Microbial Kinetics of Drug Action against Gram-Positive and Gram-Negative Organisms I: Effect of Lincomycin on *Staphylococcus aureus* and *Escherichia coli*

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Abstract □ Lincomycin-affected *Staphylococcus aureus* cultures show two phases of steady-state generation curves whose characteristics are different from those of lincomycin-affected *Escherichia coli*. In both cases, an initial (phase I) steady-state generation, expressed as $\ln N = \ln N_0 + k(\text{app}_\text{I})t$, is followed by an ultimate (phase II) steady-state generation, $\ln N = \ln N_0 + k(\text{app}_\text{II})t$, at the same dose level, where $k(\text{app}_\text{I})$ and $k(\text{app}_\text{II})$ are the apparent generation rate constants for phase I and phase II generations, respectively; N is the number of organisms per unit volume at time, t; and N_0 is the number of organisms per unit volume at some initial time, 0, during this generation phase. The $k(\text{app}_\text{I})$ was less than $k(\text{app}_\text{II})$ for lincomycin-affected *Staph. aureus*, which suggests development of organism adaptation or resistance to drug action, whereas $k(\text{app}_\text{I})$ was greater than $k(\text{app}_\text{II})$ for lincomycin-affected *E. coli*, which indicates enhanced organism inhibition during the later stages of drug-bacteria reaction at any concentration level. The generation rate constants for lincomycin-affected *Staph. aureus* in phase I and phase II generations show similar functional dependencies on drug concentration as the lincomycin-affected *E. coli* in phase I generation and suggest the same mechanism of action, which is different from that of the lincomycin-affected *E. coli* in phase II generation. The lincomycin (phase I) action on *Staph. aureus* is about 1000 times as potent (on a weight basis) as that on *E. coli* at 37.5° and pH 7.05. However, the extent of generation inhibition of *Staph. aureus* and *E. coli* by lincomycin increases as some function of pH, which indicates that the unprotonated fraction of drug concentration contributes to the activity.

Keyphrases □ Microbial kinetics—effect of lincomycin on *Staph. aureus* and *E. coli*, drug-bacteria reactions, total and viable count methods □ Lincomycin—comparison of action on *Staph. aureus* and *E. coli*, microbial kinetics, drug-bacteria reactions, generation curves □ *Staphylococcus aureus*—effect of lincomycin, compared to lincomycin effects on *E. coli*, drug-bacteria reactions, generation curves, microbial kinetics □ *Escherichia coli*—effect of lincomycin, compared to lincomycin effects on *Staph. aureus*, drug-bacteria reactions, generation curves, microbial kinetics

The quantification and prediction of antimicrobial action by microbial kinetics have been demonstrated for a number of antibiotics and chemotherapeutic agents (1). Kinetic parameters, which are derived from generation curves of drug-free and drug-affected *Escherichia coli* cultures, have been used to: *(a)* characterize drug-receptor interactions and elucidate possible mechanisms of action (2–5), *(b)* estimate minimal inhibitory concentration (MIC) (6) and relative potencies (7–9), *(c)* determine structure-activity relationships (5, 6, 9–12), and *(d)* evaluate combination effects of the drugs (5, 7–9, 13–15).

A particle-size counter is employed to monitor organism population of drug-free and drug-affected cultures (16). In all cases, coincident plots of total counts (obtained by the particle-size counter method) *versus* time with those of viable count (obtained by the pour plate method) were obtained, indicating the suitability of the particle-size counter and the total method for determining generation rates of the cultures. The technique is very rapid and permits evaluation of drug action on the cultures within 2–5 hr. It produces highly reproducible and precise kinetic parameters of drug-free and drug-affected cultures (7). Unlike conventional methods employed in the *in vitro* evaluation of antimicrobial activity, such as agar diffusion and serial dilution methods, it is not subject to restraints of drug diffusivity in agar medium or errors of definitive end-point inherent in experimental design.

E. coli has been the only test organism employed in microbial kinetic studies because it does not form clumps or chains and it produces uniform cell suspensions (17, 18) suitable for obtaining total counts. It is a classical organism employed in the evaluation of antimicrobial activity of many compounds. However, it is a typical Gram-negative organism and its use as a test organism for studies on the action of macrolide and lincosaminide antibiotics has been criticized[1]. It is claimed that these antibiotics are normally more active against Gram-positive than Gram-negative organisms (19, 20) and that the two types of organisms vary in gross morphology (17, 18). The data obtained with *E. coli* may not be readily extrapolated to other Gram-negative bacteria without experimental evidence to substantiate this implied claim. The spectrum of activity for macrolide and lincosaminide antibiotics is reason enough to select a Gram-positive coccus for the study.

It was of interest to use *Staphylococcus aureus* as a test organism representative of Gram-positive organisms in studies of drug action by microbial kinetics. This report presents the results of a study on lincomycin action on *Staph. aureus* and a comparison of the data with those obtained on *E. coli*.

EXPERIMENTAL

Test Organism—Replicate slants of *Staph. aureus* (ATCC 6538) and *E. coli* (ATCC 12407) were used in all experiments. The slants were prepared from single isolated colonies of the respective organisms and were stored in a refrigerator at 4°.

Culture Media—Antibiotic medium III[2] was prepared according to the specification of the manufacturer, filtered twice through 0.45-μm filters[3], and autoclaved at 121° for 15 min. The pH of the medium was 7.05 ± 0.05, with the exception of media used to study the antibacterial activity as a function of pH.

[1] Personal communications from Dr. J. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich., and Dr. F. Kavanagh, Eli Lilly & Co., Indianapolis, Ind., to Dr. E. R. Garrett, University of Florida, Gainesville, Fla.

[2] Difco Laboratories, Detroit, Mich.

[3] Millipore HA.

Table I—Analysis of Variance on Generation Rate Constant (k_0 in seconds^{-1}) of Drug-Free *Staph. aureus* Cultures at 37.5° and pH 7.05 within and between Day Experiments

Experiments	Days 1	2	3	4	5
	$k_0 \times 10^{-5}$ sec^{-1}				
1	39.81	40.31	39.42	40.82	41.82
2	41.69	40.13	43.65	40.68	40.32
3	40.13	39.82	40.91	42.43	42.01

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Between days	4	4.1038×10^{10}	1.0260×10^{10}
Within days	10	14.9588×10^{10}	1.4959×10^{10}
Total	14	19.0626×10^{10}	

Variance ratio (F_4^{10}) = 1.4580; [a] $p > 0.10$
Mean generation rate constant (k_0) = 40.93×10^{-5}
Standard error of the mean = 0.55×10^{-5}

[a] From Table V of "Statistical Tables for Biological Agricultural and Medical Research," 6th ed., R. A. Fisher and Yates, Eds., Hafner Publishing, New York, N.Y., 1963.

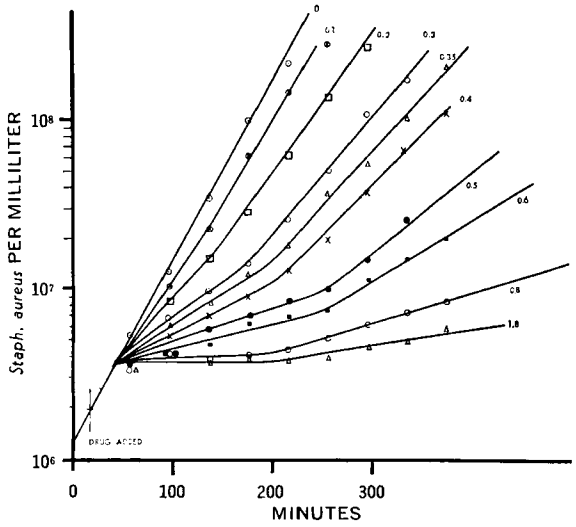


Figure 1—*Typical generation curves of* Staph. aureus *in antibiotic medium III at pH 7.05 and 37.5° in the absence and presence of various concentrations of lincomycin. The curves are labeled according to drug concentration in micrograms per milliliter.*

Antibiotic—An assayed sample of lincomycin hydrochloride[4] (860 μg base equivalent/mg) was used.

Bacterial Cultures—A 5-ml aliquot of culture medium in a screw-capped test tube was inoculated with the organism from a fresh slant and incubated at 37.5° for 12–15 hr. A sample of 0.5 ml *(E. coli)* or 1.0 ml *(Staph. aureus)* was then transferred into 50 ml fresh culture medium contained in a 125-ml loosely capped erlenmeyer flask and maintained at 37.5° in a 57-liter (15-gallon) constant-temperature shaker water bath[5]. The generation of the culture was followed up to 2.0×10^7 cells/ml. An aliquot of this culture (*i.e.*, 1.0 ml *Staph. aureus* culture/50 ml medium or 1.0 ml *E. coli* culture/100 ml medium) was added to a bulk amount of culture medium contained in a Pyrex flask fitted with a 50-ml dispenser. The "seeded" culture medium was kept in an incubator at 37.5° for 30 min with intermittent shaking. Aliquots (50 ml) of the seeded culture medium were then aseptically transferred with the aid of the dispenser into replicate erlenmeyer flasks, maintained at 37.5 ± 0.1° in the water bath.

Total Count Method—Culture samples (0.5 or 1.0 ml) were withdrawn at 20–30-min intervals and diluted to obtain counts within a range of 5000–20,000 counts/50 μl on the particle-size counter[6] equipped with a 30-μm orifice. The settings of the instrument were: 1/aperture current, 1.0; 1/amplification, 2.0; gain, 8.0; matching switch, 40K; lower threshold, 5; and upper threshold, at maximum. These operational conditions were found effective for the counting of *Staph. aureus* and *E. coli* cells without significant loss of counts or interference from the background noise. The counts were also corrected for background count on the batch of medium used when suitably diluted in the same manner as the culture samples. In general, the background count did not exceed 500 counts/50 μl. The diluent was an aqueous solution of 0.85% sodium chloride and 1.0% formaldehyde, filtered twice through a 0.45-μm filter[3].

Viable Count Method—Culture samples (0.5 or 1.0 ml) were withdrawn at 20–30-min intervals and diluted serially in sterile 0.9% NaCl solution and finally in a culture medium to obtain a concentration of 50–150 cells/ml. Aliquots of 1.0 ml from the final broth dilution were pipeted into each of three replicate sandwich agar plates. The plates were incubated at 37.5° for 48 hr, and the colonies were counted on a colony counter[7].

Effects of Antibiotic Concentration on Generation Rates—Fresh solutions of the antibiotic were aseptically prepared for each experiment. They were diluted suitably so that aliquots of 0.5 or 1.0 ml added to replicate 50 ml volumes of seeded culture medium yielded the desired concentrations of the antibiotic. The solutions were added to the cultures growing at 37.5° in the logarithmic phase at an organism concentration of 2.0×10^6 cells/ml. Samples were withdrawn every 20–30 min, and organism population was determined by the appropriate method. Generation curves for *Staph. aureus* cultures and for *E. coli* cultures affected with 0–1.0 and 0–600 μg/ml lincomycin, respectively, were obtained by the total count method. Generation curves for *Staph. aureus* cultures affected with selected concentrations of lincomycin (0, 0.4, 1.0, 1.2, and 1.4 μg/ml) were also obtained by both the total and viable count methods.

Effect of Nutrient Concentration on Drug-Affected Generation Rates—Culture media of half, normal, and twice the concentration of both ingredients were prepared and adjusted to pH 7.05 ± 0.05, where necessary. They were used for determining the generation curves at 37.5° of replicate *Staph. aureus* cultures affected with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml lincomycin as described in the previous section. The organism concentration at the time of drug addition was 2×10^6 cells/ml. Total counts were obtained on samples withdrawn every 20–30 min.

Effect of Organism Population on Drug-Affected Generation Rates—Three sets of normal strength culture media were prepared and used for determining the generation curves at 37.5° of replicate *Staph. aureus* cultures affected with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml lincomycin. The organism concentrations at the time of drug addition were 3.0×10^5, 2.0×10^6, and 3.0×10^7 cells/ml in the set of culture medium. Total counts were obtained on samples withdrawn every 20–30 min.

Effect of pH on Drug-Affected Generation Rates—Sufficient amounts of 2.0 *N* HCl and 2.0 *N* NaOH were added to culture media to obtain pH values of 6.2, 6.4, 6.8, 7.0, 7.4, and 7.7. They were used for determining generation curves at 37.5° of replicate *Staph. aureus* cultures affected with different predetermined concentrations of lincomycin at each pH level. The organism concentration at the time of drug addition was 2×10^6 cells/ml. Total counts were obtained on samples withdrawn every 20–30 min.

RESULTS

Generation Rate Constants of Drug-Free Cultures—Balanced cultures of *Staph. aureus* and *E. coli* showed an exponential phase of generation expressed as:

$$\ln N = \ln N_0 + k_0 t \qquad \text{(Eq. 1)}$$

where N is the number of organisms per unit volume at time, t: N_0 is the number of organisms per unit volume at some initial

[4] Supplied by Dr. J. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich.
[5] Model RW 65, New Brunswick Scientific Co., New Brunswick, N.J.
[6] Coulter counter, model ZB1, Coulter Electronics Co., Hialeah, Fla.
[7] Lab-line Instruments, Inc., Melrose Park, Ill.

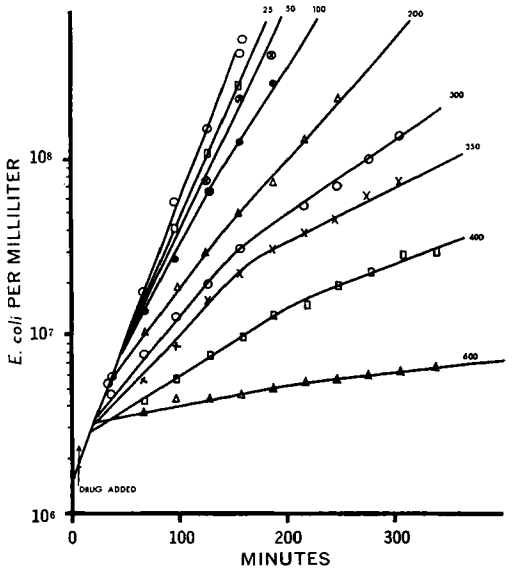


Figure 2—*Typical generation curves of* E. coli *in antibiotic medium III at pH 7.05 and 37.5° in the absence and presence of various concentrations of lincomycin. The curves are labeled according to drug concentration in micrograms per milliliter.*

time, 0, during this generation phase; and k_0 is the apparent generation rate constant. Apparent generation rate constants, k_0 in seconds^{-1}, obtained from the slopes of plots of ln N *versus* t from generation curves of *Staph. aureus* were highly reproducible in experiments performed within a day and between days on different batches of antibiotic medium III (Table I). The mean generation rate constant, k_0, obtained from the results of 15 experiments for *Staph. aureus* was 40.93×10^{-5}/sec. The generation rate constant obtained for *E. coli* in previous experiments (8, 9) and determined in the present study was 60.67×10^{-5}/sec. Thus, the mean generation time (t_{mgt}) can be calculated for each organism from the expression:

$$t_{mgt} = 0.693/k_0 \qquad \text{(Eq. 2)}$$

The values found were 28.21 and 19.04 min for *Staph. aureus* and *E. coli* cultures, respectively.

Shape of Generation Curves for Drug-Affected Cultures—The addition of graded concentrations of lincomycin to balanced cultures of *Staph. aureus* (Fig. 1) and *E. coli* (Fig. 2) decreased the generation rates after lag periods of approximately 30 and 20 min, respectively. Subsequent to the deviation from the curve of the drug-free culture, a new steady-state phase of generation (phase I) was established, followed after 1–2 generations of the drug-affected *Staph. aureus* or 3–5 generations of the drug-affected *E. coli* by an ultimate steady-state phase of generation (phase II) during the later stages of drug-bacteria reaction. Apparent generation rate constants (k_{app} in seconds^{-1}) of the drug-affected cultures were obtained from the slopes of the linear portions of the plot of ln N *versus* t in accordance with the expression:

$$\ln N = \ln N_0 + k_{app}t \qquad \text{(Eq. 3)}$$

The $k(\text{app}_\text{I})$ was less than $k(\text{app}_\text{II})$ for lincomycin-affected *Staph. aureus*, whereas the $k(\text{app}_\text{I})$ was greater than $k(\text{app}_\text{II})$ for lincomycin-affected *E. coli*, where $k(\text{app}_\text{I})$ and $k(\text{app}_\text{II})$ are the generation rate constants for the phase I and phase II generations, respectively.

Comparison of Generation Curves of Drug-Affected Cultures by Total and Viable Count Methods—Semilogarithmic plots of total and viable counts *versus* time for the action of selected concentrations of lincomycin (0, 0.4, 1.0, 1.2, and 1.4 μg/ml) against *Staph. aureus* are given in Fig. 3. Parallel generation curves were obtained from the plots of the total and viable counts for the drug-free culture and for drug-affected cultures in the subcompletely inhibitory range of lincomycin concentrations (curves A, A_1; B, B_1; and C, C_1). However, the total count was 60–80% of the viable count. At concentrations greater than 1.0 μg/ml lincomycin, complete bacteriostatic inhibition was demonstrated in 170 min for both the total and viable counts (curves D, D_1; and E, E_1). Thereafter, there was a rapid drop in the viable counts, which may indicate a bactericidal or lytic phenomenon.

Coincident generation curves were, however, obtained from plots of total and viable counts *versus* time for drug-free and drug-affected *E. coli* cultures in the subcompletely inhibitory concentration range of lincomycin (3).

Effect of Drug Concentration on Generation Rates—A plot of k_{app} *versus* concentration is given in Fig. 4. The extent of generation inhibition is directly proportional to lincomycin concentration, L, in the range 0–0.35 μg/ml for the phase I action (curve A) and 0–0.55 μg/ml for the phase II action (curve A) against *Staph. aureus* or in the range 0–100 μg/ml for the phase I action (curve C) against *E. coli*, in accordance with the expression:

$$k_{app} = k_0 - k_L L \qquad \text{(Eq. 4)}$$

where k_L is defined as the specific inhibitory rate constant. Above these concentration ranges, the k_{app} is not a linear function of the increasing drug concentration, but it asymptotically approaches zero.

On the other hand, a nonlinear decrease of the k_{app} with drug concentration is observed for the phase II lincomycin action (curve B) against *E. coli* throughout the concentration range studied. Coincidence of plots of k_{app} *versus* drug concentration is obtained (curve A) when the actual concentrations for the phase I and phase II lincomycin action against *Staph. aureus* from the data of Table I are multiplied by factors of 1.0 and 0.67, respectively. The plots for the phase I and phase II lincomycin action (curves C and B) against *E. coli* are not superimposable, which suggests different functional dependencies of the respective k_{app} values on drug concentrations.

Applicability of Saturable Receptor Site Model to Action of Lincomycin against *Staph. aureus* and *E. coli*—Figure 5 gives a plot of $L/(k_0 - k_{app})$ *versus* L in accordance with a previously derived (3) receptor site model:

$$L/(k_0 - k_{app}) = L(k_b/k_a) + 1/k_a \qquad \text{(Eq. 5)}$$

where k_a and k_b are constants of proportionality related to drug availability in the biophase and drug affinity for receptor or binding sites. Adherence to the model is observed from linear plots obtained for concentrations of lincomycin greater than 0.35 μg/ml

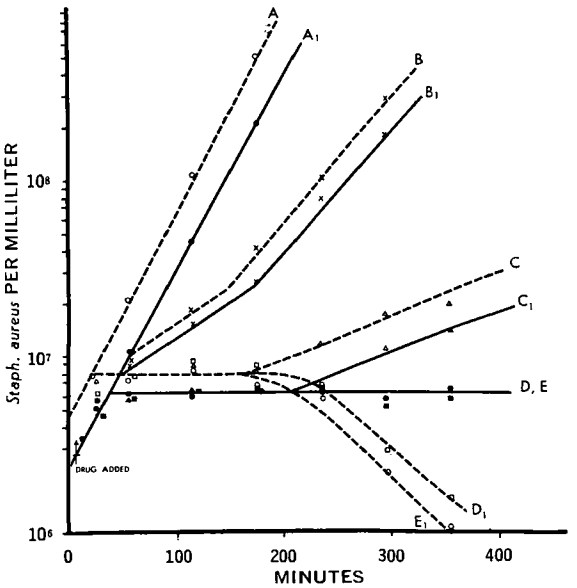


Figure 3—*Generation curves of* Staph. aureus *at pH 7.05 and 37.5° in the absence and presence of lincomycin, obtained by total* (—) *and viable* (---) *counts. The curves and respective drug concentrations (micrograms per milliliter) were: A, A_1, 0.0; B, B_1, 0.4; C, C_1, 1.0; D, D_1, 1.2; and E, E_1, 1.4.*

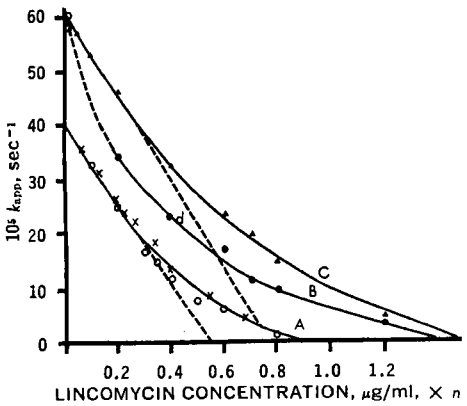


Figure 4—*Dependence of apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, for* Staph. aureus *and* E. coli *on concentrations of lincomycin (micrograms per milliliter) at pH 7.05 and 37.5°. The actual concentrations are multiplied by a factor,* n*, where* n = *1.0 in curve A for* Staph. aureus *in phase I generation* (○), n = *0.67 in a coincident curve for* Staph. aureus *in phase II generation* (×), *and* n = *0.002 in curves B and C for* E. coli *in phase I and phase II generations, respectively.*

in the phase I action (curve A) and greater than 0.55 μg/ml in the phase II action (curve B) against *Staph. aureus* or greater than 100 μg/ml in the phase I action (curve D) against *E. coli.* In all cases, deviations occur in the lower concentration ranges. Strict adherence to Eq. 5 is observed for all concentrations of lincomycin in the phase II action (curve C) against *E. coli.* The values of k_a and k_b calculated from slopes and intercepts of such plots are given in Table II. At the MIC, when there is complete bacteriostasis and $k_{app} = 0$, Eq. 5 reduces to:

$$L = k_0/(k_a - k_0 k_b) \qquad \text{(Eq. 6)}$$

where lincomycin is at the MIC. Calculated MIC values of lincomycin action against *Staph. aureus* and *E. coli* are given in Table II. The relative potency of lincomycin action (on a weight basis) calculated in accordance with Eq. 6 from the data of Table II is (*Staph. aureus* to *E. coli*) 852:1 and 611:1 for the phase I and phase II generations, respectively.

Effect of Culture Medium Variations—The plot of apparent

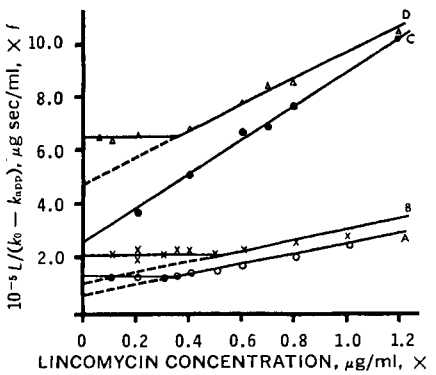


Figure 5—*Demonstration of saturation kinetics for the dependency of the apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, of* Staph. aureus *and* E. coli *at higher concentrations of lincomycin (micrograms per milliliter) at pH 7.05 and 37.5°. Key: A,* Staph. aureus*-affected culture in phase I generation; B,* Staph. aureus*-affected culture in phase II generation; C,* E. coli*-affected culture in phase II generation; and D,* E. coli*-affected culture in phase I generation. The curves were plotted in accordance with Eq. 5. The actual values of* $L/(k_0 - k_{app})$ *are multiplied by a factor,* f*, where* f = *0.01 for curves A and B and* f = *1.0 for curves C and D. The actual lincomycin concentrations,* L*, are also multiplied by a factor,* n*, where* n = *1.0 for curves A and B and* n = *0.002 for curves C and D.*

Table II—Derived[a] Kinetic Parameters from Generation Curves of Lincomycin-Affected *Staph. aureus* and *E. coli* Cultures

Kinetic Parameter	*Staph. aureus* Phase I	*Staph. aureus* Phase II	*E. coli* Phase I	*E. coli* Phase II
$10^5 k_L$ (ml/μg sec)[b]	72.73	48.73	0.16	—
$10^5 k_a$ (ml/μg sec)[c]	153.85	91.50	0.20	0.40
$10^5 k_a/k_b$ (sec^{-1})[c]	57.19	52.29	102.77	78.95
$(k_a/k_b)/k_0$[c]	1.42	1.30	1.68	1.29
$10^2 k_b$ (ml/μg)[c]	267.69	174.76	0.19	0.51
MIC (μg/ml)[d]	0.88	1.13	749.50	690.67

[a] Derived from data of Fig. 1. [b] Calculated from the slope of the plot of k_{app} *versus* concentration from 0 to 0.35 μg/ml lincomycin in phase I action on *Staph. aureus* and from 0 to 0.55 μg/ml lincomycin in phase II action of *Staph. aureus* or 0–100 μg/ml lincomycin in phase I action on *E. coli* according to the expression $k_{app} = k_0 - k_L L$, where L is the concentration of lincomycin. [c] k_a, k_a/k_b, $(k_a/k_b)/k_0$, and k_b are estimated from the slopes and intercepts of the plots of $L/(k_0 - k_{app})$ *versus* L from the expression, $L/(k_0 - k_{app}) = L(k_b/k_a) + 1/k_a$ for concentrations >0.35 μg/ml lincomycin in phase I action on *Staph. aureus* and >0.55 μg/ml lincomycin in phase II action on *Staph. aureus* or > 100 μg/ml lincomycin in phase I action on *E. coli* and all studied concentrations of lincomycin in phase II action on *E. coli.* [d] Calculated from the expression $L = k_0/(k_a - k_0 k_b)$, where lincomycin is at the MIC.

generation rate constants (k_{app}) of *Staph. aureus* affected by different concentrations of lincomycin (L) in three culture media of different compositions is given in Fig. 6. The variation of these generation rate constants among different culture media is not significantly different from that observed in the daily variation in organism generation rates. This indicates that there is no significant inactivation or binding of lincomycin by broth constituents that interferes with drug action on *Staph. aureus.* Similar evidence was established previously for lincomycin action on *E. coli* (3).

Effect of Organism Population on Drug-Affected Generation Rates—The plot of apparent generation rate constants (k_{app}) affected by various concentrations of lincomycin (L) for three different organism populations at the time of drug addition is given in Fig. 7. There are no significant differences among the generation rate constants of different organism populations at any drug concentration level studied, as was previously found for *E. coli* (3).

Effect of pH on Drug-Affected Generation Rates—The apparent generation rate constants obtained for *Staph. aureus* cultures in media of pH 6.2–7.7 in the absence and presence of graded concentrations of lincomycin are given in Fig. 8. The generation rate constant (k_0) of the drug-free culture is invariant with the pH of the culture medium in the range studied. The phase I generation rate constants [$k(\text{app}_I)$] of the drug-affected cultures are significantly decreased with increasing pH values for the same lincomycin concentration. Therefore, progressively larger amounts of lincomycin are required to produce the same generation inhibition as the pH is decreased. The calculated values of k_L obtained

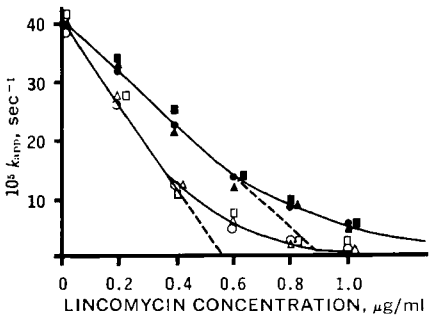


Figure 6—*Apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, of* Staph. aureus *as functions of concentration of lincomycin (micrograms per milliliter) in culture media of different compositions at pH 7.05 and 37.5°. Curve A (open symbols) is for phase I generations, and curve B (closed symbols) is for phase II generations. The culture media were: ●,○, half strength; ▲,△, single strength; and ■,□, double strength.*

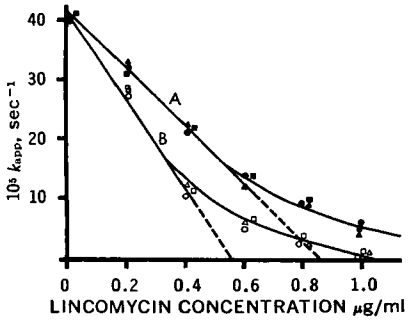


Figure 7—*Apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, of* Staph. aureus *as functions of concentration of lincomycin (micrograms per milliliter) at pH 7.05 and 37.5° for various organism populations at time of drug addition. Curve A (open symbols) is for phase I generations, and curve B (closed symbols) is for phase II generations. The organism concentrations were: ●,○, 3.0 × 10⁵ cells/ml; ▲,△, 2.0 × 10⁶ cells/ml; and ■,□, 3.0 × 10⁷ cells/ml.*

from the slopes of plots of $k(\text{app}_I)$ *versus* lincomycin concentration in accordance with Eq. 4 are plotted as a function of pH in Fig. 9. The values of k_L increase about 10-fold for a unit increase in pH over the 6.2–6.8 range, but the rate of change tends to lessen to a null slope above pH 6.8 (curve A). Similar results are obtained from plots of the data of Mielck and Garrett (3) for the action of lincomycin against *E. coli* in media with a pH range of 5.80–7.50 (curve B).

DISCUSSION

Staph. aureus is known to divide with no set pattern in the orientation of successive division planes to form irregular clumps (18). The total count method employed for monitoring organism population in broth does not differentiate between single discrete and clustered particles in counts on sample suspensions. Therefore, it was expected that the use of the particle-size counter for counting drug-free and drug-affected *Staph. aureus* would produce erratic counts and yield nonreproducible generation rates of cultures. If this were the case, it would necessitate the application of certain physicochemical concepts and basic microbiological techniques to break down clumps and disperse cells before counting or the coupling of the Channelyzer[8] and other devices to the particle-size counter to determine and characterize the distributive pattern of the cells and thereby permit an estimate of true and reproducible counts on the culture samples.

The work reported here shows that the total count method is applicable for determining the generation rates of drug-free and drug-affected *Staph. aureus* cultures with none of the anticipated problems. Although the concentrations of cells at any time, *t*, as determined by the total count method and the pour plate method were quite different (Fig. 3), the proportion of total cells to viable cells remained relatively constant in the drug-free and drug-affected cultures. The higher count obtained from viables may be

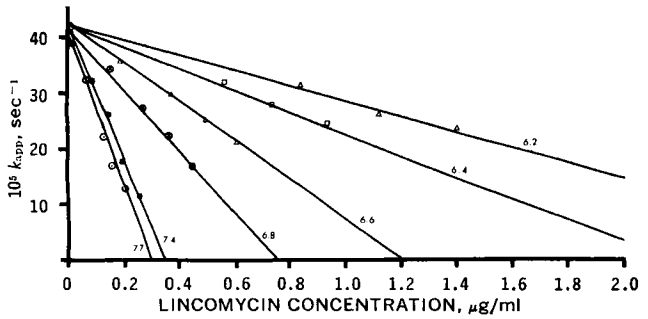


Figure 8—*Dependence of the apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, for* Staph. aureus *on concentrations of lincomycin (micrograms per milliliter) in culture media of various pH values at 37.5°. Each curve is labeled as to the pH values of the culture media.*

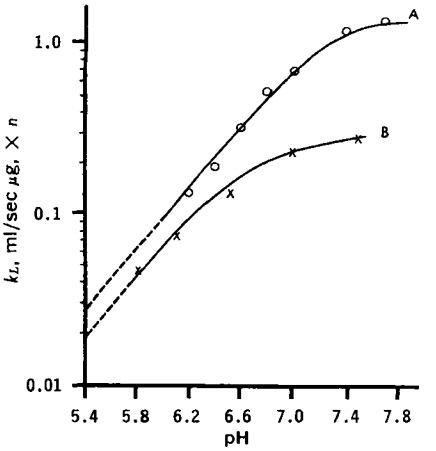


Figure 9—*Semilogarithmic plot of the apparent inhibitory rate constant,* k_L *in milliliter per microgram second, for the action of lincomycin on* Staph. aureus *(curve A) and* E. coli *(curve B) in culture media of various pH values at 37.5°. The drawn lines are consistent with Eq. 14. The actual values of* k_L *are multiplied by a factor,* n, *where* n = 10^3 *for* Staph. aureus *and* n = 10^5 *for* E. coli.

rationalized by subsequent generation of the culture samples, during the interval between withdrawal of the samples and the subsequent serial dilution procedures in saline and finally in broth before plating on agar. Alternatively, the high dilution employed to reduce the cell concentration to a countable size on the agar plate possibly caused a dispersion of randomly distributed clusters with a consequent increase in colony counts. Such effects were conceivably nonexistent in the total count procedure where the diluent contained formaldehyde, which promptly arrested generation of the culture samples which were also counted at low dilutions. It is evident that there is no kill or death of the organisms superimposed on generation inhibition by the action of lincomycin at the subcompletely inhibitory level. The generation rate constants obtained by the two methods were identical and within limits of experimental error. Size frequency analysis[8] indicates (Fig. 10) that the drug-free (curve A) and drug-affected (curve B) cultures have the same distributive pattern, except that the cell volume (μm^3) of the drug-affected culture is increased

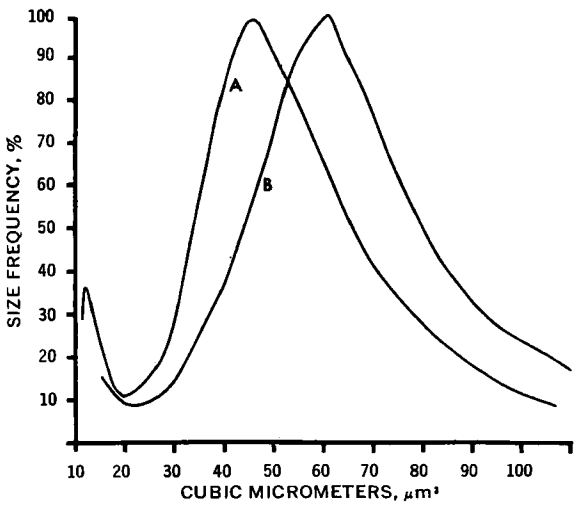


Figure 10—*Size frequency distribution curves of* Staph. aureus *cultures. Curve A is for the drug-free culture, and Curve B is for culture treated with 0.5 μg/ml lincomycin 30 min after drug addition. The curves were obtained from a Channelyzer calibrated with a threshold factor, 0.034, at base channel threshold 5 and window width 100.*

[8] Coulter Electronics Co., Hialeah, Fla.

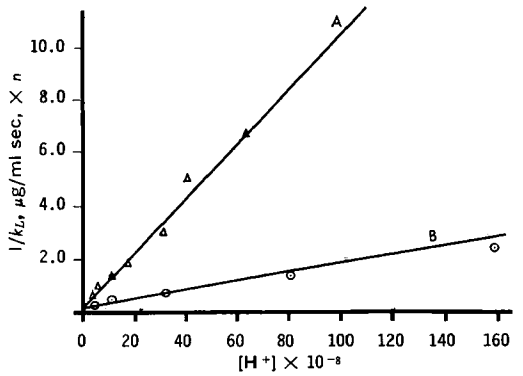


Figure 11—*Demonstration of linearity in plots of* $1/k_L$ *(micrograms per milliliter second)* versus $[H^+]$, *in accordance with Eq. 15, for the action of lincomycin on* Staph. aureus *(curve A) and* E. coli *(curve B). The curves were drawn in accordance with Eq. 15. The actual values of* $1/k_L$ *are multiplied by a factor,* n, *where* $n = 10^{-3}$ *for* Staph. aureus *and* $n = 10^{-6}$ for E. coli.

relative to that of drug-free culture, due undoubtedly to alterations in shape and form of the cells (18) by lincomycin action. There was, however, evidence of a rapid drop in the viable count (Fig. 3) for the action of lincomycin on *Staph. aureus* at concentrations above the bacteriostatic level, which may indicate a bactericidal or lytic phenomenon.

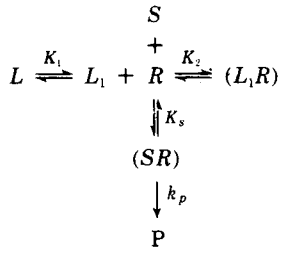
The generation rate constants of the drug-free and drug-affected *Staph. aureus* are not affected by variations in concentrations of constituents of the nutritive media (Fig. 6), indicating that no significant inactivation or binding of the lincomycin by broth constituents occurs to interfere with drug action on the cultures. Likewise, variations in inoculum size at the time of drug addition (Fig. 7) do not affect generation rate constants of cultures, which indicates that the drug is neither metabolized by the organisms, nor depleted in the medium as a result of adsorption to cellular components, nor inactivated by metabolic by-products and other interactions as functions of *Staph. aureus* numbers.

Balanced drug-free cultures of *E. coli* grow and divide at a rate 1.50 times faster than *Staph. aureus* in antibiotic medium III, which may be due to inherent variations in generation characteristics of the organisms or may suggest that the medium supports the growth of *E. coli* better than that of *Staph. aureus*. There are definite lag periods (Figs. 1 and 2) after drug addition before new steady-state generations of the cultures are established. The lag period is longer for *Staph. aureus* cultures (about 30 min) than for *E. coli* cultures (about 20 min). This may be attributed to differences in gross morphology of the organisms (17, 18) which possibly influence the rates of passage of the drug through cell membranes and formation of drug–receptor complexes that determine the onset of generation inhibition (14).

Lincomycin-affected *Staph. aureus* and *E. coli* cultures exhibit biphasic steady-state generation curves (Figs. 1 and 2) whose characteristics are, however, different. The generation rate constant $[k(\text{app}_\text{I})]$ of the phase I generation is less than the generation rate constant $[k(\text{app}_\text{II})]$ of the phase II generation of the drug-affected *Staph. aureus* and suggests development of organism resistance to drug action in the later stages of the drug–bacteria reaction. The reverse is the case with drug-affected *E. coli*, where $k(\text{app}_\text{I})$ is greater than $k(\text{app}_\text{II})$ and indicates an enhanced generation inhibition at the same concentration levels of the lincomycin.

The functional dependency of k_{app} on lincomycin concentration for the drug-affected *Staph. aureus* in phase I generation is the same as that in its phase II generation. This suggests the same mechanism (22) of lincomycin action in the phase I and phase II generations of the drug-affected cultures. The k_{app} *versus* concentration curves for the phase I and phase II generations are superimposable by a potency factor of 0.67 over the entire concentration range studied (Fig. 4). This indicates that only 67% of total drug concentration is effective against *Staph. aureus* in the phase II generation.

An operative kinetic model (Scheme I) similar to that which defined the action of many antibiotics and chemotherapeutic agents on *E. coli* (1) may be applied.



Scheme I

In this model, L is the lincomycin concentration in the medium, which is in equilibrium with L_1, the lincomycin concentration within the cell or biophase that reversibly binds with free receptor sites, R, to form a drug–receptor complex, L_1R; S is the normal substrate utilized by the receptor site, R, to synthesize the protein product, P, from the substrate–receptor complex, SR; K_1 is the drug partition constant through the cell membrane; K_2 is the drug affinity constant for the receptor site, R; K_s is the substrate affinity constant for the receptor site, R; K_s is the rate constant for protein synthesis.

The model implies that the receptor sites unreacted with lincomycin are engaged in protein synthesis. It may be assumed that a minimum fraction of protein synthesis is necessary for life-sustaining processes and that the remaining synthesis is utilized in cell growth and division. Therefore, the rate of increase in microbial numbers, dN/dt, must be proportional to a net rate of protein synthesis, dP'/dt, above a certain minimum rate, k_p', and the number of organisms, N, present:

$$dN/dt = k_m(dP'/dt)N = k_m[(dP/dt) - k_p']N = k_{app}N \quad \text{(Eq. 7)}$$

where dP/dt is the overall rate of protein synthesis, k_m is the constant of proportionality related to metabolic activity of the organism, and k_{app} is the generation rate constant of the drug-affected culture. Then:

$$dP/dt = q(1 - \theta)R_T \quad \text{(Eq. 8)}$$

where q is the constant of proportionality related to synthesis of protein from a constant supply of substrate; and θ is the fraction of total receptors, R_T, reversibly bound by lincomycin. Therefore, $(1 - \theta)\,R_T = R$ in Scheme I. Then:

$$\theta = L_1R/R_T = K_1K_2L/(1 + K_1K_2L) \quad \text{(Eq. 9)}$$

Combination of Eqs. 7–9 yields:

$$k_{app} = (qk_mR_T - k_mk_p') - qk_mR_TK_1K_2L/(1 + K_1K_2L) \quad \text{(Eq. 10)}$$

In the absence of any drug effect, Eq. 10 simplifies to:

$$k_{app} = qk_mR_T - k_mk_p' = k_0 \quad \text{(Eq. 11)}$$

where k_0 is the generation rate constant of the drug-free culture.

Substituting Eq. 11 in Eq. 10 and simplifying yield:

$$k_{app} = k_0 - k_aL/(1 + k_bL) \quad \text{(Eq. 12)}$$

where $k_a = qk_mR_TK_1K_2$, and $k_b = K_1K_2$. Therefore:

$$k_a/k_b = qk_mR_T > k_0 \quad \text{(Eq. 13)}$$

Equation 12 describes the functional dependencies of k_{app} on the drug concentration for drug-affected *Staph. aureus* in phase I and phase II generations (Fig. 4). At low drug concentrations of 0–0.35 μg/ml lincomycin in phase I action or of 0–0.55 μg/ml lincomycin in phase II action (where only 67% of the concentration is indicated to be effective), it is possible that generation inhibition is effected by reaction of the drug with only a small fraction of available receptor sites. Thus, $k_bL \ll 1$ and Eq. 12 simplifies to

Eq. 4, which is the expression for the observed linear dependence of k_{app} on drug concentrations in these concentration ranges (Fig. 4). At higher concentrations, it is possible that the already complexed receptor sites reduce the availability of the remaining sites by steric effects, protective colloid action, or other mechanisms. Therefore, it takes progressively greater concentrations of the drug to bind the remaining sites and the k_{app} does not remain a linear function of lincomycin concentration. The arithmetical transformation of Eq. 12 is Eq. 5, which is adhered to by the data of Fig. 4 from the linear plots of $L/(k_0 - k_{app})$ *versus* concentration of lincomycin greater than 0.35 μg/ml in phase I action (Fig. 5, curve A) and greater than 0.55 μg/ml in phase II action (Fig. 5, curve B) on *Staph. aureus* and may indicate a saturable mechanism similar to that describing the lincomycin phase I action on *E. coli* at concentrations greater than 100 μg/ml (Fig. 5, curve D) and that of phase II action on *E. coli* at all concentrations studied (curve C).

The phase II generation of lincomycin-affected *Staph. aureus* takes place after 1–2 successive generations of the culture in phase I generation and indicates a decreased inhibitory effect of lincomycin during the later stages of the drug–bacteria reaction at the same drug concentration level. Since the drug is neither consumed nor inactivated by the organisms during the phase I and phase II generations (Fig. 7), the organisms may have developed a resistance to drug action. Data[9] obtained from within day and between day experiments confirm that the organisms develop resistance to lincomycin action possibly by adaptation or mutation to resistant strains (23–28). Furthermore, the increased resistance to drug action in this generation phase is irreversible. The experimentally determined values of $(k_a/k_b)/k_0 > 1$ (Table II) agree with theoretical predictions in Eq. 13. However, the values remain the same for the phase I and phase II generations of drug-affected *Staph. aureus* cultures, which implies no alteration in metabolic activity of the cultures in both generation phases. Therefore, it can be argued that the development of adaptation or resistance to drug action (28) is not associated with an induction or a repression of some enzyme system resulting in overproduction of cellular RNA and nonribosomal proteins or the utilization of alternative metabolic pathways (26, 29).

On the other hand, the calculated values of k_a and k_b for the phase I generation are 1.50 times the corresponding values for the phase II generation (Table II). Since k_a and k_b are parameters derived from K_1 and K_2, as defined in Scheme I, it may be presumed that the affinity of ribosomal receptor sites for lincomycin is decreased due to structural modifications (9, 21). Alternatively, the rate of partitioning of the drug into the biophase may be decreased due to altered permeability of the cell membrane, although this is not recognized as being likely in terms of ribosomal mechanisms (29). Studies on the binding of lincomycin to ribosomes in cell-free extracts (26, 30) have shown that ribosomes of resistant strains of *E. coli*, *Staph. aureus*, and *Bacillus subtilis* have greater binding capacity than those of sensitive strains. Thus, it may be rationalized that treatment of *Staph. aureus* with subcompletely inhibitory concentrations of lincomycin results in a single mutational step to resistant phenotypes whose ribosomal components have less binding affinity for the drug than the initial strains. This may account for the observation that only 67% of the total drug concentration can be shown kinetically to be active in the phase II generation of the drug-affected cultures.

The functional dependency of k_{app} on lincomycin concentrations for drug-affected *E. coli* in phase I generation is similar to that of the drug-affected *Staph. aureus* in phase I and phase II generations but different from that of drug-affected *E. coli* in phase II generation. The k_{app} *versus* concentration curve for drug-affected *E. coli* in phase I generation is not coincident with that of its phase II generation when normalized by a potency factor (Fig. 4). This indicates that the phase I lincomycin action on *E. coli* has a different mechanism from that of the phase II action (Fig. 5). A possible kinetic model to rationalize the biphasic action of lincomycin on *E. coli* has been proposed (9). The explanation offered for the lincomycin phase II action is a blockade of additional receptor sites engaged in the synthesis of an intermediate product. The intermediate product is utilized by the receptor site, which is the binding site for lincomycin phase I action in a "sequential blocking" (31, 32) of protein synthesis. Therefore, there is an enhanced generation inhibition. The derived kinetic parameters for lincomycin action on *E. coli* (Table II) are quite consistent with the proposed model. The calculated k_b value for lincomycin-affected *Staph. aureus* in phase I generation is, however, 1400 times greater than that of the lincomycin-affected *E. coli* in phase I generation. This may imply that either the drug is more readily partitioned into the biophase of *Staph. aureus* than *E. coli* or the binding affinity of the receptors for lincomycin is greater in *Staph. aureus* than *E. coli*. Literature evidence (26, 30) supports the latter hypothesis, since it has been shown that ribosomal cell-free extracts of *Staph. aureus* bind relatively larger amounts of lincomycin than do those of *E. coli*. The observation that lincomycin is about 1000 times (on a weight basis) more active against *Staph. aureus* than *E. coli*, as indicated by the relative MIC values (Table II), may be attributed to greater binding capacity of the receptors in *Staph. aureus* for lincomycin. The decreased relative potency factor of 340 observed for the phase II generations may be due to perturbations in the kinetics of drug action on the respective organisms, as previously discussed.

The extent of generation inhibition by lincomycin action on *Staph. aureus* increases as some function of the pH value of the culture medium (Fig. 8). The constant, k_L (milliliters per microgram second), as defined in Eq. 4 for various pH values (Fig. 9, curve A) adhered to the expression:

$$k_L = k_L^* f = k_L^* K_a/(K_a + [H^+]) \qquad \text{(Eq. 14)}$$

where k_L^* is the intrinsic inhibitory rate constant of the unprotonated drug, f is the fraction of the drug unprotonated, K_a is the dissociation constant of the unprotonated base, and $[H^+]$ is the hydrogen-ion concentration. The plot of log k_L *versus* pH approaches a slope of unity when $[H^+] > K_a$ and it approaches a slope of 0 when $K_a > [H^+]$. A similar trend is observed for plots of the data (17) of lincomycin action on *E. coli* (Fig. 9, curve B). Arithmetical transformation of Eq. 4 yields:

$$1/k_L = [H^+]/(k_L^* K_a) + 1/k_L^* \qquad \text{(Eq. 15)}$$

The pKa of lincomycin, derived from the slopes and intercepts of the linear plots of $1/k_L$ *versus* $[H^+]$ (Fig. 11), is 7.70 for the data of lincomycin-affected *Staph. aureus* cultures and 7.21 for the data of lincomycin-affected *E. coli* cultures. These kinetically determined pKa values are in reasonably good agreement with the experimentally determined values of 7.6 obtained by potentiometric titration (33). The k_L^* values obtained from the intercepts of the plots are 10×10^{-3} (milliliters per microgram second) and 10×10^{-6} (milliliters per microgram second) for the lincomycin action on *Staph. aureus* and *E. coli*, respectively. Thus, it is concluded that the unprotonated fraction of lincomycin concentration contributes to antibacterial activity and that the uncharged drug has intrinsic activity against *Staph. aureus* that is about 1000 times its activity against *E. coli*.

[9] Unpublished data obtained in this laboratory.

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Improved Method for Microdetermination of Plasma Vitamin E in Laboratory Rats

MAHASEN M. MESHALI and CHARLES H. NIGHTINGALE ˣ

Abstract □ The method generally used to assay human serum for vitamin E (tocopherol) was found to be inapplicable to rat plasma. However, a modified fluorometric method was suitable for both human and rat plasma. This microassay is relatively rapid and reproducible and was utilized to monitor the onset of the tocopherol-deficiency state in rats fed a vitamin-free diet.

Keyphrases □ Vitamin E—improved microdetermination, rat plasma □ Tocopherol—improved microdetermination, rat plasma □ Fluorometry—microdetermination of vitamin E, rat plasma

Problems associated with the analysis of vitamin E (tocopherol) in biological fluids obtained from laboratory animals include the small sample size and the endogenous materials that interfere with tocopherol determinations. The present study describes a modified procedure that improves the fluorometric analysis (1) of tocopherol in humans. The modified microassay is relatively rapid and extremely reproducible, and it may be used for small laboratory animals as well as humans.

EXPERIMENTAL

Rats, Sprague-Dawley[1], weighing 156 ± 10 g, were divided into three groups of 60 each. The normal control animals were fed ordinary laboratory chow[2], while the others were fed either a tocopherol-deficient diet (deficient group) or the deficient diet supplemented with 20 μg/g of α-tocopheryl acetate (supplemented control group). Food and water were allowed *ad libitum*. The composition of the deficient diet was as suggested by Bieri and Privel (2). Both the deficient and supplemented diets[3] were prepared in powdered form in these laboratories. The rat food was assayed colorimetrically at 534 nm for tocopherol content by the method of Bieri (3).

To establish whether the decline in tocopherol plasma levels was a result of the diet and not to a lack of food intake, the rate of animal growth as well as food consumption was monitored.

The method of analysis for free and total tocopherol plasma levels in rats was a modification of the fluorometric procedure of Hansen and Warwick (1) which was suggested for humans. These authors used a 0.1-ml serum sample to which were added 1 ml of water, 1 ml of ethanol, and 2 ml of hexane. The mixture was shaken with a mechanical shaker and centrifuged; then a portion of the clear supernatant layer was assayed fluorometrically, against a reagent blank, for free tocopherol at 295 and 340 nm, the wavelengths of maximum excitation and emission, respectively. Total tocopherol (free plus acetate) was determined by redissolving 1 ml of the remaining supernate, after evaporation to dryness, in 2 ml of hexane and adding 0.5 ml of a solution containing 15.0 mg $LiAlH_4$ dissolved in 10 ml of anhydrous ether. Three milliliters of 0.1 N H_2SO_4 was added, the solution was mixed and centrifuged, and the fluorescence was measured.

In this study the assay procedure was modified by increasing the sample size to 0.2 ml of plasma and reducing the volume of

[1] Blue Spruce Farms, Altamont, New York.

[2] Ralston Purina Co., St. Louis, Mo.

[3] All dietary constituents were purchased from General Biochemicals, Chagrin Falls, Ohio, with the exception of the stripped lard which was obtained from Eastman Kodak Co., Rochester, N.Y. All materials were used as received.