A Growth-Inhibitory Effect on *Shigella dysenteriae* which Occurs with some Batches of Nutrient Agar and is Associated with the Production of Peroxide

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SUMMARY: Certain batches of nutrient agar were inhibitory to small inocula of *Shigella dysenteriae*. The inhibitory effect was abolished by catalase and by manganese dioxide. It occurred only with surface cultures incubated aerobically; poured plates or plates incubated anaerobically showed no inhibition. The inhibitor was a peroxide formed during the heating of nutrient broth and agar; it was present in minimal inhibitory concentrations since dilution with one to four volumes of saline agar removed the inhibition. It is suggested that some nutrient agars contain material which, on exposure to air, forms inhibitory concentrations of peroxide in the surface layer.

During the examination of papain digest broths for their growth-promoting properties one of us (H. P.) made the following observation. *Shigella dysenteriae*, strain CN 191 from the Wellcome Bacterial Collection, was used to test two types of broth—broth (a) an undiluted papain digest of horse muscle containing about 7.5 g. total N/l., and broth (b) which consisted of broth (a) diluted with an equal volume of 0.5 % saline. The smallest inoculum giving growth was the same with both broths. The broths were then converted into nutrient agars by the addition of 2.0 % Japanese agar. The growth from a heavy surface inoculum was about the same with both nutrient agars, but with dilute inocula more colonies grew from the agar made with the diluted broth than on the agar from the undiluted broth. Moreover, progressive dilution of the nutrient agar made from broth (a) with saline agar increased the number of colonies growing from dilute inocula. No such difference was observed when silica gel was substituted for agar as the solidifying agent.

The failure of the test organism to grow thus appeared to be due to some inhibitor. The inhibitor was not present in the broth, since the smallest inoculum which grew was the same with both broths. Similarly, the inhibitor was not present in the agar itself, since dilution of a 'bad' nutrient agar with saline agar removed the inhibition. The effect was also observed during the development of tests for standardizing bacteriological media. It was subjected to systematic study and the results are reported below.

EXPERIMENTAL

Preparation of the nutrient agars

Nutrient broth was prepared by extracting fresh minced horse muscle with hot water in the proportion of c. 700 g. muscle to 1 l. water. The meat particles were removed by filtration and to the extract was added a concentrated

peptone solution equivalent to an additional total nitrogen content of 1.5 g./l. Then 0.125 % CaCl_2 was added and the pH adjusted to 7.8. NaCl was added to a final concentration of 0.5 % and the mixture boiled, filtered and sterilized by Seitz filtration. The total nitrogen content of this broth was about 8.0 g./l. The peptone used was prepared by digesting horse muscle at pH 5.8 with papain for 2 hr. at 60°. The mixture was then boiled and sterilized by filtration. The total nitrogen content of this concentrated peptone was about 14 g./l. Nutrient agar was prepared by adding 1.25 % New Zealand agar (Davis Gelatin (N.Z.) Ltd., Christchurch, New Zealand) to nutrient broth. The nutrient agar was filtered hot and sterilized by autoclaving.

Comparison of growth on different batches of nutrient agar

A convenient test for comparing the growth on different batches of solid medium was the determination of the viable count of a bacterial suspension by the Miles & Misra (1938) technique, using an arbitrarily selected batch of medium as a standard for comparison. An overnight culture on nutrient agar of strain CN191 was emulsified in Ringer's solution and diluted to an opacity corresponding to Brown's opacity tube No. 8 (Burroughs, Wellcome Ltd.), i.e. approximately 2000×10^6 organisms/ml. Single drops, from a 0.02 ml. dropping pipette, of ten-fold serial dilutions of this suspension were dropped on agar plates and incubated in the usual manner. Four dilutions were used for each plate.

The difference between good and bad nutrient agars

Although all batches of nutrient agar used were prepared by the same standard method, two types were encountered. A 'good' nutrient agar gave confluent growth with an inoculum of a dilution of 10^{-5} of the test suspension, about 50 colonies at a dilution of 10^{-6} , and an occasional colony at 10^{-7} . On the other hand, a 'bad' nutrient agar gave confluent growth with a dilution of 10^{-1} and no growth with the subsequent dilutions. The two types of nutrient agar were indistinguishable when tested against *Escherichia coli* or *Staphylococcus aureus*, the bad nutrient agar having no inhibitory action against these organisms. This effect was shown to be a species effect and was not confined to the particular strain of *Sh. dysenteriae* used. Six other strains of *Sh. dysenteriae* were inhibited by the bad nutrient agar but no strains of the species *Sh. paradysenteriae*, *Esch. coli*, or *Staph. aureus* were affected.

Dilution of broth with saline before the preparation of nutrient agar or the dilution of a bad nutrient agar with saline agar eliminated the inhibitory effect. A typical protocol is given in Table 1, showing that relatively slight (1/3) dilution with saline agar was sufficient to remove the inhibition. Dilution with one or two volumes of saline agar was always sufficient to overcome any inhibitory effect.

Effect of various substances on the inhibition

A systematic search was made for substances which might neutralize the inhibitory effect of bad nutrient agars. A large number of growth factors, separately or together, casein acid-hydrolysate, starch, or thiolacetic acid

Table 1. *The effect of dilution of good and bad nutrient agars with saline agar on the growth of Shigella dysenteriae from small inocula*

Medium	Growth from 0.02 ml. inoculum of dilution						
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Good agar (batch M 259/48)	+	+	+	+	+	25	3
Good agar (3 vol.) + saline agar (1 vol.)	+	+	+	+	+	22	2
Good agar (1 vol.) + saline agar (1 vol.)	+	+	+	+	+	28	1
Good agar (1 vol.) + saline agar (3 vol.)	+	+	+	+	+	46	6
Bad agar (batch M 319/48)	+	—	—	—	—	—	—
Bad agar (3 vol.) + saline agar (1 vol.)	+	—	—	—	—	—	—
Bad agar (1 vol.) + saline agar (1 vol.)	+	+	+	96	8	—	—
Bad agar (1 vol.) + saline agar (3 vol.)	+	+	+	+	+	26	3

Undiluted inoculum = *c.* 2×10^9 organisms/ml. + = confluent growth or numerous colonies. Numerals = no. of colonies.

Table 2. *Neutralization of the inhibitory effect of a bad nutrient agar on Shigella dysenteriae by dilutions of purified catalase*

Medium	Growth from 0.02 ml. inoculum of dilution			
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Good agar (batch M 259/48)	+	+	37	3
Bad agar (batch M 340/48)	—	—	—	—
15 ml. bad agar + 1 ml. 0.85 % saline	—	—	—	—
15 ml. bad agar + 1 ml. catalase solution diluted 10 ⁻²	+	+	48	6
15 ml. bad agar + 1 ml. catalase solution diluted 10 ⁻⁴	+	+	51	9
15 ml. bad agar + 1 ml. catalase solution diluted 10 ⁻⁶	+	+	19	—
15 ml. bad agar + 1 ml. catalase solution diluted 10 ⁻⁸	+	88	8	—
15 ml. bad agar + 1 ml. catalase solution diluted 10 ⁻¹⁰	+	17	3	—

Undiluted inoculum = *c.* 2×10^9 organisms/ml. + = confluent growth or numerous colonies. Numerals = no. of colonies.

(0.01 % incorporated in the medium) did not neutralize the inhibitor. The inhibitory effect was, however, abolished by fresh blood, some vegetable extracts such as extract of horse radish, a purified catalase preparation and manganese dioxide, but not by salts of manganese. With the exception of manganese dioxide, the ability of these substances to neutralize the inhibitor was destroyed by heat. Table 2 shows the neutralization of the inhibitory effect by purified catalase (kindly supplied by Dr D. Herbert).

The abolition of the inhibition by high dilutions of catalase, but not by heated catalase, and also by manganese dioxide, but not by salts of manganese, can be regarded as reasonable proof that the inhibition was due to a peroxide. This agrees with the observation that catalase-producing organisms such as *Sh. paradysenteriae*, *Esch. coli*, or *Staph. aureus* were not inhibited by bad nutrient agars; *Sh. dysenteriae* does not produce catalase.

Manganese dioxide destroyed the inhibitory effect of a bad nutrient agar. This was shown either by incorporating a little solid MnO₂ in the medium (Table 3) or by placing a little MnO₂ on the surface of an agar plate. In the latter case, when the surface of a plate was inoculated with a dilute inoculum,

colonies of *Sh. dysenteriae* grew in the immediate vicinity of the MnO_2 particles but not elsewhere. Table 3 also shows that when a bad nutrient agar containing MnO_2 was filtered the filtrate was no longer inhibitory. However, when this filtered agar was autoclaved it again became inhibitory. This indicated that the inhibitor or its precursor was formed, or its formation was rapidly accelerated, during the heating of this nutrient agar.

Table 3. *The effect of MnO_2 on the inhibitory action of a bad nutrient agar on Shigella dysenteriae*

Medium	Growth of <i>Sh. dysenteriae</i> with 0.02 ml. inoculum of dilution			
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Good agar (batch 259/48)	+	180	13	1
Good agar + MnO_2 (1 g./l.)	+	210	14	4
Bad agar (batch 840/48)	—	—	—	—
Bad agar + 1 g./l. MnO_2	+	200	16	3
Bad agar + 1 g./l. MnO_2 and filtered*	+	79	12	1
Bad agar + 1 g./l. MnO_2 filtered and autoclaved†	6	—	—	—
Bad agar + 0.001 % MnSO_4	—	—	—	—

* Filtered through Seitz pads.

† Autoclaved 15 min. at 15 lb.

Undiluted inoculum = c. 2×10^9 organisms/ml. + = confluent growth or numerous colonies.
Numerals = no. of colonies.

It was possible to convert a good nutrient agar into a bad nutrient agar by continuous autoclaving. The degree of autoclaving necessary to do this varied from batch to batch. But here it was necessary to distinguish between a bad nutrient agar of the type under investigation and nutrient agars which by continued heating had lost growth-promoting properties for other species. This was done by noting whether the inhibition was removed by catalase and whether the nutrient agar was still capable of growing *Staph. aureus*.

Attempts made to extract the inhibitor were uniformly unsuccessful. Saline extracts of bad agars were made and tested in parallel with nutrient broth. The smallest inoculum to grow was the same in both cases. When these extracts were reconverted into nutrient agar by steaming with 1.25 % agar they were non-inhibitory. When the dilutions of test inoculum were made in extracts from bad nutrient agars and tested on a good nutrient agar no inhibition was observed. These results would confirm that the peroxide was present only in minimal inhibitory amounts. Chemical tests for peroxide in bad nutrient agars were negative.

The smallest inocula to grow on good and bad nutrient agars were compared by the poured plate method as well as by the surface method of Miles & Misra. No inhibition was shown with a bad nutrient agar as a poured plate. Good and bad nutrient agars were then compared when surface-inoculated plates were incubated anaerobically; under anaerobic conditions a bad nutrient agar was no longer inhibitory. Sandwich plates were made with various combinations of good and bad nutrient agars. Table 4 illustrates the results obtained. The

first layer was poured, dried and inoculated, and after the inoculum had dried the second layer of agar was then poured on top. When the two layers were both of bad nutrient agar some inhibition was obtained, but with all other combinations little inhibition was observed.

Table 4. *The effect of sandwich plates and anaerobiosis on the growth of Shigella dysenteriae on good and bad nutrient agars*

Medium	Growth of <i>Sh. dysenteriae</i> with inoculum of dilution			
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Good agar (batch M 402/48) incubated aerobically	+	+	19	6
Bad agar (batch M 605/58) incubated aerobically	—	—	—	—
Good agar (M 402/48) incubated anaerobically	+	+	25	1
Bad agar (M 605/48) incubated anaerobically	+	+	19	3
Sandwich plates: good agar upon bad agar	+	+	18	2
Sandwich plates: bad agar upon good agar	+	31	9	1
Sandwich plates: bad agar upon bad agar	19	20	4	—
Sandwich plates: good agar upon good agar	+	+	21	2

Undiluted inoculum = $c. 2 \times 10^9$ organisms/ml. + = confluent growth or numerous colonies. Numerals = no. of colonies.

The surface of some bad agar plates was removed by careful scraping or by slicing off the top layer, and then inoculated on the new surface by the usual technique. Inhibition was again observed, thus showing either that the inhibition was not confined to the surface layer or that a new inhibitor was formed on exposure of the fresh surface to air.

Attempts were made to simulate bad nutrient agar by the addition of hydrogen peroxide to good nutrient agar. In a number of experiments whenever an inhibitory concentration of peroxide was present in the medium, growth on both surface-inoculated and poured plates was inhibited. It was not found possible to produce only surface inhibition by addition of hydrogen peroxide.

DISCUSSION

The complex nature of nutrient agar and the small amount of peroxide that must be present makes the identification of the inhibitor, or of the mechanism leading to its formation, extremely difficult. The removal of the inhibition by catalase or manganese dioxide may be accepted as reasonable proof that the inhibitor is indeed a peroxide. This peroxide is present in amounts which cannot be detected by the usual chemical tests. Since dilution with one to four volumes of saline agar usually abolishes the inhibition, the peroxide present must be near the limit of its minimal bacteriostatic concentration. Various authors have shown that exposure of nutrient media to sunlight, ultraviolet radiation or X-rays may produce an inhibitory medium. Burnet (1925) demonstrated that peroxide was formed in nutrient agar exposed to sunlight. Wyss, Clark, Haas & Stone (1948) showed that organic peroxides

were formed in broth irradiated with ultraviolet light. Blank & Arnold (1935) found that irradiated carbohydrates or agar were inhibitory to *Bacillus subtilis* when incorporated into nutrient agar. These results differ in several respects from those described here. In the experiments of Burnet (1925) and Wyss *et al.* (1948) the concentration of peroxide formed was sufficient to inhibit a catalase-producing organism such as *Staph. aureus*, whereas in our experiments the concentration of peroxide formed was only sufficient to inhibit a non-catalase-producing organism. However, on one occasion, prolonged heating of a batch of bad nutrient agar produced a very dark medium which inhibited *Staph. aureus* and *Sh. dysenteriae* with both surface and poured plate cultures. This inhibition was overcome by catalase. In this respect our results may be regarded as quantitatively different from those quoted. Another difference is the mechanism by which peroxide is formed. In our experiments irradiation was not a significant factor. The peroxide was formed by reaction between material present in some broths and material normally present in agar. The production of peroxide was associated with heat and the presence of atmospheric oxygen. In fact, it was found possible to produce a bad agar by bubbling oxygen through melted good nutrient agar maintained at 47° in a water bath. In this case the agar became lighter in colour, demonstrating that the production of peroxide is not necessarily associated with the darkening of nutrient agar which occurs on prolonged heating. On the available evidence it seems that when some nutrient broths are heated with agar-agar some material is formed which on exposure to atmospheric oxygen readily produces an inhibitory concentration of peroxide. This material is distributed throughout the medium, but the peroxide is apparently confined to the surface layer. It is unlikely that peroxide is present throughout the medium since, when inhibitory concentrations of peroxide are added to good nutrient agar, it is inhibitory when inoculated by either the pour-plate or surface technique. Moreover, it may seem unlikely that peroxide would not be inhibitory to *Sh. dysenteriae* when growing under microaerophilic (or anaerobic) conditions. However, it is not possible to dismiss entirely the possibility that these low concentrations of peroxide may be distributed uniformly throughout the medium and that only the strictly aerobic metabolism of *Sh. dysenteriae* is affected.

These results are of some practical interest in connexion with the preparation of nutrient agars, particularly for surface growth from small inocula. They also have a more general appeal because of the interest which is being shown in the study of growth inhibitors and the conditions under which they arise. Peroxide is another example of such an inhibitor and the destruction of peroxide may be another function of the blood used in some bacteriological media. Since peroxide formation is always a possibility when the surface of organic material is exposed to the atmosphere the production of catalase will be an advantage to aerobic bacteria.

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