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INHIBITION OF ENZYME INDUCTION IN E. COLI BY ANODIC SILVER

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ABSTRACT

A silver anode, but not a cathode, is bactericidal at microampere current levels because of the electrochemical reactions occurring at the metal electrode surface. This has been clinically useful as a local anti-infective agent even though the mechanism of action on the bacterial cell has not been determined. We investigated the effect by inducing β-galactosidase while passing current though cultures of Escherichia coli. Enzyme induction was depressed in the silver anode chamber within twenty minutes of initiation of current (0.04 to 40 μA); induction in the connected silver cathode chamber was normal. The inhibition at the anode is not the result of electrolysis of the medium nor is the electric current itself required, since pre-anodized silver is inhibitory. The electrochemical products are effective even after derepression has occurred. They appear to act on the process of protein production itself rather than directly on the liberated β-galactoside enzyme.

INTRODUCTION

Bacterial multiplication is inhibited by metal electrodes (1-7). The bactericidal action is thought to be the result of electrochemical products formed at the electrode surfaces rather than to the electric current itself.

Previous studies (1,2,6) have investigated the effects of weak direct currents (0.02 to 20 $\mu A/mm^2$ of electrode surface area) on several bacterial species, using silver, platinum, gold, stainless steel and copper electrodes.

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At the higher current values, growth was inhibited at all electrodes, usually in conjunction with demonstrable electrolytic breakdown of the medium and severe corrosion of the metal. At the lower current values, only silver anodes were bactericidal. The silver anode effect was not associated with electrolytic breakdown of the medium nor with pH changes. The bactericidal agent was postulated to be silver ions that had been electrochemically ejected from the anode. About 5 μg silver/ml is present in the medium after four hours of treatment at 4 μA.

To study this effect we have looked at a subcellular process involved in the growth of E. coli, namely the induction of β-galactosidase. When E. coli is cultured in a glycerol-containing growth medium, the genes of the lactose operon are repressed. Addition of inducer to the culture initiates a sequence of events which includes derepression, transcription, translation and the formation of active β-galactosidase enzyme. This enzyme activity is conveniently assayed colorimetrically by the conversion of ONPG. The gratuitous inducer, IPTG, is not metabolized by the culture, thereby facilitating the study of the kinetics of the induction process.

Inhibition of enzyme induction is a potentially sensitive and early sig-ıal of cellular damage. The present paper reports the effects of the silver node upon the induction of β-galactosidase in E. coli.

MATERIALS AND METHODS

Bacterial Culture Methods. Escherichia coli B, ATCC 25922, grown in berts' C-minimal medium + 1% glycerol, was used in all experiments. Experi-ntal cultures were prepared by inoculating 5 to 10 ml of medium from an ɘrnight culture and growing it at room temperature (27°C) with aeration :ough a Pasteur pipette until exponential phase growth was achieved (about minutes doubling time). Cell concentration was determined by measurement optical density at 550 nm.

Electrical Treatment. Plexiglass chambers containing pairs of 3-ml wells used for the electrical treatment (6). Each pair of wells was connected

by a salt bridge -- a thin slot which was filled with cotton and then soaked with melted 2%-Bacto-Agar containing growth medium. Identical electrodes of acetone-cleaned, pure (99.99%), 15 mil silver wire were inserted into each pair of wells; the wires were connected to battery-operated, constant-current generators (40 μA, 4 μA, 0.4 μA, or 0.04 μA). The length of silver wire inserted into the solution was 3.2 cm (nominal area of silver surface = 0.84 cm^2). For each experiment, 2.5 to 3.0 ml of appropriately diluted exponential phase culture was placed in each well. Dilution, distribution, and initiation of current was completed in 2.5 minutes. Sufficient aeration could be obtained by diffusion due to the shallowness (< 0.8 cm) and low cell density ($< 5 \times 10^7$ cells/ml) of the culture. The content of each well was thoroughly mixed with a pipette prior to the removal of an aliquot. During the course of each experiment, the voltage across and the current through each pair of wells were periodically measured with a Keithley 602 Solid State Electrometer. Inter-electrode potentials remained well below 1.4 volts (at which voltage electrolysis of water might become important) except at the highest current level (40 μA). In one experiment the pH of the wells was also measured, and it remained at 7.0 ± 0.4, indicating the absence of any significant hydrolysis. All experiments were performed at room temperature (26.7 ± 0.3°C).

Enzyme Assay. Cultures were induced for β-galactosidase by the addition of IPTG (Isopropyl-β-D-thiogalactopyranoside, Sigma) to a final concentration of 5×10^{-4} M (8). Either 0.2 or 0.3 ml aliquots were removed to a test-tube containing one drop of toluene and sufficient growth medium to bring the final volume to 0.5 ml. These samples were incubated at 37°C for 30 minutes to promote cell lysis. Upon return of the tube to room temperature, 0.2 ml of 0.013 M ONPG (o-nitrophenyl-β-D-galactopyranoside, Sigma) dissolved in 0.25 M sodium phosphate buffer (pH 7) was added to the tube and incubated. Color development was stopped after a suitable interval, Δt, by the addition of 2.5 ml of 1 M Na_2CO_3. Optical densities were determined on either a Coleman Spec-

trophotometer, EPS-3T, or a Bausch and Lomb Spectronic 20. Correction for turbidity was made by calculating $\Delta OD = OD_{420nm} - 1.65\ OD_{550nm}$ (9). Enzyme concentrations were calculated according to equation:

Enzyme units/ml

$$= \frac{\Delta OD}{\Delta t} \times \frac{1 \text{ enzyme unit}}{1\ m\mu\text{mole ONP/min}} \times \frac{1\ m\mu\text{mole ONP/ml}}{.0075 \text{ OD units}} \times \frac{\text{final vol.}}{\text{vol. aliquot}}$$

RESULTS

Effect of Silver Electrodes on β-Galactolidase Induction. *Figure 1 shows* the results of the induction of β-galactosidase in cultures exposed to silver

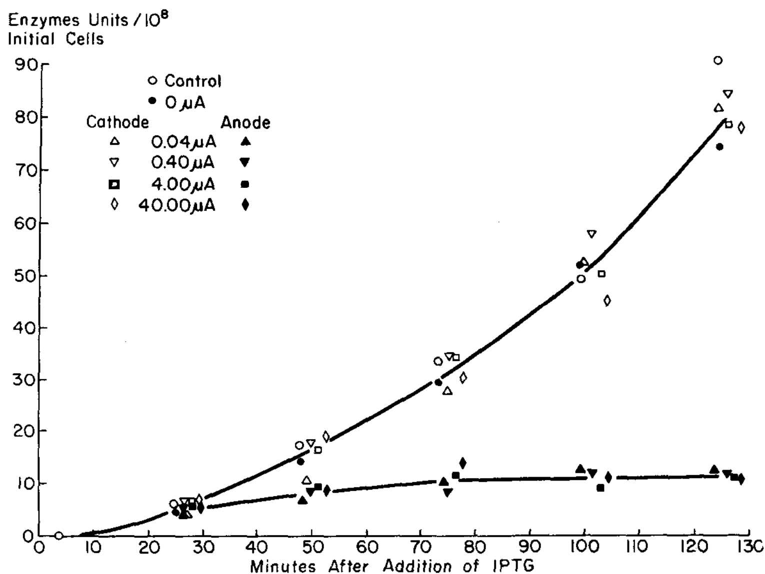

FIGURE 1

Inhibition of β-galactosidase induction at a silver anode. An E. coli culture was diluted at time zero to about 4×10^7 cells/ml in growth medium containing the inducer IPTG to a final concentration of 5×10^{-4} M. The culture was distributed to wells containing silver electrodes by 2-1/2 minutes. Aliquots were removed for enzyme assay. The ordinate represents the mean of duplicate experiments normalized to the initial cell concentration.

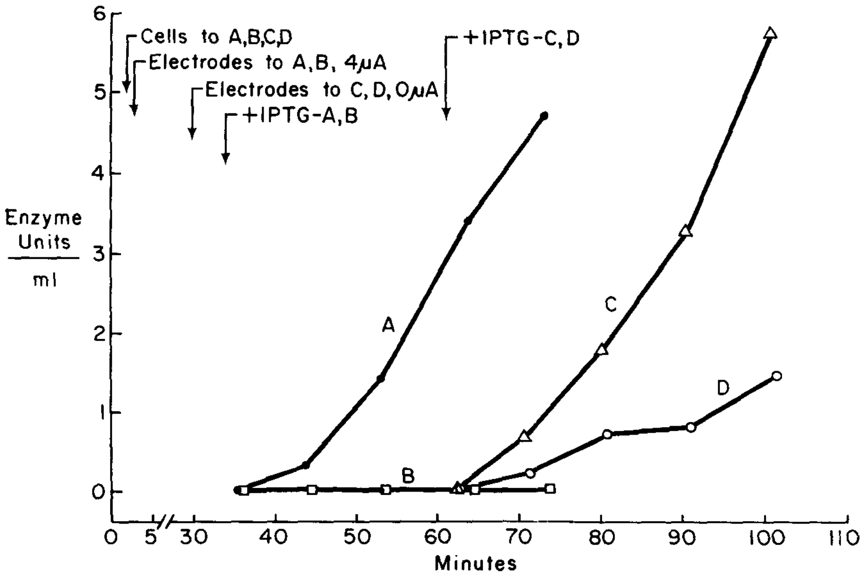

FIGURE 2

β-Galactosidase induction in the absence of concomitant current. An uninduced culture was distributed to two pairs of wells one pair (A^-, B^+) containing silver electrodes connected to a 4 μA generator. At 30 minutes these electrodes were disconnected, removed, rinsed lightly in distilled water, and placed in the other pair of wells, but with no current (C, D). Inducer, IPTG, was added to a concentration of 5×10^{-4} M at 34 minutes (A, B) and 61 minutes (C, D). Aliquots were removed for enzyme assay.

electrodes. The kinetics of induction in the cathode wells at all current levels were equivalent to control cultures, with or without silver wire. Enzyme induction in the anode wells was inhibited at all current levels. The effect was just beginning at 21 minutes after initiation of current. Thus, the cell death that resulted from several hours' treatment with a silver anode was foreshadowed by a much earlier effect on at least one inducible enzyme system.

Independence of Electric Current. We asked whether the inhibition requires simultaneous electric current (Figure 2). Exponentially growing culture was placed in four wells, one pair of which (A^-, B^+) contained silver

electrodes connected to a 4 μA generator. Thirty minutes later, the electrodes were disconnected from the generator, removed from their wells, rinsed lightly in distilled water, and then placed in the second pair of wells. Inducer was added to the first set of wells (A,B) at this time, and to the second set of wells 30 minutes later. Induction occurred in the two cathode wells, A^- and C. Induction in the anode well B^+, through which current had passed for 30 minutes prior to, but not during induction, was inhibited completely. Induction in anode well D was substantially depressed (75%) even though no current had been applied to that culture; that is, the pre-anodized silver wire was sufficient to inhibit enzyme induction. These results are consistent with the hypothesis that the inhibitory agent is an electrochemical product formed at, and loosely attached to, the silver anode.

Effect of Polarity Reversal. We studied the effect of reversing the electrical attachments to the two silver electrodes. An experiment was begin at 4 μA under the conditions of Figure 1. After 30 minutes, the polarity of the electrodes was reversed and the experiment continued for another 60 minutes. At the original cathode, induction was substantially under way at 30 minutes (following the kinetics shown in Figure 1), but it ended about 25 minutes after the polarity reversal. This demonstrated that a derepressed culture was still sensitive to the inhibitory effect. In the other well, originally anodic, induction was inhibited during the first 30 minutes; this inhibition was not relieved by reversal of polarity over the next 60 minutes.

Direct Effect on Enzyme Activity. Because the silver ion is known to be an enzyme inhibitor (10), we tested the possibility that inhibition was the results of direct inactivation of β-galactosidase by electrochemical reaction products. To do this we challenged crude enzyme preparations with various agents prior to enzyme assay. The enzyme preparations were prepared by toluene-lysis of an induced culture, grown and treated as in Figure 1, but without electrodes. The preparations were left unpurified to retain the protein-rich environment in which the anode effect occurs. Cell culture concentrations were 3-5 x 10^7 cells/ml from which were obtained final enzyme concentrations

in the range of 35-50 enzyme units/ml. The results of three experiments are presented in Table 1 in which enzyme preparations were challenged by: (a) silver-anode-treated medium, (b) silver-anode-treated/toluene-lysed culture, and (c) silver nitrate solutions.

In (a), growth medium treated by anodic silver was added to enzyme preparations and each sample was immediately assayed for enzyme activity. In (b), uninduced cultures were treated by anodic silver for 60 minutes, then lysed with toluene and added to the enzyme preparations. In this experiment por-

TABLE 1

Enzyme Activity in Crude β-Galactosidase Preparations after Treatment with Potential Inactivating Agents

ELECTRICAL TREATMENT			PRE-ASSAY CONTACT TIME WITH ENZYME PREPARATION (min)	ENZYME ACTIVITY (Enzyme Units per ml)
Material Treated	Magnitude of Current (μA)	Length of Treatment (min)		
(a)				
Growth medium	4	0	3	40
		10		36
		40		35
		70		35
(b)				
Uninduced culture	0	60	2	51
			60	59
			180	58
			1380	65
	4	60	2	49
			60	51
			180	58
			1380	61
	40	60	2	47
			60	55
			180	59
			1380	62
(c)				
Treatment with Silver Nitrate (Final Silver Concentration in μg/ml)				
	5		90	0.0
	2		90	0.71
	0.4		90	33
	0.08		90	33
	0.016		90	36
	0.0032		90	36
	0		90	31

tions of each mixture were assayed for enzyme activity periodically over 23 hours. No significant decrease in β-galactosidase activity was observed in either (a) or (b).

In (c), a series of dilutions of silver nitrate was prepared and mixed with enzyme preparations. High silver-ion concentrations abolished enzyme activity. At 5 μg silver/ml a slight white opalescence of precipitating material was visible. The concentration dependence was sharp, and the inactivating effect disappeared at silver ion concentrations below about 1 μg silver/ml. The absence of an effect on β-galactosidase below this level supports the idea that anodically dispersed silver interferes primarily with the production of galactosidase rather than its activity *per se*.

DISCUSSION

Silver anodes inhibit and kill a wide variety of bacterial species after several hours at microampere current levels. In this study we have shown an earlier effect, diminished inducibility of β-galactosidase in *E. coli*. Inhibition occurred even at the lowest current values tested. The importance of this observation is that it may relate to the mechanism of silver inhibition in many organisms. In particular, the experiments indicate the following characteristics of this phenomenon.

a) Current *per se* is not required since pre-anodized silver also had an inhibitory effect, although with reduced efficiency. It must have carried electrochemical products on its surface that inhibited enzyme induction in freely diffusing cell cultures. Electrolysis of the medium is also not necessary to produce the electrochemical agent.

b) Active β-galactosidase enzyme is relatively insensitive to silver anode products and to silver nitrate. Thus, the site of action at these current levels is *not* the enzyme itself, but rather some cellular component required for its production.

c) The induction sequence appears to be blocked at some point subsequent to derepression of the lac operon, since a derepressed culture remains sensitive to inhibition by anodic silver.

d) The 20-minute delay between initiation of anodic current and inhibition of β-galactosidase production is presumably the consequence of a number of factors: the accumulation of sufficient silver ions (or complexes), their release and diffusion throughout the chamber, and the delay between the binding of silver and the halting of protein production.

The weight of these experiments, as well as previous observations with silver anodes and bacteria, strongly implicates the silver ion or a silver ion complex as the causative agent. Assuming this, Faraday's Law predicts that at the lowest effective current tested, the maximum concentration of silver liberated was 0.02 μg silver/ml (11). This is two orders of magnitude below the concentration of silver (as nitrate) that produces a measureable decrease in β-galactosidase activity itself. It is also well below the 0.5 μg/ml (as nitrate) shown by Bragg and Rainnie (12) to inhibit respiration in E. coli. Such sensitivity leads us to suspect that the initial target of the silver ion was a structure (such as the cell membrane) which could amplify the effect of the phenomenon, a suggestion which may help explain its therapeutic effectiveness against many organisms and may delineate a possible mode of bioelectric effect.

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