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The Quantitative Ultrastructure of the Pea Shoot Apex in Relation to Leaf Initiation

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With 8 Figures

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Summary

The ultrastructure of the pea shoot apical meristem was examined quantitatively in longitudinal sections. Photographs were taken at eleven defined positions in the apex, at six developmental stages within a single plastochron. The only change in ultrastructure during the period of a single plastochron was the increase in the proportion of plastids with starch in the central regions of the apex and in the young leaf axils. This increase occurred midway in time between the emergence of successive leaves, at precisely the time that the orientation of growth changes in the region where a new leaf is to emerge. There were quantitative changes in ultrastructure associated with cell differentiation. In the sequence of cell development from the summit of the apex (central zone) to the incipient pith, cell enlargement was accompanied by an increase in the volume of endoplasmic reticulum, dictyosomes, microbodies and vacuoles per cell, an increase in the number of mitochondria, microbodies and vacuoles per cell, and an increase in the volume, but not the number, of plastids per cell. In the sequence of axillary development (before the axillary bud begins to grow) the number of mitochondria per cell decreased as cell volume decreased but the number of plastids per cell remained constant. The number of plastids per cell increased only in the developmental sequence leading to leaf development, in which the number of mitochondria and dictyosomes per cell also increased. There appeared to be no features of ultrastructure, qualitative or quantitative, which could be correlated with the different rates of cell division in different regions of the meristem. The differences in ultrastructure throughout the apex were mainly quantitative and seemed to be associated with cellular differentiation rather than with the plastochronic functioning of the apex during leaf initiation.

1. Introduction

The immediate event in the pea shoot apex which produces the change in shape of the apex and which results in the formation of a new leaf primordium is apparently a change in the direction, or orientation, of growth

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rather than a change in the rate of growth (LYNDON 1970 a, b, 1972). Apart from a reorientation of one third of the mitotic spindles (LYNDON 1972) there are no obvious differences in structure, as seen by light microscopy, which distinguish the cells at the point of leaf initiation from the cells on the opposite flank of the apical dome where a leaf is not initiated until one plastochron later. The different rates of cell division within the apex (LYNDON 1970 a) are also not related to obvious differences in structure visible with the light microscope. Differences in the amounts of DNA, RNA, and protein in the cells of the pea shoot apex have been looked for but, irrespective of their position and stage of development within the apex, or their rate of division, all the cells examined had a similar gross chemical composition as measured by quantitative histochemistry (LYNDON 1970 c). If there are differences between the cells in different parts of the apex which are at different stages in the sequence towards leaf development or between cells with different rates of growth and division then these are presumably at the ultrastructural or molecular levels.

In order to get an accurate measure of any differences there may be in the ultrastructural composition of cells in different parts of the shoot apex it is necessary that the survey be quantitative and that samples be taken in a defined and objective manner. This can be done for the pea shoot apex by an extension of the technique which has been used successfully for light microscopy (LYNDON 1968). The development of quantitative stereological methods (WEIBEL 1969) allows the calculation of the relative volumes occupied by different organelles seen on electron micrographs. When the shapes of the organelles are known and the number of profiles is also counted then the numbers of organelles can be calculated. Quantitative measurements can therefore be made not only of the relative volumes of organelles within the tissues but also of the numbers of organelles per unit volume and the numbers of organelles per cell. These methods were used in the present investigation to obtain detailed measurements of the volumes and numbers of organelles in different parts of the pea shoot apex in relation to the development of the cells, and to measure any changes in ultrastructure which might occur during the course of a single plastochron, and which would be related to the timing of leaf initiation.

2. Materials and Methods

Peas (*Pisum sativum*, cultivar Lincoln) were sown in wet sand and were grown in a controlled environment room at 23 °C in a 12 hours light/12 hours dark cycle with a light intensity of 30 $W.m^{-2}$. When the peas were 8, 9, or 10 days old the shoot tips were excised and fixed in freshly-made 2.0 percent potassium permanganate for 2 hours. They were washed in water, then dehydrated through an ethanol series and were embedded in araldite. Sections were cut on an LKB ultratome and those of a thickness of 60 to 70 nm (light gold) were taken and mounted. Fixation in glutaraldehyde was attempted but the image was poor. Fixation in permanganate, although perhaps less desirable, did result in a clearer

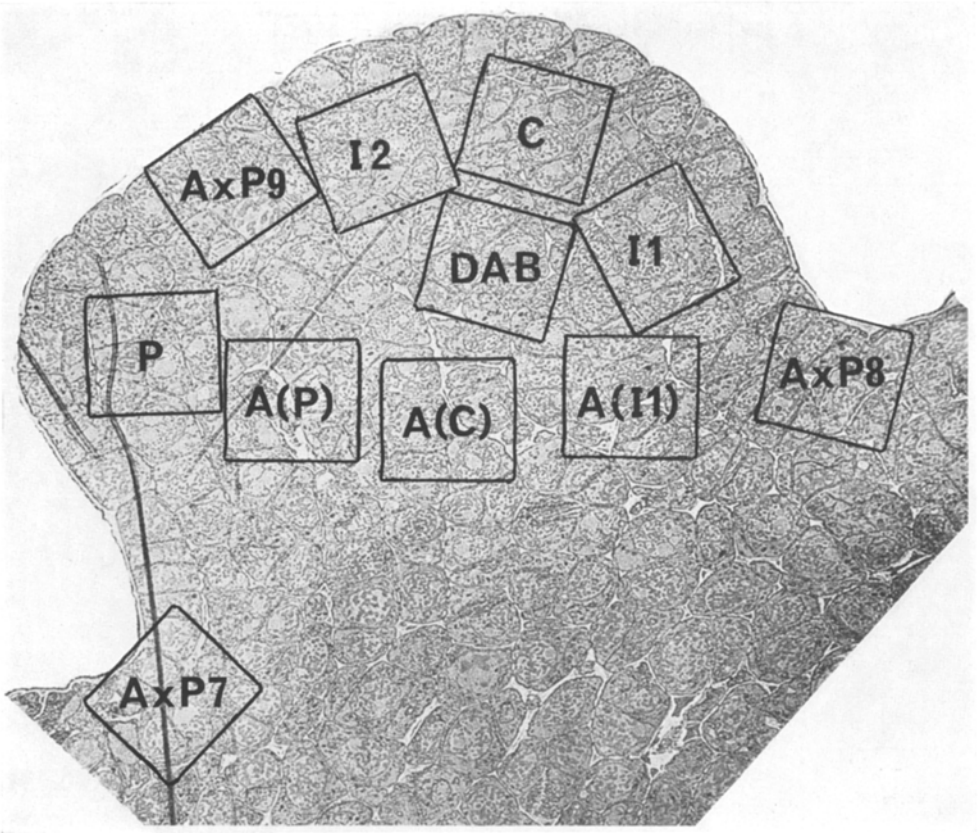

Fig. 1. Low power electron micrograph of a longitudinal section of a pea shoot apex showing the positions at which photographs were taken. C = central zone, cells at the summit of the apex. P = youngest leaf primordium, the 9th to be formed above the two reduced epicotylary leaves (LYNDON 1968). I 1, I 2 = cells which will give rise to the 10th and 11th leaf primordia respectively. DAB = cells at the base of the apical dome, on the dome/axis boundary as defined by a line joining the axils of the 8th and 9th leaf primordia. A (P), A (C), A (I 1) = regions of the stem axis near the 9th primordium, the centre of the pith, and the I 1 region respectively. AxP 9, AxP 8, AxP 7 = axils of the 9th, 8th, and 7th oldest leaves respectively. These were the only positions in which epidermal cells were included in the areas photographed. The lines at the left of the section are folds or wrinkles in the section

delineation of the organelles even though ribosomes and microtubules were lost. For the present purposes permanganate proved quite satisfactory and allowed easy identification of the organelles present in the cells.

Longitudinal sections were cut in the plane which passes through the insertions of all the leaves and also cuts medianly the positions of all future leaf primordia. Three sections were cut from each apex; the first was 10 μm from the midline, the second was median, and the third was 10 μm further into the apex. Since the cells are approximately 10 μm in diameter each of these three sections would tend to cut through different cells.

The sections were mounted on formvar/carbon films. Their ultrastructure was not examined before photography. Eleven photographs were taken of each section at the predetermined positions shown in Fig. 1. All photographs were taken on an AEI EM6 microscope at the same instrument magnification of 4,000× on the plate. A twelfth photograph was taken. This was a low power view of the whole section obtained with the microscope used in the diffraction mode. Although this produced a very low contrast image it was possible to discern the outlines of the cells and subsequently to locate the positions of the other eleven pictures on the section of the whole apex (Fig. 1). This served as a check that the eleven photographs had been taken at the correct positions.

The eleven positions were chosen so that several aspects of cell development within the apex could be followed (Table 1). The sequence central zone/DAB/A (C) represents the

Table 1. *Sequences of Cell Development Represented by Positions in the Shoot Apex*

Developmental sequence	Positions shown in Fig. 1
Cell enlargement in developing pith cells	C/DAB/A (C)
Leaf formation: successive positions of cells displaced down the apical dome	C/I 2/I 1/P
Axillary development	AxP 9/AxP 8/AxP 7
Rates of cell division: cells with slow, intermediate and fast rates of division	C/P/DAB

sequence of cell enlargement and maturation followed by the cells which become the pith. The sequence central zone/I 2/I 1/primordium represents the successive developmental stages of a cell which becomes incorporated into a young leaf primordium. The sequence AxP 9/AxP 8/AxP 7 represents the sequence of development of cells which are committed to the axillary position of the young primordium. A comparison of cells in the central zone, primordium and DAB is a comparison of cells with slow, intermediate and rapid rates of cell division respectively (LYNDON 1970 a). The lengths of the cell cycle have been shown to be approximately 69 hours in the central zone, 29 hours in the primordium, and 20 hours in the DAB region (LYNDON 1970 a, 1973).

Quantitative analysis of the ultrastructure of cells in these eleven positions in the apex at a given point in time gives information about any changes there might be in the cells as they progress along these developmental sequences, assuming the apex to be growing in a steady state. By repeating this analysis at six developmental stages during a single plastochron, defined as described elsewhere (LYNDON 1968), it becomes possible to analyse the changes in ultrastructure at each of the positions in the apex as a function of time, and particularly in relation to the timing of the events leading to the initiation of a new leaf primordium. It is already known that the change in direction of growth occurs quite abruptly about 16 hours before the new primordium appears as a bump (LYNDON 1970 b).

The electron micrographs were all enlarged and printed at a standard magnification giving a linear magnification of 9,350× and representing an area of 400 μm^2. A grid of 100 points arranged as described by WEIBEL *et al.* (1966) was placed over each photograph and the type of organelle occurring under each point was noted (Fig. 2). The organelles were classified as one of ten types. These were: 1. nucleus, 2. cytoplasm (in which no ultra-

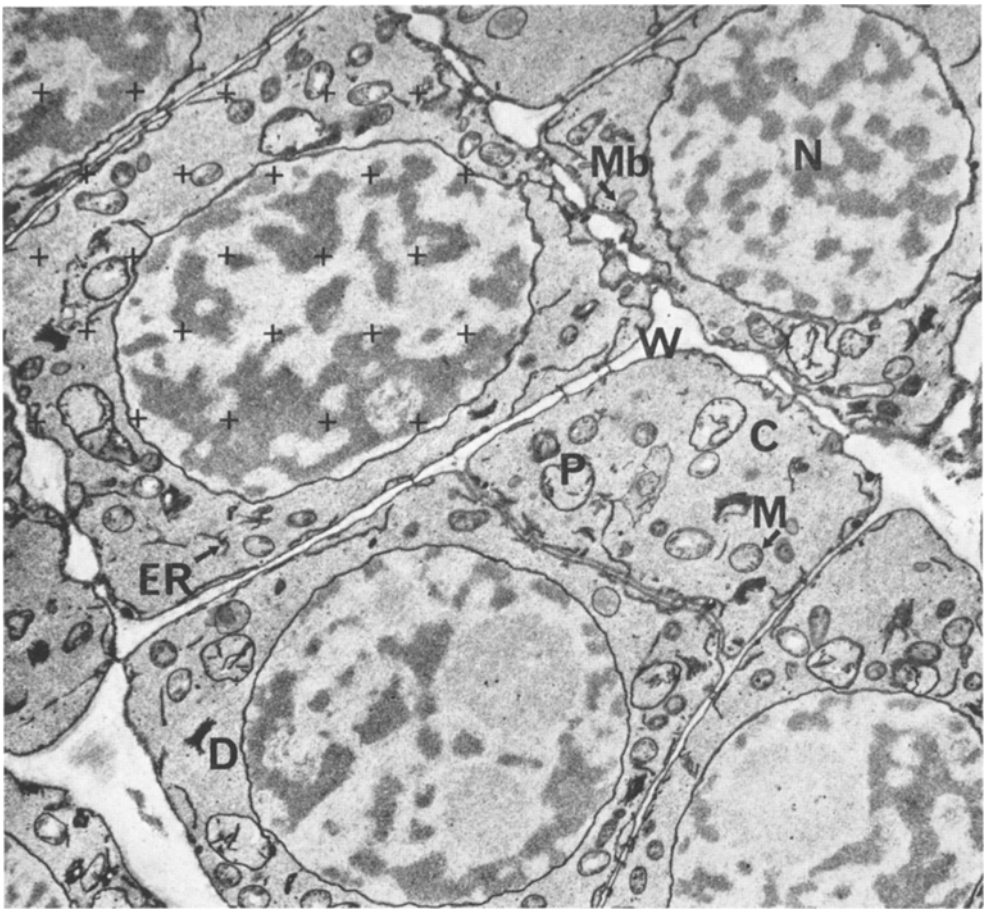

Fig. 2. Electron micrograph of I 1 region showing its ultrastructure and the positions of 25 of the 100 points which were placed in a grid over the photograph for the measurement of the relative volumes occupied by the various organelles. N = nucleus; M = mitochondrion; P = plastid; Mb = microbody; D = dictyosome; ER = endoplasmic reticulum; W = wall; C = clear cytoplasm

structure could be seen), 3. cell wall, 4. plastids, 5. mitochondria, 6. dictyosomes, 7. endoplasmic reticulum, 8. microbodies, 9. vacuoles, and 10. unidentified particles. It was always possible to distinguish plastids from mitochondria, not only by the presence or absence of clearly defined christae but also by the darker staining of the outer membrane of the plastids. The plasma membrane was included with the endoplasmic reticulum. Plasmodesmata were included with the wall. The nuclear envelope was included with the nucleus but extensions of the nuclear envelope into the cytoplasm were classified as endoplasmic reticulum. Since there were three sections for each apex and two apices for each developmental stage there were six replicates of each photograph and these were grouped together and a mean value was obtained. The values for the relative volumes of the organelles were calculated as described by Weibel *et al.* (1966).

The numbers of profiles of cells, plastids, mitochondria, microbodies, vacuoles, dictyosomes and plastid profiles with starch granules were counted on each photograph. The absolute numbers of organelles were derived from the formula:

$$N = \frac{n^{3/2}}{\beta \cdot \sqrt{\varrho}}$$

as described by Weibel and Gomez (1962), where N = number of organelles per unit volume, n = number of transections per unit area of cut surface, β = coefficient of configuration, and ϱ = fraction of volume occupied by that organelle. β was obtained by making some assumptions about the shape of each organelle, based upon the shapes of the profiles which were seen in the photographs. The cells were assumed to be isodiametric ($\beta = 1.375$). The mitochondria, plastids and microbodies were assumed to be ellipses with diameter = 0.66 × length ($\beta = 1.6$). The dictyosomes were assumed to be cylinders with diameter = 1.3 × length ($\beta = 1.5$). The vacuoles were assigned the value of $\beta = 1.5$. The numbers of cells per unit volume were calculated by taking $\varrho = 1$.

These calculations depend upon the arrangement of the organelles within the sections being random, and they also depend upon the sections themselves being random with respect to the tissue. Since there were approximately seven or so cell profiles in each photograph and each section represented different cells it was assumed that the micrographs represented essentially random cuts through the cells. It was also assumed that the arrangement of the organelles was random with respect to the plane of the section even though this was the same with respect to the positions at which leaves were initiated.

The sizes of individual organelles were obtained by dividing the volume of organelles per unit volume of tissue by the number of organelles per unit volume of tissue.

3. Results

3.1. Cellular Structure and Stage of the Plastochron

There appear to be no consistent changes in the relative volumes of the subcellular components as a function of the stage of the plastochron (Table 2). When the volume of organelles per cell, the number of organelles per cell, and the sizes of organelles were calculated, there were no clear differences between one stage of the plastochron and another in any region of the apex. In each case the data were therefore collected together and expressed as means for the whole plastochron in Figs. 5–7. There was only one clear change in ultrastructure related to plastochron stage and this was an increase in the percentage of plastids with starch. This was greatest in the regions AxP 9, A (P), and DAB (Fig. 3) and occurred between plastochron stages 9.4 and 9.65 (Fig. 4). This is the point in the plastochron when the axil of the youngest leaf (*i.e.*, AxP 9) becomes a distinct morphological entity, when periclinal divisions begin in the I 1 region of the apex at the onset of leaf initiation (Lyndon 1970 b, 1972), and when the apical dome begins to enlarge (Lyndon 1968). This sudden increase in the number of plastids containing starch is not likely to be associated with the onset of photosynthesis in these plastids, since the membrane systems are not highly developed (Fig. 8). The increase in starch is probably due to an increase in the rate of synthesis from existing

Table 2. *Percentage of the Total Tissue Volume Occupied by Each Organelle at Each Stage of the Plastochron*

	Plastochron stage						Least significant difference between means ($p = 0.05$)
	9.0	9.2	9.4	9.65	9.85	10.0	
Nucleus	36.91	36.74	36.82	34.92	35.83	35.70	3.47
Cytoplasm	34.87	32.96	35.74	35.24	32.61	33.00	2.50
Wall	8.91	10.20	8.59	11.05	10.84	11.44	1.69
Plastids	5.09	5.98	4.84	5.28	6.65	6.04	1.01
Mitochondria	5.32	4.55	4.48	5.05	5.03	4.75	0.73
Endoplasmic reticulum	6.87	7.85	6.84	5.98	6.39	6.58	0.89
Dictyosomes	1.21	1.09	1.73	1.25	1.58	1.19	0.44
Microbodies	0.15	0.08	0.19	0.16	0.26	0.15	—
Vacuoles	0.36	0.45	0.60	1.03	0.70	0.81	—
Unidentified	0.30	0.15	0.15	0.06	0.14	0.17	—

Values are means for the apex as a whole. Least significant differences were not available for microbodies and vacuoles because of zero values in the data. The developmental stages within a single plastochron from the first appearance of the 9th leaf primordium (stage 9.0) to the first appearance of the 10th primordium (stage 10.0) were defined as described by LYNDON (1968). At plastochron stage 9.0 the primordium begins to be formed as a bump on the apical dome. Each 0.1 increment in plastochron stage represents an increase of 6 μm in the height of the 9th primordium until the 10th primordium begins to be formed at plastochron stage 10.0.

precursors in the apex. The increase in starch accumulation is most marked in those cells in the central part of the apex [DAB, A (C), A (P), and A (I 1)] which are enlarging, and in the axil of leaf 9 (AxP 9), which are destined to take no major part in the growth and morphogenesis of the apical dome or the developing primordium. This is analogous to the starch accumulation which followed periods of rapid cell division in *Pelargonium* callus (WILBUR and RIOPEL 1971).

3.2. Developmental Sequences Within the Apex

Although there are no other marked changes which can be associated with the periodic functioning of the apex in the initiation of leaves, there are changes in the cells as they progress along various developmental pathways in the apex.

3.2.1. Cell Enlargement: Incipient Pith

Along the developmental sequence leading to cell maturation and enlargement [C/DAB/A (C)] (Table 1) the relative volume per cell of all organelles

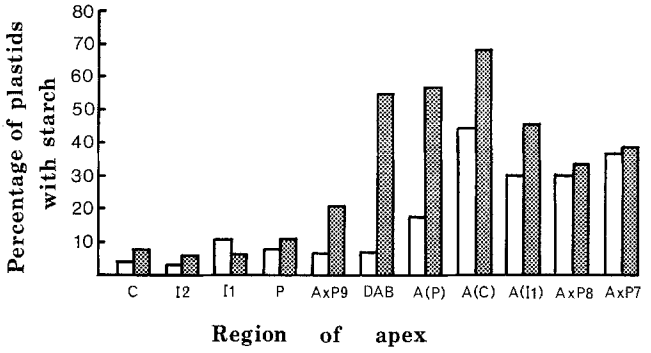

Fig. 3. Percentage of plastids with starch in the different regions of the apex a) during the first part of the plastochron (□), from plastochron stages 9.0 to 9.4 (LYNDON 1968) or 0 to 30 hours after the first appearance of the 9th leaf primordium; and b) during the second part of the plastochron (▒), from plastochron stages 9.5 to 10.0 (LYNDON 1968) or 30 to 46 hours after the first appearance of the 9th leaf primordium

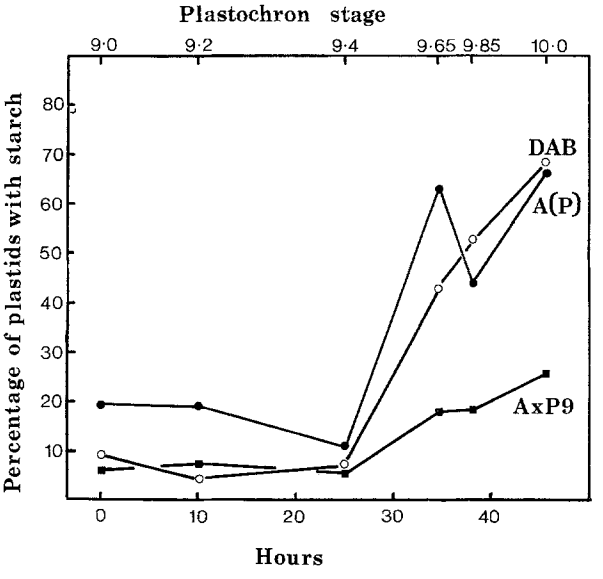

Fig. 4. Percentage of plastids with starch in the DAB, A (P) and AxP 9 regions of the apex

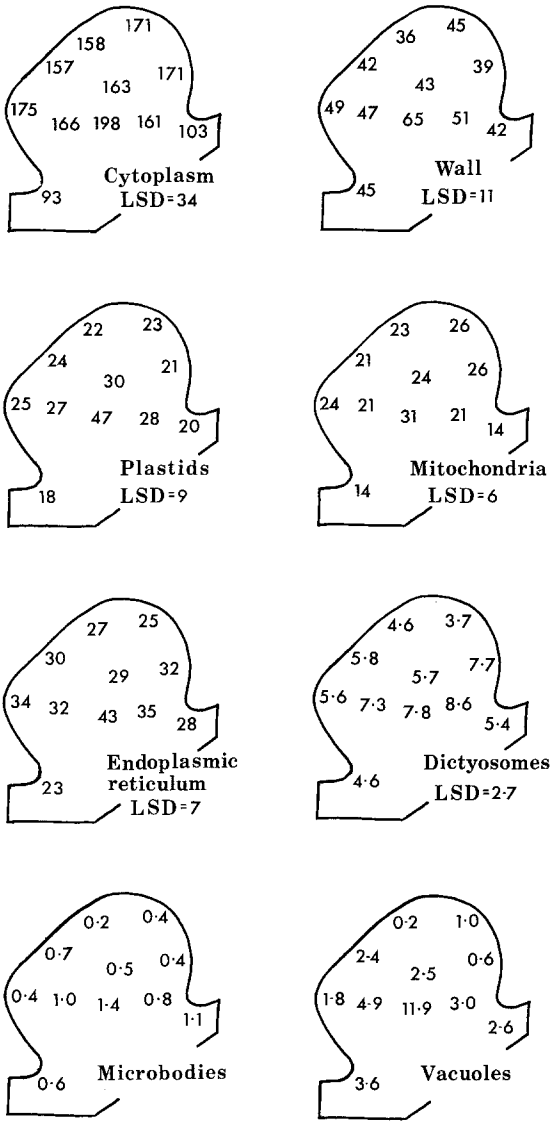

Fig. 5. Total volume (μm^3) per cell occupied by different classes of organelles. *LSD* = Least significant difference ($p = 0.05$). This is not available for microbodies and vacuoles because of zero values in the original data. Since there is only one nucleus per cell the total volume per cell occupied by the nucleus is the same as the volume per nucleus, which is shown in Fig. 7

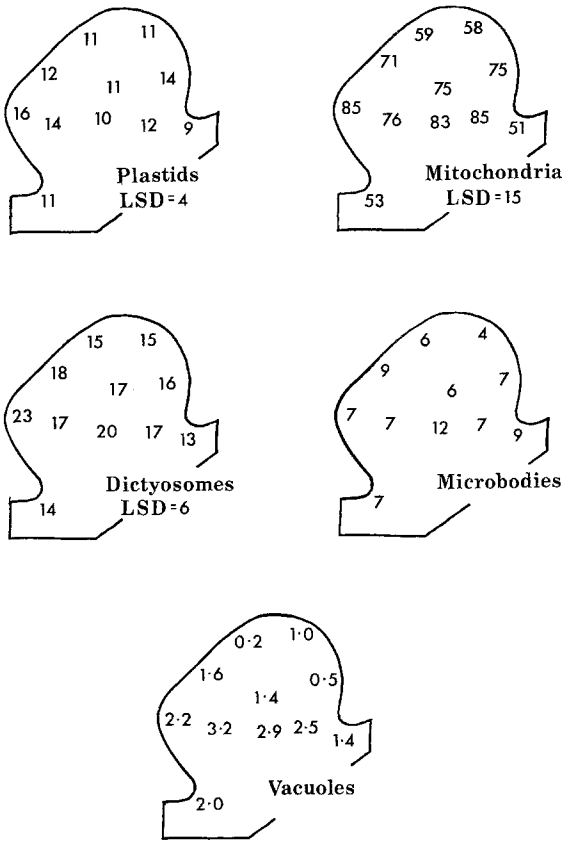

Fig. 6. Numbers of organelles per cell. *LSD* = Least significant difference (p = 0.05). This is not available for microbodies and vacuoles because of zero values in the original data

increases (Fig. 5), with the exception of the nucleus (Fig. 7). This is consistent with the continuing synthesis of cytoplasmic components as the cells enlarge but the cessation of nuclear growth as the cells pass out of division. The onset of vacuolation is shown by the increase in vacuolar volume (Fig. 5) and in vacuolar numbers (Fig. 6) and size (Fig. 7). There is also an increase in the numbers of mitochondria, microbodies, and possibly dictyosomes, but no increase in the number of plastids (Fig. 6), although the plastids increase in size (Fig. 7). With the exception of the plastids the increase in numbers of organelles implies that development along this sequence entails replication of organelles at a faster rate than replication of cells, whereas plastid division merely keeps pace with cell division.

3.2.2. Leaf Initiation

In the developmental sequence leading to leaf initiation (C/I 2/I 1/P) there is an increase in the volume of endoplasmic reticulum and vacuoles per cell

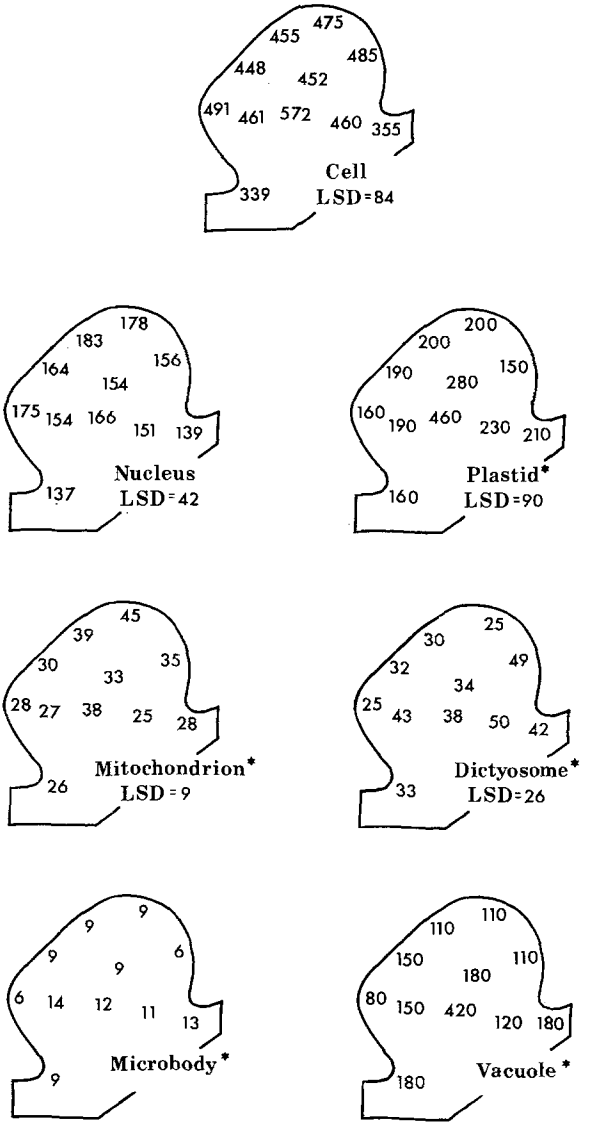

Fig. 7. Sizes of cells and of individual organelles. Volume per cell and nucleus in μm^3. * Volume per mitochondrion, dictyosome, plastid, microbody and vacuole in $\frac{\mu m^3}{100} \cdot LSD =$ Least significant difference ($p = 0.05$). This is not available for microbodies and vacuoles because of zero values in the original data

(Fig. 5), although the cells are not enlarging (Fig. 7). There is also an increase in the number of all organelles including plastids (Fig. 6). The increase in the numbers of mitochondria per cell is accompanied by a decrease in their size (Fig. 7) but no decrease in the volume of mitochondrial material per cell (Fig. 5). This implies that the replication of mitochondrial material goes on at the same rate as the replication of cellular material as a whole but the rate of division of the mitochondria outstrips the rate of cell division. Plastid

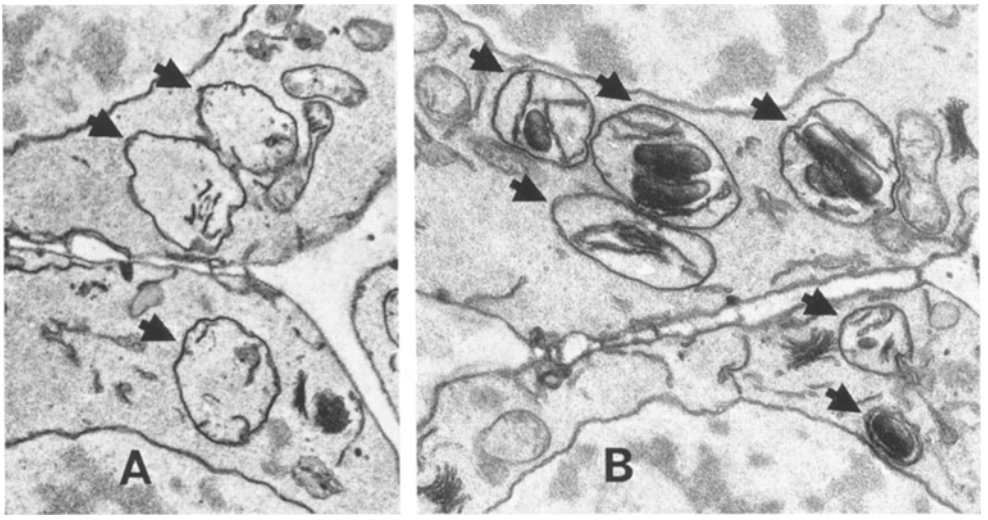

Fig. 8. Plastids in (A) the I 2 region and (B) the A (P) region of the apex at plastochron stage 9.7. Plastids indicated by arrows

number per cell also increases (Fig. 6), but plastid size remains the same (Fig. 7). In this developmental sequence, therefore, the rate of plastid replication and the synthesis of plastid constituents outstrips the rate of cell replication so that there are more plastids per cell in the incipient primordium than there are in the cells of the apical dome. The number of plastids in the cells of the apical dome of the pea is the same (about 12) as in the cells of the shoot apex of the spinach (Cran and Possingham 1972) in which the number of plastids per cell has been shown to increase during leaf development (Possingham and Saurer 1969). The increase in the number of plastids, as well as of all other organelles (Fig. 6), in the developmental sequence leading to leaf initiation in the pea shoot apex makes it different from the other developmental sequences and suggests that a release of the constraints on plastid replication is one of the first events in the differentiation of the leaf primordium.

3.2.3. Axillary Development

Along the sequence AxP 9/AxP 8/AxP 7 (the development of the axillary cells), the cells become smaller (Fig. 7) and more densely packed with organelles, shown by the decrease in the volume per cell of clear cytoplasm (Fig. 5). There are fewer mitochondria per cell (Fig. 6) and less endoplasmic reticulum (Fig. 5) as the axil develops, but the number of vacuoles tends to increase (Fig. 6). This suggests that these cells may be ceasing division and entering a more or less quiescent phase before the axillary bud begins to grow out, which it does about one or two plastochrons later, corresponding to AxP 6 or AxP 5.

Along this developmental sequence the number of mitochondria decreases and the number of plastids remains about the same. This implies that during the decrease in the cell volume (Fig. 7) the replication of plastids keeps pace with the replication of cells whereas the rate of replication of the mitochondria decreases faster than the replication of the axillary cells in which they are found.

3.2.4. Cell Division Rate

The one developmental sequence along which there seems to be no change at all is the sequence representing a threefold increase in the rate of cell division (C/P/DAB). There is no obvious change along this sequence in the volume of organelles per cell (Fig. 5), or in the numbers or sizes of organelles per cell (Figs. 6 and 7).

4. Discussion

The plastochronic functioning of the apex and the initiation of individual leaves is marked by no obvious changes in the ultrastructure of the apical cells, except for the increase in the proportion of plastids with starch when the orientation of growth in the I 1 region changes at plastochron stage 9.5. The accumulation of starch in association with morphogenesis has been noted on several occasions, in callus tissue on the initiation of shoots (Thorpe and Murashige 1968) and in the shoot apex on transition to flowering (Sadik and Ozbun 1967, Yeung and Peterson 1972, Molder and Owens 1973). Starch accumulation is stimulated in the presence of auxin (Sunderland and Wells 1968, Wozny *et al.* 1973) and cytokinin (Usciati *et al.* 1972, Hadacova *et al.* 1973) but it can be prevented, as can organ formation in callus, by gibberellin (Thorpe and Murashige 1968). This suggests the possibility that the sudden increase in the number of plastids with starch at plastochron stage 9.5 may well be indicative of a sudden change in the concentration of a growth substance in the apex at this time.

One might have expected there to be changes in the central zone correlated with its disappearance and reappearance as an obvious region, at the time a new leaf primordium emerges (LYNDON 1968), but no such changes were found.

The main changes in the ultrastructure of the cells seem to be related more to cell differentiation as seen in the development of cells along the sequences leading to development of pith cells [C/DAB/A (C)], leaf cells (C/I 2/I 1/P), and axillary cells (AxP 9, AxP 8, AxP 7). The lack of ultrastructural changes associated with the event of leaf initiation itself suggests that the morphogenetic changes in the apex which result in leaf initiation depend on changes at the molecular level rather than the ultrastructural level. The lack of any obvious correlation between ultrastructure and cell division rate in the regions of the central zone, primordium, and DAB, which encompass a threefold difference in division rate, again suggests that the differences which occur are at the molecular level.

In the developmental sequence from the central zone to the incipient pith [C/DAB/A (C)], cell enlargement is accompanied by an increase in the number of mitochondria per cell and in the development of the leaf axil (AxP 9/AxP 8/AxP 7) the number of mitochondria decreases as cell size decreases. In both cases the number of plastids remains constant (Fig. 5). The replication of plastids must therefore keep pace exactly with the replication of the cells whereas the rate of replication of the mitochondria in the enlarging cells is faster than that of the cells and in the axillary cells is slower. Plastid replication in these instances is tied to cell replication but mitochondrial replication is not. A different situation is found in the developmental sequence leading to leaf initiation (C/I 2/I 1/P). Here the numbers of both mitochondria and plastids per cell increase to almost the same extent (Fig. 5), so that neither mitochondrial nor plastid replication is tied to cell replication. The control of the replication of mitochondria and plastids is therefore not under the same control in different regions of the apex and neither are necessarily under the same control as cell replication.

The growth and enlargement of the cells of the incipient pith, as shown by the developmental sequence C/DAB/A (C), may be compared with the development of the enlarging cells of the root cap in maize (JUNIPER and CLOWES 1965). In both types of cells the amount of endoplasmic reticulum per cell and the numbers of mitochondria and dictyosomes per cell increased as the cells enlarged but the number of plastids per cell remained unchanged, or nearly so, although in both cases the plastids enlarged as the cells enlarged. Except for the lack of vacuolation in the maize root cap cells, the quantitative ultrastructural changes during cell enlargement and maturation appear to be similar in the root cap cells of the maize and the incipient pith cells of the pea shoot apex.

In the central zone of the pea shoot apex there were 11 plastids, 58 mito-

chondria, and 15 dictyosomes per cell (Fig. 6). These are very similar to the numbers of organelles in the *Epilobium* shoot meristem in which there are 11 plastids, 60 mitochondria, and 24 dictyosomes in each telophase daughter cell (ANTON-LAMPRECHT 1967). The maize root cap initial cells were similar in having 15 plastids and 20 dictyosomes per cell but had many more mitochondria, about 220 per cell (JUNIPER and CLOWES 1965).

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