THE SALIVARY CATECHOL OXIDASE/PEROXIDASE ACTIVITIES OF THE MOSQUITO *ANOPHELES ALBIMANUS*

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Summary

Salivary gland homogenates from adult female *Anopheles albimanus* mosquitoes relaxed aortic rings preconstricted with noradrenaline (NA). This relaxation is slow and is due to destruction of NA. Incubation of NA with the homogenate yielded a product with a spectrum consistent with the corresponding adrenochrome. Oxidation of NA was enhanced by a superoxide generation system and inhibited by the combined action of superoxide dismutase and catalase. Additionally, peroxidase activity on both synthetic (*o*-dianisidine) and biologically active (serotonin) substrates was also present in the salivary gland homogenates, this latter activity requiring hydrogen peroxide. Noradrenaline oxidation, serotonin and *o*-dianisidine peroxidation and vasodilation all co-elute with a heme protein of relative molecular mass 50000, as determined by molecular sieving chromatography. Peroxidase activity was localized in the posterior (female-specific) lobes of salivary glands and was also detected in nitrocellulose membranes probed by hungry mosquitoes. Protein and peroxidase activities were significantly lower in salivary glands of mosquitoes after probing and feeding on blood. It is suggested that adult female *Anopheles albimanus* mosquitoes contain a salivary heme peroxidase that functions during blood finding and blood feeding by destroying hemostatically active biogenic amines released by the vertebrate host during tissue destruction.

Introduction

When attempting to feed, blood-sucking arthropods injure their hosts and thus face a complex vertebrate defense system against blood loss, the hemostatic response. Platelet aggregation, vasoconstriction and blood coagulation are three interconnected components of the hemostatic system. Tissue destruction releases or exposes to the blood molecules such as ADP (usually millimolar concentration inside cells, but submicromolar concentrations in the extracellular compartment) and collagen that trigger platelet aggregation and platelet granule secretion. Platelets aggregate in seconds, forming a plug at the site of injury. In this process, platelets also secrete more ADP, serotonin and thromboxane A_2. These two last components induce constriction of smooth muscle around venules or arterioles, further restricting blood loss, and tightening the vessel

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against the platelet plug. Activated platelets and damaged tissue also trigger blood coagulation, leading to the formation of thrombin. Thrombin serves as an activator of platelets and catalyzes the formation of fibrin, which further reinforces the platelet plug structure at the damaged site. Blood-sucking animals thus have to counteract their host's blood coagulation, platelet aggregation and vasoconstriction to obtain a blood meal (Ribeiro, 1987; Law *et al.* 1992).

Anticoagulants have been recognized in the saliva or salivary gland homogenates of blood-sucking animals since early in this century (Cornwall and Patton, 1914), although the characterization at the molecular level of such substances has only recently begun (Law *et al.* 1992). Similarly, only within the last 10 years has antiplatelet activity been found in saliva or salivary glands of blood-sucking arthropods, leeches and bats (Ribeiro, 1987; Law *et al.* 1992). Vasodilators have also recently been found in blood-sucking arthropods, with a surprising molecular variety (Law *et al.* 1992).

From the very few arthropods studied, a large diversity of salivary vasodilatory compounds is emerging: salivary glands of ixodid ticks contain vasodilatory prostaglandins (Ribeiro, 1987; Ribeiro *et al.* 1991), those from the bug *Rhodnius prolixus* contain a nitrovasodilator (Ribeiro *et al.* 1990), the sand fly *Lutzomyia longipalpis* contains the most potent peptidic vasodilator presently known (Ribeiro *et al.* 1989; Lerner *et al.* 1991) and the mosquito *Aedes aegypti* has a peptidic vasodilator of the tachykinin family (Pappas *et al.* 1986; Ribeiro, 1992; D. Champagne and J. M. C. Ribeiro, unpublished observations). This diversity of pharmacologically active compounds appears to derive from the convergent evolution to blood-feeding behavior found in many invertebrate and vertebrate animals.

Anopheles albimanus is a mosquito vector of malaria, and laboratory colonies of this insect are readily available. Salivary vasodilator compounds from anopheline mosquitoes have never been described, although antiplatelet (Ribeiro *et al.* 1985) and anticoagulant (Yorkee and Macfie, 1924; Metcalf, 1945; Ribeiro *et al.* 1985) activities have been reported. In this paper, we describe a salivary enzyme activity that destroys catecholamines and serotonin, substances that are important vertebrate vasoconstriction agents.

Materials and methods

The Santa Tecla strain of *Anopheles albimanus* was maintained at the insectary of the University of Arizona in Tucson, at 27°C and 80% relative humidity. Adults were offered cotton swabs containing 20% sucrose in water. Salivary glands from female mosquitoes at least 3 days old were dissected and kept (20 pairs in 20 μl of phosphate-buffered saline: 10mmol1^{-1} sodium phosphate at pH7.0 with 150mmol1^{-1} NaCl) at -75°C until needed. They were then disrupted by ultrasound with a Branson sonifier (model 450) with the probe immersed for 2cm in a 100ml beaker containing 80ml of water at room temperature. The 1.5ml conical tube containing the glands was held with clamping forceps so that its conical tip was just under the tip of the probe. Power was set at 6 and a 50% cycle was run for 1min. Disruption of the glands was assessed under a stereoscope, and a new cycle was repeated if some glands appeared intact. The

homogenate was centrifuged for 2min at $10000\boldsymbol{g}$ and the supernatant was recovered for use in all assays.

Mosquito blood feeding was accomplished by anesthetizing a rat with a mixture of ketamine and xylazine and exposing mosquitoes to its shaved abdomen. To increase salivation, feeding was disturbed at 1min intervals by lifting the rat from the cage. Fully engorged mosquitoes were used to test for the loss of enzyme activity and protein with salivation.

Rabbit aorta smooth muscle ring bioassays were performed isometrically using Krebs solution at 37°C bubbled continuously with 95% O_2 and 5% CO_2 (Perry, 1968). Dexamethasone ($0.1\,\mu mol\,l^{-1}$) was added to the bath to prevent induction of NO synthase (Moncada *et al.* 1991), while EDTA ($30\,\mu mol\,l^{-1}$) was added to chelate heavy metal contaminants. Rabbit aortic strips were also mounted, in some cases, according to Ferreira and Souza-Costa (1976) in an oil-immersed, laminar-flow superfusion mode. Aortic endothelium was removed by gently rubbing the inner portion of the rings with a wooden stick. Success of endothelium removal was confirmed by the inability of acetylcholine ($1\,\mu mol\,l^{-1}$) to relax the rings preconstricted with $1\,\mu mol\,l^{-1}$ noradrenaline. The estrogenated rat uterus bioassay was performed isotonically using De Jalon solution at 30°C (Gaddum *et al.* 1949) in a laminar-flow superfusion mode. The guinea pig ileum bioassay was performed isotonically using Tyrode's solution at 37°C and bubbled with O_2 and CO_2 as for the aortic assays (Webster and Prado, 1970).

Catechol oxidase and peroxidase reaction media contained $10\,mmol\,l^{-1}$ sodium phosphate at pH7.0 (for catechol oxidase) or $10\,mmol\,l^{-1}$ Hepes at pH7.0 (for peroxidase activity, because in the presence of phosphate the peroxidase substrate tended to crystallize), $150\,mmol\,l^{-1}$ NaCl, the indicated amounts of salivary gland homogenates and either $1\,mmol\,l^{-1}$ noradrenaline for the catechol oxidase reaction or $0.2mg\,ml^{-1}$ *o*-dianisidine and $0.1\,mmol\,l^{-1}$ H_2O_2 for peroxidase activities, in a total volume of $100\,\mu l$. Indicated amounts of homogenized and diluted salivary glands, usually 0.1 pairs of glands per well, were added. Progression of the reaction, at 37°C, was followed colorimetrically at 450nm in a 96-well microplate read intermittently on a Molecular Devices Thermomax microplate reader equipped with a computer-based kinetics measurement module. For peroxidase assays, measurements were made at 15s intervals for the first 3min. For catechol oxidase measurements, a lag phase of 30–40min often occurred, followed by a linear increase in the optical density. The reaction was followed for 3h, with measurements at 15min intervals, and the rate of the reaction was measured after the lag phase. Reaction rates were determined from the slopes of the linear regression of the optical density on reaction time. Correlation coefficients used were always larger than 0.95. We define 1 unit of activity as that resulting from a change in optical density of 1 absorbance unit per minute under the conditions described.

Serotonin destruction in a solution of 1ml of phosphate-buffered saline (PBS) at pH7.0, with $1\,\mu mol\,l^{-1}$ serotonin and $10\,\mu mol\,l^{-1}$ H_2O_2 was followed fluorometrically on a Perkin Elmer model 650-10S spectrofluorimeter, with excitation set at 295nm and emission at 330nm. Salivary gland homogenates were added, and the change in the fluorescent intensity was followed in an Intel80486 processor based computer using a

Dataq analog/digital 12-bit converter. Reaction rates were calculated by linear regression of the linear portion of the fluorescent intensity change, sampled at 5s intervals.

Salivary gland histochemistry for peroxidase activity was carried out by fixing freshly dissected glands in PBS containing 2.5% glutaraldehyde and then transferring the glands to a small Petri dish containing $0.25 mg ml^{-1}$ 4-chloronaphthol and $3.6 mmol l^{-1}$ H_2O_2 in PBS. The colour developed within 2–5min.

Salivary secretion of peroxidase activity was monitored by exposing female mosquitoes to a 10cm diameter nitrocellulose membrane soaked (but not wet) with PBS containing $10 mmol l^{-1}$ sodium bicarbonate to stimulate probing (Galun *et al.* 1985) and warmed to 37°C. After 5min of exposure to mosquitoes, the membrane was transferred to a Petri dish containing $0.25 mg ml^{-1}$ 4-chloronaphthol and $3.6 mmol l^{-1}$ H_2O_2 in PBS. The membrane was carefully placed on the surface of this solution, with the side probed by the mosquitoes facing up, in order to allow diffusion of the substrates from under the membrane while preventing diffusion of the peroxidase out of the membrane. After 15min, the coloured precipitate became fixed on the nitrocellulose mesh and the membrane could be washed with water, dried and stored.

Molecular sieving HPLC was performed on salivary gland homogenates using a TSK 250 column (300mm×7.5mm) and pre-column (75mm×7.5mm, obtained through Bio Rad, USA) with $10 mmol l^{-1}$ Hepes at pH7.0 and $150 mmol l^{-1}$ NaCl at a flow rate of $0.8 ml min^{-1}$. The absorbances at 280 and 413nm were recorded, and fractions were collected every 0.4min. To assay for vasodilatory activity, gland homogenates were run with $0.2 mol l^{-1}$ ammonium formate at pH6.0. Fractions were collected as above, then $10 \mu l$ of $10 mg ml^{-1}$ bovine serum albumin was added before drying under vacuum in a centrifuge. This allowed concentration of the samples and removal of the volatile buffer. The relative molecular mass markers used were cyanocobalamin, myoglobin, ovalbumin and immunoglobulin G. Retention times of these markers did not differ in the two buffer systems used. Dried fractions were resuspended in 0.1ml of Krebs medium and assayed in the rabbit aortic ring preparation.

Light absorption spectra were measured with a Hewlett Packard model 8452A diode array spectrophotometer. Spectra were stored to a computer disk, and difference spectra were determined with a spreadsheet program.

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 homogenates of *Anopheles albimanus* were added to an aortic ring preconstricted with $1 \mu mol l^{-1}$ noradrenaline, a slow vasodilation followed (Fig. 1). In six experiments, with different rabbit aortic rings and different salivary gland homogenates, full relaxation of the aorta was obtained when four pairs of salivary glands were added per milliliter of the bioassay media. This vasodilation was independent of the presence of an intact endothelium, and was not seen if the aorta was contracted with $60 mmol l^{-1}$ KCl instead of noradrenaline. It was also destroyed by exposure to 100°C for 5min, partially destroyed by exposure to 56°C for 5min and destroyed by
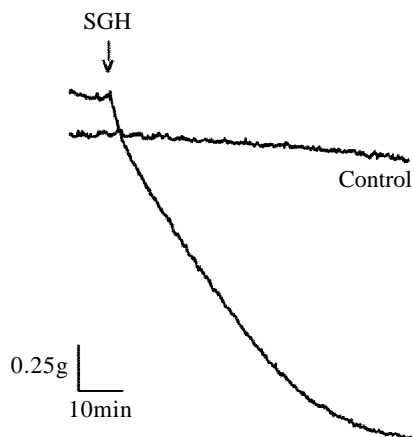
Fig. 1. *Anopheles albimanus* salivary gland homogenates induce an endothelium-independent vasodilation in a rabbit aortic ring preconstricted with noradrenaline. Aortic rings, whose endothelia have been scraped off with a wooden stick, had a resting tension of 2g and were constricted by 1 μmol l^{-1} noradrenaline. SGH indicates addition of 4 salivary gland pairs per milliliter. The control ring was from the same rabbit, and was tested simultaneously with the ring treated with the homogenate.

exposure to 90% ethanol, or 50% acetonitrile and 0.1% trifluoroacetic acid (data not shown).

The possibility that the salivary homogenate exhibited its vasodilatory activity either by slowly acting on the tissue to release some vasodilatory substance or by changing its susceptibility to noradrenaline was investigated. Aortic rings were preconstricted with noradrenaline for various periods and then 0.5ml of the Krebs medium was added to an aortic strip that was being superfused without NA. As time progressed, less vasoconstrictory activity was retained in the preparation incubated with noradrenaline and salivary homogenates (Fig. 2A). This previous finding could be explained by destruction of noradrenaline by the salivary gland homogenate. To test this hypothesis, noradrenaline in Krebs–Ringer solution was incubated with salivary gland homogenates (without aortic rings) and, at predetermined time intervals, a sample was added to the superfused aortic strip. The results indicate that the salivary homogenate was destroying noradrenaline (Fig. 2B).

It is known that noradrenaline is susceptible to oxidation, producing the corresponding adrenochrome, which has typical absorbance maxima at 300 and 490nm (Klebanoff, 1959). After incubation of 0.1mmol l^{-1} noradrenaline with salivary homogenates for 90min, the difference spectrum obtained was consistent with the oxidation of noradrenaline (Fig. 3).

Incubation of 1mmol l^{-1} noradrenaline with 1 pair of homogenized salivary glands per milliliter in phosphate-buffered saline showed a lag phase of 20–40min (Fig. 4) before the reaction started. This lag phase could be eliminated and the rate of reaction increased by the addition of both xanthine oxidase and xanthine (but not by either alone) to give a reaction producing superoxide. The oxidation of adrenaline could be totally blocked by the combined addition of superoxide dismutase and catalase. Addition of superoxide

dismutase alone increased the lag phase, which was followed by a burst of activity (Fig. 4). Salivary gland homogenates also oxidized dihydroxyphenylalanine (DOPA), which could be followed by increased absorbance at 450nm (not shown).
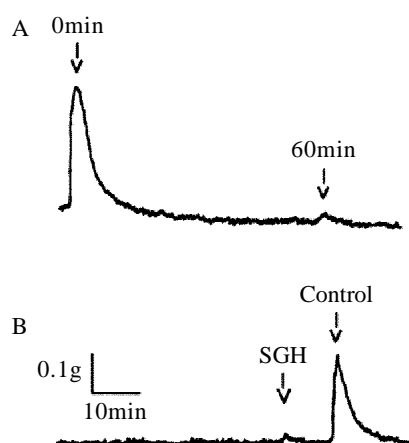

Fig. 2. Bioassay of noradrenaline at time zero and 60min after addition of *Anopheles albimanus* salivary gland homogenate (SGH) to the aortic ring preparation or to Krebs medium. 100 μl was assayed on the laminar-flow superfused aortic strip. (A) Contractions induced immediately (time 0) or 60min after SGH addition to the aortic ring shown in Fig. 1. (B) SGH indicates contraction in response to NA after 60min of exposure to SGH (without the aortic ring) or to noradrenaline incubated with Krebs medium for 60min, but without SGH (Control).
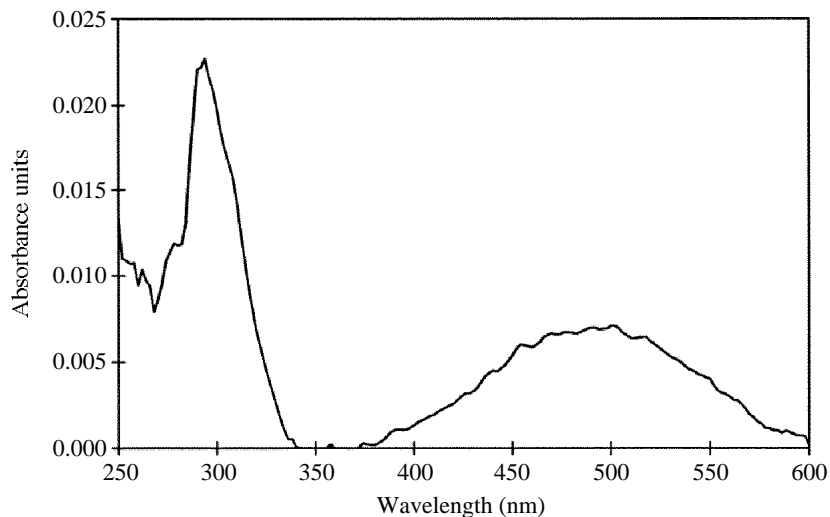

Fig. 3. Difference spectrum of noradrenaline ($0.1mmol1^{-1}$) with one pair of homogenized *Anopheles albimanus* salivary glands per milliliter at time 0 and 90min of incubation. A control noradrenaline difference spectrum without salivary glands yielded a flat difference spectrum.
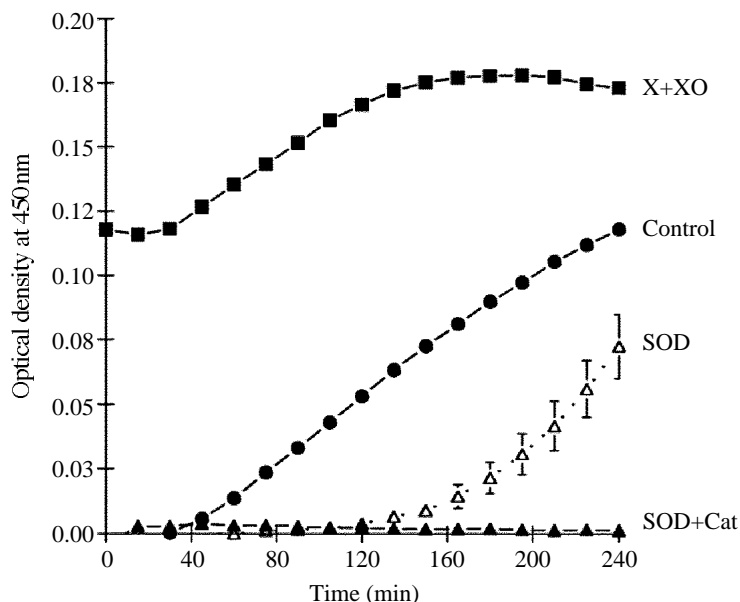
Fig. 4. Oxidation of noradrenaline by *Anopheles albimanus* salivary gland homogenates. The control trace shows the increase in absorbance at 450nm of medium containing $1\,mmol\,l^{-1}$ noradrenaline and 1 homogenized pair of salivary glands per milliliter in $0.15\,mol\,l^{-1}$ NaCl and $10\,mmol\,l^{-1}$ sodium phosphate, pH7.0. X+XO contained $1\,mmol\,l^{-1}$ xanthine and 50 milliunits of xanthine oxidase. SOD indicates the additional presence of 30 units per milliliter of superoxide dismutase. SOD+Cat contained 30 units per milliliter of superoxide dismutase and 1000 units per milliliter of catalase. The symbols and bars indicate mean ± S.E.M. of six experiments. Except for SOD, the error bars are smaller than the symbols.

Since heme peroxidases display catechol oxidase activity with kinetics similar to that shown above, including lag kinetics abbreviated by superoxide or hydrogen peroxide donors (Klebanoff, 1959; Paul, 1963), and since *Anopheles albimanus* salivary glands are a brownish-red color, suggesting the presence of a heme protein, we assayed the homogenates for peroxidase activity. The homogenates displayed a strong activity with *o*-dianisidine in the presence of hydrogen peroxide. This activity was inhibited by KCN and sodium azide, as expected of heme-containing peroxidases (Fig. 5). Additionally, the visible spectrum of 50 homogenized pairs of glands in $100\,\mu l$ of PBS indicated a typical Soret band with a maximum at 413nm, which faded upon addition of dithionite and intensified with exposure to carbon monoxide, with a shift of the maximum to 425nm (Fig. 6).

To test whether substrates of pharmacological interest could be destroyed by *Anopheles albimanus* salivary homogenates, several substances were incubated at concentrations of $1\,\mu mol\,l^{-1}$ with homogenates in the presence and absence of $10\,\mu mol\,l^{-1}$ H_2O_2. Histamine, measured in the guinea pig ileum preparation, and prostaglandin E_2, measured in the rat uterus preparation, were not destroyed by the homogenates, either in the presence and in the absence of H_2O_2 (data not shown). However, serotonin activity, monitored on the rat uterus preparation, was inhibited.
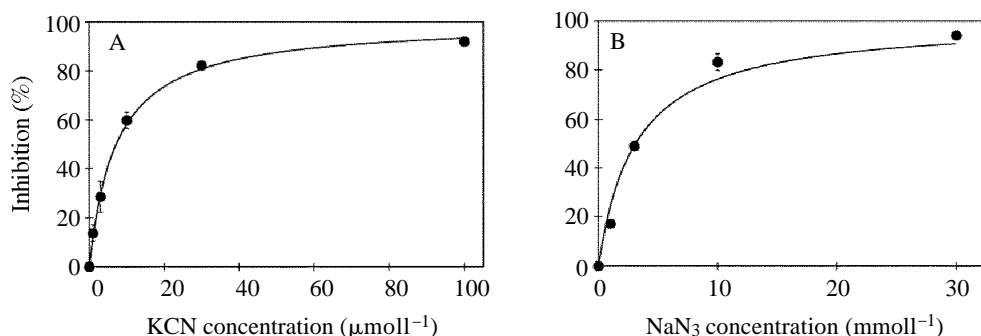
Fig. 5. Inhibition of *Anopheles albimanus* salivary peroxidase activity by KCN (A) and sodium azide (B). Reaction media contained $10 \, mmol \, l^{-1}$ Hepes buffer at pH7.0, $0.2 \, mg \, ml^{-1}$ *o*-dianisidine, $0.1 \, mmol \, l^{-1}$ H_2O_2, 1 pair of homogenized salivary glands per milliliter and the indicated concentrations of KCN or sodium azide. Symbols and bars are the mean ± S.E.M. of three experiments with different salivary gland pools. The tracings are hyperbolic functions yielding the minimum squared residues, assuming a maximum inhibition of 100%.
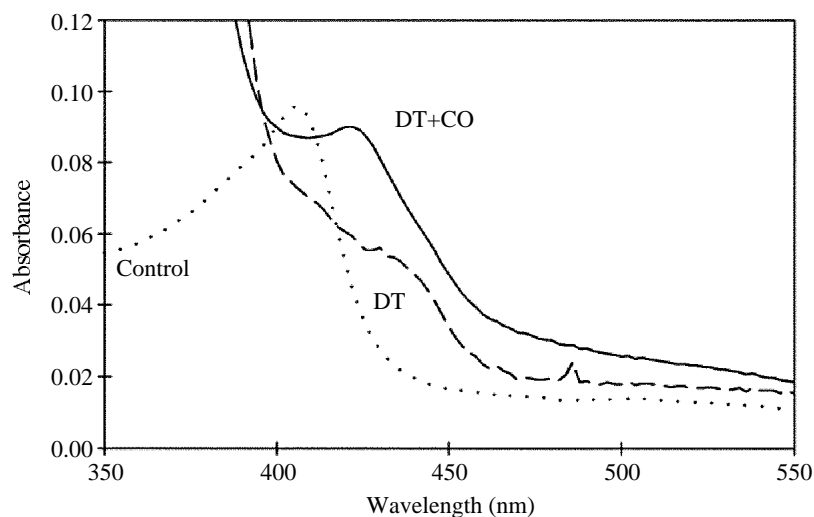

Fig. 6. Optical absorbance of *Anopheles albimanus* salivary gland homogenates in the presence of dithionite (DT) and carbon monoxide (CO). Fifty pairs of homogenized glands in 0.1ml of $0.15 \, mol \, l^{-1}$ NaCl, $10 \, mmol \, l^{-1}$ sodium phosphate, pH7.4, were used (Control). A small crystal of dithionite was added and carefully stirred, yielding the spectrum labelled DT. Carbon monoxide was bubbled into the solution, producing the spectrum labelled DT+CO.

Incubation of homogenates with serotonin and hydrogen peroxide caused a time-dependent destruction of the biological activity of serotonin (Fig. 7). The intrinsic fluorescence of serotonin was also diminished upon incubation with homogenates and H_2O_2 (Fig. 8A). The reaction was efficient in the use of H_2O_2, as indicated by the destruction of $1 \, \mu mol \, l^{-1}$ serotonin when submicromolar concentrations of H_2O_2 were added to the medium (Fig. 8B).
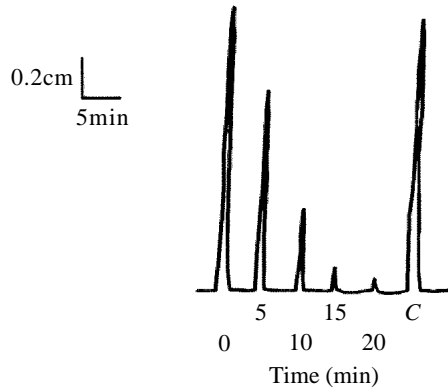
Fig. 7. Destruction of serotonin by *Anopheles albimanus* salivary gland homogenates. De Jalon solution, containing $10\,\mu\text{mol}\,\text{l}^{-1}$ H_2O_2 and $200\text{ng}\text{ml}^{-1}$ serotonin was incubated for 0, 5, 10, 15 and 20min with 0.1 pairs of homogenized glands per milliliter. Following incubation, 0.1ml was injected into a laminarly perfused rat uterus preparation. A control, incubated without salivary homogenates for 25min, is indicated by *C*.
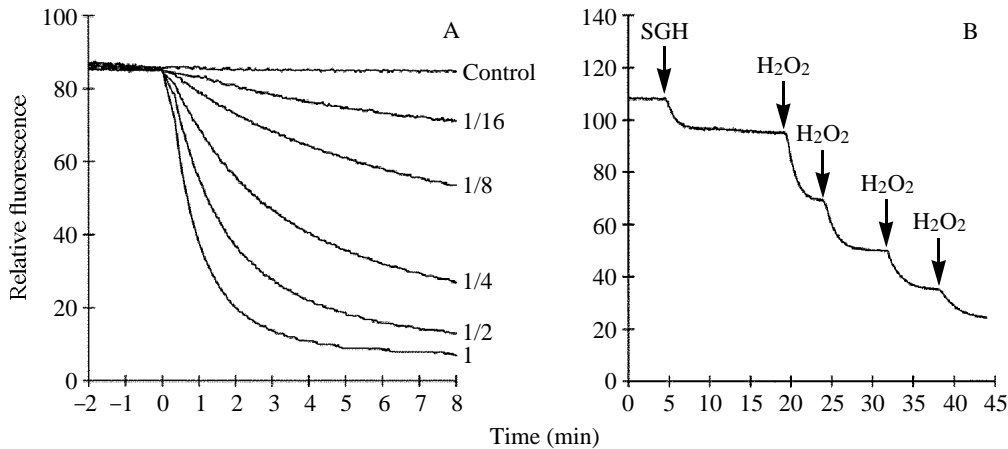

Fig. 8. Disappearance of the intrinsic fluorescence of serotonin during incubation with *Anopheles albimanus* salivary gland homogenates. (A) The numbers indicate the amount, in pairs of homogenized salivary glands, added to 1ml of $0.15\text{mol}\,\text{l}^{-1}$ NaCl, $10\text{mmol}\,\text{l}^{-1}$ sodium phosphate, pH7.0 (PBS), $1\,\mu\text{mol}\,\text{l}^{-1}$ serotonin and $10\,\mu\text{mol}\,\text{l}^{-1}$ H_2O_2. (B) The cuvette contained 1ml of PBS and $1\,\mu\text{mol}\,\text{l}^{-1}$ serotonin. SGH indicates the addition of one pair of homogenized salivary glands. H_2O_2 indicates the addition of $0.1\,\mu\text{mol}\,\text{l}^{-1}$ H_2O_2.

To test whether the catechol oxidase and peroxidase activities reside in the same molecule(s), homogenates were submitted to molecular sieving chromatography, and the eluate was monitored for the various activities. Activities for catechol oxidase, *o*-dianisidine peroxidation, serotonin peroxidation and vasodilation co-eluted with the same retention time as a peak absorbing at 413nm with an estimated relative molecular mass of 50000 (Fig. 9).
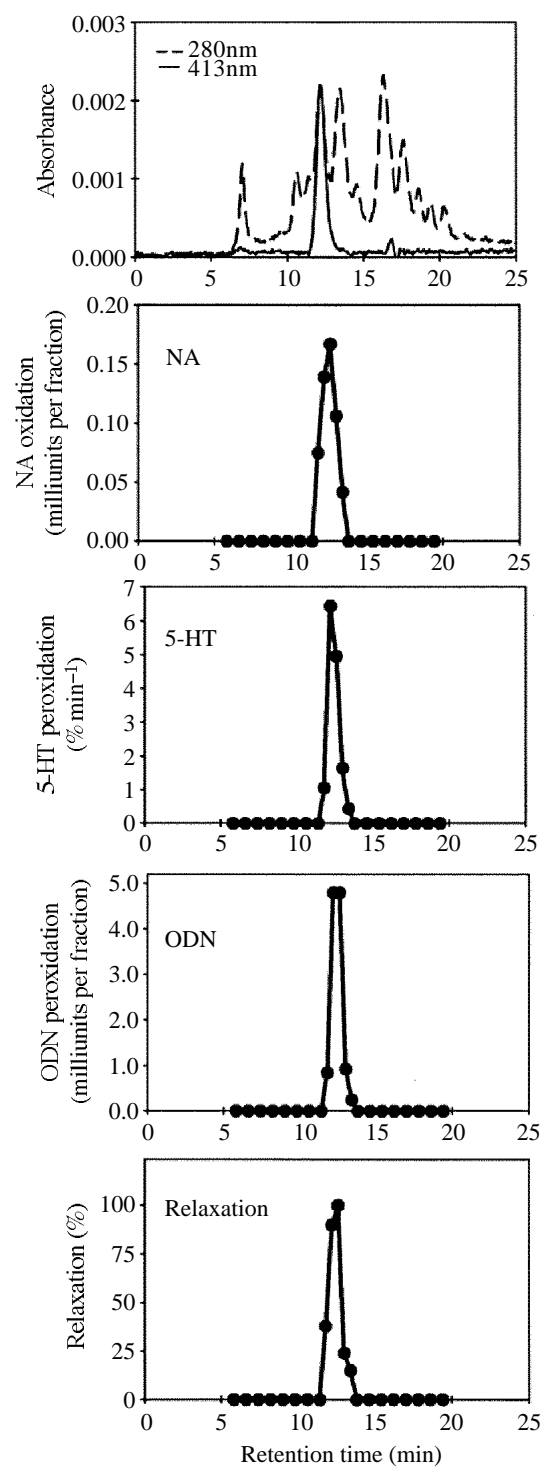
Fig. 9

The peroxidase activity was localized within the glands by fixing them in glutaraldehyde and staining for peroxidase activity. Stain localized exclusively on the distal, female-specific lobes of the glands (Fig. 10A).

To verify whether the peroxidase activity was of a secretory or a constitutive nature, mosquitoes were allowed to probe a warm membrane of nitrocellulose, which was later incubated with peroxidase substrates. Results confirmed the secretion of the enzyme activity during probing (Fig. 10B). Additionally, the total protein content and peroxidase activity levels of control and blood-fed mosquitoes were measured. The results showed a reduction of 41.9% in salivary protein and 40.5% in salivary peroxidase content following the blood meal ($P<0.01$ for both comparisons on a Student's t-test) (Fig. 11).

Discussion

The results reported above indicate that salivary gland homogenates of the mosquito *Anopheles albimanus* oxidize noradrenaline. Additionally, homogenates also demonstrate a peroxidatic reaction towards *o*-dianisidine and serotonin. The enzyme resides in the posterior, female-specific regions of the female salivary glands, and enzymatic activity can be detected in sites probed by hungry mosquitoes. Protein and peroxidase activity are significantly reduced after a blood meal, indicating the secretory fate of this activity during hematophagy. In the following paragraphs we will advance the hypothesis that both oxidase and peroxidase activities reside on a heme protein, discuss the physiological significance of these activities for blood feeding, and speculate on the question of where the superoxide or hydrogen hydroperoxide comes from.

Peroxidases can, with certain substrates, promote oxidatic (as opposed to peroxidatic) reactions. For example, horseradish peroxidase and lactoperoxidase have been shown to oxidize adrenaline (Klebanoff, 1959; Paul, 1963). Additionally, substances such as dihydroxyfumaric acid (Buhler and Mason, 1961; Dordick *et al.* 1986) or thyroxine-related compounds (Klebanoff, 1959) can be used instead of hydrogen peroxide for the co-oxidation of typical peroxidase reactions (Paul, 1963). Enzymes displaying such behavior belong to the heme peroxidase family (Paul, 1963). Interestingly, salivary glands of *Anopheles albimanus* have a distinct brownish-red color, particularly in the posterior middle lobe, and homogenates display a typical Soret absorption band. This pigment eluted as a single symmetrical peak on a TSK molecular sieving column, co-eluting with vasodilatory, noradrenaline-oxidizing and peroxidase activities. The simplest explanation for our results is that *Anopheles albimanus* salivary glands contain a heme peroxidase displaying an oxidatic reaction towards catecholamines.

Fig. 9. Molecular sieving chromatography of *Anopheles albimanus* salivary gland homogenates. Fifty homogenized pairs of salivary glands were used. The top panel indicates the absorbance of the eluate at 280 and 413nm. NA indicates noradrenaline oxidation, 5-HT indicates the disappearance (in $\%\,min^{-1}$) of the intrinsic fluorescence of serotonin when $50\,\mu l$ of the fraction was mixed with $50\,\mu l$ of $2\,\mu mol\,l^{-1}$ serotonin and $20\,\mu mol\,l^{-1}$ H_2O_2. ODN indicates peroxidation of *o*-dianisidine. Relaxation indicates vasodilatory activity (as percentage relaxation of the ring 45min after addition of samples to the aortic ring preconstricted with noradrenaline). Vasodilatory activity was from a different run, with different buffer conditions (see Materials and methods for details).
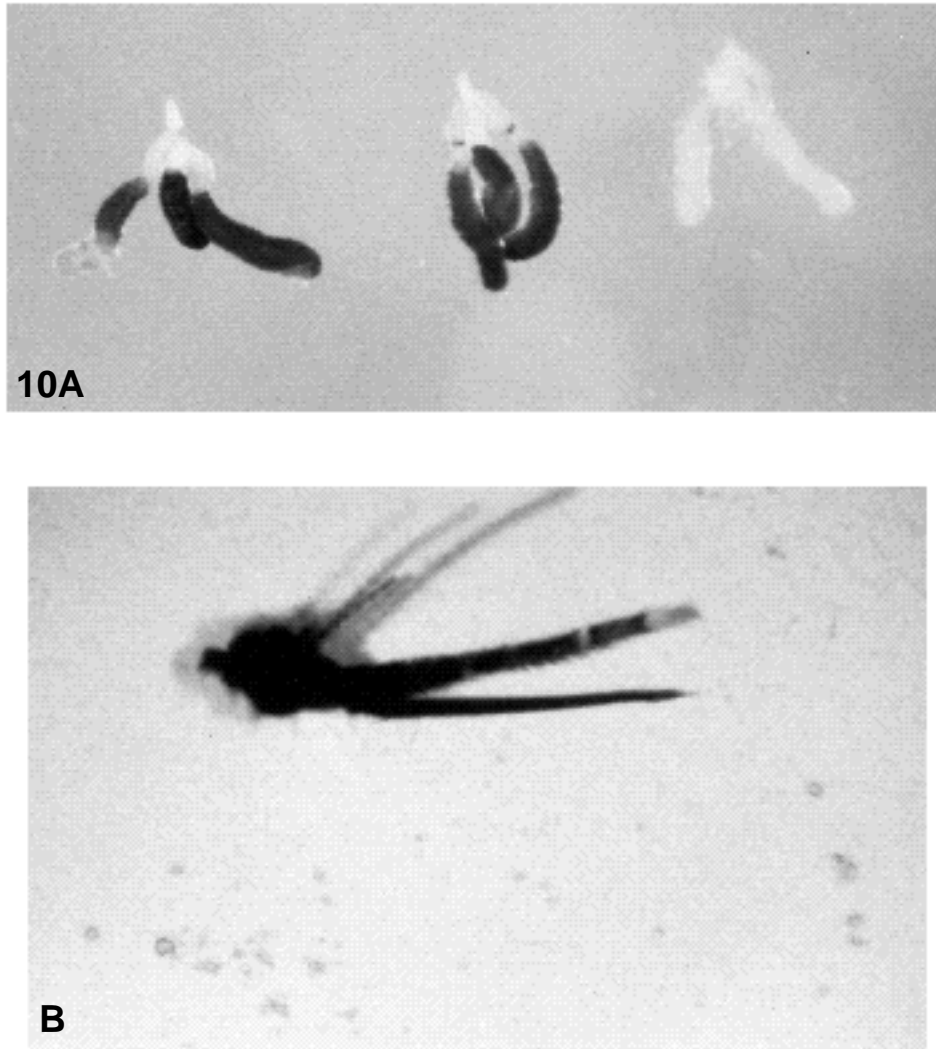
Fig. 10. Enzymatic staining of *Anopheles albimanus* salivary peroxidase. (A) Salivary glands fixed with glutaraldehyde were incubated with peroxidase substrates. The gland on the far right is a control fixed, but not incubated, with the substrate. (B) Staining of nitrocellulose probed by hungry mosquitoes. The head of a mosquito appears for size comparison.

The secretory role of these activities is apparent from the decrease in activity in the salivary glands following a blood meal and by its presence in sites probed by mosquitoes. The physiological role in feeding of these activities appears to be associated with the inactivation of vasoconstrictor substances released during the hemostatic process. Interestingly, one of the sources of vertebrate peroxidase is the eosinophil, a leukocyte that is usually found as a latecomer to inflammatory reactions and is thought to be involved in down-regulating inflammation (Bruijnzeel, 1989; Kay, 1985; Venge *et al.* 1987). Its secreted peroxidase has been implicated in the destruction of leukotrienes
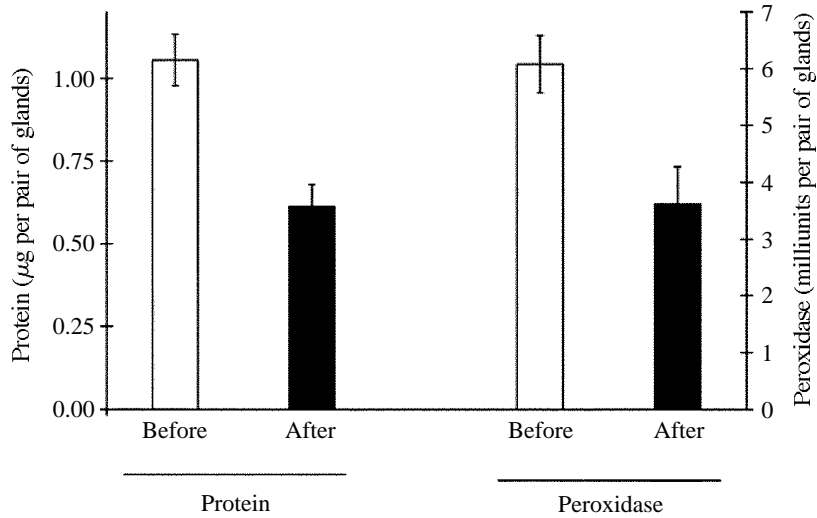
Fig. 11. Decrease in salivary protein and peroxidase activity in *Anopheles albimanus* salivary glands after probing and blood feeding. Bars represent means ± S.E.M. for 10 individuals.

(Henderson *et al.* 1982). Since heme peroxidases usually display a very large range of substrate specificity, it is possible that these enzymes also react with unknown plasma precursors, producing other biologically active substances with vasodilatory activity.

Although *Anopheles albimanus* salivary gland homogenates promote a slow vasorelaxation in noradrenaline-constricted aortic rings, this effect was seen with 1–3 pair of glands per milliliter. The volume of skin probed by mosquitoes could be conservatively estimated to be a 3mm×3mm×3mm cube, or around 30 µl. The long spectrophotometric assays were performed with 1 pair of glands per milliliter and 1 mmol l^{-1} noradrenaline, or 1000 times more noradrenaline than the pharmacological (1 µmol l^{-1}) concentrations used in the bioassay. These large amounts were needed for accumulation of enough adrenochrome to be detectable photometrically. It is probable that the concentrations of enzyme injected during feeding would be at least one or two orders of magnitude larger than those used in our assays, decreasing the time needed for its effect.

If the peroxidatic rather than the oxidatic reaction of the homogenate is the main physiological function of this activity, then superoxide, hydrogen peroxide or another suitable co-oxidizing substrate must be supplied to the feeding site while saliva is being secreted. Indeed, the effect of the homogenates on noradrenaline can be increased by orders of magnitude in the presence of superoxide. Additionally, significant destruction of serotonin was seen only in the presence of hydrogen peroxide, the decay product of the short-lived superoxide anion. Superoxide or hydrogen peroxide could be produced either by the mosquito or by the injured host tissue. Activated neutrophils, which respond to ATP and to other factors released during tissue injury by aggregation and superoxide production, could be one source of superoxide (Kuroki and Minakami, 1989; Moon *et al.* 1990; Stewart *et al.* 1990). Alternatively, superoxide or hydrogen peroxide could be supplied by the mosquito during salivation. These ideas remain to be tested.

Although this peroxidase activity is the first of its kind to be described in the saliva and salivary glands of a blood-sucking arthropod, it is interesting that both salivary peroxidase and catechol oxidase have been found in phytophagous Heteroptera (Miles and Peng, 1989). The peroxidase found in aphids is active against plant secondary compounds that are feeding deterrents to many other insects. Salivary peroxidase in phytophagous Heteroptera and in the mosquito *Anopheles albimanus* could be analogous in function, destroying host compounds that would otherwise disrupt feeding.

Many plant secondary compounds are amines, as are many vertebrate hemostatic and inflammatory mediators, and they are potential substrates for oxidases and peroxidases. Taking into consideration the enormous diversity and convergent evolution of insects towards plant-feeding and blood-feeding behavior, it is to be expected that many have found peroxidases to help the feeding process. It is possible that many may have amino-oxidases with a similar function. These enzymes are easily recognized by their FAD requirement and yellow color.

Plants and animals have sophisticated mechanisms to prevent blood, sap or tissue loss to insects. It has become apparent in the last few years that insect salivary glands contain the keys that unlock such defense responses. Since convergent evolution has resulted in the adaptation of different insects to the same mode of feeding on similar hosts, diverse solutions to the same problem may have been achieved. It is reasonable to expect that further knowledge of the biochemistry and pharmacology of arthropod salivary glands will lead to the discovery of novel compounds, to a better understanding of host/insect associations and to a new perspective in analyzing host hemostasis and injury responses.

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