been noted by him in contrast to the observations of the authors and Nylund and Westman[10]. These different data might be explained by the more or less advanced stage of infestation which can induce the death of the crayfish. Nevertheless, the encapsulating reaction does not seem to be linked to the stage of infestation since in the same group of crayfish, and for a given number of parasites, we have always observed both free organisms and others surrounded by amebocytes, while no encapsulation has been noted when a group of animals was more abundantly parasitized. Thus, these crayfish would have an activating capacity, to identify the shell of *Psorospermium* as "not self", and would be able to perform encapsulating reactions. Only some groups of *Astacus* showed these cellular reactions. It seems that the encapsulation could depend either on genetic variations between populations of different geographical origin, or on changes at the parasite level. These different reactions in *Astacus leptodactylus* are indeed very noticeable since the identification of parasites as "self" or "not self" by the cells of their hosts is probably connected with the biochemical structure of the shell and is probably concerned with a change in polysaccharides which take part in cellular immunological reactions. This might also explain the reaction of "not self" against the extraneous bodies by the hemocytes of invertebrates. Indeed, *Psorospermium haeckeli* could exist under 3 forms: the 1st free and round, the 2nd, which is the most common one, free and ovoid, and 3rd, an encapsulated one which might derive from the 2nd as a result of variations in the chemical components of the shell.

1 G. Salt, in: The cellular defence reactions of insects. Cambridge University Press, Cambridge 1970.
2 R.F. Whitcomb, M. Shapiro and R.R. Granados, in: The physiology of Insecta, vol. 5, p. 447. Ed. M. Rockstein. Academic Press, New York 1974.
3 A. Vey and C. Vago, Annls Inst. Pasteur, Paris *121*, 527 (1971).
4 T. Unestam and J.E. Nylund, J. invert. Path. *19*, 94 (1972).
5 E. Haeckel, Archs Anat. Physiol. Med. *24*, 561 (1857).
6 F. Hilgendorf, S.B. Ges. Naturf. Fr. Berlin *9*, 179 (1883).
7 O. Ljungberg and L. Monne, Bull. Off. int. Epizoot. *69*, 1231 (1968).
8 T. Unestam, in: First international symposium on freshwater crayfish 1973, p. 135. Ed. S. Abrahamsson.
9 V. Nylund and K. Westman, Fisk-Tidskr. Finl. *7*, 162 (1977).
10 V. Nylund and K. Westman, in: Fourth international symposium on freshwater crayfish 1978, p. 385. Ed. P.J. Laurent.
11 A. Vey, in: Fourth international symposium on freshwater crayfish 1978, p. 411. Ed. P.J. Laurent.
12 A. Wierzejski, Zool. Anz. *11*, 230 (1888).
13 E. Grabda, Mém. Acad. pol. Sci. Série B *6*, 123 (1934).
14 J. Krucinska and E. Simon, Przegl. zool. *12*, 288 (1968).
15 T. Unestam, in: Second international symposium on freshwater crayfish 1975, p. 327. Ed. J.W. Avault Jr.

Activity of the ciliary ampules through successive ages of the ciliate *Euplotes crassus*[1]

F. Verni, Giovanna Rosati and P. Luporini

Institute of Zoology, University of Pisa, Via Volta 4, I-56100 Pisa (Italy), and Institute of Zoology, University of Camerino, Via Camerini 2, I-62032 Camerino (Mc) (Italy), 12 May 1980

Summary. In *Euplotes crassus*, activity of the ciliary ampules is complete only in sexually mature cells. During immaturity, no change of activity occurs in cells stimulated to mate, though variations can take place during the cell cycle. At maturity, ampules become active during both vegetative and sexual reproduction.

In *Euplotes*, ciliary ampules (As) are organelles, probably secretory in nature[2], associated with the dorsal bristles and the ventral ciliary organelles[3-8].

We investigated the proportion of full or 'ripe' As (RAs) in order to appraise the degree of activity of the As system at any given moment of the cell's life. On the basis of the finding that variations in the activity occur when cells of a given mating type interact with cells of a compatible mating type, we suggested that As can play an important role in *Euplotes* interaction[7].

To test this hypothesis further we have examined the As activity in cells at different stages of their life cycle, and the variations of activity in immature and in mature cells were compared.

Materials and methods. Singlets and morphological mutant doublets have been used in order to discriminate between mixed sexually compatible cells. The singlet and doublet strains chosen were known to be homozygous for different *mt* genes, those in the former being dominant over those correspondingly carried in the latter. To be aware of this *mt* locus constitution of the 2 strains allowed us to know in advance[10] that any offspring derived by crossing singlets with doublets would have inherited the mating type of the singlet parent, that is the mating type complementary to that of the doublets.

Cells were grown in Erd-Schreiber sea water medium inoculated with *Dunaliella salina* at 22-23 °C, on a cycle of 12 h of light and 12 h of darkness.

Cells were prepared for electron microscopic observation and the percentage of RAs counted on 5 cells randomly chosen from a group of 10 cells as previously reported[7].

Results. Experiment 1. In figure 1 are reported the percentages of RAs in cells of a clone which was analyzed at

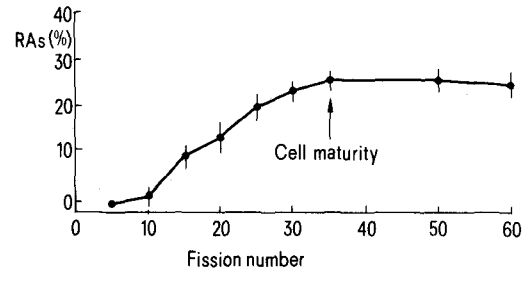

Fig. 1. Progressive increase of the proportion of RAs in cells passing from immaturity to maturity. Cell age is in number of fissions from conjugation.

5-fission intervals, from the beginning of its life cycle (reorganization from conjugation) up to the acquisition of mating capability. Each examination was performed on 48-h starved cells, since in this condition a cell population appears to be homogeneously in the macronuclear G_1 stage[11]. A singlet exconjugant obtained after crossing to a doublet was allowed to divide 5 times to obtain the 1st group of 32 cells. One of these cells was re-isolated in the presence of food to produce the next group of 32 cells. All the other cells were shifted-down to pure sea water where they usually divided once again before arresting in the G_1 stage. Groups of progressively 5-fission older cells were similarly obtained up to maturity. 10 cells of each group were processed to determine the number of RAs, while all the remaining cells were mixed with sexually reactive doublets to control their maturity. RAs (2%) appeared for the first time in cells of 10-fission age. Then the proportion of RAs increased to reach a value of about 25%; this coincided with the achievement of sexual maturity.

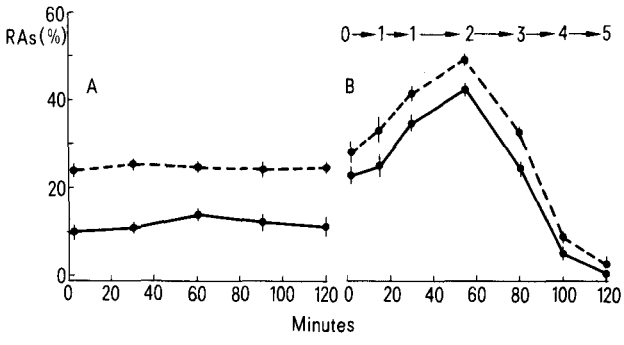

Fig. 2. Variations of the RAs activity in mixed immature singlets and mature doublets (ineffective mixture, plot A), and in mixed mature singlets and mature doublets (effective mixture, plot B). Continuous line refers to singlets, dashed line refers to doublets. The 1st cell isolation was performed from both the mixtures soon after cell mixing. The successive cell isolations were made every 30 min from the ineffective mixture, and at each of the 5 stages of the preconjugant interaction from the effective mixture. The stages are indicated by the progressive numbers at the top of plot B. The induction (or waiting) period (stage 1) ends when cells enter the visible mating reaction (stages from 2 to 4) that leads to the firm cell union (stage 5). 2 independent cell examinations were made at different times during stage 1.

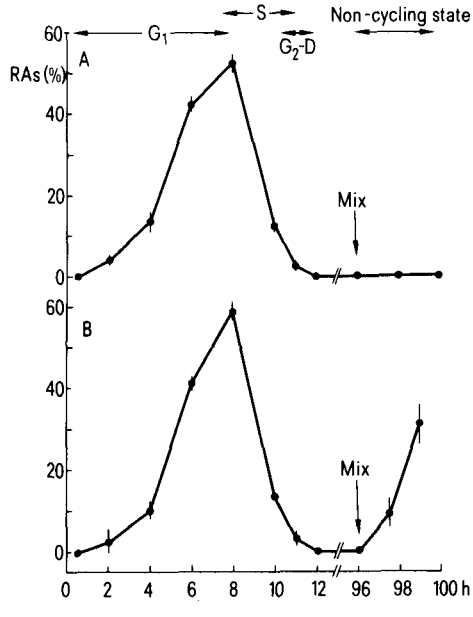

Fig. 3. From time 0 to 12 h: variations of the RAs rates through the different stages of the cell cycle. Plot A refers to immature singlets and plot B refers to mature singlets. From 96 to 100 h: variations of the RAs rates in 96-h starved immature (plot A) and mature (plot B) singlets after mixing with mating competent doublets.

Experiment 2. A singlet exconjugant, obtained as before after crossing to a doublet, was continuously cultured up to the age of 15 fissions. Then, one cell was re-isolated and grown to maturity, while all the other immature cells were shifted to pure sea water and starved for 48 h. A sample of about 10^3 starved cells in 1 ml of medium was mixed with an equal amount of complementary mating reactive doublets. No pairs formed in this mixture, because of the immature age of the singlets. Every 30 min, up to 2 h after mixing, 10 singlets and 10 doublets were isolated from this ineffective mixture and the proportion of RAs examined. Figure 2, plot A, shows that the ratio of RAs does not undergo appreciable variation in immature or in mature cells.

In order to exclude the possibility that failure of the As system to change activity represented a particular trait of the singlet clone, a 10^3 singlet sample was again mixed at maturity with doublets. As the plot B of figure 2 shows, the proportion of RAs in both singlets and doublets underwent the variations typically detectable[7] through the different stages of the preconjugant interaction.

Experiment 3. 2 samples, each consisting of 200 dividers, were successively isolated from a singlet clone. The first isolation was made from the immature clone at 15 fissions of age; the next one was made at maturity. Both samples were treated as follows. After singlets had completed their division, the sample was halved. One half was shifted to pure sea water and starved for 4 days to be used in the 2nd part of the experiment, while the other half was maintained in the presence of food and examined for the variations of the proportion of RAs through the different stages of the cells cycle. To this purpose, 20 cycling singlets were isolated every 2 h starting from the completion of cell fission. 10 singlets were examined for the proportion of RAs; the others were aceto-orcein stained and their macronuclei examined according to the scheme given by Salvano[12], in order to observe the successive stages of the cell cycle. Figure 3 shows that the trend of the As activity during the cell cycle, which was completed within 12 h, does not differ appreciably between immature and mature cells.

The lots of 4-day starved singlets were mixed with an equal number of reactive doublets and 2 mixtures were thus obtained. As in the case of experiment 2, the first mixture was ineffective and the second was effective as they involved immature and mature singlets, respectively. From the ineffective mixture, 3 isolations of singlets were performed every 2 h starting soon after cell mixing. 3 isolations were also performed from the effective mixture, but at shorter intervals from each other because the cells appeared to be able to mate despite the 4-day starvation. As it is shown in figure 3, the RAs were virtually absent in the starved cells at the time of mixing independently of their state of maturity. However, whereas mature singlets were able to start producing RAs after interaction with complementary cells, the immature ones failed to do it.

Conclusions. Results are in general consistent with the view that the system of As in *Euplotes* is functionally not complete until cells have reached maturity and that independent mechanisms of control could act on the system according to the different reproductive conditions of the cells. Immature cells were found to be completely devoid of RAs up to an age of 10 fissions. Then, RAs were produced

and their number increased as cells approached maturity (results of experiment 1). The As system of immature cells (15 fissions of age) already appeared to be functional during the cell's vegetative life. Variations in RAs numbers were in fact observed during the different stages of the cell cycle which closely matched the variations shown by the mature cells[9] (result of the 1st part of experiment 3). However, the As system of immature cells was completely unable to vary its activity in the presence of mating competent cells in which, analogously, no variation of activity took place (result of experiment 2). This inefficiency was in dramatic contrast to the result of the 2nd part of experiment 3. This showed that in mature cells the activity of the As system can be restored during the preconjugant interaction, even if it had dropped near to zero because of prolonged starvation of the cells before they were mixed.

1 This work was supported by Italian C.N.R. Preliminary data of this study have been presented at the U.Z.I. Congress held in Catania, 1978.
2 R. Dallai and P. Luporini, Eur. J. Cell Biol., in press.
3 K. Hausmann, Protistologica *14*, 459 (1978).
4 R. Gliddon, J. Cell Sci. *1*, 439 (1966).
5 E. Fauré-Fremiet and J. André, Archs Anat. microsc. Morph. exp. *57*, 57 (1968).
6 J.J. Ruffolo Jr, J. Morph. *148*, 469 (1976).
7 F. Verni, G. Rosati and P. Luporini, J. exp. Zool. *204*, 171 (1978).
8 G. Rosati and F. Verni, Trans. Am. microsc. Soc. *98*, 80 (1979).
9 G. Rosati and F. Verni, J. exp. Zool. *209*, 331 (1979).
10 K. Heckmann, Z. VererbLehre *95*, 114 (1964).
11 F. Dini and P. Luporini, Devl Biol. *69*, 113 (1979).
12 P. Salvano, J. Protozool. *22*, 230 (1975).

A method for the isolation of ommochrome-containing granules from insect eyes

K. Cölln, R. Hedemann and Eva Ojijo[1]

Institute of Zoology, University of Cologne, Cologne (Federal Republic of Germany), 31 March 1980

Summary. A routine method for the preparation of pigment granules from insect eyes is reported. When viewed using an electron microscope, the isolated granules appear to be free from different structural elements, and there is very little contamination by membranous material. Ultrastructure and extinction spectra correspond to those found in situ.

The screening pigments of insect eyes consist of pteridines and ommochromes. At least the latter are always localized in specific organelles, called pigment granules. Eye-colour mutations exhibit a broad pleiotropic expression, in which the pigment granules play an important role. Defects in pigment synthesis often cause changes in form, structure and number of the granules. The quality and quantity of these organelles determine the efficiency of the optical organ and thus influence the ability of orientation of the animal[2,3]. Hence the pigment system of the complex eyes appears to be a good model to studying the interdependence of chemo- and morphogenesis as well as the relation between structure and function. Recent knowledge about pigment granules comes from histological and histochemical studies. Only very little information about the chemistry of these organelles is available. As a basis for investigations of the chemical composition of pigment granules we have developed a routine isolation procedure.

Material and methods. Isolation procedures were worked out for *Musca domestica* and *Ephestia kühniella.* Two different strains of *Ephestia* were used: wild type *B 12* and the eye colour mutant *wa.* The characteristics of the strains, and rearing conditions, have been described by Caspari and Gottlieb[5]. *Musca* was obtained from the "Labor für Hygiene- und Vorratsforschung (BAYER, Leverkusen)". Metrizamide was obtained from NYEGAARD and Co. (Oslo). All other chemicals were purchased from MERCK, Darmstadt or SERVA, Heidelberg. The highest purity grade available was used.

The basic buffer for the isolation had the following composition: 10 mM Tris, pH 7.0 (23 °C), 1 mM $CaCl_2$, 25 mM KCl and 14 mM 2-mercaptoethanol. For homogenization and washing procedures this buffer was made 0.25 M with sucrose (= isolation buffer IB). Gradient steps (see below) in addition contained various concentrations of sucrose or metrizamide. Centrifugation was done in a Sorvall RC 2.B with HB 4 rotor, and in a Spinco L 265 B with rotor SW 40 Ti. After an initial digestion step at 400 °C[6] the quantification of nitrogen was done photometrically with ninhydrin[7]. Ommochromes were separated and determined after the method of Stratakis[8].

Suspended granules were pelleted by centrifugation and fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 90 min. After washing in cacodylate buffer containing 0.25 M sucrose for 60 min, postfixation was done with 2% OsO_4 in Michaelis buffer, pH 7.2, for 60 min, followed by several washes in the same buffer containing 0.25 M sucrose to remove excess osmium. All manipulations were performed on ice. The material was dehydrated stepwise in ethanol, then, using propylene oxide, finally embedded in Araldite[9] and examined with a Zeiss electron microscope EMS-Z.

Isolation procedure. In buffers containing divalent cations pigment granules tend to form mixed aggregates. However, these cations are essential for the maintenance of structural integrity. Furthermore, the sedimentation characteristics of granules do not differ very much from those of mitochondria. These features were taken into account in designing a separation strategy. The procedure which finally emerged is summarized schematically in the scheme and a detailed description is given below. The isolation procedure starts with fresh insect heads. Frozen material gives poor yields. Heads are homogenized in a loose fitting potter homogenizer and filtered through 3 layers of perlon gauze (100 µm). The next few steps follow the usual procedures of differential centrifugation. Slight modifications are made to increase yield and to minimize contamination with nuclei. A short treatment with Triton X-100 is necessary to dissociate aggregates of pigment granules. After addition of the detergent some more contaminations can be separated from the granules by low speed centrifugation.

The final steps involve centrifugation at 2 different gradients. The triton-treated material is distributed to 6 discontinuous gradients, containing 7 different sucrose