THE FEEDING RESPONSE OF RHODNIUS PROLIXUS TO BLOOD FRACTIONS, AND THE ROLE OF ATP

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SUMMARY

Second-instar larvae of Rhodnius prolixus will gorge on washed human erythrocytes suspended in Ringer, but not on plasma, either with or without platelets. The ED 50 of erythrocytes is 4.5 vol of packed cells per 1000 vol of Ringer. Erythrocyte ‘ghosts’ are non-stimulatory, whereas the haemolysate induces gorging. Phagostimulation by ‘ghosts’ is restored by resealing them in 1 mM adenosine triphosphate. Evidence is presented that Rhodnius responds to blood via a release of ATP from a few erythrocytes close to the epipharyngeal sense organs.

INTRODUCTION

A broad variety of blood-feeding insects have now been shown to gorge on artificial diets if the diets contain adequate concentrations of nucleoside phosphates, particularly adenosine triphosphate (ATP). The relevant literature has recently been reviewed by Galun (1975) and Friend & Smith (1977). In whole blood, ATP is present in red cells (RBCs) at a concentration of about 1 mM (Bishop, Rankine & Talbot, 1959; Lew, 1971). Platelets also contain quantities of ATP which can be released in the presence of appropriate triggers (Holmsen, 1972). Plasma normally contains little if any ATP, and indeed possesses ATPases capable of hydrolysing any ATP present (Bishop et al. 1959; Mills, 1966). Despite the presence of ATP in blood, there is little direct evidence that it acts as a gorging stimulant to an insect feeding on blood as opposed to an artificial diet. Blood cells have been reported to elicit gorging in the mosquito Culex pipiens (Hosoi, 1959), tsetse flies (Galun & Margalit, 1969), fleas (Galun, 1966), and Rhodnius prolixus (Friend, 1965). Galun & Rice (1971) present evidence that it is the platelets rather than the RBCs which trigger gorging in Aedes aegypti and suggested that in ‘normal’ separation of blood cells and plasma, the platelets are spun down with the RBCs and might therefore generally be the source of stimulation by the cellular fraction of blood. Hosoi (1959) pointed out the difficulty in the hypothesis that it is red cell ATP that induces gorging in vivo, since RBCs are usually found intact in the midgut. Indeed, the platelet release mechanism provides a means by which blood-feeding insects could respond to blood; a variety of triggers including wounding causes platelets to release ATP and ADP into plasma to produce concentrations as high as 2.8 μmol per 10^{11} platelets (Mills & Thomas, 1969). At their stated value of 4.65 x 10^8 platelets per ml of plasma, this would be equivalent to approximately 13 μM ATP.
Unfortunately, the minimal concentrations of ATP needed to induce gorging have only been adequately measured for *Rhodnius*. In this species, 3.8 \(\mu\)M ATP in a 150 mM NaCl solution will cause 50% of test insects to gorge (Smith & Friend, 1976a). For other insects, only a few doses have been tested, and these were often as high as 10 mM. Such a concentration may be substantially higher than anything blood-feeding insects experience *in vivo*.

This paper reports experiments to determine which components of blood induce gorging in *Rhodnius prolixus*.

**Material and Methods**

Second-instar larvae of *Rhodnius prolixus* from a laboratory colony were used in tests 25–30 days after their previous blood meal.

Two types of feeding apparatus were used for gorging experiments. For preliminary experiments, a simplified version of previous feeding chambers (e.g. Smith & Friend, 1976a) was used. Test insects were transferred gently into a Plexiglass cylinder (4.5 cm o.d.) closed at one end by a membrane of condom-rubber held in place by a rubber O-ring; a 1 cm wide piece of absorbent paper folded into the shape of a W and resting on its edge on the membrane provided footing for the insects, which would feed head-down through the rubber membrane. Diets were placed into siliconized Petri dishes with a diameter slightly greater than the insect chambers; the dishes were then put on a slide-warmer adjusted to maintain the diet at 36–38 °C. For testing, the insect chambers were put into the Petri dishes, membrane-end down, so that the diet was trapped under the rubber membrane. In this way, as many as 30 second-instar insects could be tested at one time on as little as 1 ml of diet.

For testing diets containing dilutions of red cells, a special apparatus was devised to ensure the cells would stay in suspension and remain evenly distributed throughout the diet. Diet chambers consisted of a Plexiglass cylinder approximately 4 cm inside diameter and 1 cm high, closed at one end with a Plexiglass plate, and covered, after filling with warmed diet, with a condom-rubber membrane secured with a rubber O-ring. Test insects were placed in feeding chambers consisting of Plexiglass cylinders closed with nylon mesh through which the insects could probe and feed. The feeding chambers and diet chambers were connected with tightly wrapped Parafilm, and then attached to the outer surface of a 30.5 cm (12 inch) kymograph drum with sticky wax (Fig. 1). The electrically driven kymograph was positioned with the axis of drum-rotation horizontal. The drum was kept warm by aiming a 250 W infra-red lamp at its inner surface. This ‘Ferris Wheel’ was rotated about once every 30 s, keeping the red cells in suspension.

For each test, insects were exposed to the diet for 20 min. The degree of feeding was estimated for each insect by eye. Insects which had not ‘sampled’ the diet showed no change in abdominal shape, and were rejected from the data; it had been found by comparing weights before and after testing that such non-sampling insects could be reliably recognized by eye. There were rarely more than 5% in this category. Insects which had sampled were either maximally (or close to maximally) gorged, or showed only the slight abdominal distension characteristic of prolonged sampling on a non-stimulatory diet (Smith & Friend, 1970). Results were therefore recorded as number
Feeding response of *R.* prolixus

Fig. 1. Apparatus for presenting diets containing dilutions of red blood cells to *Rhodnius* larvae. The diet chamber (D) is attached to the surface of a revolving kymograph drum (K) with sticky wax (arrow). The insects are housed in the feeding chamber (F) which is attached to the diet chamber with tightly wrapped Parafilm (not shown). The nylon mesh (N) and rubber membrane (R) are secured with rubber O-rings (black circles).

gorging out of number sampling. For dose–response curves, a minimum of 25 insects were tested at each dose; usually 60–120 were used. These results were analysed with a computerized probit technique.

Human blood was obtained by venipuncture and collected into 10 ml Vacutainer tubes containing 143 USP units of sodium heparin. Platelet-rich plasma (PRP) was prepared by centrifuging freshly drawn blood at approximately 400 \( g \) for 10 min. Platelet-poor plasma (PPP) was prepared by spinning PRP at about 1500 \( g \) for 10 min. Red cells were obtained by centrifuging the blood at 400 \( g \), removing the PRP and buffy coat, washing three times in twice the volume of Ringer solution and finally resuspending in the appropriate volume of Ringer. For preparation of red cell ghosts, washed red cells were prepared from fresh citrated beef blood, and were lysed in 9 vol of distilled water containing 2 mM-MgCl₂. The resulting ghosts were washed three times with this solution, then resuspended in an appropriate quantity of Ringer (Dodge, Mitchell & Hanahan, 1963). In some experiments, human red cells were lysed by adding 4 vol of distilled water to a 50% suspension of washed cells. After centrifugation, the membrane fraction was discarded, and the haemolysate was restored to isotonicity by adding concentrated Ringer.

The Ringer solution used for test diets was a standard *Rhodnius* Ringer containing 128 mM-NaCl, 8 mM-KCl, 4 mM-MgCl₂, 1.8 mM-CaCl₂, 10 mM-NaHCO₃ and 3.2 mM-NaH₂PO₄. Although this is an insect Ringer, its composition is very close to that of various mammalian Ringer solutions, and red cells were found to maintain their normal shape, fragility and internal potassium levels when suspended in it. For some diets, a simple 150 mM-NaCl solution was used. All solutions were made up in glass distilled water and adjusted to pH 7.0.
Table 1. Response of *Rhodnius prolixus* to whole blood and blood fractions.

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. sampling</th>
<th>No. gorging</th>
<th>% Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>65</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>Platelet-rich plasma</td>
<td>53</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Platelet-poor plasma</td>
<td>51</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>50% RBCs*</td>
<td>61</td>
<td>61</td>
<td>100</td>
</tr>
<tr>
<td>Ringer (control)</td>
<td>244</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>5% haemolysate</td>
<td>63</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>1% haemolysate</td>
<td>67</td>
<td>64</td>
<td>96</td>
</tr>
<tr>
<td>50% bovine RBCs</td>
<td>66</td>
<td>65</td>
<td>98</td>
</tr>
<tr>
<td>50% bovine RBC ghosts</td>
<td>121</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>50% ghosts resealed in 2 mM ATP</td>
<td>48</td>
<td>42</td>
<td>88</td>
</tr>
</tbody>
</table>

* All diluted blood fractions are in Ringer.

**RESULTS**

Table 1 shows the proportions of test insects gorging on whole blood, platelet-rich plasma (PRP), platelet-poor plasma (PPP), resuspended red cells (RBCs), and Ringer solution. All blood diets show appreciably higher potency in eliciting gorging than the Ringer control. However, only the RBC diet matched the potency of whole blood. The plasma diets were poor gorging stimulants; in contrast to the 100% response to whole blood and RBCs, the response to these diets was less than 20%. Microscopic examination of the resuspended RBCs revealed no significant contamination by platelets, although they were plentiful in the PRP.

Responses were measured to two dilutions of haemolysate obtained by spinning down lyed red cells, and to resuspended ‘ghosts’. Since bovine RBCs were used to make the ghosts, intact bovine RBCs were tested. Table 1 shows that the phagostimulatory power of the RBCs is lost with their contents. The haemolysate however is a powerful phagostimulant even at a dilution of 1% (expressed in terms of original packed cell volume). Responses to intact bovine RBCs and to intact human RBCs are similar. Microscopic examination of the ghosts revealed a few intact cells, perhaps explaining the slightly higher response compared to the Ringer control. Although the haemolysate preparation was not completely membrane-free, lack of response to the concentrated ghosts indicates that the stimulatory power of the haemolysate lies in the soluble fraction rather than associated with cell membranes. The response to ghosts resealed in the presence of 2 mM ATP and then rewashed in ATP-free Ringer is also shown in Table 1; it is almost as high as the response to intact RBCs.

Red blood cells are highly potent gorging stimulants. Since their ATP content is far higher than the level necessary to induce 50% gorging, the responses to increasing dilutions of RBCs were determined. The results are presented in Fig. 2 and Table 2. All experiments were performed using the ‘Ferris Wheel’. Aliquots of the RBC suspensions were assayed for ATP by the luciferin-luciferase method of Strehler & Totter (1954). Back-calculation showed the internal concentration of ATP of red cells as approximately 1 mM. For comparison with ATP solutions, the dose–response curve for RBCs was plotted as volume fraction x 10^-3, thus also giving ATP concentration as moles per litre of suspension. Table 2 gives calculated values for the dose giving a 50% response (ED 50) with confidence limits, and the slopes of the relationship between probit response and log dose.
Table 2. Response of second-instar Rhodnius prolixus to different doses of ATP and red blood cells in 0.15 M-NaCl and Ringer

<table>
<thead>
<tr>
<th>Diet</th>
<th>ED50 (µM)</th>
<th>Slope*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP in NaCl</td>
<td>3.1 (2.7-3.6)†</td>
<td>3.0 (2.3-3.6)</td>
</tr>
<tr>
<td>RBCs in Ringer</td>
<td>4.5 (3.9-5.2)‡</td>
<td>1.9 (1.6-2.2)</td>
</tr>
<tr>
<td>RBCs in NaCl</td>
<td>2.7 (2.2-3.2)</td>
<td>2.0 (1.6-2.3)</td>
</tr>
<tr>
<td>ATP in Ringer</td>
<td>27.6 (24.1-31.6)</td>
<td>2.4 (2.0-2.7)</td>
</tr>
</tbody>
</table>

* The slope is probit response on log dose.
† Confidence limits at P = 0.05.
‡ Doses of RBCs are expressed as equivalent ATP, assuming an endogenous concentration of 1mM ATP (see text). Thus actual concentrations of RBCs are the figures in the table x 10^-3.

Fig. 2. Response of Rhodnius to ATP and red blood cells. The RBC 'concentration' is the volume fraction x 10^-3; whole blood would be about 400 on this scale. The RBC/ATP concentration is the estimated amount of ATP per volume of total test diet, cells plus suspension medium. Curves are fitted by computerized probit techniques. Solid curves, RBCs; dashed curves, ATP; solid triangles, RBC/NaCl, open triangles, RBC/Ringer; solid circles; ATP/NaCl; open circles, ATP/Ringer.

The dose–response curve for ATP in 150 mM-NaCl had values for both ED50 and slope similar to those reported previously (Smith & Friend, 1976a, b), showing that rotation of the feeding chambers had no apparent effect on the gorging response. The ED50 for RBCs in Ringer, expressed as ATP concentration, is close to the ED50 for ATP in NaCl, although somewhat higher. The slopes of these two dose–response curves are significantly different (P = 0.05). These two diets differ in the ionic composition of the media as well as the 'packaging' of the ATP; thus the potencies of ATP in Ringer and RBCs in NaCl were also determined. Table 2 shows that the ED50 of the ATP response is affected by the nature of the medium much more than the ED50 of the RBC response. The slope of the ATP/Ringer curve is intermediate between the slopes of the ATP/NaCl and RBC/Ringer curve. The two RBC dose–response curves are parallel to each other. Statistical tests showed that the ATP/Ringer curve did not depart significantly from parallelism compared to either the two RBC curves or the ATP/NaCl curve.
DISCUSSION

It is clear from the results that it is red blood cells rather than platelets that act as gorging stimulants for *Rhodnius prolixus*. This insect thus seems to differ from *Aedes aegypti* which according to Galun & Rice (1971) responds best to the platelet fraction. The response to platelet-rich and platelet-poor plasma was slightly higher than to the Ringer control, however, although very few RBCs could be seen when samples were examined under the microscope. A low level of response is not surprising. For *Rhodnius*, the ED50 for ATP in 0.15 M-NaCl is about 4 µM. Normally there is little or no ATP in plasma (Bishop *et al.* 1959), but a level of 4 µM could presumably be reached by activation of only some of the platelets since treatment with thrombin can produce plasma ATP concentrations of approximately 13 µM (Mills & Thomas, 1969). This could occur during any stage of preparation of the blood or filling of the feeding apparatus; although the Plexiglass and rubber membranes of the feeding apparatus are technically non-wettable, they may still cause some platelet reaction. It would be of interest to assay PRP for its free ATP content during such experiments.

Because of the much greater potency of RBCs in eliciting gorging this study concentrated on the RBC fraction. Hosoi (1959) found that it was a water-soluble fraction of the contents of RBCs that induced mosquitoes to gorge, rather than the stroma. Similar results were found for *Rhodnius*. Suspensions of washed ghosts at concentrations as high as 50% were non-stimulating, whereas a 1% dilution of the isotonic haemolysate was almost as effective as the 50% intact cells. The possibility remains that the potency of the haemolysate is fortuitous, and that normally the intact cell is stimulatory; haemolysis may have changed the cell membrane in some way rendering it impotent. Some evidence against this is provided by the response to ghosts ressealed in an ATP-containing medium. Although the cell contents would be highly abnormal, the ability of a 50% cell suspension to elicit gorging was largely restored by this treatment. Nevertheless, this result should be treated with caution, since ressealed ghosts are not normal cells, and their ATP may leak out much more readily than from intact cells.

The results of the quantitative potency measurements are highly interesting. ATP has an ED50 in NaCl of 3.3 µM, slightly lower than reported previously (Smith & Friend, 1976a). The ED50 for ATP in Ringer is higher by almost an order of magnitude than the ED50 of ATP in NaCl, however. This elevation may be due to the presence of an ATPase in *Rhodnius* saliva, previously reported in Smith & Friend (1976b). The ATPase is Ca-dependent and is therefore virtually inactive in 150 mM-NaCl, but highly active in Ringer (Smith, in preparation). When an insect samples an ATP/Ringer diet, some of the ATP may be hydrolysed before it reaches the putative chemoreceptive sensilla of the epipharynx, and thus its effective concentration is reduced. Indeed, direct observation of maxillary probing into an artificial diet (Friend & Smith, 1971) reveals that saliva is continuously ejected into the diet from the closed tip of the maxillae before the first samples are taken up. Since the diet is usually sampled from the region where saliva has been added, there is a period of at least several seconds for an active ATPase to hydrolyse ATP molecules. Of course, alternative explanations for the reduced potency of ATP in Ringer are possible. For example,
Feeding response of R. prolixus

in the presence of Ca, ATP exists preferentially in a chelated form. *Rhodnius* may be less sensitive to Ca-chelated ATP than to unchelated ATP.

Although the ED50 for ATP in NaCl and Ringer differs by more than 10 times, the ED50 for RBCs in NaCl and Ringer differs by less than 2 times. It is obvious that the nature of the medium (Ringer or NaCl solution) affects the response to RBCs much less than the response to free ATP. If the ATP of the red cells were protected from the salivary ATPase almost until the moment of stimulation, then it would make little difference whether or not the salivary ATPase were active. The result supports the suggestion that ATP is released close to the sensilla and not before; the slightly higher ED50 for RBCs in Ringer may reflect a brief period between release and diffusion to the chemoreceptive cells, and hence a brief period during which the ATPase can reduce the effective concentration of ATP.

The slopes of both RBC curves are parallel, but depart significantly from parallelism compared to the ATP/NaCl curve. Dose–response curves which are not parallel are held to indicate different mechanisms at work; the slope is a measure of the standard deviation of a threshold normally distributed among the population. It is likely that the curve for ATP/NaCl reflects the threshold for response to ATP stimulating chemoreceptors of the epipharyngeal sensilla; the variation in threshold could be at the receptor site itself, or in the sensitivity of a central response mechanism linking stimulation with activation of the pumping phase. The lesser slope of the RBC curves may be due to a mechanism interposed between the RBCs and the ATP-receptive chemosensilla, for example, involved in the release of ATP from the red cells.

RBCs in NaCl and Ringer had ED50 values of $2.6 \times 10^{-3}$ and $4.5 \times 10^{-3}$ (expressed as dilutions from packed cells) respectively. These dilutions would give an ATP concentration in the total diet close to the measured ED50 for ATP in NaCl if it were distributed evenly between cell content and suspending medium. However, if the response to RBCs is due to release of ATP close to the sensilla, the ATP might have an effective concentration approaching that in intact cells, since little dilution need occur. The correlation between the ED50 of RBCs and of free ATP may then be a coincidence. If release occurred at a sufficient distance from the chemoreceptors to allow complete mixing, all the ATP would have to be released from all the RBCs at the lowest concentrations found effective. This appears unlikely for two reasons. First, the ATP would then be exposed to the ATPase in the saliva, as discussed earlier, and one could expect a greater difference in the ED50 of RBCs in Ringer and NaCl. Second, intact cells appear in the stomach after feeding, and there is no known mechanism for release of ATP without rupture of the cells. The presence of intact cells in the stomach is consistent with the hypothesis of local release, since then only a few cells would need to release their ATP, perhaps during the sampling phase of feeding described by Friend & Smith (1971).

In conclusion, it appears that red blood cells are highly potent gorging stimulants for *Rhodnius prolixus*, whereas platelets and platelet-poor plasma are not. The potency is associated with the red cell contents and not the membrane, and the potency of red cell ghosts can be restored by resealing them with an ATP-containing Ringer. The concentration of red cells necessary to elicit gorging in 50% of the population is between $2.6 \times 10^{-3}$ and $4.5 \times 10^{-3}$, which would necessitate the release of all endogenous ATP if the ED50 for ATP were to be reached in the diet generally. The data are consistent
with the possibility that ATP is only released locally, close to the chemoreceptive sensilla, from a few cells. Possible mechanisms for the release will be discussed in a further paper.

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