CHEMICAL COMPOSITION OF THE SHELL OF EXTERNAL AND INTERNAL PARASITIC EGGS (HYMENOPTERA) AND THEIR HOST EGGS AND PENETRATION OF CHEMICALS THROUGH IT

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ABSTRACT

The work has been carried out on the chemical composition of shell and embryonic membranes of eggs of *Microbracon gelechiae* Ashm., *Corcyra cephalonica* Staint., *Chilo zonellus* S., *Pyrilla perpusilla* Walk., *Trichogramma evanescens minutum* Riley and *Tetrastichus pyrillae* Craw., and penetration of chemicals through them. The eggs of the latter two insects are endoparasitic in eggs of *C. cephalonica* as well as *C. zonellus* and *P. perpusilla* respectively. The eggs of *M. gelechiae* are ecto-parasitic on larvae of *Gnorimoschema operculella* Zell. and *C. cephalonica*.

The endoparasitic eggs of *T. evanescens minutum* and *T. pyrillae* have thin non-resistant proteinaceous chorion and epembryonic membrane. They are devoid of the internal lipoid layer. They are not resistant to the penetration of hydrophilic or lipophilic substances. The poison that gets into the host-eggs is enough to kill them. In eggs of rest of the insects there is a remarkable parallelism in their fundamental structure. The shell bears respiratory pores, running through its substance. It consists of external proteinaceous cement containing loose admixture of unsaturated oily substance, proteinaceous chorion made of non-resistant exochorion and resistant endochorion, and a lipoid layer of unsaturated oily material internal to the chorion in freshly laid eggs—and of a mixture of unsaturated and saturated fatty substances in older eggs. The chorion and the lipoid layer are fully completed in the ovary. The latter is formed by ovum and the former by follicular cells. In some of the eggs of *M. gelechiae* the lipoid layer is completed after oviposition. The micropyle is closed by lipoid from this layer immediately after fertilisation and before oviposition. The cement is deposited after fertilisation when the eggs are ready for oviposition. The proteinaceous vitelline and fertilisation membranes are devoid of any oil, but the serosa and later the epembryonic membrane contain a small quantity of unsaturated-lipoid in older eggs, and it is reabsorbed by the developing larva before hatching.

The cement and the chorion do not obstruct the penetration of hydrophilic or lipophilic poisons into the eggs ; they often retain some poison to cause the death of those larvae which eat their way through while hatching. The main obstruction to the entry of water-soluble substances is given by the lipoid layer ; but it is supplemented to some extent when some fatty material appears in the epembryonic membrane in mid-development, and when some of the unsaturated-fat of the lipoid layer is converted to saturated state. The lipophilic substances penetrate quite easily through all layers of the eggs ; but some resistance encountered by them and a fumigant hydrogen cyanide during mid-development may be due to the fully developed state of epembryonic membrane, which is even resistant to the action of some corrosive chemicals at this time.

INTRODUCTION

The investigations have been carried out on eggs of *Microbracon gelechiae* Ashm., *Trichogramma evanescens minutum* Riley (Hymenoptera), *Chilo zonellus* S., *Corcyra cephalonica* Staint. (Lepidoptera), *Tetrastichus pyrillae* Craw. (Hymenoptera) and *Pyrilla perpusilla* Walk. (Homoptera). The first one is an external parasite of larvae of *Gnorimoschema operculella* Zell., a pest of potatoes (Narayanan, 1948*a*), and the second one is an internal parasite of eggs of maize stem borer, *C. zonellus* (Narayanan, 1948*b*). The larvae and the eggs of *C. cephalonica* serve as a laboratory host for the first and second insects respectively (Narayanan and

Mookherjee, 1955). *P. perpusilla* is a serious pest of sugarcane and *T. pyrillae* is the endo-parasite of its eggs (Narayanan and Kundan Lal, 1953). The work described herein is meant to be a study of differences in the chemical composition, modifications of the egg-envelopes, if any, during development because of their different environmental conditions, and to indicate how toxic materials and simple chemicals penetrate from the outside to the inner living contents. It is not intended to devise methods of control or to obtain ovicidal materials, but this study may provide a 'model' for parasitic (Hymenopterous) and host (Lepidopterous and Homopterous) eggs in the same way that previous work (Beament, 1946*a*,*b* : 1948, 1949, 1951 and Beament and Rattan Lal, 1957) has indicated the broad principles governing the mechanism of penetration through the eggs of *Rhodnius prolixus* Stähl., and *Pieris brassicae* Linn. It also explains how the host egg provides protection to the delicate egg of the parasite developing within the former. It may be possible that these recent fundamental studies and those undertaken in future may be of some value in selecting the ovicides effective against the eggs of pests, but not of parasites.

Material and Method

The eggs of *M. gelechiae* are obtained by the usual established practice of confining male and female adults in 3×2 inches glass dishes containing soaked raisens and covered on one side with muslin, the host larvae of *C. cephalonica* being kept on top of the muslin covered over with a glass plate. The insects of all the species used in this work are reared at about 30°C and 75 per cent relative humidity in an incubator. The females of *M. gelechiae* deposit eggs through the muslin-partition scattered on the surface of the larvae. The eggs can be removed gently from the host larvae with a fine camel-hair brush, as they lie loose and not gummed to the surface. In the absence of host if the eggs are crowded the newly hatched larvae suck juice from the unhatched eggs or even from the sluggish larvae. Generally the unmated females do not lay eggs ; but sometimes the unfertilised eggs are deposited by them which do not hatch at all and consequently there is no ovoviviparous development in the insect. The eggs laid by mated females are removed from the host and kept in the incubator until needed. The eggs hatch within 24 hours at 30°C of 100 per cent R.H., indicating a uniformity of development ; one can therefore presume that the eggs are fertilised immediately before laying.

The eggs of *C. cephalonica* are obtained by the usual methods of keeping gravid females in tin containers having glass top and wire-gauze bottom. These containers are placed in trays. The females oviposit through the wire-gauze and the eggs dropped in the tray below are collected. The eggs of *C. zonellus* and *P. perpusilla* are obtained by confining the gravid females in large cages containing maize and sugarcane plants respectively and kept in the field. Both the insects laid eggs in batches. The eggs of *C. zonellus* remain firmly gummed to the leaf surface, and for chemical tests they are kept soaked in water for some time and then removed from the leaf with camel-hair brush. Acetone or any other solvent cannot safely dislodge them from the leaf. Because of highly adhesive cement most of the eggs get damaged while removing them from the leaf ; hence for tests where living eggs are needed they are not dislodged from the leaf pieces. The batches of eggs of *P. perpusilla* are covered over with waxy secretion and detached hairs from the female (Narayanan, 1953). These eggs, though firmly attached to the leaf surface, can be easily removed from it with camel-hair brush soaked in acetone without damaging them. This method loosens their cement and the waxy covering. The eggs of these insects are kept in an incubator until needed. The duration of egg-stage of *C. cephalonica*, *C. zonellus* and *P. perpusilla* at 30°C and 100 per cent R.H. is about 3, 4 and 9 days respectively.

The eggs of *T. evanescens minutum* (Narayanan and Mookherjee, 1955) and those of *T. pyrillae* (Narayanan and Kundan Lal, 1953) are obtained by confining the eggs of the respective host along with the male and female adults in 2×4 inches glass-stoppered jars kept in an incubator. The parasites oviposit inside the host eggs, which are dissected to remove the parasitic eggs. The removal of parasitic eggs from the host eggs at different intervals after parasitisation indicates that the duration of egg stage of the parasites is about 24 hours at 30°C.

The technique employed is after Beament and Rattan Lal (1957) with slight modification where necessary. The experimentation with eggs is carried out at 30°C and 100 per cent R.H. The external morphology of the eggs is studied in whole mounts as well as portions of the shell mounted in glycerine, whereas the penetration experiments are conducted with the staining method. In some of the latter experiments radioactive phosphorus, P^{32}, is also used. The eggs without treatment with lipoid-solvent or after treatment for some time with petroleum-ether or chloroform are immersed for 5 and 60 minutes in 0.5 per cent aqueous solution of phosphoric acid (H_3PO_4) containing 250μc. of radioactive P^{32}. Later these eggs are washed with water and assayed for radioactivity. The conversion of some of the unsaturated material of lipoid layer of the eggs to saturated state during egg-development is studied as follows:—Fifty eggs or ova are cut into longitudinal halves, removing the inner contents along with epembryonic or vitelline membrane and thoroughly washing with distilled water. These are quickly treated with 0.1 ml. of chloroform in cavity slide. The treated shells are removed and the chloroform after evaporation leaves a deposit of fatty material on the slide. This fatty deposit is treated with 0.01 ml. of 0.1 per cent aqueous iodine solution, and the process of decolourisation of the iodine solution is noted after one hour and three hours. Each experiment is repeated six times. The air spaces in cement or respiratory pores in chorion of the eggs are studied by injection with cobalt naphthenate, the method recommended by Wigglesworth (1950) and Wigglesworth and Beament (1950). The 'transition temperature' of the eggs, i.e. the temperature at which the lipoid layer becomes markedly more permeable to water, has been determined with the technique adopted by Beament (1951) and Judge (1953). To determine the active or passive machanism of absorption and retention of moisture within the eggs, one thousand ova, living eggs and those killed with hydrogen sulphide are separately desiccated for four hours in four replications at 30°C and 0 per cent relative humidity. The difference in weight of the eggs before and after desiccation gives the amount of water lost, The desiccated eggs are then brought to 75 per cent or 100 per cent R.H. to find out whether they can regain the lost water.

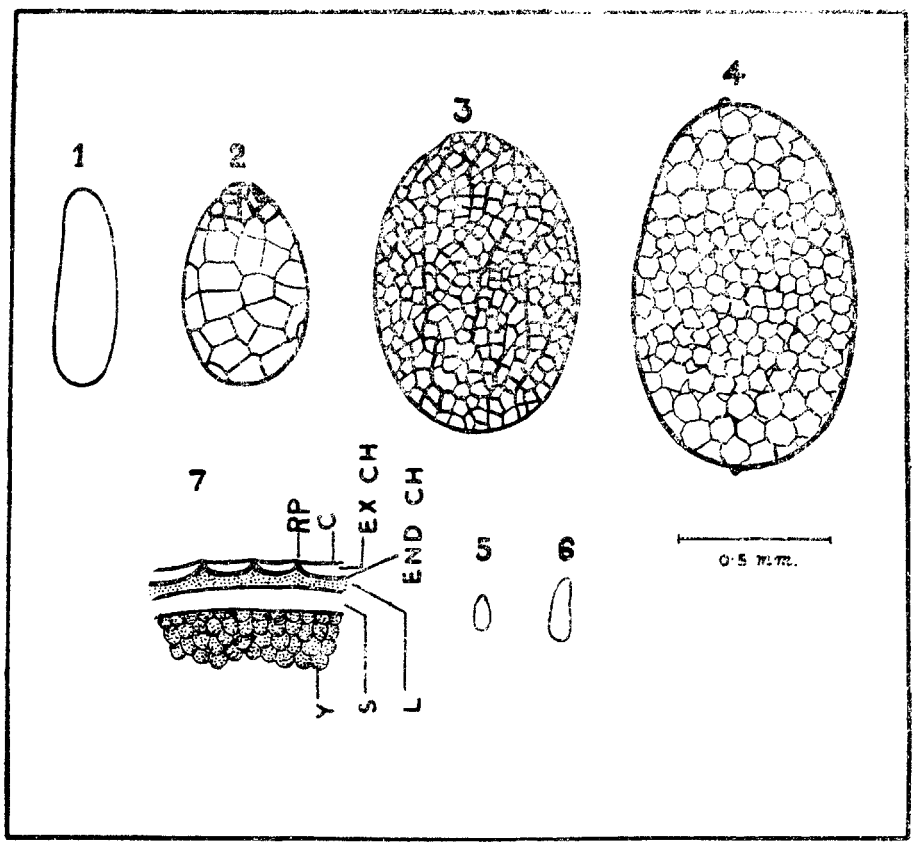
The procedure for ovicidal experiments is after Beament (1948) and the tests are performed with water-soluble salts and salts both water- and lipoid-soluble. The eggs are gently forced into the ovicidal solution with a camel-hair brush because of the presence of air in respiratory pores. They are kept dipped for five minutes and then the excess of the solution from their surface is removed with pieces of filter paper. They are then kept in an incubator. Thirty eggs per test with six replications and along a control-set constitute a single experiment.

Part I: Morphology of Eggs, Physical and Chemical Properties of Their Shell, Their Water and Oxygen Relations

(a) *Morphological notes on the eggs*

A freshly laid egg of *M. gelechiae* (Fig. 1) is dirty white, has 3.5 microns thick shell and measures 0.607 by 0.187 mm.; that of *C. cephalonica* (Fig. 2) is opaque, has 5.8 microns thick shell and measures 0.583 by 0.373 mm.; that of *T. evanescens minutum* (Fig. 5) as well as of *T. pyrillae* (Fig. 6) is transparent, has 1.2 micron

thick shell and measures 0.117 by 0.047 mm. and 0.233 by 0.07 mm. respectively; that of *C. zonellus* (Fig. 3) is light yellowish, has 4.7 microns thick shell and measures 1.003 by 0.607 mm.; and that of *P. perpusilla* (Fig. 4) is whitish, giving reddish to greenish hues, has 6.3 microns thick shell and measures 1.143 by 0.56 mm. All these eggs have a micropyle at the anterior end. They get proportionately darkened with age due to the changes in the developing embryo ; the latter is somewhat discernible through the semitransparent shell. The egg-shell of *M. gelechiae*, *T. evanescens minutum* and *T. pyrillae* is quite smooth externally, whereas that of *C. cephalonica*, *C. zonellus* and *P. perpusilla* bears lines depicting the impressions of the boundaries of follicular cells of the ovary. This pattern



TEXT-FIG 1.

Fig. 1, *Microbracon gelechiae* Ashm. egg.
Fig. 2, *Corcyra cephalonica* Staint egg.
Fig. 3, *Chilo zonellus* Zell. egg.
Fig. 4, *Pyrilla perpusilla* Walk. egg.
Fig. 5, *Trichogramma evanescens minutum* Riley egg.
Fig. 6, *Tetrastichus pyrillae* Craw. egg.
Fig. 7, Diagramatic sketch of transverse section through portion of an egg, depicting fundamental layers. C : Cement, CH : Chorion, END.CH : Endochorion, EX.CH : Exochorion, L : Lipoid layer, R.P : Respiratory pore, S : Serosa, Y : Yolk.

which is composed of non-resistant exochorion, is very mild and honey-combed type on the egg of *P. perpusilla*, but it is quite prominent and zig-zag on that o

C. zonellus and *C. cephalonica*. Further in the latter insect these lines are comparatively thicker at the anterior end in a little area round the micropyle, and this area when seen from above seems to give the appearance of a sieve-cap lying on top of the egg. Moreover the egg of *P. perpusilla* is slightly flattened dorso-ventrally. There is a small knob at its each end, the anterior one being larger than the posterior. The former and the latter measure 0.036 by 0.05 mm. and 0.02 by 0.033 mm. respectively. The micropylar canal traverses through the anterior knob and opens on the tip. The posterior knob is a solid structure.

(b) *Construction of the shell membranes*

The chorion of the eggs of *M. gelechiae*, *C. cephalonica*, *C. zonellus* and *P. perpusilla* consists of two layers : an external proteinaceous coating, the exochorion and an internal layer of tanned protein, the endochorion (Fig. 7). Internally the lipoid layer of oily material is situated between the chorion and vitelline membrane, and the latter is afterwards transformed into serosa (Fig. 7), and then into epembryonic membrane, containing a little quantity of lipoid, and is ultimately reabsorbed before hatching. On the outer surface of the egg is spread by the female with a cement (Fig. 7), a proteinaceous secretion of the accessory gland, which in the eggs of *M. gelechiae* and *C. cephalonica* dries up quickly; whereas in *C. zonellus* and *P. perpusilla* it fixes the shell firmly to the leaf surface. Moreover in the latter it appears to be firmly held by the stalks of the anterior and posterior knobs.

The chorion of the egg of *T. evanescens minutum* and *T. pyrillae* consists of only a single layer of non-resistant proteinaceous material. There is a complete absence of internal lipoid layer. The vitelline membrane as usual is transformed into serosa and then epembryonic membrane, which is devoid of the traces of lipoid and is reabsorbed before hatching. It is difficult to say whether the external cement layer (the usual lubricating layer of Hymenopterous eggs) is present or absent on these eggs; as the fully formed ova which are always devoid of the cement could not be obtained by dissecting such small insects and studied along side with the eggs. It is also not possible to dissect out the lubricating glands of these small insects even if they are present.

(1) *The cement layer*

The cement on outer surface of egg-shell of *M. gelechiae* is not present in sufficient quantity to facilitate chemical and physical tests. Most of the work has therefore been done on the paste-like contents of the cement gland (called the lubricating gland in this insect) of the female. On exposing a thin layer of this material to air, it gets transformed to a substance behaving in every way like the layer on the shell, which indicates that no chemical addition is made at the time of oviposition. It is a cement of non-adhesive type and loses only a little quantity of water on exposure to air, but gives off the entire water on desiccation and simultaneously its protein gets denatured. When the dried cement is brought back to saturated relative humidity it regains the lost water, but it does not become paste-like again. It is hygroscopic to some extent and consequently maintains a higher concentration of moisture outside the shell, thereby preventing the latter from too much drying. The contents of the gland cannot be diluted with water after they are exposed to air. It could be assumed from this that some denaturation of cement-protein has taken place by its drying in air. This process is apparently speeded up by the chemical denaturants like ethylalcohol or phosphomolybdic acid, but not by pure oxygen alone. In the gland the paste-like fluid having a *p*H of 7.5 consists of a colourless matrix containing a large number of irregular granules of dirty white colour and lipoid globules. When exposed

to air it sets into a layer; the minute granules, the fluid and the lipoid globules get mixed up and unite in such a way as to assume the appearance of a membrane. The process of denaturation affects the fluid part greatly which binds the other constituents. The lipoid globules in the cement layer do not acquire a uniform disposition, with the result that some spaces are left which are devoid of the lipoid material. The cement does not prevent the solutions of water- and oil-soluble stains from reaching the chorion surface, provided they could wet the outer surface of the egg. It may also be mentioned that the presence of lipoid material in the cement also helps in lubricating the oviduct and the ovipositor of the female, thus facilitating an easy passage of eggs through them. That is why the cement in such insects is referred to as lubricating material and the gland secreting it as the lubricating gland.

The staining reactions with fuchsin, orange-G, safranin as well as tests like xanthoproteic and ninhydrin have shown that the matrix and granules of the cement are acidophillic protein. The presence of a little reducing substance in these is indicated by argentaffin test. The cement, not only reduces osmic acid, but also gets stained with sudan fat stains. Corrosive substances such as strong nitric acid or sodium hypochlorite break down the cement, releasing oily droplets and thus indicating it to be formed of lipoprotein. But solvents like chloroform and ether can remove the lipoid component of the cement and the extracted material gives a strong reducing action. It is therefore obvious that it is an unsaturated lipoid, not chemically bound to the protein component. It does not contain any water-soluble reducing material attached to a protein. This cement is attacked by strong formic acid and sulphuric acid, a characteristic not common to most insect shell components. The treatment with lipoid solvents does not produce any marked visual change in the cement, but it becomes a little brittle on drying and shows much stronger colouring with aqueous stains. The removal of lipoid from it affects somewhat its water retentive power. The lipoid therefore is of considerable importance in maintaining a balance in the moisture content of the cement and the surrounding air.

The other gland associated with the sexual organs in a female *M. gelechiae* is the 'poison gland'. Its proteinaceous secretion is markedly acidic with *p*H 4.0 and contains a small quantity of unsaturated lipoid. The globular cells around the poison duct also secrete an unsaturated lipoid which is employed in lubricating the inner passage of the ovipositor prior to stinging the host. The secretions of the globular cells, the main poison gland and the lubricating gland soften the chorion only to a little extent.

A similar series of tests have been performed on the contents of the cement glands (accessory glands) of the females of *C. cephalonica*, *C. zonellus* and *P. perpusilla*. The chemical constituents of the cement from these insects are the same as those of the cement of *M. gelechiae*. The cement from *C. cephalonica* contains less lipoid than that of *M. gelechiae*, but it resembles the latter in physical properties. The cement from *C. zonellus* and *P. perpusilla* has air spaces in its substance like a sponge and is highly adhesive. It also contains less lipoid than that of *M. gelechiae*. The cement from all these insects does not contain any water-soluble reducing substance. Moreover the waxy covering on a batch of eggs of *P. perpusilla* helps them to resist desiccation and penetration with solutions of aqueous materials.

(2) *The chorion*

The chorion of a fully formed ovum and of a newly laid egg of *M. gelechiae* is soft and plastic. It remains so if it is kept in contact with moisture, but when exposed to low humidity or heated to 40°C there appears some irreversible hardening in it ; and this takes place in nature by normal exposure to atmosphere. The

pliability of chorion is related to its water-content and is not altered by extraction with chloroform. Chorion is composed of two layers, a comparatively thick exochorion and a thin endochorion (Fig. 7), which cannot be separated mechanically or chemically. Both these layers are made of minute particles embedded in matrix. Chorion is perforated by small pores (Fig. 7) scattered all over it. The blocking of these pores by the application of heavy petroleum oil, such as petroleum jelly, liquid paraffin, etc. on the surface of chorion actually kills the developing embryo by suffocation, thus indicating that these are respiratory pores. A study of the different stages of ovum in ovariole tube indicates that the chorion is deposited by the follicular cells.

Both the parts of chorion are basically formed of protein as is evident from staining reactions with orange-G, safranin, fuchsin, picric acid, iodine as well as from the xanthoproteic and ninhydrin tests. They do not colour with sudan fat stains or osmic acid. The fatty droplets are not liberated when these are acted upon by strong acids, etc.; and there is no intensification of staining reactions after these are extracted with chloroform. Thus, no free or chemically bound lipoid exists in them. They give argentaffin and *p*-benzoquinone reactions, but these are more pronounced in endochorion than those in exochorion, and more so in the granular parts of these layers than in the matrix. Thus they appear to be partially quinone tanned protein. Moreover, the exochorion dissolves more readily in strong acid, and is therefore regarded as less cross-linked. Strong nitric acid breaks down the chorion in cold, releasing the small particles which eventually are dissolved within a few hours. When slowly acting metarial, such as dilute mineral acids, strong ammonia, urea, strong formic acid, pepsin and trichlor-acetic acid are used they produce no effect on either layer. Trypsin on the other hand digests the exochorion only. This therefore confirms the suggestion that the endochorion is of tanned substance, whereas the exochorion is free from such bonds. A solution of lithium iodide or sodium thioglycolate does not materially affect the resistance of chorion to solution and therefore it appears unlikely that sulphur bonds play any substantial part in their construction. Both the layers of chorion do not prevent quite big molecules of the stains from penetrating into the interior of the egg, since they colour with water-soluble stains even when the entire egg is dipped in the solution, and since the developing embryo gets stained even though both the layers remain uncoloured when the entire lot is immersed in oil-soluble stains. The exochorion does not undergo any change after the egg is laid, but the tanned endochorion of ova inside the female is readily soluble in strong ammonia and 10 per cent potassium hydroxide. It seems that much of the tanning of the endochorion goes on subsequent to fertilisation, but is completed within a few hours of embryonic development. The embryo has nothing to do with it, as the tanning process of endochorion is completed even in an ovum after removal from the ovary and incubated under similar conditions. Thus the excess tanning material probably already present in the endochorion continues its action even after the eggs are laid.

A similar study of the exo- and endo-chorion of the eggs of *C. cephalonica*, *C. zonellus* and *P. perpusilla* indicates that the chemical nature resembles that of *M. gelechiae*. Like the latter the respiratory pores traversing the chorion are scattered in the eggs of *P. perpusilla*, whereas these are concentrated to some extent near the anterior end of the eggs of *C. cephalonica* and *C. zonellus*. In the eggs of *T. evanescens minutum* and *T. pyrillae*, the chorion is very thin and resembles to some extent physically and chemically the exochorion of *M. gelechiae*, except that it does not seem to have any phenolic tanning material and the respiratory pores are absent. It is just possible that these differences might be due to the fact that these eggs are never exposed to atmospheric conditions and they remain embedded in the tissue of host eggs after they are laid.

(3) *The lipoid layer*

The lipoid layer in an egg of *M. gelechiae* lies between the endochorion and the outer embryonic layer (Fig. 7). It is present on a small scale in ova but definitely in the oviposited eggs. This substance is a freely mobile material. After extraction with chloroform, the oily residue blackens deeply with osmic acid, and decolourises iodine solution. It is obviously an unsaturated material. It may be mentioned here that the lipoid from ova or the newly laid eggs decolourises the iodine solution more quickly than that obtained from the eggs in an advanced stage of development (Table I). This indicates that the ova and freshly laid eggs contain more unsaturated lipoid-material than the eggs of advanced stages; and during the course of development the embryo produces some effect on it (may be catalytic oxidation) and some of its oily material is altered to saturated oil. But this process of oxidation needs assistance of the developing embryo and does not

TABLE I

Decolourisation of iodine solution by the fatty material from the lipoid-layer of eggs of the following insects

	M. gelechiae	*C. cephalonica*	*C. zonellus*	*P. perpusilla*
	a b c d e f	a b c d e f	a b c d e f	a b *c d e f
Unsaturated lipoid decolourising iodine solution after (Hours) :—	1 1 1 3 3 3	1 1 1 3 3 3	1 1 1 3 3 3	1 1 1 3 3 3

Inference:—The delay in decolourisation of iodine solution indicates that some of the unsaturated fatty material of the lipoid layer has been altered to saturated state.

Note :—Fatty material from the lipoid layer of :

a. Ova fully formed.
b. Eggs freshly laid.
c. Ova fully formed but these have been earlier incubated at 30°C and 100 per-cent R.H. for the period equivalent to half incubation period of a normal egg.
*c. As in 'c' excepting that ova incubated for the period equivalent to about quarter incubation period of a normal egg.
d. Eggs in mid-development.
e. Eggs about to hatch.
f. Egg-shell after hatching.

appear to take place of its own accord, as there is no difference in the decolourisation of iodine solution with the lipoid taken from the ova incubated for half the incubation period of the normal eggs, from that of the lipoid taken from the fresh ova. Since it is extremely difficult to obtain this lipoid in large quantity to undertake detailed chemical analysis, the investigation on this aspect will be taken up some time later. This unsaturated lipoid giving water-proofing to eggs of *M. gelechiae* is unique so far as our present knowledge of apparently water-proofing lipoid substances of the cuticle is concerned. The lipoid layer gives chief obstruction to the penetration of only water-soluble materials into the egg and not of the oil-soluble substances. This point is further confirmed beyond doubt by experimenting with phosphoric acid containing the radioactive phosphorus, P^{32}. The eggs, which are not given prior treatment with ether or chloroform and are immersed only for 5 minutes in the solution of phosphoric acid, do not show any radioactivity, but those eggs which are immersed for 60 minutes show radioactivity in the shell as well as in the inner contents. This is probably due to the fact that phosphoric acid which is only slightly soluble in oil-solvent like ether fails to

penetrate into the eggs with 5 minutes' immersion, whereas it enters the eggs that remain dipped for an hour. On the other hand the contents of the eggs whose lipoid layer has been treated earlier with petroleum-ether or chloroform do show radioactivity whether the eggs are immersed in the phosphoric acid solution for 5 minutes or for an hour. This indicates that ether or chloroform treatment of the eggs by dislodging the lipoid layer facilitates the entry of highly water-soluble phosphoric acid. Thus the lipoid layer provides a barrier to the penetration into the eggs of water-soluble materials which are only slightly soluble in oil-solvent when the immersion is of short duration, whereas in longer immersions the little solubility of such materials in lipoid-solvent helps appreciably the process of penetration.

The formation of the lipoid layer commences in the follicular tube. Ova in different stages of growth are stained with sudan fat stains. It is noticed that the lipoid is only concentrated in the substance of the growing ovum, whereas it is present in traces in follicular cells. The lipoid appears to be deposited by ovum on the surface of vitelline membrane, whereas the chorion is formed on it simultaneously by the follicular cells. The chorion at no time is found to contain any oily material. Sometimes the formation of the lipoid layer is not completed in an ovum and it is completed within an hour or so after the oviposition. The aqueous stain in solution penetrates quite easily into the freshly laid eggs in which the lipoid layer is incomplete, whereas in those eggs in which it is already complete the penetration of such materials does not take place. The fertilisation of ovum has nothing to do with the completion of the lipoid layer, as it is even completed in ova separated from the ovary and incubated under similar conditions. The transition temperature varies between 50°C and 60°C and it is not particularly sharp. It is variable in different eggs. However, there is some increase in it as the egg advances in age. This rise in transition temperature may be due to the conversion of some of the unsaturated lipoid to saturated state, and also may be due to some loss of the more volatile component from the layer. But both these suggestions need further investigation.

In eggs of *C. cephalonica*, *C. zonellus* and *P. perpusilla* the lipoid layer internal to the chorion is also formed of freely mobile unsaturated oily material, which is deposited by the ovum before the eggs are laid. As in the eggs of *M. gelechiae*, some of its unsaturated oil gets converted into saturated oily substance in the advanced stages of eggs by the developing embryo (evident from iodine decolourisation experiments, Table I). This phenomenon also exists in the eggs of *P. brassicae* re-examined now. The transition temperature of these eggs is not sharp and falls between 60°C to 70°C. It comes nearer to the upper limit as the eggs grow older, as is the case in the eggs of *M. gelechiae*. The experiments with the dyes have shown that in these eggs also the lipoid layer is a potential barrier to the penetration of water-soluble materials. The tests with phosphoric acid containing radioactive phosphorus, P^{32}, have indicated that the water-soluble substances having a little oil-solubility can penetrate the eggs with longer immersions. In eggs of *T. evanescens minutum* and *T. pyrillae* the lipoid layer is absent, and therefore solutions of water-soluble materials can easily penetrate the shell to reach the embryo. It is just possible that since these eggs remain immersed in the fluid of host eggs, there is no need for any water-proofing in them. The solutions of oil-soluble substances also easily enter these eggs.

(c) *Embryonic covering*

It is worthwhile to probe into the nature of extra-embryonic membrane interior to the lipoid layer so as to find out whether any changes in it are running parallel with changes in the ovicidal resistance of the eggs. The ovum of *M. gelechiae*, *C. cephalonica*, *C. zonellus* and *P. perpusilla* is surrounded by vitelline membrane, which changes into fertilisation membrane in a fertilised egg. It i

proteinaceous and is devoid of lipoid in any form either free or chemically bound. It is readily permeable to water-soluble and oil-soluble materials in solution. Some time after oviposition it is progressively replaced by serosa (Fig. 7) and then by epembryonic membrane, when it becomes a little more resistant to the action of corrosive substances such as strong hydrochloric acid, 10 per cent potassium hydroxide, or 5–7 per cent sodium hypochlorite in cold, than the vitelline or fertilisation membranes. Even at this time it is quickly soluble in strong nitric acid ; and is permeable to some extent to water-soluble and oil-soluble substances. Its chemical nature is somewhat similar to that of exochorion, except that it is less resistant to the corrosive substances than the latter, contains a little unsaturated lipoid, but does not contain any tanning material. Thus at this stage it forms to some extent a secondary water-proofing mechanism. A re-examination of this membrane of *P. brassicae* now has indicated that it also contains a little quantity of unsaturated lipoid which escaped detection earlier. Moreover, when the larva is about to hatch the resistance of this membrane diminishes, probably due to the softening caused by some chemical action from the living material ; it, however, does not revert completely to the properties of the original vitelline or fertilisation membranes.

To explain the changes in the resistance of epembryonic membrane with age, the effect of pre-treatment of eggs of these insects with trichloracetic acid, and sodium thioglycolate on the action of trypsin has been studied. In the freshly laid eggs and those near hatching the membrane is soluble in cold trichloracetic acid, whereas it is insoluble in it in the intervening stages of development, thus showing the preparation for hatching carried out on the membrane by the embryo. Similarly, trypsin at *p*H 8.5–8.8 and 37°C disintegrates the early and late membrane, but not in the intervening period. When the membrane in the more resistant stage is treated first with sodium thioglycolate at 30°C and *p*H 12, it is not dissolved, but following this treatment it is readily broken down by both trypsin and trichloracetic acid. Similarly concentrated lithium iodide solution makes the membrane of the resistant stage slowly soluble in trichloracetic acid, and not-disintegrable in trypsin. In view of this the actual chemical linkage in this membrane may be a form of thioquinone. The rate of changes in its resistance is inversely proportional to the incubation period of the eggs. But owing to the small size of these eggs and the minuteness of the material under study it has not been possible to undertake detailed chemical analysis which could settle this point.

In the unfertilised eggs the vitelline membrane is applied closely and quite securely to the upper lip of the micropyle. The lipoid layer is not therefore complete round the ovum, and sperm can enter from micropyle directly into the surface of the vitelline membrane. The lipoid layer in these eggs is completed round the micropylar end immediately after fertilisation and before oviposition, consequently the deposited eggs are quite resistant to the penetration of water-soluble materials and to water-loss. The completion of the lipoid layer at micropylar end may be due to a slight shrinkage of the egg-cell leading to the breakage of the junction between micropyle and vitelline membrane and flowing of the lipoid across to seal the micropyle. In the few eggs of *M. gelechiae* having incomplete lipoid layer for some time after oviposition, the resistance to penetration of water-soluble substances is somewhat feeble at that time.

The epembryonic membrane of the eggs of *T. evanescens minutum* and *T. pyrillae*, like their shell behaves like ordinary denatured protein. It is devoid of lipoid and is reabsorbed before hatching.

(d) *Water relations of the eggs*

The eggs and newly hatched larvae of *M. gelechiae* contain about 90 per cent and 88 per cent water by weight respectively. The amount of moisture in cement

layer could not be quantitatively determined, as it is applied just like a varnish on egg's surface. The cement being hygroscopic provides moist surroundings to the chorion. The unfertilised eggs (mature ova) lose water rapidly on desiccation (Table II) and their rate of water-loss during four hours' desiccation is 5 per cent by weight per hour, whereas that of the fertilised eggs only about 1 to 1.5 per cent. There is no significant difference between the rates of loss of normal eggs and ova under the similar conditions as compared with those killed with hydrogen sulphide prior to desiccation. It appears, therefore, that death does not produce

TABLE II

Percentage loss of water in dry air at 30°C *from living eggs and ova, or those killed by fumigation with hydrogen sulphide for half an hour*

Age of eggs (Hours)	Alive		Dead	
	In 4 hours	% rate per hour	In 4 hours	% rate per hour
1–2	6.2	1.55	6.6	1.65
4	4.1	1.02	3.9	0.97
8	4.5	1.12	4.2	1.05
12	4.2	1.05	4.1	1.02
24	5.9	1.47	6.2	1.55
Mature ova from ovary	20.1	5.02	20.3	5.07

any 'break down' in the water-proofing mechanism of the eggs. But the process of fertilisation and after oviposition the subsequent stages up to mid-development are accompanied by some irreversible improvement in water-proofing. The latter may be correlated to the completion of the lipoid layer in those eggs in which it has not been completed within the ovary on the one hand, and to the conversion of some of the unsaturated lipoid of this layer to saturated state as well as to the deposition of some oily material in the epembryonic membrane with development on the other. Prior to hatching the water-proof nature reverts back to some extent to the state which is seen in fresly laid eggs, and it is correlated to the disintegration and reabsorption of the membrane by the embryo at this time. Moreover if the living or dead eggs desiccated in different stages of development are kept at 75 per cent or 100 per cent relative humidity they do not regain the lost water, indicating that there does not exist any active or passive mechanism for water absorption.

In *T. evanescens minutum* and *T. pyrillae* the eggs after removal from the host eggs do not survive desiccation even at 75 per cent R.H. and this is due to the absence of water-proofing lipoid layer.

The eggs and newly hatched larvae of *C. cephalonica*, *C. zonellus* and *P. perpusilla* contain about 86 per cent and 82 per cent water by weight respectively. The cement accounts for nearly 2 per cent of the total weight of a freshly laid egg of the first and nearly 9 per cent of that of a newly laid egg of the last two insects. The living or dead eggs of these insects in different stages of development, if desiccated and later brought to 75 to 100 per cent R.H. absorb a quantity of water which can only be accounted for by the absorption with the cement, indicating that even in these eggs there is no other active or passive mechanism for the absorption of moisture. Moreover in these eggs also there is some improvement in the water-proofed nature in the mid-development age, which disappears prior to hatching.

(e) *Oxygen relations of the eggs*

Dipping for about six hours in water or even up to 12 hours is not fatal to the eggs, which indicates that this does not interfere in their respiration. If they are kept immersed for a long period they die because of restriction in oxygen supply. They could complete development under water if the latter is kept aerated with air or even oxygen or if 5 volumes of hydrogen peroxide are mixed with 95 of water; but most of the larvae die soon after hatching. The aqueous solutions of a wetting agent (Teepol) is lethal as compared with similar volumes of distilled water if the eggs are dipped for a long period. It cannot be imagined that oxygen diffusion through either of them differs, but the solution of wetting agent slowly dislplaces air from the respiratory pores which might be acting as a physical-gill in the immersed eggs. Even the lethal effect of the obstruction of respiratory pores by short immersion in heavy petroleum-oil can be eliminated by removing it from the surface of the eggs with petroleum-ether. Thus there is a need for the respiratory pores to remain open for the normal development of the eggs. The eggs of *C. cephalonica*, *C. zonellus* and *P. perpusilla* could complete development in an atmosphere containing more than 50 per cent oxygen, whereas those of *M. gelechiae* fail to do so at such a high concentration of oxygen.

Part II : Ovicidal Experiments

The experiments given herein are only intended to give an idea about the types of poisons which can penetrate through egg-envelopes and destroy the developing embryo, and to indicate whether the parasitic-egg developing within the host-egg could be saved from such poisons or not.

(a) *Water-soluble salts of heavy metals*

The chlorides and acetates of cobalt, copper, nickel and manganese used in concentrations of 1 per cent in water do not produce any harmful effect on eggs of *M. gelechiae*, *P. perpusilla*, *C. cephalonica* and *C. zonellus*. The mortality of larvae during hatching or some time afterwards is quite common in the latter two insects, which is due to the action of poisonous materials they consume when they eat a portion of the shell for hatching. There are hardly any post-hatching casualties in the former two insects, as they do not consume the poison from the shell since they only pierce their way through for hatching. These substances do not become any more efficient either when the eggs are immersed for 5 minutes following evacuation of air from the respiratory pores and forcing the liquid into them, or when they are used in combination with a wetting agent (1 per cent Teepol). It appears that these purely water-soluble chemicals with no oil-solubility, which are potentially very toxic, are not able to cross the barrier of the lipoid layer and reach the embryo. These water-soluble poisons have also failed to kill the eggs of *T. evanescens minutum* and *T. pyrillae* developing in their respective host-eggs. The parasitic eggs are permeable to water-soluble substances due to the absence of lipoid layer in them; but the protection they get while they are within the host-eggs is due to the latter possessing a non-permeable inner lipoid layer.

The freshly laid eggs of *M. gelechiae*, *P. perpusilla*, *C. cephalonica* and *C. zonellus* are a little more susceptible, especially when the wetting agent is mixed with the solution and the immersion period is raised to six hours (Table III). The wetting agent even alone when used for such a long duration does cause some harm to these eggs as well as to endo-parasitic eggs present within them. This is probably due to the fact that in longer immersions the small quantity of the wetting agent taken up by the chorion of the host-eggs remains in contact with their lipoid layer for longer time and causes some disruption in it; but even in such conditions

the entire lipoid layer is not disintegrated. Moreover solutions of glucose and sodium chloride (potentially harmless materials) of the same concentrations as well as of the concentrations chemically equivalent to those of the solutions of the chemicals used in the ovicidal tests, do not have any detrimental effect on the eggs. This indicates that these concentrations of the chemicals in solution do not cause an adverse effect on the eggs through exosmosis during the dipping

TABLE III

Percentage kill of freshly laid eggs following immersion for six hours in 1% aqueous solutions of the chemicals

Chemicals	With or without 1% Teepol	*M. gelechiae*	*P. perpusilla*	*C. cephalonica*	*C. zonellus*	*T.evanescens minutum* eggs parasitising eggs of *C.cephalonica*	*T.pyrillae* eggs parasitising eggs of *P.perpusilla*
Cobalt acetate	a	59	58	60	58	60	59
	b	9	8	10	8	10	9
Cobalt chloride	a	59	58	58	56	59	60
	b	9	8	8	6	9	10
Cupric acetate	a	90	86	82	84	86	82
	b	20	18	16	17	18	16
Cupric Chloride	a	60	56	58	57	59	56
	b	10	6	8	7	9	6
Nickel acetate	a	70	68	69	68	67	68
	b	10	8	9	8	7	8
Nickel chloride	a	69	69	68	67	69	68
	b	9	9	8	7	9	8
Manganese acetate	a	58	56	56	58	59	59
	b	8	6	6	8	9	9
Manganese chloride	a	60	58	57	59	58	60
	b	10	8	7	9	8	10
Glucose 1%	a	42	37	40	37	38	40
	b	9	6	10	7	8	10
Glucose 1.2%	a	39	34	38	36	40	36
	b	8	8	9	6	9	6
Sodium chloride 1%	a	37	39	36	36	40	39
	b	10	6	6	8	8	8
Sodium chloride 0.36%	a	38	38	40	38	39	36
	b	9	7	7	6	10	10
Teepol only 1%		40	35	39	38	38	36
Water only		3	2	3	2	2	4
No treatment		2	3	3	2	0	0

Note :—a. With Teepol. b. Without Teepol.

period. Further copper acetate under similar conditions is more toxic to the eggs as well as to those of the internal parasites within them than copper chloride or

acetates and chlorides of the other elements; and this is probably due to the property of copper acetate being slightly soluble in lipoid-solvent (like ether), whereas the other salts are insoluble.

(b) *Chemicals—having oil- and water-solubility*

A few experiments on eggs of these insects have been conducted to compare the toxicity of aqueous solutions of mercuric chloride, bromide and nitrate, lithium chloride, arsenic trichloride (water- and lipoid-soluble compounds) and mercuric acetate (water-soluble compound). The results (Table IV) indicate that mercuric

TABLE IV

Percentage kill of eggs following immersion for 5 minutes in 0.5% aqueous solutions of the chemicals

Chemicals	Stage of eggs	*M. gelechiae*	*P. perpusilla*	*C. cephalonica*	*C. zonellus*	*T.evanescens minutum* eggs parasitising eggs of *C.cephalonica*	*T.pyrillae* eggs parasitising eggs of *P.perpusilla*
Mercuric chloride	a	100	100	100	100	100	100
	b	91	89	87	90	88	91
	c	97	95	93	96	94	97
Mercuric bromide	a	100	100	100	100	100	100
	b	87	84	83	86	96	95
	c	93	90	89	92	100	100
Mercuric nitrate	a	90	88	85	87	92	91
	b	61	57	59	58	60	56
	c	79	77	74	76	81	80
Arsenic trichloride	a	100	100	100	100	100	100
	b	85	86	83	84	87	85
	c	91	92	89	90	93	91
Mercuric acetate	a	10	8	7	9	10	11
	b	12	10	8	11	9	10
	c	10	11	10	8	9	11
Arsenic trioxide, *p*H 6–7	a	7	9	8	6	7	8
	b	8	8	7	9	7	9
	c	7	7	8	9	8	9
Arsenic trioxide, *p*H 10 or more	a	100	100	100	100	100	100
	b	89	90	88	88	87	89
	c	95	96	94	93	94	95
Lithium chloride	a	6	7	7	8	6	7
	b	7	8	6	8	7	8
	c	6	7	7	6	8	9
Water only	a	7	8	6	9	8	7
	b	9	7	9	8	7	8
	c	8	7	6	9	8	7
No treatment	a	6	7	7	8	6	6
	b	7	8	8	9	7	7
	c	6	5	7	8	7	9

Note :—a. Eggs freshly laid. b. Eggs in mid-development. c. Eggs about to hatch.

chloride, bromide and arsenic trichloride are highly toxic to the eggs of all the insects as well as to the parasitic eggs found within the host-eggs, whereas mercuric nitrate is a little less toxic. The freshly laid eggs and those about to hatch are slightly more susceptible than the eggs in mid-development. The lithium chloride does not appear to be toxic to the eggs. Moreover, arsenic trioxide (a purely water-soluble salt) at *p*H 10 or more seems to kill the eggs, whereas it is non-toxic at 6 to 7 *p*H; this might be due to the fact that the higher *p*H probably produces some effect on the chorion and the lipoid layer of the eggs and thereby facilitates the penetration of the chemical.

(c) *Fumigation of eggs*

Hydrogen cyanide at atmospheric pressure penetrates more slowly in the eggs in mid-development than in freshly laid eggs or those about to hatch. Hydrogen sulphide is equally fatal at all stages of egg-development and has a quick action. All stages of the eggs are equally susceptible to mercury vapour, but in this case a little longer exposure is necessary.

Discussion

The study of structure and chemical composition of the shell consisting of cement, chorion and lipoid, and embryonic membranes of eggs of *M. gelechiae*, *C. cephalonica*, *C. zonellus* and *P. perpusilla* as well as the information from the ovicidal experiments indicate a similarity in fundamentals with the results obtained from eggs of *P. brassicae* by Beament and Rattan Lal (1957), but some interesting variations are also evident. The external waxy covering which is only present on the batches of eggs of *P. perpusilla* gives them an additional advantage in resisting desiccation and penetration with solutions of aqueous materials. The proteinaceous cement of the eggs of all these insects resembles that of a mite, *Metatetranychus ulmi* Koch. (Beament, 1951) and *P. brassicae* in having only a physical mixture of unsaturated lipoid with its substance. It differs from that of *P. brassicae* in not containing any water-soluble reducing material linked to a protein. The cement of *M. gelechiae* like that of *Diataraxia oleracea* Linn. (Salkeld and Potter, 1953) contains more lipoid than that found in the cement of the other insects. The cement is non-adhesive and devoid of air spaces in *M. gelechiae* and *C. cephalonica*, whereas in *C. zonellus* and *P. perpusilla* it is adhesive and spongy like that of *P. brassicae*. It is not possible to say whether the cement layer is present or absent from the eggs of *T. evanescens minutum* and *T. pyrillae* as it could not be studied. It may be pointed out here that the secretion of the globular cells of poison duct does not soften the chorion of eggs of *M. gelechiae* to the extent (i.e. 1/20th of its diameter for facilitating their passage through that much size of ovipositor canal) visualised by Narayanan and Subba Rao (1955). Even the secretions of the main poison gland and the lubricating gland (cement gland) do not produce this effect. To decide this point one has to search for explanation elsewhere, especially either the mechanism of the ovipositor or something else which has escaped notice so far.

The proteinaceous chorion of eggs of these insects and that of *P. brassicae* consisting of non-resistant exochorion and resistant endochorion is slightly different from that of *D. oleracae* in not having a layer corresponding to the thin exochorion of lipoprotein which covers the latter eggs. The respiratory pores in chorion of *C. cephalonica* and *C. zonellus* like the pores in that of *D. oleracaea* and *P. brassicae* are somewhat concentrated at the anterior end of the eggs; whereas the pores are scattered all over the surface of the eggs of *M. gelechiae* and *P. perpusilla*. Like the

shell of eggs of *R. prolixus* (Beament, 1946*a*, 1948), ticks *Ixodes ricini* L. and *Ornithodorus moubata* Murray (Lees and Beament, 1948), and *P. brassicae*, which is not water-proof, the chorion of all these insects is freely permeable to quite big molecules of hydrophilic and lipophilic stains and chemicals in solutions. The non-water-proof chorion of *T. evanescens minutum* and *T. pyrillae* differs from that of all the other insects in being very thin, consisting only of non-resistant protein, and is devoid of respiratory pores.

Like eggs of *P. brassicae* there is a lipoid layer on the inner side of chorion of eggs of these insects, but it is absent from those of *T. evanescens minutum* and *T. pyrillae*. It has also been demonstrated in eggs of *Melanoplus differentialis* Thom. by Slifer (1937, 1946), *R. prolixus* by Beament (1946*b*), *Locustana pardalina* Walk. by Matthee (1951), whereas it has escaped the notice of Roonwal (1954) and Sander (1956) in eggs of *Schistocerca gregaria* Forsk. and *P. perpusilla* respectively. In *Psylla mali* Schm. (Beament, unpublished work), *P. brassicae* and the insects studied now, the lipoid layer is formed of freely mobile fatty material and not a hard wax as has been earlier noticed in eggs of a mite *M. ulmi* (Beament, 1951), *R. prolixus* and *M. differentialis*. The present investigation has shown that some of its unsaturated-lipoid is converted into saturated-lipoid by the developing embryo. This phenomenon has been observed now even in the eggs of *P. brassicae* which earlier escaped detection. Among the insects having lipoid layer the transition temperature of eggs of *M. gelechiae* lies between 50°C and 60°C, whereas that of the other insects like *P. brassicae* varies from 60°C to 70°C.

The resistance of embryonic membranes of the insects under study to the action of corrosive chemicals is lower in the early and late development, whereas it is maximum in mid-development. The latter is, however, lower than its counter part in eggs of *R. prolixus* (Beament, 1949). The only difference which the epembryonic membrane has from that of *P. brassicae* is the incorporation of a little unsaturated-lipoid in it with age of the eggs, which serves to some extent as a secondary waterproofing mechanism. A re-examination of this membrane of *P. brassicae* now has indicated that as development advances it also incorporates a little quantity of unsaturated-lipoid which escaped detection earlier. Moreover Beament (1949) and Matthee (1951) have also reported the formation of a secondary waterproofing mechanism in developing eggs of *R. prolixus* and *L. pardalina* respectively. In both these cases the secondary wax material is secreted by serosal cells, impregnating the secondary egg-membrane in the former egg, and giving a continuous film between the yellow and white cuticles in the latter egg. The epembryonic membrane of eggs of *T. evanescens minutum* and *T. pyrillae* is free from lipoid. Further in eggs of the parasitic and the host insects it is ultimately reabsorbed by the developing larva sometimes before hatching.

It may be inferred from the investigation that all layers of the endoparasitic eggs and only the cement and chorion of eggs of the other insects do not obstruct the penetration of hydrophilic or lipophilic chemicals. The lipoid layer of eggs of the other insects gives the main resistance to the entry of water-soluble materials alone, and the lipophilic substances cross this barrier quite easily. In this respect the results corroborate the earlier findings on eggs of *P. brassicae*. The appearance of oily traces in the epembryonic membrane in mid-development as well as the conversion of some unsaturated-oil of the lipoid layer to saturated-state at this time may be correlated with comparatively increased resistance to desiccation and penetration of water-soluble poisons into the eggs. Such conditions are not expected to obstruct the penetration of lipophilic substances, but some resistance offered to them and to a fumigant like hydrogen cyanide by the eggs at this stage cannot be explained clearly at present. However, the suggestion of Salkeld and Potter (1953), indicating that this resistance may be due to the fully developed state of epembryonic membrane, seems quite reasonable.

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