Enzymatic Mechanism for the Escape of Certain Moths from Their Cocoons

Abstract. *An "escape hatch" from the cocoon of* Antheraea pernyi *is established by the hydrolytic action of a virtually pure proteinase which the moth first secretes and then dissolves by means of a solvent which maintains the enzyme at optimum pH. The proteinase is both synthesized and secreted by the maxillary galeae. The solvent derives from a pair of labial glands which have the form of long convoluted tubules lying alongside the foregut and opening to the exterior through a single aperture in the head.*

In addition to the familiar commercial silkworm, *Bombyx mori*, the Lepidoptera include several hundred species of "wild silkworms" of the family Saturniidae, each of which spins a cocoon of distinctive architecture (*1*). The cocoons range in complexity from simple chambers to multilayered edifices equipped with preformed valves and escape-hatches for the exit of the adult moth. In each case the cocoon is constructed from a single thread of silk which consists, in turn, of a double filament of silk protein, fibroin, coated by a second protein, sericin. The latter ("silk jelly") provides an interstitial matrix which binds the strands of fibroin into a compact and coherent layer (*2*).

Of interest here are the simple, valveless cocoons constructed by the commercial silkworm and by numerous species of *Antheraea*, including the American species *A. polyphemus* and the Asiatic species *A. pernyi* and *A. mylitta*. These cocoons are extremely stout-walled chambers without obvious provision for the escape of the adult moths. The moth, when fully formed, secretes a fluid which moistens the cocoon's anterior end. The wet area is rapidly softened by the breakdown of sericin. The delicate moth is then able to push aside the loosened fibroin filaments, forming a large hole through which it emerges (Fig. 1).

It has generally been assumed that the fluid is a regurgitated secretion of the digestive tract (*3–5*). Trouvelot (*3*) suggested that it contained a hypothetical "bombycic acid." On the basis of potassium analyses and titration with acid, Latter (*4*) reported the active principle to be potassium hydroxide. Honda (*5*) studied the "proventricular fluid" of *B. mori* and concluded, mainly on the basis of alcoholic precipitation, that it contained an enzymic protein.

In this report we show that, at least in the case of *A. pernyi*, the fluid applied to the cocoon is dual in origin and function. The active ingredient is a remarkably pure proteinase which is synthesized and secreted by the moth's maxillae. The other component, secreted by the metamorphosed anterior ends of the silk glands, is a liquid which functions as a buffering solvent for the proteinase.

The fluid was collected directly from the cocoon. For this purpose a cocoon was cut open and the pupa temporarily removed. The internal walls of the cocoon were coated with a hydrophobic film of melted wax, and a glass capillary tube was sealed into the anterior end of the cocoon to communicate with its lumen. A moth, just prior to adult ecdysis, was placed head-down in the cocoon which was then reassembled and sealed with melted wax. When the adult initiated efforts to escape from the cocoon, the fluid ran down into the glass tube from which it was collected for analysis. Fluid so obtained was used to corroborate results on samples collected by the following more simple procedure.

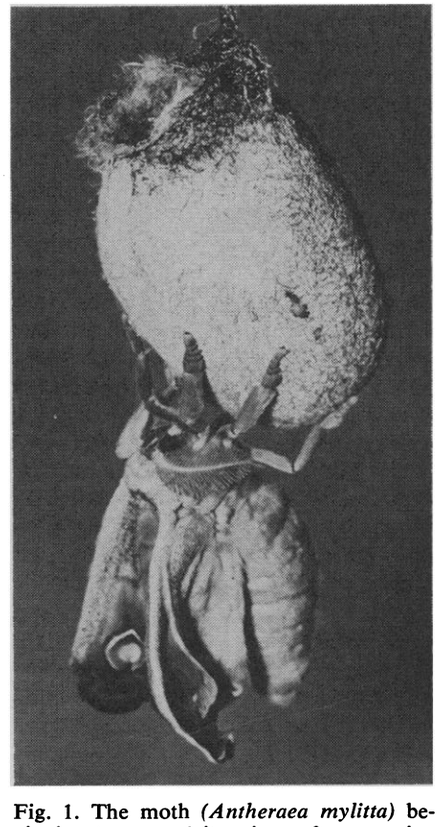

Fig. 1. The moth *(Antheraea mylitta)* beginning to expand its wings after escaping through the hole which it established in the cocoon's upper (anterior) end.

Pupae were removed from their cocoons and allowed to mature. When the moth was fully formed and ready to emerge, the old pupal skin was opened in the facial region and 7 μl of 0.05*M* physostigmine sulfate was injected dorsally into the thorax (*6*). This drug causes spastic paralysis and simultaneously induces copious secretion of the fluid, which can be collected drop-by-drop in glass capillary tubes (Fig. 2). The rate of secretion, initially high (up to 3 μl/min), gradually declines but remains considerable for over an hour; after 3 hours some fluid is still being released. As much as 0.3 ml of fluid may be collected from a single moth, amounting to over 10 percent of the animal's live weight.

The fluid is clear, colorless, and basic (*p*H 8.3 to 8.7). It reacts positively with the Folin phenol reagent as modified by Lowry (*7*) for protein measurements. The intensity of the color produced is paralleled by the fluid's ability to digest gelatin, denatured hemoglobin, sericin, and various other proteins; small peptides, however, are not cleaved within a reasonable time. Proteolytic activity is completely abolished by prolonged boiling, indicating the presence of a proteinase. When successive drops of fluid were analyzed, the Folin-positive material and the proteinase activity were found to be concentrated in the very first drops (Fig. 3). This finding led us to suspect that enzyme and solvent might have different origins.

Detailed study of moths just prior to emergence traced the proteinase to a deposit of hyaline or white semicrystalline material encrusting the surface of the two maxillary galeae, especially along their lateral margins. When dissolved in buffer, this material showed impressive proteolytic activity —as high as that of the authentic fluid.

We have established that the enzyme is synthesized and secreted by the galeae themselves. In Lepidoptera which feed as adults, these structures form the familiar proboscis (*8*); in nonfeeding Saturniidae they have been con-

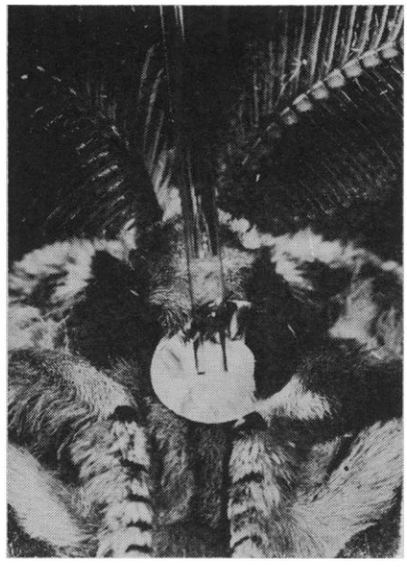

Fig. 2. By means of a capillary tube the enzymically active fluid is collected from the face of a newly emerged moth (*Antheraea pernyi*).

sidered nonfunctional vestiges. The sources of the enzyme are certain giant polyploid cells which differentiate from the simple epidermis of the galeae during the pupal-adult transformation. Until 2 days before adult ecdysis the enzyme is sequestered in large apical vacuoles in these cells. Just after the resorption of moulting-fluid has created an air space around the mouthparts, the vacuoles begin to shrink as the enzyme is secreted through fine intracellular ductules to the surface of the galeae. The secretion is first seen as small viscous droplets which gradually enlarge and coalesce, reaching maximum size a day later. Subsequently, they shrink and become more viscous as the solvent disappears. Finally, a few hours before ecdysis, the material is reduced to the dry deposit described above.

Approximately 0.1 to 0.2 mg of enzyme can be collected from each moth. Extensive studies (*9*) have been conducted on approximately 50 mg of the substance picked off with forceps from 400 individuals.

The material seems to be virtually pure protein showing slight dichromism suggestive of partial crystallinity. Its salt content is low; measurements gave values of 1.5 percent potassium, 0.1 percent sodium, and 0.01 percent each of calcium and magnesium. When treated with the Folin phenol reagent, 1 mg gave an optical density at 660 mμ equal to that of 1.6 mg of crystalline bovine serum albumen. In the ultracentrifuge it showed a single, nearly symmetrical sedimentation pattern, with only a slightly elevated baseline on the meniscus side (Fig. 4).

In its enzymic properties the material is a basic endopeptidase akin to trypsin, but with certain important distinctions. Without any purification, it is nearly as active as an equal weight of twice-crystallized trypsin when tested on denatured hemoglobin or benzoyl arginine ethyl ester. Like trypsin, it does not attack the ester substrates of chymotrypsin. We have been unable so far to resolve the enzyme into more than one active component.

The solvent exudes from the face of the moth, not through the mouth, but through a small round aperture in the midline, just ventral to the mouth opening. If this opening is blocked with melted wax no fluid appears after injection of physostigmine, until such time as the wax seal is perforated. Sealing the mouth in a similar fashion in no way impairs the secretion.

The aperture communicates internally with a pair of tubules about 0.2 mm in diameter and 100 mm long which extend beside the foregut just ventral to the longitudinal flight muscles of the thorax. They are extensively coiled and convoluted and terminate blindly on each side near the anterior end of the midgut. Their rich tracheation suggests a potential for high metabolic activity. In histological preparations, a single layer of large cells, usually two or three in any one cross section, is seen to separate the central lumen from the surrounding hemolymph.

We have established that the pair of tubules are in fact derived from the anterior ends of the larval silk glands. Therefore, on phylogenetic grounds, they are labial structures. During metamorphosis of larva into pupa, the major posterior portions of the silk glands degenerate, as previously recognized (*10*). But the anterior or duct portions are not destroyed, contrary to the common assumption; the cells survive despite the loss of most of their cytoplasm. When the pupa begins to transform into the moth, these cells, without any mitosis, undergo a spectacular metamorphosis; the end-result is a pair of glands whose cells show a fine-structure strikingly similar to that seen in the renal tubules or the salt glands of vertebrates (Fig. 5).

The fluid elaborated by the labial glands is isotonic with the surrounding hemolymph, even when the hemolymph is altered by injections of hypertonic or hypotonic solutions into the hemocoel. However, the two fluids differ radically in ionic compositions. The labial secretion is essentially an aqueous solution of 0.15*M* potassium bicarbonate. Potassium, by far the principal cation, is present in concentrations 4 to 5 times that in the blood. By contrast, the secretion contains little sodium and only traces of calcium and magnesium (less than 1 percent of the blood concentration). Bicarbonate is the major anion but not the only one present: chloride is found in concentrations comparable to that in hemolymph

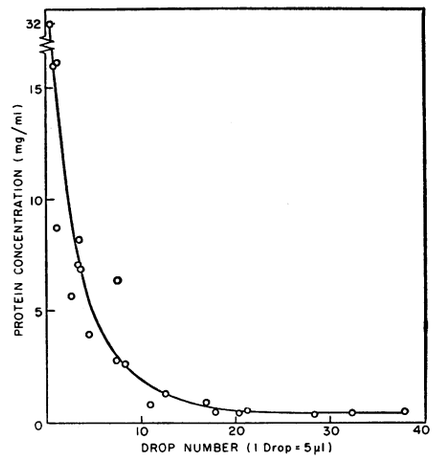

Fig. 3. The protein content of successive drops of the complete fluid. Nearly all of the protein and proteinase are recovered in the first ten drops of fluid.

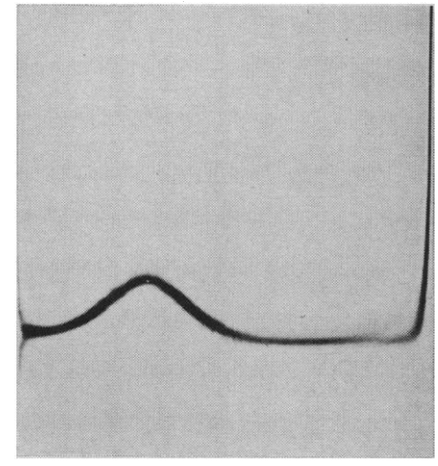

Fig. 4. Sedimentation pattern of proteinase powder recovered from the moth's galeae and dissolved in 0.002*M* potassium phosphate buffer at *p*H 7 to a final concentration of 4 mg/ml. Centrifugation at 56,100 rev/min; bar angle 45.5°; temperature 10°C; sedimentation from left to right; $S_o = 2.7$.

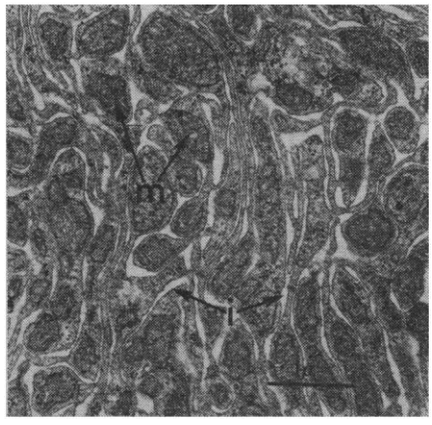

Fig. 5. Electron micrograph of a cell from a labial gland of *Antheraea pernyi* fixed shortly before adult ecdysis (× 11,500). The cell has been sectioned obliquely and the apical side, closest to the lumen, is pictured here. The surface of the cell is extensively infolded so that the apical region appears as a series of irregular, interconnected plates of cytoplasm surrounded by narrow, clear clefts of the extracellular spaces (*i*). Numerous mitochondria (*m*) are seen in the cytoplasmic plates. Fixed first in glutaraldehyde and then in 1 percent osmium tetroxide, and stained with lead citrate and uranyl acetate.

(approximately 20 meq/liter) and in isotopic experiments appears to exchange rapidly with blood chloride. Phosphate is not detectable. A substantial *p*H gradient exists across the walls of the gland (blood, about 6.5; secretion, about 8.5).

The capacity of the lumen is only a small fraction of the volume of the solvent secreted before the moth escapes from its cocoon; most of the secretion must be elaborated during a relatively brief period. The expected changes in hemolymph composition (decrease in potassium, increase in the excluded ions) are indeed observed during this interval. Our measurements suggest an active secretion of K^+ into the lumina of the glands, followed by the passive flow of water and anions (at least Cl^-). Consistent with this interpretation are determinations of electrical potentials across the secretory cells, the lumen being positive both in vivo and in vitro (*11*).

The proteinase is secreted first and coats the maxillary appendages as a dry incrustation. Then, after the moth has forced its head from the pupal exuviae, the solvent is ejected from the facial aperture between the maxillae. The first drops dissolve the enzyme and bring the latter into contact with the overlying cocoon. Subsequent secretion promotes enzymic attack on the cocoon in two ways: it wets the extensive area which must be cleared of sericin, and it serves to maintain the enzyme at optimum *p*H (approximately 8.5). Although the buffering capacity of bicarbonate is poor in this range, the fluid continuously neutralizes the carboxylic groups liberated during sericin digestion without endangering the optimization of the reaction by excessive rise in *p*H. In this sense, the labial glands resemble a *p*H-stat, continuously titrating the reaction to an end point of about *p*H 8.5.

In establishing an escape-hatch in the cocoon, the enzyme's natural substrate is the interstitial sericin which binds the fibroin filaments. We are reluctant, however, to imply limitation of its potency by adopting the name "sericinase": the enzyme is both a powerful proteinase, active on all soluble proteins so far tested, and a trypsin-like esterase. In recognition of its unusual source and remarkable biological role, we propose to call it "cocoonase."

It may be noted that the enzyme is not elaborated by *Hyalophora cecropia* or *Rothschildia orizaba*, two silkworm species whose cocoons are equipped with prominent, preformed valves. Indeed throughout the New World, Saturniidae unsealed cocoons correlate with truly vestigial maxillary galeae (*12*). However, despite the absence of cocoonase, these two species, the only ones examined thus far, can secrete a voluminous liquid which is entirely derived from the labial glands. *Samia cynthia*, which spins a cocoon with a particularly tight valve, is an intermediate form in that the galeae, though smaller than those of *A. pernyi*, are not vestigial; a small amount of cocoonase is synthesized.

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Plant Damage Caused by Irradiation of Aldehydes

Abstract. *The report that damage to petunia has been correlated with the presence of aldehydes in the atmosphere is discussed in relation to recent laboratory findings. Laboratory investigations have shown that irradiation of formaldehyde in air will not cause plant damage to the varieties of petunia, pinto bean, and tobacco wrapper used, even when nitrogen oxide is added to the system. Irradiation of propionaldehyde in air does cause damage to these plants. Addition of nitrogen oxide to the irradiated propionaldehyde-in-air system does not markedly increase damage.*

Injury to leaves of Snowstorm petunias has been associated with atmospheric levels of aldehydes (*1*). The injury is characterized by necrotic banding of the upper leaf surface and glazing of the lower leaf surface. Aldehydes were determined chemically by the bisulfite method, and plant damage occurred within 2 days after the atmospheric aldehyde content exceeded 0.20 parts per million.

Stephens and co-workers (*2*) photooxidized several aldehydes in air and concluded that the products of certain aldehydes would damage the lower leaves of pinto bean and also would damage petunia. The role of added nitrogen oxides was not investigated in this earlier work. Small concentrations of nitrogen oxides may have been present in the mixtures.

We have obtained data on the irradiation of formaldehyde and propionaldehyde in the presence of low concentrations of nitrogen oxides and in the presence of added amounts of nitrogen oxides. Composition of the mixtures, oxidant levels, and intensities of plant