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A SALIVARY NITROPHORIN (NITRIC-OXIDE-CARRYING HEMOPROTEIN) IN THE BEDBUG *CIMEX LECTULARIUS*

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Summary

Salivary gland homogenate of the bedbug *Cimex lectularius* caused vasodilation of the preconstricted rabbit aortic ring in the absence of endothelium. Vasodilation was augmented in the presence of superoxide dismutase and inhibited in the presence of Methylene Blue. Utilization of the Griess reaction indicated the presence of reactive nitrogen equivalents of the order of 337±57 pg equivalent NO_2^- per pair of salivary glands (mean ± S.E.M.; *N*=3). Salivary gland homogenates have a nitrosyl-hemoprotein that releases nitric oxide in a pH-dependent manner. The fraction containing the NO-carrying hemoprotein, when separated by HPLC, caused vasodilation of the preconstricted rabbit aortic strip. Furthermore, the presence of a nitrosyl-hemoprotein in *Cimex lectularius* salivary gland was verified by electron paramagnetic resonance spectroscopy. It is proposed that, as in the case of *Rhodnius prolixus* (Triatominae), *Cimex lectularius* salivary glands contain a hemoprotein (nitrophorin) that carries NO from the glands to the host tissues. However, because *Cimex lectularius* and *Rhodnius prolixus* belong to different hemipteran families (Cimicidae and Reduvidae) and evolved independently to blood feeding, *Cimex lectularius* and *Rhodnius prolixus* nitrophorin may be a case of convergent evolution.

Key words: vasodilator, salivary glands, feeding, hematophagy, bedbug, *Cimex lectularius*, nitric oxide, electron paramagnetic resonance, hemoproteins, nitrophorin.

Introduction

Platelet aggregation, blood clotting and vasoconstriction constitute the vertebrate hemostatic response that blood-sucking arthropods face when they attempt to feed on their vertebrate hosts. In turn, hematophagous arthropods have developed salivary anti-platelet, anti-clotting and vasodilatory factors to disarm the hemostatic apparatus of their host to facilitate the extraction of a blood meal (Ribeiro, 1987; Law *et al.* 1992).

A great diversity of vasodilators has been found in the salivary gland of blood-sucking arthropods (Law *et al.* 1992). For example, ixodid ticks possess prostaglandins (Ribeiro, 1987; Ribeiro *et al.* 1991), while the sand fly *Lutzomya longipalpis* (Ribeiro *et al.* 1989; Lerner *et al.* 1991), the mosquito *Aedes aegypti* (Champagne and Ribeiro, 1994) and the black fly *Simulium vittatum* (Cupp *et al.* 1994) contain different peptidic vasodilators. The triatomine bug *Rhodnius prolixus* has a salivary hemoprotein that carries the anti-platelet and vasodilatory compound nitric oxide (Ribeiro *et al.* 1990, 1993). This hemoprotein is abundantly expressed in *Rhodnius prolixus* salivary glands, resulting in the deep red color of the organ (Wigglesworth, 1942/43). This unique ferric hemoprotein binds reversibly with NO in a pH-dependent manner and is responsible for the storage and transport of NO, a volatile gas, from the salivary glands of the insect to its host tissues (Ribeiro *et al.* 1993). Interestingly, other triatomine bugs of the genera *Triatoma* (such as *T. rubida* and *T. infestans*) and *Panstrongylus* (*P. megistus*) have translucent glands and do not contain nitrovasodilators (J. M. C. Ribeiro, unpublished observations), suggesting that NO and NO-carrying hemoproteins have evolved uniquely in the genus *Rhodnius*.

The bedbug *Cimex lectularius* is a hematophagous hemipteran of the family Cimicidae. Its salivary glands are of an orange/red color (J. M. C. Ribeiro, unpublished results), suggesting the presence of hemoproteins. Interestingly, *Rhodnius prolixus* and *Cimex lectularius* are from different families of Heteroptera and have evolved independently to a blood-feeding mode. Triatomines belong to the family Reduvidae, a family that also contains plant-feeding and predatory bugs. Ancestors of the blood-feeding triatomines were not blood feeders, as hematophagy in these bugs evolved from either plant-feeding or predatory bugs (Sweet, 1979; Cobben, 1979). Thus, regardless of the original feeding habits of the Cimicidae, the feeding habit of bedbugs and triatomine

bugs evolved independently. Indeed, if one traces the hemipteran branch of the evolutionary tree from the Triatominae to the Cimicidae, a discontinuity on the blood-feeding mode is apparent. It is possible that the Cimicidae have evolved, independently of triatomines, a NO/nitrophorin system to assist hematophagy. The goals of this study were to look for the presence of salivary vasodilatory activity in *Cimex lectularius* and to investigate its possible association with salivary hemoproteins and nitric oxide.

Materials and methods

Cimex lectularius (L.) colonies were maintained in the insectary of the Center for Insect Science at the University of Arizona. Colonies were kept at 27 °C and 65 % humidity. Insects were fed every 10 days by exposing them to the shaved abdomen of an anesthetized rabbit. Salivary glands of insects at 8–10 days after feeding were dissected and stored in two different buffers: phosphate-buffered saline (PBS), 10 mmol l^{-1} sodium phosphate at pH 7.0 with 150 mmol l^{-1} NaCl; or sodium acetate 10 mmol l^{-1} at pH 5.0 with 150 mmol l^{-1} NaCl, at −75 °C until needed. Before use, salivary glands were thawed, disrupted with a dissection needle and the homogenate centrifuged in the cold (4 °C) at 14 000 revs min^{-1} for 5 min. The supernatant was recovered and used in the various experiments.

Rabbit aortic ring bioassays were performed as previously described (Ribeiro *et al.* 1990). The only modifications were the use of continuous bubbling of the Krebs solution with air instead of 95 % O_2 and 5 % CO_2 in order to increase the sensitivity of the aortic ring to NO and the addition 10 mmol l^{-1} Hepes buffer, pH 7.4, to the Krebs Ringer's solution. All the assays were performed with endothelium-free preparations, as demonstrated by their insensitivity to 1 μg ml^{-1} acetylcholine. Rabbit aortic ring constriction was achieved by using 200 ng ml^{-1} norepinephrine.

HPLC bioassays of rabbit aortic strips were performed as shown in Fig. 1. Briefly, an HPLC CM400 pump from Thermoseparation Products (Riviera Beach, Florida, USA) containing solution A (148 mmol l^{-1} NaCl, 6.6 mmol l^{-1} KCl, 2.8 mmol l^{-1} $CaCl_2$, 1.3 mmol l^{-1} $MgCl_2$, 11.1 mmol l^{-1} sodium acetate, pH 5.0, and 1.1 g l^{-1} glucose) with a flow rate of 1 ml min^{-1} was connected to a TSK gel G2000 SW column (C in Fig. 1) (7.5 mm×60 cm), obtained from Supelco, Inc. (Bellefonte, Pennsylvania, USA), and directed to a SM4100 dual-wavelength detector, obtained from Thermo Separation Products (Riviera Beach, Florida, USA). The signal from the detector was collected by a 80286 microcomputer with maths coprocessor, and the absorbance was recorded with a custom-made program written in QuickBasic (Microsoft, Redmond, Washington, USA). The detector solvent outlet was directed to the first inlet of a three-way connector. A second HPLC pump containing solution B (100 mmol l^{-1} Hepes, pH 7.7, with 6 mmol l^{-1} Na_2HPO_4, 6 mmol l^{-1} NaH_2HPO_4 and 2 μg ml^{-1} norepinephrine) with a flow rate of 0.1 ml min^{-1} was directly connected to the second inlet of the three-way connector. The outlet of the three-way connector sent the mixture of solution A and B directly to the aortic strip. Tension measurements were carried out by using an isometric force transducer (Harvard Apparatus, Edenbridge, Kentucky, USA) whose signal was collected by a 16-bit autoranging analog/digital converter obtained from Cole-Parmer (Niles, Illinois, USA) and then analyzed by a 80286-based microcomputer with maths coprocessor using a custom-made program written in QuickBasic. Salivary gland homogenates were injected into the TSK gel column and separated isocratically using solution A, as described above. The HPLC elution time and vasodilatory activity time were calibrated against salivary gland homogenate representing 0.1 of a pair of glands from *Rhodnius prolixus*, a well-characterized vasodilator (Ribeiro *et al.* 1990).

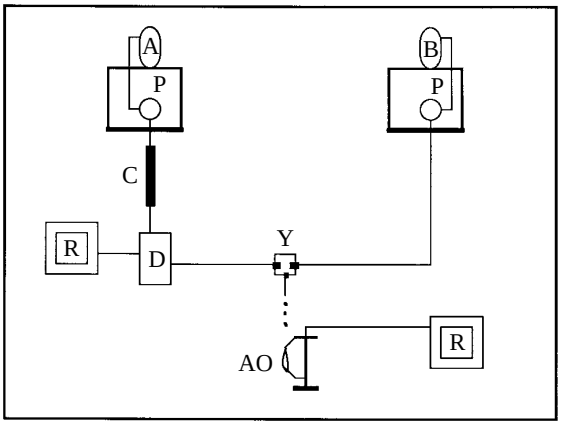

Fig. 1. HPLC bioassay. The HPLC pump (P) delivered solution A (148 mmol l^{-1} NaCl, 6.6 mmol l^{-1} KCl, 2.8 mmol l^{-1} $CaCl_2$, 1.3 mmol l^{-1} $MgCl_2$ and 11.1 mmol l^{-1} sodium acetate, at pH 5.0, and with 1.1 g l^{-1} glucose) at 1 ml min^{-1}. A was connected to a TSK gel column (C) and the column was connected to a dual-wavelength detector (D) set at 280 nm and 438 nm with the exit directed to a three-way connector (Y). The HPLC pump delivering solution B (100 mmol l^{-1} Hepes, at pH 7.7, with 6 mmol l^{-1} Na_2HPO_4, 6 mmol l^{-1} NaH_2PO_4 and 2 μg ml^{-1} norepinephrine) at 0.1 ml min^{-1}. B was directly connected to the three-way connector (Y). The outlet of the three-way connector directed the mixture of A and B to the aortic strip (AO). The aortic strip was kept at 37 °C with a glass jacket surrounding the tissue (not shown). HPLC eluent readings and aortic tension measurements were simultaneously collected using two different recording devices (R).

Optical spectroscopy assays were performed using a quartz microcuvette (Starna Cells, Atascadero, California) with a Perkin-Elmer Lambda 19 ultraviolet/visible spectrophotometer (Norwalk, Connecticut, USA). Salivary gland homogenates were prepared immediately before the optical measurements. The volume equivalent of three pairs of salivary glands (in 10 mmol l^{-1} sodium acetate at pH 5.0 with 150 mmol l^{-1} NaCl) was diluted in 50 μl of one of two buffer solutions (PBS, pH 7.0, or sodium acetate + NaCl, pH 5.0) and the spectrum was recorded between 250 and 700 nm. Different time points were measured for each sample and the data were analyzed using Lotus 123 software (Cambridge, Massachusetts, USA).

For carbon monoxide binding experiments, salivary gland

homogenate from *Cimex lectularius* was exposed to argon, to remove any volatile ligand, until a total shift in the Soret band from 438 to 388 nm was observed. Exposures to argon for longer than 1 h failed to change the spectrum any further. The Soret band is a well-defined spectral property of hemoprotein, the maximum absorption of this band ranges from 375 to 450 nm (Antonini and Brunori, 1971). In addition to being a characteristic property of hemoproteins, the changes in the absorption intensity and the wavelength of the maximum absorbance of the Soret band also provide information about the hemoprotein–ligand interactions. For example, in the presence of argon, a shift from a higher wavelength to a lower wavelength indicates dissociation of a volatile ligand from a hemoprotein containing Fe^{3+} in its heme moiety (Romberg and Kassner, 1979). After exposure to argon, salivary gland homogenate was reacted with dithionite (0.1 mmol l^{-1}), to reduce the heme iron, and exposed to carbon monoxide for 10 min. Optical measurements were again performed.

For nitric oxide binding, salivary gland homogenate at pH 7.0 was exposed to argon for 1 h or until a shift in the Soret band from 438 to 388 nm was detected spectrophotometrically. 100 mmol l^{-1} citrate buffer at pH 5.0 was then added in small amounts to the homogenate until pH 5.0 was reached. 50 μl of this solution (requiring three pairs of salivary glands) was then transferred to a 50 μl cuvette previously exposed to argon, and the mixture was exposed to argon for a further 2 h. To this solution, 0.2 μl of a saturated solution of nitric oxide (10 ml of water exposed to argon for 1 h then to nitric oxide for 2 min) was added using a 10 μl Hamilton syringe, and the spectrum was measured immediately after the addition of NO.

Determination of reactive nitrogen groups (NO_2^-) was carried out using the Griess reaction, as previously described (Bell *et al.* 1963; Ignarro *et al.* 1987) but with further modifications. Briefly, 8 μl of 40 % trichloroacetic acid (v/v) and 5 μl of sulfanilic acid (2 mg ml^{-1}) were added to 42 μl of sample containing 10 pairs of salivary glands in phosphate-buffered saline. The tube was vortexed, kept on ice for 10 min and centrifuged at 10 000 ***g*** for 1 min. To 50 μl of the clear supernatant was added 9.4 μl of *N*-(1-naphtyl)-ethylenediamine (NED, 1 mg ml^{-1}), and the spectrum of the samples was measured after 10 min at room temperature (25 °C). Nitrite concentration was determined from the absorbance at 550 nm minus the absorbance at 625 nm. Controls lacking the salivary homogenate or NED showed no absorbance at 550 nm.

For electron paramagnetic resonance assays (EPR), salivary gland homogenates of *Cimex lectularius* (100 pairs in 20 μl of 10 mmol l^{-1} sodium acetate, at pH 5.0, plus 150 mmol l^{-1} NaCl) were diluted in PBS, pH 7.0, in the presence or absence of dithionite. The solution was then transferred to an EPR quartz tube previously exposed to argon, and stored at −75 °C before analysis. Measurements were performed on a Bruker ESP-300E X-band spectrometer equipped with a helium cryostat (Oxford Instruments). Power attenuation was 15 dB; temperature 4.2 K; modulation frequency 93 kHz, modulation amplitude 2.83×10^{-4} T (2.85 G), receiver gain 1×10^5, resolution 1024 points, time constant 164 ms, and sweep width 0.17 T (1700 G).

For further information regarding heme and NO analysis, see Antonini and Brunori (1971) and Feelish (1991).

All animals used in these experiments were treated according to approved protocols reviewed by the University of Arizona Institutional Animal Care and Use Committee.

Results

When salivary gland homogenate from *Cimex lectularius* was added to the rabbit aortic ring preconstricted with 200 ng ml^{-1} norepinephrine, a reversible vasodilation was observed (Fig. 2A). The vasodilation was endothelium-independent since the endothelium was removed from the aortic ring preparations before the bioassays. The vasodilation was augmented when the aortic rings were treated with superoxide dismutase (30 units ml^{-1}) (1 unit of superoxide dismutase will inhibit the rate of reduction of cytochrome *c* by 50 % in a coupled system with xanthine oxidase at pH 7.8 at

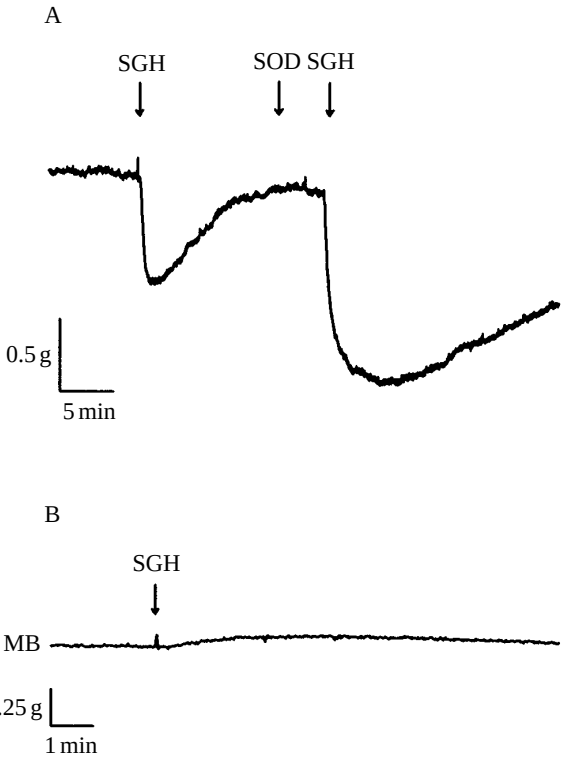

Fig. 2. (A) Vasodilatory activity of salivary gland homogenate (SGH) from five pairs of glands of *Cimex lectularius* added to a rabbit aortic ring previously contracted with 200 ng ml^{-1} norepinephrine in a 2.0 ml organ bath. SOD represents the addition of superoxide dismutase (30 units ml^{-1}) (1 unit of superoxide dismutase will inhibit the rate of reduction of cytochrome *c* by 50 % in a coupled system with xanthine oxidase at pH 7.8 at 25 °C) before the addition of the salivary gland. (B) The experimental trace marked MB represents salivary gland homogenate from five pairs of glands of *Cimex lectularius* in an aortic ring previously treated with 200 ng ml^{-1} norepinephrine and 50 μmol l^{-1} Methylene Blue.

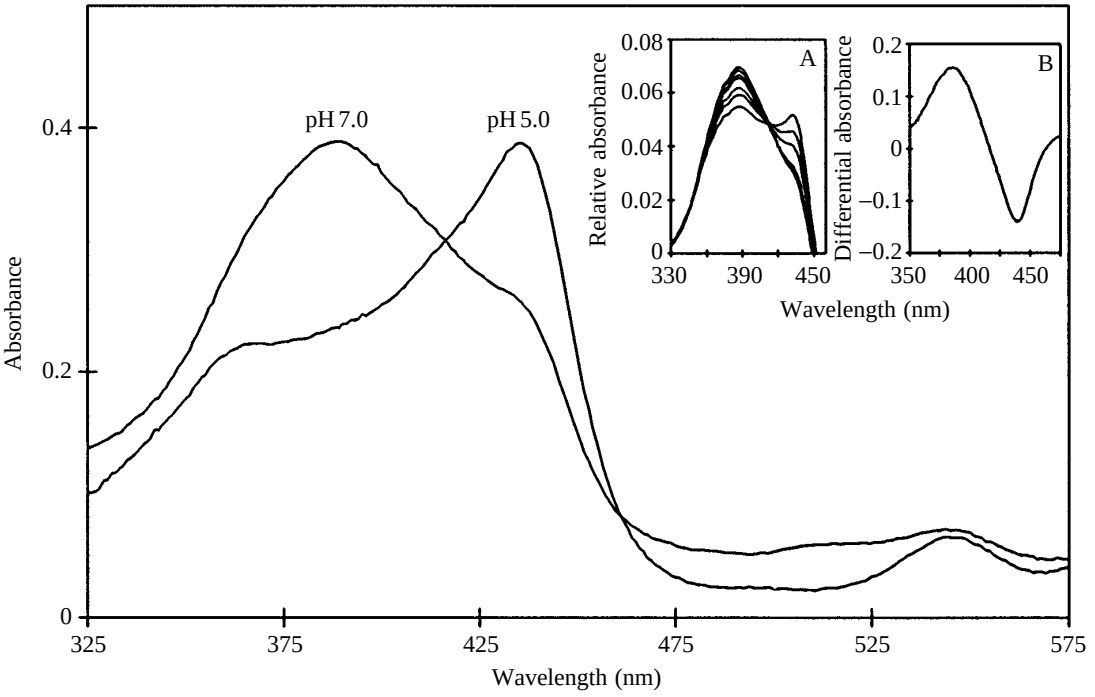

Fig. 3. Absorption spectrum of salivary gland homogenate from three pairs of glands from *Cimex lectularius* in the presence of sodium acetate ($10\,mmol\,l^{-1}$) + NaCl ($150\,mmol\,l^{-1}$), pH 5.0, or PBS, pH 7.0. Both spectra (at pH 5.0 and pH 7.0) were taken immediately after diluting the homogenate into the quartz cuvette. (A) Repetitive scans of the spectrum at pH 7.0 at different times (from 0 to 40 min, 8 min intervals). (B) Differential absorbance of the spectrum at pH 5.0 minus the spectrum at pH 7.0.

25 °C) prior to the addition of the salivary glands (Fig. 2A). Adding salivary gland homogenate to an aortic ring in the presence of $50\,\mu mol\,l^{-1}$ Methylene Blue resulted in no detectable vasodilation (Fig. 2B). These observations suggest that salivary gland homogenate contains a vasodilatory compound that behaves like nitric oxide or as an endothelium-derived relaxing factor (Ignarro *et al.* 1987). In addition, when a Griess reaction was performed in the salivary gland homogenate, it detected 337±57 pg NO_2^- equivalent per pair of salivary glands (mean ± S.E.M., N=3).

Spectrophotometric analysis of the salivary gland homogenate at pH 5.0 revealed an absorbance band with a maximum at 438 nm (Fig. 3). Changing the pH from 5.0 to 7.0 resulted in a shift of the maximum from 438 to 388 nm with a shoulder at 438 nm (Fig. 3), suggesting dissociation of a ligand at neutral pH. In addition, when the spectra were taken at different times (8 min intervals) after adjusting the solution pH to 7.0, we observed a time-dependent shift in the absorbance from 438 to 388 nm, suggesting a relatively slow dissociation of a ligand at pH 7.0 (Fig. 3, inset A). Furthermore, the differential spectrum obtained by subtracting the spectrum from the homogenate at pH 5.0 from the spectrum of the homogenate at pH 7.0 resulted in a maximum at 388 nm and a minimum at 438 nm (Fig. 3, inset B). Dissociation of the putative ligand over time (40 min) at pH 5.0 accounted for only a 10 % loss of the 438 nm shoulder observed at pH 7.0 (data not shown).

To study further the dependency of the absorbance maximum on the pH of the medium, salivary gland homogenates were measured spectrophotometrically at different pH values, and the ratio of absorbance at 388 nm to absorbance at 438 nm was plotted as a function of pH. The results showed a curve with a pKa of approximately 6.5 (Fig. 4), suggesting the presence of histidine as the ionizable group.

Because the absorption spectrum of the salivary gland homogenate is similar to that of an Fe(III) hemoprotein, where there is a shift of the Soret maximum from higher to lower wavelength when a ligand dissociates (Romberg and Kassner, 1979), we wanted to test for the presence of a hemoprotein in the salivary gland homogenate of *Cimex lectularius*. For this, the salivary homogenate at pH 7.0 was exposed to argon to remove volatile ligands; it was then reacted with dithionite in order to produce an Fe(II) heme species and finally reacted with carbon monoxide. When the salivary gland homogenate

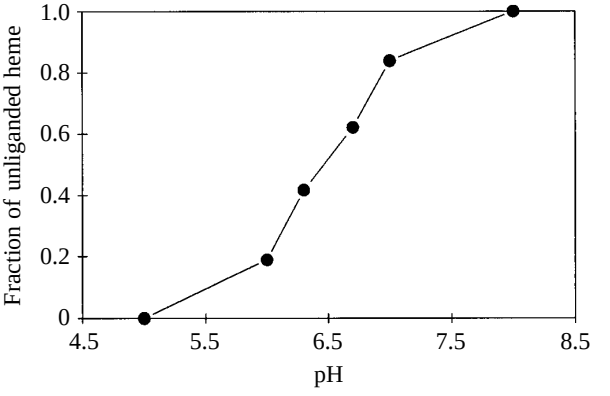

Fig. 4. pH-dependence of the fraction of the unliganded form of the salivary hemoprotein from *Cimex lectularius*. The unliganded heme fraction was measured as the ratio of the absorption minimum (388 nm) to the absorption maximum (438 nm) obtained from the difference spectrum of the individual normalized absorption spectra at different pH values at time zero minus the absorption spectrum at pH 5.0 at time zero. Points are representative of a single experiment.

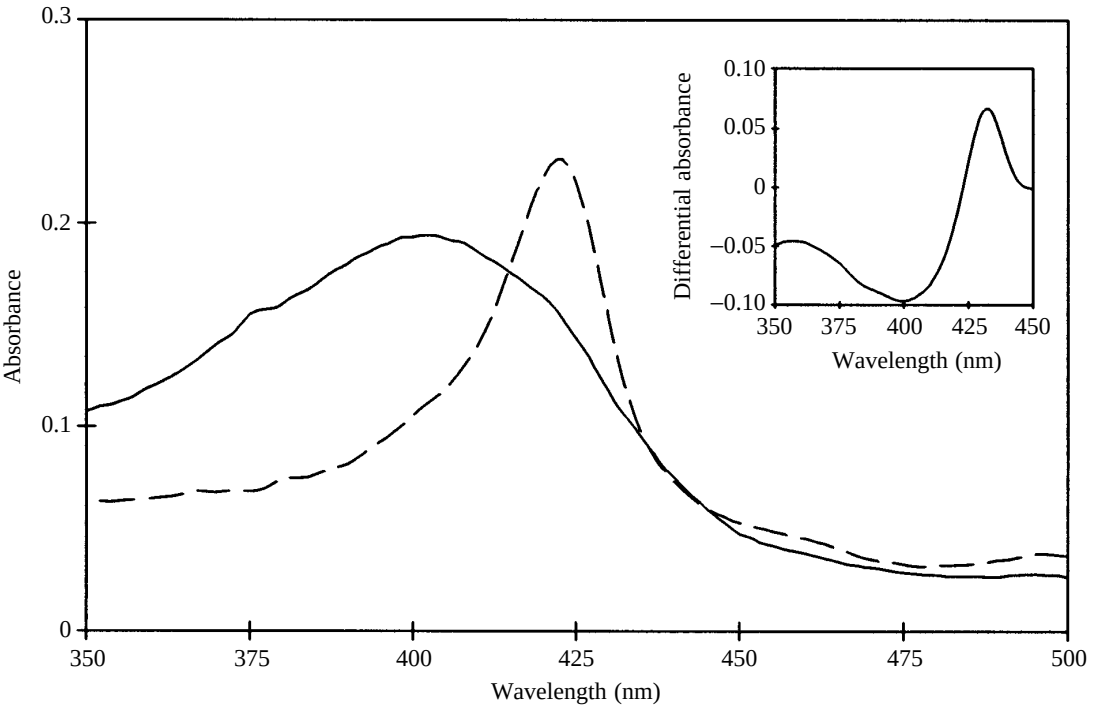

Fig. 5. Effect of CO on the light absorbance of the salivary gland homogenate from *Cimex lectularius* exposed to argon for 1 h and then reacted with dithionite. The continuous line represents the spectrum of argon-treated homogenate treated with dithionite and the dashed line represents the spectrum of salivary homogenate hemoprotein loaded with CO. The inset represents the difference spectrum of homogenate exposed to argon then treated with dithionite minus the spectrum of homogenate exposed to argon, dithionite and CO.

was reacted with dithionite, a shift in the spectrum from 388 to 400 nm was observed (Fig. 5). In the presence of carbon monoxide, the maximum absorbance band shifted from 400 to 422 nm (Fig. 5, dashed line), indicating that the protein binds to carbon monoxide and behaves as a hemoprotein.

In order to evaluate whether the vasodilatory activity was associated with the salivary hemoprotein, we ran 100 pairs of homogenized salivary glands in a molecular sieving column eluted with 10 mmol l^{-1} sodium acetate and 150 mmol l^{-1} NaCl, at pH 5.0, and tested each fraction in a rabbit aortic ring bioassay. No vasodilatory activity was found (results not shown). We interpreted this negative result, which showed no detectable vasodilatory activity, to be due to the dilution effect produced by the restricted volume of the fraction sample that can be added to the chamber where the aortic ring is submerged. If the volume of the sample is too large, it can alter the dilution of norepinephrine and it can also increase the amount of salt in the solution. In addition, column fraction samples could be degraded between the time of elution and the time of testing in the rabbit aortic ring bioassay. To eliminate the dilution effect and to preserve the vasodilatory activity of the homogenate, we performed the column chromatography at

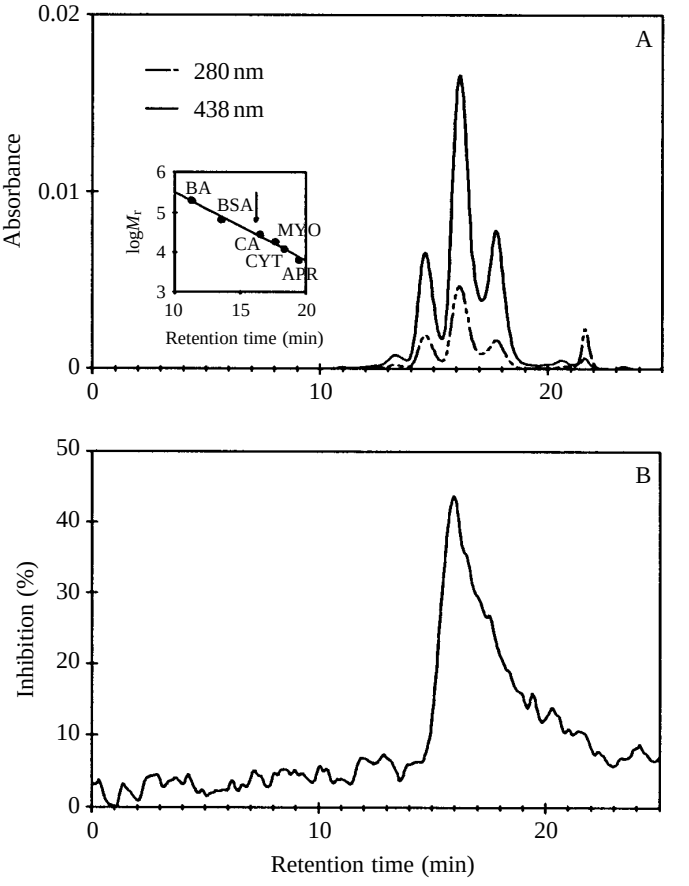

Fig. 6. Vasodilatory activity of the eluent of three pairs of salivary gland from *Cimex lectularius* separated by HPLC-TSK gel column. Salivary gland homogenate was injected into a TSK gel column and the eluent was directed to a norepinephrine- (200 ng ml^{-1}) constricted aortic strip, as described in Fig. 1. (A) HPLC chromatogram of salivary gland homogenate detected at 280 nm (dashed line) and 438 nm (solid line). The inset shows the standard curve of the molecular mass markers (BA, β-amylase; BSA, bovine serum albumin; CA, carbonic anhydrase; MYO, myoglobin, CYT, cytochrome *c*; APR, aprotinin). (B) Percentage inhibition of norepinephrine constriction of an aortic strip by the eluent of the TSK gel column homogenate from three pairs of salivary gland from *Cimex lectularius*. Constriction of the aortic strip due to norepinephrine was 1.8 g above resting tension.

pH 5.0 and the bioassay on-line immediately after neutralization of the column effluent.

When salivary gland homogenate (from three pairs of glands) was separated by HPLC using a molecular sieving column (Fig. 6A) and the eluent was directly tested on a rabbit aortic strip, the eluent containing the most abundant hemoprotein (Fig. 6A, elution time 16.2 min) detected at 438 nm caused inhibition of vasoconstriction, of the rabbit aortic strip, induced by norepinephrine (Fig. 6B). There was a significant decrease in the inhibition of vasoconstriction when salivary gland homogenate was exposed to PBS at pH 7.0 for 30 min before the HPLC bioassay (results not shown). When salivary gland homogenate (100 pairs) was separated by HPLC using a molecular sieving column as described in Fig. 6A and the fractions collected were scanned in a spectrophotometer, the fraction containing the main protein peak with a retention time at 16.2 min had an absorption spectrum the same as that shown in Fig. 3 at pH 5.0 (not shown). In addition, this hemoprotein had an apparent relative molecular mass of 28 684 when calibrated against molecular mass standards (Fig. 6A, inset). We conclude that the salivary vasodilator of *Cimex lectularius* is a molecule unstable at neutral pH and co-eluting with a hemoprotein having an apparent relative molecular mass of 28 684.

Since the ligand of *Cimex lectularius* hemoprotein was suspected of being a volatile compound and the gas nitric oxide has been shown to be involved in vasodilation, we tested whether the ligand behaved like authentic nitric oxide and could be displaced from the head space of the protein solution by a noble gas, which is unable to react with the protein but is able to change the equilibrium towards the unliganded form. For this, salivary gland homogenate at pH 7.0 was exposed to argon, resulting in a shift in the Soret band from 438 to 388 nm (Fig. 7, continuous line). This supports the hypothesis that the ligand of the salivary hemoprotein is a volatile compound. Exposure of the homogenate at pH 5.0 to argon leads to a slower, but complete, conversion of the Soret band from 438 to 406 nm. The same spectrum was observed when the homogenate at pH 7.0 was exposed to argon and then shifted to pH 5.0 (Fig. 7, dotted line). When nitric oxide was added to the salivary homogenate at pH 5.0 that had previously been exposed to argon, the Soret band shifted from 406 to 438 nm (Fig. 7, dashed line). However, when a difference spectrum was taken from the homogenate exposed to nitric oxide and the homogenate at pH 5.0 exposed to argon, it resulted in an absorption minimum at 388 nm and an absorption maximum at 438 nm (Fig. 7, inset A). The visible spectrum also showed the appearance of typical α and β bands at 580 nm and 545 nm, respectively (Fig. 7B). The absorption spectrum of the protein charged with NO was identical to the spectrum of the native salivary homogenate at pH 5.0, suggesting that nitric oxide is the ligand of this hemoprotein.

In order to test for the presence of nitric oxide in the salivary gland of *Cimex lectularius*, we measured the electron paramagnetic resonance spectrum (EPR) of 100 pairs of salivary glands in the absence and presence of dithionite (Fig. 8). The rationale for using dithionite was that Fe(III) hemoproteins have an odd number of electrons that exist in a high-spin form when pentacoordinated and, typically, give rise to EPR spectral features at about 0.11 T (1100 G) and 0.33 T (3300 G) (spectroscopic g-factors of 6.0 and 2.0, respectively) (Walker *et al.* 1984). Since nitric oxide is also an odd-electron

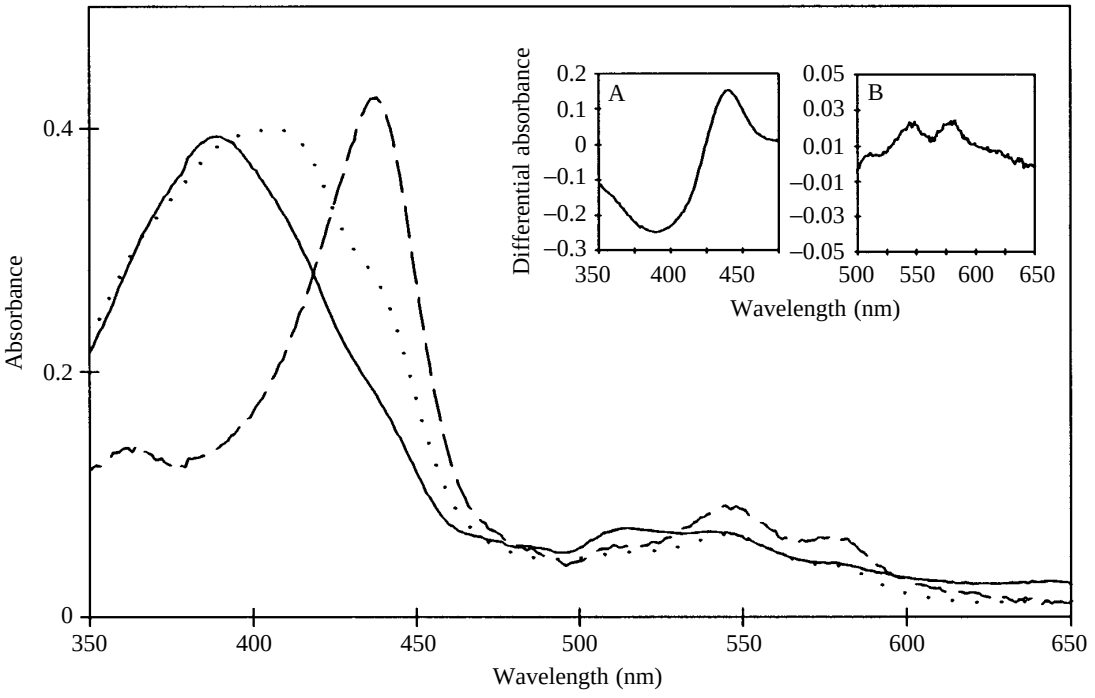

Fig. 7. Effect of NO on the absorbance by salivary gland homogenates from *Cimex lectularius* previously exposed to argon. Salivary gland homogenate was exposed to argon for 1 h and the spectrum measured (continuous line). The spectrum was then measured after the homogenate has been adjusted to pH 5.0 (dotted line). The dashed line represents the spectrum of the homogenate after the addition of NO. The insets represent the difference spectrum of the salivary gland homogenate exposed to argon minus the spectrum of the salivary gland homogenate with NO (A) from 350 to 475 nm and (B) from 500 to 650 nm.

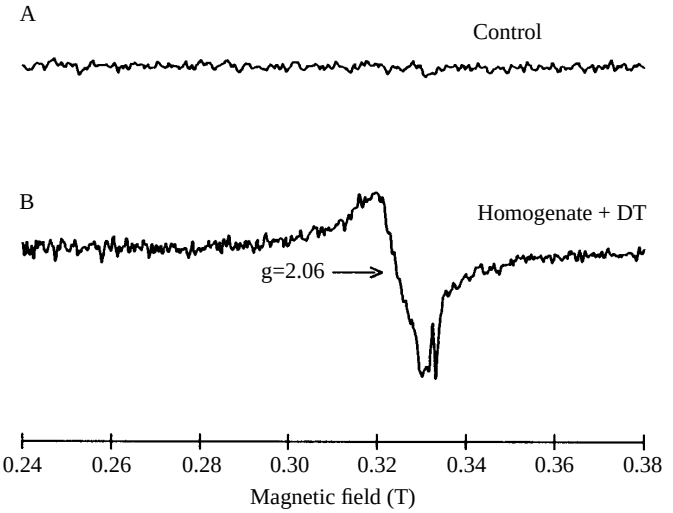

Fig. 8. EPR spectrum of 100 pairs of salivary gland homogenate of *Cimex lectularius* in the absence (A) and presence (B) of dithionite (DT). The arrow points to the *x*-axis intersection of the curve (g value). 1 G (gauss)=10^{-4} T.

species, the complexation of a high-spin Fe(III) hemoprotein with NO produces an EPR-silent species, and no signal is observed over the magnetic field range 0.1–0.4 T (1000–4000 G) (Hori *et al.* 1990; Ribeiro *et al.* 1993). If the Fe(III) hemoprotein, in the absence of NO, is treated with dithionite, Fe(III) is reduced to Fe(II), an even-electron system that also does not give an EPR signal when measured in a normally configured EPR cavity, such as the one we used. However, when NO is bound to Fe(III), reduction by dithionite produces an odd-electron species that gives rise to a characteristic Fe(II)–NO EPR signal (Ribeiro *et al.* 1993; Reisberg *et al.* 1976; Henry and Banerjee, 1973). Control reactions on the isolated salivary glands (in the absence of dithionite) resulted in a silent signal in the magnetic field range 0.05–0.38 T (500–3800 G) (only the portion of the spectrum from 0.24 to 0.38 T is shown in Fig. 8A). In the presence of dithionite, we observed a signal (Fig. 8B) characteristic of a nitrosylhemoprotein (Reisberg *et al.* 1976; Henry and Banerjee, 1973), indicating the presence of nitric oxide in the salivary gland of *Cimex lectularius*.

Discussion

We have described the presence of a vasodilatory compound in the salivary gland homogenate of *Cimex lectularius*. The vascular relaxation caused by the salivary gland homogenate was endothelium-independent, inhibited by Methylene Blue and augmented by superoxide dismutase, all of which are characteristic of the behavior of the vasodilator nitric oxide or endothelium-derived relaxing factor (EDRF) (Furchgott, 1984; Martin *et al.* 1985; Gryglewski *et al.* 1986). In addition, nitrogen-reactive groups measurable by the Griess reaction indicated the presence of 337 pg NO_2^- equivalents per pair of salivary glands. A similar salivary nitrovasodilator has been identified in *Rhodnius prolixus* (Ribeiro *et al.* 1990).

Spectrophotometric studies revealed the presence of a heme-like protein in the salivary gland homogenate of *Cimex lectularius*. The protein was confirmed to be a hemoprotein from its characteristic absorption spectra and from its affinity for carbon monoxide when in a reduced form (Fig. 5). The ligand formation of the native hemoprotein is pH-dependent and has an ionizable group with a pKa of 6.5 (Fig. 4). A similar Fe(III) hemoprotein has been identified in *Rhodnius prolixus*. However, these two proteins differ in the Soret band maximum of the liganded and unliganded forms. In *Cimex lectularius*, the maximum Soret band for the liganded form is at 438 nm and for the unliganded form at 388 nm (Fig. 3), while in *Rhodnius prolixus* the maximum Soret band for the liganded form is at 422 nm and for the unliganded form is at 404 nm (Ribeiro *et al.* 1993). Both proteins seem to have only one ligand since only two spectroscopic species were observed (unliganded and liganded) in the native protein with a very defined single isosbestic point at 413 nm (Fig. 3, inset A). In addition, the presence of a ligand in the protein is supported by the change in the absorption at 545 nm (maximum of the β band), which is decreased when the protein is unliganded (Fig. 7).

When the salivary gland homogenate was separated by HPLC in a molecular sieving column, the eluent containing a hemoprotein with an absorption spectrum similar to that described in Fig. 3 caused reversible vasodilation of the preconstricted aortic strip. The vasodilatory activity was diminished when the salivary gland homogenate was diluted in PBS at pH 7.0 and exposed to air for 30 min prior to bioassay. These data support the hypothesis that the hemoprotein carries a compound responsible for the vasodilatory activity of the rabbit aortic strip, which can be oxidized or displaced at neutral pH by exposure to air.

The ligand of this protein was shown to be a volatile compound by exposing the salivary homogenate to argon and obtaining the spectrum characteristic of the unliganded form (Fig. 7, continuous line). Furthermore, the absorption spectrum of the salivary homogenate obtained in the presence of nitric oxide (Fig. 7, dashed line) is identical to the one observed in the native salivary homogenate at pH 5.0 (Fig. 3), suggesting that the ligand of the salivary hemoprotein is nitric oxide. In addition, evidence for the presence of nitrosyl heme was obtained by EPR measurements of the dithionite-reduced native salivary gland homogenate (Fig. 8). The same compound (nitric oxide) was identified as a ligand of the hemoprotein found in the salivary gland of *Rhodnius prolixus* (Ribeiro *et al.* 1993).

Nitric oxide is a vasodilator that can be used by blood-sucking insects during feeding to avoid one of the hemostatic mechanisms of their host (vasoconstriction). *Cimex lectularius* and *Rhodnius prolixus* have in their saliva a hemoprotein with similar spectroscopic characteristics and both proteins carry nitric oxide. Interestingly, *Rhodnius prolixus* and *Cimex lectularius* are from different families of Heteroptera and have evolved independently to a blood-feeding mode (Sweet, 1979;

Cobben, 1979). Thus, their nitrophorins may have arisen by convergent evolution. Comparison of the structure and physical characteristics of the nitrophorins from these two species should yield insights into the interactions of ferric hemoproteins with nitric oxide.

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