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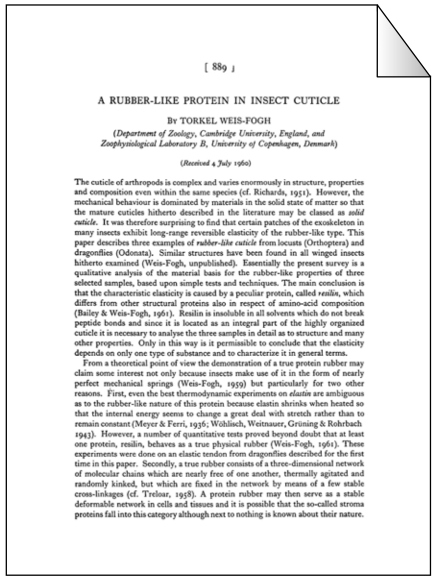
THE FIRST DESCRIPTION OF RESILIN



[889]

A RUBBER-LIKE PROTEIN IN INSECT CUTICLE

By TORKEL WEIS-FOGH

(Department of Zoology, Cambridge University, England, and Zoophysiological Laboratory B, University of Copenhagen, Denmark)

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The cuticle of arthropods is complex and varies enormously in structure, properties and composition even within the same species (cf. Richards, 1951). However, the mechanical behaviour is dominated by materials in the solid state of matter so that the mature cuticles hitherto described in the literature may be classed as *solid cuticle*. It was therefore surprising to find that certain patches of the exoskeleton in many insects exhibit long-range reversible elasticity of the rubber-like type. This paper describes three examples of *rubber-like cuticle* from locusts (Orthoptera) and dragonflies (Odonata). Similar structures have been found in all winged insects hitherto examined (Weis-Fogh, unpublished). Essentially the present survey is a qualitative analysis of the material basis for the rubber-like properties of three selected samples, based upon simple tests and techniques. The main conclusion is that the characteristic elasticity is caused by a peculiar protein, called *resilin*, which differs from other structural proteins also in respect of amino-acid composition (Bailey & Weis-Fogh, 1961). Resilin is insoluble in all solvents which do not break peptide bonds and since it is located as an integral part of the highly organized cuticle it is necessary to analyse the three samples in detail as to structure and many other properties. Only in this way is it permissible to conclude that the elasticity depends on only one type of substance and to characterize it in general terms.

From a theoretical point of view the demonstration of a true protein rubber may claim some interest not only because insects make use of it in the form of nearly perfect mechanical springs (Weis-Fogh, 1959) but particularly for two other reasons. First, even the best thermodynamic experiments on *elastin* are ambiguous as to the rubber-like nature of this protein because elastin shrinks when heated so that the internal energy seems to change a great deal with stretch rather than to remain constant (Meyer & Ferri, 1936; Wöhlisch, Weitnauer, Grüning & Rohrbach 1943). However, a number of quantitative tests proved beyond doubt that at least one protein, resilin, behaves as a true physical rubber (Weis-Fogh, 1961). These experiments were done on an elastic tendon from dragonflies described for the first time in this paper. Secondly, a true rubber consists of a three-dimensional network of molecular chains which are nearly free of one another, thermally agitated and randomly kinked, but which are fixed in the network by means of a few stable cross-linkages (cf. Treloar, 1958). A protein rubber may then serve as a stable deformable network in cells and tissues and it is possible that the so-called stroma proteins fall into this category although next to nothing is known about their nature.

Henry Bennet-Clark writes about Torkel Weis-Fogh's classic paper on resilin entitled 'A rubber-like protein in insect cuticle'. A copy of the paper can be obtained at http://jeb.biologists.org/cgi/reprint/37/4/889.

It was at one of the weekly seminars in the Department of Zoology at the University of Cambridge in the late 1950s that I heard, with great excitement, Torkel Weis-Fogh describing resilin. In his masterly first written description, Weis-Fogh explained its roles in the thorax of flying insects: as elastic tendons in dragonflies and as elastic wing hinges in locusts (Weis-Fogh, 1960). He also showed that these structures could be strained for weeks without plastic deformation and, by simple tests, that the elasticity was rubber-like. This was in contrast to commercial rubbers which are polymeric unsaturated hydrocarbons: resilin is a cuticular protein that, typically, is deposited after ecdysis. Weis-Fogh commented on the ability of resilin structures to snap back after deformation but showed that their rubbery nature was affected by their hydration and pH. He described a simple test to identify resilin: with very dilute solutions of Methylene Blue or Toluidine Blue, the protein stains a deep sapphire blue (Fig. 1B).

Within the year, John S. Edwards (Edwards, 1960) found resilin in the salivary pump of assassin bugs, used by the bugs to inject their very potent mixture of proteolytic enzymes into their prey or, as a defense, to spit at or inject into aggressors. At around this time, I too found resilin in the feeding pump of *Rhodnius prolixus* (Bennet-Clark, 1963). In both these examples, the resilin provided an elastic spring antagonist to muscle. Since then, resilin has bobbed up all over the place. With Eric Lucey, I described it as the spring that powered the high-speed catapult used in the flea jumping (Bennet-Clark and Lucey, 1967). More recently, resilin has been described in the amazingly complex wing-folding mechanism of earwigs (Dermaptera), which tuck away quite large and fully functional hindwings under tiny, hard forewings (Haas et al., 2000).

In a later paper on resilin in 1962, Jensen and Weis-Fogh explored its unique mechanical properties, showing that the energy loss, even at 200 Hz, was under 5% (Jensen and Weis-Fogh, 1962). They commented that the loss factor does not appear to increase linearly with frequency, suggesting that the losses are not due to viscous damping. The context in which Weis-Fogh discussed the dynamic properties of resilin was the comparatively slow wing-beat frequencies of locusts (c. 25 Hz) but it has become clear that resilin can act as a useful spring over far more rapid stress–release cycles. For example, in the flea *Spilopsyllus cuniculi*, the catapult releases in less than 1 ms (Bennet-Clark and Lucey, 1967) and in cicadas, where resilin acts as the elastic element in the sound-producing tymbal mechanism (Fig. 1) (Young and Bennet-Clark, 1995), the damped resonant vibration of the tymbal equates with energy losses in the whole system of under 20% (Bennet-Clark, 1997). One small cicada with largely similar resilin-containing tymbals produces sharply resonant sound pulses at over 13 kHz (Fonseca and Bennet-Clark, 1998). Thus resilin can work as a useful spring over the very wide range of speeds encountered in insect biomechanics.

Weis-Fogh had originally found that resilin, almost uniquely among biological materials, shows perfect elasticity: even when strained to over twice its original length for two weeks, a dragonfly's resilin tendon snaps back perfectly when the stress is relieved (hence the name he gave it) and that it showed neither tearing nor fatigue when stressed within its natural limits (Weis-Fogh, 1960). He pointed out that resilin was an ideal material for making elastic joints, such as hinges, that were subjected to repeated cyclical stress. Yes, indeed: in the course of its adult life, a locust may fly for 8 hours per day for about 30 days, requiring over 20 million wing beats; a cicada, singing at 4 kHz for

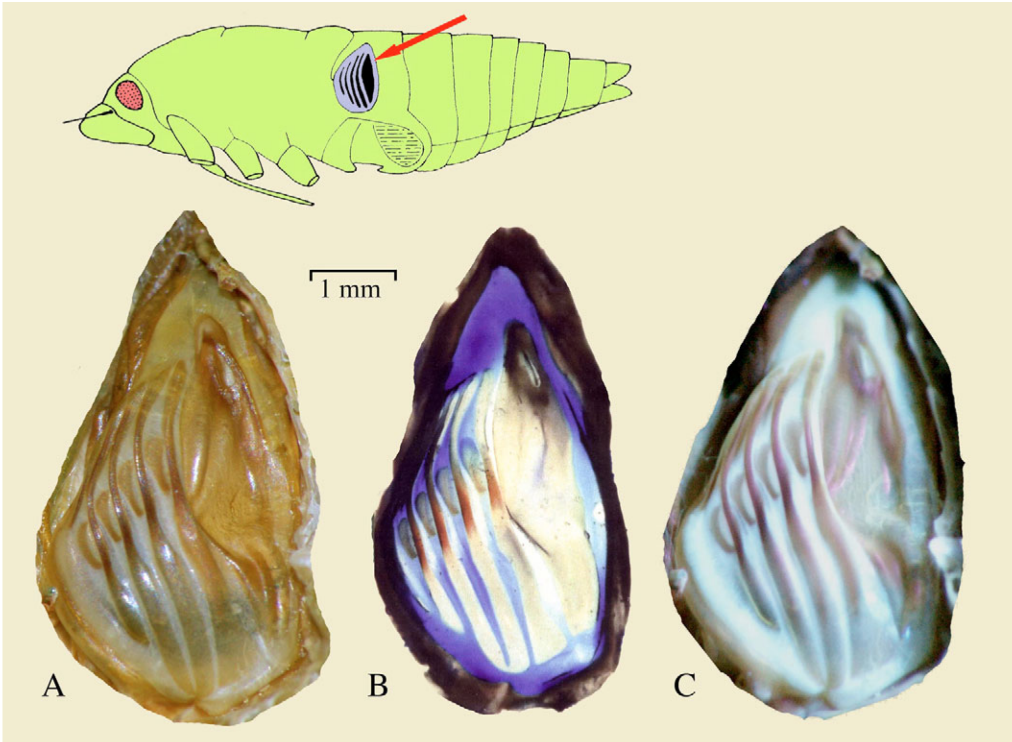

Fig. 1. The sound-producing tymbal of the Australian cicada *Cyclochila australasiae*. (Top) Diagram showing the position of the tymbal (red arrow) at the anterior end of the insect's abdomen. (A) An unstained tymbal photographed in natural light (original). (B) A different tymbal stained for 24 hours in very dilute methylene blue solution (from Young and Bennet-Clark, 1993). (C) The tymbal shown in A showing fluorescence when illuminated by an ultra-violet light-emitting diode radiating at 365 nm; the fluorescence of the resilin is not as bright as if it had been radiated at the optimal wavelength, 315 nm (original).

2 or 3 hours per day for more than 20 days, stresses the resilin in each tymbal over 400 million times, which is more than the number of cycles per year encountered by the hairspring of a mechanical watch.

Weis-Fogh was never one for hyperbole but, had he been, he would have been entitled to term resilin a 'wonder' material. In his 1960 paper he showed, with typical economy and elegance, that it was a protein. Its structure and properties remained unaffected by deep-freezing and heating to over 125°C and it was unaffected by alcohols and fixatives such as formalin and Bouin's Solution. However, it was rapidly dissolved by pepsin, trypsin and other proteolytic enzymes and also dissolved in alkaline solutions; this last snag may partly explain why Snodgrass (Snodgrass, 1946) shows gaps in the meta-pleural regions of fleas whereas I, using freshly killed fleas, observed that these regions stained brilliantly with Methylene Blue (Bennet-Clark and Lucey, 1967). The disappearance of resilin in the course of routine preparation of insect exoskeletons may also partly explain why it remained undetected for so long, but I prefer to think that Weis-Fogh knew that there must be some interesting elastic elements in insects and set out to search for them.

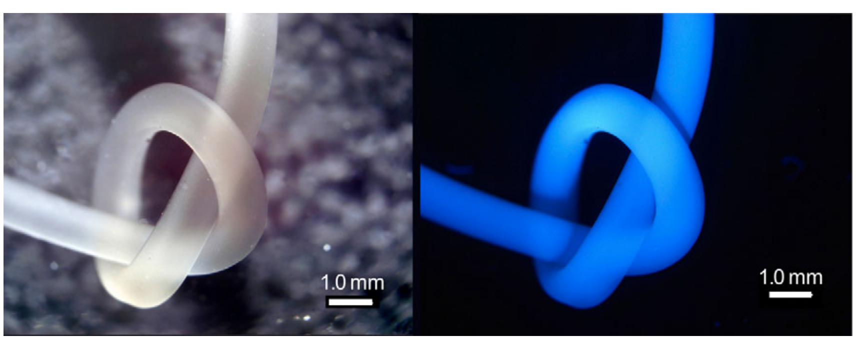

Fig. 2. Bio-synthesised resilin moulded into a flexible rod by drawing pro-resilin into a glass tube, followed by photochemical cross-linking of the precursor. Left, the rod illuminated by white light. Right, the same rod illuminated by U.V. light at 315 nm showing its fluorescence at 409 nm. Figure reproduced from Elvin et al. (Elvin et al., 2005) with permission: photograph by Dr David Merritt, UQ, Brisbane, Australia.

In later studies, with Svend Olav Andersen and others, came confirmation of the rubber-like nature of this protein and identification that the cross-links were the fluorescent amino-acids, dityrosine and trityrosine (Andersen, 1964). Dityrosine fluoresces in UV light, being maximally excited with light at 315 nm and radiating maximally at 430 nm (Andersen and Weis-Fogh, 1964; Elvin et al., 2005): this provides a useful way of identifying resilin non-invasively (see Fig. 1C).

What does the future hold? Chris Elvin and his colleagues, working in Australia (Elvin et al., 2005) have successfully inserted the gene for pro-resilin into *Escherichia coli*, obtaining the gene product and then cross-linking this product and casting it into quite large structures (Fig. 2) with remarkably high resilience: in other words, they've been able to produce resilin in potentially useful quantities and with the potential to form it into structures. Elvin suggests that applications could range from spinal disc implants and heart and blood valve substitutes to high-efficiency industrial rubbers, microactuators and nanosprings. There are serious practical problems to overcome, however, the most serious of which appear to be the ease with which resilin can be de-natured by proteases, the effects of pH and hydration on its mechanical properties and, in the context of a prosthesis, that it could create an immune response.

Nevertheless, the amazing properties of resilin will encourage further development of solutions to practical problems.

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Henry Bennet-Clark
University of Oxford
henry.bennet-clark@zoo.ox.ac.uk

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