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Quantitation of the Sensory Response in Bacterial Chemotaxis

(*Salmonella typhimurium*/receptors/stimulus-response relationship/binding *in vivo*/gradient sensing)

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ABSTRACT **A quantitative assay for the stimulus-response relationship in bacterial chemotaxis has been developed by measurement of tumble frequency. Application of the assay has shown an additive relationship between changes in receptor occupancy and recovery times. Tumble suppression is related to the change in receptor occupancy and not to its rate of change. The results can be explained in terms of varying levels of a tumble regulator.**

Bacteria have a simple sensory system that allows them to respond to stimuli and sense the direction of a chemical gradient. This system has been shown to consist of discrete chemical receptors (1–3), a transmission mechanism that relays the information from the receptors to the flagella (4, 5, 22), and a motor response that converts this information into regulation of the frequency of tumbling (6–9). Because of its amenability to chemical and genetic manipulation, the bacterial system may provide a tool for the understanding of other more complex sensory systems.

The thorough investigation of a sensory system depends on the ability to measure quantitatively the response to a defined stimulus. Quantitative methods have already been developed for the tracking of individual bacteria (7) and the migration of a colony of bacteria in a defined gradient (10). Semiquantitative tools are available in the capillary assay (11) and the response of bacteria to temporal gradients of attractants and repellents (6, 8). However, a method was needed for the precise and quantitative analysis of the immediate receptor-response relationship. The temporal gradient experiments (6) appeared to provide a basis for such a method. These experiments showed that sudden increases in attractant concentration or sudden decreases in repellent concentration led to a suppression of the spontaneous tumbling of bacteria and that this smooth swimming response gradually relaxed to the normal tumbling pattern over a period of time (6, 8). Moreover, this recovery could be influenced by conditions that altered the chemotactic response, such as methionine starvation or genetic mutation (12).

We have converted the qualitative observations into a quantitative method by determining the number of smooth swimming bacteria as a function of time after a temporal gradient stimulation. The relaxation back to the normal behavioral pattern is a simple exponential decay preceded by a slower, more complex rate of recovery. The pattern of the excitation and recovery can be analyzed and shown to be consistent with a change in the concentration of a tumble regulator in the population of bacteria. The technique thus allows the study of changing levels of the tumble regulator and assists the analysis of conditions that affect this regulator.

METHODS

Bacterial Strains and Growth Conditions. All strains used in this study are derivatives of *Salmonella typhimurium* LT2 and have been described in detail (12). Cultures were grown at 30° on a New Brunswick gyratory shaker in Vogel–Bonner citrate buffer (13) plus 1% glycerol for serine and aspartate experiments and plus 20 mM sodium nitrate for ribose and allose experiments. Bacteria were grown to midlogarithmic phase (2 to 5 $\times$ 10^8 cells per ml), centrifuged at 0° for 15 min at 3000 $\times$ *g*, resuspended in fresh medium, and kept on ice. From this suspension the bacteria were diluted into cold medium to 3 $\times$ 10^7 cells per ml, incubated for 5 min in a 30° water bath, and placed on the gyratory shaker.

Tumble Frequency Assay. A 0.9-ml aliquot of the bacterial suspension was mixed in less than one sec with 0.1 ml of attractant, and this time was recorded as time = 0. Using a semi-automatic (Finn) pipette, 8 μl of this mixture was delivered under a coverslip that was supported on two other coverslips to give a chamber of depth 60 μm. The microscope with attached camera at 500$\times$ magnification was focused on the slide surface, and an 0.8-sec exposure photograph was taken using darkfield illumination and a stroboscopic high pressure xenon arc lamp with a flashing rate of 5 Hz. An orange long pass band filter (Corning 3-69) was inserted in the light path to exclude blue light, which causes a photo response at higher intensities (14). Photographs of 0.8-sec duration were taken at periodic intervals. (Typical results are shown in Fig. 1.) The high contrast film (Kodak 2475) was developed and projected onto white paper. The number of smooth swimming bacteria was determined by counting the number of motility tracks (15) consisting of four successive bacterial images. At the end of the run, 0.9 μl of 0.2 M serine was diffused under the coverslip, causing all the bacteria to swim smoothly. The total tracks present in this final exposure were corrected for dilution and taken as 100%. A constantly smooth swimming mutant (ST20) was used to control for continued motility in the chamber as well as to determine the dilution factor (about 10%) in the final step.

RESULTS

Measurement of Recovery Curves. The results shown in Fig. 1 are typical of those obtained with this assay using a constantly tumbling mutant. On exposure to a sudden increase in attractant concentration, essentially all of the bacteria swim smoothly at the earliest measurable time after mixing (top picture, Fig. 1). The number of bacteria swimming smoothly (shown by the tracks like dotted lines) decreases steadily and the number tumbling constantly (indicated by the splotches of light) increases until, in the final picture (bottom), essentially all of the bacteria are tumbling. Unlike the tumbly mutants in Fig. 1, wild-type bacteria show a mixture of tracks and tumbles after they relax to the non-gradient tumbling frequency, but the form of the recovery curves is the same.

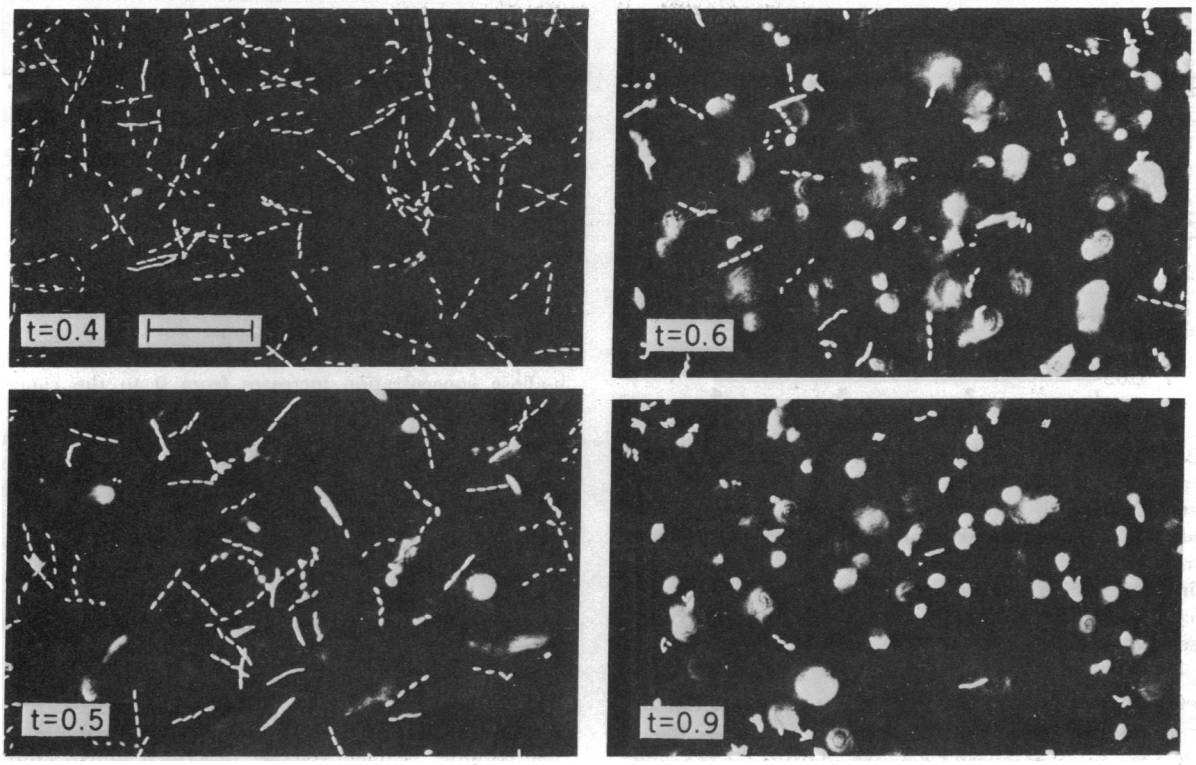

FIG. 1. Tumble frequency assay. The disappearance of tracks made by smooth swimming bacteria after an L-serine temporal gradient stimulus (0 → 0.02 mM) is shown. A 0.9-ml aliquot of a suspension of ST171 (a constantly tumbling mutant) was rapidly mixed with 0.1 ml of attractant to yield a final concentration of 0.02 mM L-serine. Photographs, at the indicated number of minutes after mixing ($t = 0$), were taken by 0.8-sec open shutter exposures to stroboscopic illumination, as described in the *text* (*Methods*). The length of the bar in the first photograph is 50 μm.

The recovery is quantitated by recording the number of tracks, which is proportional to the number of smooth swimming bacteria in the population. To be certain the bacteria have not lost motility because of experimental conditions and to obtain the maximum number of tracks, a very strong positive stimulus of serine is given at the end of the run. This causes all the bacteria to swim smoothly, and the number of tracks is then taken as 100%. Such a 100% picture looks nearly identical to that of the $t = 0.4$ min. picture in Fig. 1. Plots of the data for some typical runs are given in Figs. 2 and 3. Exponential decay preceded by a slower rate of recovery is characteristic of all recovery curves that we have measured.*

What Do the Recovery Curves Measure? When the fraction of the maximum number of tumbling mutant tracks is 0.5, (*i*) are 50% of the bacteria continuously tumbling or (*ii*) does each bacterium have a 50% probability of tumbling in the time-lapse photograph? Alternative (*i*) was found to be correct by tracking 22 individual bacteria after temporal gradient stimulation and comparing the accumulated data of the individual bacteria with the tumble frequency assay for the population. Plotting the fraction of the individually tracked bacteria that are not yet continuously† tumbling against time yielded a curve indistinguishable from the tumble frequency assay recovery curve for the same stimulus. This means that the fraction of the maximum number of tracks recorded at a given time measures the fraction of the population that is still responding to the stimulus.

Effect of Rate of Stimulation. Because bacteria travel up steeper gradients at a faster rate (10), we asked whether the smooth swimming response is a function of the change of concentration of chemoeffector $[d(c)]$ or the rate of change of chemoeffector concentration $[d(c)/dt]$. As shown in Fig. 2, the recovery curve is clearly independent of the time of mixing. Mixing times varied from 1 to 15 sec, but the curves recorded from 1 min to 3.5 min are essentially identical.

Additivity of Recovery Time. The initiating stimulus in the above experiments involved a change from an attractant concentration of 0 to a final concentration of C_f. How do the responses to two changes in concentration of 0 to C_1 and C_1 to C_f compare with a single stimulation of 0 to C_f?

If we define the recovery time, t_R, as the time it takes an individual bacterium to return to its pre-stimulus, nongradient tumbling frequency, it can be shown that the area under the recovery curves (which measure cumulative distributions of recovery times), plotted on Cartesian coordinates, is the

* For the exponential phase of the recovery curves, the distribution of recovery times is that expected from a stationary Poisson process (21), i.e., the probability of termination of the response is constant in time and equal for all members of the population. We observe the probability of response termination to decrease with increasing stimulus. This follows from the observation that the recovery curves remains log-linear and the slope increases with increasing stimulus (unpublished observations).

† In nearly every individually tracked bacterium, brief tumbles occurred prior to the onset of continuous tumbling, but their duration and frequency were evidently not sufficient to affect the photographic data.

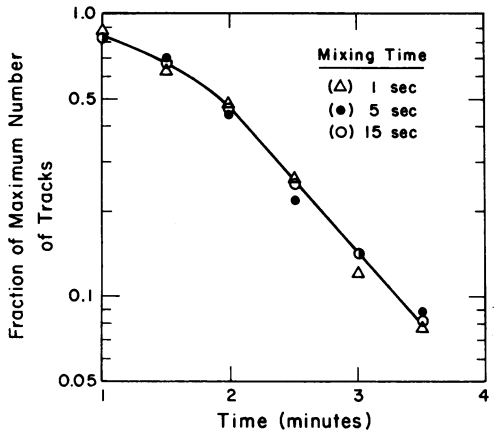

FIG. 2. Effect of mixing time on recovery curves. Serine (0.1 ml of 5 mM; gradient 0 → 0.5 mM) was delivered into 0.9 ml of a ST171 suspension with a micropipette over the period of time indicated, while the bacterial suspension was gently shaken. At 0.4 min after initiation of the stimulus, the bacteria were placed in the chamber and data were recorded by the tumble frequency assay. Points are an average of duplicates. The symbol ◑ represents coincidence of the points ○ and ●.

arithmetic mean recovery time, $\bar{t}_R$, for the population of the tumbly mutant bacteria. Examination of the data in Fig. 3 shows that the mean recovery times are additive for the additive stimuli, i.e., the areas under the two recovery curves in Exp. 1 add up to the area under the single curve in Exp. 2. Other experiments of this type, with the attractants aspartate and serine, gave similarly additive recovery times, i.e., in all cases if $\Delta(C)_{13} = \Delta(C)_{12} + \Delta(C)_{23}$, then $\bar{t}_{R_{13}} = \bar{t}_{R_{12}} + \bar{t}_{R_{23}}$.

Receptor Occupancy and Recovery Time. Previous studies on Weber Law relationships in spatial gradients (10, 16) and a correlation of *in vitro* and *in vivo* characteristics of receptor proteins (3, 17, 18) have indicated a relationship between the

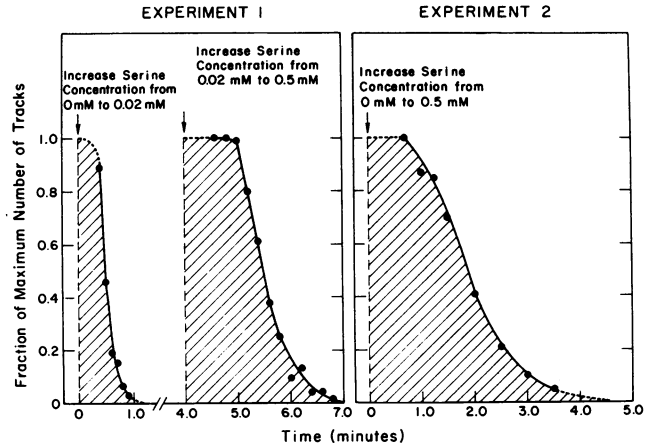

FIG. 3. Additivity experiment. In Exp. 1 the response of ST171 to a 0.02 mM serine stimulus was assayed as described in the *text*. After 4 min of incubation on a gyratory shaker in the 0.02 mM serine, the bacteria were rapidly mixed with a concentration of serine that yielded a final concentration of 0.5 mM, and the assay was initiated. In Exp. 2, the response to a single stimulus of 0.5 mM was assayed (*Methods*). The mean recovery time, $\bar{t}_R$, for each stimulus is the area under the recovery curve. The areas in Exp. 1 are 0.5 min and 1.5 min. In Exp. 2 the area is 2.0 min.

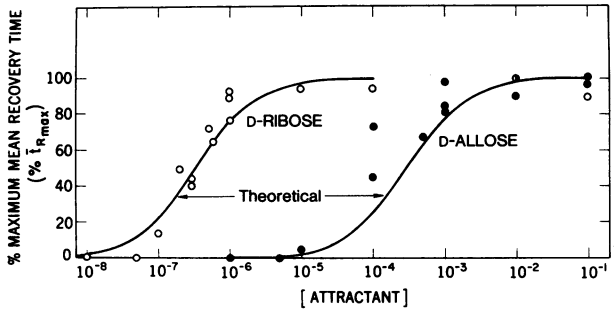

FIG. 4. Comparison of response with receptor occupancy. Points are % maximum mean recovery times ($\bar{t}_R$) for D-ribose and D-allose concentration increases from 0 to the concentration shown on the abscissa. Each point represents the average of three consecutive assays. Results are for three experiments performed on different days. $\bar{t}_{R_{max}}$ were 0.56, 0.58, and 0.62 min for ribose experiments and 0.54, 0.55, and 0.58 min for allose experiments. Theoretical curves were calculated assuming (*i*) noncooperative chemoreceptor binding constants of 3.3×10^{-7} for ribose and 3.0×10^{-4} for allose [as determined *in vitro* (17)], and (*ii*) the response $\bar{t}_R$ is proportional to the change in fraction of binding protein occupied. The symbol ◑ represents coincidence of the points ○ and ●.

occupancy of the receptor sites and the bacterial response. The tumble frequency assay described above allows us to make a quantitative analysis of the response and its correlation with receptor properties. The ribose-allose receptor was chosen for this study since this protein has been isolated in pure form and theoretical binding curves can be calculated to allow comparison with the response *in vivo* (3, 17). The results are shown in Fig. 4.

The recovery times shown in Fig. 4 for the ribose curve are a good fit to the theoretical curve plotted on the basis of assumed classical binding, i.e., $R + C \underset{}{\overset{K_d}{\rightleftarrows}} RC$ (where R = chemoreceptor and C = chemoeffector), with a dissociation constant of 3×10^{-7}, as determined *in vitro*. The points on the allose curve show greater scatter but do indeed require an approximately 1000-fold higher concentration for an equal biological effect, as would be expected from the weaker allose affinity of the pure protein ($K_d = 3 \times 10^{-4}$). Fig. 4 shows typical saturation phenomena, characteristic of binding to a receptor, and the recovery time, $\bar{t}_R$, is proportional to the change in the fraction of the receptor occupied (ΔRC) as calculated by the mass action expression.

DISCUSSION

The results described above show that (*a*) a quantitative measurement of the response to a defined chemical stimulus has been developed based on suppression of tumbling frequencies during the stimulus and relaxation, (*b*) the recovery times for such curves are a measure of the return of the tumbling frequency in a population of bacteria to pre-stimulus levels, (*c*) the suppression of tumbling of the population is proportional to the change in receptor occupancy and not to the rate of change of receptor occupancy, and (*d*) additivity is observed between the change in receptor occupancy and the recovery times.

Previous quantitative techniques for investigating chemotaxis have been based on either net migration of bacteria in spatial gradients or the tumbling patterns of individually

tracked bacteria. Both types of approaches measure chemotactic behavior resulting from an interplay of responses to continuously changing stimuli in the bacterial environment. This interplay yields data that are valuable but difficult to interpret in terms of the quantitative behavior of the tumble regulator and impossible to use for the study of the kinetics of the tumble regulator. Temporal gradient stimulation obviates this difficulty by causing the bacteria to experience a single defined stimulus. The quantification of the temporal gradient approach permits a simple, rapid, and accurate examination of the stimulus-response relationship and should be useful in determining more precisely the role of chemical agents and physical factors that affect the sensory response leading to chemotaxis.

The response in the tumble frequency assay can be explained with the assumption that an individual bacterium's motility pattern is determined by a tumble regulator whose formation and disappearance are governed by processes yielding rates v_f and v_d in equation **1**:

$$\overset{v_f}{\rightarrow} X \overset{v_d}{\rightarrow}, \quad \text{where } X = \text{tumble regulator.} \qquad [1]$$

Eq. **1** does not require assumptions of detailed mechanisms, but only that the concentration of X determines whether a bacterium tumbles, and that either or both the rate of formation and the rate of disappearance of X are susceptible to modification by attractants, repellents, and biochemical events of the cell. It is assumed in this example that increased levels of X above a threshold level inhibit tumbling and decreased levels accelerate tumbling. The choice of X as an inhibitor of tumbling has been made more plausible by the genetic studies of Parkinson on *Escherichia coli* (5a), but whether X acts as an inhibitor or an activator does not affect the analysis presented here. If it is an inhibitor, then the sudden increase in attractant concentration increases v_f (or inhibits v_d) so that X suddenly and rapidly rises to a value X_0 at times that are experimentally equivalent to $t = 0$. Subsequently, the concentration of X decreases (v_d greater than v_f) until it reaches its pre-stimulus nongradient motility level. The return to the pre-stimulus level in an individual bacterium is the recovery time (t_R), and the average recovery time ($\bar{t}_R$) measures the time that the value of X remains at tumble-inhibiting levels in the population. The $\bar{t}_R$ is found to be proportional to the change in chemoreceptor occupancy (ΔRC).

The finding that tumble suppression is proportional to the time-independent change in receptor occupancy (ΔRC) may at first seem to contradict the apparent dependence on the time rate of change in spatial gradients and in enzymatically generated temporal gradients observed by Brown and Berg (19). In the latter experiments shallow gradients are used so that the rate-limiting step in the sequence of reactions that yields v_f in Eq. **1** is the rate of change in attractant concentration. This results in a value of v_f which approaches that of v_d and, therefore, the levels of X over a short period of time depend on both rates. In the tumble frequency assay, the rate of generation of attractant is too rapid to be rate-limiting and, unlike in shallow gradient conditions, the level of X is not an integrated result of stimuli in a changing environment. This system yields a response independent of rate of delivery of the stimulus and, therefore, the recovery curves measure the relaxation to normal in much the same way as temperature jump relaxation curves (20). A conclusion from our experiments is that the total number of tumbles suppressed, when measured over a period of time that encompasses all effects of the stimulus, would depend only on the amount of chemoreceptor occupancy change.‡ The ability of the assay to achieve this separation of the stimulus-response relationship provides a tool for the analytical evaluation of the tumble regulator role.

From these experiments the receptor occupancy response relationships suggest that the receptor initiates a chain of events that lead to a transient alteration in the concentration of a tumble-regulating quantity. This tumble regulator is under the type of control we can associate with chemical compounds (which are reacting relatively slowly) or protein receptor complexes (which are dissociating relatively slowly) or with a membrane electrochemical potential that has similar temporal characteristics. It is too early to identify the tumble regulator itself, but the ability to quantitate the response allows a measurement of the regulatory kinetics and should provide a tool for further understanding of the conditions that alter tumble regulation. The similarity of recovery curves in the bacteria and action potentials in higher organisms makes this analysis of particular interest.

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‡ Shallow gradient mixing experiments, in which chemoeffector concentration changes were varied by controlling consecutive step gradients, provide added support for this conclusion (R. Macnab and D. E. Koshland, Jr., in preparation).