

ENZYMATIC BASIS OF RESISTANCE TO AUREOMYCIN
III. INHIBITION BY AUREOMYCIN OF PROTEIN-STIMULATED ELECTRON
TRANSPORT IN *Escherichia coli*

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Previous studies from this laboratory have shown that Aureomycin in low concentration inhibits the reduction of aromatic nitro groups mediated by purified extracts of *Escherichia coli* (E-26) (Saz and Marmur, 1953; Saz and Slie, 1953, 1954a). Purification and resolution of the enzyme showed that the reductase was a pyridine nucleotide-linked, loosely dissociable flavin mononucleotide-containing flavoprotein with a requirement for Mn^{++} , (Saz and Slie, 1954b; Saz and Martinez, 1956). Electron transport, measured by the capacity of the same cell-free extracts to reduce 2,6-dichlorophenol-indophenol, was also inhibited (Saz and Martinez, 1958). Similar enzymes, extracted from Aureomycin-resistant *E. coli* derived by serial passage of the parent sensitive strain in antibiotic-containing media were found to be resistant to Aureomycin (Saz *et al.*, 1956). The resistant enzyme was also a flavoprotein but in this instance, the flavin was extremely tenaciously bound to the protein. In addition it was hypothesized that a metal, if involved, was equally firmly ligated to the enzyme (Saz and Martinez, 1956; 1958).

With both enzymes, it was noted that Aureomycin at appropriate concentrations inhibited electron transport by virtue of inhibiting the reoxidation of reduced flavoprotein. In an attempt to determine the precise function of the metal in the system, it was necessary to purify the enzyme further. Since highly purified enzyme preparations were unstable, crystalline bovine serum albumin was added to stabilize the preparation. A large stimulation of enzyme activity was noted which could not be explained merely by the stabilization hypothesis. A description of this phenomenon is presented in this communication.

METHODS AND MATERIALS

E. coli (E-26) and its Aureomycin-resistant variant were grown, harvested, and washed as

previously described (Saz and Martinez, 1956). Twenty-five per cent suspensions of the cells were made in cold water and placed in a Raytheon 10 kc sonic oscillator for 30 min; the debris was centrifuged off in the cold at $18,000 \times G$ and the supernatant solution was treated with ammonium sulfate, protamine sulfate, and calcium phosphate gel as described previously. A series of negative and positive phosphate gel absorptions purified the enzyme further. In general, preparations used represented 50- to 100-fold purifications over untreated supernatant solutions arising from sonic oscillation. 2,6-Dichlorophenol-indophenol reduction and ferricyanide reduction were measured by following the decrease in absorption in a Beckman model DU spectrophotometer at 600 and 420 $m\mu$, respectively, and cytochrome *c* reduction was determined by measuring the increase in absorption at 550 $m\mu$. Aromatic nitro reductase was determined as previously described (Saz and Marmur, 1953). Cuvettes contained in a total volume of 3.0 ml, 0.05 M phosphate buffer, pH 7.5, DPNH 6.7×10^{-5} M, reducible substrate 1×10^{-5} M, enzyme preparations and various proteins as indicated in the text and in the legends of figures. The reactions were run aerobically at room temperature.

RESULTS

Figure 1 shows the effect of various concentrations of crystalline bovine serum albumin on phenol-indophenol reduction mediated by extracts of Aureomycin-sensitive *E. coli*. Similar results were observed with extracts of Aureomycin-resistant cells. It is quite clear that various concentrations of protein stimulate markedly, e. g., at 0.02 per cent albumin, stimulation up to 800 per cent is observed and at 0.002 per cent, 600 per cent is obtained. In other experiments, stimulations up to 2000 per cent have been noted. Numerous, but by no means all, other proteins tested showed similar effects. Table I

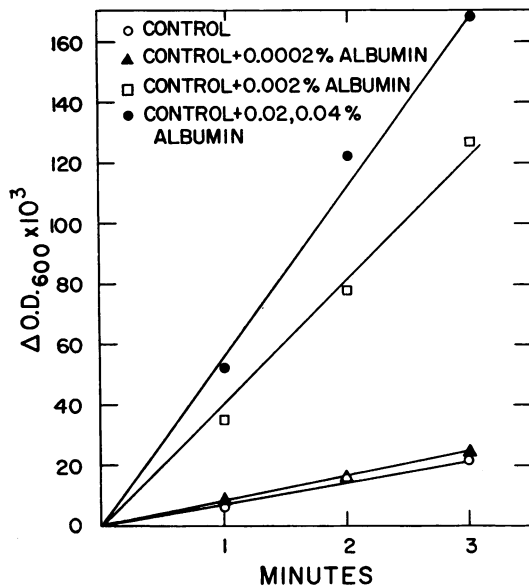


Figure 1. Effect of bovine serum albumin on 2,6-dichlorophenol-indophenol reduction. Reduced diphosphopyridine nucleotide, 6.7×10^{-5} M; 0.05 M phosphate buffer, pH 7.5; 1×10^{-5} M phenol-indophenol, extract 80 μ g protein/ml. Total volume 3.0 ml. Aerobic, 25 C. Extract prepared as previously described (Saz and Martinez, 1958) and this extract placed again on $\text{Ca}_3(\text{PO}_4)_2$ gel. Supernatant used.

is a partial list of those proteins tested and the effects on phenol-indophenol reduction. There seems to be no pattern of activity, as some globulins are as active as albumin, etc., whereas other albumins and globulins are essentially inactive. Crystalline bovine serum albumin was chosen for all further experiments.

Figure 2 shows the effect of Aureomycin on the stimulation by bovine serum albumin of phenol-indophenol reduction mediated by sensitive extracts. The nonstimulated reaction is inhibited 60 per cent by Aureomycin at a concentration of 10 μ g per ml. The 600 per cent stimulation by the protein is inhibited 90 per cent by Aureomycin. Similar results were obtained using an extract derived from Aureomycin-resistant cells. It is to be emphasized that in the latter case as has been previously shown (Saz and Martinez, 1958), the unstimulated reduction of phenol-indophenol is resistant to Aureomycin.

The degree of stimulation of the reductions is markedly pH dependent. Table 2 shows that stimulation is greatest at pH 8.0.

TABLE 1

Effect of various proteins on 2,6 dichlorophenol-indophenol reductions mediated by extracts of *Escherichia coli* (E-26)

Protein	Stimulation
	%
Human fraction V defatted.....	888
Bovine IV-4 (α - and β -globulin)...	732
Bovine serum albumin, crystalline.....	705
Whole bovine plasma.....	664
Human fraction V.....	441
Bovine fraction V.....	433
Bovine fibrinogen (fraction I)....	418
Ovine fraction V.....	417
Canine fraction V.....	334
Bovine fraction-1 (α -globulin and lipid).....	327
Equine fraction V.....	307
Turquine fraction V.....	272
Porcine fraction V.....	266
Bovine α -globulin.....	100
β -Lactoglobulin.....	50
Egg albumin, crystalline.....	10
Bovine fraction II.....	10

Conditions similar to figure 1. Protein concentration, 0.02 per cent.

A study of the physical and chemical properties of the stimulating factor was undertaken. It was established that the activity is nondialyzable, completely inactivated by boiling and acid hydrolysis, and partially inactivated by alkaline, chymotryptic or tryptic digestion. No α -amino acids or peptides or combinations of these were capable of replacing the proteins. Defatted protein was as active as the untreated and α -tocopherol and vitamin K or derivatives of these did not replace. Further, crystalline bovine serum albumin was ionophoresed in the Beckman model CP continuous flow paper electrophoresis cell at various ionic strengths in various buffers and in all instances, activity followed protein concentration. Because of this and other considerations noted above, it was concluded that the active factor is indeed the protein itself or a large hydrolytic unit of the protein. It is interesting to note that a similar conclusion was reached by Sacktor *et al.* (1958) who observed that bovine serum albumin stimulated oxidative phosphorylation in insect flight muscle.

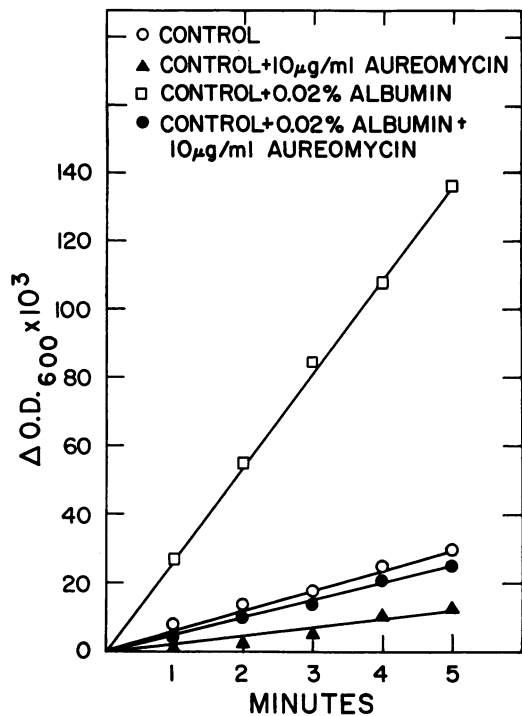


Figure 2. Effect of Aureomycin on albumin stimulation. Conditions same as figure 1.

A study was made of the inhibition of the stimulatory effect of the protein. Table 3 shows the effect of various metal binders which are the only classes of compounds tested which exhibit inhibitory activity. It is seen that Aureomycin is by far the most potent inhibitor of the stimulation. As in the case of unstimulated reductions of nitro compounds, flavins, ferricyanide and phenol-indophenol, tetracycline (Achromycin), and oxytetracycline (Terramycin), although structurally closely related to chlortetracycline (Aureomycin), are considerably less inhibitory than the latter. Cyanide inhibits to some extent as does cyclohexanediaminetetraacetic acid. Ethylenediaminetetraacetic acid does not inhibit. Other metal binders such as Cupferon, *o*-phenanthroline, sodium azide, and citrate are without effect.

Attempts to relieve the inhibition caused by metal binders by the addition of 30 metal salts and sea water which possesses more than 60 inorganic ions have been uniformly unsuccessful. Addition to the reaction mixture of the dissolved ash of the various active proteins also did not lift the inhibition. Further, none of these com-

TABLE 2
Effect of pH on albumin stimulation

pH	Optical Density ₆₀₀ × 10 ³		Stimulation %
	Control	0.02% Albumin	
5.5	43	113	163
5.9	39	146	274
6.8	14	108	671
7.5	13	134	931
8.0	4	81	1925

Conditions similar to those in figure 1. Phosphate buffer albumin at 0.02 per cent final concentration.

TABLE 3
Effect of metal binders on albumin stimulation

System	Optical Density ₆₀₀ × 10 ³	Inhibition %
Control.....	95	—
Control +		
1.7 × 10 ⁻⁵ M Aureomycin.....	2	98
1.0 × 10 ⁻² M KCN.....	53	44
1.0 × 10 ⁻² M CDTA*.....	54	43
1.0 × 10 ⁻² M EDTA†.....	94	1
1.7 × 10 ⁻⁵ M Achromycin.....	86	10
1.7 × 10 ⁻⁵ M Terramycin.....	85	11
4.0 × 10 ⁻² M Na-citrate.....	90	5

Conditions similar to figure 1. Albumin at 0.02 per cent final concentration.

* Cyclohexanediaminetetraacetic acid.

† Ethylenediaminetetraacetic acid.

pounds was able to replace the protein in stimulating reduction.

It would appear that a metal, if involved in the stimulation is attached to the enzyme, rather than to the albumin, since dialysis of the reductases versus cyanide rendered the preparations incapable of stimulation by albumin, whereas dialysis of the albumin versus cyanide still permitted stimulation by the protein.

A point of interest in this work is the observation that the proteins stimulate specifically phenol-indophenol reductase. The reduction of other 2-electron acceptors such as aromatic nitro compounds, flavin mononucleotide and flavin adenine dinucleotide are not stimulated, nor are the reductions of 1-electron acceptors such as ferricyanide and cytochrome *c*.

DISCUSSION

It is apparent from the data presented herein and from previous observations that the development of resistance to Aureomycin by *E. coli* occurs in a subtle manner and seemingly involves only limited changes in the cell's over-all metabolism. Aside from the degree of sensitivity to the antibiotic, patterns of growth and metabolism of sensitive cells and resistant variants are markedly similar. Both types of cells grow at comparable rates in identical media and both apparently exhibit the same oxidative capacities on all substrates tested. Thus far the only differences observed between the two lines of cells center about the electron transport system at a level involving reoxidation of reduced flavoproteins. Phenol-indophenol reductase in both sensitive and resistant variants of *E. coli* (E-26) is a flavoprotein. However, cell-free reductase derived from sensitive cells is probably a loosely dissociated manganoflavoprotein whereas that from the resistant variants contains tightly bound conjugated flavin and, presumably, equally firmly ligated cation. As was previously postulated (Saz and Martinez, 1956; 1958), this difference at the enzymatic level could possibly account for the sensitivity or resistance of the whole cell since reductions catalyzed by extracts derived from sensitive cells were sensitive precisely to those concentrations of antibiotic which were growth inhibitory whereas extracts derived from resistant cells were resistant to these and higher concentrations of Aureomycin and were sensitive only to those higher concentrations of antibiotic which inhibit resistant cell growth. However, as shown in this communication, even these differences are not absolute. Although applying in less pure fractions and thus presumably mirroring the events in whole cells, on higher purification a drop in reductase activity occurred which could be restored by addition of various proteins. In this latter instance, however, the structure of the reductase complex was so changed that stimulated reductions whether catalyzed by extracts derived from sensitive or resistant cells were sensitive to Aureomycin.

The manner in which the various proteins stimulate the reduction of 2,6-dichlorophenol-indophenol is unknown. One can speculate, as a result of the inhibition of stimulation by cyclohexanediaminetetraacetic acid and Aureomycin, both potent chelating agents, that a

metal is involved in the process. If so, it would appear that the putative metal is not in the ionic state but rather is in organic combination since none of 30 metals either singly or in combination, sea water, or protein ash was capable of replacing the protein in activation of the reductase. It seems clear, moreover, that the effect of the stimulating protein is upon the enzyme rather than upon the substrate since incubation of the protein with phenol-indophenol with subsequent removal of the dye by dialysis changed the properties of neither constituent insofar as the reductase was concerned. As noted above, a metal seemingly is associated with the enzyme and it is possible that this metal serves as a bridge between the reductase and the stimulatory proteins. Changes in pH would be expected to affect the binding. The data of table 2 are consistent with this hypothesis.

Other possibilities exist. There are numerous reports which indicate that bovine serum albumin or other proteins reactivate decreased oxidative phosphorylative capacity of various tissue preparations. These reports have, however, dealt primarily with the effects on mitochondria. (See, for example, Polis and Shmukler, 1957; Pullman and Racker, 1956, and Sacktor *et al.*, 1958.) The activity of bovine serum albumin as described in these reports has been ascribed to (a) preservation of a satisfactory osmolar environment which maintained the structural integrity of mitochondria, and (b) reversal of the activity of an inhibitory protein or lipid associated therewith (mitochrome) released upon aging of mitochondria. It should be emphasized that the effects reported in this communication were upon soluble rather than particulate fractions of the cell. It is unlikely that bovine serum albumin is active in this system in maintaining osmolarity since not all proteins are active. Sacktor *et al.* (1958) reached similar conclusions in their system using insect flight muscle mitochondria. Whether an inhibitory factor is released on purification of the soluble reductase which is reversed by bovine serum albumin and other proteins remains to be determined. It should also be noted that the effects of bovine serum albumin in the phenol-indophenol reductase system of *E. coli* (E-26) are probably not due to the factor reported by Donaldson *et al.* (1958a, b) since this factor could not replace bovine serum albumin and further, extracted

bovine serum albumin was as active as the untreated material.

It is of some interest that Aureomycin alone of the 3 clinically useful tetracyclines inhibits the stimulation. This indicates once again that one must view with caution the generally accepted thesis that all the tetracyclines have similar, if not identical, modes of action. It has been noted previously (Saz and Slie, 1954*a, b*; Saz and Martinez, 1958) that in the case of organic nitro reductase, ferricyanide reduction and unstimulated phenol-indophenol reduction, Aureomycin is markedly differentiated from both Terramycin and Achromycin. In all cases, 100- to 1000-fold higher concentrations of Terramycin and Achromycin were necessary to inhibit the reductions.

SUMMARY

Bovine serum albumin and certain other proteins stimulate up to 2000 per cent the rate of reduction of 2,6-dichlorophenol-indophenol mediated by highly purified extracts of Aureomycin-sensitive and -resistant *Escherichia coli*. Unstimulated reductions in extracts derived from sensitive cells are sensitive to Aureomycin, whereas those catalyzed by extracts of resistant cells are resistant to the antibiotic. No such parallelism exists in the stimulated reductions. In this instance, both sensitive and resistant extracts are sensitive to Aureomycin. The stimulation is pH dependent and is inhibited only by certain metal binders. Possible mechanisms of the stimulatory effect and the relationship of the phenomenon both to sensitivity of bacterial cells to Aureomycin and the development of resistance thereto are discussed.

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