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Mechanism by Which Hydnocarpic Acid Inhibits Mycobacterial Multiplication

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Recent work in this laboratory has shown that hydnocarpic acid (HA), a principal constituent of chaulmoogra oil, inhibits multiplication in vitro of a number of mycobacterial species. This activity of HA was not shared by several straight-chain fatty acids and by dihydrochaulmoogric acid. A study of the interaction of HA with biotin has been undertaken, based on a structural analogy between biotin and the cyclopentenyl fatty acid. The multiplication of a strain of *Mycobacterium intracellulare* susceptible to 2 μg of HA/ml was measured turbidimetrically in Dubos medium, in the presence and absence of biotin and several other compounds. Biotin and, to a lesser extent, adenine plus guanine, palmitic acid, and linoleic acid antagonized growth inhibition by HA. Desthiobiotin, thioctic acid, and succinic acid did not block inhibition of bacterial multiplication by HA. HA may act by blocking the coenzymatic activity of biotin, or it may inhibit microbial biotin synthesis. Resumption of multiplication of *M. intracellulare* after a period of inhibition by HA in broth culture was found to be accompanied by reduction of the effective concentration of the drug; this could have resulted from metabolism of HA or production of an antagonist to HA by the organisms. Also, those organisms that multiplied in the presence of HA were found to represent HA-resistant mutants of *M. intracellulare*.

Chaulmoogra oil and its major components, chaulmoogric [chemical name 13-(2-cyclopenten-1-yl)tridecanoic] and hydnocarpic [chemical name 11-(2-cyclopenten-1-yl)undecanoic] acids, were used in the treatment of leprosy for many years, apparently with some effect. However, the mechanism of action of these substances was never elucidated. Early work focused on the fatty acid character of these compounds and the important lipid content of mycobacteria. This resulted in the speculation that these compounds interfered with bacterial cell wall synthesis or function, by a decrease of surface tension or by the adsorption of a monomolecular film onto the surface of the organisms (5, 6, 8, 11). More recent work has attributed the action of chaulmoogra oil to an effect on host defense mechanisms rather than to a direct effect on the organisms (3, 4).

Recent work in this laboratory has shown that hydnocarpic acid inhibits both multiplication of *Mycobacterium leprae* in the mouse foot pad (unpublished data) and also multiplication of a number of cultivable mycobacterial strains in vitro (P. L. Jacobsen, H. Ng, and L. Levy, Amer. Rev. Resp. Dis., in press). Although an effect on host defense mechanisms cannot be excluded, there can be no doubt that hydnocarpic acid exerts a direct antimicrobial action. Adams and his co-workers (10) demonstrated that saturation of chaulmoogric acid reduced antimicrobial activity measured in vitro; the studies in this laboratory confirmed those of Adams, showing that dihydrochaulmoogric acid in a concentration of 20 μg/ml failed to inhibit multiplication of several mycobacterial strains susceptible to 2 μg of chaulmoogric acid per ml. Similarly, several saturated straight-chain fatty acids were found to be incapable of inhibiting multiplication of these organisms in the test medium used (Jacobsen, Ng, and Levy, Amer. Rev. Resp. Dis., in press).

The requirement for the cyclopentenyl ring suggests a specific mechanism of action. The structural analogy of hydnocarpic acid (HA) to biotin suggested that the mechanism by which HA inhibits mycobacterial multiplication might involve a metabolic reaction of the organism requiring biotin as a coenzyme or one responsible for biotin synthesis. The current studies were undertaken to test these possibilities. (A preliminary report of this work was presented at the 56th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April, 1972 [Jacobsen, P. L., and Levy, L., Fed. Proc. **31**: 576, 1972]. This work was submitted to the Graduate Division, Univ. of California, San Francisco, in partial ful-

fillment of the requirements for the Ph.D. degree in Comparative Pharmacology and Toxicology.)

MATERIALS AND METHODS

HA, prepared from chaulmoogra oil, was 98% pure (Jacobsen, Ng, and Levy, Amer. Rev. Resp. Dis., in press). Biotin was purchased from Calbiochem, Los Angeles, Calif. HA, palmitic, oleic, and stearic acid stock solutions were prepared in absolute ethanol; all other drugs were dissolved in distilled water. Appropriate volumes of the stock solutions were added to Dubos broth or Dubos oleic agar before autoclaving. For the experiments in which HA or biotin was added after cultures had been inoculated and incubated for 2 days, the solution of the compound to be added was first sterilized by filtration through a 0.22-μm nitrocellulose filter. The test culture, a strain of *M. intracellulare* (strain W1117 from L. G. Wayne, V.A. Hospital, Long Beach, Calif.) susceptible to 2 μg of HA/ml, was stored at 4 C on Lowenstein-Jensen medium. Plates were inoculated with 0.1 ml of a dilution of a culture of *M. intracellulare* in Dubos broth so that each plate was inoculated with approximately 100 organisms. To prepare inocula for the broth cultures, tubes of Dubos broth were inoculated and grown to an optical density of 0.400 to 0.600. One-tenth milliliter of the stock suspension was added to 5 ml of broth, and 0.1 ml of this dilution, containing from 10^5 to 10^6 organisms, was added to 7 ml of experimental broth. The optical density of each tube was measured every other day at 580 nm on a Coleman Senior spectrophotometer after the tubes were shaken on a variable-speed mixer. Results are expressed in optical density units. Plates and tubes were prepared in triplicate. Cultures were incubated at 37 C.

RESULTS

Growth inhibition by HA. To determine if the drug was merely bacteriostatic, or if it was bactericidal at some concentration, Dubos oleic agar plates containing HA in concentrations ranging from 0 to 32 μg/ml were inoculated, and the number of colonies appearing on each plate was counted. About 40% of the number of colonies appearing on drug-free plates were visible on plates containing 2 μg of HA/ml; no colonies appeared on plates containing HA concentrations of 4 μg/ml or more. Organisms were then transferred from the surface of the drug-containing plates to plates of drug-free medium by means of a sterile velvet pad (1). A few colonies appeared on the replica plates made from plates containing 4 μg of HA/ml, but no colonies were visible on those replica plates prepared from plates containing more than 4 μg of HA/ml. Thus, multiplication of this strain of *M. intracellulare* was inhibited by concentrations of HA greater than 2 μg/ml; concentrations of HA of 4 μg/ml or greater were bactericidal.

Simultaneously added biotin. The antagonism of HA inhibition by simultaneously added biotin was studied in two experiments. In the first experiment (Fig. 1), biotin was added in a concentration of 0.75 μg/ml to tubes of medium containing HA in concentrations ranging from 2 to 16 μg/ml. The tubes were then inoculated, and the multiplication of *M. intracellulare* was observed. This concentration of biotin, in the absence of HA, had no effect on bacterial multiplication. HA, in the smallest concentration studied, inhibited multiplication of *M. intracellulare* for

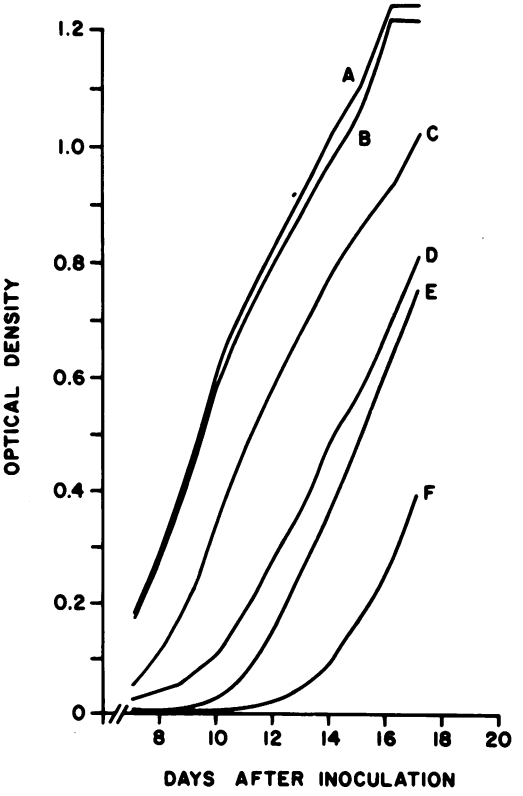

FIG. 1. *Simultaneous addition of biotin and HA. Biotin was added in a concentration of 0.75 μg/ml to tubes of Dubos medium containing HA in concentrations ranging from 2 to 16 μg/ml. The tubes were then inoculated with 10^5 M. intracellulare and incubated at 37 C. Growth was measured as the increase in optical density at 580 nm. A, Control; B, 0.75 μg of biotin per ml; C, 2 μg of HA plus 0.75 μg of biotin per ml; D, 2 μg of HA per ml; E, 4 μg of HA plus 0.75 μg of biotin per ml; F, 8 μg of HA plus 0.75 μg of biotin per ml. The tubes containing 8 or 16 μg of HA per ml and those containing 16 μg of HA plus 0.75 μg of biotin per ml demonstrated no growth, and are not represented here.*

about 8 days, after which microbial multiplication resumed at a rate approximately equal to that in drug-free medium. The larger concentrations of HA inhibited bacterial multiplication throughout the duration of the experiment. Biotin blocked the effect of HA in varying degrees. The inhibitory effects of 2 to 8 μg of HA per ml were antagonized by the single small concentration of biotin in inverse relation to the concentration of HA; the effect of 16 μg of HA per ml was not blocked at all.

In a second experiment, biotin in concentrations of 5, 25, and 50 μg/ml inhibited slightly the multiplication of the test strain of *M. intracellulare*. The effect of 2 μg of HA per ml was completely blocked by all three concentrations of biotin. The effect of 4 μg of HA per ml was antagonized equally by all three concentrations of biotin, but the antagonism was incomplete. Similarly, the inhibitory effect of 8 μg of HA per ml was antagonized equally but incompletely by all three biotin concentrations. The degree of antagonism by biotin of HA inhibition appeared to be inversely proportional to the concentration of the HA, but it did not appear to be proportional to the concentration of biotin, nor was it proportional to the mole ratio of biotin to HA.

Sequential addition. Cultures containing either HA (4 μg/ml and 8 μg/ml) or biotin (8 μg/ml) were inoculated and incubated for 2 days, at which time biotin was added to tubes originally containing HA, and HA in both concentrations was added to tubes initially containing only biotin. The results of this experiment are presented in Fig. 2. The single concentration of biotin (data not shown) did not inhibit bacterial multiplication, whereas the lower concentration of HA inhibited multiplication for about 13 days and the larger concentration did so for the duration of the experiment. HA added after preincubation with biotin had only a modest effect. On the other hand, the addition of biotin after preincubation with HA had a more profound effect. Thus, the addition of 4 μg of HA per ml to the culture preincubated with biotin produced 25% inhibition of multiplication after 8 days of incubation; 8 μg of HA per ml produced 41% inhibition under the same circumstances. By contrast, the addition of biotin after preincubation with HA produced a ninefold increase in the optical density when compared with HA alone for the smaller concentration of HA, and a sixfold increase for the large concentration. Thus, the antagonism by biotin of the inhibitory effect of hydnocarpic acid is not independent of the order in which the two compounds are added.

Effects of other compounds. Desthiobiotin in concentrations of 8 and 16 μg/ml did not antagonize the inhibitory effects of 4 or 8 μg of HA per ml, nor did this biotin analogue affect the antagonism by 8 μg of biotin per ml of the same two concentrations of HA. Thioctic acid, in a con-

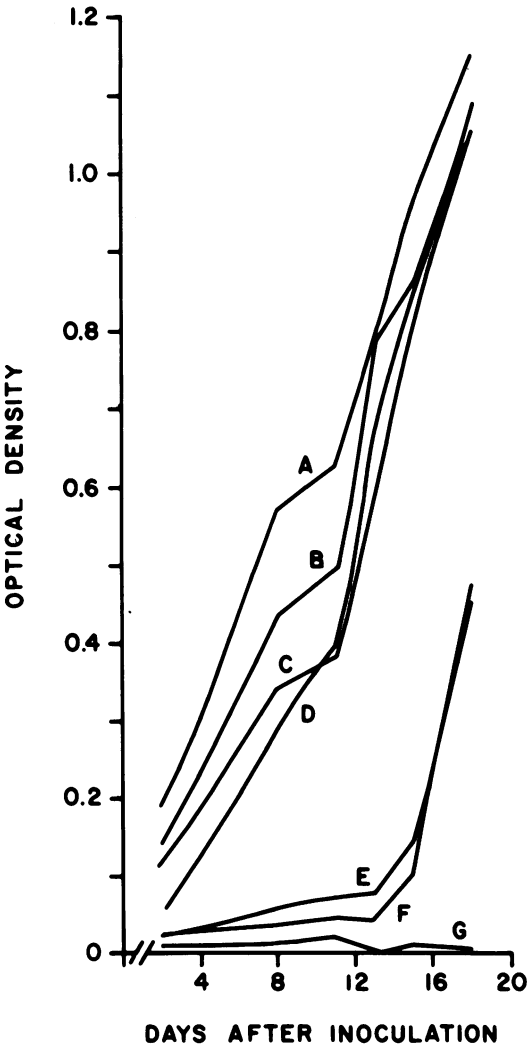

FIG. 2. *Sequential addition of biotin and HA. Tubes of Dubos medium containing either 4 or 8 μg of HA or 8 μg of biotin per ml were inoculated with M. intracellulare and incubated at 37 C. After 2 days, 8 μg of biotin per ml was added to the cultures preincubated with HA, and 4 or 8 μg of HA per ml was added to the cultures preincubated with biotin. Bacterial growth was measured turbidimetrically. A, Control; B, 8 μg of biotin per ml, then 4 μg of HA per ml; C, 8 μg of biotin per ml, then 8 μg of HA per ml; D, 4 μg of HA per ml, then 8 μg of biotin per ml; E, 8 μg of HA per ml, then 8 μg of biotin per ml; F, 4 μg of HA per ml; G, 8 μg of HA per ml.*

centration of 8 μg/ml, had no effect on the inhibition produced by 6 μg of HA per ml.

Adenine plus guanine and linoleic and palmitic acids were studied in cultures containing HA alone and also in cultures containing HA together with biotin. None of these compounds influenced multiplication of the test organism in the absence of HA. As in the experiments already described, 8 μg of biotin per ml antagonized incompletely the inhibitory effect of 8 μg of HA per ml (Fig. 3). Adenine plus guanine (each 20 μg/ml), palmitic acid (15 μg/ml), and linoleic acid (also 15 μg/ml) reversed the inhibition produced by 8 μg of HA per ml, but to a smaller degree than that produced by biotin. Similar results were obtained when the antagonism by these compounds of the inhibitory effect of 4 μg of HA per ml was studied. Adenine plus guanine, each 40 μg/ml, was no more effective than when these compounds were added in half this concentration.

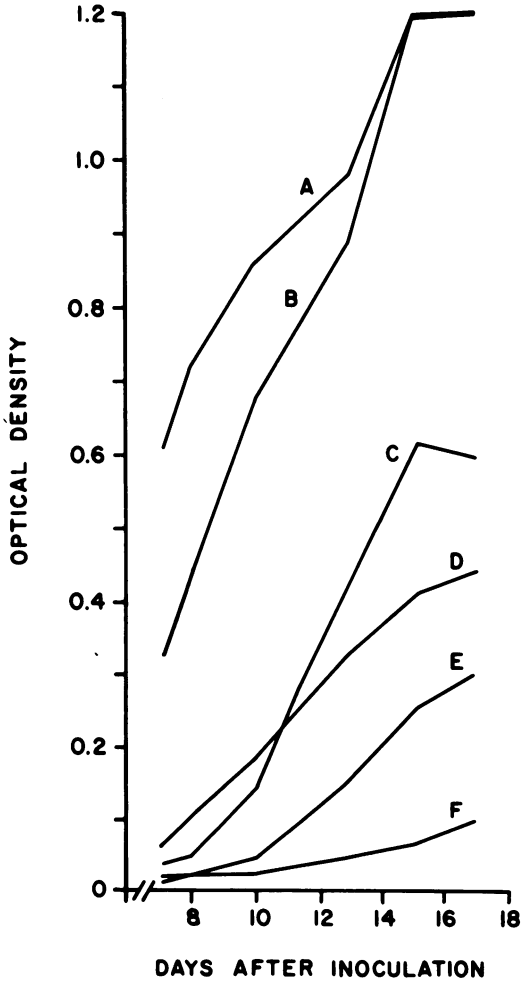

FIG. 3. *Antagonism of HA inhibition by adenine plus guanine, palmitate, and linoleate. Several compounds were added to tubes of Dubos medium containing 8 μg of HA per ml; the tubes were then inoculated and incubated, and growth was measured as for Fig. 1. A, Control; B, 8 μg of HA plus 8 μg of biotin per ml; C, 8 μg of HA plus 20 μg each of adenine and guanine per ml; D, 8 μg of HA plus 15 μg of palmitic acid per ml; E, 8 μg of HA plus 15 μg of linoleic acid per ml; F, 8 μg of HA per ml.*

Several compounds, including succinic acid and a solution of six fatty acids—oleic, stearic, palmitic, linoleic, lauric, and myristic (each 20 μg/ml)—were incapable of antagonizing the inhibition of multiplication of *M. intracellulare* caused by HA.

"Breakthrough" of growth inhibition by HA. Many of the bacterial growth curves (Fig. 1–3) showed that, after an initial period of inhibition by HA, multiplication resumed at a rate equal to that in drug-free medium, despite continued presence of the drug. Because decay of HA in medium incubated at 37 C occurred at a rate insufficient to account for the resumption of bacterial multiplication (Jacobsen, Ng, and Levy, Amer. Rev. Resp. Dis., in press), further studies of the phenomenon of resumption of bacterial multiplication in the continued presence of the drug were carried out.

Tubes of Dubos broth containing no drug or 4 or 8 μg of HA ml were inoculated. When multiplication of *M. intracellulare* was judged to have proceeded far enough, the organisms were separated from the medium by filtration through a 0.22-μm nitrocellulose membrane. Tubes of fresh medium, with or without HA, were inoculated with the filtered organisms or with an approximately equal number of "naive" organisms (not previously exposed to HA). Tubes of filtered medium were inoculated with naive *M. intracellulare*.

In Table 1, the results of inoculation of filtered and fresh media with naive *M. intracellulare* are compared. Because the individual comparisons were made at different times, the differences among the control growth curves probably reflect differences among the inocula; the inocula did not differ within each of the three experiments, however. Inhibition of multiplication in the fresh drug-containing medium demonstrates that the naive organisms were susceptible to HA; their ability to multiply in the filtered media suggests that the effective concentration of HA had been reduced. Multiplication of the organisms in the filtered media approached that in the drug-free control cultures in both experiments B and C, suggesting that the effective concentration of HA had been reduced to the same low level when

TABLE 1. *Multiplication of naive M. intracellulare in filtered and fresh media*

Time of incubation (days)	Optical density[a]							
	Expt A		Expt B			Expt C		
	0	0[b]	0	4[b]	4	0	8[b]	8
4	0.220	0.137	0.100	0.042	0.037	0.150	0.087	0.017
7	0.502	0.372	0.112	0.050	0.040	0.430	0.410	0.015
9	0.680	0.540	0.310	0.110	0.030	0.610	0.660	0.012
11	0.685	0.560	0.485	0.265	0.035	0.900	0.810	0.005
14	0.717	0.570	0.715	0.555	0.032	0.840	0.925	0.047
16	0.840	0.717	0.830	0.760	0.020	1.05	1.10	0.195

[a] The medium tested in experiment A had been filtered after 6 days of incubation, at which time optical density (OD) = 0.360. The medium tested in experiment B was filtered after 13 days of incubation (OD = 0.270). That tested in experiment C was filtered after 20 days of incubation (OD = 0.122).

[b] Initial HA concentration of filtered medium (measured in micrograms per milliliter). All other cultures were made in fresh media.

multiplication resumed, despite the twofold difference in initial drug concentration.

In Table 2, the susceptibility to HA of the *M. intracellulare* filtered from HA-containing media is compared in fresh media with that of naive organisms. Because the growth curves in drug-free medium of all three cultures were the same, only one is shown. Multiplication of naive organisms was completely inhibited by 8 μg of HA per ml, whereas multiplication of the organisms filtered from HA-containing media was only partially inhibited. The results of inoculation of medium containing 4 μg of HA per ml were similar. The organisms filtered from medium containing 8 μg of HA per ml were more resistant to the inhibitory action of HA than were the organisms filtered from medium containing 4 μg of HA per ml.

In Table 3, the multiplication of *M. intracellulare* filtered from various media and subcultured once in drug-free medium is compared with that of naive organisms both in drug-free medium and in medium containing 4 μg of HA per ml. Because the growth curves of the six cultures in drug-free medium were virtually identical, only one is shown. Both the naive organisms and those filtered from drug-free medium failed to multiply in the presence of 4 μg of HA per ml. On the other hand, multiplication of the organisms filtered from HA-containing media and subcultured once in drug-free medium was only partially inhibited by 4 μg of HA per ml; the organisms originally filtered from medium containing 8 μg of HA per ml were more resistant than those filtered from medium containing 4 μg of HA per

TABLE 2. *Multiplication of filtered and naive M. intracellulare in fresh media*

Time of incubation (days)	Optical density			
	0 μg of HA/ml	8 μg of HA/ml[a]	8 μg of HA/ml[b]	8 μg of HA/ml[c]
3	0.137	0.010	0.015	0.045
6	0.425	0.020	0.020	0.132
8	0.587	0.008	0.027	0.212
10	0.705	0.010	0.027	0.307
13	0.820	0.015	0.050	0.580
15	0.975	0.000	0.360	0.815

[a] Naive organisms.

[b] Organisms filtered from medium initially containing 4 μg of HA per ml.

[c] Organisms filtered from medium initially containing 8 μg of HA per ml.

TABLE 3. *Multiplication of filtered, subcultured M. intracellulare in fresh media*

Time of incubation (days)	Optical density				
	0 μg of HA/ml	4 μg of HA/ml[a]	4 μg of HA/ml[b]	4 μg of HA/ml[c]	4 μg of HA/ml[d]
3	0.126	0.047	0.040	0.060	0.038
4	0.230	0.045	0.040	0.066	0.038
6	0.675	0.052	0.077	0.152	0.074
7	0.857	0.062	0.130	0.262	0.065
8	1.0	0.085	0.230	0.528	0.147
9	1.12	0.094	0.341	0.626	0.217

[a] Naive organisms.

[b] Organisms filtered from medium initially containing 4 μg of HA per ml.

[c] Organisms filtered from medium initially containing 8 μg of HA per ml.

[d] Organisms filtered from medium initially containing 8 μg of HA plus 5 μg of biotin per ml.

ml. *M. intracellulare* filtered from medium containing 8 μg of HA and 5 μg of biotin per ml were intermediate in susceptibility to HA between the naive organisms and those filtered after multiplying media containing HA without added biotin.

DISCUSSION

Previous studies in this laboratory have shown that HA inhibits the multiplication of a large number of mycobacterial strains in vitro (Jacobsen, Ng, and Levy, Amer. Rev. Resp. Dis., in press). The antagonism of HA inhibition by biotin suggests a mechanism of action for this cyclopentenyl fatty acid.

The studies reported here have demonstrated that: (i) HA is a less effective inhibitor when it is added after preincubation with biotin, whereas biotin antagonism is not much reduced when the biotin is added after preincubation with HA; (ii) neither desthiobiotin nor thioctic acid may be substituted for biotin; and (iii) adenine plus guanine and linoleic and palmitic acids, added individually, block partially the inhibitory action of HA, but to a lesser degree than occurs with biotin.

The metabolic role of biotin has not been completely elucidated. Its function in bacterial and mammalian metabolism has been reviewed (7). Although most of the metabolic abnormalities observed in biotin deficiency states can be traced to deficient production of dicarboxylic acids, the abnormalities may be either direct (e.g., the function of a biotin-requiring enzyme is reduced) or indirect (e.g., an enzyme protein is not synthesized because of an insufficient supply of aspartic acid). The manifestations of these abnormalities are, therefore, many and varied. Those substances examined as possible substitutes for biotin in this study included some, e.g., succinic acid and the fatty acids, in the synthesis of which a biotin-requiring enzyme is directly involved. Adenine and guanine were included because the synthesis of purines has been shown to be profoundly decreased in rats made biotin-deficient by the feeding of avidin. Aspartic acid was not studied because it is an important constituent of the medium (2). Desthiobiotin was studied because it has been shown to be capable of substituting for the biotin requirement of *M. tuberculosis* (9). Thioctic acid was examined because it bears some structural resemblance to biotin.

The results of the experiments reported here may indicate that HA acts to inhibit microbial synthesis of biotin, or that HA inhibits in a noncompetitive fashion some biotin-requiring metabolic reaction essential to the multiplication of *M. intracellulare*. Regardless of which hypothesis is correct, HA appears to act by a mechanism unique among antimicrobial agents. HA and compounds with analogous structures may represent an interesting new class of antimicrobial substances.

An interesting phenomenon observed in the course of this work was the resumption of multiplication of *M. intracellulare* in the continued presence of HA after an initial period of inhibition. We have previously demonstrated that instability of the drug cannot be responsible for this phenomenon (Jacobsen, Ng, and Levy, Amer. Rev. Resp. Dis., in press). But the HA present in the medium might be rendered ineffective, either because the *M. intracellulare* metabolize it to inactive products or because the organisms produce a substance which antagonizes the antimicrobial effects of the drug. Alternatively, some fraction of the inoculated organisms might be resistant to HA and capable of multiplication in its presence. *M. intracellulare* filtered from HA-containing media after resumption of multiplication multiplied in the presence of ordinarily inhibitory concentrations of the drug, even after passage of the organisms through one subculture in drug-free medium. At the same time, the media from which the resistant organisms were filtered no longer contained as much HA activity as was originally present. Thus, there is evidence that changes in both the organism and the medium account for the phenomenon under discussion. It is not possible at this time to determine whether the loss of HA activity resulted from metabolism of the drug or production of some antagonist. It appears that the *M. intracellulare* that multiplied in the presence of the reduced effective concentration of HA represented a mutant strain. Whether or not the mutation involves an increased capacity to metabolize HA or to produce an antagonist remains to be studied.

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