THE CUTICULAR LIPOIDS OF INSECTS

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(With Seven Text-figures)

The ability of insects to withstand desiccation was shown by Kühnelt (1928) to be due to the epicuticular layer of the cuticle. This outermost part was found to be highly resistant to strong acids and was shown, by chemical tests, to contain fatty acids and cholesterol type molecules. Wigglesworth (1933) described a complex fatty or waxy substance in the upper layers of the cuticle which he called 'cuticulin'; Pryor (1940) found that this lipoid is entirely confined to the epicuticular layer, suggesting that it is used to impregnate a tanned protein. Lipoid material has also been extracted from the exuviae of the silkworm *Bombyx mori* (Bergmann, 1938). Bergmann found that hot alkali merely fused the epicuticle into oily droplets suggesting that saponification was taking place. The extract so obtained was a brown waxy substance, forming about 4% of the total weight of the cuticle, and it consisted of paraffins, saturated aliphatic acids and esters. A more detailed study of the waxes of certain insects specialising in wax formation has been given by Chibnall, Piper, Pollard, Williams & Sahai (1934*b*); Chibnall, Latner, Williams & Ayre (1934*a*), and Blount, Chibnall & el Mangouri (1937). All these substances were complicated mixtures of alcohols, acids, paraffins and esters with chain length of the order of C_{30}.

Hurst (1941) has suggested that the asymmetry of the insect cuticle is regulated by the 'outer free lipoid'. He postulates a cuticle surface in the form of a lipoprotein mosaic.

It is the object of this study to investigate the distribution and properties of these lipoid materials, to obtain evidence of the structure of the epicuticle by comparison of *in vitro* phenomena with those of living insect cuticle, and to indicate the function of lipoids in resisting the action of certain insecticides.

PERMEABILITY OF WHOLE CUTICLES AND OF EXUVIAE

Apparatus

For direct comparison with the figures obtained for the permeability of living insect cuticle (Wigglesworth, 1945*a*) the standard adopted throughout was the measurement of the rate of passage of water through membranes having distilled water in contact with one side, the other side being exposed to a desiccating atmosphere. The membrane holder (Fig. 1) consisted of a glass tube 1·5 cm. bore and 3 cm. long, drawn out at the upper end to approximately 5 mm. bore and having a short length of rubber tubing attached to this end by celluloid solution. A stout celluloid sheet 4 cm. square, having a central hole 1·2 cm. diameter, was sealed by celluloid solution concentrically to the lower rim of the tube. The whole apparatus was given several coats of thin celluloid solution to ensure waterproof

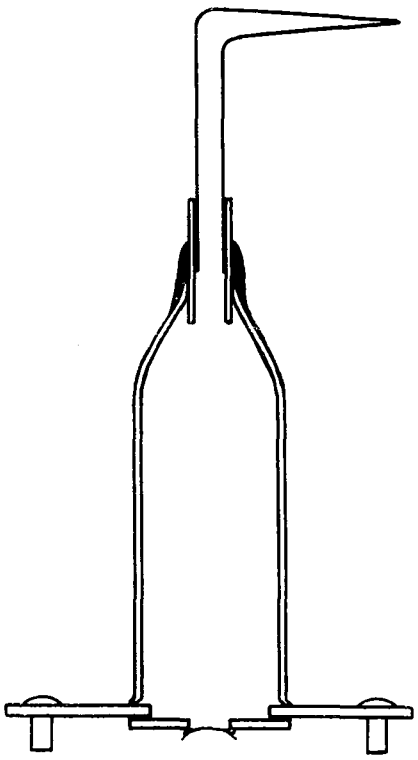

Fig. 1. Longitudinal section of a membrane holder for recording the permeability of whole cuticles of insects.

joints. Three metal studs in the perimeter of the plate supported the apparatus so that the central hole was about 1 cm. from the substrate.

Whole cuticles of *Rhodnius prolixus* were prepared for experiments by dissecting off the upper half of the abdomen of fifth-stage nymphs, 2 days after feeding. Adherent cellular material was carefully wiped out with cotton-wool, and the skin left in distilled water overnight and allowed to dry in the air. A hole 4 mm. in diameter was drilled in a stout celluloid disk 1·5 cm. diameter and the rim coated with cellulose paint. The cuticle was placed on this and the preparation left to dry. A careful examination of the seal and cuticle surface was made under a binocular microscope, and the disk mounted over the central hole of the membrane holder, using celluloid solution as a cement. The holder was then filled with water to a height of 2·5 cm., and a glass tube, drawn into a jet, placed in the rubber tube and sealed in a flame. The whole apparatus was left in a desiccator for 24 hr. before taking readings. The rate of loss of water through the membrane was obtained by weighing at intervals of 24 hr. At the end of each 3 days the sealing capillary was opened to equilibrate any pressure changes, though the amount of water lost was so small as to occupy a negligible volume. Phosphorus pentoxide was used in the desiccator and the membrane, in all experiments, was kept at approximately 5 cm. from the desiccating surface.

A sheet of celluloid, the same size as the cuticle, was sealed into a similar piece of apparatus and used as a control to check the impermeability of the cellulose paint. Pairs of holders were always used together for experiment and control; both had had the same history of exposure to humidities, and the small amount of water absorbed on the surface of the apparatus was the same in both cases. All apparatus was stored in calcium chloride desiccators when not in use.

To compare the permeability of a cast skin with preparations of the above type, the abdomen of a fifth-stage *Rhodnius* nymph, before moulting, was coated with a thin solution of gelatin in water, and allowed to dry. The bug completed its moulting processes but could not cast the abdominal part of the exuvia, which was carefully removed by cutting along the line of the spiracles. The gelatin was dissolved off by washing in several changes of water at 35° C. and the cast skin, free from excreta and undamaged, was dried and mounted as above. By reversing the disk supporting the preparation, the rate of passage of water in either direction can be measured. The exposed area was obtained by camera lucida drawings of the membrane.

Similar experiments were carried out with whole cuticles and cast skins of *Rhodnius* which had been extracted for two half-hour periods with boiling chloroform to remove the waxy substance; and with normal and extracted preparations of the pupal wing case of *Pieris brassicae* and of the wing of the t *Pieris*. The results are shown in Table 1.

In no case is the permeability of the membrane so obtained as low as that obtained for the living insect (Wigglesworth, 1945*a*). However, the table clearly shows that extraction of the various cuticles vastly increases the rate of passage of water. Whole cuticles of *Rhodnius* prepared as described have, presumably, a number of small perforations, due to the removal of the sacs of the epidermal glands during cleaning with cotton-wool, and the effect of these perforations will be magnified by the pinhole diffusion effect (Brown & Escombe, 1900). The linings of the epidermal glands are left intact in cast skins (Wigglesworth, 1933), and the gelatin coat prevents gross damage due to breaking of bristles in handling. In the wing of the imago there are no gland ducts to be considered, so that this also is a complete membrane.

Table 1. *Rate of permeation of water in mg./sq.cm./hr. through cuticular membranes at* 20° C.

Membrane and treatment	Rate of permeation
Rhodnius, 5th nymph, whole cuticle:	
With epicuticle exposed to desiccator	1·08
With endocuticle exposed to desiccator	1·7
Rhodnius, 5th nymph, whole cuticle:	
Extracted boiling chloroform	12·7
Rhodnius, 5th nymph, cast skin:	
External side exposed to desiccator	0·22
Internal side exposed to desiccator	4·6
External side re-exposed to desiccator	0·8
Rhodnius, 5th nymph, cast skin:	
Extracted boiling chloroform	17·5
Pieris pupa, wing case:	
External side exposed	0·18
Extracted boiling chloroform	5·7
Pieris imago, wing:	
Unextracted	0·29
Extracted boiling chloroform	20 approx.

These results confirm the observation of Hurst (1941) that there is an asymmetry of one hundredfold in the transmission of water through the blowfly larval cuticle. There is a suggestion of asymmetry in the whole cuticle, but in the exuviae of *Rhodnius* the ratio of rates of transmission of water in either direction are 20 : 1 in the initial period, and 5 : 1 after the membrane has been exposed to distilled water for 21 days and is very fragile. It is hoped to deal with the physical processes of this asymmetry in a separate paper (Beament, 1945*b*).

It is clear that the waterproofing layer of the cuticle lies in the exuvia of the nymph and in the pupal shell, which consist of the epicuticle and residues of the exocuticular substance; further, the structure of the epicuticular layer must be asymmetrical and cannot be represented by a regularly impregnated protein layer.

PART I. DISTRIBUTION AND PROPERTIES OF EPICUTICULAR LIPOIDS

Extraction of waterproofing substances

Exuviae of the same stage and similar size were used for the extraction of the waterproofing constituents of the cuticle. *Calliphora* pupal skins were obtained by careful removal of the delicate membrane from the vacated puparium. In each case, about one hundred clean skins were counted, weighed, and extracted with two lots of 10 c.c. of boiling chloroform under a reflux condenser for about 2 hr. The extracted solutions were filtered rapidly through no. 2 filter paper which had been thoroughly washed with hot chloroform. The waxy residues were recrystallized from boiling acetone and weighed. In Table 2 the values given are the average of at least three extractions; the variation in the calculated thickness of a wax layer of a particular example was never greater than 0·05 μ, and the agreement was usually closer. In the case of skins of *Rhodnius* nymphs, *Calliphora* puparia, *Tenebrio* larvae, and *Pieris* pupae, it was necessary to wash the skins in cold water before extraction (see p. 123) in order to get consistent values for the wax thickness. The areas of the skins were determined by camera lucida examination of a number of skins (*Rhodnius*, *Tenebrio*, *Calliphora* pupa); from geometric considerations (*Calliphora* puparia); or from the formula $S = KW^{\frac{2}{3}}$ (S = area (sq.mm.), W = weight (mg.)). The values of K used were: *Nematus* 8·5; *Pieris* larvae 9·8; *Pieris* pupae 8·4; *Rhodnius* 8·1 (Wigglesworth, 1945*a*).

Lewkowitsch & Warburton (1921) give the relative density of insect waxes as 0·96 g./c.c., and this figure was used to obtain the volume of wax and hence its thickness if present as a uniform layer over the cuticle. The thickness of the exuviae was similarly calculated on the rough assumption that their relative density was 0·8 g./c.c.; the values do not vary greatly from those obtained by microscopic observation.

It can readily be seen that there is no correlation between the cuticular thickness and the thickness of the waxy layer; in particular, the similar amounts on the very thick *Calliphora* puparium and the very delicate pupal skin are striking. While most of the cuticles have a wax thickness of 0·2–0·3 μ, the much more resistant *Pieris* pupa has a thicker layer, and in *Nematus*, which rapidly succumbs to desiccation, and whose resistance to most insecticides is low, the layer of wax is relatively thin. If we take the average length of a wax molecule as approximately 100 A. (Müller, 1930), and if the film of wax is totally orientated vertically, it represents approximately thirty monolayers.

The unextracted pupal case of *Calliphora* is white and shows interference colours; its ability to take up electrostatic charges makes it difficult to handle. After extraction, the appearance changes to a dull pale brown with no sheen or interference colours, and it does not readily become charged; all these changes are explained by the presence of a waxy film. It is doubtful if interference colours could be eliminated by the extraction of lipoid impregnating a protein. After extraction, some *Calliphora* pupal skins were heated to 140° C. with strong potash for half an hour and subjected to the 'chitosan' test. A complete layer of chitin was present.

Similar changes in the appearance of *Pieris* pupal skins were noted after extraction.

Blattids. The oily waterproofing substance of *Blatta orientalis* (Orth.) (Ramsay, 1935) was obtained by pouring cold chloroform over the abdomen of several animals, and collecting the washings. It was estimated that the thickness of oil was approximately 0·6 μ. Dust used in experiments on the desiccation of the cockroach (kindly supplied by Dr Wigglesworth) was extracted with chloroform, giving further supplies for experimental purposes.

Table 2. *Thickness of lipoid and non-lipoid material present in the exuviae of various insects*

Species and material	Treatment	Thickness of wax in μ r.d. 0·96	Thickness of exuviae in μ r.d. 0·8	Wax thickness as percentage of exuvial thickness %
Rhodnius prolixus (Hem.), exuviae, 5th nymphs	Washed water	0·25	6·5	3·8
Rhodnius prolixus (Hem.), exuviae, 5th nymphs	Unwashed	0·82 approx.	6·5	12·5
Tenebrio molitor (Col.), large larval exuviae	Washed	0·20	7·6	2·6
Calliphora erythrocephala (Dip.), puparia with pupal skin removed	Washed	0·27	47·0	0·55
Calliphora erythrocephala (Dip.), puparia with pupal skin removed	Unwashed	1·1 approx.	47·0	2·2
Calliphora erythrocephala (Dip.), pupal exuviae	Unwashed	0·18	3·1	5·8
Nematus ribesii (Hym.), exuviae of last larval stage	Unwashed	0·095	6·1	1·65
Pieris brassicae (Lep.), exuviae of last larval stage	Unwashed	0·33	7·3	4·5
Pieris brassicae (Lep.), pupal exuviae	Washed	0·4	16·7	2·4

In view of the small yield of waxes, the usual amount obtained from an extraction being a few mg., beeswax was used for the general experiments on permeability, dusts, and detergents. The pure wax was prepared from a sample kindly provided by Prof. Buxton. It was dissolved in boiling chloroform, filtered hot, and recrystallized from boiling acetone.

General properties of the lipoid extracts

With the exception of the 'soft grease' from the cockroach, all the lipoid extracts were solids, increasing in hardness and showing greater crystalline structure as one proceeded to the higher melting-point members.

The waxes from *Nematus* larvae, *Calliphora* puparia and pupae, and *Pieris* larvae were soft and without crystalline form. Those from *Nematus* and *Pieris* were a very pale yellow; the others were white. All were quite readily soluble in cold benzene, chloroform and carbon tetrachloride. *Tenebrio* larval wax, and the wax extracted from water-washed skins of *Rhodnius* nymphs were hard, white and crystalline; they were soluble in cold chloroform though less readily than the soft waxes.

On extraction, the wax from *Pieris* pupae was a deep yellow colour. The yellow fraction was much more soluble in cold chloroform and a reasonable separation was obtained, giving a hard yellow pigment with no crystalline form and making up about 30% of the total extract. The remaining, less soluble fraction was a hard white wax which crystallized in 'hedgehogs'. Without the yellow fraction this would make the thickness of the film on the pupa 0·26 μ, i.e. of the same order as that in the majority.

Table 3. *Approximate melting-points of extracted insect waxes in* ° C.

Wax	Approximate m.p. ° C.
Nematus, larval	36–42, indefinite
Calliphora, puparial	Indefinite
Calliphora, pupal	50–55
Pieris, larval	57 sharp
Tenebrio, larval	57–59
Beeswax	62–64
Rhodnius	60·5 sharp
Pieris (white wax), pupal	67 sharp
Pieris (yellow part), pupal	No change up to 100° C.

Melting-points. Complicated mixtures of waxes do not show sharp melting-points (Piper, Chibnall, Hopkins, Pollard, Smith & Williams, 1931; Piper, Chibnall & Williams, 1934). The approximate melting-points shown in Table 3 were obtained by placing a small filament of wax in a capillary tube, and heating in a flask of medicinal paraffin. An autoclave flask with flat sides was used so that the wax could be observed without optical distortion in a medium-power binocular microscope. On raising the temperature it was found that a visible change took place some degrees below the true melting-point (see Table 8).

Wigglesworth (1945*a*) shows vast increases in the permeability of the cuticle of whole insects at temperatures well below these melting-points. It would appear that some physical changes take place in the wax at this 'critical temperature', and may be associated with the changes that can be seen in the isolated waxes.

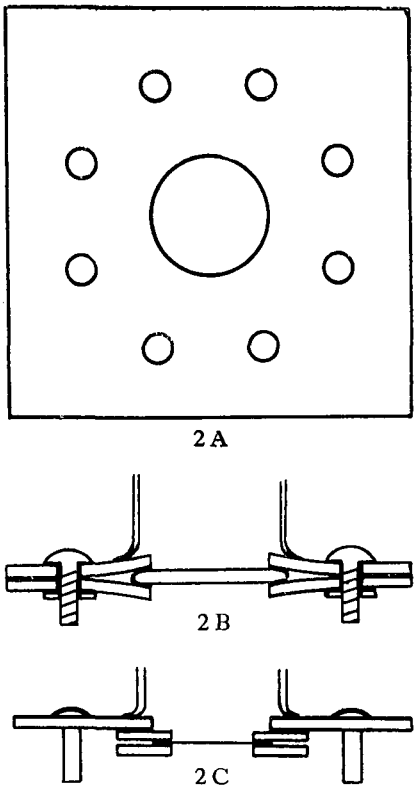

Fig. 2. A. Celluloid plate to support a tanned gelatin membrane in the holder. B. Longitudinal Section of a tanned gelatin membrane in the holder. C. Longitudinal Section of a *Pieris* wing membrane in the holder.

Waterproofing properties of the extracts

Apparatus. To test the waterproofing properties of the waxes, they must be spread on a membrane of known physical properties, and must separate a standard atmosphere from pure water. A modification of the holder shown in Fig. 1 was used. Pairs of identical plates of celluloid (Fig. 2*a*) were prepared, having eight small holes drilled in the perimeter. One of each pair was sealed to the rim of the glass tube and the other could be bolted on to it with eight 4 B.A. nuts and screws (Fig. 2*b*). In

plug of the capillary sealing tube at the top, ½ in. lengths of thermometer tubing were used so that expansion of the air could take place with temperature changes, while keeping static diffusion at a minimum.

Membranes. A standardized membrane was required which could be easily sealed into the apparatus for measuring permeabilities. Its permeability should be of the same order as that of insect cuticle after the wax has been extracted, and its surface should be suitable for laying down wax. It must resist attack by water, wax solvents (particularly chloroform) and detergents; and be unaffected by temperatures up to 80° C. The surface should approximate to the chemical type present in the insect epicuticle as this may have an orientational effect on the wax.

No membrane used satisfied all these requirements. Preparations based on paper, parchment, celluloid and porcelain were tried, but the most satisfactory artificial membrane which could be taken up to temperatures of about 60° C. was one made in the following way:

A piece of voile was placed on the bottom of a shallow tin tray and the edges secured by adhesive tape so that it was stretched flat. A hot standard solution of gelatin was poured into the tray, together with a small quantity of benzoquinone solution, so that the thickness was always the same. A number of strips of metal gauze were placed in the hot solution to strengthen the membrane and the tray was heated in a drying oven for about an hour. The soft mass was removed from the tray and allowed to dry. Circular disks were cut from it and held tightly between the two celluloid plates of the holder.

A more satisfactory natural membrane for studying the effect of temperature and of detergents on wax layers was the wing of the cabbage white butterfly, after clearing loose scales by gentle stroking with cotton-wool. All waterproofing layers were removed by two extractions with boiling chloroform for ½ hr. These membranes were sealed between two celluloid rings so that a ½ in. circle was exposed. The completed membrane was fixed over the hole in the membrane holder by cellulose paint (Fig. 2*c*).

The height of water above both types of membrane and in the control apparatus was kept at 2·5 cm., so that the volume of the air space subjected to temperature expansion was directly comparable. The exposed area of *Pieris* wing preparations was measured individually.

Deposition of wax films. A small quantity of the wax was dissolved in chloroform and placed in a pipette, the end of which had been drawn into a very fine jet so that it delivered standard size drops at a slow rate. Fifty drops were run on to a weighed watch-glass and the wax deposit weighed after evaporation. This was repeated and the thickness of wax resulting from the addition of one drop to the area of a membrane was calculated. The wax concentration was adjusted as necessary and the required number of drops added to the cup formed by the membrane and the lip of the celluloid disk. The solution was allowed to evaporate and the resultant layer examined under a binocular microscope for visible flaws. It was found that the rate of evaporation of these solutions was very variable, depending on the particular wax. Therefore, the rates of evaporation of chloroform solutions were tested under comparable conditions by timing the complete evaporation of a standard drop of solution on a glass cover-slip. All readings were taken in the open room during the course of 1 hr. The results are shown in Table 4.

Table 4. *Time for the evaporation of a standard drop of insect wax in chloroform solution*

Wax	Time of evaporation of the drop
Nematus, larval	40 sec.
Calliphora, puparial	45 sec.
Calliphora, pupal	45 sec.
Tenebrio, larval	4 min.
Pieris, larval	11 min.
Beeswax	13 min.
Rhodnius, nymphal	16 min.
Pieris, pupal (white wax)	31 min.

Chloroform solutions of cockroach grease appeared to evaporate quite quickly but the end-point could not be judged.

From Table 4 we see that the higher melting-point waxes have a greater affinity for chloroform molecules, indicating that they are more lipophilic. This is confirmed in Fig. 5. The gradation in properties is remarkably in keeping with the order of the other physical characteristics. It is well known that a slow rate of evaporation of a solvent favours crystal formation in the solute. In the case of waxes from which chloroform evaporated quickly, a small glass capsule was inverted over the membrane cup so that the high concentration of solute vapour slowed the process. The waterproofing powers of such wax films were increased, presumably because the wax layer was more highly organized (see p. 125).

The gelatin membrane had a permeability of approximately 15 mg./sq.cm./hr. when separating distilled water from a dry atmosphere at 20° C.; when coated with wax, the permeability never fell to the value of that of the intact insect from which the wax was obtained.

With the extracted *Pieris* wing, whose permeability was approximately 20 mg./sq.cm./hr., the waxed membrane frequently showed the same order of impermeability as the living insect. It was found with all the waxes, but particularly with the softer ones, that the membrane had become much less permeable after it had been taken past its 'critical

temperature' (see p. 122), and returned to room temperature. This is shown in Table 5.

Table 5. *Permeability of* 1 μ *wax films on* Pieris *wing membranes at* 25° C. *after subjection to the 'critical temperature'*

Values in mg./sq.cm./hr.

Wax	Permeability before C.T.	Permeability after C.T.
Nematus, larval	1·2	0·42
Calliphora, puparial	1·25	0·064
Calliphora, pupal	0·86	0·39
Tenebrio, larval	0·71	0·32
Pieris, larval	0·56	0·15
Beeswax	0·25	0·07
Rhodnius, nymphal	0·29	0·079
Pieris, pupal (white wax)	1·1	0·33
Pieris, pupal (yellow part)	1·6	No change
Blatta, oil	0·42	0·3

This may be due to the flow of the softened wax over small areas of the membrane not previously covered, or to an increase in the organization of the wax molecules in relation to the membrane surface, when they are in a mobile state. The phenomenon was observed with both types of membrane, but particularly on the butterfly wing. It was not necessary to take the wax up to its melting-point to observe the decrease.

The waterproofing oil from Blatta. A small drop of the oil extracted from the abdominal surface of the cockroach was applied on the end of a needle to the centre of a *Pieris* wing membrane. Over a period of 100 hr. the membrane became progressively more waterproof, and the surface shiny as the oil spread out from the centre.

Table 6. *Permeability of a* Pieris *wing membrane at intervals after a small drop of cockroach oil had been placed in the centre*

Diameter of membrane 0·7 cm. Original permeability of membrane 15 mg./sq.cm./hr. Temperature 22° C.

Time of reading hr.	Permeability of membrane
0	15·0
2	10·2
4	4·4
6	3·8
30	2·5
80	1·0
100	0·35
124	0·35

It can be seen from Table 6 that the permeability falls rapidly during the first 6 hr., by which time we suppose that an orientated and perhaps compressed monolayer has been formed over the membrane surface. Slow compacting of the monolayer, or the even distribution of the remaining oil, may account for the time taken for the value to become constant (see Table 16).

The cockroach oil spreads rapidly on water, and it would appear that 6 hr. is rather a long time for a similar spread on a membrane. However, Wigglesworth (1945*a*) shows that after rubbing the tergum of *Blatta* with dust, which removes most of the oil, recovery takes place so slowly that after 48 hr. the insect has not regained its original impermeability.

For membrane experiments it was found that the most useful thickness of wax which could be relied upon to give repeatable waterproofing values and.

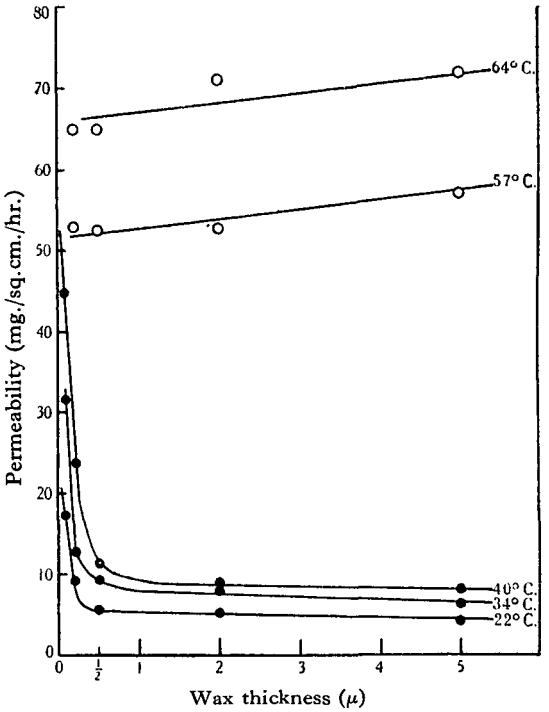

Fig. 3. Graph showing the variation of permeability of a tanned gelatin membrane coated with various thicknesses of beeswax at different temperatures.

stand up to high temperatures was 1 μ. This is vastly in excess of the average thickness on the insect (0·2–0·3 μ). Experiments on tanned gelatin confirmed the observation of Alexander, Kitchener & Briscoe (1944*a*) that the initial layer of wax has the greatest waterproofing effect, and that added thickness does not increase waterproofing regularly. Fig. 3 shows that at low temperatures, a thickness of between 0·2 and 0·3 μ is responsible for most of the impermeability of the wax film. Alexander *et al.* found a similar phenomena on celluloid membranes, occurring at a thickness of 20 mμ.

In artificial systems, the technique of depositing waxes may be such that only with a thickness of wax of 0·2 μ can one be sure of having a wax film

complete over the whole surface of the membrane. The readings are, however, reasonably reproducible and the phenomenon does not occur if *n*-paraffins are used. The orientated molecules at the membrane interface may be able to effect a similar alinement on the overlying molecules, so that, at any rate, the micelles (if not crystallites) of wax are orientated up to this thickness. Above this level, wax, although it may be partially crystalline, is present in random form, and so the effect of increasing its thickness will be small compared with the impermeability of the highly organized lower layers. The latter explanation seems to be the more probable.

Beyond the 'critical temperature', when we suppose that the orientation has broken down, the difference disappears. There is a great deal of other evidence for the presence of an orientated layer of wax at the interface. In the experiments of Alexander *et al.* (1944*a*) the smaller thickness may be due to the lesser degree of orientation induced by celluloid as compared with a tanned protein of the gelatin type; orientation induced by the insect epicuticle may be even greater. It may, therefore, be a point in the insect economy to use the average thickness of 0·25 μ of wax which seems to be the limit of induced orientation; any further increase in its wax layer would not make it vastly more impermeable. Wigglesworth (1945*a*) shows that in recovery from abrasion the waxes are laid down in a much rougher state, and that although the amount of wax present is greater than in the normal insect, the original degree of waterproofing is never recovered.

The effect of temperature on wax film permeability

If the lipoid material in the insect cuticle is present as a uniform layer it should show the same variations in permeability to water at increasing temperatures, whether on the insect or on an isolated system. The waxes were laid down on three types of membrane. Series were obtained with films of 1 μ of wax on each of unglazed porcelain, tanned gelatin and the extracted wing of the cabbage white butterfly.

Experimental procedure. The permeability of the unwaxed membrane was found by weighings after 4-hourly intervals in a desiccator. The wax was laid down from chloroform solution, and the permeability of the waxed membrane taken over periods of 24 hr. at room temperature. An initial permeability/temperature curve was then obtained using 10° intervals of temperature and 4-hourly exposures. From this curve it is possible to detect the region of the critical point although the curve did not show a very sharp break. The membrane was not taken up to the melting-point of the wax but was returned to a desiccator at room temperature and permeabilities taken until they were constant. This membrane was used for the final curve if its permeability was of a reasonably low order (Table 5). For this, 10° temperature intervals were used until the critical region was obtained, when procedure was by much smaller intervals. Readings were taken every 4 hr. until a constant value was obtained for that particular temperature. The fine capillaries at the top of the membrane holder were left open in experimental and control apparatus. Both pieces of apparatus were placed in a large desiccator having a thermometer with its bulb at the centre of the desiccating space. Phosphorus pentoxide was used as the drying agent and the whole kept inside a thermostatically controlled electric oven.

Table 7. *Temperature in* ° C. *at which the permeability of wax films,* 1·0 μ *thick on* Pieris *wing membranes reaches* 5 *mg./sq.cm./hr., compared with similar values for the source insect (Wigglesworth,* 1945*a*)

	In vitro		*In vivo*
Blatta orientalis, imago	40·0	(*Blattela*)	42·5
Calliphora eryth., puparium	41·5	(before pupation)	41·0
Calliphora eryth., pupa	54·0		57·0
Nematus ribesii, larva	39·3		43·5
Pieris brassicae, larva	46·2		46·5
Pieris brassicae, pupa	66·4		66·5
Tenebrio molitor, larva	57·1		58·5
Rhodnius prolixus, nymph	61·0		64·5
Beeswax	62·1		—

If Fig. 4 is compared with Wigglesworth's (1945*a*, figs. 1, 2 and 3), it can be seen that the changes in transpiration through the isolated lipoid films are almost identical with those given by the corresponding intact insect. The position of the critical temperature is in close agreement in every case; in Table 7 the temperatures at which the permeability reaches 5 mg./sq.cm./hr. are compared with Wigglesworth's figures for intact insects.

We can only conclude that the state of the wax in the cuticle must be very similar to that on the isolated membrane, i.e. as a continuous and discrete layer. Ramsay (1935) showed that the abrupt break at 33° C. in the rate of evaporation from the cockroach is due to a change of state in the oily film from solid to liquid. He did not, however, get any similar effects with various animal fats on membranes.

We have seen that in the experiments on the melting-points of the waxes, quite big visible changes were taking place below the melting-points. The

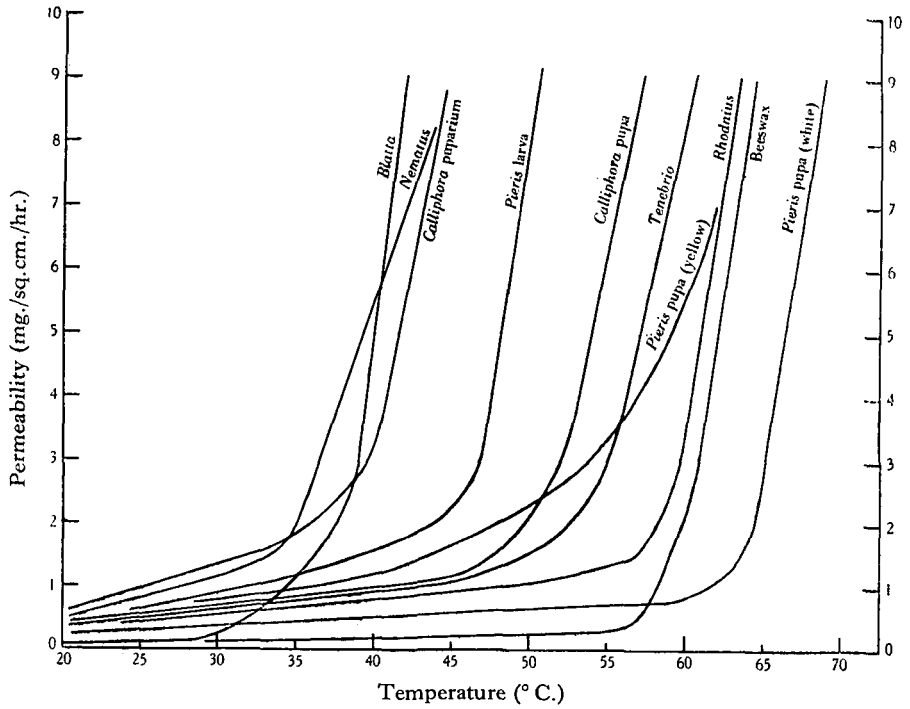

Fig. 4. Graph showing the variation of permeability with temperature of *Pieris* wing membranes covered with approximately 1 μ of wax from various insects.

increase in permeability at the critical temperature may be associated with these changes.

Fig. 4 and Table 5 also show that the waxes with higher melting-points are the more waterproof. Impermeability depends on two factors: the hydrophobic property of the individual wax molecule and the close packing of the whole layer or of a continuous plane of that layer. Fig. 5 shows that the waxes become more hydrophobic, as measured by their contact angle to water, as one goes along the series. Close packing *in vitro* depends on the rate of deposition, on the degree of crystalline structure shown by the wax, and on the changes taking place after heating to the critical temperature.

Physical changes at the critical temperature

A number of physical properties of the waxes were investigated in order to elucidate the changes taking place at the critical temperature in those characteristics relating to affinity for water.

Optical changes. Table 8 gives the changes in appearance of the wax during the melting-point observations. These changes would indicate a crystalline transition at the critical temperature. It can therefore be called a 'transition point'.

Spreading on the surface of water. A large conical funnel was supported in a water-bath so that the rim was about 1 cm. above the level of the water. Cold water could be run in through the bottom of the funnel in order to change its contents rapidly and

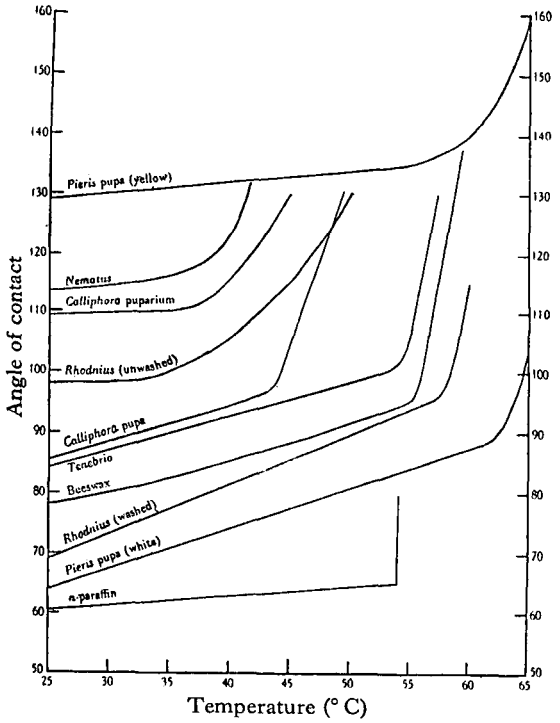

Fig. 5. Graph showing the variation in the contact angle of water against wax films from various insects at different temperatures.

to secure a clean surface. The funnel was cleaned thoroughly with soap and water, washed through for about ½ hr., and filled with water to within 1 cm. of the rim and a small quantity of lycopodium powder blown evenly over the surface. A number of fine glass rods were drawn and kept in running water to remove all surface active material. Before use each rod was tested for cleanliness by dipping into a dusted water surface. A wax filament was taken up on the end of the rod, and mounted on a vertical worm screw, so that it could be lowered into the surface of the water. This is important, as surface solution occurs only from the edge exposed to the air/water interface and not from immersed crystals (Rideal, 1926). The temperature of the bath was then raised slowly, and a thermometer in the water in the funnel gave a reading probably 1 or 2° above the true temperature of the surface. Similar experiments were carried out on waxes from washed and unwashed skins, on washed and unwashed cuticles, and on the water used for washing. The results are given in Table 9.

Table 8. *Temperatures at which crystals of wax change their appearance*

Wax	Temp. ° C.	Observation
Nematus, larval	Indef. changes from 30° C.	
Calliphora, puparial	Indef. changes from 24° C.	
Calliphora, pupal	36–46	Increasing transparency
Pieris, larval	46	Darkens
	54	Outline changes
Tenebrio, larval	51–53	Transparency
Beeswax	57–58	Transparency
Rhodnius	56–58	Darkens
Pieris (white wax), pupal	59	Transparency
Pieris (yellow part), pupal	54	Oily appearance

In the case of *Pieris* larva and *Calliphora* puparia there is spreading from the washed skin near the transition point. We must presume that in the others the affinity of the lipoid for the cuticle is greater than that for water even above the wax melting-point, or else there is a further layer isolating the wax. *Calliphora* puparia are certainly contaminated with a great deal of polar material (mostly protein and lipoid) from their larval environment, and this accounts for the high figure obtained for the thickness of wax extracted from the unwashed puparia (Table 2). There would appear to be highly polar substances present in the three highest melting-point waxes. They may be partially removed by prolonged immersion in water (see Table 2). Their effect on the spreading of wax solutions on water was tested in the following way:

Parallel experiments were carried out on two sets of equal numbers of the exuviae of *Tenebrio* larvae, *Rhodnius* fifth nymphs, and *Pieris* pupae; one set was thoroughly washed for 24 hr. in running water, the other left. They were extracted in benzene, so that the solutions would float on water. The cold extracts of these cast skins were poured on to the surface of clean water in large petri dishes, and allowed to evaporate. A fine layer of lycopodium powder was blown over the dish to show the extent of the rigid film present and its area was measured.

Table 9. *Spreading from various materials at an air/water interface, over temperature range* 20–70° C. *Room temperature*, 20° C.

Insect	Unwashed skin ° C.	Washed skin ° C.	Washing water at room temperature	Wax from unwashed skin ° C.	Wax from washed skin ° C.
Nematus, larva	None	None	None	At 24	At 29
Calliphora, puparium	Slow at 20	At 27	Rapid	At 20	At 26
Calliphora, pupa	None	None	None	At 42	At 43
Pieris, larva	Slow at 20	At 46	Slow	At 20	At 41
Tenebrio, larva	At 20	None	Rapid	At 48	At 55
Beeswax	—	—	—	At 60	—
Rhodnius, nymph	Rapid at 20	None	Rapid	At 51	At 55
Pieris, pupa (white extr.)	Very slow at 20	None	Rapid	—	At 60
Pieris, pupa (yellow extr.)	—	—	—	—	At 52
Blatta (oil)	Spreading at all temperatures				

The ratio of the areas of the extract from washed to unwashed skins was:

Tenebrio larvae	1/4
Rhodnius fifth nymphs	1/10

Washing does not seem to remove the 'spreader' in *Pieris* pupal wax, but the benzene extract of three pupae, total area about 10 sq.cm., when poured on to water, gave a rigid film of over 150 sq.cm.

It is apparent from Table 9 that the waxes in general become much more labile at a temperature a few degrees below the melting-point; this temperature corresponds with the crystalline transition temperature.

Water contact angles of wax films

The measurement of the contact angle between wax surfaces and water is one of the most useful physical properties, as it is a function of the molecular attractive force between the wax surface and water at any temperature.

Contact angles were measured by two methods: that of a water droplet on a waxed cover-slip (Beament, 1945*a*), and the method of tilting a waxed plate until the water meniscus on one side is horizontal. The wax films used were approximately 1 μ thick, laid down on clean cover-slips, and left for at least 48 hr. before use. In the case of the droplet method, readings were taken on either side of the

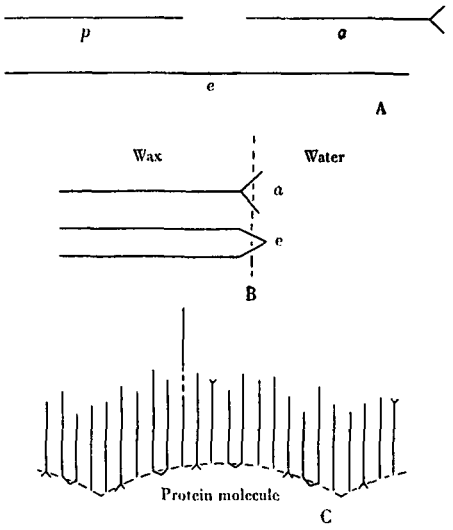

Fig. 6. A. Representation of the main components of insect waxes; p=paraffin, a=long-chain acid, e=ester. B. Diagrammatic representation of the probable orientation of wax acids and esters at a water interface above the transition temperature. C. Diagrammatic representation of the type of orientated monolayer which may be linked to a protein molecule in the insect epicuticle.

drop, and in both cases the angle was measured at every 5° of temperature over the first part of the curve, and at every degree in the region of the transition point. The change in the contact angle is shown graphically in Fig. 5.

The figure also shows the variation with temperature of the water contact angle of a film of a pure *n*-paraffin having a melting-point at 54° C. There is only a very small and regular increase in the angle up to the temperature at which the wax melts, when the water runs up between the wax and the supporting glass. In all the other waxes there is an increase in the hydrophilic properties at the transition temperature and well below the melting-point of the wax. The almost perfect gradation in properties is also seen, for the lowest melting-point waxes have the lowest contact angle, and the highest waxes are the most hydrophobic. *Rhodnius* wax extracted from unwashed skins is much more easily wetted; the transition change is much less abrupt and takes place at a lower temperature. The yellow fraction of the *Pieris* pupal extract is much more hydrophilic than the range of waxes. We thus have further evidence of the presence of a lipoid substance in the higher melting-point waxes which is much less hydrophobic.

Chibnall (1944) states that the insect waxes, having melting points in the range 40–70° C., consist of acids, esters and paraffins. The linking group at the centre of the ester is the most hydrophilic part of the molecule, while the active end of the long-chain acid is much more hydrophilic than the rest of the paraffin chain. At the transition temperature the molecules may become sufficiently mobile to orientate themselves at a water interface with their more hydrophilic groupings outwards, and such changes will increase the adhesive force of water and increase the contact angle (see Fig. 6).

Table 10. *Contact angle of* Rhodnius *wax films against water at room temperature, after various temperature treatments*

Treatment of film	Contact angle at room temperature
Laid down from chloroform	72°
Heated to 51° C.	76°
Heated to 58° C.	102°
After 24 hr.	99°

In order to gain further evidence of this, a film of *Rhodnius* wax extracted from washed skins was used. The contact angle at room temperature was taken, the film immersed in water at 51° C. for 15 min. (below the transition temperature) and the contact angle measured at room temperature. The procedure was repeated, using water at 58° C. (between transition and melting-points). The film was placed in a desiccator and the reading repeated 24 hr. later. The results are shown in Table 10. It can be seen that some permanent change has taken place in the surface wax molecules when exposed to water at the transition temperature, and that on returning to room temperature they retain a more hydrophilic orientation. This cannot, of course, happen to *n*-paraffin chains.

The various temperatures at which physical changes characteristic of the transition point take place have been brought together in Table 11.

Nature of the transition point and structure of the wax layer

Müller (1932) states that with higher *n*-paraffins, having a chain length above C_{24}, the wax molecules show crystalline changes at temperatures a few

de[redacted]s below their melting-points, and tend to be packed in a regular hexagonal system. For example, with C_{24} chains, the side spacings are 4·12 and 3·71 A. below the transition point which occurs at 43·9° C., and both values approximate to 4·10 A. at that temperature. The melting-point of the wax is 49·3° C. when the spacings suddenly increase to 4·6 A. These changes at the transition point would cause an increase in the intermolecular spaces. He suggests also that at such temperatures the amplitude of oscillation of the molecule about its long axis may amount to complete rotation. The van der Waal force between the chains would then, presumably, be partially overcome, and the wax molecules become very mobile, while yet remaining in the solid state.

Optical changes (Table 8), permeability changes (Table 5 and Fig. 4), and the variation in contact angle (Fig. 5 and Table 10), all point to similar large movements in the insect waxes at their transition points. The mobility associated with crystal changes would allow orientations of wax molecules in the presence of a suitable surface. If the intermolecular pore spaces increase, the rate of passage of water through them will also rise.

On a grosser scale, changes in the crystalline form of a homogeneous layer of wax will leave small spaces between the newly formed crystallites on first exposure to the transition temperature. We have seen that in the presence of water (Table 10) wax molecules, in a mobile state, will become permanently orientated with their hydrophilic parts exposed, and so these larger channels will have more hydrophilic linings.

The orientation of lipoid molecules at a protein surface has been postulated by Danielli (1938) and Danielli & Dayson (1943) in the structure of the membrane surrounding the erythrocyte. During the present work it has been found that the degree of waterproofing conferred by similar thicknesses of wax was greatest when laid down on a membrane most chemically akin to the insect epicuticle, namely, the *Pieris* wing membrane. Arranging the other membranes in a rough order of permeability when supporting comparable wax films: tanned gelatin, parchment, celluloid, porcelain, we see that as we descend the series, membranes are successively less able to present a surface chemically akin to the tanned protein of the epicuticle.

A preliminary X-ray investigation of waxes from these insects has suggested that when they are supported on a fine-silk meshwork, the general bulk of the wax has an orientation, probably in the form of groups of aligned micelles; but these are of random distribution. On the other hand, the wax molecules around the silk fibres appear to be in highly orientated crystallites, and this may be due to the organization of their initial layer by the silk protein.

Table 11. *Comparison of the transition point temperatures in* ° C. *obtained from various physical characteristics*

Insect	Optical changes	Spreading	Contact angle	Permeability of film		
				On tanned gelatin membranes	On *Pieris* wing membranes	*In vivo**
Nematus, larva	30	29	37	32	34	35
Calliphora, puparium	—	26	36	29	37	35
Calliphora, pupal	36–46	43	44	42	49	52
Pieris, larval	46–54	41	42	46	46	42
Tenebrio, larva	51–53	55	53	—	53	52
Beeswax	57–58	60	56	56	57	—
Rhodnius	56–58	55	58	57	57	57·5
Pieris, pupa (white)	59	60	62	—	63	60
Pieris, pupa (yellow)	54	52	—	—	—	—

* *In vivo* experiments (Wigglesworth, 1945*a*).

When the waxes are laid down from chloroform on to the membrane, the film will be crystalline, probably in the form indicated by X-ray analysis for the bulk of the wax. The persistence of chloroform molecules around the higher melting-point members (Table 4) will preclude interfacial orientation, since the hydrophobic part of the molecule is active in both processes. But in all the waxes investigated (Table 5) the lipoid layer is much more waterproof after subjection to the transition temperature, when the molecules are sufficiently mobile to orientate themselves to the tanned protein surface.

The initial layer of an insect wax is the most waterproof (Alexander *et al.* 1944*a*, and see Fig. 3). It is known that orientated monolayers on a water surface can suppress the rate of evaporation considerably (Sebba & Briscoe, 1940), and we can conclude that the extreme impermeability of the lipoid layer of insects is due essentially to an orientated and compact monolayer bound to the tanned protein of the epicuticle (Fig. 6*c*). The

additional effect of the remainder of the wax (0·25 μ) will depend on its close packing, but as most insect waxes contain molecules with chain lengths varying between C_{24} and C_{70}, the greatest organization we can expect is a general alinement, perhaps dependent on the degree of orientation in the initial layer.

The effect of solvent vapour

Insects subjected to chloroform vapour become less waterproof (Wigglesworth, 1945*a*). Competition for the wax, between solvent molecules and the substrate, disorganizes the orientated layer, and the resultant increase in permeability can be demonstrated in two ways.

An unextracted *Pieris* wing had a permeability of 0·23 mg./sq.cm./hr. After exposure for 1 hr. to concentrated chloroform vapour at room temperature this figure had risen permanently to 0·68 mg./sq.cm./hr. The *Pieris* wing is waxed on both sides, so that the inner layer will be affected only by chloroform which has migrated through the wing substance. In view of this, the increase is large and can only be accounted for by the disturbance of the orientation of the sytem by solvent molecules.

Table 6 suggests the formation of a compact orientated layer of cockroach oil on a butterfly membrane after 6 hr., while Table 16 shows that an inert dust cannot remove this monolayer. The values are compared in Table 12 with results of experiments by Wigglesworth (1945*a*) on the dead but intact cockroach.

Table 12. *Comparison of the effect of dust and chloroform vapour during* in vitro *and* in vivo *experiments on cockroach oil*

	mg./sq.cm./hr.
Steady value of membrane permeability with *Blatta* oil after 120 hr.	0·35
Value after 6 hr. spread from a drop in centre of membrane	2·9
Value after dusting (84 hr.)	2·9
	mg./sq.cm./hr.
From Wigglesworth, intact cockroach, approx. perm.	0·37
After dusting	2·5
After exposure to $CHCl_3$	3·1

The effect of chloroform vapour on the whole layer of oil has made the permeability greater than that of the monolayer left after dust adsorption. The only explanation is that the solvent molecules have disrupted the monolayer.

It is important, therefore, that wax solvents should never be used to kill insects if they are to be used for experiments on the waterproofing properties of the cuticle.

Mechanism of orientation. A protein molecule in the epicuticle may accommodate various members of the diverse mixture of acids, esters and paraffins in the wax, at different points on its surface. A diagrammatic representation of the orientated layer is given in Fig. 6*c*. Owing to the dimensions of the intermolecular spaces of the epicuticle, the wax molecules must traverse them in small micelles (unless they are made up from shorter components on the surface), and the first step in orientation will be complete. But most of the orientation will be carried out under the influence of the substrate and, since the pure waxes are solid, some form of solubilizer or emulsifier must be present. The highly polar substances present in the exuviae of insects with higher melting-point waxes may be residues of these emulsifiers (Table 4, Fig. 5, pp. 123, 124 and Table 2). A preliminary investigation has shown them to be soluble in chloroform and other good lipoid solvents; their melting-points are above 100° C. and they have high spreading powers on water. Those from *Tenebrio* larvae and from *Rhodnius* are precipitated as a minute white flocculation when acetone is added to a chloroform solution of the extracts. These properties would suggest that the substances are phospholipids or lipoproteins; they do not respond to chemical tests for cholesterol.

If cast skins of *Rhodnius* are washed with cold distilled water, and the aqueous extracts shaken with a few drops of chloroform, the substances present separate out along the interface between the two solvents and, if left overnight, form a very thin rigid membrane. This aqueous extract will, of course, contain some excretary products. The presence of these substances in *Rhodnius* wax increases the contact angle to water and generally makes the extract more hydrophilic.

The yellow fraction of *Pieris* pupal wax differs in that it is not precipitated from lipoid solvents by acetone; it is present in much larger quantities in the cuticle. Its contact angle, while smaller than that of the whole range of waxes, increases in a similar way with temperature and follows the same type of curve as its permeability changes. It spreads on water at 52° C. and shows a slight change of appearance at 54° C. This substance may be a lipochrome. On the other hand, the high melting-points and more hydrophilic properties may indicate that these small residues are akin to the keto-containing waxes shown by Chibnall *et al.* (1934*a*) to form the main bulk of the extract from *Coccus cacti*, and by Blount *et al.* (1937) from the white pine *Chermes*. In both these insects the wax is present as a filamentous coat which would be useless for waterproofing, and may be an excretary product (Chibnall, 1944) or perform a protective function. There may well be a layer of the beeswax type of wax below the keto waxes, and responsible for the waterproofing. Lipoid extraction takes out all the fat-soluble material in the cuticle, and small quantities of the simpler waxes were present in the extracts obtained from these two insects.

We can conclude that the main barrier to the

p[redacted]e of water and water-soluble substances through the insect cuticle is a highly organized wax layer some 0·25 μ thick, overlying the tanned protein of the epicuticle, as a continuous layer. This wax layer will be readily penetrated by oil and oil-soluble material, which will upset the orientation of the lowest monolayer, thus bringing about the irreversible changes in permeability reported by Hurst (1943).

PART II. ACTION OF INERT DUSTS

It is well known that certain inert dusts cause desiccation of an insect when in contact with the cuticle (Zacher & Kunicke, 1931; Chiu, 1939; Alexander *et al.* 1944*b*; Wigglesworth, 1944). From experiments on waxed celluloid membranes, Alexander *et al.* (1944*a*) suggest that 'the epicuticular fat film may be preferentially attracted by the crystalline forces at the surface of solid particles, and adhere and orientate itself on the crystal rather than on the relatively structureless cuticle surface. These may even be spread over the particles by surface migration.' We have seen above, however, that the cuticle surface is part of a highly organized physicochemical system binding down the wax molecules, and that insect waxes are mobile only at temperatures above the transition point. Wigglesworth, on the other hand, attributes the effect of these dusts to abrasion. While in the limiting case these two actions depend on the same physical processes, the experiments below were carried out to obtain additional evidence.

Method

Insect waxes were laid down on various membranes, and the steady value of the permeability was recorded. Dust (from the sample of alumina used by Wigglesworth, 1945*a*) was sprinkled thickly on to the membrane surface with the holder inverted, and left in this position for 15 min. Loose dust was gently shaken off and the apparatus returned to the desiccator. In a series of experiments such as the effect of dust over a temperature range, the membrane was redusted before each new temperature.

The presence of dust on wax films on the extracted wing of the butterfly has no effect at any temperature on the rate of transpiration through either the hard wax from *Rhodnius*, or through beeswax, over the range of thickness 0·25–1·0 μ.

Experiments on the soft wax of the *Calliphora* puparia suggested a very slight increase in permeability at room temperature, but the effect of dust at any temperature was by no means as obvious as that on the live and moving insect. On the other hand, the very slight abrasion brought about by spreading dust on the waxed membrane with a camel-hair brush is sufficient to increase transpiration by 100%. Much more dust adhered to the tanned gelatin membranes, though microscopic observation showed that there was always a fine film on *Pieris* wing preparations. We can see that dust has a considerable effect on a wax film spread on tanned gelatin, and the increases here are comparable with those obtained by Alexander *et al.* (1944*a*) using beeswax films on celluloid. It may well be that on celluloid and tanned gelatin, the wax film before the application of dust is by no means complete and continuous, seeing that the permeability is so much greater than that of the insect, or waxed *Pieris* wing. This would account for the extra amount of dust adhering to the membrane. Attraction between the dust surface and the irregular areas of the film, such as at the edges of small holes, may be sufficient to account for the increase.

It is much more likely, however, that these differences are due to the bonding force between the substrate and the wax molecules. In celluloid and tanned gelatin systems we have seen that the lipoid layer is neither so highly organized nor so strongly attached, and the surface attraction of the dust crystal forces described by Briscoe (1943) may be stronger than the interfacial bonding. It is only when abrasive action augments surface adsorption that

Table 13. *Permeability in mg./sq.cm./hr. of dusted and undusted layers of* Rhodnius *wax* (1·0 μ) *on* Pieris *wing membrane at various temperatures*

° C.	Undusted	Dusted	Redusted
20	0·31	0·34	—
30	0·68	0·8	0·60
50	1·6	2·1	1·8
58	15	12·8	10

Table 14

(*a*) Permeability in mg./sq.cm./hr. of tanned gelatin membrane (original permeability 20 mg./sq.cm./hr.) with 0·25 μ beeswax, after treatment with dust at 25° C., values in mg./sq.cm./hr.

Permeability before dusting	3·5
Add dust: After 2 hr.	6·3
After 4 hr.	7·0
After 24 hr.	10
After 48 hr.	11

(*b*) 0·25 μ beeswax on *Pieris* wing membrane (original permeability 24 mg./sq.cm./hr., 22° C.).

Permeability before dusting	1·1
Permeability after dusting (over 48 hr.)	1·2

Table 15. *Effect of dust sprinkled, or rubbed, on* 0·5 μ *of beeswax on* Pieris *wing membranes at* 20° C., *values in mg./sq.cm./hr.*

A. Before dusting	1·45	B. Before dusting	1·40
After sprinkling	1·50	Rub on dust with camel-hair brush	2·8
		Over next 6 days	3·05

It is much more likely, however, that these differences are due to the bonding force between the substrate and the wax molecules. In celluloid and tanned gelatin systems we have seen that the lipoid layer is neither so highly organized nor so strongly attached, and the surface attraction of the dust crystal forces described by Briscoe (1943) may be stronger than the interfacial bonding. It is only when abrasive action augments surface adsorption that

dusts increase transpiration through lipoid films linked to epicuticular substance.

The effect of dust on the oil extracted from the cockroach is in direct contrast (Table 16).

Table 16. *Continuation of experiment on membrane used in Table 6*

Original permeability of *Pieris* wing membrane		15·6 at 25° C.
With thin film of oil from *Blatta* abdomen		0·35
Dust added and returned at once to desiccator:	After 1 hr.	1·2
	After 2 hr.	1·75
	After 17 hr.	2·7
	After 84 hr.	2·9

Recalling Table 6, we find that the dust is able to compete for most of the oil over the membrane surface, and causes a large increase in the rate of passage of water, but that it cannot increase this beyond the value obtained when the oil has spread from a central drop for 6 hr. This value was taken to indicate the formation of a completely orientated and possibly fully compressed monolayer over the membrane surface. Hence, while the dust can remove all the superficial oil, it cannot compete for the layer of extract bound on to the epicuticular surface, even though all evidence points to the extreme mobility of this substance. It is therefore certain that stationary dust cannot remove the solid and non-mobile lipoid of the average insect, bound to the epicuticle (and perhaps protected by a cement layer).

Alexander *et al.* (1944*b*) have correlated the effectiveness of a dust with its relative hardness, and shown that dusts are still effective when coated with a few monolayers of fatty acids, though not when coated with thick films. They have also shown that angles rather than surfaces are the most important consideration next to hardness. This evidence is far more in favour of abrasion than surface action, which is very dependent on the actual molecular surface exposed.

PART III. ACTION OF EMULSIFIERS

The waxed membrane technique was used to make a preliminary investigation of the mode of action of a select series from the emulsifiers and detergents, which cause an increase in the permeability of the insect cuticle by breaking up the wax layer (Wigglesworth, 1945*a*).

An emulsifier depends for its action on having one part of the molecule highly hydrofuge and lipophil, which will attach itself to the wax molecule; the other part, hydrophil, so that when these endings are directed away from the wax molecule they provide hydrophilic channels for the migration of water. It is important, therefore, that the emulsifier itself should not unduly suppress the rate of permeation of water through a membrane. On the other hand, more strongly hydrophilic substances will not prevent the escape of water, but may have insufficient affinity for lipoid material to disperse the wax layer.

Detergents and emulsifiers used		Referred to as
Refined medium petroleum	P 31 (white oil) Shell Co.	P 31
Complex alkylated benzene ether of polyethylene glycol $R(OC_2H_4)_8OH$ (app.)	R 2206 (I.C.I.)	R 2206
Diglycol oleate	Glyco Products Co. Inc.	Diglycol oleate
Glyceryl monolaurate	Glyceryl laurate S (Glyco Co. Inc.)	Glyceryl laurate
Propylene glycol laurate	Prolaurin (Glyco Co. Inc.)	Prolaurin
Cetyl ether of polyethylene glycol $C_{16}H_{33}(OC_2H_4)_4OH$ (app.)	R 2211 (I.C.I.)	R 2211
Cetyl ether of polyethylene glycol $C_{16}H_{33}(OC_2H_4)_8OH$ (app.)	C 09993 (I.C.I.)	C 09993

Small quantities of the emulsifiers were spread on membranes with a fine camel-hair brush, and the film examined for 'completeness'. Since it was found that cellulose paint was sometimes emulsified by the detergent, celluloid solution was used to cement the butterfly wing membrane. The permeability of the detergent film was obtained from 24-hourly readings using calcium chloride desiccators at 20° C.; the results are shown in Table 17.

Table 17. *Suppression of permeability of a membrane by an emulsifier film (T.G. = tanned gelatin, P.W. = Pieris wing), expressed as percentage of steady value to original permeability (at 20° C.)*

		%		
P 31	P.W.	94		
Glyceryl laurate	P.W.	91		
Diglycol oleate	P.W.	16·5		
Prolaurin	T.G.	13		%
R 2211	T.G.	56	P.W.	52
C 09993	T.G.	25	P.W.	16

Comparing this with Wigglesworth (1945*a*, Table 13), it can be seen that R 2211, which is of

th[redacted]ighest efficiency on the insect, is itself comp[redacted]vely waterproof, while substances as far separated in action as R 2206 and C 09993 have the same order of high permeability. When this suppression effect has been allowed for we have a true measure for the action of the emulsifier on the wax layer.

The effect of the thickness of the emulsifier film was also roughly investigated. In the case of P 31 and the less polar complexes, an increase in thickness caused a proportionate drop in permeability, but thicker layers of C 09993 and R 2211 do not alter the permeability appreciably. There is, for example, only 7% difference between the value recorded for films of R 2211, 0·08 mm. thick and 1·0 mm. Thus to desiccate insects, the thinnest films on non-polar material should be applied; with highly polar substances the amount necessary for complete wax emulsification can be used, regardless of thickness.

Effect of emulsifiers on beeswax films

Membranes of known permeability were coated with standard thicknesses of wax, and the steady values of their permeabilities was taken at room temperature. Films of emulsifiers were applied and the loss of water in successive periods recorded. In the histogram (Fig. 7*a*), the effect of four detergents on beeswax films (0·25 μ) on tanned gelatin is compared with the evaporation from *Rhodnius* when covered with the same substance (Wigglesworth, 1945*a*). Fig. 7*b* shows the effect of a further series on a layer of 3 μ of beeswax on *Pieris* wing membranes. The effectiveness (D) is roughly of the same order as that on the insect (F), though the values are not as widely scattered. The amount by which the pure detergent depresses permeability is shown in column E.

It can be seen that R 2211 would be responsible for the greatest loss of water, were it not for its own impermeability; it is therefore too lipophilic. C 09993 and Prolaurin may emulsify waxes to the same extent, the greater impermeability of Prolaurin accounting for its smaller recorded effect. R 2206 and Diglycol oleate are very inefficient; they are readily permeated by water and are therefore too hydrophilic to have had a vigorous detergent action on the wax. P 31 and Glyceryl laurate are at the other end of the scale for, while they may have completely disorganized the wax layer, they are too lipophilic to allow water to escape.

Though R 2211 is more impermeable, it has a greater effect on the thick wax film than has C 09993. Since it is more lipophilic it can presumably accommodate a greater wax concentration. The effect of wax concentration on C 09993 was then investigated. A thin film of the emulsifier was added to a wing membrane, and the permeability recorded. A chloroform solution of beeswax corresponding to 0·3 μ was added and readings taken to a constant value. This was repeated, so that the amount of wax present was 0·6, 0·9, 2·0, and 6·0 μ by additions to the same membrane. The results are shown in Table 18.

Small concentrations of wax have very little effect on the permeability of the film of emulsifier, but there is a change in the system, with a wax thickness

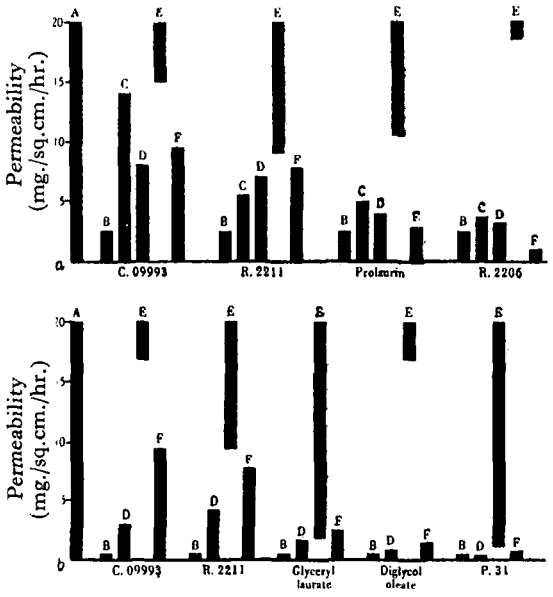

Fig. 7. Histogram showing the permeability of membranes: (*a*) 0·25 μ beeswax on tanned gelatin; (*b*) 3 μ beeswax on *Pieris* wing membranes. A. Permeability of unwaxed membranes. B. Permeability of waxed membranes. C. Permeability 4 hr. after the addition of detergent. D. Steady value of permeability 48 hr. after addition of detergent. E. Depression of the permeability of unwaxed membranes by thin films of the detergent. F. Effect of detergent on *Rhodnius* (Wigglesworth, 1945*a*). reduced to a comparative scale.

Table 18. *Effect of various thicknesses of wax on the permeability of a* Pieris *wing membrane, at* 25° C. *coated with a thin film of* C 09993

	mg./sq.cm./hr.
Permeability of membrane	17·1
,, with thin film of C 09993	15·8
,, +0·3 μ of beeswax	14·2
,, +0·6 μ of beeswax	14·0
,, +1·2 μ of beeswax	11·6
,, +2·0 μ of beeswax	3·7
,, +6·0 μ of beeswax	1·9

of between 1 and 2 μ. As the wax has been added in chloroform solution, and the solvent allowed to evaporate off, it cannot be said that these effects are due to any difficulty in removing the wax from the substrate. At the end of the experiment there was an aqueous layer over the membrane surface. This was carefully removed and spread on another

membrane; it had a permeability of approximately 5 mg./sq.cm./hr. The original membrane surface was washed with cold water and then had a permeability of 3·5 mg./sq.cm./hr., so that even in the presence of the emulsifier, some of the wax had been deposited on the membrane, and probably in a partially organized state, as it was rigidly attached. Similarly when C 09993 was added to films of 6 μ of beeswax, the permeability was not markedly increased, but after 48 hr. the upper layer of emulsifier and wax had a permeability of 4·5 mg./sq.cm./hr., while the wax layer left had increased its permeability by some 50%. It was also observed that mixtures of beeswax and C 09993 having a high wax concentration, spread on cover-slips, would deposit plates of wax at the end of 48 hr.; the 'aqueous' solution left above the plates had a permeability of approximately 5 mg./sq.cm./hr.

We can conclude that below certain concentrations, mixtures of beeswax and C 09993 are quite permeable and that the presence of wax does not vastly decrease the permeability of the detergent. Presumably all the wax molecules are surrounded by emulsifying molecules with their lipophilic parts inwards, and hydrophilic groupings outwards. The wax/emulsifier mixture is homogeneous until the amount of wax present is such that a thin layer of the mixture has a permeability of 5 mg./sq.cm./hr. This represents a state when all the lipophilic groupings are used for emulsification, and additional wax is slowly precipitated; this wax may be arranged in a partly organized way. Similar substances, if produced by the insect, could arrange the hard insect waxes in an organized form on the cuticle (see p. 126).

Wigglesworth (1945*a*) shows that the effect of a select series of detergents on the rate of permeation of water through *Rhodnius* cuticle is far more scattered than the effect on other insects. For example, C 09993 and R 2211 are of about the same effectiveness on the *Pieris* pupa, *Tenebrio* larva and *Nematus* larva. The wax of *Rhodnius* is, however, covered by a thin cement (Wigglesworth, 1945*b*), and the detergent has to penetrate this before it can break up the structure of the wax layer. We have seen (p. 126) that chloroform molecules are able to penetrate the substance of an unextracted *Pieris* wing, and to disrupt the wax layer on the opposite side to that exposed to the solvent vapour. Wigglesworth states that the water loss of a *Rhodnius*, weight 140 mg., is approximately 50% when covered over the upper abdomen with C 09993, and left in a desiccating atmosphere for 24 hr. This is equivalent to a rate of loss of 5·8 mg./sq.cm./hr., assuming that an area of approximately 0·5 sq.cm. has been covered by the detergent.

But pure C 09993 is capable of transmitting approximately 21 mg./sq.cm./hr., and is not subject to a big depression in the presence of so small a quantity of wax as that on the *Rhodnius* cuticle. The value 5·8 can therefore only be accounted for by the inability of the detergent to get at all of the wax and indeed would fit the permeability to be expected if only half of the wax layer was removed.

This is borne out in Wigglesworth's plate 2, Fig. 9, showing the amount of polyphenol staining after the action of C 09993 which has only partially removed the wax.

Similarly, the actual rate of loss from *Pieris* pupae smeared with various detergents is well below the rate to be expected if the wax were fully emulsified (Table 19).

Table 19. Pieris *pupae smeared with various detergents. Rate of loss of water calculated from Wigglesworth* (1945*a*)

Detergent	Approximate rate of loss mg./sq.cm./hr.	Available permeability of detergent mg./sq.cm./hr.
P 31	0·067	1·50
Glycol laurate	0·13	2·2
Diglycol laurate	0·13	21
R 2211	0·185	12
C 09993	0·185	21

It would appear that the effectiveness of the detergent in penetrating the cement layer is as important as its efficiency as a wax emulsifier. Some indication of this is given by experiments in which the detergents were spread on unextracted wing membranes of *Pieris*. Only in the case of C 09993 was there any increase in the rate of passage of water through the membrane. This was of the order of 10%; in all the others, the permeability fell below the starting-point value. Thus the effect of C 09993 is due to its ability to penetrate the wing substance, and disarrange the inner layer of wax as well. This is further support for the idea that the special effectiveness of C 09993 in increasing transpiration is due to its ability to penetrate or disperse the cement layer.

The relative effectiveness of a detergent in increasing transpiration through the insect epicuticle is dependent on:

(1) The amount by which the emulsifier actually disturbs the wax.

(2) The permeability of a layer of the detergent.

(3) The effect of wax molecules on this permeability; in a good emulsifier they will have no effect.

(4) The ability of the detergent to dissolve or penetrate the cement layer, when it is present.

Maximum efficiency may be obtained with a suitable balance of hydrophilic and lipophilic characteristics.

SUMMARY

The passage of water through isolated insect cuticles and exuviae, before and after extraction with lipoid solvents, has been measured. The impermeability of the cuticle is due to a thin continuous layer of lipoid over the epicuticular surface. Transmission of water through unextracted cuticle is more rapid in the direction epicuticle to endocuticle, than when reversed. This asymmetry is most marked in the exuviae.

The lipoid layer is approximately 0·25 μ thick on most of the insects investigated and is independent of cuticular thickness. The thickness of wax on a given species is very constant.

The pure extracted lipoids are solid waxes, except in the blattids, which have a mobile grease. The waxes show a gradation of physical properties corresponding to their melting-points; the hardest are most hydrofuge and are found on the most impermeable insects.

The impermeability of insect wax films, deposited on a membrane, depends on the degree of chemical orientation induced in the lowest layer of molecules by the membrane surface. The monolayer at the epicuticular interface of the insect is completely orientated and tightly packed, and is the main barrier to the passage of water.

Waxed membranes show a sudden increase in permeability at a critical temperature corresponding to that of the insect from which the wax was obtained. The waxes undergo a crystalline transition at this temperature; their molecules become mobile, and the orientated layer is disorganized. In the presence of water, wax surfaces become more hydrophilic at the transition point, and the molecules are permanently orientated in this state.

Boiling solvents must be used to extract waxes from the exuviae; the extracts are all soluble in cold chloroform. The vapour of wax solvents will destroy the orientation of the wax on the cuticle.

Substances having both hydrophilic and hydrophobic properties are present in high melting-point waxes; they may be residues of natural emulsifiers.

Inert dusts increase the transpiration of waxed membranes by adsorbing the lipoid, only if the membrane exerts little orientational force. They cannot overcome the orientational bonds of a wax layer deposited on a membrane of insect epicuticle, unless adsorption is augmented by abrasion. Dusts will adsorb all but the lowest orientated layer of the grease on the coackroach.

Some analysis is given of the mode of action of emulsifiers and detergents, which increase the permeability of waxed membranes, and of the insect cuticle. Their efficiency depends on their own permeability to water, and possibly on their ability to penetrate the cement layer on the insect, as well as on their capacity for emulsifying wax.

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