THE BEADS IN THE GOLGI COMPLEX-ENDOPLASMIC RETICULUM REGION

M. LOCKE and P. HUIE

From the Department of Zoology, University of Western Ontario, London, Ontario, Canada N6A 5B7

ABSTRACT

The region between the rough endoplasmic reticulum (ER) and the Golgi complex has been studied in a variety of insect cell types in an attempt to find a marker for the exit gate or gates from the ER. We have found that the smooth surface of the rough endoplasmic reticulum near Golgi complex transitional elements has beadlike structures arranged in rings at the base of transition vesicles. They occur in all insect cell types and a variety of other organisms. The beads can be seen only after staining in bismuth salts. They are 10–12 nm in diameter and are separated from the membrane and one another by a clear halo giving them a center to center spacing of about 27 nm. The beads are not sensitive to nucleases under conditions which disrupt ribosomes or remove all Feulgen staining material from the nucleus. Under conditions similar to those used to stain tissue, bismuth does not react in vitro with nucleic acids. The component of the beads that stains preferentially with bismuth is therefore probably not nucleic acid.

Most insect Golgi complexes (7, 16) are smaller and structurally simpler than their vertebrate counterparts (18). Nevertheless, these small Golgi complexes (GC) (Fig. 1) are involved in numerous activities. In the fat body, for example, they are concerned with the synthesis of blood proteins (10), the formation of isolation membranes related to autophagy (9, 16), and the formation of primary lysosomes necessary for both autophagy and heterophagy (16), all without gross structural differentiation. If there is a single outlet to the GC from the rough endoplasmic reticulum (RER), then the GC must be the distributing center for the various luminal proteins and membranes involved in these activities. The work described here came about as a result of searching for a marker for the exit gate or gates from the RER to the GC. The clear temporal separation of activities in a sequence made cells from the fat body and epidermis of *Calpodes* favorable experimental material for studying these aspects of structure and function. We have found a characteristic beadlike particle in the region between the RER and the GC in all insect cells as well as in cells from other organisms.

The initial objective was to try to localize the energy-dependent step in the passage of secretory material through the GC. In the guinea pig pancreas the transport of secretory proteins from the cisternae to the condensing vacuoles is an energy-dependent process that is blocked by respiratory inhibitors (N_2, cyanide, Antimycin A). The energy-dependent step lies between the transitional elements of the RER and the small, smooth-surfaced vesicles at the periphery of the GC (6). Gomori-type reactions involving the precipitation of lead phosphate after the enzymatic formation of free phosphate give a questionable localization of energy-releasing phosphatases because of the distance diffused before precipitation (4, 5). How-

ever, if phosphates are part of a cell structure, either naturally or through fixation, their presence might be detectable if they bound to or precipitated heavy metals. Bismuth oxynitrate has been used to stain plant cells where it was assumed to bind to the exposed phosphate of nucleic acids (2). We therefore treated insect tissues with bismuth salts and looked for a localization, particularly in the transition vesicle region (13). Whether or not this rationale is correct, after many trials we now have a procedure that consistently shows beadlike structures not visualized by other methods. More detailed observations on the variety of other structures stained and a rationale for the binding are discussed elsewhere. This paper is concerned with the rings of beads found only in the GC-endoplasmic reticulum transition region after bismuth staining.

MATERIALS AND METHODS

Electron Microscopy

Tissues of fifth-stage larvae of *Calpodes ethlius* (Lepidoptera) were fixed for 1 h at room temperature either in 5.0% glutaraldehyde with 2.0% sucrose in 0.05 M Na cacodylate buffer at pH 7.4, or in 5.0% formaldehyde prepared freshly from paraformaldehyde, with 4.0% sucrose in 0.05 M Na cacodylate buffer, at pH 7.4. The tissues were then washed with 0.1 M triethanolamine HCl buffer at pH 7.0 and allowed to react for 1 h at room temperature in the bismuth solution. Tissues were postfixed in cacodylate-buffered 1% osmium tetroxide, embedded in Araldite, sectioned, and observed at 80 kV on a Philips 300 electron microscope without further enhancement of contrast. In addition, nonosmicated tissue was viewed at 100 kV to show the arrangement of beads in the depth of 0.25-μm thick sections.

Preparation of Bismuth Solution

The reaction mixture was prepared by dissolving 200 mg bismuth oxynitrate ($BiONO_3 \cdot H_2O$, Alfa Inorganic, Beverly, Mass.) in 10 ml 1 N NaOH containing 400 mg Na tartrate (1). One part of this solution was added to five parts of 0.2 M triethanolamine HCl buffer at pH 7.0 and the pH adjusted with HCl to 7.0. The final mixture was clear and stable for several days when stored at 4°C.

Enzyme Treatments

Tissues freshly fixed with formaldehyde were washed for 2–3 h in Na cacodylate buffer at pH 7.4 with 10% sucrose. The tissues were then rinsed in 0.1 M Tris-HCl buffer, pH 7.2, and allowed to incubate in either an enzyme solution or a buffer control solution at room temperature for 60–75 min. DNA digestion was carried out in 2 mg/ml DNase (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M Tris-HCl buffer at pH 7.2. RNA digestion was carried out with either RNase (ICN Pharmaceuticals, Inc., Cleveland, Ohio), or RNase T_2 (Sigma) at a concentration of 2 mg protein to 1 ml 0.1 M Tris-HCl buffer, pH 7.2. To test the effects of these treatments, enzyme-incubated and buffer-incubated samples were observed by light microscopy.

The effectiveness of the DNase was determined by the Feulgen reaction. Samples were hydrolyzed (8 N HCl, at room temperature for 30 min), washed with distilled water, stained in Schiff's reagent, and counterstained in 0.5% alcoholic fast green. This DNase treatment made all nuclei Feulgen negative.

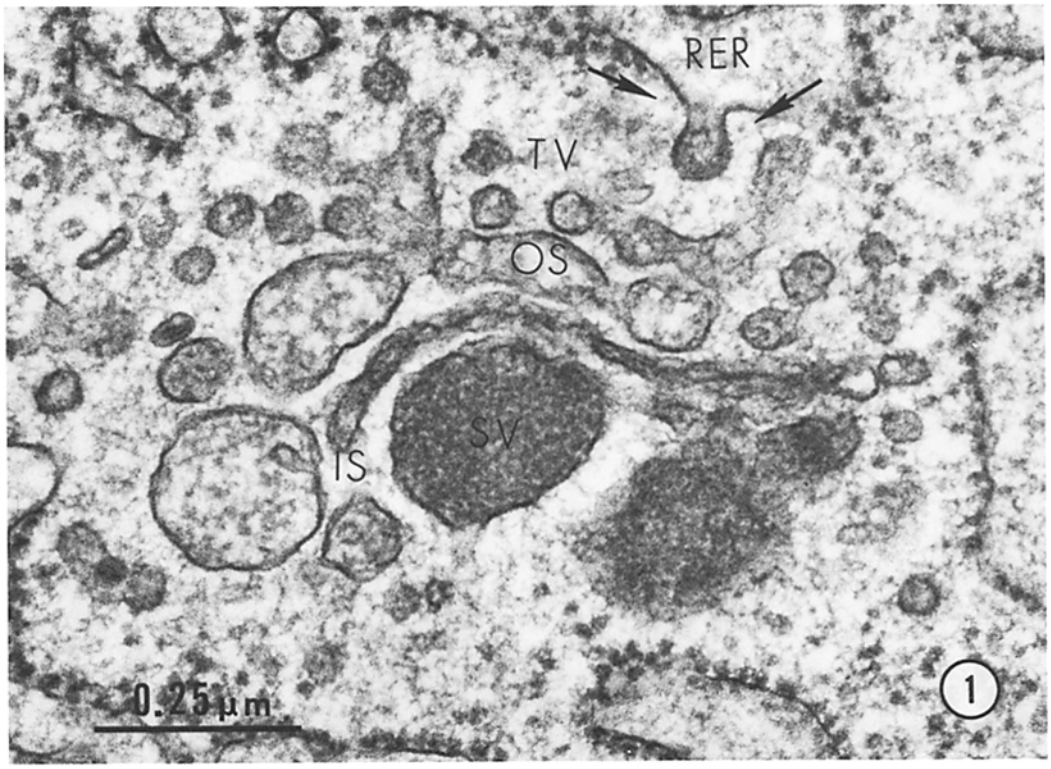
The effectiveness of RNase was determined by staining with freshly prepared methyl green-pyronin Y for 1 h at room temperature. This RNase treatment removed all pyronin-staining material. The effectiveness of the RNase was also deduced from the swollen ribosomes seen by electron microscopy.

Tissues selected for further study by electron microscopy were stained in bismuth solution as described above. Several variations of this procedure were also tried. To minimize background bismuth binding, tissues were refixed with glutaraldehyde before staining in the bismuth solution. Where it was important to note ultrastructural changes in cell components resulting from enzyme digestion, samples were en bloc stained with uranyl acetate (15) and later stained with uranyl acetate (UA) on the section.

RESULTS

The Morphology of the Beads and their Arrangement

Glutaraldehyde-fixed tissue stained with bismuth showed a similar specificity in all *Calpodes* cell types. In the region of all Golgi complexes, beadlike deposits of bismuth are arranged in subequatorial rings at the base of transition vesicles where these arise from the smooth-surfaced membrane of the RER (Figs. 2–4). The gross enlargement of particular beads in Fig. 5 shows their size relative to that of ribosomes (Fig. 6). Under these conditions which show the beads clearly, the ribosomes are not stained and the enlargements of ribosomes are from conventionally stained material. The beads are about 10–12 nm in diameter. They have an angular or irregular outline which may indicate subunits (Fig. 9). Under normal physiological conditions all beads are remarkably constant in their dimensions. Variations in the staining procedure caused little difference in the beads, which were either fully stained or without stain and invisible. We conclude that the procedure described leads to saturation of the binding



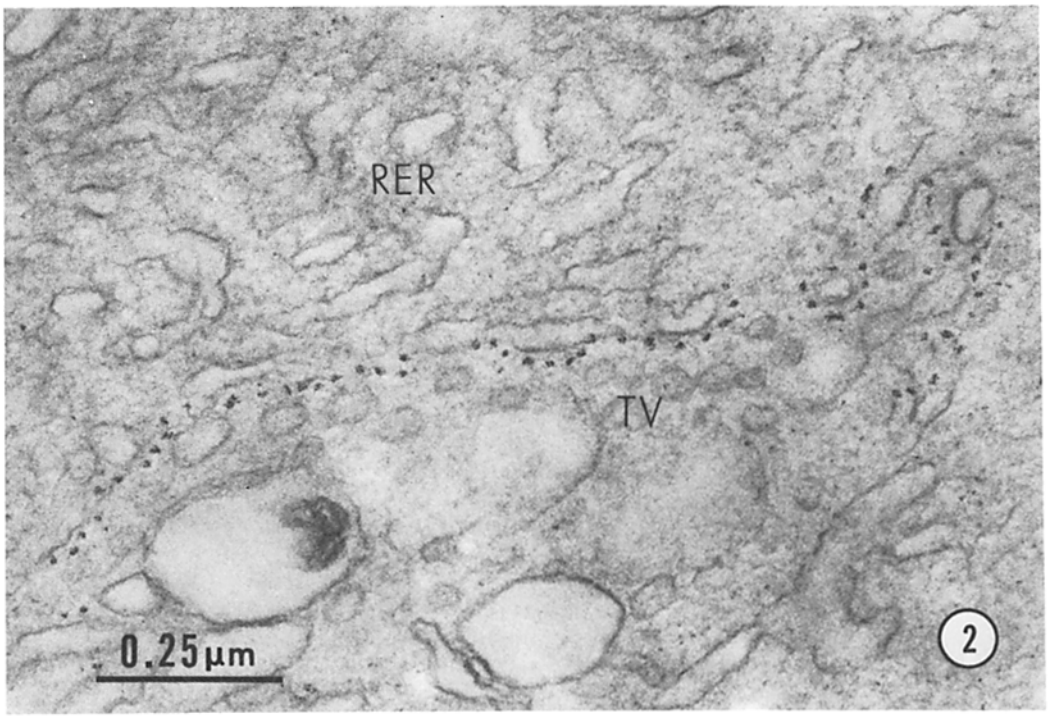
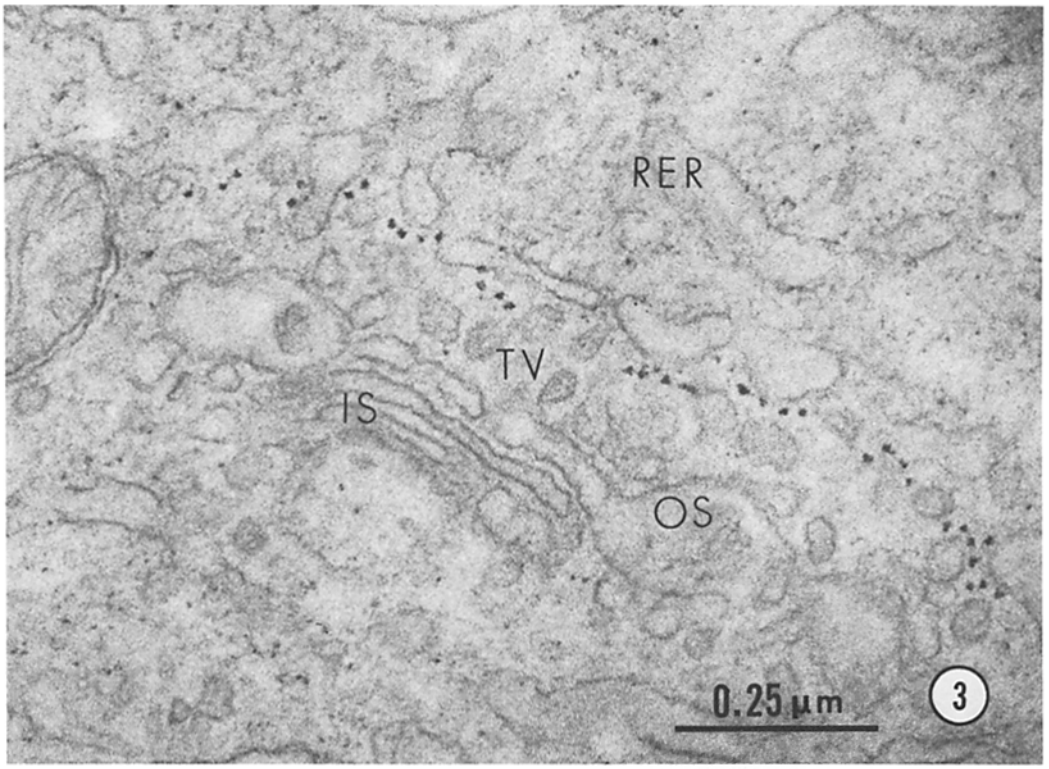


FIGURE 1 The morphology of a typical insect GC. Section stained with uranyl acetate and lead citrate. The beads may be stained slightly (the arrows point to particles in the right place) but they cannot be resolved easily against the other components. *RER* = rough endoplasmic reticulum; *TV* = transition vesicle region; *OS* = outer GC saccule; *IS* = inner GC saccule; *SV* = secretory vesicle. Fat body cell. Fixation: glutaraldehyde, osmium tetroxide. × 110,000.

FIGURE 2 The distribution and relative abundance of beads in a GC. This survey picture of a silk gland shows beads restricted to one side of the transition vesicle region *(TV)* between the RER and the first outer GC saccule. They are found nowhere else in the cell. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 100,000.



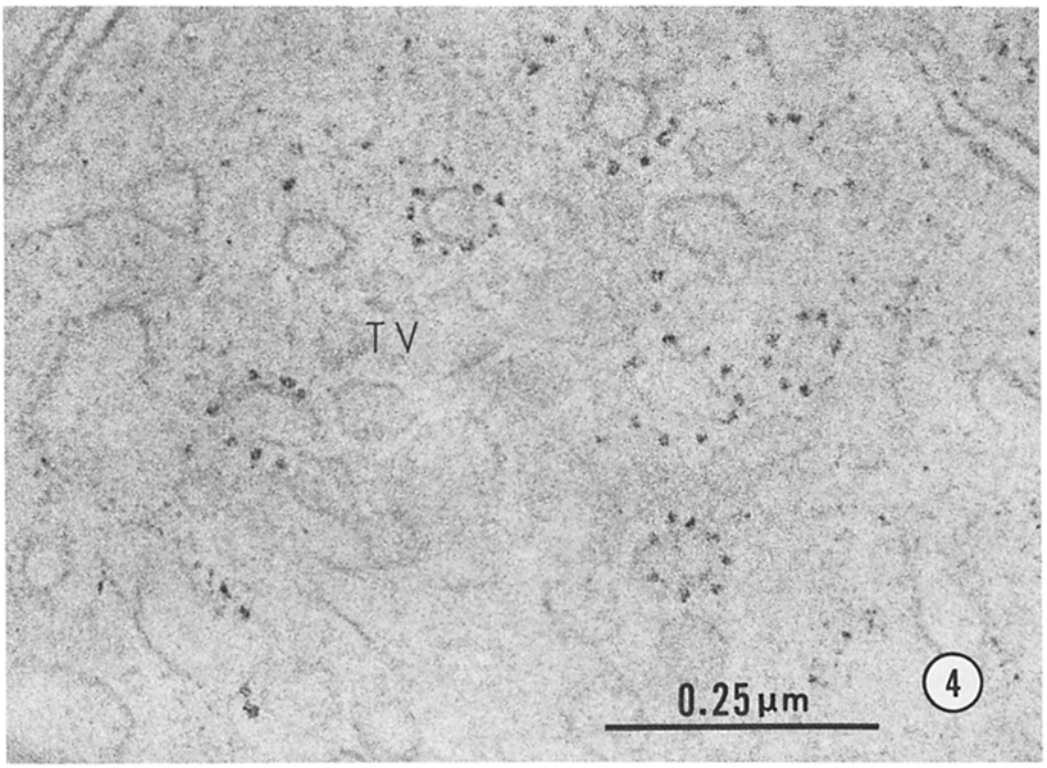


FIGURE 3 The beads are in one plane at the smooth surface of the cisterna of RER on the forming face of the GC. The section is parallel to the forming/secreting axis. Epidermal cell GC. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 110,000.

FIGURE 4 Within the plane the beads are in rings around transition vesicles where the vesicles are confluent with the smooth surface of the cisternae of RER on the forming face of the GC. This section is in a plane normal to the forming/secreting axis and through transition vesicles close to the forming face. Epidermal cell. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 150,000.

sites and the small size of the bismuth aggregates reflects a correspondingly small structure.

Bismuth staining after glutaraldehyde fixation results in virtually no general scatter of grains through the cell. The densities due to bismuth can all be correlated with particular structures. In spite of the clearness of the reaction it would be very difficult to associate the beads with a particular part of the GC if it were not for the consistency and symmetry of their spacing with respect to one another. When beads are seen near to one another in very thin sections (that is, the plane of the beads must then be very close to the plane of the section), they are separated by about 27 nm center to center (Fig. 7 and Table I). The bead center to membrane surface distance was measured when the beads were near to a normally cut membrane profile and was found to be about 14 nm, that is half the center to center separation (Fig. 8). In some preparations the beads are surrounded by a clear halo corresponding to the distance separating them from the membrane and from one another (Fig. 9). These observations suggested that the bismuth-staining beads are cores for larger unit structures found attached in strings both to one another and to the membrane. This interpretation is represented diagrammatically in Fig. 10.

Thick sections show that these strings of beads are always arranged in rings. Sections of unosmicated tissue were cut with thicknesses up to 500 nm and viewed at 100 kV. The membranes in these preparations cannot be distinguished because the lack of osmium leaves bismuth as the only significant cause of electron scattering. This allows us to view thick sections containing hundreds of contiguous beads in order to determine their three-dimensional configuration. Fig. 11 shows such a thick section which includes most of the forming face of a complete Golgi complex. The beads all appear in rings. The projections of the rings may appear circular or elliptical, or they may overlap, but single beads or open-ended strings are absent. The conclusion that the beads are all in rings was confirmed by stereopairs of photographs of these thick sections. Counts of the numbers of beads making up complete rings showed that most rings had 10 beads. In 42 rings chosen because of their complete outlines and uniform bead separation, the numbers of beads were: 8, 14%; 9, 29%; 10, 36%; and 11, 21%.

The rings of beads were always associated with

TABLE I

The Center to Center Separation of Beads

No. in ring	No. of observations	Mean separation	95% confidence interval
		nm	*nm*
8	64	28.1	26.7–29.4
9	80	27.1	25.3–28.9
10	108	24.6	23.6–25.7
11	55	28.9	27.0–30.7
12	90	26.8	25.5–28.1
	Total 397	Mean 26.8	26.1–27.4

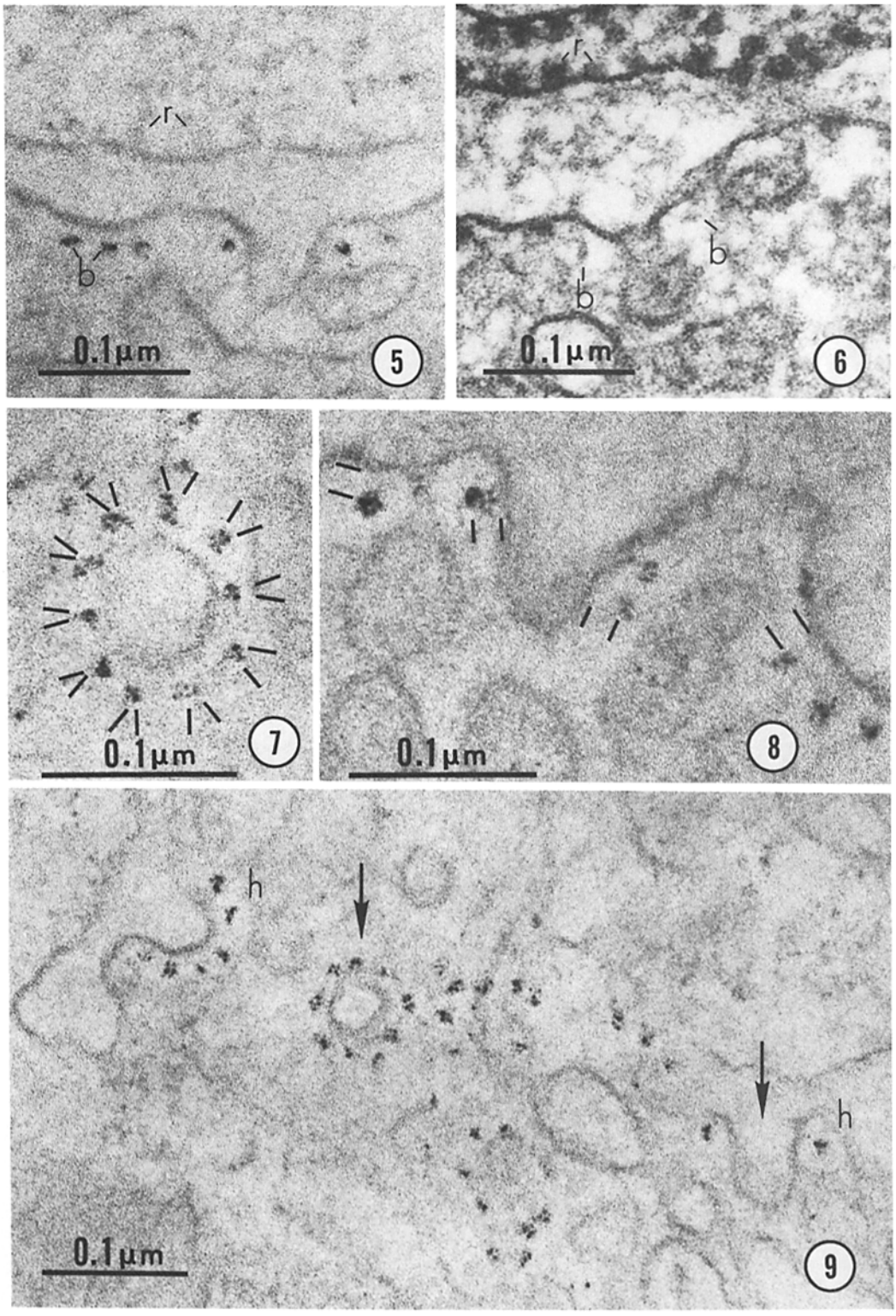
These observations were made upon about 20 different *Calpodes* larvae. The separation was similar in measurements of crayfish.

FIGURES 5 and 6 The size of the GC beads relative to ribosomes. The beads *(b)* are unlike ribosomes *(r)* in being smaller and are separate from the membrane. Comparable areas of epidermal cell RER with forming transition vesicles are shown stained by bismuth, Fig. 5 (Treatment: glutaraldehyde, bismuth, osmium tetroxide, contrast is due to bismuth and to the osmium used in postfixation), and by uranyl acetate, Fig. 6 (Treatment: glutaraldehyde, osmium tetroxide, uranyl acetate followed by uranyl acetate section staining). × 240,000.

FIGURE 7 The center to center spacing of beads in rings. Profile of beads in a ring that is very nearly in the plane of the section. The mean center to center spacing marked by parallel bars is 27 nm. Epidermal cell. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 310,000.

FIGURE 8 The center to membrane spacing of beads. The separation between bead center and membrane surface marked by parallel bars is about 14 nm, half the center to center spacing. Epidermal cell. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 290,000.

FIGURE 9 The beads are surrounded by a clear halo *(h)*. The bismuth-stained bead appears as the core of a lighter staining region that may determine both the spacing between beads and their separation from the membrane. The beads often appear to be formed from grains giving them an angular or square outline. Arrows mark views corresponding to the two section planes interpreted in Fig. 10. Epidermal cell. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 220,000.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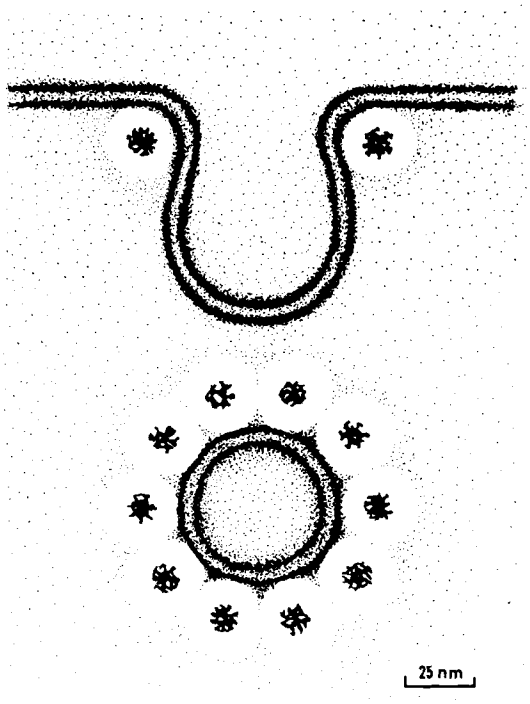


FIGURE 10 The arrangement of beads aroknd transition vesicles. Scale diagrams of the beads and their arrangement near the transition vesicles attached to the membrane of the endoplasmic reticulum.

the smooth-surfaced membrane of the RER from which transitional elements arise which then go on to make the saccules of the GC. The profiles showing circular rings around transition vesicles are probably always of vesicles still attached to the RER. The rings are subequatorially arranged at the stalk attachment of the vesicle to the membrane, rather than being equatorially arranged upon free vesicles. This association of the beads with forming vesicles suggested that they might occur in relation to other vesicles in continuity with membranes. A careful search of pinocytotic vesicles in pericardial cells and synaptic vesicles in nerve terminals showed no beads on or near coated or uncoated vesicles in parts of the cell away from the GC. In crayfish X organ neuropile the synapses had a granular bismuth deposit on the cytoplasmic face, but this was not a beaded surface. Nor were there beads near vesicles in continuity with the outer saccular face of the GC, the presumed destination of the transition vesicles. So far, we have found beads associated with forming transition vesicles and nowhere else in any cell type.

The specificity of the bismuth makes the beads easily visible. The question arose as to whether the failure to recognize the beads after other staining methods was due to their complete lack of stain or due to a failure on the part of the observer to discern such small particles on a background of similar density. A careful search of the transition vesicle region in tissue stained in block with uranyl acetate showed particles that could be beads, although they were difficult to identify. The dimensions and separation between these particles were similar to those of the particles visualized by bismuth staining. Nonspecifically stained particles that may be beads can perhaps be made out in prints of favorably oriented tissue that have already been published (see, for example, references 8, Fig. 40; 20, Fig. 16; 3, Figs. 25 and 33).

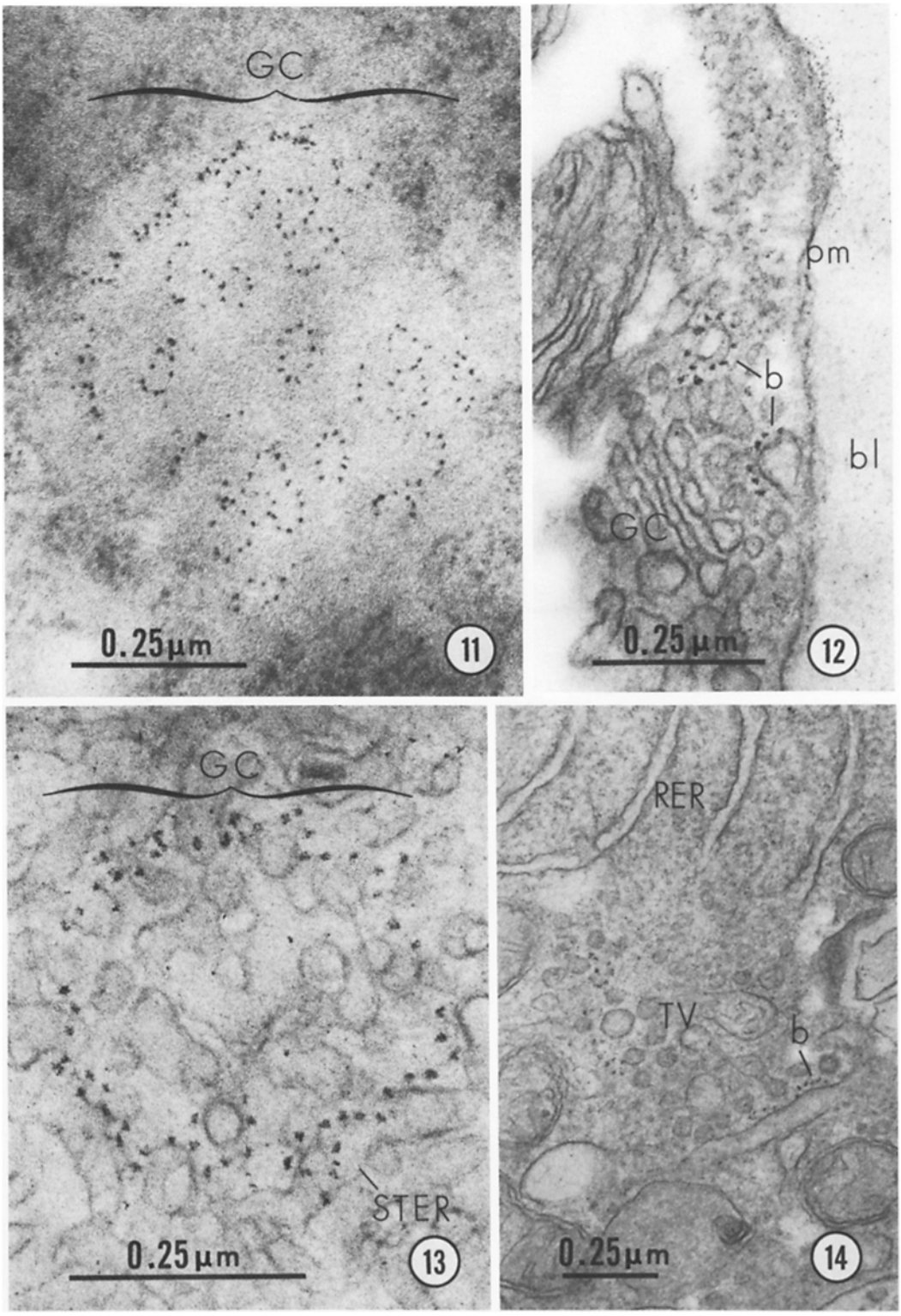
We conclude that the beads bind other heavy metals to such a limited extent that their presence would not be detected by conventional staining procedures.

FIGURE 11 The arrangement of bead rings in the GC. The pattern of rings formed by beads in a 250 nm section of the Golgi complex from an epidermal cell viewed at 100 kV. Almost all the beads are in rings. Treatment: glutaraldehyde, bismuth only. × 110,000.

FIGURE 12 Beads in the GC from muscle. Even the rather rare and diminutive GC from muscle have beads *(b)*. *pm*, plasma membrane; *bl*, basal lamina. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 110,000.

FIGURE 13 The small GC of oenocytes have beads where transition vesicles arise from the smooth tubular endoplasmic reticulum *(STER)*. Treatment: glutaraldehyde, bismuth, osmium tetroxide. × 150,000.

FIGURE 14 The beads *(b)* occur in GC from other arthropods such as those in a crayfish hepatopancreas cell. *TV*, transition vesicles; *RER*, rough endoplasmic reticulum. Treatment: glutaraldehyde, bismuth, osmium tetroxide, × 60,000.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The Distribution of the GC Beads in Various Tissues

The GC beads were first noticed in the epidermis and fat body at times when the GC was primarily concerned in distributing cuticular and blood proteins. In both these cell types the GCs are small and simple structures compared to those of vertebrates, consisting only of a few transition vesicles, one or two outer saccules, one or two inner saccules, secretory vesicles, and a few microvesicles, some of which may be coated (Fig. 1) (16, 7). We therefore surveyed GC from a number of other tissue types to confirm the generality of the localization.

The silk glands are engaged in a massive synthesis of silk proteins and the GC are large with an extensive smooth face of RER and many blebs extending into the transition vesicle region. Fig. 2 shows the beads confined to this region.

The oenocytes are giant polypoid cells engaged in steroid and wax metabolism and have an extensive smooth tubular endoplasmic reticulum. Depending upon the definition of a Golgi complex, the oenocytes have two sorts of GC, perhaps reflecting stages of development: there are aggregates of acid phosphatase-containing microvesicles presumed to be primary lysosomes, and diminutive but more typical GC with a few transition vesicles and a very few saccules and secretory vesicles (8). It is as if some GC have become mainly production centers for primary lysosomes, the end result being a cloud of microvesicles that later become functional in the massive autophagy which takes place at metamorphosis. Although the GC are small and easily overlooked, patches of beads are easily resolved (Fig. 13). These beads are at the tips of smooth tubular endoplasmic reticulum, suggesting that there may be a homology between the smooth face of RER where transition vesicles arise and these smooth tubules connecting regions with bound ribosomes to regions where microvesicles arise to make the forming face of the oenocyte GC. Such a homology may be favored by the finding that the cisternal contents of oenocyte smooth tubular endoplasmic reticulum and the contents of transition vesicles are both osmiophilic (16, 11).

GC beads have also been found in muscle (Fig. 12), nerve, glial cells, hemocytes, pericardial cells, and tracheal cells. We conclude that in *Calpodes*, at least, the GC beads are a fundamental feature of GC.

The Occurrence of the Beads in Other Organisms

The ubiquity of the beads in GC from all *Calpodes* tissues suggested that they might have an even wider distribution. We therefore examined epidermis and fat body from mealworm larvae (*Tenebrio*, Coleoptera) and adult locust (*Locusta*, Orthoptera), and digestive gland and X organ from a crayfish (*Orconectes*, Crustacea) (Fig. 14). In all of these organisms the beadlike structures were found on the smooth face of the RER juxtaposed to the forming face of the GC.

We supposed from the distribution in several different arthropods that the beads might be a fundamental feature of all or most GC. So far, we have not found them in GC from onion root tip cells, anemone tentacles, clam gills, polychaete gills, frog liver, and pancreas or mouse liver and pancreas, although other cell components such as the nucleolus show the same pattern of bismuth binding as in *Calpodes*.[1]

The Nature of the Beads

Bismuth binds to several cell components in different ways. After formaldehyde fixation many basic proteins bind bismuth through their amino groups.[2] Glutaraldehyde crosslinks proteins through their amino groups, and this fixation blocks most bismuth binding but not staining of the beads. It seemed possible that the bismuth might be binding to the exposed phosphate of a nucleic acid in the beads, since it had been claimed that chromatin and RNA in onion root tip cells could be stained with bismuth and that bismuth precipitated both DNA and RNA in vitro (2). We therefore tested the effect of nucleases upon the stability of the beads and found that neither RNase nor DNase affected the ability of the beads to bind bismuth. We also tested to see if pure nucleic acids could bind bismuth. RNA and DNA spotted onto cellulose strips were treated by the procedures used to demonstrate the beads. Neither nucleic acid bound bismuth. Current work shows that in the nucleus and ribosomes bismuth binds to proteins rather than nucleic acids (12, 14). This also makes it unlikely that bismuth bind-

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[1] *Note added in proof:* We have now found beads in uranyl-stained mouse acrosomal GC.

[2] Locke, M., and P. Huie. Bismuth staining for light and electron microscopy. Manuscript in preparation.

ing in the beads is evidence for their containing nucleic acid.

DISCUSSION

The discovery of the GC beads is a chastening reminder that there may be many more ordered structures in cells than is apparent from conventionally stained tissues viewed by transmission electron microscopy. Indeed, the beads are only one of several components that appear after bismuth staining and the appropriate fixation. It remains to be seen how widespread the beads may be in different organisms, but it is difficult to think that they are taxonomic oddities of little functional significance. They must surely be related to a basic function of the GC, and if so, their possible absence from some other GC becomes as interesting as their presence.

The only structures comparable to the beads described in the literature are the rosettes of particles seen at freeze-fractured surfaces where mucocyst granules fuse with the plasma membrane in *Tetrahymena* (19). These mucocyst granules have an annulus of 110-Å diam particles which match the distribution of a rosette of 8–11 particles 150 Å in diameter on the plasma membrane. Membrane fusion occurs only at these matching sites. The proposed location of the rosette particles in the membrane is against an exact parallel with the beads, but it will be interesting to see if bismuth stains the *Tetrahymena* rosette particles and if an additional annulus of particles shows up by freeze-etching on the transition vesicles or GC saccules. Such results would be expected if the beads mark the point of return of transition vesicles to the RER. At present, all we can say is that the beads may be related to transition vesicles leaving the RER, to transition vesicles returning to the RER from the GC saccules, or to both processes.

A striking characteristic of the beads is that they are found only near microvesicles associated with one membrane face of the GC. They are not found near microvesicles forming from or fusing with any other membrane systems. It seems unlikely that we have merely overlooked the beads in other locations, and they must therefore have something to do with a kind of vesicle shuttle (6, 17) that is peculiar to the GC. Although we have said that the beads mark the exit gate from the RER, there is no formal evidence yet whether the bead rings mark vesicles moving both ways or in only one direction.

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