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Origin of the Double-Zone Effect in the Disc-Plate Sensitivity Determinations with Proteus Species

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ORIGIN OF THE DOUBLE-ZONE EFFECT IN THE DISC-PLATE SENSITIVITY DETERMINATIONS WITH PROTEUS SPECIES

by

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LIFE

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CHAPTER I

INTRODUCTION AND HISTORY OF THE PROBLEM

The usual method of determining antibiotic-sensitivities in clinical laboratories is the disc-plate technique. When employing such tests in the Bacteriology Laboratory of Mercy Hospital, using organisms isolated from clinical material, an unusual phenomenon occasionally occurred with certain strain-antibiotic combinations. A zone of inhibition surrounding an antibiotic disc on an agar plate culture denotes a sensitive organism. In certain cases, a zone of reduced growth was detected which partially or completely filled the original inhibition zone. It appeared as if organisms inoculated on the plate near the discs had been only partially inhibited by the action of the antibiotic, or if completely inhibited, had subsequently overgrown the zone of inhibition. Areas exhibiting this phenomenon were termed "Double-Zones". The bacteria most often found to produce this effect were swarming Proteus strains.

Workers in other clinical laboratories have also reported seeing this

unexplained phenomenon. However, the literature dealing with antibiotic-sensitivity studies is surprisingly lacking in specific reference not only to the appearance of the double-zone effect, but also with regard to an explanation of the mechanism of such a reaction.

Collins et al. (1954) noted that "Occasionally during incubation some overgrowth of the test organism occurred over the initial zone formed". The organisms which produced this overgrowth were not mentioned, but it was seen most frequently with chlorotetracycline and dihydrostreptomycin. Jackson and Finland (1951) noted:

> Some of the inhibition zones were fuzzy in outline at 24 hours; others showed double-zones--inner zones with complete clearing and outer zones showing partial growth. . . After 48 hours of incubation many of the clear zones observed showed partial growth, this was particularly true of the tests with aureomycin and terramycin.

The organisms best illustrating the effect in this case were Salmonella typhosa and Klebsiella pneumoniae. When testing the antibiotic-sensitivity of Proteus cultures, Pepin (1953) stated:

> their characteristic spreading on the surface of an agar plate made it difficult to read the zone of inhibition. After a very short period, a definite zone of inhibition could be read accurately and measured, but on further incubation, spreading growth in many cases obscured this zone.

In a paper by Senn and Lundsgaard-Hansen (1956), describing Staphylococcous enterocolitis originating from pre-operative treatment with antibiotics, photographs of disc-plate sensitivities were included which could well serve as illustrations of the double-zone effect produced by Proteus organisms as described in this study. The authors did not comment on these double-ringed inhibition zones, but the photographs on page 34 show typical double-zone

formations around terramycin, aehromycin, and chloromycetin discs.

This survey of the literature, comprising only four papers, clearly shows how few publications have even incidentally referred to double-zones. A study of their occurrence and the mechanism by which they are produced seemed of interest. Although the phenomenon does not appear to be limited to any one group of bacteria, Proteus organisms, according to the writer's own experience, appear to be associated with it most frequently. This genus was therefore selected for study.

In speculating as to the mechanism of double-zone formation by Proteus, at least two possibilities present themselves: 1) Inner zone growth forms by multiplication of cells that are resistant to the antibiotic contained in the discs; 2) Peculiarities in the metabolism or some other characteristic of the genus Proteus permits growth within the inhibition zone formed by the diffusing antibiotic. Of the several possible features peculiar to this genus, the one most readily brought to mind is "swarming".

A number of reports appear in the literature describing swarming. A general summary may be found in Wilson and Miles (1955). The characteristic swarming of Proteus was described by Lominski and Lendrum (1947) as seen under low magnification, in these terms:

> On solid media Proteus grows at first without swarming, which appears some 3-8 hours after inoculation, the time of onset depending mainly on the load of inoculum, the concentration of the agar and the lag-time of the culture. . .The onset of swarming is sudden; clumps of organisms shoot out from the periphery of the colony and migrate a considerable distance-occasionally as much as 1 cm-turn slightly back and settle. Practically all migrating clumps settle more or less at the same distance from the colony and thus form a ring where stationary growth begins again. The swarming phase lasts for some 10-30 minutes and is

> followed by a period of stationary growth of some 3-6 hours; the whole process is then repeated up to the limit of available surface. Meanwhile the space between the zones of stationary growth becomes gradually filled in but never quite to the thickness of the zones themselves. . .swarming does not appear to be continuous in occurrence or haphazard in direction; it is periodical and directed away from the place of stationary growth.

As these authors stated, swarming occurs from the area occupied by normal growth and its direction of progress is away from the normal growth region. This would tend to produce the very effect noted in double-zone formation around antibiotic discs: There is the usual line of demarcation between the zone where the antibiotic inhibited growth and the outer region of normal uninhibited growth. Covering the zone of inhibition more or less completely is a thin layer of less luxuriant growth, which resembles in appearance a layer that resulted from swarming. Therefore, experiments were undertaken to determine first, the frequency of double-zones among a number of strains of the species Proteus vulgaris and Proteus mirabilis; and second, to make observations regarding the importance of antibiotic-resistance and swarming in relation to the double-zone phenomenon.

CHAPTER II

PROBLEM 1: Selection of Medium and Inoculation Method for Sensitivity Testing

The medium selected for use in the determination of the origin of the double-zone phenomenon must necessarily support swarming, a characteristic of chief concern in this study. Also, the method of inoculation should be such that double-zone effects can be easily detected.

With these considerations in mind, one strain each of *Proteus vulgaris* and *Proteus mirabilis*, known to produce good double-zones, was tested on various media, using different methods of inoculation. The following media were used:

Blood Agar (5% human bank blood in Casman Medium Base)

Casman Medium (Difco)

Mueller-Hinton Medium (Difco)

Nutrient Agar (Difco)

The inoculation methods were the following:

No. 1. Five ml melted agar (55 C) seeded with 0.1 ml culture was poured over 10 ml solid agar in a petri dish.

No. 2. Pour plates were made containing 15 ml melted agar (55 C) seeded with 0.1 ml culture.

No. 3. With a sterile bent glass rod, 0.1 ml culture was spread over the surface of an agar plate (app. 20 ml agar).

No. 4. A sterile cotton swab, saturated with a broth culture, was used to inoculate the surface of an agar plate (app. 20 ml agar).

The inoculum used in all methods was a 4 hr. Nutrient Broth (Difco) culture. BBL Desi-discs were added, 4 per plate, after inoculation. Duplicate plates were incubated at 30 and 37 C and readings were made after 24 and 48 hrs.

The results, as summarized in Tables I and II indicate that any of the combinations tested could be used to demonstrate double-zone formation. However, ease of interpretation was the deciding factor in the final choice. Of the media tested, double-zones were detected most readily on Blood Agar. Clear or colorless agar did not provide a sharp contrast between growth and medium, making it difficult to determine zone types. For this reason, and also because of the widely accepted belief that swarming occurs on it more readily, Blood Agar became the medium of choice.

Although a standard-sized inoculum, such as 0.1 ml of broth culture, would be more desirable for uniformity, the technique of spreading with a swab appeared to produce satisfactory results for this particular type of experiment. The seeded cultures did not provide a heavy enough inoculum to enable

easy reading of double-zones. Spreading with a glass rod on the other hand, gave a very heavy uneven growth, so that few double-zones were visible. The majority of zones in this case were completely filled in with either a heavy film or colony overgrowth and many could be mistaken for resistant reactions. Spreading with a swab, however, provided a uniform growth, and also allowed for easy recognition of double-zones. Using this method, if the original inhibition zone was secondarily filled in with growth, it was still detectable. The effect was that of two layers around the antibiotic disc; a heavy outer layer of normal growth and an inner area of lighter growth extending up to the disc.

Considering incubation temperatures, it was noted that although double-zones were formed at 37 C, the same antibiotic-organism combination incubated at 30 C often formed zones entirely filled in with a film overgrowth, no actual double-zones being seen. Possibly, this may be explained by enhanced swarming at 30 C. Also, because 37 C is the usual incubation temperature used in clinical laboratories, it was chosen for use in these experiments.

From these results it was decided that the procedure to be used for all subsequent "Standard" sensitivity tests would be the following: Inoculate the surface of a Blood Agar plate by spreading with a cotton swab saturated with a 4 hr. Nutrient Broth culture; add Desi-discs, four per plate; incubate at 37 C for 24 hrs. or longer if required.

CHAPTER III

PROBLEM 2. Selection and Initial Sensitivity Testing of Swarming Organisms

Organisms used for study of the double-zone phenomenon were obtained from the stock collection of the Microbiology Department. Each culture was designated by its stock number. Arbitrarily, 25 strains each of *Proteus vulgaris* and *Proteus mirabilis* were chosen and transferred to Heart Infusion Broth (Difco) plus 0.5% agar, in order to provide a set of stock cultures for further use.

Since swarming ability was a major consideration of the study, the 50 organisms were tested for this characteristic. From a 24 hr. Nutrient Broth (Difco) culture of each strain, a single center streak was made along the diameter of a fresh Nutrient Agar (Difco) plate and the culture incubated at 37 C. Organisms which swarmed actively within 24 hrs. were considered "good swarmers". In this way, 16 *Proteus vulgaris* and 23 *Proteus mirabilis* strains were chosen for subsequent study.

Sensitivity tests were then performed to ascertain which combinations of antibiotics and strains of bacteria would show the double-zone effect. The standard procedure used was as follows:

1. A cotton swab, saturated with a 4 hr. Nutrient Broth culture was used to inoculate the entire surface of a Blood Agar Plate (Casman Medium, Difco, plus 5% human bank blood).

2. BBL High-Concentration Desi-discs containing the following antibiotics were added:

Antibiotic	Concentration
Aureomycin (A)	30 mcg.
Bacitracin (B)	10 units
Carbomycin (Ca)	15 mcg.
Chloromycetin (C)	30 mcg.
Dihydrostreptomycin (DS)	60 mcg.
Erythromycin (E)	15 mcg.
Neomycin (N)	30 mcg.
Penicillin (P)	10 units
Polymixin B (PB)	30 mcg.
Terramycin (T)	30 mcg.
Tetracycline (Te)	30 mcg.
Triple Sulfa (SSS)	1 mgm.

The concentrations listed are those specified for "High Concentration Desi-discs" by the manufacturers. Discs were added four per plate.

3. Readings were made after 24 and 48 hrs. incubation at 37 C. Plates were examined for: (a) Zones of inhibition, indicating sensitivity to the antibiotic, and (b) the double-zone effect. The results can be summarized as follows:

Effect of Antibiotics on Proteus vulgaris and Proteus mirabilis

Antibiotic	Proteus vulgaris	Proteus mirabilis
Aureomycin	sensitive	insensitive
Bacitracin	insensitive	insensitive
Carbomycin	insensitive	insensitive
Chloromycetin	sensitive	sensitive
Dihydrostreptomycin	sensitive	sensitive
Erythromycin	insensitive	insensitive
Neomycin	sensitive	sensitive
Penicillin	insensitive	sensitive
Polymixin B	insensitive	insensitive
Terramycin	sensitive	insensitive
Tetracycline	sensitive	sensitive
Tripie Sulfa	insensitive	insensitive

Aureomycin, chloromycetin, dihydrostreptomycin, neomycin, terramycin, and tetracycline inhibited growth of nearly all Proteus vulgaris strains. Chloromycetin, dihydrostreptomycin, neomycin, penicillin, and tetracycline usually inhibited Proteus mirabilis strains. Each of the 39 strains tested showed double-zone formation with two or more of the antibiotics employed in the experiment. This appears to be a definite indication that such a

phenomenon is not an uncommon occurrence. All strains of both species were either resistant to the remaining antibiotics tested, or if sensitive, did not give a double-zone effect. These ineffective antibiotics were not included in subsequent experiments.

The precise appearance of the double-zones differed from case to case. It was evident at once that double-zone formations were by no means uniform. It was necessary to establish descriptions which would standardize the observations and simplify recording without concealing these differences. The zone types were therefore reduced to the following:

Type 1. Clear inhibition zone: A clear zone of inhibition is formed around the disc, indicating a sensitive organism (C). In some cases, resistant colonies were found in the inhibition zone (c).

Type 2. Double-zone: Clear zone of inhibition around the disc, then a narrow band of reduced growth, and then normal growth (DR).

Type 3. Zone with overgrowth: Lacked the band of reduced growth found in Type 2; The inhibition zone was invaded by a film of growth (Z-F) or filled by a mass of confluent colonies (Z-G).

Type 4. Double-zone with overgrowth: Combined the band of reduced growth found in Type 2 with the invasion of the inhibition zone found in Type 3, so that within the band of reduced growth there was either a film (DR-F) or a confluent mass of colonies (DR-G).

A diagrammatic illustration of the zone types is given in Figure 1.

The observations in this experiment are recorded in two tables: Table III for Proteus vulgaris and Table IV for Proteus mirabilis. It is evident from the data that the two Proteus species differed in the types of

zone characteristically produced. Proteus vulgaris strains gave a high percentage of typical double-zone formations with a persisting area of no growth near the disc. Proteus mirabilis strains more frequently exhibited film or colony overgrowth of all or nearly all of the zone of inhibition.

The majority of typical double-zones formed by Proteus vulgaris were obtained with dihydrostreptomycin: 12 of the 16 strains were positive. The inhibition zones were large and the light growth partly filling the zone was readily apparent. The double-zones appearing with chloromycetin were not as apparent as those obtained with dihydrostreptomycin. The inner zone of reduced growth was usually very narrow with indistinct outlines, and often required 48 hrs. to appear. Strains which were highly sensitive to aureomycin gave good double-zones. However, strains which were only slightly sensitive gave small zones with heavy colony overgrowth. Strains 3701 and 3353 which appeared resistant, may have been very slightly sensitive, with heavy overgrowth obscurring the original small zone. Tests with terramycin and tetracycline gave results similar to those obtained with aureomycin. Strain 2389 with tetracycline produced the only example of film overgrowth with a Proteus vulgaris organism. Neomycin produced clear zones of inhibition with 10 strains. The 6 double-zones seen were not distinct, as in the case of chloromycetin determinations.

With Proteus mirabilis, dihydrostreptomycin again gave the best results. The difference, however, was in film overgrowth, which did not appear with Proteus vulgaris, but was a common occurrence with Proteus mirabilis. This type of zone was formed within 48 hrs. by 18 of the 23 strains. It was noted that if double-zones were formed within 24 hrs., film overgrowth usually

followed within 48 hrs.; and that zones with film overgrowth at 24 hrs. were followed by double-zones when incubated an additional 24 hrs. Only 2 strains gave double-zones with chloromycetin, film overgrowth occurring in both within 48 hrs. Distinct, single colonies grew in the inhibition zones of 16 strains and 12 of these were either preceded or followed by film overgrowth. When neomycin was tested, 22 strains showed film formation, 6 of which were followed by double-zones within 48 hrs. Only one typical double-zone effect was found with this antibiotic. Results with penicillin were similar to those obtained with chloromycetin. The organisms were less sensitive, however, with small zones of inhibition, so that zone types were more difficult to determine. Fifteen of the 23 strains tested were resistant to tetracycline. It appeared as if 5 of these could have been slightly sensitive with heavily overgrown small zones, as was noted with terramycin and aureomycin sensitivities of Proteus vulgaris strains. No typical double-zones were formed using this antibiotic. Six strains were found to have film or colony overgrowth.

In summary: employing 39 strains belonging to 2 species of Proteus, and testing with 7 antibiotics, the sought-for double-zone effect was observed 146 times or 78% of the whole number of antibiotic-sensitivity determinations made. The double-zone effect was thus found to be readily demonstrable. The multiplicity of differences in detail in the appearance of the zones was unexpected and complicated the further study of the phenomenon.

CHAPTER IV

PROBLEM 3: Is the Double-zone Phenomenon Due to the Growth of Antibiotic-Resistant Organisms?

Having established in the preliminary experiments a set of organism-antibiotic combinations which would produce a double-zone effect, together with a procedure for demonstrating it, further experiments were aimed at determining the mechanism of the reaction. One possibility as to the origin of the phenomenon was that the reduced growth forming the inner zone was composed of antibiotic-resistant mutants.

To test this hypothesis, sensitivity tests were repeated, using *Proteus vulgaris* strains as the test organisms and the 6 antibiotics to which this species was sensitive. Growth from the inner zone was transplanted onto Blood Agar plates and incubated at 37 C for 24 hrs. The presence or absence of swarming was recorded and a tube of Nutrient Broth inoculated. Using this 4 hr. culture, standard sensitivity tests were run to determine if the growth so

obtained reacted as resistant mutants.

Results, as summarized in Table V, indicate: 1. The swarming characteristic was retained in all but 5 instances; 2. Cultures from the reduced growth zone were antibiotic-sensitive; 3. The majority of these cultures were found to reproduce the double-zone effect. The atypical results with dihydrostreptomycin are discussed later. The appearance of the zones was not always identical to the original type, emphasizing again the variability of the reaction. It should also be noted here that the repeat standard sensitivity tests often showed variations from the initial determinations recorded in Tables III and IV. For example, a combination originally producing a double-zone might give rise to film overgrowth when tested again. It seems possible that the different types of zones are merely details in the expression of one fundamental characteristic, being due not only to differences in degree of sensitivity, but also to differences in the setting up of the test itself. The size of the inoculum, as well as the thickness of agar in the plate, is not absolutely controlled and might lead to small variations in the diffusion and antibacterial activity of the antibiotics.

Results obtained with dihydrostreptomycin in this experiment presented an entirely different aspect. Eight of the 13 inner zone growths could not be sub-cultured on Blood Agar. Strain 851 required 48 hrs. to grow but reacted typically. Of the four cultures that grew within 24 hrs., strain 3600 did not swarm or give a double-zone effect; strain 235 was resistant; strain 3699 gave a typical reaction; and strain 3488 was resistant and non-swarming. This antibiotic was evidently bactericidal, an interesting point, but one which was not investigated further.

It was thought advisable to check the characteristics of the cultures from the reduced growth zones to see if contaminations or mutations were intruding. The various bacteriological reactions investigated are recorded in Table VI in the horizontal rows labeled A, C, DS, N, Te, T. In general, few differences were noted. The characteristic which changed most often was gelatin liquefaction, which became negative 9 times. Citrate utilization changed to negative in 7 instances, and although glucose was always fermented, gas production was lost by 6 organisms. Indole production became negative in one case. The total variations of the inner zone growths from the original inoculum were 23 or 2.2% of the 1044 test reactions that were performed. Previously, 12 differences were noted between the reactions of the cultures at the time this study began and those on record for the strains used. The total of these variations was 2.08% of the 576 test reactions performed. Comparison of these variations by statistical analysis gave a chi-square value of 0.0005. This indicates that both samples of characteristics could have come from a single population. It shows no evidence of any tendency of the present experimental conditions to change the diagnostic characteristics of these bacteria.

In the course of these tests an extremely important fact emerged. In certain instances a small number of isolated colonies develop within the zones of inhibition around an antibiotic disc. Bacteriologists in general are familiar with this and consider that these colonies are composed of resistant cells. Examples may be found in the papers of Scott (1950), Weil and Harris (1953), and Perch (1954). In the present studies, a single colony from the dihydrostreptomycin inhibition zone of the original sensitivity tests of strain 3488 was cultured and retested. The colony proved to be resistant and non-

swarming. Two strains of Proteus vulgaris, tested with polymixin B in Problem 2 had a few colonies growing within the inhibition zone and these were also shown to be resistant and non-swarming. A number of similar cases were found among the Proteus mirabilis sensitivity tests. Colonies from the inhibition zones of 13 strains, denoted in Table IV by the symbol C-c, were examined and found to be antibiotic-resistant variants. Thus, single distinct colonies appearing in a clear inhibition zone are resistant to the antibiotic, whereas the inner confluent band of reduced growth seen in the double-zone phenomenon remains antibiotic-sensitive.

Since the double-zone phenomenon could not be shown to be due to antibiotic-resistant organisms, an investigation was begun to determine if the characteristic swarming of Proteus species would offer an explanation of the formation of double-zones.

CHAPTER V

PROBLEM 4: Is the Double-Zone Phenomenon Due to Swarming?

It is generally believed that bile salt media inhibits swarming of *Proteus* species. In order to investigate the possibility that swarming can occur on supposedly "inhibitory" media, the following experiment was carried out: from 4 hr. Nutrient Broth cultures of 16 *Proteus vulgaris* strains, a single center streak was made along the diameter of plates containing the following media:

Desoxycholate Agar (Difco)

Desoxycholate Citrate Agar (BBL)

Mac Conkey Agar (BBL)

Blood Agar Control (Casman Medium, Difco, plus 5% human bank blood)

These plates were all freshly prepared in order to eliminate any possible effects resulting from dryness of the surfaces. The inoculated plates were incubated at 37 C for one week and examined daily for swarming. Table VII

summarizes the results: All but two strains were positive on Blood Agar within 24 hrs., and swarming had at least begun on Mac Conkey Agar within that time. Swarming of all strains had occurred on both media within 48 hrs. Desoxycholate and Desoxycholate Citrate Agar remained negative for one week.

This experiment shows that desoxycholate inhibits swarming, the different bile salts in Mac Conkey Agar restrain it but do not entirely prevent it, and Blood Agar definitely favors it. Therefore, if the swarming characteristic of the organisms being studied caused the double-zone effect, such an effect would be much less likely to occur on media which contained bile salts. To test this hypothesis, standard sensitivity tests were made, using the media listed above plus Levine E M B Agar (Difco). Ten strains of Proteus vulgaris were used as the test organisms and readings were made after 24 and 48 hrs. incubation at 37 C. Table VIII summarizes the results: There was insufficient growth on E M B Agar to determine inhibition zones, the medium being too inhibitory. This was also true to a certain extent of Desoxycholate Citrate Agar, although readings could be made with some strains. Other bile salt media did not prevent the formation of double-zones. When they occurred, these double-zones were usually Type DR, associated with a definitely limited amount of secondary growth within the inhibition zone.

A chi square test (Batson, 1956) was made to determine if there was a significant difference in the number of double-zones formed on the various media. The numbers of double-zones formed on each medium and the numbers of tests in which the double-zones did not develop were tabulated from Table VIII. Where no growth occurred, the result was not considered in the calculations. The observed and expected numbers for the calculations were as follows:

Medium	Double-Zones Observed	Double-Zones Expected	No Double-Zones Observed	No Double-Zones Expected	Totals
Blood	36	28.53	1	8.47	37
Desoxy	22	23.91	9	7.09	31
Des-Citrate	6	13.11	11	3.89	17
Mac Conkey	27	25.45	6	7.55	33
Totals	91	91	27	27	118

The chi square values, (O-E) 2/E, in each cell are:

Medium	Double-Zones	No Double-Zones
Blood	1.91	1.91
Desoxy	0.15	0.15
Des-Citrate	3.85	3.85
Mac Conkey	0.09	0.09
Chi square (3 degrees of freedom)		12.08**

The total chi square is 12.08, associated with 3 degrees of freedom. Chi square tables show that a value of 11.34 will be exceeded in only 1% of cases. Therefore the observed value is significant at the 1% level. This means that the medium does make a difference in testing for double-zones. It can be seen from the calculations that approximately 2/3 of the chi square is contributed by Desoxycholate Citrate Medium, in which far fewer double-zones were observed than were expected.

These experiments involve an apparent contradiction. When direct tests were made for swarming ability on bile salt media, desoxycholate salts inhibited the characteristic. When sensitivity studies were made on the same media, a limited amount of double-zone formation occurred, although statistical

treatment showed the frequency of the zones was far less than could be expected. There is probably no real discrepancy here. Different procedures were employed in setting up the two tests. To test for swarming, a small inoculum was placed along the diameter of a plate. If the growing bacteria attempted to swarm, they immediately encountered the large area of uninoculated medium containing desoxycholate which inhibited swarming. In contrast to this, sensitivity tests are set up by spreading a heavy inoculum of bacteria over the entire surface of the plate. Only the small fraction of the medium into which the antibiotic diffused, the inhibition zone, remained free of growing bacteria. Under these conditions it is probable that desoxycholate is unable to prevent swarming where the medium is covered with growing bacteria. It is a matter of general experience that heavy inoculation of Desoxycholate Agar tends to suspend the effect of the bile salt. For example, these salts tend to inhibit the growth of coliforms when feces specimens are streaked on the medium, but if the inoculation is made too heavy, coliforms are likely to develop in spite of the inhibitory property of the medium. Similarly, in the sensitivity tests on Desoxycholate Agar, the mass of bacteria mutually protect each other from the effects of the desoxycholate, and swarming occurs. But even then, once the swarming organisms began to invade the area containing the antibiotic, they would encounter active desoxycholate and swarming would be inhibited.

To make a direct test of the effect of antibiotics on a swarming culture, the technique of the antibiotic sensitivity test was modified to give what may be called the "Swarming Sensitivity Method". It seemed of interest to determine what would occur if swarming organisms, advancing across a plate from a line of inoculation, were to encounter an antibiotic diffusing out from

a disc. Blood Agar plates were inoculated with a 4 hr. Nutrient Broth culture by making a single streak across one side of the agar surface. Proteus vulgaris strains were used as the test organisms. An antibiotic disc was placed on the plate opposite to the inoculum. In this way, the swarming organisms would advance from the line of inoculation toward the disc. Standard sensitivity tests were set up at the same time for comparison. All plates were incubated at 37 C and examined after 24 and 48 hrs.

The results obtained are summarized in Table IX. Sixteen strains of Proteus vulgaris were employed and each tested against several of 6 antibiotics. The zone types formed using this method were varied. A diagrammatic illustration of the major types is given in Figure 2, and can be described thus:

Type 1. Clear inhibition zone: No double-zone or layer formation; the area surrounding the disc remained free of growth, denoting a sensitive organism (C).

Type 2. Layer formation: A heavy film of growth extended out from the original line of inoculation. A secondary thin film layer formed between the heavy layer and the disc (L). With some strains, the heavy initial swarm formed a complete zone around the disc which was partially or completely invaded by the secondary film layer (Z-F).

Type 3. Double-zone: A partial ($\frac{1}{2}$ circle) or complete (full circle) double-zone formed around the disc (DR). This gave the same appearance as the Type 2 zone formed by the standard method. See page 11.

Type 4. Double-zone with overgrowth: Combined the band of reduced growth found in Type 3 with the invasion of the inhibition zone by the secondary layer as in Type 2 (DR-F).

Although these types had to be established so that records could be kept, it is believed that the different types of zones are actually only different expressions of a single effect. The designations L, Z-F, DR, and DR-F, given in Table IX describe the different extents to which the organisms had swarmed over the surface of the agar. The advancing swarm eventually encountered the diffusing antibiotic. Organisms from the swarm, however, were able to continue advancing across the antibiotic-containing agar and depending on how far they were able to invade this area, took the form of 1: a second invading film layer (L); 2: complete invasion up to the disc (Z-F); 3: a narrow band of invasion into the inhibition area, giving the appearance of a double-zone (DR); 4: formation of the narrow band followed by complete invasion of the zone up to the disc (DR-F). Thirty Eight of the 44 swarming sensitivity determinations showed one type or another of this film invasion into the area containing the antibiotic.

It can be theorized that what took place was essentially something like this: The organisms, because of their swarming ability, are able to move out across the agar surface, eventually encountering the antibiotic which diffuses out from the disc. Although sensitive to this antibiotic, as shown by the inhibition zones formed in standard sensitivity tests, the organisms are able to continue to swarm. They form a film of growth up to, and in some cases completely surrounding the discs.

This same theory would account for the double-zones observed in the standard sensitivity tests described in Problem 2. The organisms which had been inoculated on the plate in the area of the antibiotic disc were inhibited and a clear zone was formed. Outside the zone, the normally growing culture

swarmed and the swarming cells were able to advance into the zone of inhibition.

In summary: Sensitive, swarming Proteus organisms in direct contact with an antibiotic on an agar plate will be inhibited by that antibiotic, but outside the range of diffusion of the drug, are able to continue swarming. The swarm actually invades the inhibition zone, producing the double-zone phenomenon. The precise appearance of the double-zones may vary, depending on such factors as size of inoculum, length and temperature of incubation, condition of agar surface, type of medium, diffusibility of the antibiotic, and degree of sensitivity of the organism itself. Advancing organisms remain sensitive to the antibiotic, as shown in Problem 3, since when retested by the standard method, they formed inhibition zones. The process of invasion from the normal growth swarm was repeated, giving a double-zone effect again.

CHAPTER VI

DISCUSSION

The paradoxical findings of this study give rise to a number of questions. It appears that sensitive swarming organisms are able to overcome the effect of an antibiotic. What mechanism is involved, what properties do swarmers have enabling them to accomplish this fete? We know that the cells forming the inner growth zones remain antibiotic-sensitive and viable when transferred to an antibiotic-free medium. In these experiments, the inner zones were sub-cultured within 24 or 48 hrs. Would the cells remain viable for any greater length of time? Are the organisms in what might be called simply a resting stage, and in order to grow must be removed from the antibiotic environment? Their ability to swarm could enable these cells to invade an inhibition zone, but once within the zone, do they actually multiply, carrying on their vital processes? If so, is their metabolism such that they are able to continue to grow and remain sensitive to the antibiotic.

It is known that bacteria can be made antibiotic-resistant by serial transfer through increasing concentrations of antibiotic broth. The conditions in a disc-plate sensitivity test are such that the antibiotic from a disc becomes less concentrated as it diffuses into the medium, forming an inhibition zone. Organisms moving into this zone advance from an area free of antibiotic into an area of gradually increasing concentrations of the antibiotic. Do the swarming organisms acquire a temporary resistance, permitting invasion, then revert back to sensitivity when removed from the antibiotic environment? Cells made resistant by the serial transfer method remain so, and microbiologists know of no cases in which a change from resistance to sensitivity could occur as readily as would be required by this explanation.

Do swarmers produce a substance which is antagonistic to the antibiotic, overcoming its effect much the same as the enzyme penicillinase overcomes the effect of penicillin? Or is a stimulation effect involved? In Problem 4, it was shown that Proteus vulgaris strains produced double-zones on bile salt media, and yet, the same organisms failed to swarm when cultured on those media in the absence of antibiotic discs. Does this indicate that the organisms are stimulated by the presence of antibiotic in the inhibition zone, enabling them to invade and produce the double-zone effect? Or does swarming occur outside the zone because the relatively large number of cells neutralizes the inhibiting effect of desoxycholate?

Two theories may be proposed to explain the mechanism by which Proteus organisms are able to form double-zones in the presence of an antibiotic to which they are sensitive: 1) Physical Theory: A characteristic of certain Proteus species is swarming. Due to this ability, these organisms

invade an area on an agar plate containing an inhibitory antibiotic, just by mechanically crowding into the unpopulated area. 2) Biological Theory: A metabolic difference exists between swarming and non-swarming organisms. After moving physically into an inhibition zone, swarmers are able to multiply in the presence of an inhibiting antibiotic.

The swarming sensitivity determinations made in Problem 4 support the Physical Theory. It was shown that swarming progresses into an antibiotic-containing area on an agar plate. Also, it was noted in Problem 1 that 30 C, a temperature usually considered to enhance swarming, produced a majority of zones with film overgrowth (Z-F). This finding also supports the Physical Theory, since the better the swarming conditions of the test, the larger the band of reduced growth formed by the invading organisms. No positive evidence was obtained in this study to indicate that a difference exists between swarmers and non-swarmers, which would be the basis for the Biological Theory. This theory assumes that cell division takes place within the inhibition zone, once it has been invaded by swarming organisms. The sensitivity studies indicated that zones do change in appearance over a period of time, increasing in size and thickness. This, however, does not necessarily indicate growth, since such an increase could be the result of continued invasion of swarmers from the normal outer growth.

Further studies of double-zones might have certain clinical applications. In vitro experiments have been presented which indicate that strains of Proteus vulgaris and Proteus mirabilis are able to swarm in the presence of an inhibiting antibiotic. It has been suggested that swarmers may differ metabolically from non-swarmers. Is it possible that swarming forms also

develop *in vivo*? They would then have the ability to invade tissues in spite of antibiotic therapy. Various interesting speculations now arise. What is the significance of double-zones seen in clinical laboratory work? Should organisms showing this phenomenon be reported resistant or sensitive to an antibiotic? The existance of *in vivo* swarmers could well account for the poor clinical results often obtained in the treatment of infections caused by *Proteus vulgaris* and *Proteus mirabilis*. It is evident that there is room for much further study of the double-zone phenomenon.

CHAPTER VII

CONCLUSIONS

1. The sensitivity of 16 strains of *Proteus vulgaris* and 23 strains of *Proteus mirabilis* to 12 antibiotics was determined by the disc-plate method.
2. *Proteus vulgaris* strains were usually sensitive to 6 antibiotics and *Proteus mirabilis* strains to 5.
3. There was frequent formation of double-zones in which the main inhibition zone was more or less filled by lessened growth; 78% of the zones observed produced this condition.
4. The growth in these zones took a number of different forms, but always more or less filled the area of inhibition with a layer of growth less luxurient than developed on the medium without antibiotic.
5. Sub-cultures of the reduced growth comprising the inner zone were sensitive to the antibiotic.
6. By means of a modified disc-plate test it was demonstrated that swarming

organisms were able to continue their progress across the medium over the area containing the antibiotic.

BIBLIOGRAPHY

Batson, H. C. 1956 An Introduction to Statistics in the Medical Sciences. Burgess Publishing Co., Minneapolis, Minn. p. 38.

Collins, A. M., Craig, G., Zaiman, E., and Roy, T. E. 1954 A Comparison Between Disk-Plate and Tube-Dilution Methods for Antibiotic Sensitivity Testing of Bacteria. Can. J. Pub. Health 45:450.

Jackson, G. G., and Finland, M. 1951 Comparison of Methods for Determining Sensitivity of Bacteria to Antibiotics In-Vitro. Arch. Int. Med. 88:446.

Lominski, I., and Lendrum, A. C. 1947 The Mechanism of Swarming of Proteus. J. Path. Bact. 59:688.

Pepin, J. 1953 An In-Vitro Study of the Sensitivity of Proteus Cultures to Five Antibiotics. Bios 24:213.

Perch, B. 1954 Sensitivity of Proteus and Providencia to Eight Antibiotics and Sulfathiazole. Acta Path. Microbiol. Scandinav. 35:278.

Scott, E. G. 1950 Aureomycin Sensitivity Tested by the Bondi Disc Technique. Am. J. Clin. Path. 20:65.

Senn, V. A. and Lunsgaard-Hansen, P. 1956 Zum Problem der Staphylokokkenenteritiden nach Antibiotikaverabreichung. Helv. Med. Acta 23:1.

Weil, A. J. and Harris, L. 1953 Testing for Antibiotic Sensitivity in a General Hospital. Ann. Int. Med. 38:1027.

Wilson, G. S. and Miles, A. A. 1955 Topley and Wilson's Principles of Bacteriology and Immunity, 4th Edition. Williams and Wilkins Co., Baltimore, Md. p. 738.

TABLE 1 PART 1

SYMBOLS USED IN TABLES I TO IX

These same symbols are used in all tables.

Symbols used to identify zone types:

R: Resistant organism, no visible zone of inhibition.

C: Clear zone of inhibition, no overgrowth or double-zone effect.

c: Distinct single resistant colonies within the original inhibition zone.

DR: Double-zone effect.

Z-F: Original inhibition zone completely filled in by a film of growth, no double-zone effect.

Z-G: Original inhibition zone completely filled in by a solid growth (appearing to be confluent colonies), no double-zone effect.

DR-F: Double-zone effect plus a film of growth partially or completely filling the original inhibition zone.

DR-G: Double-zone effect plus solid growth (appearing to be confluent colonies), partially or completely filling the original inhibition zone.

Symbols used to identify antibiotics:

A: Aureomycin
C: Chloromycetin
DS: Dihydrostreptomycin
N: Neomycin
P: Penicillin
T: Terramycin
Te: Tetracycline

TABLE I PART 2

EFFECT OF CULTURE MEDIUM ON DEVELOPMENT OF DOUBLE ZONES

CULTURE NO. 1298 PROTEUS VULGARIS

Inoc. Meth.	Temp. C°	MEULLER-HINTON MEDIUM A	C	DS	Te	T	CASMAN MEDIUM A	C	DS	Te	T
No. 1	37	DR	C	DR	DR	DR	Z-G	C	DR	DR	DR
	30	DR	C	DR	DR	DR	Z-G	C	DR	DR	DR
No. 2	37	DR	C	DR	DR	DR	Z-G	C	DR	DR	DR
	30	DR	C	DR	DR	DR	Z-G	C	DR	DR	DR
No. 3	37	DR-F	DR-F	DR-F	DR-F	DR-F	Z-G	DR-F	Z-F	DR-F	DR-F
	30	DR-F	Z-F	DR-F	Z-F	Z-F	Z-F	Z-F	Z-F	Z-F	DR-F
No. 4	37	Z-G	Z-F	C	DR	DR	Z-G	DR	C	DR-F	DR
	30	DR-F	C	C	Z-F	Z-F	Z-F	Z-F	Z-F	Z-F	Z-F

Inoc. Meth.	Temp. C°	NUTRIENT AGAR A	C	DS	Te	T	BLOOD AGAR A	C	DS	Te	T
No. 1	37	C	C	DR	C	C					
	30	C	C	C	C	C					
No. 2	37	Z-G	C	C	DR	DR					
	30	DR	C	C	DR	DR					
No. 3	37	Z-G	C	DR	DR	Z-F	Z-G	Z-F	R	DR	DR
	30	Z-F	R	Z-F	Z-F	Z-F	Z-G	Z-F	Z-F	Z-F	Z-F
No. 4	37	Z-G	C	Z-F	Z-F	Z-F	Z-G	C	Z-F	Z-F	DR
	30	Z-F	Z-F	Z-F	Z-F	Z-F	Z-G	C	Z-F	Z-F	DR

For explanation of symbols, see page 32.

TABLE II

EFFECT OF CULTURE MEDIUM ON DEVELOPMENT OF DOUBLE ZONES

CULTURE NO. 3722 PROTEUS MIRABILIS

Inoc. Meth.	Temp. C°	MEULLER-HINTON MEDIUM A	C	DS	P	N	CASMAN MEDIUM A	C	DS	P	N
No. 1	37	R	C	DR	DR	DR	R	C	DR	DR	DR
	30	R	C-c	DR	DR	DR	R	C-c	DR	DR	DR
No. 2	37	R	C	DR	DR	DR	R	C-c	DR	Z-G	DR
	30	R	C	DR	R	DR	R	C-c	DR	Z-G	DR
No. 3	37	R	Z-F	DR-F	C-c	Z-F	R	C-c	DR-F	Z-G	Z-F
	30	R	Z-F	Z-F	R	Z-F	R	Z-F	Z-F	R	Z-F
No. 4	37	R	Z-F	DR-F	Z-F	Z-F	R	C-c	DR	Z-G	DR
	30	R	Z-F	Z-F	R	Z-F	R	Z-F	Z-F	R	Z-F

Inoc. Meth.	Temp. C°	NUTRIENT AGAR A	C	DS	P	N	BLOOD AGAR A	C	DS	P	N
No. 1	37	R	C-C	DR	Z-G	DR					
	30	R	C-c	DR	Z-G	DR					
No. 2	37	R	C	DR	Z-G	DR					
	30	R	C-c	DR	Z-G	DR					
No. 3	37	Z-G	C-c	DR	Z-G	DR	R	C-c	Z-F	Z-G	R
	30	Z-G	Z-F	Z-F	Z-F	Z-F	R	Z-F	Z-F	Z-F	Z-F
No. 4	37	Z-G	C-c	DR	Z-F	R	R	C-c	DR	Z-G	Z-F
	30	Z-G	C-c	Z-F	Z-G	Z-F	R	Z-F	DR	Z-G	Z-F

For explanation of symbols, see page 32.

TABLE III

STANDARD SENSITIVITIES - PROTEUS VULGARIS

Cult. No.	Aureomycin		Chloromycetin		Dihydrostreptomycin		Neomycin		Tetracycline		Terramycin	
	24 hr	48hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
3701	R	R*	C	DR	DR	DR	C	C	Z-G	Z-G	R	R
3600	Z-G	Z-G	C	DR	DR	DR	DR	DR	Z-G	Z-G	R	R*
3599	Z-G	Z-G	DR	DR	DR	DR	DR	DR	Z-G	Z-G	Z-G	Z-G
3566	Z-G	Z-G	C	C	DR	DR	C	C	Z-G	Z-G	R	R
3488	DR	DR	C	C	C-c	C-c	C	C	DR	DR	R	R*
3363	R	R*	C	DR	DR	DR	C	C	DR	DR	R	R*
3260	Z-G	Z-G	C	DR	C	C	C	C	Z-G	Z-G	R	R
2389	DR	DR	C	C	C	C	C	C	Z-F	Z-F	Z-G	Z-G
1298	Z-G	Z-G	C	C	DR	DR	C	C	DR	DR	DR	DR
851	DR	DR	C	C	DR	DR	C	C	DR	DR	Z-G	Z-G
847	Z-G	Z-G	DR	DR	C	C	C	C	C	C	Z-G	Z-G
549	DR	DR	DR	DR	DR	DR	DR	DR	DR	DR	Z-G	Z-G
502	DR	DR	DR	DR	DR	DR	DR	DR	DR	DR	Z-G	Z-G
501	DR	DR	C	C	DR	DR	C	C	DR	DR	DR	DR
492	DR	DR	DR	DR	DR	DR	DR	DR	DR	DR	Z-G	Z-G
235	DR	DR	C	C	DR	DR	C	DR	C	C	DR	DR

* Possible heavy overgrowth of small zone of inhibition.

For explanation of symbols, see page 32

TABLE IV

STANDARD SENSITIVITIES - PROTEUS MIRABILIS

Cult. No.	Chloromycetin 24 hr	Chloromycetin 48 hr	Dihydrostreptomycin 24 hr	Dihydrostreptomycin 48 hr	Neomycin 24 hr	Neomycin 48 hr	Penicillin 24 hr	Penicillin 48 hr	Tetracycline 24 hr	Tetracycline 48 hr
3801	C	C	DR	DR	DR	DR-F	C	C-c	C	C-c
3797	C-c	Z-F	DR	DR-F	C	Z-F	C-c	Z-G	R	R
3770	C-c	C-c	DR	DR-F	Z-F	DR-F	C-c	Z-G	R	R
3765	DR	DR-F	DR	DR	Z-F	Z-F	C-c	C-c	R	R*
3732	C-c	C-c	DR	DR-F	DR	DR	C-c	DR-G	R	R
3727	C-c	Z-G	DR	DR-F	Z-F	Z-F	DR	Z-G	R	R
3725	C-c	Z-F	DR	DR	Z-F	DR-F	C-c	Z-F	R	R*
3722	C-c	Z-F-c	DR	DR-F	Z-F	DR-F	C-c	Z-F	C	C
3718	C-c	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	C-c	R	R
3672	DR	DR-F	R	R	Z-F	Z-F	C-c	DR-F	Z-G	Z-G
3671	C-c	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	C-c	R	R
3670	C-c	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	Z-F	R	R*
3661	Z-F	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	Z-G	R	R
3630	Z-F	Z-F	Z-F	DR-F	Z-F	Z-F	C-c	Z-G	Z-G	Z-G
3552	Z-F	Z-F	Z-F	DR-F	Z-F	Z-F	Z-F	Z-F	R	R*
3544	Z-F	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	C-c	R	R
3543	Z-F	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	C-c	R	R
2382	Z-F	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	C-c	Z-G	Z-G
2349	Z-F	Z-F-c	Z-F	DR-F	Z-F	Z-F	C-c	C-c	R	R
927	Z-F	Z-F	Z-F	DR-F	Z-F	Z-F	Z-F	Z-F	Z-F	Z-F
859	C-c	C-c	Z-F	DR-F	Z-F	DR-F	Z-F	DR-F	Z-G	Z-G
856	C	C	C	C	Z-F	DR-F	C	C	R	R*
172	C-c	C-c	Z-F	DR-F	Z-F	DR-F	Z-F	DR-F	Z-G	Z-F

* Possible heavy overgrowth of small zone of inhibition.

For explanation of symbols, see page 32

TABLE V

SUB-CULTURE OF INNER GROWTH:

SWARMING ON BLOOD AGAR AND ZONE FORMATION WITH SAME ANTIBIOTIC:

STRAINS OF PROTEUS VULGARIS

Cult. No.	From Aureomycin		From Chloromycetin		From Dihydrostreptomycin		From Neomycin		From Tetracycline		From Terramycin	
	Swarm	Zone	Swarm	Zone	Swarm	Zone	Swarm	Zone	Swarm	Zone	Swarm	Zone
3701			+	DR	0				+	DR		
3600	+	Z-F	+	Z-F	-	C	-	DR	+	Z-F		
3599	+	Z-F	+	Z-F	+	Z-F	-	DR	+	Z-F	+	Z-F
3555	+	Z-F			0				+	C		
3488	+	Z-F			-	R			+	Z-F		
3353			+	Z-F	0				+	Z-F		
3260			+	Z-F								
2389	+	Z-F							+	DR	+	Z-F
1298	+	Z-F			0				+	Z-F	+	Z-F
851					+	DR			+	C	+	DR
847			+	Z-F							+	Z-F
549					0		-	C			-	DR
502					0		-	DR			+	Z-F
501					0						+	DR
492					0		+	DR			+	Z-F
235					+	R	+	Z-F			+	Z-F

\+ Swarming on Blood Agar within 24 hrs.

\- No swarming on Blood Agar within 24 hrs.

0 No growth on Blood Agar within 48 hrs.

For explanation of other symbols, see page 32

TABLE VI PART 1

BACTERIOLOGICAL CHARACTERISTICS OF SUB-CULTURES FROM THE REDUCED GROWTH ZONES OF STANDARD SENSITIVITY TESTS.

PROTEUS VULGARIS

Culture No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		REACTIONS ON VARIOUS MEDIA																	
3701	F	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	C	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
	Te	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
3600	F	-	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	A	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	C	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	DS	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
	Te	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	N	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
3599	F	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	S	-	o	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	A	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	C	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	DS	-	-	+*	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	Te	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	N	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	T	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
3555	F	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	A	-	-	+*	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	Te	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+

TABLE VI PART 2

BACTERIOLOGICAL CHARACTERISTICS OF SUB-CULTURES FROM THE REDUCED GROWTH ZONES OF STANDARD SENSITIVITY TESTS

PROTEUS VULGARIS

Culture No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		REACTIONS ON VARIOUS MEDIA																	
3488	F	-	+	+*	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	S	-	o	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
	A	-	-	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+
	DS	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
	Te	-	-	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+
3353	F	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	C	-	-	+*	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	Te	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
3260	F	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
	C	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
2389	F	-	+	+	-	-	+	-	+	+	+	+	+	-	-	+	-	+	+
	S	-	o	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+
	A	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+
	Te	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+
	T	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+
1298	F	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	A	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	Te	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	T	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+

TABLE VI PART 3

BACTERIOLOGICAL CHARACTERISTICS OF SUB-CULTURES FROM THE REDUCED GROWTH ZONES OF STANDARD SENSITIVITY TESTS

PROTEUS VULGARIS

Culture No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		REACTIONS ON VARIOUS MEDIA																	
851	F	-	+	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	S	-	o	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	DS	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	Te	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	T	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
847	F	-	+	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	S	-	o	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+
	C	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	T	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
549	F	-	o	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
	N	-	-	+	-	-	+	-	-	+	+	+	-	+	-	-	-	+	+
	T	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
502	F	-	o	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	S	-	o	+*	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	N	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	T	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
501	F	-	o	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	o	+*	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+
	T	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	+

TABLE VI PART 4

BACTERIOLOGICAL CHARACTERISTICS OF SUB-CULTURES FROM THE REDUCED GROWTH ZONES OF STANDARD SENSITIVITY TESTS

PROTEUS VULGARIS

Culture No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		REACTIONS ON VARIOUS MEDIA																	
492	F	-	O	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	S	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+
	N	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
	T	-	-	+	-	-	+	-	-	+	+	+	+	+	-	-	-	+	+
255	F	-	O	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+
	S	-	O	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+
	DS	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	N	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+
	T	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+	+

F: Original department stock culture reactions
S: Reactions of inoculum used for standard sensitivity tests
A, C, DS, N, Te, T: Reactions of sub-cultures from reduced growth zones formed by each antibiotic
+*: Anaerogenic
O: No growth

1. Acid from Adonitol
2. Acid from Aesculin
3. Acid and Gas from Glucose
4. Acid from 10% Lactose slant
5. Acid from 0.5% Lactose
6. Acid from Maltose
7. Acid from Mannitol
8. Acid from Salicin
9. Acid from Sucrose
10. Acid from Xylose
11. Urea hydrolyzed
12. Indole produced
13. Methyl Red positive
14. Acetyl-methyl-carbinol produced
15. Citrate utilized
16. Gelatin liquified
17. Motility positive in semi-solid media
18. H_2S produced

TABLE VII

SWARMING ON BILE SALT MEDIA—PROTEUS VULGARIS

Cult. No.	24 HRS				48 HRS				1 WEEK			
	B	D	DC	M	B	D	DC	M	B	D	DC	M
3701	+s	-	-	+s	+	-	-	+	+	-	-	+
3600	+	-	-	+	+	-	-	+	+	-	-	+
3599	+	-	-	+	+	-	-	+	+	-	-	+
3555	+	-	-	+s	+	-	-	+	+	-	-	+
3488	+	-	-	+s	+	-	-	+	+	-	-	+
3353	+	-	-	+s	+	-	-	+	+	-	-	+
3260	+	-	-	+	+	-	-	+	+	-	-	+
2389	+	-	-	+	+	-	-	+	+	-	-	+
1298	+	-	-	+	+	-	-	+	+	-	-	+
851	+	-	-	+	+	-	-	+	+	-	-	+
847	+	-	-	+s	+	-	-	+	+	-	-	+
549	-	-	-	-	+	-	-	+	+	-	-	+
502	+	-	-	+	+	-	-	+	+	-	-	+
501	+	-	-	+s	+	-	-	+	+	-	-	+
492	-	-	-	-	+	-	-	+	+	-	-	+
235	+	-	-	+	+	-	-	+	+	-	-	+

B: Blood Agar Control
D: Desoxycholate Agar
DC: Desoxycholate-Citrate Agar
M: Mac Conkey Agar

-: No swarming
+: Swarming
+s: Organisms beginning to swarm out from center streak but not to much distance

TABLE VIII PART 1

ZONE FORMATION ON AGAR CONTAINING CERTAIN BILE SALTS

Cult. No.	BLOOD AGAR CONTROL						DESOXYCHOLATE AGAR					
	A	C	DS	N	Te	T	A	C	DS	N	Te	T
3701		C DR	DR		Z-G			C	DR		R	
3600	Z-G	DR	DR	C DR	Z-G		R	C	DR	DR	R	
3555	Z-G		DR		Z-G		R		DR		R	
3488	DR		DR-c		DR		Z-G		DR		Z-G	
3353		C DR	DR		Z-G			C DR	DR		R	
2389	DR				C	DR	C DR				Z-G	Z-G
1298	Z-F		DR		DR	DR	DR		DR		DR	DR
847	DR	C DR				DR	DR	DR				Z-G
501	DR		DR-F		DR	DR	DR		DR		R	DR
492	DR	C DR	DR	DR	DR	DR	O	O	O	O	O	O

O: Media too inhibitory growth

48 hr. readings given on second line if different from 24 hr.

For explanation of other symbols, see page 32.

TABLE VIII PART 2

ZONE FORMATION ON AGAR CONTAINING CERTAIN BILE SALTS

Cult. No.	MAC CONKEY AGAR						DESOXY-CITRATE AGAR					
	A	C	DS	N	Te	T	A	C	DS	N	Te	T
3701		C DR	DR		R			C	DR		Z-G	
3600	R	C DR	DR	DR	R		R	C	DR	C	R	
3565	R		DR		Z-G		R		DR		R	
3488	Z-G		DR		C		C		DR		C	
3353		C DR	DR		R			C	C		Z-G	
2389	DR				DR	DR	O				O	O
1298	O		O		O	O	O		O		O	O
847	DR	DR				Z-G	O	O				O
501	DR-G		DR		DR	DR-G	O		O		O	O
492	DR-G	DR	DR	DR	DR	DR	O	O	O	O	O	O

O: Media too inhibitory no growth

48 hr. readings given on second line if different from 24 hr.

For explanation of other symbols, see page 32.

TABLE IX PART 1

EXPLANATION OF SYMBOLS

Column 1: Appearance of zones in Standard sensitivity tests.

Explanation of symbols given on page 32

Column 2: Appearance of zones in Swarming sensitivity tests.

C: Organism appears sensitive to antibiotic, clear zone formed.

R: Organism appears resistant to antibiotic, growth advanced up to or beyond disc.

L: Thin film of growth between normal swarm and antibiotic disc.

Z-F: Complete inhibition zone formed around disc by initial swarm; zone partially or completely filled in by thin film over-growth.

DR: Partial or complete double-zone formation.

DR-F: Partial or complete double-zone formation, plus a film of growth within the inhibition zone.

O: Organism did not swarm within 48 hrs.

Fourty Eight hrs. reading on second line if different from 24 hr.

TABLE IX PART 2

COMPARISON OF ZONE FORMATIONS

(1) STANDARD METHOD — (2) SWARMING METHOD

PROTEUS VULGARIS

Cult. No.	Auromycin 1	Auromycin 2	Chloromycetin 1	Chloromycetin 2	Dihydrostreptomycin 1	Dihydrostreptomycin 2	Neomycin 1	Neomycin 2	Tetracycline 1	Tetracycline 2	Terramycin 1	Terramycin 2
3701			C DR	C R	DR	L DR			DR-F	DR-F		
3600			C DR	C R	DR	C DR-F	C	Z-F	Z-G	DR-F		
3699			Z-F	Z-F	DR	DR-F	DR	L Z-F				
3555	R	R			DR	C DR						
3488	DR	DR-F Z-F			DR-c	DR-F Z-F			DR	DR-F Z-F		
3363			C DR	C Z-F	DR	C DR						
3260	Z-G	L	C DR	L DR								
2389	DR	L Z-F										

TABLE IX PART 3

COMPARISON OF ZONE FORMATIONS

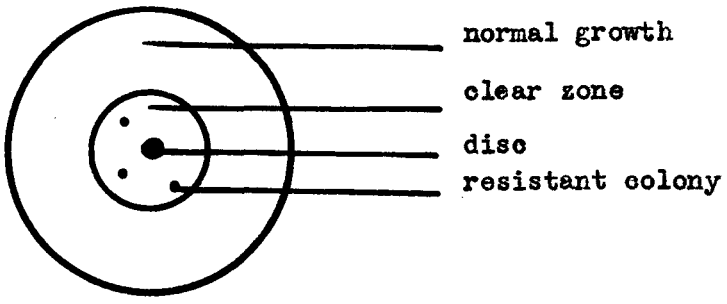
(1) STANDARD METHOD — (2) SWARMING METHOD

PROTEUS VULGARIS

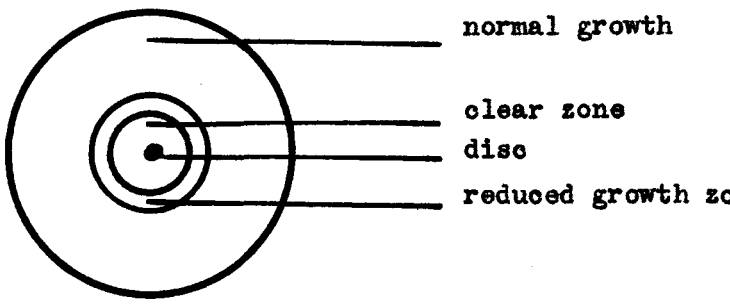
Cult. No.	Aureomycin		Chloromycetin		Dihydrostreptomycin		Neomycin		Tetracycline		Terramycin	
	1	2	1	2	1	2	1	2	1	2	1	2
1298					DR	L			DR	L	DR	Z-F
851	DR	DR-F			DR	DR-F			DR	DR-F		
847			DR-F	L							Z-G	L Z-F
549	DR	O	C	O	DR	O	DR	O	DR	O		
502	DR	DR-F	DR	DR-F	DR	DR			DR	L		
501	DR	DR			DR	DR-F			DR	Z-F DR-F	DR	Z-F
492	DR	L	DR	C	DR	C			DR	C		
235	DR	DR-F Z-F			DR	DR-F Z-F	C	DR-F			DR	Z-F

FIGURE 1
ZONE TYPES—STANDARD SENSITIVITY TESTS

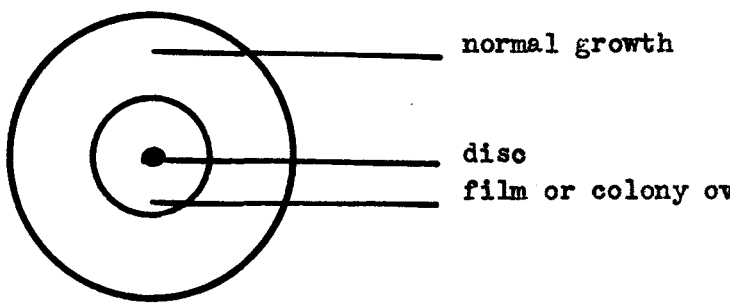
TYPE 1



TYPE 2



TYPE 3



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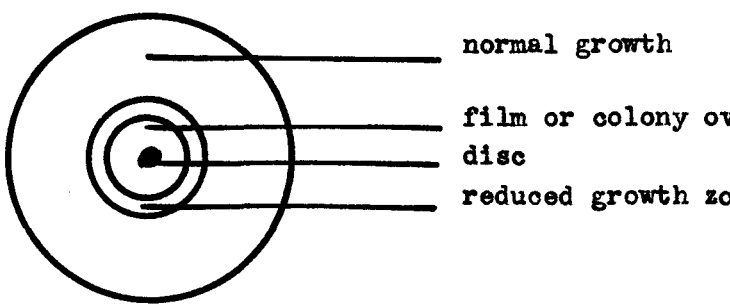
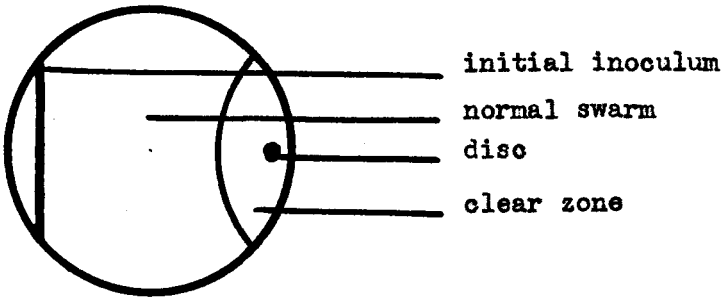


FIGURE 2
ZONE TYPES--SWARMING SENSITIVITY TESTS

TYPE 1



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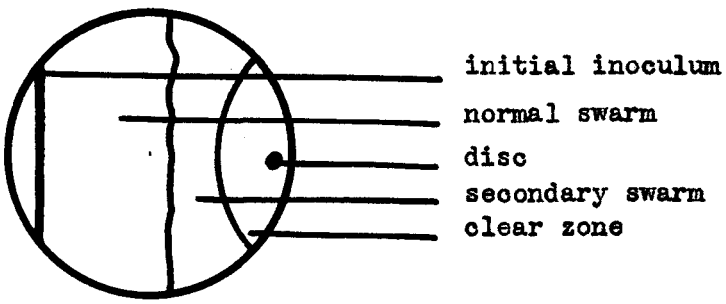

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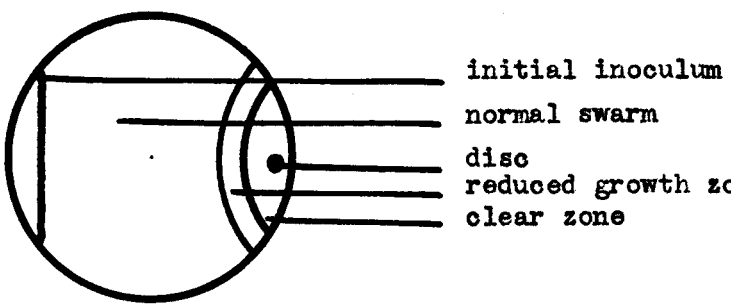

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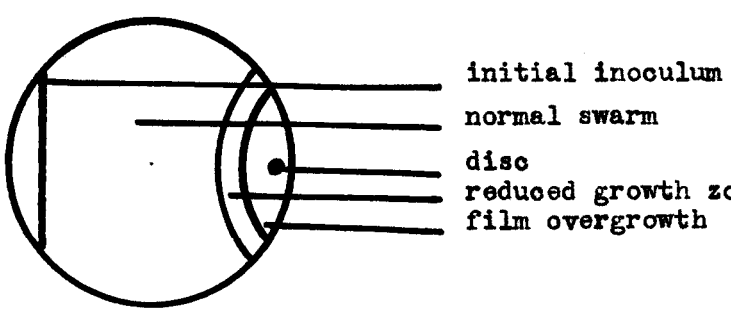

APPROVAL SHEET

The thesis submitted by Roberta Smith has been read and approved by three members of the Department of Microbiology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact than any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 28 1957
Date

McDonald Fulton
Signature of Advisor