ouabain and, except in the liver, it finally exceeded the control values by 7-48% (gastrocnemius muscle and kidney, respectively) in the presence of 10^{-3} moles/l ouabain.
Although the ouabain-induced increase of activity was similar in all tissues, the tissue sensitivity to the drug was different. The concentrations of ouabain which activated the membrane ATPase to one half of the Na^+-K^+-Mg^{2+}-dependent values were (in moles/l) 1.2×10^{-5} for the gastrocnemius muscle, 3×10^{-6} for the heart, 1.8×10^{-5} for the brain, 7×10^{-7} for the liver, and 2.3×10^{-6} for the kidney. The liver membrane fractions were thus the most sensitive.
The susceptibility of membrane Na^+-K^+-Mg^{2+} ATPase to the blocking effect of ouabain is the most common and characteristic feature of this enzyme[7,8]. In concentrations as low as 10^{-7} to 10^{-6} moles/l, ouabain inhibits the enzyme by combining with the outer part of the enzyme complex[9]. Potassium ions compete with the binding of ouabain on the outer surface of the cell membranes[10]. In the golden hamster, however, the relationship between ouabain and potassium seems to be more complicated and probably reflects some unusual characteristics of the enzyme in this animal. When K^+ is present, ouabain has a 'normal' blocking effect; the ouabain stimulation occurs only in the absence of K ions in the reaction medium. This suggest that 2 distinct binding sites may exist on the external surface of the enzyme complex, one for K^+ and another for ouabain. Separate occupation of either of them stimulates the enzyme, whereas the simultaneous occupation of both sites results in enzyme inhibition.
In the whole diaphragm of the golden hamster, we observed sodium extrusion from the muscle fibres during activation of membrane ATPase by ouabain (table 2) in the absence of K^+ in the incubation medium. 20 min after the application of ouabain, the intracellular sodium concentration decreased from 20.8 to 16.0 mmoles/l. This indicates that a) ouabain probably stimulates the enzyme from the outer side of the membrane, b) the ouabain-stimulated enzyme apparently corresponds to the ion transporting ATPase and c) ouabain activation of this enzyme is not coupled with Na^+-Na^+ exchange, but rather with the net outward transport of sodium ions.
Further comparative studies on other mammals are necessary to check whether the unusual effect of ouabain is due to unique properties of the golden hamster membrane ATPase per se or whether this phenomenon is related to the ability of the animal to hibernate.

Table 2.

Control	13.0±0.9
K^+-free	20.8±1.3
+OUA	16.1±0.7
+5 K^+	15.0±0.8
+5 K^+, OUA	20.6±2.2

Sodium content of golden hamster diaphragm in mmoles per l of muscle fibre water after immersion in standard Liley muscle bath[11] containing 5 mmoles/l K^+ for 6 h (control) or on K^+-free muscle bath before (K^+-free) and 20 min after application of 5 mmoles/l K^+ (+5 K^+), ouabain 10^{-4} moles/l (+OUA) and simultaneous application of potassium and ouabain (+5 K^+, OUA), respectively. Mean values±SEM from 6 absorption spectrophotometry estimations in each group (with correction for inulin space of diaphragm[6]) are given.

1 H. Dlouhá, Y. Donselaar, J. Teisinger and F. Vyskočil, Physiol. bohemoslov. *29*, 543 (1980).
2 A. Matsui and A. Schwartz, Biochim. biophys. Acta *128*, 380 (1966).
3 M. Ekblad, Analyt. Biochem. *83*, 694 (1977).
4 G.L. Miller, Analyt. Chem. *31*, 964 (1959).
5 S. Varon and H. McIlwain, J. Neurochem. *8*, 262 (1961).
6 N. Akaike, J. Physiol. *245*, 499 (1975).
7 H.J. Schatzmann, Helv. physiol. pharmac. Acta *11*, 346 (1953).
8 I.M. Glynn and S.J.D. Karlish, Physiol. Rev. *37*, 13 (1975).
9 R.W. Albers, G.J. Koval and G.J. Siegel, Molec. Pharm. *4*, 324 (1968).
10 R. Whittam, C. Hallam and D.G. Wattam, Proc. R. Soc. Lond. B *193*, 217 (1976).
11 A.W. Liley, J. Physiol. *134*, 427 (1956).

Platelet antiaggregating activity in the salivary secretion of the blood sucking bug *Rhodnius prolixus*

J.M.C. Ribeiro and E.S. Garcia

Departamento de Fisiologia da Universidade Federal Fluminense, Rua Prof. Hernani de Mello, 101, 24.210 Niterói (R.J., Brazil), 24 July 1980

Summary. The salivary secretion of *Rhodnius prolixus* inhibits both ADP and collagen induced platelet aggregation in human platelet-rich plasma. Fractionation studies show that at least 3 different inhibitors are present in *Rhodnius* saliva.

It has been observed among blood sucking insects that most species studied have developed mechanisms for preventing blood from clotting in the food cannals while feeding is in progress. Most of the anticoagulant activity is found in the salivary glands or in the gut contents after the meal[1-3]. The blood sucking bug *Rhodnius prolixus* presents an anticoagulant activity in its salivary glands[4] that was characterized as an antifactor VIII[5]. We recently described a powerful apyrase activity (ATP → AMP+2 orthophosphate) in the salivary secretion of *Rhodnius*[6] and suggested that this activity was physiologically important in preventing host platelet aggregation during the parasite meal. In the work recorded here we provide evidence that *R. prolixus* saliva has indeed an antithrombotic activity that is distinct from the anticoagulant activity. This is the first time the presence of such activity in blood sucking insects has been described.
Material and methods. All experiments were carried out with salivary secretion obtained from 1000-2000 adults and 5th instar nymphs starved for 30 days, which probed for 5 min a heated artificial feeder apparatus containing 10 ml water[6]. The solution yielded about 8 mg of protein; this was freeze-dried and stored at -20 °C until used. Human platelet-rich plasma (PRP) was obtained as described before[7]. Platelet aggregation was monitored by the method of Born and Cross[8]. ATPase and ADPase activities were assayed as described before[6]. 1 enzyme unit is the amount of enzyme that produces 1 µmole of orthophosphate per min at pH 7.5 and 30 °C. Agarose slab gel electrophoresis was performed in Tris Cl buffer 0.025 M pH 7.5[9]. Protein was determined by the technique of Lowry et al.[10] using bovine serum

albumin as standard. Clotting time was measured after recalcification of human citrated plasma at 38 °C[12].

Results and discussion. Figure 1 shows that the addition of *Rhodnius* saliva to PRP completely inhibited the aggregation induced by a high dose of ADP (7.5 µM) or collagen. The inhibition was effective down to 0.125 mg of salivary protein/ml of PRP and on further dilution full aggregation was observed. The concentrations of saliva used in this experiment are considered to be physiological by the following argument: 5th instar nymphs starved for 30 days contain 0.106±0.008 mg of protein/gland pair and 0.55±0.05 units of apyrase in their salivary glands (mean±SE, n=4). After a blood meal, we observe a decrease to 0.042±0.004 mg of protein/gland pair and to 0.16±0.01 units of apyrase/gland pair, the rest of the apyrase activity being recovered in the insect crop; furthermore, we have shown[6], using the apyrase activity as a marker for the salivary secretion, that the insect salivates during the whole meal. As the insects consumed an average of 0.25 ml of blood, we can find the actual concentration of saliva in the meal as 0.106–0.042/0.25 or 0.25 mg of salivary protein/ml of blood. If we consider a hematocrit of 50%, the concentration of insect salivary protein in the plasma would be 0.5 mg/ml. Figure 1 shows that this concentration of salivary proteins in plasma is highly efficient in inhibiting platelet aggregation induced by ADP or collagen. No preincubation of the saliva with PRP was necessary for the inhibition to occur. The same effect was observed if saliva was added 15 sec after collagen or immediately after ADP.

The activity was lost if saliva was boiled for 2 min and was not lost if it was subjected to dialysis. After incubation with pronase (20 mg of salivary protein/ml+1 mg pronase/ml in Tris Cl buffer 0.05 M pH 7.5+0.1 M NaCl) for 24 h a reduction in the rate of collagen induced aggregation could still be seen but it was completely lost for ADP. A pronase treatment of 1 h inactivated more than 95% of the salivary apyrase and the minimal inhibitory threshold dose was doubled for both ADP and collagen induced aggregation. These results suggest that the antithrombotic activity is not due solely to apyrase activity. After submitting *Rhodnius* saliva to a preparative agarose electrophoresis, it is observed that at pH 7.5 most of the salivary proteins are positively charged, and that at least 11 protein bands can be counted in the amido black stained gel (figure 2,A). This gel was sliced into 14 fractions as shown in figure 2, A and after extraction of salivary materials from each slice, protein was determined in each fraction by the method of Lowry et al.[10] (figure 2,B). Most of the ATPase and ADPase activities have a parallel profile, comigrating to the anode, suggesting the existence fo a true apyrase enzyme (figure 2,C). Some activity, however, is found migrating to the cathode. ADP induced platelet aggregation (figure 2,D) is inhibited mainly by the fractions containing ADPase activity and also by fraction 3 where no ADPase is found; the inhibition, however, is observed mainly in the second, rather than the first wave of aggregation (result not shown).

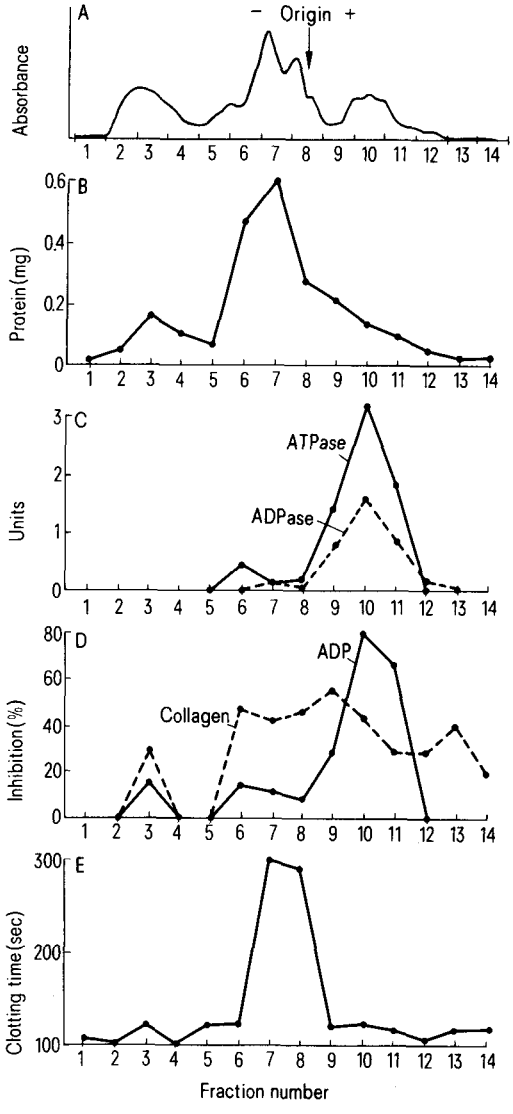

Fig. 1. ADP (left) or collagen (right) induced platelet aggregation of human PRP in the presence of salivary secretion of *Rhodnius* added to give the following protein concentrations: a) 0.5 mg/ml; b) 0.25 mg/ml; c) 0.125 mg/ml; d) no addition. Stock saliva was 20 mg protein/ml in 0.15 M NaCl. ADP final concentration was 7.5 µM. Collagen suspension was added as the minimal amount to give a maximal response.

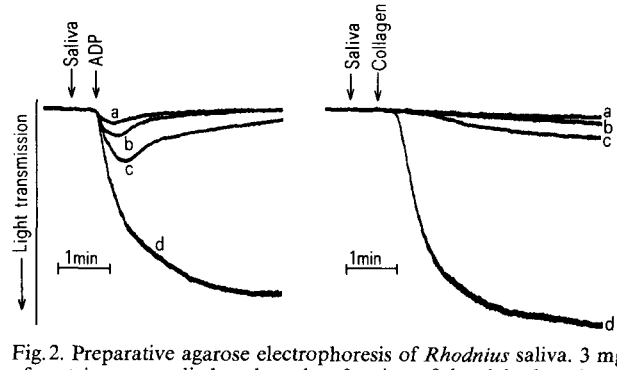

Fig. 2. Preparative agarose electrophoresis of *Rhodnius* saliva. 3 mg of protein was applied to the gel; a fraction of the slab, 6 cm long, was cut, fixed and stained with amido black. (A) shows the gel scan. The remaining gel was fractionated in 4-mm intervals as shown in A and after freezing and thawing it was force filtered through filter paper upon centrifugation. Each fraction yielded 210–260 µl of liquid which was freeze dried and diluted to 50 µl of which 1–3 µl were used for protein determination[9] (B); 1 µl was used to assay ATPase and ADPase (C); 20 µl were used for each antiaggregating activity experiment in a 0.4-ml cuvette, as described in the legend of figure 1. The amplitude of the aggregation 4 min after addition of ADP or collagen was used to measure the degree of inhibition (D). 2 µl was used for detection of anticoagulant activity measured by the clotting time after recalcification of human citrated plasma, in a final volume of 0.3 ml (E)[12].

Inhibition of collagen-induced aggregation (figure 2,D) is also observed in fraction 3 and in all fractions after fraction 6. Anticoagulant activity is found only in fractions 7 and 8 (figure 2,E). This experiment demonstrates the degree of redundancy found for the antithrombotic activity of salivary *R. prolixus* secretion. At least 2 factors inhibit ADP induced platelet aggregation and more than 2 are involved in the inhibition of collagen induced platelet aggregation. The anticoagulant activity and the apyrase activity together cannot completely explain the results shown in figure 1, which are thus to be interpreted as the concerted action of many substances. The possibility that protein bound prostaglandins could be involved was ruled out as the extraction procedure for plasma prostaglandins[11] was performed with *Rhodnius* saliva and the final evaporated ether extract was not an inhibitor of platelet aggregation. Our data indicate that a complex system for inhibiting platelet aggregation is present in the saliva of *Rhodnius prolixus*. This system together with the anticoagulant activity may provide the insect with an antihemostatic machinery important for the completion of the meal in a small lenght of time. To assess how generally such mechanisms are distributed, it would be interesting to study the presence of antithrombotic substances in other hematophagous insects.

1 J.W. Cornwall and W.S. Patton, Indian J. med. Res. *2*, 569 (1914).
2 H.M.O. Lester and L. Lloyd, Bull. ent. Res. *19*, 39 (1928).
3 R.H. Gooding, Ques. Ent. *8*, 5 (1972).
4 B.A. Baptist, Q. Jl. microsc. Sci. *83*, 91 (1941).
5 K. Hellmann and R.I. Hawkins, Nature *207*, 265 (1965).
6 J.M.C. Ribeiro and E.S. Garcia, J. Insect Physiol. *26*, 303 (1980).
7 M.A. Packham, N.G. Ardlie and J.E. Mustard, Am. J. Physiol. *217*, 1009 (1969).
8 G.V.R. Born and M.J. Cross, J. Physiol., Lond. *168*, 178 (1963).
9 C.P. Dietrich, H.B. Nader, L.R.G. Brito and M.E. Silva, Biochim. biophys. Acta *237*, 430 (1971).
10 O.H. Lowry, H.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
11 M.W. Greaves and W. McDonald-Gibson, Life Sci. *11*, 73 (1972).
12 L. Tocantins and L.A. Kazal, Blood coagulation, hemorrage and thrombosis. Methods of study. Grune Stratton, New York 1955.

The growth responses of hamster chondrocytes, dermal fibroblasts and embryo cells to whole blood serum and plasma-derived serum[1]

Y. Katoh and S. Takayama[2]

Department of Experimental Pathology, Cancer Institute, 1-37-1, Kami-Ikebukuro, Toshima-ku, Tokyo 170 (Japan), 29 April 1980

Summary. Autoradiographic studies with ^{3}H-thymidine demonstrated that the growth responses of hamster chondrocytes, dermal fibroblasts and embryo cells, respectively, differed in media containing whole blood serum (WBS) and plasma-derived serum (PDS). Dermal fibroblasts seemed to require a growth factor from platelets for growth, but chondrocytes did not. Embryo cells showed an intermediate pattern of growth response to this factor.

Recently, several investigators[3-5] have shown that platelets are the source of a growth factor in serum called the platelet-derived growth factor (PGF). Serum from whole blood (whole blood serum (WBS)) contains the factor because it is released from platelets during blood coagulation, whereas serum from plasma (plasma-derived serum (PDS)) lacks the factor because plasma is essentially free of platelets. It has been reported that PDS is unable to stimulate proliferation of several types of cells, such as primate arterial smooth muscle cells and dermal fibroblasts[6], mouse 3T3 cells[4,5], and human glial cells[7]. This paper reports differences in the growth responses of hamster chondrocytes, dermal fibroblasts and embryo cells to WBS and PDS.

Materials and methods. Chondrocytes were obtained from 1-week-old hamsters as described previously[8]. Dermal fibroblasts were obtained as follows: The skin of the back of 1-week-old hamsters was sterilized with 70% ethanol, separated and rinsed twice in Hanks' solution. The tissue fragments were minced with scissors and trypsinized with 0.25% trypsin. Then the dermal fibroblasts were cultured in Ham F-12 medium supplemented with 10% fetal calf serum. Embryo cells were obtained from embryos on the 13th day of gestation as described previously[9]. Primary cultures of these 3 types of cells were trypsinized and stored in liquid nitrogen for use as standarized cells.

Blood was collected from 8-month-old male SD rats in plastic tubes containing 1/10 vol. of 3.8% sodium citrate. The pooled blood was divided into 2 fractions for preparation of WBS and PDS by the method of Ross et al.[10]. Platelets were obtained from rats, added to the PDS, and recalcified by adding 1M $CaCl_2$ to give a concentration of 20 µmoles/ml. The resulting serum was centrifuged at 22,000 × g for 30 min at 4 °C, and the supernatant was dialyzed against Ringer solution for 24 h at 4 °C, filtered through a Millipore filter, and used as PDS + PR (platelet releasate).

Secondary cultures of the cells were trypsinized and seeded into Lab-Tek tissue culture chambers (4802: Lab Tek Products, Illinois, USA) at a density of 5×10^4 cells/chamber in Ham F-12 medium supplemented with 5% concentrations of the 3 types of serum: WBS, PDS, and PDS + PR. Then ^{3}H-thymidine (4 µCi/ml; 6.7 Ci/mmole: New England Nuclear, Massachusetts, USA) was added to each dish and the cells were incubated for 48 h at 37 °C. After incubation, the cells were fixed with methanol, washed twice with cold 5% trichloroacetic acid and processed for autoradiography. Approximately 1000 cells were counted for each point.

Results and discussion. The results are shown in the figure. As can be seen, 83% of the nuclei of chondorocytes were labelled with ^{3}H-thymidine in 5% WBS, 76% in 5%