Cockroach Leg Regeneration: Effects of Ecdysterone in vitro

Abstract. *Regenerating epithelial tissue from the cockroach leg forms two cuticular coverings. The first of these consists of a protein-carbohydrate complex that is replaced before molting. The second covering represents the cuticle containing chitin. The deposition of the cuticle can be induced in vitro by adding ecdysterone to the culture medium.*

Although the appearance of cuticle-like deposits on cultures of insect epidermal tissues have been reported (*1–6*), there has been considerable confusion over the nature of the materials being deposited (*2–6*), their relation with the epithelial cells (*3, 6*), and the conditions under which they are deposited (*1, 4*). The chemical nature of the deposits (*1–5*) and the hormonal requirements of explants (*2, 3, 6*) remain largely undefined. To elucidate some of these problems, we have made a series of parallel studies in vivo and in vitro using regenerating leg tissues from the cockroach *Leucophaea maderae*.

The mesothoracic legs of late instar nymphs were removed at the trochantero-femoral articulation 24 hours after ecdysis. At specific times, 8 to 42 days after removal of the leg, the coxae containing the regenerating legs were removed from the insects and the regenerates were dissected. Some of these regenerates were fixed, sectioned, and subjected to histochemical examination, while others were placed in Rose multipurpose tissue chambers under dialysis strips and cultured in M-18 (*7*) nutrient medium. The histochemical procedures for investigating the cuticular sheaths included the triple stain technique for demonstration of protein and carbohydrate (*8*), the thiazine red dichroic method for determination of birefringence (*9*), and the fluorescent chitinase test for chitin (*10*). In conjunction with the triple stain technique, several prior treatments were used to verify the specificity of the staining reaction. These included acetylation, deacetylation, and pepsin digestion (*11*). When dissected 25 days after leg removal, the regenerates were covered with a thin gelatinous sheath that showed a positive PAS (periodic acid–Schiff) reaction (Figs. 1 and 2). Acetylation prevented this reaction while deacetylation restored it, confirming that the PAS-positive reaction was the result of the presence of 1,2-glycols contained in carbohydrate substances. Prior treatment with pepsin also prevented the PAS reaction. These results indicate that the sheath consists of carbohydrate complexes conjugated with

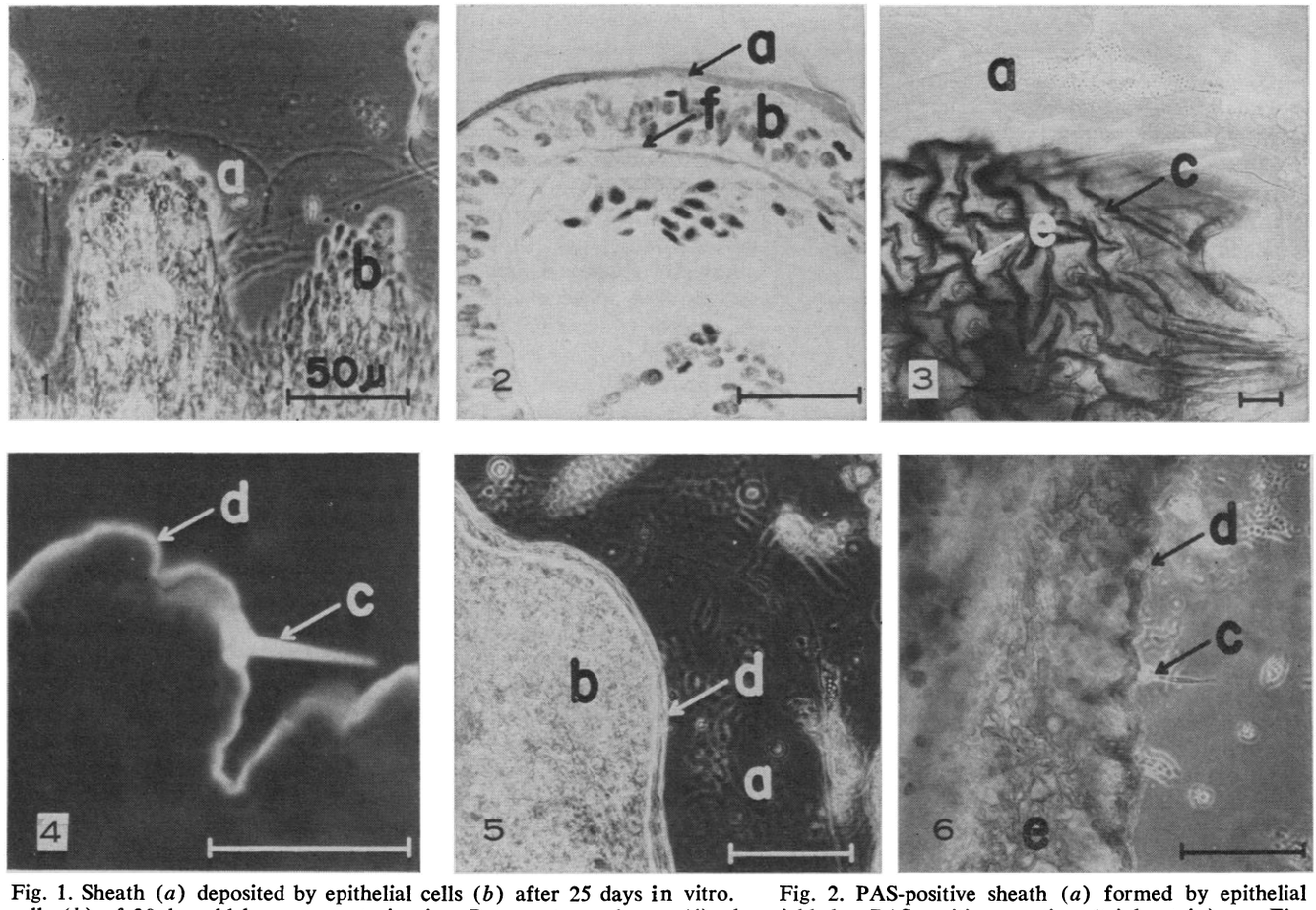

Fig. 1. Sheath (*a*) deposited by epithelial cells (*b*) after 25 days in vitro. Fig. 2. PAS-positive sheath (*a*) formed by epithelial cells (*b*) of 30-day-old leg regenerate in vivo. Basement membrane (*f*) also yielded a PAS-positive reaction (triple stain). Fig. 3. Surface of leg regenerate (44 days in vivo) just before molting shows sheath (*a*) and chitinous cuticle with setae (*c*) and rugose surface (*e*). Fig. 4. Fluorescent chitinase test indicates presence of chitin in both the cuticle (*d*) and seta (*c*) in leg regenerate just before molting. Fig. 5. Cuticular deposit (*d*) appears in vitro between sheath (*a*) and epithelial cells (*b*) in leg regenerate treated with β-ecdysone. Figure 1 shows paired control treated with water. Fig. 6. Seta (*c*), cuticular deposits (*d*), and rugose surface (*e*) formed in vitro are evident 27 days after treatment with β-ecdysone. Cuticle is pigmented. [Scale, 50 μ]

protein, possibly a glyco- or mucoprotein which dissociates upon pepsin hydrolysis. The sheath displayed neither dichroic birefringence nor affinity for the fluorescent-chitinase conjugate, an indication that chitin was not present.

When the regenerates were allowed to develop for 40 to 42 days before dissection, they were found, upon removal, to be covered with a wrinkled membrane bearing spines and small setae (Fig. 3) that had developed between the original sheath and epithelial cells. This membrane was PAS-negative, displayed dichroic birefringence, and showed affinity for the fluorescent-chitinase conjugate (Fig. 4). This layer, which had replaced the initial sheath, thus represented the early stage of the developing chitinous cuticle.

When 25-day-old leg regenerates were placed in culture, epithelial cells quickly migrated over the injured areas and a new sheath was deposited over the surface (*2*). Some of the epithelial cells developed hairlike processes that extended out into the gelatinous sheath, but in no case did a recognizable cuticle develop. These activities continued for as long as 30 days.

To investigate the hormonal induction of cuticle formation in vitro, Rose chambers were set up in pairs so that each member of the pair contained a leg from opposite sides of the insect. One chamber of the pair was treated with ecdysterone (*12*), while the other was treated either with water or with another isomer of ecdysone, 22-isoecdysone, which has been reported to be inactive in the *Calliphora* assay system (*13, 14*). When such chambers were treated 4 days after explantation with water solutions of ecdysterone (2.5 to 12 μg per milliliter of medium), evidence of deposition of cuticle was apparent by the end of 7 days. A thin refractile layer appeared between the epithelial cells and the gelatinous sheath; the epithelial cells began to round out and became filled with a granular material; and, in a few days the refractile layer thickened and became wrinkled (Fig. 5). At the same time, the hairlike epithelial cells began to thicken, and at the base a socket began to form. By day 14 after treatment, the cuticle that showed affinity for the fluorescent-chitinase conjugate began to darken, forming a rugose covering over the explant (Fig. 6 and Table 1).

All the 14 regenerates treated with ecdysterone developed a recognizable cuticle within 14 days, and 8 developed setae. Doses as low as 2.5 μg per milliliter of medium were 100 percent effective. None of the 14 control regenerates produced cuticle or setae, and there was no apparent difference between those treated with water and those treated with 22-isoecdysone.

Table 1. The effect of ecdysone analogs on the deposition of cuticle in vitro in 25-day-old cockroach leg regenerates.

Treatment	Dose (μg/ml)	Number of regenerates		
		Treated	Developing cuticle	Developing setae
Water	10*	4	0	0
22-Isoecdysone	12	10	0	0
Ecdysterone	12	4	4	3
Ecdysterone	5	6	6	2
Ecdysterone	2.5	4	4	3

* In microliters.

At the end of the 14-day period, all control chambers were washed out, filled with fresh medium, and treated with ecdysterone. After eight more days, all the original water control explants showed recognizable cuticle. Those that had been treated with 22-isoecdysone also developed cuticle but required two to five more days to do so.

The chemical composition and function of the gelatinous sheath are only partially known. We found similar sheaths in cultures of epidermal tissues from embryos of the tobacco hornworm *Manduca sexta* (Johannson) and the differential grasshopper *Melanoplus differentialis* (Thomas). Because these deposits occur only on the outer surface of epidermal tissues, they may serve to protect the delicate epithelium and developing setae, while the underlying cuticle is secreted.

The appearance of the chitinous cuticle was unmistakable because the setae and surface sculpturing were similar to that found at the time of ecdysis on regenerates that develop in vivo. The cuticle formed in vitro, however, remained thin and only partially developed. The heavy layer of endocuticle that is formed in vivo at the time of ecdysis was largely absent, a situation similar to that reported by Miciarelli *et al.* (*4*).

With the possible exception of Sengel and Mandaron (*5*), none of the earlier workers succeeded in inducing the deposition of cuticle by explants of epidermal tissue in vitro. Sengel and Mandaron obtained what seemed to be cuticular deposits on leg imaginal disks of late instar *Drosophila* larvae by treating them with ecdysone. Unfortunately, both brain and ring gland tissues were present in these cultures, and the results obtained were such that the source of stimulation and the nature of the cuticular deposits remain uncertain. Miciarelli *et al.* (*4*) and Demal (*1*) used explants of epidermal tissues from immature insects late in their developmental cycle, and cuticle deposition was spontaneous. Larsen (*3*) and Ritter and Bray (*6*) used long-term cultures of tissues of mesodermal origin. Larsen obtained spontaneous deposits of unknown composition, and Ritter and Bray found "chitin"-containing crystals that were spontaneously deposited on the glass surface in cultures of blood clots.

In our study, the secretory activities of the epidermal cells have been changed from the deposition of sheath material to that of chitinous cuticle. This change, which can be induced by ecdysterone in low doses, is not induced by much larger doses of an inactive ecdysone analog. This system, in which the epidermal cells are available for microscopic examination throughout both secretory processes, provides an excellent means of studying the effects of hormones on the process of cuticle deposition.

E. P. MARKS
R. A. LEOPOLD
Metabolism and Radiation Research Laboratory, State University Station, Fargo, North Dakota 58102

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