THE WATERPROOFING MECHANISM OF ARTHROPODS

I. THE EFFECT OF TEMPERATURE ON CUTICLE PERMEABILITY IN TERRESTRIAL INSECTS AND TICKS

BY J. W. L. BEAMENT

*Agricultural Research Council Unit of Insect Physiology,
Department of Zoology, University of Cambridge*

INTRODUCTION

In a recent paper Beament (1958a) has examined the physico-chemical problems associated with the measurement of evaporation from small animals and has described a method of investigation which gives results suitable for physical analysis; this method reveals an abrupt breakdown in the waterproofing system of the cockroach, *Periplaneta*, at a critical temperature. An analysis of the experimental data suggests that the unusually low permeability of the cockroach cuticle to water is due to an organized monolayer of grease and that this becomes disorientated at a transition temperature. Beament's paper demonstrates that a phenomenon of importance can be masked in the results obtained by simpler experimental methods. Although simple methods have revealed an apparent resemblance between the cockroach and certain other insects in respect of this property, we cannot necessarily conclude that other insects besides cockroaches have a transition phenomenon in their temperature-permeability relationship. There are important differences between the epicuticular structures of the few insects which have been examined in any detail; in particular, the cockroach has a waterproof grease (Beament, 1955), whereas many terrestrial arthropods appear to rely on hard waxes for waterproofing (Beament, 1945; Wigglesworth, 1945; Lees, 1947; etc.). A selection of terrestrial arthropods has now been investigated. A slightly modified version of the equipment previously described (Beament, 1958a) has been used; this is described in the appendix, and photographs appear in Beament (1958b). From measurements on the rate of evaporation at various temperatures, before and after the application of adsorptive dusts, it is possible to discern in more detail the form which the waterproofing system may take in some of these insects.

Adjectival nouns in the phrases 'skin temperature', 'air temperature', etc., will be used throughout; in particular 'air-cuticle temperature difference' means the difference in temperature between the surface of an animal and the surrounding air at such distances from the animal that air temperature is determined only by thermostatting equipment. 'Permeability/temperature curve' will be used for the

plot of the permeability of a cuticle at constant saturation deficiency against cuticle temperature. The layer of wax secreted on to the epicuticle surface at the time of ecdysis will be called the 'primary wax layer'.

INITIAL EXPERIMENTS

For the initial experiments the adult male of the locust, *Schistocerca gregaria*, was selected. The experimental procedure of Beament (1958*a*) was followed, but accurate readings could be taken after much shorter time intervals of desiccation because the new balance had much greater sensitivity. For an increment of a few degrees in temperature stable conditions could be obtained some 5–10 min. from the time of adjusting the temperature control, and readings of evaporative loss were taken every minute at each stable temperature until three successive readings agreed. A complete temperature/permeability curve could thus be constructed from experiments involving a very small loss of the water contained in the specimen. Dry air was flushed through the box after each resetting of the temperature control, and also whenever the accumulated water of evaporation inside the box amounted to 1% relative humidity. Information was obtained in order of increasing temperature (if the temperature is lowered during a series of observations the technique must be modified (p. 398)).

Fig. 1, curve *A*, shows the result of such an experiment on an adult locust; permeability is expressed at unit saturation deficiency gradient for various surface temperatures. The curve does not contain a sharply defined discontinuity, though the permeability of the cuticle is changing markedly with temperature and rises steeply at temperatures above 48° C. Comparing this with corresponding measurements on the cockroach (Beament, 1958*a*) we could conclude that the locust—which appears to have a hard wax on its surface—does not show a transition temperature and that we have no evidence for a special waterproofing system based on an organized monolayer of wax. But an insect which has been anaesthetized in 5% carbon dioxide in air and then killed by exposure to hydrogen sulphide gas has many of its spiracles fixed in the open position. When a living locust is ventilating its tracheal system the water loss through its spiracles is appreciable, and though no information has been found on corresponding losses from dead insects the results shown in curve *A* will certainly include some evaporative loss through the spiracles. It seemed possible that the curve for cuticular transpiration alone might still contain a discontinuity if the effect of tracheal evaporation were removed, and the experiments were therefore repeated with animals in which the spiracles had been blocked. Dr T. Weis-Fogh kindly provided specimens in which he had occluded the spiracles with wax.

Fig. 1, curve *B*, shows the result of determinations made on such an animal, which was so far as possible identical in size and weight with that used for curve *A*. Against all expectations, the rate of evaporation at all temperatures was found to be greater than from the corresponding specimen whose spiracles were open; curve *B* is also smoother and even less likely to contain a discontinuity than is curve *A*.

These experiments were repeated a number of times, and wax, paint or injected liquid was used to block the spiracles. A family of curves was obtained, each showing the property of an individual. The only general conclusion which could be reached was that the rate of water loss is lower at all temperatures if the spiracles are not touched. Though considerable variation was found from one animal to another, and though the most waterproof of the animals with blocked spiracles lost water less rapidly (at all temperatures) than the most permeable of the untreated animals, it was obvious that the least waterproof animal gave the smoothest curve.
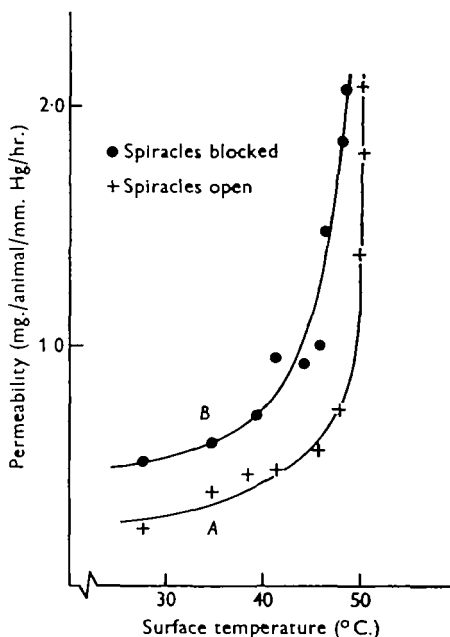

Fig. 1. Graph showing the permeability of the cuticle of the adult locust *Schistocerca gregaria* at various cuticle temperatures. *A*, an animal with some spiracles open; *B*, an animal whose spiracles have been occluded.

Similar experiments were performed on large nymphs of *Rhodnius*; and again animals with blocked spiracles showed, in general, higher rates of water loss and smoother permeability/temperature curves. But the best of the untreated animals did give a curve with a sharp break—of the kind clearly suggesting a transition point—at about 60° C. It was then found that a control experiment, in which a *Rhodnius* was handled as though to block the spiracles but in which the spiracles were left untouched, revealed high water loss and a very smooth curve. In contrast, animals which had been carefully captured, killed and transferred to the balance without having been touched by the operator's hand all gave sharp breaks in their permeability/temperature curves and were very waterproof. This series of initial experiments concluded with the demonstration of a sharp discontinuity in the curve for an adult locust when the specimen had likewise been manipulated in the most careful manner possible.

The author has not so far found any method of blocking spiracles which does not involve appreciable damage to the waterproofing system, and in the results which are here reported the apparent permeabilities must include some component due to evaporation from the tracheal system. Failure to separate the two sources of water loss has to some extent limited the accuracy with which the data can be analysed, but the discovery of the extreme delicacy of the waterproofing system has proved of much greater importance (p. 410); further, some of the divergence between the measurements made by previous authors and those described here may well be attributed to the damage incidental to blocking spiracles.

EXPERIMENTAL PROCEDURE

The animals used in subsequent experiments were selected, wherever possible, at an appropriate stage of their life history, just previous to moulting. They were transferred to clean glass containers and allowed to moult there; where possible they were also killed in these containers and transferred to the balance in ways which involved the least possible damage. In particular, the pupa of *Pieris* was selected for much of the latter work in this study because it could be manipulated entirely by its silk. We must, however, bear in mind, when considering the actual rates of water loss recorded below, that insects in nature may have sustained superficial damage to their cuticles. Wigglesworth (1945) shows that a soil-living larva of *Agriotes* has an extremely permeable cuticle, though the cuticle is very much more waterproof if the insect is allowed to moult away from the soil. He attributed this state of affairs to gross abrasion of the waterproof layer; the degree of damage caused by handling insects in the present type of experiment is probably very much smaller.

To obtain a curve (such as those of Figs. 2–5), the properties of at least four individuals of the closest equivalence in age, instar, past history, etc., were examined. The first animal of each group was used to establish the approximate form of the permeability/temperature curve, measurements being taken over short periods of time at temperature intervals of 5° C. This experiment showed the region of temperature likely to contain a sudden change in permeability, and made it possible to assess the order of water loss in relation to the total water content of the specimen. Each of the remaining individuals was then examined more critically; the range of temperature likely to contain a discontinuity was covered in small steps and the duration of desiccation was adjusted according to the depletion of the specimen's water content. In principle it is essential that each curve should be obtained from one individual (Beament, 1958a), but in fact the three individuals of each group showed only small differences in the form of the curve, and the characteristics of the particular individuals selected for Figs. 2–5 are probably representative of that species at that stage of its life history. Individual variation within the species has not been investigated here in greater detail, but changes caused by the ageing of the cuticle are shown in detail later (p. 403).

RESULTS

From Figs. 2 to 5 it is clear that all but two of the permeability/temperature curves contain sharp discontinuities; they are so clearly defined that they obviously represent the sudden breakdown in waterproofing envisaged by Wigglesworth (1945)
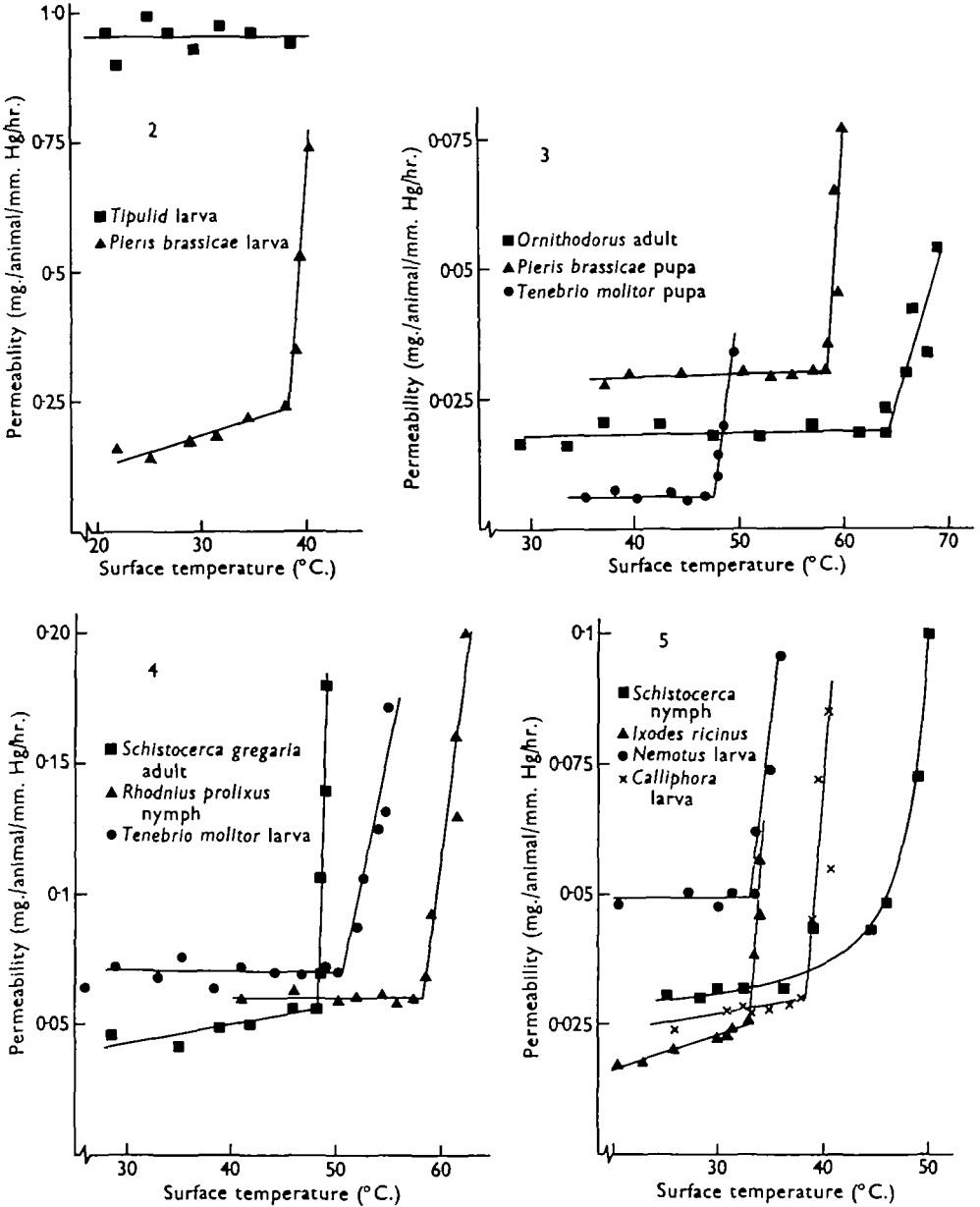
Figs. 2–5. Graphs showing the permeability to water of the cuticles of a number of arthropods at various cuticle temperatures.

and Beament (1945), to which they applied the terms 'transition point' and 'critical temperature'. These terms will again be adopted here, with the provision that they do not necessarily imply the acceptance either of the physico-chemical interpretation put on them in 1945 or of that proposed by Beament (1958a) for cockroach grease. The critical temperatures of these different species and stages in life history cover a wide range of temperature, from that of *Calliphora* to that of the argasid tick *Ornithodorus*, and if one compares them with values obtained by inspection of the published curves of earlier workers, either on whole animals or on isolated waxes, the correspondence is indeed close (Table 1). The permeabilities in Figs. 2–5 have not been reduced to a basis of unit surface area; for this reason the curves do not reveal the comparative permeabilities of different animals (see p. 418). This procedure has been adopted because of the great difficulty in estimating the true surface area of an insect. But where previous workers have not only used the same species (*Rhodnius*) as one involved in the present investigation, but have also adopted a formula (Wigglesworth, 1945) relating weight to surface area, it is justifiable to apply this formula to the new data and to compare the three investigations in the same terms.

Table 1. *A comparison of the transition temperatures* (° C.) *of the animals in the present study with those suggested by previous authors*

(V.B.W., Wigglesworth (1945); A.D.L., Lees (1947).
Figures for isolated waxes from Beament (1945).)

Animal	Present study	Previous whole animal and source	Isolated wax
Pieris larva	38	35–45 V.B.W.	42–47
Pieris pupa	58	55–60 V.B.W.	60–65
Tenebrio larva	51	52 V.B.W.	50–55
Tenebrio pupa	48	52 V.B.W.	—
Ixodes adult female	33	30–35 A.D.L.	—
Ornithodorus adult	64	65 A.D.L.	—
Nematus larva	33	30–35 V.B.W.	30–35
Calliphora larva	38	35 V.B.W.	35–40
Calliphora pupa	51	50–55 V.B.W.	47–52
Rhodnius nymph	58	55–60 V.B.W.	55–60

In Fig. 6 we have the relationships for the *Rhodnius* nymph, taken from Wigglesworth (1945), Holdgate & Seal (1956) and from Fig. 4. The information is all shown according to the previous convention which makes no correction for the change of saturation deficiency with temperature, and since both the previous workers used *air* temperatures as the abscissa, curves for both air and cuticle temperatures have been included in the information extracted from Fig. 4. These curves emphasize more than ever the advantage of expressing results of this kind on linear scales and corrected for changes in saturation deficiency (Beament, 1958a); they also show that, despite the refinements adopted in the present method of investigation, the correspondence between the results now obtained and those of Wigglesworth (1945) are very close indeed—so much so that if Wigglesworth and Beament had adopted a suitable correction for saturation deficiency much of the

more recent controversy over the existence of transition temperatures might never have occurred. But it is of some interest that the curve from Holdgate & Seal's (1956) paper shows a rate of evaporation which is consistently and at all temperatures two to three times greater than the rates represented by the other two curves, and that a discontinuity is much less obvious in their curve—indeed a transition point cannot be found with certainty even if their information is transformed to constant
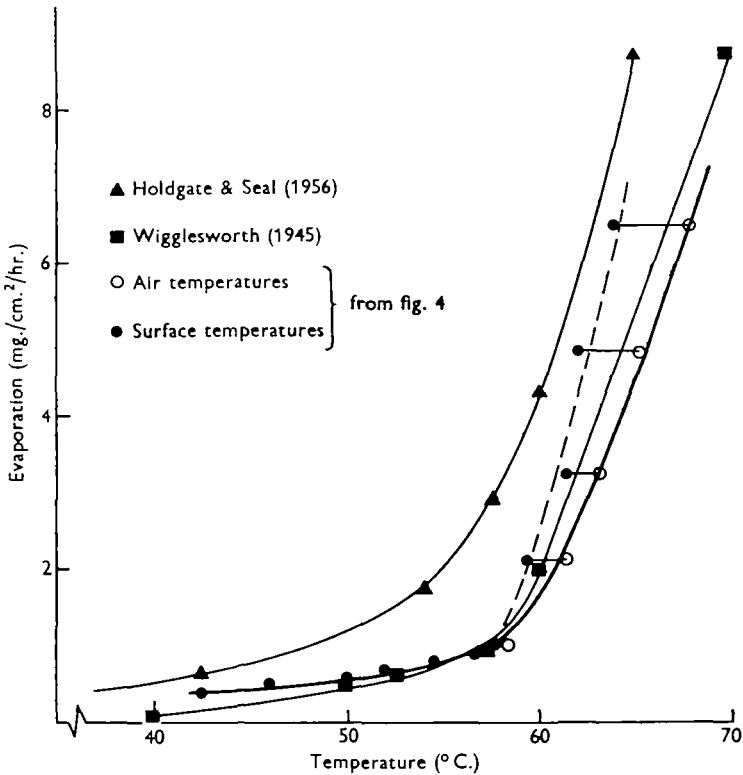

Fig. 6. Graph comparing the results of three investigations into the relationship between evaporation from a large nymph of *Rhodnius* in dry air and temperature. Values from Holdgate & Seal (1956) and Wigglesworth (1945) plotted at air temperatures. Data from the experiments giving the corresponding curve of Fig. 4 are plotted both at air, and at corresponding cuticle, temperatures.

saturation deficiency and plotted on a linear scale. It is tempting to compare this curve with those of Fig. 1, both in smoothness and in rate of loss, and to suggest that the three curves are representative of insects which have suffered superficial damage.

Particular attention has been paid in these curves (Figs. 2–5) to the region of transition; below the critical point all these curves show a small and nearly linear change of permeability with temperature, corresponding to the state of affairs at lower temperatures in the cockroach. Admittedly there are few points on these lower regions of the curves, and if we are to apply the analysis which proved of value in investigating the waterproofing system of the cockroach they must be

determined more critically. Above transition, in the cockroach, where it was assumed that the curve showed the property of completely disorientated grease, the curve was again linear with small slope. But in these new curves, so far as we have investigated higher temperatures, the curves continue to rise steeply from the point of transition. In these hard waxes then, we do not so far appear to be dealing with systems sufficiently similar to that of the cockroach for similar methods of analysis to prove fruitful at this juncture; hence we must investigate the circumstances on either side the discontinuity in various ways in order to get further information.

TEMPERATURE CYCLES

An observation by Wigglesworth (1945) forms the starting-point of the next series of experiments. He showed that if the larva of *Rhodnius* is exposed to a high temperature the impermeability of the cuticle is permanently impaired; this conclusion was based on alternate recordings of water loss at 80° and 50° C.—temperatures which he believed, and we have now confirmed, to be on either side of the transition point for this insect. The extent of the permanent damage was represented by a twofold to fourfold increase in water loss at the lower temperature, after exposure to the higher one. On the other hand exposure to temperatures above the critical point does not produce any permanent effect on the permeability of cockroach grease below the critical point (there may be some hysteresis or a slight shift in the actual critical temperature due to evaporation of solvent). The cockroach system is reversible with temperature and this is readily explained by the mobility of the grease molecules and their re-orientation against the wet surface of the epicuticle. To what extent does the behaviour of hard waxes differ?

Method

Where successive decrements of temperature are used in a series of determinations in the balance, great care must be taken to ensure that a dry atmosphere is maintained in the box. When the temperature of the equipment is raised, the enclosed air expands and some escapes; if the box is cooled, atmospheric air—which will have an appreciable humidity—will be drawn in. So, for experiments in which the temperature is lowered, a small but sufficient stream of dry air must be fed continuously through the box while it is cooling. With this particular equipment it was found that even if dry air at 20° C. was blown through the box at a rate of 3–4 l. per minute, when the control was set to maintain 40° C. the recording thermistor registered a drop of less than half a degree.

Results

Fig. 7 is a composite figure showing the effect of various temperature cycles on large nymphs of *Rhodnius*; each cycle shows the property of an individual, but the curves have been adjusted along the abscissa so that the vertical portions of the curves (*P–Q*) lie on one straight line—an adjustment involving shifts of less than 2° C. between the various curves. The sequence in which determinations in each

'cycle were made is indicated by arrow-heads. It is quite clear (e.g. curve *A*) that Wigglesworth's original observation has been confirmed, i.e. exposure to a temperature well above the transition point permanently impairs the waterproofing at lower temperatures. One must bear in mind that Wigglesworth quoted air temperatures, and that the extent to which the air temperature must rise in order to obtain values towards the upper end of the cuticle temperature line *P–Q* is much
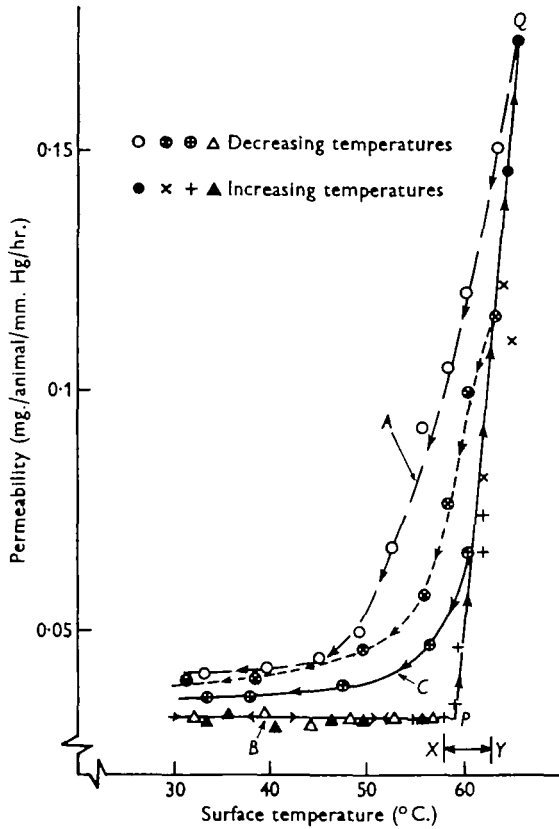

Fig. 7. Graph showing the permeability to water of the cuticle of *Rhodnius* nymphs during different temperature cycles. The information from four individuals has been adjusted along the temperature axis so that vertical portions of the curves lie on a line *PQ*. For further explanation, see text.

greater than the projection of *P–Q* on to the temperature axis. What is more important is the discovery that once the waterproofing system has been subjected to a temperature well above the transition point the sharp discontinuity also disappears permanently. Thus the heat-treated waterproofing system of *Rhodnius* has a permeability complexly related to temperature, and differs entirely in this respect from the cockroach. There is nothing particularly unusual about a permeability/ temperature curve of shape *A*, but the existence of this relationship in heat-treated

wax makes the permeability-temperature relationship of the natural cuticle seem all the more remarkable.

Now consider curve B; an untreated individual can be taken up to a temperature quite close to its transition point (subsequently determined) without causing any detectable change in permeability/temperature relationship at lower temperatures. This is in marked contrast with what happens at and above the transition temperature—for below that point the system is very temperature-stable. From curve C we learn that one has only to increase the temperature of the surface slightly above the transition point to get some permanent impairment of permeability and loss of sharpness of transition. All the curves which were obtained with decreasing temperatures (i.e. which start from the line $P-Q$), suggest that the property of that cuticle approaches the line $P-Q$ asymptotically. This is confirmed by experiments in which nymphs, exposed to temperatures above transition, have been cooled to room temperature and then measured with a series of increasing temperatures.

The general conclusion from these experiments is that they confirm the abruptness of the start of transition; they show too that the changes above the commencement of transition may be progressive with further increase in temperature until the maximum permanent impairment of waterproofing has been reached (though of course the actual temperature difference between points P and Q is small). From Fig. 7 it is certainly fair to limit the extent of the entire transition phenomenon to the interval of surface temperature indicated by $X-Y$—three or four degrees at most. Results similar to these have been obtained using both the larva and pupa of *Tenebrio* and the pupa of *Pieris*; it may be noted that although we have not demonstrated (Fig. 5) a sharp discontinuity in the curve for the locust hopper, nevertheless, exposure to temperatures along the steep upper portion of its permeability/temperature curve does lead to a permanent increase in permeability at lower temperatures, and to a less steep curve. This discovery serves to illustrate an important proposition. If we find a sharp discontinuity in a permeability/temperature curve we can call this a transition point, and it is simplest to assume that all transition points are expressions of the same type of physico-chemical change (except in the cockroach for which the curve is markedly different). But suppose we do not find a sharp discontinuity, but merely a change in slope over a particular temperature interval. If we can further demonstrate a permanent increase in permeability after exposure to a higher temperature and a marked smoothing out of the curve, we may at least assume that something similar to a transition phenomenon is taking place over that range of temperature. This argument becomes of greater importance in the experiments next reported.

PERMEABILITY AT LOWER TEMPERATURES

In the curves of Figs. 2–5 particular attention was paid to determining the transition regions, and comparatively few readings occur in the regions of lower temperature. Straight lines have been drawn through those points to construct the curves, but if we are to attempt a determination of the form and slope of the lines for physical

analysis the points must be determined with greater accuracy. The order of water loss of some of these insects at room temperatures is remarkably low; even with such sensitive apparatus as we are now using, it may be difficult to detect a sensible amount of water loss for an interval of less than 10 min.; and, surprisingly, readings may have to be extended over much more than half an hour to obtain that necessary agreement between successive values which indicates that a steady rate of evaporation has been established (see p. 406). But when one also takes account of the need to construct curves from determinations all made on one individual the complete series of measurements may extend over 3 or more days. The validity of the information so obtained rests on the assumption that the properties of the waterproofing system remain stable for this length of time after the animal has been killed (it is shown on p. 405 below that this assumption is justified). But as one of the basic measurements we are making is of a change in weight, we must guard against changes other than water loss—in particular, oxidation and bacterial decomposition—which might alter the weight of the specimen. For this reason air was replaced with nitrogen during the long-term experiments. Some of the short-term experiments were repeated with nitrogen, as a check, but this made no difference to the results obtained.
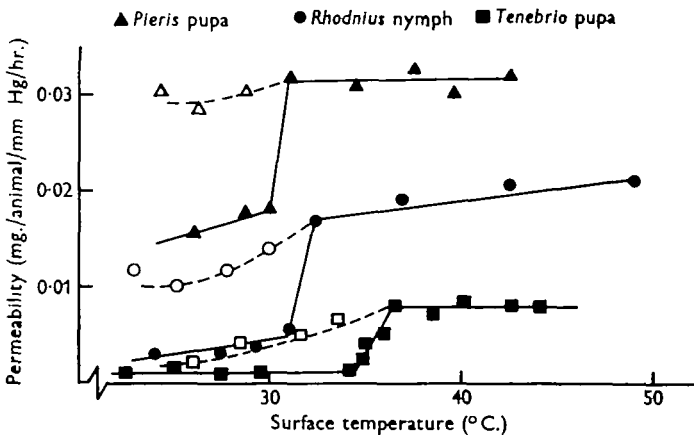

Fig. 8. Graph showing the permeability to water of three different insects at lower temperatures: *Rhodnius* nymph 1 month after moulting, *Tenebrio* and *Pieris* pupae each 5 days after moulting. The broken curves show permeabilities determined after each individual has been exposed to the highest temperature represented on the corresponding continuous line.

Results

In Fig. 8 we show the permeability of three insects at lower temperatures: the pupae of *Pieris* and of *Tenebrio*, each killed at 5 days after pupating, and of a *Rhodnius* nymph 1 month after moulting. The broken line of each curve shows the permeability redetermined after the individual has been exposed to the highest temperature for which a point of determination appears on the associated unbroken curve. None of the specimens has been allowed to approach the temperature

of transition as recorded in Figs. 3 and 4. Now each of these curves shows a discontinuity of a kind not dissimilar to a transition phenomenon; the discontinuities are not particularly sharp, but it must be remembered that the actual changes in permeability which take place across the discontinuity are very small and that they might appear much more sharp if they were followed by a long, almost vertical excursion of the curve. The suggestion of a transition phenomenon is supported by the demonstration of irreversible increase produced by exposure to higher temperature: except in respect of this irreversibility each curve closely resembles the permeability/temperature curve for the cockroach (Beament, 1958*a*) in which the curve is abruptly displaced upwards at the transition point.

On the other hand, when the lower temperature regions of the curves of the larvae of *Calliphora* and *Pieris* and of the adult *Ixodes* were investigated in detail, no indication of a second smaller discontinuity was found; the significance of these differences will be discussed later (p. 416). But where two breaks clearly occur in a curve an important issue arises. In the cockroach we visualized the transition to be the disorientation of a monomolecular layer of grease. But it is extremely difficult to visualize a monolayer with two stages of transition, especially so, for example, in *Rhodnius* where the two breaks occur some 25° C. apart and do not seem to be related in any way—disorganizing one has no effect on the other. There is enough wax on these insects (Beament, 1945) to provide for twenty-five monolayers. However, if we suggest two *independent* monolayers, the difference in their properties indicates that they must be composed of waxes having very different average molecular lengths.

Before examining this possibility, it is interesting to consider the absence of any indication of a second transition phenomenon in the results of previous workers. Though the permeability of a cuticle remains sensibly constant over a lower range of temperature (i.e. the curve as plotted on the axes chosen in this paper is sensibly parallel to the abscissa) the measured rates of evaporation over this region are rising exponentially with temperature, keeping pace with the increasing saturation deficiency of dry air. When transition occurs at a high temperature, the change in evaporation rate over a small interval of temperature is great, not only because of the change in permeability, but because it is being measured in high saturation deficiencies; smaller changes in permeability associated with lower transition points have to be measured in lower saturation deficiencies, with the result that simple apparatus cannot detect them.

TRANSITION POINT AND THE AGE OF THE ANIMAL

Wigglesworth (1945) pointed out that a constant rate of water loss was typical of each species and of each stage of the development of the species; Beament (1946, 1951) observed a similar phenomenon in the waterproofing waxes of egg-shells. On the other hand, Holdgate & Seal (1956) demonstrated a substantial difference in the water loss/temperature curves as between young pupae and older pupae. Briefly, they found that a newly moulted animal is more permeable to water than

an older one at all temperatures, and that if both curves are plotted on the same axis, the curve for the younger animal starts to rise steeply at a lower temperature than that for the older one. Holdgate & Seal drew attention to a waxy bloom on the outer surface of the cement covering the cuticle of *Tenebrio*, and they associated the differences in the curves they obtained with the secretion of the cement layer. It is of special interest to note their statement that the wax exposed on the surface of the newly moulted *Tenebrio* pupa melts at 40° C., whereas the waxy bloom appearing above the cement melts in the region of 60–80° C.
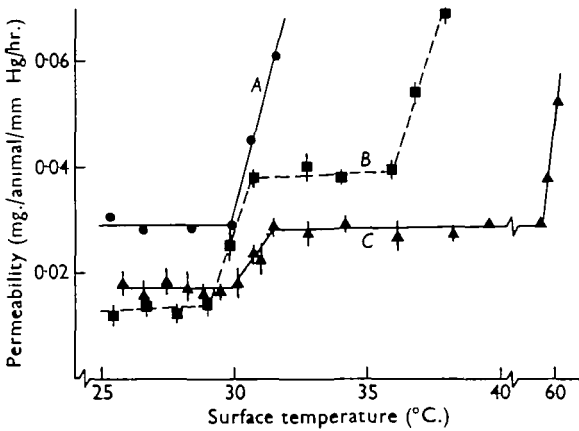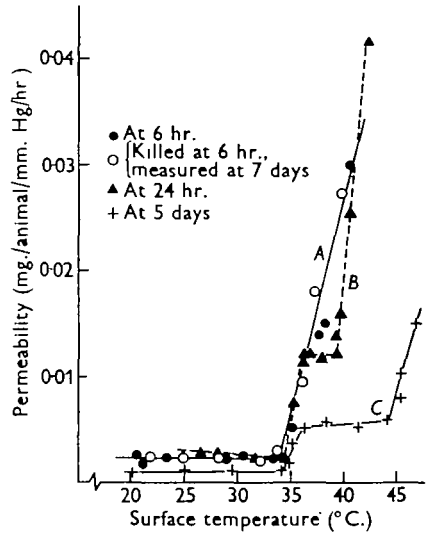

Fig. 9

Fig. 10

Fig. 9. Graph showing changes in the permeability/temperature relationship of pupae of *Pieris* with age. *A*, pupa killed 6 hr. after pupation; *B*, killed 24 hr. after pupation; *C*, killed 5 days after pupation. Vertical lines through individual values indicate the maximum limits of experimental error in the determination.

Fig. 10. Graph showing changes in the permeability/temperature relationship of *Tenebrio* pupae with age. *A*, pupae killed at 6 hr. after pupation and measured either then or 7 days later; *B*, pupae killed and measured at 24 hr. after pupation; *C*, pupae killed and measured at 5 days after pupation.

For investigation of the ageing of the waterproofing system from the time of ecdysis, the pupa of *Tenebrio* and the pupa of *Pieris* were selected. In Fig. 9, curve *A* shows the permeability/temperature relationship of a *Pieris* pupa killed with hydrogen sulphide 6 hr. after moulting; there is one sharp transition point at about 30° C. and above it the curve rises steeply for so far as it has been determined. This animal has only its primary wax layer. When, however, a pupa is killed at 24 hr. (Fig. 9, curve *B*) there are two discontinuities, at 30° C. and again at 36° C. When a pupa is killed at 5 days (curve *C*) one transition occurs at 30° C. as before but the other is not found till the skin temperature reaches 59° C. Curves *B* and *C* have been determined with greatly extended periods of desiccation, and the limits

of accuracy of the individual points have been added, to make clear that the distinction between the two discontinuities is real and to emphasize the sharpness of the lower discontinuity. One should note that it is the upper point of curve C (transition at about 60° C.) which the earlier workers referred to as 'the' transition temperature of the *Pieris* pupa.

Similar experiments of pupae of *Tenebrio* are illustrated in Fig. 10. Making due allowance for differences in technique and for the scales on which the results are expressed, there is no great discrepancy between the data of Holdgate & Seal (1956) and the results of this experiment. Certainly, the characteristics of waterproofing change appreciably with the age of the living pupa. A transition point at 35° C. remains relatively constant in position throughout the life of the instar. A second transition point (curve B) appears at about the time of secretion of the cement; at that time it is not much higher in temperature than the first transition point but there is no doubt (on grounds of experimental error) that there are two separate breaks in curve B. As the pupa ages, the temperature of the second transition point appears progressively higher (curve C). The accuracy of this experiment is such as to indicate clearly that there are still only two transition points, as distinct from a series of transition points added at successively higher temperatures with age.

As in some of the previous experiments, the measurements on a particular specimen used to provide the data in Fig. 10 often extended over 3 or 4 days. It is very important, therefore, to note that curve A is drawn through two series of determinations; one set of points has been derived from a pupa immediately after it had been killed, while the other comes from a pupa killed at an identical age but stored for 7 days at 15° C. before the measurements were made. There is no sensible difference between the curves representing the trend of the two series of points. This information considerably enhances the value of the extended experiment, for it would seem to indicate that the properties of the waterproofing system remain very stable for some time after death even though they may be changing in the living animal. Other implications of this experiment are discussed below (p. 416).

THE DISTRIBUTION OF CUTICULAR WATER DURING EVAPORATION

Beament (1948) points out that unless measurements are made under conditions of a steady rate of water loss, the results obtained from experiments on transpiration from cuticle and from models of cuticle could be most misleading; and in the present work emphasis has been laid on the need to obtain consecutive readings of close agreement at any one closely controlled temperature before adopting a measurement of permeability. But the changes which occur prior to the establishment of a constant rate of flow through the cuticle at any one temperature are not without interest, for we can deduce some information about the distribution of water in the outer cuticle from them.

Fig. 11 has been constructed from data obtained from a *Tenebrio* pupa shortly after the secretion of the cement. The permeability/temperature curve of this specimen has been drawn, but for clarity only a selection of the points which deter-

mine it has been given. In addition, there are several points which have been obtained by calculating the apparent permeability of the cuticle from the rate of water loss which is measured immediately after the apparatus has taken up a new temperature. The readings shown in the figure were obtained in alphabetical order. Consider, first, circumstances below the lower transition temperature. At about 27° C. the permeability measured when the rate of water loss is constant is indicated by *b*. The temperature is raised, and as soon as it becomes stable the rate of water loss first measured gives us a permeability *c*; it is significantly higher than the
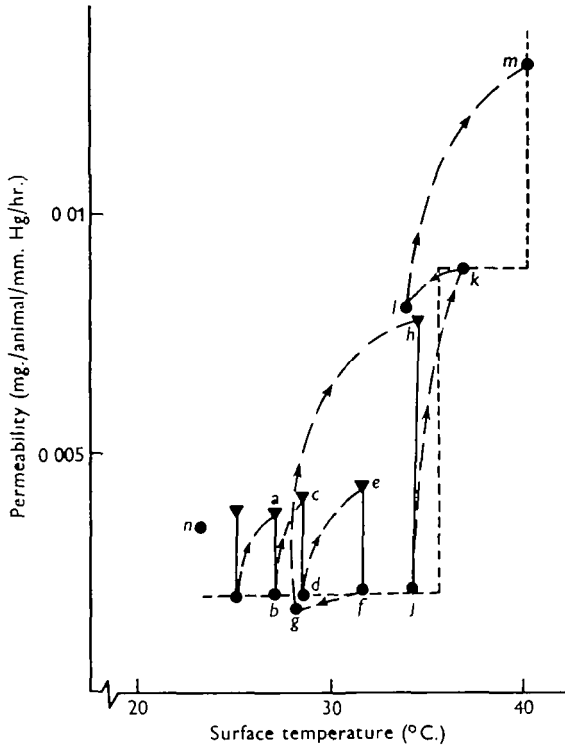

Fig. 11. The permeability/temperature relationship for a *Tenebrio* pupa at 24 hr. old. Certain experimental values have been added: *a, c, e, h* are apparent permeabilities immediately after a change in temperature; *b, d, f, j*, are corresponding true values; *g, k, l, m, n*, are true values attained immediately after a change in temperature. Determinations were obtained in alphabetical order (symbol *i* not used). For further explanation, see text.

steady rate (*d*) which obtains some 30 min. later. This phenomenon is observed with each temperature increment over the first portion of the permeability/temperature curve. Thus although the true permeability of the cuticle is not altering sensibly in this temperature range, each time the temperature is raised the specimen loses some water in addition to that which can be accounted for by its permeability. In contrast, if we lower the temperature (*f* to *g*) the initial rate of evaporation, and subsequent values at the new lower temperature, agree closely; judged by the line of the graph the permeability (*g*) is low, but the departure is hardly significant.

We see, too, that the larger the increment (g to hj) the greater is the initial rate of water loss (giving h) though the steady value (j) comes closely on to the line through b, d, g and f. Interesting as it may be to pursue this kind of experiment in greater detail, a temperature increment cannot be made in a few seconds (it does take only a few minutes) and the cuticle obviously starts to react to an external change in temperature and saturation deficiency immediately the temperature of the air surrounding it starts to change.

Now consider events at temperatures above the lower transition point. As soon as a new temperature has been obtained the rate of water loss seems to settle to a constant value at once (k); it does not take longer to adjust the temperature in this range than it did at lower temperatures. If the specimen is now cooled to a temperature lower than the lower transition point (l) we still get steady rates of evaporation simultaneously with the establishment of the new temperature; the actual permeability is of course now permanently impaired, and the proximity of points l and h is coincidental. The picture of events is completed by taking the specimen well above its higher transition point (m) and then returning it to the lowest temperature of the whole experiment (n). In both cases steady readings are obtained as soon as the new temperatures have been established.

We next examine in a similar fashion the experimental data from which curve A of Fig. 10 was obtained; here we have the freshly emerged pupa of *Tenebrio* with only the primary wax layer on its surface. We find that throughout the experiment the rates of water loss are sensibly constant at each temperature, from the time of the establishment of the new temperature. The same remarks apply to the experimental records for the *Calliphora* larva and for the newly formed pupa of *Pieris*, but variations similar in nature to those illustrated in Fig. 11 occur in the experimental records for the mature *Pieris* pupa and for the mature *Rhodnius* nymph.

These findings must now be considered in the light of two theories. Beament (1954) suggested that some of the unusual properties of insect cuticle, particularly its ability to obtain water from subsaturated atmospheres and its asymmetric behaviour when the direction of flow through it was reversed, might be more readily understood if one considered the distribution of water across the cuticle during conditions of steady flow. Across each component of a cuticle transmitting water there must be a concentration gradient, falling in the direction of flow, and the greater the rate of flow the steeper the gradient (Fig. 12A). This picture supposes an extremely simple structure for cuticle, comprising one uniform hydrophilic phase and a single layer of wax outside it. The gradients through the hydrophilic layer have been greatly exaggerated for clarity, and no account has been taken of the comparative resistance to water flow of the two components comprising the cuticle model. In fact, the wax is so much more resistant that, so long as we consider only low rates of flow entirely in the outward direction, the change in concentration at the wax-hydrophilic cuticle interface is negligible compared with a corresponding change in saturation deficiency at the outer surface of the wax (Fig. 12B). We may regard the hydrophilic cuticle as a reservoir of water. Our experiments with animals (*Calliphora* larvae and young pupae of *Tenebrio* and

Pieris) lend support to this conclusion: we believe these animals to have a hydrophilic cuticle covered only with a primary wax layer, as in this simple model. When the external saturation deficiency changes, the rate of flow changes immediately, and proportionally to it. Indeed we assume in our calculation of the permeability of the cuticle (meaning of the wax layer) that the change in gradient across the wax is the change in saturation deficiency.
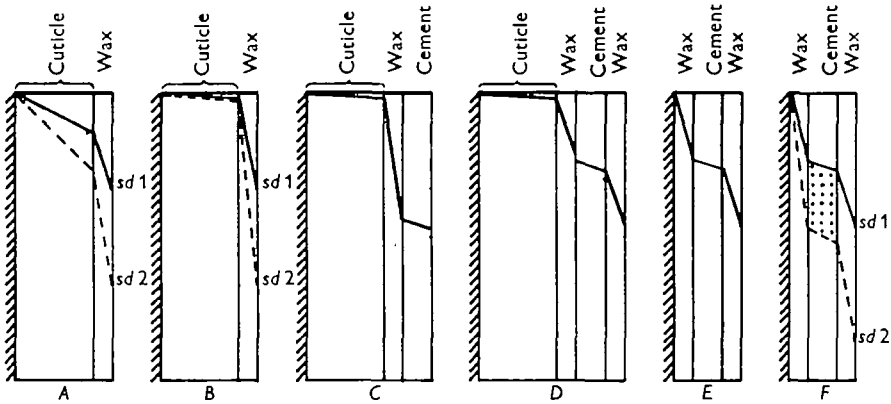

Fig. 12. Representations of sections of cuticle through which water is transpiring from the inside of an animal (left of each diagram) into dry air (right of each diagram). Diagonals through sections indicate the form of the gradient of water content causing transpiration. *sd* 1 and *sd* 2 indicate two external saturation deficiencies, of which *sd* 2 is twice *sd* 1. In section *F* the stippled area represents the amount of water which the cement must lose during the change from conditions of steady flow at *sd* 1 to similar conditions at *sd* 2. The relative thicknesses of the components of the cuticles are not to scale. For further explanation, see text.

But are we correct in assuming that the cuticle of an older pupa can be represented by this simple model? We have (p. 404 above) questioned the possibility that one wax layer could show two transition points and have suggested that two such discontinuities in the permeability/temperature curve of a single monolayer would be even less readily accepted. The upper transition temperature appears only after secretion of the cement, and to represent the cement a layer which must certainly be more hydrophilic than wax must be added to the model (Fig. 12 C). If the wax is still the real barrier to the flow of water we must still suppose that almost all the gradient causing flow must be across it; and we would expect the system to respond rapidly to changes in external conditions, for they will produce only small changes of gradient in the cement, as in the hydrophilic cuticle.

But if we assume that there is a second monolayer of wax on the outer surface of the cement (Fig. 12 D) (not the waxy bloom of Holdgate & Seal (1956), though this may well arise from such a monolayer) many more of our observations can be explained. We can now suggest that the two transition points belong respectively to the inner and outer wax layers; this correlates exactly with our experiments on animals of different ages within the instar. We can continue to regard the main inner hydrophilic cuticle as a reservoir of water and simplify our model of gradients

to Fig. 12 *E*. The resistance to flow now occurs in two wax layers and the gradients across them will be steep. When the rate of flow increases, the gradient across the cement may not change greatly in accommodating that greater flow, but the distribution of steeper gradients across the wax will cause a substantial alteration in the concentration level within the cement (Fig. 12 *F*). In other words, when the rate of flow is increased the cement must lose some water before it can establish its new slight gradient.

If we now consider Fig. 11, we can see that all stages of this argument fit with our findings. (This figure shows *permeabilities*; the rate of flow through the cuticle increases with temperature, proportionally with the increase in saturation deficiency, when permeability remains unchanged.) Each time the temperature is raised and *until* new stable conditions obtain, the actual water of evaporation which is measured can be considered as two fractions. One represents an increased saturation deficiency; the other represents a reduction in the water content of the cement. The cement is thin and its water content at saturation will not be great; if water could escape from it freely the water content of the cement would quickly reach its new value. But if the surface of the cement has a highly impermeable wax barrier on it a considerable period of time must elapse after a temperature increment before the initial rate of water loss comes down to a steady value. The greater the change in rate of flow, the greater is the alteration of the water content of the cement, as is seen by comparing *d*, *e*, *f* with *g*, *h*, *j* in Fig. 11. After a decrease in temperature the rate of water loss should be low during the period of transition while the cement is accumulating water. This is not convincingly shown; experimentally, it takes much longer to obtain a particular decrement by cooling than a corresponding increment of temperature by heating, and we may not have been able to obtain the vital reading (but see below and Fig. 13).

But these theories are of greater interest still when we consider the events above the lower transition temperature in Fig. 11. If the primary wax layer of the pupa retains its properties of permeability after the secretion of the cement, it should now be very permeable to water. Water can flow from the reservoir of the hydrophilic cuticle into the cement. If the remaining barrier with high transition temperature is on the outer surface of the cement, the system is now represented by Fig. 12 *B*, the outer wax layer representing wax, and the cement representing cuticle. We should expect, and we indeed find, that changes in the rate of flow can be accommodated very rapidly.

THE PUPARIUM MODEL

The transient conditions we have analysed in the preceding section are revealed in changes of very small magnitude. The actual amounts of water which might be lost by the cement over any one change of rate of flow are extremely small, though calculation shows their order to be reasonable. Much more satisfactory confirmation of our ideas would be given by a system, similar in essentials to the wax-cement-wax sandwich we have postulated, but in which the changes to be observed were on a greater scale. Fortunately, one such model is readily available. During

the development of *Calliphora* there is a stage where the puparium surrounds the pupa. Neither cuticle, so far as we know (Wolfe, 1954), has any cement, and thus the waterproofing system consists of two primary wax layers with a great thickness of puparial cuticle between them. There is also an air gap between the puparial cuticle and the pupal wax but its presence does not affect our arguments. The principal difference between this waterproofing system and the one we have postulated for the older pupa of *Tenebrio* is that the outer wax (on the puparium) has the lower transition temperature. Not only can we investigate this system by taking the intact animal through a wide range of temperature, but (see Wigglesworth, 1945) we can cut away a large part of the puparial cuticle without damaging the pupa and investigate the properties of the inner layer alone.
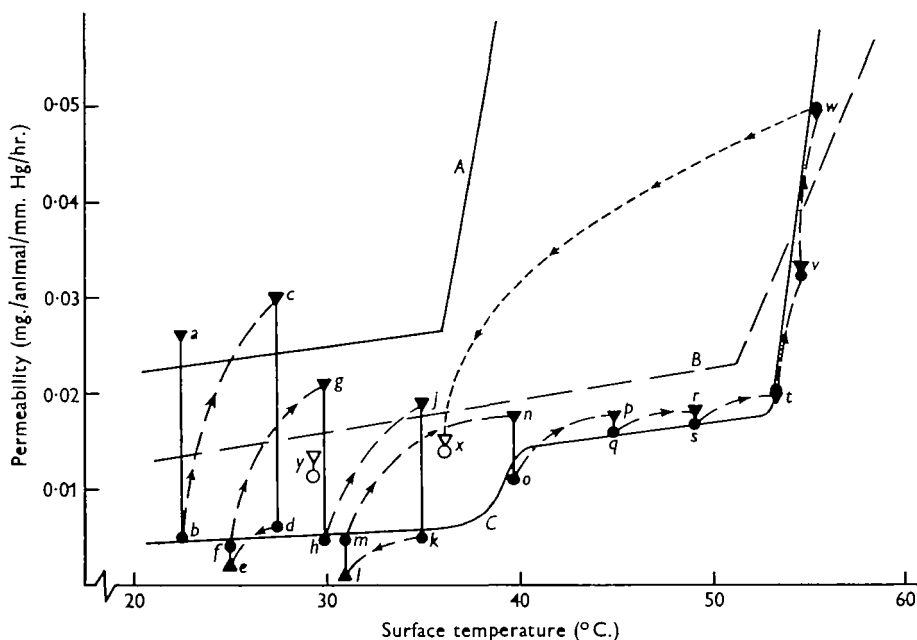

Fig. 13. Permeability/temperature curves for *Calliphora*. *A*, puparium at 24 hr., having only the larval wax layer. *B*, the pupa alone at 3 days old (obtained by dissection). *C*, the intact puparium containing a pupa at 3 days. Experimental values have been added to curve *C*; triangles indicate apparent permeabilities immediately after a change in temperature, and circles the corresponding true permeability. Information has been obtained in alphabetical order (symbols *i* and *u* not used).

The results of these experiments (Fig. 13) entirely substantiate our theory based on events in the cuticle of the older *Tenebrio* pupa (Fig. 11). When only one wax layer is present, either naturally or effectively, above the transition point of the puparial wax (or artificially, after dissection of the puparium) a steady rate of water loss is rapidly established at each new temperature. When two impermeable barriers enclose the thick cuticle of the puparium, new rates of flow are accom-

modated only very slowly, and the apparent values of permeability calculated immediately after new conditions of temperature have been established depend on the direction in which the temperature has changed.

THE ACTION OF ADSORPTIVE DUST

We have not been able to obtain *Tenebrio* pupae in which the cement has been produced and which do not show two transition points; indeed we do not know whether there is a natural stage in development at which the pupa has cement but is without the wax we have supposed to constitute an impermeable barrier above it. But since we have postulated this outer wax layer, it would be desirable to supplement the information of Figs. 11 and 13 by carrying out experiments after we have tried to remove that wax. Holdgate & Seal (1956) measured the water loss from *Tenebrio* pupae from which they had washed the waxy superficial bloom with chloroform. However rapidly washing is carried out, contamination of the cuticle with polar solvents (Beament, 1954) may produce far-reaching effects which are by no means understood. It would be unwise to draw conclusions from these experiments.

In the earliest experiments of this paper (p. 394) we showed that however gently an insect is handled its permeability is increased. We have actually observed this with a number of different species in the course of the work, but in reviewing those very qualitative results it is rather surprising to find that it is the insects *with* a cement layer which are most obviously changed by handling. It may be worth noting a previously unpublished observation that the cuticle of *Periplaneta* is apparently unaffected by gentle handling—and certainly this has no effect on the appearance of a sharp transition point—whereas handling destroys the sharpness of the transition phenomenon in some other insects; and it is particularly worth contrasting the effect of handling the new, and the mature, pupa of *Pieris* (Fig. 14). These experiments are qualitative but they clearly suggest (*a*) that the proportional increase in evaporation produced by handling an older pupa (with cement) is much greater than that produced by handling a young pupa, and (*b*) that handling an older pupa interferes with the abruptness of the upper transition point alone.

The second observation strengthens our hypothesis that the upper transition phenomenon is associated with the outer surface of the cement. The first is much more surprising; if the cement protects the primary wax layer, why should the permeability of the cuticle be increased so greatly by interference with the upper surface alone? It is clearly necessary to obtain quantitative information about this; and the experiments of Fig. 12 suggest a way in which adsorptive dusts can be used.

The mode of action of the so-called 'inert' dusts, which cause increased transpiration, has been a source of controversy for many years (see, for example, Beament, 1956). Much of the argument has been about the precise mechanism by which the dust works—whether it adsorbs wax or whether it abrades and damages the wax mechanically. But such hypotheses necessarily depend on the structure of the cuticle; they have not taken into account the possibility that there could be

a further waterproofing layer on the outside of the cement. Suppose we could provide circumstances in which a dust can act only by adsorption when applied to the surface of a mature pupa; we should get a specimen showing only the characteristics of a young pupa (except that its primary wax layer will be covered by cement alone). We can, additionally, apply such a dust to a newly emerged pupa and find its effect on a system with a single wax layer. If the experiment proves successful, we shall also throw considerable light on the long-standing problem of the mode of action of dusts.
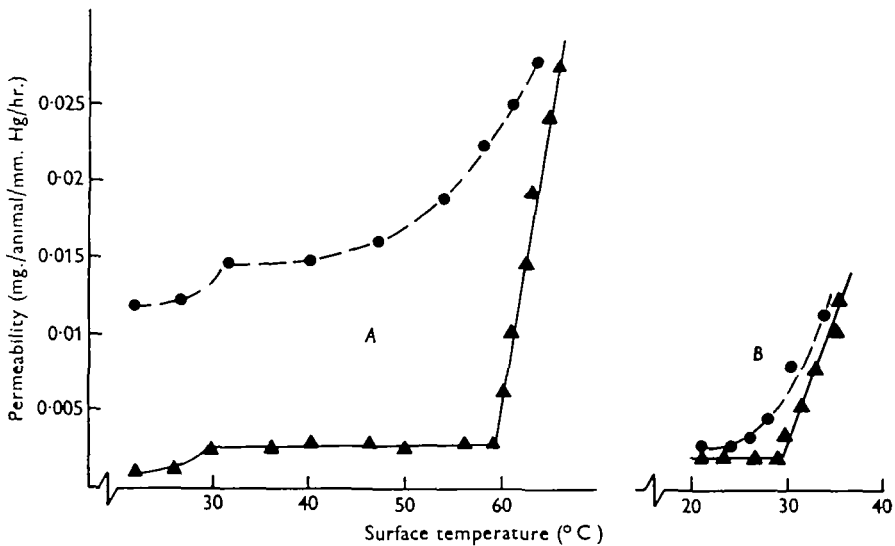
Fig. 14. The increase in permeability produced by handling pupae of *Pieris*.
A, pupa 5 days old; *B*, pupa 12 hr. old.

Animal charcoal ('activated' charcoal) was selected for these experiments; not only has it excellent adsorptive properties but it is also very soft indeed. The sample used in the experiments reported below came from the same jar as that used by Holdgate & Seal (1956). These authors rolled pupae in this dust for a stated number of times and showed an increase in evaporation correlated with the amount of rolling. We have not attempted to copy this technique exactly; on the other hand, every effort has been made to provide conditions in which the dust could adsorb without abrading.

The specimen was placed on a rubber bung at the bottom of a 3 cm. diameter glass tube, 1 m. long. This tube was mounted vertically; into its upper end was inserted a short glass tube of considerably smaller diameter which formed one limb of an inverted U of about 60° included angle. The other limb of this U-tube—about 1 m. long—thus rose at an angle from the bench. Its lower end was led into a vessel containing a sample of animal charcoal and a glass nozzle on a flexible sleeve. By blowing compressed air through the nozzle a cloud of charcoal particles could be blown up the inclined tube; heavier particles were precipitated and ran back to

the vessel. By adjusting the rate of air flow an alutrate of very fine charcoal dust was sent into the top of the vertical tube containing the specimen and settled on it. When the pupa was uniformly covered in a dense black film the bung supporting it was carefully extracted. As much superficial dust as possible was removed from the pupa by playing a fine jet of compressed air against its surface and it was then transferred to the balance; its surface was still a dull grey colour. Some animals were successfully placed on the balance merely by tipping the bung; others were manipulated by means of a minute suction tube which made contact with only a very small area of the surface.
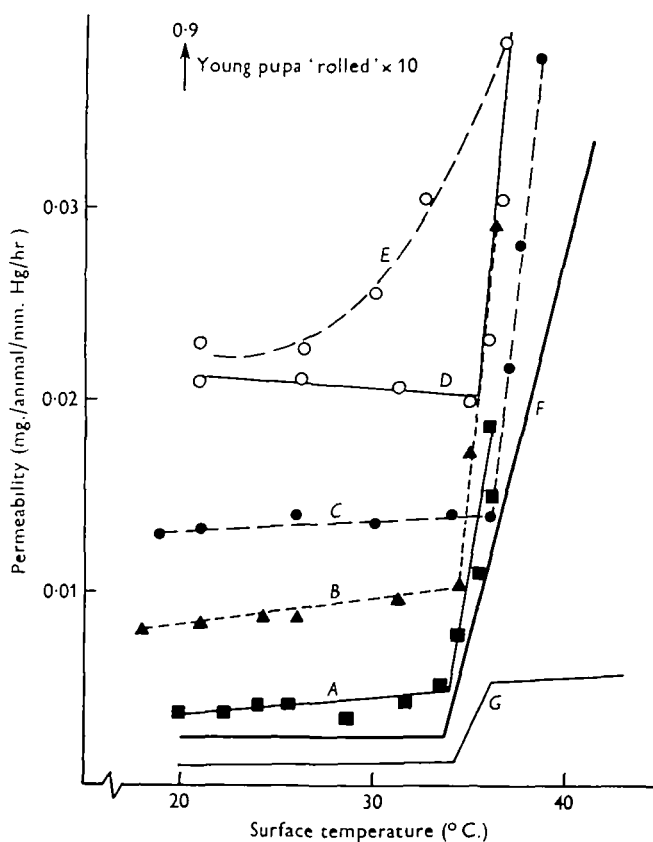

Fig. 15. Permeability/temperature curves for pupae of *Tenebrio* after various treatments with animal charcoal. *A*, pupa 12 hr. old, having a surface covered with dust (adsorption alone); *B*, pupa 5 days old, having a surface covered with dust (adsorption alone); *C*, pupa 5 days old, rolled once in dust; *D*, pupa 5 days old, rolled ten times in dust; *E*, the pupa of curve *D* measured after heating to the highest temperature indicated on curve *D*; *F*, an intact pupa 12 hr. old; *G*, an intact pupa 5 days old.

For comparative purposes other pupae were placed in the long wide tube and about a cubic centimetre of animal charcoal was added. The tube was closed at both ends with bungs and rotated very slowly so that the specimen slid gently from one end to the other along the inner surface of the glass and rolled in the dust at either end.

The permeability/temperature curves of these charcoal-treated animals are shown in Fig. 15. Apart from the curves on that figure, attention is drawn to the exceptionally high permeability of a freshly emerged pupa which had been treated by rolling it in dust for ten revolutions of the tube; the animal lost water so rapidly that one could not use present techniques to construct a permeability/temperature curve for it. Exposing the primary wax to a soft adsorptive dust with movement must lead to heavy damage. By contrast (Fig. 15, curve *A*), if a freshly emerged pupa is coated in an alutrate of charcoal but movement is avoided as far as possible, there is very little increase in permeability.

When mature pupae are treated with dust their permeability/temperature curves show three features, each of considerable significance. Where an animal has been exposed—so far as this is experimentally possible—only to adsorptive forces all sign of an upper transition point disappears (curve *B*); not unnaturally, there are no upper transition discontinuities in the curves from animals which have also been rolled in dust (curves *C* and *D*). But the lower transition point is quite clearly defined in all these curves, whether the animals have been subject to adsorptive forces alone or whether they have been rolled in dust as well. Of course, when the animal is rolled in dust some gross mechanical damage must occur, and the more the pupae are exposed to this treatment the less abrupt is the lower transition discontinuity; yet even in the curve (*D*) obtained from the most damaged cuticle one cannot mistake the transition region, and from the arguments above (p. 400) our confidence in its reality is increased by taking such a specimen through a temperature cycle (curve *E*), when we obtain a smooth curve typical of permanently impaired waterproofing.

In addition, the experimental records show that every one of the animals represented in Fig. 15 attains a steady rate of water loss almost simultaneously with the establishment of new conditions of temperature. This evidence fits excellently with our suggestion above, namely, that we are here dealing with systems containing only one waterproof barrier.

Thirdly, there remains perhaps the most puzzling feature of Fig. 15, which confirms the suggestion originally made in connexion with the handling of pupae of *Pieris*. The charcoal-treated mature cuticle shows greatly increased permeability at all temperatures, even though it retains the characteristic lower transition discontinuity so sharply that in *this* respect we might suggest that the primary wax layer is entirely undamaged. When the insect is taken through a temperature cycle (curves *D* and *E*) the relation between the two half cycles is typical, but the entire phenomenon has been translated up the ordinate. This discovery, which is discussed below, may prove of significance.

<div align="center">DISCUSSION</div>

Excepting the locust hopper, we have found at least one sharply defined discontinuity in each of the permeability/temperature curves of a range of different insect and acarine species. The nature of the discontinuity closely resembles that which Wigglesworth (1945) and Beament (1945) supposed to exist in experiments which

technically were incapable of revealing a discontinuity precisely; wherever we can compare similar species the temperatures at which some of these breaks occur also correspond closely with the temperatures originally suggested by those authors and by Lees (1947). There is no doubt that the phenomenon we now describe is the one originally called a 'transition' occurring at a 'critical temperature'. We must make it clear that there is no single experiment in the paper by Holdgate & Seal (1956) which conflicts directly with the findings of a corresponding investigation in the present work; all discrepancies can be explained by differences in technique and in the parameters and scales chosen to display and examine results. Holdgate & Seal were quite justified in concluding that they could not show the existence of transition phenomena, and they were also probably correct in suggesting that changes in the permeability of wax layers on insects could not be explained by Müllerian transitions. It is unfortunate that they attempted a mathematical treatment of data all too scanty for the purpose, but not nearly as unfortunate as the suggestion by reviewers, such as Edney (1957), that the transition phenomenon is an artefact.

The whole of the evidence we now have is consistent with the following hypothesis: if two transition points occur, the lower is associated with the primary wax layer and the upper is associated with the outer surface of the cement. The critical temperatures suggested by earlier workers must be compared in some species with the upper critical point, and in others with the single critical point we have found. Wigglesworth (1945) and Beament (1945) thought that all transition phenomena were properties of the primary wax layer and we must now attempt to associate the behaviour of the cuticle with what we know of its structure. *Calliphora* larvae, puparia, and pupae (Wolfe, 1954) and *Ixodes* (Lees, 1947) have no cement; they have only one transition point. *Rhodnius* nymphs (Wigglesworth, 1947), *Tenebrio* pupae (Holdgate & Seal, 1956) and *Pieris* pupae have cement; they have two transition points. We know nothing of the nature of the cuticle surface in larvae of *Tenebrio*, *Pieris* or *Nematus*, though there are reasons (see for example, Way, 1950) for believing that they might have a cement covering. Malek (1958) proposes that the cuticle of the locust is very similar to that of the cockroach; it has a cement (see Kramer & Wigglesworth, 1950) but Beament (1955, 1958a) suggests that the cement on the cockroach is a discontinuous structure, so that *functionally* the grease is in a single layer over the surface. If the locust is like this it also would have one functional wax layer. Finally, *Ornithodorus* (Lees, 1947) has a cement layer; it is difficult to decide whether its curve (Fig. 3) shows a small break around 35° C. With such a scanty knowledge of cuticles, and until we have information on a very large number of different species and orders, we cannot make useful suggestions about types of waterproofing systems. The material question is: if a cuticle has a cement layer, but reveals only one transition point, does this represent the property of the primary wax layer, or of a wax on the cement, or can there be two layers with identical properties? In the three insects we have examined in most detail, two critical temperatures are associated with wax on either side of the cement; those two wax layers must have very different properties, the most likely basis for which must be a difference in mean molecular weight.

Consider the *Tenebrio* pupa. A primary wax with transition around 35° C. suggests a relatively low molecular weight, and the X-ray studies of Holdgate & Seal (1956) indicate that this wax melts at about 40° C., i.e. a few degrees above its critical temperature, which is precisely the relationship first suggested by Beament in 1945. Holdgate & Seal give 60–80° C. as the melting-point of the waxy bloom over the cement, a temperature bearing a similar relationship to the transition point of about 55° C. which we now associate with the surface of the cement. We must be careful to distinguish between a bloom—which is a discontinuous forma-tion—and the imperforate layer which we think more likely to give great im-permeability and sudden transition; but there is no reason why the bloom should not be associated with a continuous layer of wax at the actual surface of the cement. Whatever the detailed structure there is on the cement a wax of high melting-point, and therefore of high molecular weight; and its origin must be established. The cement is likely to be shellac (Beament, 1955), and although commercially obtained shellac includes waxes which may well be waterproofing materials there is no evidence in Wigglesworth's studies (1947, 1948) that the epidermal glands contain or secrete any wax. Holdgate & Seal show that the wetting properties of the cement surface change continuously from the time of its secretion, corres-ponding with the gradual appearance of the bloom. All this suggests that there is no wax in the cement when it is first secreted, but that wax subsequently migrates into it and through it. The most obvious origin of that wax is the primary wax layer. We suggest that the primary wax of *Tenebrio* is a solution of the hard outer wax in a volatile solvent and that it migrates through the cement to form the outer layer by the evaporation of volatile components there. The transition point of its primary wax may appear constant over a period of a week, but this does not dis-prove the existence of a solvent; the grease of the cockroach, which is known to contain volatile solvents (Beament, 1955), shows a change of only 2–3° C. when exposed on the living animal for a month (Beament, 1958 a). Cockroach grease may change to a wax melting at 55–60 °C. on heating but such a change is only accomplished at room temperatures in about 6 months. Further comparison of the properties of the two wax layers on some of these insects with the grease of the cockroach supports this theory. *Tenebrio* pupae have transition points at 35° and 55° C.; *Rhodnius* nymphs at 30° and 58° C.; *Pieris* pupae at 30° and 60° C.; *Tenebrio* waxes melt at 40° and at 60–80° C.; cockroach grease changes from transi-tion at 30° C. to a melting-point at 55–60° C. And further, the form of a wax bloom is characteristic of a process of crystallization by slow evaporation of a solvent.

Superficially, some of the evidence of Beament (1945) is against this theory. Extracted waxes on membranes and membranes of cast skins showed charac-teristics which we now associate with the outer wax layer, but the bulk of the wax extracted from the cuticle must have come from the *inner* primary wax layer. We do not have information species for species; but the melting-points quoted by Beament (1945) (e.g. 60° C. for the total extract of *Rhodnius* wax) cannot always be reconciled with Holdgate & Seal's figures (e.g. 40° C. for the melting-point of

Tenebrio primary wax), or with the low transition temperatures of the main primary layer in many of the insects studied in this paper. But the original records show that Beament (1945) extracted waxes from cast skins which had been collected over periods of months if not years, and some of the cast skins he used as membranes were equally aged. Both the process of extracting wax and the long-term storage of skins will result in excessive loss of any solvent they may contain. The waxes used in 1945 may well have been changed by losing their solvents so as to have only the properties of the outer layer, and a repetition of these experiments in the light of present findings may show that they confirm our ideas.

THE TRANSITION PHENOMENON

It is tempting merely to point to the theory of mono-molecular organization and disorientation which explains events in the cockroach grease, and to suggest that similar processes occur in hard waxes. There are, however, very important differences between hard waxes and cockroach grease. Because of the mobility conferred by the solvent, cockroach grease can reform waterproof monolayers after temporary exposure to heat; it can spread over water, wet surfaces, and, we presume, over the entire surface of the insect. Hard waxes, so far as we know, can only spread at temperatures a few degrees below their melting-points (Beament, 1945); their properties are irreversibly changed by exposure to higher temperatures, and indeed we do not know whether in fact they do form a continuous layer over the surface of the animal. We can argue that unless the whole surface of the animal were covered with the hard wax it could not be as waterproof as our measurements indicate. Since our figures include spiracular loss, the cuticles of animals described in this paper are actually more impermeable than our figures would suggest.

But there is a more telling argument against the discontinuous distribution of these hard waxes over the cuticle surface; and it leads to an important conclusion about the transition of hard waxes. Suppose that most of the water transpired through these cuticles passed through numerous small unwaxed areas, large capillaries, etc. By the well-known pinhole diffusion theory of Brown & Escombe (1900) the contribution of small perforations to water loss will be out of all proportion to their summed area. When the waterproofing material is taken past its melting-point these areas must become covered by flow of the molten wax; indeed the reflexion electron micrographs of Holdgate & Seal (1956) actually show that the bloom of the *Tenebrio* pupa exposed to 80° C. does melt and flow into a layer over the surface. Yet the effect of exposure to temperatures above the transition point and above the melting-point is always to *increase* the permeability of the wax at *all* temperatures, as well as to remove the transition discontinuity. So we cannot associate transition with the microscopic redistribution of wax through melting; transition must surely be a molecular phenomenon.

We must next consider the shape of the permeability/temperature curves. Spiracular losses are included but it can be argued that they cannot affect the shape

of the curves; losses from the equivalent of a free water surface must give a curve parallel to the abscissa when plotted on the axes used in these figures (see Beament, 1958a, p. 496); and we cannot expect to distinguish between losses from a waxed tracheal surface and from the exposed waxed cuticle itself. Over the limited range of temperature where experiments were possible (limited, to curtail evaporation of solvents) cockroach grease has a small and constant temperature coefficient of permeability, both below and above transition. In the natural state, hard wax also exhibits a constant temperature coefficient; once it has been permanently changed by heating, the temperature coefficient apparently obeys complex exponential laws, as is the case for most substances. Heat-treated wax has no very remarkable property, then, but the naturally occurring layers have, and the degree and nature of the difference between the two underlines the peculiar state in which the natural waxes occur.
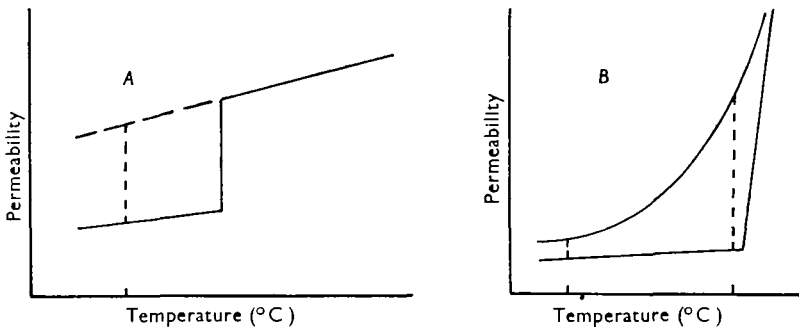

Fig. 16. Diagram showing (*A*) the form of the permeability/temperature curve for *Periplaneta* (Beament, 1958a). The permeability of the grease at the indicated temperature can be compared with the probable permeability of that grease when modified above transition, by projection of the upper curve. *B*, Typical permeability/temperature curves for insects with hard waxes; the ratio of the permeabilities of waxes in the natural state and after exposure to high temperatures depends on the temperature at which comparison is made. For further explanation, see text.

The peculiarity of cockroach grease below its transition temperature is the existence of the organized monolayer. By projecting (Fig. 16*A*) the part of the curve which represents completely disorganized grease down to temperatures where that same grease would develop the monolayer, it is possible to compare the properties of the grease in either state *at the same temperature* and thus to deduce the absolute permeabilities of the grease in monolayer and in disorientated states respectively. In characteristic curves for these hard waxes (Fig. 16*B*) the actual permeabilities of the natural state and the heat-treated (heat-stable) state can be compared. The magnitude of the change in permeability depends on the temperature at which comparisons are made; at temperatures just below the transition point the difference in permeability between the two forms of wax is so great that we can hardly attribute it merely to a change in a monolayer of a thick wax—it must surely be a change throughout the thickness of the layer. Some support for this argument can be obtained by calculating the absolute permeabilities of cuticles

having hard waxes, and comparing the figures with that for the cockroach. In doing this we must bear in mind: (1) that the thickness of the wax is known only roughly for some of the insects—and here we shall assume throughout that it is $0.25\,\mu$ (Beament, 1945); (2) that the values we calculate will be high because they are based on figures which include spiracular loss; and (3) that the surface areas of the animals have been determined only very approximately, in most cases using formulae (Wigglesworth, 1945) which relate area to weight. With all these approximations and inaccuracies detailed comparison of the permeability of different species is not profitable; comparison of figures all obtained for one species is of course altogether more satisfactory.

The absolute permeability (given in ml. $\times\ 10^{-8}$ water vapour at N.T.P. per second referred to 1 cm.^{2} of material of thickness 1 ml. subjected to a pressure difference of 1 ml. of mercury) of disorganized cockroach grease at 25° C. is approximately 2·0. The waterproof monolayer of the cockroach has an order of permeability of 0·4 absolute unit, and the whole cuticle at this temperature, 1·65 units. Corresponding figures at 25° C. for the very waterproof insects in the present study are: *Tenebrio* mature pupae, 0·15; *Pieris* mature pupae, 0·5; *Rhodnius* nymphs, 0·9. These figures invite comparison with the value for the monolayer of cockroach grease, and so support our contention that molecular organization causing a very waterproof state extends through the wax layer beyond the confines of a single monolayer. After these three insects have been heated through only the lower transition point the permeabilities (at 25° C.) are respectively 0·75, 0·9 and 1·5 absolute units. The order of these figures is more in keeping with the permeability of the whole cockroach system—a monolayer and much disorganized wax; according to our present ideas they are the permeabilities of cuticles in which the natural state of the primary layer has been destroyed but which still have a thin natural layer on the cement. After heating through both transition points the absolute permeabilities at 25° C. rise respectively to 1·3, 1·9 and 2·4, suggesting that now all the wax is disorganized. If we next consider newly moulted pupae, *Tenebrio* has an absolute permeability of 0·4 and *Pieris* of 0·7; both figures suggest that the primary wax alone is much more extensively organized than the grease of the cockroach.

On the other hand, less waterproof arthropods, such as *Ixodes* and the larvae of *Calliphora* and *Pieris*, give values of absolute permeability between 3 and 6, and the permanent increase measured at 25° C. after heat-treatment is comparatively small. We can suggest that they resemble the cockroach more closely in the degree to which their waterproofing layers are naturally organized, but so many factors affect our calculations that we must await the outcome of much further research before this picture is complete.

There remains the inevitable question of the biological importance of a double waterproofing system. The outer layer over the cement is remarkably delicate (to handling and to adsorption by dust) and we have an anomaly so far unexplained. On being handled or dusted, animals having cement may show a considerable increase in their actual water loss but no reduction in the sharpness of the lower transition point (Figs. 14 and 15). The increased water loss is much greater than

ought to be expected merely as a result of removing an outer monolayer but leaving a primary wax layer intact. It is interesting here to refer to the explanation which has been offered for the asymmetric permeability of insect cuticle (Beament, 1954).* When water was placed on the outside of isolated cuticle and dry air on the inside, high rates of water transport were recorded; this was explained by the great attraction for water of the hydrophilic part of the cuticle when it was dry; this attraction set up a large gradient across the wax. When the outer wax of a wax-cement-wax sandwich is removed (as by dust or abrasion) the cement is then excessively dried by the air, and greatly increased rates of flow are observed; there is a close parallel in behaviour between the cuticle model and the wax-cement system. Of course, the experimental findings in the present paper are far more firmly based than is this entertaining theory, and other explanations may be forthcoming; but, on the evidence of experiments alone, there is no doubt that the insect which protects its primary wax with a cement does achieve far greater impermeability if it has further wax above the cement.

CONCLUSION

It would appear that some degree of special molecular organization, and some degree of consequent abnormal impermeability, exists in the waterproofing waxes of a large number of terrestrial arthropods. Hardly anything is known of the physical chemistry of waxes composed of mixed molecular species, and we have little evidence on which to base speculation on the kind of molecular organization which may occur. The waterproofing system of the cockroach is unusual because its grease is mobile; the evidence that it has a monolayer of special properties in that grease is strong, but it is not necessarily true that the wax of an extremely waterproof insect such as the pupa of *Tenebrio* is naturally arranged in a monolayer or in a series of monolayers. On the other hand, the phenomena which occur at particular temperatures are so sharply defined that the wax must be behaving as a unit despite its heterogeneous composition; this once more points to the very special nature of the waterproofing wax. All our evidence, too, is compatible with the presence of solvents or volatile materials of lower molecular weight when these harder waxes are first secreted. The existence of such solvents makes it more easy to understand the migration of wax through the cement and the formation of special molecular arrangements in the wax; and this may have implications on the nature of the transport processes at an earlier stage still—when the waxes are metabolized and secreted.

It is to be doubted if techniques which measure permeability to water can alone take our knowledge of this waterproofing system very much further. It is more than ever necessary now to bring to the study of special organization in these waxes techniques divorced from permeability measurement.

SUMMARY

1. The relationship between the temperature of the cuticle and its permeability to water has been determined for a number of different terrestrial arthropods.

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* In that paper the second fraction of the equation on p. 109 has been printed inverted.

2. In most of these animals the waterproofing wax loses its great impermeability to water very abruptly at a particular transition temperature; that temperature varies appreciably with the individual, but appears to be characteristic of any one species at a particular age within a particular instar. No change in permeability with temperature was found in *Tipula* sp. taken from soil, in which the cuticle was greatly damaged; above 48° C. the permeability of the cuticle of *Schistocerca* nymphs changes rapidly with increasing temperature, but this change is not abrupt.

3. In the permeability/temperature curves for larvae of *Pieris, Tenebrio, Calliphora* and *Nematus*, pupae of *Calliphora* and adults of *Schistocerca, Ixodes* and *Ornithodorus* only one transition discontinuity is found. In all these animals the wax maintains almost constant permeability up to the point of transition, above which permeability increases very rapidly with temperature.

4. Immediately after moulting, *Rhodnius* nymphs and pupae of *Tenebrio* and *Pieris* also reveal only one transition point; this occurs at a comparatively low temperature. But when cement is secreted over the primary wax layer, a second and higher transition point is found which is independent of the first point. The lower transition temperature seems constant in position during the life of an instar, but the upper point occurs at progressively higher temperatures as the instar ages. The evidence suggests that these animals have two special waterproof layers of wax, the one with high transition temperature occurring on the outer surface of the cement. This outer layer could arise by the migration of wax from the primary layer (with lower transition temperature) through the cement to the outer surface, there to lose volatile components and waterproof the surface.

5. In every animal investigated a permanent increase in permeability is produced by heating a wax layer above its transition point; after such treatment no transition point can be found.

6. From calculations of the absolute permeability of the various wax layers, and from the behaviour of the wax, it is suggested that all epicuticular waxes are laid down with some special molecular arrangement and that in very waterproof insects this may extend throughout the thickness of the wax layers; in less waterproof insects only a portion of the wax layer (as in the cockroach) may be specially organized.

7. Very slight mechanical damage promotes increased transpiration through the cuticle. Stationary adsorptive dusts applied directly to a primary wax layer do not affect permeability; applied to wax above the cement, they promote rates of evaporation in excess of the value exhibited by the same cuticle having a primary wax layer alone. This phenomenon is discussed in relation to the distribution of water within the transpiring cuticle.

technical information. Finally, I must thank Prof. V. B. Wigglesworth and Dr A. D. Lees for their continuing interest and encouragement.

APPENDIX

A MODIFIED APPARATUS FOR MEASURING EVAPORATION FROM INSECTS

The apparatus used in the present paper is a modified form of that previously described (Beament, 1958a) and the essential points in modification can be seen from Fig. 17. The balance beam (b) is a complex lattice comprising two girders of Elektron metal separated by plastic struts. It is suspended (s) asymmetrically so as to detect more sensitively the weight changes occasioned by the evaporation of

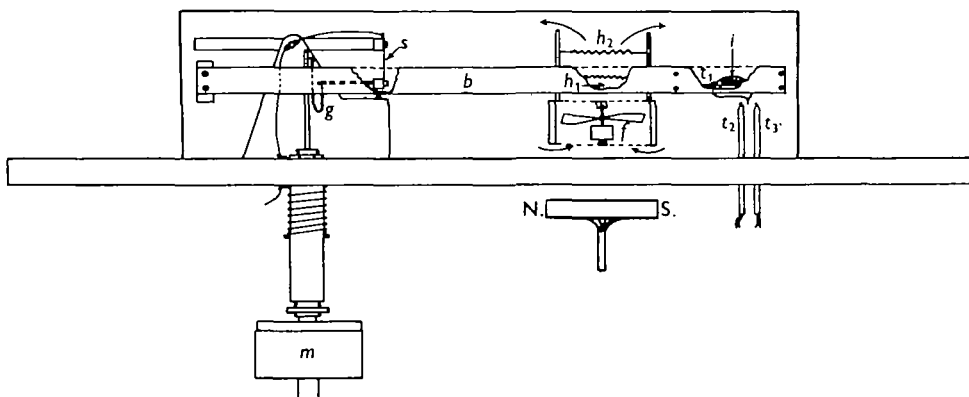

Fig. 17. Schematic drawing of balance used in the investigation.
For explanation, see text.

water from the specimen (i). A compensation mechanism has been introduced to counter most of the effect of temperature change on such an asymmetric system. The insect, with its skin in contact with one thermocouple (t_1), is supported on a fine nylon net across one end of the lattice; weight changes are measured by adjusting with a micrometer movement (m) the distribution of a gold chain (g) which is looped between the opposite end of the lattice and the spindle piston of the micrometer. Air temperature is measured and controlled via two thermistors (t_2, t_3) by the method of Beament & Machin (1958, 1959), but adapted to produce much greater heat dissipation in both the controlled (h_1) and the background (h_2) heaters. It was found necessary to enclose both heaters inside the fan housing and to place the fan (f) *under* the heaters; the direction of air circulation is thus reversed as compared with the apparatus previously described. The thickness of the plastic box for the balance has been increased to $\frac{1}{4}$ in. for better insulation, and massive brass bars have been added to prevent distortion at high temperatures. The box measures 3 in. × 3 in. × 12 in. and is sealed with a neoprene gasket. Air or nitrogen (see p. 401) was obtained dry and compressed in cylinders and could be introduced via a tap in the base of the box. Further information can be obtained from photographs in the paper by Beament (1958b).

SPECIFICATION OF EQUIPMENT

Maximum weight of specimen: 3 g. Accuracy of weight change: ±0·01 mg. Temperature measurement and control: ±0·04° C. over range, ambient − 40° C.; ±0·08° C. over range 40–80° C. Water content of air: 5 p.p.m. Temperature coefficient of balance: a change equivalent to an apparent alteration at the specimen of 2 mg. was produced by raising the temperature from 20 to 80° C. This was linearly distributed over the range and all readings have been appropriately corrected; the change is independent of load, primarily because riders and specimen are on the same side of the fulcrum. No drift could be detected under conditions of constant temperature, so that measurements of evaporation by weight change are absolute readings.

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