Changes in *Escherichia coli* Cell Envelope Structure and the Sites of Fluorescence Probe Binding Caused by Carbonyl Cyanide *p*-Trifluoromethoxyphenylhydrazone†

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ABSTRACT: (1) In cells of *E. coli* strain ML 308-225, the inhibition of [^{14}C]proline active transport by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) increases with uncoupler concentration from ~20% at 2 μM to ~100% at 5 μM. (2) The increase in the rotational relaxation time (ρ) of the fluorescent probes *N*-phenyl-1-naphthylamine (PhNap) and 8-anilino-1-naphthalenesulfonate (ANS) under these conditions shows the same functional dependence on FCCP concentration as does the decrease in active transport. The ρ values of both ANS and PhNap increase by a factor of 3–3.5 when the cells are treated with 5 μM FCCP. (3) The ρ values of PhNap and ANS in membrane vesicles are increased by 6–10 times relative to cells without FCCP and, for ANS, are decreased in D_2O, and as well by relatively high concentrations of FCCP. (4) When the cells are treated with 0.1 mM ethylenediaminetetraacetic acid (EDTA), complete inhibition of [^{14}C]proline transport is achieved at 0.3 μM FCCP. This treatment removes a permeability barrier to FCCP since active transport of [^{14}C]proline by ML 308-225 membrane vesicles is also completely inhibited by 0.3 μM FCCP. (5) EDTA treatment results in an increase in the ρ value of PhNap, approaching the value obtained in membrane vesicles, which is only slightly affected by FCCP; EDTA treatment only slightly affects the ρ value of ANS, which again increases in the range of FCCP concentrations (0.1–0.3 μM) which are inhibitory to active transport. (6) It is shown that, although changes in binding of PhNap upon addition of FCCP are significant, they do not account for changes in polarization, lifetime, and ρ value of PhNap. Changes in the fluorescence parameters of ANS are not complicated by dye binding changes. (7) It is concluded that in untreated cells PhNap and ANS monitor structural changes in the cell envelope exterior to the inner membrane, with PhNap localized outside an EDTA-sensitive permeability barrier. FCCP action to deenergize the inner membrane causes a structural change in the outer membrane which removes a permeability barrier or gate to the lipophilic PhNap. The structural change is also monitored by changes in the fluorescence parameters of amphiphilic ANS. Deenergization of the inner membrane thus results in dynamic structural changes which affect the outer membrane.

Many aspects of the static structure of the *Escherichia coli* cell envelope have been described (Schnaitman, 1971; Costerdon et al., 1974; Braun, 1976). It consists of an outer cell membrne, a peptidoglycan layer, an inner cytoplasmic membrane, and connective structures between the peptidoglycan layer and outer membrane. It might be hypothesized that the envelope also has a dynamic structure in which structural changes are associated with alterations in particular biosynthetic activities which reside in the cell membrane or envelope. The existence of connections between different layers of the envelope suggests the possibility that activity changes in one of the layers of the envelope may affect the structure of another layer.

Respiration, energy transduction, and certain transport activities are localized in the inner or cytoplasmic membrane. These activities may be perturbed and inhibited by uncouplers of oxidative phosphorylation. It is of interest to inquire whether particular uncouplers or bacteriocidal agents which inhibit the membrane energy level, and for which there is a precedent for inferring an interaction with the cell membrane, will cause structural changes in the membrane or envelope. Certain uncouplers, such as the substituted carbonyl cyanide phenylhydrazones, are thought to inhibit oxidative phosphorylation and active transport in whole cells by increasing the proton permeability of the cytoplasmic membrane (Pavlasova and Harold, 1969; Scholes and Mitchell, 1970). These compounds similarly inhibit active transport in purified inner membrane vesicles (Barnes and Kaback, 1970; Kaback et al., 1974).

Fluorescent probes are useful monitors of their environment and of relevant changes in biological membrane systems. Noncovalently bound probes have been used to measure the rotation of macromolecules in solution (Anderson and Weber, 1969), the microviscosity within lipid micelles (Shinitzky et al., 1971; Cogan et al., 1973) and lipid bilayers (Jacobson and Wobschall, 1974), the energy state of mitochondria and submitochondrial particles (Chance, 1970; Brocklehurst et al., 1970; Azzi et al., 1971), and the energy state of bacterial membrane vesicles (Reeves et al., 1972). However, with these probes the interpretation of the response can be complicated by uncertainty about the location of the probe and as well by fluorescence intensity changes which are either nonspecific or which do not correlate linearly with the physiological effects of the perturbing agent (Cramer et al., 1976). In the bacterial cell envelope, the fluorescence emission properties of the probe can change due to (a) movement of the probe from the external medium into the envelope, (b) movement of the probe from one binding site to another within the envelope, and (c) a change in the environment around the probe molecule (Weber et al., 1976). In order to deal with these problems, measurements of

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fluorescence intensity must be combined with assay of other fluorescence parameters, such as the polarization and lifetime, which relate more directly to membrane structural properties (Helgerson et al., 1974; Weber et al., 1976) and as well changes in dye binding (Cramer and Phillips, 1970; Nivea-Gomez et al., 1976). Changes in fluorescence parameters must also be correlated with changes in physiological parameters as a function of added perturbant.

It has been inferred from studies with noncovalently bound fluorescence probes that structural changes in the cell envelope are associated with deenergizing effect of both the nonfluorescent uncoupler FCCP[1] and colicin E1. FCCP causes an increase in the fluorescence intensity of cell-bound ANS (Cramer and Phillips, 1970). Colicin E1 causes an increase in the emission intensity of ANS and also of the lipophilic probe PhNap. The time courses of the intensity changes with the latter probe were found to correlate well with those of the decrease in intracellular ATP and potassium levels (Phillips and Cramer, 1973). FCCP and colicin E1 cause an increase in the rotational relaxation time of PhNap (Helgerson et al., 1974), which in the case of colicin E1 has also been measured using differential polarized phase fluorometry (Weber et al., 1976). When corrected for changes in probe binding and environment, a change in the rotational relaxation time indicates a change in the microviscosity of the environment occupied by the probe.

The experiments presented here are concerned only with dynamic structural changes in the cell envelope caused by the uncoupler FCCP. Deenergization of the inner membrane, assayed through inhibition of active transport of proline, is closely associated with structural changes exterior to the inner or cytoplasmic membrane as assayed through changes in probe rotational relaxation time. The work presented here complements that presented elsewhere on structural changes in the cell envelope caused by uncouplers and colicin E1 (Helgerson and Cramer, 1976).

Methods

(1) *Strains, Media, and Growth Conditions. Escherichia coli* ML 308-225 were grown in medium A containing (grams/liter): $(NH_4)_2SO_4$, 1.0; sodium citrate·$2H_2O$, 0.6; $MgSO_4$, 0.1; K_2HPO_4, 7.0; KH_2PO_4, 3.0; succinate (adjusted to pH 7.0 with KOH), 10.0 g; pH 7.0. *E. coli* AN120 *unc* A (ATPase$^-$) and AN180 (ATPase$^+$) were grown in M9 salts medium containing (grams/liter): NH_4Cl, 1.0; $MgSO_4$, 0.13; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; glycerol, 10.0; pH 7.0. All cultures were grown at 37 °C to a titer of $\sim 5 \times 10^8$ cells/mL, washed twice in M9 medium without carbon source, and resuspended in M9 without carbon source to the original titer.

(2) *Preparation of EDTA-Treated ML 308-225 Cells and ML 308-225 Membrane Vesicles.* ML 308-225 whole cells were treated with 0.1 mM K-EDTA (pH 7.0) for 1.5 min at room temperature essentially as described in Leive (1967). The fluorescence intensity and polarization of 2 μM PhNap were measured in all EDTA-treated cell preparations before use in transport experiments. EDTA treatment results in an increase of two- to three-fold in the intensity and a twofold increase in the polarization of 2 μM PhNap in the EDTA-treated cells relative to control cells (see text). Membrane vesicles were prepared from ML 308-225 cells as described by Kaback (1971). The membrane vesicles used in the fluorescence experiments and in some transport experiments were kindly supplied by Dr. H. R. Kaback. Membrane vesicles were stored in liquid nitrogen at 4 mg of membrane protein/mL and never refrozen for subsequent use.

(3) *Fluorescence Polarization and Lifetime Measurements.* Fluorescence polarization was measured with the front-surface fluorometer previously described (Helgerson et al., 1974). The 360-nm excitation light was defined by a Bausch and Lomb high-intensity monochromator with ultraviolet grating and deuterium source and a Corning 7-39 filter. Emission was defined by a 2-mm thick $NaNO_2$ liquid filter and a Corning 3-74 filter. The polarization, $p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, is defined relative to vertically polarized exciting light. The cuvette was thermostated at 21 °C and constantly stirred during measurements using a magnetic stirring bar. All polarization and lifetime values are corrected for the highly polarized background fluorescence and scattered light from the cells. In all cases the intensity of this background was less than 15% of the intensity after adding the probe.

Fluorescence lifetimes were measured with an subnanosecond fluorometer (SLM Instruments, Urbana, Ill.) operating at a light modulation frequency of 10 MHz (Spencer and Weber, 1969). The 360-nm excitation light was defined by a Bausch and Lomb 150-W xenon lamp and a 360-nm interference filter. Emission was defined by the same filter combination used in the fluorescence polarization measurements. The cell samples were mechanically stirred between measurements. An individual lifetime measurement took approximately 2 min to complete. The cuvette was thermostated at 21 °C.

(4) *Assay of [^{14}C]Proline Transport by ML 308-225 Whole Cells, EDTA-Treated Cells, and Membrane Vesicles.* A 1.0-mL sample containing cells (titer $\sim 5 \times 10^8$, ~0.08 mg of total protein/mL) or membrane vesicles (0.2 mg of membrane protein/mL) was incubated at room temperature with stirring. After 8 min in the presence of FCCP, D-lactate was added to a final concentration of 10 mM and the incubation continued for 5 min. Sample aliquots of 0.2 mL were then added to test tubes containing 10 μL of [^{14}C]proline (specific activity 260 mCi/mmol, final concentration 10 μM). Transport was terminated by diluting the samples with 2 mL of 0.1 M LiCl and filtering the diluted sample through a Millipore filter. The test tube and filter were washed once with 2 mL of 0.1 M LiCl. After being glued to aluminum planchets and dried, the radioactivity was counted using a Nuclear-Chicago gas flow counter operating at 10% efficiency. A control sample was prepared by first diluting the cells, adding the [^{14}C]proline, and filtering immediately. The counts obtained from this sample were subtracted from all sample counts to correct for nonspecific binding. Aliquots of each sample were added to four separate test tubes and transport was terminated at 0.5, 1.5, 3.0, and 5.0 min.

(5) *Measurement of the Fluorescent Probe Binding to Cells.* The binding of the probes was measured by centrifugation essentially as described previously (Cramer and Phillips, 1970). However, the amount of probe bound to the cell pellet and free in solution was determined by making the respective supernatant samples 3% in Triton X-100 and measuring the fluorescence intensity. In the presence of 3% Triton X-100, both ANS and PhNap are highly fluorescent with emission peaks at 490 and 430 nm, respectively. The relative fluorescence levels of the supernatant samples were read on the scanning fluorometer described previously (Phillips and Cramer, 1973). A standard curve of fluorescence intensity vs. probe concentration in 3% Triton X-100 showed that 1 μM ANS and 0.5 μM PhNap could be accurately detected. Readings were corrected for the experimentally determined decrease in intensity

[1] Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ANS, 8-anilino-1-naphthalenesulfonate; PhNap, *N*-phenyl-1-naphthylamine; EDTA, ethylenediaminetetraacetate.

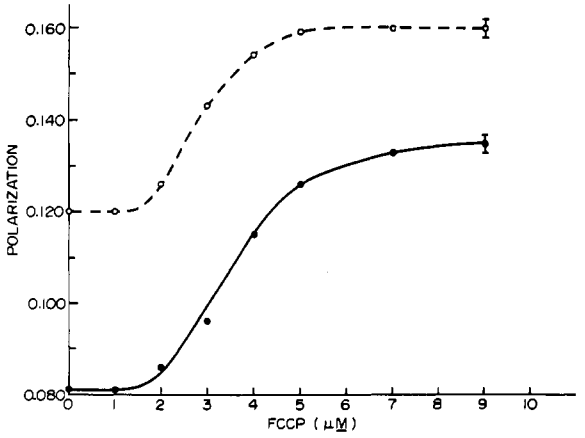

FIGURE 1: Fluorescence polarization of 50 μM ANS (open circles, dashed line) or 5 μM PhNap (closed circle, solid line) bound to the *E. coli* ML 308-225 cell envelope vs. the final FCCP concentration present in the sample mixture. FCCP was added in successive 1 μM increments to obtain the concentrations shown, and the polarization was measured 6 to 8 min after each addition. The cell titers before and after the PhNap experiment were 6.0×10^8 and 6.2×10^8, respectively. Samples incubated under the same conditions for ~1 h without the addition of FCCP showed no increase in polarization. Temperature was 21 °C. Uncertainty in measurements given by error bar on last point.

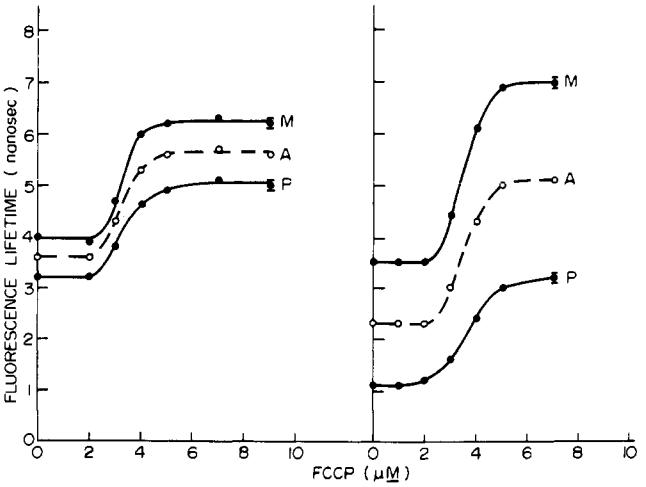

FIGURE 2: Fluorescence lifetimes of 50 μM ANS (right) or 5 μM PhNap (left) bound to the ML 308-225 cell envelope vs. final FCCP concentration. The experimental procedure is as described in Figure 1. Individual lifetime measurements are accurate to ±0.1 ns, but the lifetime varied by ±0.5 ns in different cell samples. The cell titer was 5.0×10^8. Temperature was 21 °C. *M* is lifetime measured by change in modulation; *P*, lifetimes measured through phase shift; *A*, average of phase and modulation measurements (open circles, dashed line).

due to light absorption by FCCP. Control experiments showed that no fluorescent material was released from the cells under these conditions. After correcting for the small amount of probe lost due to experimental manipulations (<2%) and for the dilutions involved in making the supernatants 3% in Triton X-100, the probe bound (%) was obtained from the ratio of the intensity of probe in the pellet to the intensity of probe in pellet plus supernatant.

Results

Effect of FCCP on Fluorescence Parameters of ANS and PhNap with Untreated Cells. The fluorescence polarization of 50 μM ANS and 5 μM PhNap in the presence of cells of strain ML-308-225 is plotted as a function of the concentration of FCCP present in the cell suspension (Figure 1). A concentration of 5 μM FCCP causes the amplitude of the fluorescence emission peak of ANS and PhNap to increase by a factor of 1.6 and 2.3, respectively, the emission peak of ANS to shift from 510 to 500 nm and that of PhNap from 415 to 405 nm (data not shown). Fluorescence from FCCP is completely negligible in these experiments, and the only effect of FCCP on the PhNap fluorescence is to cause a decrease in intensity which can be exactly predicted from the absorbance of FCCP ($\epsilon_m = 1.6 \times 10^4$ M^{-1} cm^{-1} at 360 nm in methanol). The fluoroscence polarization values of 0.120 for ANS and 0.081 for PhNap obtained in the absence of FCCP are not affected by FCCP concentrations <2 μM and increase rapidly to maximum values of 0.160 for ANS and 0.135 for PhNap at 5–7 μM FCCP. Since FCCP could cause a decrease in intracellular ATP levels, the increase in polarization might be a consequence of that action. However, the fluorescence polarization of both ANS and PhNap responds identically with FCCP in cells of strain AN120 *unc* A and AN180 (Helgerson, 1976). AN120 *unc* A is a mutant showing less than 5% of the ATPase activity of the parent strain AN180 (Butlin et al., 1971).

The fluorescence lifetimes of ANS and PhNap as determined by phase shift and modulation change, measured as a function of FCCP concentration with cells of ML 308-225, are shown in Figure 2. The average lifetime increases from 3.6 to 5.5 ns for PhNap and from 2.3 to 5.0 ns for ANS as the concentration of FCCP is increased from 2 to 5 μM FCCP. Both the fluorescence polarization and lifetime of the cell-bound probes show the same dependence on FCCP concentration. Fluorescence lifetimes measured by phase and modulation will be the same if the population of emitters is homogeneous but will differ if the emission is heterogeneous (Spencer and Weber, 1969). For example, the fluorescence lifetime of ANS in ethanol is found to be 8.1 and 8.0 ns as determined by phase and modulation change at a frequency of 10 MHz; the phase and modulation lifetime of PhNap in methanol, determined similarly, is identically 5.0 ns. In untreated cells, EDTA-treated cells, and membrane vesicles, the phase and modulation lifetimes are split indicating a heterogeneous emission. Since in all cases the phase and modulation lifetimes show the same dependence on FCCP concentration, the average value will be used in all calculations of rotational relaxation times.

The increased values of both p and τ in the presence of FCCP indicate a change in the rotational relaxation time (ρ) of the probes. The rotational relaxation time of a fluorescent molecule can be calculated using the Perrin equation (Perrin, 1926, 1929; Weber, 1953):

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right) \quad (1)$$

where p_0 is the limiting polarization of a rigid, randomly oriented population of emitters, and τ is the lifetime of fluorescence; ρ is the rotational relaxation time of the sphere, defined as the mean time required for movement of one axis of the sphere in space through an angle of arc cos $1/e$. Using the Perrin equation, the rotational relaxation times of ANS and PhNap in cells of strain ML 308-225 have been calculated and are shown in Figure 3 as a function of FCCP concentration. The rotational relaxation time of an equivalent sphere is defined as $\rho = 3\eta V/kT$ (Perrin, 1926, 1929; Weber, 1953) where η and V are the equivalent viscosities and volume, respectively. The temperature is constant in these experiments, so the threefold increase in rotational relaxation times from 2–3 to 7–8 ns shown in Figure 3 indicates that FCCP causes an increase in the microviscosity of the environment occupied by the probes.

Correction for Changes in Dye Binding. Nivea-Gomez et

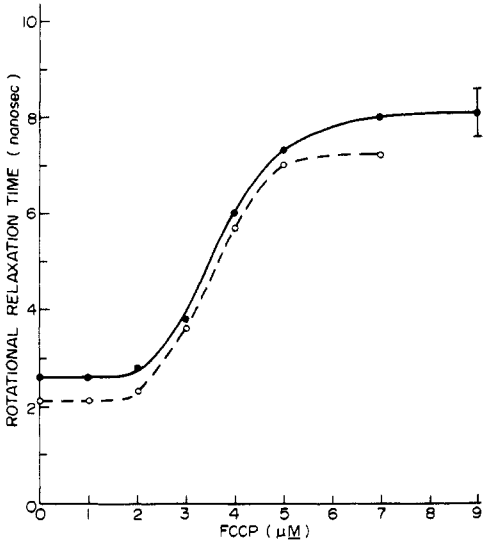

FIGURE 3: FCCP-induced increase in the rotational relaxation time (ρ) of 50 μM ANS (open circles, dashed line) or 5 μM PhNap bound to the ML 308-225 cell envelope calculated from the Perrin equation using the fluorescence polarization values shown in Figure 1 and the average lifetime values shown in Figure 2. An increase in ρ indicates an increase in the microviscosity of the environment of the probe.

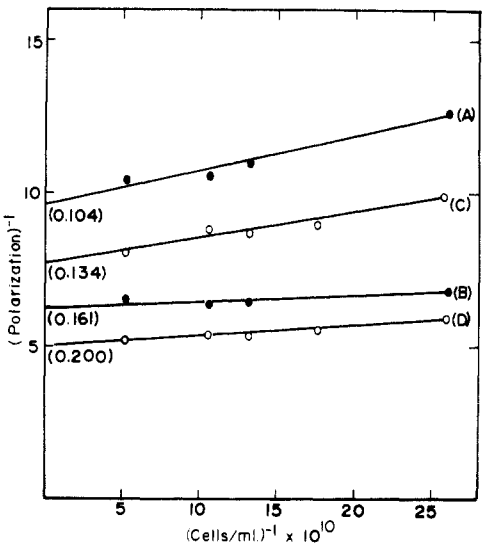

FIGURE 4: Double reciprocal plot of the fluorescence polarization of PhNap and ANS bound to ML 308-225 cells vs. cell titer. Additions are (A) 5 μM PhNap, (B) 5 μM PhNap + 5 μM FCCP, (C) 50 μM ANS, and (D) 50 μM ANS + 5 μM FCCP. Temperature was 25 °C. Polarizations were corrected for the background of scattered light from the cell suspension. The values of the polarization obtained by extrapolation to infinite cell titer are shown in parentheses. Limiting protein concentrations were 0.30–0.06 mg/mL.

TABLE I: FCCP-Induced Changes in the Binding of ANS and PhNap to ML 308-225 Whole Cells and EDTA-Treated Cells.

Sample	ANS bound (%)[a]	PhNap bound (%)[a]
Whole cells	2.8 ± 0.4	6.6 ± 1.6
Whole cells + 5 μM FCCP	3.2 ± 0.2	19.5 ± 1.1
EDTA-treated cells	3.3	20
EDTA-treated cells + 0.3 μM FCCP	3.1	*b*

[a] Standard deviation based on four separate experiments. [b] There is no change in fluorescence intensity upon adding FCCP to EDTA-treated cells.

al. (1976) have concluded that the changes in fluorescence parameters of PhNap caused by agents disrupting energy transduction can be attributed solely to an increase in dye uptake. Changes in the amount of PhNap and ANS bound to ML 308-225 cells and EDTA-treated cells due to FCCP are shown in Table I. Treatment with EDTA, according to the method of Leive (1965, 1967) is known to remove lipopolysaccharide from the outer membrane and to increase cell permeability to molecules such as actinomycin D. The small increase (10%) in binding of the probe ANS to cells caused by 5 μM FCCP is not sufficient to account for the observed intensity changes. Also, with EDTA-treated cells the addition of 0.3 μM FCCP causes no uptake of ANS even though the increase in the rotational relaxation time is the same as in untreated cells containing 5 μM FCCP. It can be concluded that uptake of ANS from the external medium into the cell envelope is not involved in the structural changes monitored by ANS. In agreement with the results of Nivea-Gomez et al. (1976), addition of 5 μM FCCP to cells causes a large increase (2.9×) in the amount of PhNap bound to the cell envelope. This same increase is also observed when EDTA-treated cells are compared with untreated cells. Although the uptake of PhNap from the external medium when cells are treated with FCCP or EDTA is an important factor in interpreting the structural changes monitored by the probe PhNap, as first pointed out by Nivea-Gomez et al. (1976), the data presented by these authors (e.g., their Table I) do not support the conclusion that the increase in PhNap polarization caused by deenergization can be explained solely in terms of dye uptake. Two other cases in which it can be shown fairly simply that the fluorescence changes caused by the deenergizing agent cannot be explained by dye uptake are (a) the fluorescence changes caused by colicin E1 acting on EDTA-treated cells, where there is no change in dye binding, and (b) the effect of FCCP in removing the apparent lipid phase transition in intact cells (Helgerson and Cramer, 1976). The analysis of changes in PhNap fluorescence parameters tends in other instances to be complicated by the problem of calculating the contribution of free and bound probe to the total fluorescence. Due to light scattering by the turbid cell suspension, comparison of the fluorescence intensity from the probe in the presence and absence of cells can be highly misleading as to the real contribution of the free probe. The extrapolation of PhNap fluorescence intensity to infinite cell titer can provide evidence for an increase in quantum yield caused by addition of the uncoupler FCCP (Helgerson and Cramer, 1976). A similar extrapolation to infinite cell titer can also provide an estimate of the polarization of bound PhNap and ANS (Figure 4). Double reciprocal plots of the polarization of both probes vs. cell concentration show an increase in the value of the polarization extrapolated to infinite cell concentration in the presence of FCCP. We will only discuss the PhNap data in detail since ANS binding in any case does not change significantly upon deenergization. It should be emphasized that all polarization measurements reported in this work were measured using a front-surface fluorometer to minimize depolarization due to light scattering within the cell suspension. The range of cell concentrations which can be used is limited on the low side by intensity and on the high side ($\sim 2 \times 10^9$ cells/mL) by anaerobiosis and scattering. The double reciprocal plots of PhNap polarization are linear over the range of concentrations used, where the extent of dye binding is large enough so that fluorescence of free dye cannot be resolved in the emission spec-

TABLE II: Calculation of the Fraction of Intensity from Probe Bound to the Whole Cell Envelope.[a]

Sample	FCCP (5 μM)	P_∞	P_F	P	f_B
Cells + 5 μM PhNap	(−)	0.108	0.009	0.090	0.82
	(+)	0.170	0.009	0.160	0.94

[a] Data from Figure 4. P, measured anisotropy at 6×10^8 cells/mL; P_∞, anisotropy at infinite cell titer; P_F, anisotropy of free probe; f_B, fraction of intensity from bound probe.

trum. The polarization values of PhNap extrapolated to infinite cell concentration are 0.104 and 0.161 in the absence and presence of 5 μM FCCP, thus demonstrating that the polarization of bound PhNap is increased in the presence of FCCP.

The polarization values extrapolated to infinite cell concentration can be used to estimate the weighted intensity of the bound dye at a particular cell concentration. At the cell concentration used for the dye binding experiments, 6×10^8 mL, the measured polarizations in Figure 4 are 0.088 and 0.153, respectively, in the absence and presence of FCCP. From these polarization values, and the fact that the polarization of the free dye in solution is 0.009, it is immediately obvious that the bound dye contributes most of the intensity. The fraction of the intensity, f_B, which arises from the bound probe can be calculated using the addition law of polarizations (Weber, 1953)

$$f_B = \frac{P - P_F}{P_\infty - P_F} \quad (2)$$

where the anisotropy, P, is defined in terms of the polarization, p, as

$$P = \left(\frac{1}{p} - \frac{1}{3}\right)^{-1} \quad (3)$$

and the subscripts are defined in Table II. As shown in Table II, the calculated fraction of intensity, f_B, from bound PhNap is found to be 0.82 in the absence of FCCP. We would emphasize that this calculation is somewhat qualitative, and that a value of 0.75 is obtained in this case if the polarization data of Figure 1 is used. Using the data of Figure 4, the value of f_B increases from 0.82 to 0.94 in the presence of FCCP. These calculations again imply that the changes in fluorescence parameters caused by FCCP arise from changes in the environment around the dye as well as dye uptake.

The values of f_B calculated above, together with the measurements of fractional dye binding shown in Table I, allow an estimate of the relative fluorescence emission yield of the bound vs. free dye, not corrected for instrument response, of 40–80. Ballard et al. (1972) have reported that the quantum yield of PhNap increases by a factor of 23.5 in hexane relative to water. Our own measurements (not shown) show that the emission yield of PhNap is enhanced 95-fold in dioxane relative to water between 380 and 500 nm, with the spectrum corrected for source output but not detector sensitivity. Turner and Brand (1968) report that the quantum yield of ANS is increased by 180 times in dioxane relative to H_2O. Thus, the calculated values of f_B are not unreasonable relative to emission yield measurements.

One could use the values of f_B derived from Figure 4 to make a self-consistent calculation to correct the measured values of the polarization in Figure 1 to values for the bound probe. The polarization values obtained in this way are 10–20% higher than the corresponding measured values. Since sample reproducibility from day to day is only about 10%, the measured polarization values will be used for calculation of rotational relaxation time.

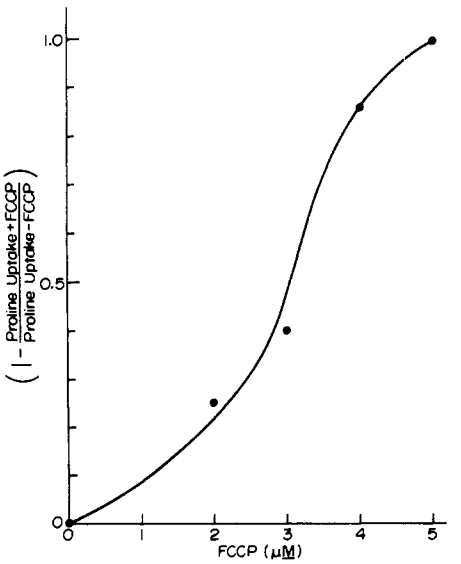

FIGURE 5: Inhibition of proline transport in ML 308-225 whole cells vs. FCCP concentration. Cells were washed twice and resuspended in M9 medium without carbon source. Cells were incubated with the indicated concentration of FCCP for 8 min. Then 10 mM D-lactate was added and the incubation continued for 5 min. The [^{14}C]proline transport assay was performed as described in Methods with the proline uptake in 30 s being measured. The initial control rate of proline transport was 7.0 nmol min^{-1} (mg protein)$^{-1}$. Inhibition of uptake measured at longer times (1.5, 3, and 5 min) gave similar results. The cell titer was 7.7×10^8. Temperature was 25 °C.

The measured average fluorescence lifetimes of PhNap and ANS in the presence and absence of 5 μM FCCP can be corrected for the contribution of background fluorescence and scattered exciting light from the cell suspension and for the contribution of the probe free in solution using the method of Cogan et al. (1973). The effect of the correction for background and scattered light is very small and that for dye binding is small (≤0.5 ns). The corrected values of polarization and lifetime can be used to compute the rotational relaxation times (ρ value) of the probe bound to the cell envelope in the presence and absence of 5 μM FCCP: PhNap alone, 3.1 ns corrected, 2.6 ns uncorrected; PhNap + FCCP, 7.9 ns corrected, 7.2 ns uncorrected. The measured ρ values in Figure 3 may underestimate the ρ values of the bound probe, but do not alter the relative values of the rotational relaxation times measured in the presence and absence of FCCP or the shape of the functional dependence of rotational relaxation time on FCCP concentration. For this reason and because of the approximate nature of the Perrin equation, the values of the rotational relaxation times calculated from the quantities measured directly will be used to discuss the changes in environment of the probes.

Dependence of Active Transport on FCCP Concentration. It is important to compare the functional dependence of the increase in ρ value on FCCP concentration (Figure 3) with that of the inhibition of active transport (Figure 5). In order to determine the effect of FCCP on the transport of a specific substrate a carbon source must be used whose oxidation is not affected by the uncoupler (Avi-Dor, 1963). FCCP (5 μM) inhibits succinate oxidase activity by 62% in whole cells while having no effect on D-lactate oxidase activity (Helgerson, 1976). D-Lactate was used to drive [^{14}C]proline transport in ML 308-225 cells and the inhibition of transport by varying concentrations of FCCP was measured. The results shown in

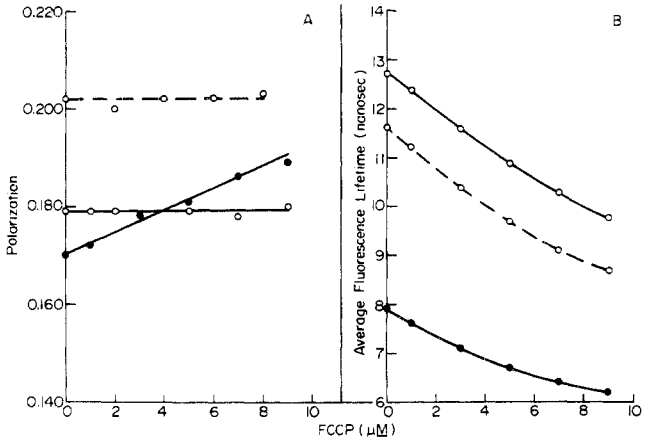

FIGURE 6: Fluorescence polarization (A) and lifetime (B) of 50 μM ANS or 5 μM PhNap bound to ML 308-225 membrane vesicles as a function of FCCP concentration. The experimental procedures are as described in Figures 1 and 2, except that membrane vesicles were used at 0.2 mg of membrane protein/mL and 10 mM D-lactate was present. Membrane vesicles were suspended in M9 buffer made with either water or deuterium oxide (Sigma Chemical Co., 99.7%) as solvent. ANS, H_2O (open circles, dashed line); ANS, D_2O (open circles, solid line); PhNap, H_2O or D_2O (closed circle, solid line). The ratio of the intensities (I_{D_2O}/I_{H_2O}) for the probes in solution with no vesicles present was found to be 2.4 for ANS and 2.0 for PhNap. When bound to membrane vesicles I_{D_2O}/I_{H_2O} was 1.6 for ANS and 1.0 for PhNap.

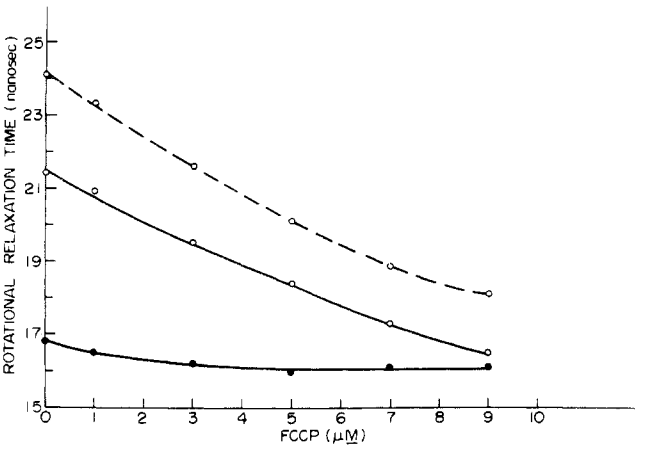

FIGURE 7: The rotational relaxation time (ρ) of 50 μM ANS or 5 μM PhNap bound to ML 308-225 membrane vesicles vs. final FCCP concentration. Values were calculated using the Perrin equation with the fluorescence polarization and lifetimes shown in Figure 6. ANS in H_2O (open circles, dashed line); ANS in D_2O (open circles, solid line); PhNap in H_2O or D_2O (closed circles, solid line).

Figure 5 indicate that the inhibition increases from 25% at 2 μM FCCP to 100% at 5 μM FCCP. It is evident that both the inhibition of active transport of [^{14}C]proline across the cytoplasmic membrane and the increase in rotational relaxation times of envelope bound ANS and PhNap show a very similar dependence on FCCP concentration.

Effect of FCCP on Fluorescence Parameters of ANS and PhNap with Inner Membrane Vesicles. All the components necessary for proline transport are present in the cytoplasmic membrane since inner membrane vesicles can be prepared which will concentrate proline when provided with an energy source (Kaback and Milner, 1970). The energy needed to achieve this transport is derived from the oxidation of the carbon source (e.g., D-lactate) via the electron transport chain (Kaback and Barnes, 1971; Klein and Boyer, 1972). The transport of proline into these vesicles is inhibited by uncouplers (Kaback et al., 1974). ML 308-225 membrane vesicles were prepared in order to determine if the fluorescent probe responses observed in whole cells were also present in the cytoplasmic membrane.

The fluorescence polarization and lifetimes of membrane vesicle-bound ANS and PhNap vs. FCCP concentration in H_2O or D_2O buffer are shown in Figure 6 and the calculated rotational relaxation times in Figure 7. The conditions of these experiments are the same as for the cells shown in Figures 1 and 3 except that membrane vesicles (0.2 mg of membrane protein/mL) with 10 mM D-lactate present were used. Addition of D-lactate caused the fluorescence intensity of ANS to drop ~5% as has been reported (Reeves et al., 1972), but had little effect (1%) on the polarization and lifetime of ANS or PhNap. In contrast to the fluorescence parameters characteristic of the probes bound to whole cells, the membrane vesicles in H_2O in the absence of FCCP exhibit (a) polarization and lifetime values of the probes that are significantly higher than in whole cells (the contribution to the fluorescence signal from the free probe is negligible), (b) rotational relaxation times that are six- and tenfold higher than obtained with cells with PhNap and ANS, respectively, (c) essentially no change in the rotational relaxation time of PhNap (16–17 ns) in the presence of FCCP concentrations up to 9 μM, and (d) a gradual lowering of the rotational relaxation time of ANS (24.1 to 18.1 ns) with increasing FCCP concentration (0 to 9 μM). Under these conditions the active transport of [^{14}C]-proline is 90% inhibited by 0.3 μM FCCP (data not shown). Thus, the structural changes caused by FCCP in whole cells which correlate with inhibition of proline transport are not observable in the inner, cytoplasmic membrane. The lack of an effect of D_2O on the fluoroescence parameters of PhNap implies that this probe, unlike ANS, is located in the core of the membrane vesicle where it is inaccessible to water. A similar comparison with H_2O and D_2O with similar results has been made with lecithin micelles and submitochondrial particles (Radda, 1971). The effect of FCCP on surface-bound ANS to gradually lower the rotational relaxation time with increasing concentration (Figure 6) suggests that FCCP may be able to disrupt the aqueous/membrane interface of these vesicles. Similar effects have been observed in submitochondrial particles (Zimmer et al., 1972) and phospholipid bilayer membranes (Chen and Hsia, 1974) using electron spin resonance spectroscopy. Such structural effects caused by this uncoupler should be kept in mind, especially if it is used at high concentrations.

Effect of FCCP with EDTA-Treated Cells on (a) Fluorescence Parameters of ANS and PhNap and (b) on Active Transport. When *E. coli* cells are treated with EDTA as described in Methods, it has been reported that 30 to 50% of the lipopolysaccharide is removed from the outer membrane (Lieve, 1965), proline and methyl β-D-thiogalactoside transport are unaffected, and a permeability barrier to small molecules such as actinomycin D is removed (Lieve, 1967). EDTA treatment has a similar effect on the relatively hydrophobic molecules PhNap and FCCP. In the absence of FCCP, EDTA treatment causes a threefold increase in PhNap fluorescence and binding (Table I) and an increase in the rotational relaxation time of PhNap from 2.6 ns (Figure 3) to 10.9 ns (Figure 8). The similar effects of EDTA treatment and FCCP addition in causing increased uptake of PhNap are illustrated by the lack of effect of FCCP addition on PhNap fluorescence parameters with EDTA-treated cells (Table I and Figure 8). The rotational relaxation time of ANS in the absence of FCCP is only slightly increased by EDTA treatment (compare Figures 3 and 8) but is affected by a 20-fold smaller concentration of FCCP than is needed in untreated cells (Figure 8), without any

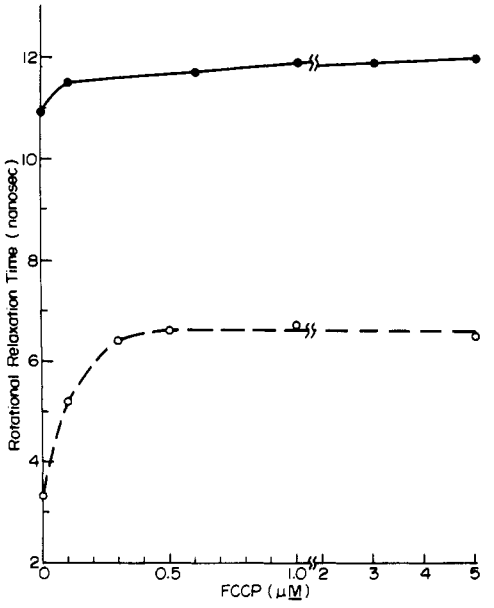

FIGURE 8: The rotational relaxation time (ρ) of 50 μM ANS (open circles, dashed line) or 2 μM PhNap bound to EDTA-treated ML 308-225 cells vs. final FCCP concentration. Values were calculated using the Perrin equation with the fluorescence polarization and lifetimes shown in Helgerson (1976).

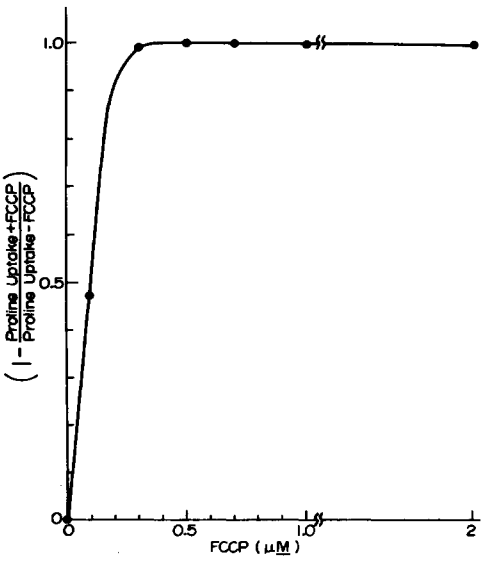

FIGURE 9: Inhibition of [^{14}C]proline transport in EDTA-treated ML 308-225 cells vs. FCCP concentration. The experimental procedure is as described in Figure 5 and Methods. The cell titer was 6.2×10^8. The initial control rate of proline transport was 5.4 nmol min^{-1} (mg of protein)$^{-1}$.

significant change in ANS binding. It is clear that a permeability barrier to FCCP has been removed by EDTA since transport in EDTA-treated cells is also completely inhibited by a 20-fold smaller concentration of FCCP (0.3 μM) than is needed in untreated cells (compare Figures 9 and 5).

Discussion

Inhibition of proline transport by FCCP is accompanied by a parallel increase in the rotational relaxation time (ρ) of the probes PhNap and ANS in untreated cells, and of ANS alone in cells treated first with EDTA. The rotational relaxation time of these probes is then a fluorescence parameter which operationally appears to correlate well with the energy level of the cell membrane assayed through active transport.

From the comparison of fluorescence intensity, dye binding, and the other fluorescence parameters in untreated cells, EDTA-treated cells, and vesicles, it is shown that another structural event which occurs during deenergization is the opening of a permeability barrier or gate in the outer membrane to PhNap. This results in increased binding of PhNap and increased fluorescence intensity, studied by Nivea-Gomez et al. (1976) in connection with studies on the mechanism of action of colicin Ia. Although this increase in fluorescence intensity appears to correlate qualitatively with membrane energy level (Phillips and Cramer, 1973; Nivea-Gomez et al., 1976), it is quite reversible under conditions where membrane energy level assayed by active transport is not (Cramer et al., 1976). In experiments on reversibility of membrane energy level, the ρ value of PhNap appears to correlate better with energy level than does intensity.

The observation of Nivea-Gomez et al. that changes in partitioning of PhNap are significant in calculation of the different fluorescence parameters is correct. Their inference that all of the fluorescence changes of PhNap can be attributed to increased binding of PhNap upon deenergization does not appear to be correct. This is shown by the analysis of polarization data extrapolated to infinite cell titer presented above, by a similar extrapolation of fluorescence intensity to infinite cell titer, which provides the relative quantum yields of bound dye in the presence and absence of FCCP (Helgerson and Cramer, 1976), and by the removal by FCCP of an apparent phase transition in the cell envelope sensed by FCCP (Helgerson and Cramer, 1976). The weighted contribution of free dye emission to the intensity measured in the presence of the turbid cell suspension can be overestimated because of scattering losses in the cell suspension. These losses are smaller in front-face measurements. Before deenergization, PhNap appears to be located outside a permeability gate of the outer membrane, which may consist of the saccharide chain of the lipopolysaccharide (Nikaido, 1976) or of a transmembrane diffusion pore formed by lipoproteins (Inouye, 1974) or protein I (Nakae, 1976) in the outer membrane. Deenergization causes movement of the dye through this gate and changes in the resultant dye environment which, when assayed through the rotational relaxation time, correlate well with energy level assayed here through active transport.

The increase in rotational relaxation time of ANS upon addition of FCCP to cells, and deenergization of the membrane, appears to show very little complication due to dye binding (Table I above; Cramer and Phillips, 1970), although the absolute degree of binding of this probe is small. An increase in binding of this negatively charged probe might be expected upon deenergization of the membrane due to collapse of the negative membrane potential. This is the interpretation of Griniuviene et al. (1975) who reported increases of ANS fluorescence intensity upon deenergization similar to those seen previously (Cramer and Phillips, 1970), as well as reversal of these intensity changes upon energization. That changes in membrane potential may play a role in the ANS response is implied by the finding of Griniuviene et al. (1975) that sonicated membranes show oppositely directed ANS fluorescence intensity changes. However, these workers did not measure dye binding, and it is clear from the response of membrane vesicles and whole cells reported here that, although changes of membrane potential may play a role in determining ANS fluorescence response, they do not affect net dye binding. The increase in rotational relaxation time of ANS upon addition of FCCP could be caused by movement of the probe within the envelope to binding sites of higher microviscosity or an increase in the microviscosity of the envelope around the probe mole-

cules. In either case, the rotational relaxation time of ANS monitors a structural change which correlates with the degree of deenergization by FCCP. The localization of ANS is not definitely known. It is known that the outer membrane is permeable to hydrophilic molecules smaller than pentalysine (Payne and Gilvarg, 1968) and polysaccharides smaller than 700 molecular weight (Nakae, 1976; Decad and Nikaido, 1976). Because (a) ANS is an amphiphilic molecule, (b) the fluorescence properties of ANS in untreated and EDTA-treated cells are different from those observed in inner membrane vesicles, and (c) the fluorescence changes caused in vesicles by FCCP are smaller and oppositely directed, it is inferred that the events monitored by ANS occur in regions of the envelope exterior to the cytoplasmic membrane.

Thus, structural changes associated with deenergization of the inner membrane appear to occur in a region of the cell envelope outside the inner membrane and, in particular, in the outer membrane. An important finding which relates to the mechanism by which the effects might be transmitted from inner to outer membrane is that changes in fluorescence parameters of PhNap appear to be caused by many agents which deenergize the inner membrane (Nivea-Gomez et al., 1976). We have argued elsewhere that such effects are not caused by minimal inhibitory concentrations of cyanide (Cramer et al., 1976). This may just be a question of the relative efficiency or completeness of deenergization caused by these agents. FCCP and colicin such as E1 and 1a appear to be more efficient deenergizing agents than cyanide or azide. Evidence has recently been presented that colicin E1 causes a depolarization of the cell membrane (Gould et al., 1976; Gould and Cramer, 1977). In the context of present ideas of energy transduction, this would be a common feature of deenergizing agents. Thus, it might be hypothesized that structural changes in the outer membrane of *E. coli*, caused by deenergization of the inner membrane, and documented here for the uncoupler FCCP, are triggered by collapse of the inner membrane potential. This large change in local potential would either affect the outer membrane directly or through its effect on connective structures between inner layers and the outer membrane.

The above measurements, together with other fluorescence probe work on this system cited above, establish the existence of dynamic structural changes which occur in the outer membrane concomitantly with deenergization of the inner membrane, and which may affect outer membrane function. One example of such a functional alteration dependent on membrane energization is colicin receptor function. The efficiency of action of colicin K, expressed through the time course of trypsin sensitivity of adsorbed colicin, appears to be decreased when the membrane is deenergized (Plate and Luria, 1972).

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Noncoding Nucleotide Sequence in the 3′-Terminal Region of a Mouse Immunoglobulin κ Chain Messenger RNA Determined by Analysis of Complementary DNA†

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ABSTRACT: The sequence preceding the 3′-terminal poly(adenylic acid) [poly(A)] tract of the immunoglobulin κ chain mRNA from mouse myeloma MOPC 41A was studied by analysis of complementary DNA (cDNA). Short ^{32}P-labeled cDNA was synthesized on the mRNA using DNA polymerase I of *Escherichia coli* and oligo(dT) as a primer. The cDNA was characterized by analyzing the oligonucleotides produced by digestion with T4 endonuclease IV. The sequence was also studied by an adaptation (Brownlee, G. G., and Cartwright, E. M. (1977), *J. Mol. Biol.* (in press)) of a rapid sequencing method using gel electrophoresis (Sanger, F., and Coulson, A. R. (1975), *J. Mol. Biol. 94*, 441). Labeled cDNA of variable length, synthesized using reverse transcriptase of avian myeloblastosis virus, was extended with this enzyme in four reactions, each of which contained only three deoxynucleoside triphosphates (dNTPs). Displaying the products of these "minus" reactions on a polyacrylamide gel permitted a tentative sequence of about 100 residues to be read directly. A "plus" gel system, based upon selective degradation of cDNA in the presence of single dNTPs, gave less satisfactory results. The combined results established the sequence for the first 59 residues adjacent to the poly(A) in the mRNA and provided a tentative sequence for the next 45 residues. The sequence does not reach the κ constant coding region, so the 3′ noncoding region in this mRNA must be more than 100 residues long. The first 60 residues are identical with the sequence reported by others for the equivalent region of a different mouse κ chain mRNA, but the subsequent sequences appear to differ; if substantiated, this result would indicate that there is more than one κ constant region gene in the mouse. There is little homology with sequences determined in 3′-terminal noncoding regions of other mRNAs.

Messenger RNAs (mRNAs) of eukaryotes contain a noncoding region before the 3′-terminal poly(A)[1] tract (for reviews, see Proudfoot and Brownlee, 1976; Adams, 1977). Determination of more sequences in this region should help to clarify its function. Direct sequence analysis of eukaryotic mRNAs has proven difficult, because it is not generally feasible to label the molecules sufficiently in vivo. Therefore attention has turned to sequencing methods based upon analysis of radioactive cDNA made on mRNA templates. Labeled cDNA of high specific activity can be prepared with α-^{32}P-labeled dNTPs in a reaction catalyzed either by a viral reverse transcriptase or by DNA polymerase I of *Escherichia coli*, which gives shorter products (Proudfoot and Brownlee, 1974; Cheng et al., 1976; Proudfoot, 1976). Since the synthesis is generally primed by oligo(dT) on the poly(A) tract of the mRNA, the cDNA sequence is complementary to the 3′-terminal portion of the mRNA.

We have analyzed cDNA made on an immunoglobulin light chain (κ) mRNA, purified from mouse myeloma MOPC 41A (Mach et al., 1973; Cory et al., 1976). A sequence of 45 residues in the equivalent region of a different mouse κ chain mRNA (MOPC 21) has been reported briefly by Milstein et al. (1974) and subsequently corrected (Proudfoot and Brownlee, 1976, and personal communication). We hoped that a comparison of the two nucleotide sequences would indicate whether the mRNAs for these two κ chains, which differ considerably in their variable regions but may have identical constant regions (Gray et al., 1967; Svasti and Milstein, 1972), possess the same sequence in their 3′-terminal noncoding regions.

We first characterized the κ cDNA by digestion with T4 endonuclease IV, which makes scissions preferentially at certain deoxycytosine residues (Sadowski and Hurwitz, 1969; Galibert et al., 1974; Proudfoot and Brownlee, 1974; Bernardi et al., 1976). To confirm and extend the partial sequence determined in this way, we then used an adaptation of the rapid gel sequencing technique of Sanger and Coulson (1974), which was developed for RNA templates by Brownlee and Cartwright (1977). In this technique, labeled cDNA of variable length is elongated in four separate reactions, each containing only three dNTPs, and the products are displayed by electrophoresis on a polyacrylamide slab gel; the resulting labeled bands on an autoradiogram then permit a tentative sequence of about 100 residues to be read directly. This technique has promise as a general approach to determining sequences in RNA molecules.

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[1] Abbreviations used are: poly(A), poly(adenylic acid); cDNA, complementary DNA; dNTPs, deoxynucleoside triphosphates; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Pu, deoxyadenosine or deoxyguanosine; Py, deoxycytidine or thymidine; P_i, inorganic phosphate.