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REQUIREMENT OF HEAT AND METABOLIC ENERGY FOR THE EXPRESSION OF INHIBITORY ACTION OF COLICIN K

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SUMMARY

Escherichia coli B, induced for β-galactoside permease, can accumulate thiomethyl-β-galactoside in the cell even at 0 °C. At this temperature, cells adsorb colicin K but the adsorbed colicin does not inhibit thiomethyl-β-galactoside uptake. Inhibition by colicin K is, however, seen at 0 °C after exposure of the colicin K-cell complex to a high temperature: a greater degree of inhibition occurs with increasing temperature or duration of exposure. There is a transition point at around 21 °C in Arrhenius plots of this colicin K activation reaction.

If inhibitors of energy yielding reactions are present during the heat treatment, the inhibitory action of colicin K (as measured by thiomethyl-β-galactoside uptake after returning the colicin K-cell complex to 0 °C and removal of the inhibitors) is prevented.

These results indicate that adsorbed colicin K is converted into the active state only in the presence of metabolic energy and that cell surface fluidity appears to be concerned in this process.

INTRODUCTION

Colicin K is a bacteriocin which, upon adsorption onto sensitive bacteria, causes composite drastic changes, such as cessation of nucleic acid and protein synthesis [1, 2], destruction of some of the sugar transport systems [3], depletion of the cellular ATP [4] and efflux of the internal K^+ [2]. All of those effects occur, as in the case of the effects of other colicins, in a single hit mode. Although the mechanism by which the colicin acts in multiple ways in a single hit mode has not been clarified, it has been postulated [5–7] that its action is exerted through bacterial membrane: a change in the cell surface at which a colicin molecule is attached is transmitted in some way and spreads over the whole cell surface, causing drastic changes in cell physiology.

Abbreviations: TMG, thiomethyl-β-galactoside; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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As a first approach in the elucidation of the mechanism of colicin action, we raised the question of whether any metabolic energy is required for colicin to exert its inhibitory action. Since many of the colicin K-sensitive reactions require metabolic energy themselves, experiments have to be carried out under the condition where energy supply is allowed, in order to examine whether or not colicin K is really-acting. This complicates interpretation of the results and determination of whether energy is a prerequisite for colicin K action per se. Plate and Luria [8] showed that colicin K-cell interaction goes through at least two successive stages, stage I and stage II, and they found, as described briefly in their discussion, that the transition from stage I to stage II is an uncoupler-sensitive process. Lusk and Nelson [9] observed that colicin K-induced efflux of Mg^{2+} is blocked by inhibitors of energy-yielding metabolism. These results imply that colicin K is able to become active only under energy-utilizing conditions.

In the present study we confirmed the fact, originally discovered by Wendt [10], that the effect of colicin K appears at a much slower rate at low temperatures. By making use of this, in combination with the fact that uptake of thiomethyl-β-galactoside (TMG) occurs at 0 °C, we obtained evidence which suggests that colicin K requires metabolic energy for the exertion of its inhibitory action. The results of these experiments are the subject of the present paper.

MATERIALS AND METHODS

Chemicals

Isopropyl-β-thiogalactoside, dithiothreitol, *p*-chloromercuriphenylsulfonate and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma Chemical Co. [^{14}C]TMG was a product of New England Nuclear Corp. All other chemicals used were of reagent grade.

Colicin K

Crude colicin K, prepared as described elsewhere [11], was used in most experiments. Extensively purified colicin K, which was prepared by the method of Kunugita and Matsuhashi [12] and kindly supplied by Dr Matsuhashi of the University of Tokyo, was also used in some experiments, and it was confirmed that essentially the same results are obtained with the purified preparation. The potency of the colicin preparation was expressed in terms of inhibition units for TMG uptake, which was determined in a way similar to that described for T4 phage ghosts [11], except that the rate of [^{14}C]TMG uptake (2 h at 0 °C) was measured instead of [^{3}H]uridine incorporation.

Bacterial growth and induction of β-galactoside permease

E. coli B062 (Bb strain) was grown at 37 °C in Medium E [13] containing 0.4 % glycerol and $5 \cdot 10^{-4}$ M isopropyl-β-thiogalactoside. When a density of the culture reached approximately $3 \cdot 10^{8}$ cells/ml, cells were collected by centrifugation, washed with plain Medium E and resuspended in the original volume of Medium E/0. 4 % glycerol.

Uptake of [^{14}C]TMG at 0 °C

Washed *E. coli* cells, either treated or untreated with colicin K at 0 °C, were

subjected to heat treatment where indicated, and [^{14}C]TMG was added. (When specified as colicin K-cell complex, the cells were treated with colicin K at 0 °C for 10 min and then unadsorbed colicin was removed by centrifugation.) After standing in ice (except where otherwise indicated) for 2h, the mixture was diluted with chilled Medium E containing 0.4 % glycerol, and immediately filtered through Whatman glass fiber filter (GF/C). The filter was washed with the same medium, dried and counted in a liquid scintillation spectrophotometer.

RESULTS

TMG uptake by E. coli cells at 0 °C

As shown in Fig. 1, cells of *E. coli* are capable of taking up [^{14}C]TMG intracellularly even at 0 °C. Under the conditions employed, the uptake continued linearly with time for about 150 min at a rate approximately 1/150 that of the cells at 30 °C. This uptake is inhibited by various metabolic inhibitors of energy-yielding reactions, such as NaF, 2,4-dinitrophenol, or CCCP, to varying degrees, comparable to those at 30 °C (Fig. 2). The uptake did not occur with the cells not induced for β-galactoside permease (Fig. 1). These results indicate that TMG uptake at 0 °C is a permease-dependent and metabolic energy-dependent reaction, essentially the same in its nature as that observed at 30 °C, and not due to a cause like non-specific diffusion into the cells.

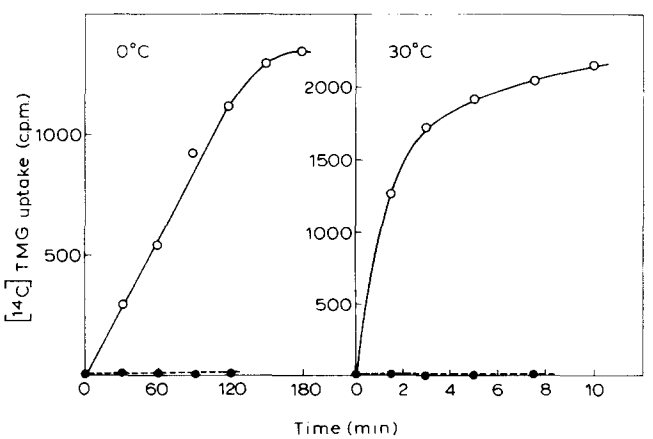

Fig. 1. Uptake of [^{14}C]TMG by *E. coli* at 0 °C and 30 °C. [^{14}C]TMG was added at time 0 to *E. coli* cells (not colicin K treated) and at intervals, 100 μl portions (containing $1.8 \cdot 10^7$ cells; 60 nCi = 26 nmol of TMG) were analyzed for TMG uptake. -○-, induced for β-galactoside permease; -●-, not induced.

Effect of colicin K on TMG uptake at 0 °C

It was reported by Wendt [10] that when ^{42}K-containing *E. coli* cells were treated with colicin K, intracellular ^{42}K is rapidly released into the medium and that this effect of colicin K is greatly reduced at low temperatures. A lack of the effect of colicin K at 0 °C was also confirmed in our TMG uptake system (Fig. 3). Is the effect of colicin K, then, always intrinsically absent at 0 °C, or does the colicin need

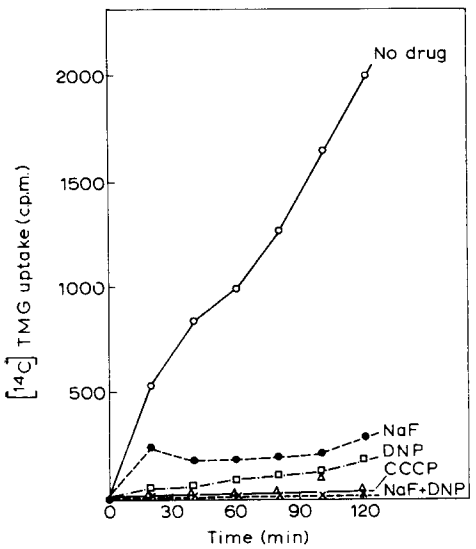

Fig. 2. Effect of inhibitors on TMG uptake at 0 °C. Inhibitors and [^{14}C]TMG were added at −10 and 0 min, respectively, and the mixtures incubated at 0 °C. At intervals, 100-μl portions (containing $3.6 \cdot 10^7$ cells, 60 nCi = 26 nmol TMG) were taken for the assay of TMG uptake. Concentrations of inhibitors: NaF, 50 mM; 2,4-dinitrophenol (DNP), 1 mM; CCCP, 20 μM.

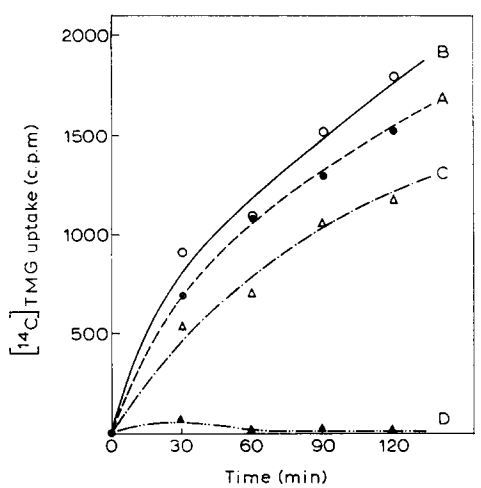

Fig. 3. Effect of heat treatment on TMG uptake of colicin K-adsorbed *E. coli*. Colicin K was added to *E. coli* culture (30 inhibition units/cell) at 0 °C and a portion was treated with heat as indicated. The [^{14}C]TMG uptake was carried out all at 0 °C. A sample (100 μl) for each point contained $2.5 \cdot 10^7$ cells and 60 nCi = 26 nmol of TMG. A, not colicin K treated; B, + colicin K (0 °C); C, + colicin K, 25 °C, 1 min; D, + colicin K, 30 °C, 3 min.

a certain temperature to start its inhibitory action and once such an inhibitory condition is established, is the effect of the colicin seen even at 0 °C? As shown in Fig. 3, when colicin K-treated cells were exposed to 25 °C for 1 min and then returned to 0 °C, the ability of TMG uptake was found to be inhibited partially, while if the cells were exposed to 30 °C for 3 min, complete loss of TMG uptake was observed after returning the cells to 0 °C. In the absence of colicin K, similar heat treatments did not cause any effect on TMG uptake. It is thus concluded that the effect of colicin K per se can be seen even at 0 °C provided that the colicin-cell complex is exposed to high temperatures for a short period.

To examine further the effect of temperature on the colicin K-cell complex, *E. coli* cells which adsorbed colicin K at 0 °C were brought to various temperatures for various periods, returned to 0 °C and then the ability of TMG uptake was examined. Fig. 4 shows the results. It is clearly seen that the higher the temperature or the longer the period of treatment, the greater the effect of colicin K. The reaction, through which the adsorbed colicin K is converted into an active form will be referred to as the "colicin K activation reaction" in this paper. Since the true rate of the colicin K activation reaction is difficult to determine because the inhibition curves (Fig. 4) are not of the "single hit" type with respect to the time of heat treatment, a reciprocal of the time at which 50 % inhibition was attained was taken as a rough index of the rate of this reaction. Arrhenius plots of the apparent rate of the colicin K activation reaction are presented in Fig. 5. It appears that there is a transition point, though not a very clear one, at around 21 °C. It was found that this relation holds over a wide range of multiplicity of colicin per cell, covering from 20 to 450 inhibition units/cell, although at higher multiplicities, the rate of the colicin K activation reaction was greatly accelerated, in agreement with Wendt's observation [10]. An apparent

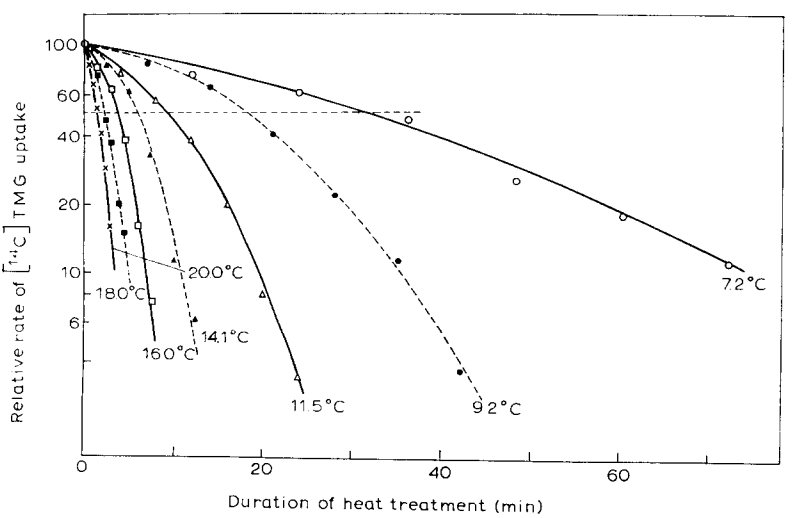

Fig. 4. Effect of heat pulse on the rate of TMG uptake of the colicin K/*E. coli* complex. The colicin K-cell complex (430 inhibition units/cell) was treated at various temperatures for various periods shown in the figure. The [^{14}C]TMG uptake was then carried out at 0 °C for 2 h. Each sample (100 μl) contained $1.7 \cdot 10^7$ cells and 60 nCi = 26 nmol of TMG. The rate of TMG uptake with the no-heat treated sample was taken as 100.

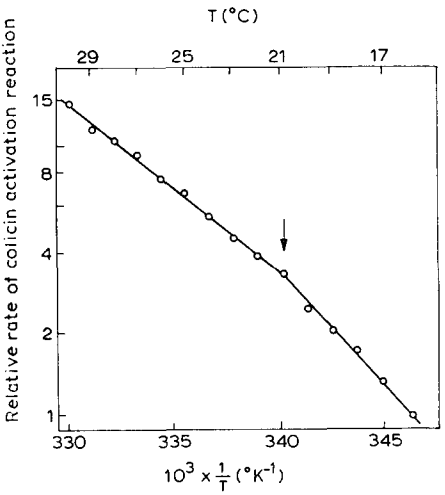

Fig. 5. Arrhenius plots of the colicin K activation reaction. The colicin K-cell complex (22 inhibition units/cell) was treated at various temperatures (16–30 °C) for various durations (5 different points at a given temperature). Periods required to cause a moderate extent of inhibition at each temperature had been determined in a preliminary experiment under the same conditions. From the figure based on the data (like Fig. 4), the time (A) required to cause 50 % inhibition was obtained at each temperature and a reciprocal of (A) was used as the relative rate of colicin K activation reaction. 1/(A) at 16 °C was taken as 1.0.

"activation energy" of the colicin K activation reaction was found to be 36.7 ± 1.8 kcal (from 4 experiments of varying multiplicities) for the temperature range below approx. 21 °C.

Effect of metabolic inhibitors during "heat pulse" on the colicin K activation reaction

The next problem challenged was that of whether the colicin K activation reaction is energy requiring or not. The following method was used. *E. coli* cells that adsorbed colicin K at 0 °C were treated with various inhibitors and subjected to heat pulse (30 °C, 3 min). After returning to 0 °C, the cells were diluted 100-fold with medium so that the effect of the inhibitors became essentially negligible and then the rate of [^{14}C]TMG uptake was measured. If the colicin K activation reaction requires metabolic energy, the presence of inhibitors during the heat pulse would keep the adsorbed colicin in a dormant state, and hence the cells should still retain a high activity of TMG uptake at 0 °C after diluting out the inhibitors. Table I summarizes the results of such experiments. It also includes appropriate control experiments to show the extent of inhibition by the drugs in these particular experiments, and the extent of recovery after 100-fold dilution without heat pulse. In the experiment shown in Table I, the uptake ability of the colicin K-adsorbed cells was reduced to 0.44 % after heat treatment (line1). 2,4-Dinitrophenol at concentration of 1 mM (line 2), inhibited the uptake ability, reducing it to 5.6 %, without heat pulse, if the drug was present throughout the period (column B). When this mixture was diluted 100-fold again without heat pulse, the effect of the inhibitor was completely reversed (121 %, column C). If, however, this mixture was exposed to heat pulse and then diluted out, the cellular activity of TMG uptake decreased only to 11 % (column D). It

TABLE I

EFFECT OF INHIBITORS DURING HEAT PULSE ON THE COLICIN ACTIVATION REACTION

10 μl ($3 \cdot 10^7$ cells) of concentrated colicin K-cell complex (35 inhibition units/cell), containing inhibitors as indicated, were subjected to heat pulse (30 °C, 3 min) (for column D only), diluted with 1 ml of chilled Medium E/glycerol (for columns C and D), and the uptake of [^{14}C]TMG (0.15 Ci/64 mmol per ml) was measured (0 °C, 2 h). For column B, the cells were diluted with Medium E/glycerol containing inhibitors of the same concentration and the TMG uptake was determined without heat pulse.

A	B	C	D	E
Inhibitor used	TMG uptake (%)			D/C × 100 (%)
	Inhibitor present throughout (No heat pulse)	Inhibitor removed		
		No heat pulse	Heat pulse	
No inhibitor	100	100*	0.44	0.44
2,4-Dinitrophenol (1 mM)	5.6	121	11.4	9.6
NaF (50 mM)	31.5	68.2	2.4	3.5
NaCN (2 mM)	21.0	92.6	9.3	10.1
CCCP (5 μM)	13.2	122	47.2	38.7
NaF + NaCN	8.1	78.7	35.7	45.3
NaF + 2,4-dinitrophenol	2.2	116	120	103

* 677 cpm. This was taken as 100 for comparison.

should be emphasized here that in the presence of 2,4-dinitrophenol the effect of heat pulse is not as great as in the drug-free control (see column E; 9.6 % in the presence of 2,4-dinitrophenol vs 0.44 % in control). Likewise, NaF has some blocking effect on colicin K-induced inhibition, if present during the heat pulse (line 3). Although either 2,4-dinitrophenol or NaF alone has only a little effect in preventing colicin K action, when these two drugs were used together (line 7), no inhibition by colicin K was seen even after heat treatment: that is, these two drugs in combination completely block the colicin K activation reaction. A similar, but lesser synergistic effect was seen between NaF and NaCN. Only CCCP, known as a potent proton conductor, had a considerable effect on the colicin K activation reaction at the concentration used in Table I. The effect of CCCP concentration during the heat pulse on this reaction was examined in a similar way (Fig. 6) and it was found that this inhibitor at concentrations above 10 μM, where TMG uptake was inhibited more than 98 % without heat pulse, completely blocks the colicin K activation reaction. These results indicate that metabolic energy is a prerequisite for adsorbed colicin K to exert its inhibitory action.

In the experiments described above, there remains the possibility that the adsorbed colicin K might be inactivated or detached from the cells during heat treatment in the presence of the inhibitors, thus causing an apparent reduction in the effect of the heat treatment. However, as shown in Table II, if the colicin K-adsorbed cells were heat treated (30 °C, 3 min) in the presence of CCCP, chilled back to 0 °C, diluted to remove the CCCP effect and then again subjected to the same heat pulse (in the absence of the drug), they were found to have lost the ability of TMG up-

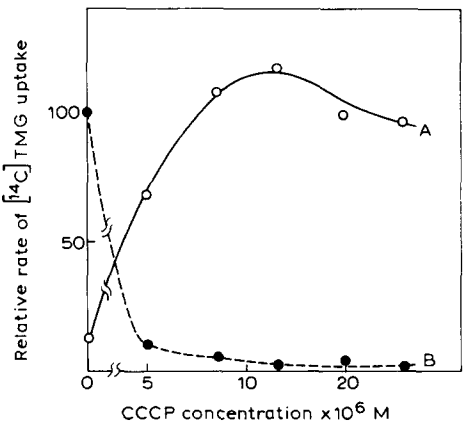

Fig. 6. Effect of CCCP concentration during heat pulse on colicin K activation reaction. 10 μl ($2.3 \cdot 10^7$ cells) of concentrated colicin K-cell complex (30 inhibition units/cell), containing various concentrations of CCCP, were subjected to a heat pulse (30 °C, 3 min), returned to 0 °C, diluted 100-fold with Medium E/glycerol containing no CCCP (A) or containing the same concentration of the inhibitor (B), and then the rate of [^{14}C]TMG uptake was measured (0 °C, 2 h). The final TMG concentration was 0.15 μCi = 64 nmol/ml.

TABLE II

POSSIBILITY OF INACTIVATION OR DETACHING OF COLICIN K DURING THE HEAT PULSE

Colicin K-cell complex (30 inhibition units/cell) was treated as shown. 80 μl ($2.2 \cdot 10^7$ cells) of the complex were then used for measurement (0 °C, 2 h) of [^{14}C]TMG uptake (0.6 Ci/0.26 mol per ml).

Treatment	Relative TMG uptake
0 °C, cf*	100**
0 °C → 30 °C, 3 min → 0 °C, cf	9.9
0 °C, + CCCP*** → cf	104
0 °C, + CCCP → 30 °C, 3 min → 0 °C, cf	114
0 °C, + CCCP → 30 °C, 3 min → 0 °C, cf → 30 °C, 3 min → 0 °C	4.2

* The colicin K-cell complex was pelleted by centrifugation in the cold and resuspended in chilled Medium E/glycerol containing no CCCP.

** 262 cpm, taken as 100.

*** CCCP, 5 μM.

take. Therefore, it is inferred that initially attached colicin K was still present in an "intact" state after these treatments.

Effect of Mercury reagent during heat pulse on the colicin K activation reaction

The method described in the preceding section can be applied not only to the inhibitors of energy-yielding reactions, but to many other drugs whose effects are reversible. The effect of a mercury reagent, *p*-chloromercuriphenylsulfonate, on the colicin K activation reaction was examined (Table III). This mercury reagent inhibited cellular TMG uptake itself, but the inhibition was mostly reversed by the addition of dithiothreitol. Thus the above method is applicable to this reagent. Heat

TABLE III

EFFECT OF MERCURY REAGENT DURING HEAT PULSE ON THE COLICIN ACTIVATION REACTION

100 μl ($3.8 \cdot 10^7$ cells) of *E. coli* cells were used for the measurement of [^{14}C]TMG uptake (0 °C, 2 h; 0.6 μCi = 0.26 μmol/ml), with additions or treatment as shown in the table. At least 10 min were allowed between additions.

Treatments	Relative TMG uptake
0 °C (no PCMPS*, no colicin K)	100 (= 871 cpm)
0 °C (+PCMPS)	6.7
0 °C (+PCMPS → +DTT**)	80.7
0 °C (+PCMPS → +colicin K*** → +DTT)	81.3
0 °C (+PCMPS → +colicin K) → 37 °C, 2 min → 0 °C (+DTT)	2.4
0 °C (+colicin K)	116
0 °C (+colicin K) → 37 °C, 2 min → 0 °C	2.5

* *p*-Chloromercuriphenylsulfonate, 0.1 mM.
** Dithiothreitol, 10 mM.
*** 23 inhibition units/cell.

treatment of the colicin K-cell complex in the presence of the mercury reagent, followed by addition of dithiothreitol after returning to 0 °C, revealed that this mercury reagent has no effect in preventing the colicin K activation reaction.

EDTA (5 mM) was also tested and found to have no effect on the colicin K activation reaction (data not shown).

DISCUSSION

As has been shown by Wendt [10] with the system which involves measurement of the efflux of intracellular K^+, the inhibitory effect of colicin K on TMG uptake was also shown to be greatly reduced at low temperatures. The effect of colicin K could, however, be seen, even at 0 °C, if colicin K-adsorbed cells were subjected to heat treatment for certain periods. This implies that an adsorbed colicin K molecule cannot become active at 0 °C but after activation by "heat", its inhibitory effect is present even at 0 °C. In the present study, the effect of temperature on the activation of the colicin-cell complex could be followed accurately since all the measurements of TMG uptake were performed after the cells were returned to 0 °C, unlike the measurements of intracellular K^+ efflux [10]. Arrhenius plots of the colicin K activation reaction thus obtained showed that there is a transition point at around 21 °C, suggesting that fluidity of the cell surface components might be concerned with this activation process. This supposition would be further substantiated if different transition temperatures were obtained by changing the cell surface fatty acid composition, by the use of, for example, *E. coli* fatty acid auxotrophic mutants.

The process of colicin K action has been resolved by Plate and Luria [8] into two successive stages; stage I, which is recoverable by trypsin and stage II, which is no longer reversible. According to these authors, transition from stage I to II is blocked at low temperatures, and by inhibitors such as 2,4-dinitrophenol or CCCP. Adsorbed colicin K at 0 °C is probably in stage I, and after heat treatment it would

shift into stage II, although in the present study trypsin rescuability was not examined.

Combination of the result that adsorbed colicin K at 0 °C becomes active only after the heat pulse, with the fact that TMG uptake can be measured at 0 °C, permitted us to analyse the requirement of metabolic energy in the colicin K activation reaction, by the use of various reversible metabolic inhibitors during the heat pulse. 2,4-Dinitrophenol, a proton conductor, or NaF, an inhibitor of the glycolytic pathway, when used separately, were shown to have only a partial effect in preventing the colicin K activation reaction, while if these were used together adsorbed colicin K was kept perfectly inactive even after the heat pulse. This would be interpreted to mean that any metabolic energy, produced either by respiration (inhibitable by 2,4-dinitrophenol) or by ATP formed via the glycolytic pathway (NaF sensitive) can be utilized for the activation of the colicin K-cell complex, so that, in order to block this activation process completely, both drugs have to be used. CCCP is a very potent proton conductor and thus this drug alone is effective in blocking the colicin K activation reaction, provided that a sufficiently high concentration is used (Fig. 6). These results are comparable with Lusk and Nelson's observation [9] that colicin K-induced Mg^{2+} loss from the cells is inhibited by the presence of metabolic inhibitors. The effect of 2,4-dinitrophenol is also in agreement with the results by Cavard et al. [14], who reported that the cellular lysis by colicin K is prevented in the presence of this inhibitor.

It may now be concluded that the adsorbed colicin K is initially inactive in blocking cellular metabolism, but is converted to the active state under the supply of metabolic energy, and that in this conversion process the fluidity of cell surface structure appears to be somehow concerned.

At present, we are still, of course, far from constructing a picture of colicin K action. Clarification of the way in which metabolic energy is involved in this activation process will be the next step toward the elucidation of the problem.

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