Protein component of the surface 'wax' bloom of a desert tenebrionid, *Zophosis testudinaria*

Many kinds of insects secrete a powdery or filamentous bloom on their cuticular surfaces. Functions attributed to the bloom include predator deterrents, camouflage and/or mimicry, thermal protection, increased reflectance and reduction of water loss.[1] Most of the studies to date have concentrated on the lipid components of this extracuticular secretion, especially the hydrocarbon fraction.[2-8] Other components have been largely ignored, except by a few investigators who have referred to the possibility of amino acids or proteins as components of the bloom.[2,9,10] We now report that the surface bloom of a Namib desert tenebrionid beetle, *Zophosis testudinaria,* contains not only lipids[7] but proteins of various molecular weights. Furthermore, the dermal glands have been shown to be involved in the production and sequestration of these proteins.

The beetles used in this study were collected at Tsondab Vlei in the eastern portion of the Namib desert in Namibia. Once transferred to laboratory conditions, the beetles were maintained at 30°C and approximately 30% relative humidity for the duration of the experiment. They were fed lettuce and oats. Initially the pink bloom on the elytral surface of all beetles was removed with cotton gauze saturated with distilled water. Beetles were then left for 24 hours to allow for bloom development. Maximal bloom production was determined visually as the pink bloom develops on an otherwise black dorsal cuticular surface.

Experimental beetles were given 1-μl injections of a tritiated amino acid mixture containing 15 amino acids (0,5 μCi; specific activity 532 200 d.p.m.; Amersham, UK; code TRK.440), diluted 1:1 with Ringer solution, by means of a 10-μl Hamilton syringe.[11] Control beetles were injected with 1 μl of Ringer solution. The needle was inserted through the soft intersegmental membrane at the base of the third leg into the haemocoel, and the wounds were sealed with beeswax.

The bloom from the experimental and control beetles was removed with gauze after 8, 24, 48, 72 and 120 h. The gauze containing the pink bloom from each individual was solubilized with 0,5 ml Soluene (Packard, UK) and left overnight at 60°C. Ten millilitres of liquid scintillation cocktail (Packard) was added to each vial and the amount of radioactivity was determined in an LKB Wallac Scintillation spectrometer at an efficiency of 35%.

At the same time one elytron from each of two beetles was prepared for autoradiography. The beetle was beheaded and the elytron removed, fixed in buffered formalin, dehydrated, cleared and embedded in Epon Araldite. One-micron-thick sections were mounted on glass slides. At least four slides containing 10 to 20 sections were prepared from each insect to ensure that a reasonable number of glands would be sectioned. The slides were dipped in Kodak NTB2 emulsion and exposed at 4°C for seven days. After development the sections were lightly stained in 1% Toluidine Blue in borax. Counts of the silver grains were made to compare the amount of labelling over the glands and the cuticle immediately dorsal to the gland cells in the experimental and control insects at fixed time intervals.

The proteins present in the bloom were separated by SDS-polyacrylamide gel electrophoresis. The bloom was first collected from 25 beetles, pooled and then extracted from the cotton gauze with distilled water or SDS sample buffer [2,3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol in 0,0625 M Tris-HCl buffer, at pH 6,8]. Samples extracted with water were lyophilized and reconstituted with SDS sample buffer. Electrophoresis was performed on 15% polyacrylamide gels.[12] Molecular weight marker proteins (Pharmacia, Sweden; low molecular weight calibration kit) were co-electrophoresed with the experimental samples. These were rabbit muscle phosphorylase *b* (M_r
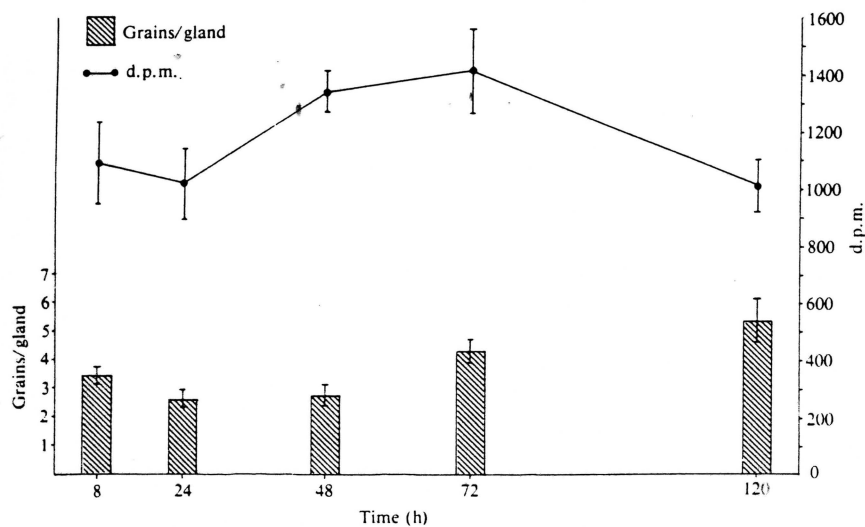
Fig. 1. Line graph showing radioactivity of bloom material expressed in disintegrations per minute removed from *Zophosis testudinaria* after an injection of [³H]amino acids ($n = 14$); the histogram shows average grain counts per gland ($n = 60$). Bar = s.e.m.

94 kilodaltons), bovine serum albumin (67 kD), ovalbumin (43 kD), bovine erythrocyte carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD) and α-lactalbumin (14 kD). These were used to calculate log molecular weights against the relative R_f values. Gels were subsequently fixed in 50% (v/v) methanol, 10% (v/v) acetic acid and stained with Coomasie Blue R-250 or silver.[13]

To increase the amount of radioactivity required for fluorography, 50 beetles were injected with [³H] amino acid mixture and fed 1 μl of the labelled mixture at 3-day intervals for 15 days. Bloom was collected from the beetles every 2 days and stored at −70°C. Tritiated wax bloom samples were processed as above. The gels were fixed, treated with EN³HANCE (New England Nuclear, USA), dried and fluorographed on Kodak X-Omat AR film at −70°C.

The bloom was continuously regenerated, and in a given 24-h period the beetles became pink. The radioactivity contained in the surface bloom increased until day 4 and then declined (Fig. 1), yet remained significantly greater than that of the controls when compared, using the t test (i.e. control $\bar{x} = 278$ d.p.m.; range = 114 − 530 d.p.m.; s.e.m. = ± 99 d.p.m.: experiment, 144 h after injection $\bar{x} = 906$ d.p.m.; range = 456 − 1895 d.p.m.; s.e.m. = ± 200 d.p.m.; $P < 0,001$).

Autoradiography showed that the radioactive material was sequestered in the 'wax' glands (Figs 1, 2a and b). From these studies it appears that the glands do not work in unison; certain glands were heavily labelled whereas others incorporated no label at all. Numerous grains were noted over some of the secretory cells 8 h after the [³H] amino acid injection. Counts in these experimental beetles (mean grain count per gland = 3,4; range = 0 − 15, s.e.m. = ± 0,3, $n = 60$) was significantly higher than in control beetles (mean grain count per gland = 0,5; range = 0 − 2, s.e.m. = ± 0,08, $n = 90$) ($P < 0,001$), when compared using the t test. However, after 24 hours and longer the grains were located mainly over the gland reservoirs (Fig. 2b). The largest number of grains over the gland reservoirs of the experimental beetles was seen at 72 h and 120 h (Fig. 1).

SDS gel electrophoresis of the bloom resolved 7 major protein bands (Fig. 3). Calculation of the log molecular weights against the relative R_f gave molecular weights between 13 000 and 36 000. Two of the surface bloom proteins were derived from the amino acids first seen in the secretory cells (Fig. 2a), then in the gland reservoirs (Fig. 2b), as the fluorography revealed two ³H-labelled protein bands (Fig. 4) that fell into the above molecular weight range.

Radioactivity was detected in both the bloom and in the dermal gland reservoirs 8 h after the injection. The radioactivity increased in the surface bloom until 72 h, after which it declined. No similar trend was seen in the gland reservoirs. Greatest incorporation in the glands was observed at 120 h, when the surface bloom radioactivity had declined. It could be that the radioactive amino acids are 'sweated' out immediately after the injection, and only at a later stage do they appear on the surface as the radioactive protein component of the bloom.
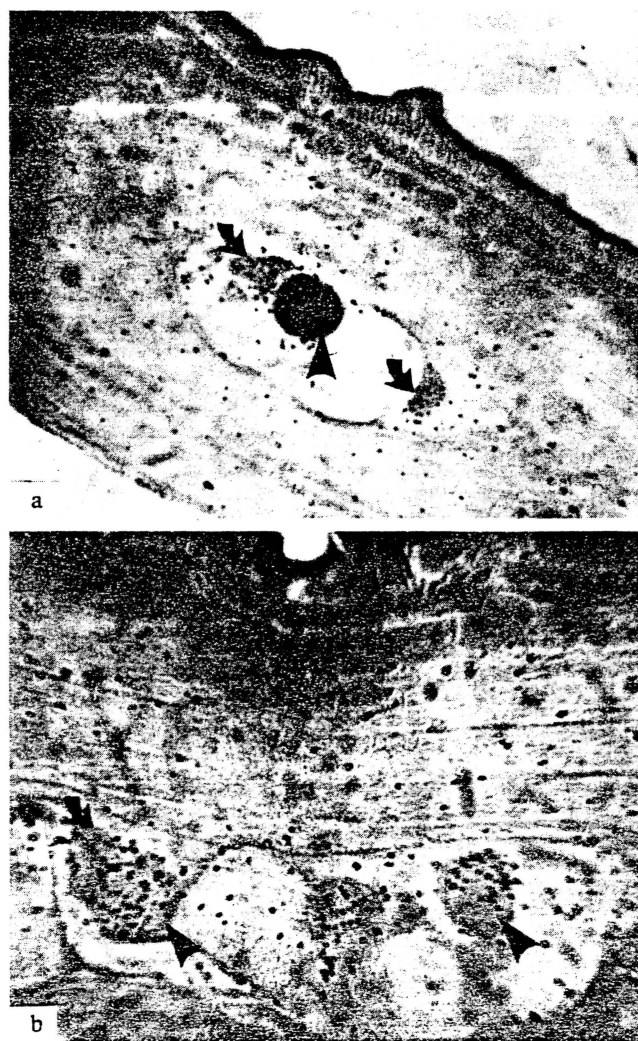

Fig. 2. Section through *Z. testudinaria* elytron showing incorporation of radioactive material in the dermal glands. Arrow head = reservoir, curved arrow = secretory cell. *a*, Eight hours after the [³H]amino acid injection. × 720. *b*, 24 hours after the [³H]amino acid injection. × 1800.
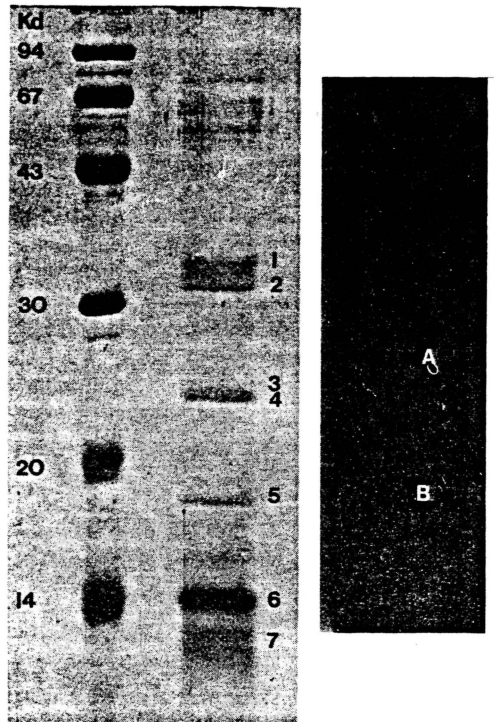
Fig. 3. (*left*) SDS polyacrylamide gel electrophoresis of the proteins from the surface bloom of *Z. testudinaria*. Fig. 4. (*right*) Fluorograph of bloom showing incorporation of radioactivity in two of the protein bands (A and B).

could also play a role in the actual secretory process. It could be that the protein in *Z. testudinaria* is involved in assembly of the filaments as they migrate up the ducts.[8] Furthermore, the bloom proteins may strengthen the filaments and act as the scaffolding that maintains the loft of the bloom off the cuticle surface. This would be important in increasing the tortuosity of the path of the water molecule as it leaves the cuticle surface and thereby enhances waterproofing.[2] This is important as these diurnal Namib desert beetles are subjected to high ambient and sand temperatures, and reduced water loss is paramount for their survival.

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Previous work on Namib desert tenebrionid beetles has shown that dermal glands were responsible for the production of bloom.[10] The gland cells described showed structural features such as rough endoplasmic reticulum that were indicative of protein secretion.[10] Protein was also found to be present in the bloom material washed from the cuticle.

We have now linked gland activity and production of the protein component of the bloom. This is the first time that proteins have been separated from a 'wax' bloom, and at least two of these proteins can be traced to the amino acids incorporated in the dermal glands and associated secretory cells. Although nothing is known of the activity of the glands or the rate of turnover of the bloom of *Z. testudinaria,* full bloom development under natural conditions in the Namib desert is complete in 8 h (McClain, in preparation). This coincides with the appearance of radioactivity in both dermal gland reservoirs and in the surface bloom. Bloom development in the other insects studied indicates much slower rates. *Calpodes* produces the long straight bloom filaments at rates of 60 μm in 12 h.[14] In coccinellid larvae of the Scymini, development of the wax bloom takes about 48 h.[4] Another arid land-adapted tenebrionid of the North American desert, *Cryptoglossa verucosa,* is reported to produce the bloom maximally only after 7 – 10 days.[2]

The functional role of the proteins in the 'wax' bloom of *Z. testudinaria* is not known, but the inclusion of proteins with the lipids may be involved in maintaining the integrity of the bloom, thus enhancing the survival of these diurnal desert beetles. Proteins may be involved in the formation of the matrix of the filament as an inner and outer core (McClain, in preparation). They

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