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Changes in ploidy level of epidermal cells during last larval instar of the tobacco hornworm, *Manduca sexta*

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Summary

The relative DNA content of *Manduca sexta* abdominal epidermal nuclei during the final larval instar was measured by cytophotometry of whole-mount preparations of the epidermis. In the middle intrasegmental region, epidermal cells showed a ploidy level of 4C to 32C on the day of ecdysis. During the subsequent period of feeding, the proportion of higher ploidy cells, such as 16C and 32C, increased. This situation remained until the day of apolysis preceding pupal cuticle formation when mitoses reduced the cells to 2C, 4C, 8C and 16C, except for the pupal pock-mark cells, which increased to 32C or 64C. Metaphase cells showed various ploidy levels, correlated with the size of their mitotic figures. By contrast, in the anterior and posterior margin of a segment where no mitoses occurred, the cells continued to increase in ploidy throughout the instar.

Key words: *Manduca sexta*, tobacco hornworm, DNA content, epidermal cells, ploidy level, mitosis.

Introduction

Many insect tissues become polyploid, often when the cells are destined to synthesize large amounts of protein. Recent studies on the cell cycle in the epidermis of the beetle *Tenebrio molitor* have shown that the cells are primarily in the G_2 stage during the feeding/growing phase of the final larval instar, then undergo mitosis before producing pupal cuticle (Besson-Lavoignet & Delachambre, 1981). Similarly, *Drosophila* imaginal discs arrest in the G_2 state before producing pupal cuticle (Graves & Schubiger, 1982; Fain & Stevens, 1982).

Previous studies indicated that the epidermal cells of the tobacco hornworm, *Manduca sexta*, are primarily 2C or 4C during the final feeding stage (Dyer, Thornhill & Riddiford, 1981) when they are producing endocuticle during growth (Wolfgang & Riddiford, 1981). In this case on the final day of feeding, a transient octaploidy in about 30% of the cells was observed (Dyer *et al.* 1981). Mitoses then occurred 2 days later just before the cells begin producing pupal cuticle (Dyer *et al.* 1981; Kato & Riddiford, unpublished data).

The transient octaploidy occurred during the time that these epidermal cells become pupally committed in response to ecdysteroid in the absence of juvenile hormone on day 3 (Riddiford, 1978). DNA synthesis and mitoses have been linked to reprogramming of the genome and cellular differentiation in some (Holtzer, Weintraub, Mayne & Mochan, 1972), but not in all, cells (for a recent review, see Blau, Pavlath, Hardeman, Chiu, Silberstein, Webster, Miller & Webster, 1985) including this epidermis in which prevention of DNA synthesis had no adverse effect on pupal commitment (Dyer *et al.* 1981). Yet it was of interest to determine whether this transient octaploidy was representative of generalized DNA synthesis or was localized to particular cells. Therefore, we began a study of the ploidy increase during the pupal commitment period using whole mounts to localize the octaploid cells. Our initial findings, however, indicated that such cells were present both before and after day 3 as well. Therefore, we have reinvestigated the changing pattern of DNA content in *Manduca* epidermis during the final larval instar using whole mounts of defined segmental regions. This technique ensures the sequential observation of

the same region and shows that cells in different regions of the segment behave differently with respect to an increase in ploidy and to mitoses.

Materials and methods

Animals

M. sexta larvae were reared individually on an artificial diet at 25 °C in a 12L: 12D photoperiod, as described by Truman (1972) and Bell & Joachim (1976). Larvae that ecdysed to the 5th instar at 00.00 AZT (arbitrary Zeitgeber time; lights off) ±2 h on day 0 were selected. Of these larvae only those destined to wander on the night of day 4 (Gate I) were selected by weight.

Cytophotometric measurement of DNA content

Integument of the fifth abdominal segment was removed and then fixed in 10 % buffered formalin (pH 7·0) in 0·25 M-sucrose for 24 h at 4 °C. After fixation, the dorsolateral part of the integument was cut, washed under running tap water for at least 30 min, cleaned of adhering muscle and fat body tissues, and then stored in 70 % ethanol at 4 °C for at least 20 h. For Feulgen staining, the integument was hydrolysed in 5 N-HCl at 25 °C for 60 min, then rinsed in distilled water and stained in Schiff's reagent for 1 h. After staining, the integument was rinsed in 10 % sodium metabisulphite for 30 min and then washed in water. Then each segment was cut into three pieces (1) anterior margin, (2) middle intrasegmental region and (3) posterior margin (Fig. 1). The epidermis of each piece was mechanically removed with a fine needle from the cuticle onto an albumenized slide. After drying, the slides were dehydrated through a graded ethanol series and mounted in Cargille oil (refractive index, 1·544).

Relative absorbance of Feulgen-stained nuclei was measured with a scanning and integrating microdensitometer (Vickers, M85) at 560 nm. Initial studies by K.K.N. used a scanning microscope photometer (SMP) (Carl Zeiss) at 570 nm. The SMP was linked to a PDP-12 computer (Digital Equipment Corp.) which facilitated scanning at predetermined intervals of 0·5 μm.

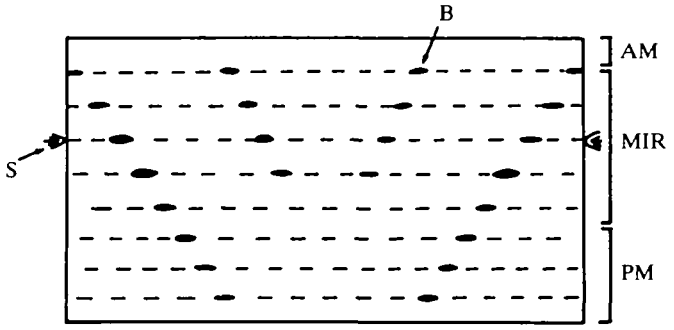

Fig. 1. Dorsolateral part of the cuticle of the fifth abdominal segment. AM, anterior margin; MIR, middle intrasegmental region; PM, posterior margin of a segment; S, site of a spiracle; and B, black marks on the cuticle. Dotted lines show cuticular grooves on the surface.

The 1C or 2C standard was estimated by measurements of spermatids (1C), brain (2C) and subtracheal (2C) cells. As another standard, chicken erythrocytes were used. These cells contain 2·5 pg per nucleus (Rasch, Barr & Rasch, 1971). A smear of erythrocytes was stained simultaneously with *Manduca* cells.

Measurement of epidermal nuclear size

Diameter of nuclei in whole-mounted epidermis was measured with an eyepiece micrometer. For elliptic nuclei,

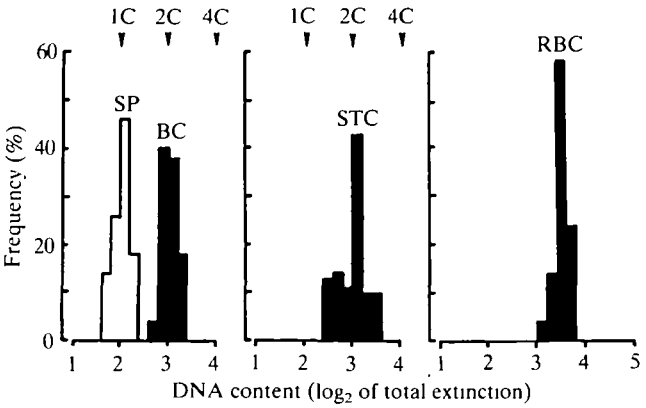

Fig. 2. Frequency distribution in DNA content of various standard cells. SP, spermatid cells (n = 50); BC, brain cells (n = 50); and STC, subtracheal cells (n = 100) of *Manduca sexta*. RBC, red blood cells of chicken (n = 100).

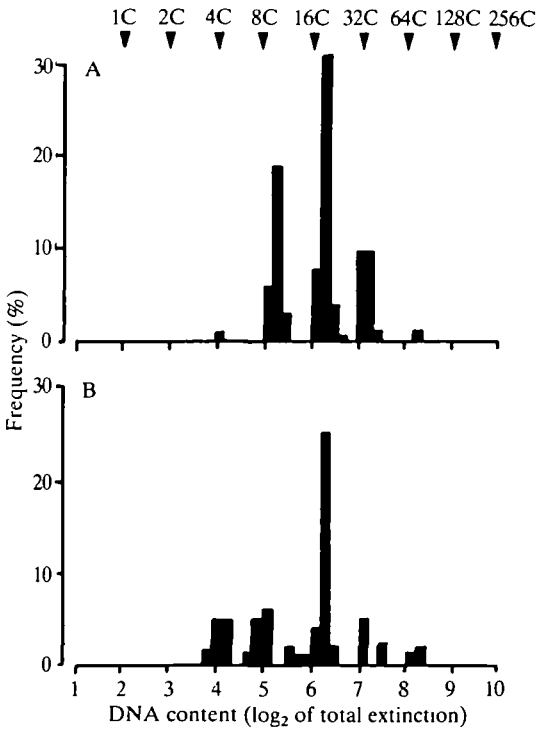

Fig. 3. Frequency distribution of DNA ploidy classes in epidermal nuclei from (A) the anterior margin and (B) the middle intrasegmental regions of a day 3 larva (n = 100 each).

both short and long diameters were measured and then the average was calculated as the nuclear diameter.

Results

Determination of standard values of DNA content

To provide standard values for the DNA measurements, nuclei of spermatids and larval brain were measured (Fig. 2). The means of absorbance values were $0{\cdot}76 \pm 0{\cdot}10\,E_{560nm}$ for the spermatids, and $1{\cdot}54 \pm 0{\cdot}18$ for larval brain. From these data, the 2C value was determined as $1{\cdot}5\,E_{560nm}$. This corresponds to 1·5 pg of DNA, which was estimated from the DNA content of chicken erythrocyte nuclei. Also, the DNA content of nuclei of larval subtracheal cells was found to be 2C (Fig. 2). These cells are located beneath the epidermis, are very small and have nuclei of about 2 μm in diameter.

Preliminary observations on the DNA content of epidermal nuclei

Initially, we measured the DNA content in different areas (Fig. 1) of the fifth abdominal segment of day 3 larvae. Fig. 3 shows that the DNA content of these epidermal nuclei ranged from 4C to 64C. A comparison of the anteriormost region (Fig. 3A) to the

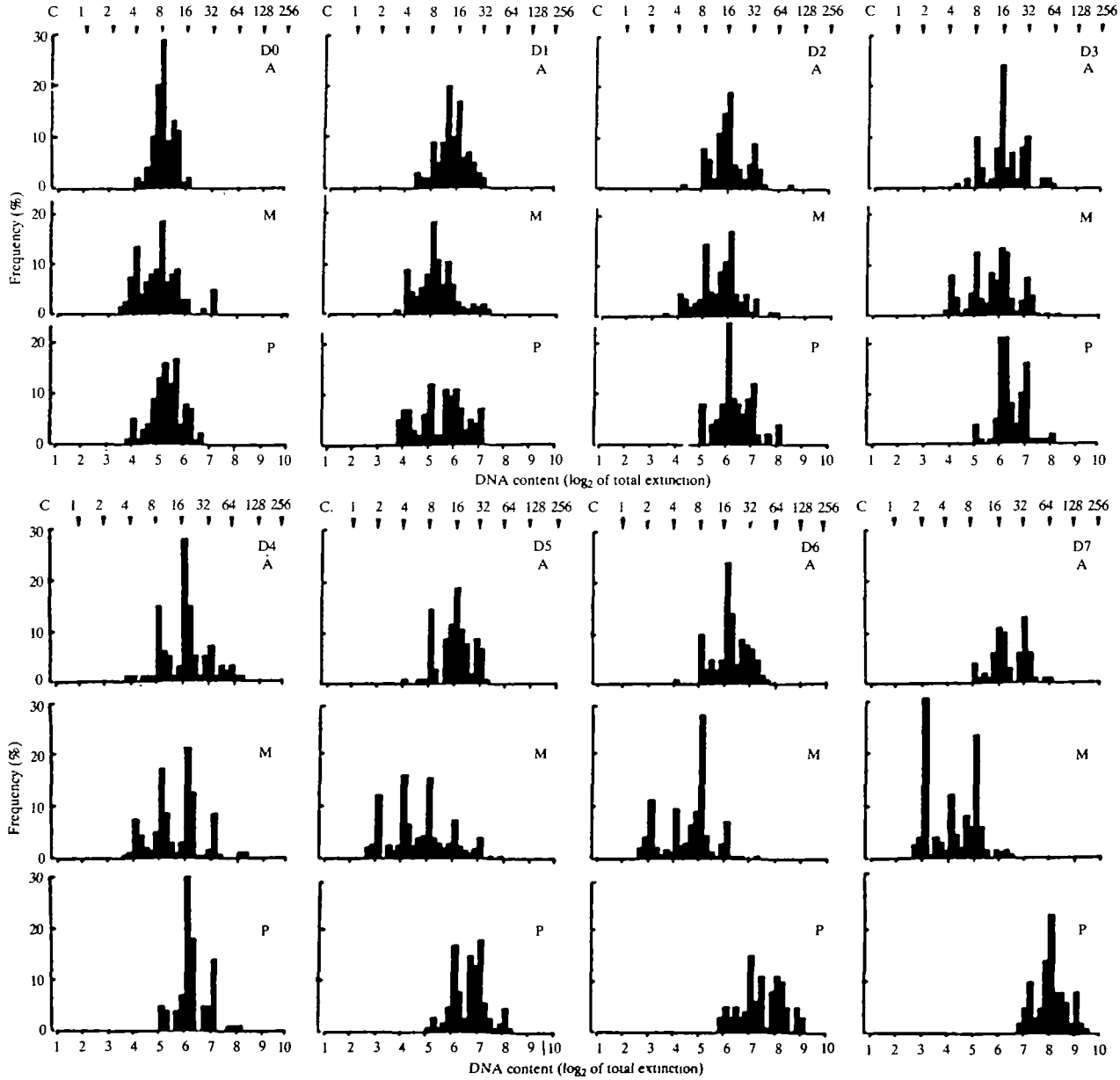

Fig. 4. Frequency distribution of DNA ploidy classes in epidermal nuclei from the anterior margin (A) (n = 100), middle intrasegmental region (M) (n = 200) and posterior margin (P) (n = 100) of the fifth abdominal segment on the designated days (D0–D7) of the final (5th) larval instar.

middle intrasegmental region (Fig. 3B) showed a tendency to slightly higher ploidy in the anterior margin. Different areas within the intrasegmental region showed only random variation in ploidy (data not shown). Similar observations were made on epidermis from day 2 and from wandering larvae (data not shown). In all cases no 2C nuclei were found, thus conflicting with the previous observations on isolated nuclei of primarily 2C and 4C nuclei during the feeding period (Dyer *et al.* 1981).

Temporal and spatial changes in DNA content of general epidermal nuclei during the 5th larval instar

Therefore, we reinvestigated the changes in ploidy in the anterior, middle intrasegmental and posterior regions (Fig. 1) of the fifth abdominal segment during the 5th larval instar using the whole-mount technique. Fig. 4 shows that on day 0 the ploidy of nuclei in all three regions ranged from 4C to 16C with the median being 8C. During the growth phase there was an increase in ploidy in all regions, particularly noticeable in the intrasegmental region and the posterior margin between days 1 and 2. By day 3 the 16C class was predominant in all regions, and there was little change on day 4 (wandering). Differences between the three regions appeared on day 5 when the ploidy level was reduced in the middle intrasegmental region due to mitoses (Kato & Riddiford, unpublished data). By day 7 when the pupal cuticle was being deposited, the cells in this region ranged from 2C to 16C with nearly 20% in the 2C class. The anterior margin remained unchanged until day 6–7 when a slight increase in ploidy was noted. By contrast, the ploidy of the posterior margin increased significantly between days 5 and 7 so that by day 7 the ploidy ranged from 32C to 128C.

Ploidy levels of pock-mark cells

The abdominal pupal cuticle in *Manduca* has many round, shallow depressions called pock marks on the surface of the intrasegmental region. Cells underlying these pock marks develop from general epidermal cells during the larval–pupal transformation. These pock-mark cells are large in size, oval in shape and arranged in a rosette (Roseland & Riddiford, 1980). On day 6, nuclei of 16C and 32C classes were predominant among these pock-mark cells (Fig. 5). By the following day 64C and 128C class nuclei appeared.

Size of general epidermal nuclei during the 5th larval instar

Manduca epidermal nuclei are quite heterogeneous in size (Fig. 6) as first described by Dyer *et al.* (1981). This heterogeneity was most remarkable in the middle intrasegmental region of a segment. In all

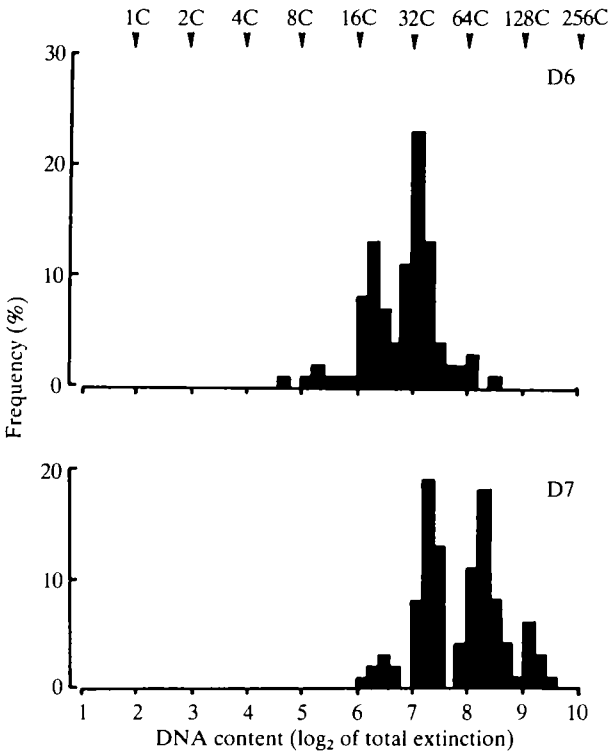

Fig. 5. Frequency distribution of DNA ploidy classes of differentiated pock-mark cell nuclei (n = 100) on day 6 (D6) and day 7 (D7).

regions nuclei increased in size from day 0 to day 4. Between days 5 and 7, nuclei in the middle intrasegmental region decreased dramatically in size whereas those in the anterior margin decreased to a lesser extent. By contrast, the nuclei in the cells of the posterior margin increased to 12–16 μm.

Dyer *et al.* (1981) showed that there was no predictable relationship between nuclear size and DNA content in *Manduca* epidermis. In studying the mitotic wave that occurs in the epidermis on day 5, we noted varying sizes of mitotic figures. To determine if this size were correlated with DNA content, we measured the length of a mitotic figure, which corresponds to the diameter of the equatorial plane since the mitotic axes are parallel to the cuticular surface (Madhavan & Schneiderman, 1977). As shown in Fig. 7, DNA content was positively correlated with the size of the metaphase figure. Metaphase nuclei of the 4C class were mostly 4 μm to 6 μm, whereas 8C, 16C and 32C nuclei were mainly 8, 10 and 12 μm, respectively.

Discussion

Using whole-mount preparations of *Manduca* abdominal epidermis we have shown that the ploidy level of the nuclei during the feeding/growing stage ranges between 4C and 64C with a gradual shift in

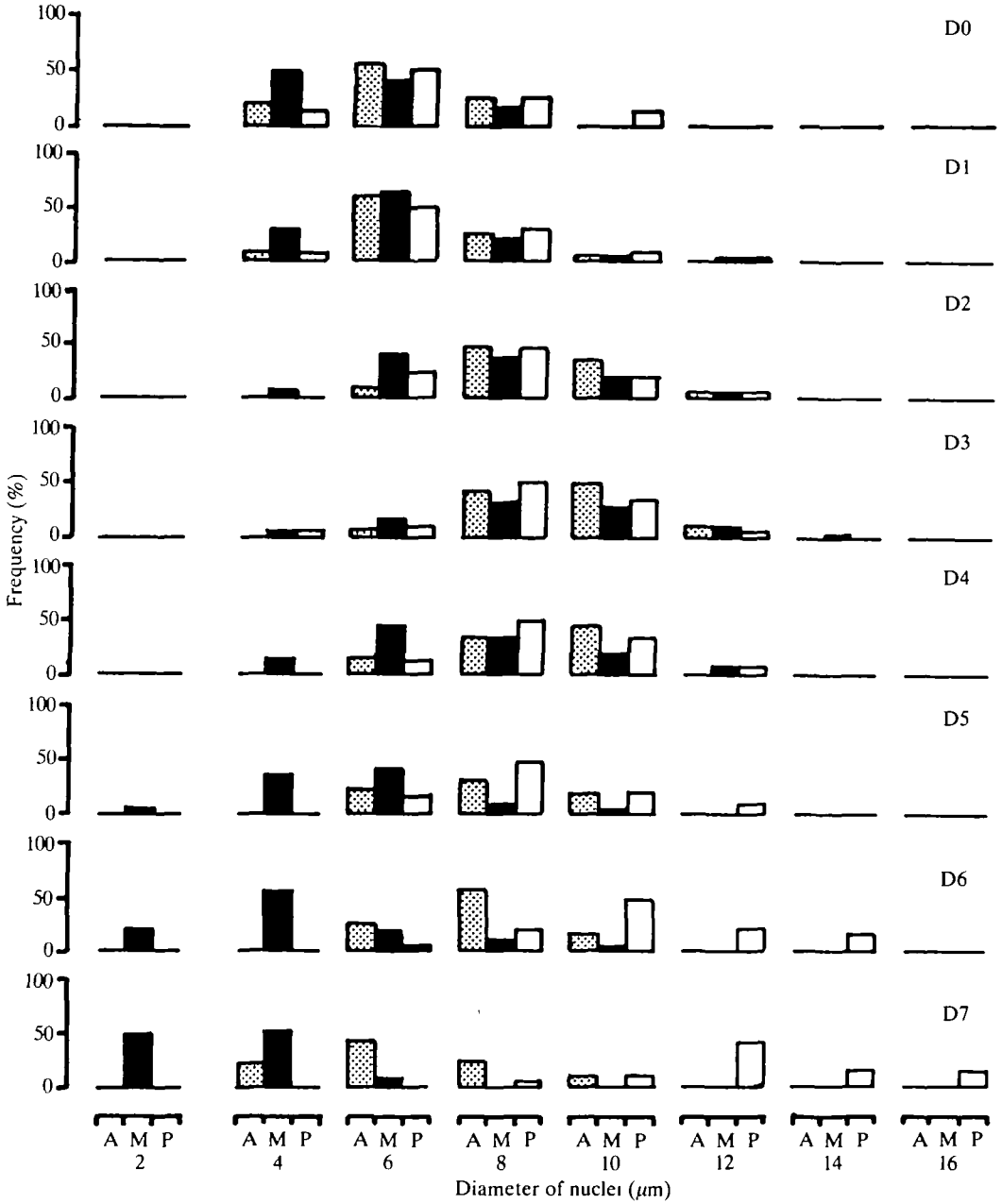

Fig. 6. Frequency distribution of epidermal nuclear size except for pock-mark cells in three regions of fifth abdominal segment. A, anterior margin (▩); M, middle intrasegmental region (■); and P, posterior margin (□) of a segment. n = 100 per region.

distribution to the higher ploidy levels as the animal gets larger. During this period little difference was noted among the different regions of the segment. On day 5 when mitoses are noted in the middle intrasegmental region (Kato & Riddiford, unpublished data), a reduction of ploidy in that region occurs and 2C cells are seen for the first time. By contrast, the cells in the anterior and posterior margins continue to increase in ploidy with some in the posterior margin attaining 128C by the onset of deposition of pupal cuticle on day 7.

These findings are not consistent with the results of previous studies on *Manduca* epidermis (Wielgus, Bollenbacher & Gilbert, 1979; Dyer *et al.* 1981). First, Dyer *et al.* (1981) reported the existence of a 2C cell population in the early 5th instar larva. They assumed the ploidy of the smallest nucleus observed (about 4 μm in diameter) to be 2C. In our whole-mount preparations we never detected such 2C cells during the early stage of the instar. Instead we found that the 2C nuclei that appeared on day 6 were about 2 μm in diameter, and those in the 4C class were about 4 μm.

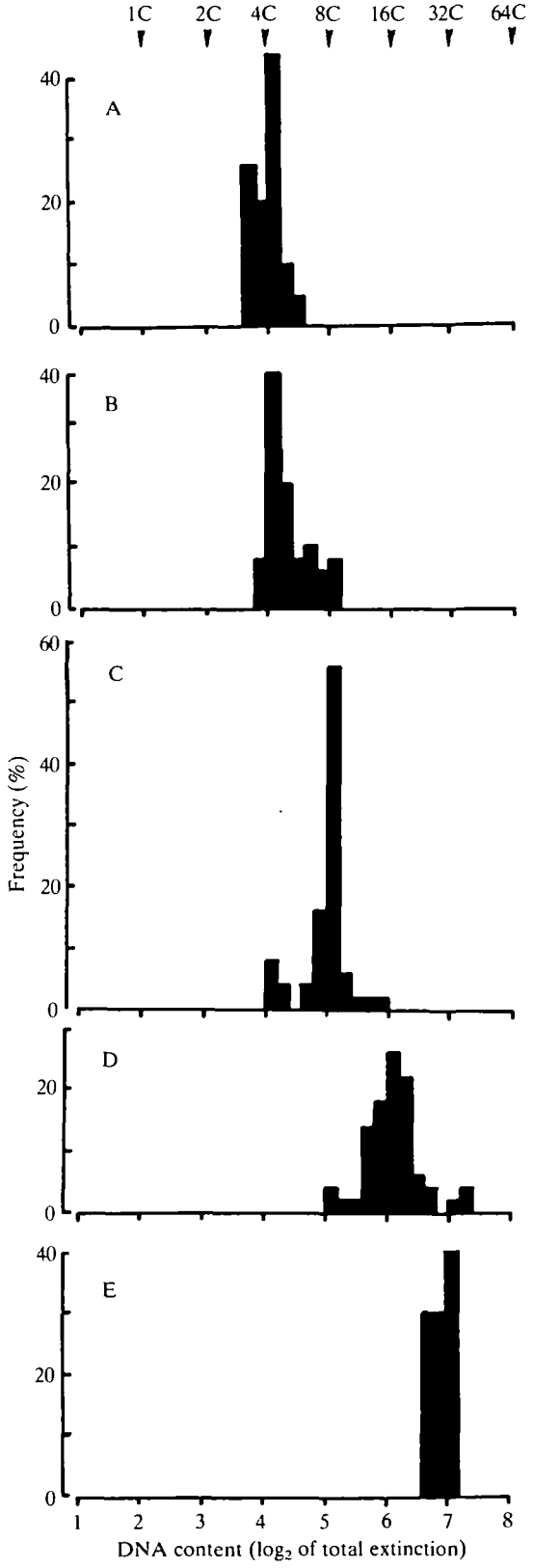

Fig. 7. Frequency distribution of DNA ploidy classes of metaphase cells of various sizes (4–13 μm). (A) 4–5 μm (n = 25); (B) 6–7 μm (n = 50); (C) 8–9 μm (n = 50); (D) 10–11 μm (n = 50); and (E) 12–13 μm (n = 10).

Therefore, the cell population that was estimated as 2C by Dyer *et al.* (1981) must have been in the 4C class. Similarly, Wielgus *et al.* (1979) assumed the lowest level of DNA content in the 5th instar epidermis to be 2C. This assumption is not always true since epidermal cells of insects usually have a long G_2 phase in the cell cycle (Kato, 1977; Besson-Lavoignet & Delachambre, 1981; Graves & Schubiger, 1982) in contrast to rapidly proliferating vertebrate cells.

Second, Dyer *et al.* (1981) described levels of ploidy only up through 8C. The large nuclei with high ploidy observed here in whole mounts may have been excluded during their isolation procedure. The whole-mount technique described here allows assessment of all cells, irrespective of size, so more accurately reflects the different ploidy levels present. Third, in our whole mounts, 2C cells with nuclei about 2 μm in diameter appeared at the time when the mitoses began. Dyer *et al.* (1981) did not report such 2C cells during this mitotic period. Since they regarded objects less than 4 μm in diameter as cellular debris, they would have excluded the 2 μm nuclei.

In *Manduca*, the epidermal cells increase in ploidy and thus must synthesize DNA during the feeding period. The noticeable shift in ploidy between day 1 and 2 coincides with the burst of DNA synthesis in the epidermis seen on day 2 (Wielgus *et al.* 1979). Little further increase in ploidy is seen on day 3 or wandering although the above authors noted a second burst of DNA synthesis on day 3. Thus, the ploidy increase is correlated with growth and the increased size of the epidermal cells as the animal grows larger. Polyploidization occurs commonly in various differentiated cells of insects to increase production of protein and other macromolecules (Brodsky & Uryvaeva, 1977). In *Manduca* these epidermal cells are directly responsible for secreting endocuticle during the rapid growth in size (tenfold) and in surface area (fourfold) over a 4-day period (Wolfgang & Riddiford, 1981). In *Tenebrio* larvae, which grow very slowly, the general epidermal cells show no DNA synthesis during the feeding period of the final larval instar and remained 4C (Besson-Lavoignet & Delachambre, 1981). Thus, the increase of ploidy in *Manduca* abdominal epidermis can ensure rapid growth by providing the machinery for synthesizing a large quantity of macromolecules. The increase in ploidy in the specialized pock cells and the posterior margin, which produces the flexible intersegmental membrane of the pupae during the prepupal period, likely reflects the specialized nature of these cells.

Our findings show that these polyploid cells can enter the mitotic phase, except for certain specialized epidermal cells in the segmental margins. Although mitoses in polyploid cells leading to a reduction in chromosome number are well known in plant tissues

(Nagl, 1978), they are rare in animal cells (D'Amato, 1976). Also, spontaneous occurrence of such mitoses is exceptional. In insects, mitoses in 4C to 64C cells have been observed in the hindgut of the mosquito *Culex pipiens* during metamorphosis (Grell, 1946), in the larval epidermis of the mosquito, *Aedes aegypti* (Risler, 1959) and in the fat body of prolongedly starved *Rhodnius* after feeding (Wigglesworth, 1967). Although the precise signal for such mitoses are not known for the above cases, in *Manduca* this reduction in ploidy by mitosis occurs in response to the prepupal ecdysteroid surge (Kato & Riddiford, unpublished data).

Thus, we have shown that the dorsal abdominal epidermal cells of the final instar *Manduca* caterpillar range in ploidy from 4C to 64C, the ploidy level continuing to increase as the animal grows fourfold in surface area. During pupal development the ploidy levels of the general cells in the middle intrasegmental region are reduced to between 2C and 8C, whereas those forming the pock marks in the pupal cuticle increase in ploidy. At this time there are also ploidy increases in the anterior and posterior margins of the segment as the cells prepare to produce the flexible intersegmental membrane.

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