TERRAMYCIN INHIBITION OF GLUCONATE OXIDATION BY *ESCHERICHIA COLI*

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The mode of action of "terramycin" (oxy-tetracycline, Pfizer), has not been described in detail, although numerous investigators have shown that the antibiotic exerts an inhibitory effect on oxidative mechanisms. Terramycin inhibition of the oxidation of Krebs cycle intermediates by *Escherichia coli* was reported by Wong and Ajl (1953). Similarly, Ajl (1953) observed the terramycin inhibition of microbial oxidation of substrates which participate in terminal respiration. Glutamate oxidation inhibition was demonstrated by Karp and Snyder (1952), in murine and epidemic typhus rickettsiae. Terramycin and "aureomycin" were also shown to disrupt oxidative phosphorylation systems in rat mitochondria (Loomis, 1950).

This study was initiated in an attempt to define the site of terramycin activity on oxidative reactions in *Escherichia coli*.

EXPERIMENTAL METHODS

Bacteriological. The organism used was *Escherichia coli* Rutgers strain S-2. The culture was sensitive to less than 1 μ g per ml terramycin when tested by antibiotic serial dilution procedures. Stock cultures were transferred weekly and maintained on brain heart infusion agar (Difco). Cell suspensions were prepared by transferring growth from stock cultures to 250 Erlenmeyer flasks containing 100 ml of brain heart infusion broth. Following incubation for 24 hr at 37 C, the cells were harvested by centrifugation and washed with distilled water. Cells were then resuspended in sufficient distilled water so that the final suspension was standardized to give an optical density of 0.0798 in the Klett-Summerson photoelectric colorimeter when using a green light filter transmitting at a wave length of 500–575 m μ .

Methods of analysis. Substrate oxidation in the presence and absence of inhibitors was measured with the Warburg respirometer, employing con-

ventional manometric techniques for oxygen uptake (Umbreit *et al.*, 1951). Each reaction flask contained a total of 4 ml, which included standardized suspension, 1 ml; 0.05 M tris-(hydroxymethyl)-aminomethane buffer (Tris), pH 7.2, 1 ml; and distilled water or inhibitors, 0.8 ml. The center well contained 30 per cent KOH, 0.2 ml, and was fitted with fluted filter paper. Substrate, 1 ml, added to the side arm, was tipped into the reaction flask after a 15-min temperature equilibration.

Chromatographic analyses were conducted on centrifuged aliquots of reaction mixtures containing gluconate, Tris buffer and standardized cell suspensions in the proportions described above. These were spotted on Whatman No. 1 filter paper and developed for the identification of 2-ketogluconate according to the paper chromatographic method of Koepsell *et al.* (1952).

Dehydrogenase activity was demonstrated and measured by use of the Thunberg technique (Umbreit *et al.*, 1951), using methylene blue as the hydrogen acceptor. Determination of dehydrogenase activity in the absence of inhibitors was conducted by adding, to standard Thunberg tubes, methylene blue, 2 ml of 1:10,000 (0.000267 M); Tris buffer, 2 ml, pH 7.2; substrate, 2 ml; and distilled water, 2 ml. Standardized cell suspension, 2 ml, was added to the side arm and the assembled tube was evacuated with a Cenco Hyvac pump for 2 min. Following a 15-min temperature equilibration at 37 C, the cells were tipped into the tube containing the reaction mixture. The rates of methylene blue reduction were then followed colorimetrically on a Bausch and Lomb spectrophotometer (Spectronic 20) at a wave length of 660 m μ . The kinetic data obtained from the rates of methylene blue reduction were plotted according to the double reciprocal plot method of Lineweaver and Burk (1934).

RESULTS AND DISCUSSION

Initially, a series of carbohydrates and chemically related compounds were tested in the Warburg respirometer to determine whether any of these were oxidatively catabolized by *Escherichia coli*. One of the compounds shown to be oxidized in a phosphate independent reaction was calcium gluconate. It can be seen in figure 1 that 5.0 μ moles of oxygen were consumed by the cells per 10 μ moles of gluconate substrate. Chromatographic analysis of the reaction mixtures at the end of the oxidation indicated that 2-ketogluconate was at least one of the products formed during the course of the oxidation.

The addition of cyanide to the reaction mixtures caused a marked inhibition in oxygen consumption (figure 1). Since oxygen was apparently the final electron acceptor in the reaction, the cyanide sensitivity indicated the possible participation of cytochrome oxidase or a closely related enzyme sensitive to cyanide. Further evidence in favor of this possibility was obtained through use of the Thunberg technique, utilizing methylene blue as the final hydrogen acceptor. In this case, the dye was used as a means of bypassing the cyanide sensitive electron transferring reaction, as described by Barron and Harrop (1928). As may be noted in figure 2

Figure 1. Oxidation of gluconate in the presence and absence of inhibitors. Flasks contained: 10^{-2} M gluconate, 1 ml; 0.05 M Tris buffer, pH 7.2, 1 ml; standard cell suspension, 1 ml; distilled water, 0.8 ml; or 10^{-3} M cyanide or terramycin solution containing 1 mg/ml terramycin.

Figure 2. Effect of supplements on the oxidation of gluconate. Thunberg tubes contained: 10^{-3} M gluconate, 2 ml; 0.05 M Tris buffer, pH 7.2, 2 ml; $1:10,000$ methylene blue, 2 ml; standard cell suspension, 2 ml; and the following supplements: 200 μ g/ml terramycin, 10^{-3} M cyanide, 10 μ g/ml diphosphopyridine nucleotide (DPN), 10 μ g/ml triphosphopyridine nucleotide (TPN).

substrate oxidation (or dehydrogenation) continued in the presence of cyanide, as evidenced by the rapid reduction of methylene blue.

Data in figure 1 also show that terramycin interfered with the oxidation of gluconate. This is in accord with reports in the literature concerning the inhibitory effects of terramycin on microbial oxidations. Although the over-all effects of the antibiotic appeared similar to that of cyanide, it was not immediately apparent as to whether they affected the same sites in the electron transfer system mediating the conversion of gluconate to 2-ketogluconate. Accordingly, Thunberg experiments were performed in which terramycin was included. From figure 2 it can be seen that terramycin inhibited the reduction of methylene blue and, therefore, apparently inhibited the reaction in at least one site other than that affected by cyanide. That terramycin did not affect gluconate dehydrogenase was demonstrated by the fact that 2-ketogluconate was formed even in the presence of the antibiotic. On the other hand, it was found that terramycin interfered with that portion of the reaction in which diphosphopyridine nucleotide (DPN) was directly involved. Evidence of this can be seen in figure 2 where the addition of DPN stimulated the reduction of methylene blue and also reversed the terramycin inhibition of the reaction. Furthermore, under the experimental conditions, the reaction appeared to be

* With reference to unsupplemented control.

TABLE 1
Rates of terramycin inhibited methylene blue reduction as affected by diphosphopyridine nucleotide (DPN)

DPN μg/ml	Rates of Methylene Blue Reduction	
	A*	B†
20	5.4×10^{-4}	5.5×10^{-4}
10	3.8×10^{-4}	5.0×10^{-4}
5	2.5×10^{-4}	3.6×10^{-4}
0	1.6×10^{-4}	1.6×10^{-4}

Thunberg tubes contained: 10^{-3} M gluconate, 2 ml; 0.05 M Tris buffer, pH 7.2, 2 ml; 1:10,000 methylene blue, 2 ml; standard cell suspension, 2 ml; terramycin and DPN, in final concentrations as listed above.

* 200 μg/ml terramycin.

† 100 μg/ml terramycin.

Figure 3. Lineweaver-Burk plot of terramycin inhibition of methylene blue reduction in the presence of substrate, diphosphopyridine nucleotide (DPN).

● -- ●, 200 μg/ml terramycin.

○ -- ○, 100 μg/ml terramycin.

DPN specific since triphosphopyridine nucleotide (TPN) did not influence the reduction of methylene blue in the presence or absence of the antibiotic. The rates of methylene blue reduction in the presence of varied terramycin and DPN concentrations were determined quantitatively and are recorded in table 1. By plotting (figure 3) these rates according to the method of Lineweaver and Burk, it can be seen that terramycin

appears to be a competitive inhibitor of the hydrogen carrier, DPN.

Schematically, the sites of interference of terramycin and cyanide in the over-all reaction involving the conversion of gluconate to 2-ketogluconate may be represented as follows:

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SUMMARY

The oxidation of gluconate by a strain of *Escherichia coli* sensitive to "terracyclin" resulted in the formation of 2-ketogluconate. This reaction was inhibited by terramycin. Evidence was presented which indicated that terramycin competitively inhibited diphosphopyridine nucleotide (DPN) in the electron transport system mediating the oxidation.

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