

**The spermatocyte membrane in *Lithobius forficatus* L.
(Myriapoda Chilopoda).
Changes induced by hormonal actions.
Preliminary results**

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Summary. The plasma membrane of the spermatocyte has been studied in the centipede *Lithobius forficatus* L. using the technique for freeze-fracture replicas. The animals were activated either by an injection of 1 µg of 20-OH-ecdysone or by an electrical stimulation of the *pars intercerebralis*, the posterior-dorsal part of the protocerebron ; both operations led to an increase in spermatocyte synthetic metabolism. The number of intramembraneous particles (IMP) was constantly lower on the E face than on the P face. In all experimentally activated animals, the number of IMP on the E face decreased. In ecdysteroid-treated animals, the total number of IMP on both faces was approximately one-half that of the controls ; nevertheless the P/E ratio of IMP was virtually the same as in the controls. In electrostimulated animals, the P/E ratio was always higher than in the controls, but in the spring series the total number of IMP was significantly higher than in the controls. It is postulated that (i) the decrease of IMP, at least on the E faces, was due to the vertical shift of IMP in the plasma membrane, this change probably being related to the increase in spermatocyte synthetic metabolism, and that (ii) the increase in IMP after electrostimulation probably occurred because of a higher neurohormone level.

Introduction.

In *Lithobius forficatus* L., spermatocyte growth can be increased by two different techniques — the injection of 20-OH-ecdysone (Descamps, 1981a) and electrical stimulation of the brain (Descamps, 1978) — which induce neurohormonal secretions. As it has been demonstrated that hormonal action (Rapoport *et al.*, 1981) or modifications in environmental factors (Funk *et al.*, 1982) can also induce a displacement of intramembraneous particles (IMP) in the plasma membrane, it was tempting to study changes in the partition coefficient and/or in the IMP density caused by increasing spermatocyte growth rate.

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It has been shown that the partition of IMP between the two layers constituting the plasma membrane does not occur at random and that it can be used as a measurement of the interactions of the opposite sides of a particle with adjacent molecules. The resulting partition coefficient of the membrane particles serves as a tool for characterizing a membrane (Satir and Satir, 1974). This partition coefficient is constant for a given type of cell or membrane (*e.g.* specialized plasma membrane, Golgi membrane, endoplasmic reticulum ; Chailley, 1979). The results ⁽¹⁾ of the use of this coefficient in relation to the spermatocyte membrane are reported in the present paper.

Material and methods.

Stimulation. — The synthetic metabolism of the spermatocyte was increased either by the injection of 1 μg of 20-OH-ecdysone (Simes) in Ringer 10 % alcoholic solution or by *pars intercerebralis* electrostimulation (Descamps, 1978). In the latter case, the cephalic cuticle was pierced and silver electrodes 7/100 mm in diameter were introduced into the holes, one in the *pars intercerebralis* area and the other in the posterior-lateral area of the head, in order not to disturb the anterior part of the dorsal blood vessel. Electrical stimulation was obtained by rectangular shocks of 1 msec at a frequency of 5 Hz for 10 min. The direction of the current was changed every 15 sec to avoid electrode polarization.

The experiments were carried out at two different periods, in winter during minimal spermatocyte growth and in spring during rapid spermatocyte growth.

Preparation of freeze-fracture replicas. — 24 h after stimulation, the testis was removed and fixed for 2 h at 4 °C with 6.25 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, prior to glycerination. Freeze-fracturing was performed in a Balzers BAF 300 apparatus equipped with an electron beam evaporation device and a quartz crystal thin-film monitor.

The samples were mounted between two gold disks, frozen in melting nitrogen and stored in liquid nitrogen. They were fractured at -120 °C using a double replica hinge device, followed by Pt-C shadowing. The replicas, cleaned with sodium-hypochlorite for 3 h, washed in distilled water and mounted on copper grids, were examined under a Jeol JEM 100 CX electron microscope.

Counting of IMP. — The surface was measured and the IMP's counted using an electronic measuring device (MOP/AMOI, Kontron) on micrographs of the same magnification ($\times 120\,000$). IMP density was measured per μm^2 .

On fracture replicas of the testis, spermatocyte diameter could be determined only by the cytoplasmic aspect (Golgi structure, number and size of mitochondria). Three classes of spermatocytes were defined according to diameter : less than 50 μm , 50 to 80 μm and more than 80 μm (Beniouri, 1983). We used only those between 50 and 80 μm in diameter which corresponded to a

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period of regular growth. It must be remembered that *L. forficatus* spermatocytes show a dramatic increase in size from about 20 to 100 μm (Descamps, 1971). 200 to 250 density measurements were made for each type of experiment in each experimental series.

Results.

The fracture faces were identified according to Branton *et al.* (1975) : the P face was the hemi-membrane lying on the cytoplasm ; the complementary hemi-membrane was the E face. The horizontal display of IMP was regular and there was always more IMP on the P face than on the E face. The partition coefficient used in this paper was the ratio of IMP density on the P face (PF) to that on the E face (EF) : $K_p = PF/EF$. The total density of IMP (FP + EF) was also an interesting parameter for the characterization of the membrane.

Winter experiments (table 1).

Controls. — The total density of IMP was high ($1\,738 \pm 301$) (fig. 1) and the P/E ratio was 4.47. IMP density was $1\,420 \pm 253$ on the P face and only 318 ± 48 on the E face.

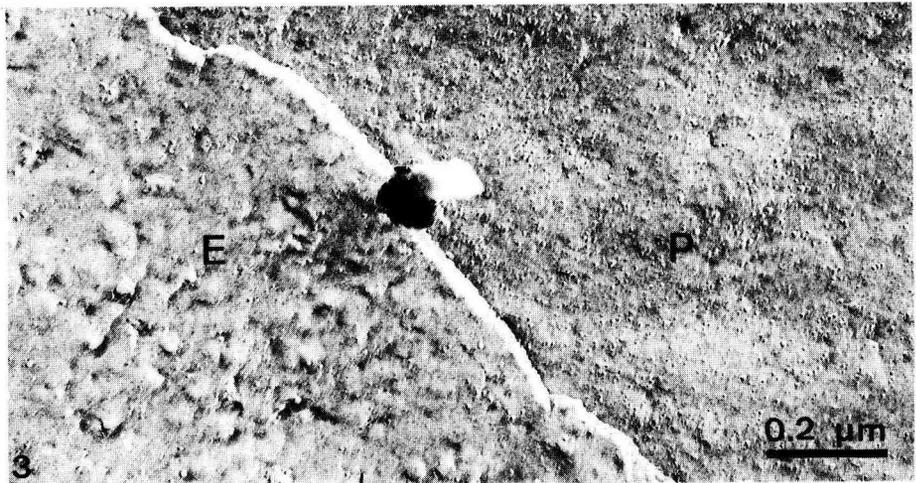
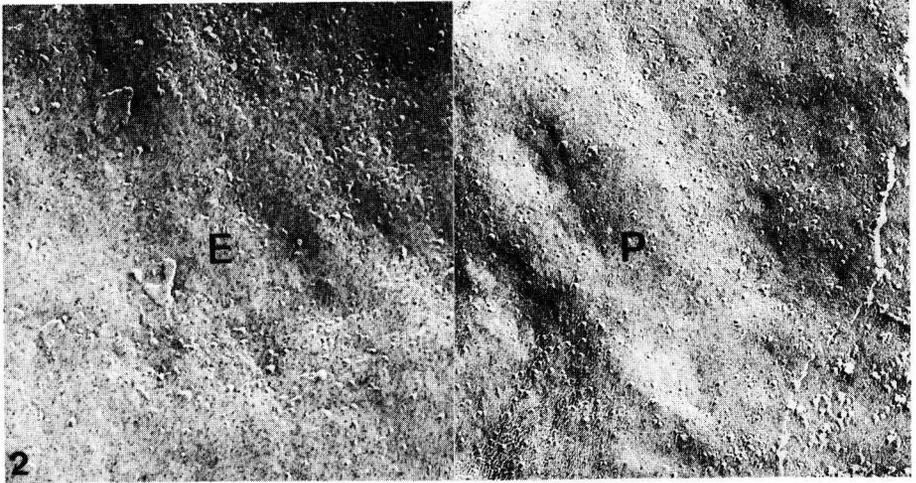
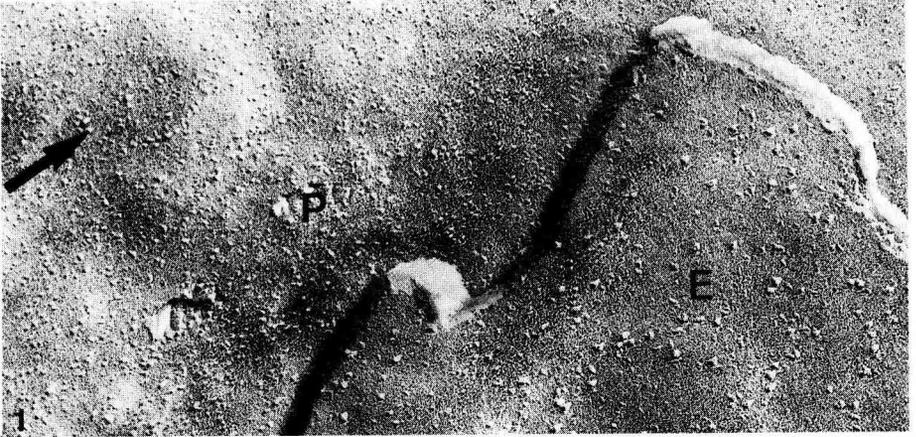
Injection of 20-OH-ecdysone. — The P/E ratio (4.38) was about the same as in the controls, but total IMP density was only $1\,010 \pm 231$ (fig. 2). The IMP distribution on the P and E faces was 822 ± 136 and 188 ± 95 , respectively. These densities were about one-half those of the controls.

Pars intercerebralis electrostimulation. — In this experiment the total density ($1\,782 \pm 268$) (fig. 3) and the P/E ratio (5.20) were respectively comparable and higher than those of the controls ($1\,738 \pm 301$; 4.47).

TABLE 1
Plasma membrane characteristics in winter experiments.

Fracture faces	Control		20-OH-ecdysone		<i>Pars intercerebralis</i> electrostimulation	
	E	P	E	P	E	P
IMP/ μm^2	318 ± 48	$1\,420 \pm 253$	188 ± 95	822 ± 136	290 ± 90	$1\,492 \pm 178$
Student's t-test	↑ S ————— ↑		↑ S ————— ↑		↑ NS ————— ↑	
Total density	$1\,738 \pm 301$		$1\,010 \pm 231$		$1\,782 \pm 268$	
P/E ratio	4.47		4.38		5.20	

S : significant ; NS : non significant.



This change in the ratio was due to a slight decrease in E face density and an increase in P face density (290 ± 90 ; $1\ 492 \pm 178$). Nevertheless, IMP density was not statistically different from that of the controls (Student's t-test : $P > 0.10$).

Spring experiments (table 2).

In the controls (fig. 4), P face density was $1\ 110 \pm 153$, whereas E face density was 468 ± 18 . Total IMP density ($1\ 577 \pm 171$) and the P/E ratio (2.37) were lower than in winter.

After p.i. electrostimulation (fig. 6), the total density ($1\ 684 \pm 242$) was higher than in the controls and the asymmetry between the two hemi-membranes increased ($P/E = 3.57$; P face : $1\ 316 \pm 190$; E face : 368 ± 52). Statistical analysis (Student's t-test) showed that there was a highly significant difference ($P < 0.001$) between the E faces and the controls but that there was no difference for the P faces ($0.05 < P < 0.10$).

TABLE 2
Plasma membrane characteristics in spring experiments.

Fracture faces	Control		20-OH-ecdysone		<i>Pars intercerebralis</i> electrostimulation	
	E	P	E	P	E	P
IMP/ μm^2	468 ± 18	$1\ 110 \pm 153$	259 ± 190	661 ± 48	368 ± 52	$1\ 316 \pm 190$
Student's t-test	↑ S ————— ↑		↑ S ————— ↑		↑ S ————— ↑	
	↑ ————— ↑		↑ ————— NS ————— ↑		↑ ————— ↑	
Total number IMP/ μm^2	$1\ 577 \pm 171$		920 ± 238		$1\ 684 \pm 242$	
P/E ratio	2.37		2.55		3.57	

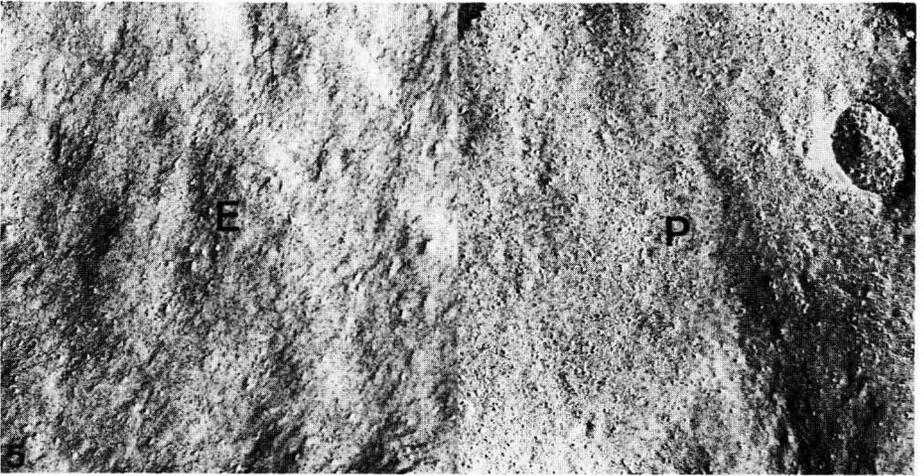
S : significant ; NS : non significant.

FIGS. 1-3. — *The spermatocyte membrane in the winter experiments.* The arrow on figure 1 indicates the direction of shadowing for all the figures on the plate. Same magnification for all images : $\times 80\ 000$.

Fig. 1. — Control. Note that most of the large IMP's are on EF (also see the other figures).

Fig. 2. — 24 h after injection of $1\ \mu\text{g}$ of 20-OH-ecdysone.

Fig. 3. — 24 h after electrostimulation of the *pars intercerebralis*. The decrease in IMP density is only significant after 20-OH-ecdysone injection.



Discussion.

This freeze-fracture study shows that there was a change in the inner structure of the membrane related to the spermatogenetic cycle or the experimental series. Experimental stimulation induced a significant decrease in the number of IMP's on the E face, except in the *pars intercerebralis* experiments in winter. Compared to the winter controls, total IMP density decreased only in the spring controls and in 20-OH-ecdysone-injected animals. *Pars intercerebralis* electrostimulation, at least in the spring, induced an increase in total IMP density.

Nevertheless, artifacts can influence density measurements and are essentially related to 1) fixation and 2) fracture.

1) Although freeze-fracture replicas of glutaraldehyde-fixed tissues reveal a good preservation of the ultrastructure (Edelmann and Margenstern, 1979), some authors prefer fixation by a glutaraldehyde-osmium fluid since the addition of osmium prevents the postfixation movement of membrane lipids (Hasty and Hay, 1978). Such movements affect the horizontal display of particles, but that does not seem to be the case here (see below). pH is also known to affect IMP distribution (Copps, Chelack and Petkau, 1976), so the pH of the fixative was rigorously controlled, ensuring the same fixation parameters for all the experimental series. 2) Plastic deformation can influence particle arrangement (Sjöstrand, 1979), but not enough to induce wide changes in density measurements. The examination of numerous replicas and statistical analysis of the data minimize this risk of error.

The IMP can be moved in the lipid bilayer of the membrane. These shifts are probably related to transport capacity or to receptor linkage. Horizontal and vertical shifts have been described (for example : Armond and Staehelin, 1979 ; Rapoport *et al.*, 1981 ; Funk *et al.*, 1982 ; Bluemink *et al.*, 1983). As the horizontal display of IMP in the spermatocyte membrane remained regular in the controls as well as in the stimulated animals, the change in IMP density on the E face, shown by our results, may be due to a vertical shift of the IMP. After 20-OH-ecdysone supply, the amplitude of this vertical shift would be wider and the IMP would go deeper into the PF hemi-membrane ; fewer IMP were detected in the replicas. However, it has also been suggested that the IMP may not be detected due to a change in bilayer thickness (Armond and Staehelin, 1979). In another connection, although most IMP's are of a proteinic nature, the existence of IMP-like particles of a lipidic nature has been demonstrated. These particles are possibly involved in intermembrane attachment (Miller, 1980 ; Verkleij, 1984). So,

FIGS. 4-6. — *The spermatocyte membrane in the spring experiments.* The arrow on figure 4 indicates the direction of shadowing for all the figures on the plate. Magnification : $\times 80\ 000$.

Fig. 4. — Control.

Fig. 5. — 24 h after injection of 1 μg of 20-OH-ecdysone.

Fig. 6. — 24 h after electrostimulation of the *pars intercerebralis*. A net decrease in IMP density is observed after 20-OH-ecdysone supply on both EF and PF. Statistical analysis shows that there is also a significant decrease in IMP density of EF after electrostimulation of the *pars intercerebralis*.

if there is a modification in intermembrane attachment, these IMP-like particles would disappear, inducing a change in IMP density.

How can we explain the differences in the results obtained using the two methods of stimulation ? After electrical stimulation, the neurohormone release into the hemolymph is more rapid (maximum : 2 h after stimulation ; Joly and Descamps, 1977) than after ecdysone injection (maximal precursor incorporation into neurosecretory cells on day 3 ; Jamault-Navarro *et al.*, 1983). As this study was conducted on animals fixed 24 h after stimulation, the increase in IMP density after electrical stimulation should be related to an increase in the hemolymphatic neurohormone level, and the vertical shift should be related to ecdysone action. At any event, as previously stated (Descamps, 1981b, c), the differences in IMP density are in agreement with a possible bifactorial control of spermatocyte growth by both ecdysteroids and hormonal factor(s) emitted by the neurosecretory cells of the *pars intercerebralis*.

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Résumé. *Action des hormones sur la membrane spermatocytaire chez Lithobius forficatus L. (Myriapode Chilopode). Résultats préliminaires.*

La membrane plasmique du spermatocyte a été étudiée en cryofracture chez des *L. forficatus* dont les synthèses ont été activées soit par l'injection de 20-OH-ecdysone, soit par électrostimulation de la *pars intercerebralis* (partie postéro médiane du protocerebron).

Chez tous les animaux (témoins et stimulés), le nombre de particules intramembranaires (PIM) de la face E est inférieur à celui constaté sur la face P. L'activation expérimentale du métabolisme provoque une diminution du nombre de PIM de la face E. Après injection d'ecdystéroïdes, la densité en PIM atteint environ la moitié de celle des témoins ; le rapport P/E est néanmoins comparable à celui des témoins. Après électrostimulation de la *pars*, le rapport P/E est toujours supérieur à celui des témoins ; de plus, dans les séries de printemps, le nombre total de PIM est supérieur à celui des témoins.

Il semblerait que (i) la diminution du nombre de PIM, constatée surtout sur la face E, soit due à leur glissement vertical dans la membrane plasmique. Le changement est probablement en relation avec l'augmentation du métabolisme de synthèse spermatocytaire (ii) l'accroissement du nombre de PIM après électrostimulation soit lié à l'augmentation supérieure du taux de neurohormones.

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