COMPONENT PERMEABILITIES AND WATER CONTENTS IN *PERIPLANETA* INTEGUMENT: ROLE OF THE EPIDERMIS RE-EXAMINED

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SUMMARY

Improved *in vivo* and *in vitro* techniques for measuring cuticular water permeability are described. Air flowing over a cuticle disc mounted in a holder, permitted elimination of unstirred layers, or corrections for them, for the first time. Conditions inside the holder were incompatible with the long-term health of the epidermal cells. Significantly, mean permeabilities of these discs did not differ from values obtained *in vivo* on the same cuticular plate.

Overall cuticular permeability was apportioned between endocuticle and combined epicuticle and exocuticle on the basis of measurements made before and after solvent extraction of lipids. Under identical activity gradients, endocuticle permeability was 35 to 40 times greater than the value for the other layer. Permeability of both component layers showed strongly non-linear relationships with ambient activity, with empirical proportionality to the reciprocal of vapour pressure lowering.

Cuticle water contents measured in activity gradient conditions showed significantly higher values *in vivo* than *in vitro*. The amount of water contained in the combined epicuticle and exocuticle was too small to measure.

We conclude that neither permeability nor water content data support the existence of a significant water barrier in the region of the epidermis.

INTRODUCTION

Over the last decade or so, several experimental studies have claimed that the epidermis plays a significant role in regulating cuticular water content. Mass changes of excised cuticle following exposure to water activities equivalent to that of the haemolymph have been interpreted as evidence that cuticular water is not in equilibrium with the body fluids (Winston & Nelson, 1965; Winston, 1967; Winston & Hoffmeier, 1968; Winston & Beament, 1969; Lindqvist, Salminen & Winston, 1972). A change in cuticle water activity might modulate mechanical properties of cuticle (Reynolds, 1975; Hillerton & Vincent, 1979) or reduce water loss by decreasing cuticle permeability or altering the activity gradient across the cuticle. It has also

been suggested that integumental permeability is in some way hormonally controlled (Treherne & Willmer, 1975a,b).

On the other hand, Riddle (1981) has attributed water activity deficits to a significant water barrier between the haemolymph and cuticle. Berridge (1970) suggested that any form of epidermal influence over cuticular water would have to be associated with a significant barrier. If so, measurable differences should be apparent when the permeability of intact integument is compared to that of the cuticle alone. The operation of passive forces favoured by Riddle (1981) would distribute integumental water according to the prevailing activity gradients, determined by the permeabilities of component layers. The active model suggests, on the other hand, that the observed water distribution would differ from that predicted from permeabilities and activity gradients. Comparisons of cuticle water content with and without an epidermis will help determine whether or not the epidermis is significantly involved.

A complete understanding of all of the forces governing the distribution of water within the cuticle, however, will not be possible until the water holding properties of the layers which make up the integument are separately investigated. Many of the relevant principles were laid down in Beament's (1981) review of insect water relations. However as Beament and far more recent reviewers such as Gilby (1980) have noted, complete component permeability values, let alone water contents, are lacking even for a single species. This paper is an attempt to rectify the deficiency. As a first step it has been practical to treat the epidermis, inner endocuticle and combined epicuticle-exocuticle as separate layers.

MATERIALS AND METHODS

We used *Periplaneta americana* because of the numerous studies on this species, including those claiming an epidermal role in regulating or determining cuticular permeability (Winston & Beament, 1969). Our study was restricted to the prothoracic shield or pronotum of *Periplaneta*, a single cuticular plate large enough to be studied *in vitro*. Our measurements are therefore uncomplicated by regional differences in cuticle permeability, or by parallel water loss through the spiracles, anus and mouth (Hadley, 1982).

Experimental animals were bred in a laboratory culture, fed *ad libitum* on lab chow and water and maintained at a regulated temperature of 24–27 °C at 43–45 % relative humidity. The cuticle preparations were taken from adult specimens without regard to sex or age. A Wescor 5100B Vapour Pressure Osmometer was used to determine haemolymph osmotic pressures of a representative sample of animals directly from the culture and following 6 h exposure to a variety of regulated ambient activities.

Permeability measurement

In vivo

Integumental water loss was measured using a technique similar to that described by Nicolson, Louw & Edney (1984). A specially designed brass cup (Fig. 1A) was fitted to the pronotum of a cockroach temporarily immobilized by chilling at 1 °C for approximately 2 h. All animals recovered from chilling and their subsequent activity

A

One of three clamps

Clamping screw

'Fun-tak'

Pronotum

1 cm

C

2 cm

B

Control stream

Brass cup held
by adjustable clamp

Dewar flask

Liquid nitrogen

Vapour traps

Cockroach

Double
flow meter
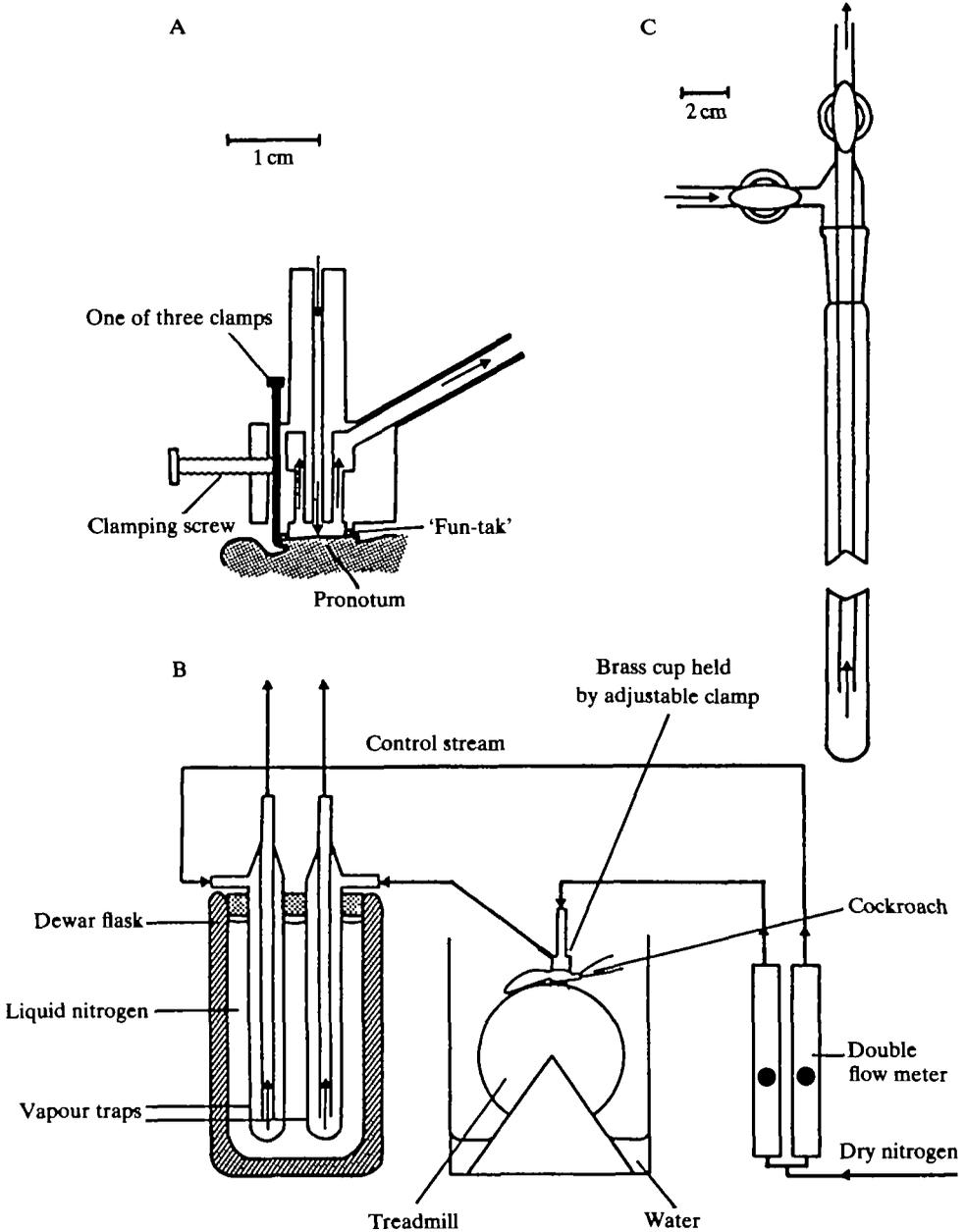
Dry nitrogen

Treadmill   Water

Fig. 1 Diagrams to show *in vivo* cuticular permeability measuring technique. (A) Section of cup clamped to cockroach pronotum. (B) Pyrex water vapour trap. The collecting thimble of the trap is approximately 20 cm long. (C) Diagram showing complete apparatus and direction of nitrogen flow.

suggested no ill effects. The base of the cup was sealed to the animal with a thin ring of 'Fun-tak' mastic paste (Lepages Ltd, Bramalea, Ontario, Canada) and secured with three blunt hooks beneath the ventral edge of the pronotum. Once the animal had recovered, a regulated stream of dry nitrogen (0·29 mmHg vapour pressure; water

activity, a_w, 0·015) at room temperature ($22\pm1\cdot0$ °C) was passed through the cup, whose dimensions matched those of the *in vitro* cuticle holder described below. A glass vapour trap (Fig. 1B) placed in a Dewar of liquid nitrogen collected the evaporated water while an identical control stream was passed directly to a second vapour trap. The difference in mass gain of the two traps after 48 h gave the net water flux from which the *in vivo* pronotal permeability was calculated. It was necessary to use nitrogen as a carrying gas rather than air because liquid oxygen, which has a higher boiling point than nitrogen, was found to collect in the traps. The use of nitrogen necessitated low flow rates (63 ml min^{-1}) which were insufficient to eliminate un-stirred layers. However the difference between permeability values before and after correction for this error was insignificant ($P>0\cdot05$). Intermittent checks for leakage from the cup were made by comparing flow rates of the two streams by bubbling the effluent nitrogen from the traps through water.

To reduce the risk of injury and stress during the 48 h of restraint the cockroaches were supported on a 9 cm diameter polystyrene 'wheel' (Fig. 1C) which was placed in a darkened container with water to increase ambient humidity.

In vitro

Cuticle permeabilities *in vitro* were measured by mounting pronotal cuticle samples in specially designed holders. Pronotal plates which had been dissected from decapitated cockroaches were cut into discs 7 mm in diameter using a specially har-dened stainless steel punch (Fig. 2A) operated by a small hand press. A sharp cutting edge working against a Teflon cutting surface minimized edge damage to the discs. After the disc had been cut, remaining pieces of the ventral folding of the pronotum were carefully removed using fine forceps. Measurements were made using intact discs of complete integument, solvent extracted integument, or cuticle discs as used for water content measurement with the epidermis removed by gentle scraping. Discs were solvent extracted by shaking in 20 ml chloroform: methanol (1:2) for 2 h at room temperature. It had been established by progressive weighing and permeability measurement that this procedure removed all extractable material from the disc. Since solvent extraction did not remove the non-lipid components of the outer layers, subsequent permeabilities were corrected to endocuticle thickness.

After preparation the discs were placed between 0·013 mm thick polyethlyene washers and clamped firmly in a stainless steel cuticle holder by a pressure ring screwed down with an open threaded cap (Fig. 2B). The pressure ring was provided with a central tubular inlet surrounded by six outlet holes. A Teflon washer, 0.5 mm thick, between the pressure ring and the cap, prevented the ring twisting against the disc as the cap was tightened. The edge clamping leaves a 6 mm diameter free disc with an area of 0·283 cm^2.

The holder was partially filled with cockroach Ringer (approximately 1 ml) which matched the ionic composition, mean osmotic pressure and pH of the haemolymph as closely as possible. The Ringer combined the 'universal buffer' at pH 7·0, of Perrin & Dempsey (1974) with commonly used ion and glucose concentrations obtained from a summary of the literature by Guthrie & Tindall (1968) (in mmol l^{-1}) NaCl, 167·7; KCl, 30.3; CaCl$_2$. 2H$_2$O, 4·5; NaHCO$_3$, 2·1; glucose, 22·2; NaH$_2$PO$_4$. 2H$_2$O, 0·1; citric acid, KH$_2$PO$_4$, sodium tetraborate and Tris, 20; and NaOH, 44·8. The

A

1 cm

Hardened
stainless steel

Hardened
cutting edge

C

2 cm

To hygrometer

Needle valve

Water level

Plexiglas air
distributing
manifold

B

1 cm

Glass
capillary

Copper wall
of chamber

O-ring

End view of
clamping assembly

Threaded
lid

Threaded cap

Rubber washer

Teflon lining

Outlet holes   Inlet tube

Cuticle disc

Polyethylene
washers

Pressure
ring

Teflon washer

Sealing O-ring

Stainless steel
cuticle holder

Cuticle clamping assembly

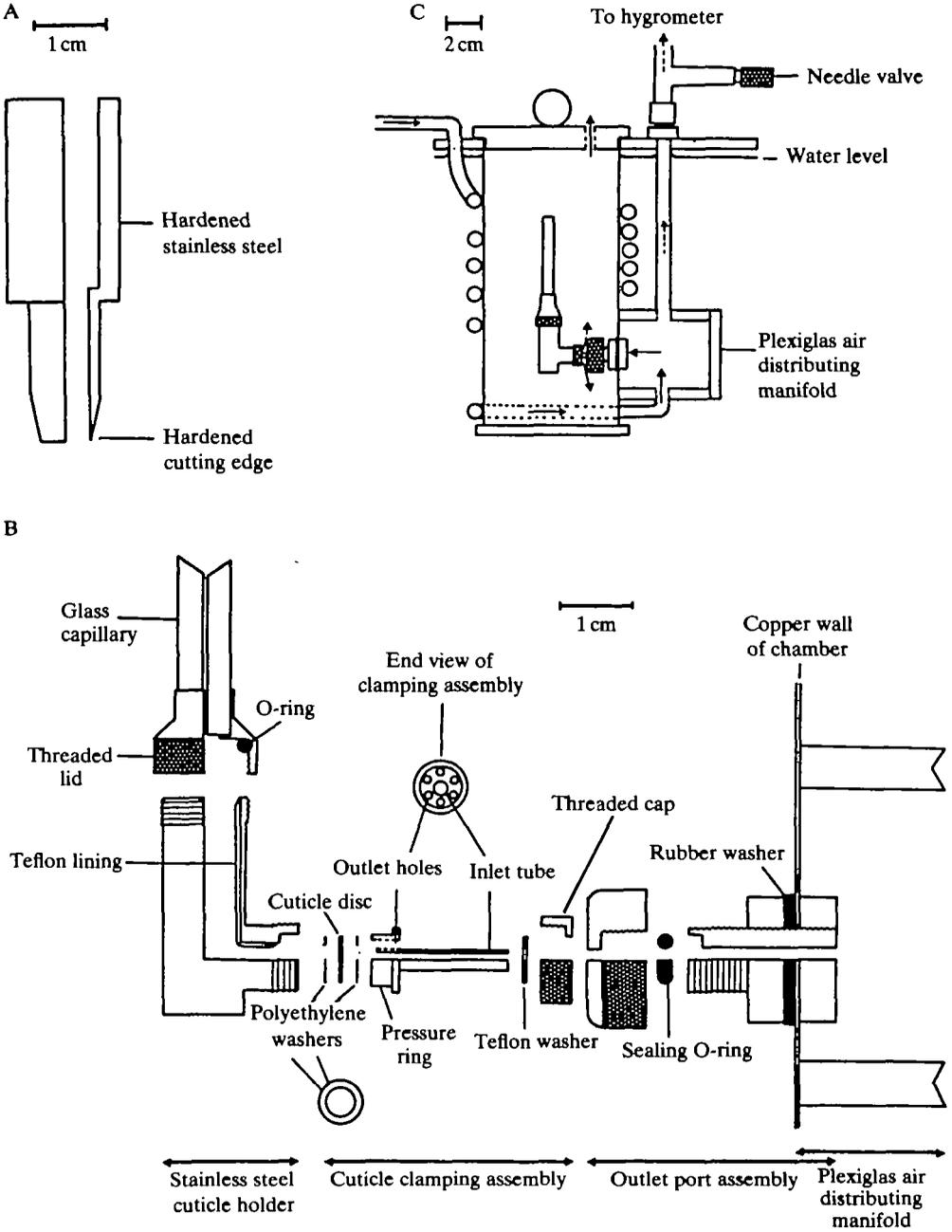Outlet port assembly

Plexiglas air
distributing
manifold

Fig. 2. Exploded sectional diagrams of *in vitro* cuticular measuring apparatus. (A) The pronotal disc cutter. (B) The cuticle holder and outlet port of the air distribution system. (C) The temperature-regulated air distribution system which is contained in a rectangular, circulating water bath.

pH was 7·0 and osmotic pressure was adjusted to 383 mosmol l^{-1} by addition of concentrated NaCl or distilled water. The solvent activity of such a solution is 0·9931 at 20 °C.

The holder was sealed with a threaded cap provided with an O-ring and a 4·5 cm glass capillary (0·055 mm bore) to allow equalization of pressure. Total leakage, measured using a double layer of aluminium foil sandwiched between polyethylene gaskets in place of cuticle, was $7·05 \times 10^{-4}$ mg h^{-1} mmHg^{-1} ($N=9$). Calculations using the coefficient of diffusion of water vapour in air (Leighly, 1937) indicated that about half ($3·2 \times 10^{-4}$ mg h^{-1} mmHg^{-1}) was apparently lost through the capillary. Leakage through the seals and capillary tube of the holder represented less than 6 % of the mean cuticle flux of 0·012 mg h^{-1} mmHg^{-1}.

Flowing air was supplied to the cuticle surface by inserting the inlet tube into one of six ports of an air-distributing manifold (Fig. 2B, C) immersed in a temperature controlled water bath at 20±0·1 °C. The air stream, dried over silica gel or regulated by temperature at any chosen humidity in the system described by Machin (1976), was brought to the experimental temperature by passing it through a 4-m copper tube associated with the distribution system. Humidity was monitored by diverting a small portion of the air stream, through a needle valve, to a Cambridge Systems thermo-electric dewpoint hygrometer.

Calculations in the manner of House (1974) indicated that errors due to unstirred layers in the Ringer were less than 0·1 % for whole cuticle and less than 5 % for endocuticle. However all fluxes were influenced by unstirred layers in the air since water fluxes through the cuticle increased with air-flow without reaching a plateau even at maximum flow rates. To quantify this error, a blank run was performed with a Millipore filter disc (0·1 μm pore) replacing the cuticle in the holder. Since the filter approximated a free water surface (Machin, 1969) the effective permeability of the air above the membrane could be calculated. At 20 °C it was found to be 42·9 mg cm^{-2} h^{-1} mmHg^{-1} at maximum air flow rates of 1·5 1 min^{-1}. This value was used to correct apparent permeability for unstirred layer errors using the following general relationship relating overall permeability (P_O) to the component permeabilities (P_1 and P_2):

$$\frac{1}{P_O} = \frac{1}{P_1} + \frac{1}{P_2},\tag{1}$$

where P_O is the uncorrected permeability, P_1 the corrected value and P_2 the apparent permeability of the unstirred layer. The greatest error due to unstirred layers was found to be a negligible 0·1 % for whole cuticle but 49·8 % for the much more permeable endocuticle.

Experiments with thermocouples established that evaporative cooling was negligible. This was probably due to the temperature stabilizing effects of flowing air and also the heat conducting properties of the metal holder.

Water fluxes were determined by weighing the cuticle holders on an analytical balance accurate to 0·05 mg. To obtain the most reliable values the holders with cuticles were first equilibrated with flowing air for 30 min before the initial weighing. They were left until they had lost about 10 mg, then weighed again. The length of time between weighings (about 48 h for untreated cuticle) was the principal limitation of

the technique. Weighing times could be reduced to 3 h, with less precision, using a continuous weighing technique combined with statistical analysis of the results. Single cuticle holders, exposed to still, dry air at 20 °C, were continuously weighed on a Mettler AK 160 balance accurate to 0·1 mg. Masses were read automatically at 60-s intervals and stored on a computer data acquisition system. The rate of mass loss was then determined by least squares regression analysis of data collected over a period of 3 h ($r^2 > 0\cdot90$).

Cuticle water content

The very high water content of the epidermis (Table 1) made it crucially important that the soft tissues were completely removed. All attached soft tissue was removed by gentle scraping with the back of a scalpel blade and rubbing with pieces of Ringer-moistened tissue. Measurements were made in two conditions. In gradient conditions, water activity was graded from the inside activity of the haemolymph or Ringer to the ambient value, and a net outward flux of water occurred. For *in vivo* determinations pronotal discs were taken directly from animals previously exposed to known ambient activities for 6 h. Cockroaches were held in small, open-ended vials covered with a layer of cheesecloth and placed in the humidity chamber used for *in vitro* permeability measurement. The discs were subsequently placed in the cuticle holders in the same ambient conditions for a further 24 h, for *in vitro* measurement.

In non-gradient or equilibrium conditions complete pronotal discs, as well as ones with endocuticle alone, were equilibrated in turn to a declining series of water activities, starting with Ringer. To prevent condensation on the samples, particularly in very high activities, exceptionally accurate temperature regulation is required (Machin, 1984). The controlling solutions, Ringer and several saturated salt solutions were therefore held in a heavy brass cell (2·6 kg mass) whose thermal mass reduced the temperature oscillations (20·0 ± 0·1 °C) of the regulating water bath to ±0·01 °C. A central well surrounded with filter paper wicks held the solutions. Before cuticle excision cockroaches were equilibrated to Ringer-saturated air for 6 h by placing them in plastic boxes lined with filter paper wicks and partially filled with Ringer. Ambient activity was checked with a Vaisala HMA 14A electronic hygrometer probe. To obtain endocuticle samples both lipid and non-lipid outer layers of the epi-exocuticle were completely removed from cuticle discs by abrasion with fine emery paper.

All water content determinations were made by weighing the cuticle preparations on a recording Mettler ME22 microbalance (accurate to 1 μg) after being blotted between filter paper. Mass loss was then recorded on a strip chart recorder for several minutes to permit exponential extrapolation to the time of blotting in order to compensate for evaporative losses. The time which elapsed between blotting and the beginning of the chart recording was carefully recorded with a stopwatch. Dry masses were determined after 48 h over dried silica gel.

RESULTS

Permeability

In transverse frozen sections of pronotal cuticle, an outer brown layer, apparently consisting of epicuticle and exocuticle (Kramer & Wigglesworth, 1950;

Table 1. *Morphometry of 7 mm diameter* Periplaneta *pronotal discs at 20 °C*

	Hydrating conditions	Total integument (cuticle + epiderms)	Cuticle	Endocuticle	Epi-exocuticle
PRIMARY DATA					
Dry mass (mg ± S.E.)		2·349 ± 0·102 (13)	1·691 ± 0·030 (58)	1·175 ± 0·024 (65)	0·434 ± 0·014 (65)
Dry volume (mm³ ± S.E.)	Gradient equilibrated				
Water content (g g⁻¹ dry mass ± S.E.)		2·140 ± 0·118 (5)	0·264 ± 0·009 (57) 0·256 ± 0·010 (15)		
Hydrated volume (mm³ ± S.E.)	Equilibrated			1·432 ± 0·035 (39)	0·498 ± 0·026 (39)
DERIVED VALUES					
Integumental water content (mg)	Equilibrated gradient	5·027	0·433 0·446	0·433	
Proportion of total integumental water		0·921	0·079	0·079	
Dry density (mg mm⁻³)			1·049	0·962	
Dry mass (mg)				1·130	0·561
Proportion of dry mass				0·668	0·332

Hydrated values in equilibrated conditions are in haemolymph activities of 0·9931 and in gradient conditions with ambient dry air activities of 0·046.
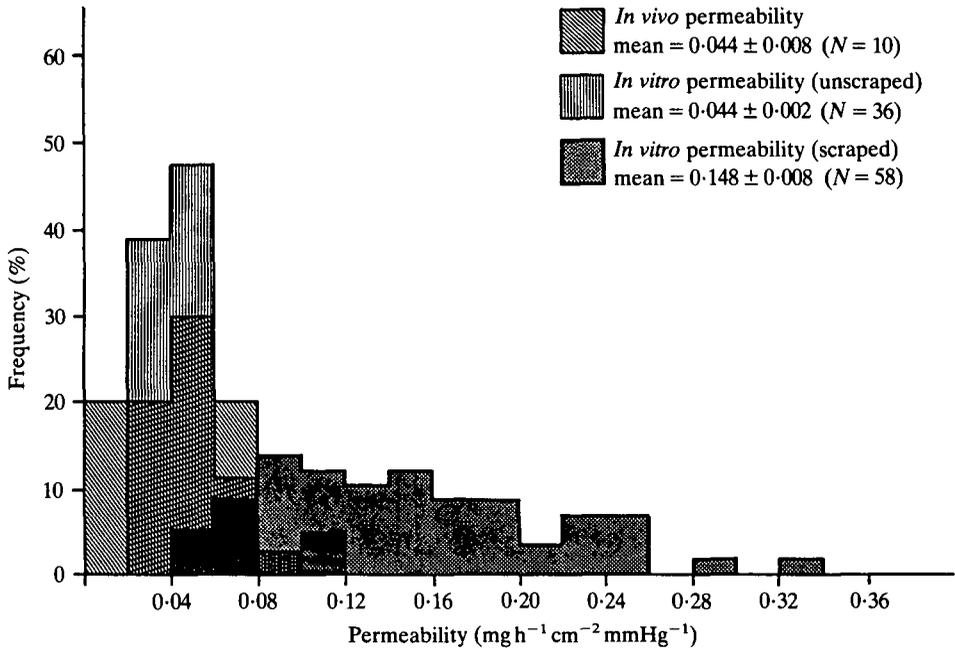
Fig. 3. Histogram showing frequency distribution of pronotal permeabilities to illustrate the effects of different methods of preparation. Mean values are in $mg\,h^{-1}\,cm^{-2}\,mmHg^{-1}$.
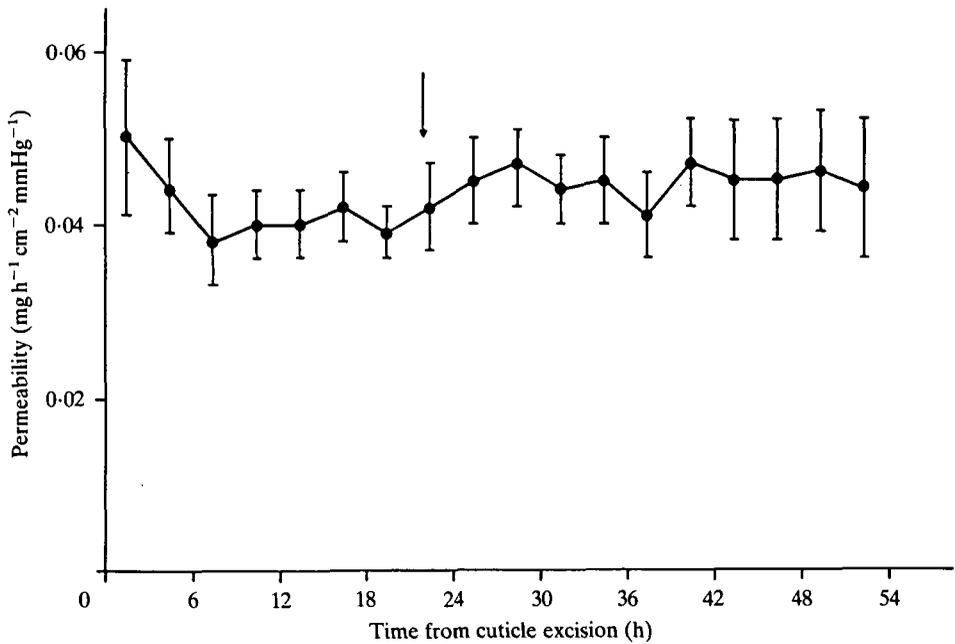

Fig. 4 Graph showing change in *in vitro* pronotal permeability with time following excision from the cockroach. The arrow marks the point at which the epidermis has become partially disintegrated.

Wigglesworth, 1975) contributed a mean hydrated thickness of $11.4 \pm 0.4 \mu m$, while the remaining white layer, making up approximately three-quarters of the 46.1 ± 1.0 μm total, was apparently endocuticle. Table 1 summarizes morphometric data for the pronotal discs.

The frequency distribution of *in vivo* and *in vitro* permeabilities indicated no significant difference ($P > 0.05$) between mean unscraped permeabilities *in vitro* and those obtained with the pronotal cup on live animals in which the epidermal layer was, of course, healthy (Fig. 3). It can be seen that scraping the underside of the cuticle, to remove epidermal tissues, skewed the permeabilities towards higher values, significantly elevating the mean value. Time course experiments, however, showed a slight permeability increase between 18 and 30 h, but none of the values calculated at successive 3-h periods up to 54 h were significantly different ($P > 0.05$) (Fig. 4). Slightly higher initial permeabilities may have been due to the evaporation of fluids squeezed from the seal by the clamping process. Histological examination of the epidermis after varying lengths of time in the cuticle holder indicated a disintegration of the epidermis which had become extensive by 22 h, probably because physiological conditions in the Ringer were inadequate to sustain the health of the epidermis. Measurements made with Ringer at the end of the experiment showed P_{O_2} and pH had been sharply reduced and P_{CO_2} considerably elevated.

Equation 1 was also used to calculate epi-exocuticle permeability (P_2) knowing overall cuticle permeability (P_O) and that of the endocuticle (P_1). It can be seen from Table 2 that solvent extraction led to considerably elevated permeabilities. Equation 3 for endocuticle is based on values from higher activities appropriate to the internal location of this layer. The permeabilities of all cuticle layers changed noticeably with the ambient humidity. The best least squares fit to the data yielded the following relationships.

$$\text{Whole cuticle } P = 0.0348 + \frac{0.254}{vp_s - vp_a} \qquad (2)$$

$$\text{Endocuticle } P = -0.892 + \frac{9.424}{vp_s - vp_a} \qquad (3)$$

$$\text{Epi-exocuticle } P = 0.0355 + \frac{0.263}{vp_s - vp_a}, \qquad (4)$$

where P is the corresponding permeability, vp_a ambient vapour pressure and vp_s saturated vapour pressure at $20\,^{\circ}C$. Values for r^2 are 0.974, 0.915 and 0.975, respectively.

Cuticle water content

Table 3 shows that *in vivo* water contents were about 20 % higher than the *in vitro* values. The differences between water contents at corresponding ambient activities were significant ($P > 0.001$). For both groups, water contents declined only slightly with ambient activity. In contrast to the comparatively small effect of external factors on cuticle water content, the effect of the composition of fluids bathing the inside of cuticle could be quite marked. In a preliminary series of experiments we found that

Table 2. *Effect of different ambient activities on* in vitro *cuticle permeability at 20 °C in vigorously flowing air*

Ambient activity (a_w)	Whole cuticle		Endocuticle		Calculated epi-exocuticle
	N	Mean permeability ± s.e. ($mg\,h^{-1}\,cm^{-2}\,mmHg^{-1}$)	N	Mean permeability ± s.e. ($mg\,h^{-1}\,cm^{-2}\,mmHg^{-1}$)	Permeability ($mg\,h^{-1}\,cm^{-2}\,mmHg^{-1}$)
0·988	–	–	12	47·858 ± 5·185	–
0·975	–	–	12	12·829 ± 0·662	–
0·963	–	–	12	9·046 ± 0·440	–
0·911	–	–	12	5·947 ± 0·203	–
0·850	5	0·130 ± 0·005	18	4·769 ± 0·206	0·134
0·540	5	0·074 ± 0·003	12	2·777 ± 0·107	0·076
0·046	36	0·044 ± 0·002	12	1·831 ± 0·063	0·045

Endocuticle values were obtained from solvent-extricated cuticle discs, corrected for endocuticle thickness and considerable unstirred layer errors.

changing the pH of the bathing Ringer from 7 to 3 significantly increased the water content from $0·265\pm0·009$ to $0·363\pm0·016\,g\,g^{-1}$ dry mass ($N = 12$). A similar phenomenon has been observed for the abdominal cuticle of *Rhodnius* (Reynolds, 1975).

The relationship between equilibrium water content and water activity in cuticle was strongly non-linear, a finding that has been well established previously with whole cuticle preparations from other species (Machin, 1979; O'Donnell, 1982). The relationship, referred to below as water affinity, is such that water content per gram dry mass is proportional to the reciprocal of vapour pressure lowering (Fig. 5). The equations describing the best least squares fit for whole cuticle ($r^2 = 0·925$) and endocuticle ($r^2 = 0·849$) are:

$$\text{whole cuticle equilibrium water content} = 0·106 + \frac{0·0234}{vp_s - vp_a}, \qquad (5)$$

$$\text{endocuticle equilibrium water content} = 0·0915 + \frac{0·0185}{vp_s - vp_a}. \qquad (6)$$

Table 3. *Comparison of* in vivo *and* in vitro *cuticle water contents under gradient conditions at 20 °C*

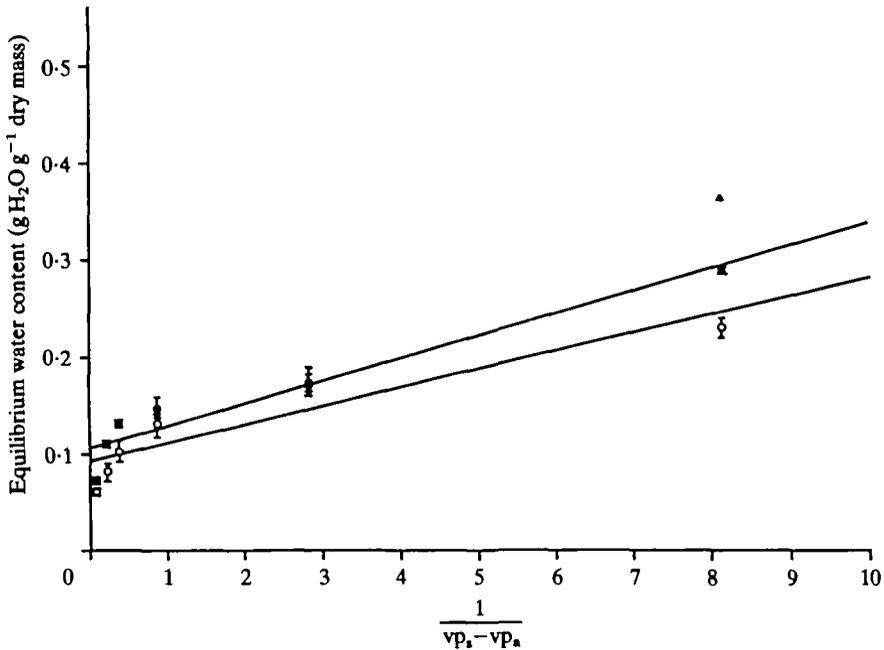| Ambient activity (a_w)	In vivo		In vitro	
	N	Mean water content ± s.e. ($g\,g^{-1}$ dry mass)	N	Mean water content ± s.e. ($g\,g^{-1}$ dry mass)
0.9931 (Ringer)	16	0·366 ± 0·009	16	0·299 ± 0·006
0·85	18	0·357 ± 0·011	18	0·307 ± 0·008
0·56	17	0·357 ± 0·014	17	0·295 ± 0·012
0·20	18	0·286 ± 0·006	18	0·243 ± 0·006
0·046	18	0·281 ± 0·012	18	0·220 ± 0·008

Fig. 5. Graph showing relationships between whole cuticle (●) and endocuticle (○) equilibrium water contents and reciprocal of vapour pressure lowering measured *in vitro*. Water contents are expressed per gram dry mass of the appropriate layer. The *in vivo* value for whole cuticle (△) at haemolymph activity is added for comparison. Error bars were smaller than the symbol.

There was no significant difference between whole cuticle and endocuticle water affinities ($P > 0.05$) indicating that epi-exocuticle water content was too small to detect.

DISCUSSION

The striking agreement between *in vivo* and *in vitro* results and lack of significant change in permeability with time provides convincing evidence that the epidermal barrier has only minor influence, if any, on the permeability of *Periplaneta* pronotum. The agreement is specially significant when it is realized that the leakage encountered in the *in vivo* method would tend to underestimate permeability whereas *in vitro* leakage would give an overestimate.

If the removal of the epidermis is not the cause of increased permeabilities following scraping, cuticle damage must be the explanation. In fact, elevated permeability of some samples and normal values of others following scraping support this view. We are reasonably certain that tissue removal did not lead to abrasion damage of the outer layers because pronotal plates subjected to the same amount of handling without scraping showed no differences in permeability between those cut with the outside surface downwards and those reversed. It appears more likely that the water conducting properties of the numerous dermal gland canals known to perforate *Periplaneta* pronotum (Scheie & Smyth, 1967; Scheie, Smyth & Greer, 1968) change as a result

of scraping. Assuming the damage results in the production of high conductance pathways, as would occur if a number of canals became air-filled pinholes, we can calculate the total area necessary to account for the permeability increase. From the diffusion coefficient of water vapour (Leighly, 1937) and a diffusion path of 0·046 mm, the mean thickness of hydrated pronotum, the area of pinholes to account for the increased permeability would be $4·8 \times 10^{-4}$ cm^2 or 0·17 % of the total disc area. For ducts of 10 μm diameter the area amounts to 610 ducts per cuticle disc, or only 0·5 % of the total thought to be present. Thus damage to only a small proportion of the ducts would be sufficient to account for permeability changes associated with scraping the inner surface of the cuticle disc.

The distribution of permeabilities among component cuticular layers conforms to the general principle (Beament, 1961) that the barrier to water is likely to be superficially located. Nevertheless, the results have contributed several new insights to the complex properties of insect cuticle. Water flux studies are rarely performed in anything other than dry, ambient air and the discovery that the permeability of the entire cuticle as well as both component layers is gradient dependent, demonstrates the complexity of the interaction between cuticle and penetrating water molecules. In addition, the increased sophistication and repeatability of the *in vitro* technique has provided new quantitative information concerning the differences between inner and outer cuticular layers. This is particularly true of the endocuticle permeabilities, which have been corrected for unstirred layer errors for the first time. The slopes of the permeability equations indicate that the endocuticle is about 35 to 40 times as permeable as the combined outer layers under identical activity gradients. The difference in the water affinities of these layers must be even greater, the endocuticle being more hydrophilic than the other layers.

Considerable difficulty in obtaining repeatable water contents was experienced in early stages of this work. Accurate water measurement depended on two important procedures, rapid but complete removal of epidermal tissues and subsequent weighing to determine water content. In our judgement it is virtually impossible to remove all traces of tissue from the entire pronotum because of cuticular folding at the edge. We feel that effective cleaning is greatly aided by punching a disc from the centre of the plate to avoid the complex edges. There is good agreement between mean water contents based on this technique, in the present study, and those of Winston & Beament (1969) (converted to g H$_2$O g^{-1} dry mass) with lower standard errors for similar sample size in this study. On the other hand, Treherne & Willmer's (1975b) water contents of complete pronotal discs after dry mass standardization are between two and three times higher.

This paper establishes that the cuticular layers differ with respect to their water affinities. It now has been quantitatively confirmed that the cuticle consists of outer hydrophobic layers of low permeability overlying the bulkier, high permeability, hydrophilic endocuticle. The sclerotization of the outer cuticular layer (Hillerton & Vincent, 1979), as well as its higher lipid content (Kramer & Wigglesworth, 1950), may be the cause of its lower water content. In natural conditions of course, differences of water distribution are exaggerated by existing water activity gradients which normally decline outwards. The relative stability of whole cuticle water contents over the widest range of ambient activities as well as the large changes

accompanying massive internal pH shifts are consistent with this distribution of properties.

Empirical dependence of both equilibrium water content and permeability on the reciprocal of vapour pressure lowering suggests that permeability might depend on cuticular water content. The possible significance of this inter-relationship is to be investigated more fully in a succeeding paper (Machin & Lampert, 1985). The principle that the water content of complex organic materials such as cuticle follows a strongly non-linear relationship with activity is well established (Beament, 1961; Skaar, 1972; Kuntz & Kaufmann, 1974). It has been shown that cuticle water contents in intact insects with a healthy epidermis are significantly higher than *in vitro* values. The possibility remains therefore that the epidermis is involved in maintaining elevated water activity in the cuticle. Since it has previously been established that the endocuticle *in vitro* is already highly hydrated, the adaptive significance of even more water is unclear. We prefer the explanation that following excision and unavoidable partial dehydration associated with weighing, slight, irreversible changes which affect water binding do occur. The hysteresis occurs only once because further dehydration does not affect the cuticle's capacity to rehydrate to typical *in vitro* levels. This latter explanation would be more consistent with earlier conclusions about the insignificance of epidermal permeability.

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