THE ORIGIN OF STIMULATION ZONES ON PENICILLIN ASSAY PLATES

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Numerous authors have observed the occurrence of zones of stimulation at the peripheries of inhibition zones on assay plates of penicillin and other antibacterial substances. Pratt and Dufrenoy[1,2] give many references. The phenomenon was ascribed by Ingram[3] to unused nutrients within the inhibition zones being available to the marginal cells. Pratt and Dufrenoy[2] expressed a different view, that "the enhanced growth evidenced by bacteria exposed to sublethal concentrations of penicillin may be ascribed to the action of penicillin *per se*, to degradation products of penicillin or to the release of metabolites by some of the organisms affected by penicillin. It is difficult at the present time to evaluate the extent to which each of these factors contributes to the enhancing action".

The experiments described below were undertaken in an attempt to demonstrate that stimulation may be caused by additional nutrients diffusing from within the zones as well as by a direct or indirect action of the penicillin itself.

Experimental and Results

The organisms used were: *Staphylococcus aureus* N.C.T.C. 4736 and a freshly isolated *Bacterium coli* Type I (44° positive) the latter being relatively resistant to penicillin. The medium generally used was a nutrient broth (oxoid peptone 1 g., Lab-Lemco 1 g., sodium chloride 0·5 g., tap water 100 ml., *p*H 7·4) solidified with 1·5 per cent. of agar, and it was bulk-seeded before pouring with about 10^7 organisms/ml. from a 24-hour agar slope culture. The mean zone diameters were established using 10 I.U./ml. for the staphylococcus and 10^4 I.U./ml. for the *Bact. coli.*

The fact that diffusion of nutrients from within a zone not occupied by bacterial growth can cause stimulation at the zone margin was demonstrated by removing a disc of medium 20 mm. in diameter from a seeded plate with sterile cork borer and filling the hole with sterile melted nutrient agar. After incubation for 18 hours at 37° C. there was a ring of denser growth at the circumference of the circle of sterile agar. The effect was slight with the staphylococcus, but with *Bact. coli* it was quite marked. It was not possible to prevent some organisms spreading on to the sterile agar during filling and scattered colonies always appeared within the "zone". A difficulty was that if the filling did not reach the level of the original surface a spurious effect was obtained, due to the reflection of light at the "step". On the other hand, if overfilling occurred the spreading agar covered organisms which would otherwise have been on the surface,

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with the result that their growth was restricted and the "zone" margin appeared ragged. By studying the reflection of light from the plates immediately after filling the holes, unsatisfactory plates could be rejected.

The next step was to determine whether this nutrient effect was sufficient to account for the growth stimulation observed around zones produced by penicillin, and for this it was necessary to prepare plates containing sterile agar circles of the same size as the zones which would be formed by the stated concentration of penicillin placed in cups in the same plate. The procedure adopted was to pour plates from bulk-seeded medium and when set divide them into 3 equal segments, from 2 of which discs were removed and the holes refilled with sterile medium. Then two 8 mm. cups were made, one in the centre of a refilled hole and the other in the third segment. Penicillin in *p*H 7·0 phosphate buffer solution was then placed in these cups and the plates incubated for 18 hours at 37° C. The diameter of the discs removed was as close as possible to the mean zone diameters in tests with the stated concentrations of penicillin against both organisms. However, as is well known, the individual replicate zones of an assay of a single concentration differ in size and this was especially so with *Bact. coli*. It was therefore necessary to prepare a considerable number of replicate plates in order to obtain sufficient cases where the zone formed by the penicillin was of exactly the same diameter as the sterile agar circles and which were suitable for photographic reproduction.

With *Bact. coli* rings of slight stimulation were formed round the circles of sterile medium (Fig. 1.C). Around the circles of sterile medium containing penicillin the rings were more strongly marked (Fig. 1.A), and were similar in density to those obtained with penicillin used in the normal way (Fig. 1.B). The additional stimulation in the presence of penicillin was clearly visible. With *Staph. aureus*, the contrast between stimulation and background growth was slight in all cases but the results were qualitatively the same. The rings were hardly discernible around the circles of sterile medium and somewhat more strongly marked around the penicillin cups, but plates suitable for photographic reproduction could not be obtained with the nutrient agar. In order to obtain enhanced differences between the degrees of stimulation the experiments were carried out using other media and it was found that enrichment of the medium with yeast extract gave more pronounced stimulations while a less nutrient medium gave smaller stimulations. Thus heavier growth and more pronounced stimulations were obtained with *Bact. coli* on a medium containing 0·5 per cent. each of peptone, beef extract and yeast extract (all Difco) (Fig. 2) than on the nutrient agar (Fig. 1). With *Staph. aureus* poor growth and slight stimulations were obtained on 0·5 per cent. each of peptone and beef extract (both Difco) (Fig. 3) but a richer growth with heavier stimulations on the nutrient agar fortified with 0· 5 per cent. of Difco yeast extract (Fig. 4).

Circles of sterile medium do not exactly represent the nutrient conditions inside a penicillin inhibition zone, which must contain dead and lysed cells. Products from these might be responsible for additional stimulation, supposing they were more readily available to the growing bacteria than

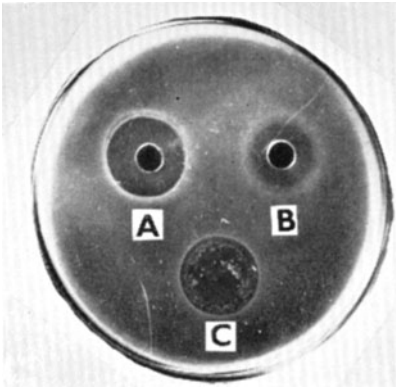

FIG. 1. *Bact. coli* grown on a medium of nutrient agar.

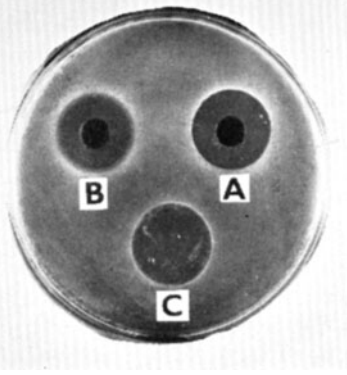

FIG. 2. *Bact. coli* grown on a medium containing 0·5 per cent. each of peptone, beef extract and yeast extract (all Difco).

A. Penicillin cup in unseeded agar disc.
B. Normal penicillin zone in seeded agar.
C. Unseeded agar disc.

(Approximately half natural size.)

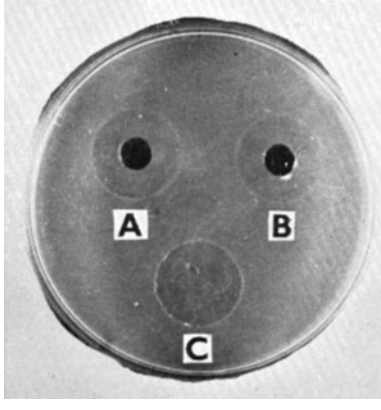

FIG. 3. *Staph. aureus* grown on a medium containing 0·5 per cent. each of peptone and beef extract (both Difco).

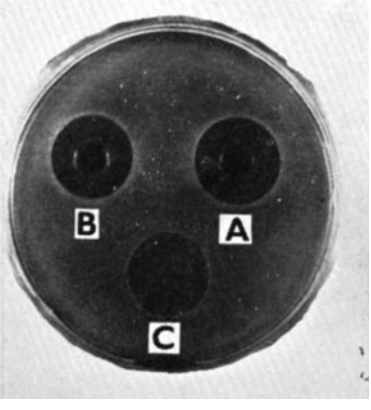

FIG. 4. *Staph. aureus* grown on a medium of nutrient agar fortified with 0·5 per cent. of yeast extract (Difco).

A. Penicillin cup in unseeded agar disc.
B. Normal penicillin zone in seeded agar.
C. Unseeded agar disc.

(Approximately half natural size.)

the nutrients of the sterile medium, so trials were made to investigate this point. The growth on a 24 hours slant culture of *Staph. aureus* 4736 was washed off in 5 ml. of nutrient broth. After incubation for 1 hour at 37° C. penicillin was added to a concentration of 0·02 I.U./ml. and the suspension incubated for a further 5 hours, by which time considerable lysis had occurred. The suspension was then centrifuged and penicillinase was added to the supernatant fluid and allowed to act for 1 hour at 37° C. Drops of the final product were then put into cups in nutrient agar plates seeded with the two organisms, but in no case was there any sign of increased growth around the cups. A control fluid was prepared in exactly the same way except that the bacteria were omitted. A staphylococcus autolysate prepared according to the method given by Todd[4] also gave negative results. It was thought that the lack of growth response to the staphylococcus products might have been due to the use of too rich a medium, so the lysates were tested against both organisms in a synthetic medium (mannitol 1 g., ammonium chloride 0·5 g. in 100 ml. of a mineral salt solution with 1·5 per cent. agar). Stimulation around cups due to lytic products was very weak or completely absent. The slight growth of *Staph. aureus* on this medium without added aneurine was probably due to the presence of small amounts of the vitamin in the mannitol.

Finally, penicillin zones were formed in the synthetic medium, using two seeding rates of 10^6 and 10^9 cells/ml. to determine whether the products from a large number of lysed cells could exert a significant nutrient effect in the poorer medium. It appeared that there might have been a more marked stimulation with the higher seeding rate, but owing to the great differences in zone size at different inoculum levels no reliable conclusions could be formed.

Discussion

It has been shown that nutrients diffusing from an uninoculated zone may cause stimulation at the periphery. The extent of the response was different with the two organisms tested, and this may be a reflection of their nutritional requirements; the non-exacting *Bact. coli* may be capable of greater and more regular stimulation where the exacting *Staph. aureus* may not respond, because growth of the latter is normally limited by the supply of various factors, a slight additional availability of which might be insufficient to produce a marked response. It has also been shown that the stimulation rings round penicillin zones are more marked than around circles of uninoculated agar. There would thus seem to be two effects, one due to increased amounts of nutrients which may be regarded as prolonging the period of active growth[5], and the other due to penicillin, a stimulation which may be regarded as an increase in metabolic rate[6]. But, for an increased metabolic rate to manifest itself in increased growth, additional nutrients must be available. The supply of nutrients from within either a penicillin zone or a disc of sterile medium to the periphery is limited by rates of diffusion, and it is difficult to see why these should be different in the two cases. The extra growth around the penicillin zone might be due to a more effective utilisation of the nutrients, but on this

point no evidence is available. There is, however, an important difference between a penicillin zone and a disc of sterile medium; the former contains dead, possibly lysed, bacteria and products diffusing from these may contribute to the nutrient status at the periphery of the zone. *Bact. coli* and *Staph. aureus* must be considered separately in this connexion, for while Pratt and Dufrenoy[7] have shown that penicillin acts on these species in essentially the same way, *Staph. aureus* is readily lysed by penicillin, possibly by accelerated autolysis[1], while *Bact. coli*, because it is not subject to autolysis[8], probably does not lyse in the presence of penicillin.

Pratt and Dufrenoy[1] have interpreted the waves of post-lytic growth observed in cultures of staphylococci by various workers, e.g., Bonét-Maury[9], in terms of release of nutrients from cells whose permeability has been increased by the action of penicillin, but since the experiments in question were all conducted in media containing abundant nutrients there is no justification for concluding that in the waves of growth nutrients derived from other bacterial cells were being used, especially since increased cell permeability could not be demonstrated by Gale and Taylor[10] with penicillin. There would, however, seem to be no reason why such nutrients should not be utilisable, and indeed it is possible that they may be of especial value, the argument being that they might be structurally very similar to units in living cells and hence easily synthesised into cell substance. Indirect evidence on this point is found in the work of Lesfargues and Delaury[11] on the elaboration by bacteria of substances capable of stimulating the growth of isolated animal tissues, while yeast cells killed by ultra-violet radiations yield materials which increase the growth rate of yeast[12,13,14,15]. The possibility of a similar action in the case of *Bact. coli* is not so well founded.

The presence or absence of stimulation due to bacterial products must, however, depend on the quantity available, and calculation shows that, considering *Bact. coli* cells to have a volume of $2\mu^3$, a specific gravity of unity and a water content of 80 per cent., the seeding rate of 10^7 cells/ml. would provide only about 0·004 mg./ml. of dry matter in the medium of which only part could be of high value. Of this 0·004 mg., nucleic acids might amount to 0·0005 mg.[16] and amino-acids might total 0·0013 mg.[17] Growth prior to lysis might increase these amounts 2 to 4 fold, but even so they are still small compared with the 15 mg. or so of nitrogenous organic nutrients/ml. of the original medium, and evidently, unless the bacterially-derived nutrients have a very high food value indeed, their influence could be only slight. This is supported by the present finding that the amount of stimulation depends on the nutrient value of the medium and by the work of Miller, Green and Kitchen[18], who found stimulatory effects to be produced most readily in a medium fortified with yeast extract. If nutrients from bacteria were of especial value it might be expected that growth rates in the post-lytic waves would be increased, but in fact these growth rates seem to be the same as, or lower than, those recorded prior to lysis[9,19,20,21].

The work of Ingram[3] and of Mitchison and Spicer[22] seems to indicate that stimulation zones are the result of greater development of individual

colonies rather than of a greater number of colonies. This would seem to indicate a direct stimulatory action of penicillin on the cells and this is also suggested by the development of complex ring patterns around assay cups, the most likely explanation for which is that the penicillin diffuses in Liesegang ring patterns[23,24]. Moreover Ericksen[25] found that staphylococci trained to penicillin resistance showed stimulation zones whose widths varied directly with the penicillin concentration.

SUMMARY

The peripheral stimulation around penicillin inhibition zones is due in part to additional nutrients which have diffused from the zones; there is also a stimulation attributable to the penicillin itself or to products of its degradation.

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DISCUSSION

The paper was read by MR. N. D. HARRIS.

MR. N. L. ALLPORT (London) referred to a note in *Nature* by Professor A. D. Gardner in 1940 in which he attributed the spherical enlargement of the organisms to possible imperfect fission. *Streptococcus pyogenes* growing at the edge of the penicillin zones were very much bigger than organisms growing normally. He also commented that in the case of some Gram-negative organisms such as *Bact. coli.* the enlargement of the bacteria may become so great that they even burst. Mr. Allport asked whether the author could throw any light on that.

MR. E. ADAMS (Plymouth) said that he had always thought that penicillin was one of the few antibacterial substances which were relatively insensitive

to differences in inoculum. Burkholder had found that a small concentration of penicillin did not stimulate the growth of organisms.

MR. G. SYKES (Nottingham) said there was no doubt that the basic medium itself was of high significance. With regard to the authors' suggestion that penicillin acted as a stimulant to the growth of the organisms, it was realised that in a diffusion plate assay a dynamic state was being dealt with, and that whilst growth was taking place there was also a potential of diffusion from the cup into the agar. In contrast to that in the turbidimetric assay there was certainly a growth of organisms. There was a definite relationship between the amount of growth and the concentration of organisms and this did not suggest that a small amount of penicillin could possibly give any stimulation.

MR. J. S. CANNELL (Ashton-under-Lyne) said that difficulty had been experienced in obtaining sharp zones when certain suspensions of *B. subtilis* spores were employed. There had been a tendency to obtain small sized zones with rather diffused edges, and it had been found that the addition of *p*-aminobenzoic acid to the medium tended to increase the sharpness of the zone as well as its diameter.

MR. R. L. STEPHENS (Brighton) asked whether it was possible that the stimulation might be due to the diffusion of inhibitory metabolites from the bacteria at the edge of the zone. The author had considered the diffusion of materials from the zone outwards, but what of the self-inhibitory substances which were produced by most micro-organisms during their own growth?

MR. N. D. HARRIS, in reply, said that the enlargement of the cells was not due solely to penicillin or other antibiotics. On enlargement the cells became more transparent. It was to be doubted whether such cells would give the same appearance of intense growth as in fact obtained at the margins of the zones. Although it had been reported that penicillin activity was independent of inoculum size, if the same experiments were carried out using different inocula, considerable differences in the zone sizes would be obtained. He was not very surprised that Burkholder did not obtain stimulation by penicillin. One did not always obtain stimulation with one particular strain of organism on different occasions. The problem of direct stimulation was complex. Even in dilution assays, as the concentration of penicillin increased the density of growth did not necessarily fall. There was a zone phenomenon whereby as the concentration increased there was decreasing growth, then increased growth followed by decreasing growth again. If *p*-aminobenzoic acid was acting as a growth factor then he would have expected smaller zones, but that did not seem to be the case. It might be that *p*-aminobenzoic acid inhibited substances in the medium which were known to inhibit the germination of the *B. subtilis* spores. The appearance of stimulation could be obtained as the result of diffusion of inhibitory substances away from the cells.