Changes in Rotational Motion of a Cell-Bound Fluorophore Caused by Colicin E1: a Study by Fluorescence Polarization and Differential Polarized Phase Fluorometry†

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ABSTRACT: The stationary fluorescence polarization and the differential phase delay of the polarized components of the fluorescence of 1-phenylnaphthylamine in *Escherichia coli* suspensions were measured before and after addition of colicin E1. Both sets of measurements register an increase in the rotational relaxation time of the fluorescent probe when colicin is present. These increases are absent in an *E. coli* mutant tolerant to colicin E1. The physical interpretation of the changes demands separate estimation of the fraction f_2 of the emitting fluorophores that change their properties upon colicin addition and of the rotational relaxation time ρ_2 of this fraction, following the colicin-induced changes. By themselves, the steady state polarization observations permit only the conclusion that f_2 must be in the range of 1–0.06 and the change in ρ_2/τ between 1.5 and a value larger than 10. Combination of the data of stationary polarization with those of differential phase fluorometry results in an important reduction in the uncertainty: f_2 must be in the range 1–0.33 and the change in ρ_2/τ in the range 1.5–2.5.

Evidence for a physical change in the cell envelope of *Escherichia coli* strain B/1,5 caused by colicin E1 closely linked to membrane deenergization has been recently reported (Helgerson et al., 1974). The steady-state polarization, p, of the hydrophobic probe *N*-phenyl-1-naphthylamine (1-NPN)[1] is increased upon addition of colicin E1. The fluorescence lifetime, τ_0, of the probe, independently measured by direct decay or phase fluorometry, is also increased in the colicin-treated cells. Introduction of p and p_0, the limiting polarization for the fluorophore, together with the values of τ_0, into the Perrin equation (Perrin, 1926; Weber, 1953) leads to the conclusion that the rotational relaxation time, ρ, of the probe included in the membrane is increased by a factor of two, and, therefore, that the viscosity of the medium in which the probe rotates rises in the same proportion. This conclusion is not unequivocal: the values of the stationary polarization and the fluorescence lifetime are average values over an unknown distribution of probe environments, and the change in polarization that follows addition of colicin may be interpreted as a transfer of the probe from one environment to another where it displays a lengthier rotational relaxation time, or alternatively, as resulting from a change in the viscosity of its surroundings, without change in the probe distribution. To stress the limitations of stationary polarization data, we can imagine that we deal in reality with a system of two components, one with $p = p_0$ or equivalently $\tau_0/\rho \ll 1$, and another with $p = 0$ or $\tau_0/\rho \gg 1$. The calculated value of ρ would then not bear any relation to the rotational relaxation time of any actual component of the system, or to the viscosity of the environment, recording only the proportions of freely rotating and immobilized molecules. The uncertainty involved in the stationary measurements may be diminished by a more direct measurement of ρ, either by the decay of the fluorescence polarization in real time (Wahl, 1966; Yguerabide et al., 1970; Tao, 1969) or by the difference in lifetime of the polarized components (Jablonski, 1961; Bauer, 1963; Spencer and Weber, 1970.)

Differential Polarized Phase Fluorometry. The sensitivity and speed of execution of the measurements of $\Delta\tau$, the difference in the lifetime of the polarized components, may be greatly increased by the direct measurement of the phase difference between the two polarized components of the fluorescence rather than independent measurements of each with respect to the exciting light. The gains are much the same as those of difference spectrophotometry over the comparison of separately obtained spectra. A brief account of this method has been given (Weber and Mitchell, 1976) and a paper on general theory and application to small fluorophores in homogeneous solution is in preparation (Mantulin and Weber, to be published). The delays $\delta_\parallel$ and $\delta_\perp$ of the polarized components of the fluorescence with respect to the polarized excitation, when the fluorophore is a sphere with rotational rate R, have been given by Spencer and Weber (1970):

$$\tan \delta_\parallel = -\omega \left(\frac{\gamma_\parallel(\Gamma^2 + \omega^2) + 2R(2\Gamma + 6R)}{(\gamma_\parallel(\Gamma^2 + \omega^2) + 2R\Gamma)(\Gamma + 6R) - 2R\omega^2} \right) \quad (1)$$

an identical equation holds for $\tan \delta_\perp$, but with $\gamma_\parallel$ replaced by $\gamma_\perp$. In these equations

$$\gamma_\parallel = \left(\frac{1 + p_0}{3 - p_0}\right); \gamma_\perp = \left(\frac{1 - p_0}{3 - p_0}\right) \quad (2)$$

where $\Gamma = 1/\tau_0$ = rate of emission; $R = \frac{1}{2}\rho$ = rate of rotation; ω = circular frequency of modulated excitation. The phase delay ϕ between the polarized components is obtained by introducing the values of $\tan \delta_\parallel$, $\tan \delta_\perp$ of eq 1 into the relation:

$$\tan (\delta_\parallel - \delta_\perp) \equiv \tan \phi = \frac{\tan \delta_\parallel - \tan \delta_\perp}{1 + \tan \delta_\parallel \tan \delta_\perp} \quad (3)$$

yielding

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[1] Abbreviation used: 1-NPN, *N*-phenyl-1-naphthylamine.

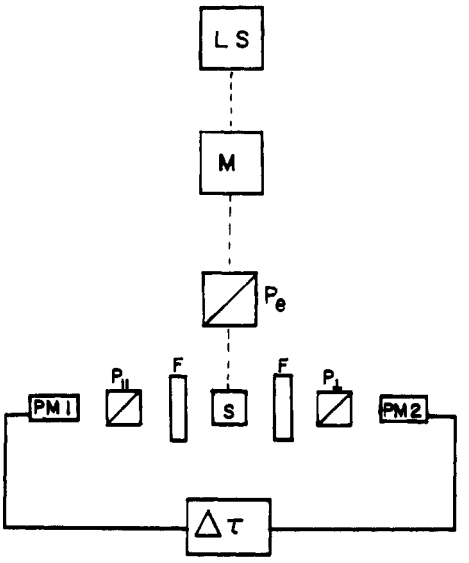

FIGURE 1: Plan of differential phase fluorometer. LS, light source, M, ultrasonic standing-wave modulator and monochromator; Pe, excitation polarizer; S, sample container; F, filters to remove exciting light; PM1, PM2, XP1023 Amperex photomultipliers; $\Delta\tau$, electronics for measurement of differential phase.

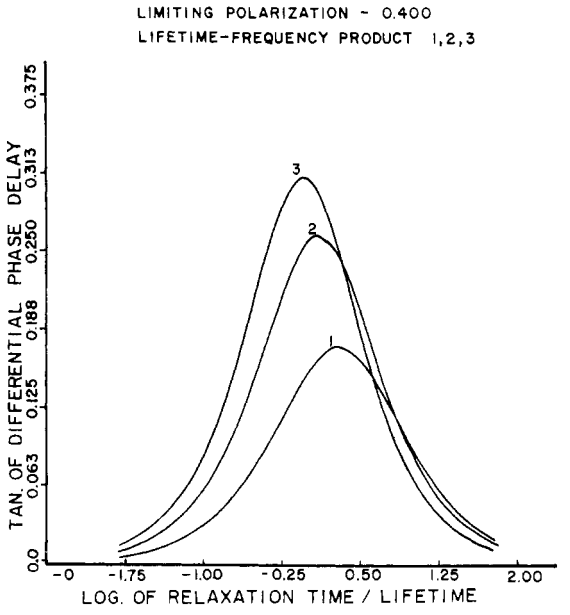

FIGURE 2: Dependence of differential delay upon α.

$$\tan\phi = -\frac{\omega(\gamma_{\parallel}-\gamma_{\perp})\alpha\tau_0}{\gamma_{\parallel}\gamma_{\perp}(1+\omega^2\tau_0^2)+(\gamma_{\parallel}+\gamma_{\perp})\alpha+\alpha^2} = \omega\Delta\tau \tag{4}$$

where $\alpha = \tau_0/\rho = 2R\tau_0$. Equation 4, a quadratic equation in α, may be conveniently written as

$$\alpha^2 + \frac{2\alpha}{3-p_0}\left(1 - \left|\frac{p_0}{\tan\phi}\right|\omega\tau_0\right) + m^2(1+\omega^2\tau_0^2) = 0 \tag{5}$$

with $m^2 = (1-p_0^2)/(3-p_0)^2$. The absolute value $|p_0/\tan\phi|$, appearing in eq 5 derives from the fact that $\tan\phi < 0$ if $p_0 > 0$, and $\tan\phi > 0$ if $p_0 < 0$. Equation 5 shows that α can be calculated from the modulation frequency ω and the experimental values of p_0, τ_0, and $\tan\phi$. These latter values can all be determined by means of the instrument shown schematically in Figure 1, and discussed in greater detail by Spencer and Weber (1969, 1970). Figure 2 shows a plot of $\tan\phi$ against log α for values of $\omega\tau_0 = 1, 2, 3$ according to eq 5. It will be observed that the quadratic dependence of $\tan\phi$ upon α results in two values of ρ, sometimes very different, for each value of $\tan\phi$. In cases of a pure fluorophore in a homogeneous environment the ambiguity is easily solved by a measurement of the steady-state fluorescence polarization, or the relative amplitude of the modulated components, or by the effect of temperature upon $\tan\phi$.

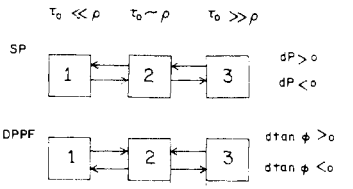

FIGURE 3: Changes in rotational rate that can be deduced from changes in stationary polarization (SP) or differential phase fluorometry (DPPF). Three possible ranges of values are assumed.

TABLE I: Combinations of Changes in $\tan\phi$ and Stationary Polarization.

	dtan $\phi > 0$	dtan $\phi < 0$
dp > 0	3 → 2	2 → 1
dp > 0	1 → 2	2 → 3

We shall be concerned here with the application of the method to complex biological systems, in cases where the aim is the characterization of an unknown environment by measurements of the rotational rate of a small fluorophore of known properties. We propose to combine the measurements of stationary fluorescence polarization with those of differential polarized phase fluorometry with the object of reducing the uncertainty of each separate observation, as follows: to account for the unknown distribution of the probe among different environments, we assume the existence of three main components, or sets of components, each corresponding to one of the boxes in Figure 3. They represent, respectively, the fluorophore populations with $\tau_0 \ll \rho$, $\tau_0 \approx \rho$, and $\tau_0 \gg \rho$. The observation of an increase in $\tan\phi$ (dtan $\phi > 0$) implies that the components of boxes 1 and/or 3 are transferred to the middle box ($\tau_0 \approx \rho$), as is easily decided by inspection of Figure 2. Conversely, a decrease in $\tan\phi$ (dtan $\phi < 0$) would imply that the components in the middle box are transferred to the boxes 1 and/or 3. An increase in stationary polarization (dp > 0) indicates transfer from 3 to 2 and/or 2 to 1. A decrease in stationary polarization (dp < 0) must result from transfers of 1 to 2 and/or 2 to 3. There are, therefore, four possible combinations of changes in $\tan\phi$ and stationary polarization, and they single out one among the possible transfers in the components of the boxes. These are shown in Table I By use of the two types of data, the uncertainty in the changes in distribution attending the sole use of one of the methods may be halved, by limiting the possible changes in distribution to those occurring in the interval $\tau_0 \gg \rho$ to $\tau_0 \approx \rho$ or in the interval $\tau_0 \approx \rho$ to $\tau_0 \ll \rho$. One may expect this reduction in uncertainty to be significant in many cases of biological interest.

Experimental Section

The cells are found to exhibit some fluorescence of their own when excited at 363 nm. This is too small in comparison with the fluorescence of the probe to affect appreciably the results,

TABLE II: Effect of Colicin E1 on Cell Envelope-Bound 1-NPN (2 μM).

Strain[a]	E1 (μg/ml)	Cell Survival	τ_0 (ns)[b]	$\Delta\tau_0$ (ns) (14 MHz)	$\Delta\tau_0$ (ns) (28 MHz)	ρ (ns)
B/1,5	0	1.0	5.6		0.25	0.98
	0.1	3.6×10^{-2}	7.4		0.45	1.90
	1.0	1.4×10^{-5}	7.4		0.48	2.05
	10	5.6×10^{-7}	7.4		0.46	1.93
B/1,5[c]	0	1.0	6.9	0.38		1.55
	1.0	6.1×10^{-5}	8.0	0.75		3.56
A586 *tol* VIII	0	1.0	6.5	0.53		2.38
	1.0	1.0	7.0	0.54		2.38

[a] Cells were grown as described previously (Helgerson et al., 1974) in M-9 minimal media on 0.1% glucose at 20 °C and concentrated 10× in M-9 to approximately 5×10^9 cells/ml. [b] Each τ_0 is the average of phase and modulation measurements made at either 14 or 28 MHz using a cross-correlation subnanosecond fluorometer (Spencer and Weber, 1969). [c] This second set of experiments with strain B/1,5 was run as a control at 14 MHz for the experiments with the *tol* mutant.

presented in Table II, in connection with which several points may be noticed: (1) There is some heterogeneity in the emission of 1-NPN in control cells, as revealed by lifetimes of 6.9 and 5.6 ns obtained at the frequencies of excitation of 14 and 28 MHz, respectively. The heterogeneity all but disappears in the emission from colicin-treated cells. (2) The differential lifetimes are quite small, ranging from 250 to 750 ps. They give unequivocally dtan $\phi > 0$, while dp > 0. Therefore, we see from Table I that the action of E1 colicin produces an increase in rotational relaxation time of 1-NPN from values smaller than τ_0 to values comparable with it. (3) The dependence of the measured differential and average lifetimes and the calculated rotational relaxation time on the presence of colicin is shown for the sensitive strain B/1,5 and tolerant mutant *tol* VIII A586 (we thank Professor S. E. Luria for kindly sending us this strain). In agreement with the static polarization measurements made previously (Helgerson et al., 1974), the rotational relaxation time, ρ, of the probe in the cell envelope is increased approximately twofold in the presence of colicin E1, although the ρ values are somewhat smaller than those determined previously (Helgerson et al., 1974) using static polarization data. It is evident from Table II that the increase in ρ is not a consequence of overloading the cells with colicin, since the final ρ value is independent of colicin concentration varied over two orders of magnitude. The activity of the colicin E1 used in these experiments is approximately 1 part in 50 by protein weight. The effect of the colicin in increasing ρ values appears related to the mechanism of transmission of lethal effects and not simply adsorption, since the $\Delta\tau$ and ρ values of the probe molecules adsorbed to the tolerant mutant are not affected by colicin. E1 also has no effect on the static polarization of the tolerant mutant (data not shown). We cannot attribute any significance in terms of altered envelope structure to the difference in ρ values of the sensitive and tolerant strains, since it is clear from Table II that the absolute ρ values of the sensitive strain B/1,5 are somewhat variable and may be a function of growth conditions or growth phase.

Characterization of the Colicin Effect: Minimal Fluorophore Population and Maximum Rotational Relaxation Times Involved. A more precise analysis of the consequences of the statement that colicin action results in a change in environment such that $\rho_{initial} < \tau_0$; $\rho_{colicin} \simeq \tau_0$, deduced from Table I, may be done as follows: we assume that the fluorophore population contains only two classes of molecules; a fraction f_2 undergoes a homogeneous change, while the complementary fraction, $1 - f_2$, does not change at all when colicin is added. We use the differential phase data to calculate f_{min}, the minimum value of f_2 required to explain the changes. The data of steady-state polarization are then employed to calculate p_{max}, the maximum value of polarization of the emission from the variable fraction f_2. Thus, we can place a lower limit to the fraction of fluorophore that is affected when colicin is added, and an upper limit to the changes in rotational relaxation time that this fraction undergoes. Let the subscripts i and c denote quantities observed initially and after addition of colicin. Then, ϕ_c, the observed phase delay after addition of colicin, arises from the superposition of two photocurrents with delays ϕ_i and ϕ_2, and amplitudes $(1 - f_2)\cos^2\phi_i$ and $f_2\cos^2\phi_2$, respectively, giving

$$\tan\phi_c = \frac{\tan\phi_i\cos^2\phi_i(1-f_2) + f_2(\tan\phi_2\cos^2\phi_2)}{(1-f_2)\cos^2\phi_i + f_2(\cos^2\phi_2)} \quad (6)$$

from which

$$\frac{1}{f_2} = 1 + \frac{\cos^2\phi_2(\tan\phi_2 - \tan\phi_c)}{\cos^2\phi_i(\tan\phi_c - \tan\phi_i)} \quad (7)$$

The maximum value of tan ϕ is observed when the coefficient of 2α of eq 5 equals the square root of the constant term, giving

$$\tan\phi_{max} = \frac{p_0\omega\tau_0}{1 + [(1+\omega^2\tau_0^2)(1-p_0^2)]^{1/2}} \quad (8)$$

This is independent of α, so that tan ϕ_{max} may be calculated from ω, τ_0, and p_0. Using $\tau_0 = 7.4$ ns, $p_0 = 0.38$ (Helgerson et al., 1974) we have

$$\begin{aligned}\tan\phi_{max}\,(14.2\text{ MHz}) &= 0.119\\ \tan\phi_{max}\,(28.4\text{ MHz}) &= 0.198\end{aligned} \quad (9)$$

If ϕ_2 is substituted by ϕ_{max} in eq 7, $f_2 = f_{min}$. From Table II, at 14.2 MHz one calculates from tan $\phi = \omega\Delta\tau$ that tan $\phi_i = 0.034$, tan $\phi_c = 0.067$ for *E. coli* B/1,5 without and with 1 μg/ml of colicin. These and the first of the tan ϕ_{max} values in eq 9 give, from eq 7,

$$f_2 > 0.39$$

For excitation at 28.4 MHz, we use tan $\phi_i = 0.045$, tan $\phi_c = 0.086$. Together with the second value in eq 9 they give

$$f_2 > 0.27$$

We conclude that the minimum fraction of 1-NPN molecules that changes its rotational rate upon colicin addition is one-third. To calculate p_{max}, we introduce a values defined by the relation

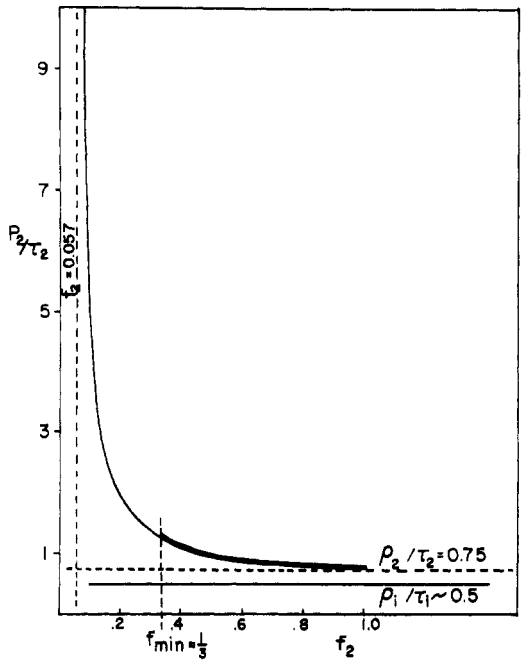

FIGURE 4: The uncertainty limits of f_2, the fraction of fluorophores that change upon colicin addition, and ρ_2/τ_2, the rotational relaxation time that has to be assigned to it from the experimental data. The bold line gives the range of values deduced by use of both methods. The values between the asymptotes $f_2 = 0.057$ and $\rho_2/\tau_2 = 0.75$ give the range of values when stationary polarizations alone are used.

$$a = (1/p - \tfrac{1}{3})^{-1} \tag{10}$$

The addition law of polarizations (Weber, 1953) gives

$$a_c = a_i(1 - f_2) + a_2 f_2 \tag{11}$$

or

$$a_2 = a_i + \delta a/f_2 \tag{12}$$

where

$$\delta a = a_c - a_i$$

If f_2 is substituted by f_{min} we get $a_2 = a_{max}$, or $\rho_2 = \rho_{max}$. The relation between f_2, the fraction of the fluorophore that changes its properties, and ρ_2, the rotational relaxation time that needs to be assigned to it to account for the experimental results, is obtained by combining eq 12 with the Perrin equation. The result is

$$\rho_2/\tau_2 = \frac{3(a_i f_2 + \delta a)}{(a_0 - a_i)f_2 - \delta a} \tag{13}$$

From eq 13 the permissible range of values of ρ_2/τ_2 and f_2 is

$$\frac{3a_c}{a_0 - a_c} < \frac{\rho_2}{\tau_2} < \infty$$
$$1 > f_2 > \frac{\delta a}{a_0 - a_i} \tag{14}$$

The values of p_i are 0.06–0.07 and these of p_c are 0.08–0.09 in preparations of cells concentrated in the same way as in the experiments quoted in Table II. Using as average values: $a_i = 0.066$, $a_c = 0.087$, together with $a_0 = 0.435$, we find

$$0.75 < \rho_2/\tau_2 < \infty$$
$$1 > f_2 > 0.054 \tag{15}$$

The possible pairs of values of ρ_2/τ_2 and f_2 are those read from any point of the curve between the asymptotes $\rho_2/\tau_2 = 0.75$ and $f_2 = 0.05$ in the plot of ρ_2/τ_2 against f_2, according to eq 13 (Figure 4). The observations of differential phase fluorometry restrict the possible values to the range covered by the bold line, namely

$$0.75 < \rho_2/\tau_2 < 1.26$$
$$1 > f_2 > \tfrac{1}{3} \tag{16}$$

The initial value of ρ_2/τ is 0.50, as calculated from the a_i values given above. Therefore, eq 16 shows that the changes affect no less than one-third of the fluorophores and that the increase in the ρ_2/τ_2 ratio of this fraction can be placed between the narrow limits of 1.5- and 2.5-fold. These considerations provide a quantitative basis for the possible interpretation of the physical changes of the cell envelope, associated with deenergization of the membrane by colicin E1.

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