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PERTURBATION OF THE CHEMOTACTIC TUMBLING OF BACTERIA

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The bacterial sensing system has been studied on three levels. First, a quantitative method has been devised for measuring the "action spectrum" of the bacterium in response to a sudden addition of attractant. Second, a technique has been developed for the rapid isolation of mutants defective in the transmission part of the sensing system. Third, a study of the effects of light on the transmission system reveals two components, one which generates tumbling and another which inhibits it.

INTRODUCTION

The bacterial sensory system is of particular interest as a model system because it can be manipulated by genetic and biochemical means. With these tools it is hoped that the fundamental biochemistry of the network which allows the bacterium to sense the direction of chemical gradients can be elucidated. Various methods which are being pursued in our laboratory to elucidate the nature of the system will be discussed in this article. The first involves a quantitative analysis of the response itself, the second a new method for the selection of mutants, and the third involves the influence of light on the sensory system.

RESULTS AND DISCUSSION

Pt. 1 — A Quantitative Assay for the Stimulus-Response Relationship

Quantitative methods had been previously developed for measuring the migration of bacteria into capillaries (1), the tracks of individual bacteria (2), and the migration of a bacteria in a defined chemical gradient (3). However, a method was needed to examine more analytically and more directly the response relationship. The temporal gradient approach of Macnab and Koshland (4) seemed appropriate for such an examination and a workable procedure, called the tumble frequency assay, has been developed by Spudich and Koshland (5).

In this method the bacteria (most usefully the constantly tumbling mutant) are subjected to a sudden temporal increase in attractant concentration. All of the bacteria swim smoothly under these circumstances and stroboscopic pictures (four flashes in 0.8 s) show all the bacteria swimming in a straight line in four successive positions.

As time goes on, increasing numbers of bacteria resume a tumbling pattern until (in the case of the constantly tumbling mutant) all of the bacteria are tumbling continuously

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(cf. Fig. 1). By counting those bacteria which are smooth swimming as a function of time it was found that an approximately exponential decay of the smooth-swimming response could be measured. Wild-type bacteria returned to a state in which only a fraction of the bacteria were tumbling in any interval of time, but their stimulation to smooth swimming and recovery to a normal state yielded curves of the same form as the constantly tumbling mutant. The advantage in using the constantly tumbling mutant was the increased accuracy.

With this procedure for quantitating the direct response, a number of individual questions could be answered. One of the first questions was whether the rate of delivery of the attractant influenced the amount of response, i.e. whether the response was proportional to dc or to $\frac{dc}{dt}$. This could be determined as shown in Fig. 2 by varying the time (5 s, 10 s, 15 s) taken to raise the attractant concentration a constant amount and then measuring the recovery time as a function of the stimuli. The results shown in Fig. 2 clearly show that the recovery time is independent of the rate of delivery of the stimulus and is related only to the amount of the change in attractant concentration, i.e. recovery time is a function of dc and not of $\frac{dc}{dt}$.

For example, a particular response was obtained when the initiating stimulus involved a change from an attractant concentration of O to a final concentration of C_f. It was of interest to see how such a response compared to the response to two changes of concentration, one from O to C_1 and a second from C_1 to C_f. Studies of this sort were carried out with the attractants serine and aspartate and in all cases the results were additive, i.e. the sum of the recovery times observed for the change from O to C_1 and the change from C_1 to C_f were equal to the recovery time of the stimulus from O to C_f. Further subdivision of the stimuli has the same result. Thus, the responses are additive in a manner which severely limits the mathematical possibilities for the behavior of the tumble regulator.

Finally, it was of interest to determine the quantitative relationship of the response to the properties of the receptor protein. This was done by using an attractant, ribose, for which the receptor had been isolated. The binding of ribose to this receptor had been measured in equilibrium dialysis experiments with the purified protein (6, 7). The dissociation constant (Kd) was 3.3×10^{-7}. Furthermore, it was known that allose served as a chemoattractant and a competitor with ribose, but had a much smaller affinity for the purified receptor (Kd equalled 3×10^{-4}).

The tumble frequency assay described above was applied to the whole bacteria to measure responses to ribose and allose. The results are shown in Fig. 3. The solid lines are those plotted for the calculated change in the amount of receptor-chemoattractant complex on the basis of the binding characteristics of the pure protein. The experimental points represent the recovery times measured using the tumble frequency assay on the whole bacteria. It can be seen that there is an excellent agreement between in vivo binding and the in vivo bacterial response, with the response measured being proportional to the change in receptor occupancy. Whatever the compound which is released into the transmission system ultimately to be converted into flagella function, its release is proportional to $\Delta(RC)$, where R is the receptor protein and C is the chemoattractant. The quantitative assay has already revealed significant features of the response-stimulus relationship and more studies are in progress to attempt to further clarify the properties of the molecular mechanism linking the receptor-chemoattractant complex and the chemotaxis response.

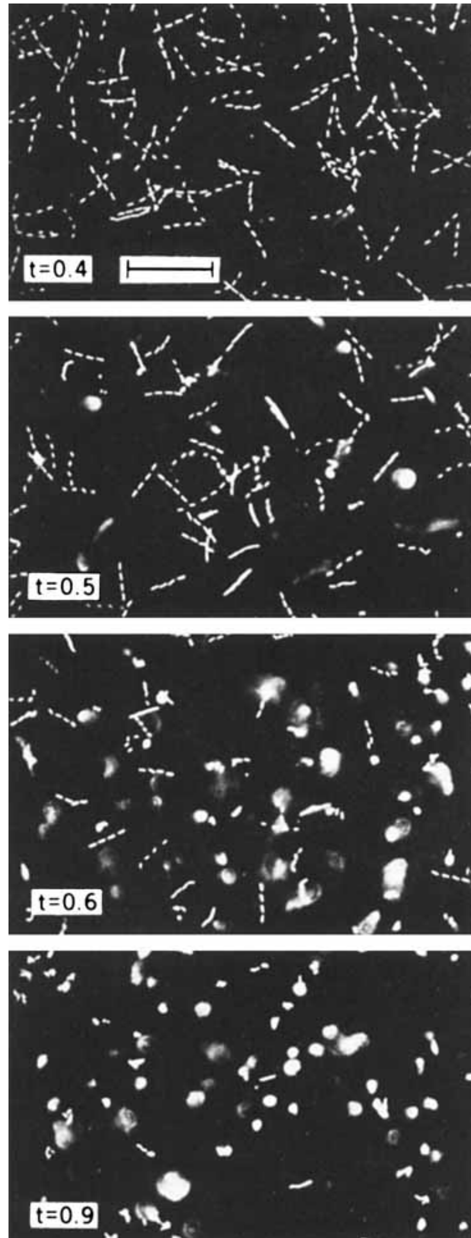

Fig. 1. Tumble frequency assay. The disappearance of tracks made by smooth-swimming bacteria after an L-serine temporal gradient stimulus (0 → 0.02 mM) is shown. A 0.9-ml aliquot of a suspension of ST171 (a constantly tumbling mutant) was rapidly mixed with 0.1 ml of attractant to yield a final concentration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

Pt. 2 – The Selection of General Nonchemotactic Mutants

Adler and his co-workers divided nonchemotactic mutants of bacteria into two types (8). Specific mutants involved alteration in the specific receptor molecules which bind the attractant. General mutants were found to be nonchemotactic for all attractants and hence presumably involved with transmission systems between the receptor and the flagella. Armstrong and Adler (9) studied 38 generally nonchemotactic mutants of Escherichia coli and found three complementation groups. Parkinson has extended these techniques and reported a fourth group (10). A generally nonchemotactic mutant of Salmonella typhimurium has recently been reported by Vary and Stocker (11).

In pursuing our studies on the transmission of information in the chemotactic system, we wished to isolate a number of nonchemotactic mutants in the Salmonella system. Since the methods published were laborious, a new procedure was developed by D. Aswad (12) which allowed us to separate nonchemotactic mutants from chemotactic wild types and nonmotile bacteria. The principle of the method is shown in Fig. 4. The bacteria were place in a test tube containing a preformed gradient of chemoattractant (serine) similar to the gradient used previously in our population migration apparatus (3). The gradient was devised in such a way that all the bacteria which respond to the chemoattractant would swim up the gradient to the top of the tube. Nonmotile bacteria would remain in the vicinity of the initial inoculum in the middle of the tube. The nonchemotactic bacteria which were motile but could not sense the direction of a gradient would theoretically swim in all directions and some would accumulate in the bottom of the tube.

The separation we obtained was even better than we had expected. The reason for the improved separation was found to be a phenomenon previously found in sperm: motile sperm tend to accumulate at the bottom of a tube (13). The migration of smooth-swimming bacteria to the bottom of the tube is not due to a simple gravity effect of settling to the bottom, since the nonmotile bacteria remained in the middle of the tube over the same interval. The most likely explanation is that the swimming bacteria assume an attitude in which the heavier body points slightly downward and they are then propelled by the flagella into a downward spiral towards the bottom of the tube. At any rate, the generally nonchemotactic bacteria were separated cleanly from the nonmotile and the chemotactic.

When the method was applied to a mutagenized culture of strain ST23, the wild-type Salmonella, 72 mutants were isolated which were completely motile but lacked the ability to sense a gradient of any attractant. Further selection of these bacteria have placed them in six complementation groups based on motility studies and preliminary mapping procedures. Hence, it now appears there may be six or more genes which are related to the transmission system. Most of these genes are in the flagella region and may be related to the final manner in which the signal is transmitted to the flagella. In some cases the genes may lie well outside the flagella region. The ability to select mutants with ease will certainly facilitate our biochemical studies of chemotaxis. An important feature of the method is that it is readily applicable to the selection of temperature-sensitive mutants.

Pt. 3 – The Effects of Light

The finding of mutants in a transmission system is not enough to determine the bio-

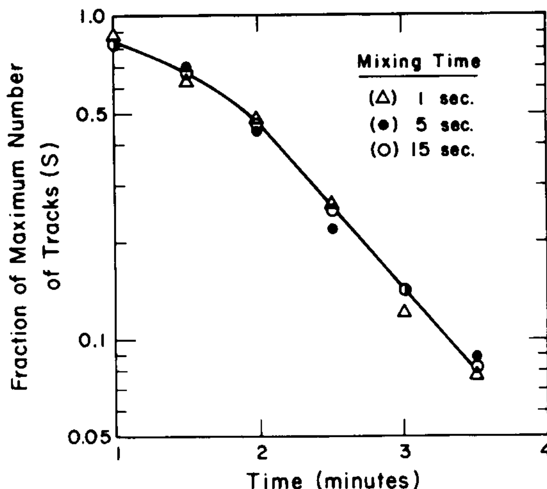

Fig. 2. Effect of mixing time on recovery curves. Serine (0.1 ml of 5 mM; gradient 0 → 0.5 mM) was delivered into 0.9 ml of a ST171 suspension with a micropipette over the period of time indicated, while the bacterial suspension was gently shaken. At 0.4 min after initiation of the stimulus, the bacteria 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 •.

chemistry of these mutations. Some clue must be obtained as to the nature of the biochemical events in order to identify the role of the proteins which are shown to undergo mutations. One of our most valuable insights into this transmission process has been through the effects of light on the tumbling responses of S. typhimurium and E. coli. Initially, the observation was made by R. Macnab (14) that free-swimming bacteria can be caused to tumble by exposure to intense light and that the tumbling response to light had the action spectrum of a flavin. Further studies of this phenomenon have now revealed three separate and interesting light effects: (a) an initial increase in tumbling caused by short exposure to light; (b) a smooth response caused by intermediate exposure to light; and (c) a paralysis or "killing effect" caused by long exposure to light.

The high-intensity light source used in these investigations was a 70-W xenon lamp (Osram XBO75W). To protect the bacteria from the effect of intense blue light, a long-pass orange filter (Corning 3-69, 50% cutoff at 530 nm) was inserted between the light source and the condensor. The influence of light on bacteria was recorded either by visual observation in which descriptions of the bacterial motion were recorded on audiotape and replayed later, or by use of videotape. By the use of a silicon diode tube the video camera was able to accomodate a wide range of light intensities.

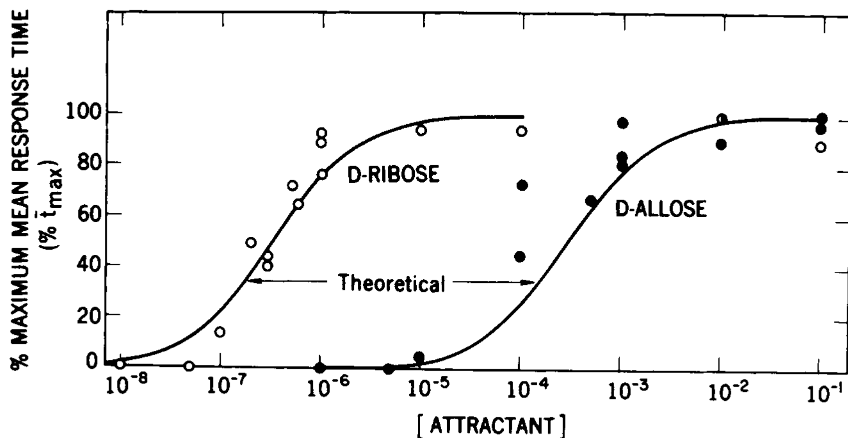

Fig. 3. Comparison of response with receptor occupancy. Points are percent maximum mean recovery times. ($\bar{t}_R$) for D-ribose and D-allose concentration increases from 0 to the concentration shown on the abscissa. Each point represents the average of three consecutive assays. Results are for three experiments performed on different days. $t_{R_{max}}$ 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 (1) noncooperative chemoreceptor binding constants of 3.3×10^{-7} for ribose and 3.0×10^{-4} for allose (as determined in vitro) and (2) the response $\bar{t}_R$ is proportional to the change in fraction of binding protein occupied. The symbol ◑ represents coincidence of the points ○ and ●.

When the long-pass filter is placed in the light path the S. typhimurium have a normal motility pattern consisting of both runs and tumbles. When the filter is removed from the light path the bacteria tumble continuously, but if the exposure is limited to a few seconds the bacteria rapidly resume a normal motility pattern after the filter is reinserted. Macnab and Koshland (14) observed that when the filter remained out for a longer time S. typhimurium continued to tumble until they became immotile. We have confirmed this paralyzing effect of light but have also observed that the cells usually become smooth swimming before they are paralyzed. The time elapsed before exposed bacteria became first smooth swimming and then paralyzed was determined by tracking individual bacteria (Table I). In different experiments the mean times ranged from 5 to 16 s for smooth swimming and 27 to 55 s for paralysis, with different cultures giving different values. The pattern was always similar to Table I and the timing of the response was not markedly changed by the growth phase of the culture. Smooth swimming and paralysis are not the result of damage to the cells by UV light. Most of the UV rays from the xenon lamp were filtered out by the glass optics of the microscope. Moreover, a long-pass filter that absorbed wavelengths of less than 390 nm caused no significant change in the photoresponses of S. typhimurium (Table I). On the other hand, a band-pass filter cutting out all wavelengths below 530 nm blocked all three photoresponses. Thus, the wavelengths inducing the smooth-swimming response and paralysis are between 390 and 530 nm, which are the wavelengths most effective in generating the tumbling response.

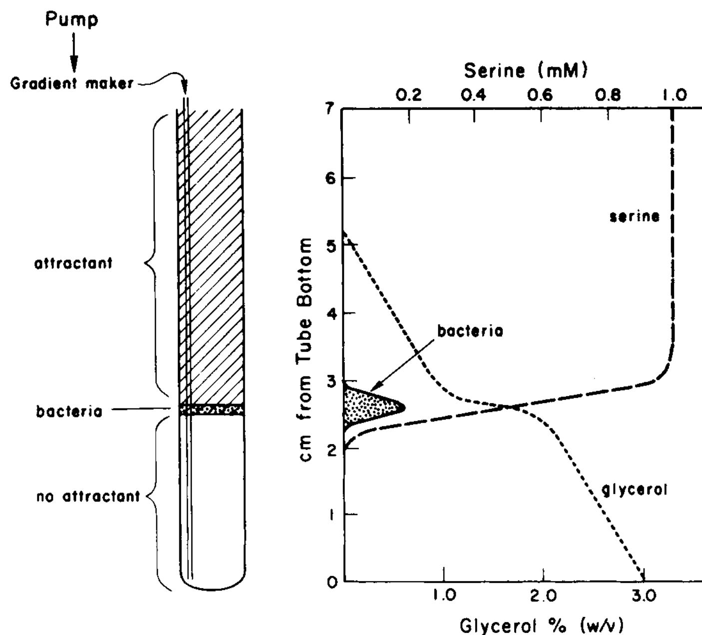

Fig. 4. Preformed liquid gradient used for isolation of chemotaxis mutants. The drawing on the left indicates how the gradient was constructed. The drawing on the right shows the distribution of the selection tube contents at the beginning of the enrichment period. All solutions were made up in minimal medium and pumped through a stainless steel inlet pipe (20 gauge hypodermic needle) which extended to the bottom of the 18 × 150-mm glass tube. First, 2.0 ml of 1.0 mM serine (attractant) was pumped in, followed by 2.5 ml of 1.0 mM serine which contained glycerol. The glycerol concentration was initially 0% wt/vol and increased linearly to 1.0% at the end of the 2.4 ml pumping segment. Next. 0.2 ml of bacteria (4 × 10^7 bacteria per ml in 0.5 ml of a solution of 0.5 mM serine and 1.5% glycerol) was pumped in, followed by 2.5 ml of glycerol which increased linearly from 2.0 to 3.0%. The glycerol gradient was necessary to stabilize the attractant gradient against convection.

TABLE I. Effect of Prolonged Illumination on Salmonella typhimurium ST1

Wavelength	Initial response	Time (s) to become (A) smooth swimming	Time (s) to become (B) paralyzed
>290	constant tumbling	5.3 ± 0.4	30.7 ± 4.1
>390	constant tumbling	5.8 ± 0.4	27.1 ± 3.1
>530	normal motility	no change	no change

Studies with the photoreducible dyes such as proflavine and riboflavin were able to increase the sensitivity of S. typhimurium to light, and studies with these receptors revealed that all three of the light effects shown above could be obtained also by irradiation of externally added dyes. Since the responses in both the presence and absence of added dyes undoubtedly operate through photosensitive pigments, it will be convenient to use

the words "intrinsic" and "extrinsic" to distinguish between them. Intrinsic will therefore refer to the response observed by Macnab and Koshland (14) in which some natural pigment within the bacterium absorbs light and generates tumbling. The extrinsic effect refers to the classical "photodynamic effect" (15) in which a dye added to the medium generates tumbling (15).

In the presence of 10^{-6} M proflavine, exponentially growing S. typhimurium were able to respond to light from a 15-W tungsten lamp (intensity 1.8×10^{-2} joules cm^{-2} s^{-1}). Stationary phase cells required considerably higher concentrations of proflavine (10^{-4} M) before they responded to light from the tungsten lamp, even though the sensitivity of the intrinsic light effect was similar for cells from stationary and exponential phases. With these concentrations it was possible to show that short exposures of light caused initial tumbling, that longer exposures caused the smooth-swimming response, and that even longer exposures caused the paralyzing effect.

In view of the effect of photosensitizing dyes, the possibility was considered that the intrinsic light responses were an artifact resulting from a contaminating dye in the medium. This was shown not to be the case by several avenues of investigation. One of these was repeated washing to eliminate any dyes in the medium. This was shown to have no influence on the intrinsic light response. The second utilized competitors such as histidine which eliminated the extrinsic effect without eliminating the intrinsic light effect described above. The third avenue of investigation examined the light response in an anaerobic environment which inhibited extrinsic but not intrinsic responses. Thus, it was clear that the effect observed by irradiating the native bacteria was due to the absorption of light by some photoreceptor which is made endogenously by the bacteria. It was clearly different from the extrinsic or photodynamic effect in which extraneous dyes were added to the medium in which the bacteria were suspended. Nevertheless, further study indicated that these two effects had many features in common.

The tumbling and smooth-swimming intrinsic light responses were shown to be closely related to the chemotactic machinery of the cell. In the first place, the chemotactic mutants isolated by the procedure described above did not show light-induced tumbling. (All cells were eventually paralyzed by light.) Moreover there is an intimate and logical relationship between temporal gradients and the light response. When bacteria are subjected to a concentration jump from 0 to 1 mM serine (a strong attractant which suppresses smooth swimming for several minutes) the bacteria showed no light effect during the initial phases of this interval. The bacteria stimulated by serine continue to swim smoothly at an intensity of light which causes tumbling to the unstimulated bacteria. However, if the light stimulus is given late in the smooth-swimming response, just before the cells are returning to the normal tumbling pattern, the light generates a tumbling response. Thus, it would seem that the light-induced tumbling is related to the level of the tumble generator. The light is capable of altering the tumble generator level to the threshold for tumbling when the level is close to the threshold but not when it has been displaced greatly. Similar experiments with added proflavine indicate that the extrinsic light response also operates on the tumble-generating mechanism. The light response from the extrinsic effect also is not seen in the nonchemotactic mutant and could be overcome by strong temporal gradients of attractants. Similar observations have been made by H. Berg for the extrinsic light effect on E. coli.

TABLE II. Response of Salmonella typhimurium to Blue and Infrared Light

Effective wavelengths	Temporal serine gradient	Response to change in light intensity	
		(a) on	(b) off
Blue (8 <530 nm)	uniform	tumbling	normal
	increasing	smooth	smooth
Infrared (>700 nm)	uniform	smooth	tumbling
	increasing	smooth	smooth

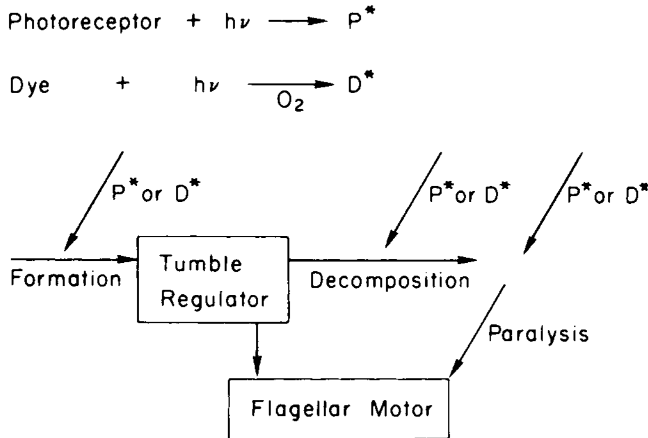

Fig. 5. Possible mechanism of blue light effects in Salmonella typhimurium. Double arrows indicate that one or more intermediate steps may be involved. The primary reaction with light activates a photoreceptor either via an endogenous light acceptor (P*) or an externally added dye (D*). The photoreceptor can then modify (a) a molecule depleting the supply of tumble generator, (b) a second molecule increasing the supply of tumble generator, or (c) destroy molecules which cause paralysis.

Once the initial light quantum has been received by the photoreceptor, it would appear that there are three different pathways for the tumbling, smooth-swimming, and paralysis effects. In the first place, the time sequence indicates intermediate steps. Studies on the mechanism of the photodynamic action indicate that photosensitive dyes such as proflavine are activiated by light to a triplet state which decays rapidly (16). However, in the presence of proflavine, S. typhimurium ST1 continues to tumble for up to 12 s after exposure to a brief pulse of light, indicating a species considerably more stable than a

triplet state. Similarly, the reversal of the intrinsic response in E. coli may require up to 2 s after illumination ceases. Second, the effect of a temporal gradient of attractant is not the same for all effects. A sudden sharp temporal gradient of serine can prevent the initial generation of tumbling, but it does not alter the time required to induce paralysis. Hence, it would seem that at least these two effects are operating through different intermediates. Finally, the timing of the responses indicates three separate pathways. The tumbling response occurs very soon after exposure to light and in most cases is rapidly reversed at the end of the exposure. Light-induced smooth swimming requires longer intervals to initiate and longer intervals for reversal, a recovery time of 45.4 s being observed for ST171. Paralysis requires the longest exposure and is irreversible.

An attractive hypothesis (shown in Fig. 5) is to consider formation of a more stable species as a point of convergence for the extrinsic and intrinsic responses. For example, the intrinsic light effect might operate through the activation of a flavin molecule which photo-oxidizes another molecule leading to the response. When an extrinsic dye is added a small amount leaks into the membrane and as a result of light and oxygen, photo-oxidizes the same molecule. In this scheme the initial photoreception will be different for the extrinsic and intrinsic light effects, but the pathways for the three different responses would be common.

The initially activated photoreceptors would presumably act on three different molecules, one of which would generate more rapid tumbling, another of which would generate less tumbling, and a third which would eventually induce paralysis.

That the light-induced effect directly perturbs the tumble generator and the chemotactic system seems clear. The two short-term effects are immediately transmitted so that an alternation of the tumbling frequency occurs but the effects are reversible. Moreover che I and che II mutants which have disturbed apparatuses for responding to gradients of attractants do not exhibit the light response. Obviously, this light effect is complex since it perturbs the chemotactic machinery in opposite directions. Initially there is an increase in tumbling and this is followed by a decrease in the tumbling rate on longer exposure to light. Thus, light cannot simplistically destroy the tumble regulator itself, but must be involved either in the enzymes that are forming and degrading this tumble regulator or on some low molecular weight compounds which affect the levels of the tumble regulator. Since the action spectra and proteins involved in such light effects can be pursued, this light effect provides an avenue for further understanding of the sensing system.

A response to infrared light that is opposite in effect to the tumbling response to blue light has recently been detected in S. typhimurium and E. coli and is shown in Fig. 5. When these bacteria are exposed to infrared light they show a short smooth-swimming response and when exposure to infrared light is terminated the bacteria tumble continuously for 1–4 s. It is possible that the infrared response is a manifestation of thermotaxis and this is being investigated. What is clear is that the infrared stimulus feeds into the same response system that is involved in chemotaxis and the blue light response. Cells swimming smoothly in response to an attractant gradient do not tumble on cessation of infrared illumination. Similarly, general chemotactic mutants that are detected in the apparatuses for responding to chemical gradients do not tumble in response to cessation of infrared illumination. In view of these results studies of the mechanism of the infrared light effect may also provide insight into the mechanism of chemotaxis.

In summary, therefore, the chemotactic system of bacteria has become a system in which certain features are clearly discernible. There is an initial receptor which binds attractant in which the purified protein appears to have specificity and binding affinity identical to those of the protein in the living bacterium. The signal that is transmitted to the flagella operates through a general transmission system which can be perturbed chemically and genetically. Selection for genetic mutants has revealed six probable gene locations and possibly more will be found. The transmission system of the wild type can also be perturbed by light intensities which alter the levels of the tumble regulator. Some features of this tumble regulator are now discernible, but the precise manner in which it operates must remain the subject of future study.

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