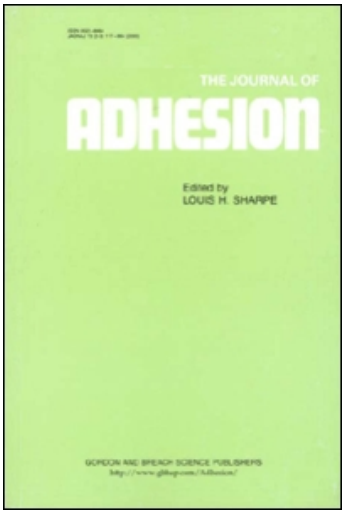


**The Journal of Adhesion**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713453635

# If it's Tanned it Must be Dry: A Critique

Julian F. V. Vincent[a]
[a] Centre for Biomimetic and Natural Technologies, The University, Bath, UK

Online publication date: 12 November 2009

**To cite this Article** Vincent, Julian F. V.(2009) 'If it's Tanned it Must be Dry: A Critique', The Journal of Adhesion, 85: 11, 755 — 769
**To link to this Article: DOI:** 10.1080/00218460903291296
**URL:** http://dx.doi.org/10.1080/00218460903291296

*The Journal of Adhesion*, 85:755–769, 2009

ISSN: 0021-8464 print/1545-5823 online
DOI: 10.1080/00218460903291296

Taylor & Francis
Taylor & Francis Group

# If it's Tanned it Must be Dry: A Critique

**Julian F. V. Vincent**
Centre for Biomimetic and Natural Technologies,
The University, Bath, UK

*The mechanism of phenolic tanning of insect cuticle, and other extracellular protein structures such as byssus and perisarc, has been supposed to be due to specific covalent cross links. Yet cuticle can be swollen in a strong H-bond breaker, an observation that instantly and irrevocably disproves this theory. Physicochemical expulsion of water is a much more robust mechanism which accords more closely with experimental observation, and suggests stabilisation mechanisms which would give advantages for technical composite materials.*

**Keywords:** Hyrdrogen bonding; Hyrophobicity; Insect cuticle; Sclerotisation; Stiffness; Water plasticisation



## INTRODUCTION

I can't remember when I first met Herb Waite, but I already knew about the phenolic tanning processes of mussel byssus and other materials molluscan, since I had been interested in insects and the materials of which they are made since the age of 6, and did my Ph.D. (some years later!) on the hormonal control of tanning of locust cuticle. It was natural, therefore, that I, with an interest in the engineering side of nature, should be interested in the mechanical correlates of the "hardening and darkening" of insect cuticle. The story that unfolds highlights the difficulties that can be generated when the mechanical properties of a system are inferred from their biochemistry. As far as I am concerned the matter is still not resolved: chemistry is not a sufficient science for the prediction of mechanical

Received 2 October 2008; in final form 15 April 2009.

One of a Collection of papers honouring J. Herbert Waite, the recipient in February 2009 of *The Adhesion Society Award for Excellence in Adhesion Science, Sponsored by 3 M.*

Address correspondence to Julian F. V. Vincent, Centre for Biomimetics and Natural Technologies, The University, Bath BA2 7AY, UK. E-mail: j.f.v.vincent@bath.ac.uk

properties. Is the mussel byssus thread stabilised by DOPA-derived cross-links, or is the DOPA driving the water out? And what part does the protein matrix play (it isn't inert)? Herb and his students have shown that there is commercial interest in the outcome of the processes involved, but until sufficient understanding of the basic mechanism has been acquired, its control will remain unsteady and the technology unreliable.

## BIOCHEMICAL BACKGROUND

In the early part of the last century, the understanding of the process of stiffening insect cuticle was confused by the presence of chitin, and was called "chitinisation" in the absence of any knowledge of the biochemistry of the process. Pryor showed that the oötheca of the cockroach, *Periplaneta americana*, also stiffened and went brown (the two main characteristics of chitinisation) despite the absence of chitin [1]. He showed that the prime mover of this process is the introduction of dihydroxyphenols. His model was based on the tanning of leather by benzoquinone; in 1940 it was accepted that benzoquinone tans by linking, covalently, two adjacent protein chains. Some of the effect of tanning is due to polymerised complexes of tanning agents formed within the matrix which then dehydrate the matrix. Pryor showed that this can happen to gelatin, a cube of which he left in concentrated benzoquinone for 2 months and which shrank to about half its original size and became as hard as wood. The reason for thinking that benzoquinone was cross-linking the proteins was that it was not polymerising very extensively and so, Pryor thought, presumably exerted its major effect by covalent cross-links.

About 6 months after Pryor's paper was published, Fraenkel and Rudall proposed that since the most dramatic change in the tanning blowfly (*Calliphora vomitoria*) puparium (another common model system, chosen because of cheapness, ease of timing, and lack of growth of the cuticle) is the loss of water (Fig. 1: water content down from 70% [0.41 g/g] to 40% [0.29 g/g] as a chemically driven process; down to 12% [0.11 g/g] as physical evaporation) then it is the most likely to affect stiffness of the cuticle [2]. The fact that dry material is stiffer than the same material when wet is common experience, but the relative magnitude of dehydration and supposed quinone cross-links in stiffening insect cuticle was estimated, not measured. But the consequences of dehydration on the protein had not been adequately considered. Fraenkel and Rudall considered that the dehydration leads to the closer packing of the components of the puparium leading to a "mixed crystallisation of chitin and protein". Referring

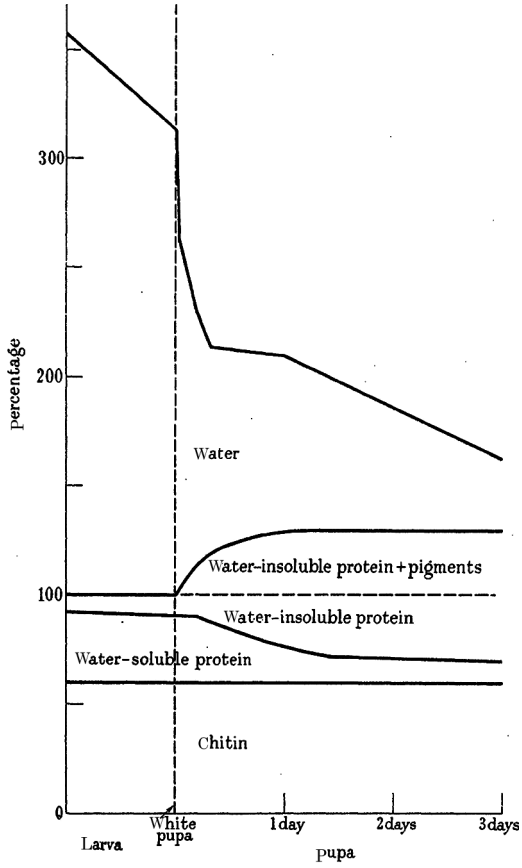


**FIGURE 1** The significant water loss measured by Fraenkel and Rudall, from Ref. [2] with permission.

to Pryor's paper in an Appendix added in proof they pointed out that "the introduction of aromatic cross-linkages in the protein . . . would help to explain the mechanism (of stiffening) but for a complete understanding . . . it is necessary to consider also other factors that are associated with the process" presumably meaning changes in water content.

## A MECHANICAL APPROACH

In the late 1970s I started to look at the tanning of insect cuticle since I was interested in the physico-chemical changes that were taking place, most particularly the mechanical changes and consequences, none of

which had been measured properly. I started these experiments in the expectation that Pryor was right (he had been my tutor in college, after all), and that my study would be more confirmatory than challenging. However, the closer I looked at the phenolic cross-linking theory, the weaker it seemed. My very simple experiments were punching big holes in the commonly accepted stories [3].

The most widely used of the experiments purporting to show that covalent cross-links exist in insect cuticle was insolubility of the cuticle proteins in any solvent less disruptive than 1 N NaOH at 100°C. What was never explained by those who used this criterion is how the chitin, itself "only" hydrogen bonded [4], could resist this treatment. Clearly experiments that use a series of solvents to distinguish bond types were not taking into account any co-operative effects that can effectively shield bonds from the action of solvents. Additionally, as well as removing proteins, 1,2-diaminoethane (a breaker of H-bonds) removes a considerable proportion of the brown colour from *C. vomitoria* puparial and *P. americana* oöthecal cuticles, suggesting that a large proportion of the "tanning" agent is not covalently linked to the proteins. These extractions were carried out under the most completely anhydrous conditions possible to prevent hydrolysis of covalent bonds [5]. The results from extracting cuticles with various solvents raised doubts as to how much of the cuticular matrix is stabilised by covalent cross-links; insolubility in a mild reagent is not evidence for covalent cross-linking, since the bonds may simply be hidden within a cooperative structure that is "only" H-bonded. When it comes to stiffness it is the *global* bond energy that is important, and a few H-bonds are a mechanical match for a covalent bond.

Even more destructive of the theory of covalent cross-linking is that when sclerotised cuticle from the metathoracic femur of the locust, *Locusta migratoria migratorioides*, is swollen in an effective protein solvator, such as pure formic acid, it swells reversibly without much protein being extracted [6], and the swollen cuticular pieces have long-range elasticity, indicating that the protein chains have considerable kinetic freedom. In the theory of cross-linking of macromolecules, the state of solvation or swelling obtained when any cross-links were formed dictates the degree of swelling that can be obtained when the material is at equilibrium with a swelling medium [7]. Therefore, if a material is cross-linked when it is swollen, as biochemists say must happen when the soft hydrated cuticle begins to be sclerotised, those cross-links cannot restrict the entry of free solvent at a later time, let alone cause solvent to be expelled. Therefore, it is not the phenolic cross-links that restrict the entry of water but secondary, non-covalent, links formed when the water between the proteins is removed. It

is, therefore, not possible for covalent cross-linking on its own to provide the stiffness observed. This is the main argument against all authors who show that covalent cross-linking is significant since even the most sophisticated biochemistry [8] says nothing about the mechanical consequences of any cross-linking which is observed. And the intimate biochemistry is done in relatively dilute solution, a very different environment from the near-solid state of the cuticle matrix. This is understandable, since the stabilised proteins are difficult to extract from cuticle, and you cannot be sure that what you have in solution is a reasonable representation of what is present in the cuticle.

Why, then, do they go on doing it, these biochemists? The reason is probably that the phenolics are reactive so that elegant biochemistry is possible. By contrast, experiments on the water content of the cuticle and its control are difficult to perform. Pryor's model gained almost universal acceptance largely because it is easy to manipulate with available techniques. It is clear that none of the biochemists so far mentioned had measured any mechanical properties, yet they discussed them with ease. The basic problem was that in all the cuticles investigated the two processes of quinone tanning and dehydration occur together: it is a classic case of having two variables in a single experiment. The proper measurement was obviously to compare changes in stiffness, mechanically measured, as phenolic tanning and/or dehydration increased.



## WATER IN DETAIL

In the locust, *L. m. migratorioides*, the water content of the cuticle of the metathoracic femur drops during the first 6 h of sclerotisation from 43% [0.19 g/g] to 32% [0.16 g/g] [6]. In 1981, Andersen said that although this degree of dehydration may contribute to the stabilisation of cuticle, it could not account for the observed differences. He thought that the properties of mature cuticle could best be explained by the assumption (*sic*) that covalent cross-links are present between protein molecules. However, in a later review [9] he said, using the same data from the same paper, that the differences in mechanical properties were mainly due to the loss of cuticular water and the incorporation of aromatic residues and cross-links in the proteins during sclerotisation. The short-range elasticity may mainly be due to the flexibility of the protein molecules, as they can hardly move relative to each other, due to the dehydration and the aromatic cross-links and filling material between the proteins. In other words, he had finally accepted that a relatively small reduction of water content

could cause a large change in stiffness, typical (diagnostic, even) of a hydrogen-bonded material [10]. This had already been shown to occur in insect cuticle [11] where a small increase in water content (from 26% [0.15 g/g] to 31% [0.16 g/g]) in the extensible abdominal cuticle of larval *Rhodnius prolixus* is associated with a drop in stiffness from 62 to 2.5 MPa. In this insect the driver is most probably a drop in pH that increases the water-binding by the charged groups on the cuticular proteins.

Is there any evidence that phenols are hydrophobic? In tea and wine the astringency and dry "mouthfeel" are largely due to polyphenols by their interactions with proline-rich proteins in the saliva. Astringency arises from precipitation of polyphenol peptide complexes. Intermolecular binding is dominated by the stacking of polyphenolic rings onto planar hydrophobic surfaces and is strengthened by multiple cooperative binding of polyphenolic rings and peptides becoming increasingly coated with polyphenol. When the coating is sufficiently extensive to provide cooperative polyphenol bridges, the peptides precipitate, probably as a phase separation process [12]. This may be akin to the cross-linking and dehydration mechanism in cuticle.



Water is not just "water". Mostly we appreciate it in the bulk. When it interacts with a substrate it can form different structures depending on the substrate and how much water there is already present. In simple terms, the most tightly bound water is present as a nonfreezable monolayer (the Langmuir or BET monolayer), the next most free is "absorbed" water, and the most free is "capillary" water. The expectation [10] is that stiffness increases as the unbound water disappears; as a corollary, plasticisation needs only a monolayer of free water for effective lubrication between the layers of bound water, so that a change of a relatively small amount of water can induce a large change in stiffness.

Using differential scanning calorimetry, the amount of nonfreezable water (measured by weighing) in maggot cuticle, tanned or untanned, is shown to be $0.375 \pm 0.02$ g/g of the protein [13], although the amounts of water in the other compartments vary according to the degree of tanning (Fig. 2). NMR experiments gave the same result for the amount of non-freezable water [13]. These experiments suggested that whilst tanning reduces the water-holding capacity of the cuticle it does not affect the sites to which the water would bind strongly. Wetting-and-drying experiments of tanned and untanned cuticles confirmed this—tanning reduces the amount of water that can be absorbed, but so does simply drying in the air, and the amount of water retained in "air-dried" cuticle is the same in both cases [13]. Simple calculations then showed that each amino acid residue in

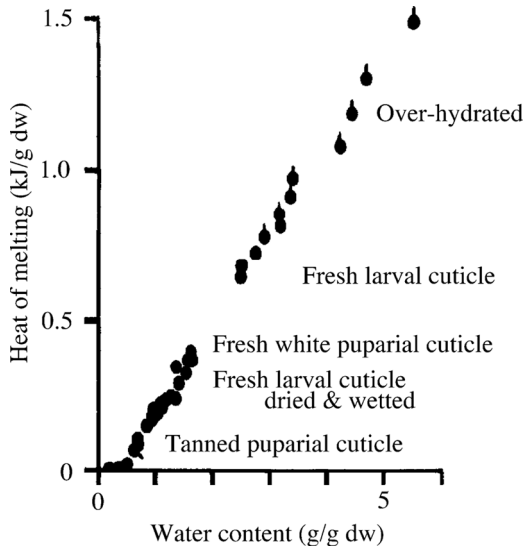


**FIGURE 2** The amount of freezable water in maggot and puparial cuticle of *Calliphora* prepared in various ways. From Vincent and Ablett, Ref. [13], with permission.



*C. vomitoria* maggot cuticle has, on average, four water molecules strongly associated with it. The peptide link has been shown to associate 1.5 molecules of water [14] and a maximum of two to three water molecules per side chain is reasonable [15], which gives a total amount of water which is acceptably close to that observed. So the 0.375 g/g represents the BET monolayer.

The sites most commonly favoured for covalent links in blowfly puparial cuticle are lysine and histidine [16]. These two residues account for about 22% of the total water associated with amino groups, which is about one-eighth of the total water associated with the cuticle. This water would be displaced by the formation of covalent cross-links, leading to a predictable decrease in the amount of unfreezable water. In experimental terms, this would be about 0.05 g/g of water. This is outside the observational error of Vincent and Ablett [13], suggesting that these groups are either not masked (*i.e.*, are not involved in cross-links after all) or that the cross-linking phenolics are binding as much water as they are displacing, which is unlikely since phenolics contain at the most a single free amino group and are, in general, hydrophobic. So are lysine and histidine *really* interacting with the phenols under the conditions within the cuticle?

## DOES "STIFF" IMPLY "DRY?"

That the stiffness of hydrated materials increases when they dry is irrefutable; the questions are by how much do they increase, and what is the proportionality of stiffness to water content? A simple experiment measuring the tensile stiffness of tanned (puparial) and untanned (maggot) *C. vomitoria* cuticle, dry, gave the figures of $3.05 \pm 0.49$ and $2.2 \pm 0.41$ GPa (means $\pm$ standard error; no significant differences), respectively [3]. However, the chitin in the naturally tanned cuticle is orientated in a preferred direction parallel to the direction of stretching, whereas the maggot cuticle has the chitin effectively randomly orientated. Thus, the naturally tanned material would be expected to be stiffer when measured in tension, although we had found no difference. These experiments were crude.

A more sophisticated set of experiments was performed using a torsional pendulum [17] which measures the shear modulus, $G$, rather than the Young's modulus, $E$, and so is less dependent on the orientation of the chitin. This approach has the advantages that displacements are small and, therefore, linear—the specimen can have its experimental conditions changed, so that direct comparisons are possible; the stiffness of a single specimen can be followed as its water content changes.

A strip of *C. vomitoria* maggot cuticle was glued into a freely-oscillating torsional pendulum suspended by a tungsten wire about 30 cm long and 60 μm diameter (Fig. 3). A similar piece of cuticle was

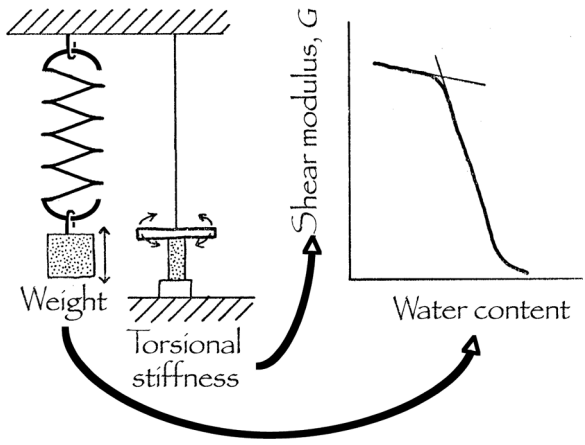


**FIGURE 3** Torsional pendulum for measuring changes in shear modulus ($G$) with changing water content. From Vincent, Ref. [17], with permission.

stretched over a platinum wire frame with a hook, and hung from a fine calibrated silica glass spring as close to the torsional pendulum as possible. The assumption was that both pieces of cuticle would dry at the same rate. The length of the spring as the cuticle dried was followed using a travelling microscope with sensitivity of 0.02 mm, which corresponds to a weight sensitivity of the spring of 11 μg. At the same time the shear modulus of the sample mounted in the torsional pendulum was obtained by gently moving the bob weight through no more than 10 degrees, then recording the time taken for ten oscillations. Readings were repeated every minute. Thus, the spacing of the data points gives information about the rate of the processes occurring. The elastic shear modulus ($G'$) of the cuticle was calculated [18].

A fresh cuticle typically shows a change of stiffness of about an order of magnitude over the range of water content of 0.4 to 1 g/g (Fig. 4). This non-linearity of variation of stiffness with water content shows that the change is a transition. Several factors can change the position and slope of this transition. Simply drying the cuticle, wetting it again, and repeating the measurements (which can, conveniently, be done without removing the sample from the torsional pendulum) reduces the amount of water absorbed and moves the transition to a lower water content. Whether or not the cuticle is tanned, and by how much, also affects it. Thus, tanning the cuticle with 2% catechol affects both the slope of the transition and the stiffness of the dry cuticle (Fig. 5), characteristics which are very like those of the white



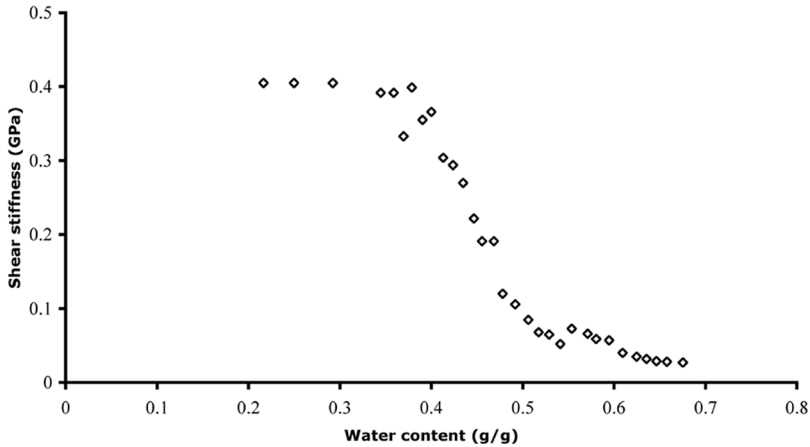


**FIGURE 4** Shear modulus of fresh maggot cuticle changing with moisture content. From Vincent, Ref. [17], with permission.

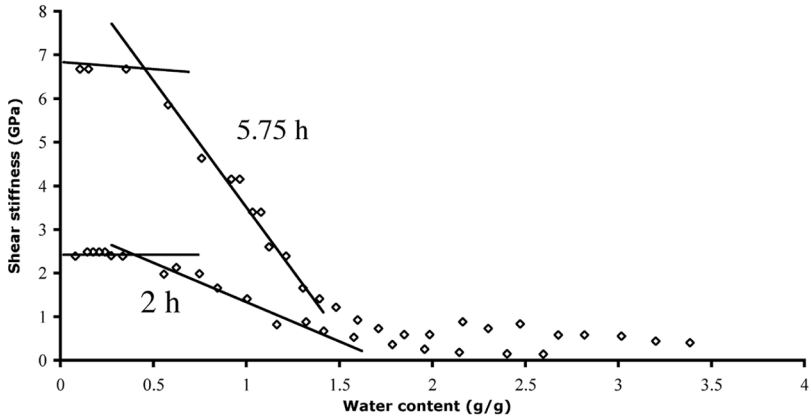


**FIGURE 5** Shear modulus of maggot cuticle, tanned in 2% catechol for 5.75 h (upper graph) and 2 h (lower graph) changing with moisture content. From Vincent, Ref. [17], with permission.



puparium cuticle. The slope of the transition can be affected in a minor fashion by wetting and drying.

Thus, when maggot cuticle is dried it becomes stiff, but in a process that shows three distinct phases. When the cuticle is saturated with water its stiffness is relatively low. As it loses water the stiffness increases, but at a slow rate. At a certain point, often well defined in terms of slope change and commonly around 1.0 g/g water content, the stiffness begins to increase relatively rapidly. At the end of this rapid phase the rate of change of stiffness with water content once again reduces. These changes in modulus can be explained in general terms as a function of the way water interacts with the protein [10]. At low water content, where the stiffness is high, the water is supposedly present in a relatively immobile form. Co-operative interactions are important where the modulus changes rapidly with water content. In this section, the steeper the slope the fewer the water molecules involved in co-operativity and the more flexible the polymer (protein) molecules must be.

## MOLECULAR MECHANISMS

The cooperative interactions are most probably between beta structures of the protein, since so much is observed in the dried protein removed from larval cuticle [5,19]. Dried cast films of *C. vomitoria* larval cuticular protein, extracted in 8 M urea and dialysed against 0.1 M ammonium acetate, were examined by infrared spectroscopy

and found to possess massive amounts of beta-sheet conformation in cast films, shown by the asymmetry of the Amide II peak at $1622\,cm^{-1}$, the reduced height of the peak at $1648\,cm^{-1}$ and a slight shoulder at $1695\,cm^{-1}$. The proteins crystallised; the films were birefringent around the edges. Thin films examined by electron diffraction were also found to be crystalline although they are polycrystalline, suggesting that crystallisation is a general property of these cuticular proteins [5]. So it seems that at least some cuticular proteins destined to become 'sclerotised' can form considerable amounts of stable secondary structure. This is in addition to the beta structures reacting with the chitin [20–22]. This ability to form secondary structures was also shown in isoelectrofocused gels across a urea gradient of 0 to 8 M. Several of the protein bands showed complexing as the urea content reduced [23].

The mechanical transition has its high point at about 0.4 g/g of water, which is the water content above which the water is freezable and, therefore, free or interstitial and capable of plasticising the protein matrix [13]. The mechanical transition upon drying is presumably, therefore, due to the removal of the plasticising fraction of the water. Water adsorption isotherms of tanned and untanned cuticle were deconvolved [24] into tightly bound (Langmuir layer) and multilayer water fractions. These isotherms [17] suggest a multilayer fraction existing at contents below about 0.4 g/g that is also plasticising the cuticle, but not so much as the freezable water does (Fig. 6). But since the isotherms show a maximum hydration at about the level at which freezable water disappears, the indication is that the freezable



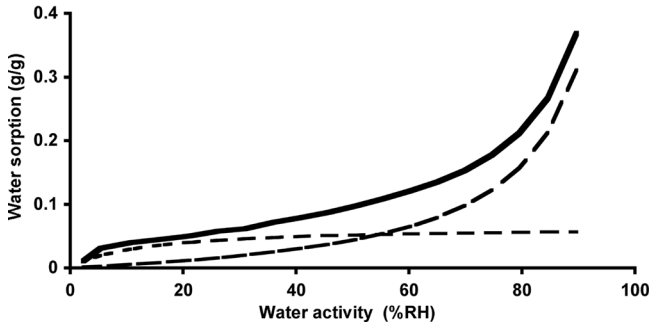


**FIGURE 6** Deconvolved isotherm of *C. vomitoria* larval cuticle showing strong water binding (Langmuir monolayer—horizontal dashed line) and weak water binding (rising dashed line), together making up the solid line. From Vincent, Ref. [17], with permission.

water is provided only by contact with liquid water and not by water vapour. Further removal of water increases the stiffness, but at a relatively low rate, indicated both by the experiments on the maggot cuticle, and confirmed in tensile tests on locust wing cuticle, where an increase from 13.5 to 16.5 GPa was reported over a drop in RH from 75 to 35% [25]. Over this range of RH the water being lost is categorized as bound multilayer—in other words, not free water but nonetheless water capable of plasticisation. The remainder of the detail derived from the analysis of the water sorption isotherms is too confusing to be presented here—there seems to be no convincing relation between the water content at 95% RH and the amount of water that can be ascribed to the tightly-bound (Langmuir) layer. All that can be said is that the tanned cuticle absorbs much less water from humid air, and so has been waterproofed by the addition of phenols.

## WHAT HAPPENS WITH REAL CROSS-LINKS?

The most interesting result from the torsional pendulum experiments is the modification of the mechanical transition by the action of glutaraldehyde, which was chosen as a histological fixative which will introduce unequivocal covalent cross-links into the protein. My interpretation of this curve is that by stabilising the protein in its hydrated state the glutaraldehyde is inhibiting it from changing its conformation as it loses water. This hinders it in forming secondary bonds co-operatively since the protein chains are held apart or in the wrong



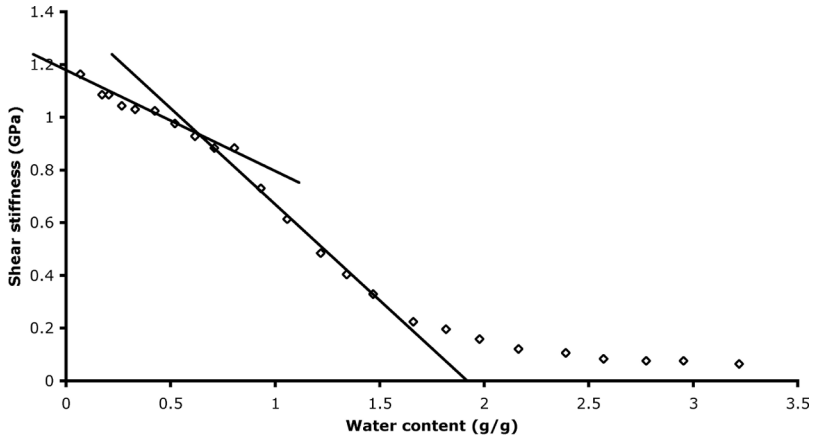


**FIGURE 7** Shear modulus of maggot cuticle, cross-linked with glutaraldehyde, changing with moisture content. From Vincent, Ref. [17], with permission.

positions and so cannot interact. Hence, the transition is suppressed (Fig. 7) but not eliminated. The deduction can only be that either the phenols cross-link the proteins in a manner that does not hinder their mobility to self-associate and form higher structures, perhaps even crystalline as observed on extracted *C. vomitoria* protein, or that they are not cross-linking the proteins at all since they would only get in the way of the reorganization of the proteins and formation of beta structures as the water is expelled.

The prime function of the phenol-based tanning mechanism, at least in the maggot, seems therefore not as a primary cross-linking mechanism. The phenols reduce the water content and keep the cuticle waterproof. This drives the matrix proteins towards a condition of hydrogen-bonded self-assembly, and keeps water content low in the presence of external moisture. Indeed, cross-linking of the phenols to the proteins may be more important to retain the phenols within the protein matrix and, thus, maintain the hydrophobicity.



## A WIDER VISION

The main problem with this model is that it has been developed mostly with the maggot-into-puparium transition, which is only one of the possible sclerotisation systems. In order to generalize it there needs to be more experimentation on other types of cuticle, which can have a wide variation of protein hydrophobicity [26] though perhaps all the proteins contain beta structures [27] which would be important for co-operative interactions. The prerequisites for insect cuticular sclerotisation are that the proteins should be self-assembling in some way and that the dehydration mechanism should not hinder the spatial reorganization of the proteins as they self-assemble from their highly solvated state in the Schmidt layer [28]. The main test is that a hydrogen-bond breaker will cause the proteins in sclerotised cuticle to solvate and swell. In byssus threads the range of proteins is much wider [29] (and perhaps the silk-like domains are providing a large part of the DOPA-driven co-operative interactions) and the mechanical demands are greater [30]. The fact that the DOPA is incorporated into the secreted protein is probably a trivial difference, since insect cuticle is underlain with a layer of cells which continually minister to the cuticle and secrete the phenols. Byssus is without cellular contact when it tans and so needs all the components *in situ* when it is formed. However, phenolic tanning is much more widely spread [31–33] so there is ample material for examination and experiment. The first test would be to expose some byssus threads to formic acid!

## APPLICATIONS

There are implications in this work for the production of composite materials using the standard sort of epoxy matrix. Water is an enemy of fibrous composites, as it is of many technical materials that do not include water as a primary component. However, biological composites, using water not only as the medium of synthesis, but also as part of the assembly processes, can be highly stable and durable. Bits of beetle elytron can be found, pristine, in drift deposits of a million or more years old. So water is not intrinsically destructive, nor is it uncontrollable. If the matrix materials could use some of the techniques seen in insect cuticle proteins to generate the requisite cross-linking then water, and the associated advantages of a self-assembly mechanism using hydrophobicity as a driving force, could be used to drive a more robust, lower energy, stabilising system.



## REFERENCES

[1] Pryor, M. G. M., *Proc. R. Soc. B* **128**, 378–398 (1940).
[2] Fraenkel, G. and Rudall, K. M., *Proc. R. Soc. B* **129**, 1–35 (1940).
[3] Vincent, J. F. V. and Hillerton, J. E., *J. Insect Physiol.* **25**, 653–658 (1979).
[4] Minke, R. and Blackwell, J., *J. Mol. Biol.* **120**, 167–181 (1978).
[5] Hillerton, J. E. and Vincent, J. F. V., *J. Insect Physiol.* **25**, 957–963 (1979).
[6] Andersen, S. O., *J. Insect Physiol.* **27**, 393–396 (1981).
[7] Treloar, L. R. G., *The Physics of Rubber Elasticity*, (Clarendon Press, Oxford, 1975).
[8] Suderman, R. J., Dittmer, N. T., Kanost, M. R., and Kramer, K. J., *Insect Biochem. Mol. Biol.* **36**, 353–365 (2006).
[9] Andersen, S. O., Hojrup, P., and Roepstorff, P., *Insect Biochem. Mol. Biol.* **25**, 153–176 (1995).
[10] Nissan, A. H., *Macromols.* **9**, 840–850 (1976).
[11] Reynolds, S. E., *J. Exp. Biol.* **62**, 69–80 (1975).
[12] Charlton, A. J., Baxter, N. J., Khan, M. L., Moir, A. J. G., Haslam, E., Davies, A. P., and Williamson, M. P., *J. Agric. Food. Chem.* **50**, 1593–1601 (2002).
[13] Vincent, J. F. V. and Ablett, S., *J. Insect Physiol.* **33**, 973–979 (1988).
[14] Puffr, R. and Sebenda, J., *J. Polym. Sci.* **16**, 79–93 (1967).
[15] Kuntz, I. D. and Kauzmann, W., *Adv. Prot. Chem.* **28**, 239–245 (1974).
[16] Wybrandt, G. B. and Andersen, S. O., *Biochim. Biophys. Acta* **1201**, 15–18 (1994).
[17] Vincent, J. F. V., *J. Bionic Eng.* **1**, 4–8 (2004).
[18] Ward, I. M., *An Introduction to the Mechanical Properties of Solid Polymers*, (Wiley, New York, 2004).
[19] Hackman, R. H. and Goldberg, M., *Insect Biochem.* **9**, 557–561 (1979).
[20] Fraenkel, G. and Rudall, K. M., *Proc. R. Soc. B* **134**, 111–143 (1947).
[21] Rebers, J. E. and Willis, J. H., *Insect Biochem. Mol. Biol.* **31**, 1083–1093 (2001).
[22] Iconomidou, V. A., Willis, J. H., and Hamodrakas, S. J., *Insect Biochem. Mol. Biol.* **35**, 553–560 (2005).
[23] Hillerton, J. E. and Vincent, J. F. V., *Entom. Gen.* **11**, 1–9 (1985).
[24] Christie, J. H., Woodhead, I. M., Krenek, S. H., and Sedcole, J. R., *Textile Res. J.* **72**, 273–278 (2002).

[25] Smith, C. W., Herbert, R., Wootton, R. J., and Evans, K. E., *J. Exp. Biol.* **203**, 2933–2943 (2000).
[26] Hillerton, J. E. and Vincent, J. F. V., *Int. J. Biol. Macromol.* **5**, 163–166 (1983).
[27] Karouzou, M. V., Spyropoulos, Y., Iconomidou, V. A., Cornman, R. S., Hamodrakas, S. J., and Willis, J. H., *Insect Biochem. Mol. Biol.* **37**, 754–760 (2007).
[28] Weis-Fogh, T., in *Insect Ultrastructure*, A. C. Neville (Ed.) (The Royal Entomological Society, London, 1970), "Structure and formation of insect cuticle," pp. 165–185.
[29] Qin, X.-X., Coyne, K. J., and Waite, J. H., *J. Biol. Chem.* **272**, 32623–32627 (1997).
[30] Bell, E. C. and Gosline, J. M., *J. Exp. Biol.* **199**, 1005–1017 (1996).
[31] Knight, D. P., *Nature* **218**, 585–586 (1968).
[32] Brunet, P. C. J. and Coles, B. C., *Proc. R. Soc. B* **187**, 133–170 (1974).
[33] Waite, J. H., *Biol. Bull.* **183**, 178–184 (1992).