Biochimica et Biophysica Acta, 506 (1978) 111—118

BBA 77892

CHANGES IN FLUORESCENCE OF 8-ANILINO-1-NAPHTHALENE SULFONATE AFTER BACTERIOPHAGE T5 INFECTION OF *ESCHERICHIA COLI*

INITIAL FLUORESCENCE RISE COINCIDES WITH ONSET OF RUBIDIUM EFFLUX

E. OLDMIXON and V. BRAUN

Mikrobiologie II, Institut für Biologie II, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen (G.F.R.)

(Received April 4th, 1977)
(Revised manuscript received September 13th, 1977)

Summary

Escherichia coli cells pre-loaded with $^{86}Rb^+$ begin to lose $^{86}Rb^+$ immediately after phage T5 addition. The loss proceeds with negative-exponential (first-order) kinetics for up to approximately 15 min after phage addition. The constant which characterizes the rate of loss increases with increasing numbers of infecting phage per cell.

It is known that anaerobic, fermenting cells of *E. coli* show a two-step increase in 8-anilino-1-naphthalene sulfonate (ANS) fluorescence upon infection with bacteriophage T5; the first rise begins immediately upon phage addition, the second 6 min later.

The onset of $^{86}Rb^+$ release, therefore, is correlated with the first fluorescence rise with respect to timing and response to the multiplicity of infection.

Introduction

The fluorescence of 8-anilino-1-naphthalene sulfonate (ANS), *N*-phenyl-naphthyl-1-amine, or 5-dimethylamino-1-naphthylene sulfonyl ethylamine (dansyl ethylamine) rises in two steps after infection with T5 bacteriophage of *Escherichia coli* cells which possess a functional (Ca^{2+} + Mg^{2+})-dependent, membrane-bound adenosine triphosphatase (ATPase) complex and which are held anaerobically in the presence of a fermentable substrate [1—3]. The first rise occurs immediately after phage addition and can be seen under both

Abbreviation: MOI, average number of infecting phages per cell.

aerobic and anaerobic conditions [2]. The second rise is seen only under conditions where the electron transport chain is not operating and seems to indicate the cessation of proton-pumping by the membrane-bound ATPase complex [2,3]. This paper describes experiments which show that the permeability barrier to potassium breaks down upon phage addition, that this effect, as with the first-step fluorescence rise, occurs both aerobically and anaerobically and that its magnitude is proportional to the multiplicity of infection, that is, the average number of infecting phages per susceptible cell.

Materials and Methods

Cell strains. Wild-type *E. coli* B and *E. coli* K-12 Ymel (*rha fadA but*-12 *lacI supE*-57 *supF*-58 λ^+) were used.

Bacteriophage. Wild-type T5 from the departmental collection was used. The growth and purification methods have been described [2].

Media. Cells were grown in a modified M9 [4] medium in which KH_2PO_4 had been replaced mol for mol with NaH_2PO_4. Where indicated, sufficient KCl was added to bring the final K^+ concentration to 4 mM. Glucose, maltose, or succinate was used as energy source at 0.4% final concentration.

Chemicals. All chemicals were analytical grade from Merck, Darmstadt, unless otherwise noted.

Radioisotopes. ^{86}RbCl was obtained from Amersham Buchler as a sterile aqueous solution with a typical initial specific activity of about 500 Ci/mol of rubidium. ^{86}Rb has a radioactivity half-life of 18.66 days. Using the information given on the packaging label (exact time and date when activity had been determined), we calculated what the activity at the time of phage infection would be and adjusted the amounts of isotope accordingly.

Experimental conditions. Cells from single colonies of *E. coli* B or *E. coli* K-12 Ymel were picked from nutrient agar plates and grown overnight in low-potassium M9 medium with 4 mM K^+ and maltose as carbon source. Cells were grown in flasks with an indented mixing vane and were shaken at 37°C at 200 cycles/min in a New Brunswick Scientific Co. rotary shaking water bath. Flasks were not filled more than one-quarter full. The next morning, portions of the overnight culture were diluted into fresh, low-potassium M9 medium with glucose or succinate as carbon source to an A_{578nm} = 0.006 (1.00 cm light path) and grown with shaking at 37°C to an absorbance of approx. 0.15. The amounts of culture, fresh medium with carbon source, and ^{86}RbCl were calculated which would give 100 ml of culture at A_{578nm} = 0.0063 with 3 μCi $^{86}Rb^+$ per ml, and the radioactive culture and a pilot culture, identical except for being non-radioactive (used for A_{578nm} determinations and fluorescence experiments), were grown to an A_{578nm} between 0.10 and 0.13. The cultures were harvested by centrifugation in 30-ml Corex tubes (trademark Corning Glass Co., Corning, New York) in a Sorvall RC-5 centrifuge, SS-34 angle rotor, 4°C, 7500 rev./min, for 20 min. The radioactive supernatant was carefully decanted to radioactive waste. The cell pellets were resuspended in a total of 10 ml chilled M9 medium with neither potassium nor carbon source, and centrifuged again. This wash was repeated once more. After the second wash, the cells were resuspended in enough chilled M9 medium (no K^+, no carbon

source) to give a final A_{578nm} = 1.00, and the growth carbon source was re-added. Under these conditions, A_{578nm} = 1.0 is equivalent to approx. $1.65 \cdot 10^9$ cells per ml. For anaerobic experiments, 5-ml Hamilton gas-tight syringes with square-tipped needles and 50-step dispensing devices allowed rapid mixing of phage and cells, holding at 37°C, and fast, precise sampling, all anaerobically. The desired amount of T5 phage was diluted up to 0.1 ml with phage buffer (0.5 M NaCl, 0.1 M Tris, pH 8.0, 0.01% gelatin, 1 mM $CaCl_2$) and held under argon longer than an hour. The resuspended, $^{86}Rb^+$-labeled cells were bubbled with water-saturated argon at 37°C for 10 min. The syringe was completely flushed three times with argon, and then the phage were taken up under argon. To start the experiment, the cells (4.9 ml) were taken into the syringe. This mixes the contents thoroughly and does not introduce bubbles. Samples (500 μl) were dispensed onto moist, soaked-and-rinsed cellulose filters (0.46 μm pore size, Schleicher and Schuell, Co.) on a vacuum filtering apparatus and washed once with 5 ml of 0.1 M LiCl (aqueous). The first sample was taken 30 s after starting to draw the cells into the syringe; the following samples were taken at 1 min, 2 min, etc., or at intervals of 3 min, depending on experimental design. The syringe was held at 37°C between samples by submerging the barrel in water in a cylinder standing in a 37°C bath, warming the barrel and keeping the mechanism dry. Gas exchange or loss of sample is negligible, owing to the cannula's small bore.

For aerobic experiments, 8.2 ml of $^{86}Rb^+$-labeled cells were held in 30-ml Corex tubes in a 37°C water bath and bubbled with water-saturated air. Phage were added to start the experiment and mixed by bubbling. Samples were removed by a mechanical microliter pipet (500 or 1000 μl) and filtered as described above.

The amount of $^{86}Rb^+$ remaining in the cells was determined by transferring the filters to scintillation vials, adding 5 ml of distilled water, and counting the flashes of Cerenkov radiation in a scintillation counter with a fully-open counting window.

The results were plotted as (Rb_t/Rb_0) versus time of sampling and as $-\ln(Rb_t/Rb_0)$ versus time of sampling where: Rb_t = scintillations per min in sample taken at time t; Rb_0 = scintillations per min at zero-time, as estimated by a least-squares fit of an exponential curve of the form $Rb_t = Rb_0(e^{-kt})$ to the data, where k is a constant characterizing the fitted curve and also the slope of the (linearized) fitted curve in the second kind of plot (this treatment must assume that all the $^{86}Rb^+$ in the cell at zero-time is potentially available for efflux); t = time of sampling after phage infection, in min.

ANS fluorescence curves were also taken with the unlabeled cells to ensure that the T5-*E. coli* interaction was typical for this system, using cells at A_{578nm} = 1.0 and multiplicity of infection (MOI) = 3. The experimental conditions have been described previously [2].

Results

Fig. 1 shows the changes in ANS fluorescence that occur after T5 infection of *E. coli Ymel* at an average of three phages per cell (MOI = 3) under four different conditions: fermentable substrate and anaerobicity, fermentable

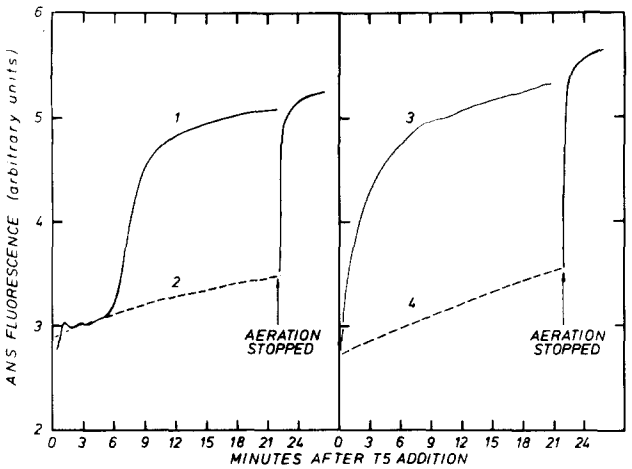

Fig. 1. Changes in *E. coli* ANS fluorescence after T5 addition under four different conditions. Left-hand portion. Cells resuspended in low-potassium M9 medium with 4 mM KCl and 0.4% glucose at a density of $1.65 \cdot 10^9$ cells per ml. T5 phage added at MOI = 3 at zero time. Curve 1, solid line (———): anaerobic, except for approx. 1 min following T5 addition. Curve 2, dashed line (- - - - - -): contents of cuvette aerated continuously. Right-hand portion. Cell sample as in left-hand portion of figure, except with 0.4% succinate instead of glucose. Curve 3, ———, anaerobic, except for short time after T5 addition at zero time. Curve 4, - - - - - -, aerated throughout. The pre-phage fluorescence levels for curves 1, 2, and 4 lie at 2.7—2.9 units and for curve 3 at 4.7—4.9 units.

substrate and aerobicity, non-fermentable substrate and anaerobicity, and non-fermentable substrate and aerobicity. (The analogous curves with *E. coli* B are essentially identical.) The fluorescence does not rise when T5 phage are added to an ANS solution (not shown).

Some properties of similar curves have been discussed previously [2]. What is important to notice is that in curves 1, 2, and 4 an increase in the fluorescence occurs immediately after phage addition. Curve 3 shows the reduction of ANS fluorescence due to the introduction of air into the cuvette during mixing and the quickly following rise in fluorescence to a level somewhat higher than the anaerobic level seen without phage as the introduced oxygen is consumed.

The sizes of the small immediate fluorescence rise and the second, larger, delayed fluorescence rise seen in curve 1 are related to the multiplicity of infection [2].

The left part of Fig. 2 shows the fraction of $^{86}Rb^+$ remaining in the cells at various times after phage infection under the conditions of curves 1 and 2 in Fig. 1, that is, in the presence of a fermentable substrate, glucose, under anaerobic and aerobic conditions. The slopes of these curves are reminiscent of negative-exponential curves associated with first-order or pseudo first-order processes. Therefore, the data were re-plotted as described in Materials and Methods. A negative-exponential curve (left-hand part) becomes a straight line with positive slope, passing through the origin (right-hand side).

In Fig. 3 are plotted the $^{86}Rb^+$ efflux data obtained after T5 infection (3 phages per cell) in the presence of a non-fermentable substrate, succinate, anaerobically and aerobically (compare Fig. 1, curves 3 and 4).

All the data indicate that, while the rate of efflux may vary under the several

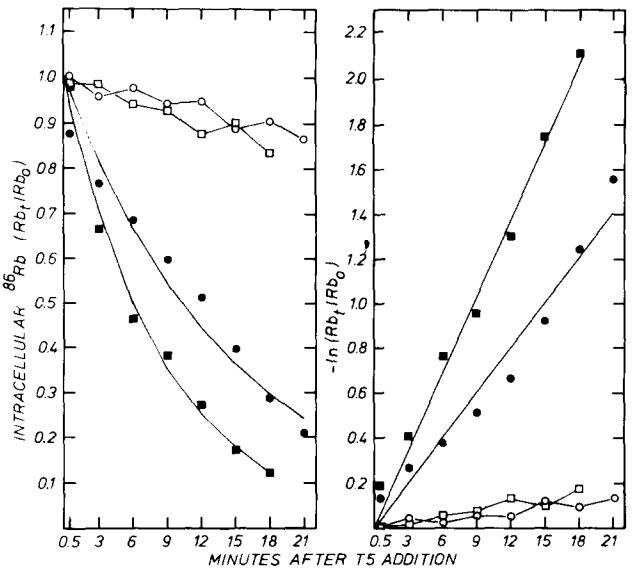

Fig. 2. Release of $^{86}Rb^+$ from cells of *E. coli* K-12 *Ymel* in the presence of glucose (fermentable substrate), with and without added T5 phage and with and without continuous aeration. The cells were resuspended in M9 medium to a cell density of $1.65 \cdot 10^9$ cells per ml. The experimental design is described in Materials and Methods. Empty symbols indicate control samples, not infected with phage. Filled symbols indicate samples infected with T5 phage at 0 min. Anaerobic samples are indicated by circles (○, no phage; ●, T5 at MOI = 3). Aerobic samples are shown as squares (□, no phage; ■, T5 at MOI = 3). Left portion. Radioactivity remaining in cells normalized to the amount in cells at zero-time (amount calculated by back-extrapolation of the fitted line shown in the right-hand portion of the figure). Right portion. Data curves from the left-hand portion of the figure transformed as described in Materials and Methods so that a loss of intracellular label proceeding with first-order kinetics would become a straight line with positive slope.

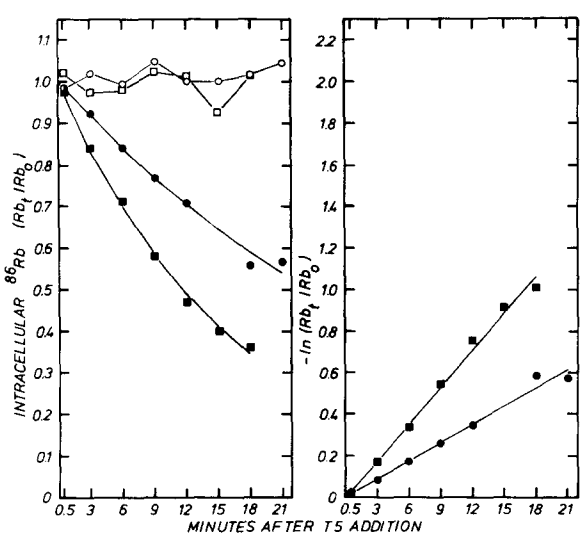

Fig. 3. Release of $^{86}Rb^+$ from cells of *E. coli* K-12 *Ymel* in the presence of succinate (non-fermentable substrate), with and without added T5 phage at MOI = 3 and with and without continuous aeration. Except for the carbon source, the conditions and the symbols used are as in Fig. 2. In the right-hand portion of the figure, the control curves (uninfected cells: empty symbols) have been omitted for the sake of clarity; they would lie scattered narrowly about the abscissa, as in Fig. 2.

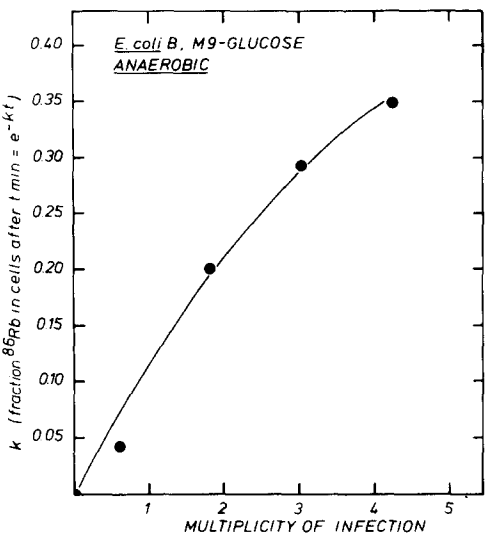

Fig. 4. Dependence of the constant describing the instantaneous rate-of-loss of $^{86}Rb^+$ from the intracellular space of *E. coli* K-12 *Ymel* after T5 phage infection versus the average number of phage infecting per cell (MOI). The cells were resuspended to a density of $1.65 \cdot 10^9$ cells per ml in M9 medium with 0.4% glucose. The experiments were performed under anaerobic conditions as described in Materials and Methods. The Materials and Methods section describes how the experimental data were handled to give this curve, and the Discussion the possible significance of this curve.

conditions, the same kind of mechanism operates in all four cases.

The effect of varying the multiplicity of infection was investigated with cells held anaerobically in the presence of a fermentable substrate, and Fig. 4 shows the results, expressed as the apparent efflux constant, k, versus the multiplicity. The constant k is also the slope of, for instance, the lines in the right-hand sides of Figs. 2 and 3. The larger k is, the steeper the line, and the greater the fraction of intracellular $^{86}Rb^+$ released into the medium in a given time interval.

Discussion

Fig. 1 shows that there are (at least) three forms the fluorescence curve observed after T5 infection of *E. coli* can take, one form being produced aerobically (curves 2 and 4) and two forms anaerobically, depending on whether the substrate can be fermented (curve 1) or not (curve 3).

Anaerobically, a large fluorescence rise occurs either immediately (as with a non-fermentable substrate and an aerobic-to-anaerobic transition, with or without added phage) or delayed by approx. 6 min (as with a fermentable substrate, after addition of T5 phage). Aerobically, no large fluorescence rise is observed. Anaerobically, a small, phage-caused rise can be seen clearly from the time of phage addition to fermenting cells and by comparing the fluorescence intensities under anaerobic conditions before and after phage addition to cells without a fermentable substrate. Aerobically, the small, phage-caused rise in fluorescence can always be observed. Thus, T5 infection of susceptible *E. coli* cells under these four conditions brings about a small rise in fluorescence.

From Figs. 2 and 3, it is evident that under the four conditions outlined above, $^{86}Rb^+$ efflux from pre-loaded cells starts very shortly after phage addition. The onset of this efflux, therefore, correlates with the occurrence of the small, early fluorescence rise. As noted above, the ANS fluorescence curve of anaerobic, fermenting cells may show a later change starting approx. 6 min and finishing approx. 12 min after T5 addition, but the $^{86}Rb^+$ efflux starts as a negative-exponential curve and remains so, under all four conditions, for at least 10 min. Therefore, whatever triggers the large fluorescence rise does not have a major, simultaneous influence on the $^{86}Rb^+$ efflux. Not only is the efflux correlated with the small fluorescence step and independent of the large step, but because the data fall on the same negative-exponential curve for as long as 20 min after phage infection, there does not seem to be anything similar to a "sealing reaction" [5—7] operating on Rb^+ (or K^+) ion permeability in this system.

Both the apparent efflux constant, k, and the size of the early fluorescence rise are positively related to the multiplicity of infection when the cells are anaerobic with a fermentable substrate (ref. 2 and Fig. 4).

Similar events occur in other systems. Changes in potassium permeability of whole *E. coli* cells are caused by colicin E1 action [8], colicin K action [9,12], colicin Ia action [13], bacteriophage T3 action [14], and bacteriophage T7 action [14,15]. Cellular permeability to Mg^{2+} and Co^{2+} increases after either colicin K or colicin E1 action [16,17]. These permeability changes are also correlated with changes in the fluorescence of added probes. Colicin E1 attachment to *E. coli* brings about increases in *N*-phenylnaphthyl-1-amine and ANS fluorescence, which have been intensively studied [18—21]. Increases in fluorescence after colicin K attachment have been using chlorotetracycline [22] and 3,3′-dihexyloxacarbocyanine [12]. Increases in ANS and *N*-phenylnaphthyl-1-amine fluorescence in the colicin I system have been described also [23]. Perturbations in the *E. coli* membrane after phage T3 or T7 attachment have been mentioned in the literature [14].

Colicins E2 and E3, whose modes of action are different from those of E1, I, or K (for review, see ref. 24) and which have not been reported to alter the permeability of cells membranes to small ions, have been found not to cause an increase in ANS fluorescence [20], reinforcing the idea that a process which perturbs the ion permeability of *E. coli* also causes an increase in ANS cell fluorescence. The nature of this common cause has not yet been demonstrated.

Acknowledgments

We would like to thank Dr. Karlheinz Altendorf for describing the rubidium efflux method to us, and for his participation in interesting discussions. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 76) to whom we would also like to extend thanks.

References

1 Hantke, K. and Braun, V. (1974) Virology 58, 310—312
2 Oldmixon, E. and Braun, V. (1977) submitted for publication

3 Oldmixon, E. and Braun, V. (1976) Abstr. 8th Meeting of the North West European Microbiological Group, p. 144
4 Clowes, R.C. and Hayes, W. (1968) Experiments in Microbial Genetics, p. 187, Blackwell Scientific Publications, Oxford
5 Puck, T.T. and Lee, H.H. (1954) J. Exp. Med. 99, 481
6 Puck, T.T. and Lee, H.H. (1955) J. Exp. Med. 101, 151
7 Silver, S., Levine, E. and Spielman, P. (1968) J. Virol. 2, 763—771
8 Gould, J.M., Cramer, W.A. and van Thienen, G. (1976) Biochem. Biophys. Res. Commun. 72, 1519—1525
9 Nomura, M. and Maeda, A. (1965) Zent. Bakteriol. Parasitenkd. Abt. I. Orig. 196, 216—239
10 Hirata, H,., Fukui, S. and Ishikawa, S. (1969) J. Biochem. 65, 843—847
11 Wendt, L. (1970) J. Bacteriol. 104, 1236—1241
12 Brewer, G.J. (1976) Biochemistry 15, 1387—1392
13 Gilchrist, M.J.R. and Konisky, J. (1975) J. Biol. Chem. 250, 2457—2462
14 Ponta, H., Altendorf, K.-H., Schweiger, M., Hirsch-Kaufmann, M., Pfennig-Yeh, M.-L. and Herrlich, P. (1976) Mol. Gen. Genet. 149, 145—150
15 Britton, J.R. and Haselkorn, R. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2222—2226
16 Lusk, J.E. and Nelson, D.L. (1972) J. Bacteriol. 112, 148—160
17 Lusk, J.F. (1976) Biochim. Biophys. Acta 448, 189—191
18 Helgerson, S.L., Cramer, W.A., Harris, J.M. and Lytle, F.E. (1974) Biochemistry 13, 3057—3061
19 Cramer, W.A., Phillips, S.K. and Keenan, T.W. (1973) Biochemistry 12, 1177—1181
20 Cramer, W.A. and Phillips, S.K. (1970) 104, 819—825
21 Cramer, W.A. and Keenan, T.W. (1974) Biochem. Biophys. Res. Commun. 56, 60—67
22 Brewer, G.J. (1974) Biochemistry 13, 5038—5045
23 Nieva-Gomez, D., Konisky, J. and Gennis, R.B. (1976) Biochemistry 15, 2747—2753
24 Reeves, P. (1972) The Bacteriocins, pp. 60—61, Springer-Verlag, New York