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THE EFFECT OF BRAIN HOMOGENATES ON DIRECTLY MEASURED WATER FLUXES THROUGH THE PRONOTUM OF *PERIPLANETA AMERICANA*

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Summary

Measurements of tritiated water (THO) efflux using a cup, ventilated with dry air, attached to the pronotum of *Periplaneta*, have been used to obtain unambiguous measurements of the permeability of this structure. Permeability values were 53 % of those determined gravimetrically. Our results support the proposal that cockroach cuticle permeability is hormonally controlled, to the extent that compounds extracted from the brain have been shown to be capable of inducing permeability change over relatively short periods. Fresh brain homogenate injections from hydrated donors produced a 28 % increase in pronotal permeability in 5 h, rising to 46 % the day after. Injections of saline or of fresh brain extracts from dehydrated and normally hydrated cockroaches had no effect. However, previously frozen brain homogenates, from donors at all hydration levels, significantly increased pronotal permeability the day following injection.

The capacity of the pronotum to undergo increases in permeability over relatively short periods was also evident in other experiments. THO permeabilities of excised pronotal discs ($5.16\times10^{-10}\pm0.31\times10^{-10}$ m s^{-1}) were an order of magnitude higher than those of intact pronota ($0.49\times10^{-10}\pm0.06\times10^{-10}$ m s^{-1}). *In vivo* permeability of pronota of accidentally injured cockroaches was significantly higher than that of uninjured animals, even though the pronota themselves were not damaged.

We argue that the observed changes in cuticle permeability are too small primarily to serve osmoregulatory functions. We suggest, instead, that the changes might be associated with the control of secondary processes in which the cuticle is involved and which unavoidably promote water loss.

Introduction

Noble-Nesbitt and Al-Shukur (1987, 1988*a*,*b*) have argued that cuticle permeability in *Periplaneta* is under hormonal control. The proposed mechanism involves different brain factors which increase water permeability in hydrated

Key words: *Periplaneta americana*, tritiated water efflux, pronotal permeability, hormonal control.

animals and decrease it when they are dehydrated. This complex mechanism is based on observed changes in whole-animal water loss following decapitation and after experimental injection of brain and abdominal ganglion homogenates into intact and headless recipients. In most instances, experimentally induced changes in water loss might reasonably be attributed to changes in cuticle permeability because most non-motile insects lose more water through the cuticle than through the spiracles (Edney, 1977). Quiescent *Periplaneta* are no exception to this general principle (Machin *et al.* 1991). However, this species appears to be unusually sensitive to human interference in some experimental situations, even when the cockroaches are enclosed in containers to avoid direct handling. Following introduction into a balance, spiracular losses can be as much as 3.3 times higher than cuticular fluxes in hydrated animals and 2.1 times in dehydrated individuals (Machin *et al.* 1991). These, and other periods of elevated and often changing rates of weight loss, were associated with exploration following introduction into an unfamiliar weighing chamber, grooming and other activities leading to increased metabolic rate.

Machin *et al.* (1991) also found that long-term average water losses during spiracular constriction were 60 % higher in hydrated cockroaches compared with dehydrated individuals. While the identification of spiracular constriction in continuous weight recordings may be used in a general way to estimate the relative importance of cuticular and spiracular water losses (Kestler, 1985), the technique may not be absolutely reliable in all circumstances. For example, it is not possible to conclude with certainty that the changes in cuticular permeability described above were not the result of slight, hydration-related differences in the effectiveness of spiracular sealing. Tests of hormonal effect on the cuticle must, of course, ultimately rest on unambiguous measurements of cuticle permeability. To this end we chose the ventilated cup technique pioneered by Nicolson *et al.* (1984) and also by Hadley *et al.* (1986) using tritiated water (THO) to measure pronotal water flux *in vivo*. We also used the excised pronotal disc technique of Machin *et al.* (1985) to compare THO and gravimetrically determined water fluxes. This paper reports the results of such measurements and the effect of injecting brain homogenates into dehydrated recipients.

Materials and methods

Adult *Periplaneta americana* of both sexes were obtained from a laboratory culture, provided with unlimited dry laboratory chow and water and maintained at a regulated temperature of 24–27 °C and approximately 44 % relative humidity (RH). At least 3 days before experimentation cockroaches were removed from the culture and placed in small jars, again with free access to dry food and water. Initial water content of all experimental animals was assumed to be equivalent to that of a sample of animals kept under the same conditions, which produced water contents between 68 and 71 % of wet mass (mean 69.3±0.3 %, N=14) with no significant difference between the sexes (P>0.7).

THO efflux in vivo

For THO efflux measurement, cockroaches were first anaesthetised by exposure to CO_2 for 20 s, then a ventilated brass cup, specially designed to fit onto the pronotum (internal diameter 6 mm), was attached with mastic sealant (Fun-tak) (Machin *et al.* 1985). The animal was then inverted and injected, using a Hamilton 7000 series syringe, with 25 μCi of THO in 5 μl of distilled water. The blunt tip of the needle was inserted between the abdominal sternites and through the intersegmental membrane. Slow withdrawal of the needle after injection gave time for the haemolymph to clot at the point of insertion. This minimized leakage and made the application of wound sealants unnecessary. Cockroaches were then placed on a treadmill (Machin *et al.* 1985) and continuously flowing (120 ml min^{-1}) dry air of constant water activity was passed through the cup. Dry air was produced by passing air from an aquarium pump through a column of silica gel (a_w=0.046). A soap bubble flowmeter, comparing air flow upstream and downstream of the cup, was routinely used at the beginning of each experiment to check for leaks in the seal with the pronotum. Preparations were rejected unless the flows were equal, measured to an accuracy of 2 %. THO passing across the pronotum was collected twice daily for 2-h periods in a series of three scintillation vials, each containing 10 ml of Aquasol (Biotechnology Systems, NEN Research Products, Boston, MA, USA) and immersed in an ice bath (Nicolson *et al.* 1984). We confirmed their observation that radioactivity in the third vial never exceeded background levels. Samples were counted for 5 min in a Beckman model LS350 liquid scintillation counter. Blood specific activity was measured daily by collecting a small sample in a calibrated 5 μl microcapillary placed over the end of a cut antenna.

THO equilibration

In order to measure the effects of various experimental procedures on pronotal permeability, several days of relatively stable, predictable THO efflux were required. Serial sampling of haemolymph following injection showed a steady increase in THO activity reaching a plateau by about 4 h (Fig. 1). Following this period separate, longer-term experiments (up to 80 h) established that relatively stable values held until 28 h when a slow, more or less linear decline in activity became apparent, presumably the linear initial slope of an exponential wash-out curve. Average biological THO half-life determined from these values was 366.7 h or 15.3 days, compared with the physical half-life of tritium, 12.3 years. The bi-modal THO equilibration curve described by Nicolson *et al.* (1984), after injecting the beetle *Onymacris plana*, may have been due to differences in sampling technique. Although not specified, it seems likely that their sites of sampling and injection were quite close. By contrast, the antennal sampling site used in this study was well removed from the point of injection.

After finding that the time to reach relatively stable THO effluxes was tens of hours, we experimented with injecting cockroaches several days prior to attaching

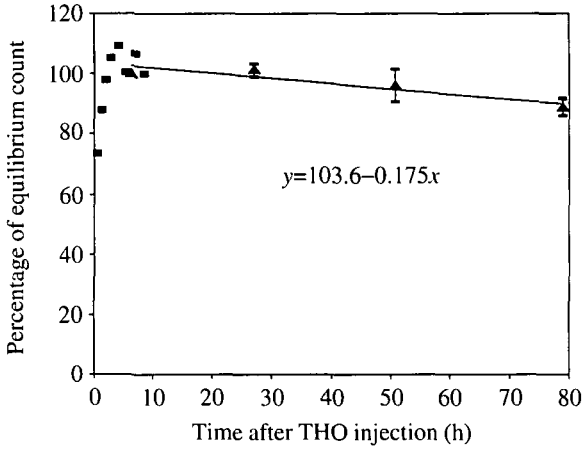

Fig. 1. Graph showing changes in haemolymph radioactivity (expressed as cts min^{-1} as a percentage of the first relatively stable value) following THO injection. The graph is a composite showing the rapid rise and stabilization immediately following injection (■) followed by the average trend based on longer-term measurements in 25 experimental animals (▲). Error bars are ±S.E.

the cup and collecting the tracer. However, the rapid decline in collected counts was still evident, apparently associated with equilibration in the collecting apparatus external to the animal (Fig. 2). A 24-h equilibration period following THO injection, with the ventilated cup in place and dry air flowing, was found to be sufficient to avoid this problem. Tracer collections were made at approximately 25, 30, 49, 54, 73 and 78 h following THO injection, the time being calculated at the mid-point of the 2-h collection period. Brain homogenates or saline were injected between the third and fourth collection, at approximately 50 h. Wet and dry masses were obtained for all donor cockroaches in order to calculate their water content at the time of brain dissection.

Water loss and hydration

Handling, injection, attaching the cup and physical restraint associated with experimentation resulted in elevated total water losses in recipient cockroaches. So far as we were able to observe, defecation during the experiments was rare, probably because of the dehydrated state of the animals (Tucker, 1977). Preliminary measurements established that average water loss was initially nearly 30 times higher than the losses from quiescent animals given in Machin *et al.* (1991). However, the cockroaches rapidly adjusted to experimental conditions with losses falling to 3.7 times the quiescent baseline by the first THO collection at 25 h and to 2.1 times the baseline by 78 h, the usual end of the experiment. Despite this adjustment in water loss, we estimated that it would be impossible to keep cockroaches in a hydrated state [water content (WC)>72 %]. For example, we calculated that even an initial water content of 75 % would fall to 70 % by the end of the experiment. An attempt was made to provide the cockroach with drinking

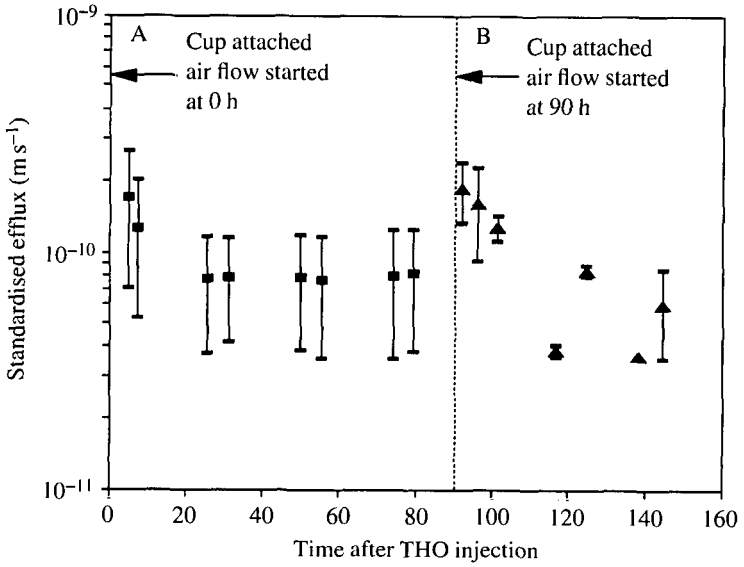

Fig. 2. Graph comparing standardized THO efflux with different delays following tracer injection. (A) The ventilated cup was installed immediately following injection and collection began 4 h later, at the beginning of quasi-stable haemolymph activities. (B) In another group of cockroaches, the cup was installed 90 h after injection and collection began 20 min later, the minimum period necessary for leak testing. To compare individuals and groups, effluxes were standardized by dividing by haemolymph specific activity. Although the units are the same, standardized effluxes cannot be considered as accurate measurements of permeability until reasonably stable effluxes are established. Error bars are ±S.E., N=2.

water during the experiment; however, with the necessary mechanical restraint, cockroaches could not drink easily and the technique was abandoned. With an initial culture water content of 69.3 % we calculated that mean water content of recipient cockroaches, based on live mass, just before injection of brain homogenates or saline, was 64.8±0.3 %, falling to 63.5±0.4 % (N=19) by the end (78 h after THO injection), below the 66 % upper limit for dehydrated cockroaches defined by Noble-Nesbitt and Al-Shukur (1987).

Brain homogenate preparation

Since decapitation is reported only to affect water loss in hydrated animals we abandoned this procedure as a test of hormonal effects. Noble-Nesbitt and Al-Shukur (1988*a*) reported marked differences in the effects of hydrated and dehydrated brain homogenate injections on water loss from dehydrated cockroaches and we chose to repeat this experiment as our test of hormonal effects on cuticle permeability. We followed the saline recipe given in Noble-Nesbitt and Al-Shukur (1988*a*) for preparing the brain homogenates and for control injections. It was sometimes convenient to store donor brains for short periods before experimentation. A cryogenic tissue freezer (Kelvinator Commercial Products,

Manitowoc, USA) held at −80°C was used for this purpose. It was also thought that freezing and thawing might enhance tissue homogenization (Evans *et al.* 1985). The effects of both fresh and pre-frozen brain homogenates were compared.

Donor animals for brain homogenate injections, designated according to Noble-Nesbitt and Al-Shukur (1987), 'dehydrated' (WC<66%), 'normal' and 'hydrated' (WC>72%), were obtained as described in Machin *et al.* (1991). The brain (excluding the corpus cardiacum and corpus allatum) was dissected from a weighed, pre-conditioned donor cockroach. 'Fresh' brains were immediately homogenized in 50 μl of cold saline in a Kontes micro-ultrasonic cell disrupter (Mandel Scientific Company Ltd, Guelph, Canada) for approximately 20 s, then centrifuged for 5 min at 3200 revs min^{-1}. 'Pre-frozen' brains were stored after dissection at −80°C, in 50 μl of saline, then thawed before being homogenized and centrifuged. The complete procedure, from dissection to injection, excluding the period of freezing, took less than 20 min during which time, wherever possible, the preparation was kept cool with crushed ice. In each case the supernatant was injected into the experimental animal with a 50 μl Hamilton syringe, following the technique described previously. Control injections of 50 μl of saline were administered in the same manner.

Analysis of results

Total counts in the three collection vials, minus background, were converted to permeability in units of $m\,s^{-1}$ after Croghan and Noble-Nesbitt (1989). Allowances were made for the decline in haemolymph specific activity during the course of the experiment. The small size of haemolymph samples taken from the antenna made volume measurement and resulting specific activity variable and inaccurate. To minimize the effect of these errors in our permeability calculations we first determined the average rate of haemolymph specific activity decline, during the time of the experiment, using all experimental values. The rate of decline (Fig. 1) was (0.175% h^{-1}). Again, to avoid basing calculations on a single measurement, haemolymph specific activities were averaged for each animal before adjusting for declining activity at the time of each THO collection. The nominal unweighted mean haemolymph specific activity was considered to correspond to the time midway between the first and last haemolymph sample collections. The adjustments were 1.056, 1.046, 1.007, 0.997, 0.958 and 0.947 times mean specific activity for the three collections preceding and following brain homogenate injection.

Permeabilities measured before brain homogenate injection, at 25, 30 and 49 h, were compared with those measured immediately after, at 54 h, and the day after, at 73 and 78 h. Experiments were continued until changes in statistical significance resulting from further replication (Student's *t*-test) seemed unlikely. In order to account for the sometimes large differences between experimental animals, data were also subjected to two- and three-factor analysis of variance (ANOVA). A two-factor ANOVA was sufficient to detect differences between sampling times while accounting for animal variability; however, a three-factor ANOVA was

necessary to distinguish differences between types of brain homogenate injection, accounting for the effects of both animal variability and sampling time.

In vitro *experiments*

A series of *in vitro* permeability measurements was also made with pronotal discs dissected from cockroaches injected with fresh brain homogenates or saline. Discs were excised 24 h after injection and clamped in cuticle holders containing cockroach Ringer's solution, following the method of Machin *et al.* (1985). Fluxes were determined gravimetrically over a period of 48 h at 20°C and in dry flowing air (43.3 ml min^{-1}). Water contents of the excised discs was also measured at the end of the experiment after the method of Machin and Lampert (1987). Retention of epidermal tissue until just prior to wet weight measurement has no effect on the permeability of the preparation but water contents comparable to *in vivo* values are ensured.

Comparisons between *in vitro* permeability determined from THO flux and gravimetry were made by adding 5 μl of the previously described THO solution to the Ringer's solution then passing the effluent air, at 120 ml min^{-1}, from the cuticle holder through a series of four scintillation vials. Higher THO activities and *in vitro* water fluxes made it necessary to use four vials to ensure complete tracer collection. This system was tested for leakage following the procedure described previously. We also compared permeabilities, determined by the two methods, of freshly excised pronotal discs and those that had first been washed with chloroform:methanol (2:1) for approximately 2 min to remove waxy layers. Preliminary studies indicated that equilibration of THO within the cuticle discs was complete after 6 h, thus establishing that incomplete THO equilibration could not account for differences in permeability determined by the two methods. Calculations determined that unstirred layer errors were 0.15 % at air flow rates of 120 ml min^{-1} and 0.25 % at flow rates of 43.3 ml min^{-1}. In both the ventilated cup and excised pronotal disc holders, the diameter of exposed cuticle was 6 mm, giving an area of 0.283 cm^2.

Results

Differences between the effects of fresh and pre-frozen brain homogenates on cuticle permeability were highly significant at all three hydration levels ($P<0.001$, in all cases). The effects of fresh and pre-frozen homogenates will therefore be presented separately. Table 1 summarizes mean pronotal permeabilities in experimental groups before, 5 h after and 1 day after injection of brain homogenates from donors in various states of hydration. Our results indicate that at least some brain homogenate injections produce an increase in pronotal permeability of dehydrated cockroaches, whereas saline injections do not. One day after injection, fresh hydrated, and pre-frozen hydrated, normal and dehydrated brain homogenates produced 46, 27, 67 and 54 % increases over pre-treatment permeabilities, respectively. None of the injections produced a significant permeability decrease.

Table 1. *Effect of brain homogenate and saline injections on* in vivo *cuticle permeability of dehydrated cockroaches*

		$10^{11}\times$mean permeability ($m\,s^{-1}$)		
Donor type	*N*	Before injection	5 h after	1 day after
Saline	4	5.385±0.607	5.127±1.108	4.928±0.644
Fresh brain homogenates				
Hydrated	6	6.125±0.739	7.847±1.484	8.968±1.158
Normal	4	7.005±0.776	6.833±1.435	7.955±1.074
Dehydrated	6	5.195±0.655	4.990±0.958	5.536±0.706
Pre-frozen brain homogenates				
Hydrated	6	4.850±0.435	4.450±0.558	6.176±1.012
Normal	2	3.406±0.218	3.779±0.671	5.676±0.984
Dehydrated	3	5.625±1.036	5.787±1.980	8.680±1.516

Before injection values are means of those measured 25, 20 and 1 h prior to brain homogenate injection.

1 day after values are means of those measured 23 and 30 h following injection.

Values are means±S.E.

N refers to number of recipient animals.

Statistical analysis, summarized in Table 2, also established that, with the exception of fresh hydrated brain homogenate injections, where the 5 h post-injection permeability was also significantly elevated, the response to brain homogenates was delayed until the day after injection. In addition to the differences between the responses to saline and fresh hydrated brain homogenate injections already noted, differences between the effects of injection were observed between normal and dehydrated homogenates. In the pre-frozen series, the effects of all brain homogenate injections were different from one another, as were the effects of the dehydrated homogenate compared with the saline injection.

During some of the *in vivo* experiments it was noticed that THO flux was unusually high and close inspection usually revealed that the thin cuticle underneath the pronotum had been torn by the hooks which clamp the cup into place. In some cases, the animal died before the end of the experiment, we suspect from excessive dehydration. The injury was always in the neck region and never extended to the area of measurement on the pronotum. Mean permeability of injured or dying animals was $10.228\times10^{-11}\pm1.769\times10^{-11}\ m\,s^{-1}$ ($N=5$) compared with $5.513\times10^{-11}\pm0.261\times10^{-11}\ m\,s^{-1}$ ($N=31$) for uninjured animals, before brain homogenate injection. The results are significantly different from each other ($P<0.01$).

In vitro permeability measurements produced no significant differences between animals injected with brain homogenates or saline, nor were there significant differences in pronotal water contents (Table 3). THO-derived pronotal permeabilities measured *in vitro* were consistently about an order of magnitude

Table 2. *Summary of analysis of variance (ANOVA)*

Donor type	Time after injection	*F*-value	*P*-value
A Two-factor ANOVA comparing permeabilities at all sampling times, accounting for variability due to animal differences			
Saline		0.50	0.616
Fresh brain homogenates			
Hydrated		8.79	0.001
Dehydrated		0.54	0.588
Normal		0.95	0.407
Pre-frozen brain homogenates			
Hydrated		5.52	0.012
Dehydrated		30.01	<0.001
Normal		6.49	0.021
B Two-factor ANOVA comparing pre-homogenate injection permeabilities with values at each post-injection sampling time, accounting for variability due to animal differences			
Fresh brain homogenates			
Hydrated	5 h	4.66	0.045
	1 day	16.06	0.001
Pre-frozen brain homogenates			
Hydrated	5 h	1.51	0.236
	1 day	7.24	0.013
Dehydrated	5 h	0.58	0.467
	1 day	55.48	<0.001
Normal	5 h	0.54	0.495
	1 day	10.94	0.013
C Three-factor ANOVA comparing effect of donor type on permeability, accounting for variability due to animal differences and sampling time			
Fresh brain homogenates			
Hydrated–dehydrated		16.42	<0.001
Hydrated–normal		0.26	0.615
Dehydrated–normal		29.55	<0.001
Hydrated–saline		16.22	<0.001
Normal–saline		32.58	<0.001
Dehydrated–saline		0.93	0.342
Pre-frozen brain homogenates			
Hydrated–dehydrated		13.93	<0.001
Hydrated–normal		5.32	0.028
Dehydrated–normal		47.29	<0.001
Hydrated–saline		0.40	0.530
Normal–saline		3.36	0.080
Dehydrated–saline		15.26	0.001

Table 3. *Gravimetrically determined permeabilities and water contents of isolated pronotal discs excised from dehydrated cockroaches (WC=65.25±0.628 %, N=33) injected with fresh brain homogenates and saline*

	10^{10}×permeability ($m s^{-1}$)		Water content ($g g^{-1}$ dry mass)	
Donor type	*N*	Mean±S.E.	*N*	Mean±S.E.
Saline	17	9.552±0.899	11	0.283±0.010
Hydrated	8	11.084±0.717	9	0.258±0.016
Dehydrated	8	9.938±1.144	2	0.341±0.055
P-values				
Hydrated–saline		>0.1		>0.2
Dehydrated–saline		>0.7		>0.3
Hydrated–dehydrated		>0.4		>0.1

Mean water content of hydrated donors was 75.21±0.973 % and of dehydrated donors 64.46±1.597 %.

Table 4. *Summary of simultaneous THO and gravimetrically determined permeabilities comparing intact and solvent-treated excised pronotal discs*

		10^{10}×permeability ($m s^{-1}$)			
Disc type	*N*	THO Mean±S.E.	Gravimetric Mean±S.E.	*P*	THO/ gravimetric
Intact	4	5.158±0.305	9.663±0.937	<0.01	0.534
Solvent-treated	3	137.385±13.519	143.691±14.228	>0.9	0.955

greater than corresponding values determined with intact cockroaches. For instance, pronotal permeability in intact cockroaches 1 day after saline injection was $0.493\times10^{-10}\pm0.064\times10^{-10}$ m s^{-1} (N=4) (Table 1) compared with $5.158\times10^{-10}\pm0.305\times10^{-10}$ m s^{-1} (N=4) (Table 4) in isolated discs. Simultaneously measured THO flux and gravimetric water loss from the same pronotal disc (Table 4) yielded THO permeabilities which were 53.4 % of values determined by weighing. This difference disappeared when the cuticle discs were treated with chloroform: methanol (2:1).

Discussion

In addition to providing unambiguous measurements of the effects of injected brain homogenates on cuticle permeability, our results provide information on the accuracy and compatibility of cuticle permeabilities, measured using THO, with those based on other techniques. Although THO and gravimetrically determined permeabilities are generally comparable, simultaneous measurements using the

two methods on the same preparation reveal consistent differences. The THO method yielded permeability values which were, on average, 53% of those determined gravimetrically. Nicolson *et al.* (1984) also reported THO permeabilities of small areas of cuticle in *Onymacris plana* beetles which were about half those based on gravimetrically determined losses of the whole animal (Edney, 1971). Nagy and Costa (1980) have discussed the effects of unequal rates of evaporation of H_2O and THO on the proportions of these forms of water remaining in the body fluids (i.e. isotopic fractionation) in structures where intermixing is limited. Our method avoids the effects of isotopic fractionation in the haemolymph by allowing for changes in THO-specific activities during each experiment. However, our data do show that the waterproofing layers in the cuticle discriminate between THO and H_2O flux because the permeability differences disappear after solvent extraction. The observed discrimination was much greater than the 8% or 23% difference due to reduced vapour pressure of THO quoted by Nagy and Costa (1980) or the 5% due to lower gas diffusion coefficients calculated from Graham's law.

This study has also demonstrated that THO-determined permeability of the intact pronotum is about an order of magnitude lower than that of the excised pronotum. Comparisons between gravimetrically determined intact pronotal permeabilities, taken from the literature (1.156×10^{-10} m s^{-1}, Machin *et al.* 1985) since none was performed in this study, and values for excised pronota ($9.552\times10^{-10}\pm0.899\times10^{-10}$ m s^{-1}, saline controls in Table 3) determined using similar techniques also show the same difference. Electrical conductance (G) measurements using *Periplaneta* pronotum, which seem likely to parallel the water-conducting properties of this structure, also conform to this pattern, producing intact values that are tenfold less than those measured after excision (*in vivo* $G=3\times10^{-5}$ mhos cm^{-2}, Scheie and Smyth, 1968; excised $G=3\times10^{-4}$ mhos cm^{-2}, Scheie and Smyth, 1967). Since electrical conductance measurements do not depend on the formation of an effective seal between the excised cuticle and its holder, these results support the claim of Machin *et al.* (1985) that higher *in vitro* permeabilities are not the result of mechanical damage or other technical limitations of the *in vitro* techniques. Higher pronotal permeabilities in injured animals, when the pronotal cuticle was undamaged, also suggest that the pronotum may be capable of permeability changes over relatively short time intervals.

Since our measurements of the effects of brain homogenate injections are limited to the responses of dehydrated recipients, they provide only a partial test of the proposed model of hormonal control of cuticle permeability in *Periplaneta* (Noble-Nesbitt and Al-Shukur, 1988*a*). The results we have obtained qualitatively, but not quantitatively, conform to their model. Pronotal permeabilities were significantly increased by 28% 5 h following the injection of fresh hydrated brain homogenate and then by 46% 1 day after. Noble-Nesbitt and Al-Shukur (1988*a*, their Fig. 1) recorded a 66% increase in total water loss in a similar experiment. However, in absolute rather than relative terms (using their method of comparison

and correcting for the above-mentioned THO-based underestimation of net cuticular flux), we estimate that fresh hydrated brain extract injections in this study produced a 17.3 $\mu g\,cm^{-2}\,h^{-1}$ increase in cuticular water loss (54.6 minus 37.3 $\mu g\,cm^{-2}\,h^{-1}$). By comparison, Noble-Nesbitt and Al-Shukur (1988*a*) reported increased respiratory plus cuticular losses, resulting from the same injection, of 74.5 $\mu g\,cm^{-2}\,h^{-1}$ (186.5 minus 112.0 $\mu g\,cm^{-2}\,h^{-1}$). Similar hydration-related differences in whole-cuticle water loss from intact cockroaches of 22.3 $\mu g\,cm^{-2}\,h^{-1}$ (54.9 minus 32.6 $\mu g\,cm^{-2}\,h^{-1}$) (Machin *et al.* 1991) to those of pronotum alone suggest that permeability change extends beyond the pronotum to other regions of the cuticle. To attribute most of the hydration-dependent differences in whole-animal water loss during periods of spiracular constriction to changes in cuticle permeability would mean also that there are no systematic differences in the effectiveness of spiracular sealing, as described in the Introduction. This phenomenon could, however, still account for the abrupt short-term changes in cuticular flux noted by Machin *et al.* (1991).

As in the earlier study, injections of saline or extracts of fresh brains from dehydrated animals failed to have any effect on the permeability of dehydrated individuals. The failure of dehydrated individuals to show permeability decreases in response to any injection further supports the Noble-Nesbitt and Al-Shukur (1988*a*) model. However, all brain homogenates from pre-frozen hydrated, normal and dehydrated donors produced significant pronotal permeability increases by the day following injection of 27, 67 and 54%, respectively. This response could at least be partially explained by a conventional dose–response effect in which pre-frozen homogenates contain higher concentrations of the active factor because of superior brain tissue extraction. However, the slower response to pre-frozen homogenate injections may indicate a more complex control, perhaps involving additional factors sensitive to freezing. Our failure to confirm the effects of hydrated brain homogenates in excised pronotal discs may have been because the changes were obscured by the much larger permeability increases associated with the excision itself.

The nature of structural changes responsible for any of the permeability changes described above are as yet unknown. Their relative rapidity seems to eliminate the possibility of extensive change in chemical composition of the epicuticular barrier. Croghan and Noble-Nesbitt (1990), who apparently favour changes in layers of the cuticle arranged in series, have already discussed some possible mechanisms of permeability modulation, but direct evidence that the proposed changes in the physiological state of the endocuticle influence epicuticle permeability is lacking. Calculations based on cuticle permeability–water content models (Machin and Lampert, 1987) suggest that measurable changes in water content would probably arise from changes in the physiological state of the endocuticle (which holds most of the cuticular water) or from permeability changes in any other series layer. However, changes in the conducting properties of parallel diffusion pathways occupying a small proportion of the total area would have no effect on cuticle water content. The estimated change in water content following our observed

change in pronotal permeability is unfortunately too small to be reliably detected. With the larger permeability changes resulting from pronotal excision, predicted *in vitro* water contents should be 7.6% lower than *in vivo* values. Machin and Lampert (1987) observed no differences between *in vivo* and *in vitro* water content, suggesting that changes in cuticle permeability result from alterations in the conducting properties of shunt pathways arranged in parallel. If changes in the pathways were profound enough, the observed changes in permeability could be accounted for by structures occupying only a small proportion of the total cuticular area.

Noble-Nesbitt (1990, 1991) proposed that the functional significance of hormonal regulation of cuticle permeability is to provide an energy-free method of removing excess water. However, the much reduced changes in water loss suggested by permeability measurements in this study are more consistent with our earlier conclusions that ventilation through the spiracles, not cuticular flux, represents the most effective and variable avenue for water loss in *Periplaneta* (Machin *et al.* 1991). With respect to variable losses through the cuticle, we speculate that other activities of intermoult cuticle, such as cement deposition or pheromone release, are inevitably associated with increased water loss and that dehydrated cockroaches are able to limit these activities to conserve water. The idea that water loss through supplementary pathways of the cuticle, such as secretory or even sensory structures, might vary with hydration status of the cockroach is now under active investigation.

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