

LOCALIZATION OF THIAMINE
PYROPHOSPHATASE ACTIVITY
IN THE GOLGI APPARATUS OF
A MOLLUSC, *HELIX ASPERSA*

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ABSTRACT

Spermatids of the snail *Helix aspersa* were studied after fixation in buffered osmium tetroxide and after applying Novikoff and Goldfischer's method (15) for demonstrating thiamine pyrophosphatase (TPPase) activity both with the light and the electron microscope. The appearance of cells in the light microscope after localizing the enzyme is very similar to the appearance after the application of classical Golgi techniques. The electron microscope shows the "dictyosomes" to consist of non-granular membranes, vesicles, and vacuoles typical of the ultrastructure of the Golgi apparatus. Sites of TPPase activity are localized by deposits of lead phosphate, and are found between the membranes of the Golgi apparatus, in the small vesicles, in multivesicular bodies often found associated with it, but not within the large Golgi vacuoles. Heavy deposits are found on the caudal part of the nuclear envelope, but not in the acrosomal granule. It is suggested that TPPase may act as an intermediary in acrosome formation by the Golgi apparatus or "acroblast" of this cell. The finding of diphosphatase activity in the Golgi apparatus of an invertebrate is suggested as additional evidence for the existence of a homology between the Golgi apparatus of all animal cells.

INTRODUCTION

Novikoff and Goldfischer (15) have shown that diphosphatase activity in a number of cells of rat, mouse, and frog is confined to the Golgi apparatus. On the basis of this activity they have devised a staining procedure based on the Gomori acid phosphatase technique, which permits demonstration of the Golgi apparatus in these cells. Tissues fixed in cold formaldehyde were incubated in a medium buffered near neutrality and containing either a nucleoside diphosphate or thiamine pyrophosphate (TPP) as substrate together with lead ions and activating ions. Phosphate ions liberated by the action of diphos-

phatases were trapped at the sites of origin as insoluble lead phosphate, which can be visualized very readily in the electron microscope after subsequent treatment with a tissue-contrasting agent such as osmium tetroxide, followed by embedding and sectioning.

If diphosphatases are confined to the Golgi apparatus of vertebrate cells, then demonstration of this activity in invertebrate tissues would strengthen the evidence for homology between the Golgi apparatus in all cells throughout the animal kingdom. The following work is an account of the demonstration of sites of thiamine pyro-

phosphatase activity in the Golgi apparatus of spermatids of the snail, *Helix aspersa*.

The Golgi apparatus or "idiosome" of the spermatid of *Helix* appears in sections for the optical microscope as a hyaline "internum" approximately 2μ in diameter, partially or com-

nology that has been applied to this cytoplasmic inclusion by optical cytologists since it was first described in 1874, the reader is referred to Nath (14). However, if the Golgi apparatus is examined in an uncompressed living cell (19), these rods are clearly seen, on altering the level of focus of the

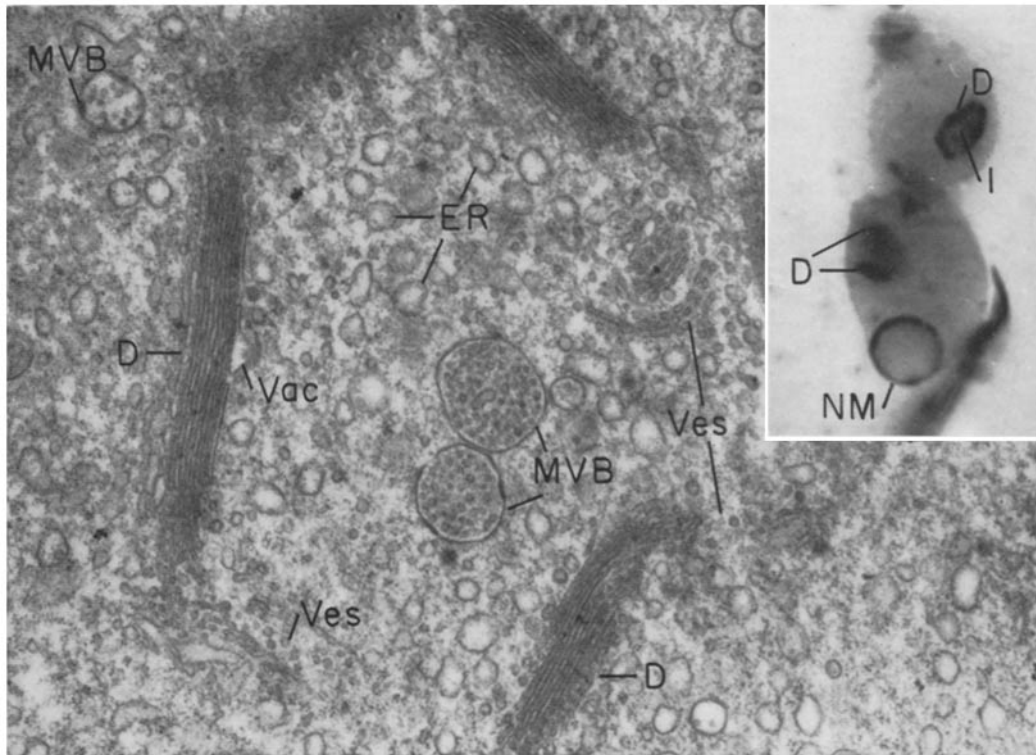


FIGURE 1 (inset)

Photomicrograph of two free, uncompressed early spermatids after inosine diphosphatase visualization. The dictyosomes (*D*) are heavily stained; the internum (*I*) appears lighter than the surrounding cytoplasm. The nuclear membrane (*NM*) also shows staining. Approximately $\times 2000$.

FIGURE 1

Electron micrograph of the typical Golgi complex or acroblast of a spermatid after osmium tetroxide fixation. The dictyosomes are lamellar arrays of non-granular membranes with vacuoles (*Vac*) and vesicles (*Ves*); the latter appear to be pinching-off from the closed ends of the membrane pairs of the dictyosomes (*D*). Multivesicular bodies (*MVB*) are frequently found in the cytoplasm of the internum, which does not otherwise differ from the external cytoplasm in its content of profiles of rough-surfaced endoplasmic reticulum (*ER*) and ribonucleoprotein granules. Permanganate stained. $\times 29,000$.

pletely surrounded by an "externum" made up of straight or slightly curved rods about 1μ in length and 0.25μ in thickness. These rods have been variously described in spermatocytes and spermatids as "batonnettes" or "banana-shaped bodies" (for a review of the intricate and confusing termi-

objective lens, to be in fact flattened, discoid bodies, described by Parat (17) as *éléments en écaille* or "scale-bodies." No structure can be observed by phase contrast in these highly refractile scale-bodies, or "dictyosomes," as they are most frequently termed. They become impreg-

nated very heavily with silver or osmium salts after application of the classical Golgi techniques, although the internum of the structure shows no reaction. In sections of insect spermatids after Golgi impregnation, Bowen (2) has described a lamellar structure, but no such lamellation has

phologically the agranular endoplasmic reticulum of the typical Golgi apparatus of animal cells (4). Large vacuoles are frequently closely associated with these stacks, and small vesicles are frequently seen apparently being pinched off from the closed ends of the membrane pairs. A similar phenome-

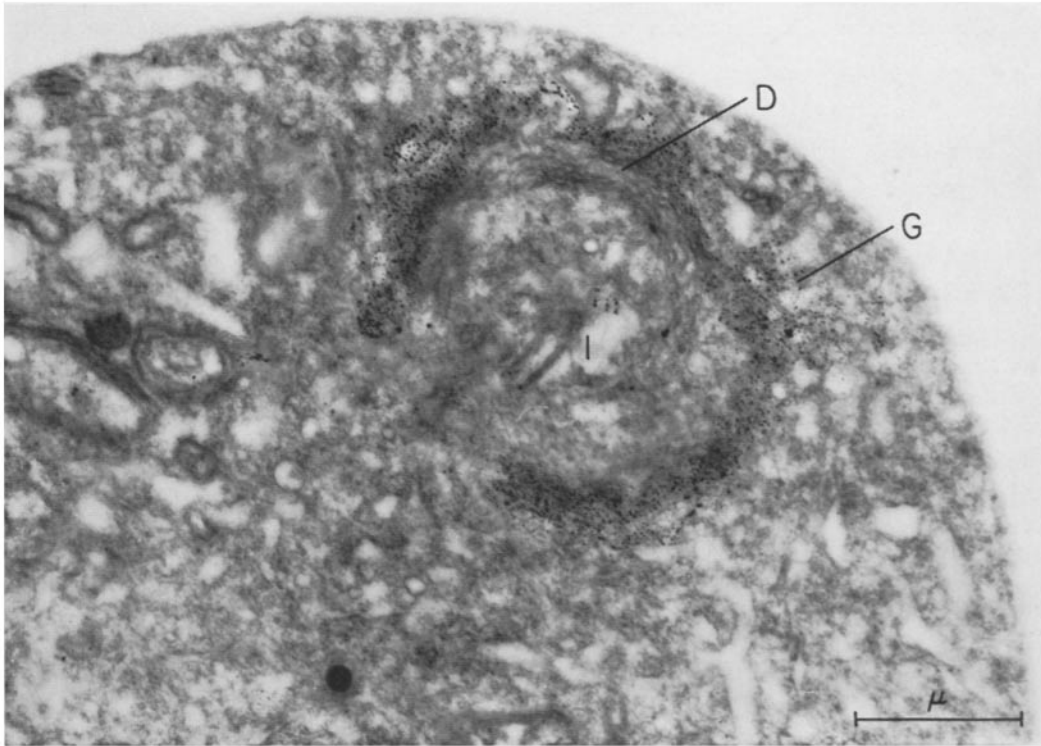


FIGURE 2

The acroblast of a spermatid after the thiamine pyrophosphatase procedure. The general fixation of the cytoplasm is reasonably good; dense granules (*G*) of lead phosphate are seen in the outer membranes of the dictyosomes (*D*); there is little deposit in the internum (*I*). Unstained. $\times 16,000$.

been described in the snail. The function of the Golgi complex, consisting of dictyosomes and internum, in the spermatid is now agreed by most authors to be the secretion of the acrosome (6, 14); we therefore propose to refer to it as the "acroblast," a term first proposed by King (9).

The ultrastructure of the acroblast in spermatids of *Helix aspersa* was first described by Beams and Tahmisian (1) using the electron microscope, and that of the closely related snail, *Helix pomatia*, has been described in great detail by Grassé and his coworkers (7). They have shown the dictyosomes to consist of stacks of flattened paired membranes or saccules closely resembling mor-

non has been reported in vertebrate spermatids (13). The dictyosome of the snail spermatid fulfills all the ultrastructural and classical cytological criteria of a typical and undeniable Golgi apparatus in an invertebrate. We used it therefore as a test object in order to determine whether the technique suggested by Novikoff and Goldfischer as a specific histochemical reaction for the Golgi apparatus in vertebrates might also be specific for the invertebrate Golgi apparatus.

MATERIALS AND METHODS

Well-grown specimens of the common British garden snail, *Helix aspersa*, were collected locally during

June and July, previous experience having proved this season to be best for showing all stages of spermatogenesis, and were kept in large glass jars in the laboratory until required. The animals were killed by decapitation and the upper part of the shell dissected away as rapidly as possible. The ovotestis was removed, cleared of the surrounding digestive gland tissue, and fixed either by using Palade's cold fixative, at pH 7.3 for $\frac{1}{2}$ hour, or by following Nov-

The medium was centrifuged before use to remove a slight precipitate which formed. Initially, the original procedure of Novikoff *et al.* (15) was followed, and 40 μ frozen sections of the tissue were cut after formaldehyde fixation. The very small size of the ovotestis and its diffuse nature made this a very difficult procedure. The results obtained were very capricious; cells close to the edge of the section were poorly preserved and heavily over-impregnated, while

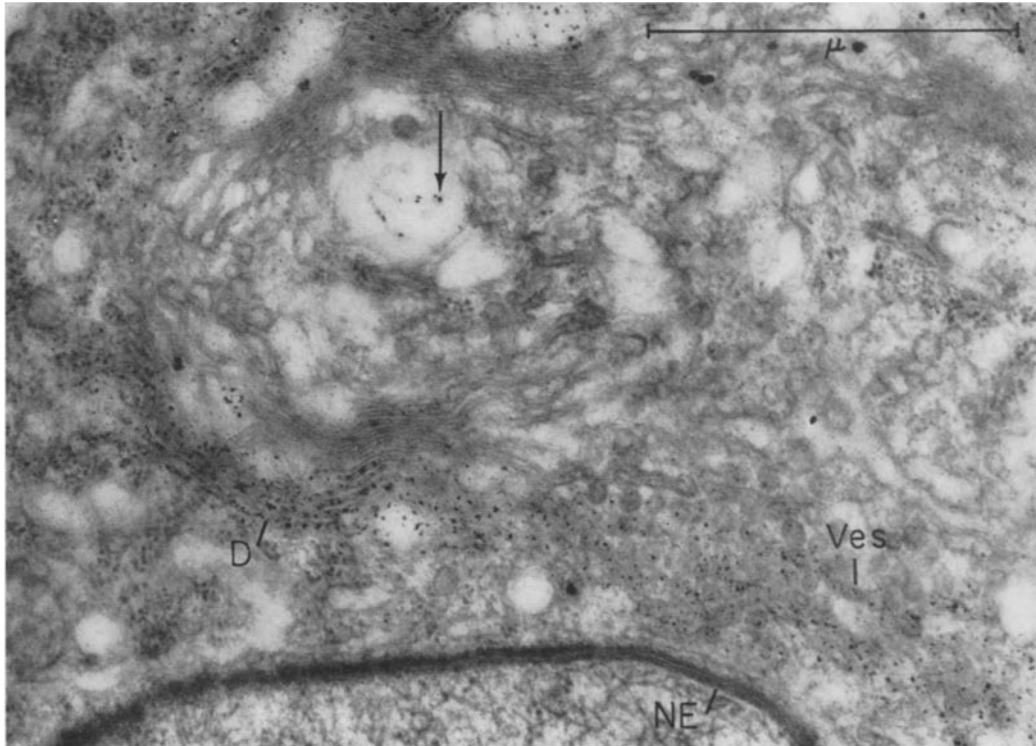


FIGURE 3

Lead phosphate reaction product is seen localised within the dictyosome (*D*) membrane pairs; in the small vesicles in the internum (arrow); and in the small vesicles (*Ves*) associated with the free ends of the dictyosomes. The nuclear envelope (*NE*) is thickening prior to acrosome formation, but no reaction product is seen in association with it. Lead stained. $\times 49,000$.

ikoff and Goldfischer's procedure (15), in which case material was fixed in formol-calcium overnight in the refrigerator. After fixation the ovotestis was teased in cold distilled water and the free cells and fragments of tissue were sedimented by gentle centrifugation and incubated for 15 minutes at 37°C in the incubation medium. This consisted of:

TPP (chloride), 0.03 M in water	0.5 ml
Distilled water	0.2 ml
Tris-maleate buffer, pH 7.2, 0.2 M	1.0 ml
Lead nitrate (1 per cent)	0.3 ml
Manganese chloride 0.025 M	0.5 ml

cells towards the centre of the frozen section showed little or no reaction product. Gentle teasing of the material prior to incubation and its conservation by light centrifugation after the subsequent preparative steps was found to yield a far more homogeneous preparation.

Following incubation the cells were rinsed in distilled water, postfixed for electron microscopy in Palade's fixative for 1 hour at room temperature, dehydrated in the usual graded alcohols followed by epoxy propane, and then embedded in British Ciba Araldite. Samples were taken for optical microscopy

before postfixation in osmium tetroxide, treated with dilute ammonium sulphide for 1 minute, rinsed, and mounted in Farrants' medium. Araldite blocks were sectioned at about 60 $m\mu$ in either a Porter-Blum or a Huxley microtome, the sections being picked up on either bare 200-mesh grids or formvar/carbon-coated grids. Sections were stained with either Millonig's lead stain (12) or Lawn's permanganate stain (10) prior to examination in a modified (11)

carried out and will shortly be reported elsewhere (3).

RESULTS

1. Light Microscope

The appearance of free spermatids after the inosine diphosphate procedure is shown in Fig. 1

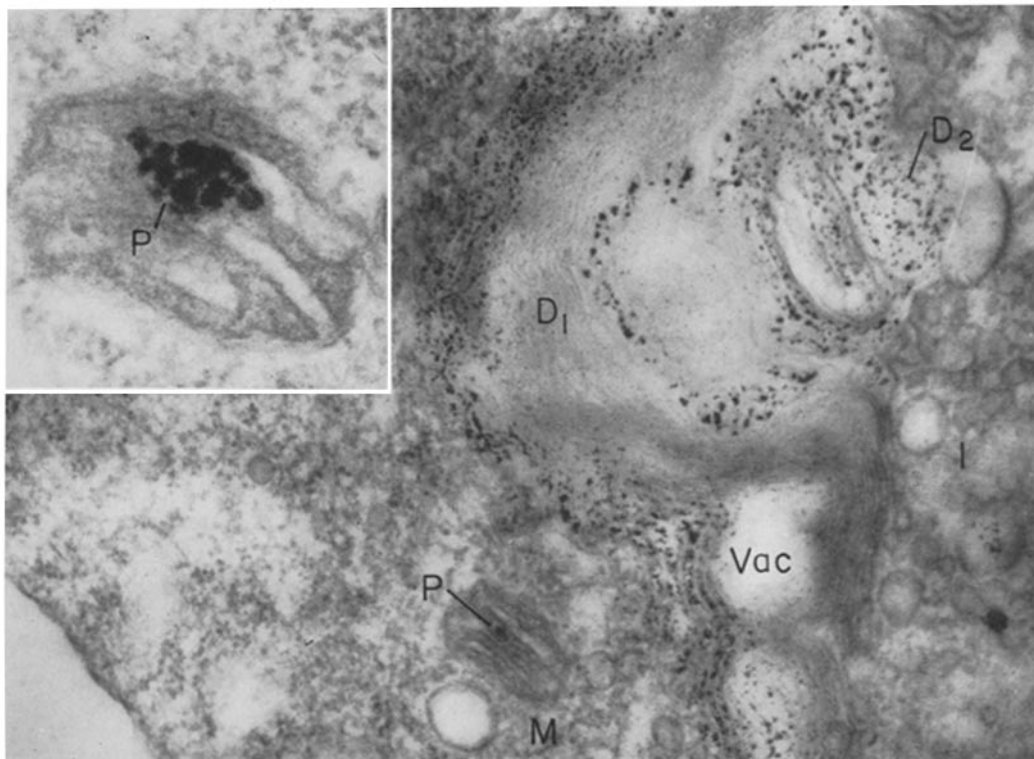


FIGURE 4

Dense granules of lead phosphate are deposited within the saccules of the dictyosomes, shown sectioned perpendicular at D_1 and parallel at D_2 . No deposit occurs within the large vacuoles (Vac), and very little within the dictyosome membranes facing the internum (I). A mitochondrion (M , and inset) contains very electron-opaque granules (P) in its matrix; lead phosphate is possibly deposited at this site. Lead stained. $\times 59,000$; inset $\times 92,000$.

Siemens Elmiskop I electron microscope at 60 or 80 kv, using the double condenser system.

In all cases, parallel controls were prepared in which the cells were incubated in a medium identical with the medium described above, except that the thiamine pyrophosphate was omitted. A few preparations have also been made and examined in which inosine diphosphate (IDP) was used as substrate in the same concentration. Detailed histochemical studies on the ovotestis of *Helix aspersa* have also been

inset. The staining is darker and more intense if IDP is used as the substrate instead of TPP; the preparations yield more satisfactory photographs and hence have been used for illustration. No difference other than in staining intensity can be seen at the light microscope level. The appearance is virtually identical with that after a standard Mann-Kopsch procedure, as far as the dictyosomes are concerned; with diphosphatase preparations

the nuclear envelope is frequently seen to be stained. The dictyosomes stain very heavily and appear as irregularly curved, discoid bodies, as the objective focus is altered. The substance of the internum, enclosed by the dictyosomes, is not stained by diphosphatase methods or by the

curved. In permanganate-fixed material, and sometimes in osmium-fixed material, each membrane can be resolved as a pair of electron-opaque lines approximately 25 Å thick separated by a less dense space approximately 30 Å wide, the unit membrane of Robertson (18). Each mem-

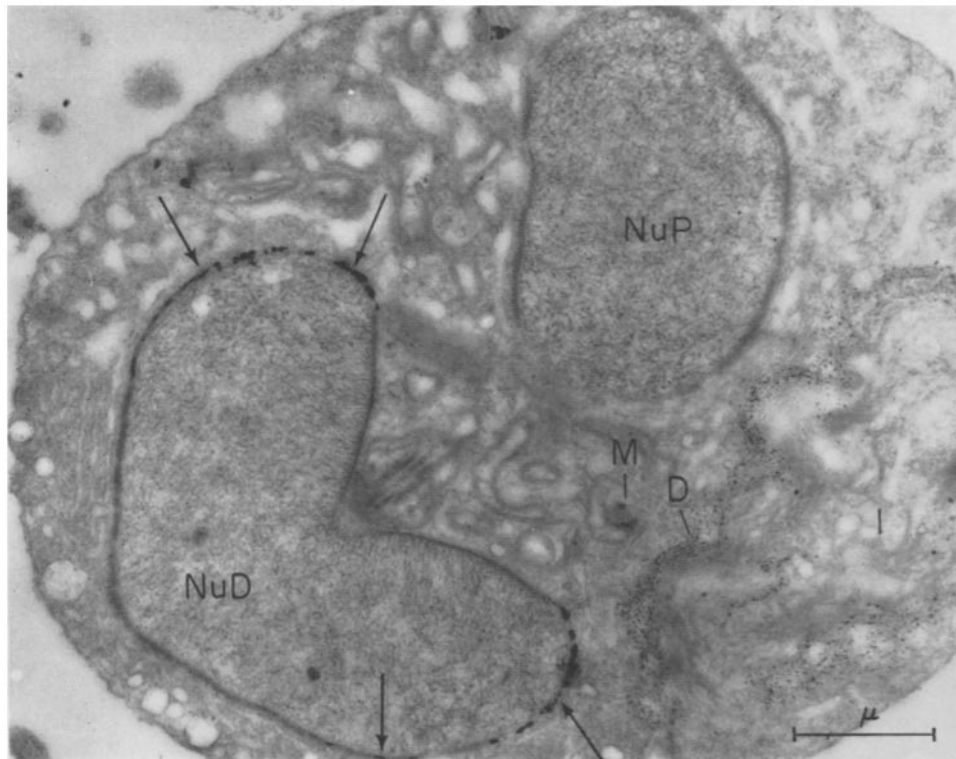


FIGURE 5

A section through a late spermatid of *Helix aspersa*, showing the proximal part (*NuP*) of the sperm head together with the distal part (*NuD*), and the Golgi apparatus *D* and *I*. A very heavy deposit of reaction product (arrows) is seen within the membranes of the nuclear envelope of the distal part; none is deposited in the proximal part in spite of the close apposition of the Golgi apparatus. Reaction product is also associated with the dense granules of the mitochondrion, *M*. Unstained. $\times 19,000$.

Golgi procedures. Control preparations showed no staining.

2. Electron Microscope

In spermatids fixed in buffered osmium tetroxide the Golgi apparatus closely resembles the structure described by Grassé (7) in the related species, *Helix pomatia*. The dictyosomes, when sectioned exactly normal to the axis of the disc, appear as stacks of 8 to 12 paired, non-granular membranes in parallel array (see Fig. 1), usually arranged in straight lines roughly 1.5 μ long, but sometimes

brane pair forms an enclosed "saccule" 15 to 20 $m\mu$ in thickness, containing material which is more electron-opaque than that in which the saccules lie. The profiles of the closed ends of the saccules are frequently spatulate, and numerous vesicles 20 to 50 $m\mu$ in diameter are associated with the free ends of the dictyosomes. These small vesicles appear to arise by a "pinching-off" process from the spatulate ends of the membrane pairs, and they collect in the cytoplasm of the internum. This cytoplasm differs little from the general cytoplasm of the spermatid in its content

of RNP granules and granule-studded, closed membrane profiles, but it is frequently seen to contain multivesicular bodies, which appear as numbers of circular profiles of vesicles of about the same diameter as those seen at the ends of the dictyosomes, enclosed in a vacuole 0.5μ or

appear to be swollen (see Figs. 2 to 4). The most important feature is the appearance of heavy deposits of small, very electron-opaque granules of irregular shape and size, but of the order of 100 A or less in diameter. These granules are, according to Novikoff and Goldfischer, insoluble

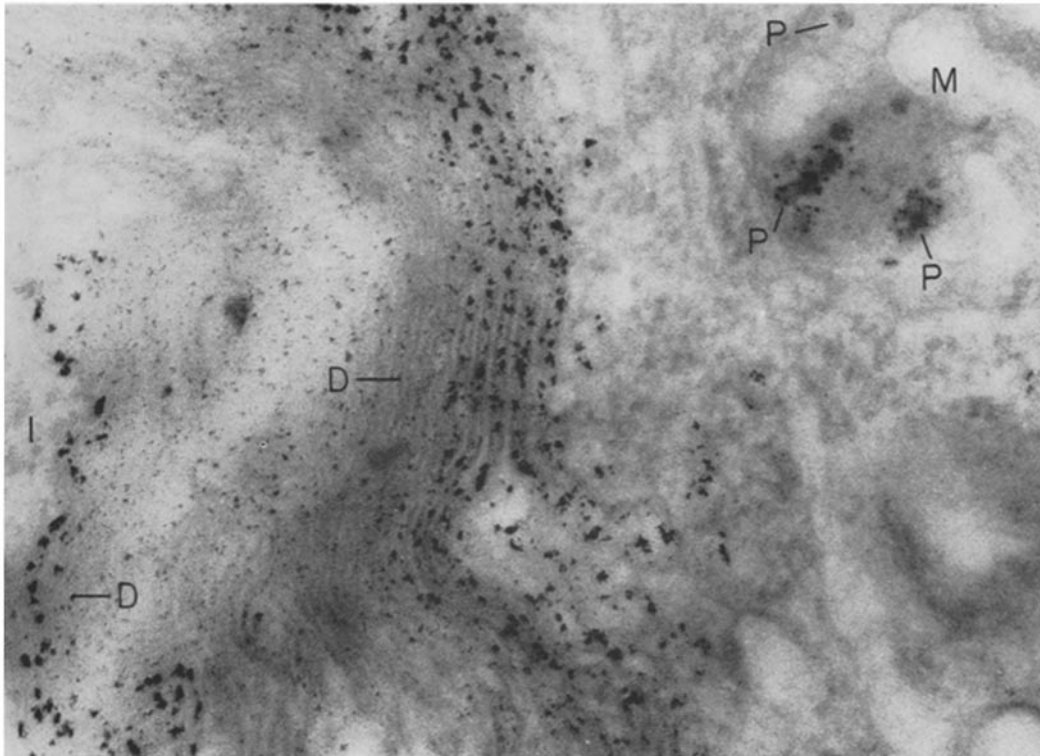


FIGURE 6

A higher power micrograph of the Golgi membranes (*D*) and mitochondrion (*M*) shown in Fig. 5. Reaction product is seen to be concentrated in the electron-opaque material of the Golgi saccules, and is clearly seen to be in association with the dense granules (*P*) of the mitochondrion (*M*). *I*, internum. Unstained. $\times 105,000$.

more in diameter, bounded by a single membrane. The cytoplasm of the internum rarely contains mitochondria.

In the case of spermatids fixed in formaldehyde overnight, incubated in Novikoff and Goldfischer's medium, and postfixed in buffered osmium tetroxide, the electron microscope shows the basic cellular structures to be well preserved, closely resembling the familiar structures seen after fixation with buffered osmium tetroxide alone. The membranes of the nucleus, endoplasmic reticulum, Golgi apparatus and mitochondria are readily recognisable, although the vesicles and vacuoles

lead phosphate deposited at the site of release of orthophosphate ions formed by the hydrolysis of the thiamine pyrophosphate substrate by the enzyme. The granules of reaction product are found among the paired membranes of the dictyosomes (Figs. 2 to 6); on the nuclear envelope of the caudal part of the spermatid nucleus or developing sperm head (Figs. 5 and 7), this being the site of heaviest deposition of reaction product; in the free vesicles pinched off from the free ends of the dictyosomes (Fig. 3); in or on the paired membranes occasionally found in the internum (Figs. 3 and 9); in some multivesicular bodies

(Fig. 10); and possibly associated with the electron-opaque granules found in the mitochondrial matrix (Figs. 4 to 6) and which are a prominent feature of the mitochondria of these cells. The electron-opacity of these mitochondrial granules increases very markedly after Araldite sections are stained with Millonig's solution and also after incubation in the diphosphate medium,

from any intracellular reaction product, although non-specific deposits of electron-opaque material resembling reaction product, but generally more flocculent in nature, are sometimes found on isolated scraps of cell debris and on the plasma membranes of damaged cells (see Fig. 11). No reaction product has been found on the Golgi apparatus, nuclear envelope or mitochondria.

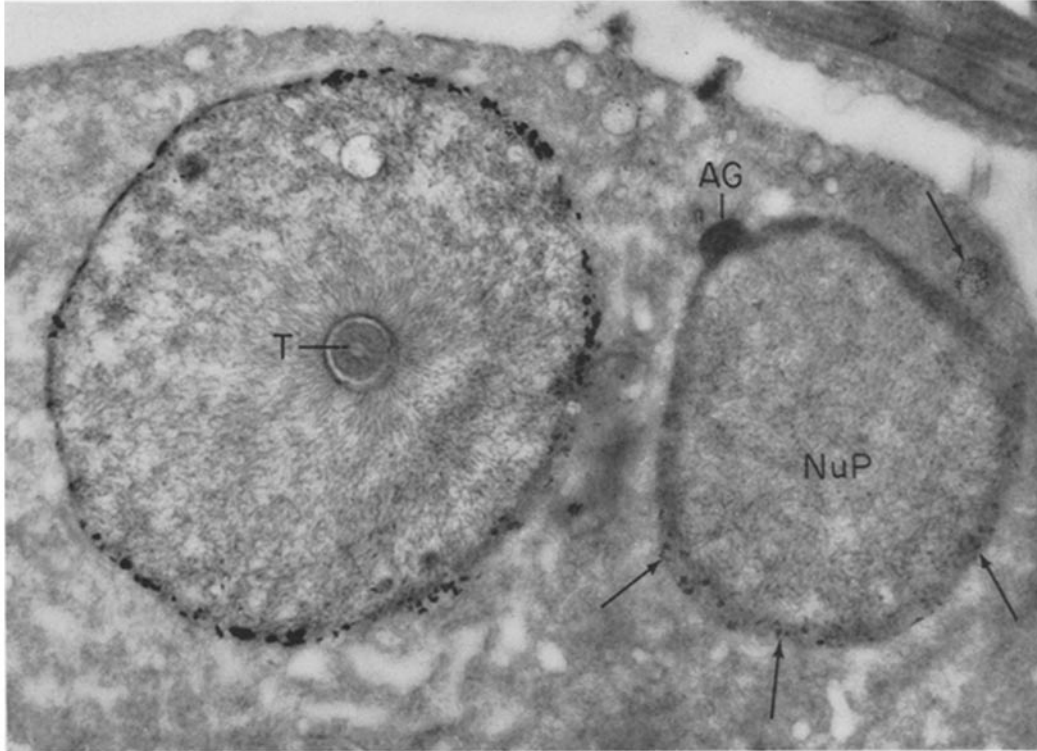


FIGURE 7

A section through a late spermatid in which the sperm head is doubled back upon itself. The proximal part (*NuP*) shows the acrosomal granule (*AG*); few grains of reaction product (arrows) are seen in the nuclear envelope of this part, but none are in association with the acrosomal granule. The distal part containing the tail filaments (*T*) is seen to have a heavy deposit in association with the nuclear envelope. Unstained. $\times 20,000$.

which contains lead ions. Since these mitochondrial granules appear so very dense after treatment with lead salts both in the incubation medium and after section staining, it is difficult to be certain whether or not they are specific sites for hydrolysis of the substrate by TPPase. No lead phosphate has been found deposited within the large Golgi vacuoles frequently associated with the dictyosomes (see Fig. 4).

Control preparations were, in general, free

We have ignored any cells showing reaction product on the plasma membrane, and have discarded as "over-incubated" any preparation showing a high proportion of such cells. "Clean" TPP preparations can be recognised by the well defined deposition of reaction product on the Golgi apparatus and by the absence of reaction product on cell debris and isolated scraps of cell membranes which inevitably accompany cells prepared by teasing.

The results we have obtained with the inosine diphosphate material agree with those published by Novikoff *et al.* (15, 16), and Essner and Novikoff (5), who used vertebrate material. The enzyme or enzymes concerned in the dephosphorylation of IDP are more widely distributed among the organelles of *Helix* spermatids than those which dephosphorylate TPP; there is a corresponding

present an account of the mitochondrial localisation of nucleoside diphosphatase activity in these cells.

DISCUSSION

The finding of deposits of lead phosphate reaction product in electron micrographs of Golgi elements and nuclear envelope was to be expected after

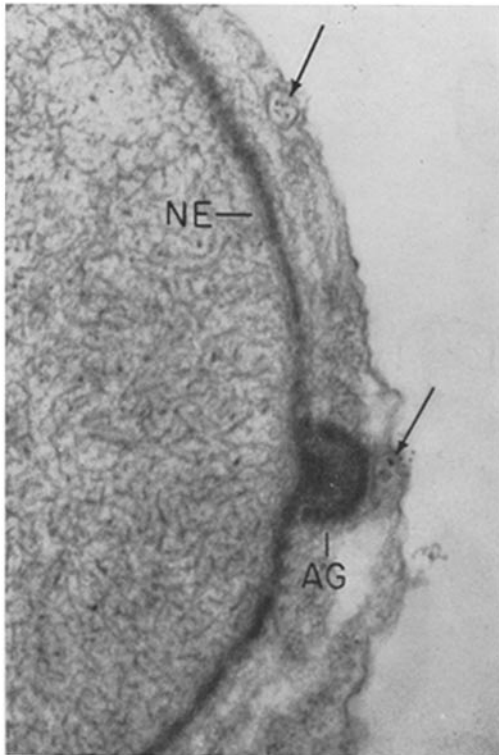


FIGURE 8
The acrosomal granule (AG) of a late spermatid. A few possible grains of reaction product are shown (arrows). There is no deposit associated with the acrosomal granule (AG) or the thickened nuclear envelope (NE). Lead stained. $\times 40,000$.

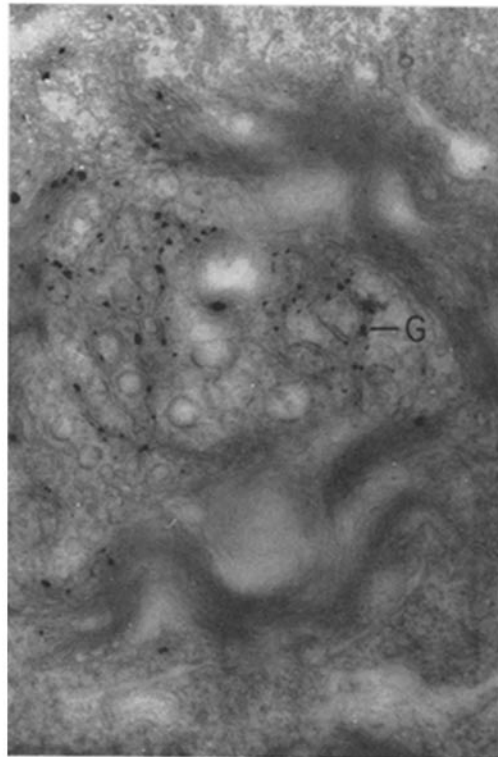


FIGURE 9
The Golgi apparatus of a spermatid showing granules of reaction product (G) within free membrane pairs contained within the internum. Unstained. $\times 27,000$.

lesser specificity towards the Golgi apparatus (see Fig. 12). In particular, heavy deposits of reaction product are found on the membranes of the endoplasmic reticulum, as well as on all the sites on which TPP is localised. If incubation time is increased, the plasma membrane also shows IDPase activity. In particular, the mitochondria show greatly increased activity. The dense granules are now a mass of reaction product, which is also deposited in the electron-opaque material between the cristae. We hope shortly to

the initial results with the preparations for optical microscopy; deposits in mitochondria were unexpected. In the dictyosomes, the lead phosphate deposit appears to be confined to the electron-opaque material between the paired membranes of the Golgi lamellae rather than in the less dense material surrounding them (see Figs. 2 to 6). More deposit occurs in the lamellae nearer the cytoplasm than in those facing the internum (see Figs. 2, 3, and 5). A possible explanation for this may be that the substrate diffuses more readily into the outer membranes of the

dictyosomes from the cytoplasm. However, the acroblast has a very open structure (see Fig. 1) and there seems to be no reason why the concentration of substrate in the internum should be any different from the concentration in the cytoplasm surrounding the acroblast. Even in very heavily impregnated cells which show deposits on the plasma membrane, the dictyosome membranes

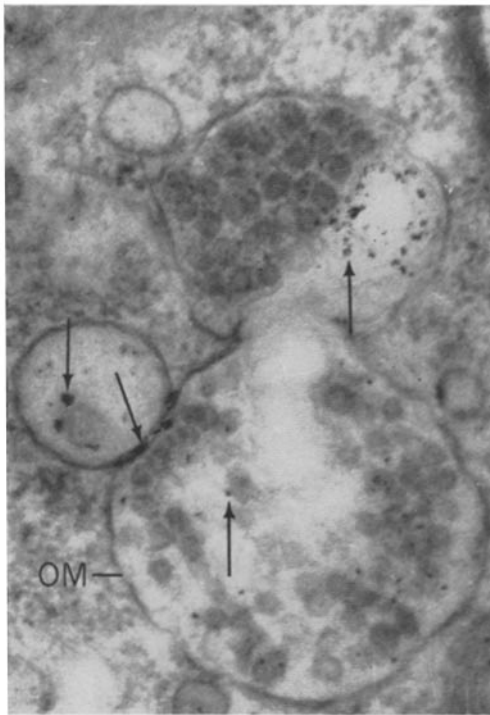


FIGURE 10

A multivesicular body showing granules of reaction product (arrows) enclosed within the outer membrane (*OM*) and in the small vesicles. Lead stained. $\times 80,000$.

facing the internum show much less activity than those facing the cytoplasm.

The small vesicles which Grassé suggested arise from the free ends of the dictyosome by a "beading-off" process contain reaction product (see Fig. 3), as do the scattered membrane pairs of the internum (Figs. 3 and 9) and some of the small vesicles contained within the multivesicular bodies of the internum (Fig. 10). All these sites are associated with the Golgi complex, and the findings lend weight to the hypothesis that the small vesicles

and multivesicular bodies arise from the membranes of the dictyosomes. The finding of heavy deposits of reaction product around the nuclear envelope at the base of the sperm head must be examined in the light of the postulated function of the Golgi apparatus in this cell, namely, the secretion of the acrosome. Acrosome formation is, according to Nath (14), a very short-lived stage in the spermiogenesis of pulmonate molluscs. This may account for the fact that the acrosomal granule is rarely seen in the electron microscope. When it is seen in the thiamine pyrophosphatase preparations, however, there appears to be no reaction product associated with it (Figs. 7 and 8). Neither is reaction product associated with the characteristic thickening of the nuclear envelope by deposition of electron-opaque material on either side of the two nuclear membranes (Fig. 3) which precedes acrosome formation. It is possible, therefore, that the enzyme acts as an intermediary in the formation of the material of the acrosomal granule or acrosome, and, then having performed its function, passes down the developing sperm head towards the tail, to be cast off along with the remainder of the spermatid cytoplasm. It is also possible that this specific enzyme is not associated with acrosome formation at all. Further work is in progress in an attempt to resolve this problem.

The association of reaction product with the electron-opaque granules of mitochondria is not established with certainty. There is definite deposition associated with the dense granules in the mitochondrion shown in Figs. 5 and 6, but in the mitochondrion in Fig. 4 and inset there is less certainty.

The evidence obtained from invertebrate material and presented here is in agreement with Novikoff and Goldfischer's findings (15, 16) that thiamine pyrophosphatase reaction product was found in or between the paired Golgi membranes in rat, mouse, and frog tissues. This strengthens the evidence for the existence of a homology between the Golgi apparatus in cells throughout the animal kingdom.

The possibility exists that the deposits of reaction product found on the nuclear envelope are an artifact. Holt (8) points out that nuclear staining takes place with the related Gomori acid phosphatase technique as an artifact associated with lead depletion in the incubation medium. We do not think that nuclear staining

in the spermatid is an artifact, because we frequently observe nuclear staining in spermatids when neighbouring cells at earlier stages (spermatogonia and spermatocytes) show staining of the Golgi apparatus only. We are at present investigating this problem further.

The finding of diphosphatase activity in the nuclear envelope and endoplasmic reticulum of

may be peculiar to the strikingly well developed cristae generally found in spermatid mitochondria immediately prior to sperm maturation, or it may possibly be a species difference.

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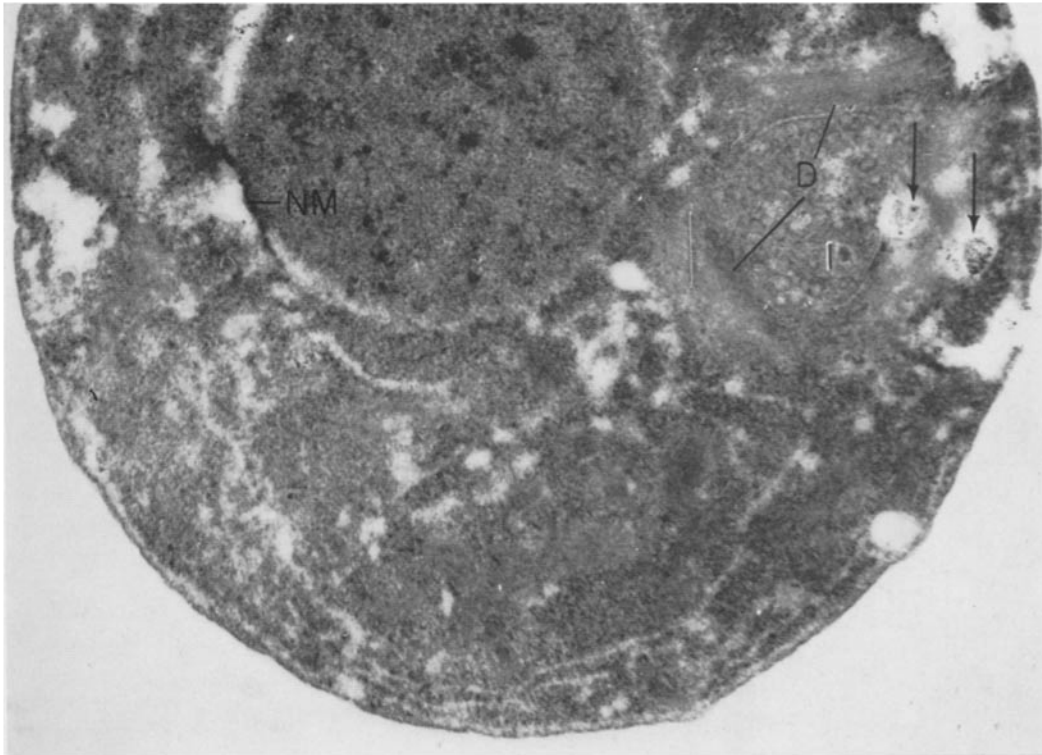


FIGURE 11

A spermatid from a control preparation incubated in a medium from which the substrate was omitted. No deposit can be seen on the dictyosomes (*D*), internum (*I*), nuclear envelope (*NM*) or any other cell organelle. Slight non-specific deposits (arrows) are sometimes found in the control cells, frequently associated with sites of damage to the cell membrane. Compare with Figs. 2 and 12. Un-stained. $\times 18,000$.

these invertebrate cells, together with the greater specificity of TPP for the Golgi membranes, is in agreement with the latest results of Novikoff *et al.* (16) on vertebrate material. The diphosphatase activity of the dense granules and cristae of the mitochondria has not previously been reported; it

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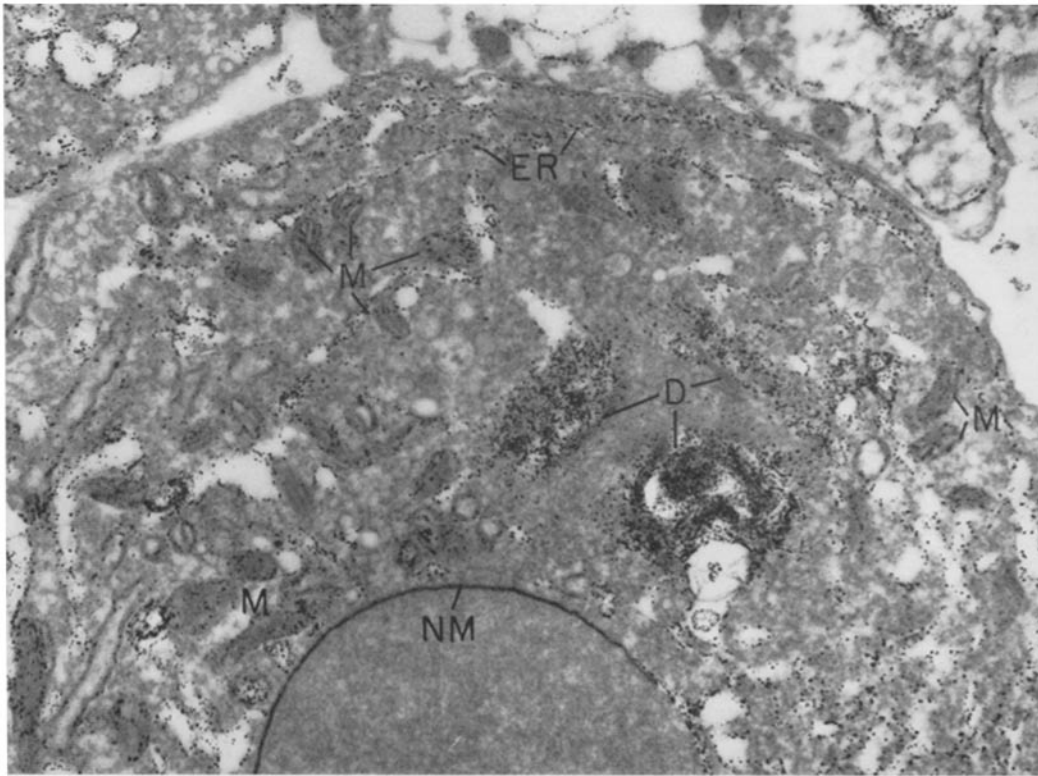


FIGURE 12

A spermatid from a preparation using IDP as substrate. Note the lesser specificity of this medium for the Golgi apparatus in these cells. Heavy deposits are found on the endoplasmic reticulum membranes (ER) and on the mitochondrial cristae (M) as well as on the dictyosome membranes (D) and nuclear envelope (NM). Lead stained. $\times 18,000$.

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