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NITRIC OXIDE LOADING OF THE SALIVARY NITRIC-OXIDE-CARRYING HEMOPROTEINS (NITROPHORINS) IN THE BLOOD-SUCKING BUG *RHODNIUS PROLIXUS*

ROBERTO H. NUSSENZVEIG[1], DAVID L. BENTLEY[2] AND JOSÉ M. C. RIBEIRO[1,*]

[1]*Department of Entomology and Center for Insect Sciences* and [2]*Division of Biotechnology, University of Arizona, Forbes Building, Room 410, Tucson, AZ 85721, USA*

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Summary

The salivary glands of the blood-sucking bug *Rhodnius prolixus* are formed by a single layer of binucleated epithelial cells surrounded by a double layer of transversely oriented smooth muscle cells. The epithelial cells are rich in rough endoplasmic reticulum and mitochondria and have abundant microvillar projections towards the gland lumen. This cell layer surrounds a relatively large cavity where abundant secretory material is stored. Epithelial cells produce an intense and generalized NADPH diaphorase reaction, in contrast to other tissues such as brain, Malpighian tubules and skeletal muscle. Ultrastructural analysis of the osmiophilic reaction product indicates that it is localized within cytoplasmic vacuoles, a similar location to that of NADPH diaphorase (NO synthetase) activity in neuronal cells of vertebrates. Measurements of the time course of protein accumulation, NADPH diaphorase activity and the degree of nitrosylation of hemoproteins (nitrophorins) in the salivary glands of *Rhodnius prolixus* nymphs after a blood meal indicate that the nitrophorins are synthesized and accumulate when NO production is low (with a 25 % loading of the nitrophorins during the fourth- to fifth-instar molt). NO loading of the nitrophorins increases to 90 % after the molt, concomitant with a large increase in the salivary NADPH diaphorase activity. It is concluded that synthesis of NO occurs within the epithelial cells while the nitrophorins are stored extracellularly. It is hypothesized that the luminally oriented microvilli may serve as a diffusion bridge to direct intracellularly produced NO into the luminal cavity, where the nitrophorins are stored.

Key words: saliva, salivary glands, NO synthetase, nitric oxide, diaphorase, ultrastructure, *Rhodnius prolixus*, triatomine.

Introduction

The blood-sucking bug *Rhodnius prolixus* contains a salivary nitrovasodilator (Ribeiro *et al.* 1990) that, in concert with other activities, helps the insect to locate blood (Ribeiro and Garcia, 1981). This vasodilator is a nitrosylhemoprotein (named nitrophorin; D. Champagne, R. H. Nussenzveig and J. M. C. Ribeiro, in preparation) that stores and delivers nitric oxide to the host tissues during probing and feeding. Nitrophorins display an intense Soret band absorbing maximally at 422 nm in the presence, or at 404 nm in the absence, of nitric oxide (Ribeiro *et al.* 1993).

Rhodnius prolixus salivary glands have a nitric oxide synthetase that is similar to the vertebrate neural constitutive enzyme in its cofactor requirements (Ribeiro and Nussenzveig, 1993). Indeed, NADPH, FAD, tetrahydrobiopterin, calmodulin, calcium and arginine were required for NO production. Additionally, like the vertebrate enzyme, NO synthetase activity co-eluted with a single peak of NADPH diaphorase activity when submitted to molecular sieving high-performance liquid chromatography. Again resembling the vertebrate enzyme, *Rhodnius prolixus* NADPH diaphorase was active in the presence of NADPH and dye acceptor, and did not require the other cofactors for the full NO synthetase activity (Ribeiro and Nussenzveig, 1993). This salivary nitric oxide synthetase is probably involved in loading the salivary nitrophorins with nitric oxide.

NADPH diaphorase activity involves the enzymatic transport of two electrons from NADPH to suitable acceptors such as Nitro Blue Tetrazolium. On the basis of kinetic and primary sequence analysis of cloned vertebrate enzymes, NO synthetases have been shown to contain a domain analogous to cytochrome *P*-450 reductase (Mayer *et al.* 1991; Hope *et al.* 1991; Dawson *et al.* 1991; Bredt *et al.* 1991), which is responsible for the NADPH diaphorase reaction. Because the purple formazan product of such reactions can react with

*Author for correspondence.

osmium to produce an electron-dense compound, NADPH diaphorase activity can be localized by optical and electron microscopy (Van Noorden and Butcher, 1984; Hope and Vincent, 1989). *Rhodnius prolixus* salivary NADPH diaphorase is a good marker of salivary NO synthetase because the salivary NADPH diaphorase activity co-elutes with NO synthetase, with a molecular mass of approximately 160 kDa (Ribeiro and Nussenzveig, 1993).

Since most of the nitrophorins secreted by *Rhodnius prolixus* are contained within the large reservoir formed by the lumen of the salivary gland, and the NO synthetase activity is observed in the cellular pellet (Ribeiro and Nussenzveig, 1993), and also because NO is an unstable gas (Kiechle and Malinski, 1993), the question arises as to whether the nitrophorins are charged with NO intracellularly or whether the proteins are secreted uncharged, followed by NO secretion to the gland lumen. In this paper, we have investigated the ultrastructural localization of *Rhodnius prolixus* salivary NADPH diaphorase, as well as the temporal kinetics of NO loading into salivary nitrophorins.

Materials and methods

Insects were taken from a colony of *Rhodnius prolixus* Ståhl maintained at 27 °C and 70 % relative humidity. Salivary glands were dissected from starved fifth-instar nymphs directly in 4 % paraformaldehyde and 1 % glutaraldehyde in 0.1 mol l^{-1} sodium phosphate buffer, pH 7.0, and maintained at 4 °C for 30 min. After fixation, the glands were washed three times with 0.1 mol l^{-1} phosphate buffer followed by incubation for 30 min at 37 °C with, or without, 1 mmol l^{-1} NADPH, 1 mg ml^{-1} Nitro Blue Tetrazolium (NBT) and 0.01 % Triton X-100 in phosphate-buffered saline (PBS) containing 0.15 mol l^{-1} NaCl and 10 mmol l^{-1} sodium phosphate at pH 7.5. The glands were rinsed three times in PBS and postfixed with 1 % osmium tetroxide (OsO_4) for 2 h to form an electron-dense reaction product acting as a marker for NADPH diaphorase activity. Tissue was then dehydrated rapidly through an ethanol series, rinsed in propylene oxide (PO) and embedded in Spurr's epoxy resin. Ultrathin sections were cut on an ultramicrotome and examined in a Philips 420 electron microscope at 80 kV.

For ultrastructural studies, the fixed glands were incubated in PBS at pH 7.5 with 0.01 % Triton X-100 for 30 min at 37 °C, followed by postfixation with 1 % OsO_4 for 2 h, dehydration with ethanol and PO and embedding in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in the electron microscope at 80 kV.

Histological studies of the salivary glands were performed by fixing the glands in alcoholic Bouin's fixative for 48 h followed by washing with lithium carbonate for 1 h and dehydration in an ethanol series. Glands were infiltrated with paraffin for 72 h and embedded. Sections 8 μm thick were placed on a glass slide and stained using Harris' hematoxylin followed by alcoholic Eosin Y.

For studies related to the time course of synthesis of salivary proteins, fourth-instar nymphs were fed on a rabbit, and the salivary glands of 10 insects were individually dissected and transferred to 300 μl of a solution containing 10 mmol l^{-1} sodium acetate, pH 5.0, and 150 mmol l^{-1} NaCl. This pH was chosen because the NO–hemoprotein is known to be stable at this value (Ribeiro *et al.* 1993). After homogenization of the glands with a plastic pestle and centrifugation for 3 min at 14 000 ***g***, the light absorbance spectrum of the supernatant from 700 to 250 nm was recorded in a lambda 19 Perkin Elmer spectrophotometer for each of 10 individual pairs of glands at a selected number of days post-feeding. The absorbance at 280 nm was taken as an indication of total protein content. The absorbance at 413 nm (isosbestic point between nitrosylated and non-nitrosylated hemoprotein species, Ribeiro *et al.* 1993) was taken as a measure of hemoprotein content. The ratio of absorbance at 422 and 404 nm was used to estimate the degree of NO loading of the hemoprotein, based on spectra of totally nitrosylated and totally non-nitrosylated homogenates, assuming that the combined spectrum is the sum of these two forms (Ribeiro *et al.* 1993).

The time course of NADPH diaphorase activity following a blood meal by the fourth-instar nymphs was measured by individually dissecting out the salivary glands of 10 insects into 50 μl of phosphate-buffered saline at pH 7.4, homogenizing and centrifuging as above, and adding the supernatant to 50 μl of a reaction mixture to give final concentrations of 50 mmol l^{-1} sodium phosphate, pH 7.4, 1 mmol l^{-1} NADPH, 0.5 mg ml^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.1 mol l^{-1} NaCl, 1 mmol l^{-1} $CaCl_2$, 0.1 % Triton X-100 and 5 μmol l^{-1} FAD. The increase in absorbance at 550 nm was followed at 37 °C for 25 min in an automated ELISA plate reader. The activity is expressed as milliabsorbance units per minute per gland pair.

Results and discussion

Rhodnius prolixus salivary glands are made up of a single epithelial layer of binucleate cells (Baptist, 1941) and a double layer of smooth muscle cells surrounding a large secretory cavity (Figs 1, 2A). Using electron microscopy, the transverse arrangement of the two smooth muscle layers was evident from the arrangement of the myofibrils (Fig. 2B). Occasionally a nerve was observed in the muscular layer (Fig. 2B). The epithelial cells are rich in endoplasmic reticulum, vesicles and mitochondria, and have microvillar projections into the glandular lumen (Fig. 2A,C,D). When dissected into a saline solution, the glands were observed to contract with a peristaltic-like motion, which was sometimes followed by extrusion of the stored secretory materials.

Following incubation of the fixed salivary glands with NADPH and Nitro Blue Tetrazolium, a purple coloration was observed within 5 min. No color developed if either of the two substrates was omitted. The precipitate formed within the cells and not within the secretory cavity, because rupture of the glands to remove the stored secretions showed that the reaction product was within the cell layer and not in the secretory material (Fig. 3). Under the same conditions, there was no

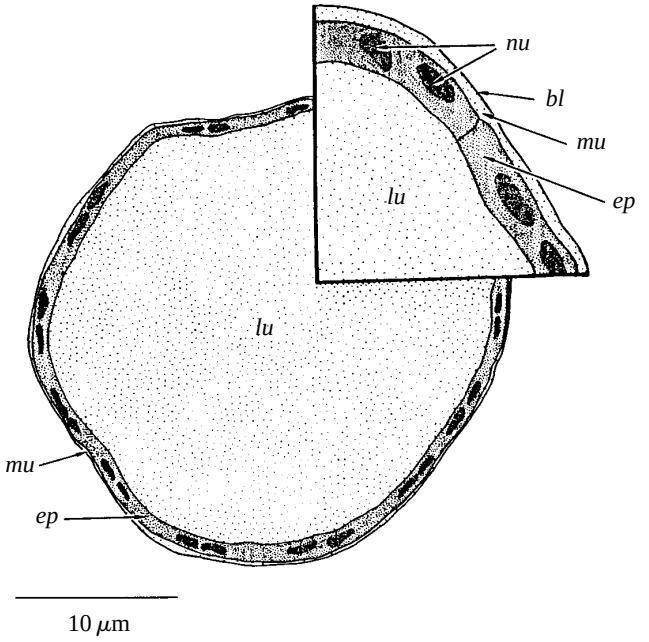

Fig. 1. Schematic drawing of a cross section of salivary glands showing the basal layer (*bl*) enveloping a muscle layer (*mu*) that surrounds epithelial (*ep*) binucleated (*nu*) cells and a large luminal (*lu*) cavity which contains abundant hemoproteins.

generalized Formazan color development in fixed samples of Malpighian tubules, striated muscle and brain. Lack of color development in non-salivary tissues does not reflect a complete absence of NADPH diaphorase, as increased incubation times led to eventual color development in non-salivary tissues (not shown).

Ultrastructural analysis of the Formazan product indicated an almost exclusive association with vacuolar structures of the epithelial cells (Fig. 4A,C). Control preparations showed no precipitated material (Fig. 4B,D). Similar vacuolar structures have been implicated as the site for NO synthesis in vertebrate neuronal cells (Hope and Vincent, 1989; Wolf *et al.* 1992).

Although there are many enzymes capable of producing a NADPH diaphorase reaction (Tracey *et al.* 1993), it was proposed recently that NO synthetase is the main enzyme responsible for the NADPH diaphorase reaction in fixed tissues (Matsumoto *et al.* 1993). Indeed, the histochemical distribution of this enzyme in vertebrates is consistent with the results of biochemical and physiological experiments (Dawson *et al.* 1991; Hope *et al.* 1991; Mitchell *et al.* 1992; Young *et al.* 1992). In the case of *Rhodnius prolixus* salivary glands, we have previously shown that most NADPH diaphorase activity co-chromatographed with NO synthetase activity in a molecular sieving column (Ribeiro and Nussenzveig, 1993). The presence of the Formazan precipitate in vesicular structures in the salivary glands of *Rhodnius prolixus* thus probably reflects the compartmentalization of NO synthetase in this insect cell.

Because the localization of the Formazan product indicated that NO in *Rhodnius prolixus* salivary glands may be produced

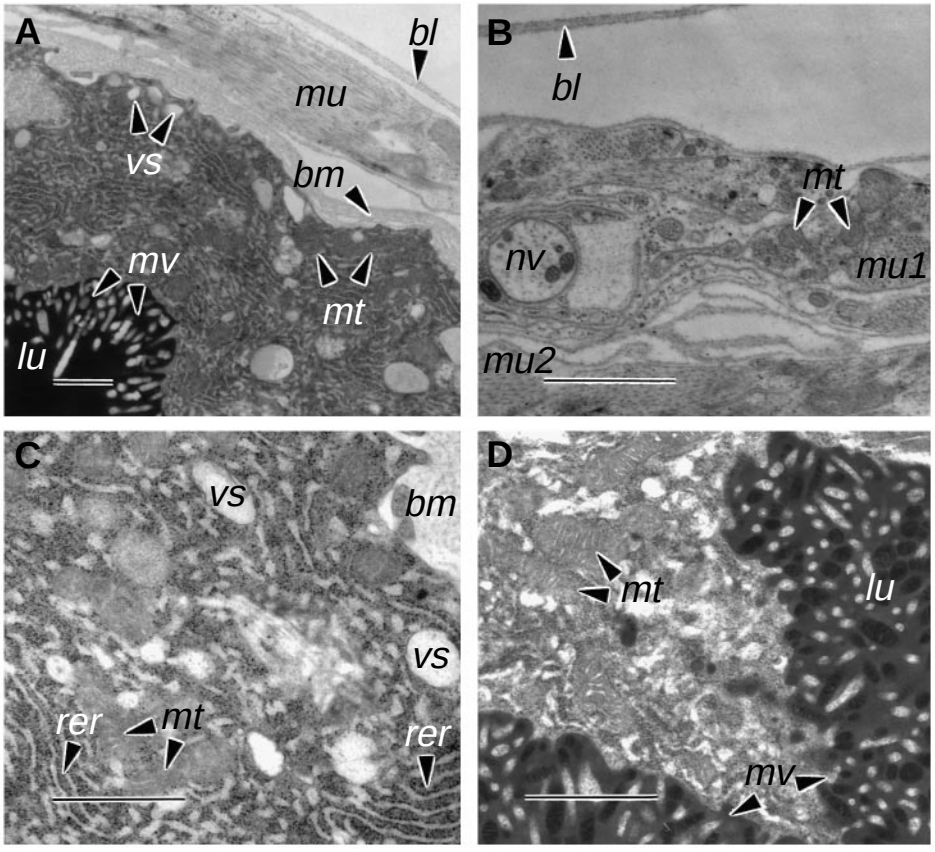

Fig. 2. Electron micrographs of salivary glands stained with uranyl acetate and lead citrate. (A) A cell layer with a outer basal layer (*bl*) surrounding a layer of muscle (*mu*); a basal membrane (*bm*) separates the muscle layer from a single layer of epithelial cells. The epithelial cells are rich in mitochondria (*mt*) and vesicles (*vs*); microvillae (*mv*) extend from the cell and penetrate into the lumen (*lu*). Two layers of muscle (*mu1* and *mu2*) are observed to run transversely to each other. A nerve (*nv*) is also occasionally apparent (B). The epithelial cells (C) are rich in rough endoplasmic reticulum (*rer*). Abundant microvillae projects into the luminal cavity (D). Scale bars, 1 μm.

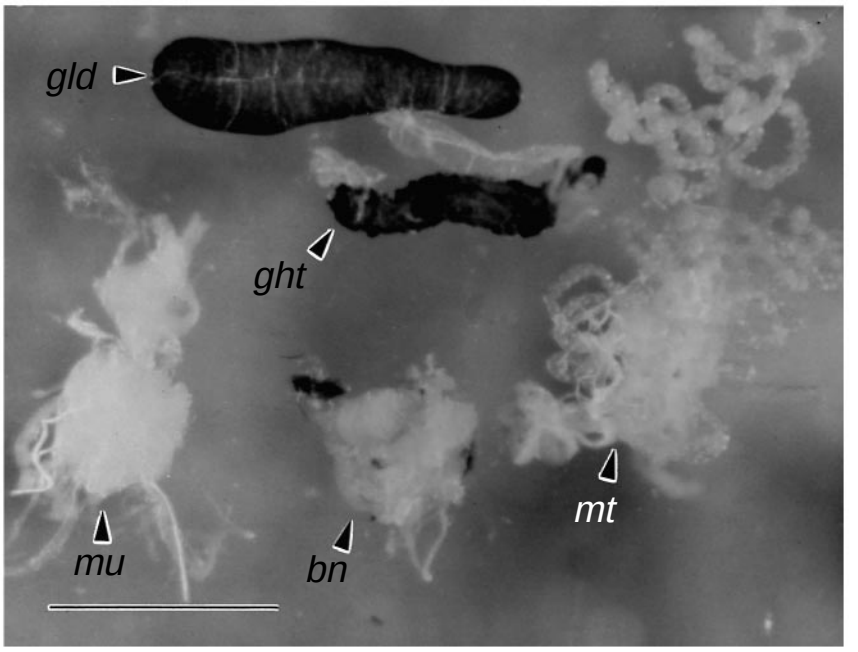

Fig. 3. Light microscopy of different tissues incubated with Nitro Blue Tetrazolium and NADPH showing considerable activity in the salivary glands (*gld*) compared with tissues such as brain (*bn*), muscle (*mu*) and Malpighian tubules (*mt*). Formazan precipitation was shown to be cell-specific, as demonstrated by the presence of large amounts of reaction product in a gland devoid of secretory contents (*ght*). Scale bar, 100 μm.

intracellularly, the question arises as to whether NO and the NO carrier hemoprotein (Ribeiro *et al.* 1993) could be synthesized in the same compartment and the NO carried to the gland lumen in a bound state with the hemoprotein. To address this issue, we investigated the kinetics of resynthesis of salivary proteins after a blood meal. *Rhodnius prolixus* takes large blood meals of about ten times its own body mass, during which most of the salivary proteins are secreted (Ribeiro and Garcia, 1980). This single blood meal allows the insect to molt to the next stage (usually 12–14 days for the fourth- to the fifth-instar molt at 27 ˚C), when the next feed (21 days) occurs (the insect will usually refuse to take a meal until 3–5 days after molting). The results indicate that after a blood meal *Rhodnius prolixus* loses about half of its salivary proteins, which are

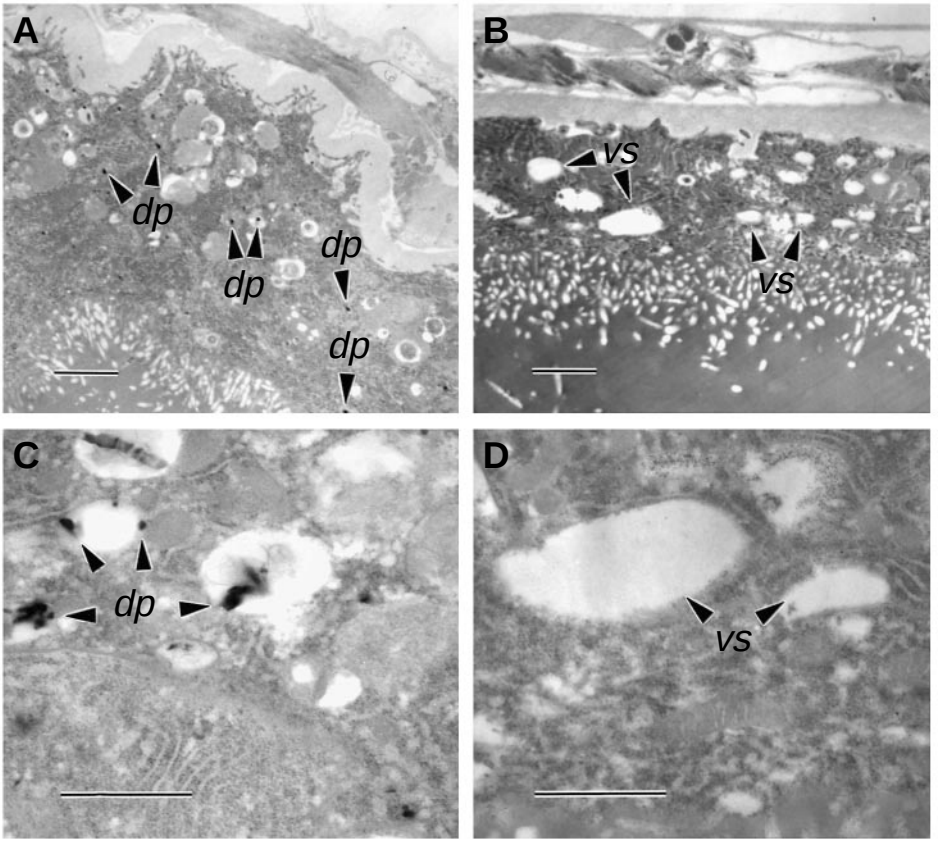

Fig. 4. Visualization of Formazan deposits in unstained electron microscopic preparations of salivary gland sections following NADPH diaphorase cytochemistry, incubated in the presence (A,C) or absence (B,D) of NADPH. Diaphorase (*dp*) activity in A and C is largely restricted to vesicles (*vs*). Controls without NADPH show no Formazan deposit. Scale bar, 1 μm.

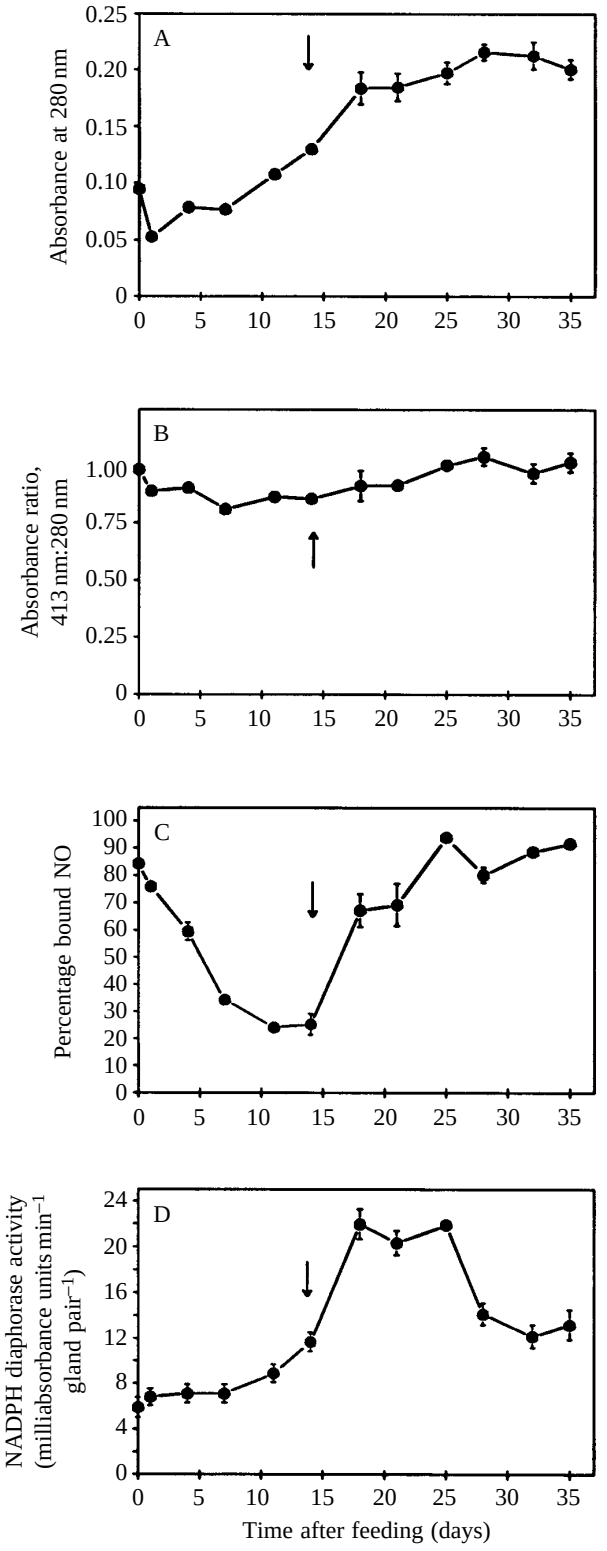

Fig. 5. (A) Kinetics of total salivary protein content (measured by ultraviolet absorbance at 280 nm), (B) hemoprotein to protein ratio, (C) percentage of nitric oxide bound to hemoproteins and (D) NADPH diaphorase activity in fourth-instar *Rhodnius prolixus* nymphs fed on day 1. Day 0 represents the pre-blood meal values. Symbols and bars are the mean ± S.E.M. (N=10). In some cases, the error bars are smaller than the symbols used and are not visible. Arrows indicate the molt from the fourth to the fifth instar. Insects are ready to feed 21 days after a blood meal.

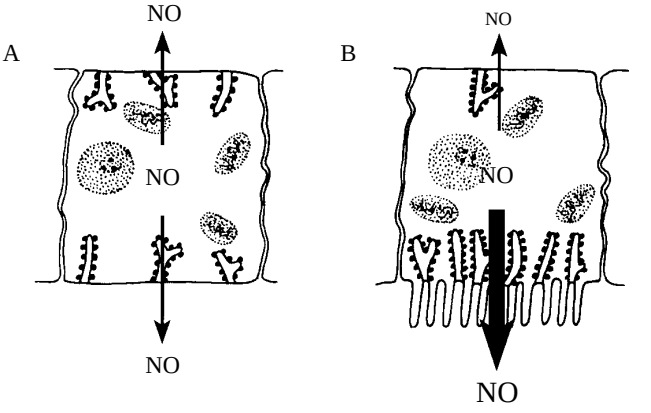

Fig. 6. Diagram depicting diffusion of NO from a symmetrical cell (A) and from a cell containing increased membrane projections on one side (B).

gradually resynthesized over a period of 4 weeks (Fig. 5A). During this period, the hemoproteins are a constant fraction of the total proteins, as measured by the 413 nm to 280 nm absorption ratio, indicating that nitrophorins are synthesized and exported to the lumen as holoproteins (Fig. 5B). As more hemoprotein is synthesized, the fraction of NO loading decreases, reaching a minimum at about the time of molting, but NO content increases rapidly after the molt (Fig. 5C). This sharp increase in NO loading of the hemoproteins is paralleled by a similar sharp increase in the salivary gland NADPH diaphorase activity (Fig. 5D), which more than doubles within 4 days. The results thus indicate that most of the nitrophorins are already synthesized (and in a luminal position) when NO is produced. Accordingly, NO is probably synthesized intracellularly and then diffuses to the gland lumen, where it binds to the nitrophorins.

If NO is produced intracellularly in a perfectly symmetrical epithelial cell, it follows that the probability of NO diffusion to either side of the cell would be equal (Fig. 6A). However, NO solubility in water is very limited, a saturated solution being approximately 2 mmol l^{-1}, but solubility in lipids is much greater. Accordingly, it has been postulated that NO could partition and diffuse with higher efficiency in lipid membranes than in the aqueous cytoplasm (Kiechle and Malinski, 1993). If this is the case, then an asymmetry of the membranes of a cell could direct NO diffusion preferentially to one side of the cell (Fig. 6B). The presence of abundant microvilli protruding into the luminal cavity of *Rhodnius prolixus* salivary glands (Fig. 2A,D) could thus provide an efficient gateway mechanism to direct the flow of NO from an intracellular compartment towards the gland lumen. We cannot conclude from this work that the Formazan-positive vesicle membranes are connected to the microvilli membranes. Microvilli are usually associated with a role in increasing the

cellular absorptive surface, but in this case we suggest that they can also work in the reverse direction, providing an increased diffusion surface for NO, a lipid-soluble gas secreted by the salivary glands of *Rhodnius prolixus*.

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