of b.wt as ice (n = 6). Schmid[2] reported a lower amount, 35%, for *Hyla versicolor*.

The accumulation of cryoprotectants is an important adaptation for survival amongst cold hardy invertebrates[4,5]. Glycerol is the most common cryoprotectant but other polyols and various sugars also occur in some species. Schmid[2] reported 0.3 M glycerol in urine and muscle samples of the gray tree frog, *H. versicolor*. Cryoprotectants were measured in blood samples of warm and cold acclimated frogs and in those frozen at −4 °C for 4 days. Glucose is the cryoprotectant accumulated by *R. sylvatica* (table). Only minor amounts of glycerol were produced and sorbitol, fructose and mannose were not found. Amounts of glucose of up to 325 µmol/ml were found in blood with an average of 185 µmol/ml (3.33 g%). Glucose accumulated only in frogs which had experienced subzero temperatures. No glucose accumulation was found in cold acclimated frogs even after 12 weeks at 3 °C. Lack of an anticipatory response, as is found for cryoprotectant synthesis in many insect species[4,5], suggests that frogs synthesize cryoprotectant only when immediately threatened with freezing temperatures. Wood frogs hibernate under forest leaf litter; with a deep snow covering most may never experience subzero temperatures. However, if temperatures at hibernation sites do drop below 0 °C the frogs can quickly turn on their cryopreservation mechanisms. Glucose is perhaps an optimal cryoprotectant to use in this regard. Synthesis from glycogen requires only 3 enzymatic steps (phosphorylase, phosphoglucomutase, glucose-6-phosphatase) which are normally present in all tissues.

Effect of low temperature acclimation and freezing exposure on glucose and glycogen metabolism in the wood frog, *R. sylvatica*

		Control	Cold acclimated	Freezing exposure
		Blood metabolites (µmol/ml)		
Glucose		2.4 ± 0.3	2.0 ± 0.3	185.0 ± 39.7
Glycerol		0.1 ± 0.03	0.1 ± 0.05	1.1 ± 0.2
		n = 9	n = 7	n = 6
		Tissue metabolites (µmol/g wet weight)		
Liver:	Glycogen	904.3 ± 59.8	738.3 ± 75.0	100.7 ± 45.5
	Glucose	3.3 ± 0.6	4.1 ± 1.3	344.4 ± 34.7
Muscle:	Glycogen	48.5 ± 4.6	36.4 ± 8.3	38.1 ± 9.3
	Glucose	1.1 ± 0.2	0.8 ± 0.1	26.0 ± 4.1
		n = 5	n = 5	n = 6

Results are means ± SEM with n as shown. Control frogs were sampled after 3 weeks at 23 °C. Cold acclimated frogs were sampled at intervals between 1 and 12 weeks at 3 °C; no significant differences were found with time. Freezing exposed frogs underwent a 1 °C per day decrease in temperature from 3 to −4 °C followed by 4 days frozen at −4 °C. Frogs were killed by double pithing. Blood samples were removed from the severed aorta using a heparinized capillary tube. Tissues were rapidly dissected out and frozen in liquid nitrogen. Perchloric acid extracts of blood and tissues were prepared and metabolites were measured enzymatically as described by Storey and Storey[6,7]. Glycogen is quantitated as glucose units.

To further investigate cryoprotectant metabolism in *R. sylvatica* tissue glycogen and glucose levels were measured (table). Liver of *R. sylvatica* contained large amounts of glycogen; accumulation of glycogen during autumn months has been well documented for other anuran species as a preparation for winter survival[8,9]. Glycogen content of the liver decreased somewhat over the 12 weeks of cold acclimation, no doubt supporting basal metabolism. Liver glycogen content dropped dramatically, however, over the 8 days of exposure to subzero temperature. The corresponding rise in liver glucose levels indicates that liver glycogen reserves are converted to glucose. Muscle glycogen reserves were not affected by freezing exposure despite an increase in muscle glucose levels. This suggests that liver glycogen may be the source of all glucose (tissue and blood) produced with glucose distributed from liver to all other tissues via the blood. The 638 µmol/g wet weight decrease in liver glycogen (measured as glucose units) more than accounts for the 340 µmol/g rise in liver glucose levels. Although muscle glucose levels were elevated in freezing exposed animals, they were not in equilibrium with blood glucose concentration. This may perhaps be due to a restriction of blood flow to the extremities when subzero temperatures are encountered and/or an early freezing of extremities.

The present study shows that wood frogs can rapidly alter their intermediary metabolism when faced with freezing temperatures and initiate a rapid synthesis of glucose for use as a cryoprotectant. The accumulation of high levels of glucose in animals which have been exposed to freezing suggests major alterations in the mechanisms of control of blood glucose levels by pancreatic hormones (insulin, glucagon) in the freeze tolerant animal.

1 Acknowledgments. I thank Dr J. Ballantyne, Dr F. Schueler and I. McMurray for help with frog collections and Dr. W. Schmid, Dr J. Bogart and Dr F. Cook for helpful discussions. Supported by an N.S.E.R.C. operating grant and by a grant from the Atkinson Charitable Foundation.

2 Schmid, W.D., Science *215* (1982) 697.
3 MacArthur, D.L., and Dandy, J.W.T., Comp. Biochem. Physiol. *72A* (1982) 137.
4 Storey, K.B., and Storey, J.M., Trends biochem. Sci. *8* (1983) 242.
5 Ring, R.A., in: Low Temperature Preservation in Medicine and Biology, p. 187. Eds M.J. Ashwood-Smith and J. Farrant. Pitman Medical Publishing Co., Tunbridge Wells 1980.
6 Storey, K.B., and Storey, J.M., J. comp. Physiol. *131* (1979) 311.
7 Storey, K.B., and Storey, J.M., J. comp. Physiol. *149* (1983) 495.
8 Farrar, E.S., and Dupre, R.K., Comp. Biochem. Physiol. *75A* (1983) 255.
9 Koskela, P., and Pasanen, S., Comp. Biochem. Physiol. *50A* (1975) 723.

0014-4754/84/111261-02$1.50 + 0.20/0

Fluid and protein clearance in the rat endometrium. Part I: Ultrastructural proof of the absence of an intrinsic lymphatic system from the rat endometrium

J.M. Lauweryns[1] and F.J. Cornillie

Katholieke Universiteit te Leuven, School of Medicine, Laboratory of Histopathology, 12 Minderbroedersstraat, B-3000 Leuven (Belgium), 5 December 1983

Summary. An integrated histological and ultrastructural study of the endometrial microcirculation in rats reveals that lymphatic capillaries are absent from the superficial uterine mucosa. Blood capillaries are fenestrated, and their basement membrane may be poorly developed.

Key words. Rat, uterus; rat, endometrium; uterus, rat; microcirculation, endometrial; clearance, non-lymphatic; fluid-drainage physiology; lymph drainage; capillaries, lymphatic.

Lymphatic capillaries drain excessive interstitial proteins and fluid from the tissues[2,3]. The cyclical formation of an endometrial edema during pre-estrus is a well known physiological event in Rodentia[4,5]. It is suggested that endometrial lymphatic capillaries remove these proteins and fluid from the interstitium[6]. In the murine endometrium Fabian[7] described well developed subepithelial lymphatic capillaries that could be traced very easily by an intra-uterine instillation of Patent Blue Violet. She also observed a marked dilatation of the lymphatic capillaries in the middle endometrium during estrus. However, other experiments with interstitial injections of Japanese ink, vascular perfusion fixation and histological studies[8] or vascular perfusion fixation and light optical investigations without any tracer injection[9] demonstrated that endometrial lymphatics were absent throughout the entire uterine mucosa in mice. Still other micro-anatomical studies[6,10] could not demonstrate lymphatic capillaries in the superficial endometrium of rabbits and rats; however, a well developed lymphatic plexus was shown in the basal endometrium of these species.

Blood and lymphatic capillaries can be differentiated morphologically with certainty only by correlated light-microscopic and ultrastructural studies[11].

The present contribution deals with the lymph-drainage in the endometrium of the rat and tends to rely specifically on ultrastructural data. So, for the first time, this paper offers clear morphological proof of the absence of endometrial lymphatics in the rat. In the following accompanying paper the presence and ultrastructure of alternative, non-lymphatic endometrial clearance mechanisms is described and discussed with special attention to endometrial fluid-drainage physiology.

Material and methods. 52 virgin and 3 pregnant (day 14) WISTAR rats were anesthetized with sodium pentobarbital (30 mg/Kg i.p.). The estrus cycle of virgin rats was determined by vaginal cytology. 40 biopsies (20 from each uterine horn) per animal were processed for light and/or electron microscopy. Histological specimens were prepared following routine schedules. For ultrastructural study, the uterus was fixed either by vascular perfusion with 1.25% glutaraldehyde (0.1 M phosphate buffer; pH 7.2; 350–360 mosm) at room temperature or by immediate immersion of thin (1 mm) transverse slices in cold 2.5% glutaraldehyde solution (650–700 mosm). Perfusion fixation was followed by further immersion in the same primary fixative or directly by a post-fixation in phosphate-buffered 1% OsO_4. The fixed specimens were dehydrated and embedded in epoxy resin. Semithin (1 micron) sections were stained with toluidine blue; thin (50 nm) sections were contrasted with uranyl acetate and lead citrate[12] and studied with a Philips 300 electron microscope.

All specimens were screened histologically for the presence of endometrial lymphatics in paraffin or plastic sections. More than 1000 putative or 'probable' endometrial lymphatic capillaries were selected on semithin sections. They were taken for ultramicrotomy and fine structural study. These small vessels of questionable nature were devoid of intraluminal red cells, had a diameter that was larger than that of the smallest typical blood capillaries and usually lacked apparent pericytes. These histological characteristics are used sometimes to indicate such capillaries light-optically as true lymphatic capillaries.

Results. Correlated light and electron microscopical study of the 'questionable' or 'probably lymphatic' small vessels reveals that all of them are unmistakeable blood capillaries. During pre-estrus, estrus and pregnancy the thin endothelial lining is provided with many fenestrations sometimes occurring in series (fig. 1). In thicker endothelia, vesicular fusion at times creates transendothelial thoroughfare channels (fig. 2). Neither fenestrations nor endothelial channels are found during di-estrus and met-estrus. The morphological appearance of the endothelial basement membrane is variable. Very often a typical basement membrane is lacking (fig. 1) or displaced by a fibrogranular pericapillary substance; elsewhere it may be poorly developed or discontinuous. Usually, thin pericyte processes can be observed in the pericapillary region; they are enclosed by a diverging basement membrane or the pericapillary fibrogranular material (fig. 2). A few rare lymphatic capillaries are present at the endomyometrial junction. Serial sectioning reveals that they are blind beginnings of myometrial

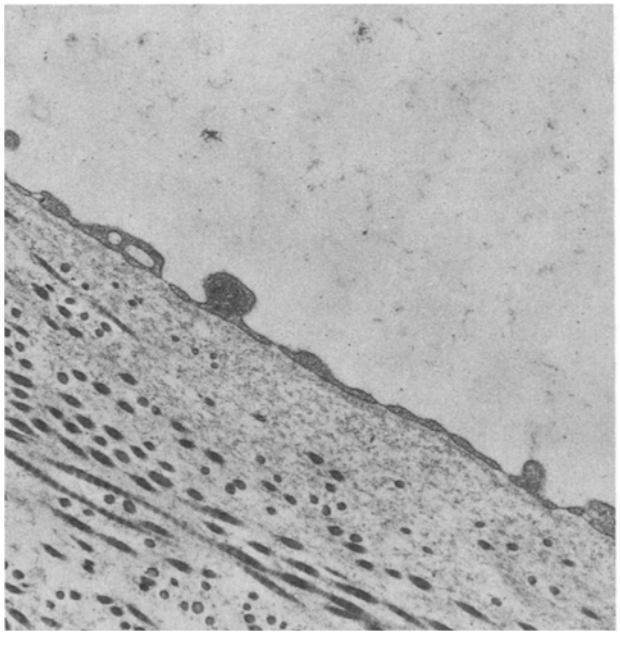

Figure 1. Serial fenestrations (with diaphragm) in the thin endothelium of an endometrial blood capillary. A basement membrane is lacking. Pre-estrus, perfusion fixation. × 23,300.

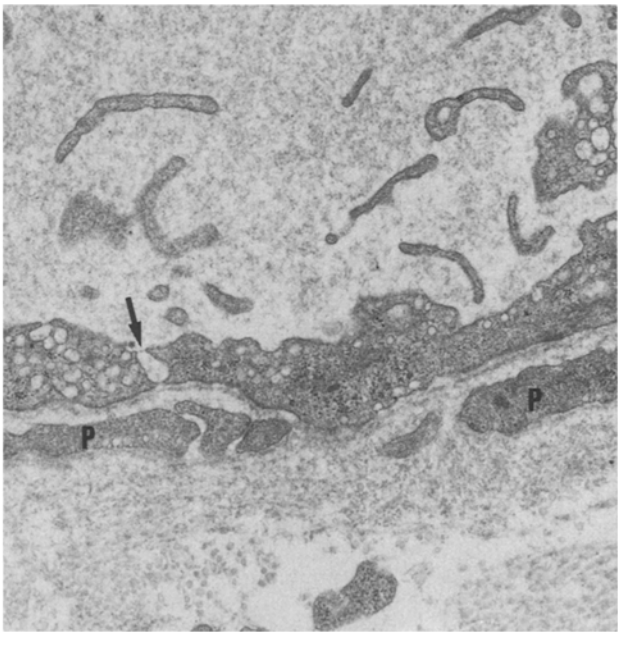

Figure 2. Transendothelial thoroughfare channel (arrow) in an endometrial blood capillary. Processes of a pericyte (P) are surrounded by a basement membrane-like material. Estrus, immersion fixation. × 19,000.

lymphatics; they never traverse the entire mucosa towards the more superficial endometrial regions.

Discussion. Our integrated light and electron microscopic study agrees with the micro-anatomical results obtained in rabbits[6,10], rats[6] and mice[8,9]. They are, however, contradictory to those of Fabian[7] who described a very rapid filling (within a few seconds!) of endometrial lymphatics after intra-uterine injection of Patent Blue Violet. The uterine lumen is lined by epithelial cells that are closely connected with relatively impermeable tight junctions[13]. Tracer experiments with intra-luminal application of ferritin[14,15,16] or thorotrast[17] never demonstrated such a rapid trans- or intercellular passage of particles. However, Fabian noted that the injection of tracer was usually performed in post mortem isolated uteri. Erroneous interstitial injection or a post mortem increase of the epithelial permeability could result in an artefactual access and interstitial accumulation of the tracer. Since Fabian[7] never substantiated the results of injection experiments by morphological studies of the putative, tracer-filled lymphatic capillaries, her description of endometrial lymphatics in mice remains unconvincing.

The absence of an intrinsic endometrial lymphatic system in rats poses the problem of the clearance of fluid and proteins in the uterine mucosa. Because cyclical edemas are removed very efficiently under physiological conditions we presume that at least one (or more?) alternative clearance mechanism(s) is (are) active in the rat endometrium. Clearance along low-resistance connective tissue channels or prelymphatics on the one hand and venous drainage of fluid after interstitial proteolysis on the other hand are 2 such mechanisms suggested to be active in tissues lacking lymphatic capillaries[18,19]. The second part of this contribution will therefore investigate the presence of such non-lymphatic clearance mechanism(s) in the endometrium of the rat.

1 Supported by a grant from the Nationaal Fonds voor Wetenschappelijk Onderzoek-Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Belgium).

2 Földi, M., Experientia *32* (1976) 817.
3 Huth, F., and Bernhardt, D., Lymphology *10* (1977) 54.
4 Ham, K., Hurley, I., Lapota, L., and Ryan, G., J. Endocr. *46* (1970) 71.
5 Martin, L., Hallowes, R., Finn, C., and West, D., J. Endocr. *56* (1973) 309.
6 Leak, L., Anat. Rec. *175* (1973) 396.
7 Fabian, G., Lymphology *9* (1976) 78.
8 Waugh, A., Bennett, N., and Scothorne, R., J. Anat. *127* (1978) 642.
9 Malaney, T., Reid, O., and Scothorne, R., J. Anat. *131* (1980) 754.
10 McLean, J., and Scothorne, R., J. Anat. *107* (1970) 39.
11 Lauweryns, J., and Boussauw, L., Lymphology *2* (1969) 108.
12 Reynolds, E., J. Cell Biol. *17* (1963) 208.
13 Murphy, C., Swift, J., Mukherjee, T., and Rogers, A., Cell Tissue Res. *223* (1982) 281.
14 Parr, M., and Parr, E., Biol. Reprod. *11* (1974) 220.
15 Parr, M., and Parr, E., J. Reprod. Fert. *50* (1977) 151.
16 Parr, M., and Parr, E., J. Reprod. Fert. *52* (1978) 183.
17 Enders, A., and Nelson, P., Am. J. Anat. *138* (1973) 277.
18 Casley-Smith, J., in: Progress in Lymphology, p.5. Eds H. Weissleder, V. Bartos, L. Clodius and P. Malek. Avicenum Czechoslovak medical press, Prague 1981.
19 Casley-Smith, J., Földi-Börcsök, E., and Földi, M., Br. J. exp. Path. *57* (1976) 179.

0014-4754/84/111262-03$1.50 + 0.20/0

Fluid and protein clearance in the rat endometrium. Part II: Ultrastructural evidence for the presence of alternative, non-lymphatic clearance mechanisms in the rat endometrium

F.J. Cornillie and J.M. Lauweryns[1]

Katholieke Universiteit te Leuven, School of Medicine, Laboratory of Histopathology, 12 Minderbroedersstraat, B-3000 Leuven (Belgium), 5 December 1983

Summary. In the rat endometrium, resident macrophages and exudate phagocytes ensure proteolysis by means of phagocytosis, macro- and micropinocytosis. Using exogenous tracer particles no ultrastructural evidence could be obtained for the occurrence of endometrial prelymphatics. It is suggested that the free tissue fluid may be drained via the fenestrated (probably venous) blood capillaries.

Key words. Rat, uterus; rat, endometrium; endometrium, rat; uterus, rat; microcirculation, endometrial; clearance, non-lymphatic; fluid-drainage physiology; lymph drainage; macrophages; phagocytes.

Absence of lymphatic capillaries from the superficial endometrium[2,3] brain[4-7], bone marrow[5,6], retina[5,6,8] and pulmonary interalveolar septa[9-11] has been shown in various species. This absence poses the problem of the clearance of interstitial proteins and fluid in these tissues. In the brain of rabbits and cats Casley-Smith et al.[7] demonstrated a local accumulation of interstitially injected carbon in the basement membrane-region of blood capillaries and in the tunica adventitia of larger vessels. They postulated that transport of proteins occurs in non-endothelialized, low resistance tissue channels, some of which conduct to lymphatic capillaries and therefore were named prelymphatics. Some preliminary morphological characterization of prelymphatics was provided by scanning electron microscope studies of corrosion cast specimens of the rabbit brain microcirculation[12].

Macrophages, however, play an important role in the clearance of interalveolar septa, as was shown with protein tracers such as carbon[13] and ferritin[14,15].

The present paper reports the results of a clearance study in the rat endometrium using colloidal ferritin and carbon as protein tracers and attempts to answer the question which clearance mechanism(s) is (are) present in this tissue, which lacks an intrinsic lymphatic drainage system.

Material and methods. 23 virgin WISTAR rats were used; the anesthesia and fixation procedures were identical to those described earlier[2]. Ferritin (horse spleen ferritin, 2 × crystalline, cadmium free, Fluka) was instillated into the uterine lumen at the ovarian end, using a tuberculin syringe, in 5 rats. Carbon (C11/1431a, Günther Wagner, Hanover) was administered in the same way to 8 animals. Usually 0.15 cc of a 10 x diluted