STUDIES ON MECHANISM OF THE STREPTOMYCIN REACTION

I. Phosphate Reversal of the Dihydrostreptomycin Inactivation of *Escherichia coli*[1]

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Several investigators have recently studied the effect of streptomycin and its derivative dihydrostreptomycin on the viability of *Escherichia coli*. Paine and Clark (1954) showed that the killing effect of dihydrostreptomycin was directly related to the degree of metabolic activity of the cells and was not related to the various effects of this drug on respiration. These workers therefore discounted the hypothesis proposed by Oginsky *et al.* (1949) that streptomycin acts by inhibiting terminal oxidation. Rosenblum and Bryson (1953) found that streptomycin was most effective as a sterilization agent when the bacterial cells were in a medium in which they were potentially capable of multiplication. Inhibitors of cell multiplication, such as iodoacetate, arsenite, azide, or cyanide antagonized the action of streptomycin. When these growth inhibitors were present in amounts calculated to permit some cell multiplication during the test period, the lethal activity of streptomycin was enhanced. They believed that the inhibitory mechanism may be more closely related to nitrogen assimilation than to respiration. Wasserman (1953) found that streptomycin stimulated the total oxygen consumption of cellular suspensions of *E. coli* when oxidizing a number of substrates. In the presence of streptomycin, more CO_2 was formed from glycerol and the respiratory quotient was closer to the theoretical value for total glycerol oxidation than when the antibiotic was absent. On the basis of these results, he proposed that streptomycin acted in a manner similar to dinitrophenol or azide to uncouple assimilatory processes from oxidative dissimilation. Since the bacteria would be unable to use any of the carbon for biosynthesis, more complete terminal respiration would result. This theory appears unlikely since azide was reported by Rosenblum and Bryson (1953) to block rather than augment the lethal action of streptomycin.

In a previous study from this laboratory (Hurwitz *et al.*, 1955) it was found that aerated, metabolizing but nonproliferating *E. coli* cells were more resistant to dihydrostreptomycin than were unaerated cellular suspensions. Since these results were obtained in the presence of phosphate buffer and lactate, a study of the effect of phosphate on the bactericidal action of dihydrostreptomycin on cellular suspensions of *E. coli* seemed to be warranted. An additional factor pointing to the need for more knowledge about the effect of phosphate metabolism on the dihydrostreptomycin mechanism was the reported inhibition of activity of the antibiotic by phosphorylation inhibitors, such as iodoacetate and azide (Rosenblum and Bryson, 1953). Also, Donovick *et al.* (1948) have reported that an increase in phosphate concentration increased the minimal inhibitory dose of streptomycin for growth of *Klebsiella pneumoniae*.

METHOD

E. coli strain B was grown for 18 hr in unaerated nutrient broth and then washed with saline by centrifugation. Approximately 5×10^8 viable bacteria were added to a final volume of 10 ml in 17 by 150 mm test tubes. Except where noted, the bacteria were suspended in 0.9 per cent NaCl. When phosphates were added, they were in the ratio of two parts of anhydrous Na_2HPO_4 to one part of anhydrous KH_2PO_4. The substrate used in these experiments was 0.1 per cent (0.011 M) lactate. The pH value was adjusted to 7.0 where necessary and the dihydrostreptomycin concentration used was 10 μg/ml as the streptomycin base. The suspensions were aerated during the course of incubation by bubbling sterile air through the tubes except where

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noted. Incubation was at 37 C and samples were removed at intervals for surface plating on nutrient agar after appropriate dilution to determine the number of bacteria capable of producing macrocolonies in 48 hr at 37 C.

RESULTS

Earlier results (Hurwitz *et al.*, 1955) had shown that unaerated cellular suspensions were more sensitive to the bactericidal action of dihydrostreptomycin than were aerated suspensions of *E. coli*. This order of sensitivity was found in the presence of .067 M phosphate buffer, saline and 0.1 per cent lactate. When a similar experiment was performed in the absence of phosphate (saline plus 0.1 per cent lactate), the susceptibility to dihydrostreptomycin of both systems increased and aerated suspensions were found to be as susceptible as unaerated cells (table 1). This indicated that phosphate metabolism was possibly implicated in the mechanism of action of dihydrostreptomycin since aeration in the presence of phosphate and substrate made suspensions of sensitive *E. coli* cells more resistant to the bactericidal effect of the drug while similar aeration in the absence of inorganic phosphate did not protect the cells.

TABLE 1

Effect of dihydrostreptomycin on viability of unaerated and aerated suspensions of Escherichia coli cells in presence and absence of phosphate

Treatment	Number of Viable Bacteria/ml	
	0 hr	5 hr
Unaerated in phosphate buffered saline		
With dihydrostreptomycin	5.6×10^7	2.0×10^3
Without dihydrostreptomycin	8.3×10^7	7.6×10^7
Unaerated in unbuffered saline		
With dihydrostreptomycin	8.2×10^7	2.2×10^2
Without dihydrostreptomycin	4.8×10^7	4.0×10^7
Aerated in phosphate buffered saline		
With dihydrostreptomycin	5.5×10^7	4.0×10^7
Without dihydrostreptomycin	8.3×10^7	8.1×10^7
Aerated in unbuffered saline		
With dihydrostreptomycin	4.3×10^7	2.8×10^2
Without dihydrostreptomycin	4.8×10^7	3.9×10^7

Phosphate buffer consisted of Na_2HPO_4 and KH_2PO_4, .067 M, pH 7.0. Substrate was 0.011 M lactate. Dihydrostreptomycin-SO_4 (Pfizer) present in 10 μg/ml concentration expressed as streptomycin base. The pH value even in unbuffered saline did not change during incubation. Counts of viable bacteria were made by spreading 0.1 ml aliquots of treated suspensions on nutrient agar, after proper dilution, and incubating plates for 48 hr at 37 C.

The effect of phosphate concentration on the susceptibility to dihydrostreptomycin of aerated cells was then studied. A basal solution containing 0.9 per cent NaCl, 0.1 per cent lactate, and 10 μg streptomycin base per ml as the dihydrostreptomycin-SO_4 (Pfizer) was prepared. Two parts of Na_2HPO_4 and one part of KH_2PO_4 was added to a portion of this basal solution to a concentration of 6.7×10^{-2} M phosphate and a pH value of 7.0. This phosphate solution was then diluted with basal solution to make a 6.7×10^{-3} M, 6.7×10^{-4} M, and a 6.7×10^{-5} M phosphate solution in 0.9 per cent saline, 0.1 per cent lactate, and 10 μg dihydrostreptomycin-SO_4 per ml. A control, consisting of basal solution, and controls consisting of basal solution containing KCl instead of 0.9 per cent NaCl were also prepared. These solutions were Seitz filtered and added aseptically in 10 ml amounts to 17 by 150 mm aeration tubes. The tubes were inoculated with a washed suspension of *E. coli* strain B grown for 18 hr in unaerated nutrient broth. The suspension was prepared so that each tube after inoculation contained approximately 5×10^7 culturable bacteria per ml. The tubes were aerated and viable counts were made at designated intervals by removing aliquots and spreading quadruplicate 0.1 ml samples on nutrient agar plates by means of a bent glass rod, after proper dilution of the suspension in sterile saline. The maximum amount of dihydrostreptomycin present per agar plate was 1 μg expressed as the streptomycin base. This concentration had no demonstrable effect on growth of our strain on nutrient agar. Measurements of pH before and after incubation in unbuffered saline solution showed that no significant change oc-

TABLE 2

Effect of phosphate concentration on survival of Escherichia coli cells exposed to dihydrostreptomycin

Concentration				Number of Survivors	
Phosphate Buffer	P	K	Na	0 hr	5 hr
moles/L	*g/L*	*g/L*	*g/L*		
6.7×10^{-2}	2.08	1.02	5.23	3.1×10^{7}	2.1×10^{7}
6.7×10^{-3}	2.08×10^{-1}	1.02×10^{-1}	3.53	4.0×10^{7}	1.9×10^{5}
6.7×10^{-4}	2.08×10^{-2}	1.02×10^{-2}	3.36	4.5×10^{7}	3.2×10^{3}
6.7×10^{-5}	2.08×10^{-3}	1.02×10^{-3}	3.34	3.5×10^{7}	5.8×10^{2}
0	0	0	3.34	4.3×10^{7}	2.8×10^{2}
0	0	10.46	0	3.1×10^{7}	2.1×10^{6}
0	0	5.23	0	2.4×10^{7}	1.6×10^{4}
0	0	5.23×10^{-1}	0	2.5×10^{7}	1.3×10^{2}
0	0	5.23×10^{-2}	0	2.5×10^{7}	8×10^{1}

The cellular suspension consisted of a saline-washed, 18-hr old culture from nutrient broth. Substrate was 0.011 M lactate. Dihydrostreptomycin-SO_4 (Pfizer) concentration was 10μg ml expressed as the streptomycin base. Suspensions were aerated at 37 C and 0.1 ml aliquots were spread on the surface of nutrient agar plates after proper dilution in saline. Plate counts of survivors were made after 48 hr incubation.

curred when lactate was used as the substrate. When glucose was used as substrate in an unbuffered saline solution, pH value was lowered to 5.2 during incubation. This increased acidity might account for the lack of sensitivity of cellular suspensions of *E. coli* to dihydrostreptomycin in glucose-saline solution reported by Rosenblum and Bryson (1953).

Table 2 shows the effect of dihydrostreptomycin on viability at different phosphate concentrations under these conditions. Figure 1 shows the relationship of concentration of phosphate to number of survivors after exposure to this drug.

Under the conditions of this experiment, varying the phosphate concentration only slightly affected the Na^+ concentration, whereas the Cl^- concentration remained constant. The K^+ concentration, however, varied directly as did the phosphate concentration. It might therefore be argued that the number of survivors was a function of the K^+ concentration rather than of the phosphate concentration, since the number of survivors increased with the KCl concentration in the absence of phosphate (table 2). However, addition of 6.7×10^{-2} M per L of phosphate containing 1.02 g of K per L almost completely inhibited dihydrostreptomycin activity, whereas 5.23 g of K per L as the chloride in the absence of phosphate enabled survival of less than 0.1 per cent of the bacteria.

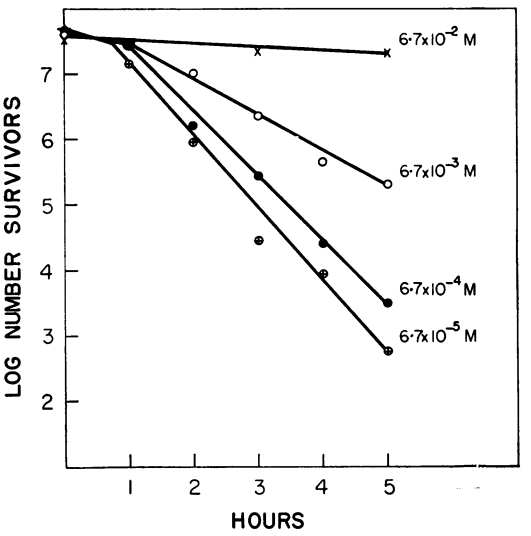

Figure 1. Effect of phosphate concentration on the number of survivors of aerated suspensions of *Escherichia coli* exposed to 10 μg/ml dihydrostreptomycin. Substrate, 0.011 M lactate in saline, pH 7.0.

Chlorides were eliminated as a substitute for phosphate in the previous experiment since the phosphate effect occurred in the presence of a constant concentration of chloride. Donovick *et al.* (1948) have reported that sulfate was even more effective than phosphate in increasing the

minimum inhibitory dose of streptomycin necessary to prevent growth of *K. pneumoniae* in tryptone broth. However, when the previous experiment was repeated with sulfate replacing equimolar concentrations of phosphate, the maximum percentage of survivors found at 6.7×10^{-2} M per L of sulfate was 0.28. At 6.7×10^{-2} M per L of phosphate, about 70 per cent of the bacteria remained viable. In addition, with sulfate instead of phosphate, no difference was found between aerated and unaerated systems, whereas in the presence of phosphate, the dihydrostreptomycin effect on viability could be almost completely reversed in the aerated, but not in the unaerated system.

From the results of these two experiments, it would appear that several cations and anions other than phosphate are able to affect dihydrostreptomycin activity, but that these effects are clearly different from the phosphate effect.

That an interrelationship between dihydrostreptomycin and phosphate concentrations exists is shown by table 3. In these experiments, the phosphate inhibition of the drug was studied at 1, 3, and 10 μg/ml. As phosphate concentration decreased, less drug was needed for inactivation of the bacteria.

TABLE 3

Interrelationship between dihydrostreptomycin and phosphate concentrations

Conc. Phosphate (Moles/L)	Number of Survivors/ml after 5 hr Aeration		
	Dihydrostreptomycin/ml		
	1 μg	3 μg	10 μg
6.7×10^{-2}	4.6×10^{7}	4.5×10^{7}	2.1×10^{7}
6.7×10^{-3}	4.8×10^{7}	2.8×10^{6}	1.9×10^{5}
6.7×10^{-4}	2.0×10^{7}	1.4×10^{6}	3.2×10^{3}
6.7×10^{-5}	1.8×10^{6}	4.0×10^{5}	5.8×10^{2}

TABLE 4

Effect of iodoacetate on the bactericidal action of dihydrostreptomycin

Concentration			Number of Viable Bacteria	
Iodoacetate	Phosphate	Dihydrostreptomycin	0 hr	5 hr
M	M	μg/ml		
0	0.0067	10	4.6×10^{7}	4.8×10^{4}
0.0002	0.0067	10	4.6×10^{7}	1.1×10^{7}
0.0002	0.0067	0	4.5×10^{7}	4.0×10^{7}

Substrate 0.011 M lactate, pH 7.0.

Rosenblum and Bryson (1953) found that the phosphorylation inhibitor, iodoacetate, in a concentration sufficient to prevent growth, would prevent the streptomycin effect on viability of *E. coli*. To further implicate phosphate metabolism in the streptomycin mechanism, this effect of iodoacetate was repeated with suspensions of resting cells. Although our results differed somewhat quantitatively, iodoacetate was found to reverse the action of dihydrostreptomycin. At 6.7×10^{-3} M phosphate concentration, 10 μg dihydrostreptomycin/ml allowed only one bacteria per thousand to produce a macrocolony. When 0.0002 M iodoacetate was added, approximately 24 per cent of the bacteria were found to be viable. Iodoacetate alone in this concentration had no effect on viability. Our results are reported in table 4.

DISCUSSION

The results clearly show that phosphate metabolism is implicated in some way in the mechanism of bactericidal action of dihydrostreptomycin. Aeration in the presence, but not in the absence of phosphate, protected *E. coli* cells against the lethal action of the antibiotic. When the cellular suspension was not aerated, phosphate had little effect on the bactericidal action. In the absence of substrate, the drug at the 10 μg/ml level had no effect on viability of either aerated or unaerated cells.

The interrelationship of dihydrostreptomycin and phosphate concentrations in the aerated suspension seems to indicate that some intermediate which inhibits the bactericidal action of the drug is produced as a result of phosphate metabolism. Furthermore, it would appear that the rate of formation of the intermediate is a function of the phosphate concentration since as phosphate concentration increases, more of the antibiotic is needed to produce the same lethal effect.

Since aeration of a cellular suspension of *E. coli* in the presence of substrate and inorganic phosphate should favor oxidative phosphorylation, the possibility that oxidative phosphorylation may be implicated in the mechanism of action of dihydrostreptomycin is being further investigated.

The reversal of the bactericidal effect of dihydrostreptomycin by iodoacetate is puzzling. If the iodoacetate acts as a phosphorylation inhibitor, it should have the same effect as decreasing phosphate concentration, i.e., the activity of this antibiotic should be enhanced rather than lessened. It should be noted, however, that iodoacetate, being a potent sulfhydryl reagent may act in various ways in addition to inhibiting phosphorylation. The effect of iodoacetate on the activity of the antibiotic points up the complexity of the bacterial mechanism.

SUMMARY

The lethal effect of dihydrostreptomycin on aerated cellular suspensions of *Escherichia coli* strain B has been shown to be inversely proportional to the phosphate concentration of the external medium. Phosphate has little if any effect on activity of this drug when the cellular suspension is not aerated. Substrate is required for the bactericidal effect of dihydrostreptomycin in both aerated and unaerated cellular suspensions. In the aerated suspension, as phosphate concentration increases, more of the drug is required to maintain the same bactericidal action.

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