Postmolt cuticle growth in a cockroach: in vitro deposition of multilamellate and circadian-like layered endocuticle

F. Weber*

Zoologisches Institut der Universität, Badestrasse 9, D-4400 Münster (Federal Republic of Germany), 26 March 1984

Summary. Pieces of the hind legs of freshly molted *Blaberus craniifer* adults were cultivated in vitro. The deposition of multilamellate and circadian-like layered endocuticle was observed. In multilamellate cuticle, the number of double lamellae was frequently much greater than the number of days in culture. In circadian-like layered cuticle, the number of double layers corresponded to the numbers of days in culture, or it was smaller.
Key words. Cockroach; endocuticle; in vitro growth; multilamellate structure; circadian-like structure.

In many Pterygota, the endocuticle which is secreted by the epidermis after molt grows with a daily rhythm[1]. Each day two layers are formed which differ in the orientation of chitin microfibrils. Thus, the periodic structure of the endocuticle can easily be investigated with a polarizing microscope. For example, in cross-sectioned cuticle from the legs of the cockroach *Blaberus craniifer,* broad isotropic layers alternate with anisotropic layers, which are thinner (with the exception of the first one), and which are partly composed of a few anisotropic and isotropic lamellae (fig. 1). In some species, including *Blaberus craniifer,* it has been demonstrated that the daily growth of the endocuticle is controlled by an endogenous circadian clock[1,2]. Moreover, experimental findings suggest that the clock which controls this rhythm is not localized in the nervous system or in a hormone gland, but perhaps in the epidermis itself[3]. To test this hypothesis, postmolt cuticle growth in isolated *Blaberus craniifer* leg pieces was investigated in vitro.

Method. 0.5–24 h after the imaginal molt the hind legs were cut off and sterilized in Na-hypochlorite solution. After rinsing in Ringer solution, pieces (full cylinders or cylinders lengthwise bisected) of the tibia were transferred into plastic vessels (16 mm diameter), which were filled with 0.6 ml MARKS' medium M14 or M20 (GIBCO products) (+7.5% newborn calf serum+antibiotic/antimycotic; ecdysone was not applied). In

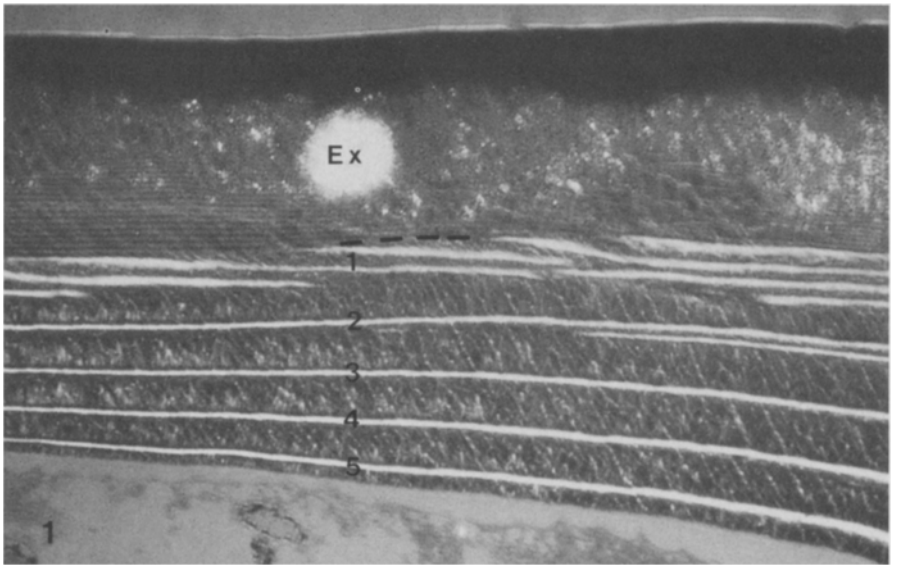

Figure 1. Endocuticle deposited in vivo. Ex; exocutile. 1, 2, ... 5: anisotropic layers. The leg piece was fixed five days after molt. The first anisotropic layer is composed of several lamellae, the second layer partly of two lamellae. × 900.

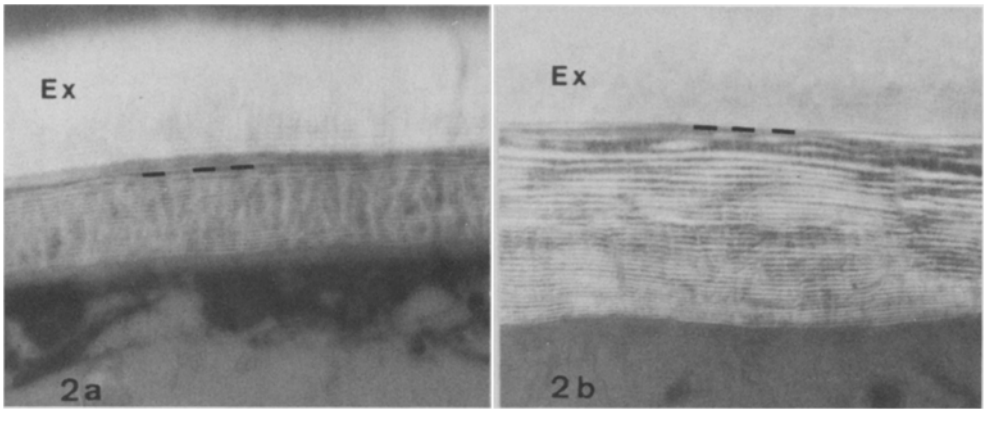

Figure 2. Multilamellate endocuticle deposited in vitro. ——, border between material secreted in vivo and in vitro. *a* 10 days in vitro, cultivated together with fat body. *b* 12 days in vitro, without fat body. × 900.

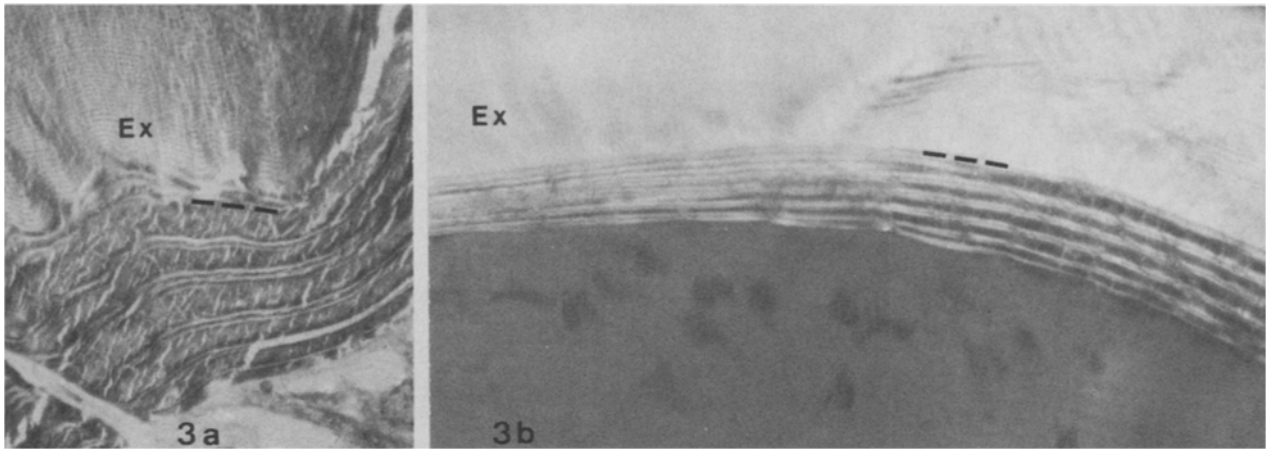

Figure 3. Circadian-like layered endocuticle deposited in vitro. ——, border between material secreted in vivo and in vitro. *a* Five days in vitro, cultivated without fat body. *b* Six days in vitro, without fat body. × 900.

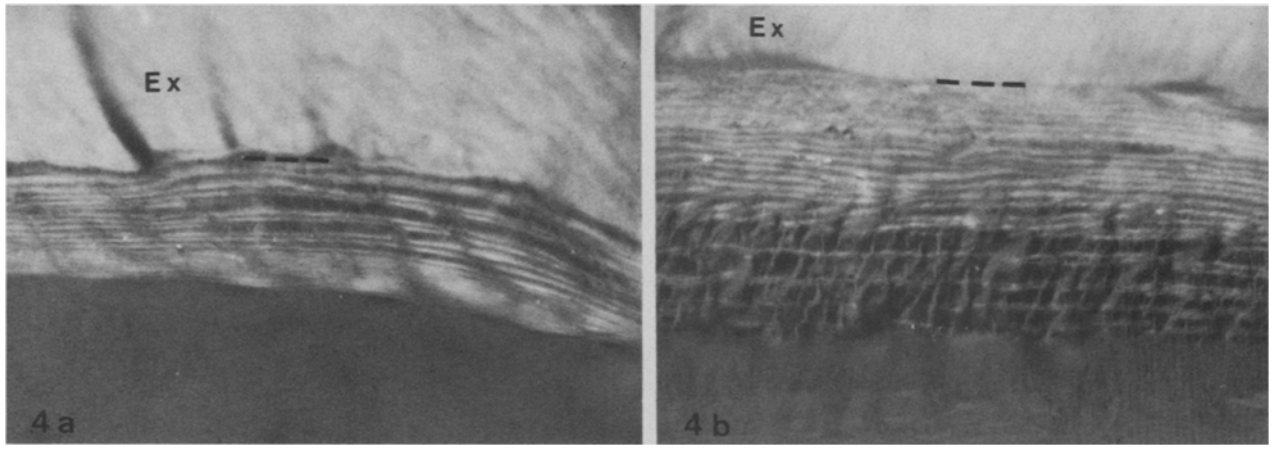

Figure 4. Transition between multilamellate and circadian-like layered endocuticle deposited in vitro. ——, border between material secreted in vivo and in vitro. *a* 12 days in vitro, cultivated together with fat body. *b* 14 days in vitro, without fat body. × 900.

one vessel either one full cylinder of ~ 3mm length, or two full cylinders (~ 2 mm) of the same tibia, or both halves of a lengthwise bisected cylinder (~ 3 mm) were cultured. In some cases fat body was added (~ 8 mm^3 per vessel). As control, a leg piece from each animal was fixed at the moment of preparation. The cultures were incubated at 25 °C; they were slowly shaken; air exchange by diffusion was possible.

After 5–10 days in vitro one piece was fixed (the 3 mm full cylinders were cross-divided), and the medium was renewed. 6–7 days later the other piece was fixed. The pieces were fixed in glutaraldehyde (1.5%, pH 7.4, 12 h), embedded in Epon (5:5), and sectioned with an ultramicrotome (~ 2 µm). The sections were stained with toluidine blue (0.1% in borate buffer, pH 9.22, 60 °C)[4], and analyzed with a polarizing microscope. Four cultures, which are infected by fungi, were eliminated. 34 cultures without fat body and 19 cultures with fat body were examined for cuticular growth in vitro. No attempt was made to characterize the in vitro secreted cuticle chemically.

Results. In about 92% of the cultured leg pieces deposition of endocuticle was observed. The growth was not influenced by the choice of the medium (M14 or M20) nor by presence of fat body (compare figs 2a, b and 4a, b). However, within the pieces the quantity and structure of the secreted material varied extremely. The endocuticle was deposited in two different forms. It mainly consisted of thin isotropic and anisotropic lamellae of nearly equal thickness (fig. 2). More rarely, circadian-like layered endocuticle was observed: isotropic layers alternated with thinner anisotropic layers, the latter of which were partly composed of a few isotropic and anisotropic lamellae (fig. 3). In some cases circadian-like cuticle was deposited at first, but multilamellate material later (fig. 4a). In other cases first multilamellate material and then circadian-like cuticle was formed (fig. 4b). Circadian-like layered cuticle also changed laterally into multilamellate packets (figs 3b and 4a).

In multilamellate endocuticle the number of double lamellae was frequently much greater than the number of days in culture (fig. 2). In circadian-like endocuticle the number of double layers corresponded to the number of days in culture, or it was smaller (fig. 3). Growth in vitro was independent of the age of the donor. Leg pieces that were taken from cockroaches which were still white (0.5–1.0 h after molt) sometimes darkened slightly around the edges of the cut. Areas of darkening and areas of cuticle deposition in vitro did not correspond. Cuticle deposition was observed in non-darkened areas, too. However, in the middle of full cylinders no or only little cuticle was deposited.

Discussion. Endocuticle deposition in an ecdysone-free medium has already been reported of several other in vitro systems: the larval epidermis of *Manduca sexta*[5] and the larval tracheae of

Calpodes ethlius[6] continued intermolt endocuticle growth in an ecdysone-free medium; in isolated imaginal discs of *Drosophila melanogaster*[7] and *Plodia interpunctella*[8] a pulse of ecdysone followed by cultivation in the absence of the hormone induced the deposition of a complete cuticle (including well formed endocuticle). Cuticle formation in vitro has also been observed in the presence of ecdysone; however, in such cases endocuticle was not or only incompletely deposited (for reviews, see Marks and Sowa[9] and Oberlander[10]).

The present results demonstrate that multilamellar as well as circadian-like endocuticle was deposited in cultured leg pieces which were taken from freshly molted imaginal cockroaches. It could be that in cockroaches postmolt deposition of endocuticle is triggered *before* or *during* molt. Thereafter, endocuticle deposition does not seem to depend on further hormonal stimulation. However, the leg pieces contained not only epidermal cells, but also certain quantities of haemocytes, fat body cells and other cells. The efficiency of these nonepidermal cells for the observed endocuticle deposition remains obscure, particularly with regard to the synthesis of cuticle proteins[11].

In the cultured leg pieces mainly multilamellate endocuticle was deposited. The reason for the formation of such endocuticle, which was never found in tibiae developed in vivo, is unknown. In vitro deposition of circadian-like layered endocuticle has not been observed before. Whether the frequency of double layer formation in vitro is really controlled by a circadian (temperature-compensated) clock has to be examined by further experiments.

* I am indebted to Mrs Hassenrück for excellent assistance. – Supported by the Deutsche Forschungsgemeinschaft (We 389/12-2).

1 Neville, A.C., J. Insect Physiol. *29* (1983) 211.
2 Lukat, R., Experientia *34* (1978) 477.
3 Neville, A.C., J. Insect Physiol. *13* (1967) 933.
4 Caveney, S., J. Insect Physiol. *16* (1970) 1087.
5 Mitsui, T., and Riddiford, L.M., Devl Biol. *62* (1978) 193.
6 Ryerse, J.S., and Locke, M., J. Insect Physiol. *24* (1978) 541.
7 Fristrom, J.W., Doctor, J., Fristrom, D.K., Logan, W.R., and Silvert, D.J., Devl Biol. *91* (1982) 337.
8 Dutkowski, A.B., Oberlander, H., and Leach, C.E., Roux' Arch. *183* (1977) 155.
9 Marks, E.P., and Sowa, B.A., in: The Insect Integument, p.339. Ed. H.R. Hepburn. Elsevier, Amsterdam 1976.
10 Oberlander, H., in: Cuticle Techniques in Arthropods, p.253. Ed. T.A. Miller. Springer, New York 1980.
11 Geiger, J.G., Krolak, J.M., and Mills, R.R., J. Insect Physiol. *23* (1977) 227.

0014-4754/85/030398-03$1.50 + 0.20/0

Imprinting of the Peking duck *(Anas platyrhynchos)* and dependence on exposure to light during ontogenesis

G. Teuchert and A. Kretschel[1]

Abt. Neuroanatomie, Fakultät für Biologie der Universität, D-4800 Bielefeld (Federal Republic of Germany), 16 January 1984

Summary. Embryos of Peking ducks were either incubated in complete darkness up to hatching or were put into light one week before hatching. Control embryos were incubated under dim light conditions which corresponded broadly to the natural conditions. Under standardized imprinting conditions the controls and both groups of the light deprived ducklings showed the 'following response'. Most of the dark-incubated embryos, however, did not distinguish between imprinting and test objects of different shapes. Since most of the embryos kept in darkness only for 21 days also failed to develop the capacity for shape discrimination, there is apparently a critical period for light influences on the development of this capacity at some time during the early prenatal period.

Key words. Prenatal light deprivation; imprinting in ducks.

Visual experience influences the development of various brain regions in a variety of vertebrate species[2-6]. Observations on birds similar to those on other vertebrate groups indicate that exposure to normal light optimizes the development of behavior with respect to early learning phenomena such as the imprintability of chickens, while dark-rearing leads to behavrioral deficits[7]. However, the role of the prenatal influence of sensory input on the posthatch pattern of behavior has not attracted any attention, with the exception of acoustic stimulation of chickens in ovo[8-10].

The question investigated here was whether the influence of light during incubation of the avian embryo is essential for the structural differentiation of behavioral patterns which evolve during early postnatal life. The 'filial imprinting' of precocial birds like Peking ducks turned out to be suitable for answering this question because imprintability is influenced by visual stimuli and imprinting takes place during a 'sensitive period' in early postnatal life[11,12].

Material and method. So far we have studied 37 control animals which were exposed to light in ovo and 80 light-deprived animals. The control embryos were incubated (incubator type 'VOMO') for 28 days at 37.8 °C in dim light. Light-deprived embryos were incubated in another incubator of the same type in complete darkness. Within the group of light-deprived embryos 64 animals hatched in darkness, while 16 eggs were put into light 1 week before hatching, i.e. on the 21st day of incubation. After hatching, all ducklings were reared in diffuse light in separate plain gray boxes, which were protected from any visual stimuli. The training and testing was done under standard conditions in the imprinting apparatus which has been constructed by Pröve[1] based on the model of the Hess imprinting-apparatus[12]. The imprinting object (fig. 1) was a green ball moving and emitting sounds. About 20 h after hatching, each duckling was trained for 15 min and was then put back into its box. 24 h after the training the animal was tested with a green duck for 10 min. For the test the duckling was placed in the arena at about the same distance away from the two models while they were stationary (position a, a' in fig. 2). After the duckling had started off towards one or the other of the models, the models started to move and the duckling was allowed to follow for a few min. Then it was pushed away cautiously and the test was repeated twice after changing the position of both models and stopping the sounds. Only when the choice was correct three times did we assume that the imprinting had been successful; this is recorded in columns above the baseline of the table (fig. 3). Even if only one choice