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An investigation of some matrix protein components critical to the extensible properties of insect cuticle

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Extensible abdominal cuticles from both nymphal *Rhodnius* and nymphal *Triatoma* are very similar in mechanical properties: ultimate tensile strength ~ 10 $\mathrm{MNm^{-2}}$, stiffness ~ 100 $\mathrm{MNm^{-2}}$ and ultimate strain ~ 15%. The cuticles are also similar in composition, minor differences found do not appear to be sufficient to allow different mechanical properties. Some proteins can be identified, tentatively, as having a contribution to the extensible properties of the cuticles. There appears to be no contribution to the change in extensibility by stress-softening.

1. Introduction

Insect cuticle is largely a two-phase composite of a polysaccharide fibrous element (chitin) embedded in an amorphous protein matrix. One of the many functions of this material is mechanical: it forms the external supportive structure of an insect. The mechanical properties of cuticle may be expected to depend upon the relative amounts, distribution and composition of each phase. Studies of unsclerotized, (i.e. not containing any cross-linked components and having an extractable matrix) extensible cuticle from different insects have tended to emphasize the role of the protein composition of the matrix in the mechanical properties of these materials [1, 2]. Protein composition seems to vary considerably from cuticle to cuticle, whereas the fibrous phase of most cuticles consists of 2.8 nm diameter fibrils of chitin that appear for practical purposes to be physically and biochemically identical [3]. The orientation of fibrils in a majority of cuticles is also similar. Sheets of parallel fibres lie parallel to the surface of the cuticle, the fibre axis in each sheet being regularly displaced from those above and below it by a small angle. This arrangement is known as helicoidal cuticle (see Fig. 1). Such a structure was first described and explained, for crustacean cuticle, by Bouligand [5] and was subsequently shown to occur in many other animal integuments.

If it is true that the composition of the matrix is important in determining the mechanical properties of the composite, care must be taken when exploring this relationship to ensure that other variables in the system are not confusing the issue. For example, Hackman [1] compares the protein composition of extensible cuticles from the bloodsucking bug *Rhodnius* to that of the tick *Boophilus,* both of which stretch their abdominal cuticles greatly when feeding as they take a blood meal many times their unfed body weight. He concludes that both animals have arrived at the same solution to the problem of producing a cuticle that will stretch. However, this comparison is highly confused by the fact that (a) the two cuticles have different chitin fibre contents (11.2% by weight for *Rhodnius*, 3.8% by weight for *Boophilus*), a factor which would be expected to greatly affect mechanical behaviour, and that (b) their feeding styles are dissimilar; they feed at very different rates. This last point is an important factor because these materials are viscoelastic.

In this paper an attempt is made to resolve the question of just how closely matrix protein composition is correlated with mechanical properties by examining the cuticle of two very similar bugs,

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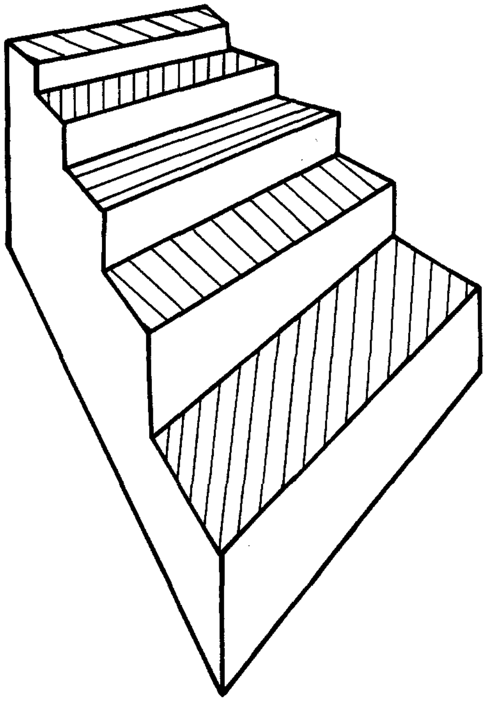

Figure 1 Schematic representation of the helicoidal arrangement of cuticle. The orientation of the parallel chitin fibres in any one lamina is drawn in on the top surface of each lamina. After Gubb [4].

Rhodnius and *Triatoma*. These two insects both have a helicoidal cuticular structure [6] and the chitin contents of the cuticles are very similar (see Section 3). Their feeding techniques are similar in manner and rate. Thus other factors of cuticle composition can be neglected and the relationship between mechanical properties and protein composition of the matrix may be studied.

Insect cuticle is secreted by an epidermis which maintains active control of the cuticle composition and mechanical properties. Following appropriate stimulation e.g. starting feeding, the extensible cuticle of *Rhodnius* is plasticized probably by a reduction in pH controlled by the epidermis [7, 8]. A reduction in stiffness from 10^8 to 10^7Nm^{-2} occurs to allow a rapid stretch of the cuticle to accommodate a blood meal ten times the unfed weight of the insect in 15 min. Without plasticization a similar cuticular strain may be achieved but requires an additional order of magnitude of time. As these insects are ectoparasites it is advantageous to feed quickly before being discovered and dislodged or even killed.

Mechanical tests described in this paper aim to show how similar *Triatoma* cuticle is to *Rhodnius* cuticle in tensile properties, and evidence has been gathered to show that similar strains and strain rates are achieved by a similar plasticization. Additional cyclic loading tests are designed to show that other effects, such as the stress-softening phenomenon which facilitates high extensibility in the locust intersegmental membrane [2], do not contribute to the change in extensibility of *Triatoma* cuticle. Similarities in protein constituents of the two cuticles are examined by gel-electrophoresis and correlated with similarities in mechanical function.

2. Materials and methods

Laboratory cultures of *Rhodnius prolixus* Stål and *Triatoma phyllosoma pallidipennis* Stål were maintained as described by Hillerton [9]. Unfed fifth instar *Rhodnius* and third and fifth instar *Triatoma* were used. The abdomens of fifth instar *Rhodnius* and the third instar *Triatoma* are of similar size and the cuticles are of a comparable thickness so making mechanical comparisons easier. The cuticle was dissected and muscle and epidermis scraped off, cuticle was then rinsed in distilled water to remove the remaining debris and the samples were then tested in saline (1% NaCl) or in liquid paraffin. Plasticization was induced by the injection of 5-hydroxytryptamine (5HT) as described by Reynolds [10].

Load extension tests on abdominal cuticle from fifth instar *Rhodnius* and on fifth instar *Triatoma* were carried out as described by Hillerton [11] where indicated in the results tables. Further tests on third instar *Triatoma* cuticle were performed on parallel-sided strips of cuticle stuck by cyanoacrylate glue to the stirrups of the load–extension machine described by Vincent [2]. The width of each strip was measured with calipers and its thickness with a screw-gauge micrometer. The stirrups were set at a constant displacement before glueing each specimen, resulting in a gauge length of 2.5 mm. An average specimen was 1 to 2 mm wide and 60 to 65 μm thick; a constant extension rate of 15.9 mm min^{-1} was used. The *limit Youngs modulus, E*, which is the stiffness or elasticity of the cuticle, was calculated from the gradient of the load–extension curve in its upper reaches where the relationship between load and extension is approximately linear. Ultimate tensile strength (*UTS*) was calculated from the maximum sustainable load and ultimate percentate elongation

TABLE I Mechanical properties of abdominal tergites from *Rhodnius* and *Triatoma* (mean ± 1 standard error)

Insect	*UTS* (MNm^{-2})	*E* (MNm^{-2})	*UPE* (%)	Number of samples (*n*)
Rhodnius	13.1 ± 0.4*	111 ± 12*	12.4 ± 0.9†	8
Triatoma‡	12.3 ± 0.5	137 ± 5.7	8.1 ± 0.9	5
Triatoma§	5.4 ± 1.07	67.4 ± 10.5	29.6 ± 3.2	6

*From Hillerton [10].
†Calculated from Hillerton [10].
‡Determined by the method of Hillerton [10].
§Determined by the method described here.

(*UPE*) was taken from the extension at which the maximum load occurred.

For cyclic testing, tests were carried out on *Triatoma* cuticle as described above except that load and extensions were limited to the regions where the material is elastic, i.e. where full recovery occurred when the load was removed. Ten loading–unloading cycles were performed on each specimen and the limit Youngs modulus in the loading protion of each cycle noted.

To determine the extension of cuticle occuring during feeding *Triatoma* nymphs were fed on the ears of half-lop rabbits [9]. Prior to feeding each insect was weighed and the dorsal surface of the abdomen photographed with a scale. Immediately after feeding and before defaecation each insect was reweighed and rephotographed. The circumferential and longitudinal strains in the abdomen brought about by feeding were estimated from the photographs. The change in distance between two muscle insertion sites on either side of the mid-dorsal line was used for circumferential strain and two muscle insertion sites along the mid-dorsal lines were for longitudinal strain. These plane-projected measurements of distances on a surface that is initially flat but becomes curved on feeding are subject to some error, but as the muscle insertion sites are close together and as most curvature is near the lateral margins these errors are (probably) quite small.

Cuticle composition was determined and polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS), which separates according to molecular weight, was performed exactly as described by Hillerton [9].

3. Results

The mechanical properties of the extensible abdominal cuticle of nymphal *Rhodnius* appear to be very similar to those of the same cuticle from *Triatoma* (Table I). The differences shown, although well within the range of experimental errors found in testing most biological materials, may also be ascribed to the different test methods used. The third and fifth instar *Triatoma* appeared very similar in mechanical properties even though different test methods were used.

Triatoma extensible cuticle, just like *Rhodnius* extensible cuticle, can be plasticized by 5-hydroxytryptamine (Table II). The changes found in the stiffness and the ultimate percentage elongation between the two species are very similar. It can be inferred that *Triatoma* nymphs also plasticize their cuticle during feeding from the observation that the maximum strain observed in the feeding experiments (Fig. 2) was far greater than that possible in the mechanical tests on unplasticized cuticle (Table I). The maximum strain measured in *Triatoma* cuticle as a result of feeding was 75% (Fig. 2) compared with a possible maximum of 100% determined by mechanical tests on artificially plasticized cuticle. Cyclic loading tests on *Triatoma* cuticle are shown in Fig. 3. Fig. 4 shows the stiffness in the upper

TABLE II Effect of 5-hydroxytryptamine (5HT) on the machanical properties of the abdominal tergites from *Rhodnius* and *Triatoma* (mean ± 1 standard error). Determined by the method of Hillerton [10]

Insect	*UTS* (MNm^{-2})	*E* (MNm^{-2})	*UPE* (%)	Number of samples (*n*)
Rhodnius control	13.1 ± 0.4*	111 ± 12*	11.8 ± 1.7	8
5HT plasticized	14.3 ± 2.2*	0.5 ± 0.7*	100*	6
Triatoma control	12.3 ± 0.5	137 ± 5.7	8.1 ± 0.9	5
5HT plasticized	9.5 ± 1.4	6.4 ± 0.3	96 ± 12	3

*From Hillerton [10].

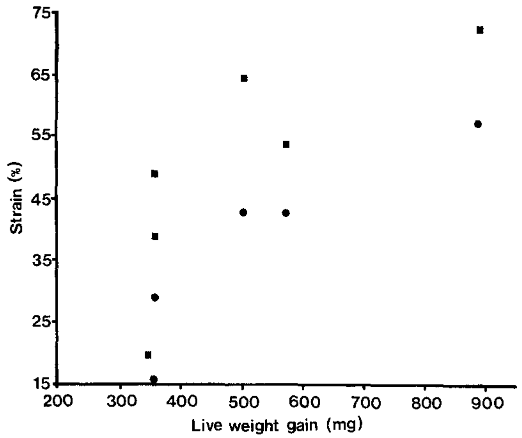

Figure 2 Longitudinal (■) and circumferential (●) strain in the abdominal tergites of *Triatoma* upon feeding. Each point represents one insect.

portion of each loading cycle of a similar test. The plateau region of this curve indicates that no stress-softening occurs as the same stress is required to achieve a given strain in subsequent cycles. There is no apparent difference between the behaviour of cuticle stretched circumferentially as opposed to longitudinally.

Rhodnius extensible cuticle has been found to contain 11.5% (dry wt) of chitin and *Triatoma* extensible cuticle 11.6% (dry wt) of chitin. Extraction of cuticle with 8 M urea removed 72.0% (dry wt) from *Rhodnius* cuticle and 71.4% (dry wt) from *Triatoma* cuticle.

The urea extracted proteins from *Rhodnius* and *Triatoma* when separated by SDS PAGE appear to have some similarities and some differences although it is difficult to resolve these simply by comparing electrophoretograms (Fig. 5). However, running a mixture of equal proportions of the *Rhodnius* and *Triatoma* extracts alongside the separate fractions allows a more detailed comparison to be made. If the proteins were totally different the number of bands found would probably be the sum of those in the two separate fractions. This is not so and several of the bands from the two insects merge and run as one (Fig. 5). There are some concentration differences and some bands are absent from one or other of the insects. From Fig. 5 it may be seen specifically that *Triatoma* has little of the protein MW 78 000 (Band a) found in *Rhodinius* but has a much higher concentration of proteins MW 68 000 (Band b) and 58 000 (Band c). Protein MW 51 000 (Band d) found in *Rhodnius* is absent from *Triatoma* extracts. There is a much higher concentration of protein MW 46 000 (Band e) in *Triatoma* than in *Rhodnius* and the protein MW 41 500 (Band f) found in *Triatoma* is absent from *Rhodnius* samples. The rest of the bands appear to be common to both species.

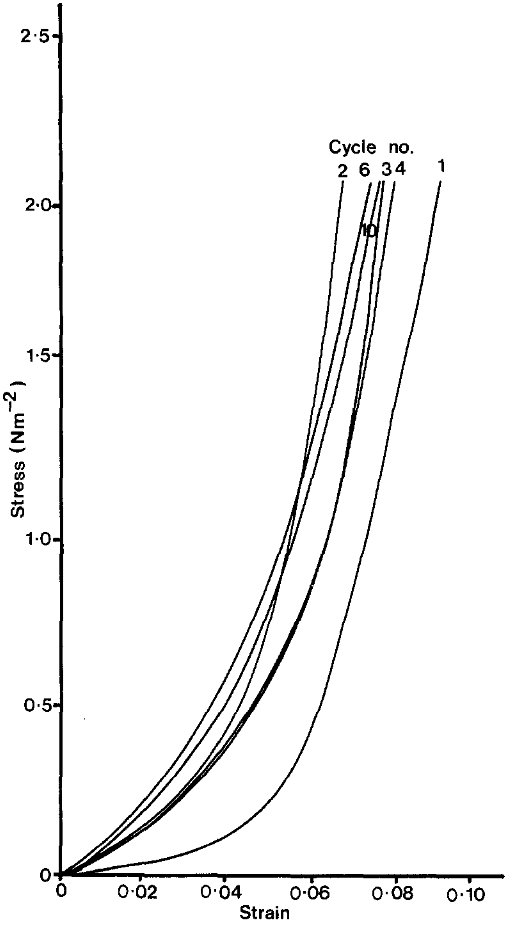

Figure 3 Loading portions of typical cyclic stress–strain curves for the abdominal tergites of *Triatoma*.

4. Discussion

The major mechanical properties of *Rhodnius* extensible cuticle have been reported previously [10, 11] and the results from here are similar. The results determined for homologous cuticle from *Triatoma* are also very comparable. The only measurements showing a significant difference are the ultimate percentage elongations for the two instars of *Triatoma* tested; this may result from the very different strain rates used. The method described above used a strain rate ten times lower than that used for the other tests and so creep may contribute in increasing the breaking strain determined in these experiments as this cuticle is

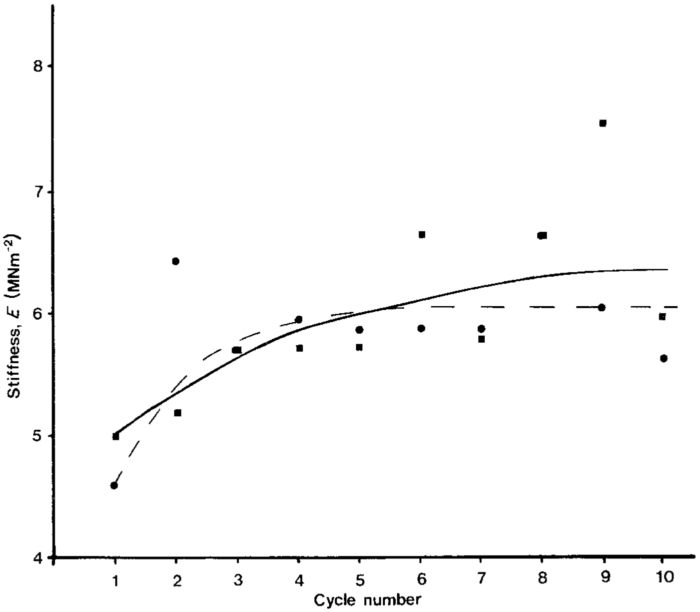

Figure 4 Stiffness against load–extension cycle number for the abdominal tergites of *Triatoma*. ●-----● longitudinal samples, ■-----■ circumferential samples. This is a typical result.

viscoelastic [12]. The similarity of extensible cuticle from the related bugs is further enhanced by the finding that they are both plasticized by the same mechanism.

Bennett-Clark [13] found that *Rhodnius* abdominal cuticle rarely, if ever, reaches its maximum sustainable strain during feeding. It was found here that *Triatoma* does not approach the maximum possible strain in the cuticle either.

The extension of the abdominal cuticle of these bugs which occurs to accommodate the blood meal is facilitated by plasticization. Another pliant cuticle, the abdominal intersegmental membrane of the female locust, is greatly extended during oviposition and in this case the cuticle displays stress-softening [2]. Vincent [14] considered that the composition of the locust intersegmental membrane was advantageous for stress-softening as it

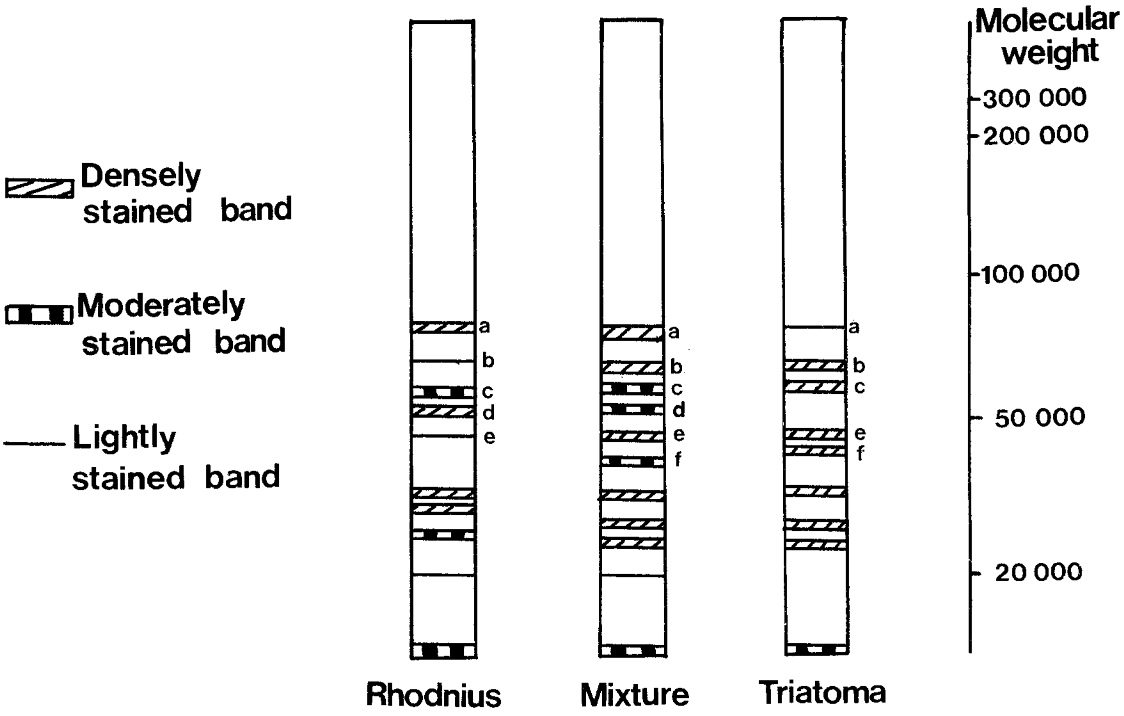

Figure 5 SDS electrophoretograms of urea extractable proteins from *Rhodnius* and *Triatoma* abdominal cuticle.

has a high chitin content and there is some primary cross-linking forming a loose but entangled network. He also suggested that in other extensible cuticles, with lower chitin content, stress-softening would be less likely to occur. The extensible cuticles of *Rhodnius* (and *Triatoma* by inference) might be expected to show some stress-softening even though it has a low chitin content, no apparent covalent cross-linking [9] and shows much reduced secondary bonding when plasticized [12]. However stress–strain curves produced for *Triatoma* cuticle cycled at a constant strain rate showed no detectable hysteresis and so stress-softening does not appear to be a major contribution to the change in extensibility of the cuticle during feeding. The cyclic loading experiments also showed that there is no significant difference in the mechanical properties of the cuticle when tested circumferentially as opposed to longitudinally which confirms the identical values for stiffness found for the two orientations in *Rhodnius* cuticle by Hillerton [11]. The extensible cuticles are isotropic in the plane of the cuticle.

As well as being similar in mechanical properties the two cuticles are extremely similar in composition, chitin content and protein extractability. The only differences occur between a few of the proteins. These are concentration differences except for the direct swap of the protein MW 51 000 in *Rhodnius* for that of MW 41 500 in *Triatoma*. At the simplest level it would appear that these differences are of no consequence to the mechanical properties of the cuticle. Perhaps it is not differences which should be considered but, as the mechanical properties are so similar, the ways in which the compositions are similar. They have the same chitin content and the ultrastructure of the two cuticles is similar (Hillerton, [6]) and a similar proportion of extractable protein is found. This indicates that they have approximately the same amount of secondary bonding in the protein matrix which can be manipulated during stretch of the cuticle and that, as it has been shown that the amount of protein matrix in *Rhodnius* extensible cuticle affects the ultimate percentage elongation [11], the mechanical response during feeding should be similar. The matrix of insect cuticle consists of a great many proteins, as demonstrated by Hillerton [9] for *Rhodnius* cuticle. The cuticle also has many functions other than mechanical ones, and it is therefore reasonable to assume that only a few of the proteins will be crucial to the small range of mechanical properties tested here. Which of the proteins found in each cuticle are responsible for their similar mechanical behaviour? It would be tempting to assume that those proteins found to be the same or very similar in these two cuticles were those that determined their observed mechanical similarities. However, these two insects are very closely related and it may be expected that their cuticles contain identical proteins which confer similar non-mechanical properties on them, or even that they contain similar non-functional proteins for common evolutionary reasons. Fortunately there is another piece of evidence which allows us to decide which of the proteins common to both cuticles are likely to have mechanical significance. Hillerton [15] found that a fraction of cuticular protein in both *Rhodnius* and *Triatoma* has a negative temperature coefficient of solubility. This change in solubility is similar to that shown by many other structural proteins such as tubulin, actin and various virus coats. The implication is that this change in solubility upon the supply of energy is a gross manifestation of changes in secondary bonding which would allow plasticization and its reverse to occur. This specific characteristic of these proteins identify them as being significant to the mechanical behaviour of these cuticles. In Fig. 5 the protein Bands b, h and k (which are common to both cuticles) have negative coefficients of solubility and can therefore be identified as those protein elements conferring similar mechanical properties on the two cuticles.

In conclusion, *Rhodnius* and *Triatoma* nymphal abdominal cuticles appear to be very similar in both composition and mechanical properties which might be expected because of their similar method of feeding and close phyllogenetic relationship. It appears that these similarities might be due to conservatism in the cuticle matrix proteins of the cuticle.

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