Increased Bacterial Density at the Edge of Antibiotic Zones of Inhibition

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Since the introduction of antibiotic sensitivity testing, it was frequently noted that a heavier bacterial growth was found to be present at the demarcating line of the inhibition zone. This was particularly evident when penicillin discs were employed against staphylococci. It has been suggested that this ring of increased growth at the edge of inhibition zones was due to a stimulation of growth by subinhibitory concentrations of antibiotic, and has come to be known as a growth-stimulation phenomenon attributed to a stimulation effect induced by penicillin at subinhibitory levels.

According to P. M. Waterworth (Ann. Inst. Pasteur 75:95–98, 1948), once penicillinase-producing staphylococci start to grow around a zone of inhibition produced by a penicillin disc, then multiplication continues as the bacteria destroy the diffusing penicillin and an abrupt margin with a ring of stronger growth delineates the inhibition zone. This phenomenon was considered by Waterworth to be characteristic of penicillinase-producing microorganisms as the results of more nutrient available at the margin of the inhibition zone.

This study was performed to determine whether a ring of increased bacterial density, as noted at the edge of antibiotic zones of inhibition, could be reproduced independent of antibiotics or by "subinhibitory levels" of antibiotics when a source of extra nutrient was not available.

To illustrate these points, the following experiments were prepared. A sterile waxed-paper disc (diameter, 25 mm) was placed on a plate of brain-heart-blood agar. With a nebulizer, a 24-hr broth culture of Staphylococcus aureus (FDA 209) was sprayed to produce an almost confluent growth. An antibiotic sensitivity disc such as cephalothin, bacitracin, kanamycin, or vancomycin was placed

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**FIG. 1.** Plate with Staphylococcus aureus (FDA 209) and the inhibition zone produced with a vancomycin sensitivity disc (left) and the not-planted zone (right), both showing the "ring of increased growth" phenomenon.

**FIG. 2.** Plate with Staphylococcus aureus (FDA 209) showing one zone of inhibition (penicillin, 0.5 μ) and no zone and no rings of growth stimulation at subinhibitory levels of penicillin.
on the opposite side of the plate. The waxed-paper disc was then removed, and the plate was incubated for 20 hr. Two growth-free zones were observed (Fig. 1). One of these was a circular area surrounding the antibiotic sensitivity disc; the other was that area protected by the circular waxed-paper disc, which, of course, was never planted.

Both zones were delineated by a surrounding ring of heavier bacterial growth than that which occurred over the rest of the plate. When the same experiment was repeated in a less rich culture medium (Nutrient Agar, Difco) or with a smaller inoculum, the ring of heavier growth was reduced or absent. The ring of heavier growth was produced with all the antibiotics mentioned above and seemed to be stronger with those that produced a sharper zone limit.

In a second set of experiments, after spraying the surface with a 24-hr culture of a Staphylococcus aureus (FDA 209), holes of 8 mm in diameter were punched in the surface of brain-heart-blood-agar. Penicillin, 0.001 to 0.5 units/ml, was introduced in the holes (0.1 ml per hole) and was incubated for 24 hr at 37 C. The results show either no inhibition zone and no stimulation zone or the usual zone of inhibition where the penicillin concentration was high enough to produce inhibition of growth (Fig. 2).

These experiments show that the relatively richer medium available at the outer edge of either a nongrowth or of an antibiotic-protected zone (compared with the medium exhausted elsewhere on the plate) can produce an identical "ring of growth stimulation." In contrast, subinhibitory or barely inhibitory concentrations of penicillin failed to produce such rings unless enough antibiotic was present to produce a zone of inhibition also.

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