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# Ultracytochemical Demonstration of Several Phosphatases in the Human Corpus Luteum of Early Pregnancy by a Cerium-Based Method in Combination with a Microslicer

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## ABSTRACT

Acid phospatase (AcPase), alkaline phosphatase (AlPase), 5'-nucleotidase (AMPase), Mg-activated adenosine triphospatase (ATPase), thiamine pyrophospatase (TPPase) and glucose-6-phospatase (G6Pase) were investigated in the human corpus luteum of early pregnancy using a cerium-based method in combination with a microslicer.

Ultrastructurally, cerium phosphate reaction product appears as a very fine electron dense precipitate. The AcPase reaction products were exclusively localized in the lysosomes and in two cisternae of the Golgi lamellae on the trans-side. The AlPase and AMPase reaction products were observed in the plasma membrane. The Mg-activated ATPase reaction products were found in the matrix of the mitochondria as well as in the plasma membrane. The TPPase reaction products were present in two cisternae of the Golgi lamellae on the trans-side. The G6Pase reaction products were found in the rough endoplasmic reticulum. This technique can be applied routinely for the demonstration of phosphatase activity because of its reproducibility and specificity when cerium is used as the capturing agent, and yields better results than the lead-based method. In the luteal cells, these several phosphatases might serve to maintain a constant metabolic activity in the early pregnancy.

In phosphatase cytochemistry, lead has been generally used as the capturing agent for the localization of enzyme activity. This lead-based method, first adapted by Sheldon et al[14] is employed to demonstrate acid phosphatase (AcPase) by Barka and Anderson[1], 5-nucleotidase (AMPase) by Wachstein and Meisel[18], Mg-activated adenosine triphospatase (ATPase) by Wachstein and Meisel[18], thiamine pyrophosphatase (TPPase) by Novikoff and Goldfischer[8] and glucose-6-phosphatase (G6Pase) by Hugon et al[4] and Leskes et al[6]. However, some troubles as to the tendency of the formation of non-specific deposits in the lead-based method cytochemistry remain to be more improved.

Recently it was shown that several phosphatases could be demonstrated using an improved cytochemical cerium-based method in guinea pig and rat (Hulstaert et al[5] and Robinson and Karnovsky[10,11]).

The present study concerns the applicability of a cerium-based method in combination with a microslicer for the demonstration of AcPase, AlPase, AMPase, ATPase, TPPase and G6Pase in the human corpus luteum of early pregnany and compares results obtained with cerium to those with lead.

## MATERIALS AND METHODS

Ten cases of the human corpus luteum of early

**Table 1.** Incubation media used in this study

| | |
|---|---|
| AcPase | 0.1 M acetate buffer (pH 5.0)<br>1 mM β-glycerophosphate (disodium salt, Sigma)<br>2 mM $CeCl_3$ |
| AlPase | 0.1 M Tris-maleate buffer (pH 8.0)<br>1 mM β-glycerophosphate (disodium salt, Sigma)<br>2 mM $CeCl_3$ |
| AMPase | 70 mM Tris-maleate buffer (pH 7.2)<br>5 mM Mg $(NO_3)_2$<br>1.3 mM AMP<br>2 mM $CeCl_3$ |
| Mg-activated ATPase | 70 mM Tris-maleate buffer (pH 7.2)<br>5 mM $MgSO_4$<br>2.3 mM ATP<br>2 mM $CeCl_3$ |
| TPPase | 0.1 M Tris-maleate buffer (pH 7.2)<br>1 mM thiamine pyrophosphate chloride (Sigma)<br>2 mM $MnCl_2$<br>2 mM $Cecl_3$ |
| G6Pase | 0.1 M cacodylate buffer (pH 6.6)<br>1 mM glucose-6-phosphate (monosodium salt, Sigma)<br>2 mM $CeCl_3$ |

pregnancy were used in this study. The specimens were fixed for 30 min with 1.5% glutaraldehyde solution adjusted to pH 7.4 with 0.1 M cacodylate buffer containing 5% sucrose and immersed overnight at 4°C in the same buffer containing 5% sucrose to remove the fixative. Thirty to 40 μm thick sections were cut on a microslicer (DTK-1000, Dosaka EM Co., Ltd.). These microslicer sections were preincubated for 1 hr in media containing 2 mM CeCl3 as the capturing agent for the localization of AcPase, AlPase, AMPase, Mg-activated ATPase, TPPase and G6Pase. For the demonstration of AcPase, AlPase, AMPase, TPPase and G6Pase, the incubation media according to Robinson and Karnovsky[10,11] were used. For the demonstration of Mg-activated ATPase, the medium according to Hulstaert et al[5] was modified. This medium consisted of 2 mM CeCl3, 2.3 mM ATP, 5 mM MgSO4 and 70 mM Tris-maleate buffer (pH 7.2). The incubation media used in the present study were shown in the Table 1. All of the cytochemical reactions were carried out for 30 min at 37°C with constant gentle mixing. According to Unsitalo-Karnovsky[15], some specimens were treated in parallel for the cytochemical localization of AMPase with 1.8 mM lead nitrate as the capturing agent.

After incubations, these materials were rinsed overnight at 4°C in 0.1 M cacodylate buffer containing 5% sucrose, dehydrated in ethanol and embedded in Poly/Bed 812. Sections cut on a Porter-Blum MT-1 ultramicrotome, stained uranyl acetate and Reynolds' lead solution and coated with carbon were examined with a Hitachi H-300 type electron microscope. A control incubation was done by omitting the substrate from each medium.

## RESULTS

All reaction products with cerium as the capturing agent were clearly observed as the fine granular precipitates in the human corpus luteum of early pregnancy. The AcPase reaction products were exclusively localized in the lysosomes and in two cisternae of the Golgi lamellae on the trans-side (Figs. 1 and 2 a,b). The AlPase and AMPase reaction products were present in all plasma membrane of the luteal cells (Fig. 3). The Mg-activated ATPase reaction products were found in the matrix of the mitochondria as well as in all plasma membrane (Figs. 4 and 5). The TPPase reaction products were cleary localized in two cisternae of the Golgi lamellae on the trans-side (Fig. 6). The localization of AcPase and TPPase activities were partially overlapping. The G6Pase reaction products were distributed in the all cisternae of the rough endoplasmic reticulum (Fig. 7).

On the other hand, the reaction products with lead nitrate as the capturing agent were often recognized as non-specific deposits showing accumulation or diffusion of electron dense material (Fig. 8). No reaction products were seen in these cells incubated in the control medium without the substrate.

## DISCUSSION

Recent improved cerium-based techniques (Veenhuis et al[16], Blok et al[2], Hulstaert et al[5], Robinson and Karnovsky[10,12]) have shown that cerium phosphate reaction products for several phosphatases are clearly demonstrated, and also cerium appears to be a better capturing agent for inorganic phosphate than lead in both its reproducibility and specificity. However, cerium

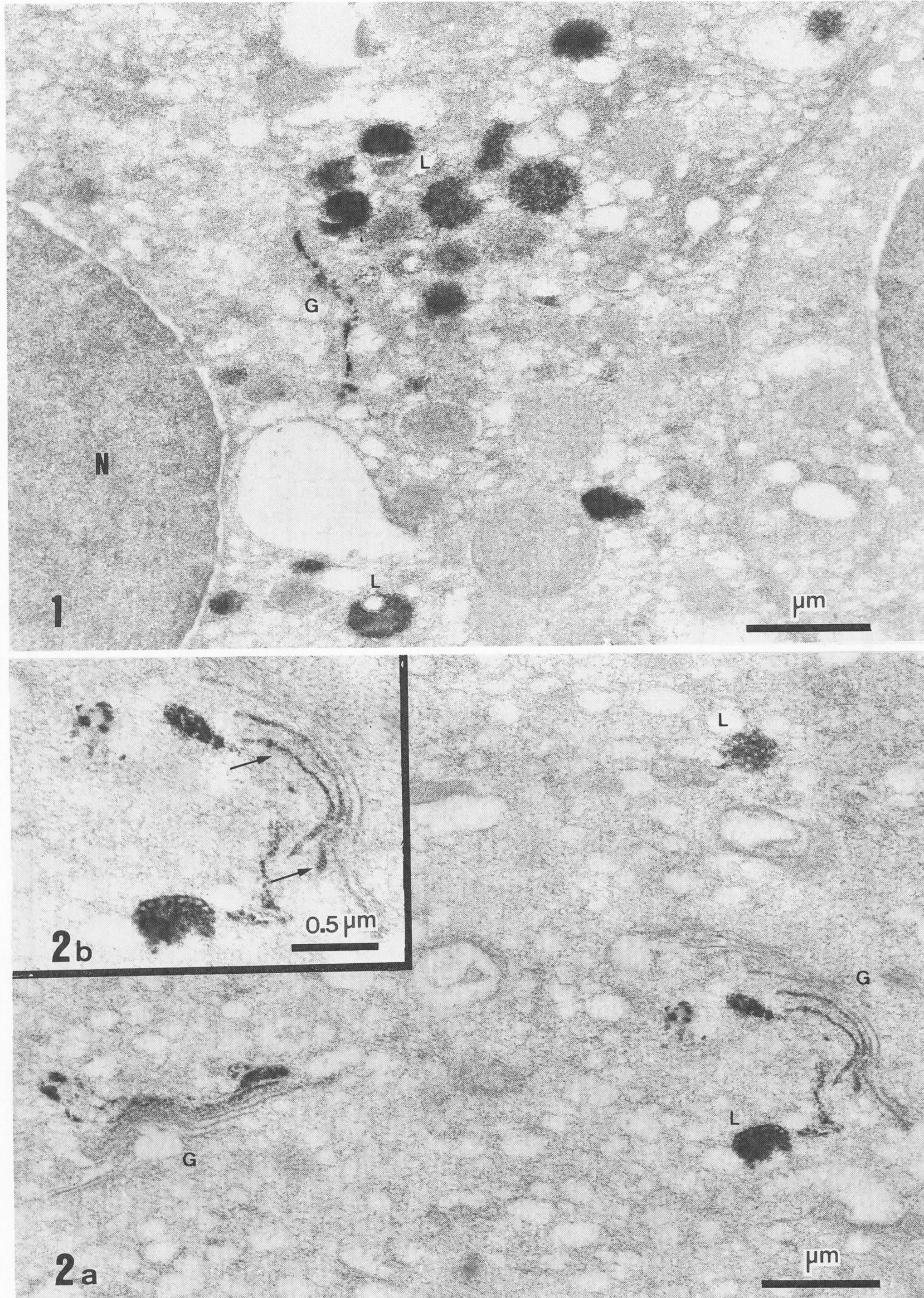



**Fig. 1.** Reaction products for AcPase activity in the lysosomes (L) and in the Golgi complex (G). N: nucleus. Cerium-based method. bar = 1 $\mu$m.

**Fig. 2a and 2b.**

**2a.** Reaction products for AcPase activity in the lysosomes (L) and in two cisternae of the Golgi lamellae on the trans-side (G). Cerium-based method. bar = 1 $\mu$m.

**2b.** Higher magnification of the Golgi complex in Fig. 2a. Arrows indicate two cisternae of the Golgi lamellae on the trans-side. Cerium-based method. bar = 0.5 $\mu$m.

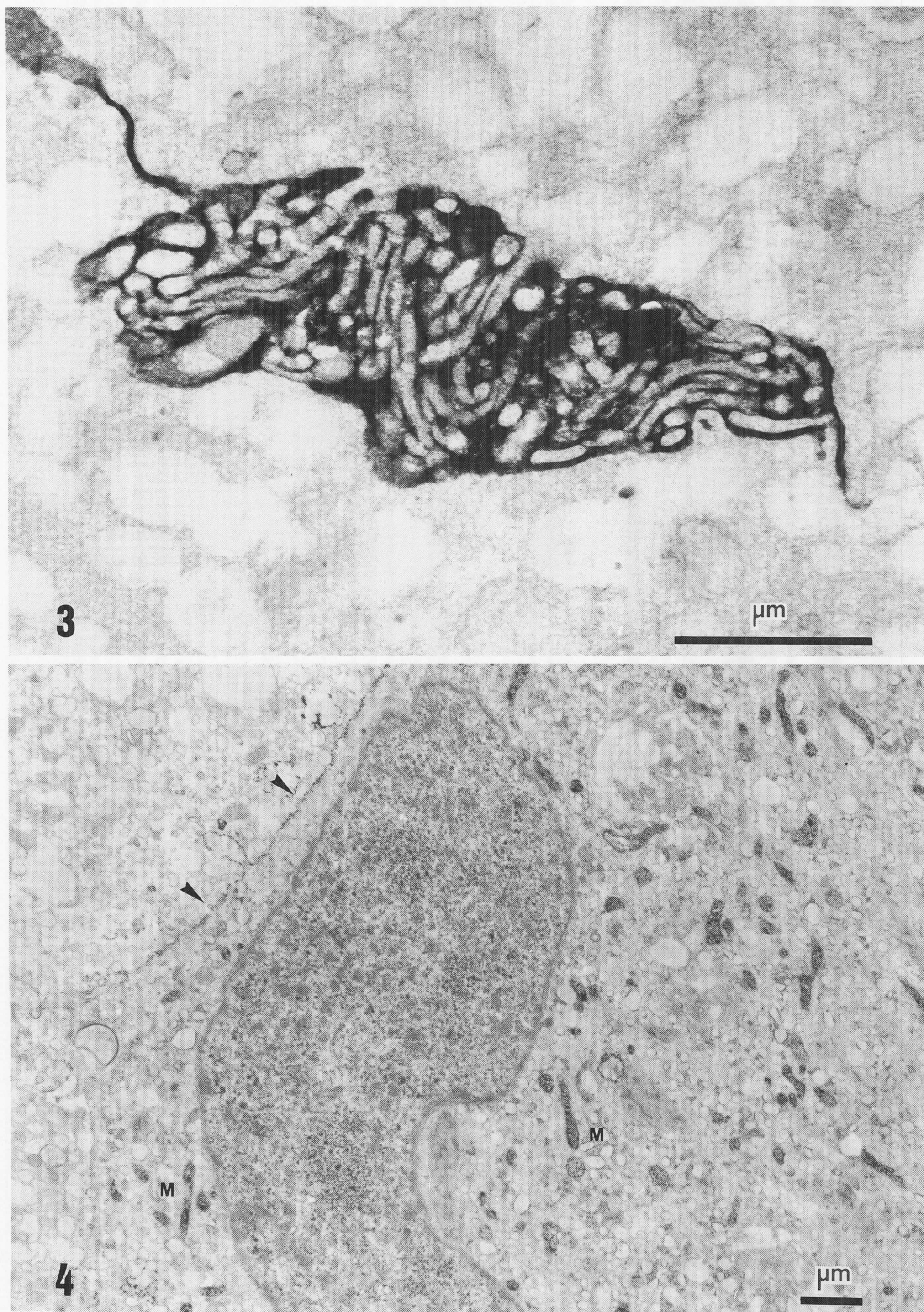
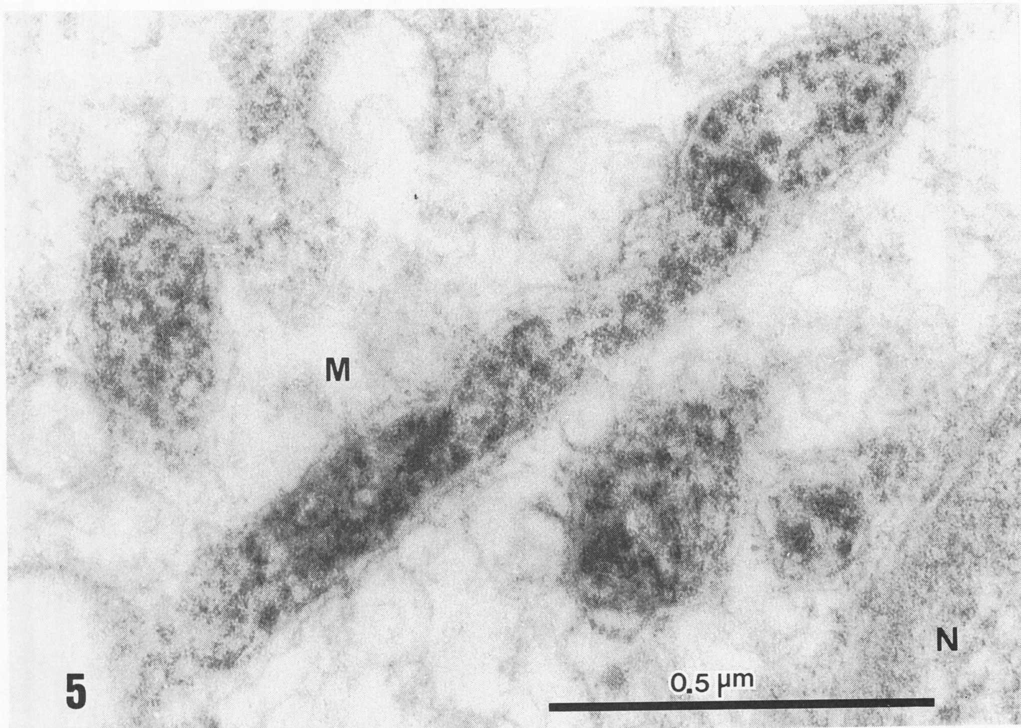



**Fig. 3.** Reaction products for AMPase activity in the plasma membrane. Cerium-based method. bar= 1 $\mu$m.
**Fig. 4.** Reaction products for Mg-activated ATPase activity in the matrix of the mitochondria (M) as well as in the plasma membrane (arrowheads). Cerium-based method. bar= 1 $\mu$m.



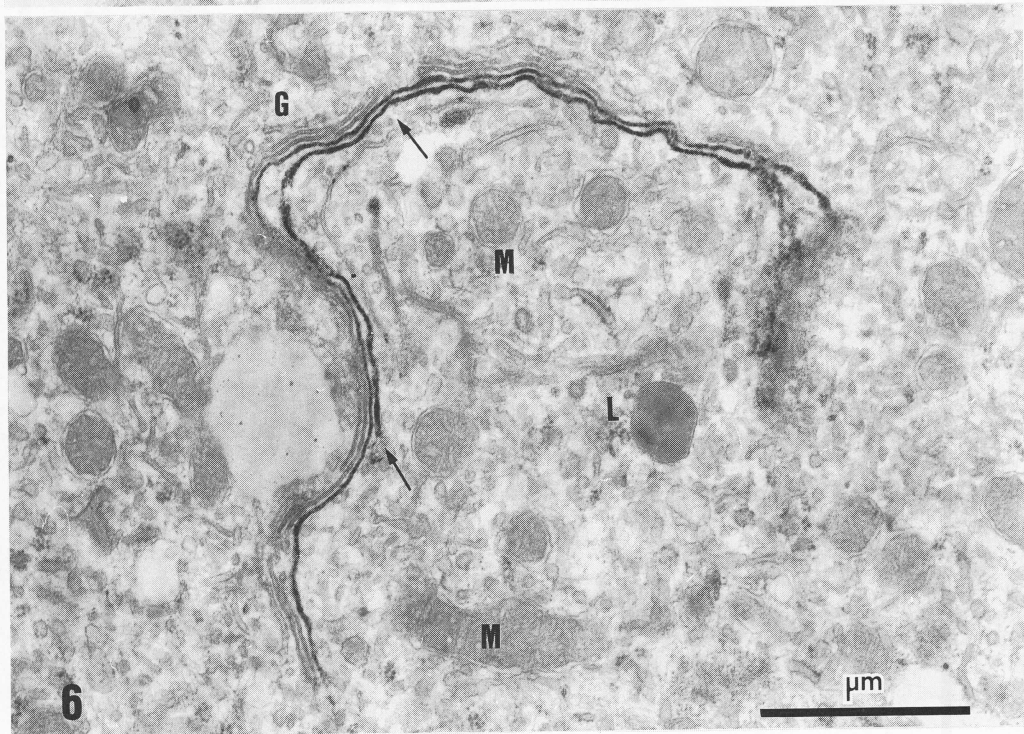
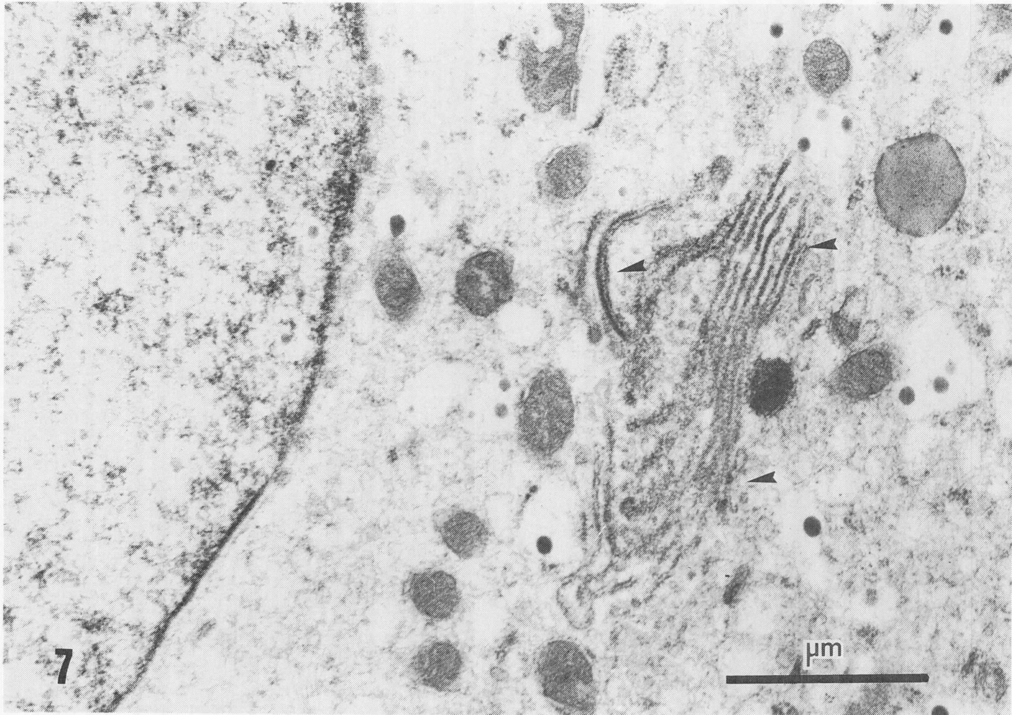




**Fig. 5.** Higher magnification of the mitochondria in Fig. 4. Cerium-based method. bar= 0.5 μm.
**Fig. 6.** Reaction products for TPPase activity in two cisternae of the Golgi lamellae (G) on the trans-side (arrows). L: lysosome, M: mitochondria. Cerium-based method. bar= 1 μm.



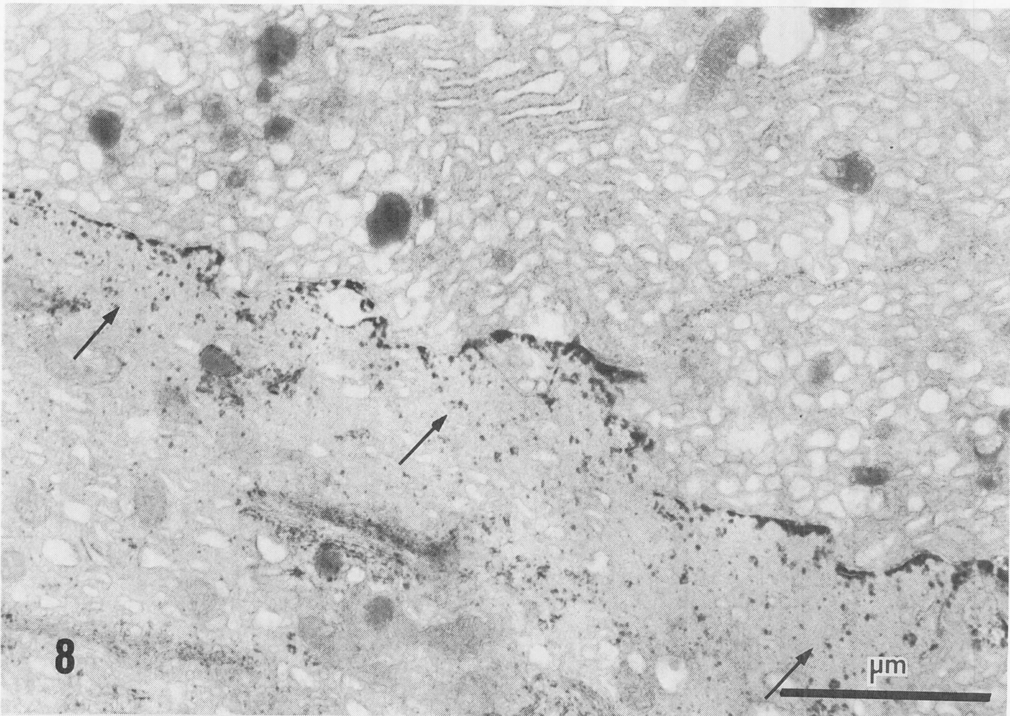




**Fig. 7.** Reaction products for G6Pase activity in all rough endoplasmic reticulum (arrowheads). Cerium-based method. bar = 1 μm.

**Fig. 8.** Reaction products for AMPase activity. Note non-specific deposits showing accumulation or diffusion of electron dense material from the plasma membrane into the cytoplasm (arrows). Lead-based method. bar = 1 μm.

**Table 2.** Ultracytochemical localization of AcPase, AlPase, AMPase, Mg-activated ATPase, TPPase and G6Pase with cerium in the human corpus luteum of early pregnancy

| | |
|---|---|
| AcPase | lysosome<br>two cisternae of the Golgi lamellae on the trans-side |
| AlPase | plasma membrane |
| AMPase | plasma membrane |
| Mg-activated ATPase | matrix of the mitochondria<br>plasma membrane |
| TPPase | two cisternae of the Golgi lamellae on the trans-side |
| G6Pase | rough endoplasmic reticulum |

does not completely penetrate the tissue sections, that is, even in a tissue section of 40-75 μm, there will be the areas where the cerium has not penetrated (Robinson and Karnovsky[12]). In order to overcome its inadequate penetration of tissue sections, 30-40 μm thick sections, cut on a recently improved new instrument for making non-frozen sections "microslicer" (Mayahara et al[7]), were preincubated in the medium without the substrate.

In these ultracytochemical study described above, cerium phosphate reaction product appears as very fine electron dense precipitate. The AcPase, AlPase, AMPase, ATPase, TPPase and G6Pase were clearly observed in the human corpus luteum of early pregnancy using a cerium-based method in combination with a microslicer (Table 2). The localizations in the luteal cells are in accordance with the earlier observations in other organs[12,17].

The localizations of phosphatases have been demonstrated in the liver, spleen, neutrophil, and kidney. These enzymes have an important role in maintaining the metabolic activity in the cell. However, in the human corpus luteum of early pregnancy few reports have been published.

The AcPase in one of the lysosomal enzymes and the lysosome has many biological function in cytodifferentiation, absorption, digestion, metabolism of protein, lipid and nucleic acid and secretion[13]. The lysosomes are formed in rough endoplasmic reticulum-Golgi system, so the AcPase activity was observed in rough endoplasmic reticulum, the nuclear envelope, the Golgi complex and some granules in developing guinea pig neutrophils[12]. However, in the rat liver[5] and in the luteal cells at the present study, the AcPase activity was observed only in the lysosomes and in two cisternae of the Golgi lamellae on the trans-side.

The AlPase is believed to be important in the material transport of plasma membrane and is observed in almost all cells. In this study, the reaction products were found in all plasma membrane of the luteal cells as other cells[12].

The AMPase is the ectoenzyme localized in the plasma membrane and called as the marker enzyme of the plasma membrane[11], and this enzyme has been believed as the important one to regulate the cell function by changing the absorption of the nucleoside. In the luteal cells, the activity is also observed in all plasma membrane.

The Mg-activated ATPase is positive in the plasma membrane of various cells[5]. On the localization of intramitochondrial ATPase activity, Ogawa and Mayahara reported that the ATPase activity was demonstrated in the matrix of the mitochondria[9], and this reaction was activated by $Mg^{++}$. In this study, the activity is found in the matrix of the mitochondria as well as in the plasma membrane and control incubation revealed no precipitates. The intramitochondrial localization of ATPase in the present study is in accordance with the results by Ogawa and Mayahara[9].

The areas positive for the TPPase activity is believed to be limited in the trans-side of the Golgi complex and the areas stained for the TPPase and AcPase reactions are partially or completely overlapping though there are minor variations among them[13]. In the luteal cells the TPPase activity is limited in two cisternae of the Golgi lamellae on the trans-side and both TPPase and AcPase activities have partial overlap.

The G6Pase is an enzyme to produce the glycogen and release it to the vessels in the liver. In the liver, the activity was seen in all cisternae of the rough and smooth endoplasmic reticulum and of the nuclear envelope[5]. In this study, the activity is localized in all rough endoplasmic reticulum but not in the smooth endoplasmic reticulum in the luteal cells.

Although the sites of phosphatase activity by the cerium-based method are not essentially

different from the results by the lead-based method, non-specific deposits showing accumulation or diffusion of electron dense material are often recognized in the lead-based method, especially for the demonstration of AMPase. With the cerium-based method as described above, there is no tendency of the formation of non-specific deposits. In addition, its fine granularity of reaction product shows a precise localization of phosphatase activity.

In conclusion, the cerium-based method as an ultracytochemical probe for phosphatase activity overcomes the above mentioned problems and provides additional insights into the cell structure and function. And this technique in combination with the microslicer is superior to the traditional lead-based method because of its reproducibility and specificity and can be applied routinely for the demonstration of phosphatase activity, especially for AMPase activity. Furthermore, these several phosphatases positive in the luteal cells might serve to maintain a constant metabolic activity in the early pregnancy.

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