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THE ROLE OF THE SALIVARY GLANDS IN FEEDING IN *RHODNIUS PROLIXUS*

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SUMMARY

The effect of salivarectomy on feeding in *Rhodnius prolixus* was studied. Salivarectomized insects drew less blood and at a lower rate than controls when fed on a rabbit. Operated insects also pierced host skin much more often (10 times in 5 min) than controls (1 or 2 times in 5 min). None of these differences was observed when feeding was performed artificially through latex membranes. Intradermal injection of salivary secretion in rat tails increased the duration of bleeding induced by small cuts made on the injection site. It is suggested that the antihaemostatic action of saliva is important for positioning of the maxillae inside blood vessels.

INTRODUCTION

The blood-sucking bug *Rhodnius prolixus* requires a single large meal for each moult and continues to feed on blood throughout adult life. Hosts are located by means of heat-sensitive antennal sensors (Wigglesworth & Gillet, 1934). Once on the host, *Rhodnius* uses its mandibles to penetrate the skin in a series of rapid movements; the maxillae are then thrust into the tissues in a back-and-forth motion and finally rest inside a blood vessel (Lavoipierre, Dickerson & Gordon, 1959), when pumping of the blood begins. Friend & Smith (1971), by observing insects feeding through artificial membranes and measuring changes in electrical resistance between the bug and the diet, showed that once the maxillae are in the meal, probing of the diet and salivation occurs and, if a feeding stimulant is present, pumping begins at a high rate. Ribeiro & Garcia (1980) showed that salivation takes place during the whole gorging phase. It is also known that the salivary secretion contains anticoagulant activity (Baptist, 1941; Hellmann & Hawkins, 1964, 1965) and apyrase activity (Ribeiro & Garcia, 1979, 1980; Smith, Cornish & Wilkes, 1980). These substances may enhance the ability of the insect to draw blood from the host.

In the present work, we report the effect of salivarectomy on feeding of adult *R. prolixus* and discuss the role of salivary secretion in feeding.

METHODS

Insects

All insects were obtained from a colony of *R. prolixus* reared and maintained in the laboratory at a relative humidity of 50–60% and at 25 ± 2 °C. They were artificially fed on human citrated blood every 25–30 days.

Salivarectomy

Adult insects, which had moulted 7–10 days earlier, were operated on as described before (Ribeiro & Garcia, 1980). Insects were fixed for the operation ventral side up on a Petri dish filled with paraffin wax. Two rubber bands extended over the insect prosternum and the abdomen was held in place by entomological pins fixed to the wax. A U-shaped incision was made with a scalpel in the metasternum, limited by the leg bases and extending to the posterior limits of the metasternum. This U-shaped cuticle was lifted with fine forceps and the cherry-coloured salivary glands were usually just below. Each gland with its small accessory gland was lifted with fine forceps and when the salivary duct was apparent, it was cut with ophthalmological scissors; the glands were then removed from the insect. We avoided any unnecessary traction on the duct. The cuticle was then restored to its original place and the wound sealed with paraffin wax, which was melted over the cuticle with a heated pin. The operation took less than 5 min per insect. Sham-operated insects had their cuticles cut open and then closed as described above.

Feeding

Insects were fed either on citrated human blood through a small feeding apparatus using latex membranes (Garcia *et al.* 1975) or over a shaved area of a rabbit abdomen. The insects were kept individually in small jars 4 cm in diameter covered by gauze cloth held in place by rubber bands. Insects were weighed before and immediately after the meal to estimate the amount of ingested food. Observations of feeding in the transilluminated mouse ear were performed as described in Lavoipierre *et al.* (1959).

Measurement of the rate of feeding

This was done by joining a small feeding apparatus (Garcia *et al.* 1975) through tygon tubing to a 0·3 ml pipette. This was held horizontally in order to keep the pressure constant on the feeding membrane, preventing it from mechanical displacement during the experiment. The surface area of the latex membrane was not greater than 1 cm^2. A three-way valve was connected between the feeder and the pipette to allow the pipette to be refilled quickly for the next experiment. A 1 mm movement of the meniscus in the pipette corresponded to a 1 μl volume change, the resolution of this potometer. The feeder was warmed to 38 °C. The vertical distance of the latex membrane was about 2 cm below the pipette. The insects were presented individually in a 25 ml beaker covered by gauze cloth. The volume in the pipette was noted at each 30 s, starting when the insect pierced the membrane. For these measurements either plasma or saline (0·15 M-NaCl) containing 0·1 mM ATP was used, avoiding the problem of sedimentation of red blood cells inside the apparatus when using whole citrated blood.

Bleeding time

A modification of the rat tail preparation (Cruz, 1965) was used for these measurements. The isogenic lineage RA (Amsterdan) was used. In the same tail, at different places but not at the same time, 1 μl of saline and 1 μl of salivary secretion diluted in saline were injected with a Hamilton syringe 1–2 cm from the tail end. The syringe was not removed until 30 s elapsed from injection time. After 90 s, a small cut on the injection site was made with a razor blade, 1 mm deep and 5 mm wide. Each 30 s the blood accumulated at the edge of the wound was carefully removed with filter paper, and the time of bleeding arrest was noted. Half of the individual experiments began with the injection of saline and half with the injection of salivary secretion. The second injection was made immediately after the bleeding of the preceding cut had stopped. The results were tested by mean of paired *t* tests.

Salivary secretion

This was obtained as described before (Ribeiro & Garcia, 1980). From 600 to 1000 5th-stage and adult bugs were allowed to probe water for 5 min through a thin latex membrane in a heated feeding apparatus. The solution containing the salivary secretion was freeze dried and later reconstituted with 0·15 M-NaCl.

Protein determination

The technique of Lowry *et al.* (1951) was used, with bovine serum albumin as a standard.

Probing behaviour

In some experiments the probing behaviour (defined as a set of sequenced actions that include the extension of the rostrum, the contact of the labium with the host skin, piercing of host skin by the mandibles and the maxillary movements inside host tissues) was evaluated by measuring the number of piercing events inflicted by an insect to its host as a function of time. A piercing was easily identified by the rapid alternate movements of the head that pushes the mandibles inside the host skin.

RESULTS

Preliminary observations and precautions

Preliminary observations showed that in artificial feeders salivarectomized insects could feed on blood normally. In order to select for the experiments those insects that did not present post-surgical complications, the following scheme was adopted throughout the work: insects were operated between 7 to 10 days after the imaginal moult and were fed artificially on blood 4–5 days after surgery. In the fourth day after the surgery, mortality in both salivarectomized and sham-operated insects was not greater than 20%. One month after this blood meal, survival of both groups of operated insects was usually 60% (control was *ca.* 95%). If this population of operated insects was then allowed to feed regularly, the insects survived normally when compared to the controls. We thus employed throughout the work insects that had been

operated on 30–36 days before and had been artificially fed 4–5 days after the surgery. Care was taken to include the same number of males and females in each of the salivarectomized, sham-operated and control groups. The average insect weight of each group at the time of the experiment was not statistically different when tested by analysis of variance.

Observations with the transilluminated mouse ear preparation

The detailed observations of Lavoipierre *et al.* (1959) were confirmed with normal insects. After penetration of the skin, the flexible golden maxillae extend from the mandibles and freely move through the host tissue. In this movement, small vessels can be punctured and more or less extensive haemorrhages several times wider than the vessel radius can be formed. This is the striking feature that is *not* observed when using salivarectomized insects. These probe in an identical manner to the controls and their maxillae move inside host tissue to the same extent and frequency, but when puncturing small vessels, *very small* haemorrhages are collected. This difference can be seen with the naked eye. When control or sham-operated insects probe the mouse ear for 20–30 s, a small reddish area becomes immediately visible at the site of probing. This was never seen with salivarectomized insects (8 different insects probing at least 5 times each insect, each probing lasting from 15 s to 1 min). Finally, in all groups of insects studied, we observed that the maxillae occasionally rested in a blood vessel (arteriole or venule) and then pumping could be followed by the almost complete occlusion of the vessel, which seems to beat regularly. Eventually a reverse pumping is observed, as described by Lavoipierre *et al.* (1959).

Observation of feeding on rabbits

The mouse ear preparation, although well suited for microscopical observations, is unsuitable for large-scale work. We therefore observed feeding on the shaved abdomen of the rabbit to study the behaviour and the amount of blood ingested by salivarectomized insects compared to controls. The insects did not differ in their readiness to probe the skin. However, one striking feature of salivarectomized insects was their difficulty in initiating pumping. In contrast to the controls (normal as well as sham-operated bugs), which usually pierced the host skin only once or twice before beginning to gorge, salivarectomized insects usually pierced several times for several minutes. These findings could be easily quantified by measuring the number of piercings per insect as a function of time (Fig. 1). Note that salivarectomized insects pierced the rabbit skin on average once each 30 s for at least 5 min. Sometimes, however, a salivarectomized bug after probing several minutes seems to find a vessel and sucks for several minutes. Control insects completed the meal in 8–12 min. To study the amount of food ingested by each insect, we presented the insects to the rabbit in small jars that were weighed (jar + insect) immediately before the meal; we then exposed the insects for 10 min and immediately after we weighed each jar + insect to assess the amount of food taken. Time was registered after the first piercing (detected by the rapid alternate movements of the head that pushes the mandibles inside host skin) and in all cases the insects were actively engaged in probing or sucking. Insects were discarded if they stayed for more than 30 s without the labium contacting th

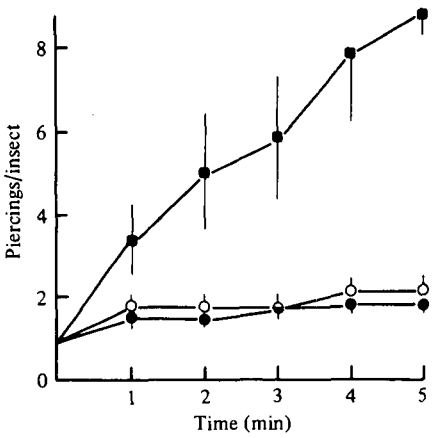

Fig. 1. Number of piercing events as a function of time in salivarectomized (■), sham-operated (○) and control (●) insects fed on a rabbit. Zero time was arbitrarily fixed on the first piercing. The symbols and bars are the mean ± S.E. of five determinations.

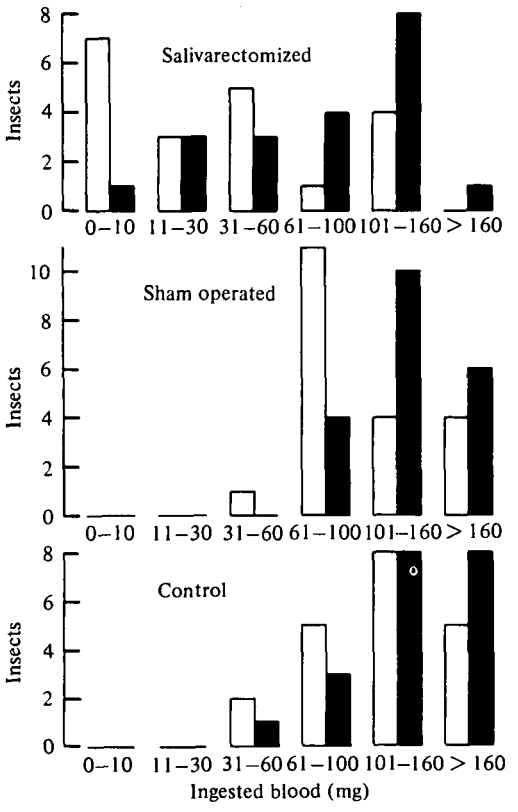

Fig. 2. Amount of blood ingested by salivarectomized, sham-operated and control insects fed on a rabbit. Left (light) bars represent frequency of insects that fed on indicated ranges of blood in a 10 min exposure. Right (dark) bars represent data obtained after a second exposure of 20 min, with a total exposure time of 30 min. Interval between exposures was less than 2 min. Twenty insects were used in each group.

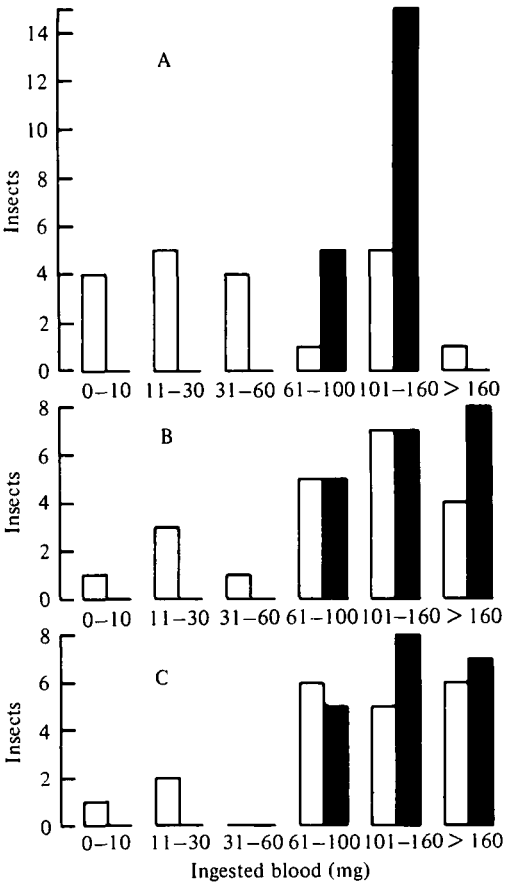

Fig. 3. Amount of blood ingested by salivarectomized (light bars) and non-operated control insects (dark bars) fed daily for 3 days on a rabbit. The bars represent frequency of insects that fed on indicated ranges of blood in a 30 min exposure. (A) First meal; (B) sum of first and second meals; (C) sum of the three meals.

rabbit skin after the first piercing; these comprised less than 10% of the insects and occurred in all groups tested. Two minutes after the initial 10 min period, all the insects were exposed again to the rabbit for an additional period of 20 min. Although many of the salivarectomized bugs were still feeding or trying to feed after the end of this second exposure, we did not increase the duration of the experiment in an attempt to avoid errors due to urine losses (Wigglesworth, 1931). The frequency distribution of the different groups of insects as a function of the amount of blood ingested is shown in Fig. 2. It is clear that salivarectomized insects drew less blood from a rabbit than did their controls. Note that even after the two exposures (total of 30 min), they took less blood than the controls did in 10 min. To test the significance of the distribution differences, we divided the three distributions obtained at the higher exposure time into two groups – those below and those above 100 mg of blood intake, and applied a χ^2 test (7·549; 2 degrees of freedom; $P < 0{\cdot}025$). This significant difference is obviously attributed to salivarectomy. A single, continuous exposure of 30 min wit a different lot of insects is shown in Fig. 3A.

If repeated feeding opportunities are given to salivarectomized insects, they may take a 'normal' amount of blood from a living rabbit. We exposed operated and control insects to the rabbit for 30 min once a day for a period of three days, and the additional ingested blood in each meal was added to the amount accumulated the day before. We observed (Fig. 3) that after the third meal the operated insects drew more additional blood than the controls, the amount taken approaching that taken by the controls.

Artificial feeding

The effect of salivarectomy on the amount of blood ingested was studied using artificial feeders (see Methods). Salivarectomized insects probed normally and ingested the same amount of blood as their controls (Fig. 4). We also studied the kinetics of feeding of these two groups of insects, using a potometer that presented plasma (0·1 mM-ATP added) through a latex membrane. Fig. 5 shows the kinetic profile of six insects for each of the two groups. In all cases, an initial phase lasting from 0·5 to 2 min could be observed with a slow pumping rate, probably related to phase I (probing and sampling), as described by Friend & Smith (1971). This phase was characterized by intermittent antennal movements, seen in both groups. A second phase followed, with a rather constant pumping rate, ranging from 22 to 36 μl/min, corresponding to phase II of Friend & Smith. The insects were apparently at complete rest and did not move the antennae. Sometimes a third phase was seen, with a slower rate and lasting a few minutes or less. Again, antennal movements were observed. Similar results were obtained when saline instead of plasma was used. The pumping rates of the second phase were $29{\cdot}3 \pm 1{\cdot}7$ and $27{\cdot}9 \pm 1{\cdot}7$ $\mu l.min^{-1}$ for control and salivarectomized insects respectively (mean $\pm$ S.E., $n = 6$). This experiment demonstrates that the kinetics of feeding on artificial diets is not changed by the salivarectomy and is also important as a control of the surgical procedure, showing that no post-surgical trauma affected the pump function or the food canals.

This set of experiments showed that no differences could be detected between salivarectomized and control insects when they were fed through artificial membranes, which is in clear contrast with the results observed when the insects were fed on a rabbit. One hypothesis that could explain the differences observed in the two feeding

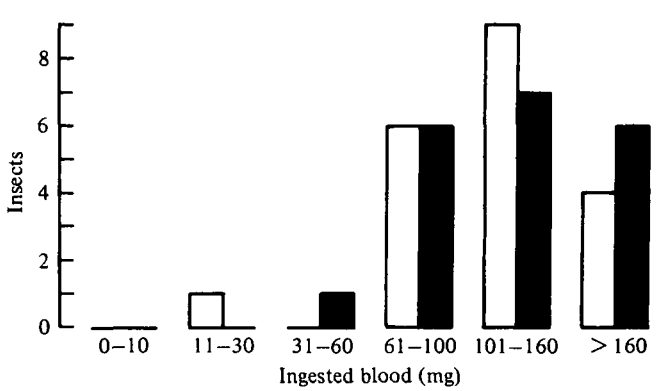

Fig. 4. Frequency distribution of salivarectomized (light bars) and non-operated controls (dark bars) that fed on indicated ranges of blood through an artificial feeder. Exposure time was 30 min.

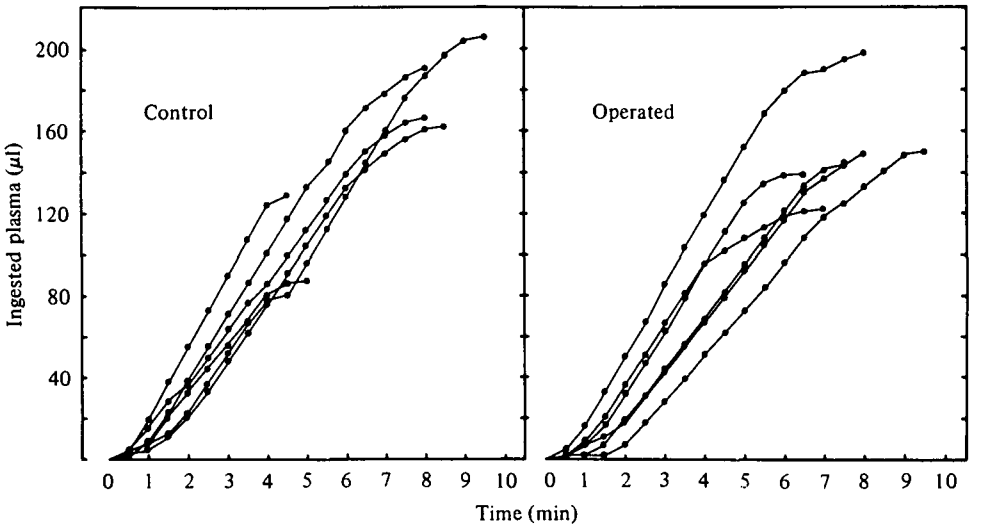

Fig. 5. Plasma ingestion profile as a function of time in 6 non-operated controls and 6 salivarectomized insects fed on a potometer.

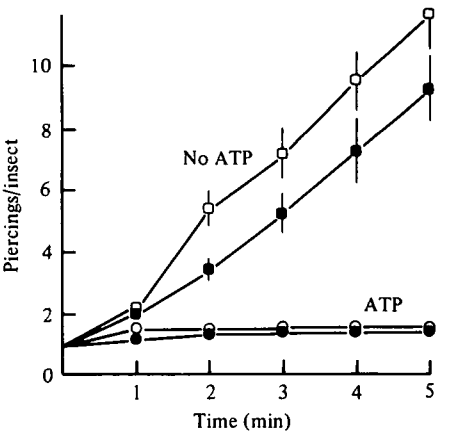

Fig. 6. Number of piercing events as a function of time in salivarectomized (open symbols) and non-operated control (closed symbols) insects artificially fed on saline (0·15 M-NaCl) with (circles) or without (squares) 1 mM-ATP. The symbols and bars are the mean ± S.E. of six determinations.

systems is that the salivary secretion should be important in the formation of haemorrhages after punctures of the vessels by the maxillary probing and thus exposing the gorging stimulants (Friend, 1965) necessary for triggering the pumping of the diet. To test this hypothesis we exposed normal controls and salivarectomized bugs to an artificial feeder containing either 0·15 M-NaCl or 0·15 M-NaCl plus 1 mM-ATP (Fig. 6). It is clearly seen that repeated piercing is observed for both groups only in the absence of ATP. The small difference in piercing frequency between salivarectomized and control insects in the absence of ATP was not statistically significant when tested by a *t* test. This experiment shows that the absence of gorging factors in th

Table 1. *Effect of the salivary secretion on duration of bleeding in rat tails*

Rat	Control (min)	Saliva 20 mg/ml (min)
1	2·5	5·5
2	1·5	4·5
3	5·5	6·5
4	3·0	10·5
5	2·5	5·0
6	2·5	4·0
$\bar{X} \pm$ S.E. ...	2·9 ± 0·6	6·0 ± 1·0
Rat	**Control (min)**	**Saliva 100 mg/ml (min)**
7	3·5	> 30
8	2·0	> 30
9	3·0	> 30
10	4·5	> 30
$\bar{X} \pm$ S.E. ...	3·3 ± 0·5	–

artificial feeder induces probing behaviour for normal as well as salivarectomized insects which mimics that observed for the operated insects fed on a rabbit; this suggests that salivary secretion exposes the phagostimulants when feeding on a rabbit. This hypothesis will be further developed in the discussion of this work.

Salivary secretion and bleeding time

The small haemorrhages seen when normal insects probe host skin, together with our recent finding of platelet-anti-aggregating activity in the salivary secretion of *Rhodnius prolixus* (Ribeiro & Garcia, 1981) prompted us to examine the effects of the salivary secretion on bleeding time. Our freeze-dried salivary secretion was reconstituted with saline to give a protein concentration of 100 mg/ml or 20 mg/ml and was injected intradermally in rat tails (see Methods). At the lower concentration, bleeding time was doubled ($p < 0{\cdot}025$) (Table 1), and at the higher concentration bleeding time was greatly increased and was always longer than 30 min. In these cases, bleeding stopped after 45 min or more but could begin again spontaneously or with gentle manipulation of the wound. If the blood was collected from the wounds into glass tubes, either immediately after the cut was done or at any other time, it could coagulate normally after a few minutes.

Observation of the crop contents following blood sucking in the rabbit

It is known that the ingested blood does not coagulate in the crop of *Rhodnius prolixus* (Wigglesworth, 1972). Hellmann & Hawkins (1965) detected an anti-factor VIII in the salivary glands of *Rhodnius* that was also found in the stomach, after the meal. They also found in the crop a specific anti-thrombin not found in the salivary glands. In order to verify the relative role of the salivary anticoagulant in the state of the ingested blood in the crop, we observed the crop contents following blood sucking in a rabbit. We used salivarectomized insects that were operated on 2 months before and fed on the fourth and on the 32nd day after surgery. On the 60th day they were exposed to a rabbit, and only those insects that took enough blood to distend the abdomen

were used. Four insects were studied at each interval of 1, 24 and 48 h after the meal. The crop was dissected out and then opened. In all cases the contents were fluid and easily pipetted with a 50 μl pipette. When transferred to a test tube containing 0·5 ml 0·15 M-NaCl and gently shaken, the contents dispersed easily. No sign of a clot was observed in the twelve insects. These results could not be explained by the remains of previous meals containing salivary anticoagulin, because the insects were fed twice without the salivary glands before the experiment. We conclude that the presence of the crop anti-thrombin (Hellmann & Hawkins, 1965) may explain this result. We never found in these insects any remains of the salivary glands: this excludes the possibility of regeneration.

DISCUSSION

The main findings in this work may be summarized as follows. Salivarectomized insects could feed with difficulty in a rabbit, ingesting less blood than controls when a 'normal' length of time was allowed for the meal. However, they could feed normally in an artificial feeder. The difficulty was clearly related to the beginning of the meal, as indicated by the repeated piercing of the rabbit's skin (Fig. 1). Normal insects, unlike the salivarectomized, could produce more or less extensive haemorrhages during probing. Saliva was shown to increase the duration of bleeding in rats. Finally, normal and salivarectomized insects probed in a similar manner when presented to an artificial feeder with or without phagostimulants.

Role of the salivary secretion

Lavoipierre *et al.* (1959) reported that *Rhodnius prolixus*, as well as other triatomines, are vessel feeders, i.e. they suck blood directly from blood vessels (venules or arterioles). Haemostasis of these small vessels is well known today to be the consequence mainly of platelet aggregation and also possibly the muscular response of the vessel (Mustard & Packam, 1977). Although coagulation is important in the stabilization of the platelet plug, preventing coagulation does not increase the bleeding time of small wounds. However, if platelet aggregation is impaired, bleeding time increases (Mustard & Packham, 1977). We recently demonstrated that the salivary secretion of *Rhodnius* prevents platelet aggregation *in vitro*, and that this activity was associated with more than 3 substances, including the apyrase activity, which can deplete di- and trinucleotides released by platelets and injured cells (Ribeiro & Garcia, 1979, 1980, 1981). In the present work we demonstrate that the salivary secretion can induce an increase in bleeding time, as expected. The concentrations of salivary secretion used are considered to be physiological: Smith *et al.* (1980) measured the protein concentration of saliva removed from salivary glands and found a value of 250 mg/ml. We found a higher value of 324 ± 17 mg/ml (mean $\pm$ S.E., $n = 8$), when measuring the concentration of saliva drawn from single salivary glands using micropipettes of 70–150 nl.

The antihaemostatic action of the salivary secretion, together with the observation of Lavoipierre *et al.* (1959) that *Rhodnius prolixus* is a vessel feeder, and the probing behaviour of salivarectomized insects, suggest that the salivary secretion is important in that it produces more or less large haemorrhages that make blood available for ingestion in the probing phase. This indicates for the insect that a vessel exists in th

probing area, encouraging the insect to continue maxillary probing in that particular region and finally find the vessel. It should be noted here that if the mouse ear is considered to be a flat surface, the area occupied by venules and arterioles is a very small fraction of the total and the probability that random maxillary probing would discover a vessel is small. When the insect does not, after a given length of time (probably 20–40 s), find the phagostimulants, they discontinue probing and begin again in a different region. This hypothesis is supported by data given in Figs. 1 and 6, where the insects are artificially fed and the probing behaviour of normal and salivarectomized insects is shown to depend exclusively on the presence of phagostimulants. This hypothesis also explains the flat distribution of blood ingestion in salivarectomized insects (Fig. 2) when compared to the more centrally dispersed distribution in normal insects. If by chance a salivarectomized insect finds a suitable vessel, it could then ingest a large amount of blood in a small amount of time.

The antihaemostatic activity in the salivary secretion may also be important in the maintenance of feeding by assuring a continuous flow of blood. The 'reverse pumping' bursts observed in Lavoipierre *et al.* (1959) are perhaps important in this respect. We showed (Ribeiro & Garcia, 1980) that saliva is injected continuously during the whole meal and then, in a reverse burst, saliva may be ejected further along the feeding vessel and, together with the increase in hydrostatic pressure (seen by the sudden enlargement of the vessel), remove the platelet plugs that may be obstructing the flow.

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