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Miscellanea.

The Prerequisites for the Formation of a Peritrophic Membrane in Culicidae Females.

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Introduction.

In agreement with other authors (CLEMENTS, STOHLER) we postulated in a previous paper (5) that the peritrophic membrane (P.M.) in female mosquitoes is produced, at least in part, from the material secreted by the epithelial cells of the stomach immediately after a blood meal. We also expressed the opinion that this secretion is provoked by a mechanical factor—i.e. by distension of the midgut epithelium—associated with the ingestion of food.

To test the correctness of these two postulates, we undertook two types of experiment. In the first, we "fed" mosquitoes with water or air instead of blood in order to establish the effect of purely mechanical distension of the midgut epithelium and also to assess the importance of the role played by the chemical properties of the ingested "food" in the formation, if any, of a P.M. In the second series of experiments, we allowed the mosquitoes to ingest varying quantities of blood, so as to find out whether the extent to which the midgut epithelium is distended has any effect on the formation of a P.M.

In the course of the investigations referred to above (5), it had been found that *Aedes aegypti* and *Anopheles gambiae* form a P.M. following the ingestion of blood serum. Similar experiments with *Anopheles stephensi* had failed to yield clear-cut results. We have since repeated these experiments and shall likewise report briefly on them in the present paper.

Material and methods.

The experiments were conducted chiefly with *Aedes aegypti* and *Anopheles stephensi*. We also worked with *Anopheles gambiae* in a few cases. All the mosquitoes belonged to strains which we had often employed before. Detailed information on the methods used for breeding and keeping these mosquitoes will be found in our previous paper (5).

The *dissection of the P.M.* in freshly killed mosquitoes and its evaluation in the light microscope and phase-contrast microscope were likewise described in that paper (5).

Weighing. All the female mosquitoes were handled separately. We first blew them into an aluminium foil bag the size of a finger and weighed them in the fasting state, without anaesthesia, on a Mettler Balance Mod. M5 SA (reproducible degree of accuracy: 2 μg). The mosquitoes were then allowed to ingest blood from a guinea-pig, the duration of the meal being adapted to the requirements of each particular experiment. After the meal the mosquitoes were weighed again and the amount of blood ingested and retained was calculated from the difference between the two weighings (cf. p. 150).

"Feeding" with water or air. We first offered hungry female mosquitoes water of body temperature placed under the skin of a guinea-pig—a procedure which has already been employed by a number of authors (1, 9). Despite the occasional addition of 0.1% alanine which—admittedly, in combination with other substances—has been described as one of the attractants in blood

(SCHAERFENBERG, 8; SCHUSCHUKOW), the mosquitoes usually refused to accept this water. We therefore changed over to compulsory "feeding", i.e. to pumping water or physiological saline into the midgut through the rectum ("enema method"). For this purpose, we briefly anaesthetised each mosquito separately with ether or carbon dioxide, introduced a micropipette into the rectum, and injected the liquid into the stomach with a micrometer syringe (Mod. Agla, Burroughs Wellcome & Co.). To make it easier to follow the progress of the fluid, we also coloured the latter with methylene blue. The quantity injected was sufficient to distend the gut to the same extent as after a normal blood meal. "Feeding" with air was carried out in basically the same way. Instead of using a micrometer syringe, we blew the air into the gut through the micropipette.

Compulsory feeding of mosquitoes has already been applied by earlier authors (6, 7), who made use of the sucking reflex which frequently occurs following a minor injury to the mosquito's proboscis—removal of the tip of the proboscis or detachment of the labium. The food offered in this way thus follows the normal route and may therefore also be mixed with saliva or with secretion from the anterior portion of the midgut before entering the stomach. No such mixing occurs with the enema method, and we considered this to be particularly advantageous in experiments designed to establish what influence the chemical composition of the intestinal contents may have on the formation of the P.M. As regards "feeding" with air, this method is probably in any case the only one that can be employed.

Feeding with blood serum. The serum from guinea-pig blood is too viscous to be administered through a micropipette via the rectum, and in this instance therefore we adopted the method of KADLETZ & KUSMINA (6). However, instead of inserting the cut proboscis into a thin glass tube, we simply laid it in a drop of serum. The mosquitoes were anaesthetised to begin with; to prevent them flying away subsequently, we placed a cover slip over them.

Van Wisselingh test. Van Wisselingh's chitosan-iodine test for the detection of chitin was performed in the manner described by WATERHOUSE & WIGGLESWORTH. In order to find the very fine P.M. more easily following heating with KOH solution, we placed 5 midguts in 0.3 ml. KOH solution at a time in an ignition tube, and sealed the tube by melting. The tubes were then heated to approx. 155°C in an oil bath for one hour.

Results.

P.M. formation as a function of the quantity of blood ingested.

Aedes aegypti. In this species of mosquito, as we know (5), the P.M. persists from the 8th to the 48th hour following ingestion of a complete blood meal. Following an incomplete blood meal—as BOISSEZON (2) demonstrated in the case of *Culex pipiens*—the entire process of digestion is more rapid than after a complete meal. Consequently, we may expect to find the P.M., provided one has been formed, if we submit the insects to autopsy some time between 8 hours after the blood meal and shortly before the end of digestion. Even where the mosquitoes are fed extremely small quantities of blood, digestion, with very few exceptions, lasts more than 16 hours. We therefore dissected the insects between 14 and 16 hours after ingestion of blood meal.

The results are summarised in *Table 1.* As regards the terms used to qualify the P.M., we would point out that we consider a P.M. to be present only if we can dissect it at autopsy and demonstrate it in the phase-contrast microscope.

TABLE 1.

Aedes aegypti: P.M. formation as a function of the quantity of blood ingested.

Amount of blood in mg.	No. of mosquitoes	P. M.			
		Complete	Incomplete	Incoherent	Absent
0.01–0.09	3	–	–	–	3
0.1 –0.19	4	–	–	3	1
0.2 –0.29	8	–	1	5	2
0.3 –0.39	11	–	3	6	2
0.4 –0.49	7	–	5	2	–
0.5 –0.59	13	1	8	4	–
0.6 –0.69	5	–	4	–	1
0.7 –0.79	10	4	3	3	–
0.8 –0.89	8	1	4	3	–
0.9 –0.99	10	6	2	2	–
1.0 –1.09	12	9	3	–	–
1.1 –1.19	4	3	1	–	–
1.2 –4.0	13	13	–	–	–
Total	108	37	34	28	9

The P.M. is described as complete if it surrounds the blood coagulum on all sides, and as incomplete if one end—usually the rostral end—is missing; it is regarded as incoherent if only loose shreds can be found on the surface of the blood coagulum; if no trace of a P.M. can be discovered, the P.M. is considered to be absent.

It should also be stated that the average weight (100 weighings) of an *Aedes aegypti* of our strain is 2.4 mg (minimum: 1.6 mg; maximum 3.4 mg), and that a complete blood meal weighs on the average about 3.2 mg. This figure relates to the amount of blood which is retained in the midgut; that the mosquito may in fact ingest more blood, but excretes some of it again immediately following the meal (3) is a possibility that need not concern us here.

The measurements show that it takes a minimum quantity of 0.5 mg blood for a complete P.M. to form and that no P.M. at all develops following an intake of less than 0.1 mg. Although the figures in the four columns of Table 1 overlap, their distribution nevertheless clearly suggests that, in the range 0-1.2 mg, the more blood the mosquito ingests, the more complete is the P.M. If the amount of blood ingested exceeds 1.2 mg, the P.M. usually develops quite "normally".

Anopheles stephensi. Unlike the P.M. of *Aedes aegypti,* that of *Anopheles stephensi* remains visible until the last remnants of blood have disappeared from the midgut; on the other hand, it cannot be demonstrated until at least 32 hours have elapsed after a complete blood meal (5). Following an incomplete blood meal digestion often takes no more than 24 hours. This comparatively short time might be insufficient for a P.M. to form, and, it would thus be pointless to look for the membrane too early. We therefore dissected our mosquitoes 24 hours after the ingestion of quantities ranging from 0.01 to 0.39 mg blood, and 30 hours after ingestion of amounts exceeding 0.4 mg.

In the fasting state, an *Anopheles stephensi* female weighs on the average (64 weighings) 1.5 mg (minimum: 0.7 mg; maximum: 2.2 mg); it ingests about

TABLE 2.

Anopheles stephensi: P.M. formation as a function of the quantity of blood ingested.

(The figures in italics refer to Anopheles gambiae.)

Amount of blood in mg.	No. of mosquitoes	P. M.			
		Complete	Incomplete	Incoherent	Absent
0.01–0.09	7	5	2	–	–
0.1 –0.19	19 *4*	8 *1*	9 *3*	1 –	1 –
0.2 –0.29	12 *3*	5 *3*	4 –	3 –	– –
0.3 –0.39	12 *3*	5 *3*	6 –	1 –	– –
0.4 –0.49	2	–	1	1	–
0.5 –0.59	9	5	2	2	–
0.6 –0.69	3	2	–	1	–
0.7 –0.79	5	4	1	–	–
0.8 –0.89	3	2	1	–	–
0.9 –0.99	3	3	–	–	–
1.0 –1.09	3	2	1	–	–
Total	78	41	27	9	1

1.5 mg blood in a single normal meal. The results of our experiments (see *Table 2)* show that at least an incomplete P.M. is formed following ingestion of even the smallest quantities of blood. Even under normal conditions it is sometimes impossible to demonstrate a P.M. in as many as 20% of the mosquitoes (5); the fact that 10 of the 78 Anopheles mosquitoes studied in this experiment displayed either an incoherent P.M. or none at all can therefore be disregarded. Hence, in *Anopheles stephensi,* in contrast to *Aedes aegypti,* no correlation could be observed between the quantity of blood ingested and the degree to which a P.M. was formed.

Anopheles gambiae. As regards this species of mosquito, only very few results are available (see *Table 2*). As far as can be judged, however, they tally with those obtained with *Anopheles stephensi.*

P.M. formation following a water "feed".

"Oral feeding." Although oral feeding was tried many times with various species of mosquito, it proved successful only in 10 *Anopheles stephensi.* Significantly enough, these successes coincided almost invariably with the approach of a thunderstorm (cf. 4, p. 244). It was confirmed that pure water passes directly into the midgut and that the diverticula remain empty (cf. CHRISTOPHERS). One mosquito was sacrificed immediately after the water "meal", whereas the other 9 were examined after 24 hours. In 3 of the mosquitoes the gut was already empty—they had probably ingested only a little water. In the 6 others, some of the water was still visible in the gut. A P.M. was found in these 6 mosquitoes; although it was thin, it could none the less be dissected out as a coherent membrane and demonstrated in the phase-contrast microscope.

Enema method. It was found that if pure water was used, the mosquitoes were severely damaged and died of cytolysis of the epithelial cells of the gut within 5 hours after the rectal injection. When physiological saline was

employed, however, the mosquitoes made a good recovery from the anaesthesia, remained alive, and behaved normally. As after "oral feeding", the water in the gut appeared upon dissection to be mixed with a granulate. We treated altogether 25 *Aedes aegypti* females in this way. In 23 we found a P.M. round a remnant of water after 2-6 hours. The membrane was thin, but coherent and visible in the phase-contrast microscope. In 4 cases the membrane was placed in an aqueous solution, in which it remained intact for more than 30 hours.

We also carried out the same experiment with 15 *Anopheles stephensi* females. All of them displayed a P.M. 15-20 hours following the enema. On the basis of the criteria employed, it was impossible to distinguish these P.M.s from those observed following "oral feeding". When placed in an aqueous solution, the membranes dissolved within 20-30 minutes.

P.M. formation following "feeding" with air.

11 *Aedes aegypti* females were used in this experiment. Two of the mosquitoes were dissected for control purposes within 2 hours after "feeding"; examination showed that the insufflated air was in the gut. 16 hours after insufflation only 3 mosquitoes were still alive; two others had only just died. A P.M. was found in the gut of each of these 5 mosquitoes. It did not surround the air, but was folded lengthwise, like a cloth lifted out of a basin of water with one hand. In the phase-contrast microscope the P.M. presented a normal appearance. It remained insoluble in an aqueous solution.

The same experiment was performed on 13 *Anopheles stephensi* mosquitoes. 5 of them were dissected after 7 hours. In 3 the air had passed into the body cavity, whereas in the other 2 it was in the gut; both these two displayed a P.M. After 21 hours only 2 of the remaining mosquitoes were still alive, but both of them had likewise developed a P.M. The 4 P.M.s found hardly differed at all in appearance from those observed in *Aedes aegypti,* but in an aqueous medium they dissolved within 20-30 minutes.

P.M. formation in Anopheles stephensi following feeding with blood serum.

In four experiments blood serum was ingested by a total of 45 females. After 27-32 hours, the time at which we had intended to dissect the mosquitoes, only 7 were still alive. The method of compulsory feeding employed appears therefore to cause considerable damage to a large proportion of the insects. On the other hand, a complete P.M. was observed in all 7 surviving mosquitoes. In the phase-contrast microscope this P.M. presented the usual picture. It dissolved within 20-30 minutes in physiological saline. It was thicker than after water "feeding", but thinner than after the ingestion of whole blood.

Results of the Van Wisselingh test.

Since authors such as CLEMENTS, WATERHOUSE, and WIGGLESWORTH consider it essential to demonstrate the presence of chitin in a P.M., we carried out Van Wisselingh's chitosan-iodine test on the membranes we found. In the case of *Aedes aegypti,* we tested the P.M. 16 hours after a blood, physiological saline, or air "meal", while in *Anopheles stephensi* we tested it 48 hours after a blood meal, 36 hours after a serum feed, and 16 hours after the injection of physiological saline or the insufflation of air. For control purposes, the guts of unfed mosquitoes of both species were also examined.

In both species of mosquito the test yielded clear-cut results following a

blood or serum "meal": after heating with KOH solution the P.M. was invariably still clearly recognisable as such; it turned a violet-red colour following the addition of iodine; the addition of acetic acid caused this stain to disappear immediately. The results were ill-defined or negative following "feeding" with physiological saline: in *Aedes aegypti,* a coarse material was left, which assumed an orange colour that could not be removed with acetic acid; in *Anopheles stephensi,* striae still adhered to the wall of the ignition tube after heating; these striae stained a violet colour which persisted despite attempts to remove it. Following "feeding" with air, on the other hand, fragments of a membrane were found which showed the typical staining reaction—i.e. the violet stain induced by the addition of iodine disappeared in response to acetic acid. The control tests yielded negative results.

Discussion and conclusions.

As we have seen, a P.M. is formed both after "feeding" with air and after "feeding" with water or physiological saline, irrespective of whether the liquid is absorbed through the proboscis or administered via the rectum. Hence, it is not necessary for the formation of a P.M. that the "meal" should be mixed with saliva or with the secretion from the anterior portion of the midgut. "Food" that enters in the normal way through the proboscis is in fact mixed with secretions; if this were not so, it would be impossible to understand why the mosquitoes survive the "oral" absorption of pure water, but die of cytolysis of the epithelial cells of the gut following rectal injection of pure water.

After "feeding" with air or water the P.M. is thinner than after a blood meal. The membranes, however, do not differ from one another in respect of their other properties. They can be dissected in a coherent form from freshly killed mosquitoes, and they present the same picture in the phase-contrast microscope. The P.M. of *Aedes aegypti* is invariably insoluble in an aqueous medium, whereas that of *Anopheles stephensi* is always soluble. (In this connection, the question arises as to how a P.M. can develop at all in *Anopheles stephensi* following a water or saline "meal". The answer is probably that this is due to the effect of certain stomach secretions which prevent the P.M. from being dissolved at the same time as it is being formed. We did indeed notice in our preparations that the water in the gut was no longer pure, but was mixed with a granular material.) Finally, the Van Wisselingh test yielded positive results even after "feeding" with air—a finding which suggests that the P.M. contains the same chemical substances as after a normal blood meal. The ill-defined results obtained with this test following "feeding" with saline are due in our opinion to the difficulties of working with such small quantities. We should like to point out here that the Van Wisselingh test yields positive results even in Anopheles mosquitoes (cf. also 5), whose P.M. is water-soluble. Chitin, however, is said to be insoluble in water. We therefore wonder whether the Van Wisselingh test is really a specific test for chitin.

As regards the questions posed at the beginning of this paper, we can now come to the following conclusions:

The secretion of the epithelial cells of the stomach is induced solely by distension of the cells. In the case of *Aedes aegypti,* when the amount of food involved is very small, there is a correlation between the quantity absorbed and the extent of the secretion. In *Anopheles stephensi,* on the other hand, no such correlation appears to exist, and secretion obeys the "all or nothing" principle. This difference may possibly be due to the cytological structure of the epithelial cells and to their secretion mechanism (STAEUBLI et al.).

The P.M. develops independently of the chemical composition of the food and is formed in the main from the secretions of the epithelial cells. The physical condition of the gut contents is immaterial in this respect; these contents can be gaseous (air), liquid (water), or solid (coagulated or agglutinated blood). This answers the question asked by CLEMENTS.

In the presence of air or water the P.M. appears thinner than when it surrounds blood or serum. The explanation for this finding may be that no proteins which might become superimposed on the membrane are released during the "digestion" of air or water. Consequently, the only contribution the food makes to the P.M. would consist in adding degradation products, especially proteins, to it.

Following an incomplete blood meal, and following the absorption of serum or water, "digestion" proceeds more rapidly than after a complete blood meal. Although the secretion of the epithelial cells in the gut is normal, it is conceivable that the time available is not sufficient for this material to harden into a P.M. From the experiments described, however, we know that the P.M. can also be formed in a shorter time than normal. Its formation is thus connected chronologically with the course of the digestive process.

The experiments in which *Anopheles stephensi* females were fed with blood serum were a repetition of previous experiments that had failed to yield clear-cut results. The findings obtained in this second series show that in *Anopheles stephensi,* too, a P.M. is formed following the ingestion of serum—a result which was to be expected in view of what has been said above.

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(This list includes only those references which were not mentioned in our previous paper [5]).

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