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Comparative study of structure and function of blood cells from two *Drosophila* species

Michel Brehélin*

Laboratoire de Pathologie Comparée (L.A. 43), U.S.T.L., Montpellier, France

Summary. Hemocytes of *Drosophila melanogaster* and *Drosophila yakuba* larvae have been defined in terms of their ultrastructure and functions in "coagulation", wound healing, encapsulation, phenol-oxydase activity, and phagocytosis. The position of these cells among the classical hemocyte types of insects is determined. We distinguish two plasmatocyte types (macrophage-plasmatocytes and lamellocytes) which do not seem to belong to the same lineage, and oenocytoids which are the crystal cells of the literature.

Key words: Hemocytes – *Drosophila* – Ultrastructure – Phagocytosis

Despite the fact that the blood cells of *Drosophila* have been the subject of several studies in recent years, their terminology remains controversial. For example, Rizki and Rizki (1980) observe plasmatocytes, podocytes, lamellocytes and crystal cells in *Drosophila melanogaster* larvae, whereas, in the same species, Yu et al. (1976) describe prohemocytes, plasmatocytes, granular cells, crystal cells, and oenocytoids.

In the course of our study on the defense reaction of *Drosophila* larvae against the parasitoid *Leptopilina boulardi* (Nordlander 1980) we have been confronted by this confusion in the nomenclature of *Drosophila* blood cells. The aim of the present study is to define the hemocyte types observed in *D. melanogaster* and *D. yakuba* larvae, in terms of their structural and functional features, and to determine the position of these cells among the well known hemocyte types of insects.

Materials and methods

We have used the strains n° 173-1 of *D. melanogaster* (from Petit-Bourg, Guadeloupe) and n° 185-3 of *D. yakuba* (from Kounden, Cameroun) provided by Dr Y. Carton, to whom we are grateful. Twenty pairs of adults are allowed to lay eggs for 1 h on medium for *Drosophila* (David 1959). Larvae are reared on this medium at 26° C ± 1. Larval life lasts for 136 h ± 5 for *D. yakuba* and 149 h ± 8 for *D. melanogaster*.

Send offprint requests to: Dr. M. Brehélin, Laboratoire de Pathologie Comparée, Université des Sciences et Techniques du Languedoc, Place E. Bataillon, 34060 Montpellier, France

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Injections are performed on second or third larval instars, according to the method of L'Héritier (1952). Iron saccharate (12.5 mg in 1 ml) is diluted in the following "Ringer" solution for insects: NaCl 7.5 g, KCl 0.35 g, $CaCl_2$ 0.21 g, distilled water 1 liter.

The number of hemocytes per mm^3 is counted in a Piette cell (depth 0.02 mm) without dilution of the blood. For differential hemocyte counts, blood films are fixed in formaldehyde vapour then stained with Giemsa. Presence of iron saccharate has been evidenced by Perl's reaction for iron (Martoja and Martoja 1967).

For electron microscopy, hemolymph is directly collected, after puncturing of the cuticle, in a 5% glutaraldehyde solution in phosphate buffer (pH 7.4), then centrifuged for 5 min (900 g) at 4° C; the pellets are rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated, and embedded in Epon 812. Ultrathin sections are contrasted with uranyl acetate and lead citrate and examined in a Hitachi or Jeol electron microscope operated at 80 kv.

Results

Morphology and ultrastructure

Prohemocytes (Pr). They appear as rounded cells with a high nucleocytoplasmic ratio, as in other insect species. They possess numerous free ribosomes; their RER and Golgi apparatus are poorly developed. They are rare in the circulating blood.

Plasmatocytes (Pl). These cells are larger than the Pr, with a less regular shape, but it is seldom difficult to separate them from Pr. At the end of the third larval instar they contain numerous inclusions that have led some authors to call them "granulocytes" (Yu et al. 1976, Srdič and Gloor 1978). However they are true Pl as shown by their ultrastructural features (Fig. 1a): numerous digitations and pinocytotic vesicles, well developed RER and Golgi apparatus, small lysosome-like bodies. At the end of the third instar they contain numerous phagosomes and large bodies of resorptive nature (Fig. 1b). We have never observed in these cells large inclusions of endogenous origin such as the granules of true granulocytes or coagulocytes. In the two *Drosophila* species studied, the hyaloplasm of Pl appears very opaque to electrons.

Crystal cells (CC). These cells are slightly larger than Pl and of more regular shape. With phase contrast microscopy they are easily recognizable for they contain large inclusions. These inclusions have a crystalline aspect in *D. melanogaster* (see Rizki and Rizki 1959, Srdič and Gloor 1979). In the strain of *D. yakuba* we have used, inclusions of CC appear more globular than those in *D. melanogaster* (compare Fig. 5a with Fig. 6a).

Other differences between CC of *D. melanogaster* and *D. yakuba* appear at the ultrastructural level. The paracrystalline pattern of inclusions is much less evident in *D. yakuba* than in *D. melanogaster* (Figs. 5b, 6b). In the latter species, the typical organelles other than free ribosomes are rare in CC; but in *D. yakuba* these cells sometimes contain numerous mitochondria and Golgi bodies, and a well devoloped RER. In some cases the ultrastructure of *D. yakuba* CC recalls that of Pl and the typical ("crystal") inclusions are small and rare (Fig. 7).

In *D. melanogaster* and in *D. yakuba* the typical CC inclusions are never membrane bounded.

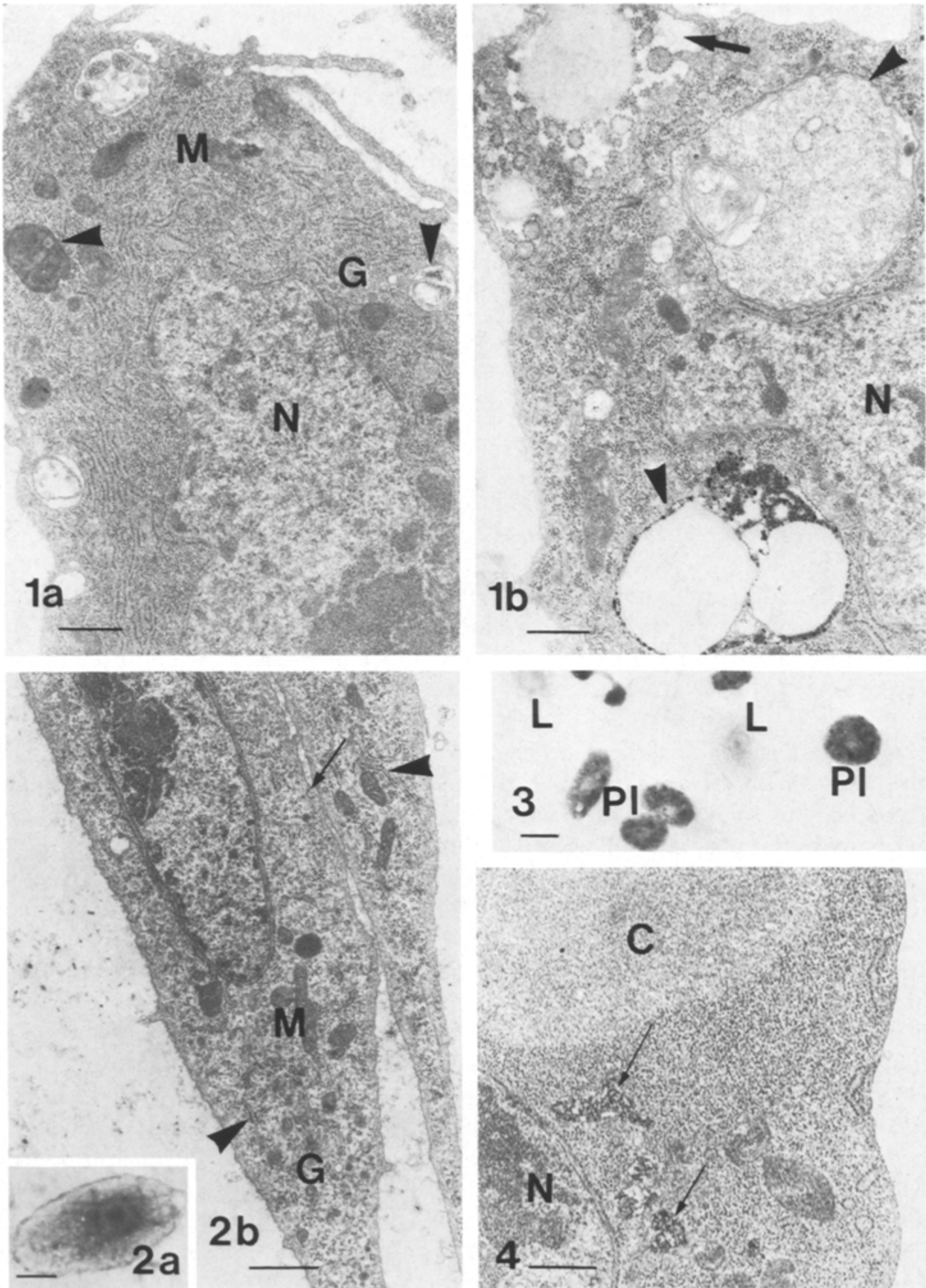

Fig. 1a, b. Plasmatocytes of *D. melanogaster*. **a** From larva 80 h old (third instar). Note pseudopodia, heterogeneous bodies of resorptive nature (*arrowheads*) and distended cisternae of RER. This cell is a macrophage type plasmatocyte. Bar = 0.5 µm. ×16,000. **b** From larva 140 h old: note large phagosomes (*arrowheads*), extracellular material (*arrow*) in process of being engulfed by Pl (histolysing fat body cells). These Pl are erroneously called "granulocytes" by some authors. Bar = 0.5 µm. ×16,000. For all figures: *N* nucleus, *M* mitochondrion, *G* Golgi complex, *Cyt* cytoplasm

Fig. 2a, b. Lamellocytes of *D. yakuba* (120 f old larva). Shape regular and flattened. Note absence of phagosomes; cisternae of RER short and narrow (*arrowheads*), pinocytotic vesicle (*arrow*). Bar: **a** = 5 µm. ×1,000, light microscopy; **b** = 1 µm. ×9,000, TEM

Fig. 3. Hemocytes of *D. yakuba*, 6 h after injection of iron saccharate: Perl's reaction for iron. *Pl* strongly stained whereas two *L* are not. Bar = 10 µm. ×500

Fig. 4. CC of *D. yakuba* 3 h after injection of iron saccharate; vesicles filled with inert powder (*arrows*). Bar = 0.5 µm. ×18,000

Lamellocytes (L). They are the largest blood cells of *D. melanogaster* and *D. yakuba* larvae. Light microscopically they exhibit a very flat and regular shape, and their cytoplasm appears very little contrasted (Fig. 2a). At the ultrastructural level (Fig. 2b) they show short and narrow vesicles of the RER, a well developed Golgi apparatus, and some free ribosomes; the matrix of their elongated mitochondria is very opaque to electrons; in some cases microtubules are numerous. They never exhibit multivesicular bodies or large inclusions of resorptive nature, as observed in Pl and to a lesser extent in CC. These cells are more numerous in the blood of normal larvae of *D. yakuba* (up to 20%), than in *D. melanogaster* (less than 6%).

Functions

Characteristics in vitro. After blood removal in the absence of fixative, CC transform in vitro. They lose their typical inclusions and, under phase contrast microscopy, look like coagulocytes of other insect species. This phenomenon has been well studied by Srdič and Gloor (1979) and we only want to underline that some CC never transform. Between slide and coverslip, most CC discharge their inclusions in 2 to 3 min but some CC are intact more than 30 min after blood removal.

Wound healing. In this rapid process the role of hemocytes seems to be very restricted. Wounds are immediately plugged up by the neighbouring tissues (epidermis, muscles, tracheae) in which few hemocytes belonging to the three main types are entrapped. A small irregular plug is sometimes built up by Pl and L. In larvae 96 h old (at 26° C) reconstitution of epidermis and cuticle is accomplished in less than 12 h.

Capsule formation. Pl as well as L are concerned with the formation of capsules around foreign bodies. This reaction is not useful in the discrimination of these two hemocyte types. The result of our study on encapsulation of parasitoid egg and inert implants will be reported elsewhere (Brehélin and Carton, in preparation).

Phagocytosis of inert particles. Half an h after injection of iron saccharate, hemocytes of all types have engulfed the inert powder but only Pl are overloaded with it and remain in this state for more than 48 h. The iron saccharate content of L appears very low at any time after injection (Fig. 3). At the ultrastructural level, despite the fact that L exhibit pinocytotic vesicles, we have not been able to detect amounts of inert powder in these hemocytes.

CC engulf the inert powder to a higher extent than L. From half an h to 48 h after injection, CC that contain iron saccharate are numerous; but in each case their load in phagocytosed material appears less prominent than in Pl (Fig. 4).

Hemocyte counts performed on *D. melanogaster* larvae after injection of iron saccharate show a heavy and lasting increase in the number of hemocytes of all types (Table 1). This rise is first evident for Pl. In our experimental series, 3 h after injection of iron saccharate, the Total Hemocyte Count per mm^3 (THC), grows from 3078 to 8169. However the number of L and CC remains stable; only the

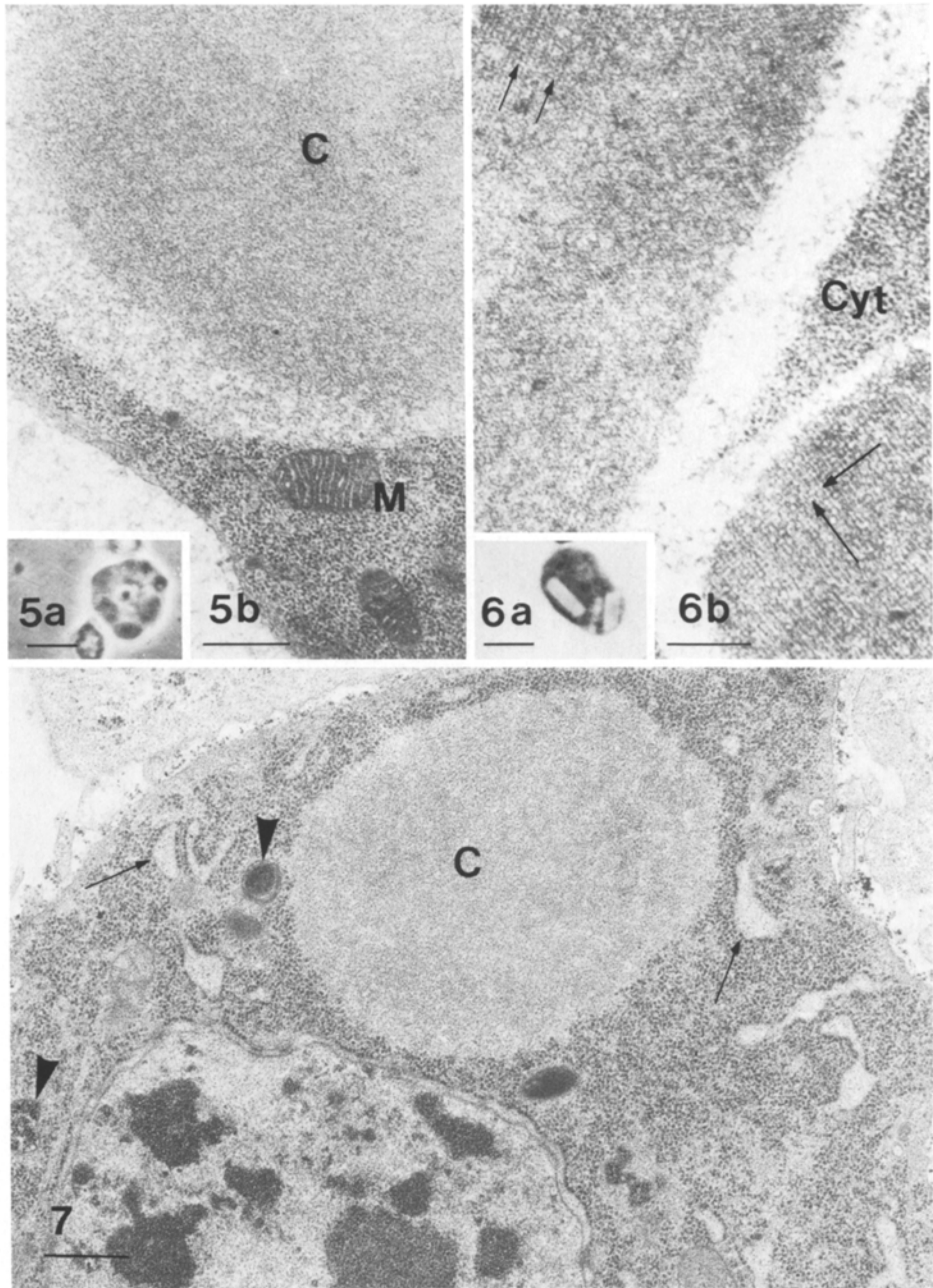

Fig. 5a, b. Crystal cells of *D. yakuba*. **a** Light microscopy. Cell resembles spherule cells of other insect species. Bar = 8 μm. × 800. **b** TEM. Paracrystalline nature of inclusion *C* not evident (compare with **Fig. 6b**); inclusion not membrane bounded. Bar = 0.5 μm. × 23,000

Fig. 6a, b. Crystal cells of *D. melanogaster*. **a** Phase optics. Note sharp contour of inclusions. Bar = 8 μm. × 800. **b** TEM. Note paracrystalline nature of inclusions (*arrows*). Bar = 0.25 μm. × 44,000

Fig. 7. Hemocyte from *D. yakuba* (120 h old larva), with intermediate features between Pl and CC; note small size of "crystal" *C*, remnants of phagosomes (*arrowheads*) and enlarged cisternae of RER (*arrows*). Bar = 0.5 μm. × 22,000

Table 1. Hemogram modifications after injections in larvae of *D. melanogaster* (third instar, 96 h old)

	NTH	Pl %	Pl	L%	L	CC%	CC	NL
N 96h	3078 ± 1706	91.3 ± 3.8	2785 ± 1475	5.9 ± 2.8	198 ± 175	2.8 ± 1.0	94 ± 84	12
P 3h	3437 ± 1341	93.0 ± 1.9	3182 ± 1119	4.3 ± 1.2	157 ± 86	2.6 ± 1.7	97 ± 77	10
R 3h	4393 ± 1936	91.4 ± 2.3	4076 ± 1928	7.2 ± 2.6	295 ± 133	1.4 ± 0.9	64 ± 52	12
IS 3h	8169 ± 2994	95.8 ± 1.5	7841 ± 2923	2.8 ± 1.2	213 ± 103	1.3 ± 1.0	111 ± 109	13
N 120h	3450 ± 1048	91.5 ± 2.7	3138 ± 901	5.7 ± 1.5	206 ± 104	2.9 ± 1.5	106 ± 65	10
P 24h	4925 ± 1905	73.4 ± 11.9	3537 ± 1278	23.1 ± 12.3	1219 ± 936	3.4 ± 1.7	170 ± 89	10
R 24h	5000 ± 1250	66.6 ± 20.0	3352 ± 1404	30.7 ± 19.9	1518 ± 1022	2.6 ± 1.2	128 ± 63	10
IS 24h	25900 ± 4793	81.1 ± 12.0	21273 ± 6186	17.4 ± 11.6	3050 ± 1712	1.5 ± 0.6	377 ± 103	12
IS 48h	22750 ± 5200	82.4 ± 7.6	18751 ± 3728	15.5 ± 7.3	3526 ± 1670	2.1 ± 1.1	477 ± 255	8

Differential hemocyte counts after single injection in larvae of *D. melanogaster* (third instar, age 96 h).
N 96 h: time of injection; normal larvae 96 h old (at 26° C).
P 3 h, R 3 h, IS 3 h: larvae 3 h after puncturing of cuticle (P) or injection of "Ringer" (R) or of iron saccharate (IS).
N 120 h: normal larvae 120 h old.
P 24 h, R 24 h, IS 24 h: larvae 120 h old, 24 h after puncturing of cuticle or injection of "Ringer" or of iron saccharate.
IS 48 h: larvae 144 h old, 48 h after injection of iron saccharate.
NTH: number of hemocytes per mm^3 of hemolymph.
Pl%, L%, CC%: percentages of plasmatocytes, lamellocytes, and crystal cells.
Pl, L, CC: number of plasmatocytes, lamellocytes and crystal cells per mm^3 of hemolymph.
NL: number of larvae in experiment.
Arrows: comparisons of means: 0 = not significant; * = significant at 5 % level; ‡ = significant at 1 % level; Each mean given with standard deviation.

Table 2. Hemogram modifications after injections in larvae of *D. yakuba* (third instar, 114 h old). (other comments, see Table 1)

	NTH	Pl %	Pl	L%	L	CC %	CC	NL
N 114h	3395 ± 1397	81.9 ± 5.9	2779 ± 200	14.4 ± 6.6	487 ± 223	3.9 ± 1.8	135 ± 62	12
IS 18h	18211 ± 5591	66.9 ± 7.9	12175 ± 1433	31.6 ± 8.7	5758 ± 1582	1.5 ± 1.2	268 ± 225	11

number of Pl per mm^3 increases (from 2785 to 7841). Twenty four h after injection, this increase, evident in the three hemocyte types, is relatively more important for L than for Pl or CC; the percentage of L grows from 5.7 % in normal larvae 120 h old, to 17,4 % in injected larvae of the same age. The rise in THC and L is still evident 48 h post-injection.

Puncturing of the cuticle challenges a slight rise of THC and an important increase in the percentage of L, which are evident only 24 h later. Injection of saline solution alone provokes a change of the hemogram in the same way than injection

of iron saccharate, but to a lesser extent. For example, 24 h post-injection, the THC rises to a main value of 5000 cells/mm^3 instead of 23000 in the case of iron saccharate injection. Hemogram modifications after injection of iron saccharate in larvae of *D. yakuba* go in the same direction (Table 2).

Different series of normal animals (*D. melanogaster* as well as *D. yakuba*), reared under standard conditions, sometimes show notable differences in THC (and not in DHC). For example, in one series we have measured a THC of 9732 ± 1740 hemocytes per mm^3 for normal larvae 96 h old (*D. melanogaster*) instead of about 3000 to 4000 cells/mm^3 in most cases.

Discussion

Hemocyte types. Among the three main types of hemocytes found in *D. melanogaster* and *D. yakuba*, only Pl are recognized by different authors as having the same ultrastructural and functional features as in other insect species. They are phagocytic cells with high endocytotic capabilities. We think that "granulocytes" observed by some authors (see Yu et al. 1976) in *Drosophila* species are in fact Pl engaged in phagocytosis of degenerating tissues, for they are more numerous at the end of the third instar. Moreover, no ultrastructural evidence exists for true granulocytes. This has been already stated by Zachary and Hoffmann (1973) in *Calliphora erythrocephala*, another species of Diptera. The other main hemocyte types described in the literature for *D. melanogaster* and *D. yakuba* larvae, seem to be more specific for *Drosophila* species.

Light microscopically, L look like oenocytoids of *Melolontha melolontha* (Brehélin 1977); they have a very flattened shape and show a low contrast in phase contrast microscopy. But the ultrastructure of L is quite different from that of true oenocytoids (Brehélin et al. 1978); typical cytoplasmic organelles are very scarce in oenocytoids whereas they are well developed in L. Ultrastructurally, L look like what some authors call "typical plasmatocytes" (Akaï and Sato 1973; Ratcliffe and Gagen 1977) especially in lepidopterans. As L, these "typical plasmatocytes" seem to have restricted capacities of endocytosis in vivo (Akaï and Sato 1973; Neuwirth 1974; Brehélin unpublished observations); they are essentially concerned with encapsulation (Akaï and Sato 1973). We think that a partition has to be made between 2 Pl types in insects, based on morphological and functional criteria. We distinguish (1) Pl-macrophages with numerous pseudopodia, large phagosomes, enlarged cisternae of the RER, and endowed with endocytotic capabilities, and (2) typical Pl with a regular shape, devoid of peculiar inclusions and with narrow cisternae of the RER; Pl of this second type have restricted capacities of endocytosis in vivo but make capsules around foreign bodies. Pl of *Drosophila* larvae belong to the first type of Pl, and L to the second. We agree with Rizki (1980) to affirm that L exist apart from CC, which is in opposition to Srdič and Gloor (1978, 1979).

CC are present in *D. melanogaster* as well as in *D. yakuba* larvae, in the same proportion and with the same behaviour in vitro, but they exhibit some structural differences (Figs. 5, 6) and, in *D. yakuba* the term CC is not adequate. In both species some CC transform in vitro to look like coagulocytes (which they are not) of other insect species. What is the place of CC in the classical hemocyte terminology? For Srdič and Gloor (1979) they are "spherule cells" before the in vitro transformations, and "coagulocytes" after these transformations have

occurred. But in other insect species, true coagulocytes can be characterized before any transformation and not merely thereafter (Brehélin et al. 1978); the morphological features of coagulocytes are not found in CC and the term coagulocyte has to be discarded concerning CC. Intact CC, especially in *D. yakuba*, look like spherule cells of other insect species, but morphological similarities are not sufficient to define a hemocyte type. Moreover, inclusions in spherule cells are membrane bounded, which is not the case in CC. CC have been shown to contain phenol-oxydase (Rizki and Rizki 1959, and personal observation) and this enzyme has never been found in spherule cells (see Crossley 1975, 1979). But phenol-oxydases have been evidenced in oenocytoids (Vercauteren and Aerts 1958, Beaulaton and Monpeyssin 1977). The paucity in cell organelles other than free ribosomes in most CC recalls that of oenocytoids. Moreover, most authors are in agreement that oenocytoids are produced by transformation of hemocytes belonging to different hemocyte types and do not come directly from stem cells (Beaulaton and Monpeyssin 1977; Brehélin 1977). In the same way, CC of *D. yakuba* larvae seem to be transformed Pl (Fig. 7). For all these reasons we tend to place CC among to oenocytoids.

Hemogram modifications and hemocyte relationships

The origin of *Drosophila* hemocytes remains controversial. Srdič and Gloor (1978, 1979) consider L as transformed CC and state that CC "might branch off from Pl". On the other hand, according to Rizki and Rizki (1980) Pl transform into L, and CC come from a different lineage.

Our observations on *D. yakuba* hemocytes suggest that Pl may synthesize substances that lead to formation of CC "crystals" (Fig. 7). Furthermore, CC as well as Pl, possess a hyaloplasm which is very opaque to electrons, enlarged cisternae of the RER and are capable of endocytosis (this is in opposition to Rizki and Rizki 1980 but in agreement with Srdič and Gloor 1978). This suggests that Pl may transform into CC.

Concerning L, our results are contradictory. L never contain the large amounts of endocytotic material seen in Pl. Their behaviour concerning pinocytosis of inert powder is quite different from that of Pl; if Pl transform into L as asserted by Rizki and Rizki (1980), we have to admit that after injection of iron saccharate, Pl lose their large and numerous phagosomes before becoming L. Moreover, ultrastructurally we have never seen hemocytes with intermediate features between Pl and L.

On the other hand, results of counts show that injection of iron saccharate provokes an increase of Total Hemocyte Count per mm^3 of hemolymph. This increase, which is very important for L 24 h after injection, is preceded by an increase of Pl alone, as soon as 3 h after injection. This suggests that Pl may transform into L after they are discharged into the circulation. This conclusion would be in agreement with that of Nappi (1969) in *D. euronotus* and that of Carton and Kitano (1979). But in our experiments we cannot discard the hypothesis that L are produced by stem cells (and not by Pl) in hematopoietic tissues and discharged into the circulating blood later than are Pl; a liberation of L later than that of Pl should produce a decrease in the percentage of Pl as shown in our experiments and those of others (see Nappi 1969).

It seems that in *Drosophila* larvae, sessile hemocytes are numerous; their liberation 3 h post-injection provokes an increase in THC. This increase cannot be

ascribed to a decline of hemolymph volume since (1) experimental larvae are more turgescent than normal larvae, and (2) only Pl are affected.

We have to underline that injections of saline solution or puncturing of cuticle provoke, 24 h later, modifications of the hemogram in the same way as injection of iron saccharate; but these modifications are less evident than those challenged by iron saccharate injections. In conclusion, every intervention performed on *D. melanogaster* or *D. yakuba* larvae induces an increase in L percentage. Furthermore, injection of particulate material induces a precocious liberation of sessile Pl, the hemocyte type endowed with active endocytotic capacity.

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