AN IMPROVED MEDIUM FOR THE CULTIVATION OF HEMOLYTIC STREPTOCOCCUS[1]

ALAN W. BERNHEIMER, WILLARD GILLMAN, G. A. HOTTLE AND A. M. PAPPENHEIMER, JR.

Department of Bacteriology, School of Medicine, University of Pennsylvania

Received for publication July 7, 1941

In the preceding paper (Bernheimer and Pappenheimer, 1942) the factors necessary for massive growth of group A hemolytic streptococcus on a medium of essentially defined chemical composition were discussed. The medium has been adapted for heavy growth in large volumes. Although worked out specifically for the C203S strain, the medium has proved useful for the cultivation of other strains of group A streptococcus.

The following solutions are prepared:

Solution I: 10 liters of distilled water (Note 1), 7.5 grams $MgSO_4 \cdot 7H_2O$, 150 mgm. uracil, 150 mgm. adenine sulfate and 110 mgm. phenol red are placed in a 20 liter Pyrex carboy and sterilized at 15 lbs. for 90 minutes in the autoclave.

Solution II: To 3 liters of 10 per cent casein hydrolysate (Note 2) are added 2 grams cystine (dissolved separately in water with the aid of a little concentrated HCl), 45 grams KCl, 37.5 grams KH_2PO_4, 15 grams $Na_2HPO_4 \cdot 12H_2O$, and 2 ml. of 0.4 per cent phenol red. The pH is adjusted with 20 per cent NaOH to 7.5–7.8 and the mixture boiled gently for 5 minutes and filtered. To the clear filtrate is added 0.015 mgm. biotin (Note 3), 15 mgm. nicotinic acid, 15 mgm. pyridoxine (vitamin B_6) and 300 mgm. tryptophane (Note 4). This solution is then distributed into liter flasks, plugged and covered with paper caps and autoclaved at 15 lbs. for 20 minutes (Note 5).

Solution III: 300 ml. of 10 per cent $NaHCO_3$ is sterilized by filtration.

Solution IV: 20 ml. of 10 per cent thioglycollic acid solution is sterilized at 15 lbs. for 10–20 minutes.

Solution V: 60 mgm. calcium pantothenate, 15 mgm. thiamine

[1] The expenses of this work have been defrayed by a generous grant from the Commonwealth Fund.

(vitamin B_1), 7 mgm. riboflavin and 3 grams of glutamine (Note 6) are made up to 50 ml. with distilled water and sterilized by filtration.

Solution VI: To 1000 ml. of 50 per cent glucose containing 150 mgm. $CaCl_2$ is added 30 ml. of salt mixture (Note 7). Sterilization is carried out in the Arnold for 1 hour.

Before inoculation the carboy containing solution I is allowed to warm up to 35°C. The other solutions are then added in the order given and the contents thoroughly mixed. The inoculum consists of a saline suspension of the centrifuged organisms from 100 ml. of a 6–8-hour broth culture of streptococcus (Note 8). The carboy is then fitted with a mechanical stirring device sealed with mercury (we have used mercury-sealed stirrers made of stainless steel) and placed in the incubator at 35°C. at about 5 p.m. The following morning continuous stirring at *ca.* 70 r.p.m. is commenced and the culture is kept near pH 7 as judged by the color of the phenol red, by continuously dropping in 5N NaOH from a buret. Stirring and neutralisation are continued until growth ceases (30–48 hours). (Note 9.)

NOTES

1. The amount of water added to the carboy is chosen so that the final volume after mixing all the solutions will be 15 liters.

2. Technical casein is hydrolysed with 5 liters of 5N H_2SO_4 per kilogram of casein at the boiling point for 24 hours and the sulfate removed with barium hydroxide. It is important to remove most of the anions ($SO_4^{=}$) in order that the salt concentration in the final medium may be as low as possible. The hydrolysate is decolorized with charcoal and stock solutions stored over a little chloroform. Any precipitate of tyrosine which may form on standing redissolves on heating. We have adjusted our final hydrolysate concentration on a basis of the amount of casein hydrolysed. A typical "16 per cent" stock hydrolysate contained 17.3 mgm. nitrogen per milliliter.

3. We have used a biotin preparation obtained from the S.M.A. Corporation, Chagrin Falls, Ohio. The preparation is reported to contain 100 gamma of biotin per milliliter. Its purity is given as 0.05 per cent. It should be pointed out that many strains do not appear to require biotin, but it is included in the present medium because it has been shown essential for growth of strain C203S.

4. The present list of growth factors is probably not complete for many strains. For example we have found that growth of the erythrogenic toxin-producing strain, No. 594 is accelerated by the addition of 300 mgm. asparagine per liter. p-Aminobenzoic acid and inositol may also be included on general grounds if desired.

5. All the solutions in flasks are sterilized with paper-capped cotton plugs so that they may be poured into the carboy with less danger of introducing contaminants. We have had no serious trouble with contamination of carboys to date.

6. Glutamine may be purchased from the S.M.A. Corporation. We have made 100 grams in this laboratory, at very small expense, from common beets following the procedure of Vickery, Pucher, and Clark (1935).

7. The salt mixture used contains 50 mgm. $CuSO_4 \cdot 5H_2O$, 50 mgm. $ZnSO_4 \cdot 7H_2O$, 50 mgm. $FeSO_4 \cdot 7H_2O$, and 20 mgm. $MnCl_2 \cdot 4H_2O$ and 1 ml. concentrated HCl in 100 ml. of distilled water.

8. The size and condition of the inoculum are of great importance and the optimum inoculum may vary with each strain from day to day. It is desired to produce definite growth by the following morning. If too heavy an inoculum is used the cultures may become so acid after 12–15 hours growth that they will no longer continue growing even though neutralized.

An alternative procedure which we have used successfully and which is probably more suitable than the arbitrary procedure described above, is to add only 40 ml. of the glucose solution VI at the time of inoculation. The remainder may be added the following morning. With such a low glucose concentration there is no danger of overproduction of acid.

9. The rate of growth and yield vary with the strain. In general the rate of neutralization and the amount of NaOH added are a good indication of growth. As much as 800 ml. of 5N NaOH may be used up by a single carboy in 30–36 hours. The amount of growth may vary from about 1 gram dry weight of bacteria per liter to more than 2 grams per liter for the strains which we have used. Using a medium similar to that described above, we have obtained massive growth of strains C203S, 1048M, NY No. 5, and No. 594 in carboy lots. Strain C203S has yielded more than 200 mgm. bacterial nitrogen per liter with correspondingly high hemolytic titer and strain No. 594 has yielded 1 gram dry weight bacteria per liter with 12 Lf units per milliliter (360,000 Skin Test Doses per milliliter) of scarlet fever toxin found in the culture filtrate. Strain 1048M has yielded more than 1 gram dry weight of bacteria per liter.

SUMMARY

A medium of essentially defined composition has been described which supports heavy growth of several strains of Group A hemolytic streptococcus in large volumes. The cost of materials is about $0.32 per liter of medium.

REFERENCES

BERNHEIMER, A. W., AND PAPPENHEIMER, A. M., JR. 1942 Factors necessary for massive growth of group A hemolytic streptococcus. J. Bact., **43**: 481–494.

VICKERY, H. B., PUCHER, G. W., AND CLARK, H. E. 1935 The preparation of glutamine. J. Biol. Chem., **109**, 39.