# ON THE ISOLATION FROM AGAR OF AN INHIBITOR FOR NEISSERIA GONORRHOEAE

HERBERT L. LEY, JR., AND J. HOWARD MUELLER

*Department of Bacteriology and Immunology, Harvard Medical School and School of Public Health, Boston*

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The separation of an inhibitor for *Neisseria gonorrhoeae* from commercial agars was attempted on the basis of previous work by Mueller and Hinton (1941) and Gould, Kane, and Mueller (1944). In addition, this inhibitor was compared with certain organic compounds of a similar nature by biologic tests of inhibitory activity.

Until the medium of Mueller and Hinton (1941) was developed, it was thought impossible to grow *N. gonorrhoeae* on a simple, well-defined medium, but these investigators showed that casein hydrolyzate, meat extract, agar, and starch supported growth of the organisms as well as chocolate blood agar or ascitic fluid agar. Gould, Kane, and Mueller (1944) further showed that a fluid medium containing casein hydrolyzate, sodium chloride, magnesium sulfate, and phosphate buffer would support growth of *N. gonorrhoeae*, and that a similar medium with agar added would fail to grow the organisms. The addition of starch to this last medium again permitted growth, which led the authors to the hypothesis that starch neutralized an inhibitor present in the agar. Frantz (1942) also developed a similar simple fluid medium for a closely related organism, *Neisseria intracellularis*, and observed inhibition of growth of the organisms upon addition of agar to the medium. The experimental work which follows was begun in order to prove the hypothesis of inhibition of *N. gonorrhoeae* by agar, or a substance in it, that was proposed by Gould, Kane, and Mueller.

## EXPERIMENTAL

*Separation of the inhibitor from agar.* In the following preparations Merck's U.S.P. agar-agar shreds were used throughout. It was decided to attempt the separation of the inhibitor from the agar by the customary methods of dialysis and extraction. Hydrated agar was dialyzed through viscose tubing against running tap water for 3 days, and a similar sample of agar was electrodialyzed, using viscose partitions, for a similar length of time. Solid hydrated agar, cut in 1.0-cm cubes, was extracted for 7 days by direct contact with large volumes of distilled water, 0.1N HCl, 0.1N NaOH, and 95 per cent ethanol. A series of 5.0-g lots of dry, shredded agar was extracted for 3 days each in an all-glass Soxhlet apparatus, having an extraction chamber of 100-ml capacity and 40-mm diameter, with 250 ml of each of the following freshly distilled cp solvents: diethyl ether, acetone, benzene, chloroform, ethanol, and methanol.

The treated agar samples were incorporated into the medium of Mueller and Hinton (1941) without starch in the following proportions:

| | |
|---|---|
| Agar | 2.5 g |
| Distilled water to make | 46 ml |

Neutralize this mixture to litmus with 1 N NaOH or HCl and autoclave 15 minutes at 15 pounds' pressure to put the agar into solution. (In hydrated agar the amount of water present may be determined by weighing, and a suitable amount added to bring the total volume to 46 ml.)

| | |
|---|---|
| Casein hydrolyzate, Difco | 2.58 g |
| Meat infusion[1] | 44 ml |

Add this mixture to the warm fluid agar, bring to pH 7.4 by the addition of 1 N NaOH, tube in 13.5-ml amounts, and autoclave 10 minutes at 10 pounds' pressure.

When the medium was to be used, the tubes were heated in boiling water for 5 minutes to melt the agar; 1.5 ml of 0.9 per cent sterile saline or 1.5 per cent starch sol[2] were added as required; and the contents of the tubes were poured into sterile petri dishes, which were rotated to mix the addition and the agar. After the medium had hardened, it was inoculated in sectors with one loop of a heavy saline suspension of a 24-hour growth of the test strain of gonococcus from a starch agar slant.[3] The sector was streaked with a loop in the customary manner. The plates were incubated in a candle jar at 35 C for 24 hours, and then were examined.

Through experience it has been found that colony size is a poor criterion of the efficacy of the medium or, to put it another way, of the presence of inhibition. The pattern of growth furnishes a much better criterion. The organism may not have grown at all; it may have grown only at the start of the streak in the region of large inoculum; or it may have grown along the entire streak in the area of small as well as of large inoculum. These three responses are indicated, respectively, by the letters O, L, and S in tables 1 and 2 below. In general, it may be said that where there is no inhibition the growth is S; where there is moderate inhibition, L; and where there is much inhibition, O.

Table 1 demonstrates that neither dialysis nor electrodialysis is effective in removing the hypothetical inhibiting agent from agar. Extraction by contact with water, acid, and alkali, as well as by ethanol, is similarly ineffective. In all cases the organisms grew well on the treated agar with added starch, indicating that the procedures did not introduce further inhibiting substances not neutralized by starch.

Table 2 demonstrates that with the exception of diethyl ether, the organic

[1] Meat infusion may be prepared by suspending 1 pound of chopped beef heart in 500 ml of tap water, which is brought to active boiling, and then filtered through cheesecloth and filter paper.

[2] Starch sol may be prepared by making a paste of 1.5 g of corn starch (Argo) in 10 ml cool, distilled water, and pouring the paste with stirring into 90 ml boiling distilled water. Autoclave 10 minutes at 10 pounds' pressure.

[3] Through the course of this experiment 19 strains of *N. gonorrhoeae* were used, all supplied by the Boston Dispensary. All were checked by colonial and microscopic morphology and fermentation reactions.

solvents did not introduce additional inhibitory substances, for growth was excellent on the media containing the added starch. Without the starch, however, growth was poor in all agars extracted with organic solvents except methanol. In that one instance growth was almost equivalent to that on the

TABLE 1

*The effect of dialysis and simple extraction of agar on the growth of gonococci*

| PREPARATION OF AGAR | GONOCOCCUS STRAINS | | | | | |
|---|---|---|---|---|---|---|
| | 3 | 5 | 7 | 3 | 5 | 7 |
| | Starch added | | | Saline added | | |
| None | S | S | S | L | L | L |
| Dialysis, tap water | S | S | S | L | 0 | 0 |
| Electrodialysis | S | S | S | 0 | 0 | 0 |
| Extraction, $H_2O$ | S | S | S | 0 | S | L |
| Extraction, HCl | S | S | S | L | L | L |
| Extraction, NaOH | S | S | S | 0 | L | L |
| Extraction, EtOH | S | S | S | 0 | S | L |

TABLE 2

*The effect of extracting agar with organic solvents on the growth of gonococci*

| EXTRACTING AGENT* | GONOCOCCUS STRAINS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| | Starch added | | | | | | | | |
| None | S | S | S | S | S | S | S | S | S |
| Ether | S | S | S | S | 0 | 0 | 0 | S | 0 |
| Acetone | S | S | S | S | S | S | S | S | S |
| Benzene | S | S | S | S | S | S | S | S | S |
| Ethanol | S | S | S | S | S | S | S | S | S |
| Methanol | S | S | S | S | S | S | S | S | S |
| | Saline added | | | | | | | | |
| None | 0 | 0 | S | S | 0 | S | 0 | S | 0 |
| Ether | 0 | 0 | S | L | 0 | S | 0 | L | 0 |
| Acetone | 0 | 0 | L | L | 0 | S | 0 | L | L |
| Benzene | L | 0 | S | S | L | S | 0 | S | 0 |
| Ethanol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | L |
| Methanol | S | S | S | S | S | S | S | S | 0 |

* The chloroform-extracted agar was omitted from the experiment because it did not solidify, presumably because of acid hydrolysis by the decomposing solvent.

same medium with added starch. It may be concluded that methanol extraction removes the inhibiting agent for *N. gonorrhoeae* that is present in commercial agar.

At this point in the investigation it was decided that the easiest method of

quantifying the inhibitory activity of a methanol extract of agar would be to use a suitable fluid medium which would reproducibly grow the organisms, and to which could be added decimal dilutions of the extract. In this manner the minimum amount of extracted material inhibiting growth could be determined. The medium of Mueller and Hinton (1941) seemed an excellent one to modify; the agar and starch were omitted from it, giving it the following composition:

| | |
|---|---|
| Distilled water | 300 ml |
| Meat infusion[4] | 150 ml |
| Casein hydrolyzate, Difco | 8.75 g |

Bring this mixture to pH 7.4 with 1 N NaOH, and, after placing in suitable containers, autoclave for 10 minutes at 10 pounds' pressure.

Ten ml of the medium were first placed in 20-mm test tubes, but inconsistent results in terms of reliable growth following a 0.5-ml inoculum of a 24-hour fluid culture directed attention to other forms of containers. A 10-ml volume of fluid in a 50-ml Erlenmeyer flask was then tried, with consistent growth following a 0.5-ml inoculum. These flasks were carefully cleaned in a hot conc. $H_2SO_4$ = $HNO_3$ bath and rinsed thoroughly with distilled water each time before use. They, like the plates previously described, were incubated for 24 hours at 35 C in a candle jar.

When the organisms did grow in the test tubes, they were viable at 35 C for as long as 8 days, whereas those in the flasks were viable only 3 days. The main difference in the two cases was in the surface-volume ratio for the medium, so the phenomena observed may have been due to a difference in gas exchange at the gas-liquid interface. This hypothesis has not yet been investigated. It may be pointed out, however, that Frantz (1942) encountered a similar difficulty in cultivating the meningococcus in fluid media. In the flasks, the Mueller-Hinton fluid modification was a quite satisfactory and useful tool with which further work on extraction could be pursued.

*Characterization and estimation of activity of inhibitor.* Forty g of Merck's shredded agar were extracted in a Soxhlet apparatus with cp methanol for three days, and the volume of the extract was reduced to 80 ml by distillation. This extract has a light brown color and no recognizable odor. When 10 ml of the extract were added to an equal volume of water, a curdy, white precipitate formed, which dissolved upon heating and the addition of NaOH, and which reappeared upon acidifying with $H_3PO_4$. Upon extraction of the acid mixture with petroleum ether, the precipitate disappeared from the aqueous phase. This information indicated that the methanol extract contained an appreciable concentration of a fatty-acid-like compound.

Further extraction of the compound from acid and alkaline aqueous phases by petroleum ether was unsatisfactory because of highly persistent and troublesome emulsions of the two phases. However, the biologic activity of the product of the methanol extraction was tested by evaporating a volume containing the extract

[4] See footnote 1.

of 2.5 g of agar to dryness on the steam bath, adding 2.0 ml of water to the residue, and neutralizing the solution to phenol red indicator with NaOH and $H_3PO_4$. The solution was then evaporated to one-half its volume over an open flame, a procedure which effectively sterilized it. A series of flasks containing 9.0 ml of fluid medium was set up as noted in the protocol below, and after inoculation with 0.5 ml of a 12-hour fluid culture of gonococcus strain 11, which was known to be sensitive to agar inhibition, the flasks were incubated for 24 hours in a candle jar at 35 C. This was followed by streaking a loop from each of the flasks on a sector of a Mueller-Hinton starch agar plate to demonstrate the growth of the inoculum. The results following incubation of the plates at 35 C for 24 hours in a candle jar are shown in table 3.

From these data it may be concluded that a methanol extract of agar contains a substance which, in suitable concentrations, inhibits completely the growth of gonococcus strain 11 and which is biologically neutralized by the addition of starch.

After removing an aliquot containing 1.0 mg solids, the methanol extract of 30 g of agar containing 90 mg of solid material was made alkaline with NaOH and

TABLE 3

*The effect of products of methanol extraction of agar on the growth of gonococcus strain 11 in fluid medium*

| ADDITION | INOCULATED | STARCH SOL 1.5% 4.5 ML | PLATE GROWTH |
|---|---|---|---|
| None | − | − | 0 |
| None | − | + | 0 |
| None | + | − | ++++ |
| None | + | + | ++++ |
| Methanol extract 1.25 g agar | + | − | 0 |
| Methanol extract 1.25 g agar | + | + | ++++ |

reduced to dryness on a steam bath. The residue was treated as indicated in the following flow sheet.

90 mg solid material
|
Heat 98 C for 4 hr
to char polysaccharides
|
Extract 3x with 1.5 ml
petroleum ether ——— Extract: discard
|
Residue: acidify with
2.0 ml 0.1 N HCl
|
Extract 3x with 2.0 ml
petroleum ether ——— Residue: discard
|
Extract: heat to dryness
on steam bath, yield 3.0 mg
of white solid

The 3.0 mg of white solid was dissolved in 50 per cent aqueous ethanol and titrated with $4 \times 10^{-2}$ N NaOH, using phenolphthalein as an indicator. The calculated equivalent molecular weight, based on the assumption of one carboxylic group per molecule, was $3.7 \times 10^2$. This would suggest that the compound contains 24 carbon atoms per molecule, if it is a straight chain, aliphatic compound.

Because it was felt that the inhibiting compound obtained from agar was similar to a fatty acid, a crude mixture of butter fatty acids was prepared by alkaline hydrolysis of domestic butter. In addition, cp stearic acid (Eastman) and oleic acid (Merck) were procured for comparison with the inhibitor. These acids and the inhibitor were put into solution in suitable concentrations in 2.0 ml of water adjusted to approximate pH 7.4 with phenol red indicator by the addition of 0.5 N $Na_2CO_3$ and 1 M $H_3PO_4$. The solutions were reduced to one-half their volume by boiling, which effectively sterilized them, and were added with bacteriologic technique to a series of 50-ml Erlenmeyer flasks containing 9.0 ml of fluid medium. Each of the fatty acids was serially diluted in the same quantity

TABLE 4

*Relative growth of gonococcus strain 11 in the presence of various concentrations of fatty acids*

| MATERIAL ADDED | CONCENTRATION OF ADDITION (Dry wt, μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 100 | 50 | 10 | 5.0 | 1.0 | 0.5 | 0.1 | 0.05 |
| 1 mg aliquot of orig. extract | | 0 | | ++++ | | ++++ | | |
| Purified white solid | | 0 | | 0 | | 0 | | ++++ |
| Butter fatty acids | 0 | | 0 | | 0 | | ++ | |
| Oleic acid | 0 | | 0 | | 0 | | ++++ | |
| Stearic acid | 0 | | 0 | | + | | ++++ | |

of medium so that a wide range of final concentrations of the fatty acids was obtained. All the flasks were inoculated with 0.5 ml of a 12-hour fluid culture of gonococcus strain 11, and were incubated 12 hours at 35 C in a candle jar. One loop of fluid from each flask was then removed and streaked on a starch agar plate to test for growth of the inoculum. These plates were examined, following 24 hours of incubation at 35 C in a candle jar (table 4).

On the basis of these data, it may be concluded that the procedure of purification of the methanol extract concentrated the inhibiting fraction approximately 100 times. If the number of units of inhibition in the original extract and in the purified product is calculated (defining a unit as the minimum amount inhibiting growth under the conditions of the experiment), it is found that the original extract contained $1.8 \times 10^3$ units, and the product, $6.0 \times 10^5$. For the crude biologic assay employed, the agreement is excellent, but even more important is the implication that very little active material was lost in purification. Oleic, stearic, and butter fatty acids may be seen to have an inhibitory action similar to that of the purified agar extract in essentially the same concentrations. It has

been shown, in addition, that the activity of both the fatty acids tested and the agar inhibitor is fully retained following refluxing for 1½ hours with 2.5 N NaOH.

*The role of starch.* The work of Mueller and Hinton (1941) did not explain the protective action of starch in their medium, but Schoch and Williams (1944) have demonstrated what appears to be the probable mechanism of action. These authors described the adsorption of fatty acids by the linear component of corn starch; the adsorption was reversible, and by the use of either 80 per cent aqueous dioxane or methanol the acids could be recovered quantitatively from the starch. The Corn Products Refining Company kindly supplied samples of various commercially available starch products, which were tested for activity in the Mueller-Hinton medium. It was found that mildly hydrolyzed derivatives and the linear component of whole starch were as effective as the unmodified product. All strongly hydrolyzed starches and glycogen were ineffective in the medium. All effective products gave a blue color when tested with iodine, but one starch which was not satisfactory for the medium also gave a blue color. This confirms the impression of Mueller and Hinton (1941) that commercial starch lost its activity when hydrolyzed by ptyalin to the point at which it no longer gave a blue color with iodine.

## SUMMARY AND DISCUSSION OF RESULTS

The development of the starch agar medium of Mueller and Hinton (1941) and the further clarification of the growth requirements of the gonococcus by Gould, Kane, and Mueller (1944) seemed to indicate that commercial agar contained an inhibitory compound that rendered growth of the gonococcus difficult without starch. Not all strains of gonococcus were sensitive to the inhibitor, and, indeed, of the 19 strains used in the work reported in this paper, one-half were not sensitive to the inhibitor in its usual concentration in 1.75 per cent agar media. Separation of this inhibitor from the agar was successfully accomplished by continuous methanol extraction, and the compound was found to have many of the properties of fatty acids. It was further concentrated by extraction with petroleum ether to the point that 0.5 μg per ml was sufficient to inhibit growth completely. Oleic, stearic, and butter fatty acids were shown to have similar inhibitory properties in equivalent concentrations. The actual chemical composition of this inhibitor has not been determined, but its solubility properties, its approximate equivalent molecular weight, and its biologic similarity to two of the higher fatty acids would indicate that it resembles these fatty acids very closely.

The mechanism of neutralization of the inhibition by starch is postulated to be an adsorption phenomenon on the basis of the work of Schoch and Williams (1944). Glass and Kennet (1939) have shown in addition that certain varieties of blood and sugar charcoals, as well as graphite, permitted growth of gonococci in the media which they described. Charcoals have long been known to be excellent adsorbers of fatty acids, and Linner and Gortner (1935) employed "norite" to check the Langmuir adsorption equation for 31 organic compounds, including 6 fatty acids. The actual mechanism of inhibition of growth of the

gonococcus by fatty acids is unknown at present. Several authors, Dubos (1945), Dubos and Davis (1946), and Wyss, Ludwig, and Joiner (1945), have described the inhibiting effects of fatty acids on other microorganisms, so that it is possible that other organisms may be found equally susceptible to this inhibiting agent in agar. This does not mean that the fatty acid has possibilities as a new chemotherapeutic agent; the varying susceptibility of the gonococcus and the ease with which the material may be neutralized suggest that its use in clinical medicine would be extremely limited. It is felt, too, that extraction of all bacteriologic agar to remove the inhibiting acids is not justified in view of the favorable results with the addition of starch to a medium like the Mueller-Hinton.

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