SODIUM DIETHYLDITHIOCARBAMATE AND OXINE IN THE DIFFERENTIATION OF BRUCELLA SPECIES

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(With Plates 10–12)

In the course of studies on the mode of action of 2:3-dimercaptopropanol (BAL) on brucellae and on the sulphur metabolism of these organisms, Renoux (1952) made certain observations that led to the development of a test for the differentiation of brucella species. The object of the present work was to examine this test more closely, and in particular to try to determine the basis of the differences observed.

The carbamate test

The best results were obtained with liver agar, which was melted and decanted from the rather heavy sediment that tends to occur in this medium. The plates were inoculated by flooding them with a thick suspension of the brucellae to be tested. The excess inoculum was pipetted off and the plates dried in the incubator. A 1% (w/v) solution of sodium diethyldithiocarbamate in sterile distilled water was prepared. Sterile disks of filter-paper (Whatman no. 42) 9 mm. in diameter were dipped in the solution, drained on the side of the bottle and placed on the centre of the plates. The plates were examined after incubation for 2 days at 37° C. in 10% CO_2.

The most usual appearances produced by brucella strains are shown in Pl. 10, figs. 1*a*, *b*, 2*a*, *b* and 3. These have been regarded as typical of *Brucella melitensis*, *Br. abortus* and *Br. suis* respectively (Renoux, 1952; Pickett, Nelson & Liberman, 1953). Pickett and his colleagues incorporated the carbamate in tablets. The 'typical melitensis' appearance, reading from the centre towards the periphery, comprises a zone of inhibition of growth adjoining the disk, surrounded by a ring of growth of increasing intensity ceasing abruptly 5–6 mm. from the edge of the disk; beyond this, growth is totally inhibited over a zone about 2 mm. wide. The whole effect is that of a target. In the 'typical abortus' appearance, there is a central zone of complete or partial inhibition surrounded by a zone of growth which appears, by transmitted light, bounded peripherally by a narrow brown line and an outer white line; beyond this, there is a narrow zone of inhibition, 1·5–2 mm. in depth. Typical strains of *Br. suis* show a simple clear zone of inhibition 4–6 mm. wide surrounding the carbamate disk.

Behaviour of a series of strains with carbamate

Source of the strains

The strains of *Br. abortus* comprised 9 isolated from cows' milk in this country, 4 isolated by blood culture from cases of undulant fever and 3 typical stock strains,

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including the official standard strain (544) of the World Health Organization. There were also 3 strains isolated from milk, of the type sensitive to all the usual test dyes, and 5 strains that behaved biochemically as *Br. abortus* and serologically as *Br. melitensis*: these were isolated from cows' milk.

The strains of *Br. melitensis* comprised 11 isolated from cows' milk in this country, 2 from blood culture from cases of brucellosis, and 5 stock strains, including W.H.O. strain 16M. There were also 3 strains, isolated by blood culture from patients in Italy, of the type that behaved biochemically as *Br. melitensis* and serologically as *Br. abortus*.

The strains of *Br. suis* comprised 5 stock strains kept in this laboratory, the W.H.O. strain 1330, 4 strains kindly sent by Dr I. F. Huddleson, Michigan State College, U.S.A. (1776B, 1808, 1811, 1814) and a collection of 20 strains kindly sent by Dr M. J. Pickett, University of California, U.S.A. The latter consisted of strains isolated from man or animals, and submitted from various States (California, Texas, Minnesota, Indiana, Georgia, Maryland, Montana, Columbia) and from Rio de Janeiro and Frankfurt.

Carbamate disk test

Tests were made on 25 strains of *Br. abortus*, 21 strains of *Br. melitensis* (including in each instance the atypical strains mentioned above) and 13 strains of *Br. suis*. The distances from the edge of the carbamate disk to the outer limit of the zone of inhibition of growth are summarized in Table 1.

Table 1. *Width of total zone of inhibition produced by different brucella strains in the carbamate test (measured from the disk to the outer limit of inhibition)*

	Millimetres									
	3–	4–	5–	6–	7–	8–	9–	10–	11–	
	Number of strains									Total
Br. abortus	—	—	—	1	5	12	4	2	1	25
Br. melitensis	—	—	—	3	3	4	6	4	1	21
Br. suis	2	5	4	1	1	—	—	—	—	13

All the strains of *Br. suis* produced a simple zone of inhibition around the disk, usually 4·5–5 mm. in width: some strains had zones as narrow as 3 mm. or as wide as 7 mm. There was sometimes slight enhancement of growth beyond the zone of inhibition.

The strains of *Br. melitensis* almost all produced the 'target' appearance described above. The outer zone of inhibition, with most strains, stopped abruptly 6·5–10 mm. from the edge of the disk. The inner zone of growth ended very sharply, usually 5·5–6·5 mm. from the disk, so that the width of the outer zone of inhibition was 1·5–2·5 mm. With a few strains the zones of growth and of inhibition extended a few mm. farther from the disk.

The strains of *Br. abortus* gave a somewhat similar appearance to that of

Br. melitensis. The limit of the outer zone of inhibition was, with most strains, 7·5–9·5 mm. from the disk and differed from that of *Br. melitensis* in being gradual and less defined. The zones of growth mostly stopped 6–6·5 mm. from the disk.

When the plates were observed by transmitted light, the inner zones of growth of *Br. abortus* and *Br. melitensis* appeared to show a narrow white ring at the periphery with a brown ring just inside it. This, as is discussed later, proved to be due to the presence of an underlying precipitate in the medium. The precipitate was absent or much less marked on plates inoculated with *Br. suis*.

The aberrant strains behaved in the same way as the type which they resembled biochemically.

Sensitivity of brucellae to carbamate

It was of interest, especially in view of the curious appearances found in the carbamate disk test, to determine the sensitivity of the various brucella strains to carbamate. Plates of liver agar were prepared, containing a series of successive dilutions of sodium diethyldithiocarbamate. These were ruled into squares and inoculated by spreading a loopful of a heavy suspension in broth, from a 48 hr. liver-agar culture, over a circular area 1·5 cm. in diameter. The plates were incubated for 48 hr. at 37° C. in 10 % CO_2.

Table 2. *Sensitivity of brucellae to carbamate*

(Number of strains inhibited by different concentrations.)

	1/4000	1/6000	1/8000	1/12,000	1/16,000	1/24,000	1/32,000	Total
Br. abortus	1	7	5	2	4	2	3	24
Br. melitensis	3	10	7	2	—	—	—	22
Br. suis	16	3	—	—	—	—	—	19

The results are summarized in Table 2, which shows the number of strains of each species whose growth was inhibited by different concentrations of carbamate. All of the 19 strains of *Br. suis* were inhibited by concentrations of 1/6000 or 1/4000. The inhibitory concentration for 22 strains of *Br. melitensis* was between 1/4000 and 1/12,000; none of the strains were inhibited by dilutions greater than 1/12,000. The 24 strains of *Br. abortus* had a wider range of sensitivity, scattered between 1/4000 and 1/32,000; 9 of the strains were inhibited by dilutions higher than 1/12,000.

Chelating effects of carbamate

The inhibitory action of certain compounds on bacteria has been attributed to their activity as chelating agents in rendering traces of essential metals unavailable for bacterial metabolism. Albert, Rubbo, Goldacre & Balfour (1947) studied a large number of chelating agents, including sodium diethyldithiocarbamate, recorded their antibacterial activity against a range of organisms, and found that the inhibitory effect in culture could be reversed by the addition of certain metallic ions. Carbamate chelated strongly with Mn, Zn, Co, Cd, Cu and Pb, and less strongly with Fe. In later work (Rubbo, Albert & Gibson 1950;

Albert, Gibson & Rubbo 1953) it was shown that the action of these chelating agents was not simple. Working with 8-hydroxyquinoline (oxine) and *Staphylococcus aureus*, they found that the action of this agent was dependent on the presence of one or more of three metallic ions (cupric, ferrous, ferric) and that the metal-oxine complexes are the true toxic agents. The antibacterial action of these complexes can be antagonized by trace amounts of cobalt or a large excess of certain other metallic ions, such as ferrous, nickel, zinc and cadmium. The possible mechanism of these antagonisms, based partly on the comparative avidity of the metallic ions for oxine, is fully discussed in the above papers.

For the purposes of the present work, the action of various metals in reversing the antibacterial effect of sodium diethyldithiocarbamate in liver agar was simply demonstrated. Plates were poured containing concentrations of carbamate that were just completely, or almost completely, inhibitory for the brucella species concerned. The plates were inoculated by flooding them with a thick suspension of the organism and pipetting off the excess fluid. They were then dried in the incubator and filter-paper disks, dipped in 1 %-solutions in distilled water of ferrous sulphate, copper sulphate, manganese sulphate, magnesium sulphate, zinc sulphate and cobalt chloride, were placed on different sections of each plate. The plates were inspected after incubation for 2 and 4 days at 37° C.

With plates containing an inhibitory concentration of carbamate, reversal of inhibition was shown by such appearances as are seen in Pl. 11, fig. 5. A wide zone of bacterial growth around one disk (cobalt) and a narrower zone around another (copper) indicate reversal by these two metals. Where the concentration of carbamate was not quite inhibitory, appearances such as that shown in Pl. 11, fig. 6, were observed. The organism in this instance is *Br. suis* and zones of inhibition of growth, due to toxicity of the metal, and of enhanced growth, due to the reversal effect, may be clearly seen. Reading from the top of the plate in a clockwise direction, the plate shows: Fe (no effect), Cu (inhibition surrounded by zone of enhancement), Mn (enhancement), Mg (no effect), Co (slight inhibition and marked enhancement), Zn (marked inhibition). The inner white ring round the Cu disk is a zone of precipitation in the medium.

The conclusion drawn from a considerable number of tests was that, with all strains of each of the three species examined, reversal of inhibition by carbamate was obtained with cobalt, copper and zinc and less strongly with iron and manganese. The effect was more readily observed with *Br. suis*, which grows more quickly than *Br. abortus* or *Br. melitensis*.

The target or zone effect

The total width of the zones of inhibition of the brucella species round the carbamate disk was found to accord with the sensitivity of the organism as determined by dilution methods, but an explanation was sought to account for the inner ring of vigorous growth with *Br. melitensis* and *Br. abortus*. This was found to be related to the presence or absence of a precipitate in the underlying medium. On an uninoculated plate, the disk is surrounded by a whitish zone of precipitation

(Pl. 10, fig. 4) which is faint near the disk but appears, about 5 mm. from the edge of the disk, as a dense ring, white internally and brown peripherally.

On plates sown with *Br. melitensis* and *Br. abortus* this zone of precipitation is seen, and its colour probably accounts for the white and brown rings noted with *Br. abortus* by Renoux (1952). Further, the inner zone of growth of these two organisms, specially *Br. melitensis*, is found to correspond exactly with the area of precipitation; the inhibitory agent is clearly not effective in this area. In plates inoculated with *Br. suis* there is little or no precipitate, and inhibition is observed in the zone surrounding the disk. This absence of precipitate can be well seen in gradient plates made as described by Szybalski & Bryson (1952). On the side of the plate containing the high concentration of carbamate there is a dense precipitate in the medium, but this is cleared around colonies of *Br. suis* growing on the surface of the plate, and not around colonies of the other two species.

The nature of the precipitate was discussed with Prof. Adrian Albert, who suggested that the sodium diethyldithiocarbamate might be oxidized to tetraethylthiuram disulphide which is insoluble in water. Its presence or absence might thus be related to the reducing activity of the organism under test. It is therefore of interest to note the observations of Tuttle & Huddleson (1934) who, during a study of the mechanism of the selective action of dyes, determined the oxidation-reduction potentials associated with the growth of the three brucella species in liver infusion broth. They found that *Br. suis* consistently produced a lower Eh than *Br. abortus* and *Br. melitensis*. The formation of the inactive precipitate would thus be less likely to occur with that species.

In the course of the present work the behaviour in the carbamate test of a few organisms of other genera was examined. Strains of *Bacterium coli* showed a wide clear zone of inhibition round the disk (Pl. 12 , fig. 7), whereas *Bacillus subtilis* (Pl. 12, fig. 8), *Corynebacterium diphtheriae* and *Staph. aureus* all showed the target appearance. The electrode potentials of these and other species growing in different media have been studied by various workers. Their results have been summarized by Hewitt (1950). Although figures for the different species can be properly compared only when the organisms are growing in the same cultural conditions, it is perhaps worth noting that an intense reducing activity is uniformly recorded for *Bact. coli* and not for *B. subtilis*.

The sensitivity of brucellae to oxine

In view of the above results, it was of interest to determine the sensitivity of brucella species to another commonly used chelating agent, 8-hydroxyquinoline (oxine), which has been studied in detail by Rubbo *et al.* (1950) and Albert *et al.* (1953). They found that it chelated strongly at pH 7·3 with Mn, Zn, Cd, Co, Pb and Cu, and less strongly with Fe. In general, Gram-positive bacteria were highly sensitive to oxine and the effect was reversible by cobalt. Gram-negative bacteria were much more resistant, and the antibacterial action was reversed by zinc and iron. However, *Haemophilus pertussis* and *Neisseria meningitidis* were highly sensitive, and *Br. abortus* was much more sensitive than most other Gram-

negative bacteria. The mechanism of action of oxine has been mentioned above and is fully discussed by Albert and his colleagues, and by Gale & Mitchell (1949).

Observations on the effect of oxine on brucellae

Strains of the three brucella species were examined by the same methods as were used with carbamate, and the results can be briefly summarized.

In liver agar, copper and zinc were effective in promoting the growth of all three brucella species in the presence of inhibitory concentrations of oxine. A small effect was noted with cobalt and iron, specially for *Br. suis*.

Strong concentrations of oxine can only be made in acid solution so disk tests with 1/200 solutions are of limited value. Strains of *Br. suis* were inhibited for distances from 1 to 3 mm. round the disk, those of *Br. abortus* for 3–6 mm., and those of *Br. melitensis* for 4–9 mm. No precipitation or zone effects were noted.

The sensitivity of 22 strains of *Br. abortus*, 20 strains of *Br. melitensis* and 28 strains of *Br. suis* was tested in the same way as with carbamate, loopfuls of thick suspensions of the organisms being spread over an area of 1 cm. diameter on liver agar plates containing concentrations of oxine from 1/8000 to 1/256,000. The concentration inhibiting growth was noted after 3 days' incubation at 37° C. in 10 % CO_2. The results are summarized in Table 3. The strains of *Br. abortus* were inhibited by concentrations of oxine within the range 1/64,000 to 1/192,000, the *Br. melitensis* strains within the range 1/64,000 to 1/256,000 and the *Br. suis* strains at 1/32,000, with the exception of two strains both inhibited at 1/48,000.

Table 3. *Sensitivity of brucellae to oxine*

(Number of strains inhibited by different concentrations.)

	1/32,000	1/48,000	1/64,000	1/96,000	1/128,000	1/192,000	1/256,000	Total
Br. abortus	—	—	8	7	3	4	—	22
Br. melitensis	—	—	5	10	1	3	1	20
Br. suis	26	2	—	—	—	—	—	28

The high resistance of *Br. suis* to oxine was regularly observed with strains derived from widely scattered sources, and could be used as an additional test for identification of this species. Plates from one experiment are shown in Pl. 12, fig. 9. The control plates on the right, without oxine, show the 28 available strains of *Br. suis* and 8 strains each of *Br. abortus* and *Br. melitensis*. On those on the left, which contain oxine in a concentration of 1/32,000 the *Br. abortus* and *Br. melitensis* strains are completely suppressed and the *Br. suis* strains are growing. From tests made with different basic media, such as Albimi and Tryptose agar, it is clear that the actual critical dilution of oxine for any medium must be ascertained by trial.

DISCUSSION

The appearances produced by *Br. abortus*, *Br. melitensis* and *Br. suis* in the carbamate test are sufficiently distinct and consistent as to make the test of some value in the differentiation of the species. The test appears to be based simply on the

different sensitivity of the organisms to carbamate, which acts as a chelating agent. *Br. melitensis* and *Br. abortus*, being more sensitive than *Br. suis*, produce a wider zone of inhibition, and they grow freely in the inner zone, probably because the sodium diethyldithiocarbamate is rendered ineffective by oxidation to the insoluble tetraethylthiuram disulphide. The greater reducing activity of *Br. suis* prevents this oxidation, and a simple zone of inhibition results. As with the other differential characters of the brucellae, there is no doubt a gradation of sensitivity so that types with intermediate degrees of resistance exist.

The same pattern of sensitivity of the brucellae is seen with oxine, and here also the main difference is between *Br. suis* and the other two species. With the considerable number of strains examined, this difference was sufficiently distinct to have some value in identification, since all the *Br. suis* strains resisted oxine in a concentration of 1/48,000 (in the liver agar medium used) and all the members of the other two species tested were inhibited.

SUMMARY

The effect of two chelating agents, sodium diethyldithiocarbamate and 8-hydroxyquinoline (oxine), on 70 strains of brucellae (24 *Br. abortus*, 22 *Br. melitensis* and 28 *Br. suis*) has been examined.

The pattern of growth and inhibition of the three species as it appears in the carbamate test described by Renoux is of some value in the differentiation of species. *Br. suis* is more resistant to carbamate than the other two species.

The three species also differ in their sensitivity to oxine, and strains of *Br. suis* from various sources were consistently more resistant than the other species. This observation may be of use in the identification of *Br. suis*.

My thanks are due to Prof. Adrian Albert and Dr J. H. Marshall for helpful discussion of the chemical aspects of this work, and to Mr B. Madge, A.I.M.L.T., for technical assistance.

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EXPLANATION OF PLATES 10—12

PLATE 10

Figs.1–4. Appearance of brucellae in the carbamate disk test.

Figs. 1*a*, *b*. *Br. melitensis*.
Figs. 2*a*, *b*. *Br. abortus*.
Fig. 3. *Br. suis*.
Fig. 4. Uninoculated plate.

PLATE 11

Figs. 5, 6. Reversal of chelation on carbamate plates.

Fig. 5. *Br. suis* on a liver agar plate containing an inhibitory concentration of sodium-diethyldithiocarbamate.
Fig. 6. *Br. suis* on a liver agar plate containing a concentration of sodium diethyldithiocarbamate not quite inhibitory.
The disks were impregnated with the metal salts indicated.

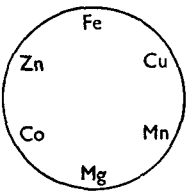
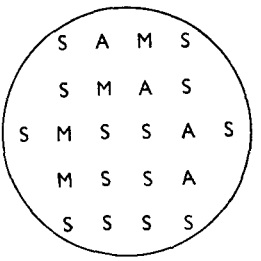


PLATE 12

Fig. 7. Carbamate disk test with *Bact. coli*.
Fig. 8. Carbamate disc test with *B. subtilis*.
Fig. 9. Selective action of oxine for *Br. suis*.
The two liver agar plates on the right contain no oxine. Those on the left contain 1/32,000 oxine. In each pair of plates, the location of the different brucella strains is as indicated below, the test comprising in all 8 strains of *Br. abortus*, 8 of *Br. melitensis* and 28 of *Br. suis*.



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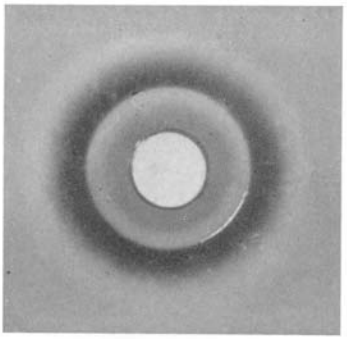

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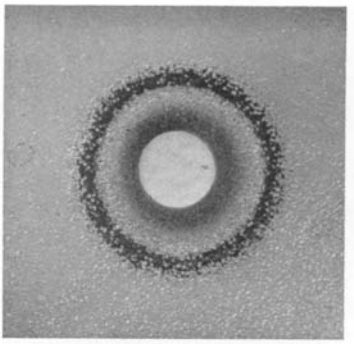
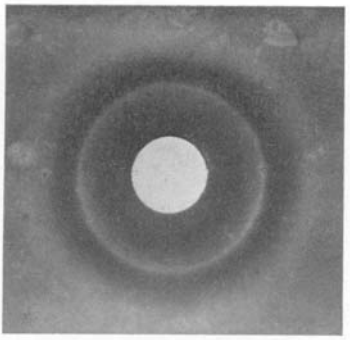
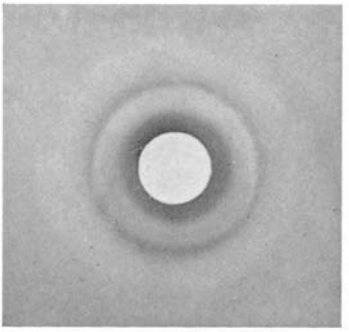


1*b*



2*a*



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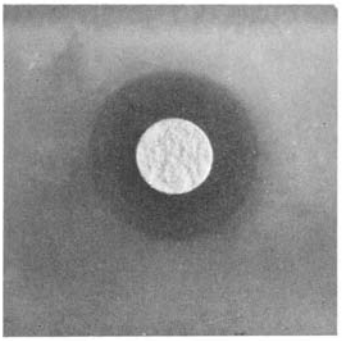

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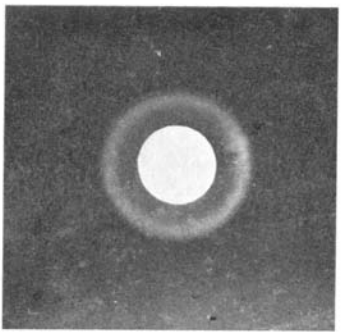

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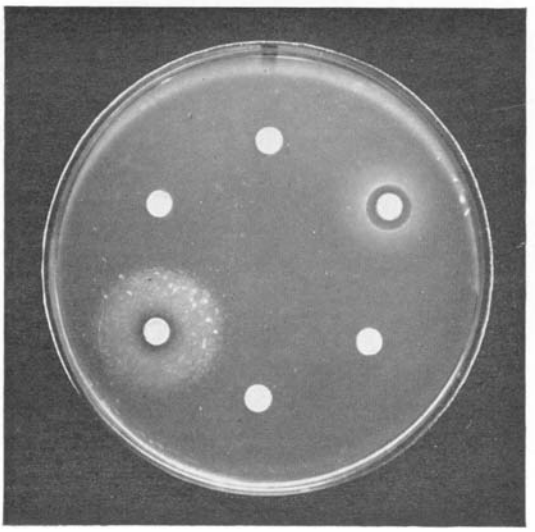

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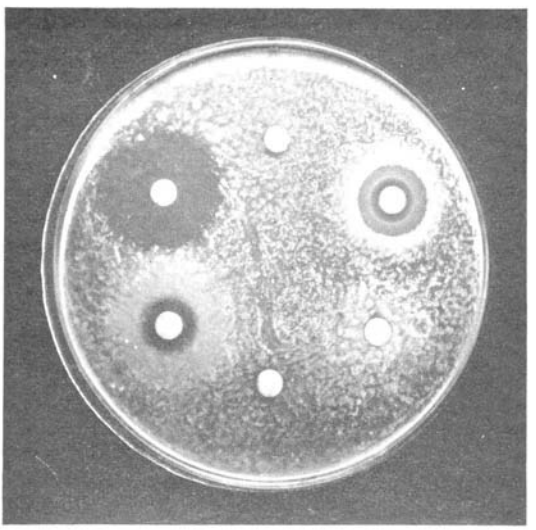

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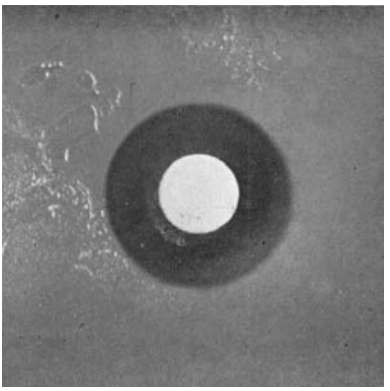

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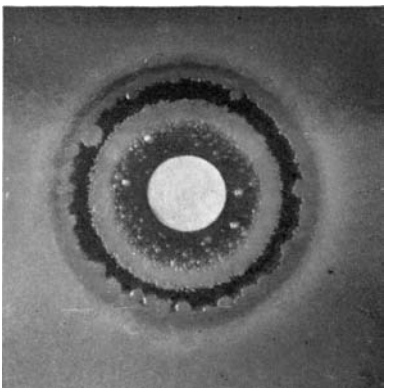

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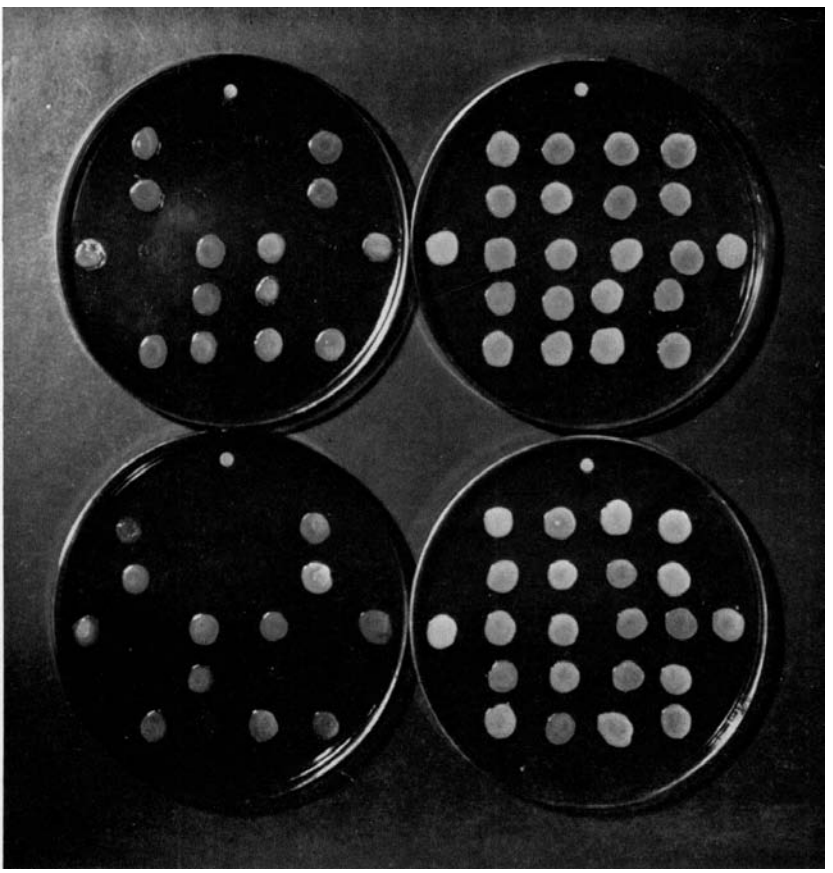

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