Effect of Mutation, Electric Membrane Potential, and Metabolic Inhibitors on the Accessibility of Nucleic Acids to Ethidium Bromide in *Escherichia coli* Cells†

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ABSTRACT: The uptake of ethidium bromide by *Escherichia coli* K 12 cells has been studied by using ^{14}C-labeled ethidium and spectrofluorometry on three *E. coli* strains: the first one (AB1157) has an ethidium-resistant phenotype; the second one derives from the first one after a single mutation (at 10 min on the *E. coli* genetic map) and has an ethidium-sensitive (Ebs) phenotype; the third one is the *acrA* strain which appeared to have the same phenotype as the Ebs strain. When the cells are in exponential growth, no ethidium enters wild-type cells, and a very limited amount of ethidium enters Ebs and *acrA* cells. Massive quantities of ethidium enter AB1157, Ebs, and *acrA* cells treated by uncouplers and respiring Ebs cells treated by the membrane ATPase-inhibitor dicyclohexylcarbodiimide. A small amount of ethidium enters cells treated in M9 succinate medium by metabolic inhibitors such as KCN or cells starved with oxygen in the same M9 medium. The amount of ethidium and ethidium dimer retained at equilibrium by either type of cell, and by cells infected by T5 phage, as well as the kinetics of influx and efflux, has been measured under a variety of situations (membrane energized or not, and/or membrane ATPase inhibited or not). Furthermore, it was shown that ethidium binds to both RNA and DNA when it enters CCCP-treated wild-type *E. coli* cells, whereas it binds mainly to DNA when it enters Ebs and *acrA* cells in exponential growth. As it will be discussed, it is difficult to account for the EthBr uptake by invoking only membrane functions and active transport. Therefore, it is proposed that the variations of the nucleic acid accessibility in *E. coli* cells might play a role in the control of this uptake. Accordingly, in ethidium-sensitive cells, the mutation would have caused a significant part of the chromosomal DNA (10–20%) to become accessible to ethidium. Hansen [Hansen M. T. (1982) *Mutat. Res. 106*, 209–216], after a study of the photobinding of psoralen to nucleic acids in the *acrA* mutant, also suggested that DNA environment was modified in *acrA* cells.

Most of the bacterial strains developed for testing the mutagenicity of organic compounds have mutations which alter the permeability of the membrane. Such mutants are *rfa*, or *envA* (Ames et al., 1973; Moreau et al., 1976). Unfortunately these strains, which were made permeable to hydrophobic substances, retain an apparent impermeability to hydrophilic substances. They are completely insensitive to many hydrophilic antibiotics (Nikaido, 1972; Coleman & Leive, 1979; Lambert & Le Pecq, 1982). As a consequence, these strains cannot be used to test the mutagenicity of hydrophilic and/or charged compounds, among them the new antitumor derivatives of ellipticine under study in this laboratory (Le Pecq et al., 1974, 1975; Paoletti et al., 1980). This has led us to try to isolate *Escherichia coli* mutants sensitive to hydrophilic substances. Ethidium bromide, a positively charged quaternary ammonium derivative, was chosen as the model compound. We have then isolated, after mutagenesis, several ethidium-sensitive strains, which we described recently (Lambert & Le Pecq, 1982). One of these strains (BL101) become ethidium sensitive (Ebs) after a single mutation, located at 10 min on the *E. coli* genetic map. It appeared that the *acrA* mutation was located very close. We observed that this *acrA* strain had a phenotype very similar to that of our Ebs strain and was also sensitive to ethidium. We selected this Ebs strain and the parent strain as well as the *acrA* strain to study the factors which control the apparent permeability barrier to hydrophilic substances in *E. coli*. Since ethidium holds a positive charge, we were naturally led to study what could be the importance of the electric membrane potential in the uptake of ethidium by the *E. coli* cells. Since, in *E. coli*, there is a membrane potential of about 100 mV, negative inside, it could be expected that the ethidium cation might be concentrated by the bacteria, according to the Nernst law, in the same way as are tetraphenylphosphonium and triphenylmethylphosphonium (Schuldiner & Kaback, 1975; Weiss & Luria, 1978; Hirota et al., 1981). The unexpected observation was then made that depolarization of *E. coli* membrane by uncouplers apparently triggered a massive influx of ethidium inside the bacterial cells. A study of this phenomenon was thus initiated. The results of this study are presented and discussed in the present paper.

Materials and Methods

Materials. Ethidium bromide (EthBr)[1] was from Sigma. [^{14}C]EthBr was from C.E.A. (France). Its specific activity was 17.8 mCi/mmol. Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was from Boehringer, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was from Aldrich. FCCP and CCCP solutions were extemporarily prepared. They were first solubilized in ethanol at 10 mg/mL and further diluted in 10 mM NaOH. The final concentration of ethanol in medium was less than 0.1%. Control experiments showed that, at this concentration, ethanol had no significant effect on bacterial growth and viability. Triphenylmethylphosphonium ($TPMP^+$) was from K & K, and [^{14}C]$TPMP^+$ (24 mCi/mmol) was provided by Dr. Nguyen-Dat-Xuong. CO was from Air Liquide, and KCN was from Prolabo (France). *N,N'*-Dicyclohexylcarbodiimide (DCCD) was from Baker. Polycarbonate filters (Nuclepore) were from Serlabo (France). Ethidium dimer (Gaugain et al., 1978a) was a gift

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; EthBr, ethidium bromide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; H 33258, Hoechst 33258 or bisbenzimide; $TPMP^+$, triphenylmethylphosphonium; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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from Dr. Roques. 4',6-Diamidino-2-phenylindole (DAPI) was from Serva, bisbenzimide or Hoechst 33258 (H 33258) was from Riedel-de Haen Ag, and pancreatic deoxyribonuclease I (EC 3.1.4.5), pancreatic ribonuclease I (EC 3.1.4.22), and calf thymus DNA were from Boehringer.

Bacterial Strains, Phage, and Media. *E. coli* K 12 strain AB1157 (*thr*-1, *leu*-6, *proA*-2, *his*-4, *thi*-1, *arg*E3, *lac*Y1, *gal*K2, *ara*-14, *xyl*-5, *mtl*-1, *tsx*-33, *str*A31, *sup*37) (Howard-Flanders & Boyce, 1966) was kindly provided by Dr. N. Otsuji. The *acrA* strain [MH 321 from Hansen (1982)] was kindly provided by Dr. Hansen. BL101 strain derives from AB1157 after a single mutation. This strain was isolated and selected for its sensitivity toward ethidium bromide (Lambert & Le Pecq, 1982). The phenotype name "Ebs" was given to this strain. T5 phage was kindly provided by Dr. A. Jacquemin-Sablon.

M9 medium contains per liter the following: Na_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; $MgSO_4$, 1 mM; $CaCl_2$, 0.1 mM; vitamin B_1, 1.0 mg; casamino acids (Difco), 3.0 g. M9 glucose is M9 medium plus glucose at 2.0 g/L. M9 succinate is M9 medium plus succinate at 16 mM. For T5 experiments, bacteria were grown in MGM medium according to Lanni (1961).

Methods. (*i*) *Measurement of* [^{14}C]*Ethidium Retention by Bacteria.* Bacteria were grown in M9 glucose medium at 37 °C up to an absorbance of 0.6 at 650 nm (4×10^8 cells/mL). They were then centrifuged and resuspended in prewarmed M9 glucose medium. After a 5-min incubation, various amounts of [^{14}C]EthBr were added. At different times, aliquots (0.25 mL) were filtered on polycarbonate filters, washed twice with 2.0 mL of LiCl 0.1 M at 0 °C, and finally rinsed with 2.0 mL of ethanol at 0 °C. Filters were dried and put in vials with scintillation liquid (Permafluor III Packard). Radioactivity was measured in an Intertechnique counter.

(*ii*) *Fluorescence Measurements.* Fluorescence was measured on an SLM 800 spectrofluorometer (Urbana, IL) with a double monochromator for excitation and a single monochromator for emission and equipped with a thermostated cell holder. This instrument was associated with a 9315 Ortec photon counter, interfaced through a 779 Ortec interface controller to a Minc Digital computer. The fluorescence intensity could be plotted either from the computer on a 4662 Tektronix or, after digital analog conversion by a 449 Ortec rate meter, on a Servogor analog plotter. Fluorescence measurements, on bacterial suspensions, were all done at 37 °C in quartz cuvettes (Hellma) 0.4 or 0.2 cm wide, in order to minimize inner filter effect. When correction for such an effect was necessary (Figure 11), the measurements were done in the 0.2-cm cuvette. For cuvettes of small width, all the fluorescence emitted along the light path is registered. In that case, the total emitted fluorescence is proportional to

$$\int_0^u I_0 \times 10^{-Du}\, du = I_0(1 - 10^{-Du})/2.3D \qquad (1)$$

with u = the cuvette width, D = the absorbance measured in 1-cm cuvette, and I_0 = the light beam intensity reaching the cuvette. In the absence of absorption, fluorescence would be proportional to I_0u. Therefore, the correction factor for the inner filter effect is in these conditions:

$$K_c = 2.3Du/(1 - 10^{-Du}) \qquad (2)$$

Control measurements (fluorescence determination of EthBr in suspension of AB1157 cells at various absorbance) showed that for the 0.2-cm cuvette, fluorescence was accurately corrected according to this formula.

EthBr and ethidium dimer fluorescences were excited at 545 nm and recorded at 610 nm. H 33258 fluorescence was excited at 380 nm and recorded at 450 nm.

Fluorescence lifetime was measured by the time-correlated single photon counting technique, as previously described (Wahl et al., 1970; Yguerabide, 1972), with an instrument built in this laboratory.

Fluorescence excitation spectrum of EthBr retained by bacteria was measured in a 0.2-cm cuvette. The absorbance of the bacterial suspension was first measured at the excitation wavelength to correct for the inner filter effect according to eq 2. In our conditions, this correction factor never exceeded 1.6. Finally the spectrum was corrected for the variation of the lamp excitation intensity as previously described (Le Pecq & Paoletti, 1967).

(*iii*) *Sonication.* Bacteria were sonicated with a Branson sonicator for three periods of 30 s, at 0 °C, separated by 1-min interruptions. DNA was sonicated as previously described (Le Pecq et al., 1974).

(*iv*) *EDTA Treatment of Bacterial Cells.* Bacteria were treated as described by Leive (1968). Bacteria at a density of 4×10^8 cells/mL were washed twice with 0.12 M Tris-HCl buffer, pH 8, and resuspended in the same buffer at a density of 4×10^9 cells/mL. Bacteria were treated with EDTA at a final concentration of 0.3 mM, for 2 min, at 37 °C. Then the bacteria were diluted 10-fold, in prewarmed growth medium, before measurements of [^{14}C]TPMP$^+$ and ethidium uptake.

(*v*) *Oxygen Uptake.* Oxygen uptake by bacteria in M9 succinate medium was measured with a Clark electrode by using a Gilson oxygraph.

(*vi*) [^{14}C]*TPMP$^+$ Uptake.* Wild-type cells were prepared as described in the EDTA treatment section and diluted in prewarmed growth medium containing 0.002 mM tetraphenylborate. After 5 min of incubation at 37 °C, [^{14}C]-TPMP$^+$ was added at a final concentration of 0.010 mM. At intervals, samples (0.250 mL) were removed, filtered, and washed with 1 mL of growth medium contaning 0.010 mM cold TPMP$^+$. Radioactivity retained by filters was then measured. Ebs cells were treated in the same way, except that EDTA treatment was omitted. Membrane potential was calculated by using the Nernst equation, with the bacterial volume estimated according to Hirota et al. (1981).

(*vii*) *ATP Measurement.* ATP level was estimated by the luciferin luciferase bioluminescence assay by using the test combination ATP bioluminescence CLS from Boehringer. ATP was extracted from bacteria as described by Lundin & Thore (1975). The bioluminescence was measured in the SLM photocounting spectrofluorometer. The emitted light from the sample was directly focused on the photomultiplier. For each series, assays of blanks and ATP standards were performed. Control measurements with internal ATP standard were done to check for the presence of interfering factors.

(*viii*) *T5 Infection.* Bacteria were grown in MGM medium at 37 °C. At an absorbance of 0.6 at 650 nm, $CaCl_2$ was added (1 mM). Then bacteria were infected with T5 phage at a multiplicity of 10.

(*ix*) *Effect of CCCP on EthBr Organic Solvent Solubility.* Isoamyl acetate was shown to be an appropriate solvent for organic-phase partition analysis (Waring et al., 1975). [^{14}C]EthBr in aqueous solution (1 μg/mL) was added to isoamyl acetate (v/v) and shaken. Radioactivity was determined in aliquots of the organic and aqueous phases. These determinations were done in the presence of various concentrations of CCCP or tetraphenylborate.

(*x*) *Computations.* A Minc Digital computer was used. Nonlinear regression estimations of kinetic parameters were

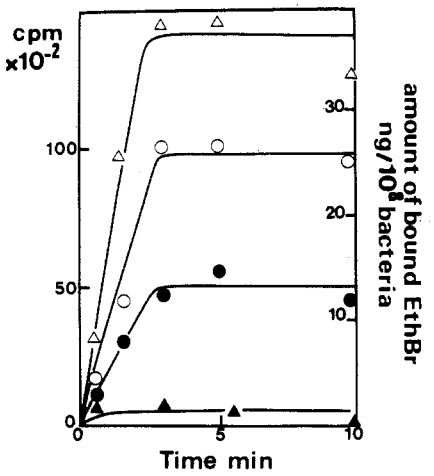

FIGURE 1: Kinetics of [^{14}C]ethidium bromide uptake by *E. coli* cells. Bacteria (4×10^8 cells/mL) in M9 glucose medium were incubated for various times with different concentrations of [^{14}C]EthBr. At indicated time, bacteria were filtered and radioactivity was counted as described under Methods. (●) Ebs BL101 + 1 μg/mL [^{14}C]EthBr; (▲) AB1157 + 1 μg/mL [^{14}C]EthBr; (○) Ebs BL101 + 2 μg/mL [^{14}C]EthBr; (△) Ebs BL101 + 4 μg/mL [^{14}C]EthBr.

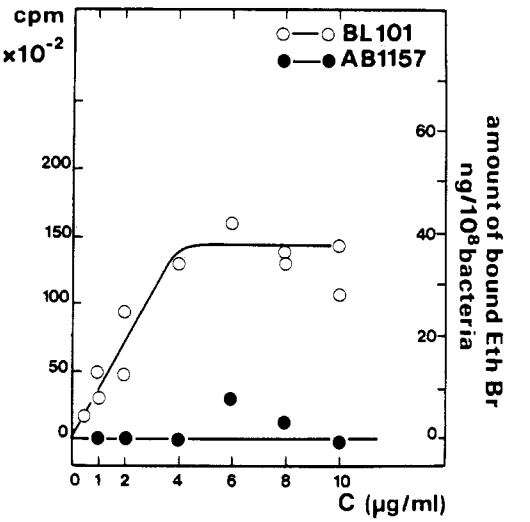

FIGURE 2: Amount of [^{14}C]ethidium bromide retained at equilibrium by *E. coli* cells as a function of ethidium concentration. Bacteria (4×10^8 cells/mL) were incubated with various concentrations of [^{14}C]EthBr for 10 min at 37 °C in M9 glucose medium. [^{14}C]EthBr retained by bacteria was measured as in Figure 1. (○) Ebs BL101; (●) AB1157.

performed by using the Marquardt algorithm (Marquardt, 1963). A Fortran program was kindly provided by Dr. Nilsson.

Results

In this work, three *E. coli* strains, BL101, AB1157, and *acrA*, are used. The BL101 strain, which has an ethidium-sensitive phenotype (Ebs), derives from the AB1157 strain after a single mutation, located at 10 min on the bacterial chromosome (Lambert & Le Pecq, 1982). Several *E. coli* strains, with a mutation in the same region of the chromosome, have similar phenotypes (Imae, 1968; Nakamura, 1968; Otsuji, 1968; Tonnesen & Friesen, 1973; Nakamura et al., 1975; Coleman & Leive, 1979; Higashi et al., 1981). As discussed recently, all these mutations are probably allelic to *acrA* (Coleman & Leive, 1979). In addition, it has been shown that the *acrA* mutation causes the membrane ATPase to become sensitive to DCCD (Hirota et al., 1981). The BL101 strain is also DCCD sensitive, as will be shown later in this work. Ethidium, as well as other DNA binding substances such as bisbenzimide and the antitumor derivatives of ellipticine (Le Pecq et al., 1974, 1975; Paoletti et al., 1980), inhibits the growth of the BL101 strain. The growth inhibition is easily reversed by adding an excess of DNA to the medium. Ethidium does not elicit any cytotoxic effect on this strain and acts as a purely bacteriostatic agent.

To understand the causes of the ethidium sensitivity of the Ebs and *acrA* strains, we were led to study first the uptake of ethidium by these strains and second the physicochemical nature of the ethidium binding structures in the *E. coli* cells.

(I) Study of the Ethidium Uptake by E. coli Cells. Ethidium is a singly charged cation. The electric membrane potential of the bacteria, which is close to 100 mV (negative inside) might then cause a concentration of ethidium inside the bacteria, following the Nernst law (Felle et al., 1980; Hirota, 1981). In addition, the inactivation of the membrane ATPase could also influence ethidium uptake, if active transport is involved.

In order to determine how the electric membrane potential and various metabolic inhibitors affect this uptake, we were then led to run a comparative study on ethidium uptake in both strains.

As described under Materials and Methods, two different methods have been used to measure EthBr uptake. In the first one, the amount of [^{14}C]EthBr retained by bacteria is directly measured after filtration of the cells. In the second one, an apparent amount of EthBr retained by bacteria is computed from fluorometric measurements. This calculation is possible because the fluorescence of EthBr retained by bacteria decays as a single exponential, with a lifetime of 23 ± 1 ns (results not shown). The fluorescence lifetimes of EthBr bound to DNA and RNA are 23–24 and 26 ns, respectively (Olmsted & Kearns, 1977; Tao et al., 1970; Wahl et al., 1970). The first method measures the total amount of EthBr retained by bacteria, while the second one measures the amount of EthBr bound to fluorescent sites inside the cells.

(a) Uptake of Ethidium by Ebs, AcrA and Resistant Cells with Fully Energized Membranes. Uptake studies of radioactive drugs by cells are often complicated by their nonspecific adsorption on membranes. In order to minimize this effect, we have been led to set up a methodology described under Materials and Methods. In this technique, cells are incubated with [^{14}C]EthBr for different times, then collected on polycarbonate filters (these filters do not absorb EthBr), and washed. It appears that when the filters are washed with LiCl solution alone, as described by Weiss & Luria (1978) in their study of triphenylmethylphosphonium uptake, an almost instantaneous binding of EthBr to cells is observed. The amount of EthBr retained by the wild-type cells under these conditions is small (6 ng/10^8 bacteria for an external EthBr concentration of 1 μg/mL). Furthermore, the fluorescence of this retained EthBr is not enhanced. If the filters are rinsed with ethanol for 5 s, after the LiCl wash, no significant amount of [^{14}C]-EthBr is retained by wild-type cells. On the contrary, under the same conditions, a time- and concentration-dependent uptake of EthBr is observed in the sensitive Ebs BL101 strain.

The kinetics of [^{14}C]EthBr uptake by the Ebs BL101 strain for different external concentrations of EthBr are shown in Figure 1. The amount of retained [^{14}C]EthBr at equilibrium, as a function of EthBr external concentration, is shown in Figure 2 for the Ebs BL101 and the parent AB1157 strains. It is observed that only the sensitive Ebs strain is able to retain a significant amount of EthBr.

The efflux kinetics of EthBr can be measured for the Ebs

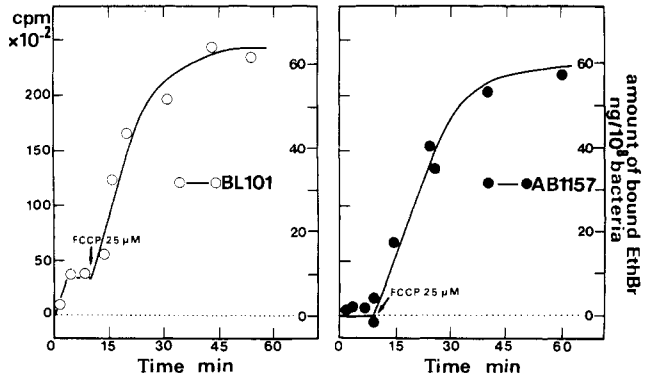

FIGURE 3: Effect of uncoupler (FCCP) on the [^{14}C]ethidium bromide uptake by Ebs and ethidium-resistant *E. coli* cells. [^{14}C]EthBr (1 μg/mL) was added to bacteria (4×10^8 cells/mL) in M9 glucose medium at 37 °C. At different times radioactivity retained by the cells was determined as in Figure 1 before and after FCCP (0.025 mM) treatment. (○) Ebs BL101; (●) AB1157.

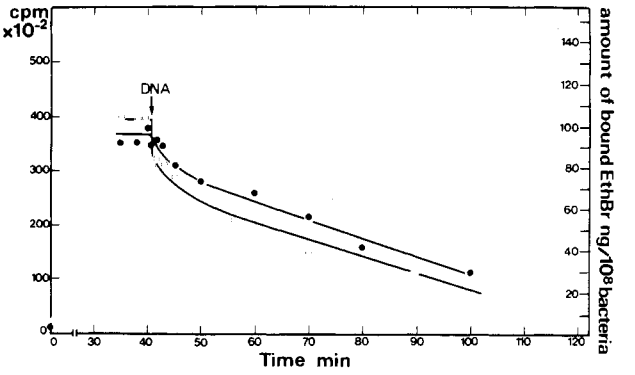

FIGURE 4: Kinetics of [^{14}C]ethidium bromide efflux in FCCP-treated Ebs and ethidium-resistant *E. coli* cells. Bacteria (4×10^8 cells/mL) in M9 glucose medium were treated with FCCP (0.025 mM) and incubated with [^{14}C]EthBr (1 μg/mL) at 37 °C. At the indicated time, sonicated calf thymus DNA was added at 0.050 mg/mL. Then aliquots were taken at different intervals, and radioactivity was counted as before. (○) Ebs BL101; (●) AB1157.

strain by adding an excess of DNA to the medium. DNA binds EthBr and displaces it from the cells. Under these conditions, it is observed that EthBr is released from bacteria according to a single exponential process with a lifetime of 2–3 min (results not shown).

The fluorescence measurements of the maximum amount of EthBr retained by the Ebs strain (shown later in this work; Figure 6 and 7) and by the *acrA* cells (results not shown) give values almost identical with those obtained from [^{14}C]EthBr determinations (40 ng/10^8 bacteria or 0.1 nmol/10^8 bacteria). This demonstrates that most of the EthBr retained by the sensitive strains is bound to structures enable to enhance its fluorescence quantum yield as much as DNA or RNA.

Ebs bacteria apparently behave as structures able to reversibly bind EthBr according to the simple scheme

$$\text{ethidium} + \text{bacteria} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} (\text{ethidium–bacteria})$$

From the data in Figure 1, we can compute k_f. The initial rate of uptake, taken from Figure 1, is divided by the EthBr external concentration and by the total concentration of EthBr bacterial sites at the initial time (computed from the value 0.1 nmol/10^8 bacteria; Figure 2). Then

$$k_f = 4 \times 10^4\ \text{M}^{-1}\ \text{min}^{-1} \qquad k_b = 0.3\ \text{min}^{-1}$$

Thus, the efflux rate appears to be the limiting step in the equilibrium process.

These rates correspond to an apparent binding constant K:

$$K = k_f/k_b = 1.2 \times 10^5\ \text{M}^{-1}$$

This value is in agreement with the equilibrium data shown in Figures 2, 6, and 7.

(*b*) *Effects of Uncouplers (FCCP or CCCP) on the Ethidium Uptake by Ebs and Wild-Type Cells of E. coli.* FCCP or CCCP are uncouplers of oxidative phosphorylation. They act by making the membrane permeable to H^+ ions. They cause the collapse of the electric membrane potential. In aerobic *E. coli* cells, this potential mainly results from the efflux of protons coupled with the oxidation of respirable substrates (Mitchell, 1967). At the beginning of this work, FCCP was used but was later replaced by CCCP because of the latter's greater stability. In several experiments it was observed that FCCP and CCCP gave identical results.

(*i*) [^{14}*C*]*EthBr Uptake Measurement.* In Figure 3 the uptake of EthBr by wild-type (AB1157) and Ebs (BL101) cells is shown before and after the addition of the uncoupling agent FCCP. An unexpected result is obtained. The uncoupler induces in wild-type cells (AB1157) a massive time-dependent uptake of EthBr, as no significant amount of EthBr was retained by the cells before the addition of FCCP. In the case of the Ebs strain, a limited amount of EthBr is retained by the cells, in the absence of FCCP, as already shown. The addition of FCCP triggers a secondary massive time-dependent uptake of EthBr. Once the equilibrium has been reached, EthBr can be displaced from the bacteria by adding an excess of DNA to the medium. EthBr is then very slowly displaced from the cells (Figure 4).

(*ii*) *Fluorometric Measurement of EthBr Uptake.* The effect of FCCP on the EthBr uptake by *E. coli* cells, measured by fluorometry, is qualitatively and quantitatively similar to that measured with [^{14}C]EthBr.

The measurements were made under two different conditions: (1) Cells were preequilibrated with EthBr, and fluorescence was recorded after the addition of the uncoupler FCCP (results not shown). (2) Cells were preequilibrated with the uncoupler FCCP, and the fluorescence was recorded after the addition of EthBr. The kinetics of the fluorescence increase were identical in both cases for either *E. coli* strain. They could be fitted by a single exponential with a time constant of 0.1 min^{-1}. The results obtained in the case of the Ebs strain are shown in Figure 5.

It can therefore be concluded that the limiting rate for the EthBr uptake is not the rate of the appearance of structures able to bind EthBr inside the cells upon FCCP treatment. The limiting step could be related to the rate of equilibration of EthBr across the membrane and/or the rate of equilibration of EthBr with its binding sites inside the cell.

The amount of EthBr bound to bacteria after FCCP treatment can be measured as a function of the external concentration of EthBr. The results are shown in Figure 6. The fluorescence titration of FCCP-pretreated cells by EthBr is also compared with that of sonicated bacteria in this figure. The structures able to bind EthBr and enhance its fluorescence in sonicated bacteria are DNA (35%) and RNA (65%). This is deduced from EthBr fluorescence measurements in the presence of sonicated bacteria before and after DNase and/or RNase treatment according to Le Pecq (1971).

The data shown in Figure 6 can be interpreted according to the Scatchard equation:

$$r/c = K(n - r) \qquad (3)$$

where r is the amount of EthBr retained by 10^8 bacteria, c =

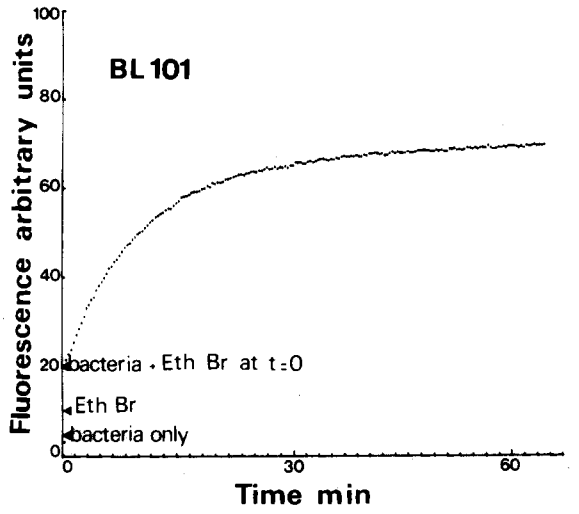

FIGURE 5: Fluorometric measurement of ethidium bromide uptake by Ebs *E. coli* cells after FCCP treatment. Bacteria (1×10^8 cells/mL) in M9 glucose medium were treated with FCCP (0.025 mM) for 5 min at 37 °C. Then EthBr (1 μg/mL) was added and the fluorescence recorded. A time constant of 0.1 min^{-1} was computed by nonlinear regression for a single exponential.

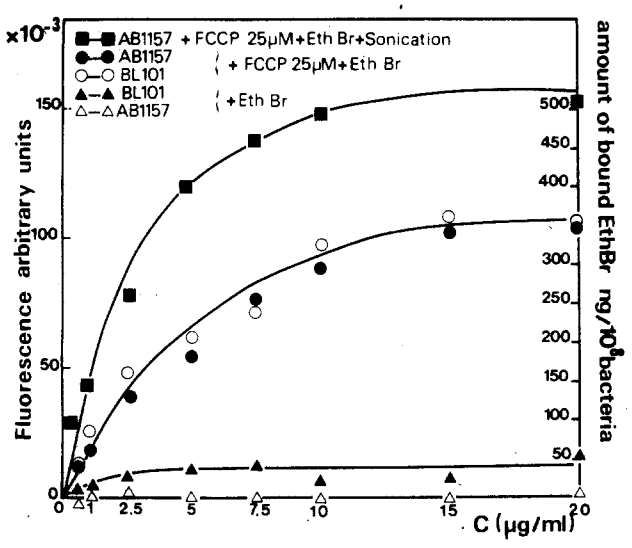

FIGURE 6: Fluorometric measurement of ethidium bromide retained by Ebs and resistant *E. coli* cells at equilibrium. Bacterial suspension (1×10^8 cells/mL) in M9 glucose medium was separated in to three samples: (i) Bacteria were incubated with various concentrations of EthBr. Fluorescence was measured after 10 min of incubation at 37 °C. (▲) Ebs BL101; (△) AB1157. (ii) Bacteria were incubated with various concentrations of EthBr and with FCCP (0.025 mM). Fluorescence was measured after 60 min of incubation. (○) Ebs BL101; (●) AB1157. (iii) Bacteria, incubated as in the preceding sample (ii), were sonicated after 60 min, and fluorescence was measured. (■) AB1157 All incubations and measurements were done at 37 °C.

the molar free concentration of ethidium, K = the apparent binding constant, and n = the maximum number of EthBr sites per 10^8 bacteria. The results are shown in Figure 7.

The apparent binding constants of EthBr for energized Ebs cells, deenergized wild-type and Ebs cells, and sonicated bacteria are very similar (1.5×10^5 M^{-1}).

It should be noted that the collapsing of the electric potential of the cell membrane by uncoupler caused an effect opposite to what could have been expected had the ethidium cation been distributed across the membrane according to the electric potential. It was observed that *E. coli* cells were able to concentrate hydrophobic cations such as tetraphenylphosphonium or triphenylmethylphosphonium when their

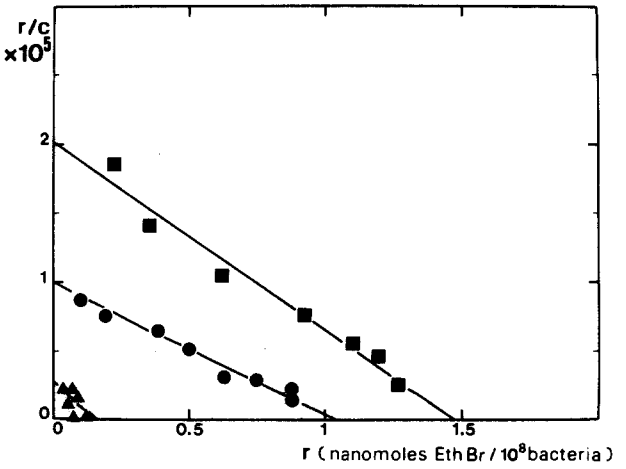

FIGURE 7: Scatchard representation of the results of Figure 6. The symbols are the same as in Figure 6.

membranes were energized and to release these cations when their membranes were deenergized (Hirota et al., 1981; Schuldiner & Kaback, 1975; Weiss & Luria, 1978). Indeed, in control experiments, it was observed that [^{14}C]TPMP$^+$ was concentrated about 60-fold by both AB1157 and BL101 cells grown in M9 succinate medium after EDTA treatment. The Ebs cells concentrated the same amount of [^{14}C]TPMP$^+$ (without EDTA treatment) as did the *acrA* cells (Hirota et al., 1981). The membrane potential corresponding to a 60-fold concentration is −105 mV, similar to the result of Hirota et al. (1981). Furthermore, after CCCP treatment, AB1157 and Ebs cells were unable to concentrate a significant amount of [^{14}C]TPMP$^+$, indicating that the cell electric membrane potential has been completely abolished by CCCP.

The reversibility of the uncoupler effect could easily be observed. Resistant cells were preequilibrated with EthBr and CCCP or FCCP until equilibrium was reached. Then the cells were centrifuged and resuspended in a prewarmed medium containing various EthBr concentrations (from 0 to 50 μg/mL) and no uncoupler. The fluorescence intensity of the solutions, or the [^{14}C]EthBr retained by the cells, was then measured as a function of time (Figure 8). It is observed that, after a short lag period, EthBr is very quickly and completely released from the cells. Upon resuspension in the growth medium, the ATP pool of the cells treated by CCCP and EthBr was completely restored after a few minutes (Figure 8). In all the cases, the latter part of the fluorescence curves should be fitted with a single exponential, the time constant of which was 0.01 s^{-1}. All these results indicate that the resistant cells are able to expel EthBr completely and, at the same rate, whatever the EthBr gradient may be.

The same experiments were performed with the Ebs cells. After CCCP treatment, cells were resuspended in fresh growth medium containing no or 1 μg/mL EthBr. The EthBr release and the ATP pool restoration curves (not shown) were qualitatively the same than those observed for the resistant cells, except that the latter part of the EthBr release curve could now be fitted with an exponential of a slightly lower time constant (0.0077 s^{-1}). Furthermore, when the resuspending medium contained no EthBr, EthBr was completely expelled. When the resuspending medium contained 1 μg/mL EthBr, the cells retained at the limit the same quantity of EthBr as the non-CCCP-treated cells incubated in a medium containing the same concentration of EthBr. Experiments could not be performed by using resuspending medium containing higher EthBr concentrations because, in these conditions, the difference between fluorescence at zero time and at infinite time becomes too small to be measured.

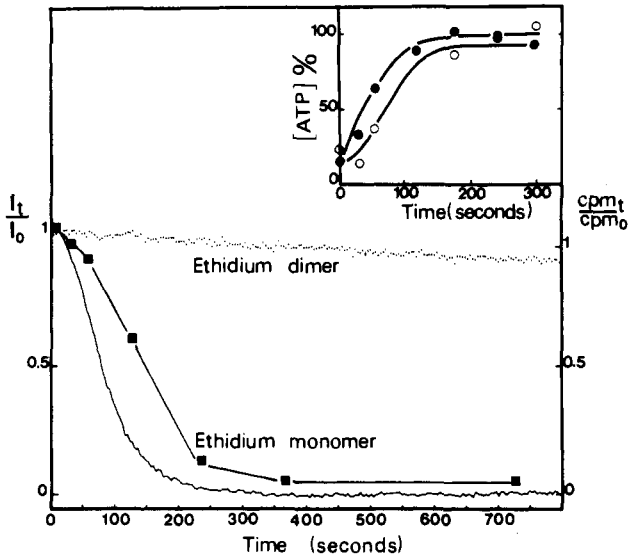

FIGURE 8: Kinetics of ethidium bromide and ethidium dimer efflux upon reenergization of bacteria. AB1157 cells (1×10^8 cells/mL) were treated in M9 glucose medium with CCCP (0.040 mM) and EthBr (1 μg/mL) or ethidium dimer (1 μg/mL). After 60 min of incubation at 37 °C, bacteria were centrifuged and resuspended in prewarmed (37 °C) M9 glucose medium containing 1 μg/mL EthBr or 1 μg/mL ethidium dimer but no CCCP. Then fluorescence was recorded. Experimental data are represented by dots for ethidium dimer and a continuous line for ethidium monomer. The same experiment was repeated with [^{14}C]EthBr (1×10^8 cells/mL). The results are shown by filled square (■). For comparison, all the results were normalized to the values obtained at the initial time, and fluorescence intensities were subtracted from the fluorescence intensity of the free dye. In the upper right, the ATP pool variation upon reenergization of the cells, in the same conditions as above, is shown. (○) Ethidium; (●) ethidium dimer.

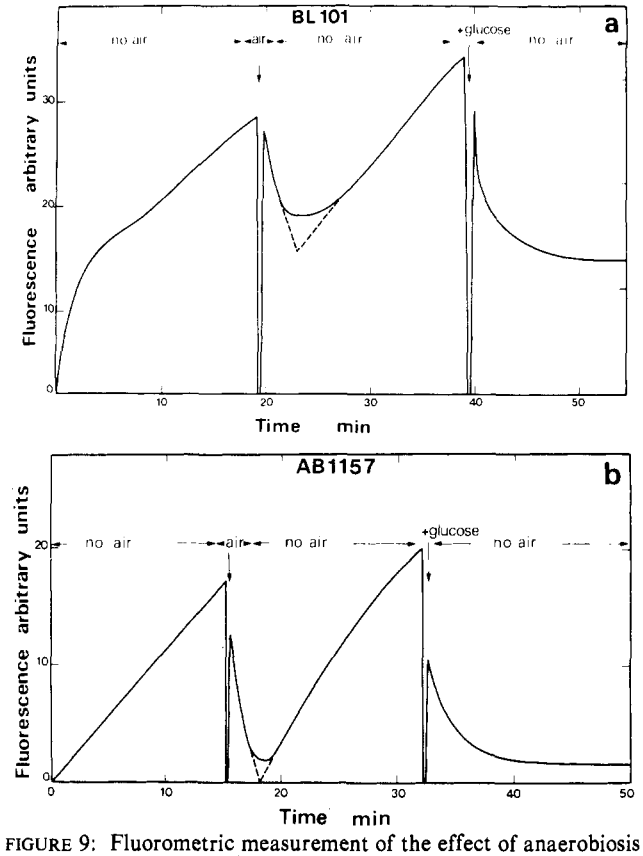

FIGURE 9: Fluorometric measurement of the effect of anaerobiosis on ethidium bromide influx and efflux in *E. coli* cells. Bacteria in M9 succinate medium at an absorbance of 0.15 at 650 nm were bubbled with argon in the fluorescence cuvette. Then EthBr (2 μg/mL) was added and fluorescence recorded while the cuvette was kept closed. At the indicated time, air was bubbled in the cuvette, or glucose was added under argon. (Panel a) Ebs BL101 cells; (panel b) AB1157 cells.

For reasons which will be discussed later, the same experiment was done with ethidim dimer. Ethidium dimer consists of two ethidium cations linked by a polyamine chain. It has been shown to bind to DNA and RNA with a very high binding affinity (Gaugain et al., 1978a,b; Reinhardt et al., 1982). Ethidium dimer like EthBr is not retained by wild-type energized *E. coli* cells. Its uptake by *E. coli* cells is also triggered by the action of uncouplers (FCCP or CCCP). As shown in Figure 8, when cells were preincubated with ethidium dimer and CCCP and then resuspended in a fresh medium containing the same concentration of ethidium dimer as the preincubation medium, but no CCCP, no significant release of ethidium dimer was observed upon resuspension of the cells, whereas the ATP pool was well restored.

(*c*) *Effect of Anaerobiosis on EthBr Uptake.* Although FCCP and CCCP are very efficient uncouplers at low concentrations, they are known to induce several secondary effects by inhibiting various enzymes at higher concentrations (Weigel & Englund, 1977). On the other hand, they are hydrophobic anionic compounds, and they could form a complex with EthBr and consequently modify its uptake. To check for this possibility, the effect of various concentrations of CCCP on the [^{14}C]EthBr partition in organic phase (isoamyl acetate) was studied. The effect of tetraphenylborate which is known to increase the membrane permeability of organic cations was also studied. It was observed that (i) in a v/v isoamyl acetate–water mixture, EthBr was not extracted by the organic phase. (ii) More than 95% of EthBr was extracted by the organic phase as soon as the tetraphenylborate concentration reached 2.5×10^{-6} M. Although tetraphenylborate did form a complex with EthBr and increased its solubility in organic phase, it did not modify the kinetics and the amount of EthBr uptake up to a concentration of 10^{-4} M. (iii) CCCP did not cause any significant EthBr organic phase extraction up to a concentration of 2×10^{-4} M, a concentration much higher than that used for cell deenergization (4×10^{-5} M). To further characterize the effects of deenergization of membranes on EthBr uptake, the effect of cell deenergization induced in a physiological way was studied. This could be performed on bacteria grown on a nonfermentable carbon source. If these cells are deprived of oxygen, the electric membrane potential collapses. It can be very rapidly restored when oxygen is added again or, in the absence of oxygen, when a fermentable carbon source is added (i.e., glucose). In Figure 9, it is observed that, when the resistant strain AB1157 was deprived of oxygen in a medium containing only a nonfermentable carbon source, EthBr fluorescence increased and therefore uptake was induced. It was further observed that when air was bubbled into the solution and oxygen was supplied again, EthBr fluorescence decreased rapidly, almost to the level of free dye, and started to increase again when oxygen was completely consumed (oxygen concentration in the medium was followed by using a Clark electrode). Addition of glucose prevented fluorescence from increasing in the absence of oxygen. Furthermore, the cells were equilibrated in anaerobic conditions (under a layer of paraffin oil) with various concentrations of EthBr. The amount of EthBr retained by the cells was then deduced from fluorescence measurements, as described in the legend of Figure 6. This amount was found to be proportional to the EthBr concentration in the incubation medium (80 ng/10^8 cells for an external EthBr concentration of 10 μg/mL). It was further shown that all this EthBr was released from the cells

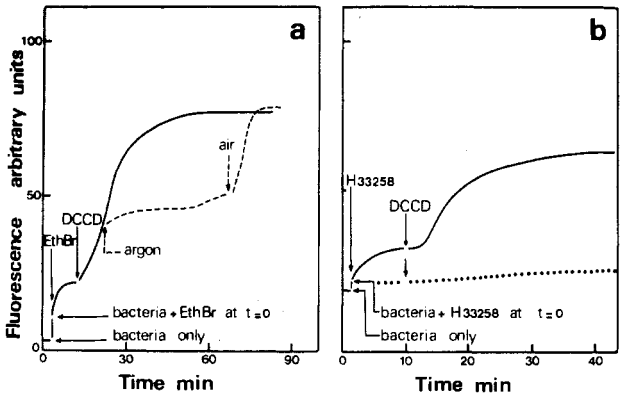

FIGURE 10: Fluorometric measurement of the effect of membrane ATPase inhibition by DCCD on ethidium bromide and bisbenzimide (H 33258) uptake by *E. coli* Ebs cells. (Panel a) Ebs cells were grown in M9 succinate medium up to an absorbance of 0.15 at 650 nm. Then EthBr (1 μg/mL) was added, and fluorescence was recorded. At the indicated time, DCCD (0.1 mM) was added. The dashed line shows the same experiment, but at the indicated time, argon was bubbled and paraffin oil was layered on the sample in order to keep the cells anaerobic. At the indicated time air was bubbled. (Panel b) Ebs and resistant AB1157 cells were grown and treated with DCCD as above but bisbenzimide (H 33258) at 2 μg/mL replaced EthBr. Fluorescence was measured in a 0.2-cm cuvette. (—) Ebs BL101; (···) AB1157.

after air bubbling. It must be emphasized that the amount of EthBr retained by the anaerobic cells is much smaller than the amount retained by the FCCP- or CCCP-treated cells (Figure 6). The same experiment performed with the sensitive strain showed two types of fluorescence increase: one which is oxygen independent and corresponds to the fluorescence observed in glucose medium and the other one which is oxygen dependent and which follows, both qualitatively and quantitatively, the fluorescence curve observed with the resistant strain AB1157.

(*d*) *Effect of Membrane ATPase Inhibition by DCCD on EthBr Uptake.* Recently it has been shown that the membrane ATPase of an *acrA* mutant was sensitive to DCCD without any prior treatment of the cells. To reveal the DCCD sensitivity of ATPase, wild-type cells must be pretreated with EDTA (Hirota et al., 1981). The Ebs BL101 strain has the same properties as the *acrA* mutant and is directly DCCD sensitive. In Figure 10, the effect of DCCD on the uptake of EthBr is shown. The effect is similar to that of uncouplers, but the uptake is blocked if cells are deprived of oxygen. The uptake is restored if oxygenation is resumed. In the presence of oxygen, the DCCD treatment of Ebs cells caused a progressive slowdown of oxygen consumption, no change of $TPMP^+$ uptake (results not shown), and a drastic decrease of ATP cell content (Table I). An apparently striking difference between the action of DCCD and uncouplers on EthBr uptake was observed. With DCCD, EthBr uptake took place as the membrane was energized, as shown by the $TPMP^+$ uptake, while with uncouplers or oxygen depletion, it occurred as the membrane was deenergized.

The same experiment was repeated with the DNA-specific dye Hoechst 33258 (bisbenzimide). This dye does not bind to RNA but binds preferentially to AT-rich DNA. Its fluorescence is then strongly enhanced (Weisblum & Haenssler, 1974; Comings, 1975; Latt & Wohlleb, 1975; Muller & Gautier, 1975; Jensen et al., 1977; Brunk et al., 1979). The results are shown in Figure 10, where they can be compared to those obtained in the same conditions with EthBr. As is shown later, the comparison of the results obtained with the two dyes will permit an estimate of the respective contributions of DNA and RNA to the EthBr fluorescence increase.

Table I: ATP Cell Content

media	strains	ATP[a] content (%) control[c]	CCCP (40 μM)	KCN (5 mM)	CO[b]	DCCD (100 μM)
M9 succinate	AB1157	100	16	6	10	
	BL101	100	13	5	4	22
M9 glucose	AB1157	100	127			
	BL101	100	121			

[a] All ATP assays were performed after 5 min of treatment. [b] CO was bubbled 1 min, and ATP assay was performed 5 min later. [c] ATP content of AB1157 and BL101 cells was found to be 350 and 230 pmol/10^8 cells in M9 succinate medium and 280 and 305 pmol/10^8 cells in M9 glucose medium, respectively. These results are in agreement with those previously found [reviewed in Karl (1980)].

Ebs cells were pretreated with DCCD with air regularly supplied. Upon EthBr addition, EthBr fluorescence increased according to a single exponential process with a time constant of 0.063 min^{-1} at 37 °C (results not shown).

(*e*) *Effect of Metabolic Inhibitors on EthBr Uptake.* Resistant and sensitive cells were treated in M9 succinate medium with 5 mM KCN. Alternatively, CO was bubbled in the same medium. In both cases, the ATP content of the cells was drastically decreased (Table I) and cell oxygen consumption stopped. AB1157 cells, in M9 succinate medium (4×10^8 cells/mL), were equilibrated with various concentrations of EthBr, in the presence of 5 mM KCN. The amount of EthBr retained by the cells was then measured by fluorometry, as described in the legend of Figure 6. Under these conditions, the cells retained a quantity of EthBr proportional to the EthBr concentration in the medium (35 ng/10^8 cells for an EthBr concentration of 10 μg/mL in the medium). This value is much smaller than the quantity of EthBr retained by the FCCP- or CCCP-treated cells (Figure 6).

(*f*) *Effect of EDTA Treatment on EthBr Uptake.* EDTA treatment of *E. coli* cells is known to make these cells permeable to many chemicals such as actinomycin D (Leive, 1968) and positively charged compounds such as $TPMP^+$ (Schuldiner & Kaback, 1975). It was observed that EDTA treatment of AB1157 cells made them effectively permeable to $TPMP^+$ but did not trigger any significant EthBr uptake (results not shown).

(*II*) *Nature of the Ethidium Receptor Structures in E. coli Cells.* The fluorescence lifetime of EthBr retained by bacteria was found to be close to that of EthBr bound to nucleic acids in vitro (23 ns). This already indicated that DNA and/or RNA might be the EthBr binding structures inside the cells. In order to establish this point with greater precision, several experiments were performed.

(*a*) *Fluorescence Excitation Spectra of EthBr Retained by Bacteria in the UV Part.* When the fluorescence excitation spectrum of EthBr–nucleic acid complexes was measured in vitro in the UV region, the quantum yield of EthBr increased by a factor of 3–6, because the energy absorbed by DNA and/or RNA is transferred to EthBr by a singlet–singlet energy-transfer process. The variation of the quantum yield of EthBr follows the variation of the DNA and/or RNA absorption spectrum. This phenomenon has been taken to be evidence of intercalation, because it can be computed that, in this case, energy transfer can only take place if a close contact between base pair and dye exists (Le Pecq & Paoletti, 1967; Rayner et al., 1980; Reinhardt et al., 1982). Reinhardt et al.

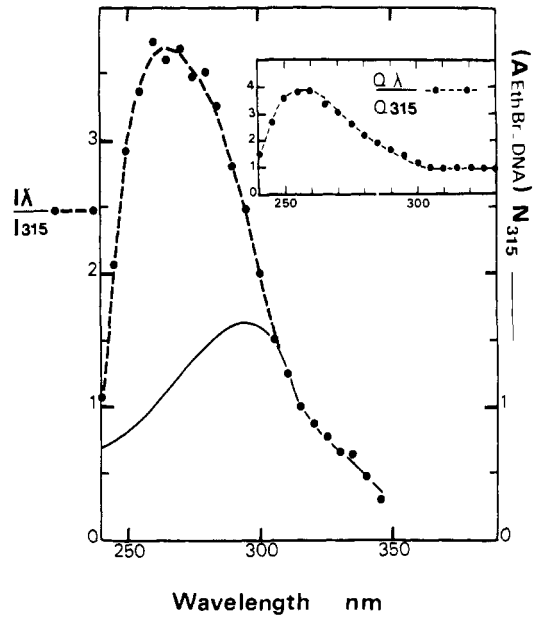

FIGURE 11: Fluorescence excitation spectrum of EthBr retained by *E. coli* Ebs cells. Bacteria were treated by DCCD and EthBr, as described in panel a of Figure 10, and equilibrated for 60 min. Fluorescence excitation spectrum was recorded and corrected as described under Methods. This corrected spectrum (●--●) and the absorption spectrum of DNA-bound EthBr [measured according to Le Pecq & Paoletti (1967) and shown by a continuous line] were normalized for comparison at 315 nm. In the insert, the ratio of the fluorescence quantum yield at wavelength λ to the fluorescence quantum yield at 315 nm for EthBr retained by bacteria (Q_λ/Q_{315}) is shown.

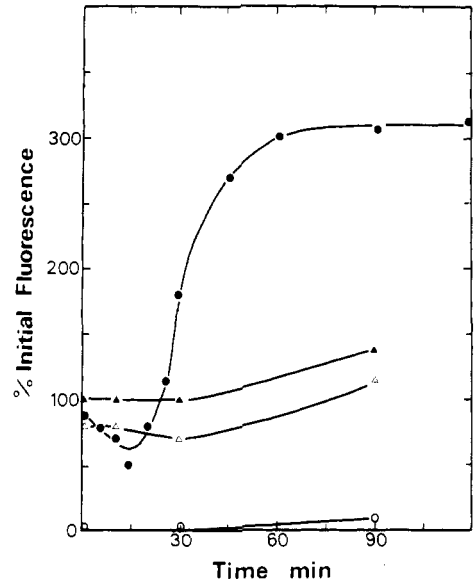

FIGURE 12: Effect of phage T5 infection on ethidium bromide retention by *E. coli* AB1157 cells after CCCP treatment. Four different series were studied: (i) Noninfected bacteria were treated with nalidixic acid (0.050 mg/mL) in order to prevent further DNA replication. At different times, aliquots were taken and incubated 60 min with EthBr (2 μg/mL) + 0.040 mM CCCP at 37 °C before fluorescence measurement. The data were then corrected for the fluorescence measured on bacteria treated in identical conditions but without CCCP. The fluorescence intensity obtained at time 0 was set to 100 to normalize all further measurements (▲). (ii) T5-infected bacteria were treated with nalidixic acid and at different times after with EthBr and CCCP as described above. Fluorescence measurements were corrected and normalized as before (△). (iii) T5-infected bacteria were incubated for different times and then were treated as above with nalidixic acid, EthBr, and CCCP. Fluorescence was measured as before (●). (iv) T5-infected bacteria were treated as in the preceding series (iii), but CCCP was omitted. Fluorescence was measured as before (○).

(1982) have further shown that this phenomenon does not take place with fluorescent dyes which are bound but not intercalated in nucleic acids. The results of Figure 11 clearly show that such a singlet–singlet energy-transfer process occurs when EthBr is retained by bacteria. This is a strong argument in favor of the EthBr intercalation in nucleic acids inside cells.

(*b*) *Uptake of Ethidium by T5-Infected Cells after Uncoupler Treatment.* A simple way to determine whether DNA is able to bind EthBr inside the cells is to try to change the DNA cell content. This can be done simply by T5 phage infection. Soon after infection, T5 induces a DNase activity which almost completely hydrolyzes the host DNA. After 10 min the host DNA hydrolysis is completed and T5 DNA synthesis begins. Sixty minutes later T5 DNA synthesis levels off. The total amount of synthesized T5 DNA is about 3 times greater than the amount of host DNA present before infection (Lanni, 1969). At different times after T5 infection, cells were treated with nalidixic acid to block any further DNA replication and with the uncoupler CCCP to trigger the EthBr uptake (Figure 12). The bacteria treated with nalidixic acid, immediately after T5 infection, were incubated for 45 min and then sonicated. Their DNA content was determined fluorometrically according to Brunk et al. (1979). No significant amount of DNA was measured in these bacteria. In these nalidixic acid treated cells, T5 phage can induce the host DNA degradation but cannot replicate its DNA. In these cells the remaining EthBr fluorescence is therefore related to RNA only, since all DNA was degraded.

These experiments show that (i) T5 DNA like *E. coli* DNA is not accessible to EthBr unless cells have been pretreated with CCCP and (ii) EthBr fluorescence after CCCP treatment follows closely the T5 DNA synthesis as described by Lanni (1969).

Discussion

The interaction of several membrane probes with intact *E. coli* cells has been studied. Most of these probes were hydrophobic, neutral or negatively charged molecules such as *N*-phenyl-1-naphthylamine, pyrene, 1,6-diphenyl-1,3,5-hexatriene, and 8-anilino-1-naphthalenesulfonate (Helgerson et al., 1974; Nieva-Gomez et al., 1976; Nieva-Gomez & Gennis, 1977). These molecules interact specifically with membranes and apparently with no other structures in the cell. They could therefore be used to probe the state of energization of the cell membrane. Ethidium, a DNA-intercalating compound, is a well-known fluorescent probe of nucleic acid structure [reviewed in Le Pecq (1971)]. Its uptake by *E. coli* cells is expected to depend not only on the cell membrane functions but also on the state of accessibility of nucleic acids inside the bacteria.

It is clear from our results that a large number of EthBr binding sites is present inside *E. coli* cells. When these sites are apparently fully accessible, they drive into the cells a huge concentration of EthBr [for an external EthBr concentration of $(2–5) \times 10^{-5}$ M, the internal concentration is 5 mM]. Before the mechanisms of EthBr influx and efflux are discussed, it is necessary to establish the chemical nature of the EthBr receptor structures inside the cells.

Chemical Nature of Ethidium Binding Structures in E. coli. The observations relevant to this problem are the following: (i) Fluorescence excitation and emission spectra and fluorescence lifetime of EthBr inside bacteria are very similar to that of EthBr bound to nucleic acids in vitro.

(ii) The excitation fluorescence spectrum of EthBr in bacteria in the ultraviolet region (<300 nm; Figure 11) is similar to that observed for EthBr–nucleic acid complexes. This indicates that EthBr is intercalated in nucleic acids because energy transfer between base pairs and dye occurs.

(iii) The amounts of EthBr retained by bacteria, measured by [^{14}C]EthBr retention or by fluorometry, are the same.

(iv) The maximum amount of EthBr which can be retained by *E. coli* cells is of the same order as the amount of EthBr which binds to nucleic acids in sonicated cells.

(v) Experiments (unpublished results of our laboratory) with 8-azidoethidium, a photoreactive analogue of EthBr (Hixon et al., 1975), show that after irradiation, the azide toxicity in Ebs cells is almost completely dependent on the activity of the *uvrB* gene and that EthBr in excess can suppress this toxicity. In the wild-type strain the same is observed, but the 8-azidoethidium entrance must first be made possible by uncoupler treatment. Furthermore, Yielding et al. (1979) showed that in a *Salmonella* ethidium-sensitive strain, [^{14}C]EthBr azide remained, after irradiation, associated 50% with DNA and 50% with RNA.

(vi) When the quantity of DNA in cells changes after T5 infection, the number of EthBr binding sites varies in parallel (Figure 12). It is important to notice in this experiment that T5 DNA, like nucleic acids in *E. coli* cells, is only accessible to EthBr after uncoupler treatment.

(vii) The dye Hoechst 33258 (bisbenzimide) is a compound which does not bind to RNA and binds specifically to AT-rich DNA. Its fluorescence is then strongly enhanced (Weisblum & Haenssler, 1974; Comings, 1975; Latt & Wohlleb, 1975; Brunk et al., 1979). The parental strain AB1157 was found resistant to this compound, while Ebs strain BL101 was found sensitive. It was observed that like EthBr, this compound does not apparently enter AB1157-resistant cells in exponential growth (Figure 10). A small amount of this dye enters these cells, when they are deprived of oxygen in succinate medium. When oxygen is resupplied, this dye is apparently released, like EthBr in the same conditions (results not shown). In Figure 10, it is further shown that a small amount of this compound enters Ebs cells in exponential growth and that DCCD triggers in these cells a secondary entry of the dye. The use of this dye is of particular interest, because its fluorescence enhancement is DNA specific, whereas EthBr fluorescence is enhanced by both RNA and DNA. The comparison of the measurements made in the same conditions with EthBr and bisbenzimide allows us to estimate the fraction of DNA and RNA accessible to EthBr in Ebs cells. In Figure 10, it is immediately apparent that the ratio of the fluorescence increase after DCCD to the fluorescence increase before DCCD treatment is larger for EthBr than for bisbenzimide. This already indicates that after DCCD treatment, RNA, which is not detected by bisbenzimide, constitutes a sizable fraction of the nucleic acids detected by EthBr in DCCD-treated cells. A quantitative interpretation can be made by comparing the fluorescence increases of bisbenzimide and of EthBr observed with bacteria and DNA in the same conditions (M9 succinate medium at 37 °C). The respective fluorescence increases can then be transformed into apparent amount of DNA. With bisbenzimide the apparent DNA amounts computed are 0.1 $\mu g/10^8$ bacteria before DCCD treatment and 0.4 $\mu g/10^8$ bacteria after DCCD treatment and with EthBr 0.07 $\mu g/10^8$ bacteria before DCCD treatment and 0.6 $\mu g/10^8$ bacteria after DCCD treatment.

These results indicate that for the Ebs cells, in exponential growth, DNA constitutes most of the structures accessible to EthBr and that 10–20% of the total chromosomal DNA has become apparently accessible in these Ebs mutant cells.

For the cells treated by uncouplers and DCCD, these results and those obtained in the T5 experiment (Figure 12) indicate that 30–40% of EthBr binding sites in *E. coli* cells are represented by DNA and the remaining fraction by RNA.

RNA able to bind EthBr in cells must mainly be tRNA since ribosomal RNA is not accessible to EthBr in ribosomes (Bollen et al., 1970). Although the tRNA concentration in *E. coli* cells is much larger than that of DNA, its relative contribution as an EthBr receptor is much less than that of DNA. tRNA has only one EthBr binding site per molecule, that is, one per about 80 nucleotides (Bittman, 1969; Urbanke et al., 1973; Wells & Cantor, 1977), while DNA has one EthBr site per five nucleotides (Le Pecq & Paoletti, 1967).

In conclusion, it can clearly be established that DNA and RNA constitutes the structure able to bind EthBr inside *E. coli* cells. The factors which control the access of ethidium to DNA and RNA will now be discussed.

Control of the Ethidium Access to Nucleic Acids inside E. coli Cells. When the bacteria are in exponential growth, ethidium bromide is apparently unable to enter fully energized wild-type *E. coli* K 12 cells. A small amount of EthBr enters the ethidium-sensitive strain BL101 and *acrA* cells in exponential growth (about 0.1 nmol/10^8 bacteria at the maximum).

Massive quantities of EthBr enter wild-type or Ebs *E. coli* cells (1 nmol/10^8 cells at the maximum; Figure 6) (1) when cells are poisoned by uncouplers such as FCCP or CCCP (deenergized membrane) and (2) when the cell membrane ATPase is blocked in the Ebs strain by DCCD in the presence of oxygen (energized membrane).

Small quantities of EthBr enter cells (about 10 times less than in the previous two cases (1) when the cells are deprived of oxygen in a growth medium containing only a nonfermentable carbon source and (2) when the cells are treated by metabolic inhibitors such as KCN.

These results demonstrate that the state of energization of the membrane has no influence on the EthBr permeability of cell membranes. The rates of EthBr uptake are very similar, when uptake is triggered by CCCP (deenergized membrane, $k = 0.1$ min^{-1}) and when uptake is triggered by DCCD (energized membrane, $k = 0.063$ min^{-1}). Furthermore, when the cell membrane potential is abolished by CCCP treatment, the $TPMP^+$ cation is released from the cells, as expected, whereas a massive cell uptake of the ethidium cation is triggered. These results clearly show that both the EthBr membrane permeability and the EthBr cell uptake are not under the direct dependence of the electric membrane potential.

Only two factors can influence the uptake of EthBr by *E. coli* cells: (i) the permeability of the cell membrane and/or the existence of transport systems and (ii) the state of accessibility of nucleic acids inside the cells.

If membrane functions are involved in the control of EthBr uptake by *E. coli* cells, these functions must obligatorily involve an active transport phenomenon. Indeed, when massive quantities of EthBr have entered CCCP-treated cells, a complete expulsion of EthBr from cells takes place upon cell reenergization. This expulsion occurs even against a gradient and despite the tight binding of EthBr to nucleic acids inside cells. An active transport system, able to expel the massive quantities of EthBr which have then entered the cells, would need to be obligatorily invoked. Accordingly, when the active efflux of EthBr is inhibited, for instance by deenergization of the cells, EthBr would enter the cells. Upon reenergization of the cells, active efflux would be reactivated. EthBr would be expelled from the cells, as actually observed. In the mutant cells, the active efflux would be somewhat deficient because

of the membrane alteration. Several active efflux systems, as a cause of resistance to compounds such as tetracycline and arsenate, have been characterized (McMurry et al., 1980; Mobley & Rosen, 1982; Silver & Keach, 1982). Therefore, such a hypothesis involving active efflux of EthBr does not seem at first unlikely. Indeed, it could well account for several of the data presented in this paper. Nevertheless, the EthBr uptake mechanisms studied here differ drastically in several aspects from the active efflux system studied for tetracycline and arsenate. In addition, it could not account for several observations reported here.

(1) Cells grown in M9 succinate medium and treated by KCN or CO are ATP depleted, have no more membrane electric potential, and have no available energy source. Therefore, in this deenergized state, these cells should be unable to perform any active transport. EthBr should then freely enter the cells and fully saturate the nucleic acids inside the cells. Although some EthBr enters KCN-treated cells, the amount of EthBr bound to nucleic acids inside KCN-treated cells is 10 times less than the maximum amount of potential binding sites.

(2) All the drugs which could enter the cells upon inactivation of this active transport should be ejected from the cell after reactivation of this active transport. This is clearly not observed in the case of ethidium dimer, although the ATP pool is well restored even in the presence of this dye (Figure 8).

(3) Ebs and *acrA* cells should be somewhat deficient in this transport system. One then expects that this deficiency will become more and more pronounced, as the external EthBr concentration increases. Consequently, the EthBr uptake by these mutant cells should increase cooperatively with the external EthBr concentration. Indeed, it is the opposite situation which is observed, since the quantity of EthBr which enters the Ebs and *acrA* cells levels off at high external EthBr concentrations (Figure 2).

(4) Disruption of the cell membrane permeability by EDTA could be expected to alter the EthBr uptake as it does for other ions such as $TPMP^+$. This clearly does not happen.

(5) The variation of the membrane permeability and/or the change of activity of a transport system is not expected to influence the cellular distribution of the drug once entered in the cells. In this paper, it was observed that upon deenergization of the cells, EthBr binds to both RNA and DNA inside the cells. On contrary, when EthBr enters energized Ebs cells, EthBr binds mainly to DNA. Hansen (1982) made a comparative study of the photoaddition of radiolabeled psoralen to nucleic acids inside wild-type and *acrA* cells. He made the critical observation that in *acrA* cells the amount of psoralen covalently bound to nucleic acids after irradiation increased relatively to wild-type cells. In addition, he observed that in *acrA* cells, psoralen had mainly reacted with DNA. Therefore, he suggested that the mutation had modified the DNA environment in the *acrA* cells.

These two last experiments suggest strongly that change in the state of accessibility of nucleic acids inside the cells might be an important factor in controlling the uptake of DNA-reacting compounds. It was first thought that in prokaryotic cells, DNA was naked and not associated with proteins such as in eukaryotic cells. But many recent investigations show that indeed, prokaryotic chromosome DNA is associated with polyamines, RNA, and proteins. Under electron microscope, chromatin-like fibers were seen coming out from disrupted *E. coli* cells (Griffith, 1976). Several proteins have been found associated with the chromosomal DNA (Varshavsky et al., 1977). Several histone-like proteins have been characterized in *E. coli* (Rouviere-Yaniv & Gros, 1975; Haselkorn & Rouviere-Yaniv, 1976; Berthold & Geider, 1976; Hubscher et al., 1980; Lathe et al., 1980). DNA binding protein II forms nucleosome-like structures with double-stranded DNA (Rouviere-Yaniv et al., 1979). Plasmids have been isolated from *E. coli* cells as DNA–protein complexes (Wu et al., 1982). Furthermore, it has been shown that *E. coli* chromosome could be isolated as a supercoiled folded structure attached to the membrane. This structure is able to fold and unfold under certain conditions (Stonington & Pettijohn, 1971; Worcel & Burgi, 1972; Pettijohn et al., 1973; Worcel & Burgi, 1974; Portalier & Worcel, 1976; Drlica et al., 1978). The arguments in favor of the DNA packaging in a chromatin-like structure in prokaryotes have recently been reviewed (Geider & Hoffmann-Berling, 1981; Pettijohn, 1982).

Similarly, gene D5 of phage T5 codes for a protein produced in large quantities (2% of total cell protein). This protein binds to double-stranded DNA with a very high affinity and has an amino acid composition similar to that of *E. coli* histone-like DNA binding proteins (McCorquodale et al., 1979; Rice et al., 1979). All these data strongly suggest that indeed *E. coli* DNA is not naked inside the cells. Thus, its reactivity with DNA binding substances is expected to be severely reduced in cells, because of its packaging in a chromatin-like structure. Therefore, one can expect that changes in the DNA packaging and/or DNA accessibility resulting from various treatments or mutations might alter drastically the cell EthBr uptake.

The fact that reagents which are known to alter membrane functions such as CCCP and DCCD dramatically modify the EthBr uptake could have been misleading. Indeed, CCCP has been shown to interfere with many cell functions. Its action on the cell growth was shown to be related to DNA polymerase inhibition rather than to cell deenergization (Weigel & Englund, 1977).

In conclusion, we would like to suggest that EthBr uptake by *E. coli* cells in various physiological conditions reflects the state of nucleic acid accessibility. Of course, this does not rule out that membrane properties do not affect EthBr uptake. In particular, it is likely that both membrane permeability and DNA environment are modified in Ebs and *acrA* cells, which have indeed a very complex phenotype.

Nevertheless, the Ebs *E. coli* strain might prove to be an interesting tool for further studies. Indeed, if this mutation really alters the DNA packaging, a genetic approach of this process could be envisioned. The increased sensitivity of this strain to DNA binding compounds could be very useful in testing the biological effects on bacteria of a variety of compounds with potential carcinogenic or antitumor properties.

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Registry No. ATP, 56-65-5; ATPase, 9000-83-3; ethidium bromide, 1239-45-8; oxygen, 7782-44-7.

References

Ames, B. N., Durston, W. E., Yamasaki, E., & Lee, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2281–2285.

Berthold, V., & Geider, K. (1976) *Eur. J. Biochem. 71*, 443–449.

Bittman, R. (1969) *J. Mol. Biol. 46*, 251–268.

Bollen, A., Herzog, A., Favre, A., Thibault, J., & Gros, F.

(1970) *FEBS Lett. 11*, 49–54.
Brunk, C. F., Jones, K. C., & James, T. W. (1979) *Anal. Biochem. 92*, 497–500.
Coleman, W. J., Jr., & Leive, L. (1979) *J. Bacteriol. 139*, 899–910.
Comings, D. E. (1975) *Chromosoma 52*, 229–243.
Drlica, K., Burgi, E., & Worcel, A. (1978) *J. Bacteriol. 134*, 1108–1116.
Felle, H., Porter, J. S., Slayman, C. L., & Kaback, H. R. (1980) *Biochemistry 19*, 3585–3590.
Gaugain, B., Barbet, J., Oberlin, R., Roques, B. P., & Le Pecq, J.-B. (1978a) *Biochemistry 17*, 5071–5078.
Gaugain, B., Barbet, J., Capelle, N., Roques, B. P., & Le Pecq, J.-B. (1978b) *Biochemistry 17*, 5078–5088.
Geider, K., & Hoffman-Berling, H. (1981) *Annu. Rev. Biochem. 50*, 233–260.
Griffith, J. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 563–567.
Hansen, M. T. (1982) *Mutat. Res. 106*, 209–216.
Haselkorn, R., & Rouviere-Yaniv, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1917–1920.
Helgerson, S. L., Cramer, W. A., Harris, J. M., & Lytle, F. E. (1974) *Biochemistry 13*, 3057–3061.
Higashi, T., Suzuki, K., & Otsuji, N. (1981) *J. Bacteriol. 146*, 1117–1123.
Hirota, N., Matsuura, S., Mochizuki, N., Mutoh, N., & Imae, Y. (1981) *J. Bacteriol. 148*, 399–405.
Hixon, S. C., White, W. E., Jr., & Yielding, K. L. (1975) *J. Mol. Biol. 92*, 319–329.
Howard-Flanders, P., & Boyce, R. P. (1966) *Radiat. Res. 6*, 156–184.
Hubscher, U., Lutz, H., & Kornberg, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5097–5101.
Imae, Y. (1968) *J. Bacteriol. 95*, 1191–1192.
Jensen, R. H., Langlois, R. G., & Mayall, B. H. (1977) *J. Histochem. Cytochem. 25*, 954–964.
Karl, D. M. (1980) *Microbiol. Rev. 44*, 739–796.
Lambert, B., & Le Pecq, J.-B. (1982) *C. R. Hebd. Seances Acad. Sci., Ser. C 294*, 447–450.
Lanni, Y. T. (1961) *Virology 15*, 127–135.
Lanni, Y. T. (1969) *J. Mol. Biol. 44*, 173–183.
Lathe, R., Buc, H., Lecocq, J. P., & Bautz, K. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3548–3552.
Latt, S. A., & Wohlleb, J. C. (1975) *Chromosoma 52*, 297–316.
Leive, L. (1968) *J. Biol. Chem. 243*, 2373–2380.
Le Pecq, J.-B. (1971) *Methods Biochem. Anal. 20*, 41–86.
Le Pecq, J.-B., & Paoletti, C. (1967) *J. Mol. Biol. 27*, 87–106.
Le Pecq, J.-B., Nguyen-Dat-Xuong, Gosse, Ch., & Paoletti, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5078–5082.
Le Pecq, J.-B., Gosse, Ch., Nguyen-Dat-Xuong, & Paoletti, C. (1975) *C. R. Hebd. Seances Acad. Sci., Ser. D 281*, 1365–1367.
Lundin, A., & Thore, A. (1975) *Anal. Biochem. 66*, 47–63.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.
McCorquodale, D. J., Gossling, J., Benzinger, R., Chesney, R., Lawhorne, L., & Moyer, R. W. (1979) *J. Virol. 29*, 322–327.
McMurry, L., Petrucci, R. E., Jr., & Levi, S. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3974–3977.
Mitchell, P. (1967) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26*, 1370–1379.
Mobley, H. L. T., & Rosen, B. P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6119–6122.
Moreau, P., Bailone, A., & Devoret, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3700–3704.
Muller, W., & Gautier, F. (1975) *Eur. J. Biochem. 54*, 385–394.
Nakamura, H. (1968) *J. Bacteriol. 96*, 987–996.
Nakamura, H., Tojo, T., & Greenberg J. (1975) *J. Bacteriol. 122*, 874–879.
Nieva-Gomez, D., & Gennis, R. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1811–1815.
Nieva-Gomez, D., Konisky, J., & Gennis, R. B. (1976) *Biochemistry 15*, 2747–2753.
Nikaido, H. (1972) *Biochim. Biophys. Acta 433*, 118–132.
Olmsted, J., III, & Kearns, D. R. (1977) *Biochemistry 16*, 3647–3654.
Otsuji, N. (1968) *J. Bacteriol. 95*, 540–545.
Paoletti, C., Le Pecq, J.-B., Nguyen-Dat-Xuong, Juret, P., Garnier, H., Amiel, J.-L., & Rouesse, J. (1980) *Recent Results Cancer Res. 74*, 107–123.
Pettijohn, D. E. (1982) *Cell (Cambridge, Mass.) 30*, 667–669.
Pettijohn, D. E., & Hecht, R. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 31–41.
Portalier, R., & Worcell, A. (1976) *Cell (Cambridge, Mass.) 8*, 245–255.
Rayner, D. M., Szabo, A. G., Loutfy, R. O., & Yip, R. W. (1980) *J. Phys. Chem. 84*, 289–293.
Reinhardt, C., Roques, B. P., & Le Pecq, J.-B. (1982) *Biochem. Biophys. Res. Commun. 104*, 1376–1385.
Rice, A. C., Ficht, T. A., Holladay, L. A., & Moyer, R. W. (1979) *J. Biol. Chem. 254*, 8042–8051.
Rouviere-Yaniv, J., & Gros, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3428–3432.
Rouviere-Yaniv, J., Yaniv, M., & Germond, J. E. (1979) *Cell (Cambridge, Mass.) 17*, 265–274.
Schuldiner, S., & Kaback, H. R. (1975) *Biochemistry 14*, 5451–5460.
Silver, S., & Keach, D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6114–6118.
Stonington, G. O., & Pettijohn, D. E. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 6–10.
Tao, T., Nelson, J. H., & Cantor, C. R. (1970) *Biochemistry 9*, 3514–3524.
Tonnesen, T., & Friesen, J. D. (1973) *Mol. Gen. Genet. 121*, 177–186.
Urbanke, C., Romer, R., & Maass, G. (1973) *Eur. J. Biochem. 33*, 511–516.
Varshavsky, A. J., Nedospasov, S. A., Bakayev, V. V., Bakayeva, T. G., & Georgiev, G. P. (1977) *Nucleic Acids Res. 4*, 2725–2745.
Wahl, Ph., Paoletti, J., & Le Pecq, J-B. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 417–421.
Waring, M. J., Wakelin, L. P. G., & Lee, J. S. (1975) *Biochim. Biophys. Acta 407*, 200–212.
Weigel, Ph., & Englund, P. T. (1977) *J. Biol. Chem. 25*, 1148–1155.
Weisblum, B., & Haenssler, E. (1974) *Chromosoma 46*, 255–260.
Weiss, M. J., & Luria, S. E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2483–2487.
Wells, B. D., & Cantor, C. R. (1977) *Nucleic Acids Res. 4*, 1667–1680.
Worcel, A., & Burgi, E. (1972) *J. Mol. Biol. 71*, 127–147.
Worcel, A., & Burgi, E. (1974) *J. Mol. Biol. 82*, 91–105.
Wu, F. Y. H., Kolb, A., & Buc, H. (1982) *Biochim. Biophys. Acta 696*, 231–238.
Yguerabide, J. (1972) *Methods Enzymol. 26*, 498–578.
Yielding, L. W., Graves, D. E., Brown, B. R., & Yielding, K. L. (1979) *Biochem. Biophys. Res. Commun. 87*, 424–432.