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THE MECHANISM OF PLASTICIZATION OF THE ABDOMINAL CUTICLE IN *RHODNIUS*

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SUMMARY

1. The mechanism of plasticization of the abdominal cuticle in *Rhodnius* larvae has been investigated, using the properties of loops of cuticle under varying test conditions as a model for the behaviour of the cuticle *in vivo*.

2. It is supposed that plasticization is effected by a change in the intracuticular environment. A number of model mechanisms for plasticization may be proposed, which suppose that the epidermis is capable of regulating
(*a*) pH,
(*b*) ionic strength,
(*c*) Ca and/or Mg,
(*d*) urea,
within the cuticle.

3. Analyses of cuticle ash show that models (*b*) and (*c*) are not responsible for plasticization *in vivo*. The levels of inorganic ions within the unplasticized cuticle are not sufficiently high to allow plasticization upon their removal.

4. No evidence for model (*d*) has been found; urea does not occur in the cuticle in detectable quantities.

5. Exact measurements of the intracuticular pH have not been achieved but staining experiments strongly suggest that a change in pH occurs within the cuticle on plasticization. This pH change is probably large enough to account for the increased extensibility shown by plasticized cuticle.

INTRODUCTION

The abdominal cuticle of the blood-sucking bug *Rhodnius prolixus* becomes more extensible when the insect feeds (Bennet-Clark, 1962) and also when 5-hydroxytryptamine (5-HT) is injected into the haemocoel (Reynolds, 1974). This plasticization of the cuticle is evidently under the control of the underlying epidermal cells, which respond to nervous or to pharmacological stimulation.

The ready reversibility of plasticization suggests that the severance of primary (covalent) bonds between cuticle macromolecules is not involved, but rather a reduction of the extent to which those molecules interact by means of weaker, secondary bonds. The mechanical properties of normal and plasticized cuticle are consistent with this interpretation (Reynolds, 1975).

How is such a reduction in the extent of secondary bonding achieved? In Part I of this paper ways in which the epidermis might exercise control over the mechanical

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properties of the cuticle are considered. The experiments reported there have made particular use of loops of cuticle which have been stripped of their parent epidermis. The behaviour of such loops in artificial media of known composition has been considered to be a model for the behaviour of the cuticle *in vivo*. In Part II some experiments designed to assess the importance of these hypothetical plasticizing mechanisms to the living animal are described.

MATERIALS AND METHODS

The insects used were taken from culture as 5th-instar larvae, 1–2 weeks after their last ecdysis.

Where samples of plasticized cuticle were required, plasticization was induced by the injection of 10 μl of 10^{-4} M 5-HT solution, made up in the Ringer solution described by Maddrell (1969). This amount of 5-HT is more than sufficient to induce maximal plasticization of the abdominal cuticle (Reynolds, 1974).

The samples of cuticle used for analysis were the tergites and sternites of abdominal segments 3, 4 and 5. The samples were scraped free of epidermal cells (which are pink and easily visible) using a blunt scalpel blade. To ensure the removal of as much as possible of the epidermis, the samples were rubbed briefly against filter paper. As Hackman & Goldberg (1958) comment, such a procedure is almost certain to result in the loss of some of the innermost layers of the procuticles, but this is unavoidable if contamination from the cells is to be avoided.

Such small samples of tissue (1–2 mg usually) lose water very rapidly in air, and it is necessary to make a correction for this loss. It was found that the weight loss over the first 2 min could be regarded as a simple exponential function of time, and so for each sample a number of timed weighings were made, and a plot of $(\text{weight})^{-1}$ against time was extrapolated to zero time.

For the determination of water content, samples prepared and weighed wet as described above were transferred to clean aluminium foil pots and dried over P_2O_5 for 5 days (found by control experiment to be sufficient to dry to constant weight), after which the samples were reweighed. A correction was not found to be necessary for this weighing. The water content was estimated by difference.

For the determination of inorganic ions, the dry weight of the samples was determined as above, and the samples, pooled in batches of five whole abdominal cuticles, were ashed in a Tracerlab low temperature ashing device. The cuticle samples remained in their aluminium foil pots during this procedure. The ash was taken up in either doubly deionized water or 10^{-2} M nitric acid (made up with doubly deionized water). These procedures gave similar results. The resulting solutions were transferred straight away to polystyrene containers, to minimize contamination from the aluminium containers. Cuticle contents of Na and K were determined from the sample solutions thus prepared by emission flame photometry. Ca and Mg contents were determined by absorption flame photometry. Blank runs were used to check contamination. A Pye Unicam SP90A flame spectrophotometer was used for the determinations.

Buffers used were 0·01 M citrate/phosphate in the range pH 5–7; 0·01 M Tris, pH 7–9; 0·05 M-$NaHCO_3$, pH 9–11; 0·05 M-Na_2CO_3/NaOH, pH 11 and above.

For mechanical testing, loops of cuticle were cut from the abdomens of insects as

Table 1. *The water content of* Rhodnius *abdominal cuticle, expressed as* % *of the total weight before dehydration* (*means* ± S.E.)

Unplasticized	25·8 ± 0·9 %
Plasticized	31·3 ± 0·5 %

described in Reynolds (1975). The epidermal cells on the inside of these loops were removed before the loops were transferred to the experimental medium. They remained there for 30 min before testing took place. The loops were subjected to a creep test, in which extension under a maintained load of 5 g was continuously monitored using a displacement transducer. The testing apparatus is described by Reynolds (1975). The rate of creep of the cuticle samples 30 sec after the imposition of the load was taken as a measure of the extensibility of the cuticle.

RESULTS AND DISCUSSION

Part I

(i) *Hydration of the cuticle*

Bennet-Clark (1961) and Maddrell (1966) have both suggested that an increase in the hydration of the cuticle might accompany plasticization.

The results presented in Table 1 show that this is indeed the case. The difference between plasticized and unplasticized cuticle is highly significant and represents an increase in water content during plasticization of more than 20 %.

However, we do not know why the cuticle takes up extra water. Bennet-Clark (1962) points out that when the epidermis is damaged (as by pinching with forceps) then the overlying cuticle is subsequently unable to plasticize. If the mechanism of plasticization were, as suggested by Maddrell (1966), 'merely . . . to cause an increase in the permeability of the epidermal cells so that water may more easily pass into the cuticle', one would expect that any damage to the epidermis would lead to plasticization rather than prevent it.

Maddrell's idea presupposes that the abdominal cuticle of *Rhodnius* is normally less hydrated than it would be if it were at equilibrium with the blood. This is not the case; samples of cuticle do not swell if placed in *Rhodnius* Ringer solution (which is known to have much the same ionic composition and osmotic strength as the insect's blood), nor do they become more extensible. Bennet-Clark (1961) noted that cuticle loops soaked in Ringer at pH 7 for 10 min showed a 'marked decrease in stretchability'. I have confirmed this, and find that even in distilled water (maintained at pH 7 by citrate/phosphate buffer at a final concentration of only 0·01 M) cuticle loops are less extensible than they are when tested immediately after excision from the living insect.

If increased hydration *is* the cause of plasticization, then it is not clear why water should enter the cuticle. A simple permeability change is not enough to explain the increased water content.

(ii) *pH within the cuticle*

It has been noted by Beament (1965) that by controlling the ambient pH within the cuticle, the epidermis might control the hydration of the cuticle.

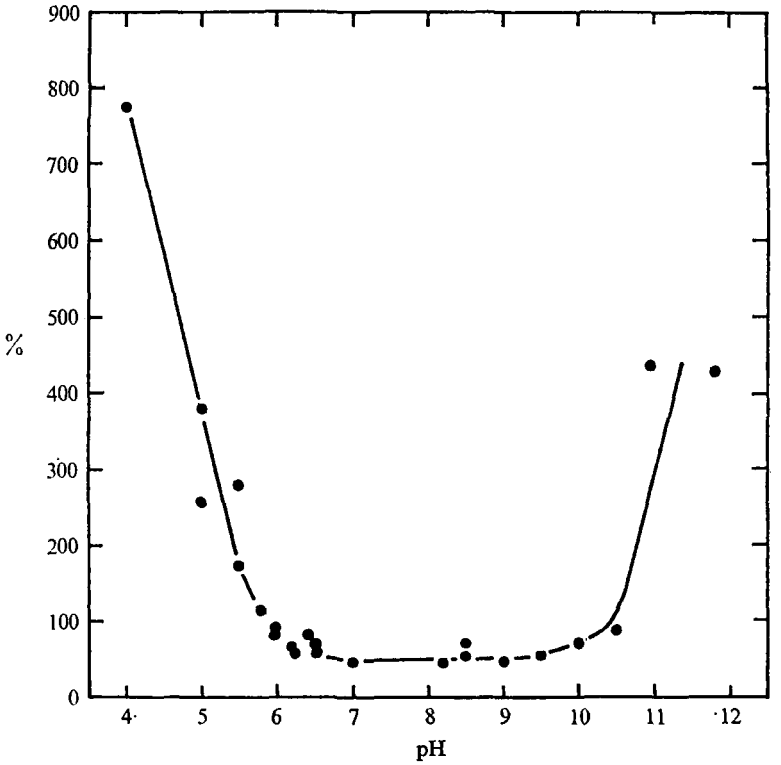

Fig. 1. Swelling behaviour of the abdominal cuticle of *Rhodnius* in dilute buffer solutions (0·01 M). The ordinate shows the water content of the cuticle expressed as a percentage of its dry weight.

The effect of changing the ambient pH on the swelling properties of *Rhodnius* abdominal cuticle samples in very dilute buffer solution is shown in Fig. 1. As can be seen, there is a very sharp change in the extent of hydration at pH 6 and another at pH 10. Polyelectrolyte gels show minimum capacity for swelling at their isoelectric points. When displaced from this point by a change in pH, they become increasingly more hydrated as they acquire a larger net charge on the surface of their molecules. Fig. 1 seems to show that the polyelectrolytes of the *Rhodnius* abdominal cuticle (the matrix proteins mainly) show very little net charge over a wide range of pH, with a sharp increase in net surface charge at pH 6 and at pH 10.

Some staining experiments with the dye Prussian Blue support this interpretation. Yonge (1932) used the extent to which ferrocyanide ions ($Fe(CN)_6^{4-}$) were bound to the 'chitin' lining of the foregut of decapods under different pH conditions to determine the isoelectric point of the material. These ions bind to fixed positive charges and can subsequently be 'developed' with ferric chloride solution, which precipitates the dye Prussian Blue (ferric ferrocyanide); the intensity of Prussian Blue staining thus corresponds to the density of positive fixed charges.

The staining solutions were prepared as described by Yonge; buffers were as described in the Methods section. It was found necessary to time operations very exactly in order to obtain consistent results. It was found that pieces of cuticle could be stained at pH values up to pH 8–8·5, provided that the cuticle samples were left

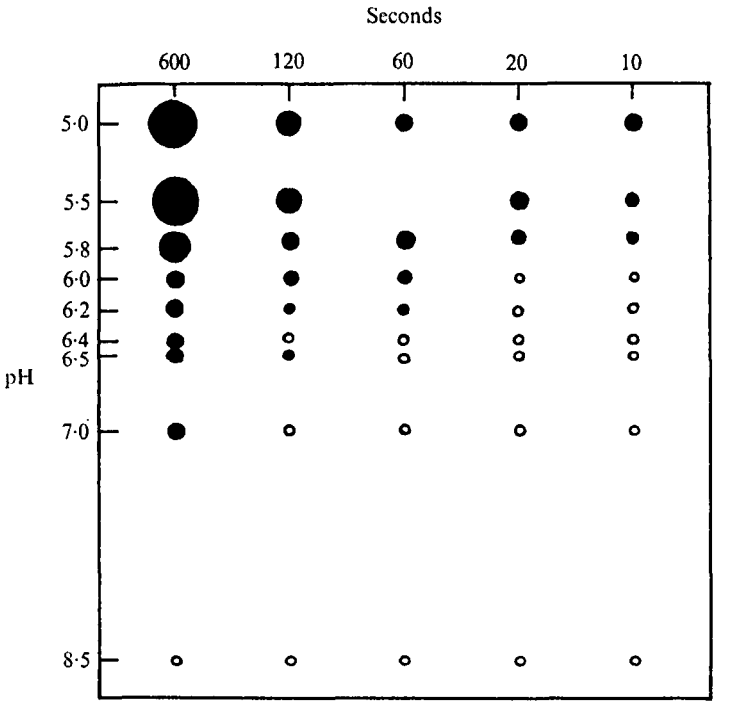

Fig. 2. Staining with Prussian blue. Cuticle samples were soaked in a dilute solution of $K_4Fe(CN)_6$ solution, buffered at the pH shown. The length of the exposure to $Fe(CN)_6^{4-}$ ions is shown. The buffer was 0·01 M citrate/phosphate. The intensity of staining which resulted on 'developing' in $FeCl_3$ solution is indicated by the size of the points.

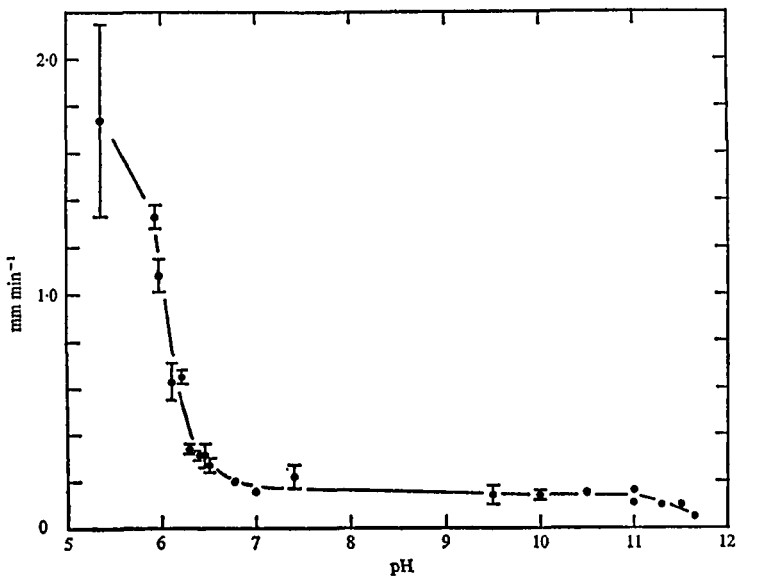

Fig. 3. The relation between the ambient pH and the extensibility of cuticle loops. Dilute buffer solutions (0·01 M). Means ±S.E.

Table 2. *The effect of reduced pH on the extensibility of cuticle loops: reversibility*

	Creep rate at 30 sec
(i) pH 7·0 (60 min)	0·18 ± 0·01 mm min^{-1}
(ii) pH 5·5 (30 min) pH 7·0 (60 min)	0·19 ± 0·01 mm min^{-1}
(iii) pH 5·5 (60 min)	1·45 ± 0·14 mm min^{-1}

The loops were soaked in the media for the times shown before testing. The buffer solution was 0·01 M maleate. Means ± S.E.

to soak in the ferrocyanide solution for periods of 10 min or more. At pH values higher than this staining never occurred. Only the procuticle took up the stain; the epicuticle did not appear to be stained under these conditions.

Briefer immersions in the ferrocyanide solution resulted in staining only at lower pH values: thus, 1 min immersion resulted in staining up to pH 6·2, whereas 30 sec immersion gave staining up to pH 5·8. A number of experiments of this kind built up a qualitative picture of a slow increase in the density of fixed positive charges within the cuticle as the pH was lowered from pH 8–8·5 down to about pH 6, when the charge density increased much more rapidly. This is illustrated in Fig. 2. This picture confirms what the swelling data of Fig. 1 tell us about the behaviour of the cuticle proteins in this region of pH.

Bennet-Clark (1961) described some experiments in which he investigated the mechanical properties of the abdominal cuticle when subjected to changes in the pH of a bathing medium of Ringer solution. I have repeated these experiments using cuticle loops with an intact epidermis as Bennet-Clark did, and have confirmed that there is a change in the mechanical properties of the loops at about pH 6.

This change is much accentuated if the epidermal cells are removed before soaking the loops in the test medium. The inflexion of the curve is much sharper and is seen to be at about pH 6·2 (Fig. 3). In these experiments the rate of creep at 30 sec after the imposition of a 5 g load is taken as a measure of extensibility rather than Bennet-Clark's criterion of the force required to stretch the loop to twice its original length.

The change in extensibility caused by immersion in a medium of low pH is reversible, provided that the pH is not too low (Table 2). At very low pH (pH 4 or less), however, the cuticle may lose some of its material into solution if the treatment is prolonged.

The abrupt change in mechanical properties at pH 6·2 is what we would expect from our interpretation of the influence of pH changes in this region on the fixed charge density and the swelling properties of the cuticle. With increasing surface charge as the ambient pH is lowered, the net electrostatic repulsive forces between molecules increase, making their close approach, and hence the formation of van der Waals' bonds, more difficult. The increased number of fixed charges within the material increases the tendency of a polar solvent like water to solvate the material. The presence of an increased number of fixed charges also increases the size of the pool of 'free' ions (the 'Donnan excess') within the material which act osmotically to draw water into the material and swell it, thus further separating the molecules and reducing the extent of their secondary interaction. All these influences tend to plasticize the material. (For a discussion of the influence of fixed charges on swelling, etc., see Robinson, 1965.)

However, this interpretation also predicts that a similar abrupt change in the mechanical properties of the cuticle loops should be seen at about pH 10. It is obvious from Fig. 3 that this does not occur. If anything, such high pH values result in decreased extensibility, though it may be noted that above pH 11 the cuticle samples were very brittle (after 30 sec soaking) so that at pH 11·9 it was impossible to test them at all, the loops breaking immediately on the imposition of a load. At such high pH values the cuticle samples show severe weight losses and it is clear that material is passing into solution; the results of tests at such unphysiologically high pH are probably not very meaningful and will not be considered further.

Another way in which a relatively small pH change might affect the mechanical properties of the cuticle, in addition to the effects of increased surface charge as outlined above, might involve disruptive effects on particular cross-links between molecules. Hackman (1955*a*, *b*) has described the formation of weak bonds, between chitin and proteins from the insect cuticle, which are disruptible by small pH changes 'in the biological range'. It was suggested that these weak bonds involved the formation of Schiff base linkages between sugar residues of the chitin and free amino groups on the protein chains.

Hackman & Goldberg (1958) estimated that about 3% of the protein in the soft larval cuticle of *Agrianome spinicollis* was bound by bonds like these. Bennet-Clark (1961) using the same procedure found that the abdominal cuticle of larval *Rhodnius* contained no significant amount of protein which corresponded to this fraction. These results seem to indicate that Schiff base type bonds cannot be very important in maintaining the integrity of the cuticle. However, it must be pointed out that Bennet-Clark's results do not necessarily mean that this type of bonding does not occur in the *Rhodnius* abdominal cuticle: proteins which are multiply bonded would appear to be extracted only by those methods which disrupted the strongest bonds by which they were bound.

In this context it is interesting to note that Harkness (1970) has found that the strength of loops of skin cut from the tails of young rats is sharply reduced at pH values lower than pH 7, and that much of this reduction in strength is attributable to the rupture of Schiff base links by the lowered pH. Such linkages are thought to be important in the formation of intermolecular cross-links in collagen, though in older animals these linkages may become stabilized in some way (Bailey, Peach & Fowler, 1970). The breaking strength of the loops used by Harkness is determined largely by the collagenous framework present in the skin.

Regardless of the means by which it is achieved, the existence of a sharp change in extensibility at about pH 6 provides a hypothetical mechanism for the plasticization of the abdominal cuticle which occurs *in vivo*, just as pointed out by Bennet-Clark (1961). The plasticization of the cuticle can be achieved over a limited range of pH which is close to that normally considered 'biological'. The present study shows that the change in mechanical properties caused by pH change is large enough to account for the increased extensibility shown by plasticized cuticle in the living insect. 30 sec after the imposition of a 5 g load, loops of cuticle from the abdominal integument of normal insects show a creep rate of $0{\cdot}26 \pm 0{\cdot}03$ mm min^{-1} when tested under oil. Loops of plasticized cuticle taken from insects injected with 5-HT show a creep rate of $1{\cdot}11 \pm 0{\cdot}09$ mm min^{-1}. In terms of the model behaviour of Fig. 2, this corresponds to a change in ambient pH within the cuticle of only half of one unit, from pH 6·5 to pH 6·0.

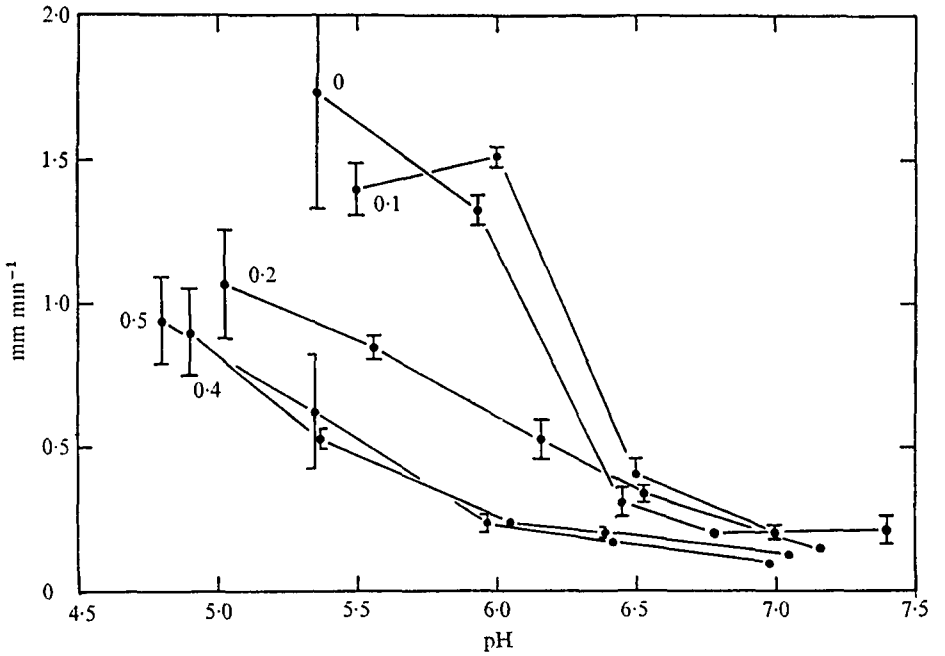

Fig. 4. The effect of changes in ionic strength on the relation between pH and the extensibility of cuticle loops. Buffer about 0·01 M in all cases. Ionic strength is represented as equivalent to the molar concentration of NaCl. Means ± S.E.

The fact that the pH dependency of extensibility is more marked for cuticle loops which have been stripped of their epidermal cells than for those which retain some epidermal influence suggests that the cells may be able to regulate the intracuticular pH to some extent.

(iii) *Ionic strength within the cuticle*

Other factors, in addition to changes in pH, are known to affect the way in which protein molecules interact with one another by means of secondary bonds. In particular, variations in ionic strength are important; their role in determining the properties of polyelectrolytes in solution has been considered by Katchalsky (1964) as a model for the behaviour of connective tissues.

If the effects of varying both the pH and the ionic strength of the bathing medium on the extensibility of cuticle loops are investigated, a family of curves may be drawn up which describe the behaviour of the cuticle under a wide variety of conditions. In Figs. 4 and 5 two such families of curves are shown, plotted in two different ways. Ionic strength is indicated in these plots as the molar concentration of NaCl in the test solution; for monovalent ions, the ionic strength, μ, is equal in value to the molarity.

It may be seen that at pH values of 6·5 and above, changing the ionic strength has little effect on the extensibility of the cuticle loops, but at pH 6 and below, the effects are marked. For most pH values, the effect of increasing ionic strength is biphasic; in the range μ = 0–0·1, increased ionic strength leads to increased extensibility; in the range μ = 0·2–0·5, increased ionic strength leads to decreased extensibility (Fig. 5). The latter effect is particularly marked; at pH 6·0 the 30 sec creep rate is reduced

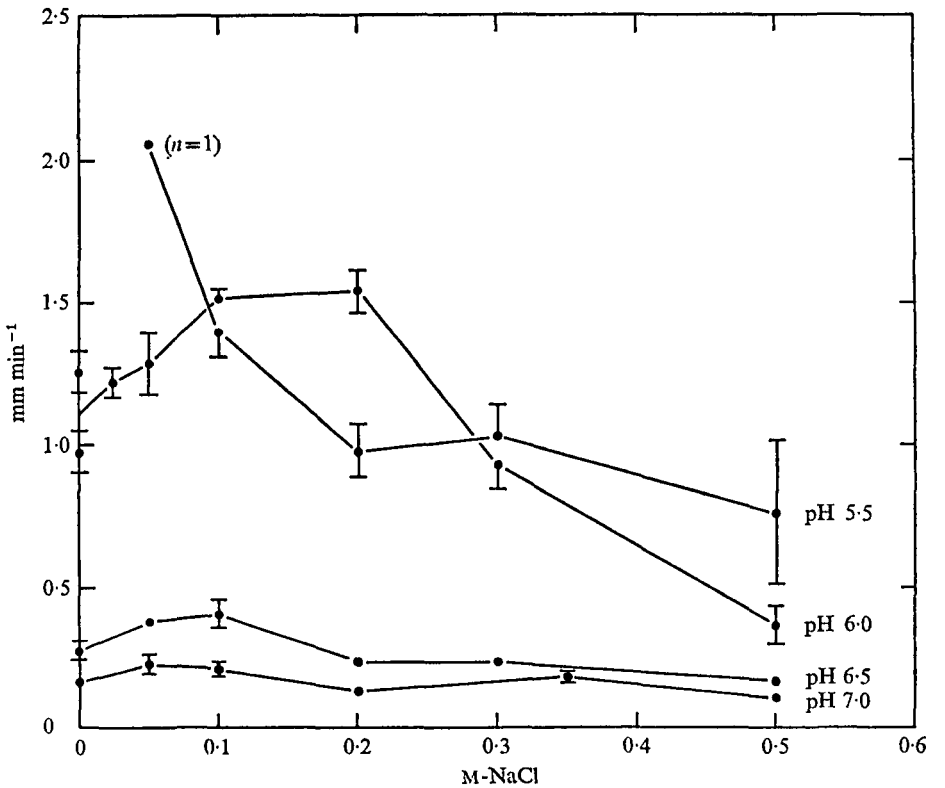

Fig. 5. The effect of changes in pH on the relation between ionic strength and the extensibility of cuticle loops. Buffer about 0·01 M in all cases. Ionic strength is represented as equivalent to the molar concentration of NaCl. Means ±S.E.

from $1{\cdot}54 \pm 0{\cdot}17$ mm min^{-1} at an ionic strength of 0·2, to $0{\cdot}37 \pm 0{\cdot}07$ mm min^{-1} at an ionic strength of 0·5. At very low pH values, the biphasic nature of the effect of increased ionic strength is lost, and only a progressive reduction in extensibility is seen as the ionic strength is increased.

It is clear from Figs. 4 and 5 that the reduction in extensibility caused by high ionic strengths is quantitatively much more important than the increase seen in the range $\mu = 0$–0·1. At a constant low pH of 6·0, it would be possible to account for the changes in mechanical properties which are observed to occur during plasticization of the cuticle *in vivo* in terms of a change in the ionic strength of the intracuticular environment alone. Such a model of the plasticization process would require the epidermal cells to maintain a high intracuticular ionic strength, in the order of $\mu = 0{\cdot}5$ under normal circumstances, and to withdraw ions from the cuticle until the ionic strength within it were about 0·3 in order to produce plasticization. The intracuticular pH would be low, in the region of pH 6, all the time.

The means by which a change in the ionic strength affects cuticular extensibility are probably complex. An increased ionic strength is known to decrease the distance over which point charges in dilute solution may interact, as described by the Debye–Hückel relationship. The situation for the interaction of fixed charges on the surface of protein molecules in what may be regarded as an extremely concentrated colloidal

solution is obviously not comparable to this except in very general terms, but it might be said that a high ionic strength can 'mask' charged groups and reduce the extent of their interaction. Non-specific interaction between the net surface charges on two similar polyelectrolyte molecules must always result in a net repulsive force between the molecules; considered from this point of view, higher ionic strengths within the cuticle might be likely to result in decreased repulsion, and hence a greater possibility for non-electrostatic, cross-linking interactions between molecules, such as van der Waals' bonds. This would lead to a reduced extensibility.

High ionic strengths are also known to cause a reduction in the apparent osmotic pressure shown by polyelectrolytes in colloidal solution (Robinson, 1965). This is because a considerable proportion of the osmotic pressure shown by such a system is due to the presence of a 'Donnan excess' of diffusible ions from solution, whose mobility is restricted by the presence of fixed charges within the colloidal system. At high ionic strengths, however, the significance of the 'Donnan excess' is reduced with the general increase in the concentration of diffusible ions, so that the apparent osmotic pressure exerted by the colloid now approaches more closely the appropriate value for a high molecular weight substance in solution, and the colloidal gel tends to shrink. Applying this to the *Rhodnius* cuticle, we might expect that higher ionic strengths will be associated with reduced hydration and an increased extent of secondary interaction between the matrix macromolecules. Consequently extensibility will be reduced.

These points are both in accord with observations on the effects of changing ionic strength in the range μ = 0·2–0·5, where increased ionic strength does lead to reduced cuticular extensibility. However, in the range μ = 0–0·1, increased ionic strength causes increased extensibility. This may be associated with effects on attractive interactions between particular charged groups on the surface of the macromolecules of the matrix. Katchalsky (1964) has considered the co-precipitation from solution of two differently charged polyelectrolytes which occurs at the point of minimum net charge to be a model for the kind of electrostatic interaction which might occur between fixed charges of opposite polarity on the surface of biological macromolecules. Here, co-precipitation is favoured by low ionic strength, as we would predict. From this it could be argued that specific, attractive, electrostatic forces between long chain molecules in the cuticle might be reduced by an increased ionic strength, with a consequent increase in extensibility, as occurs between μ = 0 and 0·1.

A suitable combination of effects like those discussed above (so that disruption of attractive secondary interactions occurred in the range of ionic strengths, μ = 0–0·1, while the effects on repulsive secondary interactions and on the osmotic relations of the cuticle took place in the range of ionic strengths, μ = 0·2–0·5), would explain the biphasic behaviour of the cuticle loops when subjected to a progressively increasing ionic strength in the bathing medium. Such biphasic behaviour recalls the 'salting in' and 'salting out' of proteins in solutions of progressively higher ionic strength. Such explanations remain purely hypothetical, however, in the absence of more definite information about the properties of the (unknown) proteins which make up the cuticle matrix, and of the bonds by which they are held together.

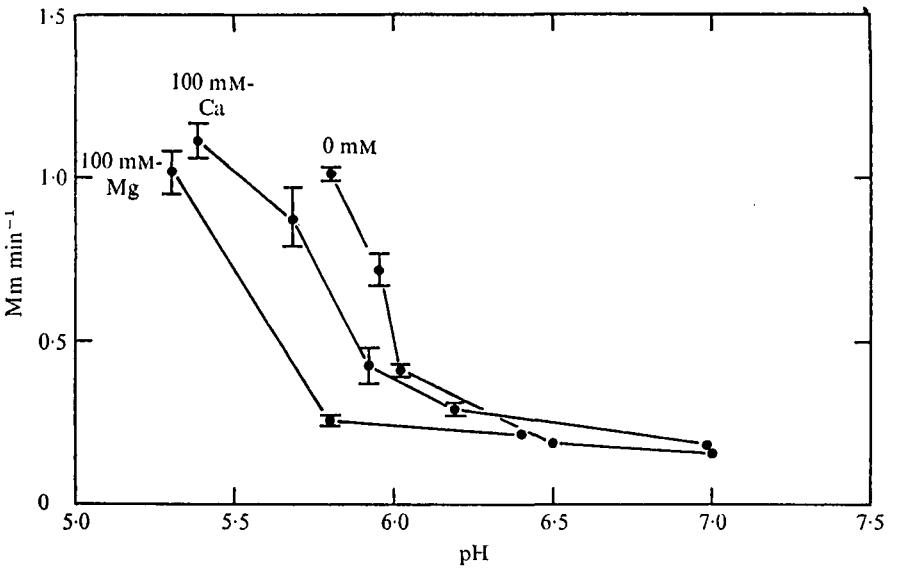

Fig. 6. The effect on the extensibility of cuticle loops of changes in the concentration of $CaCl_2$ and of $MgCl_2$. Buffer strength about 0·01 M. Means ±S.E.

(iv) *The effects of divalent cations*

In addition to the general effects outlined above, associated with increased ionic strength, high concentrations of particular ions within the cuticle might result in particular effects on the mechanical properties of the cuticle. Divalent cations like Ca^{2+} and Mg^{2+} are known to act as ionic cross-bridges in some biological systems, being chelated by multiple negative charges fixed appropriately in space by the tertiary structure of a large molecule. Katchalsky (1964) has considered the role Ca and Mg may play as cross-linking agents in connective tissue matrices.

The effects on extensibility of soaking cuticle loops in solutions of $CaCl_2$ and $MgCl_2$ were compared (Fig. 6). Both Ca and Mg appear more effective in reducing the extensibility of the cuticle than Na (Fig. 4). This is partly due to the greater ionic strength of solutions of salts of divalent ions ($\mu = \frac{1}{2} m_1 z_1^2$; where m = molarity, z = charge, for each ionic species). Were $CaCl_2$ and $MgCl_2$ completely ionized in solution, μ would be equal to that for 3 times the concentration of NaCl. The situation is complicated by the fact that in the range of concentrations in which we are interested, salts of Ca and Mg are far from completely ionized, and thus show much less than full potential ionic strength. For these reasons it is difficult to judge how much of the extra effect of these ions in reducing the extensibility of the cuticle is due to specific binding. It is, however, quite certain that low concentrations of these ions (in the order of 10^{-6} M) do not have marked effects on the mechanical properties of the cuticle, as they do on the mechanical properties of muscle (Ebashi & Endo, 1968) and the contractile stalk of the protozoan, *Vorticella* (Amos, 1972).

(v) *Breaking hydrogen bonds*

Hydrogen bonds are important in maintaining the integrity of the abdominal cuticle in *Rhodnius*; Bennet-Clark (1961) estimated that some 78% of the protein in

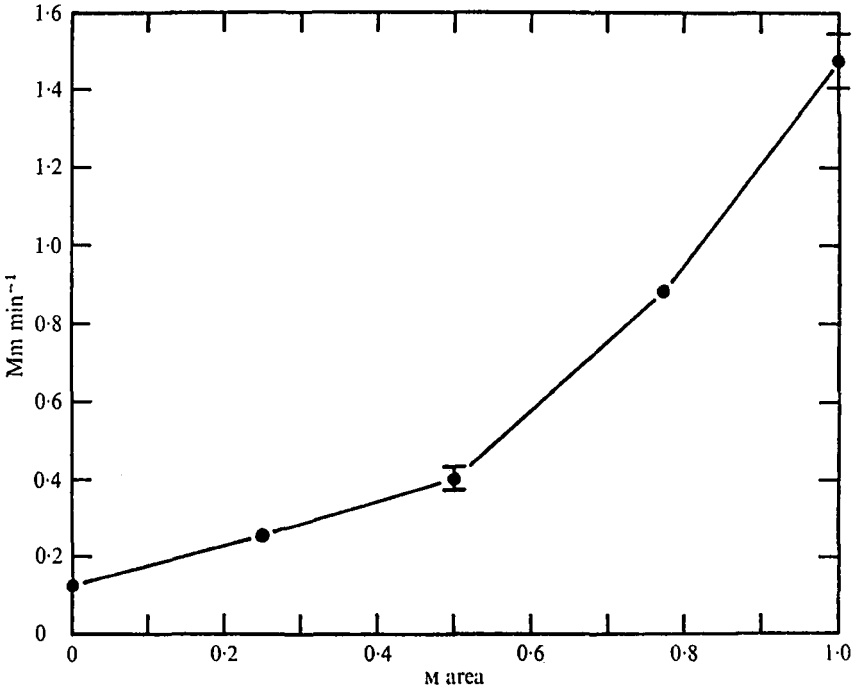

Fig. 7. The effect on the extensibility of cuticle loops of changes in the concentration of urea. pH 7·0. Buffer was 0·01 maleate. Means ±S.E.

Table 3. *The effect of urea on the extensibility of cuticle loops: reversibility*

	Creep rate at 30 sec
(i) 0 M urea (30 min)	0·13 ± 0·01 mm min^{-1}
(ii) 1·0 M urea (30 min) 0 M urea (30 min)	0·18 ± 0·01 mm min^{-1}
(iii) 1·0 M urea (30 min)	1·48 ± 0·07 mm min^{-1}

The loops were soaked in the media for the times shown before testing. pH was constant at 7·0. Buffer was 0·02 M maleate. Means ±S.E.

the cuticle was hydrogen bonded to other cuticle components on the basis of its solubility in 7 M urea solution at 4 °C, according to the extraction regime of Hackman & Goldberg (1958).

The effect of soaking cuticle loops in urea solutions of various concentrations at pH 7 is shown in Fig. 7. Evidently, urea is a plasticizing agent for the cuticle. The concentrations of urea used were low compared to the 7 M solution used by Bennet-Clark for extraction and this relatively mild treatment did not result in the dissolution of the cuticle. The effects on the extensibility of the cuticle are reversible (Table 3).

Urea is well known to be a hydrogen bond disrupting agent and it seems likely that this plasticizing effect is due to the rupture of interchain hydrogen bonds in the cuticle protein(s). The action of urea on the cuticle provides an additional mechanism whereby the epidermal cells might plasticize it. The release of a relatively small amount of urea into the cuticle could change the mechanical properties of the cuticle to the extent observed on plasticization *in vivo*. Fig. 7 shows that an intracuticular concentration of 0·85 M would suffice.

Table 4. *The contents of major cuticle cations in the abdominal cuticle of* Rhodnius

	Unplasticized	Plasticized
Na	27·5 ± 1·2	18·7 ± 1·9
K	21·5 ± 0·4	17·8 ± 0·7
Mg	2·97 ± 0·12	3·17 ± 0·23
Ca	1·64 ± 0·11	2·79 ± 0·10

μMoles per g dry cuticle. Means ± S.E.

Part II

The properties of the cuticle which were investigated in Part I have suggested the following methods by which the epidermis might effect plasticization of the overlying cuticle:

(*a*) intracuticular pH might be controlled,
(*b*) intracuticular ionic strength might be controlled,
(*c*) intracuticular Ca and/or Mg might be controlled,
(*d*) intracuticular urea might be controlled.

The models may be distinguished by their starting requirements for the value of the intracuticular pH. Models (*a*) and (*d*) both assume that under normal conditions the pH within the cuticle is such that extensibility is low (i.e. pH is greater than 6·2). Models (*b*) and (*c*) both require that under normal conditions intracuticular pH is low (i.e. pH is less than 6·2) so that extensibility is only maintained at a low level by the presence within the cuticle of high concentrations of ions. Each of the models is capable, alone, of accounting for plasticization as observed *in vivo*.

It is now obvious that, in order to understand how plasticization comes about, we must know the concentrations of the major cuticle ions and the pH within the cuticle; even if this information alone does not account for the changes in mechanical properties seen on plasticization, it is a necessary background to them.

(i) *Inorganic ions*

The cuticular contents of Na, K, Mg and Ca were estimated by flame photometry from ashed samples of cuticle. The results are shown in Table 4, where they are referred to the dry weight of the original cuticle samples.

The contents of Mg and Ca in the cuticle are low, in contrast to the situation in the larval cuticles of *Sarcophaga* (Richards, 1956) where Mg was the major cation, and of *Agrianome* (Hackman & Goldberg, 1958) where Ca and Mg were present at higher levels than other cations. It does not seem likely that either of these ions can make much contribution to the cross-linking of cuticular protein(s) in the *Rhodnius* abdomen. Calculation from the water content determined above shows that the effective concentration in the cuticle water would be about 10 mM for Mg and about 5 mM for Ca, assuming 25% water content for unplasticized cuticle. These concentrations are far too low to have much effect on the cuticle's mechanical properties, and the hypothetical mechanism of model (*c*) must be abandoned.

The position for Na and K is rather different. These are present in the cuticle in fair quantity. Calculation of their concentrations in the aqueous phase of the

unplasticized cuticle, again assuming 25% water content, gives concentrations of 92 mM for Na and 72 mM for K. It is necessary to point out that these figures can only be approximations to the true activity, which is probably depressed by the high density of fixed charges which exists within the cuticle. Also some Na and K might be present as insoluble salts, e.g. urates. Nevertheless, it is clear that the ionic strength due to Na and K within the cuticle is appreciable, and this must be taken into account in any proposal concerning the mechanism of plasticization which operates in life.

On plasticization, the cuticular content of both Na and K is reduced. The differences are highly significant in both cases, and represent the removal from the cuticle of some 32% of its Na and 27% of its K content. The calculated concentrations in the cuticle water now become 43 mM for Na and 41 mM for K, assuming 30% water content in the plasticized cuticle. The increased water content of the plasticized cuticle accentuates the effect of the withdrawal of these ions, of course, and the ionic strength due to Na and K is apparently halved.

Is it possible that these changes in the levels of Na and K can account for plasticization alone? Referring back to Figs. 3 and 4, it may be seen that the most pronounced effects of changes in ionic strength are those between $\mu = 0{\cdot}2$ and $0{\cdot}5$. It seems likely that the ionic strength due to the major cations of the cuticle is not sufficiently high to take the cuticle's aqueous phase into this range. It is concluded that plasticization is probably not effected by a simple withdrawal of ions from the cuticle.

(ii) *Intracuticular pH*

The measurement of the ambient pH inside the cuticle seems not to have been attempted before, probably for the very good reason that the measurement of pH in a hard extracellular matrix is fraught with difficulty. Ideally the pH would be measured *in vivo* while still subject to the regulating influence of the epidermis. However, since the tissue is hard and, what is worse, covered with a highly waterproof layer of wax, it is possible neither to insert an electrode made of H^+ ion-sensitive glass, nor to equilibrate the inside of the cuticle with an indicator solution without removing the epidermis from inside, or the wax layer from the outside. Some attempts were made to abrade the wax layer of the cuticle and render the inside of the cuticle accessible to externally applied indicator solutions, but I was unable to convince myself that there was effective contact between the indicator on the outside and the cuticle water on the inside.

The approach which was adopted to measure the intracuticular pH was to remove samples of cuticle from the abdomens of living insects, to remove the epidermal cells as quickly as possible, and to immerse the samples in solutions of suitable indicators or stains, following a strictly timed protocol, so that results were as uniform as possible. It was found possible to show a difference between plasticized and unplasticized cuticle samples in their behaviour towards the indicator *p*-nitrophenol and the stain Prussian Blue.

A concentrated solution of *p*-nitrophenol in ethanol was diluted with doubly deionized water until the colour of the indicator could just be seen in a drop of solution. A number of drops (all approx. 50 μl) were placed under the surface of light mineral oil. The solution was unbuffered except for the indicator itself, and had a pH of about pH 6 as judged by the colour of the drops. Small pieces of cuticle were taken from

both untreated insects and those injected with 5-HT to cause plasticization of the abdominal cuticle. The epidermal cells were scraped as quickly as possible from these cuticle samples which were then placed in the drops of indicator under the oil. Three observations were made. First, that pieces of unplasticized cuticle quickly took up the indicator as a stain, and became yellow in colour. Pieces of plasticized cuticle did not become yellow. Secondly, that the plasticized cuticles became swollen in the drop of indicator, whereas the pieces of normal cuticle did not. Thirdly, that the indicator solution remained yellow in colour in the drops which were in contact with the pieces of unplasticized cuticle, but became decolourized when in contact with the pieces of plasticized cuticle quite quickly, the effect being noticeable after 1–2 min.

The colourless molecule of p-Nitrophenol ionizes to give a highly coloured anion (yellow) and H^+, the dissociation having a pK of about 6. The effective range of the indicator is therefore about pH 5–7 (colourless to yellow).

The interpretation for the above observations suggested here is that the intracuticular pH of the plasticized cuticle samples was considerably lower than that of the unplasticized cuticle samples. This is suggested by the increased swelling shown by the plasticized cuticle, and by its decolorization of the indicator solution. An intracuticular pH below 6 is suggested by both these observations.

This is confirmed by staining experiments with $K_4Fe(CN)_6$ solution. Rapidly prepared samples of cuticle, as described above, were immersed in a dilute solution of $K_4Fe(CN)_6$ for exactly 1 min, rinsed very briefly in distilled water and 'developed' for exactly 1 min in $FeCl_3$ solution. The blue staining of the plasticized cuticle was much more intense than that of the unplasticized cuticle sample, although both took up the stain to some extent. This suggests that the plasticized cuticle is at a lower pH than cuticle in the normal state, and thus possesses a higher density of positive fixed charges.

These experiments do not allow any definite values of pH to be ascribed to the cuticle in either the normal or the plasticized state. However, these experiments considered together constitute strong evidence that the intracuticular pH changes on plasticization. The pH within the unplasticized cuticle is probably higher than pH 6, while within the plasticized cuticle it is probably less than pH 6.

It is difficult to assess the importance of this pH change in the plasticizing mechanism of the intact insect, because its magnitude is not known. However, we have already noted that the movements of ions out of the cuticle on plasticization do not oppose the plasticizing effect, but rather go some way toward assisting it. Bearing this in mind, and referring back to Fig. 3, it is pointed out that a relatively small change in the ambient pH within the cuticle would appear to be sufficient to account for plasticization, perhaps in the order of 0·5–1 pH unit. This is the order of change which we have observed above to occur in the cuticle, and is also in the right range of around pH 6.

It is concluded, on the basis of the evidence presented here, that there is a difference in the intracuticular pH between normal and plasticized cuticle, and that this difference is probably large enough to account for the differences in mechanical properties shown by the cuticle in these states.

(iii) *Urea*

Chromatographic tests on aqueous extracts of normal and plasticized abdominal cuticle failed to show the presence of urea in the cuticle. Plasticization does not seem to be caused by the secretion into the cuticle of urea. Traces of uric acid were found in the cuticle extracts; this is probably a contaminant from the epidermis, where it is known to occur in quantity (Wigglesworth, 1933). Uric acid is not a good hydrogen bond disrupting agent, and cuticle loops do not become plasticized even when soaked in a saturated solution of uric acid at pH 7.

These tests do not, of course, rule out the possibility that another hydrogen bond disrupting agent might cause plasticization of the cuticle *in vivo*.

GENERAL DISCUSSION

The results reported in Part II of this paper have led to the conclusion that, of the possible mechanisms of plasticization proposed in Part I, the lowering of the intracuticular pH is most likely to operate *in vivo*. Alternative methods by which the epidermal cells might achieve the same end have either been shown not to operate in life (reduction in intracuticular ionic strength; removal of divalent cations) or may be dismissed as unlikely in the absence of any evidence in their support (hydrogen bond disruption by urea), and in any case unnecessary by the finding that there appears to be a pH change within the cuticle on plasticization. It is likely that this pH change alone is large enough to account for the increased extensibility shown by plasticized cuticle.

The increased swelling power shown by the cuticle in its plasticized state (both predicted from model experiments with altered pH and directly observed in samples of plasticized cuticle), may explain the apparently anomalous results of Maddrell (1966), in which the extensibility of the abdominal cuticle was related to the osmolarity of the haemolymph. In one series of experiments it was found that when oxblood was injected into the haemocoel, the extent of the plasticization which was produced could be modulated by the osmolarity of the Ringer injected at the same time. It is very likely that the action of injected oxblood is pharmacological (Reynolds, 1974), inducing the epidermis to plasticize the cuticle in the normal way. In the plasticized state, at low pH, the cuticle may act as a kind of osmometer, swelling more when the contents of the haemocoel are dilute than when they are concentrated, with corresponding effects on mechanical properties. The similar dependence observed by Maddrell of cuticular extensibility on the osmolarity of the haemocoel contents when the cuticle is in the *unplasticized* state is more difficult to explain. Perhaps, it may be suggested, one action of the injections of diluted haemolymph or distilled water was to cause plasticization by an indirect route, viz. by causing the release from the epidermal nerve terminals of the neurohumour which normally initiates plasticization. Such a release might well be caused by osmotic shock.

It might be pointed out that the alteration of intracuticular pH is energetically an extremely economic way of effecting plasticization. A pH change of one unit, from pH 6 to pH 5 represents a change within the cuticle of 9×10^{-5} M in the concentration of hydrogen ions. This would be more than sufficient to account for plasticization.

using the removal of other cations to achieve the same end would require a change in concentration within the cuticle of about 10^{-1} M, so that 10^3 times more ions would have to be moved.

Thus, the suggestion of Bennet-Clark (1961) as to the mechanism of plasticization in the abdominal cuticle of *Rhodnius* is probably correct. Harkness (1965) has suggested that a similar mechanism employing a change in extracellular pH might be employed by cells to alter the mechanical properties of connective tissues in vertebrates, 'either to move through them or to mould them'. To the best of my knowledge, the abdominal integument of *Rhodnius* is the first example where evidence has been found that cells may actually use this remarkably simple method to control the mechanical properties of an extracellular structure.

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