The Culture of *Streptococcus pneumoniae*

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SUMMARY

In the fluid culture of *Streptococcus pneumoniae* in glucose+serum broth the part played by the serum appeared to be solely the regulation of pH value, and may be substituted by a suitable amount of sodium carbonate. The reason for the autolysis experienced in the fluid culture of pneumococci would appear to be due to hydrogen peroxide production. The addition of cysteine to a glucose+carbonate broth precluded the autolytic effect either by the cysteine giving an anaerobic condition of growth or by destroying H_2O_2 .

INTRODUCTION

In the growth of pneumococci in fluid media it is common practice to use Wright's broth (Wright, 1933) or its equivalent, supplemented with serum and glucose. This medium has two disadvantages: (a) the serum is expensive and contaminates the microbes with antigenic foreign protein; (b) the incubation of the culture has to be closely watched since at a certain stage of growth marked autolysis takes place. This presents two problems: what part is played by the serum in the culture medium and what is the reason for the autolysis?

METHODS

Pneumococci, Types I, II and III were grown in modifications of the classical Wright's broth+serum+glucose medium. Note was taken of the changes in pH value and of the effect of the addition of H_2O_2 on the counts in cultures made in these different media.

RESULTS

The serum effect

When serum was omitted from the supplemented Wright's broth, growth began but soon ceased; the addition of serum, after the apparent cessation of growth, resulted in a marked increase in count. At the stage of apparent cessation of growth the medium was found to be at about pH 4, and that the addition of the usual 10% (v/v) of serum changed the reaction to about pH 7, when growth began again. It seemed, therefore, that the part played by the serum might be no more than to control the reaction of the medium. This was tested as follows. Volumes (100 ml.) of 1% (w/v) Casamino acids+0.02% (w/v) tryptophan+5% (v/v) yeast extract (Stokes, 1955) were seeded with Types I, II and III pneumococci in closed 100 ml.

bottles and incubated for 18 hr. at 34°. In no case was the growth heavy. To each bottle of medium was then added 1 ml. sterile 10% (w/v) glucose and incubation continued for 7 hr. The growth increased appreciably and the cultures became acid again (about pH 4) and required 2 ml. of 3% (w/v) Na₂CO₃ solution for neutralization. The neutralized cultures were incubated for a further 18 hr. The amount of growth again increased. Again the cultures became acid and required a second 2 ml. of 3% (w/v) Na₂CO₃ solution for neutralization. These observations were followed up, using Wright's broth, where the main consideration was to observe the effect of pH control on the count. Samples (100 ml.) of broth were prepared as follows:

- (a) Control; Wright's broth with no modification.
- (b) Wright's broth + 1 ml. 10% (w/v) glucose.
- (c) Wright's broth + 1 ml. 10% (w/v) glucose + 0.5 ml. 18.5% (w/v) Na₂CO₃.
- (d) (c) + 0.2 ml. stock solution 4 as used for the culture of *Haemophilus influenzae* (Holt, 1961; uracil, hypoxanthine, cysteine).
- (e) (c) + 0.1 ml. normal horse blood (as source of catalase).

All bottles were seeded with Type I pneumococcus and incubated for 18 hr. at 34° with the results shown in Table 1.

Autolysis. Pneumococci are thought by some workers to produce peroxide during growth, the peroxide being responsible for the green coloration surrounding colonies on blood agar. It seemed that peroxide production might also be related to the autolysis which takes place in fluid culture since this occurs only after incubation for some hours. On blood agar cultures the young colony is domed and only later assumes the typical draughtsman appearance, and at a time when the growing microbes are spatially more removed from any protective action afforded by the embedded red cells. This possibility was investigated. Eight 100 ml. bottles of Wright's broth were seeded with *Pneumococcus* Type III and incubated overnight at 34°; the growth was poor. To these bottles the following additions were made:

Bottle no.	Addition	Relative opacity after 3 hr. further incubation
1	None (primary control)	++
2	+ 5 ml. '20 vols.' H ₂ O ₂	+
3	+ 0.1% (w/v) glucose	+++
4	+ glucose and peroxide	+
5	+ 5 ml. horse serum	+++
6	+ serum and peroxide	Almost clear
7	+ serum and glucose	+++
8	+ serum, glucose and peroxide	+

The bottles were then incubated a further 3 hr. with the results shown above.

These observations were confirmed with *Pneumococcus* Types I and II and extended in order to exclude the possibility that the hydrogen peroxide only prevented growth and did not simply have a direct lytic effect. Three bottles of Wright's broth were seeded with *Pneumococcus* Type II and incubated at 34° overnight. Next morning to two of the three bottles were added 10 ml. horse serum and 0.2 ml. 50% (w/v) glucose; incubation was continued for 3 hr. The growth in the reinforced broth increased appreciably whereas there was no apparent change in the control.

At this stage 5 ml. of '20 vols.' H_2O_2 were added to one of the broth + serum + glucose mixtures and incubation continued for a further 3 hr. By this time the bottle containing added peroxide was less opaque than after incubation for 3 hr., indeed less than the control; whereas the culture containing serum + glucose without peroxide had increased considerably in opacity. It had already been noted, when working on the part played by serum, that the addition of solution 4 (Holt, 1962) which contained cysteine, to the medium resulted in good growth (see above). Cysteine might have a protective action against the effect of peroxide in two ways: (a) by making the medium anaerobic, in which circumstance peroxide would not be formed; (b) by its ready oxidation to cystine by any peroxide that might develop, then

Table 1. *Influence of pH control on count (by opacity) of Streptococcus pneumoniae grown in Wright's broth*

Pneumococcus Type I incubation 18 hr. at 34°		
Medium	pH value after 18 hr.	Count (million/ml.)
(a) Control	7	About 300
(b) +0.1 % (w/v) glucose	4	1900
(c) +0.1 % (w/v) glucose +0.09 % (w/v) carbonate	7	1000
(d) +glucose +carbonate and 0.2 % (v/v) stock solution 4	6.5	2000
(e) +glucose +carbonate +0.1 % (v/v) blood	6.5	About 2500

Table 2. *Components of a medium for the culture of Streptococcus pneumoniae*

(1) Basal medium	
NaCl	2.5 g.
KH_2PO_4	0.5 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.4 g.
CaCl_2 (dried)	1 ml. 1 % (w/v) soln.
Proteose peptone (Difco)	20 g.
L-Cysteine HCl	0.15 g.
Yeast extract (Difco)	5 g.
Distilled water	1000 ml.
Adjusted to	pH 7.2
(2) 50 % (w/v) glucose in distilled water	
(3) 20 % (w/v) Na_2CO_3 solution	

decomposing the latter. The appearance of the colonies of pneumococci when grown on the especially enriched blood plate (Holt, 1962) which contains cysteine, is in keeping with this interpretation; the colonies were not umbilicated and the green halo was virtually absent although grown under fully aerobic conditions.

These two findings, namely the substitution of sodium carbonate for serum and the protective action of cysteine against autolysis, were adopted to give an excellent cultural technique as follows. The medium finally adopted is given in Table 2.

The basal medium was brought to the boil and filtered, and dispensed in 100 ml. and 1 l. volumes in screw-cap bottles. These and the glucose and carbonate solutions were sterilized by autoclaving at 121° for 15 min. Litre volumes of the basal

medium were placed in the incubator (34°) and seeded next morning with 100 ml. of overnight culture in the same medium; at the same time 2 ml. of the sterile 50 % (w/v) glucose was added. Incubation was continued for 2 hr. and then 5 ml. of 20 % (w/v) Na₂CO₃ solution added; incubation was continued for a further 5 hr.

The yield of pneumococci under these conditions was about 2000 million/ml. for Types I and III and about 8000 million for Type II. Incubation may be continued for a further 18 hr. without evidence of autolysis. Haemolytic streptococci also grew more profusely in this medium than in normal glucose broth.

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