2 H.N.B. Gopalan, Chromosoma *44*, 25 (1973).
3 M. Robert and H.N.B. Gopalan, Kenya Nat. Acad. J. Sci. and Technol., in press (1979).
4 H. Kroeger, Chromosoma *15*, 36 (1964).
5 A.L. Politoff, S.J. Socolar and L.R. Lowenstein, J. gen. Physiol. *53*, 498 (1969).
6 M. Robert, Chromosoma *36*, 1 (1971).
7 M.T. Franze-Fernandez and A.O. Pogo, Proc. natl. Acad. Sci. USA *68*, 3040 (1971).
8 C.N. Murthy and H. Sidransky, Biochim. biophys. Acta *262*, 328 (1971).
9 M. Oravec and A. Korner, Biochim. biophys. Acta *247*, 404 (1971).
10 J. Vesely and A. Cihak, Biochim. biophys. Acta *204*, 614 (1970).
11 R. Shields and A. Korner, Biochim. biophys. Acta *204*, 521 (1970).
12 G. Arcaya, N.E. Pantoja, M. Pieber, C. Romero and J.C. Toha, Z. Naturforsch. *26b*, 1026 (1971).
13 J.L. Dimicoli and C. Helene, Biochimie *53*, 331 (1971).
14 C. Helene, Nature, New Biol. *234*, 120 (1971).

Microperoxisomes in steroidogenic cells of the rat ovary: Interstitial, thecal and luteal cells[1]

G. Familiari, G. Franchitto, S. Correr and P. Motta

Department of Anatomy, Faculty of Medicine, University of Rome, Viale Regina Elena 289, I-00100 Roma (Italy), 8 December 1978

Summary. Microperoxisomes are present in luteal, interstitial and thecal cells. They are in close relation with smooth endoplasmic reticulum and lipids. Their probable role in steroid biosynthesis is discussed.

Peroxisomes (or microbodies) are found in a variety of plant and animal cells[2,3]. Biochemically peroxisomes are characterized by the presence of oxidative enzymes generating H_2O_2 and catalase, which are able to break down H_2O_2[4].

Morphologically, by electron microscopy, they were originally noted in the cells of the proximal convoluted tubule of the kidney[5] and in liver cells[6,7]. In these cells they appear as rather round organelles of about 0.5–1.0 µm in diameter, bounded by a unit membrane and contain a dense matrix in which a cristalline nucleoid is evident. Although, morphologically, it is sometimes difficult to differentiate them from primary lysosomes, cytochemically they can be easily distinguished from lysosomes because they lack acid phosphatase.

More recently, smaller peroxisomes (0.1–0.45 µm in diameter) have been described in a large variety of tissues. These organelles, considered by some authors as progenitors of the larger peroxisomes[8], are commonly called 'microperoxisomes' but lack a central nucleoid and may sometimes be easily confused with primary lysosomes. Cytochemically they can be identified as microperoxisomes because they do not contain acid phosphatase as do lysosomes, and better because they can be easily identified by their specific staining with 3–3′ diaminobenzidine (DAB)[9].

More recently, a number of microperoxisomes have been identified in a number of steroid-secreting cells and their possible significance in steroid biosynthesis has been discussed.

Among steroidogenic tissues studied, microperoxisomes have been cytochemically identified in adrenal cortex[10–12], in Leydig cells[13–15] and in corpus luteum of late pregnancy[16–18]. In all these cells, they have been reported as frequently being closely associated with membranes of smooth endoplasmic reticulum. The purpose of this paper is to identify, by DAB staining, microperoxisomes in steroidogenic cells of adult ovary (luteal, thecal and interstitial cells) and to describe their possible relationship with other cellular organelles.

Materials and methods. Morphology. 5 anesthetized healthy young adult albino rats were used in this study. Ovaries were perfused with a solution of 3% glutaraldehyde buffered to pH 7.4 with 0.1 M sodium cacodylate for 4–6 h at 4 °C.

The tissues, cut into small pieces, were then washed in the same buffer, postfixed in 1.0% osmium tetroxide, for 1–2 h, dehydrated in alcohol, and embedded in Epon 812[19]. Thin sections cut on a Porter-Blum MT-1 ultramicrotome were stained with uranyl acetate[20] followed by lead citrate[21] and examined in a Zeiss EM9A electron microscope.

Cytochemistry. Small pieces of tissue (1–2 mm^3) were fixed for 4–6 h in 3% glutaraldehyde buffered to pH 7.4 with 0.1 sodium cacodylate, then rinsed overnight in the same buffer at 4 °C. For cytochemical identification of microperoxisomes, the pieces were incubated in DAB oxidation medium at pH 9.0[9]. In the control experiments, the pieces were incubated in medium lacking H_2O_2.

In other control tissue, both the preincubation and incubation medium contained 0.2 M aminotriazole. After incubating in the medium with DAB, the pieces were washed 3 times (5 min each) in 0.05 M 2-amino-2-% methyl-1,1-propandiol buffer (pH 9.0), postfixed in 1% cacodylate buffered osmium tetroxide for 1 h, dehydrated in a graded series of alcohol, and embedded in Epon 812. Thin sections, either unstained or stained with uranyl acetate and

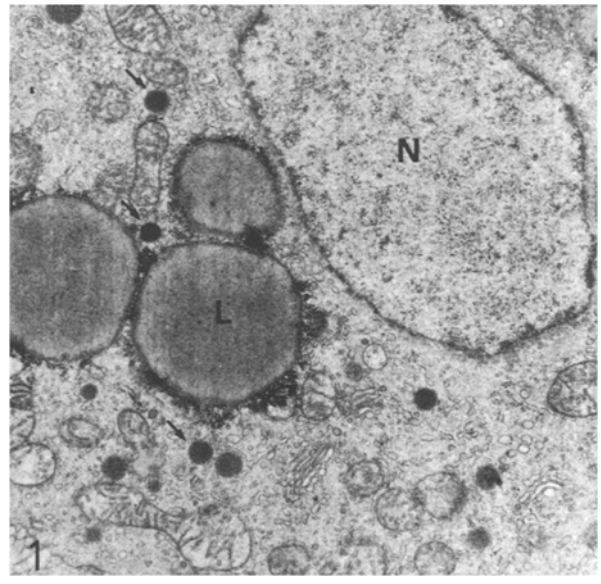

Fig. 1. Rat's ovary: corpus luteum. N, nucleus; L, lipids droplets; arrows, microperoxisomes.

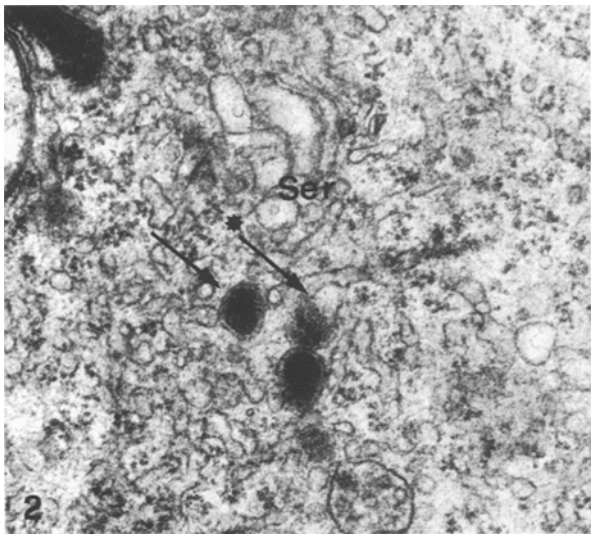

Fig. 2. Rat's ovary: interstitial gland. SER, smooth endoplasmic reticulum; → microperoxisomes; *→ continuity of microperoxisome with SER.

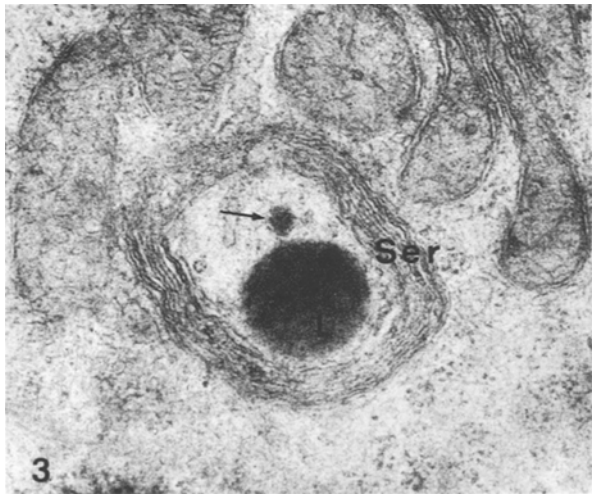

Fig. 3. Rat's ovary: corpus luteum. SER, smooth endoplasmic reticulum; L, lipids droplets; → microperoxisome.

lead citrate, were studied with a Zeiss EM9A electron microscope.

Results. Microperoxisomes of steroidogenic cells of the rat, when incubated in DAB and fixed in aldehyde, appear as small round organelles (0.1–0.5 μm in diameter) bounded by a unit membrane which is highly electron opaque (figure 1).

Microperoxisomes have the same relationship in luteal, interstitial and thecal cells: they are in close proximity with the membranes of smooth endoplasmic reticulum (ER) (figure 2).

In other cases, when the steroidogenic cells are filled with smooth ER and lipids, microperoxisomes are usually associated with membranes of smooth endoplasmic reticulum having a rounded shape and lipids droplets (figure 3). Moreover microperoxisomes were observed in close relation only with lipids droplets (figure 4, a), especially when regressive changes occur in the cytoplasm of steroidogenic cells. In these cases, at high magnification, the limiting membrane of the microperoxisomes appears contiguous

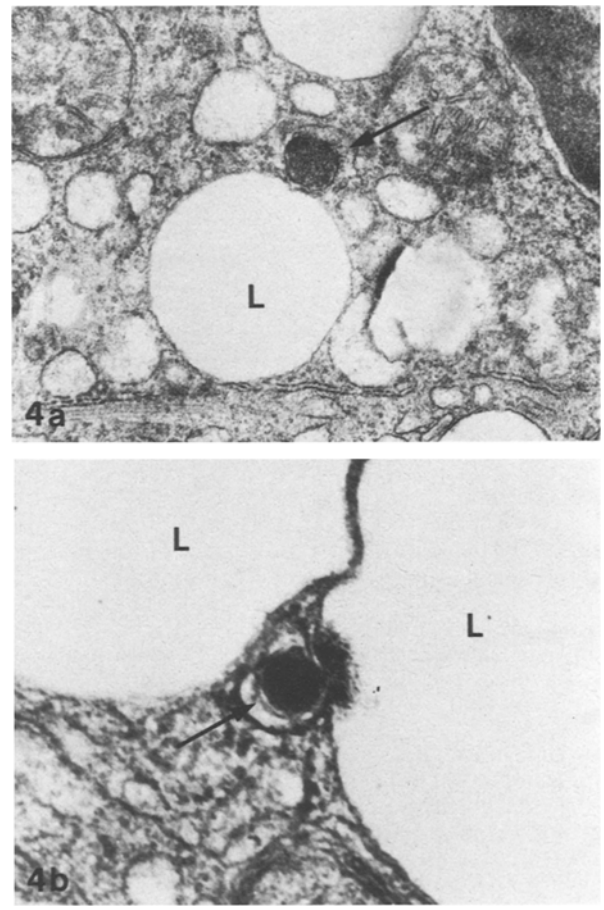

Fig. 4. Rat's ovary: thecal cells in regressive phose. *a* L, lipid droplets; → microperoxisome. *b* L, lipids droplets; → microperoxisome. Note the continuity of microperoxisome with the lipids droplets.

with a lipid droplet. In addition the membrane appears to be discontinuous in some areas and the material contained in the peroxisomes (enzymes?) seems to be involved in a process of diffusion (figure 4, b).

Discussion. Organelles with a paracrystalline nucleoid (0.2–0.3 μm in diameter) (peroxisomes), were previously described in interstitial, luteal and granulosa cells of pregnant and nonpregnant mice[22]. The relationship of these organelles with smooth endoplasmic reticulum has not been considered, and only their catalytic activity with peroxidase has been demonstrated.

Gulyas and Yuan[17] studied microperoxisomes by means of DAB staining in granulosa and luteal cells of Rhesus monkey. Their close association with both smooth endoplasmic reticulum and lipid droplets was observed. Recently the same authors[18] demonstrated in corpus luteum of Rhesus monkey incubated with DAB the continuity of microperoxisomes with membranes of smooth endoplasmic reticulum by means of serial sections. Using tilted sections they also demonstrated various kinds of continuity between smooth endoplasmic reticulum and microperoxisomes. Microperoxisomes are generally considered to be organelles of subcellular respiration containing catalase and flavinoxidase[23,24].

Particulary, in Leydig cells[25], it was proposed that peroxisomes through catalase might regulate intracellular cholesterol and its utilization in the synthesis of androgens.

The presence of microperoxisomes in different steroidogenic cells of rat ovary confirm their close relation with smooth

endoplasmic reticulum and lipid droplets. Moreover, the turnover of these cellular organelles in relation to the cycle of interstitial, luteal and thecal cells of rat ovary should also be considered.

The close association of microperoxisomes with membranes of smooth endoplasmic reticulum and lipid droplets, which in active steroidogenic cells are considered respectively as sites of steroid enzymes and material precursor of the hormones[26], might suggest a probable role of these organelles in the synthesis of steroid products. Further the close association between microperoxisomes and partially empty lipids in some cases might be related with the anabolic phase of the cell, more than with the secretion of steroid material[27].

1 This work was supported by funds from CNR (Contract N.CT-77.01921.4).
2 J.F. Hogg, Ann. N.Y. Acad. Sci. *168*, 209 (1969).
3 Z. Hruban and M. Rechcigl, Jr. Int. Rev. Cytol. suppl. 1, 20 (1969).
4 C. de Duve, Proc. R. Soc., Biol. *173*, 71 (1969).
5 J. Rhodin, Thesis, University of Stockholm, Stockholm 1954.
6 H. Gansler and C. Rouiller, Schweiz. Z. allg. Paht. Bakt. *19*, 217 (1956).
7 P. Baudhuin, H. Beaufay and C. de Duve, J. Cell Biol. *26*, 219 (1965).
8 A.B. Novikoff and P.M. Novikoff, J. Histochem. Cytochem. *21*, 963 (1973).
9 A.B. Novikoff and S. Goldfischer, J. Histochem. Cytochem. *17*, 675 (1969).
10 M.M. Magalhoes and M.C. Magalhoes, J. Ultrastruct. Res. *37*, 563 (1971).
11 M.E. Beard, J. Histochem. Cytochem. *20*, 173 (1972).
12 V.H. Black and B.H. Bogart, J. Cell Biol. *57*, 345 (1973).
13 J.K. Reddy and D. Svoboda, J. Histochem. Cytochem. *20*, 140 (1972).
14 J.K. Reddy and D. Svoboda, Lab. Invest. *26*, 657 (1972).
15 V.H. Black, Anat. Rec. *178*, 506 (1974).
16 Z. Hruban, E.L. Vigil, A. Slesers and E. Hopkins, Lab. Invest. *27*, 184 (1972).
17 B.J. Gulyas and L.C. Yuan, J. Histochem. Cytochem. *23*, 359 (1975).
18 B.J. Gulyas and L.C. Yuan, Cell Tissue Res. *179*, 357 (1977).
19 J.H. Luft, J. biophys. biochem. Cytol. *9*, 409 (1961).
20 M.L. Watson, J. biophys. biochem. Cytol. *4*, 475 (1958).
21 E.S. Reynolds, J. Cell Biol. *17*, 208 (1963).
22 P. Bock, Z. Zellforsch. *133*, 131 (1972).
23 E. Mc Groarty and N.E. Tolbert, J. Histochem. Cytochem. *21*, 949 (1973).
24 C. de Duve, J. Histochem. Cytochem. *21*, 941 (1973).
25 J.K. Reddy, J. Hystochem. Cytochem. *21*, 967 (1973).
26 A.K. Christensen and S.W. Gillim, in: The gonads, p.415. Ed. I. McKerns. Appleton-Century, New York 1969.
27 A.B. Novikoff, in: Ciba Found. Symp. on Lysosomes, p.36. Ed. A.V.S. DeRenck and M.P. Cameron. Little Brown, Boston 1963.

Effects of low temperatures on survival of frozen-thawed mouse embryos

H. Miyamoto and T. Ishibashi

Department of Animal Science, College of Agriculture, Kyoto University, Kyoto 606 (Japan), 23 January 1979

Summary. Almost no damage to mouse morulae was observed between 0 and −40 °C, and freezing damage to embryos in DMSO, glycerol or ethylene glycol occurred after exposure to −60, −50 or −50 °C, respectively. Cooling embryos in DMSO or glycerol to temperatures below −50 to −60 °C increased freezing damage. To the contrary, in the presence of ethylene glycol, no more damage occurred after exposure to temperatures below −50 °C.

Although mouse embryos stored for 200 days or longer at −88 °C or −196 °C develop into viable fetuses, embryos stored at −44 °C are not viable after 3 days in storage[1]. Storage of embryos at −88 °C or lower is, therefore, necessary for practical application. A major cause of freezing damage is the formation of intracellular ice crystals during cooling and their growth by recrystallization during thawing[2]. Intracellular freezing of unfertilized mouse ova cooled in the presence of 1 M dimethyl sulphoxide (DMSO) generally occurs at about −40 to −45 °C[3]. The survival and fertilizability of unfertilized mouse ova after freezing and thawing are dependent upon the temperature to which they are exposed[4]. This report examines the resistance to cooling of mouse embryos to various low temperatures in the presence of different cryoprotectants.

Materials and methods. Female ICR mice were induced to superovulate and mated[5]. Morulae were flushed from the reproductive tracts with a modified Dulbecco's phosphate-buffered salt solution (PBS)[5] at 78–82 h after an injection of HCG. 10–20 embryos were transferred to each test-tube containing 0.1 ml PBS. The tubes, suspended in ethanol in a Dewar flask, were then cooled to 0 °C and the cryoprotectant in 0.05 ml PBS kept at 0 °C was added to samples in 3 increments at 10-min intervals. The cryoprotectants used were 1.2 M DMSO, 1 M glycerol and 1.2 M ethylene glycol. All samples were equilibrated for 10 min at 0 °C after the addition of the cryoprotectant and seeded with an ice crystal at −5 °C. They were cooled to −79 °C at 0.5 °C/min by adding dry ice to ethanol in a Dewar flask and then a Dewar flask containing samples was placed into liquid nitrogen to be cooled from −79 to −120 °C at 1–2 °C/min. After the temperature of samples was lowered to −120 °C, samples were transferred into liquid nitrogen. They were recovered for survival estimation from a Dewar flask or

Survival of mouse morulae after freezing to various low temperatures in the presence of DMSO, glycerol or ethylene glycol

Freezing temperature (°C)	Percentage of morulae developing to expanded blastocysts after culturing for 36 h Cryoprotectant		
	DMSO (1.2 M)	Glycerol (1 M)	Ethylene glycol (1.2 M)
0	96 (77)	100 (79)	96 (74)
− 10	100 (84)	100 (83)	100 (80)
− 20	94 (82)	97 (73)	95 (91)
− 30	91 (93)	93 (89)	92 (77)
− 40	93 (88)	91 (77)	93 (88)
− 50	91 (80)	79 (86)	86 (95)
− 60	86 (90)	74 (92)	84 (90)
− 70	87 (95)	68 (79)	86 (77)
− 79	87 (77)	70 (88)	88 (81)
−100	83 (80)	60 (75)	87 (79)
−120	78 (96)	53 (95)	85 (96)
−196	67 (95)	43 (81)	88 (91)

Figures in parentheses represent the number of thawed embryos recovered.