J. exp. Biol. 108, 1–7 (1984)
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ROLE OF MOSQUITO SALIVA IN BLOOD VESSEL LOCATION

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Accepted 1 August 1983

SUMMARY

In order to ascribe a blood feeding function to the saliva of mosquitoes, we determined whether this secretion may limit the initial probing phase of biting behaviour. The probing of hosts was indeed prolonged when the salivary ducts were severed, but this prolongation was absent when mosquitoes were fed on an artificial meal contained beneath a membrane. *In vitro*, turbidometric assays demonstrated that saliva inhibits the ADP- and collagen-mediated aggregation of platelets. ATP and ADP were hydrolysed by saliva, and this apyrase activity explains, in part, the observed effect upon platelets. We conclude that the saliva of mosquitoes functions by facilitating location of blood vessels.

INTRODUCTION

Although the saliva of mosquitoes provides the all-important vehicle for transmission of pathogens to vertebrate hosts, its pharmacological function in blood feeding has not been conclusively established. Without doubt, it serves as a solubilizer for solid sugar (Eliason, 1963), a role, in nature, that would contribute to ingestion of sugar sources such as honeydew. Less persuasive evidence ascribes some anticoagulant activity which may assist blood ingestion (Yorke & Macfie, 1924; Metcalf, 1945; Hudson, 1964), but mosquitoes surgically deprived of saliva are able to ingest blood (Hudson, Bowman & Orr, 1964; Mellink & van den Bovenkamp, 1981; Rossignol & Spielman, 1982).

Observations on movements of mosquito mouthparts within living tissue provide a basis for assigning salivary function. After penetrating the skin, mosquitoes thrust their stylets back and forth before locating a blood vessel and beginning to feed. During this probing phase, mosquitoes salivate copiously (Griffiths & Gordon, 1952), an activity that is difficult to interpret in terms of blood-ingestion. Such extravascular secretion could not inhibit coagulation of ingested blood, and this creates a paradox.

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Key words: Mosquito, saliva, haemostasis.

It may be that the main function of mosquito saliva is to facilitate vessel location, a hypothesis that has not yet been examined. Accordingly, we determined whether saliva influences the ability of mosquitoes to locate blood vessels, thus shortening the early probing phase of biting behaviour.

MATERIALS AND METHODS

A strain of *Aedes aegypti*, originating from material collected in 1969 on Grand Bahama Island, was used throughout this work. Rearing and surgical procedures used were those described by Rossignol & Spielman (1982). Mosquitoes were deprived of sugar the night before treatment.

Disodium salts of ADP and ATP from equine muscle (99 % pure) were obtained from Sigma Chemical Co. (U.S.A.). Collagen (2 %) suspension in saline (Collagen type II, Sigma) was prepared from homogenized bovine achilles tendon and centrifuged at 1000 ***g*** for 5 min. The supernatant was stored at 4 °C. All other chemicals were of analytical grade. Distilled and deionized water was used throughout.

Blood (9 ml) from a healthy human volunteer was collected in plastic syringes containing 1 ml of 3·8 % sodium citrate. Blood was centrifuged at 200 ***g*** for 15 min. The resulting platelet-rich plasma was collected and used within 5 h.

Guinea pigs were anaesthetized with Nembutal and Ketamine administered intraperitoneally. We presented individual mosquitoes to guinea pigs in small (1·5×3×6 cm) cages covered with nylon net (30 mesh) and recorded duration of time between stylet penetration and the appearance of blood in the gut as observed through the thin abdominal pleura. When mosquitoes withdrew their mouthparts before taking blood, timing stopped and began anew following subsequent penetration. Mosquitoes were observed until 420 s had elapsed, but a few which ceased probing before this time were discarded, provided that no blood was ingested. Each insect was used only once. For artificial feeding, insects were exposed to a feeder containing a warm diet (150 mM-NaCl, 1 mM-ATP, and phenol red added for colour) and covered by a Baudruche membrane. In all cases, ducted and sham-operated insects were observed alternately in order to minimize possible bias.

Saliva was collected in oil in a device modified from that of Rossignol & Spielman (1982). Mosquitoes were chilled on wet ice for 20 s and their legs and wings removed. Using a dissecting microscope, the stylets were exposed and placed in a section of PVC tubing (i.d. 0·28 mm) filled with approximately 0·5 μl of mineral oil. As described for *Calliphora* (Hansen Bay, 1978), we observed that serotonin induces salivation by *Aedes aegypti* (unpublished). Thus, 0·1 μl of a serotonin solution (20 μM) in saline was injected into the haemocoel. Mosquitoes were then allowed to salivate for 10–15 min and the tubes were emptied into 1·5 ml conical plastic tubes. Samples obtained from 25 mosquitoes were pooled and stored at −70 °C until use. Before assay, 25 μl of 0·15 M-NaCl was added per sample (1 μl for each mosquito). Following shaking, the tube was centrifuged for 1 min at 10 000 ***g***. The resulting aqueous layer was used in the assays.

A turbidimetric method for measuring platelet aggregation was used (Born & Cross, 1963), employing siliconized glass cuvettes. The transmittance of the suspension at 550 nm was recorded during continuous stirring at 37 °C.

Apyrase activity was assayed by measuring the release of inorganic phosphate from ADP and ATP (Fiske & Subbarow, 1925). One milliunit of activity is defined as the amount of enzyme that releases one nanomole of inorganic phosphate per minute from a defined standard medium held at 30 °C.

RESULTS

Behavioural studies

First we determined whether saliva serves to shorten probing time and whether probing aids in locating blood vessels. We reasoned that if blood vessel location is an

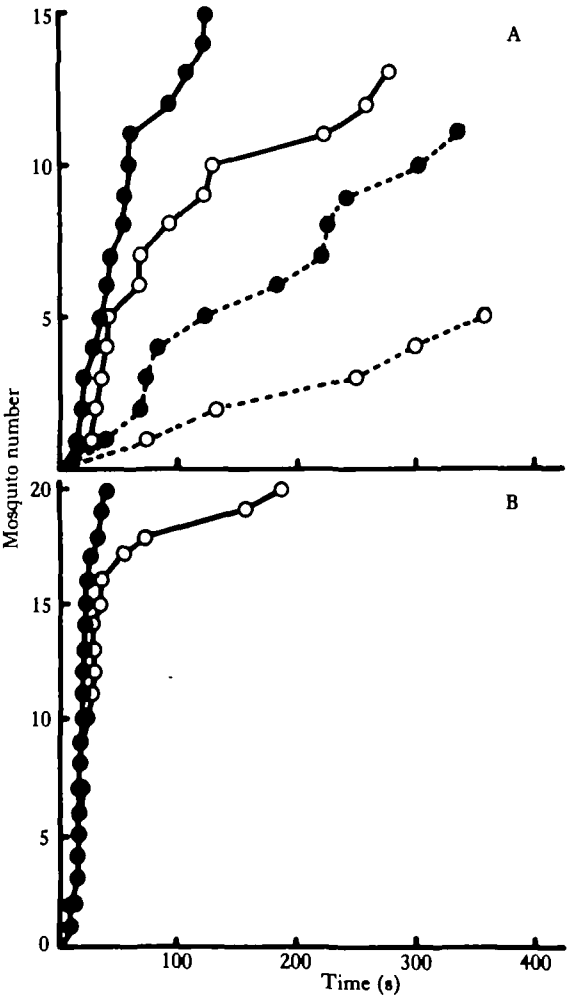

Fig. 1. (A) Duration of probing by neck-pierced (unbroken line) and duct-sectioned (dashed line) mosquitoes exposed to the ear (black circles) or back (white circles) of a guinea pig. Each treatment represents the cumulation of 15 mosquitoes tested up to 420 s. Mosquitoes probing more than 420 s are not represented on the graph. (B) Duration of probing by neck-pierced (dark circles) and duct-sectioned (white circles) mosquitoes exposed to a membrane-covered saline solution containing ATP (1 mM). Each treatment represents the cumulation of 20 mosquitoes.

important variable in blood-feeding, then host tissues having different vascularization should yield correspondingly different probing periods. In addition, if saliva plays a role in facilitating vessel location, we would anticipate that the duration of probing of salivating mosquitoes would differ from that of non-salivating mosquitoes. Duct-sectioned (saliva-deprived) and neck-pierced (sham-operated) mosquitoes were placed on the shaved back or ear of an anaesthetized guinea pig. We recorded the duration of time between the initial penetration of skin and the appearance of blood in the midgut, as gauged visually under 3× magnification. Probing time of saliva-deprived mosquitoes was prolonged (Fig. 1A) on both the ear and the back (median time 223 s and >420 s). In addition, salivating mosquitoes feeding on sparsely vascularized skin (on the back) probed longer than did mosquitoes feeding on the ear (median time 95 s and 44 s) (Fig. 1A). These experiments demonstrate the usefulness of our measure of probing time in describing the duration of blood vessel location, and prove that the presence of saliva reduces the duration of probing.

Because saliva seems to be important when mosquitoes attempt to feed on skin, it follows that saliva would not facilitate feeding from a pool of medium. Accordingly, we measured probing time of duct-sectioned and neck-pierced mosquitoes placed over a membrane-covered feeding apparatus containing a dyed solution of normal saline and 1 mM-ATP (Fig. 1B). Probing times were brief and nearly identical in both treatment groups, thereby supporting the hypothesis that saliva is important to mosquitoes mainly when blood vessels are sparse.

Biochemical studies

In seeking an explanation for this finding, we suggested that saliva may assist mosquitoes in placing their mouthparts by promoting haematoma formation around blood vessels. Platelet aggregation provides the main limit for such haemorrhage (Mustard & Packham, 1977). Accordingly, we sought evidence that the saliva of

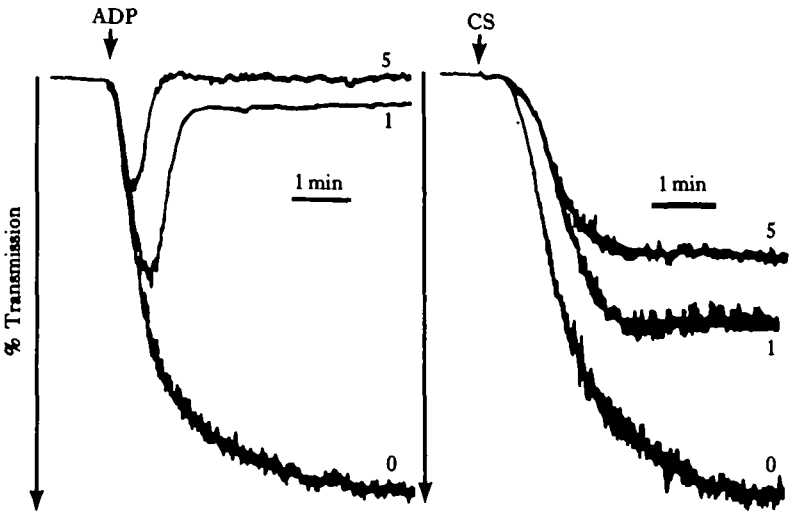

Fig. 2. Aggregometer tracings of ADP- (left) and collagen-induced (right, CS) platelet aggregation of 100 μl human citrated platelet-rich plasma in the presence of *Aedes aegypti* saliva. Inhibition results from the addition of saliva harvested from one or from five mosquitoes as indicated.

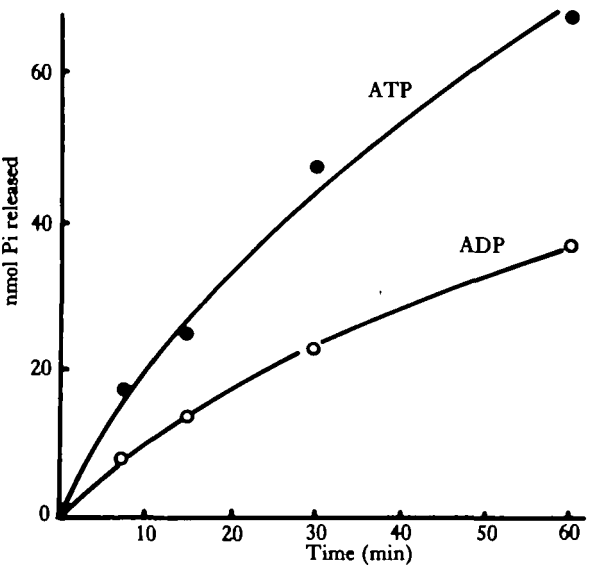

Fig. 3. Orthophosphate released from ATP or ADP by the saliva secreted by a single mosquito. Reaction media contained 100 mM-NaCl, 4 mM-KCl, 2 mM-$MgCl_2$, 2 mM-$CaCl_2$ and 50 mM-Hepes buffer pH 7·5.

mosquitoes may inhibit platelet aggregation. The effect of mosquito saliva on ADP and on collagen-triggered platelet aggregation was assayed in turbidimetric *in vitro* tests employing citrated platelet-rich human plasma. Saliva collected for 10 min from a single insect was sufficient to inhibit platelet aggregation induced by either ADP or collagen in 100 μl of platelet-rich plasma (Fig. 2). Similar results were obtained with homogenized salivary glands. These results demonstrate, for the first time, that mosquito saliva inhibits platelet aggregation, a discovery that supports our hypothetical anti-haemostasis mechanism.

ADP is one of the main physiological factors triggering platelet aggregation (Mustard & Packham, 1977; Vargaftig, Chignard & Benvemiste, 1981), and apyrases (enzymes that break ATP and ADP to AMP and orthophosphate) antagonize ADP stimulation of that process. Accordingly, we sought evidence of apyrase activity in mosquito saliva. Both ATP and ADP were hydrolysed by saliva (Fig. 3), demonstrating apyrase activity in saliva. This enzymatic activity is consistent with inhibition of platelet aggregation.

Preliminary biochemical characterization demonstrated a divalent cation requirement for hydrolysis of both ATP and ADP ($Ca > Mg = Sr$) and an optimum pH plateau for both substrates at pH 8·5–10. Activity was reduced rapidly in saline, but stabilized in the presence of albumin (1 mg ml^{-1}) or detergent (Triton X-100, 0·003 %). No hydrolysis was detected when AMP, glycerol-3-phosphate, *p*-nitrophenyl-phosphate, or pyrophosphate were used as substrates.

DISCUSSION

These experiments demonstrate that mosquito saliva reduces the duration of probing that is required for blood vessel location. We have shown that saliva inhibits

platelet aggregation and have identified apyrase activity in saliva that may fulfill such a function. We suggest that saliva may promote haematoma formation around blood vessels, thereby facilitating location of such vessels. A haematoma would augment the volume of blood in probed tissue and thus present an enlarged target for detection.

A previous report suggested that duct-sectioned mosquitoes experience difficulty in penetrating skin (Mellink & van den Bovenkamp, 1981), an effect that progressively disappears as mosquitoes repeatedly feed at the same site. These observations were attributed to gradual recovery from surgical trauma rather than to salivary deprivation. In contrast, our duct-sectioned mosquitoes appeared to be unimpaired because there was no difference in the way that neck-pierced and duct-ablated mosquitoes probed membrane-covered solutions.

The kissing bug, *Rhodnius prolixus*, possesses a complex anti-haemostasis machinery. Because haemostatis presents redundant pathways to platelet aggregation, its inhibition requires the blocking of two or more pathways. In addition to apyrase activity (Ribeiro & Garcia, 1980, 1981), kissing bug saliva contains anti-serotonin activity (Ribeiro, 1982) and anti-thromboxane activity (Ribeiro & Sarkis, 1982). These properties of saliva allow kissing bugs to inhibit platelet aggregation and shorten probing time. Mosquito saliva must possess another factor or factors in addition to apyrase activity to inhibit platelet aggregation.

Similarity of salivary function between kissing bugs and mosquitoes suggests a common strategy wherein saliva promotes location of blood vessels by inhibiting haemostasis. In contrast to their identical function, the apyrase moieties of triatomine and culicine saliva differ in their biochemical properties. Apyrase activity of triatomines is strictly Ca^{2+}-dependent: that of mosquitoes will react with Mg^{2+} and Sr^{2+} as well. In addition, the optimal pH of mosquito apyrase activity is more alkaline. This evidence of convergent evolution further supports the hypothesis that blood vessel location, through inhibition of platelet aggregation, is a vital function of saliva in these blood-feeding insects. Such a function may be common to other vessel feeders. Indeed, *Glossina* saliva has factors which inhibit platelet aggregation (Mant & Parker, 1981), suggesting that probing may be prolonged in saliva-deprived tsetse flies. Previous studies have shown that salivariectomized *Glossina* fed with difficulty (Lester & Lloyd, 1928).

Salivary apyrase activity in mosquitoes raises a conflict with current concepts on phagostimulation. Both ADP and ATP have been demonstrated to be potent phagostimulants in artificial media (Friend & Smith, 1977), implying that these compounds are the *in vivo* phagostimulants. However, both compounds are degraded by apyrase activity. Interestingly, AMP is a potent phagostimulant of the mosquito *Culex pipiens* (Hosoi, 1958). Recently, 2,3-diphosphoglycerate (DPG), which is unaffected by apyrase activity, has been demonstrated to be a potent phagostimulant of *Rhodnius prolixus* (Smith & Friend, 1982; Macarini, 1983). Such a broad response may provide redundancy or result from adaptation and evolution to a specific mode of feeding. This reasoning implies that AMP or DPG may function as the effective phagostimulant *in vivo*.

Mosquitoes must rapidly engorge on their hosts, and a slow-feeding mosquito may not complete engorgement before irritating its host (Gillett, 1967). Thus, brief

periods of host contact increase survival. Anti-haemostatic components of saliva play such a role by facilitating blood vessel location, thus shortening the duration of vector-host contact.

The work was supported by contract DAMD 17-78-C-8026 from the U.S. Army Medical Research and Development Command, by Grant No. AI-18694 from the NIH, and by Grant No. RF-80019 from the Rockefeller Foundation.

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