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Perception of breath components by the tropical bont tick, *Amblyomma variegatum* Fabricius (Ixodidae)

II. Sulfide-receptors

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Summary. Wall-pore sensilla in the capsule of Haller's organ on foreleg tarsi of the tick, *Amblyomma variegatum*, show multicellular responses upon stimulation with human and bovine breath. Filtering breath through charcoal removes the stimulant for some of these receptors. Analysis by gas chromatography coupled with olfactory sensillum electrophysiological recordings indicates that an ethanol extract of the breath components trapped on charcoal contains a major stimulant eluting at the same retention time as H_2S. Two types of H_2S-sensitive receptors have been identified. They are housed in separate sensilla, and are called sulfide-receptor 1 and 2.

Although, both receptor types are characterized by a high sensitivity to H_2S with an estimated threshold of ca. 0.1 ppb and a response range covering 5–6 log orders of magnitude, their overall response to sulfides and mercaptans is nevertheless dissimilar. The type 1 receptor fires slightly more upon stimulations with H_2S than type 2, whereas ethylmercaptan induces a stronger response from type 2, and dimethyl sulfide activates only receptor 2.

In a bioassay, H_2S tested at concentrations of ca. 0.02 ppm and 1 ppm equally arouses 60% of resting ticks. Two-thirds of these ticks quest the air with their first pair of legs, and the remainder start active search. By contrast, H_2S at ca. 1 ppm in a mixture with CO_2 severely diminishes the locomotor stimulating effect of CO_2.

Key words: Tick – Host-finding – Vertebrate breath – Hydrogen sulfide – Sulfide-receptors

Introduction

Several investigators have demonstrated that volatiles of vertebrate origin, other than CO_2, act as cues for host-finding by haematophagous anthropods. Despite this, chemical analysis of breath components has been made only in a few cases; 1-octen-3-ol and acetone in cow breath are used for host-location in tsetse flies (Hall et al. 1984; Vale and Hall 1985). Furthermore, octenol and/or acetone improve attractiveness of CO_2 in other blood-sucking Diptera (Culicidae: Kline et al. 1990; *Stomoxys calcitrans:* Warnes and Finlayson 1985a, b; Tabanidae: French and Kline 1989). It is still not clear whether breath components other than CO_2 elicit a response in ticks, although human subjects and CO_2 traps attract roughly the same numbers of *Amblyomma americanum* in the field (Mount and Dunn 1983). Besides, 1-octen-3-ol and acetone do not attract *Amblyomma hebraeum* (Norval et al. 1987). However, there may be other vertebrate associated volatiles which are relevant for *Amblyomma* sp. Indeed, studies in this laboratory on all wall-pore sensilla on the tarsus of the foreleg of *Amblyomma variegatum* have revealed that some olfactory cells in Haller's organ respond to components of vertebrate odour (Steullet, unpublished). Three other sensilla in the capsule of Haller's organ also bear cells which are stimulated by breath (multicellular responses). One of the activated cells is a CO_2-excited receptor, another a CO_2-inhibited receptor (Steullet and Guerin 1992), and the response of two other cells to sulfur components of breath is described here.

Materials and methods

Ticks and electrophysiology

Experiments were undertaken for the most part with unfed *Amblyomma variegatum* males, nevertheless, some unfed females were also used for comparison. Rearing methods, preparation of ticks, electrophysiological set-up, as well as stimulus-delivery system are already described in Steullet and Guerin (1992).

Stimulants

Human breath. Human breath was blown via the needle connexion into the barrel of a 5-ml polypropylene syringe whose other end

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bore a rubber stopper, and was immediately used as stimulus. Breath was collected as a rule in the morning.

Bovine breath. A personal sampling pump (SKC Inc., USA) sucked air into a Tedlar sampling bag (SKC, USA) at a rate of 250 ml/min via a teflon tube placed in the mouth of a 200 kg Simmental steer held in a rearing pen at the Agricultural Research Station of Ciba-Geigy (St-Aubin, Switzerland). Breath was thus transported to the laboratory and used for stimulation 1–2 h later by venting from the Tedlar bag into the barrel of 5-ml syringes which were immediately used as stimulus cartridges.

Porapak conditioning and filtering human breath. Porapak Q, a porous polymer which selectively desorbs water while retaining a large spectrum of volatiles was used to collect breath-borne odours. Conditioning of Porapak Q (50–80 mesh, Milipore Corporation, USA) was carried out by: 1) heating under N_2 (1 l/min) at 200 °C for 24 h, 2) extraction with dichloromethane (Merck, analytical grade) in a Soxhlet extractor for 24 h, and 3) drying under N_2 (1 l/min) at 110 °C for 2 h. About 600 mg of conditioned Porapak Q was then packed into the barrel of a Pasteur pipette (70 mm long, 5 mm i.d.) and human breath was blown directly through this adsorbant trap for 30 s. A portion of the filtered breath was introduced in the barrel of a 5-ml syringe to be used as stimulus.

Filtering human breath through charcoal. Commercially available charcoal air-sampling tubes (coconut-base 50/100 mg in a 6 mm OD × 70 mm long trap, SKC, USA) were also used to collect breath-borne volatiles. Charcoal traps a wide range of volatiles like Porapak Q, but its adsorbtion capacity for small molecular weight compounds is higher. Human breath was blown through the trap for 30 s and a portion of the filtered breath was entrained in a 5-ml syringe to be used as stimulus.

Hydrogen sulfide. Two methods of generating H_2S vapours were employed. A certified H_2S-permeation tube (Dynacal, VICI Metronics, USA) liberating H_2S at 546 ng/min ± 2% at 30 °C was used. Different concentrations of H_2S were obtained by mixing flows of charcoal-filtered air with that passing from a 500-ml gas-wash bottle containing the H_2S-permeation tube held at 30 °C in a constant T°C bath. Precise concentrations were introduced into the stimulus cartridge to cover 4 log orders of magnitude. Considering the dilution which occurred in the stimulus-delivery-tube, H_2S concentrations at the level of the preparation (5 mm from outlet of tube) ranged from ca. 0.003 ppm to ca. 2.6 ppm. Continuous or pulsed stimulations with H_2S were made by passing charcoal-filtered air through the 500-ml flask containing the H_2S-permeation tube, and then directly into the main air stream flowing over the preparation. Air flows were controlled by voltage-pressure converters and the duration of stimulation by solenoid valves. The T° increase (ca. 1 °C) which ensued in the main air stream during a stimulation had no effect on the sulfide-receptor response.

The second method employed to generate H_2S was the use of aqueous solutions of Na_2S. This method was particularly useful for providing H_2S doses higher than that obtainable from the permeation tube. Ten µl aliquots of various Na_2S solutions (10^{-5} mg/10 µl to 10^{-1} mg/10 µl H_2O) were applied to filter paper strips which were then enclosed in the 5-ml syringe. After allowing 3 min for evaporation of H_2S vapour, 2 ml of the stimulus cartridge volume were injected in 1 s into the main air stream flowing over the preparation. The quantity of H_2S leaving the lower Na_2S concentrations was calibrated by comparing the sulfide-receptor responses to these solutions with those obtained with the reference H_2S values from the certified permeation tube.

Other sulfides. A certified ethylmercaptan permeation tube (452 ng/min at 30 °C, Dynacal, VICI Metronics, USA) was employed in the same way as the H_2S-permeation tube to provide a graded concentration series of ethylmercaptan. Dimethyl sulfide (> 99% GC, Fluka, Switzerland) was successively diluted in paraffin oil to provide 10^{-1} to 10^{-4} molar solutions. Ten µl of these solutions were applied to filter paper strips, enclosed in the barrel of a 5-ml syringe, and used as stimulus source.

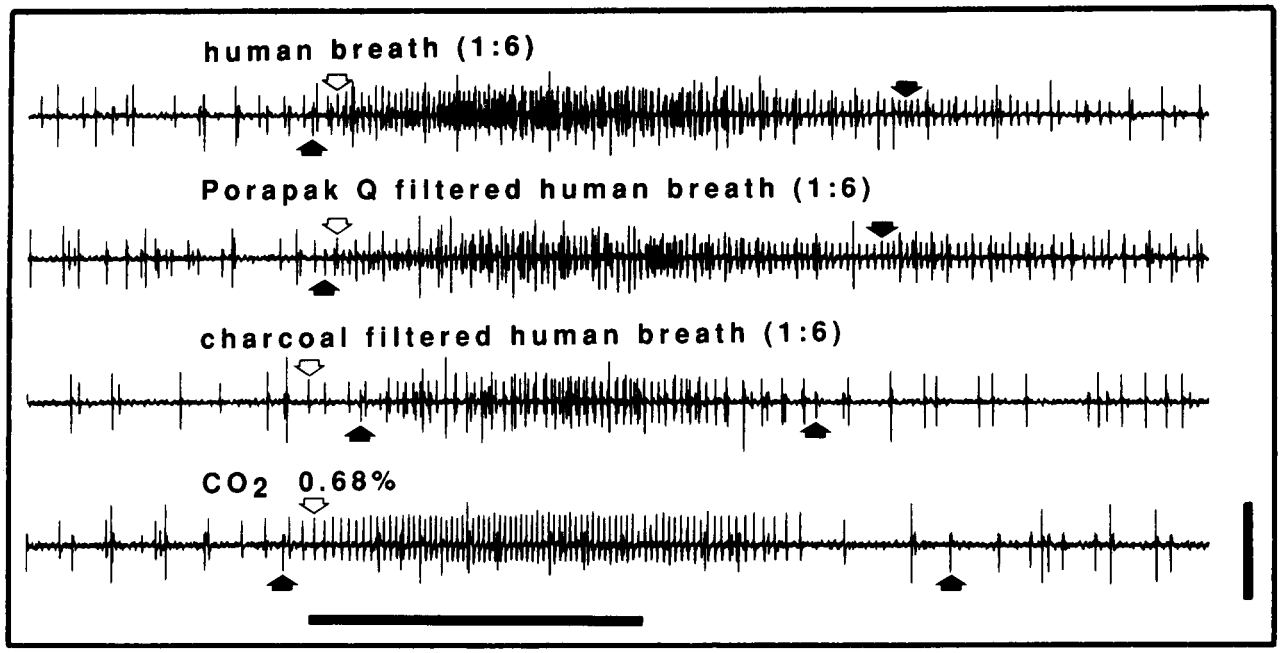

Fig. 1. Representative responses of the *Amblyomma variegatum* breath sensillum 2 to human breath, Porapak-filtered human breath, charcoal-filtered human breath, and CO_2. Filtered and unfiltered breath was diluted by a factor of 6 in the air stream conveying the stimulus to the preparation to give, for unfiltered breath, an equivalent of 0.68% CO_2. Stimuli were introduced, in this case, at 25 cm from the outlet of the stimulus-delivery-tube. *Bold arrow*, sulfide-receptor 2 (cell 4 in Fig. 2), which displays a negative going spike at the beginning of a stimulation, but then becomes biphasic. *White arrow*, CO_2-excited receptor (cell 1 in Fig. 2). Horizontal bar 1 s stimulation; vertical bar 1 mV

Other volatiles tested. The following volatiles were also tested to screen sulfide-receptor specificity: methane, CO_2, ammonia, acetone, 3-pentanone, 4-heptanone, hexanal, pentanol, 1-octen-3-ol, 1-octen, propionic acid, n-butyric acid, isobutyric acid, n-valeric acid, iso-valeric acid, heptanoic acid, L-lactic acid, γ-butyrolactone, γ-valerolactone, γ-caprolactone (all vertebrate odours), nonanoic acid, 2-nitrophenol, 2,6-dichlorophenol, methylsalicylate (tick pheromone components), dichloromethane and distilled water (solvents). Chemical purity of trade products was >99% as indicated by GC. Chemicals were dissolved in analytical grade CH_2Cl_2 or in distilled water, depending on their nature, at 10^{-3} and 10^{-2} *M*, and prepared for stimulation as for dimethyl sulfide (above). Methane from the mains was entrained in the barrel of a 5-ml syringe for stimulation; for CO_2 stimulation see Steullet and Guerin (1992). A concentrated aqueous solution of 35% NH_4OH was diluted 10 and 100 times in distilled H_2O and used as the ammonia stimulus source.

Coupled gas chromatography – single unit recording

Human breath was blown through a coconut-charcoal trap for 15 min. The trap was then eluted with either dichloromethane or ethanol (Merck, analytical grade). From the first drop of the eluate, 1.5 µl was immediately injected on-column onto a DBWAX fused silica capillary gas chromatography column (30 m, 0.32 mm i.d., 0.25 µm film thickness, G&W Scientific, USA) with H_2 (0.5 m/s) as carrier gas; oven temperature: 30 °C for 5 min, then programmed at 10 °C/min to 230 °C. The column effluent was divided with a glass Y-piece splitter in the ratio 2:1 over, respectively, the flame ionisation detector (FID) and the biological detector. The latter consisted of an electrophysiological preparation of a capsular sensillum known to have cells responding to breath. An air stream, maintained at ca. 80% RH and 22±1 °C in a water-jacketed tube (3 mm i.d.), swept a third of the column effluent from the heated (250 °C) transfer line of the chromatograph to the tick preparation at a speed of 1.5 m/s. The outlet of the tube was 5 mm from the tick tarsus. Column effluent was thus simultaneously monitored by both the FID detector and sensillum in order to locate any electrophysiologically active component in the extract (Wadhams 1982). A 1.5 µl aliquot of the first solvent drop eluting from an unused charcoal trap served as the blank control. A standard H_2S stimulus was obtained by injection of 10 µl of headspace from an aqueous solution of Na_2S (1 g/10 ml) moderately acidified with few drops of 1 N HCl. All spikes were sorted from noise with a discriminator level incorporated in the amplifier (UN-03 Syntech, The Netherlands) and the frequency was converted into a voltage. This converted signal, the receptor potential and FID responses were simultaneously printed on a chart recorder and stored on video tapes (Steullet and Guerin 1992). Play back of parts of the recording where an increase in spike frequency occurred was necessary to identify the responding cell(s). Discrimination for different spike types was made by eye (as described in Steullet and Guerin 1992). Identification of a stimulant in the extract was based on 1) the response of the biological detector to both a component of the extract and the standard eluting at the same retention time on the capillary column, and 2) on the type of cell activated.

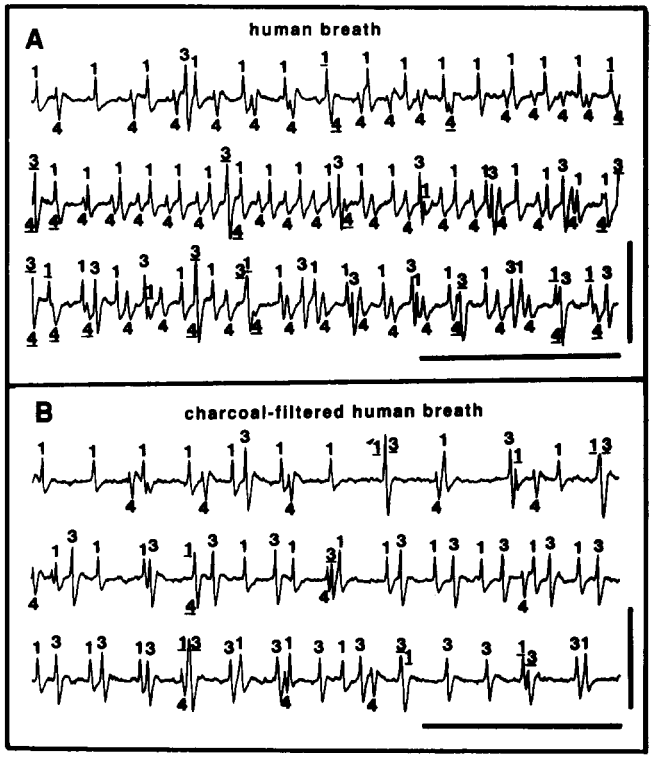

Fig. 2A, B. Detail of recordings from breath sensillum 2 illustrated in Fig. 1. **A** Response to human breath. **B** Response to human breath filtered through charcoal. Spike discrimination was made by eye. Note that unfiltered human breath elicits higher firing rates than charcoal-filtered human breath in cell 1 (CO_2-excited receptor, white arrow in Fig. 1) and cell 4 (sulfide-receptor 2, bold arrow in Fig. 1). Note also that spike 4 has a strong negative phase at the lower frequency in **B**, but becomes biphasic at the higher frequency in **A**. Cell 3, in this particular case, is slightly more excited in **B** than in **A**, but no statistical difference exists as a rule (Fig. 3). Cell 2 fires at **A** very low frequency. It is absent from this example. Enumeration corresponds to cell numbers in Fig. 3, and underlined spikes are overlapping events. Horizontal bars 100 ms; vertical bars 1 mV

Behavioral bioassay

A behavioral bioassay based on the activity level of ticks was developed. During first steps in host-searching by adult *A. variegatum*, 3 distinct phases are observed: 1) resting, where fully inactive ticks keep their legs folded under their body; 2) questing, where

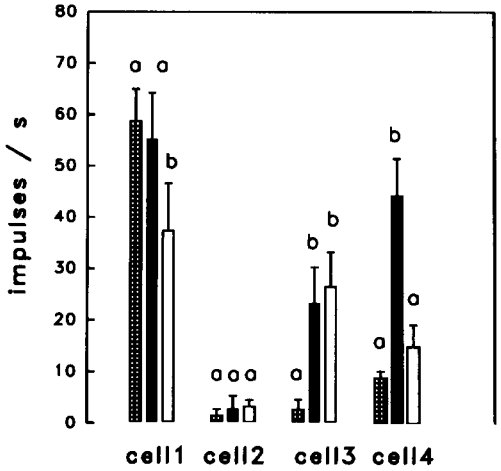

Fig. 3. Responses of cells of breath sensillum 2 in the capsule of Haller's organ of 6 different male *A. variegatum* to human breath, charcoal-filtered human breath, and to CO_2 at a concentration equivalent to the level in unfiltered breath. Stimuli were introduced into the main air stream at 25 cm from the outlet of the stimulus-delivery-tube. Cell frequencies were determined on spike trains of 1 s from the beginning of the response. For each cell, blocks labelled *a* and *b* are significantly different from one another ($P<0.05$) (Wilcoxon's paired comparison test). Bars associated with each block are standard deviations. See also Fig. 2. ■ carbon dioxide (breath conc.), ■ human breath, □ charcoal-filtered human breath

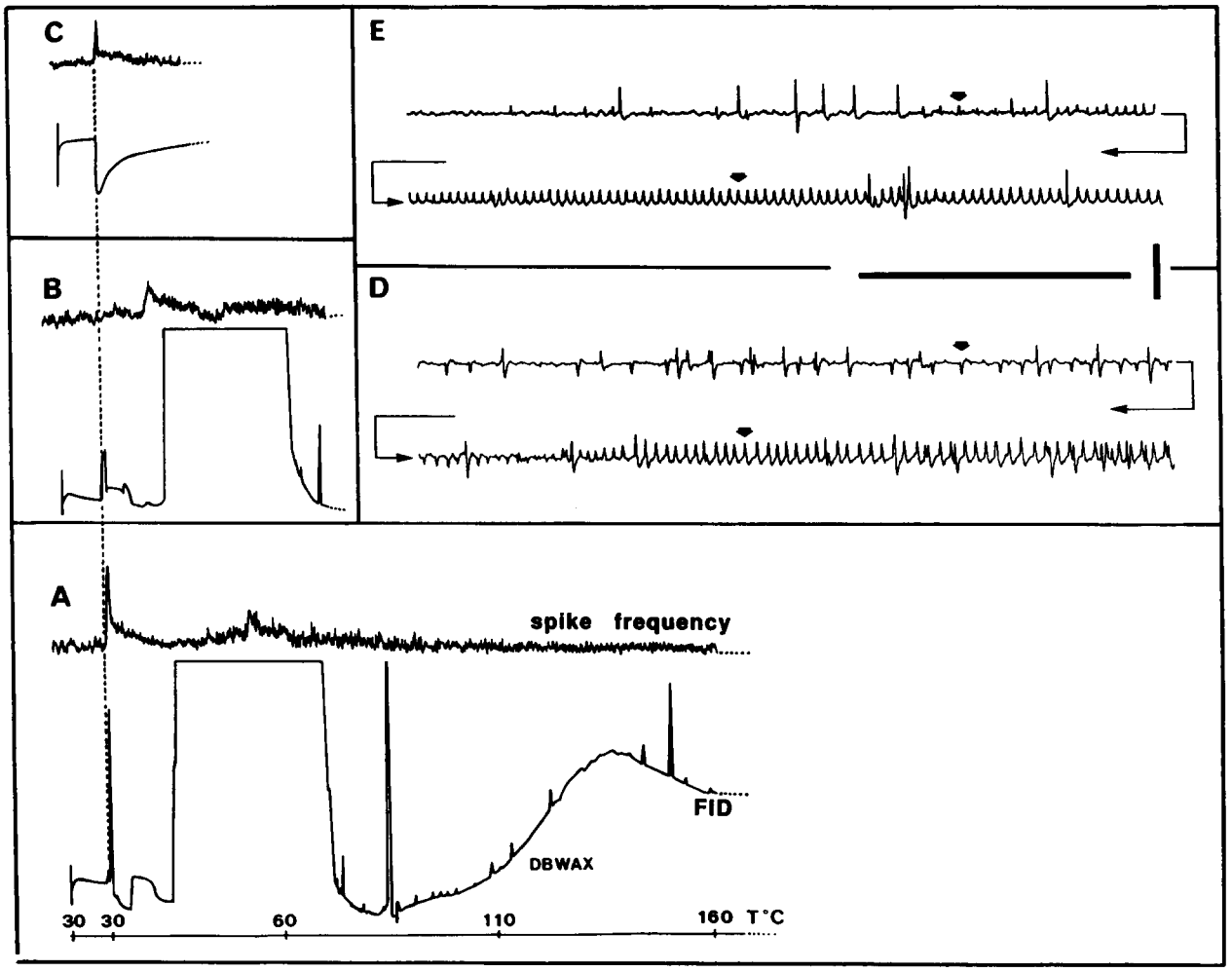

Fig. 4A–E. Analysis of human breath by gas chromatography coupled with breath sensillum 2 electrophysiology recordings from the capsule of Haller's organ of a male *A. variegatum*. **A, B,** and **C** spike activity of breath sensillum 2 (*upper trace,* frequency to voltage converted signal) recorded simultaneously with chromatogram (*lower trace* obtained with a flame ionisation detector, *FID*). **A** Breath components adsorbed on charcoal and extracted with ethanol, **B** blank control, i.e. an ethanol extract of an unused charcoal trap, and **C** 10 µl of the headspace over an acidified aqueous solution of Na_2S which generates H_2S. Note that the breath component, which causes an increase in spike frequency of the breath sensillum in **A**, elutes at the same retention time as H_2S (negative FID response) in **C**, where a corresponding increase in spike frequency is also observable. *Column: DBWAX* (J & W Scientific, USA) high resolution fused silica capillary column (30 m, 0.32 mm i.d., 0.25 µm film thickness) was temperature programmed after 5 min at 30 °C at 10 °C/min to 230 °C. Temperature scale same for **A, B,** and **C**. **D** Expanded trace of the breath sensillum 2 response, before and during elution of the active breath component in **A**. **E** Expanded trace of the breath sensillum 2 response, before and during elution of H_2S in **C**. For both **D** and **E**, horizontal bar is 1 s; vertical bar 1 mV. *Bold arrow:* sulfide-receptor 2 (cell 4 in Figs. 2 and 3). Note how the spike of this receptor changes sign on stimulation in **D**, and remained biphasic after strong stimulation. This explains why in **E** (experiment made after **D**) the sulfide-receptor 2 is already biphasic even before arrival of H_2S at the sensillum

ticks raise at least one of the forelegs; 3) walking, where ticks finally rise to their feet and begin locomotion. Groups of 25 male *A. variegatum* (7–8 months old, all fed on the same steers) were placed in 100-ml glass gas-wash bottles. A charcoal-filtered and humidified air stream (80% RH, 23±2 °C, 3.3 ml/s) passed continuously through each bottle. Blanks or stimuli, contained in 10-ml syringes, were injected at 0.8 ml/s for 10 s and thus diluted by a factor of 5 in the humidified air stream before entering the bottle at 2 cm from the floor, where most of ticks lay. Cigarette smoke indicated that the stimuli were distributed throughout the bottle for ca. 40 s before being flushed out. Only one stimulus was tested per day on the same group of ticks. Ticks had thus a day to return to the resting position (most do so <1 h after stimulation). On successive days, a series of stimulations was applied to each group of ticks in a different sequence, to avoid any influence of the order in which the stimuli were tested. The number of resting, questing, and walking ticks was recorded just before and after stimulation. Data concerning the same stimulus were pooled and compared by Chi-square with data for other stimuli. The following stimuli enclosed in 10-ml syringes were tested: 1) H_2S produced by 10 µl of an aqueous solution of Na_2S at either 10^{-4} or 10^{-3} mg/10 µl deposited on a filter paper strip and enclosed in the syringe in an atmosphere of either N_2, 5% CO_2, or 1% CO_2; 2) dimethyl sulfide (10 µl of a 10^{-2} *M* solution in paraffin oil) enclosed in a syringe with N_2; 3) 5% CO_2; 4) 1% CO_2; 5) human breath collected in the morning; N_2 (blank control).

Results

Breath-sensitive cells

Three functionally different types of sensilla, whose cell activity was captured with different recording electrode

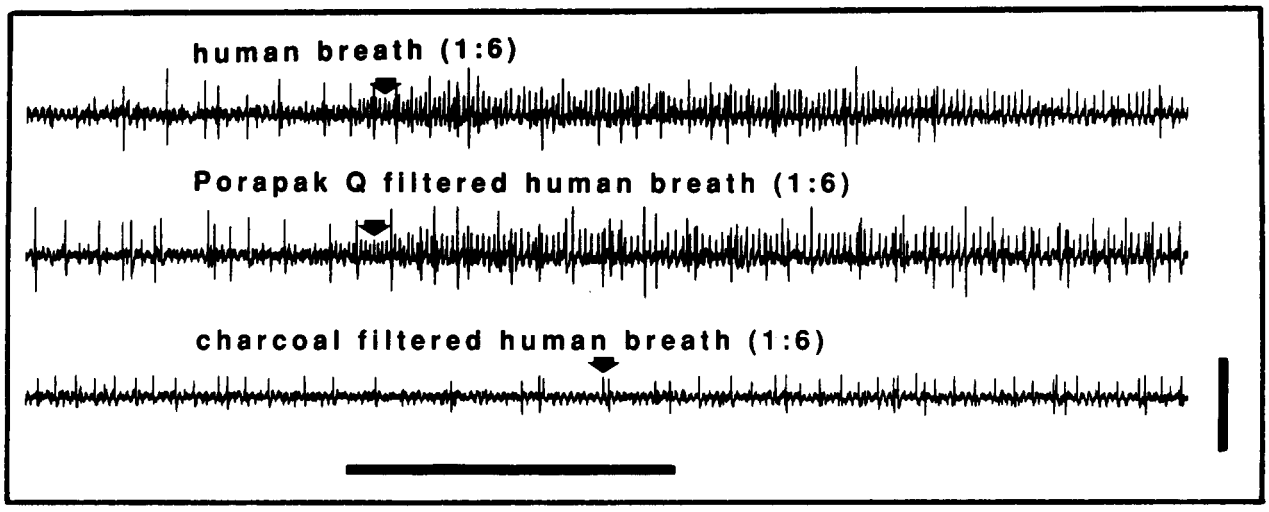

Fig. 5. Representative responses of sulfide-receptor 1 *(bold arrow)* of breath sensillum 1 in the capsule of Haller's organ of a male *A. variegatum* to human breath, Porapak-filtered human breath, and charcoal-filtered human breath. Filtered and unfiltered breath samples were diluted by a factor of 6 in the air stream conveying the stimulus to the preparation. Stimuli were introduced at 25 cm from the outlet of the stimulus-delivery-tube. Horizontal bar 1 s stimulation; vertical bar 1 mV

orientations within the capsule, carry cells responding to breath (Steullet and Guerin 1992). For convenience, these sensilla types are termed here breath sensillum 1, 2, and 3. As previously demonstrated, breath sensillum 2 has a CO_2-excited receptor and breath sensillum 3 a CO_2-inhibited receptor (Steullet and Guerin 1992). Results described in this paper deal with 2 other cells, respectively found in breath sensilla 1 and 2, which were activated by human and bovine breath. These results are based on 37 recordings from breath sensillum type 1 and 65 recordings from breath sensillum type 2 from different ticks. For practical reasons, most experiments were undertaken with human breath. Responses of these cells to breath strongly decreased over the working day, suggesting the active component(s) in human breath was much more prevalent in the morning.

Sulfide-sensitive cell in breath sensillum 2. In breath sensillum 2, Porapak-filtered breath elicited a multicellular response, involving 4 different cells, which was hardly different from that produced on stimulation with human breath. By contrast, the pattern of cell activity induced by charcoal-filtered breath was much simpler (Fig. 1 and details in Fig. 2). Recordings from sensilla of 6 different males indicated that cells 1 and 4 of this sensillum (Figs. 1, 2, and 3) were significantly less activated with charcoal-filtered breath than with unfiltered breath ($P<0.05$, Wilcoxon's paired comparison test), whereas no difference in the response of cell 3 was observed (Fig. 3). Cell 1 is the CO_2-excited receptor described in Steullet and Guerin (1992). A slight retention of CO_2 on charcoal is responsible for the weaker response of this cell to charcoal-filtered breath. On the other hand, the breath component activating cell 4 was not adsorbed by Porapak, but was retained on charcoal. But even on the latter, some breakthrough occurred as evidenced by the fact that air displaced from the charcoal trap, through which breath had been blown for a few minutes, excited cell 4. This suggested that the active breath component was very volatile.

A dichloromethane extract of breath components adsorbed on charcoal did not stimulate cell 4, but an ethanol extract did so strongly. In coupled gas chromatography and breath sensillum 2 recordings, one early eluting component of the latter extract elicited a response as evidenced by an increase in cellular activity (Fig. 4A) and a clear receptor potential. This active component had a retention time of 1 min, eluting before the solvent. A careful examination of the associated spikes established that this response was due to a strong activation of cell 4 (bold arrow in Fig. 4D), characterized by a quite small spike amplitude. An extract of an unused charcoal trap did not stimulate any cell of breath sensillum 2 (Fig. 4B). The retention time of the active breath component matched that of H_2S, which also activated cell 4 (Fig. 4C, bold arrow in Fig. 4E). Figure 6 illustrates the response of this sulfide-receptor to increasing concentrations of H_2S.

Sulfide-sensitive cell in breath sensillum 1. Unfiltered or Porapak-filtered breath was equally effective as a stimulant for a cell in breath sensillum 1, whereas charcoal-filtered breath failed to stimulate the same cell in this sensillum (bold arrow in Fig. 5). However, this cell responded strongly to an ethanol extract of the charcoal trap. Like cell 4 of breath sensillum 2, this cell also responded selectively to H_2S.

Properties and specificity of the two sulfide-sensitive cells

Both the activated cell of breath sensillum 1 (bold arrow in Fig. 5) and cell 4 of breath sensillum 2 (bold arrow in Fig. 1) are sulfide-sensitive. Nevertheless, they differ in important respects and will be termed sulfide-receptor

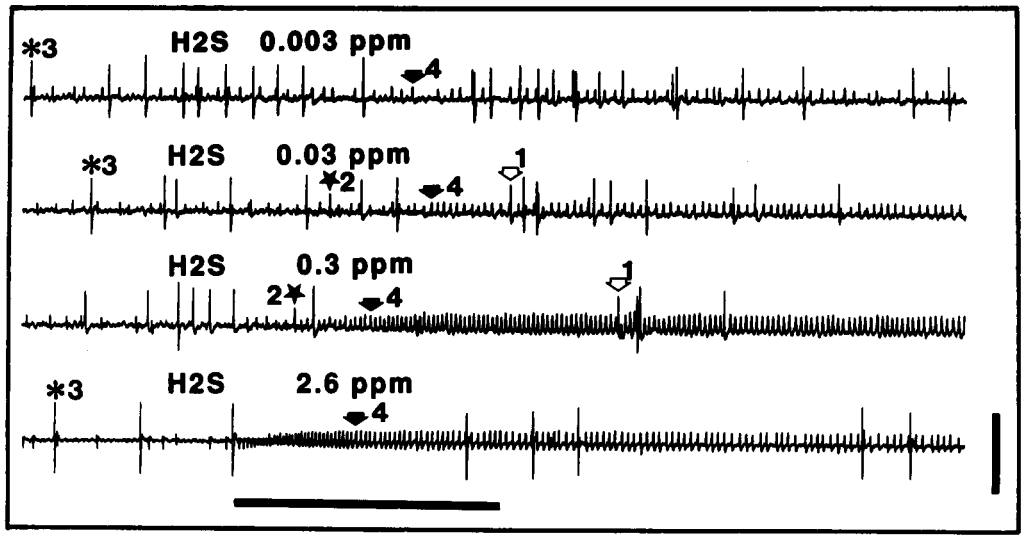

Fig. 6. Representative responses of sulfide-receptor 2 (*bold arrow*, and cell 4 in Fig. 2) of breath sensillum 2 in the capsule of Haller's organ of a male *A. variegatum* to 4 concentrations of H_2S. Stimulus concentration was estimated at the level of the preparation. H_2S vapours were introduced into the stimulus-delivery-tube at 25 cm from its outlet. *White arrow:* CO_2-excited receptor (cell 1 in Fig. 2); *asterisk:* cell 3 in Fig. 2; *star:* cell 2 in Fig. 2. Note the change in the sign of the sulfide-receptor spikes following stimulation with high concentrations of H_2S (see text). Horizontal bar 1 s stimulation; vertical bar 1 mV

1 and 2, henceforth. Spike shapes of both cells were quite similar, characterized by a small amplitude (0.1–1 mV) and a duration of 5–6 ms. Interestingly, the spikes were often negative going at low frequency but became biphasic at a higher frequency (bold arrow in Fig. 4D, and Fig. 6). They occasionally remained biphasic after strong excitation.

Figure 7 illustrates the relationship between H_2S dose and the phasic part of the response of the sulfide-receptor 2 with two different stimulation methods. In Fig. 7a, the H_2S vapour was flushed into the stimulus-delivery-tube at 25 cm from its outlet to the preparation, so that the stimulus onset was not very sharp. This resulted in an attenuation of the phasic part of the response (slope of the logarithmic relation $m = 0.11$). In Fig. 7b, the stimulus was introduced 3 cm from the outlet of the stimulus-delivery-tube, so that the stimulus onset was much sharper. Here, the phasic portion of the response was clearly most modulated by concentration changes between ca. 10^{-2} and ca. 10^{-1} ppm H_2S, and these responses were significantly higher than the responses obtained with the graded stimulus onset ($P < 0.05$, ANOVA test with the General Linear Models Procedure on SAS, Australia). However, the slope of the logarithmic relationship between H_2S dose and the phasic part of the response was also rather flat ($m = 0.21$). With either method of stimulation, the H_2S response curve covered about 6 log orders of magnitude, from an estimated threshold at < 0.1 ppb to over 10 ppm. Continuous or repetitive stimulation with ca. 0.1 ppm H_2S is extremely well monitored (Fig. 8); phasic bursts in spike activity followed 1 s repetitive stimulations, and little adaptation occurred over the 10 s continuous stimulation.

Both types of sulfide-receptors show dissimilarity in their respective responses to the 3 sulfides tested (Table 1). Whereas both were very sensitive to H_2S, the firing

Table 1. Responses of sulfide-receptors 1 and 2 to 3 sulfides: hydrogen sulfide, dimethyl sulfide, and ethylmercaptan. Response magnitude was determined on spike trains of 400 ms after the first 200 ms of the response, which corresponded to the period of strongest firing. Responses of sulfide-receptors were compared by ANOVA with the General Linear Models Procedure on SAS: on the right side of the table, **a** and **b** signify that responses of receptors 1 and 2 were significantly different ($P < 0.05$) for a given sulfide. § indicates that the response frequency of a receptor varies significantly with the concentration of a given compound ($P < 0.05$)

Sulfide-receptor	*n*	Amounts of Na_2S per cartridge				
		10^{-4} mg [imps/s]	10^{-3} mg [imps/s]	10^{-2} mg [imps/s]	10^{-1} mg [imps/s]	
Type 1	12	38.0 ± 21.5	55.4 ± 18.2	68.8 ± 12.4	70.4 ± 15.4	**a** §
Type 2	12	30.6 ± 16.5	49.2 ± 18.0	61.3 ± 10.8	62.5 ± 12.7	**b** §

Sulfide-receptor	*n*	molar conc. of dimethyl sulfide in cartridge				
		10^{-4} *M* [imps/s]	10^{-3} *M* [imps/s]	10^{-2} *M* [imps/s]	10^{-1} *M* [imps/s]	
Type 1	9	12.5 ± 7.3	17.8 ± 8.6	14.7 ± 5.7	23.9 ± 9.4	**a**
Type 2	9	22.9 ± 11.6	23.4 ± 7.9	44.2 ± 13.5	57.8 ± 13.5	**b** §

Sulfide-receptor	*n*	Ethylmercaptan conc. at preparation			
		10^{-2} ppm [imps/s]	10^{-1} ppm [imps/s]	1.2 ppm [imps/s]	
Type 1	4	13.1 ± 2.4	25.0 ± 16.6	25.0 ± 11.4	**a** §
Type 2	4	24.4 ± 13.9	36.3 ± 16.6	44.4 ± 11.6	**b** §

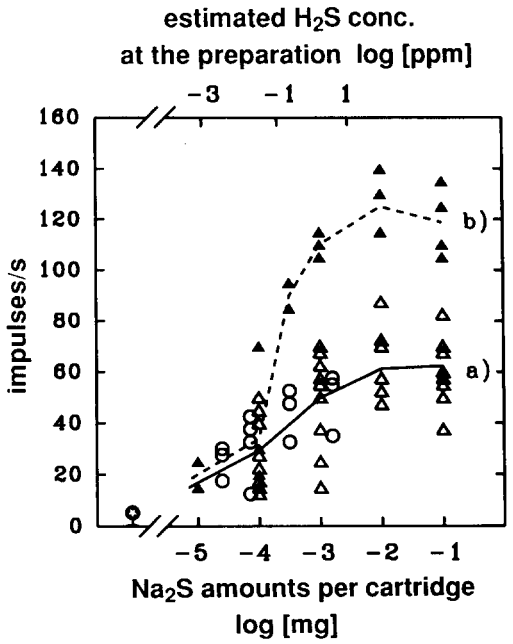

Fig. 7. Relationship between dose of H_2S and the phasic part of the response of the sulfide-receptor 2 of male *A. variegatum* obtained by two methods of stimulation: a) graded stimulus onset was obtained by introducing the H_2S vapour 25 cm from the outlet of the stimulus-delivery-tube to the preparation, b) much sharper stimulus onset was obtained by introducing H_2S vapour 3 cm from the outlet of the stimulus-delivery-tube. Response magnitude was determined for the period when spike frequency was maximal: for curve *a* for 400 ms after 200 ms from the beginning of the response, and for *b* for the first 200 ms of the response. In curve *a*, hollow triangles ($n=12$) refer to responses obtained with aqueous solutions of Na_2S as stimulus source *(bottom abscissa)*, and *hollow circles* ($n=4$) are responses obtained with the certified H_2S-permeation tube which provided a maximal concentration of 3 ppm *(top abscissa)*. Aqueous solutions of Na_2S as stimulus source were used for curve *b (filled triangles)* ($n=4$). The amounts of H_2S produced by the aqueous solutions of Na_2S was calibrated with the responses of the same receptors to H_2S from the certified permeation tube. *Star (lower left)* is the response to H_2S free air ($n=12$). *Trend lines* connect mean values

rate of the type 1 was significantly higher than that of the type 2 over the concentration range tested, suggesting that type 1 is more sensitive (Table 1). On the other hand, ethylmercaptan as well as dimethyl sulfide elicited a significantly stronger response in the type 2 than in the type 1 over the range of concentrations tested; the firing rate of the type 1 was not modified significantly by increasing dimethyl sulfide concentration (Table 1). None of the other synthetic volatiles listed in Materials and methods activated either of these two sulfide-receptors. Although, the number of animals tested were sufficient to detect only large differences in sulfide-receptor responses between males and females, these did not appear.

Behavioral bioassay

Either 0.02 ppm or 1 ppm H_2S blown into the bottle containing the ticks aroused ca. 60% from rest, approximately 40% raised only their forelegs, and ca. 20% began walking (Table 2). A slightly weaker behavioral response, though not significantly different, was recorded for 10^{-2} *M* dimethyl sulfide (Table 2). Behavior triggered by 10^4 or 2000 ppm CO_2 (1% or 0.2%, respectively) in this bioassay was different: although about 60% of ticks were likewise aroused from rest with CO_2, only ca. 10% remained with raised forelegs, but >50% started to walk

Table 2. Behavioral response of *A. variegatum* males to CO_2, H_2S, mixtures of these two volatiles, dimethyl sulfide, and human breath (sampled early in the morning). Activity states observed (numbers of resting, questing, and walking ticks) following each stimulus are compared pairwise, and are assigned different letters when significantly different ($P<0.05$, Chi-square test). Data are presented here in percentages to make results more comprehensible. H_2S was generated by aqueous solutions of Na_2S at either 10^{-4} or 10^{-3} mg/10 µl, generating vapours in the stimulus cartridge corresponding to ca. 0.1 ppm and ca. 5 ppm H_2S, respectively. Before arriving in the bottle containing the ticks, stimuli were diluted by a factor of 5 in the main humidified air stream

Stimulus in the cartridge	*n*	% ticks activated	% ticks questing	% ticks walking	
Blank	300	18	17	1	a
CO_2 5%	200	63	7	56	b
CO_2 1%	100	62	10	52	bc
CO_2 5% + H_2S 0.1 ppm	50	72	10	62	bc
CO_2 5% + H_2S 5 ppm	50	58	18	40	cd
CO_2 1% + H_2S 0.1 ppm	50	50	22	28	de
CO_2 1% + H_2S 5 ppm	50	78	52	26	f
H_2S 0.1 ppm	100	61	41	20	efg
H_2S 5 ppm	100	53	38	15	eg
Dimethyl sulfide 10^{-2} *M*	50	48	38	10	g
Human breath	50	66	54	12	fg

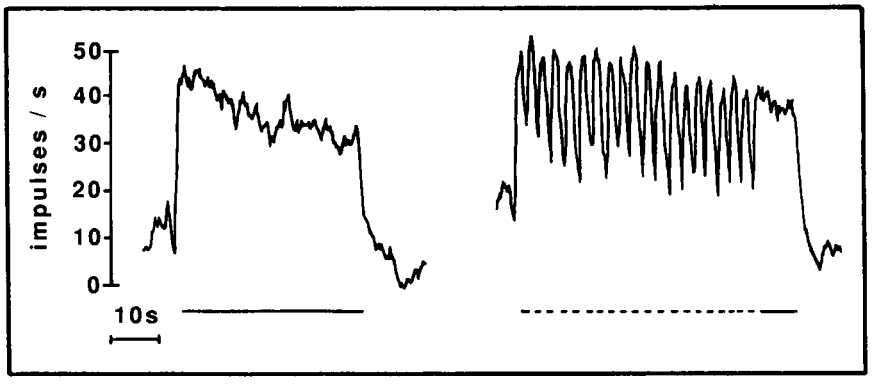

Fig. 8. Representative response of a sulfide-receptor 2 in the capsule of Haller's organ of a male *A. variegatum* to continuous *(left)* and repeated stimulation *(right)* with ca. 0.1 ppm H_2S. The traces are the frequency to voltage converted response of the sulfide-receptor which could be sorted with a window discriminator from other spikes because of its small amplitude. Horizontal bar, stimulation. Responses illustrated here are typical for both types of sulfide-receptors present in the capsule, and were highly reproducible

(Table 2). In mixtures, CO_2 and H_2S appeared to act antagonistically on *A. variegatum* behavior. Depending upon the relative concentrations of CO_2 and H_2S in the mixture, activated ticks tended to either raise their forelegs or begin walking. With CO_2 at 10^4 ppm and H_2S at ca. 0.02 ppm in the mixture, ticks tended to respond as for stimulation with CO_2 alone. By contrast, with a mixtures of CO_2 at 2000 ppm and H_2S at ca. 1 or even as low as 0.02 ppm, ticks responded as for stimulation with H_2S alone (Table 2). Finally, human breath collected early in the morning, when it normally strongly activated sulfide-sensitive cells, evoked a similar response to that of H_2S alone, or to that of a CO_2 and H_2S mixture with a relatively high concentration of H_2S (Table 2).

Discussion

Sulfide-sensitive cells responding to breath have been found in two wall-pore sensilla located in the capsule of Haller's organ of adult *A. variegatum*. Both cells are very sensitive to H_2S, but the type 1 is slightly more sensitive. The H_2S dose-response covers a wide range of concentrations, from <0.1 ppb (estimated threshold) to >10 ppm. The phasic part of the response to H_2S is significantly different depending on whether the stimulus is introduced at 25 cm or 3 cm from the outlet of the stimulus-delivery-tube and proves, as expected, to depend on the sharpness of the stimulus onset. The log/log H_2S dose and phasic part of the response relation does not have a steep slope: 0.21 with a sharp stimulus onset and 0.11 with a graded stimulus onset. Interestingly, the subjective H_2S dose-response relation for humans also has a similar slope (0.11), and the absolute threshold is ca. 10^{-3} ppm (Lindvall 1977). This may suggest equivalent primary transduction processes for H_2S perception in both ticks and humans, an equally high sensitivity most probably imposed by the extreme toxicity of H_2S for eucaryotypes.

The concentration of sulfides in human breath, produced in situ by anaerobic bacteria living on decomposing food remains and saliva in the buccal cavity, is highly variable during the day, ranging from ca. 0.007 ppm to 0.7 ppm for H_2S. Peak levels depend on prolonged periods of reduced saliva flow and abstinence from food or liquid uptake (Tonzetich 1977). Thus, sulfur compound concentrations are generally much higher early in the morning than later in the day. This corroborates our findings that human breath collected from the mouth in the morning stimulated the sulfide-sensitive cells much more than the same collected after lunch. Ruminants, the commonest hosts of adult *A. variegatum*, produce large quantities of H_2S, with concentrations up to about 100 ppm in the rumen (Hungate 1966). The amount of H_2S expelled from the rumen will then vary according to the frequency of eructation events, which is in turn linked to the digestion state. The tick can most probably detect H_2S liberated by an eructating host from significant distances as sulfide-receptors are still sensitive to H_2S levels 10^6 times lower than that found in the rumen.

Concentrations of H_2S expired from vertebrates cover more than 3–4 log orders of magnitude, a range within which the sulfide-receptors of *A. variegatum* can easily discriminate (<0.1 ppb to >10 ppm). This contrasts with the narrow sensitivity range of the CO_2-excited receptor which can only discriminate over 2–3 log concentrations of CO_2, or just 1–2 log concentrations for the CO_2-inhibited receptor (Steullet and Guerin 1992). Moreover, sulfide-receptors are comparatively much more sensitive than the CO_2-receptors (estimated threshold for the sulfide-receptors: <0.1 ppb H_2S, against ca. 10–20 ppm CO_2 for the CO_2-inhibited receptor, and ca. 50–100 ppm CO_2 for the CO_2-excited receptor). Thus, both the CO_2-receptors and the sulfide-receptors are adapted for the perception of CO_2 and H_2S levels given off by vertebrates.

Stimulation with low molecular weight sulfur compounds reveals that the specificity of the two sulfide-receptors differs. The type 1 responds significantly stronger to H_2S, but significantly weaker to ethylmercaptan than the type 2. On the other hand, dimethyl sulfide elicited a strong response in the type 2, but not at all in the type 1. Thus, replacement of one or both of the hydrogens on the sulfur atom by another group (e.g. methyl- or ethyl-) alters perception by sulfide-receptor 1. Although many sulfur compounds other than H_2S have been identified in human breath such as ethyl-3-mercaptoproprionate, methyl-n-propyl sulfide, n-hexylmercaptan, dimethyl sulfide, methyl sulfide, and di-tert-butyl-disulfide (Krotoszynski et al. 1977; Tonzetich 1977), no further active breath component other than H_2S was found for either of the sulfide-receptors during gas chromatography coupled with breath sensillum recordings. Concentrations of sulfides other than H_2S (e.g. dimethyl sulfide) in the breath extracts tested here might have been too low to be detected. Nevertheless, presence of two types of receptors which differ in their specificity suggests that the tick may discriminate for various sulfur compounds. These products abound not only in vertebrate breath but also in general vertebrate body odours (O'Connell et al. 1979; Natynczuk et al. 1989), and a response of these sulfide-receptors to cotton pads impregnated with human axillary secretions was recorded (Steullet, unpublished). Such receptors could provide the tick with specific information about hosts and possibly predilection sites.

Little is known about sulfur compound perception in arthropods. Some carrion beetles (*Necrophorus* sp.) bear carrion sensilla excited by a wide variety of volatiles including H_2S and butylmercaptan (Waldow 1973). On the other hand, some phytophagous insects are attracted by disulfides as well as by thiosulfinates and thiosulfonates (Al Rouz and Thibout 1988; Auger et al. 1989a, b). However, H_2S has never figured to date among the host-finding cues listed for haematophagous arthropods. *Glossina pallidipes* showed no electroantennogram response to H_2S in this laboratory, but recordings in the capsule of Haller's organ of the camel tick, *Hyalomma dromedarii*, provided preliminary evidence for the presence of at least one sulfide-receptor. Kneidel (1984) observed that some American dog ticks, *Dermacentor variabilis*, were attracted by carrion. This behavior could possibly be mediated by sulfides and mercaptans.

In *A. variegatum*, H_2S as well as dimethyl sulfide seem to act in the arousal phase of host-finding, i.e. ticks raise

their forelegs in the air but few start walking. Thus, unlike CO_2, H_2S cannot be considered as a strong locomotor stimulant. In fact, H_2S and CO_2 act antagonistically with regard to activation of *A. variegatum*. Arousal depends greatly on the relative concentration of H_2S in the mixture. A high amount of the latter diminishes the locomotor stimulant effect of CO_2, whereas a smaller quantity of H_2S does not very much alter the response of *A. variegatum* to CO_2. Human breath sampled early in the morning strongly stimulates sulfide-receptors, but does not initiate the same level of locomotion as an equivalent concentration of CO_2 alone. Sulfides as well as other components of vertebrate body odour perceived by the tick (as indicated by gas chromatography coupled to recordings from other olfactory sensilla, Steullet, in preparation) could subsequently intervene as cues bestowing a certain host specificity on *A. variegatum*.

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