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Kinetics and Mechanisms of Drug Action on Microorganisms XXI: Effect of Quinacrine on *Escherichia coli* and Its Possible Complexation with Components of Nutrient Growth Medium

A. J. RICHARD* and EDWARD R. GARRETTˣ

Abstract □ The steady-state generation of *Escherichia coli* (ATCC 12407) was inhibited by the antimalarial quinacrine in the drug concentration range of 0–100 μg/ml at pH 6.9 in Anton's medium, and the rate of decrease of generation constants increased with drug concentration. The drug activity was affected by the concentration of amino acids in the nutrient broth and indicated that the inactivation of quinacrine may be explained by its saturable complexation to the amino acids; methods were applied to estimate concentrations of complexing sites. Studies of generation inhibition at various pH values showed that the diprotonated quinacrine exerted no significant antibacterial activity. Quinacrine was bactericidal at concentrations above 120 μg/ml. Equipotent combinations of quinacrine with tetracycline or chloramphenicol were indifferent.

Keyphrases □ Quinacrine—inhibition of *E. coli* generation in Anton's medium, amino acid concentration effect on quinacrine inactivation □ *Escherichia coli* generation—inhibition by quinacrine, in Anton's medium, amino acid concentration effect on quinacrine inactivation □ Microorganisms, inhibition of *E. coli*—quinacrine in Anton's medium, amino acid concentration effect on quinacrine inactivation

The antimalarial (1) quinacrine, 6-chloro-9-[[4-(diethylamino)-1-methylbutyl]amino]-2-methoxyacridine, has been reported to be clinically effective and synergistic *in vivo* (2) and *in vitro* (3–5) in combinations with other antibiotics to delay or prevent the emergence of antibiotic-resistant strains of Gram-negative and Gram-positive bacteria. Quinacrine, a known nucleic acid intercalating agent, inhibits DNA replication and prevents the transfer of R-factors to result in (6, 7) antibacterial activity.

This paper reports on the quantitative microbial kinetics of the Gram-negative *Escherichia coli* (ATCC 12407) as affected by quinacrine. A general theory, recently developed (8) to rationalize the steady-state microbial generation of *E. coli* affected by bacteriostatic drugs, is applied to quinacrine-affected generation rates. Specifically, it is shown how microbial kinetics may be used to quantify the protein binding or nutrient complexation of a bacteriostatic agent when such binding is a saturable process.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* (ATCC 12407) were used in all experiments. This nonclumping strain is well suited for colony counts and for small particle monitoring[1].

Culture Medium—Only Anton's medium (9) was used in this study. Its preparation and the variations of the broth employed were described previously (8).

Antibiotics—The antibiotics used in combination experiments were all assayed antibiotics and included quinacrine hydrochloride[2] USP (hereafter referred to as quinacrine), chloramphenicol[3] (purity grade), and tetracycline hydrochloride[4] USP.

[1] Coulter counter.
[2] Winthrop Laboratories, New York, N.Y.
[3] Thomae GMBH, Germany.
[4] The Upjohn Co., Kalamazoo, Mich.

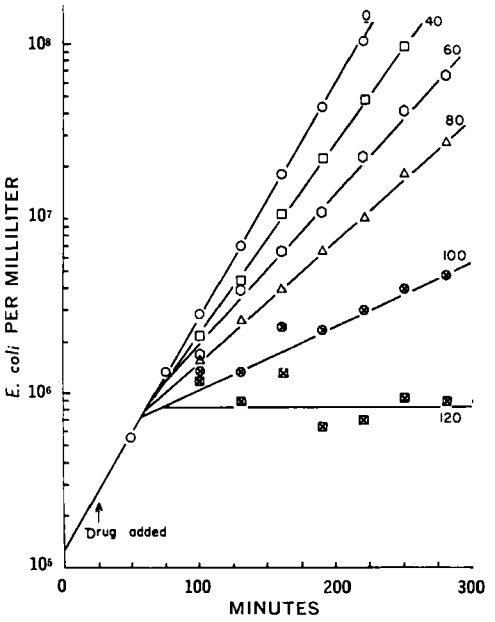


Figure 1—*Typical generation curves of* E. coli *in Anton's medium (pH 6.9) in the absence and presence of various concentrations of quinacrine, obtained by total counts. The curves are labeled according to the quinacrine concentrations in micrograms per milliliter.*

Bacterial Generation and Monitoring—Methods used in this laboratory for bacterial culture, total count monitoring, viable counting, and size frequency distribution studies were described in detail (8, 10, 11).

Reversibility of Drug Action—Reversibility of drug action can be shown by taking aliquots of organism cultures containing about 10^7 *E. coli*/ml during the drug-affected steady-state growth at 37.5° and diluting into fresh broth with no added drug and also into fresh broth containing predetermined drug concentrations (11). Reversibility of drug action is established if the diluted cultures grow at the control rate in the fresh broth and at the predicted rates when drug is present in the diluent. Reversibility was studied at 0 and 90 μg/ml of quinacrine at pH 6.9 in Anton's medium.

Effect of Equipotent Mixtures of Quinacrine and Other Growth Inhibitors—Replicate 49.5-ml volumes of cultures containing 5×10^5 *E. coli*/ml generating in the logarithmic phase at pH 6.9 and 37.5° were treated with 0.5 ml of equipotent mixtures of antibiotics (12). The mixtures consisted of 100, 80, 60, 40, 20, and 0% of the equipotent quinacrine solution and the residual percentage of the other antibiotic solution to maintain equipotency at two different dosage levels. If the drugs precipitated on mixing, the mixture was diluted with Anton's medium until the precipitate dissolved; the appropriate volume was withdrawn from each culture flask before addition of drug solutions.

RESULTS AND DISCUSSION

Effect of Drug Concentration on Generation Rates—The generation rates of *E. coli* in graded quinacrine concentrations measured by the total count and the viable count methods in Anton's medium at pH 6.9 were coincident (Fig. 1), and no bactericidal activity was observed up to 100 μg/ml of quinacrine. It was, therefore, justified to use total counts to determine generation rates up to a limiting quinacrine concentration of 100 μg/ml. At 150 μg/ml, a significant kill of bacteria was observed. The drug was rapidly equilibrated between the growth medium and the biophase, as indicated by a lag time of 25 min or less between the time of drug addition and the appearance of deviation from the control logarithmic phase growth rate. The slopes of the linear semilogarithmic plots of the drug-affected organisms obtained from the curves in Fig. 1 represent the steady-state generation rate constants (k_{app}). The plots of k_{app} values against drug concentrations are shown in Fig. 2 for three inoculum sizes: 1×10^5, 5×10^5, and 1×10^6 *E. coli*/ml at the time of drug addition. No difference in drug effectiveness was observed when the quinacrine was added over this 10-fold range in the bacterial population.

Effect of pH and Nutrient Broth Components on Drug-Affected Generation Rates—The quinacrine-affected generation rates and rate constants (k_{app}) were reduced markedly with increased pH of the growth medium (Fig. 3), whereas pH variation in the region studied had no significant effect on drug-free generation. Since the phosphate buffer of the growth medium has reduced capacity above pH 7.8, and since *E. coli* generation is significantly affected in drug-free medium above pH 8, the former is an upper limit for the pH range. Quinacrine is a dication (13) at neutral pH values with pKa's of 7.69 and 10.18. When the total quinacrine concentrations were multiplied by the fraction, *f*, of drug singly charged to obtain monocation concentrations, the various k_{app} concentration curves were coincident (Fig. 3, insert).

Variations of the ammonium ion (NH_4^+), magnesium ion (Mg^{+2}), citrate, and dextrose components of Anton's broth did not modify the drug-affected generation rates. When the broth amino acid concentration was halved, the potency of quinacrine increased; when the amino acid concentration was doubled, the potency of the quinacrine decreased (Fig. 4). This indicates significant drug inactivation by the broth amino acids of effective quinacrine in the medium. Identical effects were observed when the broth concentrations were doubled and halved. When the concentration abscissa for the doubled concentration is halved and when the concentration abscissa for the half concentration is doubled, the resultant plots are close but not exactly coincident to that for the normal amino acid concentration, indicating that the extent of inactivation is related to the amino acid concentration.

Reversibility of Drug Action—The effects of quinacrine on *E. coli* were reversible after exposure of the organisms up to concentrations of 90 μg/ml of quinacrine for 200 min at pH 6.9. A 10-fold dilution of the culture at 10^7 organisms/ml with fresh medium 225 min after the addition of 90 μg/ml of quinacrine immediately resulted in a new generation rate close to that observed in a drug-free culture. Higher drug concentrations were not studied for reversibility because of the kill effect observed at drug levels of 150 μg/ml.

Drug Effects on Size of *E. coli*—There was no difference in the size of generating *E. coli* up to quinacrine levels of 50 μg/ml as compared to organisms generating in drug-free medium. However, the average cell size was 40% greater than the control cells at 55 μg/ml of quinacrine and almost double at 95 μg/ml of quinacrine, indicating that cell division occurs at larger cell sizes with higher drug concentrations.

Effects of Drug Combinations with Quinacrine—The observed k_{app} values for variously presumed equipotent combinations of quinacrine with other bacteriostatic drugs against the volume fraction of quinacrine solution in mixtures of solutions

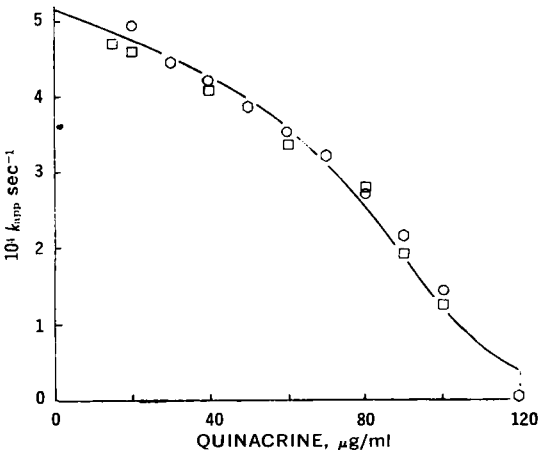


Figure 2—*Dependency of the apparent generation rate constant,* k_{app}, *at pH 6.9 of* E. coli *on drug concentration of quinacrine in micrograms per milliliter. The solid line through the data points was calculated from the theory (8) which accounts for the complexation of quinacrine with amino acids in the nutrient broth. The inoculum sizes were:* ○, 10^5 E. coli/ml; ○, 5×10^5 E. coli/ml; *and* □, 10^6 E. coli/ml.

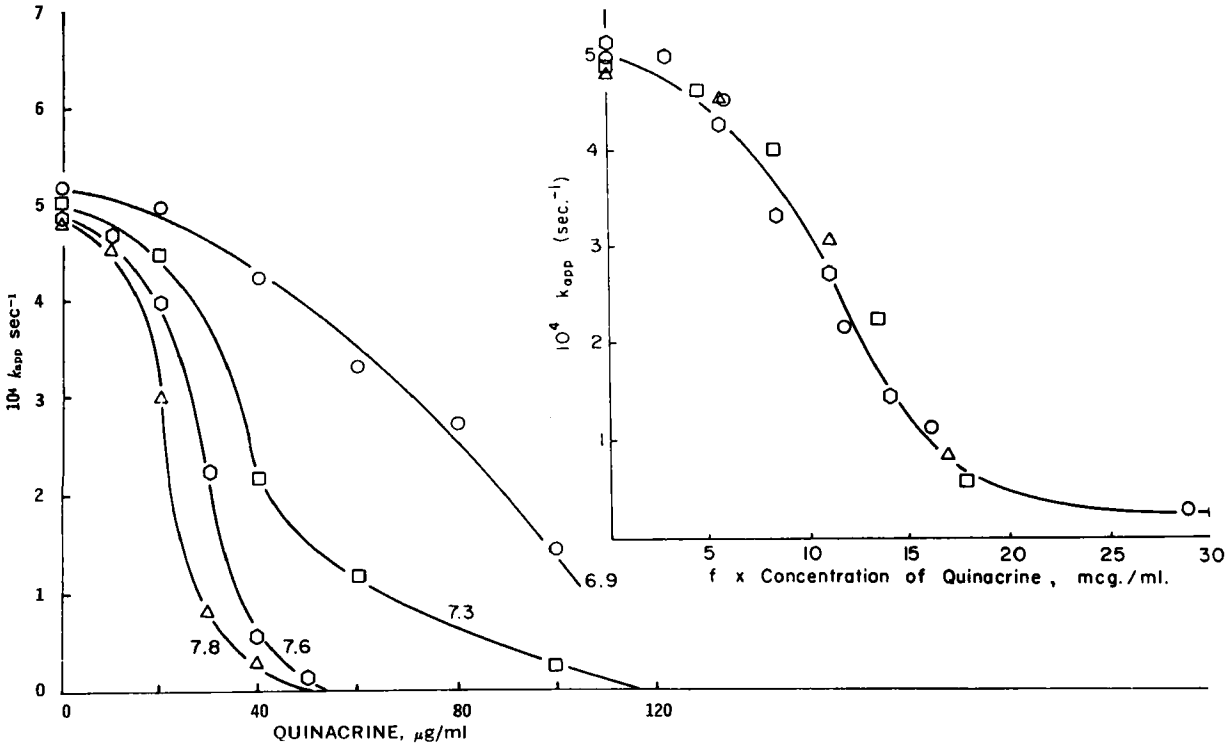


Figure 3—*Effect of pH upon the dependency of* k_{app} *on drug concentration. Only one curve (insert) is required when the quinacrine concentrations were multiplied by the fraction,* f, *of singly charged drug with a pKa' of 7.7. The curves are labeled with the pH value of the study.*

equipotent in quinacrine and tetracycline or chloramphenicol showed no significant difference in k_{app} for any mixture. Thus, it can be concluded that chloramphenicol and tetracycline act independently of the action of quinacrine.

Evaluation of Drug Inactivation in Medium by Microbial Kinetics—A significant fraction of the quinacrine is rendered inactive by the amino acid component of the nutrient broth at pH 6.9 (Fig. 4). The activity of quinacrine increases with pH (Fig. 3) and indicates improved transport of the drug across the bacterial cell wall with decreasing charge and/or decreased inactivation of drug by the broth amino acids with increasing pH.

Scheme I represents a model consistent with the observed dependency of the steady-state rate constants (k_{app}) for microbial generation on the concentration of quinacrine in the nutrient medium. This model postulates that free or effective drug, C_u, permeates the cell to result in a steady-state biophase concentration, C', which competes with the reaction of a metabolite precursor, M, for those receptor sites, R, vital to microbial generation. Steady-state generation of bacteria can result when the concentration, C', of drug in the biophase is a constant. It is assumed that biophase interactions and possible inactivation of drug do not significantly affect the external concentration of drug, C_u, and that the equilibria of M and C' with receptor sites, R, are instantaneous.

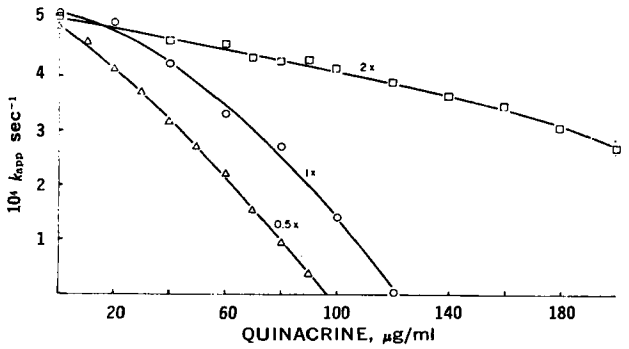


Figure 4—*Effect of varying the concentration of casamino acids of Anton's medium upon the* k_{app} *dependency on quinacrine concentration. The curves are labeled as to the multiples of casamino acid concentration. No such effects were observed when other components of Anton's medium were each varied in turn.*

An expression is available (8, 14, 15) to calculate the concentration of free or effective drug in the nutrient broth for this system when there are saturable drug complexing or reacting sites, $n[P]$, in the medium:

$$C_u = \frac{C_t}{1 + \dfrac{n[P]}{K_4 + C_u}} \qquad \text{(Eq. 1)}$$

where C_t is the total drug concentration in the medium, K_4 is the dissociation constant for complexation, and n is the number of sites per molecule of complexing agent, P. The value of C_u can be expressed explicitly as the solution of a quadratic equation when the parameters $n[P]$ and K_4 are known. It has been shown (8) that consideration of the saturable receptor site model of Scheme I with the assumption of nonsaturable inactivation of the drug in the biophase, *i.e.*, where k_d is a first-order rate constant, leads to:

$$k_{app} = k_0 - \frac{k_0 K_1' K_2 C_u}{1 + K_1' K_2 C_u} \qquad \text{(Eq. 2)}$$

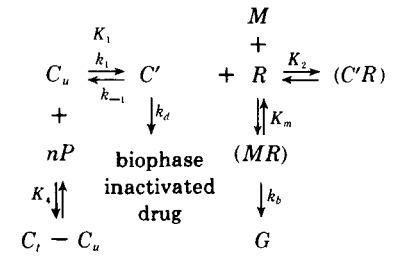
so that:

$$\lim_{C_u \to \infty} \frac{k_0 - k_{app}}{k_{app}} = K_1' K_2 C_t - K_1' K_2 n[P] \qquad \text{(Eq. 3)}$$

and:

$$\lim_{C_u \to 0} \frac{k_0 - k_{app}}{k_{app}} = \frac{K_1' K_2 C_t}{1 + \dfrac{n[P]}{K_4}} \qquad \text{(Eq. 4)}$$

where k_0 is the microbial generation rate constant in the absence of drug, and $K_1' = k_1/(k_{-1} + k_d)$ reduces to $K_1 = k_1/k_{-1}$ for the drug-affected steady-state generation if there is no biophase inactivation of drug. A plot (Fig. 5) of $(k_0 - k_{app})/k_{app}$ against drug concentration will provide a slope, S_2, at high drug concentrations to estimate preliminarily $K_1'K_2$, and an initial slope, S_1, at low



Scheme I—Steady-state model for permeation of free drug of concentration C_u *in nutrient medium into the biophase of the microorganism of concentration* C′, *and the net result is the observed inhibition of the microbial generation rate which is postulated to be proportional to the production of* G. *Capitalized* K's *represent equilibrium constants for the particular reactions; lower case* k's *are rate constants.*

drug concentrations to estimate $K_1'K_2/(1 + n[P]/K_4)$. The intercept of the extrapolated terminal linear portion of the curve on the abscissa, C_1, will provide an estimate of $n[P]$, and the ratio of the terminal slope, S_2, to the initial slope, S_1, gives $1 + n[P]/K_4$. It is then possible to calculate K_4 from the ratio:

$$\frac{C_1}{n[P]/K_4} = \frac{n[P]}{n[P]/K_4} = K_4 \qquad \text{(Eq. 5)}$$

These initial estimates of these parameters can be refined by the methods given previously (8) to give the best fit for the plot of the experimental k_{app} against drug concentration. These refined parameters of $n[P]$ and K_4 then permit the calculation (8) of C_u as a function of total drug concentration, C, by:

$$C_u = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C + n[P])^2 - 4n[P]C}}{2} = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C - n[P])^2 + 4n[P]K_4}}{2} \qquad \text{(Eq. 6)}$$

The obtained values for quinacrine in normal Anton's broth are $K_4 = 0.49$ μg of equivalent quinacrine/ml or 0.93×10^{-6} mole/liter and $n[P] = 105$ μg of equivalent quinacrine/ml or 2.06×10^{-4} mole/liter. The curve drawn through the points in Fig. 2 was calculated from Eq. 6 using these parameters. An appropriate plot (Fig. 6, curve A) shows a decreasing rate of change of k_{app} with the increasing calculated C_u values consistent with the saturable receptor site model (Scheme I) for drug action that was postulated for the estimates of C_u. The minimum inhibitory concentration (MIC) was about 15 μg/ml of unbound quinacrine at pH 6.9 compared to that based on total drug of about 100 μg/ml (Fig. 2). In terms of the concentration of the monoprotonated quinacrine concentration (Fig. 6, curve B), the MIC is about 2.5 μg/ml.

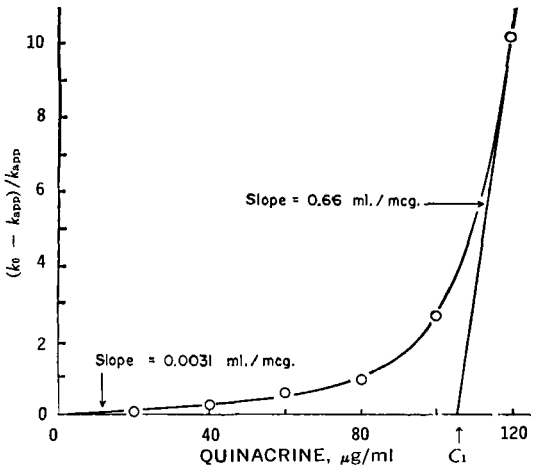


Figure 5—*Plot of* $(k_0 - k_{app})/k_{app}$ versus *concentration of quinacrine in micrograms per milliliter at pH 6.9 according to Eqs. 3 and 4, where the asymptotic terminal slope,* S_2, *is an estimate of* $K_1'K_2$, *and the initial slope estimates* $K_1'K_2/(1 + n[P]/K_4)$. *The intercept,* C_1, *on the abscissa estimates* $n[P]$.

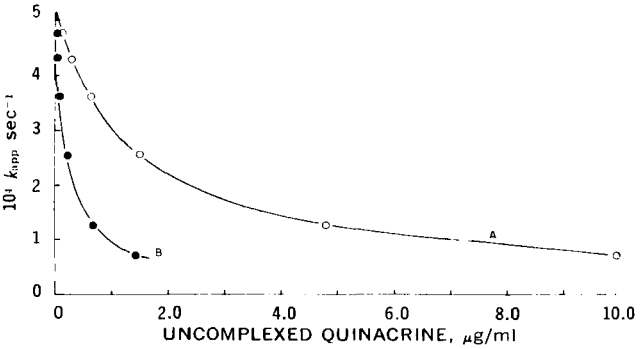


Figure 6—*Plot of* k_{app} versus *a presumed uncomplexed quinacrine concentration at pH 6.9 to the amino acids of the nutrient broth (curve A) and* versus *a presumed uncomplexed quinacrine concentration that is singly charged (curve B), i.e., the quinacrine concentrations multiplied by the fraction,* f, *of singly charged drug with a pKa′ of 7.7.*

It must be realized that these estimates of the constants for complexation of quinacrine to the media were based on Scheme I and assumed nonsaturable biophase inactivation of drug and saturable binding of drug to receptor sites so that $K_1'K_2$ of Eq. 3 for the data of Fig. 5, as deduced from such a model (8), is 0.66 ml/μg of equivalent quinacrine or 0.33 liter/mole. This may not necessarily be true since the terminal points of the k_{app} *versus* concentration plots of Figs. 2–4 also could be approximated reasonably by straight lines, which would indicate essentially nonsaturable binding of receptor sites in the biophase. The fitting of the data to a model that presumes this with saturable complexation to the components of the medium would give different values of $n[P]$ and K_4 (8).

It has been shown for a model based on such premises (8), where:

$$k_{app} = k_0 - k_0K_1'K_2C_u \qquad \text{(Eq. 7)}$$

so that $K_1'K_2C_u \ll 1$ in Eq. 2, that plots of $(k_0 - k_{app})/k_0$ against total drug concentration will provide a slope, S_2', at high drug concentrations to estimate preliminarily the $K_1'K_2$ of Eq. 7 for the data of Fig. 7, which is 77 ml/μg of equivalent quinacrine or 39 liters/mole, and an initial slope, S_1', at low drug concentrations to estimate $K_1'K_2/(1 + n[P]/K_4)$ from:

$$\lim_{C_t \to 0} (k_0 - k_{app})/k_0 = K_1'K_2C_t/(1 + n[P]/K_4) \qquad \text{(Eq. 8)}$$

$$\lim_{C_t \to \infty} (k_0 - k_{app})/k_0 = K_1'K_2C_t - K_1'K_2n[P] \qquad \text{(Eq. 9)}$$

where, when $(k_0 - k_{app})/k_0 = 0$, the intercept of the extrapolated terminal linear portion of the curve on the abscissa, C_1, will provide an estimate of $n[P]$. It would be also possible from the ratio of the terminal slope, S_2', to the initial slope, S_1', to estimate $1 + n[P]/K_4$ so that the K_4 value may be estimated by Eq. 5. Such plots (Fig. 7) in accordance with Eqs. 8 and 9 estimate $n[P] = 40$ μg of equivalent quinacrine/ml or 0.79×10^{-4} mole/liter for normal Anton's medium (Fig. 7, curve A). At half the concentration of Anton's medium, the estimated $n[P]$ is approximately half this value (Fig. 7, curve B), which is consistent with the premise that the number of complexation sites for quinacrine is proportional to the concentration of components of the nutrient medium.

The additional fact that plots of k_{app} against concentration of monoprotonated quinacrine are coincident for all studied pH values in Anton's medium (Fig. 3, insert) indicates that the extent of complexation to the components of the medium is not significantly pH dependent, that the active species in the medium are the monoprotonated and neutral molecules, and that monoprotonation of the tertiary nitrogen of the side chain (pKa′ = 10.18) has little effect on the ability of quinacrine to exercise its action, where the dication with the protonated amine of pKa′ = 7.69 (13) appears to exercise no action.

Although the inactivation of quinacrine effectiveness by amino acids can be rationalized by the postulated complexation of

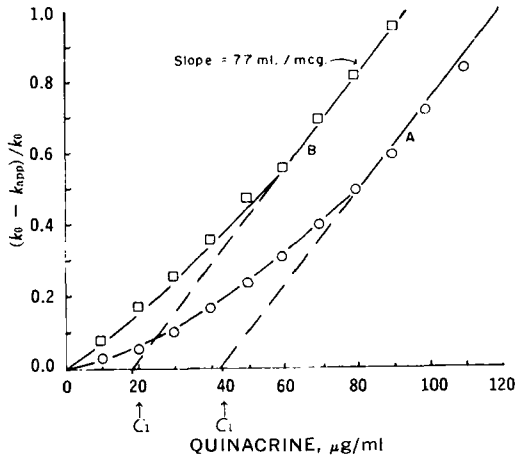


Figure 7—*Plot of* $(k_0 - k_{app})/k_0$ versus *concentration of quinacrine in micrograms per milliliter at pH 6.9 according to Eqs. 8 and 9, where the asymptotic terminal slope,* S_2, *is an estimate of* $K_1'K_2$. *The intercept,* C_1, *on the abscissa estimates* $n[P]$. *Curve A is for normal Anton's medium, and curve B is for a medium with half the concentrations of the components.*

Scheme I, where the complexed drug is presumed to be unavailable to the biophase, no direct evidence of such complexation is available as yet. Alternative explanations may be that increased amino acid concentrations decrease the rate of permeation to the biophase, characterized by the rate constant k_1 by modifying the cell wall, or increase the rate of biophase inactivation characterized by the rate constant k_d of Scheme I. However, if such processes exist, they must be saturable in that either the amino acid inhibition of the rate of permeation has a limited effect or the rate of biophase inactivation has a maximum value related to the amino acid concentration.

If the increase in concentration of a nutrient such as the amino acids has the effect of permitting a biophasic or metabolic increase in the rate of bacterial repair of damage caused by the antibacterial insult, this could also account for the observed phenomenon of amino acid antagonism of drug action. Again, such a process must be saturable and the rate of repair cannot be directly proportional to the amino acid concentration but must approach a maximum value related to the amino acid concentration.

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