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Biosynthesis of Salivary Prostaglandins in the Lone Star Tick, *Amblyomma americanum*

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Dopamine-induced saliva from ticks fed [^{3}H]arachidonic acid contained the radiolabelled prostaglandins E_2, $F_{2\alpha}$, D_2, and B_2, the latter probably derived from PGE_2 owing to the alkalinity of tick saliva. Prostaglandin synthetase (PGS) activity in the salivary gland homogenate from the lone star tick, *Amblyomma americanum*, could not be detected by standard radiometric methodologies successfully employed for tissues from many animal species, including numerous arthropods. Modifications to the assay conditions had no effect. The presence of a PGS-inhibitor in the salivary glands was ruled out. It is postulated that the PGS in *A. americanum* salivary glands may be considerably different from that found in other animals, including vertebrate hosts.

Arachidonic acid Biosynthesis Prostaglandins Prostaglandin synthetase Saliva Ticks

INTRODUCTION

In contrast to other blood-feeding arthropods, ixodid ticks remain attached to their host for many days where they alternate between imbibing blood components infiltrating into the feeding lesion and returning excess fluid and ions back to the host via the saliva. Sustained feeding presents problems for the successful completion of the meal: prolonged attachment risks an immune and inflammatory response from the host and the capillaries must remain patent. Saliva contains substances able to facilitate feeding: immuno-suppressants (Ribeiro, 1987; Ramanachandra and Wikel, 1992); anti-coagulants (Sauer *et al.*, 1995); anti-hemostatics (Ribeiro, 1987) and analgesics (Ribeiro, 1987). Prostaglandins of the 2-series possess many of these properties and have been identified in tick saliva by bioassay (Dickinson *et al.*, 1976; Higgs *et al.*, 1976; Ribeiro *et al.*, 1985) and by bioassay/gas chromatography–mass spectrometry (Ribeiro *et al.*, 1992). It is now thought that prostaglandins secreted into the host in tick saliva may be vital for the accomplishment of a successful bloodmeal (Sauer *et al.*, 1993).

Prostaglandins of the 2-series are synthesized from the precursor arachidonic acid via the cyclooxygenase pathway (Needleman *et al.*, 1986; Smith, 1989) following the generation of unesterified arachidonic acid from phospholipid, most commonly by the action of phospholipase A_2 (PLA_2) (Holtzman, 1991). The salivary glands of the lone star tick, *Amblyomma americanum*, contain a high concentration (~8%) of arachidonic acid which is present only in the phospholipid fraction (Shipley *et al.*, 1993a). Arachidonic acid levels in salivary glands increase dramatically (40×) during feeding (Shipley *et al.*, 1993b) due solely to sequestration from the bloodmeal as *A. americanum* is incapable of synthesizing arachidonic acid from any precursor (Bowman *et al.*, 1995). The salivary glands possess a calcium-sensitive PLA_2 activity many fold higher than other tick tissues (Bowman *et al.*, 1993) which is capable of generating increased free arachidonic acid levels in salivary gland explants following calcium ionophore stimulation (Bowman *et al.*, 1994). Dopamine-induced saliva of *A. americanum* contained extremely high concentrations of PGE_2 (500 ng/ml) and $PGF_{2\alpha}$ ($\geq$50 ng/ml) (Ribeiro *et al.*, 1992).

Taken overall, we have evidence that the salivary glands of the lone star tick possess many of the attributes of a prostaglandin producing organ. However, demonstrating *A. americanum* salivary glands are capable of synthesizing prostaglandins has remained elusive to date, though microsomes of the whole tick may have some eicosanoid producing capacity (Pedibhotla and Stanley-Samuelson, personal communication). In this paper, we present findings on prostaglandin synthetase (PGS) activity in *A. americanum* salivary gland homogenates as assessed by the formation of prostaglandins from radiolabelled arachidonic acid using standard methodologies. Employing a novel approach, we report radiolabelled prostaglandins were isolated from the

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saliva of *A. americanum* which had been fed or injected with tritiated arachidonic acid.

MATERIALS AND METHODS

Materials

Thin-layer chromatography plates 20 × 20 cm, 250 μm thickness channelled silica gel G with a pre-absorbent zone were purchased from Analtech (Newark, DE). Silicic acid (BioSil-A; 100–200 mesh) was acquired from BioRad (Richmond, CA). Arachidonic acid [5,6,8,9,11,12,14,15-^{3}H(N)]- (80–100 Ci/mmol) and prostaglandin E_2 [5,6,8,11,12,14,15-^{3}H(N)]- (154 Ci/mmol) were obtained from Du Pont-New England Nuclear (Wilmington, DE). Prostaglandin standards and unlabelled arachidonic acid were purchased from Cayman Chemical Company (Ann Arbor, MI). High-performance liquid chromatography grade ethyl acetate and 2,2,4-trimethyl pentane were purchased from EM Science (Gibbstown, NJ). Chloroform and methanol were supplied by Fisher Scientific (Pittsburgh, PA) and were glass-redistilled before use. All other chemicals were obtained from Sigma (St Louis, MO). Bovine seminal vesicles were from Oxford Biomedical Research Inc. (Oxford, MI).

Preparation of salivary gland homogenate

Adult female lone star ticks, *Amblyomma americanum* (L.), were reared on sheep according to the methods of Patrick and Hair (1976). Ticks were removed from the sheep either during the "slow-feeding" phase (<200 mg) or the "fast-feeding" phase (>200 mg). Salivary glands were dissected into ice-cold 0.1 M morpholinopropane sulfonic acid (MOPS), pH 6.8, containing 0.02 M ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and rinsed three times in the same. Glands from 10 ticks were manually homogenized in 150 μl 100 mM Tris–HCl, pH 7.8 containing 10 mM EGTA (Buffer A). The homogenate was transferred to the incubation tubes with a further 150 μl Buffer A.

Bovine seminal vesicle homogenates (~3 mg/replicate) were prepared as for the salivary glands.

Preparation of salivary gland microsomes

Approximately 175 pairs of frozen salivary glands from slow feeders were homogenized in ice-cold 10 mM Tris–HCl, 0.25 M sucrose, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.2 containing 0.05% (w/v) *p*-amino-benzamidine. The homogenate was centrifuged at 900 ***g*** for 10 min, and that supernatant was centrifuged at 11,500 ***g*** for 10 min. The 11,500 ***g*** supernatant was centrifuged at 100,000 ***g*** for 60 min and the resultant microsome-rich pellet reserved for PGS activity assay.

Prostaglandin synthetase assay

An equal volume Buffer A containing bovine hemoglobin and reduced glutathione was added to the salivary gland or bovine seminal vesicle preparations to give final concentrations of 0.4 μM hemoglobin and 5 mM glutathione. The reaction was initiated by the addition of 1.0 μCi [^{3}H]arachidonic acid, giving a final substrate concentration of 0.02 μM and incubated at 37°C for 20 min. A variety of assay conditions were tested and are discussed in the Results.

The reaction was terminated by the addition of 0.1 M HCl to acidify the mixture to pH 3–4.5. Lipids were extracted into 2 ml ethyl acetate and the phases separated by centrifuging for 5 min at 2500 ***g***. The upper organic layer was collected and the aqueous phase re-extracted as before. The pooled lipid extract was dried under nitrogen and re-dissolved in 150 μl ethyl acetate. Glass pasteur pipettes (0.5 × 6 cm) were plugged with glass wool and packed with activated BioSil-A silicic acid to a height of 3 cm. The lipid extract was transferred to the column and the prostaglandins eluted with 6 ml ethyl acetate–acetic acid (99:1, v/v), whilst the phospholipid fraction remained on the column.

The extracted prostaglandin samples were mixed with authentic standards and applied in ethyl acetate to prewashed and activated silica gel G plates with pre-absorbent zones. The plates were developed in one of two solvent systems. Solvent system A: the organic phase of ethyl acetate–2,2,4-trimethyl pentane–acetic acid–water (110:50:20:100, v/v/v/v) after drying with anhydrous magnesium sulphate (Salmon and Flower, 1982) used in non-equilibrated developing tanks. Solvent system B: chloroform–methanol–acetic acid–water (90:8:1:0.8, v/v/v/v) (Salmon and Flower, 1982) used in equilibrated developing tanks. The position of prostaglandin standards was determined following exposure to iodine vapor. Distribution of the radioactivity was assessed by radioscanning (BioScan 2000, BioScan, Washington, DC).

In vivo salivary prostaglandin biosynthesis

Biosynthesis of prostaglandins and their subsequent secretion in the saliva of *A. americanum* was investigated following [^{3}H]arachidonic acid administration by ingestion or injection. Between 10 and 15 partially fed female *A. americanum* (50–150 mg) were used in each experiment. For the injection studies, [^{3}H]-arachidonic acid was dried under nitrogen in a 0.5 ml microfuge tube and resuspended to a concentration of 2 μCi/μl in 68 mM NaCl containing 0.25 mg/ml bovine serum albumin with warming and vigorous vortexing. Approximately 2 μl of the injectate was administered into the hemocoel of the ticks with a 10 μl syringe fitted with a 22 gauge needle. For the ingestion studies, [^{3}H]arachidonic acid was resuspended as above to a concentration of 4 μCi/μl and fed to *A. americanum* via 25 μl glass capillary tubes placed over the mouthparts. The microfuge tube was rinsed twice with a further 20 μl diluent and the rinses fed to the ticks. By this method, more than 85% of the label was ingested as assessed by the amount of radioactivity remaining

in the microfuge tube and capillary tubes. Following [^{3}H]arachidonic acid administration, ticks were maintained at room temperature in a humidity chamber.

After 6–10 h, ticks were induced to salivate by injecting 10 μl buffered tick saline (Needham and Sauer, 1979) containing 4 mM dopamine, 4 mM theophylline and 3% dimethyl sulfoxide into the hemocoel at 20 min intervals. Saliva was collected for up to 4 h into glass capillary tubes placed over the mouthparts.

All glassware used from this point was siliconized (Sigmacote, Sigma) to minimize the loss of prostaglandins through adsorption. The saliva was diluted to approximately 1.0 ml with distilled water and then acidified to pH 3–4.5 with 3% (v/v) formic acid and vortexed vigorously for 90 s with 4 ml ethyl acetate. Phase separation was achieved by centrifugation at 2500 ***g*** for 5 min and the upper organic phase saved. The lower phase was extracted twice more with 3 ml ethyl acetate, and the organic layers pooled. The ethyl acetate extract was reduced in volume under nitrogen to approximately 1 ml and passed through a small amount of anhydrous magnesium sulfate in a glasswool plugged pasteur pipette. The column was washed with a further 2 ml ethyl acetate and the lipid extract dried under nitrogen. Prostaglandins were separated by TLC in solvent systems A and B and the radioactivity quantified as described above.

Dehydration of PGE_2 in tick saliva

Aqueous solutions of PGE_2 readily dehydrate at acidic pH values to PGA_2, or dehydrate and isomerize to PGB_2 at basic pH values (Stehle, 1982). Extraction of prostaglandins from the highly basic (pH ~ 9.5, Bowman, unpublished observations) tick saliva involves a preliminary acidification (pH ~ 3–4.5) step. It was of interest to determine if the PGA_2/B_2 in tick saliva could be attributed to either of the pH extremes.

Dopamine-induced saliva was obtained from four groups of approximately 15 partially fed *A. americanum*, as described above. Fifty microliters of saliva from each group was pooled and heated in a boiling water-bath for 5 min in a sealed microfuge tube and then cooled. From each of the four groups and the boiled pooled saliva sample, 150 μl aliquots were transferred to 0.5 ml microfuge tubes and vortexed with 1.0 μl (0.1 μCi) [^{3}H]PGE_2 in ethanol. The samples were kept at room temperature for 60 min and then stored at −20°C overnight. Distilled water samples, in triplicate, were treated in a similar manner.

Saliva and water samples were transferred to siliconized glass centrifuge tubes with two rinses of 425 μl buffered tick saline (Needham and Sauer, 1979) without the bovine serum albumin. The samples were carefully acidified to pH 3.5–4.0 with 3% (v/v) formic acid and extracted once with 4 ml and twice with 3 ml ethyl acetate. The prostaglandins were separated by TLC in solvent system B and the radioactivity quantified, as above.

RESULTS

Prostaglandin synthetase activity in tick salivary glands

Bovine seminal vesicle preparations exhibited high PGS activity [Fig. 1(A)], but *A. americanum* salivary gland material failed to convert [^{3}H]arachidonic acid to [^{3}H]prostaglandins either as crude homogenate [Fig. 1(C)] or any of the subcellular fractions tested, including a microsome-rich fraction. Addition of salivary gland homogenate to bovine seminal vesicle homogenate had no effect on the prostaglandin production [Fig. 1(B)]. Several assay parameters were altered without any effect on the PGS activity of the salivary gland homogenate including: (1) incubation times, 3–60 min; (2) pH, 6.2–7.8; (3) buffers, Tris–HCl and KH_2PO_4–NaOH; (4) amount of [^{3}H]arachidonic acid, 1–10 μCi; (5) arachidonic acid concentration, 0.02–10 μM; (6) various co-factors: reduced glutathione, 3–5 mM; tryptophan, 1 mM; hemoglobin, 0.4 μM; hematin, 1 μM; hydroqinone, 0.25 mM; (7) phospholipase A_2 inhibitors: EGTA, 1–20 mM, and oleyl oxy-ethyl phosphorylcholine, 10–100 μM; (8) 15-hydroxyprostaglandin dehydrogenase and lysophosphatide acyl transferase inhibitor: *N*-ethyl maleimide, 0.25 mM; and (9) dopamine, 10 μM. PGS activity could not be detected in the salivary glands of slow or fast feeding ticks under any of the conditions tested.

In vivo salivary prostaglandin biosynthesis

Dopamine-induced saliva collected from ticks 6–10 h after being fed or injected [^{3}H]arachidonic acid contained between 2–5% of the administered radioactivity. The majority of the radioactivity in the saliva was neither ethyl acetate nor chloroform extractable and was presumed to be water derived from the β-oxidation of [^{3}H]arachidonic acid. Typically, about 0.05–0.20% of the injected radioactivity was recovered in the ethyl acetate extract and contained several [^{3}H]prostaglandins as well as unconverted [^{3}H]arachidonic acid (Fig. 2). The prostaglandins were identified by the co-migration with authentic standards in both solvent system A and B. Prostaglandin E_2 was always the major product together with appreciable quantities of $PGF_{2\alpha}$ and PGD_2. The radioactivity peak which co-migrated with the PGA_2/B_2 standards was assumed to be derived from [^{3}H]PGE_2. In none of the samples did radioactivity co-migrate with the 6-keto-$PGF_{1\alpha}$ standard in solvent system A.

Dehydration of PGE_2 in tick saliva

Incubation of [^{3}H]PGE_2 in distilled water for 60 min at room temperature and overnight at −20°C followed by the acidification step (pH 3.5–4) in the extraction procedure resulted in marginal (<3%) PGA_2 production. However, similar treatment of [^{3}H]PGE_2 in tick saliva (pH ~ 9.5) resulted in higher levels (~15%) of PGA_2/B_2, significantly greater than in distilled water ($P < 0.001$). Boiled saliva had high levels (12.5%) of PGA_2/B_2.

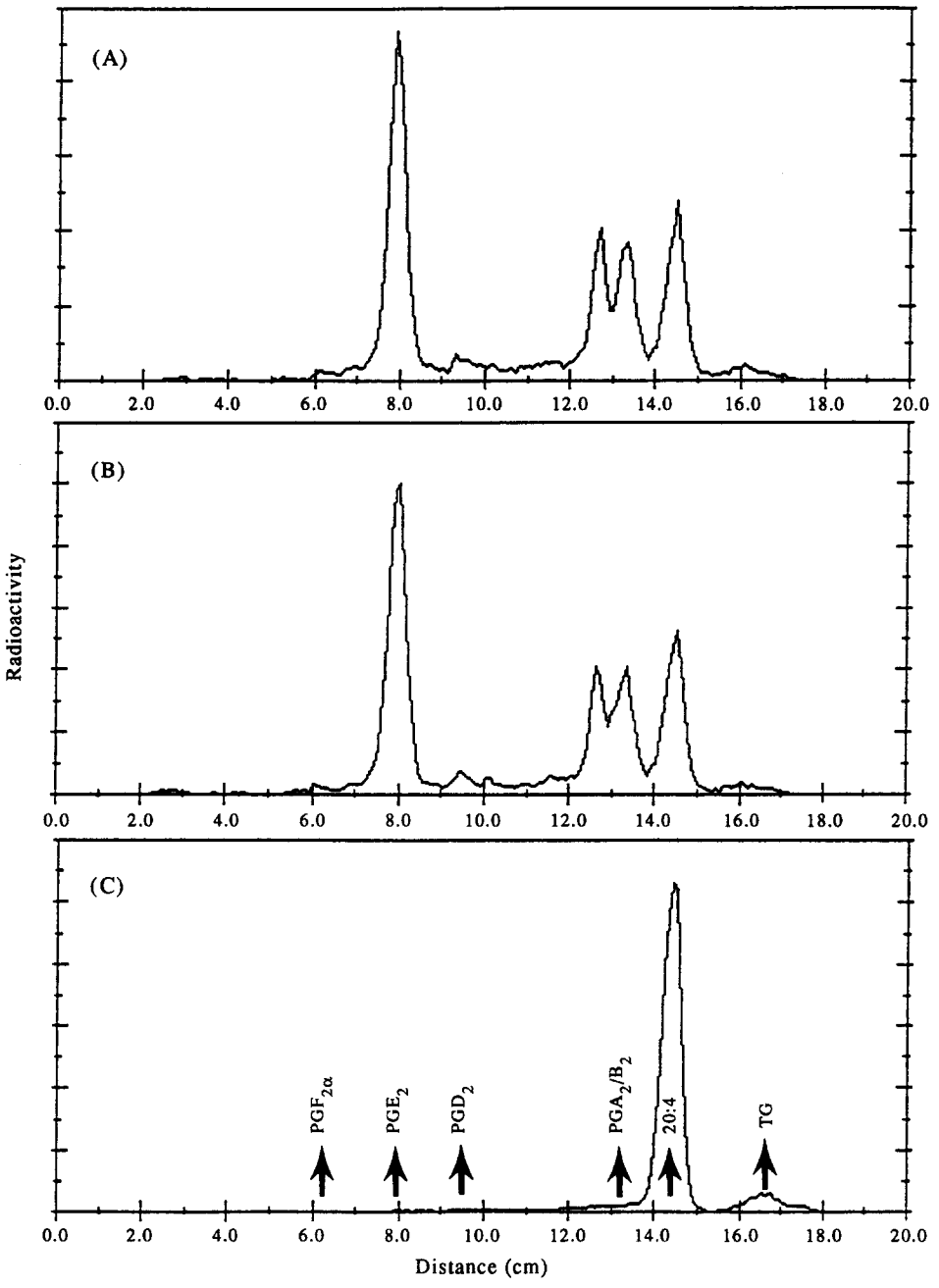

FIGURE 1. Radio-chromatogram of TLC separated ethyl acetate extracted products in solvent system B from incubations of [^{3}H]arachidonic acid with homogenates of (A) bovine seminal vesicles, (B) bovine seminal vesicles and *A. americanum* salivary glands, (C) *A. americanum* salivary glands. See text for experimental details. Arrows indicate position of co-migrating prostaglandins, arachidonic acid (20:4), and triglyceride (TG) standards.

DISCUSSION

Evidence is rapidly accumulating that the *A. americanum* salivary gland has evolved to be a potent prostaglandin producing organ (Shipley *et al.*, 1993a, b, 1994; Bowman *et al.*, 1993a, b) capable of producing saliva with prostaglandin levels many fold higher than vertebrate inflammatory exudates (Ribeiro *et al.*, 1992). Seemingly, demonstrating PGS activity in the salivary gland should be a trivial matter. Using assay conditions similar to those employed in this paper, PGS activity has been demonstrated in many arthropods including: *Acheta domesticus* (Destephano and Brady, 1977); *Teleogryllus commodus* (Tobe and Loher, 1983); *Musca domestica* (Wakayama *et al.*, 1986); *Aedes aegypti* (Stanley-Samuelson and Petzel, 1993) and a plethora of vertebrate species. No PGS activity was detected in *A. americanum* salivary gland homogenate under any conditions tested. *Acheta domesticus* male reproductive tracts have high PGS activities in a 12,100 *g* pellet which is 95% abolished by the re-addition of the 12,100 *g* supernatant (Destephano and Brady, 1977). No PGS activity was detected in any of the *A. americanum* sub-cellular fractions tested. In addition, combining salivary gland homogenate with bovine

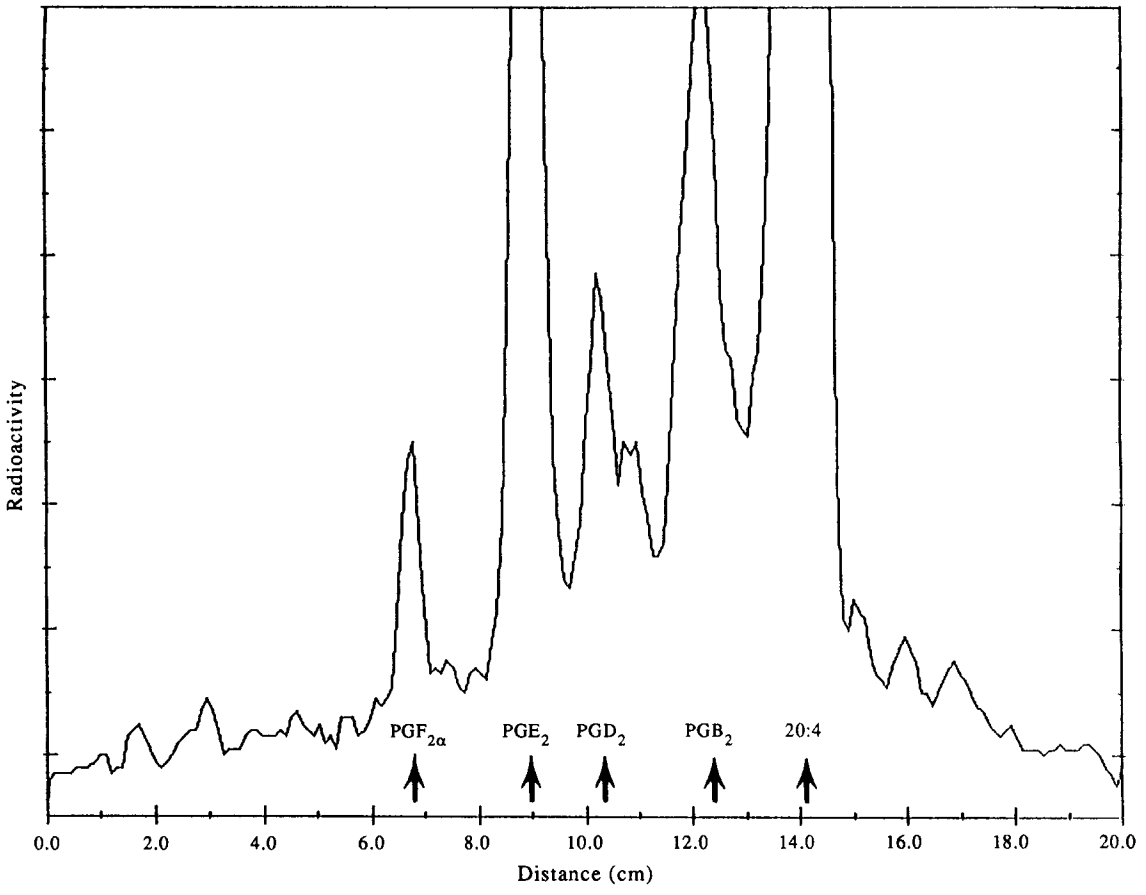

FIGURE 2. Radio-chromatogram of TLC separated ethyl acetate extracted products in solvent system B of dopamine-induced saliva from partially fed female *A. americanum* 8 h after ingesting [^{3}H]arachidonic acid. Arrows indicate the position of prostaglandin and arachidonic acid (20:4) standards. Similar results were obtained with solvent system B.

seminal vesicle homogenate had negligible effect on the [^{3}H]prostaglandin produced, indicating the gland homogenate does not possess a PGS-inhibitor nor do they rapidly metabolize any [^{3}H]prostaglandins produced. The PGE_2 content of salivary glands in female cattle ticks, *Boophilus microplus*, was found to be greatest in the fast-feeding stage (Dickinson *et al.*, 1979). In the present study no PGS activity was detected in either the slow- or fast-feeding *A. americanum*.

The possibility that prostaglandins are synthesized elsewhere in the tick body and transported by the hemolymph and then secreted in saliva is unlikely. Prostaglandins are accepted to act as autocoids, i.e. local hormones, owing to the extremely short half-lives of prostaglandins, as demonstrated in mammals. In addition, there is growing evidence that the salivary gland of *A. americanum* has many adaptations regarding the metabolism of arachidonic acid (see above). Amounts of PGE_2 and $PGF_{2\alpha}$ in cultured *Hyalomma anatolicum excavatum* increased during a 72 h incubation period, as determined by radioimmunoassay (RIA) (Shemesh *et al.*, 1979), indicating that tick salivary glands, at least in this species, can synthesize prostaglandins.

We have previously reported that ingested [^{3}H]-arachidonic acid is readily sequestered by the salivary glands of *A. americanum* and incorporated into phospholipids by a selective mechanism different from that exerted upon other ingested fatty acids (Bowman *et al.*, 1995). In this paper, we demonstrated that, following dopamine stimulation, such ingested [^{3}H]arachidonic acid is converted into PGE_2, $PGF_{2\alpha}$, and PGD_2 and salivated. This is the first report where a dietary constituent has been traced through its ingestion, conversion to an autocrine component and subsequent secretion where its site of action is remote from the organism, i.e. the host. Such a pathway elegantly demonstrates the parasite/host relationship. Whether dopamine causes synthesis of prostaglandins or simply the secretion of saliva containing prostaglandins is unknown. Dopamine stimulated the production of PGE_2 in cultured rat inner medullary collecting duct cells apparently through Ca^{2+} mobilization and the subsequent activation of PLA_2 (Huo and Healy, 1991). *Amblyomma americanum* salivary glands possess a Ca^{2+}-sensitive PLA_2 (Sauer *et al.*, 1993) capable of generating increased arachidonate levels in isolated glands following Ca^{2+}-ionophore stimulation (Bowman *et al.*, 1994). Dopamine's stimulation of fluid secretion in isolated salivary glands is abolished in the absence of Ca^{2+} or the presence of Ca^{2+}-channel blockers (Needham and Sauer, 1979), indicating dopamine causes an influx of Ca^{2+}. The role

of dopamine in salivary gland prostaglandin production is currently under investigation.

The major [^{3}H]prostaglandin secreted in the saliva was PGE_2 followed by $PGF_{2\alpha}$ in similar ratios as reported by Ribeiro *et al.* (1992). The roles ascribed to PGE_2 in tick feeding including vasodilation, anti-hemostasis, anti-inflammatory and immunosuppressive are reviewed by Sauer *et al.* (1993). A role for $PGF_{2\alpha}$ is less clear. The conversion of PGE_2 to PGB_2 owing to the alkalinity of tick saliva suggests that previous determinations of PGE_2 in tick saliva are underestimates. This is the first report of PGD_2 in tick saliva. Work to establish verification of PGD_2 in tick saliva by GC–MS is currently in progress. PGD_2 could be beneficial to tick feeding as it is a potent inhibitor of platelet-aggregation (Smith *et al.*, 1974) and a vasodilator (Heavey *et al.*, 1984). Absence of [^{3}H]6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, was unexpected as 6-keto-$PGF_{1\alpha}$ was reported in pilocarpine-induced saliva of *Ixodes dammini* at seven times the level of PGE_2, as determined by RIA (Ribeiro *et al.*, 1988). This may be due to differences in species, salivation stimulants or methods of determination.

In summation, using standard methodologies PGS activity could not be detected in the homogenates of *A. americanum* salivary glands. It has been successfully demonstrated that *A. americanum* is indeed capable of synthesizing and orally secreting PGE_2, $PGF_{2\alpha}$, and PGD_2 originating from ingested arachidonic acid. We hypothesize that the PGS enzyme in *A. americanum* salivary glands is considerably different from the PGS studied in other animals, including the hosts of ticks, and may be a potential candidate for control strategies.

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