zyme activities in fetal and neonatal tissues has been studied (*16*), little is known about the development of plasma peptidases or proteases. However, plasma renin activity in sheep (*17*) and plasma vasopressinase activity in monkeys (*18*) are reported to be higher in fetal and newborn animals than in maternal or nonpregnant animals. In addition, the proteases involved in clotting activity are also present in the plasma of newborn mammals. In view of the possible neurotropic functions of TRH, it will be of interest to determine the time of appearance of plasma TRH peptidase activity and to compare it with the development of the central nervous system of the rat, most of which occurs after birth (*19*).

In regard to a possible physiological function of TRH degradation in plasma, TRH travels from the hypothalamus to the pituitary via the hypophyseal portal vessel (*20*), and hypophyseal portal blood inactivates TRH (*8*). Plasma inactivation of TRH could be one means of controlling the activity of TRH at the level of the pituitary or at other as yet unknown tissue sites. The lack of an active TRH degradation system in neonatal rats and the later development of an active degradation system suggests some physiological function for degradation of TRH by plasma. The specificity of the TRH peptidase is not known, but a high degree of specificity would lend further weight to a physiological role for the plasma inactivation of TRH. The role of plasma peptidases in controlling the activity of polypeptide hormones warrants further study.

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Mg^{2+},Ca^{2+}-Dependent Adenosine Triphosphatase as Receptor for Divalent Cations in Bacterial Sensing

Abstract. *The Mg^{2+},Ca^{2+}-dependent adenosine triphosphatase responsible for interconversion of the energized membrane state to adenosine triphosphate is the receptor for divalent metal ions in bacterial chemotaxis. The receptors controlling bacterial behavior show a common pattern of dual functions.*

Bacteria can respond to chemical stimuli by means of a prototypic sensory system. This system comprises a number of distinct chemoreceptor proteins, a mechanism to analyze and transmit chemical information, and a motor response requiring energy transduction (*1, 2*). Although it is contained in a single cell, the bacterial system is analogous in many ways to those of higher organisms.

In cells of higher species electrochemical gradients play a key role in the propagation of action potentials; the receptor-chemoeffector complex which is formed ultimately signals a depolarization involving massive changes in cation gradients (*3*). In lower species such as paramecium, ciliary activity is controlled by Ca^{2+} gradients (*4*).

Cation gradients have also been implicated in energy transduction processes in microorganisms and higher species. A central role for proton and cation gradients is proposed in the chemiosmotic hypothesis of Mitchell (*5*). Kashket and Wilson (*6*) have correlated the transport of sugars with proton gradients in artificial membranes and in intact bacteria. Racker and Stoeckenius (*6*) have shown that a light-induced proton gradient in *Halobacterium halobium* can generate adenosine triphosphate (ATP) in a reconstructed membrane system (*7*). Alternative approaches of Boyer (*8*), Ernster (*9*), and others also invoke an energized membrane state, but differ in the way it is generated. A central role in the conversion of the energized membrane state to ATP molecules in bacteria is played by the membrane-bound Mg^{2+},Ca^{2+}-dependent adenosine triphosphatase (Mg^{2+},Ca^{2+}-ATPase) protein, which is extensive and highly conserved throughout all living systems (*10*). This protein is involved in the synthesis of ATP in the final steps of oxidative phosphorylation (*11*), energy-dependent transhydrogenation (*12*), and the coupling of the hydrolysis of ATP to the transport of certain amino acids and sugars (*13*). The role of the ATPase is of particular interest since Larsen *et al.* (*14*) have established that the energized state is required for motility and that ATP is required for chemotaxis.

Two key questions can be asked in relation to bacterial chemotaxis: The first is whether the general chemotactic signal is generated by a pure Nernst-type electrochemical gradient across the membrane, or whether it operates through a membrane-bound protein. The second is whether a cation gradient is a general means of signal transmission from recep-

tor to flagella. Initial results by R. Macnab, in our laboratory, had indicated a response of bacteria to Mg^{2+}. This work was designed to throw light upon these questions.

Strains used in this study are ST171, a constantly tumbling mutant derived from *Salmonella typhimurium* LT2 (*15*); AN120, an *Escherichia coli* K12 *uncA* mutant, and AN180, its isogenic parent (*11*); SW46 and BG-31, two other *E. coli unc* mutants, and their parent, 1100 (*16*). Bacterial cultures were grown at 30°C on a New Brunswick gyratory shaker in Vogel-Bonner citrate medium (VBC) supplemented with histidine and thymine in the case of ST171, and with 0.3 percent glycerol, 20 m*M* sodium nitrate, arginine, and thymine in the cases of AN120 and AN180. Bacteria were grown to early logarithmic phase (10^8 cells per milliliter), centrifuged at 0°C for 15 minutes at 3000*g*, resuspended in either VBC lacking Mg^{2+}, or 10 m*M* imidazole buffer, *p*H 7.0 (selected for its high solubility for divalent cations), and stored on ice. From this suspension, bacteria were diluted to 4×10^7 cells per milliliter and incubated for 15 minutes at 30°C on a gyratory shaker. Recovery times from temporal gradients of added attractants and salts were determined by the quantitative assay for chemotaxis described by Spudich and Koshland (*17*). The use of a tumbly mutant did not affect the qualitative conclusions of the study and made the quantitation simpler. Checks with wild-type bacteria were performed to establish that the mutant properties did not alter the response relationships to cations.

Bacteria control their direction of migration by suppressing tumbling while traveling in a favorable direction and increasing tumbling while traveling in an unfavorable direction (*18, 19*). This is achieved by a temporal sensing system which was demonstrated by subjecting the bacteria to a rapid increase in attractant or repellent concentrations and then observing the relaxation of the tumbling response until motility returned to normal (*18, 20*). The recovery times so observed can be used as qualitative or quantitative measures of the sensory response (*17, 20*).

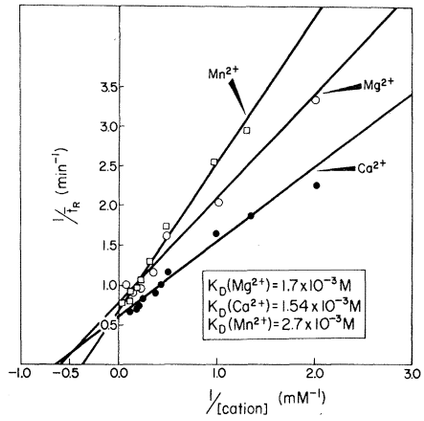

Fig. 1. Dose-response plots for Mg^{2+}, Ca^{2+}, and Mn^{2+} taxis in *S. typhimurium*. Data are shown for ST171, a *cheT* uncoordinated mutant, in VBC medium lacking Mg^{2+}, *p*H 7.0, at 30°C; $\bar{t}_R$, average recovery time; K_D, dissociation constant.

The response times of a tumbling mutant of *S. typhimurium*, subjected to temporal gradients of some representative cations and some known attractants, are shown in Table 1. Suppression of tumbling is caused by increases in concentrations of Mg^{2+} and Ca^{2+} and also to Zn^{2+}, Mn^{2+}, and Co^{2+}. Varying the anion for a given salt resulted in no change in the observed recovery time, a result which indicates that the bacteria are responding specifically to the divalent cation added. Weak responses were observed at high concentrations (100 m*M*) of K^+ but not Na^+. The absence of an effect of Na^+ indicates that the positive results in the case of the divalent cations are not caused by a generalized ionic strength effect. Moreover, the normal swimming pattern observed after preincubation in various Mg^{2+} concentrations in the range 0 to 10.0 m*M* shows that the bacteria respond to changes in divalent cation concentrations, rather than absolute values. Wild-type *E. coli* K12 (Table 1), gave essentially the same pattern of responses as *S. typhimurium*.

The suggestion of a saturation effect was examined by studying the recovery times of the intact bacteria over a concentration range of metal ion gradients and analyzing the results by double reciprocal plots. As can be seen in Fig. 1, the data fall on straight lines that indicate a Michaelis-Menten binding for the metal to a receptor. The binding constants are calculated by assuming the recovery time is proportional to the change in receptor occupancy, as shown for the attractants ribose and allose (*17*). Thus the dissociation constants for metal binding can be determined. The resulting values for K_D were 1.54 m*M* for Ca^{2+}, 1.7 m*M* for Mg^{2+}, 2.0 m*M* for Zn^{2+}, 2.7 m*M* for Mn^{2+}, and 14 m*M* for Co^{2+}. The demonstration of distinct saturation curves and a specificity pattern indicates that the metal ions operate through binding to a protein receptor molecule and are not acting solely as a membrane gradient, in which the response would be simply a function of the difference between external and internal cation concentrations.

The response maxima for different metals measured for the intact bacteria and their variation with *p*H are similar to those reported for the purified F1 portion of the membrane-bound Mg^{2+},Ca^{2+}-ATPase protein complex from *E. coli* (*21*) and checked by us for the purified *Salmonella* F1 protein. The possibility that this ATPase was the receptor protein was therefore investigated. The *E. coli uncA* mutant AN120, lacking the Mg^{2+},Ca^{2+}-ATPase activity (*11*) but exhibiting normal Mg^{2+} transport (*22*), was tested for the divalent cation response (Table 1). The mutant, grown in VBC with EDTA (ethylenedinitrilotetraacetic acid) and glycerol and resuspended in VBC lacking Mg^{2+} or in 10 m*M* sodium phosphate

Table 1. Response of *S. typhimurium* ST171 to temporal gradients of mono- and divalent cations. Bacteria were grown in Vogel-Bonner citrate medium (VBC) supplemented with L-histidine (50 μg/ml) and thymine (30 μg/ml) for ST171 and with arginine (50 μg/ml) and thiamine hydrochloride (10^{-4}*M*) for AN120 at 30°C and harvested by centrifugation at early logarithmic growth (10^8 cells per milliliter). The bacterial pellet was resuspended in VBC (*p*H 7.0) lacking Mg^{2+} or in 10 m*M* sodium phosphate buffer (*p*H 7.0) containing 10^{-4}*M* EDTA. All measurements were made at 30°C; ND, not done.

Chemical and gradient (m*M*)	Recovery time (minutes)				
	Tumbly mutant of *S. typhimurium* (ST171)		Wild-type *E. coli* (K12)	ATPase mutant of *E. coli* (AN120)	ATPase mutant of *E. coli* (SW46)
	Control	+DCCD			
$MgCl_2$ (0 → 10)	1.03	<0.2	1.2	<0.2	<0.2
$CaCl_2$ (0 → 10)	1.45	<0.2	1.5	<0.2	<0.2
$ZnCl_2$ (0 → 5)	1.9	<0.2	0.8	<0.2	<0.2
NaCl (0 → 100)	<0.2	ND	<0.2	ND	ND
KCl (0 → 100)	0.55	ND	0.6	ND	ND
L-Serine (0 → 0.1)	1.2	1.3	4.2	4.1	4.4
Ribose (0 → 1)	0.8	0.7	1.0	0.5	0.55
$+Mg^{2+}$ (0 → 10)	0.8	0.8	1.0	0.8	0.8
Galactose (0 → 1)	<0.2	<0.2	1.1	0.6	0.5
$+Mg^{2+}$ (0 → 10)	<0.2	<0.2	1.1	1.2	1.2

buffer, *p*H 7.0, containing $10^{-4}M$ EDTA exhibits chemotaxis toward serine, ribose, and galactose but does not respond to Mg^{2+} or Ca^{2+}. The parent strain, AN180 (which has wild-type ATPase activity), responds to serine, these sugars, and divalent cations with the same recovery times as wild-type *E. coli* K12. Similarly, a second ATPase-deficient strain, SW46, lacks the response to cations (Table 1); this strain exhibits the same recovery times for cations, amino acids, and sugars as does AN120. The parent strain of SW46, *E. coli* 1100, does respond to all divalent cations tested with the same recovery times as K12.

The divalent cation response was also tested on *E. coli unc* mutant BG-31, isolated by Simoni and Shandell (*16*). Mutant cells of this strain are unable to couple the energy derived from the hydrolysis of ATP to the active transport of proline or to transhydrogenase activity, but exhibit normal levels of Mg^{2+},Ca^{2+}-ATPase activity. This strain responds to all amino acids, sugars, and divalent cations tested with the same recovery times as wild-type K12, a result consistent with the presence of ATPase activity in this bacterium. Thus, in all five *E. coli* strains studied, there is a positive correlation between the presence of ATPase enzymatic activity and divalent cation taxis.

Finally, metal taxis was tested in *S. typhimurium* ST171 in the presence of *N*,*N'*-dicyclohexylcarbodiimide (DCCD) (Table 1), a potent and highly selective inhibitor of ATPase activity in the bacterial membrane-bound ATPase protein (*23*). In the presence of $10^{-4}M$ DCCD, a concentration of the inhibitor which completely eliminates ATPase activity in intact bacteria, ST171 showed no response upon additions of 10 m*M* $MgSO_4$ or 10 m*M* $CaCl_2$, whereas under these same conditions the bacteria exhibited a normal response to serine and a weak response to ribose.

These results present strong prima facie evidence that the Mg^{2+},Ca^{2+}-ATPase or a portion of it is the chemotaxis receptor for the divalent metal ions. Considerable evidence from studies with antibodies to the soluble F1 portion of the ATPase complex indicates that the F1 component, which catalyzes the phosphohydrolase reaction and binds the metals, is on the inside of the cytoplasmic membrane (*24*). Direct interaction between divalent cations such as Mg^{2+} and the ATPase protein could occur in either of two ways: (i) the cations interact with some small fraction of the F1 which might be exposed on the outer surface of the membrane, or (ii) there exists some mechanism by which the cations or the F1 oligomer, or one of its constituents, can transverse the membrane. Two systems for the transport of magnesium have now been identified, both with Michaelis constants of 15 to 50 μM (*25*, *26*). Both of these systems would be saturated at Mg^{2+} concentrations well below 1 m*M*. However, recent experiments (*26*) show that uptake of Mg^{2+} in the millimolar range occurs by passive diffusion. Such a mechanism could account for the observed Mg^{2+} taxis, with the ATPase as the chemoreceptor.

The special role of metal ions raises the question as to whether the metal ion stimulus is part of a common signaling system. A test of additivity between responses was studied (data not shown). The quantitative responses to three different stimuli, serine, aspartate, and ribose, which are known to bind to three different chemoreceptors, were studied independently and in conjunction with the Mg^{2+} stimulus. The recovery times were algebraically additive. Thus, the signal from cations feeds into the central response system just as do the signals for sugar and amino acid attractants and repellents (*20*, *27*).

To establish that the cation responses interact through a protein receptor mechanism, competition studies based on the constants determined from Fig. 1 were pursued. High saturating levels of a divalent metal such as Mg^{2+} or Ca^{2+} were added to the preincubation medium, and then temporal gradients of other metal ions were administered to the bacteria. In all cases the gradient response was obliterated, just as would be expected if the two metal ions were competing for the same site. Sodium chloride does not inhibit the response at concentrations up to 50 m*M* and KCl diminishes the response by only 30 percent at 20 m*M*.

These experiments answer several questions in regard to the role of cations in bacterial behavior. They establish that the Mg^{2+}-Ca^{2+} effect operates through a protein receptor in the same way that ribose gradients modify the behavior of bacteria by operating through the ribose receptor. The form of the function leading to saturation is not that expected from a simple Nernst-type gradient. The fact that the signal from the Mg^{2+} gradient is additive with signals from other receptors, moreover, indicates that the signals feed into a common system that is processed and ultimately affects flagellar function. The lack of a substantial effect of K^+ and Na^+ gradients either on the Mg^{2+} or Ca^{2+} gradient, or on the behavior of the bacterium, indicates that these cations are not directly involved in the sensing function of the bacteria. Another cation gradient, the proton gradient, has been implicated in the mediation of repellent signals in the case of *Bacillus subtilis* by Ordal and Goldman (*28*).

The second question as to whether the Mg^{2+},Ca^{2+}-ATPase is involved in a central processing signal after the initial chemoeffector receptor signal is also answered by these studies. The mutant lacking the Mg^{2+},Ca^{2+}-ATPase responded to other attractants such as serine, ribose, and galactose. If the enzyme were the central source of processing for these other signals, altered behavior would be observed. Nonchemotactic mutants that destroy the ability of the organism to transmit information from receptor to flagella are known, and that type of behavior would be expected if the central transmission system were blocked.

The Mg^{2+},Ca^{2+}-ATPase, a central protein in the conversion of energy in the bacterium, is utilized as a chemoreceptor. On reexamination of previously identified chemoreceptors, each is seen to have a dual function. The largest single group appears to be the periplasmic proteins and some membrane-bound proteins that serve as proteins for transport systems and for chemotaxis. Adler and Epstein (*29*) identified enzyme II of the phosphotransferase system of Kundig and Roseman (*30*) as the chemoreceptor for glucose taxis in *E. coli*. We have identified a protein in the Mg^{2+},Ca^{2+}-ATPase complex as the Mg^{2+} receptor. It might be argued that there is economy in energy by producing a protein for two purposes. Yet nature is profligate in the synthesis of proteins or their degradation if these add to control properties of an organism. It is unlikely that economy in protein synthesis could be a justification. Rather it would seem indicative of the way in which sensing systems evolved; that is, that modification of an existing function is the beginning of a sensing process. Some compounds are needed for the nutrition and metabolism of the organism and hence are transported across the cell membrane. The Mg^{2+} and Ca^{2+} ions are needed for many enzymatic processes. A modification that improves the response of the organism to these influences would give it survival value over other organisms that could not respond to these conditions. The important step achieved was not that increased Mg^{2+} concentrations were more favorable to the organism, but that the organism developed a process by which it could sense a gradient of Mg^{2+} and modify its

behavior accordingly. It appears therefore that a sensing function becomes attached to a function which has survival value, and the sensing function is then processed through a central system to affect behavior of the organism.

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Phosphoribosylpyrophosphate Synthetase Is Elevated in Fibroblasts from Patients with the Lesch-Nyhan Syndrome

Abstract. *In subconfluent cultures of fibroblasts from patients with complete or partial deficiencies of hypoxanthine-guanine phosphoribosyltransferase, phosphoribosylpyrophosphate synthetase activity is elevated. The abnormally high catalytic activity of the synthetase appears to account for the overproduction of purines by the cultured mutant cells and presumably for that by the patients.*

A deficiency of the catalytic activity of the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), has been described in tissues and cultured cells from patients with the Lesch-Nyhan syndrome, a heritable syndrome of cerebral palsy, self-mutilation, and hyperuricosuria (*1, 2*).

The variable clinical and biochemical descriptions of HGPRT deficiencies in man (*3, 4*) include one consistent finding—the excessive urinary excretion of uric acid, a result of overactive de novo purine biosynthesis (*4–6*).

The biochemical basis of purine overproduction in human HGPRT deficiencies appears to be the increased intracellular concentrations of phosphoribosylpyrophosphate (PRPP) (*7, 8*), which serves as both a substrate and allosteric regulator (*9*) of de novo purine synthesis. The increased cellular concentration of PRPP has been ascribed to decreased utilization by HGPRT, the deficient purine salvage enzyme that has been considered to be a major intracellular consumer of PRPP. However, males with partial deficiencies of HGPRT enzyme activity overproduce purines and uric acid to extents (on the basis of body mass) equal to or even greater than many patients with the complete enzyme deficiency (*3, 5*). Thus, there is not a strong correlation between the degree of deficiency of HGPRT in the erythrocytes and the degree of purine overproduction.

We have observed increases of apparently normal PRPP synthetase enzyme in cultured rat hepatoma (HTC) cells with chemically induced HGPRT deficiency (*10*). We proposed that the enhanced rate of de novo purine synthesis in these mutant cells was due to increased PRPP synthetase activity rather than to the loss of activity of the HGPRT and any reduced PRPP utilization (*10*).

Our study was undertaken to determine whether mechanisms similar to those in mutant rat hepatoma cells might be responsible for the purine overproduction in cultured fibroblasts from patients completely or partially deficient in HGPRT activities. We demonstrate that the PRPP synthetase activity is greater in the mutant than in normal fibroblasts. This abnormality is present when the cultures are subconfluent but not when confluent. The rates of de novo purine synthesis measured during the growth phases of the cells change in parallel with the levels of PRPP synthetase activity. Furthermore, immunotitration data from two mutant cell cultures suggest that the increase of PRPP synthetase activity results from increased numbers of PRPP synthetase molecules.

The specific catalytic activities of PRPP synthetase were significantly elevated in gel-filtered extracts of cultured fibroblasts from patients with the Lesch-Nyhan syndrome when compared to those from normal young males (Fig. 1). In experiments in which cells were harvested 4 and 5 days after subculture, seven of the eight cultures from affected individuals had significantly greater PRPP synthetase than cultures from the matched controls (Fig. 1). The fibroblasts from patient S.S. were reexamined in a growth cycle experiment (see below) and were not exceptional. The levels of adenosine kinase, another soluble cytoplasmic enzyme, were the same in both the mutant and control cells.

However, further investigation indicated that the measurable levels of PRPP synthetase activity fluctuated in both mutant and normal fibroblasts and that the elevations in the mutant fibroblasts were not consistent. The previously described induction of PRPP synthetase during the activation of quiescent mouse spleen lymphocytes (*11*) suggested that the harvesting of fibroblasts at different stages of growth might be responsible for these fluctuations.

In three experiments we harvested and assayed mutant and control cultures simultaneously at three or four different stages of growth, where each stage was a fixed period after subculturing (by trypsinization and dilution) (Figs. 2 and 3). The consistent differences of activity in the examined mutant cells and that of their respective controls occurred between 2 and 5 days after dilution. However, the specific catalytic activities of the next enzyme in the sequence of the de novo purine pathway, PRPP glutamyl amidotransferase, varied by less than 25 percent both between mutant and normal cells and between cells harvested throughout the growth cycle. The rates of de novo purine synthesis were examined at various times throughout the growth cycle and compared to the rates in normal fibroblasts at similar times.