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Inhibition of Growth of T-Strain Mycoplasmas by Hydroxamic Acids and by Aurothiomalate

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Sorbyl-, benzoyl-, and 3-amino-benzoyl hydroxamic acids inhibited the development of an alkaline *p*H by T-strain cultures grown in broth containing 0.05% urea and phenol red. The specificity of this urease inhibition was demonstrated by the inhibition, by 10^{-4} M sorbyl-hydroxamic acid, of the release of $^{14}CO_2$ from ^{14}C-urea by washed T-strain mycoplasmas in 4 hr of incubation. Sorbyl-, benzoyl-, and 3-amino-benzoyl hydroxamic acids at a concentration of 10^{-3} M markedly inhibited the multiplication of T-strain 354 during 18 hr of incubation; this inhibition was not corrected by thymidine at a concentration of 500 μg per ml. Aurothiomalate was 20 times more inhibitory to *Mycoplasma hominis* DC-63 than to T-strain 354; equivalent inhibitory concentrations were 50 μg per ml for *M. hominis* and 1,200 μg per ml for T strains.

T-strain mycoplasmas have not yet received official nomenclature and their resemblances to, and distinguishing features from, the classified mycoplasmas still need further definition. This paper describes two characteristics of T-strains by which they can be differentiated from *Mycoplasma hominis*.

The cultural and colonial characteristics of T strains originally described by Shepard (12) were later supplemented by the biochemical characteristic of urea metabolism (14). In this laboratory, the metabolism of urea was studied (6, 7), but the utilization of chemical methods for measuring NH_3 and of ^{14}C-urea for the measurement of CO_2 did not reveal the purpose of the hydrolysis of the urea by the organisms. It was therefore decided to determine whether, in fact, T-strain urease activity was closely related to replication of the organisms. Since Kobashi (10) first demonstrated in 1962 that hydroxamic acids were highly specific inhibitors of urease, other workers, notably Fishbein (4) and Gale (8) have extended the observations. Gale (8) reported in 1969 on a study of 61 different hydroxamic acid derivatives and found 21 to be inhibitory to cell-free urease from the jack bean and also from *Proteus morganii*. The effects of sorbyl-, benzoyl-, and 3-amino-benzoyl hydroxamic acids on urease activity and on multiplication of T-strain mycoplasmas were therefore studied and comparisons were made with *M. hominis*.

A distinguishing feature of mycoplasmas is their sensitivity to gold compounds as first noted by Sabin (11) and Findlay (3). In defining the properties of T-strain mycoplasmas, the effect of aurothiomalate (Myochrysine) on the multiplication of T-strains was also evaluated, and the resistance of T-strains and *M. hominis* to the inhibitory activity of gold was compared.

MATERIALS AND METHODS

Cultures and cultural methods. T-strain 354, previously isolated and utilized in this laboratory, and *M. hominis* DC-63 were employed for the study. T strains were grown in PPLO broth (Difco) supplemented with 10% horse serum, 1% yeast, (Oxoid), and 0.05% urea. *M. hominis* was grown in PPLO broth supplemented with 20% horse serum, 10% yeast extract (Microbiological Associates, Inc.), and 1% arginine. Both media contained 0.002% phenol red and 1,000 units of penicillin per ml. Determinations of the number of viable mycoplasmas were made by the terminal dilution method, with the use of the Reed and Muench calculations. Serial 10-fold dilutions of cultures were made in broth at *p*H 6.0 containing 1% urea and 0.002% phenol red; six 1-ml samples of each dilution were incubated at 37 C for 3 days. The development of an alkaline *p*H from NH_3 production indicated the presence of T-strain mycoplasmas, and the maintenance of the original *p*H of 6.0 indicated the absence of T strains in that sample. The inhibitory effect of the compounds under study was determined by initiating a culture with a dilution of a fresh broth culture estimated to contain approximately 10^3 organisms per ml; the precise number of organisms in these cultures at the onset of the experiments was obtained 3 days later from the results of the terminal dilution counts as described above. Both T strains and *M. hominis* were incubated at 37 C for these experiments. The duration of the incubation for T strains was 18 hr, because of the previously described lability of these

organisms (5, 6), whereas *M. hominis*, which is somewhat slower in multiplication and more stable, was incubated for 24 hr in the hydroxamic acid experiments and for 48 hr in the aurothiomalate experiments. At the termination of the incubation periods, repeat counts were made by the terminal dilution method, and the initial and postincubation counts were compared.

Inhibitory compounds. The hydroxamic acids were kindly provided by G. R. Gale of the Medical College of South Carolina. They were incorporated into the broth medium in 1% dimethyl sulfoxide (DMSO) at concentrations varying from 10^{-3} to 10^{-5} M. Standard pharmaceutical 50-mg vials of intramuscular sodium aurothiomalate (Myochrysine, Merck & Co., Inc.) were employed, and a survey of batches of five different lot numbers showed no differences in inhibitory properties. The concentrations of aurothiomalate are given in amounts of elemental gold.

Urease activity. The techniques employed were similar to those used in the previously reported studies of urea metabolism (6, 7). For the specific demonstration of inhibition of the enzyme urease, 250-ml broth cultures of T strains, incubated for 18 hr at 30 C, were centrifuged at 10,000 × *g* for 30 min; the T-strain pellet was resuspended in phosphate buffer at *p*H 6.4 and then recentrifuged at 10,000 × *g* for 30 min. This washed pellet was resuspended in 5 ml of phosphate buffer and added to 50 ml of Earle's solution containing 0.1% urea and 5 μCi of ^{14}C-urea in an Erlenmeyer flask, attached to three KOH traps, of the apparatus previously used. The flask was placed in a water bath at 37 C, and N_2 was bubbled through the apparatus for 4 hr; then 2 ml of 50% trichloroacetic acid was added, and N_2 was bubbled through for a further 15 min. The scintillation counting procedures were identical to those previously used (7).

RESULTS AND DISCUSSION

Initial experiments readily demonstrated that the three hydroxamic acids inhibited the development of an alkaline *p*H by T-strain mycoplasmas in the broth medium containing 0.05% urea and 0.002% phenol red. This inhibition of urease was therefore examined by the use of ^{14}C-urea, and sorbyl-hydroxamic acid was selected for study. Table 1 shows the effect of sorbyl-hydroxamic acid on the release of $^{14}CO_2$ from ^{14}C-urea by washed T-strain mycoplasmas in 4 hr. This experiment was designed to prevent the multiplication of T-strain mycoplasmas during the study period, and thus confined the observations exclusively to the enzymatic activity of T-strain urease. The virtually complete cessation of urease activity with 10^{-4} M sorbyl-hydroxamic acid was demonstrated by the ^{14}C-label remaining in the medium and not appearing in the KOH traps.

TABLE 1. *Effect of sorbyl-hydroxamic acid (S-HA) on release of $^{14}CO_2$ from ^{14}C-urea by washed T-strain mycoplasma cells in 4 hr*

Determination	^{14}C label in medium[a] and released CO_2 (%)	
	Control	10^{-4} M S-HA
Zero time	100	100
Residual medium	1.6	92
KOH traps	88.3	2.3

[a] Earle's solution (*p*H 6.4) containing 5 μCi of ^{14}C-urea.

Figure 1 shows comparisons between the

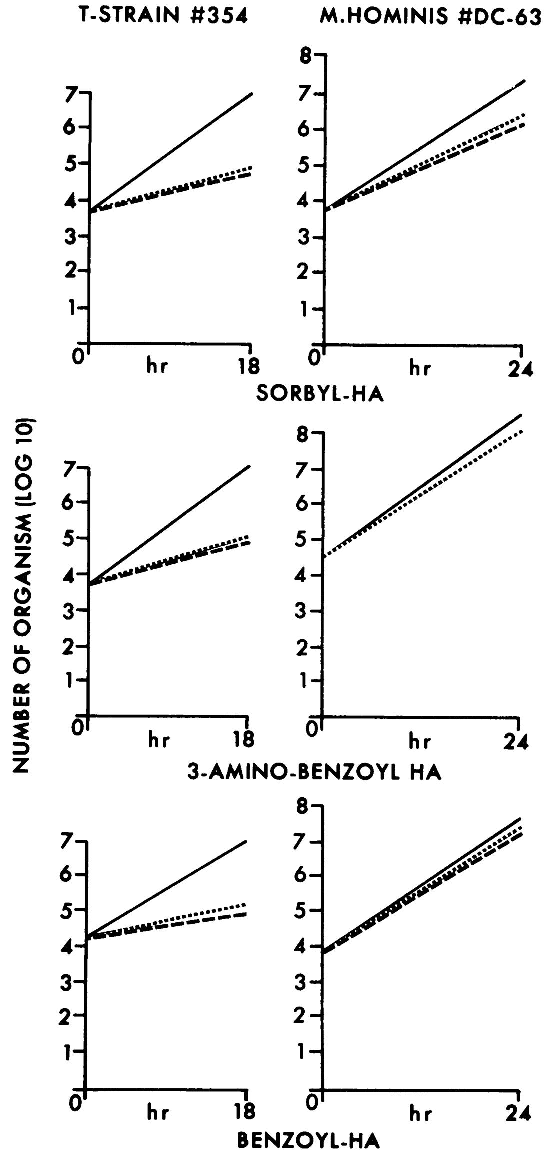

FIG. 1. *Growth of T-strain 354 and Mycoplasma hominis DC-63 in 10^{-3} M sorbyl-, 3-amino-benzoyl-, and benzoyl-hydroxamic acids (HA), with and without thymidine (500 μg/ml). Solid lines, growth in 1% DMSO; dotted lines, growth in 1% DMSO plus 10^{-3} M HA; dashed lines, growth in 1% DMSO plus 10^{-3} M HA plus thymidine.*

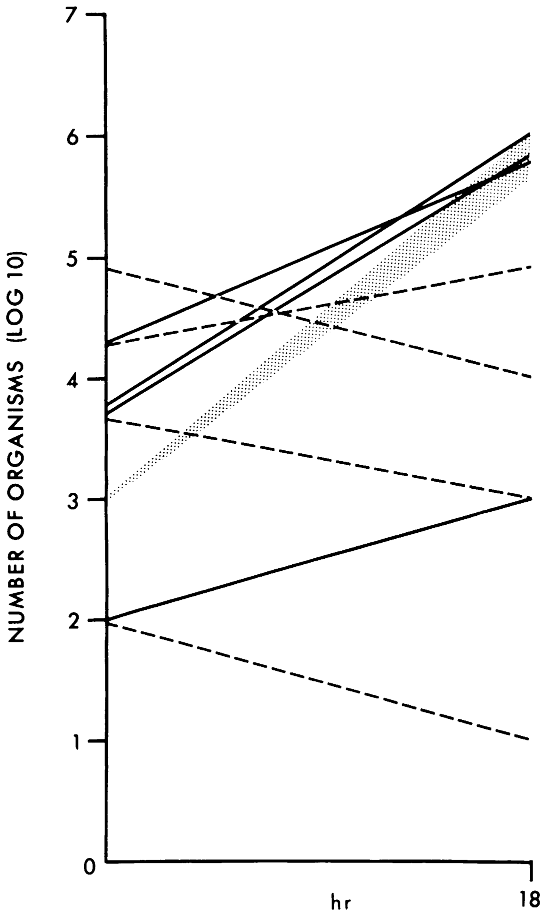

FIG. 2. *Growth of T-strain 354 in aurothiomalate at concentrations of 1,200 μg/ml (solid lines) and 1,500 μg/ml (dashed lines). The shaded area shows growth in control broth.*

growth of T strains and *M. hominis* in the presence of sorbyl-hydroxamic acid, 3-amino-benzoyl-hydroxamic acid, and benzoyl-hydroxamic acid, and the influence of thymidine on the inhibition of multiplication. All three compounds at a concentration of 10^{-3} M markedly inhibited the growth of T-strain mycoplasmas. In contrast, the benzoyl- and 3-amino-benzoyl compounds had no effect on multiplication of *M. hominis*. Under the particular experimental conditions used, multiplication of *M. hominis* was slightly inhibited by sorbyl-hydroxamic acid, the reduction being from 1,000-fold multiplication to 100-fold multiplication in 24 hr. In no case was the inhibition of mycoplasma multiplication reversed by the presence of thymidine at a concentration of 500 μg per ml. The influence of thymidine was examined because sorbyl-hydroxamic acid has been observed to cause a selective inhibition of deoxyribonucleic acid (DNA) synthesis (9). The slight inhibition of *M. hominis* by the sorbyl derivative was not corrected by thymidine, but Gale (9) has observed that thymidine does not entirely restore DNA synthesis to normal under these circumstances.

The specific inhibition of T-strain urease by hydroxamic acid compounds was therefore correlated with a specific inhibition of multiplication of the organisms by these same compounds. It was concluded, therefore, that the urease activity produced by the organisms was a requirement for multiplication.

Figures 2 and 3 show the effects of sodium aurothiomalate on the multiplication of T strains and *M. hominis*. There was some variability in the response of the organisms to gold, as would be expected from the previous observations (2) on the inverse correlation between inoculum size and the degree of inhibition by gold. The growth curves, however, demonstrated an approximate 20-fold difference in the sensitivity of these two organisms to gold, and the relative resistance of

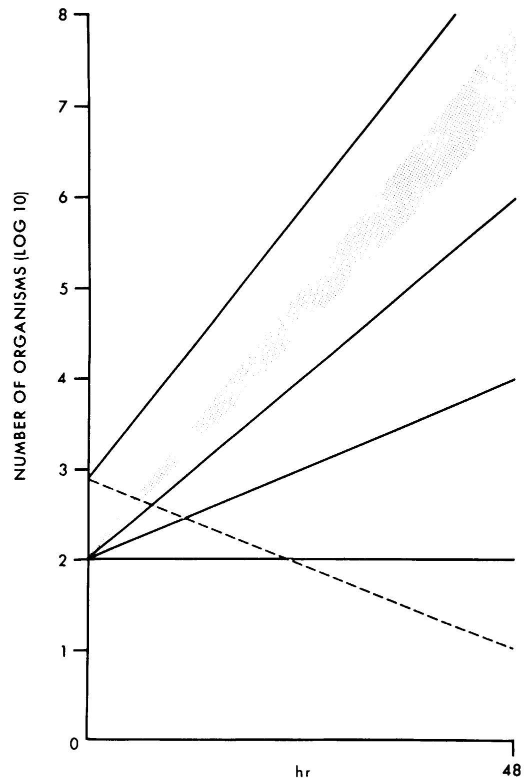

FIG. 3. *Growth of Mycoplasma hominis DC-63 in aurothiomalate at concentrations of 50 μg/ml (solid lines) and 100 μg/ml (dashed line). The shaded area shows growth in control broth.*

T-strains to gold is readily apparent. Equivalent inhibitory concentrations of aurothiomalate were 50 μg per ml for *M. hominis* and 1,200 μg per ml for T-strain 354.

In comparing the susceptibility of T strains and *M. hominis* to antimicrobial agents, unequivocal differences were thus found, and T strains were significantly more susceptible to thallium acetate and erythromycin (13), but significantly more resistant to lincomycin (1) and gold. These characteristics of T strains can be added to the cultural and biochemical characteristics by which T strains may be defined as a distinct group of mycoplasmas with a number of unique properties.

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