phenoxymethyl penicilloic acid was added to furnish concentrations of 0, 0.25, and 0.5 mg./ml. These solutions, together with standard phenoxymethyl penicilloic acid preparations as in Procedure D, were submitted to Procedures C and D. Recoveries ranged from 92 to 100%. Similar results were obtained with phenethicillin and phenoxyethyl penicilloic acid.

Extraction Efficiency—Under the described conditions the extraction of phenoxymethyl and phenoxyethyl penicilloic acids from aqueous solutions by chloroform was between 88 and 92% complete. This was confirmed by assaying, by nitration, samples containing 0.5 mg./ml. and 1 mg./ml. directly and after extraction.

DISCUSSION

The yellow quinoid radical produced by nitration of these compounds that originate from the phenolic group could be produced if phenol or its derivatives were present in the sample used for nitration.

During numerous assays of urine samples without penicillin or penicilloic acid, the blank value of the chromatographed chloroform extract was close to or similar to the normal blank value, indicating that phenolic compounds present in urine do not interfere with this assay. The difference in R_f values for penicillins and for the corresponding penicilloic acids probably derives from the fact that the former are monobasic acids, which generally have high values compared with dibasic acids to which class the latter belong. The R_f value of phenoxyacetic acid in the method used is higher than that of penicillin; possible interference by this acid, if present in the extract, is thus eliminated.

The data given in Tables I and II provide an example only of the information obtainable using this method. No attempt has been made to correlate the results with liquid intake, excretion volumes, or other controllable factors.

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Kinetics and Mechanisms of Drug Action on Microorganisms X: Action of Spectinomycin on *Escherichia coli* by Microbial Kinetics

JOBST B. MIELCK and EDWARD R. GARRETT*

Abstract □ The steady-state growth of *Escherichia coli* cultures, $N = N_0 e^{k_0 t}$, is slowly inhibited by spectinomycin to a new steady state with a new rate constant, k_{app}. The $k_{app.}$ is linearly dependent on drug concentration, S, above a certain minimum concentration of spectinomycin, S^*; *i.e.*, $k_{app.} = k_0 - k_S(S - S^*)$. This minimum concentration is a function of the concentration of the media and can be assigned to binding or removal of microbiologically effective spectinomycin as protonated material by the components of the media. The logarithm of the inhibitory constant, k_S, linearly increases with the pH of the media to pH 7.6, and this implies that only uncharged material is biologically active. The slow rate of achievement of a drug-equilibrated, steady-state, microbial generation rate can be reconciled with a relatively rapid reequilibrated rate on dilution with fresh media by postulating depletion of a cell-generated vital metabolite linked to the growth rate of the microorganism.

Keyphrases □ Microorganisms—mechanism, kinetics, drug activity □ Kinetics, mechanism—spectinomycin activity □ Spectinomycin action, *E. coli*—kinetics, mechanism □ pH, organism population, nutrient concentration, effects—spectinomycin-affected generation rates □ *E. coli* generation rates—spectinomycin concentration

The aminoglycoside spectinomycin has antibacterial activity against a variety of Gram-positive and Gram-negative microorganisms (1). The inhibitory action is bacteriostatic and is reversed by washing the drug-affected cells (2). The antibiotic forms a stoichiometric 1:1 complex with the 30-S ribosomal subunit extracted from *Escherichia coli*. The formation of this complex blocks some steps in the translocation of the peptidyl-RNA from the acceptor site for aminoacyl *t*-RNA to the peptidyl donor site. Protein synthesis is thus inhibited (2).

The determination of the generation rates of *E. coli* as a function of the concentrations of a variety of antibiotics and chemotherapeutic agents has established a useful procedure to quantify the effects of drugs on the time course of bacterial generation (3–8).

The purposes of this study are to determine kinetically the inhibitory effects of spectinomycin on the generation rate of *E. coli* in defined concentration ranges and to evaluate the effects of variable inoculum size, concentration, and pH of the culture medium on drug activity.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* strain B/r were used in all experiments. The slants had been prepared from a single colony and were stored in a refrigerator at 4°.

Culture Media—Antibiotic medium 3[1] was rehydrated according to the specifications of the manufacturer. The media were filtered twice through Millipore 0.45 μ HA filters and autoclaved at 120° for 15 min. The pH of the media was 7.05 ± 0.05. Various amounts of Millipore-filtered 1.7 *N* HCl and 2.0 *N* NaOH, respectively, were added aseptically after the sterilization to obtain pH values within a range of 7.50 to 5.87 for the investigation of the anti-

[1] Bacto Antibiotic Medium 3, Difco Laboratories, Detroit, Mich.

bacterial activity of spectinomycin as a function of the pH. Media with half and twice the amount of nutrients, respectively, were prepared and adjusted to a pH of 7.14 ± 0.02 for the study of the binding capacity of the medium for the antibiotic.

Antibiotic—An assayed sample of spectinomycin sulfate[2] was used (640 ± 35 mcg. base/mg.).

Bacterial Generation—A 5-ml. aliquot of culture medium was inoculated from a fresh slant, and the culture was allowed to grow for 12 hr. at 37.5° in an incubator. A 1.0-ml. sample was then diluted with 9.0 ml. broth at 37.5°, which was diluted 100-fold into a fresh medium after 60 min. The growth of this culture was followed up to 10^7 *E. coli*/ml. Samples of this culture were finally appropriately diluted in a single step, or in several steps, into replicate volumes of 49.0 ml. broth in 125-ml. loosely capped conical flasks to achieve the desired organism concentrations. The cultures were maintained at 37.5 ± 0.1° in a 50-gal. constant-temperature water bath equipped with a shaker. All pipets and media used for the dilutions of the cultures were kept at 37.5° to protect the organisms from temperature shocks.

Total Count Method—One-milliliter samples were withdrawn at 20-min. intervals from the cultures. They were diluted to obtain counts within a range of 10,000 to 30,000 counts/50 μl. on the Coulter counter, model B.[3] The diluent used was a Millipore 0.45 μ HA-filtered aqueous solution of 0.85% NaCl and 1% formaldehyde. The instrument was equipped with a 30-μ orifice; the settings were: an aperture current of 5, amplification of 8, gain of 10, lower threshold of 13, and upper threshold at maximum. The total counts were corrected for the background count of the particular batch of medium used, diluted in the same way as the sample. The background counts in general did not exceed 1000 counts/50 μl.

Viable Count Method—Samples of 0.50 ml. were withdrawn from the cultures and appropriately diluted into sterilized 0.85% saline solution so that 50 to 150 colonies per plate would result. From these dilutions, aliquots of 1.00 ml. were pipeted onto each of three replicate agar plates. The plates were incubated for 48 hr. at 37.5° and the colonies counted on a Colony counter model C-110.[4]

Effect of Antibiotic Concentration on Generation Rates—Fresh solutions of spectinomycin in distilled water were aseptically prepared for each experiment. They were sufficiently diluted so that aliquots of 1.00 ml. added to the 49-ml. culture volumes yielded the desired graded drug concentrations between 6.00 and 20.0 mcg./ml. The solutions were added to the cultures growing at 37.5° in the logarithmic growth phase at determined organism concentrations. Samples were withdrawn every 20 min. and counted by the respective method. A culture without drug was studied in each experiment to obtain the generation rate constant in absence of drug as a reference. The generation curves for 21.9, 35.0, and 43.8 mcg./ml. spectinomycin at pH 7.05 were obtained by both the viable and total cell count methods (Fig. 1).

Effect of Organism Population on Drug-Affected Generation Rates—Each flask of four sets of four replicate 49-ml. volumes of culture medium of pH 7.05 was inoculated with 1.00 ml. of appropriately diluted culture growing in the logarithmic growth phase. The organism concentrations in the respective sets were 5×10^3, 5×10^4, 5×10^5, and 5×10^6 *E. coli*/ml. at the time of addition of drug. Drug solutions were added to achieve spectinomycin concentrations of 15.0, 24.0, and 30.0 mcg./ml., respectively. One culture in each set contained no drug. Total cell counts were obtained from samples drawn every 20 min.

Reversibility of Drug Action—A 49-ml. volume of culture growing in the logarithmic growth phase at 37.5° contained at time zero a population of 10^6 *E. coli*/ml. (Curve A in Fig. 2). Drug was added to achieve a concentration of 28 mcg./ml. spectinomycin (Curve B in Fig. 2) at time zero. An aliquot of 2.00 ml. of this culture was diluted into 49 ml. of fresh medium at 50 min. after the addition of drug to give 1.12 mcg./ml. (Curve C in Fig. 2). At the same time, 5.00 ml. of the culture of Curve B was diluted into 45.0 ml. of broth that contained 28.0 mcg./ml. spectinomycin (Curve D in Fig. 2), and 2.00 ml. of the drug-free culture of Curve A was diluted into 49 ml. of drug-free broth (Curve A_1 in Fig. 2). At 240 min., 0.80 ml. of the culture of Curve B was diluted into each of two replicate 49-ml. volumes of fresh medium to give 4.48 mcg./ml. (Curve E in Fig. 2). When these cultures containing 4.48 mcg./ml. drug reached the steady-state growth phase, at 355 min. and 6×10^6 *E. coli*/ml., a 1.00-ml. aliquot of spectinomycin solution was added to one of the replicate cultures to establish a drug concentration of 30.5 mcg./ml. (Curve F in Fig. 2). The total number of organisms in the various cultures was obtained from samples withdrawn every 20 min.

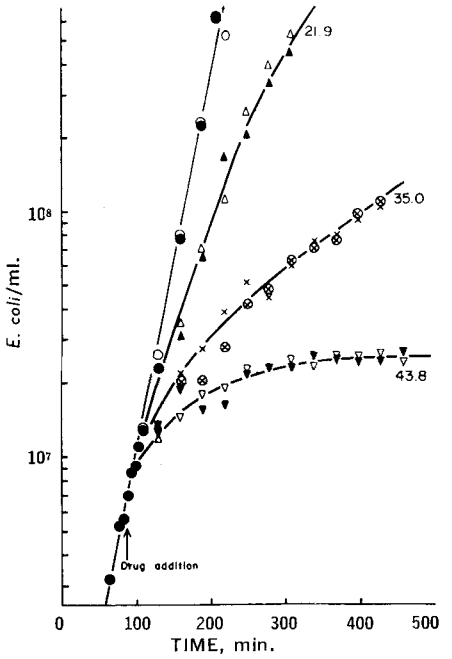

Figure 1—*Generation rate curves of* E. coli *at 37.5° and pH 7.05 obtained by total (closed symbols) and viable (open symbols) cell counts. The curves are labeled with the respective concentrations of spectinomycin (mcg./ml.).*

Effect of pH on Drug-Affected Generation Rates—Sufficient amounts of 1.7 *N* HCl and 2.0 *N* NaOH were added to the culture media to obtain pH values of 5.87, 6.20, 6.53, 6.55, 6.72, 6.87, 7.13,

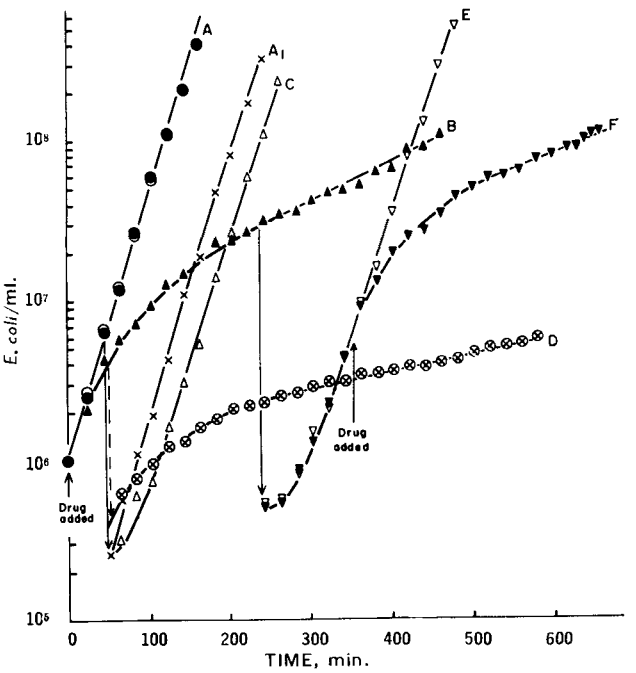

Figure 2—*Generation rate curves of* E. coli *at 37.5° and pH 7.10 on addition and dilution of spectinomycin. The curves, final drug concentrations (mcg./ml.), and apparent steady-state generation rate constants (10^4 $k_{app.}$ in $sec.^{-1}$) were A, 0, 6.20; A_1, 0, 6.24; B, 28.0, 0.96; C, 1.12, 5.95; D, 28.0, 0.42; E, 4.48, 5.90; and F, 30.5, 0.79.*

[2] The Upjohn Co., Kalamazoo, Mich.
[3] Coulter Electronics Co., Hialeah, Fla.
[4] New Brunswick Scientific Co., New Brunswick, N. J.

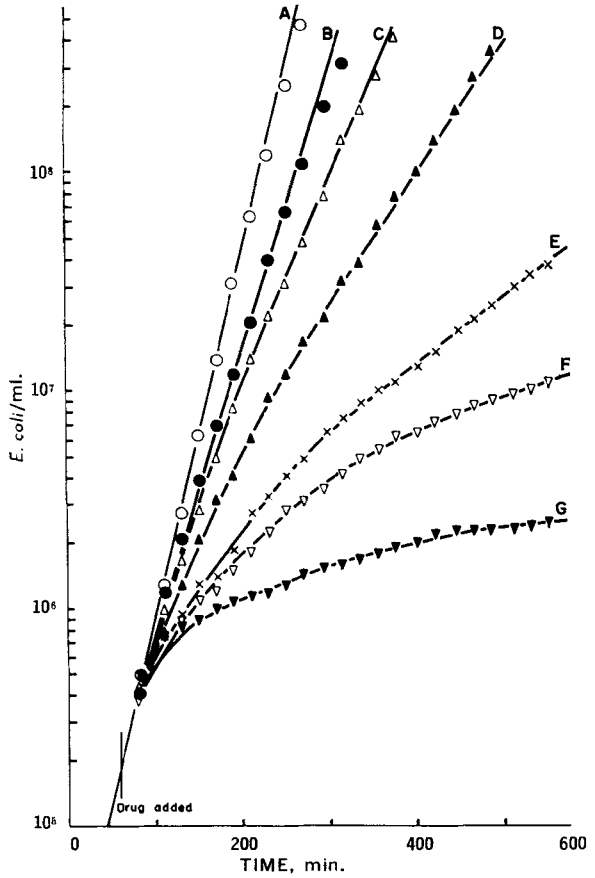

Figure 3—*Typical generation curves for* E. coli *at 37.5° and pH 6.72. The curves, spectinomycin concentrations (mcg./ml.), and generation rate constants (10^4 $k_{app.}$, $sec.^{-1}$) were: A, 0, 6.30; B, 32.0, 4.56; C, 40.0, 3.58; D, 53.3, 2.37; E, 66.7, 1.18; F, 72.7, 0.572; and G, 80, 0.*

7.22, 7.30, and 7.50, respectively. The pH values of these studies were read at 10^7 *E. coli*/ml., using a Schott model U combination glass electrode and a Beckman Zeromatic pH meter with an expanded scale. From a single slant, separate cultures for each pH were prepared as previously described. At organism concentrations in the range from 8×10^4 to 8×10^5 *E. coli*/ml., 1.00-ml. aliquots of spectinomycin solutions were added to achieve the desired drug concentrations. A typical example is given in Fig. 3. In addition to the data obtained for the total number of organisms as a function of time, the pH in some of the cultures was monitored as a function of the organism concentration.

Table I—Bacteriostatic Activity Constants for Spectinomycin against *E. coli* at Various Media pH Values

pH[a]	$10^5(k_S \pm SD)$,[b] ml. mcg.$^{-1}$ sec.$^{-1}$	$10^4\ k_0$,[c] sec.$^{-1}$	$10^4\ k'_0$,[d] sec.$^{-1}$	S^*,[e] mcg. ml.$^{-1}$
5.87	0.142 ± 0.017	5.90	6.16	18.3
6.30	0.294 ± 0.012	6.40	6.26	−4.76
6.30	0.389 ± 0.015	6.30	6.68	9.77
6.53	0.869 ± 0.017	6.55	8.01	16.8
6.55	0.562 ± 0.034	6.29	7.29	17.8
6.72	0.956 ± 0.028	6.30	7.52	12.8
6.87	1.32 ± 0.12	6.09	7.05	7.27
7.13	2.20 ± 0.10	6.23	7.75	6.91
7.22	2.98 ± 0.14	6.50	6.80	1.01
7.30	2.42 ± 0.10	5.91	6.51	2.48
7.50	4.88 ± 0.11	6.19	7.89	3.48

[a] pH of the culture medium at 10^7 *E. coli* $ml.^{-1}$. [b] Slope of a plot of $k_{app.}$ in $sec.^{-1}$ *versus* drug concentration, S, in mcg. $ml.^{-1}$ obtained by a least-squares fit of the data to $k_{app.} = k'_0 - k_S S$, and the standard deviation of that slope. [c] Experimentally observed generation rate constant at $S = 0$. [d] Calculated generation rate constant at $S = 0$. [e] Calculated drug concentration, S, at $k_{app.} = k_0$.

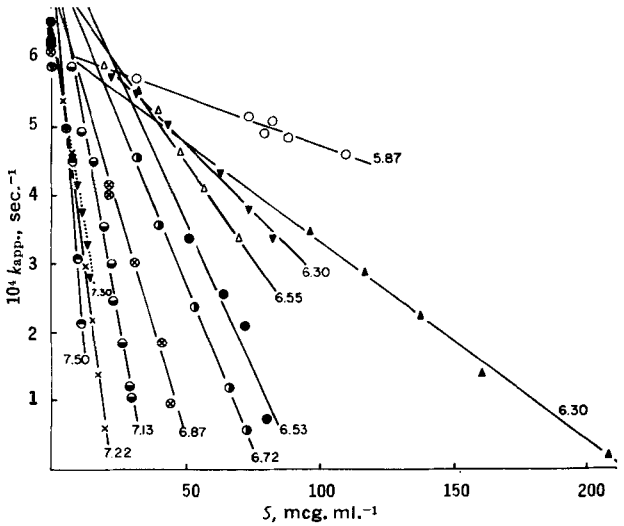

Figure 4—*Apparent first-order generation rate constants,* $k_{app.}$ *in* $sec.^{-1}$, *as functions of the spectinomycin concentration,* S, *in mcg./ml. The curves are labeled with the pH of the media.*

Effects of Nutrient Concentration on Drug-Affected Generation Rates—Culture media of half and twice the normal concentration of nutrients were prepared and adjusted to pH 7.14 ± 0.02 and 6.87 ± 0.02, respectively. Microbial cultures were prepared in the previously described manner using these media separately. The respective relative nutrient concentrations, pH values, and the range of spectinomycin concentrations (mcg./ml.) used were: (*a*) for half the nutrient concentration: at pH 7.10 from 8 to 20 mcg./ml., at pH 7.16 from 6.7 to 12 mcg./ml.; and at pH 6.87 from 23 to 32 mcg./ml.; and (*b*) for twice the nutrient concentration: at pH 7.16 from 7 to 16 mcg./ml. The drug-free and drug-affected generation rate constants were obtained by total cell counts.

RESULTS

Effect of Drug Concentration on Generation Rates—The number of *E. coli* per milliliter obtained by both total and viable cell counts is plotted on a semilogarithmic scale against time in Fig. 1. The numbers from the respective methods are coincident; thus no kill or death of microorganisms is observed between 0 and 44 mcg./ml. over the time period studied. Thus, this study can be based upon total cell counts obtained within this range of drug concentration.

A typical family of generation curves obtained over a wide range of graded drug concentrations and based on total cell counts is given in Fig. 3. Upon addition of drug to the culture growing in the logarithmic phase, the generation rate is decreased slowly until a new spectinomycin-affected steady-state phase is established. Apparent first-order generation rate constants, $k_{app.}$ in $sec.^{-1}$, are obtained from the slopes of this linear portion in accordance with the equation

$$\log N = \log N_0 + \frac{k_{app.}}{2.303}\, t \qquad \text{(Eq. 1)}$$

where N is the number of microorganisms. A plot of the $k_{app.}$ values obtained at various pH values against spectinomycin concentration, S (Fig. 4), demonstrates the linear dependence of $k_{app.}$ on S at lower $k_{app.}$ values in accordance with the equation

$$k_{app.} = k_0' - k_S S;\ k_{app.} < k_0 \qquad \text{(Eq. 2)}$$

where k_0' in $sec.^{-1}$ is the extrapolated value for $k_{app.}$ at $S = 0$, the slope k_S in ml./mcg.-sec. is the inhibitory constant of the antibiotic, and k_0 is the generation rate constant in the absence of the antibiotic. The values for k_S and k_0', calculated from least-squares fits, and the experimentally determined k_0 in $sec.^{-1}$ are listed in Table I.

Effect of pH on Drug-Affected Generation Rate—The apparent first-order generation rate constants, $k_{app.}$, obtained at pH values from 5.87 to 7.50 in the culture media in the absence and presence

of graded spectinomycin concentrations, S, are plotted against S in Fig. 4. The drug-free generation rate constants are independent of pH in this range [Table I and (5)], while the drug-affected generation rate constants decrease with increasing pH at comparable drug concentrations. The calculated inhibitory constant, k_S, and intercepts, k_0', are listed in Table I. A plot of the log k_S against pH is given in Fig. 5. The drug concentrations, $S = S^*$ (Table I), that produce $k_{app.} = k_0$, the drug-free generation rate constant, can be calculated from Eq. 2. The pH values of the media in drug-free and drug-affected growing cultures were constant to within 1% of their initial values up to 10^8 *E. coli*/ml. (5).

Effect of Organism Populations on Drug-Affected Generation Rates—Apparent first-order generation rate constants, $k_{app.}$, for the logarithmic portion of the growth curves obtained in the presence of three different spectinomycin concentrations over a 1000-fold range in inoculum size at the time of drug addition are given in Table II. Although the data show a trend toward smaller rate constants at lower inoculum sizes, the differences appear insignificant compared to the observed values for $k_{app.}$ at $S = 0$. At high organism concentrations ($N > 10^8$ *E. coli*/ml.) or after long periods of drug-affected generation ($t > 600$ min.), the generation rate of the drug-affected cultures increased markedly and approached a new steady state. Apparently, a very small fraction of the bacteria was resistant to spectinomycin. The estimated fraction of the inoculum that was not inhibited was less than 0.01 when the growth curves of the new steady state were extrapolated to the time of inoculation.

On the basis of these observations, it cannot be concluded that the antibiotic is consumed by the organism or that a drug antagonist is excreted as a function of the numbers of organisms (6). It is, therefore, assumed that the drug concentration is in sufficient excess to remain constant over the time period of observation.

Reversibility of Drug Action—Addition of drug to a culture growing in the logarithmic growth phase (Curve A in Fig. 2, $k_0 = 6.20 \times 10^{-4}$ sec.$^{-1}$) to establish 28.0 mcg./ml. spectinomycin (Curve B in Fig. 2) resulted in a gradual decrease in the rate of generation over a period of 200 min. until a new steady state ($k_{app.} = 0.96 \times 10^{-4}$ sec.$^{-1}$) was achieved. Curve A_1 in Fig. 2 ($k_0 = 6.24 \times 10^{-4}$ sec.$^{-1}$) demonstrates that the technique of diluting the cultures does not significantly affect the generation rate constant. Upon dilution of the culture of Curve B in Fig. 2 into fresh medium where the drug concentration of 28.0 mcg./ml. was reduced to 1.12 mcg./ml. (Curve C in Fig. 2), the generation rate gradually increased over a period of 60 min. until a new logarithmic phase with a generation rate constant of 5.95×10^{-4} sec.$^{-1}$ was established.

Table II—Effect of Inoculum Size[a] on Spectinomycin-Affected Growth of *E. coli*

Inoculum Size, *E. coli*/ml.	[Spectinomycin], mcg./ml. — $10^4 k_{app.}$, sec.$^{-1}$				S^*,[b] mcg./ml.
	0	15.0	24.0	30.0	
5×10^3	6.18	3.81	0.96	—	7.7
5×10^4	6.00	3.92	1.37	0.173	5.2
5×10^5	6.00	4.05	1.60	0.238	7.3
5×10^6	6.09	4.04	—	—	—

[a] At time of drug addition. [b] Calculated drug concentration, $S^* = S$, at $k_{app.} = k_0$ from linear relation $k_{app.} = k_0' - k_S S$.

When the culture of Curve B was diluted 10-fold into a fresh medium where the original drug concentration was maintained, the continuous decrease in the generation rate was not interrupted. However, the generation rate constant, 0.42×10^{-4} sec.$^{-1}$ established at 220 min., was lower than that of Curve B. The expected value for this rate constant was that for Curve B in Fig. 2, 0.96×10^{-4} sec.$^{-1}$. The difference in these rate constants could suggest that the activity of spectinomycin is dependent on the concentration of microorganisms. However, the results from the studies of the influence of inoculum size on the generation rate constants over a much wider range of organism concentrations did not show a significant dependence. Dilution of the culture of Curve B at the beginning of its steady-state phase at 240 min. into drug-free medium (Curve E in Fig. 2) yielded a gradual increase in the generation rate over a period of 80 min. as compared to the 60 min. of Curve C in Fig. 2, whereafter a new steady-state phase with a rate constant of 5.90×10^{-4} sec.$^{-1}$ was established.

When drug was added to a replicate culture of that of Curve E in Fig. 2 to yield a drug concentration of 30.5 mcg./ml., a gradual decrease in the generation rate over 160 min. was observed, when a new logarithmic phase with a generation rate constant of 0.79×10^{-4} sec.$^{-1}$ was achieved.

Effect of Nutrient Concentration on Drug-Affected Generation Rates—The concentration of nutrients in the culture media has a significant effect on the drug-affected generation rate constants (Table III and Fig. 6). The calculated values for the spectinomycin concentration, $S = S^*$ when $k_{app.} = k_0$, increase nearly twice when the nutrient concentration is doubled. These S^* values were calculated from least-squares fits of the generation rate constants to the drug concentrations in accordance with Eq. 2.

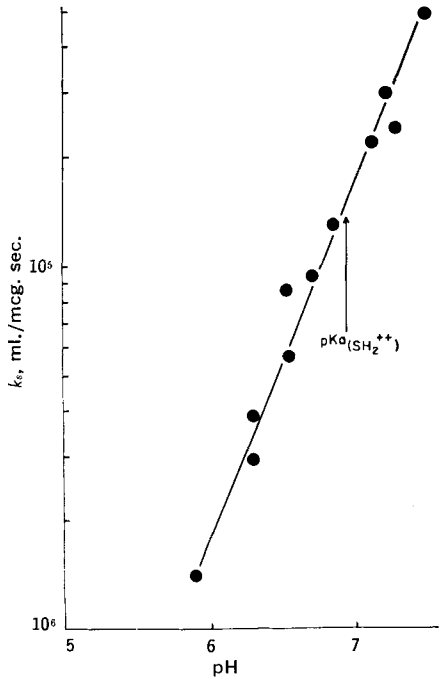

Figure 5—*Dependence of the log of the inhibitory constant,* k_S, *in ml./mcg.-sec. of spectinomycin on the pH of the culture media.*

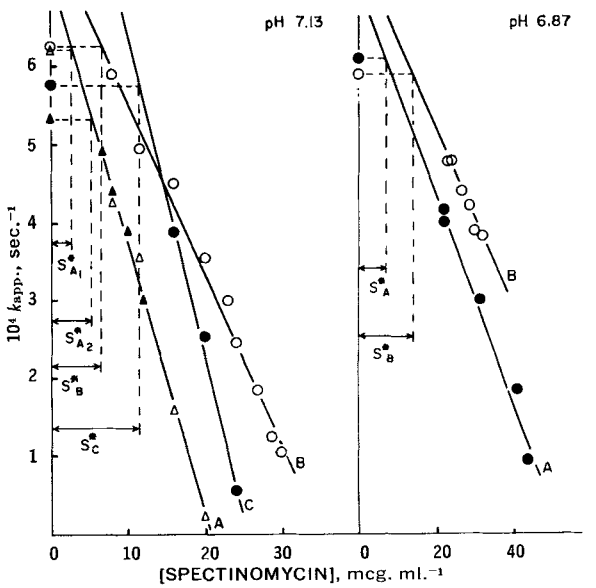

Figure 6—*Effect of various nutrient concentrations on the dependence of the generation rate constants of* E. coli *on the spectinomycin concentration. The curves, relative nutrient concentrations and calculated spectinomycin concentration,* $S = S^*$ *(mcg./ml.) at* $k_{app.} = k_0$, *were at pH 7.13: A, 0.5, 2.96, and 5.49 respectively; B, 1, 6.9; C, 2, 12.0, and at pH 6.87: A, 1, 7.27; and B, 2, 14.1.*

Table III—Effect of Nutrient Concentration on the Biologically Ineffective Spectinomycin Concentration, S^*, against *E. coli*

Nutrients[a]	pH	$10^5\ k_S$[b]	$10^4\ k_0'$[c]	$10^4\ k_0$[d]	S^*[e]
0.5	7.10	0.348	7.23	6.20	2.96
	7.16	0.346	7.23	5.33	5.49
1	7.13	0.260	7.75	6.23	6.91
2	7.16	0.416	10.7	5.73	12.0
1	6.87	0.129	7.05	6.09	7.27
2	6.87	0.118	7.56	5.90	14.1

[a] Relative nutrient concentration with regard to normal antibiotic medium 3. [b] Least-squares slope from the linear relation $k_{app.} = k_0' - k_S S$. [c] Intercept calculated from this relation. [d] Experimentally determined $k_{app.}$ at $S = 0$. [e] Calculated drug concentration, S, at $k_{app.} = k_0$.

DISCUSSION

The drug-affected, apparent first-order generation rate constant, $k_{app.}$, is linearly related to spectinomycin concentration (Fig. 4 and Eq. 2). Similar equations have been successfully employed to define the antibacterial activity of chloramphenicol (9, 10), tetracycline (9, 10), and, in part, lincomycin (5). They are based on the assumption that the extracellular spectinomycin concentration, S, equilibrates with the drug concentration, S', in the receptor site compartment or biophase. Here the antibiotic reversibly binds to receptor sites, R, to form the drug–receptor complex, RS':

$$S \overset{K_1}{\rightleftharpoons} S';\ S' + R \overset{K_2}{\rightleftharpoons} RS' \qquad \text{(Eq. 3)}$$

The equilibria are characterized by the respective equilibrium constants K_1 and K_2 during the drug-affected steady state. The constant K_2 is the affinity constant of spectinomycin for its receptor site, and the product:

$$K_1K_2 = k_b(\text{ml./mcg.}) \qquad \text{(Eq. 4)}$$

is a quantitative measure of the antibacterial activity. It is implied that the fraction of drug-free receptor sites is proportional to the rate of protein synthesis. A certain fraction of this protein synthesis would be proportional to the rate of generation of bacteria, while a large fraction may be necessary for the maintenance of viability (9). The rate of increase in the number of bacteria may be defined in accordance with this concept (9) by

$$\frac{dN}{dt} = \{q\,(k_p - k_p') - qk_pK_1K_2[S]/(1 + K_1K_2[S])\}N \qquad \text{(Eq. 5)}$$

where k_p is the overall rate constant of protein synthesis; k_p' is the minimum rate constant of protein synthesis necessary for bacterial generation; and q is a proportionality constant linking the rate of population increase of N organisms, dN/dt, to the net rate of protein synthesis and to the total number of organisms, N, in a balanced culture. If only a small fraction of the total number of receptor sites has to be reacted with spectinomycin to achieve complete inhibition of bacterial generation,

$$K_1K_2[S] \ll 1 \qquad \text{(Eq. 6)}$$

As a consequence, Eq. 5 reduces to

$$\frac{dN}{dt} = \{q\,(k_p - k_p') - qk_pK_1K_2[S]\}N \qquad \text{(Eq. 7)}$$

Combining the constants in Eq. 7 yields

$$\frac{dN}{dt} = (k_0 - k_S[S])N \qquad \text{(Eq. 8)}$$

where $k_{app.}$ is defined as

$$k_{app.} = (k_0 - k_S[S]) \qquad \text{(Eq. 9)}$$

which is identical with Eq. 2 when k_0' is substituted for k_0. The calculated $k_{app.}$ (Eq. 2) equals k_0 at a concentration S^* (Table I) of spectinomycin. This implies that it takes a definite concentration of antibiotic to be exceeded before antibacterial activity is manifested. It is possible that this amount is bound either by the microorganisms or by the constituents of the culture media. If the bacteria were responsible for this binding, the calculated value for S^* would be a function of the inoculum size. The studies conducted with different inoculum sizes showed no such dependency for S^* (Table II), and permitted the conclusion that the organisms were not responsible for the binding of spectinomycin. If the media were responsible for this binding, S^* would vary with the concentration of nutrients. The experiments carried out with culture media that contained half and twice the normally used concentration of nutrients did show that S^* definitely increased with the nutrient concentration; these experiments permitted the conclusion that the constituents of the media effectively inhibited a definite amount of spectinomycin from exercising biological action (Table III). The relative amounts bound, *i.e.*, the ratio of the estimated S^* values, are of the same order of magnitude as the relative nutrient concentrations for studies conducted at the same pH values (Fig. 6). The S^* values also decreased with increasing pH (Table I and Fig. 4), although the data showed great variability. The nature of the dependency of logarithm S^* on pH (Fig. 7) implied that the charged species were preferably bound to the media.

The inhibitory constant, k_S (Eq. 2 and Table I), varied as a function of pH. The linear plot of log k_S against pH (Fig. 5) has a calculated slope of 1.053 ± 0.056. The pH region studied includes the pKa of the doubly protonated spectinomycin, $pKa_2 = 6.95$ (1). The linearity of this plot is maintained up to significantly higher pH values, *i.e.*, pH 7.5. The pKa of the singly protonated spectinomycin is 8.70 (1). It is, therefore, expected that the pKa_1 influences the antibacterial activity and that the uncharged fraction of the total spectinomycin concentration is the only antibacterially active species. The k_S values cannot be obtained at extracellular pH values higher than 7.5. Beyond this pH, the drug-free generation rate constant changes and the generation rate-determining step may be different from that within a range of pH 5.8 to 7.5 where k_0 is independent of pH. It is expected that the curve in Fig. 5 would approach a pH-independent value for k_S at extracellular pH values that exceed the pKa_1. An intrinsic activity constant, k_S^*, may be defined with regard to the concentration of unprotonated spectinomycin. Thus, it may be calculated (11) from the unprotonated fraction, f_S, of the total antibiotic concentration and the respective $[H^+]$ and the k_S values (Table I):

$$k_S^* = k_S/f_S = k_S([H^+]^2 + K_1[H^+] + K_1K_2)/K_1K_2 \qquad \text{(Eq. 10)}$$

The literature values for the dissociation constants (1) are $K_1 = 1.01 \times 10^{-7}$ and $K_2 = 2.00 \times 10^{-9}$. The mean value obtained for k_S^* was $(2.66 \pm 0.49) \times 10^{-3}$ ml./mcg.-sec.

The rate of achievement of the drug-affected steady-state generation rate with spectinomycin is a slower process than with tetracycline (4), chloramphenicol (7), and lincomycin (5). This is apparent from inspection of Figs. 1–3 where time in excess of 200 min. after drug addition is necessary to obtain a linear semilogarithmic plot of the number of organisms against time. There are several possible explanations for this phenomenon. The assumption of the model of Eq. 3 could imply that the rate of diffusion of drug, S, into the biophase (the compartment where antibiotic concentration, S', is in instantaneous equilibration with receptor sites, R) is a slow process and is rate limiting. However, when the drug-affected culture media are diluted with fresh media (Curves G and E, Fig. 2), the resumption of the new steady-state generation rate occurs in the relatively short time interval of less than 50 min. Although differences in rates of diffusion of spectinomycin in and out of the biophase, *i.e.*,

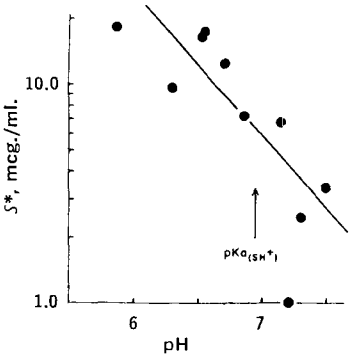

Figure 7—*Correlation of the log of* S*, *the spectinomycin concentration,* S, *at* $k_{app.} = k_0$ *calculated from the linear dependence of* $k_{app.}$ *on* S *with the pH of the culture media.*

a possible "active" process, could be postulated, it appears more reasonable to be critical of the assumption of rate-determining drug diffusion into the biophase.

An alternate explanation may be that drug action, as manifested by the degree of inhibition of generation rate, increases the sensitivity of the organism to further drug action until the steady-state generation rate is achieved. This "feedback" phenomenon is explainable on the premise that the rapidly equilibrated drug concentration in the biophase is competitive for receptor sites with a metabolic intermediate produced by the growing organism.

An analogy can be drawn to the sulfonamide–*p*-aminobenzoic acid competition. The initial fraction of receptor sites that is drug–receptor complex is reflected by an initial decrease in generation rate. This results in a diminution of the production of a vital metabolic intermediate. Subsequent depletion of excess stores of this intermediate in the normal metabolic or generation processes of the organism results in less amounts to compete with drug concentrations in the biophase, greater fractions of drug–receptor complex, and, consequently, further slowing of generation rates. On dilution of the drug with fresh medium, the drug in the organism's biophase rapidly reequilibrates with the consequence of less drug-bound receptor sites. A new steady-state production of metabolic intermediate may occur. This results in the observed reasonably rapid increase in microbial generation to new steady-state conditions (Fig. 2). A kinetically equivalent phenomenon is that the feedback is mediated by decreasing the number of available receptor sites concomitant with decreasing growth rates.

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Pharmacokinetic Evidence for Saturable Renal Tubular Reabsorption of Riboflavin

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Abstract □ Pharmacokinetic relationships have been developed to characterize a multicompartment drug distribution and elimination model which includes a saturable renal tubular reabsorption process. The derived expressions have been applied to serum concentration and urinary excretion data obtained after rapid intravenous administration of riboflavin to man and dog. The mathematical relationships and experimental data demonstrate the dependence of renal clearance on the serum concentration of the drug and on urine flow rate. The results of this study indicate that the renal excretion of riboflavin, like that of several other water-soluble vitamins, involves saturable tubular reabsorption as well as tubular secretion.

Keyphrases □ Riboflavin—saturable renal tubular reabsorption □ Renal tubular reabsorption, secretion—riboflavin □ Flavin-inulin—clearance ratio □ Pharmacokinetics—riboflavin renal clearance

A number of natural substances are known to be reabsorbed from renal tubules by a saturable process. Among these are the water-soluble vitamins: thiamine (1), pantothenic acid (2), and ascorbic acid (3). The renal excretion of another water-soluble vitamin, riboflavin, has been shown (4) to involve tubular secretion in man, and it has recently been suggested (5) that tubular reabsorption of riboflavin occurs in the chicken. In addition, an analysis of published data (6) indicates that the renal clearance of riboflavin in man decreases at lower serum levels of the vitamin.

A substance that undergoes saturable renal tubular reabsorption will characteristically yield higher renal clearances with increasing serum concentrations (7). The kinetics of this process have not been studied in detail, particularly over a wide concentration range such as is obtained after rapid intravenous injection of the substance. The single-injection technique for the study of renal clearance is often considered unsuitable (7) because of the difficulty of characterizing and accounting for the effect of rapid flux of the drug between plasma and tissue. Thus, this technically simpler method is often rejected in favor of the commonly used constant intravenous infusion method where plasma and tissue levels of the drug are maintained relatively constant. However, the constant infusion method will not reveal a possible concentration dependence of renal clearance unless the study is carried out at several, widely different infusion rates.

The purpose of this report is to present mathematical relationships which may be utilized for pharmacokinetic