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.

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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

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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.

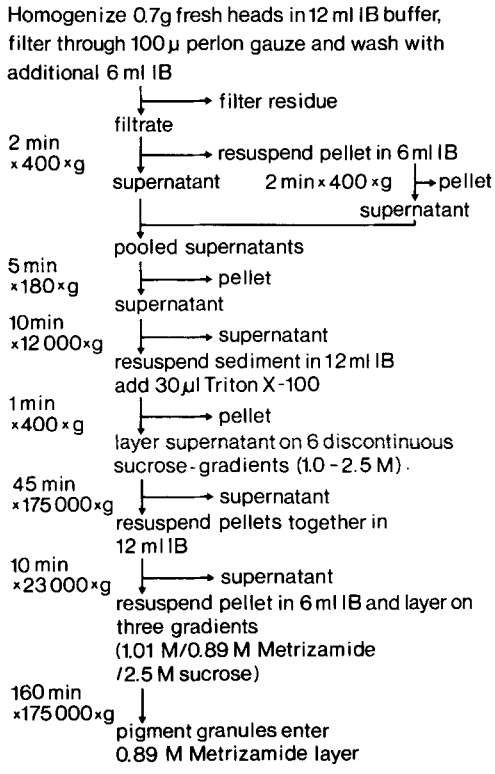
Isolation procedure. In buffers containing divalent cations pigment granules tend to form mixed aggregates. However, these cations are essential for the maintenance of structural integrity. Furthermore, the sedimentation characteristics of granules do not differ very much from those of mitochondria. 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.

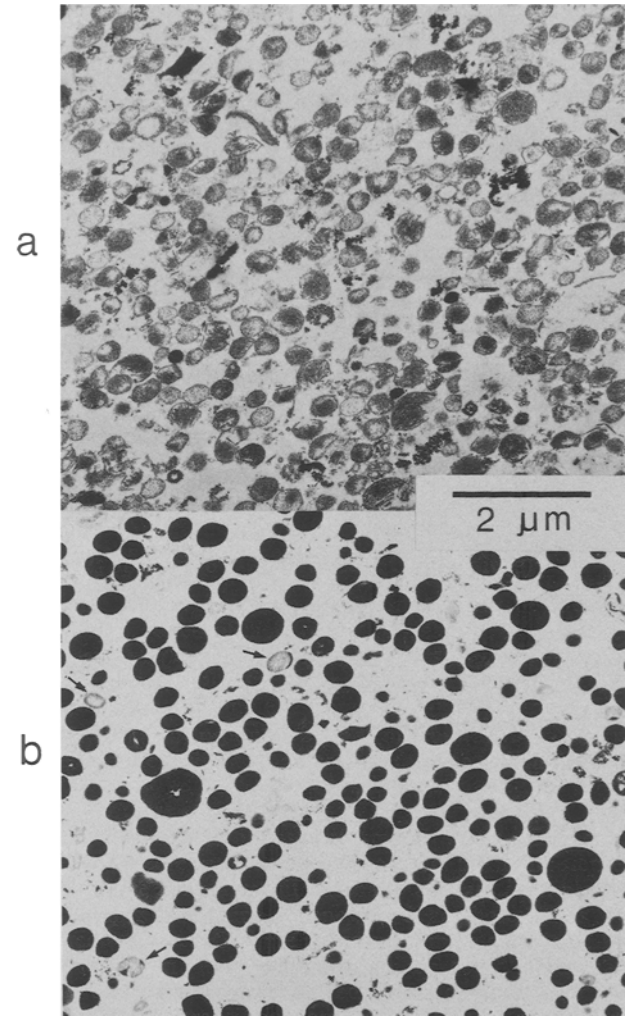
The final steps involve centrifugation at 2 different gradients. The triton-treated material is distributed to 6 discontinuous gradients, containing 7 different sucrose

concentrations in a range between 1.0 M and 2.5 M. Most of the pigment pellets out during centrifugation. Only a small portion remains floating at the 2.0/2.5 M interphase of the gradients even after prolonged centrifugation. The combined pellets are resuspended and distributed on 3 gradients containing from bottom to top 1.01 M and 0.89 M metrizamide and 2.5 M sucrose. The pigment granules float in the 0.89 M metrizamide layers, which represents their buoyant density. They are harvested by perforating the tubes, pelleted, stored and frozen in liquid nitrogen or at −20 °C.

Comments on the isolation procedure. The selection of the isolation medium is crucial in the purification of pigment granules. Hypotonic conditons should be avoided. Phosphate buffers, as used by other authors[10-12] extract large amounts of ommochromes. This can be prevented to some extent by strongly reducing conditions, but even 0.43 M mercaptoethanol[10] is not sufficient to stabilize these organelles. Besides this, Ca^{++} and Mg^{++} are important for the binding of ommochromes. In our a.m. medium, which takes account of these facts, the loss of ommochromes is negligible. The promoting effect of Ca^{++} and Mg^{++} on the aggregation tendency of pigment granules can be overcome by a brief treatment with Triton X-100. During centrifugation in the sucrose gradients (scheme) most of the granules pellet out leaving a lot of non-granular material at the 2.0/2.5 M interphase. This is a particular step in the preparation. It is shown by the fact that a mutant *(wa)* without pigment granules[13] does not yield any pellet material when subjected to this procedure. The last step,

Summary of procedures for the preparation of ommochrome granules





Electron microscopy pictures of isolated granules in *a Musca; b Ephestia* (arrows indicate the special type of granules, presumably coming from primary pigment cells).

Purification of pigment granules from *Musca* and *Ephestia*

	Ommochromes (µg)	Total nitrogen (µg)	Ommochromes: residual nitrogen	Purification	Recovery (%)
Starting material	4604.04 (4212.00)	13,812.12 (25,560.00)	0.35 (0.18)	1.0 (1.0)	100.0 (100.0)
12,000× g pellet	1270.08	873.18	1.70	4.9	27.6
Pellet of sucrose gradient	1058.40	490.57	2.75	7.9	23.0
0.89 M layer of metrizamide gradient	449.08 (635.04)	71.44 (212.70)	17.00 (4.26)	48.6 (23.7)	9.8 (15.1)

Ephestia values parentheses, 420 *Musca* heads and 1800 *Ephestia* heads were used.
The recovery figure is based on ommochromes.

the recentrifugation of the pellets in metrizamide gradients (scheme), gives a highly purified granule preparation in the 0.89 M layer. Debris and some pigment, stays at the upper border of this layer.

The overall purification, as determined by the ratio ommochromes: residual nitrogen, is about 49-fold for *Musca* and 24-fold for *Ephestia,* and the recoveries are 10 and 15% respectively (table). For *Ephestia* identical results are obtained, when the calculation is based on pterorhodin. This pteridin pigment is an additional component of the granules[14]. Electronmicroscopic studies reveal, that the preparations are virtually clean with only very little membraneous contamination (figure).

The granules of *Musca* represent a type containing only xanthommatin. They show a fibrillar or granular matrix surrounded by a membrane (figure, a). This picture seems to be typical for xanthommatin granules having lost some pigment during fixation[15]. It has been found also for *Calliphora* in situ[4] and after isolation[10]. Under reducing conditions the extinction spectrum of isolated *Musca* granules is identical with that reported for 'red granules' in situ by Strother[16].

In preparations from *Ephestia,* the eyes of which have a heterogenous ommochrome spectrum consisting of xanthommatin and ommin[17], most granules (about 99%) are electron dense. Their appearance is very similar to that found in secondary pigment and retinula cells[18]. About 1% of isolated organelles show a fibrous structure typical of depigmented primary pigment cell granulae (figure 1, b). Microspectroscopic measurements on isolated granula preparations under reducing conditions give extinction spectra identical to those of secondary pigmentcells[19], which contain the mass of ommochromes. Some successful experiments with *Manduca sexta* and *Calliphora erythrocephala* seem to indicate that the method here reported is also suitable for other species.

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2 I. Ziegler, Adv. Genet. *10,* 349 (1961).
3 B. Linzen, Adv. Insect Physiol. *10,* 117 (1974).
4 H. Langer, in: Photoreceptor optics, p. 429, Eds. A.W. Snyder and R. Monzel. Springer, Berlin 1975.
5 E.W. Caspari and F.J. Gottlieb, in: Handbook of genetics, vol. 3, p. 125, Ed. R.C. King. Plenum Press, New York 1976.
6 C.A. Lang, Analyt. Chem. *30,* 1692 (1958).
7 S. Jacobs, Nature, London *183,* 262 (1959).
8 E. Stratakis, Insect Biochem. *6,* 29 (1976).
9 A. Fischer and G. Horstmann, Z. Zellforsch. *116,* 175 (1971).
10 H.E. Hehl, thesis, University of Munich (1974).
11 J.P. Phillips, H.S. Forrest and A.D. Kulkorni, Genetics *73,* 45 (1973).
12 I. Ziegler and L. Jeanicke, Z. VererbLehre *90,* 53 (1959).
13 G. Hanser, Z. VererbLehre *82,* 74 (1948).
14 K. Cölln, Verh. dt. zool. Ges. *292* (1976).
15 C.B. Boschek, Z. Zellforsch. *118,* 369 (1971).
16 G.K. Strother, J. gen. Physiol. *49,* 1087 (1966).
17 A. Kühn and A. Egelhaaf, Z. VererbLehre *90,* 244 (1959).
18 G. Horstmann, thesis, University of Cologne (1973).
19 K. Cölln, Verh. dt. zool. Ges., 288 (1977).

The sternal gland and recruitment communication in the primitive ant *Aneuretus simoni*[1]

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Summary. In the primitive ant *Aneuretus simoni,* the only living aneuretine species, recruitment communication is mediated by the secretions of the sternal gland, whereas alarm substances are produced in the pygidial gland. This represents the first demonstration of chemical communication in this species.

Trail communication is nearly ubiquitous in species of the advanced subfamilies of ants, and the source of pheromones releasing trail following behavior varies between groups. In most myrmicine and formicine species trail substances are produced in glands associated with the sting, and the hindgut, respectively[2], while dolichoderine trail pheromones originate in the ventral organ (Pavan's gland)[3]. The only living representative of the subfamily Aneuretinae, *Aneuretus simoni,* possesses a glandular epithelium in the 7th sternum similar in structure to the dolichoderine ventral organ[4], now termed the sternal gland[5]. Here we report for the first time on the role of the sternal gland in recruitment communication in *Aneuretus simoni.*

During the course of field studies in Sri Lanka, one of us (AJ) repeatedly observed workers of *A. simoni* traveling along well-defined trails to fallen fruit. Trails were also used during nest emigrations. In the laboratory, scout ants returned to the nest in what appeared to be trail laying posture after discovering a large food source. Inside the nest the scout encountered workers with a motor display which produced arousal. Additional ants soon arrived at the food source.

Trail communication was analyzed by extracting the contents of the hindgut, Dufour's gland, poison gland, pygidial gland, and sternal gland in ethanol, and artificial trials drawn out with a microsyringe were offered to groups of workers. The results (table) show that only sternal gland trails were effective in eliciting trail following behavior. In addition, trails drawn out from nest entrances induced recruitment even when aged 1–2 h prior to testing.

The number of workers following artificial trails 10 cm in length composed of extracts of different glands. Responses were recorded in a 10-min period

Extract tested	Trial					
	I	II	III	IV	V	VI
Sternal gland	38	44	52	37	43	47
Pygidial gland	0	0	1	1	0	0
Dufour's gland	0	0	0	0	0	0
Poison gland	0	0	0	0	0	0
Hindgut	3	1	2	0	0	1