Modified Degrees of Streptomycin Dependence and Resistance in *Escherichia coli*

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SUMMARY

For three streptomycin-dependent (S-dependent) strains of *Escherichia coli*, the streptomycin concentration necessary for optimal growth during incubation for 24 hr in a nutrient broth medium was about 10 µg./ml. Less than 5 µg./ml. was sufficient for fairly heavy growth and the minimal streptomycin concentration permitting appreciable growth was 0.40 µg./ml. Division of S-dependent bacteria was inhibited at streptomycin concentrations greater than 20 µg./ml. and small inocula gave no visible growth in 24 hr at more than 30 µg. streptomycin/ml.

Following addition of any of several salts to the growth medium at 0.05M growth occurred over a wide range of streptomycin concentrations and the optimum was increased from twofold to as much as 1000-fold. Maximal concentration of streptomycin in which growth of S-dependent *Escherichia coli* was possible increased to as high as 20,000 µg. streptomycin/ml. in some instances, and the minimal concentration which supported growth was increased in the presence of several of the salts. Salts also increased the degree of resistance of a streptomycin-resistant *E. coli* mutant from 20 to as much as 10,000 µg. streptomycin/ml. In 0.10M phosphate-buffered nutrient broth, maximal and optimal concentrations of streptomycin increased with increasing acidity; at pH 5.8 heavy growth of an S-dependent strain of *E. coli* occurred at 200,000 µg. streptomycin/ml.

INTRODUCTION

Growth rates of streptomycin-dependent (S-dependent) bacteria in a mineral medium are proportional to streptomycin concentration (Spotts, 1962) and fairly high streptomycin concentrations, 100 µg. or more/ml., have commonly been used to obtain optimal growth. The activity of streptomycin as an antibiotic is markedly decreased in the presence of salts or in salt-containing defined media however (see Henry & Hobby, 1949; Wasserman, Lessner & West, 1954), and its function as a growth factor for dependent strains may be subject to the same salt effect (Engelberg & Artman, 1962). The 'true' streptomycin optimum for such strains may then be well below reported values. The amount of streptomycin necessary for growth has, in fact, been found to vary with the culture medium (Hashimoto, 1959; Goldschmidt, Matney & Bausum, 1962). We had noted that several S-dependent strains of *Escherichia coli*, routinely grown in nutrient broth at rather low streptomycin concentrations, grew well in the chemically defined minimal medium of Davis & Mingioli (1950) only at concentrations of streptomycin up to 100 times as high. We have therefore attempted to define the optimal streptomycin concentration, as well as the minimal and maximal values for growth of three S-dependent

strains of *E. coli* in an ordinary nutrient medium, and to determine the extent to which these values can be modified by individual salts. Also some study has been made of the influence of salts on the degree of resistance of a low-level streptomycin-resistant mutant.

METHODS

Bacterial strains. Three S-dependent strains of *Escherichia coli* were used: Sd-4 was originally obtained from Dr M. Demerec and has been maintained here as a stock culture for several years; strains HB and D-R (Funk & Plunkett, 1960; Plunkett, 1962) were isolated in this laboratory. The resistant strain, SR, was derived here from *E. coli* strain ATCC 11887.

Media. Difco Nutrient Broth, free of added salt, was the basal growth medium; nutrient agar was used for plating. For studying salt effects the broth was made up in 0.05M-salt solutions before sterilization. Streptomycin sulphate was added to the medium to give the desired concentrations just before use.

Cultural conditions. S-dependent bacteria for the initial inoculum in each experiment were grown overnight at 37.5° in nutrient broth containing 10 µg. streptomycin/ml., resistant bacteria in nutrient broth alone. Samples (5 ml.) of the various growth media which contained graded concentrations of streptomycin were then inoculated with 2×10^3 to 5×10^3 bacteria of either the S-dependent or S-resistant type. The inoculated cultures, in small flasks, were shaken at 37.5° for 24 hr. The extent of growth in each flask was then determined in terms of light extinction (E) readings at 650 mµ, with uninoculated medium as a reference. For a few experiments larger starting inocula, 10^4 to 10^5 bacteria taken directly from stock cultures, were used. For determining their response to streptomycin on a solid medium, bacteria were spread on series of nutrient agar plates containing different concentrations of streptomycin. Degrees of resistance or dependence were judged by the time at which colonies first became visible, by comparative counts, and by the final size and overall appearance of the colonies on continued incubation.

RESULTS

Optimal, maximal and minimal streptomycin concentrations for growth

In salt-free nutrient broth at streptomycin concentrations increasing in 5 µg./ml. increments from 0 to 60 µg./ml., turbidity at the end of 24 hr was highest at 10 µg./ml. There was, however, nearly equivalent growth at 5 and also at 15 and 20 µg./ml. for strains HB and D-R, and for Sd-4 at 5 and 15 µg./ml. At concentrations higher than 25 µg./ml. extinction readings declined rapidly and there was little or no visible turbidity in 24 hr at streptomycin concentrations above 30 µg./ml. When cultures at higher streptomycin concentrations were incubated for an additional 48 hr growth became evident up to 100 µg./ml., but rarely at concentrations greater than 150 µg./ml. The lowest tested streptomycin concentration that would support appreciable growth in nutrient broth was 0.40 µg./ml. for all three S-dependent strains. Above this concentration the growth response improved rapidly and 2 or 3 µg./ml. were sufficient to produce heavily turbid cultures. Strain SR possessed only a slight resistance to streptomycin; the maximal streptomycin concentration which permitted visible growth in nutrient broth in 24 hr was 20 µg./ml.; on further incubation to 72 hr growth occurred up to 40 but seldom above 50 µg./ml.

On solid media, optimal and maximal streptomycin concentrations were considerably higher than in broth. As judged by the time colonies first became visible on the plates and by their final size, the optimal streptomycin concentration for the S-dependent strains on nutrient agar was 50–100 $\mu\text{g./ml.}$ Outside of this range, rate of growth and overall size of the colonies were diminished. This was true regardless of the concentration of streptomycin in the broth in which the bacteria had been grown originally. S-dependent bacteria which grew well in nutrient broth at the optimal concentration, 10 $\mu\text{g. streptomycin/ml.}$, gave only barely visible colonies in 24 hr on 10 $\mu\text{g. streptomycin/ml. agar}$ while, at the same time, large and clearly distinct colonies appeared on agar containing 50–100 $\mu\text{g. streptomycin/ml.}$ On continued incubation colonies appeared on agar up to 700 $\mu\text{g. streptomycin/ml.}$ Similarly, the resistance of strain SR was higher on agar than in broth, with some colony growth becoming visible at streptomycin levels up to 400 $\mu\text{g./ml.}$

Effect of salts

Table 1 gives minimal, maximal, and optimal streptomycin concentrations for growth of S-dependent *Escherichia coli* as well as maximal concentrations for the resistant strain (SR) in salt-containing nutrient broth, when streptomycin was present at concentrations between 0 and 20,000 $\mu\text{g./ml.}$ All the salts tested increased the maximal streptomycin concentration which permitted appreciable growth of S-dependent and S-resistant strains; the minimal requirement for S-dependent growth was also increased in several instances. The streptomycin concentration at which the heaviest growth of S-dependent bacteria took place was, in every case, increased. In many of these instances growth was almost equally heavy over a wide range of streptomycin concentrations; this is shown in Fig. 1, which gives specific data for the growth of strain HB in nutrient broth in comparison with growth in media containing each of four of the tested salts. Most salts had the additional effect of stimulating overall growth so that extinction readings at the optimal streptomycin concentrations in the salt-containing media were generally higher than those in nutrient broth controls at the 10 $\mu\text{g./ml.}$ optimum for this medium. Even where overall growth was inhibited, as with K_2HPO_4 , the optimal and maximal streptomycin concentrations were again at values higher than those for nutrient broth alone.

Effects of phosphate buffer

Most of the salts had little effect on the pH value of the medium at the concentration used. Nutrient broth alone had pH values between 6.8 and 7.0; the salt-containing broths ranged from pH 6.5 to 7.3 except for K_2HPO_4 with a value near pH 8.0. When optimal and maximal streptomycin concentrations were determined for nutrient broth media made up in 0.10M-phosphate buffer at three pH values, growth responses corresponding to those shown in Fig. 2 were obtained. The acid-buffered medium was especially effective in increasing growth at high streptomycin concentrations. In one experiment heavy growth of strain HB was observed in this medium even with streptomycin 200,000 $\mu\text{g./ml.}$ At pH 5.8 and 7.0 there was no appreciable growth at streptomycin 10 $\mu\text{g./ml.}$, the optimum for nutrient broth, or even at 50 $\mu\text{g./ml.}$ Optimal growth occurred only at concentrations of the order of several hundred $\mu\text{g./ml.}$ In the alkaline medium growth of S-dependent bacteria,

Table 1. *Minimal, optimal, and maximal streptomycin concentrations for growth of streptomycin-dependent and -resistant strains of Escherichia coli in nutrient broth containing 0.05M-salts*

Streptomycin concentrations: 0, 10, 20, 50, 100, 500, 1000, 5000, 10,000, 20,000 $\mu\text{g./ml.}$ Extinction (E) at 650 m μ was recorded after growth for 24 hr at 37.5°. Minimal and maximal values are, respectively, the lowest and highest concentrations of streptomycin which permitted visible growth (E > 0.08), the optimal concentration is that at which the extinction at 24 hr was highest and is given as a range where turbidity readings were within ± 0.03 of this value.

Salt	Streptomycin concentration ($\mu\text{g./ml.}$)												Strain sr.
	Strain HB			Strain Sd-4			Strain D-R						
	Min.	Opt.	Max.	Min.	Opt.	Max.	Min.	Opt.	Max.	Min.	Opt.	Max.	
NaCl	10	20-100	100	10	10-20	100	10	10-20	500	10	10-20	500	100
K ₂ HPO ₄	10	50-100	500	20	100	100	50	100-500	500	50	100-500	500	1,000
(NH ₄) ₂ SO ₄	50	500-10,000	> 20,000	50	500-10,000	> 20,000	10	500-5,000	> 20,000	10	500-5,000	> 20,000	10,000
NH ₄ Cl	10	50-100	1,000	10	20	1,000	10	20-100	500	10	20-100	500	500
MgSO ₄	50	500-1,000	1,000	50	20-500	1,000	20	50-1,000	1,000	20	50-1,000	1,000	1,000
MgCl ₂	100	500-1,000	5,000	50	500	5,000	20	500	10,000	20	500	10,000	1,000
NH ₄ NO ₃	10	20-500	1,000	10	20	1,000	10	20-50	1,000	10	20-50	1,000	1,000
Na citrate	500	1,000	1,000	50	500-1,000	1,000	500	500	500	500	500	500	1,000

although limited, occurred again only at streptomycin concentrations well above those required for growth in plain nutrient broth. A phosphate-buffered medium also increased the degree of streptomycin resistance of strain SR. At pH 5.9 heavy growth of strain SR took place at all streptomycin concentrations to 24,000 $\mu\text{g./ml.}$ At pH 7.0 resistance was increased to over 12,000 and at pH 7.8 to 3200 $\mu\text{g. strepto- mycin/ml.}$

Fig. 1

Fig. 2

Fig. 1. Effect of streptomycin concentration on growth of *Escherichia coli* strain HB in nutrient broth in the presence of 0.05M-salts. □, Nutrient broth control; ○, NaCl; △, K_2HPO_4 ; ●, NH_4Cl ; ▲, MgCl_2 .

Fig. 2. Effect of streptomycin concentration on growth of *Escherichia coli* strain HB in phosphate-buffered nutrient broth. ○, pH 5.80; △, pH 7.00; □, pH 7.75.

DISCUSSION

The rather common practice of supplementing nutrient broth with 0.5% NaCl may account for some of the high optimal streptomycin values that have been reported by others for streptomycin dependent growth in this medium. Our results indicate that, for three S-dependent strains of *Escherichia coli*, appreciable growth in ordinary nutrient broth was possible even at streptomycin 0.40 $\mu\text{g./ml.}$, and heavy growth at less than 5 $\mu\text{g./ml.}$ In fact, in the absence of added salt, streptomycin actually inhibited the rate of growth of S-dependent bacteria at values only slightly in excess of the optimal 10 $\mu\text{g./ml.}$ This inhibition suggests that streptomycin retains some of its antibiotic action even when it is required for growth and it may give some support to the assumption that streptomycin functions at a site within the cell rather than its surface. It would be of interest in this respect to compare the effects of excess streptomycin on S-dependent bacteria, with those resulting from streptomycin treatment of sensitive bacteria, as a possible means of distinguishing primary from secondary streptomycin effects.

Engelberg & Artman (1961) calculated that as little as 0.60 $\mu\text{g. streptomycin/ml.}$ medium should satisfy the requirement of *Escherichia coli* strain Sd-4, provided all the streptomycin was absorbed. Sodium chloride decreased streptomycin uptake however, and in a defined medium the amount of the antibiotic bound by the S-dependent bacteria was only a small fraction of that required for growth. It seems likely, therefore, that salt interference with streptomycin uptake may be in some part responsible for the highly elevated streptomycin requirements we have found in salt-containing media. However, the effect is of a greater magnitude than might be expected solely on the basis of competition for cell binding sites. The

rather narrow range of effectiveness of streptomycin in nutrient broth alone and the inhibition of growth of S-dependent bacteria that occurred at a streptomycin concentration only 2.5 to 5 times the optimal value is in sharp contrast to the continued effectiveness of streptomycin as a growth factor over a wide range of concentrations, and up to values over 50 times the optimum in the presence of salts. Although the salt effect was a fairly general one, there was considerable variation in the effect of different salts even at equivalent ionic strengths. Specific ions are known to modify the activity of streptomycin against S-sensitive bacteria (Ørskov & Ørskov, 1960; Hurwitz & Rosano, 1958, 1962; Mager, Benedict & Artman, 1962; Bragg & Polglase, 1963; Willick & Polglase, 1963) and similar effects might account in part for this variation. Magnesium, for example, is notably effective as a streptomycin antagonist. Since a function at the bacterial ribosome is postulated both for the magnesium ion (Hershko, Amoz & Mager, 1961) and for streptomycin (Davies, 1964; Cox, White & Flaks, 1964), competition for a binding site here could explain the high streptomycin requirement of S-dependent bacteria in the presence of magnesium salts. Other cations might be expected to have a similar effect.

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