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AN EVALUATION OF *N*-PHENYL-1-NAPHTHYLAMINE AS A PROBE OF MEMBRANE ENERGY STATE IN *ESCHERICHIA COLI*

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SUMMARY

Colicin E1 and the uncoupler of oxidative phosphorylation, trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP), cause an increase in the fluorescence intensity of *N*-phenyl-1-naphthylamine bound to whole cells of *Escherichia coli*. It has been shown elsewhere that this fluorescence increase correlates well with de-energization. Addition of glucose causes a large cyanide-sensitive decrease of intensity, tentatively associated with energization, with the emission spectrum almost returning to the original trace with a peak at 417 nm. These data suggest that there may be a measurable competition between de-energization and energization of the cell membrane, and that the probe fluorescence intensity may be a general indicator of membrane energy level.

The conclusions reached about cellular energy level from measurements of the probe fluorescence intensity correlate partly (a, b below, not c) with the energy level assayed physiologically through rates of active transport: (a) FCCP is found to be a poor inhibitor of proline transport if cells are first incubated with glucose, showing either competition between the processes of energization and de-energization or an increase in the envelope permeability barrier to FCCP caused by glucose addition. (b) Cyanide blocks the fluorescence decrease caused by glucose and inhibits proline and serine transport, consistent with the decrease in probe fluorescence intensity indicating an increase in membrane energization. However, (c) it appears that the amplitude of the fluorescence intensity decrease caused by glucose addition in the presence of FCCP and colicin E1 greatly exaggerates the extent of real membrane energization. Glucose added after uncoupler can cause only a small increase, and after colicin, a negligible increase in the proline transport rate, indicating that the magnitude of the fluorescence intensity decrease after glucose addition is not a true measure of membrane energization, but rather seems to amplify this energization greatly. Glucose addition does not cause a decrease in fluorescence intensity in cells treated with EDTA to remove lipopolysaccharide and an apparent barrier to the probe.

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; PhNap, *N*-phenyl-1-naphthylamine. FCCP, *p*-trifluoromethoxy-carbonylcyanidephenylhydrazone, CCCP, *m*-chloro-carbonylcyanidephenylhydrazone.

The rotational relaxation time of the probe in intact cells appears to correlate somewhat better with the cellular energy level than does intensity.

INTRODUCTION

Derivatives of acridine [1, 2], anilinonaphthalene sulfonate [3], merocyanine [4, 5] and dansyl galactoside [6] have been used as qualitative indicators of the level of membrane energization or energy-linked parameters in bacterial membrane particles and vesicles.

Derivatives of anilinonaphthalene (ANS [7, 8] and PhNap [9–13]) as well as cyanine dye [14] have been used as indicators of energy level in whole cells of *Escherichia coli*. The fluorescence parameter which has been most often used [1–5, 7–10, 13, 14] to indicate energy level is the intensity of fluorescence. In this work, we will concentrate on the question of whether changes in the fluorescence intensity of a particular probe, *N*-phenyl-1-naphthylamine (PhNap), quantitatively reflect changes in membrane energy level in whole cells.

It has been demonstrated that the uncoupler of oxidative and photophosphorylation *p*-trifluoromethoxy-carbonylcyanide-phenylhydrazone (FCCP) and colicin E1, which is one of a class of colicins interfering with energy-requiring cellular processes, both cause an increase in the fluorescence of the lipophilic probe *N*-phenyl-1-naphthylamine [10–12]. It has been shown that the time course of the fluorescence intensity increase caused by the colicin parallels very closely that of biochemical events associated with the de-energization, the decrease in intracellular potassium and ATP pools [10]. Thus, it is documented with respect to these parameters that the fluorescence probe is a reliable monitor of the time course of the cellular de-energization caused by colicin E1. The time course of the fluorescence intensity change caused by FCCP has not yet been correlated with that of biochemical events associated with de-energization. However, the extent of the increase in rotational relaxation time of the probe PhNap caused by FCCP correlates quantitatively with FCCP inhibition of proline transport in whole cells and EDTA-treated cells (Helgerson, S. L. and Cramer, W. A., unpublished). The association of the increase in fluorescence of cell-bound PhNap with a de-energization process is strengthened by the observation that the addition of glucose [13, 14] or succinate or D-lactate [13] to starved or washed cells causes a decrease in the fluorescence intensity. It will, however, be shown in the present work that a decrease in fluorescence intensity associated with apparent reenergization caused by glucose addition does not correlate well with real energization assayed through the ability to carry out active transport.

METHODS

(1) *The strains.* Those used were *Escherichia coli* A_{428} (F^-, *pro*$^-$, *lac*$_1^-$, T_6^R, *gal*$_2^-$, *ara*$^-$, *his*$^-$, *xyl*$^-$, *man*$^-$, B_1^-, *Str*^{R}), *E. coli* ML-308-225, B/1,5 and K12 1100 obtained, respectively, from Dr. D. Gutnick, Dr. H. R. Kaback, Dr. S. Silver and Dr. R. D. Simoni.

(2) *Media and growth conditions.* Cells were inoculated from overnight cultures into medium A containing (g/l): $(NH_4)_2SO_4$, 1; K_2HPO_4, 10.5; KH_2PO_4, 4.5;

$MgSO_4 \cdot 7H_2O$, 0.1, and addition of 1 μg/ml thiamine and 20 μg/ml proline and histidine to A_{428}, 1 μg/ml thiamin to 1100, with 0.2 % glucose as a carbon source, except where indicated. Cultures were grown to mid-logarithmic phase (2–5 × 10^8 cells/ml). The cells were washed once or twice in an equivalent volume of A medium without carbon source, and resuspended in the washing medium.

(3) *Colicin E1.* It was prepared according to the procedure of Schwartz and Helinski [15].

(4) *The time course of cellular de-energization by colicin E1 or uncoupler.* This was monitored by the increase in fluorescence of the envelope (inner plus outer membrane)-bound hydrophobic probe *N*-phenyl-1-naphthylamine(2–5 μM) generally as described previously [10]. Changes in fluorescence intensity were monitored with a Perkin-Elmer Hitachi MPF-2 or MPF-4 spectrofluorimeter with the excitation beam at 352 nm (2–3 nm half band width and the emission measured at 410 or 420 nm (7–10 nm half band width). The cell titer of the stirred cell suspension thermostatted at 30° or 37 °C was approximately 5 · 10^8/ml in a medium without carbon source, amino acids, or vitamins. Additions to the cuvette during the course of fluorescence measurement were made with the photomultiplier tube temporarily shielded.

(5) *Fluorescence lifetime and polarization.* Phase lifetime measurements were made at a modulation frequency of 10 MHz using a cross-correlation phase fluorimeter [17] made by SLM Instruments (Urbana, Illinois, U.S.A.). Polarization measurements were made in a stirred cuvette using a double-ended detector system also designed by SLM. Lifetime and polarization values for different energy states were made on single runs with a common stirred suspension while following fluorescence intensity on an external recorder. The results were calculated with an on-line computer from the average of 3 sets of phase lifetime or polarization measurements, each set of 3 taken in a time of approximately 1 min. Thus, lifetimes and then polarizations were measured before addition of uncoupler, 10 min after uncoupler addition, and then 2 min after subsequent addition of glucose. Rotational relaxation times, calculated from the Perrin equation [18] were determined on-line along with each polarization value.

(6) *Assay of active transport.* Log phase cells were grown for approximately 5 h at 37 °C to a titer of 3–5 · 10^8/ml in the medium with 0.2 % glucose. 10 μl of proline (260 C/mol) were added at a final concentration of 9 μM to 0.2 ml of cells incubated at room temperature (20–22 °C). The cells were removed from suspensions stirred under appropriate conditions. The amino acid cell mixture was diluted with 2 ml of 0.1 M LiCl to stop amino acid uptake, and the diluted cell suspension collected on 0.45 μm membrane filters. The filters were counted on a Nuclear Chicago Model 181B gas flow counter.

(7) *Reagents. p*-trifluoromethoxy-carbonylcyanidephenylhydrazone (FCCP) and *m*-chloro-carbonylcyanidephenylhydrazone (CCCP) were kindly provided by Dr. P. G. Heytler. *N*-phenyl-1-naphthylamine was obtained from Eastman. Amino acids were purchased from Schwarz-Mann.

RESULTS AND DISCUSSION

Fluorescence changes caused by colicin, uncoupler, and glucose; effect of cyanide. The data of Fig. 1A, B show that the addition of the uncoupler FCCP or colicin El

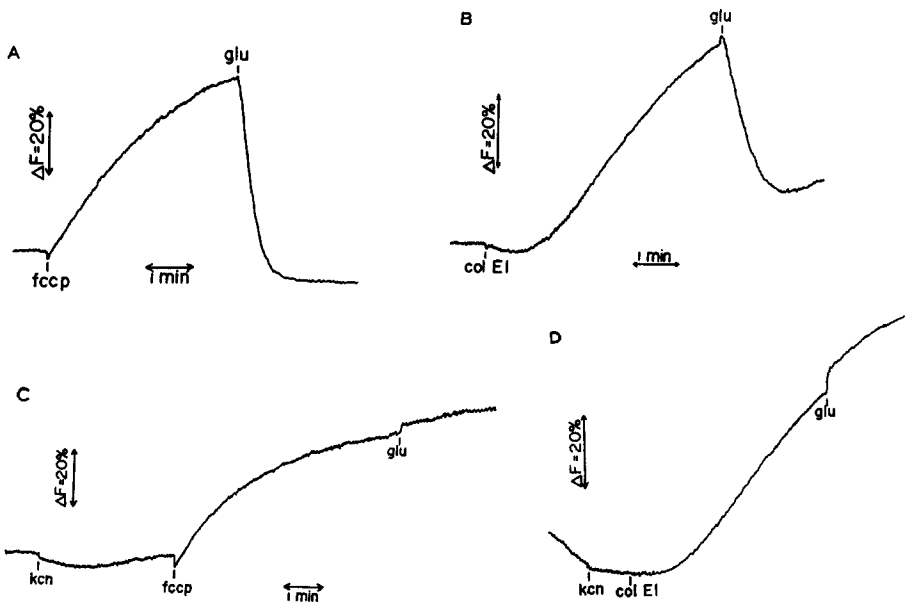

Fig. 1. Reversible changes in the fluorescence of bound *N*-phenyl-1-naphthylamine caused by FCCP or colicin E1 (increasing fluorescence) and glucose (decreasing fluorescence) shown in traces A and B. Effect of cyanide on the glucose reversal, shown in traces C and D. (A) Strain A_{428}, 2 μM FCCP, 5 mM glucose. (B) Strain A_{428}, 0.5 μg/ml colicin E1, 5 mM glucose. Cell survival, <0.01; (C) Strain A_{428}, 0.3 mM KCN (inhibits respiration by over 90 % after 3 min) 2 μM FCCP, 5 mM glucose; (D) Strain A_{428}, 0.6 mM KCN, 0.5 μg/ml colicin E1, 5 mM glucose. *N*-phenyl-1-naphthylamine concentration, 3 μM for uncoupler, and 5 μM for colicin experiments. Cells grown on 0.3 % glucose.

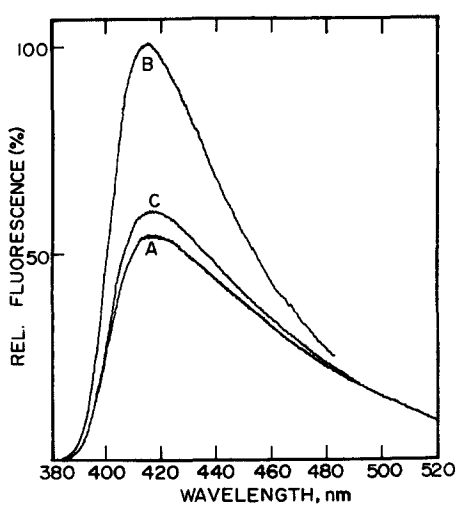

Fig. 2. Fluorescence emission spectrum of *N*-phenyl-1-naphthylamine (5 μM) with strain A_{428} before colicin addition (A), after addition of 0.1 μg/ml colicin E1 and incubation for 10 min with cell survival of $1.0 \cdot 10^{-3}$ (B) and approximately 2 min after subsequent addition of glucose (C). Cell concentration, $3 \cdot 10^8$/ml. Temp., 25 °C.

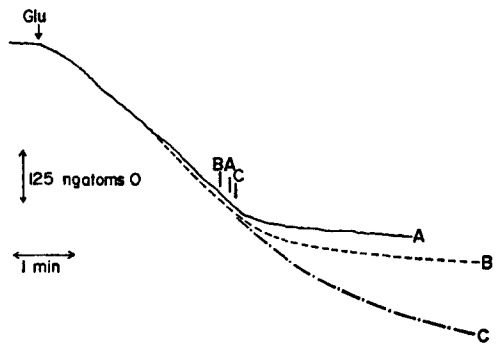

Fig. 3. Time course of the inhibition of respiration of strain *E. coli* K12 1100 by KCN added at concentrations of 0.1 mM (A), 0.3 mM (B), and 1 mM (C).

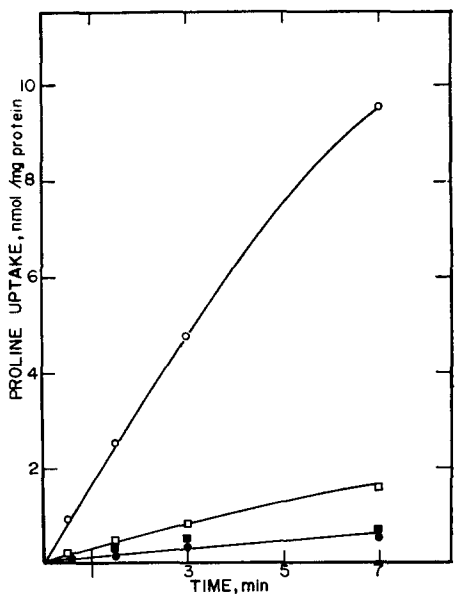

Fig. 4. Inhibition of proline transport by cyanide in strain ML-308-225. 5 min aerobic incubation with (circles) or without (squares) 10 mM glucose, followed by 3 min incubation with 0.5 mM KCN (filled symbols). Aliquots from these four suspensions were then incubated with labelled proline for the indicated times as described in Methods.

to washed cells of wild type strain A_{428} or the ATPase$^-$ mutant N_{144} (not shown) in the absence of a carbon source causes the fluorescence increase of bound *N*-phenyl-1-naphthylamine associated with cellular and membrane de-energization [10, 11]. Subsequent addition of glucose caused the fluorescence intensity level to return at least half-way, and in some cases completely to the original baseline, suggesting that the fluorescence level of bound PhNap may be an indicator of competing processes of energization and de-energization. Qualitatively similar effects of glucose addition on fluorescence intensity have also been shown with a cyanine probe which is supposed to be a specific monitor of membrane potential [14]. The fluorescence emission spec-

trum, increased in amplitude and slightly blue-shifted after addition of colicin E1, returns to its original form with a peak at 417 nm after glucose addition (Fig. 2). We note here, without attempting an explanation, that the effect of glucose is in this respect quite different from that reported by others [13] for the effect of oxygen added as an analogous energizing agent for anaerobic cells. The addition of oxygen in ref. 13 caused a large shift in the emission spectrum to longer wavelengths, resulting in a peak at 445 nm compared to the emission maxima of the initial aerobic or anaerobic suspensions, which are at approximately 405–410 nm.

The data of Figs. 1C, D show that glucose does not cause a decrease in fluorescence intensity in the presence of cyanide. The concentration of cyanide used in the experiments of Fig. 1 is sufficient to inhibit respiration in strain K12 1100 as well as A_{428} by 90 % within 2 min after addition (Fig. 3). The traces of Fig. 1 imply that the fluorescence response caused by colicin E1 is slower in the presence of excess energy (added glucose) than in washed cells without glucose. This glucose effect and the effect of cyanide on the colicin response are of interest since it has been proposed that energy is required to initiate colicin action [18, 19]. From the fact that an amount of cyanide sufficient to inhibit respiration (Fig. 3) and transport (Fig. 4) by approximately 90 % does not inhibit the rate of the fluorescence response to the colicin E1 initially added, we would conclude that the amount of cellular energy necessary to initiate the colicin transmission response is very small. Figs. 1C and D also show that the fluorescence change caused by addition of strongly inhibitory though low concentrations of cyanide cause a very small or negligible change in fluorescence intensity. It is of interest to compare the lack of effect of minimal inhibitory cyanide concentrations used in this work with the small fluorescence intensity changes produced by somewhat higher cyanide concentrations in ref. 13, since it was proposed in ref. 13 that the signal changes produced by KCN, although small, are significant.

The ability of low concentrations of cyanide to inhibit proline transport by 80–90 % (Fig. 4) is consistent with the inference that the cyanide-sensitive fluorescence decrease caused by glucose addition (Fig. 1) is associated with a re-energization. The data in Fig. 4 were obtained with the strain ML-308-225 used in the studies of Berger [20] and Berger and Heppel [21]. Proline transport is appreciably more sensitive to cyanide in our studies than in those of refs. 20 and 21 in which cells previously starved in dinitrophenol were used in order to distinguish between the kind of cellular energization caused by different carbon sources. Exactly the same results shown in Fig. 4 were obtained with the strain A_{428} used in Figs. 1 and 2. We also found that transport of serine was inhibited as strongly as that of proline by 1 mM cyanide. Inhibition of serine transport by 10 mM cyanide had previously been demonstrated [22]. Proline and serine are classified as shock-resistant transport systems [20, 21]. Aerobic proline transport is known to be coupled principally to respiration from earlier work [23]. We have found that glutamine transport, as a shock-sensitive system [20, 21], is inhibited by only 50 % by 1 mM cyanide (data not shown). Thus, it would seem possible that the decrease in fluorescence intensity caused by glucose addition may be a measure of the membrane energy level which can be used to drive the transport of shock-resistant transport systems.

Additional transport studies do lend some support to the existence of a measurable competition between the processes of energization and de-energization. When cells are incubated in glucose for several minutes before addition of the uncouplers

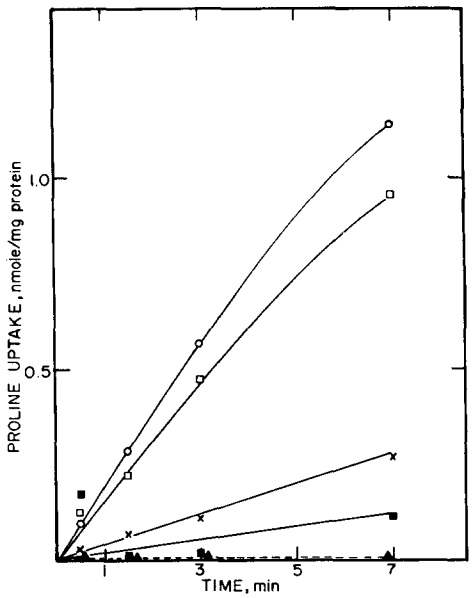

Fig. 5. Effect of glucose added after uncoupler in stimulating proline transport by strain A_{428}: transport was measured by taking 0.2-ml aliquots from a single 5-ml stirred cell suspension ($3 \cdot 10^8$ cells/ml) firstly containing no carbon source (×), after addition of 10 mM glucose and incubation for 5 min (○), and then after addition of 4 μM CCCP and incubation for 15 min (□); in a second set of assays again made with a single 5 ml stirred cell suspension, transport was measured with aliquots taken after a 15 min incubation with 4 μM CCCP (▲), and then 5 min after addition of 11 mM glucose (■).

FCCP or CCCP, the extent of inhibition of transport by the uncoupler is greatly diminished (compare open circles, transport with glucose, and open squares, transport after incubation with glucose and subsequent addition of CCCP, in Fig. 5). An alternate explanation of this glucose effect may reside in the existence of a permeability barrier in the outer membrane to the CCCP compounds (Helgerson, S. L. and Cramer, W. A., unpublished). De-energization appears to cause a decrease in the permeability barrier to molecules like PhNap. It would then be consistent to infer that energization would cause an increase in the extent of a barrier to hydrophobic molecules and that this is the reason that CCCP is less effective when added after glucose in Fig. 5. In any case, it seems of some importance to note this limitation on the efficacy of these commonly used uncouplers.

Further transport experiments show that the fluorescence decrease caused by glucose addition greatly overestimates the level of real membrane energization assayed through transport experiments. The logical analogue of the fluorescence experiment with glucose is to assay transport firstly in the absence of a carbon source, then in the presence of FCCP or colicin which should inhibit the transport, and finally a third assay of the same cell suspension after a short incubation with 10 mM glucose. This experiment is shown in Fig. 5. The transport rate in the absence of glucose in twice washed cells is approximately 20 % of that assayed in its presence. CCCP added in the absence of glucose causes complete inhibition. There is a small and variable in-

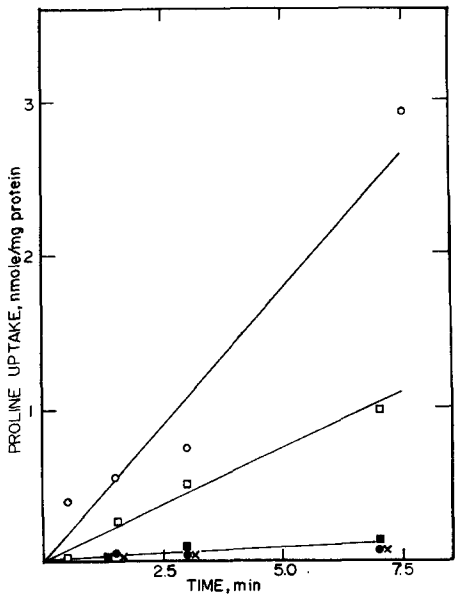

Fig. 6. Effect of glucose added after colicin E1 in stimulating active transport by strain A_{428}. Proline transport by strain A_{428}: aliquots were taken from a stirred cell suspension as in Fig. 3 for assay of transport in the absence of carbon source (×), after addition of glucose (○) and then after addition of colicin (□). Transport was also assayed after a 10-min incubation with colicin (●) and then 3 min after subsequent addition of 10 mM glucose to this sample (■). Survival after colicin (100 ng/ml) treatment, 0.01.

crease in the transport rate when glucose is added after CCCP. This effect of added glucose is quite variable and we have seen transport restored to as much as 50 % of the control rate with glucose. However, the initial rate of proline transport when glucose is added after CCCP is generally of the magnitude shown in Fig. 5, which, at 10 % of the control rate with glucose, is much smaller than the 100 % reversal of the fluorescence intensity caused by glucose addition (Fig. 1A).

In the case of colicin E1, we have never seen any significant restoration of transport caused by glucose added after incubation of the cells for 5–10 min with colicin E1 (Fig. 6). Transport was assayed 3 min after glucose addition in order to see any momentary or transient energization. Thus, the decrease in fluorescence intensity (70 % of the initial rise) caused by glucose addition in the analogous fluorescence experiment (Fig. 1B) greatly exaggerates any real membrane energization. It is noted that it is not surprising that glucose addition cannot reenergize the membrane after 10 min of colicin action. It has been inferred by Nieva-Gomez et al. [13] that addition of glucose to starved cells which have been treated with colicin I_a, results in a momentary energization.

Although the increase in fluorescence intensity of PhNap appears to correlate well with deenergization (ref. 10 and Helgerson, S. L. and Cramer, W. A. unpublished), the decrease in intensity upon glucose addition appears to greatly amplify or exaggerate the extent of real energization. Only the direction of the fluorescence decrease of the probe is indicative of a possible change in energy level upon glucose

TABLE I

Effect of glucose added after colicin E1 (0.1 μg/ml) on the polarization, lifetime, and calculated rotational relaxation time (ρ) of PhNap (5 μM) bound to strain A_{428}. Cell survival, 0.01.

	Lifetime (ns)	polarization	ρ (ns)
A_{428}, no additions	3.5	0.099	3.1
A_{428}, +E1, 10 min	5.3	0.180	11.9
A_{428}, +E1, 10 min, +glucose, 3 min	4.3	0.178	9.4

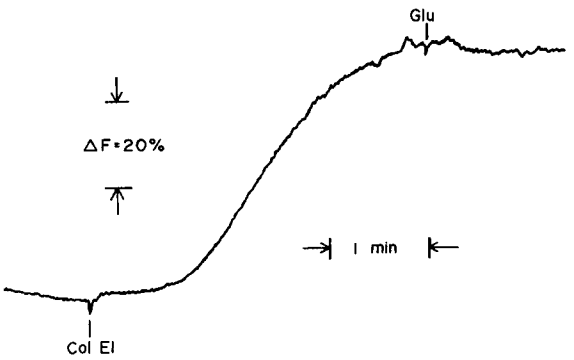

Fig. 7. Comparative effect of glucose in causing a decrease in fluorescence of *N*-phenyl-1-naphthylamine in the presence of colicin E1 using EDTA-treated strain B/1,5. Cells were grown with 1 % glycerol as carbon source. Cells were washed twice with 0.12 M Tris · HCl buffer, pH 8, concentrated three-fold and resuspended in the same Tris buffer. Cells were treated for 1.5 min with 0.1 mM EDTA at pH 8.0. EDTA treatment was stopped by adding a tenfold excess of M9 (Mg^{2+}-containing) medium, and washed twice in the same medium. Colicin concentration, 0.1 μg/ml. Dye concentration, 2 μM. Cells grown and handled identically except for the EDTA-treatment show the fluorescence trace of Fig. 1B.

addition. Another fluorescence parameter, the rotational relaxation time of the probe, derived from lifetime and polarization measurements, appears to correlate somewhat better but still not really quantitatively with the energy level of cells treated with colicin and glucose (Table 1). The lifetime, polarization, and calculated rotational relaxation times of the fluorescence probe are found to be 3.5 ns, 0.099, and 3.1 ns with strain A_{428}, before any additions are made. Addition of 0.1 μg/ml colicin E1 causes an increase in the lifetime to 5.3 ns, an increase of approx. 50 %, while the increase in fluorescence intensity was 80–90 % during this time. The addition of colicin E1 caused the polarization and rotational relaxation time to increase to 0.180 and 11.9 ns. This increase in rotational relaxation time caused by colicin has been measured previously by the steady-state polarization technique [12], and as well by differential polarized phase fluorimetry [24]. The addition of glucose in this experiment caused approximately 75 % of the colicin-induced fluorescence intensity increase to be reversed (as in Fig. 1). The decrease in fluorescence lifetime to 4.3 ns reversed slightly more than half of the original increase with colicin. There was, however, very little change

in the measured polarization and the decrease in calculated rotational relaxation time (to 9.4 ns) was only about 30 % of the original change caused by colicin. That the polarization does not change after glucose addition, and that the calculated rotational relaxation moves only 30 % of the way toward the original value before colicin addition shows, in spite of the intensity and emission spectra data of Figs. 1B and 2, that glucose does not cause the cell envelope to return to the original state which existed before colicin addition.

The *N*-phenyl-1-naphthylamine appears to occupy sites in the cell envelope where the fluorescence intensity effectively amplifies energization events which occur in the inner membrane. Other studies involving comparison with EDTA-treated cells (Helgerson, S. L. and Cramer W. A., unpublished) indicate that the lipopolysaccharide barrier of the outer membrane forms a barrier to *N*-phenyl-1-naphthylamine. De-energization by FCCP may remove much of the outer membrane barrier to PhNap, resulting in dye movement toward the inner membrane. Colicin E1 may affect the barrier to the dye in a similar way, though not to as large an extent as does FCCP. The fluorescence decrease caused by glucose may then be caused by dye moving outward through the permeability barrier. EDTA-treated cells, which have lost the lipopolysaccharide and the permeability barrier, do not show a decrease in PhNap fluorescence intensity upon glucose addition (Fig. 7). The sites of dye binding in EDTA-treated cells would seem to be less variable than in untreated cells. Thus, the probe fluorescence intensity of the EDTA-treated cells seems to report more closely the real level of energization of the inner membrane.

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REFERENCES

1 Eilermann, L. J. M. (1970) Biochim. Biophys. Acta 216, 231–233
2 Nieuwenhuis, F. J. R. M., Kanner, B. I., Gutnick, D. L., Postma, P. W. and Van Dam, K. (1973) Biochim. Biophys. Acta 325, 62–71
3 Reeves, J. P., Lombardi, F. J. and Kaback, H. R. (1972) J. Biol. Chem. 247, 6204–6211
4 Laris, P. C. and Perhadsingh, W. A. (1972) Biochem. Biophys. Research Commun. 57, 620–626
5 Kashket, E. R., and Wilson, T. H. (1974) Biochem. Biophys. Research Commun. 59, 879–886
6 Schuldiner, S., Spencer, R. D., Weber, G., Weil, R. and Kaback, H. R. (1975) J. Biol. Chem. 250, 8893–8896
7 Aithal, H. N., Kalra, V. K. and Brodie, A. F. (1974) Biochemistry 13, 171–177
8 Griniuviene, B., Dzheia, P. and Gsinius, L. (1975) Biochem. Biophys. Res. Commun. 64, 790–796

9 Cramer, W. A. and Phillips, S. K. (1970) J. Bacteriol. 104, 819–825
10 Phillips, S. K. and Cramer, W. A. (1973) Biochemistry 12, 1170–1176
11 Cramer, W. A., and Phillips, S. K., and Keenan, T. W. (1973) Biochemistry 12, 1177–1181
12 Helgerson, S. L., Cramer, W. A., Harris, J. M. and Lytle, F. E. (1974) Biochemistry 13, 3057–3061
13 Nieva-Gomez, D., Konisky, J. and Gennis, R. B. (1976) Biochemistry 15, 2747–2753
14 Brewer, G. (1976) Biochemistry 15, 1387–1392
15 Schwartz, S. and Helinski, D. R. (1971) J. Biol. Chem. 246, 6318–6327
16 Spencer, R. D. and Weber, G. (1969) Ann. N.Y. Acad. Sci. 158, 361–376
17 Weber, G. (1953) Adv. Prot. Chem. 8, 416–456
18 Plate, C. A. and Luria, S. E. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2030–2034
19 Jetten, A. M. and Jetten, M. E. R. (1975) Biochim. Biophys. Acta 387, 12–22
20 Berger, E. A. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1514–1518
21 Berger, E. A. and Heppel, L. A. (1974) J. Biol. Chem. 249, 7747–7755
22 Van Thienen, G. and Postma, P. W. (1973) Biochim. Biophys. Acta 323, 429–440
23 Klein, W. L. and Boyer, P. D. (1972) J. Biol. Chem. 247, 7257–7265
24 Weber, G., Helgerson, S. L., Cramer, W. A. and Mitchell, G. (1976) Biochemistry, in the press
25 Lieve, L. (1967) J. Biol. Chem. 243, 2373–2380