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On the Growth Requirements of Pneumococci.

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A semi-synthetic medium developed for the CHA strain of type III pneumococcus[1] failed to support optimal growth of other types and strains. This led to a study of the nutritional requirements of different strains and resulted in certain modifications of the semi-synthetic medium. This modified medium, described below, supported the growth of some 25 strains other than the original CHA pneumococcus.

Preparation of the Medium. The constituents of the improved medium, listed in Table I, are combined as follows. The cystine, adenine, guanine, and uracil are dissolved in the acid casein hydrolysate by heating slightly. This solution is diluted with 500 ml distilled water after which the acetate, $MgSO_4$, and KH_2PO_4 are added. The pH is adjusted to 7.6 to 7.8 with NaOH and the volume made up to 800 ml. This basal medium is then heated to boiling, filtered while hot through filter paper, and aged in the refrigerator at least 3 days before the growth factors are added.

TABLE I.
Constituents of the CHA and the Improved Media for Pneumococci.

	CHA medium	Improved medium
Casein hydrolysate*	0.5 g N	1.25 g N
l-Cystine	25.0 mg	20.0 mg
Adenine sulfate		10.0 "
Guanine hydrochloride		10.0 "
Uracil		10.0 "
$NaC_2H_3O_2 \cdot 3H_2O$		200.0 "
$MgSO_4 \cdot 7H_2O$	400.0 "	400.0 "
KH_2PO_4	5.0 g	6.0 g
Biotin	2.0 μg	2.0 μg
Choline chloride	5.0 mg	6.0 mg
Calcium pantothenate	1.0 "	2.5 "
Nicotinic acid	1.0 "	5.0 "
Thiamin hydrochloride	2.0 "	2.0 "
Asparagine	1.0 "	1.0 "
Ascorbic acid	300.0 "	350.0 "
Glucose†	5.0 g	5.0 g
Distilled water to make 1 liter		

* 500 g SMACO Vitamin-Free Casein was hydrolyzed for 18 hours in 3 liters of 1:1 HCl. The hydrolysate was concentrated twice *in vacuo*, diluted to 2.5 liters, and treated with Darco charcoal until water clear (about 5 times with 50 g charcoal).

† Autoclaved separately and added aseptically after medium is autoclaved.

To the aged basal medium are added the choline, nicotonic acid, pantothenate, biotin, thiamin, asparagine, and ascorbic acid. Except for the ascorbic acid, these growth factors are added from stock solutions kept in the refrigerator. The volume is made up to 1000 ml with distilled water, and the resulting medium is tubed in 5 ml portions and autoclaved at 15 lb for 10 minutes. 0.1 ml of a 25% solution of sterile glucose is added to each tube which is then carefully shaken to insure homogeneity. The complete medium is kept in the dark at 5 to 9°C until it is used.

Organisms and Inoculum. Throughout the major part of this investigation 5 strains of pneumococci were employed, including SV-1 and McGovern strains of type I, the CH strain of type II and Wistuba and CHA strains of type III. For a period of 7 years these strains had been passed through mice at least every other day and cultured in beef-infusion broth enriched with 2% defibrinated rabbit blood. The other strains tested had been stored 2 to 5 years in a dehydrated state. Prior to testing for growth in the improved medium, these latter strains were cultured in beef-infusion broth enriched with blood.

The inoculum was prepared by subculturing one drop of the stock culture in 5 ml of the semi-synthetic medium and incubating 5 hours at 37°C. This young culture was centrifuged and the cells washed once and diluted 1000 times in sterile basal medium. 0.1 ml of this dilution added to each tube gave an inoculum of 1000 to 10,000 organisms per ml. The inoculated tubes were incubated at 37°C and turbidity resulting from growth was measured

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1 Badger, E., *J. Bact.*, 1944, **47**, 509.

TABLE II.
Effect of Adenine, Guanine, and Uracil on the Growth of Pneumococci.

	Turbidity readings after 12 and 14 hrs incubation									
	I SV		I McGovern		II CH		III Wistuba		III CHA	
Purine or pyrimidine*	12	14	12	14	12	14	12	14	12	14
None	6†	79	12	22	0‡	0	8	64	51	135
Adenine	45	170	30	125	0	0	27	168	106	179
Guanine	98	170	30	104	0	0	152	172	81	150
Uracil	8	81	12	24	0	40	99	183	65	153
Adenine + guanine	103	182	25	113	0	11	164	176	147	170
" + uracil	70	174	37	160	143	159	164	185	74	155
Uracil + guanine	126	186	122	151	67	143	178	188	95	157
Adenine + " + uracil	133	192	115	158	115	145	177	190	159	175

* Added to basal medium as adenine sulfate, guanine hydrochloride and uracil; 10 μg per ml.
† Uninoculated medium reads zero.
‡ Grew out after 24 hours' incubation.

in a Klett-Summerson photoelectric colorimeter equipped with a 540 mμ filter.

Response of the 5 Strains to Modifications of the CHA Medium. In Table I are listed the constituents of the CHA medium and the improved medium. A comparison of the 2 media shows, first of all, certain changes in concentrations of the original constituents. These changes were instituted after the following observations were made. Better growth of all strains was obtained by increasing the casein hydrolysate from 0.5 to 1.25 mg N per ml. Lowering the concentration of cystine from 25 to 20 μg and raising the concentration of ascorbic acid from 300 to 350 μg per ml provided better growth of II CH but had no effect on the other strains. Raising the concentration of KH_2PO_4 from 5 to 6 mg per ml increased the rate of growth of Wistuba, II CH, and McGovern. It was not possible to replace the KH_2PO_4 with sodium glycerophosphate.

The minimum effective concentration of each of the 4 essential growth factors, choline, nicotinic acid, pantothenic acid and biotin agreed with those determined for CHA.[1] However, to insure the presence of an excess but still optimal concentration of the growth factors, choline chloride was raised from 5 to 6 μg per ml, calcium pantothenate from 1 to 2.5 μg per ml, and nicotinic acid from 1 to 5 μg per ml.

The second difference in the 2 media is the addition of adenine, guanine, uracil, and acetate. It was found that adenine, guanine, and uracil are not essential for growth, but they do have an accelerating effect, which is most apparent in the early hours of incubation and with small inocula. They also appear to be more effective when added to the basal medium than when added later with the growth factors, suggesting an interaction with the basal medium constituents. These factors probably explain why the accelerating effect of adenine, guanine, and uracil was not observed in the original studies of CHA. From the data in Table II it is seen that the response to the individual purines and the pyrimidine and their various combinations differs with each strain. Type III CHA seemed least affected by any combination, while the growth of II CH was markedly accelerated by the addition of adenine and uracil but was inhibited by guanine.

The addition of synthetic thymine† alone or in combination with adenine, guanine, and/or uracil produced no measurable effect. Xanthine gave no further acceleration of growth in the presence of all 3 but could partially replace guanine. Bohonos and Subbarow[2] have also included adenine, guanilic acid, and uracil in their medium for pneumococcus.

The accelerating action of sodium acetate was observed during preliminary studies of the effect of alkaline-treated peptone on the growth of pneumococcus. The addition of

† We are indebted to Dr. J. L. Stokes of Merck and Co., Inc., for the thymine.

2 Bohonos, N., and Subbarow, Y., *Arch. Biochem.*, 1943, **3**, 257.

sodium acetate in a concentration of 100 μg per ml of medium accelerated the growth of CHA and Wistuba. The response of strain Wistuba to sodium acetate is shown in Table III. That this accelerating effect is not due to a buffering action is apparent from the relatively small concentration required as compared to the phosphate present. Neutralized glacial acetic acid is as effective as the sodium acetate, suggesting that the acetate radical is the active factor rather than some contaminating ion. The acetate could not be replaced by succinate, lactate, citrate, pyruvate, or glycine. Further study is indicated on the role of acetate in pneumococcal metabolism.

TABLE III.
The Response of Type III Strain Wistuba to Sodium Acetate.

Sodium acetate μg per ml	Turbidity readings*
0	77
10	103
50	128
100	175
200	175
500	133

* Readings made after 12 hours' incubation.

The response of the pneumococci to thiamin and asparagine varied. Thiamin accelerated growth of all 5 strains when present in a concentration of 1 to 3 μg per ml. The effect on the growth of strains II CH, CHA, and Wistuba was much more pronounced than upon the two type I strains. These type I strains were also least affected by asparagine as is seen in Table IV. From the data presented here and similar experiments the optimum concentration of asparagine is seen to be 1 μg per ml. This optimum concentration is quite sharp, since 0.5 or 1.5 μg per ml provides slower growth. Concentrations greater than 2 μg are definitely inhibitory for all 5 strains. This concentration of asparagine is exceedingly small compared with that used by Bohonos and Subbarow[2] or Roe and Adams,[3] who included asparagine in their media for pneumococcus in a concentration of 250 and 100 μg per ml respectively.

That asparagine cannot be replaced by glutamine is also shown in Table IV. In high concentrations glutamine stimulated the growth of II CH, Wistuba, and CHA, but inhibited the growth of the two type I strains. When the asparagine and glutamine were sterilized by filtration the results were the same as given in Table IV. With *Streptococcus lactis*, Niven[4] found that asparagine was more satisfactory in casein hydrolysate media, while glutamine was more effective in media containing pure amino acids.

Growth of Other Strains in the Improved Medium. Having developed a simplified medium for the 5 strains of pneumococci mentioned above, it was interesting to test its ability to support the growth of other strains. Twenty-four additional strains, not recently passed through mice, were tested in the improved medium. Growth after 5 successive subcultures from an initial small washed inoculum was obtained with 21 out of 24 strains. Those that gave an early rapid growth included types I Lederle, I 1811, IV NYS, V NYC, VI NYC, IX Ramey, XI NYC, XIX NYS, XXIII NYS, XXIII Marvin, XXVII NYC, XXIX NYS, and XXXII NYC; while those which grew out more slowly included types II 2118, III H, VII CHA, XIV Halse, XV Leichliter, XXVII Glacken, XXXI NYS, and XXXIII League. Strains XXXI NYC, X NYC, and X NYS failed to grow out in 48 hours. However, slow growth of the two type X strains was obtained in the medium without guanine.

Variation in the growth requirements of the strains of pneumococci was indicated by the different rates of growth in the simplified medium. Some of the factors affecting this variation were demonstrated in an experiment in which growth in the complete medium was compared with that in the medium from which one of the growth factors had beeen omitted. The results of this study are summarized in Table V. The omission of a factor resulted in either (1) no growth in 48 hours, (2) delayed growth, (3) growth the same as the control, or (4) improved accelerated growth. All strains tested required biotin, choline, nicotinic acid, and pantothenic acid for growth,

[3] Roe, A. S., and Adams, M. H., *J. Bact.*, 1944, **47**, 432.

[4] Niven, C. F., *J. Bact.*, 1944, **47**, 343.

TABLE IV.
Response of Pneumococci to Glutamine and Asparagine.

Glutamine or asparagine	μg per ml	Turbidity readings*				
		I SV	I McGovern	II CH	III Wistuba	III CHA
None		85	135	0	11	71
Asparagine	0.1	69	125	0	60	90
	1.0	80	125	85	185	102
	2.0	60	100	25	170	85
	10.0	0	20	0	36	0
	100.0	0	14	0	0	0
Glutamine	0.1	85	90	0	0	85
	1.0	80	93	0	0	87
	10.0	7	45	0	16	85
	100.0	0	27	65	41	107

* Readings made after 12 hours' incubation; uninoculated medium reads zero.

TABLE V.
Effect of Omitting Certain Factors from the Complete Medium on Growth of 26 Strains of Pneumococci.

Factor omitted	No growth*	Delayed growth	Growth not affected	Accelerated growth
Biotin	26			
Choline	26			
Nicotinic acid	26			
Pantothenic acid	26			
Asparagine	6	12	3	5
Thiamin		24		2
Adenine		21	1	4
Guanine		20	4	2
Uracil	3	14	3	6

* No growth after 48 hours' incubation.

confirming the observations of Rane and Subbarow[5] and Bohonos and Subbarow[2] with other strains. The effect of asparagine, thiamin, adenine, guanine, and uracil on the growth of these pneumococci varied from being essential for growth of some strains to actually inhibiting the growth of others.

That this improved medium is still incomplete is indicated by the accelerating action of small amounts of peptone. Of the growth-stimulating substances tested, none was able to replace this peptone action.

5 Rane, L., and Subbarow, Y., *J. Bact.*, 1940, **40**, 695.

Conclusions. A simplified medium has been described which supported the growth of 26 out of 29 strains of pneumococci. All strains required biotin, choline, nicotinic acid, and pantothenic acid for growth, but varied in their response to thiamin, asparagine, adenine, guanine, and uracil. The extent of this variation makes apparent the necessity of considering the growth requirements of each strain in the study of pneumococcal nutrition. In such a study the importance of concentration and interaction of medium constituents, size of inoculum, and the time of measurement of growth cannot be overemphasized.