Negative Chemotaxis to Ammonia and Other Weak Bases by Migrating Slugs of the Cellular Slime Moulds

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Ammonia is known to be a repellent gas for rising sorogens of *Dictyostelium discoideum*, and it has been suggested that it is also a repellent gas for migrating slugs of the same species. Here we present evidence that migrating slugs of *D. discoideum* and two related species, *D. mucoroides* and *Polysphondylium violaceum*, indeed orient away from high concentrations of NH_3 . In *D. discoideum*, brief exposure of a slug to an NH_3 gradient of about 1 p.p.m. mm^{-1} (10^{-5} atm cm^{-1} or 0.00076 mmHg mm^{-1}) was sufficient to alter the direction of its migration. The gases of other weak bases, such as methylamine, trimethylamine, ethylamine and pyrrolidine, were also effective.

INTRODUCTION

The typical natural habitat of the dictyostelid slime moulds is soil (reviewed in Bonner, 1967; Raper, 1984). Although their behaviour in the natural environment has not been observed, simulation experiments in the laboratory show that at least the asexual developmental cycle of some dictyostelids proceeds essentially in the same manner in the soil as under ordinary laboratory conditions (Higuchi *et al.*, 1983; K. Inouye, unpublished observations).

The behaviour of the pseudoplasmodium, or 'slug' (the precursor structure of the fruiting body), is generally thought to have significant effects on the survival and dispersal of the spores. For example, migrating slugs of most species are oriented by light and by a temperature gradient in such a way that they would tend to migrate towards the soil surface where they would fruit, thus ensuring seemingly optimal conditions for spore dispersal (Bonner *et al.*, 1950, 1985; Whitaker & Poff, 1980). It has been reported that slugs of *Dictyostelium discoideum* are also oriented by 'slug turning factor', a diffusible metabolite produced by slug cells (Fisher *et al.*, 1981). However, since slugs in their natural habitats most probably move around in a tiny space surrounded by soil particles, the possibility arises that the course of slug migration is also affected by gaseous substances, which would diffuse much less quickly under such circumstances than in the open air.

To examine the effects of gases on slug orientation, we have tried to observe the behaviour of migrating slugs placed in a concentration gradient of various gases. However, because of the general difficulty in building up stable concentration gradients of gases and also in verifying the existence of the gradients, we will restrict the scope of this report to experiments using weak bases such as ammonia. Taking advantage of their extremely high solubilities in water, we were able to build up gradients of these gases by using a point source of the gas and an agar plate as sink. Such gradients lasted for a sufficiently long time, and could be visualized with appropriate pH indicators. The results clearly show that migrating slugs of *D. discoideum* orient away from all the weak bases that have been tested.

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METHODS

Organisms and culture conditions. Wild-type strains of *Polysphondylium violaceum* (strain no. 6), *Dictyostelium mucoroides* (H-1) and *D. discoideum* (NC4 and V12M2) were used. Cells grown on *Klebsiella aerogenes* on a modified SM medium (Inouye, 1988b) were collected and washed in 20 mM-potassium phosphate buffer, pH 6.0 (PB), by repeated centrifugation. All incubations were conducted at 22 °C.

Plate method. Agar plates were prepared by pouring 8 ml molten agar (in distilled water containing 0.01% phenol red) into 9 cm diameter plastic Petri dishes. Thick suspensions (10 µl) of washed cells (about 1.6×10^8 cells ml⁻¹ in PB supplemented with 2 mM-MgSO₄) were deposited at a position 1 cm from the centre of each agar plate. After the cells had settled on the agar substratum, excess fluid was removed. The plates were then placed in a light box so that unilateral light came in from the opposite side of the cell deposit (with respect to the centre of the plate). Within a day of deposition the plated cells aggregate to form slugs, which subsequently migrated away from the original cell deposits towards the light source. An Eppendorf tube (0.4 ml) with a hole (diameter 1 mm) in its side was then placed in each plate as a gas source so that the hole was positioned 3 cm from the centre in a direction perpendicular to the light direction (Fig. 1a). The tube contained either H₂O or 1 M-NaOH (200 µl) with various amounts of NH₄Cl or trimethylamine hydrochloride. Care was taken that the liquid in the tube did not leak onto the agar surface. The plates, now carrying migrating slugs and a gas source, were then sealed with Parafilm, placed in a light-tight metal can, and incubated for another 24 h. After this incubation period, the plates were opened up, the gradation of the colour of each plate recorded, and the slugs observed using a dissecting microscope. The slime tracks left behind by the slugs were then transferred onto round polyethylene sheets, which were subsequently stained with Coomassie blue solution (0.3% in ethanol/acetic acid/water, 5:1:4, by vol.) for permanent records (Fisher *et al.*, 1981).

The 'orientation index' (*I*) was defined as

$$I = \frac{n_+ - n_-}{n_+ + n_-}$$

where *n*₊ and *n*₋ denote the numbers of slugs found in the nearer side of the plate to the source and in the distant side from the source, respectively. Those which remained in the central zone (width 1 cm for *Dictyostelium*, 0.5 cm for *Polysphondylium*) were not included.

Micropipette method. Thick suspensions of washed vegetative cells were streaked on plain agar plates and incubated in the dark. Next day, a small agar block (about 2 × 3 × 1 mm) carrying a migrating slug (normally about 1 mm long, for all three species used) was cut out from the plate and placed near the centre of a freshly prepared plain agar plate. The lid of the plate was replaced with another lid with a hole and a piece of wet filter paper attached inside. The plate was then put on the stage of an inverted microscope (Nikon TMD), and the movement of the slug was recorded using a high-sensitivity TV-camera (National WV-1850) and a timelapse videotape recorder (National NV-8030 or AG-6011). A glass micropipette filled with NH₃ solution (0.1–10%, w/v) was introduced into the plate through the hole so that its tip was positioned about 100 µm above the agar surface and at various horizontal distances from the anterior region of the slug (Fig. 2). For accurate measurement of the angles, slugs migrating flat on the agar surface were used. It was assumed that the magnitude of the NH₃ gradient was proportional to the concentration of NH₃ solution in the micropipette and to the cross-section of its tip. In the experiments designed to eliminate the influence of substratum pH, slugs on the agar blocks were allowed to migrate further so that their tips extended beyond the edge of the agar blocks. A micropipette filled with the gas source was introduced when more than a quarter of the length of the slug protruded from the edge of the agar block

Fig. 1. The plate method. (a) Schematic representation showing the stage when an Eppendorf tube containing NH₃-generating solution has just been put in place. (b) A concentration profile of NH₃ gas at 8 h after setting up the NH₃ source as shown in (a); the concentration of NH₃ at different points is given in p.p.m. (10⁻⁶ atm).

Fig. 2. Schematic representation of the micropipette method. L, red light for observation; SY, 2.5 ml syringe; R, release (a hole of 0.5 mm diameter) for preventing flows of gas or liquid through the tip; P, glass micropipette; F, wet filter paper; S, slug; B, agar block; A, agar plate; OB, objective (normally $\times 10$), VTR, videotape recorder. A manipulator holding the micropipette is omitted in the figure.

(Fig. 2, inset). The gas was considered to be a repellent if the slug kept advancing but the direction of its migration had deviated from its original direction away from the micropipette by at least 30° after a period of 15 min.

The glass micropipettes were made of pyrex glass tubing (1 mm outer diameter) using a vertical electrode puller (Narishige) and a pipette-tip grinder (Chatani). The inner diameter of the tips of the micropipettes used was 13–20 μm .

Since slugs of the cellular slime moulds show strong phototaxis but are relatively unresponsive to red light of wavelength over 600 nm (Francis 1964; Poff *et al.*, 1973), very weak red light was used for observation and video-recording to minimize any effect of light. The light source was a halogen microscope lamp and red light was selected with sharp cut-off filters (610 nm) made of acrylic board (total thickness 8 mm); light intensity was further cut down with a neutral filter ($\times 16$). Since the image was hardly visible through the oculars, a TV-monitor screened with a red acrylic board was used for observation. When room illumination was needed for handling the plates and micropipettes, a photographic red light bulb was used.

Calibration of NH_3 concentration. For the plate method, the gradient of the phenol red colour (yellow to red) of each plate was recorded at the end of the experiments. The colour before introducing the source was yellow throughout the plate except for the central region (where the slugs were migrating), which showed a reddish tint. The colours were compared with standard colours made by injecting a known amount of NH_3 gas into tissue culture flasks (60.0 ml) containing 3.0 ml phenol red agar (' NH_3 -standard flasks'). To verify the NH_3 gradient in the air, 57 small pieces of wet pH-indicator paper (cresol red, 3.5×3.5 mm with 2.5 μl distilled water) were attached at the lattice points (1 cm period) of the underside of the lids of Petri dishes (9 cm). The lids were then placed on agar plates containing NH_3 sources prepared in exactly the same way as in the chemotaxis experiments. The colour of the pH-paper at various times was compared with the standard colours. The partial pressure of NH_3 (p) in the ' NH_3 -standard flasks' was calculated from the solubility constant of NH_3 in water and the pH values, using the formula

$$p = \frac{0.0112}{1 + 10^{(9.38 - \text{pH})}} \times [\text{NH}_3 + \text{NH}_4^+]_{\text{in solution}}$$

where p is in atm and ammonia concentration in mol l^{-1} (Denmead *et al.*, 1982). The pH values of the agar in the ' NH_3 -standard flasks' were determined using 'pH-standard flasks' which had been made by including buffer at various pH values (6.0–11.6) in phenol red agar. As an example, a concentration profile of NH_3 at 8 h after the introduction of the NH_3 source estimated by this method is shown in Fig. 1(b).

For the micropipette method, a micropipette filled with 10% NH_3 solution was placed 100 μm above the surface of the phenol red (0.02%) agar (3.0 ml) made up in a tissue culture flask (60.0 ml, with a hole) placed on the inverted microscope, and photographs were taken at intervals with a constant voltage. Green light was selected with a band pass filter (509–562 nm). The pH of the agar at various distances from the pipette tip at increasing times after its introduction was determined by measuring the absorbance of the negatives of the photographs. A

Fig. 3. Concentration profiles of NH₃ gas. (a) Profile produced by a micropipette filled with 10% (w/v) NH₃ solution. The distance is that measured from the point on the agar surface immediately beneath the tip. Concentration profiles at 1 min (○), 3 min (●), and 6 min (△) after the introduction of the pipette are shown. (b) Magnitude of the NH₃ gradient after 6 min at different points from the pipette tip. Differences between pairs of consecutive points in (a) were divided by the distance between the points (40 μm).

standard curve was constructed by measuring the absorbance of the negatives of the 'NH₃-standard flasks' and the 'pH-standard flasks' (see above). A microscope equipped with a photometer (Reichert) was used for the measurements of absorbance. The area of measurement at each point was a circle of diameter 200 μm on the negative (corresponding to 8 μm in the real scale). Fig. 3(a) shows profiles of NH₃ gas concentrations measured in this way at 1, 3, and 6 min after the introduction of a micropipette filled with 10% NH₃ solution, and Fig. 3(b) the dependence of the gradient of NH₃ concentration on the distance from the pipette tip measured at 6 min.

RESULTS

Chemotaxis observed with a population of slugs (plate method)

Chemotactic response of migrating slugs. When an NH₃ source was placed at one end of a plate carrying migrating slugs and the plate incubated in the dark, there was an obvious tendency for the slugs to orient towards the opposite side of the plate. This was the case for all three species (four strains) of dictyostelid examined. Trimethylamine, another gaseous weak base, was equally effective. Fig. 4 shows an example of such an experiment for *D. discoideum* NC4, in which the slime tracks of slugs migrating in a control plate and in a plate containing an NH₃ source are shown. To quantify this tendency, we used an 'orientation index', *I* (see Methods). Fig. 5 shows the results of experiments, which, by the use of this index, clearly indicate the above tendency.

An alternative method to quantify the direction of chemotaxis in *D. discoideum* made use of the pulsatile nature of slug migration in this species. Often, but not always, *D. discoideum* slugs migrate in such a way that their tips continue to advance in the air to the extent that almost the whole slug is supported only at its rearmost part until it falls down in an indeterminate direction to resume migration (Inouye & Takeuchi, 1979). Consequently, the track of such a slug is more or less zigzag. Since most of the slug body is in the air immediately before falling down, it is reasonable to assume that the gradient of the gas, if it is a repellent or an attractant, would affect

Fig. 4. Slime tracks obtained with the plate method. (a) Control (1 M-NaOH without NH_4Cl), (b) 20 mM- NH_3 (1 M-NaOH with 4 μmol NH_4Cl). The positions of the gas sources are indicated by a cross. The orientation index, I , was 0.13 for (a) and -0.57 for (b).

the direction of falling. We concluded therefore that the number of turnings towards and away from the gas source could be a good measure for the taxis. Measurements were made only for a few plates that showed very clear zigzag tracks. It was found that 67% and 89% of the turns seen in NH_3 plates with 2.0 and 0.2 μmol NH_3 in the source solution, respectively, were in the direction away from the source, while only 53–57% were away from the source for the NH_3 plates containing less than 0.02 μmol NH_3 .

Visualization of NH_3 gradients. Gradients of NH_3 and other weak bases were visualized by including phenol red in the agar. That the gradients were produced by the gradient of NH_3 gas existing in the air was verified by the use of many small pieces of pH-indicator paper (cresol red) attached to the underside of the lid of the Petri dishes (see Methods). With this method, it was possible to observe the gradual spreading of NH_3 gas to form a concentration gradient in the air (Fig. 1b). The NH_3 gradient around the centre of a plate that showed negative chemotaxis was estimated to be around 0.1 p.p.m. mm^{-1} (10^{-6} atm cm^{-1}) at 8 h after the introduction of an NH_3 .

Fig. 5. Chemotaxis of migrating slugs represented by the orientation index. (a) *P. violaceum*, (b) *D. mucoroides*, (c) *D. discoideum* NC4, (d) *D. discoideum* V12M2. The gas source was 200 μl 1 M-NaOH (●) or 200 μl H_2O (○) containing the amount of NH_4Cl indicated. Each point represents the mean of two to four experiments involving 31–117 slugs. Bars indicate 80% confidence intervals.

source when the gradient reached a maximum. It is noteworthy that a small gradient still existed at 16 h.

Possibility of pH-taxis. Raper (1939) and, more convincingly, Bonner *et al.* (1985) have shown that *D. discoideum* slugs tend to move towards the acid side of a pH gradient in the substratum. In our experiments, pH gradients always arose due to dissolution of the weak bases. Therefore one might imagine that the pH gradient resulting from the dissolution of the NH_3 could have been the cause of the taxis of the slugs. This possibility is unlikely because the course of slug migration was little affected ($I = 0.02 \pm 0.15$ for *D. discoideum* V12M2) by a pH gradient of the non-volatile base KOH approximately equal in magnitude to the greatest pH gradient produced by NH_3 gas, namely about 0.2 pH unit cm^{-1} around pH 8 at the centre of the plate at 8 h. This is in agreement with the results of Bonner *et al.* (1985), who estimated that a pH gradient greater than 3 pH units cm^{-1} is needed for significant chemotactic response of slugs. In addition, the inclusion of a buffer (10 mM-phosphate, pH 7) in the agar, which substantially reduced and delayed the generation of a pH gradient, affected the chemotactic responses of slugs only very slightly (about 6% reduction in the absolute value of I). Buffer was not included in the standard condition because it shortened the migration period of the slugs. The direct involvement of the gases in slug chemotaxis was further verified in the experiments using a glass micropipette as a gas source described in the following section.

Chemotaxis observed with single slugs (micropipette method)

Chemotactic responses in slugs to various weak bases. To demonstrate the effect of a concentration gradient of the gases more directly, we observed the behaviour of individual slugs in a concentration gradient of various gases. Glass micropipettes were used as the gas source. To minimize the possible effect of a pH gradient on slug behaviour, we applied a gradient of the weak bases to the aerial part of the slug (Fig. 2, inset; see Methods). Fig. 6 shows an example of

Fig. 6. Repeated turns by a *D. discoideum* slug induced by alternating the direction of a gradient of ethylamine. The anterior region of the slug extends in the air from the agar block which is seen in the lower right corner of each photograph (see Fig. 2 inset). The gas source was a glass micropipette containing 3% (w/v) ethylamine solution. The tip diameter is 13 μm . (a) 0 min, (b) 3 min, (c) 4.7 min, (d) 8.4 min, (e) 15.4 min, (f) 17 min, (g) 27.3 min. Bar, 0.1 mm.

Fig. 7. Repeated turns of a *P. violaceum* slug. The vertical dark line seen in the middle of each photograph is the edge of the agar block on which the slug had been sitting. The slug is completely in the air in (f)–(h). The water condensed on the micropipette near the tip did not obturate the tip opening. The micropipette contained 10% (v/v) NH_3 gas. (a) 0 min, (b) 11 min, (c) 19.2 min, (d) 60 min, (e) 62.7 min, (f) 80.8 min, (g) 81.2 min, (h) 98 min. Bar, 0.1 mm.

Table 1. Effect of various weak bases on the orientation of migrating slugs of *P. violaceum*, *D. mucoroides* and *D. discoideum*

Micropipettes containing dilute NH_3 gas (1.0–10%, w/v) or aqueous solutions (3–40%, v/v) of other weak bases were used as gas source (see Fig. 2 inset). Deviation of orientation of slugs was measured after 15 min.

Species/gas	Number of slugs		
	Deviation towards source ($> 30^\circ$)	No deviation ($< 30^\circ$)	Deviation away from source ($> 30^\circ$)
<i>P. violaceum</i>			
Air	0	4	0
Ammonia	0	0	7
Trimethylamine	0	1	4
<i>D. mucoroides</i>			
Air	0	3	0
Ammonia	0	0	10
<i>D. discoideum</i>			
Air	0	8	3
Ammonia	0	1	14
Methylamine	0	1	4
Trimethylamine	0	0	5
Ethylamine	0	0	5
Pyrrolidine	0	1	5

such an experiment with a *D. discoideum* slug. As shown in these photographs, it was often possible to make the slugs repeat right and left turns by alternating the position of the gas source. Such alternating turns in the air could be induced many times with *D. mucoroides* and *P. violaceum* because the slugs of these species can migrate in the air for a long period by virtue of the fact that stalk is continuously formed during migration (Fig. 7).

Fig. 8. Chemotactic responses of migrating slugs of *D. discoideum* (NC4) to NH_3 gradients of various magnitudes. A micropipette filled with NH_3 solution was introduced at $t = 0$ min and removed at $t = 6$ min. The angles between the directions of the slugs at $t = 0$ min and at $t = 12$ min were measured (a) and related to the NH_3 gradients at $t = 6$ min (b). In (a), the NH_3 gradient around the slug tip at $t = 6$ min was $6.6 \text{ p.p.m. mm}^{-1}$. The time is shown in the lower left corner of each photograph in min : s; bar, 0.1 mm .

Several gaseous and volatile weak bases were examined using this method. Table 1 summarizes the results. It is evident that migrating slugs of *D. discoideum* showed negative chemotaxis to all the weak bases tested.

Chemotaxis with different NH_3 gradients. To estimate the magnitude of the NH_3 gradient sufficient to elicit a significant turn of migrating slugs, we measured the angles of deviation of the course of migration of slugs over a 12 min period induced by a brief exposure to various magnitudes of NH_3 gradient. The magnitude of the NH_3 gradient was determined separately by measuring the pH of the agar plate (see Fig. 3). As shown in Fig. 8, half-maximal response was observed at approximately 1 p.p.m. mm^{-1} ($10^{-5} \text{ atm cm}^{-1}$).

DISCUSSION

This work was started with a view to searching for volatile or gaseous substances that are chemotactic for migrating slugs of the cellular slime moulds. In preliminary tests, the only gas that elicited unequivocal chemotactic responses was NH_3 . Therefore we concentrated on NH_3 and related compounds for further study.

The relationship between NH_3 and slug migration has been examined by Bonner and his coworkers. Having shown that migrating slugs of *D. discoideum* orient away from the alkaline side of a pH gradient, Bonner *et al.* (1985) suggested that the effect of the pH gradient might be mediated by a local difference in NH_3 concentration due to a different extent of ionization of the

NH_3 molecules produced by the slugs. This proposal has since been strengthened by the findings that rising sorogens of *D. discoideum* orient away from high concentrations of NH_3 (Bonner *et al.*, 1986; Feit & Sollitto, 1987) and that slugs migrate faster in a uniform atmosphere of NH_3 (Bonner *et al.*, 1988). The present study not only confirms the proposal that NH_3 is a repellent gas for migrating slugs but also establishes the minimum NH_3 gradient required.

It was found in this study that slugs orient away not only from NH_3 but also from other weak bases. The possibility therefore arises that the effects of NH_3 , and of the other weak bases, are mediated by a rise in cytoplasmic pH (pH_i), because these weak bases elevate the pH_i of dissociated slug cells (Inouye, 1988*a*; and unpublished observations). It is also possible that a rise in the pH of some intracellular vesicles (Yamamoto & Takeuchi, 1983) is involved in the transduction of the weak base effect.

From the ecological point of view, the negative chemotaxis to NH_3 may have important functions in the production and dispersal of spores. As Bonner *et al.* (1985) have suggested, it may help the slugs move towards the soil surface, just as phototaxis and thermotaxis are thought to do. We would further suggest that the amount of NH_3 produced by a slug itself, estimated to be of the order of 10^{-13} – 10^{-12} mol min^{-1} for a medium-sized slug consisting of 10^4 cells (see Sussman *et al.*, 1977; Bonner *et al.*, 1988), is large enough to guide the slug towards a more open space in the soil. For instance, 1 p.p.m. NH_3 contains only about 4×10^{-14} mol NH_3 in 1 mm^3 , and an NH_3 gradient of 1 p.p.m. mm^{-1} was shown in this study to be sufficient for a significant chemotactic response of the slug. In addition, since NH_3 has an inhibitory effect on the transition from slug to fruiting body (Schindler & Sussman, 1977), the avoidance by slugs of high concentrations of NH_3 would lessen the risk of their failing to form fruiting bodies. It should also be noted that a decrease in ambient NH_3 concentration induces fruiting (Schindler & Sussman, 1977; Inouye, 1988*b*). Finally, slugs formed in a single amoebal population will be encouraged to disperse by the negative NH_3 taxis. As discussed above, the amount of NH_3 produced by a single slug will be sufficient to affect the course of migration of nearby slugs, and this will cause mutual repulsion of slugs. In preliminary experiments, we found that migrating slugs of *D. discoideum* repel each other (unpublished observations).

In summary, migrating slugs of some cellular slime mould species show negative chemotaxis to ammonia gas, and it is suggested that this may have important functions in the ecology of these organisms.

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