Endocytosis of transcobalamin in male rabbit germ cells: electron microscope radioautography study

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ABSTRACT The binding of ^{125}I-iodinated transcobalamin to a suspension of isolated rabbit germ cells was studied by Scatchard plot. The number of binding sites was evaluated to about 1000 per cell, and its association constant (Kass) in order of 14.6 l/nmole. The distribution of structures related to endocytosis was determined by ultrastructural histomorphometric studies. Both coated and uncoated structures were present regardless of maturation stage. The number of coated vesicles was at its highest in the initial maturation steps, whereas the number of uncoated vesicles was highest in the final maturation steps. The endocytosis of ^{125}I-iodinated transcobalamin by the suspension of germ cells was studied by electron microscope radioautography. The tracer was mostly detected over the plasma membrane, coated vesicles and multivesicular bodies of germ cells. The grains were observed mainly over spermatocytes and round spermatids; 31.6% and 32% of these cells, respectively, were labeled. In contrast the tracer was detected in only 8.11% of elongated spermatids. In conclusion, iodinated transcobalamin is internalized in rabbit germ cells by receptor-mediated endocytosis. This phenomenon was predominant in the early stages of germ cell maturation.

KEY WORDS: *rabbit, germ cells, transcobalamin, binding, internalization*

Transcobalamin (TC) is a cobalamin (Cbl, vitamin B12) transport protein found in plasma of man and other mammals (Hall, 1975). It facilitates Cbl uptake by various cell types *in vivo* and *in vitro*. The uptake of the TC-Cbl complex is mediated by a receptor on the cell membrane (Youngdahl-Turner *et al.*, 1979).

Gametogenesis depends on hormonal (Parvinen, 1982; Huang and Nieschlag, 1986) and non-hormonal factors (Saez *et al.*, 1989; Skinner, 1991). Vitamin deficiencies can have a dramatic effect on the morphology of the seminiferous tubules and can disturb spermatogenesis. Disturbance of fertility has been reported in cobalamin deficiencies such as pernicious anemia (Watson, 1962; Jackson *et al.*, 1967). Seminal plasma has been demonstrated to be the most concentrated site of transcobalamin, compared to other body fluids (Carmel and Bernstein, 1984; Hansen and Nexø, 1993). Little is known about the transport of cobalamin in the initial segments of the reproductive tract, such as the testis and epididymis. The purpose of the present study was to analyze the interactions of TC with the spermatogenic cells. We have studied the specific binding of radiolabeled rabbit TC on rabbit germ cells by Scatchard plot and its subsequent uptake within these cells by electron microscope radioautography.

The initial suspension of germ cells contained 4%±1.5 spermatogonia, 32%±2.5 primary and secondary spermatocytes, 36%±0.56 round spermatids, 20%±0.12 elongated spermatids,

5%±1.8 residual bodies, 1%±0.5 interstitial cells, and 2%±1.15 damaged cells (Giemsa stained smears analysis). The cell composition was not significantly modified after *in vitro* binding studies and the viability of the cells was more than 95% in all experiments. The ultrastructural study of these cells did not show morphological alterations.

Binding of the [125]I- labeled TC to isolated cells

As shown in Fig. 1, a specific binding of ^{125}I-iodide labeled TC to the mixed isolated germ cells was found. The Scatchard plot analysis showed a single class of binding site with an association constant Ka of 14.6 l/nmole and 1032 sites per cell.

Previous studies have found specific binding sites in rat pachytene spermatocytes for transferrin (Holmes *et al.*, 1983; Steinberger *et al.*, 1984; Sylvester and Griswold, 1984) and for steroid binding protein (Steinberger *et al.*, 1984; Felden *et al.*, 1992). The present data show that germ cells also have a specific binding site for transcobalamin, another transport protein from seminal plasma. The binding was calcium dependent since incubation of germ cells with EDTA resulted in a sharp decrease in cellular uptake of the

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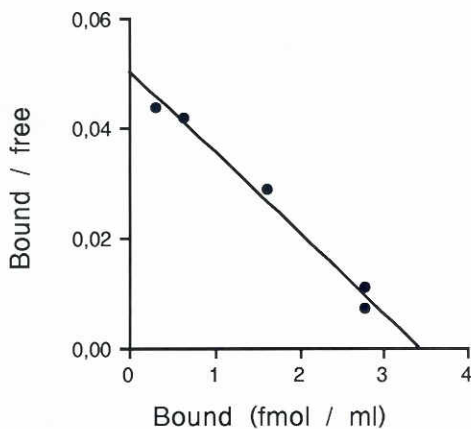
Fig. 1. Scatchard plot of the interaction between germ cells and ^{125}I-iodinated transcobalamin (TC). *Binding studies were performed at 4°C for 1 hour; each point is the mean of three different experiments.*

tracer (Fig. 4). In a previous study, we observed 40% inhibition of the TC binding to germ cells by EDTA, when using cyano ([57]Co) cobalamin TC (TC saturated with labeled vitamin B_{12}) (Boukhzer *et al.*, 1992).

Morphological evidence of an endocytic apparatus in rabbit germ cells

Most of isolated rabbit germ cells exhibited coated and uncoated structures in the peripheral cytoplasm (Fig. 2a,b). Coated pits were frequently arranged as paired structures (Fig. 2a). Coated vesicles of 113±67 nm in diameter were observed just below the plasma membrane (Fig. 2b). Uncoated endocytic vesicles were also present below the plasma membrane in all germ cells (Fig. 2a). Their shape was somewhat irregular and they measured 283±84 nm in diameter. As shown in Fig. 3, the mean number of coated vesicles per 100 μm linear plasma membrane was highest in the early stages of germ cell maturation, whereas the mean number of uncoated vesicles was highest in the final stages of germ cell maturation.The involvement of coated structures in receptor-mediated endocytosis has been established by ultrastructural studies in numerous cells (Goldstein *et al.*, 1985). Our study is the first report of the existence of coated structures in rabbit germ cells and makes these cells a good candidate for receptor-mediated endocytosis. Such structures have been also described in monkey germ cells (Gerard *et al.*, 1991). Two other endocytosis related structures were scattered in the whole cytoplasm: 1) small, empty, vesicular structures with smooth membrane, 150-300 nm in diameter. They have been referred to as early endosomes (Fig. 2c). 2) Multivesicular bodies 1500-3000 nm in diameter, located in the perinuclear cytoplasm

(Fig. 2e). They were referred to as late endosomes and considered as prelysosomal elements (Gerard *et al.*, 1991).
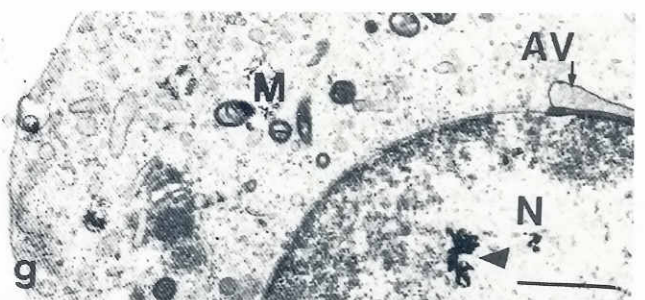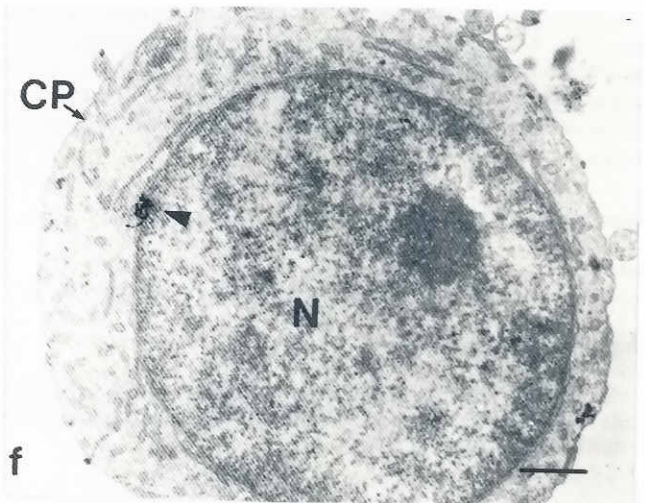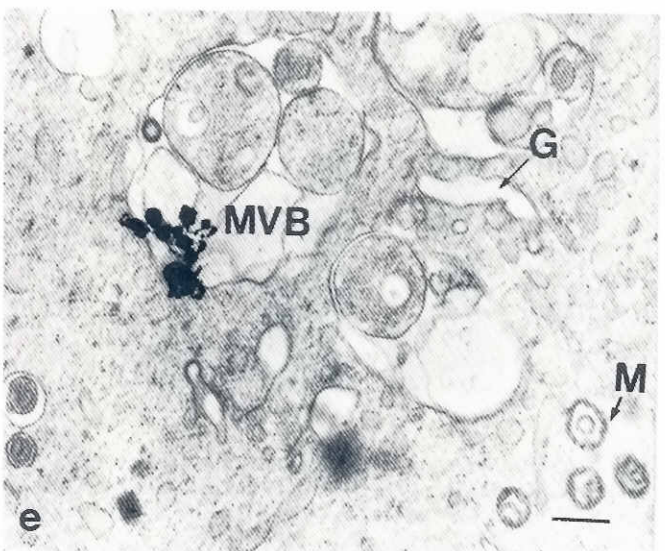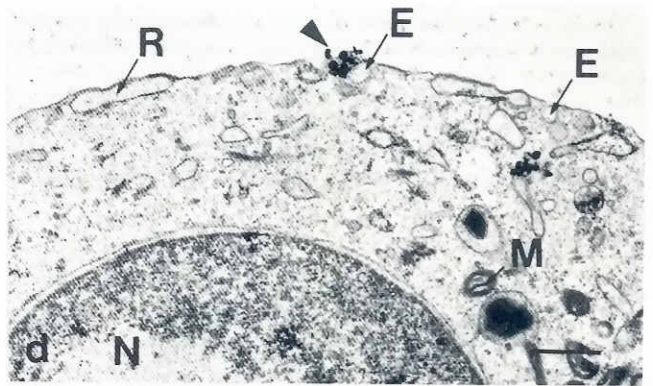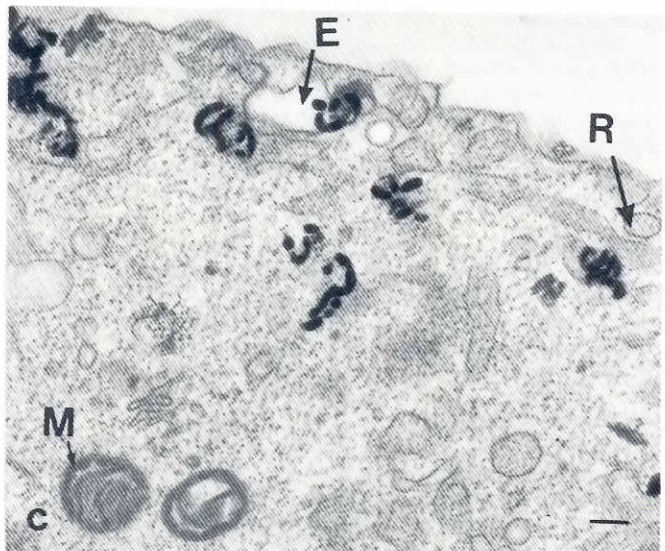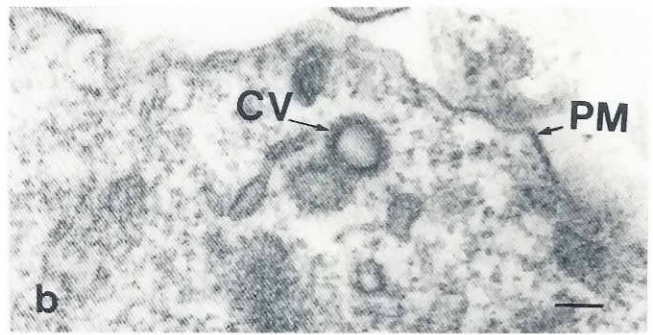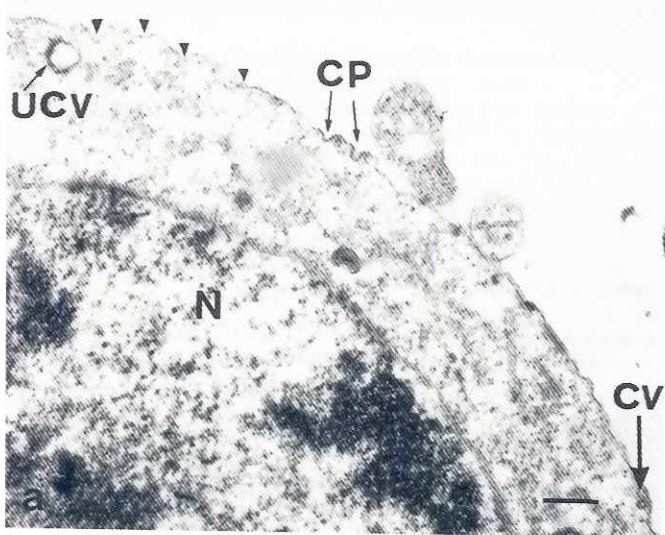
Cellular localization of transcobalamin

The results should be interpreted with caution since we do not know if the iodine label remained attached to the TC molecule as it travelled through the different cellular compartments. The cell labeling was significantly different from the background activity since the number of silver grains was 5.46, 14.84, 2.48, respectively per 1000 μm^2 of spermatocytes, round spermatids and elongated spermatids, versus 0.83 per 1000 μm^2 of extracellular compartment. The proportion of labeled spermatocytes, round spermatids and elongated spermatids was 31.6%, 32%, 8.11% respectively (Fig. 3). EDTA pretreatment reduced significantly the proportion of labeled spermatocytes and round spermatids to 8.9, 14.17 respectively (Fig. 4). HD analysis demonstrated that only 15.8%, 3.3% and 14.3% of the silver grains were located within one HD of the plasma membrane in spermatocytes, round spermatids and elongated spermatids respectively (Fig. 5). At higher magnification silver grains were seen in association with the plasma membrane (Fig. 2d), and with small coated pits and vesicles (Fig. 2c). At the intracellular level, clusters of silver grains were observed over endosomes and multivesicular bodies (Fig. 2e).The tracer was detected over the delineating membrane rather than over the lumen. Finally, some grains were also located over the nuclear membrane (Fig. 2f) and the nucleus (Fig. 2g).

Numerous studies have shown the importance of TC to facilitate cobalamin uptake by cells (Youngdhal-Turner *et al.*, 1979). A specific receptor for TC has been demonstrated in target cells from liver and placenta, in human fibroblasts and in intestinal epithelial cells. After internalization of TC, the protein is degraded by lysosomal proteases (Youngdahl-Turner *et al.*, 1979).

Our *in vitro* study showed clearly that purified radio-iodinated rabbit TCII was not only bound to rabbit germ cell plasma membrane but also internalized by cells, as confirmed by half distance analysis. Since the distribution of the labeling according to the maturation stage correlated positively with the distribution of coated vesicles (Fig. 3), it is suggested that the internalization of TC required coated structures. The labeling density was the highest in spermatocytes and round spermatids. These data are close to those reported for transferrin and cationic ferritin (Segretain *et al.*, 1992), and indicate that TC is required during the early stages of spermatogenesis, but not in the late stages. It may therefore be suggested that the transcobalamin could play a major role in the maturation of pachytene spermatocytes. Similar results were obtained by Holmes *et al.* (1983) with transferrin. Many vitamins utilize binding proteins in the circulatory system to facilitate their transport and localization. Preliminary evidence exists for the production of vitamin binding proteins by Sertoli cells, such as

Fig. 2. Transmission electron microscope observation of isolated rabbit germ cells. (a-b) Endocytic structures; standard preparation. (c-d-e-f-g) Internalization fate of transcobalamin; autohistoradiographic preparation after incubation with [125]I- iodinated transcobalamin (TC) for 60 min. (a) *Primary spermatocyte. Arrowhead: plasma membrane indentations, CP: coated pit, CV: coated vesicle, UCV: uncoated vesicle, N: nucleus. Bar= 1 μm.* **(b)** *Primary spermatocyte. CV: coated vesicle, PM: plasma membrane. Bar= 0.1 μm.* **(c)** *Round spermatid. Silver grains are located in the peripheral cytoplasm associated with endosomes (E). R: smooth endoplasmic reticulum, M: mitochondria. Bar= 0.3 μm.* **(d)** *Round spermatid. A silver grain (arrowhead) is located on the plasma membrane in close relation with an endosome (E). R: smooth endoplasmic reticulum, M: mitochondria. Bar= 1 μm.* **(e)** *Elongated spermatid. A cluster of silver grains is located on a multivesicular body (MVB) near the Golgi area (G). M: mitochondria. Bar= 0.3 μm.* **(f)** *Type B spermatogonia. One cluster of silver grains (arrowhead) is associated with the nuclear envelope. N:nucleus, CP: coated pits. Bar= 1 μm.* **(g)** *Acrosome phase spermatid. One Cluster of silver grains (arrowhead) is located within the nucleus (N). AV: acrosomal vesicle, N: nucleus. Bar= 0.5 μm.*
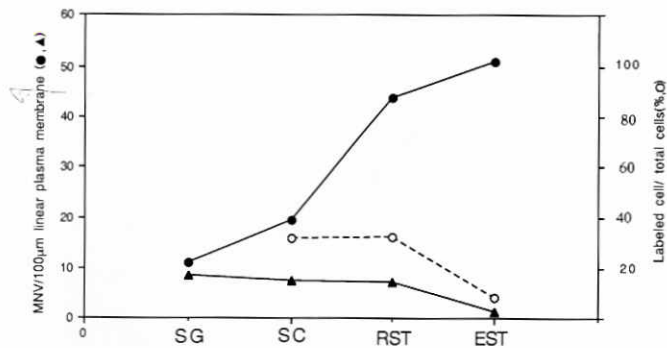
Fig. 3. Distribution of uncoated and coated vesicles in the peripheral cytoplasm compared to the labeling frequency within different germ cell maturation stages. *The distribution of uncoated (filled circles) and coated (filled triangles) vesicles is expressed as the mean number of the vesicles (MNV) per 100 µm linear plasma membrane. The labeling frequency is expressed as the labeled cells/total cells ratio (open circles). SG: spermatogonia, SC: spermatocytes, RST: round spermatids, EST: elongated spermatids.*

folate-binding protein and biotin-binding protein (Skinner, 1991). In the case of vitamin A, Sertoli cells synthesize and secrete Retinol-binding protein that binds and transports vitamin A to germ cells (Fetterolf and Skinner, 1987; Ong et al., 1987). TC may play a similar role in the development of the male gametes by facilitating the uptake of cobalamin in the spermatogenic cells.

In conclusion, rabbit germ cells share a specialized endocytic apparatus involving coated pits and vesicles which internalize TC by receptor-mediated endocytosis. It remains to be determined whether this endocytosis is required for internalization of cobalamin by germ cells or whether cobalamin itself plays a direct role in spermatogenesis.

Experimental Procedures

Chemicals

Culture media were obtained as described recently (Gueant et al., 1991).

Ligand and cell preparation
Purification

Transcobalamin was purified from rabbit blood plasma, using a sequence of Cbl-sepharose thermolabile affinity chromatography and Sephadex G-200 gel filtration (Nexø et al., 1977).

Labeling

Iodination of purified transcobalamin was carried out according to the method of Markwell (1982) as previously described by Gueant et al. (1985) for intrinsic factor. The specific activity of the tracer was estimated at 0.2 µCi/nmole.

Isolation of germ cells

Rabbits (Fauve de Bourgogne, mean age 22 weeks and mean weight 1.5 kg) were anesthetized with sodium phenobarbital (3 mg/kg), and the testes were removed and rinsed carefully in the RPMI culture medium (pH 7.4). The seminiferous tubules were dissected out of interstitial and connective tissue under a stereomicroscope, and placed in the culture medium. Germ cells were isolated from seminiferous tubules using mechanical method with a potter homogenizer. The resultant cell suspension was filtered on cotton wool, and centrifuged at 150 g for 10 min. The pellet was washed twice and resuspended in fresh culture medium. Germ cells were counted under a phase contrast microscope, and the viability of the cells was

checked by trypan blue (Sigma) exclusion test. Cell smears were stained with May-Grunwald Giemsa in order to quantitate the different cell types.

Binding of labeled TC with isolated germ cells

Binding of rabbit TC to the isolated cells was studied as described (Gueant et al., 1991; Boukhzer et al., 1992) by incubating increasing amounts (10-300 fmol) of ^{125}-Iodide labeled transcobalamin (aprox. 5000 cpm to 60,0000 cpm) with 10^6 cells per test tube in Tris-HCl buffer (pH 7.4) containing 0.12 mole/l NaCl, 1mmole/l CaCl$_2$. Non-specific binding of the tracer to germ cells was tested in presence of 100-fold excess of non-labeled Transcobalamin. Non-specific binding was determined at 18%.

After each series of experiments, cells were fixed for ultrastructural control of their integrity.

Cellular uptake

Aliquots of 10^6 cells in 100 µl RPMI medium were incubated in the presence of ^{125}I-TCII (15000 cpm in 100 µl RPMI) at 34°C in a shaking waterbath for 60 min. For inhibition studies, aliquots were incubated with EDTA (10 mmole/l) for 15 min before adding ^{125}I-TCII (9 pmoles/100 µl RPMI, 15000 cpm) for 60 min at 34°C. After incubation, cells were centrifuged at 150 g for 10 min, washed, and fixed for electron microscope radioautography.

TEM procedures

Cells were fixed for 1 h in glutaraldehyde 2.5% in 0.1 M cacodylate buffer pH 7.4 at 4°C (vol/vol), and then for 1 h in 5% glutaraldehyde in the same buffer and routinely embedded (Gerard et al., 1991). Specimens were examined under Zeiss EM 952 or Philips CM12 transmission electron microscope.

Morphometric parameters of germ cells were determined, using the Philips computer software connected to the CM12 Philips microscope.

Radioautography

Ultrathin sections of radiolabeled samples were processed as described by Larra and Droz (1970). The sections (80 nm) were placed on celloidin coated glass slides and contrasted with 2.5% uranyl acetate and lead citrate (vol/vol). The slides were coated with an Ilford L4 nuclear emulsion and radioautographs were developed as described (Gerard et al., 1982).

Silver grain quantification

Silver grains were counted in randomly observed material.

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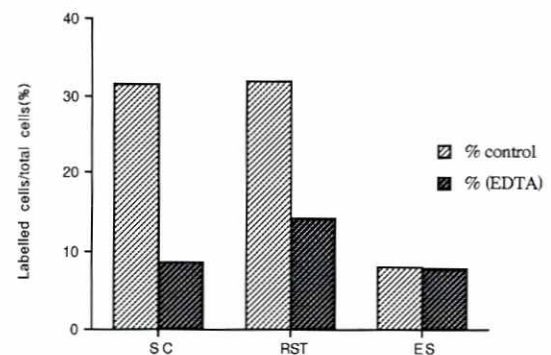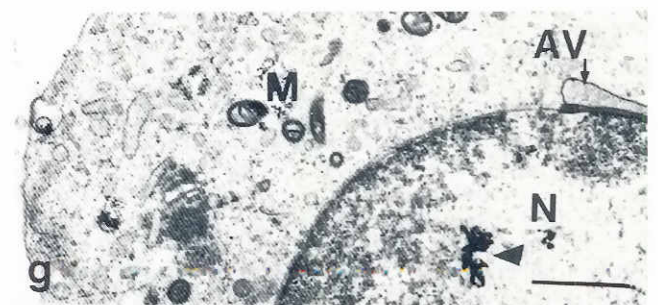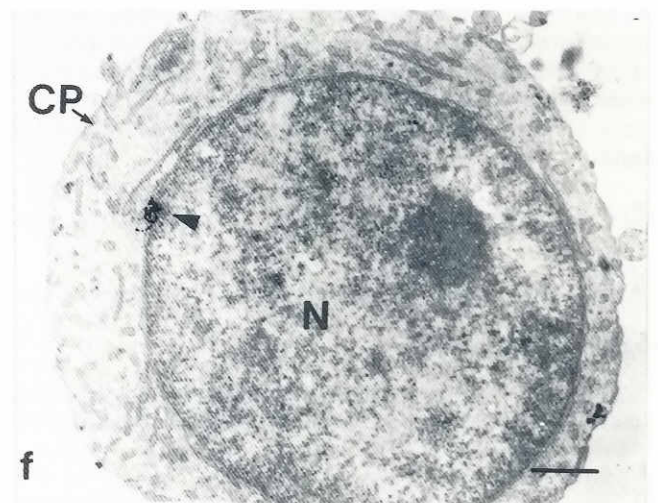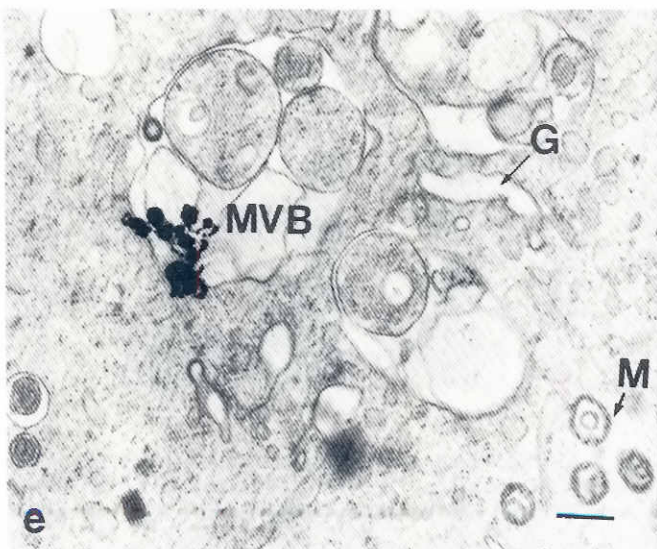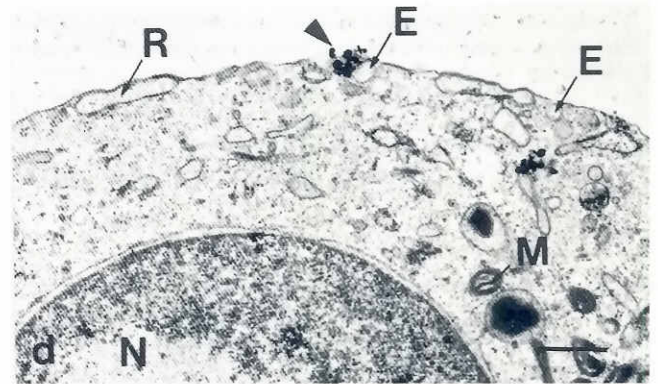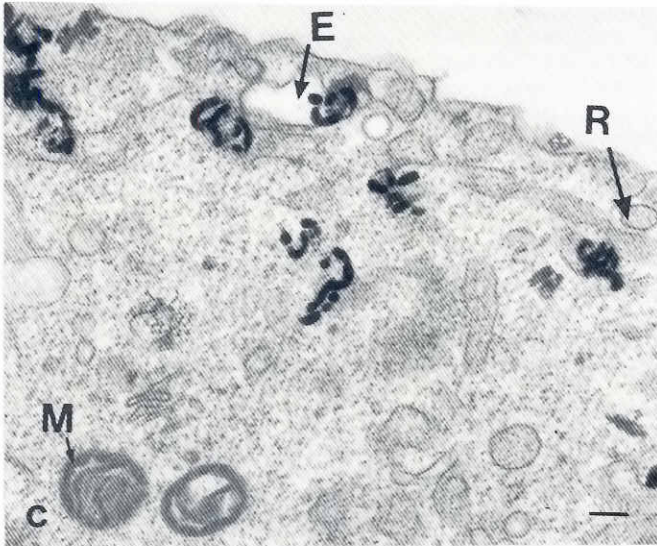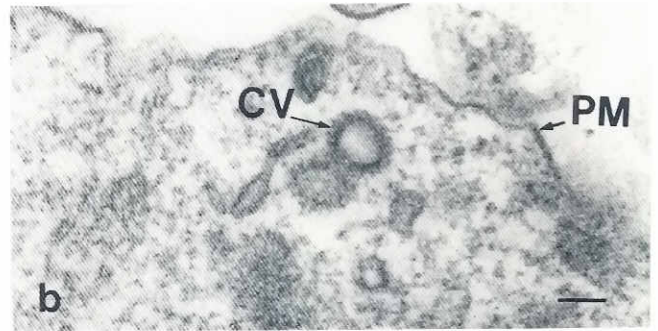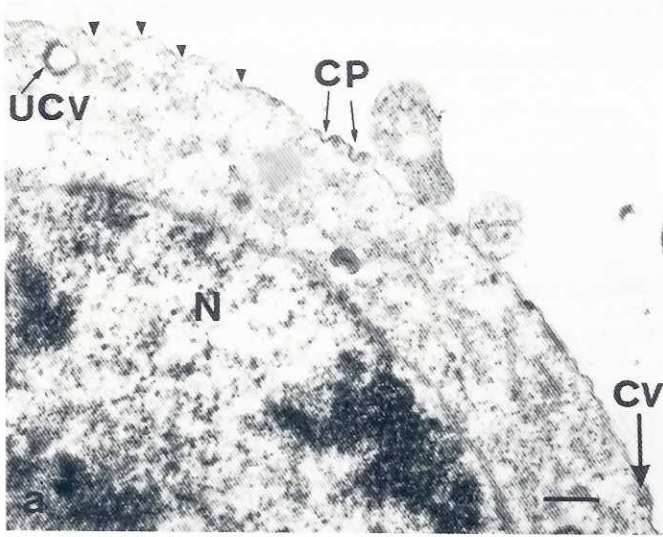

Fig. 4. Relative labeling of the isolated rabbit germ cells following incubation in presence of ^{125}I-iodinated transcobalamin (TC) (9 pmol/ml; 15000 cpm) with or without EDTA pretreatment (10 mM for 15 min). *SC: spermatocytes, RST: round spermatids, EST: elongated spermatids.*
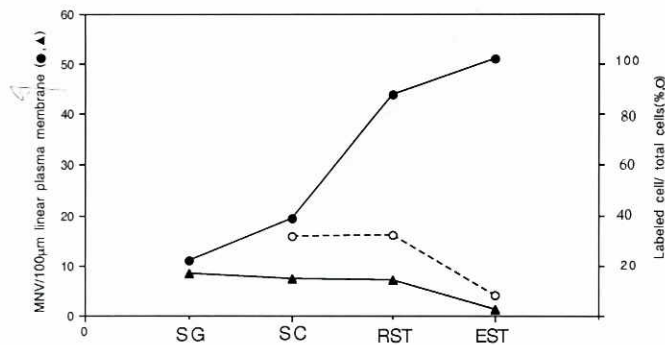
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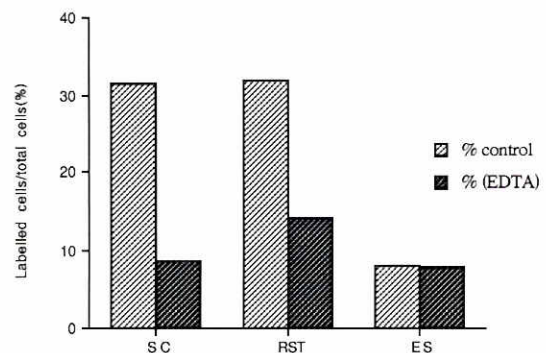

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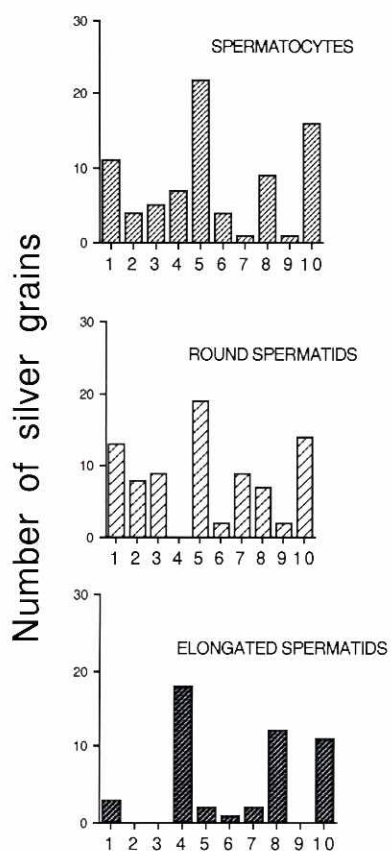
Fig. 5. Half-distance (HD) analysis for each cell type (spermatocytes, round spermatids and elongated spermatids). *Silver grains were scored on the EM screen at a magnification of x8000. The HD axis origin was located on the plasma membrane. One HD= 140 nm.*

1000 µm² of cell section, in each category. The number of silver grains counted over celloidin in random fields at some distance from the cell was estimated at 0.83 grain/1000 µm² and was considered to be the background index.

2) The cell labeling frequency was determined at x8000 magnification by counting the number of labeled and unlabeled spermatocytes, round spermatids and elongated spermatids, and expressed as the labeled/total cells ratio, in each category. A cell was considered labeled as soon as at least one silver grain was located on it. The label was determined by counting the incidence of at least 100 grains in each cell type.

3) The cellular localization of the radioactive sources was determined by the half-distance (HD) analysis (Salpeter *et al.*, 1978) as used in a previous study (Gerard *et al.*, 1988). With regard to the section size (80 nm), the nuclear emulsion (L4) and the radioelement (^{125}I), one HD was calculated to be at 140 nm (Salpeter *et al.*, 1978). For this analysis the HD axis origin was located on the plasma membrane only.

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