Studies on Negative Chemotaxis and the Survival Value of Motility in *Pseudomonas fluorescens*

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SUMMARY

Multiplication of aerobic piliated fla^+ and fla^- *Pseudomonas fluorescens* growing in aerated mixed culture gave $\text{fla}^+:\text{fla}^-$ ratios approximately 1:1 over a 24 hr growth period. When fla^+ and fla^- were cultured simultaneously in non-aerated media, fla^+ then outgrew the fla^- mutant to a final (24 hr) ratio of 10:1 or greater, thereby lending supporting evidence to the view that motility is a survival factor in environments where 'nutrients' are limited or discontinuous.

Gravity, magnetism, and light did not influence a negative chemotactic response of fla^+ *P. fluorescens* to hydrogen ions. Temperature variations, pH, or viscosity alterations either inhibited or stimulated motility, thereby making impossible a determination of their specific effects. Various antibiotics, membrane-active agents, and protein or DNA synthesis inhibitors were employed in attempts to interfere with the chemotactic response, but none completely inhibited chemotaxis without also affecting motility. The cytoplasmic membrane may act as a transducer of environmental stimuli in chemotaxis.

INTRODUCTION

The positive value of motility as a survival factor for bacteria in environments where 'nutrients' or 'harmful agents' are discontinuously distributed seems fairly obvious; however, little verification of this view exists outside of a few theoretical speculations. Carlson (1962) suggested that no matter how inefficient the motility mechanism, an organism could always achieve a net gain in collection of nutrients by moving. Piliation also is reported to favour survival of aerobic bacteria by allowing surface film formation in static cultures (Brinton, 1959). The experimental results presented here were obtained from a study on a system wherein the factor favouring multiplication was oxygen, which has long been known to invoke an aerotactic response in several kinds of bacteria including *Pseudomonas* (Beijerinck, 1893; Baracchini & Sherris, 1959).

Tactic responses involve not only mechanisms by which bacteria move, but imply the (possible) existence of sensing and transducing devices which direct bacteria towards conditions most suitable for survival and multiplication (Doetsch & Hageage, 1968).

Little in the way of postulated mechanisms of tactic responses has been published for bacterial systems. Links (1955) maintained that any factor which can suddenly and reversibly increase the quantity or consumption of energy-supplying substance

to the motor apparatus can exercise a chemotactic effect. Clayton (1958) modified Links's hypothesis and proposed that the phototaxis in *Rhodospirillum rubrum* involved an ATP-mediated reaction closely associated with photosynthetic phosphorylation.

Recently Armstrong, Adler & Dahl (1967) isolated non-chemotactic mutants of *Escherichia coli*, but were unable to determine the reason for the lack of chemotactic activity.

Hydrogen ions, which stimulate negative chemotaxis in *fla*⁺ *Pseudomonas fluorescens*, provide a means for investigation which is rapid, repeatable, and simple (Smith & Doetsch, 1968). The present paper reports experiments attempting to uncover the basis of this negative chemotactic response. In our view, 'negative chemotaxis' is characterized by an abrupt change in the direction of translational motility. This behaviour is postulated to be caused by a critical change in the membrane potential (the membrane serving as a non-specific environmental monitor) when a bacterium encounters a chemical stimulus. The energy of such stimuli is transduced into a signal causing a reversal in direction of flagellar motion. A mechanism whereby this might be accomplished has been suggested by Doetsch & Hageage (1968). All chemotactic responses by bacteria may thus be considered movements *away* from environmental conditions which induce a critical change in membrane potential.

METHODS

Bacteria studied. *Pseudomonas fluorescens* (University of Maryland 401), an obligately aerobic, actively motile bacterium (*fla*⁺) with a single polar flagellum approximately 5 μ long was selected for this investigation.

Mutant isolation. Non-motile (*fla*⁻) mutants were obtained by a method similar to that of Armstrong *et al.* (1967) by growing the *fla*⁺ organisms at 30° in 10 ml. Trypticase Soy Broth (TSB) (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.) overnight, inoculating one loopful of culture on to the centre of a Tryptone Semisolid Agar (TSSA) (Difco Laboratories, Detroit, Mich., U.S.A.) plate prepared with 1% (w/v) Bacto-Tryptone and 0.35% (w/v) Bacto-Agar. Plates were sealed with masking tape to prevent drying and incubated overnight at 30°. Growth was harvested daily with a Pasteur pipette from the centre of the large colony which developed, and washed once in 3 ml. TSB; one loopful of this suspension then was used to inoculate a fresh TSSA plate. The procedure was repeated successively for 10 days at which time the suspension was streaked on to several Trypticase Soy Agar (TSA) (Baltimore Biological Laboratories) plates for colony development and isolation. These plates were incubated 24 hr at 30°, colonies were picked and each inoculated by stabbing into tubes containing 10 ml. Sulfide Indole Motility (SIM) medium (Difco Laboratories). After overnight incubation at 30°, the tubes were examined for the presence of non-motile isolates. These were checked for: Gram-negative staining reaction (Hucker), formation of small (1 mm. diameter) colonies on TSSA, growth in phenol-red + sucrose broth (Difco), weak acidic reaction in dextrose + phenol-red broth (Difco), basic reaction in litmus milk (Difco), nitrate reduction, inability to liquefy gelatin, presence of cytochrome oxidase using Taxos N Neisseria-Pseudomonas detection discs (Baltimore Biological Laboratories), and a growth rate in TSB comparable to the parent strain. All tests requiring incubation were made at 30° for 24 hr. The mutant chosen formed

small (1 mm.), shiny, discrete colonies on TSA spread plates, whereas the parent strain formed larger, less opaque, colonies on the same medium. O antiserum prepared in rabbits against the *fla*⁻ isolate was tested against the parent *fla*⁺ strain and gave a positive rapid slide agglutination test reaction. When stained by Rhodes (1958) method, the mutant was non-flagellated. Both cultures had polar Type VI pili when grown 16–18 hr at 30° in TSB and stained with 1 % (w/v) sodium phosphotungstate (pH 7.0) on carbon-formvar coated copper grids and examined with an electron microscope (RCA EMU 3 f).

Selection experiments

Culture preparation. *Fla*⁺ and *fla*⁻ cultures were grown 16–18 hr at 25° in 100 ml. TSB contained in 150 ml. prescription bottles. Forty ml. of each culture were removed and aseptically washed twice in TSB after centrifuging at 2000 *g* for 15 min. each washing. Each suspension was adjusted with TSB to a 1.0 absorbance at 425 m μ .

Determination of survival. *Fla*⁺ and *fla*⁻ cultures were distributed into five 250 ml. cotton-stoppered sidearm flasks containing 100 ml. TSB in the following manner: flask 1, 2 ml. *fla*⁺ suspension; flask 2, 2 ml. *fla*⁻ suspension; flasks 3, 4 and 5 contained 1 ml. *fla*⁺ and 1 ml. *fla*⁻. Flasks 1, 2 and 3 were shaken at 20 rev./min. on an incubator-shaker at 30°, and flasks 4 and 5 were incubated without shaking. Colony counts were made from flasks 3, 4 and 5 after 8 and 24 hr by spreading 0.1 ml. of a diluted suspension on to TSA plates which had been dried overnight at 37°, then incubated at 30° for 24 hr. Multiplication of the culture also was followed by measuring absorbance of flasks 1 and 2 for 8 hr at 425 m μ . Flasks 4 and 5 were not shaken except for sampling, each being sampled only once to minimize any effect shaking would have on the results if only a single flask were used. After 24 hr incubation at 30° on TSA spread plates, *fla*⁺:*fla*⁻ ratios at the different time intervals tested were determined.

Chemotaxis experiments

Culture preparation. Forty ml. of a *fla*⁺ *Pseudomonas fluorescens* culture grown in 100 ml. TSB in a 150 ml. prescription bottle for 16–18 hr at 30° were washed once in pH 7.0 buffer (modified from Adler, 1966b), and resuspended to the original volume to give an absorbance of 0.25 at 425 m μ or approximately 3.0×10^8 bacteria/ml. The buffer consisted of 10^{-2} M-mono- and di-basic K phosphates, 10^{-3} M-MgSO₄ and 10^{-4} M-EDTA. When chemicals were added to the buffer, this was done first and then drops of a dense suspension of bacteria (50 ml. washed as previously described and resuspended to 3 ml.) were added with a Pasteur pipette to give the above-mentioned absorbancy.

Flat glass capillary preparation. Flat glass capillaries prepared from 8 mm. internal diameter flint-glass tubing segments by the method of Wright & Colebrook (1921) were cut to 4 cm. lengths. Approximate inside dimensions were 1.2 mm. wide by 0.3 mm. deep.

Demonstration of negative chemotaxis. Capillary tubes were filled from Pasteur pipettes containing the test suspension; one end was plunged twice into plain (1.5 % w/v) 'Ionagar' (Oxoid) No. 2 (Consolidated Laboratories, Inc., Chicago, Ill.) so that a 1 cm. length agar plug was formed; the other end was plunged once into either acidic Ionagar (titrated to pH 1.0 at 55° with N-HCl) or basic Ionagar (pH 12.0 with N-NaOH), depending upon the experiment done. A bit of 'Vaseline' (Chesebrough Manuf. Co.) was placed at either end of the capillary to allow secure mounting on a glass

slide (Fig. 1). Observations were made with a Bausch & Lomb Stereozoom Variable Power microscope, utilizing the substage concave mirror and a Nicholas Variable Illuminator mounted in the substage lamp receptacle. The mirror was adjusted to give a 'dark-field' effect. Using an ocular micrometer, calibrated to read directly in mm. for different magnifications, formation and migration of bacterial 'acid response bands' (a.r.b.) or basic response bands (b.r.b.) were observed and recorded as $\mu\text{m.}$ moved per unit time. Active motility of suspensions sealed in capillary tubes for 3 hr revealed that oxygen depletion would not be a factor affecting these experiments during the 30 min. experimental time period. (The *fla⁺ Pseudomonas fluorescens* used here is aerotactic and motility ceased rapidly in very dense suspensions held under conditions of limited aeration.)

Measurement of motility. Motility estimates made by the method of Shoesmith (1960) were based on bright-phase observation of wet-mount preparations examined with a Zeiss photomicroscope equipped with $\times 12.5$ oculars and a $\times 63$ Neofluar phase objective. The right eyepiece was fitted with a black cardboard diaphragm with a centre opening 1 mm. in diameter. Motility of the suspensions was estimated by counting the number of organisms swimming by the aperture during a 1 min. interval. An average of five observations was used to estimate the 'per cent motile' for any given sample.

Investigations of factors possibly influencing acid response bands

Temperature. Washed suspensions were divided into 3 ml. portions and incubated 30 min. at 4°, 20° and 37°. Acid response band formation and migration then were followed for 30 min. after capillary tubes were prepared in triplicate on glass slides. Experiments were made in 4° and 37° incubators and at room temperature ($\approx 20^\circ$), and where appropriate, a rapid visual estimation of motility was made.

Magnetic fields. Washed bacterial suspensions in the process of forming a.r.b. were subjected to the effect of a 12,000 gauss horseshoe magnet (Alnico V) to determine the influence of a strong non-fluctuating magnetic field on it. Capillary tubes prepared in triplicate were placed both parallel and at right angles to the lines of force and at both the north and south poles. Capillary tubes parallel to lines of force were positioned with one end of the tube touching the magnetic pole, whereas tubes at right angles to the lines of force were placed with the centre 5 mm. from one of the poles. Migration of a.r.b. was followed for a period of 30 min. The effect of a fluctuating magnetic field was observed by placing the capillary tube preparation on the centre of the platform of a Mag-mix magnetic stirrer, Model 11-V-3 (Precision Scientific Co., Chicago, Ill.). The capillaries were mounted 3 mm. above the surface of the platform to obviate any effects of heat generated by the stirrer motor. The dissecting microscope optical system was mounted above the platform so the a.r.b. migration could be followed, and a Nicholas Illuminator was mounted on the adjustable lamp base. The slide preparation was positioned so that the centre of the capillary tubes was at the centre of rotation of the magnetic stirrer. Migration of a.r.b. when viewed from above was from left to right; the stirrer rotation was counterclockwise. Migration of a.r.b. formed was followed for 30 min. with the stirrer set at maximum output.

Gravity. Migration rates were determined in triplicate capillary tubes by mounting the slide preparations (1) in a horizontal position, (2) with the a.r.b. ascending, and (3) with the a.r.b. descending.

Viscosity. Buffered solutions (pH 7) of polyvinylpyrrolidone, (type NPJ-K 90:PVP) and Carbowax 4000, both 5 % (w/v), were tested for their ability to affect band formation and motility rate. Relative viscosity of each solution and the buffer was measured by noting the time necessary for the meniscus of the solution to drop a 4 cm. length in a vertically held Pasteur pipette.

Light. Capillary tubes again were prepared in triplicate. Migration rates were plotted in unfiltered light using the Nicholas Illuminator at full brightness (35 W.) and in the dark, except for brief illumination of the specimen so that readings could be made at 5 min. intervals. Wratten gelatin filters were mounted on a transparent glass

Fig. 1. Diagram of capillary tube experiment. A = acidic agar plug, B = plain Ionagar plug, C = ARB, D = zone of non-motile organisms, and E = motile organisms.

Fig. 2. 'Grid' used to measure effect of direct electric current on bacterial cells and acid response bands. A = capillary tube, B = saline agar, C = 3 cm. space cut in plastic Petri dish, D = cathode, and E = anode.

microscope stage; the capillary preparation then was placed on top of the filter. Formation and migration of a.r.b. was compared for 30 min. against the same experiment done in a darkroom. Wratten filters no. 58 (green, dominant wavelength $540\cdot2\text{ m}\mu$), no. 25 (red, $615\cdot1\text{ m}\mu$), no. 15 (yellow, $579\cdot3\text{ m}\mu$), and no. 47 (blue, $463\cdot7\text{ m}\mu$) were used.

Direct electric current. A Thomas Electrophoresis Unit, Model no. 21, was attached to a 'grid' of the design shown in Fig. 2. Capillary tubes were placed on the grid and saline agar (1 %, w/v, NaCl) was dropped on each end to ensure electrical contact with the electrodes. The voltage necessary to cause migration of heat-killed (60° for 15 min.)

bacteria was determined in cell suspensions in buffers of pH 5, 7 and 8 using Ionagar of pH 7.0 as capillary plugs. Ionagar plugs of pH 1 and 8 were used in the anode side of the capillary when the voltage necessary to disrupt any a.r.b. which formed in response to each type of plug was determined. Voltage necessary to cause migration of heat-killed bacteria with a pH 1 acid agar plug also was determined.

Influence of pH. Demonstration of the applicability of Weber's Law to negative chemotaxis was attempted by suspending washed bacteria in buffer adjusted to pH values of 5, 6, 7 and 8, and determining the reaction of each suspension to Ionagar plugs of pH 1, 2, 3 and 4. *Pseudomonas fluorescens* grown in bottles of TSB (100 ml.) at pH 6, 7 and 8 were washed and suspended in pH 7 buffer and judged for their ability to form a.r.b. to agar plugs of pH 1, 2, 3 and 4.

Table 1. *Compounds tested for their effect on acid response bands and motility*

Compound	Concentration or range used*	Compound	Concentration or range used*
Dicoumarol	1.5×10^{-3} M	Chloramphenicol	1.2×10^{-2} to 1.2×10^{-4} M
Eserine SO ₄	1×10^{-3} M	'Triton'	1 to 10 mg./ml.
Acetylcholine Cl	1×10^{-3} M	Benzalkonium Cl	1 to 0.001 mg./ml.
Atabrine	2.5×10^{-3} M	p-Nitrophenyl glycerol (PNPG)	5×10^{-2} to 5×10^{-4} M
Indole acetic acid	2×10^{-3} M	Potassium cyanide	2.5×10^{-3} M
Sodium dodecyl SO ₄	2.2×10^{-3} M	Polymyxin B SO ₄	2100 units/ml.
Dimethyl sulphoxide (DMSO)	1 to 9.8×10^{-4} M	Phenethyl alcohol	1×10^{-1} to 5×10^{-4} M
Cetylpyridinium Cl	2.6×10^{-3} to 2.6×10^{-6} M	n-Butanol	1×10^{-1} to 5×10^{-4} M
DL-p-Fluorophenylalanine	5.5×10^{-2} to 5.5×10^{-3} M	Tyrocidine HCl	5×10^{-6} to 1×10^{-7} M
Urea	1.67×10^{-1} to 1.67×10^{-3} M	Digitonin	1×10^{-6} M
Sodium barbital	9.7×10^{-3} to 9.7×10^{-5} M	Serotonin creatinine SO ₄	5×10^{-6} to 1×10^{-7} M
Morphine SO ₄	5.3×10^{-3} to 2.7×10^{-5} M	Bacitracin	5×10^{-6} to 5×10^{-7} M
Cocaine SO ₄	1.28×10^{-3} to 5.9×10^{-4} M	Strychnine SO ₄	4.7×10^{-3} to 4.7×10^{-5} M
Sanguinarine SO ₄	3.8×10^{-3} to 3.8×10^{-4} M	Pentachlorophenol	1.5×10^{-3} to 1.5×10^{-4} M
Amobarbital	1.2×10^{-3} to 1.77×10^{-4} M	p-Chloromercuribenzoate	1.5×10^{-3} M

* When a range of concentrations was tested, the increments were 0.5 M or 0.5 mg./ml.

Chemical agents. Various compounds reported to be inhibitors of nerve impulse transmission in 'higher' organisms, DNA- and protein synthesis inhibitors, and materials able to destroy membrane integrity were incorporated into the buffer system at various concentrations. Compounds such as dimethyl sulphoxide (DMSO) also were used if they showed some promise from reports of effects on other organisms. Concentrations are given in Table 1.

Each compound was screened for ability to inhibit a.r.b. formation, and if any was noted it was further checked to determine its effect on motility as compared with a control of plain buffer. Any compounds which showed little effect on motility and that seemed at the same time to affect a.r.b. formation were studied further.

Several compounds were tested for activity in the presence of non-inhibitory amounts of buffered (pH 7) DMSO (0.0312 M) to see whether permeability and, hence, the action of these compounds, was intensified. All compounds were prepared in buffer in as small an amount as possible on the same day they were tested. Solid compounds were weighed and dissolved in 3 ml. buffer for the desired concentration; dilutions of fluid compounds were made to the desired concentration in buffer, 3 ml. of which was

used as a test sample in the experiment. Bacteria were added only after the dissolution of the solid material, and the mixtures were allowed to incubate at room temperature (20°) in the solutions for 30 min. before any experiment was performed.

RESULTS AND DISCUSSION

Selection experiments

The ratio of $\text{fla}^+:\text{fla}^-$ organisms remained constant when they were grown together as mixed aerated cultures, fla^- colony counts being always slightly higher than fla^+ counts

Fig. 3

Fig. 4

Fig. 3. Multiplication of fla^+ and fla^- *Pseudomonas fluorescens* over a 24 hr period in two different experiments; showing $\text{fla}^+:\text{fla}^-$ ratios in aerated mixed cultures. $\circ$, fla^+ ; $\bullet$, fla^- . —, Expt. 1; ---, Expt. 2.

Fig. 4. Multiplication of fla^+ and fla^- *Pseudomonas fluorescens* over a 24 hr period in two different experiments; showing $\text{fla}^+:\text{fla}^-$ ratios in non-aerated mixed cultures. $\circ$, fla^+ ; $\bullet$, fla^- . —, Expt. 1; ---, Expt. 2.

(Fig. 3). $\text{fla}^+:\text{fla}^-$ ratios changed remarkably from a 1:1 ratio in non-aerated media: after 24 hr the fla^+ colony count was 10–33 times greater than the fla^- count (Fig. 4).

Selective multiplication of aerobic fla^+ bacteria under non-aerated conditions supports the view that motility is a positive factor in survival. If a bacterium is living in an environment where favourable conditions are discontinuous or limited it is able to facilitate contact with more satisfactory conditions if it is motile. An actively motile organism could accomplish this in two ways: (1) by migrating through the medium to more favourable areas; and (2) by causing the medium to circulate in the immediate vicinity of the organism. Operation of either factor would enable the organism to increase the amount of material contacting the cell surface, thereby increasing the flux of material across the membrane, and resulting in greater availability of nutrients and dissipation of waste or growth-inhibitory substances present in the medium or secreted by the organism itself. Our data thus agree with Carlson's (1962) analysis since oxygen is a 'nutrient' in the broad sense.

The results demonstrate furthermore that motile piliated bacteria have an advantage over non-motile piliated forms in static (non-aerated) conditions. Although positive

aerotaxis and piliation admittedly both play a role in survival of aerobic forms, motility is considered the greater advantage under the particular experimental conditions used here since it allows for the expression of this response.

Chemotactic experiments

Demonstration of negative chemotaxis. Fla⁺ *Pseudomonas fluorescens* suspensions in capillary tubes developed a single 0.1 mm. deep band in which the bacteria migrated away from the acidic Ionagar plug. The pH values of plugs tested ranged from less than 1.0 to 4.0. No band developed at the opposite end of the tube which was plugged

Fig. 5. Migration of acid response bands as related to pH of acidic Ionagar plug.

with plain Ionagar and served as a control. At pH 1.0 band migration was as rapid as 200 μ /min. over a period of 30 to 40 min., after which the migration rate then decreased. A sharp boundary was always observed on the side of the band directed to the origin of the stimulus, the opposite side being more diffuse (Plate 1). Phase-microscopic examination ($\times 630$) of the band showed that bacteria moving to the sharp boundary immediately reversed direction in a manner similar to the 'Schreckbewegung' described by Englemann (1883). Bacteria entering the acid zone became quickly immobilized. Basic response bands formed in response to the stimulus from basic agar plugs were not as sharply defined as those in response to acid and therefore were not further considered. An a.r.b. did not develop if either deflagellated (10 ml. suspension exposed for 5 min. at 16,000 rev./min. in a Sorvall Omni-Mixer) or heat-killed (60° for 15 min.) bacteria were used. Incorporation of 1 mg./ml. *m*-cresol purple and 10 mg./ml. bromthymol blue in the buffer revealed that an a.r.b. formed between pH 5.5 and 6.0, and the organisms therein moved as the acid diffused from the agar plug to always remain in this pH range, which

covered a zone approximately 2 mm. wide. Neither dye affected the motility of the organism over at least 1 hr. A mixture (1:1:1) of *Pseudomonas fluorescens*, *Spirillum serpens* and *Agrobacterium tumefaciens* suspensions gave a single a.r.b. composed of all three organisms migrating as a unit; these organisms are equally sensitive and negatively chemotactic. Microscope examination and electron micrographs revealed that bacteria remaining on the acid side of the retreating band were not motile and lacked flagella. Varying the concentrations of HCl in Ionagar plugs resulted in response curves in which migration rate was proportional to total distance traversed from the acidic agar plug (Fig. 5). If an acidic agar plug was placed in both ends of a capillary tube, two a.r.b.'s developed which migrated towards each other to disappear immediately upon contact, leaving only non-motile bacteria throughout the tube.

Studies of factors influencing acid response bands

Temperature. Bacteria incubated at 4° and then transferred to capillary tubes were non-motile, therefore no a.r.b. was formed. Acid response bands formed at 20° migrated at the same rate as controls, but subsequent tubes set up from that portion of the culture incubated for more than 1 hr at 37° failed to show a.r.b. formation, since the organisms became non-motile because of temperature effects.

Table 2. *Effect of direct electric current on heat-killed bacteria and on acid response band of living bacteria*

Stimulus	Cell condition*	Buffer pH	Migration (units/min.)†	Volts to move cells‡	Volts to disperse a.r.b.§
None	Dead	5	.	10	No band
None	Dead	7	.	7	No band
None	Dead	8	.	10	No band
pH 1 agar	Dead	7	.	10	No band
pH 1 agar	Live	5	.	.	No band
pH 1 agar	Live	7	0.33	.	23
pH 1 agar	Live	8	0.23	.	32.5
pH 8 agar	Live	5	No band	.	No band
pH 8 agar	Live	7	No band	.	No band
pH 8 agar	Live	8	No band	.	No band

* Cells in buffer (adjusted to indicated pH by titration of 10⁻² M-phosphate buffer).

† One unit is equal to 0.071 mm. (71 μ) on ocular micrometer.

‡ Indicates reading on voltmeter of Thomas Electrophoresis Unit when bacteria began migrating towards anode.

§ Indicates reading on voltmeter of Thomas Electrophoresis Unit causing dispersion of acid response band and migration towards anode.

Magnetic fields, gravity, light and age. No effect on a.r.b. was observed in the experiments on magnetic fields, gravity and light. In addition, organisms incubated in TSB for 48 hr and 1 week gave an a.r.b. similar to that of the 16–18 hr culture.

Viscosity. Comparative rates of flow of the solutions used to test for viscosity were 0.3 sec./cm. for buffer, 0.4 sec./cm. for 5% Carbowax, and 6.65 sec./cm. for 5% PVP. No band was formed with PVP in the buffer, and the band formed with Carbowax was less dense than that in the buffer control. Motility counts revealed that the reason for these differences lay in somewhat inhibited motility by the more viscous

solutions. Although motility was not completely halted (motility counts were 14 % of the buffer control in PVP and 72 % in Carbowax), the decrease in the case of PVP was sufficient to completely eliminate band formation.

Direct electric current. Voltage required to cause migration of dead cells towards the anode differed considerably from that required when living organisms were used (Table 2). Migration of dead cells occurred with an input of 10 V. whether the acid stimulus was present in the tube or not, whereas at least 23 V. were necessary to cause disruption of a.r.b. moving away from the acid stimulus. As might be expected, no a.r.b. was formed either with dead organisms with or without a stimulus, or with living

Table 3. *Effect of varying the pH of the buffer on acid response band formation*

Cell suspension pH*	Agar plug pH†	a.r.b. formation‡	Cell suspension pH*	Agar plug pH†	a.r.b. formation‡
5	1	+ -	7	1	+
5	2	+ -	7	2	+
5	2.5	+ -	7	2.5	-
5	3	-	7	3	-
5	3.5	-	7	3.5	-
5	4	-	7	4	-
6	1	+	8	1	+
6	2	+	8	2	+
6	2.5	+	8	2.5	-
6	3	+	8	3	-
6	3.5	-	8	3.5	-
6	4	-	8	4	-

* Cells in buffer (adjusted to indicated pH by titration of 10^{-2} M-phosphate buffer).

† 'Ionagar' titrated to indicated pH at 50° with N-HCl.

‡ a.r.b. formed within 15 min. after insertion of acid agar plug into capillary tube.

+ = formation of acid response band equivalent to control; + - = weak acid response band; - = no acid response band formed.

organisms when no stimulus was present. Acid response bands also were not formed with living organisms if the pH of the buffer was as low as 5, or when the Ionagar plug was pH 8.0. It is interesting to speculate upon whether the increase in voltage necessary to 'force' the living organisms into the acid causing dispersion of the a.r.b. is related in some manner to the phenomenon of negative chemotaxis.

Influence of pH. The effect of pH of the suspending medium on a.r.b. formation is depicted in Table 3. *Pseudomonas fluorescens* grown at different pH values and resuspended in buffer of pH 7 showed no difference in ability to form a.r.b. with acid agar plugs; an a.r.b. formed at any pH below 3.0.

Chemical agents. Compounds found not to inhibit motility or a.r.b. formation were: urea, indole acetic acid, digitonin, cocaine SO_4 , amobarbital, eserine SO_4 , 'Triton', serotonin creatinine SO_4 , morphine SO_4 , chloramphenicol, and dicoumarol. Compounds which inhibited motility or a.r.b. formation were: atabrine, benzalkonium Cl, sanguinarine SO_4 , pentachlorophenol, polymyxin, *n*-butanol, sodium dodecyl SO_4 , cetyl pyridinium Cl, sodium barbital, strychnine SO_4 , *p*-chloromercuribenzoate, phenethyl alcohol, and tyrocidine HCl. All concentrations employed are given in the methods section of this paper. Those compounds which at some concentration partially inhibited motility and a.r.b. formation were: potassium cyanide, bacteria 56 %

as motile as control at 2.5×10^{-3} M; DL-*p*-fluorophenylalanine, 75 % at 5.5×10^{-2} M; PNPG, 94–59 % at 1.5 to 3×10^{-5} M; and DMSO, 90–70 % at 3.12×10^{-2} to 1.25×10^{-1} M; however, at a 10-fold greater DMSO concentration, the motility rate was only 45.5 % of the control.

Since temperature, viscosity, and pH variations all exerted inhibitory or stimulatory effects on motility of *Pseudomonas fluorescens*, any measurement, therefore, of influence on negative chemotaxis, was compromised.

The effect of variation of environmental pH on the a.r.b. initiated by varying the pH of agar plugs, did not give evidence of any conformation with Weber's Law in that fla⁺ organisms failed to respond in a manner which indicated that the threshold response was a constant fraction of the environmental stimulus under the conditions described. It is possible that concordance under Weber's Law was not observed because: (1) motility rate is dependent upon pH (Schuetze & Doetsch, 1967) and any threshold response was masked; and (2) the response may be to pH increments finer than those chosen for measurement in this series of experiments, indeed, finer than the measuring capability of the experimental system.

Since we have found that migration rate of an a.r.b. is related to factors such as hydrogen ion concentration, degree of ionization of the acid, and ionic strength of the buffer, including that to which the chemical agents have been added, it is desirable that any compound which inhibits or stimulates the chemotactic reaction manifests itself by preventing or intensifying band formation. At the same time, such an agent ought to exhibit little effect on motility rate, particularly if motility *per se* and chemotactic responses are distinct as found by Adler (1966a) in the case of *Escherichia coli*.

The difficulty of 'proving' inhibition of chemotaxis lies in the technical difficulty of demonstrating that motility is not affected at the same time that chemotaxis is repressed. Measurement of motility by crude visual methods has several shortcomings, but it seems the most feasible preliminary method available. The motility rate in control organisms is not initially stable, and varies considerably, so that one must allow the suspension to stand for 10 min. after preparation; care also must be taken to wait at least one min. before counting any slide preparation, since motility rate again stabilizes after this time.

Failure to observe inhibitory effects of chemical agents on negative chemotaxis may have several explanations: (1) inhibitory agents exist but were not found; (2) both motility and chemotaxis were simultaneously affected; (3) the action of agents was not observable under the conditions imposed in the experiment; and (4) the agents did not gain access to the membrane or postulated receptors.

The idea of hydrogen ions acting as a stimulus in a negative chemotactic reaction in the organism studied is appealing when viewed in the light of possible alteration of electrical potentials along the membrane. The theoretical number of hydrogen ions 'displaced' by the volume of one *Pseudomonas fluorescens* cell ($0.96 \mu^3$) at pH 5.5 is 2×10^8 or one H⁺ for a 1 m μ thick cross-section of the organism. It is interesting to speculate as to whether this is, in fact, the 'signal' to which this organism responds.

Mechanisms which control flagellar responses of bacteria remain, as yet, unknown. Any 'neurological' system, however primitive, described in flagellated eucaryotic organisms seems completely lacking in procaryotic forms; in addition, the exact

nature of excitations (if any) elicited by stimuli also is unknown (Doetsch & Hageage, 1968).

A theory developed by us, in the course of laboratory experiences with various flagellated bacteria (Doetsch, 1966*b*; Doetsch, Cook & Vaituzis, 1967) suggested that the flagellum or flagellar fascicle is part of an excito-motor system developed for spacial accommodation. Stimuli are considered to be 'sensed' by the cytoplasmic membrane acting as a 'monitor' or signal receptor. The entire membrane may serve this function, or possibly there are specialized receptor areas which may or may not lie near the origin or point of insertion of the flagellum (Doetsch, 1966*a*). The mechanism of sensing environmental stimuli is visualized as involving an induced change in membrane potential by which means the signal is transduced and transmitted to a 'motor unit' (possibly a differentiated membrane immediately surrounding the flagellum). A gap, synapse, or an equivalent thereof, and a primitive 'muscle-membrane' or contractile element, may be coupled with, and activate, a rigid or semi-rigid non-contractile flagellum. The chemical events may be similar to those found in higher animals, based upon acetylcholine (Doetsch & Hageage, 1968).

Successful experiments influencing and interfering with the postulated sensing function of the membrane would add considerable support to this view of the nature of sensory processes in bacteria. The response may be, on the other hand, a result of some change, induced by the stimulus, in the general metabolic state of the organism, and controlled by a mechanism which is linked to one or several energy-yielding pathways. This possibility has yet to be ruled out in the phenomenon of negative chemotactic responses.

Studies with bacterial systems might yield valuable clues into the general problem of how the energy of stimuli is transduced into the language of the sensory code at the unicellular level. The implications of such studies are exciting to contemplate and undoubtedly would open new horizons for development of a theory of bacterial behaviour and motility.

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EXPLANATION OF PLATE

Photomicrograph of an acid response band ($\times 290$); stimulus is on the left

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(Facing p. 391)