tine predominantly of $\Delta^3$-carene; (*b*) generally taller trees with conical crowns; foliage distinctly bluish, the needles thicker than in (*a*); cones with the apophyses more rounded on the outer side and generally less spiny than in (*a*); and turpentine predominantly $\alpha$-pinene.

Unfortunately, the lineage of the New Zealand specimens cannot be traced back to a known provenance in California. Nevertheless, the scheme suggested not only fits the chemical facts so far, but also agrees closely with the morphological work of Duffield (27). His conclusion was that the mainland populations of *P. muricata* could well be divided into two varieties, one of which, including the Huckleberry Hill population and the typical form near San Luis Obispo, stretched from the Annapolis area southward, while the other, including the Fort Bragg population, stretched from Annapolis northward.

Further close collaboration between botanists and chemists studying this species is highly desirable; particularly useful would be a detailed population analysis in the Annapolis area where, according to Duffield (27), the trees show a morphological transition between the typical variety and the northern one.

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# Amino Ketones: Kinetics of *In Vitro* Antibacterial Activity

By SHU-SING CHENG†, SIGURDUR JONSSON‡, and FRED T. SEMENIUK

**$\alpha$- and $\beta$-Amino ketone analogs of amino acids were tested *in vitro* against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus anthracis, Saccharomyces cerevisiae,* and *Candida* spp. by serial broth dilution and agar diffusion methods and found to be active as growth-inhibitors at concentration levels which varied with the test culture. The study of the growth curve of a test bacterium in a liquid medium with and without the presence of the drug provides an accurate means for the *in vitro* evaluation of the antibacterial activity of potential chemotherapeutic agents. It serves also as a means for studying the dynamics as well as the mode of antibacterial action. Since the generation time and the numbers of generations of microbial growth occur before stationary phase can be calculated, the inhibitory activity of different drugs may be compared on this basis.**

THE growth-inhibitory activity of $\beta$-amino ketone analogs of $\beta$-alanine against *Escherichia coli* and *Staphylococcus aureus* has been reported recently (1). This leads to speculation that $\alpha$-amino ketone analogs of $\alpha$-amino acids might possess similar activity. This study was then undertaken in order to investigate the potential antimicrobial action of these two categories of amino ketone analogs of amino acids.

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## PROCEDURES AND RESULTS

There are three general methods for determining drug sensitivity of bacteria currently in use: the serial broth dilution, agar diffusion, and agar plate dilution methods. For the *in vitro* evaluation of antimicrobial activity of amino ketone hydrochlorides, the first two methods were selected.

**Agar Diffusion Method.**—*Staphylococcus aureus*, *Bacillus anthracis*, and *Pseudomonas aeruginosa* were used as test organisms. Stock culture was maintained on slants of peptone-casein agar and transferred to fresh slants about once a week. The assay plates and inocula were prepared according

TABLE I.—ANTIMICROBIAL ACTIVITIES OF AMINO KETONE HYDROCHLORIDES TESTED BY AGAR DIFFUSION METHOD

| Hydrochloride[a] | Diameter, mm., of Inhibitory Zone against Microorganism Exhibited by the Indicated Quantity of Drug | | | |
|---|---|---|---|---|
| | *Bacillus anthracis* | | *Pseudomonas aeruginosa* | |
| | 1 mg. | 2 mg. | 1 mg. | 3 mg. |
| *n*-Hexyl β-aminoethyl ketone | 9 | 11 | .. | .. |
| *n*-Heptyl β-aminoethyl ketone | 13 | 32 | .. | .. |
| Phenyl β-aminoethyl ketone | 0 | 0 | 0 | 16 |
| *p*-Tolyl β-aminoethyl ketone | 0 | 0 | 0 | 10 |
| 2,5-Dimethylphenyl β-aminoethyl ketone | 8 | 11 | 0 | 11 |
| Phenyl aminomethyl ketone | 10 | 16 | .. | .. |
| *o*-Tolyl aminomethyl ketone | 0 | 13 | 0 | 11 |
| *p*-Tolyl aminomethyl ketone | 7 | 16 | .. | .. |
| 3,4-Dimethylphenyl aminomethyl ketone | 9 | 17 | 0 | 14 |
| *p*-Tolyl α-aminoethyl ketone | 0 | 7 | 0 | 10 |
| 3,4-Dimethylphenyl α-aminoethyl ketone | 0 | 8 | .. | .. |
| 4-Phenyl-4-keto-2-amino-butyric acid-(1) | 8 | 11 | .. | .. |
| 2-Amino-1-indanone | 16 | 22 | 10 | 18 |

[a] See references (1) and (7) for the preparation of these compounds.

to U.S.P. XV method for penicillin microbial assay (2). The medicated disk method was used instead of cylinder-plate method. Peptone agar and peptone-casein agar were used for preparing the supporting agar layer and seeding layer, respectively.

The medicated paper disks were gently placed on the surface of the inoculated agar plate. For reference, sulfanilamide (5 mg.), sodium sulfamerazine (3 mg.), and sodium sulfapyridine (3 mg.) were included. In the case of sulfanilamide, the drug was placed directly on the surface of the inoculated agar plate without using the paper disk. All the plates were incubated at 37° for 16 to 18 hours, after which the growth responses were examined and the diameters of the clear zones of growth inhibition were measured on a bacterial colony counter. The results are summarized in Table I. 2-Amino-1-indanone hydrochloride exhibited a rose-red inhibitory zone on the surface of agar plate inoculated with *B. anthracis*, whereas phenyl aminomethyl ketone and *p*-tolyl aminomethyl ketone hydrochlorides each exhibited a rose-red inhibitory zone with a surrounding zone of growth-stimulation when tested under the same conditions. *o*-Tolyl aminomethyl ketone and 3,4-dimethylphenyl aminomethyl ketone hydrochlorides each showed yellow to brownish inhibitory zone surrounded by a zone of growth-stimulation when tested against *B. anthracis* and *P. aeruginosa*.

Of the 17 compounds tested, 2-amino-1-indanone and *n*-heptyl β-aminoethyl ketone hydrochlorides (2 mg. each) exhibited the widest zones of inhibition against the growth of *B. anthracis*. Sulfanilamide (5 mg.) produced very slight or no inhibition on the test organisms. Sodium sulfapyridine (3 mg.) and sodium sulfamerazine (3 mg.) were inactive.

**Serial Broth Dilution Method.**—*Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538 P, *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida krusei* were used as test organisms. Stock cultures of *E. coli* and *Staphylococcus aureus* were maintained on trypticase-soy agar slant, while the stock cultures of yeast and fungi were maintained on Sabouraud dextrose agar slants. The culture media used in the test were a synthetic liquid medium (3) for *E. coli;* nutrient broth for *Staphylococcus aureus;* and Sabouraud liquid medium for *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida krusei*.

The inocula were prepared from 18- to 24-hr. bacterial broth cultures or from 3-day fungus and yeast broth cultures. For broth cultures, the test organisms were transferred into the appropriate liquid medium and incubated at 37° for bacteria, and 30° for yeast and fungi. One drop (0.05 ml.) of 1:100 dilution of the broth culture in sterile distilled water was used to inoculate the medium. Viable cell counts were made from the serial dilutions of these inocula by the pour-plate method for bacteria, and by the surface-spreading method for yeast and fungi.

The stock solutions of amino ketone hydrochlorides were prepared by dissolving the drug in sterile distilled water to make a concentration of 5000 mcg./ml., and were sterilized by filtering through a Millipore filter.

For the conduct of the test, 10 15-ml. test tubes, each containing 5 ml. of the sterilized double-strength broth, were divided into duplicate series. A 1-ml. quantity of the stock solution of amino ketone hydrochloride was added to the first tube; 0.5 ml. of the same solution was added to the second; 0.5 ml. of a 1:2 and 1:4 dilution of the stock solution, respectively, was added to the third and fourth. The volume was made up to 10 ml. in each tube. The final concentration of the amino ketone hydrochloride in each tube was 500, 250, 125, 62.5, and 0 mcg. per ml., respectively. The last tube, without drug, served as organism growth control. Each tube was inoculated with 1 drop of a 1:100 dilution of the broth culture. Incubation was at 37° overnight for the bacteria, and at 30° for 3 days for yeast and fungi. The tubes were examined for growth inhibition of the organism at the end of each incubation period. The least amount of drug resulting in complete inhibition of growth was recorded as minimum inhibitory concentration. The results are summarized in Table II.

**Mode of Antibacterial Action.**—In order to investigate the mode of antibacterial action, a study of the growth curve of the test organism in a liquid medium, with and without the presence of drug, was then performed. The results are shown in Figs. 2 and 3 for the response of *Staphylococcus aureus* toward *o*-tolyl aminomethyl ketone and *n*-heptyl β-aminoethyl ketone hydrochlorides, respectively. The growth curve was based on viable cell counts obtained by the pour-plate technique.

Examination of the control growth curve Ia (the curve resulting from the growth of *Staphylococcus aureus* in the absence of drug) in Figs. 2 and 3 reveals that the curves follow the pattern of a typical growth curve of bacterium as shown in Fig. 1, except for the lack of a lag phase. This is explainable by the active physiological state of the bacterial cells (19-hr. culture).

In Fig. 2, curves IIa, IIIa, and IVa record the growth response of 14,600 cells of *Staphylococcus aureus* in nutrient broth in the presence of 125, 250, and 1000 mcg. of *o*-tolyl aminomethyl ketone hydrochloride per ml. of culture medium. Curves IIa and IIIa indicate that, at drug concentrations of 125 mcg. and 250 mcg./ml., 14,600 cells from the inoculum multiply by four to five generations to reach a final population of $3 \times 10^5$ cells, 8 hr. and 36 min. after inoculation and reach the stationary phase. The calculation involves solving the equation $b = a \times 2^n$, where $b$ is the number of cells of the final population, $a$ is the number of cells introduced into the medium from the inoculum, $n$ is the number of generations by which the initial number of cells multiply to yield the final population (4). The calculation is shown as follows

$$b = a \times 2^n$$

$$n = \frac{\log_{10} b - \log_{10} a}{\log_{10} 2}$$

$$= \frac{\log_{10} 3 \times 10^5 - \log_{10} 14{,}600}{\log_{10} 2}$$

$$= 4.368 \text{ generations}$$

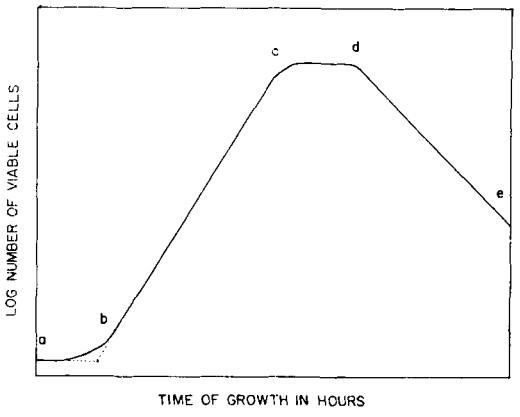


Fig. 1.—Typical growth curve of bacteria.

In contrast to curves IIa and IIIa, Ia indicates that, in the absence of drug, 14,600 cells continue the multiplication for 20.32 generations to yield a final population of $192 \times 10^8$ cells before the phase of decline is reached at 8 hr. and 36 min. after the inoculation was made

$$n = \frac{\log_{10} 192 \times 10^8 - \log_{10} 14{,}600}{\log_{10} 2}$$

$$= 20.32 \text{ generations}$$

On the other hand, as shown by curve IVa, representing a drug concentration of 1000 mcg./ml., 14,600 cells of *Staphylococcus aureus* did not increase in number, but decreased logarithmically even after brief contact with the drug. Eighty-eight minutes after inoculation and contact with the drug, the population dropped to 9000 cells, contrasting with the respective figures of $105 \times 10^3$, $213 \times 10^3$, and $696 \times 10^3$ cells in the presence of 250, 125, and 0 mcg. of *o*-tolyl aminomethyl ketone hydrochloride/ml. of culture medium, respectively. In the logarithmic phase, the curve IVa has a negative slope of −0.00239 in comparison with +0.00973, +0.01322, and +0.0197 for curves IIIa, IIa, and Ia, at respective drug concentrations of 250, 125,

TABLE II.—ANTIMICROBIAL ACTIVITIES OF AMINO KETONE HYDROCHLORIDES TESTED BY BROTH SERIAL DILUTION METHOD

| Hydrochloride[a] | Minimum Inhibitory Concn. against Microorganism, mcg./ml. of Medium | | | | |
|---|---|---|---|---|---|
| | *E. coli* | *Staph. aureus* | *C. albicans* | *C. krusei* | *Sacch. cer.* |
| *n*-Butyl β-aminoethyl ketone | 500 | ... | ... | ... | ... |
| *n*-Hexyl β-aminoethyl ketone | 500 | 1000 | 2000 | ... | 1000 |
| *n*-Heptyl β-aminoethyl ketone | 250 | 500 | ... | 1000 | ... |
| Phenyl β-aminoethyl ketone | 500 | 1500 | ... | ... | ... |
| *p*-Tolyl β-aminoethyl ketone | 500 | ... | ... | ... | 2000 |
| 2,5-Dimethylphenyl β-aminoethyl ketone | 250 | 250 | 1000 | 2000 | 2000 |
| Phenyl aminomethyl ketone | 250 | 250 | 500 | ... | 2000 |
| *o*-Tolyl aminomethyl ketone | 250 | 250 | ... | ... | ... |
| *p*-Tolyl aminomethyl ketone | 250 | 250 | ... | ... | ... |
| 3,4-Dimethylphenyl aminomethyl ketone | 250 | 250 | ... | ... | ... |
| 2-Furyl β-aminoethyl ketone | 250 | ... | ... | ... | ... |
| 2-Thienyl β-aminoethyl ketone | 125 | ... | ... | ... | ... |
| *p*-Hydroxyphenyl α-aminoethyl ketone | 125 | ... | ... | ... | ... |
| *p*-Tolyl α-aminoethyl ketone | ... | ... | 2000 | ... | 2000 |
| 3,4-Dimethylphenyl α-aminoethyl ketone | 125 | 200 | ... | ... | ... |
| Phenyl α-aminoethyl ketone | ... | 200 | ... | ... | ... |
| 4-Phenyl-4-keto-2-amino-butyric acid | 1000 | ... | ... | ... | ... |
| 2-Amino-1-indanone | 125 | 63 | 500 | 250 | ... |

[a] See references (1) and (7) for the preparation of these compounds.

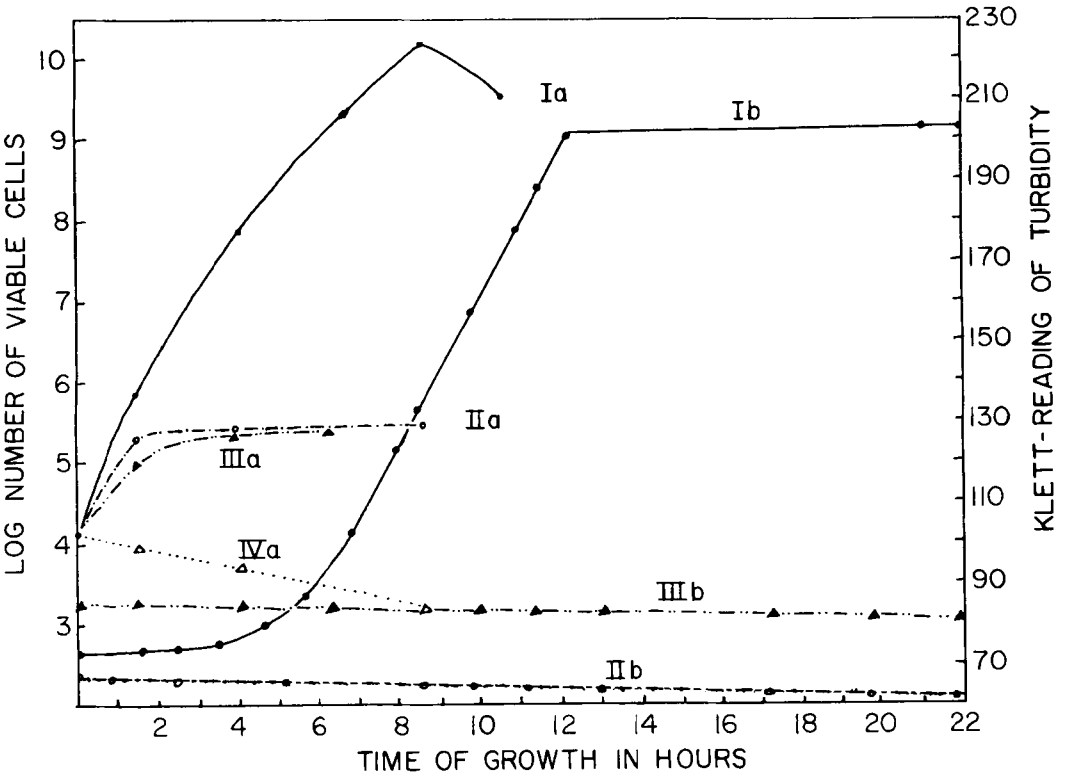


Fig. 2.—Growth response of *Staphylococcus aureus* toward *o*-tolyl aminomethyl ketone hydrochloride.

and 0 mcg./ml. of culture medium. The slopes of the straight lines represent the growth rate, $R$, during the logarithmic growth. They are simply calculated by solving the equation for logarithmic growth in terms of the variables of the growth curve

$$R = \frac{\log_{10} b_2 - \log_{10} b_1}{t_2 - t_1}$$

where $t_2$ and $t_1$ is the time at which the culture has viable cell count of $b_2$ and $b_1$, respectively. The data were taken from Table III at the end of 88 min. of incubation. The positive value of $R$ represents the growth rate, whereas the negative value of $R$ represents the death rate. From growth rate, the generation time, $g$, can be calculated by solving the following equation

$$b = a \times 2^n$$

$$\log_{10} b = \log_{10} a + n \log_{10} 2$$

$$\log_{10} b = \log_{10} a + \frac{t}{g} \log_{10} 2$$

$$\log_{10} b = \log_{10} a + \frac{0.301}{g} t$$

then

$$\text{slope} = R = \frac{0.301}{g}$$

and

$$g = \frac{0.301}{R}$$

TABLE III.—GROWTH RESPONSE OF *Staphylococcus aureus* TOWARD *o*-TOLYL AMINOMETHYL KETONE HYDROCHLORIDE IN NUTRIENT BROTH

| Growth Time | Calculated Log No. Viable Cell/30 ml. —Drug Concentration mcg./ml. Medium— 0 | 125 | 250 | 1000 |
|---|---|---|---|---|
| Zero (Inoculation) | 4.1644 | 4.1644 | 4.1644 | 4.1644 |
| 1 hr. 28 min. | 5.8426 | 5.3284 | 5.0212 | 3.9542 |
| 4 hr. 3 min. | 7.9258 | 5.4771 | 5.4771 | ... |
| 6 hr. 20 min. | 8.9484 | ... | ... | ... |
| 8 hr. 36 min. | 10.2833 | ... | ... | ... |
| 10 hr. 23 min. | 9.4771 | ... | ... | ... |

The averaged generation time calculated for cultures in the presence of *o*-tolylaminomethylketone hydrochloride at concentration of 0, 125, and 250 mcg./ml. is 15.78, 22.77, and 30.94 min., respectively. The elongation in generation time due to the presence of drug indicates the bacteriostatic effect of *o*-tolyl aminomethyl ketone hydrochloride.

Curve IVa reveals the bactericidal action of *o*-tolyl aminomethyl ketone hydrochloride at concentration of 1000 mcg./ml. The rate constant, $k$ of the bactericidal action at this concentration is equal to 0.05726 min.$^{-1}$. This is calculated from the equation for logarithmic order of death (5)

$$k = \frac{2.303}{t} \log_{10} \frac{a}{b}$$

where $a$ is the initial population and $b$ is the final number of bacteria after contact with the drug for time period $t$. The death rate follows the rate of a

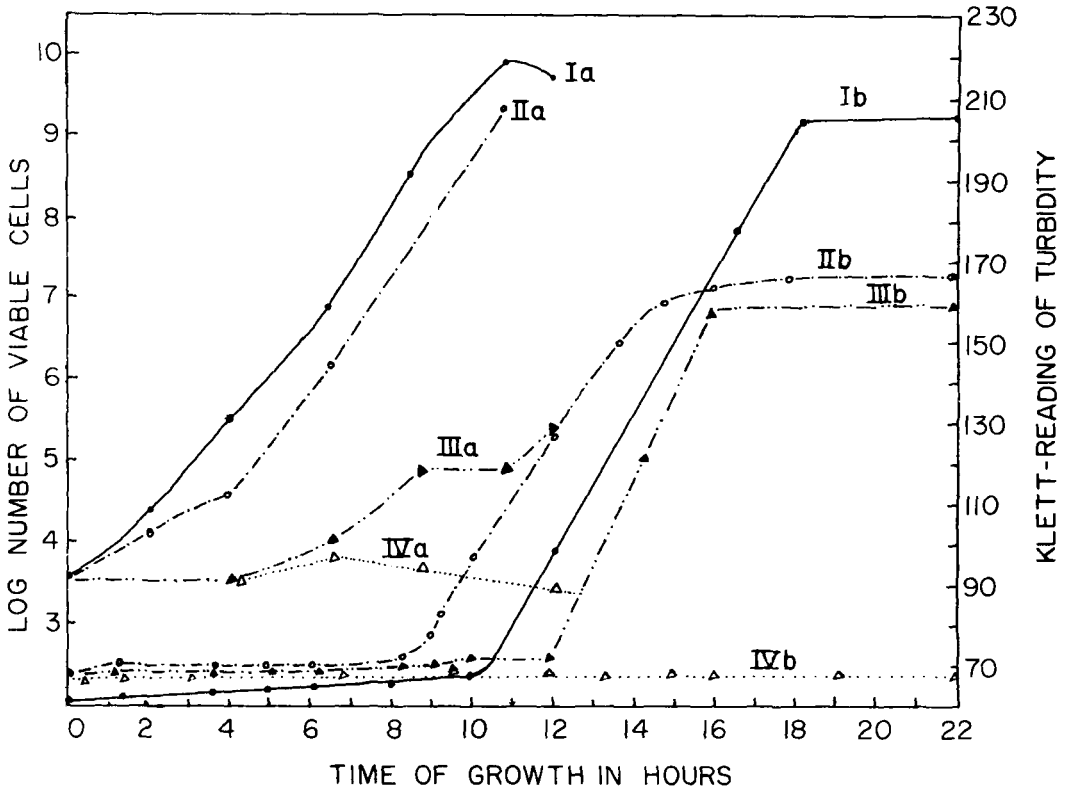


Fig. 3.—Growth response of *Staphylococcus aureus* toward *n*-heptyl β-aminoethyl ketone hydrochloride.

first-order reaction, which is usually observed for many bactericidals (6). It is still an open question whether this implies a simple inhibition of the rate of a single reaction sequence or is merely a coincidence.

From the mathematical treatment, it is obvious that *o*-tolyl aminomethyl ketone hydrochloride acts as a bacteriostat at low concentrations (*ca.* 250 mcg./ml.), and as a bactericide at higher concentrations (*ca.* 1000 mcg./ml.). This conclusion is true also for *n*-heptyl β-aminoethyl ketone hydrochloride, an amino ketone analog of β-alanine. The growth curves in Fig. 3 confirm this statement. In the above experiment, the growth responses were checked also by the turbidity reading of the culture. The turbidity curves are plotted in Figs. 2 and 3 and are designated with subscript *b*, in contrast to the use of subscript *a* for the viable population curve resulting from colony counting.

In order to investigate the mechanism by which β-aminoethyl ketone hydrochlorides act as antibacterial agents, an inhibition-reversal study with β-alanine and calcium L-pantothenate was performed. The results show that at inhibitor: metabolite molar ratio of 1.101:1 or 11.48:1, neither β-alanine nor calcium L-pantothenate possess any reversal activity in antagonizing the growth-inhibitory effect of *n*-heptyl β-aminoethyl ketone hydrochloride against *E. coli*.

## DISCUSSION

The reversal of the growth-inhibitory effect of a compound by a structurally or biochemically related metabolite implies that the inhibitor in question prevents growth by interfering with a biochemical pathway involving that metabolite. The preliminary experiment on the inhibition-reversal study of *n*-heptyl β-aminoethyl ketone hydrochloride by either β-alanine or calcium pantothenate failed to demonstrate the antagonism at an inhibitor:metabolite molar ratio of 1.101:1 or 11.48:1 against *E. coli*. At a molar ratio of 0.805:1 glycine also failed to nullify the growth-inhibition of an amino ketone analog of glycine on a glycine dependent organism. These facts suggest that amino ketone analogs of amino acids may be valuable chemotherapeutic agents, and they may warrant further study.

In contrast to α-aminomethanesulfonic acid, the amino ketone analogs of glycine prepared for this work (7) are active in growth-inhibition against *E. coli* at concentration levels of *ca.* $1 \times 10^{-3}$ mole. α-Aminomethanesulfonic acid has been found to be a glycine antagonist on test systems such as: the bacteriophage of *E. coli* (8), vaccina virus (9), and of different species of bacteria (10), but is not active against *E. coli* (11). This implies that amino ketones

TABLE IV.—GROWTH KINETICS OF *Staphylococcus aureus*[a] IN THE PRESENCE OF *o*-TOLYL AMINOMETHYL KETONE HYDROCHLORIDE

| Drug Concn., mcg./ml. | Growth Rate, min.$^{-1}$ | Generation Time, min. | Final Population, cell | Rate Constant of Bactericidal Action, min.$^{-1}$ |
|---|---|---|---|---|
| 0 | +0.01907 | 15.78 | $696 \times 10^3$ | ... |
| 125 | +0.01322 | 22.77 | $213 \times 10^3$ | ... |
| 250 | +0.00973 | 30.94 | $105 \times 10^3$ | ... |
| 1,000 | −0.00239 | ... | $9 \times 10^3$ | 0.05726 |

[a] Initial population, $14.6 \times 10^3$ cells of *Staphylococcus aureus* 6538P; culture medium, nutrient broth; incubation temperature, 37°. Data are average figures calculated for the first 88 min. of incubation.

may be more reliable than amino sulfonic acids as growth inhibitors when they are structurally related to natural amino acids. The observations that amino ketone analogs, $H_2NCH_2CH_2COR$, of β-alanine inhibit the growth of many microorganisms, whereas taurine, $H_2NCH_2CH_2SO_3H$, the amino sulfonic acid analog of β-alanine, functions as an essential metabolite in vertebrates and invertebrates have lent support to this viewpoint.

The study of the growth curve of *Staphylococcus aureus* in liquid medium, with and without the presence of drug, reveals the pattern of growth-inhibition. Two types of response to an inhibitor have been observed for *o*-tolyl aminomethyl ketone and *n*-heptyl β-aminoethyl ketone hydrochloride, respectively; these are referred to as types A and B. In type A, the plot of log viable cell count against time is linear up to 88 min. after inoculation, as shown by curves Ia, IIa, and IIIa in Fig. 2. This linear relationship is in agreement with the exponential growth law. The growth rate of a test bacterium is reduced by amino ketone hydrochloride; the degree of depression increases with increase in concentration of drug. Type A growth response of *Staphylococcus aureus* toward *o*-tolyl aminomethyl ketone hydrochloride corresponds to the type 1 response described by Moore and Boylen (12) for *E. coli* toward 5-bromouracil; while type B growth response of *Staphylococcus aureus* to *n*-heptyl β-aminoethyl ketone hydrochloride is equivalent to type 3 growth response of *E. coli* toward pyridine-3-sulfonic acid.

The study of the growth curves of test bacteria suggests an accurate method for evaluating the *in vitro* antibacterial activity of drugs with mathematical implication. It serves also, as a means for studying the kinetics as well as the mode of antibacterial action, and may be generally applicable in the screening of antimicrobial agents.

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