Mitchell, P. (1966), *Biol. Rev. 41*, 41.
Mitchell, P., and Moyle, J. (1967), *Nature (London) 213*, 137.
Panet, R., and Selinger, Z. (1972), *Biochim. Biophys. Acta 255*, 34.
Pressman, B. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 1898.
Racker, E. (1972), *J. Biol. Chem. 247*, 8198.
Scarpa, A., and Inesi, G. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 22*, 273.
Yamamoto, T., and Tonomura, Y. (1967), *J. Biochem. (Tokyo) 62*, 558.

Chlorotetracycline as a Fluorescent Probe for Membrane Events in the Action of Colicin K on *Escherichia coli* †

Gregory J. Brewer‡

ABSTRACT: The mechanism of action of the protein antibiotic, colicin K, was investigated with a fluorescent probe, chlorotetracycline (CT). *Escherichia coli* cells when added to medium containing CT cause an enhancement of system fluorescence. Subsequent addition of colicin K causes a twofold increase in fluorescence. This fluorescence effect of colicin K is manifested concurrently with loss of activity of the cellular galactoside transport system and with cell death. Studies with metabolic inhibitors indicate that some form of energized membrane state is necessary for colicin to act. With lactate as carbon source, anoxia prevents colicin action. In the presence of oxygen and lactate, CT fluorescence caused by colicin K is reversible by anoxia while loss of cell viability is irreversible. Characterization of the fluorescence response indicates dependence upon magnesium. Use of [^{3}H]tetracycline as a tracer shows that the colicin-stimulated fluorescence results from an increased cellular uptake of probe from the medium rather than from an increased quantum yield. Fluorescence anisotropy studies suggest that, in the absence of colicin, CT is associated with the membrane. Colicin K causes CT to fluoresce from a more fluid environment, presumably the cytoplasm of the cell. A model is presented that interprets the findings on CT fluorescence with and without colicin in terms of interactions between CT, membrane, and magnesium ions.

Colicin K, a protein antibiotic, is capable of interacting with sensitive *Escherichia coli* with kinetics consistent with a single molecule per cell being sufficient to cause death as measured by inability to form colonies. Other effects are inhibition of macromolecular syntheses [DNA, RNA, protein, polysaccharide, and lipid (Jacob *et al.*, 1952; Nomura, 1963; Nomura and Maeda, 1965)], leakiness to internal potassium (Nomura and Maeda, 1965; Hirata *et al.*, 1969; Feingold, 1970; Wendt, 1970; Dandeu *et al.*, 1969), inability to accumulate amino acids and galactosides (Fields and Luria, 1969a), and a lowering of ATP levels (Fields and Luria, 1969b). These physiological changes caused by colicin point to a generalized structural change in the membrane of the cell. Cell morphology is not noticeably changed and the cells retain their ability to concentrate substances that are transported by the phosphoenolpyruvate system (Fields and Luria, 1969a).

In this study, fluorescent probes were employed in an attempt to monitor continuously colicin-caused changes in the membrane. Light emission by these probes, when excited at a suitable wavelength, is weaker in aqueous solution and stronger in a hydrophobic environment. Cramer and Phillips (1970) have shown that colicin E1 caused a fluorescence enhancement of the probe ANS.[1] Their data demonstrated an all-or-none phenomenon, suggesting that ANS monitors a conformational change of the membrane placing the probe into a more hydrophobic environment. Phillips and Cramer (1973) found a similar colicin-caused fluorescence effect with a similar fluorescent probe, *N*-phenylnaphthylamine, and showed that the rate of colicin-caused fluorescence was dependent on both temperature and the fatty acid composition of the membrane (Cramer *et al.*, 1973).

A new fluorescent probe, chlorotetracycline (CT), was employed in the present study of the mechanism of action of colicin K. CT is bacteriostatic by its ability to bind to ribosomes and to inhibit protein synthesis (Franklin, 1963). CT fluorescence is 20 times higher when it chelates magnesium or calcium in an aqueous solvent and is 100–200 times higher in the presence of magnesium or calcium in a hydrophobic environment such as a biological membrane (Caswell and Hutchison, 1971). Caswell (1972) has used this probe to monitor changes in divalent cation distribution in the mitochondrial membrane upon energization. The data presented here suggest that colicin K, in a reaction that re-

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received April 22, 1974.* This investigation was supported by research grants from the National Science Foundation (GB 30575) and National Institutes of Health (AI 03038) to Dr. S. E. Luria and was conducted during the tenure of a Damon Runyon Cancer Research Fellowship.

‡ Present address: Department of Microbiology, University of Southern California School of Medicine, Los Angeles, Calif. 90033.

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonic acid; CT, chlorotetracycline; TMG, methyl β-D-thiogalactoside; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

quires an energized membrane, causes a redistribution of and an altered membrane permeability to CT.

Materials and Methods

Cells and Growth Conditions. E. coli strain W-6 (pro$^-$) from this laboratory's collection was made resistant to the tetracyclines by the introduction of an R factor (RP4) from strain J53 (F$^-$pro$^-$met$^-$str^S) (Datta *et al.*, 1971), the gift of Harry Meade. This strain was grown on medium M9A (38 mM Na_2HPO_4–22 mM KH_2PO_4–8.6 mM NaCl–18.7 mM NH_4Cl) with 0.2 mM $MgSO_4$, or Ozeki medium (Nagel de Zwaig and Luria, 1967), 0.4% sodium D(L)-lactate (pH 7.0), 0.01% L-proline, 3 μM thiamine at 37° by reciprocal shaking to a density of 100 Klett units (green filter, 5×10^8 cells/ml). Cells were harvested by centrifugation at 20°, resuspended, and stored at 16°. *E. coli* G-6 *unc-A*, constructed in this laboratory (Plate *et al.*, 1974) from an *unc-A* strain (N144), obtained from Dr. L. Gutnick, was grown on Ozeki medium with 0.4% glucose, 0.01% L-histidine, and 2 μM thiamine.

Colicin K Preparation and Cell Killing Assay. Colicin K judged to be more than 98% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis and with a specific activity of 7 molecules per killing unit was prepared by a modification of the method of Kunugita and Matsuhashi (1970) in which the diethylaminoethyl-Sephadex chromatography was omitted and colicin was eluted from the harvested cells by gentle stirring. The amount of cell death caused by colicin (survival ratio S/S_0) was expressed in terms of a multiplicity (m) of killing units per cell ($S/S_0 = e^{-m}$) after dilution at room temperature in the buffer from which the cells were removed and plating on LB agar (Luria *et al.*, 1960).

Fluorescence measurements were performed with a Perkin-Elmer MPF-3 equipped with a thermostated cell holder at 37°. Cell suspensions (1.5 ml) were magnetically stirred from above employing a Teflon-coated magnetic stirring flea (P73, Precision Cells, Hicksville, N.Y.) and oxygenated through 28-gauge stainless steel tubing (Hamilton, Reno, Nev.). CT fluorescence was excited at 385 nm (10-nm slit) and measured at 515 nm (10-nm slit) with a filter in the emission path which blocks light below 430 nm. Scatter was less than 10% and not important because relative measurements are reported and colicin K does not change cell scattering. For polarization measurements of fluorescence anisotropy, polarizing filters were placed in the excitation and emission beams whose slits were enlarged to 20 nm. Limiting anisotropy was determined for 20 μM CT, 10 mM Tris-Cl (pH 7.4), and 1 mM $MgCl_2$ in 99% glycerol at 4°.

CT excited state lifetime measurements (Yguerabide *et al.*, 1970; Yguerabide, 1972) were kindly conducted by Professor Lubert Stryer (Yale University). A cell suspension at 10^9 cells/ml in M9A, 0.7 mM $MgCl_2$, and 0.4% lactate was stirred and oxygenated at 37°. The excitation wavelength was limited by a Corning 7-51 filter and the emission by a 3-71 filter. Colicin K was added at a multiplicity of 14. The observed fluorescence decay curves did not correspond to a single lifetime and are reported as average values for a least-squares best fit to a single exponential decay.

Chemicals. Chlorotetracycline-HCl was used as supplied by Nutritional Biochemicals Corp. (Cleveland, Ohio). Aqueous solutions (1.5 mM) were made fresh weekly and stored protected from light. [^{14}C]TMG (2.2 Ci/mol) and [^{3}H]tetracycline (660 Ci/mol) were obtained from New England Nuclear (Boston, Mass.). DCCD was from Aldrich Chemical Corp. (Milwaukee, Wis.) and FCCP was the gift of P. Heytler (E.I. duPont de Nemours and Co., Wilmington, Del.). Trypsin (two times crystallized) was from Worthington Biochemical Corp. (Freehold, N.J.).

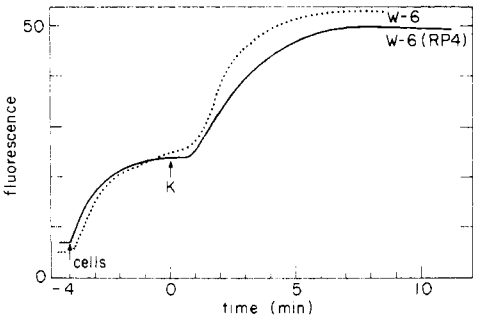

FIGURE 1: Colicin K caused CT fluorescence in *E. coli* W-6 and W-6(RP4). Cells grown in Ozeki medium were added at 2×10^9 cells/ml at −4 min to 1.5 ml of Ozeki medium plus 0.4% sodium D(L)-lactate and 20 μM CT. At 0 time colicin K was added at a multiplicity of 7. Fluorescence of cells alone, in the absence of CT, was 5 and was affected by colicin less than 2% (measured on an expanded scale).

Results

Study of CT fluorescence in an *E. coli* strain bearing a tetracycline resistance factor made it possible to follow the effects of colicin on cell viability. Resistance results from reduced drug permeability (Franklin, 1967); the drug inside the cell is chemically unchanged (Franklin and Higginson, 1970). CT fluorescence was monitored at 37° in oxygenated minimal medium containing lactate as carbon source. In the absence of cells, CT in this magnesium-containing medium fluoresces relatively weakly. When cells are added (Figure 1), the CT fluorescence rises in a few minutes to a new steady-state level. Addition of purified colicin K produces, after a short lag, a further rise in system fluorescence to a new steady-state level roughly double the pre-colicin level. With increasing colicin concentrations the lag period is shortened to a minimum of about 20 sec and the rate of colicin-stimulated rise is increased. Figure 1 also shows that the CT fluorescence response to colicin is not affected by the resistance factor on the time scale of the experiment. In separate experiments at low colicin multiplicities, the maximum level of fluorescence stimulated by colicin K parallels the fraction of cells killed by the colicin. This implies that the colicin-stimulated fluorescence monitors an event closely correlated to the lethal event. At colicin multiplicities above two killing units per cell (survival e^{-2}), the steady-state amount of colicin-stimulated fluorescence is approximately constant (Figure 2), indicating that the increase in fluorescence reflects an all-or-none event.

In order to relate the CT fluorescence response to a membrane function altered by colicin, fluorescence was monitored simultaneously with the amount of [^{14}C]methyl thiogalactoside ([^{14}C]TMG) retained by cells preloaded with [^{14}C]TMG (Fields and Luria, 1969a). As shown in Figure 3, the colicin-stimulated efflux of TMG parallels the rise in fluorescence. TMG efflux, half-maximal at 1.3 min, proceeds more rapidly than the CT fluorescence rise, half-maximal at 4.2 min. When the amount of active colicin is limited at various times by the addition of trypsin (Plate and Luria, 1972), there is a one-to-one correspondence between cell killing, TMG retention, and CT fluorescence production (Figure 3, inset).

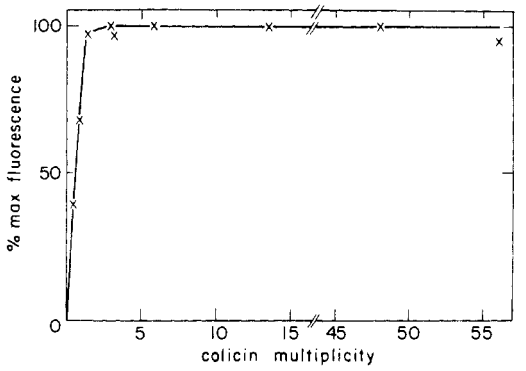

FIGURE 2: CT maximum fluorescence dependence on colicin multiplicity. Conditions are as described in Figure 1.

TABLE I: Specificity of CT Fluorescence Response to Colicin.

E. coli Strain	Viability Response	Colicin-Stimulated Fluorescence
W	Sensitive	+
K-12	Sensitive	+
C-600	Sensitive	+
C-600 tol II	Tolerant	−
K-235 (col K)	Immune	−
Other colicins, strain W-6		
E1	Sensitive	+
E2	Sensitive	−
E3	Sensitive	−

The specificity of the colicin-stimulated CT fluorescence was examined with regard to other colicins and cells (Table I). The colicin-stimulated CT fluorescence is absent in cells that do not exhibit physiological changes in response to colicin K, such as tol II mutants (Nagel de Zwaig and Luria, 1967) or immune strains like K235, from which colicin K was isolated. Colicin E1, which in sensitive cells causes physiological changes similar to those caused by colicin K, also produces an increase in CT fluorescence. Colicins E2 and E3, that have as their primary targets cellular DNA (Nomura, 1963) or rRNA (Bowman *et al.*, 1971; Senior and Holland, 1971), kill bacteria without eliciting increased CT fluorescence.

Energy Relationships. Colicin K appears to inhibit certain transport systems by interfering with their energy coupling mechanism (Fields and Luria, 1969a; Luria, 1973; Plate *et al.*, 1974). The CT fluorescence response might serve as a system for detecting changes in the energy state of the membrane. These relationships can be investigated by the use of metabolic inhibitors (Table II). The ATPase inhibitor DCCD, which blocks the colicin-induced fall in cellular ATP without interfering with the action of colicin on transport (Feingold, 1970), has no effect on the CT fluorescence response. This implies no direct link between ATP levels and the fluorescence response. A normal CT fluorescence response is in fact observed for a mutant *E. coli unc-A* (see Figure 5, + O_2), which has a greatly diminished membrane ATPase activity. Arsenate does not affect the maximum level of colicin-stimulated CT fluorescence, although it slowed the rate of rise in fluorescence. Among the uncouplers that supposedly act by abolishing the membrane proton gradient (Hopfer *et al.*, 1968), 1 μM FCCP does not block the colicin-stimulated CT fluorescence, but 5 μM FCCP inhibits it completely without affecting the pre-colicin fluorescence rise. Dinitrophenol could not be used for fluorescence studies because of its strong absorption in the region of CT excitation.

It should be noted that although colicin K causes a stimulation of CT fluorescence as well as a stimulation of ANS fluorescence, the two probes behave differently in response to FCCP or CCCP. CCCP by itself causes a rise in cellular ANS fluorescence (Cramer and Phillips, 1970) but it does not enhance CT fluorescence.

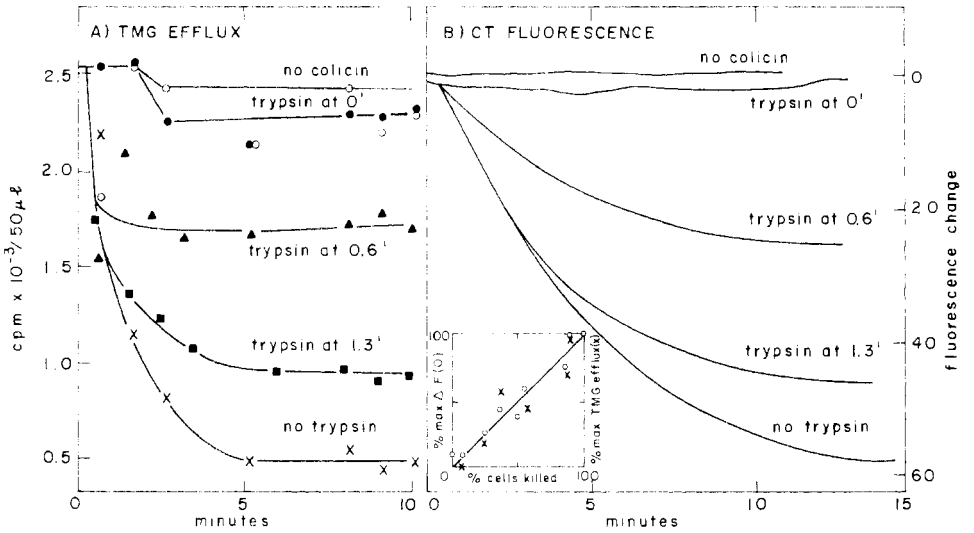

FIGURE 3: Simultaneous measurement of colicin-caused TMG efflux and CT fluorescence. W-6(RP4) cells grown in Ozeki medium containing also 0.5 mM isopentyl thiogalactoside were resuspended at 5×10^9 cells/ml in 0.45 ml of Ozeki medium plus 0.4% lactate and 20 μM CT at 8°. 2.5 μl of [^{14}C]TMG (20 mM, 43.5 μCi/ml) was added and half the solution was removed to a tube containing colicin K at a multiplicity of 3.5 at 8°. After 25 min for adsorption of colicin, 0.20 ml was transferred to the fluorescence cuvet containing 1.8 ml of Ozeki medium, 0.4% lactate, 20 μM CT, and 10 μl of [^{14}C]TMG at 37° (0 time). Recording of fluorescence was interrupted mementarily at the indicated times for transferring 50-μl aliquots to 0.45-μm pore membranes, followed by a 2-ml wash with 37° Ozeki medium and 0.4% lactate. Filters were dried and counted. When indicated, 20 μl of freshly dissolved trypsin at 40 mg/ml was added. Survival measured after 11 min is indicated in the inset (data from several experiments). Note the inverted ordinate for fluorescence.

TABLE II: Effects of Inhibitors on CT Fluorescence and Transport.

	Inhibition of CT Fl.		Protection from Colicin Killing (Trypsin)	Inhibition of Respiration-Dependent Transport
	−K	+K		
Uncoupled oxidative phosphorylation				
DCCD (0.1 mM)	−	−	−	−
Absence of ATPase (*unc-A* mutant)	−	−	−	−
Arsenate (50 mM)	−	−	−	−
FCCP (1–5 μM)	−	±	+	+
Inhibited respiration				
KCN (2 mM)	−	+	+	+
Azide (10 mM)	±	+		+
Anoxia	−	+	+	+
Control	−	−	−	−

The observation that neither uncouplers of oxidative phosphorylation nor respiratory inhibitors block the pre-colicin CT fluorescence implies that CT is not being actively transported in the absence of colicin. Since the respiratory inhibitors cyanide and azide block the colicin-stimulated fluorescence, respiration driven electron transport appears to be required for colicin to act (see Plate, 1973).

Anoxia. If the respiratory inhibitors are acting solely by blocking electron transport, then omitting oxygen from a cell suspension utilizing lactic acid should produce the same effects. Allowing a suspension of cells to go anoxic by stopping oxygenation has essentially no effect on the pre-colicin CT fluorescence rise (Figure 4). Upon addition of colicin K there is only a small rise in fluorescence, probably associated with the introduction of some oxygen. This dramatic inhibition of the colicin-initiated fluorescence rise under anoxia is associated with protection from colicin killing as shown by treatment with trypsin, and is observed with cells grown and tested on lactate or succinate but not on glucose (data not shown, but see Figure 5). This finding confirms the unpublished observation of Levinthal (cited in Luria, 1970) that anoxia prevents the inhibitory effects of colicin E1. Upon return to aerobic conditions, the CT fluorescence response returns, and the cycle could be repeated by the addition and removal of oxygen. Viability remains low after the first exposure to oxygen.

When glucose is the energy source, anoxia does not inhibit the colicin-induced fluorescence rise (see Figure 5). This is attributed to energization of the membrane through the membrane Ca^{2+}- and Mg^{2+}-activated ATPase. Similar experiments were conducted with the mutant *E. coli unc-A,* which lacks the membrane ATPase activity. Addition of colicin K to cells under anoxia in the presence of glucose yields no fluorescence increase, although interpretation is complicated by an elevated base line (Figure 5). Again, these cells are effectively rescued from cell death by trypsin.

It appears, therefore, that agents that block the physiological events caused by colicin K also block the CT fluorescence response. The interesting exception to this generalization is the oxygen reversibility of fluorescence for cells

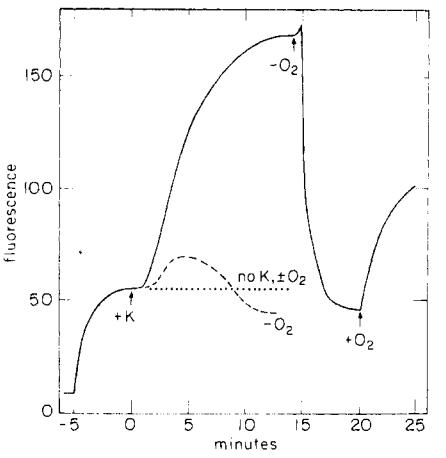

FIGURE 4: Effect of anoxia on W-6(RP4). Cells grown in Ozeki medium were added at 1×10^9 cells/ml at −5 min to 1.5 ml of Ozeki medium plus 0.4% lactate and 20 μM CT in the presence (—, ...) or absence (- - -, . . .) of oxygen. At 0 time colicin K was added as 20 μl to equal a multiplicity of 1.0 as measured in the presence of oxygen at 5 min (survival = 36%) ($-O_2$, survival = 37%). At 5 min freshly dissolved trypsin was added at 0.4 mg/ml to each cuvet. Cell viability was measured at 15 min ($+O_2$, survival = 37%) ($-O_2$, survival = 114%). At 15 min oxygen was removed and reintroduced at 20 min. Cell viability at 20 min was 40%.

grown on lactate. Phillips and Cramer (1973) have reported that anoxia does not affect *N*-phenylnaphthylamine fluorescence caused by colicin E1, but their experiment was conducted on cells grown on glucose and tested in its absence, leading to equivocal interpretation.

Characterization of Fluorescence. (A) MAGNESIUM EFFECTS. The pre-colicin and post-colicin rises in fluorescence could be due to an increased uptake of CT by the cells and/or an increased quantum yield for bound CT molecules. Divalent cations such as magnesium could be involved in either process. For this reason, experiments similar to that in Figure 1 were conducted at different magnesium concentrations. Figure 6 shows that the steady-state fluorescence of CT in the presence of *E. coli* cells (without colicin) depends on the magnesium concentration in a bell-shaped fashion with a maximum fluorescence change at 0.4 mM magnesium. The fact that the cell-caused fluorescence

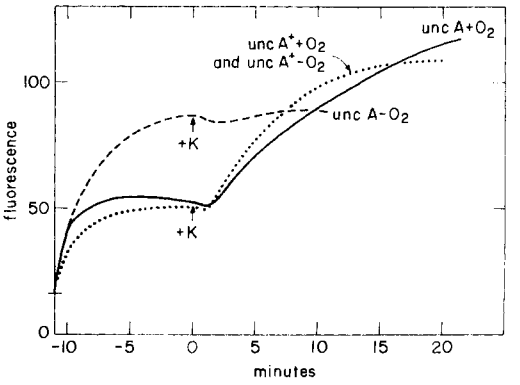

FIGURE 5: Effect of anoxia on *unc-A* and *unc-A*$^+$ in glucose. G-6 *unc-A* (RP4) or G-6 *unc-A*$^+$ grown in Ozeki medium with glucose were added at 1×10^9 cells/ml at −11 min to 1.5 ml of Ozeki medium plus 0.4% glucose and 20 μM CT in the presence (—, . . .) of oxygen. At 0 time colicin K was added as 20 μl to equal a multiplicity of 3 as measured in the presence of oxygen at 5 min (survival = 6.4%) ($-O_2$, survival = 55%). At 5 min trypsin was added. Cell viability was measured at 15 min ($+O_2$, survival = 6.9%) ($-O_2$, survival = 72%). Viability was not determined for *unc-A*$^+$ (. . . .).

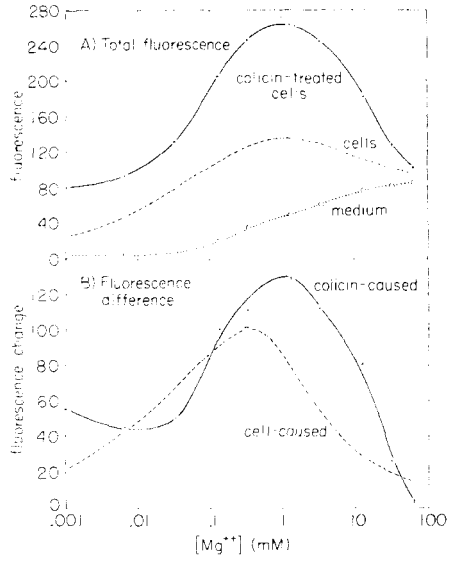

FIGURE 6: Effect of magnesium on CT fluorescence. W-6(RP4) cells grown in M9A medium were harvested and washed with one-half culture volume of 10 mM Tris-Cl (pH 7.4). Fluorescence was determined in 1.5 ml of 10 mM Tris-Cl (pH 7.4), 0.4% lactate, 20 μM CT, and the desired concentration of $MgCl_2$ (medium), followed by the addition of cells to 1×10^9/ml. After 8 min, colicin K was added at a multiplicity of 15. (A) Steady-state fluorescence for pre-colicin (- - -) and colicin-treated cells (—) was obtained by extrapolation to infinite time in a fluorescence *vs.* time double reciprocal plot. (B) Fluorescence difference attributed to cells (- - -) and the colicin-caused difference (—, derived from panel A: colicin treated − untreated cells).

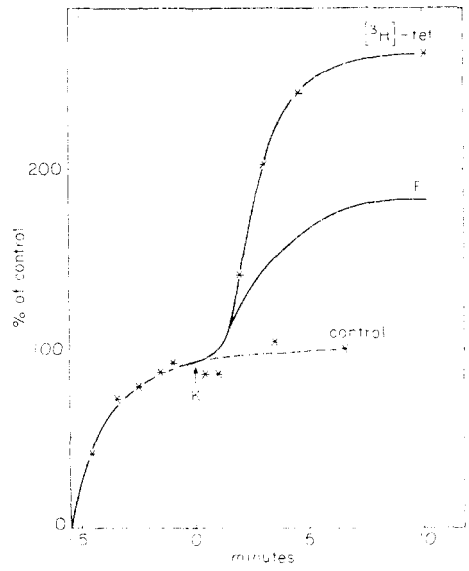

FIGURE 7: Uptake and fluorescence of [^{3}H]tetracycline. W-6(RP4) cells grown in Ozeki medium were added at 2×10^9 cells/ml at −6 min to 1.8 ml of Ozeki medium plus 0.4% lactate and 20 μM [^{3}H]tetracycline (660 Ci/mol). At the indicated times (*) 50-μl aliquots were withdrawn, filtered, and washed quickly with 2 ml of 37° Ozeki medium plus lactate. Filters were dried and counted. To another cuvet, colicin K was added at 0 time at a multiplicity of 4 and aliquots were treated similarly; 100% control ^{3}H cpm (5×10^4 cpm/50 μl) was normalized to fluorescence (—, - - -) in the absence of colicin.

curve is shifted toward lower magnesium concentrations relative to that of the medium (Figure 6A) implies a stronger association of CT with cellular magnesium than with magnesium in the medium. The difference between steady-state levels for colicin-stimulated fluorescence and the levels for the untreated cells is also dependent on the magnesium concentration in the medium, but with a maximum at 1 mM (see Figure 6B). Attempts to measure binding constants by determination of cell-associated CT gave inconsistent results, presumably because of a fast equilibrium of CT between cells and medium and of the difficulty of extracting cellular CT into conditions of constant quantum yield.

To explore the possibility that colicin K causes an increased uptake of magnesium, which in turn allows more CT to bind to the cells, an experiment was conducted in the presence of EDTA to chelate extracellular magnesium. When bacteria are incubated in 10 mM EDTA (10 mM Tris-Cl (pH 7.1), 0.4% lactate, and 20 μM CT) the steady-state fluorescence is 30% of that in the absence of EDTA. However, when colicin K is added to the cells in EDTA, the steady-state fluorescence rises to the maximum level of the control without EDTA. This experiment implies that 70% of the cellular CT fluorescence is due to EDTA-accessible magnesium sites that are not involved in the fluorescence response to colicin.

It should be noted that a colicin K-stimulated efflux of magnesium was reported by Lusk and Nelson (1972) in cell preparations that had been stored on ice before use, but only at a later time than TMG efflux. Our initial preparations, similar to those of Lusk and Nelson, exhibited only a transient colicin-initiated rise in CT fluorescence. Addition of 5 mM magnesium to the medium blocked the falling phase of the response, suggesting that cold-treated cells leaked magnesium down a concentration gradient a few minutes after colicin treatment. To avoid this complication, no further experiments were done with ice chilled stored cells. However, the fact that conditions which remove magnesium from the cells result in a drop in fluorescence suggests that the normal rise in fluorescence may be due to an increased uptake of CT into magnesium rich cells (since the EDTA experiment precludes an uptake of magnesium being responsible for the elevated fluorescence).

(B) PROBE UPTAKE: [^{3}H]TETRACYCLINE. To test whether colicin causes increased binding of probe to the cell, uptake studies were conducted simultaneously with fluorescence measurements using [^{3}H]tetracycline (Figure 7). Before addition of colicin, the uptake of radioactive probe could be normalized to correspond with fluorescence, indicating that the average quantum yield of the system is unchanged during the course of cell-caused fluorescence. Upon addition of colicin, fluorescence rises as usual, validating the substitution of the [^{3}H]tetracycline for its chlorinated derivative CT. Colicin K causes the cellular uptake of probe to increase at a faster rate than the fluorescence and reach a higher plateau. This means that the increase in system fluorescence in response to colicin K is more than accounted for by an increased cellular uptake of probe. Based on the specific activity of the [^{3}H]tetracycline, the average concentration of probe in the cell is 170 μM (assuming a cell volume of 8×10^{-13} cm^3). Colicin causes this value to increase to 440 μM, 22-fold concentrated over the medium, but only 2% of the total probe in the system.[2]

(C) PROBE ENVIRONMENT: FLUORESCENCE ANISOTROPY. To test the possibility that substantial amounts of probe are traversing the membrane and forming a complex with magnesium in the cytoplasm, steady-state

[2] The amount of [^{3}H]tetracycline taken up into a single cell after colicin is approximately 2×10^5 molecules. Based on a lipid composition of 10% of the cell dry weight (2×10^{-13} g), the cell has 2×10^7 molecules of phospholipid (average molecular weight 700). Therefore, the mole ratio of probe to phospholipid is 1/100.

fluorescence polarization measurements were conducted. The steady-state fluorescence anisotropy can be defined in terms of the fluorescence intensity when measured in a plane parallel ($F_\parallel$) *vs.* a plane perpendicular ($F_\perp$) to the exciting light (eq 1). High anisotropy ($F_\parallel \gg F_\perp$) is suggestive of fluorescence originating from a relatively immobilized probe while low anisotropy ($F_\parallel \approx F_\perp$) suggests a probe in a more fluid environment, providing the excited-state lifetime of the probe does not change. Quantitatively, the anisotropy is related to probe immobility by the spherical rotational correlation time, ϕ, and the excited-state lifetime, τ

$$\bar{A} = (F_\parallel - F_\perp)/(F_\parallel + 2F_\perp) \quad (1)$$

$$\bar{A} = A_0/(1 + \tau/\phi)$$

or (2)

$$\phi = \tau/[(A_0/\bar{A}) - 1]$$

where A_0 is the limiting anisotropy (no motion). Figure 8 shows that aqueous CT has a very low anisotropy ($\bar{A}$ = 0.02) as expected for unbound aqueous probe. Measurement of the excited-state lifetime of aqueous CT–magnesium is at the limit of the instrument's sensitivity, $\tau < 1$ nsec. Upon addition of cells, the anisotropy rises rapidly (Figure 8), then reaches a steady state ($\bar{A}$ = 0.15). The measured average excited-state lifetime for this condition is τ = 1.6 nsec. After colicin addition, the anisotropy falls to a new steady state ($\bar{A}$ = 0.06), and τ drops to 1.2 nsec. Referring to eq 2, the colicin-induced drop in anisotropy combines with a drop in average excited-state lifetime, to indicate a significant drop in the average rotational correlation time of the probe. Making the (unwarranted) assumption that the probe is a rigid sphere with a limiting anisotropy, A_0 = 0.25, the pre-colicin rotational correlation time would be 2.4 nsec (eq 2) which colicin lowers to an average 0.4 nsec. Since the rotational correlation time is linearly dependent on both the microviscosity and the size (molecular weight) of the CT complex, colicin causes CT either to experience a region of lower viscosity, that is, more fluid, or to become bound as a smaller complex.[3] Either of these possibilities is consistent with CT leaving a membrane-bound state to form a free CT–magnesium complex in the cytoplasm.

If the fluorescing probe is initially bound to the cell membrane and colicin stimulates further fluorescence by increasing the amount of CT bound to a more fluid membrane, then both pre-colicin and post-colicin fluorescence anisotropy as a function of temperature may be expected to behave as it would in a lipid extract. A sharp change in anisotropy is expected at the "phase-transition" temperature of the fatty acid chains from fluid to liquid crystalline. If, on the other hand, either pre- or post-colicin fluorescence is due primarily to an unbound CT–magnesium complex (*e.g.*, CT–magnesium in the cytoplasm), then no break in the anisotropy *vs.* temperature curve is expected, and the anisotropy would mimic an aqueous solution of CT and magnesium. Figure 9 shows the effects of temperature on CT anisotropy. Untreated cells follow closely the temperature-dependent anisotropy of a sonicated lipid extract with a transition temperature 20° < T_t < 25°. In sharp contrast, the colicin-treated cells mimic more closely the buffered aqueous magnesium. Thus, before colicin addition CT fluoresces from an environment not unlike that of sonicated cell lipids. After addition of colicin, the majority of CT fluorescence is from an environment with slightly higher anisotropy than that of CT in buffer, consistent with the conclusion that CT fluoresces from the cellular cytoplasm.

Previous studies have characterized fluorescent probe environment by describing colicin-caused changes in the fluorescence emission spectrum (Phillips and Cramer, 1973). No such changes were observed for CT.

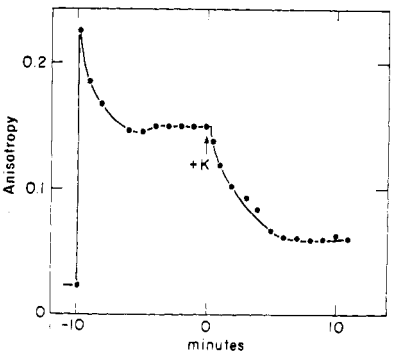

FIGURE 8: CT fluorescence anisotropy. W-6(RP4) cells grown in M9A medium were added at 8 × 10^8 cells/ml at −10 min to 1.5 ml of M9A with 0.4% lactate, 20 μM CT, and 0.2 mM $MgCl_2$. The polarized fluorescence was measured by rotating the emission polarizer every minute from a parallel to a perpendicular alignment with the excitation polarizer. Steady-state anisotropy was calculated by eq 1.

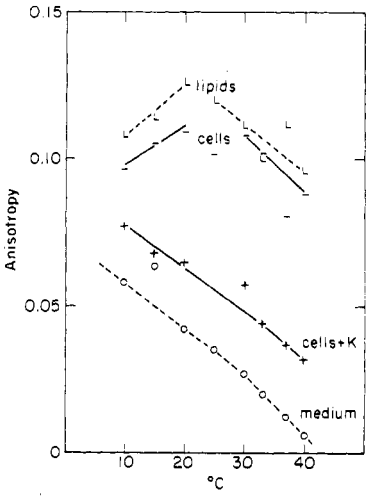

FIGURE 9: Dependence of fluorescence anisotropy on temperature. W-6(RP4) cells grown on M9A were incubated at 1 × 10^{10} cells/ml at 37° in 10 mM Tris-Cl (pH 7.4), 0.4% lactate, and 20 μM CT for 5 min and then diluted 1/15 into the fluorescence cuvet at the desired temperature in the same medium (—). An additional preparation was treated with colicin K for 8 min at a multiplicity of 7 before similar transfer to the desired temperature (+). The anisotropy of the medium alone containing 5 mM $MgCl_2$ and 20 μM CT was determined separately (O). Lipids (L) were prepared by a Folch *et al.* (1957) extract from the same cells grown under the same conditions; 1 mg of lipid extract was suspended in 0.5 ml of 10 mM Tris-Cl (pH 7.4) and 0.1 mM $MgCl_2$ and sonicated at 20 W under nitrogen for 2 min total time (Sonifier Cell Disruptor, Heat Systems Co., Melville, N.Y.). These liposomes were made 20 μM in CT and placed at 37°. Fluorescence anisotropy was determined by diluting 1/10 into 10 mM Tris-Cl (pH 7.4) and 20 μM CT at the desired temperature.

[3] The pre-colicin anisotropy corresponds to that of CT in 60% glycerol at 37° (viscosity = 5 P). This is of the order of phospholipid viscosity in *E. coli* membranes as judged by spin-labeling studies (Sinensky, 1974). The colicin-caused anisotropy corresponds to that of 15% glycerol at 37° (viscosity = 1 P), which is approximately the cytoplasmic viscosity for *E. coli* at 37° (Keith and Snipes, 1974). Water has a viscosity of 0.007 P at 37°. However, these correlations must not be taken too literally because the excited-state lifetimes for glycerol solutions were not measurable ($\tau < 1$ nsec).

Discussion

Addition of *E. coli* cells to CT causes a rise in fluorescence that is further heightened upon addition of colicin K to sensitive but not to colicin-insensitive bacteria. The colicin-induced rise in CT fluorescence, like that in ANS fluorescence (Cramer and Phillips, 1970), is an all-or-none response of a bacterial cell. Viable counts and measurements of active transport of β-D-galactoside show that the three effects are exerted concurrently on those cells that make the transition from stage I to stage II of colicin K action (Plate and Luria, 1972). All treatments that prevent the transition also prevent the colicin-induced rise in CT fluorescence. These findings are expected if the increased fluorescence is a consequence of changes occurring in stage II of colicin action.

It is known that FCCP and respiratory inhibitors block the stage I to stage II transition. Likewise, they block colicin-stimulated CT fluorescence, suggesting that some form of membrane energized state created by electron transport is necessary for colicin to act on the membrane or to link its action to other membrane processes. The surprising observation that colicin-induced CT fluorescence is reversible with anoxia in cells utilizing lactate suggests that after colicin action CT fluorescence remains coupled to respiration while the linkage of respiration to substrate transport is uncoupled. Characterization of CT fluorescence suggests the involvement of magnesium in the cellular and colicin-caused fluorescence. Studies with radioactive tetracycline show that colicin K causes an increased uptake of extracellular probe rather than a change in the quantum yield of previously adsorbed probe. Fluorescence anisotropy studies suggest that CT is associated with the cell membrane before colicin action and that colicin K causes a shift of CT from the membrane and medium to a more fluid region of the cell, such as the cytoplasm.

What do the properties of CT fluorescence say about the mode of action of colicin K? Without claiming its validity, one can propose a heuristic model based primarily on the interactions between CT and magnesium. CT is in equilibrium between the free species and a complex with magnesium, both in the membrane of the cell and in intra- and extracellular aqueous phases. The fact that magnesium enhances the pre-colicin cellular fluorescence of CT suggests that magnesium binds to sites accessible to CT that had been depleted of magnesium by washing the cells. The anisotropy studies imply that these sites are on the membrane. Addition of magnesium beyond a maximal concentration would tend to pull CT off the cell into the medium, lowering the net fluorescence because of the lower quantum yield of CT-Mg^{2+} in aqueous environment *vs.* membrane-bound.

Uptake measurements with [^{3}H]tetracycline have shown that colicin, which is known to act on the cytoplasmic membrane, stimulates uptake of the probe. The anisotropy studies in this case indicate that the cytoplasm rather than the membrane may be the reservoir for the accumulated probe. The high intracellular concentration of magnesium (25–30 mM, Silver and Clark, 1971; Lusk *et al.*, 1968), together with the increased probe concentration and a higher quantum yield of fluorescence at higher viscosities (G. J. Brewer, unpublished), could explain the increased fluorescence in the presence of colicin. The decrease in colicin-caused fluorescence at high magnesium concentrations may be due to the magnesium pulling CT out of the cell into the medium where a lower quantum yield would be experienced due to lower viscosity. This mechanism explains the bell-shaped profile of fluorescence *vs.* external magnesium. An alternative explanation may be that the colicin-caused drop in anisotropy is due to increased energy transfer resulting in randomization of emission because of higher probe concentrations. This process could explain the reversed slope of anisotropy observed as a function of temperature for the lipids and cells below 20°, under which conditions the probe might be concentrated in ever smaller domains of fluid membrane. Conversely, a nonmembrane localization for CT in colicin-treated cells is suggested by the absence of this phenomenon.

Concerning energy relationships to colicin-stimulated CT fluorescence, the model must include two separate functions: one dependent on colicin and the other dependent on some form of energized state of the membrane. Before colicin, CT fluorescence is not dependent on energy in these experiments, except in the case of *unc-A* where the altered membrane ATPase may exert changes either directly or indirectly through defective energization (Rosen, 1973). Colicin action requires energy and after colicin action CT fluorescence changes are reversible, dependent on oxygen. In colicin-treated cells the membrane may be more permeable to CT. Energization and de-energization of the membrane may alter the partitioning of magnesium among the cytoplasm, the membrane, and the medium. In this regard, the recent work of Träuble and Eibl (1974) is of interest. These authors showed that in phospholipid bilayers, the addition of magnesium could induce a liquid to liquid-crystalline phase transition of the fatty acyl chains. Their work, based on electrostatic charge interactions at the membrane surface, suggests the possible role of a transmembrane potential or pH gradient in control of the fluidity of the membrane or the so-called energized state. Work is in progress to determine the effect of colicin K on these parameters.

Acknowledgments

The author expresses his deepest appreciation for the stimulation and guidance of Dr. S. E. Luria in whose laboratory this work was conducted. The author also benefited from many helpful discussions with Dr. Charles Plate and Dr. Paul Schimmel.

References

Bowman, C. M., Dahlberg, J. E., Ikemura, T., Konisky, J., and Nomura, M. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 964.

Caswell, A. H. (1972), *J. Membrane Biol. 7,* 345.

Caswell, A. H., and Hutchison, J. D. (1971), *Biochem. Biophys. Res. Commun. 42,* 43.

Cramer, W. A., and Phillips, S. K. (1970), *J. Bacteriol. 104,* 819.

Cramer, W. A., Phillips, S. K., and Keenan, T. W. (1973), *Biochemistry 12,* 1177.

Dandeu, J.-P., Billault, A., and Barbu, E. (1969), *C. R. Acad. Sci. 269,* 2044.

Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. B., and Richmond, M. H. (1971), *J. Bacteriol. 108,* 1244.

Feingold, D. S. (1970), *J. Membrane Biol. 3,* 372.

Fields, K. L., and Luria, S. E. (1969a), *J. Bacteriol. 97,* 57.

Fields, K. L., and Luria, S. E. (1969b), *J. Bacteriol. 97,* 64.

Folch, J., Lees, M., and Stanley, G. H. S. (1957), *J. Biol. Chem. 266,* 497

Franklin, T. J. (1963), *Biochem. J. 86,* 449.

Franklin, T. J. (1967), *Biochem. J. 105,* 371.
Franklin, T. J., and Higginson, B. (1970), *Biochem. J. 116,* 287.
Hirata, H., Fukui, S., and Ishikawa, S. (1969), *J. Biochem. 65,* 843.
Hopfer, U., Lehninger, A. L., and Thompson, T. E. (1968), *Proc. Nat. Acad. Sci. U. S. 59,* 484.
Jacob, F., Siminovitch, L., and Wollman, E. (1952), *Ann. Inst. Pasteur, Paris 83,* 295.
Keith, A. D., and Snipes, W. (1974), *Science 183,* 666.
Kunugita, K., and Matsuhashi, M. (1970), *J. Bacteriol. 104,* 1017.
Luria, S. E. (1970), *Science 168,* 1166.
Luria, S. E. (1973), *in* Bacterial Membranes and Walls, Leive, L., Ed., New York, N.Y., Marcel Dekker, p 293.
Luria, S. E., Adams, J. N., and Ting, R. C. (1960), *Virology 12,* 348.
Lusk, J. E., and Nelson, D. L. (1972), *J. Bacteriol. 112,* 148.
Lusk, J. E., Williams, R. J. P., and Kennedy, E. P. (1968), *J. Biol. Chem. 243,* 2618.
Nagel de Zwaig, R., and Luria, S. E. (1967), *J. Bacteriol. 94,* 1112.
Nomura, M. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28,* 315.
Nomura, M., and Maeda, A. (1965), *Zentralbl. Bakteriol., Parasitenk., Infektionskr. Hyg., Abt. 1: Orig. 196,* 216.
Phillips, S. K., and Cramer, W. A. (1973), *Biochemistry 12,* 1170.
Plate, C. A. (1973), *Antimicrob. Agents Chemother. 4,* 16.
Plate, C. A., and Luria, S. E. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2030.
Plate, C. A., Suit, J. L., Jetten, A. M., and Luria, S. E. (1974), *J. Biol. Chem.* (in press).
Rosen, B. P. (1973), *Biochem. Biophys. Res. Commun. 53,* 1289.
Senior, B. W., and Holland, I. B. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 959.
Silver, S., and Clark, D. (1971), *J. Biol. Chem. 246,* 569.
Sinensky, M. (1974), *Proc. Nat. Acad. Sci. U. S., 71,* 522.
Träuble, H., and Eibl, H. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 214.
Wendt, L. (1970), *J. Bacteriol. 104,* 1236.
Yguerabide, J. (1972), *Methods Enzymol. 26,* 498.
Yguerabide, J., Epstein, H. F., and Stryer, L. (1970), *J. Mol. Biol. 51,* 573.

Penicillin-Induced Secretion of a Soluble, Uncross-Linked Peptidoglycan by *Micrococcus luteus* Cells†

David Mirelman,* Rivka Bracha, and Nathan Sharon

ABSTRACT: Incubation of *Micrococcus luteus* cells in the presence of penicillin G leads to accumulation in the culture medium of a linear uncross-linked peptidoglycan. The amount of secreted peptidoglycan was dependent on the concentration of penicillin and paralleled the rate of cell wall synthesis, and the secretion was not accompanied by any cell lysis. Analysis of the peptidoglycan revealed that over 84% of its weight could be accounted for by *N*-acetylglucosamine, *N*-acetylmuramic acid, Ala, Glu, Gly, and Lys in the molar ratios of 2:2:3:1:1:1. The product was digested by lysozyme to yield mainly the disaccharide GlcNAc-β-(1→4)-MurNAc and the disaccharide-hexapeptide GlcNAc-β-(1→4)-MurNAc-L-Ala-D-*i*-Glu(Gly)-L-Lys-D-Ala-D-Ala. The molecular weight of the secreted peptidoglycan, estimated by ultracentrifugation, was found to be 38,000, corresponding to a chain length of approximately 50 disaccharide units, half of which are substituted by the hexapeptide on their *N*-acetylmuramic acid residues. A similar value for the molecular weight of the peptidoglycan was obtained from periodate oxidation and β-elimination studies. Muramic acid was found at the reducing end of the peptidoglycan. In addition to the uncross-linked peptidoglycan, penicillin also promoted the secretion of a hexapeptide in equimolar amounts to that of unsubstituted muramic acid residues in the secreted peptidoglycan. It is concluded that the secretion of the peptidoglycan is due to the inhibition by penicillin of the attachment of newly synthesized peptidoglycan strands by transpeptidation to the preexisting cell wall. The secretion of the free hexapeptide is assumed to be the result of the action of a penicillin-insensitive amidase which acts on the newly synthesized peptidoglycan strands.

Studies on the biosynthesis of bacterial cell walls have indicated that two types of enzymatic reactions are involved in the attachment of newly synthesized peptidoglycan to the preexisting one in the wall: (1) penicillin-insensitive transglycosylation reactions which build up linear peptidoglycan strands by a series of transfers from the two nucleotide precursors, UDP-GlcNAc and UDP-MurNAc-pentapeptide, and a lipid carrier (Strominger, 1970) and (2) penicillin-sensitive transpeptidation reactions which bind the newly synthesized linear peptidoglycan chains to suitable acceptor sites on the preexisting peptidoglycan of the cell wall (Mirelman *et al.,* 1972, 1974a; Fiedler and Glaser, 1973).

If in fact both types of reactions operate in the biosynthesis and assembly of the bacterial cell wall peptidoglycan, it could be expected that in the presence of penicillin, bacterial cells will continue to synthesize linear peptidoglycan

† From the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel. *Received May 20, 1974.* This investigation was supported by a grant from the Volkswagen Stiftung, Germany.