ELECTRON MICROSCOPE STUDIES ON SALIVARY GLAND CELLS

I. The Nucleus of *Bradysia mycorum* Frey (Sciaridae), with Special Reference to the Nucleolus

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ABSTRACT

Salivary glands were fixed in cold 1 per cent osmium tetroxide in veronal-acetate buffer containing sucrose and embedded in methacrylate mixture or Araldite. The salivary gland nuclei of sciarids show a continuous production of nucleoli, which remain multiple and not consolidated into a single structure. The earliest recognizable nucleoli, which we call "elementary nucleoli," are aggregations of a few paired 40 A fibrils and a few 150 A particles, at many points within chromosome bands. Further development consists of the detachment of the elementary nucleoli from their points of origin and their subsequent mutual coalescence. As a result, dense patches of nucleolar material are formed which become large nucleoli at the surface of chromosomes, either attached to the band or free. The fully formed nucleoli have a characteristic dual structure with a narrow dense periphery and a broader less dense internum. Fibrils and particles are present in both regions, and the difference in density reflects differences in the packing of the two structural elements. The duality in structure is lost in later stages. The nucleolar fibrils appear to be similar to the chromosomal fibrils. The 150 A particles in nucleoli, chromosomes, and nuclear sap seem identical. The significance of these observations is discussed for nucleologenesis in general.

INTRODUCTION

The salivary gland nuclei of sciarids have multiple nucleoli (Fig. 1). Under the light microscope, these nucleoli are Feulgen-negative. Other cytochemical tests (1) reveal that these nucleoli contain two or three forms of ribonucleoproteins (RNP's); *i.e.*, perichromosomal RNP and RNPs of the pars amorpha and of the parachromatin of the nucleolus described by other authors (1). The distribution of the ribonucleic acid (RNA) label in autoradiograms of a related sciarid nucleus (2) shows a close correspondence with the distribution of the nucleoli in electron micrographs of the present material. The work presented here deals with the origin, development, and later transformations of the nucleoli at the ultrastructural level, aspects which for nucleoli in general have not received all the attention they deserve.

MATERIAL AND METHODS

Bradysia mycorum Frey (= *Lycoriella solani* (Winn), det. Tuomikoski), was first found in laboratory cultures of the midge *Smittia sp.* and has been thereafter maintained separately. The larval salivary glands of *B. mycorum* are strikingly differentiated into a short,

flattened, anterior portion and a much longer, tubular, posterior portion. As the cells in the anterior portion are much larger, they have been mainly used for the present investigation. The glands were dissected and fixed for 1 to 1½ hours in cold (4°) 1 per cent osmium tetroxide, buffered at pH 7.4 with acetate-veronal (3), containing sucrose (4). After dehydration in ethanol, the tissue was embedded in *n*-butyl-methyl methacrylate mixture (5) or Araldite epoxyresin (6). Thin (400 to 800 A) and thick (up to 0.25 micron) sections were cut on a Porter-Blum Servall microtome with glass knives, and the sections were mounted on grids with collodion or formvar and carbon films. All Araldite sections were "stained" by floating the grids on a mixture of 1 per cent potassium permanganate and 2.5 per cent uranyl acetate (A. E. G. Dunn, unpublished data). Apart from the addition of uranyl acetate, the technique was as described by Lawn (7). According to Dunn, the uranyl acetate "stains" earlier than the permanganate and this accounts for the better preservation of general structure than in a plain permanganate-treated section. The mixture does not keep and was freshly prepared. The electron microscopes used were Philips E.M. 75 and Siemens Elmiskop I. The electron micrographs were made at original magnifications of 2000 to 20,000 and further enlargements were obtained photographically.

OBSERVATIONS

In methacrylate-embedded tissue the electron opacity is highest in nucleoli, intermediate in chromosome bands, and lower in interbands and nuclear sap (Fig. 1). In Araldite-embedded and subsequently stained tissue, the resolution is generally better and the same order of density prevails (Figs. 2 and 13).

The Nuclear Envelope

This is clearly double (Fig. 4) and with closely spaced "pores" (Fig. 3). The appearance of the nuclear envelope varies in different cells and this aspect will be considered in a later publication.

The Chromosomes

The fibrillar nature of the chromosomes is evident, both in the bands and interbands. The greater electron opacity of the bands is probably due to the closer packing of more abundant fibrils than in the interbands. On close examination, especially of the bands, short lengths (straight or curved) of paired 40 A fibrils can be detected (Figs. 5, 8, and 11).

The parallel lying fibrils together seem to form a unit 100 A in diameter. This presumably corresponds to the "elementary chromosome fibril," or the morphological unit generally seen in electron micrographs of chromosomes (8). Occasionally, the 40 A fibrils show a beaded appearance with minute (20 to 30 A) particles apparently adhering to them. This aspect needs further study. In the thin sections studied, the paired fibrils generally appear at the bands in various profiles which are open at one or two points (Fig. 5). These are interpreted as oblique sections of extremely short lengths of the paired fibrils, which are probably coiled in a complex way. These profiles enable us to distinguish the fibrils to some extent from the particles, which are next referred to.

Unlike the fibrils, the particles do not occur constantly. They are a variable component even in the bands; in some bands they are sparse and scattered, while in others (which as described later produce nucleoli) they are abundant. The particles are dense and have a diameter of about 150 A. Particles of characteristically similar size and density occur also in nucleoli, nuclear sap, at the nuclear membranes, and in the cytoplasm.

Occasionally, in some chromosome regions,

FIGURE 1

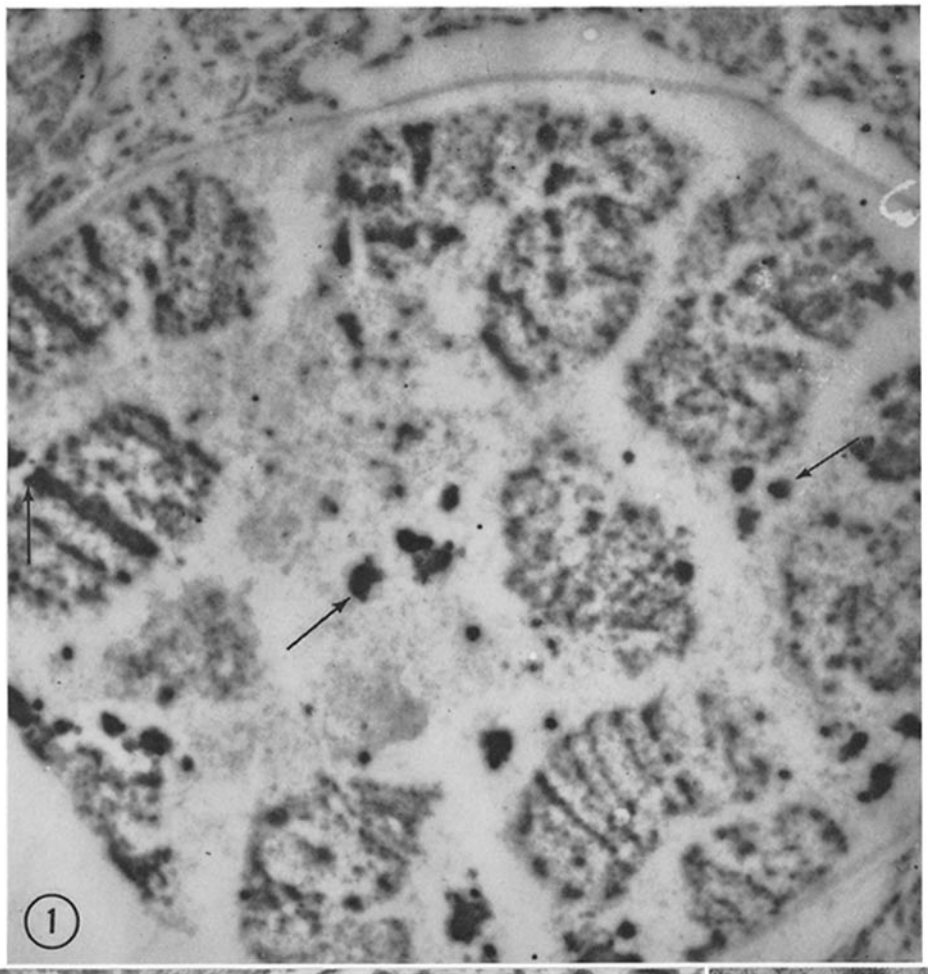
Electron micrograph of an entire nucleus of a salivary gland cell showing the multiple nucleoli (arrows). From a thick section of methacrylate-embedded tissue. × 4,500.

FIGURE 2

Micrograph of part of a nucleus showing the nucleoli (arrows) attached to the chromosome band. The nucleoli are the most electron-opaque structures in the nucleus (see also Figs. 1 and 13). From a thick section of Araldite-embedded tissue. × 10,000.

FIGURE 3

Part of the nuclear envelope with its closely spaced pores. From a thin section of Araldite-embedded tissue. × 24,000.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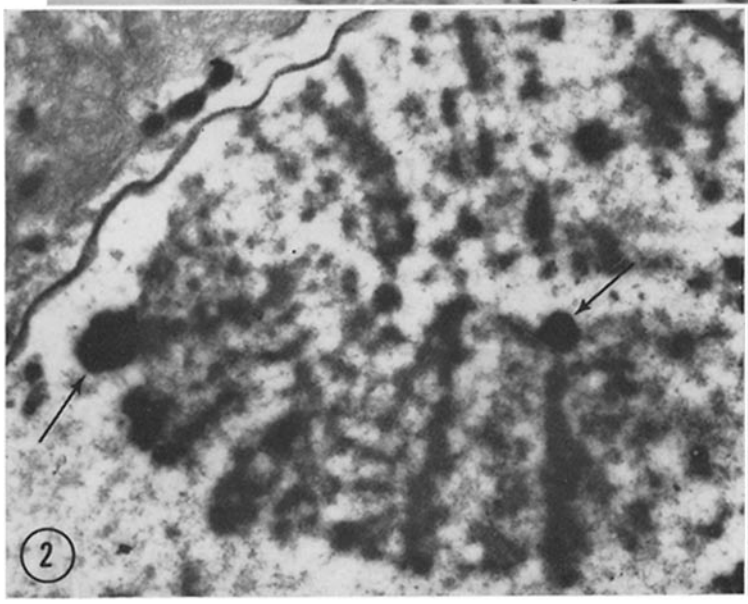
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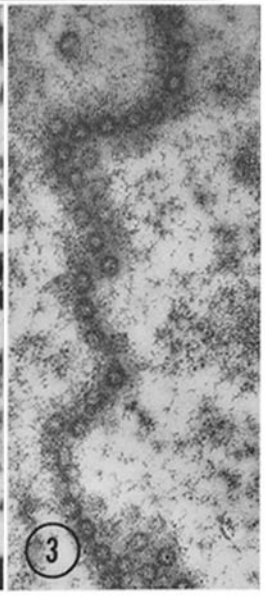
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dense particles of larger dimensions (about 400 A) are seen (Fig. 8). These large particles are also found in the nuclear sap (Fig. 11) and in nucleoli adjacent to the chromosome regions which contain them. Some of these particles appear to be composed of two or three smaller particles.

The Nuclear Sap

The nuclear sap generally presents an amorphous aspect with fine, apparently coiled, filaments of very low electron opacity. Sometimes, these filaments give the impression of strings of beads (Figs. 4 and 8). Amidst these filaments are found particles similar to those in chromosomes, nucleoli, and elsewhere in the cells, which were mentioned before.

The Nucleoli

The numerous nucleoli occur either associated with the chromosomes or free. Those associated with the chromosomes are restricted to the bands (Figs. 2 and 13). The nucleoli are variable in size and shape, although many tend to be spherical. The free nucleoli are usually the largest and some of these measure 1.5 microns in diameter (the nuclei have an average diameter of 25 microns).

Development of the Nucleoli

The earliest recognizable nucleoli occur at many points within the chromosome bands (Fig. 6). Due to the limitations inherent in the analysis of thin sections, it is not possible to define the exact size and shape of these earliest indications of nucleoli, but it seems probable that they are less than about 2000 A in diameter and more or less spherical, as indicated in certain thick sections (Fig. 7). For reasons to be stated later, we have called them elementary nucleoli. Many bands have elementary nucleoli and their later stages of development, but other bands or series of bands do not have them. Thus, at any given time, not all bands produce nucleoli.

Ultrastructurally, in thin sections, the elementary nucleolus resolves into an aggregation of a few 100 A fibrils and a few 150 A particles (Fig. 6). These structural components of nucleoli appear to

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FIGURE 4

Part of a nucleus and adjoining cytoplasm. The double nature of the nuclear envelope is clear. Note the amorphous aspect of the nuclear sap with apparently coiled, low-density filaments and with dense particles. The small masses (arrows) are believed to be the internum of disintegrating nucleoli (see text). From a thin section of Araldite-embedded tissue. × 24,000.

FIGURE 5

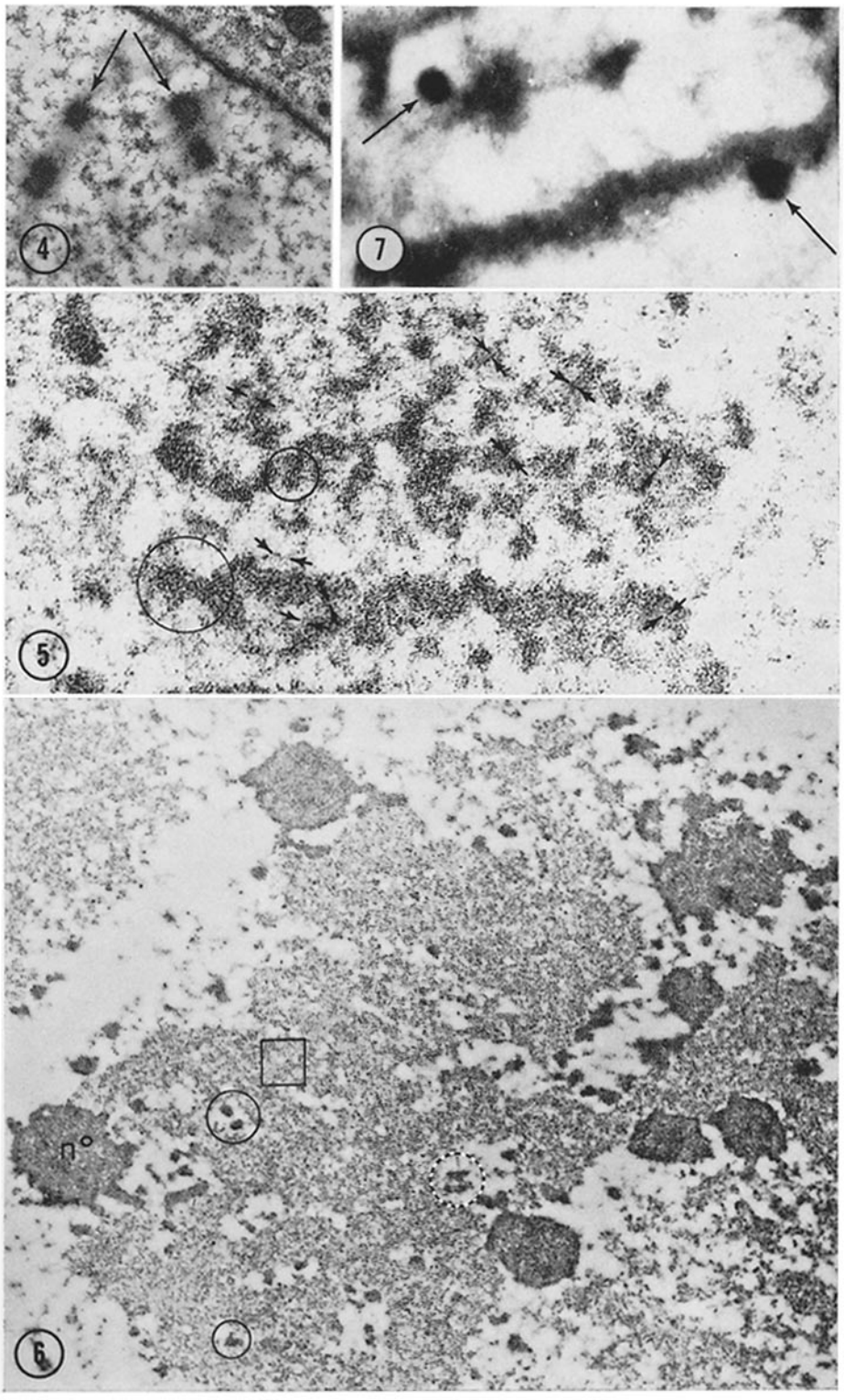
A small area of a chromosome is shown. Bands and interbands are clearly discernible (see also Fig. 13). Short lengths of parallel 40 A fibrils (arrows) together seem to form a unit 100 A in diameter. Predominantly in the bands various profiles are seen (circles) which are interpreted as oblique sections of the paired fibrils. From a thin section of Araldite-embedded tissue. × 30,000.

FIGURE 6

A chromosome band showing many elementary nucleoli (circles) at different points (see text). Large nucleoli (n^o) are at the periphery. Short lengths of parallel fibrils and closely aggregated, dense 150 A particles are seen in the elementary nucleolus (broken circle). Particles similar in size and density are also scattered in the chromosome band. Profiles of the fibrils may be noted in another elementary nucleolus (circle). The fibrils and their profiles seen in nucleoli are similar to those elsewhere in the chromosome band (square, and also see Fig. 5 *et seq.*), but in nucleoli they appear to be slightly more compactly packed and are probably a little denser. From a thin section of methacrylate-embedded tissue. × 25,000.

FIGURE 7

Part of a chromosome showing early nucleoli (arrows) appearing as nodules at the bands. From a thick section of methacrylate-embedded tissue. × 24,000.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be similar to those in the chromosomes, but the 100 A fibrils (which like the chromosomal fibrils consist of two 40 A fibrils and show similar profiles) are slightly more compactly packed. This would account for the over-all greater density of the elementary nucleoli, although possibly the nucleolar fibrils themselves are denser than the chromosomal fibrils.

Sections through bands which are actively producing nucleoli suggest a sequence of events leading finally to the formation of large nucleoli. The scattered elementary nucleoli seem to move from their points of origin and to coalesce mutually to form large patches of nucleolar material within chromosomes and on their surfaces. These patches are uniformly dense (Figs. 8, 11, and 12) and contain numerous tightly packed fibrils and particles. At the surface of the chromosomes, it would appear that the nucleolar patches round off as more or less spherical masses and in this way give rise to the large nucleoli. Frequently, the large nucleoli formed are in contact with the nucleolar patches (Figs. 11 and 12), which thus appear to add continuously to the growing nucleoli. The nucleoli remain attached to the chromosomes or may become free. In either case, these large nucleoli, which are considered to be fully formed, show a characteristic dual structure consisting of a narrow dense periphery surrounding a broader, less dense internum (Figs. 8 to 10); both regions contain fibrils and particles. The density of the periphery corresponds to that of the nucleolar patches, which might suggest that the peripheral material is a more recent addition to the growing nucleolus. Together with the observed sparser distribution of elements in the internum, this also suggests that as material is added at the periphery it is dispersed into the internum. The characteristic dual structure of the large nucleoli indicates a certain degree of organization, perhaps as the result of some sort of equilibrium between the addition of new material and its dispersal.

In later stages, all nucleoli do not appear alike. Some nucleoli show dense areas within the internum, either small and scattered (Fig. 14), or occupying almost the whole internum (Fig. 15). Vacuoles or optically empty spaces appear within these dense areas (Fig. 14). In terms of density the over-all appearance of such nucleoli has become very complex, and the changes that have occurred probably indicate incipient disintegration. In other nucleoli different changes seem to occur, judging from the reconstruction of a series of observations. Some of these nucleoli appear stellate, suggesting a dispersal of the constituent elements. In what is probably a next stage the nucleoli appear homogeneous, as though consisting of only the internum. The final stage could be the breaking up of the internum into smaller parts in the nuclear sap, as in Fig. 4.

DISCUSSION

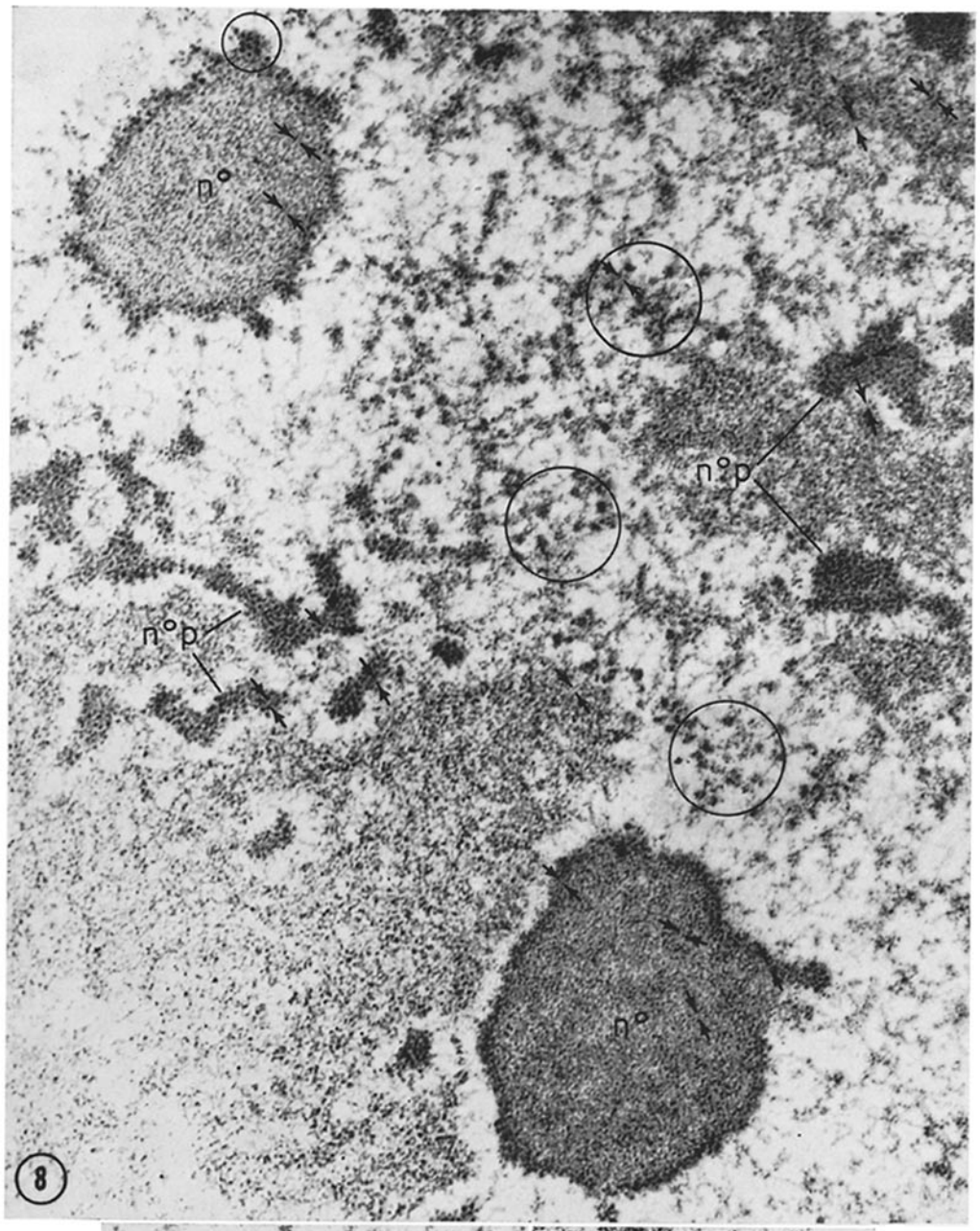
The occurrence of numerous nucleoli and their continuous production make the present material favourable for the study of nucleolar origin and development. The earliest recognizable nucleoli are probably less than about 2000 A in diameter and occur scattered within some bands of the polytene chromosomes. Reports of what were regarded as the earliest indications of nucleolar material under the electron microscope are available (9–11). The prenucleolar material or substance, as it has been termed, is described as loose clusters or irregular layers lying between the

FIGURE 8

This micrograph shows large nucleoli (n^o), patches of nucleolar material ($n^o p$), and also areas of large 400 A particles (circles), some of which appear compound. Note the dual structure of the large nucleoli, with a narrow periphery (corresponding in density to that of the nucleolar patches) and a broader less dense internum. Particles and fibrils (arrows indicate paired fibrils) are present in both regions of the nucleoli and appear similar to those in chromosomes. From a thin section of Araldite-embedded tissue. × 60,000.

FIGURES 9 AND 10

Large, free nucleoli showing the dual structure: short lengths of paired fibrils (arrows) and their profiles (circles) may be detected amidst the particles. From thin sections of Araldite-embedded tissue. × 32,000 and 30,000, respectively.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chromosomes and which later appear as more distinct prenucleolar bodies. In our material, we think that the equivalents to these two stages would be the nucleolar patches and the subsequent large nucleoli, respectively (see also later). The earliest indications of nucleoli that we have detected are within the chromosome and in this respect they are antecedent to any stage so far reported; we have therefore called them elementary nucleoli. Dense areas of "chromosomal ribonucleoproteins" described by Swift (16) appear to us to correspond to the nucleolar patches in our material.

Due to the intimate association of elementary nucleoli and chromosomes, a discussion of the development of the nucleolus and its ultrastructure is helped by a prior consideration of chromosomal ultrastructure which, to that end, follows. Although there have been many investigations of salivary gland chromosomes, a final elucidation of their fine structure is still difficult. However, it has been proposed that in the interbands the chromosomal fibrils run more or less straight and parallel to the chromosome axis, while in the bands they are extensively coiled and closely packed (12–14). It has also been noticed that the fibrils tend to associate in pairs (8, 12, and 13). In the present material the association in parallel of two 40 A fibrils measures 100 A in diameter, as pointed out earlier. This diameter compares well with the lower range of dimensions previously reported (*cf.* references 13 and 15) for fibrils in polytene chromosomes.

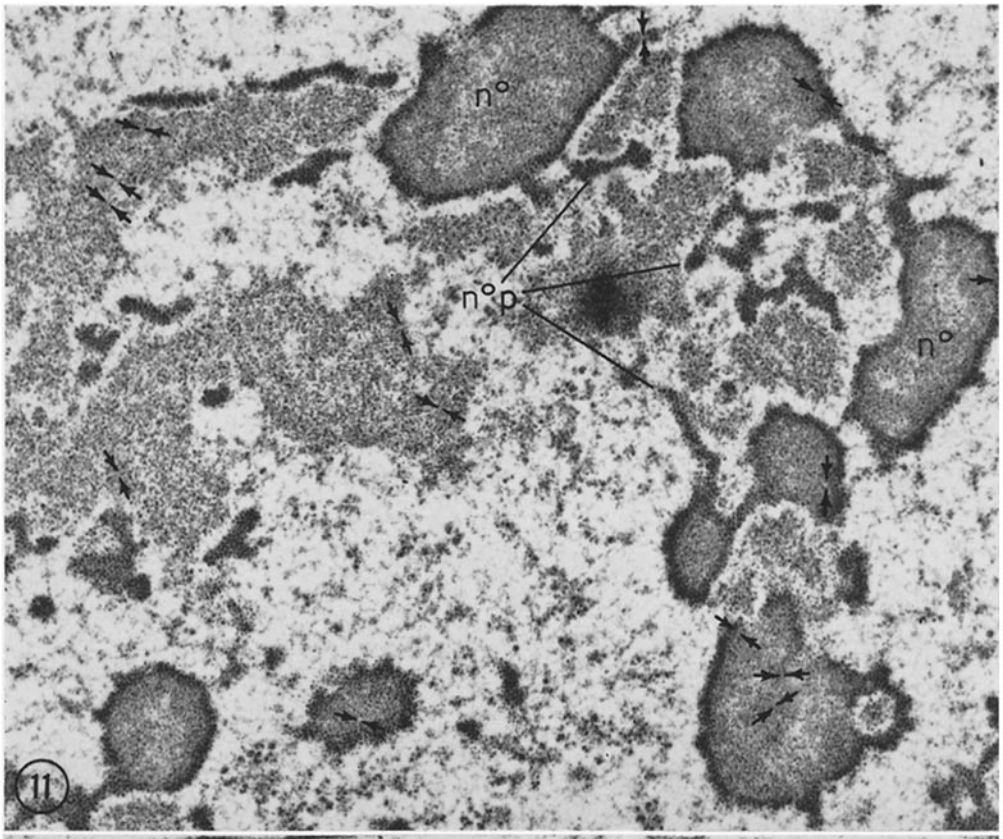
Scattered within bands are the dense 150 A particles similar to those present elsewhere in the nucleus. Dense particles have been previously demonstrated both in salivary gland chromosomes (17, 18) and in nucleoli (19, 20, and others). It now seems well established that these particles, similar in appearance to the particles in cytoplasm (21), are ribonucleoprotein in nature (22). They are also observable in many active genetic loci such as puffs and Balbiani rings of polytene chromosomes and loops of lampbrush chromosomes (*cf.* 22 for references). The large 400 A particles occasionally noticed in our material may correspond to those reported in certain special chromosome regions (14, 17, 20, and 23).

Fibrils and particles identical to those in chromosomes are present in all stages of nucleolar development beginning with the elementary nucleoli. When the elementary nucleoli coalesced and formed dense patches of nucleolar material, the greater electron opacity of these patches was ascribed mainly to a tight packing of the fibrils and particles. The subsequent formation of the large nucleoli with a characteristic dual structure has already been described. In these nucleoli, it was suggested that as material is added to the periphery it is dispersed into the internum. This assumption agrees with the observations in an autoradiographic study on a related sciarid nucleus (2) in which the first labeled nucleolar RNA appears in the periphery and later permeates the whole of the nucleolus. It has been shown biochemically that nucleolar particles are the seat of an intense synthesis of RNA (22).

The nucleolus is well known from light microscope studies to be extremely plastic and with variable morphology. But at the ultrastructural level the observations of many authors permit some generalization. Although the nucleolus is known not to have a characteristic ultrastructural organization, two regions are usually detected; *i.e.*, one largely particulate and the other rather diffuse and amorphous. Several authors, beginning with Borysko and Bang (24) and Bernhard *et al.* (25), have reported a central thread-like or reticulate region, and other authors (26 and 27) have noted this region to be surrounded by a particulate region. These regions would seem to correspond to the "nucleolonema" and "pars amorpha," respectively, described earlier (28) with the light microscope. In other instances this central structure is not present and the nucleoli appear relatively homogeneous. However, although not universally

FIGURES 11 AND 12

These micrographs show the dense patches of nucleolar material ($n^o p$) in the chromosomes. These patches are in contact with large nucleoli (n^o) formed at the surface of chromosomes. Short lengths of fibril pairs are indicated (arrows) both in nucleoli and in the rest of the chromosome. In the nuclear sap at the bottom of Fig. 11, large 400 A particles are present (see also Fig. 8). From thin sections of Araldite-embedded tissue. × 32,000 and 37,000, respectively.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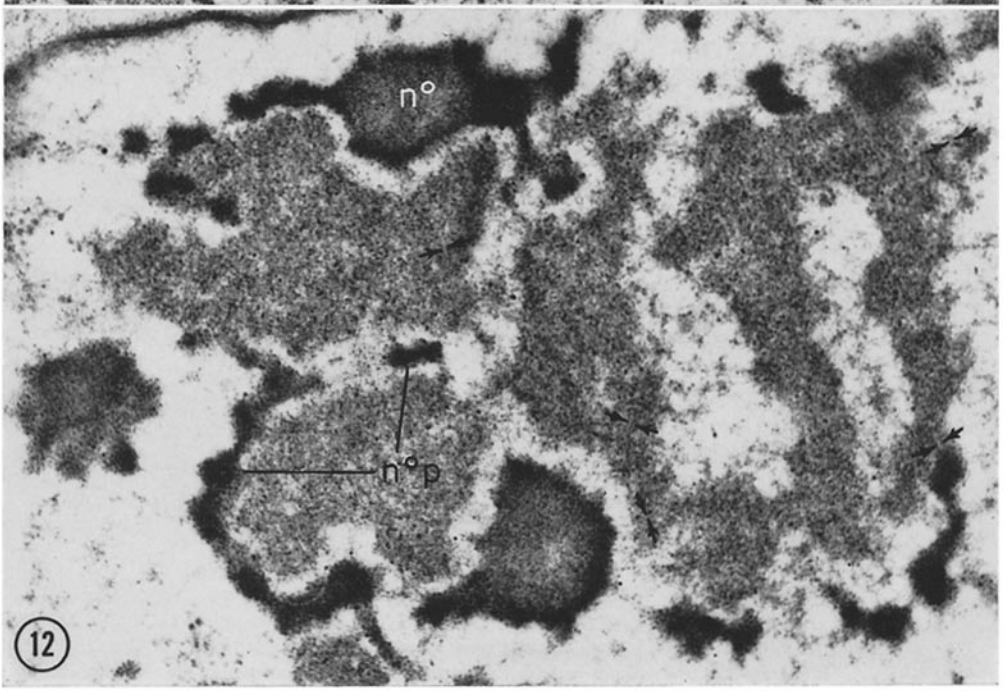
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present, the existence of a nucleolonema-like structure has been confirmed in many cases. There has been no agreement among earlier authors (26, 29, and 30) as to its structural composition, but recently it has been claimed (27) that there is no constant ultrastructural difference between nucleolonema and pars amorpha except that both the particles and the fibrils are densely packed in the former. It was also observed that the proportion of these two elements may change markedly in either region, and that this could explain the observed variation in nucleolar morphology (27) or nucleolar type (9). We have observed a single large nucleolus (Fig. 16) showing a nucleolonema-like structure which can be similarly accounted for by assuming a closer aggregation of the elements (particularly the fibrils, as suggested in the micrograph) normally more dispersed in the internum. Taken together, these observations lend support to the view of Serra (31) that, when present, the nucleolonema should be considered a normal physicochemical "artefact."

It has of late become increasingly evident that the typical interphase nucleolus characteristic of most cell nuclei is formed from prenucleolar bodies derived from anaphase-telophase chromosomes. References to these bodies can be found in the older literature, and recently Lafontaine (9) first demonstrated them with the electron microscope. The prenucleolar bodies accumulate and consolidate at a particular chromosome locus, the nucleolus organizer. In this respect, an occurrence of unorganized, multiple nucleoli may be regarded as primitive to the typical organized condition, assuming an evolutionary acquisition of the organizer (32). The salivary gland nucleus of *Bradysia* with a non-functional organizer (it is functional in other tissues of the larvae) then resembles the primitive condition. This condition shows many loci producing elementary nucleoli, which finally develop into large multiple nucleoli. On our previous assumptions the large nucleoli in our material would strictly correspond to the prenucleolar bodies in typical nuclei taken to represent the more evolved condition. We infer from these therefore that the initial formative events described in *Bradysia* occur in typical nuclei as well, and that in general there is a coparticipation of many genes in nucleolar formation. This implies that the nucleolus functions as an intermediary in the transfer of information from genes.

The similarity of structural elements in nucleoli and chromosomes found in the present study agrees with the findings of other workers in different materials (9, 27, 33, and 34). In our material, however, the similarity has been traced to what we believe is an earlier stage of nucleologenesis (*i.e.*, the elementary nucleoli) than hitherto observed

FIGURE 13

Part of a chromosome with bands, interbands, and large nucleoli (n^o). The fibrillar nature of the chromosome is apparent. The electron opacity is highest in nucleoli, intermediate in chromosome bands, and lower in interbands and nuclear sap. The dual structure of the large nucleoli in this and in previous figures may be compared with the later nucleolar stages shown in Figs. 14 and 15. From a thin section of Araldite-embedded tissue. × 16,000.

FIGURE 14

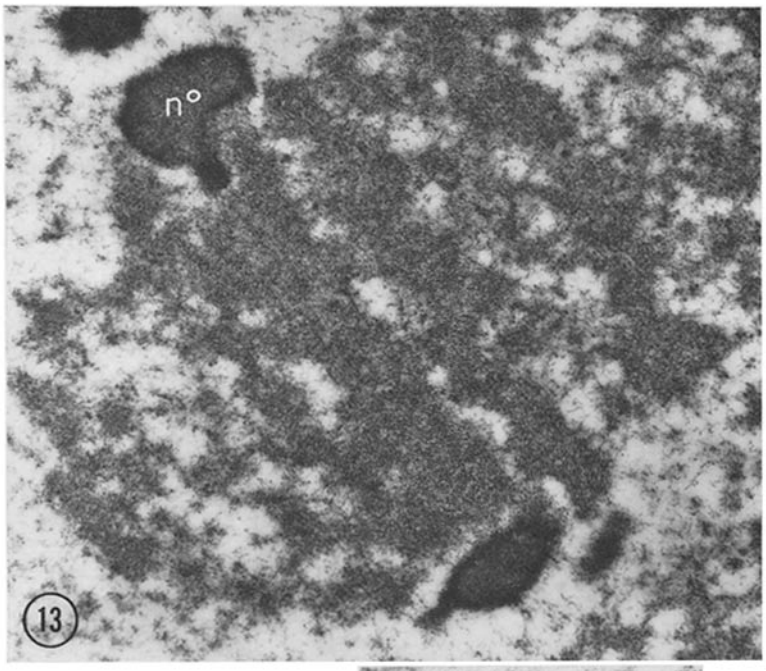
A late nucleolus attached to a chromosome band. Dense areas (one of which has a vacuole) have appeared in the internum of this nucleolus which is apparently disintegrating. From a thin section of methacrylate-embedded tissue. × 42,000.

FIGURE 15

A late nucleolus with almost the entire internum appearing secondarily dense. From a thin section of Araldite-embedded tissue. × 37,000.

FIGURE 16

One exceptional nucleolus with a central nucleolonema-like structure. The paired fibrils (arrows) and particles appear to be more closely aggregated in this structure than normally. From a thin section of methacrylate-embedded tissue. × 24,000.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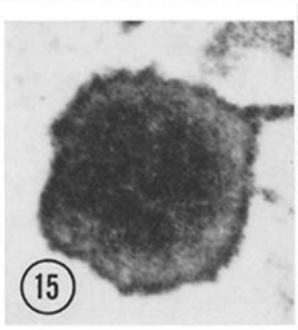
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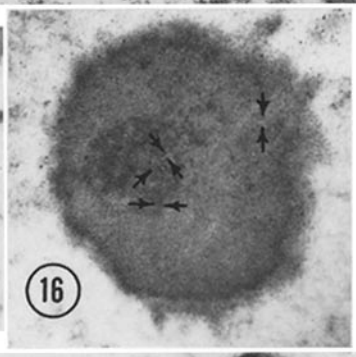
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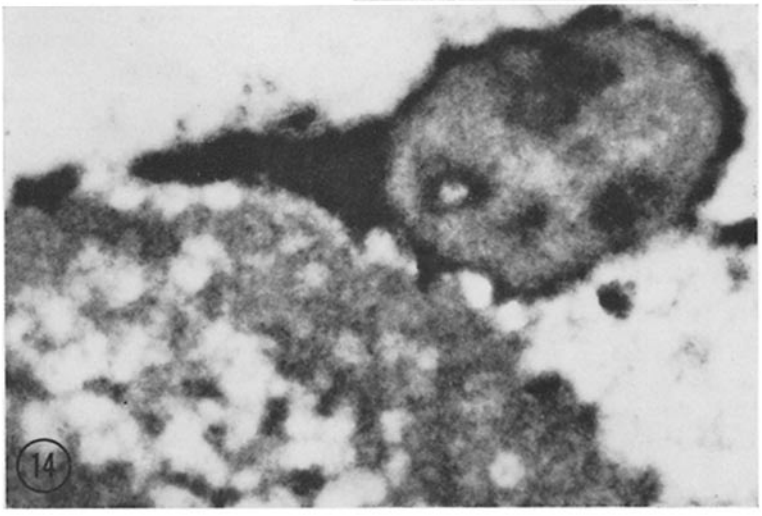
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(see references 9–11). The elementary nucleoli are in intimate association with the chromosome, and the fibrils in both are essentially similar; both contain particles, presumably of ribonucleoprotein. The increasing evidence for the general occurrence of fibrils in nucleoli perhaps justifies a consideration of their nature. Two possible derivations of the fibrils might be envisaged: (1) The first is that certain chromosome fibrils in bands initiate the formation of elementary nucleoli and later become part of the developing nucleoli. While this paper was being revised a similar hypothesis was put forward (35) based solely on observations on grown nucleoli not including the formative stages as studied here. Two implications resulting from the first hypothesis are: (*a*) that nucleoli should contain DNA in the chromosome fibrils that have become nucleolar, which must be weighed against the general lack of demonstrable DNA in nucleoli; (*b*) that the continuity of certain chromosome fibrils is broken, at least momentarily, during release of the prospective nucleolar fibrils. (2) A second hypothesis would be that the nucleolar fibrils consist of RNA newly synthesized (perhaps as strands) in the genes concerned. This would agree with recent cytochemical observations with the electron microscope, which indicate that nucleolar fibrils are DNAase-resistant and that they presumably contain high polymer RNA (36, 37). A decision between these two alternative hypotheses depends on further work.

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