A PARTIALLY DEFINED MEDIUM FOR CULTIVATION OF PNEUMOCOCCUS

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A chemically defined medium capable of supporting the growth of pneumococcus would be of value in studies on the metabolism and sulfonamide resistance of strains of this organism and would simplify the isolation of metabolic products. Before a medium may be considered to satisfy the nutritional requirements of a microorganism it must meet two qualifications—it must permit a heavy growth of the organism starting with a reasonably small inoculum, and it must permit unlimited subculture of the organism.

Rane and Subbarow (1940) have developed a gelatine hydrolyzate medium which supported growth of four strains of pneumococcus, but failed to permit growth of a fifth strain, and no report of subculture on this medium was made. Cohen, Halbert, and Perkins (1942) reported poor growth and "failure of growth in successive transplants" on the medium of Rane and Subbarow. In our own experience none of ten strains tried would grow on subculture in this medium. Rane and Subbarow demonstrated the essentiality of pantothenic acid, nicotinic acid, and choline for the strains they tested. A mixture of known amino acids could replace the gelatine hydrolyzate as a medium for three of the strains but not for the fourth. Asparagine was included in the amino acid mixture but was not added to the gelatine hydrolyzate. Glutamic acid was added to both media. This addition is of interest in view of the observation of Fildes and Gladstone (1939) that in the absence of glutamine, growth of a strain of pneumococcus was greatly delayed or failed completely, whereas with glutamine growth occurred overnight. They noted that glutamine could be replaced by a high concentration of glutamic acid. Bohonos and Subbarow (1943) recently demonstrated that pneumococcus requires biotin for growth, a fact predicted by Landy *et al.* (1942) from observations on a single strain. Bohonos and Subbarow used an acid hydrolyzate of casein supplemented with, among other substances, glutamine, choline, and asparagine. They noted difficulty in the subculture of certain strains on this medium.

Badger (1944) also found the medium of Rane and Subbarow to be inadequate for several strains of pneumococcus, and in investigating the nutritional requirements of a strain of pneumococcus type III developed a medium similar to that of Bohonos and Subbarow and similar in most respects to media developed for the cultivation of group A hemolytic streptococci by Bernheimer *et al.* (1942) and by Woolley (1941).

All of these media for pneumococcus contain a reducing agent such as ascorbic acid (Badger, 1944), or thioglycollic acid (Bernheimer *et al.*, 1942), in accordance with the findings of Dubos (1929) that a lowered redox potential is essential for

the initiation of growth of the pneumococcus. None of these media for pneumococcus contains added CO_2 despite the findings of Kempner and Schlayer (1942) that the lag phase in the growth of pneumococci is an inverse function of the CO_2 concentration in the medium, being infinite at concentrations below 0.1 per cent. The observation of Badger (1944) that the "ageing of the basal medium (for two days in the refrigerator) shortened the lag period considerably" is undoubtedly explained by the solution of CO_2 from the air during this "ageing."

EXPERIMENTAL

Medium. Since the medium of Rane and Subbarow (1940) proved to be unsuitable for the cultivation of our strains of pneumococcus, we turned to the medium of Bernheimer *et al.* (1942), designed for the growth of the C203S strain of hemolytic streptococcus group A. This medium was prepared using an acid hydrolyzate of Harris' vitamin-free casein as a base in place of the acid digest of technical casein used for streptococcus. This medium failed to support growth of pneumococcus, but on addition of all the usual growth factors growth was obtained, and by a process of elimination the essential factors missing from the C203S medium were found to be choline and asparagine. The essentiality of choline has been demonstrated both by Badger and by Rane and Subbarow, but the essentiality of asparagine for pneumococci has not been demonstrated, although it was included in the media both of Badger and of Bohonos and Subbarow. Apparently not all strains of pneumococcus require asparagine as well as glutamine. The complete medium is described in detail in table 1 and will hereafter be referred to as the pneumococcus medium.

Inoculum. Eight-hour cultures of pneumococci in beef heart infusion broth were centrifuged and resuspended in sterile saline. Each 10-ml tube of the pneumococcus medium was inoculated with one loopful of washed organisms, and at the same time a tube of neopeptone broth received an identical inoculum as a control on the viability of the seeding culture. The inoculum for serial subcultures in the pneumococcus medium was one loopful of unwashed culture. Broth controls were set up simultaneously using an inoculum of the same size.

Test of Medium. Ten strains of pneumococci were tested on the casein hydrolyzate medium of Bernheimer *et al.* (1942), made up as above, but lacking choline and asparagine. Growth was absent or scanty on first inoculation in the medium, and no strain grew on second transfer. However, on the addition of as little as 0.1 ml of meat infusion neopeptone broth to 10 ml of the casein hydrolyzate medium fair growth was obtained, and with 1 ml, excellent growth. This would indicate that failure of growth in the medium of Bernheimer *et al.* is due to lack of some nutrient rather than to the presence of a toxic component which might render it an unsuitable pablum for pneumococcus. Accordingly, a "shotgun" mixture of amino acids and growth factors consisting of glutathione, methionine, norleucine, hydroxy proline, beta alanine, threonine, pimelic acid, inositol, folic acid, sodium oleate, asparagine, choline, and *p*-aminobenzoic acid was added to the Bernheimer medium, and fairly good growth of pneumococcus occurred.

TABLE 1

The pneumococcus medium

Basal Medium—for one liter of medium

Acid hydrolyzate of casein	200	ml of 10% solution
l-Cystine	150	mg
l-Tryptophane	20	mg
KCl	3	gm
$Na_2HPO_4 \cdot 12H_2O$	7.5	gm
$MgSO_4 \cdot 7H_2O$	0.5	gm
Distilled water to make	900	ml

Adjust pH to 7.5, heat to boiling, filter, and tube in 9 ml amounts or appropriate multiple. Autoclave.

Solution I—vitamin mixture for 12.5 liters

Biotin	0.015	mg
Nicotinic acid	15.0	mg
Pyridoxine	15.0	mg
Calcium pantothenate	60.0	mg
Thiamine	15.0	mg
Riboflavin	7.0	mg
Adenine sulfate	150.0	mg
Uracil	150.0	mg

Dissolve in 100 ml of distilled water and sterilize by filtration. Store in refrigerator.

Solution II—salt mixture for 50 liters

$FeSO_4 \cdot 7H_2O$	50 mg
$CuSO_4 \cdot 5H_2O$	50 mg
$ZnSO_4 \cdot 7H_2O$	50 mg
$MnCl_2 \cdot 4H_2O$	20 mg
HCl concentrated	1 ml

Dissolve in 100 ml of distilled water and sterilize by boiling.

Addition mixture per liter of medium

Vitamin mixture (solution I)	8.0	ml
Salt mixture (solution II)	2.0	ml
Glucose (20% solution)	10.0	ml
Glutamine	200	mg
Asparagine	100	mg
Choline	10	mg
$CaCl_2 \cdot 2H_2O$	10	mg
Distilled water to make	50	ml

Sterilize by filtration and store in refrigerator. Add 0.5 ml to each 9 ml of basal medium. This addition mixture should not be kept longer than a few weeks as the glutamine is unstable. Solutions I and II appear to keep indefinitely.

Bicarbonate—thioglycollate mixture

Thioglycollic acid	10%

Add 1 ml of thioglycollic acid to 9 ml of sterile distilled water, mix well and heat in boiling water bath for 10 minutes.

Bicarbonate. Weigh 200 mg samples of sodium bicarbonate into test tubes and autoclave. Add 10 ml of sterile distilled water to a test tube containing bicarbonate and dissolve the latter. Then add 0.2 ml of 10% thioglycollic acid and mix well immediately. Add 0.5 ml of the mixture to each 9.5 ml of medium. This bicarbonate-thioglycollate mixture is unstable and must be made up and added to the medium just prior to inoculation.

The nonessential growth factors were excluded by a process of elimination. The crucial experiment of the series is given in detail as follows: The medium was that of Bernheimer, to which had been added all the previously mentioned additional growth factors except choline, asparagine, and PABA. The latter were added both singly and together. To control tubes, 0.1 ml of neopeptone broth was added instead of the three substances under test. All tubes were set up in duplicate. Visible growth was estimated roughly after 18 hours of incubation, and final pH was determined as an additional indication of growth. The test organism was a rough variant of pneumococcus type I. The inoculum was one loopful in 10 ml of medium, equivalent to approximately 10^{-3} ml of whole culture. The results of these tests are given in table 2.

TABLE 2

Test for essentiality of choline, asparagine, and PABA

NUMBER	ADDITION	VISIBLE GROWTH	FINAL pH
1	None	0	7.6
1a	None	0	7.55
2	Infusion peptone broth, 0.1 ml	+++	6.8
2a	Infusion peptone broth, 0.1 ml	++	6.9
3	Choline, one loopful of 0.1% solution	+	7.2
3a	Choline, one loopful of 0.1% solution	++	7.1
4	PABA, one loopful of 0.1% solution	0	7.5
4a	PABA, one loopful of 0.1% solution	0	7.6
5	Asparagine, 2 mg	±	7.4
5a	Asparagine, 2 mg	±	7.3
6	Choline, PABA, and asparagine	+++	6.8
6a	Choline, PABA, and asparagine	++++	6.6

As shown in table 2, both asparagine and choline possess growth-promoting qualities for pneumococcus. PABA is inert in this respect. When all three substances were present growth was excellent.

Further experiments eliminated PABA, the amino acids, and growth factors from consideration, indicating that with an inoculum of 10^{-3} ml or larger, choline and asparagine are the only essential growth requirements for this strain of pneumococcus which are not present in the medium of Bernheimer *et al.*

In order to rule out the possibility that the growth-promoting effect of asparagine might be due to some contaminating substance, the asparagine (cp) was twice recrystallized from water and on comparative titration proved slightly more effective in promoting growth than the original material. The pneumococcus medium was used except that asparagine was added separately and the glucose concentration doubled to permit heavier growth and thus accentuate

any difference between cp and recrystallized asparagine. The data are presented in table 3.

The results of the experiment outlined in table 3 indicate that for this strain of pneumococcus at least asparagine is an essential growth factor in a glutamine-containing medium. The strain used by Badger apparently required neither glutamine nor asparagine, but either substance shortened the lag phase and accelerated growth.

The essentiality of glutamine was demonstrated for three freshly isolated strains of pneumococci, types III, V, and IX. The medium used was the complete medium described in table 1 but with glutamine omitted. The organisms were centrifuged, washed once in saline, and resuspended in saline to the original volume. After inoculation with 0.1 ml of washed culture into 10 ml of glutamine-free medium, types V and IX grew well in 16 hours whereas type III grew well

TABLE 3

*Comparison of cp asparagine and twice recrystallized asparagine as growth factors for IR strain of pneumococcus**

TUBE	ADDITION	VISIBLE GROWTH	FINAL pH
1	None	+	6.8
2	None	+	6.8
3	0.1 ml neopeptone broth	++++	5.2
4	0.1 ml neopeptone broth	++	6.5
5	1 mg asparagine cp	++++	5.1
6	1 mg asparagine (2× recrystallized)	++++	5.0
7	0.1 mg asparagine cp	++++	5.25
8	0.1 mg asparagine (2× recrystallized)	++++	5.50
9	0.01 mg asparagine cp	++	6.3
10	0.01 mg asparagine (2× recrystallized)	++	6.8
11	0.001 mg asparagine cp	+	6.9
12	0.001 mg asparagine (2× recrystallized)	+	7.0

* Tubes were seeded with one loopful of a broth culture of IR pneumococci which had been centrifuged and resuspended in saline to one-fifth the original volume.

only after 48 hours. None of the three strains grew in subculture on the glutamine-free medium. In other experiments the lag period before initiation of growth was found to be a function of the size of the inoculum and the amount of glutamine in the medium, becoming infinite with small inocula and reduced amounts of glutamine. The limiting quantities in each case are a characteristic of the strain used. It is evident, however, from the work of Badger that one strain of pneumococcus at least can be carried for 20 transfers in a glutamine-free medium.

Subculture in medium. The pneumococcus medium, using an acid hydrolyzate of vitamin-free casein as a base, was tubed in 10 ml amounts. The culture was a mouse-virulent strain of pneumococcus type I (SVI). Using an inoculum of 0.1 ml, the culture was readily carried in duplicate through 9 transfers in the medium, full growth taking place after incubating for about 5 hours. With an

inoculum of one loopful (10^{-3} ml), both tubes in the duplicate series did not always show growth within 24 hours, but by the inoculation of two tubes from the tube in which growth had occurred after 24 hours, the culture was carried through 6 transfers in the medium.

For many purposes a vitamin-free base is not necessary. Accordingly, the pneumococcus medium was prepared using a commercially available acid hydrolyzate of casein, "casamino acids" (Difco). On this medium two freshly isolated strains of pneumococci as well as an old laboratory strain were readily carried for 10 transfers, using 0.1 ml inoculum for each transfer in 10 ml of medium. In addition 23 other strains of pneumococci have been grown for one or more transfers in the medium. No attempt was made to carry these strains for many transfers. In fact, we have found only one strain of pneumococcus, a markedly degraded rough variant of type III (M3R), which did not grow well on this medium. Failure of this strain to grow indicates that a medium satisfactory for all strains of pneumococci is not yet available.

Use of medium in investigations of sulfonamide resistance of pneumococci. As MacLeod (1940) has pointed out, it is most important in studying sulfonamide susceptibility of strains of pneumococci to employ a medium free of sulfonamide inhibitors. Meat infusion and the various peptones are heavily contaminated with inhibitors, and as a consequence routine bacteriological media are unsuited for this purpose. The pneumococcus medium, prepared with vitamin-free casein hydrolyzate base, is inhibitor-free. Certain lots of casamino acids (Difco) were found to be very low in inhibitor, but other lots contained so much that they were unusable for studies of sulfonamide susceptibility. It might be well to note at this point that different lots of casamino acids vary greatly also in the content of lactic acid and fermentable carbohydrates. If these substances are to be avoided it is necessary to use specially purified casein for preparing the acid hydrolyzate.

Tillett, Cambier, and Harris (1943) used the pneumococcus medium, prepared with casamino acids low in inhibitor, in testing sulfonamide-fast strains of pneumococcus for their ability to synthesize sulfonamide inhibitors. They refer to it as the medium of Bernheimer *et al.* (1942). It should be noted that choline and asparagine were also present in the medium which they used.

When testing for sulfonamide susceptibility or resistance by determining the ability of an organism to grow in various concentrations of sulfonamides, it is important that small inocula be used. With the pneumococcus medium, growth is uncertain and the lag phase long when inocula smaller than 10^{-3} ml of whole culture are used. However, the addition of fresh rabbit serum, shown by MacLeod (1940) to be sulfonamide-inhibitor-free, makes it possible to initiate growth with inocula as small as 10^{-8} ml of culture, containing 1 to 5 organisms, as determined by pour plates. The pneumococcus medium containing casamino acids and enriched with 0.25 ml of rabbit serum per 10 ml was used to compare the growth of a sulfonamide-susceptible strain, SVI, with a sulfonamide-resistant strain, P86, in the presence of various concentrations of sulfathiazole. The results of these tests are shown in table 4.

As shown in table 4, the sulfonamide-susceptible strain, SVI, grows poorly in M/16,000 and not at all in M/8000 sulfathiazole. The resistant strain, P86, however, grows well in M/1000 sulfathiazole. One sample of each culture was washed before dilution to remove sulfonamide inhibitors derived from the meat infusion peptone broth. Washed and unwashed cultures behaved identically in the test, as might be expected, since the inoculum was 2×10^{-6} ml, too small a volume to contain significant amounts of inhibitor.

Different lots of medium were tested directly for the presence of sulfonamide inhibitors by the method of MacLeod (1940). In this method, the effect of added substances suspected of containing inhibitors of sulfonamide bacteriostasis of *Escherichia coli* grown in a defined medium is determined. The pneumococcus medium made with one lot of casamino acids contained inhibitor equivalent to a 10^{-6} M PABA solution, a negligible amount. As is noted above, some lots

TABLE 4

Growth of sulfonamide-susceptible and sulfonamide-resistant strains of pneumococci in the presence of various concentrations of sulfathiazole

STRAIN OF PNEUMOCOCCI	CONCENTRATION OF SULFATHIAZOLE							
	M/1,000	M/2,000	M/4,000	M/8,000	M/16,000	M/32,000	M/64,000	None
SVI	−	−	−	−	++	++++	++++	++++
SVI washed culture	−	−	−	−	+	++++	++++	++++
P86	++++	++++	++++	++++	++++	++++	++++	++++
P86 washed culture	++++	++++	++++	++++	++++	++++	++++	++++

Medium: The pneumococcus medium enriched with 2.5% rabbit serum.
Culture: An 18-hour broth culture diluted in the pneumococcus medium. Washed culture is the same culture centrifuged and resuspended to original volume in the pneumococcus medium and then diluted.
Inoculum: 2×10^{-6} ml per 10 ml of medium; with SVI, 300 organisms, and with P86, 600 organisms by plate count.
Incubation: 22 hours at 37 C.

of casamino acids contain so much inhibitor as to be unusable for this purpose. Vitamin-free casein hydrolyzates contain no detectable inhibitor.

Use of medium for growth of hemolytic streptococcus. The medium of Bernheimer *et al.* (1942) was designed for growing the C203S strain of hemolytic streptococcus and appears to be a complete medium for this strain. In our hands, some freshly isolated group A strains grew well, whereas other strains failed to grow even in the medium supplemented with choline and asparagine. However, when the complete pneumococcus medium was supplemented with 2.5 per cent rabbit serum, all strains of group A hemolytic streptococci which were tested grew well from small inocula. Such an enriched medium has been used by Wilson (1944) for investigations on the sulfonamide resistance of group A strains freshly isolated from individuals treated with sulfonamides prophylactically or therapeutically.

Use of the medium for massive growth of pneumococcus. Bernheimer *et al.* (1942) report the massive growth of several strains of group A hemolytic streptococci on the medium devised by them. In an experiment based on this procedure, massive growth of the SVI strain of pneumococcus type I was obtained using the medium of table 1 prepared with casamino acid base. To one liter of this medium and to one liter of meat infusion neopeptone broth as a control were added 5 mg of phenol red as an indicator. The flasks were inoculated with 5 ml of an 18-hour culture of pneumococci and incubated at 37 C. As soon as acid production became evident, as judged by a marked change in the indicator

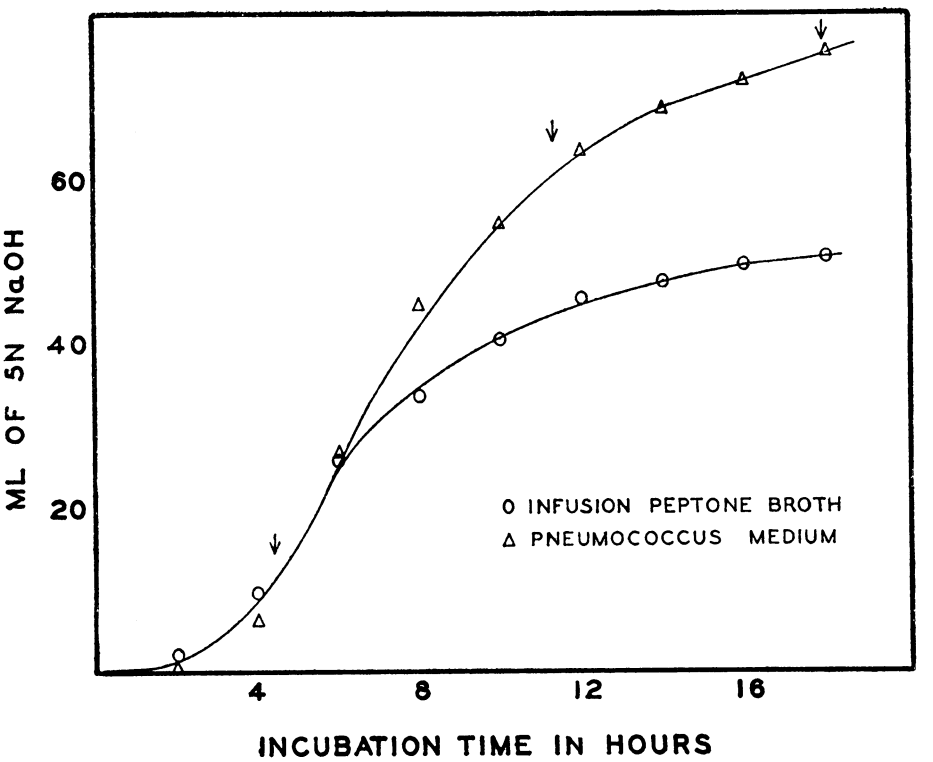

FIG. 1. Amount of sodium hydroxide required to maintain at pH 7.5 cultures of pneumococci growing in infusion peptone broth and in the pneumococcus medium.

color, additional glucose to a final concentration of 5 per cent was added to each flask. The pH was maintained at about 7.5 by frequent additions of 5N NaOH. In a period of 17 hours (figure 1) 75 ml of 5N NaOH were required to maintain the pH of the casamino acid medium, whereas only 50 ml of 5N NaOH were required for the broth control (figure 1). It is evident that the casamino acid medium permits a heavier growth of pneumococci than does the broth. According to Bernheimer *et al.*, the C203S strain of hemolytic streptococcus required about 50 ml of 5N NaOH per liter of medium when grown under similar conditions. Smears were made at the times indicated in the figure to determine the

degree of autolysis of the organisms in the two media. In general there was less autolysis in the casamino acid medium than in broth. If intact organisms are desired, smears should be stained at intervals during the course of growth so that the organisms can be harvested before autolysis has progressed too far. The pneumococcus medium, being free of nondialyzable substances, facilitates the isolation of such metabolic products as the capsular polysaccharides and bacterial enzymes.

SUMMARY

A medium is described which permits growth of most strains of pneumococcus in repeated subculture. The medium is completely dialyzable and can be prepared free of sulfonamide inhibitors. The utility of this medium for obtaining massive growth of pneumococcus and for studies on the sulfonamide resistance of pneumococcus and hemolytic streptococcus has been demonstrated.

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