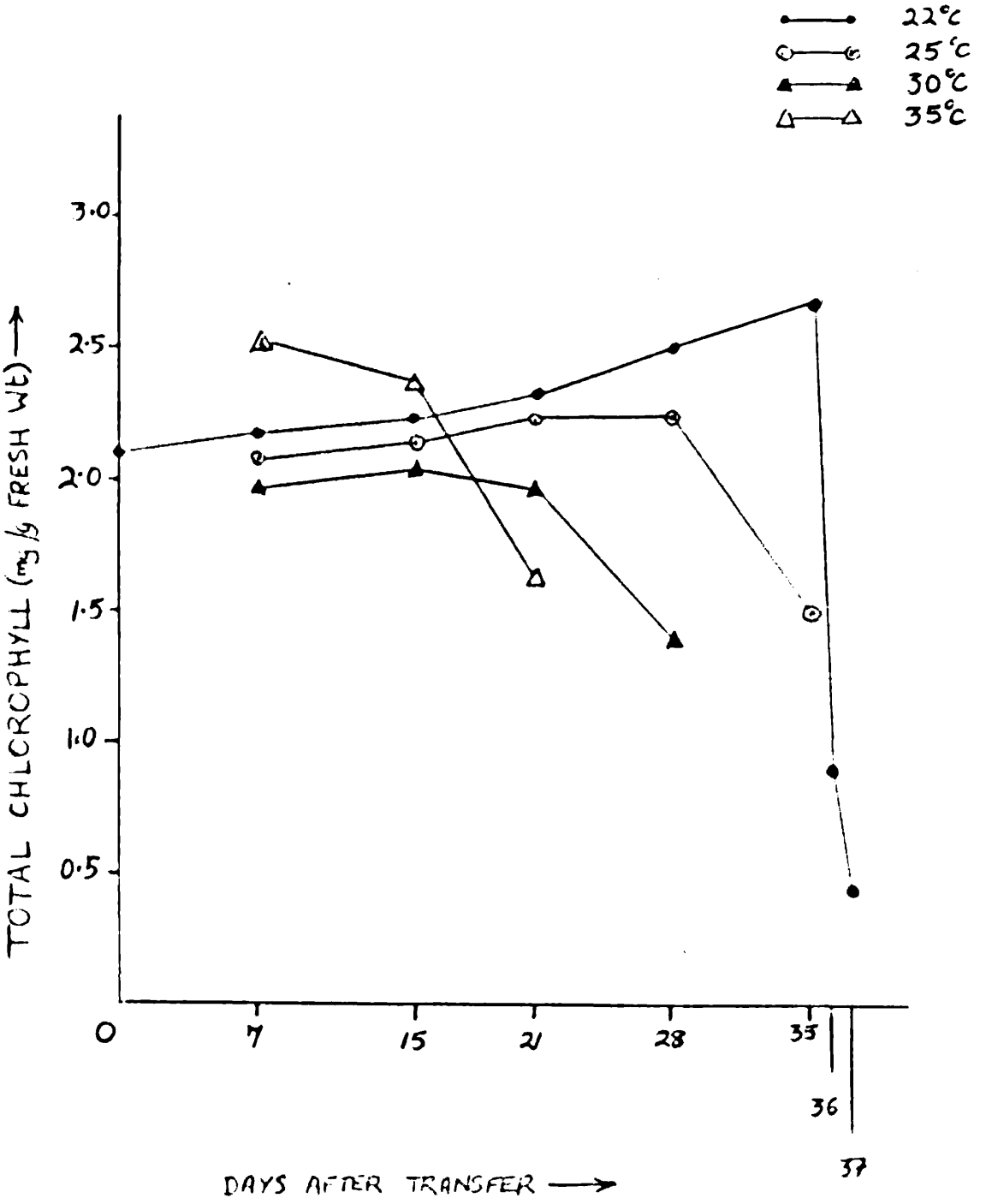
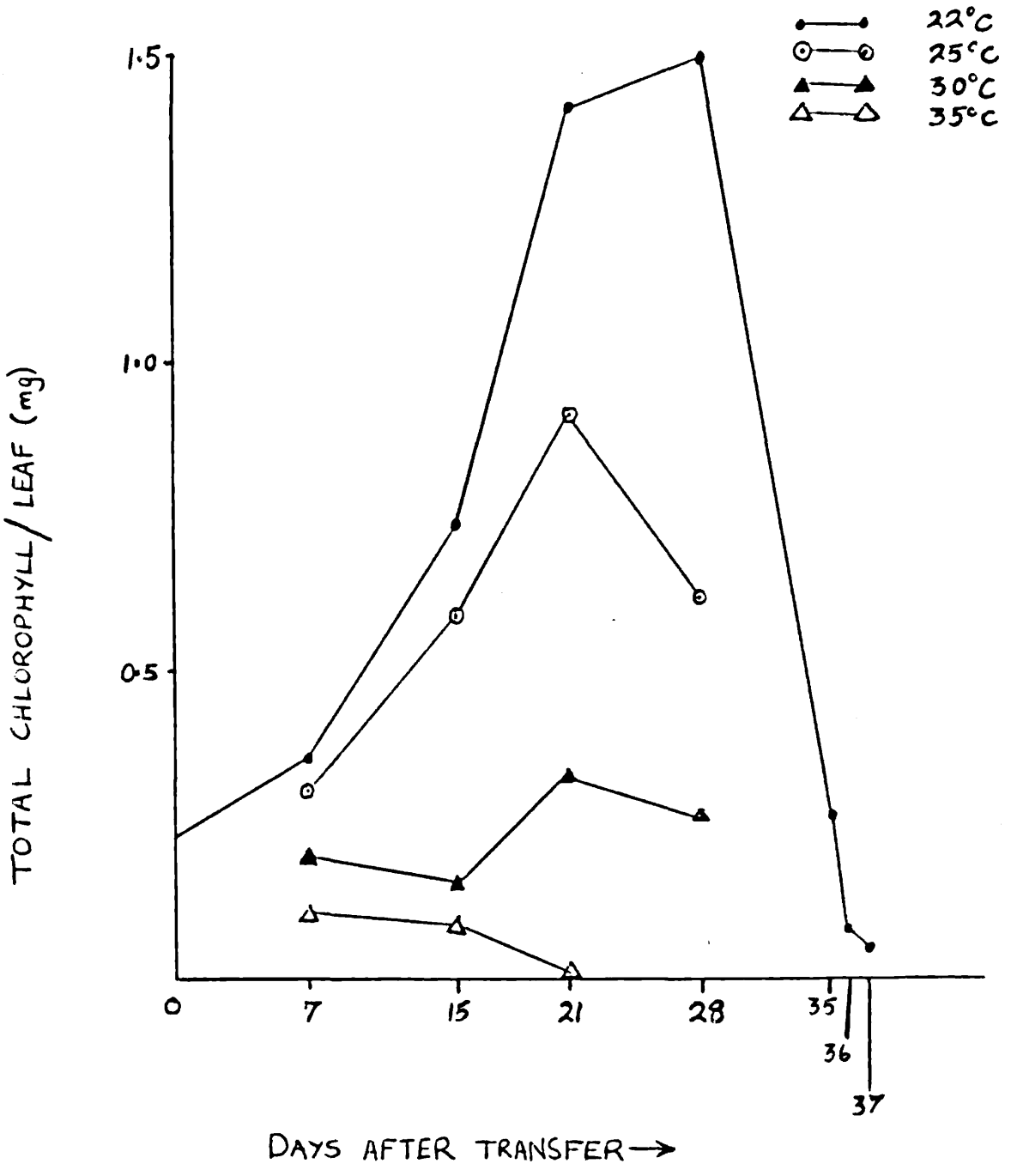

22°C
25°C
30°C
35°C
TOTAL CHLOROPHYLL (mg/g FRESH WT) ⟶
3·0
2·5
2·0
1·5
1·0
0·5
0
7
15
21
28
35
36
37
DAYS AFTER TRANSFER ⟶

TABLE 5 - Total chlorophyll (mg/leaf, fresh weight) during growth of the leaf at different temperatures

TEMPERATURE	No. of days at indicated temperature since transfer from 22°C							
	0 DAY	7 DAYS	15 DAYS	21 DAYS	28 DAYS	35 DAYS	36 DAYS	37 DAYS
* 22°C	0.234	0.363	0.746	1.419	1.498	0.273	0.090	0.046
25°C	-	0.311	0.591	0.918	0.625	-	-	-
30°C	-	0.204	0.158	0.344	0.258	-	-	-
35°C	-	0.106	0.906	0.016	-	-	-	-

* Original growing temperature

TEXT Figure 9. *Total chlorophyll (mg/leaf, fresh weight) during growth of leaf at different temperatures.*


22°C
25°C
30°C
35°C
1·5
1·0
0·5
TOTAL CHLOROPHYLL / LEAF (mg)
0
7
15
21
28
35
36
37
DAYS AFTER TRANSFER →

DISCUSSION

The data in the present investigations have been collected to evaluate the effect of a range of temperatures on the chloroplast ultrastructure during different stages of leaf growth and senescence. From the micrographs it becomes apparent that young spinach plants subjected to continuous high temperature conditions soon show a considerable disruption of the leaf chloroplast ultrastructure and this degradative process increases with the increase of temperature to which the plant is subjected. Under normal temperature conditions (22°C in the present investigations) the process of chloroplast degradation occurs as a feature of leaf senescence.

The changes in the ultrastructure of the chloroplast which take place during normal ageing and senescence of spinach leaves do not differ in many fundamental ways from those which have been reported from the leaves of other plants (Barton, 1966; Hurkman and Kennedy, 1975; Hurkman, 1979). In each case the chloroplasts are usually the first organelles to show some changes in the structure but are also the most persistent and could still be identified when all other cell organelles had degenerated. The pattern of events is slightly different in the artificially induced senescence of wheat leaves where the endoplasmic reticulum and the mitochondria appear to be the first organelles to show structural changes (Shaw and Manocha, 1965).

The plastids of spinach leaves show that during earlier stages of leaf growth, in fully green leaves, temperatures of 22°C and 25°C

do not produce any significant change in the chloroplast size and shape, whereas the onset of senescence under normal growth temperature and exposure to the higher temperatures (30°C and 35°C) both lead to reduction in the size of the chloroplasts as well as changing the shape of the chloroplasts. As the breakdown processes progress the shape of the plastid changes to elliptical and spherical. Under all temperature conditions the fully disrupted plastids seem to be much smaller than those of normal plastids. The reduction in size and change in shape of chloroplasts of yellow-green and fully senescent leaf have also been earlier reported in Betula verrucosa (Dodge, 1970), Nicotiana tabaccum (Hurkman and Kennedy, 1975), Triticum aestivum (Hurkman, 1979). Presumably, this reduction can be accounted for by the loss from the chloroplast of most of the stroma and chloroplyll and some of the proteins from the membrane systems. Heating also causes a partial destruction of the chloroplast membrane lipids, presumably owing to the activation of lipases (Molotkovsky and Zheskova, 1965). The recorded dearrangement of the spinach chloroplast structure results not so much to the destruction of the lipid ingredients of the membranes as to the direct action of free fatty acids so formed as described by Molotkovsky and Zheskova (1965). But Santarius (1980) has shown that the heat treatment of intact leaves and of isolated thylakoid membranes from spinach (Spinacia oleracea L.) caused inactivation of photochemical processes such as electron transport through photosystem II and photophosphorylation. According to him the heat induced damage to thylakoids is not caused by chemical alterations in the lipids such as oxidation of unsaturated fatty

acids, or release of free fatty acids due to hydrolysis of lipids. He showed that during high temperature treatment changes in the lipid-lipid interactions and/or delocalization of specific lipids within the thylakoids might be responsible for the disorganization of the functional integrity of the membranes. Since, as also shown by Molotkovsky and Zheskova (1965), thermostability of chloroplast membranes is decreased when they are exposed to free fatty acids, small amounts of membrane lipids which become hydrolyzed during extended heat treatment may partly contribute to the primary heat damage of spinach chloroplasts.

The rapid build up of starch in chloroplasts during the early stages of leaf growth appears to cease as the leaf approaches maturity. However, the further increase in temperature over normal growth temperature of plants enhances starch destruction as is clear from the electron micrographs of the present study. At 22°C the spinach leaf chloroplasts show starch grains up to the 28th day of the observations at 25°C, up to 15 days (Figs. 10, 11); at 30°C up to 7 days (Fig. 5); and at 35°C no starch grain is seen in any chloroplast after 7 days. A similar loss of starch has also been seen in cultured spinach leaf discs in high light intensity (Cran and Possingham, 1974b). They explained this loss of starch as resulting from its consumption as an energy source since leaf discs lack an efficient system for the transport of metabolites. In the attached spinach leaf, with the loss of chloroplast structure due to maturity of leaf under normal temperature conditions and because of quicker chloroplast destruction under high temperature conditions, the photosynthetic capacity is

likewise reduced and the accumulated starch used as an energy source.

Lichtenthaler (1968) suggested that plants can be divided into two groups according to whether senescence of the chloroplast results in an increased number of osmiophilic globules or a similar number of much larger globules. According to this division of plants, which is accepted by many workers, Dodge (1969) has shown that Betula clearly belongs to the second group where it joins a number of other trees and perennial plants. But on the contrary the results of this investigation show that the osmiophilic globules in spinach chloroplasts have increased both in number and size with advancement of the senescence or during the plastid destruction, because of high temperature conditions. This increase in both number and size of osmiophilic globules with the onset of senescence has also been reported earlier in many other plants, e.g. Vicia faba (Greenwood et al., 1963), Nicotiana tabacum (Hurkman and Kennedy, 1975), Triticum aestivum (Hurkman, 1979).

The studies conducted earlier by Ikeda and Ueda (1964), Barton (1966), Dennis et al. (1966), Lichtenthaler (1968), Hurkman and Kennedy (1975), Hurkman (1979) have shown that the osmiophilic globules are repositories for lipids resulting from membrane breakdown products. In addition, studies of lipid composition of osmiophilic globules have shown a correlation between lipoquinone and carotenoid content and number of globules in ageing plastids (Barr and Arntzen, 1969; Lichtenthaler, 1967, 1968, 1969a, b). Hurkman (1979) has also shown that when lamellar breakdown of primary wheat leaf chloroplast is induced artificially by detaching the leaf from the plant, the number

of osmiophilic globules formed is far less than in the normally senescing leaf chloroplast. However, our studies with spinach reveal that when the plant is subjected to high temperature conditions, the quicker breakdown of chloroplast lamellae is accompanied by a corresponding increase in the osmiophilic globules. Therefore this proportionate increase in the globules with lamellar breakdown of chloroplast induced by high temperature lends support to the idea that the globules are lipid repositories. A further confirmation of this suggestion of Ikeda and Ueda (1964) comes from the work of Mukohata (1973). Mukohata (1973) showed that when isolated spinach chloroplasts are warmed within the temperature range of 30°C - 55°C, for five minutes, it appears to induce irreversible thermal denaturation of, for example, enzyme proteins or lipid architecture. However, even when the intactness of internal structure of the chloroplast is lost, enzymes or redox components related to the Ferricyanide-Hill reaction are not inactivated. Furthermore, since the activity for the acid-base ATP formation is only lost on warming over 50°C, enzymes related to the ATP synthesis might not be fully inactivated. Therefore it is most likely that thermal denaturation takes place in the lipid architecture, and the activities are changed due to such structural modifications. Further it has also been shown that the globules of mature chloroplasts of Vicia faba (Greenwood et al., 1963) and spinach (Bailey and Whyborn, 1963) contain a mixture of lipids and other substances, not all of which are to be found in chloroplast membranes. The evidence would thus seem to indicate that although the globules in senescent chloroplasts may in part be membrane breakdown products or precursors they

probably also represent a general store of insoluble lipid material, not necessarily connected with membrane formation or breakdown (Bailey et al., 1966).

The accumulation of osmiophilic material in the cytoplasm adjacent to chloroplasts is not seen in spinach in the present investigation in any cell undergoing senescence or chloroplast destruction due to high temperature conditions. Such lipid depositions between chloroplasts and plasmamembranes have previously been described by Mittelheuser and Van Steveninck (1971) in wheat leaves. However, osmiophilic deposits adjacent to chloroplast envelope as observed by Mlodzianowski and Kninthiewicz (1973) in detached ageing leaves of Kohlarabi are seen in a few cells of the yellow-green spinach leaves at 25°C. These deposits of osmiophilic material on the surface of organelles are attributed by these authors to the action of lipophanerase.

Changes in the chloroplast shape and volume are comparatively slow in the chloroplasts of green spinach leaves but more abrupt as the chloroplast approaches senescence or in chloroplasts subjected to increased temperature, and are associated with significant changes in the chloroplast lamellae. These changes take place continuously throughout the leaf age. Most of the studies conducted before (Barton, 1966; Hurkman and Kennedy, 1975; Hurkman, 1979) show only the structural changes of the chloroplasts as the leaf reaches maturity and finally fully senesced. But the results of the present investigation present the comparative effect of different temperatures on the chloroplast ultrastructure throughout the growth of the spinach leaf and finally

the disorganization of the whole chloroplast ultrastructure. Electron micrographs show that in fully green spinach leaves, the increased temperature conditions help in the building up of both the stroma and grana lamellae, as it is clear from the electron micrographs that the highest number of plastid stroma lamellae and size of grana is seen up to 28 days after the spinach plant is kept at 22°C; 21 days at 25°C; 15 days at 30°C and only 7 days at 35°C. After these respective leaf growths at the respective temperatures the lamellae start showing slight disorganization which mark the start of chloroplast breakdown processes. Therefore from these studies we can conclude that in the intact leaf of Spinacia oleracea, the temperature of 35°C is most effective both in synthetic and degradative processes. The initial increase in chloroplast lamellae and size of grana have also been described earlier by many workers (Dodge, 1970; Hurkman, 1979). These results thus lead us to a general interpretation that the higher the temperature under which the spinach plant is kept, the faster is the increase in the number of chloroplast lamellae and size of grana and the shorter is the life of the chloroplast.

In spinach plastids an extensive development of stroma lamellae as a result of formation of lamellar sheets by the inner limiting membrane of the plastid takes place in the very early stages of the naturally senescing leaves and very rarely under high temperature conditions (30°C and 35°C). This extensive development of an irregularly-shaped lamellar system of Spinacia oleracea leaves showing peculiarly interesting structure has rarely been reported earlier in the normally senescing plastids of higher plants. However, Schötz et al. (1968)

have reported a somewhat similar irregularly-shaped lamellar system in the chloroplast of Oenothera hybrids (lamarckiana x hookeri) as a result of disharmony between genome and plastom. This disharmony between genome and plastom does not only prevent unspecifically the chloroplast differentiation but it may also lead to a shape of the lamellar system which is exceptional for higher plants but common in the cells of various Cyanophyceae. A characteristic ring-shaped, cup-shaped, bladder-like or loop-like lamellae, or the lamellae forming large bowl-shaped regions, or the lamellae connecting together forming bridge-like connections in the plastid stroma of the naturally senescing leaves of Spinacia oleracea are similar to those observed in the plastids of dark grown Capsella bursa - pastoris leaves (Sharma, 1977).

Clewell and Schmid (1969) have reported a chloroplast from a yellow plant of Lespedeza procumbens in which the secondary multiplication of thylakoids have produced what appears to be channels. Such channels have also been reported earlier in normal green leaves of Cassia obtusifolia (Homann, 1967) and Rhodopseudomonas palustris (Tauschel and Drews, 1967) in which the secondary lamellar multiplication has been reported. But none of these species shows an extensive development of the abnormal lamellar system.

Diers and Schötz (1969) used serial sections to elucidate the three-dimensional configuration of the lamellar system which is the basis for ring-shaped lamellae often seen in spinach plastids. They have shown that these configurations can be more or less deep, cup-

shaped single lamellae or stacks of lamellae. The lamellae either occupy only the small parts of stroma space and sometimes they divide the interior of the stroma space in large bowl-shaped regions. The bridge-like connections between lamellae as seen in some of the electron micrographs of the naturally senescing spinach leaf chloroplasts of the present investigation actually reveal a characteristic alteration in the course of the lamellar direction (Schötz et al., 1968), it turns by about 90° above and below the respective junction.

Contrary to the results of Dennis and co-workers (1967) who showed that in Brussels sprout leaf chloroplast the loosening of the grana structures is the first indication of senescence, our results show that in normally senescing leaf of Spinacia oleracea an extensive and abnormal development of the stroma lamellae of the chloroplast is the first sign of senescence and is followed by loosening of the grana and splitting of lamellar membrane structures. A characteristic swelling, adjacent to the osmiophilic globules occurs at the terminus of each thylakoid, which appears as a club-shaped structure in section (Figs. 28, 52, 53). This distortion is mostly observed in the naturally senescing cells and has been interpreted by Barton (1966), who also found such structures in the naturally senescing leaf chloroplasts of Phaseolus, as being a stage in weakening of the thylakoid membrane prior to breakdown.

Loosening of grana and splitting of the lamellar membranes are however, not very prominent under increased temperature conditions in spinach plastids whereas the club-shaped structures developed at

the terminus of each thylakoid as seen in naturally senescing leaf chloroplasts can also be seen under very high temperature conditions (35°C), (Fig.28). A peculiar feature of the chloroplasts of spinach leaves under high temperature conditions is the breaking up of the stroma lamellae joining the grana which latter appear as patches of membranes lying in the fully senescent chloroplast. However, in chloroplasts of naturally senescing spinach leaves, the loosening of grana is such that no difference remains between stroma and grana lamellae. No membrane vesicles are seen in any chloroplast of fully senescent spinach leaf or in any fully disrupted chloroplast under the effect of high temperature conditions. Mlodzianowski and Ponitka (1973), Cran and Possingham (1974b), Hurkman (1979) have shown that during the senescence process the lamellar system of chloroplast is reduced to membrane vesicles which along with the osmiophilic globules constitute the whole content of the fully senescent chloroplast.

The loss of plastid envelope at the terminal stages of senescence is not considered as a characteristic feature of spinach chloroplasts, since at no stage of the senescence is the rupture of plastid envelope noticed in the present investigation. However, the presence of envelope-free chloroplast contents as seen in fully senescent spinach chloroplasts at 25°C as observed might be an artefact of fixation as has earlier been described by Colquhoun and associates (1975), Hurkman (1979). Perhaps senescent membranes cannot withstand the chemical changes caused by fixation. Among the several consistent changes of spinach cell senescence, plasmolysis of cells is a very common phenomenon. The other changes being swelling of chloroplasts

and dilation of chloroplast lamellae (Figs. 52, 53). Anderson and Schaelling (1970) suggested that alteration of membrane permeability causes chloroplasts to become spherical and turgid. The results of present work in spinach suggest that the appearance of many small dark coloured deposits around the outer surface of chloroplasts (Fig. 56) might be the broken down products of the membrane lipids of the envelope, which thus increase the permeability of the membranes. Another possible explanation which can be given to this is that at the terminal stages of senescence the whole plastid contents is pushed to one side of the chloroplast leaving a very big stroma space (Fig. 56).

In chloroplasts of spinach leaves under very high temperature conditions (35°C) the chloroplast envelope is lost before the complete lamellar breakdown. This difference indicates an earlier change in membrane permeability caused by very high temperature and suggests that membrane integrity is necessary for the complex sequence of changes leading to lamellar breakdown in chloroplasts of naturally senescing leaves. Since less time is involved for chloroplast to deteriorate under very high temperature conditions, perhaps the enzymes necessary for lamellar breakdown are not synthesized or are destroyed before they could act on chloroplast membranes. Such a quick loss of chloroplast envelope has also been noticed previously by Hurkman (1979) in detached ageing leaves of Triticum aestivum.

The results of TEXT Figure 8 show that the spinach leaves growing under normal temperature conditions have a constant increase in the chlorophyll content until the onset of senescence. These results also

show that up to 35 days of leaf age, the chlorophyll content is increasing and electron micrographs also show a normal structure of the chloroplast up to this stage of leaf growth. The abrupt reduction in the level of chlorophyll occurs when the chloroplasts show even a small disorganisation of the lamellar structure and the chlorophyll content falls continuously afterwards. Schmid and co-workers (1966) and Dennis and co-workers (1967) have shown a correlation between the rapid loss of chlorophyll and the breakdown of grana, which is the most significant event especially in the early senescence, since the grana are the location of the chlorophyll in the chloroplast. Our results of the chlorophyll content of spinach leaf and the electron micrographs also provide a similar correlation.

The striking feature of very high temperature treatment (35°C) of the spinach plant is that when this temperature treatment is given only for seven days, the chlorophyll content shows a tremendous increase and is much more above the level of chlorophyll of leaf of this age under normal temperature conditions. Comparing the chlorophyll content of the spinach leaf under 35°C temperature condition and the structure of the chloroplast from electron micrographs, we see that the chloroplasts also show an extensive increase in the number and size of the grana. Such an initial increase in the chlorophyll accumulation under high temperature conditions has also been shown by McWilliam and Naylor (1967). The sensitivity of chlorophyll metabolism to temperature is also shown by Friend (1960). According to him the failure of chlorophyll development at high temperature in Marquis wheat seedlings has been attributed to a block either in the

pathway of chlorophyll synthesis immediately before protochlorophyll or in the hydrogen donor system involved in the protochlorophyll - chlorophyll transformation. A similar phenomenon also takes place in some strains of Euglena where chlorophyll formation is prevented at a temperature of 34°C - 35°C (Brawerman and Chargodd, 1959). Pearcy et al. (1977) have found that in Atriplex lentiformis high growth temperatures induce a substantial increase in the thermal stability of the photosynthetic apparatus. The apparent increase in chlorophyll content of the spinach leaf in this experiment might also be a result of the rapid loss of water from the leaf since the chlorophyll content is calculated here on fresh weight basis as is usual.

Temperature affects the growth of the whole plant mainly through its effect on respiration and on morphology. Woodward (1979) has shown that in Phleum bertolonii DC and Phleum alpinum L., an increase in temperature results in a decline in cell area and an increase in cell number, also the period of extension for a particular leaf decreases with increasing temperature, through the higher frequencies of cell division. The effect of temperature on the number and area of the leaves alters the leaf area ratio, and changes in the leaf thickness alter the rate of photosynthesis per unit area (Friend et al., 1962; Woodward, 1979). The net effect of an increase in temperature will therefore depend on the extent of dry matter loss through increased respiration, and dry matter increase through increase in the leaf area ratio. Feierabend and Schrader-Reichhardt (1976) working on rye leaves and Feierabend and Mikus (1977) working on wheat and barley found that although growth of leaves (elongation and expansion) is

not severely affected under the high temperature conditions, light grown leaves had lower dry weight and contained less protein at 32°C than at 22°C. This was expected because chloroplast protein synthesis is highest at 22°C in light. Furthermore it appears that the lack of photosynthetic carbohydrate production becomes a major limiting factor at 32°C. The reduction of photosynthate under high temperature conditions can also be related to the biochemical interpretation that heating causes free fatty acids to accumulate in the chloroplasts and unsaturated fatty acids are strong inhibitors of oxidative phosphorylation in mitochondria (Woftczak and Lehninger, 1961) and photochemical reduction in chloroplasts (Krogman and Jagendorf, 1959).

C H A P T E R V

THE EFFECTS OF PROLONGED HIGH TEMPERATURE CONDITIONS ON CHLOROPLAST ULTRASTRUCTURE IN THE YOUNG EXPANDING LEAF, WITH A COMPARISON OF EFFECTS IN LEAVES AT SUCCESSIVELY HIGHER NODES

INTRODUCTION

In the previous chapter we have seen the changes in the chloroplast structure in the individual leaf as it grows and ages at both normal and abnormally high temperatures. A further experiment described in this chapter was designed to investigate more fully the effects of prolonged high temperature on the very young leaf tissues. The only practical way of doing this seemed to be to sample successive leaves as they emerge from the apical bud. In the control conditions of 22°C, we may thus observe at the same time the ultrastructural development in successively formed leaves and see whether they differ in any significant way from the changes described for the individual leaf in Chapter IV.

METHOD

Seedlings were raised at 22°C under 14h light/10h dark conditions. When the plants were four weeks old they were divided into four groups of 20 plants each. One group was left at 22°C while the remaining three groups were transferred to cabinets at 25°C, 30°C and 35°C respectively; the L:D regime being maintained for all.

At the apex of four weeks old plant grown at 22°C there are 4 to 5 leaf primordia as yet unexpanded (TEXT Figure 5). Within a further week the outermost of these has expanded from the bud and is about 6 - 8 mm long: this new leaf provided the 'Day 7' sample. During the sixth week a further new leaf has expanded and this provided

the second ('Day 15') sample. In plants that have been transferred to higher temperature conditions at the four week stage this second expanded leaf, though at a comparable stage of growth to the first leaf sampled ('Day 7') will obviously have spent a longer period during its early development at the higher temperature. We may, thus, compare the effect of different temperatures at each node. We must exercise caution in comparing the effects of any one temperature condition at successive nodes since, with the exception of the control (22°C), the length of time that the plant has been exposed to the higher temperature is confounded with node number.

OBSERVATIONS

0 Day sampling

The zero day sampling of the youngest leaf of four week old plants of this experiment is equivalent to the zero day sampling of the youngest leaf of four week plants of the experiment of Chapter IV. Therefore additional zero day sample was not taken for this experiment.

7 Day sampling

22°C

The plastid ultrastructure of the 6 - 8 mm long leaf differentiated at 22°C shows a well ordered array of grana with maximum stacking of up to 15 thylakoids, interconnected by long fret membranes (Fig. 58). Most of the chloroplasts have starch grains of varying sizes and numbers (usually not more than three per chloroplast). The stroma

comprising the internal matrix material has a granular appearance. The chloroplasts lie appressed to the inner wall of the cell in the form of a ring (Fig. 57). These are usually bi-convex, plano-convex or concavo-convex in form and each chloroplast is surrounded by a bi-membranous envelope. Osmiophilic globules are not many in these chloroplasts and are of very small size.

$25^{o}C$

The plastids of the leaf newly expanded after seven days at $25^{o}C$ do not show the normal plastid differentiation. They are variously shaped, each with very long grana, having maximum stacking up to 10. Stroma lamellae are very short and sometimes even broken (Figs. 59, 60). Starch grains are not very common in these chloroplasts and they are of very small size. Osmiophilic globules are also few.

$30^{o}C$

The plastids of the leaf at this temperature do not seem to differ much from those differentiated under $22^{o}C$ temperature condition. They are also elongated, bi-convex, plano-convex or concavo-convex in shape and each chloroplast is surrounded by a resistant double-membraned envelope. Such chloroplasts have fewer grana and each granum is composed of a maximum of 6 - 8 thylakoids stacked together and are interconnected by few, short fret membranes (Figs. 61, 62). Few small densely stained globules are present in the chloroplasts lying against the lamellae or free in the stroma. There are no starch grains. In the cell cytoplasm there can be seen some mitochondria and Golgi body (Fig. 62).

35°C

The chloroplasts of about 7 mm long leaf differentiated under 35°C temperature conditions still retain their elongated structure showing normal grana-fretwork system with very well developed grana having maximum stacks up to 20. Very few, short fret membranes connect the two grana (Fig. 64). The peculiar feature of chloroplast under this temperature condition is that the free ends of the lamellae become hollow and slightly swollen at their respective ends. The stroma of such chloroplast has a granular appearance and there is no starch, but contains 8 - 12 densely stained osmiophilic globules which are largest (0.07 μm) among all the chloroplasts studied in all temperature conditions at this stage of leaf differentiation. The cytoplasm of the cell contains many mitochondria (Fig. 65) clustered together and appearing normal in structure. Endoplasmic reticulum can also be seen in the cytoplasm of the cell.

15 Day sampling

22°C

At this stage of leaf differentiation under normal conditions of growth, the chloroplast has stroma comprising internal matrix material which has a granular appearance, presumably because of fraction I protein. The grana are rarely highly developed (20 stacks). Usually they are composed of 3 - 10 thylakoids and are mostly connected by short fret membranes. Presence of starch grains in the stroma is a consistent feature of chloroplasts under these conditions of differentiation. However, the size of starch grains do not show any consistency and

are sometimes very big (Fig. 66). Normally the chloroplasts are bi-convex or plano-convex in shape, but sometimes the grana-fretwork system appears arc-shaped or deep arc-shaped, until it becomes 'S'-shaped (Fig. 67). The cytoplasm of the cells contain many mitochondria which vary greatly in size. Few endoplasmic reticulum are also present in the cytoplasm. Osmiophilic globules are very few and very small in size.

$25^{o}C$

The plastids of spinach leaves differentiated under these conditions do not show the normal plastid shape. The grana are long and are interlinked by short fret membranes. Some of the plastids possess very big starch grains which disrupt the whole plastid structure (Fig. 69). In some chloroplasts very big bands of protein crystals can be seen (Fig. 68). Osmiophilic globules are many but of small size. The plastid division by constriction is very common under these conditions of leaf differentiation.

$30^{o}C$

The stroma of chloroplasts contain ribosomes which do not show a well ordered distribution. The lamellae become slightly distorted from their original arrangement and show a characteristic splitting with hollow regions along their length (Figs. 70, 71). Some of the lamellae are broken and the free end of each lamella shows a little club-like swelling. Both the number and size of grana show a slight increase over those chloroplasts of leaves differentiated seven days after the plant is kept at $30^{o}C$. Shape and size of such chloroplasts also show a sudden change. These become smaller in size with much variation in shape. Osmiophilic globules are also fewer and smaller. All

these structures are held by a rather resistent double membraned envelope. Cytoplasm of the cell contains a large number of mitochondria near the vicinity of chloroplasts and show normal structure (Fig. 70). No starch grain is seen in any chloroplast under these conditions of leaf differentiation. Few small vesicles also appear in some of the chloroplasts (Fig. 71).

35°C

The characteristic feature of spinach leaf chloroplasts differentiated 15 days after the plant is kept at 35°C is that in regions adjacent to dense globules a characteristic swelling occurs at the terminus of each thylakoid, which appears as a club-shaped structure in section (Figs. 72, 73). Since this distortion is only observed adjacent to globules it is interpreted as being a stage in weakening of the thylakoid membrane prior to breakdown. These plastids show a great reduction of size (Fig. 72). Osmiophilic globules show a much greater increase in size (0.2 μm). The grana which are very big in size and many in number are interlinked by few small fret membranes. Some of the lamellae also show characteristic splitting as shown by chloroplasts of spinach leaves differentiated after the plant is kept for 15 days at 30°C (Fig. 73). Ribosomes do not show much uniformity in distribution. Mitochondria are present in the cell cytoplasm and appear to be normal.

21 Day sampling

22°C

The plastids of the leaves differentiated after the plant is kept

at 22°C for 21 days show a slight reduction in size. These plastids assume various shapes, for example, bi-convex, plano-convex or concavo-convex (Figs. 74, 75). Starch grains are present in most of the plastids. Both stroma and grana lamellae are well developed. The maximum stacking of thylakoids per plastid observed in these plastids is 15. Osmiophilic globules are few and of very small size.

25°C

Under this temperature treatment the leaf chloroplasts become much shorter than the normal chloroplasts and are usually bi-convex in shape. The stroma is granular and each plastid possesses few grana which are interlinked by long fret membranes (Fig. 76). Most of the grana are comprised of 3 - 10 thylakoids each. Osmiophilic globules are few and small. Starch grains are seen only in few plastids. Plastid division by constriction mechanism is also a common process especially in the younger plastids (Fig. 77). The cytoplasm of the cells is reduced much in quantity and lies appressed to the inner wall of the cells and it contains endoplasmic reticulum (Fig. 76) and some cytoplasmic whorls (Fig. 77).

30°C

The plastids of the leaves differentiated at this stage of plant growth and under this temperature condition show a further reduction in size than the correspondingly differentiated plastids at 22°C and 25°C. These become swollen and disc-shaped. The stroma of each such plastid is filled with ribosomes which do not show a greater degree of uniformity in its distribution. The most striking feature of chloroplasts under these conditions of development is the most elaborate and

complex system of grana and stroma lamellae. These lamellae show a characteristic splitting with hollow regions along their length (Figs. 78, 79). The osmiophilic globules show a much greater increase in size and number and appear to be very distinct. The cytoplasm of the cell shows a further reduction and appears to be in the form of a thin film lying close to the inner wall of the cell. The cell cytoplasm contains mitochondria (Fig. 78) which resemble the normal mitochondria and also contains endoplasmic reticulum.

35°C

The plastids of leaves differentiated after the plant is kept at 35°C for 21 days almost lose their original shape and become more or less spherical in appearance. The ultrastructure of such plastids shows the complete distortion of the internal structures. The splitting of the lamellae increase to such an extent that in slightly overstained sections it gives the appearance of a long chain (Fig. 81). The grana which are sometimes cut off from each other look like small patches (Fig. 80). Osmiophilic globules show a further increase both in number and size. The double-membraned envelope appears rather resistant and holds all the internal matrix materials together.

28 Day sampling

22°C

The leaf differentiated at this stage of plant growth has plastids which are mostly plano-convex and sometimes concavo-convex and/or bi-convex in shape. However, in some of them the grana fretwork system appears arc-shaped or deep arc-shaped, until it becomes 'sickle-shaped' (Fig. 83) or 'U'-shaped. The stroma of the plastids contain

grana which do not show any consistency of their number and size. Sometimes the grana are many, each composed of a maximum stack of up to 12 and are interconnected by short fret-membranes (Fig. 82) while in others the grana are few, each having a maximum stack of up to 6 and are interconnected by long fret-membranes (Fig. 83). The most characteristic feature of most plastids under these conditions is the presence of protein body/bodies (P) (Fig. 82) which are of variable size and are usually bounded by lamellae on all sides. Large starch grain/grains though present in most of the chloroplasts is not a consistent feature. Osmiophilic globules are few and small. The cytoplasm of the cells contains many mitochondria which appear to have normal structure. Endoplasmic reticulum is also present in the cytoplasm.

$25^{o}C$

The chloroplasts of the corresponding leaf after 28 days at this temperature have similar shape, size and ultrastructure to those of leaves differentiated at $22^{o}C$. 'Sickle-shaped' and 'U'-shaped chloroplasts are common due to curving up of grana fretwork system. The presence of protein bodies (Figs. 84, 85) is a consistent feature of all the chloroplasts at this stage of leaf differentiation. In all the 'sickle-shaped' chloroplasts observed, which are similar in ultrastructure to each other in many ways, are shown the presence of protein bodies at exactly the same places. These protein bodies are surrounded by lamellae. The number and size of grana do not show much consistency. Starch grains are present in some of the chloroplasts and are of variable sizes. Many mitochondria are present in the cytoplasm of the cells near the vicinity of the chloroplasts. Rarely, chloroplast division by constriction can be seen (Fig. 86).

Osmiophilic globules show a slight increase both in number and size.

30°C

The plastid ultrastructure at this temperature is closely similar to that of the previously sampled leaves after 21 days at 35°C. Besides the splitting and disorganization of lamellae and increased osmiophilic globules in the stroma of the chloroplast, this stage represents the weakening of the envelope membranes. The chloroplasts, each of which acquires a more or less spherical shape, seem to swell on the sides (Figs. 87 - 90). This change in shape and swelling of the chloroplast may be because of the increased internal pressure of the plastid contents. Some of the chloroplasts are seen bursting at particular points and liberate the contents in the cell cytoplasm (Figs. 88, 89, 90). Cell cytoplasm which shows a greater degree of reduction of its contents, contains few mitochondria with distorted cristae. Some of the mitochondria observed are without envelopes (Fig. 88). No starch grain is seen in any chloroplast.

35°C

At this temperature, the leaves differentiated after 28 days possess chloroplasts which consist of a complicated system of lamellae lying either free in the cell (Figs. 91, 92) or enclosed in envelope membranes (Fig. 91) and having a large number of small darkly stained depositions on the outer surface of the envelope. These depositions may presumably be the lipid globules of envelope membrane because of the high temperature effect. The chloroplast envelope membrane may disappear completely and all the internal matrix material of the chloroplast is dispersed in the cell. Most of the osmiophilic globules are still

attached to the lamellae while others are dispersed into the cell. The splitting of the lamellae at various places is so much that the space created by this splitting occupies most of the internal space of the chloroplast (Fig. 92). Cell cytoplasm contains some mitochondria with abnormal structure.

DISCUSSION

One aim of this experiment has been to study the reletive effect of different temperatures on the type of chloroplast formed in the newly differentiated leaves at different stages of plant growth. All the stages of plant growth observed are before the onset of leaf senescence. The chloroplasts produced in the leaf tip of the spinach plant at 22°C are usually long with large grana and very long fret membranes. The shape and ultrastructure of the chloroplasts produced at 22°C are more or less the same irrespective of the stage of the plant growth before the onset of senescence. The chloroplasts produced at all the stages of plant growth (before senescence) at 25°C are comparable to those at 22°C. However, the production of chloroplasts at elevated temperatures (30°C and 35°C) at every growth stage show considerable differences both in shape and ultrastructure when we compare them with those at 22°C and 25°C.

The size of the spinach leaf chloroplasts produced both at 22°C and at 25°C show a slight reduction in the leaves differentiated closer to the onset of senescence. Starch grains also show a similar pattern of reduction. In contrast, under high temperature conditions

(30°C and 35°C), in addition to a continuous reduction in the size of chloroplasts, no starch grain is seen in any chloroplast of the leaf differentiated at any stage of the plant growth. The shape of such chloroplasts observed under high temperature conditions shows a change from the elliptical to spherical as the leaves differentiate at the later growth stages of plant growth near senescence. The visual observations (TEXT Figure 6) on the plant growth show that with the increase of temperature the newly differentiated leaves show a reduction in size and this reduction also increases with the age of the plant. The net effect of an increase in temperature will depend on the extent of dry matter loss through increased respiration and dry matter increase through increase in leaf area ratio (Friend et al., 1962; Woodward, 1979). Therefore the loss of starch observed in spinach chloroplasts at high temperatures might have been due to its ultilization by the metabolically active chloroplasts to overcome the lack of photosynthetic carbohydrate production which becomes a major limiting factor at high temperature conditions. This has also been shown by Feierabend and Schrader-Reichhardt (1976) in rye leaves and Feierabend and Mikus (1977) in wheat and barley. Since the chloroplasts of newly differentiated leaves are metabolically active therefore the synthetic processes are actively taking part in the chloroplasts and are enhanced to some extent by the increase in temperature. The justification also comes from the results of the present investigation on spinach. The electron micrographs of spinach chloroplasts of newly differentiated leaves show that increase in temperature has resulted in the increase in both the number and size of grana of

the newly developed chloroplasts. On the contrary, according to Wehrmeyer (1964) and Perner (1965) the shape of the grana or intergrana, or the variability of the size and number of grana has no functional meaning. According to them, many grana layers are not necessary organelles for photosynthesis and unstacked lamellae in a loosely structured chloroplast may work even better and the lamellae in such chloroplasts can do more photochemistry. They also found that widely spaced lamellae permit more chlorophyll to react with enzymes or more enzymes to reach an excited chlorophyll in its life time.

Smillie and co-workers (1978) have shown three levels of temperature effects on chloroplast development in barley. At temperatures from 11°C to 31°C there is acclimation to growth temperature in that photosynthetic electron transfer capacity, and to a lesser extent chloroplast structure, is altered depending upon the growth temperatures. Outside this range both cold and very warm temperatures can result in the production of chloroplasts showing abnormal properties and structure. Finally there are ranges of low and high temperature in which chloroplast biogenesis, but not other cellular growth process, is inhibited.

Coupled with the synthetic processes, the spinach leaves differentiated at the later growth stages also show some destructive processes like splitting of the lamellae and increase in both number and size of osmiophilic globules and this destructive effect increases with the increase of growth temperature under which the leaf is allowed to

differentiate. As the increase in osmiophilic globules and the splitting of the lamellae go hand in hand it can be concluded that the lipid architecture of the lamellae is destroyed by high temperature conditions and is being utilized in the building of the osmiophilic globules. It has been discussed in more detail in Chapter IV.

The characteristic swelling at the terminus of each thylakoid which appears as a club-shaped structure, as described by Barton (1966) only in the plastids of the senescent cells are also observed in the plastids of the newly differentiated spinach leaves under high temperature conditions. These structures are observed only in the plastids which show lamellar breakdown and appear to be a stage in the weakening of the thylakoid membrane prior to breakdown. Such structures are more pronounced under 35°C temperature. The cells of the spinach leaves under these conditions of differentiation possess mitochondria with distorted cristae and the cytoplasm remains as a very thin film. However, the most resistent structure appears to be the plastid envelope which holds the plastid organelles together. When the plant is subjected to 30°C temperature treatment for 28 days or 35°C for 21 days, each chloroplast of the newly differentiated leaf assumes an elliptical or spherical shape and the whole chloroplast structure is distorted with broken lamellae which take the shape of sheets of varying sizes. Plastids in the leaf primordia at the shoot apex are in the proplastid state and these develop into the plastid type (partially differentiated plastids) at the base of the newly differentiated leaf and subsequently into fully differentiated chloroplasts (Cran and Possingham, 1972). In the newly differentiated

leaf of the mature plant under high temperature conditions, it is possible that the development of the proplastid into partially differentiated chloroplasts or their further differentiation into mature chloroplasts, or both these processes, are arrested. It is clear from the electron micrographs of the present investigation that the type of plastids present in the newly differentiated spinach leaf after the four week old plant is subjected to 30°C for the further 28 days, or 35°C treatment for 21 days, has either no granal organization or if grana are present, these seldom exceed 5 thylakoids. The reason for the arrest of one or more of these differentiation stages only in the mature spinach plant under high temperature conditions and not in the younger plants may be that the cells at the shoot apex of the mature spinach plant under high temperature stress have low metabolic activity compared to those of the younger plant, and are therefore deficient of essential material required by the proplastid to differentiate fully into the chloroplast.

The observed plastid swelling in spinach leaves under the influence of high temperature may be due to an increase in free fatty acids of the chloroplast which results in conformational changes in an actomyosin-like protein (Molotkovsky and Zhestkova, 1965). Anderson and Schaelling (1970) suggested that alteration of membrane permeability causes chloroplasts to become round and turgid. At the same time the breaking up of the membrane lipids of the envelope results in the weakening of the envelope and the increased internal pressure of such plastids exerted by the increased volume of the chloroplast makes it burst, liberating the contents free in the cell. The electron

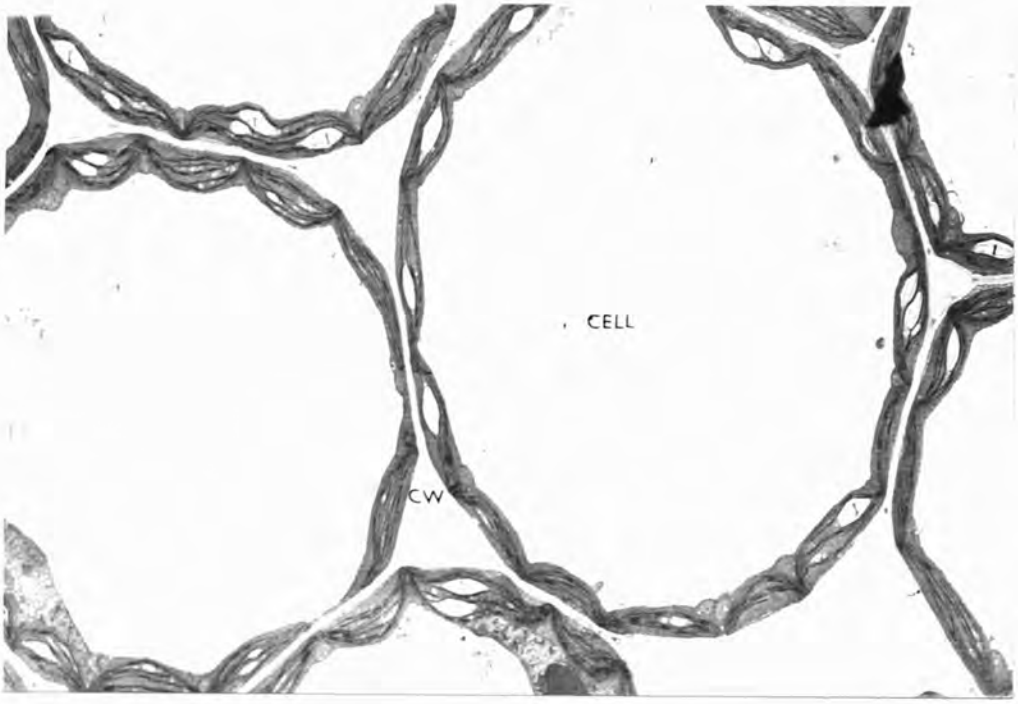
micrographs of the present investigation (Figs. 88, 89, 90) show that under high temperature conditions the bursting of the chloroplast resembles exactly the bursting of a balloon which has a small weak area. In chloroplasts of spinach leaves differentiated after 28 days at 35°C the chloroplasts have lost their respective envelopes before complete lamellar breakdown. A similar effect is observed also at this temperature during the ageing of leaves (Chapter IV). Since less time is involved for the chloroplast to deteriorate under very high temperature conditions, perhaps the enzymes necessary for the complete lamellar breakdown are not synthesized or are destroyed before they can act on chloroplast membranes.

Besides the ultrastructural changes of the plastid of the newly differentiated spinach leaves as influenced by the elevated temperatures, the division of the spinach chloroplast is also very much affected by high temperature conditions. As shown by the electron micrographs, the plastids of newly differentiated leaves at all growth stages of the plant show division at both 22°C and 25°C. However, at high temperatures (30°C and 35°C) no plastid division is seen in any cell at any stage of the leaf differentiation.

PLATE 36 *Section through the cells of the tip of the youngest, fully expanded leaf of 5-week old plant grown under normal temperature conditions.*

Fig. 57. *Section showing the general cell structure. Chloroplasts are lying at the periphery of each cell forming a continuous ring.*
X 36,450.

Fig. 58. *A bi-convex chloroplast with normal stroma and grana lamellae and large starch grains.*
X 60,750.


CELL
CW

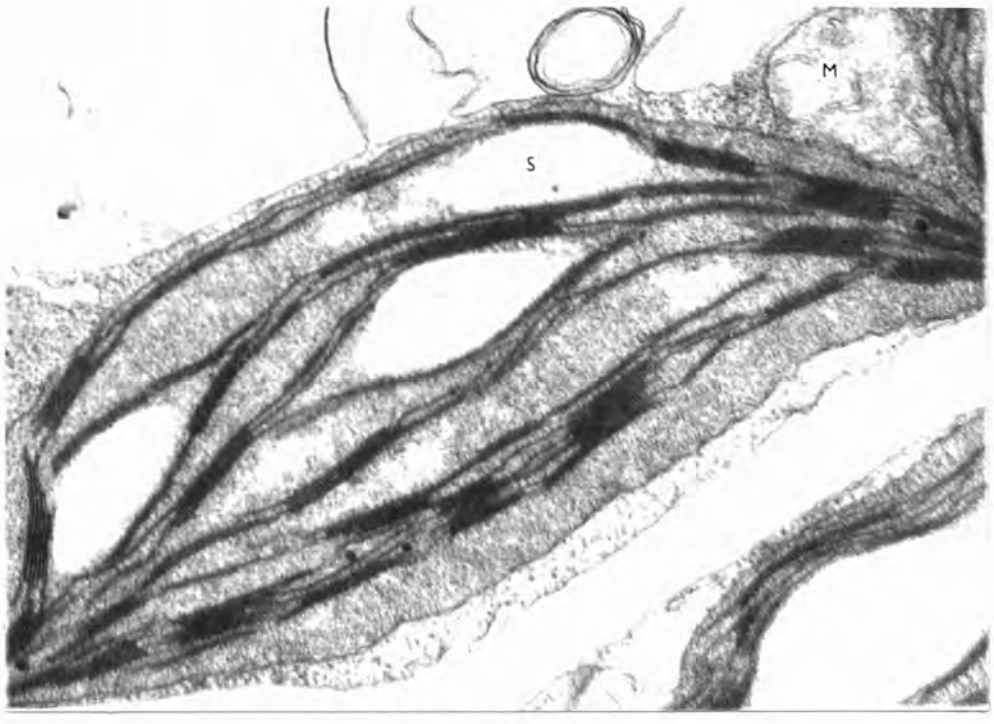
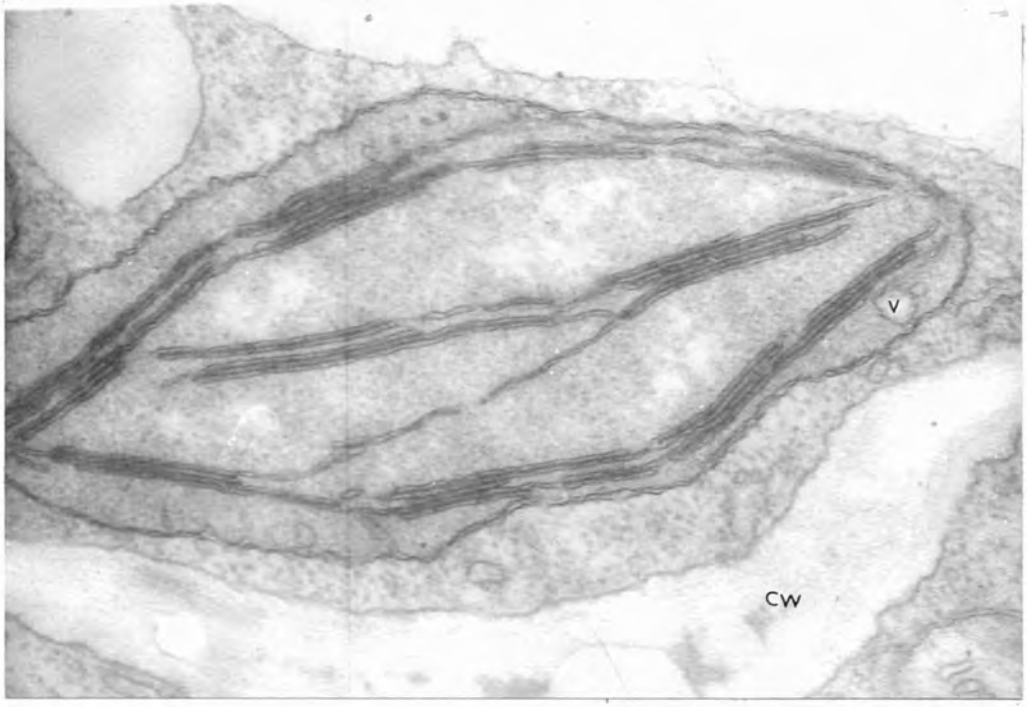

M
S

PLATE 37 *Chloroplasts of the youngest, fully expanded leaf of the plant grown under normal temperature condition for 4 weeks and then kept at 25°C for 7 days.*

Fig. 59. *Chloroplast section shows only few grana each composed of few but long thylakoids. It also shows some broken lamellae and few vesicles. X 60,750.*

Fig. 60. *Chloroplast shows very long grana, interconnected by only a single short lamella. A small starch grain, few broken lamellae and few vesicles are also present in the chloroplast. X 97,200*


V
CW

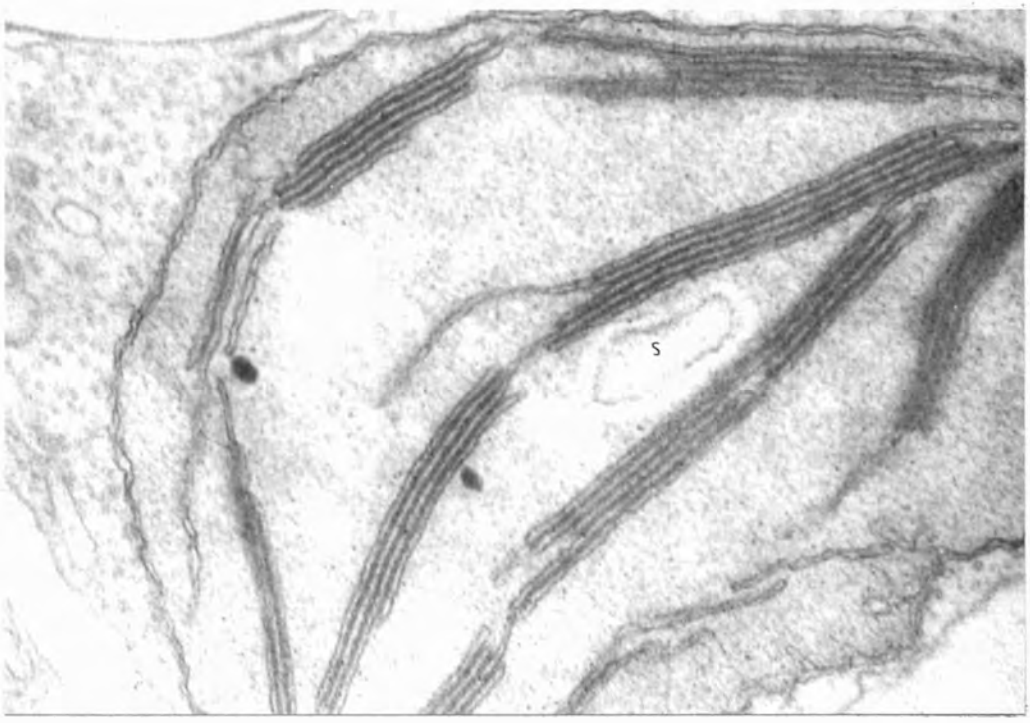

S

PLATE 38 *Section through the cell of the youngest leaf of the plant grown at 22°C for 4 weeks followed by 7 days at 30°C.*

Fig. 61. *Chloroplasts with few but long grana interconnected by very short fret membranes. Each granum consists of a stack of up to 6 thylakoids.*
X 36,450.

Fig. 62. *Elongated, concavo-convex plastid showing the presence of a few vesicles. Cell cytoplasm contains Golgi bodies and mitochondria.*
X 60,750.

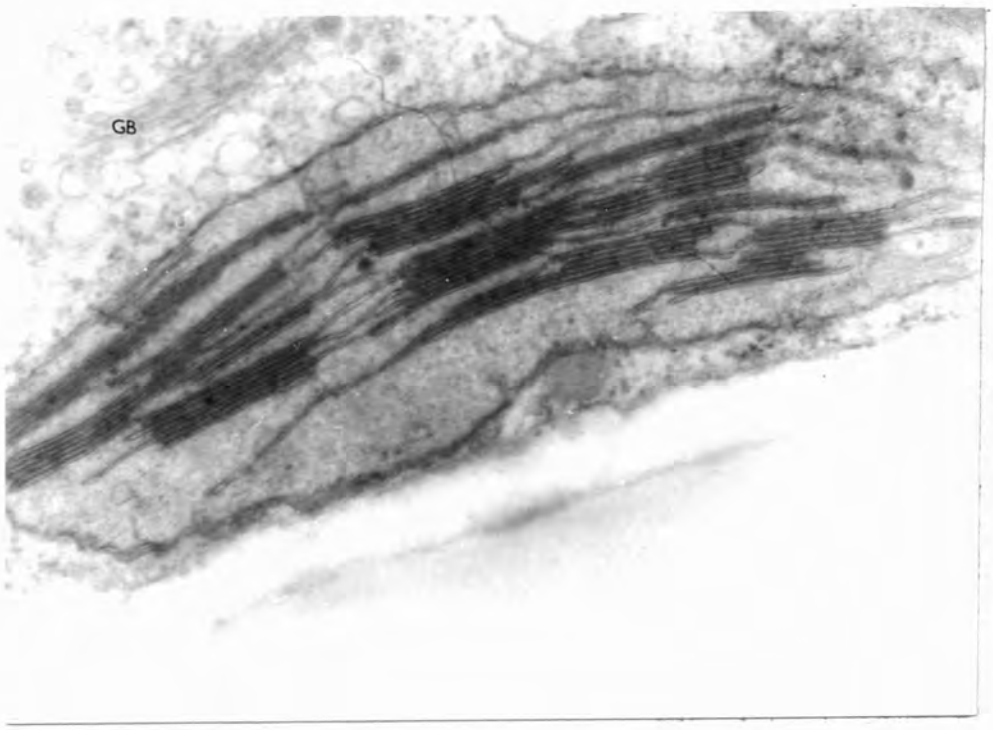

GB

PLATE 39 *Section through the cells of the youngest leaf of the plant grown at 22°C for 4 weeks followed by 7 days at 35°C.*

Fig. 63. *Section showing the general cell structure. Each cell has only one plastid and many mitochondria.*
X 14,580.

Fig. 64. *An elongated chloroplast having many, mutithylakoidal grana which are interconnected by short fret-membranes. Chloroplast also shows the presence of many, big osmiophilic globules.*
X 36, 450.

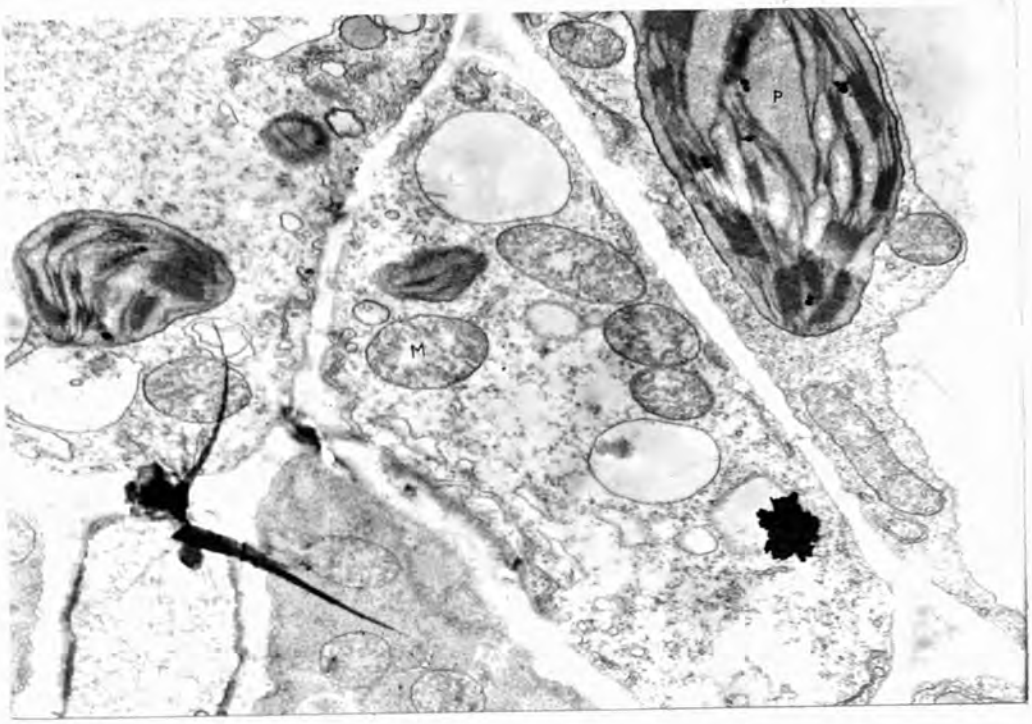

P
M

PLATE 40 *Section through the cell of the youngest leaf of the plant grown at 22°C for 4 weeks followed by 7 days at 35°C.*

Fig. 65. *A portion of the cell cytoplasm showing the presence of many mitochondria and endoplasmic reticulum.*
X 60,750.

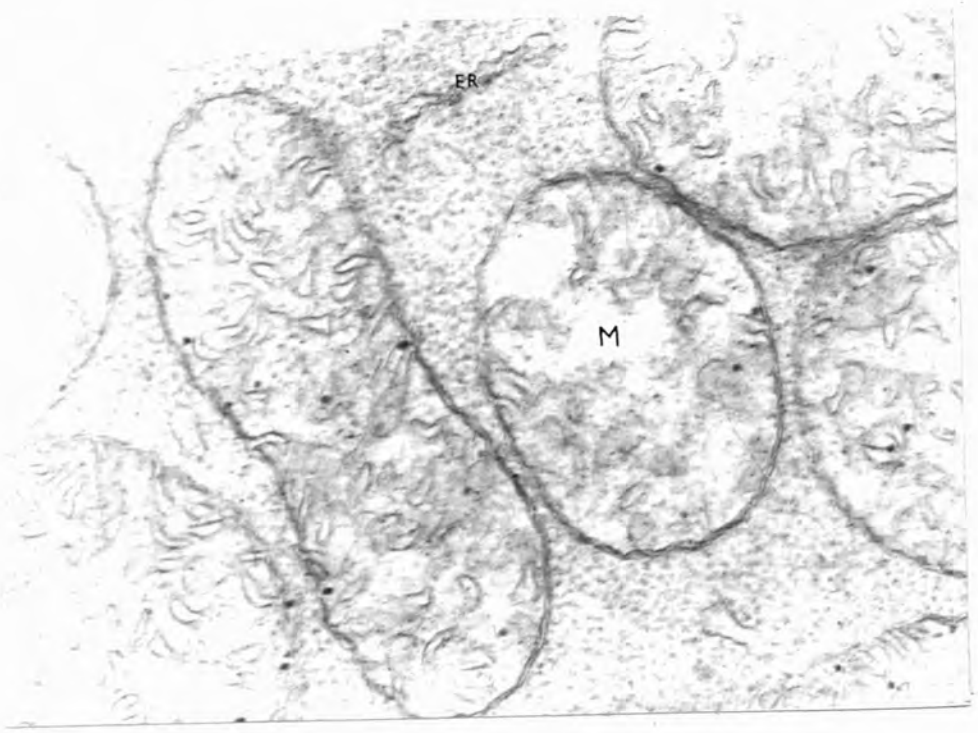
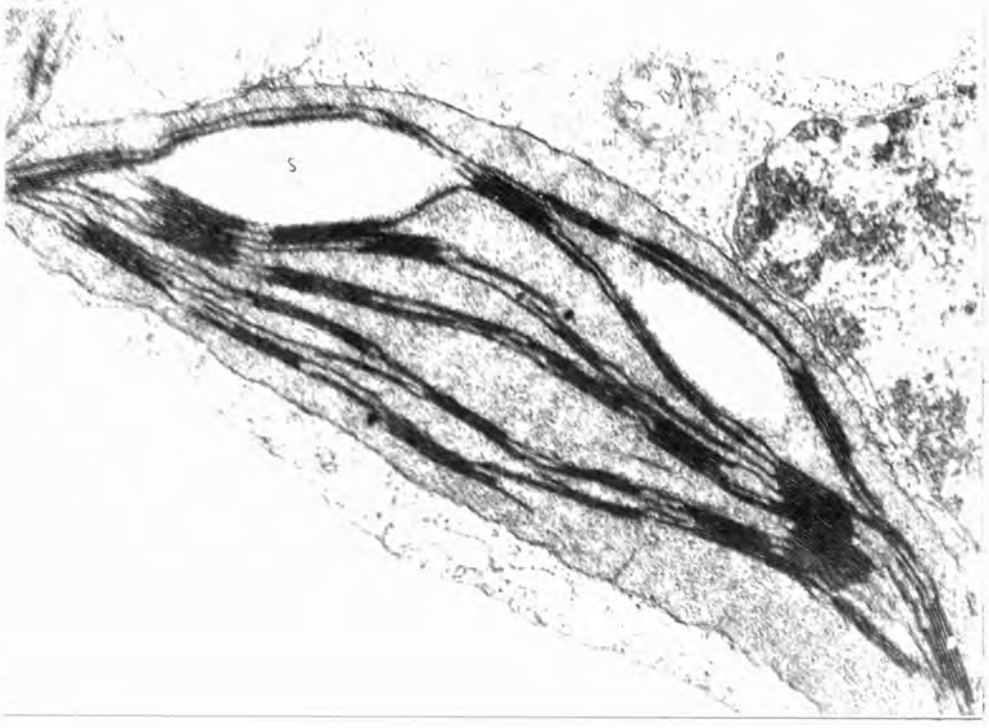

ER
M

PLATE 41 — *Chloroplasts of the newly differentiated leaf of 6-weeks old plant growing under normal temperature conditions.*

Fig. 66. — *A plano-convex chloroplast having well developed grana interconnected by short fret-membranes and two large starch grains.*
X 36,450.

Fig. 67. — *Chloroplast showing the presence of a small crystalline body and a small starch grain.*
X 24,300.


S

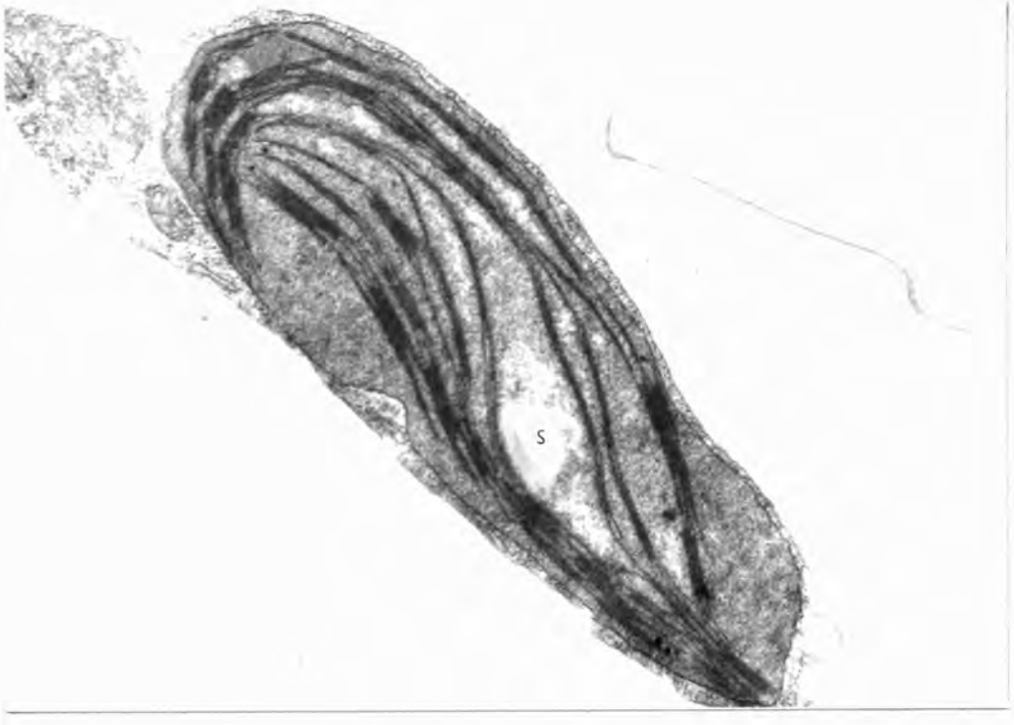
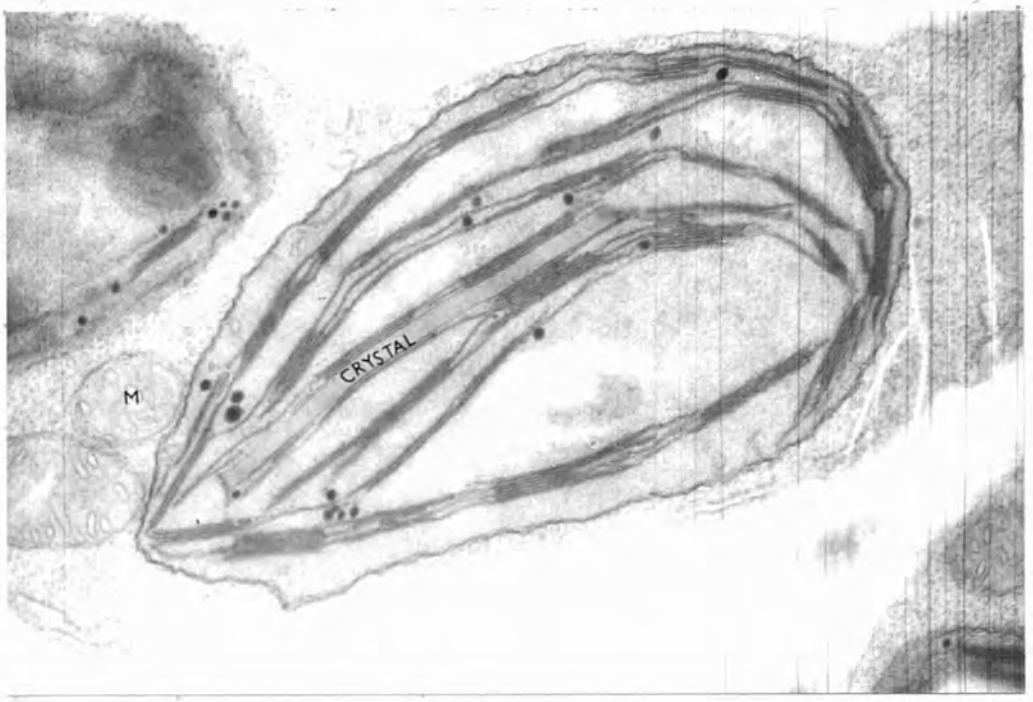

S

PLATE 42 *Section through the cells of the youngest leaf of 6-weeks old plant grown at 22°C for the first 4 weeks followed by 2 weeks at 25°C.*

Fig. 68. *A chloroplast having many long grana interconnected by short fret-membranes. The chloroplast also has a long membrane-bound crystal and several osmiophilic globules.*
X 36,450.

Fig. 69. *A chloroplast apparently at the later stage of division by constriction. A single (?) large starch grain is included in one half.*
X 24,300.

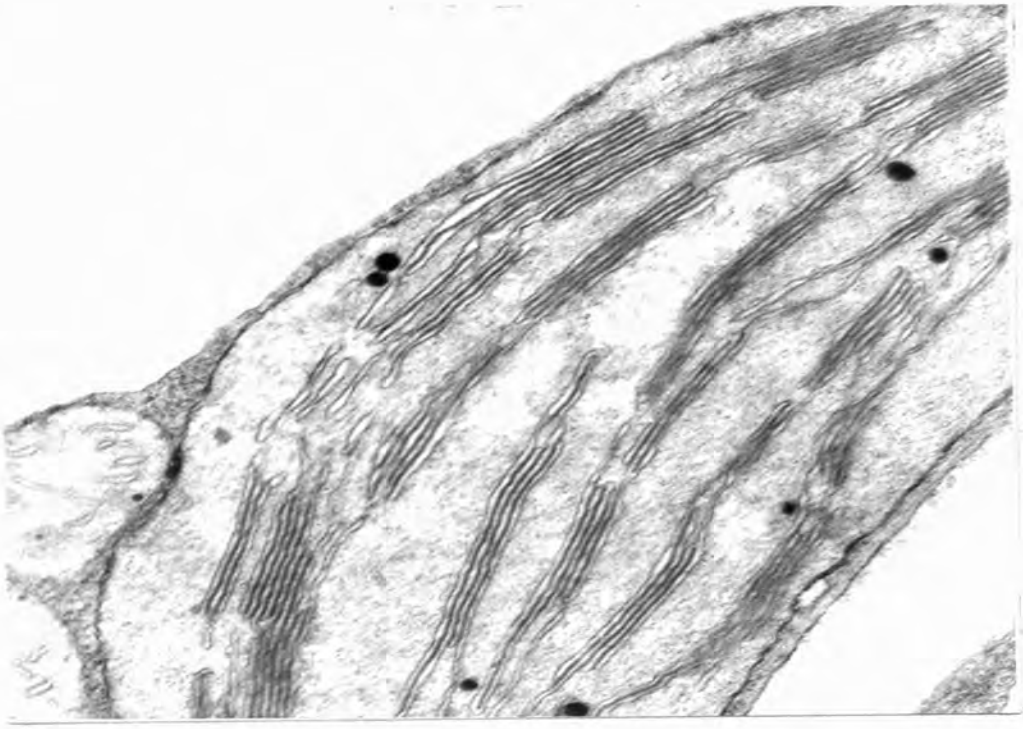

CRYSTAL
M


S

PLATE 43 — *Chloroplasts of the newly differentiated leaf of 6-week old plant grown at 22°C for the first 4 weeks followed by 2 weeks at 30°C.*

Fig. 70. — *A chloroplast showing characteristic splitting of the lamellae which are broken at various places and have club-shaped swellings at their free ends. X 60,750.*

Fig. 71. — *Similar to Fig. 70 but also showing some vesicles. X 36,450.*



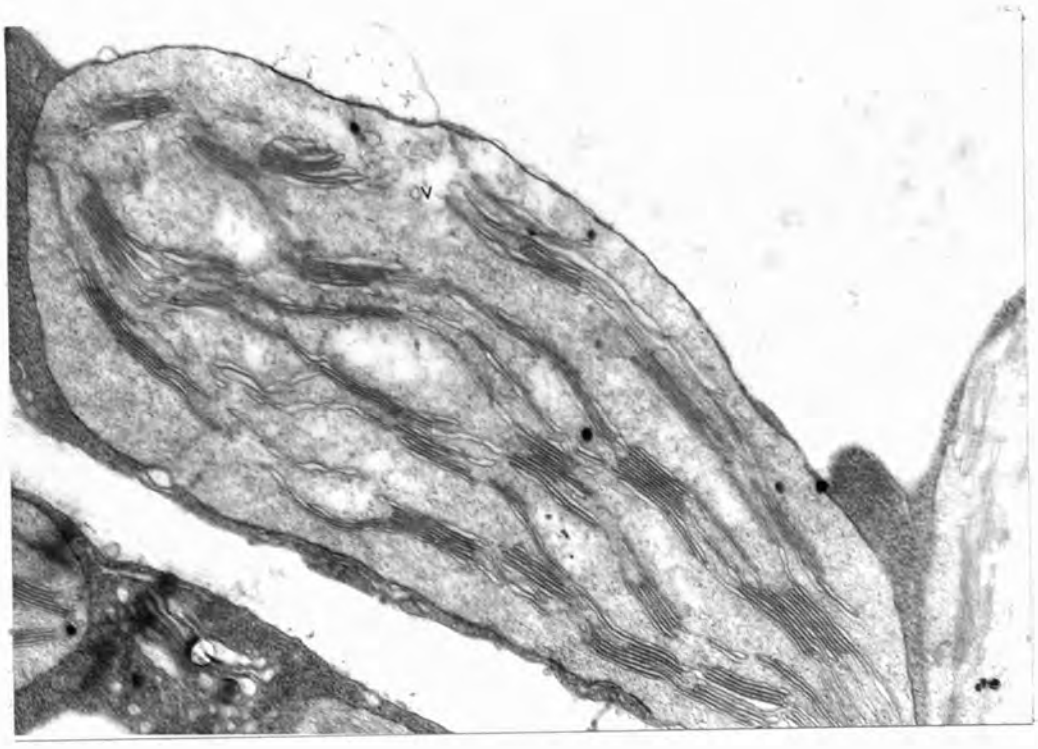
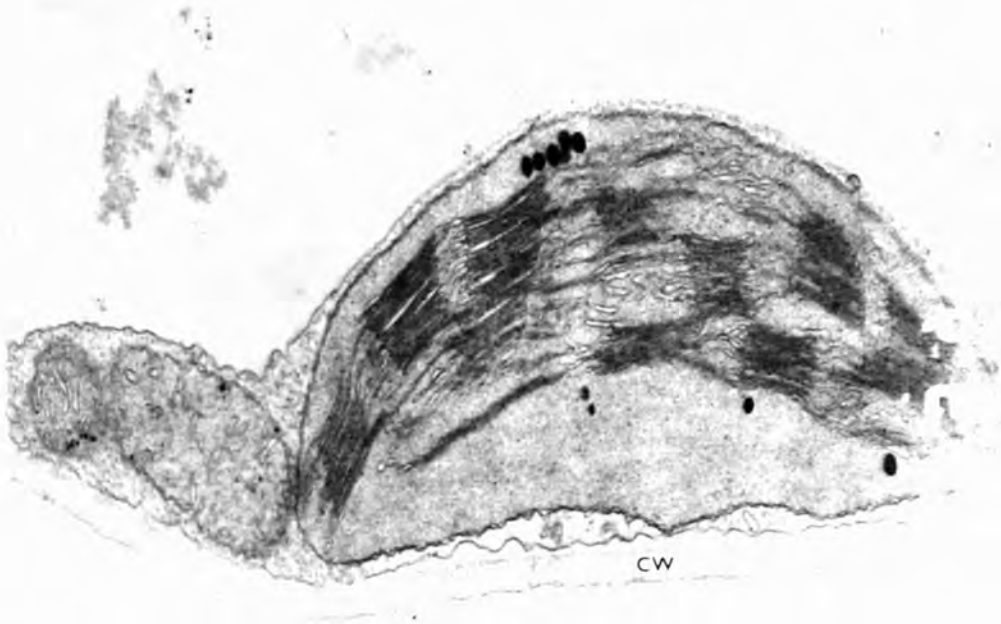


PLATE 44

Chloroplasts of the newly formed leaves of 6-week old plant grown at 22°C for the first 4 weeks followed by 2 weeks at 35°C.

Fig. 72. *A shrunken chloroplast with many, multi-thylakoidal grana interconnected by a large number of short fret-membranes. The terminal ends of the thylakoids show characteristic club-shaped swellings and some of the thylakoids are even broken off from the inter-connecting lamellae. Osmiophilic globules are present.*
X 36,450.

Fig. 73. *Section through the chloroplast clearly showing the characteristic swellings at the terminal ends of the thylakoids. It also shows big osmiophilic globules.*
X 60,750.


CW

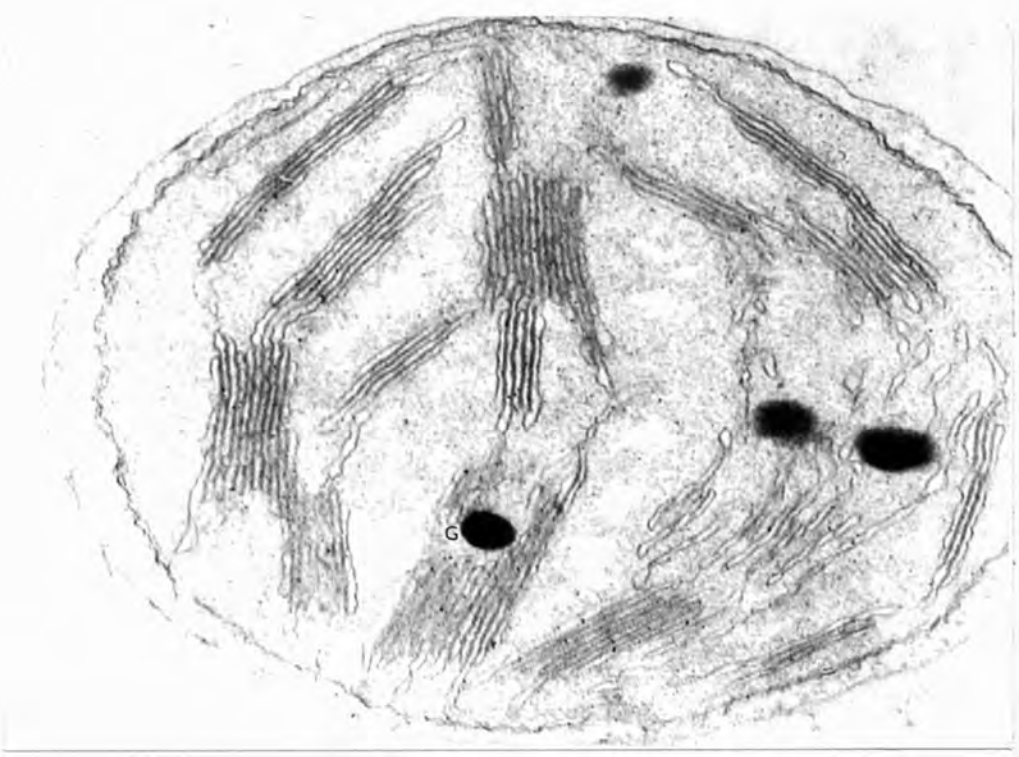
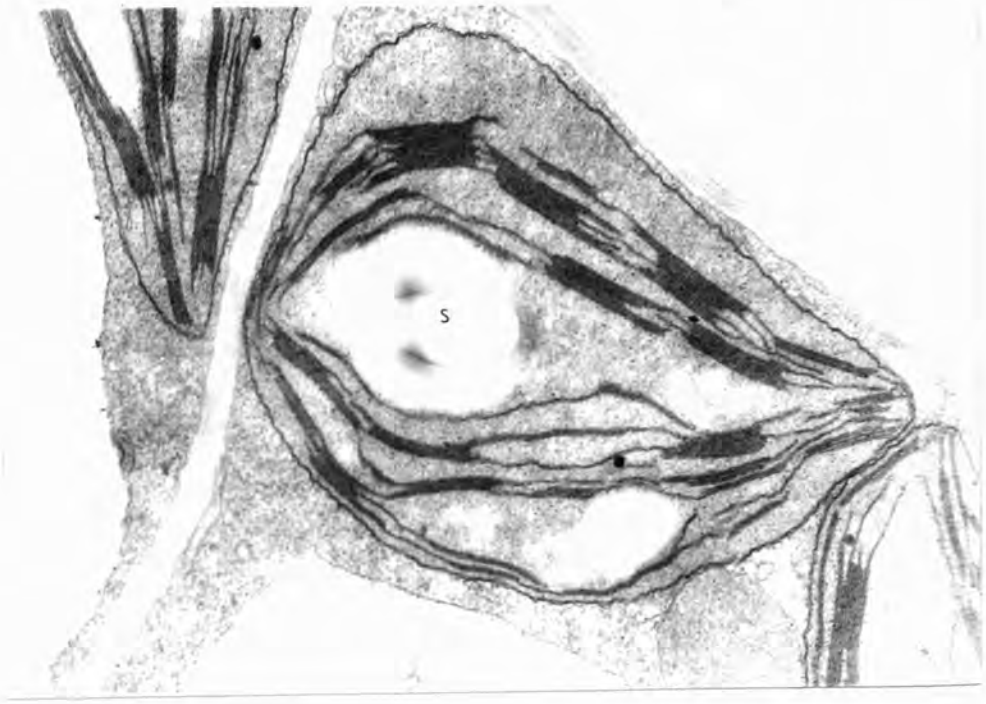

G

PLATE 45 — *Section through the newly differentiated leaf of 7-week old plant grown at 22°C.*

Fig. 74. — *Biconvex chloroplasts having well developed lamellar system and large starch grains.*
X 36,450.

Fig. 75. — *A plano-convex plastid. Some of the grana are connected by very long lamellae.*
X 36,450.


S

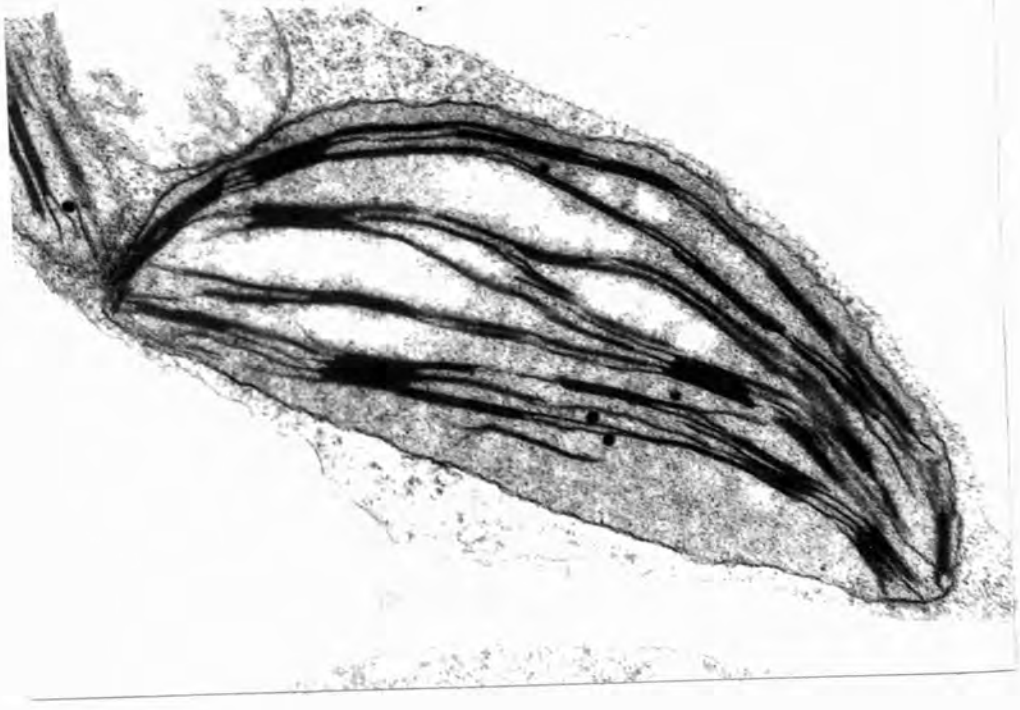


PLATE 46 *Section through the cells of the youngest leaf of 7-week old plant grown at 22°C for the first 4 weeks followed by 3 weeks at 25°C.*

Fig. 76. *Chloroplast shows well developed stroma and grana lamellae. Some of the grana are connected by very long fret-membranes.*
X 36,450.

Fig. 77. *A chloroplast which appears to be at the later stage of division by constriction.*
X 24,300.


CW

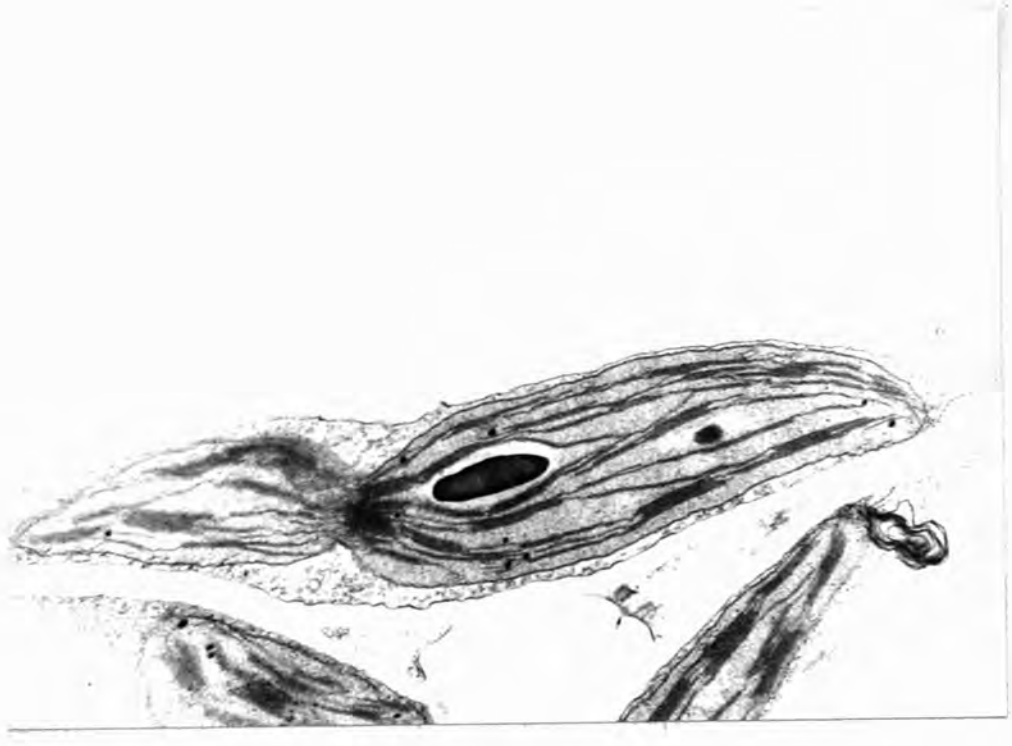
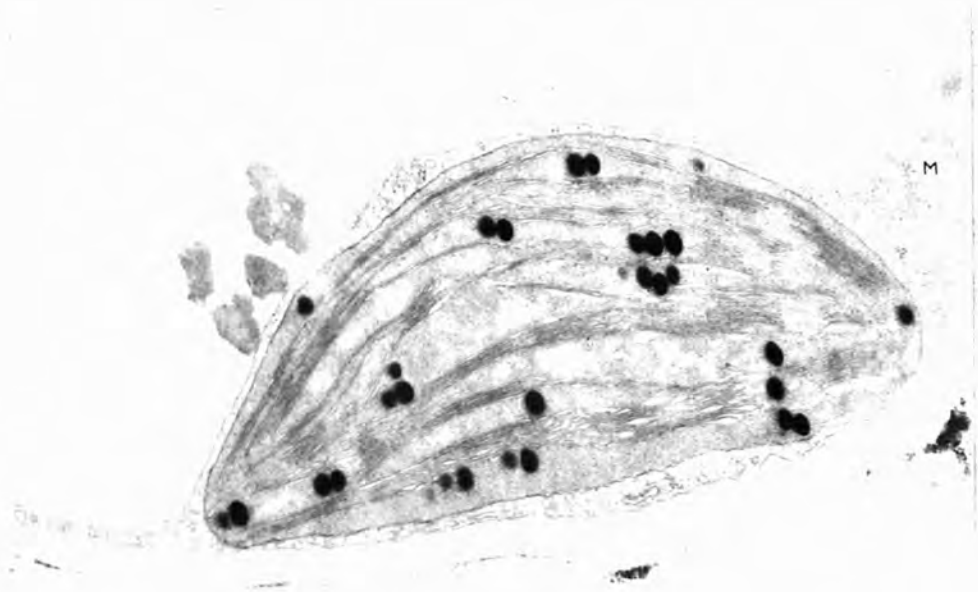


PLATE 47 *Chloroplasts of the newly differentiated leaf of 7-week old plant grown at 22°C for the first 4 weeks followed by 3 weeks at 30°C.*

Fig. 78. *A chloroplast showing reduction in size and abnormal organization of lamellar system. Lamellae show characteristic splitting at many places. Many large densely stained osmiophilic globules appear in the chloroplast.*
X 24,300.

Fig. 79. *An enlarged part of chloroplast showing abnormal organization of the lamellae and their characteristic splitting at various places.*
X 60,750.


M

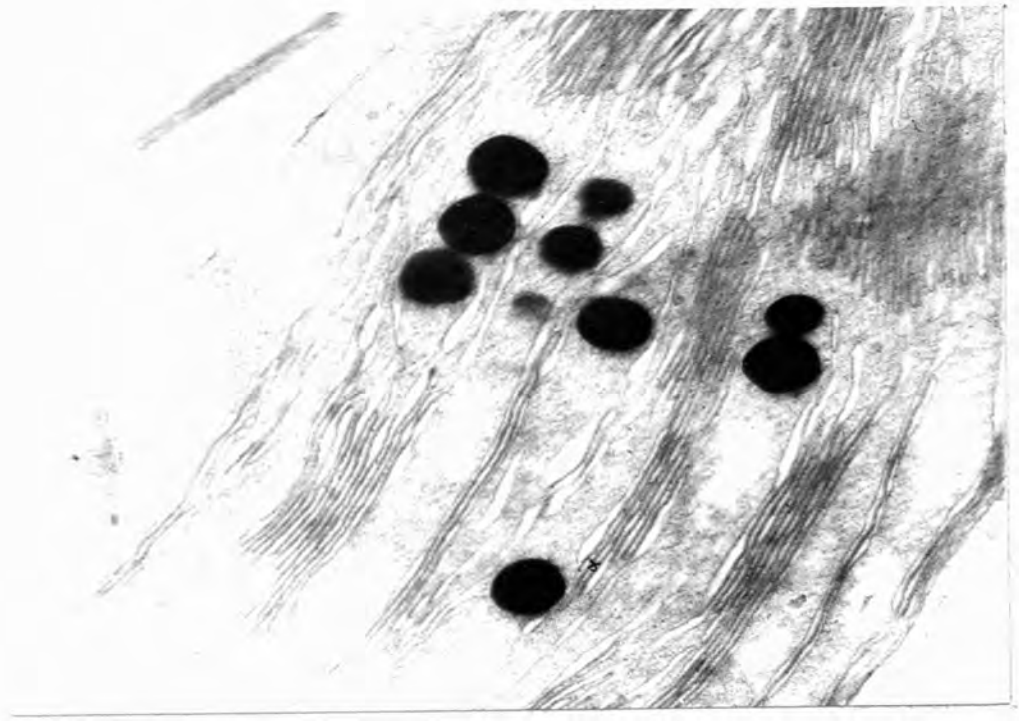
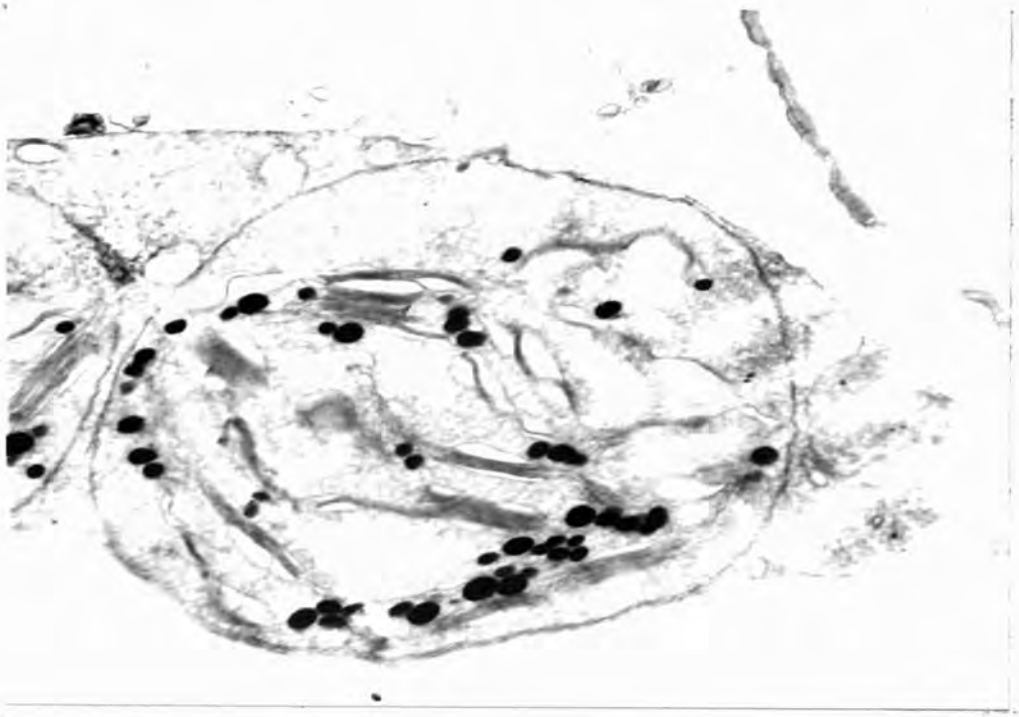


PLATE 48 *Rounded plastids of the newly differentiated leaf of 7-week old plant grown at 22°C for the first 4 weeks followed by a further 2 weeks at 35°C.*

Figs. 80. & 81. *Chloroplasts showing the complete disorganization of the lamellar system. Some grana still present show split thylakoids mostly without interconnecting lamellae. Osmiophilic globules are numerous and are densely stained.*
X 36,450 each.



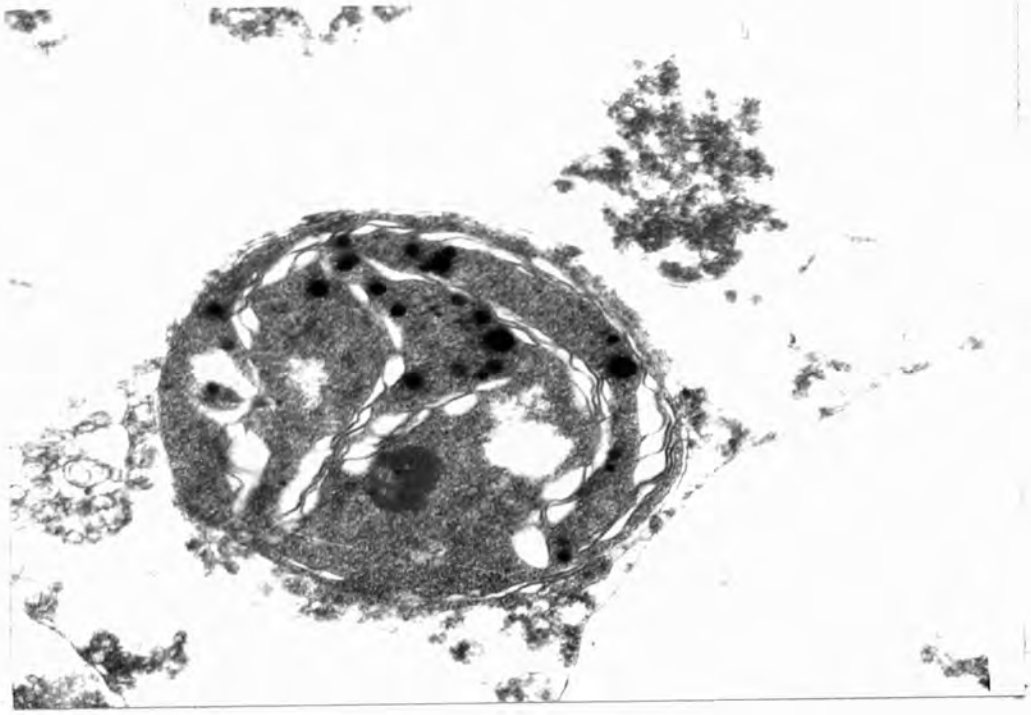
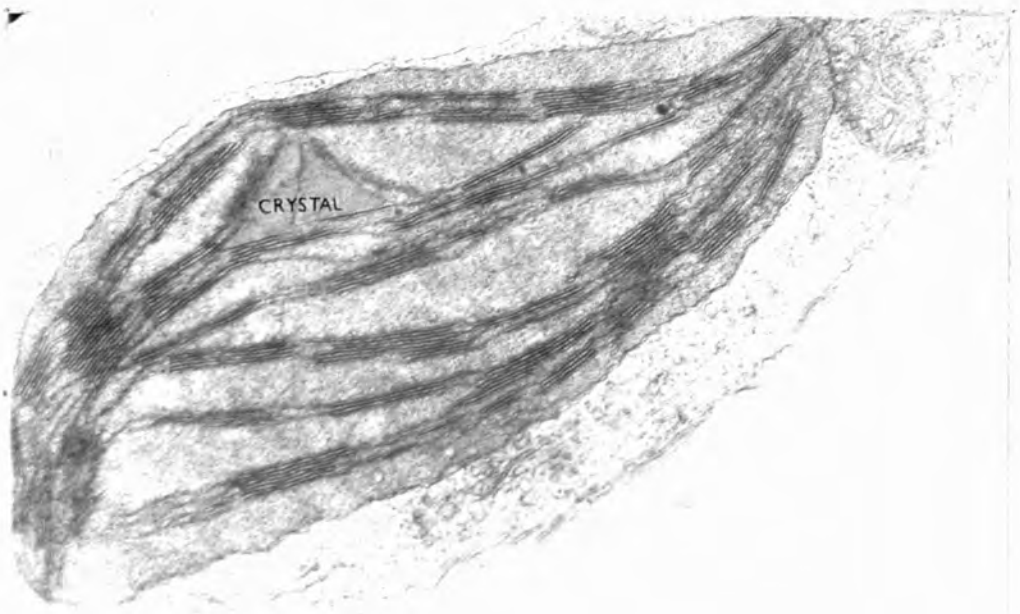


PLATE 49 *Section through the youngest leaf of 8-week old plant grown at 22°C.*

Fig. 82. *Chloroplast shows many long grana connected by short stroma lamellae. A membrane-bound crystal also appears in the chloroplast. X 36,450.*

Fig. 83. *A 'sickle'-shaped chloroplast. X 36,450.*


CRYSTAL

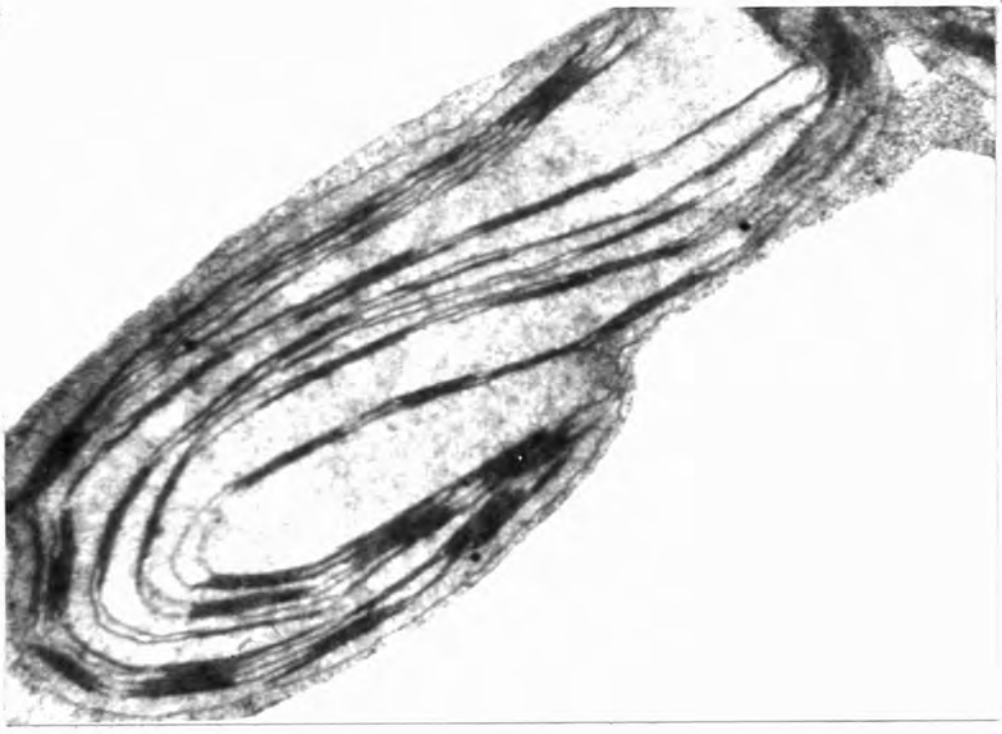
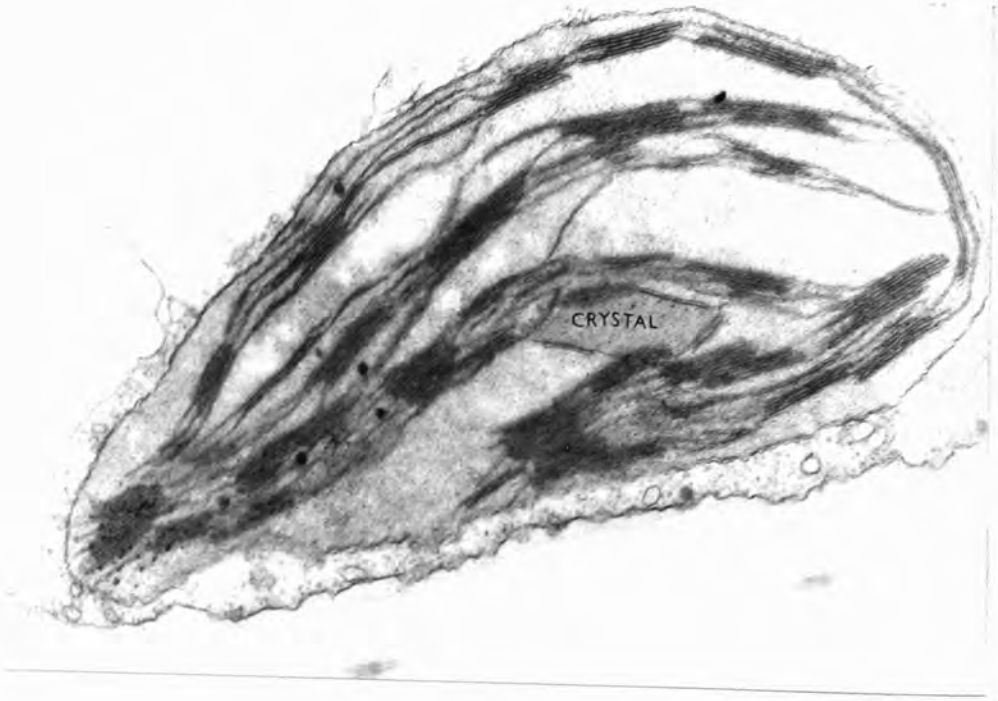


PLATE 50 *Chloroplasts of the youngest leaf of 8-week old plant grown at 22°C for the first 4 weeks followed by another 4 weeks at 25°C.*

Fig. 84. *An oval-shaped plastid showing a characteristic 'sickle'-shaped curving of the lamellar system. It also shows the presence of a membrane-bound crystal.*
X. 36,450.

Fig. 85. *An elongated chloroplast with a small membrane-bound crystal and a small starch grain.*
X 36,450.


CRYSTAL

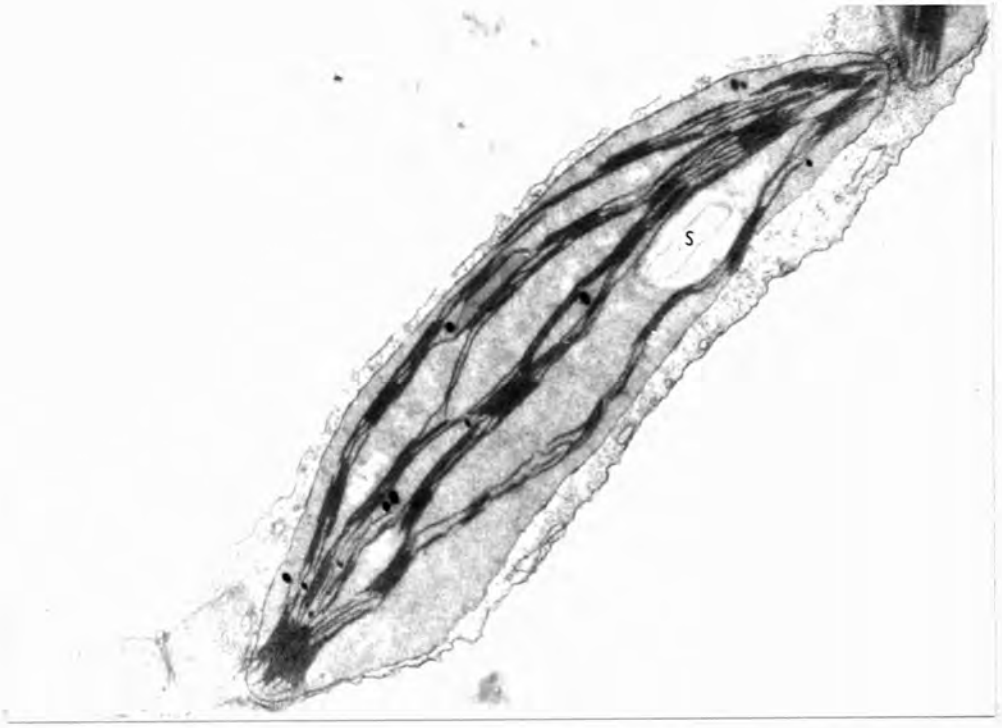

S

PLATE 51

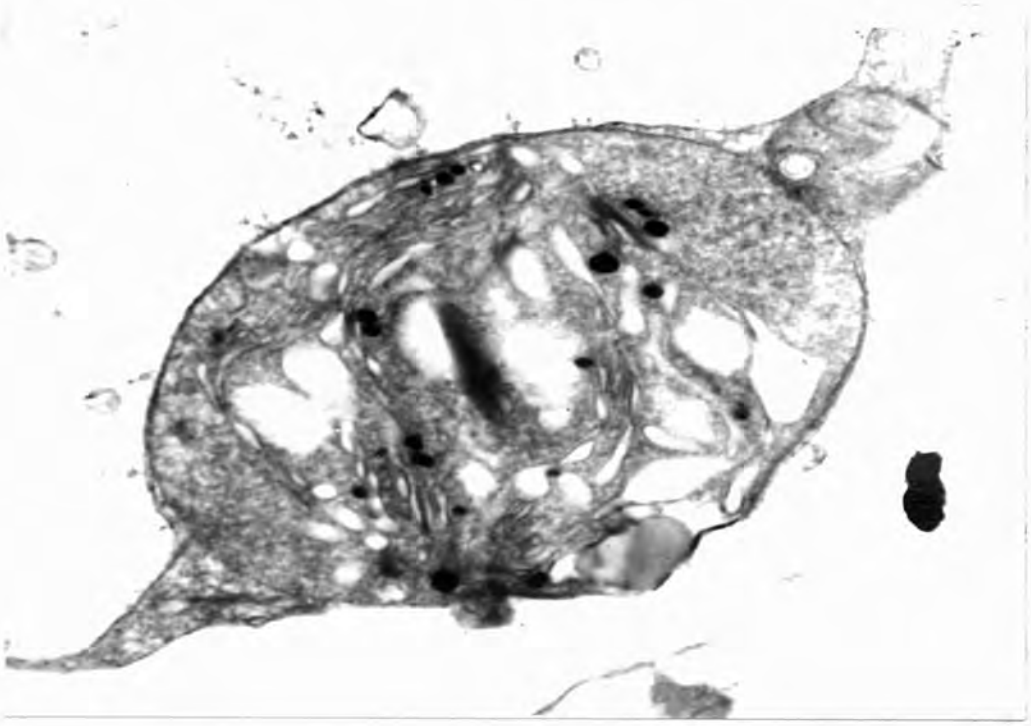
Fig. 86. *Section through a dividing chloroplast of the youngest leaf of 8-week old plant grown at 22°C for the first 4 weeks followed by another 4 weeks of 25°C.*

X 145,800.

PLATE 52 *Chloroplasts of the newly differentiated leaf of the 8-week old plant grown at 22°C for the first 4 weeks followed by another 4 weeks at 30°C.*

Fig. 87. *Rounded plastid showing a reduction in size and complete disorganization of the lamellar system.*
X 36,450.

Fig. 88. *Plastid showing bursting at a single point.*
X 36,450.



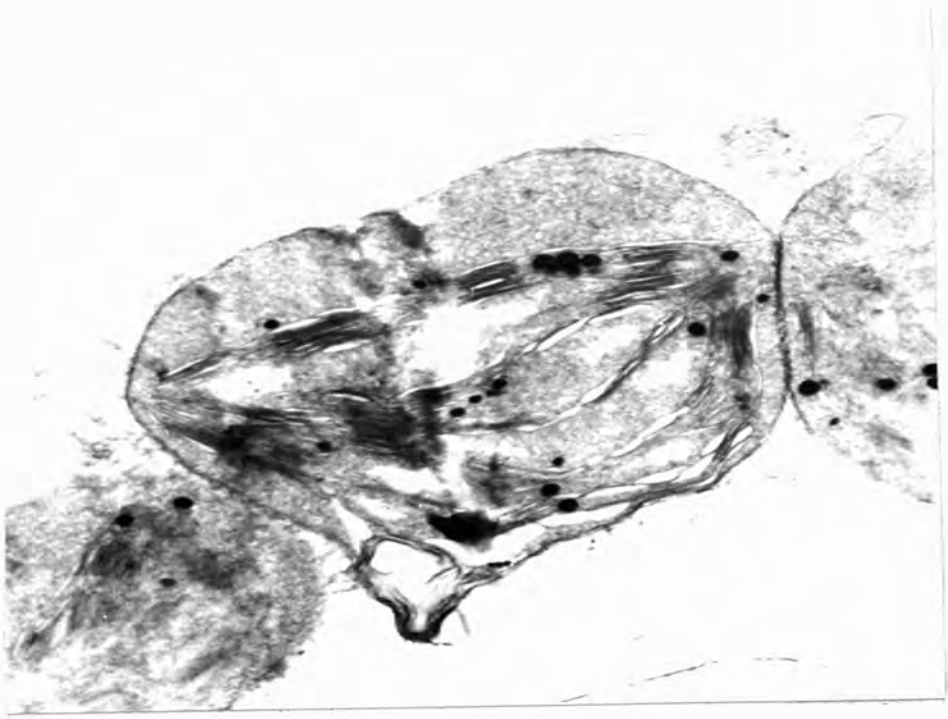
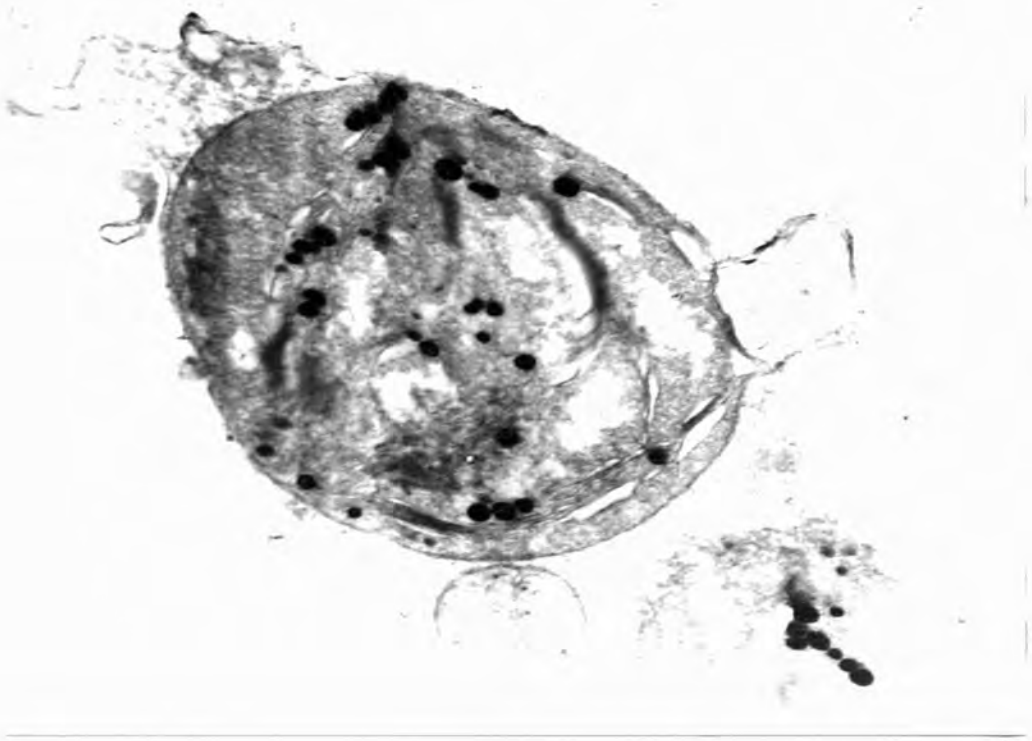


PLATE 53 *Chloroplasts of the newly differentiated leaf of the 8-week old plant grown at 22°C for the first 4 weeks followed by another 4 weeks at 30°C.*

Figs. 89. & 90. *Plastids showing typical bursting at a single point.*
X 36,450 each.



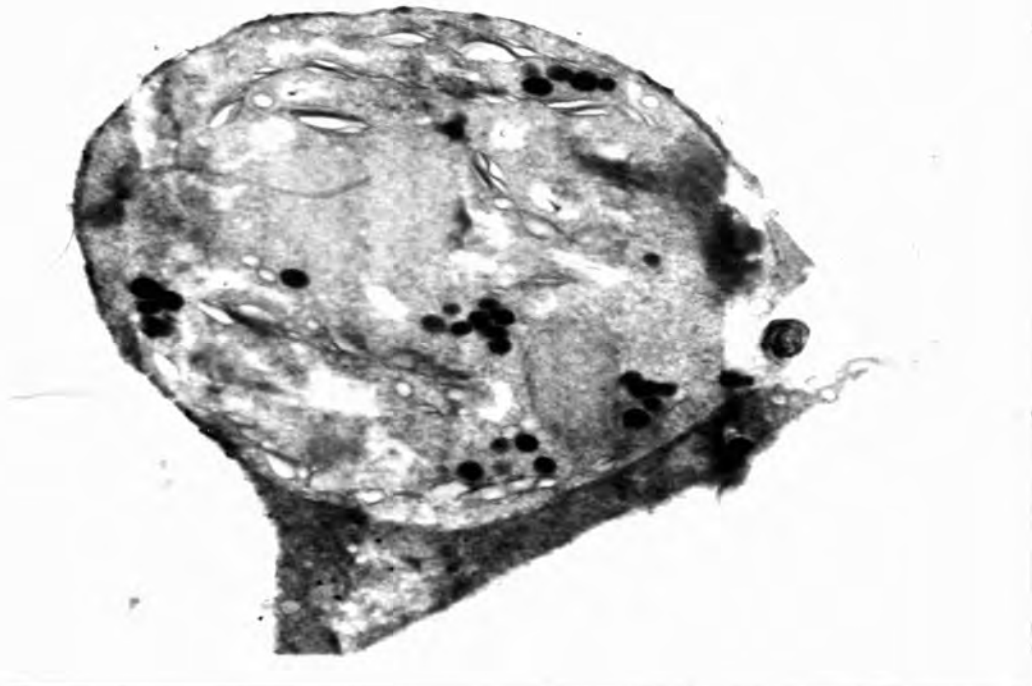
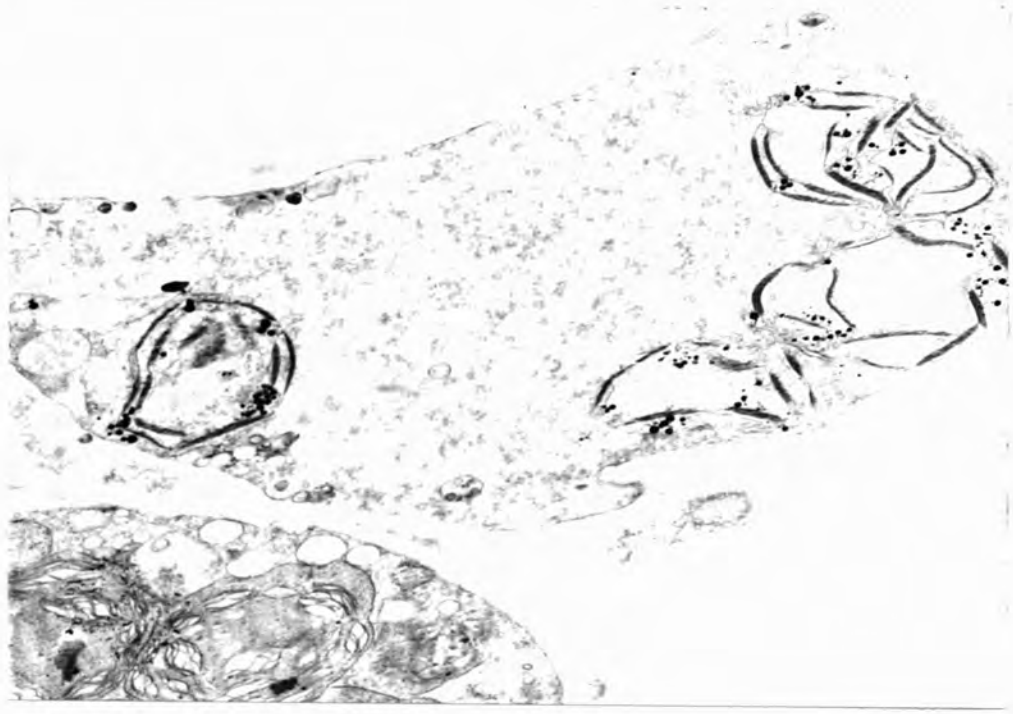
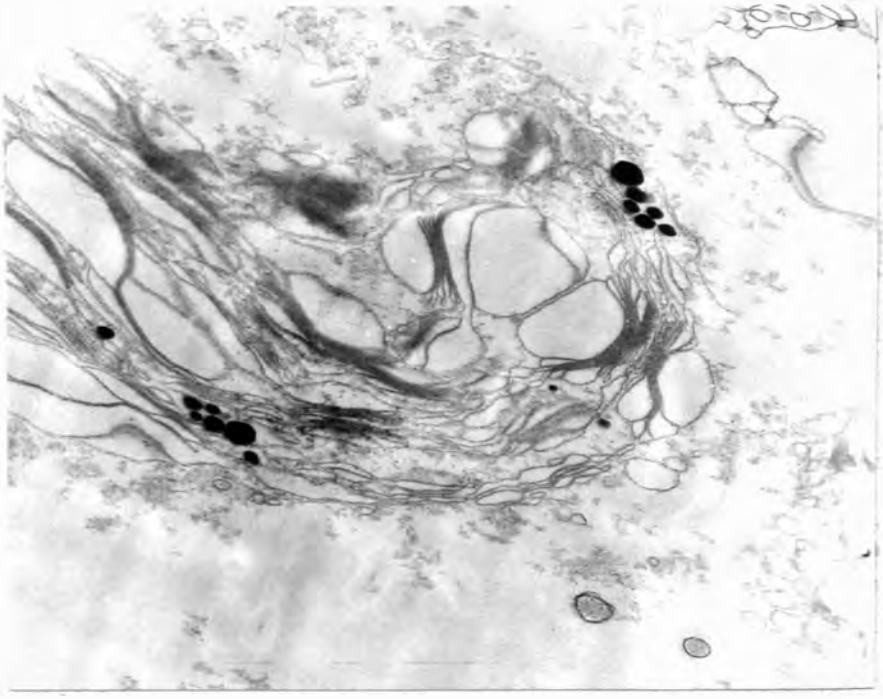


PLATE 54 *Section through the cells of the youngest leaf of 8-week old plant grown at 22°C for the first 4 weeks followed by another 4 weeks at 35°C.*

Fig. 91. *Plastids showing either the complete or partial absence of the plastid envelopes. Some of the plastids have a more or less intact lamellar system.*
X 9,720.

Fig. 92. *Disrupted plastid lamellar system lying free in the cell cytoplasm. Lamellae show swellings of much greater degree.*
X 36,450.





C H A P T E R VI

THE INFLUENCE OF TEMPERATURE ON THE FORMATION OF PROLAMELLAR BODIES IN ATTACHED LEAVES OF SPINACH IN DARKNESS

INTRODUCTION

As observed in spinach leaf chloroplasts (Chapters III, IV, and V of this thesis) and also shown earlier by Barton (1966), Hurkman (1979), high temperature disrupts the plastid ultrastructure mainly by its effects on plastid lamellae. Cran and Possingham (1972b) have shown the possibility that the prolamellar bodies in spinach leaf discs are produced in dark either as the result of outgrowths of thylakoids or formed de novo as a result of continued membrane synthesis. During the course of the present investigations it was, therefore, considered of interest to find out the effect of high temperature conditions on prolamellar body formation in spinch leaf chloroplasts in dark. An attempt is also made to trace out the process of prolamellar body formation in spinach leaf chloroplasts under normal growing temperature.

METHOD

Spinach seeds were sown and plants were raised in growth cabinets maintained at 22°C and at 14h light/10h dark conditions as described earlier in Chapter II. When the plants were four weeks old, one group of ten plants was transferred to another cabinet maintained at 30°C. After a further 24 hours, during which time the transferred plants could become acclimated to the higher temperature conditions, the plants at both temperatures were subjected to darkness. The first fully opened leaf of each plant (TEXT Figure 5) was tagged in both sets, and the samples for electron microscopy were collected

after 3 hours, 9 hours, 30 hours, 80 hours, and 128 hours of uninterrupted darkness from both the sets. The samples were collected from the tip of the leaves under a green safe light.

OBSERVATIONS

At zero hour

Zero hour sampling of this experiment is exactly the same as that of zero hour sampling of the experiment conducted in Chapter IV. Therefore the electron micrographs of zero hour sampling of the experiment of Chapter IV is also considered as the starting point for this experiment.

After 3 hours of darkness

22°C

The grana are well developed with normal grana fretwork system, having maximum stack up to 20 (Fig. 93). The stroma lamellae which connect the two grana are very long, especially near the envelope. The plastids under these treatments possess various shapes, e.g. biconvex, plano-convex or concavo-convex. The starch grains are present in most of the plastids and in some plastids these are very big in size and their number is more than three which therefore alter the shape of the plastid (Fig. 94). Osmiophilic globules are very few in number and are of very small size.

30°C

The plastids of the newly differentiated intact leaf of four weeks old spinach plant placed in dark for three hours at 30°C show very

little difference from those at 22°C in dark. Here, the well developed grana with maximum stacks up to 20 are interlinked by comparatively shorter fret-membranes. In most chloroplasts the grana fretwork is slightly pushed towards one surface leaving a space towards the other surface between the double membraned plastid envelope and the grana fretwork. This space which is filled with stroma is granular in appearance and may be called 'stroma space' (Fig. 95). Osmiophilic globules show a little increase both in number and size over those plastids at 22°C after being three hours in dark. Starch grains are also present in most of the plastids but their size is much smaller. In a few plastids the presence of many starch grains alters the shape of the plastid and also disorganizes the lamellar system of the plastid (Fig. 96).

After 9 hours of darkness

22°C

The spinach plastids under this treatment at 22°C temperature condition do not show much variation from those which are given three hours dark treatment at the same temperature. The only differences observed after nine hours in dark are: the size and number of starch grains show a much greater reduction and in most plastids the osmiophilic globules show a little increase both in their number and size (Fig. 97). Both grana and stroma lamellae show a normal structure. The plastids assume various shapes (Figs. 97, 98).

30°C

Under 30°C after nine hours in dark, the most prominent change which the plastids have shown is a loosening of the plastid lamellae resulting

in an increase in area of the thylakoids and hence of grana (Figs. 99, 100). In some of the grana lamellae some clear areas can also be seen. In section, stroma lamellae at many places show unstained regions which at certain points are broken off from the grana. Stroma space is still a common feature of these plastids. Starch grains if present are not more than one per plastid. Osmiophilic globules show a further increase in size.

After 30 hours of darkness

22°C

Under normal conditions these plastids do not show any significant change in ultrastructure from those after three hours or nine hours in dark. The only change observed is a little loosening of the plastid lamellae which thus increases the area of each granum (Figs. 101, 102). However, in some immature plastids (or proplastids) which have not yet been fully differentiated into mature chloroplasts, some small vesicles are budded off from the plastid membranes and accumulate at certain places in the plastid which give the appearance of pro-lamellar bodies (Fig. 102). Starch grains are present only in very few of the plastids and are of very small size.

30°C

Under high temperature conditions (30°C) the intact spinach leaf plastids after 30 hours in dark show a little reduction in the number of thylakoids per granum and the maximum stack observed is 15. Very few of such plastids show the presence of starch grains and these are of small size. These plastids also show a little reduction in

their size and are much swollen in comparison to the normal plastids (Fig. 103).

After 80 hours of darkness

22°C

The plastids under this treatment have become comparatively smaller in size but the plastid ultrastructure does not show much difference from that after 30 hours at 22°C. Some of the plastids possess a large stroma space which is granular in appearance. At this stage of dark treatment the spinach chloroplasts under normal temperature conditions show the appearance of prolamellar bodies which in most of the plastids is a simple structure and does not possess the well developed paracrystalline structure (Figs. 105, 106) shown by typical etioplasts. The prolamellar bodies which are randomly arranged in the plastid are very small in size. The number of thylakoids per granum does not show any reduction. Starch grains are present only in a few chloroplasts. The osmiophilic globules show a little increase (Figs. 105, 106).

30°C

The plastid ultrastructure of spinach leaves at this stage of development under high temperature conditions show a much greater variation. Besides the tremendous swelling of the chloroplast, the number of thylakoids per granum is reduced considerably. The maximum stacking observed is 10. Starch grains are present only in a few of these chloroplasts. Many small vesicles can be seen within the plastid (Figs. 107, 108). It is clear that the vesicles originate from

swelling of the plastid membranes, since many vesicles have the little remains of lamella attached to their outer surface (Fig. 108). In some partially differentiated plastids there is a large prolamellar body which has paracrystalline structure (Fig. 108).

After 128 hours of darkness

22°C

At this stage of dark treatment, under normal temperature conditions, the prolamellar body formed in spinach plastids is small but possesses more or less paracrystalline structure, resembling that of a typical etioplast (Figs. 109, 110). The thylakoid stack per granum at this stage is considerably reduced. No starch grain is observed in any of the plastids at this stage of dark treatment.

30°C

The loosening of plastid lamellae takes place to a very high extent under high temperature conditions. Many small vesicles appear to be chopped off from the lamellae and in some chloroplasts they seem to accumulate to give the appearance of a very simple type of prolamellar body (Fig. 112). Most of the stroma lamellae are broken down and therefore, grana seem to lie randomly in the chloroplast (Figs. 111, 112). Osmiophilic globules are either absent or are very few in number. Starch grains are present only in a few of the plastids. Some of the plastids also show division by constriction mechanism (Fig. 111).

DISCUSSION

In general when plants are placed in darkness, two separate processes appear to occur with regard to the membranes of the chloroplasts; firstly, a reduction of the thylakoids originally present in the chloroplast and secondly, the formation of new membrane structures in the form of prolamellar bodies. When we compare these results with the results of experiments described here we find that at 22°C and 30°C temperature conditions both the number and size of thylakoids increase up to 28 days and 21 days respectively. But in this experiment only after 128 hours of dark treatment do the number and size of thylakoids show a reduction. At 22°C prolamellar bodies appear after 128 hours of darkness whereas at 30°C the lamellae are loosened and broken at various places with the production of vesicles which do not, however, appear to be formed from the outgrowths of the inner membranes of plastid envelope as described by Hodge et al. (1956), Mühlenthaler and Frey-wyssling (1959), Röbbelen (1959), von Wettstein (1959), Erriksson et al. (1961)

The loss of thylakoids of spinach plastids under prolonged dark treatment might result from the excessive vesicular outgrowth. The observations of Cran and Possingham (1972b) on prolamellar body formation within the plastids of dark grown spinach leaf discs support the suggestion of Gunning and Jagoe (1965) that this structure is not formed from vesicular outgrowths of the plastid since such vesicle formation only occurs after four days of growth in the dark. The prolamellar bodies appear to be formed in close association with

changes in the stroma metabolism of dark grown plastids. The absence of osmiophilic globules or, at least, their number and size remaining constant during the prolamellar body formation in spinach plastids, gives an indication that both destructive and constructive processes are taking place simultaneously.

A partially differentiated, dividing plastid under normal growth temperature conditions after 30 hours in dark is seen to possess a very small prolamellar body (Fig. 102), having paracrystalline structure resembling that of a typical etioplast. A similar plastid structure is also observed (Fig. 108) at 30°C temperature condition after being 80 hours in dark. This early development of prolamellar bodies typical of etioplasts may be because the plastids which are not yet fully differentiated have a very high metabolic activity which results in quicker structural changes. This is further strengthened from the work of Cran and Possingham (1972b) who describe that partially differentiated spinach chloroplasts have considerably high plasticity. According to them the plastids of spinach can dedifferentiate into proplastid-like organelles when placed in dark and these, on illumination, can again differentiate into chloroplasts.

According to Gunning (personal communication quoted by Sharma, 1977) if a seedling is germinated in light and then transferred to darkness, the fully differentiated chloroplasts that were in existence at the time of transfer would not be expected to develop prolamellar bodies. If the seedlings are sufficiently healthy to continue growing (utilising food reserves) then the new growth might contain etioplasts,

or if the existing green tissue (leaves or cotyledons) is young enough to continue lamellar synthesis, then again prolamellar bodies might develop. We could thus explain the formation of rudimentary prolamellar bodies or no prolamellar body at all in the chloroplasts of the young leaves, which have differentiated in light and then transferred to darkness. As the spinach plastids under high temperature conditions start showing senescence (lamellar disorganization) after a few days in the darkness, it is therefore clear that the leaves are not healthy enough to continue growing in darkness possibly either due to the inability of leaves to utilize food reserves when transferred from light to darkness or due to high temperature conditions.

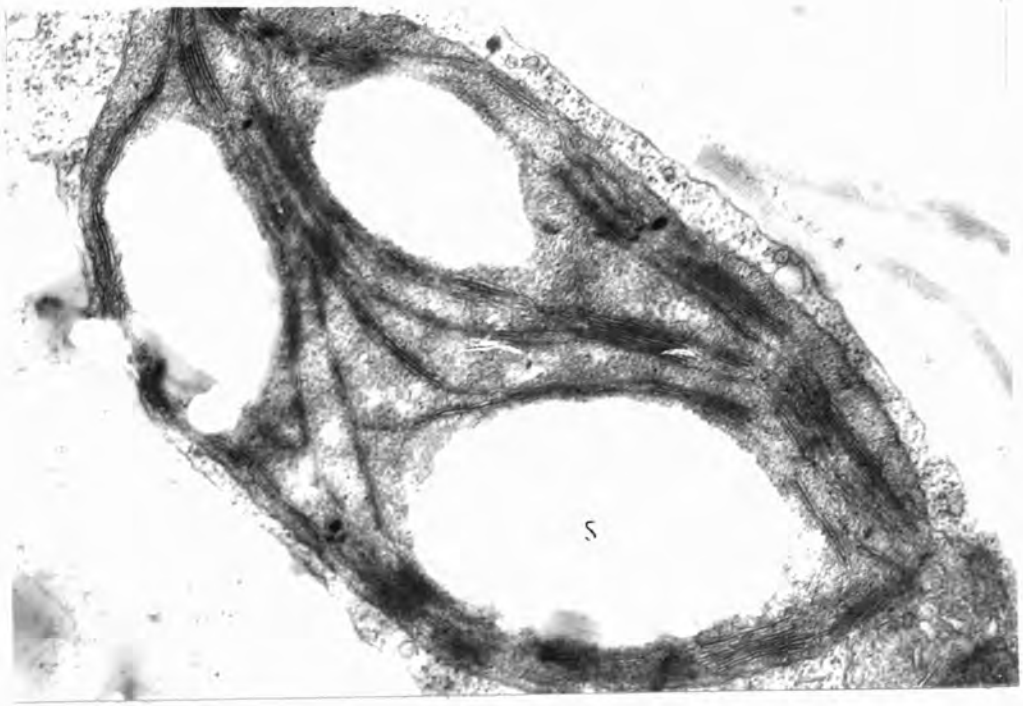
The etioplasts formed in the present experiments show the prolamellar body with a central core built of an array of interconnected tubules which are arranged in the crystalline or concentric (Wehrmeyer, 1965a, b, c) pattern. The prolamellar bodies in etioplasts show that the tubules that get branched and regularly cross connected to form quasi-crystalline lattice are cut at various angles in different parts of the crystal. It is now known, that the pattern of tubules seen in sections of prolamellar body depend on the angle of sectioning and the thickness of the sections, as well as the regularity of the lattice (Ikeda, 1968; Weier and Brown, 1970). As shown in Fig. 108 some small circular profiles of interconnecting tubules appear to lack connection with other tubules. Such prolamellar body appears as zigzag lines and dots in a linear fashion, which is formed as a result of breaking down of the combination of hexagons (Ikeda, 1968).

This pattern is explained as being due to sections that are sufficiently thin to include only interconnecting tubules or only tubules which lie in the plane of peripheral lamellae (Weier and Brown, 1970). In thicker sections these patterns are not seen but appear as recti-hexagonal type, in which each hexagon is equivalent in size within a prolamellar body exhibiting a characteristic honeycomb-like arrangement of tubules (ordered hexagonal).

PLATE 55 *Chloroplasts of youngest leaf on 4-week old plant after 3 hours in dark at 22°C.*

Fig. 93. *A biconvex chloroplast showing well developed lamellar system. The grana are connected by short fret-membranes. X 36,450.*

Fig. 94. *Chloroplast shows large starch grains. X 36,450.*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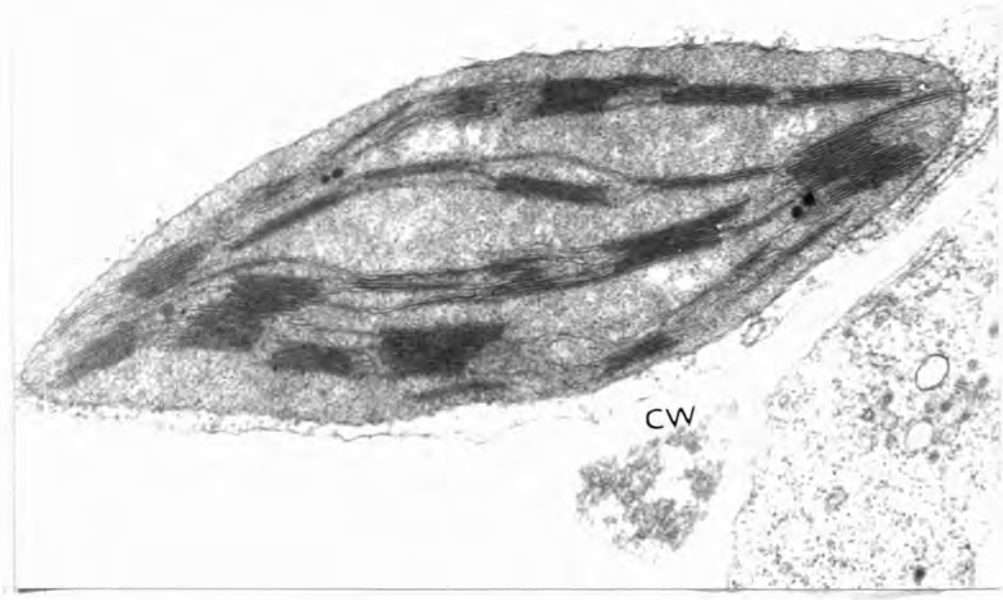
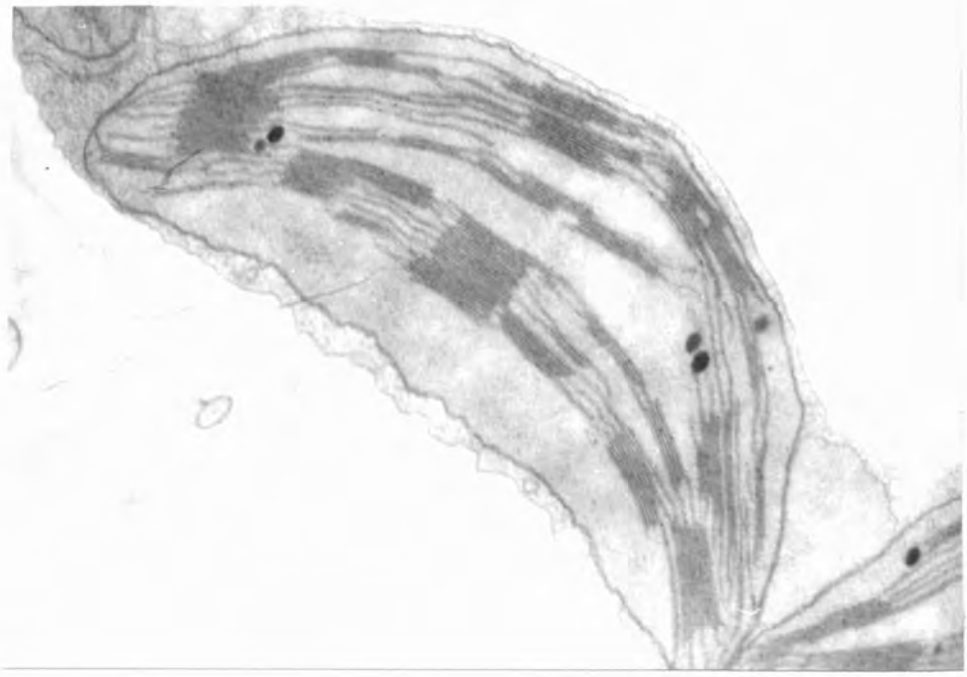

CW

PLATE 56 *Chloroplasts of youngest leaf on 4-week old plant grown at 22°C. After 3 hours dark treatment at 30°C.*

Fig. 95. *Concavo-convex chloroplast with well developed grana fretwork system. Osmiophilic globules show a little increase in size. X 36,450.*

Fig. 96. *Chloroplast containing many starch grains and disorganized lamellar system. X 60,750.*



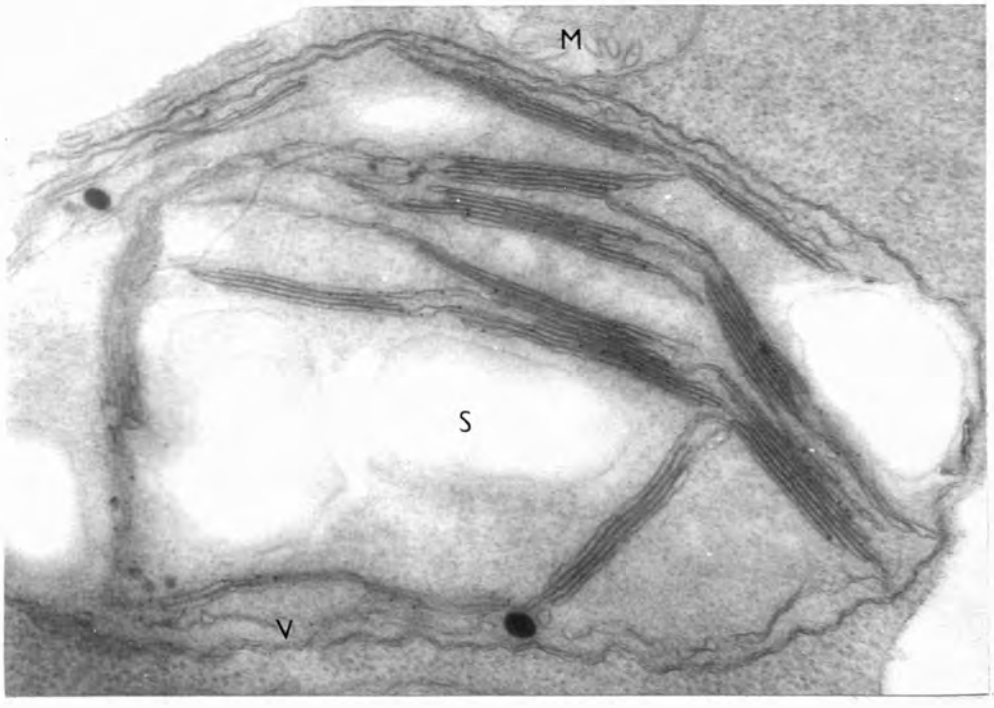
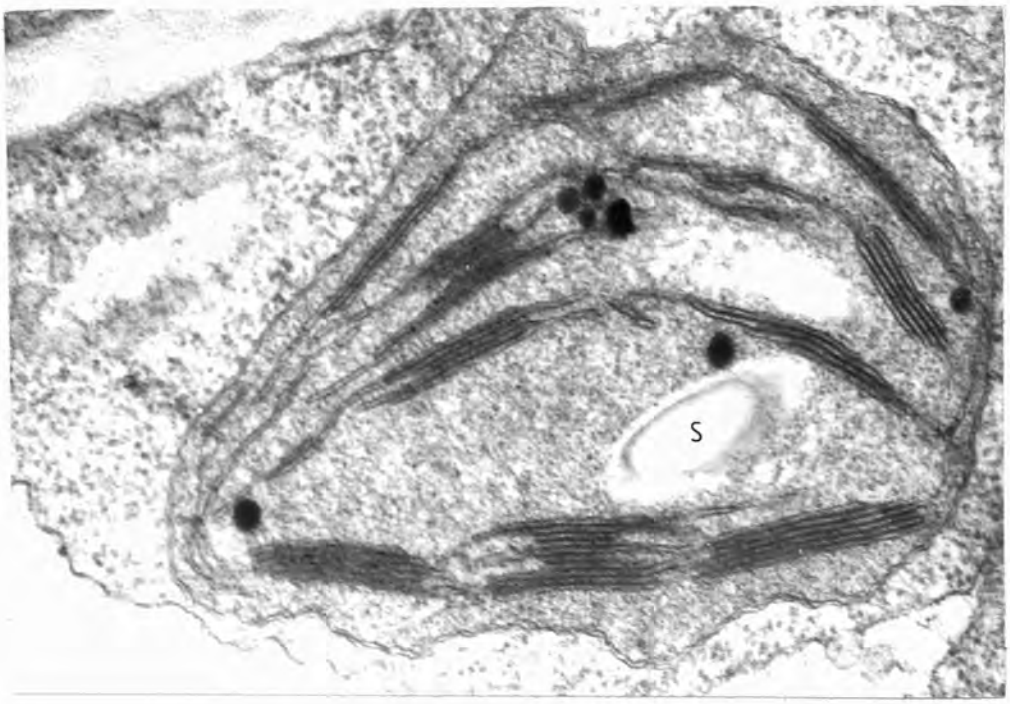

M
S
V

PLATE 57 *Chloroplast of youngest leaf on 4-week old plant after 9 hours in dark at 22°C.*

Fig. 97. *An irregularly oval-shaped chloroplast showing the presence of reduced starch grain and large osmiophilic globules.*
X 60,750.

Fig. 98. *A rounded chloroplast with a single large starch grain.*
X 36,450.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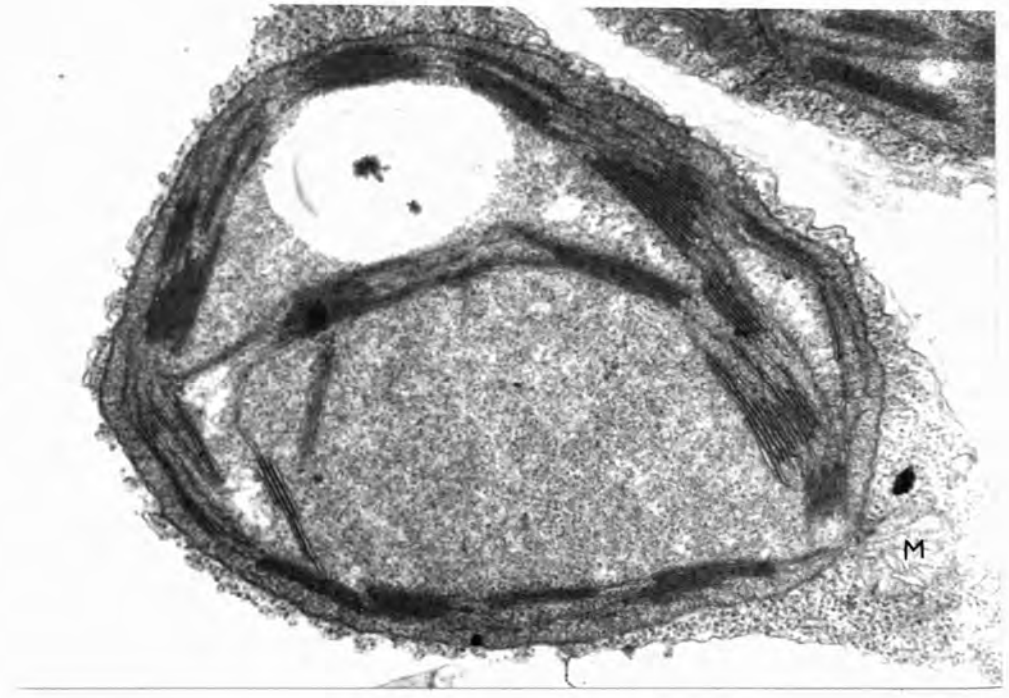
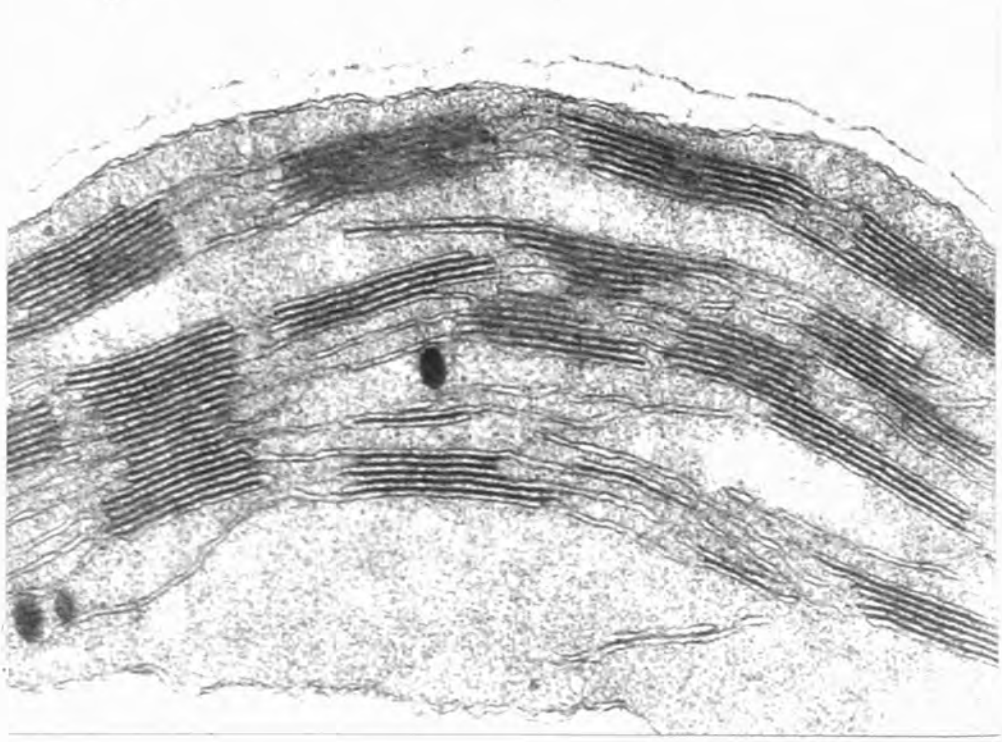

M

PLATE 58 *Chloroplasts of the youngest leaf on 4-week old plant grown at 22°C. After 9 hours dark treatment at 30°C.*

Fig. 99. *Chloroplast showing slight loosening of lamellae. Some of the lamellae are also broken.*
X 60,750.

Fig. 100. *A magnified part of a chloroplast. Starch grains are also present.*
X 97,200.



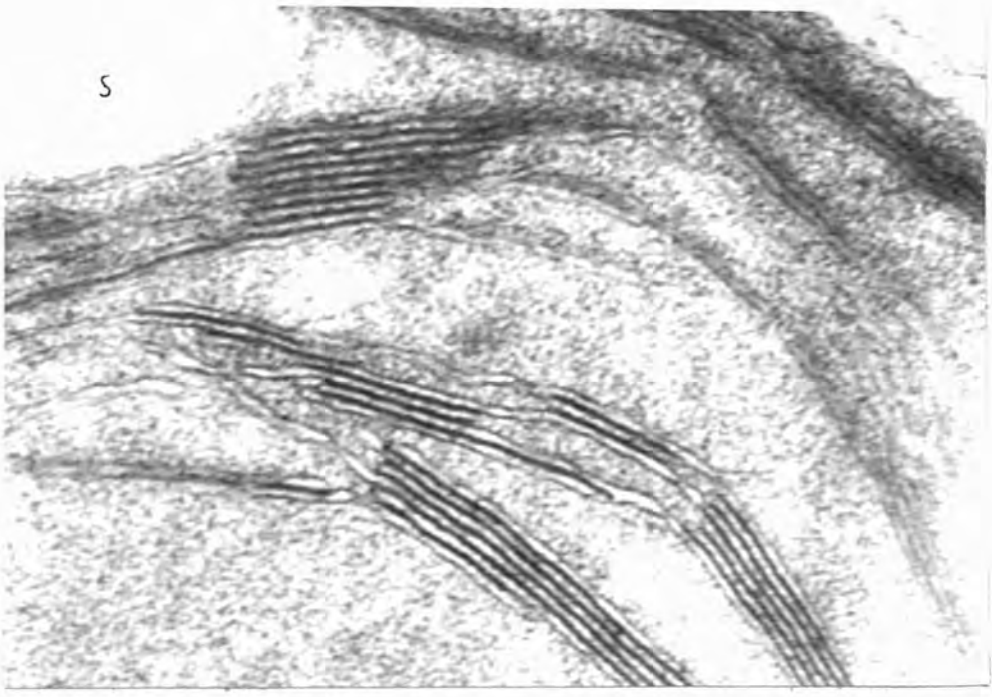
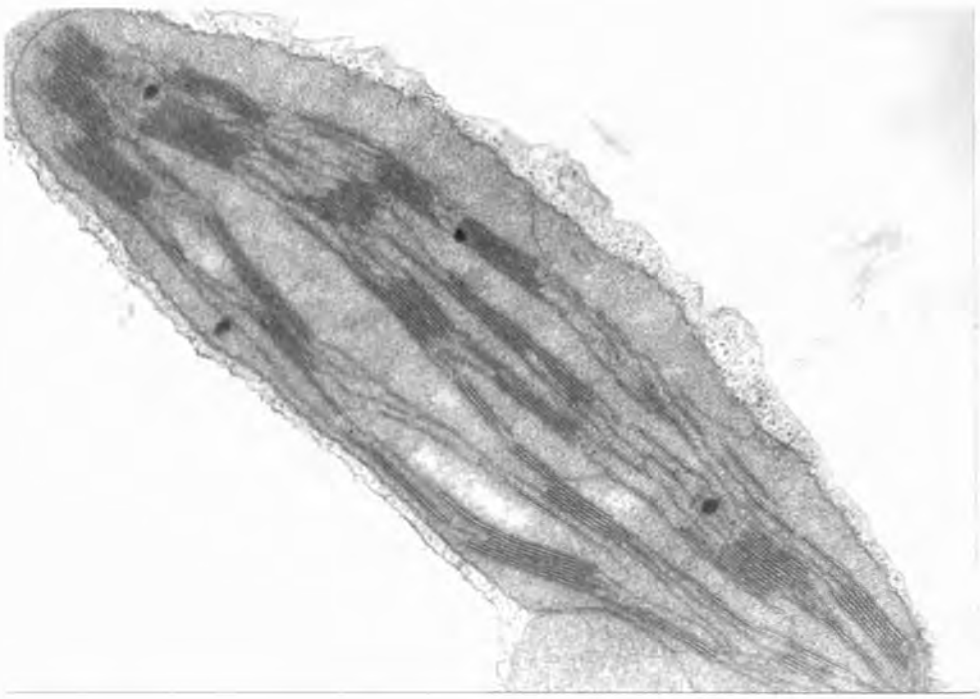

S

PLATE 59 — *Chloroplast of youngest leaf on 4-week old plant subjected to 30 hours in dark at 22°C.*

Fig. 101. — *Chloroplast with many multithylakoidal grana with slightly loosened lamellae.*
X 36,450.

Fig. 102. — *A dividing, partially differentiated plastid showing the presence of a small prolamellar body.*
X 36,450.



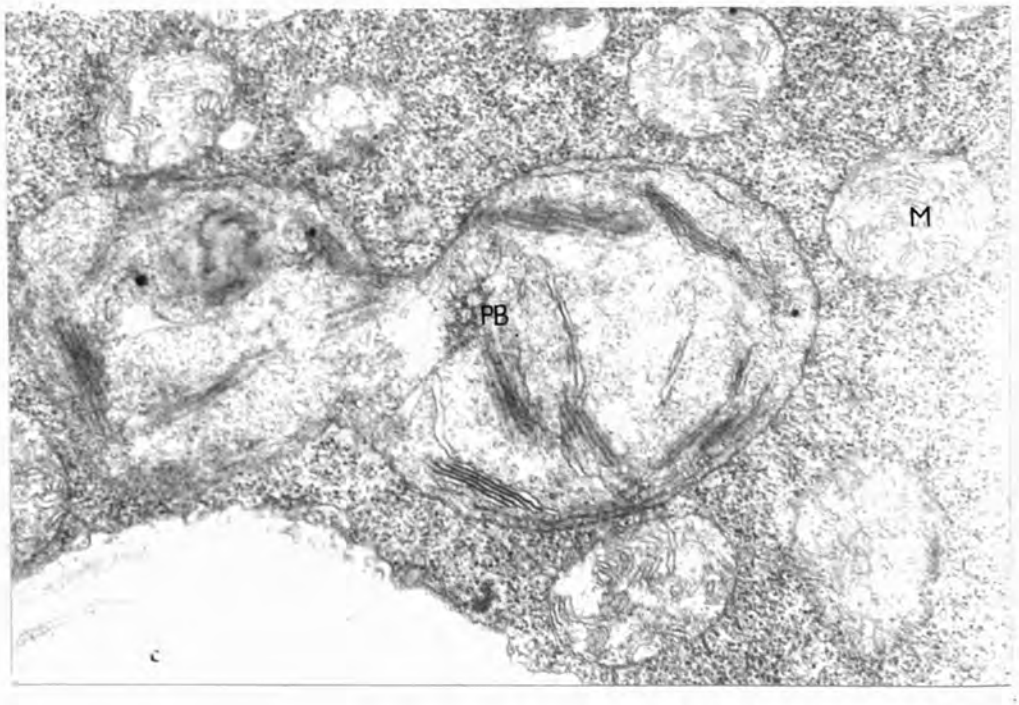
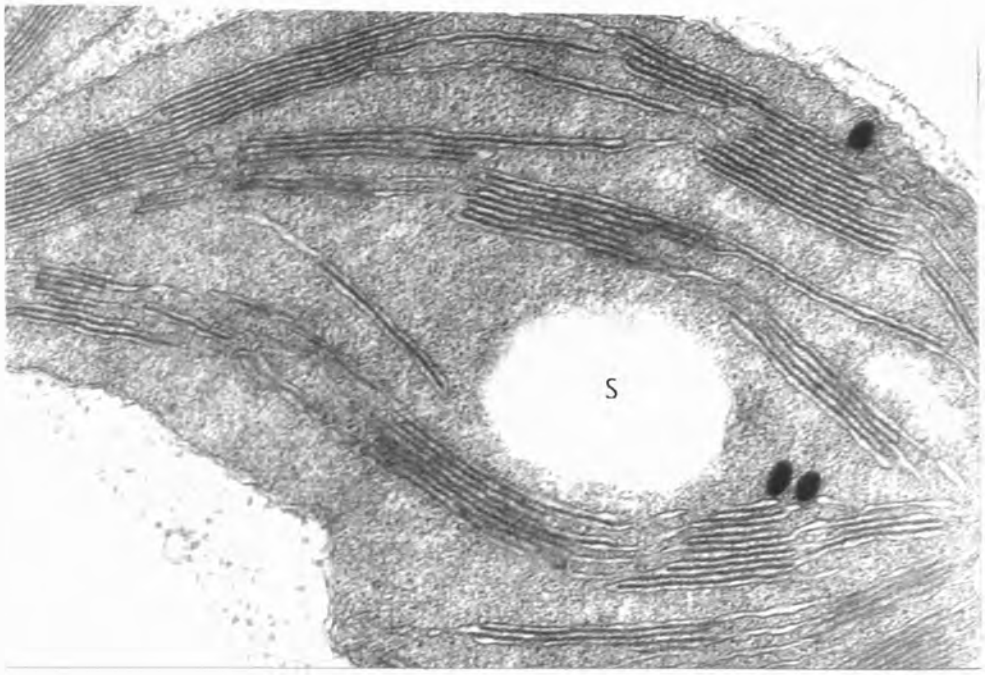

M
PB

PLATE 60 *Chloroplast of youngest leaf on 4-week old plant grown at 22°C. After 30 hours dark treatment at 30°C.*

Figs. 103. & 104. Swollen plastid with loosened lamellae. Each plastid shows a single starch grain and few osmiophilic globules.
Fig. 103 - X 36,450
Fig. 104 - X 97,200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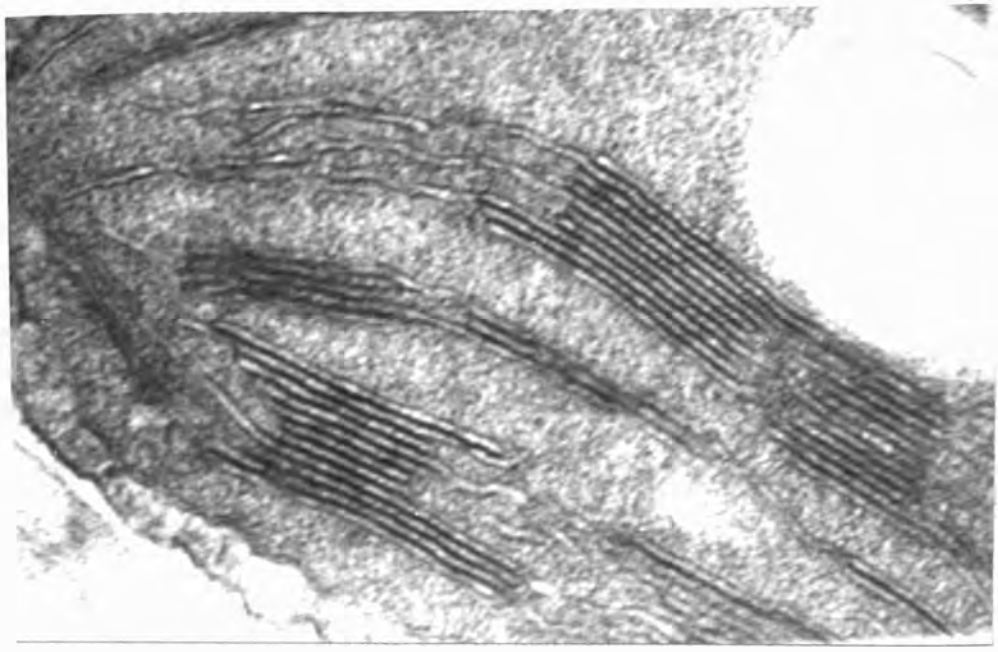
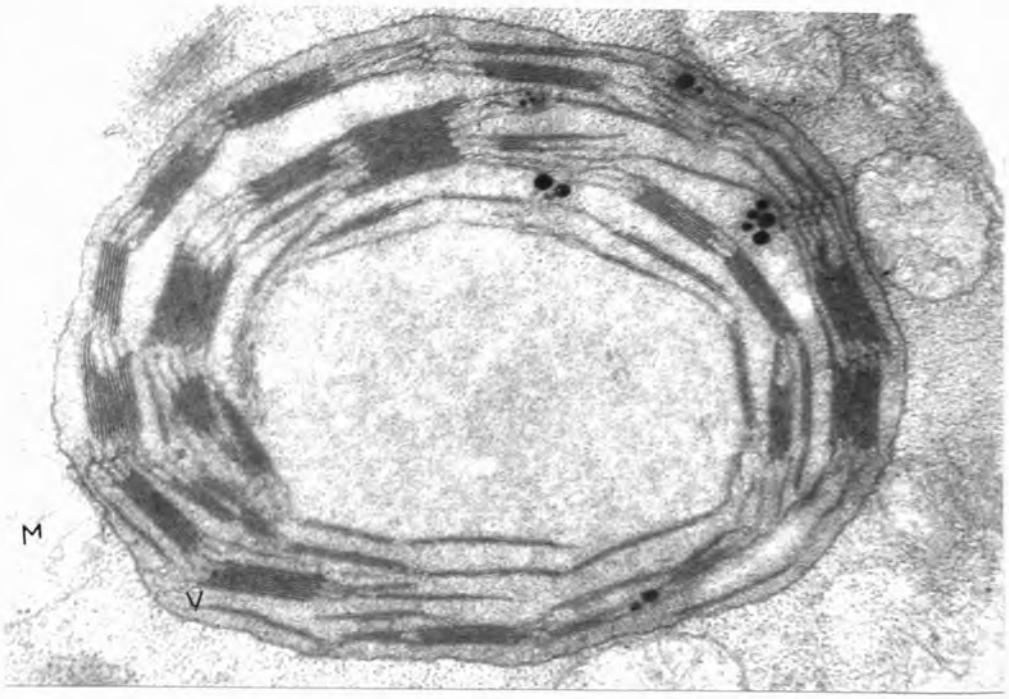


PLATE 61 *Chloroplast of youngest leaf on 4-week old plant after 80 hours in dark at 22°C.*

Figs. 105. & 106. *Plastids show well developed thylakoidal systems and accumulation of few vesicles at certain places which appear to be prolamellar bodies. Osmiophilic globules are large and numerous. (These plastids are probably of similar shape but sectioned in planes at right angles to one another).*
X 36,450 each.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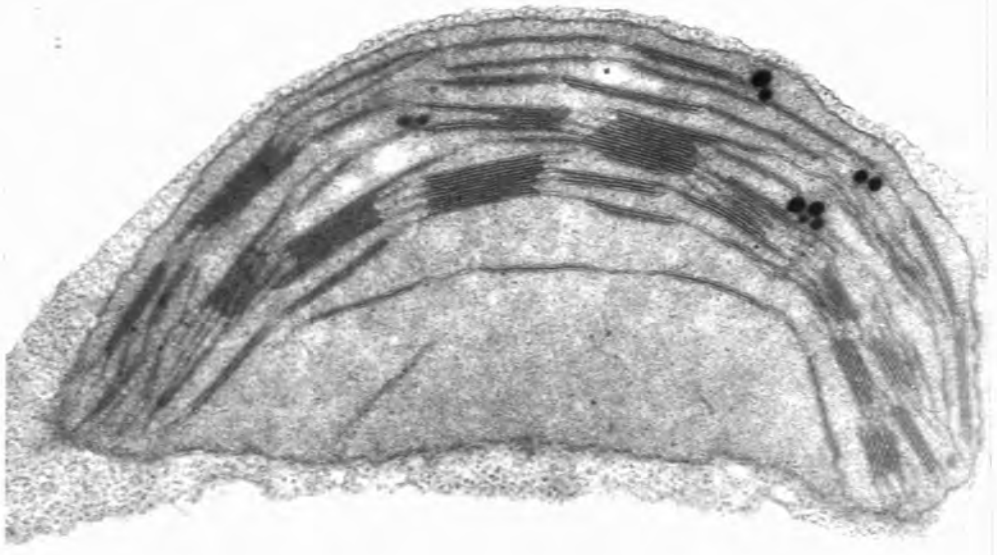
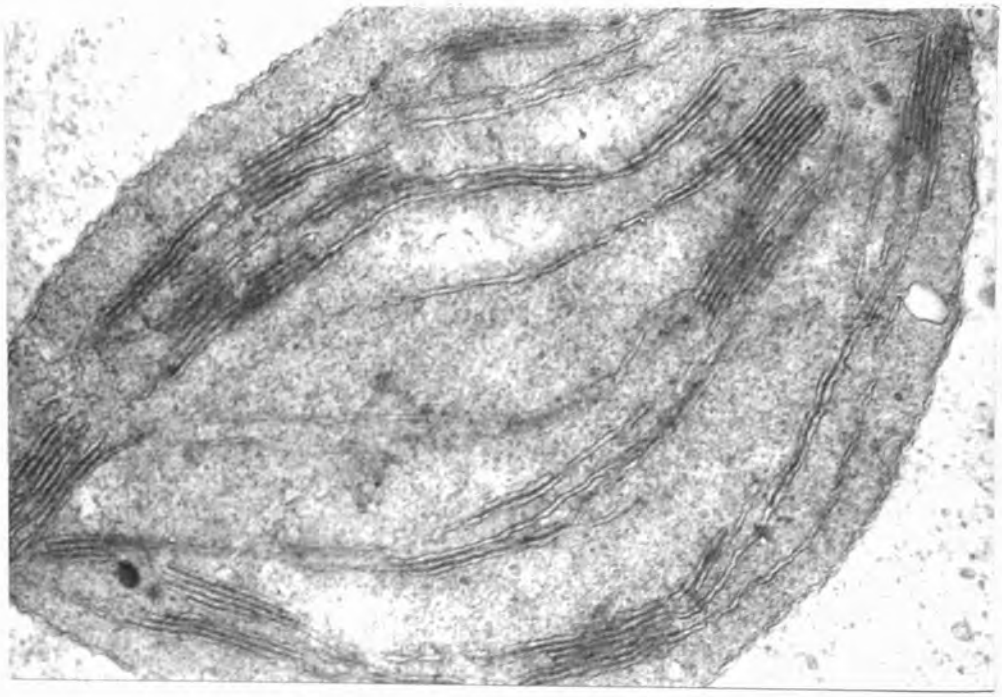


PLATE 62 — *Plastids of the youngest leaf on plant grown at 22°C for 4 weeks. After 80 hours dark treatment at 30°C.*

Fig. 107. — *A swollen plastid with reduced lamellar system. Many small vesicles have appeared in the plastid.*
X 60,750.

Fig. 108. — *A partially differentiated plastid showing the presence of a pro-lamellar body having paracrystalline structure. At certain places a few short tubules are attached to the prolamellar body and are cut off from the remaining plastid lamellae.*
X 97,200.



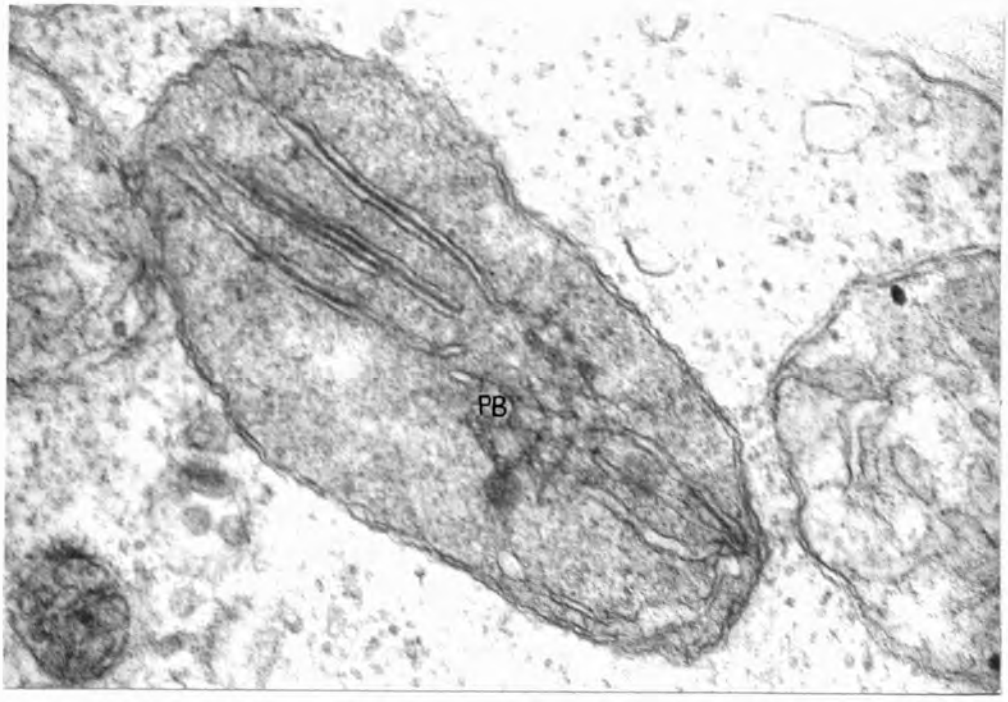
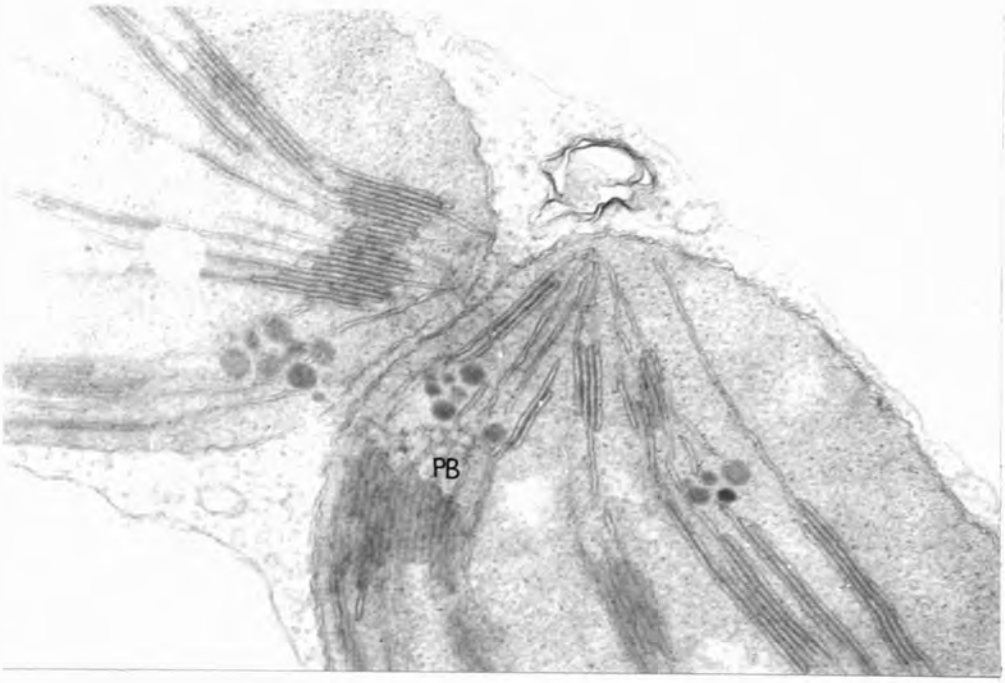

PB

PLATE 63 *Etioplasts of the youngest leaf on 4-week old plant after 128 hours in dark at 22°C.*

Figs. 109. & 110. The etioplasts show a reduction in the number of thylakoids per granum. Prolamellar bodies appear to be of crystalline form.

Fig. 109. - X 60,750.

Fig. 110. - X 97,200.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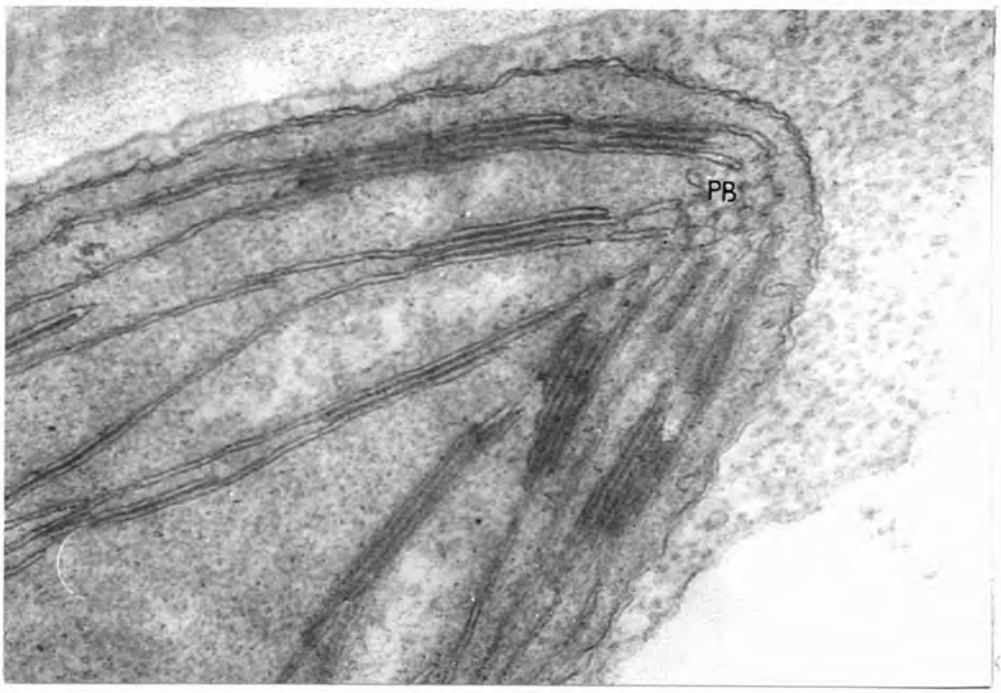
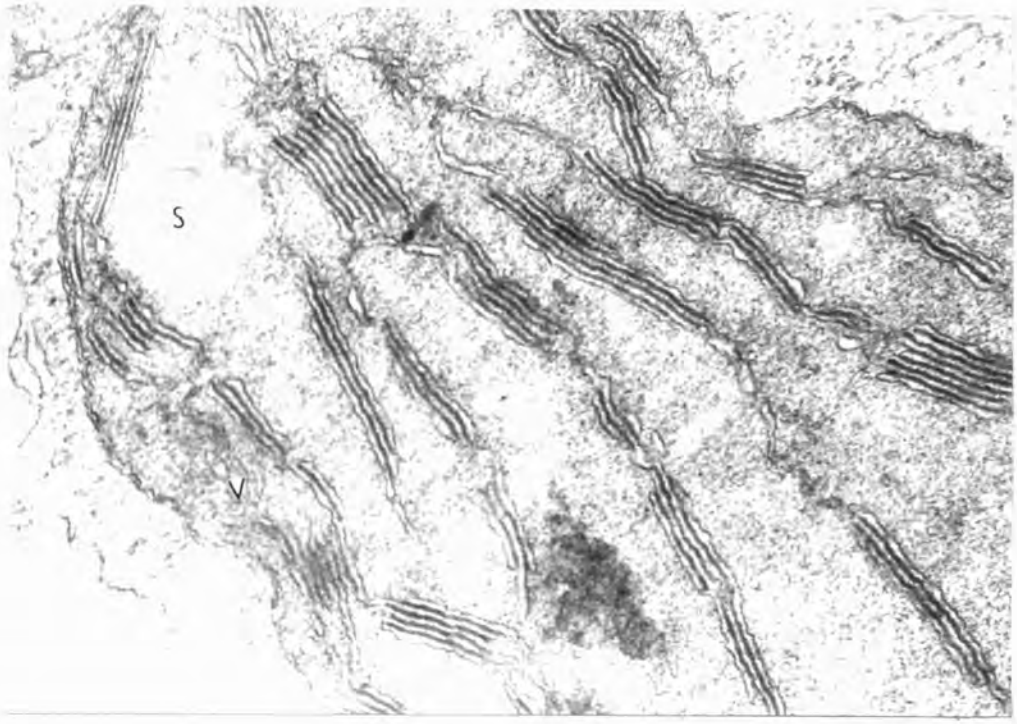

PB

PLATE 64 *Plastids of youngest leaf on 4-week old plant grown at 22°C. After 128 hours of dark treatment at 30°C.*

Figs. 111. & 112. *Plastids are of reduced size. The number of thylakoids per granum is reduced and lamellae show characteristic splitting. Some lamellae are broken and their free ends show little swellings. Many vesicles appear in the plastids. X 60,750 each.*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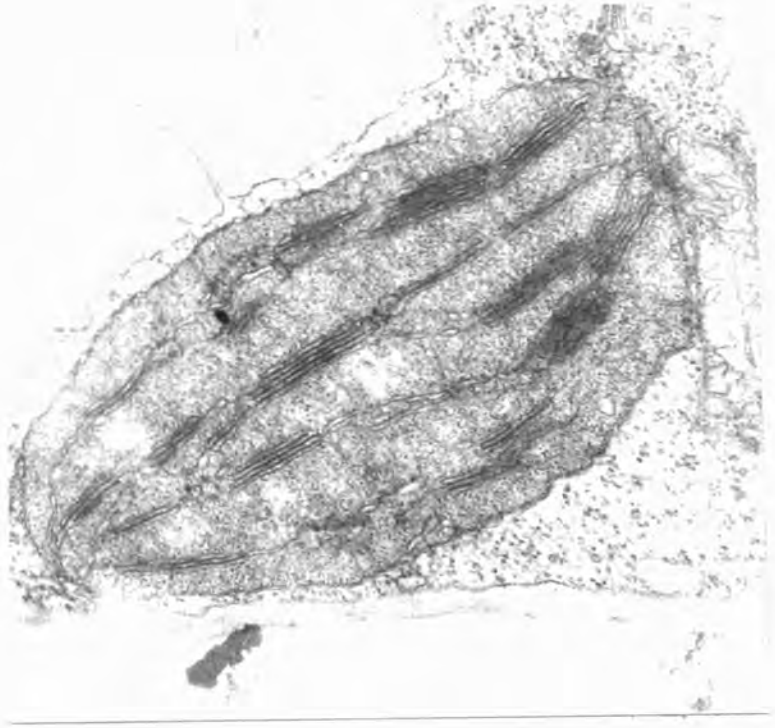

SUMMARY

1. In the primary leaf of the spinach seedling the chloroplasts appear to be formed from amyloplasts. The present investigation reveals that all the developing plastids exhibit similar ultrastructure up to the fourth day of germination under the light/dark conditions at 22°C and 30°C and in complete darkness at 22°C.

A possible significant association between plastids and endoplasmic reticulum is observed at the fourth day stage only under light/dark conditions at 22°C.

2. Lamellae in the form of small segments begin to appear in the developing plastids at the four day amyloplast stage under the three conditions studied, and increase in number and length as the plastid development proceeds. Under light/dark conditions the lamellae form incipient grana which show a much greater degree of thylakoidal swelling in 30°C temperature conditions than at 22°C. The grana at this stage do not exceed more than three thylakoids per granum. In darkness no incipient grana formation is observed up to the six day stage of plastid development.

3. Peculiar 'amoeboid' plastids are observed at the six day stage at 22°C under light/dark conditions. These plastids are elongated with irregular outlines and internally possess many small randomly distributed lamellae; they do not contain any starch grains.

4. Prolamellar bodies appear only at the five day stage in seedlings

grown at 22°C in light/dark conditions whereas in complete darkness they are present from the five day stage onwards and the structure of the prolamellar bodies appears to be sometimes non-crystalline and at other times crystalline. Etioplasts produced in the dark at the seven day stage possess typical paracrystalline prolamellar bodies. At 30°C under light/dark conditions prolamellar bodies were not seen at any stage.

5. Plastid division was observed in the cells of the primary leaves from the very early stages of the plastid development at 22°C temperature condition in complete darkness as well as under light/dark condition. Plastids developing at 30°C did not show division at any stage of development.

6. The most characteristic features of the plastids at the seven day stage of development under 30°C temperature condition are that they become swollen and the plastid envelope appears corrugated; some of the plastids burst.

7. Vesicles are produced in the developing plastids under all the conditions studied, but the vesicles produced in complete darkness and under light/dark conditions at 30°C are much bigger than those produced at 22°C under light/dark condition.

8. The chloroplast exhibits normal ultrastructure up to 28 days of leaf age at 22°C temperature conditions; up to 21 days at 25°C; up to 15 days at 30°C and only up to 7 days at 35°C. The granal stackings which show a gradual increase during the leaf growth are

maximum at these respective ages of leaves. Subsequently the chloroplasts start showing irregularities in both stroma and grana lamellae (splitting of lamellae and forming loops, hooks and bridges by breaking and curving at various places). The shape of the plastid changes to sub-spherical or spherical as it becomes swollen.

9. A characteristic club-shaped swelling is observed at the terminus of each thylakoid during the intermediate stages of senescence and also during chloroplast disruption by the effect of high temperature. Such characteristic swellings are assumed to be a stage of weakening of the thylakoids during senescence. An accumulation of small densely stained globules is observed on the outer surface of the plastid envelope during the intermediate stages of senescence.

10. In completely senesced leaf tissues the chloroplasts become rounded and swollen. Internally, the stroma and grana lamellae completely lose their original structure and lie in the form of patches which appear to have formed from the broken lamellae. No vesicle formation is observed in any of the senescent plastids. Osmiophilic globules which show a continuous increase in both number and size as the leaf matures, are much bigger in completely senesced leaf tissue.

11. Chlorophyll content and fresh weight of leaves growing at $22^{o}C$ and $25^{o}C$ temperature conditions show a sudden decline during the early stages of senescence and remain constant at the later stages. At $35^{o}C$, an apparent initial increase in chlorophyll content on a fresh weight basis is followed by a continuous decrease throughout.

12. It is inferred from the present investigation that the high temperature effects of plastid disruption are very similar to those of natural senescence of the chloroplast. High temperature enhances the natural senescence of the chloroplasts and this enhancement increases with the increase of temperature.

13. The plastids of the leaves which emerge in succession from the apical buds of the spinach plant do not show the same ultrastructure at all stages of the plant growth. Under normal growing temperature of the plant ($22^{o}C$) the plastids of the successively formed leaves show normal ultrastructure only up to 50 days of plant age after which the leaves which emerge show a general reduction of the plastid size, increase in number and size of grana and reduction in length of the interconnecting stroma lamellae. Such plastids also assume various shapes and most common are 'sickle-shaped', 'S'-shaped and biconvex. Some of the plastids differentiated at the later stages of the plant growth also include membrane-bound crystals. Starch grains were observed in these plastids even up to 58 days of the plant age.

14. Under higher growing temperature conditions of the plant the structure of the plastids which are differentiated in the newly emerged leaves depends upon two conditions: (i) the temperature to which the plant is subjected and, (ii) the time which the plant has spent at a particular temperature before the apical bud gives rise to leaf.

15. The present investigation shows that the higher the temperature

to which the spinach plant is subjected, the sooner the plastids show changes in the structure and ultrastructure in the successively differentiated leaves. The electron micrographs of this study reveal that at 25°C temperature condition the plastids show normal ultrastructure in the newly emerged leaves even up to 58 days of plant growth. However, at 30°C and 35°C the plastids of newly differentiated leaves start showing structural changes after just seven days. The structural changes are more abrupt at 35°C.

16. The general structural changes observed in the plastids of the plants under 30°C and 35°C temperature conditions up to seven days are as follows: changes in the shape of the plastids, reduction in plastid size, increase in size of grana, reduction in length of the interconnecting stroma lamellae, disappearance of starch and appearance of few vesicles and increase in number and size of the osmiophilic globules.

17. In leaves which are differentiated after the growing plant has spent 15 days at either 30°C or 35°C there is a further reduction of the plastid size and also swelling. The free ends of the thylakoids show characteristic club-shaped swellings and the interconnecting lamellae are broken at many places. Osmiophilic globules show a much greater increase in size. Starch grains are completely absent in such plastids.

18. The leaves differentiated after 21 days at 30°C possess plastids which show many structural abnormalities. The lamellae become swollen and are split at many places. Osmiophilic globules increase both in

number and size. The plastids do not show any particular arrangement of grana and stroma lamellae and the grana are also not very distinct. At 35°C the leaves show the presence of greatly swollen plastids more or less round in shape. Grana are very few with swollen thylakoids and do not follow any particular arrangement. Most of the stroma lamellae show complete disruption.

19. The plastids in leaves differentiated after the plant has spent 28 days at 30°C show almost the same structural changes as those in leaves differentiated after 21 days at 35°C. In addition the former also show rupture of the envelope membranes and liberation of the contents. At 35°C after 28 days the plastid envelope disappears completely even before the lamellae have disintegrated. At 25°C plastid division is still observed in the leaves which are differentiated after 28 days.

20. Prolamellar bodies appear in etioplasts of young leaves of four weeks old plant after 128 hours in the dark at 22°C but do not form at 30°C. The plastids at 30°C which show an initial increase in the size of grana up to nine hours of dark treatment show a sudden decrease which is followed by the formation of a large number of vesicles and breaking up of the lamellae connecting the grana. At 22°C the plastids show a continuous reduction in the number of thylakoids per granum and an increase in number and size of the osmiophilic globules. Starch grains remain up to 30 hours in darkness at both normal growing temperature and at the higher temperature.

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