Cytologia (1991) 56:567-575
https://doi.org/10.1508/cytologia.56.567

Isolation of single viable polytene cells from larval oenocytes of *Acricotopus lucidus* (Diptera, Chironomidae)

Wolfgang Staiber and Irmgard Wech

Universität Hohenheim, Institut für Genetik,
Garbenstrasse 30, D-7000 Stuttgart 70, F. R. G.

Summary

Oenocytes explanted and collected from prepupae of *Acricotopus lucidus* (Diptera, Chironomidae) were enzymatically dissociated into single cells by treatment with elastase. The isolated cells were kept viable for days over HeLa cells as feeder culture. Viability of the isolated cells was tested with flourescein diacetate and Trypan Blue. Carmine-orcein staining demonstrated that the oenocyte nuclei contain polytene chromosomes with distinct banding patterns. The DNA-content and polytenic level of prepupal oenocyte nuclei were measured using flow cytometry at 128 C. The morphology of oenocytes was investigated in differential interference contrast and with transmission electron microscopy.

Introduction

Many tissues of dipteran larvae are composed of cells developing polytene chromosomes in their nuclei (Beermann 1962). In this special type of interphase chromosomes, gene activity can be directly observed in the expression of puffs and Balbiani rings. Polytene chromosomes in dipterans originate by somatic pairing of the homologous chromosomes and by endomitosis. Giant polytene chromosomes are formed in the larval salivary glands of chironomids. Also in the Malpighian tubules, in the midgut, and in the rectum, the chromosomes reach remarkable polytenic degrees, allowing analysis of banding and puffing patterns (Beermann 1952, Staiber and Behnke 1985, Sorsa 1988).

Within the larval salivary glands of the chironomid *Acricotopus lucidus*, cell types with very different puffing and Balbiani ring patterns border each other (Mechelke 1953, Staiber and Behnke 1985). In very rare cases, spontaneous fusions between these different cell types occur

(Staiber 1986). A very interesting phenomenon after fusion is the mutual influence of the gene activity patterns of the different nuclei. Up to now no way has been found to induce such fusions experimentally within the salivary gland organ.

A possible way to investigate the mutual influence of nuclei with different gene activity patterns in a common cytoplasm may be to dissociate polytene tissues into single viable cells, and then to fuse different types of isolated cells experimentally. Preliminary experiments to dissociate larval salivary glands of *A. lucidus* enzymatically into single viable cells were not successful. Better results in the enzymatic isolation of single viable polytene cells were achieved with larval oenocytes.

Materials and methods

Isolation and culture of the cells

Large prepupae (size 7-8.5 mm), mainly females, of *Acricotopus lucidus* (Diptera, Chironomidae) were dissected in drops of Firling medium (Firling and Kobilka 1978) on a slide with a concave grinding under a stereo microscope at 16x magnification. Groups of oenocytes (=GOEs) were isolated with fine tweezers and collected with a micropipette. They were stored at room temperature in a 5 cm Petri dish in a 4:1 mixture of Firling medium and Dulbecco's MEM over a subconfluent or a completely confluent monolayer of HeLa cells as feeder culture. HeLa cells were routinely cultured in DMEM at pH 6.9 supplemented with 10% fetal calf serum (=FCS) and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) at 37°C and 5% CO_2 in a humidified incubator. For enzymatic treatment the GOEs were transfered to a bored slide (10 mm diameter) on which a siliconized coverslip was mounted with paraffin at the bottom side (Macgregor and Varley 1983), containing a 1:1 mixture of an insect ringer (Satmary and Bradley 1984) and Firling medium (without FCS) with 4 mg/ml elastase (from porcine pancreas, lyophil., about 120 U/mg; Serva). The slide with the GOEs was incubated in a moist chamber at room temperature. The process of enzymatic digestion into single cells was controlled and photographed with an inverted microscope IM 35 (Zeiss) equipped with Nomarsky interference contrast and with Neofluar 40/0.75 and Planapo 63/1.4 objectives. After 1.5-2 hr incubation the dissociation of the GOEs into single cells was completed by a gentle sucking with a micropipette. The single oenocytes (=OEs) were collected, washed in Firling medium, and stored in Petri dishes over HeLa cells, or in the above mentioned flat bottomed well slides in a moist chamber.

Determination of viability

Two methods were used to test the viability of the isolated cells:

1. A drop of 0.1% Trypan Blue in phosphate buffered saline (PBS) was added to a drop with the isolated cells. After about 10 min, the staining behavior of the cells was examined. The exclusion of Trypan Blue indicated an intact cell membrane and thus a viable cell (Holmberg 1961).

2. A drop of a fluorescein diacetate (=FDA, Serva) solution (3.5 µl of a stem solution of 5 mg FDA in 1 ml acetone in 1ml PBS) was mixed with the dissociated cells in an insect ringer (Rotman and Papermaster 1966, Netuschil 1983). Fluorescein fluorescence was examined on an IM 35 equipped with a Neofluar 40/0.75 objective under excitation with UV light of 450-490nm (filter combination BP 450-490, FT 510, LP 520, Zeiss; mercury lamp HBO 50W). Intact and viable cells showed an increasing bright green fluorescence indicating an accumulation of fluorescein resulting from esterase activity.

Electron microscopy

Explanted GOEs were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.3, for 1hr at 4°C. After postfixation with 1% OsO4, 1 hr at 4 °C, and dehydration with ethanol, the GOEs were embedded in ERL-4206 (Biorad). Ultrathin sections (50 nm) were cut with an Ultratom IV (LKB, Stockholm). Double staining was done with uranyl acetate and lead citrate solutions. The sections were examined and photographed, using a Zeiss electron microscope EM 10A.

Flow cytometry

Immediately after explantation GOEs, brain ganglia and fat bodies of female *A. lucidus* prepupae were quickly frozen on dry ice in Firling medium (without FCS) in 2 ml microfuge tubes (Eppendorf) and stored until further preparation in a refrigerator at -80 °C. After thawing, ganglia were dissected into small pieces under a stereomicroscope with fine tweezers. 1.5 ml of a solution of 1 mg of the fluorochrome 4', 6-diamidino-2-phenylindole dihydrochloride (=DAPI) in 0.6% Triton X-100 in 100 ml PBS, pH 7.5, was added to the non-fixed probes suspended in 250-500 µl Firling medium. The samples were stirred for 1 min and were then allowed to stand for 3 min. Probes of brain ganglia and fat body were filtered through nylon gauze of 20 µm mesh. The probes of the OE nuclei were not filtered. The samples of the detergent-isolated and fluorochrome-stained nuclei were analysed with a PAS-II pulse cytophotometer (Partec, Switzerland) as described by Ulrich et al. (1988).

Chromosome preparation

Isolated single OEs were fixed in acetic-ethanol (1:3 , 10 min), subsequently stained in acetocarmine for about 30 min and in acetolactoorcein for about 5 min, and were then squashed in a small drop of the latter stain.

Results

In chironomids, the larval oenocytes (=OEs) occur only in the first seven abdominal segments and are segmentally arranged in groups of cells laterally near the cuticle in the peripheral layer of the fat body (Zavrel 1935) . In *A. lucidus* the groups of oenocytes (=GOEs) consisted of 2-7 cells

(Fig. 1a) , which are ensheeted by a common envelope . The clear majority are groups of 4 cells exhibiting a lozenge-shaped arrangement (Fig. 1a). GOEs of the lozenge-arranged type from young prepupae are 110 -150 µm long. Their widths are 90-120 µm. Their nuclei have diameters of 15-20 µm and the spherical nucleoli (only one in each nucleus) diameters of 7-10 µm.

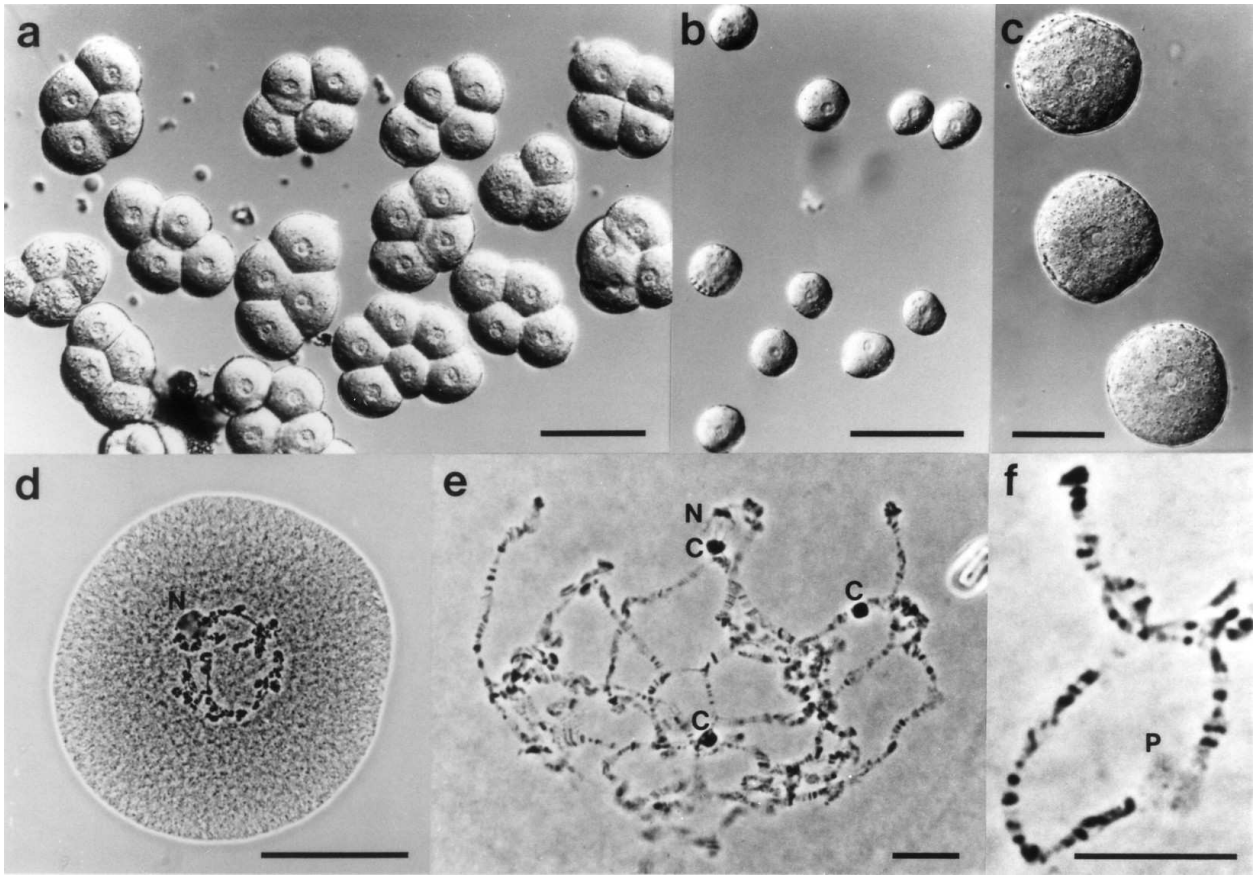

Fig. 1. Groups of oenocytes of *A. lucidus* before (**a**) and two hours after (**b**, **c**) enzymatic treatment. (**d**) Carmine and orcein-stained isolated oenocyte with polytene nucleus and nucleolus. (**e**) Polytene chromosome set of an oenocyte. (**f**) Part of a polytene chromosome exhibiting a clear band-interband pattern and a well developed puff. Interference contrast (**a**-**c**), bright field (**d**) and phase contrast (**e**, **f**) micrographs. C, centromeric region; N, nucleolus; P, Puff. Bars represent 100 µm (**a**, **b**), 50 µm (**c**, **d**) and 10 µm (**e**, **f**).

Table 1 shows the composition of 600 GOEs collected from 80 larvae. Calculating with 14 GOEs per larva, the number of GOEs amount to 53.5% of the total number of GOEs of the dissected larvae. From individual larvae, up to 13 GOEs could be collected.

Table 1. Composition of GOEs from 80 prepupae used for FCM-DNA-analysis OE nuclei

Number of cells per GOEs	2	3	4	5	6	7	8	Total
Number of GOEs	18	19	530	5	27	1	/	600

For obtaining single cells, the common surrounding basal lamella of the GOEs was removed by elastase digestion. The separation into single cells was completed by mild agitation. The isolated cells were collected with a micropipette, washed in Firling medium, and stored in a Petri dish over a monolayer of HeLa cells or in flat bottomed well slides in a moist chamber at room temperature. For determination of viability of the isolated cells, two tests were used: the exclusion of Trypan Blue through intact cell membranes, and the increasing fluorescence resulting from the splitting of FDA by cellular esterases and the accumulation of fluorescein (Rotman and Papermaster 1966).

Exemplified in one of the experiments from 12 larvae, yielding 101 GOEs composed of 408 cells, a total of 190 single intact and viable cells (=46.5%) were isolated. The rounded cells have diameters of 50-70 µm (Fig. 1b, c).

The storage of isolated OEs in Petri dishes over a layer of HeLa cells resulted in longer survival times for the cells than the storage in siliconized flat bottomed well slides. In an experiment, two samples of freshly isolated OEs were stored over HeLa cells and in flat bottomed well slides. After 24 hr 83% of the cells of the sample stored over HeLa cells, and only 17% of the cells of the sample stored in flat bottomed well slides were viable, showing strong esterase activity and exclusion of Trypan Blue. After 48 hr incubation, only the OEs stored over HeLa cells (without loss against 24 hr) were still intact and viable. In other experiments comparable results were received. For a longer time than 48 hr, the storage of isolated OEs was not tested.

The fixed and carmine/orcein-stained nuclei of isolated OEs showed fibrous chromatin and one spherical nucleolus (Fig. 1d). In squash preparations of OE nuclei, distinct patterns of darkly stained bands and slightly colored interbands, characteristic for polytene chromosomes, are clearly visible (Fig. 1e, f). The centromeric regions of the three polytene chromosomes are easily recognized as intensely stained heterochromatic blocks (Fig. 1e). They serve as reference points for the maps and the band nomenclature of the polytene chromosomes of *A. lucidus* (Staiber and Behnke 1985). In chromosome no. III the nucleolus is expressed directly adjacent to the centromeric block. On various sides the bands are decondensed and form puffs representing active gene loci. In Fig. 1e such a puff is indicated.

In *A. lucidus* the GOEs are ensheeted by a capsule-like basal lamella (Fig. 2a, c, d), with a thickness of about 25 nm in prepupal stage (Fig. 2d). For comparison, the salivary gland cells of *A. lucidus* are enclosed in this developmental stage by a 80 nm basal sheet. With the light microscope the ensheeting basal lamella of the GOEs is clearly visible when changing strongly the osmotic situation of the culture medium (Fig. 2e).

The larval oenocytes increase progressively in size during larval development and enlarge especially at moulting (Locke 1969).

GOEs dissected from second instar larvae of *A. lucidus* are already surrounded by a common membrane and are about 30 µm long and about 25 µm wide. This is in contrast to the description

of Zavrel (1935) on *Syndiamesa* (*Pseudodiamesa*), where the cells lie in a group, together but separated from each other.

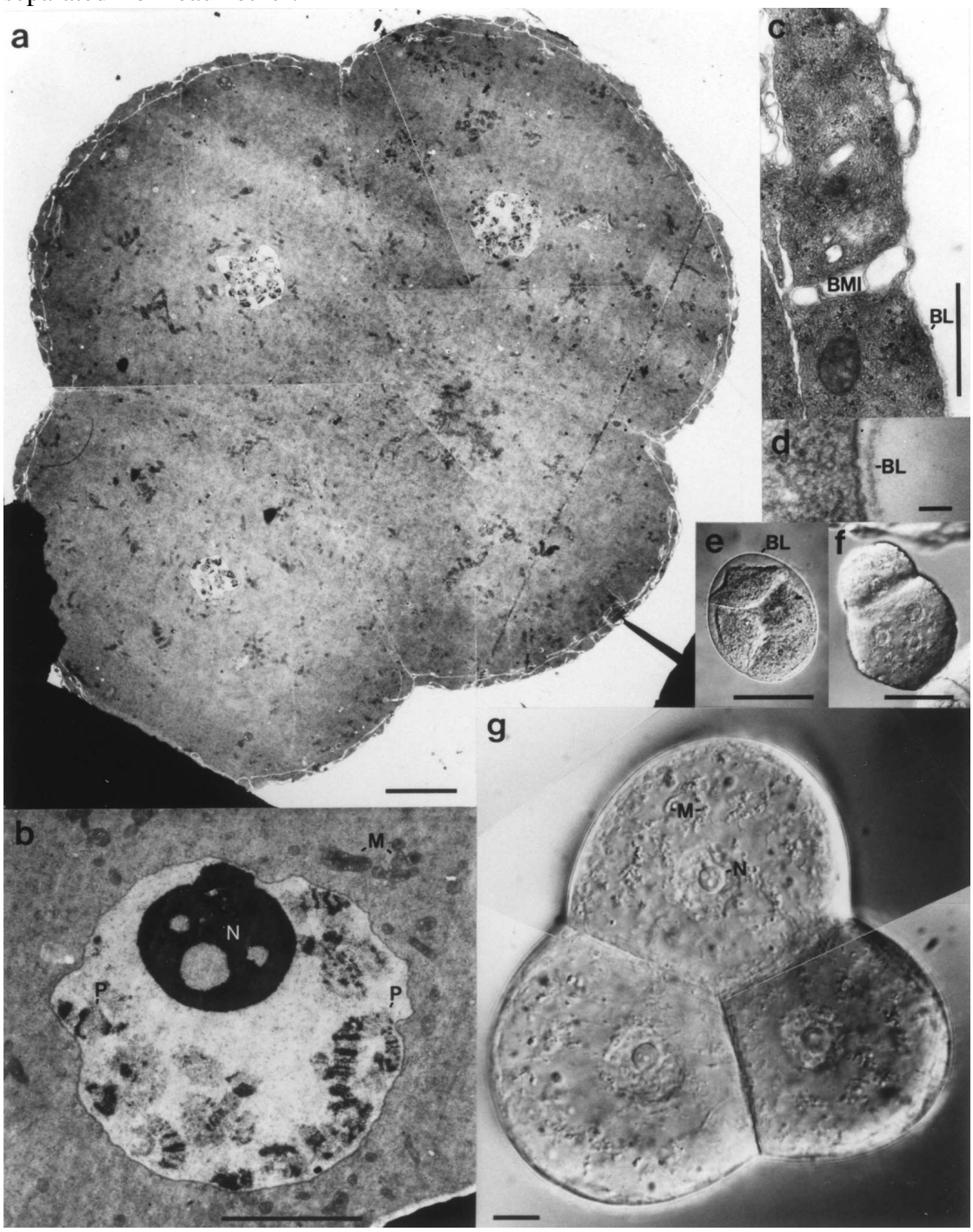

Fig. 2 (**a**) Composite electron micrograph showing a group of four oenocytes from a prepupa of *A. lucidus*. Three nuclei are sectioned. (**b**) Electron micrographs of an oenocyte nucleus with sectioned nucleolus and with polytene chromosomes exhibiting puffs and (**c**) of the peripherous region of an oenocyte showing basal membrane infoldings and the basal lamella. (**d**) Enlarged view of the basal lamella. (**e**) Micrograph of a group of oenocytes after hypotonic treatment, clearly showing the ensheating basal lamella. (**f**) Spontaneous fusion of three cells of a group of four oenocytes shortly after explantation from a larva. (**g**) High resolution interference contrast micrograph of a group of three oenocytes. Mitochondria appear as tubule-like structures. BL, basal lamella; BMI, basal membrane infoldings; M, mitochondria; N, nucleolus; P, puff. Bars represent 10 µm (**a**), 5 µm (**b**), 1 µm (**c**), 0, 1 µm (**d**), 100 µm (**e**), 50µm (**f**) and 10 µm (**g**).

In rare cases the spontaneous fusions of two or three cells in freshly collected GOEs were observed producing polykaryons of polytene nuclei (Fig. 2f).

In the peripherous region of an OE, the basal plasma membrane is infolded, forming extracellular channels which extended about 2-4 µm deep into the cytoplasm (Fig. 2a, c). The cytoplasm exhibits a smooth surfaced, tubular endoplasmic reticulum, which extends uniformly from the nuclear membrane to the periphery of the cell. The mitochondria are evenly distributed in the cytoplasm. Sometimes a few mitochondria are aggregated in groups. The cristae are vertical to the longitudinal axis of the mitochondria. In the high resolution interference contrast micrograph of Fig. 2g, the mitochondria are clearly visible as short, tubule-like structures.In the electron micrograph of the cell nucleus in Fig. 2b, the chromosome sections show clear patterns of dark bands and slightly stained interbands characteristic for polytene chromosomes. Strongly contrasted and clearly separated from the homogenous karyoplasm is the ca. 5 µm sized nucleolus. In *A. lucidus* the nucleolus organizer is located on chromosome no. III (Mechelke 1953, Staiber and Behnke 1985). Resulting from somatic pairing of the homologous chromosomes in polytene nuclei in diptera, only one nucleolus is formed. The nucleolus is composed of regions proximal to the nucleolus-organizer, exhibiting fine fibrillar components representing RNA transcripts, and of regions with granular components containing ribosomal precursor particles (Fig. 2b). Several slightly contrasted vacuoles of different size containing granular components are dispersed in the nucleolus. These vacuoles are already to be seen in differential interference contrast (Fig. 2f).

On some regions of the chromosome sections in Fig. 2b the structure of bands is disintegrated, the bands are decondensed, and the chromosome is swollen, forming a puff. Within the puffs high concentrations of ribonucleoprotein particles are present - messenger RNA precursors, packed together with protein components.

The DNA-binding fluorescent dye DAPI, base-specific for A-T, was used for flow cytometric (=FCM)-measurements in combination with the detergent Triton X-100 for cell membrane lysis. The channel number of the histograms resulted from FCM-DNA analysis is proportional to the nuclear DNA-content (Fig. 3a, b). For the first step of getting the polytenic level of the prepupal OE nuclei of *A. lucidus*, it was necessary to determine the 2C-value. This was done by using the nuclei of prepupal brain ganglia. Because of the high DNA-content of the OE nuclei, it was not possible to measure in one step their C-niveau in reference to the ganglia nuclei. Therefore, fat body nuclei, which were found to have a lower polytenic degree (so DNA-content) than the OE nuclei, were used as intermediate references between ganglia and OE nuclei. First ganglian nuclei, and immediately afterwards, with the same adjustment on the flow cytometer, fat body nuclei of females were measured (Fig. 3a). The same procedure was then used with fat body and OE nuclei (Fig. 3b). The polyteny of female prepupal fat body nuclei was found to range from 16C to 64C. FCM-measurements of fat body nuclei and OE nuclei yielded a polytenic level of 128C for the OEs.

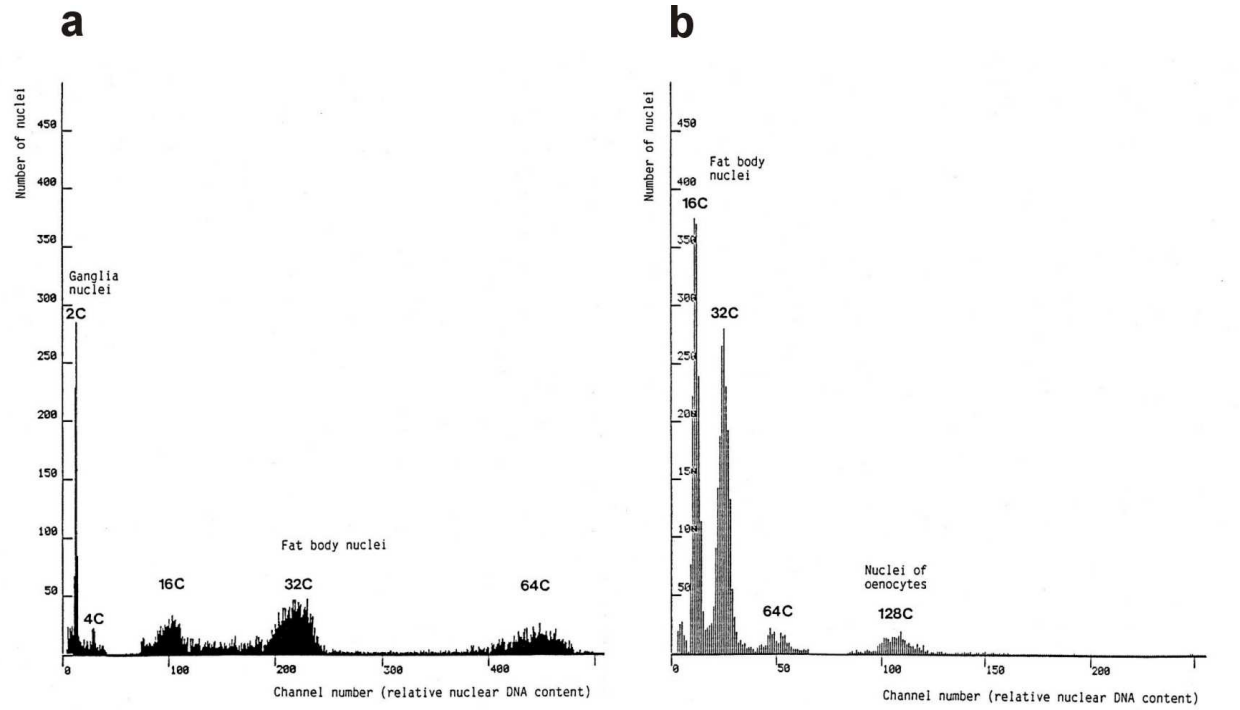

Fig. 3a, b. DNA-histograms of DAPI-stained nuclei from brain ganglia and fat body (a) and from fat body and OEs (b) of female prepupae of *A. lucidus*

Discussion

The OEs are cells of ectodermal origin. Two types of OEs, larval and imaginal OEs (=synoenocytes), are formed (Zavrel 1935). Their functions are as yet not fully understood. Generally, they are regarded as organs of intermediary metabolism and of internal secretion. Some authors see the possible function of the larval OEs in the production of components of the cuticle, such as lipids and lipoproteins of the cuticular layer or the cuticular wax (Diehl 1973, Wigglesworth 1988). Thichy (1980) sees the OEs as the haemoglobin synthesizing tissue in *Chironomus*. This was doubted by investigations of Vafopoulou-Mandalos and Laufer (1984). OEs of *Tenebrio* larvae are demonstrated by organ culture to be involved in ecdysteroid metabolism (Romer et al. 1974). They oxidize α-ecdysone synthesizied by the prothoracic glands to β-ecdysone. This view is supported by EM-investigations of Locke (1969) and Gnatzy (1970), who found morphological parallels between OEs and vertebrate cells engaged in steroid hormone synthesis. Both cell types show a densly packed, smooth, tubular endoplasmic reticulum, characteristic for steroid synthesizing tissues (Romer et al. 1974, Treadgold 1976). In aphids, which lack Malpighian tubules, the OEs are assumed to have excretory functions (Breider 1952).

A detailed description of the light microscopical morphology of larval OEs and their changes during larval and pupal development of the chironomid *Syndiamesa* (=*Pseudodiamesa*) *braniki* was given by Zavrel (1935). As tissues, apparently only necessary during larval development, the larval OEs degenerate during metamorphosis, as also do the salivary glands. During

metamorphosis, the cells of a GOEs first fuse forming a syncytium and then disintegrate (Zavrel 1935).

Speiser (1973) determined the 2C-value of *A. lucidus* to 0.20-0.24 pg on Feulgen-stained ganglian nuclei, using a scanning photometer. Speiser also measured the polytenic levels of different tissues of *A. lucidus* during the development from first larval instar to the imago. In females, the tissues reach higher polytenic degrees than in males. In *A. lucidus* no under-replication was determined during polytenization (Speiser 1973). In female prepupae the highest polyteny degrees were found in the salivary glands (4096-8192 C), in the Malpighian tubules (512-1024 C), and in the rectum (256-1024 C; Speiser 1973). The OEs, not investigated by Speiser, reach a polytenic level of 128C as determined in this study by flow cytometry.

Satymary and Bradley (1984) developed a technique for dissociation of Malpighian tubules of the hemipteran *Rhodnius* and the dipteran *Aedes* into single cells using elastase. The enzymatic treatment removes the surrounding basal lamella of the tubules. The same technique has been successfully applied to larval GOEs of *A. lucidus*.

The isolation of single polytene cells of different larval tissues, e. g. of OEs and Malpighian tubules, opens the possibility of fusing different polytene cell types and of observing directly with the light microscope the mutual influence of the different gene activity (= puffing) patterns (Staiber and Behnke 1985, Staiber 1986).

The time of two days for which viability of the isolated single OEs was demonstrated is surely enough to investigate the mutual influence of gene activities between different polytene nuclei after cell fusion.

An example of a similar type of interaction, and of the time in which such processes proceed, may be the induction of prematurely condensed chromosomes. Within one hour after fusion of a mitotic and a interphasic cell, premature chromosome condensation is induced in the interphase partner by factors derived from the metaphase partner (Cervenka and Camargo 1987).

Changes in the gene activity of the Balbiani rings BR 3 and BR 4 on the polytene chromosomes of *A. lucidus* induced by the moulting hormone ecdysone proceed within 6 hr after administration of the hormone (Panitz 1964). First signs of the induced regression of the Balbiani rings are visible 15 min after ecdysone injection (Panitz et al. 1972).The fusion of the cells may be induced, for example, by electrofusion or by laser fusion. These techniques have the advantage that one can observe the fusion process of definite cells under the light microscope and then collect the fusion products with a micropipette for further investigations (Vienken and Zimmermann 1982, Teissie and Rols 1986, Wiegand et al. 1986).

Acknowledgements

The authors are greatly indepted to Professor F. Mechelke for his support and to Professor H. Rahmann, Institute of Zoology, University of Hohenheim, for the use of his electron microscope facilities. Thanks are due to Mrs. E. Beck for friendly and expert help in EM-sectioning and for

advise on technical problems. It is a pleasure to thank our colleague Dr. Barbara Fritz for the flow cytometric measurements.

References

Beermann, W. 1952. Chromomerenkonstanz and spezifische Modifikationen der Chromomerenstruktur in der Entwicklung und Organdifferenzierung von *Chironomus tentans*. Chromosoma **5**: 139-198.

- 1962. Riesenchromosomen. Protoplasmatologia VI D. Springer, Wien.

Breider, H. 1952. Beitrage zur Morphologie und Biologie der Reblaus *Dactylosphaera vitifolii* Shim., Z. angew. Ent. **33**: 517-547.

Cervenka, J. and Camargo, M. 1987. Premature chromosome condensation induced by electrofusion. Cytogenet. Cell. Genet. **45**: 169-173.

Firling, C. E. and Kobilka, B. K. 1979. A medium for the maintenance of *Chironomus tentans* salivary glands in vitro. J. Insect Physiol. **25**: 93-103.

Holmberg, G. 1961. On the permeability to lissamine green and other dyes in the couse of cell injury and cell death. Exp. Cell Res. **22**: 406-414.

Locke, M. 1969. The ultrastructure of the oenocytes in the molt/intermolt cycle of an insect. Tissue and Cell **1**: 103-154.

Macgregor, H. and Varley, J. 1983. Working with Animal Chromosomes. John Wiley & sons, p. 134.

Mechelke, F. 1953. Reversible Strukturmodifikationen der Speicheldrüsenchromosomen von *Acricotopus lucidus*. Chromosoma **5**: 511-543.

Netuschil, L. 1983. Vitalfärbung von Plaque-Mikroorganismen mit Fluoresceindiacetat und Ethidiumbromid. Dtsch. zahnärztl. Z. **38**: 914-917.

Panitz, R. 1964. Hormonkontrollierte Genaktivitiaten in den Riesenchromosomen von *Acricotopus lucidus*. Biol. Zbl. **83**: 197-230.

Panitz, R, Wobus, U. and Serfling, E. 1972. Effect of ecdysone and ecdysone analogues on two Balbiani rings of *Acricotopus lucidus*. Exp. Cell Res. **70**: 154-160.

Romer, F., Emmerich, H. and Nowock, J. 1974. Biosynthesis of ecdysones in isolated prothoracic glands and oenocytes of *Tenebrio molitor* in vitro. J. Insect Physiol. **20**: 1975-1987.

Rotman, B. and Papermaster, B. W. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Natl. Acad. Sci. U. S. A. **55**: 134-141.

Sorsa, V. 1988. Polytene Chromosomes in Genetic Research. Ellis Horwood Limited, Chichester.

Speiser, C. 1973. Quantitative DNS-Bestimmungen im Verlauf der Ontogenese von *Acricotopus lucidus* (Chironomide). Dissertation, Universitat Hohenheim.

Staiber, W. 1986. Intermediate puffing patterns as evidence of spontaneous fusions of two different polytene cell types in the *Acricotopus salivary* gland. Eur. J. Cell Biol. **42**: 171-175.

Staiber,W. and Behnke, E. 1985. Developmental puffing activity in the salivary gland and Malpighian tubule chromosomes of *Acricotopus lucidus* (Diptera, Chironomidae). Chromosoma **93**: 1-16.

Satmary, M. W. and Bradley, T. J. 1984. Dissociation of insect Malpighian tubules into single viable cells. J. Cell Sci. **72**: 101-109.

Threadgold, L. T. 1976. The Ultrastructure of the Animal Cell. 2nd edition. Pergamon Press.

Teissie, J. and Rols, M. P. 1986. Fusion of mammalian cells in culture is obtained by creating the contact between cells after their electropermeabilization. Biochem. Biophys. Res. Comm. **140**: 258-266.

Tichy, H. 1980. The haemoglobin synthesizing tissue of *Chironomus*. In: Chironomidae Ecology, Systematics, Cytology and Physiology. Proceedings of the Seventh International Symposium on Chironomidae, Dublin, Ireland. Murray DA, ed. Pergamon Press, New York, pp. 43-50.

Ulrich, I., Fritz, B. and Ulrich, W. 1988. Application of DNA fluorochromes for flow cytometric DNA analysis of plant protoplasts. Plant Sci. **55**: 151-157.

Vafopoulou-Mandalos, X. and Laufer, H. 1984. Tissue-specificity of hemoglobin synthesis: Localization of heme synthesis in the subdermal fat body of *Chironomus thummi* (Diptera). Arch. Ins. Biochem. and Physiol. **1**: 191-197.

Vienken, J. and Zimmermann, U. 1982. Electric field-induced fusion: Electro-hydraulic procedure for production of heterokaryon cells in high field. FEBS Letters **137**: 11-13.

Wiegand, R., Zimmermann, K. Monajembashi, S., Schäfer, H., Hansch, G. M., Greulich, K. O. and Wolfrum, J. 1986. Laser-induced fusion of myeloma cells and B lymphocytes. Immunobiology **173**: 320.

Wigglesworth, V. B. 1988. The source of lipids and polyphenols for the insect cuticle: The role of fat body, oenocytes and oenocytoids. Tissue and Cell **20**: 919-932.

Zavrel, J. 1935. Endokrine Hautdrüsen von *Syndiamesa branicki* Now. (Chironomidae). Publ. Fac. Sci. Univ. Masaryk, Brno **213**: 1-19.