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The Formation of the Peritrophic Membrane in Culicidae.

By THIERRY A. FREYVOGEL and WILLY STÄUBLI

Contents.

1. Introduction.

Thanks to improved methods of investigation it has been possible to show that some insects, contrary to previous assumptions, do in fact display a peritrophic membrane (P.M.). This is true, in particular, of the females of various types of mosquito, in which the P.M. is formed periodically as a function of food intake (2, 3, 10, 11, 14, 15). It is therefore necessary to re-examine PAL's (6) query regarding the significance of the midgut structure for the transmission of plasmodia and, especially, to consider the role played by the P.M. in this transmission. STOHLER (10) demonstrated that the P.M. of *Aedes aegypti* hardens to such an extent 30 hours after the blood meal that the ookinetes of *Plasmodium gallinaceum* can no longer pass through it. It is conceivable that a similar situation obtains in the case of the plasmodia transmitted by Anopheles and that this might perhaps explain in part why the various types of Anopheles differ in their ability to transmit malaria. Questions of this nature will be examined in further studies. The present investigation was undertaken principally to establish whether the P.M. is formed in the same way and at the same time in various types of mosquito. It was also desired to expand PAL's histological findings with regard to the midgut epithelium. We have dealt with the cytological aspects of the subject in a paper which, though published separately (9), is none the less closely connected with the present work.

2. Material and methods.

The mosquitoes investigated were *Anopheles gambiae, An.*[1] *stephensi, An. maculipennis atroparvus,* and *Aedes aegypti.* All the specimens used were bred in the Swiss Tropical Institute; the strains of *An. gambiae* and *An. maculipennis atroparvus* were obtained originally from the London School of Hygiene and Tropical Medicine, while that of *An. stephensi* came from the Tropical Institute, Hamburg, and that of *Ae. aegypti* from the Congo. The mosquitoes were bred as described by GEIGY & HERBIG (5).

The female mosquitoes were allowed to feed as a rule on guinea-pigs, and also, in some cases, on chicken, Rhesus monkeys, or man. At various intervals after the blood meal they were briefly anaesthetised with ether and dissected in physiological saline or Ringer's solution. The P.M. was dissected free under a magnifying glass with the aid of watchmaker's tweezers and fine glass needles. In doubtful cases, the P.M. was examined in the phase-contrast microscope in order to distinguish it from epithelium or any inclusion bodies in the gut. Either the midgut alone or the entire abdomen of the mosquitoes was used for the histological preparations. The specimens were fixed initially in Carnoy's fluid, and later in Dubosq's fluid. Paraffin was employed for embedding purposes. The width of the slices was 7 μ. Azan was used as a general stain. The drawings were prepared with a Wild drawing tube at a magnification of 1,250 $\times$. The photographs show portions of the drawings. Both the photographs and the drawings have the same scale.

3. Preliminary experiments.

3. 1. We were unable to work throughout with mosquitoes of similar age. We therefore had to establish at the outset whether mosquitoes form a P.M. in the same way irrespective of age or of the number of blood meals previously ingested. Table 1 summarizes the results of the experiments conducted with *Ae. aegypti* and *An. stephensi.* For each experiment approx. 100 freshly hatched females, together with males, were removed from the breeding cages and allowed to feed on guinea-pigs at one-week intervals. After every blood meal 5-10 females were examined to see whether a P.M. was present.

These results show that neither the age of the mosquitoes nor the number of blood meals they have previously ingested has any effect on P.M. formation. It is also apparent that in both the types of mosquito studied approximately one-fifth of the females invariably form an incomplete P.M. In the 2 cases in which, by way of exception, no P.M. developed, the entire digestive process appeared to be disturbed, the blood in the gut being virtually intact.

3. 2. It also had to be established whether the formation of a P.M. is dependent on the species of the blood donor. For this

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[1] To avoid confusion, the abbreviation "An." is used to denote Anopheles, and "Ae." to denote Aedes.

TABLE 1.

P.M. formation as a function of the age of the mosquitoes and the number of previous blood meals.

	Blood meal	Date	State of P. M. (17–18 hours after the blood meal)		
			Complete	Incomplete	Absent
Ae. aegypti	1	13. 2.	7	3	
	2	19. 2.	7	3	
	3	26. 2.	5	5	
	4	3. 3.	7	3	
	5	11. 3.	9	1	
	6	17. 3.	8	2	
	7	23. 3.	9	1	
	8	1. 4.	8	2	
	9	8. 4.	7	3	
			(45–48 hours after the blood meal)		
An. stephensi	1	24. 2.	14	5	1
	2	3. 3.	4	3	
	3	10. 3.	3	1	
	4	18. 3.	4	1	
	5	23. 3.	3	1	1
	6	1. 4.	4	1	

purpose, specimens of *Ae. aegypti* and *An. stephensi*—from the same brood in each case—were allowed to feed on man, rabbits, guinea-pigs, and chicken. The mosquitoes were dissected and their P.M. examined 15-16 hours after the blood meal in the case of *Ae. aegypti* and 48 hours after in that of *An. stephensi*. The results are listed in Table 2. No essential differences were found; the formation of the P.M. thus appears to bear no relation to the species of the blood donor.

4. Findings based on dissections of fresh material.

4. 1. In view of the possible importance of the P.M. for the passage of ookinetes, it is of particular interest to know whether it is formed in the same way in all types of mosquito, and at what time it forms and hardens. Examination of freshly killed female mosquitoes, performed at various intervals after the blood meal, showed that, as in the case of *Ae. aegypti,* a P.M. is produced during the digestive process in both *An. gambiae* (Fig. 1) and *An. stephensi* (Fig. 2). No P.M., however, was ever demonstrated in *An. maculipennis atroparvus* (Fig. 3). In this species, the most

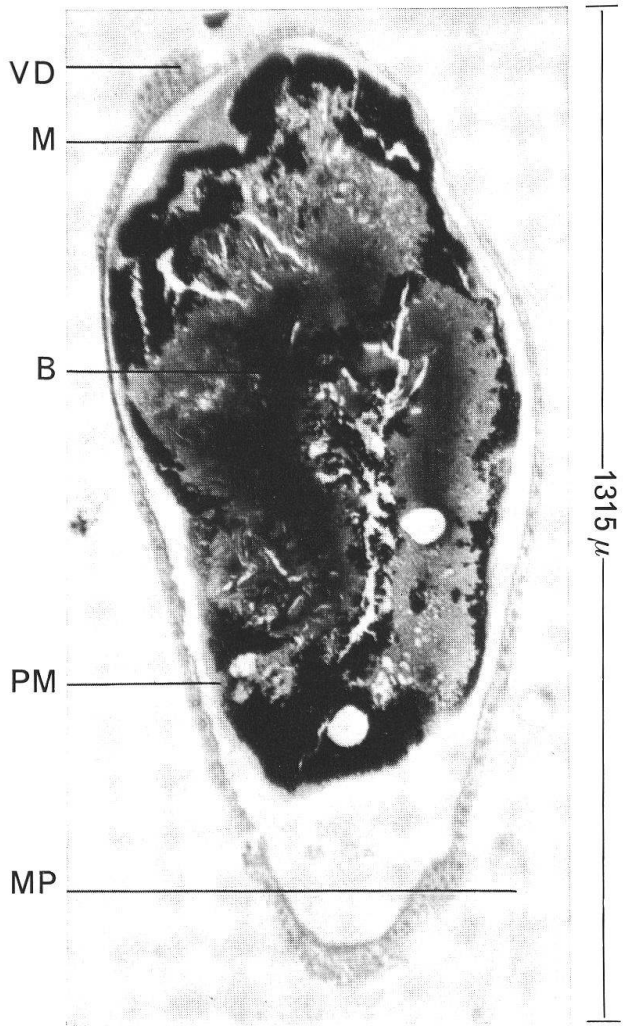

Fig. 1.

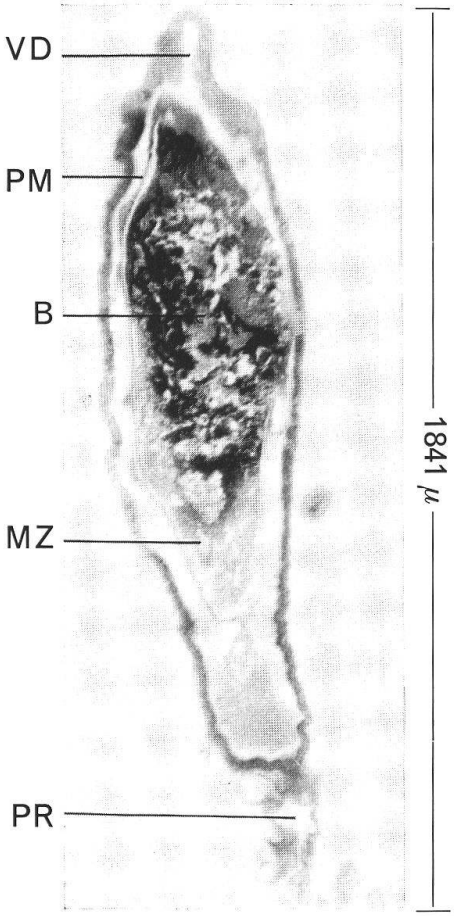

Fig. 2.

Figs. 1-3. Longitudinal sections through filled mosquito midguts.

Fig. 1. Anopheles gambiae: 30½ hours after the blood meal.

Fig. 2. Anopheles stephensi : 49 hours after the blood meal.

Key: B = blood; M = mucus-like material from the anterior portion of the midgut; MP = malpighian vessels; MZ = mixed zones of digestive secretion and erythrocytes in the process of being broken down; PM = peritrophic membrane; PR = pyloric region; VD = anterior portion of the midgut.

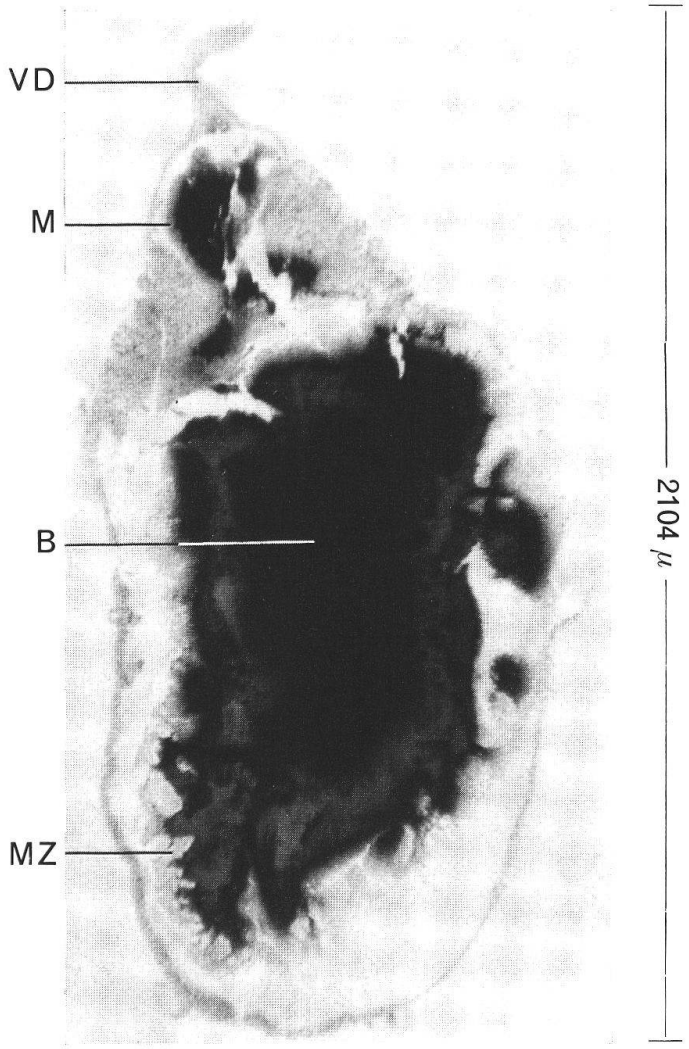

Fig. 3. Anopheles maculipennis atroparvus: 30 hours after the blood meal.

that could be seen in the peritrophic space under the phase-contrast microscope was striae of a viscous material, such as were occasionally observed in other types of mosquito prior to the development of a coherent membrane. In *An. maculipennis atroparvus,* in contrast to *An. stephensi* (see p. 119), digestion of the blood meal proceeded normally despite the absence of a P.M.

4. 2. As regards the time at which the P.M. develops, only an approximate estimation can be given on the basis of the post-mortem examinations. This time is species-specific, may possibly vary a little between mosquitoes of the same species, and perhaps depends to some extent on the amount of blood ingested. As described by STOHLER (10), the P.M. is already apparent as a rule between 5 and 8 hours after the blood meal in the case of

TABLE 2.

P.M. formation as a function of the species of the blood donor.

Blood donor	*Aedes aegypti*		*Anopheles stephensi*	
	No. of mosquitoes	State of P. M.	No. of mosquitoes	State of P. M.
Man	6	Complete in 4 Incomplete in 2	6	Complete in 3 Incomplete in 2 Absent in 1
Rabbit	5	Complete in 2 Incomplete in 3	5	Complete in 5
Guinea-pig	5	Complete in 3 Incomplete in 2	5	Complete in 3 Incomplete in 1 Absent in 1
Chicken	5	Complete in 4 Incomplete in 1	5	Complete in 3 Incomplete in 2

Ae. aegypti. It then persists until digestion is practically completed some 48 hours after the blood meal. In aberrant cases, however, the formation and degradation of the P.M. may proceed much more quickly, the membrane may appear only 2 hours after the blood meal and disappear 35 hours or so after—i.e. long before digestion is completed. This observation was invariably made in groups of Aedes females which had been allowed to feed together on the same host animal. The assumption that the host's blood is responsible for this aberrant behaviour appears unlikely for two reasons: firstly, because—as we know from the preliminary experiments we conducted—the exact composition of the blood does not seem to exert any great influence on the P.M., and secondly, because the Anopheles allowed to feed on the same host simultaneously developed a P.M. after the usual period of time. So far no explanation for this aberrant P.M. formation has been found. In *An. gambiae,* the P.M. can be demonstrated at the earliest after 13 hours; in some mosquitoes it still persists in the form of an extremely delicate structure after 60 hours, i.e. at a time when the gut no longer contains any blood. In *An. stephensi* a coherent P.M. cannot be demonstrated until 32 hours after the blood meal, and in some mosquitoes of this type it still persists 72 hours after — i.e. at a time when only very slight remnants of blood are present in the gut.

4. 3. The degree of solidification which the P.M. undergoes is more difficult to define. It is probably best to follow STOHLER's

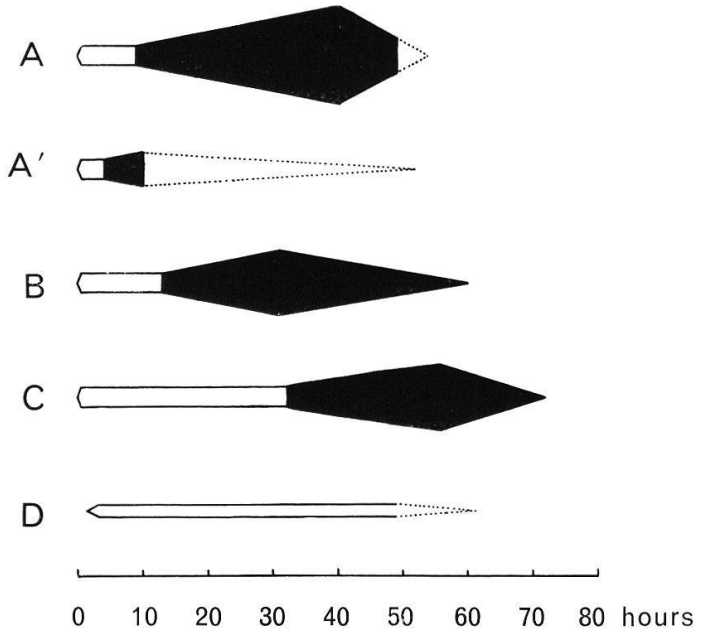

Fig. 4. Appearance of the P.M. in terms of time. A: *Aëdes aegypti*, A': *Aëdes aegypti*, aberrant type, B: *Anopheles gambiae*, C: *Anopheles stephensi*, D: *Anopheles maculipennis atroparvus*. For explanations see text.

(10) suggestion and employ the terms viscous, elastic, solid, and fragile This sequence reflects the various stages in the development of the P.M. and is applicable to all those species of mosquito investigated which form a P.M. The P.M. is strongest in *Ae. aegypti,* while in *An. gambiae* and *An. stephensi* it never develops beyond the stage of a delicate membrane which is difficult to handle. The diagram in Fig. 4 is designed to show the chronological development of the P.M. and the degree of solidification attained at various intervals after the blood meal. As confirmed by histological examination and electron-microscope studies, the material which is believed to give rise subsequently, in whole or in part, to the peritrophic membrane, begins to be secreted immediately after the blood meal in *Ae. aegypti, An. gambiae,* and *An. stephensi.* In *An. maculipennis atroparvus,* as will be explained below, the secretion of the midgut cells is delayed and is less marked. Secretion is followed by a liquid, viscous phase, which appears as a white area when no coherent membrane can yet be distinguished. The coherent P.M. phase is shown in black, the width of the strip indicating approximately the thickness of the membrane. Finally, the dotted area represents the last phase in the digestion of the blood meal; in the case of *Ae. aegypti,* blood is still present in the gut during this phase, but only fragments of P.M. are visible. In *An. gambiae* and *An. stephensi,* the P.M. may even persist for a little longer than the blood.

5. Changes in the midgut cells following the blood meal.

Numerous histological preparations were made at various intervals after the blood meal. We shall first describe single cells from the central portion of the posterior, stomach-like part of the midgut.

5. 1. Aedes aegypti.

5. 1. 1. *Unfed mosquitoes.* By "unfed mosquitoes" we mean female mosquitoes which have not ingested a blood meal for at least 3 days.

Fig. 5a and b. In the typical case, the midgut epithelium consists of high columnar cells, measuring up to 50 μ in height and approx. 10 μ in width. These cells rest on a clearly apparent base-

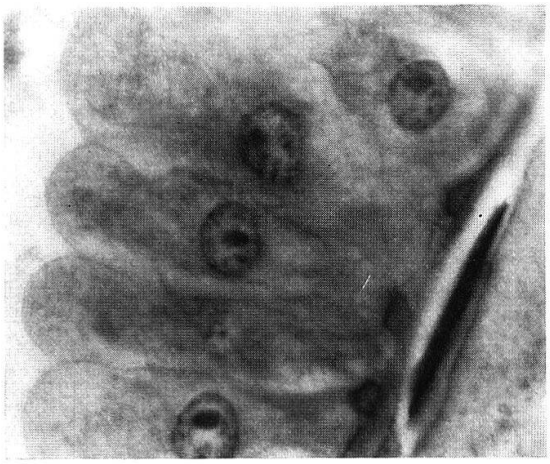

Fig. 5 a.

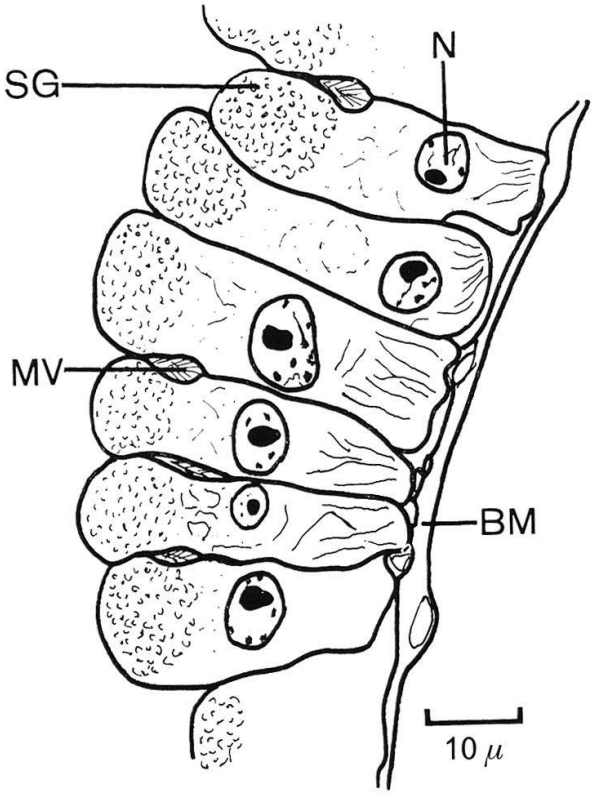

Fig. 5 b.

Fig. 5 a and b. Aëdes aegypti, unfed mosquito.

Figs. 5-14. Sections from the middle portion of the "stomach".

Key: B = blood, some of it in the process of being broken down; BM = basement membrane; BS = "blue border", secretion material from the midgut cells; M = mucus-like material from the anterior portion of the midgut; MF = muscle fibres; MV = microvilli, brush border; N = nucleus; PM = peritrophic membrane; SG = secretion granules; V = vacuoles.

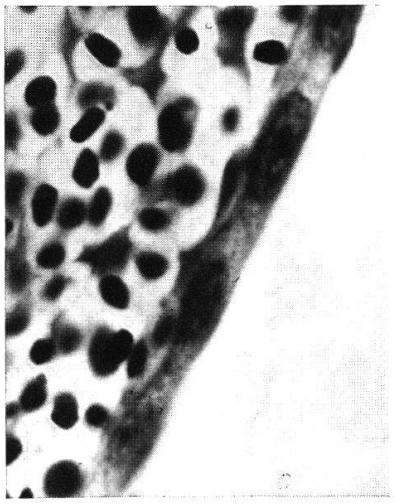

Fig. 6 a.

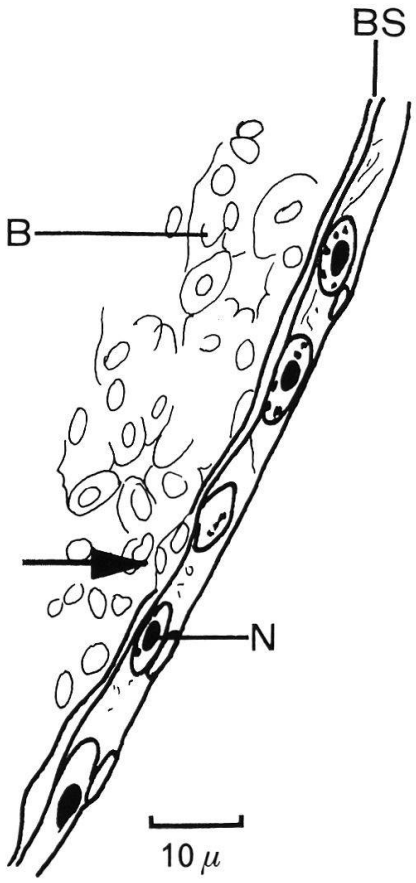

Fig. 6 b.

Fig. 6 a and b. Aëdes aegypti, 50 minutes after a blood meal, chicken.

ment membrane, which is covered on its outside by a network of muscle fibres. The nucleus is usually situated in the centre of each cell and has a diameter of 7-8 μ. The nucleolus is strikingly large, measuring 3 μ in diameter. Irregular clumps of chromatin are visible at the margin of the nucleus. The protoplasm basal to the nucleus has a "thread-like" structure, the "threads" running vertically to the basement membrane. Apical to the nucleus, the protoplasm does not reveal any clear structure. The pole of the cell at the end facing the lumen of the gut has a swollen, cobble-stone-like appearance. It contains comparatively coarse granules which stain orange with azan. The cell is constricted beneath the apex. The brush border is wedged into the resultant intermediate spaces; at the pole itself no brush border can be seen. In the light of the findings reported by other authors (1, 7, 9) we consider the swelling of the cell apices and the distribution of the brush border as an artefact. No P.M. is present.

5. 1. 2. *Freshly fed mosquitoes:* Fig. 6a and b. The two illustrations show the extent to which the midgut epithelium has been

stretched. At places, the cells measure only 3 μ in height. Their width is impossible to determine with accuracy because their borders cannot be seen. At this stage the basement membrane cannot be distinguished from the epithelium. The nuclei are considerably flattened. Similarly, the nucleoli appear ovoid and measure $2 \times 1\frac{1}{2}$ μ. No definite structure can be detected in the protoplasm. Nothing can be seen of the brush border. Above the epithelium, between it and the erythrocytes, there is a thin border which stains blue with azan. As the orange granules encountered in the unfed mosquito have disappeared it may be assumed that the blue border is the product of these granules; it would then also be a precursor of the P.M. As can be shown with the electron-microscope, at this stage, the blue border mingles with the brush border and, upon dissection, does not yet display any degree of solidity. It is interesting to note that the blue border may be interrupted and contain erythrocytic elements, as is shown at one point in Fig. 6b (arrow).

5. 1. 3. *Mosquito after 30½ hours:* Fig. 7a and b. The wall of the gut consists of pavement epithelium. The cells have become raised, and the nuclei are rounded again. The basement membrane can be differentiated from the epithelial cells. The brush border, approximately 3 μ thick, is clearly apparent. It stains a light blue colour, the colour becoming denser towards the lumen of the gut. In parts, the brush border is covered with a thin layer, consisting presumably of remnants of secretion material or blood degradation products (BS). Towards the inside of the gut, this layer is followed first of all by a mixed zone of secretion and partially digested erythrocytes (B). Very frequently, intermediate spaces, which we consider to be artefacts, are found between this zone and the brush border. Further towards the inside of the gut the mixed zone is followed by areas of progressive erythrocytic degradation. The inside of the gut still contains at this time a remnant of coagulated, but otherwise apparently intact red blood corpuscles. Dissection of the gut at this stage reveals as a rule a coherent P.M., which, however, very often, cannot be demonstrated in histological preparations. In the histological specimen illustrated, for instance, nothing corresponding to such a membrane can be observed.

Fig. 8 a and b show a section from a preparation obtained from another mosquito, likewise 30½ hours after the blood meal. Judging by the height of the epithelial cells, this gut was in a physiologically more advanced state. A striking feature is the vacuole

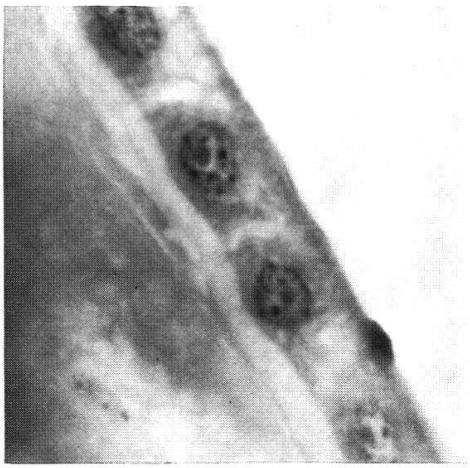

Fig. 7 a.

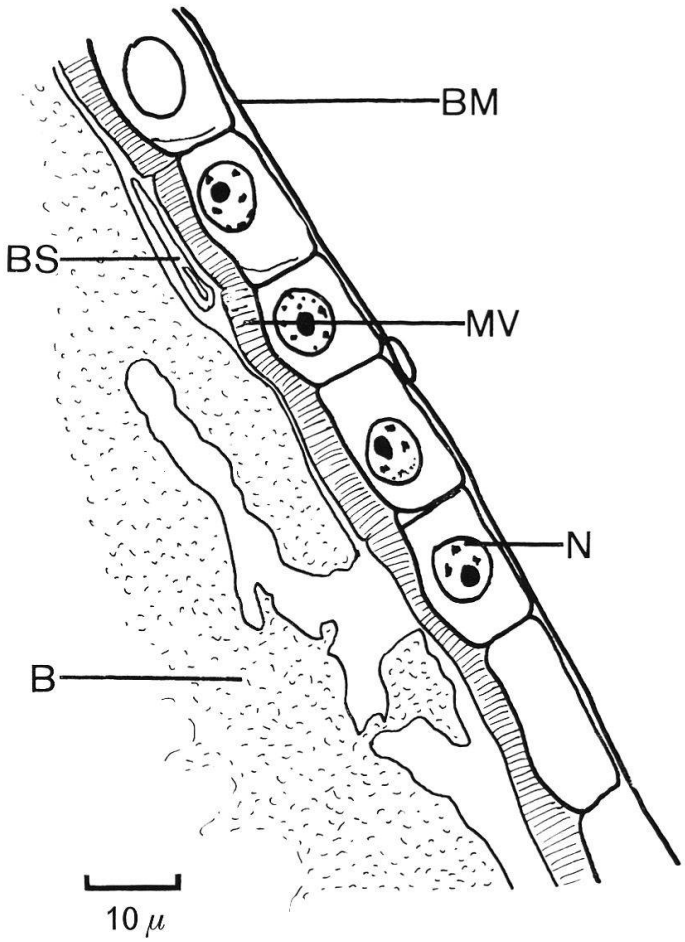

Fig. 7 b.

Fig. 7 a and b. Aëdes aegypti, 30½ hours after a blood meal, guinea-pig.

formation in the protoplasm of the cells. The P.M. is clearly detached from the brush border, although here, too, it is not continuous. It is followed by a zone containing remnants of erythrocyte nuclei. Further towards the inside of the gut there is an area of half-digested red blood corpuscles.

5. 1. 4. *Mosquito after 48 hours.* The blood meal has been fully digested. The gut ist empty and relaxed, and its wall is heavily wrinkled. In the section illustrated, the epithelium has almost returned to the state seen in the unfed mosquito. The clumps of chromatin in the nuclei stain very vividly. Similarly, secretion granules again show up orange. In line with the dissection findings, the P.M. is no longer to be seen.

5. 2. Anopheles gambiae.

5. 2. 1. *Unfed mosquito:* Fig. 9a and b. The gut is so heavily wrinkled that the brush border of the one side is frequently super-

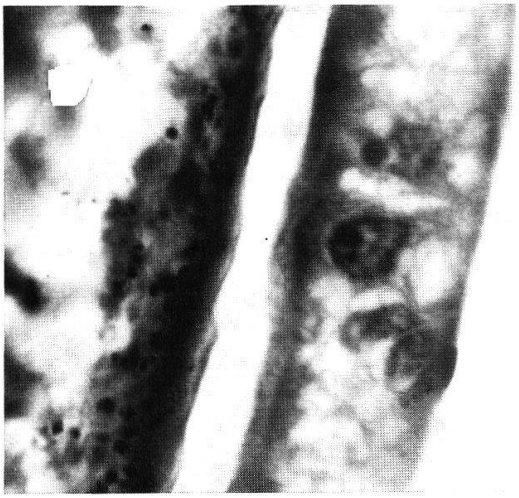

Fig. 8 a.

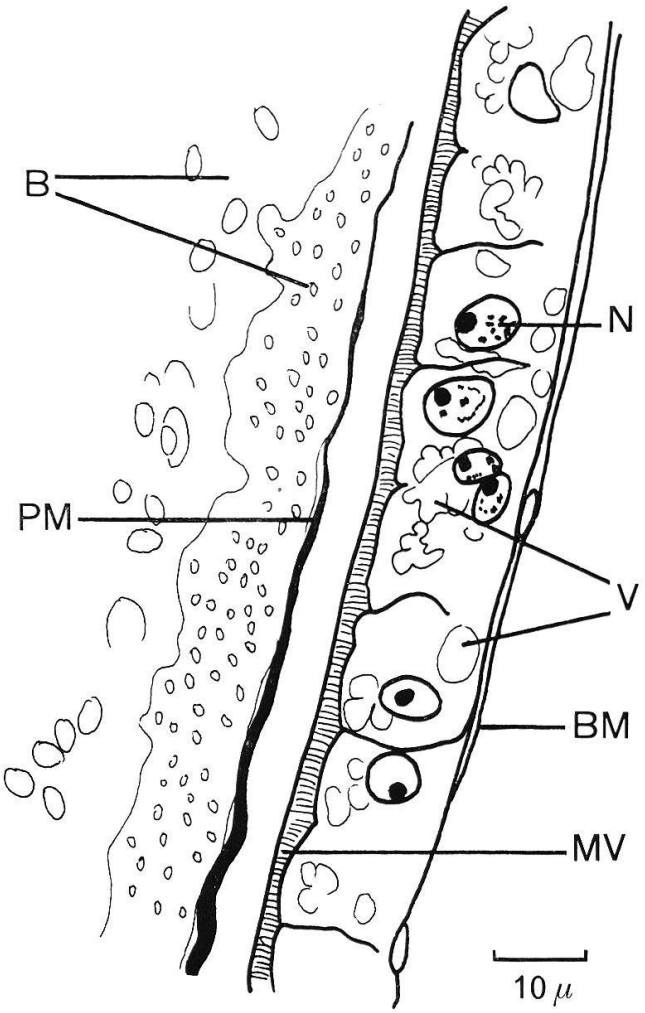

Fig. 8 b.

Fig. 8 a and b. Aëdes aegypti, 30½ hours after a blood meal, chicken.

posed on the brush border of the other side. Here, too, the epithelial cells are typically columnar in shape. The cells are less high than in *Aedes aegypti,* measuring only between 20 and 30 μ; the width of the cells is about 7 μ. The basement membrane is clearly visible as a fine violet line. The cell nuclei are not quite spherical; they are 8-10 μ long and 4-6 μ wide. The nucleoli measure 2-3 μ and stain orange. There are no actual clumps of chromatin. Basal to the nucleus the protoplasm shows a very fine thread-like structure. On the apical side no particular structure can be observed. The brush border, much stronger than in Aedes, is vertical, and measures up to 4 μ. In contrast to *Aedes aegypti, Anopheles gambiae* displays neither tapering of the apical pole nor any granulation. There is no P.M.

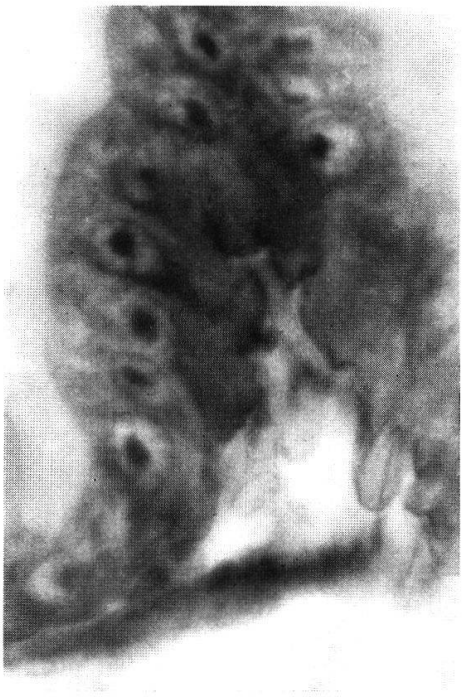

Fig. 9 a

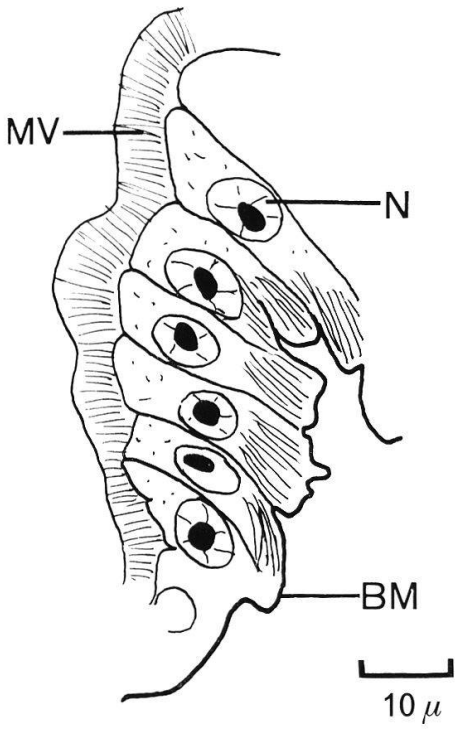

Fig. 9 b.

Fig. 9 a and b. Anopheles gambiae, unfed mosquito.

5. 2. 2. *Freshly fed mosquito:* Fig. 10a and b. As in *Ae. aegypti,* here, too, the epithelium of the gut has been stretched to quite a considerable extent. In places, the cells measure only 2 μ in height. On the basis of the distance between the nuclei the cell width must be between 15 and 35 μ. The basement membrane cannot be seen. The nuclei are flat and measure approximately $10 \times 3\,\mu$. A particularly striking feature is the blue secretion border. It does not lie directly on the epithelial cells. The brush border, though not identifiable as such, is probably located between the epithelium and the blue border. Towards the inside of the gut the blue border is followed by a lighter staining zone containing partially broken down erythrocyte membranes. In this case, too, we consider the blue border to be a product of the epithelial cells and a precursor of the P.M.

5. 2. 3. *Mosquito after 30½ hours:* Fig. 11a and b. The cells have increased considerably in height; they now measure at least 10 μ. The basement membrane is clearly recognizable. The nucleoli in the nuclei also seem to have increased somewhat in size. The protoplasm, as already described in connection with *Ae. aegypti,*

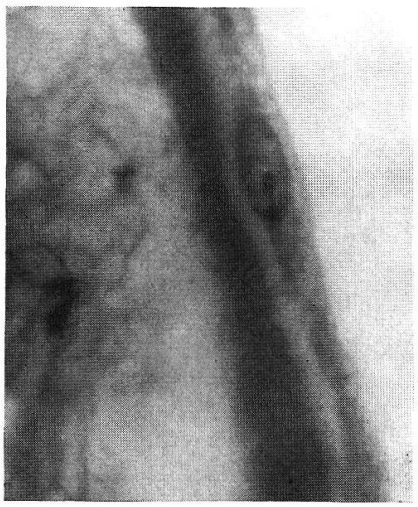

Fig. 10 a.

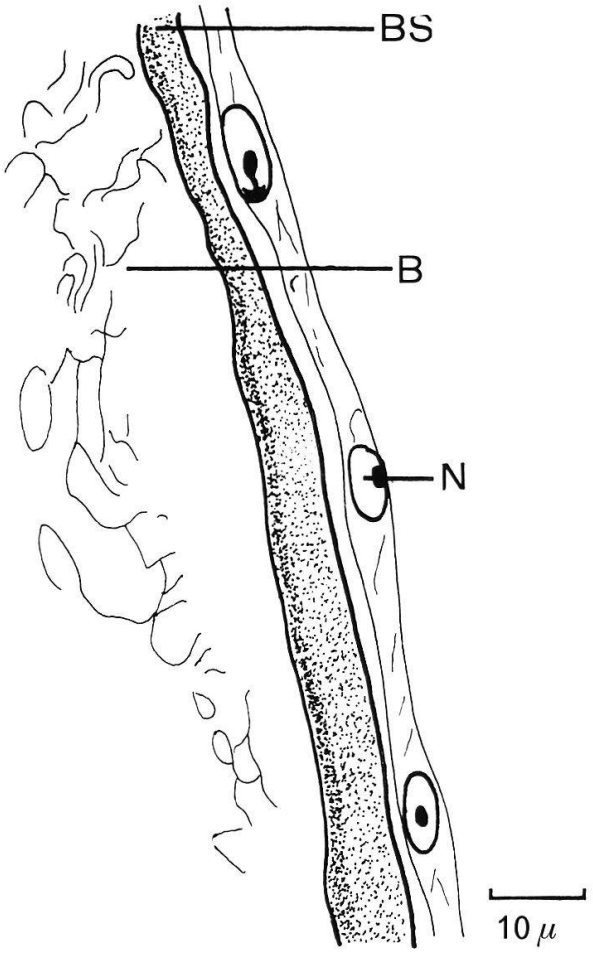

Fig. 10 b.

Fig. 10 a and b. Anopheles gambiae, 25 minutes after a blood meal, guinea-pig.

displays marked vacuolation. The brush border, 3 μ thick, is now very conspicuous; even individual microvilli can be dectected. The marginal zones of the blood stain black with azan. The blood coagulum is surrounded by the very compact blue border (cf. Fig. 1), the thickness of which varies between 3 and 17 μ. In places, the material of the border becomes compressed into line-like structures. We believe this material to be the P.M.

5. 2. 4. *Mosquito after 48 hours.* The gut may already be empty by this time. The epithelium is in a transitional stage—i.e. in a stage midway between that of the fed mosquito and that of the unfed mosquito. The degree to which the epithelium is stretched, as well as the height of the cells, varies greatly. The vacuoles have disappeared from the higher cells, but can still be seen in the lower ones. Here, the P.M. has disappeared completely.

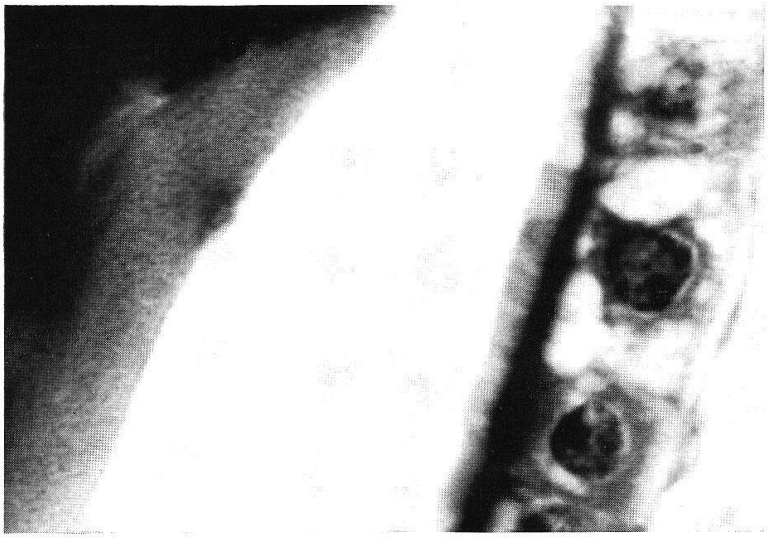

Fig. 11 a.

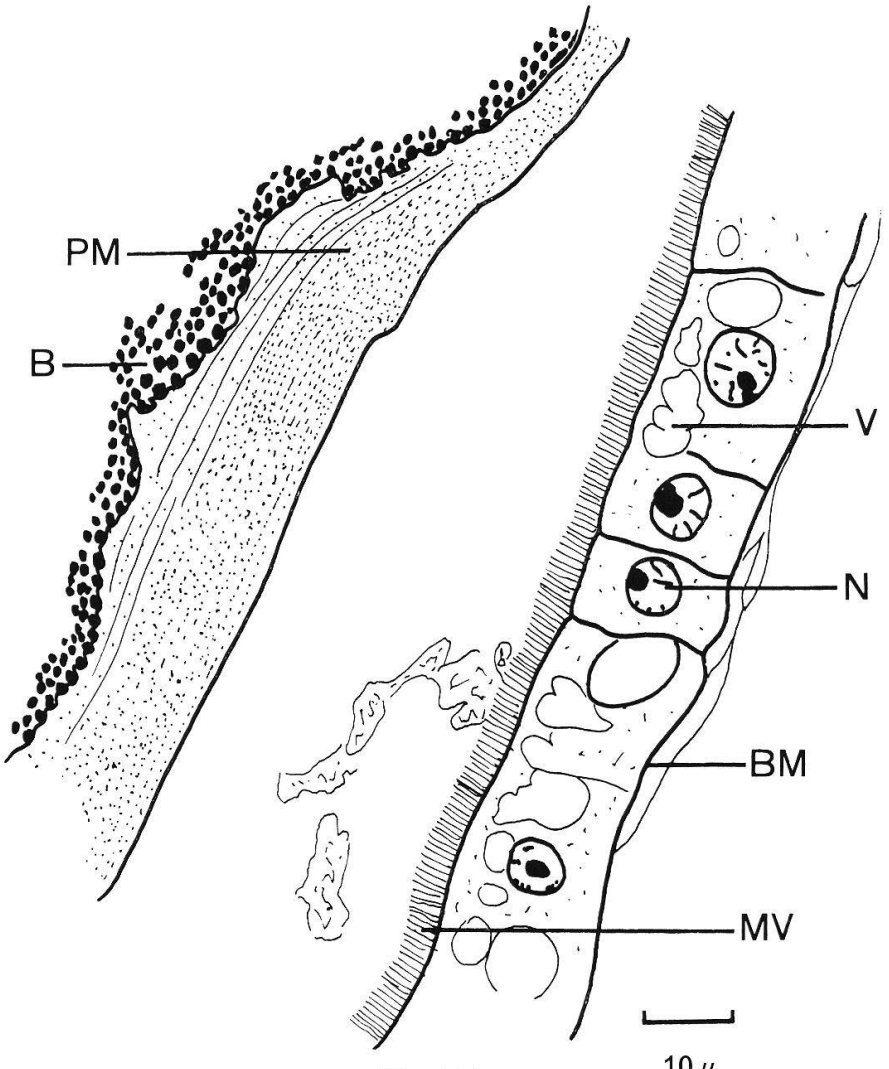

Fig. 11 b.

Fig. 11 a and b. Anopheles gambiae, 30½ hours after a blood meal, guinea-pig.

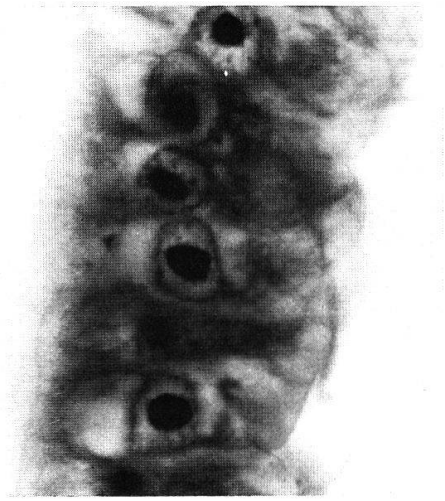

Fig. 12 a.

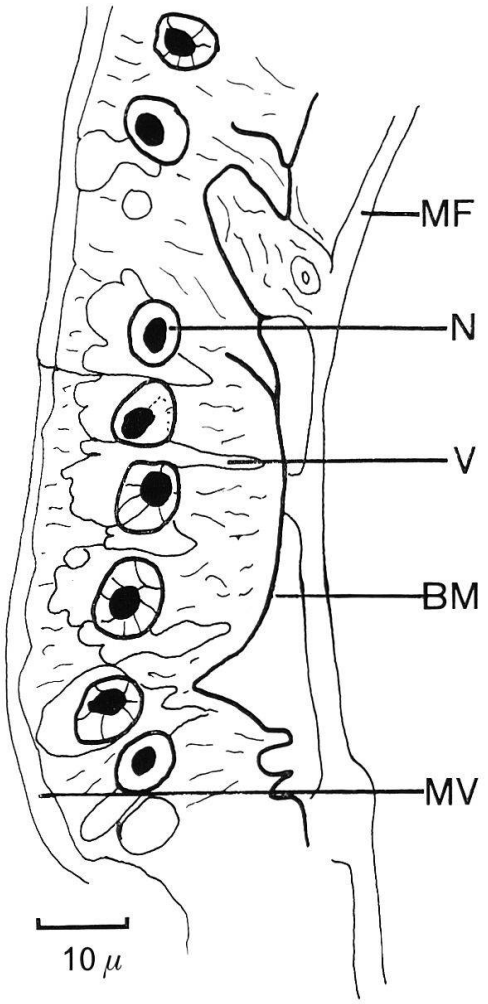

Fig. 12 b.

Fig. 12 a and b. Anopheles maculipennis atroparvus, unfed mosquito.

5. 3. Anopheles stephensi.

The histological pictures of the gut of this type of mosquito are practically identical with those of *An. gambiae,* and do not therefore need to be described separately. The digestive processes of the two mosquitoes seem to differ only in respect of their chronological pattern. Although secretion likewise begins in the first few minutes after the blood meal in *An. stephensi,* the entire process lasts longer than in *An. gambiae*, frequently taking up to 72 hours (cf. Fig. 4).

5. 4. Anopheles maculipennis atroparvus.

5. 4. 1. *Unfed mosquito:* Fig. 12a and b. The gut and its cells have, in the unfed state, approximately the same appearance as in *An. gambiae* after 30½ hours, except of course that the lumen is

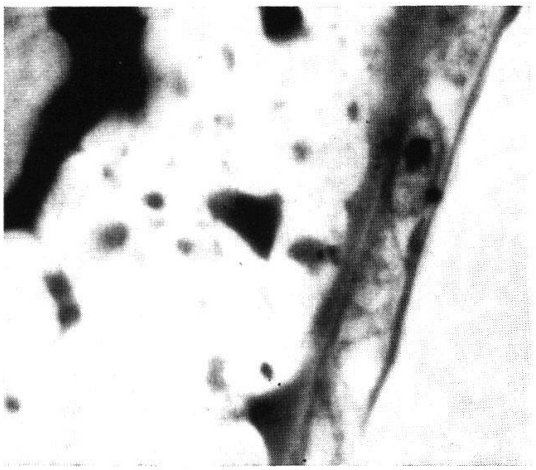

Fig. 13 a.

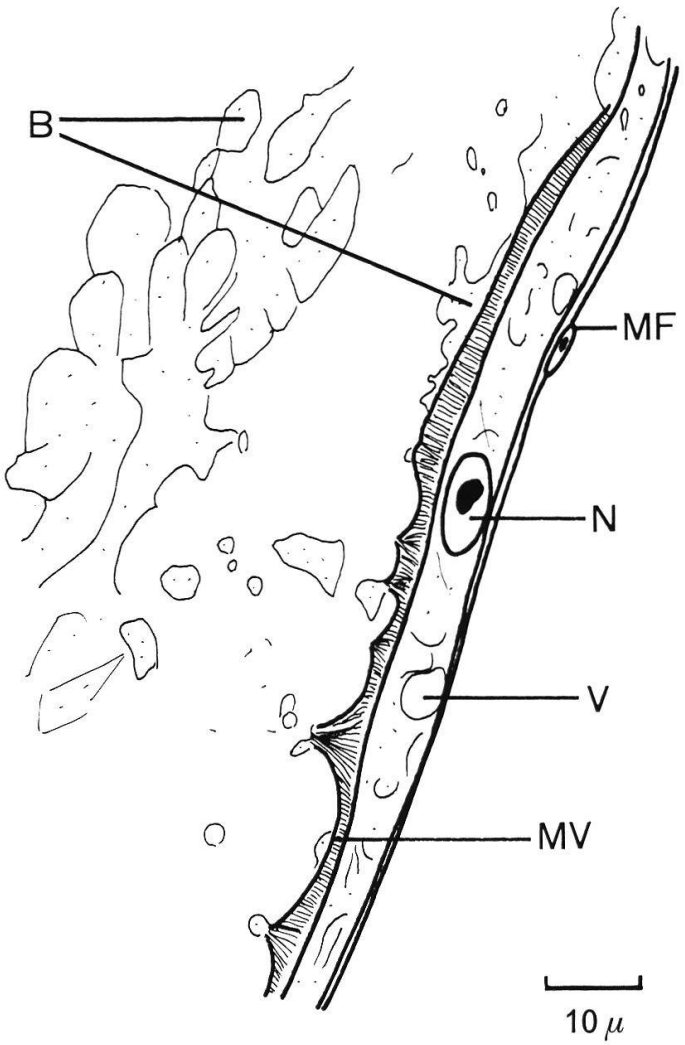

Fig. 13 b.

Fig. 13 a and b. Anopheles maculipennis atroparvus, 25 minutes after a blood meal, guinea-pig.

empty. Although the cells form a high columnar epithelium, the cell walls and the basal membrane are indistinct, the nucleoli are fairly large (3 μ), and, in particular, the protoplasm round the nuclei exhibits extensive vacuoles. For the rest, examination reveals a thread-like structure, especially basal to the nucleus. The brush border is upright, but can hardly be discerned in the microscopic picture.

5. 4. 2. *Freshly fed mosquito:* Fig. 13a and b. As far as the stretching of the epithelium of the gut is concerned, *Anopheles maculipennis atroparvus* behaves in the same way as the other mosquitoes. The dimensions of the cells are approximately the same as in the case of *An. gambiae*. The basement membrane as such cannot be seen. The muscles lie close to the epithelium. The

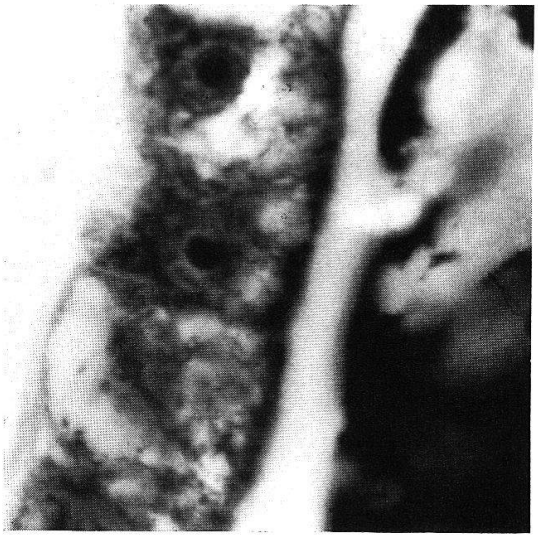

Fig. 14 a.

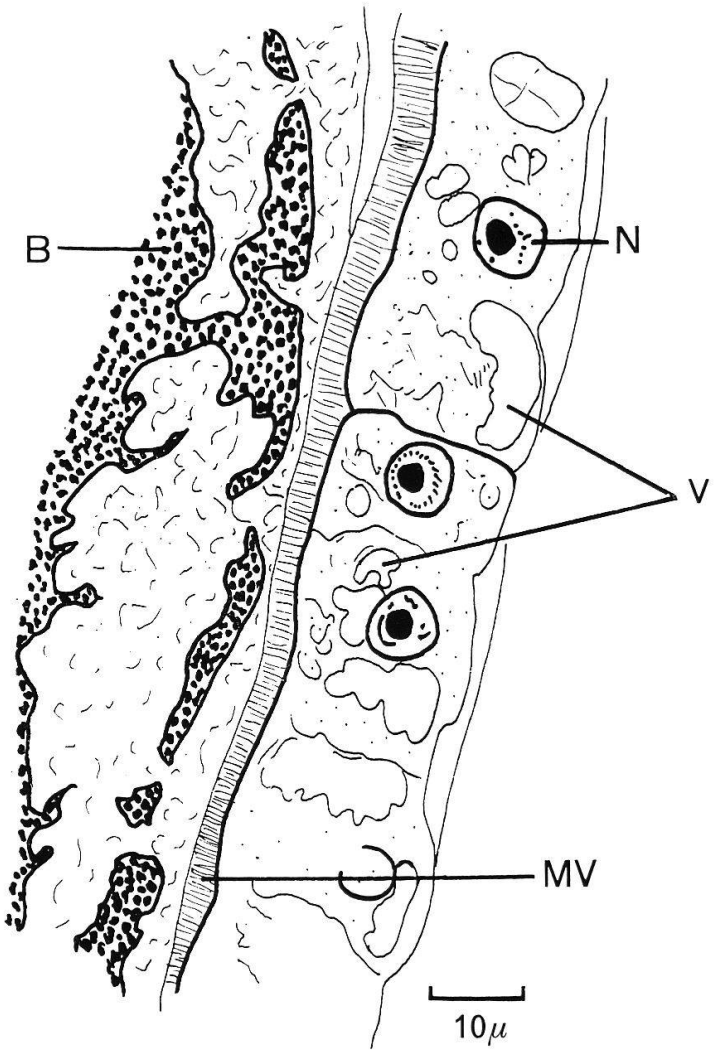

Fig. 14 b.

Fig. 14 a and b. Anopheles maculipennis atroparvus, 30½ hours after a blood meal, guinea-pig.

nuclei are flat. In contrast to the two other types of Anopheles, *An. maculipennis atroparvus* has a recognizable brush border. At the base, the border is a very light colour in most places, while towards the lumen it appears to be studded with a little "blue" secretion. On the other hand, there is no actual "blue border"—nor, for that matter, any P.M. The outer portions of the blood, which are in the process of being broken down, come into direct contact with the brush border—provided they have not been displaced artificially by the handling of the specimen.

5. 4. 3. *Mosquito after 30½ hours:* Fig. 14a and b. As in the other types of Anopheles, here, too, the epithelium has increased considerably in height. The basement membrane cannot yet be

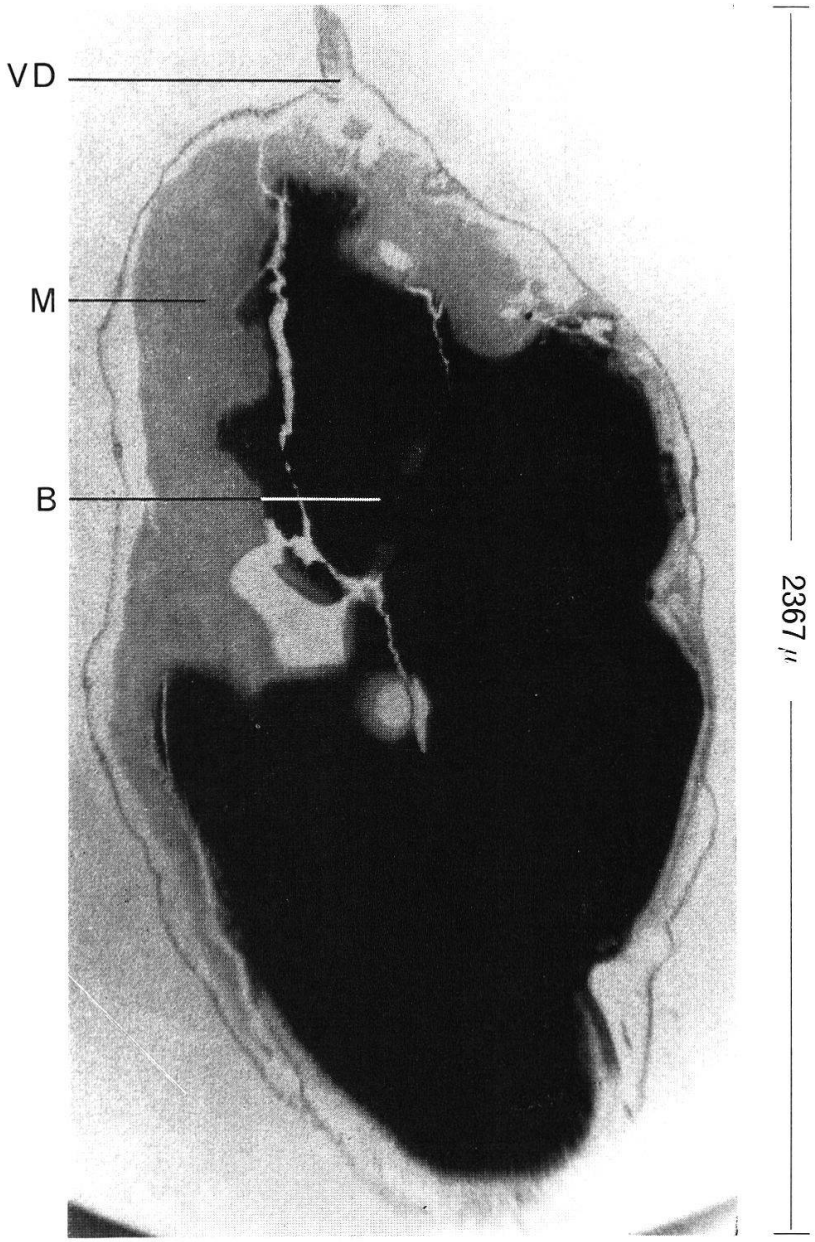

Fig. 15. Anopheles maculipennis atroparvus. Longitudinal section through a filled midgut, 30 minutes after a blood meal. For key to abbreviations, see Figs. 1-3, p. 121.

clearly seen. The protoplasm displays large vacuoles. The brush border is clearly recognizable only in parts. The firmly packed mass of blood is surrounded by an irregular zone which probably contains blood degradation products and secretions from the gut wall.

5. 4. 4. *Mosquito after 48 hours.* Even before the gut is quite empty, the wall already exhibits wrinkles. The vacuoles in the epithelial cells have become even bigger. The epithelium has the same appearance as in the unfed mosquito.

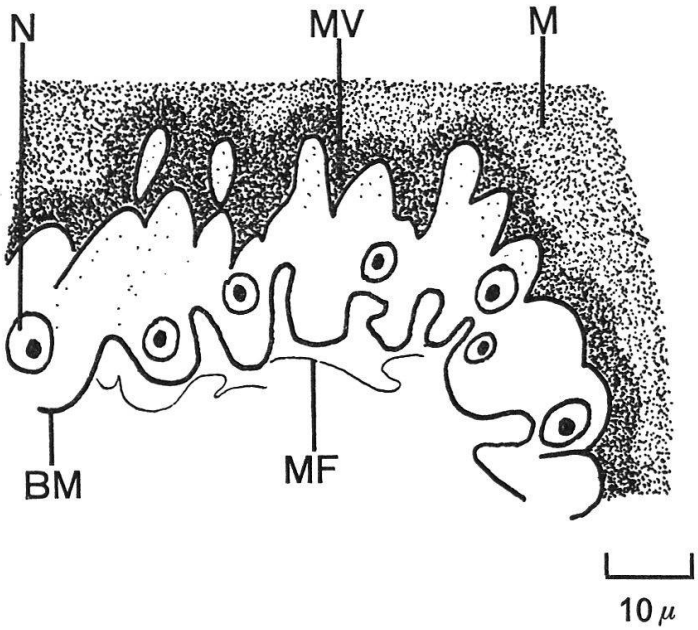

Fig. 16. Anopheles maculipennis atroparvus. Section from the anterior portion of the midgut during full secretion activity, 25 minutes after a blood meal. For key to abbreviations, see Figs. 5-14, p. 125.

5. 5. Further remarks.

5. 5. The cells described above represent only a small portion of the dilated midgut, the so-called "stomach". However, all the cells in this region look alike. Hence, what has been said in connection with the examples chosen, also applies to the other cells of the stomach, except that the rostral and caudal cells at the entrance and exit to the gut are never so markedly elongated after the blood meal as those of the central portion. They largely retain their columnar configuration at all times. Whether this is accompanied by a decrease in secretion cannot be decided on the basis of our preparations. Attempts at reconstruction with the aid of serial sections show that the jacket of secretion material, which forms round the blood within a few minutes of its ingestion, frequently contains gaps, at least in the first few hours following the blood meal. The distribution of these gaps, however, does not follow any regular pattern, so that we would be inclined to conclude that rostral and caudal, as well as dorsal, ventral, and lateral gut cells exert largely an identical function.

6. The secretion of the anterior portion of the midgut.

One of the more important differences between *An. maculipennis atroparvus,* on the one hand, and *An. gambiae* and *An. stephensi,* on the other, is that the secretion of the midgut cells is

much less in the former and— as electron-microscope examinations have shown—sets in later. It is therefore interesting to note that in *An. maculipennis atroparvus* the production of a mucus-like material by the anterior portion of the midgut is considerably more abundant and starts earlier than in the other two types of Anopheles. In the histological preparations of *An. maculipennis atroparvus,* a shining blue-stained mass becomes visible only 7 minutes after the blood meal; this mass is secreted in the anterior portion of the midgut (cf. Figs. 15 and 16), is discharged into the midgut, and forms a peculiar "cap" over the blood cake there. The borders of this cap sometimes extend in a thin layer right round the blood cake as far as the exit to the pylorus, where a second, thinner "cap" is frequently found. This second cap is probably due to the fact that the blood passing through the anterior portion of the midgut encounters mucus and pushes this mucus in front of it as far as the pyloric region. The entrance and exit to the midgut are to some extent blocked with plugs of mucus. The lateral portions of the cap disappear after only a few hours; the caudal cap likewise rapidly diminishes in size, but the rostral cap can be observed for up to 44 hours after ingestion of the blood meal. This cap in particular can become irregularly mingled with the blood. It does not appear to exert any important effect on digestion, as the erythrocytes in its vicinity are hardly broken down at all.

In *An. gambiae,* a shining blue rostral plug becomes clearly apparent in the preparation 24 hours after the blood meal. Its colour distinguishes it from the secretion of the midgut cells, which is pink-grey-blue. There may be a certain secretion from the anterior portion of the midgut immediately after ingestion of the blood meal. This secretion, however, would seem to last only a short time and to produce little material in comparison with the secretion in *An. maculipennis atroparvus.* In the sections of fresh material examined 25-60 hours after the blood meal, the rostral plug adheres to the P.M. In the phase-contrast microscope the plug material displays a certain granulation and thus differs from the material of the P.M. In addition, the P.M. dissolves in an aqueous fluid after some time (cf. below, p. 141), whereas the plug remains undissolved. Hence, the material of the plug and the material of the P.M. are doubtless of different origin and possess different properties. The findings in *An. stephensi* are similar to those in *An. gambiae;* in *An. stephensi,* however, the plugs appear to be less strongly formed. In *Ae. aegypti,* a corresponding material can likewise be observed in histological preparations and in fresh

TABLE 3.

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

Quantity of blood	*Aedes aegypti* (14 h. after blood meal)			*Anopheles gambiae* (18 and 24 hours after blood meal)			*Anopheles stephensi* (24 and 34 hours after blood meal)		
	P. M. complete	incomplete	absent	P. M. complete	incomplete	absent	P. M. complete	incomplete	absent
Very little	1	9	7	1	15	6	1	9	2
Little	4	28	3	10	10	1	4	11	0
Approx. half normal amount	2	9	4	2	5	1	4	4	0

sections. We consider the rostral whorl, which STOHLER (10) drew in his Fig. 1 and regarded as a "part of the P.M. originally protruding into the narrow portion of the midgut", to be part of the plug from the anterior portion of the midgut.

7. P.M. formation under unnatural conditions.

7. 1. In order to find out something about the *prerequisites* necessary for P.M. formation, a few preliminary experiments were conducted in which mosquitoes were fed under unnatural conditions. For instance, we first prevented mosquitoes from ingesting a complete blood meal by removing them from the host soon after they had begun to suck. After a suitable lapse of time (cf. Table 3)—digestion and all the processes connected therewith are quickened up after the ingestion of small quantities of blood—the mosquitoes were examined to see whether or to what extent a P.M. had formed. The amount of blood ingested was estimated with the naked eye upon dissection of the mosquitoes, so that the results cannot be evaluated quantitatively. Nevertheless, in all three types of mosquito the overriding impression was that the P.M. is either not formed at all or is incomplete following the ingestion of very small quantities of blood.

7. 2. *Heparinized blood.* A chicken was given an intracardiac injection of 0.4 ml heparin. As a result, the bird's blood failed to coagulate. *Ae. aegypti, An. gambiae,* and *An. stephensi* were al-

lowed to feed on this bird and were then examined. The P.M. formed normally in all three species of mosquito.

7. 3. *Defibrinated blood.* The blood of a guinea-pig was defibrinated by running it over glass beads and then offered to the mosquitoes on a heated plate (37°C) under a membrane (sausage skin). 5 *Ae. aegypti* and 5 *An. stephensi* ingested a full meal of this blood, and all of them developed a complete P.M. within the normal space of time.

7. 4. *Blood serum.* Serum obtained from guinea-pig blood was offered to mosquitoes in the same way as the defibrinated blood. 17 *Ae. aegypti,* 5 *An. gambiae,* and 7 *An. stephensi* ingested the serum. Of the *Ae. aegypti* all females formed a P.M. The same applied to *An. gambiae.* As for *An. stephensi,* dissected 48 hours after the serum intake, one female formed a very thin P.M.; in one case, a clear-cut colourless layer was observed round the contents of the gut, but this layer could not be demonstrated in the phase-contrast microscope; in five cases, dissected 33, 38, and 48 hours after the serum intake, no P.M. could be shown at all.

7. 5. These few experiments seem to indicate that formation of the P.M. is dependent to some extent on the quantity of blood ingested. The effect exerted by the quantity of blood may possibly be due simply to the fact that the cells of the gut do not start to secrete until they are sufficiently stretched. As to the quality of the substances ingested, it does not seem to be of major importance for the formation of a P.M. Whether, or to what extent, this also applies to *An. stephensi*—which showed no P.M. after a serum meal—needs further investigation. The P.M. could perhaps be due to the combined effect of gut cell secretion and certain substances in the blood meal. To obtain answers to these questions, further tests will have to be carried out.

7. 6. *Fractionated feeding. Ae. aegypti* females were first allowed to ingest an incomplete meal on guinea-pigs. 10 hours later the same mosquitoes were given the opportunity of feeding again on chicken. This choice of hosts (erythrocytes with and without nuclei) made it possible to distinguish the one blood meal from the other in the subsequent examination. Upon dissection and in the histological slices it was found that a P.M. had formed round each of the two half-meals. The anterior, more recently formed P.M. was fairly thin. This finding is in contrast to that reported by YAGUJINSKAIA (15), who discovered remnants of earlier membranes inside a P.M. in *An. maculipennis messeae.*

8. The chemical nature of the P.M.

8. 1. *Solubility of the P.M. in an aqueous solution.* The P.M. of *Ae. aegypti* keeps for days in physiological saline or Ringer's solution. The same is true of the P.M. of *Ae. togoi* (4). In the same medium at room temperature, the peritrophic membranes of *An. gambiae* and *An. stephensi* dissolve within 2-3 hours.

8. 2. *Van Wisselingh tests.* Van Wisselingh's chitosan-iodine test mentioned in WIGGLESWORTH (14) yields positive results for the P.M. of *Ae. aegypti, An. gambiae,* and *An. stephensi.* However, there is a slight difference in colour between the stained P.M. of *Ae. aegypti* (dark reddish violet) and that of the two species of Anopheles (reddish rust-brown). In all cases, the colour disappears rapidly upon addition of 3% acetic acid.

8. 3. Consequently, provided the Van Wisselingh test is really specific for chitin, it must be assumed that the P.M. of Anopheles, like that of Aedes (11), contains chitinous material. Then, however, it is difficult to understand why it is soluble in an aqueous solution. In other respects, however, the chemical structure of the P.M. in the two species is likely to display certain differences, on which histochemical studies may possibly shed some light.

9. Discussion.

Our histological findings agree in the main with those of previous authors, including especially STOHLER (10), BERTRAM & BIRD (1), SCHUSCHUKOW (7) *(Ae. aegypti),* and PAL (6) *(An. stephensi, An. culicifacies,* and *An. subpictus).* A reservation must, however, be made in respect of the "cuticula" drawn by PAL in these three species of Anopheles; a "cuticula" of this nature does not occur in the endodermal midgut portion (8, 12); we consider it to be the brush border covered with secretion. What was hitherto not known, so far as we are aware, is the fact that there are genus-specific and species-specific differences in cellular structure and secretion mechanism. These differences are most clearly apparent in unfed mosquitoes. Let us compare first of all *Aedes aegypti* with the three types of Anopheles investigated: the cells and nuclei of *Ae. aegypti* are larger; the basement membrane is thicker; particularly conspicuous are the coarse secretion granules which are very numerous in the apical portion of the cell and are not found in Anopheles. The occurrence of the cobblestone artefact (p. 126) in Aedes, but not in Anopheles despite similar treat-

ment of the material suggests a difference in the substance or state of the cell. That all these differences cannot simply be due to a specific peculiarity of *Aedes aegypti* is shown by an unpublished work (4) on *Aedes togoi;* in both species of Aedes the gut epithelium displays the same characteristic features.

The difference between *Anopheles gambiae* and *An. stephensi* on the one hand, and *An. maculipennis atroparvus* on the other, is considerably less marked. Histologically, it consists in the fact that the gut cells of *An. maculipennis atroparvus* exhibit relatively large vacuoles in the unfed state and thus appear similar to the cells of *An. gambiae* 30 hours after a blood meal. The delay in secretion activity and possibly also the non-formation of a P.M. are presumably connected therewith. Perhaps the reason is that the cells show not so much a fundamental difference in structure and function as a shift in the chronological pattern of their activity. The secretion of "mucus" from the anterior, narrow portion of the midgut, which varies both quantitatively and chronologically, has already been referred to above (p. 138).

The electron-microscope findings tally well as a rule with the results obtained in histological studies and confirm the genus-specific and species-specific differences mentioned above. In addition, the use of the electron-microscope affords a much deeper insight into the organization of the cells and reveals a fundamental difference between Aedes and Anopheles in respect of their secretion mechanism. A detailed discussion of this aspect will be found in STÄUBLI et al. (9). Since the appearance of the cellular elements in the histological preparation differs widely from their appearance under the electron-microscope, the terms used to describe these elements are not the same in some respects. It is sometimes difficult, particularly in the case of the smaller organelles, to say exactly which term corresponds to which. This is especially true as regards our investigations because, for the sake of clarity, we confined ourselves to using only the azan method for staining purposes. The terms "nucleus, "nucleolus", "brush border", and "basement membrane" refer to the same elements in both histological and electron-microscope preparations. The protoplasmic "threads", described as occurring in the basal half of the cells in unfed mosquitoes, probably correspond to the invaginations of the cellular wall seen under the electron-microscope. They are largely smoothed out when the gut epithelium is extended accordingly, the protoplasm in the histological preparation loses its "thready" appearance as the epithelium is stretched. The "blue border" in the histological preparations represents, in our view, the fine granular secretion material first

observed between the microvilli of the brush border in the electron-microscope pictures.

With regard to the orange-staining "granules" located in the apical portion of the cells of *Ae. aegypti* we suggested (p. 127) that they are possibly related to the production of the "blue border". According to the electron-microscope findings, however, *Ae. aegypti* does not display any secretion granules. The ergastoplasmic, fingerprint-like whorls, whose appearance coincides chronologically with that of the orange granules, appear in the light microscope as compact, nucleus-like structures (1). They remain practically invisible when the cells are stained with azan. Mitochondria are to be seen in all cells at any time and cannot therefore provide an explanation for the orange granules. The situation as regards the lysosomes is similar: the lysosomes attain their maximum density in freshly fed mosquitoes, the orange granules, however, in unfed mosquitoes. There still remains the possibility that certain organelles may stain differently depending on their physiological state. We know too little about this; at the moment, we cannot find any morphological equivalent to the orange granules in the electron-microscope picture. Similar difficulties are encountered in the interpretation of the "vacuoles" which appear in the histological preparations at various times during the digestion of the blood meal. A comparison with the electron-microscope pictures shows that these cannot be membrane-bound vacuoles. Zones which appear optically empty in the histological preparation really contain many kinds of submicroscopic structures. The appearance and disappearance of the "vacuoles"— or, more correctly, the varying stainability of the protoplasm—would seem at the most to be an indication of internal physiological changes in the cells. The P.M., too, cannot be distinguished with certainty in the electron-microscope pictures. Admittedly, a texture-less mass can be seen over the epithelial cells in some photographs and this mass has been considered to be a P.M. by BERTRAM & BIRD (1). However, the only evidence to support this assumption is the position of the material between the epithelial cells and the blood coagulum. On no occasion, though, has an electron-microscope picture revealed the type of structure which would be expected on the basis of STOHLER's findings (10). Moreover, the relevant material is found only in a few pictures—even at times when, upon dissection, the P.M. appears to be visible to the naked eye—and even then it is incomplete and of irregular thickness. Fairly frequently, too, erythrocytes are seen to be resting directly on the epithelial cells. Similarly, in the description of the histological preparations reference has been made to the fact that the

P.M. does not always occur and may be incomplete, although in some slices it did seem to possess a certain structure. Hence, we are confronted with the strange situation that our only evidence for the existence of the P.M. is based on dissections of fresh material. An actual P.M. cannot be detected with certainty in either the histological or the electron-microscope preparations alone. The reason might be that—as shown by the preliminary experiments—approximately one-fifth of the mosquitoes form an incomplete P.M. It might be a coincidence that the areas examined by us had, in the majority of cases, no P.M. A further reason might be found in the methods of fixation employed, although these methods produced good results with the P.M. of larval stages. There is little evidence to support either of these possible explanations. Hence, the question arises as to whether the P.M. is not simply a kind of condensation product due to the disturbance caused in the physico-chemical state of the contents of the gut by the dissection—in other words, whether the P.M. in its solid form is not an artefact. Two facts seem to militate against this view: the chitin-positive results of the Van Wisselingh test (p. 141) and, in particular, the accumulation of ookinetes of *Plasmodium gallinaceum* on the P.M. of *Ae. aegypti,* observed by STOHLER (10) 30 hours after the ingestion of infected blood. If the P.M. did not constitute a genuine obstacle for the ookinetes in the intact gut of the Aedes mosquitoes, it would be impossible to understand why the ookinetes should form such accumulations; the P.M. must consequently have existed prior to dissection. There can be no doubt of the existence of the P.M. during digestion of the blood meal in various Culicidae; on the other hand, its degree of completeness and, perhaps also of consistency, might vary.

YAGUJINSKAIA (15) found a P.M. in *An. maculipennis* in 1940. This author has informed us that she was then working on *An. maculipennis messeae. An. maculipennis atroparvus,* as we have shown, does not form a P.M.—a fact which may be connected with the working rhythm of the midgut cells in this mosquito (p. 138). Experiments would have to be conducted to discover whether the secretion activity of *An. maculipennis messeae* displays the same chronological pattern as that of *An. gambiae* or *An. stephensi.* The contradictory results of YAGUJINSKAIA and ourselves seem to support BATES's (quoted in WEYER) and WEYER's (13) view that the known varieties of the *An. maculipennis* group in fact form distinct species.

In conclusion, we should like to discuss as well the possible function of the P.M. in mosquitoes. *An. maculipennis atroparvus* does not display a P.M. However, it forms upon ingestion of a

blood meal plugs of "mucus" at the entrance and exit to the midgut, the material of these plugs originating from the anterior portion of the midgut. The pyloric aperture can presumably be effectively closed by the sphincter muscle. On the other hand, there is no anatomical device capable of blocking the passage from the anterior portion of the midgut to the middle portion. The rostral plug certainly helps to prevent reflux of the partly liquid meal from the middle portion. In *An. gambiae* and *An. stephensi,* the secretion of "mucus" is much less marked; moreover, this secretion very probably does not start until considerably later. On the other hand, the blood is immediately enveloped in a jacket of secretion which solidifies in the course of a few hours to form a P.M. If some device is necessary to prevent evacuation of the gut, the P.M. in its viscous and subsequently solid stages might well constitute such a device. By analogy with the rostral plug in *An. maculipennis atroparvus,* therefore, the function of the P.M. in the other mosquitoes investigated might simply be to perform a mechanical task, i.e. to keep the liquid portion of the blood meal in the gut. Admittedly, there are three objections to such a hypothesis: the P.M. hardens relatively late on in the course of blood digestion; it hardens from the rear towards the front (10); in cases where the P.M. is incomplete, the gaps are usually to be found in the anterior portion of the midgut.

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Zusammenfassung.

1. Bei den Stechmücken *Aedes aegypti, Anopheles gambiae, An. stephensi* und *An. maculipennis atroparvus* werden die Veränderungen der Zellen der Magenwand während der Verdauung des Blutes und die Entstehung der peritrophischen Membran (PM) verfolgt.

2. Wo die Bildung einer PM statthat, erfolgt sie unabhängig vom Alter der Mücke, von der Anzahl der vorangegangenen Fütterungen und von der Art des Blutspenders.

3. Die PM kann bei *Ae. aegypti* in der Regel frühestens 5 bis 8, bei *An. gambiae* 13 und *An. stephensi* 32 Stunden nach der Blutmahlzeit nachgewiesen werden. Bei allen drei Arten bleibt sie bis gegen das Ende der Blutverdauung bestehen. *An. maculipennis atroparvus* bildet keine PM.

4. Histologisch lassen sich im Sekretionsmechanismus der Magenzellen von *Aedes* und *Anopheles* grundsätzliche Unterschiede feststellen. Unter den Anophelinen ist das Magenepithel gleich aufgebaut; hingegen scheint die Aktivität der Zellen von *An. maculipennis atroparvus* im Vergleich zu den andern untersuchten Anopheles-Arten zeitlich verlagert zu sein.

5. Beim Einfließen des Blutes in den Magen von *An. maculipennis atroparvus* wird vor allem dessen Eingang von schleimartigem Material aus dem vordern Mitteldarmabschnitt verschlossen. Eine solche Schleimproduktion erfolgt bei den andern untersuchten Mückenarten in weit geringerem Maße und zum Teil erst viel später im Verlaufe der Blutverdauung.

6. Die PM von *An. gambiae* und *An. stephensi* ist im Gegensatz zu derjenigen von *Ae. aegypti* in wäßrigem Medium löslich.

7. Die Chitosan-Jod-Probe erweist für die PM aller untersuchten Arten die Anwesenheit von Chitin.

8. In der Diskussion werden die vorliegenden Ergebnisse mit frühern Arbeiten anderer Autoren und insbesondere mit unsern eigenen, andernorts publizierten elektronenoptischen Untersuchungen verglichen. Die Natur der PM wird erörtert. Ihre Funktion bei den Stechmücken sehen wir in einer einfachen mechanischen Aufgabe: sie verhindert ein vorzeitiges Ausfließen der Nahrungssubstanzen aus dem Magen.

Résumé.

1o Les auteurs ont étudié chez *Aedes aegypti, Anopheles gambiae, An. stephensi* et *An. maculipennis atroparvus* les cellules de l'intestin moyen pendant la digestion et la formation de la membrane péritrophique (MP).

2o La formation de la MP est indépendante de l'âge du moustique, du nombre des repas sanguins précédents et de l'espèce du donneur.

3o Généralement la MP s'observe : chez *An. aegypti* au plus tôt 5 à 8 heures après le repas sanguin, chez *An. gambiae* au plus tôt 13 heures après le repas sanguin et chez *An. stephensi* au plus tôt 32 heures après le repas sanguin. Chez ces 3 espèces elle reste visible jusqu'à la fin de la digestion. *An. maculipennis atroparvus* ne forme pas de MP.

4o Il existe des différences essentielles dans le mécanisme de sécrétion des cellules intestinales d'*Aedes* et d'*Anopheles*. Parmi les Anophèles, l'épithélium intestinal est partout de même apparence. Par contre, il semble que l'activité des cellules d'*An. maculipennis atroparvus* soit retardée par rapport à l'activité des cellules des autres espèces d'Anophèles.

5o Après l'arrivée du sang dans l'estomac d'*An. maculipennis atroparvus,* l'entrée de celui-ci est fermée d'un bouchon de mucus produit par la partie antérieure de l'intestin moyen. Une telle production de mucus n'intervient, chez les autres espèces de moustiques examinées, que dans des proportions moindres et beaucoup plus tardivement au cours de la digestion.

6o La MP d'*An. gambiae* et d'*An. stephensi* est, au contraire de celle d'*Ae. aegypti,* soluble dans un milieu aqueux.

7o La réaction de « chitosan-iode » dénonce la présence de chitine chez toutes les MP étudiées.

8o Au cours de la discussion, on compare les résultats du présent travail avec ceux d'autres auteurs, et en particulier, avec ceux que nous avons publiés ailleurs sur la MP vue par l'entremise du microscope électronique. La nature de la MP est alors discutée. Son rôle est simplement mécanique : en les enfermant dans un sac, la MP empêche les substances nutritives de couler hors de l'intestin.