

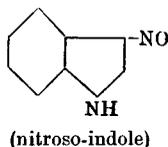
ON THE INHIBITION OF THE CHOLERA-RED REACTION BY CERTAIN NITRITE-DESTROYING ORGANISMS AND ON THE MUTUAL INHIBITION OF *B. DYSENTERIAE* (FLEXNER) AND *V. CHOLERAE* WHEN GROWN TOGETHER.

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TOBEY (1908) has published a brief account of a research which he undertook with a view to determining what contaminating organisms interfere with the cholera-red reaction. He found that "four races of *B. coli communis*, *B. pyogenes fetidus*, *B. acidi lactici*, *B. mucosus capsulatus* and *B. enteritidis* Gaertner always prevented the reaction," while *B. typhosus*, *B. faecalis alkaligenes*, *B. icteroides*, *B. dysenteriae* (Flexner) and certain spirilla (Milleri, Deneke, Finkler-Prior) did not affect it. These results have been for the most part confirmed by the present author, and the experiments which follow were undertaken to explain them.

As Poehl (1886), Bujwid (1888), Dunham (1888) and others have shown a 8-48 hours' growth of *V. cholerae* in 1 per cent. peptone water gives on the addition of a few drops of strong sulphuric acid a pink or "Burgundy wine red" colour. This, as was proved by the researches of Briege (1887), Salkowski (1887), and Petri (1889), is due to the presence in the culture of both indole and nitrites and to the consequent formation of "nitroso-indole" on the addition of any acid capable of



liberating nitrous acid from its compounds. Obviously the absence of either indole or nitrites is sufficient to abolish the reaction.

In the case of such a mixed growth as that of *B. coli* and *V. cholerae*, indole is not likely to be absent since both organisms produce it. On the other hand nitrites may be absent since *B. coli*, *B. enteriditis* Gaertner, and a large number of other organisms destroy nitrites. This may be shown to be the cause of the failure of the cholera-red reaction in mixed growths in several ways. In the first place, two similar amounts of the same medium may be inoculated with both *B. coli* and *V. cholerae*, and after these cultures have been incubated for 24 hours at 37° C., a small quantity of nitrite may be added to one of them, and then both may be at once tested with sulphuric acid. It will be found that the culture to which nitrite has been added gives the nitrite reaction while the other does not. By a somewhat different method the following experiment proves the same point. Nine tubes were taken, each containing 5 c.c. peptone water (peptone 1 per cent., sodium chloride 0.5 per cent.) and of these, three were inoculated with *B. coli* alone, three with *V. cholerae* alone, and three with both *B. coli* and *V. cholerae* together. The nine cultures were placed in the same incubator for 24 hours at 37° C. and at the end of that period one tube of each set was tested for nitrite, one for indole and one with sulphuric acid for the cholera-red reaction.

The test used for nitrite was that with  $\alpha$ -naphthylamine-acetate and sulphanilic acid, for indole Ehrlich's reagent, p-dimethyl-amido-benzaldehyde, was applied according to Boehme's method, and the cholera-red reaction was tested for in the usual way by adding to the culture a few drops of strong nitrite-free sulphuric acid. The results are presented in the following table :

*Results of testing for*

	Nitrite	Indole	Cholera-red
<i>B. coli</i>	-	+	-
<i>V. cholerae</i>	+	+	+
<i>B. coli</i> + <i>V. cholerae</i>	-	+	-

It is evident from these results that in the mixed growth as in the pure *B. coli* culture the failure to give a cholera-red reaction is due to the absence of nitrite. That the absence of nitrite is not due to its non-formation may be proved by taking samples of the mixed growth at various stages and testing them for nitrites by means of the delicate test with sulphanilic acid and  $\alpha$ -naphthylamine-acetate. At first the

samples, like similar ones from pure growths of *B. coli* or *V. cholerae*, show no development of nitrite, but after a few hours nitrites appear in the culture and steadily increase in amount till a strong reaction is given. The amount of nitrite then rapidly diminishes until finally the culture is nitrite-free. In the case of a mixed growth of *B. coli* and *V. cholerae* the disappearance of nitrite occurs in from 15 to 24 hours.

That many micro-organisms destroy nitrites has of course been known for some considerable time. Not only do certain "de-nitrifying" organisms occur in the soil, where they are of some interest from an agricultural point of view, though their denitrifying power seems to be less in the soil than in fluid cultures, but many pathogenic and other organisms possess the same power. Maassen (1901) investigated 109 organisms with respect to their action on nitrites and nitrates, and of these he found that 85 reduced nitrates to nitrites while only 50 reduced nitrites to ammonia. Many of the organisms which reduced nitrates to nitrites failed to destroy nitrites, while some that could destroy nitrites failed to reduce nitrates. *V. cholerae* of course belongs to the group which reduces nitrates to nitrites but fails to reduce nitrites. The present author (Logie, 1910) investigated fourteen strains of dysentery bacilli and found that of these all but one reduced nitrates to nitrites while only seven destroyed nitrites. The five "Shiga" strains examined failed to reduce nitrites and one of them (obtained from Prof. Neisser) failed even to reduce nitrates. Of the nine mannite-fermenting strains, only two (*B. dysenteriae* Jürgens and one of the Neisser strains) failed to destroy nitrites while all attacked nitrates. *B. dysenteriae* (Jürgens) is remarkable inasmuch as it forms both nitrites and indole and consequently gives the cholera-red reaction.

Since *B. coli communis* and *B. enteritidis* Gaertner possess the power of destroying nitrites it is not remarkable that when grown with *V. cholerae* in peptone water they abolish the cholera-red reaction; but since *B. typhosus*, *B. paratyphosus* (A and B) and many strains of *B. dysenteriae* (Flexner) also possess the power of destroying nitrites, it is surprising to find that they do not likewise abolish the cholera-red reaction. *B. dysenteriae* (Flexner) when grown in peptone water in pure culture reduces all the nitrate naturally present to nitrite and destroys the nitrite in less than 24 hours; yet if it is grown along with *V. cholerae* a cholera-red reaction may still be obtained in a 24 hours' growth. This can only be explained on the supposition that the action of *B. dysenteriae* (Flexner) is in some way inhibited by the presence of *V. cholerae*. As a matter of fact the author has found in

repeated experiments that the total number of both organisms in a mixed growth of *B. dysenteriae* (Flexner) and *V. cholerae* is much less than the number of organisms in the same quantity of a pure *B. dysenteriae* (Flexner) culture. This is strikingly shown in the following experiment.

A series of test-tubes, each containing 5 c.c. of peptone water (peptone 1 per cent., sodium chloride 0.5 per cent.) were prepared in the usual way and of these two were inoculated, the one with *B. dysenteriae* (Flexner), the other with *V. cholerae*. From these after 24 hours' incubation at 37° C., three other tubes were inoculated, one with a loopful of *B. dysenteriae* (Flexner), one with a loopful of *V. cholerae*, and one with a loopful of *B. dysenteriae* and also a loopful of *V. cholerae*. This last mentioned test-tube should therefore have contained to begin with as many organisms as the other two together; at all events it should have had more than either singly. After incubation for 24 hours at 37° C. these cultures were plated on Endo-agar in a dilution of one to ten thousand. The plates were made by pouring sterile Endo-agar into sterile Petri dishes (of 4½ inches diameter) and allowing the agar to cool under sterile filter paper till a firm moistureless surface was produced. This usually occurred in a few hours.

The measured quantity (0.025 c.c.) of diluted culture was discharged from a sterile pipette upon the surface of the agar and smeared by means of a sterile platinum needle over the whole available area. To avoid injury to the organisms from possible contact with the hot wire, the platinum wire was plunged into cold sterile water immediately after sterilisation. The plates dried rapidly so that the lids could be replaced and the capsules inverted as soon as the manipulations were complete. The colonies thus obtained were, of course, all surface growths. The following table gives the counts of six plates (two from each of the three cultures) after they had been incubated at 37° C. for 48 hours:

	<i>B. dys.</i> (Flexner)	<i>V. cholerae</i>	<i>B. dys.</i> (Flex.) + <i>V. cholerae</i>
Plate 1	351	140	32
Plate 2	324	147	73
Total	675	296	105

It will be observed that the pure *B. dysenteriae* (Flexner) culture contains by far the largest number of organisms while the mixed culture contains fewest. Indeed it is always possible to arrange the plates in the order *B. dysenteriae* (Flexner), *V. cholerae* and mixed

growth, without reading the labels at all, judging merely by the number of colonies on the plates. If the Endo-agar be made with peptone water in place of bouillon, *V. cholerae* often refuses to grow and a very striking contrast can be obtained between the numerous colonies on the pure Flexner plates and the scanty growth on those from the mixed culture. It is evident, therefore, that both *B. dysenteriae* (Flexner) and *V. cholerae* are inhibited in their growth when grown together.

The growth on the plates from the mixed culture consists of colonies of both organisms and the colonies can be distinguished by their naked eye appearance. Those of *B. dysenteriae* (Flexner) are more delicate and more sculptured, showing at first a pitted centre in which a papilla develops, which ultimately fills the pit completely so that the colony shows a central peak, from which spurs run out towards the indented margins. There is thus a radial striation. A concentric striation is also distinctly seen, its curves following the indentations of the margin, and the whole colony presents the appearance of a delicately sculptured film. When viewed by transmitted light, the colony shows a dark point at the centre which gives it somewhat the appearance of a flea-bite on Endo-agar plates. *V. cholerae* colonies, on the other hand, present an unpitted convex surface and have smooth circular margins. The centre is granular and appears darker than the marginal portion when viewed by transmitted light, but the dark part occupies a larger area proportionally than the central spot of the Flexner colony and the two colonies can be easily distinguished by this means. •

As somewhat intermediate forms occur, it is best, if a differential count is desired, to make a sub-culture from each colony on peptone water and confirm the result by testing the sub-cultures for cholera-red after 24 hours' incubation at 37° C. Such a count was made in the case of the experiment quoted above and the result of enumerating the colonies of the two organisms in the mixed plate is given in the following table:

	<i>B. dysenteriae</i> (Flexner)	<i>V. cholerae</i>
Plate 1	16	16
Plate 2	29	44
Total	45	60

It would thus appear that *B. dysenteriae* (Flexner) is inhibited to a greater extent than *V. cholerae*.

Numerous instances, of course, are known of similar inhibition of one organism by another. In many cases a particular medium is

necessary. Indeed the selective influence of a particular medium is made use of for the isolation of organisms, for example, the well-known case of the use of blood serum for the isolation of *B. diphtheriae*. In the present instance the marked inhibition of the growth of *B. dysenteriae* in peptone solution by the cholera organism is noteworthy in connection with the special suitability of this medium for the isolation of *V. cholerae* from the stools. That it is not an unfavourable medium for *B. dysenteriae* (Flexner) is shown by the large number of organisms in the pure Flexner culture—(about 270 million per c.c.).

In the case of organisms like *B. faecalis alkaligenes* which do not reduce nitrite, one does not of course expect the cholera-red reaction to disappear.

#### SUMMARY.

1. It has been shown that certain nitrite-destroying organisms when grown along with *V. cholerae* prevent the appearance of the cholera-red reaction. This is not due to the non-formation of nitrite, but to its rapid destruction by the nitrite-destroying organisms.

2. Certain nitrite-destroying organisms fail to prevent the cholera-red reaction.

3. It has been shown in the case of the *B. dysenteriae* (Flexner) that the failure to prevent the cholera-red reaction is due to an inhibition of the growth of both organisms when grown together.

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