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Spheroplasts of *Haemophilus influenzae* Induced by Cell Wall-Active Antibiotics and Their Effect upon the Interpretation of Susceptibility Tests

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The interpretation of in vitro susceptibility tests of *Haemophilus influenzae* performed by the agar or broth dilution methods with Levinthal enrichment was found to be markedly influenced by the production of spheroplasts by this species. Using an inoculum of 10^7 organisms/ml, this phenomenon was frequently evident macroscopically as a haziness on agar substrates and in broths containing cell wall-acting agents, such as ampicillin, cephalothin, and penicillin, but was not noted with chloramphenicol. Phase-contrast microscopic examination of the haze from these sources revealed numerous spherical bodies in contrast to the typical cocco-bacillary forms observed in growth controls. With this inoculum size, minimal bactericidal concentrations could not be determined since subculture of 0.1 ml of the hazy broths or the surface haze onto chocolate agar resulted in most instances in the development of a small number of colonies which, upon smear and gram stain, revealed typical *Haemophilus* morphology. An inoculum of 10^4 organisms/ml abolished the haziness on agar surfaces and in broths and resulted in clear-cut end points. Also, although spherical bodies were still present, they were distinctly less in number as contrasted to tests performed with an inoculum of 10^7 organisms/ml. It is recommended that minimal inhibitory concentration end points in antibiotic susceptibility tests be determined by microscopic, rather than macroscopic, observation of the growth milieu to determine the presence or absence of morphologically typical bacilli which, when observed, is indicative of true in vitro resistance.

With the increasing documentation of ampicillin failures and bacteriological relapse in patients with *Haemophilus influenzae* meningitis (1-4, 8, 13), the microbiology laboratory is under particular obligation to establish the true ampicillin susceptibility status of any *H. influenzae* clinical isolate. The disc-agar diffusion method, the most commonly utilized procedure in diagnostic laboratories, has been reported to be misleading and unreproducible for determining antibiotic susceptibility of this bacterial species (6, 12, 14), and for this reason other techniques, such as broth and agar dilution methods using Levinthal or Fildes enrichment as growth supplements, had to be employed. However, various investigators have also encountered difficulty in determining the exact end points of inhibition with the latter methods. Khan and co-workers (5), using a broth dilution method, attributed this difficulty to the dark straw color of brain heart infusion Fildes broth, which precluded visual observation of clear-cut end points indicative of the minimal inhibitory concentration (MIC). More recently, Khan and colleagues (4) adapted a microtiter serial dilution method with the same enrichment broth but added phenol red as a growth indicator to facilitate end point reading. On the other hand, McLinn and co-workers (6) reported similar difficulties in establishing the exact MIC of ampicillin with the agar dilution method. In their experience, this determination was complicated by the occurrence of a "hazy partial growth" that persisted through several of the lower concentrations of ampicillin and abruptly terminated in distinct unequivocal growth comparable to controls.

In our laboratory, we became increasingly aware that results with disc-agar diffusion antibiotic susceptibility studies of *H. influenzae* did not always correlate with the Levinthal broth dilution method or patient clinical response, particularly with cell wall-active antimicrobial agents, such as ampicillin. In the broth dilution method, because of the haziness that frequently developed in tubes containing these agents, difficulties were encountered with the visual determination of inhibitory end

points, which resulted in questionable interpretations.

This study describes the production of spheroplasts of *H. influenzae* under the influence of antibiotics whose activity is directed against the cell wall which, because of their stability, produce a haziness in broth and on agar surfaces that markedly interferes with the reading of end points of susceptibility to these agents.

MATERIALS AND METHODS

Organisms. The initial observation of haziness in Levinthal broths containing ampicillin (5 and 10 μg/ml), cephalothin (7.5 and 15 μg/ml), and penicillin (1 and 10 U/ml) was noted with 30 *H. influenzae* and 3 *H. parainfluenzae* isolates derived from clinical material. Of the former strains, 24 were type b. Six were recovered from blood, 10 from cerebrospinal fluid, 4 from conjunctiva, 3 from nose and throat culture, and 1 from a vaginal culture. One strain each of *H. parainfluenzae* was recovered from the nose, throat, and eye. All strains were originally isolated on chocolate agar (BBL) and characterized by virtue of their X (hemin) and V (nicotinamide adenine dinucleotide) growth factor requirements, using X and V strips (BBL) superimposed on lawns of the test organism on Trypticase soy agar (BBL). Additional criteria of identification included lack of growth on sheep blood agar in the absence of V factor and microscopic morphology of a small, gram-negative coccobacillus exhibiting some pleomorphism.

More detailed investigation of the above-noted haziness was carried out with six ampicillin-sensitive type b blood and cerebrospinal fluid isolates of *H. influenzae*, which were also beta lactamase negative, as determined by the method of Thornsberry and Kirven (10). For comparative purposes, a more ampicillin-resistant (6.25 μg/ml), beta lactamase-positive, nontypable, sputum isolate of *H. influenzae* was included.

Antibiotics. Concentrated antibiotic solutions were freshly prepared from dehydrated, commercially available vials using sterile distilled water as a diluent. Subsequent dilutions were made in broth.

Susceptibility tests. The two-tube broth dilution method of Schneierson and Amsterdam (9) was used initially, employing Levinthal enrichment (Scott Laboratories, Inc.) diluted 1:5 in glucose broth. However, because the development of the observed spherical forms might be attributed to the high serum content in Levinthal medium, subsequent tests were performed in 5 ml of glucose or Trypticase soy broth (BBL), which do not contain any serum as contrasted to the Levinthal medium, supplemented with a strip containing X and V factors (BBL). All strains grew readily with the latter, despite the absence of any serum. The inoculum consisted of 0.1 ml of a 6-h Levinthal glucose broth culture diluted 10^{-2}, which contained 10^6 organisms/ml, as determined by viable count on chocolate agar.

The MIC of ampicillin was assayed by the standard serial twofold broth dilution method, using either Levinthal enrichment or XV strips. Dilutions ranged from 50 to 0.049 μg/ml. The influence of inoculum size on MIC was studied by adding 1 ml of either 10^7 or 10^4 organisms/ml to the antibiotic dilutions brought to a final volume of 5 ml. Susceptibility results were determined by both macroscopic and microscopic examination of each broth tube after 24 h of incubation at 37 C. The minimal bactericidal concentration (MBC) was determined by subculturing onto chocolate agar all broth tubes that were optically clear or hazy. The lowest concentration of ampicillin that resulted in 100% loss of viability on subculture was considered the MBC.

Agar dilution tests were performed by incorporating twofold dilutions of ampicillin into Levinthal brain heart infusion agar (1:10). Inocula consisted of both 10^7 and 10^4 organisms/ml.

RESULTS

With the two-tube broth dilution method using Levinthal enrichment, upon visual inspection for growth inhibition a distinct haziness was frequently observed in some of the tubes containing ampicillin, cephalothin, and penicillin as contrasted to the optical clarity noted in tubes containing non-cell wall-active agents, such as chloramphenicol, and to the marked turbidity seen in the growth control tube (Fig. 1). Because of this haziness, results for these antibiotics were initially recorded as resistant since 100% inhibition of growth as observed visually is normally considered as the indicator of susceptibility. Phase-contrast microscopic examination of 0.1- to 0.2-ml samples derived from tubes showing haziness disclosed the presence of numerous spherical bodies approximately 3 to 5 μm in diameter (Fig. 2) in contrast to the typical coccobacillary forms observed in growth controls. Gram-stained smears of sedimented spherical bodies revealed only their presence and confirmed the absence of bacillary forms. Similar spherical bodies were occasionally observed in optically clear broths containing cell wall-active agents but were distinctly less in number. Spherical bodies were not seen in any of the preparations derived from optically clear broths containing antibiotics that are not directed against cell wall synthesis, such as chloramphenicol.

Haziness and spheroplasts were also observed when Trypticase soy or glucose broths were used to which X and V strips had been added as growth supplements and which did not contain any serum enrichment. Hence, their persistence could not be attributed to the presence of serum.

Subculture of 0.1 ml of the hazy, ampicillin-, penicillin-, or cephalothin-containing broths onto chocolate agar resulted in most instances

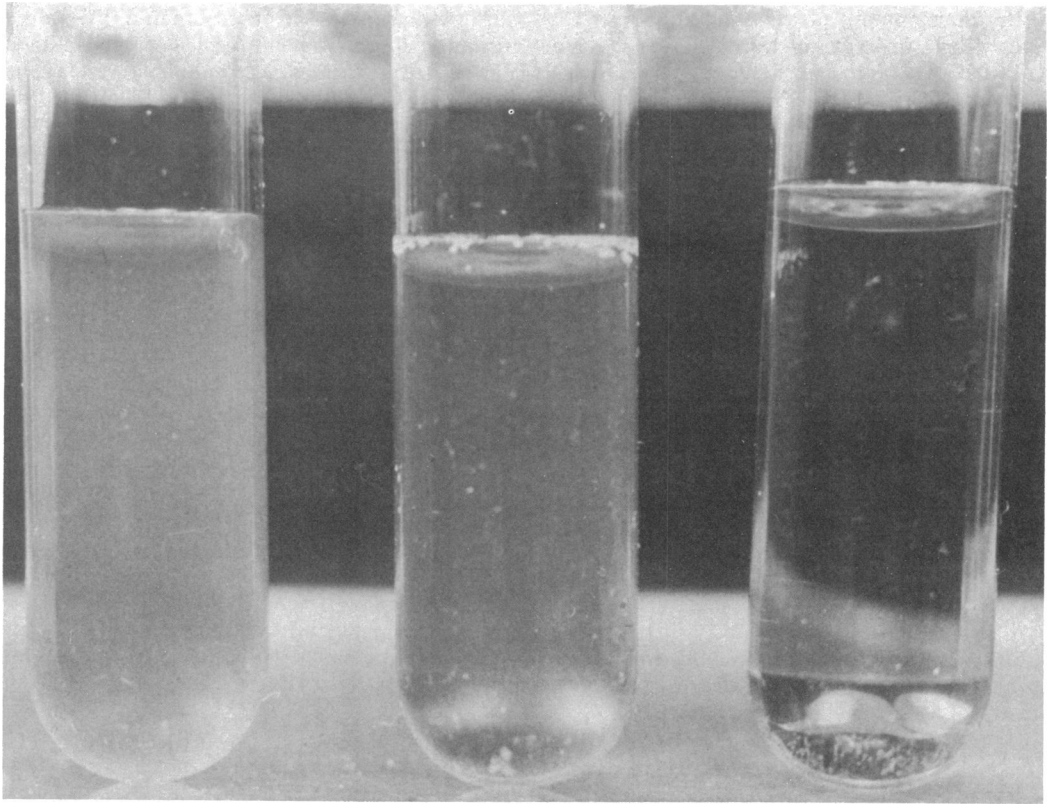

FIG. 1. *(Left) Distinct turbidity in ampicillin-free H. influenzae broth control tube. (Middle) Diffuse haziness in tube containing ampicillin. (Right) Optically clear broth in tube containing chloramphenicol.*

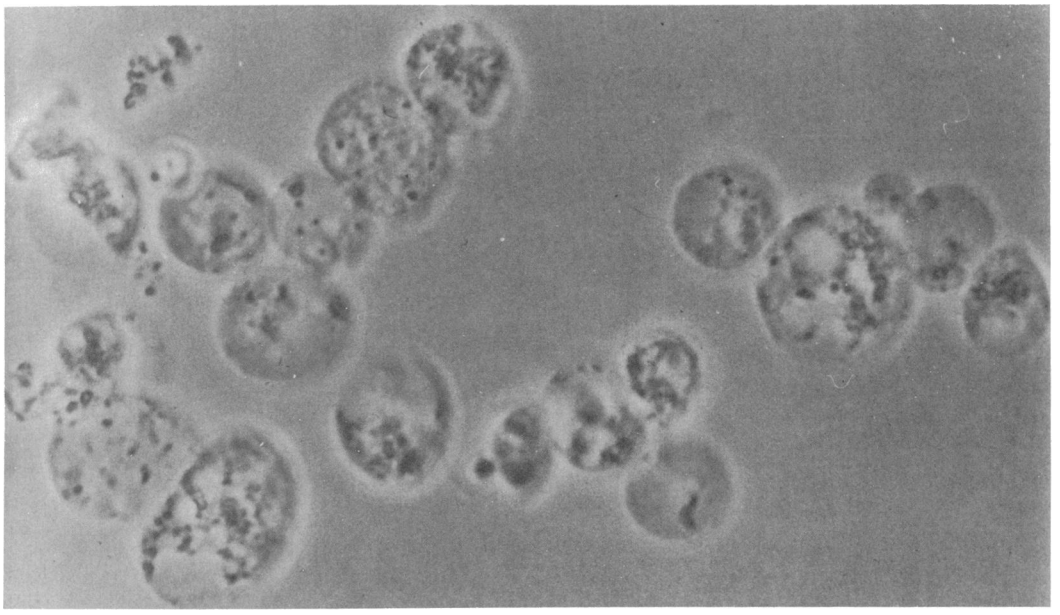

FIG. 2. *Large, spherical bodies of H. influenzae observed microscopically in hazy broth containing ampicillin.*

in the development of a small number of colonies which, upon gram-stained smear, demonstrated reversion to typical *Haemophilus* coccobacillary morphology (Fig. 3). This was contrasted to the confluent growth that developed from subculture of control broths devoid of antibiotic.

With 8 of 14 *Haemophilus* strains tested, subculture of the spheroplast-containing broths onto PPLO agar produced "fried egg" colonies (Fig. 4), which upon gram stain showed amorphous forms. Subsequent subculture of these colonies to chocolate agar gave rise to typical *Haemophilus* colonies with typical microscopic morphology.

Using an inoculum of 10^7 organisms/ml in either Levinthal broth or XV factor-enriched broth, the exact MIC of ampicillin to four of six *H. influenzae* strains tested was difficult to determine since a haziness was noted in tubes containing ampicillin ranging from 50 to 0.39 μg/ml. Microscopic examination of these broths showed many spherical bodies. At 0.195 μg/ml and lower, turbidity gradually increased to equal that of growth controls and, microscopically, a gradient of morphological variation was observed ranging from numerous filament forms at 0.195 μg/ml to typical coccobacilli at lower concentrations. Minimal bactericidal end points for these four strains exceeded 50 μg/ml since from 4 to 30 colonies developed on chocolate agar from subculture of the broths showing spherical bodies and confluent growth resulted from broths showing filament forms (Table 1).

An inoculum of 10^4 organisms/ml resulted in the abolition of haziness in tubes containing increasing amounts of ampicillin above the MIC, which was usually 0.195 μg/ml or lower for all six strains and evidenced by turbidity. Spherical bodies were also observed microscopically, but the number present were distinctly less than when the tests were performed with an inoculum of 10^7 organisms/ml. Also, filament forms were usually observed at lower concentrations of ampicillin of 0.098 or 0.049 μg/ml. With the smaller inoculum, the minimal bactericidal end points were clear-cut and either coincided with the MIC or differed by one tube dilution.

With the agar dilution method, an inoculum of 10^7 organisms/ml resulted in a haziness on the agar surface of plates containing 50 to 0.039

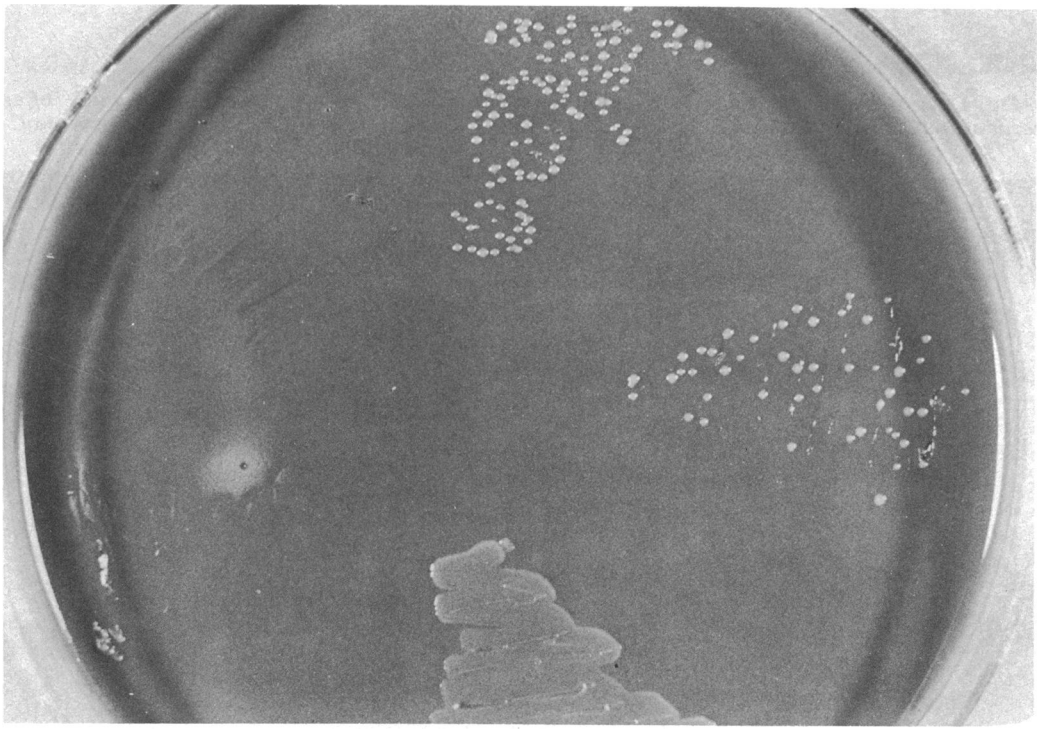

FIG. 3. *(Top and right) Development of a small number of Haemophilus colonies on chocolate agar following subculture of hazy broths containing spherical bodies after exposure to a cell wall-active antibiotic. (Bottom) Dense, confluent growth obtained from subculture of growth control broth. (Left) No growth from subculture of optically clear broth containing chloramphenicol.*

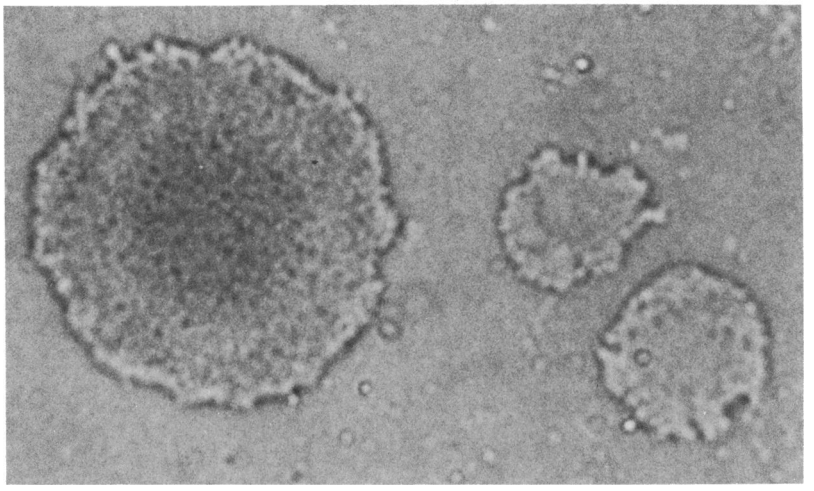

FIG. 4. *"Fried egg" colony of H. influenzae that developed on PPLO agar after subculture of spheroplast containing broth.*

TABLE 1. *Production of osmotically stable spherical bodies of H. influenzae by ampicillin, as observed in broth and agar dilution methods using an inoculum of 10^7 organisms/ml*

Determination	Ampicillin (μg/ml)											
	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.098	0.049	0
Status of visual clarity in Levinthal broth	Haziness								Increasing turbidity			Uniform turbidity
Microscopic morphology in broth	Spherical bodies								Pleomorphic forms		Coccobacillus	Coccobacillus
No. of colonies after broth subculture to chocolate agar	4	10	30	20	22	30	22	30	100	300	Confluent	Confluent
Growth appearance on Levinthal agar containing ampicillin	Surface haze								Confluent			Confluent
Microscopic morphology on agar surface	Spherical bodies								Pleomorphic forms		Coccobacillus	Coccobacillus

μg of ampicillin per ml and confluent growth at 0.195 μg/ml. Gram-stained smears of the surfaces showed spherical bodies similar to those observed in broth, whereas at 0.195 μg/ml numerous filament forms and coccobacilli were present. Subculture of all of these surfaces onto chocolate agar resulted in the recovery of *H. influenzae* with typical colonial and microscopic morphology.

An inoculum of 10^4 organisms/ml with the agar dilution method, as was the case with the broth dilution method, abolished the surface haze and resulted in sharp MIC and MBC end points of 0.098 and 0.195 μg/ml, respectively, for all six strains.

With one beta lactamase-producing, resistant *H. influenzae* strain tested, an inoculum of 10^7 organisms/ml produced haziness in broths containing ampicillin from 50 to 12.5 μg/ml. Beginning at 6.25 μg/ml, a degree of turbidity developed that was equivalent to that observed with the growth controls. Microscopic examination of the hazy broth showed numerous spherical bodies at 50 and 25 μg/ml and, at 12.5 μg/ml, pleomorphic forms were also present. Typical coccobacilli interspersed with occasional spherical bodies and aberrant forms were observed at 6.25 μg/ml. The MBC of this strain also exceeded 50 μg/ml as subculture from all of the hazy broth tubes resulted in the recovery

of the inoculated organisms. In contrast, on the basis of overt turbidity, an inoculum of 10^4 organisms/ml resulted in an MIC of 3.12 μg/ml (although microscopically, some atypical bacilli were observed with the 6.25 μg/ml tube) and an MBC of 12.5 μg of ampicillin per ml. Essentially, similar results were obtained with the agar dilution method.

DISCUSSION

Determination of antimicrobial susceptibility of *H. influenzae* is complicated because of the fastidious growth factor requirements of this species and is markedly influenced by such factors as media composition, incubation time, and inoculum size (6, 11). Regarding the latter parameter, McLinn and co-workers (6) have shown that inocula of 10^5 organisms/ml or greater resulted in an increase in the MIC, particularly with ampicillin, where a three- to fourfold increase over an inoculum size of 10^4 or less organisms was noted. These results, which were observed in tests performed by the agar dilution method, were attributed by the latter investigators to the difficulty in establishing clear-cut end points of inhibition because of a persistent hazy partial growth on the agar surfaces. They assigned the name "minimal partial inhibitory concentration" to this phenomenon, which was most pronounced with an inoculum of 10^5, as compared to 10^2, organisms/ml. No explanation for this observation was presented by the authors who recommended that susceptibility testing, particularly to cell wall-active agents such as ampicillin, be performed by the broth dilution method.

Thornsberry and Kirven (11), using a microdilution method, also noted an effect of inoculum size and observed as much as a 256-fold increase in ampicillin MIC based upon a change in inoculum size from 10^5 to 10^6 *Haemophilus* cells per ml. Although the investigators recorded end points as the complete absence of growth as observed macroscopically, they did observe "trailing" end points, particularly with ampicillin-resistant strains. Such "trailing" was not seen with ampicillin-sensitive strains nor in tests with non-cell wall-active antimicrobial agents

In the present study, we also encountered difficulties with ampicillin end point determinations, especially with large inocula. In contrast to the previous investigators (6, 11), however, who were unable to explain this effect, we could show that hazy or "trailing" end points were due to the formation of numerous spherical bodies generated by cell wall-acting agents, such as ampicillin. These bodies impart a surface haze on agar substrates and a uniform light opacity to broth. The occurrence and persistence of these spheroplasts, even in the absence of hyperosmolar environmental conditions such as existed in diluted Levinthal and glucose broth (1:100) (which had an osmolality of 405 mOsm/kg) and glucose broth supplemented with an XV strip (358 mOsm/kg), as well as on agar surfaces, have been attributed by Roberts and associates (7) to the marked osmotic stability of *Haemophilus* L forms (which is even greater than that of *Proteus mirabilis*), which are capable of withstanding osmotic lysis in a medium with an osmolality as low as 144 mOsm/kg.

The production of spherical bodies appears to be strain variable. Spherical bodies were not observed microscopically with two of six *H. influenzae* strains tested using an inoculum of either 10^7 or 10^4 organisms/ml. This coincides with our preliminary observation that haziness was not a constant finding with all strains in susceptibility tests performed by the two-tube broth dilution method.

Another feature associated with the spherical bodies was their variability in being able to revert to their natural form after subculture of the antibiotic broths onto chocolate or PPLO agars. Reversion also appeared to be inoculum size dependent. Given a strain capable of reversion, colonies developed only from subculture of ampicillin broths inoculated with 10^7, but not with 10^4, organisms/ml. This lack of growth under the latter conditions might be attributed to the smaller number of spherical bodies capable of reversion present in these broths. Still unexplained, however, is the lack of reversion of the spherical bodies after concentration of the 10^4 broths by centrifugation and subsequent subculture of the sediment to chocolate agar, a procedure which, when applied to the tubes containing 10^7 organisms/ml, increased the number of colony-forming units.

In view of the occurence and persistence of spheroplasts, which may render a haziness to broths and agars containing cell wall-acting agents, such as ampicillin, cephalothin, and penicillin, it is recommended that MIC end points be determined by microscopic (as well as macroscopic) observation of the growth milieu to determine the presence or absence of morphologically typical bacilli which, when observed, is indicative of true in vitro resistance. The role of such spheroplasts and their possible conversion to L-phase variants after ampicillin therapy of meningitis due to *H. influenzae* remains to be established.

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