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Electron Microscopic Autoradiographic Studies on Polytene Nuclei of *Drosophila melanogaster* : Part I — Replication & Its Relationship with Nuclear Membrane

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DNA replication patterns and the relationship between the sites of DNA synthesis and nuclear membrane in the polytene cells of salivary glands of *D. melanogaster* have been examined by electron microscope autoradiography. The salivary glands of 90 hr old larvae (after hatching), grown at 24°C, have been pulse labelled *in vitro* with ^{3}H-thymidine for 1 or 10 min, double fixed with glutaraldehyde and osmium tetroxide and processed for EM autoradiography. In the EM autoradiographs, all the different types of continuous and discontinuous labelling patterns, as seen in conventional squashed preparations, have been identified. No specific labelling of chromosomes or chromosomal regions near the nuclear membrane has been noted in any of the labelled nuclei. It is concluded that in salivary gland polytene cells of *D. melanogaster* larvae, replication is not restricted to the nuclear membrane at any stage of the S-period.

POLYTENE chromosomes of the Diptera are commonly used for studies on replication and transcription in higher organisms. Classically, these chromosomes are examined under light microscope (LM) in squashed preparations of larval salivary glands. Electron microscopic (EM) studies on these chromosomes have not been as common, much less EM autoradiographic investigations. We have examined the processes of replication and transcription in polytene cells of *Drosophila melanogaster* by EM autoradiography. In these studies, ultrathin sections of unsquashed glands rather than squashed preparations have been used to enable us to study the metabolic activities without disrupting the topographical relationships within the cells. The present paper describes ^{3}H-thymidine labelling patterns of polytene chromosomes in EM autoradiographs of salivary glands of late third instar larvae of *D. melanogaster*. The relationship between chromosome replication and nuclear membrane in these cells has also been analysed. A preliminary report on this was presented earlier[1].

Materials and Methods

Eggs from a wild-type stock (Oregon K$^+$) of *D. melanogaster* were collected at hourly intervals and allowed to grow on the standard *Drosophila* food medium at 24°C. Salivary glands from late third instar larvae (about 90 hr after hatching) were dissected out in *Drosophila* Ringer and pulse labelled with ^{3}H-thymidine (1mCi/ml; sp. act. 23.7 Ci/mM; Radiochemical Centre, Amersham) for 1 or 10 min. Larvae of this age have been selected, because their salivary glands are known to have a significant number of nuclei, in which the last polytenic replication cycle is beginning[2,3]. This assures that at least some of the nuclei examined would be in the initial phase of the S. After the pulse, the glands were immediately fixed in 2.5% glutaraldehyde in 0.1 *M* sodiumcacodylate buffer (*p*H 7.2) at room temperature following the technique of Peracchia and Mittler[4]. The glands were then washed in the cacodylate buffer (with 2% sucrose added) for 44-48 hr at 4°C. Post-fixation was in 1% osmium tetroxide in the same buffer at room temperature for 2 hr, after which the glands were dehydrated and embedded in an Epon-Araldite mixture. Sections, showing silver to pale-gold interference colours (about 800-1,000 Å thick), were mounted on Formvar-Carbon coated 200 mesh nickel grids and covered with a monolayer of Ilford L^4 emulsion by the loop method[5]. After appropriate exposure period (45 to 60 days for 1 min and 10 days for 10 min pulse experiments), the preparations were developed in undiluted Kodak D 19 b for 3 min at 17°C, fixed in Kodak F 24 and washed in distilled water. In some batches of tissue, stained with uranium and lead, a precipitate developed with time over certain tissue components. This could be avoided if the preparations were stained just before examination. For EM autoradiography, it has been suggested that staining be carried out prior to emulsion coating[6], but in the present material, it was found necessary to postpone staining till after photographic processing for the aforementioned reason. The processed autoradiographs were double stained with aqueous uranyl acetate and lead citrate; alcoholic uranyl acetate was avoided, as this tends to coagulate the gelatin of the emulsion[7].

Results

General features of ^{3}H-thymidine labelling patterns in EM autoradiographs — The incorporation of ^{3}H-thymidine in polytene cells, as visualized by EM autoradiography of unsquashed glands, is restricted to chromosomal regions. In these preparations, background and cytological labelling were negligible. In accordance with the observations in LM autoradiographs that the different chromosomes of a labelled nucleus show similar type of labelling

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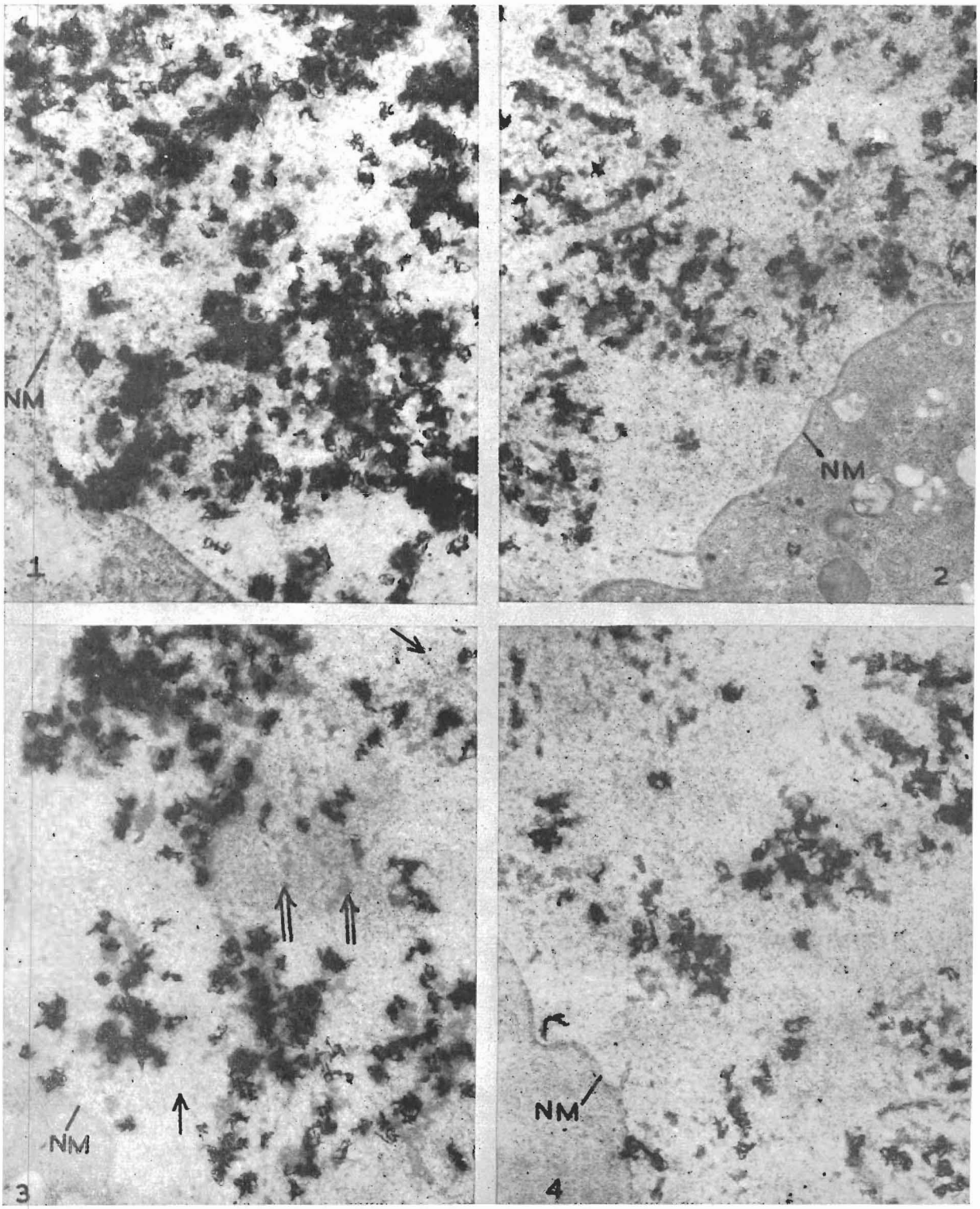

Plate I — EM autoradiographs of sections of larval salivary glands of *D. melanogaster*, pulse labelled for 10 min with ^{3}H-thymidine. In all the figures, some chromosome regions are near the nuclear membrane and others are away, but the labelling intensity as well as the distribution of labelled sites is similar in all regions. NM=nuclear membrane, NO=nucleolus, P=puff. Fig. 1 — Labelling pattern in this nucleus is heavy continuous ('3C') type ; all bands and interbands are labelled along their entire length. Note the relatively low grain density on interband regions × 9000. Fig. 2 — Medium continuous ('2C') type labelling pattern with all bands and most of interbands labelled ; grains are situated along the entire length of bands × 6000. Fig. 3 — Heavy discontinuous ('3D') type labelling ; most of the electron dense bands are labelled heavily ; majority of light bands (⇒) and interbands (→) are poorly labelled or unlabelled × 9000. Fig. 4 — Medium discontinuous ('2D') type labelling. Frequency of labelled sites and the intensity of labelling is lower than in the '3D' pattern ; the labelled bands clearly show grains along their entire length × 9000

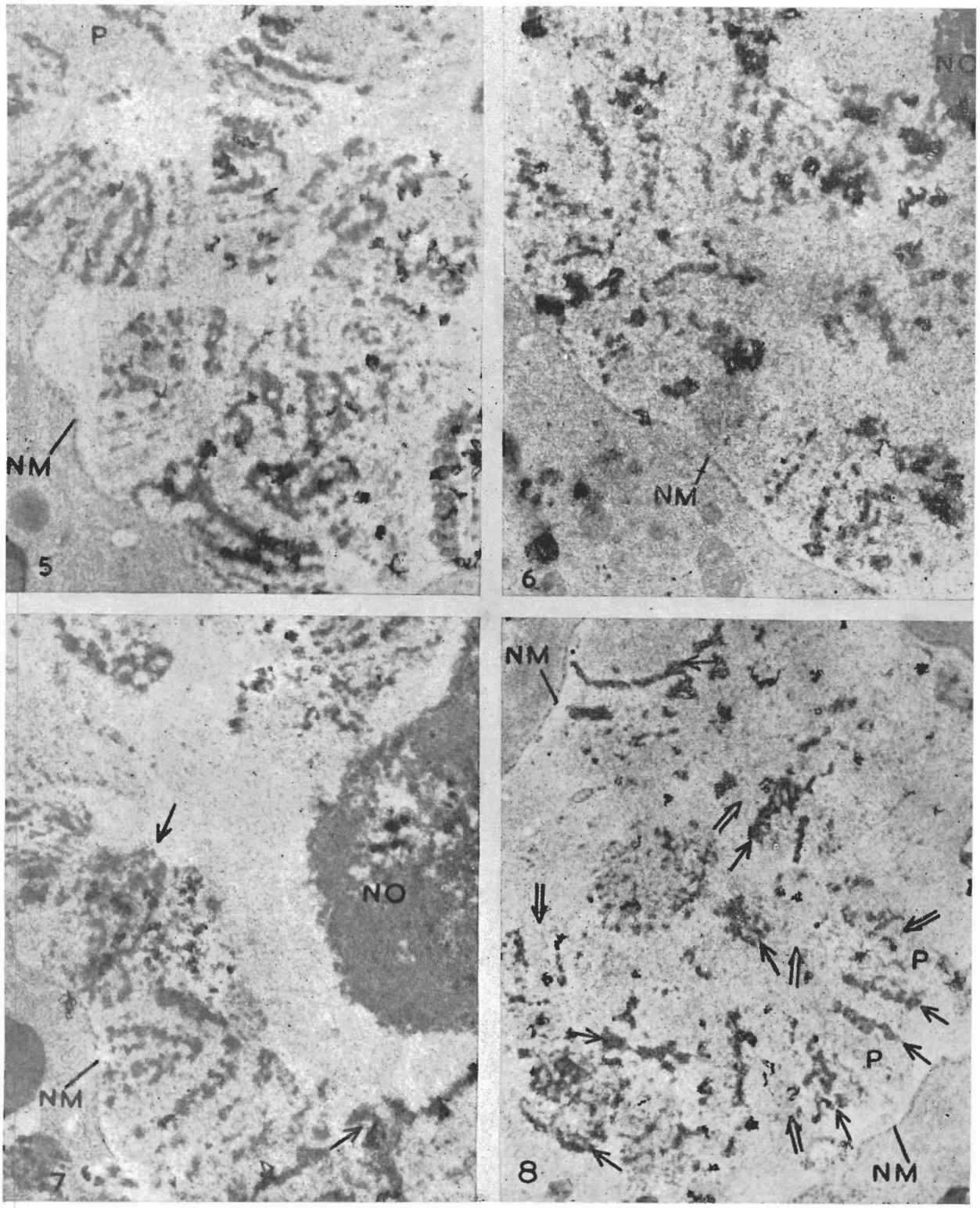

Plate II — EM autoradiographs of sections of larval salivary glands of *D. melanogaster*, pulse labelled for 10 min with ^{3}H-thymidine. Details same as in Plate I. Fig. 5 — Medium-to-low discontinuous ('2D'—'1D') type labelling with fewer silver grains and labelled sites than in Fig. 4 × 6000. Fig. 6 — Low discontinuous ('1D') type of labelling, restricted to only a few electron dense bands × 6000 Fig. 7 — Another example of low discontinuous ('1D') type ; the labelled sites are fewer than in Fig. 6. Note the arrangement of grains in rows over the labelled bands (→) × 6000. Fig. 8 — An example of 'interband labelling'. The labelling intensity in this case is low, but many of the electron dense bands are unlabelled (→), while several puffs and interbands (⇒) are clearly labelled × 3750

patterns[3], it was noted that serial sections of a labelled nucleus exhibited the same overall labelling pattern. The grains distributed over band or interband regions are usually in a row along their whole length (Figs 1-7); this probably means that all the lateral strands in a given polytene chromosome region are replicating in unison. The labelling patterns obtained after 1 or 10 min pulses are similar except that the labelling intensity is lower in preparations from 1 min pulse experiments. For this reason, the illustrations in this paper are from 10 min pulse preparations.

On the basis of LM autoradiography of polytene chromosome squashes, Rodman[3] grouped the different ^{3}H-thymidine labelling patterns into several broad categories of continuous ('3C' and '2C' types) and discontinuous ('3D', '2D' and '1D') types depending on the number of chromosomal sites labelled and the intensity of labelling. In our EM autoradiographic preparations, it was not possible to identify the different chromosome regions because of the limited stretches of chromosome regions visible in a given section. However, the autoradiographic labelling patterns observed in different nuclei could be grouped into the various continuous and discontinuous types similar to those identified at the LM level[3]. Again, as at LM level, it was not always possible to assign unequivocally some of the labelled nuclei to one or the other type of labelling pattern because of the intermediate nature of the labelling pattern in these nuclei.

The following description of these broad categories of ^{3}H-thymidine labelling patterns seen at EM level in the euchromatic regions of polytene nuclei is based on an examination of nearly 100 EM autoradiographs. Nuclei, showing labelling of all or almost all the chromosomal regions visible in a section, have been classified as the continuous type, while nuclei with distinct labelled and unlabelled segments identifiable in a section have been considered as discontinuous type. These classes are further subdivided on the basis of silver grain density on the labelled sites and the number of these labelled sites. Typical examples of the different labelling patterns observed are shown in Figs 1-8. In these figures, only a small region of the nuclear area seen in a section is shown, since the overall pattern of labelling in different areas of a section is similar, as it is in different sections of a nucleus.

Two broad types of continuous labelling have been seen. At EM level, the heavy continuous type of labelling, corresponding to the '3C' class of LM autoradiographs[3], is characterized by a very dense labelling of bands with the individual grains not distinguishable (Fig. 1); the interbands are also labelled but invariably with lower grain density than on bands. In this respect, this pattern differs from the typical '3C' pattern of LM autoradiographs of squashed preparations, where the bands and interbands are uniformly heavily labelled[3]. This difference between the two levels of analysis can be explained by the increased optical and autoradiographic resolution offered by the EM method. The second category of continuous labelling, namely, the medium continuous type ('2C') resembles the '3C' type in having all bands and most of the interbands labelled, but in this instance, the labelling intensity of band regions is not as high (Fig. 2). A few interbands may be seen to be unlabelled in these patterns.

In the discontinuous types of labelling, only the electron dense band regions are seen to be labelled, the interbands and puff regions appearing unlabelled. On the basis of labelling intensity and the number of sites labelled, the discontinuous types of labelling can be grouped into 3 categories similar to those identified in LM autoradiographs[3]. In the heavy discontinuous ('3D') type, the unlabelled regions are fewer, and the grain density on the labelled regions is nearly as high as in the '3C' type (Fig. 3). In the medium discontinuous ('2D') type, the unlabelled segments are larger than in the '3D' types and the labelling intensity is also lower (Figs 4, 5). The low discontinuous ('1D') types of labelling patterns show only a few of the more electron dense bands labelled; the grain density too is very low in this category of labelling patterns (Figs 6, 7).

The labelling pattern represented in Fig. 8 is rather unusual and very rare; only one more nucleus was seen to have this type of labelling. In this case, the labelling intensity is low to medium, but unlike the previous types, it is mainly the more disperse regions (interbands and puffs) that are labelled with ^{3}H-thymidine, while the electron dense bands are clearly unlabelled. Possibly, these patterns correspond to the rare 'interband labelling' described earlier in LM autoradiographs of these chromosomes[8,9].

Localization of ^{3}H-thymidine labelling in relation to the nuclear membrane — Several autoradiographs of each of the different types of labelling patterns have been analysed to see if in any pattern, there is a specific labelling of chromosome regions nearer the nuclear membrane. This possible relationship was examined only in non-peripheral sections of the nucleus. This ensured that any area in the centre of the section, being studied, is away from the membrane that could otherwise be present directly below or above the plane of the section. Furthermore, in this type of analysis, the width of the polytene chromosomes and the view that all the lateral strands of a given region replicate in unison, have to be taken into consideration. On this basis, if any part of a band or interband is attached to or is near the nuclear membrane, the whole of that segment is to be regarded as being associated with the membrane. In each of the Figs 1-8, it may be noted that some stretches of the chromosomes are lying parallel or close to the nuclear membrane, while others are further away from it. This is true of all the EM autoradiographs examined in this study with 1 or 10 min pulses of ^{3}H-thymidine. Chromosome regions, completely free of any association with the nuclear membrane, appear to be labelled as often as those apparently associated with it, irrespective of the type of labelling pattern. Further, it may be added that in any given pattern of labelling, the intensity of labelling also appears to be more or less the same, whether the stretch of the chromosome is close to the nuclear membrane or not.

Discussion

Replication in *Drosophila* polytene chromosomes has been studied autoradiographically by a number of workers. These studies, carried out on squashed preparations at LM level, have provided detailed information on the overall labelling patterns and on the replicative organization of some specific chromosome sites[10,11]. So far, however, replication in

polytene chromosomes has not been studied by EM autoradiography. In the present investigation, we have used ultrathin sections of entire glands rather than ultrathin sections of squashed chromosomes for studying replication in these chromosomes. Although the use of sections of glands limits the extent of chromosomes that can be seen in a section, we have been able to identify in our material all the different labelling patterns described previously in conventional squashed preparations[3]. This similarity of labelling patterns, even with the increased optical and autoradiographic resolution of the EM method, reaffirms our belief that these different patterns of labelling after a short pulse of ^{3}H-thymidine are representative of specific phases of the S-period in these nuclei. Rodman[3] discussed the significance of these labelling patterns and suggested that 2C→3C→3D→2D→1D labelling pattern sequence represents the S-period from the initiation to termination in the late third instar larval salivary glands of *D. melanogaster*.

The availability of specific patterns to identify various phases of the S, and the possibility of preserving intracellular spatial relationships in our material make it feasible to analyse one of the important aspects of the regulation of chromosome replication in higher organisms, namely the relationship of nuclear membrane with replicating chromosomes. A regulatory role of nuclear membrane on replication and chromosome organization in eukaryotes has been frequently discussed[12,15]. In prokaryotes, it is believed that replication is initiated and continued in close association with the cell membrane[16,17]. Several investigators have looked for a similar relationship between the nuclear membrane and replication in higher organisms, mostly using mammalian cells in culture and the techniques of cell fractionation and/or EM autoradiography. However, the results of these studies have not been unequivocal. The majority of cell fractionation experiements[18,23] and the earlier EM autoradiographic studies[12,24,25] suggest that the replication is initiated at the membrance and possibly also continued at this location. On the other hand, most of the EM autoradiographic and some cell fractionation studies [26-31] indicate that there is no spatial relationship between the nuclear membrane and the initiation or continuation of replication. Recently, Comings and Okada[32] and O'Brien *et al.*[33] have withdrawn their earlier conclusions about the involvement of the nuclear membrane in chromosome replication, and now believe that the replicating chromosomes need not be associated with the nuclear membrane. Obviously, the question of the involvement of nuclear membrane in chromosome replication in higher organisms is far from settled. Information on this aspect has not so far been sought in *Drosophila* cells. Our observations in this respect are important, firstly because a new cell type has been studied, and secondly, the information obtained in the present analysis reflects more closely the situation in living cells, as the glands have not been kept *in vitro* for a long duration and no pretreatment for the synchronization of cells had to be employed.

The process of chromosome replication in the polytene cells may be related to the nuclear membrane in one of the following ways : (i) only the beginning of DNA synthesis is dependent on the initiation sites on chromosomes being associated in some manner with the membrane, or (ii) all replication, viz. initiation and continuation, progresses in association with the nuclear membrane, or (iii) only the 'late' replicating sites, i.e. the sites which are still replicating in the later part of the S, are near the nuclear membrane, or finally (iv) there is no spatial relationship between the sites of replication and the nuclear membrane. The second alternative that all replication proceeds near the nuclear membrane can obviously be ruled out in the present material, since ^{3}H-thymidine incorporation has been seen to occur into chromosome regions situated far away from the nuclear periphery; this is the case irrespective of the duration of the pulse (1 or 10 min).

To check on the first possibility that the initiation of replication occurs on the nuclear membrane, one should ideally know the precise moment when the S begins. In the absence of such precise knowledge, however, we may proceed on the basis of assumptions relating specific labelling patterns to the beginning of the S in these nuclei. Previous studies on replication in polytene chromosomes of *Drosophila* have suggested three possible types of ^{3}H-thymidine labelling patterns to characterize the beginning of the S. The first and most widely held view is that a continuous type of labelling occurs at or very close to the beginning of the S-period. In other words, all the replicating units, within the limits of autoradiographic resolution, are believed to initiate their DNA synthesis at the same time or within a very short interval[3,10,31-36]. A second alternative, only slightly different from the first, is that replication is initiated in interband and other diffuse chromosome regions[8,11,37]. The other possibility[38] is that a low discontinuous labelling pattern represents the beginning of the S in these nuclei. If the initiation of replication is dependent on an association with the nuclear membrane, the 3 models of temporal order of replication outlined above, would have different consequences. If the continuous type of labelling ('2C' or '3C' type) represents the beginning of the S, one would expect to find all the chromosomes in such a nucleus to lie more or less parallel to the membrane or in a similar association. The second model, suggesting the 'interband labelling' as the beginning of the S, would predict all the labelled interbands and puffs to be associated with the membrane. On the other hand, the last alternative mentioned above would suggest that in some specific type of discontinuous labelling, all the labelled bands would be attached to the nuclear membrane. However, as has been noted earlier, during our study we have not seen any nucleus in which the labelled chromosome regions are specially oriented along the membrane only. This may indicate that in the polytene nuclei studied, the initiation of replication does not occur exclusively in association with the nuclear membrane.

In the polytene chromosomes of *Drosophila*, the 'late' replicating sites have been well characterized. These chiefly include some dark bands and the heterochromatic chromocentre region; in other words, sites still labelled in '1D' type of labelling patterns, are considered to be 'late' replicating[3,10,11, 38-40]. Some studies in mammalian cells have shown that the 'late' replicating heterochromatic sites tend to be associated with the nuclear membrane[26,27,30]. In our preparations, we have seen that all of the 'late' replicating band regions (pattern '1D') are not

specifically oriented near the nuclear membrane. However, it is not possible to know if a given labelled band, associated with the nuclear membrane in a particular '1D' type nucleus, is always similarly positioned in all the other nuclei having this type of labelling pattern. The only 'late' replicating region, identified to be consistently near the nuclear membrane, is the heterochromatic chromocentre region[41].

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