THE ACTION OF COLICINS E_1 AND K ON PROLINE TRANSPORT IN ISOLATED MEMBRANE VESICLES OF E. COLI

by

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(1964)

SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF

PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF

TECHNOLOGY

June, 1971

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Department of Biology, March , 1971

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Chairman, Departmental Committee on Graduate Students

ABSTRACT

The Action of Colicins E_1 and K on Proline Transport in Isolated Membrane Vesicles of E. coli, by Jonathan P. Kabat. Submitted to the Department of Biology on March 23, 1971 in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

This study has made use of the system of isolated membrane vesicles from osmotically shocked E. coli spheroplasts described by Kaback as a subcellular system to analyze the mechanism of action of colicin inhibition at the level of membrane functions. It was determined that: succinate can serve as an efficient energy source for the accumulation of proline by membrane vesicles through its dehydrogenation to fumarate; crude colicins E_1 and K preparations inhibit the accumulation of proline in vesicles from wild-type cells; purified colicin E_1 inhibits proline uptake, but not as efficiently as crude colicin; vesicles made from colicin-resistant strains are somewhat sensitive to colicin inhibition by the specific colicin, but not as sensitive as vesicles from wild-type cells; vesicles made from a Tol II mutant strain (Tol K, E_1, E_2, E_3, A) are not sensitive to colicin E_1 inhibition, while membranes from a Tol III mutant strain (Tol K, E_2, E_3, A) are sensitive to colicin E_1; vesicles made from an E_1-immune strain are sensitive to colicin E_1; the rate and extent of conversion of succinate to fumarate is not affected by colicin in wild-type vesicles, implying that colicin essentially uncouples the dehydrogenation of succinate from the energy-requiring process of proline accumulation; uncouplers and inhibitors completely abolish proline uptake with little effect on the conversion of succinate to fumarate; neither colicin nor uncouplers have any effect on the rate of substrate-dependent respiration in membrane vesicles. Respiration in these vesicles is completely dependent on exogenous substrate. Electron transport inhibitors reduce respiration, but do not abolish it. Colicin has no effect on respiration in starved whole cells, while uncouplers produce a 2- to 5-fold increase in respiration rate. Purified colicin inhibits energy-linked NADH transhydrogenase activity in modified membrane vesicles when it is driven by respiratory substrates, but not when it is driven by ATP. There is at least one protein species missing from the spectrum of membrane proteins from a Tol II amber mutant, as shown by double-label SDS-polyacrylamide gel electrophoresis. No missing species were detected in a Tol VIII amber mutant. The missing peak in the Tol II amber mutant membranes has been shown to be a major component of the outer membrane of E. coli, implicating the outer membrane in the transmission process involved in the mechanism of colicin inhibition.

Thesis Supervisor: Salvador E. Luria

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ACKNOWLEDGMENT

Thanks to

Nancy Ahlquist
David Baltimore
Josie Calareso
John Carson
Chuck Cole
Bob Fisher
Costa Georgopoulos
Sonia Guterman
Camille Horkun
Ron Kaback
David Kabat
Elvin Kabat
Geoffrey Kabat
Sally Kabat
Christina Kennedy
Salvador Luria
Joan Lusk
Ken Manly
Rosa Nagel de Zwaig
Mike Newlon
Chuck Plate
Helen Revel
Betty Russell
Linda Saxe
Annie Scali
Terek Schwarz
Moshe Siev
Erica Sodergren
Linc Sonenshein
Myla Zinn

The author was a trainee under a Microbiology Graining Grant (No. GM 00602) from the National Institute of General Medical Sciences, National Institutes of Health, to the Department of Biology, Massachusetts Institute of Technology, during the period 1964 to 1971.

he who dies before he dies does not die when he dies

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INTRODUCTION

The inhibition of cellular processes in bacteria by colicins can be viewed as a model system for analyzing the class of control mechanisms in which a "touching" event occurring at the outside surface of the cell regulates internal functions distant from the surface. Possible examples of such control include chemotaxis in bacteria, where a small molecule is involved ("sensed"), and contact inhibition in mammalian cells, in which the surface event seems to be one cell boundary coming into physical proximity to another.

Colicins are protein molecules, synthesized under certain conditions by certain strains of bacteria (called colicinogenic). These molecules attach to receptor sites on the surface of sensitive strains of coliform bacteria and cause inhibition of specific and different metabolic processes, depending on the type of colicin (Nomura, 1963). Colicin inhibition can occur in the absence of cellular protein synthesis. Genetic loss of a receptor leads to resistance of the cell to one or more colicins, which can no longer absorb to the cell.

Colicins have been classified according to their attachment sites (as revealed by mutational loss of adsorption capacity) and by the biochemical inhibition observed (Table A). These classifications are not consistent with each other. Thus, the E class of colicins are said to share a common receptor site with the phage BF-23, because resistance to all comes in a single mutational step, yet it contains three functional subspecies, E_1, which inhibits macromolecular syntheses, E_2, which inhibits DNA synthesis specifically, and E_3, which inhibits protein synthesis. Another colicin, termed colicin K, absorbs to a different receptor, but its biochemical effects are similar to those of E_1.

Table A

Classification of Colicins*

Mode of Action** (biochemical target)	Common cellular receptors***			
1. Interference with energy metabolism	A	E_1	K	Ib
2. DNA damage		E_2		
3. Ribosomal damage		E_3		

* Taken from Luria, in "Membrane Models and the Formation of Biological Membranes," Bolis and Pethica (Eds.), Amsterdam, 1968.

** The colicins in each row have similar killing action of susceptible bacteria.

*** The colicins in each column are those whose attachment sites on the bacterial cell wall can be lost by a single mutation.

Colicinogenic strains are protected by an unknown mechanism from the inhibitory effects of their homologous colicin (except at very high colicin multiplicities) even though they have receptors and absorb the colicin. They are said to be immune to their specific colicin (Nomura, 1967; Reeves, unpublished manuscript).

The first study of the inhibitory effect of colicin (Jacob et al., 1952) showed that colicin E_1 arrests macromolecular syntheses (protein and nucleic acids) in sensitive cells and that the time course of inhibition depends on the multiplicity of colicin molecules per cell. The kinetics are characteristic of a single-hit process, indicating that the lethal event results from the action of a single colicin particle without cooperative reinforcement. However, the probability of a single absorbed colicin molecule producing a successful "hit" can be quite low, for reasons that are poorly understood, and one killing unit often turns out to correspond to several hundred colicin particles. Colicin killing is not accompanied by cell lysis.

The intriguing observation that for colicin K the killing process could be reversed up to a certain time by treating the cells with high concentrations of the proteolytic enzyme trypsin (Nomura and Nakamura, 1962) gave experimental proof that colicin remains at the surface of the inhibited cell and suggested the necessity of maintaining the colicin at the cell surface in its intact absorbed state to produce the lethal event. The action of other colicins was also at least partially reversible by trypsin (Nomura, 1963; Reynolds and Reeves, 1963). Using radioactive colicin E_2, it was also shown that the colicin is associated with the envelope fraction after disruption

of the cells and differential centrifugation (Maeda and Nomura, 1966).

A Generalized Model of Colicin Action

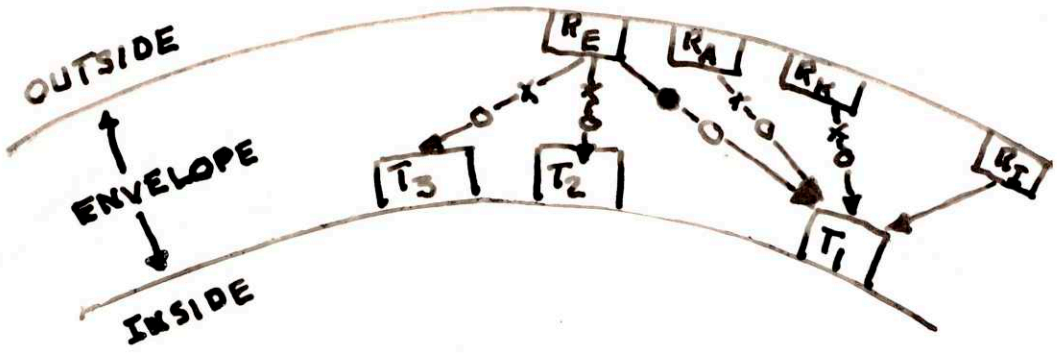
To conceptualize the overall phenomenon, a generalized model was proposed (Nomura, 1964; Nomura and Maeda, 1965) in which the cytoplasmic membrane is invoked to play a central role in "transmitting" a signal initiated by colicin absorption at the cell surface to specific biochemical "target(s)" inside the cell (Figure A). The transmission is envisaged as a spreading effect initiated at the receptor site and radiating in the 2-dimensional membrane lattice through specific channels, in effect amplifying the attachment event until the envelope is perturbed in regions distant from the absorption site. Increasing the number of colicin molecules absorbed adds to the efficiency of the spreading and thus, increases the rate of inhibition. The "target" is the primary metabolic event inhibited by the specific colicin.

This model was intended to unify schematically what was known about how different colicins act, i.e., to explain how colicins can affect internal metabolic processes while remaining at the cell surface. It does not specify mechanistically what the individual events are. It leaves unanswered fundamental questions about the nature of the transmission process, the participating elements, and the mechanism by which the target is affected.

However, historically the model led directly to the prediction and discovery of a new type of resistance to colicin. It was reasoned that if the membrane does play a fundamental role in the expression

Figure A. Relation between colicin receptors and biochemical targets in the bacterial cell envelope.

The boxes labeled R_A, R_E, ... represent the receptors for colicins of groups A, E, ... The boxes labeled T1, T2,... correspond to the biochemical targets of colicin action (see Table A). The arrows represent the formal interconnections between receptors and targets deduced from the information in Table A. The tol mutations in Table B uncouple various sets of interconnections (o: blocked by tol II mutation; x: blocked by tol III mutation; •: blocked by tol VIII mutation).
(Taken from Luria, in "Membrane Models and the Formation of Biological Membranes," Bolis and Pethica (eds.), Amsterdam, 1968.


OUTSIDE
ENVELOPE
INSIDE
R_E
R_A
R_B
R_I
T_3
T_2
T_1

of colicin lethality, mediating between receptors and targets, then there might exist classes of mutants altered in their membranes, or in their targets, which would still absorb the colicin molecule (therefore not classical resistants) but might no longer be able (a) to initiate the signal at the receptor-membrane junction, or (b) to transmit the primary event via the membrane lattice, or (c) to receive the impulse, due to an insensitive target (Nomura and Witten, 1967).

Target mutants have not been found, but membrane mutants were isolated and characterized (Table B) (Nomura and Witten, 1967; Nagel de Zwaig and Luria, 1967). These so-called "tolerant" mutants were found to have altered membrane properties in addition to colicin insensitivity. Thus, Tol II and Tol III, mapping near gal, have an increased sensitivity to deoxycholate and to EDTA, as well as a cellular fragility which leads to spontaneous lysis, even during growth. They are tolerant to a gamut of colicins which spans both receptor and target classifications. Tol VIII, tolerant only to E_1 and mapping near the his locus, is even more sensitive than Tol II and Tol III to deoxycholate; it is insensitive to EDTA, but highly sensitive to dyes such as methylene blue and acridine. This was shown to be due to an alteration of the normal permeability barrier in Tol VIII, allowing these compounds entry into the cell, where they interfere with metabolic processes (Nagel de Zwaig and Luria, 1967).

The altered membrane properties of the tolerant mutants indicate that the hypothetical "transmission" system involved in colicin sensitivity in wild-type cells contains at least some components essential for the structural properties of the membrane, such as its

Table B

Mutations to Colicin-Tolerance in E. coli K-12

Mutant class	Tolerant to colicins	Sensitive to colicins
tol II	A, E_1, E_2, E_3, K	I, B, V
tol III	A, E_2, E_3, K	E_1, I, B, V
tol VIII	E_1	E_2, E_3, K, I, B, V

Taken from Luria, in "Membrane Models and the Formation of Biological Membranes," Bolis and Pethica (eds.), Amsterdam, 1968.

stability, permeability, and sensitivity to reagents that affect interactions between membrane components (Nagel de Zwaig and Luria, 1967).

The discovery of tolerant mutations reinforced the thinking behind the generalized model that the membrane plays a key role in the expression of colicin inhibition, but it did not shed much light on the mechanisms involved in the transmission phenomenon. Partial diploids showed that tolerance is recessive to wild type, implying a missing function in the tolerants (Nagel de Zwaig and Luria, 1967). Since many tolerant strains are tolerant to colicins with different receptor sites and different biochemical targets, the missing components in these cases must be common to the transmission "channels" of the different colicins, even though the "channels" themselves have to be independent, going from specific receptors to specific targets (Figure A). A study of temperature-sensitive tolerant mutants revealed some classes in which the lesion seemed to be in the membrane itself (Nagel de Zwaig and Luria, 1967) and in one case, essential for normal cell growth (Nomura and Witten, 1967).

Membrane Structure and the Generalized Model of Colicin Action

In Gram-negative bacteria like E. coli, the envelope is composed of two membrane layers in addition to the rigid cell wall layer. These are referred to as the inner and outer membranes. The latter contains lipopolysaccharide, 5'-nucleotidase, UDPG hydrolase, and presumably some colicin receptors (J. Carson, personal communication). The inner membrane contains the enzymes of LPS synthesis, and the cytochromes and is presumably the site of electron transport and oxidative phosphorylation. A technique developed by John Carson

has permitted the separation of inner and outer membrane fragments in sucrose on the basis of their buoyant densities, so that the proteins of each fraction can be analyzed by electrophoresis on polyacrylamide gels.

The isolation of suppressible colicin-tolerant mutants (Nagel de Zwaig and Luria, 1969; Terek Schwarz, unpublished) introduced the experimental possibility of looking for missing protein components in tol membranes by double-label analysis using polyacrylamide gel electrophoresis of a mixture of wild type and amber tolerant membranes. This led to the finding that Tol II amber mutants are missing a major protein component. In collaboration with John Carson, and employing the above technique, we could demonstrate that the missing protein in the Tol II amber mutant is a major component of the outer membrane (see Results). This finding suggests that the Tol II protein, residing in the outer layer of the cell envelope, may be the membrane component with which the absorbed colicin molecule makes physical contact and that it acts as a switch to initiate the signal which eventually results in a lesion at the metabolic target site.

The possibility that physical changes occur within the membrane structure provides a realm for speculation about the mechanism of transmission of the colicin event initiated at the surface through the 2-dimensional membrane lattice. Because of the singular solubility properties of phospholipids, dictated by the presence of conflicting solubility properties in the same molecule, they evolved as the fundamental component of all biological membranes, allowing them to assume self-enclosing 2-dimensional structures in an aqueous environment. Recent evidence suggests that phospholipids in biological

membranes might exist in various organized states and undergo discrete phase changes (Reiss-Husson and Luzzati, 1967). Their free fatty acid chains create a "liquid-crystalline" state permitting the solubilization of apolar lipids such as are found in membranes of higher organisms, and conceivably also hydrophobic regions of membrane proteins, which might penetrate the lipid layer rather than be relegated to a sandwich role on the charged surface. Such evidence has generated models of membrane structure that are highly complex and no longer restricted to the simple bilamellar conformation of the classical Davson-Danielli model. An effort has been made to construct a theoretical model for allosteric type transitions through a simplified membrane structure consisting of identical protomer subunits which assume one of two conformational states (Changeux et al., 1967), and this model has been evoked to explain the colicin transmission phenomenon (Changeux and Thiery, 1967).

A recent experimental finding also suggests the possibility of a conformational change in a membrane component after colicin treatment. A large increase in the fluorescence of the dye 8-anilino-1-naphthalenesulfonate (ANS) is observed when cells to which this dye has been complexed are treated with purified colicin E_1. Such an increase also occurs when complexed cells are treated with the uncoupler p-CF_3-OCCP at 10^{-6} M. Neither colicins E_2 nor E_3 have any noticeable effect, nor has E_1 on ANS-complexed colicinogenic cells (Cramer and Phillips, 1970).

Further understanding of the transmission phenomenon involved in the expression of the colicin event awaits a more detailed picture of the components of the membrane and of structural relationships within

the membrane lattice.

We now turn to the other major locus of inquiry concerning the action of colicin, namely the nature of the target site and the metabolic events observed after colicin treatment.

The Physiological Consequences of Colicin E_1 and K on Whole Cells

This thesis is specifically concerned with the mode of action of colicins E_1 and K. They were originally described as inhibiting DNA, RNA and protein synthesis (Jacob et al., 1952). More recently cell motility, polysaccharide synthesis, the conversion of ornithine to citrulline, and the active transport of potassium ions and β-galactosides have been included among the whole cell functions arrested after treatment with these colicins (Fields and Luria, 1969b). Since all these functions are energy-consuming, these colicins became thought of as inhibitors of generalized energy production or utilization.

An observation that seemed to connect these inhibitions to a common event was made by Fields, who found a rapid decline in the cellular ATP level after colicin treatment (Fields and Luria, 1969a). No ATP was detectable in the medium, suggesting increased degradation or decreased synthesis rather than loss by leakage. Since the inhibition of the different macromolecular syntheses takes place simultaneously rather than sequentially, it was suggested that the inhibition is due to this drop in the ATP level, which might itself be caused by a primary effect of the colicin at the level of oxidative phosphorylation. The finding that cells grown under strict anaerobic conditions are protected from the inhibition of macromolecular

syntheses by E_1 and K (F. Levinthal and C. Levinthal, unpublished experiments) lent weight to this line of reasoning.

Since the ATP level does not go to zero, but is decreased to 25 to 30% of normal, the macromolecular inhibitions were postulated to be in response to sensitive allosteric type control through the ratios ATP/ADP, or ATP/AMP, as are various enzymatic functions in the catabolic pathway of glucose (Atkinson, 1966). Why synthesis, transport, and accumulation cannot run off the residual ATP concentration is unclear.

Recently, however, it has been demonstrated experimentally that at least some effects of colicin E_1 are not attendant on the drop in ATP levels. Using cells treated with an inhibitor of membrane ATPase, N,N'-dicyclohexylcarbodiimide (DCCD), Feingold has shown that E_1-induced leakage of K^{42} from preloaded cells, and the inhibition of RNA and protein synthesis by E_1 treatment, can occur in the presence of high levels of ATP (Feingold, 1971). Thus, the drop in ATP level cannot be invoked to account for the colicin effects <u>per se</u>, although it might itself be a consequence of a primary or secondary colicin effect on the DCCD-inhibitable membrane-bound ATPase. This does not negate the possibility that colicins E_1 and K affect oxidative phosphorylation or oxidative energy mobilization at a level other than ATP, perhaps between the electron transport chain and its coupling to ATP synthesis. We will return to this possibility in a later section.

Colicin Effects on Transport Functions and on Membrane Properties in Whole Cells

The early studies of the effect of colicins E_1 and K on transport (Luria, 1964) showed that the accumulation of β-galactosides and isoleucine and potassium were blocked by these colicins in direct proportion to their killing activity. Nomura (1963, 1964) showed that colicin K prevented the accumulation of K^{42} from the medium and induced leakage of K^{42} from cells which had accumulated it inside. He also reported that no leakage of P^{32}-substances from P^{32}-labeled cells, or of β-galactosidase from fully induced cells, took place after treatment with colicin K at a multiplicity as high as 50, demonstrating that generalized leakage from cells is not a consequence of colicin action. These observations supported the idea that the colicins were interfering with energy metabolism in some way, since the accumulation and maintenance of high concentrations of substrates by active transport requires energy coupling.

Fields (Fields and Luria, 1969a) made an elegant and important experimental distinction between transport systems that are or are not inhibited by colicin on the basis of their energy sources. They showed that E_1 and K completely inhibit the accumulation of β-galactosides such as TMG by whole cells, but have no effect on the accumulation of α-methyl glucoside in the same cells. Fields had also shown that E_1 and K do not affect the rate of ONPG hydrolysis by intact cells containing β-galactosidase, indicating that the barrier to this compound remains intact and that the colicin does not interfere with the carrier-mediated facilitated transport step across the membrane, necessary for ONPG hydrolysis. This suggests that the colicin works

to inhibit the TMG uptake by interfering with the energy coupling used to drive accumulation.

While the mechanism of this coupling is still unknown, work by Kennedy (Scarborough et al., 1968), Wilson and Winkler (1966), and by Kaback (Barnes and Kaback, 1970) indicates that the energy coupling for β-galactosides derives from electron-transport linked oxidation. The energy-coupling for α-methyl glucoside accumulation has been well characterized and is known to involve PEP as the unique energy donor (Kundig et al., 1964). Since PEP is a product of substrate-level phosphorylation, the above difference in colicin sensitivity of the active transport mechanisms for β-galactosides and α-methyl glucosides supports the idea that only oxidative energy pathways are targets of colicin action, and that transport systems that are not coupled to oxidative energy to pump substrate are not affected by the colicin.

This leads us to the question of the mechanism by which colicin inhibits oxidative energy-driven accumulation. Recent work by Feingold (1971) has shown that potassium leakage and the inhibition of syntheses produced by colicin E_1 can occur in cells which have a high internal concentration of ATP due to treatment of the cells with the inhibitor DCCD. The rapid loss of K^{42} under these conditions suggests the possibility that colicin might cause the specific leakage of potassium from the cell, and that this loss alone might account for colicin inhibition, in a way analogous to the inhibition of growth and transport systems by valinomycin in Strep. faecalis (Harold and Baarda, 1967).

This hypothesis supposes that the colicin event is equivalent to a "hole" punched in the cell membrane, specific for potassium. This suffices to explain the amplification phenomenon, since one hole should be sufficient to ultimately deplete the cell's entire potassium load.

Alternatively, the colicin might affect the membrane in such a way as to uncouple or hydrolyze an intermediate necessary for the energy-dependent pumping of potassium and of other transport systems. This too would result in loss of potassium with all its attendant consequences and also an inhibition of all reactions involving this energy coupling.

The first hypothesis predicts that a high exogenous potassium concentration might reverse the inhibitory effects of colicin. This is known to be true for valinomycin inhibition in Strep. faecalis (Harold and Baarda, 1967). However, many observations of colicin inhibition, including those in this thesis, have been made in high potassium medium, and thus, it is unlikely that loss of K^+ is a sufficient explanation of the mechanism of action of these colicins.

To analyze further the effect of colicin on cation permeability, Feingold sought to determine whether colicin disrupts the hydrogen ion permeability barrier as well as that for potassium. According to the chemiosmotic theory of energy mobilization (Mitchell, 1966), an intact barrier to hydrogen ion must be maintained to separate oxidizing from reducing power and thus maintain a potential across the membrane which can be tapped to drive energy-consuming reactions or to synthesize ATP. In this model, uncouplers prevent synthesis of

ATP by disrupting the hydrogen ion barrier, thereby dissipating the pH potential across the membrane.

Using the method of continuous pH monitoring of a slightly buffered cell suspension (Harold and Baarda, 1968), Feingold confirmed that, after addition of an acid load to increase the hydrogen ion concentration of the medium, the pH will rise if the H^+ barrier of the cell is disrupted as hydrogen ion flows into the cells. The magnitude of this pH rise depends on counter-ion flow from inside the cell into the medium to preserve electroneutrality. Feingold then found that treatment of cells under such conditions (slightly buffered, with acid load) with colicin E_1 by itself did not raise the pH of the medium. But if the cells were then treated with the uncoupler CCCP, which is known to increase the proton conductivity of membranes, then the presence of E_1 caused a dramatic rise in pH in the medium. This is explained by assuming that the colicin increases cation permeability, in this case K^+, but specifically not hydrogen ion permeability. When potassium leaks out as a result of colicin treatment, hydrogen ions can go in and restore electroneutrality, but only if the hydrogen ion permeability barrier is dissipated by an uncoupler. Therefore, it is clear that in this respect at least, colicin does not act like a classical uncoupler.

Recently, the system of K^{42} leakage induced by colicin K in preloaded cells has been used to obtain experimental verification that a transmission phenomenon does occur in the membrane between the event of colicin absorption and the event of potassium leakage (Wendt, 1970). It was observed that as the temperature is lowered, a delay can be detected before potassium leaks out, the extent of which is a function of the temperature. If the colicin multiplicity is

increased, the delay can be reduced. These observations were interpreted as evidence that a temperature-dependent physical change occurs in the membrane structure that protects it against the colicin action by impeding the signal to the site of action.

Purpose and Design of This Thesis - Properties of Kaback Membrane Vesicles

The multiplicity and diversity of the events initiated in the physiology of whole cells by colicins E_1 and K, and the lack of readily interpretable findings when this thesis was initiated, underscored the desirability of a subcellular system that could be used to analyze the mechanism of action of these colicins. The requirements were (1) that it be a membrane system that absorbs the colicin molecule; (2) that it perform a measurable energy-dependent physiological function, and (3) that this function be inhibitable by the absorbed colicin.

The report that cell-free preparations of isolated membrane vesicles made from osmotically shocked E. coli spheroplasts retained the ability to concentrate proline from the medium (Kaback and Stadtman, 1966) provided an attractive candidate for such a system, once it was demonstrated (see Results) that such vesicles are susceptible to colicin inhibition. The advantages of using an active transport system for studying these particular colicins are evident: the transport system itself is localized in the membrane; it is readily assayed; it is coupled to energy through a mechanism which is also associated with the membrane. The advantages of a subcellular system are equally clear: it obviates the complexity of the cytoplasmic

responses to colicin; it is self-contained, from the colicin-absorption step to the inhibition of uptake; it is susceptible to experimental manipulation, such as preloading with normally impermeable compounds; and it responds to exogenous energy sources to drive the transport machinery.

Kaback's original work (Kaback and Stadtman, 1966) described preparations of membrane vesicles made either from penicillin or lysozyme-EDTA spheroplasts by osmotic shock, followed by repeated washings in EDTA-phosphate buffer. In 10 mM Mg^{++} and 14 mM glucose, vesicles from wild type E. coli W took up amounts of C^{14}-proline increasing with time until roughly a 50-fold concentration of proline over the external medium was effected. Both Mg^{++} and glucose were required for maximal stimulation, and inhibition was observed with some inhibitors and uncouplers, suggesting that the proline pump was in some way dependent on oxidative metabolism.

During the past few years, Kaback has demonstrated a number of different transport systems in similar vesicles (for review, see Kaback, 1970a). The PEP-transferase system for hexose transport is functional and dependent on exogenous PEP in the presence of fluoride (to inhibit enolase) (Kaback, 1970b). Transport activities for amino acids other than proline have also been demonstrated, many of which respond with marked stimulation to exogenous D-(-)lactate and/or succinate (Kaback and Milner, 1970; Kabat and Luria, 1970), which are both far better substrates than glucose. Barnes and Kaback (1970) have recently shown that the concentrative uptake of lactose or TMG in membrane vesicles, mediated by the M-protein β-galactoside permease system, also responds to D-(-)lactate and succinate.

The lactate- (or succinate-) driven uptake systems are completely inhibited by uncouplers such as DNP and CCCP and by inhibitors such as azide, amytal, and cyanide. They are reported to be insensitive to high concentrations of arsenate at low phosphate concentration (Boyer et al., 1970; Barnes and Kaback, 1970), an indication that oxidative phosphorylation of ADP is not involved in driving the proline pump. Boyer has tried without success to detect an ADP-phosphorylating activity in these membranes.

The membrane vesicles used in this work constitute the so-called particulate or envelope fraction recovered after breaking spheroplasts by osmotic dilution. In Gram-negative bacteria, the cell membrane is closely associated with the rigid cell wall, parts of which can be removed from the cell by treatment with lysozyme-EDTA or by growth in penicillin, resulting in spheroplasts when the cells are maintained in hypertonic medium. Spheroplasts broken by osmotic shock or otherwise may be contaminated by parts of the cell wall as well as by internal soluble proteins. Membrane vesicles prepared according to the method of Kaback, using either spheroplasting method and multiple homogenizations and washings, are reported to have about 10% of the diaminopimelic acid of the whole cells, indicating the presence of some cell wall material (Kaback and Stadtman, 1966). Disc electrophoresis, according to Kaback, showed that almost all the soluble contents of the cell are lost, and less than 1% of the activities of glutamine synthetase, fatty acid synthetase, and leucine-activating enzyme was detectable. They have less than 5% of the cell DNA and RNA, 15 to 20% of the protein, and about 70% of the lipid of the spheroplasts from which they are made. According to

Kaback (1969) the composition of the membranes is 60 to 70% protein, 30 to 40% phospholipid, 1% carbohydrate. The predominant lipid is phosphatidyl ethanolamine (65 to 70%), but there are significant amounts of diphosphatidylglycerol (15%), phosphatidylglycerol (10 to 15%), phosphatidic acid (5 to 10%), and phosphatidyl serine. Light scattering measurements showed that the vesicles act as osmometers. Presumably the membrane ruptures during osmotic shock of the spheroplast, and the fragments, rich in lipid, reform self-enclosing spaces which retain the salient permeability characteristics of the whole cell membrane.

There was cause for initial optimism that E_1 and K might work on such vesicles from preliminary studies on spheroplasts. Nomura had reported that although spheroplasting protected against the inhibitory action of colicins E_2 and E_3, it had little effect on inhibition by colicin K (Nomura and Maeda, 1965). The same experiment was repeated using E_3, E_1, and K and it was found that whereas E_3 worked much less well on spheroplasts than on whole cells, E_1 was as potent on spheroplasts as on whole cells in preventing β-galactosidase induction, and colicin K almost as potent.

The archetypal experiment we had originally hoped to perform with the membrane vesicles was to compete against the inhibition of proline uptake caused by the colicin with various compounds, especially ATP, on the assumption that the inhibition of ATP synthesis was central to the mechanism of colicin action.

Preliminary studies were done comparing the behavior of membrane vesicles made from colicin-sensitive, resistant, tolerant, and colicinogenic cells to establish the reproducibility of the system

and to insure that the inhibition of proline uptake was the effect of the normal physiological action of the colicin. During this work, it was found that succinate provided a far better source of energy for the proline pump than glucose. I showed that the vesicles were sensitive to colicin inhibition and that the colicin appeared to uncouple proline accumulation from succinate oxidation. To approach the mechanism by which colicin produces this uncoupled situation, the action of uncouplers and inhibitors of electron transport was compared with that of the colicin on proline uptake, succinate oxidation, and general respiration. It was hoped in this way to outline the processes involved between respiration and the generation of high energy for driving the proline pump, and to point to possible levels at which inhibition would explain the colicin effect. Central to this consideration is the problem of the role of ATP, and whether synthesis of ATP or ATP utilization occurs in membrane vesicles.

Adopting current mitochondrial models to supplement our present understanding of oxidative phosphorylation and energy mobilization in bacteria, and exploiting another energy-consuming reaction in the vesicles, the energy-linked NADH-transhydrogenase activity, a consistent picture emerged relating to how the proline pump might be driven, and at what levels it might be inhibited by uncouplers, inhibitors, and colicin.

MATERIALS AND METHODS

Bacteria

E. coli W6, a proline autotroph of strain W, and its derivative W157, which lacks the ability to concentrate proline, were obtained from H. R. Kaback and originate from M. Lubin (1960, 1962). C600 Tol II and Tol III were isolated by Nagel de Zwaig (1967). The amber tolerants used in this study were isolated by Terek Schwarz in E. coli K-12 M72, obtained from Ethan Signer. Colicinogenic strains for preparing colicin stocks and for testing the colicin-related properties of different strains are from the Luria collection; all bacterial strains used in this study are listed in Table 1.

Isolation of colicin-resistant mutants and tolerant mutants from W6

Individual colonies are picked from an LB plate, resuspended in 1 ml LB and grown to about 1×10^8/ml. 0.5 ml of this culture is added to 2 ml of soft agar and 3 to 6 drops spotted on top of a stab of a colicin-producing strain which has been chloroform-killed after overnight growth. After overnight incubation at 37°C, resistant and tolerant colonies are visible inside the halo of growth inhibition around the stabbed colicin-producing culture. Candidates to be tested are picked with sterile toothpicks, grown in 1 ml of LB, purified by streaking, and again tested against colicin stabs as above and for growth on 1% DOC-LB plates (DOC = desoxycholate). Those isolates resistant to one or more colicins and sensitive to 1% DOC are classified as tolerants. Resistants are insensitive to DOC, as is the wild type parent.

Table 1

Bacterial Strains

Luria Stock Collection No.	Strain	Properties	Source
	E. coli W		
L-618	W6	pro^-	M. Lubin
L-619	W157	$pro^- pro^-_{upt}$	M. Lubin
	E. coli K-12		
L-104	C600	$F^- thi^- thr^- leu^- lac^- (i^+z^+y^-)$	
L-A592	C600 Tol II	"	
L-A590	C600 Tol III	"	
L-A606	W602 Tol II_a-K5	$F^- leu^- bio^- gal^- thi^-$	
L-A619	W602 Tol II_a-K5(Col E_1 Y20)	"	
L-A674	M72	$lac^-_{am} trp^-_{am}$	E. Signer
L-A685	M72 Tol II am	"	
L-A676	M72 Tol VIII am	"	
	Y20(E_1)	$F^- thi^- thr^- leu^-$(Col E_1)	F. Levinthal
	other *E. coli*		
C-28	K-235	prototroph (Col K)	P. Fredericq
	Salmonella typhimurium		
L-437	Sal cys 3 6 (ColE_2)		

Media

LB broth contains 1% Bactotryptone (Difco), 0.5% yeast extract (Difco) and 0.5% NaCl at pH 7.0. LB agar is LB broth plus 1% Bacto-agar (Difco). Soft agar consists of 0.8% Bactonutrient broth (Difco), 0.5% NaCl, and 0.65% agar.

A medium (Davis and Mingioli, 1950) contains per liter: 7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.5 g Na_3citrate, 0.1 g $MgSO_4$, 1 g $(NH_4)_2SO_4$, sterilized before addition of growth and supplements. Glucose as carbon source is added at 0.4%.

Penicillin spheroplasting medium (Kaback and Stadtman, 1966) contains per liter: 17.5 g Penassay broth (Difco), 200 g sucrose, 2 g $MgSO_4$, 100 units/ml Penicillin G (Squibb) and proline, 500 µg/ml.

63 minimal medium base contains per liter: 5.3 g KH_2PO_4, 10.6 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 3 ml 1 N KOH; $MgSO_4$ added to 1 mM final concentration.

Colicin killing assay

The killing titer of a colicin preparation is determined by incubating growing cells, usually C600 or W6, in LB for 10 minutes at 37°C with various dilutions of colicin. Each sample is then diluted and plated for viable bacteria and compared with the control cells, to which no colicin has been added. The number of killing particles is calculated from the multiplicity (m) of killing particles per bacterium obtained from the first term of the Poisson distribution $S/S_o = e^{-m}$ (where S is the number of surviving bacteria, and S_o is the original number of bacteria). The procedure is most accurate below a multiplicity of 4. The colicin titer is expressed as killing units/ml in the preparation.

Preparation of crude colicin extracts

Colicinogenic bacteria were grown in 200 ml LB broth to about 2×10^8/ml, centrifuged, resuspended in 10 ml of 0.85% saline, and UV-irradiated in petri dishes (5 ml/dish) for 60 seconds (General Electric Germicidal Lamp, 15 w, 50 cm distance) with reciprocating motion. The cells were then transferred to 400 ml of 63 minimal medium at 37°C containing 10 mM $MgSO_4$ and 0.5% tryptose (Difco) and incubated with shaking for 3 hours at 37°C. Cells were then collected by centrifugation, resuspended in 3 ml of 63 minimal buffer, and sonicated 2 to 3 minutes in short (20-second) bursts in the cold. The sonicated suspension was centrifuged to remove debris, and the supernatant was dialyzed against phosphate buffer (A minimal or 63 minimal) overnight in the cold with several buffer changes. The sonicate was then titered, distributed in small aliquots, frozen, and stored at about -20°C. Aliquots were thawed as needed, obviating repeated freezing and thawing. Colicin killing titers on a sensitive strain are about 10^{13} k.u./ml by this method, as measured by the standard survival assay. Control crude extracts from non-colicinogenic strains are made in exactly the same way, including the UV irradiation.

Preparation of purified E_1 - Method of Helinski (private communication)

An overnight culture of E. coli Y20(E_1) in LB is diluted 1:1000 into 4 liters of LB and grown to a Klett of 110 (no. 54 filter) with good aeration at 37°C. Colicin induction is achieved by adding Mitomycin C (Sigma) to 1 µg/ml final concentration. The culture is incubated for an additional 14 hours under growth conditions, for the first few hours under light-shielding conditions. The cells are

harvested by centrifugation and the colicin is extracted at 4°C by suspending the cells in 50 ml of 0.01 K phosphate (pH 7.0) containing 1 M NaCl, and mixing at very low speed in a rheostat-controlled Waring blendor for 30 minutes to 1 hour. The cells are spun down and this extraction procedure is repeated two more times with 50 ml of the above salt buffer each time. The supernatants are pooled and assayed. The colicin is recovered in a 40 to 60% ammonium sulfate cut. The ammonium sulfate pellet is spun down and resuspended in 5 ml of 0.01 M KPO_4 (pH 7.0), 0.85% NaCl, and dialyzed against the same overnight in the cold. (When a residual precipitate is found after dialysis, this is removed by centrifugation at 48,000 x g). The clarified ammonium sulfate fraction is further purified by chromatography on a DEAE-sephadex (A-50) column which has been equilibrated with the above saline-phosphate buffer. The column is developed under starting conditions using saline phosphate buffer and collecting 6 ml fractions. The active fractions, as measured by spot dilutions on a lawn of sensitive cells, from the OD_{280} peak are pooled and dialyzed against distilled water, and lyophilized. This peak comes off early in the elution, since the colicin does not stick to DEAE-sephadex.

The lyophilized protein is dissolved in approximately 15 ml of 0.05 M sodium borate titrated to pH 9.5 with sodium hydroxide and dialyzed in the cold against the same until complete solubility is obtained. This preparation is then subjected to chromatography on a CM-sephadex (C-50) column (2.5 cm x 25 cm) equilibrated with the pH 9.5 sodium borate buffer, and eluted using a linear gradient

consisting of 700 ml of borate buffer and 700 ml of borate buffer plus 0.3 M KCl. 8-ml fractions are collected and tested as above for activity and OD_{280}. The active fractions are again pooled, dialyzed against distilled water, and either lyophilized or concentrated and frozen and stored at -20°C until used. Killing titers are about 10^{13} k.u./ml.

Preparation of membrane vesicles by penicillin method (Kaback and Stadtman, 1966; Kaback, 1968)

Cells were grown overnight (from a single colony isolate) in A medium plus supplements, 0.4% glucose as carbon source, and > 200 µg/ml proline. The overnight culture was diluted into fresh A medium, grown to late log phase (close to 1 x 10^9/ml by Petroff-Hauser count), and diluted 1:4 into spheroplasting medium at 37°C, containing 20% sucrose in Penassay broth, $MgSO_4$, 1000 units/ml penicillin G and 500 µg/ml proline. Usual volumes were 1 to 2 liters of spheroplasts, at about 2 x 10^8/ml. This incubation is continued for 2.5 hours at 37°C with occasional gentle swirling, and the progress of spheroplast formation is followed microscopically. By 2.5 hours, more than 99.9% of spheroplasting is routinely observed. The spheroplasts are chilled in ice, centrifuged at low speed (10,000 x g/15 min), and washed once with cold 0.1 M K phosphate buffer (pH 6.6) containing 20% sucrose and 0.2% $MgSO_4$. The pellet is then resuspended in a small volume (3 to 6 ml) of the same buffer containing DNAase, and diluted into 500 ml of 0.05 M K phosphate buffer (pH 6.6) at 37°C and stirred rapidly by a magnetic stirrer. The DNAase concentration after dilution should be about 20 µg/ml.

The osmotic lysate is stirred vigorously for 10 minutes at 37°C; then EDTA is added to 5 mM with continued stirring. After 5 minutes,

$MgSO_4$ is added to 10 mM and the stirring continued another 5 minutes. At this point, debris can be seen floating in the lysate. The lysate is centrifuged at 16,000 x g for 30 minutes in the cold. The pellet is recovered and resuspended in cold 0.1 M K phosphate, 10 mM EDTA, pH 6.6, homogenized in the cold in a glass-teflon homogenizer, and centrifuged in this same buffer at 37,000 x g for 20 minutes. This wash is followed by a low speed spin, 120 x g, for 15 minutes, the pellet is discarded and the supernatant again centrifuged at 37,000 x g for 20 minutes. The pellet is finally resuspended in 0.5 M K phosphate, pH 6.6, containing 10 mM Mg^{++}, and in the early experiments, 14 mM glucose, at approximately 3 mg membrane protein/ml.

Preparation of membrane vesicles by lysozyme-EDTA method (After Kaback, 1968).

An overnight culture in A medium with 0.4% glucose, 400 µg/ml proline and specific growth supplements is diluted 1:10 into 1 liter of the same medium and grown at 37°C with shaking to a Klett of about 150 (no. 54 filter). The cells are harvested, washed two times with cold Tris buffer, 0.01 M, pH 8.0, and resuspended in a final volume of 400 ml cold 0.03 M Tris, pH 8.0, containing 20% sucrose. It is important that the cell density in the next step not exceed about 1 x 10^9/ml to avoid spontaneous lysis during spheroplasting. Spheroplasting is confirmed by microscopic observation after 30 minutes. The spheroplasts are centrifuged at 16,000 x g for 30 minutes in the cold and resuspended in a total volume of 5 ml in 0.1 M K phosphate (pH 6.6), 20% sucrose, 20 mM Mg^{++} plus DNAase so that the final concentration will be about 10 µg/ml after the osmotic dilution step.

A teflon-glass homogenizer is used in the cold to resuspend the spheroplasts, which are then rapidly diluted into 950 ml of 0.05 M K phosphate (pH 6.6) at 37°C with rapid stirring. The lysate is stirred vigorously for 10 minutes. Then EDTA is added to 5 mM, and after a further 5 minutes stirring at 37°C, $MgSO_4$ is added to 10 mM, followed by another 5 minutes incubation. The lysates are centrifuged at 27,000 x g for 30 minutes. The pellets are scraped out of steel centrifuge cans with a spatula and resuspended in cold 0.1 M K phosphate (pH 6.6) containing 10 mM EDTA (henceforth referred to as KPO_4-EDTA), and homogenized in an ice-water bath and washed in a total volume of 50 ml KPO_4-EDTA at 37,000 x g for 20 minutes. This washing is repeated three times with 50 ml buffer each time and a teflon-glass homogenization. Then the membranes are resuspended in 10 ml of KPO_4-EDTA containing 20% sucrose, at 0°C. 5-ml portions are layered on 30 ml columns of cold 60% sucrose-KPO_4-EDTA in polyallomer tubes, and centrifuged at 25,000 rpm at 4°C for 90 minutes in the SW27 rotor. This spin results in a membrane band floating at the 20 to 60% sucrose interface, and a pellet at the bottom of the tube. The band is removed with a syringe, diluted into KPO_4-EDTA and centrifuged at 37,000 x g for 1 hour to pellet the membranes. The membranes are finally resuspended in about 4 ml 0.5 M KPO_4 (pH 6.6), 10 mM Mg^{++}, yielding about 3 mg membrane protein per ml by Lowry determination. Aliquots are frozen in liquid nitrogen and stored at -90°C.

The visible pellet in the sucrose flotation step can be eliminated by submitting the washed membranes to two low speed spins (3,000 rpm in SS-34 rotor for 10 minutes). After the first spin, the pellet is resuspended and spun again. The pooled supernatants of the two spins

are equivalent to sucrose-floated membranes in that no pellet is recovered if membranes which have had the two low speed spins are spun over 60% sucrose.

Proline uptake assay

Two methods were used which gave comparable results: (a) large volume assays: 0.1 ml of membranes in 0.5 M KPO_4 (pH 6.6), 10 mM $MgSO_4$ are preincubated in 0.2 ml volumes with substrate and substances to be tested for their effect on uptake, i.e. colicin, uncouplers, etc. 0.5 ml of C^{14}-proline diluted 1:50 in phosphate buffer (0.5 M, pH 6.6), 10 mM Mg^{++} is added at time zero to give a total volume of 0.7 ml, and a final proline concentration of approximately 1 µg/ml. The C^{14}-proline stock is uniformly labeled, 205 mC/mmole, at approximately 50 µg/ml, obtained from New England Nuclear. The temperature of the uptake assay is 27°C unless otherwise stated. At various times during the incubation, 0.1 ml samples are withdrawn from the reaction mixture, diluted into 2 ml of 0.5 M KPO_4 (pH 6.6), 10 mM Mg^{++}, also at 27°C, filtered rapidly under aspirator suction on a Millipore filter (0.45 µ), and washed two times with 2 ml of the same buffer. The filtering and washing are accomplished within 20 seconds after the sample is removed from the incubation mixture. The filters are dried top-side-up on a paper towel under I.R. lamps, placed in 2 ml Omnifluor scintillation fluid, and counted in a Nuclear Chicago Scintillation counter with a C^{14} window setting.

(b) single point assays in small volume: Kinetic and single point assays were also done using small volume reaction mixtures and diluting the contents of each tube at the time of assay. For kinetic

studies, each time point is a separate but identical reaction, assayed at different times. Preincubation of membranes in either a 50-λ or a 100-λ volume were used with similar results. It was originally thought that preincubation with colicin in a smaller volume might increase the absorption and inhibition, but this was not observed.

A 20-λ sample of membranes is preincubated with substrate and with other substances to be tested at 27°C, and at t = 0, C^{14}-proline is added, either in a large (0.1 ml) volume of 0.5 M KPO_4-Mg to the small volume preincubation, or as 2 λ of the undiluted stock to the large volume preincubation. Additions of C^{14}-proline are staggered by 1 minute. At the time of sampling, 2 ml of 0.5 M KPO_4 (pH 6.6), 10 mM Mg^{++} at 27°C is pipetted into the reaction, followed by rapid filtration and two washes with 2 ml of the same buffer. Filters are then dried and counted as above.

Some of the uptake assays with the lysozyme-EDTA vesicles were done in 0.25 M KPO_4 final concentration instead of 0.5 M. This does not greatly affect the level of proline taken up, but it does improve the level of colicin inhibition and was used for this reason.

Measurement of succinic dehydrogenase activity by thin layer chromatography

50-λ aliquots of W6 membranes in 0.5 M KPO_4 (pH 6.6), 10 mM Mg^{++} are diluted 1:2 with 50 λ water or pure colicin, and incubated alone or with uncouplers or inhibitors at 27°C for 15 minutes. C^{14}-Succinate (50 μC/ml, 40 mM) is then added (5 λ) to give a final concentration of 2 mM. At various times, 2-λ samples are removed, spotted onto silica gel G thin layer plates (Analtech, Inc., 250 μ thickness) and dried

immediately. For the kinetic experiment in Figure 3, samples are removed from membranes incubated without colicin or uncouplers at 0, 5, 10, and 20 minutes of incubation. In studying the effect of uncouplers, inhibitors, and colicin on the reaction, samples were taken at 5 and 20 minutes of incubation. The TLC plates are chromatographed with water-saturated ethyl ether:formic acid (7:1, v/v) in a vapor-saturated tank, and dried. The spots are localized by scanning with a strip scanner, then they are scraped into scintillation vials and counted in 20 ml of Omnifluor. Fumarate was identified by running a mixture of C^{14}-succinate and C^{14}-fumarate standards.

Polarographic measurement of respiration

Measurements are made on a recording polarograph in conjunction with a Clark electrode cell maintained at 37°C. The volume of the cell is 1.4 ml. Full deflection on the scale is calibrated from the solubility of O_2 in water at 37°C. 0.2-ml samples of either lysozyme-EDTA vesicles of W6 (9.5 mg/ml) or starved whole cells of W6 (at about 10^{10}/ml) in 0.25 M KPO_4 (pH 6.6), 10 mM Mg^{++} are preincubated 5 minutes at 37°C with an equal volume of buffer or colicin, then placed in the electrode's cell which is filled to 1.4 ml total volume with buffer. A stirring bar in the cell keeps the suspension mixed. Substrate, uncouplers, and inhibitors are injected with a microsyringe at various times, and the rate of consumption of oxygen is followed by continual monitoring and recording. The full scale deflection is calibrated from the solubility of O_2 in water at 37°C and expressed in n-atoms of oxygen. The rate of oxygen consumption is expressed in n-atoms of oxygen consumed/minute/mg of protein.

Transhydrogenase assay

(a) Oxidative reaction: activity is measured in a medium containing 50 µmoles Tris sulfate at pH 7.8, 700 µmoles sucrose, 0.3 µmole NAD, 2 mg bovine serum albumin, 100 µg crystalline yeast alcohol dehydrogenase, 90 µmoles ethanol, and membrane vesicles or sonicated particles (0.5 to 1.0 mg protein) in 2.9 ml total volume. Colicin was preincubated with the vesicles for 5 minutes at 38°C before the other materials are added in cases where its effect was being measured. Then the medium was incubated for 2 minutes at 38°C, and the reaction started by the addition of 1.5 µmoles NADP. The reaction is carried out in a temperature-controlled cuvette and the increase of absorbance at 340 mµ is followed in a Gilford multiple sample absorbance recorder.
(b) ATP reaction: contains the same ingredients plus 6 µmoles ATP and 10 µmoles $MgCl_2 \cdot 7\ H_2O$. This measures the sum of the oxidative plus the ATP reaction. The ATP-driven activity is expressed as the difference in activity between (b) and (a).

Sonicated particles of W6 were made by the procedure of Fisher et al. (1970). Cells were grown in minimal A medium, harvested, and washed once with 50 mM Tris-SO_4, 10 mM $MgSO_4 \cdot 7\ H_2O$ at pH 7.8, then resuspended in the same buffer (1.0 g wet weight per 10 ml buffer) and disrupted in a Branson sonifier operating at full power for 2 minutes. The suspension was centrifuged at 105,000 x g for 1 hour, and the resultant pellet resuspended in 50 mM Tris-SO_4, 10 mM $MgSO_4 \cdot 7\ H_2O$ and bovine serum albumin (10 mg/ml) at pH 7.8. The particles from one gram of original cells were in 2 ml of this buffer.

Double-label membranes and SDS gel analysis

Preparation of samples: Wild type M72 and colicin-tolerant amber strains (isolated by Terek Schwarz) were grown from fresh overnight cultures in A medium plus glucose, 0.4%, tryptophan (20 µg/ml), plus H^3-leucine (wild type) or C^{14}-leucine (mutant) for at least 2 generations (H^3 at 4 µC/ml; C^{14} at 0.8 µC/ml; leucine at 10 µg/ml total), to a density of > 5 x 10^8/ml. The cultures were then centrifuged and washed once with buffer containing 100 µg/ml C^{12}-leucine, resuspended together in the same flask in penicillin spheroplasting medium, and incubated 2.5 hours at 37°C with occasional swirling. The procedure then follows the same steps as that for non-radioactive penicillin membrane preparations, until the washed membranes are finally resuspended in 0.5 M KPO_4 (pH 6.6), 10 mM Mg^{++}. The membranes were then solubilized for electrophoresis by adding SDS to 1% and urea to 3%, heating 10 minutes at 70°C, and dialyzing against SMASH buffer (2 times with 250 ml at room temperature). Aliquots of solubilized, dialyzed membranes are then counted for each isotope in a Scintillation counter. Samples (usually 20 to 50 λ) are made 10% in sucrose, methylene blue is added to mark the moving front on the gel, and they are layered under buffer on top of the gel column.

Electrophoresis (method of Jacobson and Baltimore; 1968): gels are 7% acrylamide, with ethylene diacrylate as cross-linking agent (0.33%) and ammonium persulfate at 0.1%. They contain 0.1% SDS and 3% urea, and measure 0.4 cm x 8 cm. Electrophoresis was run at 4 milliamps per gel. After electrophoresis until the methylene blue band hit at the bottom of the gel, the gels are sliced into approximately 80 fractions, and each slice is dissolved in Choules reagent (0.4 ml) for

1 hour with shaking, and then counted in 20 ml of Kinnard's scintillation fluid for dissolved gels. Counting was in a Beckman scintillation counter with ticker-tape printout. The data were corrected for isotope spillover, normalized to the highest peak, and plotted.

Solutions:

Choules solvent: 45 ml piperidine, 50 ml hyamine, in 500 ml water.

SMASH buffer: 60 g urea, 1.08 g $NaH_2PO_4 \cdot H_2O$, 1.7 g Na_2HPO_4 anhydrous, dissolved in 1800 ml distilled water; then brought up to 1978 ml, and 20 ml 10% SDS, and 2 ml mercaptoethanol added. Final volume: 2000 ml, pH 7.1.

Electrophoresis tray buffer: 0.1 M phosphate, pH 7.2, 0.1% SDS.

Kinnard's scintillation fluid for dissolved gels: 520 g naphthalene, 32.5 g PPO, 0.325 g POPOP, 2500 ml xylene, 2500 ml dioxane, 1512 ml absolute ethanol.

RESULTS

Part I. The Action of Colicins on Uptake Systems in Kaback-type Membrane Vesicles

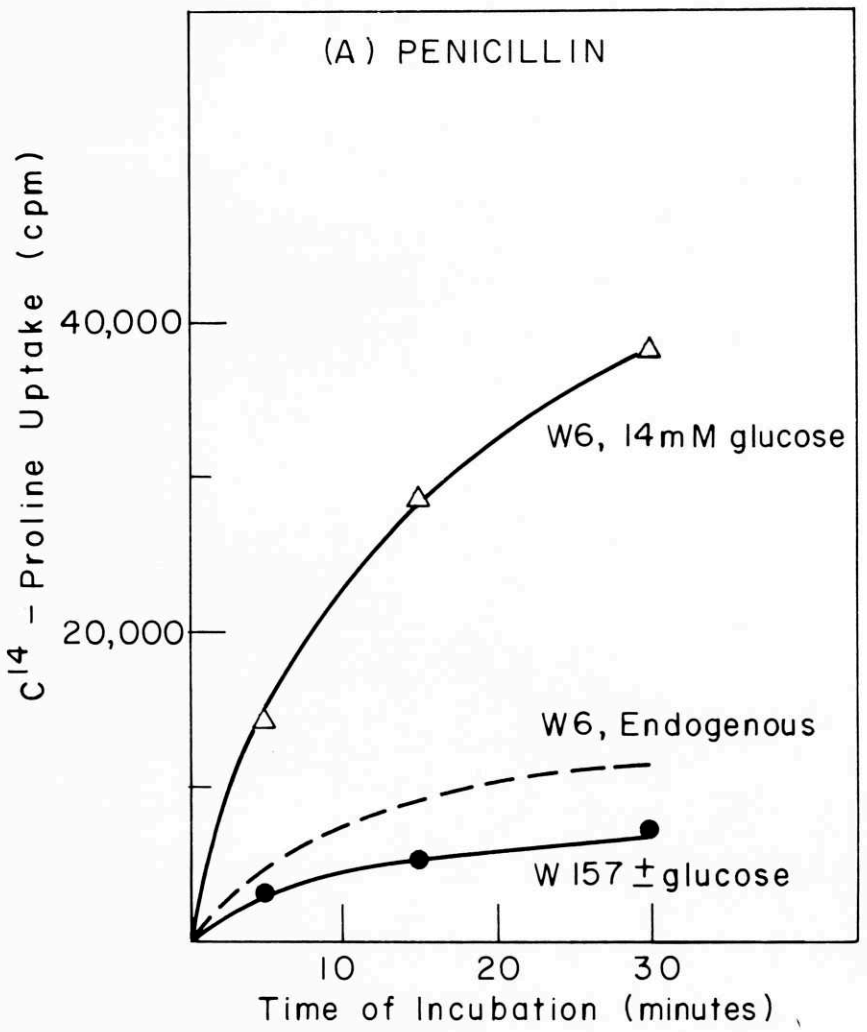
Properties of the Proline Uptake System in Membrane Vesicles

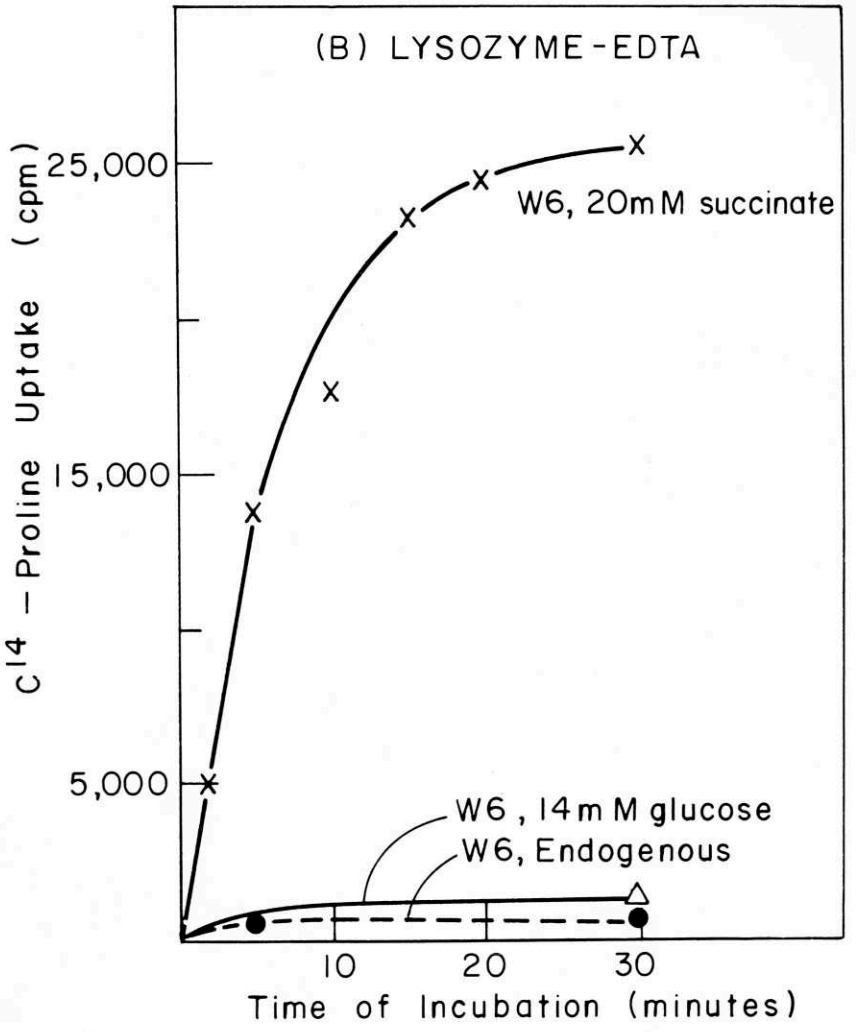
The initial objective of this study was to reproduce the Kaback membrane system (Kaback and Stadtman, 1966) to see whether colicin could inhibit the uptake phenomenon, and thus provide a subcellular membrane system through which the mechanism of action of the colicin inhibition could be approached and perhaps fruitfully analyzed.

Figure 1A shows a typical time course of C^{14}-proline uptake in E. coli W membrane vesicles made from penicillin spheroplasts according to Kaback's modification of his original procedure (Kaback, 1968, 1970). W6 is a proline auxotroph which has a normal proline transport system and requires low concentrations of external proline for growth (10 µg/ml). W157 is a mutant strain (Kessel and Lubin, 1962) which has lost the ability to concentrate proline, and only grows on high exogenous concentrations of proline (500 µg/ml). As Kaback and Stadtman had originally observed, membrane preparations from these two strains exhibit proline uptake behavior characteristic of the corresponding whole cells: W6 concentrates proline readily from the medium in the presence of 14 mM glucose, whereas W157 vesicles only accumulate low levels of proline even by the end of a 30-minute incubation. The uptake by W6 is at least partially dependent on glucose. When glucose is omitted from the incubation mixture, both the rate and final level of proline taken up by W6 vesicles are reduced two to three-fold. No glucose-dependent behavior was observed for the W157 curve, indicating that these vesicles do not use exogenous substrate for accumulation.

Figure 1. C^{14}-Proline uptake by W6 and W157 membrane vesicles made by (a) penicillin and (B) lysozyme-EDTA methods.

Membranes were suspended in 0.5 M potassium phosphate, pH 6.6, plus 10 mM $MgSO_4$ plus additions as noted: (----) endogenous; (Δ) glucose, 14 mM; (x) succinate, 20 mM. Membranes were at approximately 0.5 mg/ml as measured by Lowry determination. The conditions and procedure for assaying samples are as described in Materials and Methods. In (A), samples were taken from one reaction vessel; in (B), each point represents one 100-λ reaction mixture. Membranes were preincubated for 15 minutes at 27°C before addition of C^{14}-proline at time zero.


(A) PENICILLIN
C14 – Proline Uptake (cpm)
40,000
20,000
W6, 14mM glucose
W6, Endogenous
W 157 ± glucose
10
20
30
Time of Incubation (minutes)


(B) LYSOZYME-EDTA
C14 – Proline Uptake (cpm)
25,000
15,000
5,000
W6, 20mM succinate
W6, 14mM glucose
W6, Endogenous
10
20
30
Time of Incubation (minutes)

Rather, the level of proline uptake is the result of passive diffusion and does not represent energy-dependent accumulation against a gradient.

Many of the experiments to be reported were done, like that of Figure 1A, using membranes prepared by the original penicillin method of Kaback and Stadtman. Such penicillin preparations were not washed extensively (3 washes) during their preparation, nor were they subjected to routine methods to reduce contamination by whole cells and unbroken spheroplasts (Materials and Methods). Late in this work, a switch was made to membrane preparations made from lysozyme-EDTA spheroplasts; these were more extensively washed (> 5 washes) and routinely floated on 60% sucrose (see Materials and Methods). Such preparations are much cleaner, in that their endogenous activity is 20 times lower than that of the penicillin vesicles, and is almost indistinguishable from the background of the assay. This is shown in Figure 1B. Therefore, any uptake activity observed is really the result of addition of exogenous substrate. As in the penicillin vesicles of Figure 1A, the glucose stimulation in lysozyme membranes is approximately twice the endogenous level, but in this case, that means 20 times less in specific activity than in the penicillin case. Expressed in nmoles proline taken up per mg membrane protein per 20 minutes, for glucose-stimulated uptake, the value is 0.0815 for the lysozyme-EDTA vesicles, as opposed to approximately 2 for the penicillin vesicles (Table 2). This clearly indicates that glucose-stimulated uptake itself is a minor effect in terms of the amount of proline accumulated.

The reason for the large endogenous activity in the penicillin membranes is not clear, but the fact that it is not observed in the lysozyme-EDTA preparation implies that it is either a basic difference due

Table 2

Energy Substrates for Proline Uptake

(A) penicillin W6 vesicles	uptake cpm at 30 min	specific activity[1]
endogenous, no additions	11,474	0.725
glucose, 170 mM	30,624	1.93
succinate, Na, 85 mM	90,150	5.75
ATP, 14 mM	5,973	-
3,5-cyclic AMP, 3 mM	8,919	-
pyruvate, 140 mM	11,421	-
citrate, 140 mM	3,745	-
α-ketoglutarate, 140 mM	293	-
(B) lysozyme-EDTA W6 vesicles	**uptake cpm at 20 min**	**specific activity[2]**
endogenous, no additions	567	0.0348
succinate, Na, 20 mM	26,713	1.64
D-(-)lactate, Li, 20 mM	9,057	0.557
DPNH, 6 mM	9,051	0.556
glucose, 200 mM	1,329	0.0815
ATP, 1 mM	473	-
ATP, PEP, pyruvate kinase	491	-

1 nmoles proline/mg protein/30 minutes.

2 nmoles proline/mg protein/20 minutes.

Assays are in general as described in Materials and Methods. In (A), each assay is done on 20 λ of membranes in a total volume of 140 λ. In (B), each assay is on 20 λ of membranes in a total volume of 100 λ and a final phosphate concentration 0.25 M. In each case results are expressed as cpm proline taken up per assay in the given time, then converted to nmoles proline/mg protein/time.

to the method of spheroplasting, or that it is the result of less washing and/or contamination by whole cells or spheroplasts. This problem has not been completely resolved, but since the basic effects studied in this system are similar whether penicillin or lysozyme-EDTA membrane vesicles are used, it is felt that the high endogenous activity in the former is not a critical limitation. Since the colicin studies to be described were done on both these systems, it will be specified for each experiment what method of preparation was used.

Physical State of the Accumulated Proline

If W6 penicillin membranes that have taken up proline for 30 minutes in the presence of glucose and thus have a high level of accumulated proline are diluted into cold 5% TCA instead of phosphate buffer, kept in ice for 30 minutes, and then filtered on a Millipore filter and washed with TCA, less than 1% of the proline counts are retained on the filter, indicating that the proline accumulated by the membranes during the incubation remains associated with the vesicles in free form and is not incorporated into acid-precipitable material by a residual protein-synthesizing machinery.

Succinate Stimulation -- Respiration-driven Proline Uptake

During the course of this work it was discovered that if succinate was substituted for glucose, W6 membrane preparations from penicillin spheroplasts took up 8 to 10 times the endogenous level of proline. This represents an uptake of about 6 nmoles proline per mg protein per 30 minutes incubation (Table 2A). W157 membranes were not stimulated. In lysozyme-EDTA vesicles of W6, the net stimulation over endogenous is greater, since the endogenous is very low. With 20 mM

succinate, about 2 nmoles proline per mg protein per 20 minutes incubation is taken up by W6 lysozyme-EDTA membranes, which is a 40- to 50-fold stimulation over the endogenous level (Table 2B and Figure 1B).

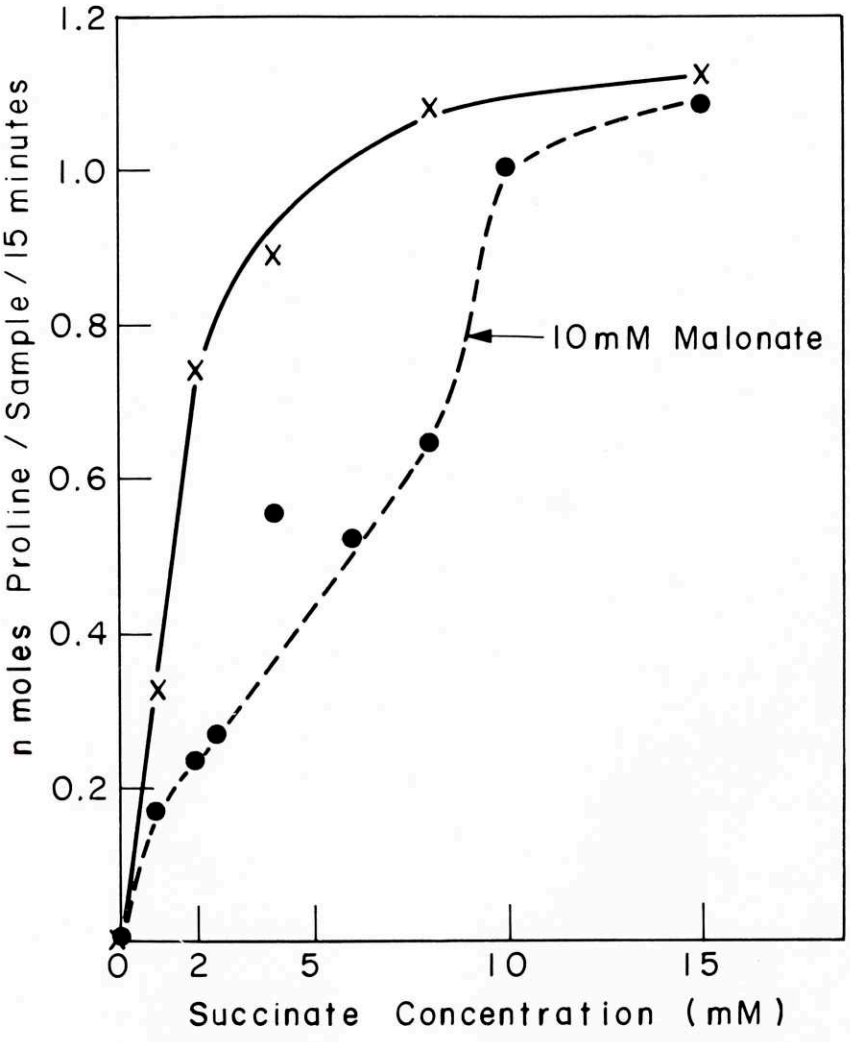
Since the pathway of succinate metabolism in E. coli involves its dehydrogenation to fumarate in the TCA cycle, it seemed reasonable to postulate that succinate was serving as an energy source for the proline pump in membrane vesicles through this dehydrogenase activity linked to the electron transport chain and oxidative energy production. To see if succinate dehydrogenase, an enzyme known to be membrane-bound in E. coli, plays a role in the succinate stimulation of proline uptake, proline accumulation in membrane vesicles was measured as a function of succinate concentration in the presence and absence of 10 mM malonate.

Without malonate proline accumulation increases linearly with succinate concentration and saturates at about 5 mM (Figure 2). The presence of 10 mM malonate markedly increases the concentration of succinate at which maximal proline uptake is reached. The curve does not plateau until 10 mM succinate, and becomes S-shaped, reflecting a competitive inhibition due to the malonate, which can be overcome by increasing the succinate concentration. The original plateau level is regained, indicating full recovery of activity. This competitive inhibition of the proline uptake in vesicles by malonate suggests that succinate-driven uptake does indeed involve the metabolic mobilization of energy via succinate dehydrogenase, the classic malonate-inhibited enzyme.

The presence of succinate dehydrogenase in membrane preparations was then confirmed by direct analysis. When C^{14}-succinate is incubated

Figure 2. Malonate inhibition of succinate-stimulated proline uptake in penicillin vesicles of W6.

20 λ of membrane vesicles in a total volume of 160 λ were preincubated with various concentrations of succinate with (•) or without (x) 10 mM Na malonate for 15 minutes, after which C^{14}-proline was added (31 µg/ml, 2.97 µC/ml), and the amount taken up in 15 minutes of incubation at 27°C was measured (Materials and Methods). The results are expressed as nmoles proline taken up/20-λ membrane sample/15 minutes, as a function of succinate concentration. (The protein concentration of this membrane preparation was not determined).


1.2
1.0
0.8
0.6
0.4
0.2
n moles Proline / Sample / 15 minutes
10mM Malonate
0
2
5
10
15
Succinate Concentration (mM)

with membranes under conditions similar to those measuring stimulated proline uptake, but at lower succinate concentration, it is converted stoichiometrically to fumarate, as measured by scintillation counting of a thin-layer chromatogram of the incubation (Figure 3). At 27°C, 690 nmoles of succinate were converted to fumarate by 1 mg of membranes in 20 minutes.

Putting this together with the malonate inhibition, it appears that succinate is converted to fumarate and only fumarate in membrane vesicles, and that this conversion is coupled to the mechanism of proline transport to drive concentrative uptake.

Kaback confirmed the stimulatory effect of succinate on proline uptake, and extended the finding to D-(-)lactate, which he found to stimulate 20- to 30-fold over the endogenous activity in lysozyme-EDTA membranes prepared from glycerol-grown cells, while succinate stimulated only 10-fold.* Since then, succinate and D-(-)lactate have been shown to drive not just proline uptake, but a variety of amino acid transport systems, and also TMG accumulation in membrane vesicles (Kaback and Milner, 1970; Barnes and Kaback, 1970). D-(-)lactate is converted stoichiometrically to pyruvate by a stereospecific lactic dehydrogenase, just as succinate is converted to fumarate (Kaback and Milner, 1970).

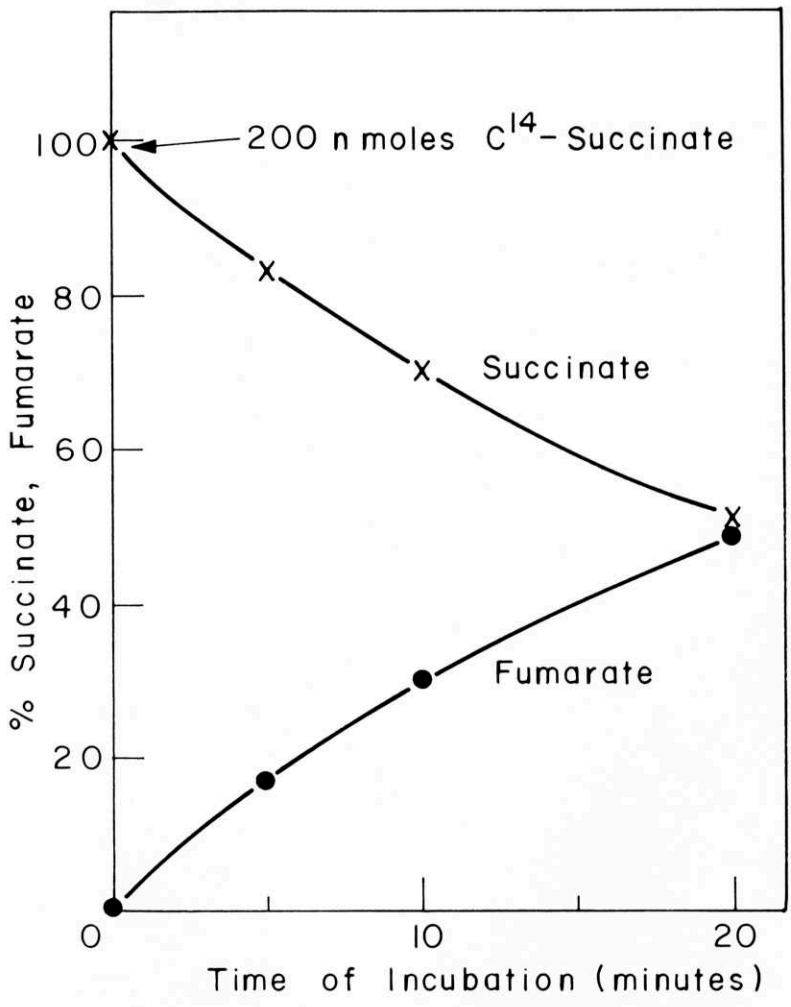
It seemed plausible that other compounds which can furnish electrons directly to the respiratory chain would be able to drive the proline pump if they could get inside the vesicles. As indicated

---

* The differences in relative stimulatory activity of succinate and D-(-)lactate between my membrane preparations and Kaback's are probably due to differences in strains and growth conditions.

Figure 3. Stoichiometry of the conversion of C^{14}-succinate to C^{14}-fumarate by lysozyme-EDTA vesicles from W6.

The experiment was performed as described in Materials and Methods.


200 n moles C14 - Succinate
100
80
60
40
20
0
% Succinate, Fumarate
Succinate
Fumarate
10
20
Time of Incubation (minutes)

in Table 2B, DPNH can also serve as an energy source for proline uptake. Under my experimental conditions, it was about as effective as D-(-)lactate, stimulating about 15-fold over the endogenous level (or about 0.5 nmoles proline/mg/20 min) at a concentration of 6 mM.

Other intermediates of the TCA cycle, such as citrate and α-ketoglutarate, were assayed for proline uptake stimulating activity but none was found.

So far, all the significant stimulatory activities for proline accumulation in membrane vesicles come from oxidation of "respiratory" compounds. Repeated attempts to detect an ATP-dependent proline uptake activity failed, in spite of varied conditions to allow the ATP to get inside the vesicles in sufficiently high concentrations. Table 2 shows the levels for ATP and for an ATP generating system using pyruvate kinase and PEP. Raising the temperature to 46°C in the presence of these substrates and then assaying uptake at 27°C (data not shown) did not contribute to any measurable stimulation, though such a procedure should make the vesicles permeable to the substrates (Kaback, 1970a).

It is not clear at this juncture whether ATP-stimulated uptake is not observed for technical or for physiological reasons, if not both. Several laboratories have reported that oxidative phosphorylating activity is not detected in such membrane preparations (Kaback, personal communication; Klein et al., 1970). Klein et al. cite the insensitivity of proline uptake to high arsenate-low phosphate conditions as evidence that the transport is not dependent on ATP synthesis. Further discussion of the mechanism by which the proline pump might be driven and of the lack of ATP stimulation will be found in later sections.

Colicin Sensitivity of Proline Uptake in Membrane Vesicles

Prior to the study of colicin effects on membranes, a cellular function in whole spheroplasts was looked at to establish whether colicins E_1 and K could absorb and inhibit after spheroplasting. The procedure followed was that described by Nomura and Maeda (1965), who had reported that the conversion of intact cells to spheroplasts by lysozyme-EDTA did not reduce the absorption of colicin molecules, but did protect against the actions of colicins E_2 and E_3, not against colicin K. The parameter measured in their experiment was induction of the enzyme β-galactosidase by IPTG after 20 minutes pretreatment of either spheroplasts or whole cells with colicin. Repeating their experiment using colicins E_1, K and E_3, I confirmed that spheroplasting greatly protects against inhibition of induced β-galactosidase synthesis by colicin E_3, but has only a small protective effect against colicin K. In the case of E_1, no significant difference between inhibition in spheroplasts and whole cells was detected (Table 3).

The sensitivity of spheroplasts to colicins E_1 and K was encouraging in that it allowed at least the possibility that osmotically broken spheroplast fragments might still be sensitive to colicins E_1 and K.

The original observations of colicin sensitivity of the membrane vesicles were made on the glucose-stimulated system of Figure 1A and Table 2A using crude colicin preparations (Materials and Methods).

Preincubation of W6 membrane vesicles made from penicillin spheroplasts with crude colicin E_1 for 15 minutes before addition of C^{14}-proline abolishes the ability of the vesicles to concentrate the proline beyond the level of the W157 membranes. If colicin is added at a time when the vesicles have taken up a high level of proline,

Table 3

β-Galactosidase formation by colicin-treated spheroplasts or whole cells

Colicin	Multiplicity	Spheroplasts % induction	cells % induction
None	0	100	100
E_1	8.3	> 1	> 1
	830	> 1	2.7
E_3	4.12	103	2.4
	412	86.5	1
K	5.1	78.5	> 1
	510	> 1	> 1

C600 was grown to 2 x 10^8/ml, centrifuged, and washed twice with Tris(121) buffer, then resuspended in 1 ml of Tris-sucrose (0.03 M Tris, pH 8, 20% sucrose). To 0.5 ml of this preparation, lysozyme (0.025 ml at 2 mg/ml) and EDTA (0.01 ml of 1%) was added. To the other 0.5 ml, only EDTA (0.01 ml of 1%) was added. After 10 minutes incubation at room temperature, spheroplasts and cells were diluted up to 8.0 ml each with λS medium (λ broth with 20% sucrose, 10 mM $MgCl_2$) and distributed in 1 ml aliquots to tubes (7 cell tubes, 7 spheroplast tubes) at 37°C. At t = 0, colicin was added at appropriate multiplicities. After 20 minutes incubation, IPTG was added to 5 mM final concentration, and the cultures were incubated another 100 minutes at 37°C with gentle shaking. β-Galactosidase was then assayed on toluenized samples. Results are normalized to the control with no colicin.

the label is rapidly lost from the membranes until the same level as the colicin-pretreated membranes is attained. These results are shown in Figure 4. Similar findings were made using crude colicin K. The results indicate that the colicin can act rapidly, and underscore the TCA result that showed that the proline taken up is only loosely associated with the vesicles and can be released back into the medium.

The results presented in Table 4A indicate that crude colicins E_1 or K also inhibit the high endogenous level of proline uptake as well as the succinate-stimulated uptake.

The extent of inhibition of proline uptake is dependent on the colicin concentration, as shown in Figure 5. In vesicles treated with various concentrations of crude colicin E_1, the uptake becomes increasingly inhibited with increasing colicin concentration until a limit is reached beyond which more colicin does not inhibit further. In Figure 5A, concentrated crude colicin E_1 and a 10-fold dilution have the same effect on W6 proline uptake kinetics. A 100-fold dilution inhibits less than maximally. A similar concentration dependency using dilutions of colicin K is shown in Figure 5B.

An extract prepared identically to the colicin-containing extract, but from the non-colicinogenic bacterium (Materials and Methods) was incubated with the membranes under the usual conditions of proline uptake. It had no inhibitory effect, as shown in Table 4B. This increased the likelihood that the observed inhibition of proline uptake in this system was due to the binding of colicin to the membranes rather than to some other phenomenon.

The role of colicin was conclusively demonstrated by showing that the inhibition of proline uptake in membrane vesicles could be

Table 4

Effect of crude colicin on proline uptake
in W6 penicillin vesicles

	Additions	uptake, cpm at 30 min	
(A)	endogenous	9,523	
	" + E_1	5,824	
	" + K	7,640	
	glucose, 200 mM	39,675	
	" + E_1	7,426	
	" + K	6,801	
	succinate, 100 mM	83,013	
	" + E_1	17,914	
	" + K	16,516	
(B)	glucose, 14 mM	24,077	
	" + A606 extract	28,905	no colicin
	" + A606 (1:10)	24,080	" "
	" + A619 extract	8,949	E_1 9 x 10^{12}/ml
	" + A619 (1:10)	11,970	E_1 9 x 10^{11}/ml

(A) 20-λ aliquots of W6 penicillin membranes in 0.5 M K phosphate (pH 6.6), 10 mM Mg^{++} were preincubated 15 minutes at 27°C with the additions as indicated. E_1 titer was 2.13 x 10^{13}/ml; K was 3.75 x 10^{13}/ml. 20-λ volumes of colicin were added. At t = 0, 100 λ of C^{14}-proline in 0.5 M K phosphate (pH 6.6), 10 mM Mg^{++} was added to final proline concentration of 1 µg/ml. After 30 minutes incubation, samples were diluted 1:20 with the same buffer at 27°C and assayed as described in Materials and Methods.

(B) 50-λ aliquots of W6 penicillin membranes (not the same preparation as in (A)) were preincubated with no additions, 50 λ of A606 extract (noncolicinogenic), or 50 λ of A619 extract (E_1 titer undiluted 9.3 x 10^{12}/ml). At t = 0, C^{14}-proline was added to a final volume of 300 λ at 1 µg/ml proline in 0.5 M KPO_4. See

Table 4, continued

Materials and Methods for preparation of A606 and A619 extracts. After 30 minutes, 200-λ samples were assayed. The counts were low because the membranes were stored at too high a temperature.

Figure 4. Effect of crude colicin E_1 on proline uptake

100-λ aliquots of W6 penicillin membranes in 0.5 M KPO_4 (pH 6.6), 10 mM Mg^{++}, 14 mM glucose were placed in each of 3 tubes. 100 λ of A medium was added to tube 1 (●); 100 λ of crude colicin E_1 (dialyzed against A medium; see Materials and Methods), killing titer 2.7 x 10^{13}/ml, was added to tube 2 (△). Tube 3 contained only 100 λ of W6 (▽). All tubes were incubated 15 minutes at 27°C. At t = 0, 0.5 ml of C^{14} proline in 0.5 M phosphate buffer (pH 6.6), 10 mM Mg^{++}, 14 mM glucose was added to each tube. 50-λ samples were taken with time and assayed as described in Materials and Methods. At t = 11 minutes, 100 λ of the crude E_1 was added to tube 3. Samples for tube 3 after 11 minutes are corrected for volume change. Results are expressed as cpm/sample x 2 for comparison with standard experiments in which 100-λ samples were taken. (■) proline uptake by W157 membranes under identical conditions (from a different experiment).

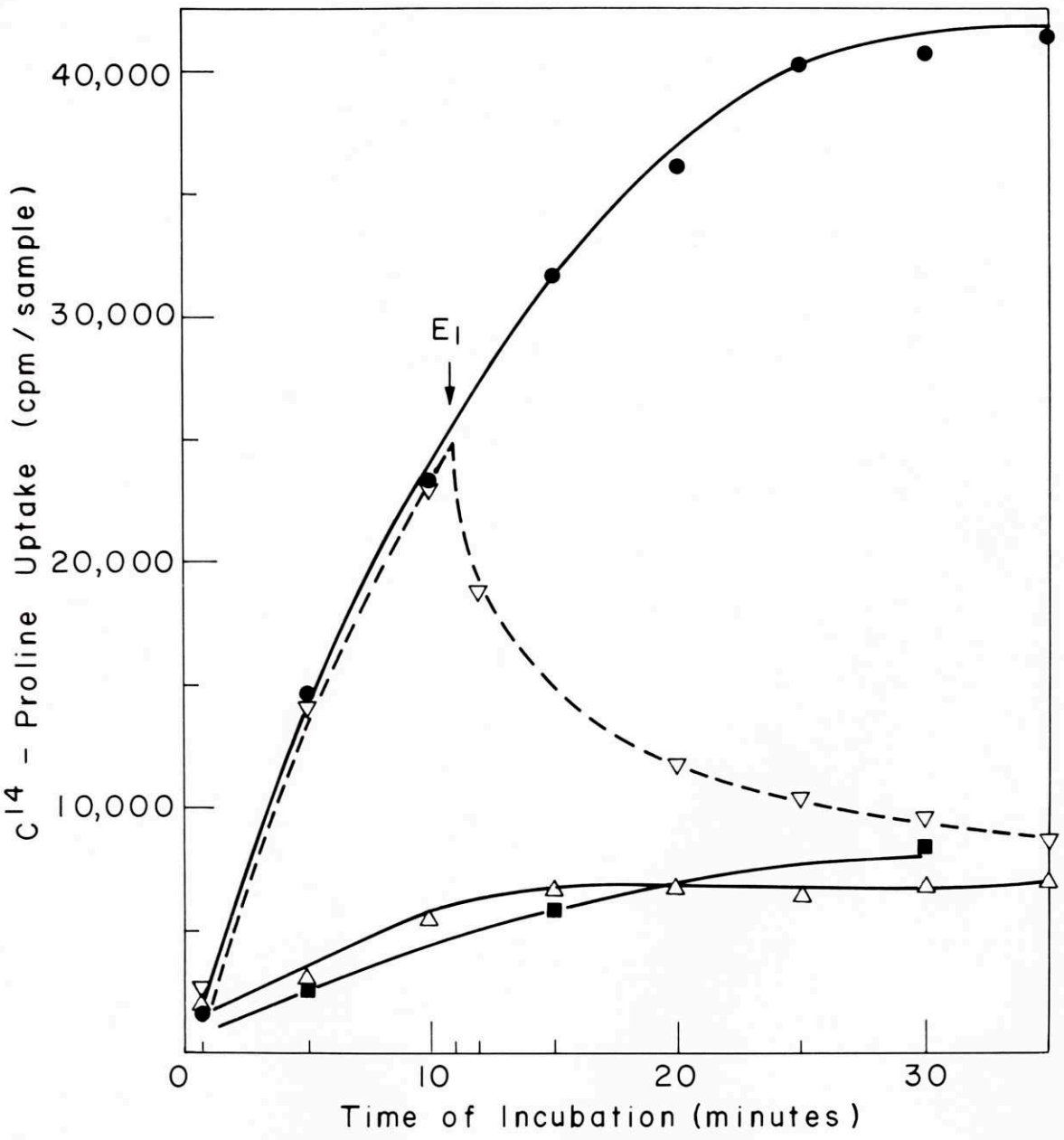
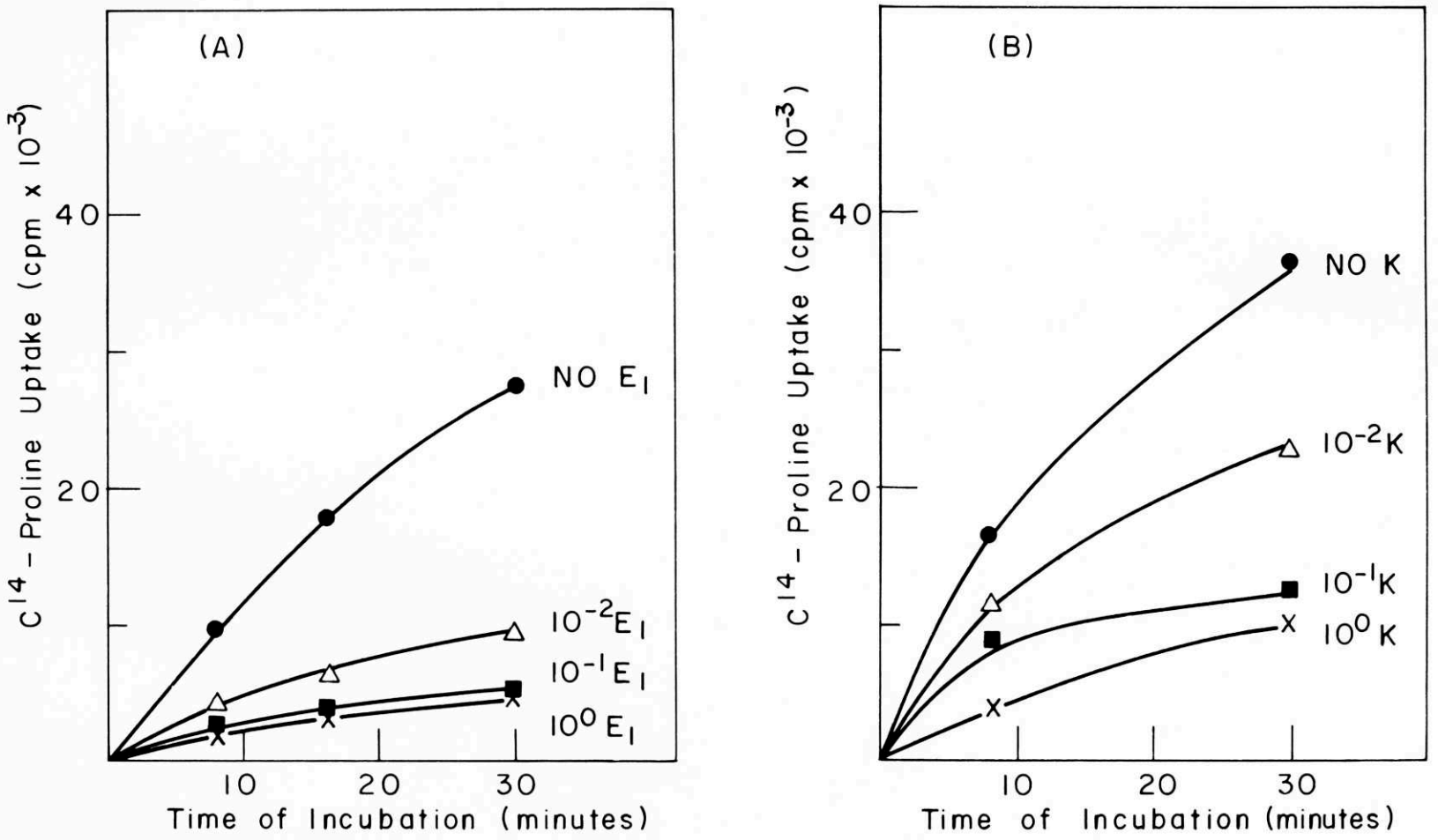

40,000
30,000
20,000
10,000
C14 - Proline Uptake (cpm / sample)
E1
0
10
20
30
Time of Incubation (minutes)

Figure 5. C^{14}-Proline uptake as a function of the amount of colicin (A) crude E_1, (B) crude K

(A) 100-λ aliquots of W6 penicillin membranes in 0.5 M K phosphate (pH 6.6), 10 mM Mg^{++}, 14 mM glucose were preincubated 15 minutes at 27°C with 100 λ of A medium (●), or 100 λ of crude colicin E_1 undiluted (x), diluted 1:10 (■), and diluted 1:100 in A medium (Δ). E_1 titer undiluted was 1.86×10^{13}/ml. At t = 0, C^{14}-proline was added in 0.5 M K phosphate (pH 6.6), 10 mM Mg^{++}, 14 mM glucose to a final proline concentration of 1 µg/ml. 0.1 ml samples were taken at 8, 16, and 30 minutes of incubation and assayed as described in Materials and Methods. Uptake is expressed in cpm/sample.

(B) Same as above, except that crude colicin K was used, titer 2.48×10^{13}/ml.


(A)
C14 - Proline Uptake (cpm x 10-3)
40
20
NO E1
10-2E1
10-1E1
10^0 E1
10
20
30
Time of Incubation (minutes)
(B)
C14 - Proline Uptake (cpm x 10-3)
40
20
NO K
10-2K
10-1K
10^0 K
10
20
30
Time of Incubation (minutes)

accomplished by highly purified colicin E_1 (Materials and Methods). Figure 6 shows succinate-stimulated proline uptake in K-12 vesicles made from penicillin spheroplasts as a function of purified colicin E_1 concentration. Various concentrations of purified E_1 were pre-incubated with membranes for 15 minutes, and then the proline taken up after 30 minutes incubation under the usual conditions was measured. The amount of proline taken up decreases monotonically with increasing colicin concentration but inhibition does not go beyond 50% even at the highest colicin concentration, 2.9×10^{12} k.u./ml in this experiment. This behavior is different from that of crude colicin, in that the crude colicin at the same killing titer routinely reduces uptake by 70 to 80%. This difference between pure and crude colicin is reproducible, but so far, the reason for it remains obscure.

Properties of Membranes from Colicin-resistant Cells

Colicin-resistant mutants of W6 were isolated (Materials and Methods) and their membranes were tested for proline uptake and colicin sensitivity. W6/E_1 refers to the E_1-resistant and W6/K to the K-resistant strain.

The resistant phenotype in whole cells is not absolute, breaking down at high colicin multiplicities. Thus, uracil incorporation into W6/E_1 whole cells is insensitive to crude colicin E_1 inhibition at a multiplicity of 6 while this multiplicity completely inhibits incorporation of uracil into W6 itself. At a multiplicity of 500, the resistance of W6/E_1 is no longer apparent, and inhibition occurs to the same level as W6 (Table 5).

It was anticipated that conditions might be found (i.e., less than maximum colicin concentrations) under which membranes from resistant

Figure 6. Inhibition of proline uptake by C600 penicillin vesicles as a function of purified colicin E_1 concentration.

20-λ aliquots of C600 penicillin vesicles (3 mg/ml) in K phosphate (pH 6.6), 10 mM Mg^{++}, were preincubated 15 minutes at 27°C with succinate and various dilutions of purified E_1 in water (1:10 dilution is relative concentration 128 on graph). At t = 0, 100 λ of C^{14}-proline was added to give final concentrations of 1 μg/ml proline, 0.5 M K phosphate, 10 mM Mg^{++} and 7 mM succinate. After 30 minutes of incubation, the reaction mixtures were diluted and assayed as described in Materials and Methods. Results are expressed as percent of total uptake in the control without colicin.

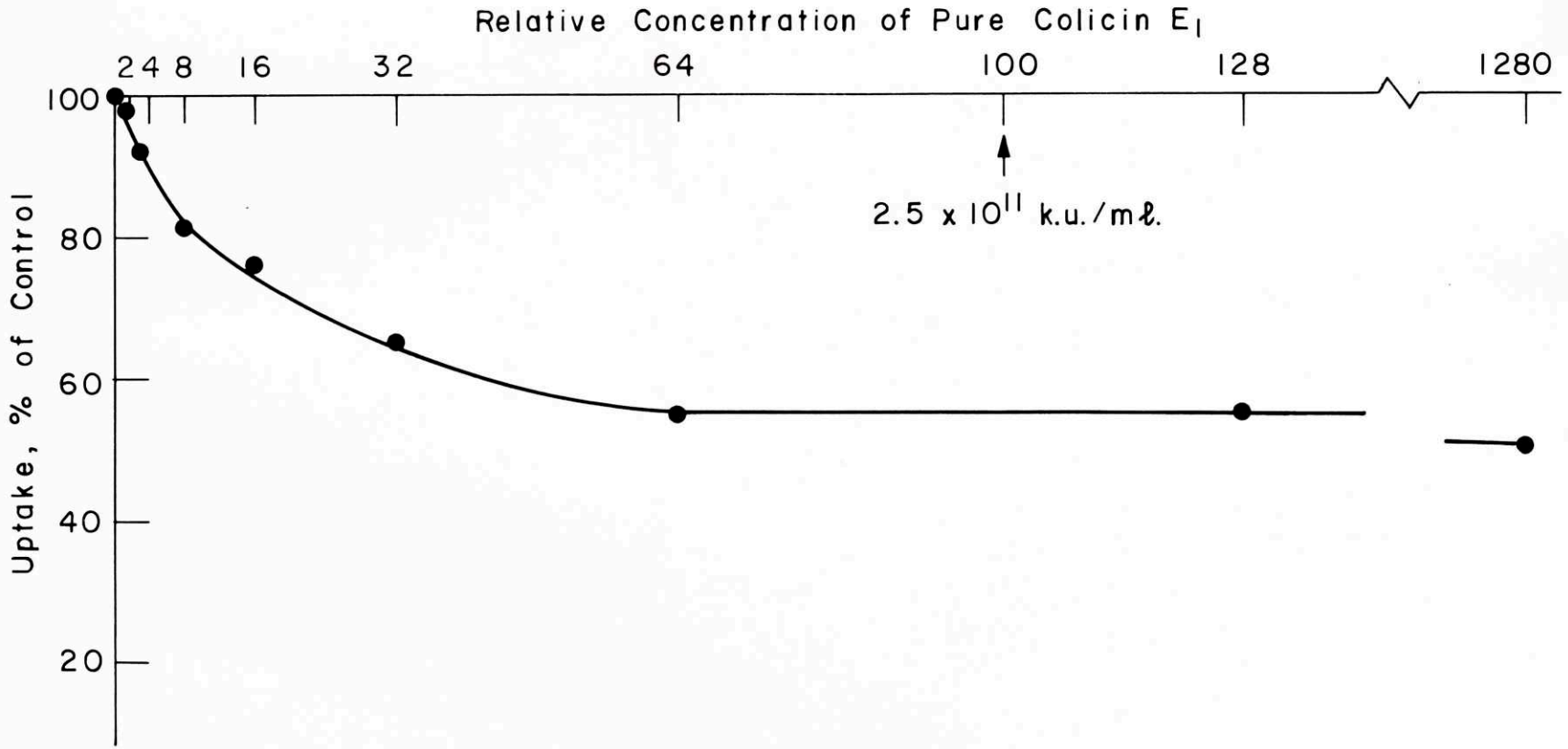

Relative Concentration of Pure Colicin E1
2 4 8 16 32 64 100 128 1280
100
80
60
40
20
Uptake, % of Control
2.5 x 10^11 k.u./mℓ.

Table 5

Properties of whole cells of W6 and W6/E_1

	cpm C^{14}-uracil incorporated in 15 minutes		
	W6	W6/E_1 [1]	multiplicity
no E_1	2,507	3,055	-
pure E_1	30	110	500
no E_1	4,026	3,345	-
10^{-2} E_1	92	4,885	6

[1] 7 independent isolates were tested and all behaved similarly.

W6 and W6/E_1 were grown in A medium (plus 0.2% glucose, 100 µg/ml proline) from fresh overnight cultures to about 2 x 10^8/ml. After distribution to tubes in 1-ml aliquots, either buffer or colicin was added, and the cells were incubated 10 minutes at 37°C for absorption. At t = 0, C^{14}-uracil (200 µg/ml, 5 µC/ml) was added to give a final concentration of 2 µg/ml. At t = 15, 0.2-ml samples were taken, precipitated in 3 ml cold 5% TCA, incubated 30 minutes in the cold, filtered and washed twice with 5 ml TCA, dried, and counted in scintillation counter. E_1 titer was 1.3 x 10^{12} k.u./ml.

mutants would be less sensitive to inhibition of proline uptake by the colicin against which they were selected than the parent W6. This turned out to be the case.

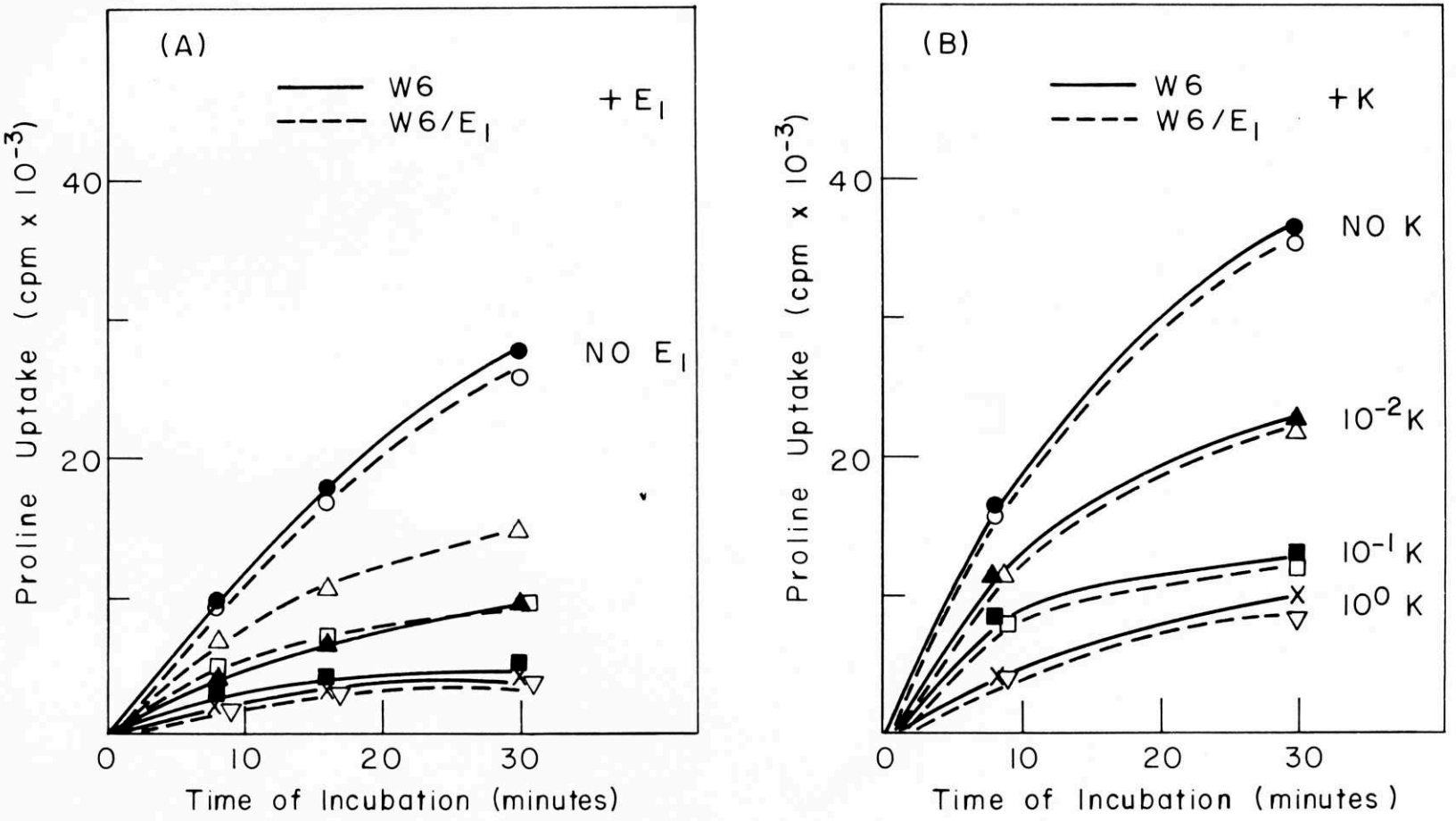
In Figure 7A the kinetics of proline uptake by penicillin vesicles of W6 and W6/E_1 are plotted at various colicin concentrations: undiluted crude E_1, 10-fold diluted, and 100-fold diluted. All curves in this figure refer to glucose-stimulated uptake. Untreated, both membrane preparations exhibit the same kinetics and amount of proline uptake, and at high colicin concentrations (undiluted), both are inhibited to the same extent. But at lower E_1 concentrations, W6/E_1 is clearly less sensitive to a given colicin concentration than the W6 membranes. The 10-fold dilution of E_1 affects the W6/E_1 to the same extent that the 100-fold dilution affects W6. The level of proline taken up by W6/E_1 at the 100-fold dilution of E_1 is higher than that of W6 at the same colicin concentration. I interpret this as evidence that the colicin is responsible for the observed inhibition, and that resistant membranes conserve the property of the cells from which they derive, which makes them more resistant to the colicin inhibition than the wild type. As with whole cells, this resistance can be overcome by a sufficiently high colicin concentration. When the colicin K sensitivities of these same vesicles are compared in Figure 7B, the differences between the two membranes at various colicin concentrations are no longer observed. The colicin E_1-resistant membranes are fully sensitive to colicin K, as expected.

A more quantitative measure of colicin inhibition of proline uptake was then made in the range of colicin concentrations where the difference between wild type and resistant membranes is most pronounced.

Figure 7. Effects of crude colicins on W6 and W6/E_1 membranes.
(A) plus colicin E_1 at various concentrations
(B) plus colicin K at various concentrations

For protocol, see Figure 5, which shows same data for W6 membranes alone.

Closed symbols are W6; open symbols are W6/E_1. (●, o) no colicin; (▲, △), 10^{-2} dilution colicin; (■, □) 10^{-1} dilution colicin; (x, ▽), undiluted colicin. Titers: E_1 1.86×10^{13} k.u./ml
K 2.48×10^{13} k.u./ml

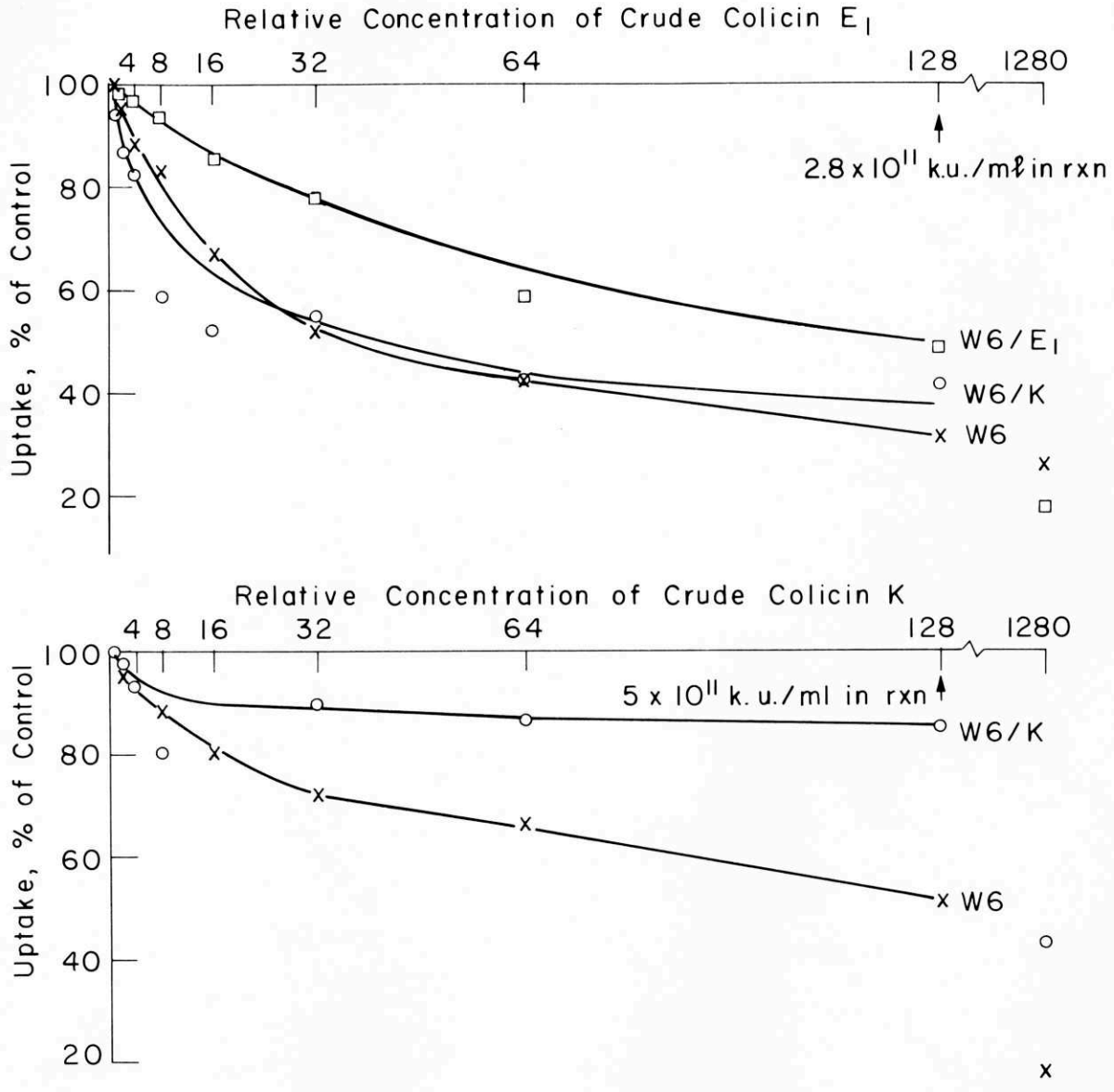

(A)
W6
W6/E1
+E1
NO E1
Proline Uptake (cpm x 10-3)
40
20
0
10
20
30
Time of Incubation (minutes)
(B)
W6
W6/E1
+K
NO K
10-2 K
10-1 K
10^0 K
Proline Uptake (cpm x 10-3)
40
20
0
10
20
30
Time of Incubation (minutes)

Vesicles of W6, W6/E_1 and W6/K from penicillin spheroplasts were pre-incubated 15 minutes at 27°C with various dilutions of crude colicin E_1 or K, and the level of glucose-stimulated proline uptake was measured after 30 minutes of incubation. In Figure 8 the percent total uptake, calculated from the level of proline taken up without colicin in the same incubation time, is plotted as a function of increasing colicin concentration. It can be seen that the resistant membrane vesicles are sensitive to the colicin along the entire concentration range tested. As the colicin concentration is increased, the resistants exhibit a consistently lower sensitivity to their specific colicin than do the wild type vesicles. In W6/E_1 the level of inhibition increases as the colicin E_1 concentration increases. W6/K behaves differently. The level of inhibition remains at 15% as the colicin K concentration increases from less than 5×10^{10} k.u./ml to 5×10^{11} k.u./ml, and it is only at 5×10^{12}/ml that a greater inhibition is seen. In neither case do the resistants display a higher threshold concentration than the wild type, above which they are sensitive and below which they are resistant. W6/K is much more sensitive to colicin E_1 than to colicin K; with E_1, the sensitivity increases with increasing E_1 concentration.

These measurements do not explain the mechanism of colicin resistance or resolve the level at which it is maintained. They just show that membrane vesicles from colicin-resistant strains are quantitatively less sensitive at a given colicin concentration to inhibition than membrane vesicles from the wild type parent, although qualitatively they are sensitive.

Figure 8. Effect of crude colicins E_1 and K on proline uptake by penicillin vesicles of W6, W6/E_1, and W6/K.

20 λ of vesicles in K phosphate 0.5 M, containing 10 mM Mg^{++} and 150 mM glucose, were preincubated with various dilutions of crude colicin in A medium, for 15 minutes at 27°C. C^{14}-Proline was added at t = 0, as in Figure 6, and uptake was measured after 30 minutes of incubation, as described in Materials and Methods. Results are expressed as percent uptake of control with no colicin. Colicin E_1 was at 2.13×10^{13} k.u./ml, and colicin K was at 3.75×10^{13} k.u./ml. (In the reaction mixture, a value of 128 relative units corresponds to 2.8×10^{11} k.u./ml for E_1, and 5×10^{11} k.u./ml for K). Vesicles were at approximately 0.04 mg/ml in the reaction.


Relative Concentration of Crude Colicin E1
4 8 16 32 64 128 1280
100
80
60
40
20
Uptake, % of Control
2.8 x 10^11 k.u./ml in rxn
W6/E1
W6/K
W6
Relative Concentration of Crude Colicin K
4 8 16 32 64 128 1280
100
80
60
40
20
Uptake, % of Control
5 x 10^11 k.u./ml in rxn
W6/K
W6

Bhattacharyya et al. (1970) have made similar observations on the qualitative sensitivity of membranes from resistant cells (see Discussion).

Membrane Vesicles from Colicin-tolerant Cells

The tolerant phenotypes include alterations of membrane permeability and in particular an increased sensitivity to surface active agents (Nagel de Zwaig and Luria, 1967). It was doubtful whether closed vesicles capable of pumping proline could be prepared from such strains. For these studies, a switch was made from E. coli W to the C600 strain of E. coli K-12 (see Figure 6) in which tolerant mutants had already been isolated and characterized (Nagel de Zwaig and Luria, 1967). Tol II and Tol III mutants were looked at because they differ only in their sensitivity to colicin E_1. Tol II is tolerant to colicin E_1 as well as to E_2, E_3, and K; Tol III is tolerant to E_2, E_3, and K but is sensitive to E_1.

It was found that membranes made from penicillin spheroplasts from both tolerant strains accumulated proline in the uptake system, although to a lesser extent than did the wild type, and they were stimulated by succinate. Using highly purified colicin E_1 and measuring succinate-stimulated proline uptake, Tol III membranes and wild-type membranes were inhibited, although the curves as a function of E_1 concentration were not identical. Tol II membranes were insensitive to the colicin, even at the highest concentration, as shown in Figure 9. It is not clear why the Tol III vesicles do not follow the same sensitivity curve as the wild type, nor why the maximum level of inhibition in the wild type is always markedly less when purified

Table 6

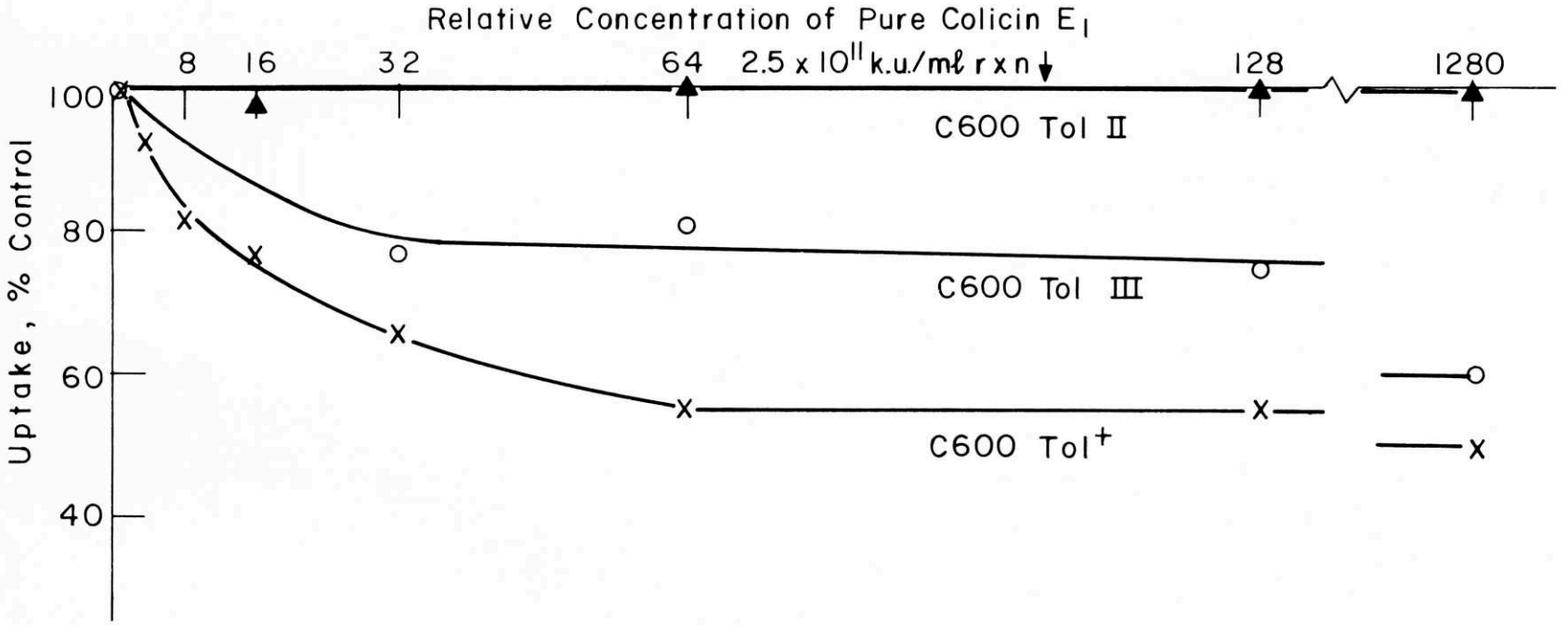
Properties of lysozyme-EDTA vesicles from different sources

Membranes from	cpm C^{14}-proline, 20 min., 27°C						
	Uptake, endogenous	Uptake, 20 mM succ.	Uptake, 20 mM succ. + pure E_1	% inhib.	E_1 k.u./mg membrane protein	Uptake, 20 min. 20 mM succ. + E_2	viable cells/ml
W6	544	28,600	15,244	46	6.6×10^{12}	27,000	3.3×10^5
C600	1,229	27,627	17,033	38	6.3×10^{12}	-	-
C600 Tol II	622	3,453	3,440	0	6.6×10^{12}	-	-
C600(E_1)	587	5,643	3,636	36	1.1×10^{13}	-	-
W6	537	20,477	crude E_1 4,246	79			
			crude K 2,092	90			

Vesicles were prepared as described in Materials and Methods, including the sucrose-flotation step. Pure E_1 was at 2×10^{13} k.u./ml, and the E_2 was approximately 1×10^{13} k.u./ml. Crude E_1 was at 1.84×10^{14} k.u./ml, and crude K at 6×10^{13} k.u/ml.

Figure 9. Effect of purified colicin E_1 on proline uptake by penicillin vesicles from various *E. coli* K12 derivatives.

The experimental procedure is the same as described in Figure 6.


Relative Concentration of Pure Colicin E1
8
16
32
64
2.5 x 10^11 k.u./ml rxn
128
1280
100
80
60
40
Uptake, % Control
C600 Tol II
C600 Tol III
C600 Tol+

colicin is used rather than crude, even though the killing titers as measured by sensitive cell viability are comparable.

The observations made on penicillin membrane vesicles using glucose and succinate as energy sources for proline uptake, and using both crude and pure colicin have been confirmed by qualitatively similar findings in lysozyme-EDTA membrane preparations. Table 6 summarizes the proline uptake of such vesicles from various strains and their sensitivities to colicin. Measurements of uptake levels were made after 20 minutes of incubation. In the usual way, colicin was pre-incubated with the vesicles for 15 minutes before addition of C^{14}-proline. In this system, as mentioned earlier, purified E_1 inhibits about 50%. Purified colicin E_2 does not inhibit succinate-stimulated uptake at all.

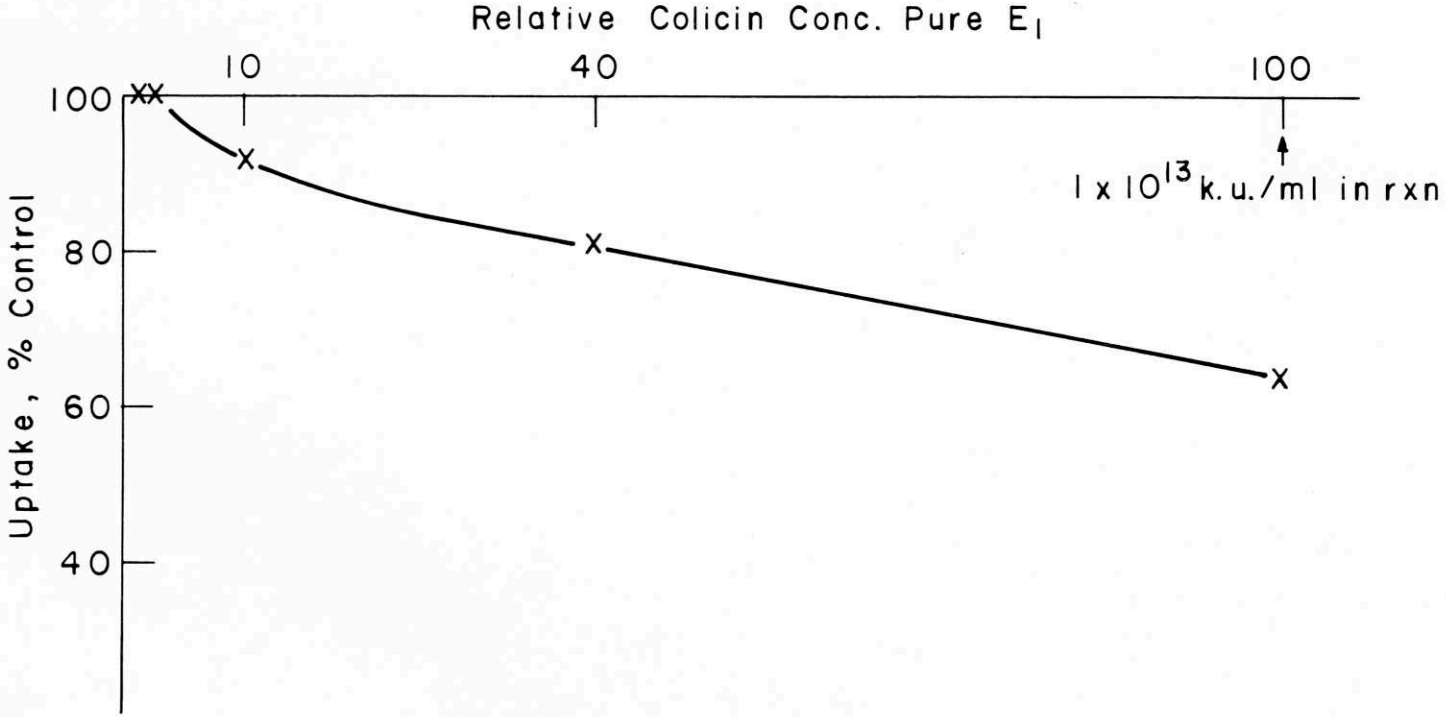
The reason for the lower level of proline taken up by the Tol II vesicles is not understood. A lower uptake was also noted in penicillin vesicles of Tol II. The kinetics of proline uptake by Tol II lysozyme vesicles are similar to those of the wild type. Proline uptake by Tol II vesicles shows no sensitivity to colicin E_1 inhibition at any colicin concentration.

Vesicles from Immune Cells

The nature of the immunity to homologous colicin by colicinogenic cells is intriguing, especially as regards how maintenance of the immune state is related to the presence and functioning of the colicinogenic factor. Only one preparation of membranes from C600(ColE_1) strain was examined and it proved to be sensitive to colicin E_1 in its uptake of proline (Table 6, Figure 10). This isolated result is

Figure 10. Effect of purified colicin E_1 on an immune strain C600(E_1).

50-λ aliquots of vesicles were mixed with an equal volume of colicin at various dilutions, and preincubated 15 minutes at 27°C with 20 mM succinate. C^{14}-Proline taken up was measured after 20 minutes of incubation. Final phosphate concentration of the reaction was 0.25 M (pH 6.6).


Relative Colicin Conc. Pure E_1
10
40
100
100
80
60
40
Uptake, % Control
1×10^{13} k.u./ml in rxn

inadequate to justify speculations. Further studies on intact cells, spheroplasts, and membranes from immune cells are greatly desirable.

Summary

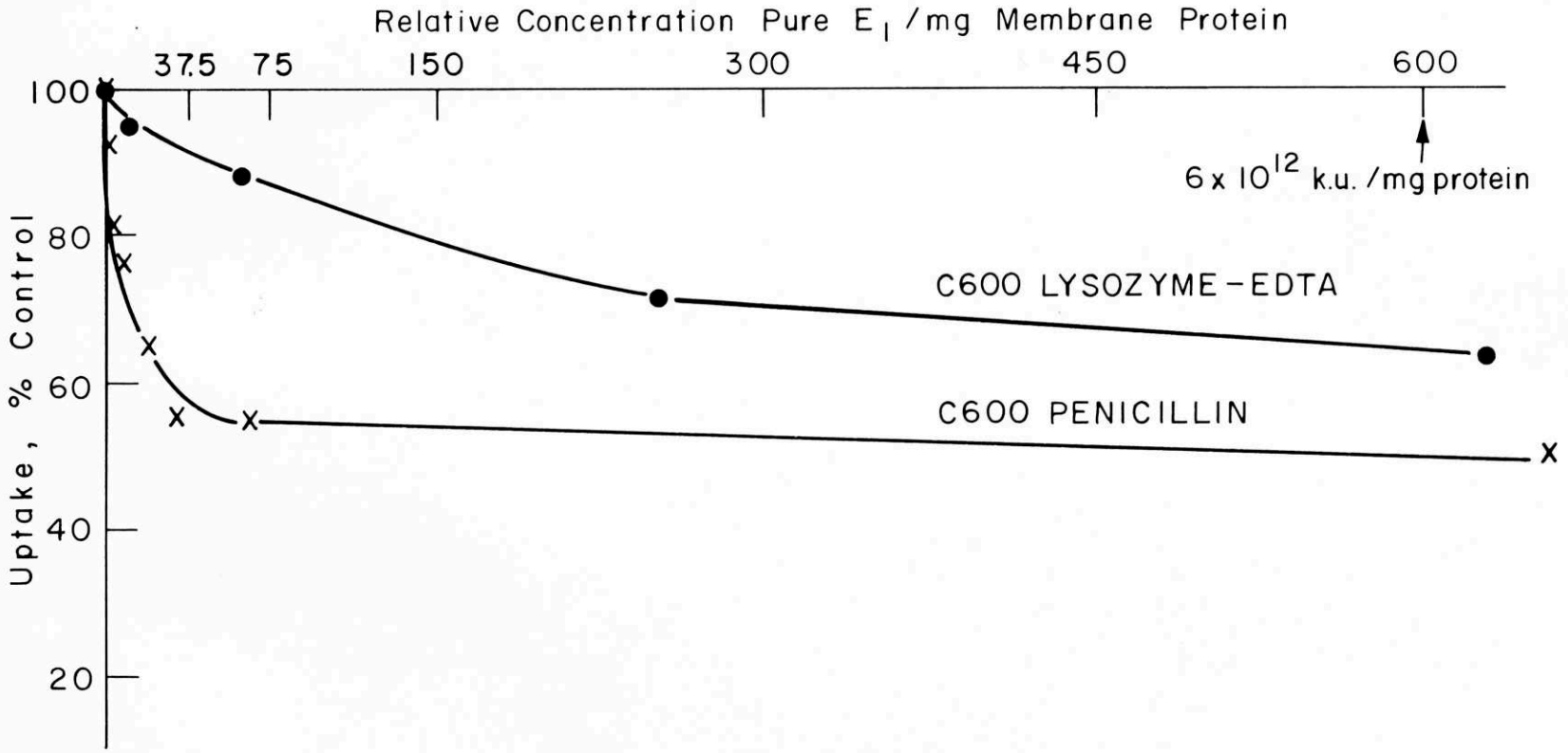
The possibility has not been excluded that the high endogenous activity of the penicillin membranes is due to contamination by whole cells or spheroplasts, rather than just a characteristic of membranes prepared by this method. If wild type C600 vesicles made by the lysozyme-EDTA method are compared to vesicles made by the penicillin method with regard to their sensitivities to purified E_1 in a range of concentrations, one finds that the penicillin vesicles are more sensitive at any given colicin concentration (Figure 11). This might be because: (1) the penicillin membranes are more intact and therefore the colicin can function with greater efficiency, (2) the penicillin membranes are larger and therefore are better targets, i.e., have higher multiplicities at any given colicin concentration, (3) the penicillin membranes contain a fraction of cells or spheroplasts not present in the lysozyme preparation, and which cause the high endogenous activity and the greater colicin sensitivity.

Neither class of vesicle made from Tol II cells is sensitive to colicin E_1.

I have not systematically analyzed the differences between penicillin and lysozyme-EDTA vesicles, and the above possibilities have not been distinguished by this work. What can be said, in summary, is that membrane vesicles as prepared, especially by the lysozyme-EDTA method, do take up high levels of C^{14}-proline, that they do so only in response to exogenous substrate, such as succinate,

Figure 11. Effect of purified colicin E_1 on vesicles prepared by lysozyme-EDTA or by penicillin.

The protocol was as indicated in Figures 6 and 9. Results are plotted as the percent of uptake of the control, as a function of the colicin concentration divided by the membrane protein concentration.


Relative Concentration Pure E_1 /mg Membrane Protein
37.5
75
150
300
450
600
100
80
60
40
20
Uptake, % Control
6×10^{12} k.u. /mg protein
C600 LYSOZYME-EDTA
C600 PENICILLIN

which becomes oxidized to fumarate and in some way drives the proline pump; that these vesicles are inhibitable by colicins E_1 and K, that resistant vesicles are more resistant than wild type vesicles, although they are partially sensitive, and that membranes from Tol II cells are insensitive.

There are complications and limitations in this system that have been alluded to in the text; for one, the difference in inhibitory power between crude and pure colicin E_1 of equal killing titer.

The following section deals with efforts to analyze the events that occur in these vesicles in an attempt to understand how the colicin works.

Part II. Comparison of Colicin with Other Inhibitors - Possible Loci of Colicin Inhibition

Effects on Proline Uptake and the Conversion of Succinate to Fumarate

In his early work Kaback noted that the proline uptake in glucose-stimulated vesicles was completely abolished by certain uncouplers and inhibitors of electron transport, namely FCCP, DNP, and amytal, while azide inhibited only 33% at 5 mM and 70% at 10 mM, and KCN at 10 mM had no inhibitory effect (Kaback and Stadtman, 1966). It was always problematic what the mechanism of the glucose stimulation might be, since the soluble enzymes of glucose catabolism are likely to be greatly depleted in the membranes, and since glucose utilization requires ATP activation in any case. The discovery of the succinate stimulation, with its much tighter coupling to proline active transport, showed the glucose phenomenon to be a minor effect and encouraged efforts to conceptualize steps between the oxidation of succinate to fumarate and the transport work performed by the proline pump. The use of uncouplers and inhibitors to map this respiratory aerobic complex is based on extensive analyses that have been made on the mitochondrial system (summarized in Lehninger, 1964).

It was reasoned that by comparing the effects of colicin, uncouplers, and inhibitors of electron transport and oxidative phosphorylation on proline uptake, on the conversion of succinate to fumarate and on overall respiratory activity some differences might appear which could point to a unique inhibitory site for colicin action. Table 7 compares the effects of colicin and of various compounds on succinate-driven proline uptake in lysozyme-EDTA vesicles of W6 and on the conversion

Table 7

Effect of inhibitors and uncouplers on lysozyme-EDTA membranes of W6

(A) proline uptake; (B) conversion of succinate to fumarate

	Comparison with effect of colicin			
	A		B	
Compound	Final concentration	Proline uptake, % inhibition	Final concentration	At T=20 min % conversion to fumarate
None	-	0	none	48
CCCP	10^{-5} M	98	2×10^{-6} M	52
	10^{-6} M	98		
NaN_3	10^{-2} M	> 98		
	10^{-3} M	95	2×10^{-3} M	39
Na amytal	10^{-2} M	80		
	10^{-3} M	9	not done	
DNP	$< 10^{-3}$ M	98		
	$< 10^{-4}$ M	77	$< 2 \times 10^{-3}$ M	39
KCN	10^{-2} M	96		
	10^{-3} M	97	2×10^{-3} M	41
Valinomycin	not done		2.7×10^{-6} M	51
pure E_1	1×10^{13}/ml	53	1×10^{13}/ml	55

For protocols, see Materials and Methods.

of C^{14}-succinate to fumarate in the same vesicles. Proline uptake is measured under the usual conditions, i.e. in the presence of 20 mM succinate, which is saturating; succinate dehydrogenase activity is measured at an initial concentration of 2 mM succinate, using thin layer chromatography to separate succinate from fumarate (Materials and Methods).

Contrary to Kaback's original findings, both azide and KCN inhibited proline uptake completely at 1 mM final concentrations, as did the uncouplers CCCP and DNP at 1 µM and less than 100 µM respectively. In the same experiment, crude colicin E_1 inhibited proline uptake 78%, crude colicin K 89% and pure E_1 53%.

Purified colicin E_1 had no measurable effect on the rate or amount of succinate converted to fumarate, when under identical conditions it inhibited proline uptake 53%. This implies that the colicin effects a dissociation or uncoupling somewhere between the dehydrogenase and the proline pump.

It was thought that uncouplers might give results comparable to the colicin and distinguishable from the inhibitors in their effect on the conversion of succinate to fumarate. Inhibitors would be expected to prevent extensive electron flow towards oxygen and therefore block the flavoprotein-linked conversion by piling up reduced intermediates of electron transport, whereas uncouplers would be predicted to allow electron flow and therefore dehydrogenation of succinate, but would prevent it from driving production of high energy compounds. But, as seen in Table 7, neither the uncouplers nor the inhibitors had much effect on the conversion of succinate to fumarate. It was reduced

from 48% to 41% with 1 mM KCN and to 39% with 1 mM N_3, but DNP at less than 0.1 mM also reduced it to 39% in this experiment. CCCP and valinomycin had no effect or slightly increased the conversion. We conclude that for all cases where the compound inhibits proline uptake more than 95%, there is more than enough conversion of succinate to fumarate to get full active transport. Stoichiometrically the dehydrogenase is loosely coupled to the proline pump in the sense that only 1 to 2 n-moles of proline is accumulated in 20 minutes by 1 mg of membranes while 690 nmoles of succinate are converted to fumarate in the same time.

Oxygen Consumption in Membrane Vesicles

Respiratory activity in the membrane vesicles was looked at as another, more inclusive monitor of electron transport. These experiments were done in collaboration with Dr. Robert Fisher. The results are summarized in Table 8.

The rate of oxygen consumption is expressed in n-atoms of oxygen per minute per mg membranes, measured polarographically using a Clark electrode (Materials and Methods). The first observation of note is that in the absence of exogenous substrate the membrane vesicles prepared by the lysozyme method do not consume oxygen. Addition of DPNH, succinate, or D-(-)lactate results in an immediate initiation of oxygen consumption. Addition of pyruvate, the endproduct of D-(-)lactate oxidation, gives no respiratory activity, as expected.

When colicin E_1, either pure or crude, is preincubated with the vesicles for 5 minutes at 37°C and these vesicles are then measured for respiratory activity with added substrate, no effect on the rate of oxygen uptake is observed. Addition of KCN to 1 mM reduces

Table 8

Respiration by membrane vesicles*

Additions	rate of O_2 consumption n-atoms O/min/mg membranes
None (endogenous)	0
20 mM succinate	28.6, 25.2, 24.8, 28.6
" + pure E_1	22.2
" + HOQNO (20 µg or 40 µg)	7.9
" + " + KCN (1 mM)	7.9
" + KCN (1 mM)	15.9
" + " + HOQNO (50 µg)	4.8
" + CCCP (6×10^{-6} M)	20.6
" + Na N_3 (1 mM)	17.4
20 mM D-(-)lactate	17.5
" + pure E_1	19.1
20 mM pyruvate	0
4 mM DPNH	47.5
20 mM succinate	22.
" + crude E_1	22
" + pure E_1	22

* Lysozyme-EDTA vesicles at 9.5 mg/ml in 0.25 M K phosphate (pH 6.6), 10 mM Mg^{++}.

For protocol, see Materials and Methods.

respiration by about 25%. It does not shut it off completely. The same is observed with 1 mM azide. The more potent inhibitor hydroxyquinoline N-oxide (HOQNO) works somewhat better. However, it seems almost impossible to shut off substrate-driven electron transport tightly enough to prevent all oxidative activity, as measured by the decrease in oxygen tension. But in view of the lack of inhibition of the conversion of succinate to fumarate by inhibitors which completely block proline accumulation, it seems likely that the oxygen-consuming activity we observe in the presence of electron transport inhibitors is not due to flow through the cytochrome but perhaps to a by-pass of electrons through some oxidase activity in the membranes acting as an electron shunt to molecular oxygen. This would account for the continuing conversion of succinate to fumarate in the presence of inhibitors without positing that they are acting like the uncouplers.

The powerful uncoupler CCCP does not inhibit the rate of respiration in these vesicles, an indication that the flow of electrons to oxygen is unimpeded. But, in a similar experiment using starved whole cells, shown in Table 9, uncouplers stimulated a dramatic increase in the rate of oxygen consumption, both in the case of endogenous respiration and for succinate-enhanced respiration. This is the well documented phenomenon known as release of respiratory control (see Discussion). It is noteworthy that pure colicin E_1 does not release respiratory control in the starved cell system, although at the multiplicity used, it reduced viability of the cells by more than three orders of magnitude (proving that it was adsorbed). This is a clear difference between the behavior of colicin and of classical uncouplers.

Table 9

Respiration in starved whole cells[1]

Additions	Rate of O_2 consumption n-atoms O/min/mg dry wt.	RCR[2]
endogenous	104	
" + 6.6×10^{-6} M FCCP	234	2.3
endogenous + crude E_1	139	
" " + 6.6×10^{-6} M FCCP	400	3.9
endogenous + A606 extract[3]	104	
" " + 6.6×10^{-6} M FCCP	523	5.0
endogenous + pure E_1	104	
" " + 6.6×10^{-6} M FCCP	348	3.3
20 mM succinate	208	
" + pure E_1	174	
" " + 6.6×10^{-6} M FCCP	520	3.1
20 mM succinate	244	
" + 6.6×10^{-6} M FCCP	330	1.4

1 Strain W6. For protocol, see Materials and Methods.

2 RCR = release of respiratory control = $\frac{\text{respiration with uncoupler}}{\text{respiration without uncoupler}}$

3 crude cell extract made from non-colicinogenic strain (Materials and Methods).

The finding that CCCP does not increase the rate of respiration in the membrane vesicles is an indication that the vesicles are in a highly uncoupled state to begin with. That the respiratory control characteristic of mitochondria and whole cells is missing in the membrane vesicles is consistent with the studies of Klein et al., who were unable to detect any level of oxidative synthesis of ATP in similar membrane preparations (Klein et al., 1970).

Energy-linked Transhydrogenase Activity in Membrane Vesicles as an Analytical Tool

If no ATP is being synthesized by these membrane vesicles, what is causing the proline pump to concentrate proline against a gradient in response to succinate or lactate oxidation? How can we interpret the inhibitions by malonate, and by the inhibitors and uncouplers of electron transport? Moreover, since colicin seems to divorce succinate oxidation from proline accumulation and does not inhibit respiration, at what level could it be working, especially if there is no ATP synthesis?

At this juncture a useful line of evidence emerges from current work on oxidative phosphorylation and energy mobilization in mitochondria. It has been found that ATP itself is not the exclusive energy-providing molecule for processes which require energy. There is substantial evidence that at least one intermediate between the electron transport chain and ATP can be utilized directly to drive energy-consuming reactions. Two processes in mitochondria which have been shown to utilize this so-called high energy intermediate are the active translocation and accumulation of cations against a gradient,

and the energy-linked nicotinamide nucleotide transhydrogenase reaction (Danielson and Ernster, 1963).

Both reactions are relevant to the problem of the source of energy for the proline pump. The first indicates that at least certain transport systems can be driven by respiration-generated intermediates on the pathway to ATP synthesis. The second is an enzymatic activity also found in particulate fractions of E. coli (Murthy and Brodie, 1964; Bragg and Hou, 1968; Fisher et al., 1970).

The transhydrogenase reaction has been described by the overall equation

$$NADH + NADP^+ + I \sim X \longrightarrow NAD^+ + NADPH + I + X$$

where $I \sim X$ is a high energy non-phosphorylated intermediate of oxidative phosphorylation which is generated either aerobically by the oxidation of NADH (or succinate) through the energy coupling sites of the respiratory chain (this reaction requires oxygen) or from ATP, referred to as the anaerobic reaction because $I \sim X$ can be generated from ATP independently of respiratory activity.

This particulate energy-linked transhydrogenase activity in E. coli clearly satisfies all the criteria discussed in the introduction for a useful subcellular bacterial system for analyzing the mechanism of action of colicins E_1 and K, and has the additional attractive property of being driven by ATP as well as by the respiration of reduced substrates. An attempt was therefore made to use this system to test the idea that colicin inhibition involves an effect at the level of aerobically generated high energy intermediate, since this is a possible mechanism by which succinate oxidation might be coupled to proline transport in membrane vesicles. The ATP reaction provides

the opportunity to look at the effect of colicin on the same energy-consuming reaction when driven by anaerobically generated intermediate. Presumably, under strictly anaerobic conditions, only glycolytically generated high energy intermediate is available for running energy-consuming reactions, including the anaerobic concentration of proline. This system therefore allows the behavior of colicin on the anaerobic reaction to contribute to defining the level at which the colicin can inhibit.

The effect of colicin E_1 on the activities of the NADH reaction and the ATP reaction are shown in Table 10 (see Materials and Methods for details of the reactions). Various conditions were tried as stated in the table. High activity is observed in a particulate fraction made by extensive sonication of whole cells of W6, but this activity is not affected by colicin, presumably because the membranes are highly dispersed and do not absorb colicin. Normal Kaback vesicles show only a low activity, which can be somewhat improved upon by procedures to make them more leaky, such as indicated in Table 10. In all cases it appears that the respiratory reaction is inhibited by pure E_1 and crude E_1, and that the ATP-driven reaction is not substantially inhibited by the colicin. Further manipulations are necessary to increase the activities sufficiently to measure accurately the magnitude of the colicin inhibition.

Table 10

Transhydrogenase activities in particulate fractions of W6

Preparation	n-moles NADPH/min/mg protein		
	NADH reaction	ATP + NADH	anaerobic
Vesicles of W6 made without EDTA washes	0	0	0
Sonic particles from whole cells	168		
Sonic particles from whole cells + pure E_1	168		
Vesicles in Tris, 50 mM, pH 7.8, 10 mM Mg^{++}	4.5	4.7	
Vesicles in Tris, 50 mM, pH 7.8, 10 mM Mg^{++} + crude E_1	2	5.4	
Vesicles sonicated at pH 7.8	4.8	9.6	4.8
Vesicles sonicated at pH 7.8 + E_1	1.2	5.4	4.2
Vesicles washed with 0.1 M Tris (pH 8.0), sonicated 1 min, resuspended in 50 mM Tris, 10 mM Mg^{++}	12.8	13.5	
Vesicles washed with 0.1 M Tris (pH 8.0), sonicated 1 min, resuspended in 50 mM Tris, 10 mM Mg^{++} + pure E_1	8	12	

Part III. Preliminary Experiments on Membrane Architecture

Whatever the mechanism of the events following colicin attachment to sensitive cells, the inhibitory signal must be transmitted to the site where it can uncouple the transport process from the energized intermediate. This transmission undoubtedly involves membrane components. Evidence for this comes from the pleiotropic effects of colicin-tolerant mutations on the membrane (see Introduction).

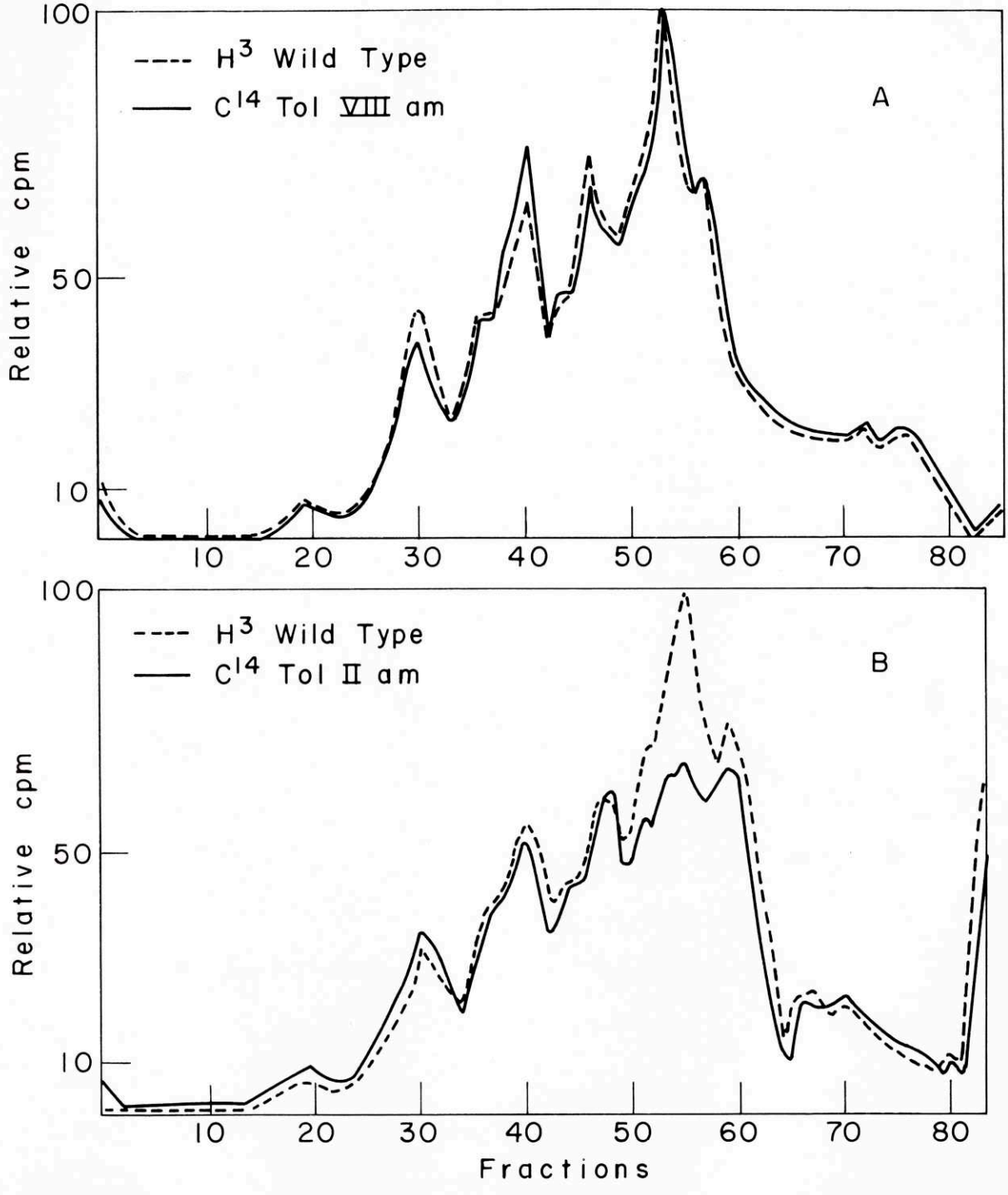
If individual components of the membrane could be identified by function and by spatial location in the membrane, one might begin to piece together the ways in which such components are synthesized and incorporated into the overall system. Towards this end an attempt was initiated to identify the membrane alteration in colicin-tolerant mutants using the experimental approach of Inouye and Guthrie (1969).

A double-label C^{14}/H^3 mixture of membrane vesicles from a tol amber mutant and its wild type parent was made, solubilized with detergent, and electrophoresed on SDS polyacrylamide gels to display the protein components in a spectrum according to size (Materials and Methods). If the amber mutation is in a membrane protein, this protein either would not be synthesized, or would not be incorporated into the membrane, or would run at a position different from the wild type species because of its abbreviated size, and a difference peak in the isotope ratio would identify the component present in the wild type and missing in the tolerant amber membranes.

Normalized profiles for mixtures of Tol II and Tol VIII with wild type are shown in Figure 12. Prelabeled cells were washed and diluted into the same flask with penicillin-containing spheroplasting

Figure 12. Gel electrophoresis of double-label membrane mixture.
(A) C^{14} = Tol VIII amber mutant; H^3 = wild type.
(B) C^{14} = Tol II amber mutant; H^3 = wild type.

Electrophoresis was performed on 7% polyacrylamide, 0.1% SDS. Gels measured 8 cm x 0.4 cm. Preparation of the membranes and of the gels is as described in Materials and Methods. Counts are corrected for isotope spillover and normalized to the highest peak in the gel.


100
50
10
Relative cpm
---- H3 Wild Type
—— C14 Tol VIII am
A
10
20
30
40
50
60
70
80
100
50
10
Relative cpm
---- H3 Wild Type
—— C14 Tol II am
B
10
20
30
40
50
60
70
80
Fractions

medium (Materials and Methods) and incubated for 2.5 hours at 37°C. The spheroplasts were then broken by osmotic dilution, washed as in the preparation of membrane vesicles, and prepared for electrophoresis (see Materials and Methods). The gel patterns in Figure 12b clearly shows that a major peak is missing in the Tol II profile, whereas in the Tol VIII (Figure 12A) no significant differences are in evidence. These gels are 7% polyacrylamide. At 10% polyacrylamide, the major difference peak in the Tol II preparation is resolved into two components, both of which are missing. The reverse-label experiment shows the same differences.

One major limitation of this procedure is that, because of the altered membrane properties associated with colicin tolerance (Nagel de Zwaig and Luria, 1967), the mixture of double-label membranes contains membranes with different physical properties. Thus it might be expected that some proteins might be differentially lost during washing. Also, the amber mutation may not affect a membrane protein and may be only indirectly responsible for any change in membrane proteins observed. In this particular penicillin preparation, the long spheroplasting period of the mixture of C^{14}- and H^{3}-labeled cells admits the possibility of label mixing due to breakdown and turnover, if any such occurs. But lysozyme-EDTA spheroplasting either separately or after mixing gave similar results (unpublished results by J. Lusk and J. Carson).

To localize further the missing component(s) in the Tol II amber mutant, double-label lysozyme-EDTA spheroplasts were sonicated, washed with 0.3 M KCl, 5 mM EDTA, pH 7.5, and spun on sucrose to effect a separation of inner and outer membrane fractions, according to the

method of J. Carson, modified from the procedure of Miura and Mizushima (1968). Inner and outer fractions were run on 7.5% polyacrylamide, 2% SDS gels after fractionation, and compared with the unseparated membrane profile in Figure 13A. The inner membrane spectrum has minor differences in various regions of the gel (Figure 13B). In the outer membrane gel, one of the two protein peaks is completely missing (Figure 13C). This species runs in the same place as the difference peak in the unseparated membranes (Figure 13A).

Further work with this system is necessary to assess the possibility of artifactual behavior resulting from the experimental methods. Possible approaches will be using other independent amber isolates, suppressing the amber with a phage-carried suppressor gene, using a temperature-sensitive Tol II mutant, and perhaps using newly developed labeling reagents (Berg, 1969) which differentially bind to protein accessible from the outside of cells only, or from both inside and outside, depending on the lipid solubility properties of the molecules.

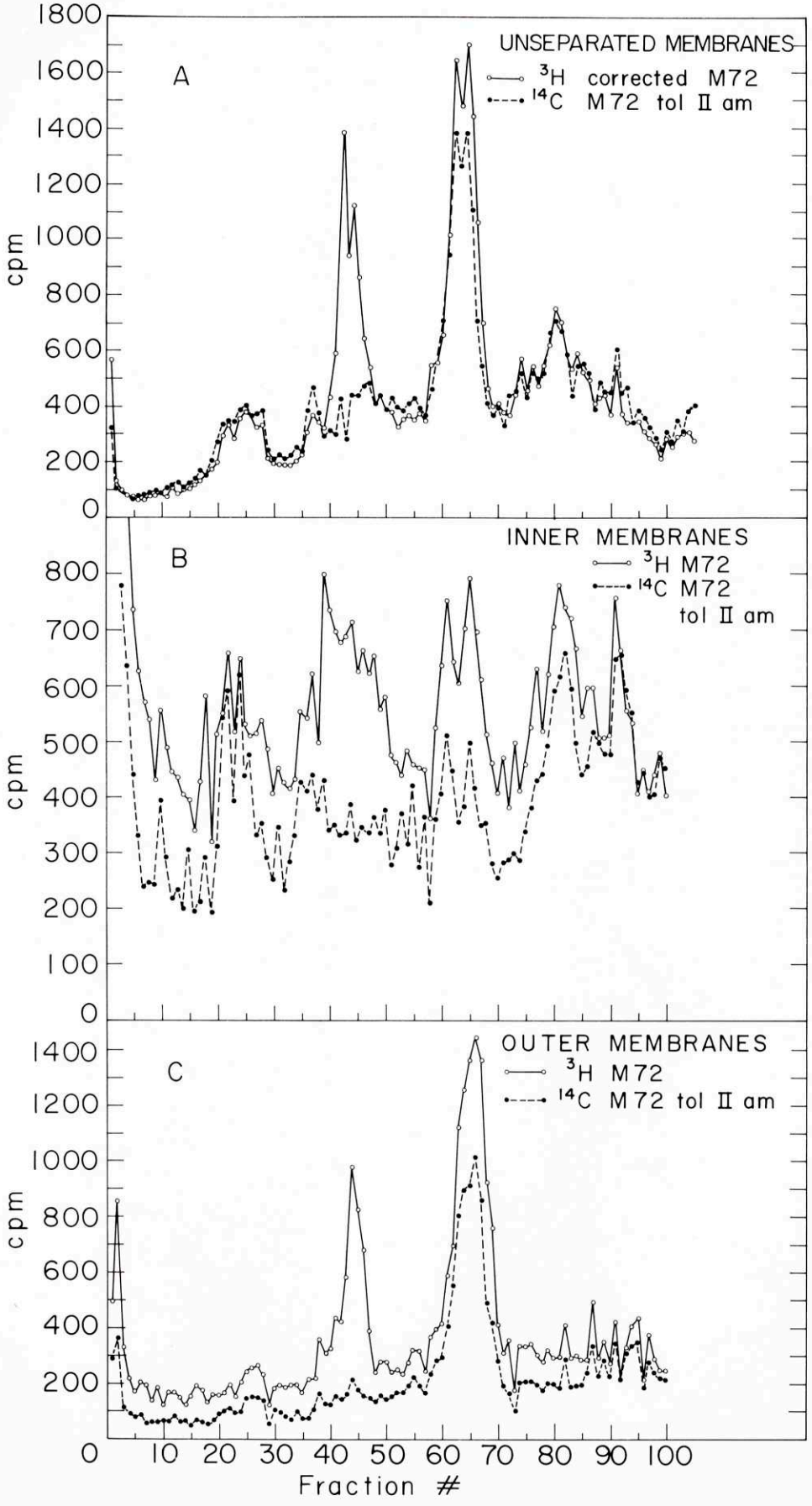
Figure 13. Gel electrophoresis of membrane fractions.

(A) unseparated membranes.

(B) inner membrane fraction

(C) outer membrane fraction.

The outer membrane fraction was made by the procedure of J. Carson, as described in Materials and Methods. Gels are 2% SDS, 7.5% polyacrylamide. C^{14} = Tol II amber mutant; H^{3} = wild type. Counts are corrected for isotope spillover and plotted as corrected cpm/gel slice. This experiment was performed in collaboration with John Carson.


A
UNSEPARATED MEMBRANES
^{3}H corrected M72
^{14}C M72 tol II am
B
INNER MEMBRANES
^{3}H M72
^{14}C M72
tol II am
C
OUTER MEMBRANES
^{3}H M72
^{14}C M72 tol II am
cpm
Fraction #

DISCUSSION

I. The Inhibition of Proline Uptake by Colicin

The data presented in this work confirm the usefulness of Kaback's preparations of isolated membrane vesicles from E. coli for the study of accumulation of substrates through membranes. Preparations of membrane vesicles can be obtained which perform concentrative uptake of proline in response to exogenous substrate. Both this work and studies from Kaback's laboratory have demonstrated that respiratory substrates, succinate in particular, but notably also D-(-)lactate and DPNH, are capable of stimulating a high steady state level of proline uptake. For succinate at 20 mM this is of the order of 40- to 50-fold over the endogenous activity.

Thus, in some way the transport machinery for proline uptake is connected to the oxidation of these substrates.

Succinate was stoichiometrically converted to fumarate by membrane vesicles, presumably via the membrane-bound flavoprotein, succinate-dehydrogenase. The fact that malonate caused a succinate-reversible competitive inhibition of proline uptake makes the involvement of succinate dehydrogenase highly probable and underlines the coupled relationship between transport and the conversion of succinate to fumarate.

This coupling is dissociated by the action of purified colicin E_1, which partially inhibits proline uptake, but does not affect the conversion of succinate to fumarate.

The inhibition of proline uptake by colicin is not as complete as the inhibition obtained with uncouplers or inhibitors of electron transport in the membrane system. Both uncouplers and inhibitors produce more than 95% inhibition; crude colicin only inhibits 70 to

80%, even at the highest colicin concentrations. Although this might be explained by the presence in the preparation of a subclass of membrane vesicles too small to absorb colicin but contributing to the proline uptake, it is not known whether this is actually the case. The broad size distribution of vesicles in a sucrose velocity gradient (unreported experiments) suggests this as a possibility.

Pure colicin E_1 inhibits proline uptake to a lesser extent (about 50%) than crude colicin at comparable killing titers. This observation is unexplained. It could be due to aggregation of the purified colicin but no real evidence exists to warrant speculation. There are no known cofactors required for colicin E_1 absorption.

Colicin-resistant whole cells absorb colicin much less well than sensitive strains, if at all. Membranes made from resistant strains are somewhat sensitive to inhibition of proline uptake but require much higher concentrations of colicin than membranes from sensitive cells. In the case of W6/E_1 the difference corresponds to approximately a factor of 10 in colicin concentration. The colicin K-resistant membranes used in this work are even less sensitive to colicin K than W6/E_1 membranes are to E_1.

Membrane vesicles made from Tol II and Tol III strains both take up proline in response to succinate, although with lower activity than wild-type vesicles. Tol II vesicles are completely insensitive to purified colicin E_1 inhibition, even at very high concentrations of E_1. Tol III vesicles are sensitive to E_1, as are the whole cells, but are not as sensitive as wild-type vesicles, for reasons which are not understood.

Membrane vesicles from immune cells, on the basis of a single preparation, appear to be sensitive to homologous colicin inhibition.

Bhattacharyya et al. (1970) reported experiments on the effects of crude colicins E_1 and K on proline uptake in membrane vesicles. Using glucose-stimulated proline uptake in lysozyme vesicles of E. coli K-12, they showed that uptake can be inhibited in wild-type membranes by preincubation with colicin, and that the level of inhibition depends on the colicin concentration. They reported that addition of crude colicin E_1 after the membranes have reached a high steady state concentration of proline caused release of the proline in the case of vesicles from wild-type and resistant strains, but had no effect on the level maintained by membranes from a Tol VIII strain. They conclude that the receptor sites for the colicin are on the cell membrane and that resistance in whole cells is due to a change in the cell wall which blocks access to the membrane receptor. This would explain why the membranes from resistant cells are sensitive to colicin. However, their determination of sensitivity of membranes from resistant cells was made on the basis of one colicin concentration only producing a loss of label from membranes in the steady state of proline uptake. This method permits the detection of qualitative sensitivity to the colicin, but does not measure relative sensitivities and therefore fails to reveal the fact that resistant membranes are still considerably less sensitive to colicin inhibition than wild type membranes. The total picture suggests that resistance to colicins E_1 and K is more complicated than it appears in the model of Bhattacharyya et al. Until the topology and the composition of the membrane vesicles, and of the cell envelope in general, are better known it is likely that the details of

the mechanism of colicin resistance will continue to be elusive. This is especially true in the light of evidence on the location of certain proteins affected by the tol mutations (see Section III below).

The finding that Tol VIII vesicles are insensitive to colicin E_1 supplements my finding of a similar insensitivity in Tol II vesicles.

II. Comparison of Colicin Action with that of Uncouplers and Inhibitors. The Interrelation between Substrate Respiration, Energy Coupling for Transport, and ATP.

Although purified E_1 partially inhibits proline uptake in membrane vesicles, it does not inhibit the conversion of succinate to fumarate. Nor do uncouplers such as CCCP or DNP or inhibitors such as KCN or NaN_3 have a significant inhibitory effect on this reaction, although they completely abolish proline uptake.

Differences in the action of colicin, uncouplers and inhibitors did appear when the overall oxidase activity of the membranes was studied polarographically. Neither colicin nor uncouplers affect the rate of substrate-dependent consumption of oxygen, whereas inhibitors of electron transport reduce the rate, without, however, abolishing it. The residual rate in the presence of inhibitors is probably due to an oxidase shunt to oxygen bypassing the cytochromes, which are undoubtedly inhibited by 1 mM concentrations of KCN, NaN_3, and HOQNO. This could explain why the inhibitors do not block the conversion of succinate to fumarate.

Uncouplers have a dramatic effect on the rate of respiration of starved whole cells, increasing both endogenous and substrate-stimulated oxygen consumption 2- to 5-fold. This is evidence for the existence of

"respiratory control" in the whole cells, a state well characterized in mitochondria, and recently shown to exist in bacteria (John and Hamilton, 1970). In this state, the rate of electron transport to oxygen is not maximal, but is controlled by the rate of ATP synthesis. Addition of uncoupler releases the respiratory chain from this control, presumably by hydrolyzing an intermediate on the pathway to ATP.

The respiratory control experiments bring out a clear difference between uncoupler action and colicin action. Even under conditions where survival due to colicin treatment is very low (less than 10^{-3}) no increase in the rate of respiration is observed in starved whole cells.

The fact that uncouplers do not produce an increase in the rate of oxygen consumption by membrane vesicles indicates that the membranes are in an uncoupled state due to the method of preparation. Clearly their state is different from that of whole cells since it is reported that they do not perform oxidative phosphorylation (Klein et al., 1970). Nevertheless, they must be "coupled" to some extent since they pump proline and perform at least a low rate of transhydrogenase reaction in response to substrate-driven respiration.

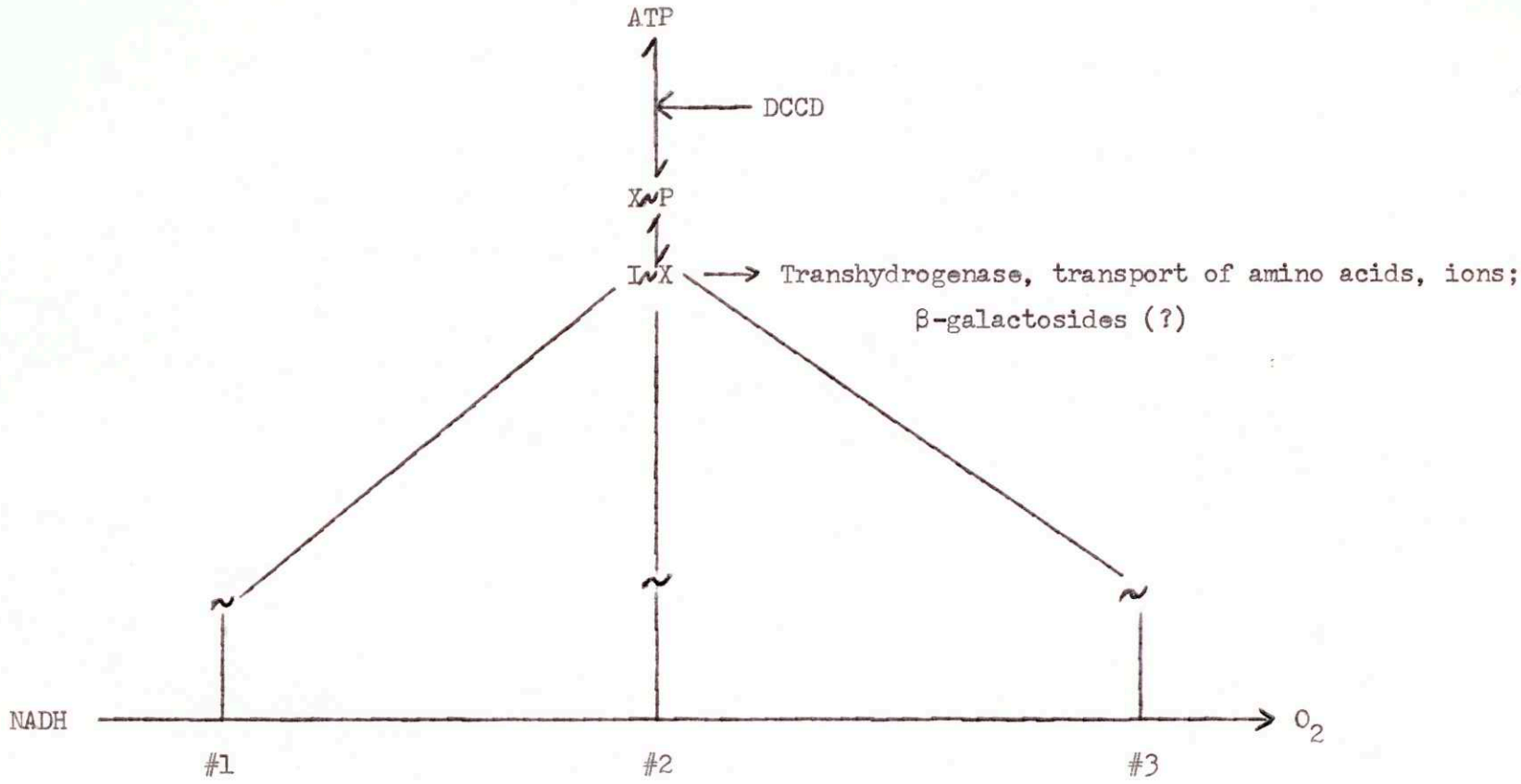
The use of the energy-linked transhydrogenase activity to look at both the glycolytic and the respiratory pathways by which membrane vesicles can mobilize energy can provide a unifying picture of the interrelation between substrate respiration, energy coupling and ATP and suggest a level at which colicin action might be exerted.

As discussed in the Results section, the transhydrogenase studies support the idea that a non-phosphorylated high energy intermediate between the respiratory chain and ATP can directly drive energy-consuming processes in the bacterial membrane. This intermediate, designated

$I_{\sim}X$ in Figure 14, can be generated either from the coupling sites on the electron transport chain in response to substrate-driven respiration, or anaerobically from ATP. In mitochondria, according to this model, addition of ADP stimulates the synthesis of ATP from $I_{\sim}X$ and the increased turnover of $I_{\sim}X$ accounts for the observed increase in the rate of respiration. Similarly, addition of valinomycin permits potassium accumulation, which drains off $I_{\sim}X$ and again causes an increase in the rate of respiration. Uncouplers behave as if they promote the hydrolysis of $I_{\sim}X$ (possibly by abolishing a H^+ gradient across the lipid by layer boundary; Hopfer et al., 1968; Pavlasova and Harold, 1969), thereby preventing ATP synthesis and releasing respiratory control. Inhibitors, of course, work at the level of the electron transport chain to prevent the flow of electrons down the potential gradient, thereby preventing the generation of aerobic $I_{\sim}X$.

In view of the evidence that synthesis of ATP does not occur in these membrane vesicles, we might postulate that $I_{\sim}X$ drives the proline pump, and that it is generated by the oxidation of succinate, D-(-)lactate, or DPNH. Presumably the proline pump could also be driven by ATP-generated $I_{\sim}X$. To date, such an activity has not been demonstrated, although this may be due to technical reasons. Clearly W6 pumps proline during anaerobic growth, and this is presumably accomplished via glycolytically generated energy. ATP-driven proline uptake measured either on membrane vesicles or, more conveniently, on anaerobic whole cells as a KCN-resistant uptake activity, should provide interesting information. Preliminary experiments on such an activity in anaerobic cells have shown it to be

Figure 14. Schematic representation of pathways to high energy intermediate.


ATP
DCCD
X~P
L~X
Transhydrogenase, transport of amino acids, ions;
β-galactosides (?)
NADH
O_2
#1
#2
#3

resistant to colicin inhibition at high colicin multiplicity (Fisher and Kabat, unpublished experiments).

Although the activities of the transhydrogenase reactions are low in membrane vesicles, both the aerobic and the anaerobic reactions are important in delimiting the mechanism of action of the colicin. It appears that the respiratory transhydrogenase activity is inhibited by purified colicin to about the same extent that succinate-driven proline uptake is inhibited in the same membranes. Colicin seems to have no inhibitory effect on the ATP-driven reaction, although confirmation of this is needed.

The current characterization of colicins E_1 and K that they exert their primary effect on oxidative phosphorylation does not specify at what level this could be occurring. This thesis suggests that succinate oxidation via succinate dehydrogenase and the electron transport chain can generate energy to pump proline in isolated membrane vesicles even though the vesicles supposedly make no ATP, and that colicin effectively prevents this energy mobilization for active transport, and other aerobic energy consuming processes in the membrane, at the level of non-phosphorylated high energy intermediate.

Feingold has already demonstrated that colicin can inhibit in the presence of high intracellular ATP levels maintained by treating the cells with DCCD. This means that the drop in ATP level caused by E_1 is secondary to the initial action of E_1 and is probably associated with the replenishing of ATP from I~X and the exhaustion of ATP in cellular reactions. Uncouplers are known to cause a similar drop in the ATP pool, presumably by promoting the hydrolysis of I~X and consequently releasing respiratory control. Since E_1 does not effect this increase of respiration rate in starved whole cells, it is unlikely

that it acts to promote the hydrolysis of I~X. We also know that it is not acting like an electron transport inhibitor.

These considerations are consistent with the view that colicin inhibits either the formation or the utilization of aerobically generated non-phosphorylated high energy intermediate, I~X. This intermediate is presumably necessary in the cell for the retention of potassium, for proline transport, and for energy-linked transhydrogenase.

If we assume that colicin affects the utilization of I~X, and if the colicin inhibits the respiratory but not the ATP-driven transhydrogenase, as the data suggest, this would mean that I~X generated from ATP is insensitive to colicin action, but when generated aerobically, is sensitive and cannot drive energy-consuming reactions. To explain this, one might invoke the existence of two high energy intermediates, functionally equivalent, except that the respiratory one is sensitive to colicin and the anaerobic one is not. Since no hydrolysis of high energy intermediate is involved, we would not expect the release of respiratory control seen with uncouplers.

The alternative possibility, that the colicin blocks the formation of I~X or a precursor of it from the respiratory side, perhaps by inhibition at the coupling sites, avoids postulating two high energy intermediates. Again, we would not expect respiratory release if no synthesis of I~X was occurring. I~X generated from ATP would be normal and would drive energy-consuming reactions.

The insensitivity of strictly anaerobic cells to colicin supports these possibilities, since in both cases the glycolytic pathway is uninhibited. Anaerobic cells are sensitive to uncouplers, which can still hydrolyze I~X, (Pavlasova and Harold, 1969).

Many observations remain unexplained by this scheme. The ATP level in colicin-inhibited cells drops only to 25 to 30% of normal (Fields and Luria, 1969a), yet this residual level does not allow accumulation or biosynthesis to occur, although by the above scheme, some high energy intermediate generated from this ATP should be available to drive energy-consuming reactions, and the ATP itself might still be used for synthesis. Perhaps the loss of essential pools due to the energy drain after colicin treatment prevents the residual ATP from being used.

A related problem is the sensitivity of hemin-less strains of E. coli to colicin inhibition, even though they lack a cytochrome system. We know that only very stringent anaerobic conditions will protect wild-type E. coli from colicin inhibition. Perhaps hemin-less strains possess the site one coupling system (which does not involve cytochrome) if grown under less than strictly anaerobic conditions, and the presence of even a minor component of this "respiratory state," perhaps associated with a particular membrane conformation, may be sufficient to allow the colicin to inhibit, in spite of the fact that the major energy mobilization is occurring through glycolytic pathways.

The entire question of the role of oxygen remains unresolved. It is not even clear that the oxygen requirement is related to terminal electron transport at all, and Feingold has proposed that it might be related to an oxidase activity associated with the event of colicin binding which initiates a transmittable change through the membrane structure (Feingold, 1971).

III. Identification and Location of the Tol II Protein

The discovery of one or more missing proteins in the outer membrane of a Tol II amber mutant provides an experimental handle for analyzing a component of the membrane which is part of the colicin transmission system and is also necessary for maintaining the structural properties of the membrane. Its synthesis and incorporation into the membrane can now be explored. The fact that wild-type membrane vesicles are sensitive to colicin implies that the method of preparation does not strip the outer membrane from the vesicles or even greatly disrupt its relationship to the inner membrane. It also suggests that, if the missing protein is really related to tolerance, the outer layer of the envelope must be intimately involved in the transmission of the colicin stimulus. Localization in the outer membrane implies that this protein is close to the colicin receptor site. Since the Tol II mutation involves tolerance to colicins K and A as well as to the E class, this protein must be accessible from the different classes of receptor. Since it appears to be a principal component of the outer membrane, it might be expected to exist under different receptor loci. Analysis of non-amber Tol II membranes will tell whether the Tol II protein is generally missing from the membrane or whether it is present in an inactive form.

Although this study could detect no significant differences in the Tol VIII amber membrane, further work on this and on Tol III membranes might uncover further membrane components involved at various levels in the colicin transmission systems. The findings that Tol II mutants of male strains of E. coli do not support the growth of filamentous male phages (J. Brown, unpublished results) and that

f1-infected cells become tolerant to colicin (H. Smilowitz, unpublished results) add an intriguing dimension to this story.

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BIOGRAPHICAL SKETCH

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Publications:

Kabat, J. P., and Luria, S. E. Bacteriol. Proc., 1970, p. 62.