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ENERGY REQUIREMENT FOR THE INITIATION OF COLICIN ACTION IN *ESCHERICHIA COLI*

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SUMMARY

1. Starved cells of a strain of *Escherichia coli* and its mutant *unc*A, treated with colicin K, E2 or E3, remained fully rescuable upon trypsin treatment (stage I in colicin action). The transition to stage II in colicin action (cells no longer rescuable by trypsin) was promoted by the addition of either glucose or D-lactate.

2. Aerobically glucose-grown cells of the normal strain were irreversibly killed by colicin K, E2 or E3 under anaerobic conditions, while similarly treated cells of its mutant *unc*A remained fully rescuable. The stage I-stage II transition in colicin action was blocked in normal cells under anaerobic conditions when succinate was the sole carbon source.

3. Arsenate alone had little effect on the progression of the stage I-stage II transition in normal cells, treated with colicin K. However, this transition was abolished in the presence of both arsenate and anaerobic conditions.

4. The initiation of colicin action could be coupled to the anaerobic electron transfer systems formate dehydrogenase-nitrate reductase and α-glycerophosphate dehydrogenase-fumarate reductase.

5. These results indicate that an energized state of the cytoplasmic membrane is required for the initiation of colicin action and that no high-energy phosphorylated compounds are necessary.

INTRODUCTION

Colicins are a class of protein antibiotics that adsorb to specific receptors on the cell surface and kill sensitive cells of *Escherichia coli* by affecting quite different biochemical processes [1]. Thus, colicin K inhibits primarily those active transport systems that are energized by the high-energy state of the membrane [2–4]; colicin E2 causes breakdown of the DNA [5]; and colicin E3 inhibits specifically protein synthesis [6].

After binding of colicin K to the receptor site, one can distinguish at least two stages in colicin action [7]. In the first stage (stage I) no detectable physiological damage occurs and cell viability can be restored by treatment with trypsin. In stage II physiological damage becomes noticeable and the cells can no longer be rescued by

trypsin. The transition from stage I to stage II is influenced by several conditions such as temperature and the overall fatty acid composition of the membrane [8] in a manner that suggests that the degree of fluidity of the lipid components of the membrane is important to colicin K action. A similar dependence is indicated for colicin E1 from the results of fluorescence studies [9].

It has been known that addition of respiratory inhibitors, such as cyanide or the uncouplers of oxidative phosphorylation, such as dinitrophenol and carbonyl-cyanide *m*-chlorophenylhydrazone prior to colicin treatment prevents the occurrence of irreversible cellular damage by colicin K [8], E3, or E2 [10, 11]. This suggests that energy may be required for the initiation of irreversible colicin action in vivo. Until now, however, the question of the specific energizing mechanism required for colicin action remained unexplored. It is known that, among the bacterial functions that require energy, some use ATP or other high-energy phosphorylated compounds directly, while other functions, such as mobility [12], transport of some sugars and amino acids [13, 14], and energy-dependent transhydrogenase [15] require what has been called an energized state of the membrane. This high-energy state can be generated either by electron transport or from ATP by means of the (Mg^{2+}, Ca^{2+})-dependent adenosinetriphosphatase (ATPase) [16, 17].

In the present study the ability of different carbon sources to provide energy for the initiation of colicin action under various conditions was investigated in normal cells of *E. coli* and in cells of a mutant *unc*A that lacks (Mg^{2+}, Ca^{2+})-ATPase activity. The results indicate that the stage I-stage II transition for several colicins specifically requires the existence of an energized state of the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The bacterial strains used throughout this study were *E. coli* G6 *his*$^-$ and its derivative G6 *unc*A [4] constructed from N144 *unc*A, which had been obtained from Dr D. Gutnick. The bacteria were grown aerobically at 37 °C in Ozeki medium base [18], supplemented with 3 μM thiamine, 1 mM L-histidine and 0.5 % glucose or 1 % succinate as carbon source. For anaerobic cultures, Erlenmeyer flasks were completely filled with medium, tightly stoppered, and the contents were stirred by means of a magnetic stirrer. The anaerobic cultures were grown in Ozeki medium base supplemented with 0.1 % yeast extract and, as a carbon source, 0.5 % glucose or 0.5 % glycerol which induces α-glycerophosphate dehydrogenase. For the induction of nitrate reductase, 50 mM potassium nitrate, 1 μM selenic acid and 1 μM sodium molybdate [19] were also added. For the induction of fumarate reductase 20 mM sodium fumarate [20] was added. The bacteria were harvested during the exponential phase of growth, washed twice in Ozeki medium base containing 100 μg of chloramphenicol per ml and resuspended in the same medium.

Colicin preparation and assay

Colicin K was prepared by a modification of the method of Kunugita and Matsuhashi [21] in which the DEAE-Sephadex chromatography was omitted and colicin was eluted from the harvested cells by gentle stirring. Colicin E2 and E3 were purified from *E. coli* QR 47 (E2) and CA 37 (E3), respectively, according to the method

of Herschman and Helinski [22]. The number of killing units in the colicin preparation was calculated from the equation $S/S_0 = e^{-m}$, where m is the multiplicity of killing units and S/S_0 the survival ratio.

Multiplicities were calculated from viability assays done after a 5-min incubation with colicin at 37 °C. Viability assays were performed by plating appropriate dilutions of cultures on LB-agar [23]. Dilutions were done in Ozeki medium base at room temperature. To determine trypsin rescuability, cells treated with colicin K were incubated with trypsin (250 μg/ml) for 5 min prior to plating: a 30-min incubation with trypsin was used for cells treated with colicins E2 and E3.

Amino acid uptake

Washed cells ($5 \cdot 10^8$ per ml) were incubated at 37 °C in Ozeki medium base containing 100 μg of chloramphenicol per ml and a carbon source. After addition of L-[U-^{14}C]proline (10.5 Ci/mol, 50 μM final concentration) or L-[U-^{14}C]glutamine (10.1 Ci/mol, 50 μM final concentration) aliquots (0.5 ml) were removed at various time intervals, filtered on nitrocellulose filters (0.45 μM, Matheson-Higgins, Inc., Woburn, Mass.) and washed with 7 ml of Ozeki medium base. The filters were dried and counted in a liquid scintillation counter.

Anaerobic studies

For anaerobic experiments incubations were carried out in tubes flushed with oxygen-free argon (less than 1 ppm oxygen) and in the presence of 1 mM sodium dithionite. A control tube received methyl viologen (−0.446 V, 5 μM final concentration) as indicator of anaerobiosis [19]. The results were similar to those obtained when the experiments were carried out under oxygen-free argon only.

Chemicals

Chloramphenicol was obtained from Parke-Davis and Co., Detroit, Mich. Trypsin was purchased from Worthington Biochemicals, Freehold, N.J. Methyl viologen was purchased from Aldrich Chemical Co., Cedar Knolls, N.J. L-[U-^{14}C]-Proline and L-[U-^{14}C]glutamine were obtained from New England Nuclear, Boston, Mass.

RESULTS

Effect of colicins on starved cells

To study the influence of a particular carbon source on colicin action the endogenous energy reserves must be sufficiently low so that the measured effect is primarily caused by the added compound. For adequate depletion of the endogenous energy reserves in cells of *E. coli* G6 and G6 *unc*A a method described by Berger [16] was used. Cells, starved by such procedure, were totally blocked in the progression of the stage I-stage II transition in colicin K action (Table I). Subsequent addition of either glucose or D-lactate restored the irreversible killing by colicin K in both G6 and G6 *unc*A. Comparable results were obtained for colicin E2 and E3. The initial uptake rates for proline and glutamine in the case of G6, and for proline in the case of G6 *unc*A, responded to the addition of the carbon sources in the same way as did the transition from stage I to stage II in colicin action. The uptake of glutamine in

TABLE I

COLICIN KILLING AND AMINO ACID UPTAKE IN STARVED CELLS OF *E. COLI* G6 AND G6 *unc*A

Cells growing aerobically in glucose, were washed, resuspended in Ozeki medium base, and vigorously aerated at 37 °C in the presence of 5 mM dinitrophenol, followed by extensive washing and resuspension in the same medium containing 100 μg of chloramphenicol per ml [16]. These starved cells were incubated for 10 min at 37 °C in the presence of glucose (20 mM), D-lactate (20 mM) or without any addition before colicins or radioactive amino acids were added. The percent survival of colicin-treated cells after trypsin treatment and amino acid uptake was determined. The percent survival of colicintreated cells without trypsin treatment is given in parentheses.

Strain	Addition	Percent survival at 10 min			Initial rate of uptake (nmol/min per mg protein)	
		K	E2	E3	Proline	Glutamine
G6	None	99 (0.2)	100 (0.1)	91 (0.2)	0.22	0.17
	Glucose	3 (0.3)	2 (0.1)	11 (0.3)	4.56	3.91
	D-Lactate	11 (0.2)	7 (0.1)	9 (0.2)	3.60	3.12
G6 *unc*A	None	96 (0.6)	99 (0.2)	103 (0.2)	0.31	0.21
	Glucose	7 (0.5)	5 (0.3)	12 (0.3)	4.23	3.56
	D-Lactate	12 (0.5)	8 (0.3)	12 (0.3)	3.09	0.33

starved cells of G6 *unc*A was restored by glucose but not by D-lactate, in agreement with Berger's report [16]. These results indicate that the stage I-stage II transition in colicin action is similar to proline transport in that neither of these activities require the formation of phosphate-bond energy.

The effect of anaerobiosis

Under aerobic conditions and with glucose as carbon source, the loss of trypsin rescuability in colicin K-treated cells of G6 and G6 *unc*A follows first-order kinetics [4]. When colicin K-treated cells of G6 *unc*A were transferred to anaerobic conditions by the addition of dithionite, the progression of the stage I-stage II transition was immediately blocked (Fig. 1). When aerobic, glucose-grown cells of G6 were placed under anaerobic conditions prior to the addition of colicin K, the cells were still killed, albeit at a slower rate than under aerobic conditions (Fig. 2a). In contrast, cells of G6 *unc*A, treated with colicin K under anaerobic conditions, were fully rescuable upon trypsin treatment and underwent a rapid decrease in viability upon the subsequent addition of oxygen (Fig. 2b). When succinate or D-lactate was the carbon source instead of glucose, anaerobiosis blocked the killing by colicin K in cells of both G6 *unc*A and G6 (Figs 2c and 2d). These results are in agreement with findings obtained from fluorescence studies [24], which show that with lactate as carbon source the chlorotetracycline fluorescence response caused by colicin K is prevented by anaerobic conditions.

A similar effect as for colicin K was observed with colicins E2 and E3 (Figs 3a and 3b). For colicin E3 the loss of trypsin rescuability was preceded by a lag, which was shifted with the time at which oxygen was added.

These results suggest that an energized state of the membrane is required for the initiation of colicin action in vivo and are in agreement with the dual input model

[16, 17] which allows the energized state to be generated via either electron transport or ATP through the mediation of the (Mg^{2+}, Ca^{2+})-ATPase.

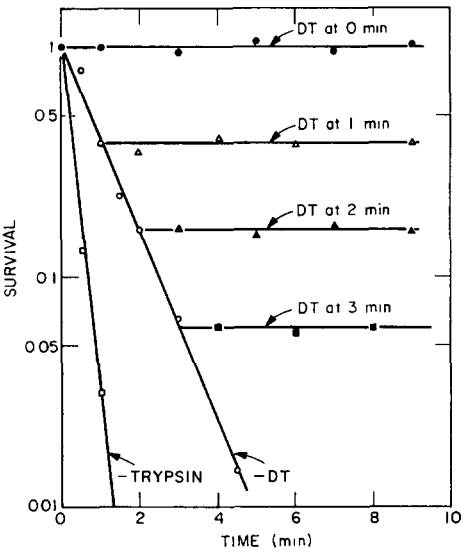

Fig. 1. Effect of anaerobiosis on the stage I-stage II transition in aerobically grown cells of G6 *unc*A treated with colicin K. Cells were incubated with aeration in Ozeki medium base containing glucose and chloramphenicol. At time zero, colicin K (m = 10.5) was added, samples were taken at various time intervals and plated directly (□) or treated with trypsin and then plated (○). Simultaneously, aliquots removed at 0 (●), 1 (△), 2 (▲), and 3 (■) min received dithionite (DT) and were sampled in the same way.

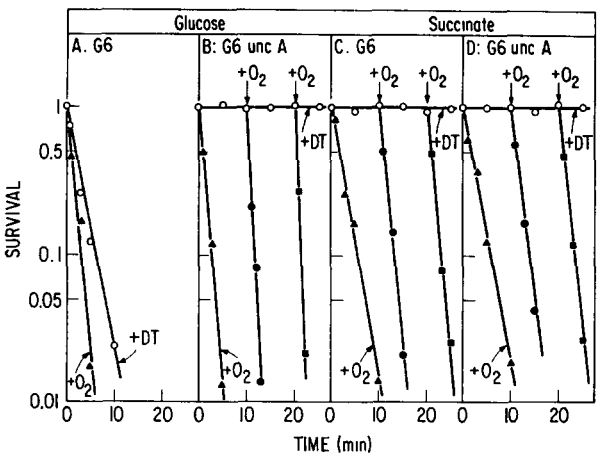

Fig. 2. Effect of anaerobiosis on the stage I-stage II transition in aerobically grown cells of G6 and G6 *unc*A treated with colicin K. Cells were incubated under anaerobic conditions (plus dithionite) in Ozeki medium base containing glucose (A–B) or succinate (C–D) and chloramphenicol. At time zero colicin K (m = 9.6) was added and samples were taken at various time intervals, treated with trypsin and plated (○). Simultaneously, aliquots were removed at 0 (▲), 10 (●) and 20 (■) min, vigorously aerated and sampled in the same way.

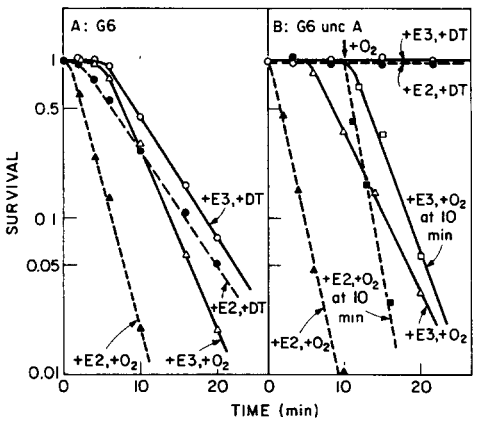

Fig. 3. Effect of anaerobiosis on the stage I-stage II transition in aerobically grown cells of G6 and G6 *unc*A treated with colicin E2 or E3. Cells were incubated under anaerobic conditions in Ozeki medium base containing glucose and chloramphenicol. At time zero colicin E2 (dotted lines) or E3 (solid lines) was added, samples were taken at various time intervals, treated with trypsin and plated (○, ●). Simultaneously, aliquots were removed at 0 and 10 min, vigorously aerated and sampled in the same way. △, ▲, oxygen added at 0 min; □, ■, oxygen added at 10 min.

The effect of arsenate

To verify the above conclusions, the effect of arsenate on the trypsin rescuability of colicin K-treated cells of *E. coli* G6 was investigated. Incubation of *E. coli* cells with arsenate causes a drastic reduction of the intracellular ATP levels [17]. Systems that use high-energy phosphorylated compounds directly as energy source are severely inhibited by arsenate, whereas those driven by an energized membrane state are relatively resistant unless this state is generated from ATP. Under aerobic conditions and with glucose as carbon source, arsenate only slightly enhanced the trypsin rescuability of colicin K-treated cells of G6. Anaerobiosis, as established by the addition of dithionite, had a somewhat larger effect. However, the combination of anaerobiosis and arsenate resulted in a total inhibition of the stage I-stage II transition (Fig. 4). Under all conditions the adsorption of the colicin was unaffected. Like in cells of G6, arsenate had hardly any influence on colicin K action in cells of G6 *unc*A.

Anaerobic electron transfer systems

Cells of strain G6 can grow anaerobically on glucose alone or with glucose plus nitrate [19, 25] as electron acceptor. With glycerol as a carbon source an external electron acceptor such as fumarate [20] or nitrate is a prerequisite for active growth. Cells of the mutant G6 *unc*A only grow actively under anaerobic conditions when an electron acceptor is available either with glucose or glycerol as a carbon source. In order to investigate more precisely the coupling between the initiation of colicin action and electron transport, cells were grown under the conditions described above and treated with colicin. Anaerobically grown cells of G6 and G6 *unc*A induced for the synthesis of nitrate reductase were normally killed by colicin K when glucose and nitrate were present. In the absence of nitrate the stage I-stage II transition was blocked in colicin K-treated cells of G6 *unc*A, but not in cells of G6. Subsequent addition

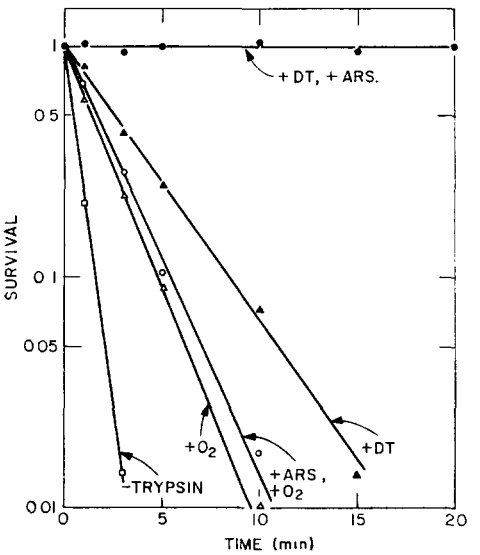

Fig. 4. Effect of arsenate on the stage I-stage II transition in aerobic cells of G6 treated with colicin K. Cells were incubated in 0.05 M Tris · HCl, pH 7.0, containing 1 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.5 % of glucose and 100 μg of chloramphenicol per ml. The cells were subsequently treated with colicin K (m = 9.9) under various conditions; samples were taken at various time intervals and the viable count was determined after trypsin treatment: △, with aeration; ○, with aeration and in the presence of sodium arsenate (20 mM arsenate); ▲, in the presence of dithionite (DT); ●, in the presence of both dithionite and arsenate. □, represents the viable count without trypsin treatment under all conditions.

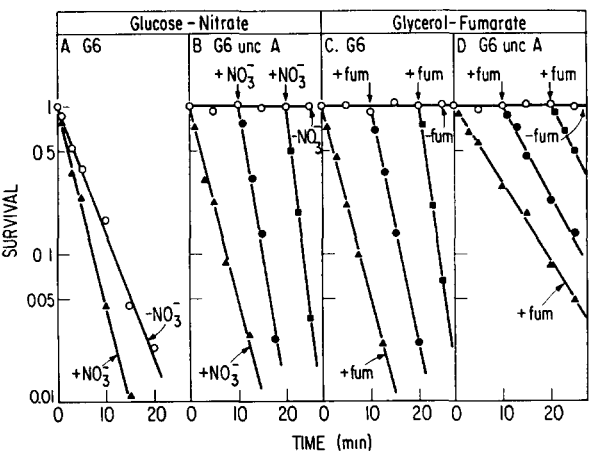

Fig. 5. Effect of colicin K on cells of *E. coli* G6 and G6 *unc*A grown anaerobically in the presence of glucose plus nitrate (A–B) or glycerol plus fumarate (C–D). (A–B) Cells were incubated under anaerobic conditions (in the presence of dithionite) in Ozeki medium base containing glucose and chloramphenicol. At time zero colicin K (m = 11) was added, samples were taken at various intervals, treated with trypsin and plated (○). Simultaneously, aliquots removed at 0, 10 and 20 min received nitrate and were sampled in the same way. (C–D) A similar procedure was followed for glycerol and fumarate. ▲, nitrate or fumarate (fum.) added at 0 min; ●, at 10 min; ■, at 20 min.

of nitrate promoted the loss of trypsin rescuability of colicin-treated cells of G6 *unc*A (Figs 5a and 5b). When glycerol was present instead of glucose, the transition was blocked both in G6 and G6 *unc*A in the absence of nitrate because glycerol could no longer be used under these conditions.

Cells of G6 and G6 *unc*A induced for the synthesis of α-glycerophosphate dehydrogenase and fumarate reductase were killed by colicin K when glycerol and fumarate were present. In the absence of fumarate, colicin-treated cells of both G6 and G6 *unc*A remained totally rescuable by trypsin treatment (Figs 5c and 5d). Subsequent addition of fumarate restored the progression of the stage I-stage II transition. The killing of G6 *unc*A occurred at a slower rate than that of G6, though the adsorption of the colicin was the same in both cases. Similar results in the α-glycerophosphate-fumarate system were obtained for colicins E2 and E3. These results show that the initiation of colicin action can be coupled to two specific inducible electron transfer systems: α-glycerophosphate dehydrogenase-fumarate reductase or formate dehydrogenase-nitrate reductase. This has also been shown for the uptake of lactose [14].

Glutamine and proline transport

The energy requirements for colicin action were compared with that for proline and glutamine uptake. The uptake of either amino acid in the presence of glucose was reduced in cells of G6 in the absence of an electron acceptor, oxygen or nitrate, but there was still a considerable amount of uptake (Fig. 6). When glycerol was used as a carbon source instead of glucose an external electron acceptor was required for active uptake of either amino acid. In G6 *unc*A with glucose as carbon source, the uptake of proline was severely inhibited in the absence of an electron acceptor, while the uptake of glutamine was much less affected (Fig. 6). The results obtained for proline transport are in agreement with previous findings of Yamamoto et al. [25] and Or et al. [26].

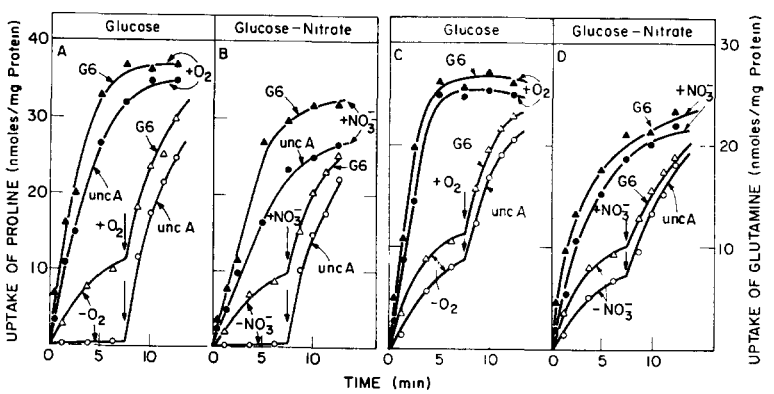

Fig. 6. Effects of anaerobiosis on the uptake of proline and glutamine in cells of G6 and G6 *unc*A. Cells of G6 (triangles) or G6 *unc*A (circles) were incubated under anaerobic conditions (by bubbling argon) in Ozeki medium base containing 0.5 % of glucose and 100 μg of chloramphenicol per ml. At time zero proline (A–B) or glutamine (C–D) was added and at time zero (closed symbols) or 7.5 min (open symbols) oxygen (A–C) or nitrate (B–D). Samples were taken at various time intervals and measured for uptake.

The uptake studies with glutamine were the only ones which gave different results when anaerobic conditions were established either by sodium dithionite in combination with bubbling argon or by bubbling argon alone. Both anaerobic conditions reduced the ATP levels to about 50 % of the ATP level in the control which was kept under aerobic conditions. In the absence of an electron acceptor either oxygen or nitrate the uptake of glutamine in G6 and G6 *unc*A was much more reduced when the anaerobic condition was established by sodium dithionite combined with bubbling argon than by bubbling argon alone. This discrepancy could be due to a specific effect of dithionite on the glutamine uptake system or due to a more anaerobic condition created by sodium dithionite.

DISCUSSION

The results obtained in this study are consistent with the scheme shown in Fig. 7, and indicate that energy is a prerequisite for the progression of the stage I-stage II transition in colicin action. Since the results with colicin K, E2 and E3 are quite similar, the existence of a general requirement for energy for the initiation of colicin action is apparent.

Some bacterial functions use high-energy phosphorylated compounds directly, while other functions are dependent on an energized state of the cytoplasmic membrane, derived either from electron transport or from ATP through the intermediary of the (Mg^{2+}, Ca^{2+})-ATPase [16, 17]. ATP or another high-energy phosphorylated compound is not a prerequisite for the initiation of colicin action since D-lactate promotes the progression of the stage I-stage II transition in starved cells of G6 *unc*A, which lack ATPase activity and cannot carry out oxidative phosphorylation. Furthermore, arsenate has little effect on the initiation of colicin action in normal cells. The stage I-state II transition in normal cells is inhibited only when both the electron transport is blocked and the ATP levels are reduced. Thus the energized state of the membrane appears to be the driving force for the progression of the stage I-stage II transition in colicin action (Fig. 7). Both aerobic and anaerobic electron transfer systems can provide an energized state of the membrane required for the initiation of colicin action and also for proline transport [25] and lactose uptake [14]. In G6 *unc*A electron transfer is the primary pathway for the establishment of the energized state and consequently is the driving force for the progression of the stage I-stage II

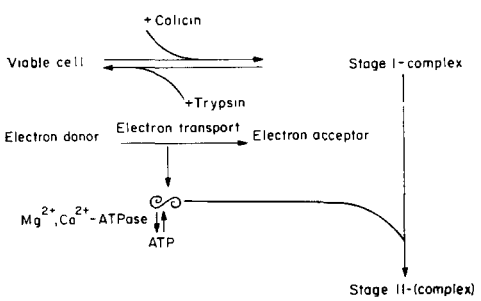

Fig. 7. A scheme of colicin action in which the role of trypsin rescue and energy requirement are illustrated. ~ represents energized state of the cytoplasmic membrane.

transition. The influence of temperature and fatty acid composition of the membrane on the initiation of colicin K action [8] may reflect an effect of these conditions on the energized state.

The phenomena obtained when the energized state is blocked resemble the observations on some tolerant mutants [18, 27, 28]. In both circumstances the colicin can attach to the receptor, but no irreversible physiological damage occurs. Some of the tolerant mutants may be affected in the coupling of the energized state to the transition of one or more colicins.

An unanswered question is: What is the significance of the energy requirement for the initiation of colicin action? Early theories on colicin action [29–31] postulated that one molecule of colicin was sufficient to kill a cell and that the membrane acted as a mediator, which spread the colicin effect by means of conformational changes. This spreading through the membrane may be an energy requiring process. Yet, in vitro studies with colicin E3 showed that the colicin can interact directly with the ribosome [32, 33]. If colicin E3 must make specific contact with intracellular ribosomes it must therefore at least partially penetrate the cytoplasmic membrane. The energy requirement for colicin E3 could indicate that energy is necessary for colicin penetration. In the case of colicin K, the cytoplasmic membrane appears to be the ultimate target of an action that leads to a dissipation of the energized state [4] and this could be caused by an energy-dependent binding to or penetration of the colicin into the cytoplasmic membrane. Further studies to resolve the nature of the energy requirement are in progress.

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Recent results [34] indicate that the accessibility of the lac carrier protein is dependent on the energized state of the membrane. This prompts us to suggest that a similar role of the energized state of the membrane may be involved in colicin action.

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