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Resilin.
A Rubberlike Protein in Arthropod Cuticle

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I. Introduction

Resilin is a structural protein whose name is derived from the Latin *resilire*, to jump back. It was discovered recently as a major constituent of certain elastic hinges and tendons in the cuticle of locusts and dragonflies (Weis-Fogh, 1960) but, as we shall see, it occurs widely and exhibits properties which are unusual and of general interest to students of proteins and elastomers and to biologists. We shall therefore discuss the results obtained so far from many different points of view and also include some unpublished material.

The wings of locusts are suspended elastically from the strong thoracic box; it was at first thought that the elastic recoil of a wing which is moved away from its equilibrium position (Fig. 1A) was due exclusively to elastic deformations of the solid cuticle of the box, but it turned out that one-quarter to one-third of the energy is stored in the elastic hinge

material itself. It was the experiments illustrated in Fig. 1 which led to the discovery of resilin (Weis-Fogh, 1959, 1961c, and unpublished). A large part of the suspending ligaments and hinges consists of a colourless

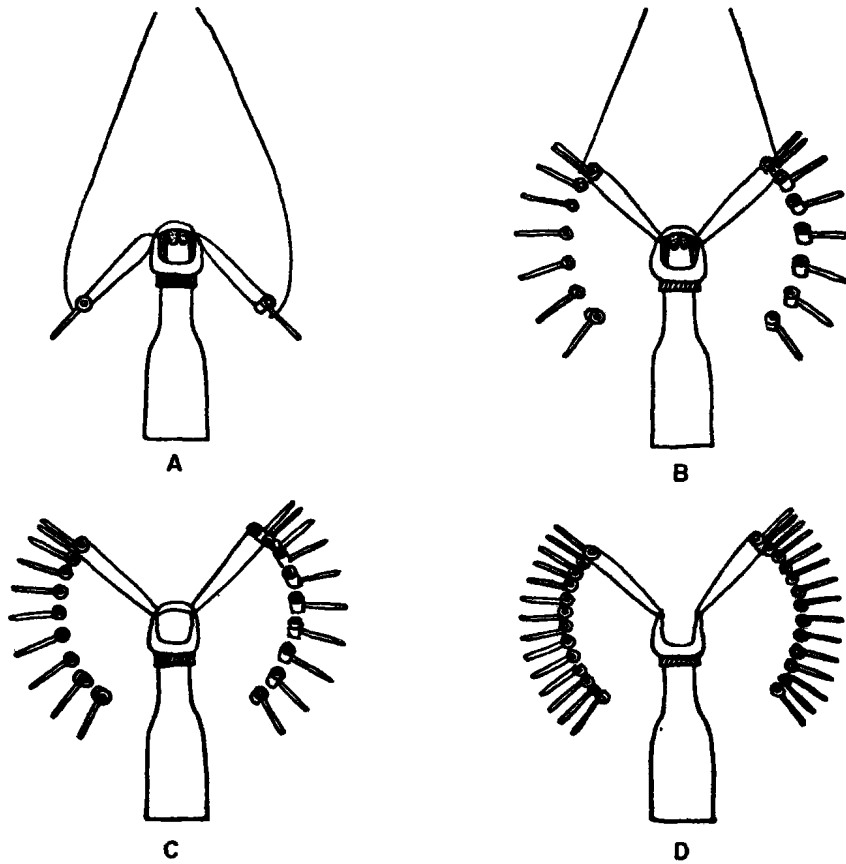

FIG. 1. The elastic recoil of the forewings in the isolated thorax of a desert locust, drawn from multiple-flash photographs. (A) Unstrained intact thorax; (B) recoil in intact thorax; (C) recoil after removal of the wing muscles; (D) recoil of the rubber-like wing-hinges. The wings are mutilated and provided with an artificial mass (from Weis-Fogh, 1961c).

transparent protein, *resilin*, with rubberlike properties (Weis-Fogh, 1960) and with a characteristic amino acid composition (Bailey and Weis-Fogh, 1961). In certain places it is present in pure isotropic form which is suitable for physical and chemical analyses without further purification.

In many respects it resembles *elastin* from vertebrates and may well be called "arthropod elastin" but, as will be shown, it differs from elastin in many important ways and lends itself more easily to studies of the rubberlike state in proteins, the number and nature of cross-links, and the formation of three-dimensional networks in biological structures.

II. Identification, Occurrence and Function

Resilin is present as an insoluble gel-like component in certain patches of the cuticle of insects and crayfish. In order to identify it we must have some criteria by means of which it can be characterized and distinguished from other members of these compound structures. In view of the detailed treatment in succeeding parts of this review, we shall only give a qualitative working definition here (based mainly on Weis-Fogh, 1960; Bailey and Weis-Fogh, 1961; Andersen, 1961, 1963; Elliott *et al.*, 1964).

A. WORKING DEFINITION

1. Both *in situ* and when dissected free, pure unstrained resilin appears as a mass of optically and mechanically isotropic protein. In the dry state it is hard and brittle and is insoluble in all solvents which do not break peptide bonds. In aqueous media and also in many anhydrous hydrophilic liquids it swells isotropically and reversibly; it then becomes rubbery and exhibits typically long-range deformability and complete elastic recovery. In water the swelling depends on pH, with minimum swelling about pH 4. It is devoid of colour, is transparent and shows no visible structure under the light microscope or electron microscope.

2. Swollen resilin becomes birefringent when strained, being positive in the direction of extension.

3. In water resilin stains with methylene blue and toluidine blue but shows no metachromasia. It stains red with the histological colour reactions of Masson and Mallory.

4. In ultraviolet light (UV-light), resilin fluoresces with a strong bluish colour with maximum intensity of about 420 mμ. The intensity increases in alkaline media.

5. Both *in situ* and when free, resilin is easily digested by all proteinases. After complete acid or basic hydrolysis it yields only amino acids, of which fifteen are ordinary amino acids (but no sulphur-containing ones and no tryptophan or hydroxyproline) and two are unusual and specific. The latter are responsible for the characteristic fluorescence both of native resilin and of the hydrolyzate.

6. So far resilin has only been found in specific parts of the cuticle in insects and crustacea, i.e. as extracellular deposits secreted by the epidermis. In certain structures it is secreted in a pure form but in most cases together with chitin and fibrous proteins.

When resilin is mixed with other substances, the bulk properties of the cuticle in question may of course differ appreciably from those of the pure material, but in most cases its presence can be established by means of simple tests, such as mechanical behaviour, strain birefringence, colour reactions, swelling and fluorescence. However, it is not safe to use only one or two of these tests.

B. RUBBERLIKE CUTICLE

Arthropod cuticle is an extracellular product of the single-layered epidermis. The thin epicuticle, the often hard and coloured exocuticle, the softer endocuticle and the flexible membranes are made up mainly of materials in the solid state of matter (chitin, lipoprotein, tanned or fibrous proteins, inorganic crystals; for reviews see Richards, 1958; Wigglesworth, 1957; Dennell, 1960). These products are therefore called solid cuticle, but besides water some soluble protein is often present. In contrast to this, La Greca (1947) described the thick wing-hinge ligaments in locusts as being highly elastic, transparent and without colour. An analysis of these and similar structures disclosed that in all cases the elasticity and the great deformability is due to the presence of a large amount of resilin (Weis-Fogh, 1960) and that swollen resilin is an almost ideal rubber in the physical sense of this word (Weis-Fogh, 1961a). Cuticle which shows long-range elasticity and a large content of resilin is therefore called *rubberlike cuticle* (Weis-Fogh, 1960). It is easy to demonstrate that the elasticity is caused by resilin since the structures become soft and flabby when treated with proteases and loose their swelling properties, fluorescence and strain birefringence.

1. *How to find rubberlike cuticle: a simple colour test*

The reasons why rubberlike cuticle was not discovered much earlier are undoubtedly due to the smallness of the patches, their transparency, lack of colour and softness—all of which remain unchanged after treatment with heat, alcohols and most other fixatives. However, it is easy to stain resilin almost selectively in fresh cuticle and then apply the other tests for the final identification.

A living insect or crayfish is killed by immersion in hot buffered water for a few minutes (pH 6·7, 95–100°C). After opening the body, the soft

TABLE I

Colour reactions of locust cuticle dyed in a mixture of 5 mg toluidine blue and 5 mg light green in 100 ml buffer solution (M/20) for 40 h and examined after 6 h washing in pure buffer solutions

Type of cuticle	pH 4·6	Colour at pH 6·1	pH 7·1
Rubberlike:			
Hinges and ligaments, wing system	Sapphire	Deep sapphire	Deep sapphire
Transitional:			
Clypeo-labral spring	Sky blue	Sapphire	Sapphire
Outer endocuticle of ocelli and ommatidia	Green-blue (faint)	No or faint green-blue	Faint sky blue
Inner endocuticle of hind margin of abdominal tergites	Blue	Blue	Blue
Tough ligaments:			
Wing system and mandible	Green	Green	Green, tinge of blue
Arthrodial membranes:			
Outer part	Green	Green	No
Inner part	Green (faint)	Green (faint)	No
Thin inner lamina	No or greenish	Purple	Purple
Tough inextensible tendons: (hardened)	Green	Green	Green
Sclerotized cuticle:			
Exocuticle	No or faint	No	No
Outer endocuticle	No or blue (faint)	No or blue (faint)	No or blue (faint)
Inner endocuticle	No or green	Green	Green
Most hairs and bristles:	Green or green-blue	Green or green-blue	Green

parts are removed and the cuticle freed from the epidermis (hypoderm) by means of a thin, strong jet of tap water. The rinsed cuticle is placed for 24–48 h at room temperature in dilute phosphate buffer (M/20, pH about 7) to which is added 5 mg toluidine blue (British Drug Houses) and 5 mg light green (E. Gurr) per 100 ml buffer. A crystal of thymol is

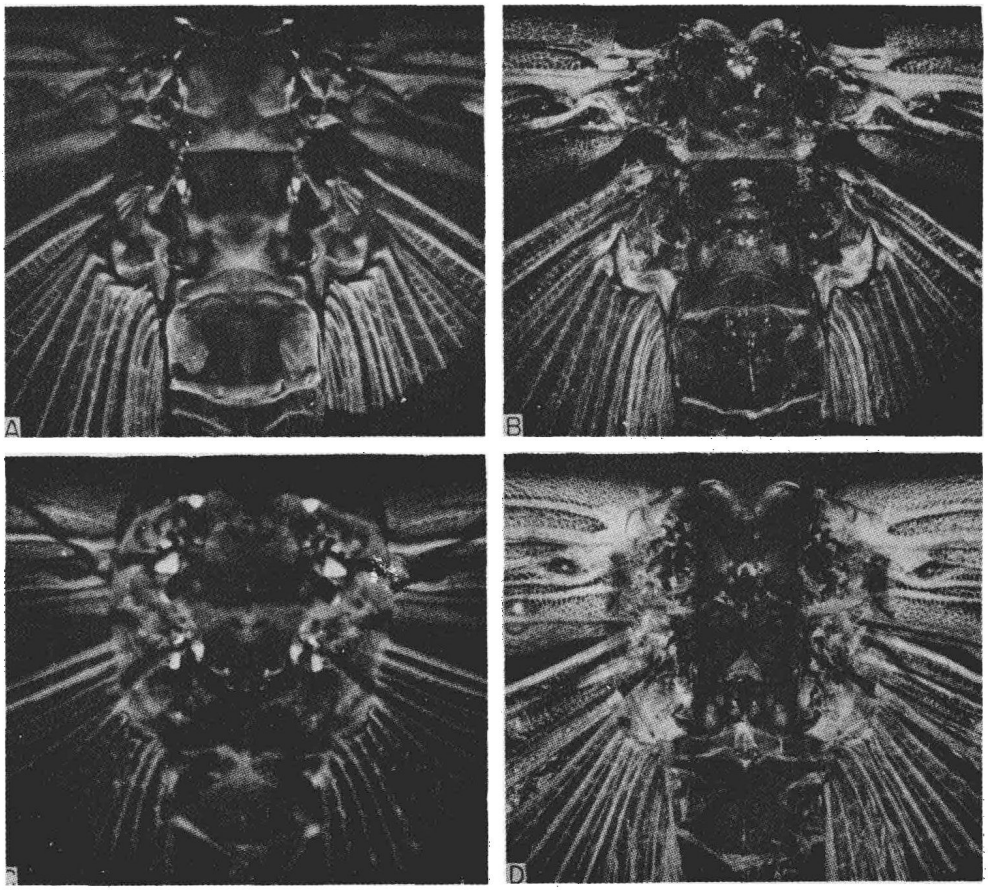

FIG. 2. The fluorescent rubberlike ligaments in the wing system of the desert locust, photographed in UV-light through a 420 mμ interference filter (A and C), as compared with ordinary light (B and D). The upper figures show the dorsal cuticle from the outside and the lower figures from the inside.

sufficient to prevent microbial growth. After rinsing in pure buffer for some hours, typical rubberlike cuticle appears as translucent, brilliant blue patches. Table I indicates that the staining reaction of locust cuticle

does not depend much on pH and that the typical sapphire blue colour at pH 6–7 is found only in patches where additional tests showed the presence of large amounts of resilin, i.e. in typical rubberlike cuticle where there is no cover of tanned exocuticle but only the thin epicuticle. The term *transitional cuticle* is used only tentatively. In the case of the clypeo-labral spring, which is described later, the thickened part fluoresces strongly and complies with all the tests for resilin, including the presence of the specific amino acids. The hind margins and the midline of the abdominal tergites also fluoresce, they stain blue and contain the two unusual amino acids. There is therefore no doubt about the presence of resilin in these parts. Some of the transparent endocuticle which covers the compound eyes and the ocelli stained faintly blue and showed some elasticity and the usual swelling behaviour, but since it did not fluoresce resilin may not be present or its fluorescence may be quenched by other materials.

Since the strong blue colour is confined to the patches which contain resilin, the method is reasonably selective but it should always be supplemented by additional tests. For anatomical work, it is sometimes convenient simply to use a mixture of glycerol and water 1 : 1 to which is added a few drops of a methylene blue solution (British Drug Houses) and a crystal of thymol. The rubberlike parts stain deep blue and the tough ligaments green while the rest remains unstained. The wide distribution of rubberlike pads and ligaments in the wing machinery and tergites of locusts, thus made visible, corresponds in detail to the picture obtained by photographing the untreated cuticle in UV-light through a 420 mμ interference filter (Fig. 2).

It is characteristic that resilin is stained selectively by the two basic dyes, methylene blue and toluidine blue (coloured cations), at least from pH 3 to pH 9·5, while basic fuchsin is useless and none of the acid dyes tested stained selectively if at all (coloured anions; acid fuchsin, eosin, light green).

It is obvious that resilin-containing cuticle is widespread in the locust but, before we discuss the occurrence of the protein in more detail, it is reasonable to describe the three test pieces upon which most of the analytical work has been done.

2. *Three test pieces*

As far as resilin is concerned, the simplest structure in Fig. 3 is the elastic tendon of dragonflies (C) and the most complicated is the wing-hinge (B). It is noticeable that the structures are readily deformed and snap back immediately to their original shape when unloaded. The

descriptions are based upon Weis-Fogh (1960), unless otherwise stated, and in each case the presence of resilin has been established by all methods available, including amino acid analyses (Bailey and Weis-Fogh, 1961; Andersen, 1963).

Elastic tendon. Most wing muscles in dragonflies run in the dorsal-ventral direction and are attached directly to the ventral wall. They insert on cuticular tendons, which are hollow air-filled invaginations from the dorsal cuticle, the so-called cap tendons. The elastic tendon for the

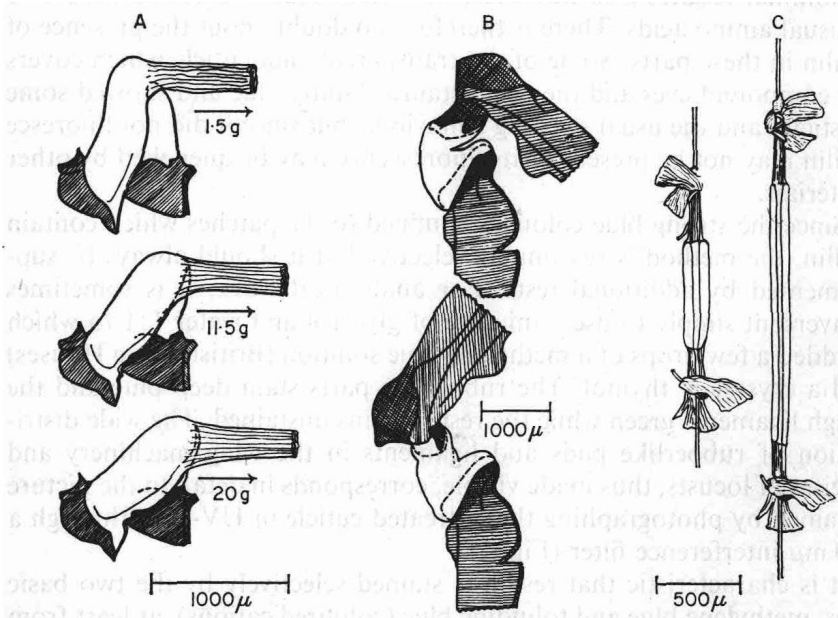

FIG. 3. The three test pieces used in most experiments. The prealar arm (A) and the main wing-hinge of the forewing (B) from the desert locust (*Schistocerca gregaria*). (C) The elastic tendon from the hindwing of a dragonfly (*Aeshna cyanea*). All the samples were placed in dilute buffer at pH 7 and are drawn both in the unstrained and in the strained state. (From Weis-Fogh, 1960.)

pleuro-subalar muscle (the third subalar) is no exception, but in contradistinction to the remaining tendons the middle part is swollen like a sausage and extremely extensible while both ends consist of the usual tough and almost inextensible cuticle (see Fig. 6). Being an invagination, the air-filled central canal is lined with a thin folded epicuticle and the periphery is covered by a single layer of the epidermal cells which formed the tendon, apparently in the course of the 3 days prior to the final moult (Neville, 1963a). The tough ends are composed mainly of chitin and

protein different from resilin and they resemble ordinary lamellate arthrodial cuticle (Fig. 4A). At the transitions between the tough and the elastic parts, resilin begins to appear in the form of amorphous masses between the other elements (B). A few microns nearer the swelling, the other proteins disappear and we are left with a bell-shaped concentric system of chitin lamellae separated by and glued together with isotropic

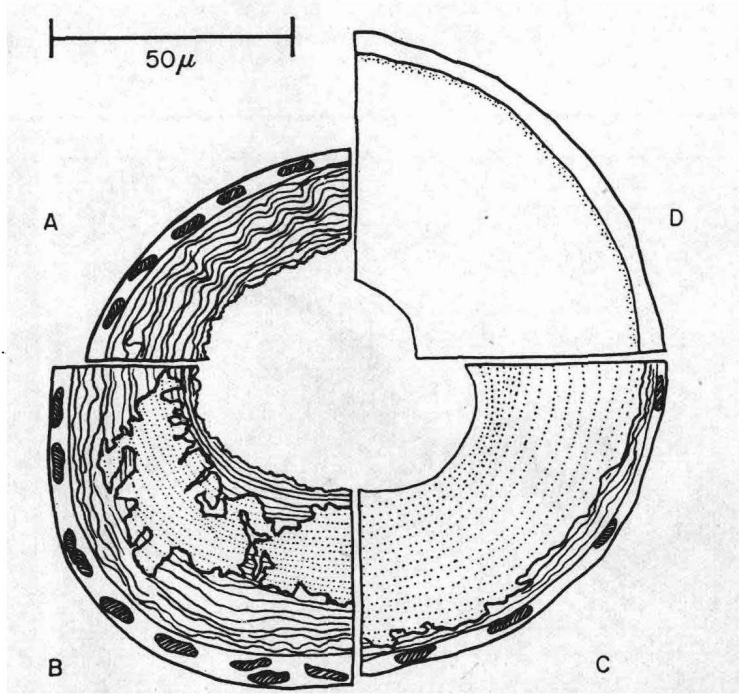

FIG. 4. Transverse paraffin sections (4 μ thick) of the dragonfly tendon, stained with Masson's triple stain in which resilin becomes red (here white). (A) Through tough tendon dorsal to the elastic part. (B) Transitional zone in which "flakes" of resilin appear. (C) Anchoring zone with resilin and chitin lamellae. (D) Pure resilin devoid of structure. The epidermal cells are indicated. (From Weis-Fogh, 1960.)

and structureless masses of resilin. This is the anchoring zone (C) and it is confined to the ends. The major part of the swelling consists of a cylindrical piece of pure resilin covered towards the central canal by a thin buckled epicuticle and by an equally thin but badly defined cover towards the peripheral cells (D). Neither of these membranes convey any mechanical strength to the tendon, as is easily seen when resilin is removed by digestion with trypsin or other proteases. When the cells are stripped