Effects of Colicins E1 and K on Cellular Metabolism

KAY L. FIELDS[1] AND S. E. LURIA

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Colicins E1 and K inhibited a whole series of energy-dependent reactions in *Escherichia coli* cells, including motility, biosynthesis of nucleic acids, proteins and polysaccharides, and the conversion of ornithine to citrulline. Respiration was only partially affected, and substrates such as glucose continued to be catabolized through the normal pathways, albeit with reduced CO_2 production. The soluble products of aerobic glucose catabolism by colicin-treated cells were analyzed. Pyruvate replaced acetate as the major excreted product, and the following intermediates of glycolysis were excreted in significant amounts: glucose-6-phosphate, fructose-1,6-diphosphate, dihydroxyacetone phosphate, and 3-phosphoglycerate. Anaerobically growing cells manifested a somewhat enhanced tolerance to the colicins. This protection by anaerobiosis appeared to depend on the exclusion of oxygen more than on the extent of fermentative catabolism versus catabolism of the respiratory type. These results are interpreted in terms of possible functions of colicin in lowering the adenosine triphosphate (ATP) content of the cells and in terms of the role of lowered ATP levels in inhibiting many of the energy-requiring reactions.

In the preceding paper (16), which described studies of the effects of colicins E1 and K on active transport systems in *Escherichia coli*, it was concluded that the inhibition of the accumulation of thiomethyl galactoside (TMG) and certain other substrates (27, 31) was due to the effect of these colicins on energy metabolism, reflected by the sharp reduction in adenosine triphosphate (ATP) levels. The fact that accumulation of α-methyl-D-glucoside (αMG) was only slightly affected by colicins or by NaN_3 was attributed to the fact that αMG accumulation is carried out by a phosphoenolpyruvate-dependent phosphotransferase system (26), which was presumably less affected by colicin treatment. While colicin causes ATP levels to fall, oxygen consumption continues (21). Levinthal and Levinthal (*unpublished data*, cited in 27) made the significant discovery that under conditions of strict anaerobiosis colicin E1 did not inhibit biosynthetic reactions in cells of *E. coli* K-12 strain C600. This suggested a selective inhibition of oxidative phosphorylation.

The findings with transport systems led us to study further certain aspects of catabolism and of some other energy-requiring cellular processes in colicin-treated *E. coli* cells. The results, presented in this paper, reveal some novel features of colicin action and suggest possible mechanisms of this action.

[1] Present address: Université de Génève, Institut de Biologie Moléculaire, Geneva, Switzerland.

MATERIALS AND METHODS

Bacteria. The bacterial strains used are listed in Table 1.

Media. Media and several procedures used were described in the preceding paper (16). The minimal phosphate medium of Kornberg et al. (24) was used for growth of cells with acetate as sole carbon source.

For experiments at a low *p*H, medium 63 was adjusted to *p*H 6.2 and supplemented with 2×10^{-6} M $FeSO_4$.

For anaerobic growth, cells were grown in 50-ml tubes fitted with bubbling tubes. Nitrogen, nitrogen-5% CO_2, argon, or argon-5% CO_2 gas was used for vigorous bubbling.

Incorporation of radioactive substrates in acid-insoluble form. Uptake of ^{14}C-labeled leucine, isoleucine, uracil, thymidine, or glucose by cell suspensions was measured by placing samples in 5% trichloroacetic acid at 0 C. After 20 min, the acid-insoluble material was collected on cold filters (Millipore Corp., Bedford, Mass.), washed with cold acid, dried, and counted in a gas-flow counter.

Incorporation of ^{14}C-glucose into glycogen-like polymers was measured according to the method of Abraham and Hassid (1) by resuspending cells in 30% KOH, boiling for 20 min, and precipitating with ethyl alcohol. The precipitate was collected on Whatman filters, dried, and counted. Total ^{14}C-glucose uptake was measured by filtering a chilled sample and washing it with phosphate buffer containing cold glucose (20 μg/ml).

Incorporation of ^{14}C-acetate was measured by adding cold 2.5% acetic acid, filtering the preparations, and washing them with 5% acetic acid.

Respiration and gas production. Oxygen uptake and

CO_2 evolution were measured in a Warburg-type respirometer with standard manometric techniques. Unless otherwise stated, cells were harvested during exponential growth, washed, and concentrated to an optical density (OD) at 500 nm of 1.0 to 2.0. The rate of oxygen uptake is expressed as microliters of O_2 per minute per OD unit (500 nm) of cells. (For *E. coli* K-12 cells, one OD unit corresponds to 5×10^8 cells, or 0.1 mg of protein per ml.)

Anaerobic conditions in Warburg vessels were established by flushing with a stream of nitrogen or argon for 10 min. Hydrogen production was measured in flasks containing KOH, and combined CO_2 and H_2 production was calculated by assuming that equal amounts of the two gases were made.

Analytical methods. Glucose was determined with Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.), buffered when necessary with medium 63.

Citrulline was determined on the supernatant fractions of centrifuged cultures by the method of Archibald (4), and pyruvate was measured by the method of Friedemann and Haugen (18).

The enzymatic assays used were those given by Bergmeyer (10) with minor modifications. The enzymes were the best grade available and were obtained as ammonium sulfate suspensions from C. F. Boehringer and Soehne, Mannheim, Germany. The samples were the supernatant fractions of centrifuged cell suspensions. In all assays, the final volume was 1.0 ml, and oxidation or reduction of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) was measured at room temperature in a Zeiss spectrophotometer. Enzyme concentrations are given in international units (1 unit = 1 μmole of product per min at 25 C).

Pyruvate was measured by the oxidation of reduced NAD (NADH) mediated by lactic dehydrogenase. Each assay contained: tris(hydroxymethyl)amino-methane (Tris), pH 7.5, 30 μmoles; NADH, 0.05 μmole; and lactic dehydrogenase, 0.2 unit.

Pyruvate, phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate were determined in a single assay which coupled each compound in succession to the oxidation of NADH by lactic dehydrogenase. Each assay contained, in micromoles: triethanol-amine-ethylenediaminetetraacetate (EDTA) buffer (pH 7.6), 50; $MgSO_4$, 10; KCl, 70; NADH, 0.1; and adenosine diphosphate (ADP), 0.5. Pyruvate was determined first by adding 2 units of lactic dehydrogenase; phospholnolpyruvate was assayed by the further addition of 0.5 unit of pyruvate kinase; 2-phosphoenolpyruvate, by the addition of 0.4 unit of enolase (Sigma Chemical Co., St. Louis, Mo.); and 3-phosphoglycerate, by the addition of 0.1 unit of phosphoglycerate mutase (Sigma Chemical Co.) and about 0.2 μmole of 2,3-diphosphoglycerate.

1,3-Diphosphoglycerate and 3-phosphoglycerate were determined by the oxidation of NADH mediated by 3-phosphoglycerate kinase and glyceraldehyde-phosphate dehydrogenase. Each assay contained, in micromoles: triethanolamine-EDTA buffer (pH 7.6), 50; $MgSO_4$, 8; glutathione, 2.5; hydrazine, 3; ATP, 7.5; and NADH, 0.05. 1,3-Diphosphoglycerate was determined first by adding 0.4 unit of glyceraldehyde-phosphate dehydrogenase; 3-phosphoglycerate was determined by the subsequent addition of 0.8 unit of 3-phosphoglycerate kinase. This assay contained no activity for pyruvate or dihydroxyacetone-phosphate.

Dihydroxyacetone-phosphate, glyceraldehyde-phosphate, and fructose-diphosphate were determined by the oxidation of NADH mediated by glycerol-1-phosphate dehydrogenase. Each assay contained, in micromoles: Tris buffer (pH 7.5), 30; and NADH, 0.05. Dihydroxyacetone-phosphate was determined by the addition of 0.1 unit of α-glycerophosphate dehydrogenase; glyceraldehyde-phosphate, by the subsequent addition of 0.4 unit of triose-phosphate iso-

TABLE 1. *Bacterial strains*

Luria stock collection no.	Strain	Relevant genotype[a]	Source
	E. coli K-12		
L-A279a	3000	Prototroph	J. Monod
L-104	C600	*thi thr leu*	
—	C600 *hmn*	*thi thr leu hmn*	M. Beljanski
L-A452	U160	(highly motile)	L. Fischer-Fantuzzi
L-A632	AB1302B	*argG argK* (derepressed)	G. Jacoby
L-A630	HP6	*glpA*	T. H. Wilson
L-A631	D7002	*aceA32*	J. Beckwith
L-A633	Y20(Col E1)	*thi thr leu* (Col E1-K30)	F. Levinthal
L-620	*E. coli* W	Prototroph	
L-622	*E. coli* W22-64	*cts*	C. A. Hirsch
L-621	*E. coli* PDC⁻	*aceD*	L. P. Hager
L-89	*E. coli* B		
—	*E. coli* B *hmn*	*hmn*	M. Beljanski
L-28	*E. coli* K235	Prototroph (Col K)	P. Frédéricq

[a] Symbols: *ace* = acetate requirement (pyruvic dehydrogenase defect); *arg* = arginine; *cts* = citrate synthetase; *glp* = glucose-6-phosphate permease; *hmn* = hemin; *leu* = leucine; *thi* = thiamine; *thr* = threonine.

merase; and fructose-diphosphate, by the addition of 0.1 unit of aldolase.

Glucose-6-phosphate and fructose-6-phosphate were determined by the reduction of NADP mediated by glucose-6-phosphate dehydrogenase. Each assay contained, in micromoles: Tris (pH 7.5), 30; $MgCl_2$, 5; and NADP, 0.1. Glucose-6-phosphate was determined by addition of 0.5 unit of glucose-6-phosphate dehydrogenase (Sigma Chemical Co., type V); fructose-6-phosphate by subsequent addition of 0.4 unit of yeast phosphoglucose isomerase (Calbiochem, Los Angeles, Calif.).

6-Phosphogluconate was determined by the reduction of NADP mediated by phosphogluconate dehydrogenase. Each assay contained: triethanolamine (pH 7.6), 370 μmoles; $MgSO_4$, 5 μmoles; NADP, 0.2 μmole; and 0.5 unit of 6-phosphogluconate dehydrogenase. The assay was free from activity of glucose-6-phosphate.

Analysis of glucose degradation products. Washed bacterial suspensions (about 5×10^8 glucose-grown cells/ml), either colicin-treated or controls, were placed in Warburg vessels with KOH papers in the center well. ^{14}C-glucose (10^{-3} M, 3.3 μc/ml, uniformly labeled, unless otherwise noted) was added from the side compartment, and incubation was continued until O_2 consumption showed a sharp break. The KOH paper, which trapped the evolved CO_2, was counted in 3 ml of ethyl alcohol and 6 ml of toluene scintillation fluid (12).

Some samples of the cell suspensions were used to determine incorporation into acid-insoluble products, and other samples were prepared for column chromatography as described by Dobrogosz (15). The sus-suspensions were added to a carrier mixture (final content: ethyl alcohol, acetate, pyruvate, formate, lactate, and succinate, each 0.05 M, with enough H_2SO_4 to give a pH of 1.7). After centrifugation, the supernatant fluid, containing all acid-soluble products, was kept at -20 C.

Partition chromatography was carried out with silicic acid columns prepared according to Ramsey (33). Silicic acid was separated from fine particles and dried overnight at 100 C. The exact proportion of solvent to silicic acid had to be determined for each batch. The column was prepared in a tube (12-mm diameter) with a pierced sintered-glass disc and layers of glass wool, sand, and Celite Super-Cel. Silicic acid (6 g) was mixed with as much 0.5 N H_2SO_4 as could be added without loss of its powdery consistency (about 3.2 ml), and the mixture was added to the column tube filled with benzene. Each column was 15 cm long and was used only once.

The acidified, carrier-containing supernatant sample (0.5 ml) was mixed with 1.5 g of silicic acid, and this powder was added to the top of the prepared column. The column was developed with a series of solvents similar to that used by Dobrogosz (15). Acid-washed chloroform (150 ml) was followed by a linear gradient of chloroform (300 ml in the mixing chamber) and 5% t-butyl alcohol in chloroform (300 ml; 4% t-butyl alcohol in chloroform in the second chamber was used for clean separation of pyruvate and formate). In the first experiments, lactate was eluted near the end of the

gradient, and remaining lactate was eluted with 50 ml of 5% t-butyl alcohol in chloroform. If the gradient was discontinued as soon as formate had been eluted, then all the lactate could immediately be eluted with 5% t-butyl alcohol in chloroform; 50 to 100 ml of 10% t-butyl alcohol in chloroform was then used to elute succinate. Finally, 50 ml of 30% t-butyl alcohol in chloroform was passed through, followed by distilled water. The chloroform-based solvents were used at a rate of 2 ml/min. Water flow was very slow and added pressure was necessary.

Fractions (8 to 10 ml) were collected, and the amount of acid in each fraction was determined by titrating a 4-ml sample under nitrogen with ethanolic KOH (33). This titration established the peaks of carrier acids. The distribution of radioactivity was determined by scintillation counting according to Dobrogosz (15) in a Nuclear-Chicago counter, model 724, with corrections applied for chemical quenching.

Paper chromatography and electrophoresis. Materials that were eluted from the silicic acid columns with water were partially characterized by paper chromatography. Radioactive compounds were detected by using a Nuclear-Chicago strip counter; standard methods were used for detection of reference compounds (11). The sensitivity was insufficient to detect minor radioactive components. To compare the eluted material with known compounds and to test whether the chromatographic properties changed upon incubation with alkaline phosphatase (type III, E. coli; Sigma Chemical Co.), the following solvent systems were used: ethyl methyl ketone-methylcellosolve-3 M NH_3, 2:7:3 (29); methanol-NH_3-water, 60:10:30 (8); and butyric acid-0.85% NaOH in water, 69:31, v/v (35).

Radioactive samples from the silicic acid columns were subjected to paper electrophoresis at pH 1.8 (4% formic acid) and pH 3.2 (9.2% butyric acid in 0.85% NaOH; 35), and in borate buffer, pH 9.2 (17). The migrations of the radioactive samples were compared (before and after treatment with alkaline phosphatase) with those of known phosphate esters.

RESULTS

Effects of colicins on biosynthetic reactions. It is known that colicins E1 and K halt biosynthesis of protein and ribonucleic acid (RNA) very quickly, as illustrated in Fig. 1. The findings were similar for a variety of colicin-sensitive E. coli strains. Interestingly, colicins K and E1 caused an arrest of protein or RNA synthesis in Shigella dysenteriae Sh only after a lag of 10 to 15 min. Resistant mutants, which do not adsorb a colicin, do not show any of the effects described.

Table 2 illustrates the effects of the colicins on incorporation of ^{14}C from glucose or acetate into E. coli cells. Practically complete inhibition occurred, not only of incorporation into acid-insoluble form but also of incorporation into any not readily washable compounds. Chloram-
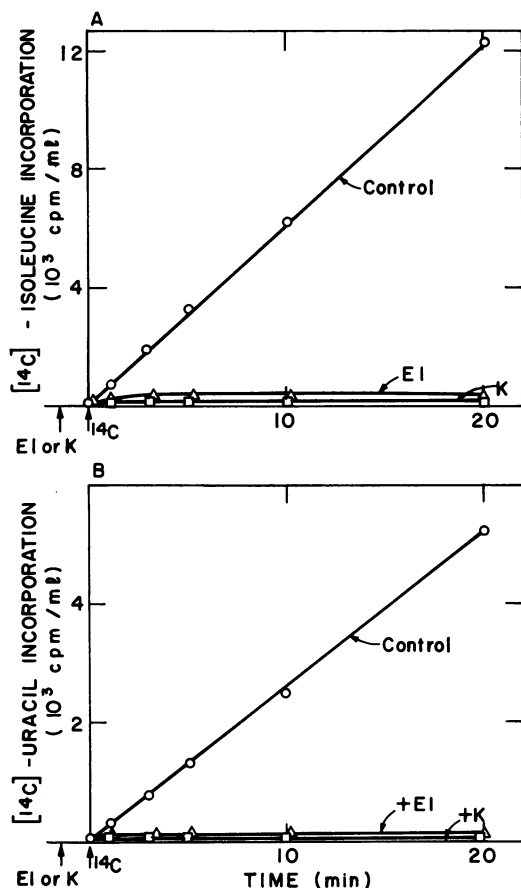
FIG. 1. *Effect of colicin E1 or K on incorporation of isoleucine or uracil. Cells of E. coli 3000 were grown in medium 63 with 0.4% glycerol and treated with colicin or buffer for 3 min; then ^{14}C-labeled isoleucine (A) or uracil (B) was added. Survival at 4 min was 0.3% for colicin E1 and 1% for colicin K.*

phenicol did not antagonize the effect of colicin on incorporation.

Effect of colicin on motility. Since a low level of aerobic metabolism is sufficient to support movement in *E. coli* (2, 3), we tested whether colicin-treated cells would retain their motility.

E. coli K-12 U160 is actively motile when grown aerobically in broth. Colicin E1 was added at room temperature at a multiplicity of 7.5 (determined by survival after complete adsorption), and small hanging drops of control and colicin-treated cells were observed in a phase-contrast microscope. Control cells remained vigorously motile, whereas colicin-treated cells slowed down, and by 6 min after addition of colicin nearly all cells had stopped

moving. Thus, motility is as sensitive to colicin as are other energy-requiring reactions.

Conversion of ornithine to citrulline. Conceivably, the interference of colicins with macromolecular biosyntheses and with motility may not result from their effect on ATP levels, but may be a product of some other effect, possibly related to membrane association. It seemed desirable to test the effect of colicins on an energy-requiring reaction involving soluble enzymes and nonpolymeric substrates. The reaction chosen for testing was the conversion of ornithine to citrulline. This reaction requires carbamyl phosphate, made by carbamyl phosphate synthetase from bicarbonate, ammonia (or glutamine), and ATP.

The strain used was an arginine-requiring mutant AB1302B (22), which lacks argininosuccinate synthetase, the enzyme that converts citrulline and ATP to the argininosuccinate. This strain is also derepressed for the enzymes of arginine biosynthesis, because of a second mutation in the regulator gene for the arginine enzymes. Intact cells of AB1302B, when provided with exogenous ornithine in the absence of arginine, synthesize carbamyl-phosphate, transfer it to ornithine, and excrete the citrulline thus formed into the medium. Thus, the intact cells serve as an in vivo assay system for the synthesis of carbamyl phosphate.

As shown in Table 3, the control cells of AB1302B readily converted ornithine into citrulline. If glucose was omitted, there was little or no production of citrulline; thus, the reaction appears to be dependent upon an exogenous energy supply. Addition of colicin E1 reduced excretion of citrulline below any significant level.

This effect of colicin does not appear to be a by-product of the effects of colicin on protein synthesis since chloramphenicol did not reduce citrulline excretion. It is also unlikely that colicin prevented the formation of citrulline by inhibiting the accumulation of ornithine since the equilibrium of the transcarbamylase reaction greatly favors citrulline formation; hence, as with o-nitrophenyl-β-D-galactoside (ONPG) hydrolysis (27), the reaction should not be subject to interference at the level of accumulation. It is likely, therefore, that this reaction is prevented by colicin through its effect on ATP levels.

Effects of colicins E1 and K on respiration. Jacob et al. (21) and Nomura (30) reported that colicins E1 and K allowed continued respiration. Catabolism of glucose by colicin-treated cells, if it occurs through the normal *E. coli* pathway, requires at least one ATP-linked phosphorylation catalyzed by phosphofructokinase. [The first

TABLE 2. *Effect of colicins on incorporation of glucose or acetate[a]*

Expt	Organism	Labeling compound	Type of cell material collected	Colicin	Incorporation (counts per min per sample)
1	3000[b]	^{14}C-glucose	Trichloroacetic acid-precipitable	—	65,000
				+E1	335
				+K	305
2	3000[c]	^{14}C-glucose	Total	—	2,080
				+E1	−80
			Glycogen	—	1,204
				+E1	−40
3	W[d]	^{14}C-acetate	Acetic acid-precipitable	—	17,500
				+CM	15,900
				+E1	95
				+E1 + CM	120

[a] Samples were treated with colicin or buffer for 5 to 7 min, the ^{14}C-labeled carbon source was added, and incorporation was measured 30 min later. Survival after colicin treatment was 0.1 to 1%. Sample volume: experiment 1, 0.05 ml; experiment 2, 0.5 ml (total cell material) or 5 ml (glycogen); experiment 3, 0.5 ml.

[b] Cells grown in 63-glucose, washed, resuspended in medium 63.

[c] Cells grown in 63-glucose, washed, and resuspended in phosphate buffer + 10^{-3} M MgSO$_4$.

[d] Cells grown in acetate medium, resuspended in buffer, and starved for 60 min. CM = chloramphenicol (100 µg/ml).

phosphorylation of glucose-6-phosphate can probably be carried out by a phosphoenol-pyruvate-linked phosphotransferase (34)]. If the phosphofructokinase reaction continued, the colicin-treated cells would be an example of an ATP-requiring function not inhibited by these colicins.

A typical experiment is shown in Fig. 2. Under growth conditions with glucose as carbon source, colicin E1 had no effect on O$_2$ consumption. When growth was prevented by removal of required amino acids, respiration by control cells was depressed, but colicin actually stimulated respiration. In the absence of growth, glucose catabolism may be slowed down by excess ATP, and colicin may restore a faster rate by reducing ATP levels.

The effect of colicin E1 on respiration was studied on several *E. coli* strains with a variety of substrates and with suspensions of cells grown on different substrates. The significant results of these experiments can be summarized as follows. Respiration rate is least affected by colicin when glucose is the substrate; the total amount of O$_2$ taken up per mole of glucose, however, is always reduced by colicin treatment. The rate of CO$_2$ evolution is more strongly affected than the rate of O$_2$ consumption. Respiration with galactose, arabinose, glycerol, or α-glycerol-phosphate is partially inhibited; respiration with succinate is more strongly affected.

TABLE 3. *Inhibition of conversion of ornithine to citrulline by colicin E1[a]*

Additions	Citrulline production
	µg/ml
0.4% glucose	20
0.4% glucose + chloramphenicol	20
0.4% glucose + colicin E1	4
0.4% glucose + colicin E1 + chloramphenicol	3
None	2

[a] Cells of strain AB1302B growing in medium 63 (with glucose, threonine, leucine, and arginine) were harvested, resuspended in medium without arginine for 15 min, and then again collected and resuspended in medium 63 with threonine, leucine, and 20 µg of L-ornithine per ml. Glucose (0.4%), colicin E1 (survival 0.2%), and chloramphenicol (50 µg/ml) were added in various combinations. After 60 min, citrulline was assayed in the supernatant fluid of centrifuged samples.

The only substrate for which colicin E1 completely abolished respiration was acetate; the rate of O$_2$ consumption by suspensions of growing bacteria with acetate as substrate was reduced to that of colicin-treated cells without substrate. (In some experiments, in which very concentrated suspensions of starved cells were treated with colicin E1, O$_2$ consumption in the
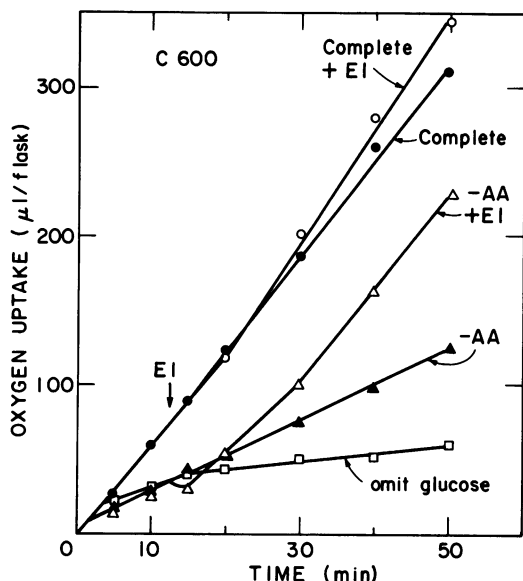
FIG. 2. *Effect of colicin E1 on oxygen uptake by E. coli C600 with glucose. Cells of E. coli C600 grown in medium 63 with glucose, threonine, and leucine were collected, resuspended in medium without amino acids, and shaken for 30 min at 37 C. Then samples of the suspension were resuspended in fully supplemented medium (complete), or without glucose, or without amino acids (−AA), and placed in Warburg flasks for O_2 uptake measurements. Colicin E1 or buffer was added from the side arm at 12 min. The survival of colicin-treated cells was about 0.1% (measured at 50 min).*

presence of acetate continued for about 20 min before inhibition set in. Incorporation of uracil or of counts from labeled acetate into acid-insoluble materials stopped as promptly as with lighter cell suspensions. The reason for the delayed onset of inhibition of respiration remains unclarified.)

Taken as a whole, the results of the respiration studies indicate that catabolism of many carbon sources continues after colicin E1 treatment. Colicin K, whenever tested, gave comparable results. The lower degree of inhibition observed for glucose than for other carbon sources may be due to the fact that glucose activation can be mediated by the phosphoenolpyruvate-dependent phosphotransferase, whereas other substrates require ATP for entry or for activation. The very strong inhibition of respiration with acetate by acetate-grown cells may reflect some effect of colicin on the functioning of the Krebs cycle or on the uptake of acetate from the medium.

Products of aerobic glucose catabolism. Even though substantial glucose oxidation by colicin-treated cells does occur, its catabolic fate is

clearly altered. The final levels of O_2 consumption are lower, and the production of CO_2 is also reduced. Data from a typical experiment are shown in Table 4. In this and similar experiments, light suspensions of washed bacteria from growing cultures were allowed to oxidize a limited amount of ^{14}C-glucose in Warburg flasks. O_2 consumption was monitored to determine when the substrate had been used up, and then the suspensions were analyzed for ^{14}C-containing compounds as described in Materials and Methods. Figure 3 illustrates a silicic acid column fractionation of products from labeled glucose obtained with control and E1-treated cells in an experiment similar to that of Table 4.

The overall effects of colicin E1 can be summarized as follows. The uptake of O_2 was reduced by 40 to 50% and CO_2 production was reduced by 70 to 80%. As expected, trichloroacetic acid-precipitable material went from about 40% of the added carbon in the controls to less than 1% in the colicin-treated cells. Production of acetate, the major nonvolatile product of normal cells, was strongly diminished, whereas pyruvate, absent from the supernatant fluid of the control suspensions, became a major product. In normal cells, a small variable amount of the ^{14}C from glucose was found as materials eluted from the silicic acid column by water; this fraction was greatly increased by colicin treatment.

Thus, the alterations of glucose catabolism caused by the colicins are, first, the replacement of CO_2 and acetate by pyruvate as main products and, second, a substantial increase in substances that are not fractionated by silicic acid column chromatography.

To test whether the pyruvate produced by colicin-treated cells was formed from glucose via the glycolytic pathway, as is the case for normal *E. coli* cells (36), a comparison was made between uniformly labeled and C-1 labeled glucose. The distribution of carbon 1 into pyruvate, acetate, and CO_2 was consistent with glycolytic degradation and not with production of pyruvate by either the Entner-Doudoroff pathway or the hexose-phosphate shunt.

Analysis of the water-eluted fraction. Among the soluble substances produced from glucose by colicin-treated cells, the fraction that was eluted with water from the silicic acid column accounted for 45 to 75% of the carbon in different experiments. Paper chromatography showed that this fraction did not consist of simple carbohydrates; neither did it form derivatives characteristic of ketones and aldehydes. In chromatography with alkaline solvents, a distinct peak was observed, whose position was altered by pretreatment with alkaline phosphatase. Likewise, the electro-

TABLE 4. *Effect of colicins E1 and K on glucose degradation by E. coli 3000*[a]

Colicin	Time of incubation with ^{14}C-glucose (min)	Viable cells/ml	Percentage of ^{14}C input					Column separation (percentage of total ^{14}C)					
			$^{14}CO_2$	Trichloroacetic acid-precipitable material	Acid-soluble material	Recovery of ^{14}C input (%)	Unused glucose (%)	Eluted before acetate	Acetate	Pyruvate	Lactate	Eluted with water	Lost on column
Control	30	3.5×10^8	14	33	47	95	4	1	22	ND	ND	17	9
K	30	$<10^4$	3.5	0.1	95	98	5	1.6	6	12.4	<1	77	4
E1	30	$\sim 10^4$	4	0.2	105	109	30	2.5	5.5	22	<1	66	4
E1	60	$\sim 10^4$	4.2	0.4	99	104	0	4	8	27	2.3	56	2

[a] Cells of *E. coli* 3000 growing in medium 63 with glucose were harvested, washed, and resuspended at 5×10^8 cells/ml. After 5 min of treatment with buffer or colicin (survival 0.6%), the suspensions were added to Warburg flasks (2.8 ml/flask). ^{14}C-glucose (0.8 μmoles, 3.3 μc/ml, uniformly labeled) was added and O$_2$ consumption was measured. Samples from three flasks were taken at 30 min, when glucose had not yet been completely used up; a fourth flask, with colicin E1, was sampled at 60 min, after exhaustion of glucose was revealed by a sharp break in respiration rate. The samples were analyzed and the acid-soluble material was fractioned on a silicic acid column as described in Materials and Methods. ND = none detected.
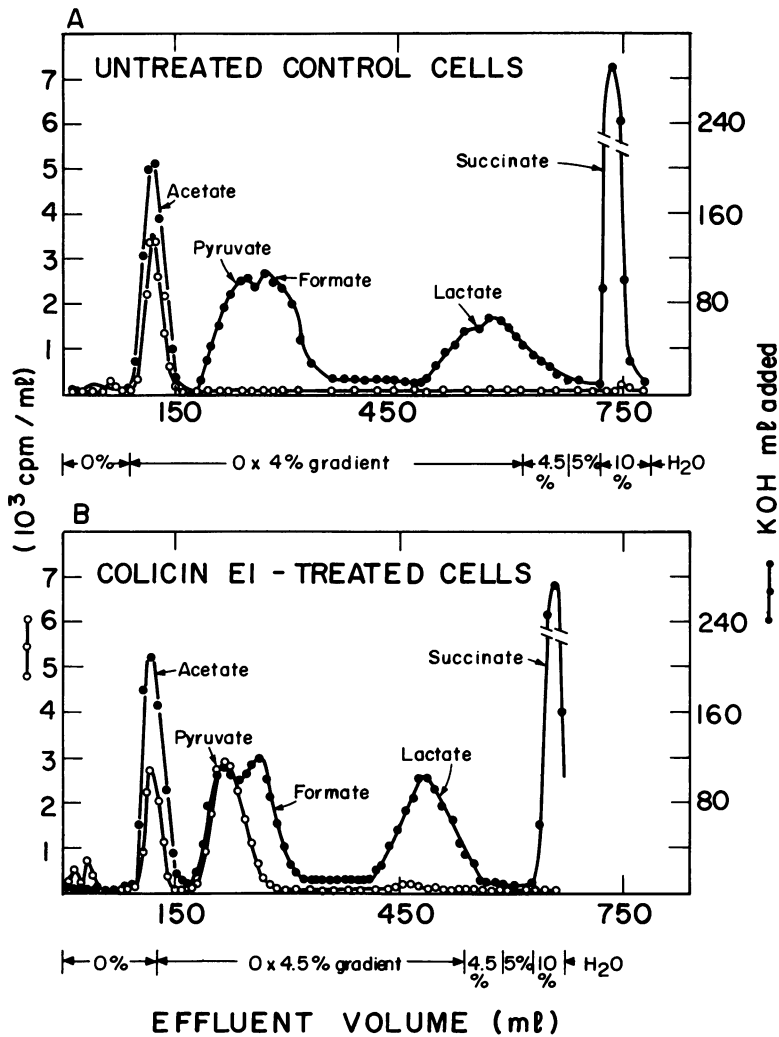
FIG. 3. *Chromatographic separation of soluble products from utilization of ^{14}C-glucose by E. coli 3000. Samples of the supernatant fluids from an experiment similar to the one in Table 4 were fractionated on a silicic acid column as described in Materials and Methods. (A) Control cells; (B) E1-treated cells. Fractions of 10 ml each were collected, counted for radioactivity (○), and titrated with 0.085 N KOH (●) to determine the positions of the various acids added as internal standards. The abscissa gives the effluent volumes. The solvents are indicated below each graph as follows: 0% = chloroform; 0 × 4% = a 300 × 300 ml gradient of chloroform and 4% t-butyl alcohol in chloroform; 4.5%, 5%, 10% = increasing proportions of t-butyl alcohol in chloroform; H₂O = distilled-water wash.*

phoretic mobility of the labeled materials was altered by phosphatase treatment. To identify these materials, enzyme analyses were carried out on one portion of supernatant fluid, and another portion was analyzed by column chromatography. The results (Table 5) confirmed the presence of pyruvate as a major product (one-third of the total carbon) and also revealed that colicin-treated cells, but not normal cells, ex-

creted the following compounds: glucose-6-phosphate, fructose diphosphate, dihydroxyacetone phosphate, and 3-phosphoglycerate. Table 5 lists a series of other compounds which were tested for but were not found.

In view of the limited precision of some of the measurements in this kind of experiment, it is not certain whether the identified compounds represent the full range of labeled products made

TABLE 5. *Products made from glucose by E. coli 3000[a]*

Determination	Control	Colicin E1	
Materials from flask	*calculated percentage of ^{14}C from glucose*		
CO_2	12	7	
Trichloroacetic acid-precipitable	25	1	
Acid-soluble	56	(122)	
Column fractionation of acid-soluble material			
Acetate	23	8	
Pyruvate	<1	29	
Eluted with water	31	~42	
Lost in column	~2	~9	
			percentage of input carbon
Enzymatic assays on supernatant fluid	*μmoles/ml*	*μmoles/ml*	
Pyruvate	≤0.005	0.58	20
Glucose-6-phosphate	≤0.003	0.09	9
Dihydroxyacetone-phosphate	≤0.01	0.14 ± .02	7
Fructose diphosphate	≤0.01	0.16	6
3-Phosphoglycerate	≤0.003	0.3 ± 0.05	15
Not found			
Fructose-6-phosphate	≤0.003	≤0.02	
Gluconate-6-phosphate	NM[b]	≤0.003	
Glyceraldehyde-phosphate	≤0.01	≤0.02	
Phosphoenolpyruvate	≤0.05	≤0.05	
2-Phosphoglycerate	≤0.003	≤0.03	
1,3-Diphosphoglycerate	≤0.003	≤0.003	
Glucose			<2

[a] This experiment was similar to that of Table 4. The suspensions were allowed to utilize ^{14}C-glucose (1 μmole/ml) for 60 min in the presence of chloramphenicol (60 μg/ml). Survival of colicin-treated cells was 0.3%. A sample of the acid-soluble material was analyzed by silicic acid column chromatography. A sample of the suspension, without acid treatment, was centrifuged, and the supernatant fluid was subjected to a series of enzymatic tests.

[b] Not measured.

by the colicin-treated cells. The labeled materials eluted by water in the column fractionation of the control suspension fluid were not identified by the enzymatic analysis.

Once some products made from glucose by colicin-treated cells were identified, it was possible to follow the course of their excretion. All the products listed above started accumulating immediately after addition of glucose, continued to increase until all glucose had been consumed, and did not disappear thereafter. This was true for both colicin E1 and colicin K treatments. Dinitrophenol (2 × 10^{-4} M), whose action on *E. coli* cells resembles that of these colicins in allowing continued respiration while inhibiting biosyntheses and the accumulation of galactosides, did not cause excretion of either pyruvate or any of the glycolytic intermediates.

Tests on *E. coli* K-12 mutants. Because the colicin-treated cells excreted mainly pyruvate instead of acetate, specific effects of colicins on pyruvic dehydrogenase or the Krebs cycle were

suspected. Therefore, the effect of colicins E1 and K on various relevant mutants was tested.

Two mutants, *E. coli* K-12 D7002, with an absolute acetate requirement due to an amber mutation in pyruvic dehydrogenase, and *E. coli* W PDC⁻, lacking pyruvic dehydrogenase, were still fully sensitive to colicins; addition of succinate, glutamate, and acetate did not prevent the blocking of protein synthesis by colicins.

E. coli W 22-64, which lacks citrate synthetase and requires glutamate (19), was fully sensitive to killing by colicin E1, and pyruvate production from glucose was increased by colicin treatment.

Since glucose-6-phosphate is excreted by colicin-treated cells, *E. coli* K-12 HP6, a mutant lacking glucose-6-phosphate permease (37), was tested. When treated with colicin E1, it still excreted glucose-6-phosphate when utilizing glucose, an indication that this excretion is not specifically mediated by the glucose-6-phosphate transport system.

In summary, the experiments on glucose

catabolism by colicin-treated cells revealed that this catabolism did continue, as far as pyruvate, by the normal pathway, but that pyruvate as well as several phosphorylated intermediates were excreted into the medium. Excretion of pyruvate was coupled with reduced production of acetate, the main normal product of aerobic glucose catabolism. The continued production of pyruvate supported the conclusion, from the experiments on αMG accumulation, that production of phosphoenolpyruvate continues in colicin-treated cells.

Effect of colicins on anaerobic catabolism of glucose. As already mentioned, Levinthal and Levinthal found that *E. coli* K-12 strain C600, growing on glucose under strict anaerobiosis, continued to synthesize protein and RNA after treatment with colicin E1; admission of oxygen promptly stopped biosynthesis. This observation first pointed to oxidative phosphorylation as a target for colicin action. In the present work, some preliminary studies of the effects of colicin E1 on anaerobic glucose catabolism and on accumulation of galactosides were done without, however, taking extreme precautions to exclude traces of O_2.

Cells of various *E. coli* strains growing in a glucose minimal medium under N_2 or argon (with or without 5% CO_2) were still sensitive to colicins E1 and K, in the sense that accumulation of TMG and incorporation of amino acids or uracil were always reduced. This reduction was never as complete as under aerobic conditions. It varied from 65 to 90%, being more pronounced in those experiments where anaerobic conditions were less carefully maintained. Yet, glucose metabolism was fermentative rather than respiratory, as shown by analysis of fermentation products (Table 6; see below). Thus, the presence of a predominantly fermentative glucose catabolism was not sufficient to confer full resistance to colicin.

A study of hemin-deficient mutants was illuminating in this respect. These *hmn* mutants (9) require hemin for aerobic growth. When growing without added hemin, they lack cytochromes and catalase and require glucose, amino acids, and partially anaerobic conditions. Even with the *hmn* mutants of *E. coli* C600 or B growing anaerobically, colicins E1 and K were fully bactericidal and inhibited biosynthetic activities even more (75 to 97%) than in the parent strains.

The catabolic fate of glucose used by anaerobically grown bacteria, normal or colicin-treated, was analyzed for *E. coli* C600, C600 *hmn*, and 3000, with the use of washed suspensions re-

turned as rapidly as possible to anaerobic conditions. The rate of glucose disappearance was not significantly altered by colicin treatment. In tests with cells grown at *pH* 6.2, which have an active formic hydrogenlyase, the evolution of H_2 and CO_2, in equal volumes, was the same for control and colicin-treated cells of strain C600. (The *hmn* mutants when grown without hemin produce little or no gas, probably because the formic hydrogenlyase system includes some porphyrin components.)

The products from ^{14}C-labeled glucose were then analyzed by use of cells grown and tested anaerobically at *pH* 7; under these conditions, formate replaces H_2 and CO_2. The normal cells gave the typical products of *E. coli* fermentation: formate, ethyl alcohol, and acetate (in ratios reasonably similar to the theoretical 2:1:1 values); in addition, a substantial amount of lactate and variable quantities of succinate were produced (Table 6). Colicin E1 did not significantly alter the pattern of fermentation, within the limits of reproducibility of the experimental results.

An interesting exception was experiment 3 in Table 6. In this experiment, during fermentation of glucose by C600 *hmn*, anaerobiosis was not well maintained. Here, colicin caused an almost complete disappearance of the products of the clastic reaction and the appearance of pyruvate; lactate production was high and unaffected by colicin.

This finding led us to test the effect of the deliberate introduction of air to fermenting suspensions of anaerobically grown cells (Table 6, experiment 4). In control cells, air caused a shift from clastic products to acetate and CO_2 and to a large increase in lactate. In colicin-treated cells, air caused formation of pyruvate instead of acetate and CO_2, while lactate continued to be made. Other experiments showed that glucose-6-phosphate also appeared among the products from colicin-treated anaerobic cells, but not from control cells.

The significant results of this limited study on anaerobically grown cells appear to be, first, that colicin can cause inhibition in anaerobically grown cells that are carrying out a fermentative, glycolytic type of catabolism; and, second, that in the presence of O_2 the colicin causes even anaerobically grown cells to excrete pyruvate instead of acetate and CO_2, but does not diminish the production of lactate. Hence, since pyruvate is available in colicin-treated cells as a substrate for lactate production, the excretion of substantial amounts of pyruvate cannot be due simply to its leaking out as fast as it is made, but must be attributed to a failure of pyruvate oxidation.

TABLE 6. Effect of colicin E1 on fermentation of glucose by E. coli strains[a]

Expt	Strain	Sample	Percentage of calculated input to flask			Percentage of total counts/min applied to column								
			CO_2	Trichloroacetic acid ppt	Soluble	Ethyl alcohol	Acetate	Formate	Pyruvate	Lactate	Succinate	Water eluate	Not recovered	Unused glucose
1	C600	Control	13	NM[b]	64	34	35	16	—	2	1	NM	12	≤2
		+ E1	2	NM	66	17	37	25	≤2	9	—	NM	10	≤2
2	C600 hmn	Control	2	3	100	28	18	17	≤2	7	10	3	14	≤5
		+ E1	1.4	1.3	86	27	16	25	—	4	9	75	10	≤5
3	C600 hmn	Control	2	NM	100	10	16.5	14	—	54	7.5	NM	3	≤10
		+ E1	0.4	NM	100	1.6	3	—	17.5	65	—	NM	13	≤10
4	3000	Control, −O₂	4	6	90	26	17.5	17	2	9	11	1	16	≤2
		Control, +O₂	15	16	78	2	45	—	1	49	—	11	—	≤2
		+ E1, −O₂	4	1.5	94	12	17	16	2	8	9	15	19	≤2
		+ E1, +O₂	3	0.5	97	5	3	—	37	38	—	18	—	≤2

[a] In these experiments, cells grown anaerobically in a tryptone-phosphate-glucose medium at pH 7.2 were washed, resuspended in deaerated medium 63 without glucose, treated with colicin, and transferred to Warburg flasks. Anaerobiosis was reestablished and ¹⁴C-glucose was added. Incubation was continued for 60 min, and the labeled products were measured as described above for aerobic suspensions. In experiment 4, parallel samples were allowed to consume glucose in aerobiosis and in anaerobiosis.

[b] Not measured.

This effect of colicin is presumably a block, direct or indirect, of pyruvate dehydrogenase.

DISCUSSION

The results of the present study, while not providing definite explanations for the mode of action of colicins E1 and K, suggest some interesting interpretations. First, they show that these colicins, which under aerobiosis lower the ATP levels and block most energy-requiring reactions, allow continuing oxidation of many substrates, including glucose, by pathways which require ATP. Hence, the residual amounts of ATP remaining in colicin-inhibited cells are indeed significant, since they are available and sufficient for at least some reactions.

Two new sets of findings emerge. First, there is the excretion of pyruvate by colicin-treated cells catabolizing glucose, and the corresponding failures to convert pyruvate to acetate and CO_2 and to utilize exogenous acetate (even with cells adapted to grow with acetate as sole carbon source). Second, one observes a selective excretion of certain phosphorylated intermediates of glycolysis by colicin-treated cells, even though there is no general damage to the permeability barrier (16).

These findings should be considered in relation to the main questions to be answered: how do colicins E1 and K cause a reduction in ATP levels, and how does the lowered ATP level lead to a practically complete block of biosynthesis?

To take the second question first, one plausible suggestion comes from the "adenylate control hypothesis" (5, 6), according to which critical regulation of many catabolic enzymes, and also of certain anabolic enzymes, would be exerted by the adenosine monophosphate (AMP)-ATP or ADP-ATP ratios, so that even a small drop of ATP or increase in AMP (or ADP) may bring about strong shifts in enzyme activity by allosteric effects (7). Continued production of fructose diphosphate by colicin-treated cells would not be unexpected, since phosphofructokinase has a low K_m for ATP and is stimulated by ADP (20). On the other hand, glycogen synthesis would be expected to stop because the ADP-glucose pyrophosphorylase of *E. coli* is inhibited by AMP and ADP (32).

Support for this idea comes from the striking resemblance between the effects of colicins on sensitive *E. coli* and the effects of high temperatures on a temperature-sensitive mutant T28 of *E. coli* K-12 described by Kohiyama et al. (23) and by Cousin (13) and Cousin and Belaich (14). A rapid but incomplete decrease in ATP is coupled with a complete arrest of deoxyribo-nucleic acid, RNA, and protein synthesis. Cousin (*personal communication*) has found that the mutant has an altered, temperature-sensitive adenylate kinase. Thus, at the higher temperature AMP would accumulate and ATP levels drop. Colicins may produce the same effect on adenylate compounds through a different mechanism.

Next we must consider the possible mechanisms by which colicins lower ATP levels. It is conceivable that the effect may be simply an inhibition on adenylate kinase or the activation of an adenosine triphosphatase, but it is difficult to reconcile such mechanisms with three sets of data: the dependence of colicin inhibition on the presence of O_2, the selective excretion of certain intermediates of glycolysis, and the failure of conversion of pyruvate to acetate.

The excretion of pyruvate cannot by itself explain the reduction in ATP levels, since oxygen consumption continues and, if oxidative phosphorylation were normal, substantial amounts of ATP could be made from the electrons derived from triose phosphate dehydrogenation. The findings suggest a functional block in pyruvate dehydrogenase, but this is probably not the primary action of colicin since dehydrogenase-less mutants are still inhibited. Neither is the failure to split pyruvate due only to its excretion, because when lactic dehydrogenase is present in the cells it continues to function after colicin treatment. More likely, the nonfunctioning of pyruvate dehydrogenase is an indirect effect of a block in oxidative phosphorylation or of a functional alteration of the cytoplasmic membrane.

In addition to pyruvate, a series of phosphorylated intermediates of glycoslysis—phosphoglycerate, dihydroxyacetone phosphate, glucose-6-phosphate, and fructose diphosphate—are excreted by colicin-treated cells. Such loss of phosphorylated intermediates from *E. coli* in response to treatments that affect energy metabolism had not previously been reported. The cytoplasmic membrane is not freely permeable to phosphorylated compounds, and we know that colicin-treated cells are not generally permeable since ONPG does not leak in, αMG (or its phosphate) does not leak out rapidly, and residual ATP remains inside the cells (16). Furthermore, the loss of phosphorylated intermediates from colicin-treated cells is always partial; more glucose-6-phosphate is metabolized than is lost from the cells.

Two possibilities may be considered: either the phosphorylated intermediates, as well as pyruvate, are excreted from colicin-treated cells

as a result of the lowered levels of cellular ATP, or they are excreted because of an alteration of the cytoplasmic membrane. These two mechanisms may be complementary rather than alternative; low ATP levels may contribute to membrane dysfunction, and excretion of intermediates must contribute to lowering ATP production.

It is doubtful that loss of metabolic intermediates is by itself sufficient to explain the lowered ATP levels in colicin-treated cells. The balance of products made from glucose by colicin-treated cells indicates that, if both oxidative and substrate level phosphorylation were still functional, the production of ATP would be more than sufficient to convert all the glucose to fructose diphosphate. If, on the other hand, oxidative phosphorylation did not take place, there would be barely enough ATP made to produce the mixture of glucose-6-phosphate, fructose diphosphate, and triose-phosphate that is actually observed.

The basic observation pointing to an effect of colicins E1 and K on oxidative phosphorylation was that of Levinthal, who showed that anaerobiosis protected glucose-grown cells against inhibition by colicin E1. Our experiments on active transport of TMG and αMG (16) indicated that the action of colicins E1 and K resembled in some respects those of azide or dinitrophenol and were consistent with a primary effect of colicins on ATP production.

The findings with anaerobic cells reported in the present paper suggest that the protection afforded by strict anaerobiosis against the inhibition by colicins is not due only to independence of oxidative phosphorylation. First, hemin-negative mutants proved extremely sensitive to colicins. Second, protection from colicin treatment required very strict anaerobiosis; when anaerobiosis was not complete colicin became inhibitory, even though the fermentation products indicated that glucose was fermented normally.

Kovac and Kuzela (25) reported that dinitrophenol, azide, and carbonyl-cyanide p-trifluoromethoxyphenylhydrazone were even more potent inhibitors of growth with anaerobic than with aerobic cells of *E. coli* ML. It is possible that the experiments of Kovac and Kuzela were done under conditions of incomplete anaerobiosis, like those in which we found colicins to be effective inhibitors.

An interesting possibility to explain the action of colicins on energy metabolism is suggested by the chemiosmotic theory of Mitchell (28), an essential feature of which is that the membrane acts as an osmotic and electric barrier, driving an anistropic adenosine triphosphatase toward

the synthesis of ATP by maintaining a pH gradient. Mitchell suggests that some uncouplers of oxidative phosphorylation act by promoting leakage of protons through the membrane, thereby short-circuiting the charge separation and dissipating the energy needed for ATP synthesis.

Colicins E1 and K, which as we have seen do produce selective changes in membrane permeability, may act as suggested by Mitchell's theory. Alternatively, they may affect oxidative phosphorylation by allowing destruction or loss of some (hypothetical) intermediate in oxidative phosphorylation. The apparently essential role of oxygen in colicin action remains unexplained. Studies with isolated bacterial membrane preparations will probably be needed to understand the mode of action of colicins on oxidative phosphorylation and on other membrane-associated functions.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Abraham, S., and W. Z. Hassid. 1957. The synthesis and degradation of isotopically labeled carbohydrates and carbohydrate intermediates, p. 489–560. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 4. Academic Press, Inc., New York.

2. Adler, J. 1966. Chemotaxis in bacteria. Science 153:708–715.

3. Adler, J., and B. Templeton. 1967. The effect of environmental conditions on the motility of *Escherichia coli*. J. Gen. Microbiol. 46:175–184.

4. Archibald, R. M. 1944. Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. J. Biol. Chem. 156:121–142.

5. Atkinson, D. E. 1965. Biological feedback control at the molecular level. Science 150:851–857.

6. Atkinson, D. E. 1966. Regulation of enzyme activity. Ann. Rev. Biochem. 35:85–124.

7. Atkinson, D. E., and L. Fall. 1967. Adenosine triphosphate conservation in biosynthetic regulation. *Escherichia coli* phosphoribosyl pyrophosphate synthase. J. Biol. Chem. 242:3241–3242.

8. Bandurski, R. A., and B. J. Axelrod. 1951. The chromatographic identification of some biologically important phosphate esters. J. Biol. Chem. 193:405–410.

9. Beljanski, M., and M. Beljanski. 1957. Sur la formation d'enzymes respiratoires chez un mutant d'*Escherichia coli* streptomycine-résistant et auxotrophe pour l'hémine. Annal. Inst. Pasteur 92:396–412.

10. Bergmeyer, H.-U. (ed.). 1963. Methods of enzymatic analysis. Academic Press, Inc., New York.

11. Block, J. R., E. L. Durrum, and G. Zweig. 1955. A manual of

paper chromatography and paper electrophoresis. Academic Press, Inc., New York.

12. Buhlen, D. R. 1962. A simple scintillation counting technique for assaying $^{14}CO_2$ in a Warburg flask. Anal. Biochem. 4:413–417.

13. Cousin, D. 1967. Mutants thermosensibles d'*Escherichia coli* K-12 II. Étude d'une mutation létale affectant une réaction génératrice d'énergie. Ann. Inst. Pasteur 113:309–325.

14. Cousin, D., and J. Belaich. 1966. Sur une mutation thermosensible d'*Escherichia coli* affectant une fonction énergetique. Compt. Rend. 263:886–888.

15. Dobrogosz, W. J. 1966. Altered end-product patterns and catabolite repression in *Escherichia coli*. J. Bacteriol. 91:2263–2269.

16. Fields, K. L., and S. E. Luria. 1969. Effects of colicins E1 and K on transport systems. J. Bacteriol. 97:57–63.

17. Frahn, J. L., and J. A. Mills. 1957. Paper ionophoresis of carbohydrates. I. Procedures and results for four electrolytes. Australian J. Chem. 12:65–89.

18. Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. 147:415–442.

19. Gilvarg, C., and B. D. Davis. 1956. The role of the tricarboxylic acid cycle in acetate oxidation in *Escherichia coli*. J. Biol. Chem. 222:307–319.

20. Griffin, C. C., B. N. Houck, and L. Brand. 1967. Purification of *Escherichia coli* phosphokinase. Biochem. Biophys. Res. Commun. 27:287–293.

21. Jacob, F., L. Siminovitch, and E. Wollman. 1952. Sur la biosynthèse d'une colicine et sur son mode d'action. Ann. Inst. Pasteur 83:295–315.

22. Jacoby, G. A., and L. Gorini. 1967. Genetics of control of the arginine pathway in *Escherichia coli* B and K. J. Mol. Biol. 24:41–50.

23. Kohiyama, M., D. Cousin, A. Ryter, and F. Jacob. 1966. Mutants thermosensibles d'*Escherichia coli* K-12. I. Isolement et caracterisation rapide. Ann. Inst. Pasteur 110:465–486.

24. Kornberg, H. L., P. J. R. Phizackerley, and J. R. Sadler. 1960. The metabolism of C_2 compounds in micro-organisms. 5. Biosynthesis of cell materials from acetate in *Escherichia coli*. Biochem. J. 77:438–445.

25. Kovac, L., and S. Kuzela. 1966. Effect of uncoupling agents and azide on the synthesis of β-galactosidase in aerobically and anaerobically grown *Escherichia coli*. Biochim. Biophys. Acta 127:355–365.

26. Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Proc. Natl. Acad. Sci. U.S. 52:1067–1074.

27. Luria. S. E. 1964. On the mechanisms of action of colicins. Ann. Inst. Pasteur 107:67–73.

28. Mitchell, P. 1966. Metabolic flow in the mitochondrial multiphase system: an appraisal of the chemi-osmotic theory of oxidative phosphorylation, p. 64–84. *In* J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (ed.), Regulation of metabolic processes in mitochondria. Elsevier, Amsterdam.

29. Mortimer, D. C. 1952. Paper chromatographic separation of some biologically important phosphate esters. Can. J. Chem. 30:653–660.

30. Nomura, M. 1963. Mode of action of colicins. Cold Spring Harbor Symp. Quant. Biol. 28:315–324.

31. Nomura, M., and A. Maeda. 1965. Mechanism of action of colicines. Zentr. Bakteriol. Parasitenk. Abt. I Orig. 196:216–239.

32. Preiss, J., L. Shen, E. Greenberg, and N. Gentner. 1966. Biosynthesis of bacterial glyogen. IV. Activation and inhibition of the adenosine diphosphate glucose pyrophosphorylase of *Escherichia coli* B. Biochemistry 5:1833–1845.

33. Ramsey, H. A. 1963. Separation of organic acids in blood by partition chromatography. J. Dairy Sci. 46:480–483.

34. Tanaka, S., D. G. Fraenkel, and E. C. C. Lin. 1967. The enzymatic lesion of strain MM-6, a pleiotropic carbohydrate-negative mutant of *Escherichia coli*. Biochem. Biophys. Res. Commun. 27:63–67.

35. Wade, H. E., and D. M. Morgan. 1955. Fractionation of phosphates by paper ionophoresis and chromatography. Biochem. J. 60:264–270.

36. Wang, C. H., I. Stern, C. M. Gilmour, S. Klungsoyr, D. J. Reed, J. J. Bialy, B. E. Christensen, and V. H. Cheldelin. 1958. Comparative study of glucose catabolism by the radiorespirometric method. J. Bacteriol. 76:207–216.

37. Winkler, H. H. 1966. A hexose phosphate transport system. Biochim. Biophys. Acta 117:231–240.